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A glimpse into the effect of sulfur supply on metabolite profiling, glutathione and phytochelatins in *Panicum maximum* cv. Massai exposed to cadmium

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ABSTRACT

Sugar, amino and organic acid, glutathione (GSH) and phytochelatin (PC) content in plant tissues can be altered by S nutrition, and these metabolites have the potential to lower a plant's susceptibility to cadmium (Cd) toxicity. 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) used for Cd phytoextraction, since this forage grass shows fast growth, high biomass production, and adaptation to soil and climatic adversities. We evaluated in a greenhouse 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}) in nutrient solution, within a single growth period. The tissues of plants exposed to Cd showed distinct responses related to the primary metabolism. Tryptophan, lysine, and histidine were more accumulated in all tissues when Massai grass was exposed to Cd and was grown with 1.9 and/or 3.7 mmol L^{-1} S, which indicates that these amino acids are probably involved in Cd accumulation and detoxification in this plant. Among the sugars and sugar derivatives detected, galactinol appears to be the most active in decrease Cd-induced oxidative stress. Although there was no effect of Cd/S combinations on the expression of the genes encoding *GSH1* and *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 the majority of metabolites evaluated in this study was mostly induced when Cd-exposed Massai grass was supplied with 1.9 mmol L^{-1} S, but more studies in field conditions are necessary to define the optimal S concentration for the plants grown in soil.

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, 2012), and it can be taken up by plants and thereby enter the human food chain (Sanità di Toppi and Gabbrielli, 1999). Recent studies show that human Cd intake was above the limits recommended by the US Agency for Toxic Substances and

Abbreviations: GSH, glutathione; h-GSH, homoglutathione; γ-ECS, γ-glutamylcysteine synthetase; *GSHS*, glutathione synthetase; *hGSHS*, homoglutathione synthetase; PCs, phytochelatins; h-PCs, homophytochelatins; des Gly-PC, desglycine phytochelatins; cys-PCs, isoforms of phytochelatins; *PCS*, phytochelatin synthase; ROS, reactive oxygen species; NPTs, non-protein thiols; ICP-OES, inductively coupled plasma optical emission spectrometry; GC–TOF–MS, high performance gas chromatography with mass spectrometer; HPLC, high performance liquid chromatograph; PCR, polymerase chain reaction; qPCR, reverse transcription PCR in real-time; RNA, ribonucleic acid; RNAse, ribonuclease; cDNA, complementary DNA; dsDNA, double-stranded DNA; DNAse, deoxyribonuclease; MIQE, Minimum Information for publication of Quantitative real-time PCR Experiments; PCA, principal component analysis; OAS, O-acetylserine; OAA, oxaloacetate; 2-OG, 2-oxoglutarate or α-Ketoglutarate; GABA, gamma-aminobutyric acid or 4-Aminobutanoate; Glu-6P, glucose-6-phosphate; Ala, alanine; ß-Ala, ß-alanine; Asn, asparagine; Asp, aspartate; Lys, lysine; Met, methionine; Ser, serine; Gly, glycine; Thr, threonine; Cys, cysteine; Trp, tryptophan; Phe, phenylalanine; Tyr, tyrosine; Leu, leucine; Ile, isoleucine; Val, valine; Glu, glutamate; Gln, glutamine; His, histidine; Pro, proline; Arg, arginine; ND, non-detected

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Disease Registry (ATSDR) and the European Food Safety Authority (EFSA; Clemens et al., 2013). Given that Cd can cause a number of human diseases including hypertension, pulmonary emphysema and cancer (World Health Organization, 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 et al., 2016). However, very few plants are tolerant to Cd toxicity (Sanità di Toppi and Gabbrielli, 1999), with merely nine plants (*Picris divaricata*, *Nocceae caerulescens*, *Noccaea praecox*, *Arabidopsis haller*, *Arabis paniculata*, *Sedum alfredii*, *Phytolacca americana*, *Potentilla griffithii* and *Viola boashanensis*) being considered as Cd hyperaccumulators (Meyer and Verbruggen, 2012). In addition, these plants grow slowly and as a result do not produce high biomass (Meyer and Verbruggen, 2012), thereby decreasing their efficiency of phytoextraction (Sheoran et al., 2016). As an alternative, the use of forage grasses for Cd phytoextraction has been shown to be promising (Xie et al., 2014; Rabêlo and Borgo, 2016; Rabêlo et al., 2017a, 2017b, 2017c, 2018a, 2018b) 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 et al., 2016) and can likely be optimized with a well-balanced sulfur (S) supply (Rabêlo et al., 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 and 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 $(\gamma\text{-Glu-Cys})_n$-Gly (with n = 2–11) are synthesized from GSH by phytochelatin synthase (PCS) (Cobbett and Goldsbrough, 2002) and show structural variations such as desglycine phytochelatins [desGly-PC, $(\gamma\text{-Glu-Cys})_n$] (Bernhard and 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 et al., 1986). Phytochelatins act on Cd chelation and are involved both in its transport from the cytosol to vacuoles (Cobbett and 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 et al., 2006). Jozefczak 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 ($O_2^{\bullet -}$), 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 and 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 and 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 et al., 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 and 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 et al., 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.

2. Methods

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 (22°42′ south latitude and 47°38′ 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 of nutrient solution used in the study is shown in Table 1. The pots used in the study were placed in a randomized block design, consisting of four biological replicates per condition.

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
$MgSO_4{\cdot}7H_2O$ (1 mol L^{-1})	0.1	0.1	0.1	1.9	1.9	1.9	2	2	2
$MgCl_2{\cdot}6H_2O$ (1 mol L^{-1})	1.9	1.9	1.9	0.1	0.1	0.1	–	–	–
$CaSO_4{\cdot}2H_2O$ (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[a]	1	1	1	1	1	1	1	1	1
Fe(III) – EDTA[b]	1	1	1	1	1	1	1	1	1

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

[b] Fe(III)-EDTA = 100 μmol L^{-1}.