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Sulfur supply as a sustainable technology for phytoextraction: its effects on cadmium uptake and detoxification in *Panicum maximum* Jacq. cv. Massai

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“The task is, not so much to see what no one has seen yet; but to think what nobody has thought yet, about what everybody sees.”

Arthur Schopenhauer

ABSTRACT

RABÊLO, F.H.S. **Sulfur supply as a sustainable technology for phytoextraction**: its effects on cadmium uptake and detoxification in *Panicum maximum* Jacq. cv. Massai. 2018. 189 f. Tese (Doutorado) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2018.

Cadmium (Cd) concentration in the environment has increased in most recent decades, which represents a serious socio-environmental problem, since Cd is toxic to most living beings. Thus, it is important to reduce the concentration of this metal in contaminated environments, and the use of plants properly supplied with sulfur (S) can contribute to this (phytoextraction), since S is a component of metabolites involved in defense responses against stress caused by Cd. Therefore, our aim with this study was to evaluate the effect of S on i) Cd uptake kinetics, ii) root development and nutrient uptake, iii) metabolic profiling and thiol peptides synthesis, and iv) activity of antioxidant and photosynthetic system of Massai grass (*Panicum maximum* Jacq. cv. Massai) used for Cd phytoextraction. The studies were carried out in a greenhouse conditions using pots of 0.5 and 2.0 L for development of the study about Cd uptake kinetics (treatments represented by combinations of S: 0.1 and 1.9 mmol L^{-1} and Cd concentrations: 0.1 and 0.5 mmol L^{-1}) and Cd detoxification mechanisms (treatments represented by combinations of S: 0.1, 1.9 and 3.7 mmol L^{-1} and Cd concentrations: 0.0, 0.1 and 0.5 mmol L^{-1}), respectively. Pots were distributed in randomized blocks, with four replications. Plants used in Cd kinetics study were exposed to treatments for 14 days after they remained in solutions containing only S for 42 days, while plants used in Cd detoxification study were exposed to treatments for 9 days after growing in solutions containing only S for 44 days. At the end of studies, plants used were harvested and sent for analysis. Maximum uptake rate (V_{max}) and Cd apoplastic influx of Massai grass exposed to highest Cd concentration was highest when the plants were supplied with highest S concentration. The root development of Massai grass was strongly inhibited when plants were exposed to 0.5 mmol L^{-1} Cd, but proper (1.9 mmol L^{-1}) S supply allowed highest Cd uptake, while excessive S supply (3.7 mmol L^{-1}) decreased iron plaques formation in the roots of plants. Lysine, cysteine, ornithine, arginine, tryptophan and histidine were accumulated in more than one tissue in plants exposed to Cd, as well as galactinol. Glutathione (GSH), phytochelatins (PCs) and their homologues were strongly induced by Cd, whereas plants supplied with 1.9 and/or 3.7 mmol L^{-1} S showed the highest concentrations of these peptides. Plants supplied with highest S concentration showed lowest lipid peroxidation and highest photosynthetic rate, which evidences that antioxidant system of these plants was more efficient in mitigating the damages caused by Cd. In view of these results, it is clear that Massai grass properly supplied with S shows greatest Cd tolerance, as well as phytoextraction potential.

Keywords: Amino acids. Anatomical changes. Antioxidant enzymes. Environmental pollution. Gene expression. Glutathione. Metabolic profile. Nutritional status. Organic acids. Oxidative stress. Photosynthetic system. Phytochelatins. Root morphology. Sugars. Thiol compounds. Uptake kinetics.

RESUMO

RABÊLO, F.H.S. **Suprimento de enxofre como tecnologia sustentável para fitoextração:** efeitos na absorção e detoxificação de cádmio em *Panicum maximum* Jacq. cv. Massai. 2018. 189 f. Tese (Doutorado) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2018.

A concentração de cádmio (Cd) no ambiente aumentou em décadas recentes, o que representa sério problema sócio-ambiental, visto que o Cd é tóxico para a maioria dos seres vivos. Por isso, é importante reduzir a concentração desse metal em ambientes contaminados e o uso de plantas adequadamente supridas com enxofre (S) pode contribuir para isso (fitoextração), uma vez que o S é componente de metabólitos envolvidos no combate ao estresse causado pelo Cd. Assim, o nosso objetivo com esse estudo foi avaliar o efeito do S: i) na cinética de absorção do Cd; ii) no desenvolvimento radicular e na absorção de nutrientes; iii) no perfil metabólico e síntese de compostos tiol, e iv) na atividade do sistema antioxidante e fotossintético do capim-massai (*Panicum maximum* Jacq. cv. Massai), utilizado para fitoextração de Cd. Os estudos foram conduzidos em casa de vegetação utilizando-se vasos de 0,5 e 2,0 L para a condução do estudo de cinética de absorção de Cd (tratamentos representados pelas combinações das doses de S de 0,1 e 1,9 mmol L^{-1} e Cd de 0,1 e 0,5 mmol L^{-1}) e para o estudo dos mecanismos de detoxificação de Cd (tratamentos representados pelas combinações das doses de S de 0,1; 1,9 e 3,7 mmol L^{-1} e Cd de 0,0; 0,1 e 0,5 mmol L^{-1}), respectivamente. Os vasos foram distribuídos em blocos ao acaso, com quatro repetições. As plantas utilizadas no estudo de cinética foram expostas aos tratamentos durante 14 dias após as mesmas terem permanecido em soluções contendo apenas S durante 42 dias, enquanto as plantas utilizadas no estudo de detoxificação de Cd foram expostas aos tratamentos pelo período de 9 dias após terem crescido em soluções contendo apenas S por 44 dias. Ao final dos estudos, as plantas utilizadas foram colhidas e encaminhadas para análises. A velocidade máxima de absorção (V_{max}) e o influxo apoplástico de Cd do capim-massai exposto à maior dose de Cd foram maiores quando as plantas foram supridas com a maior dose de S. O desenvolvimento radicular do capim-massai foi fortemente inibido quando as plantas foram expostas à dose de Cd de 0,5 mmol L^{-1}, mas o adequado (1,9 mmol L^{-1}) suprimento de S permitiu maior absorção de Cd, enquanto o suprimento excessivo (3,7 mmol L^{-1}) de S diminuiu a formação de placas de ferro nas raízes das plantas. A lisina, cisteína, ornitina, arginina, triptofano e histidina foram acumulados em mais de um tecido nas plantas expostas ao Cd, assim como o galactinol. A glutationa (GSH), as fitoquelatinas (PCs) e seus homólogos foram fortemente induzidos pelo Cd, sendo que as plantas supridas com as doses de S de 1,9 e/ou 3,7 mmol L^{-1} apresentaram as maiores concentrações desses peptídeos. As plantas supridas com as maiores doses de S apresentaram menor peroxidação lipídica e maior taxa fotossintética, o que demonstra que o sistema antioxidante dessas plantas foi mais eficiente na atenuação dos danos causados pelo Cd. Diante desses resultados, fica claro que o capim-massai suprido adequadamente com S apresenta maior tolerância ao Cd, assim como maior potencial de fitoextração.

Palavras-chave: Ácidos orgânicos. Açúcares. Alterações anatômicas. Aminoácidos. Cinética de absorção. Compostos tiol. Enzimas antioxidantes. Estado nutricional. Estresse oxidativo. Expressão gênica. Fitoquelatinas. Glutationa. Morfologia radicular. Perfil metabólico. Poluição ambiental. Sistema fotossintético.

SUMMARY

1 INTRODUCTION 17

2 LITERATURE SUPPORT 19
2.1 Cadmium contextualization in the environment and problems caused to living beings 19
2.2 Uptake, transport, distribution and localization of cadmium in plants 20
2.3 Changes caused by cadmium in plants 21
2.3.1 Changes in nutrients, organic acids and amino acids metabolism 21
2.3.2 Changes in photosynthetic and antioxidant systems and in sugar synthesis 22
2.3.3 Morpho-anatomical and productive changes 24
2.4 Technologies to mitigate cadmium impact on the environment and on the plants 25
2.4.1 Phytoextraction 25
2.4.1.1 *Panicum maximum* Jacq. cv. Massai 26
2.4.2 Sulfur supply 27
2.5 Uptake, transport and assimilation of sulfur in plants 27
2.6 Cadmium-sulfur relation and synthesis of GSH, PCs and other thiol compounds 28
2.7 Cadmium-sulfur relation and the activity of antioxidant system 29
2.8 Cadmium-sulfur relation and the activity of photosynthetic system 30
References 31

3 A GLIMPSE INTO THE SYMPLASTIC AND APOPLASTIC Cd UPTAKE BY MASSAI GRASS MODULATED BY SULFUR NUTRITION: PLANTS WELL-NOURISHED WITH S AS A STRATEGY FOR PHYTOEXTRACTION 43
Abstract 43
3.1 Introduction 43
3.2 Materials and methods 45
3.2.1 Plant material and treatments 45
3.2.2 Development of kinetics study 48
3.2.3 Determination of kinetic parameters 51
3.2.4 Determination of Cd concentrations in apoplast and symplast and S 52
3.2.5 Determination of biomass production 53
3.2.6 Statistical analysis 53
3.3 Results 53
3.3.1 Biomass production and root/shoot ratio 53
3.3.2 Depletion of Cd in nutrient solution 54
3.3.3 Kinetic parameters 55
3.3.4 Symplastic and apoplastic Cd influx 56
3.3.5 Concentrations of Cd (apoplast and symplast) and S 57
3.3.6 Pearson’s correlation among the studied variables 58
3.3.7 Relationship between Cd influx and accumulation site 58
3.4 Discussion 60
3.5 Conclusions 66
References 66

4 PROPER S SUPPLY REDUCES THE DAMAGE OF HIGH Cd EXPOSURE IN ROOTS AND INCREASES N, S AND Mn UPTAKE FAVORING PHYTOREMEDIATION WITH MASSAI GRASS 73
Abstract 73
4.1 Introduction 73
4.2 Materials and methods 75
4.2.1 Plant material and treatments 75
4.2.2 Massai grass growth and plant material collection for analysis 75
4.2.3 Determination of dry matter production and shoot:root ratio 78
4.2.4 Determination of root length, root surface and Cd tolerance index (TI) 80
4.2.5 Anatomical analyses and regions of S, Ca and Fe accumulation 80
4.2.6 Determination of concentrations and content of nutrients and Cd 81
4.2.7 Cd translocation factor (TF) 81
4.2.8 Statistical analysis 81
4.3 Results 82
4.3.1 Biomass partition of Massai grass supplied with S and exposed to Cd 82
4.3.2 Root length, root surface and Cd tolerance index of Massai grass 83
4.3.3 Morpho-anatomical changes in root system of Massai grass 85
4.3.4 Concentration of nutrients and Cd in root and shoot tissues of Massai grass 86
4.3.5 Correlation among elements concentrations and morphological parameters 88
4.3.6 Cd translocation factor (TF) 88
4.3.7 Content of nutrients and Cd in the root and shoot tissues of Massai grass 88
4.3.8 Localization of S, Ca and Fe in the root tip of adventitious roots of Massai grass 90
4.4 Discussion 93
4.5 Conclusions 98
References 98

5 METABOLITE PROFILING REVEALS THAT SUFFICIENT SULFUR SUPPLY IS ESSENTIAL FOR INCREASED Cd PHYTOEXTRACTION IN MASSAI GRASS 107
Abstract 107
5.1 Introduction 107
5.2 Materials and methods 110
5.2.1 Plant material and exposure to Cd and S 110
5.2.2 Growth and harvest of Massai grass 110
5.2.3 Determination of dry mass production and root/shoot ratio 111
5.2.4 Determination of S and Cd contents 111
5.2.5 Metabolite profiling 111
5.2.6 Determination of non-protein thiols (NPTs) 112
5.2.7 Gene expression analyses related to thiol compounds biosynthesis 112
5.2.8 Statistical analyses 113
5.3 Results 114
5.3.1 Dry mass production, root/shoot ratio and S and Cd contents in Massai grass 114
5.3.2 Metabolic responses to Cd and S 116
5.3.3 Effect of S and Cd combinations on non-protein thiols (NPTs) 129
5.3.4 Expression of genes related to non-protein thiols (NPTs) biosynthesis 132
5.4 Discussion 133
5.5 Conclusions 140
References 140

6 PROPER SULFUR-SUPPLY NOT IMPROVES THE PERFORMANCE OF ASCORBATE-GLUTATHIONE CYCLE AGAINST CADMIUM-INDUCED STRESS BUT INCREASES THE PHOTOSYNTHETIC RATE OF MASSAI GRASS USED FOR Cd PHYTOEXTRACTION 149
Abstract 149
6.1 Introduction 149
6.2 Materials and methods 152
6.2.1 Plant material and treatments 152
6.2.2 Massai grass growth and plant material collect to analyses 152
6.2.3 Determination of plant growth inhibition 153
6.2.4 Determination of S and Cd concentrations 153
6.2.5 Determination of H_2O_2 and malondialdehyde (MDA) concentrations 153
6.2.6 Determination of GSH and GSSG concentrations 154
6.2.7 Determination of DHA concentration 155
6.2.8 Extraction and quantification of proteins 155
6.2.9 Determination of enzymatic activity 155
6.2.10 Gene expression analyses related to antioxidant enzymes biosynthesis 157
6.2.11 Light and transmission electron microscopy 158
6.2.12 Determination of morphological and photosynthetic parameters 158
6.2.13 Statistical analyses 159
6.3 Results 159
6.3.1 Growth inhibition and concentrations of S and Cd in Massai grass 159
6.3.2 Effect of S and Cd on H_2O_2 and MDA concentrations of Massai grass 161
6.3.3 GSH, GSSG and DHA concentrations and GSH/GSSG ratio of Massai grass 161
6.3.4 Activity of enzymes related to oxidative stress in Massai grass 165
6.3.5 Expression of the genes related to enzymes of ascorbate-glutathione cycle 169
6.3.6 Visual, anatomical and ultrastructural changes in the leaf blades of Massai grass 171
6.3.7 Effect of S and Cd on the photosynthetic parameters of Massai grass 172
6.4 Discussion 175
6.5 Conclusions 181
References 181

7 GENERAL CONCLUSIONS 189

1 INTRODUCTION

Cadmium (Cd) concentration in the environment has increased in recent decades due to anthropic activities (e.g. industrial waste disposal and use of sewage sludge as an alternative source of nutrients for plants), which is a serious socio-environmental problem (CLEMENS et al., 2013; KHAN et al., 2017). Initially, Cd competes with other nutrients for the same uptake sites located in the roots of plants, decreasing the uptake of essential elements to plant growth (RABÊLO; BORGO, 2016). Then, Cd changes the metabolism of nutrients, amino acids and carbohydrates, degrades proteins and lipids, inhibits enzymatic activity which increasing the formation of reactive oxygen species (ROS), damages photosynthetic apparatus, and degrades chlorophyll and carotenoids, among other damages, which decrease the biomass production of plants (SANITÁ DI TOPPI; GABBRIELLI, 1999; CUYPERS et al., 2010; KEUNEN et al., 2013; BASHIR et al., 2015a). Besides to decrease the biomass production of plants, Cd intake can cause several diseases in animals (e.g. bone fractures and kidney stones) and in humans (e.g. hypertension and cancer) (HUFF et al., 2007; BHATTACHARYYA, 2009). Therefore, it is fundamental to study new technologies to decrease Cd concentration in the environment (KRÄMER, 2005; SHEORAN; SHEORAN; POONIA, 2016).

Among the technologies currently used for remediation of soils contaminated with Cd, phytoextraction is the technology of lowest cost and the most socially accepted (SHEORAN; SHEORAN; POONIA, 2016). Cadmium is removed from contaminated site through harvest of aboveground plants established in the environment, and then the contaminated material can be used for energy production, among other uses (SHEORAN; SHEORAN; POONIA, 2017). However, plants used for Cd phytoextraction show low biomass production (eg, *Noccaea caerulenscens*), which decrease the Cd phytoextraction efficiency (SHEORAN; SHEORAN; POONIA, 2016). Thus, the study of plants with extensive roots (increases soil exploitation), high biomass production, successive shoot emissions after harvest and fast growth, such as grasses (e.g. *Panicum maximum* cv. Massai) may contribute to advances in Cd phytoextraction (RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b; RABÊLO; JORDÃO; LAVRES, 2017). However, Cd damage is unavoidable, even in forage grasses that are considered tolerant of adversities imposed by environment, but Cd damage can be mitigated if the plants are grown with proper sulfur (S) supply (RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b).

Sulfur is a structural component of metabolites involved in Cd detoxification, such as methionine, glutathione (GSH), and phytochelatins (PCs) (COBBETT; GOLDSBROUGH, 2002; CAPALDI et al., 2015). Glutathione acts as Cd-chelator and antioxidant, and it is used as substrate for PCs synthesis, which act in sequestration and transport of free Cd ions from cytosol to vacuoles (COBBETT; GOLDSBROUGH, 2002; NOCTOR et al., 2011). Sulfur also increases chlorophyll synthesis and participates in the formation of photosynthetic system and electron transport system, since it is a component of iron-sulfur proteins and thioredoxin systems (IMSANDE, 1998), besides to show relation with phytohormones, that are essential in plants adaptation to environmental changes (CAPALDI et al., 2015). In this sense, proper S supply to plants used in remediation of soils contaminated with Cd is essential (SETH et al., 2012; CAPALDI et al., 2015). However, few studies evaluated the potential of heavy metals phytoextraction of forage grasses grown with proper S availability (GILABEL et al., 2014; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b; RABÊLO; JORDÃO; LAVRES, 2017). Thus, it is fundamental to analyze the influence of S on Cd uptake and detoxification mechanisms of these plants to optimize this strategy of phytoextraction.

In view this context, it is evident that use of forage grasses supplied with S can increase Cd phytoextraction efficiency. Thus, our hypothesis with this study was that proper S supply can protect organelles more sensitive to Cd due to increase in thiol peptides concentrations such as cysteine, methionine, GSH and PCs. These peptides are involved in defense responses against stress caused due to Cd toxicity. High GSH and PCs associated to high amino acids synthesis and an efficient antioxidant system probably mitigates the damage caused by Cd on photosynthetic system, which allows highest biomass production and Cd phytoextraction. Therefore, our aim with this study was to evaluate the effect of S on: i) Cd uptake kinetics in Massai grass; ii) root development and nutrient uptake; iii) metabolic profiling and synthesis of GSH and PCs; as well as the iv) activities of antioxidant (ascorbate-glutathione cycle) and photosynthetic systems of Massai grass exposed to Cd.

2 LITERATURE SUPPORT

2.1 Cadmium contextualization in the environment and problems caused to living beings

Uncontaminated soils show Cd concentrations between 0.2 and 1.1 mg kg^{-1} soil, but there are variations in this concentration due to soil texture. For example, sandy soils show Cd concentrations between 0.01 and 0.3 mg kg^{-1} soil, and clay soils show concentrations between 0.2 and 0.8 mg kg^{-1} soil (KABATA-PENDIAS, 2011). However, the concentration of this metal in the soils of several places in the world increased in recent decades due to annual addition of 13,000 tons of Cd through anthropic activities such as application of phosphate fertilizers and waste disposal of municipal sewage, industrial processes and mining (IRFAN et al., 2013; RANI et al., 2014). Until 2005, about 235 million hectares of agricultural soils in the world had high Cd concentrations (GIORDANI; CECCHI; ZANCHI, 2005), but this area continues to increase according to World Health Organization - WHO (WHO, 2011).

Cadmium can be taken up by plants in inorganic forms in soil solution (e.g. Cd^{+2}, $CdCl^{+}$, $CdCl_2$, $CdSO_4$) and for control purposes, Cd concentrations in soil solution between 0.04 and 0.32 μmol L^{-1} solution are considered normal, while concentrations between 0.33 and 1.00 μmol L^{-1} solution indicate moderate pollution (WAGNER, 1993; McLAUGHLIN et al., 1996; SANITÁ DI TOPPI; GABBRIELLI, 1999). Even in soils considered contaminated, Cd uptake by plants can be low due to their Cd tolerance mechanisms and vice-versa (KUTROWSKA; SZELAG, 2014; VIEHWEGER, 2014). Thus, depending on Cd-concentration and localization in plant tissues, this metal can alter the metabolism of nutrients, amino acids, organic acids and sugars, degrade chlorophyll, carotenoids, proteins and lipids, damage the photosynthetic apparatus, stimulate ROS formation, and cause morpho-anatomical and productive changes, among other damages (NAGAJYOTI; LEE; SREEKANTH, 2010; NAZAR et al., 2012; PARMAR; KUMARI; SHARMA, 2013; BASHIR et al., 2015a).

Cadmium is one of most toxic elements present in the environment and plants represent the main entry route of this heavy metal in the food chain of animals and humans (WAGNER, 1993; BHATTACHARYYA, 2009). Animals that feed of plants with high Cd concentrations can develop pathologies related to bone fracture and kidney stones, among others pathologies (HUFF et al., 2007; BHATTACHARYYA, 2009). In turn, humans who ingest plants and/or animals contaminated with Cd may develop several diseases such as anemia, hypertension, gastric dysfunctions, pulmonary emphysema and cancer (HUFF et al., 2007; FAO/WHO, 2011). In more recent years, Cd weekly intake by humans (between 2.8

and 4.2 μg kg^{-1} body weight) was above the limits recommended by Agency for Toxic Substances and Disease Registry - ATSDR (0.8 μg kg^{-1} body weight) and European Food Safety Authority - EFSA (2.5 μg kg^{-1} body weight) (CLEMENS et al., 2013). Thus, the study of Cd and technologies to reduce its concentration in the environment is extremely important to current global scenario (FAO/WHO, 2011; CLEMENS et al., 2013; RANI et al., 2014; GHOSH et al., 2015).

2.2 Uptake, transport, distribution and localization of cadmium in plants

Normally Cd is present at low concentrations in soil solution (< 0.32 μmol L^{-1}) in most sites and predominantly in ionic form Cd^{+2}, which presents low diffusion, suggesting that mass flow is the predominant mechanism of ion-root contact in uptake of this metal by plants (LUX et al., 2011). Cadmium uptake is controlled by difference of electrochemical potential of Cd^{+2} activity in cytosol and root apoplast (IRFAN et al., 2013). Then, Cd transport in root cells occurs by means of transporters of ZIP family (zinc regulated transport/iron regulated transport-like protein) and Nramp (natural resistance-associated macrophage protein) or non-selective cation channels, while some transporter proteins localized in vascular vessels (e.g. YSL family - yellow stripe-like) carry Cd from roots to shoots of plants (LUX et al., 2011; MENDOZA-CÓZATL et al., 2011; IRFAN et al., 2013).

In some species Cd is quickly transported from roots to shoots, but its distribution varies between tissues (roots, stems and leaves) and plants (CLEMENS, 2006). Originally, Cd distribution in plants is conditioned to its retention by cell wall of roots, but the activity of other heavy metals sequestration routes (e.g. organic acids, amino acids and PCs) probably plays a fundamental role in Cd translocation from roots to shoots (HAQ; MAHONEY; KOROPATNICK, 2003; BENAVIDES; GALLEGO; TOMARO, 2005; PAL; RAI, 2010; KUTROWSKA; SZELAG, 2014). Other factors such as mobilization of sequestered metal, the efficiency of passage of metal through roots and loading activity of xylem also change Cd distribution in plant tissues (CLEMENS, 2006; MENDOZA-CÓZATL et al., 2011).

The change in Cd distribution in plant tissues is associated with the site of accumulation of this metal, which occurs differently in Cd-tolerant and non-tolerant plants (BENAVIDES; GALLEGO; TOMARO, 2005). To exemplify, 40% of Cd taken up by sunflower (*Helianthus annuus*) was accumulated in shoots (LOPES JÚNIOR; MAZZAFERA; ARRUDA, 2014), while 93% of Cd taken up by lettuce (*Lactuca sativa*) was accumulated in roots (AKHTER et al., 2014). Cadmium accumulation in roots occurs preferentially at cell walls of apoplast, but this may change according to species (WÓJCIK et al., 2005).

Akhter et al. (2014) reported that Cd was more accumulated at apoplast in lettuce and at symplast in barley (*Hordeum vulgare*). It is important to emphasize that main site of Cd accumulation in tolerant plants is the root apoplast (WÓJCIK et al., 2005; BUCKLEY; BUCKLEY; HUANG, 2010; AKHTER et al., 2014). However, even Cd-tolerant plants show metabolic (e.g. nutrients and amino acids), physiological (e.g. stomatal conductance and assimilation of carbon dioxide - CO_2), antioxidants (e.g. enzymatic activity), anatomical (e.g. vascular vessels), morphological (e.g. foliar area) and productive changes due to Cd accumulation (LÓPEZ-BUCIO et al., 2000; MA; PETERSON, 2003; GRATÃO et al., 2005; PARMAR; KUMARI; SHARMA, 2013; ZEMANOVÁ et al., 2013; 2015).

2.3 Changes caused by cadmium in plants

2.3.1 Changes in nutrients, organic acids and amino acids metabolism

Often Cd decreases the uptake of divalent cations (e.g. Ca^{+2}, Mg^{+2}, Cu^{+2}, Fe^{+2}, Mn^{+2}, Ni^{+2} and Zn^{+2}) due to competition for the same uptake sites localized at plasma membrane, besides to change the uptake, transport and assimilation of nitrogen (N), phosphorus (P) and S (GALLEGO et al., 2012; RABÊLO; BORGO, 2016). Firstly, Cd changes the water relations of root cells and inhibits the activity of enzyme Fe^{+3} reductase, causing nutritional deficiency of Fe^{+2}, which seriously affects the photosynthesis (PARMAR; KUMARI; SHARMA, 2013; CHOPPALA et al., 2014). Cadmium decreases the uptake and transport of nitrate (NO_3^-) from roots to shoots due to inhibition caused in nitrate reductase activity, besides interfering in the process of ammonium (NH_4^+) assimilation (HERNÁNDEZ; CARPENA-RUIZ; GARATE, 1996; BALESTRASSE; GALLEGO; TOMARO, 2004). Skrebsky et al. (2008) observed that acid phosphatase activity in *Pfaffia glomerata* decreased in Cd presence, which resulted in lowest P uptake by this plant. ATP-sulfurylase activity also decreased in Cd presence, which interfered negatively in sulfate (SO_4^-) assimilation (ASTOLFI; ZUCHI; PASSERA, 2004). It is clear that nutritional changes caused by exposure to Cd affect the normal development of plants, mainly by decreasing some nutrients concentrations in the most of time, which may limit, for example, the synthesis of organic acids and amino acid precursors (LÓPEZ-BUCIO et al., 2000; SHARMA; DIETZ, 2006).

Organic acids (e.g. citrate and malate) play important functions in the metabolic route of solutes activation for osmotic adjustment and cationic balance, besides to participate of components involved in the control of nutritional deficiencies and tolerance to heavy metals (LÓPEZ-BUCIO et al., 2000; CLEMENS, 2001). For example, concentration of citrate and malate in the leaves and citrate, malate and succinate in the roots of corn (*Zea mays*) exposed

to Cd increased (DRESLER et al., 2014). Organic acids act as Cd-quelators, as well as PCs and amino acids, especially when Cd is transported to vacuole, which decreases the toxicity caused by this metal to plants (SANITÁ DI TOPPI; GABBRIELLI, 1999; LÓPEZ-BUCIO et al., 2000). It has been reported that organic acids concentrations in root apoplast is usually higher than in other tissues, especially when the plants are exposed to Cd, indicating that there is a close relationship between organic acid metabolism and Cd tolerance (LÓPEZ-BUCIO et al., 2000; WÓJCIK et al., 2005; BUCKLEY; BUCKLEY; HUANG, 2010; LUX et al., 2011; JAVED et al., 2013). Besides to reported functions, organic acids are also involved in amino acids synthesis, which also act on heavy metals detoxification (VELÍŠEK; KUBEC; CEJPEK, 2006; ZEMANOVÁ et al., 2015).

Amino acids are precursors of proteins and play important roles in Cd detoxification in plants (JEŽEK et al., 2011). Although Cd exposure normally changes the concentrations of nutrients (e.g. N, P and S) of plants, some amino acids are synthesized in greater amounts and are used as signaling molecules and osmolytes, which regulate ions transport and assist in Cd detoxification (XU et al., 2012; ZEMANOVÁ et al., 2013). In this sense, S-rich amino acids (e.g. cysteine and methionine) are fundamental for attenuation of Cd toxicity in plants, since they are components of peptides involved in Cd detoxification (MENDOZA-CÓZATL et al., 2005; RAUSCH; WACHTER, 2005; GILL; TUTEJA, 2011). Therefore, cysteine content usually increases in plants exposed to Cd (RAUSCH; WACHTER, 2005). Other amino acids, such as proline, histidine, serine, and threonine also act on heavy metals tolerance, which may result in high content of these amino acids (HALL, 2002; SHARMA; DIETZ, 2006). Serine often decreases lipid peroxidation caused by Cd at membrane and threonine can complex Cd^{+2} in cytosol (HALL, 2002; SHARMA; DIETZ, 2006; XU et al., 2012). Therefore, changes in concentrations of nutrients, organic acids and amino acids may assist to detect some tolerance mechanism developed by plants in response to stress caused by Cd (SUN et al., 2010).

2.3.2 Changes in photosynthetic and antioxidant systems and in sugar synthesis

In the leaves of plants, Cd can degrade chlorophyll and carotenoid molecules, modify fluorescence emission, and reduce the quantum yield of photosynthesis (SANITÁ DI TOPPI; GABBRIELLI, 1999; WANG et al., 2014). Fluorescence yield of chlorophyll can informs the level of energy excitation in pigment system that controls photosynthesis, allowing to estimate the inhibition or the damage in electron transfer process of photosystem II (PSII) (BOLHÀR-NORDENKAMPF et al., 1989). Cadmium presence usually decreases quantum efficiency of

PSII and electron transport rate in many plants, and in presence of high Cd concentrations can occur inactivation of PSII and electron transport chain that would originate ATP (adenosine triphosphate) (PARMAR; KUMARI; SHARMA, 2013; WANG et al., 2014; SEBASTIAN; PRASAD, 2014). Stomatal conductance (g_s) is also changed in Cd presence, which decreases CO_2 entry in the stomatal cavity and, consequently, intercellular CO_2 concentration (c_i) in the leaves (FARID et al., 2013). Depending on Cd concentration into cells, carbon assimilation by plants may also decrease due to inhibition of enzymes involved in CO_2 fixation (FARID et al., 2013; PARMAR; KUMARI; SHARMA, 2013). These changes in photosynthetic system of plants caused by Cd usually result in leaf rolling and chlorosis, which can be easily diagnosed (BENAVIDES; GALLEGO; TOMARO, 2005).

Alterations in electron transfer process in chloroplasts represents one of main causes of ROS generation in plants cultivated in environments contaminated by Cd (GRATÃO et al., 2005; MØLLER; JENSEN; HANSSON, 2007). During oxygen (O_2) reduction in H_2O, the transfer of 1, 2 or 3 electrons to O_2 can occur, resulting in generation of radicals superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-), respectively (GRATÃO et al., 2005; GILL; TUTEJA, 2010). Energy transfer at photosynthetic processes also allows the formation of other radicals, such as singlet oxygen ($^1O_2$), dioxygen ($^3O_2$) and peroxide (O_2^{2-}) (GILL; TUTEJA, 2010). Among these ROS, the radicals O_2^-, OH^- and $^1O_2$ are extremely reactive and can cause lipid peroxidation and proteins oxidation, among other damages (GILL; TUTEJA, 2010; FARMER; MUELLER, 2013), mainly when Cd concentration is high (AIBIBU et al., 2010). Hydrogen peroxide is moderately reactive compared to radicals mentioned above, but this ROS inactivate enzymes (eg, glutathione reductase - GR) by thiol groups (-SH) oxidation, which may decrease the efficiency of Cd detoxification mechanisms (GILL; TUTEJA, 2010, 2011). To scavenging ROS, plants synthesize non-enzymatic antioxidants (e.g. GSH) and enzymatic antioxidants (e.g. superoxide dismutase - SOD, catalase - CAT, guaiacol peroxidase - GPOX and ascorbate peroxidase - APX) (GRATÃO et al., 2005; GILL; TUTEJA, 2010).

Among non-enzymatic antioxidants (e.g. carotenoids, ascorbic acid and proline), GSH is the most active in cellular defense against ROS (GILL; TUTEJA, 2010; NOCTOR et al., 2011). Glutathione reduced can be oxidized (GSSG) by radicals O_2^- and OH^-, minimizing the excessive oxidation of sensitive cellular components, and to restore normal plant functions, GSSG is restored to GSH by GR (NOCTOR et al., 2011). Anjum et al. (2011) reported that mung bean (*Vigna radiata*) exposure to Cd increased H_2O_2 and GSSG concentrations and GR activity. Similar results were also described in other studies

(YANNARELLI et al., 2007; YU et al., 2013; HOREMANS et al., 2015). In relation to enzymatic antioxidants, the activities of SOD, CAT, GPOX and APX are fundamental to reduce oxidative stress in plants exposed to Cd (GRATÃO et al., 2005; RABÊLO; BORGO, 2016). Superoxide dismutase catalyzes the dismutation of O_2^- in H_2O_2, and it is considered the first defense line against ROS (GRATÃO et al., 2005). Catalase, GPOX and APX act at reduction of H_2O_2 generated by SOD, forming $H_2O + O_2$, H_2O + GSSG and two H_2O, respectively (GILL; TUTEJA, 2010). Thus, antioxidant activity is usually high when plants are exposed to Cd, which is essential to mitigate the toxic effects caused by this metal, as observed in ryegrass (*Lolium perenne*) (LUO et al., 2011), corn and rice (*Oryza sativa*) (GOEL et al., 2012).

Besides ROS causes lipid peroxidation of membrane and protein oxidation, ROS can also alter carbohydrate metabolism, affecting directly sugars (e.g. raffinose and galactinol) synthesis (GILL; TUTEJA, 2010; FARMER; MUELLER, 2013; KEUNEN et al., 2013). Verma and Dubey (2001) reported that reduced sugars concentrations increased and unreduced sugars concentrations decreased in roots and shoots of rice plants exposed to Cd, which can indicate that photosynthetic process of these plants was compromised. On the other hand, total sugars concentrations increased with exposure to Cd (VERMA; DUBEY, 2001), which is essential to help maintain the reduced state of cells and avoid oxidative stress (MISHRA et al., 2014). It is important to note that sugars act at chloroplasts protection and at photosynthesis stabilization under stress conditions (KEUNEN et al., 2013; MISHRA et al., 2014), which may reduce Cd damage and increase Cd-tolerance.

2.3.3 Morpho-anatomical and productive changes

Changes in nutrient, organic acid, amino acid and sugar metabolism and in antioxidant and photosynthetic systems caused by cultivation of plants in environments contaminated by Cd contribute directly to change the plant cell structures (LÓPEZ-BUCIO et al., 2000; MA; PETERSON, 2003; GRATÃO et al., 2009; LUX et al., 2011; KEUNEN et al., 2013). The most pronounced changes occur in roots, where Cd content is usually highest (LUX et al., 2011; AKHTER et al., 2014). Gratão et al. (2009) reported that root diameter of tomato plants (*Solanum lycopersicum*) exposed to Cd decreased and there was disintegration of epidermis in relation to treatment without Cd. In plants more tolerant to adversities, such as forage grasses, heavy metals uptake also causes anatomical changes (GOMES et al., 2011). These authors described that cell wall thickness of root xylem and root cortical parenchyma of *Brachiaria decumbens* exposed to heavy metals increased, while root metaxylem area decreased when

compared to treatment without heavy metals exposure. Changes caused by Cd exposure also may result in shorter roots and lower surface area, impairing water and nutrients uptake (MA; PETERSON, 2003; LUX et al., 2011; GOMES; SÁ E MELO MARQUES; SOARES, 2013).

The most pronounced changes occur in root vessels (e.g. xylem and phloem), although when Cd is transported to shoots there are also anatomical and/or ultrastructural changes (GRATÃO et al., 2009; LUX et al., 2011). Cadmium presence in cell medium changed the format and number of granules in the chloroplasts of leaf blades of cotton plants (*Gossypium hirsutum*) (DAUD et al., 2013; 2015). Changes observed in shoots (eg, chloroplast format, and thylakoid and stromal disorganization) of plants decrease leaf area, number of leaves and, depending of Cd localization, the number of tillers in grasses (VOLLENWEIDER et al., 2006; HERATH et al., 2014). Thus, morpho-anatomical changes due to Cd exposure may decrease plants biomass production (BENAVIDES; GALLEGO; TOMARO, 2005; FARID et al., 2013) and the phytoextraction efficiency (SHEORAN; SHEORAN; POONIA, 2016).

2.4 Technologies to mitigate cadmium impact on the environment and on the plants

2.4.1 Phytoextraction

Phytoextraction is a technology that consists of pollutants removal from contaminated environments by harvesting aboveground plants grown in these environments (BARCELÓ; POSCHENRIEDER, 2003; ALKORTA et al., 2004). Then, harvested plant material can be used for energy production, among other uses, which makes phytoextraction a technology of lower cost and lower environmental impact in relation to other technologies (SHEORAN; SHEORAN; POONIA, 2017). Plants used for this purpose, ideally, must present fast growth associated to high biomass production and high capacity to accumulate metals (BARCELÓ; POSCHENRIEDER, 2003). However, Cd hyperaccumulator plants (accumulation above 100 mg kg^{-1} DW) show low dry mass production (e.g. *Noccaea caerulenscens*), which reduces phytoextraction efficiency (SHEORAN; SHEORAN; POONIA, 2016). Therefore, the study of plants that show high biomass production, fast growth, adaptation to soil and climatic adversities and successive emissions of apical meristem after shoot harvesting, such as grasses (e.g. Massai grass), may increase Cd phytoextraction efficiency (RABÊLO; BORGO, 2016; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b; RABÊLO; JORDÃO; LAVRES, 2017). It should be noted that forage grasses also have an extensive root system, which is fundamental for phytoextraction, since the exploitation area of contaminated soils by heavy metals increases (GARBISU et al., 2002; MONTEIRO et al., 2011). In addition, forage grasses growth is highest under tropical conditions, where temperature and rainfall are usually

ideal for these plants (KRÄMER, 2005; MONTEIRO et al., 2011). In this sense, even if these plants shows mechanisms to reduce root-to-shoot Cd translocation, the high biomass production can compensate the lowest Cd concentrations in shoots, justifying the evaluation of forage grasses for phytoextraction (BARCELÓ; POSCHENRIEDER, 2003; ALKORTA et al., 2004; KRÄMER, 2005; RABÊLO; BORGO, 2016).

2.4.1.1 *Panicum maximum* Jacq. cv. Massai

Massai grass is a caespitose small plant (< 0.6 m) with narrow and erect leaves that present medium pilosity. The stems of this plant not show cerosity and the inflorescences are of intermediate type, between a panicle and a racemo (JANK et al., 2010). This forage grass is a C_4 plant, which gives it high efficiency in the use of solar radiation, nutrients and water (TOLEDO-SILVA et al., 2013). To exemplify, under suitable management and fertilization conditions, Massai grass can produce more than 19 t ha^{-1} $year^{-1}$ DW (JANK et al., 1994). This plant has been cultivated in Brazil for feed of ruminant animals due to high acceptability and bromatological composition (JANK et al., 2010), but this plant also presents characteristics that are desirable for environmental pollutants phytoremediation (CARMO et al., 2008). Although Massai grass presents potential for heavy metals phytoextraction, such as Cd, there are no studies available in the literature on their use for this purpose.

Carmo et al. (2008) reported that Massai grass presented tolerance to residual activity of picloram in Oxisol, and indicated this grass for evaluation in phytoremediation programs of this herbicide. Forage grasses usually are considered tolerant of environmental adversities, but *Panicum maximum* exposure to metals has resulted in damages, such as lipid peroxidation of membranes (GILABEL et al., 2014; NASCIMENTO et al., 2014; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b). Thus, some strategies can be used to minimize the damage caused by Cd to forage grasses, such as S supply (SAITO, 2000, 2004; MENDOZA-CÓZATL et al., 2005; RAUSCH; WACHTER, 2005; TAKAHASHI et al., 2011; GILABEL et al., 2014; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b). It is important to highlight that Massai grass produces more biomass when it is supplied with S, besides to be less exigent in soil fertility in relation to other cultivars of *Panicum maximum* (VOLPE et al., 2008; JANK et al., 2010).

2.4.2 Sulfur supply

Sulfur is essential for antioxidant metabolism of plants because it is a component of compounds such as GSH, which is able to increase plant tolerance to heavy metals (SEHT et al., 2012). Glutathione is a key component in Cd detoxification due to affinity of this element for -SH groups of cysteine contained in GSH (JOZEFCZAK et al., 2014; SOBRINO-PLATA et al., 2014). Glutathione is also a precursor of PCs, which are cysteine-rich peptides acting on sequestration of free Cd^{+2} ions in cytosol (COBBETT; GOLDSBROUGH, 2002). Several studies have shown that toxicity caused by Cd increases sulfate (SO_4^{2-}) uptake by plants in order to increase cysteine synthesis, which is a structural component of GSH (γ-Glu-Cys-Gly) and PCs [$(\gamma\text{-Glu-Cys})_n$-Gly (n = 2-11)] (COBBETT; GOLDSBROUGH, 2002; CAPALDI et al., 2015). Rabêlo, Azevedo and Monteiro (2017a) reported that GSH synthesis in Tanzania guinea grass (*Panicum maximum* cv. Tânzania) exposed to Cd increased with proper S supply, which reduced tiller mortality during the regrowth of this plant used for Cd phytoextraction. Bashir et al. (2015b) reported that proper S supply increased the synthesis of GSH and PCs in mustard (*Brassica juncea*) exposed to Cd. Besides to be a structural component of antioxidant metabolites (e.g. GSH) and Cd-chelators (e.g. PCs), S is also involved in metabolic pathways of antioxidant enzymes and in formation of photosynthetic system (IMSANDE, 1998; SUN et al., 2007; MARSCHNER, 2012; CAPALDI et al., 2015). Thus, S supply to plants established in environments contaminated with Cd is essential, mainly due to fact that majority of soils in the world shows low SO_4^{2-} concentration (SCHERER, 2001; GILL; TUTEJA, 2011).

2.5 Uptake, transport and assimilation of sulfur in plants

On average, S-total concentration in the earth's crust is between 0.6 and 1.0 g kg^{-1} soil, although in more weathered soils (e.g. Oxisols and Ultisols), where adsorption and immobilization of S are highest, this nutrient availability in soil solution is low (SCHERER, 2001; HAVLIN et al., 2005). Mass flow is the main mechanism of ion-root contact in S uptake (MARSCHNER, 2012). Sulfur is taken up by plants predominantly via SO_4^{2-}, but also can be taken up through the stomata of leaves in oxidized (SO_2) and reduced (H_2S) form or through of gases carbonyl sulfide (COS), carbon disulfide (CS_2), sulfide dimethyl (CH_3SCH_3) and methyl mercaptan (CH_3SH) (EPSTEIN; BLOOM, 2005). Sulfate uptake and transport in plants are mediated by SULTRs (sulfur transporters), that can be divided into ATP-dependent ABC type transporters, $Na^+(H^+)$/sulfate symporters, and/or sulfate (Cl^-, CO_3^{2-}, oxalate) antiporters (GIGOLASHVILI; KOPRIVA, 2014). Sulfate uptake into roots occurs

through H^+ co-transporters, whereas low affinity transporters are involved in SO_4^{2-} distribution, storage (vacuoles) and redistribution (HAWKESFORD, 2012). Then, SO_4^{2-} is transported through xylem to leaves to reduction and incorporation into carbon skeletons (GIGOLASHVILI; KOPRIVA, 2014).

Sulfur assimilation occurs by SO_4^{2-} activation by ATP, where ATP-sulfurylase enzyme catalyzes the substitution of two phosphate groups of ATP by sulfuryl group, which results in formation of adenosine phosphosulfate (APS) and pyrophosphate (HAWKESFORD, 2012). Activated SO_4^{2-} (APS) may serve as a substrate for SO_4^{2-} esters synthesis or SO_4^{2-} reduction. For esters synthesis, APS kinase catalyzes the formation of phosphoadenosine phosphosulfate (PAPS) in an ATP-dependent reaction. From PAPS, activated SO_4^{2-} can be transferred to an OH^- group forming an SO_4^{2-} ester (CAPALDI et al., 2015). The activated SO_4^{2-} of APS is reduced to sulfite (SO_3^{2-}) by APS reductase, requiring two electrons from GSH. Then, six electrons from ferredoxin are required to reduce SO_3^{2-} to sulfide (S^{2-}), catalyzed by sulfite reductase (HAWKESFORD, 2012). Resulting S^{2-} reacts with O-acetylserine (OAS) to form cysteine and acetate, in reactions catalyzed by serine O-acetyltransferase and OAS thiolyase (GIGOLASHVILI; KOPRIVA, 2014). Cysteine is the first stable product of the assimilatory SO_4^{2-} reduction and it acts as precursor to synthesis of other compounds containing reduced S (CAPALDI et al., 2015). Adenosine phosphosulfate sulfation is an alternative pathway where APS kinase catalyzes the reaction of APS with ATP to form PAPS, and sulfotransferases transfer SO_4^{2-} from PAPS to various compounds including flavonoids, glucosinates, peptides and polysaccharides (LEUSTEK; SAITO, 1999). Methionine (another S-containing amino acid) is synthesized in plastids from cysteine. After synthesis of cysteine and methionine, S can be incorporated into proteins and other compounds, such as S-adenosylmethionine and acetyl-CoA (CAPALDI et al., 2015).

2.6 Cadmium-sulfur relation and synthesis of GSH, PCs and other thiol compounds

Plants exposed to Cd usually taken up more SO_4^{2-} to increase the synthesis of cysteine, GSH, PCs and other thiols (GILL; TUTEJA, 2011; CAPALDI et al., 2015). Glutathione is synthesized from glutamate, cysteine and glycine in two ATP-dependent reactions, where enzyme γ-glutamylcysteine synthetase catalyzes the formation of a peptide bond between the carboxyl group of glutamate and the amino group of cysteine, to yield γ-glutamylcysteine (γ-EC) (YADAV, 2010). Then, glutathione synthetase ligates a glycine residue with γ-EC, to form GSH (YADAV, 2010; NOCTOR et al., 2011). Glutathione is the non-enzymatic antioxidant most active in plants exposed to Cd, so the synthesis of this metabolite usually

increases with Cd exposure, especially when there is proper S supply to plants (N OCTOR et al., 2011; BASHIR et al., 2015b; RABÊLO; AZEVEDO, MONTEIRO, 2017a). Glutathione also show correlation with expression of genes linked to cell defense (e.g. *GSH1*, *PCS1* and *GR1*), since many signaling pathways are changed by GSH synthesis, before and during some plant stress condition (SEHT et al., 2012; JOZEFCZAK et al., 2014; CAPALDI et al., 2015). In addition, GSH is precursor of PCs, which are cysteine-rich peptides acting on free Cd^{+2} ions sequestration in cytosol (COBBETT; GOLDSBROUGH, 2002).

Phytochelatins are synthesized from GSH in a reaction catalyzed by PC synthase, and based on number of γ-Glu-Cys units, PCs can be classified into PC_2, PC_3, PC_4 and so on until PC_{11} (COBBETT; GOLDSBROUGH, 2002; PAL; RAI, 2010). However, the mainly PCs synthesized by plants exposed to Cd are PC_2, PC_3 and PC_4 (VÁZQUEZ; GOLDSBROUGH; CARPENA, 2006). Akhter, McGarvey and Macfie (2012) reported that PC_2 was the most synthesized PCs in the roots of barley exposed to Cd, followed by PC_4 and PC_3, respectively. Phytochelatins synthesis is essential to increase the tolerance of plants to Cd and the potential of extraction of this metal in contaminated areas, and proper S supply to plants can contribute to improve this process (SEHT et al., 2012; BASHIR et al., 2015b). Glutathione and PCs are the major thiol compounds produced by plants exposed to Cd, but the synthesis of other compounds (e.g. γ-EC) is also important to mitigate the damage caused by Cd (HARADA et al., 2002; SUÁREZ et al., 2010), mainly because thiol compounds usually act together with antioxidant enzymes (MOHAMED et al., 2012; DIXIT et al., 2015).

2.7 Cadmium-sulfur relation and the activity of antioxidant system

Cysteine participates in the formation of sulfhydryl (-SH) and disulfide bonds (-SS), which are the active centers of some antioxidants, such as GSH, which is the non-enzymatic antioxidant most active in plants (SAITO, 2004; NOCTOR et al., 2011). In this sense, it is essential that plants grown in environments contaminated by Cd receive proper S supply in order to mitigate the oxidative damages caused by Cd (GILL; TUTEJA, 2011; CAPALDI et al., 2015). Wu et al. (2005) reported that GSH concentration and glutathione-S-transferase (GST) activity in the leaves and roots of rice exposed to Cd increased when there was proper S supply, which reduced the oxidative stress caused by Cd to plant. Glutathione-S-transferase catalyzes the conjugation of GSH with products that cause oxidative stress to plant cells, such as OH^- radicals and products of oxidative degradation of DNA (DIXON et al., 2002). Changes in S metabolism caused by Cd exposure affect directly GST activity and indirectly the activity of other antioxidant enzymes, since ROS scavenging occurs in an integrated way

(NOCITO et al., 2007; NIKOLIĆ et al., 2008; HERNÁNDEZ et al., 2015; TARHAN; KAVAKCIOGLU, 2016). To exemplify, Bashir et al. (2015b) reported that proper S supply to mustard exposed to Cd increased GSH concentration and SOD, CAT, APX and GR activity, which minimized the oxidative damage caused by Cd. It is important to mention that ascorbate-glutathione cycle is the most important cycle in ROS scavenging and the proper S supply can optimize this cycle, mitigating the damage caused by Cd mainly to photosynthetic system (CUYPERS et al., 2010; GILL; TUTEJA, 2010, 2011; CAPALDI et al., 2015).

2.8 Cadmium-sulfur relation and the activity of photosynthetic system

Cadmium may degrade chlorophyll, modify the electron transfer process between the photosystem I - PSI and PSII, reduce the stomatal opening and activity of enzymes involved in carbon fixation, among other damages, decreasing the photosynthetic rate (*A*) and the biomass production of plants (PARMAR; KUMARI; SHARMA, 2013; BASHIR et al., 2015a). Li et al. (2015) reported that g_s and *A* values of *Elsholtzia argyi* plants exposed to Cd decreased in relation to control treatment. Although Cd changes the photosynthetic process of most plants, some studies have suggested that proper S supply can mitigate the damages caused by Cd in the photosynthetic apparatus (CHEN; HUERTA, 1997; MASOOD; IQBAL; KHAN, 2012; BASHIR et al., 2015b). Sulfur act indirectly in chlorophyll synthesis, since the membranes of chloroplasts are composed of galactolipids and sulfolipids, and directly in electron transport system and photosynthetic system formation, since it is a component of iron-sulfur proteins and thioredoxin system (IMSANDE, 1998). Bashir et al. (2015b) reported that chlorophyll *a* and *b* syntheses in mustard exposed to Cd increased when there was proper S supply. Chen and Huerta (1997) reported that barley plants exposed to Cd and grown with proper S supply showed higher *A* and g_s than plants cultivated with low S supply. These results demonstrate that proper S supply is essential to increase chlorophyll synthesis and to mitigate the damage caused by Cd, which is extremely important for plants used in Cd phytoextraction (RABÊLO, 2014). In addition, plants of species *Panicum maximum* are responsive (eg, highest biomass production) to S (SHMIDT; DE BONA; MONTEIRO, 2013; SCHMIDT; MONTEIRO, 2014), which allows greater heavy metal phytoextraction (GILABEL et al., 2014; RABÊLO; AZEVEDO, MONTEIRO, 2017a, 2017b).

References

AIBIBU, N.; LIU, Y.; ZENG, G.; WANG, X.; CHEN, B.; SONG, H.; XU, L. Cadmium accumulation in *Vetiveria zizanioides* and its effects on growth, physiological and biochemical characters. **Bioresource Technology**, Oxford, v. 101, p. 6297-6303, 2010.

AKHTER, M.F.; McGARVEY, B.; MACFIE, S.M. Reduced translocation of cadmium from roots is associated with increased production of phytochelatins and their precursors. **Journal of Plant Physiology**, Jena, v. 169, p. 1821-1829, 2012.

AKHTER, M.F.; OMELON, C.R.; GORDON, R.A.; MOSER, D.; MACFIE, S.M. Localization and chemical speciation of cadmium in the roots of barley and lettuce. **Environmental and Experimental Botany**, Oxford, v. 100, p. 10-19, 2014.

ALKORTA, I.; HERNÁNDEZ-ALLICA, J.; BECERRIL, J.M.; AMEZAGA, I.; ALBIZU, I.; GARBISU, C. Recent findings on the phytoremediation of soils contaminated with environmentally toxic heavy metals and metalloids such as zinc, cadmium, lead, and arsenic. **Reviews in Environmental Science and Bio/Technology**, Berlin, v. 3, p. 71-90, 2004.

ANJUM, N.A.; UMAR, S.; IQBAL, M.; KHAN, N.A. Cadmium causes oxidative stress in mung bean by affecting the antioxidant enzyme system and ascorbato-glutathione cycle metabolism. **Russian Journal of Plant Physiology**, New York, v. 58, p. 92-99, 2011.

ASTOLFI, S.; ZUCHI, S.; PASSERA, C. Role of sulphur availability on cadmium-induced changes of nitrogen and sulphur metabolism in maize (*Zea mays* L.) leaves. **Journal of Plant Physiology**, Jena, v. 161, p. 795-802, 2004.

BALESTRASSE, K.B.; GALLEGO, S.M.; TOMARO, M.L. Cadmium-induced senescence in nodules of soybean (*Glycine max* L.) plants. **Plant and Soil**, Dordrecht, v. 262, p. 373-381, 2004.

BARCELÓ, J.; POSCHENRIEDER, C. Phytoremediation: principles and perspectives. **Contributions to Science**, Barcelona, v. 2, p. 333-344, 2003.

BASHIR, H.; QURESHI, M.I.; IBRAHIM, M.M.; IQBAL, M. Chloroplast and photosystems: Impact of cadmium and iron deficiency. **Photosynthetica**, Dordrecht, v. 53, p. 321-335, 2015a.

BASHIR, H.; IBRAHIM, M.M.; BAGHERI, R.; AHMAD, J.; ARIF, I.A.; BAIG, M.A.; QURESHI, M.I. Influence of sulfur and cadmium on antioxidants, phytochelatins and growth in Indian mustard. **AoB Plants**, Oxford, v. 12, 2015b. doi: 10.1093/aobpla/plv001.

BENAVIDES, M.P.; GALLEGO, S.M.; TOMARO, M.L. Cadmium toxicity in plants. **Brazilian Journal of Plant Physiology**, Campos dos Goytacazes, v. 17, p. 21-34, 2005.

BHATTACHARYYA, M.H. Cadmium osteotoxicity in experimental animals: Mechanisms and relationship to human exposures. **Toxicology and Applied Pharmacology**, New York, v. 238, p. 258-265, 2009.

BOLHÀR-NORDENKAMPF, H.R.; LONG, S.P.; BAKER, N.R.; ÖQUIST, G.; SCHREIBER, U.; LECHNER, E.G. Chlorophyll fluorescence as probe of the photosynthetic competence of leaves in the field: a review of current instrument. **Functional Ecology**, Hoboken, v. 3, p. 497-514, 1989.

BUCKEY, W.T.; BUCKEY, K.E.; HUANG, J. Root cadmium desorption methods and their evaluation with compartmental modeling. **New Phytologist**, London, v. 188, p. 280-290, 2010.

CAPALDI, F.R.; GRATÃO, P.L.; REIS, A.R.; LIMA, L.W.; AZEVEDO, R.A. Sulfur metabolism and stress defense responses in plants. **Tropical Plant Biology**, New York, v. 8, p. 60-73, 2015.

CARMO, M.L.; PROCOPIO, S.O.; PIRES, F.R.; CARGNELUTTI FILHO, A.; BARROSO, A.L.L.; SILVA, G.P.; CARMO, E.L.; BRAZ, G.B.P.; SILVA, W.F.P.; BRAZ, A.J.B.P.; PACHECO, L.P. Plant selection for phytoremediation of soils contaminated with picloram. **Planta Daninha**, Viçosa, v. 26, p. 301-313, 2008.

CHEN, Y.; HUERTA, A.J. Effects of sulfur nutrition on photosynthesis in cadmium-treated barley seedlings. **Journal of Plant Nutrition**, Philadelphia, v. 20, p. 845-856, 1997.

CHOPPALA, G.; SAIFULLAH; BOLAN, N.; BIDI, S.; IQBAL, M.; RENGEL, Z.; KUNHIKRISHNAN, A.; ASHWATH, N.; OK, Y.S. Cellular mechanisms in higher plants governing tolerance to cadmium toxicity. **Critical Reviews in Plant Sciences**, Boca Raton, v. 33, p. 374-391, 2014.

CLEMENS, S. Molecular mechanisms of plant metal tolerance and homeostasis. **Planta**, Berlin, v. 212, p. 475-486, 2001.

CLEMENS, S. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. **Biochimie**, Paris, v. 88, p. 1707-1719, 2006.

CLEMENS, S.; AARTS, M.G.M.; THOMINE, S.; VERBRUGGEN, N. Plant science: the key to preventing slow cadmium poisoning. **Trends in Plant Science**, London, v. 18, p. 92-99, 2013.

COBBETT, C.; GOLDSBROUGH, P. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. **Annual Review of Plant Biology**, Palo Alto, v. 53, p. 159-182, 2002.

CUYPERS, A.; PLUSQUIN, M.; REMANS, T.; JOZEFCZAK, M.; KEUNEN, E.; GIELEN, H.; OPDENAKKER, K.; NAIR, A.R.; MUNTERS, E.; ARTOIS, T.J.; NAWROT, T.; VANGRONSVELD, J.; SMEETS, K. Cadmium stress: an oxidative challenge. **Biometals**, Dordrecht, v. 23, p. 927-940, 2010.

DAUD, M.K.; ALI, S.; VARIATH, M.T.; ZHU, S.J. Differential physiological, ultramorphological and metabolic responses of cotton cultivars under cadmium stress. **Chemosphere**, Oxford, v. 93, p. 2593-2602, 2013.

DAUD, M.K.; QUILING, H.; LEI, M.; ALI, B.; ZHU, S.J. Ultrastructural, metabolic and proteomic changes in leaves of upland cotton in response to cadmium stress. **Chemosphere**, Oxford, v. 120, p. 309-320, 2015.

DIXIT, G.; SINGH, A.P.; KUMAR, M.; SINGH, P.K.; KUMAR, S.; DWIVEDI, S.; TRIVEDI, P.K.; PANDEY, V.; NORTON, G.J.; DHANKHER, O.P.; TRIPATHI, R.D. Sulfur mediated reduction of arsenic toxicity involves efficient thiol metabolism and the antioxidant defense system in rice. **Journal of Hazardous Materials**, Amsterdam, v. 298, p. 241-251, 2015.

DIXON, D.P.; DAVIS, B.G.; EDWARDS, R. Functional divergence in the glutathione transferase superfamily in plants. **Journal Biological Chemistry**, Baltimore, v. 277, p. 30859-30869, 2002.

DRESLER, S.; HANAKA, A.; BEDNAREK, W.; MAKSYMIEC, W. Accumulation of low-molecular-weight organic acids in roots and leaf segments of *Zea mays* plants treated with cadmium and copper. **Acta Physiologia Plantarum**, Heidelberg, v. 36, p. 1565-1575, 2014.

EPSTEIN, E.; BLOOM, A.J. **Mineral nutrition of plants:** principles and perspectives. 2. ed. Massachussets: Sinauer, 2005. 380 p.

FARID, M.; SHAKOOR, M.B.; EHSAN, S.; ALI, S.; ZUBAIR, M.; HANIF, M.A. Morphological, physiological and biochemical responses of different plant species to Cd stress. **International Journal of Chemical and Biochemical Sciences**, Dschang, v. 3, p. 53-60, 2013.

FARMER, E.E.; MUELLER, M.J. ROS-mediated lipid peroxidation and RES-activated signaling. **Annual Review of Plant Biology**, Palo Alto, v. 64, p. 429-450, 2013.

GALLEGO, S.M.; PENA, L.B.; BARCIA, R.A.; AZPILICUETA, C.E.; IANNONE, M.F.; ROSALES, E.P.; ZAWOZNIK, M.S.; GROPPA, M.D.; BENAVIDES, M.P. Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. **Environmental and Experimental Botany**, Oxford, v. 83, p. 33-46, 2012.

GARBISU, C.; HERNÁNDEZ-ALLICA, J.; BARRUTIA, O.; ALKORTA, I.; BECERRIL, J.M. Phytoremediation: A technology using green plants to remove contaminants from polluted areas. **Reviews on Environmental Health**, Tel Aviv, v. 17, p. 75-90, 2002.

GHOSH, D.; SAHAB, R.; GHOSH, A.; NANDI, R.; SAHA, B. A review on toxic cadmium biosorption from contaminated wastewater. **Desalination and Water Treatment**, Philadelphia, v. 53, p. 413-420, 2015.

GIGOLASHVILI, T.; KOPRIVA, S. Transporters in plant sulfur metabolism. **Frontiers in Plant Science**, Lausanne, v. 5, p. 442-458, 2014.

GILABEL, A.P.; NOGUEIROL, R.C.; GARBO, A.I.; MONTEIRO, F.A. The role of sulfur in increasing Guinea grass tolerance of copper phytotoxicity. **Water, Air and Soil Pollution**, Dordrecht, v. 225, p. 1806-1815, 2014.

GILL, S.S.; TUTEJA, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. **Plant Physiology and Biochemistry**, Paris, v. 48, p. 909-930, 2010.

GILL, S.S.; TUTEJA, N. Cadmium stress tolerance in crop plants probing the role of sulfur. **Plant Signaling and Behavior**, Austin, v. 6, p. 215-222, 2011.

GIORDANI, C.; CECCHI, S.; ZANCHI, C. Phytoremediation of soil polluted by nickel using agricultural crops. **Environmental Management**, New York, v. 36, p. 675-681, 2005.

GOEL, S.; MALIK, J.A.; AWASTHI, R.; SANDHIR, R.; NAYYAR, H. Growth and metabolic responses of maize (C_4 species) and rice (C_3 species) genotypes to cadmium toxicity. **Cereal Research Communications**, Budapest, v. 40, p. 225-234, 2012.

GOMES, M.P.; SÁ E MELO MARQUES, T.C.L.L.; NOGUEIRA, M.O.G.; CASTRO, E.M.; SOARES, A.M. Ecophysiological and anatomical changes due to uptake and accumulation of heavy metal in *Brachiaria decumbens*. **Scientia Agricola**, Piracicaba, v. 68, p. 566-573, 2011.

GOMES, M.P.; SÁ E MELO MARQUES, T.C.L.L.; SOARES, A.M. Cadmium effects on mineral nutrition of the Cd-hyperaccumulator *Pfaffia glomerata*. **Biologia**, Warsaw, v. 68, p. 223-230, 2013.

GRATÃO, P.L.; POLLE, A.; LEA, P.J.; AZEVEDO, R.A. Making the life of heavy metal-stressed plants a little easier. **Functional Plant Biology**, Melbourne, v. 32, p. 481-494, 2005.

GRATÃO, P.L.; MONTEIRO, C.C.; ROSSI, M.L.; MARTINELLI, A.P.; PERES, L.E.P.; MEDICI, L.O.; LEA, P.J.; AZEVEDO, R.A. Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium. **Environmental and Experimental Botany**, Oxford, v. 67, p. 387-394, 2009.

HALL, J.L. Cellular mechanisms for heavy metal detoxification and tolerance. **Journal of Experimental Botany**, Oxford, v. 53, p. 1-11, 2002.

HAQ, F.; MAHONEY, M.; KOROPATNICK, J. Signaling events for metallothionein induction. **Mutation Research**, Amsterdam, v. 533, p. 211-226, 2003.

HARADA, E.; YAMAGUCHI, Y.; KOIZUMI, N.; SANO, H. Cadmium stress induces production of thiol compounds and transcripts for enzymes involved in sulfur assimilation pathways in *Arabidopsis*. **Journal of Plant Physiology**, Jena, v. 159, p. 445-448, 2002.

HAVLIN, J.L.; BEATON, J.D.; TISDALE, S.L.; NELSON, W.L. **Soil fertility and fertilizers:** An introduction to nutrient management. 7. ed. New Jersey: Pearson Prentice Hall, 2005. 528 p.

HAWKESFORD, M.J. Sulfate uptake and assimilation - whole plant regulation. **Proceedings of the International Plant Sulfur Workshop**, Freiburg, v. 1, p. 11-24, 2012.

HERATH, H.M.D.A.K.; BANDARA, D.C.; WEERASINGHE, P.A.; IQBAL, M.C.M.; WIJAYAWARDHANA, H.C.D. Effect of cadmium on growth parameters and plant accumulation in different rice (*Oryza sativa* L.) varieties in Sri Lanka. **Tropical Agricultural Research**, Rajarata, v. 25, p. 432-442, 2014.

HERNÁNDEZ, L.E.; CARPENA-RUIZ, R.; GARATE, A. Alterations in the mineral nutrition of pea seedlings exposed to cadmium. **Journal of Plant Nutrition**, Philadelphia, v. 19, p. 1581-1598, 1996.

HERNÁNDEZ, L.E.; SOBRINO-PLATA, J.; MONTERO-PALMERO, B.; CARRASCO-GIL; FLORES-CÁCERES, M.L.; ORTEGA-VILLASANTE, C.; ESCOBAR, C. Contribution of glutathione to the controle of cellular redox homeostasis under toxic metal and metalloid stress. **Journal of Experimental Botany**, Oxford, v. 66, p. 2901-2911, 2015.

HOREMANS, N.; VAN HEES, M.; VAN HOECK, A.; SAENEN, E.; DE MEUTTER, T.; NAUTS, R.; BLUST, R.; VANDENHOVE, H. Uranium and cadmium provoke different oxidative stress responses in *Lemna minor* L. **Plant Biology**, Hoboken, v. 17, p. 91-100, 2015.

HUFF, J.; LUNN, R.M.; WAALKES, M.P.; TOMATIS, L.; INFANTE, P.F. Cadmium-induced cancers in animals and in humans. **International Journal of Occupational Environmental Health**, Burlington, v. 13, p. 202-212, 2007.

IMSANDE, J. Iron, sulfur, and chlorophyll deficiencies: A need for a integrative approach in plant physiology. **Physiologia Plantarum**, Lund, v. 103, p. 139-144, 1998.

IRFAN, M.; HAYAT, S.; AHMAD, A.; ALYEMENI, M.N. Soil cadmium enrichment: Allocation and plant physiological manifestations. **Saudi Journal of Biological Sciences**, Amsterdam, v. 20, p. 1-10, 2013.

JANK, L.; SAVIDAN, Y.H.; SOUZA, M.T.; COSTA, J.C.G. Evaluation of the *Panicum maximum* germiplasm introduced from Africa. I. - Forage production. **Brazilian Journal of Animal Science**, Viçosa, v. 23, p. 433-440, 1994.

JANK, L.; MARTUSCELLO, J.A.; EUCLIDES, V.P.B.; VALLE, C.B.; RESENDE, R.M.S. *Panicum maximum*. In: FONSECA, D.M.; MARTUSCELLO, J.A. (Ed.). **Plantas forrageiras**. Viçosa: Universidade Federal de Viçosa, 2010. chap. 5, p. 166-196.

JAVED, M.T.; STOLTZ, E.; LINDBERG, S.; GREGER, M. Changes in pH and organic acids in mucilage of *Eriophorum angustifolium* roots after exposure to elevated concentrations of toxic elements. **Environmental Science and Pollution Research**, Heidelberg, v. 20, p. 1876-1880, 2013.

JEŽEK, P.; HLUŠEK, J.; LOŠÁK, T.; JŮZL, M.; ELZNER, P.; KRÁČMAR, S.; BUŇKA, F.; MARTENSSON, A.M. Effect of foliar application of selenium on the content of selected amino acids in potato tubers (*Solanum tuberosum* L.). **Plant, Soil and Environment**, Prague, v. 57, p. 315-320, 2011.

JOZEFCZAK, M.; KEUNEN, E.; SCHAT, H.; BLIEK, M.; HERNÁNDEZ, L.C.; CARLEER, R.; REMANS, T.; BOHLER, S.; VANGRONSVELD, J.; CUYPERS, A. Differential response of *Arabidopsis* leaves and roots to cadmium: Glutathione-related chelating capacity *vs* antioxidant capacity. **Plant Physiology and Biochemistry**, Paris, v. 83, p. 1-9, 2014.

KABATA-PENDIAS, A. **Trace elements in soils and plants**. 4. ed. Boca Raton: CRC Press, 2011. 505 p.

KEUNEN, E.; VANGRONSVELD, J.; van den ENDE, W.; CUYPERS, A. Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. **Plant, Cell and Environment**, Oxford, v. 36, p. 1242-1255, 2013.

KHAN, M.A.; KHAN, S.; KHAN, A.; ALAM, M. Soil contamination with cadmium, consequences and remediation using organic amendments. **Science of the Total Environment**, Amsterdam, v. 601-602, p. 1591-1605, 2017.

KRÄMER, U. Phytoremediation: novel approaches to cleaning up polluted soils. **Current Opinion in Biotechnology**, London, v. 16, p. 133-141, 2005.

KUTROWSKA, A.; SZELAG, M. Low-molecular weight organic acids and peptides involved in the long-distance transport of trace metals. **Acta Physiologia Plantarum**, Heidelberg, v. 36, p. 1957-1968, 2014.

LEUSTEK, T.; SAITO, K. Sulfate transport and assimilation in plants. **Plant Physiology**, Rockville, v. 120, p. 637-643, 1999.

LI, S.; YANG, W.; YANG, T.; CHEN, Y.; NI, W. Effects of cadmium stress on leaf chlorophyll fluorescence and photosynthesis of *Elsholtzia argyi* - A cadmium accumulating plant. **International Journal of Phytoremediation**, Philadelphia, v. 17, p. 85-92, 2015.

LOPES JÚNIOR, C.A.; MAZZAFERA, P.; ARRUDA, M.A.Z. A comparative ionomic approach focusing on cadmium effects in sunflowers (*Helianthus annuus* L.). **Environmental and Experimental Botany**, Oxford, v. 107, p. 180-186, 2014.

LÓPEZ-BUCIO, J.; NIETO-JACOBO, M.F.; RAMÍREZ-RODRÍGUEZ, V.; HERRERA-ESTRELLA, L. Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. **Plant Science**, Georgetown, v. 160, p. 1-13, 2000.

LUO, H.; LI, H.; ZHANG, X.; FU, J. Antioxidant responses and gene expression in perennial ryegrass (*Lolium perenne* L.) under cadmium stress. **Ecotoxicology**, Dordrecht, v. 20, p. 770-778, 2011.

LUX, A.; MARTINKA, M.; VACULÍK, M.; WHITE, P.J. Root responses to cadmium in rhizosphere: a review. **Journal of Experimental Botany**, Oxford, v. 62, p. 21-37, 2011.

MA, F.; PETERSON, C.A. Current insights into the development, structure, and chemistry of the endodermis and exodermis of roots. **Canadian Journal of Botany**, Ottawa, v. 81, p. 405-421, 2003.

MARSCHNER, P. **Mineral nutrition of higher plants**. 3. ed. New York: Elsevier, 2012. 651 p.

MASOOD, A.; IQBAL, N.; KHAN, N.A. Role of ethylene in alleviation of cadmium-induced photosynthetic capacity inhibition by sulphur in mustard. **Plant, Cell and Environment**, Oxford, v. 35, p. 524-533, 2012.

McLAUGHLIN, M.J.; TILLER, K.G.; NAIDU, R.; STEVENS, D.P. Review: the behaviour and impact of contaminants in fertilizers. **Australian Journal of Soil Research**, Melbourne, v. 34, p. 1-54, 1996.

MENDOZA-CÓZATL, D.; LOZA-TAVERA, H.; HERNÁNDEZ-NAVARRO, A.; MORENO-SÁNCHEZ, R. Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, protists and plants. **FEMS Microbiology Reviews**, Amsterdam, v. 29, p. 653-671, 2005.

MENDOZA-CÓZATL, D.G.; JOBE, T.O.; HAUSER, F.; SCHROEDER, J.I. Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. **Current Opinion in Plant Biology**, London, v. 14, p. 554-562, 2011.

MISHRA, B.; SANGWAN, R.S.; MISHRA, S.; JADAUN, J.S.; SABIR, F.; SANGWAN, N.S. Effect of cadmium stress on inductive enzymatic and nonenzymatic responses of ROS and sugar metabolism in multiple shoot cultures of Ashwagandha (*Withania somnifera* Dunal). **Protoplasma**, Vienna, v. 251, p. 1031-1045.

MOHAMED, A.A.; CASTAGNA, A.; RANIERI, A.; SANITÁ DI TOPPI, L. Cadmium tolerance in *Brassica juncea* roots and shoots is affected by antioxidant status and phytochelatin biosynthesis. **Plant Physiology and Biochemistry**, Paris, v. 57, p. 15-22, 2012.

MØLLER, I.M.; JENSEN, P.E.; HANSSON, A. Oxidative modifications to cellular components in plants. **Annual Review of Plant Biology**, Palo Alto, v. 58, p. 459-481, 2007.

MONTEIRO, F.A.; NOGUEIROL, R.C.; MELO, L.C.A.; ARTUR, A.G.; ROCHA, F. Effect of barium on growth and macronutrient nutrition in Tanzania guineagrass grown in nutrient solution. **Communications in Soil Science and Plant Analysis**, New York, v. 42, p. 1510-1521, 2011.

NAGAJYOTI, P.C.; LEE, K.D.; SREEKANTH, T.V.M. Heavy metals, occurrence and toxicity for plants: a review. **Environmental Chemistry Letters**, Heidelberg, v. 8, p. 199-216, 2010.

NASCIMENTO, S.S.; SILVA, E.B.; ALLEONI, L.R.F.; GRAZZIOTTI, P.H.; FONSECA, F.G.; NARDIS, B.O. Availability and accumulation of lead for forage grasses in contaminated soil. **Journal of Soil Science and Plant Nutrition**, Temuco, v. 14, p. 783-802, 2014.

NAZAR, R.; IQBAL, N.; MASOOD, A.; IQBAL, M.; KHAN, R.; SYEED, S.; KHAN, N.A. Cadmium toxicity in plants and role of mineral nutrients in its alleviation. **American Journal of Plant Sciences**, Georgetown, v. 3, p. 1476-1489, 2013.

NIKOLIĆ, N.; KOJIĆ, D.; PILIPOVIĆ, A.; PAJEVIĆ, S.; KRSTIĆ, B.; BORIŠEV, M.; ORLOVIĆ, S. Responses of hybrid poplar to cadmium stress: photosynthetic characteristics, cadmium and proline accumulation, and antioxidant enzyme activity. **Acta Biologica Cracoviensia Series Botanica**, Krakow, v. 50, p. 95-103, 2008.

NOCITO, F.F.; LANCILLI, C.; GIACOMINI, B.; SACCHI, G.A. Sulfur metabolism and cadmium stress in higher plants. **Plant Stress**, Tel Aviv, v. 1, p. 142-156, 2007.

NOCTOR, G.; QUEVAL, G.; MHAMDI, A.; CHAOUCH, S.; FOYER, C.H. Glutathione. **The Arabidopsis Book**, Riverside, v. 9, p. 1-32, 2011.

PAL, R.; RAI, J.P.N. Phytochelatins: peptides involved in heavy metal detoxification. **Applied Biochemistry and Biotechnology**, Totowa, v. 160, p. 945-963, 2010.

PARMAR, P.; KUMARI, N.; SHARMA, V. Structural and functional alterations in photosynthetic apparatus of plants under cadmium stress. **Botanical Studies**, New York, v. 54, p. 45-51, 2013.

RABÊLO, F.H.S. **Enxofre na atenuação dos efeitos tóxicos do cádmio no capim-tanzânia**. 2014. 103 p. Dissertação (Mestrado em Solos e Nutrição de Plantas) - Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, 2014.

RABÊLO, F.H.S.; BORGO, L. Changes caused by heavy metals in micronutrient content and antioxidant system of forage grasses used for phytoremediation: an overview. **Ciência Rural**, Santa Maria, v. 46, p. 1368-1375, 2016.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. Proper supply of S increases GSH synthesis in the establishment and reduces tiller mortality during the regrowth of Tanzania guinea grass used for Cd phytoextraction. **Journal of Soils and Sediments**, Heidelberg, v. 17, p. 1427-1436, 2017a.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. The proper supply of S increases amino acid synthesis and antioxidant enzyme activity in Tanzania guinea grass used for Cd phytoextraction. **Water, Air and Soil Pollution**, Dordrecht, v. 228, 2017b. doi: 10.1007/s11270-017-3563-6.

RABÊLO, F.H.S.; JORDÃO, L.T.; LAVRES, J. A glimpse into the symplastic and apoplastic Cd uptake by Massai grass modulated by sulfur nutrition: Plants well-nourished with S as a strategy for phytoextraction. **Plant Physiology and Biochemistry**, Paris, v. 121, p. 48-57, 2017.

RANI, A.; KUMAR, A.; LAL, A.; PANT, M. Cellular mechanisms of cadmium induced toxicity: a review. **International Journal of Environmental Health Research**, Oxon, v. 24, p. 378-399, 2014.

RAUSCH, T.; WACHTER, A. Sulfur metabolism: a versatile platform for launching defense operations. **Trends in Plant Science**, London, v. 10, p. 503-509, 2005.

SAITO, K. Regulation of sulfate transport and synthesis of sulfur-containing amino acids. **Current Opinion in Plant Biology**, London, v. 3, p. 188-195, 2000.

SAITO, K. Sulfur assimilatory metabolism. The long and smelling road. **Plant Physiology**, Jena, v. 136, p. 2443-2450, 2004.

SANITÀ DI TOPPI, L.; GABBRIELLI, R. Response to cadmium in higher plants. **Environmental and Experimental Botany**, Oxford, v. 41, p. 105-130, 1999.

SCHERER, H.W. Sulfur in crop production - invited paper. **European Journal of Agronomy**, Montrouge Cedex, v. 14, p. 81-111, 2001.

SCHMIDT, F.; DE BONA, F.D.; MONTEIRO, F.A. Sulfur limitation increases nitrate and amino acid pools in tropical forages. **Crop and Pasture Science**, Melbourne, v. 64, p. 51-60, 2013.

SCHMIDT, F.; MONTEIRO, F.A. Sulphur supply affects morphology, growth and nutritional status of Tanzania Guinea grass and Mineirão stylo. **Grass and Forage Science**, Oxford, v. 70, p. 439-450, 2014.

SEBASTIAN, A.; PRASAD, M.N.V. Photosynthesis mediated decrease in cadmium translocation protect shoot growth of *Oryza sativa* seedlings up on ammonium phosphate - sulfur fertilization. **Environmental Science and Pollution Research**, Heidelberg, v. 21, p. 986-997, 2014.

SEHT, C.S.; REMANS, T.; KEUNEN, E.; JOZEFCZAK, M.; GIELEN, H.; OPDENAKKER, K.; WEYENS, N.; VANGRONSVELD, J.; CUYPERS, A. Phytoextraction of toxic metals: a central role for glutathione. **Plant, Cell and Environment**, Oxford, v. 35, p. 334-346, 2012.

SHARMA, S.S.; DIETZ, K.J. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. **Journal of Experimental Botany**, Oxford, v. 57, p. 711-726, 2006.

SHEORAN, V.; SHEORAN, A.S.; POONIA, P. Factors affecting phytoextraction: a review. **Pedosphere**, Beijing, v. 26, p. 148-166, 2016.

SHEORAN, V.; SHEORAN, A.S.; POONIA, P. Phytomining: A review. **Minerals Engineering**, Oxford, v. 22, p. 1007-1019, 2017.

SOBRINO-PLATA, J.; MEYSSEN, D.; CUYPERS, A.; ESCOBAR, C.; HERNÁNDEZ, L.E. Glutathione is a key antioxidant metabolite to cope with mercury and cadmium stress. **Plant and Soil**, Dordrecht, v. 377, p. 369-381, 2014.

SKREBSKY, E.C.; TABALDI, L.A.; PEREIRA, L.B.; RAUBER, R.; MALDANER, J.; CARGNELUTTI, D.; GONÇALVES, J.F.; CASTRO, G.Y.; SHETINGER, M.R.C.; NICOLOSO, F.T. Effect of cadmium on growth, micronutrient concentration, and δ-aminolevulinic acid dehydratase and acid phosphatase activities in plants of *Pfaffia glomerata*. **Brazilian Journal of Plant Physiology**, Londrina, v. 20, p. 285-294, 2008.

SUÁREZ, C.; TORRES, E.; PÉREZ-RAMA, M.; HERRERO, C.N.; ABALDE, J. Cadmium toxicity on the freshwater microalga *Chlamydomonas moewusii* Gerloff: biosynthesis of thiol compounds. **Environmental Toxicology and Chemistry**, Hoboken, v. 29, p. 2009-2015, 2010.

SUN, X.M.; LU, B.; HUANG, S.Q.; MEHTA, S.K.; XU, L.L.; YANG, Z.M. Coordinated expression of sulfate transporters and its relation with sulfur metabolites in *Brassica napus* exposed to cadmium. **Botanical Studies**, New York, v. 48, p. 43-54, 2007.

SUN, X.; ZHANG, J.; ZHANG, H.; NI, Y.; ZHANG, Q.; CHEN, J.; GUAN, Y. The responses of *Arabidopsis thaliana* to cadmium exposure explored via metabolite profiling. **Chemosphere**, Oxford, v. 78, p. 840-845, 2010.

TAKAHASHI, H.; KOPRIVA, S.; GIORDANO, M.; SAITO, K.; HELL, R. Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. **Annual Review of Plant Biology**, Palo Alto, v. 62, p. 157-184, 2011.

TARHAN, L.; KAVAKCIOGLU, B. Glutathione metabolism in *Urtica dioica* in response to cadmium based oxidative stress. **Biologia Plantarum**, Prague, v. 60, p. 163-172, 2016.

TOLEDO-SILVA, G.; CARDOSO-SILVA, C.B.; JANK, L.; SOUZA, A.P. De Novo transcriptome assembly for the tropical grass *Panicum maximum* Jacq. **PLoS One**, San Francisco, v. 8, p. 70781, 2013.

VÁZQUEZ, S.; GOLDSBROUGH, P.; CARPENA, R.O. Assessing the relative contributions of phytochelatins and the cell wall to cadmium resistance in white lupin. **Physiologia Plantarum**, Lund, v. 128, p. 487-495, 2006.

VELÍŠEK, J.; KUBEC, R.; CEJPEK, K. Biosynthesis of food constituents: Amino acids: 4. Non-protein amino acids - a review. **Czech Journal of Food Science**, Prague, v. 24, p. 93-109, 2006.

VERMA, S.; DUBEY, R.S. Effect of cadmium on soluble sugars and enzymes of their metabolisme in rice. **Biologia Plantarum**, Prague, v. 44, p. 117-123, 2001.

VIEHWEGER, K. How plants cope with heavy metals. **Botanical Studies**, New York, v. 55, p. 35-47, 2014.

VOLLENWEIDER, P.; COSIO, C.; GÜNTHARDT-GOERG, M.S.; KELLER, C. Localization and effects of cadmium in leaves of a cadmium-tolerant willow (*Salix viminalis* L.) Part II Microlocalization and cellular effects of cadmium. **Environmental and Experimental Botany**, Oxford, v. 58, p. 25-40, 2006.

VOLPE, E.; MARCHETTI, M.E.; MACEDO, M.C.M.; LEMPP, B. Forage accumulation and plant and soil characteristics according to soil base saturation, rates of phosphorus and nitrogen in the establishment of massaigrass. **Brazilian Journal of Animal Science**, Viçosa, v. 37, p. 228-237, 2008.

WAGNER, G.J. Accumulation of cadmium in crop plants and its consequences to human health. **Advances in Agronomy**, San Diego, v. 51, p. 173-212, 1993.

WANG, Y.; JIANG, X.; LI, K.; WU, M.; ZHANG, R.; ZHANG, L.; CHEN, G. Photosynthetic responses of *Oryza sativa* L. seedlings to cadmium stress: physiological, biochemical and ultrastructural analyses. **Biometals**, Dordrecht, v. 27, p. 389-401, 2014.

WÓJCIK, M.; VANGRONSVELD, J.; D'HAEN, J.; TUKIENDORF, A. Cadmium tolerance in *Thlaspi caerulescens*. II. Localization of cadmium in *Thlaspi caerulescens*. **Environmental and Experimental Botany**, Oxford, v. 53, p. 163-171, 2005.

WHO. **Evaluation of certain food additives and contaminants:** seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: Joint FAO/WHO Expert Committee on Food Additives, 2011. 228 p.

WU, Z.; ZHANG, C.; YAN, J.; YUE, Q.; GE, Y. Effects of sulfur supply and hydrogen peroxide pretreatment on the responses by rice under cadmium stress. **Plant Growth Regulation**, Dordrecht, v. 77, p. 299-306, 2015.

XU, J.; ZHU, Y.; GE, Q.; LI, Y.; SUN, J.; ZHANG, Y.; LIU, X. Comparative physiological responses of *Solanum nigrum* and *Solanum torvum* to cadmium stress. **New Phytologist**, London, v. 196, p. 125-138, 2012.

YADAV, S.K. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. **South African Journal of Botany**, Amsterdam, v. 76, p. 167-179, 2010.

YANNARELLI, G.G.; FERNÁNDEZ-ALVAREZ, A.J.; SANTA-CRUZ, D.M.; TOMARO, M.L. Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress. **Phytochemistry**, Oxford, v. 68, p. 505-512, 2007.

YU, F.; LIU, K.; LI, M.; ZHOU, Z.; DENG, H.; CHEN, B. Effects of cadmium on enzymatic and non-enzymatic antioxidative defences of rice (*Oryza sativa* L.). **International Journal of Phytoremediation**, Philadelphia, v. 15, p. 513-521, 2013.

ZEMANOVÁ, V.; PAVLÍK, M.; PAVLÍKOVÁ, D.; TLUSTOŠ, P. The changes of contents of selected free amino acids associated with cadmium stress in *Noccaea caerulescens* and *Arabidopsis halleri*. **Plant, Soil and Environment**, Prague, v. 59, p. 417-422, 2013.

ZEMANOVÁ, V.; PAVLÍK, M.; PAVLÍKOVÁ, D.; KYJAKOVÁ, P. Changes in the contents of amino acids and the profile of fatty acids in response to cadmium contamination in spinach. **Plant, Soil and Environment**, Prague, v. 61, p. 285-290, 2015.

3 A GLIMPSE INTO THE SYMPLASTIC AND APOPLASTIC Cd UPTAKE BY MASSAI GRASS MODULATED BY SULFUR NUTRITION: PLANTS WELL-NOURISHED WITH S AS A STRATEGY FOR PHYTOEXTRACTION[1]

Abstract

To date, there have been no studies demonstrating the influence of sulfur (S) on cadmium (Cd) uptake kinetics, which limits the understanding of mechanisms involved in uptake of this element. Therefore, this study was carried out in order to quantify the contribution of symplastic and apoplastic uptakes of Cd (0.1 and 0.5 mmol L^{-1}) by Massai grass (*Panicum maximum* cv. Massai) grown under low and proper S supply (0.1 and 1.9 mmol L^{-1}) by measuring Cd concentration in nutrient solution (V_{max}, K_m, and C_{min}) along the plant's exposure time (108 h) and determining Cd concentration in root symplast and apoplast. V_{max} of Cd influx in Massai grass exposed to highest Cd and S concentrations was 38% higher than that plants supplied with lowest S concentration. K_m and C_{min} of plants exposed to highest Cd concentration was higher than that plants subjected to lowest Cd concentration, although values were not affected by S supply. Symplastic influx of Cd in plants subjected to lowest Cd and S concentrations was 20% higher as compared to plants supplied with highest S concentration, whereas the apoplastic influx of Cd was higher when there was highest S supply, regardless of Cd concentration in solution. This result indicates that proper S supply decreases the contribution of symplastic Cd uptake and increases the contribution of apoplastic Cd uptake when the toxicity caused by Cd is lowest.

Keywords: environmental pollution, kinetics, K_m, *Panicum maximum*, plant nutrition.

3.1 Introduction

Cadmium (Cd) concentration in the environment has increased in the last decades due to the disposal of industrial and municipal waste in inappropriate areas and the application of phosphate fertilizers and sewage sludge, among other factors (CATALDO; GARLAND; WILDUNG, 1983). This fact represents a major socio-economic problem, since Cd is toxic to plants, animals, and humans (STRITSIS; CLAASSEN, 2013). For this reason, several strategies to decrease Cd concentration in the environment have been investigated, most

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notably phytoextraction (SHEORAN; SHEORAN; POONIA, 2016). However, to this date, there are few known species of plants considered Cd hyperaccumulators, which fosters the study of other plants, as forage grasses (ZHAO et al., 2002; RABÊLO; BORGO, 2016). These plants can offset lowest Cd concentrations with their high biomass production, mainly when plants grown with proper sulfur (S) supply (RABÊLO; BORGO, 2016; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b). Sulfur is a component of metabolites that act in chelation and defense against the damage caused by Cd [e.g. amino acids, reduced glutathione (GSH) and phytochelatins (PCs)], and proper supply of this nutrient can increase Cd extraction potential (SETH et al., 2012; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b). However, so far, there have been no studies demonstrating the influence of S on Cd uptake kinetics, which limits the understanding of mechanisms involved in uptake of this element and the application of genetic breeding to optimize the phytoextraction.

Cadmium can enter cells from roots through transporters of ZIP (Zinc-regulated transporter/Iron-regulated transporter-like Protein) family, non-selective cation channels, or in form of Cd-chelates through YSL (Yellow-Stripe 1-Like) proteins (LUX et al., 2011). By this pathway, Cd can reach the Casparian strips via symplast, through the cytoplasm of cells from roots connected by plasmodesmata (high-affinity transport system - HATS) (REDJALA; STERCKEMAN; MOREL, 2009). However, uptake-kinetics studies have shown that Cd influx, when at high concentrations, follows the Michaelis-Menten constant with a linear component (COSTA; MOREL, 1994; HARRIS; TAYLOR, 2004; PERRIGUEY; STERCKEMAN; MOREL, 2008). This linear component has been attributed to strong binding of Cd to cell wall, but it may also represent the apoplastic Cd flow towards the xylem through transporters and/or channels (low-affinity transport system - LATS) (LUX et al., 2011). These uncertainties hamper a more precise characterization of symplastic and apoplastic participations in Cd uptake, especially in situations in which plants have been exposed to Cd for short periods (STERCKEMAN; REDJALA; MOREL, 2011). In any event, the uptake kinetics of metals normally includes two stages: the first stage is rapid and occurs after contact of metal with roots (passive system - apoplastic uptake), while the second is slowest and depends of metabolic activity (active system - symplastic uptake) (SLOOF; VIRAGH; van der VEER, 1995). However, the importance of each stage varies according to species and concentration of heavy metal and other ions (eg, SO_4^{2-}) in solution (REDJALA; STERCKEMAN; MOREL, 2009, 2010).

Although sulfate (SO_4^{2-}) does not compete with Cd for the same uptake sites, proper S supply may increase the synthesis of thiol compounds (-SH) in apoplast and alter the influx

of Cd in this pathway, since Cd has a high affinity with thiol compounds (NOCITO et al., 2002; ANJUM et al., 2015). Thus, it is possible that S supply results in a greatest accumulation of metal in this site, which is desirable, since the main site of Cd accumulation in tolerant plants is root apoplast (WÓJCIK et al., 2005; AKHTER et al., 2014). On the other hand, S is a component of PCs [$(\gamma\text{-GluCys})_n\text{-Gly}$, in which *n* ranges from 2 to 11], which act in Cd sequestration from cytosol to vacuole (COBBETT; GOLDSBROUGH, 2002). In this regard, S supply may increase the synthesis of PCs and consequently modify Cd influx via symplast (HART et al., 1998; MENDOZA-CÓZATL et al., 2008). It should be stressed that when exposed to Cd, some plants taken up more S (NOCITO et al., 2002) and, consequently, proper supply of this nutrient may change Cd uptake kinetics in relation to plants growing under low S supply because of synthesis of thiol compounds (bonding and/or chelation of Cd). In view of this scenario, our aim with this study was to identify the symplastic and apoplastic contributions in Cd uptake by Massai grass (*Panicum maximum* cv. Massai) exposed to high Cd concentrations (0.1 and 0.5 mmol L^{-1}) under lower (0.1 mmol L^{-1}) and proper (1.9 mmol L^{-1}) S supplies, since plants of this species have been evaluated in Cd phytoextraction studies as a function of S supply (RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b).

3.2 Materials and methods

3.2.1 Plant material and treatments

The study to evaluate the effect of S on Cd uptake by roots of *Panicum maximum* cv. Massai was carried out in a greenhouse (Figures 1 and 2), using plastic pots with 0.6 L capacity containing 0.5 L of a nutrient solution. Treatments were represented by combinations of two S concentrations (0.1 and 1.9 mmol L^{-1}) and two Cd concentrations (0.1 and 0.5 mmol L^{-1}), in nutrient solutions modified from solution of Epstein and Bloom (2005). Plastic pots used in the study were distributed as random blocks with four replications. Cadmium concentrations tested in this study were high in relation to other studies of Cd uptake kinetics (LOMBI et al., 2001; HE et al., 2007), but relatively high and potentially toxic Cd concentrations are required to investigate the existence of Cd transport via LATS (HART et al., 2002), mainly in plants that are possibly Cd hyperaccumulator, such as Massai grass.

Figure 1 - Greenhouse (A), aeration system of nutrient solution (B, C, D, E and F), greenhouse cooling system (G and H), expanded vermiculite (I), plastic tray for seed germination (J), mean length of seedlings (K) and size of plastic containers (L) used in the study to evaluate the effect of S on Cd uptake by roots of Massai grass

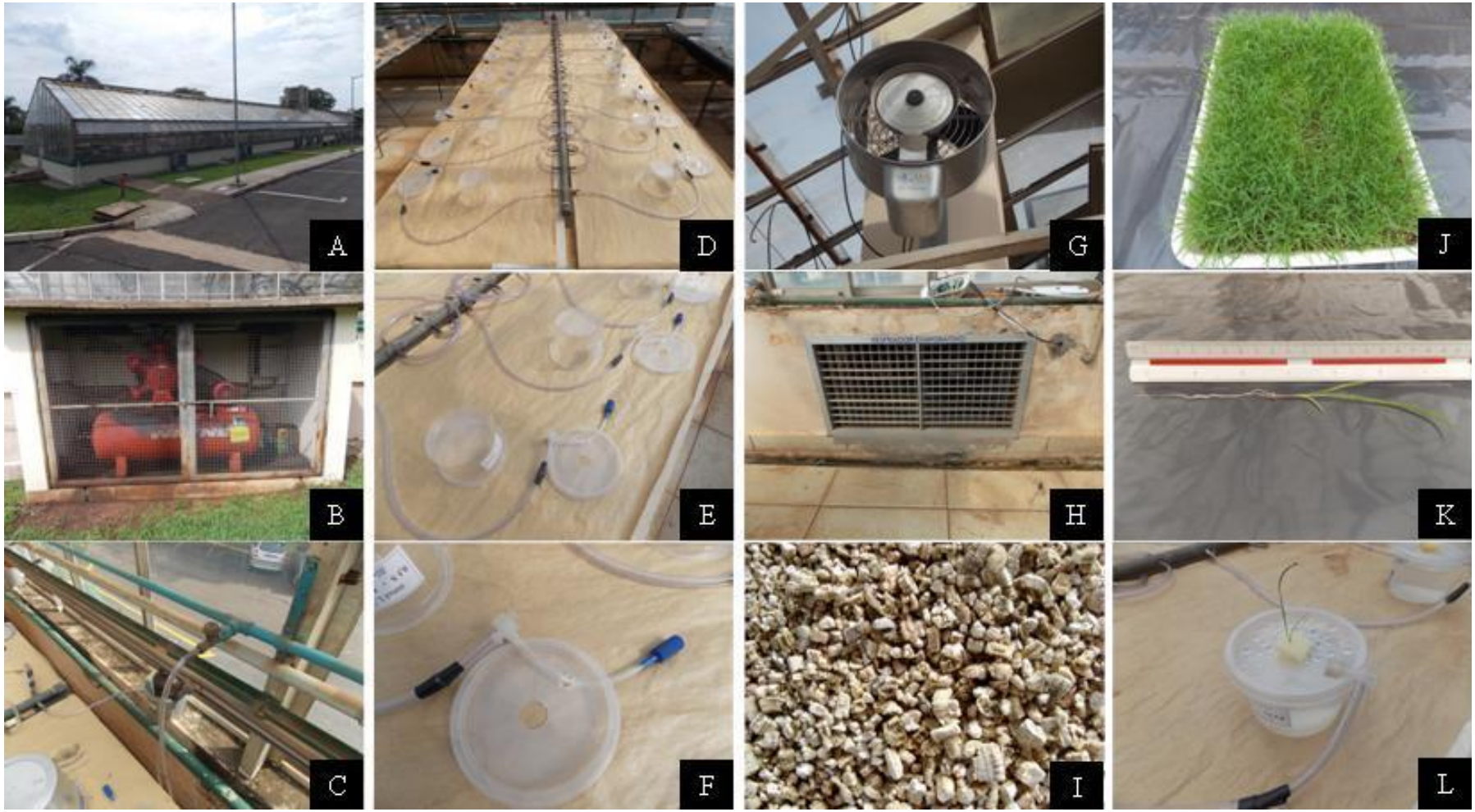
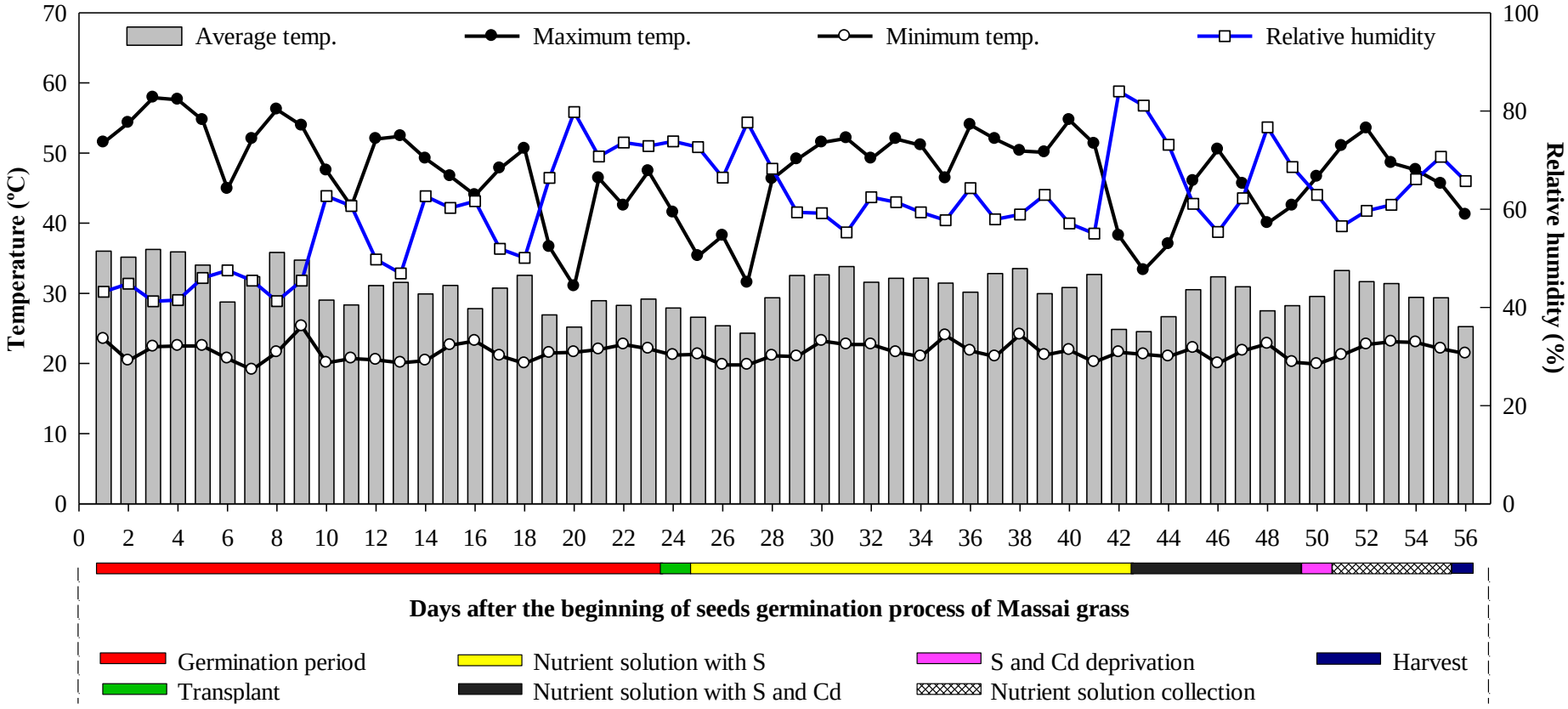


Figure 2 - Climatic dates (average temperature, maximum temperature, minimum temperature and relative humidity) recorded in the greenhouse during Cd uptake kinetics study in the roots of Massai grass, describing the steps between the beginning and the end of study (germination period, transplant, addition of undiluted and modified nutrient solution to meet S concentrations, addition of undiluted and modified nutrient solution to meet S and Cd concentrations, S and Cd deprivation in nutrient solution, nutrient solution collection, and harvest of plants)



3.2.2 Development of kinetics study

Seeds were set to germinate in a tray containing expanded vermiculite, which was irrigated with deionized water in the first 14 days, and nutrient solution modified to meet S concentration of 0.1 mmol L^{-1} (diluted at 25% ionic strength) in the following nine days (days 15-23) to acclimatize the plants to nutrient solution (RABÊLO; AZEVEDO; MONTEIRO, 2017a). After the 23 days of seeding, one seedling (± 10 cm height) was transferred to each pot containing undiluted nutrient solutions modified to meet S concentrations of 0.1 and 1.9 mmol L^{-1} (100% ionic strength) for 19 days (days 24-42) to acclimatize the plants to S concentrations (RABÊLO; AZEVEDO; MONTEIRO, 2017a). Then, the solutions modified to meet S and Cd concentrations were provided for seven days (days 43-49) to acclimatize the plants to concentrations of S and Cd (RABÊLO; AZEVEDO; MONTEIRO, 2017a). On the next day (day 50), the nutrient solutions were modified to prevent the supply of S and Cd aiming to increase uptake capacity of these two elements, as has been demonstrated for other elements (EPSTEIN; RAGEN, 1952; LEE; RUDGE, 1986). After 24 h of S and Cd deprivation, the solutions modified to meet S and Cd concentrations were supplied again, for five days (days 51-55) to carry out the study of kinetics (Figure 3). The nutrient solutions provided in each period of kinetics study were kept constantly aerated (day and night) and renewed when the composition of nutrient solution was modified (days 24, 43, 50, and 51) and when the same composition was used for more than seven consecutive days (15-23 and 24-42 periods). Solutions maintained for periods of more than seven days were renewed each seven days, and solutions maintained for periods of less than seven days (solutions supplied between the days 43-49, 50, and 51-55) were renewed only once, on days 43, 50, and 51.

The composition and initial pH of nutrient solutions used in the study is shown in Table 1, and the chemical speciation of nutrient solution used between days 51-55 (period of collection of nutrient solution for calculation of kinetic parameters), calculated using Visual MINTEQ® software v. 3.0 (GUSTAFSSON, 2012), is described in Table 2. Initial pH values of nutrient solutions used during the study ranged from 5.78 to 5.83 (Table 1). Xu et al. (2012) demonstrate that, in model phytoplankton species, modest variations in pH (within 0.4 pH units of average seawater) have a negligible effect on Cd uptake system.

Figure 3 - Representation of solutions used in Cd kinetic study (A) [Adapted from Bucher et al. (2014)] and Cd compartmentalization in apoplast an d symplast in the roots of Massai grass (B) [Adapted from Mori et al. (2009)]

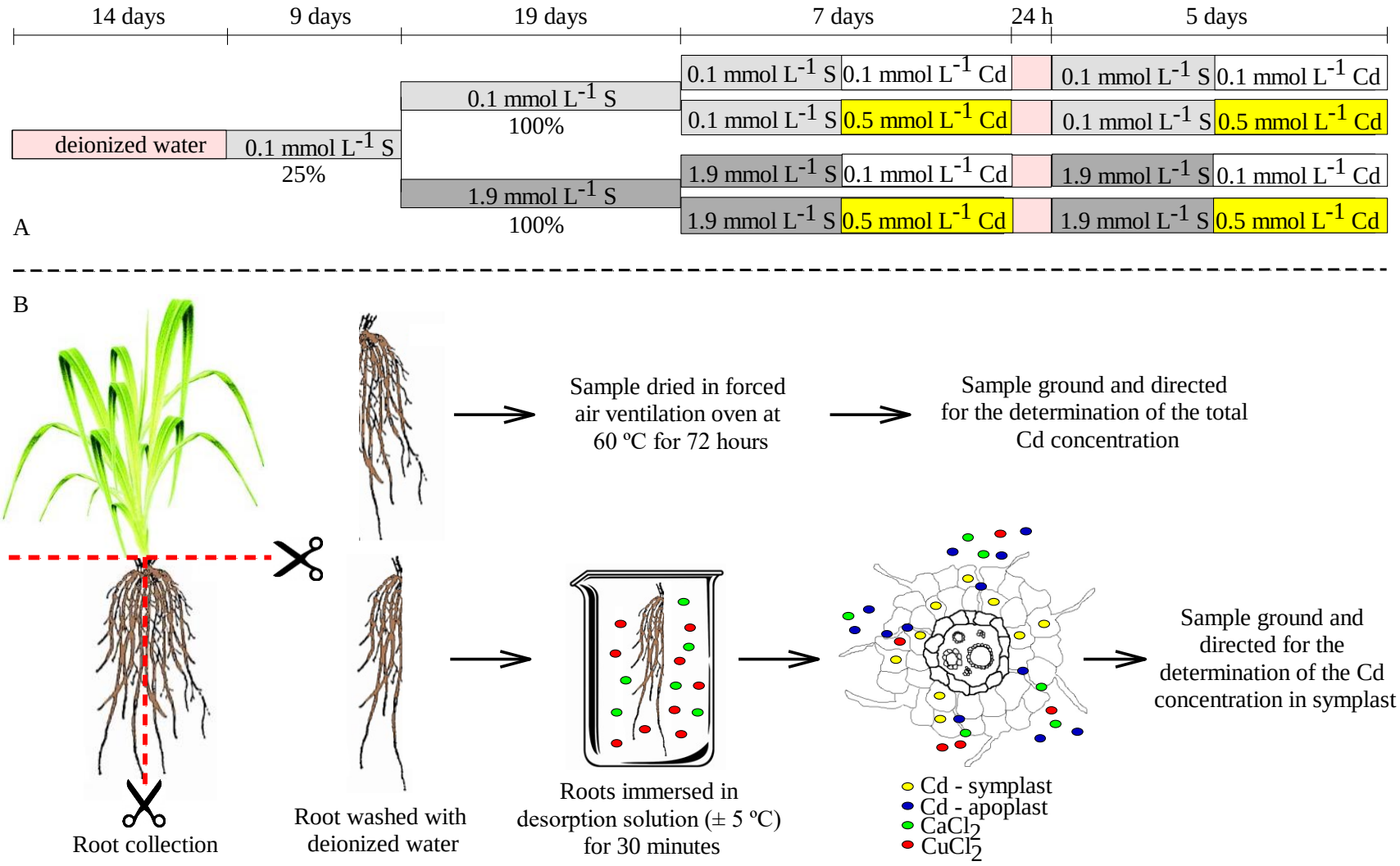


Table 1 - Volumes and initial pH of stock solutions used in preparation of nutrient solutions provided in each period of study of Cd uptake kinetics

S (mmol L^{-1})	0.1	0.1	1.9	1.9	0.1	0.1	1.9	1.9
Cd (mmol L^{-1})	0.1	0.5	0.1	0.5	0.1	0.5	0.1	0.5
Stock solution	Volume used (mL L^{-1})							
	Days 0-14				Days 15-23			
$CdCl_2$ (1 mol L^{-1})	-	-	-	-	-	-	-	-
KH_2PO_4 (1 mol L^{-1})	-	-	-	-	0.25	0.25	0.25	0.25
NH_4NO_3 (1 mol L^{-1})	-	-	-	-	1.13	1.13	1.13	1.13
KNO_3 (1 mol L^{-1})	-	-	-	-	1.50	1.50	1.50	1.50
KCl (1 mol L^{-1})	-	-	-	-	0.25	0.25	0.25	0.25
$MgSO_4.7H_2O$ (1 mol L^{-1})	-	-	-	-	0.03	0.03	0.03	0.03
$MgCl_2.6H_2O$ (1 mol L^{-1})	-	-	-	-	0.48	0.48	0.03	0.03
$CaCl_2$ (1 mol L^{-1})	-	-	-	-	1.25	1.25	1.25	1.25
Micronutrients - Fe[1]	-	-	-	-	0.25	0.25	0.25	0.25
Fe(III) - EDTA[2]	-	-	-	-	0.25	0.25	0.25	0.25
pH (H_2O)	5.89	5.89	5.89	5.89	5.80	5.80	5.80	5.80
Stock solution	Days 24-42				Days 43-49			
$CdCl_2$ (1 mol L^{-1})	-	-	-	-	0.1	0.5	0.1	0.5
KH_2PO_4 (1 mol L^{-1})	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
NH_4NO_3 (1 mol L^{-1})	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
KNO_3 (1 mol L^{-1})	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
KCl (1 mol L^{-1})	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$MgSO_4.7H_2O$ (1 mol L^{-1})	0.1	0.1	1.9	1.9	0.1	0.1	1.9	1.9
$MgCl_2.6H_2O$ (1 mol L^{-1})	1.9	1.9	0.1	0.1	1.9	1.9	0.1	0.1
$CaCl_2$ (1 mol L^{-1})	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Micronutrients - Fe[1]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Fe(III) - EDTA[2]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
pH (H_2O)	5.80	5.80	5.80	5.80	5.79	5.83	5.78	5.79
Stock solution	Day 50				Days 51-55			
$CdCl_2$ (1 mol L^{-1})	-	-	-	-	0.1	0.5	0.1	0.5
KH_2PO_4 (1 mol L^{-1})	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
NH_4NO_3 (1 mol L^{-1})	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
KNO_3 (1 mol L^{-1})	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
KCl (1 mol L^{-1})	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$MgSO_4.7H_2O$ (1 mol L^{-1})	-	-	-	-	0.1	0.1	1.9	1.9
$MgCl_2.6H_2O$ (1 mol L^{-1})	2.0	2.0	2.0	2.0	1.9	1.9	0.1	0.1
$CaCl_2$ (1 mol L^{-1})	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Micronutrients - Fe[1]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Fe (III)-EDTA[2]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
pH (H_2O)	5.83	5.83	5.83	5.83	5.79	5.83	5.78	5.79

[1]Composition of micronutrient solution (µmol L^{-1}): KCl = 50; H_3BO_3 = 25; $MnSO_4.H_2O$ = 2; $ZnSO_4.7H_2O$ = 2; $CuSO_4.5H_2O$ = 0.5; H_2MoO_4 (85 % MoO_3) = 0.5. [2]Fe(III)-EDTA = 100 µmol L^{-1}.

After the period of deprivation of S and Cd in nutrient solution (day 50), we began the supply of modified solutions to meet combinations of S and Cd concentrations (days 51-55), as well as the immediate sampling of nutrient solution to determine the kinetic parameters according to method proposed by Claassen and Barber (1974). Aliquots of 10 mL were collected every 15 min for the first 2 h; every 30 min for the following 4 h; every 60 min during the subsequent 6 h; and, lastly, every 12 h until the end of 108-h experiment. Volume of nutrient solutions in the pots was kept constant (0.5 L) during collection period by adding deionized water (CLAASSEN; BARBER, 1974). At the end of last solution collection, plants were separated into shoot and root to determine S and Cd concentrations (apoplast and symplast) and measure the plant biomass production.

Table 2 - Chemical speciation of nutrient solution (pH 5.5) by Visual MINTEQ® software

0.1 mmol L^{-1} S + 0.1 mmol L^{-1} Cd				0.1 mmol L^{-1} S + 0.5 mmol L^{-1} Cd			
S - Species	(%)	Cd - Species	(%)	S - Species	(%)	Cd - Species	(%)
SO_4^{-2}	66.55[2]	Cd^{+2}	58.77[3]	SO_4^{-2}	69.87	Cd^{+2}	52.88
HSO_4^-	5.04	$CdCl^+$	38.45	HSO_4^-	0.01	$CdCl^+$	36.16
$CdSO_4$ (aq)[1]	0.23	$CdCl_2$ (aq)	1.54	$CdSO_4$ (aq)	1.07	$CdCl_2$ (aq)	1.51
$MgSO_4$ (aq)	6.26	$CdSO_4$ (aq)	0.23	$MgSO_4$ (aq)	6.42	$CdSO_4$ (aq)	0.21
$CaSO_4$ (aq)	18.54	$CdNO_3^+$	0.96	$CaSO_4$ (aq)	19.09	$CdNH_3^{+2}$	0.01
KSO_4^-	1.81	$CdHPO_4$ (aq)	0.02	KSO_4^-	1.90	$CdNO_3^+$	0.86
$NH_4SO_4^-$	1.56	$CdEDTA^{-2}$	0.01	$NH_4SO_4^-$	1.63	$CdHPO_4$ (aq)	7.96
-	-	$CdHEDTA^-$	0.02	-	-	$CdEDTA^{-2}$	0.40
1.9 mmol L^{-1} S + 0.1 mmol L^{-1} Cd				1.9 mmol L^{-1} S + 0.5 mmol L^{-1} Cd			
S - Species	(%)	Cd - Species	(%)	S - Species	(%)	Cd - Species	(%)
SO_4^{-2}	71.91	Cd^{+2}	51.63	SO_4^{-2}	71.50	Cd^{+2}	51.18
HSO_4^-	0.01	$CdCl^+$	33.51	HSO_4^-	0.01	$CdCl^+$	34.56
$CdSO_4$ (aq)	0.21	$CdCl_2$ (aq)	1.33	$CdSO_4$ (aq)	1.03	$CdCl_2$ (aq)	1.44
$MgSO_4$ (aq)	6.16	$CdSO_4$ (aq)	4.00	$Cd(SO_4)_2^{-2}$	0.04	$CdSO_4$ (aq)	3.90
$CaSO_4$ (aq)	18.11	$Cd(SO_4)_2^{-2}$	0.07	$MgSO_4$ (aq)	6.05	$Cd(SO_4)_2^{-2}$	0.07
KSO_4^-	1.93	$CdNH_3^{+2}$	0.01	$CaSO_4$ (aq)	17.82	$CdNH_3^{+2}$	0.01
$NH_4SO_4^-$	1.66	$CdNO_3^+$	0.84	KSO_4^-	1.91	$CdNO_3^+$	0.82
-	-	$CdHPO_4$ (aq)	7.80	$NH_4SO_4^-$	1.64	$CdHPO_4$ (aq)	7.62
-	-	$CdEDTA^{-2}$	0.79	-	-	$CdEDTA^{-2}$	0.40

[1]aq - aqua complex. [2]Variation < 5% in SO_4^{-2} availability (pH 3.5 to 5.5). [3]Variation < 10% in Cd^{+2} availability (pH 3.5 to 5.5).

3.2.3 Determination of kinetic parameters

Kinetic parameters (maximum uptake rate - V_{max}; Michaelis-Menten constant - K_m; minimum concentration below which plants cease to uptake the element - C_{min}; and uptake capacity - α) were determined following the method proposed by Claassen and Barber (1974), whose principle is the quantification of reduction in the concentration of element of interest of solution over time as a function of its uptake by plant root system. V_{max} and K_m values were

calculated by graphic-mathematical approach (RUIZ, 1985) using Cinetica Windows® software v. 1.0 (RUIZ; FERNANDES FILHO, 1992). C_{min} value was estimated from the moment that Cd concentration in exhaust solution remained constant, and α was obtained by V_{max}/K_m ratio (KELETI; WELCH, 1984). Cadmium depletion curves (decrease in Cd concentration in nutrient solution over time) were adjusted by using the graphic-mathematical model proposed by Ruiz (1985). Symplastic Cd influx was calculated by equation of Claassen and Barber (1974):

$$I_s = \frac{V_{max} \times [Cd]}{K_m + [Cd]} \quad (1)$$

Where I_s is symplastic Cd influx (mmol g^{-1} FW h^{-1}), V_{max} is maximum Cd uptake rate (mmol g^{-1} FW h^{-1}), K_m is Michaelis-Menten constant (mmol L^{-1}), and [Cd] represents Cd concentration in nutrient solution. Afterwards, apoplastic Cd influx was obtained by equation described by Lombi et al. (2001):

$$I_a = \frac{V_{max} \times [Cd]}{K_m + [Cd]} + a[Cd] \quad (2)$$

Where I_a is apoplastic influx of Cd (mmol g^{-1} FW h^{-1}) and *a* is the decline of linear component. It is noteworthy that this equation has been used to calculate Cd symplastic influx (REDJALA; STERCKEMAN; MOREL, 2009), but there is evidence that linear component of this equation can represent Cd influx via apoplast (LUX et al., 2011). After the calculations, the curves of Cd symplastic and apoplastic influxes were adjusted using SigmaPlot® software v. 13.0 (SYSTAT SOFT INC., 2015).

3.2.4 Determination of Cd concentrations in apoplast and symplast and S

Concentrations of Cd (apoplast and symplast) and S were determined only in the root system of plants. Cadmium concentration in apoplast and symplast was determined according to methods described by Lavres Junior et al. (2008) and Mori et al. (2009), with modifications. The root system of Massai grass was divided into two longitudinal sections with equivalent masses; one of sections was immersed in a container with 150 mL of "desorption" solution (2 mmol L^{-1} $CuCl_2$, 0.5 mmol $L^{-1}CaCl_2$, and 100 mmol L^{-1} HCl) at 5 °C for 30 min (Figure 3B). $CaCl_2$ 0.5 mmol L^{-1} was used to maintain cell wall integrity during

Cd desorption and $CuCl_2$ 2 mmol L^{-1} for release of Cd (Cd^{2+}) connected to root apoplast exchange sites (adapted from WANG, 2003). After desorption process, the sectioned part of root system was washed in deionized water and dried in a forced-air oven at 60 ºC for 72 h, and so was the sectioned part that was not immersed in desorption solution. Subsequently, plant material was ground (Wiley mill) and subjected to nitric-perchloric digestion, as described by Malavolta, Vitti and Oliveira (1997). Concentrations of S and Cd in the extract obtained after digestion were quantified by optic emission spectrophotometry with inductively coupled plasma (ICP-OES, Model iCAP 7000 SERIES, Thermo Scientific, Waltham, Massachusetts, USA). Later, Cd concentration in apoplast was calculated as total Cd concentration (part sectioned from root system not immersed in desorption solution) minus Cd concentration in symplast (part sectioned from roots immersed in desorption solution).

3.2.5 Determination of biomass production

The biomass production of shoots and root system was obtained after plant material was dried in a forced-air oven at 60 ºC for 72 h. Later, with biomass production values, root/shoot ratio was calculated.

3.2.6 Statistical analysis

The data were subjected to analysis of variance (F test) and means were compared by Tukey's test ($P < 0.05$) using the Statistical Analysis System v. 9.2 (SAS INSTITUTE, 2008). Results were expressed as means ± standard error of mean (SEM). Later, Pearson's correlation studies were performed among the analyzed variables (SAS INSTITUTE, 2008).

3.3 Results

3.3.1 Biomass production and root/shoot ratio

The highest biomass productions (shoot and root) of Massai grass occurred under lower Cd availability (0.1 mmol L^{-1}) and higher S supply (1.9 mmol L^{-1}) in nutrient solution (Table 3). Biomass production of shoot and root of plants supplied with highest S concentration was 73 and 130% higher compared with biomass production of plants grown with lowest S concentration, under reduced exposure to Cd (0.1 mmol L^{-1}). There was no significant effect ($P > 0.05$) of S supply on biomass production of plants exposed to highest Cd concentration in solution. Biomass production of shoots of plants grown with highest Cd availability was 40 and 74% lower than plants exposed to lowest Cd concentration when they were grown receiving S supplies of 0.1 and 1.9 mmol L^{-1}, while in the root system there was

decrease of 70 and 96%, respectively. There was significant effect (P <0.05) only for Cd concentrations on root/shoot ratio; in plants subjected to lowest Cd concentration, this ratio was 100 and 280% higher than plants exposed to highest Cd concentration, with lowest and highest S supplies, respectively.

Table 3 - Biomass production (shoots and roots) and root/shoot ratio of Massai grass supplied with S and exposed to Cd

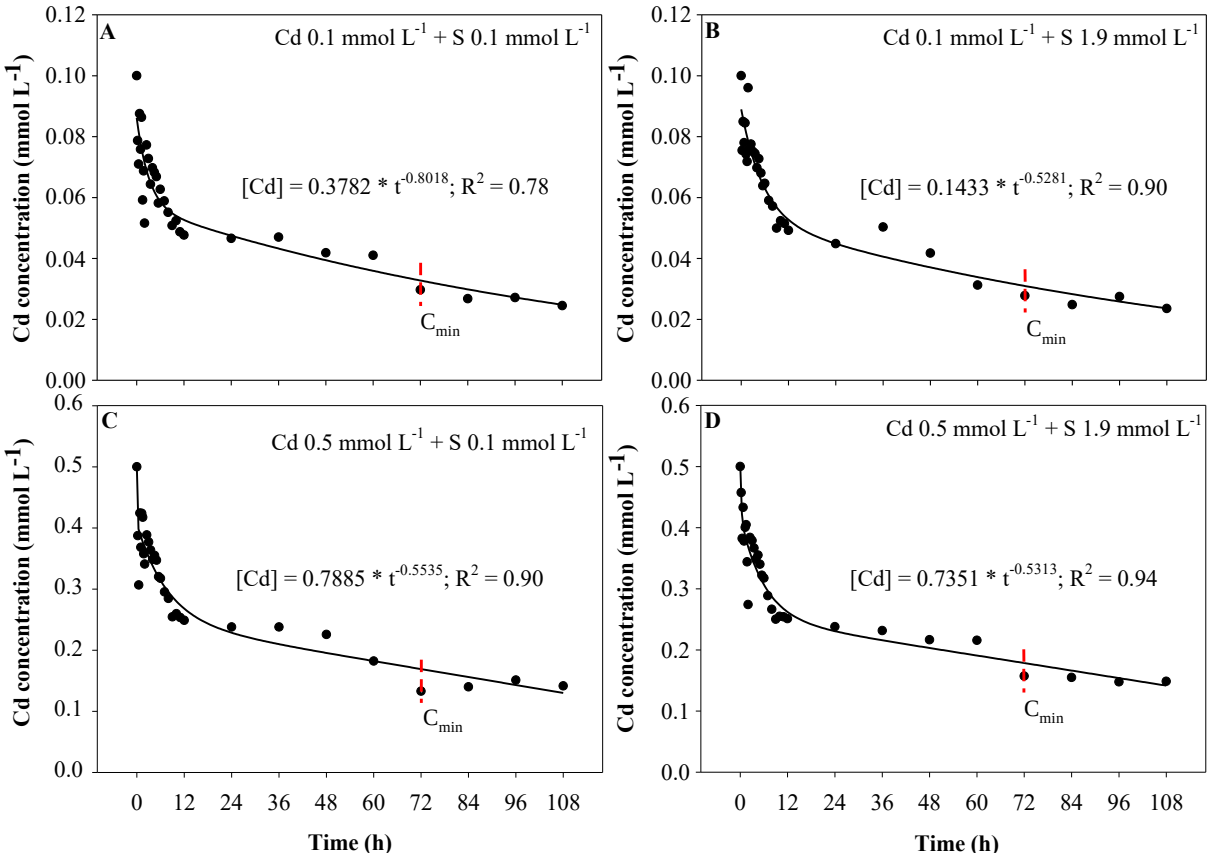
Cd (mmol L^{-1})	S (mmol L^{-1})	Shoot (g/plant)	Root (g/plant)	Root/shoot ratio
0.1	0.1	0.70 ± 0.055 b	0.10 ± 0.010 b	0.14 ± 0.013 a
	1.9	1.21 ± 0.082 a	0.23 ± 0.015 a	0.19 ± 0.014 a
0.5	0.1	0.42 ± 0.035 c	0.03 ± 0.003 c	0.07 ± 0.006 b
	1.9	0.32 ± 0.031 c	0.01 ± 0.003 c	0.05 ± 0.011 b

Means ± SEM followed by different letters in the column differ by Tukey's test (P <0.05).

3.3.2 Depletion of Cd in nutrient solution

Cadmium depletion in nutrient solution fit the potential model better, irrespective of concentration of Cd and S (Figure 4). In all combinations of Cd and S concentrations evaluated, Cd concentration in solution was reduced practically to half after 12 h of exposure of Massai grass to treatments. Cadmium concentration in nutrient solution of plants exposed to lowest Cd and S concentrations was 0.051 mmol L^{-1} (Figure 4A), while in nutrient solution of plants subjected to lowest Cd concentration and highest S concentration, Cd concentration was 0.038 mmol L^{-1} (Figure 4B). When Massai grass was exposed to highest Cd concentration, Cd concentration in nutrient solution after 12 h was 0.199 mmol L^{-1} (Figure 4C) and 0.196 mmol L^{-1} (Figure 4D), when S was supplied in amounts of 0.1 and 1.9 mmol L^{-1}, respectively. Cadmium concentration in nutrient solution remained constant after 72 h of exposure of plants to combinations of Cd and S concentrations, and C_{min} value was estimated at that moment.

Figure 4 - Cadmium depletion curves as a function of combination of Cd and S concentrations of 0.1 mmol L^{-1} + 0.1 mmol L^{-1} (A); 0.1 mmol L^{-1} + 1.9 mmol L^{-1} (B); 0.5 mmol L^{-1} + 0.1 mmol L^{-1} (C); and 0.5 mmol L^{-1} + 1.9 mmol L^{-1} (D), and of time of collection of nutrient solution aliquots



3.3.3 Kinetic parameters

Kinetic parameters evaluated here (V_{max}, K_m, C_{min}, and α) were changed significantly ($P < 0.05$) by combination of Cd and S concentrations (Table 4). Highest V_{max} was observed in Massai grass exposed to highest Cd concentration and supplied with S concentration of 1.9 mmol L^{-1}. This value was 38% higher than V_{max} of plants exposed to highest Cd concentration and grown under lowest S supply. Plants exposed to highest Cd concentration displayed a 27 and 45 times higher V_{max} than those exposed to Cd concentration of 0.1 mmol L^{-1} when S was supplied at 0.1 and 1.9 mmol L^{-1}. K_m values of Massai grass cultivated with highest Cd concentration were 493 and 500% higher in relation to K_m of plants exposed to lowest Cd concentration when S supply was 0.1 and 1.9 mmol L^{-1}, respectively. Similar results were found for C_{min}, which was 508 and 435% higher in Massai grass exposed to highest Cd concentration with lowest and highest S supplies, as compared with plants exposed to Cd concentration of 0.1 mmol L^{-1}. Thus, as occurred for V_{max}, highest α value was found in

plants exposed to Cd concentration of 0.5 mmol L^{-1} and to S concentration of 1.9 mmol L^{-1}, which showed a 30% greater Cd uptake capacity (α) than plants grown under highest Cd concentration and lowest S concentration. Uptake capacity of plants exposed to highest Cd concentration was 376 and 668% greater than plants exposed to 0.1 mmol L^{-1} Cd with lowest and highest S supplies in nutrient solution, respectively.

Table 4 - Kinetic parameters V_{max}, K_m, C_{min} and uptake capacity ($\alpha = V_{max}/K_m$) estimated from Cd uptake by Massai grass supplied with S (mmol L^{-1}) and exposed to Cd (mmol L^{-1})

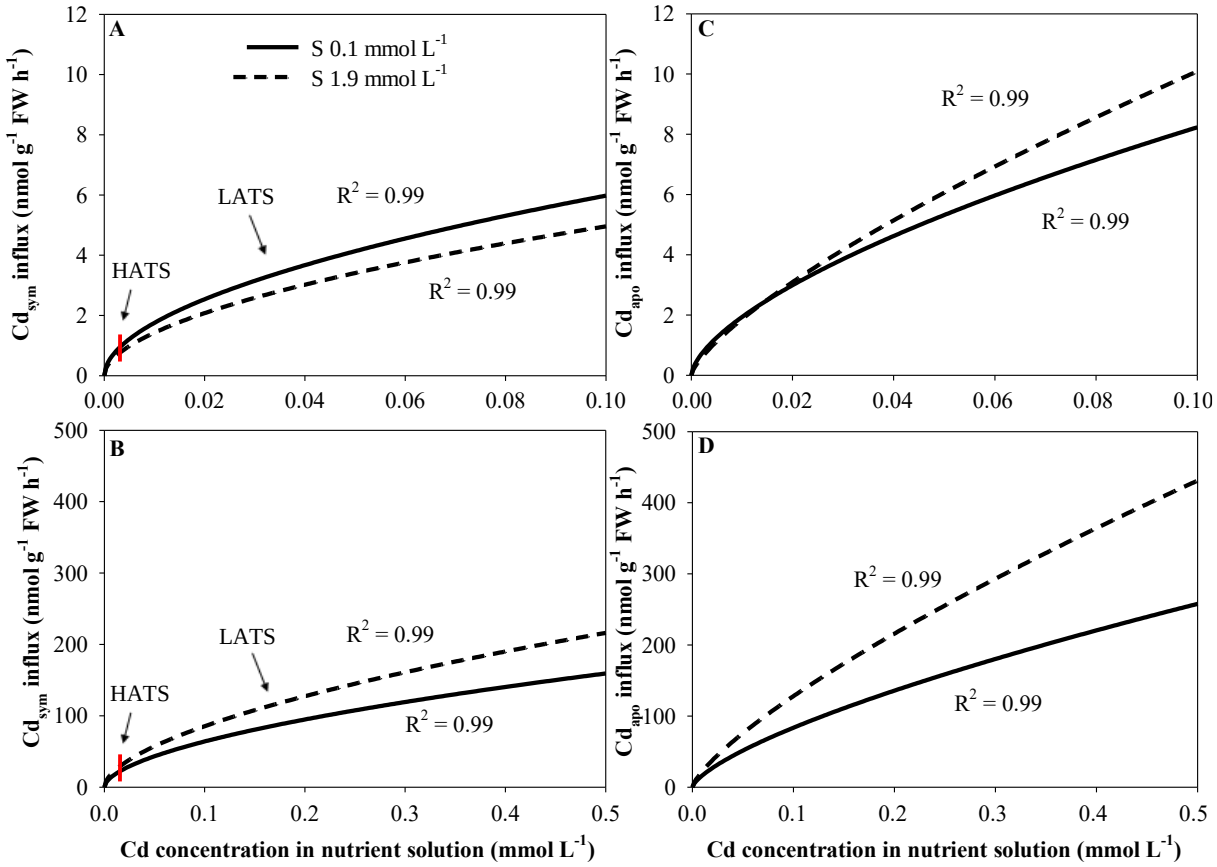
Cd	S	V_{max}	K_m	C_{min}	α
		µmol g^{-1} FW h^{-1}	µmol L^{-1}	µmol L^{-1}	----------
0.1	0.1	0.0082 ± 0.0003 c	0.045 ± 0.002 b	0.012 ± 0.001 b	0.184 ± 0.008 c
	1.9	0.0069 ± 0.0005 c	0.047 ± 0.002 b	0.014 ± 0.001 b	0.148 ± 0.013 c
0.5	0.1	0.2319 ± 0.0078 b	0.267 ± 0.016 a	0.073 ± 0.001 a	0.876 ± 0.056 b
	1.9	0.3212 ± 0.0047 a	0.282 ± 0.004 a	0.075 ± 0.001 a	1.137 ± 0.055 a

Means ± SEM followed by different letters in the column differ by Tukey's test (P <0.05).

3.3.4 Symplastic and apoplastic Cd influx

Symplastic influx of Cd from Massai grass grown under lowest S concentration was 20% higher than plants supplied with highest S concentration when they were exposed to 0.1 mmol L^{-1} Cd (Figure 5A). However, when Massai grass was exposed to highest Cd concentration, the apoplastic influx of plants supplied with lowest S concentration was 26% lower than plants supplied with highest S concentration (Figure 5B). Symplastic influx of Cd from Massai grass under highest Cd concentration was 26 and 44 times greater than plants exposed to lowest Cd concentration when S was available at 0.1 and 1.9 mmol L^{-1} in nutrient solution (Figures 5A-B). Highest apoplastic influxes of Cd from Massai grass exposed to Cd concentrations of 0.1 (Figure 5C) and 0.5 mmol L^{-1} (Figure 5D) were observed when plants were supplied with S concentration of 1.9 mmol L^{-1}. Highest supply of this nutrient resulted in 24 and 68% higher apoplastic Cd influx in relation to plants under lowest S, when Massai grass was exposed to Cd concentrations of 0.1 and 0.5 mmol L^{-1}, respectively. Cadmium influx via apoplast of plants exposed to 0.5 mmol L^{-1} Cd was 32 and 43 times higher than apoplastic influx of Massai grass exposed to lowest Cd concentration when S was supplied in amounts of 0.1 and 1.9 mmol L^{-1}, respectively (Figures 5C-D).

Figure 5 - Symplastic influx (A and B) and apoplastic influx (C and D) of Cd by root system of Massai grass exposed to Cd concentrations of 0.1 mmol L^{-1} (A and C) and 0.5 mmol L^{-1} (B and D) and supplied with S concentrations of 0.1 and 1.9 mmol L^{-1} in the nutrient solution. HATS = high affinity transport system; LATS = low affinity transport system



3.3.5 Concentrations of Cd (apoplast and symplast) and S

Cadmium concentrations significantly changed (P <0.05) Cd concentration in apoplast, symplast, and total Cd concentration, whereas S concentrations changed S concentration. Cadmium concentration in apoplast of plants exposed to highest Cd concentration was 110 and 126% higher than plants exposed to lowest Cd concentration when S was supplied at 0.1 and 1.9 mmol L^{-1}, respectively, whereas Cd concentration in symplast was 89 and 102% higher for the same comparison. Cadmium concentration in apoplast was 46, 60, 62, and 79% higher than Cd concentration in symplast when plants were grown with Cd (mmol L^{-1}) and S (mmol L^{-1}) combinations of 0.1 + 0.1, 0.1 + 1.9, 0.5 + 0.1, and 0.5 + 1.9, respectively. By contrast, total Cd concentration of plants exposed to highest Cd concentration was 101 and 116% greater than plants exposed to Cd concentration of 0.1 mmol L^{-1} in lowest and highest S supply in nutrient solution. Massai grass supplied with highest S supply showed 94 and

58% higher S concentration in the root system in relation to plants supplied with S concentration of 0.1 mmol L^{-1} when plants were exposed to Cd concentrations of 0.1 and 0.5 mmol L^{-1}, respectively (Table 5).

Table 5 - Cadmium concentrations in apoplast, symplast, and apoplast + symplast (total), and S concentration in the roots of Massai grass supplied with S (mmol L^{-1}) and exposed to Cd (mmol L^{-1})

Cd	S	Cd concentration (mg kg^{-1} DW)			S concentration
		Apoplast	Symplast	Total	(g kg^{-1} DW)
0.1	0.1	388.34 ± 24.48 b	266.26 ± 00.17 b	654.61 ± 24.31 b	1.50 ± 0.19 b
	1.9	400.55 ± 18.64 b	250.89 ± 09.07 b	651.44 ± 09.57 b	2.91 ± 0.10 a
0.5	0.1	814.58 ± 20.00 a	503.58 ± 24.11 a	1318.17 ± 04.10 a	1.79 ± 0.08 b
	1.9	903.81 ± 13.75 a	505.73 ± 31.33 a	1409.54 ± 17.58 a	2.83 ± 0.12 a

Means ± SEM followed by different letters in the column differ by Tukey's test ($P < 0.05$).

3.3.6 Pearson's correlation among the studied variables

Highest correlation coefficients (r) are highlighted in Table 6 (bold numbers). As can be observed in the table, correlations between V_{max} and Cd concentration in apoplast ($r \geq 0.97$); V_{max} and Cd concentration in symplast ($r \geq 0.94$); K_m and Cd concentration in apoplast ($r \geq 0.96$); K_m and Cd concentration in symplast ($r \geq 0.98$); C_{min} and Cd concentration in apoplast ($r \geq 0.94$); C_{min} and Cd concentration in symplast ($r \geq 0.98$); α and Cd concentration in apoplast ($r \geq 0.98$); and α and Cd concentration in symplast ($r \geq 0.92$) revealed that there is a close relationship between Cd influx and Cd accumulation site in the root system of Massai grass. It is also noteworthy that S concentration did not have a negative correlation ($P > 0.05$) with variables of study of Cd uptake kinetics by Massai grass.

3.3.7 Relationship between Cd influx and accumulation site

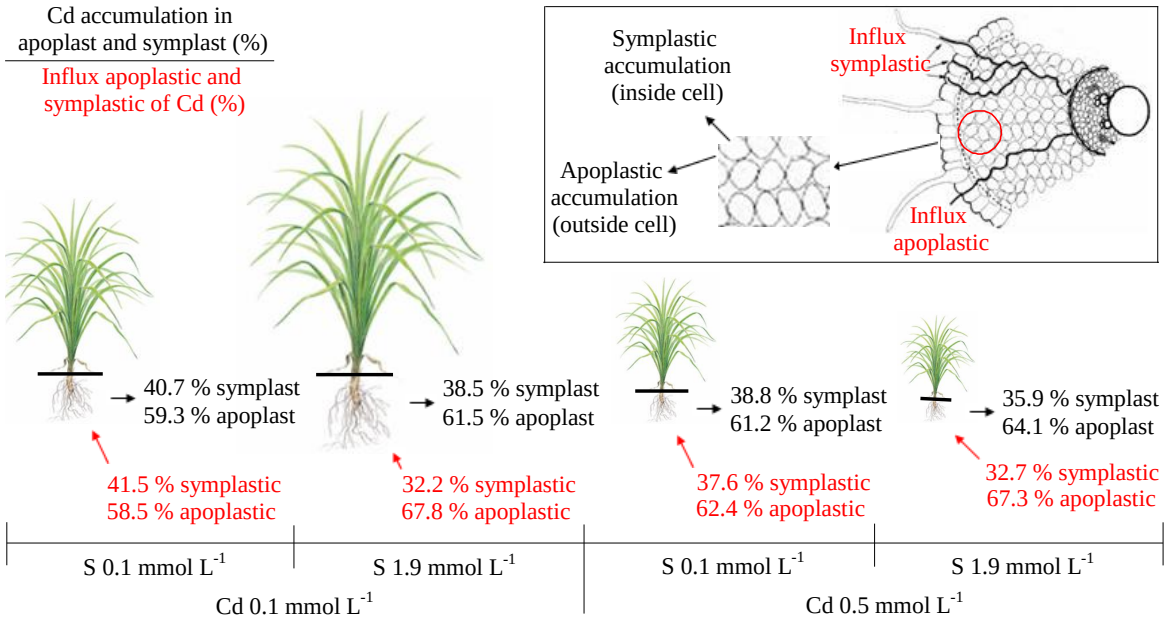
Symplastic Cd influx was 41.5, 32.2, 37.6, and 32.7% when Massai grass was exposed to combinations of Cd (mmol L^{-1}) and S (mmol L^{-1}) concentrations of 0.1 + 0.1, 0.1 + 1.9, 0.5 + 0.1, and 0.5 + 1.9, respectively. Similarly, Cd accumulation in the root symplast was 40.7, 38.5, 38.8, and 35.9% when plants were grown with Cd (mmol L^{-1}) and S (mmol L^{-1}) combinations of 0.1 + 0.1, 0.1 + 1.9, 0.5 + 0.1, and 0.5 + 1.9, respectively (Figure 6).

Table 6 - Pearson's correlation among shoot biomass production (SBP), root biomass production (RBP), maximum uptake rate (V_{max}), Michaelis-Menten constant (K_m), minimum concentration below which the plant ceases to uptake the element (C_{min}), Cd uptake capacity (α), Cd concentration in apoplast (Cd_{apo}), Cd concentration in symplast (Cd_{sym}), total Cd concentration (Cd_{tot}), and S concentration (S_{con}) of Massai grass supplied with S and exposed to Cd (n=16)

	SBP	RBP	V_{max}	K_m	C_{min}	α	Cd_{apo}	Cd_{sym}	Cd_{tot}	S_{con}
SBP										
RBP	**0.96****									
V_{max}	-0.76**	-0.82**								
K_m	-0.75**	-0.82**	**0.97****							
C_{min}	-0.74**	-0.81**	**0.94****	**0.98****						
α	-0.78**	-0.83**	**0.99****	**0.94****	**0.92****					
Cd_{apo}	-0.72**	-0.78**	**0.97****	**0.96****	**0.94****	**0.98****				
Cd_{sym}	-0.72**	-0.80**	**0.94****	**0.98****	**0.98****	**0.92****	**0.94****			
Cd_{tot}	-0.73**	-0.79**	**0.97****	**0.98****	**0.97****	**0.97****	**0.99****	**0.97****		
S_{con}	0.36^{ns}	0.29^{ns}	0.18^{ns}	0.10^{ns}	0.06^{ns}	0.15^{ns}	0.20^{ns}	0.09^{ns}	0.16^{ns}	

*p<0.05; **p<0.01; ^{ns}-not significant.

Figure 6 - Percentage contribution of symplastic and apoplastic mechanisms in influx (red color) and accumulation site (black color) of Cd in the root system of Massai grass supplied with S and exposed to Cd. Contribution of Cd influx and accumulation site was calculated using the data presented in Figure 5 and Table 5, respectively. The image located on upper right side (adapted from TINKER; NYE, 2000) shows the symplastic and apoplastic mechanisms



3.4 Discussion

Biomass production (shoots and roots) of plants decreased with highest Cd exposure (Table 3), which was also reported in other studies (HE et al., 2007; STRITSIS; CLAASSEN, 2013). This occurs because Cd changes the metabolism of nutrients, amino acids, and carbohydrates; degrades proteins and lipids; and damages the photosynthetic apparatus, among other factors (BENAVIDES; GALLEGO; TOMARO, 2005). However, biomass production of root of Massai grass supplied with highest S concentration was 130% higher as compared with biomass production of grass cultivated with lowest S concentration, when 0.1 mmol L^{-1} Cd was provided in nutrient solution, which is associated with highest synthesis of amino acids, GSH, and PCs found under proper S supply in other study with Massai grass (Figure 3, p. 127; Figure 4, p. 128; Figure 5, p. 129; Figures 6A-F, p. 131), since these metabolites are involved in Cd detoxification (COBBETT; GOLDSBROUGH, 2002; SETH et al., 2012). Furthermore, S metabolism is involved in the synthesis of essential hormones for adaptation of plants to adverse conditions, such as Cd exposure (CAPALDI et al., 2015). On the other hand, highest S supply increased the damage caused by largest Cd concentration to Massai grass (Table 3), in some sort of double toxicity (Cd and S). The toxicity caused by S may occur when plant metabolism is altered (WARD, 1976) and, in this situation, the synthesis of GSH is also highest, but is aimed at signaling the lowest need for S uptake (HERSCHBACH; RENNENBERG, 1994).

Changes in the root biomass production of Massai grass resulted in Cd depletion over time. This depletion in solution of plants supplied with highest S concentration (highest biomass production) was 26% higher as compared with depletion in solution of plants grown under lowest concentrations of S and Cd (lowest biomass production) (Figures 4A-B), while there was no effect of S on Cd depletion in solution of plants exposed to highest Cd concentration (biomass production unchanged) after 12 h of exposure (Figures 4C-D). Cadmium uptake can occur either passive (LATS) or active (HATS) transport, and Cd can cross the plasma membrane through channels of divalent cations (e.g. Ca^{+2} and Zn^{+2}) or by Cd-specific transporter proteins (SLOOF; VIRAGH; van der VEER, 1995; CHEN et al., 2008). This uptake can be simulated by Michaelis-Menten constant (V_{max} and K_m). V_{max} (defined as the product between the number of uptake sites per root unit by its working velocity; EPSTEIN; BLOOM, 2005) of Massai grass exposed to lowest Cd concentration was not changed ($P > 0.05$) by S supply, unlike V_{max} of Massai grass exposed to highest Cd concentration (Table 4). Higher V_{max} observed in Massai grass exposed to highest Cd and S concentrations in relation to lowest S concentration may be related to

alteration in the root architecture (PERRIGUEY; STERCKEMAN; MOREL, 2008; REDJALA; STERCKEMAN; MOREL, 2009), since the uptake area (more uptake sites) of root system in these plants was larger than elongation area (fewer uptake sites). Plants supplied with highest S concentration possibly had a higher number of cortical cell layers compared to plants cultivated with lowest S concentration, wich could lead to a difference in ratio between the exposed plasmalemma area and the root biomass, which in turn would produce a difference in uptake rate (V_{max}) (ZELKO; LUX; CZIBULA, 2008; REDJALA; STERCKEMAN; MOREL, 2009). Bouranis et al. (2006) related that S-deprivation resulted in shorter lateral roots in upper sectors and in a limited extension of lignified layers towards the next lateral root carrying sector in maize.

Although V_{max} of Massai grass exposed to highest Cd concentration was changed by S concentrations ($P < 0.05$), K_m remained constant (Table 4), indicating that affinity of Cd by uptake sites of root system of grass is not modified by S supply, just as Cd does not change the affinity of S by root uptake sites (NOCITO et al., 2002). In addition, K_m of Massai grass exposed to Cd concentration of 0.1 mmol L^{-1} was 84 and 83% lower in relation to K_m of plants exposed to highest Cd concentration when S was supplied at 0.1 and 1.9 mmol L^{-1}, but the amount of Cd absorbed (Cd concentration x biomass production) was 66 and 963% higher for the same comparison. It is important to note that in studies carried out with relatively high Cd concentrations (> 90 μmol L^{-1}), physical uptake parameters (diffusion and irreversible binding) tend to be accentuated, whereas active transport (HATS and LATS) characteristics are minimized (CATALDO; GARLAND; WILDUNG, 1983). This indicates that K_m cannot be used as the main factor to explain the elevated Cd uptake by plants grown in contaminated environments (REDJALA; STERCKEMAN; MOREL, 2009). In this regard, Cd uptake (high concentrations) probably is more deeply affected by architecture than by uptake capacity (V_{max} and K_m) of plant root (REDJALA; STERCKEMAN; MOREL, 2009, 2010). This assumption becomes even clearer as we observe the negative correlation between α (V_{max}/K_m) and biomass production of root system (Table 6) and high C_{min} values (Table 4). High C_{min} values (concentration in which influx is equal to efflux) are indicative of serious damage to plasma membrane that may lead to cell death (MARSCHNER, 1995). V_{max} values verified in this study are relatively smaller and K_m values are relatively higher than those pointed out in other studies (CATALDO; GARLAND; WILDUNG, 1983; HART et al., 1998, 2002). This result probably occurred by use of complete nutritive solution (Table 1), since the increase of concentration of other divalent cations may inhibit Cd^{2+} influx due to reduction of plasma membrane

electronegativity, non-competitive inhibition of influx by a variety of cations, or direct competition for specific cation transporters such as observed between Cd^{2+} and Zn^{2+} (COSTA; MOREL, 1993; KINRAIDE, 2001; HART et al., 2002; HARRIS; TAYLOR, 2004).

Distinguishing uptake into symplast from apoplastic binding is a major problem in studies of root uptake of divalent cations, such as Cd (HARRIS; TAYLOR, 2004). At concentrations above 5 μmol L^{-1} Cd, the physical uptake (Cd irreversibly bound to root cell wall) can seriously distort any kinetic interpretation of Cd uptake in plants, which can explains the similarities in symplastic uptake (Figure 5B) between the most damaged root tissues (1.9 mmol L^{-1} S + 0.5 mmol L^{-1} Cd) and less damaged (0.1 mmol L^{-1} S + 0.5 mmol L^{-1} Cd) (CATALDO; GARLAND; WILDUNG, 1983). Influx of Cd across the plasma membrane of root cells has been shown to occur via a concentration-dependent process exhibiting saturable kinetics, that Cd uptake by roots is controlled by a transport protein in plasmalemma (LUX et al., 2011). Several results indicate that Cd gets through the plasmalemma of root cells in an opportunistic way, via other divalent cation carriers or channels, such as those for Zn^{2+}, Cu^{2+}, Fe^{2+} or Ca^{2+}, that show poor selectivity (CATALDO; GARLAND; WILDUNG, 1983; COSTA; MOREL, 1993; ZHAO et al., 2002; HAN et al., 2006). A saturable symplastic influx (Figures 5A-B) component supports the existence of membrane transporter-mediated uptake, which in low external concentrations can be regarded as HATS (REDJALA; STERCKEMAN; MOREL, 2009). Cadmium species transported through the symplasm are unknown, but could include Cd^{2+} (predominant specie in nutrient solution used in this study; Table 2) or Cd-chelates (LUX et al., 2011). Plants grown under lowest S concentration showed higher symplastic influx of Cd as compared with those supplied with highest S concentration, when Cd was supplied at 0.1 mmol L^{-1} (Figure 5A). Plants well nourished in S usually present highest synthesis of PCs when exposed to Cd (BASHIR et al., 2015). Phytochelatins act in sequestration of free Cd^{2+} ions in cytosol, transporting them to organelles less sensitive to damage caused by Cd, such as vacuoles (COBBETT; GOLDSBROUGH, 2002). Thus, Cd accumulation in vacuole of plants well nourished in S tends to be largest and in plants grown with low S supply the accumulation tends to be smallest, which facilitates Cd transport to xylem by symplastic via. Moreover, plants grown with proper S supply display detoxification mechanisms, such as Cd efflux from cytoplasm to apoplast, which is more efficient in comparison with plants cultivated with low S supply, such that symplastic influx of Cd is usually lowest under such conditions (NOCITO et al., 2002; JASINSKI et al., 2003; VAN BELLEGHEM et al., 2007). This fact becomes clearer as we

observe that difference between Cd concentration in the root symplast and root apoplast of Massai grass supplied with lowest S concentration was lower than plants provided with highest S concentration (Table 5). In this way, proper S supply benefits Cd accumulation in the root apoplast of Massai grass, which is desirable, since the main accumulation site of Cd in tolerant plants is root apoplast (WÓJCIK et al., 2005; AKHTER et al., 2014). By contrast, symplastic influx of Cd of plants exposed to highest Cd concentration was highest when there was highest S supply (Figure 5B). This result is possibly linked to partial loss of selectivity of plasma membrane (high C_{min} values), which may occur under high toxicity conditions (toxicity by Cd and S) and lead to greater symplastic uptake (ZHAO et al., 2002). In all treatments the linear component verified in Figures 5A-B probably reflects a second pathway for Cd into symplast (LATS) as demonstrated by Redjala, Sterckeman and Morel (2009). The linear component of Cd uptake system is likely related to that Cd uptake may be passive (COSTA; MOREL, 1994) through channels of other divalent cations like Ca which are permeable to Cd (LOMBI et al., 2001; ZHAO et al., 2002). This LATS for Cd uptake was shown in concentration-dependent kinetics on plant organelles (LUX et al., 2011). The similarity of slope for all treatments (Figures 5A-B) suggests that S not change the Cd uptake by LATS. The transition concentration that determines the switch between HATS and LATS depends on ion (MARSCHNER, 1995). For Cd, it seems that this switch is much lower for non-accumulator plants than for hyperaccumulator plants (REDJALA; STERCKEMAN; MOREL, 2009). Massai grass probably is a Cd hyperaccumulator plant, but the transition concentration that determined the switch between HATS and LATS was lower (~1 μmol L^{-1}) for Massai grass exposed to Cd concentration of 0.1 mmol L^{-1} (Figure 5A) than switch related by Redjala, Sterckeman and Morel (2009) for *Noccaea caerulescens* (10 μmol L^{-1}) exposed to 50 μmol L^{-1}, suggesting that Cd uptake by Massai grass occurs predominantly through the LATS and the apoplastic via in high Cd concentrations.

Because of high Cd concentrations to which Massai grass was exposed, we chose to use the equation described by Lombi et al. (2001) to calculate the apoplastic influx, since there are indications that linear component of this equation can represent the apoplastic influx of Cd (LUX et al., 2011). The adoption of this equation for this purpose is not usual, but employing inadequate protocols to remove Cd from root apoplast may cause high efflux of Cd from symplast, mainly in cases when plasma structure is seriously damaged (high C_{min} values; Table 4), which may compromise the reliability of results (LOMBI et al., 2001; HARRIS; TAYLOR, 2004). In this sense, apoplastic uptake function that could be useful for modeling Cd transport is not available from literature, since in most studies of Cd uptake kinetics,

desorption processes were performed, which more or less eliminated the apoplastic uptake (REDJALA; STERCKEMAN; MOREL, 2009). However, the contribution of apoplast to Cd total root uptake is very important, mainly when Cd concentration is high. If apoplastic barriers (particularly Casparian bands and suberin deposits) can blocking the cation flow towards stele and xylem (LUX et al., 2011), the apoplastic influx will accumulate Cd in the root only, while the sole symplastic influx would contribute to shoot content (REDJALA; STERCKEMAN; MOREL, 2009). However, it is also possible for Cd^{2+} and Cd-chelates to reach the xylem solely via an extracellular, apoplasmic pathway in regions of the root lacking a Casparian band (LUX et al., 2004). In this situation, the apoplastic root influx should be considered as an important mechanism in understanding the phytoaccumulation of this metal (REDJALA; STERCKEMAN; MOREL, 2009). Apoplastic influx of Cd by root system of Massai grass exposed to Cd concentrations of 0.1 and 0.5 mmol L^{-1} was higher when plants were grown with highest S supply (Figures 5C-D). The amount and composition of suberin are among the major factors that limit apoplastic displacement of water and inorganic ions (SCHREIBER et al., 2005). In this sense, it is possible that Massai grass supplied with highest S concentration tested in this study shows lowest deposition of suberin between epidermis and cortex (apoplastic via), since the synthesis of suberin can be regulated by ethylene, and plants well nourished with S show lowest ethylene synthesis when exposed to Cd (BARBERON et al., 2016; KHAN et al., 2016). In this context, lowest deposition of suberin (mainly in regions near the Caspary strips) in plants grown with lowest S supply may facilitate the apoplastic transport of Cd to shoots, but this fact can increase the damage caused by Cd (REDJALA; STERCKEMAN; MOREL et al., 2009). Redjala, Sterckeman and Morel (2010) stated that apoplastic uptake of Cd by corn plants ranged from 15 to 82% as a function of increase in Cd concentration, whereas apoplastic uptake of Cd by Massai grass exposed to lowest Cd concentration ranged from 58.5 (0.1 mmol L^{-1}) to 67.8% (1.9 mmol L^{-1}) as S concentration was increased (Figure 6).

The reduced slope of linear component in intact roots of plants supplied with highest S concentration in relation to lowest S concentration (Figures 5C-D) was therefore probably the result of reduced Cd binding to cell wall constituents (e.g. cellulose, hemicellulose, and proteins) due the lowest deposition of suberin between epidermis and cortex (apoplastic via) (HART et al., 1998). Costa and Morel (1993) verified that exchangeable fraction represented about 5% of total Cd absorbed in the roots of *Lupinus albus* due to presence of anionic charges on cell wall (carboxylic groups). Cell wall, intercellular spaces and outer surface of plasmalema delimit the apparent free space (AFS), where the element can enter by mass flow,

diffusion, ion exchange and Donnan equilibrium (MARSCHNER, 1995). However, the elements can also leave the AFS, since this process is reversible. Thus, physical interactions (eg, electrostatic forces) such as cation exchange capacity (CEC) of carboxylic groups may influence apoplastic uptake of Cd (REDJALA; STERCKEMAN; MOREL, 2009). Although CEC is not an essential step in passage of ions across the plasma membrane to cytoplasm, it has an indirect effect, which is increase of cations concentration in apoplast (MARSCHNER, 1995). There is a rapid Cd accumulation in apoplasm which is freely accessible to outer solution when Cd concentration in nutrient solution is high (REDJALA; STERCKEMAN; MOREL, 2010). The relative contribution of apoplastic via increased when Massai grass was cultivated with highest S concentration (Figure 6), what suggest that S changes the composition of cell wall and, consequently, the CEC of roots of Massai grass exposed to Cd. Retention of Cd in the roots is a factor that contributing to low Cd accumulation in shoots (HARRIS; TAYLOR, 2004), but in other study we verified that Cd content in shoots of Massai grass (Table 7, p. 91) was very high what suggest that Cd^{2+} and Cd-chelates to reach xylem via an extracellular, apoplasmic pathway in regions of root lacking a Casparian band in this plant. In this sense, it is possible that Cd uptake through PCs-Cd in AFS has contributed to highest Cd apoplastic uptake in plants cultivated with highest S concentration, since plants properly supplied with S present high synthesis of PCs when exposed to Cd (BASHIR et al., 2015). Mendoza-Cózatl et al. (2008) described that PCs are involved in Cd transport from roots to shoots in *Brassica napus*.

Based on conditions presented here, we observed that irrespective of toxicity caused by highest S supply to plants exposed to highest Cd concentration, the contribution of apoplastic pathway in Cd uptake by Massai grass is a little higher (Figure 6) under higher concentrations of S in the root tissue (Table 5) in relation to lowest concentration, and that part of Cd absorbed via apoplast by these plants is accumulated in symplast (Figure 6), which suggests that Cd not binding to cell wall of apoplast may enters the cells through transporters of ZIP family located in root apoplast (GUERINOT, 2000; LUX et al., 2011). After the entering into cells, Cd is complexed to S-containing molecules, such as Cd-PCs, Cd-PCs- sulfide and/or Cd-sulfide (VAN BELLEGHEM et al., 2007). Thus, analyzing the values in absolute terms (Cd concentration x mass production), we note that greatest S supply to plants exposed to Cd concentration of 0.1 mmol L^{-1} increased the amount of Cd absorbed by 129% in relation to plants grown under lowest S supply, indicating that regardless of predominant mechanism in Cd uptake (symplastic or apoplastic), S supply is essential for phytoextraction process, possibly due to greatest synthesis of PCs (RABÊLO; AZEVEDO; MONTEIRO, 2017a).

To exemplify, Larsson, Asp and Bronman (2002) described that Cd uptake by wild *Arabdopsis thaliana* plants was approximately 40% higher in relation to uptake by plants containing the mutant *cad1-3* (lowest activity of PC synthase enzyme, which controls the synthesis of PCs).

3.5 Conclusions

Massai grass exposure to high Cd concentrations for longer periods indicated that this forage grass does not feature efficient detoxification mechanisms to be used in phytoextraction processes in environments with Cd concentrations higher than 0.1 mmol L^{-1}. However, Massai grass grown under proper S supply showed tolerance to Cd (0.1 mmol L^{-1}), and thus we recommend sulfate fertilization for forage grasses used in Cd extraction process.

Proper S supply decreased symplastic and increased apoplastic Cd uptake by Massai grass exposed to lowest Cd concentration. On the other hand, part of Cd taken up via apoplast was accumulated in symplast at the end of growth of Massai grass. Therefore, the influence exerted by Cd transition (symplast-apoplast-symplast) upon the tolerance of plants cultivated under proper S supply should be better investigated.

References

AKHTER, M.F.; OMELON, C.R.; GORDON, R.A.; MOSER, D.; MACFIE, S.M. Localization and chemical speciation of cadmium in the roots of barley and lettuce. **Environmental and Experimental Botany**, Oxford, v. 100, p. 10-19, 2014.

ANJUM, N.A.; HASANUZZAMAN, M.; HOSSAIN, M.; THANGAVEL, P.; ROYCHOUDHURY, A.; GILL, S.S.; RODRIGO, M.A.M.; ADAM, V.; FUJITA, M.; KIZEK, R.; DUARTE, A.C.; PEREIRA, E.; AHMAD, I. Jacks of metal / metalloid chelation trade in plants - an overview. **Frontiers in Plant Science**, Lausanne, v. 6, 2015. doi: 10.3389/fpls.2015.00192.

BARBERON, M.; VERMEER, J.E.M.; DE BELLIS, D.; WANG, P.; NASSER, S.; ANDERSEN, T.G.; HUMBEL, B.M.; NAWRATH, C.; TAKANO, J.; SALT, D.E.; GELDNER, N. Adaptation of root function by nutrient-induced plasticity of endodermal differentiation. **Cell**, Cambridge, v. 164, p. 447-459, 2016.

BASHIR, H.; IBRAHIM, M.M.; BAGHERI, R.; AHMAD, J.; ARIF, I.A.; BAIG, M.A.; QURESHI, M.I. Influence of sulfur and cadmium on antioxidants, phytochelatins and growth in Indian mustard. **AoB Plants**, Oxford, v. 7, 2015. doi: 10.1093/aobpla/plv001.

BENAVIDES, M.P.; GALLEGO, S.M.; TOMARO, M.L. Cadmium toxicity in plants. **Brazilian Journal of Plant Physiology**, Campos dos Goytacazes, v. 17, p. 21-34, 2005.

BOURANIS, D.L.; CHORIANOPOULOU, S.N.; KOLLIAS, C.; MANIOU, P.; PROTONOTARIOS, V.E.; SIYIANNIS, V.; HAWKESFORD, M.J. Dynamics of aerenchyma distribution in the cortex of sulfate-deprived adventitious roots of Maize. **Annals of Botany**, Oxford, v. 97, p. 695-704, 2006.

BUCHER, C.A.; SANTOS, L.A.; NOGUEIRA, E.M.; RANGEL, R.P.; SOUZA, S.R.; FERNANDES, M.S. The transcription of nitrate transporters in upland rice varieties with contrasting nitrate-uptake kinetics. **Journal of Plant Nutrition and Soil Science**, Weinheim, v. 177, p. 395-403, 2014.

CAPALDI, F.R.; GRATÃO, P.L.; REIS, A.R.; LIMA, L.W.; AZEVEDO, R.A. Sulfur metabolism and stress defense responses in plants. **Tropical Plant Biology**, New York, v. 8, p. 60-73, 2015.

CATALDO, D.A., GARLAND, T.R., WILDUNG, R.E. Cadmium uptake kinetics in intact soybean plants. **Plant Physiology**, Rockville, v. 73, p. 844-848, 1983.

CHEN, W.; LI, L.; CHANG, A.C.; WU, L.; KWON, S.; BOTTOMS, R. Modeling uptake kinetics of cadmium by field-grown lettuce. **Environmental Pollution**, Oxford, v. 152, p. 147-152, 2008.

CLAASSEN, N., BARBER, S.A. A method for characterizing the relation between nutrient concentration and flux into roots of intact plants. **Plant Physiology**, Rockville, v. 54, p. 564-568, 1974.

COBBETT, C.; GOLDSBROUGH, P. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. **Annual Review of Plant Biology**, Palo Alto, v. 53, p. 159-182, 2002.

COSTA, G.; MOREL, J.L. Cadmium uptake by *Lupinus albus* (L.): cadmium excretion, a possible mechanism of cadmium tolerance. **Journal of Plant Nutrition**, Philadelphia, v. 16, p. 1921-1929, 1993.

COSTA, G.; MOREL, J.L. Efficiency of H^+-ATPase activity on cadmium uptake by four cultivars of lettuce. **Journal of Plant Nutrition**, Philadelphia, v. 17, p. 627-637, 1994.

EPSTEIN, E.; HAGEN, C.E. A kinetic study of the absorption of alkali cation by barley roots. **Plant Physiology**, Rockville, v. 27, p. 457-474, 1952.

EPSTEIN, E.; BLOOM, A.J. **Mineral nutrition of plants: principles and perspectives**. 2. ed. Sunderland: Sinauer Associates, 2005. 380 p.

GUERINOT, M.L. The ZIP family of metal transporters. **Biochimica et Biophysica Acta**, Amsterdam, v. 1465, p. 190-198, 2000.

GUSTAFSSON, J.P. **Visual MINTEQ**. Version 3.0. Stockholm, 2012. Available at: <http://www2.lwr.kth.se/English/OurSoftware/vminteq/Stockholm-Sweden>. Access in: oct. 25. 2017.

HAN, F.; SHAN, X.; ZHANG, S.; WEN, B.; OWENS, G. Enhanced cadmium accumulation in maize roots - the impact of organic acids. **Plant and Soil**, Dordrecht, v. 289, p. 355-368, 2006.

HARRIS, N.S.; TAYLOR, G.J. Cadmium uptake and translocation in seedlings of near isogenic lines of durum wheat that differ in grain cadmium accumulation. **BMC Plant Biology**, London, v. 4, 2004. doi: 10.1186/1471-2229-4-4.

HART, J.J.; WELCH, R.M.; NORVELL, W.A.; SULLIVAN, L.A.; KOCHIAN, L.V. Characterization of cadmium binding, uptake, and translocation in intact seedlings of bread and durum wheat cultivars. **Plant Physiology**, Rockville, v. 116, p. 1413-1420, 1998.

HART, J.J.; WELCH, R.M.; NORVELL, W.A.; KOCHIAN, L.V. Transport interactions between cadmium and zinc in roots of bread and durum wheat seedlings. **Physiologia Plantarum**, Lund, v. 116, p. 73-78, 2002.

HE, J.Y.; ZHU, C.; REN, Y.F.; JIANG, D.A.; SUN, Z.X. Root morphology and cadmium uptake kinetics of the cadmium sensitive rice mutant. **Biologia Plantarum**, Prague, v. 51, p. 791-794, 2007.

HERSCHBACH, C.; RENNENBERG, H. Influence of glutathione (GSH) on net uptake of sulphate and sulphate transport in tobacco plants. **Journal of Experimental Botany**, Oxford, v. 45, p. 1069-1076, 1994.

JASINSKI, M.; DUCOS, E.; MARTINOIA, E.; BOUTRY, M. The ATP-binding cassette transporters: structure, function, gene family comparison between rice and *Arabidopsis*. **Plant Physiology**, Rockville, v. 131, p. 1169-1177, 2003.

KHAN, M.N.A.; ASGHER, M.; PER, T.S.; MASSOD, A.; FATMA, M.; KHAN, M.I.R. Ethylene potentiates sulfur-mediated reversal of cadmium inhibited photosynthetic responses in mustard. **Frontiers in Plant Science**, Lausanne, v. 7, 2016. doi: 10.3389/fpls.2016.01628.

KELETI, T.; WELCH, G.R. The evolution of enzyme kinetic power. **Biochemical Journal**, London, v. 223, p. 299-303, 1984.

KINRAIDE, T.B. Ion fluxes considered in terms of membrane-surface electrical potentials. **Australian Journal of Plant Physiology**, Victoria, v. 28, p. 605-616, 2001.

LARSSON, E.H.; ASP, H.; BORNMAN, J.F. Influence of prior Cd^{2+} exposure on the uptake of Cd^{2+} and other elements in the phytochelatin-deficient mutant, *cad1-3*, of *Arabidopsis thaliana*. **Journal of Experimental Botany**, Oxford, v. 53, p. 447-453, 2002.

LAVRES JUNIOR, J.; MORAES, M.F.; CABRAL, C.P.; MALAVOLTA, E. Genotypic influence on the absorption and toxicity of manganese in soybean. **Revista Brasileira de Ciência do Solo**, Viçosa, v. 32, p. 173-181, 2008.

LEE, R.B.; RUDGE, R.A. Effects of nitrogen deficiency on the absorption of nitrate and ammonium by barley plants. **Annals of Botany**, Oxford, v. 57, p. 471-486, 1986.

LOMBI, E.; ZHAO, F.J.; McGRATH, S.P.; YOUNG, S.D.; SACCHI, G.A. Physiological evidence for a high-affinity cadmium transporter highly expressed in a *Thlaspi caerulescens* ecotype. **New Phytologist**, London, v. 149, p. 53-60, 2001.

LUX, A.; ŠOTTNÍKOVÁ, A.; OPATRNÁ, J.; GREGER, M. Differences in structure of adventitious roots in Salix clones with contrasting characteristics of cadmium accumulation and sensitivity. **Physiologia Plantarum**, Lund, v. 120, p. 537-545, 2004.

LUX, A.; MARTINKA, M.; VACULÍK, M.; WHITE, P.J. Root responses to cadmium in rhizosphere: a review. **Journal of Experimental Botany**, Oxford, v. 62, p. 21-37, 2011.

MALAVOLTA, E.; VITTI, G.C.; OLIVEIRA, S.A. **Avaliação do estado nutricional das plantas**: princípios e aplicações. 2. ed. Piracicaba: Potafos, 1997. 201 p.

MARSCHNER, H. **Mineral nutrition of higher plants**. San Diego: Academic Press, 1995. 889 p.

MENDOZA-CÓZATL, D.G.; BUTKO, E.; SPRINGER, F.; TORPEY, J.W.; KOMIVES, E.A.; KEHR, J.; SCHROEDER, J.I. Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. **Plant Journal**, Hoboken, v. 54, p. 249-259, 2008.

MORI, S.; KAWASAKI, A.; ISHIKAWA, S.; ARAO, T. A new method for evaluating symplastic cadmium absorption in the root of *Solanum melongena* using enriched isotopes ^{113}Cd and ^{114}Cd. **Soil Science and Plant Nutrition**, Oxon, v. 55, p. 294-299, 2009.

NOCITO, F.F.; PIROVANO, L.; COCUCCI, M.; SACCHI, G.A. Cadmium-induced sulfate uptake in maize roots. **Plant Physiology**, Rockville, v. 129, p. 1872-1879, 2002.

PERRIGUEY, J.; STERCKEMAN, T.; MOREL, J.L. Effect of rhizosphere and plant-related factors on the cadmium uptake by maize (*Zea mays* L.). **Environmental and Experimental Botany**, Oxford, v. 63, p. 333-341, 2008.

RABÊLO, F.H.S.; BORGO, L. Changes caused by heavy metals in micronutrient content and antioxidant system of forage grasses used for phytoremediation: an overview. **Ciência Rural**, Santa Maria, v. 46, p. 1368-1375, 2016.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. Proper supply of S increases GSH synthesis in the establishment and reduces tiller mortality during the regrowth of Tanzania guinea grass used for Cd phytoextraction. **Journal of Soils and Sediments**, Heidelberg, v. 17, p. 1427-1436, 2017a.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. The proper supply of S increases amino acid synthesis and antioxidant enzyme activity in Tanzania guinea grass used for Cd phytoextraction. **Water, Air and Soil Pollution**, Dordrecht, v. 228, 2017b. doi: 10.1007/s11270-017-3563-6.

REDJALA, T.; STERCKEMAN, T.; MOREL, J.L. Cadmium uptake by roots: contribution of apoplast and of high- and low-affinity membrane transport systems. **Environmental and Experimental Botany**, Oxford, v. 67, p. 235-242, 2009.

REDJALA, T.; STERCKEMAN, T.; MOREL, J.L. Determination of the different components of cadmium short-term uptake by roots. **Journal of Plant Nutrition and Soil Science**, Weinheim, v. 173, p. 935-945, 2010.

RUIZ, H.A. A graphico-mathematical method for the estimation of the kinetic parameters, K_m and V_{max}. **Revista Ceres**, Viçosa, v. 32, p. 79-84, 1985.

RUIZ, H.A.; FERNANDES FILHO, E.I. Cinética: Software para estimar as constantes V_{max}, K_m da equação de Michaelis-Menten. In: REUNIÃO BRASILEIRA DE FERTILIDADE DO SOLO E NUTRIÇÃO DE PLANTAS, 20., 1992, Piracicaba. **Proceedings...** Campinas: Fundação Cargill, 1992. p. 124-125.

SAS INSTITUTE. **SAS User's guide**: Statistics. Version 9.2. Cary, NC, 2008. Available at: <http://support.sas.com/software/92/ Cary-United States of America>. Access in: oct. 25. 2017.

SCHREIBER, L.; FRANKE, R.; HARTMANN, K.D.; RANATHUNGE, K.; STEUDLE, E. The chemical composition of suberin in apoplastic barriers affects radial hydraulic conductivity differently in the roots of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix). **Journal of Experimental Botany**, Oxford, v. 56, p. 1427-1436, 2005.

SEHT, C.S.; REMANS, T.; KEUNEN, E.; JOZEFCZAK, M.; GIELEN, H.; OPDENAKKER, K.; WEYENS, N.; VANGRONSVELD, J.; CUYPERS, A. Phytoextraction of toxic metals: a central role for glutathione. **Plant, Cell and Environment**, Oxford, v. 35, p. 334-346, 2012.

SHEORAN, V.; SHEORAN, A.S.; POONIA, P. Factors affecting phytoextraction: a review. **Pedosphere**, Beijing, v. 26, p. 148-166, 2016.

SLOOF, J.E.; VIRAGH, A.; van der VEER, B. Kinetics of cadmium uptake by green algae. **Water, Air and Soil Pollution**, Dordrecht, v. 83, p. 105-122, 1995.

STERCKEMAN, T.; REDJALA, T.; MOREL, J.L. Influence of exposure solution composition and of plant cadmium content on root cadmium short-term uptake. **Environmental and Experimental Botany**, Oxford, v. 74, p. 131-139, 2011.

STRITSIS, C.; CLAASSEN, N. Cadmium uptake kinetics and plants factors of shoot Cd concentration. **Plant and Soil**, Dordrecht, v. 367, p. 591-603, 2013.

SYSTAT SOFTWARE INC. **Sigmaplot**: guide of personal computers. Version 13.0. San Jose, CA, 2015. Available at: <http://www.sigmaplot.co.uk/London-UnitedKingdon>. Access in: oct. 25. 2017.

TINKER, P.B.; NYE, P.H. **Solute movement in the rhizosphere**. New York: Oxford University Press, 2000. 444 p.

VAN BELLEGHEM, F.; CUYPERS, A.; SEMANE, B.; SMEETS, K.; VANGRONSVELD, J.; d'HAEN, J.; VALCKE, R. Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. **New Phytologist**, London, v. 173, p. 495-508, 2007.

WANG, J.J. Kinetics of manganese uptake by excised roots of sensitive and tolerant tobacco genotypes. **Journal of Plant Nutrition**, Philadelphia, v. 26, p. 1439-1450, 2003.

WARD, G.M. Sulphur deficiency and toxicity symptoms in greenhouse tomatoes and cucumbers. **Canadian Journal of Plant Science**, Ottawa, v. 56, p. 133-137, 1976.

WÓJCIK, M.; VANGRONSVELD, J.; D'HAEN, J.; TUKIENDORF, A. Cadmium tolerance in *Thlaspi caerulescens*. II. Localization of cadmium in *Thlaspi caerulescens*. **Environmental and Experimental Botany**, Oxford, v. 53, p. 163-171, 2005.

XU, Y.; SHI, D.; ARISTILDE, L.; MOREL, F.M.M. The effect of pH on the uptake of zinc and cadmium in marine phytoplankton: Possible role of weak complexes. **Limnology and Oceanography**, Hoboken, v. 57, p. 293-304, 2012.

ZELKO, I.; LUX, A.; CZIBULA, K. Difference in the root structure of hyperaccumulator *Thlaspi caerulescens* and non-hyperaccumulator *Thlaspi arvense*. **International Journal of Environment and Pollution**, Geneva, v. 33, p. 123-132, 2008.

ZHAO, F.J.; HAMON, R.E.; LOMBI, E.; McLAUGHLIN, M.J.; McGRATH, S.P. Characteristics of cadmium uptake in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens*. **Journal of Experimental Botany**, Oxford, v. 53, p. 535-543, 2002.

4 PROPER S SUPPLY REDUCES THE DAMAGE OF HIGH Cd EXPOSURE IN ROOTS AND INCREASES N, S AND Mn UPTAKE FAVORING PHYTOREMEDIATION WITH MASSAI GRASS

Abstract

There are no studies showing S effect on root development and nutrient uptake in forage grasses used for Cd phytoextraction. In this sense, our aim in this study was to evaluate in an integrated manner the effect of S supply (0.1, 1.9, and 3.7 mmol L^{-1}) on root development and nutrient uptake of *Panicum maximum* Jacq. cv. Massai (Massai grass) exposed to Cd (0.1, and 0.5 mmol L^{-1}), and it implications for Cd phytoextraction. After 44 days of growth in nutrient solution, Massai grass was exposed to combinations of S and Cd concentrations for 9 days. Plants were then harvested and separated into leaf blades, stems and sheaths, and roots. Our results showed that root development of Massai grass was strongly inhibited when plants were exposed to 0.5 mmol L^{-1} Cd, but proper S supply (1.9 mmol L^{-1}) allowed higher Cd uptake, while excessive S supply (3.7 mmol L^{-1}) decreased Fe plaques formation in plant roots. On the other hand, there was a greater development of roots when Massai grass was exposed to 0.1 mmol L^{-1} Cd, while some strategies to restrict Cd entry, such as deposition of suberin and lignin in endodermis and G-layer development, were observed. Proper S supply to these plants increased root length, root surface and N, S, and Mn concentrations. Thus, it seems evident that Massai grass plants well supplied with S present a great potential to cope with Cd toxicity and fulfill the phytoextraction strategy.

Keywords: anatomical changes, biomass partition, ionomics, root development.

4.1 Introduction

Cadmium (Cd) concentrations in soils of many regions of the world have increased in recent decades mainly due to anthropogenic activity, such as inappropriate municipal and industrial waste disposal and the application of agricultural products (e.g. phosphate fertilizer and sewage) of poor quality (HE et al., 2015). This fact is a serious socio-environmental problem, since Cd is toxic to plants, animals and humans (CLEMENS; FENG MA, 2016). Therefore, it is fundamental to decrease Cd concentrations in contaminated soils, for which a low cost alternative and low environmental impact is phytoextraction. Phytoextraction involves Cd removal from soil by harvesting the shoots of plants established in these environments (MAHAR et al., 2016; SHEORAN; SHEORAN; POONIA, 2016). However,

few plant species have capacity to hyperaccumulate Cd, which stimulates research on alternative species, such as forage grasses (RABÊLO; BORGO, 2016; RABÊLO; AZEVEDO; MONTEIRO, 2017a) for this purpose. Forage grasses probably can offset the lower Cd concentration in relation to Cd hyperaccumulators plants due to higher biomass production, especially when grown with proper sulfur (S) supply (RABÊLO; BORGO, 2016; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b). Sulfur is essential to mitigate the damage caused by Cd to plants, since this nutrient is a component of several metabolites (e.g. reduced glutathione - GSH and phytochelatins - PCs), which act on detoxification of this metal (CAPALDI et al., 2015; RABÊLO; AZEVEDO; MONTEIRO, 2017a). In addition, proper S supply can at least partially restore ionic equilibrium and enhance macronutrient accumulation in plants exposed to Cd, alleviating Cd-induced toxicity (MATRASZEK et al., 2016a; 2017).

The first negative impact caused by Cd to plants is related to nutrient uptake, since this metal competes for the same nutrient uptake sites as Ca, Cu, Fe, Mn and Zn (CLEMENS, 2006). Azevedo, Pinto and Santos (2005) reported that Ca, Mg, Cu, Fe and Mn concentrations in the root system of sunflower (*Helianthus annuus*) exposed to Cd concentrations of 0.05 and 0.5 mmol L^{-1} were lower than in non-exposed plants. The decrease in nutrients concentrations can seriously compromise the development of plants and limit the phytoextraction of elements such as Cd (NAZAR et al., 2012). After being taken up, Cd can alter root morphology and limit uptake and transport of nutrients from root to shoot (LUX et al., 2011). Gratão et al. (2015) reported that P, Ca and Mg concentrations in the root and shoot of tomato (*Solanum lycopersicum*) exposed to 1 mmol L^{-1} Cd were lower than control treatment. However, P, K and Mg concentrations in soybean (*Glycine max*) (DRAŽIĆ; MIHAILOVIĆ; STOJANOVIĆ, 2004), Fe and Zn in mustard (*Brassica juncea*) (JIANG et al., 2004), and Cu, Fe, Mn and Zn in tomato (GRATÃO et al., 2015) increased when plants were grown in Cd presence. This variation in nutrients concentration in plants exposed to Cd is related, among other factors, to Cd content *in situ* and Cd detoxification mechanisms (VAN BELLEGHEM et al., 2007).

Proper S supply to Massai grass (*Panicum maximum* Jacq. cv. Massai) resulted in highest Cd concentration in root apoplast (Table 5, p. 58), which is very significant since highest Cd content in apoplasmic space has been reported in metal-tolerant plants (WÓJCIK et al., 2005). Many studies have described Cd accumulation sites in roots (VAN BELLEGHEM et al., 2007; AKHTER et al., 2014; ZHANG et al., 2014) and the beneficial effect of S on Cd tolerance (BABULA et al., 2009; BASHIR et al., 2015; RABÊLO; AZEVEDO; MONTEIRO, 2017b), but little is known about how S interferes in

nutrient uptake and root development (MATRASZEK et al., 2016b; 2017), especially in plants used in Cd phytoextraction processes (DEDE; OZDEMIR, 2016). Plants grown under Cd exposure usually exhibit reduced root development and formation of root iron (Fe) plaques, which limits Cd phytoextraction (SEBASTIAN; PRASAD, 2016). In this sense, Fan et al. (2010) reported that excessive S supply decreased root Fe plaques formation in rice (*Oryza sativa*) exposed to Cd, resulting in highest Cd content in the roots. Hence, our aim was to evaluate in an integrated manner the effect of S supply on root development and nutrient uptake in Massai grass exposed to Cd, and it implications in Cd phytoextraction.

4.2 Materials and methods

4.2.1 Plant material and treatments

The study to evaluate S effect on attenuation of Cd damage in *Panicum maximum* Jacq. cv. Massai was conducted under greenhouse conditions (Figures 1 and 2), using a hydroponic system with pots containing 2 L of nutrient solution. The treatments consisted of combinations three S concentrations (0.1, 1.9 and 3.7 mmol L^{-1}) and two Cd concentrations (0.1 and 0.5 mmol L^{-1}), in nutrient solutions modified from Epstein and Bloom (2005) nutrient solution. The composition and chemical speciation of nutrient solution used in the study is shown in Table 1 and Table 2, respectively. Pots used in the study were placed in a randomized block design, with four replications.

4.2.2 Massai grass growth and plant material collection for analysis

To germinate, seeds were placed in a tray containing expanded vermiculite, which was watered with deionized water during the initial 14 days, followed by a modified nutrient solution to provide 0.1 mmol L^{-1} S (diluted to 25% ionic strength) in the subsequent 9 days. Twenty-three days after sowing, five seedlings (± 10 cm of height) were transplanted into each pot containing the undiluted nutrient solution (100% of the ionic strength) modified to supply only S for 21 days. After this period, the nutrient solutions were modified to supply S and Cd (100% ionic strength), for 9 days. The solutions were replaced every 7 days and remained continuously aerated. During the conduction of the study (53 days), the average temperature in the greenhouse was 30.5 °C and the relative humidity of air was 60.5%.

Figure 1 - Greenhouse (A), aeration system of nutrient solution (B, C, D, E and F), greenhouse cooling system (G and H), expanded vermiculite (I), plastic tray for seed germination (J), mean length of seedlings (K) and size of plastic containers (L) used in the study to evaluate the effect of S on Cd detoxification mechanisms by Massai grass

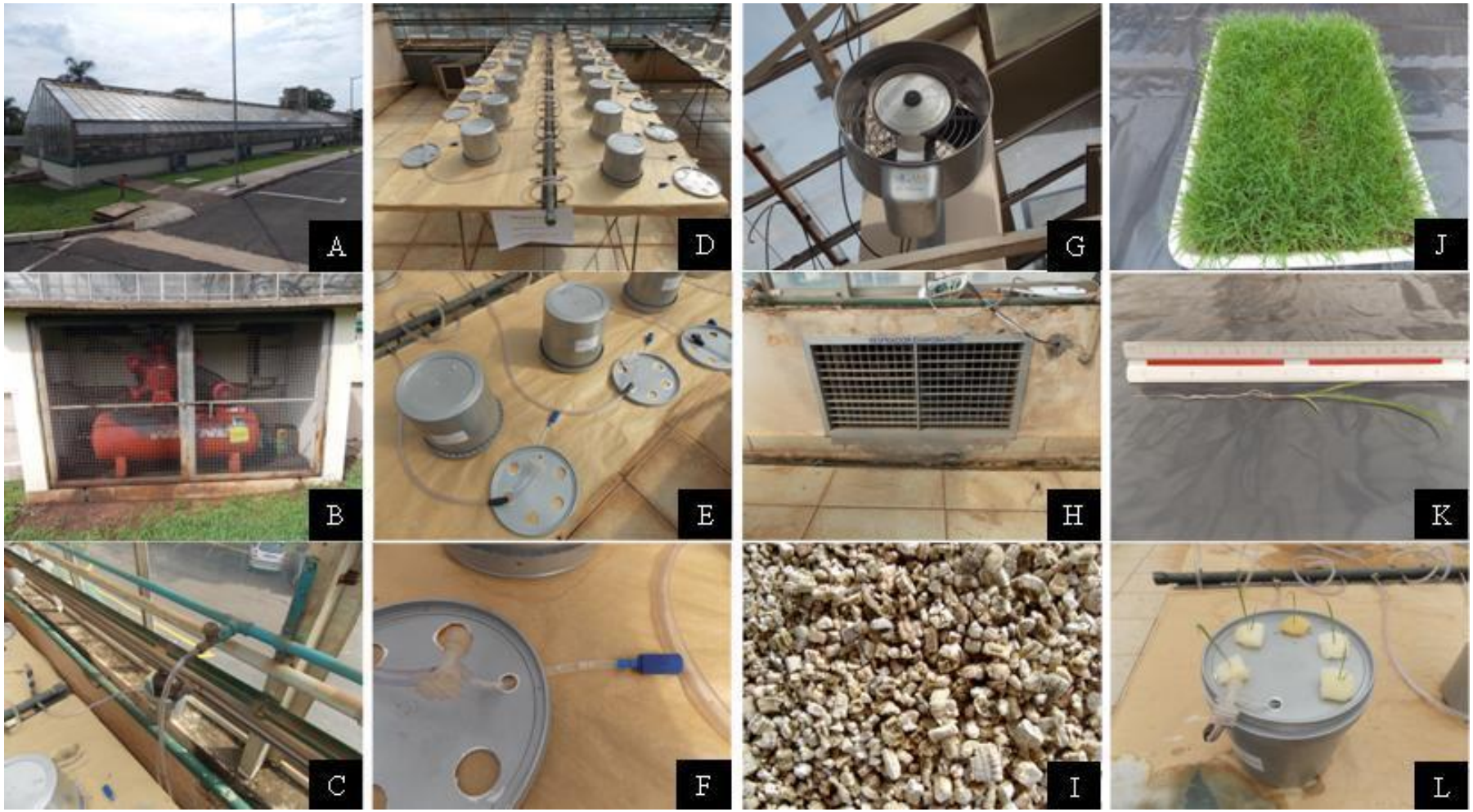


Figure 2 - Climatic dates (average temperature, maximum temperature, minimum temperature and relative humidity) recorded in the greenhouse during Cd detoxification mechanisms study in Massai grass, describing the steps between the beginning and the end of study (germination period, transplant, addition of undiluted and modified nutrient solution to meet S concentrations, addition of undiluted and modified nutrient solution to meet S and Cd concentrations, photosynthetic measurements, and harvest of plants)

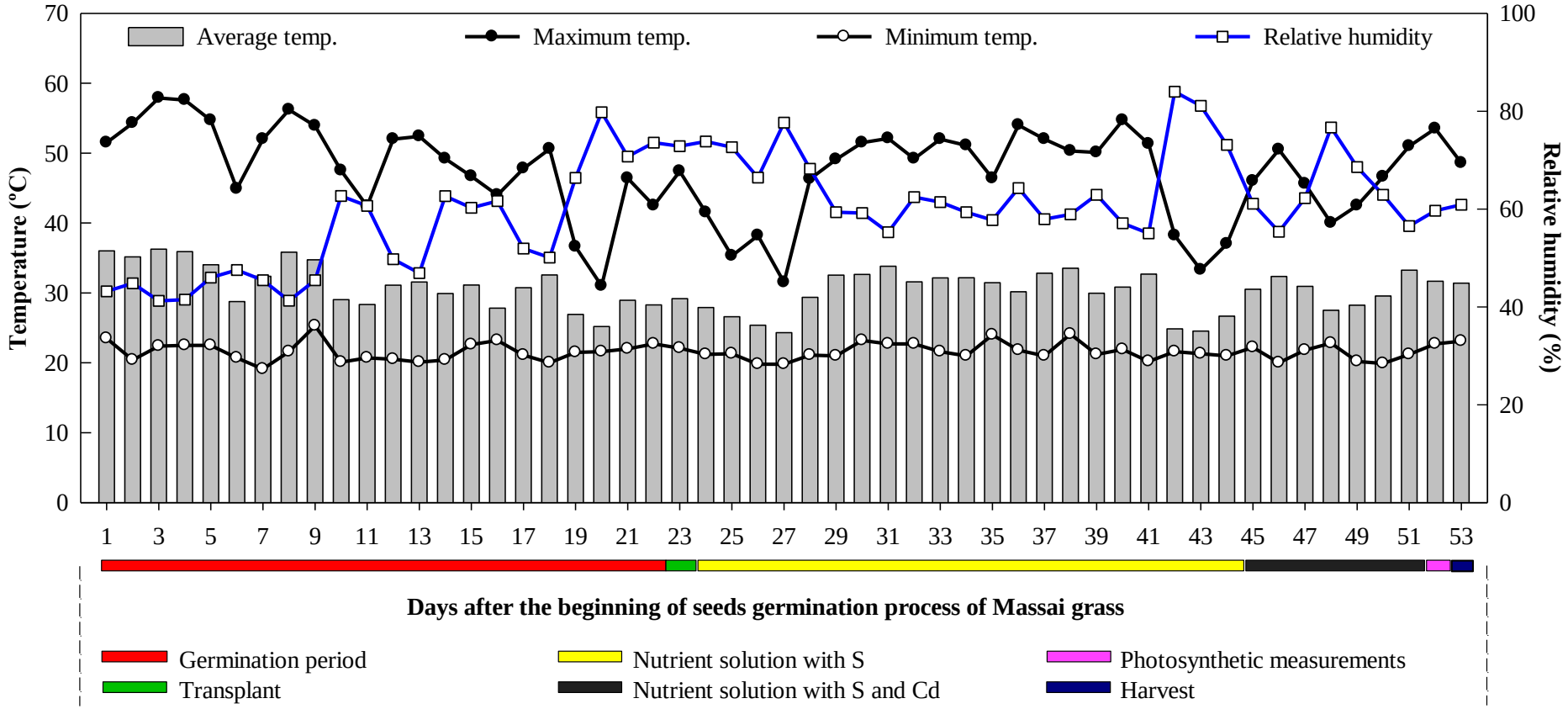


Table 1 - Volumes of stock solutions used in preparation of nutrient solutions provided during Cd detoxification mechanisms study in Massai grass

S (mmol L^{-1})	0.1	0.1	0.1	1.9	1.9	1.9	3.7	3.7	3.7
Cd (mmol L^{-1})	0.0	0.1	0.5	0.0	0.1	0.5	0.0	0.1	0.5
Stock solution	Volume (mL L^{-1})								
$CdCl_2$ (0.1 mol L^{-1})	0	1	5	0	1	5	0	1	5
KH_2PO_4 (1 mol L^{-1})	1	1	1	1	1	1	1	1	1
NH_4NO_3 (1 mol L^{-1})	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
KNO_3 (1 mol L^{-1})	6	6	6	6	6	6	6	6	6
KCl (1 mol L^{-1})	1	1	1	1	1	1	1	1	1
$MgSO_4.7H_2O$ (1 mol L^{-1})	0.1	0.1	0.1	1.9	1.9	1.9	2	2	2
$MgCl_2.6H_2O$ (1 mol L^{-1})	1.9	1.9	1.9	0.1	0.1	0.1	-	-	-
$CaSO_4.2H_2O$ (0.01 mol L^{-1})	-	-	-	-	-	-	170	170	170
$CaCl_2$ (1 mol L^{-1})	5	5	5	5	5	5	3.3	3.3	3.3
Micronutrients - Fe[1]	1	1	1	1	1	1	1	1	1
Fe(III) - EDTA[2]	1	1	1	1	1	1	1	1	1

[1]Composition of micronutrient solution (µmol L^{-1}): KCl = 50; H_3BO_3 = 25; $MnSO_4.H_2O$ = 2; $ZnSO_4.7H_2O$ = 2; $CuSO_4.5H_2O$ = 0.5; H_2MoO_4 (85 % MoO_3) = 0.5. [2]Fe(III)-EDTA = 100 µmol L^{-1}.

At 53rd day of experiment, adventitious root tips of plants exposed to following treatments: 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, 1.9 mmol L^{-1} S + 0.5 mmol L^{-1} Cd and 3.7 mmol L^{-1} S + 0.5 mmol L^{-1} Cd, were sampled. The anatomical changes were evaluated, as well as S, Ca, and Fe accumulation. In addition, at the last day of exposure of Massai grass to different S and Cd combinations, plants were harvested and separated into roots and shoots. The root system of one plant from each pot was collected to determine root length, surface and dry matter production. The root system of two plants from each pot was used to determine N, P, K, Ca, Mg, S, Cu, Fe, Mn, Zn, and Cd concentrations. Massai grass shoot was separated into leaf blades and stems and sheaths, and then each part of one plant from each pot was used to determine dry matter production and the leaf blades and the stems and sheaths of two plants from each pot were used for elemental profiling.

4.2.3 Determination of dry matter production and shoot:root ratio

The dry matter production of leaf blades, stems and sheaths and root system was obtained after weighing the plant material, which remained in a forced ventilation oven at 60 ºC for 72 hours. The shoot:root ratio was calculated by dividing total dry weight of leaf blades and stems and sheaths by total root dry matter.

Table 2 - Chemical speciation of nutrient solution (pH 5.5) by Visual MINTEQ® software (GUSTAFSSON, 2012)

0.1 mmol L^{-1} S + 0.0 mmol L^{-1} Cd				0.1 mmol L^{-1} S + 0.1 mmol L^{-1} Cd				0.1 mmol L^{-1} S + 0.5 mmol L^{-1} Cd			
S - Species	(%)	Cd - Species	(%)	S - Species	(%)	Cd - Species	(%)	S - Species	(%)	Cd - Species	(%)
SO_4^{-2}	70.33[2]	-	-	SO_4^{-2}	66.55	Cd^{+2}	58.77[3]	SO_4^{-2}	69.87	Cd^{+2}	52.88
HSO_4^-	0.01	-	-	HSO_4^-	5.04	$CdCl^+$	38.45	HSO_4^-	0.01	$CdCl^+$	36.16
$MgSO_4$ (aq)[1]	6.56	-	-	$CdSO_4$ (aq)	0.23	$CdCl_2$ (aq)	1.54	$CdSO_4$ (aq)	1.07	$CdCl_2$ (aq)	1.51
$CaSO_4$ (aq)	19.50	-	-	$MgSO_4$ (aq)	6.26	$CdSO_4$ (aq)	0.23	$MgSO_4$ (aq)	6.42	$CdSO_4$ (aq)	0.21
KSO_4^-	1.93	-	-	$CaSO_4$ (aq)	18.54	$CdNO_3^+$	0.96	$CaSO_4$ (aq)	19.09	$CdNH_3^{+2}$	0.01
$NH_4SO_4^-$	1.66	-	-	KSO_4^-	1.81	$CdHPO_4$ (aq)	0.02	KSO_4^-	1.90	$CdNO_3^+$	0.86
-	-	-	-	$NH_4SO_4^-$	1.56	$CdEDTA^{-2}$	0.01	$NH_4SO_4^-$	1.63	$CdHPO_4$ (aq)	7.96
-	-	-	-	-	-	$CdHEDTA^-$	0.02	-	-	$CdEDTA^{-2}$	0.40
1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd				1.9 mmol L^{-1} S + 0.1 mmol L^{-1} Cd				1.9 mmol L^{-1} S + 0.5 mmol L^{-1} Cd			
S - Species	(%)	Cd - Species	(%)	S - Species	(%)	Cd - Species	(%)	S - Species	(%)	Cd - Species	(%)
SO_4^{-2}	72.02	-	-	SO_4^{-2}	71.91	Cd^{+2}	51.63	SO_4^{-2}	71.50	Cd^{+2}	51.18
HSO_4^-	0.01	-	-	HSO_4^-	0.01	$CdCl^+$	33.51	HSO_4^-	0.01	$CdCl^+$	34.56
$MgSO_4$ (aq)	6.18	-	-	$CdSO_4$ (aq)	0.21	$CdCl_2$ (aq)	1.33	$CdSO_4$ (aq)	1.03	$CdCl_2$ (aq)	1.44
$CaSO_4$ (aq)	18.18	-	-	$MgSO_4$ (aq)	6.16	$CdSO_4$ (aq)	4.00	$Cd(SO_4)_2^{-2}$	0.04	$CdSO_4$ (aq)	3.90
KSO_4^-	1.94	-	-	$CaSO_4$ (aq)	18.11	$Cd(SO_4)_2^{-2}$	0.07	$MgSO_4$ (aq)	6.05	$Cd(SO_4)_2^{-2}$	0.07
$NH_4SO_4^-$	1.66	-	-	KSO_4^-	1.93	$CdNH_3^{+2}$	0.01	$CaSO_4$ (aq)	17.82	$CdNH_3^{+2}$	0.01
-	-	-	-	$NH_4SO_4^-$	1.66	$CdNO_3^+$	0.84	KSO_4^-	1.91	$CdNO_3^+$	0.82
-	-	-	-	-	-	$CdHPO_4$ (aq)	7.80	$NH_4SO_4^-$	1.64	$CdHPO_4$ (aq)	7.62
-	-	-	-	-	-	$CdEDTA^{-2}$	0.79	-	-	$CdEDTA^{-2}$	0.40
3.7 mmol L^{-1} S + 0.0 mmol L^{-1} Cd				3.7 mmol L^{-1} S + 0.1 mmol L^{-1} Cd				3.7 mmol L^{-1} S + 0.5 mmol L^{-1} Cd			
S - Species	(%)	Cd - Species	(%)	S - Species	(%)	Cd - Species	(%)	S - Species	(%)	Cd - Species	(%)
SO_4^{-2}	72.71	-	-	SO_4^{-2}	72.57	Cd^{+2}	58.20	SO_4^{-2}	72.03	Cd^{+2}	57.58
HSO_4^-	0.01	-	-	HSO_4^-	0.01	$CdCl^+$	21.23	HSO_4^-	0.01	$CdCl^+$	22.76
$MgSO_4$ (aq)	6.06	-	-	$CdSO_4$ (aq)	0.24	$CdCl_2$ (aq)	0.47	$CdSO_4$ (aq)	1.18	$CdCl_2$ (aq)	0.55
$CaSO_4$ (aq)	17.56	-	-	$Cd(SO_4)_2^{-2}$	0.02	$CdSO_4$ (aq)	9.03	$Cd(SO_4)_2^{-2}$	0.08	$CdSO_4$ (aq)	8.75
KSO_4^-	1.97	-	-	$MgSO_4$ (aq)	6.03	$Cd(SO_4)_2^{-2}$	0.32	$MgSO_4$ (aq)	5.92	$Cd(SO_4)_2^{-2}$	0.31
$NH_4SO_4^-$	1.68	-	-	$CaSO_4$ (aq)	17.48	$CdNH_3^{+2}$	0.01	$CaSO_4$ (aq)	17.18	$CdNH_3^{+2}$	0.01
-	-	-	-	KSO_4^-	1.96	$CdNO_3^+$	0.95	KSO_4^-	1.93	$CdNO_3^+$	0.93
-	-	-	-	$NH_4SO_4^-$	1.68	$CdHPO_4$ (aq)	8.91	$NH_4SO_4^-$	1.65	$CdHPO_4$ (aq)	8.67
-	-	-	-	-	-	$CdEDTA^{-2}$	0.86	-	-	$CdEDTA^{-2}$	0.42

[1]aq - aqua complex. [2]Variation < 5% in SO_4^{-2} availability (pH 3.5 to 5.5). [3]Variation < 10% in Cd^{+2} availability (pH 3.5 to 5.5).

4.2.4 Determination of root length, root surface and Cd tolerance index (TI)

The roots of one plant from each pot were collected for root surface and root length measurements. After collection, the roots were rinsed in running water, immersed in gentian violet solution (50 mg L^{-1}), for 5 min, to increase the contrast of roots and then placed on paper towel to remove the excess solution. Then, the roots were placed on transparency sheets (transparent polystyrene film), without overlapping, and scanned in grayscale with 200 dpi resolution (HP Scanjet 2400, Hewlett-Packard Comp., Houston, USA). The digitalized images obtained were processed individually through the software Safira© v. 1.1 (JORGE; RODRIGUES, 2008), with threshold set at 170 (threshold value for adjusting the pixel values of images). The length and surface were separated and calculated by software Safira© v. 1.1 according to root diameter (< 3 mm, 3.1-6 mm, 6.1-9 mm and > 9 mm). Then, the data of root length were used to calculate Cd tolerance index (TI), according Wilkins (1978):

$$\text{TI (\%)} = \left(\frac{\text{root length in Cd treatment}}{\text{root length in control}} \right) \times 100 \quad (1)$$

in which control treatment is represented by 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd.

4.2.5 Anatomical analyses and regions of S, Ca and Fe accumulation

The anatomical alterations of root tip of adventitious root were analyzed by scanning electron microscopy after freeze-fracture. The samples were fixed in a modified Karnovsky solution (KARNOVSKY, 1965) (2% glutaraldehyde, 2% paraformaldehyde in 0.05 mol L^{-1} sodium cacodylate buffer, pH 7.2), for 48 h, at 4 °C. The samples were then transferred to a 10% glycerol solution for a period of 30 min and then "fractured" in N_2 liquid. Post-fixation was done in 1% osmium tetroxide in water for 1 h and 30 min at room temperature. Dehydration was done in an acetone series (25 to 100%), 15 min each. Subsequently, the samples were critical point dried in liquid CO_2 (MS CPD 300, Leica, Diegem, Belgium) mounted on metal stubs, and sputter coated with 80 nm gold (MED 010, Balzers Union, Balzers, Liechtenstein). Micrographs were obtained under a Zeiss electron microscope (LEO 435 VP, Zeiss, Cambridge, UK), at 20 kV and digital images recorded. Part of samples were mounted on carbon tape and taken to x-ray microfluorescence apparatus (Orbis PC, EDAX-AMETEK Inc., Mahwah, USA) to determine the approximate concentration and the areas of S, Ca, and Fe accumulation in the root tip of adventitious root.

4.2.6 Determination of concentrations and content of nutrients and Cd

After drying in a forced air ventilation oven at 60 °C for 72 h, plant material was ground in a Wiley type mill. Nitrogen concentration was determined using semimicro Kjeldahl method after sulfuric digestion, while P, K, Ca, Mg, S, Cu, Fe, Mn, Zn (MALAVOLTA; VITTI; OLIVEIRA, 1997) and Cd concentrations were determined after nitric-perchloric digestion (HNO_3 65% and $HClO_4$ 70%). The extracts were analyzed for determination of elements concentration by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7000 SERIES, Thermo Fisher Scientific, Waltham, USA). The content of individual elements was obtained by multiplying the concentration of element in the tissue by dry matter production of respective tissue (leaf blade, stems and sheaths and root).

4.2.7 Cd translocation factor (TF)

The translocation factor (TF) was calculated for leaf blades and stems and sheaths as described in the equations below:

$$TF = \frac{C_{leaf\ blades}}{C_{root}} \quad (2)$$

$$TF = \frac{C_{stems\ and\ sheaths}}{C_{root}} \quad (3)$$

Where $C_{leaf\ blades}$, $C_{stems\ and\ sheaths}$ and C_{root} are Cd concentration in the leaf blades (mg kg^{-1} DW), stems and sheaths (mg kg^{-1} DW) and root (mg kg^{-1} DW) of Massai grass, respectively.

4.2.8 Statistical analysis

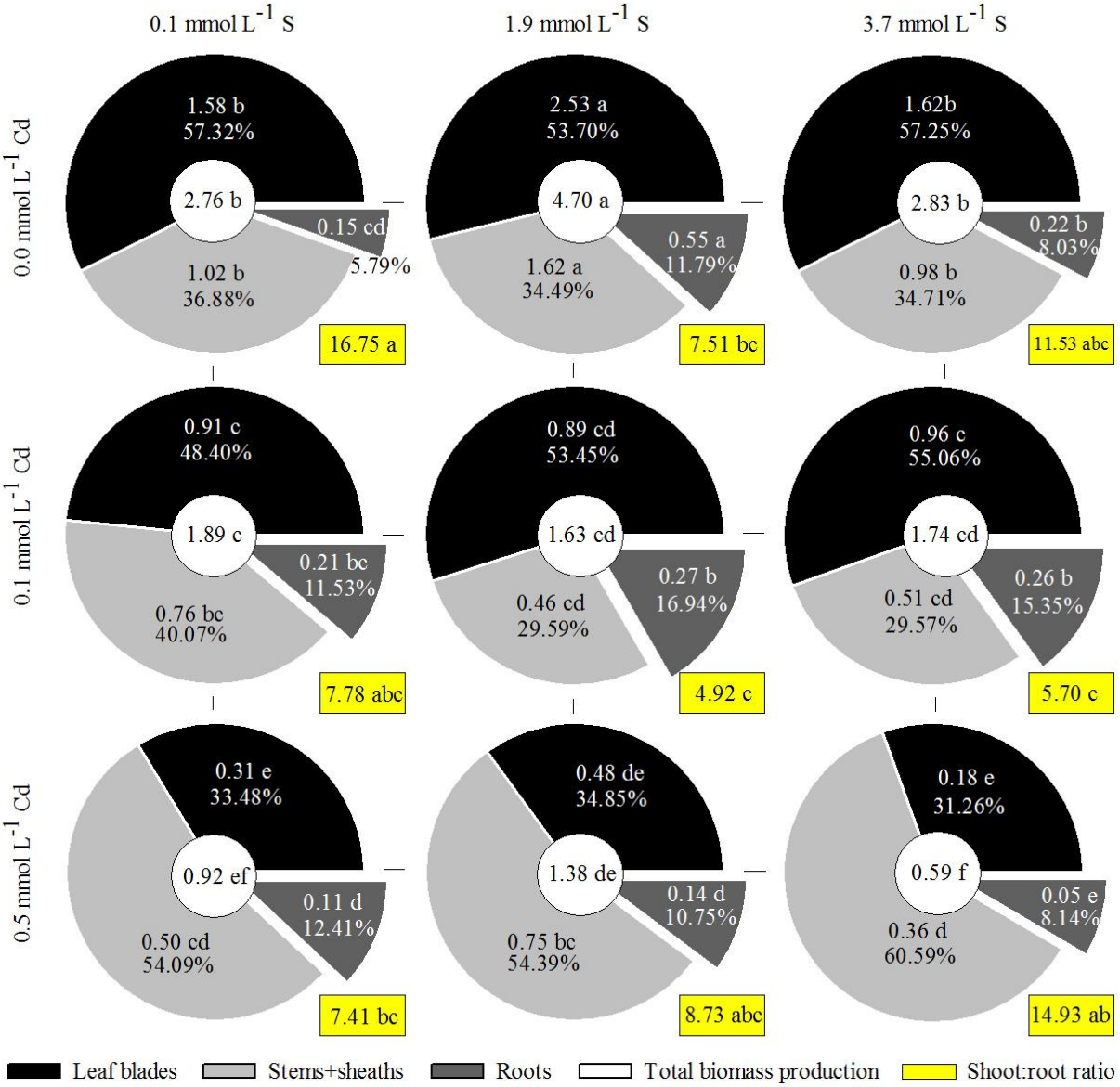
The data were submitted to analysis of variance (F test) and to comparison of means by Tukey test ($P < 0.05$) through Statistical Analysis System v. 9.2 (SAS INSTITUTE, 2008). Later, Pearson's correlation tests were performed among the analyzed variables (SAS INSTITUTE, 2008).

4.3 Results

4.3.1 Biomass partition of Massai grass supplied with S and exposed to Cd

The highest total dry matter production was observed when Massai grass was cultivated without Cd and with 1.9 mmol L^{-1} S (control treatment, Figure 3).

Figure 3 - Dry matter production (g/plant) and shoot:root ratio of Massai grass supplied with S and exposed to Cd. Different letters on each color (black, dark gray, light gray, yellow, and white) indicate significant difference by Tukey test (P <0.05)



When plants where exposed to Cd, S supply of 1.9 mmol L^{-1} resulted in the highest dry matter production in comparison to other S concentrations, especially at highest Cd exposure. The same results occurred with stems and sheaths and roots production. It is important to mention that percentage contribution of roots to biomass production of Massai grass exposed to 0.1 mmol L^{-1} Cd increased in comparison to plants not exposed to Cd, while the percentage contribution of leaf blades in the biomass production of Massai grass exposed to highest Cd concentration decreased. Plants supplied with 0.1 mmol L^{-1} S and not exposed to Cd presented higher shoot:root ratio in comparison to plants exposed to 0.1 mmol L^{-1} Cd and supplied with the two highest S concentrations and in comparison to plants exposed to highest Cd concentration and the lowest S concentration (Figure 3).

4.3.2 Root length, root surface and Cd tolerance index of Massai grass

Massai grass of control treatment presented the highest root length and root surface (Table 3). However, when plants were exposed to 0.1 mmol L^{-1} Cd, the highest root length occurred with the highest S concentration, while the highest root surfaces were present with S supply of 1.9 and 3.7 mmol L^{-1}. The length and total root surface of plants exposed to 0.5 mmol L^{-1} Cd were not changed by S supply, but the length and surface as a function of diameter were. The supply of 1.9 and S 3.7 mmol L^{-1} S to Massai grass cultivated with 0.5 mmol L^{-1} Cd resulted in the higher participation of roots with diameter lower than 3 mm as compared to other plants, but these plants did not present roots with a diameter higher than 6.1 mm. On the other hand, plants cultivated with 0.1 mmol L^{-1} Cd presented highest participation of roots with a diameter between 6.1 and 9.0 mm when plants were supplemented with 0.1 mmol L^{-1} S. Sulfur concentrations did not change ($P > 0.05$) the participation of roots with diameter higher than 9 mm in plants exposed to 0.1 mmol L^{-1} Cd, but in Cd absence, S supply of 1.9 mmol L^{-1} resulted in a greatest participation of roots with a diameter higher than 9 mm. The TI of plants exposed to 0.1 mmol L^{-1} Cd increased linearly with S supply, but the TI of plants exposed to 0.5 mmol L^{-1} Cd and supplied with 0.1 mmol L^{-1} S was higher than TI of plants supplied with 3.7 mmol L^{-1} S.

Table 3 - Root length, root surface and Cd tolerance index of Massai grass supplied with S and exposed to Cd as a function of root diameter

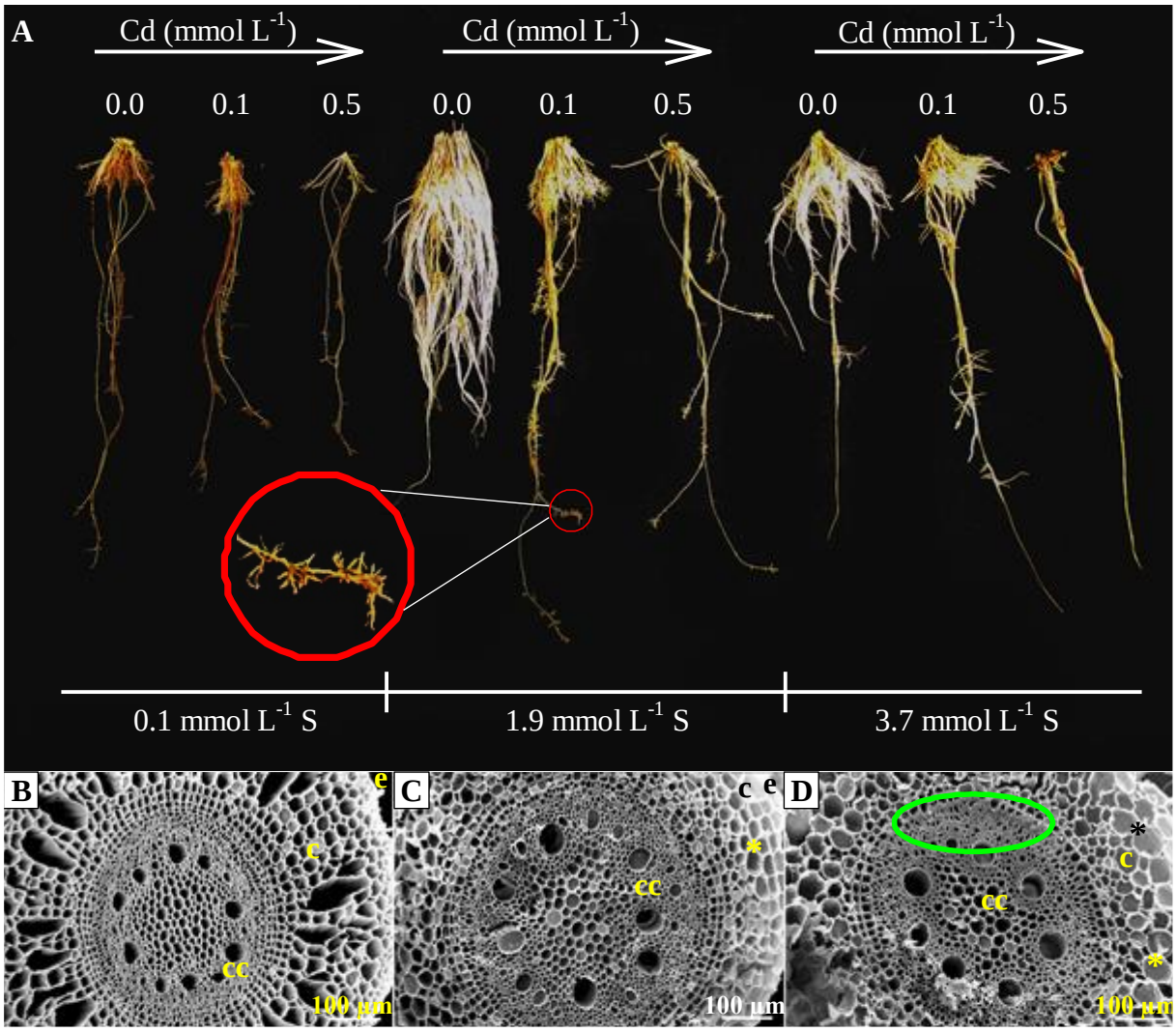
Cd (mmol L^{-1})	0.0			0.1			0.5		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7	0.1	1.9	3.7
					Root length				
Total (mm)	4184 ± 271 e	11815 ± 295 a	8769 ± 218 b	4020 ± 203 e	5447 ± 158 d	6698 ± 365 c	2503 ± 114 f	2381 ± 40 f	1538 ± 105 f
< 3 mm (%)	87.25 ± 0.58 de	85.40 ± 0.93 ef	88.69 ± 0.48 cd	84.49 ± 0.88 f	84.22 ± 0.33 f	90.07 ± 0.32 bc	91.96 ± 0.22 b	96.23 ± 0.10 a	96.73 ± 0.27 a
3.1 to 6 mm (%)	12.23 ± 0.54 ab	12.94 ± 0.90 a	10.46 ± 0.46 bc	14.03 ± 0.88 a	14.65 ± 0.32 a	9.52 ± 0.29 c	8.03 ± 0.22 c	3.76 ± 0.10 d	3.26 ± 0.27 d
6.1 to 9 mm (%)	0.43 ± 0.04 d	1.31 ± 0.05 a	0.78 ± 0.06 c	1.37 ± 0.06 a	1.05 ± 0.04 b	0.32 ± 0.03 d	0.00 ± 0.00 e	0.00 ± 0.00 e	0.00 ± 0.00 e
> 9 mm (%)	0.07 ± 0.01 bc	0.34 ± 0.02 a	0.05 ± 0.00 c	0.09 ± 0.00 b	0.07 ± 0.00 bc	0.09 ± 0.00 bc	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 d
					Root surface				
Total (mm^2)	24406 ± 1699 d	71961 ± 2162 a	49149 ± 1225 b	25662 ± 1652 d	31497 ± 981 c	32551 ± 680 c	10262 ± 314 e	11216 ± 355 e	6886 ± 103 e
< 3 mm (%)	74.98 ± 1.37 c	64.78 ± 2.49 e	79.01 ± 0.97 c	66.33 ± 1.39 de	73.67 ± 0.66 cd	74.14 ± 1.50 cd	80.65 ± 2.67 bc	87.93 ± 1.38 ab	89.88 ± 1.51 a
3.1 to 6 mm (%)	24.43 ± 1.41 ab	28.33 ± 2.11 a	17.72 ± 0.66 bc	27.84 ± 1.31 a	24.16 ± 0.70 ab	24.01 ± 1.29 ab	19.34 ± 2.67 bc	12.06 ± 1.38 cd	10.11 ± 1.51 d
6.1 to 9 mm (%)	0.52 ± 0.37 d	5.02 ± 0.26 a	2.97 ± 0.39 b	5.74 ± 0.25 a	2.09 ± 0.08 bc	1.79 ± 0.23 c	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 d
> 9 mm (%)	0.05 ± 0.06 bc	1.86 ± 0.14 a	0.29 ± 0.03 b	0.07 ± 0.01 bc	0.06 ± 0.00 bc	0.04 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
					Tolerance index				
(%)	-	-	-	34.00 ± 1.72 c	46.00 ± 1.33 b	56.75 ± 3.09 a	21.25 ± 0.96 d	20.25 ± 0.34 de	13.00 ± 0.89 e

Means ± SEM followed by different letters in rows differ significantly from each other by Tukey test ($P < 0.05$).

4.3.3 Morpho-anatomical changes in root system of Massai grass

The number and the length of roots of Massai grass exposed to Cd decreased in comparison with control treatment. The roots were brown and the number of lateral roots formed close to root tip increased (Figure 4A).

Figure 4 - Morpho-anatomical changes (root tip) in roots of Massai grass supplied with S and exposed to Cd. A: Massai grass roots of plants supplied with S at different exposures of Cd; B-D freeze-fractured roots observed under the scanning electron microscope. B: 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd; C: 1.9 mmol L^{-1} S + 0.5 mmol L^{-1} Cd; D: 3.7 mmol L^{-1} S + 0.5 mmol L^{-1} Cd. c = cortex; cc = central cylinder; e = epidermis; asterisk indicates cortical cells with increased diameter in relation to treatment without Cd (B). G-layer highlighted inside green circle (D)



After addition of S the damage caused by Cd in the roots of Massai grass was attenuated, and this effect was most evident in plants exposed to 0.1 mmol L^{-1} Cd (Figure 4A). Plants of control treatment developed highest number of roots with highest length and surface (Figure 4A). The roots of these plants were used to compare the anatomical changes caused by Cd. The central cylinder in the roots of Massai grass of control treatment presented typical structure of root characteristic for this species (Figure 4B). In plants exposed to Cd occurred degeneration of epidermis, absence of aerenchyma, and increased cortical parenchyma diameter with less cell layers (Figures 4C-D), especially when the plants were supplied with 3.7 mmol L^{-1} S (Figure 4D). Thick cell walls characteristic for gelatinous layer (G-layer) can be found in the cells of vascular parenchyma within central cylinder (Figures 4C-D). G-layer was observed in this species under Cd treatment by Parrota et al. (2015).

4.3.4 Concentration of nutrients and Cd in root and shoot tissues of Massai grass

Sulfur concentrations in the leaf blades, stems and sheaths and roots of Massai grass supplied with 3.7 mmol L^{-1} S were higher than in plants supplied with 0.1 mmol L^{-1} S, regardless of Cd concentrations (Table 4). The concentrations of N, Cu and Fe in the leaf blades and Cu and Mn in the stems and sheaths of plants not exposed to Cd and supplied with 3.7 mmol L^{-1} S were higher than in plants cultivated with 0.1 mmol L^{-1} S, but the concentrations of Ca in the leaf blades, N and Ca in the stems and sheaths and N in the roots of plants supplied with 0.1 mmol L^{-1} S were higher when compared to plants cultivated with 3.7 mmol L^{-1} S. When analyzing the nutrient concentrations of Massai grass cultivated with 1.9 mmol L^{-1} S it is essential to note that concentrations of N and P in the leaf blades, N, P and Cu in the stems and sheaths and N, P, Cu and Fe in the roots of plants exposed to 0.1 mmol L^{-1} Cd were higher than those not exposed to Cd, while Mn concentrations in the stems and sheaths and Ca, Mg, Mn and Zn in the roots were lower. Plants cultivated with 0.5 mmol L^{-1} Cd presented lower concentrations of K, Ca and Mn in the stems and sheaths and K, Ca, Mg, Mn and Zn in the roots and higher concentrations of P, K, Mg, Fe and Mn in the leaf blades, P, Cu and Zn in the stems and sheaths and Cu and Fe in the roots when compared to plants not exposed to Cd and cultivated with 1.9 mmol L^{-1} S. When plants were exposed to 0.1 mmol L^{-1} Cd, S supply of 3.7 mmol L^{-1} S increased N and Mn concentrations in the leaf blades, N, Mg, Cu, Mn and Zn in the stems and sheaths and K, Mg, Fe and Mn in the roots in relation to Massai grass cultivated with 0.1 mmol L^{-1} S, but the opposite happened with concentrations of Ca and Fe in the leaf blades and stems and sheaths and Zn in the roots.

Table 4 - Nutrients and Cd concentration in the leaf blades, stems and sheaths, and roots of Massai grass supplied with S and exposed to Cd

Cd (mmol L^{-1})	0.0			0.1			0.5		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7	0.1	1.9	3.7
					Leaf blades				
N (g kg^{-1} DW)	10.05 ± 0.19 bc	6.87 ± 0.28 d	17.25 ± 0.79 a	8.02 ± 0.36 cd	11.17 ± 0.38 b	16.57 ± 0.69 a	6.77 ± 0.13 d	6.45 ± 0.16 d	2.90 ± 0.35 e
P (g kg^{-1} DW)	3.59 ± 0.05 a	2.50 ± 0.18 b	4.06 ± 0.11 a	3.43 ± 0.10 a	3.57 ± 0.16 a	4.08 ± 0.10 a	4.08 ± 0.21 a	4.08 ± 0.16 a	3.96 ± 0.10 a
K (g kg^{-1} DW)	32.77 ± 0.66 c	30.21 ± 0.84 c	33.95 ± 0.18 c	32.63 ± 1.10 c	34.51 ± 0.41 c	34.57 ± 0.85 c	46.73 ± 1.22 a	39.93 ± 0.87 b	45.56 ± 1.57 a
Ca (g kg^{-1} DW)	5.29 ± 0.10 b	4.02 ± 0.05 def	3.65 ± 0.11 ef	6.38 ± 0.12 a	3.37 ± 0.14 f	4.23 ± 0.24 cde	4.75 ± 0.21 bcd	4.74 ± 0.14 bcd	4.88 ± 0.26 bc
Mg (g kg^{-1} DW)	1.69 ± 0.09 de	1.65 ± 0.04 e	1.99 ± 0.13 cde	2.35 ± 0.04 c	1.70 ± 0.07 de	2.39 ± 0.22 c	4.42 ± 0.10 a	2.14 ± 0.03 cd	3.25 ± 0.03 b
S (g kg^{-1} DW)	2.10 ± 0.04 ef	2.36 ± 0.13 def	2.34 ± 0.13 ab	2.00 ± 0.08 f	2.76 ± 0.09 cd	2.87 ± 0.06 bc	2.53 ± 0.09 cde	2.65 ± 0.10 cd	3.58 ± 0.14 a
Cu (mg kg^{-1} DW)	7.76 ± 0.05 c	6.03 ± 0.13 e	10.34 ± 0.04 b	6.76 ± 0.23 de	6.35 ± 0.13 de	6.58 ± 0.26 de	13.37 ± 0.25 a	6.54 ± 0.09 de	7.04 ± 0.35 cd
Fe (mg kg^{-1} DW)	101.16 ± 0.33 ef	91.79 ± 0.19 f	170.50 ± 8.37 c	148.15 ± 10.39 cd	110.82 ± 0.48 ef	118.80 ± 2.85 ef	329.66 ± 8.02 a	121.80 ± 8.06 de	284.00 ± 3.12 b
Mn (mg kg^{-1} DW)	85.46 ± 1.59 d	96.32 ± 1.06 d	91.68 ± 1.58 d	99.75 ± 1.68 d	84.24 ± 0.86 d	186.77 ± 1.88 b	233.35 ± 5.85 a	131.97 ± 8.80 c	154.82 ± 11.11 c
Zn (mg kg^{-1} DW)	15.72 ± 0.53 de	14.19 ± 0.27 e	17.76 ± 0.85 de	23.13 ± 0.90 bc	16.04 ± 0.13 de	19.65 ± 0.59 cd	37.59 ± 1.58 a	14.41 ± 0.23 e	26.98 ± 1.49 b
Cd (mg kg^{-1} DW)	-	-	-	159.32 ± 2.97 d	135.40 ± 7.60 d	123.06 ± 5.89 d	818.98 ± 15.02 c	983.39 ± 41.76 b	1068.79 ± 25.63 a
					Stems and sheaths				
N (g kg^{-1} DW)	26.10 ± 0.74 a	10.20 ± 0.74 cd	13.25 ± 0.97 bc	7.93 ± 0.17 d	8.87 ± 0.36 d	13.42 ± 0.75 bc	9.00 ± 0.31 d	8.76 ± 0.37 d	16.40 ± 1.10 b
P (g kg^{-1} DW)	2.40 ± 0.13 bc	1.97 ± 0.05 c	2.62 ± 0.13 b	2.30 ± 0.10 bc	2.62 ± 0.05 b	2.74 ± 0.08 ab	3.22 ± 0.16 a	2.74 ± 0.10 ab	2.62 ± 0.19 b
K (g kg^{-1} DW)	40.36 ± 0.83 ab	40.73 ± 1.38 ab	45.35 ± 0.53 a	31.95 ± 0.70 cd	36.59 ± 1.24 bc	33.70 ± 1.04 cd	31.71 ± 1.44 cd	30.95 ± 1.06 d	31.61 ± 1.02 cd
Ca (g kg^{-1} DW)	5.45 ± 0.17 a	4.54 ± 0.21 b	3.57 ± 0.15 cde	4.13 ± 0.29 bcd	4.38 ± 0.21 bc	3.23 ± 0.11 e	3.88 ± 0.16 bcde	3.47 ± 0.08 de	3.58 ± 0.14 cde
Mg (g kg^{-1} DW)	2.60 ± 0.19 a	2.76 ± 0.15 a	3.23 ± 0.20 a	1.55 ± 0.16 b	2.58 ± 0.21 a	2.82 ± 0.32 a	2.33 ± 0.18 ab	2.65 ± 0.14 a	2.46 ± 0.13 ab
S (g kg^{-1} DW)	1.41 ± 0.14 c	2.32 ± 0.11 b	3.88 ± 0.29 a	1.31 ± 0.06 c	2.16 ± 0.10 b	2.69 ± 0.12 b	2.35 ± 0.17 b	2.26 ± 0.13 b	4.07 ± 0.07 a
Cu (mg kg^{-1} DW)	3.77 ± 0.28 e	3.30 ± 0.09 e	7.80 ± 0.12 a	4.45 ± 0.17 de	5.48 ± 0.62 cd	6.34 ± 0.10 bc	7.14 ± 0.27 ab	5.67 ± 0.24 cd	7.48 ± 0.26 ab
Fe (mg kg^{-1} DW)	156.77 ± 2.41 b	175.24 ± 6.38 b	141.50 ± 11.45 b	280.44 ± 8.07 a	180.74 ± 6.01 b	170.30 ± 11.15 b	167.64 ± 12.12 b	165.12 ± 11.00 b	148.15 ± 12.66 b
Mn (mg kg^{-1} DW)	41.08 ± 1.40 d	82.02 ± 1.10 a	62.18 ± 2.66 b	39.81 ± 1.10 d	49.57 ± 1.08 c	57.42 ± 1.43 b	63.17 ± 1.25 b	43.59 ± 0.94 cd	61.28 ± 0.90 b
Zn (mg kg^{-1} DW)	15.31 ± 0.94 e	16.28 ± 0.18 de	20.38 ± 0.85 cde	21.16 ± 1.22 cd	24.86 ± 1.17 c	30.99 ± 1.49 b	43.17 ± 1.08 a	24.81 ± 1.95 c	25.49 ± 1.01 bc
Cd (mg kg^{-1} DW)	-	-	-	155.11 ± 15.27 d	172.46 ± 8.70 d	248.88 ± 13.77 c	871.97 ± 28.68 b	933.03 ± 9.31 b	1117.72 ± 27.67 a
					Roots				
N (g kg^{-1} DW)	15.46 ± 1.32 a	8.43 ± 0.31 cd	10.40 ± 0.31 bc	9.92 ± 0.51 bc	12.12 ± 0.70 b	10.15 ± 0.45 bc	5.86 ± 0.27 d	10.93 ± 0.34 bc	6.10 ± 0.18 d
P (g kg^{-1} DW)	2.46 ± 0.17 bc	1.80 ± 0.12 c	2.11 ± 0.10 bc	2.75 ± 0.07 ab	2.59 ± 0.16 ab	3.15 ± 0.18 a	2.42 ± 0.21 bc	2.24 ± 0.09 bc	2.37 ± 0.05 bc
K (g kg^{-1} DW)	32.15 ± 1.40 abc	30.84 ± 1.22 abc	35.55 ± 1.30 a	27.91 ± 1.10 c	29.12 ± 0.81 bc	34.13 ± 1.33 ab	9.76 ± 0.07 e	14.99 ± 1.15 de	17.40 ± 1.09 d
Ca (g kg^{-1} DW)	4.18 ± 0.05 b	6.00 ± 0.16 a	4.03 ± 0.12 b	2.58 ± 0.18 c	2.54 ± 0.14 c	2.78 ± 0.14 c	2.17 ± 0.05 c	2.63 ± 0.15 c	2.64 ± 0.14 c
Mg (g kg^{-1} DW)	4.57 ± 0.43 b	6.84 ± 0.12 a	4.23 ± 0.21 b	2.53 ± 0.21 cd	2.55 ± 0.13 c	5.15 ± 0.02 b	1.54 ± 0.11 e	2.06 ± 0.15 cde	1.59 ± 0.14 de
S (g kg^{-1} DW)	1.69 ± 0.12 f	4.53 ± 0.13 b	5.34 ± 0.10 a	2.60 ± 0.15 e	4.22 ± 0.25 bc	5.36 ± 0.11 a	1.19 ± 0.12 f	2.97 ± 0.14 de	3.56 ± 0.08 cd
Cu (mg kg^{-1} DW)	7.18 ± 0.23 f	8.49 ± 0.17 ef	7.41 ± 0.23 f	15.77 ± 1.04 b	21.01 ± 0.91 a	15.32 ± 1.49 bc	5.66 ± 0.25 f	12.09 ± 0.32 cd	11.33 ± 0.27 de
Fe (mg kg^{-1} DW)	146.49 ± 12.04 f	171.67 ± 16.45 f	154.50 ± 16.63 f	671.77 ± 12.67 e	1042.30 ± 11.49 b	933.10 ± 11.32 c	1309.35 ± 26.35 a	858.73 ± 9.13 d	858.86 ± 10.01 d
Mn (mg kg^{-1} DW)	147.81 ± 16.24 b	260.66 ± 14.00 a	153.11 ± 16.45 b	64.57 ± 1.84 c	157.03 ± 14.60 b	155.50 ± 17.67 b	187.25 ± 6.36 b	163.32 ± 15.42 b	149.02 ± 2.77 b
Zn (mg kg^{-1} DW)	44.20 ± 0.78 b	57.44 ± 2.35 a	45.34 ± 1.17 b	44.83 ± 1.51 b	39.41 ± 1.43 b	31.10 ± 1.64 c	39.71 ± 1.22 b	40.40 ± 1.48 b	28.14 ± 1.09 c
Cd (mg kg^{-1} DW)	-	-	-	422.02 ± 22.51 d	447.10 ± 20.98 d	440.64 ± 14.15 d	1093.80 ± 11.43 c	1230.96 ± 29.54 b	1580.95 ± 32.74 a

Means ± SEM followed by different letters in rows differ significantly from each other by Tukey test ($P < 0.05$).

Plants supplied with 1.9 mmol L^{-1} S presented highest concentrations of Cu and Fe in the roots, when exposed to 0.1 mmol L^{-1} Cd (Table 4). When Massai grass was exposed to 0.5 mmol L^{-1} Cd, S supply of 3.7 mmol L^{-1} resulted in lower N, Mg, Cu, Fe, Mn and Zn concentrations in the leaf blades, P and Zn in the stems and sheaths and Ca, Fe and Zn in the roots compared to plants supplied with 0.1 mmol L^{-1} S, but the inverse was verified for N concentrations in the stems and sheaths and K and Cu in the roots. Cadmium concentrations in the leaf blades, stems and sheaths and roots of Massai grass exposed to 0.5 mmol L^{-1} Cd and supplied with 3.7 mmol L^{-1} S increased in 30, 28 and 44% in relation to plants cultivated with 0.1 mmol L^{-1} S.

4.3.5 Correlation among elements concentrations and morphological parameters

The correlation coefficients (r) are highlighted in Table 5. In general, P, K, Mg, Fe, Mn, Zn, and Cd concentrations in the leaf blades of Massai grass showed negative correlation with morphological and productive parameters evaluated. In the stems and sheaths, this pattern was maintained for P, Cu, Zn, and Cd. On the other hand, there was a positive correlation between morphological and productive parameters and K and Mn concentrations. Morphological and productive parameters showed positive correlation with K, Ca, Mg, S, Mn, and Zn concentrations, and negative correlation with P, Fe, and Cd concentrations in the roots of Massai grass.

4.3.6 Cd translocation factor (TF)

The highest Cd TF from roots to leaf blades in Massai grass occurred in plants exposed to 0.5 mmol L^{-1} Cd, but there was no significant effect ($P > 0.05$) of S supply (Table 6). However, Cd TF from roots to stems and sheaths in plants exposed to 0.1 mmol L^{-1} Cd increased with S supply of 3.7 mmol L^{-1} S, differently of plants exposed to 0.5 mmol L^{-1} Cd, where there was no significant effect ($P > 0.05$) of S supply.

4.3.7 Content of nutrients and Cd in the root and shoot tissues of Massai grass

The highest S content in the leaf blades, stems and sheaths and roots of Massai grass were observed with S supply of 1.9 and 3.7 mmol L^{-1} and in Cd absence in nutrient solution (Table 7).

Table 5 - Pearson's correlation among elements concentrations and morphological parameters of Massai grass (*n*=36)

	N	P	K	Ca	Mg	S	Cu	Fe	Mn	Zn	Cd
						Leaf blades					
Leaf blades biomass	0.31^{ns}	-0.67***	**-0.79*** **	-0.27^{ns}	-0.67***	-0.28^{ns}	-0.24^{ns}	-0.62***	-0.58**	-0.59**	**-0.77*** **
Stems and sheathsbiomass	0.04^{ns}	-0.69***	-0.61***	-0.07^{ns}	-0.52**	-0.33*	-0.16^{ns}	-0.50**	-0.51**	-0.48**	-0.51**
Root biomass	0.21^{ns}	-0.73***	-0.67***	-0.36*	-0.53**	-0.29^{ns}	-0.36*	-0.58**	-0.37*	-0.49**	-0.63***
Shoot:root ratio	-0.15^{ns}	0.11^{ns}	0.03^{ns}	0.16^{ns}	-0.02^{ns}	0.09^{ns}	0.01^{ns}	0.11^{ns}	-0.23^{ns}	-0.01^{ns}	0.09^{ns}
Root surface (< 3 mm)	0.49**	-0.51**	**-0.71*** **	-0.48*	-0.59**	-0.04^{ns}	-0.18^{ns}	-0.54**	-0.49**	-0.53**	**-0.75*** **
Root surface (3.1 - 6 mm)	0.24^{ns}	**-0.74*** **	**-0.74*** **	-0.32^{ns}	-0.55**	-0.26^{ns}	-0.31^{ns}	-0.56**	-0.44**	-0.48**	**-0.72*** **
Root surface (6.1 - 9 mm)	0.08^{ns}	**-0.75*** **	-0.64***	-0.19^{ns}	-0.45**	-0.23^{ns}	-0.27^{ns}	-0.41*	-0.43**	-0.37*	-0.57**
Root surface (> 9 mm)	-0.13^{ns}	**-0.77*** **	-0.43**	-0.26^{ns}	-0.32*	-0.19^{ns}	-0.24^{ns}	-0.32^{ns}	-0.27^{ns}	-0.33*	-0.34*
Root length (< 3 mm)	0.49**	-0.51**	-0.69***	-0.50*	-0.54**	-0.03^{ns}	-0.17^{ns}	-0.51**	-0.38*	-0.48**	**-0.72*** **
Root length (3.1 - 6 mm)	0.37*	-0.63***	**-0.74*** **	-0.42*	-0.59**	-0.18^{ns}	-0.25^{ns}	-0.56**	-0.50**	-0.49**	**-0.79*** **
Root length (6.1 - 9 mm)	0.13^{ns}	**-0.76*** **	-0.68***	-0.31^{ns}	-0.53**	-0.21^{ns}	-0.29^{ns}	-0.48**	-0.53**	-0.45**	-0.64***
Root length (> 9 mm)	-0.05^{ns}	**-0.76*** **	-0.51**	-0.25^{ns}	-0.39*	-0.21^{ns}	-0.31^{ns}	-0.41*	-0.29^{ns}	-0.37*	-0.44**
						Stems and sheaths					
Leaf blades biomass	0.18^{ns}	-0.61***	**0.74*** **	0.43**	0.25^{ns}	-0.17^{ns}	-0.55**	-0.03^{ns}	0.41*	-0.64***	**-0.80*** **
Stems and sheathsbiomass	0.07^{ns}	-0.63***	0.59**	0.41*	0.25^{ns}	-0.21^{ns}	-0.58**	0.02^{ns}	0.37*	-0.62***	-0.56**
Root biomass	-0.25^{ns}	-0.58**	0.46**	0.21^{ns}	0.14^{ns}	-0.18^{ns}	-0.55**	0.14^{ns}	0.54**	-0.40*	-0.64***
Shoot:root ratio	0.55**	0.03^{ns}	0.12^{ns}	0.18^{ns}	0.18^{ns}	0.13^{ns}	0.05^{ns}	-0.27^{ns}	-0.10^{ns}	-0.28^{ns}	0.08^{ns}
Root surface (< 3 mm)	-0.05^{ns}	-0.52**	**0.73*** **	0.18^{ns}	0.38*	0.07^{ns}	-0.28^{ns}	-0.08^{ns}	0.53**	-0.49**	**-0.76*** **
Root surface (3.1 - 6 mm)	-0.12^{ns}	-0.62***	0.56**	0.27^{ns}	0.17^{ns}	-0.14^{ns}	-0.53**	0.12^{ns}	0.56**	-0.48**	**-0.73*** **
Root surface (6.1 - 9 mm)	-0.27^{ns}	-0.65***	0.48**	0.17^{ns}	0.07^{ns}	-0.07^{ns}	-0.46**	0.23^{ns}	0.60**	-0.46**	-0.59**
Root surface (> 9 mm)	-0.15^{ns}	-0.56**	0.40*	0.22^{ns}	0.17^{ns}	-0.02^{ns}	-0.49**	-0.02^{ns}	**0.73*** **	-0.39*	-0.36*
Root length (< 3 mm)	-0.09^{ns}	-0.45**	0.68***	0.09^{ns}	0.41*	0.09^{ns}	-0.28^{ns}	-0.08^{ns}	0.60***	-0.41*	**-0.72*** **
Root length (3.1 - 6 mm)	-0.10^{ns}	-0.56**	0.67***	0.31^{ns}	0.27^{ns}	-0.09^{ns}	-0.44**	0.06^{ns}	0.52**	-0.47**	**-0.80*** **
Root length (6.1 - 9 mm)	-0.23^{ns}	-0.63***	0.57**	0.26^{ns}	0.13^{ns}	-0.08^{ns}	-0.48**	0.16^{ns}	0.56**	-0.51**	-0.66***
Root length (> 9 mm)	-0.13^{ns}	-0.59**	0.40*	0.25^{ns}	0.16^{ns}	-0.08^{ns}	-0.54**	0.02^{ns}	0.69***	-0.40*	-0.46**
						Roots					
Leaf blades biomass	0.31^{ns}	-0.39*	0.68***	**0.90*** **	**0.88*** **	0.34*	-0.23^{ns}	**-0.80*** **	0.42**	**0.74*** **	**-0.85*** **
Stems and sheathsbiomass	0.23^{ns}	-0.51**	0.41*	**0.87*** **	**0.72*** **	0.15^{ns}	-0.38*	**-0.77*** **	0.47**	**0.81*** **	-0.64***
Root biomass	0.06^{ns}	-0.27^{ns}	0.52**	**0.73*** **	**0.77*** **	0.48**	0.07^{ns}	-0.43**	0.49**	0.68***	-0.65***
Shoot:root ratio	0.17^{ns}	-0.16^{ns}	-0.02^{ns}	0.11^{ns}	0.00^{ns}	-0.22^{ns}	-0.37*	-0.38*	-0.06^{ns}	-0.09^{ns}	0.03^{ns}
Root surface (< 3 mm)	0.15^{ns}	-0.34*	0.72***	**0.78*** **	**0.82*** **	0.63***	-0.09^{ns}	-0.66***	0.44**	0.66***	**-0.79*** **
Root surface (3.1 - 6 mm)	0.10^{ns}	-0.30^{ns}	0.60***	**0.82*** **	**0.83*** **	0.48**	-0.02^{ns}	-0.58**	0.46**	**0.72*** **	**-0.74*** **
Root surface (6.1 - 9 mm)	-0.08^{ns}	-0.37*	0.47**	**0.77*** **	**0.70*** **	0.44**	-0.06^{ns}	-0.56**	0.38*	**0.74*** **	-0.60***
Root surface (> 9 mm)	-0.16^{ns}	-0.51**	0.25^{ns}	**0.84*** **	**0.70*** **	0.30^{ns}	-0.24^{ns}	-0.49**	0.66***	**0.70*** **	-0.42*
Root length (< 3 mm)	0.10^{ns}	-0.32^{ns}	0.67***	**0.78*** **	**0.85*** **	0.66***	-0.11^{ns}	-0.60***	0.50**	0.62***	**-0.75*** **
Root length (3.1 - 6 mm)	0.15^{ns}	-0.28^{ns}	0.69***	**0.77*** **	**0.81*** **	0.53**	0.01^{ns}	-0.58**	0.40*	0.68***	**-0.80*** **
Root length (6.1 - 9 mm)	0.02^{ns}	-0.40*	0.53**	**0.79*** **	**0.70*** **	0.46**	0.00^{ns}	-0.57**	0.40*	**0.76*** **	-0.67***
Root length (> 9 mm)	-0.10^{ns}	-0.45**	0.35*	**0.82*** **	**0.76*** **	0.35*	-0.15^{ns}	-0.49**	0.63***	0.68***	-0.49**

*p<0.05; **p<0.01; ***p<0.001; ^{ns}-non-significant.

Table 6 - Cadmium translocation factor (TF) in Massai grass supplied with S and exposed to Cd

Cd (mmol L^{-1})	0.1			0.5		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
$TF_{leaf\ blades}$	0.38 ± 0.026 b	0.30 ± 0.023 b	0.27 ± 0.006 b	0.74 ± 0.017 a	0.80 ± 0.048 a	0.67 ± 0.026 a
$TF_{stems\ and\ sheaths}$	0.36 ± 0.035 c	0.38 ± 0.027 c	0.56 ± 0.043 b	0.79 ± 0.029 a	0.75 ± 0.025 a	0.70 ± 0.009 a

Means ± SEM followed by different letters in rows differ significantly from each other by Tukey test ($P < 0.05$).

The highest contents of N, K, Ca, Mg, Cu, Fe, Mn and Zn in the leaf blades, K, Ca, Mg, Cu, Fe and Mn in the stems and sheaths and N, K, Ca, Mg, Mn and Zn in the roots occurred in Cd absence and with S supply of 1.9 and/or S 3.7 mmol L^{-1} S (Table 7). When plants were exposed to 0.1 mmol L^{-1} Cd, the highest S content in the roots occurred with S supply of 1.9 and 3.7 mmol L^{-1}, and the highest contents of K and Fe in the stems and sheaths occurred with S supply of 0.1 mmol L^{-1}, while the highest contents of N and Cu in the roots were observed with S supply of 1.9 mmol L^{-1} and the highest contents of N and Mn in the leaf blades and Mg in the roots occurred with S supply of 3.7 mmol L^{-1}. Plants exposed to 0.5 mmol L^{-1} Cd showed highest Cu content in the leaf blades with S supply of 0.1 mmol L^{-1} and highest N and Cu contents in the roots with S supply of 3.7 mmol L^{-1}. The highest Cd content in the leaf blades, stems and sheaths and roots occurred when plants were exposed to 0.5 mmol L^{-1} Cd and were supplied with 1.9 mmol L^{-1} S.

4.3.8 Localization of S, Ca and Fe in the root tip of adventitious roots of Massai grass

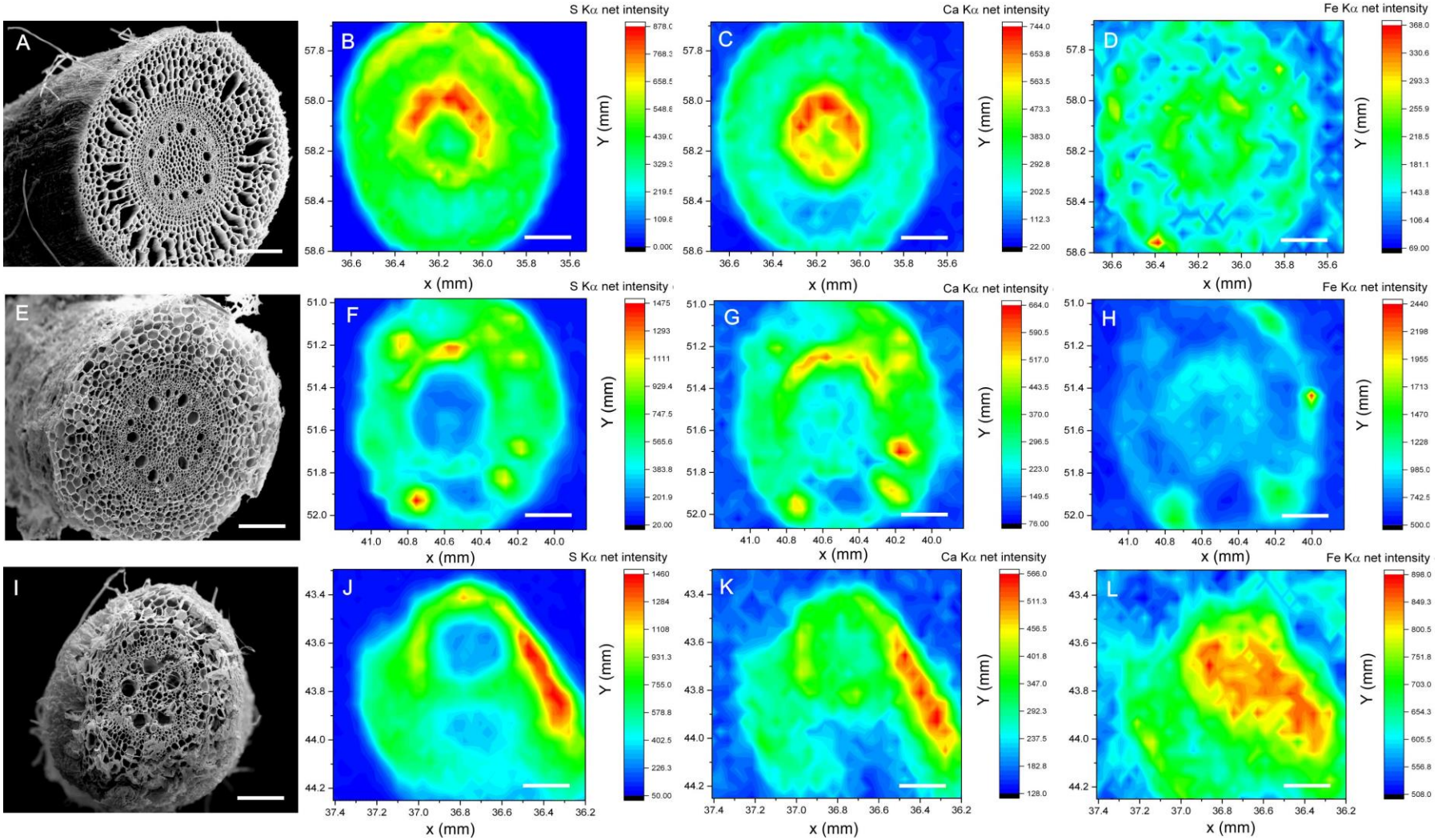
The highest S concentrations in plants of control treatment was observed around the central cylinder and exodermis (Figures 5A-B), while the highest Ca concentrations were observed in the central cylinder, mainly around the phloem and xylem (Figure 5C). There was no specific site for Fe accumulation under these conditions (Figure 5D). However, when plants supplied with 1.9 mmol L^{-1} S were exposed to 0.5 mmol L^{-1} Cd, S concentration was highest in the cortex region (Figures 5E-F), whereas there was highest Ca concentration around the endodermis (Figure 5G). Iron concentration in the roots of these plants was higher than in plants not exposed to Cd, but there was also no specific site for Fe accumulation (Figure 5H). When plants exposed to 0.5 mmol L^{-1} Cd were grown with 3.7 mmol L^{-1} S, the highest S (Figures 5I-J) and Ca (Figure 5K) concentrations were present in the cortex. The highest Fe concentrations in these conditions were observed around the medullar parenchyma, xylem, phloem and pericycle (Figure 5L).

Table 7 - Nutrients and Cd content in the leaf blades, stems and sheaths and roots, of Massai grass supplied with S and exposed to Cd

Cd (mmol L^{-1})	0.0			0.1			0.5		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7	0.1	1.9	3.7
				Leaf blades					
N (mg/plant)	15.85 ± 0.67 b	17.36 ± 0.66 b	27.88 ± 1.41 a	7.37 ± 0.47 cd	9.97 ± 1.78 c	15.87 ± 2.06 b	2.09 ± 0.09 e	3.07 ± 0.26 de	0.56 ± 0.17 e
P (mg/plant)	5.68 ± 0.33 a	6.35 ± 0.61 a	6.56 ± 0.18 a	3.14 ± 0.04 bc	3.13 ± 0.44 bc	3.90 ± 0.48 b	1.25 ± 0.03 d	1.84 ± 0.15 cd	0.73 ± 0.16 d
K (mg/plant)	51.70 ± 2.11 b	76.60 ± 4.37 a	54.96 ± 1.95 b	29.85 ± 0.49 cd	30.76 ± 5.36 cd	33.07 ± 3.85 c	14.54 ± 1.08 e	19.08 ± 1.75 de	8.29 ± 1.46 e
Ca (mg/plant)	8.37 ± 0.43 b	10.16 ± 0.35 a	5.92 ± 0.29 c	5.85 ± 0.20 c	3.00 ± 0.53 de	4.00 ± 0.35 cd	1.48 ± 0.12 ef	2.27 ± 0.25 ef	0.87 ± 0.11 f
Mg (mg/plant)	2.66 ± 0.12 bc	4.18 ± 0.10 a	3.23 ± 0.27 b	2.16 ± 0.09 cd	1.50 ± 0.23 de	2.23 ± 0.16 c	1.36 ± 0.04 e	1.02 ± 0.09 ef	0.59 ± 0.12 f
S (mg/plant)	3.33 ± 0.19 b	5.99 ± 0.41 a	5.39 ± 0.26 a	1.83 ± 0.09 cde	2.49 ± 0.50 bcd	2.78 ± 0.44 bc	0.78 ± 0.02 e	1.28 ± 0.17 de	0.64 ± 0.09 e
Cu (µg/plant)	12.29 ± 0.79 b	15.23 ± 0.19 a	16.75 ± 0.70 a	6.18 ± 0.08 c	5.66 ± 0.99 cd	6.28 ± 0.71 c	4.15 ± 0.26 cd	3.12 ± 0.29 de	1.25 ± 0.16 e
Fe (µg/plant)	160.17 ± 9.46 b	232.22 ± 7.00 a	275.52 ± 14.09 a	135.32 ± 7.29 bc	99.28 ± 18.35 cd	113.74 ± 13.52 bc	102.54 ± 7.45 cd	58.28 ± 6.11 d	51.76 ± 9.03 d
Mn (µg/plant)	135.04 ± 6.94 bc	243.75 ± 8.71 a	148.27 ± 4.27 b	91.33 ± 0.93 cd	75.66 ± 14.36 de	181.11 ± 27.65 b	72.27 ± 3.45 de	62.28 ± 3.68 de	27.33 ± 2.99 e
Zn (µg/plant)	24.78 ± 1.12 bc	35.86 ± 0.78 a	28.63 ± 0.69 b	21.23 ± 1.14 c	14.38 ± 2.69 de	18.76 ± 2.08 cd	11.66 ± 0.74 ef	6.95 ± 0.82 fg	4.96 ± 0.99 g
Cd (µg/plant)	-	-	-	146.25 ± 6.49 bc	121.10 ± 22.66 c	119.64 ± 19.72 c	253.62 ± 10.75 b	470.33 ± 48.65 a	195.04 ± 34.91 bc
				Stems and sheaths					
N (mg/plant)	26.48 ± 1.26 a	16.49 ± 0.95 b	12.86 ± 0.66 b	6.01 ± 0.13 c	4.13 ± 0.35 c	6.92 ± 1.52 c	4.49 ± 0.16 c	6.54 ± 0.69 c	5.96 ± 0.89 c
P (mg/plant)	2.44 ± 0.19 abc	3.20 ± 0.09 a	2.60 ± 0.31 ab	1.74 ± 0.08 cde	1.22 ± 0.10 e	1.38 ± 0.22 de	1.60 ± 0.05 cde	2.07 ± 0.28 bcd	0.92 ± 0.03 e
K (mg/plant)	41.15 ± 2.84 b	66.14 ± 2.62 a	44.62 ± 3.50 b	24.28 ± 1.15 b	16.92 ± 0.92 cd	17.25 ± 3.77 cd	15.80 ± 0.46 cd	23.43 ± 3.26 c	11.45 ± 1.49 d
Ca (mg/plant)	5.54 ± 0.32 b	7.35 ± 0.21 a	3.52 ± 0.32 c	3.16 ± 0.34 cd	2.06 ± 0.27 de	1.68 ± 0.36 e	1.93 ± 0.06 de	2.61 ± 0.32 cde	1.30 ± 0.18 e
Mg (mg/plant)	2.68 ± 0.35 bc	4.49 ± 0.24 a	3.17 ± 0.27 b	1.17 ± 0.11 d	1.22 ± 0.19 d	1.53 ± 0.45 cd	1.16 ± 0.10 d	2.01 ± 0.33 bcd	0.87 ± 0.05 d
S (mg/plant)	1.41 ± 0.07 b	3.76 ± 0.17 a	3.85 ± 0.53 a	0.99 ± 0.05 b	1.00 ± 0.08 b	1.36 ± 0.25 b	1.18 ± 0.10 b	1.68 ± 0.17 b	1.46 ± 0.17 b
Cu (µg/plant)	3.87 ± 0.44 bc	5.35 ± 0.14 b	7.65 ± 0.48 a	3.39 ± 0.19 c	2.54 ± 0.31 c	3.26 ± 0.64 c	3.55 ± 0.07 bc	4.32 ± 0.62 bc	2.70 ± 0.32 c
Fe (µg/plant)	159.65 ± 10.01 bc	284.74 ± 13.59 a	140.96 ± 21.68 cd	213.00 ± 9.97 b	84.18 ± 7.48 de	90.50 ± 23.37 de	83.56 ± 5.40 de	124.69 ± 18.25 cd	52.38 ± 3.81 e
Mn (µg/plant)	42.18 ± 4.35 c	133.22 ± 4.03 a	60.71 ± 2.97 b	30.33 ± 1.93 cd	23.04 ± 1.73 d	29.08 ± 4.73 cd	31.55 ± 0.90 cd	33.06 ± 4.64 cd	22.07 ± 2.47 d
Zn (µg/plant)	15.72 ± 1.91 bcd	26.41 ± 0.24 a	20.07 ± 1.76 ab	16.16 ± 1.44 bcd	11.55 ± 1.03 cd	15.65 ± 2.45 bcd	21.57 ± 0.80 ab	18.33 ± 1.65 bc	9.18 ± 1.11 d
Cd (µg/plant)	-	-	-	117.37 ± 11.63 c	81.30 ± 10.74 c	130.47 ± 29.73 c	435.68 ± 17.50 b	704.54 ± 88.36 a	400.56 ± 37.78 b
				Roots					
N (mg/plant)	2.41 ± 0.16 c	4.68 ± 0.23 a	2.35 ± 0.07 c	2.15 ± 0.14 cd	3.31 ± 0.10 b	2.65 ± 0.10 c	0.68 ± 0.10 e	1.55 ± 0.04 d	0.31 ± 0.10 e
P (mg/plant)	0.38 ± 0.04 cd	1.00 ± 0.09 a	0.48 ± 0.04 cd	0.60 ± 0.04 bc	0.71 ± 0.06 b	0.82 ± 0.04 ab	0.27 ± 0.04 de	0.38 ± 0.01 de	0.12 ± 0.04 e
K (mg/plant)	5.08 ± 0.41 c	17.07 ± 0.57 a	8.08 ± 0.48 b	6.06 ± 0.30 c	7.99 ± 0.39 b	8.95 ± 0.55 b	1.12 ± 0.14 d	2.13 ± 0.14 d	0.89 ± 0.30 d
Ca (mg/plant)	0.65 ± 0.04 bcd	3.33 ± 0.18 a	0.92 ± 0.06 b	0.56 ± 0.04 cde	0.70 ± 0.07 bcd	0.72 ± 0.02 bc	0.24 ± 0.03 ef	0.37 ± 0.03 def	0.13 ± 0.05 f
Mg (mg/plant)	0.71 ± 0.04 cd	3.79 ± 0.11 a	0.95 ± 0.05 c	0.55 ± 0.05 de	0.70 ± 0.05 cd	1.35 ± 0.06 b	0.17 ± 0.02 f	0.29 ± 0.02 ef	0.07 ± 0.03 f
S (mg/plant)	0.26 ± 0.02 cd	2.52 ± 0.13 a	1.21 ± 0.06 b	0.57 ± 0.05 c	1.15 ± 0.06 b	1.41 ± 0.09 b	0.14 ± 0.03 d	0.42 ± 0.02 cd	0.17 ± 0.05 d
Cu (µg/plant)	1.12 ± 0.05 de	4.70 ± 0.10 b	1.69 ± 0.11 d	3.41 ± 0.21 c	5.78 ± 0.40 a	3.98 ± 0.29 bc	0.65 ± 0.10 e	1.72 ± 0.05 d	0.55 ± 0.16 e
Fe (µg/plant)	22.85 ± 1.21 d	94.67 ± 7.70 c	35.06 ± 3.68 d	146.47 ± 8.89 b	286.33 ± 14.02 a	244.60 ± 9.57 a	150.50 ± 19.53 b	122.34 ± 1.92 bc	43.33 ± 14.05 d
Mn (µg/plant)	23.25 ± 2.81 cd	144.42 ± 7.92 a	34.58 ± 3.53 bc	14.04 ± 0.74 d	43.71 ± 6.03 b	40.34 ± 3.82 bc	21.45 ± 2.73 cd	23.36 ± 2.57 cd	7.44 ± 2.40 d
Zn (µg/plant)	6.97 ± 0.40 cd	31.89 ± 1.72 a	10.30 ± 0.54 bc	9.71 ± 0.26 bc	10.87 ± 0.89 b	8.18 ± 0.67 bcd	4.57 ± 0.60 de	5.76 ± 0.28 d	1.38 ± 0.41e
Cd (µg/plant)	-	-	-	92.19 ± 7.77 b	122.08 ± 2.30 b	115.68 ± 6.83 b	125.77 ±15.76 b	175.31 ± 3.88 a	78.74 ± 24.22 b

Means ± SEM followed by different letters in rows differ significantly from each other by Tukey test (P <0.05).

Figure 5 - Localization of S, Ca, and Fe in cross sections of adventitious roots tip of Massai grass supplied with S and exposed to Cd. A-D: 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd; E-H: 1.9 mmol L^{-1} S + 0.5 mmol L^{-1} Cd; I-L: 3.7 mmol L^{-1} S + 0.5 mmol L^{-1} Cd. A, E, I: freeze-fractured roots observed under the scanning electron microscope; B-D, F-H, J -L: X-ray microfluorescence. Bars = 200 μm



4.4 Discussion

The lowest biomass production of Massai grass exposed to Cd (Figure 3) is a typical symptom of phytotoxicity, and this result occurs because Cd can change the nutrient uptake, stomatal opening and leaf transpiration, decrease photosynthetic concentration, degrade chlorophyll and cause oxidative stress, among other damages (CLEMENS, 2006; ŠIMONOVÁ et al., 2007; NAZAR et al., 2012; RIZWAN et al., 2017). Although there are studies showing that S attenuates injuries caused by Cd (BASHIR et al., 2015; KHAN et al., 2015, 2016; LIANG et al., 2016; RABÊLO; AZEVEDO; MONTEIRO, 2017a), the biomass production of Massai grass exposed to Cd did not significantly increase with increased S supply (Figure 3). This can be attributed to lowest water use efficiency observed in these plants (Figures 11H-1, p. 174), since other parameters such as chlorophyll concentration, liquid photosynthetic rate, and carboxylation efficiency increased (Figures 11C-D and 11G, p. 174) and the lipid peroxidation induced by reactive oxygen species (ROS) decreased (Figures 3E-F, p. 162) with proper S supply. Proper S supply can improve water use efficiency by grasses not exposed to toxic elements (ARTUR; GARCEZ; MONTEIRO, 2014), but when the plants are exposed to Cd, which changes the plant water relations (POSCHENREIDER; GUNSE; BARCELÓ, 1989), there may be no positive effect of S on water use efficiency.

The reduced root development and changes on root tissue and cell level represent other typical symptoms of phytotoxicity caused by Cd (LUX et al., 2011; MARTINKA; VACULÍK; LUX, 2014). The root length of Massai grass exposed to 0.5 mmol L^{-1} Cd was reduced by more than 40% and the root surface was reduced by more than 50% compared to plants not exposed to Cd, regardless of S supply (Table 3). Cadmium concentrations that inhibited root growth around 50% increased root hair development near the root apex in *Sorghum bicolor* (KURIAKOSE; PRASAD, 2008) and *Chloris gayana* (KOPITTKE; BLAMEY; MENZIES, 2010), suggesting that Cd accelerates the differentiation of these cells (LUX et al., 2011). However, exposure to high Cd concentrations may reduce the development of root hairs and disintegrate the rhizoderm and outer cortical layers (KURIAKOSE; PRASAD, 2008; GRATÃO et al., 2009), as verified in Massai grass exposed to highest Cd concentration (Figures 4C-D, 5E, and 5I). In addition, Massai grass exposed to 0.5 mmol L^{-1} Cd presented degradation of epidermis and greater diameter of cortical parenchyma, especially when plants were grown with 3.7 mmol L^{-1} S (Figure 4D). The increase in parenchyma cell size caused by Cd, especially when Cd concentrations do not cause significant root necrosis, may result in larger root diameter (MAKSIMOVIĆ et al., 2007; LUX et al., 2011). However, Massai grass grown in Cd presence showed lower root

diameter in relation to plants not exposed to Cd, especially when it was supplied with the two highest S concentrations (Table 3). Plants exposed to Cd showed formation of brownish lateral primordia near the root apex (Figure 4A). Lunáčková et al. (2003) also verified formation of lateral primordia near the root apex, and suggested that Cd accelerates the production of lateral roots. The development of roots of smaller diameter and lateral roots occurred in conditions of S supply of 0.1 mmol L^{-1}, when there was no exposure to metals (Table 3) (ZHAO et al., 2014). When Massai grass was exposed to Cd, S supply of 1.9 and 3.7 mmol L^{-1} reduced root diameter (Table 3). It is probable that highest S availability decreased ethylene synthesis in roots of Massai grass and, consequently, decreased the root diameter, since this hormone acts as a regulator of morphological responses related to abiotic stress, such as the growth of primary and lateral roots (REMANS et al., 2012; KHAN et al., 2016; ABOZEID et al., 2017). However, the TF of Massai grass exposed to 0.1 mmol L^{-1} Cd increased with S supply of 3.7 mmol L^{-1} (Table 3), which is probably related to increase of Cd bound to soluble fractions in the roots of these plants, which allows greater Cd transport from roots to shoots, as observed in rice (ZHANG et al., 2014).

Besides the smallest diameter and degradation of outer cortical cells layers observed in roots of plants exposed to Cd (Figures 4C-D, 5E and 5I), it is possible to observe fewest cortical cell layers and increased cell wall thickening in the endodermis and central cylinder (Figures 4C-D, 5E and 5I). This thickening usually occurs due to deposition of polymers, such as suberin and lignin (PARROTTA et al., 2015). Suberins and lignins form the impregnation material of Casparian bands developed on endodermal cell wall (SCHREIBER et al., 1999; WHITE, 2001). The deposition of suberin and lignin in the endodermis strongly restricts Cd entry in the xylem, so that this process can be interpreted as a response against Cd transport into root cells (LUX et al., 2004; 2011; PARROTTA et al., 2015). This process occurs closer to root apex in plants exposed to high concentrations of potentially toxic metals (VACULÍK et al., 2009; LUX et al., 2011), and indicates a greater ability to block Cd apoplasmic flow to xylem, since thickening of cell wall increases the area of metal retention (WÓJCIK et al., 2005; GOMES et al., 2011). These results are in accordance with our study, in which approximately 60% of Cd taken up by Massai grass was retained in the root apoplasmic space (Figure 6, p. 59). Gomes et al. (2011) also reported that there was greater deposition of suberin and lignin in the root endodermis in *Brachiaria decumbens* exposed to Cd, Cu, Pb and Zn. In addition, Massai grass presented gelatinous layer (G-layer; PARROTTA et al., 2015) when exposed to Cd (Figure 4D). This type of wall is characterized by presence of a thick layer of crystalline cellulose, usually associated with vascular tissues of plants grown under

stress conditions (GUERRIERO; SERGEANT; HAUSMAN, 2013), mostly in tissues of organs exposed to tension stress (MELLEROWICZ; GORSHKOVA, 2012). There is not much information about the functions of this layer in plants exposed to Cd, but it is known that G-layer is formed in conditions of high transpiration during the day (HAYASHI; KAIDA, 2011), suggesting that this layer can be synthesized in order to decrease the transport of Cd^{2+} to shoot in plants grown in Cd presence, since Cd is mainly taken up by mass flow and this process is governed by transpiration (STERCKEMAN et al., 2004).

Several factors such as the composition of cell wall of roots, biomass production and the presence of other ions affect the uptake and transport of metals (EKVALL; GREGER, 2003; CLEMENS, 2006). To exemplify, Cd concentration in all tissues of Massai grass grown under highest Cd concentration increased when the highest S concentration was supplied (Table 4), while the highest Cd contents were observed in plants grown with 0.5 mmol L^{-1} Cd and 1.9 mmol L^{-1} S (Table 7). According to Ekvall and Greger (2003), Cd concentration and Cd content are correlated with biomass production (Table 5), which corroborates our results, since the highest Cd concentrations occurred in plants that showed lowest biomass production (Table 4, Figure 3) and the highest Cd contents occurred in plants exposed to 0.5 mmol L^{-1} Cd, and which presented highest biomass production (Table 7; Figure 3). The supply of highest S concentrations (6 and 9 mmol L^{-1}) also resulted in greater Cd content in wheat (MATRASZEK et al., 2017), lettuce (MATRASZEK et al., 2016a) and mustard (MATRASZEK et al., 2016b), which is dangerous for food production but desirable for Cd phytoextraction (SHEORAN; SHEORAN; POONIA, 2016). Excessive S supply increases Cd content in the roots due to reduction in Fe plaques formation (FAN et al., 2010) and allows highest Cd transport from roots to shoot (ZHANG et al., 2013), possibly due to PCs synthesis, since PCs act in Cd transport from roots to shoot (MENDOZA-CÓZATL et al., 2008). Sulfur supply of 3.7 mmol L^{-1} decreased Fe plaques formation in the roots of plants exposed to 0.5 mmol L^{-1} Cd (Tables 4 and 7, Figures 5H and 5L), while S supply of 3.7 mmol L^{-1} allowed greater Cd transport from roots to stems and sheaths in plants exposed to 0.1 mmol L^{-1} Cd (Table 6). In this sense, it is possible that S supply of 3.7 mmol L^{-1} decreased Cd fraction bound to cell wall and increased Cd fraction bound to soluble fractions in the roots of these plants, which allows greater Cd transport from roots to shoots (ZHANG et al., 2014). Besides the change in root cytology and anatomy (LUX et al., 2011; MARTINKA; VACULÍK; LUX, 2014), Cd can cause nutritional disturbances in plants due to: i) competition of Cd^{2+} using the same nutrient uptake sites (CLEMENS; FENG MA, 2016), ii) change in the distribution of these nutrients in the metabolic processes (CLEMENS,

2006, NAZAR et al., 2012, GOMES; SÁ E MELO MARQUES; SOARES, 2013), and iii) change in the water relations of plant (POSCHENREIDER; GUNSE; BARCELÓ, 1989).

In general, Massai grass exposed to 0.1 mmol L^{-1} Cd presented lower Cu concentration in the leaf blades, N and K in the stems and sheaths and Ca and Mg in the roots, while plants exposed to 0.5 mmol L^{-1} Cd showed lower concentrations of N in the leaf blades, K in the stems and sheaths and N, K, Ca, Mg and S in the roots, compared to plants not exposed to Cd (Table 4). Cadmium decreases nitrate reductase activity and, consequently, nitrate (NO_3^-) uptake and its transport from root to shoot, decreasing N concentration in shoots (KHAN et al., 2015). Tomato plants exposed to Cd also showed lowest N concentrations in shoots (NOGUEIROL et al., 2016). Astolfi, Zuchi and Passera (2005) reported that Cd inhibits the activity of H^+-ATPase (ion-carrying protein across the plasma membrane) and changes the membrane electrochemical gradient, which decreases the nutrients uptake such as K and Mg (LINDBERG; WINGSTRAND, 1985). In addition, Cd may cause changes in the root vascular system (e.g. reduction in the number and diameter of xylem vessels) that decreases nutrient transport, such as K, to shoots (OUZOUNIDOU et al., 1994; LUX et al., 2011). Cadmium can also alter ion balance by competition of Cd^{2+} by Ca transporters, decreasing Ca concentrations (TRAN; POPOVA, 2013). Cadmium exposure also resulted in lowest K concentration in shoots of corn (OBATA; UMEBAYASHI, 1997) and lowest Ca and Mg concentrations in roots of sunflower (AZEVEDO; PINTO; SANTOS, 2005) and tomato (GRATÃO et al., 2015). Yamaguchi et al. (2016) reported that Cd stimulated sulfate (SO_4^{2-}) transport from roots to shoots in *Arabdopsis thaliana*, which resulted in lowest S concentrations in roots. This process occurs by higher expression of *SULTR2;1* and *SULTR3;5* genes, which recover and transport SO_4^{2-} from root apoplasmic space to shoots (TAKAHASHI et al., 2000; KATAOKA et al., 2004). Although S concentration in roots of plants exposed to highest Cd concentration decreased, S concentration in adventitious root tip (cortex region) of these plants increased (Figures 5F and 5J). This suggests that S may have bound to Cd (Cd-S) in the cortex region (VAN BELLEGHEM et al., 2007), forming Cd-PCs, Cd-PCs-sulphide and/or Cd-sulphide associations (VÖGELI-LANGE; WAGNER, 1996) in order to reduce the damage caused by this metal.

The exposure of Massai grass to Cd also resulted in lowest nutrient content (Table 7), with exception of Cu and Fe contents in the roots of plants exposed to 0.1 mmol L^{-1} Cd. Plants exposed to Cd usually exhibit lowest nutrient contents because Cd decreases plant biomass production, while nutrient content is positively correlated to biomass production (EKVALL; GREGER, 2003; CLEMENS, 2006; MATRASZEK et al., 2016a, 2016b).

Highest Fe content occurs by Fe plaques formation on root surface of plants exposed to Cd due to change in the Fe^{2+}/Mn^{2+} availability, redox potential and concentration of radial oxygen loss in the root (MENDELSSOHN; KLEISS; WAKELEY, 1995; SEBASTIAN; PRASAD, 2016). The plaque is formed when oxygen released from the aerenchyma oxidizes Fe^{2+} to Fe^{3+}, and Fe^{3+} oxide precipitates on root surface (SEBASTIAN; PRASAD, 2015). Iron plaques can adsorb Cu, resulting in highest concentration (Table 4) and content (Table 7) of this nutrient in the roots of plants that show Fe plaques (YE et al., 1997). The exposure of Massai grass to lowest Cd concentration also resulted in highest concentrations of Zn in the stems and sheaths and P in the roots (Table 4), while Massai grass exposure to 0.5 mmol L^{-1} Cd resulted in highest concentrations of K, Mg, Fe, Mn, and Zn in the leaf blades, P, Cu, and Zn in the stems and sheaths and Fe in the roots (Table 4). High P concentration in the roots may occur from Cd association with phosphates in the root cortex [$Cd_3(PO_4)_2$] (VAN BELLEGUEM et al., 2007), in order to reduce Cd transport from root to shoot (SELA et al., 1990). The increase in concentrations of P, K, Mg, Fe, Mn, and Zn in shoots of Massai grass exposed to Cd (Table 4) is associated to lowest biomass production of these plants (Figure 3; Table 5) (EKVALL; GREGER, 2003). The effect of nutrient concentration as a function of lowest biomass production of plants exposed to Cd has been reported in several studies (DRAŽIĆ; MIHAILOVIĆ; STOJANOVIĆ, 2004; JIANG et al., 2004; GHNAYA et al., 2005; GRATÃO et al., 2015; NOGUEIROL et al., 2016). Concentrations of N, S and Mn in the leaf blades, N, Mg, S and Mn in the stems and sheathsand S, Fe and Mn in the roots of Massai grass exposed to 0.1 mmol L^{-1} Cd increased with S supply of 1.9 and 3.7 mmol L^{-1} (Table 4). Khan et al. (2015) reported that exposure of wheat to Cd resulted in lowest nitrate reductase activity and N concentration, but this effect was eliminated with proper S supply. The authors attributed the result to highest N allocation to Rubisco in S well-nourished plants, which resulted in highest photosynthetic rate and plant growth, and consequently, in highest N concentration. The increase in S concentration is a result of highest S supply (DAVIDIAN; KOPRIVA, 2010). It should be remembered that S well-nourished plants are more tolerant to Cd, which is essential for Cd phytoextraction (BASHIR et al., 2015; CAPALDI et al., 2015; DEDE; OZDEMIR, 2016; MATRASZEK et al., 2016a, 2017; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b). Sulfur can reduce Fe oxidation in the roots of plants exposed to Cd, which decreases Fe plaques stability (GAO et al., 2010), and possibly favors Mn^{2+} uptake, which is essential to decrease the oxidative stress induced by Cd, since Mn acts on ROS detoxification in mitochondria and peroxisomes (Mn-SOD) (GILL; TUTEJA, 2010).

4.5 Conclusions

Massai grass root development was strongly inhibited when plants were exposed to 0.5 mmol L^{-1} Cd, but proper S supply allowed highest Cd content, while excessive S supply decreased Fe plaques formation in the roots of plants. On the other hand, there was a greater development of Massai grass roots exposed to 0.1 mmol L^{-1} Cd, taking into account that this species presented strategies to restrict Cd entry (e.g. deposition of suberin and lignin in the endodermis and G-layer development). Proper S supply to these plants increased root length and root surface and concentrations of some nutrients, such as N, S and Mn. In view of this context, it seems evident that Massai grass properly supplied with S presents great potential for Cd phytoextraction.

References

ABOZEID, A.; YING, Z.; LIN, Y.; LIU, J.; ZHANG, Z.; TANG, Z. Ethylene improves root system development under cadmium stress by modulating superoxide anion concentration in *Arabidopsis thaliana*. **Frontiers in Plant Science**, Lausanne, v. 8, 2017. doi: 10.3389/fpls.2017.00253.

AKHTER, M.F.; OMELON, C.R.; GORDON, R.A.; MOSER, D.; MACFIE, S.M. Localization and chemical speciation of cadmium in the roots of barley and lettuce. **Environmental and Experimental Botany**, Oxford, v. 100, p. 10-19, 2014.

ARTUR, A.G.; GARCEZ, T.B.; MONTEIRO, F.A. Water use efficiency of marandu palisadegrass as affected by nitrogen and sulphur concentrations. **Revista Ciência Agronômica**, Fortaleza, v. 45, p. 10-17, 2014.

ASTOLFI, S.; ZUCHI, S.; PASSERA, C. Effect of cadmium on H^+-ATPase activity of plasma membrane vesicles isolated from roots of different S-supplied maize (*Zea mays* L.) plants. **Plant Science**, Clare, v. 169, p. 361-368, 2005.

AZEVEDO, H.; PINTO, C.G.G.; SANTOS, C. Cadmium effects in sunflower: nutritional imbalances in plants and calluses. **Journal of Plant Nutrition**, Philadelphia, v. 28, p. 2221-2231, 2005.

BABULA, P.; RYANT, P.; ADAM, V.; ZEHNALEK, J.; HAVEL, L.; KIZEK, R. The role of sulphur in cadmium(II) ions detoxification demonstrated in *in vitro* model: *Dionaea muscipula* Ell. **Environmental Chemistry Letters**, Heidelberg, v. 7, p. 353-361, 2009.

BASHIR, H.; IBRAHIM, M.M.; BAGHERI, R.; AHMAD, J.; ARIF, I.A.; BAIG, M.A.; QURESHI, M.I. Influence of sulfur and cadmium on antioxidants, phytochelatins and growth in Indian mustard. **AoB Plants**, Oxford, v. 7, 2015. doi: 10.1093/aobpla/plv001.

CAPALDI, F.R.; GRATÃO, P.L.; REIS, A.R.; LIMA, L.W.; AZEVEDO, R.A. Sulfur metabolism and stress defense responses in plants. **Tropical Plant Biology**, New York, v. 8, p. 60-73, 2015.

CLEMENS, S. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. **Biochimie**, Paris, v. 88, p. 1707-1719, 2006.

CLEMENS, S.; FENG MA, J. Toxic heavy metal and metalloid accumulation in crop plants and foods. **Annual Review of Plant Biology**, Palo Alto, v. 67, p. 1-12, 2016.

DAVIDIAN, J.C.; KOPRIVA, S. Regulation of sulfate uptake and assimilation - the same or not the same? **Molecular Plant**, Cambridge, v. 3, p. 314-325, 2010.

DEDE, G.; OZDEMIR, S. Effects of elemental sulphur on heavy metal uptake by plants growing on municipal sewage sludge. **Journal of Environmental Management**, London, v. 166, p. 103-108, 2016.

DRAŽIĆ, G.; MIHAILOVIĆ, N.; STOJANOVIĆ, Z. Cadmium toxicity: the effect on macro- and micro-nutrient contents in soybean seedlings. **Biologia Plantarum**, Prague, v. 48, p. 605-607, 2004.

EKVALL, L.; GREGER, M. Effects of environmental biomass-producing factors on Cd uptake in two Swedish ecotypes of *Pinus sylvestris*. **Environmental Pollution**, Oxford, v. 121, p. 401-411, 2003.

EPSTEIN, E.; BLOOM, A.J. **Mineral nutrition of plants**: principles and perspectives. 2. ed. Sunderland: Sinauer Associates, 2005. 380 p.

GUSTAFSSON, J.P. **Visual MINTEQ**. Version 3.0. Stockholm, 2012. Available at: <http://www2.lwr.kth.se/English/OurSoftware/vminteq/Stockholm-Sweden>. Access in: oct. 25. 2017.

FAN, J.L.; HU, Z.Y.; ZIADI, N.; XIA, X.; WU, C.Y.H. Excessive sulfur supply reduces cadmium accumulation in brown rice (*Oryza sativa* L.). **Environmental Pollution**, Oxford, v. 158, p. 409-415, 2010.

GAO, M.X.; HU, Z.Y.; WANG, G.D.; XIA, X. Effect of elemental sulfur supply on cadmium uptake into rice seedlings when cultivated in low and excess cadmium soils. **Communications in Soil Science and Plant Analysis**, New York, v. 41, p. 990-1003, 2010.

GHNAYA, T.; NOUAIRI, I.; SLAMA, I.; MESSEDI, D.; GRIGNON, C.; ABDELLY, C.; GHORBEL, M.H. Cadmium effects on growth and mineral nutrition of two halophytes: *Sesuvium portulacastrum* and *Mesembryanthemum crystallinum*. **Journal of Plant Physiology**, Jena, v. 162, p. 1133-1140, 2005.

GILL, S.S.; TUTEJA, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. **Plant Physiology and Biochemistry**, Paris, v. 48, p. 909-930, 2010.

GOMES, M.P.; MARQUES, T.C.L.L.S.M.; NOGUEIRA, M.O.G.; CASTRO, E.M.; SOARES, A.M. Ecophysiological and anatomical changes due to uptake and accumulation of heavy metal in *Brachiaria decumbens*. **Scientia Agricola**, Piracicaba, v. 68, p. 566-573, 2011.

GOMES, M.P.; SÁ E MELO MARQUES, T.C.L.L.; SOARES, A.M. Cadmium effects on mineral nutrition of the Cd-hyperaccumulator *Pfaffia glomerata*. **Biologia**, Warsow, v. 68, p. 223-230, 2013.

GRATÃO, P.L.; MONTEIRO, C.C.; ROSSI, M.L.; MARTINELLI, A.P.; PERES, L.E.P.; MEDICI, L.O.; LEA, P.J.; AZEVEDO, R.A. Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium. **Environmental and Experimental Botany**, Oxford, v. 67, p. 387-394, 2009.

GRATÃO, P.L.; MONTEIRO, C.C.; TEZOTTO, T.; CARVALHO, R.F.; ALVES, L.R.; PETERS, L.P.; AZEVEDO, R.A. Cadmium stress antioxidant responses and root-to-shoot communication in grafted tomato plants. **Biometals**, Paris, v. 28, p. 803-816, 2015.

GUERRIERO, G.; SERGEANT, K.; HAUSMAN, J.F. Integrated-omics: a powerful approach to understanding the heterogeneous lignification of fibre crops. **International Journal of Molecular Science**, Basel, v. 14, p. 10958-10978, 2013.

HAYASHI, T.; KAIDA, R. Functions of xyloglucan in plant cells. **Molecular Plant**, Cambridge, v. 4, p. 17-24, 2011.

HE, S.; HE, Z.; YANG, X.; STOFFELLA, P.J.; BALIGAR, V.C. Soil biogeochemistry, plant physiology, and phytoremediation of cadmium-contaminated soils. **Advances in Agronomy**, San Diego, v. 134, p. 135-225, 2015.

JIANG, X.J.; LUO, Y.M.; LIU, Q.; LIU, S.L.; ZHAO, Q.G. Effects of cadmium on nutrient uptake and translocation by Indian Mustard. **Environmental Geochemistry and Health**, Dordrecht, v. 26, p. 319-324, 2004.

JORGE, L.A.C.; RODRIGUES, A.F.O. **Safira**: sistema de análise de fibras e raízes. São Carlos: Embrapa Instrumentação Agropecuária, 2008. 20 p. (Boletim de Pesquisa e Desenvolvimento, 24).

KARNOVSKY, M.J. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. **Journal of Cell Biology**, New York, v. 27, p. 137-138, 1965.

KATAOKA, T.; HAYASHI, N.; YAMAYA, T.; TAKAHASHI, H. Root-to-shoot transport of sulfate in Arabidopsis: Evidence for the role of *SULTR3;5* as a component of low-affinity sulfate transport system in the root vasculature. **Plant Physiology**, Rockville, v. 136, p. 4198-4204, 2004.

KHAN, M.I.R.; NAZIR, F.; ASGHER, M.; PER, T.S.; KHAN, N.A. Selenium and sulfur influence ethylene formation and alleviate cadmium induced oxidative stress by improving proline and glutathione production in wheat. **Journal of Plant Physiology**, Jena, v. 173, p. 9-18, 2015.

KHAN, M.N.A.; ASGHER, M.; PER, T.S.; MASSOD, A.; FATMA, M.; KHAN, M.I.R. Ethylene potentiates sulfur-mediated reversal of cadmium inhibited photosynthetic responses in mustard. **Frontiers in Plant Science**, Lausanne, v. 7, 2016. doi: 10.3389/fpls.2016.01628.

KOPITTKE, P.M.; BLAMEY, F.P.C.; MENZIES, N.W. Toxicity of Cd to signal grass (*Brachiaria decumbens* Stapf.) and Rhodes grass (*Chloris gayana* Kunth.). **Plant and Soil**, Dordrecht, v. 330, p. 515-523, 2010.

KURIAKOSE, S.V.; PRASAD, M.N.V. Cadmium stress affects germination and seedling growth in *Sorghum bicolor* (L.) Moench by changing the activities of hydrolyzing enzymes. **Plant Growth Regulation**, Dordrecht, v. 54, p. 143-156, 2008.

LIANG, T.; DING, H.; WANG, G.; KANG, J.; PANG, H.; LV, J. Sulfur decreases cadmium translocation and enhances cadmium tolerance by promoting sulfur assimilation and glutathione metabolism in *Brassica chinensis* L. **Ecotoxicology and Environmental Safety**, San Diego, v. 124, p. 129-137, 2016.

LINDBERG, S.; WINGSTRAND, G. Mechanisms for Cd^{2+} inhibition of (K^{+}+Mg^{2+}) ATPase activity and uptake in roots of sugar beet (*Beta vulgaris*). **Physiologia Plantarum**, Lund, v. 63, p. 181-186, 1985.

LUNÁČKOVÁ, L.; ŠOTTNÍKOVÁ, A.; MASAROVIČOVÁ E.; LUX, A.; STREŠKO, V. Comparison of cadmium effect on willow and poplar in response to different cultivation conditions. **Biologia Plantarum**, Prague, v. 47, p. 403-411, 2003.

LUX, A.; ŠOTTNÍKOVÁ, A.; OPATRNÁ, J.; GREGER, M. Differences in structure of adventitious roots in Salix clones with contrasting characteristics of cadmium accumulation and sensitivity. **Physiologia Plantarum**, Lund, v. 120, p. 537-545, 2004.

LUX, A.; MARTINKA, M.; VACULÍK, M.; WHITE, P.J. Root responses to cadmium in rhizosphere: a review. **Journal of Experimental Botany**, Oxford, v. 62, p. 21-37, 2011.

MAHAR, A.; WANG, P.; ALI, A.; AWASTHI, M.K.; LAHORI, A.H.; WANG, Q.; LI, R.; ZHANG, Z. Challenges and opportunities in the phytoremediation of heavy metals contaminated soils: A review. **Ecotoxicology and Environmental Safety**, San Diego, v. 126, p. 111-121, 2016.

MAKSIMOVIĆ, I.; KASTORI, R.; KRSTIĆ, L.; LUKOVIĆ, J. Steady presence of cadmium and nickel affects root anatomy, accumulation and distribution of essential ions in maize seedlings. **Biologia Plantarum**, Prague, v. 51, p. 589-592, 2007.

MALAVOLTA, E.; VITTI, G.C.; OLIVEIRA, S.A. **Avaliação do estado nutricional das plantas**: princípios e aplicações. 2. ed. Piracicaba: Potafos, 1997. 201 p.

MARTINKA, M.; VACULÍK, M.; LUX, A. Plant cell responses to cadmium and **zinc**. In: NICK, P.; OPATRNÝ, Z. (Ed.). **Applied plant cell biology**. Cellular tools and approaches for plant biotechnology. Heidelberg: Springer, 2014. chap. 7, p. 209-246.

MATRASZEK, R.; HAWRYLAK-NOWAK, B.; CHWIL, S.; CHWIL, M. Interaction between cadmium stress and sulphur nutrition level on macronutrient status of *Sinapis alba* L. **Water, Air and Soil Pollution**, Dordrecht, v. 227, 2016a. doi: 10.1007/s11270-016-3059-9.

MATRASZEK, R.; HAWRYLAK-NOWAK, B.; CHWIL, S.; CHWIL, M. Macroelemental composition of cadmium stressed lettuce plants grown under conditions of intensive sulphur nutrition. **Journal of Environmental Management**, London, v. 180, p. 24-34, 2016b.

MATRASZEK, R.; CHWIL, S.; HAWRYLAK-NOWAK, B.; KOZŁOWSKA-STRAWSKA, J. Effect of sulphur and cadmium on macronutrient balance in spring wheat. **Proceedings of the National Academy of Sciences, Section B-Biological Sciences**, Allahabad, v. 87, p. 927-936, 2017.

MELLEROWICZ, E.J.; GORSHKOVA, T.A. Tensional stress generation in gelatinous fibres: a review and possible mechanism based on cell-wall structure and composition**. Journal of Experimental Botany**, Oxford, v. 63, p. 551-565, 2012.

MENDELSSOHN, I.A.; KLEISS, B.A.; WAKELEY, J.S. Factors controlling the formation of oxidized root channels: a review and annotated bibliography. **Wetlands**, Dordrecht, v. 15, p. 37-46, 1995.

MENDOZA-CÓZATL, D.G.; BUTKO, E.; SPRINGER, F.; TORPEY, J.W.; KOMIVES, E.A.; KEHR, J.; SCHROEDER, J.I. Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. **Plant Journal**, Hoboken, v. 54, p. 249-259, 2008.

NAZAR, R.; IQBAL, N.; MASOOD, A.; IQBAL, M.; KHAN, R.; SYEED, S.; KHAN, N.A. Cadmium toxicity in plants and role of mineral nutrients in its alleviation. **American Journal of Plant Sciences**, Georgetown, v. 3, p. 1476-1489, 2013.

NOGUEIROL, R.C.; MONTEIRO, F.A.; GRATÃO, P.L.; SILVA, B.K.A.; AZEVEDO, R.A. Cadmium application in tomato: nutritional imbalance and oxidative stress. **Water, Air and Soil Pollution**, Dordrecht, v. 227, 2016. doi: 10.1007/s11270-016-2895-y.

OBATA, H.; UMEBAYASHI, M. Effects of cadmium on mineral nutrient concentrations in plants differing in tolerance for cadmium. **Journal of Plant Nutrition**, Philadelphia, v. 20, p. 97-105, 1997.

OUZONIDOU, G.; CLAMPORAVA, M.; MOUSTAKAS, M.; KARATAGLIS, S. Responses of maize (*Zea mays* L.) plants to copper stress - I. Growth, mineral content and ultrastructure of roots. **Environmental and Experimental Botany**, Oxford, v. 35, p. 167-176, 1994.

PARROTTA, L.; GUERRIERO, G.; SERGEANT, K.; CAI, G.; HAUSMAN, J. Target or barrier? The cell wall of early- and later-diverging plants *vs* cadmium toxicity: differences in the response mechanisms. **Frontiers in Plant Science**, Lausanne, v. 6, 2015. doi: 10.3389/fpls.2015.00133.

POSCHENRIEDER, C.; GUNSE, B.; BARCELÓ, J. Influence of cadmium on water relations, stomatal resistance, and abscisic acid content in expanding bean leaves. **Plant Physiology**, Rockville, v. 90, p. 1365-1371, 1989.

RABÊLO, F.H.S.; BORGO, L. Changes caused by heavy metals in micronutrient content and antioxidant system of forage grasses used for phytoremediation: an overview. **Ciência Rural**, Santa Maria, v. 46, p. 1368-1375, 2016.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. Proper supply of S increases GSH synthesis in the establishment and reduces tiller mortality during the regrowth of Tanzania guinea grass used for Cd phytoextraction. **Journal of Soils and Sediments**, Heidelberg, v. 17, p. 1427-1436, 2017a.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. The proper supply of S increases amino acid synthesis and antioxidant enzyme activity in Tanzania guinea grass used for Cd phytoextraction. **Water, Air and Soil Pollution**, Dordrecht, v. 228, 2017b. doi: 10.1007/s11270-017-3563-6.

REMANS, T.; THIJS, S.; TRUYENS, S.; WEYENS, N.; SCHELLINGEN, K.; KEUNEN, E.; GIELEN, H.; CUYPERS, A.; VANGRONSVELD, J. Understanding the development of roots exposed to contaminants and the potential of plant-associated bacteria for optimization of growth. **Annals of Botany**, Oxford, v. 110, p. 239-252, 2012.

RIZWAN, M.; ALI, S.; ADREES, M.; IBRAHIM, M.; TSANG, D.C.W.; ZIA-UR-REHMAN, M.; ZAHIR, Z.A.; RINKLEBE, J.; TACK, F.M.G.; OK, Y.S. A critical review on effects, tolerance mechanisms and management of cadmium in vegetables. **Chemosphere**, Oxford, v. 182, p. 90-105, 2017.

SAS INSTITUTE. **SAS User's guide**: Statistics. Version 9.2. Cary, NC, 2008. Available at: <http://support.sas.com/software/92/ Cary-United States of America>. Access in: oct. 26. 2017.

SCHREIBER, L.; HARTMANN, K.; SKRABS, M.; ZEIER, J. Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. **Journal of Experimental Botany**, Oxford, v. 50, p. 1267-1280, 1999.

SEBASTIAN, A.; PRASAD, M.N.V. Trace element management in rice. **Agronomy**, Basel, v. 5, p. 374-404, 2015.

SEBASTIAN, A.; PRASAD, M.N.V. Iron plaque decreases cadmium accumulation in *Oryza sativa* L. and serves as a source of iron. **Plant Biology**, Hoboken, v. 18, p. 1008-1015, 2016.

SELA, M.; FRITZ, E.; HUTTERMANN, A.; TEL-OR, E. Studies on cadmium localization in the water fern *Azolla*. **Physiologia Plantarum**, Lund, v. 79, p. 547-553, 1990.

ŠIMONOVÁ, E.; HENSELOVÁ, M.; MASAROVIČOVÁ E.; KOHANOVÁ, J. Comparison of tolerance of *Brassica juncea* and *Vigna radiata* to cadmium. **Biologia Plantarum**, Prague, v. 51, p. 488-492, 2007.

SHEORAN, V.; SHEORAN, A.S.; POONIA, P. Factors affecting phytoextraction: a review. **Pedosphere**, Beijing, v. 26, p. 148-166, 2016.

STERCKEMAN, T.; PERRIGUEY, J.; CAËL, M.; SCHWARTZ, C.; MOREL, J.L. Applying a mechanistic model to cadmium uptake by *Zea mays* and *Thlaspi caerulescens*: consequences for the assessment of the soil quantity and quality factors. **Plant and Soil**, Dordrecht, v. 262, p. 289-302, 2004.

TAKAHASHI, H.; WATANABE-TAKAHASHI, A.; SMITH, F.W.; BLAKE-KALFF, M.; HAWKESFORD, M.J.; SAITO, K. The role of three functional sulfate transporters involved in uptake and translocation of sulfate in *Arabidopsis thaliana*. **Plant Journal**, Hoboken, v. 23, p. 171-182, 2000.

TRAN, T.A.; POPOVA, L.P. Functions and toxicity of cadmium in plants: recent advances and future prospects. **Turkish Journal of Botany**, Ankara, v. 37, p. 1-13, 2013.

VACULÍK, M.; LUX, A.; LUXOVÁ, M.; TANIMOTO, E.; LICHTSCHEIDL, E. Silicon mitigates cadmium inhibitory effects in young maize plants. **Environmental and Experimental Botany**, Oxford, v. 67, p. 52-58, 2009.

VAN BELLEGHEM, F.; CUYPERS, A.; SEMANE, B.; SMEETS, K.; VANGRONSVELD, J.; d'HAEN, J.; VALCKE, R. Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. **New Phytologist**, London, v. 173, p. 495-508, 2007.

VÖGELI-LANGE, R.; WAGNER, G.J. Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves - implication of a transport function for cadmium-binding peptides. **Plant Physiology**, Rockville, v. 92, p. 1086-1093, 1990.

WHITE, P.J. The pathways of calcium movement to the xylem. **Journal of Experimental Botany**, Oxford, v. 52, p. 891-899, 2001.

WILKINS, D.A. The measurement of tolerance to edaphic factors by means of root growth. **New Phytologist**, London, v. 80, p. 623-633, 1978.

WÓJCIK, M.; VANGRONSVELD, J.; D'HAEN, J.; TUKIENDORF, A. Cadmium tolerance in *Thlaspi caerulescens*. II. Localization of cadmium in *Thlaspi caerulescens*. **Environmental and Experimental Botany**, Oxford, v. 53, p. 163-171, 2005.

YAMAGUCHI, C.; TAKIMOTO, Y.; OHKAMA-OHTSU, N.; HOKURA, A.; SHINANO, T.; NAKAMURA, T.; SUYAMA, A.; MARUYAMA-NAKASHITA, A. Effects of cadmium treatment on the uptake and translocation of sulfate in *Arabidopsis thaliana*. **Plant and Cell Physiology**, Oxford, v. 57, p. 2353-2366, 2016.

YE, Z.H.; BAKER, A.J.M.; WONG, M.H.; WILLIS, A.J. Copper and nickel uptake, accumulation and tolerance in *Typha latifolia* with and without iron plaque on the root surface. **New Phytologist**, London, v. 136, p. 481-488, 1997.

ZHANG, W.; LIN, K.F.; ZHOU, J.; ZHANG, W.; LIU, L.L.; HAN, X.L. Spatial distribution and toxicity of cadmium in the joint presence of sulfur in rice seedling. **Environmental Toxicology and Pharmacology**, Amsterdam, v. 36, p. 1235-1241, 2013.

ZHANG, W.; LIN, K.; ZHOU, J.; ZHANG, W.; LIU, L.; ZHANG, Q. Cadmium accumulation, sub-cellular distribution and chemical forms in rice seedling in the presence of sulfur. **Environmental Toxicology and Pharmacology**, Amsterdam, v. 37, p. 348-353, 2014.

ZHAO, Q.; WU, Y.; GAO, L.; MA, J.; LI, C.; XIANG, C. Sulfur nutrient availability regulates root elongation by affecting root indol -3- acetic acid levels and the stem cell niche. **Journal of Integrative Plant Biology**, Hoboken, v. 56, p. 1151-1163, 2014.

5 METABOLITE PROFILING REVEALS THAT SUFFICIENT SULFUR SUPPLY IS ESSENTIAL FOR INCREASED Cd PHYTOEXTRACTION IN MASSAI GRASS

Abstract

Improving sugar, amino and organic acid, glutathione (GSH) and phytochelatin (PC) content in plant tissues has the potential to lower a plant's susceptibility to cadmium (Cd) toxicity. Since the above-mentioned metabolites can be altered by S nutrition supplying plants with sulfur (S) has been suggested as a way to improve Cd tolerance. In the current study, our aim was to analyze the effect of S nutrition on the metabolic profile and the synthesis of GSH and PCs in *Panicum maximum* cv. Massai (Massai grass) which is used for Cd phytoextraction. We evaluated combinations of three S (0.1, 1.9 and 3.7 mmol L^{-1}) and two Cd concentrations (0.1 and 0.5 mmol L^{-1}) within a single growth period. Plants were subsequently harvested and separated into leaf blades, a sample comprising both stem and sheath material, and roots. The tissues of plants exposed to Cd showed distinct responses related to primary metabolism. The amino acids lysine, cysteine, ornithine, arginine, tryptophan and histidine accumulated in more than one tissue, indicating that they are involved in Cd accumulation and/or detoxification in Massai grass. Among the sugars and sugar derivatives detected, galactinol appears to be the most active in Cd detoxification. As opposed to amino acids and sugars, organic acids were unaffected by Cd exposure. Although there was no effect of Cd/S combinations on the expression of the genes encoding γ-glutamylcysteine synthetase (*GSH1*) and phytochelatin synthase (*PCS2*), the levels of GSH and PCs were strongly increased by Cd, mainly in roots and samples comprising both stem and sheath material of Massai grass and independently of different metabolic changes occurring in these tissues. Synthesis of most metabolites evaluated in this study was mostly induced when Cd-exposed Massai grass was supplied with 1.9 mmol L^{-1} S. Thus, proper S supply improves Cd detoxification mechanisms such as sequestration by PCs and amino acids in Massai grass used for Cd phytoextraction.

Key words: metabolomics, *Panicum maximum*, thiol compounds, tolerance mechanisms.

5.1 Introduction

Over recent decades, the concentrations of cadmium (Cd) in agricultural soils of several countries have increased (KHAN et al., 2017). This represents a serious socio-environmental problem since Cd is one of the most toxic metals present in the environment (AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY - ATSDR, 2012), and it can be taken

up by plants and thereby enter the human food chain (SANITÁ DI TOPPI; GABBRIELLI, 1999). Recent studies show that human Cd intake was above the limits recommended by ATSDR and the European Food Safety Authority (EFSA) (CLEMENS et al., 2013). Given that Cd can cause a number of human diseases including pulmonary emphysema and cancer (WHO, 2011), it is fundamental to reduce its concentration in soils in order to decrease its entry into in the food chain. Among the several techniques used to lower soil Cd concentrations, phytoextraction is one displaying the lowest environmental impact and lowest costs, and as such is often regarded as the most socially acceptable (SHEORAN; SHEORAN; POONIA, 2016). However, few plants are tolerant to Cd toxicity (SANITÁ DI TOPPI; GABBRIELLI, 1999), with merely nine plants being considered as Cd hyperaccumulators (MEYER; VERBRUGGEN, 2012). In addition, these plants grow slowly and as a result do not produce high biomass (MEYER; VERBRUGGEN, 2012), thereby decreasing their efficiency of phytoextraction (SHEORAN; SHEORAN; POONIA, 2016). As an alternative, the use of forage grasses for Cd phytoextraction has been shown to be promising (XIE et al., 2014; RABÊLO; BORGO, 2016; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b; RABÊLO; JORDÃO; LAVRES, 2017) due to their flexible adaptation to soil and climatic adversities, high biomass production, extensive root systems and rapid growth. These characteristics are essential for Cd phytoextraction (SHEORAN; SHEORAN; POONIA, 2016) and can likely be optimized with a well-balanced sulfur (S) supply (RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b).

Among other processes, S is associated with tolerance to abiotic stress, secondary metabolism, photosynthetic oxygen production and electron transport in plants (CAPALDI et al., 2015). In addition, S is present in glutathione (GSH) and phytochelatin (PC) molecules, which are among the main compounds involved in Cd detoxification (COBBETT; GOLDSBROUGH, 2002; NOCTOR et al., 2012). Bashir et al. (2015) reported that GSH and PCs levels in leaves and roots of Cd-exposed *Brassica juncea* increased when there was proper S supply, thereby mitigating Cd-induced damage. Glutathione (γ-Glu-Cys-Gly), whose biosynthesis is catalyzed by two ATP-dependent enzymes [γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSHS)], is the main non-enzymatic antioxidant of plants. Amongst other functions, it regulates sulphate assimilation, confers abiotic stress resistance and acts as a substrate for PCs (NOCTOR et al., 2012). These functions can also be fulfilled by GSH analogs, where the C-terminal residue is different from glycine, such as homoglutathione (h-GSH; γ-Glu-Cys-ß-Ala) (KLAPHECK, 1988). Phytochelatins $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (with n=2-11) are synthesized from GSH by phytochelatin synthase (PCS)

(COBBETT; GOLDSBROUGH, 2002) and show structural variations such as desglycine phytochelatins [desGly-PC, $(\gamma\text{-Glu-Cys})_n$] (BERNHARD; KÄGI, 1987), isoforms of PCs [cys-PC, Cys-$(\text{Glu-Cys})_n$-Gly] (FERNÁNDEZ et al., 2013) and homophytochelatins [h-PCs; $(\gamma\text{-Glu-Cys})_n$-ß-Ala] (GRILL; WINNACKER; ZENK, 1986). Phytochelatins act on Cd chelation and are involved both in its transport from the cytosol to vacuoles (COBBETT; GOLDSBROUGH, 2002) and its translocation from roots to shoots (MENDOZA-CÓZATL et al., 2008). Furthermore, PC_2, PC_3 and PC_4 are the major thiol compounds induced by Cd (VÁZQUEZ; GOLDSBROUGH; CARPENA, 2006). Jozecfzak et al. (2014) reported that PC_2, PC_3, PC_4 and PC_5 induction in roots and leaves of *Arabidopsis thaliana* rapidly increased upon Cd exposure. Nevertheless, because of the use of GSH for PCs synthesis, oxidative damage occurred in the roots of these plants.

The oxidative damage generated by reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) the hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$) can be minimized by organic acids, amino acids and sugars, which can act as chelators, antioxidants and/or osmoprotectants during Cd stress (SHARMA; DIETZ, 2006; VILLIERS et al., 2011; KEUNEN et al., 2013). In this context, the study of metabolite profiles is an important tool to better understand stress responses in plants (VILLIERS et al., 2011; OBATA; FERNIE, 2012). Xie et al. (2014) reported that the levels of malate, citrate, oxoglutarate, glycerate, glycine, proline, norvaline, serine, threonine, glutamate, gluconate, xylulose, galactose and thalose were higher in the leaves of a Cd-tolerant *Cynodon dactylon* genotype (WB242) than those in a non-tolerant genotype (WB144). Furthermore, the Cd-tolerant genotype displayed a greater accumulation of trehalose upon Cd exposure. Accumulation of the above-mentioned metabolites thus appears to be fundamental for plants used in Cd phytoextraction. For example, proline acts as a radical scavenger, electron sink and macromolecule stabilizer (MATYSIK et al., 2002), malate and citrate can form complexes with Cd in vacuoles after Cd transport by PCs (KROTZ; EVANGELOU; WAGNER, 1989) and trehalose can act as a signaling molecule in pathways related to abiotic stress (KEUNEN et al., 2013). Although there are studies showing specific accumulation of amino acids (e.g. proline), organic acids (e.g. malate) and sugars (e.g. sucrose) in plants exposed to Cd (HÉDIJI et al., 2010; SUN et al., 2010; KEUNEN et al., 2016), further studies with forage grasses are needed to better understand how these metabolites are involved in their Cd tolerance (XIE et al., 2014). Such studies also need to take into consideration the supply of different S concentrations.

It is important to note that S is a component of cysteine, which is required for methionine (CAPALDI et al., 2015), GSH (NOCTOR et al., 2012) and PCs (COBBETT; GOLDSBROUGH, 2002), synthesis. Therefore, plants grown under different S concentrations may present different responses to Cd (ZHANG et al., 2013; BASHIR et al., 2015; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b), at the level of their metabolite profile. In this context, our aim here was to analyze the effect of low, medium and high S concentrations on the metabolite profiles including non-protein thiols (NPTs) and in particular GSH and PCs, in *Panicum maximum* Jacq. cv. Massai (Massai grass), which is used for Cd phytoextraction, in order to better understand Cd detoxification mechanisms in forage grasses.

5.2 Materials and methods

5.2.1 Plant material and exposure to Cd and S

Panicum maximum Jacq. cv. Massai plants were grown in a hydroponic system (described in item 2.2) using 2.2 L plastic pots containing 2 L of nutrient solution arranged in a greenhouse (22º42' south latitude and 47º38' west longitude). The treatments were represented by combinations of three S concentrations (0.1, 1.9 and 3.7 mmol L^{-1}) and two Cd concentrations (0.1 and 0.5 mmol L^{-1}), in nutrient solutions modified from the solution described by Epstein and Bloom (2005). The exact composition and chemical speciation of nutrient solution used in the study is shown on pages 78 and 79, respectively. The pots used in the study were placed in a randomized block design, consisting of four biological replicates per condition.

5.2.2 Growth and harvest of Massai grass

Seeds were sown in a tray containing expanded vermiculite, which was irrigated with deionized water in the first 14 days and nutrient solution modified in order to provide 0.1 mmol L^{-1} S (diluted to 25% ionic strength) over the following nine days. After 23 days, five seedlings (± 10 cm high) were transplanted to each pot containing the undiluted nutrient solutions (100% ionic strength) modified to provide one of the three different experimental S concentrations for a further 21 days. After this period, modified nutrient solutions were provided to obtain different S and Cd concentrations (100% ionic strength) during nine days. Solutions were replaced every week and remained constantly aerated. The average temperature in the greenhouse was 30.5 ºC, with a relative air humidity of 60.5% (more details on page 77). After the last day of S and Cd exposure, plants were harvested to determine dry mass production, root/shoot ratio, S and Cd contents, metabolite profiles,

NPTs concentrations and gene expression. After harvesting, shoots were separated into leaf blades and a sample comprising both stem and sheath material. All samples were snap frozen in liquid nitrogen and stored at -80 °C until further analyses.

5.2.3 Determination of dry mass production and root/shoot ratio

The dry mass production was obtained after weighing the plant material, which remained in forced air ventilation oven at 60 ºC for 72 h. Subsequently, the root/shoot dry mass ratio (with the shoot dry weight equaling that of leaf blades and the sample comprising both stem and sheath material) was calculated.

5.2.4 Determination of S and Cd contents

After drying in a forced ventilation oven at 60 °C for 72 h, dried plant material was milled in a Wiley type mill and subsequently subjected to nitric-perchloric digestion prior to the determination of S (MALAVOLTA; VITTI; OLIVEIRA, 1997) and Cd concentrations by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7000 SERIES, Thermo Fisher Scientific, Waltham, USA). Sulfur and Cd contents were obtained by multiplying the concentration of the element in tissues (leaf blades, the sample comprising both stem and sheath material and roots) by the dry mass of the respective tissue.

5.2.5 Metabolite profiling

Polar metabolites were extracted from 50 mg of plant material, following the method described by Giavalisco et al. (2011). After extraction, samples were derivatized as described (LISEC et al., 2006) and analyzed by gas chromatography mass spectrometery (GC-TOF-MS, LECO Instruments, St. Joseph, USA). The acquisition parameters used were identical to those described by Weckwerth, Wenzel and Fiehn (2004). Peak detection, alignment of retention times and library searching were performed using the TargetSearch package of Bioconductor (CUADROS-INOSTROZA et al., 2009). Metabolites were quantified by peak intensity of selected mass, normalized by fresh weight of samples, followed by sum of peak intensities (total ion count) and logarithmic transformation in base 2. Note that metabolite profiling was not performed on plants exposed to 0.5 mmol L^{-1} Cd due to their low biomass.

5.2.6 Determination of non-protein thiols (NPTs)

Extraction of NPTs was performed in a cold room at 4 °C, following the method described by Fernández et al. (2012). The supernatant obtained was injected into a high performance liquid chromatography Waters 600 (HPLC) (Waters Corp., Milford, USA). Then, post-column derivatization was performed with Ellman's reagent (ELLMAN, 1959). Derivatized thiols were detected at 412 nm using the Waters 996 photodiode array detector (Waters Corp.). The resultant peaks were compared to external GSH standards and a variety of PCs kindly provided by Professor Meinhart Hans Zenk (FRIEDERICH; KNEER; ZENK, 1998). Quantitative changes in NPTs obtained were analyzed using integrated areas of absorbance at 412 nm and quantified in terms of GSH equivalents. As for the metabolite profiling, NPTs levels could not be determined in plants exposed to 0.5 mmol L^{-1} Cd because of too low biomass

5.2.7 Gene expression analyses related to thiol compounds biosynthesis

The expression of *GSH1* and *PCS2* genes in the leaf blades of plants was measured by real-time reverse transcription PCR (RT-qPCR) as described by Keunen et al. (2015). We also tried to determine the gene expression of the genes *GSH2* and *PCS1*, but we could not get suitable primers to do this. Extraction of RNA was performed using the RNAqueous® Total RNA Isolation Kit (Ambion, Life Technologies, Merelbeke, Belgium), followed by treatment with DNAse I Kit (Invitrogen, ThermoScientific, Waltham, USA). Then, extracted RNA was quantified in RNAse-free PCR tubes containing QuantiFluor® RNA System (Promega Corp., Madison, USA) and 1 µL of RNA samples using a portable fluorometer (Quantus™ Fluorometer, Promega Corp., Madison, USA). Random primers and SuperScript™ III RT Kit (Invitrogen) were used to convert RNA into cDNA. After obtaining cDNA, new concentration measurements were carried out in RNase-free PCR tubes containing QuantiFluor® dsDNA System (Promega Corp.) and 1 µL of cDNA samples using the Quantus Fluorometer™. After this step, cDNA was diluted 10 times in 1/10 diluted Tris-EDTA (TE) buffer (Tris-HCl 1 mmol L^{-1}, Na_2-EDTA 0.1 mmol L^{-1}, pH 8.0) and subsequently stored at -20 °C.

Real-time PCR quantification was performed in 96-well optical plates using the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Gent, Belgium) and the Fast SYBR® Green Master Mix (Applied Biosystems). Amplification occurred under universal cycling conditions (20 s at 95 ºC, 40 cycles of 3 s at 95 ºC and 30 s at 60 ºC), followed by the generation of a dissociation curve to verify amplification specificity. Forward and reverse primers (300 nmol L^{-1}) were designed and optimized for Massai grass

(Table 1) using Primer3 (ROZEN; SKALETSKY, 2000). Then, gene expression data were calculated by the $2^{-\Delta Cq}$ method in relation to the sample with the highest expression (minimum Cq). All data were normalized using the expression of three stable reference genes (REMANS et al., 2008) (Table 2; GIMENO et al., 2014) selected using the GrayNorm (REMANS et al., 2014). All procedures were performed according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines (BUSTIN et al., 2009) as described in Table 3.

Table 1 - Forward and reverse primers (300 nmol L^{-1}) designed and optimized for Massai grass

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Primer efficiency
GSH1	AAGCTCCCACAAGTTCCTGG	GCAGGCAAAGCACACAATCT	149	106%
PCS2	TCTCATCGCCTCCTACCACA	AAGCGAGCGACATCCAAGAT	117	116%

Table 2 - Summary of the reference genes used in determination of gene expression in leaf blades of Massai grass

Gene name[a]	Gene description	GenBank acession number db EST[b]	*Arabdopsis* ortholog locus	Rice TIRG identifier[c]
CYP5	Cyclophilin 5	FE633090	At2g29960	LOC_Os06g49480
SAMDC	S-adenosyl methionine decarboxylase	FL72288	At3g02470	LOC_Os02g39790
U2AF	Splicing factor U2af	FL907910	At5g42820	LOC_Os05g48960

[a]All *Panicum virgatum* (Switchgrass) sequences were named according on similarity to *Arabidopsis thaliana* proteins determined with BLASTX. [b]Accession number of the most similar EST to the rice protein according to the Switchgrass GenBank dbEST. [c]TIRG rice genome identifier of the rice proteins used to identify Switchgrass reference genes sequences via TBLASTN among the Switchgrass GenBank dbEST (GIMENO et al., 2014).

5.2.8 Statistical analyses

The unsupervised multivariate statistical analyses of metabolite profiling data was performed by Bioconductor pcaMethods package (STACKLIES et al., 2007) and the heatmap was performed using the d3heatmap package (https://cran.r-project.org/web/packages/d3heatmap/d3heatmap.pdf) of R. Data of dry mass production, root/shoot ratio, S and Cd contents, metabolic profiling, NPTs and gene expression (normalized relative quantities were log transformed before statistical analysis) were submitted to analysis of variance (F test) and Tukey test ($P < 0.05$) through Statistical Analysis System v. 9.2 (SAS INSTITUTE, 2008).

Table 3 - Quantitative real-time PCR parameters according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines derived from Bustin et al. (2009)

Sample/Template	
Source	Leaf blades of *Panicum maximum* plants grown in hydroponics
Method of preservation	Liquid nitrogen
Storage time	Ten months at -80 °C
Handling	Frozen
Extraction method	Phenol-free Total RNA isolation: RNAqueous® Total RNA Isolation Kit* (Ambion, Life Technologies, Merelbeke, Belgium)
RNA: DNA-free	As stated in the Materials and Methods section
Concentration	Portable fluorometer (Quantus™ Fluorometer, Promega Corp., Madison, USA)
Assay optimisation and validation	
Accession number	Table 2
Amplicon details	Product size: Table 1
Primer sequences	Table 1
In silico	Primers were blasted using the BLAST tool at https://phytozome.jgi.doe.gov/pz/portal.html#
Empirical	A primer concentration of 300 nmol L^{-1} was used Annealing temperature: 60 °C
Priming conditions	Combination of oligodT-primers and random hexamers
PCR efficiency	Dilution series (slope, y-intercept and r^2)
Linear dynamic range	Samples are situated within the range of the efficiency curve
Reverse transcription - PCR	
Protocols	As stated in the Materials and Methods section
Reagents	As stated in the Materials and Methods section
No template control (NTC)	Cq and dissociation curve verification
Data analysis	
Specialist software	7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Belgium) Software v 2.0.1
Statistical justification	Four biological replicates Outliers were eliminated after statistical validation using the extreme studentized deviate analysis (Statistical Analysis System v. 9.2) at significance level 0.05 Log transformation of the data Analysis of variance (F test) and Tukey test
Normalisation	Three reference genes were selected (Table 2)

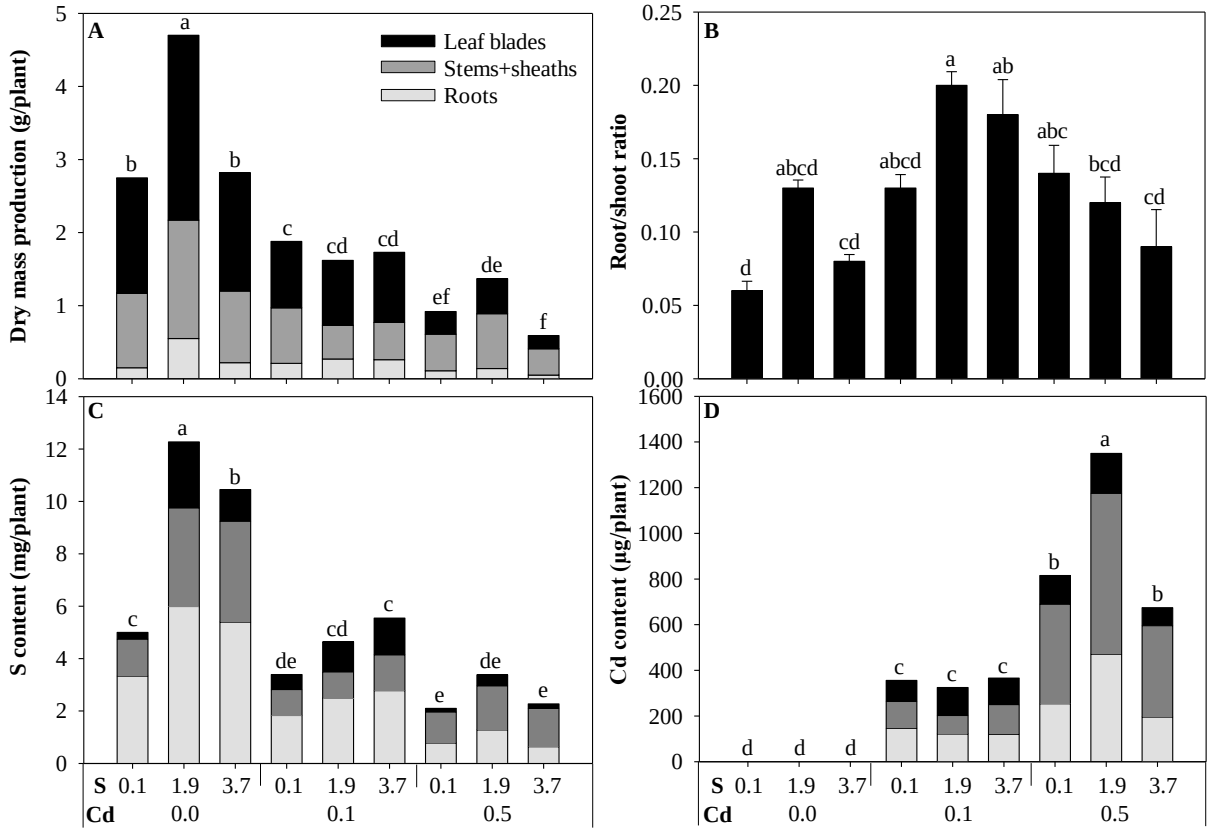
*All procedures were performed according to the manufacturer's protocols.

5.3 Results

5.3.1 Dry mass production, root/shoot ratio and S and Cd contents in Massai grass

Dry biomass production was the highest when Massai grass was grown without Cd and with 1.9 mmol L^{-1} S (control condition, Figure 1A). It decreased when plants were exposed to Cd, mainly with 0.5 mmol L^{-1}.

Figure 1 - Dry mass production (A), root/shoot ratio (B), S content (C) and Cd content (D) in Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.0, 0.1 and 0.5 mmol L^{-1}). Different letters on the bars indicate significant statistical differences by Tukey test (P <0.05)



Sulfur supply did not change the dry mass production of plants exposed to 0.1 mmol L^{-1} Cd, which corresponded to less than 40% of dry mass production being observed in the control treatment. By contrast, plants supplied with an intermediate S concentration displayed higher dry mass when Massai grass was grown in the presence of 0.5 mmol L^{-1} Cd. The highest root/shoot ratios occurred in plants exposed to the lowest Cd concentration that were cultivated with 1.9 and 3.7 mmol L^{-1} S (Figure 1B). The highest S content in the leaf blades, samples comprising both stem and sheath material and roots of Massai grass were observed with the supply of the two highest S concentrations and in the absence of Cd (Figure 1C). When the plants were exposed to the lower Cd concentration, the highest S content in the roots occurred with the supply of the two highest S concentrations. By contrast, the highest Cd content in the leaf blades, samples comprising both stem and sheath material, and roots occurred when plants were exposed to the highest Cd concentration and supplied with 1.9 mmol L^{-1} S (Figure 1D).

5.3.2 Metabolic responses to Cd and S

A total of 69 (in leaf blades), 63 (in samples comprising both stem and sheath material) and 66 (in roots) metabolites were identified by GC-TOF-MS and classified as organic acids, sugars, amino acids or other (Tables 4-6). In the leaf blades of Massai grass, the levels of 34 of 69 metabolites were altered ($P < 0.05$) in response to combinations of altered S and Cd concentrations (Table 4), similarly 39 of 63 metabolites in samples comprising both stem and sheath material (Table 5) and 40 out of 66 metabolites in the roots (Table 6), were altered. Principal component analysis (PCA) highlighted that metabolite levels in the leaf blades (Figure 2A) and sample comprising both stem and sheath material (Figure 2B) of Massai grass were more altered by S than by Cd, whereas in the root samples the effects of S and Cd was similar (Figure 2C). When comparing all conditions to the control condition (1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd), we observed that levels of amino acids in the leaf blades of Massai grass mostly increased whereas those of organic acids, sugars and other metabolites generally decreased (Figure 2D). This pattern was also seen for amino acids and organic acids in the samples comprising both stem and sheath material (Figure 2E) and roots (Figure 2F). By contrast, sugars and other metabolites in samples comprising both stem and sheath material (Figure 2E) and roots (Figure 2F) were more increased than in the leaf blades (Figure 2D). To summarize the results, schematic metabolic networks are presented depicting changes in the main metabolites induced by Cd and S in the leaf blades (Figure 3), samples comprising both stem and sheath material (Figure 4) and roots (Figure 5).

In leaf blades, the levels of trehalose, sucrose, shikimate and tryptophan decreased while the level of glycine increased under the highest S supply in relation to the control condition (Figure 3).

Table 4 - Metabolites levels in the leaf blades of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1})

Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Organic acids						
Citrate	12.85 ± 0.17 a	13.50 ± 0.25 a	13.23 ± 0.19 a	13.20 ± 0.30 a	13.35 ± 0.25 a	13.55 ± 0.17 a
Fumarate	7.77 ± 0.11 a	8.09 ± 0.23 a	7.97 ± 0.11 a	7.89 ± 0.04 a	7.97 ± 0.11 a	7.96 ± 0.05 a
Isocitrate	7.95 ± 0.28 a	6.76 ± 0.23 b	7.06 ± 0.16 ab	8.02 ± 0.26 a	7.89 ± 0.21 ab	7.24 ± 0.26 ab
Itaconate	6.17 ± 0.06 a	6.44 ± 0.19 a	4.87 ± 0.13 b	6.34 ± 0.22 a	5.96 ± 0.38 ab	6.43 ± 0.16 a
α-Ketoglutarate (2-OG)	6.89 ± 0.15 a	7.23 ± 0.18 a	7.20 ± 0.13 a	6.99 ± 0.11 a	7.28 ± 0.10 a	7.27 ± 0.03 a
Lactate	6.50 ± 0.16 a	6.86 ± 0.15 a	6.74 ± 0.13 a	6.45 ± 0.11 a	6.45 ± 0.06 a	6.56 ± 0.14 a
Malate	11.49 ± 0.15 ab	12.25 ± 0.19 b	11.65 ± 0.20 ab	11.23 ± 0.31 a	11.46 ± 0.07 ab	11.44 ± 0.31 ab
Malonate	11.49 ± 0.15 ab	12.25 ± 0.19 b	11.65 ± 0.20 ab	11.23 ± 0.31 a	11.46 ± 0.07 ab	11.44 ± 0.31 ab
Oxalate	8.74 ± 0.29 a	10.79 ± 0.28 b	11.38 ± 0.11 b	8.76 ± 0.19 a	10.98 ± 0.27 b	8.79 ± 0.31 a
Oxaloacetate (OAA)	8.36 ± 0.26 a	8.77 ± 0.26 ab	9.27 ± 0.16 b	8.24 ± 0.12 a	8.75 ± 0.09 ab	8.32 ± 0.12 a
Succinate	8.12 ± 0.34 a	8.95 ± 0.15 b	8.36 ± 0.16 ab	7.94 ± 0.11 a	8.27 ± 0.05 ab	7.88 ± 0.11 a
Tartrate	8.00 ± 0.17 ab	8.31 ± 0.12 a	8.00 ± 0.09 ab	7.82 ± 0.07 ab	7.78 ± 0.10 b	7.86 ± 0.06 ab
Sugars						
Cellobiose	5.04 ± 0.19 ab	5.84 ± 0.09 a	4.87 ± 0.38 ab	5.44 ± 0.17 ab	5.56 ± 0.09 ab	4.39 ± 0.40 b
Erythritol	6.65 ± 0.18 a	6.60 ± 0.18 a	6.47 ± 0.06 a	6.71 ± 0.13 a	6.37 ± 0.08 a	6.50 ± 0.18 a
Fructose	11.57 ± 0.13 a	11.43 ± 0.20 a	11.42 ± 0.17 a	12.32 ± 0.40 a	12.68 ± 0.31 a	12.52 ± 0.47 a
Fucose	7.85 ± 0.10 ab	8.07 ± 0.06 b	7.88 ± 0.04 ab	7.64 ± 0.07 a	7.54 ± 0.10 a	7.56 ± 0.09 a
Galactinol	8.10 ± 0.10 a	8.27 ± 0.11 a	8.06 ± 0.10 a	8.27 ± 0.10 a	8.22 ± 0.08 a	8.32 ± 0.12 a
Galactitol	11.09 ± 0.25 ab	10.66 ± 0.10 ab	10.02 ± 0.10 b	11.43 ± 0.58 ab	11.74 ± 0.16 a	11.67 ± 0.36 a
Glucose	11.95 ± 0.29 ab	11.42 ± 0.11 ab	10.72 ± 0.12 b	12.49 ± 0.57 a	12.65 ± 0.19 a	12.55 ± 0.41 a
Glucose-6-phosphate (Glu-6P)	6.71 ± 0.36 a	7.21 ± 0.21 a	7.66 ± 0.22 a	6.60 ± 0.31 a	6.84 ± 0.18 a	7.44 ± 0.08 a
Glycerate	11.43 ± 0.26 a	12.02 ± 0.23 a	12.04 ± 0.29 a	11.82 ± 0.11 a	12.13 ± 0.11 a	12.23 ± 0.10 a
Idose	11.95 ± 0.29 ab	11.42 ± 0.11 ab	10.72 ± 0.12 b	12.49 ± 0.57 a	12.65 ± 0.19 a	12.55 ± 0.41 a
Maltose	7.83 ± 0.14 a	8.39 ± 0.20 a	7.94 ± 0.13 a	8.12 ± 0.18 a	7.69 ± 0.19 a	7.84 ± 0.04 a
Raffinose	9.74 ± 0.22 b	10.87 ± 0.14 a	10.24 ± 0.11 ab	10.81 ± 0.26 a	10.95 ± 0.19 a	10.65 ± 0.15 a
Rhamnose	10.13 ± 0.30 a	10.14 ± 0.24 a	9.47 ± 0.20 a	9.65 ± 0.14 a	9.67 ± 0.17 a	9.40 ± 0.13 a
Ribose	11.01 ± 0.22 a	11.42 ± 0.14 a	10.88 ± 0.12 a	10.83 ± 0.10 a	10.87 ± 0.11 a	10.82 ± 0.12 a
Sorbose	11.57 ± 0.08 a	11.67 ± 0.16 a	11.54 ± 0.27 a	12.26 ± 0.40 a	12.44 ± 0.26 a	12.35 ± 0.42 a
Sucrose	10.64 ± 0.17 bc	11.53 ± 0.22 a	10.48 ± 0.04 c	11.47 ± 0.16 a	11.35 ± 0.11 ab	11.39 ± 0.24 ab
Talose	7.43 ± 0.00 a	7.94 ± 0.17 a	7.55 ± 0.14 a	8.05 ± 0.08 a	7.91 ± 0.19 a	8.00 ± 0.05 a
Trehalose	4.29 ± 0.15 a	4.62 ± 0.15 a	2.38 ± 0.09 b	4.91 ± 0.17 a	4.43 ± 0.22 a	3.98 ± 0.72 ab

Table 4 - Continuation...

Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Xylitol	9.59 ± 0.16 ab	9.90 ± 0.12 a	9.33 ± 0.11 b	9.37 ± 0.09 ab	9.38 ± 0.09 ab	9.38 ± 0.10 ab
Xylose	11.01 ± 0.22 a	11.42 ± 0.14 a	10.88 ± 0.12 a	10.83 ± 0.10 a	10.87 ± 0.11 a	10.82 ± 0.12 a
Amino acids						
5-Oxoproline	7.35 ± 0.14 a	7.27 ± 0.41 a	8.03 ± 0.12 a	7.86 ± 0.24 a	7.85 ± 0.21 a	7.89 ± 0.14 a
Alanine	15.28 ± 0.11 a	15.09 ± 0.21 a	15.79 ± 0.24 a	15.21 ± 0.16 a	15.15 ± 0.21 a	15.18 ± 0.17 a
Asparagine	14.62 ± 0.14 a	12.23 ± 0.58 b	12.80 ± 0.39 b	14.53 ± 0.20 a	14.54 ± 0.07 a	14.38 ± 0.25 a
Aspartate	14.30 ± 0.09 a	13.57 ± 0.70 a	14.49 ± 0.07 a	14.23 ± 0.10 a	14.40 ± 0.03 a	14.37 ± 0.07 a
ß-Alanine	7.78 ± 0.20 a	8.19 ± 0.28 a	8.81 ± 0.33 a	7.80 ± 0.26 a	8.03 ± 0.50 a	7.72 ± 0.26 a
Cysteine	12.38 ± 0.12 a	9.70 ± 0.54 b	9.64 ± 0.21 b	12.11 ± 0.05 a	12.09 ± 0.16 a	11.55 ± 0.27 a
Glutamate	7.80 ± 0.13 a	7.76 ± 0.43 a	8.56 ± 0.13 a	8.35 ± 0.25 a	8.32 ± 0.24 a	8.41 ± 0.14 a
Glutamine	12.46 ± 0.47 a	10.30 ± 0.43 b	10.88 ± 0.19 ab	12.35 ± 0.45 a	11.83 ± 0.33 ab	11.01 ± 0.43 ab
Glycine	10.74 ± 0.13 cd	9.96 ± 0.29 ac	10.88 ± 0.09 d	9.16 ± 0.15 ab	9.33 ± 0.17 ab	8.91 ± 0.22 b
Histidine	9.63 ± 0.35 a	8.45 ± 0.02 bc	8.00 ± 0.08 b	9.94 ± 0.29 a	9.77 ± 0.17 a	9.17 ± 0.15 ac
Homoserine	2.75 ± 0.13 a	2.55 ± 0.43 a	2.42 ± 0.08 a	3.28 ± 0.34 a	2.32 ± 0.01 a	2.58 ± 0.14 a
Isoleucine	9.50 ± 0.09 a	9.51 ± 0.14 a	9.78 ± 0.10 a	9.58 ± 0.16 a	9.49 ± 0.08 a	9.29 ± 0.25 a
Leucine	8.96 ± 0.05 a	8.98 ± 0.12 ab	9.72 ± 0.16 b	9.49 ± 0.17 ab	9.27 ± 0.18 ab	9.35 ± 0.20 ab
L-proline	7.70 ± 0.14 a	4.81 ± 0.11 bc	4.18 ± 0.14 b	7.29 ± 0.21 a	6.41 ± 0.14 d	5.26 ± 0.15 c
Lysine	10.55 ± 0.27 ab	9.80 ± 0.06 b	10.17 ± 0.24 ab	10.88 ± 0.23 a	10.83 ± 0.23 a	10.12 ± 0.25 ab
Methionine	3.49 ± 0.34 a	3.52 ± 0.19 a	3.79 ± 0.21 a	3.43 ± 0.25 a	4.03 ± 0.22 a	3.57 ± 0.14 a
Norleucine	12.40 ± 0.16 a	12.56 ± 0.17 a	12.72 ± 0.18 a	12.21 ± 0.17 a	12.41 ± 0.13 a	12.55 ± 0.12 a
O-acetylserine (OAS)	9.46 ± 0.16 ab	9.55 ± 0.36 ab	9.04 ± 0.16 b	10.16 ± 0.30 a	9.53 ± 0.18 ab	9.84 ± 0.20 ab
Ornithine	10.27 ± 0.20 ab	7.18 ± 0.42 c	7.26 ± 0.22 c	10.64 ± 0.41 a	9.85 ± 0.17 ab	8.98 ± 0.24 b
Phenylalanine	10.34 ± 0.37 a	10.37 ± 0.14 a	10.02 ± 0.18 a	10.35 ± 0.15 a	10.16 ± 0.18 a	10.00 ± 0.27 a
Proline	13.08 ± 0.55 a	11.62 ± 0.35 a	12.39 ± 0.29 a	13.15 ± 0.35 a	12.63 ± 0.17 a	13.01 ± 0.23 a
Serine	13.72 ± 0.20 a	11.99 ± 0.22 c	12.41 ± 0.29 cd	13.52 ± 0.10 ab	13.21 ± 0.19 ab	12.65 ± 0.20 bcd
Threonine	11.45 ± 0.17 a	10.94 ± 0.41 a	11.41 ± 0.13 a	11.39 ± 0.14 a	11.29 ± 0.21 a	10.78 ± 0.27 a
Tryptophan	10.06 ± 0.08 a	9.94 ± 0.12 a	8.98 ± 0.27 b	10.12 ± 0.27 a	10.04 ± 0.10 a	10.02 ± 0.07 a
Tyrosine	9.96 ± 0.13 a	10.02 ± 0.09 a	9.73 ± 0.13 a	10.16 ± 0.21 a	10.07 ± 0.15 a	9.86 ± 0.22 a
Valine	11.85 ± 0.14 a	11.62 ± 0.10 a	11.62 ± 0.09 a	11.73 ± 0.17 a	11.64 ± 0.07 a	11.37 ± 0.19 a
Others						
4-Aminobutanoate (GABA)	11.76 ± 0.31 ab	12.14 ± 0.30 ab	12.74 ± 0.20 b	11.66 ± 0.13 a	12.20 ± 0.10 ab	11.72 ± 0.14 a
Benzoate	7.76 ± 0.10 ab	7.95 ± 0.19 b	7.80 ± 0.12 ab	7.26 ± 0.06 a	7.26 ± 0.14 a	7.55 ± 0.08 ab

Table 4 - Conclusion

Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Cis-aconitate	13.79 ± 0.16 a	13.99 ± 0.19 a	13.79 ± 0.24 a	13.62 ± 0.20 a	13.77 ± 0.11 a	13.91 ± 0.15 a
Gluconate	7.04 ± 0.04 ab	7.51 ± 0.14 b	7.05 ± 0.08 ab	6.92 ± 0.17 a	6.94 ± 0.12 a	6.76 ± 0.05 a
Glycerol	9.88 ± 0.13 a	10.12 ± 0.17 a	10.18 ± 0.23 a	10.01 ± 0.11 a	9.81 ± 0.12 a	9.92 ± 0.20 a
Putrescine	10.81 ± 0.11 a	11.15 ± 0.15 a	11.08 ± 0.17 a	10.96 ± 0.18 a	11.00 ± 0.25 a	10.94 ± 0.14 a
Pyruvate	8.25 ± 0.19 a	8.76 ± 0.15 a	8.77 ± 0.26 a	8.03 ± 0.16 a	8.20 ± 0.19 a	8.39 ± 0.13 a
Shikimate	13.42 ± 0.08 ab	14.32 ± 0.19 b	13.24 ± 0.34 a	12.94 ± 0.05 a	13.51 ± 0.12 ab	13.22 ± 0.25 a
Glycerol 3-phosphate	8.43 ± 0.24 a	8.53 ± 0.35 a	8.97 ± 0.22 a	8.31 ± 0.17 a	8.47 ± 0.16 a	8.60 ± 0.09 a
Sinapate	8.25 ± 0.21 a	8.61 ± 0.21 a	8.40 ± 0.15 a	8.86 ± 0.18 a	8.81 ± 0.10 a	8.87 ± 0.19 a
Spermidine	11.02 ± 0.10 a	11.39 ± 0.20 a	11.34 ± 0.12 a	11.15 ± 0.09 a	11.43 ± 0.12 a	11.52 ± 0.18 a

Mean ± SEM followed by distinct letters in the lines differed by Tukey test ($P < 0.05$).

Table 5 - Metabolites concentrations in the sample comprising both stem and sheath material of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1})

Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Organic acids						
Citrate	12.54 ± 0.18 a	13.34 ± 0.21 a	12.48 ± 0.21 a	13.01 ± 0.30 a	13.27 ± 0.37 a	13.14 ± 0.24 a
Fumarate	6.85 ± 0.13 a	7.45 ± 0.12 b	7.11 ± 0.13 ab	7.03 ± 0.15 ab	7.19 ± 0.15 ab	7.21 ± 0.06 ab
Isocitrate	9.75 ± 0.08 a	9.72 ± 0.19 a	9.52 ± 0.12 a	9.34 ± 0.14 a	9.64 ± 0.15 a	9.37 ± 0.15 a
Itaconate	6.28 ± 0.06 a	6.67 ± 0.13 ab	6.51 ± 0.07 ab	6.93 ± 0.07 bc	7.32 ± 0.02 cd	7.80 ± 0.14 d
α-Ketoglutarate (2-OG)	9.34 ± 0.03 a	9.77 ± 0.07 a	9.70 ± 0.13 a	9.66 ± 0.13 a	9.71 ± 0.11 a	9.67 ± 0.05 a
Lactate	4.70 ± 0.05 b	5.19 ± 0.15 abc	4.78 ± 0.10 bc	5.32 ± 0.04 ac	5.70 ± 0.19 a	5.60 ± 0.06 a
Malate	12.54 ± 0.11 a	13.34 ± 0.19 b	12.01 ± 0.06 a	12.24 ± 0.14 a	12.32 ± 0.19 a	12.31 ± 0.20 a
Malonate	8.59 ± 0.22 a	8.84 ± 0.29 a	8.89 ± 0.20 a	8.69 ± 0.25 a	9.20 ± 0.16 a	8.95 ± 0.04 a
Oxalate	2.75 ± 0.08 a	3.18 ± 0.04 a	4.72 ± 0.23 bc	3.02 ± 0.10 a	4.02 ± 0.04 b	5.21 ± 0.09 c
Oxaloacetate (OAA)	2.59 ± 0.04 b	3.23 ± 0.19 ab	3.35 ± 0.04 ab	3.68 ± 0.18 a	3.78 ± 0.05 a	3.70 ± 0.08 a
Succinate	11.65 ± 0.16 b	12.21 ± 0.14 a	12.36 ± 0.09 a	11.81 ± 0.13 ab	12.04 ± 0.13 ab	11.95 ± 0.12 ab
Tartrate	7.49 ± 0.12 a	7.79 ± 0.06 a	7.76 ± 0.09 a	7.43 ± 0.20 a	7.51 ± 0.11 a	7.43 ± 0.04 a
Sugars						
Cellobiose	4.87 ± 0.21 a	4.94 ± 0.14 a	4.39 ± 0.11 a	*ND	4.79 ± 0.07 a	5.03 ± 0.13 a
Fructose	14.54 ± 0.12 a	14.80 ± 0.36 a	14.99 ± 0.17 a	14.50 ± 0.02 a	14.76 ± 0.08 a	14.83 ± 0.07 a
Fucose	7.97 ± 0.07 a	8.15 ± 0.11 abc	8.02 ± 0.04 ac	8.36 ± 0.12 bcd	8.42 ± 0.05 bd	8.59 ± 0.06 d
Galactinol	10.43 ± 0.21 a	10.81 ± 0.23 ac	10.16 ± 0.24 a	11.96 ± 0.20 b	12.41 ± 0.06 b	11.51 ± 0.25 bc
Glucose	13.61 ± 0.10 a	13.94 ± 0.13 a	14.02 ± 0.14 a	13.64 ± 0.15 a	13.78 ± 0.13 a	13.71 ± 0.06 a
Glucose-6-phosphate (Glu-6P)	7.04 ± 0.10 b	7.55 ± 0.10 ab	7.83 ± 0.19 a	7.24 ± 0.10 ab	7.44 ± 0.22 ab	7.86 ± 0.13 a
Glycerate	8.71 ± 0.05 a	8.93 ± 0.06 ab	8.56 ± 0.11 a	9.53 ± 0.18 bc	9.59 ± 0.12 bc	9.63 ± 0.17 c
Raffinose	8.25 ± 0.08 b	8.79 ± 0.19 ab	8.48 ± 0.14 ab	9.02 ± 0.11 a	9.07 ± 0.07 a	8.58 ± 0.17 ab
Rhamnose	7.28 ± 0.12 a	6.89 ± 0.06 a	6.92 ± 0.15 a	7.31 ± 0.18 a	7.21 ± 0.05 a	7.24 ± 0.07 a
Ribose	8.89 ± 0.17 a	8.81 ± 0.09 a	8.84 ± 0.20 a	9.50 ± 0.31 a	9.29 ± 0.14 a	9.55 ± 0.21 a
Sorbose	13.97 ± 0.20 a	13.92 ± 0.27 a	14.41 ± 0.25 a	13.91 ± 0.04 a	14.21 ± 0.18 a	14.44 ± 0.13 a
Sucrose	11.05 ± 0.22 ab	11.33 ± 0.04 ab	10.42 ± 0.10 b	11.69 ± 0.36 a	10.74 ± 0.31 ab	11.11 ± 0.11 ab
Talose	13.38 ± 0.04 a	13.69 ± 0.22 a	13.83 ± 0.11 a	13.52 ± 0.17 a	13.68 ± 0.14 a	13.61 ± 0.09 a
Trehalose	5.35 ± 0.08 ab	5.24 ± 0.10 ab	5.08 ± 0.10 b	5.29 ± 0.03 ab	5.53 ± 0.03 ac	5.74 ± 0.07 c
Xylitol	7.34 ± 0.15 a	7.26 ± 0.12 a	7.19 ± 0.19 a	7.96 ± 0.29 a	7.78 ± 0.10 a	8.03 ± 0.20 a
Xylose	7.77 ± 0.31 ab	7.82 ± 0.20 ab	6.83 ± 0.17 b	8.53 ± 0.14 a	8.14 ± 0.30 a	7.66 ± 0.25 ab

Table 5 - Continuation...

Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Amino acids						
5-Oxoproline	15.70 ± 0.04 a	14.95 ± 0.47 a	14.56 ± 0.46 a	15.65 ± 0.03 a	15.38 ± 0.02 a	15.48 ± 0.15 a
Alanine	15.65 ± 0.17 a	14.95 ± 0.23 a	15.42 ± 0.19 a	15.54 ± 0.16 a	15.51 ± 0.06 a	15.30 ± 0.11 a
Arginine	8.96 ± 0.12 a	7.65 ± 0.23 b	7.79 ± 0.20 b	8.92 ± 0.27 a	8.43 ± 0.10 ab	8.78 ± 0.06 a
Asparagine	10.35 ± 0.18 abc	9.85 ± 0.27 bd	9.34 ± 0.03 d	11.17 ± 0.15 a	10.86 ± 0.23 ac	10.25 ± 0.03 bcd
Aspartate	14.44 ± 0.11 a	14.36 ± 0.33 a	14.67 ± 0.11 a	14.46 ± 0.09 a	14.58 ± 0.13 a	14.51 ± 0.04 a
ß-Alanine	8.53 ± 0.26 ab	6.72 ± 0.12 c	7.18 ± 0.30 ac	7.35 ± 0.19 ac	8.63 ± 0.18 ab	8.53 ± 0.23 b
Citrulline	8.62 ± 0.13 a	7.98 ± 0.07 a	8.02 ± 0.21 a	7.90 ± 0.22 a	8.10 ± 0.19 a	7.98 ± 0.14 a
Glutamate	14.40 ± 0.09 a	14.69 ± 0.07 a	14.70 ± 0.08 a	14.36 ± 0.15 a	14.48 ± 0.09 a	14.44 ± 0.04 a
Glutamine	14.09 ± 0.16 a	13.72 ± 0.36 a	13.48 ± 0.48 a	13.90 ± 0.07 a	13.88 ± 0.07 a	13.86 ± 0.04 a
Glycine	12.31 ± 0.04 a	11.25 ± 0.30 a	11.57 ± 0.16 a	12.04 ± 0.35 a	11.47 ± 0.08 a	11.25 ± 0.38 a
Histidine	10.79 ± 0.13 a	9.36 ± 0.24 b	9.46 ± 0.31 b	11.30 ± 0.17 a	10.88 ± 0.02 a	10.97 ± 0.04 a
Isoleucine	10.68 ± 0.05 ab	10.35 ± 0.16 b	10.36 ± 0.13 b	10.99 ± 0.06 a	10.99 ± 0.08 a	10.88 ± 0.13 a
Leucine	10.30 ± 0.04 a	10.10 ± 0.15 a	10.23 ± 0.15 a	10.85 ± 0.12 b	10.87 ± 0.09 b	10.83 ± 0.11 b
L-proline	6.56 ± 0.12 a	4.94 ± 0.10 c	5.10 ± 0.03 c	6.33 ± 0.20 ab	5.89 ± 0.05 bd	5.63 ± 0.11 d
Lysine	11.40 ± 0.22 a	10.21 ± 0.16 b	10.61 ± 0.19 ab	11.15 ± 0.24 ab	11.24 ± 0.19 a	11.30 ± 0.20 a
Methionine	9.62 ± 0.13 a	8.78 ± 0.29 b	9.15 ± 0.23 ab	9.53 ± 0.06 ab	9.53 ± 0.01 ab	9.63 ± 0.05 a
Norleucine	4.06 ± 0.14 a	2.74 ± 0.10 b	3.78 ± 0.15 a	4.23 ± 0.15 a	4.09 ± 0.17 a	4.19 ± 0.06 a
O-Acetylserine (OAS)	7.73 ± 0.16 ab	6.65 ± 0.14 a	8.68 ± 0.10 bc	7.97 ± 0.11 ab	8.02 ± 0.07 ab	9.62 ± 0.08 c
Ornithine	7.90 ± 0.23 ab	7.07 ± 0.16 b	7.59 ± 0.18 ab	8.23 ± 0.24 a	7.84 ± 0.10 ab	8.00 ± 0.10 a
Phenylalanine	10.40 ± 0.13 a	10.42 ± 0.16 a	10.14 ± 0.28 a	10.62 ± 0.15 a	10.74 ± 0.09 a	10.79 ± 0.09 a
Proline	13.24 ± 0.42 a	11.35 ± 0.12 b	12.12 ± 0.37 ab	13.54 ± 0.37 a	12.90 ± 0.16 ab	13.15 ± 0.38 a
Serine	14.82 ± 0.15 a	14.37 ± 0.23 a	14.46 ± 0.20 a	14.81 ± 0.10 a	14.83 ± 0.12 a	14.80 ± 0.06 a
Threonine	13.19 ± 0.05 a	12.44 ± 0.20 bc	12.29 ± 0.25 c	13.10 ± 0.14 ab	12.93 ± 0.08 abc	12.87 ± 0.09 abc
Tryptophan	11.06 ± 0.03 a	10.51 ± 0.07 c	9.76 ± 0.08 d	11.51 ± 0.11 b	10.83 ± 0.05 ac	11.59 ± 0.08 b
Tyrosine	11.15 ± 0.12 ab	10.58 ± 0.05 bc	10.05 ± 0.05 c	11.45 ± 0.28 a	10.99 ± 0.08 ab	11.18 ± 0.16 ab
Valine	12.91 ± 0.08 a	12.10 ± 0.21 b	12.23 ± 0.18 bc	12.94 ± 0.13 a	12.79 ± 0.04 ac	12.65 ± 0.13 abc
Others						
4-Aminobutanoate (GABA)	10.25 ± 0.17 a	9.99 ± 0.10 a	10.20 ± 0.18 a	10.67 ± 0.25 a	10.18 ± 0.22 a	10.23 ± 0.12 a
Benzoate	6.65 ± 0.04 a	7.30 ± 0.06 b	6.93 ± 0.18 ab	6.89 ± 0.13 ab	6.96 ± 0.11 ab	7.09 ± 0.04 ab
cis-Aconitate	13.82 ± 0.13 a	14.28 ± 0.09 a	13.89 ± 0.14 a	13.75 ± 0.17 a	13.94 ± 0.16 a	13.85 ± 0.07 a

Table 5 - Conclusion.

Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Glycerol	9.97 ± 0.32 a	9.91 ± 0.20 a	9.89 ± 0.15 a	9.52 ± 0.06 a	9.98 ± 0.18 a	11.09 ± 0.45 a
Glycerol 3-phosphate	5.94 ± 0.01 b	6.49 ± 0.10 ab	6.70 ± 0.13 ab	6.98 ± 0.21 a	7.19 ± 0.21 a	7.14 ± 0.09 a
Putrescine	11.55 ± 0.08 a	11.84 ± 0.10 ab	12.26 ± 0.11 b	11.76 ± 0.22 ab	12.07 ± 0.11 ab	12.12 ± 0.14 ab
Pyruvate	4.44 ± 0.06 a	5.10 ± 0.10 c	4.89 ± 0.08 bc	4.48 ± 0.04 a	4.56 ± 0.04 ab	4.74 ± 0.07 ab
Shikimate	13.72 ± 0.04 ab	14.46 ± 0.11 b	13.90 ± 0.21 ab	13.71 ± 0.20 a	14.07 ± 0.28 ab	13.62 ± 0.15 a
Spermidine	7.34 ± 0.06 a	7.25 ± 0.27 a	7.61 ± 0.12 ab	8.01 ± 0.05 bc	8.26 ± 0.14 bc	8.58 ± 0.09 c

Mean ± SEM followed by distinct letters in the lines differed by Tukey test ($P < 0.05$).
*ND = non-detected.

Table 6 - Metabolites levels in the roots of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1})

Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Organic acids						
Citrate	11.31 ± 0.63 ab	11.48 ± 0.29 ab	10.33 ± 0.19 b	12.26 ± 0.20 a	11.57 ± 0.19 ab	11.97 ± 0.12 a
Fumarate	6.03 ± 0.21 a	8.31 ± 0.65 b	6.07 ± 0.26 a	6.14 ± 0.09 a	5.91 ± 0.24 a	6.24 ± 0.14 a
Isocitrate	8.97 ± 0.42 a	7.31 ± 0.30 b	8.05 ± 0.30 ab	8.66 ± 0.30 ab	7.47 ± 0.36 ab	7.65 ± 0.11 ab
α-Ketoglutarate (2-OG)	5.19 ± 0.37 a	4.36 ± 0.09 a	4.30 ± 0.21 a	4.96 ± 0.07 a	5.15 ± 0.22 a	5.32 ± 0.06 a
Lactate	3.84 ± 0.23 a	3.59 ± 0.06 a	4.49 ± 0.16 a	4.28 ± 0.06 a	3.52 ± 0.44 a	4.32 ± 0.05 a
Malate	11.03 ± 0.32 ab	12.97 ± 0.09 c	9.88 ± 0.20 a	10.94 ± 0.18 ab	10.07 ± 0.30 ab	11.11 ± 0.35 b
Malonate	11.03 ± 0.32 ab	12.97 ± 0.09 c	9.88 ± 0.20 a	10.94 ± 0.18 ab	10.07 ± 0.30 ab	11.11 ± 0.35 b
Oxalate	4.00 ± 0.10 a	2.67 ± 0.23 b	4.47 ± 0.15 a	4.26 ± 0.12 a	4.25 ± 0.21 a	4.23 ± 0.13 a
Oxaloacetate (OAA)	9.86 ± 0.15 a	10.56 ± 0.32 a	10.49 ± 0.22 a	10.12 ± 0.23 a	10.62 ± 0.33 a	10.53 ± 0.25 a
Succinate	7.58 ± 0.14 a	7.63 ± 0.13 a	7.80 ± 0.20 a	7.35 ± 0.07 a	7.25 ± 0.05 a	7.27 ± 0.08 a
Tartrate	5.99 ± 0.11 a	6.65 ± 0.27 a	6.21 ± 0.32 a	6.17 ± 0.18 a	6.26 ± 0.08 a	6.83 ± 0.31 a
Sugars						
Cellobiose	3.54 ± 0.13 a	2.80 ± 0.15 a	3.50 ± 0.17 a	3.70 ± 0.08 a	3.87 ± 0.05 a	3.92 ± 0.10 a
Fructose	14.83 ± 0.26 a	15.21 ± 0.08 a	14.54 ± 0.37 a	15.16 ± 0.06 a	15.07 ± 0.05 a	15.12 ± 0.07 a
Fructose-1,6-diphosphate	3.36 ± 0.04 a	2.78 ± 0.23 a	3.81 ± 0.23 a	2.77 ± 0.29 a	3.17 ± 0.10 a	2.70 ± 0.20 a
Fucose	6.22 ± 0.37 b	4.68 ± 0.12 a	5.11 ± 0.18 a	5.65 ± 0.21 ab	4.80 ± 0.25 a	4.75 ± 0.11 a
Galactinol	9.00 ± 0.16 b	7.78 ± 0.25 c	9.31 ± 0.27 b	10.92 ± 0.20 a	11.33 ± 0.15 a	11.51 ± 0.27 a
Glucose	13.38 ± 0.23 a	13.38 ± 0.11 a	13.25 ± 0.24 a	13.42 ± 0.07 a	13.35 ± 0.09 a	13.48 ± 0.04 a
Glucose-6-phosphate (Glu-6P)	6.17 ± 0.62 ab	4.02 ± 0.16 c	5.96 ± 0.18 b	7.37 ± 0.18 a	6.83 ± 0.18 ab	7.14 ± 0.02 ab
Glycerate	7.08 ± 0.19 a	6.90 ± 0.16 a	6.93 ± 0.21 a	7.30 ± 0.12 a	7.01 ± 0.11 a	7.50 ± 0.23 a
Gulose	7.05 ± 0.73 ab	7.24 ± 0.26 ab	6.14 ± 0.21 b	8.01 ± 0.18 a	7.38 ± 0.19 ab	7.74 ± 0.12 a
Lyxose	4.68 ± 0.13 b	3.60 ± 0.06 a	3.99 ± 0.20 a	4.18 ± 0.14 ab	3.76 ± 0.07 a	3.71 ± 0.08 a
Mannose	8.82 ± 0.09 a	9.00 ± 0.03 ab	8.69 ± 0.11 a	9.41 ± 0.16 b	9.13 ± 0.07 ab	9.44 ± 0.11 b
Raffinose	8.12 ± 0.56 a	7.18 ± 0.31 a	7.53 ± 0.30 a	10.25 ± 0.22 b	10.19 ± 0.11 b	10.00 ± 0.12 b
Rhamnose	5.71 ± 0.32 ab	5.72 ± 0.17 ab	6.12 ± 0.31 b	5.36 ± 0.28 ab	4.71 ± 0.29 a	5.55 ± 0.21 ab
Ribose	8.33 ± 0.14 a	8.20 ± 0.08 a	8.42 ± 0.28 a	8.17 ± 0.08 a	8.10 ± 0.29 a	7.87 ± 0.14 a
Sorbose	14.37 ± 0.38 a	14.79 ± 0.11 a	14.14 ± 0.39 a	14.83 ± 0.11 a	14.77 ± 0.06 a	14.82 ± 0.10 a
Sucrose	9.22 ± 0.40 a	10.05 ± 0.58 a	9.12 ± 0.25 a	9.25 ± 0.36 a	8.85 ± 0.16 a	10.14 ± 0.59 a
Talose	13.09 ± 0.19 a	13.10 ± 0.06 a	13.18 ± 0.19 a	13.17 ± 0.03 a	13.04 ± 0.07 a	13.23 ± 0.04 a
Trehalose	8.90 ± 0.50 a	8.50 ± 0.29 a	9.32 ± 0.31 a	9.58 ± 0.12 a	9.56 ± 0.26 a	9.38 ± 0.10 a
Xylitol	5.67 ± 0.05 a	7.42 ± 0.37 a	7.14 ± 0.55 a	6.06 ± 0.37 a	7.81 ± 0.05 a	7.47 ± 0.45 a

Table 6 - Continuation...

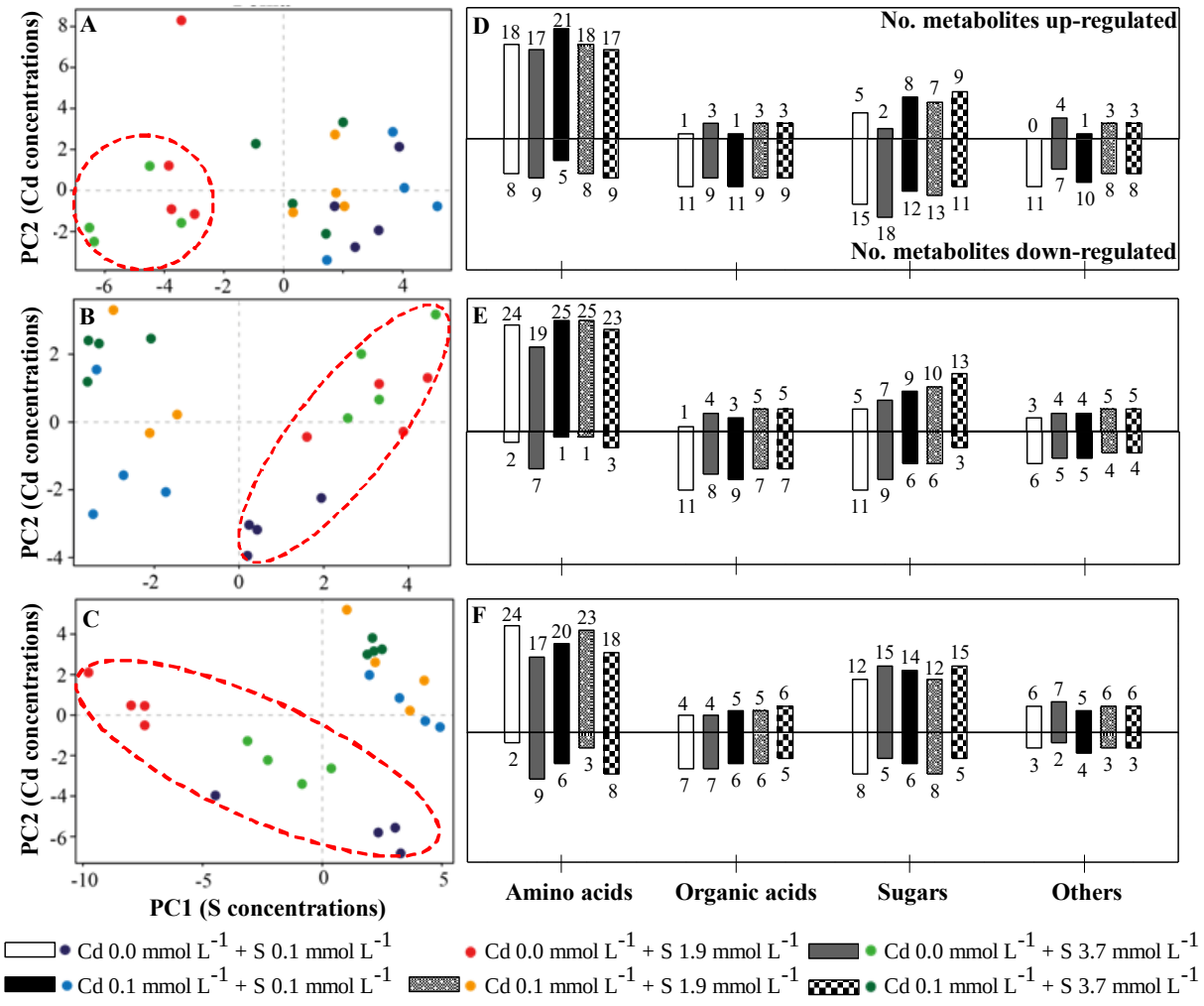
Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Xylose	8.16 ± 0.33 b	5.60 ± 0.19 a	6.79 ± 0.26 ab	5.61 ± 0.12 a	5.69 ± 0.07 a	5.25 ± 0.02 a
Amino acids						
5-Oxoproline	12.98 ± 0.18 a	12.22 ± 0.18 a	12.73 ± 0.17 a	12.86 ± 0.17 a	12.33 ± 0.29 a	12.51 ± 0.10 a
Alanine	15.49 ± 0.08 a	15.66 ± 0.37 a	15.46 ± 0.10 a	15.22 ± 0.04 a	15.41 ± 0.18 a	15.44 ± 0.09 a
Arginine	7.44 ± 0.16 a	6.63 ± 0.21 b	6.76 ± 0.10 ab	7.11 ± 0.14 ab	7.32 ± 0.16 a	6.90 ± 0.07 ab
Asparagine	11.04 ± 0.57 c	7.85 ± 0.28 ab	6.84 ± 0.27 b	9.32 ± 0.18 ac	8.67 ± 0.42 ab	8.06 ± 0.15 ab
Aspartate	12.82 ± 0.55 a	11.03 ± 0.28 b	12.70 ± 0.22 ac	11.97 ± 0.36 abc	11.21 ± 0.38 bc	11.25 ± 0.19 abc
ß-Alanine	8.20 ± 0.06 a	7.81 ± 0.13 a	8.07 ± 0.04 a	7.94 ± 0.17 a	7.82 ± 0.21 a	7.70 ± 0.07 a
Citrulline	7.62 ± 0.19 a	7.09 ± 0.15 ab	7.25 ± 0.14 ab	7.12 ± 0.14 ab	7.24 ± 0.10 ab	6.89 ± 0.07 b
Cysteine	5.49 ± 0.56 a	4.80 ± 0.24 a	5.65 ± 0.17 a	7.05 ± 0.18 b	7.61 ± 0.05 b	7.32 ± 0.08 b
Glutamate	13.92 ± 0.29 a	12.83 ± 0.22 b	13.50 ± 0.15 ab	13.57 ± 0.20 ab	12.96 ± 0.34 ab	13.09 ± 0.16 ab
Glutamine	12.60 ± 0.38 a	11.12 ± 0.38 a	11.72 ± 0.31 a	12.29 ± 0.31 a	11.39 ± 0.45 a	11.24 ± 0.11 a
Glycine	10.97 ± 0.14 a	10.12 ± 0.26 b	10.35 ± 0.07 ab	10.37 ± 0.05 ab	10.48 ± 0.13 ab	10.43 ± 0.15 ab
Histidine	7.62 ± 0.73 ab	4.18 ± 0.04 c	6.49 ± 0.06 b	8.05 ± 0.17 a	8.28 ± 0.21 a	7.55 ± 0.15 ab
Isoleucine	10.94 ± 0.07 a	10.74 ± 0.16 a	10.39 ± 0.07 a	10.51 ± 0.20 a	10.78 ± 0.13 a	10.43 ± 0.14 a
Leucine	11.06 ± 0.10 a	10.77 ± 0.20 a	10.60 ± 0.10 a	10.74 ± 0.28 a	11.09 ± 0.21 a	10.81 ± 0.18 a
L-proline	10.00 ± 0.07 a	10.80 ± 0.38 a	10.86 ± 0.26 a	10.55 ± 0.28 a	11.35 ± 0.47 a	11.08 ± 0.33 a
Lysine	10.50 ± 0.14 a	9.64 ± 0.24 b	10.03 ± 0.15 ab	10.09 ± 0.18 ab	10.46 ± 0.15 a	10.06 ± 0.10 ab
Methionine	9.40 ± 0.25 a	9.20 ± 0.09 ad	9.03 ± 0.09 abd	8.35 ± 0.14 bc	8.57 ± 0.20 bcd	8.29 ± 0.08 c
O-acetylserine (OAS)	4.59 ± 0.18 a	2.50 ± 0.24 c	3.56 ± 0.11 b	4.16 ± 0.16 ab	4.21 ± 0.15 ab	4.02 ± 0.13 ab
Ornithine	7.53 ± 0.21 b	6.00 ± 0.13 c	6.94 ± 0.21 ab	6.89 ± 0.02 ab	6.78 ± 0.15 a	6.83 ± 0.08 a
Phenylalanine	8.79 ± 0.20 a	7.95 ± 0.21 b	8.11 ± 0.19 ab	8.36 ± 0.13 ab	8.47 ± 0.12 ab	7.99 ± 0.16 ab
Proline	10.86 ± 0.35 ab	10.21 ± 0.27 b	10.16 ± 0.16 b	11.25 ± 0.18 a	11.53 ± 0.07 a	10.99 ± 0.04 ab
Serine	14.05 ± 0.21 c	12.46 ± 0.31 b	13.02 ± 0.13 ab	13.47 ± 0.12 ac	13.24 ± 0.16 abc	13.19 ± 0.10 ab
Threonine	11.10 ± 0.32 a	9.82 ± 0.22 b	9.96 ± 0.11 b	10.14 ± 0.06 b	10.21 ± 0.20 b	9.74 ± 0.06 b
Tryptophan	9.63 ± 0.16 a	7.90 ± 0.26 c	7.67 ± 0.14 c	8.94 ± 0.04 ab	8.89 ± 0.27 ab	8.25 ± 0.09 bc
Tyrosine	9.81 ± 0.23 a	8.98 ± 0.14 b	9.36 ± 0.22 ab	9.37 ± 0.15 ab	9.40 ± 0.14 ab	9.13 ± 0.09 ab
Valine	12.48 ± 0.15 b	12.06 ± 0.14 ab	11.67 ± 0.06 a	11.76 ± 0.12 a	11.91 ± 0.13 a	11.58 ± 0.12 a
Others						
4-Aminobutanoate (GABA)	13.45 ± 0.21 a	14.27 ± 0.43 a	14.09 ± 0.25 a	13.75 ± 0.26 a	14.36 ± 0.39 a	14.21 ± 0.28 a
Benzoate	6.61 ± 0.35 a	6.44 ± 0.11 a	7.39 ± 0.38 a	6.77 ± 0.21 a	6.54 ± 0.21 a	6.53 ± 0.22 a
cis-Aconitate	7.12 ± 0.06 ab	6.69 ± 0.09 b	8.03 ± 0.42 abc	9.08 ± 0.55 ac	8.40 ± 0.44 abc	9.86 ± 0.17 c

Table 6 - Conclusion.

Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Gluconate	3.81 ± 0.03 ab	3.12 ± 0.17 a	3.80 ± 0.18 ab	3.82 ± 0.05 ab	3.79 ± 0.19 ab	3.91 ± 0.04 b
Glycerol	9.68 ± 0.10 a	9.59 ± 0.01 a	10.14 ± 0.10 a	10.11 ± 0.11 a	9.73 ± 0.15 a	10.01 ± 0.03 a
Glycerol 3-phosphate	6.58 ± 0.77 ab	4.30 ± 0.16 c	5.58 ± 0.13 bc	7.81 ± 0.18 a	7.44 ± 0.19 a	7.86 ± 0.07 a
Putrescine	11.39 ± 0.32 ab	11.22 ± 0.16 ab	12.01 ± 0.40 b	10.75 ± 0.32 ab	10.42 ± 0.43 a	11.07 ± 0.21 ab
Pyruvate	4.75 ± 0.17 a	4.76 ± 0.11 a	5.35 ± 0.27 a	4.74 ± 0.03 a	4.70 ± 0.18 a	4.78 ± 0.04 a
Shikimate	10.15 ± 0.11 ab	10.86 ± 0.17 a	9.95 ± 0.19 b	10.52 ± 0.18 ab	10.42 ± 0.21 ab	10.38 ± 0.12 ab

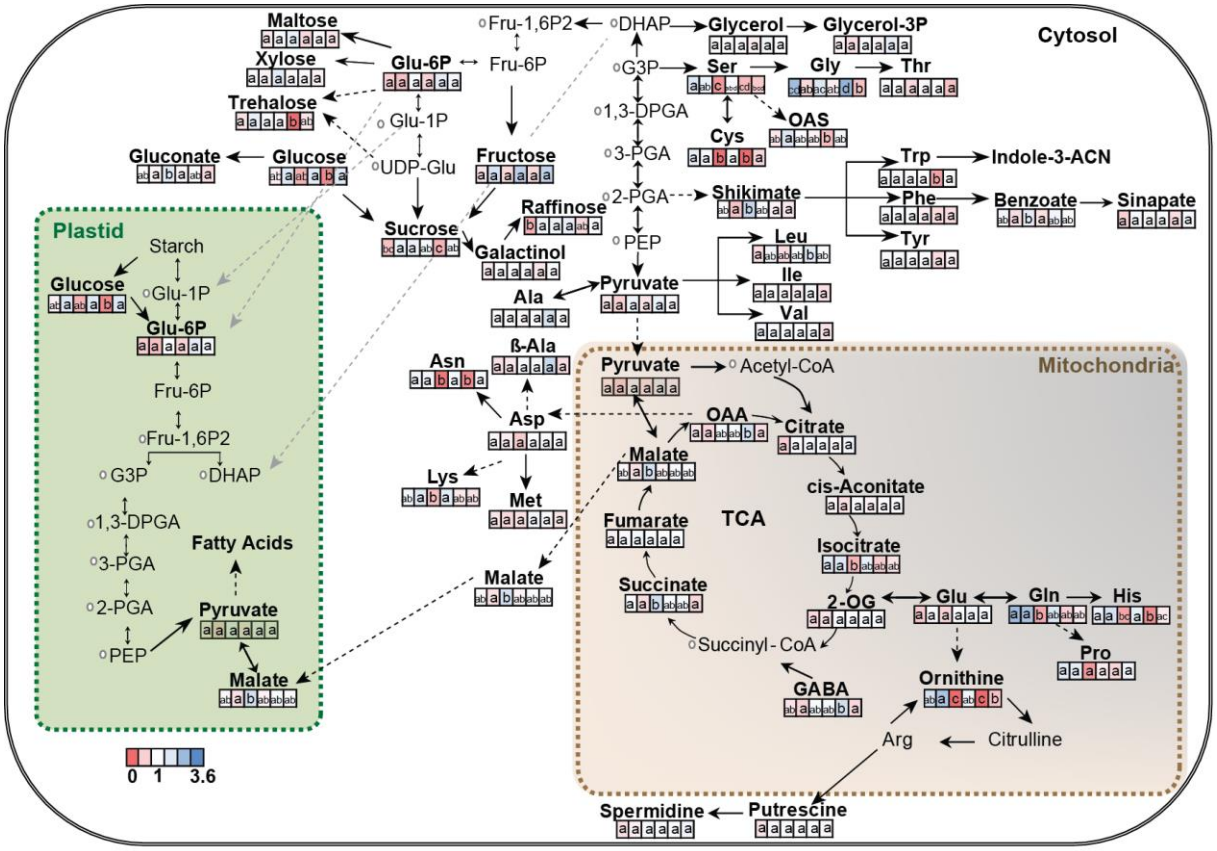
Mean ± SEM followed by distinct letters in the lines differed by Tukey test ($P < 0.05$).

Figure 2 - Principal components analysis (PCA; A, B and C) and overall comparison (D, E and F, in relation to control treatment: 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd) of metabolic profiling in the leaf blades (A and D), a sample comprising both stem and sheath material (B and E) and roots (C and F) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1}). Below the figure, legends in circular form refer to parts A, B and C, while rectangular forms refer to parts D, E and F



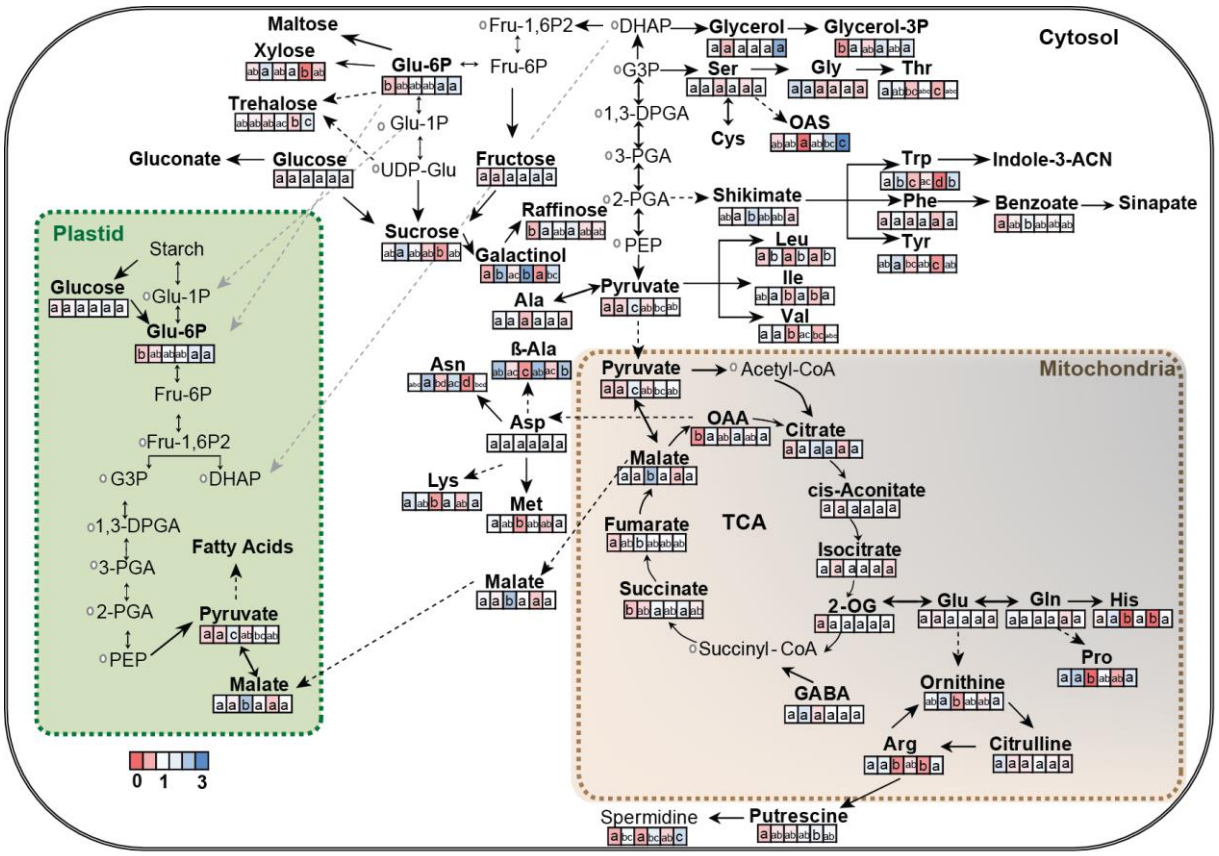
In leaf blades, the levels of trehalose, sucrose, shikimate and tryptophan decreased while the level of glycine increased under the highest S supply in relation to the control condition (Figure 3). In general, Cd exposure caused increased levels of glucose, sucrose, asparagine, lysine, cysteine, histidine and ornithine and decreased gluconate and glycine levels in relation to the control, mainly when the plants were supplied with 1.9 and 3.7 mmol L^{-1} S (Figure 3).

Figure 3 - Metabolic changes in leaf blades of Massai grass supplied with S and exposed to Cd. The boxes located below each metabolite represent the respective exposure conditions (from left to right): 0.1 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, 0.1 mmol L^{-1} S + 0.1 mmol L^{-1} Cd, 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, 1.9 mmol L^{-1} S + 0.1 mmol L^{-1} Cd, 3.7 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, and 3.7 mmol L^{-1} S + 0.1 mmol L^{-1} Cd. Different letters located in the boxes below each metabolite indicate significant statistical differences by Tukey test ($P < 0.05$)



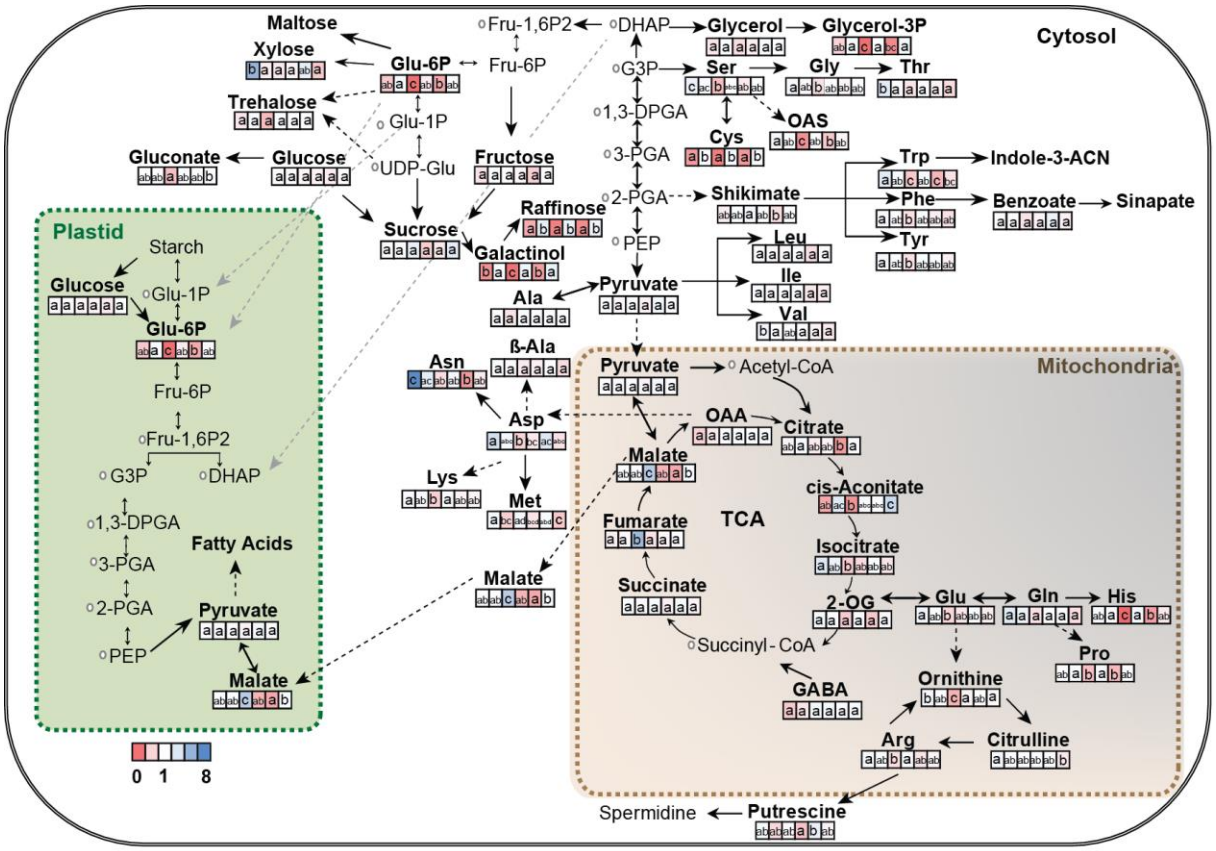
Levels of glucose-6-phosphate (Glu-6P) and O-acetylserine (OAS) in the samples comprising both stem and sheath material of plants supplied with 3.7 mmol L^{-1} S and not exposed to Cd increased in comparison to the control, whereas malate and tryptophan levels decreased (Figure 4). Galactinol, ß-alanine, tryptophan, lysine, isoleucine, leucine, tyrosine, histidine, arginine and spermidine levels increased in a sample comprising both stem and sheath material of plants exposed to Cd, mainly when Massai grass plants were grown in the presence of the two highest S concentrations. By contrast, levels of pyruvate and malate decreased in plants exposed to Cd, regardless of S supply (Figure 4).

Figure 4 - Metabolic changes in the sample comprising both stem and sheath material of Massai grass supplied with S and exposed to Cd. The boxes located below each metabolite represent the respective exposure conditions (from left to right): 0.1 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, 0.1 mmol L^{-1} S + 0.1 mmol L^{-1} Cd, 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, 1.9 mmol L^{-1} S + 0.1 mmol L^{-1} Cd, 3.7 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, and 3.7 mmol L^{-1} S + 0.1 mmol L^{-1} Cd. Different letters located in the boxes below each metabolite indicate significant statistical differences by Tukey test ($P < 0.05$)



Levels of Glu-6P, galactinol, OAS, histidine and ornithine increased in the roots of Massai grass not exposed to Cd and supplied with 3.7 mmol L^{-1} S in relation to the control, while fumarate, malate and shikimate decreased (Figure 5). In general, when the plants were exposed to Cd, the levels of Glu-6P, raffinose, galactinol, lysine, glycerol 3-phosphate, OAS, cysteine, tryptophan, histidine, proline, ornithine and arginine increased and the levels of fumarate and malate decreased as compared to the control condition (Figure 5).

Figure 5 - Metabolic changes in the roots of Massai grass supplied with S and exposed to Cd. The boxes located below each metabolite represent the respective exposure conditions (from left to right): 0.1 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, 0.1 mmol L^{-1} S + 0.1 mmol L^{-1} Cd, 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, 1.9 mmol L^{-1} S + 0.1 mmol L^{-1} Cd, 3.7 mmol L^{-1} S + 0.0 mmol L^{-1} Cd, and 3.7 mmol L^{-1} S + 0.1 mmol L^{-1} Cd. Different letters located in the boxes below each metabolite indicate significant statistical differences by Tukey test (P <0.05)



5.3.3 Effect of S and Cd combinations on non-protein thiols (NPTs)

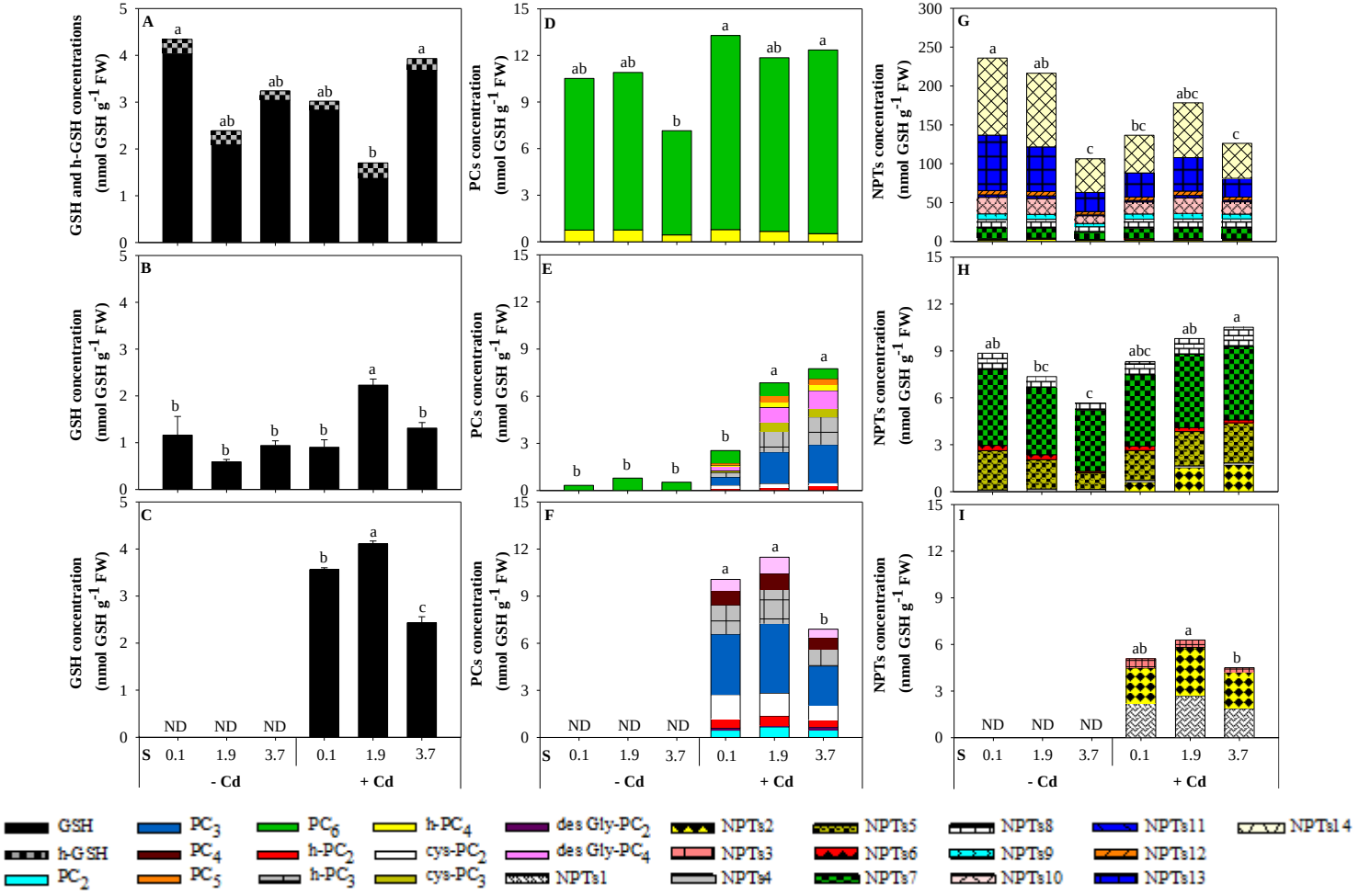
A total of 15 (leaf blades), 16 (a sample comprising both stem and sheath material) and 12 (roots) NPTs were detected by HPLC in Massai grass tissues, but only 4 (leaf blades), 10 (samples comprising both stem and sheath material) and 9 (roots) compounds were identified as GSH, PC or their analogs (Table 7; Figure 6). The GSH and h-GSH levels in the leaf blades (Figure 6A) and GSH in samples comprising both stem and sheath material (Figure 6B) of plants not exposed to Cd were unchanged (P >0.05) by S concentrations. Otherwise, Massai grass exposed to Cd showed higher GSH levels in the leaf blades (Figure 6A), samples comprising both stem and sheath material (Figure 6B) and roots (Figure 6C) when the plants were cultivated with 3.7, 1.9 and 1.9 mmol L^{-1} S, respectively.

Table 7 - Concentrations of non-protein thiols in the leaf blades, a sample comprising both stem and sheath material and roots of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1})

Cd (mmol L^{-1})	0.0			0.1		
S (mmol L^{-1})	0.1	1.9	3.7	0.1	1.9	3.7
Non-protein thiols (nmol GSH g^{-1} FW)						
			Leaf blades			
GSH	4.03 ± 0.10 a	2.09 ± 0.10 ab	3.04 ± 0.71 ab	2.83 ± 0.51 ab	1.37 ± 0.10 b	3.68 ± 0.66 a
h-GSH	0.31 ± 0.02 a	0.30 ± 0.01 a	0.20 ± 0.03 a	0.19 ± 0.11 a	0.33 ± 0.01 a	0.25 ± 0.06 a
NPTs2	1.98 ± 0.29 a	2.62 ± 0.10 a	1.70 ± 0.31 a	1.76 ± 0.47 a	1.83 ± 0.19 a	1.65 ± 0.31 a
h-PC_4	0.75 ± 0.07 a	0.76 ± 0.01 a	0.44 ± 0.04 a	0.78 ± 0.20 a	0.67 ± 0.01 a	0.52 ± 0.18 a
NPTs4	0.41 ± 0.03 ab	0.32 ± 0.03 ab	0.22 ± 0.08 b	0.26 ± 0.02 ab	0.48 ± 0.06 a	0.45 ± 0.07 ab
PC_6	9.77 ± 0.50 ab	10.14 ± 0.18 ab	6.71 ± 0.25 b	12.50 ± 1.92 a	11.18 ± 0.50 a	11.83 ± 0.99 a
NPTs6	1.23 ± 0.09 ab	1.34 ± 0.06 ab	0.70 ± 0.04 b	1.62 ± 0.38 a	1.45 ± 0.03 ab	1.31 ± 0.23 ab
NPTs7	13.73 ± 0.15 a	13.06 ± 0.28 a	9.60 ± 0.43 b	13.00 ± 1.03 a	13.50 ± 0.35 a	13.54 ± 0.69 a
NPTs8	11.06 ± 0.84 ab	11.20 ± 0.36 ab	6.79 ± 0.45 b	12.11 ± 1.39 a	11.71 ± 0.30 a	12.09 ± 1.68 a
NPTs9	7.06 ± 0.07 a	6.31 ± 0.11 ab	4.09 ± 0.28 b	6.52 ± 1.08 ab	6.99 ± 0.15 a	6.15 ± 0.74 ab
NPTs10	21.33 ± 0.21 a	20.26 ± 0.74 a	9.96 ± 1.51 b	15.45 ± 1.60 ab	19.97 ± 1.16 a	15.75 ± 2.41 ab
NPTs11	3.18 ± 0.38 ab	3.65 ± 0.12 a	1.76 ± 0.30 b	1.96 ± 0.41 ab	3.25 ± 0.05 ab	2.18 ± 0.67 ab
NPTs12	5.40 ± 0.06 a	5.46 ± 0.13 a	3.68 ± 0.13 a	4.58 ± 1.59 a	5.57 ± 0.10 a	4.06 ± 1.10 a
NPTs13	71.44 ± 7.97 a	57.44 ± 1.12 ab	24.50 ± 3.44 c	30.95 ± 10.42 bc	43.06 ± 1.62 abc	24.01 ± 8.05 c
NPTs14	99.08 ± 2.43 a	94.78 ± 3.63 a	43.29 ± 4.58 b	48.37 ± 16.29 b	70.59 ± 1.21 ab	45.03 ± 11.10 b
			Sample comprising both stem and sheath material			
h-PC_2	ND	ND	ND	0.12 ± 0.04 b	0.18 ± 0.00 ab	0.32 ± 0.07 a
cys-PC_2	ND	ND	ND	0.22 ± 0.08 a	0.27 ± 0.07 a	0.18 ± 0.02 ab
NPTs2	ND	ND	ND	0.59 ± 0.21 b	1.53 ± 0.24 a	1.74 ± 0.16 a
PC_3	ND	ND	ND	0.52 ± 0.24 b	2.00 ± 0.15 a	2.42 ± 0.16 a
h-PC_3	ND	ND	ND	0.28 ± 0.19 b	1.31 ± 0.17 a	1.76 ± 0.13 a
cys-PC_3	ND	ND	ND	0.11 ± 0.07 b	0.58 ± 0.13 a	0.54 ± 0.02 a
des Gly-PC_4	ND	ND	ND	0.29 ± 0.13 b	0.95 ± 0.19 a	1.14 ± 0.05 a
h-PC_4	ND	ND	ND	0.08 ± 0.04 b	0.34 ± 0.05 a	0.38 ± 0.02 a
PC_5	ND	ND	ND	0.13 ± 0.09 b	0.41 ± 0.08 a	0.38 ± 0.03 a
NPTs4	0.11 ± 0.04 a	0.17 ± 0.01 a	0.14 ± 0.01 a	0.13 ± 0.01 a	0.12 ± 0.01 a	0.10 ± 0.03 a
PC_6	0.33 ± 0.22 a	0.79 ± 0.04 a	0.53 ± 0.01 a	0.79 ± 0.03 a	0.81 ± 0.06 a	0.63 ± 0.21 a
NPTs5	2.51 ± 0.28 a	1.87 ± 0.07 ab	1.06 ± 0.02 b	1.91 ± 0.06 ab	2.19 ± 0.24 a	2.56 ± 0.35 a
NPTs6	0.34 ± 0.02 a	0.32 ± 0.01 ab	0.10 ± 0.03 c	0.28 ± 0.01 ab	0.25 ± 0.05 ab	0.19 ± 0.02 bc
NPTs7	4.85 ± 0.31 a	4.31 ± 0.21 a	3.89 ± 0.13 a	4.56 ± 0.21 a	4.70 ± 0.42 a	4.70 ± 0.05 a
NPTs8	1.04 ± 0.07 ab	0.69 ± 0.12 bc	0.47 ± 0.08 c	0.85 ± 0.12 abc	1.00 ± 0.09 ab	1.22 ± 0.08 a
			Roots			
PC_2	ND	ND	ND	0.46 ± 0.06 b	0.67 ± 0.03 a	0.49 ± 0.08 ab
des Gly-PC_2	ND	ND	ND	0.14 ± 0.07 ab	0.10 ± 0.04 b	0.18 ± 0.07 a
h-PC_2	ND	ND	ND	0.58 ± 0.14 a	0.59 ± 0.08 a	0.43 ± 0.11 a
NPTs1	ND	ND	ND	2.21 ± 0.28 ab	2.69 ± 0.16 a	1.87 ± 0.23 b
cys-PC_2	ND	ND	ND	1.55 ± 0.11 a	1.46 ± 0.11 a	0.95 ± 0.09 b
NPTs2	ND	ND	ND	2.27 ± 0.27 b	3.12 ± 0.22 a	2.34 ± 0.30 ab
PC_3	ND	ND	ND	3.85 ± 0.47 a	4.43 ± 0.44 a	2.50 ± 0.27 b
h-PC_3	ND	ND	ND	1.85 ± 0.25 ab	2.18 ± 0.22 a	1.07 ± 0.44 b
PC_4	ND	ND	ND	0.92 ± 0.09 a	1.01 ± 0.15 a	0.74 ± 0.13 a
des Gly-PC_4	ND	ND	ND	0.71 ± 0.18 a	1.04 ± 0.27 a	0.53 ± 0.13 ab
NPTs3	ND	ND	ND	0.60 ± 0.10 a	0.47 ± 0.02 ab	0.29 ± 0.11 b

Mean ± SEM followed by distinct letters in the lines differed by Tukey test ($P < 0.05$). GSH = glutathione; h-GSH = homoglutathione; $NPTs_{(1-14)}$ = non-protein thiols; h-$PC_{(2-4)}$ = homophytochelatin; $PC_{(2-6)}$ = phytochelatin; cys-$PC_{(2-3)}$ = isoforms of phytochelatin; des Gly-$PC_{(2\ and\ 4)}$ = desglycine phytochelatin; ND = non-detected.

Figure 6 - Concentrations of GSH (A, B and C), PCs (D, E and F) and non-identified non-protein thiols - NPTs (G, H and I) peptides in the leaf blades (A, D and G), a sample comprising both stem and sheath material (B, E and H) and roots (C, F and I) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and either or not exposed to Cd (0.1 mmol L^{-1}). Different letters on the bars indicate significant statistical differences by Tukey test ($P < 0.05$). ND = non-detected

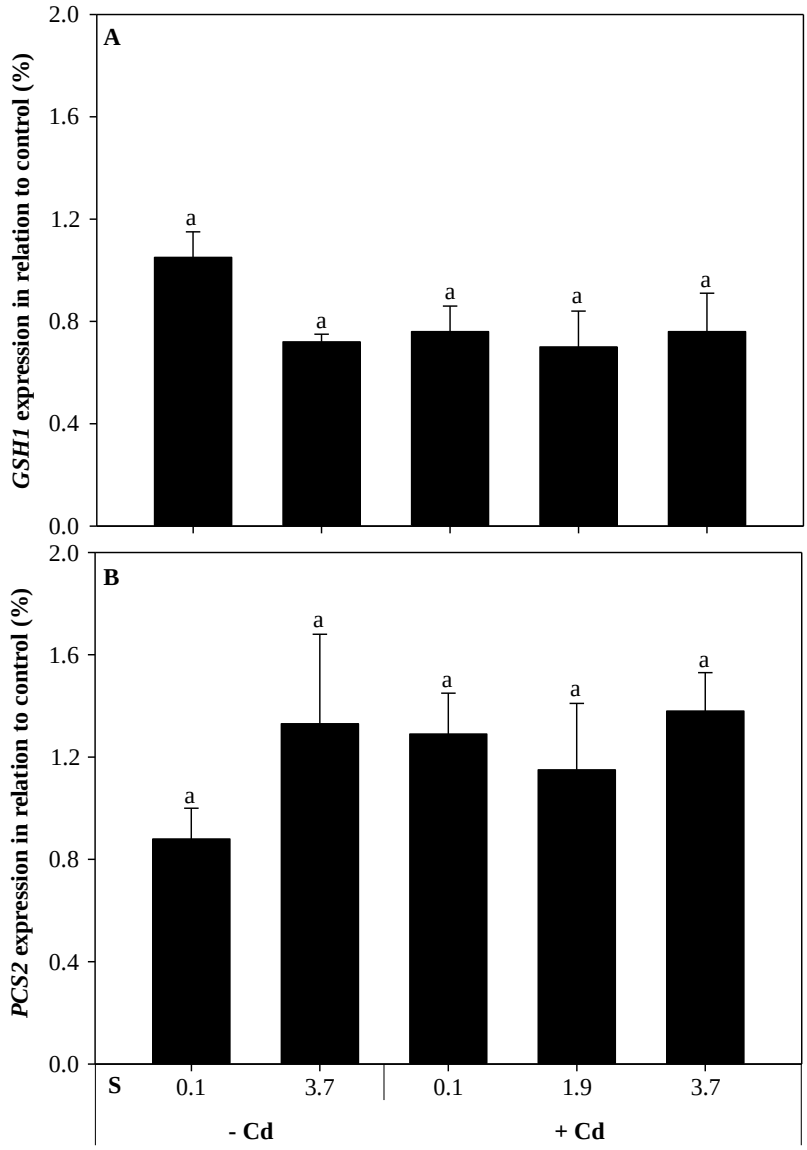


In general, the total concentration of PCs in the leaf blades of Massai grass was unchanged by the exposure conditions ($P > 0.05$), with the exception of plants grown in the presence of the highest S concentration, which displayed higher PCs concentration when exposed to Cd (Figure 6D). There was no effect of the exposure conditions ($P > 0.05$) on concentrations of h-PC_4 and PC_6 in the leaf blades of Massai grass (Table 7). Similarly, there was no effect ($P > 0.05$) of S concentrations on PCs concentration in samples comprising both stem and sheath material of plants grown without Cd. However, plants exposed to Cd displayed higher PC concentrations when they were grown in the presence of the two highest S concentrations (Figure 6E), which can be attributed to the increase in concentrations of h-PC_2, PC_3, h-PC_3, cys-PC_3, desGly-PC_4, h-PC_4 and PC_5 observed under these conditions (Table 7). No PCs were detected in the roots of plants not exposed to Cd, but eight different PCs were detected in the roots of plants grown in Cd presence (Table 7). The roots of Massai grass exposed to Cd and supplied with highest S concentration presented the lowest PCs concentration (Figure 6F), due to lower concentrations of cys-PC_2, PC_3 and h-PC_3 (Table 7). Eleven unidentified NPTs were detected in the leaf blades of Massai grass (Table 7), with the total NPTs concentration in this tissue of plants not exposed to Cd decreased by supplying the highest S concentration (Figure 6G). However, there was no effect of S supply ($P > 0.05$) on NPTs concentration in the leaf blades of plants exposed to Cd (Figure 6G). The NPTs in a sample comprising both stem and sheath material of plants not exposed to Cd decreased with increasing S supply, but there was no effect ($P > 0.05$) of S concentrations on NPTs in Massai grass exposed to Cd (Figure 6H). Notably, concentrations of unidentified NPTs in a sample comprising both stem and sheath material (Figure 6H) were around 20 times smaller than in leaf blades (Figure 6G). No unknown NPTs were detected in the roots of Massai grass grown without Cd, but three of them were detected in plants exposed to Cd (Table 7; Figure 6I). Plants grown under 1.9 mmol L^{-1} S supply presented higher NPTs concentrations in the roots as compared to plants supplied with the highest S concentration (Figure 6I).

5.3.4 Expression of genes related to non-protein thiols (NPTs) biosynthesis

Although NPTs concentrations changed ($P < 0.05$) in different tissues of Massai grass as a function of S and Cd combinations (Table 7; Figure 6), there was no effect of either element on the expression levels of γ-ECS (*GSH1*, Figure 7A) and PCS (*PCS2*, Figure 7B) in the leaf blades of Massai grass.

Figure 7 - Expression levels of γ-glutamylcysteine synthetase (*GSH1*, A) and phytochelatin synthase (*PCS2*, B) relative to control treatment (1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd) in the leaf blades of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and either or not exposed to Cd (0.1 mmol L^{-1}). Different letters on the bars indicate significant statistical differences by Tukey test (P <0.05)



5.4 Discussion

When Massai grass was exposed to Cd, biomass production decreased (Figure 1A), which can be attributed to nutritional imbalance, alteration of water relations, oxidative stress and damage to the photosynthetic system caused by Cd (SANITÀ DI TOPPI; GABBRIELLI, 1999). On the other hand, biomass production of plants exposed to Cd did not increase significantly (P >0.05) with supply of the two highest S concentrations (Figure 1A), probably due to lower water use efficiency observed in these plants (Figures 11H-1, p. 174).

Typically, Cd inhibited root growth more strongly than shoot growth, thus decreasing the root/shoot ratio (BARCELÓ; POSCHENRIEDER, 1990). Indeed, the root/shoot ratio of Massai grass grown with the two highest S concentrations was lower in plants exposed to 0.5 mmol L^{-1} Cd than in plants exposed to 0.1 mmol L^{-1} Cd (Figure 1B). It is also clear that root/shoot ratio of Massai grass exposed to 0.1 mmol L^{-1} Cd increased when plants were supplied with two highest S concentrations (Figure 1B). It is probable that under conditions of lower Cd content (plants exposed to 0.1 mmol L^{-1} Cd, Figure 1D), Massai grass prioritize to allocate S in the roots (Figure 1C), which is the tissue containing the highest Cd concentration in these plants (Table 4, p. 87), in order to reduce the damage caused by Cd (GILL; TUTEJA, 2011). This process allows more root growth and, therefore, higher root/shoot ratios. This fact suggests that S supply changes the physiological homeostasis of Massai grass exposed to Cd, which is supported by PCA analyses of metabolic changes. Indeed, metabolites identified by GC-TOF-MS in leaf blades and a sample comprising both stem and sheath material were more altered by S than by Cd concentrations, while in the roots the effect of these elements was similar (Figures 2A-C).

Metabolites (amino acids, organic acids and sugars) have many functions in plants exposed to Cd, besides the functions of intermediary metabolism, since they act as signaling/regulating agents and antioxidants amongst other functions (SHARMA; DIETZ, 2006; SUN et al., 2010; KEUNEN et al., 2013). In this sense, the specific accumulation of amino acids, organic acids and/or sugars can be used to identify important pathways of Cd detoxification in forage grasses (XIE et al., 2014; RABÊLO; AZEVEDO; MONTEIRO, 2017b). Cysteine, lysine, histidine, asparagine, ornithine, glucose and sucrose levels in the leaf blades of Massai grass exposed to Cd increased in relation to the control condition, mainly when there was S availability of 1.9 and 3.7 mmol L^{-1} (Table 4; Figure 3). The increase in cysteine concentration is critical for Cd tolerance, since this amino acid is required for methionine, GSH and PCs synthesis (COBBETT; GOLDSBROUGH, 2002; NOCTOR et al., 2012; CAPALDI et al., 2015). Lysine and asparagine are synthesized from aspartate derived of oxaloacetate (OAA) and histidine and ornithine are synthesized from glutamate derived from α-ketoglutarate (2-OG) (SHARMA; DIETZ, 2006), suggesting that these amino acid synthesis pathways are involved in Cd detoxification in the leaf blades of Massai grass. Asparagine is involved in Cd detoxification through the chelating peptide synthesis pathway (COSTA; SPITZ, 1997). Histidine is considered to be the most important free amino acid involved in metal hyperaccumulation, since it can act as a tridentate binding by means of its carboxylate, amine and imadazole functions (OZEN; YAMAN, 2017).

Xu et al. (2012) reported that histidine accumulation in *Solanum nigrum* favored Cd uptake and translocation from roots to shoots, which increased Cd accumulation in the leaves of these plants. In this study, we observed that all tissues (leaf blades, a sample comprising both stem and sheath material and roots) of plants exposed to Cd showed higher histidine levels in relation to the control (Tables 4-6; Figures 3-5), suggesting that histidine may also favor Cd uptake and translocation in Massai grass. Ornithine acts on signaling pathways regulating the synthesis of proline, arginine, putrescine and aminobutyric acid (GABA) (MAJUMDAR et al., 2013), making this amino acid important for Cd hyperaccumulators as verified by Zemanová, Pavlík and Pavlíková (2017) in two species of *Noccaea*. Rabêlo, Azevedo and Monteiro (2017b) reported that only cysteine concentration increased in shoot (first growth) of *Panicum maximum* cv. Tânzania (Tanzania guinea grass) exposed to Cd, suggesting again that amino acids synthesized from OAA and 2-OG are important for Cd tolerance of Massai grass, since these plants presented higher Cd contents and lower toxicity than Tanzania guinea grass. Moreover, the increase in glucose and sucrose levels in the leaf blades of Massai grass exposed to Cd contributes to a reduction of oxidative stress, since both sugars are involved in ROS scavenging (KEUNEN et al., 2013, 2016). Besides acting on ROS, glucose activates root apical meristem for growth (XIONG et al., 2013), which may also contribute to increased root/shoot ratio of plants grown with the two highest S concentrations and 0.1 mmol L^{-1} Cd (Figure 1B).

As was observed in the leaf blades, some amino acids (ß-alanine, lysine, isoleucine, arginine, tryptophan, leucine and tyrosine) accumulated more in the sample comprising both stem and sheath material of plants exposed to Cd, especially when the two highest S concentrations were supplied (Table 5, Figure 4). Beta-alanine can fulfill osmoprotectant functions under stress conditions (HANSON et al., 1994) and, like lysine and asparagine, is also synthesized from aspartate derived of OAA, indicating again that this pathway of amino acid synthesis is involved in mitigation of Cd damage in shoots of Massai grass, as observed in *A. thaliana* (SUN et al., 2010). Increases in isoleucine and leucine levels (Table 5, Figure 4) indicate that these branched-chain amino acids play important functions in the sample comprising both stem and sheath material of Massai grass during Cd stress, such as promotion of protein synthesis induced by stress (JOSHI et al., 2010) and maintenance of amino acid homeostasis (ARAÚJO et al., 2010). Arginine is the major organic nitrogen storage in plants and is a precursor of polyamines and nitric oxide (WINTER et al., 2015), which makes this amino acid essential within the metabolism of metal-exposed plants. Nasibi et al. (2013) reported that arginine decreased nickel toxicity and increased its translocation from roots to

shoots by chelation or polyamines production in *Hyoscyamus niger*. Like arginine, tryptophan plays an important role in regulating plant development and defense responses and is precursor of indol-eacetic acid, which is a plant hormone necessary for cell expansion (ZEMANOVÁ et al., 2014). According to Li and Yang (2003), there is an interaction between tryptophan and metal ions. Therefore, the exposure of plants to Cd can increase tryptophan synthesis (ZEMANOVÁ; PAVLÍK; PAVLÍKOVÁ, 2017). Tyrosine, as well as tryptophan, is synthesized from shikimate, and both amino acids are required for protein synthesis and production of secondary aromatic metabolites (e.g. anthocyanin), which are important in mitigation of oxidative damage caused by Cd (RICE-EVANS; MILLER; PAGANGA, 1996; NIKIFOROVA et al., 2006). Although tyrosine mitigates Cd-induced damage, its concentration was not increased in other grasses exposed to Cd (XIE et al., 2014; RABÊLO; AZEVEDO; MONTEIRO, 2017b), showing that tyrosine accumulation in forage grasses is specific. In view of the observed changes in amino acid metabolism in leaf blades (Figure 3) and a sample comprising both stem and sheath material (Figure 4), it is clear that low S supply may limit amino acid synthesis as described by Nikiforova et al. (2006), and thereby compromise some mechanisms of Cd detoxification (e.g. chelation) (VILLIERS et al., 2011). Although proper S supply favors Cd detoxification (BASHIR et al., 2015), levels of galactinol and spermidine in the a sample comprising both stem and sheath material of plants exposed to Cd increased independently of S concentrations (Table 5, Figure 4). Galactinol is as efficient as GSH in scavenging of OH^- (NISHIZAWA; YABUTA; SHIGEOKA, 2008) and, therefore, accumulation of this sugar could decrease Cd-induced oxidative stress (SUN et al., 2010). Spermidine is a polyamine extremely important for plants grown under stress conditions, since this metabolite can acts as signaling/regulating agent and antioxidant (KASUKABE et al., 2004). In addition, spermidine appears to be related to processes favoring Cd accumulation by plants used for Cd phytoextraction (GONG et al., 2016). This corroborates our results, where a sample comprising both stem and sheath material were the main site of Cd accumulation in Massai grass (Figure 1D), although the roots are the site of higher Cd concentration (Table 4, p. 87).

The roots are the first site of Cd-plant contact, so tissue defense mechanisms should be activated quickly (JOZEFCZAK et al., 2014). Chaffei et al. (2004) suggested that increases in the proportion of amino acids with high N/C ratio (e.g. arginine and proline) preserves the roots from Cd toxicity, which occurred in our study as well (Table 6, Figure 5). Proline can acts as osmolyte, radical scavenger, electron sink, macromolecule stabilizer and cell wall component (MATYSIK et al., 2002), which makes this amino acid fundamental for plants to

respond to Cd exposure (SHARMA; DIETZ, 2006). Besides increased proline and arginine levels, proper S availability (1.9 mmol L^{-1}) also resulted in the highest levels of OAS, lysine, cysteine, histidine, ornithine and tryptophan in the roots of plants exposed to Cd (Table 6, Figure 5). In general, the highest OAS levels were found in S deprivation conditions associated with lowest levels of cysteine and GSH (NIKIFOROVA et al., 2006). Nevertheless, levels of OAS, cysteine (Table 6, Figure 5), and GSH (Figure 6C) increased simultaneously in the roots of Massai grass. This fact suggests that synthesis of S-rich metabolites in the roots of plants exposed to Cd is not limited when there is proper S supply, a key asset for plants used in Cd phytoextraction (GILL; TUTEJA, 2011; SETH et al., 2012; SHEORAN; SHEORAN; POONIA, 2016). On the other hand, the roots of Massai grass cultivated under proper S supply presented the lowest levels of malate (as in the sample comprising both stem and sheath material; Table 5, Figure 4) and fumarate (Table 6, Figure 5), which can be associated with high iron (Fe) levels observed in the roots of these plants (Table 4, p. 87). Malate, which is synthesized from fumarate hydration, complexes Fe in the root apoplast (ROSCHZTTARDTZ et al., 2011). Therefore, high Fe concentrations in the roots can result in lower malate and fumarate levels. In addition, GSH (Figures 6B-C) and PCs (Figures 6E-F) synthesis was relatively the highest in plants grown with 1.9 mmol L^{-1} S, which may result in lowest organic acids levels, since GSH and PCs show highest affinity for Cd^{2+} during chelation of this metal as compared to organic acids (KROTZ; EVANGELOU; WAGNER, 1989; COBBETT; GOLDSBROUGH, 2002; NOCTOR et al., 2012). Regardless of S supply, levels of Glu-6P, glycerol 3-phosphate, raffinose and galactinol increased in roots of plants exposed to Cd (Table 6, Figure 5). This could be attributed to the acidification caused by Cd in the cellular cytosol, thereby reducing root respiration (GARMASH; GOLOVKO, 2009), leading to Glu-6P and glycerol 3-phosphate accumulation (van DONGEN et al., 2009). On the other hand, to mitigate the damage caused by cytosolic acidification, plants can accumulate galactinol and raffinose, since these sugars are involved in attenuation of oxidative stress caused by Cd (NISHIZAWA; YABUTA; SHIGEOKA, 2008), as observed by Sun et al. (2010) and Keunen et al. (2016) in *A. thaliana*. Kieffer et al. (2008) also reported that levels of Glu-6P and raffinose may increase for osmotic adjustment and protection of cellular constituents against Cd-induced damage.

Some metabolites detected in this study evidently act in processes related to detoxification (e.g. galactinol) and accumulation (e.g. histidine) of Cd in Massai grass, as well as acting as a substrate for GSH and PCs synthesis (e.g. glutamate, cysteine and glycine). Therefore, as these metabolites participate in a complex detoxification network, changes in

their levels may directly impact GSH and PCs synthesis in plants exposed to Cd (DUCRUIX et al., 2006; VILLIERS et al., 2011). It is important to note that Cd binds strongly to N-rich compounds (e.g. lysine) and, mainly, to S-rich compounds (e.g. cysteine) (NIEBOER; RICHARDSON, 1980). Thus, GSH and PCs are fundamental for Cd tolerance in plants (GRILL; WINNACKER; ZENK, 1986; BERNHARD; KÄGI, 1987; KLAPHECK, 1988; KLAPHECK et al., 1988, KLAPHECK; SCHLUNZ; BERGMANN, 1995; COBBETT; GOLDSBROUGH, 2002; VÁZQUEZ; GOLDSBROUGH; CARPENA, 2006; NOCTOR et al., 2012; FERNÁNDEZ et al., 2013; JOZECFZAK et al., 2014; BASHIR et al., 2015). In this study, GSH and PCs synthesis was strongly induced by Cd (Figures 6A-F), mainly in a sample comprising both stem and sheath material (Figures 6B and 6E) and roots (Figures 6C and 6F) of Massai grass, which were the tissues that presented the highest Cd content (Figure 1D). Seth et al. (2012) reported that plants exhibiting high GSH levels when exposed to Cd are more tolerant and efficient to be used for Cd phytoextraction purposes. In this context, proper S supply can increase Cd phytoextraction efficiency, since GSH and PCs levels in a sample comprising both stem and sheath material (Figure 6B) and roots (Figure 6C) of Massai grass supplied with 1.9 mmol L^{-1} S were higher than plants supplied with 0.1 mmol L^{-1} S. Nocito et al. (2006) reported that plants exposed to Cd take up more S to increase GSH and PCs synthesis. Thus, when S supply is not limited, GSH and PCs synthesis tend to increase (GILL; TUTEJA, 2011). Positive effects of S on GSH synthesis in plants exposed to Cd have also been demonstrated by Bashir et al. (2015) and Rabêlo, Azevedo and Monteiro (2017a). Massai grass also showed a GSH (h-GSH) homologue in the leaf blades (Figure 6A). The synthesis of GSH and h-GSH occurs in two stages, where the first stage is the same for the two metabolites (acting of γ-ECS), but the second stage is catalyzed by specific enzymes [GSHS for GSH and homoglutathione synthetase (hGSHS) for h-GSH] (KLAPHECK et al., 1988; NOCTOR et al., 2012). As γ-ECS is encoded by the *GSH1* gene (NOCTOR et al., 2012), and there was no difference ($P > 0.05$) in the expression of this gene in the leaf blades of Massai grass (Figure 7A), it is clear that GSH and h-GSH synthesis in this plant is related to expression of genes that encode GSHS and hGSHS and/or to posttranscriptional processes. Thus, the decrease in glycine concentration could limit GSH synthesis, and β-alanine could limit h-GSH synthesis (GRILL; WINNACKER; ZENK, 1986). However, glycine concentration in the leaf blades of Massai grass supplied with 3.7 mmol L^{-1} S was lower than in plants supplied with 0.1 and 1.9 mmol L^{-1} S, when plants were exposed to Cd (Table 4, Figure 3), but GSH and PCs synthesis in these plants were not compromised (Table 7).

Phytochelatins mostly induced by Cd in a sample comprising both stem and sheath material (Figure 6E) and roots (Figure 6F) of Massai grass were PC_3 and h-PC_3, while in the leaf blades (tissue with the lowest Cd content, Figure 1D) only PC_6 and h-PC_4 were detected (Fig. 6D). Oven et al. (2001) suggested that the occurrence of h-GSH in a particular plant determines the formation of h-PCs during exposure to Cd. However, there was synthesis of h-PCs in a sample comprising both stem and sheath material and roots of Massai grass, but h-GSH was not detected in these tissues (Table 7), which can be attributed to h-GSH depletion for h-PCs synthesis (KLAPHECK; SCHLUNZ; BERGMANN, 1995) and Cd translocation from roots to shoots (KLAPHECK, 1988; MENDOZA-CÓZATL et al., 2008). Further studies are needed to understand how this process occurs. Besides h-PCs, other homologues of PCs (desGly-PCs and cys-PCs) were found in a sample comprising both stem and sheath material and roots (Table 7) of Massai grass exposed to Cd, as observed in *Zea mays* (WÓJCIK; TUKENDORF, 1999) and *Dittrichia viscosa* (FERNÁNDEZ et al., 2013). The high levels and variety of PCs (Figures 6D-F, Table 7) found in Massai grass exposed to Cd suggest that synthesis of these peptides is essential for Cd tolerance in this forage grass. Gonzalez-Mendoza, Moreno and Zapata-Perez (2007) reported that genes encoding PCS (*PCS1* and *PCS2*; COBBETT; GOLDSBROUGH, 2002) are closely associated with plant tolerance mechanisms, mainly in the leaves. However, *PCS2* expression in the leaf blades of Massai grass was not altered ($P > 0.05$) by the different S and Cd concentrations (Figure 7B). Jozefczak et al. (2014) also observed that *PCS2* expression in *Arabidopsis* leaves was not altered by exposure to Cd, although the *PCS1* gene was more expressed and PC_{2-4} levels increased. These results indicate that PCs synthesis in *Arabidopsis* leaves is controlled by *PCS1* gene and/or by post-transcriptional processes, which could also be the case in Massai grass. Besides GSH and PCs, other NPTs may also form Cd-S complexes, with NPTs potentially increasing plant tolerance to Cd (ZHANG et al., 2013). In this sense, we observed that NPTs levels in the leaf blades (Figure 6G), a sample comprising both stem and sheath material (Figure 6H) and in the roots (Figure 6I) of Massai grass exposed to Cd were mainly the highest when plants were grown with 1.9 mmol L^{-1} S. Zhang et al. (2013) also observed that proper S supply increased NPTs concentration in rice plants exposed to Cd. Besides to decrease Cd toxicity by the formation of Cd-S complexes, NPTs reduce the oxidative damage caused by Cd due their redox properties (GILL; TUTEJA, 2011). In view of these results, it is clear that Massai grass properly supplied with S shows a high potential for Cd phytoextraction in relation to other plants (MEYER; VERBRUGGEN, 2012) used for the same purpose.

5.5 Conclusions

Different tissues of Massai grass showed different responses in the primary metabolism when plants were exposed to Cd. Nevertheless, cysteine, ornithine, arginine, tryptophan, lysine, and histidine accumulated in more than one tissue, indicating that these amino acids are probably involved in Cd accumulation and detoxification in this plant. Among the sugars, galactinol is most likely to active in Cd detoxification processes in Massai grass. Organic acids were not as affected by Cd exposure as amino acids and sugars. Despite of the lack of changes in the expression of genes encoding γ-ECS (*GSH1*) and PCS (*PCS2*), glutathione, PCs and their homologous were strongly increased by Cd, mainly in the roots and a sample comprising both stem and sheath material of plants, independently of different metabolic changes occurring in these tissues. Synthesis of most metabolites evaluated in this study was highest when Massai grass exposed to Cd was grown with 1.9 mmol L^{-1} S. Thus, proper S supply improves Cd detoxification mechanisms of Massai grass used for Cd phytoextraction, such as sequestering Cd by PCs and amino acids.

References

AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY - ATSDR. **Draft toxicological profile for cadmium**. Atlanta, 2012. Available at: <http://www.atsdr.cdc.gov/toxprofiles/tp5.pdf>. Access in: 15 Sep. 2017.

ARAÚJO, W.L.; ISHIZAKI, K.; NUNES-NESI, A.; LARSON, T.R.; TOHGE, T.; KRAHNERT I.; WITT, S.; OBATA, T.; SCHAUER, N.; GRAHAM, I.A.; LEAVER, C.J.; FERNIE, A.R. Identification of the 2-hydroxyglutarate and isovaleryl-CoA dehydrogenases as alternative electron donors linking lysine catabolism to the electron transport chain of Arabidopsis mitochondria. **The Plant Cell**, Rockville, v. 22, p. 1549-1563, 2010.

BARCELÓ, J.; POSCHENRIEDER, C. Plant water relations as affected by heavy metal stress: a review. **Journal of Plant Nutrition**, Philadelphia, v. 13, p. 1-37, 1990.

BASHIR, H.; IBRAHIM, M.M.; BAGHERI, R.; AHMAD, J.; ARIF, I.A.; BAIG, M.A.; QURESHI, M.I. Influence of sulfur and cadmium on antioxidants, phytochelatins and growth in Indian mustard. **AoB Plants**, Oxford, v. 7, 2015. doi: 10.1093/aobpla/plv001.

BERNHARD, W.R.; KÄGI, H.R. Purification and characterization of atypical cadmium-binding polypeptides from *Zea mays*. **Experientia Supplementum**, Basel, v. 52, p. 309-315, 1987.

BUSTIN, S.A.; BENES, V.; GARSON, J.A.; HELLEMANS, J.; HUGGETT, J.; KUBISTA, M.; MUELLER, R.; NOLAN, T.; PFAFFL, M.W.; SHIPLEY, G.L.; VANDESOMPELE, J.; WITTWER, C.T. The MIQE guidelines: Minimum Information for publication of Quantitative realtime PCR Experiments. **Clinical Chemistry**, Washington, DC, v. 55, p. 611-622, 2009.

CAPALDI, F.R.; GRATÃO, P.L.; REIS, A.R.; LIMA, L.W.; AZEVEDO, R.A. Sulfur metabolism and stress defense responses in plants. **Tropical Plant Biology**, Nova York, v. 8, p. 60-73, 2015.

CHAFFEI, C.; PAGEAU, K.; SUZUKI, A.; GOUIA, H.; GHORBEL, M.H.; MASCLAUX-DAUBRESSE, C. Cadmium toxicity induced changes in nitrogen management in *Lycopersicon esculentum* leading to a metabolic safeguard through an amino acid storage strategy. **Plant and Cell Physiology**, Oxford, v. 45, p. 1681-1693, 2004.

CLEMENS, S.; AARTS, M.G.M.; THOMINE, S.; VERBRUGGEN, N. Plant science: the key to preventing slow cadmium poisoning. **Trends in Plant Science**, London, v. 18, p. 92-99, 2013.

COBBETT, C.; GOLDSBROUGH, P. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. **Annual Review of Plant Biology**, Palo Alto, v. 53, p. 159-182, 2002.

COSTA, G.; SPITZ, E. Influence of cadmium on soluble carbohydrates, free amino acids, proline content of *in vitro* cultured *Lupinus albus*. **Plant Science**, Shannon, v. 128, p. 131-140, 1997.

CUADROS-INOSTROZA, A.; CALDANA, C.; REDESTIG, H.; KUSANO, M.; LISEC, J.; PEÑA-CORTÉS, H.; WILLMITZER, L.; HANNAH, M.A. TargetSearch - a Bioconductor package for the efficient preprocessing of GC-MS metabolite profiling data. **BMC Bioinformatics**, London, v. 10, 2009. doi: 10.1186/1471-2105-10-428.

DUCRUIX, C.; JUNOT, C.; FIÉVET, J.B.; VILLIERS, F.; EZANA, E.; BOURGUIGNON, J. New insights into the regulation of phytochelatin biosynthesis in *A. thaliana* cells from metabolite profiling analyses. **Biochimie**, Paris, v. 88, p. 1733-1742, 2006.

ELLMAN, G. Tissue sulfhydryl groups. **Archives of Biochemistry and Biophysics**, New York, v. 82, p. 70-77, 1959.

EPSTEIN, E.; BLOOM, A.J. **Mineral nutrition of plants**: principles and perspectives. 2. ed. Sunderland: Sinauer Associates, 2005. 380 p.

FERNÁNDEZ, R.; BERTRAND, A.; GARCÍA, J.I.; TAMÉS, R.S.; GONZÁLEZ, A. Lead accumulation and synthesis of non-protein thiolic peptides in selected clones of *Melilotus alba* and *Melilotus officinalis*. **Environmental and Experimental Botany**, Oxford, v. 78, p. 18-24, 2012.

FERNÁNDEZ, R.; FERNÁNDEZ-FUEGO, D.; RODRÍGUEZ-GONZÁLEZ, P.; ALONSO, J.I.G.; BERTRAND, A.; GONZÁLEZ, A. Cd-induced phytochelatin synthesis in *Dittrichia viscosa* (L.) Greuter is determined by the dilution of the culture medium. **Environmental Science and Pollution Research**, Heidelberg, v. 21, p. 1133-1145, 2013.

FRIEDERICH, M.; KNEER, R.; ZENK, M.H. Enzymic synthesis of phytochelatins in gram quantities. **Phytochemistry**, Oxford, v. 49, p. 2323-2329, 1998.

GARMASH, E.V.; GOLOVKO, T.K. Effect of cadmium on growth and respiration of barley plants grown under two temperature regimes. **Russian Journal of Plant Physiology**, New York, v. 56, p. 343-347, 2009.

GIAVALISCO, P.; LI, Y.; MATTHES, A.; ECKHARDT, A.; HUBBERTEN, H.; HESSE, H.; SEGU, S.; HUMMEL, J.; KOHL, K.; WILLMITZER, L. Elemental formula annotation of polar and lipophilic metabolites using ^{13}C, ^{15}N and ^{34}S isotope labelling, in combination with high-resolution mass spectrometry. **Plant Journal**, Hoboken, v. 68, p. 364-376, 2011.

GILL, S.S.; TUTEJA, N. Cadmium stress tolerance in crop plants probing the role of sulfur. **Plant Signaling and Behavior**, Austin, v. 6, p. 215-222, 2011.

GIMENO, J.; EATTOCK, N.; VAN DEYNZE, A.; BLUMWALD, E. Selection and validation of reference genes for gene expression analysis in Switchgrass (*Panicum virgatum*) using quantitative real-time RT-PCR. **PLoS One**, San Francisco, v. 9, 2014. doi: 10.1371/journal.pone.0091474.

GONG, G.X.; LIU, Y.; HUANG, D.; ZENG, G.; LIU, S.; TANG, H.; ZHOU, L.; HU, X.; ZHOU, Y.; TAN, X. Effects of exogenous calcium and spermidine on cadmium stress moderation and metal accumulation in *Boehmeria nivea* (L.). **Environmental Science and Pollution Research**, Heidelberg, v. 23, p. 8699-8708, 2016.

GONZALEZ-MENDOZA, D.; MORENO, A.Q.; ZAPATA-PEREZ, O. Coordinated responses of phytochelatin synthase and metallothionein genes in black mangrove, *Avicennia germinans*, exposed to cadmium and copper. **Aquatic Toxicology**, Amsterdam, v. 83, p. 306-314, 2007.

GRILL, E.; WINNACKER, E.L.; ZENK, M.H. Synthesis of seven different homologuous phytochelatins in metal-exposed *Schizasaccharomyces pombe* cells. **FEBS Letters**, Amsterdam, v. 197, p. 115-120, 1986.

HANSON, A.D.; RATHINASABAPATHI, B.; RIVOAL, J.; BURNET, M.; DILLON, M.O.; GAGE, D.A. Osmoprotective compounds in the Plumbaginaceae: a natural experiment in metabolic engineering of stress tolerance. **Proceedings of the National Academy of Sciences of the USA**, Washington, DC, v. 91, p. 306-310, 1994.

HÉDIJI, H.; DJEBALI, W.; CABASSON, C.; MAUCOURT, M.; BALDET, P.; BERTRAND, A.; ZOGHLAMI, L.B.; DEBORDE, C.; MOING, A.; BROUQUISSE, R.; CHAÏBI, W.; GALLUSCI, P. Effects of long-term cadmium exposure on growth and metabolomic profile of tomato plants. **Ecotoxicology and Environmental Safety**, San Diego, v. 73, p. 1965-1974, 2010.

JOSHI, V.; JOUNG, J.G.; FEI, Z.; JANDER, G. Interdependence of threonine, methionine and isoleucine metabolism in plants: accumulation and transcriptional regulation under abiotic stress. **Amino Acids**, Wien, v. 39, p. 933-947, 2010.

JOZEFCZAK, M.; KEUNEN, E.; SCHAT, H.; BLIEK, M.; HERNÁNDEZ, L.C.; CARLEER, R.; REMANS, T.; BOHLER, S.; VANGRONSVELD, J.; CUYPERS, A. Differential response of *Arabidopsis* leaves and roots to cadmium: Glutathione-related chelating capacity *vs* antioxidant capacity. **Plant Physiology and Biochemistry**, Paris, v. 83, p. 1-9, 2014.

KASUKABE, Y.; HE, L.; NADA, K.; MISAWA, S.; IHARA, I.; TACHIBANA, S. Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. **Plant and Cell Physiology**, Oxford, v. 45, p. 712-722, 2004.

KEUNEN, E.; PESHEV, D.; VANGRONSVELD, J.; van den ENDE, W.; CUYPERS, A. Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. **Plant, Cell and Environment**, Oxford, v. 36, p. 1242-1255, 2013.

KEUNEN, E.; SCHELLINGEN, K.; STRAETEN, D.V.D.; REMANS, T.; COLPAERT, J.; VANGRONSVELD, J.; CUYPERS, A. ALTERNATIVE OXIDASE1a modulates the oxidative challenge during moderate Cd exposure in *Arabidopsis thaliana* leaves. **Journal of Experimental Botany**, Oxford, v. 66, p. 2967-2977, 2015.

KEUNEN, E.; FLOREZ-SARASA, I.; OBATA, T.; JOZEFCZAK, M.; REMANS, T.; VANGRONSVELD, J.; FERNIE, A.R.; CUYPERS, A. Metabolic responses of *Arabidopsis thaliana* roots and leaves to sublethal cadmium exposure are differentially influenced by ALTERNATIVE OXIDASE1a. **Environmental and Experimental Botany**, Oxford, v. 124, p. 64-78, 2016.

KHAN, M.A.; KHAN, S.; KHAN, A.; ALAM, M. Soil contamination with cadmium, consequences and remediation using organic amendments. **Science of the Total Environment**, Amsterdam, v. 601-602, p. 1591-1605, 2017.

KIEFFER, P.; PLANCHON, S.; OUFIR, M.; ZIEBEL, J.; DOMMES, J.; HOFFMANN, L.; HAUSMAN, J.F.; RENAUT, J. Combining proteomics and metabolite analyses to unravel cadmium stress-response in poplar leaves. **Journal of Proteome Research**, Washington, DC, v. 8, p. 400-417, 2008.

KLAPHECK, S. Homoglutathione: isolation, quantification and occurrence in legumes. **Physiologia Plantarum**, Lund, v. 74, p. 727-732, 1988.

KLAPHECK, S.; ZOPES, H.; LEVELS, H.G.; BERGMANN, L. Properties and localization of the homoglutathione synthetase from *Phaseolus coccineus* levels. **Physiologia Plantarum**, Lund, v. 74, p. 733-739, 1988.

KLAPHECK, S.; SCHLUNZ, S.; BERGMANN, L. Synthesis of phytochelatins and homo-phytochelatins in *Pisum sativum* L. **Plant Physiology**, Rockville, v. 107, p. 515-521, 1995.

KROTZ, R.M.; EVANGELOU, B.P.; WAGNER, G.J. Relationships between cadmium, zinc, Cd-peptide, and organic acid in Tobacco suspension cells. **Plant Physiology**, Rockville, v. 91, p. 780-787, 1989.

LI, Y.; YANG, C.M. A rationally designed novel receptor for probing cooperative interaction between metal ions and bivalent tryptophan side chain in solution. **Chemical Communications**, Cambridge, v. 23, p. 2884-2885, 2003.

LISEC, J.; SCHAUER, N.; KOPKA, J.; WILLMITZER, L.; FERNIE, A.R. Gas chromatography mass spectrometry-based metabolite profiling in plants. **Nature Protocols**, London, v. 1, p. 387-396, 2006.

MAJUMDAR, R.; SHAO, L.; MINOCHA, R.; LONG, S.; MINOCHA, S.C. Ornithine: the overlooked molecule in regulation of polyamine metabolism. **Plant and Cell Physiology**, Oxford, v. 54, p. 990-1004, 2013.

MALAVOLTA, E.; VITTI, G.C.; OLIVEIRA, S.A. **Avaliação do estado nutricional das plantas**: princípios e aplicações. 2. ed. Piracicaba: Potafos, 1997. 201 p.

MATYSIK, J.; ALIA; BHALU, B.; MOHANTY, P. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. **Current Science**, Bangalore, v. 82, p. 525-532, 2002.

MENDOZA-CÓZATL, D.G.; BUTKO, E.; SPRINGER, F.; TORPEY, J.W.; KOMIVES, E.A.; KEHR, J.; SCHROEDER, J.I. Identification of high levels of phytochelatins glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. **Plant Journal**, Hoboken, v. 54, p. 249-259, 2008.

MEYER, C.; VERBRUGGEN, N. The use of the model species *Arabidopsis halleri* towards phytoextraction of cadmium polluted soils. **New Biotechnology**, Amsterdam, v. 30, p. 9-14, 2012.

NASIBI, F.; HEIDARI, T.; ASRAR, Z.; MANSOORI, H. Effect of arginine pre-treatment on nickel accumulation and alleviation of the oxidative stress in *Hyoscyamus niger*. **Journal of Soil Science and Plant Nutrition**, Temuco, v. 13, p. 680-689, 2013.

NIEBOER, E.; RICHARDSON, D.H.S. The replacement of the nondescript term ‘heavy metal’ by a biologically and chemically significant classification of metal ions. **Environmental Pollution Series B**, London, v. 1, p. 2-26, 1980.

NIKIFOROVA, V.J.; BIELECKA, M.; GAKIÈRE, B.; KRUEGER, S.; RINDER, J.; KEMPA, S.; MORCUENDE, R.; SCHEIBLE, W.R.; HESSE, H.; HOEFGEN, R. Effect of sulfur availability on the integrity of amino acid biosynthesis in plants. **Amino Acids**, Wien, v. 30, p. 173-183, 2006.

NISHIZAWA, A.; YABUTA, Y.; SHIGEOKA, S. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. **Plant Physiology**, Rockville, v. 147, p. 1251-1263, 2008.

NOCITO, F.F.; LANCILLI, C.; CREMA, B.; FOURCOY, P.; DAVIDIAN, J.C.; SACCHI, G.A. Heavy metal stress and sulfate uptake in maize roots. **Plant Physiology**, Rockville, v. 141, p. 1138-1148, 2006.

NOCTOR, G.; MHAMDI, A.; CHAOUCH, S.; HAN, Y.; NEUKERMANS, J.; MARQUEZ-GARCIA, B.; QUEVAL, G.; FOYER, C.H. Glutathione in plants: an integrated overview. **Plant, Cell and Environment**, Oxford, v. 35, p. 454-484, 2012.

OBATA, T.; FERNIE, A.R. The use of metabolomics to dissect plant responses to abiotic stresses. **Cellular and Molecular Life Sciences**, Basel, v. 69, p. 3225-3243, 2012.

OVEN, M.; RAITH, K.; NEUBERT, R.H.H.; KUTCHAN, T.M.; ZENK, M.H. Homo-phytochelatins are synthesized in response to cadmium in Azuki beans. **Plant Physiology**, Rockville, v. 126, p. 1275-1280, 2001.

OZEN, S.A.; YAMAN, M. Examination of correlation between histidine, sulfur, cadmium, and cobalt absorption by *Morus* L., *Robinia pseudoacacia* L., and *Populus nigra* L. **Communications in Soil Science and Plant Analysis**, New York, v. 48, p. 1212-1220, 2017.

RABÊLO, F.H.S.; BORGO, L. Changes caused by heavy metals in micronutrient content and antioxidant system of forage grasses used for phytoremediation: an overview. **Ciência Rural**, Santa Maria, v. 46, p. 1368-1375, 2016.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. Proper supply of S increases GSH synthesis in the establishment and reduces tiller mortality during the regrowth of Tanzania guinea grass used for Cd phytoextraction. **Journal of Soils and Sediments**, Heidelberg, v. 17, p. 1427-1436, 2017a.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. The proper supply of S increases amino acid synthesis and antioxidant enzyme activity in Tanzania guinea grass used for Cd phytoextraction. **Water, Air and Soil Pollution**, Dordrecht, v. 228, 2017b. doi: 10.1007/s11270-017-3563-6.

RABÊLO, F.H.S.; JORDÃO, L.T.; LAVRES, J. A glimpse into the symplastic and apoplastic Cd uptake by Massai grass modulated by sulfur nutrition: Plants well-nourished with S as a strategy for phytoextraction. **Plant Physiology and Biochemistry**, Paris, v. 121, p. 48-57, 2017.

REMANS, T.; SMEETS, K.; OPDENAKKER, K.; MATHIJSEN, D.; VANGRONSVELD, J.; CUYPERS, A. Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. **Planta**, Berlin, v. 227, p. 1343-1349, 2008.

REMANS, T.; KEUNEN, E.; JAN BEX, G.; SMEETS, K.; VANGRONSVELD, J.; CUYPERS, A. Reliable Gene Expression Analysis by Reverse TranscriptionQuantitative PCR: Reporting and Minimizing the Uncertainty in Data Accuracy. **The Plant Cell**, Rockville, v. 26, p. 3829-3837, 2014.

RICE-EVANS, C.A.; MILLER, N.J.; PAGANGA, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. **Free Radical Biology and Medicine**, New York, v. 20, p. 933-956, 1996.

ROSCHZTTARDTZ, H.; SEGUELA-ARNAUD, M.; BRIAT, J.F.; VERT, G.; CURIE, C. The FRD3 citrate effluxer promotes iron nutrition between symplastically disconnected tissues throughout *Arabidopsis* development. **The Plant Cell**, Rockville, v. 23, p. 2725-2737, 2011.

ROZEN, S.; SKALETSKY, H. ***Primer3 on the WWW for general users and for biologist programmers***. In: MISENER, S.; KRAWETZ, S.A. (Ed.). **Bioinformatics methods and protocols**. Totowa: Humana Press, 2000. chap. 20, p. 365-386.

SANITÀ DI TOPPI, L.; GABBRIELLI, R. Response to cadmium in higher plants. **Environmental and Experimental Botany**, Oxford, v. 41, p. 105-130, 1999.

SAS INSTITUTE. **SAS User's guide**: Statistics. Version 9.2. Cary, NC, 2008. Available at: <http://support.sas.com/software/92/ Cary-United States of America>. Access in: oct. 25. 2017.

SEHT, C.S.; REMANS, T.; KEUNEN, E.; JOZEFCZAK, M.; GIELEN, H.; OPDENAKKER, K.; WEYENS, N.; VANGRONSVELD, J.; CUYPERS, A. Phytoextraction of toxic metals: a central role for glutathione. **Plant, Cell and Environment**, Oxford, v. 35, p. 334-346, 2012.

SHARMA, S.S.; DIETZ, K. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. **Journal of Experimental Botany**, Oxford, v. 57, p. 711-726, 2006.

SHEORAN, V.; SHEORAN, A.S.; POONIA, P. Factors affecting phytoextraction: a review. **Pedosphere**, Beijing, v. 26, p. 148-166, 2016.

STACKLIES, W.; REDESTIG, H.; SCHOLZ, M.; WALTHER, D.; SELBIG, J. pcaMethods a bioconductor package providing PCA methods for incomplete data. **Bioinformatics**, Oxford, v. 23, p. 1164-1167, 2007.

SUN, X.; ZHANG, J.; ZHANG, H.; NI, Y.; ZHANG, Q.; CHEN, J.; GUAN, Y. The responses of *Arabidopsis thaliana* to cadmium exposure explored via metabolite profiling. **Chemosphere**, Oxford, v. 78, p. 840-845, 2010.

van DONGEN, J.T.; FRÖHLICH, A.; RAMÍREZ-AGUILAR, S.J.; SCHAUER, N.; FERNIE, A.R.; ERBAN, A.; KOPKA, J.; CLARK, J.; LANGER, A.; GEIGENBERGER, P. Transcript and metabolite profiling of the adaptive response to mild decreases in oxygen concentration in the roots of *Arabidopsis* plants. **Annals of Botany**, Oxford, v. 103, p. 269-280, 2009.

VÁZQUEZ, S.; GOLDSBROUGH, P.; CARPENA, R.O. Assessing the relative contributions of phytochelatins and the cell wall to cadmium resistance in white lupin. **Physiologia Plantarum**, Lund, v. 128, p. 487-495, 2006.

VILLIERS, F.; DUCRUIX, C.; HUGOUVIEUX, V.; JARNO, N.; EZAN, E.; GARIN, J.; JUNOT, C.; BOURGUIGNON, J. Investigating the plant response to cadmium exposure by proteomic and metabolomic approaches. **Proteomics**, Weinheim, v. 11, p. 1650-1663, 2011.

WECKWERTH, W.; WENZEL, K.; FIEHN, O. Process for the integrated extraction, identification and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks. **Proteomics**, Weinheim, v. 4, p. 78-83, 2004.

WINTER, G.; TODD, C.D.; TROVATO, M.; FORLANI, G.; FUNCK, D. Physiological implications of arginine metabolism in plants. **Frontiers in Plant Science**, Lausanne, v. 6, 2015. doi: 10.3389/fpls.2015.00534.

WÓJCIK, M.; TUKENDORF, A. Cd - tolerance of maize, rye and wheat seedlings. **Acta Physiologia Plantarum**, Heidelberg, v. 21, p. 99-107, 1999.

WHO. **Evaluation of certain food additives and contaminants: seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives**. Geneva: Joint FAO/WHO Expert Committee on Food Additives, 2011. 228 p.

XIE, Y.; HU, L.; DU, Z.; SUN, X.; AMOMBO, E.; FAN, J.; FU, J. Effects of cadmium exposure on growth and metabolic profile of Bermudagrass [*Cynodon dactylon* (L.) Pers.]. **PLoS One**, San Francisco, v. 9, 2014. doi: 10.1371/journal.pone.0115279.

XIONG, Y.; McCORMACK, M.; LI, L.; HALL, Q.; XIANG, C.; SHEEN, J. Glc-TOR signalling leads transcriptome reprogramming and meristem activation. **Nature**, London, v. 496, p. 181-186, 2013.

XU, J.; ZHU, Y.; GE, Q.; LI, Y.; SUN, J.; ZHANG, Y.; LIU, X. Comparative physiological responses of *Solanum nigrum* and *Solanum torvum* to cadmium stress. **New Phytologist**, London, v. 196, p. 125-138, 2012.

ZEMANOVÁ, V.; PAVLÍK, M.; PAVLÍKOVÁ, D.; TLUSTOŠ, P. The significance of methionine, histidine and tryptophanin plant responses and adaptation to cadmium stress. **Plant, Soil and Environment**, Prague, v. 60, p. 426-432, 2014.

ZEMANOVÁ, V.; PAVLÍK, M.; PAVLÍKOVÁ, D. Cadmium toxicity induced contrasting patterns of levels of free sarcosine, specific amino acids and selected microelements in two *Noccaea* species. **PLoS One**, San Francisco, v. 12, 2017. doi: 10.1371/journal.pone.0177963.

ZHANG, W.; LIN, K.; ZHOU, J.; ZHANG, W.; LIU, L.; HAN, X. Spatial distribution and toxicity of cadmium in the joint presence of sulfur in rice seedling. **Environmental Toxicology and Pharmacology**, Amsterdam, v. 36, p. 1235-1241, 2013.

6 PROPER SULFUR-SUPPLY NOT IMPROVES THE PERFORMANCE OF ASCORBATE-GLUTATHIONE CYCLE AGAINST CADMIUM-INDUCED STRESS BUT INCREASES THE PHOTOSYNTHETIC RATE OF MASSAI GRASS USED FOR Cd PHYTOEXTRACTION

Abstract

Cadmium (Cd) can cause oxidative stress, halt metabolism, injuries on the photosynthetic apparatus of plants and exhibit phytotoxity when this metal is taken up. However, plants grown with proper sulfur (S) supply can be more tolerant to Cd, since this nutrient is component of antioxidants and photosynthetic system. Therefore, our aim with this study was to evaluate the effect of S supply (0.1, 1.9 and 3.7 mmol L^{-1}) on the antioxidant (ascorbate-glutathione cycle) and photosynthetic system of *Panicum maximum* cv. Massai (Massai grass) exposed to Cd (0.0, 0.1 and 0.5 mmol L^{-1}) as a prerequisite for studies of Cd phytoextraction. Plants supplied with 3.7 mmol L^{-1} S showed the lowest growth and the highest Cd concentrations when Massai grass was exposed to 0.5 mmol L^{-1} Cd. However, Massai grass grown without S deprivation showed the highest glutathione (GSH + GSSG) concentrations in all tissues and the lowest lipid peroxidation in the stems and sheaths and roots. In general, the activities of enzymes of ascorbate-glutathione cycle did not increase in plants exposed to Cd, even with proper S supply, probably because the expression of the genes involved in the synthesis of these antioxidants did not increase either. High cadmium exposure halted the photosynthetic system of Massai grass, although well-nourished plants in S show greatest photosynthetic rate due to highest rafinose concentration. In conclusion, Massai grass well-nourished in S showed tolerance to Cd concentration of 0.1 mmol L^{-1}.

Key words: antioxidants, chloroplasts, forage grasses, gene expression, *Panicum maximum*.

6.1 Introduction

Currently, agriculture soils of several countries shows high cadmium (Cd) concentrations due mainly to anthropogenic activities (representing more than 90% of Cd added to the environment), such as the inappropriate disposal of municipal and industrial waste and the use of fertilizers (e.g. phosphate fertilizer) of poor quality (KHAN et al., 2017). This fact is a serious socio-environmental problem since Cd is toxic for plants, animals and humans (KHAN et al., 2017; HE et al., 2017). One of the main consequences induced by Cd

stress in plants is the increase in the production of reactive oxygen species (ROS), which results in oxidative damages that induce lipid peroxidation (GRATÃO et al., 2005; CUYPERS et al., 2010; LOIX et al., 2017), as observed in the leaves of *Panicum virgatum* (TAI et al., 2017). Lipid peroxidation can cause the rupture of tonoplast and change the structure and amount of chloroplasts in the cells (PARMAR; KUMARI; SHARMA, 2013). In addition, Cd can cause stomatal closure resulting in lowest intracellular CO_2 concentration (c_i) and disturbances in the plant water relations, such as degrade chlorophyll and decrease the activity of Calvin cycle enzymes, resulting in lowest photosynthetic rate (*A*) (OUZOUNIDOU; MOUSTAKAS; ELEFTHERIOU, 1997; PARMAR; KUMARI; SHARMA, 2013). Xie et al. (2014) related that chlorophyll concentration in *Cynodon dactylon* exposed to Cd was lower than in plants not exposed to Cd, while Zhang, Gao and Xia (2014) reported that *Vetiveria zizanoides* exposed to Cd showed low *A*.

To scavenge ROS and decrease the damage caused by Cd to the photosynthetic apparatus, the plants increase the synthesis of enzymatic and non-enzymatic antioxidants, mainly of the ascorbate-glutathione cycle (GRATÃO et al., 2005; LOIX et al., 2017). Among the enzymes involved in this cycle, superoxide dismutase (SOD) acts on dismutation of superoxide (O_2^-) in hydrogen peroxide (H_2O_2), while ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (GPOX) reduce H_2O_2 to H_2O (CUYPERS et al., 2010). The action of CAT and GPOX on the reduction of H_2O_2 depends of glutathione reductase (GR), which restores the oxidized glutathione (GSSG) by GPOX to reduced glutathione (GSH) and dehydroascorbate reductase (DHAR), which restores the dehydroascorbate (DHA) generated by APX to ascorbate (GRATÃO et al., 2005). Tai et al. (2017) noted that *P. virgatum* grown with Cd showed lower *A*, stomatal conductance (g_s) and c_i and higher activity of SOD and CAT in relation to plants not exposed to Cd. High antioxidant enzymes activities indicate adaptive response of plants to Cd stress, as well as high expression of genes that encoding enzymes of the ascorbate-glutathione cycle (KULAEVA; TSYGANOV, 2011). Luo et al. (2011) noted that expression of genes encoding APX, GR and the isoforms of SOD (Mn-SOD and Cu/Zn-SOD) in the leaves of *Lolium perenne* exposed to Cd increased in order to decrease the oxidative damages caused by Cd in these plants. Another typical adaptation response of plants to Cd stress is related to increase of GSH synthesis (NOCTOR et al., 2012), as verified in the shoots of *Panicum maximum* cv. Tânzania by Rabêlo, Azevedo and Monteiro (2017a). Glutathione reduced binds to products that may cause oxidative stress (e.g. hydroxyl - OH^-) in a reaction catalyzed by glutathione-S-transferase (GST) enzyme, which makes this peptide the main non-enzymatic antioxidant of plants (NOCTOR et al., 2012).

In this sense, plants well nourished in sulfur (S) may be more tolerant to Cd, since S is a component of cysteine, which in turn is a component of GSH (γ-Glu-Cys-Gly) (GILL; TUTEJA, 2011; HENDRIX et al., 2017).

Sulfur incorporation into thiol compounds such as GSH and phytochelatins (PCs) [$(\gamma\text{-Glu-Cys})_n$-Gly, where n = 2-11] is crucial for plant defense against oxidative stress induced by Cd (GILL; TUTEJA, 2011). Bashir et al. (2015) reported that GSH and PCs concentrations and the activity of SOD, CAT and APX in *Brassica juncea* exposed to Cd increased with proper S supply, which was important to mitigate the oxidative damages caused by Cd. Rabêlo, Azevedo and Monteiro (2017b) also observed that APX activity in the shoots of *P. maximum* cv. Tânzania grown with Cd increased when there was proper S supply. Sulfur is also involved in the synthesis of essential phytohormones (e.g. ethylene) for plant adaptation to Cd stress (CAPALDI et al., 2015; KHAN et al., 2015). In addition, S acts directly in the electron transport system and in the formation of photosynthetic apparatus as a component of iron-sulfur (Fe-S) proteins and thioredoxin system, and acts indirectly in the chlorophyll synthesis, since the membranes of chloroplasts are composed by galactolipids and sulfolipids (IMSANDE, 1998). Bagheri et al. (2017) described that GSH, GSSG and DHA concentrations and the activity of SOD, CAT, APX and GR in the leaves of *Spinacia oleracea* plants exposed to Cd increased with proper S supply in relation to plants grown with S deprivation, which allowed these plants to present highest chlorophyll concentration. Khan et al. (2015) noted that *Triticum aestivum* exposed to Cd showed lowest ethylene synthesis and highest activity of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), A, g_s, c_i, leaf area and biomass production when plants were well nourished in S.

In view of this context it is clear that cellular demand for S is correlated with the mechanisms of adaptation to Cd, such as it is evident the need to reduce Cd concentration in contaminated soils in order to reduce the damage caused to plants and the food contamination (GILL; TUTEJA, 2011; KHAN et al., 2017). It is important to mention that when Cd is taken up by plants, this heavy metal can enter the human food chain and cause a series of diseases such as pulmonary emphysema and cancer (KHAN et al., 2017). In order to minimize this serious socio-environmental problem, several technologies have been used to reduce Cd concentration in contaminated soils, with emphasis for phytoextraction, which show low cost and environmental impact (SHEORAN; SHEORAN; POONIA, 2016). However, few plants can uptake high amounts of Cd due to their toxicity (REEVES et al., 2017), stimulating the study of plants normally used for other purposes (e.g. animal feed), such as forage grasses. Forage grasses show desirable characteristics for Cd phytoextraction, such as high biomass

production, fast growth, extensive root system and tolerance to soil and climatic adversities, mainly when these plants are grown with proper S supply (RABÊLO; BORGO, 2016; SHEORAN; SHEORAN; POONIA, 2016; RABÊLO; AZEVEDO; MONTEIRO, 2017a, 2017b; RABÊLO; JORDÃO; LAVRES, 2017). Therefore, our aim with this study was to evaluate the effect of low, medium and high S supply on the antioxidant (ascorbate-glutathione cycle) and photosynthetic system of *P. maximum* cv. Massai (Massai grass) exposed to Cd as a prerequisite for studies of Cd phytoextraction.

6.2 Materials and methods

6.2.1 Plant material and treatments

The study of S effect on the attenuation of Cd damage in *Panicum maximum* Jacq. cv. Massai was conducted in a hydroponic system using plastic pots containing 2 L of nutrient solution arranged in a greenhouse (22º42' south latitude and 47º38' west longitude). The treatments were represented by combinations of three S concentrations (0.1, 1.9 and 3.7 mmol L^{-1}) and two Cd concentrations (0.1 and 0.5 mmol L^{-1}), in nutrient solutions modified from solution of Epstein and Bloom (2005). The composition and chemical speciation of nutrient solution used in the study is shown on pages 78 and 79, respectively. The pots used in the study were placed in a randomized block design, with four replicates.

6.2.2 Massai grass growth and plant material collect to analyses

To germinate, seeds were placed in a tray containing expanded vermiculite, which was irrigated with deionized water in the first 14 days and modified nutrient solution to provide 0.1 mmol L^{-1} S (diluted to 25% ionic strength) over the following nine days. After 23 days, five seedlings (± 10 cm high) were transplanted to each pot containing the undiluted nutrient solutions (100% ionic strength) modified to provide only different S concentrations for 21 days. After this period, modified nutrient solutions were provided to obtain different S and Cd concentrations (100% ionic strength) during nine days. Solutions were replaced every week and remained constantly aerated. The average temperature in the greenhouse was 30.5 ºC, with a relative air humidity of 60.5% (more details on page 77).

During the last day of exposure of Massai grass to S and Cd we count the number of tillers and leaves and measured some photosynthetic parameters (chlorophyll concentration, A, g_s, c_i, carboxylation efficiency - k, instantaneous water use efficiency - WUE and intrinsic water use efficiency - WUE_i). We also collect samples (middle part) from the first newly expanded leaf of plants grown with 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd,

1.9 mmol L^{-1} S + 0.5 mmol L^{-1} Cd and 3.7 mmol L^{-1} S + 0.5 mmol L^{-1} Cd in order to analyze the anatomical and ultrastructural changes occurred in this tissue. Then, one plant of each pot was used to determine the leaf area, specific leaf area and dry mass production, two plants were used to determine S and Cd concentration and two plants were used to analyze compounds of ascorbate-glutathione cycle and gene expression. After harvesting, shoots were separated into leaf blades and a sample comprising both stems and sheaths. All samples were snap frozen in liquid nitrogen and stored at -80 °C until further analyses. It is important to note that biomass production of plants exposed to 0.5 mmol L^{-1} Cd was very low, so that the analyses involving the antioxidant system were not performed in these plants.

6.2.3 Determination of plant growth inhibition

The dry mass production was obtained after weighing the plant material, which remained in forced air ventilation oven at 60 ºC for 72 h. Then, with dry mass production data, we calculated the plant growth inhibition (%) relative to control treatment (1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd) as described in equation 1.

$$\text{Plant growth inhibition } (\%) = \left[\left(\frac{\text{dry mass production in the S and Cd treatment} \times 100}{\text{dry mass production in the control treatment}} \right) - 100 \right] \quad (1)$$

6.2.4 Determination of S and Cd concentrations

After drying in a forced air ventilation oven at 60 °C during 72 h, plant material was ground in a Wiley type mill. Sulfur (MALAVOLTA; VITTI; OLIVEIRA, 1997) and Cd concentrations were determined after nitric-perchloric digestion (HNO_3 65% and $HClO_4$ 70%), by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7000 SERIES, ThermoScientific, Waltham, USA).

6.2.5 Determination of H_2O_2 and malondialdehyde (MDA) concentrations

The measurement of H_2O_2 concentration was performed in the leaf blades, stems and sheaths and roots of plants, following the method described by Alexieva et al. (2001), with modifications. Firstly, 0.2 g of frozen samples was macerated in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) in the presence of 20% (w/w) of polyvinyl polypyrrolidone (PVPP). After complete homogenization, 1.4 mL of extract was centrifuged at 10,000 rpm for 5 min at 4 °C. An aliquot of 0.2 mL was withdrawn from supernatant, and then in this aliquot

was added 0.2 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 7.0) and 0.8 mL of 1 mol L^{-1} potassium iodide. Then, the solution was left for 1 h in the dark for stabilizing the reaction and the readings were taken in a spectrophotometer at 390 nm. Three independent replicates from each sample were used.

Lipid peroxidation was also determined in the leaf blades, stems and sheaths and roots of Massai grass, where metabolites that were reactive to 2-thiobarbituric acid (TBA) were used to estimate the MDA concentration (HEATH; PACKER, 1968). The initial procedures for MDA measurements were the same for H_2O_2 measurements as described above. Following centrifugation, 0.25 mL of supernatant was added to 1 mL of 20% (w/v) TCA containing 0.5% TBA. The mixture was placed in a water bath at 95 °C for 30 min and then on ice. After 20 min on ice, the samples were centrifuged at 10,000 rpm for 10 min in order to separate some residue formed during heating and to clarify the samples. Readings at 535 and 600 nm were measured using a spectrophotometer, and MDA concentration was determined using the following equation 2 (ALCÂNTARA et al., 2015). Three independent replicates from each sample were used.

$$\text{Concentration} = \left(\frac{\text{Absorbance}\,(535 - 600)}{155{,}000} \right) \times 10^3 \qquad (2)$$

6.2.6 Determination of GSH and GSSG concentrations

The concentrations of GSH and GSSG in the leaf blades, stems and sheaths and roots of Massai grass were determined by spectrophotometry, according to Anderson (1985), with some modifications. The fresh tissue (100 mg) was homogenized with 1 mL of 50 mmol L^{-1} sulfosalicylic acid and centrifuged at 10,000 rpm for 20 min at 4 °C. Then, 0.2 mL of supernatant was added to 1.8 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 7.0) containing 0.5 mmol L^{-1} ethylene diamine tetraacetic acid (EDTA) and 100 μL of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The mixture was kept in the dark for 5 min and then taken to spectrophotometer at 412 nm to measure GSH concentration. Then, were added to mixture 100 μL of 0.4 mmol L^{-1} nicotinamide adenine dinucleotide phosphate (NADPH) and 2 μL of GR (205 units/mg). The mixture was kept in the dark for 20 min and taken again to spectrophotometer at 412 nm to measure GSH + GSSG concentration. Concentration of GSSG was obtained by difference between GSH + GSSG and GSH values. Then, with GSH and GSSG data we calculate the redox state (GSH/GSSG).

6.2.7 Determination of DHA concentration

Polar metabolites were extracted from 50 mg of plant material (leaf blades, stems and sheaths and roots), following the method described by Giavalisco et al. (2011). After extraction, samples were derivatized (LISEC et al., 2006) and analyzed by high performance gas chromatography with mass spectrometer (GC-TOF-MS, LECO Instruments, St. Joseph, USA). The used acquisition parameters of chromatograms were identical to those described by Weckwerth, Wenzel and Fiehn (2004). Chromatograms were exported from Leco ChromaTOF v. 3.25 to statistical program R-2.12.2 (https://cran.r-project.org/bin/windows/base/old/2.12.2/). Peak detection, alignment of retention times and library searching were performed using the TargetSearch package of Bioconductor (CUADROS-INOSTROZA et al., 2009). Dehydroascorbate (DHA) were quantified by peak intensity of selected mass, normalized by fresh weight of samples, followed by sum of peak intensities (total ion count) and logarithmic transformation in base 2.

6.2.8 Extraction and quantification of proteins

The samples were homogenized in 100 mmol L^{-1} potassium phosphate buffer (pH 7.5) (ratio of 1 g of leaf blade and stems and sheaths tissues to 3 mL of buffer and 1 g of root tissue to 2 mL of buffer) containing 1 mmol L^{-1} EDTA, 3 mmol L^{-1} dithiothreitol (DTT) and 4% (w/v) PVPP (AZEVEDO et al., 1998). The homogenate was centrifuged at 10,000 rpm for 30 min at 4 °C, and the supernatant was stored in 0.2 mL aliquots at -80 °C for antioxidant activity determination. Total protein concentration was measured by method of Bradford (1976), using bovine serum albumin (BSA) as standard.

6.2.9 Determination of enzymatic activity

The activity of SOD was carried out under non-denaturing conditions in 14% polyacrylamide gels using 30 μg of proteins for electrophoretic separation, with constant current per plate of 20 mA for 6 h, according to Laemmli (1970), without sodium dodecyl sulfate (SDS). Gel was developed following the method of Beauchamp and Fridovich (1971), with modifications of Azevedo et al. (1998). Gel was rinsed in deionized water and then incubated at room temperature in the dark for 30 min in 50 mmol L^{-1} potassium phosphate buffer (pH 7.8), 1 mmol L^{-1} EDTA, 0.05 mmol L^{-1} riboflavin, 0.1 mmol L^{-1} tetrazolium-nitrobenzyl chloride (NBT) and 0.3% N,N,N′,N′-tetramethylethylenediamine (TEMED). At the end of this period, the reaction mixture was poured off, the gel rinsed with deionized water and then illuminated in water until the development of colourless bands of

SOD. For the bands fixation, the gels were immersed in 7% (v/v) acetic acid solution for 15 min. After this step, the developed gels were scanned by ImageJ software, version 1.47 (National Institutes of Health, Bethesda, USA; http://imagej.nih.gov/ij), and the relative SOD activity was calculated based on the activity of standard in the gel (bovine SOD, Sigma Chemical Co., St. Louis, USA), according to Alcântara et al. (2015). Then, the isoforms were classified as Mn-SOD, Fe-SOD or Cu/Zn-SOD, according to Azevedo et al. (1998).

Catalase activity was determined according to method described by Kraus, McKersie and Fletcher (1995), with modifications of Azevedo et al. (1998). The reaction medium was composed of 1 mL of 30 mmol L^{-1} H_2O_2 solution in 100 mmol L^{-1} potassium phosphate buffer (pH 7.5). The reaction started with the addition of 25 μL of plant extract and the activity was determined following the decomposition of H_2O_2 at 10 s intervals for 1 min at the absorbance at 240 nm.

Ascorbate peroxidase activity was determined as described by Nakano and Asada (1981). The reaction medium was composed of 15 μL of sample extract and 80 mmol L^{-1} potassium phosphate buffer solution (pH 7.0) containing 5 mmol L^{-1} ascorbate, 1 mmol L^{-1} EDTA and 1.45 mmol L^{-1} H_2O_2. The reading was performed for 1 min in a spectrophotometer at 290 nm and the enzymatic activity determined using the molar extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Guaiacol peroxidase activity was measured as described by Matsuno and Uritani (1972). The assay was conducted in a reaction mixture containing 0.78 mL of phosphate-citrate buffer (0.2 mmol L^{-1} dibasic sodium phosphate solution and 0.1 mmol L^{-1} citric acid, pH 5.0), 50 μL of 0.5% guaiacol and 25 μL of protein. The mixture was incubated at 30 °C for 15 min and then placed on ice with addition of 50 μL of 2% sodium metabisulfite solution. After this step, the mixture was allowed to stand for 10 min for reading in spectrophotometer at 450 nm.

Glutathione reductase activity was determined following the method described by Smith, Vierheller and Thorne (1988), with modifications of Azevedo et al. (1998). The reaction medium (1 mL) was composed of 1 mmol L^{-1} DTNB; 1 mmol L^{-1} GSSG, 0.1 mmol L^{-1} NADPH and 50 μL protein in 0.1 mmol L^{-1} potassium phosphate buffer solution (pH 7.5). The reading was performed during 1 min at 412 nm.

Glutathione-S-transferase activity was determined as proposed by Habig, Pabst and Jakoby (1974), with modifications. The determination was conducted at 30 °C in a reaction mixture containing 0.9 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 6.5), 50 μL of 100 mmol L^{-1} GSH (analytical product), 25 μL of 40 mmol L^{-1} 2,4-dinitrochlorobenzene (CDNB) and 25 μL of protein. Then, the absorbance measurement was initiated due to

formation of GSH-CDNB conjugate. The reading was performed in a spectrophotometer at 340 nm, carrying out readings every 10 s during 2 min, and the enzymatic activity determined using the molar extinction coefficient of 9.6 mmol cm^{-1} (HABIG; JAKOBY, 1981).

6.2.10 Gene expression analyses related to antioxidant enzymes biosynthesis

The expression of *CSD2, CSD3, FSD2, MSD1*, *APX1*, *GR1*, *GR2*, *CAT1* and *CAT2* genes in the leaf blades of plants was measured by reverse transcription PCR real-time (RT-qPCR) as described by Keunen et al. (2015). We also tried to determine the gene expression of these genes in the stems and sheaths and roots, but we could not get suitable primers to do this. Extraction of RNA was performed using the RNAqueous® Total RNA Isolation Kit (Ambion, Life Technologies, Merelbeke, Belgium), followed by treatment with DNAse I Kit (Invitrogen, ThermoScientific, Waltham, USA). Then, RNA extracted was quantified in RNAse-free PCR tubes containing QuantiFluor® RNA System (Promega Corp., Madison, USA) and 1 μL of RNA samples by a portable fluorometer (Quantus™ Fluorometer, Promega Corp., Madison, USA). Random primers and SuperScript™ III RT Kit (Invitrogen) were used to convert RNA into cDNA. After obtaining cDNA, new concentration measurements were carried out in RNase-free PCR tubes containing QuantiFluor® dsDNA System (Promega Corp.) and 1 μL of cDNA samples using the Quantus Fluorometer™. After this step, cDNA was diluted 10 times in 1/10 diluted Tris-EDTA (TE) buffer (Tris-HCl 1 mmol L^{-1}, Na_2-EDTA 0.1 mmol L^{-1}, pH 8.0) and subsequently stored at -20 °C.

Real-time PCR quantification was performed in 96-well optical plates using the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Gent, Belgium) and the Fast SYBR® Green Master Mix (Applied Biosystems). Amplification occurred under universal cycling conditions (20 s at 95 ºC, 40 cycles of 3 s at 95 ºC and 30 s at 60 ºC), followed by the generation of a dissociation curve to verify amplification specificity. Forward and reverse primers (300 nmol L^{-1}) were designed and optimized for Massai grass (Table 1) using Primer3 (ROZEN; SKALETSKY, 2000). Then, gene expression data were calculated by the $2^{-\Delta Cq}$ method in relation to the sample with the highest expression (minimum Cq). All data were normalized using the expression of three stable reference genes (REMANS et al., 2008) for *P. virgatum* (Table 2, p. 113; GIMENO et al., 2014) selected using the GrayNorm (REMANS et al., 2014). All procedures were performed according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines (BUSTIN et al., 2009) as described in the Table 3, p. 114.

Table 1 - Forward and reverse primers (300 nmol L^{-1}) designed and optimized for Massai grass

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Primer efficiency
CSD2	ACCTTCACGAGTTTGGCGAT	TGGCGGACTTCATCTTCTGG	103	98%
CSD3	GCTGGAGAAGATGGTGTTGC	CTGGATCGGCATGGACAACT	106	105%
FSD2	CAGCAGCCACTTCCCTATCC	TCATGCCGTTGAGCCTATCC	115	85%
MSD1	GATGGGTGTGGCTTGCTTTG	ACATTGTCGTACACCTCTCCG	148	97%
APX1	ATTGTTGCCCTCTCTGGTGG	GCCCTCTTTGTCACCACTCA	138	88%
GR1	CCTTCTGCTGTGTTCTCCCA	AGAGAGAGTGGCCCTGAGAG	126	103%
GR2	CCTTCTGCTGTGTTCTCCCA	GAGTGGCTTTGAGAGGTCTGA	121	117%
CAT1	TCAAGAAGGAGAACAACTTCAAGC	GGACAGCGCATCGATCCAT	101	110%
CAT2	CCAACGACATCTCCCACCTC	TAGAACTTGACGGCGAACCC	148	111%

6.2.11 Light and transmission electron microscopy

Anatomical and ultrastructural analyses of transversal sections of leaf fragments of first newly expanded leaf of plants were analyzed by light and transmission electron microscopy. The samples were fixed in a modified Karnovsky solution (KARNOVSKY, 1965) (2% glutaraldehyde, 2% paraformaldehyde in 0.05 mol L^{-1} sodium cacodylate buffer, pH 7.2), for 48 h, at 4 °C. Then, the samples were rinsed in 0.1 mol L^{-1} sodium cacodylate buffer and post-fixed for 1 h with 1% osmium tetroxide in the same buffer. The post-fixed samples were then dehydrated in a graded acetone series and embedded in Spurr epoxy resin (EMS, Electron Microscopy Sciences), for 48 h. Semi-thin sections (120-200 nm) were collected in glass slides, stained with 2.5% toluidine blue for 5 min, rinsed in distilled water and air-dried. The sections were permanently mounted in Entellan resin, observed and documented using an upright light microscope (AxioPlan, Zeiss, Jena, Germany). Ultra-thin sections (60-90 nm) were obtained on an ultra-microtome (Sorvall Porter Blum MT2, Norwalk, USA), placed on copper grids (300 mesh) and stained with 2.5% uranyl acetate for 20 min followed by lead citrate (REYNOLDS, 1963) for 12 min to be examined under electron microscope JEM 1400 (JEOL Co. Ltd, Tokyo, Japan) operating at 80 kV.

6.2.12 Determination of morphological and photosynthetic parameters

Leaf area was determined in leaf area integrator model LI 3100 (Li-Cor Inc., Lincoln, USA). The specific leaf area was obtained by quotient between the leaf area and the dry mass production of leaf blades (WITKOWSKI; LAMONT, 1991). Chlorophyll concentration was measured on the first newly expanded leaves of plants through of Chlorophyll Meter SPAD-502 (Soil-Plant Analysis Dev., Section, Minolta Camera Co., Osaka, Japan). The measurement of photosynthetic parameters (A, g_s and c_i) was performed on the first

newly expanded leaf of the same plant used to determine the chlorophyll concentration using an infrared gas analyzer (Li-6400, Li-cor Inc.). The parameters were measured under photosynthetic photon flux density of 1,400 μmol m^{-2} s^{-1}, air CO_2 concentration of 400 ± 20 μmol mol^{-1}, and natural conditions of temperature and humidity. The measurements were performed between 12:00 and 14:00 h, following the procedures recommended by Long and Bernacchi (2003). Then, the *k* ($k = A/c_i$) (FARQUHAR; SHARKEY, 1982), WUE (WUE = *A*/transpiration rate) and WUE_i ($WUE_i = A/g_s$) (WANG; WANG; SHANGGUAN, 2016) were calculated.

6.2.13 Statistical analyses

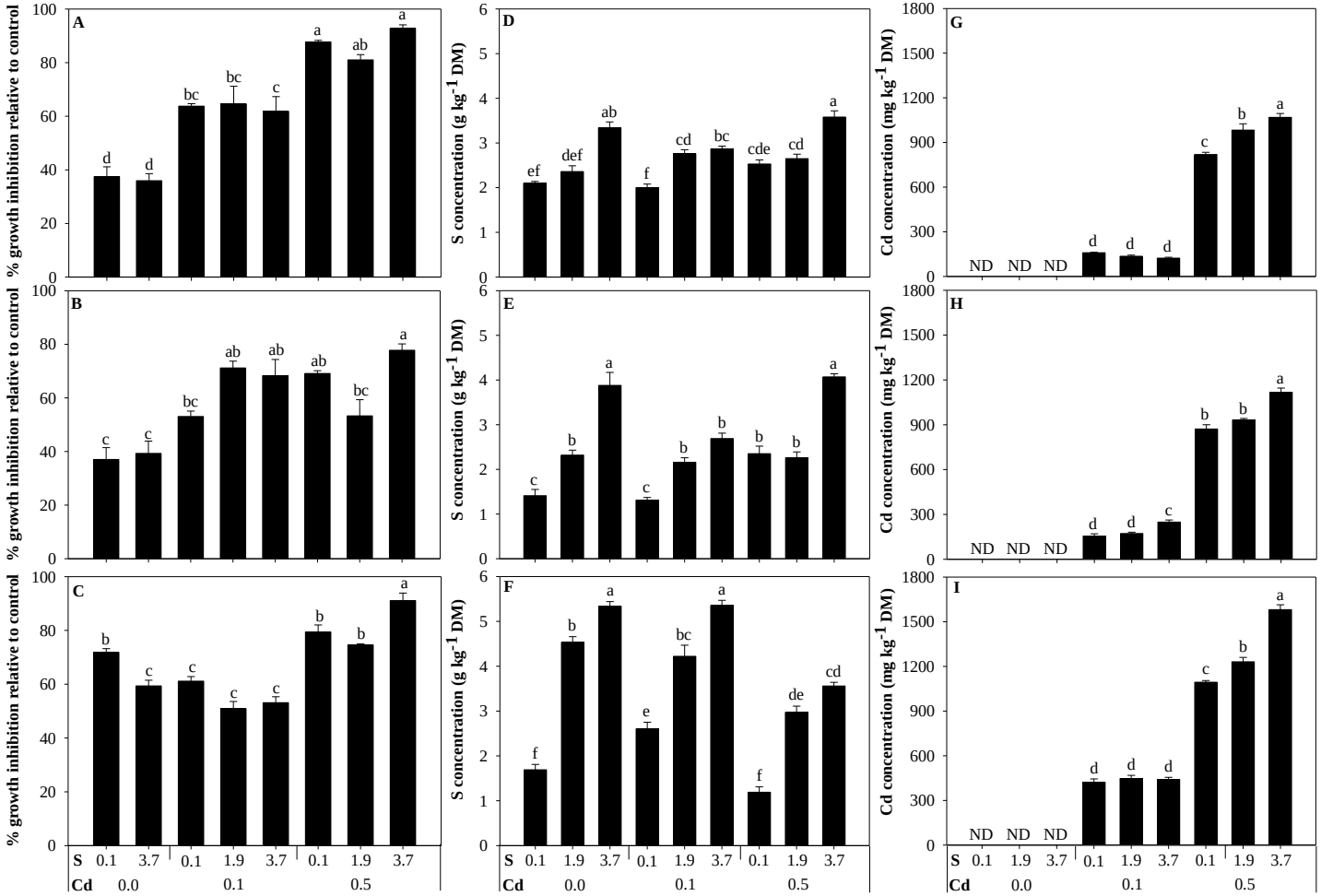
The unsupervised multivariate statistical analyses of DHA data was realized by Bioconductor pcaMethods package (STACKLIES et al., 2007). All data were submitted to analysis of variance (F test) and Tukey test ($P < 0.05$) through Statistical Analysis System v. 9.2 (SAS INSTITUTE, 2008). Results were expressed as mean ± standard error of the mean (SEM).

6.3 Results

6.3.1 Growth inhibition and concentrations of S and Cd in Massai grass

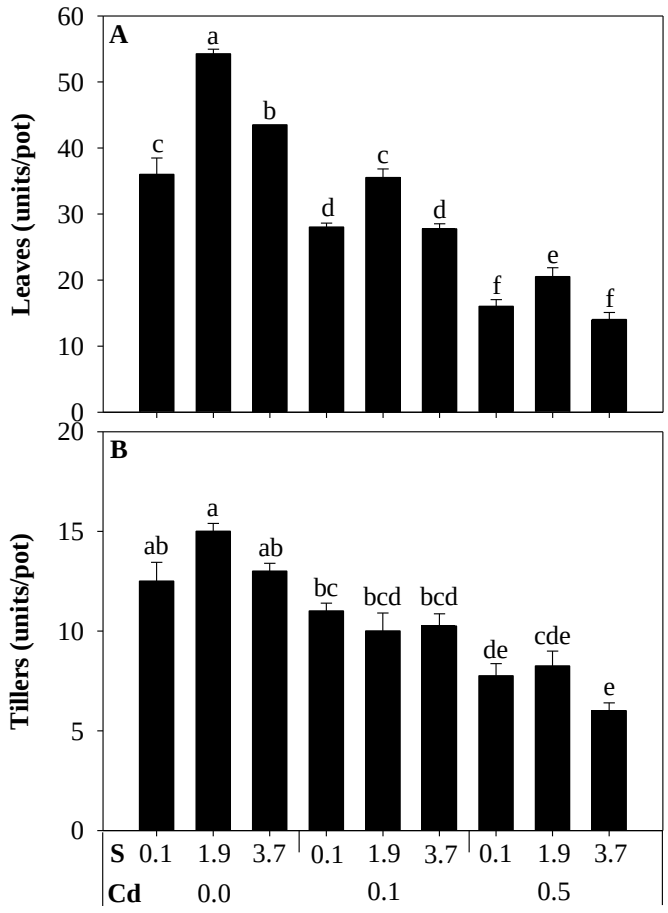
The leaf blades production of Massai grass was inhibited by more than 60 and 80% in relation to control treatment when the plants were exposed to 0.1 and 0.5 mmol L^{-1} Cd, respectively, regardless of S supply (Figure 1A), which can be attributed to the lower number of leaves observed in the plants exposed to Cd (Figure 2A). Massai grass exposure to Cd also resulted in lower stems and sheaths production (Figure 1B) due to lower tillering (Figure 2B), but the S supply of 1.9 mmol L^{-1} to Massai grass allowed higher stems and sheaths production in relation to S supply of 3.7 mmol L^{-1} when the plants were exposed to 0.5 mmol L^{-1} Cd (Figure 1B). Root production of plants supplied with 0.1 and 1.9 mmol L^{-1} S was inhibited by more than 70% in relation to control treatment, while this inhibition was higher than 90% to Massai grass supplied with 3.7 mmol L^{-1} S when the plants were exposed to 0.5 mmol L^{-1} Cd (Figure 1C). Rabêlo and Borgo (2016) reported that the changes caused by Cd exposure in the forage grass growth affects directly the concentration of nutrients and Cd. In this sense, we can observe that S concentrations in the leaf blades (Figure 1D) and stems and sheaths (Figure 1E) of Massai grass exposed to 0.5 mmol L^{-1} Cd were relatively higher than in plants exposed to 0.1 mmol L^{-1} Cd.

Figure 1 - Growth inhibiton (%) relative to control treatment (1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd) (A, B and C), S concentration (D, E and F) and Cd concentration (G, H and I) in the leaf blades (A, D and G), stems and sheaths (B, E and H) and roots (C, F and I) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 and 0.5 mmol L^{-1}). Different letters on the bars indicate significant statistical difference by Tukey test (P <0.05). ND = non-detected



In general, S concentrations in the leaf blades and stems and sheaths of plants supplied with 3.7 mmol L^{-1} S were higher than in plants supplied 0.1 mmol L^{-1} S, regardless of Cd concentration (Figures 1D-E). On the other hand, S concentrations in the roots of plants exposed to 0.5 mmol L^{-1} Cd was lower than in plants exposed to 0.1 mmol L^{-1} Cd (Figure 1F). Cadmium concentrations in all tissues of Massai grass exposed to 0.5 mmol L^{-1} Cd were higher than in plants exposed to 0.1 mmol L^{-1} Cd, and the plants supplied with 3.7 mmol L^{-1} S showed higher Cd concentrations in relation to plants supplied with 0.1 mmol L^{-1} S when Massai grass was grown with 0.5 mmol L^{-1} Cd (Figures 1G-H).

Figure 2 - Number of leaves (A) and tillers (B) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 and 0.5 mmol L^{-1}). Different letters on the bars indicate significant statistical difference by Tukey test (P <0.05).

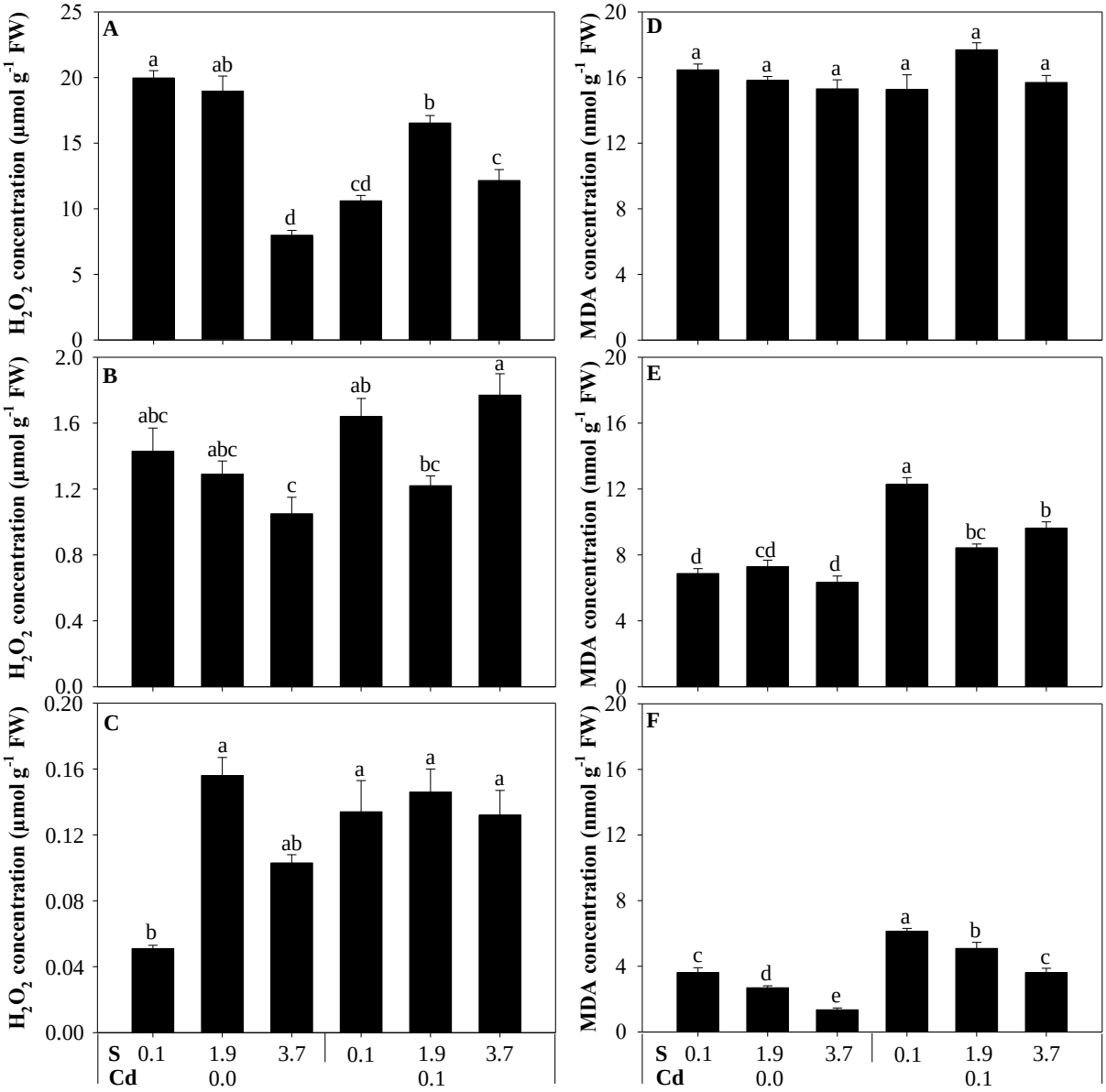


6.3.2 Effect of S and Cd on H_2O_2 and MDA concentrations of Massai grass

Hydrogen peroxide concentration in the leaf blades of Massai grass grown with 0.1 mmol L^{-1} S was higher than in plants supplied with 3.7 mmol L^{-1} S in Cd absence (Figure 3A). However, H_2O_2 concentrations in the stems and sheaths (Figure 3B) and roots

(Figure 3C) of Massai grass supplied with 0.1 and 3.7 mmol L^{-1} S did not differ (P >0.05) each other in plants not exposed to Cd.

Figure 3 - Concentrations of hydrogen peroxide (H_2O_2) (A, B and C) and malondialdehyde (MDA) (D, E and F) in the leaf blades (A and D), stems and sheaths (B and E) and roots (E and F) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1}). Different letters on the bars indicate significant statistical difference by Tukey test (P <0.05)



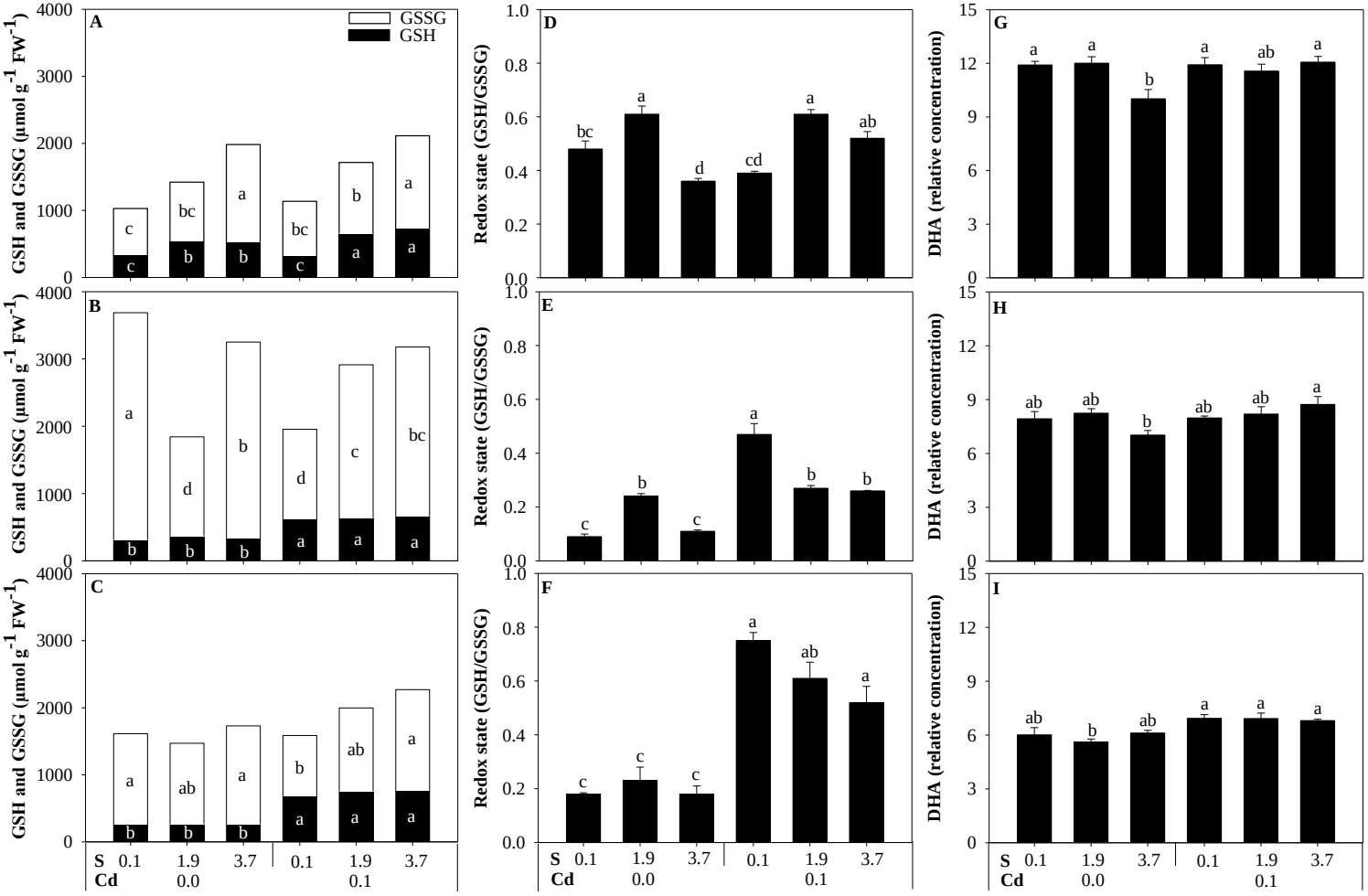
Hydrogen peroxide concentrations in all tissues of plants supplied with 0.1 and 3.7 mmol L^{-1} S also did not differ (P >0.05) each other when Massai grass was grown with 0.1 mmol L^{-1} Cd (Figures 3A-C). The concentrations of MDA in the leaf blades of Massai grass

were not changed (P >0.05) by S and Cd concentrations (Figure 3D). However, MDA concentrations in the stems and sheaths (Figure 3E) and in the roots (Figure 3F) of plants exposed to Cd were higher than in plants not exposed, and the plants supplied with 3.7 mmol L^{-1} S showed lower MDA concentrations in the stems and sheaths and roots in relation to plants grown with 0.1 mmol L^{-1} S when Massai grass was exposed to Cd (Figures 3E-F).

6.3.3 GSH, GSSG and DHA concentrations and GSH/GSSG ratio of Massai grass

Oxidized glutathione concentrations were higher than GSH concentrations in all tissues of Massai grass grown with different S and Cd concentrations (Figures 4A-C). The concentrations of GSH and GSSG in the leaf blades of plants supplied with 3.7 mmol L^{-1} S were higher than in plants supplied with 0.1 mmol L^{-1} S, regardless of Cd concentration (Figure 4A). In the stems and sheaths (Figure 4B) and roots (Figure 4C) of Massai grass, GSH concentrations of plants exposed to Cd were higher than in plants not exposed to Cd, and GSSG concentrations of plants supplied with 3.7 mmol L^{-1} S was higher than in plants grown with 0.1 mmol L^{-1} S when the plants were exposed to 0.1 mmol L^{-1} Cd. The highest GSH/GSSG ratio in the leaf blades (Figure 4D) and stems and sheaths (Figure 4E) of Massai grass grown in Cd absence was observed with S supply of 1.9 mmol L^{-1}. In the leaf blades of Massai grass exposed to Cd, the highest GSH/GSSH ratio also occurred with S supply of 1.9 mmol L^{-1} (Figure 4D), unlike what was observed in the stems and sheaths, where the highest GSH/GSSG ratio occurred in the plants supplied with 0.1 mmol L^{-1} S (Figure 4E). There was no effect of S supply (P >0.05) on the GSH/GSSG ratio in the roots of Massai grass, but the plants exposed to Cd showed higher GSH/GSSG ratio than plants grown in Cd absence (Figure 4F). The concentrations of DHA in the leaf blades (Figure 4G) and stems and sheaths (Figure 4H) of Massai grass exposed to Cd were higher than in plants grown in Cd absence when there was S supply of 3.7 mmol L^{-1}. In the roots, DHA concentration of plants exposed to Cd was higher than in plants not exposed when there was S supply of 1.9 mmol L^{-1} (Figure 4I).

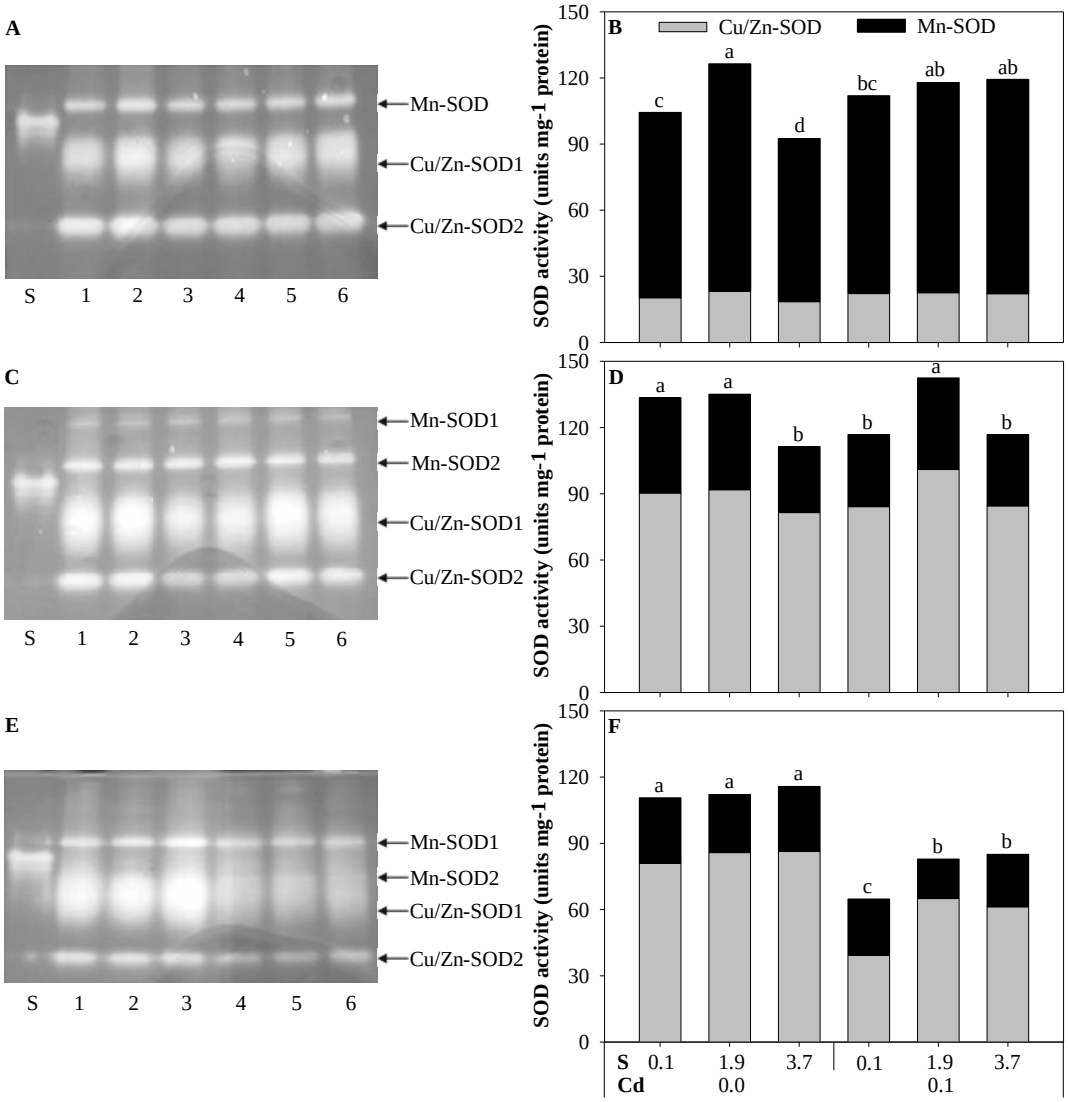
Figure 4 - Glutathione reduced (GSH) and oxidize (GSSG) concentrations (A, B and C), redox state (D, E and F) and dehydroascorbate (DHA) concentration (G, H and I) in the leaf blades (A, D and G), stems and sheaths (B, E and H) and roots (C, F and I) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1}). Different letters on the bars indicate significant statistical difference by Tukey test ($P < 0.05$). DHA concentration was expressed relative to median of metabolite



6.3.4 Activity of enzymes related to oxidative stress in Massai grass

Two SOD isoforms (Mn-SOD and Cu/Zn-SOD) were identified in the leaf blades of Massai grass (Figure 5A), and the Mn-SOD isoform activity was higher than Cu/Zn-SOD isoform activity (Figure 5B).

Figure 5 - Analysis of superoxide dismutase (SOD) in non-denaturing PAGE (A, C and E) and in densitometer (B, D and F) in the leaf blades (A and B), stems and sheaths (C and D) and roots (E and F) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1}). The lanes listed in A, C and E are as follows: (S) bovine SOD standard; (1) 0.1 mmol L^{-1} S + 0.0 mmol L^{-1} Cd; (2) 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd; (3) 3.7 mmol L^{-1} S + 0.0 mmol L^{-1} Cd; (4) 0.1 mmol L^{-1} S + 0.1 mmol L^{-1} Cd; (5) 1.9 mmol L^{-1} S + 0.1 mmol L^{-1} Cd; and (6) 3.7 mmol L^{-1} S + 0.1 mmol L^{-1} Cd. The arrows indicate SOD isoforms identified in PAGE in each tissue. Different letters on the bars indicate significant statistical difference by Tukey test ($P < 0.05$)



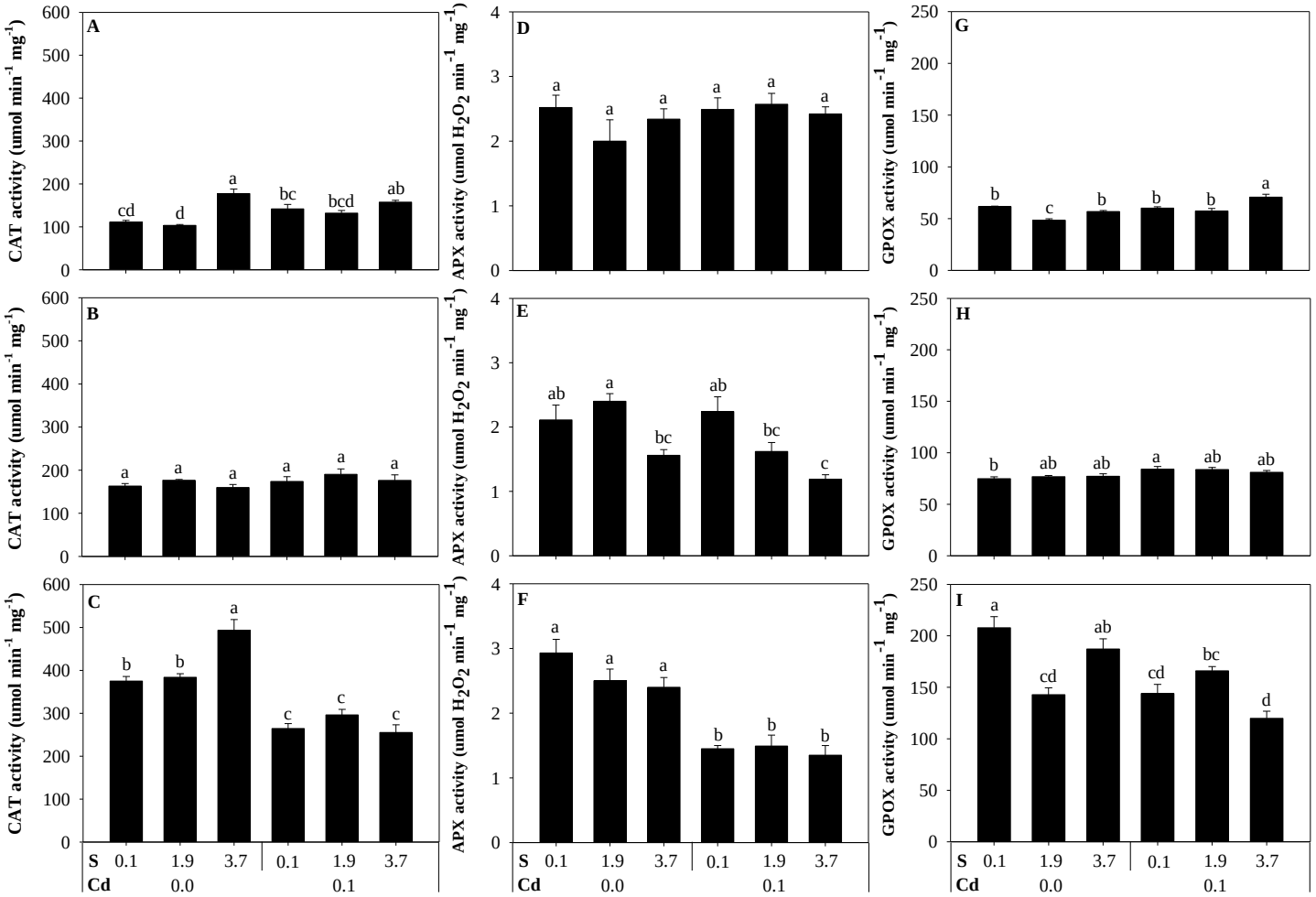
Total SOD activity in the leaf blades of Massai grass exposed to Cd was not changed (P >0.05) by S supply, but the S supply of 1.9 mmol L^{-1} resulted in highest activity in plants grown in Cd absence (Figure 5B). In the stems and sheaths of Massai grass two SOD isoforms (Mn-SOD and Cu/Zn-SOD) (Figure 5C) also were identified, but in this issue Cu/Zn-SOD showed higher activity than Mn-SOD isoform (Figure 5D). The highest total SOD activity in the stems and sheaths of plants exposed to Cd was verified with S supply of 1.9 mmol L^{-1} (Figure 5D). The same two SOD isoforms (Mn-SOD and Cu/Zn-SOD) observed in the leaf blades and stems and sheaths were detected in the roots of Massai grass grown with different S and Cd concentrations (Figure 5E). In the roots, the Cu/Zn-SOD isoform activity was higher than Mn-SOD activity (Figure 5E) like in the stems and sheaths. Total SOD activity in the roots of plants not exposed to Cd was higher than in plants exposed to Cd (Figure 5F). Sulfur supply of 1.9 and 3.7 mmol L^{-1} to Massai grass resulted in higher SOD activity in relation to S supply of 0.1 mmol L^{-1} when the plants were exposed to Cd (Figure 5F).

Catalase activity in the leaf blades (Figure 6A), stems and sheaths (Figure 6B) and roots (Figure 6C) of Massai grass exposed to Cd were not changed (P >0.05) by S supply, but CAT activity in the leaf blades (Figure 6A) and roots (Figure 6C) of plants not exposed to Cd were highest with S supply of 3.7 mmol L^{-1}.

The activity of APX in the leaf blades of Massai grass was not changed (P >0.05) by S and Cd supply (Figure 6D). However, in the stems and sheaths the APX activity of plants supplied with 1.9 mmol L^{-1} S was higher than in plants supplied with 3.7 mmol L^{-1} S when Massai grass was not exposed to Cd, and when the plants were exposed to Cd the APX activity of plants supplied with 0.1 mmol L^{-1} S was higher than in plants grown with 3.7 mmol L^{-1} S (Figure 6E). The activity of APX in the roots of Massai grass exposed to 0.1 mmol L^{-1} Cd was lower than in plants not exposed to Cd, regardless of S supply (Figure 6F).

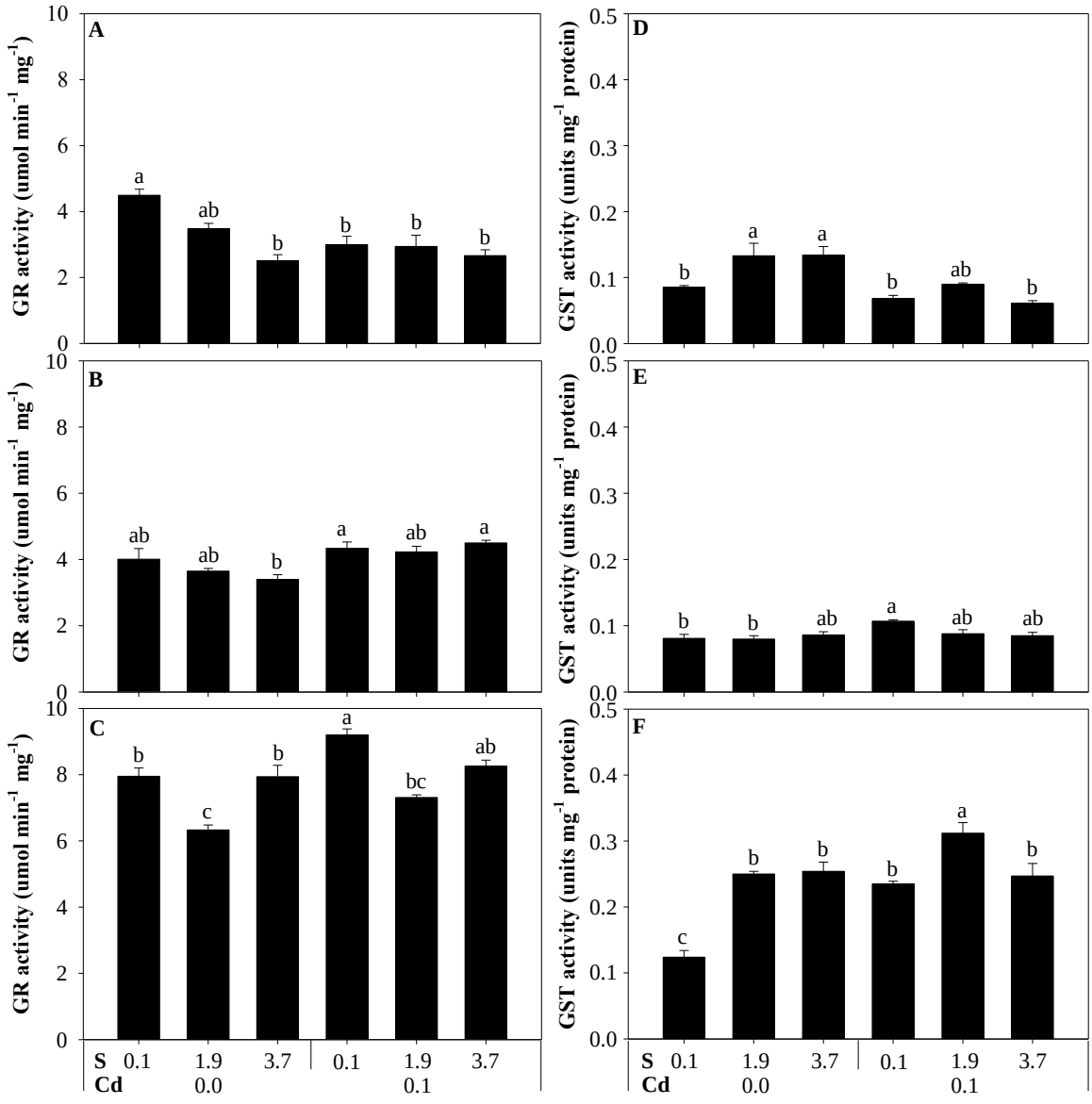
In the leaf blades of plants exposed and not exposed to Cd, the supply of 3.7 mmol L^{-1} S resulted in higher GPOX activity in relation to plants supplied with 1.9 mmol L^{-1} S (Figure 6G). Guaiacol peroxidase activity in the stems and sheaths of plants exposed to Cd was higher than in plants not exposed to Cd when there was S supply of 0.1 mmol L^{-1} (Figure 6H). The roots of plants supplied with 1.9 mmol L^{-1} S showed lowest GPOX activity when Massai grass was not exposed to Cd and highest GPOX activity when the plants were Cd-grown (Figure 6I).

Figure 6 - Activity of catalase (CAT) (A, B and C), ascorbate peroxidase (APX) (D, E and F) and guaiacol peroxidase (GPOX) (G, H and I) in the leaf blades (A, D and G), stems and sheaths (B, E and H) and roots (C, F and I) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1}). Different letters on the bars indicate significant statistical difference by Tukey test ($P < 0.05$)



The highest GR activity in the leaf blades of Massai grass occurred in the plants not exposed to Cd and grown with 0.1 mmol L^{-1} S (Figure 7A). There was no effect of S supply (P >0.05) on the GR activity in the leaf blades (Figure 7A) and stems and sheaths (Fig. 6B) of plants exposed to Cd. Sulfur supply of 1.9 mmol L^{-1} resulted in the lowest GR activities in the roots of plants exposed and not exposed to Cd (Figure 7C).

Figure 7 - Activity of glutathione reductase (GR) (A, B and C) and glutathione-S-transferase (GST) (D, E and F) in the leaf blades (A and D), stems and sheaths (B and E) and roots (E and F) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1}). Different letters on the bars indicate significant statistical difference by Tukey test (P <0.05)

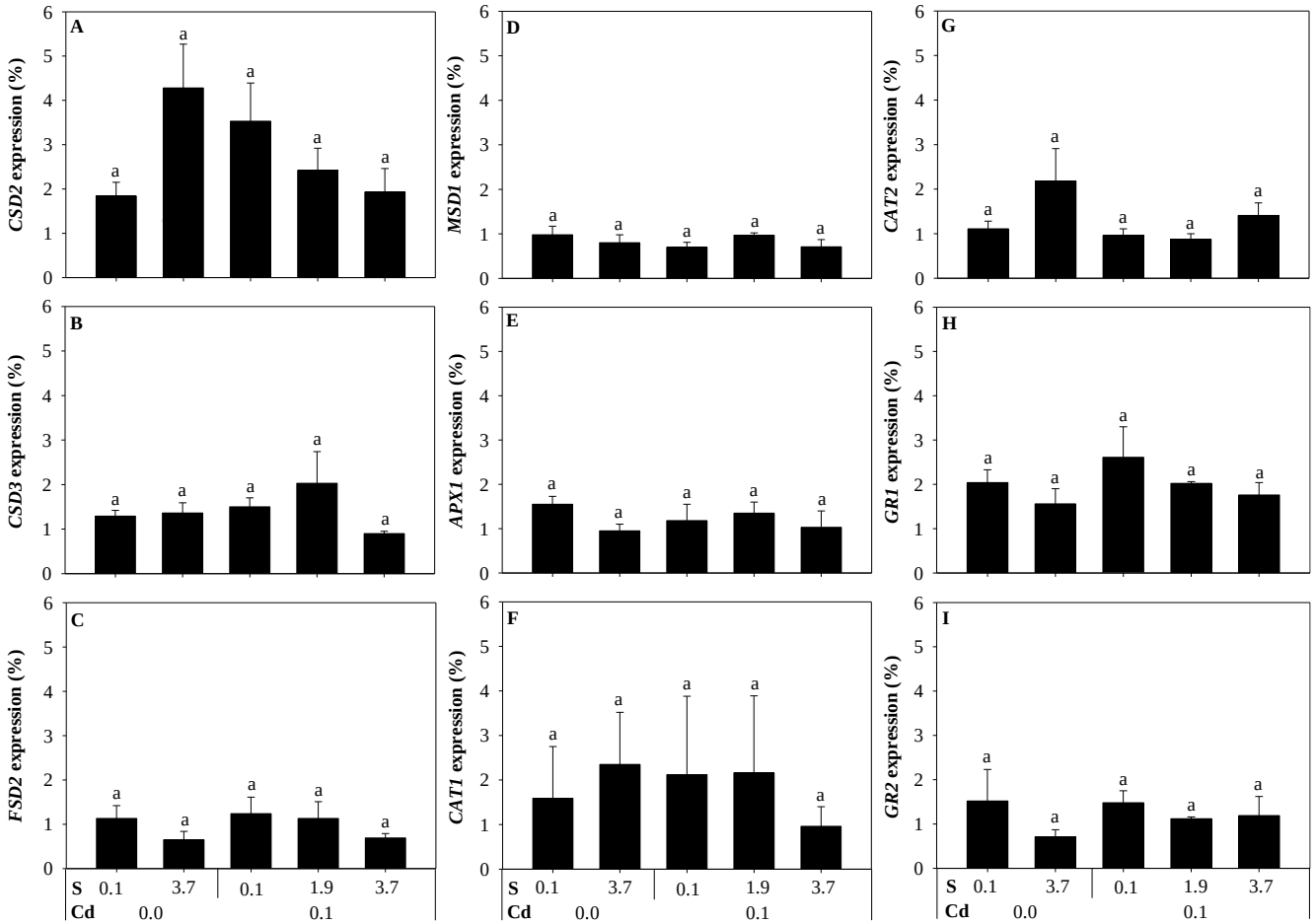


The highest GST activities in the leaf blades of Massai grass were observed when the plants were not exposed to Cd and were supplied with 1.9 and 3.7 mmol L^{-1} S (Figure 7D). There was no effect of S supply (P >0.05) on the GST activity in the leaf blades (Figure 7D) and stems and sheaths (Figure 7E) of plants exposed to Cd. The highest GST activities in the roots of Massai grass not exposed to Cd occurred with S supply of 1.9 and 3.7 mmol L^{-1}, while in plants exposed to Cd the highest GST activity occurred in plants grown with 1.9 mmol L^{-1} S (Figure 7F).

6.3.5 Expression of the genes related to enzymes of ascorbate-glutathione cycle

Expression levels of *CSD2* (Figure 8A), *CSD3* (Figure 8B), *FSD2* (Figure 8C), *MSD1* (Figure 8D), *APX1* (Figure 8E), *CAT1* (Figure 8F), *CAT2* (Figure 8G), *GR1* (Figure 8H) and *GR2* (Figure 8I) relative to control treatment (1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd) in the leaf blades of Massai grass were not changed (P >0.05) by S and Cd concentrations.

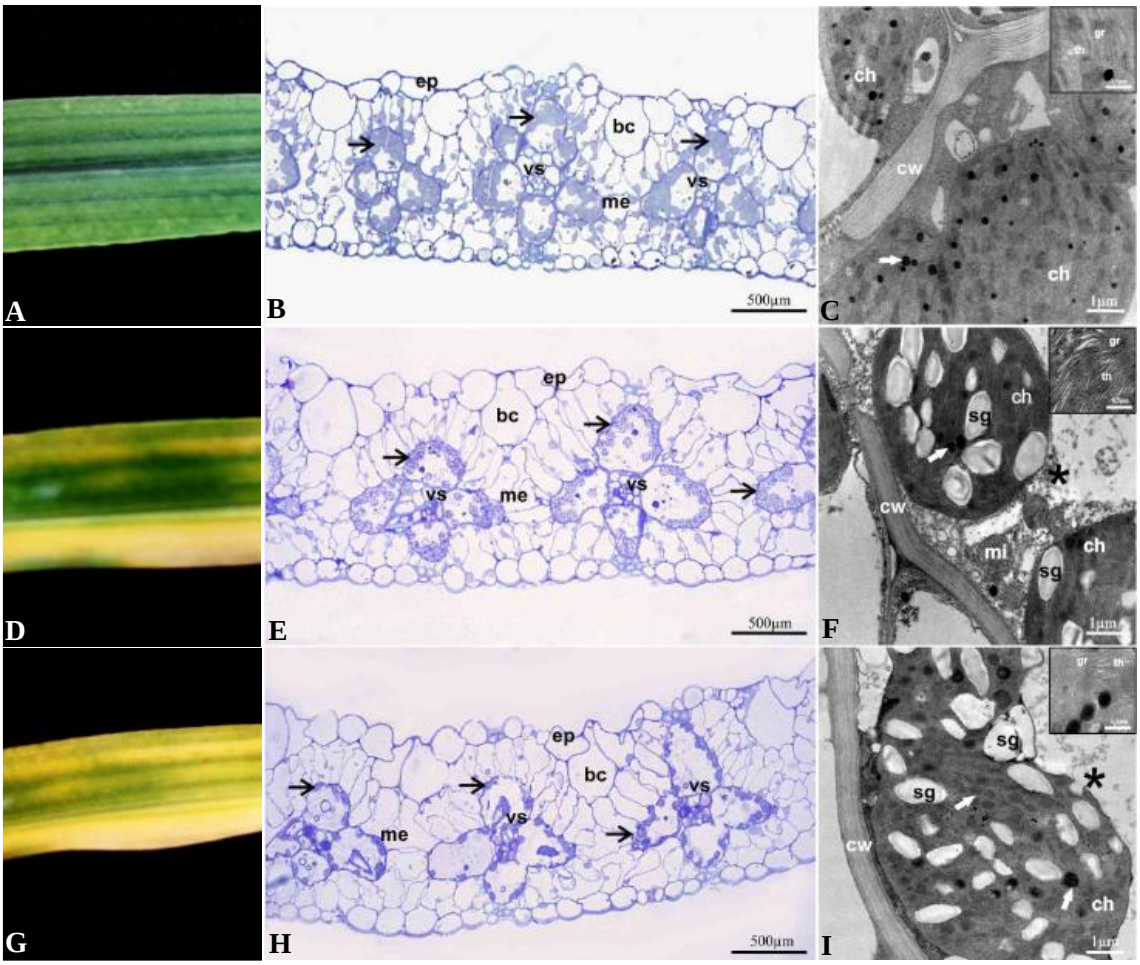
Figure 8 - Expression levels of copper/zinc superoxide dismutase [*CSD2* (A) and *CSD3* (B)], iron superoxide dismutase [*FSD2* (C)], manganese superoxide dismutase [*MSD1* (D)], ascorbate peroxidase [*APX1* (E)], catalase [*CAT1* (F) and *CAT2* (G)], and glutathione reductase [*GR1* (H) and *GR2* (I)] relative to control treatment (1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd) in the leaf blades of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 mmol L^{-1}). Different letters on the bars indicate significant statistical difference by Tukey test ($P < 0.05$)



6.3.6 Visual, anatomical and ultrastructural changes in the leaf blades of Massai grass

Massai grass grown with 1.9 mmol L^{-1} S and not exposed to Cd did not present any type of chlorosis in the first newly expanded leaf (Figure 9A). This plant showed high amount of chloroplasts (Figure 9B) containing well compartmentalized grana and organized thylakoids (Figure 9C), and low starch grains accumulation (Figure 9C).

Figure 9 - Visual (A, D and G), anatomical (transverse sections of mesophyll) (B, E and H) and ultrastructural (C, F and I) changes in the first newly expanded leaf of Massai grass supplied with S and exposed to Cd. B-E-H (light microscopy); C-F-I (transmission electron microscopy). A-B-C: 1.9 mmol L^{-1} S + 0.0 mmol L^{-1} Cd; D-E-F: 1.9 mmol L^{-1} S + 0.5 mmol L^{-1} Cd; G-H-I: 3.7 mmol L^{-1} S + 0.5 mmol L^{-1} Cd. ep = epidermis; bc = buliform cell; vs = vascular system; me = mesophyll; ch = chloroplast; sg = starch grain; cw = cell wall; mi = mitochondria; gr = grana; th = thylakoids. Black arrows show chloroplasts; white arrows show plastoglobules; asterisk show rupture of tonoplast



However, Massai grass cultivated with 1.9 mmol L^{-1} S showed marginal yellowing and necrosis in the first newly expanded leaf (Figure 9D), low amount of chloroplasts (Figure 9E) and high starch grains and mitochondria accumulation associated to rupture of tonoplast and disorganization in the internal structures of chloroplasts in plants exposed to 0.5 mmol L^{-1} Cd (Figure 9F). The same changes presented by plants supplied with 1.9 mmol L^{-1} S and exposed to 0.5 mmol L^{-1} Cd (Figures 9D-F) were verified in the first newly expanded leaf of Massai grass grown with 3.7 mmol L^{-1} S when the plants were exposed to 0.5 mmol L^{-1} Cd (Figures 9G-I). However, the yellowing and necrosis were more severe under high S-supply conditions (Figure 9G) compared to plants exposed to 0.5 mmol L^{-1} Cd and supplied with 1.9 mmol L^{-1} S (Figure 9D) due to lower amount of chloroplasts (Figure 9H) and to higher starch grains accumulation associated to rupture of tonoplast (Figure 9I). Massai grass exposed to 0.5 mmol L^{-1} Cd showed premature senescence when the plants grown under high S-supply (Figure 10).

6.3.7 Effect of S and Cd on the photosynthetic parameters of Massai grass

The leaf area of Massai grass decreased when the plants were exposed to Cd (Figure 11A), which can be attributed to the lowest number of leaves (Figure 10) and to damage caused by Cd in the leaf blades of these plants (Figures 9D-I). The highest specific leaf areas occurred in the plants exposed to 0.5 mmol L^{-1} Cd when there was S supply of 0.1 and 1.9 mmol L^{-1} (Figure 11B), indicating that these leaves are thinner than the leaves of plants exposed to other S and Cd combinations. The lowest chlorophyll concentration were observed in plants grown with 0.5 mmol L^{-1} Cd, regardless of S supply, but chlorophyll concentration of Massai grass exposed to 0.1 mmol L^{-1} Cd was similar to chlorophyll concentration of plants not exposed to Cd (Figure 11C). The *A* of Massai grass exposed to Cd decreased in relation to plants grown in Cd absence, but plants exposed to Cd showed highest *A* when the plants were supplied with 3.7 mmol L^{-1} S (Figure 11D). Sulfur supply of 3.7 mmol L^{-1} to Massai grass also resulted in highest g_s in plants exposed to 0.1 mmol L^{-1} Cd, but there was no effect of S supply ($P > 0.05$) on the g_s of plants grown with 0.5 mmol L^{-1} Cd (Figure 11E). Intracellular CO_2 concentration of plants exposed to Cd decreased by more than 50% in relation to plants grown in Cd absence, but plants grown with 1.9 mmol L^{-1} S showed higher c_i than plants grown with 0.1 and 3.7 mmol L^{-1} S when Massai grass was exposed to Cd (Figure 11F). Carboxylation efficiency of plants exposed to Cd did not decrease in relation to plants not exposed to Cd (Figure 11G). Sulfur supply of 3.7 mmol L^{-1} to plants exposed to Cd resulted in the highest *k* (Figure 11G). Plants grown with 3.7 mmol L^{-1} S showed lower

WUE in relation to plants grown with 0.1 mmol L^{-1} S when Massai grass was exposed to Cd (Figure 11H). Massai grass exposed to Cd also showed lower WUE when compared to plants not exposed to Cd (Figure 11H). Intrinsic water use efficiency of plants grown with 0.1 mmol L^{-1} S also was higher than in plants grown with 3.7 mmol L^{-1} S when Massai grass was exposed to Cd (Figure 11I).

Figure 10 - Symptoms of Cd toxicity in the shoots of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to 0.0 (A), 0.1 (B) and 0.5 mmol L^{-1} (C)

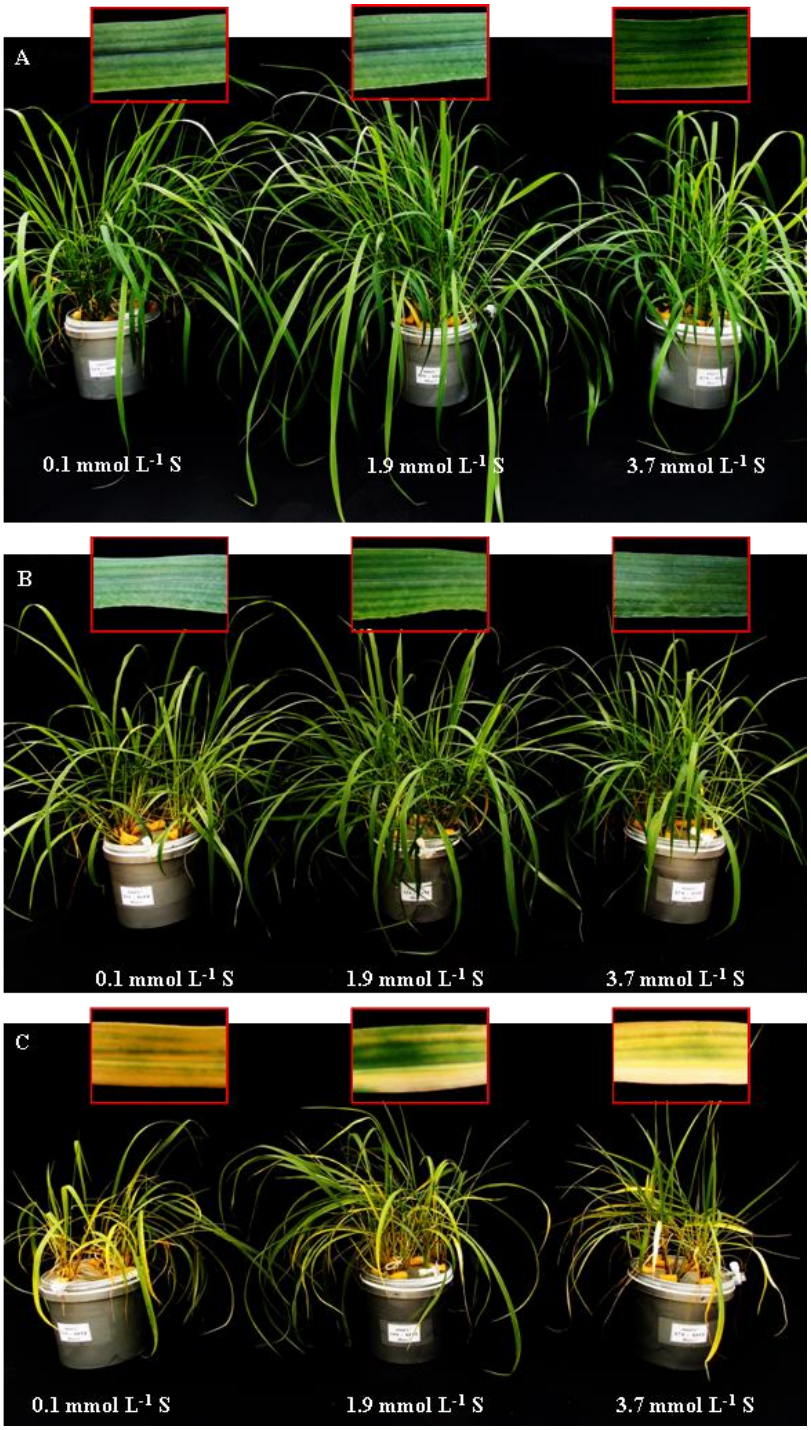
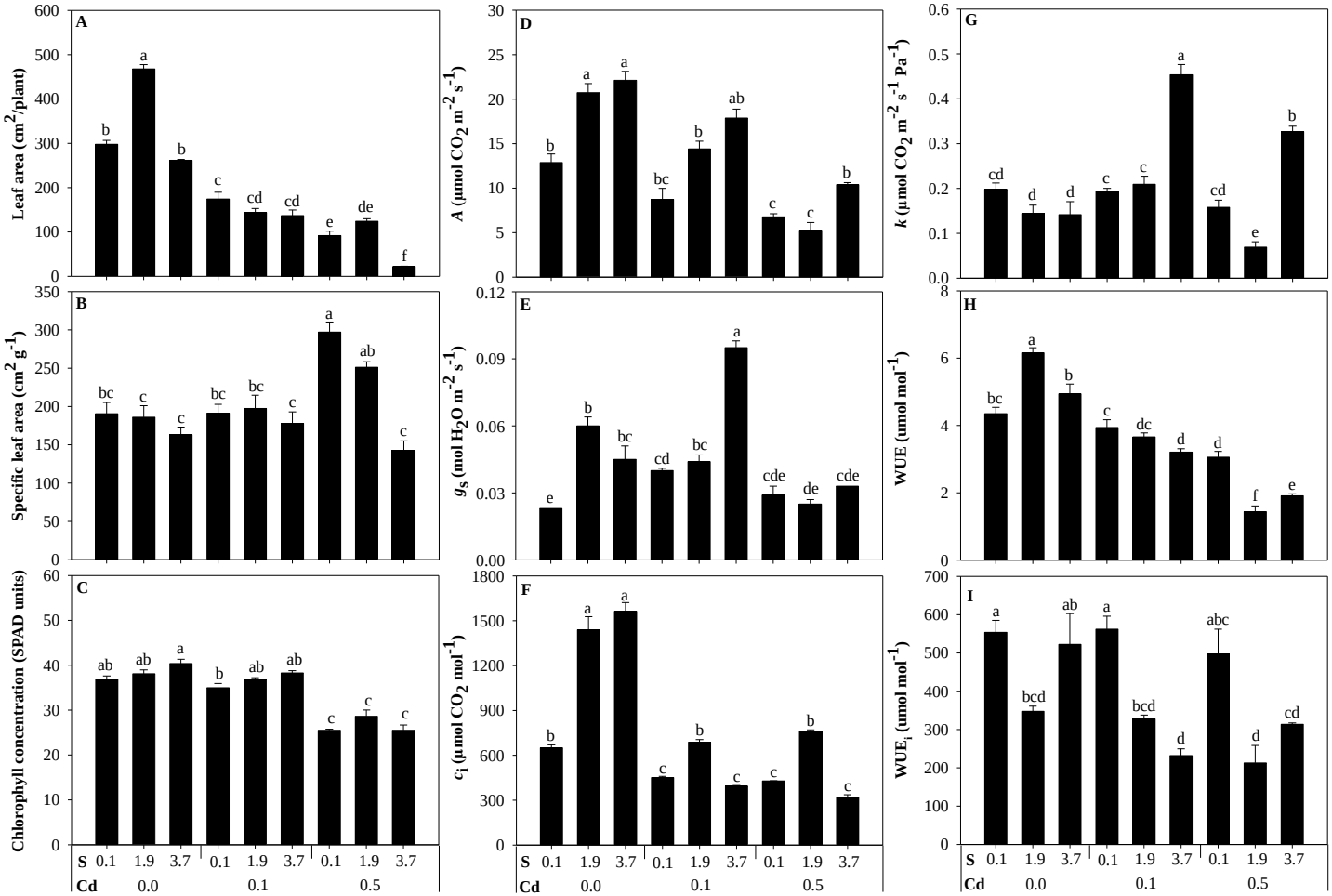


Figure 11 - Leaf area (A), specific leaf area (B), chlorophyll concentration (C), photosynthetic rate - *A* (D), stomatal conductance - g_s (E), intracellular CO_2 concentration - c_i (F), carboxylation efficiency - *k* (G), instantaneous water use efficiency - WUE (H) and intrinsic water use efficiency - WUE_i (I) of Massai grass supplied with S (0.1, 1.9 and 3.7 mmol L^{-1}) and exposed to Cd (0.1 and 0.5 mmol L^{-1}). Different letters on the bars indicate significant statistical difference by Tukey test ($P < 0.05$)



6.4 Discussion

In general, the growth of Massai grass exposed to Cd was inhibited by more than 50% in relation to control treatment (Figures 1A-C), regardless of S concentrations (Figures 1D-F), which can be attributed to high Cd concentrations observed in their tissues (Figures 1G-I). High Cd concentrations can change the processes of uptake, transport and assimilation of nutrients, water uptake, respiration and photosynthesis of plants, which decreases the growth and biomass production (HE et al., 2017). Moreover, the most part of Cd taken up by grasses is accumulated at the base of plants, which changes negatively physiological processes related to tillers and leaves emission (FUJIMAKI et al., 2010), as observed in this study (Figure 2). Cadmium accumulation in the roots, at the base or shoots of plants depends of capacity of Cd retention in the root cells, mainly by PCs, and of Cd translocation efficiency from roots to shoots (MENDOZA-CÓZATL et al., 2011). In this study, we observed that Massai grass grown with 0.1 and 1.9 mmol L^{-1} S showed higher synthesis of PCs in the roots (Figure 6F, p. 131) and lower Cd translocation from roots to shoots than Massai grass grown with 3.7 mmol L^{-1} S, when the plants were exposed to 0.1 mmol L^{-1} Cd (Table 6, p. 90). These results show that S excess can decrease the efficiency of the mechanisms of Cd detoxification in plants, as reported by Masood, Iqbal and Khan (2012). This conclusion is more evident when we observed that plants supplied with 3.7 mmol L^{-1} S showed the lowest growth (Figures 1A-C) and the highest Cd concentrations (Figures 1G-I) when Massai grass was exposed to 0.5 mmol L^{-1} Cd.

The lowest synthesis of PCs observed in the roots of Massai grass supplied with 3.7 mmol L^{-1} S (Figure 6F, p. 131) represents a big problem because free Cd^{2+} ions in the cytosol can cause oxidative damage to cellular organelles due to imbalance between pro-oxidants (e.g. ROS) and antioxidants (CUYPERS et al., 2010). This fact is clear when we observed that H_2O_2 concentration increased with Cd exposure only in plants grown with 3.7 mmol L^{-1} S (Figures 3A-B). On the other hand, H_2O_2 concentrations in the leaf blades (Figure 3A) were more than 100 times higher than in the roots (Figure 3C), regardless of Cd concentration. This result can be attributed to functions of H_2O_2 as opening and closing of stomata (DESIKAN et al., 2004) and regulation of genes encoding antioxidant enzymes in the leaves of plants exposed to stress conditions (NEILL et al., 2002). Although H_2O_2 acts in processes related to cellular defense, this ROS can cause lipid peroxidation (LOIX et al., 2017). However, there was no difference ($P > 0.05$) in lipid peroxidation in the leaf blades of Massai grass exposed or not to Cd (Figure 3D), differently that was observed in the stems and sheaths (Figure 3E) and roots (Figure 3F), where plants exposed to Cd showed higher lipid

peroxidation. It should be noted that lipid peroxidation can be caused by others ROS (e.g. O_2^- and OH^-) and this is the main factor for existence of difference in reactions of peroxidation in the roots and shoot tissues (FARMER; MUELLER, 2013). Although Cd induces lipid peroxidation in plant tissues, this process was attenuated in the stems and sheaths (Figure 3E) and roots (Figure 3F) of Massai grass grown without S deprivation (0.1 mmol L^{-1} S), as reported in other studies (HASSAN; WANG; ZHANG, 2005; ANJUM et al., 2008; BASHIR et al., 2015; KHAN et al., 2015, 2016; LIANG et al., 2016; BAGHERI et al., 2017). Hassan, Wang and Zhang (2005) noted that proper S supply increased the synthesis of GSH in *Oryza sativa* (cultivar Bing 97252) exposed to Cd, which contributed to decrease the lipid peroxidation caused by Cd, since GSH acts as a scavenger of H_2O_2 and singlet oxygen ($^1O_2$) (NOCTOR et al., 2012).

Although the proper S supply allows highest synthesis of GSH, this occurred only in the leaf blades of Massai grass grown with or without Cd in the nutrient solution (Figure 4A), which can be attributed to the lowest synthesis of PCs observed in this tissue (Figure 6D, p. 131), since GSH is used as substrate for synthesis of PCs (MENDOZA-CÓZATL et al., 2011). The concentrations of GSH in the stems and sheaths (Figure 4B) and roots (Figure 4C) of Massai grass exposed to Cd did not increase ($P > 0.05$) with S supply of 1.9 and 3.7 mmol L^{-1}, which can be attributed to the use of GSH for synthesis of PCs (MENDOZA-CÓZATL et al., 2011) in the stems and sheaths (Figure 6E, p. 131) and the lowest GR activity (Figure 7C) and the highest GST activity (Figure 7F) observed in the roots. Glutathione reductase restores GSSG to GSH (GRATÃO et al., 2005), while GST binds GSH with Cd (Cd-GSH) and/or products that cause oxidative stress (e.g. OH^-) (NOCTOR et al., 2012). Thus, lowest GR activities and highest GST activities decrease GSH concentrations. On the other hand, GSH + GSSG concentrations of plants exposed to Cd were highest when there was no S deprivation (Figures 4A-C), which is in agreement with the results observed by Bashir et al. (2015) and Liang et al. (2016). Although the plants grown with 1.9 and 3.7 mmol L^{-1} S showed highest GSH + GSSG concentrations, the most part of glutathione was in the oxidized state (Figures 4A-C). The oxidation of GSH to GSSG probably contributed to decrease the lipid peroxidation induced by Cd in the stems and sheaths (Figure 3E) and roots (Figure 3F) of Massai grass grown with 1.9 and 3.7 mmol L^{-1} S, but low GSH/GSSG ratios (Figures 4D-F) limit the performance of ascorbate-glutathione cycle (LIANG et al., 2016; HENDRIX et al., 2017), which can increase cell sensitivity to Cd (JOZEFCZAK et al., 2015). However, the oxidized status of glutathione (GSSG) is associated with the activation of antioxidant defense systems, such as SOD and CAT (JOZEFCZAK et al., 2015).

Superoxide dismutase and CAT are involved in O_2^- and H_2O_2 scavenging mainly in chloroplasts, in reactions involving successive oxidations and reductions of ascorbate and GSH by enzymes such as CAT, GPOX, GR and DHAR (GRATÃO et al., 2005). Superoxide dismutase activity in the leaf blades of Massai grass exposed to Cd did not increase (Figures 5B, 5D and 5F), probably because the expressions of *CSD2*, *CSD3*, *FSD2* and *MSD1* genes (Figures 8A-D) that are involved in the syntheses of isoforms Cu/Zn-SOD, Fe-SOD and Mn-SOD did not increase either ($P > 0.05$). Thus, the highest lipid peroxidation observed in the stems and sheaths (Figure 3E) and in the roots (Figure 3F) of plants exposed to Cd may have been caused by O_2^- (FARMER; MUELLER, 2013) due to low SOD activity. Luo et al. (2011) reported that Cd induced the expression of genes involved in the synthesis of Cu/Zn-SOD, Fe-SOD and Mn-SOD in *L. perenne*, which resulted in highest SOD activity in this plant. These results indicate that Massai grass has a limited capacity to catalyze the dismutation of O_2^- in H_2O_2 when exposed to high Cd concentrations. In addition, SOD activity in plants exposed to Cd was limited by low S supply in relation to plants supplied with 1.9 mmol L^{-1} S (Figures 5B, 5D and 5F). Sulfur deprivation may limit the activity of SOD and other antioxidants due to the preferential use of cysteine for synthesis of GSH and PCs (BAGHERI et al., 2017). Cysteine are involved in the formation of sulfhydryl (-SH) and disulfide (-SS) bonds, which are the active centers of numerous antioxidants (GILL; TUTEJA, 2011; CAPALDI et al., 2015). Only two isoforms of SOD (Cu/Zn-SOD and Mn-SOD) were identified in Massai grass tissues (Figure 5). Although *FSD2* gene expression has occurred (Figure 8C), Fe-SOD isoform was not detected in Massai grass probably by changes in post-transcriptional or post-translational processes. The isoform Fe-SOD is often not detected in plants (FERREIRA et al., 2002). The isoform Cu/Zn-SOD showed lower activity in the leaf blades (Figure 5B) and higher activity in the stems and sheaths (Figure 5D) and roots (Figure 5F) in relation to isoform Mn-SOD. Ogawa, Kanematsu and Asada (1997) suggested that highest activity of isoform Cu/Zn-SOD is associated with lignification process of tissues, which is in agreement with our study, since the stems and sheaths and roots show higher lignification than leaf blades. The stems and sheaths and roots were the main sites of Cd accumulation in Massai grass (Table 7, p. 91), which suggests that isoform Cu/Zn-SOD may be indirectly involved in Cd accumulation in this plant.

The activities of CAT (Figures 6A) and APX (Figures 6D) in the leaf blades of Massai grass grown with and without Cd were very similar, probably because there was no difference ($P > 0.05$) in the expression of *APX1* (Figure 8E), *CAT1* (Figure 8F) and *CAT2* genes (Figure 8G). Luo et al. (2011) reported that expression of the genes involved in synthesis of

APX by *L. perenne* increased with exposure to Cd, which increased APX activity. The activities of CAT and GPOX in the stems and sheaths of Massai grass grown with and without Cd were also similar (Figures 6B and 6H). Although the activities of CAT, APX and GPOX did not increase (P >0.05) in the leaf blades or stems and sheaths of plants exposed to Cd, only the plants grown with 3.7 mmol L^{-1} S presented higher H_2O_2 concentrations in relation to plants not exposed to Cd (Figures 3A-B). Besides the lowest synthesis of PCs observed in these plants (Figures 6D-F, p. 131), this result may be associated with a low DHAR activity in the leaf blades and stems and sheaths and with the low APX activity in the stems and sheaths of Massai grass grown with 3.7 mmol L^{-1} S when the plants were exposed to Cd (Figure 6E). The low DHAR activity may limit the ascorbate-glutathione cycle, since ascorbate is used as a substrate to restore other antioxidants (JOZECFZAK et al., 2015). To exemplify, DHAR restores DHA to ascorbate, which is used as reducing agent by APX during H_2O_2 scavenging (GRATÃO et al., 2005). The conclusion that the low DHAR activity may have contributed to increase H_2O_2 concentrations becomes clearer when we observe that DHA concentrations in the leaf blades and in the stems and sheaths of plants grown with 3.7 mmol L^{-1} S increased when Massai grass was exposed to Cd (Figures 4G-H). These results indicate again that S excess may decrease the efficiency of Cd detoxification by plants (MASOOD; IQBAL; KHAN, 2012). The activities of CAT, APX and GPOX in the roots of Massai grass exposed to Cd decreased in relation to plants not exposed to Cd (Figures 6C, 6F and 6I), as occurred with SOD (Figures 5B, 5D and 5F), regardless of S supply. Bashir et al. (2015) verified that CAT and APX activity decreased and SOD activity increased in *B. juncea* exposed to Cd in relation to control treatment. The balance between the activities of SOD, CAT, APX and GPOX is essential for O_2^- and H_2O_2 scavenging, so when the activity of one enzyme decreases the activity of another enzyme should increase (GRATÃO et al., 2005). However, this balance was not observed in the roots of Massai grass, which may be associated with synthesis of other antioxidants (e.g. galactinol and raffinose) or with the high Cd concentrations verified in this tissue (Figure 1I), which may damage the enzymes and decrease their activities (LUO et al., 2011). Rabêlo, Azevedo and Monteiro (2017b) observed that SOD and CAT activities decreased in the roots of *P. maximum* cv. Tanzania due to high Cd concentrations.

In general, the activities of SOD (Figure 5), CAT (Figures 6A-C), APX (Figures 6D-F), GPOX (Figures 6G-I) and GR (Figures 7A-C) in the tissues of Massai grass exposed to 0.1 mmol L^{-1} Cd were similar or lower than in plants not exposed to Cd. However, there were no visual symptoms of Cd toxicity (Figure 10) in plants exposed to 0.1 mmol L^{-1} Cd,

suggesting that others antioxidants (e.g. galactinol and raffinose) are indispensable to attenuate the oxidative stress caused by Cd in these plants. Massai grass exposed to 0.1 mmol L^{-1} Cd presented higher concentrations of galactinol in the roots and stems and sheaths and higher concentrations of raffinose in the leaf blades than Massai grass not exposed to Cd (Figure 3, p. 127; Figure 4, p. 128; Figure 5, p. 129). Galactinol and raffinose act on O_2^- and OH^- scavenging, but are more efficient on OH^- scavenging (KEUNEN et al., 2013). Nishizawa, Yabuta and Shigeoka (2008) observed that galactinol and raffinose exhibit similar capacity to GSH to scavenge OH^- radicals, and that raffinose is as important as ascorbate and GSH on ROS scavenging in chloroplasts. Although the plants exposed to 0.1 mmol L^{-1} Cd not showed visual symptoms of Cd toxicity (Figure 10), the high Cd concentrations observed in Massai grass exposed to 0.5 mmol L^{-1} Cd (Figures 1G-I) damaged the photosynthetic system of these plants, resulting in chlorosis and leaf necrosis (Figures 9D and 9G; Figure 10). Massai grass exposed to 0.5 mmol L^{-1} Cd showed low amount of chloroplasts, rupture of tonoplast and high starch grains accumulation (Figures 9E-F and 9H-I). High starch grains accumulation damages the thylakoids of chloroplasts which impairs the photosynthesis process and reduces the *A* (DELUCIA; SASEK; STRAIN, 1985). Gratão et al. (2009) reported that *Lycopersicon esculentum* plants exposed to Cd presented disorganized stroma and thylakoid system in relation to plants not exposed to Cd. Ouzounidou, Moustakas and Eleftheriou (1997) observed that *T. aestivum* plants exposed to Cd showed dilation of thylakoid membranes, chlorophyll degradation and lower contents of Fe, Mg, K and Ca in the shoots in relation to control plants, inducing the premature senescence of these plants.

In this study, we observed that plants exposed to 0.5 mmol L^{-1} Cd also presented premature senescence, especially when Massai grass was supplied with 3.7 mmol L^{-1} S (Figure 10), which is explicit by highest plastoglobules accumulation in chloroplasts of these plants (Figure 9I). High plastoglobules accumulation occurs due to increase of oxidative stress in the photosynthetic apparatus and the process of leaf senescence (AUSTIN et al., 2006). Besides to cause oxidative stress, Cd decreased the chlorophyll concentration (Figure 11C), *A* (Figure 11D) and, consequently, the leaf area (Figure 11A) and Massai grass growth (Figures 1A-C). Similar results were reported in other studies with forage grasses (XIE et al., 2014; ZHANG; GAO; XIA, 2014; RABÊLO; AZEVEDO; MONTEIRO, 2017a; TAI et al., 2017). Cadmium decreases chlorophyll concentration by inhibiting its synthesis or by replacing Mg^{2+} in chlorophyll molecules, which causes the degradation of these molecules (PARMAR; KUMARI; SHARMA, 2013). Moreover, plants exposed to Cd exhibit low

activity of Fe^{3+} reductase enzyme and formation of Fe plaques in the root system, which may cause Fe^{2+} deficiency in the shoots of plants and compromise the performance of photosynthetic system (IMSANDE, 1998; PARMAR; KUMARI; SHARMA, 2013, HE et al., 2017). It is important to highlight that Massai grass exposed to Cd presented Fe plaques in the roots (Figures 5H and 5L, p. 92) and lower Mg and Fe contents in the shoots (Table 7, p. 91) in relation to plants grown in Cd absence, which contributed to induce premature senescence in these plants (OZOUNIDOU; MOUSTAKAS; ELEFTHERIOU, 1997). Although Cd decreased chlorophyll concentration and *A* of plants, these processes were mitigated when Massai grass was grown without S deprivation (Figures 11C-D). This is because S participates of synthesis of chlorophyll, membrane of thylakoid and Fe-S proteins that are involved in transport of electrons between photosystems I (PSI) and PSII (IMSANDE, 1998), besides to be associated with production of photosynthetic oxygen (GILL; TUTEJA, 2011), and to be component of several antioxidants (e.g. GSH) that act to protect enzymes related to photosynthetic process (CAPALDI et al., 2015). Anjum et al. (2008) reported that *Brassica campestris* plants showed a close correlation between *A* and GSH concentration in plants exposed to Cd, and that proper S supply increased *A* of these plants.

Besides to decrease the chlorophyll concentration (Figure 11C) and *A* (Figure 11D), Cd decreased the g_s (Figure 11E) and c_i (Figure 11F) in the leaf blades of Massai grass, mainly of plants exposed to 0.5 mmol L^{-1} Cd. The stomatal closure may occur due to Cd entry into guard cells in competition with Ca^{+2} (PERFUS-BARBEOCH et al., 2002) and K^+ (POSCHENRIEDER; GUNSE; BARCELÓ, 1989). It should be noted that Massai grass exposed to 0.5 mmol L^{-1} Cd showed lower Ca and K contents than plants not exposed to Cd (Table 7, p. 91). Cadmium also decreases the amount of stomata on the leaf surface (PIETRINI et al., 2010), which associated with stomatal closure results in lower c_i and *A* (PARMAR; KUMARI; SHARMA, 2013). Perfus-Barbeoch et al. (2002) also observed that Cd exposure decreased the g_s and c_i in *Arabdopsis thaliana* plants. Another typical change caused by Cd is related to water use by plants (POSCHENRIEDER; GUNSE; BARCELÓ, 1989). We can verify that WUE (Figure 11H) and WUE_i (Figure 11I) by Massai grass exposed to Cd decreased, mainly in plants supplied with 1.9 and 3.7 mmol L^{-1} S. This result can be attributed to higher resistance caused by Cd to water flow from stems to leaves and inside the guard cells (BARCELÓ; VAZQUEZ; POSCHENRIEDER, 1988; POSCHENRIEDER; GUNSE; BARCELÓ, 1989), and to the increase of transpiration rate (data not shown) caused by highest S concentrations (WANG; WANG; SHANGGUAN, 2016). On the other hand, the highest *k* observed in the leaf blades of plants occurred in plants

exposed to 0.1 mmol L^{-1} Cd that were grown with 3.7 mmol L^{-1} S (Figure 11G), which can be attributed to high *A* (Figure 11D) and to low c_i (Figure 11F) observed in these plants, since *k* is calculated by A/c_i ratio (FARQUHAR; SHARKEY, 1982). This result associated with the absence of toxicity symptoms caused by 0.1 mmol L^{-1} Cd (Figure 10) suggests that Cd did not change the integrity of photosynthetic apparatus of Massai grass, such as reported in *A. thaliana* by Perfus-Barbeoch et al. (2002). Chen and Huerta (1997) also reported that *Hordeum vulgare* plants exposed to Cd presented highest *k* when the plants were grown without S deprivation. From these results, it is clear that Massai grass shows potential for Cd phytoextraction (RABÊLO; JORDÃO; LAVRES, 2017), mainly when compared to others plants (RABÊLO; BORGO, 2016; REEVES et al., 2017) that have been studied for this purpose.

6.5 Conclusions

The low S supply limits the synthesis of glutathione and the photosynthetic process of Massai grass exposed to Cd, while the high S supply to plants exposed to 0.5 mmol L^{-1} Cd decreases the water use efficiency and increases the size of plastoglobules and the starch grains accumulation. Therefore, the low and high S supply decreases the efficiency of Cd detoxification. On the other hand, phytochelatins and sugars such as galactinol and raffinose are more effective to attenuate the oxidative stress caused by Cd to Massai grass than enzymes related to ascorbate-glutathione cycle, although there are findings that isoform Cu/Zn-SOD is indirectly involved in Cd accumulation in this plant. Cadmium accumulation by Massai grass resulted in less growth, but this plant did not show visual symptoms of Cd toxicity when was exposed to 0.1 mmol L^{-1} Cd. Thus, Massai grass can be a Cd hypercumulator plant with potential for Cd phytoextraction, mainly when supplied with 1.9 mmol L^{-1} S.

References

ALCÂNTARA, B.K.; MACHEMER-NOONAN, K.; SILVA JÚNIOR, F.G.; AZEVEDO, R.A. Dry priming of maize seeds reduces aluminum stress. **PLoS One**, San Francisco, v. 10, 2015. doi: 10.1371/journal.pone.0145742.

ALEXIEVA, V.; SERGIEV, I.; MAPELLI, S.; KARANOV, E. The effect of drought and ultravioleta radiation on growth and stress markers in pea and wheat. **Plant, Cell and Environment**, Oxford, v. 24, p. 1337-1344, 2001.

ANDERSON, M.E. Dertermination of glutathione and glutathione disulfide in biological samples. **Methods in Enzymology**, San Diego, v. 113, p. 548-555, 1985.

ANJUM, N.A.; UMAR, S.; AHMAD, A.; IQBAL, M.; KHAN, N.A. Sulphur protects mustard (*Brassica campestris* L.) from cadmium toxicity by improving leaf ascorbato and glutathione. **Plant Growth Regulation**, Dordrecht, v. 54, p. 271-279, 2008.

AUSTIN, J.R.; FROST, E.; VIDI, P.A.; KESSLER, F.; STAEHELIN, L.A. Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contains biosynthetic enzymes. **The Plant Cell**, Rockville, v. 18, p. 1693-1703, 2006.

AZEVEDO, R.A.; ALAS, R.M.; SMITH, R.J.; LEA, P.J. Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. **Physiologia Plantarum**, Lund, v. 104, p. 280-292, 1998.

BAGHERI, R.; AHMAD, J.; BASHIR, H.; IQBAL, M.; QURESHI, M.I. Changes in rubisco, cysteine-rich proteins and antioxidant system of spinach (*Spinacia oleracea* L.) due to sulphur deficiency, cadmium stress and their combination. **Protoplasma**, Wien, v. 254, p. 1031-1043, 2017.

BARCELÓ, J.; VAZQUEZ, M.D.; POSCHENRIEDER, C. Cadmium-induced structural and ultrastructural changes in the vascular system of bush bean stems. **Botanica Acta**, Stuttgart, v. 101, p. 254-261, 1988.

BASHIR, H.; IBRAHIM, M.M.; BAGHERI, R.; AHMAD, J.; ARIF, I.A.; BAIG, M.A.; QURESHI, M.I. Influence of sulfur and cadmium on antioxidants, phytochelatins and growth in Indian mustard. **AoB Plants**, Oxford, v. 7, 2015. doi: 10.1093/aobpla/plv001.

BEAUCHAMP, C.H.; FRIDOVICH, I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. **Analytical Biochemistry**, San Diego, v. 44, p. 276-287, 1971.

BRADFORD, M.M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing principle of protein-dye-binding. **Analytical Biochemistry**, San Diego, v. 72, p. 248-254, 1976.

BUSTIN, S.A.; BENES, V.; GARSON, J.A.; HELLEMANS, J.; HUGGETT, J.; KUBISTA, M.; MUELLER, R.; NOLAN, T.; PFAFFL, M.W.; SHIPLEY, G.L.; VANDESOMPELE, J.; WITTWER, C.T. The MIQE guidelines: Minimum Information for publication of Quantitative realtime PCR Experiments. **Clinical Chemistry**, Washington, DC, v. 55, p. 611-622, 2009.

CAPALDI, F.R.; GRATÃO, P.L.; REIS, A.R.; LIMA, L.W.; AZEVEDO, R.A. Sulfur metabolism and stress defense responses in plants. **Tropical Plant Biology**, New York, v. 8, p. 60-73, 2015.

CHEN, Y.; HUERTA, A.J. Effects of sulfur nutrition on photosynthesis in cadmium-treated barley seedlings. **Journal of Plant Nutrition**, Philadelphia, v. 20, p. 845-856, 1997.

CUADROS-INOSTROZA, A.; CALDANA, C.; REDESTIG, H.; KUSANO, M.; LISEC, J.; PEÑA-CORTÉS, H.; WILLMITZER, L.; HANNAH, M.A. TargetSearch - a Bioconductor package for the efficient preprocessing of GC-MS metabolite profiling data. **BMC Bioinformatics**, London, v. 10, 2009. doi: 10.1186/1471-2105-10-428.

CUYPERS, A.; PLUSQUIN, M.; REMANS, T.; JOZEFCZAK, M.; KEUNEN, E.; GIELEN, H.; OPDENAKKER, K.; NAIR, A.R.; MUNTERS, E.; ARTOIS, T.J.; NAWROT, T.; VANGRONSVELD, J.; SMEETS, K. Cadmium stress: an oxidative challenge. **Biometals**, Dordrecht, v. 23, p. 927-940, 2010.

DELUCIA, E.H.; SASEK, T.W.; STRAIN, B.R. Photosynthetic inhibition after long-term exposure to elevated levels of atmospheric carbon dioxide. **Photosynthesis Research**, Dordrecht, v. 7, p. 175-184, 1985.

DESIKAN, R.; CHEUNG, M.K.; CLARKE, A.; GOLDING, S.; SAGI, M.; FLUHR, R.; ROCK, C.; HANCOCK, J.; NEILL, S. Hydrogen peroxide is a common signal for darkness- and ABA-induced stomatal closure in *Pisum sativum*. **Functional Plant Biology**, Melbourne, v. 31, p. 913-920, 2004.

EPSTEIN, E.; BLOOM, A.J. **Mineral nutrition of plants: principles and perspectives**. 2. ed. Sunderland: Sinauer Associates, 2005. 380 p.

FARMER, E.E.; MUELLER, M.J. ROS-mediated lipid peroxidation and RES-activated signaling. **Annual Review of Plant Biology**, Palo Alto, v. 64, p. 429-450, 2013.

FARQUHAR, G.D.; SHARKEY, T.D. Stomatal conductance and photosynthesis. **Annual Review of Plant Physiology**, Palo Alto, v. 33, p. 317-345, 1982.

FERREIRA, R.R.; FORNAZIER, R.F.; VITÓRIA, A.P.; LEA, P.J.; AZEVEDO, R.A. Changes in antioxidant enzyme activities in soybean under cadmium stress. **Journal of Plant Nutrition**, Philadelphia, v. 25, p. 327-342, 2002.

FUJIMAKI, S.; SUZUI, N.; ISHIOKA, N.S.; KAWACHI, N.; ITO, S.; CHINO, M.; NAKAMURA, S. Tracing cadmium from culture to spikelet: noninvasive imaging and quantitative characterization of absorption, transport and accumulation of cadmium in an intact rice plant. **Plant Physiology**, Rockville, v. 152, p. 1796-1806, 2010.

GIAVALISCO, P.; LI, Y.; MATTHES, A.; ECKHARDT, A.; HUBBERTEN, H.; HESSE, H.; SEGU, S.; HUMMEL, J.; KOHL, K.; WILLMITZER, L. Elemental formula annotation of polar and lipophilic metabolites using ^{13}C, ^{15}N and ^{34}S isotope labelling, in combination with high-resolution mass spectrometry. **Plant Journal**, Hoboken, v. 68, p. 364-376, 2011.

GILL, S.S.; TUTEJA, N. Cadmium stress tolerance in crop plants probing the role of sulfur. **Plant Signaling and Behavior**, Austin, v. 6, p. 215-222, 2011.

GIMENO, J.; EATTOCK, N.; VAN DEYNZE, A.; BLUMWALD, E. Selection and validation of reference genes for gene expression analysis in Switchgrass (*Panicum virgatum*) using quantitative real-time RT-PCR. **PLoS One**, San Francisco, v. 9, 2014. doi: 10.1371/journal.pone.0091474.

GRATÃO, P.L.; POLLE, A.; LEA, P.J.; AZEVEDO, R.A. Making the life of heavy metal-stressed plants a little easier. **Functional Plant Biology**, Melbourne, v. 32, p. 481-494, 2005.

GRATÃO, P.L.; MONTEIRO, C.C.; ROSSI, M.L.; MARTINELLI, A.P.; PERES, L.E.P.; MEDICI, L.O.; LEA, P.J.; AZEVEDO, R.A. Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium. **Environmental and Experimental Botany**, Oxford, v. 67, p. 387-394, 2009.

HABIG, W.H.; PABST, M.J.; JAKOBY, W.B. Gluathione-S-transferases. The first enzymatic step in mercapturic acid formation. **Biological Chemistry**, Berlin, v. 249, p. 7130-7139, 1974.

HABIG, W.H.; JAKOBY, W.B. Assays for differentiation of glutathione-S-transferases. **Methods in Enzymology**, San Diego, v. 77, p. 398-405, 1981.

HASSAN, M.J.; WANG, Z.; ZHANG, G. Sulfur alleviates growth inhibition and oxidative stress caused by cadmium toxicity in rice. **Journal of Plant Nutrition**, Philadelphia, v. 28, p. 1785-1800, 2005.

HE, S.; HE, Z.; YANG, X.; STOFFELLA, P.J.; BALIGAR, V.C. Soil biogeochemistry, plant physiology, and phytoremediation of cadmium-contaminated soils. **Advances in Agronomy**, San Diego, v. 134, p. 135-225, 2015.

HEATH, R.L.; PACKER, L. Photoperoxidation in isolated chloroplasts. I. kinetics and stoichiometry of fatty acid peroxidation. **Archives of Biochemistry and Biophysics**, San Diego, v. 125, p. 189-198, 1968.

HENDRIX, S.; SCHRÖDER, P.; KEUNEN, E.; HUBER, C.; CUYPERS, A. Molecular and cellular aspects of contaminant toxicity in plants: the importance of sulphur and associated signalling pathways. In: CUYPERS, A.; VANGRONSVELD, J. (Ed.). **Phytoremediation**. London: Elsevier, 2017. chap. 6, p. 223-276.

IMSANDE, J. Iron, sulfur, and chlorophyll deficiencies: A need for a integrative approach in plant physiology. **Physiologia Plantarum**, Lund, v. 103, p. 139-144, 1998.

JOZEFCZAK, M.; BOHLER, S.; SCHAT, H.; HOREMANS, N.; GUISEZ, Y.; REMANS, T.; VANGRONSVELD, J.; CUYPERS, A. Both the concentration and redox state of glutathione and ascorbate influence the sensitivity of arabidopsis to cadmium. **Annals of Botany**, Oxford, v. 116, p. 601-612, 2015.

KARNOVSKY, M.J. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. **Journal of Cell Biology**, New York, v. 27, p. 137-138, 1965.

KEUNEN, E.; PESHEV, D.; VANGRONSVELD, J.; van den ENDE, W.; CUYPERS, A. Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. **Plant, Cell and Environment**, Oxford, v. 36, p. 1242-1255, 2013.

KEUNEN, E.; SCHELLINGEN, K.; STRAETEN, D.V.D.; REMANS, T.; COLPAERT, J.; VANGRONSVELD, J.; CUYPERS, A. ALTERNATIVE OXIDASE1a modulates the oxidative challenge during moderate Cd exposure in *Arabidopsis thaliana* leaves. **Journal of Experimental Botany**, Oxford, v. 66, p. 2967-2977, 2015.

KHAN, M.I.R.; NAZIR, F.; ASGHER, M.; PER, T.S.; KHAN, N.A. Selenium and sulfur influence ethylene formation and alleviate cadmium-induced oxidative stress by improving proline and glutathione production in wheat. **Journal of Plant Physiology**, Jena, v. 173, p. 9-18, 2015.

KHAN, N.A.; ASGHER, M.; PER, T.S.; MASOOD, A.; FATMA, M.; KHAN, M.I.R. Ethylene potentiates sulfur-mediated reversal of cadmium inhibited photosynthetic responses in Mustard. **Frontiers in Plant Science**, Lausanne, v. 7, 2016. doi: 10.3389/fpls.2016.01628.

KHAN, M.A.; KHAN, S.; KHAN, A.; ALAM, M. Soil contamination with cadmium, consequences and remediation using organic amendments. **Science of the Total Environment**, Amsterdam, v. 601-602, p. 1591-1605, 2017.

KRAUS, T.E.; McKERSIE, B.D.; FLETCHER, R.A. Paclobutrazol-induced tolerance of wheat leaves to paraquat may involve increased antioxidant enzyme activity. **Journal of Plant Physiology**, Jena, v. 145, p. 570-576, 1995.

KULAEVA, O.A.; TSYGANOVA, V.E. Molecular-Genetic basis of cadmium tolerance and accumulation in higher plants. **Russian Journal of Genetics**, New York, v. 1, p. 349-360, 2011.

LAEMMLI, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature**, London, v. 227, p. 680-685, 1970.

LIANG, T.; DING, H.; WANG, G.; KANG, J.; PANG, H.; LV, J. Sulfur decreases cadmium translocation and enhances cadmium tolerance by promoting sulfur assimilation and glutathione metabolism in *Brassica chinensis* L. **Ecotoxicology and Environmental Safety**, San Diego, v. 124, p. 129-137, 2016.

LISEC, J.; SCHAUER, N.; KOPKA, J.; WILLMITZER, L.; FERNIE, A.R. Gas chromatography mass spectrometry-based metabolite profiling in plants. **Nature Protocols**, London, v. 1, p. 387-396, 2006.

LOIX, C.; HUYBRECHTS, M.; VANGRONSVELD, J.; GIELEN, M.; KEUNEN, E.; CUYPERS, A. Reciprocal interactions between cadmium-induced cell wall responses and oxidative stress in plants. **Frontiers in Plant Science**, Lausanne, v. 8, 2017. doi: 10.3389/fpls.2017.01867.

LONG, S.P.; BERNACCHI, C.J. Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. **Journal of Experimental Botany**, Oxford, v. 4, p. 2393-2401, 2003.

LUO, H.; LI, H.; ZHANG, X.; FU, J. Antioxidant responses and gene expression in perennial ryegrass (*Lolium perenne* L.) under cadmium stress. **Ecotoxicology**, Dordrecht, v. 20, p. 770-778, 2011.

MALAVOLTA, E.; VITTI, G.C.; OLIVEIRA, S.A. **Avaliação do estado nutricional das plantas**: princípios e aplicações. 2. ed. Piracicaba: Potafos, 1997. 201 p.

MASOOD, A.; IQBAL, N.; KHAN, N.A. Role of ethylene in alleviation of cadmium-induced photosynthetic capacity inhibition by sulphur in mustard. **Plant, Cell and Environment**, Oxford, v. 35, p. 524-533, 2012.

MATSUNO, H.; URITANI, I. Physiological behavior of peroxidase isozymes in sweet potato root tissue injured by cutting or with black rot. **Plant and Cell Physiology**, Oxford, v. 13, p. 1091-1101, 1972.

MENDOZA-CÓZATL, D.G.; JOBE, T.O.; HAUSER, F.; SCHROEDER, J.I. Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. **Current Opinion in Plant Biology**, London, v. 14, p. 554-562, 2011.

NAKANO, Y.; ASADA, K. Hydrogen peroxide is scavenged by ascorbate-especific peroxidase in spinach chloroplasts. **Plant and Cell Physiology**, Oxford, v. 22, p. 867-880, 1981.

NEILL, S.J.; DESIKAN, R.; CLARKE, A.; HURST, R.D.; HANCOCK, J.T. Hydrogen peroxide and nitric oxide as signalling molecules in plants. **Journal of Experimental Botany**, Oxford, v. 53, p. 1237-1247, 2002.

NISHIZAWA, A.; YABUTA, Y.; SHIGEOKA, S. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. **Plant Physiology**, Rockville, v. 147, p. 1251-1263, 2008.

NOCTOR, G.; MHAMDI, A.; CHAOUCH, S.; HAN, Y.; NEUKERMANS, J.; MARQUEZ-GARCIA, B.; QUEVAL, G.; FOYER, C.H. Glutathione in plants: an integrated overview. **Plant, Cell and Environment**, Oxford, v. 35, p. 454-484, 2012.

OGAWA, K.; KANEMATSU, S.; ASADA, K. Generation of superoxide anion and localization of CuZn-Superoxide dismutase in the vascular tissue of Spinach hypocotyls: their association with lignification. **Plant and Cell Physiology**, Oxford, v. 38, p. 1118-1126, 1997.

OUZOUNIDOU, G.; MOUSTAKAS, M.; ELEFTHERIOU, E.P. Physiological and ultrastructural effects of cadmium on Wheat (*Triticum aestivum* L.) leaves. **Archives of Environmental Contamamination and Toxicology**, New York, v. 32, p. 154-160, 1997.

PARMAR, P.; KUMARI, N.; SHARMA, V. Structural and functional alterations in photosynthetic apparatus of plants under cadmium stress. **Botanical Studies**, New York, v. 54, p. 45-51, 2013.

PERFUS-BARBEOCH, L.; LEONHARDT, N.; VAVASSEUR, A.; FORESTIER, C. Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. **Plant Journal**, Hoboken, v. 32, p. 539-548, 2002.

PIETRINI, F.; ZACCHINI, M.; IORI, V.; PIETROSANTI, L.; FERRETTI, M.; MASSACCI, A. Spatial distribution of cadmium in leaves and on photosynthesis: examples of different strategies in willow and poplar clones. **Plant Biology**, Hoboken, v. 12, p. 355-363, 2010.

POSCHENRIEDER, C.; GUNSE, B.; BARCELÓ, J. Influence of cadmium on water relations, stomatal resistance, and abscisic acid content in expanding bean leaves. **Plant Physiology**, Rockville, v. 90, p. 1365-1371, 1989.

RABÊLO, F.H.S.; BORGO, L. Changes caused by heavy metals in micronutrient content and antioxidant system of forage grasses used for phytoremediation: an overview. **Ciência Rural**, Santa Maria, v. 46, p. 1368-1375, 2016.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. Proper supply of S increases GSH synthesis in the establishment and reduces tiller mortality during the regrowth of Tanzania guinea grass used for Cd phytoextraction. **Journal of Soils and Sediments**, Heidelberg, v. 17, p. 1427-1436, 2017a.

RABÊLO, F.H.S.; AZEVEDO, R.A.; MONTEIRO, F.A. The proper supply of S increases amino acid synthesis and antioxidant enzyme activity in Tanzania guinea grass used for Cd phytoextraction. **Water, Air and Soil Pollution**, Dordrecht, v. 228, 2017b. doi: 10.1007/s11270-017-3563-6.

RABÊLO, F.H.S.; JORDÃO, L.T.; LAVRES, J. A glimpse into the symplastic and apoplastic Cd uptake by Massai grass modulated by sulfur nutrition: Plants well-nourished with S as a strategy for phytoextraction. **Plant Physiology and Biochemistry**, Paris, v. 121, p. 48-57, 2017.

REEVES, R.D.; BAKER, A.J.M.; JAFFRÉ, T.; ERSKINE, P.D.; ECHEVARRIA, G.; van der ENT, A. A global database for plants that hyperaccumulate metal and metalloid trace elements. **New Phytologist**, London, 2017. doi: 10.1111/nph.14907.

REMANS, T.; SMEETS, K.; OPDENAKKER, K.; MATHIJSEN, D.; VANGRONSVELD, J.; CUYPERS, A. Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. **Planta**, Berlin, v. 227, p. 1343-1349, 2008.

REMANS, T.; KEUNEN, E.; JAN BEX, G.; SMEETS, K.; VANGRONSVELD, J.; CUYPERS, A. Reliable Gene Expression Analysis by Reverse TranscriptionQuantitative PCR: Reporting and Minimizing the Uncertainty in Data Accuracy. **The Plant Cell**, Rockville, v. 26, p. 3829-3837, 2014.

REYNOLDS, E.S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. **Journal of Cell Biology**, New York, v. 17, p. 208-212, 1963.

ROZEN, S.; SKALETSKY, H. Primer3 on the WWW for general users and for biologist programmers. In: MISENER, S.; KRAWETZ, S.A. (Ed.). **Bioinformatics methods and protocols**. Totowa: Humana Press, 2000. chap. 20, p. 365-386.

SAS INSTITUTE. **SAS User's guide**: Statistics. Version 9.2. Cary, NC, 2008. Available at: <http://support.sas.com/software/92/ Cary-United States of America>. Access in: oct. 26. 2017.

SHEORAN, V.; SHEORAN, A.S.; POONIA, P. Factors affecting phytoextraction: a review. **Pedosphere**, Beijing, v. 26, p. 148-166, 2016.

SMITH, I.K.; VIERHELLER, T.L.; THORNE, C.A. Assay of glutathione reductase in crude tissue homogenates using 5,5'-dithilbis (2-nitrobenzoic acid). **Analytical Biochemistry**, San Diego, v. 175, p. 408-413, 1988.

STACKLIES, W.; REDESTIG, H.; SCHOLZ, M.; WALTHER, D.; SELBIG, J. pcaMethods a bioconductor package providing PCA methods for incomplete data. **Bioinformatics**, Oxford, v. 23, p. 1164-1167, 2007.

TAI, Z.; YIN, X.; FANG, Z.; SHI, G.; LOU, L.; CAI, Q. Exogenous GR24 alleviates cadmium toxicity by reducing cadmium uptake in Switchgrass (*Panicum virgatum*) seedlings. **International Journal of Environmental Research and Public Health**, Basel, v. 14, 2017. doi: 10.3390/ijerph14080852.

WANG, X.; WANG, L.; SHANGGUAN, Z. Leaf gas exchange and fluorescence of two winter wheat varieties in response to drought stress and nitrogen supply. **PLoS One**, San Frrancisco, v. 11, 2016. doi: 10.1371/journal.pone.0165733.

WECKWERTH, W.; WENZEL, K.; FIEHN, O. Process for the integrated extraction, identification and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks. **Proteomics**, Weinheim, v. 4, p. 78-83, 2004.

WITKOWSKI, E.T.F.; LAMONT, B.B. Leaf specific mass confounds leaf density and thickness. **Oecologia**, New York, v. 88, p. 486-493, 1991.

XIE, Y.; HU, L.; DU, Z.; SUN, X.; AMOMBO, E.; FAN, J.; FU, J. Effects of cadmium exposure on growth and metabolic profile of Bermudagrass [*Cynodon dactylon* (L.) Pers.]. **PLoS One**, San Francisco, v. 9, 2014. doi: 10.1371/journal.pone.0115279.

ZHANG, X.; GAO, B.; XIA, H. Effect of cadmium on growth, photosynthesis, mineral nutrition and metal accumulation of bana grass and vetiver grass. **Ecotoxicology and Environmental Safety**, San Diego, v. 106, p. 102-108, 2014.

7 GENERAL CONCLUSIONS

The highly cadmium accumulation in apoplast, development of lignified root barriers, and the synthesis of GSH, PCs, amino acids and sugars (notably, raffinose and galactinol) are one of the main Cd detoxification mechanisms of Massai grass. On the other hand, ascorbate-glutathione cycle is apparently not as important for Massai grass as Cd detoxification mechanisms cited in the phrase earlier.

Massai grass showed more efficient Cd detoxification mechanisms when was supplied with 1.9 mmol L^{-1} S in relation to 0.1 and 3.7 mmol L^{-1} S. Although the growth of this forage grass decreased with Cd exposure, there were no visual symptoms of Cd toxicity caused by 0.1 mmol L^{-1} Cd, which suggest that Massai grass shows potential for Cd phytoextraction. In this sense, since Massai's physiology and biochemistry were deeply investigated in this current study, it is really worth carrying out new studies with this genotype/phenotype in order to evaluate its Cd phytoextraction capacity in *soils*, since in these substrates there are many others factors, as well as soil-plant-microorganisms interactions to be considered in studies of phytoextraction.