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

Silicon and tanzania guinea grass tolerance to stress by copper toxicity

Leandro Otavio Vieira Filho

Dissertation presented to obtain the degree of Master in Science. Area: Soil and Plant Nutrition

Piracicaba
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1. Elemento benéfico 2. Estresse oxidativo 3. Fitorremediação 4. Forrageiras 5. Interação Cu × Si 6. Toxidez por metal I. Título

DEDICATION

To my missed father,
to my dear mom,
to my supportive godparents Leandrea and Raul,
to my niece Terezinha and my sister-in-law Claizete,
to all those who believed on me,
I dedicate**.**

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EPIGRAPH

“Today is hard, tomorrow will be worse, but the day after tomorrow will be sunshine.”.

Jack Ma

SUMMARY

RESUMO

Silício e a tolerância do capim-tanzânia ao estresse pela toxidez por cobre

O cobre (Cu) é um elemento essencial para as plantas, porém, quando em excesso, pode causar danos irreversíveis às plantas. Este metal induz a produção excessiva de espécies reativas de oxigênio (ERO), que danificam organelas causando a disfunção delas. Uma possível maneira de aumentar a tolerância de plantas aos metais é o fornecimento de silício (Si). Um experimento foi conduzido com o objetivo de avaliar o papel do Si (0, 1 e 3 mmol L^{-1}) nas respostas morfológicas, nutricionais, metabólicas e fisiológicas do *Panicum maximum* cv. Tanzânia sob doses de Cu (0,3, 250, 500 e 750 μmol L^{-1}). Esse capim foi cultivado hidroponicamente em casa de vegetação por dois períodos de crescimento (33 e 30 dias). Treze dias após a semeadura, plântulas foram transplantadas para solução nutritiva, fornecendo-se apenas as doses de Si por 25 dias. A exposição ao cobre foi realizada apenas no primeiro crescimento das plantas e durou sete dias. O segundo corte ocorreu 31 dias após o primeiro corte. O experimento consistia de seis blocos completos ao acaso: três para avaliações de produção, morfologia e análises nutricionais e três para análises metabólicas e fisiológicas. A produção, a morfologia e o metabolismo das plantas foram quantificados na parte aérea e nas raízes. O índice de conteúdo de clorofila (valores SPAD) e as análises fisiológicas foram determinados nas lâminas diagnósticas (LD), e as concentrações de Cu e Si nas LD e nas raízes. Para o cálculo dos acúmulos de Cu e Si levou-se em consideração toda a biomassa da planta. Plantas expostas a doses de Cu acima de 0,3 μmol L^{-1} apresentaram menores valores de produtividade, parâmetros morfológicos e de SPAD. Plantas supridas com Si apresentaram menor concentração e acúmulo de Cu, e maiores valores de produtividade, parâmetros morfológicos e SPAD do que aquelas que não receberam o fornecimento de Si. A concentração e o acúmulo de silício foram maiores nas plantas expostas ao excesso de Cu do que nas expostas à dose controle de Cu (0,3 μmol L^{-1}). Os parâmetros de trocas gasosas das plantas no primeiro crescimento foram afetados positivamente pelo Si e negativamente pelo incremento nas doses de Cu. No segundo crescimento, observou-se evento de eustresse em que plantas expostas à dose de Cu residual apresentaram os valores mais altos de parâmetros de troca gasosa e os valores mais baixos de indicadores de estresse. As atividades de enzimas antioxidantes foram reduzidas com o incremento nas doses de Cu. O suprimento de silício resultou em incremento na atividade da superóxido dismutase (SOD). O capim tanzânia suplementado com Si foi capaz de suportar melhor a toxicidade do Cu, mostrando um aumento na produção de biomassa da planta, e em parâmetros morfológicos e de trocas gasosas. As plantas suplementadas com Si reduziram a absorção de Cu e, consequentemente, plantas expostas a altas taxas de Cu e suplementadas com Si ainda foram capazes de produzir uma biomassa apreciável na rebrota.

Palavras-chave: Elemento benéfico; Estresse oxidativo; Fitorremediação; Forrageiras; Interação Cu × Si; Toxidez por metal

ABSTRACT

Silicon and tanzania guinea grass tolerance to stress by copper toxicity

Whist copper (Cu) is an essential element for plants, when this element is present in excess quantities it can cause irreversible damage. This metal induces excessive production of reactive oxygen species (ROS), which damages organelles causing dysfunction. A possible means for the promotion of metal tolerance in plants is the adition of the element silicon (Si). The current study was conducted with the aim of evaluating the role of Si (0, 1 and 3 mmol L^{-1}) on the morphologic, nutritional, metabolic and physiological responses of *Panicum maximum* cv. Tanzania under different Cu rates (0.3, 250, 500 and 750 µmol L^{-1}). The grass was grown in a greenhouse under hydroponic conditions for two growth periods (33 and 30 days). Thirteen days after sowing, the seedlings were transplanted to a nutrient solution and supplied just with the Cu rate of 0.3 µmol L^{-1} and the set Si rates for 25 days. The remaining Cu rates were only added for a seven day period during the first growth stage. The second harvest took place 31 days after the first harvest. The experiment had six randomized blocks: three for yield, morphology and nutritional analyses and three for metabolic and physiological analyses. Plant yield, morphology and metabolic parameters were quantified in shoots and roots. Chlorophyll content index (SPAD values) and gas exchange parameters were determined in diagnostic leaves (DL), and Cu and Si concentrations were analysed from the DL and roots. The calculation of Cu and Si contents took into account the whole plant biomass. Plants exposed to Cu rates above 0.3 µmol L^{-1} showed low values of plant yield, morphologic parameters and SPAD, in both growth periods. Silicon supplied plants showed lower Cu concentration and content, and higher values of plant yield, morphlogic parameters and SPAD than the ones with no Si application. Silicon concentration and content were higher in plants exposed to excess Cu compared to those exposed to the control rate (0.3 µmol L^{-1}). Gas exchange parameters in plants of the first growth were positively affected by Si supply and negatively affected by Cu rates. In the second growth, an eustress event was observed, in which plants exposed to stressing rates of residual Cu showed the highest values of gas exchange parameters and the lowest values of stress indicators. The activities of antioxidant enzymes were reduced with the increment in Cu rates. Silicon supply resulted in an increment in superoxide dismutase (SOD) activity. Tanzania guinea grass supplied with Si was able to better deal with Cu toxicity, showing increases in plant yield, morphologic and gas exchange parameters. Silicon supplied plants reduced their absorption of Cu and consequently, plants exposed to high Cu rates were still able to produce considerable biomass in the regrowth.

Keywords: Beneficial element; Cu × Si interaction; Forage grass; Metal toxicity; Oxidative stress; Phytoremediation

1. INTRODUCTION

The total area of permanent pasture in Brazil is about 197 million hectares, corresponding to 71% of the country's agricultural area and 23% of total land area (FAO 2012). Due to the fact that most of Brazil's land area is located within tropical climate zones, tropical pastures are responsible for feeding the majority of the national herd. *Panicum maximum* is a tropical forage grass species that is grown throughout ~20% of the 100 million hectares of cultivated pastures in Brazil (Torres et al. 2016). This species has a number of cultivars, including tanzania grass, which is characterized by its rusticity, high leaf/stem ratio, high seed production, high regrowth rate and low seasonality (Jank et al. 2010, Jank et al. 2013). Besides the use of this grass for animal feeding and ground cover, its potential for phytoextraction of contaminant metals has been invetigated in a number of studies (Monteiro et al. 2011, Gilabel et al. 2014, Rabêlo et al. 2016).

Plant species that are used for recovery of contaminated soils have the ability to absorb metals from within the environment. However, the efficiency of such plants can be limited by the presence of these chemicals within the substrate. A benefit of tanzania guinea grass in phytoremediation is that it is relatively tolerant to excess metals such as copper (Cu), cadmium (Cd) and barium (Ba), and has the ability to maintain high productivity in highly contaminated environments (Monteiro et al. 2011, Gilabel et al. 2014, Rabêlo et al. 2016). Additionally, the regrowth of the forage grasses results in a greater phytoextraction of the metal as the plant does not require resowing.

Heavy metals have adverse effects on human health and therefore the contamination of the food chain by these metals deserves special attention (Ali et al. 2013, Zeng et al. 2015). Some metals such as Cu, are essential for plants, classified as a micronutrient due to the low concentration in plant tissue, which reflects the low demand in most cultivated plants. Copper acts in many functions in the plant, mainly related to photosynthesis, respiration, metabolism of reactive oxygen species (ROS), remodelling of the cell wall and stacking of thylakoids (Burkhead et al. 2009). The excess of this metal in plants induces excessive production of reactive oxygen species (ROS), which damage mitochondria, chloroplasts and peroxisomes of the cells, causing dysfunction (Chandna et al. 2012). Plant processes are affected by excess Cu because these organelles are responsible for the main metabolic processes, including the production of chemical energy, photosynthesis, photorespiration, oxidative phosphorylation, β-oxidation and tricarboxylic acid cycle. Copper is naturally present in soils as rocks, sediments and minerals (Baker 1990). In addition to weathering, other additions of anthropic Cu to the soil can occur via fertilizers, sewage sludge, pesticides (sulfate, oxychloride, Bordeaux syrup, among others) and atmospheric deposition by dust, rain and industrial smoke (Malavolta 2006).

Research aiming to enhance the phytoremediation potential of the plants and to modulate the phytoextraction of the metal has been conducted by adding nutrients and beneficial elements such as silicon (Si) to the growth medium. The beneficial effects of Si in the relief of abiotic stress, especially those caused by heavy metals, are attributed to its deposition mainly in the cell walls of roots, leaves and stems, which creates binding sites for metals. In addition, Si can reduce apoplastic flow and consequently, the translocation of toxic metals (Ma &Yamaji 2006). Silicon may also contribute to the compartmentalization of heavy metals within the cell vacuole, as it can attach to the metal and carry it further into the compartment (Wang et al. 2015). Although Si has already been shown to be effective in reducing toxicity effects of excess Cu in certain species, this effect has not yet been proven for tropical grass, such as tanzania guinea grass.

In this study, the main objective was to evaluate the effect of Si application on the metabolic, physiological, nutritional and productive attributes of *Panicum maximum* cv. Tanzania exposed to differing Cu rates in nutrient solution in greenhouse conditions.

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2. SILICON MODULATES COPPER ABSORPTION AND INCREASES YIELD OF TANZANIA GUINEA GRASS UNDER COPPER TOXICITY

ABSTRACT

Silicon (Si) is a beneficial element which has proved to enhance the tolerance of plants to excess metal in a given growth medium. However, the efficacy of Si in mitigating Cu toxicity in plants can vary between plant species and with the amount of copper (Cu) present in the soil/medium. An experiment was performed to investigate the role of Si (0, 1 and 3 mmol L^{-1}) in alleviating Cu toxicity (0.3, 250, 500 and 750 µmol L^{-1}) in Tanzania guinea grass (*Panicum maximum* cv. Tanzania). The grass was grown for 63 days in a greenhouse under hydroponic conditions. Thirteen days after sowing, seedlings were transplanted to pots and grown for a further 25 days, and then exposed to the set Cu rates for seven days. Plants in the second growth period were also evaluated in order to test the influence of residual Cu and Si supply on the regrowth of the plants. Plant yield and morphology were determined in shoots (dry weight, and number of tillers and leaves) and roots (dry weight, surface area and length). Chlorophyll content, Cu and Si concentrations were determined in the diagnostic leaves (DL) and roots, and calculations of Cu and Si contents took into account the whole shoots and roots. The results confirmed that the supply of Si to Tanzania guinea grass can alleviate the effects of excessive Cu. Plant yield and chlorophyll contents increased with Si supply and decreased with the increment of Cu rates in both growth periods. Copper concentration in DL and in roots, and Cu content in shoots and roots were higher in plants exposed to Cu of 750 µmol L^{-1} with no Si application than in other combinations. Silicon concentration in shoots and roots were highest in plants that were supplied with 3 mmol L^{-1} of Si and exposed to 750 µmol L^{-1} of Cu. However, Si content in roots and shoots increased as the Cu concentration decreased and Si supplementation increased. Besides reducing Cu concentration in plant tissues, the most important Si role was to reduce the transport of Cu from roots to shoots.

Keywords: Forage grass; Interaction Cu × Si; Phytoremediation; Trace elements

2.1. Introduction

Copper is an essential element for plants, being directly involved in the photosynthetic electron transport chain (Maksymiec 1997). Despite its importance, Cu in excess may cause irreversible damages to plants. This element has been widely used in the formation of pesticides for the control of fungal diseases in crops around the world (Megateli et al. 2013), which has led to numerous cases of Cu toxicity in plants. Along with the increased Cu from pesticides, other forms of anthropogenic Cu contamination include: (1) mining and smelting of other metals; (2) industrial activity and; (3) improper waste disposal in soil (Nogueirol et al. 2010). In order to remediate the contaminated environment, some plant species with phytoextraction potential are being studied.

Phytoremediation is a sustainable and eco-friendly technique that can be applied in metal contaminated soils. Besides having a considerable metal tolerance, the chosen species must have phytoextraction potential. *Panicum maximum* cv. Tanzania possesses these characteristics and studies are beginning to confirm its potential as a promising species for phytoextraction (Monteiro et al. 2011, Gilabel et al. 2014, Rabêlo et al. 2016, Souza Junior et al. 2018). Compared to hyperaccumulating species, Tanzania guinea grass has higher Cu, Cd and Ba extraction potential because it is able to accumulate more of the metal due to its higher biomass production. Also, this grass can regrow after the harvest of its biomass, thus alleviating the need for resowing, simplifying and accelerating the environment recovery.

Plants contain dozens of enzymes and proteins containing Cu, and this metal acts as an electron carrier in photosynthesis and as a receptor of chemical energy derived from proteins and oxidative enzymes (Epstein & Bloom 2004). Excess copper induces excessive production of reactive oxygen species (ROS), which damage mitochondria, chloroplasts and peroxisomes of the cells, causing the dysfunction of these organelles that are responsible for the main metabolic processes, including the production of chemical energy, photosynthesis, photorespiration, oxidative phosphorylation, β-oxidation and tricarboxylic acid cycle (Chandna et al. 2012).

Studies that aim to enhance the phytoremediation potential of plants and to modulate the phytoextraction of metals have been conducted by adding chemical elements to the growth medium. Silicon (Si) is widely reported as a beneficial growth-promoting element, which promotes protection against biotic (Van Bockhaven et al. 2013, Vulavala et al. 2016) and abiotic stresses (Kim et al. 2014, Abdula et al. 2016, Cooke & Leishman 2016). Plants well-nourished with Si are able to better deal with metal contamination due to the capacity of Si in holding the metal in the cell wall by providing binding sites (Li et al. 2008). Silicon also acts on lowering apoplastic efflux inducing less metal transport (Ma & Yamaji 2006), and metal compartmentalization in cell vacuole (Wang et al. 2015). Silicon has presented satisfactory results on improving Cu tolerance in plants, but this has not yet been proven for tropical grasses such as tanzania guinea grass.

It is believed that Si plays an important role on alleviating Cu toxicity in tanzania guinea grass. Silicon may contribute to the increase in the tolerance of tanzania guinea grass to Cu toxicity, especially in relation to the protection of the photosynthetic apparatus as a consequence of the reduction of Cu transport from roots to shoot (Adrees et al. 2015). The reduction of Cu transport in shoots may sound counterintuitive for the purpose of phytoextraction of this metal from the substrate but reducing the rate of uptake prevents the plant from absorbing excessive concentrations of Cu which leads to a reduction in biomass production and therefore a reduction in overall phytoextraction from the plant as a whole.

The aim of the present study was to evaluate the role of Si on the enhancement of phytoextraction potential of tanzania guinea grass exposed to stressing Cu rates by measuring plant yield, chlorophyll contents, and concentrations and contents of Cu and Si.

2.2. Material and Methods

2.2.1. Experimental conditions

The experiment was carried out in greenhouse conditions and tanzania guinea grass (*Panicum maximum* cv. Tanzania) was used. This grass was chosen based on previous studies, which evidenced its tolerance to metal stress. Seedlings were obtained in sand washed with deionized water. Thirteen days after sowing, the seedlings reached 4-5 cm height and they were transplanted to 3.5 L pots (15 seedlings per pot) filled with ground quartz as substrate. In the first five days after transplantation the Hoagland and Arnon (1950) solution was used with 20% of ionic force in order to adapt the seedlings to the growth medium. Afterward the nutrient solution was used with 100% of ionic force.

The experiment was set in complete randomized block design in a 3x4 factorial, with three replications. Based on preceding researchs, it was adopted three Si rates (0, 1 and 3 mmol L^{-1}) and four Cu rates (0.3, 250, 500 and 750 µmol L^{-1}). After the first week of seedlings adaptation to hydroponic condition, thinning was started and proceeded until five seedlings remained per pot. Copper rates were applied at the 25th day after transplantation. Except for the seven days of Cu exposure period, the concentration of Cu was unique, 0.3 µmol L^{-1}, but plants in the experimental units were supplied with their respective Si rates.

Aiming to evaluate the effects of Cu on the regrowth of tanzania guinea grass, plants were evaluated in two growth periods. The harvesting time was determined in the first growth period by severe Cu toxicity and in the second one by the plant senescence. After seven days of metal exposure, part of the plants was severely intoxicated and the first harvest was immediately done. At the regrowth period, the harvest occurred 30 days after the first harvest.

2.2.2. Visual observation

Daily observations of the plants were made throughout the experiment aiming to detect visual symptoms due to Cu excess in the plants, and also, for favorable responses by the Si application in the nutrient solution.

2.2.3. Relative chlorophyll index (SPAD value)

The chlorophyll quantification was performed in the end of each growth period, by indirect measurement obtained from the determination of SPAD units. For this determination the portable device SPAD 502 was used and the procedure consisted of 10 measures in the middle third of the blade of the newly expanded leaves (diagnostic leaves), in all experimental units (Lavres Júnior et al. 2010).

2.2.4. Morphogenesis and plant yield

The number of tillers and the number of leaves were counted right after the plant being cut down. The shoots were collected in the two growth periods and the roots were collected just at the end of the second growth period. After drying the collected plant material, it was determined the dry mass production of the plant parts. For the root surface measurement, it was separated approximately 20% of the fresh roots and immersed in 50 mg L^{-1} gentian violet solution. After drying, this fraction of the root system was blushed, scanned and transformed into a digital file in order to quantify the surface and root length by the software SIARCS 3.0 (Crestana et al. 1994).

2.2.5. Concentration and accumulation of Si and Cu

For the determination of Cu concentration in the plant tissue, it was used the methodology described by Sarruge and Haag (1974), which consists of nitric-perchloric digestion of the ground plant material and subsequent analysis of the extract in an atomic absorption spectrophotometer. Silicon concentration in the plant tissue was determined following the method described by Elliott and Snyder (1991), starting with the digestion of ground plant material using sodium hydroxide and hydrogen peroxide, after autoclaving and analyzing in photo-colorimeter with wavelength of 410 ηm. Both Si and Cu concentrations were determined in the two most recently expanded leaves called diagnostic leaves (DL).

Silicon and Cu accumulations in the plant parts were calculated from the product of the concentration of these elements by the respective dry masses produced in each plant part.

2.2.6. Statistical analysis

All data were statistically analyzed using the software "Statistical Analysis System" (SAS Institute 2008). The analysis of variance was performed firstly by the GLM procedure. It was used the F test to verify if there is significance in the interaction among Si rates × Cu rates. In case of significant interaction, it was carried out an outspread of this interaction. When there was no significance in the interaction, a comparison of means for Si rates and regression analysis by the REG procedure for Cu rates was done. Comparison of means was done by Tukey test at 5%.

2.3. Results

2.3.1. Symptoms of toxicity and signs of amelioration

The severity of Cu toxicity increased with the rate of Cu added to the nutrient solution in both growth periods (Fig. 1a and 1c). The main visual symptoms were: reduced growth, leaf rolling, chlorosis and lodging. Silicon supply resulted in greater plant tolerance to Cu toxicity in both growth periods (Fig. 1b and 1d). The Si supplied plants showed fewer and less severe symptoms in erected and expanded leaves. Also, plants exposed to Cu rates of 750 μmol L^{-1} with no Si added in the nutrient solution did not regrow after cutting (Fig. 1b), whilst plants supplied with Si to regrew regardless of Cu rates (Fig. 1d).

During first growth, symptoms of Cu toxicity advanced from temporary rolling of leaves during the hotter hours of the day to a permanent rolling (Fig. 2a). The next stage saw an increase leaf chlorosis and under Cu rates of 500 and 750 μmol L^{-1} there was plant lodging (Fig. 2b). In the second growth, plants in general did not show rolling leaves and the chlorosis was less severe (Fig. 1b and 1d). The biggest difference among the Si and non-Si supplied plants of tanzania guinea grass during the second growth was the speed of plant growth. In other words, it was possible to distinguish the Si rates by the size of the plants (Fig. 1).

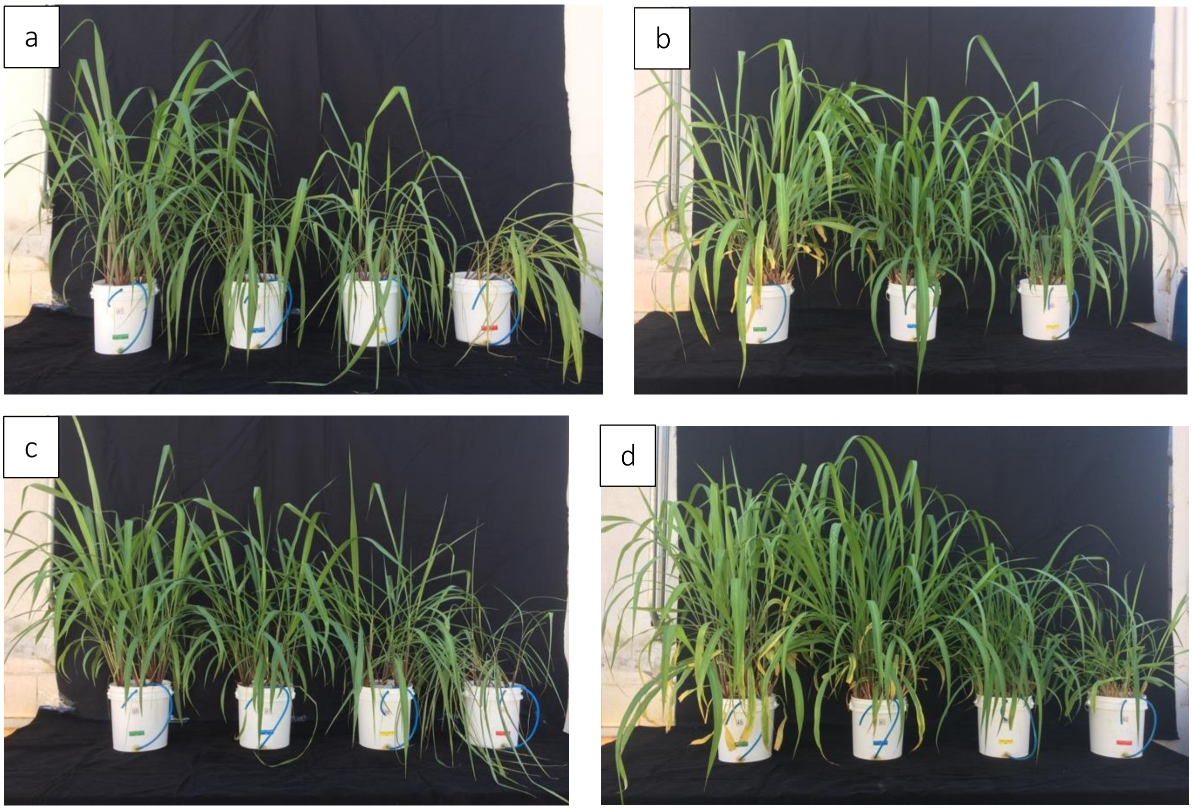


Fig. 1 Plants of *Panicum maximum* cv. Tanzania exposed to Cu rates of 0.3, 250, 500, and 750 µmol L^{-1} with no Si supply (a – first growth; b – second growth) and with Si supply of 3 mmol L^{-1} (c – first growth; d – second growth). The pictures were taken just before the harvest.

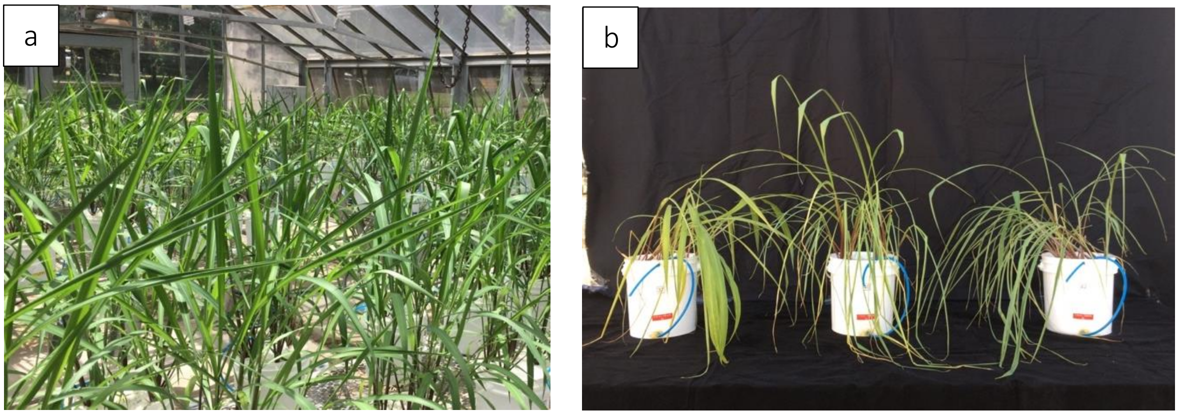
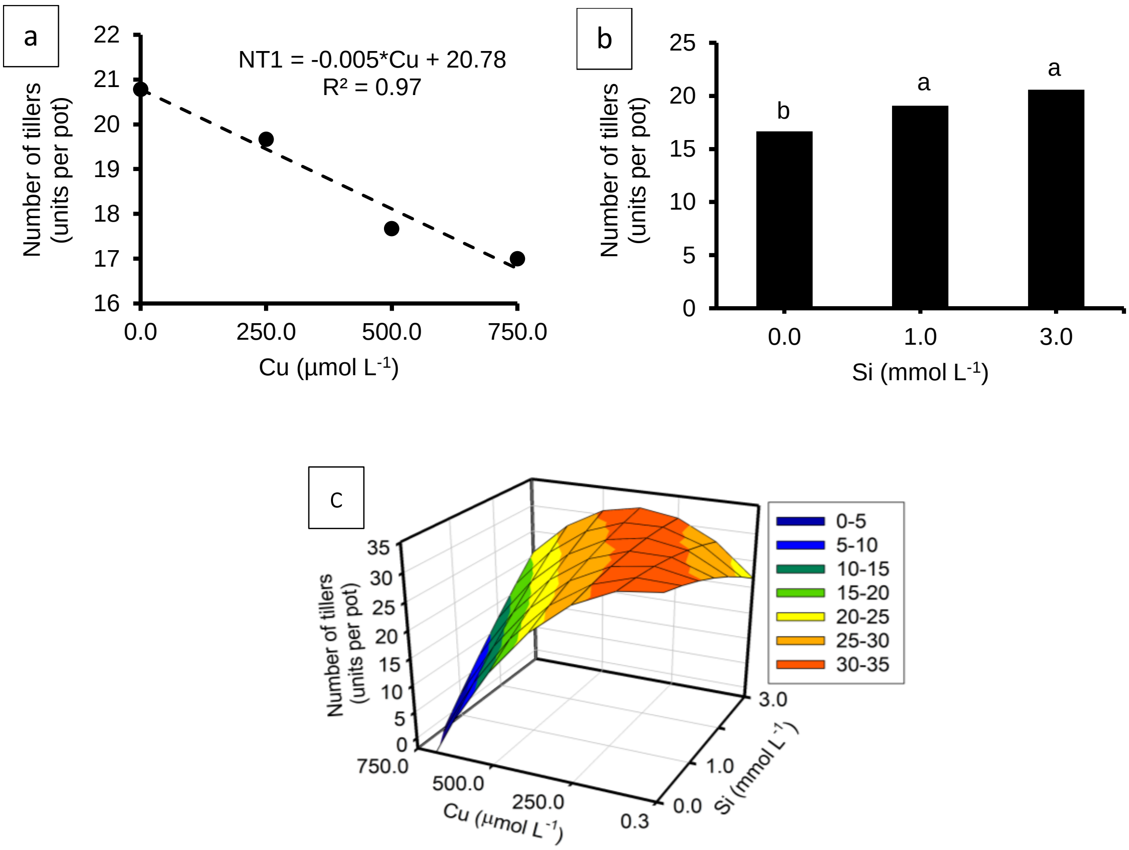


Fig. 2 Plants of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates with Cu toxicity symptoms in the initial (overview - a) and final stages (plants exposed to Cu of 750 µmol L^{-1} in the first growth - b).

2.3.2. Plant yield, morphology and chlorophyll content

The yield characteristics of the tanzania guinea grass were influenced by the exposure rates of both Cu and Si. The number of tillers during the first growth was affected by both Si and Cu rates, without significant interaction. As Cu rates increased the number of tillers decreased

(Fig. 3a) with the plants exposed to 750 µmol L^{-1} and 0.3 µmol L^{-1} of Cu differing by about 18%. Silicon supply increased the number of tillers by about 14% compared to plants that were grown in no-Si supply condition, but there was no significant difference between plants grown with 1 and 3 mmol L^{-1} of Si (Fig. 3b). In the second growth, the interaction Cu rates × Si rates was significant for the number of tillers (Fig. 3c). The number of tillers declined with increasing Cu rates and increased with Si rates. Si supply lowered the number of tillers in plants in the lowest Cu rate (0.3 µmol L^{-1}); however, as the toxicity increased, Si supply resulted in higher values.

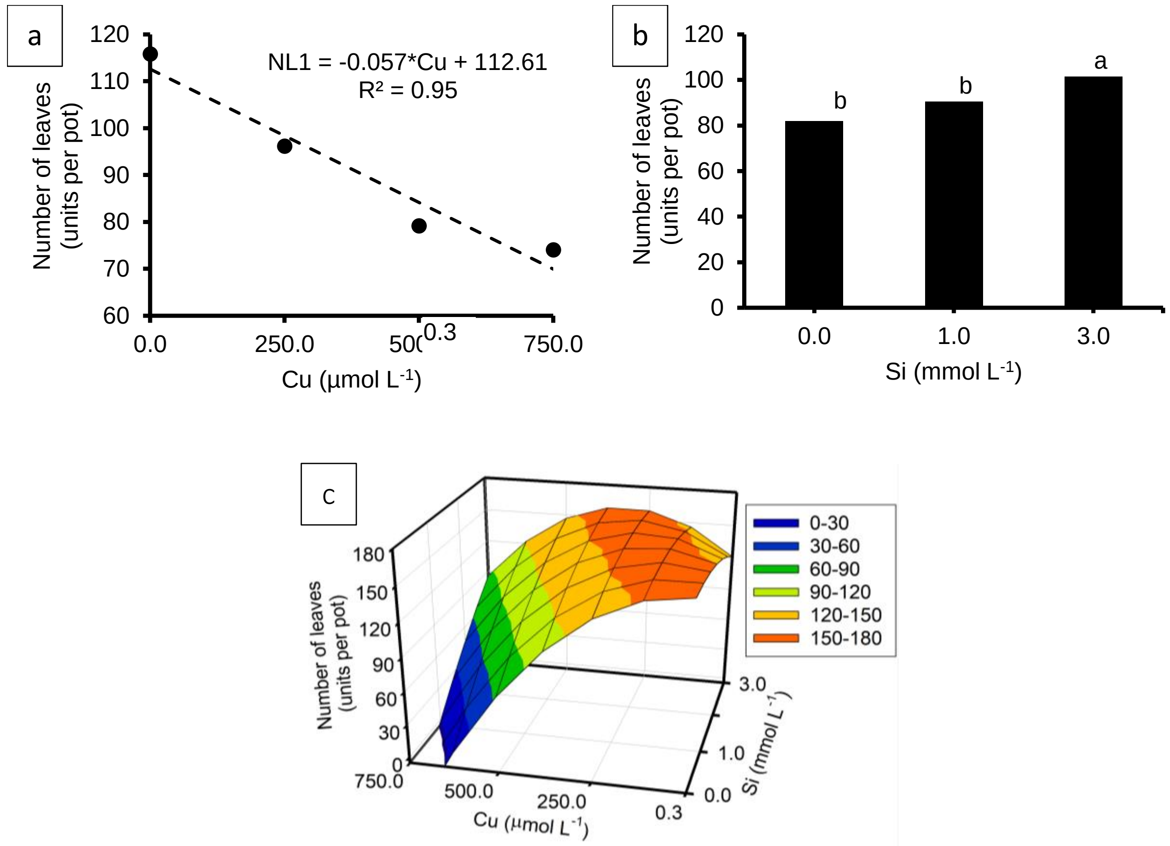


$$NT2 = 33.35 - 2.36*Si + 0.0024*Cu - 0.51*Si^2 + 0.018*Cu*Si - 0.00008*Cu^2$$

$$R^2 = 0.84$$

Fig. 3 Number of tillers per pot in the first (a and b) and second growth period (c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. In the figure 3b, different letters show significant difference by Tukey test.

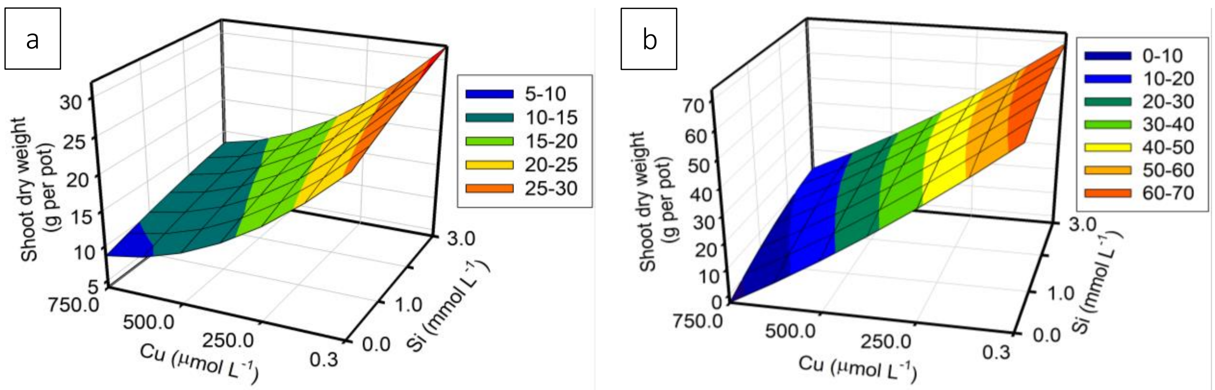
The number of leaves were also affected by Si and Cu rates separately, without significant interaction in the first growth. The increasing rate of Cu resulted in lower leaf production (Fig. 4a). Plants treated with 750 µmol L^{-1} had 36% less leaves than those exposed to 0.3 µmol L^{-1} of Cu. Plants exposed to 3 mmol L^{-1} of Si showed the highest number of leaves and there was no significant difference between 0 and 1 mmol L^{-1} (Fig. 4b). In the second growth, the interaction Cu rates × Si rates was significant (Fig. 4c). As the Cu rates increased, the number of leaves decreased. When combined with Si, the number of leaves increased with Cu rates. Again, Si was responsible for lower number of leaves when there was low or no Cu toxicity.



NL2 = 159.52 – 0.26*Si +0.0076*Cu – 4.22*Si^2 + 0.0808*Cu*Si – 0.0004*Cu^2

$R^2 = 0.84$

Fig. 4 Number of leaves per pot in the first (a and b) and second growth period (c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. In the figure 4b, different letters show significant difference by Tukey test.



SDW1 = 25.96 + 2.43*Si – 0.0419*Cu – 0.15*Si^2 – 0.0006*Cu*Si + 0.00002*Cu^2

$R^2 = 0.96$

SDW2 = 63.68 + 4.99*Si – 0.105*Cu – 1.01*Si^2 – 0.004*Cu*Si + 0.00002*Cu^2

$R^2 = 0.96$

Fig. 5 Shoot dry weight per pot in the first (a) and second growth (b) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates.

The interaction Cu rates × Si rates was significant for the shoot dry weight of Tanzania guinea grass, in both growths (Fig. 5). The highest shoot dry weight was obtained with the combination of maximum Si rate and with the Cu rate of 0.3 μmol L^{-1}. The lowest values were acquired with the maximum Cu rate combined with no-Si supply. In both growth periods, Si supply was responsible for the highest shoot dry weight in every Cu rate. Furthermore, plants exposed to the highest Cu rate did not regrow in the absence of Si (Fig. 3c, 4c and 5b).

Root dry weight (Fig. 6a) and root surface area of tanzania guinea grass (Fig. 6b) showed a similar performance with a significant interaction Cu rates × Si rates. Both parameters were improved by Si supply in all Cu rates, with the highest values for plants exposed to Cu of 0.3 μmol L^{-1} combined with 3 mmol L^{-1} of Si and the lowest values in plants treated with 750 μmol L^{-1} of Cu and with no-application of Si. Root length was significantly affected by Cu rates but not by Si rates (Fig. 6b). Comparing the more contrasting Cu rates (0.3 and 750 μmol L^{-1}), the Cu toxicity led to a reduction of 82% in root length.

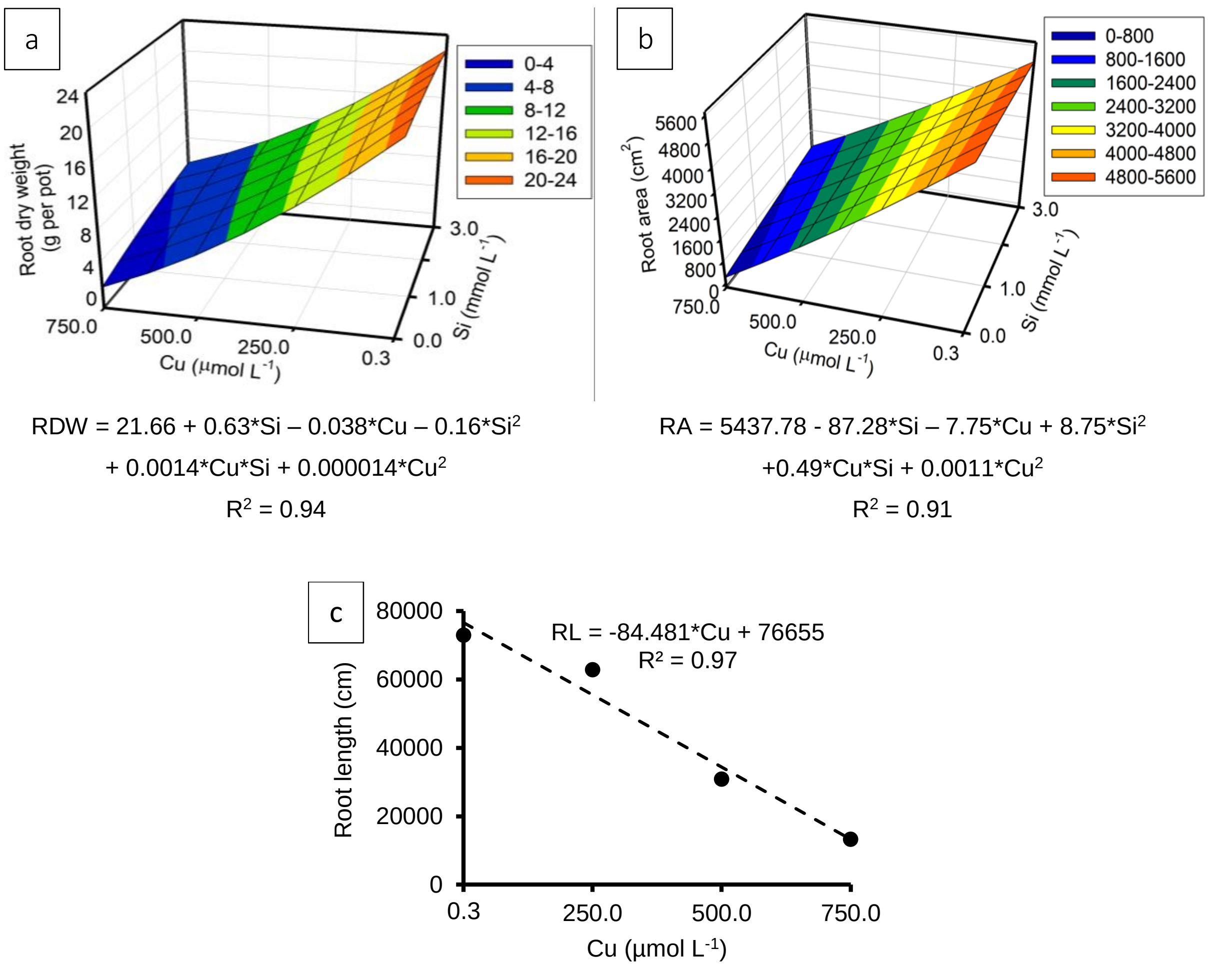


Fig. 6 Dry weight (a), surface area (b) and length of roots (c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates.

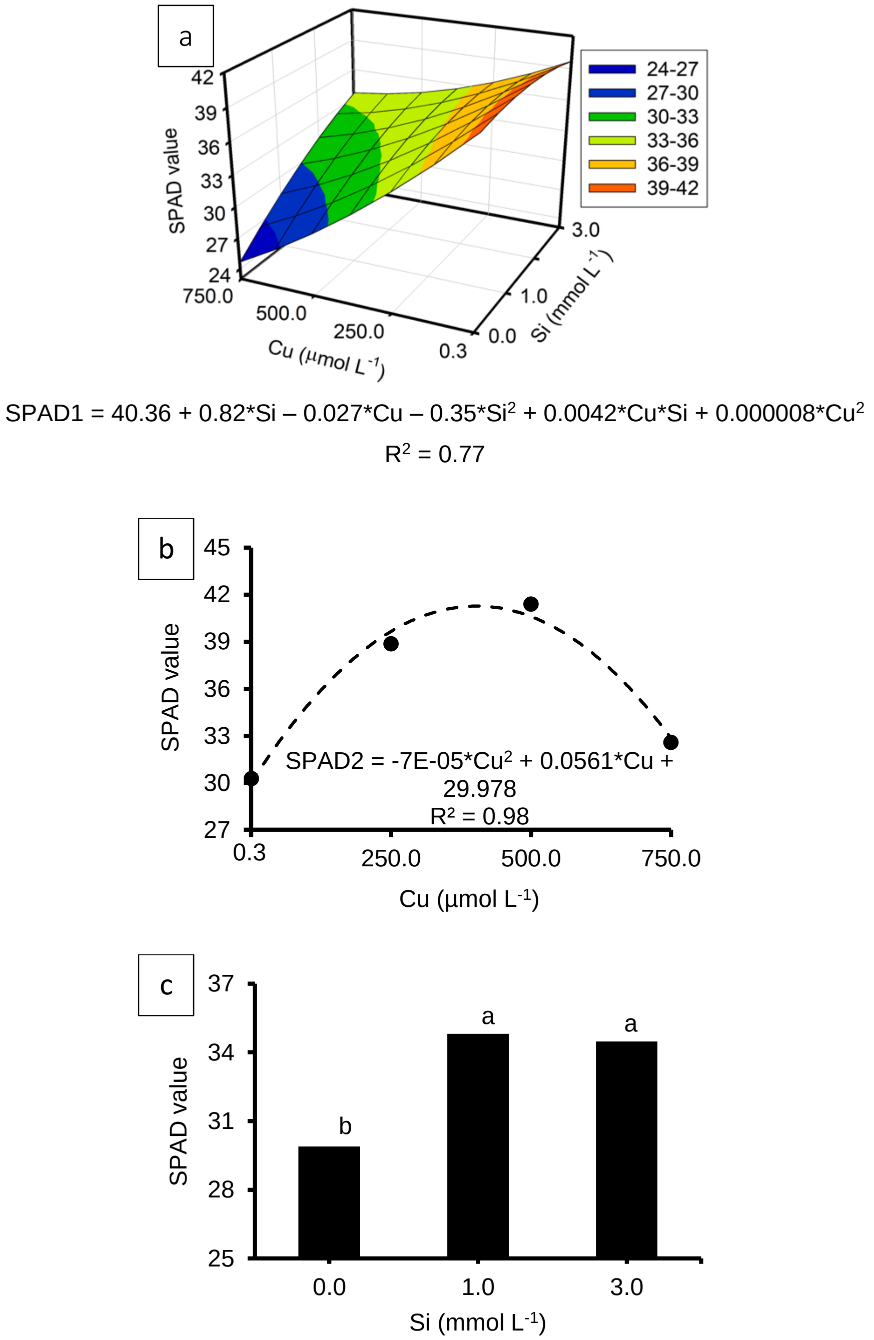


Fig. 7 SPAD values in diagnostic leaves in the first (a) and in the second growth period (b and c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. In the figure 7c, different letters show significant difference by Tukey test.

Tanzania guinea grass supplied with Si showed higher values for SPAD in all Cu rates (Fig. 7a), resulting in a significant interaction Cu rates × Si rates in the first growth period. Plants supplied with Si of 3 mmol L^{-1} and exposed to Cu of 750 µmol L^{-1} had a SPAD value 36% higher than plants exposed to the same Cu rate without Si supply. There was no significant interaction Cu rates × Si rates in the second growth, however, both factors separately affected SPAD value (Fig. 7b and 7c). Copper rates positively influenced SPAD values up to 500 µmol L^{-1}, with an increment of 36% compared to the Cu rate of 0.3 μmol L^{-1} (Fig. 7b). Copper rates above 500 μmol L^{-1} reduced SPAD values by about 21%. Silicon supply also positively influenced SPAD values (Fig. 7c), even though there was no difference for Si between 1 and 3 mmol L^{-1} but there was significant difference between application and no-supply of Si. Silicon application in the nutrient solution resulted in an increase of 15% in SPAD values.

2.3.3.Cu and Si concentrations and contents

In the first growth period, the interaction Cu rates × Si rates was significant for Cu concentrations in diagnostic leaves (DL) and Cu content in shoots of tanzania guinea grass (Fig. 8a and 8b). Plants supplied with Si showed less Cu in DL (Fig. 8c) and accumulated less Cu in shoots (Fig. 8d) in the first growth, regardless of the Cu rate applied. In the second growth, Cu concentration in DL (Fig. 8c) and Cu content in shoots (Fig. 8d) were only affected by Cu rates. Plants exposed to Cu of 750 μmol L^{-1} had 34 times more Cu in shoots than plants exposed to Cu of 0.3 μmol L^{-1}. Copper content in shoots increased as the concentration of this metal in the nutrient solution increased up to 500 μmol L^{-1}; after which, Cu content decreased. To Cu concentration and content in roots, the interaction Cu rates × Si rates was significant. In roots, the highest Cu concentration occurred in combinations with no-Si application, decreasing under Si supply (Fig. 8e). The opposite was observed for Cu content in roots, where combinations with the highest Si rates showed the highest Cu content (Fig. 8f).

The interaction Cu rates × Si rates was significant to Si concentration in DL of tanzania guinea grass during the first growth period (Fig. 9a). In the first growth, plants supplied with Si had higher Si concentration in DL as the Cu rate increased. In the second growth, Si concentration in DL increased with the Si supply and was only significantly affected by Si rates (Fig. 9c). In both the first and the second growth periods, the interaction Cu rates × Si rates was significant to Silicon content in shoots (Fig. 9b and 9d). Silicon content in shoots in both growth periods increased with Si supply and decreased under Cu rates. In roots, the interaction Cu rates × Si rates was significant for Si concentration and content. Silicon concentration increased with the increasing of Cu rates (Fig. 9e). The opposite occurred for Si content in roots (Fig. 9f), which decreased with the increase in Cu rates.

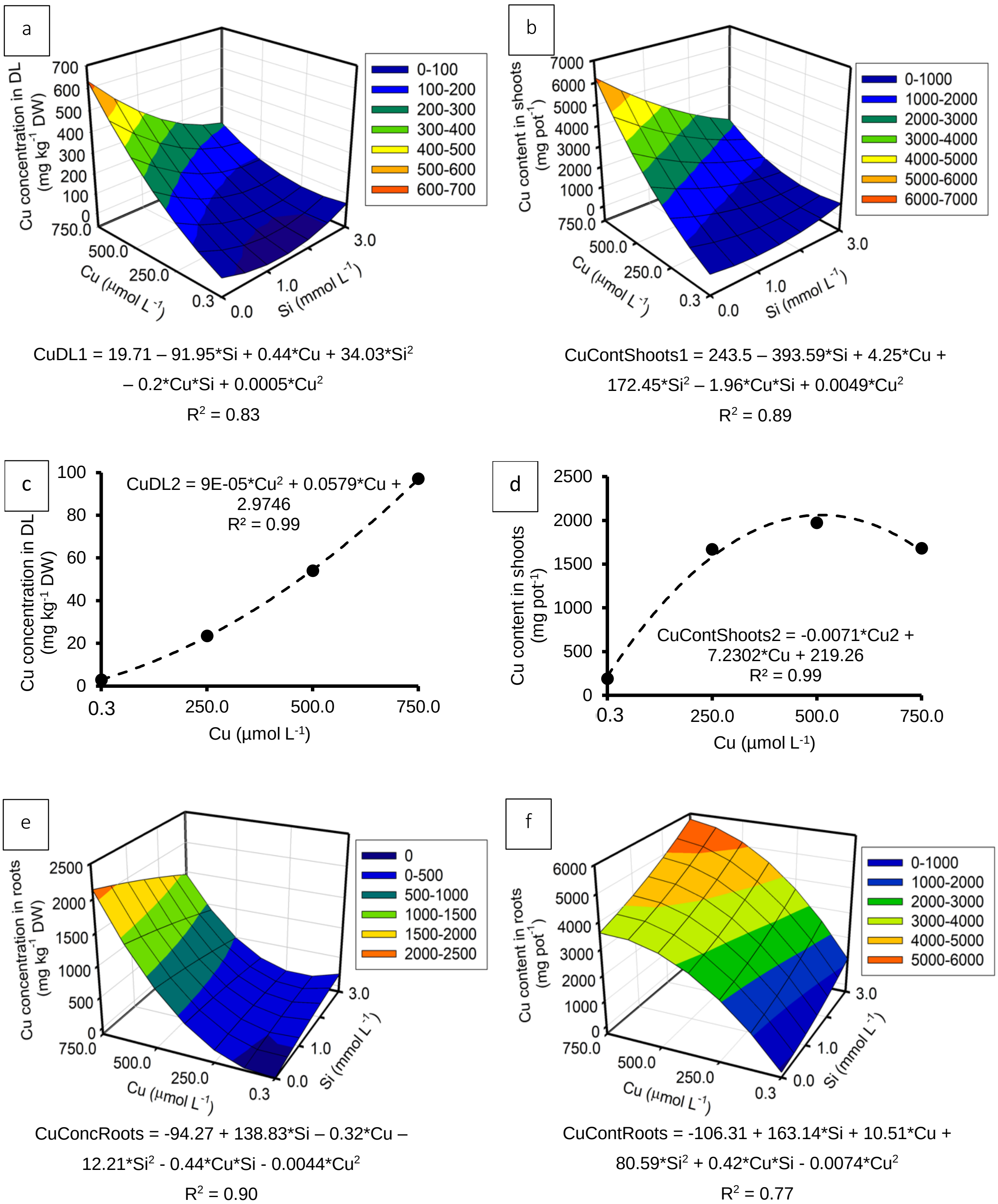


Fig. 8 Copper concentration in diagnostic leaves (mg per kg of dry weight) (a – first growth; c – second growth) and in roots (e), and Cu content in shoots (b – first growth; d – second growth) and in roots (f) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. The original data of figures 8a, 8b and 8c was transformed to $\sqrt{x}$, and in the figure 8d to $\log x$, aiming to adjust to a normal distribution.

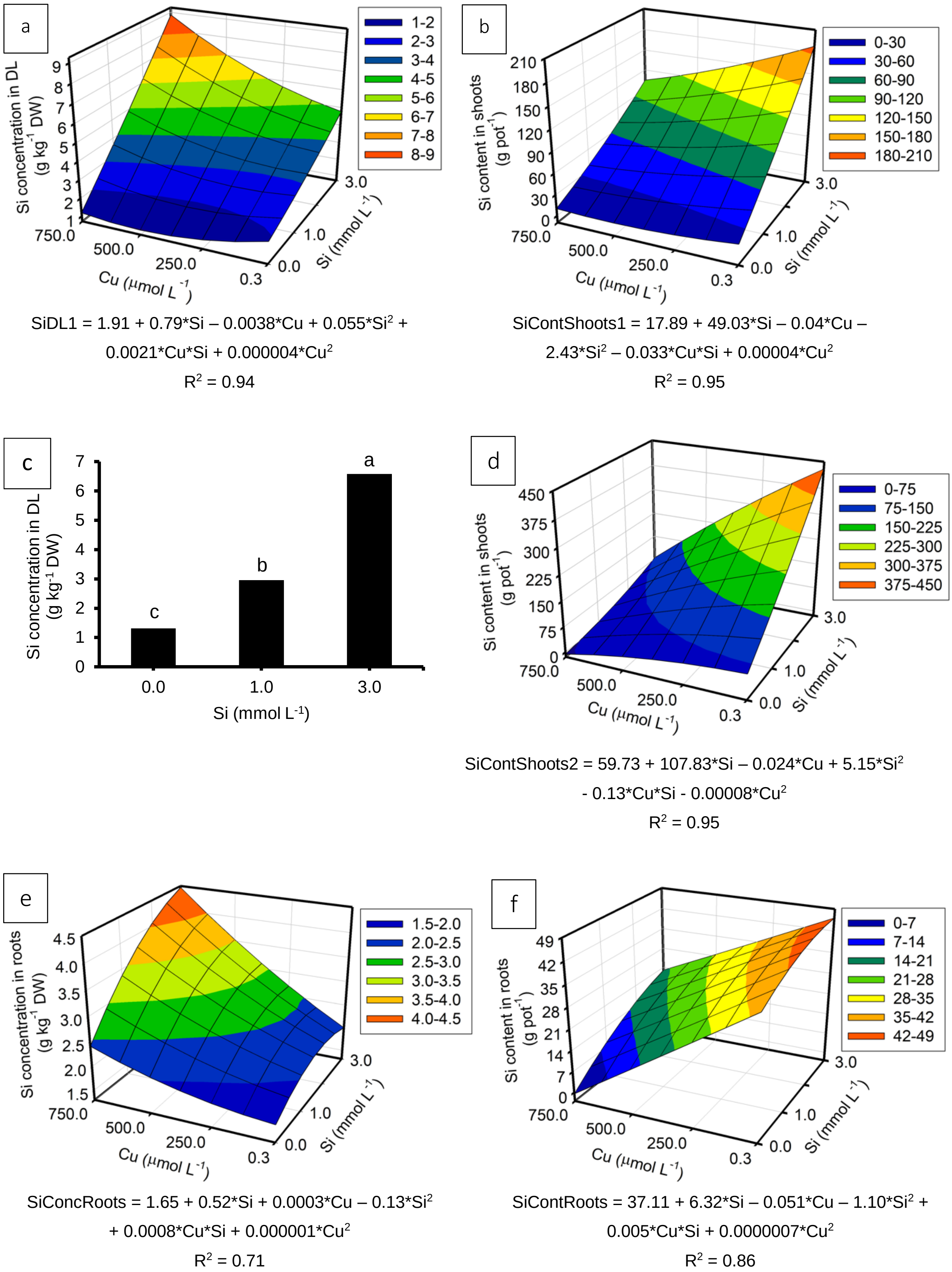


Fig. 9 Silicon concentration in diagnostic leaves (g per kg of dry weight) (a – first growth; b – second growth) and roots (e), and Cu content in shoots (c – first growth; d – second growth) and roots (f) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. In the figure 9c, different letters show significant difference by Tukey test. In the figures 9b and 9c, the original data was transformed to $\sqrt{x}$ aiming to adjust to a normal distribution.

2.4. Discussion

Forage grasses are well known for their potential for high biomass production, characterized by a considerable number of leaves and tillers (Langer 1979). However, these characteristics are strongly affected when forages are exposed to metal toxicity. Tanzania guinea grass was greatly affected by Cu rates above 0.3 μmol L^{-1}, which culminated in a low production of tillers, leaves and shoot dry weight. Gilabel et al. (2014) also verified low production of aboveground biomass in Tanzania guinea grass as Cu rates increased above 0.3 μmol L^{-1}. Nevertheless, the scenario changed when plants were supplied with Si. Plants that were exposed to various doses of Cu showed a higher number of tillers, leaves and shoot dry weight when supplied with Si. Despite the fact that Si is not an essential element for higher plants, it has been already proven that Si can favor the growth and development of many plant species (Epstein 1994). Nowakowski and Nowakowska (1997) exposed wheat (*Triticum aestivum*) seedlings to Cu rates of 10, 20 and 40 μg cm^{-3} in combination with 500 μg cm^{-3} of Si, also reported greater increment in biomass production. In the current experiment, the tanzania guinea grass also produced less below ground biomass when exposed to Cu excess in nutrient solution, but Si positively affected root dry weight and root area. Similar results were reported for cotton (*Gossypium hirsutum*) under Cu rates of 0, 25 and 50 μmol L^{-1} and Si rates of 0 and 1 mmol L^{-1} (Ali et al. 2016). The chlorophyll content of tanzania guinea grass (indirectly quantified by SPAD values) during first growth period dropped with the increment of Cu rates above 0.3 μmol L^{-1}, but this reduction was less evident in plants supplied with Si. Rice (*Oryza sativa*) seedlings had reduced chlorophyll concentration when exposed to Cu toxicity with this reduction also being alleviated by the supply of Si (Kim et al. 2014).

Even though tanzania guinea grass produced less biomass as the Cu rates increased, Cu concentration in DL was also increased. Healthy Cu concentrations in the younger leaves of *Panicum maximum* range from 4 to 14 mg kg^{-1} (Werner et al. 1996). In this study, the values reached over 600 mg kg^{-1} of Cu in the combinations of 750 μmol L^{-1} with no Si supply during the first growth. The low biomass production of Tanzania guinea grass intoxicated with Cu is strictly related to the concentration of this metal in plant tissues. The nutritional imbalance caused by excess Cu can induce destabilization of lipid components of the plasma membrane, causing increases in permeability, which induces an increase in Cu absorption itself (Maksymiec 1997). This membrane disruption can also reduce biomass production and chlorophyll contents in plants exposed to the highest Cu rate. The supply of Si reduced Cu concentrations in DL and in aboveground biomass in the first growth period. Collin et al. (2014) also found similar results when evaluating Cu concentration in aboveground biomass of bamboo (*Phyllostachys fastuosa*) exposed to Cu of 1.5 and 100 μmol L^{-1} and treated with 1.1 mmol L^{-1} of Si. Due to the reduction in Cu absorption when treated with Si, plants exposed to high Cu rates survived and still provided an appreciable biomass production in the second growth period. Plants exposed to 750 μmol L^{-1} and some combinations of 500 μmol L^{-1} of Cu did not survive in the absence of Si. In the second growth, the low Cu content in shoots of plants exposed to 750 μmol L^{-1} is a consequence of their low aboveground biomass production. Copper concentration in roots also increased with the increment in Cu rates with no-Si supply, however, Cu content in roots was higher in plants supplied with Si than those not supplied with Si. This can be explained by the substantially high root biomass and reduction in the transport of Cu to the shoots in plants supplied with Si. Silicon supply similarly reduced Cu concentrations in plants of zinnia (*Zinnia elegans*) and snapdragon (*Antirrhinum majus*) exposed to Cu concentrations up to 150 μmol L^{-1} and supplied with Si of 1.7 and 3.4 mmol L^{-1} (Frantz et al. 2011).

Low Cu uptake in the presence of Si may be explained by the relationship between the plant exposure to copper toxicity and exogenous Si which is not yet fully understood. Previous studies have shown interesting results among the positive effect of Si on metal intoxicated plants. A likely explanation for this is the reduction in apoplastic transport of metals by decreasing free metal concentration in the apoplast, which has already been confirmed in plants exposed to excess manganese (Iwasaki et al. 2002, Rogalla & Romheld 2002). According to Adrees et al. (2015), Si deposition may reduce metal concentration in xylem as a consequence of the formation of a physical barrier in the endoderm proximity, thus reducing the cell wall porosity. Moreover, these authors stated that the concentrations of Cu in shoots are lower than in the roots as Si decreases metal translocation. This decrease in metal translocation might be a result of alterations in the structure in shoots and roots, co-precipitation, chelation and compartmentation of metals in plants. In the present study, once the Cu concentration and transport was low in the grass supplied with Si, these plants were exposed to a lower level of toxicity than those that did not receive Si supply.

Silicon concentrations in DL were largely influenced by the increasing Cu rates in the first growth. Bamboo plants exposed to Cu rates of up to 100 μmol L^{-1} and supplied with Si of 1.1 mmol L^{-1} also showed higher Si concentrations in shoots than those also supplied with Si in the absence of Cu. It suggests that this grass absorbs more silicon in an attempt of better deal with Cu toxicity. In the present study, concentrations of Si in roots showed a similar pattern to the shoots, the concentration raised as Cu rates increased. This goes against the findings of Mateos-Naranjo et al. (2015) that obtained lower Si concentration in roots of *Spartina densiflora* exposed to an excessive Cu rate (15 mmol L^{-1}) than those exposed to the Cu rate of 0.5 μmol L^{-1}.

Kim et al. (2014) studied rice seedlings exposed to 100 μmol L^{-1} of Cu and found results that corroborate with the findings of the present study, wherein plants supplied with 1 mmol L^{-1} of Si, had higher Si concentrations in their roots than the ones with the Cu rate of 0.5 μmol L^{-1}. In both growth periods, Si content was incresed with decreasing Cu rates. The accumulation of Si in belowground biomass also increased with Si supply and reduced by Cu rates. This is once more explained by the considerable large biomass of plants less intoxicated with Cu due to Si supply.

This study improves the current knowledge in regards to *Panicum maximum* cv. Tanzania and its potential for phytoextraction. Copper toxicity is becoming a worldwide problem and sustainable and eco-friendly solutions have to be investigated. Tanzania guinea grass performed well absorbing metal from the nutrient solution though phytoextraction even when not supplied with Si. However, this performance can be drastically improved when exogenous Si is applied. Plants supplied with Si were able to control the absorption of Cu and promote regrowth after cutting. The grass exposed to the highest Cu rate showed the highest values of Cu concentration when not treated with Si and presented a lower biomass in both growth periods. In other words, under Cu toxicity, the main benefit of Si treatment to this grass is to maintain prolonged productivity and thus continued phytoextraction of Cu from the substrate.

2.5. Conclusion

Excess Cu negatively affected the biomass production and chlorophyll contents of *Panicum maximum* cv. Tanzania, and increased Cu concentration. Silicon supply improved biomass production and chlorophyll content by decreasing Cu concentration. Besides reducing Cu absorption, the most important role of Si was to reduce the transport of Cu from roots to shoots. Although Si supply did not increase Cu phytoextraction, it allowed successive harvesting of the aboveground biomass, which could be an interesting approach in phytoremediation programs.

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3. SILICON IMPROVES PHOTOSYNTHETIC ACTIVITY AND INDUCES SOD ACTIVITY IN TANZANIA GUINEA GRASS UNDER COPPER TOXICITY

ABSTRACT

Copper (Cu) toxicity reduces plant photosynthetic activity and causes oxidative stress in plants, which impairs plant growth. A possible solution to overcome such toxicity is the supply of silicon (Si), which may promote higher tolerance of plants to Cu stress. The present study evaluated the influence of different Si rates (0, 1 and 3 mmol L^{-1}) on metabolic and physiological responses of *Panicum maximum* cv. Tanzania exposed varying levels of Cu stress (0.3, 250, 500 and 750 µmol L^{-1}). Plants had two growth periods: the first grown for 32 days after seedlings were transplanted and the second grown for 30 days after the harvest of foliage. In the first growth period, plants were exposed to Cu for seven days, 25 days after being transplanted. The second harvest had the objective of evaluating the regrowth of the grass under the residual Cu rates. Gas exchange parameters were determined in the diagnostic leaves (DL) at the end of each growth period. Concentrations of stress indicators malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and proline, and activities of antioxidant enzymes were determined in shoots and roots. Silicon rates positively and Cu rates negatively affected gas exchange parameters and concentrations of stress indicators in the first growth. In the second growth period, an eustress event was observed where the highest values of gas exchange parameters and lowest malondialdehyde (MDA) concentrations were observed in the combinations with high Cu rates. Antioxidant system enzymes had their activities considerably reduced by the raising of Cu rates. Superoxide dismutase (SOD) activity was positively affected by Si supply to plants in both growth periods. The results provide evidence of the positive influence of Si treatment on the photosynthetic parameters and SOD activity of tanzania guinea grass under stress by Cu toxicity.

Keywords: Forage grass; Heavy metal; Interaction Cu × Si; Reactive oxygen species

3.1. Introduction

Demand for food in the world is increasing each year, thus, crop yields and viable crop area needs to be increased. This increases pressure on the environment by expanding agricultural land and the intense use of agrochemicals. These anthropogenic activities can be detrimental to the environment and productivity. Heavy metal pollution is an increasing problem around the world caused by the intense use of agrochemicals such as that from Copper (Cu) which is used in pesticides that provide antimicrobial protection for many crops (Megateli et al. 2013). Copper can also be released into croplands from fertilizers, sewage sludge, mining activities, pesticides, atmospheric deposition of dust, rain, industrial smoke or naturally from soil weathering

(Malavolta 2006). As a result, contamination of soil and water sources by Cu is widespread and is a threat to crop yield and human health.

Copper is an essential metal for plants, classified as a micronutrient due to the low concentration in plant tissue, which reflects in low demand by most cultivated plants. This metal acts in many plant functions, the main ones being related to photosynthesis, respiration and metabolism of reactive oxygen species (ROS) (Burkhead et al. 2009). However, when in excess it can causes disorders, not only in plants, but also in the animals that consume these plants. To cope with oxidative stress, plants activate certain mechanisms in their metabolisms.

In order to decrease the synthesis of ROS, plants under stress activate enzymes that can convert the superoxide anion and peroxide radicals into less toxic and non-toxic forms, thus minimising damage to plant cells. The most important enzymes in the antioxidant system are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR) (Heldt and Piechulla, 2005). Superoxide dismutase acts as an initial defense against ROS and is responsible for catalyzing the dissociation of O_2^- to H_2O_2 and O_2, and CAT and the conversion of H_2O_2 to H_2O or O_2 (Mittler 2002). Ascorbate peroxidase and guaiacol peroxidase enzymes also convert H_2O_2 to H_2O, with APX using ascorbate as substrate whilst GPX uses guaiacol (Mittler 2002). The enzyme GR is important as it reduces glutathione (GSH) to aid the performance of APX, and also works as a chelating agent of free toxic metals in the cytoplasm (Paulose et al. 2013, Deng et al. 2015). Even though plants activate these enzymes during stress events, this mechanism seems to be limited to conditions of high rates of redox reactive metals such as Cu (Schutzendubel & Polle 2002).

A sustainable and eco-friendly solution to treat contaminated farm fields is phytoremediation. For this technique to be successful, the selected species must be metal tolerant with high phytoextractor potential and provide considerable biomass in which the metal will be stored. Nevertheless, the agronomic management, in particular to fertilization, of such plants require further study. Fertilization can be used to boost performance of phytoremediating plants, and research related to the improvement of this technique has been carried out with a number of nutrients and beneficial elements. Silicon is a beneficial element for plants that can relieve abiotic stress, especially that caused by heavy metals (such as Cu). This relief effect is attributed to its deposition mainly in the cell walls of roots, leaves and stems, which creates binding sites for metals (Ma & Yamaji 2006). Although the effect of Si supply on the attenuation of Cu toxicity has been already reported for some species, it varies according to plant species, plant age, concentration of the metal under consideration and period of exposure to contaminating metal (Cooke & Leishman 2016).

Tanzania guinea grass is an important cultivar of the species *Panicum maximum* known for its characteristics of rusticity, high leaf/stem ratio, high seed production, high regrowth rate and low crop seasonality (Jank et al. 2010, Jank et al. 2013). In particular, *Panicum maximum* cv. Tanzania has demonstrated metal phytoextraction potential for Cu, Ba and Cd (Monteiro et al. 2011b, Gilabel et al. 2014, Rabêlo et al. 2016, Souza Junior et al. 2018). However, there is a scarcity of information regarding the effect of Si on photosynthetic parameters of this grass, as well as on its antioxidant system enzymes. Therefore, the objective of this study was to evaluate the influence of Si on metabolic and physiological responses of *Panicum maximum* cv. Tanzania exposed to varying degrees of Cu stress by measuring gas exchange parameters, concentrations of stress indicators and the activities of antioxidant system enzymes.

3.2.Material and Methods

3.2.1. Experimental conditions

Tanzania guinea grass (*Panicum maximum* cv. Tanzania) was grown in hydroponic conditions in a greenhouse. According to preceding research, this grass has an appreciable level of tolerance to metal stress. The experiment was conducted in a 3x4 factorial using complete randomized block design replicated three times. Three Si rates (0, 1 and 3 mmol L^{-1}) and four Cu rates (0.3, 250, 500 and 750 μmol L^{-1}) were used based on earlier studies. All other nutrients were provided based on the Hoagland and Arnon (1950) solution.

Thirteen days after transplant, the seedlings were transferred from sand washed with deionized water to ground quartz. Seedlings with an average height of 4-5 cm were transplanted in number of fifteen per pot (3.5 L of capacity). All the pots were supplied with nutrient solution with 20% of its ionic force for seven days. A thinning was performed in the second week after transplantation until just five plants remained per pot and the nutrient solution with 100% of its ionic force was provided.

Silicon rates were implemented since day one and Cu rates (0.3, 250, 500 and 750 μmol L^{-1}) were applied in the 21st day after transplantation and were used during seven days. Before and after the exposure period, Cu rate was settled with 0.3 μmol L^{-1}. It was analyzed two growth periods in order to study if tanzania guinea grass would respond to the residual Cu toxicity during the regrowth. The second harvest took place 30 days after the first one. Severe toxicity determined the harvesting time in first growth period, and leaf senescence determined in the second. In each harvest, samples were frozen at -80 ºC right away for further biochemical analysis.

3.2.2. Gas exchange parameters

Photosynthetic activity was evaluated by measuring CO_2 and H_2O changes in newly expanded leaves (diagnostic leaves - DL). Twenty-four hours before starting the measurement, the pots with plants were moved to a growth chamber intending to provide uniform and constant environment conditions: temperature of 27 ºC, luminous intensity of 400 μmol m^{-2} s^{-1} PAR, and average atmospheric CO_2 of 350 mg L^{-1}. Measurement was performed with IRGA WALZ-GFS-3000 photosynthesis gas exchange fluorescence analyzer at the end of each growth period. It was assessed transpiration rate (TR), stomatal conductance (g_s), assimilation rate (A_N), quantum yield of photosystem II (ϕ_{PSII}) and electron transport rate (ETR).

3.2.3. Oxidative stress indicators (MDA and H_2O_2)

The lipid peroxidation was evaluated with the TBA (2-thiobarbituric acid) test and the concentration of acid reactive substances was measured. The final product of peroxidation (malondialdehyde - MDA) was detected in spectrophotometer using wavelengths of 535 and 600 ηm (Gratao et al. 2005). Malondialdehyde concentration was determined according to the specific equation of reaction (Mihara et al. 1980). Hydrogen peroxide (H_2O_2) was quantified according to the method described by Gay et al. (1999), wherein hydrogen peroxide donates electrons to iron and binds to xylenol in 30 minutes. Then the spectrophotometer readings were made at 390 ηm wavelength.

3.2.4. Proline concentration

The methodology used for proline determination was that described by Bates et al. (1973). Sulfosalicylic acid solution (30 g L^{-1} - in water) was added to the plant sample for metabolic evaluations (300 mg), then centrifuged at 10,000 rpm for 20 minutes in 15 ºC. The supernatant was placed in tubes containing ninhydrin solution and glacial acetic acid, and heated to 100 ºC in water bath for 1 hour. Prior to the spectrophotometer readings, the tubes were shaken for 15 seconds and placed aside for 10 minutes for ceasing phase separation.

3.2.5. Proteins extraction and quantification

Protein extraction followed the method used for enzymatic analysis described by Monteiro et al. (2011a), using the frozen tissue macerated in potassium phosphate buffer solution (100 mg L^{-1}, pH 7.5), ethylenediamine tetraacetic acid (EDTA, 1 mmol L^{-1}), dithiothreitol (DTT, 3 mmol L^{-1}) and polyvinylpolypyrrolidone (PVPP, 40 mg L^{-1}). The extract was then centrifuged at 10,000 rpm at a temperature of 4 ºC for 30 minutes and the supernatant was extracted, which was stored in a freezer with a temperature of -80 ºC. For protein quantification, bovine serum albumin (BSA) was used as buffer, according to Bradford (1976). Extractions for enzymatic analyses followed the same procedure used for protein extractions.

3.2.6. Antioxidant enzymes

3.2.6.1. Catalase

The determination of catalase (CAT) activity was carried out in accordance with Monteiro et al. (2011a), using a mixture of protein extract, potassium phosphate buffer solution (100 mmol L^{-1}, pH 7.5) and H_2O_2 (30%). After waiting one minute for the decomposition of H_2O_2, the CAT activity was determined by a spectrophotometer with a wavelength of 240 ηm at a temperature of 25 ºC.

3.2.6.2. Ascorbate peroxidase

The activity determination of this enzyme was performed based on the mixture of the protein extract at 30 °C (in water bath), potassium phosphate buffer solution (100 mmol L-1, pH 7.5), ascorbate (5 mmol L^{-1}) and EDTA (1 mmol L^{-1}). Then H_2O_2 (1 mmol L^{-1}) was added waiting one minute to start the spectrophotometer readings at 290 ηm wavelength (Cakmak & Horst 1991). Ascorbate peroxidase (APX) activity was calculated using the ascorbate extinction coefficient of 2.8 mmol cm^{-1}.

3.2.6.3. Glutathione reductase

The methodology used to determine glutathione reductase (GR) activity was that described by Gratão et al. (2008), wherein protein extract was used for reaction with potassium phosphate buffer (100 mmol L^{-1}, pH 7.5), 2-nitrobenzoic acid, oxidized glutathione (1 mmol L^{-1})

and NADPH (0.1 mmol L^{-1}). The activity of this enzyme was measured by spectrophotometer readings at 412 ηm wavelength.

3.2.6.4. Guaiacol peroxidase

Guaiacol peroxidase (GPX) activity was quantified using the Matsuno and Uritani (1972) methodology. It was proceeded a mixture of protein extract and sodium phosphate buffer (28.4 g L^{-1}, pH 5.0) containing citric acid (21 g L^{-1}), guaiacol and H_2O_2 with heating to 30 °C for 15 minutes. After incubation, the samples were positioned in ice bath and sodium metabisulphite (20 g L^{-1}) was added in order to stop the reaction. Readings were carried out immediately in a spectrophotometer at 450 ηm wavelength.

3.2.6.5. Superoxide dismutase

This enzyme was evaluated by non-denaturing polyacrylamide gels at 9% (PAGE) following the methodology of Azevedo et al. (1998). Subsequently to the non-denaturing separation, the gel was washed with deionized water and incubated in the dark with potassium phosphate buffer (50 mmol L^{-1}, pH 7.8) containing EDTA (1 mmol L^{-1}), nitroblue tetrazolium (NBT, 0.1 mmol L^{-1}), riboflavin (0.05 mmol L^{-1}) and N, N, N ', N'-tetramethyl ethylenediamine (TEMED, 0.3 g L^{-1}). Deionized water was then used to wash the gels, which were exposed to light until the achromatic bands of superoxide dismutase (SOD) activity became visible in purple color. Bovine SOD was the standard used.

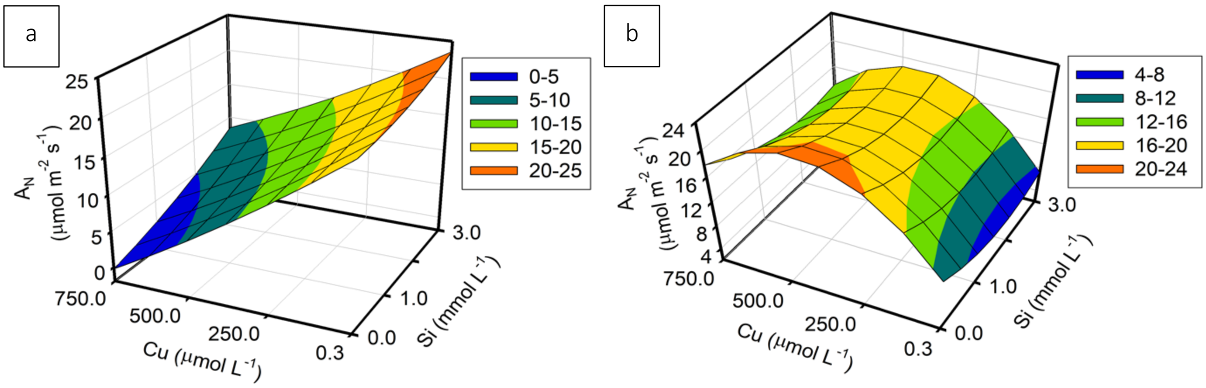
3.2.7.Statistical analysis

Data were analyzed using the software "Statistical Analysis System" (SAS Institute 2008). The analysis of variance started with the GLM procedure. The F test was performed to verify the significance of the interaction Cu rates × Si rates was significant. On condition of significant interaction, it was done an outspread of this interaction. In case of no significance in the interaction, the means for Si rates were compared and implemented a regression analysis for Cu rates by REG procedure. Means were compared by Tukey test at 5%.

3.3. Results

3.3.1. Gas exchange parameters

The interaction Cu rates × Si rates was significant for net photosynthetic rates (net CO_2 assimilation rate – A_N) in plants from both first and second growth periods (Fig. 1). Increasing Cu rates drastically reduced A_N in the first growth period, however, this reduction was less pronounced in plants supplied with Si (Fig. 1a). The scenario changed in the second growth period, when plants exposed to residual Cu showed high A_N values and Si supply reduced these values (Fig. 1b). Net photosynthetic rates increased until the Cu rate of 500 µmol L^{-1}, after which, A_N started declining.



$A_N1 = 20.11 + 0.1*Si – 0.031*Cu + 0.36*Si^2 + 0.002*Cu*Si + 0.000005*Cu^2$

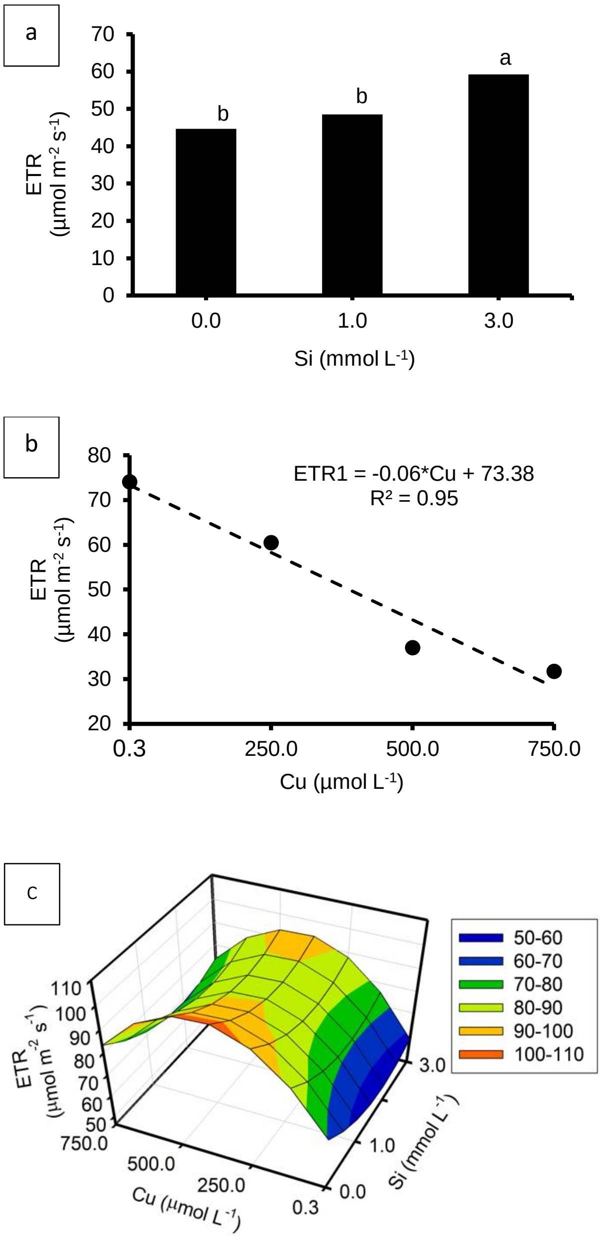
$R^2 = 0.79$

$A_N2 = 20.11 – 4.42*Si + 0.054*Cu + 1.12*Si^2 – 0.0007*Cu*Si – 0.00006*Cu^2$

$R^2 = 0.47$

Fig. 1 Net CO_2 assimilation rate (A_N) in DL in the first (a) and second growth period (b) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates.

Electron transport rate (ETR) in the first growth was affected by Si and Cu rates, albeit, with no significant interaction. There was no significant difference between plants not treated with Si and those treated with 1 mmol L^{-1} Si, with the difference appearing just in the plants treated with 3 mmol L^{-1} of Si (Fig. 2a). Plants supplied with Si of 3 mmol L^{-1} showed ETR values 20% higher than those treated with no or 1 mmol L^{-1} of Si. Copper excess reduced ETR, reducing by 67% when comparing plants exposed to 750 µmol L^{-1} of Cu, to those treated with the 0.3 µmol L^{-1} of Cu (Fig. 2b). The opposite happened in plants from the second growth with ETR values increasing with Cu rates and the lowest value being identified in the plants supplied with Si and not exposed to Cu excess (Fig. 2c). Plants exposed to rates of residual Cu higher than 500 µmol L^{-1} showed reduced ETR values.



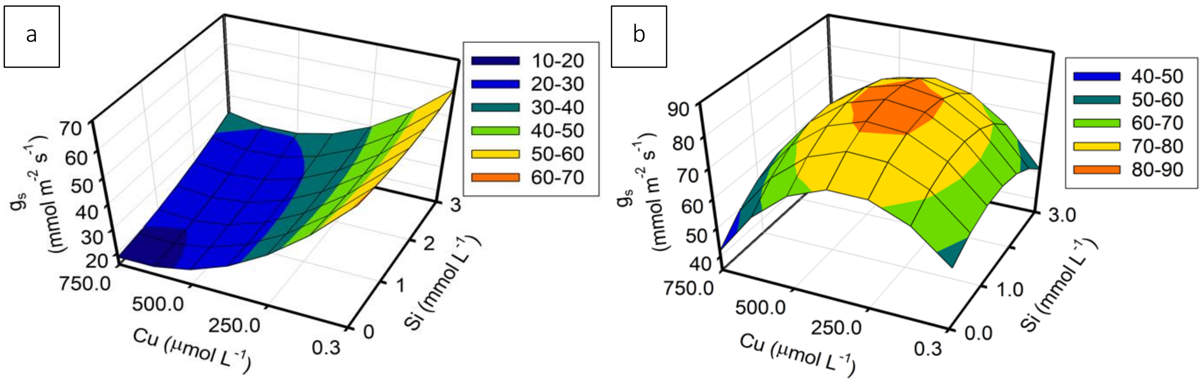
$$ETR2 = 69.26 - 16.55*Si + 0.17*Cu + 4.2*Si^2 + 0.0008*Cu*Si - 0.0002*Cu^2$$

$$R^2 = 0.50$$

Fig. 2 Electron transport rate (ETR) in diagnostic leaves in the first (a and b) and second growth period (c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. In the figure 2a, different letters show significant difference by Tukey test.

In both growth periods, the interaction Cu rates × Si rates was significant for stomatal conductance (g_s). In the first growth period, g_s was positively influenced by the supply of Si and negatively influenced by exposure to Cu (Fig. 3a). Tanzania guinea grass exposed to the maximum Cu rate with no application of Si showed g_s values 30% lower than those exposed to the same Cu rate with the maximum Si supply. In the second growth, high g_s values were identified in plants supplied with Si around 1 mmol L^{-1} and exposed to Cu of 250 μmol L^{-1} (Fig. 3b).

Transpiration rate (TR) of tanzania guinea grass was not significantly affected by any of the factors studied in the first growth period. In the second growth, TR was influenced by Si rates alone (Fig. 4). The lowest TR was found in the plants supplied with 3 mmol L^{-1} of Si whilst the plants with no Si aplication and Si of 1 mmol L^{-1} did not show significant difference. Transpiration rates of plants supplied with 3 mmol L^{-1} of Si were 6% lower than plants with Si of 1 mmol L^{-1} and no Si application.



$g_s1 = 62.49 – 3.56*Si – 0.13*Cu + 0.91*Si^2 + 0.008*Cu*Si + 0.00009*Cu^2$

$R^2 = 0.71$

$g_s2 = 10.41 – 4.42*Si + 0.054*Cu + 1.12*Si^2 – 0.0007*Cu*Si – 0.0006*Cu^2$

$R^2 = 0.50$

Fig. 3 Stomatal conductance (g_s) in diagnostic leaves in the first (a) and second growth period (b) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates.

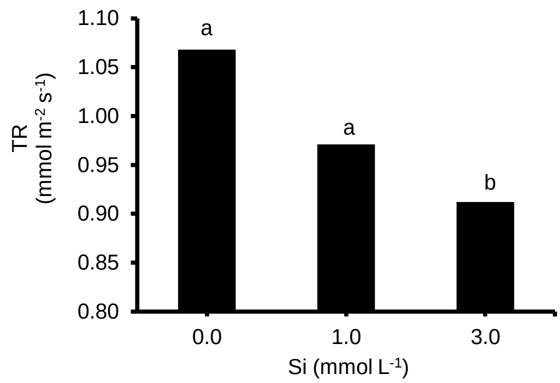
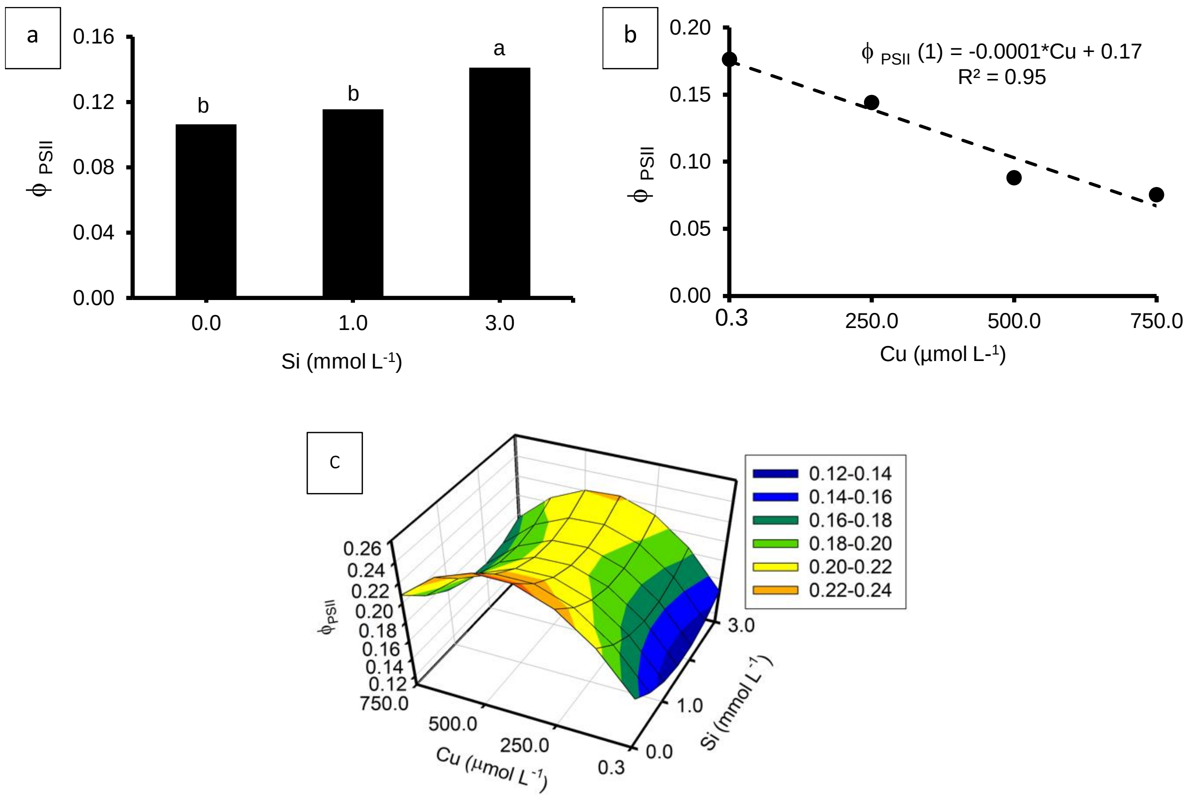


Fig. 4 Transpiration rate (TR) in diagnostic leaves in the second growth period of Panicum maximum cv. Tanzania as influenced by Si rates. Different letters show significant difference by Tukey test.

Quantum efficiency of photosystem II (ϕ_{PSII}) values were not influenced by Si rates and Cu rates in the first growth with any significant interaction. There was significant difference in ϕ_{PSII} between plants treated with 3 mmol L^{-1} of Si and those with no Si treatment and Si of

1 mmol L^{-1} (Fig. 5a). Plants treated with 3 mmol L^{-1} of Si increased ϕ_{PSII} in 20% compared to those treated with other rates of Si. The ϕ_{PSII} reduced with the rising of Cu rates (Fig. 5b), and the difference between the lowest and the highest Cu rates was more than 50%. In the second growth period, ϕ_{PSII} increased with Cu rates, peaking at 500 μmol L^{-1}, then declining (Fig. 5C). Plants with no Si supply showed the highest ϕ_{PSII} values.



ϕ_{PSII} (2) = 69.26 – 16.55*Si + 0.17*Cu + 4.2*Si² + 0.0008*Cu*Si – 0.0002*Cu²

$R^2 = 0.50$

Fig. 5 Quantum efficiency of photosystem II (ϕ_{PSII}) in diagnostic leaves in the first (a and b) and second growth period (c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. In the figure 3a, different letters show significant difference by Tukey test.

3.3.2.Stress indicators and proline

The interaction Cu rates × Si rates was not significant for MDA concentration in shoots of tanzania guinea grass in the first and second growth periods. Nevertheless, Cu rates significantly affected MDA concentration in shoots in both growth periods. In the first growth, MDA concentration in shoots increased with Cu rates, with the difference of 54% between the highest and lowest exposure rates of Cu (Fig. 6a). Plants exposed to the residual Cu responded differently, with MDA concentrations in shoots in the plant regrowth being lower in plants with

Cu rates of 250 and 500 µmol L^{-1}, compared to those with Cu rates of 0.3 µmol L^{-1} (Fig. 6b). However, plants from the combinations of Si rates that received Cu of 750 µmol L^{-1} still showed the highest MDA concentrations. The interaction Cu rates × Si rates was significant for MDA concentration in roots (Fig. 6c). Silicon and Cu together increased MDA concentration in roots, and the highest values were found in the plants exposed to the highest Cu rate and supplied with the highest Si rate.

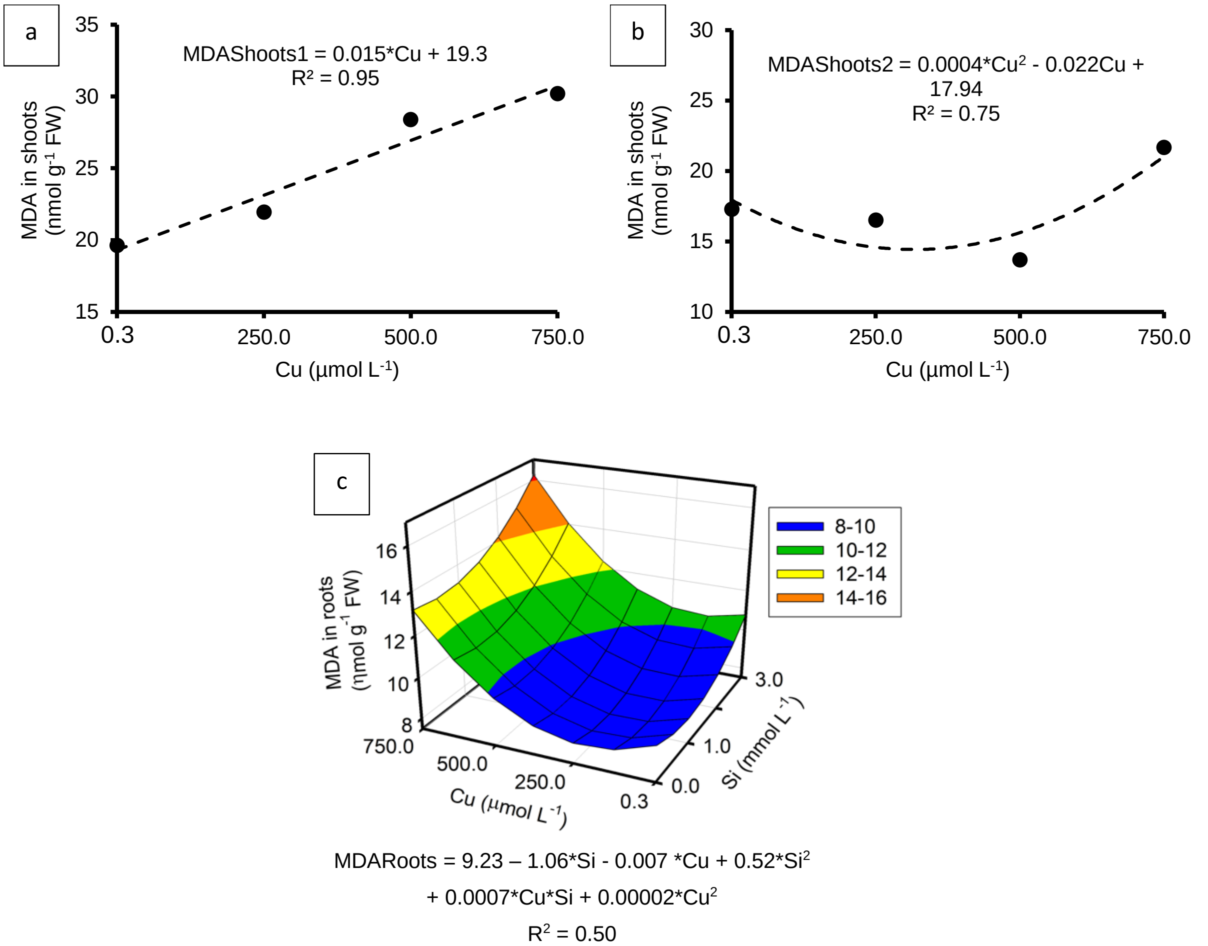
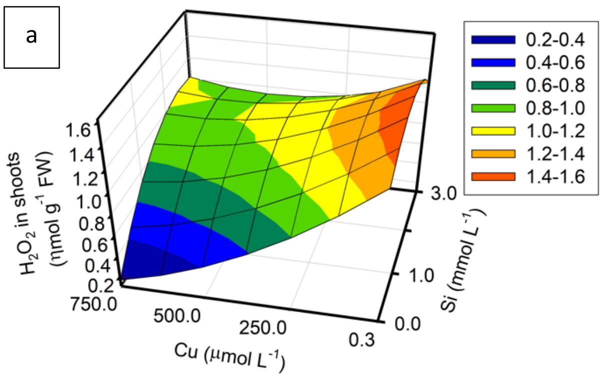


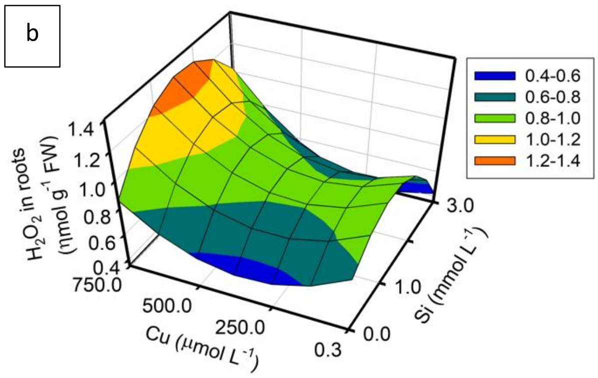
Fig. 6 Malondialdehyde (MDA) concentrations in shoots in the first (a) and second growth (b), and roots (c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. The concentrations are expressed in ηmol per gram of fresh weight of each part.

The H_2O_2 concentrations in plants at the first growth were not significantly affected by any of the factors studied. In the second growth period of the grass, H_2O_2 concentrations increased with the rasing of Si supply and decreasing of Cu rates (Fig. 7a). There was a slight difference in H_2O_2 concentrations among Si rates when the grass was exposed to Cu of 0.3 µmol L^{-1}. However, when it was exposed to Cu of 750 µmol L^{-1} the H_2O_2 concentrations increased with the increment in Si rates. In the roots, the interaction between Cu rates and Si rates was significant for H_2O_2 concentrations and the highest concentrations were detected in plants exposed to 750 µmol L^{-1} Cu and supplied with 1 mmol L^{-1} Si (Fig. 7b). Plants with no-Si application and supplied with Si of 3 mmol L^{-1} showed the lowest H_2O_2 concentrations.



$H_2O_2Shoots2 = 1.33 + 0.34*Si - 0.002 *Cu – 0.13*Si^2 + 0.0004*Cu*Si + 0.000001*Cu^2$

$R^2 = 0.52$

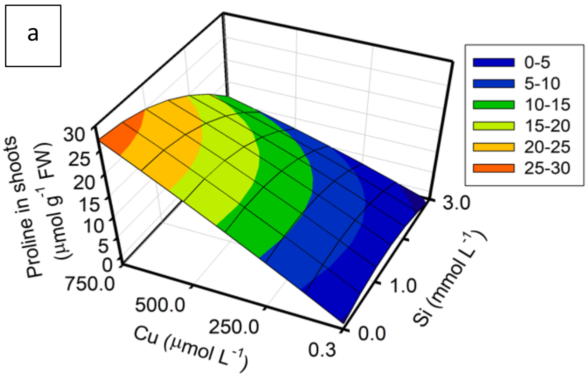


$H_2O_2Roots = 0.75 + 0.39*Si - 0.001 *Cu – 0.16*Si^2 + 0.0002*Cu*Si + 0.000002*Cu^2$

$R^2 = 0.50$

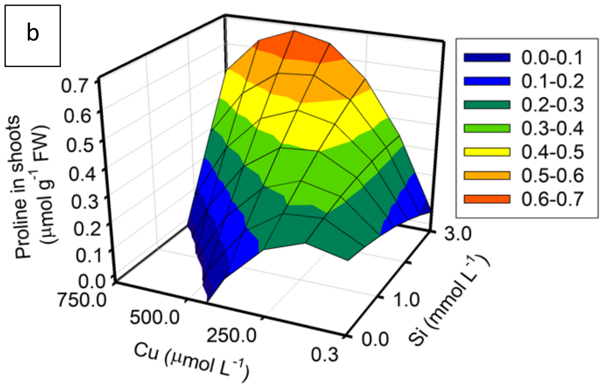
Fig. 7 Hydrogen peroxide (H_2O_2) concentrations in shoots in the second growth (a) and roots (b) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. The concentrations are expressed in ηmol per gram of fresh weight of each part.

To proline concentrations in plant parts in both growth periods, the interaction Cu rates × Si rates was significant (Fig. 8). Copper rates increased and Si supply reduced proline concentrations in shoots in the first growth (Fig. 8a). In the second growth, proline concentrations in shoots increased with Cu and Si rates (Fig. 8b). Proline concentrations in the roots followed the pattern of the shoots during the first growth (Fig. 8c). The highest proline concentration was found when the highest Cu rate was combined to the lowest Si rate.



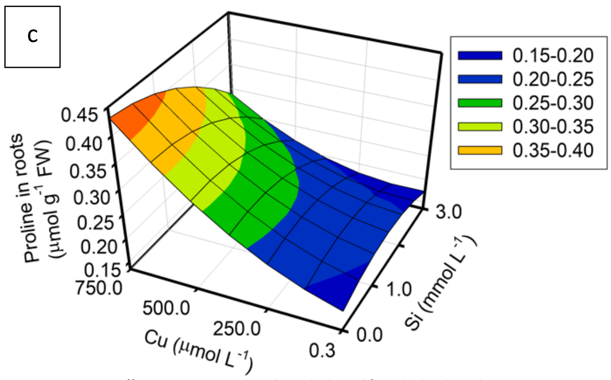
ProlineShoots1 = -0.55 + 3.91*Si + 0.04*Cu – 1.53*Si^2 – 0.0061*Cu*Si – 0.000005*Cu^2

R^2 = 0.70



ProlineShoots2 = 0.24 – 0.029*Si + 0.0006*Cu – 0.011*Si^2 + 0.0007*Cu*Si – 0.000003*Cu^2

R^2 = 0.62



ProlineRoots = 0.18 + 0.04*Si + 0.0001*Cu – 0.014*Si^2 – 0.00006*Cu*Si + 0.0000002*Cu^2

R^2 = 0.50

Fig. 8 Proline concentrations in shoots in the first (a) and second growth (b), and roots (c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. The concentrations are expressed in µmol per gram of fresh weight of each part.

3.3.3. Antioxidant enzymes

Superoxide dismutase (SOD) activity was more evident in combinations in which the grass received Si supply, in both growth periods (Fig. 9a and 9b). Also, SOD activity increased with the raising in Cu rates in the nutrient solution. In general, the activity of this enzyme was higher in the first. In the roots, SOD activity was higher in plants supplied with 1 mmol L^{-1} of Si than in plants with no Si application or supplied with 3 mmol L^{-1} of Si (Fig. 9c).

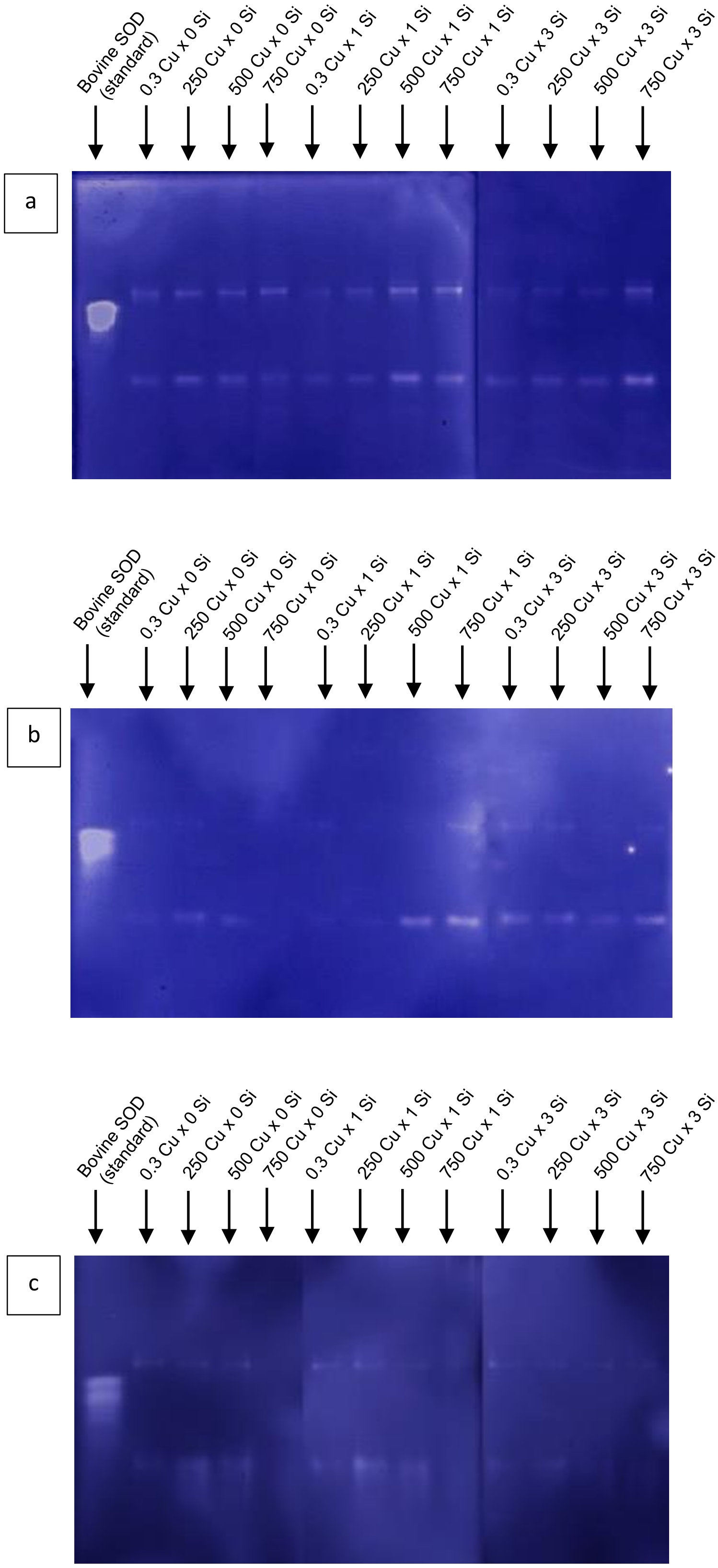


Fig. 9 Superoxide dismutase (SOD) activity in shoots (first growth – a and second growth – b) and roots (c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates.

In the first growth, there was no significant difference among combinations in the activity of catalase (CAT) in shoots of tanzania guinea grass. Catalase activity in shoots in the second growth of tanzania guinea grass was only affected by Cu rates, with no significant interaction between Cu rates and Si rates (Fig. 10a). Compared to plants receiving 0.3 μmol L^{-1} of Cu, tanzania guinea grass exposed to 750 μmol L^{-1} of Cu showed a reduction of 65% in CAT activity. In the roots, the interaction between Cu rates and Si rates was significant for CAT activity (Fig. 10b). Copper rates increased and Si rates decreased CAT activity in roots.

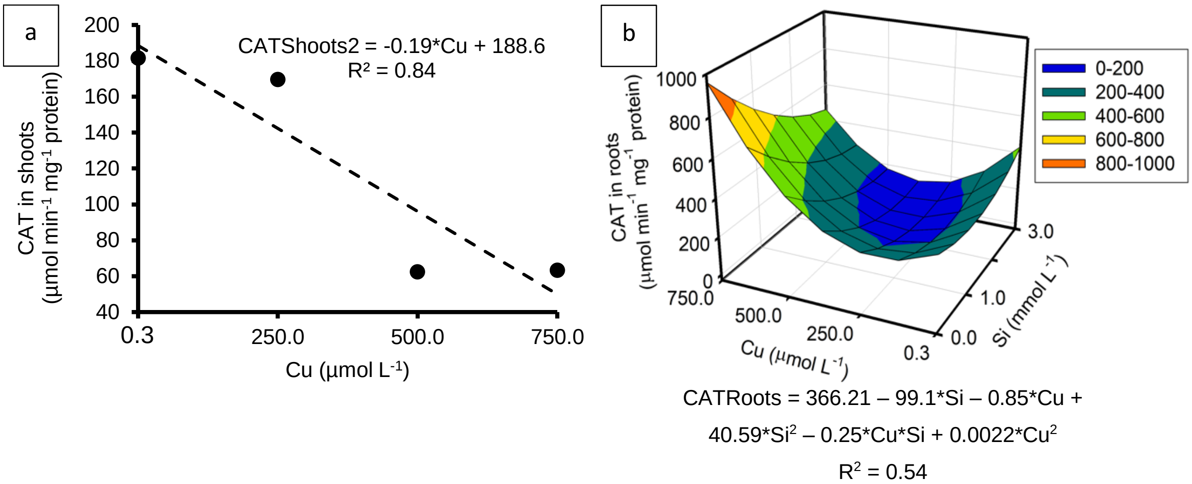


Fig. 10 Catalase (CAT) activity in shoots in the second growth (a), and roots (b) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates.

Ascorbate peroxidase (APX) activity was not affected in any of plant parts of the grass by any of the factors studied. The interaction Cu rates × Si rates was significant for guaiacol peroxidase (GPX) activity only in shoots in the first growth (Fig. 11a). Guaiacol peroxidase increased with the increasing of Cu rates and decreasing of Si rates. In the roots, Cu and Si rates separately affected the activity of GPX, with no significant interaction between them. Plants that did not receive Si had statistically significant higher GPX activity in roots than those that received Si treatment (Fig. 11b). The highest GPX activity in roots occurred in plants exposed to 750 μmol L^{-1} of Cu. The lowest GPX activity was identified in plants exposed to 250 μmol L^{-1} of Cu, being slightly less than plants exposed to 500 μmol L^{-1} of Cu.

Glutathione reductase (GR) activity in shoots was not significantly affected by silicon supply but was affected by exposure to Cu (Fig. 12a and 12b). The increment in Cu rates reduced GR activity in shoots in first and second growth. Comparing the more contrasting Cu rates (0.3 and 750 μmol L^{-1}), GR had its activity reduced in shoots by 33% and 77% in the first and second growth period, respectively. In roots, the interaction Cu rates × Si rates was significant with Cu decreasing GR activity and Si increasing the activity (Fig. 12c). The highest GR activity was identified in treatments with the Cu rate of 0.3 μmol L^{-1} combined with Si supply of 3 mmol L^{-1}. Except for plants exposed to Cu of 750 μmol L^{-1}, plants supplied with Si had higher GR activities than the ones with no-application of this beneficial element.

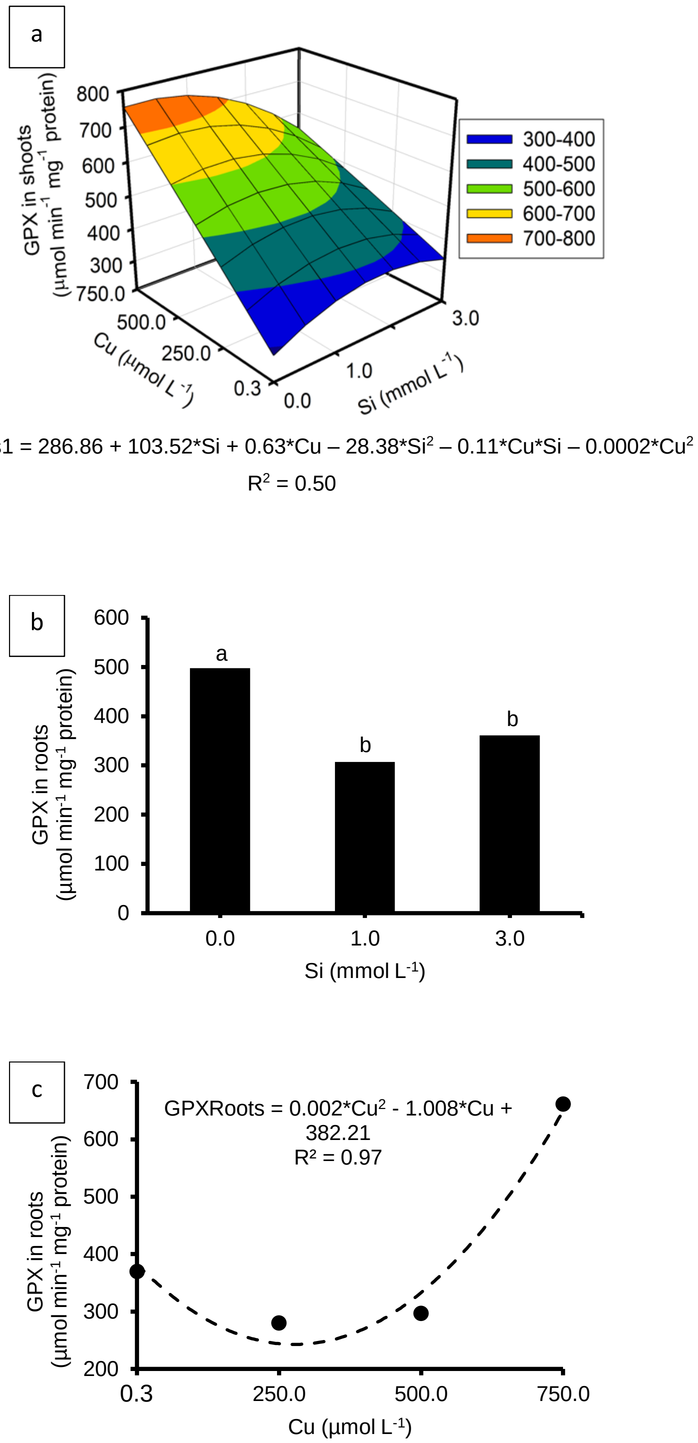
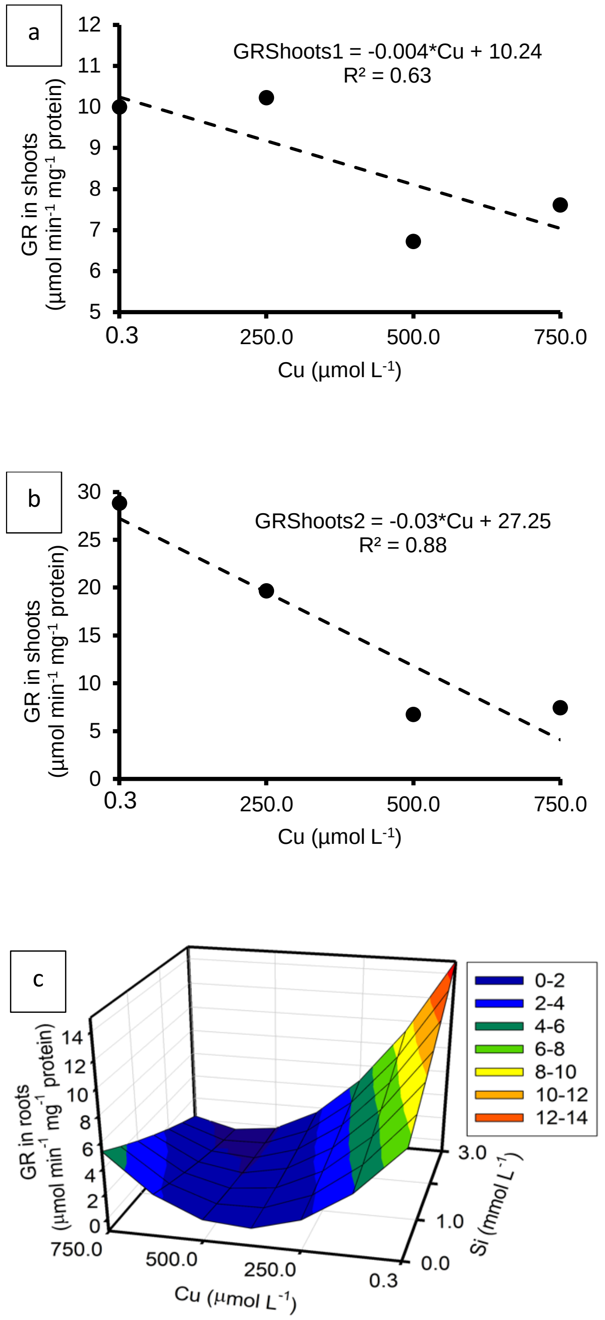


Fig. 11 Guaiacol peroxidase (GPX) activity in shoots in the first growth (a), and roots (b and c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates. In the figure 11b, different letters show significant difference by Tukey test.



GRRoots = 7.61 + 2.08*Si – 0.035*Cu + 0.12*Si^2 – 0.0055*Cu*Si + 0.00004*Cu^2

R^2 = 0.91

Fig. 12 Glutathione reductase (GR) activity in shoots in the first growth (a) and second growth (b), and roots (c) of *Panicum maximum* cv. Tanzania as influenced by Cu and Si rates.

3.4. Discussion

High Cu rates changed tanzania guinea grass metabolism regardless of its proved tolerance (Gilabel et al. 2014, Souza Junior et al. 2018). Gas exchange parameters of tanzania guinea grass were affected by both Cu exposure and Si treatment. Except for transpiration rate (TR), in the first growth all the analyzed photosynthetic parameters decreased as Cu rates increased: net photosynthetic rate (A_N), stomatal conductance (g_s), electron transport rate (ETR) and quantum efficiency of photosystem II (ϕ_{PSII}). Similar results were reported for plants of wheat (*Triticum aestivum*) cultivated in nutrient solution containing Cu of 400 μmol L^{-1}, showing low values of A_N, g_s, ETR and ϕ_{PSII} (Noreen et al. 2018). Cucumber plants (*Cucumis sativus*) exposed to Cu rates of up to 50 μmol L^{-1} (much lower than the ones applied in this study with tanzania guinea grass) also showed a reduction in A_N, g_s, and ϕ_{PSII} (Burzynski & Klobus 2004).

Plants contain dozens of enzymes and proteins containing Cu, and this metal acts as an electron carrier in photosynthesis and as a acceptor of chemical energy derived from proteins and oxidative enzymes (Epstein & Bloom 2004, Heldt & Piechulla 2005). This metal is very reactive and essential in many metabolic routes, justifying the fact that its homeostasis is strictly controlled in all organisms (Burkhead et al. 2009). Copper toxicity is directly related to the favoring of oxidative stress, as high concentrations in the plant tissue induces the excessive synthesis of reactive oxygen species (ROS) (Stadtman & Oliver 1991). As an active redox transition element, Cu can catalyze the overproduction of ROS by stimulating the auto-oxidation and Fenton reactions (Fidalgo et al. 2013). The ROS excess in plants damages mitochondria, chloroplasts and peroxisomes of cells, causing the dysfunction of these organelles that are responsible for the main metabolic processes, including the production of chemical energy, photosynthesis, photorespiration, oxidative phosphorylation, β-oxidation and tricarboxylic acid cycle (Chandna et al. 2012). In the chloroplast, the most pronounced damage is caused by lipid peroxidation in the thylakoid membranes, which occurs when electrons from light cannot be carried in the transport chain by the reaction centers. These loose electrons react to form ROS, thus inducing low photosynthetic efficiency in plants and low metabolic activity.

Silicon supply alleviated the stress induced by excess Cu in the first growth by promoting increases in A_N, g_s, ETR and ϕ_{PSII}. Even when plants were exposed to the recommended Cu rate (0.3 μmol L^{-1}), the high A_N was found in plants supplied with Si. These results are similar to those found by Mateos-Naranjo et al. (2015), in which C4 grass (*Spartina densiflora*) was exposed to 15 mmol L^{-1} of Cu and supplied with 500 μmol L^{-1} of Si and found that Si had a positive effect on A_N, g_s, and ϕ_{PSII}. Cotton plants (*Gossypium hirsutum* L.) supplied with

1 mmol L^{-1} of Si and exposed to Cu rates up to 50 μmol L^{-1} showed higher A_N, g_s and TR values than the ones cultivated in the absence of Si.

The beneficial effects of Si on the relief of abiotic stress, especially those caused by heavy metals (such as Cu), are attributed to the deposition of this mineral mainly on the cell walls of roots, leaves and stem, which creates binding sites for metals. In addition, Si can promote a reduction in the passage of the apoplastic flow and consequently reduce the translocation of toxic metals (Ma and Yamaji, 2006). Silicon may also contribute to the compartmentalization of heavy metals to the cell vacuole, since, through its binding to the metal, it can be further carried into this compartment (Wang et al., 2015). Silicon plays an important role in reducing the reactivity of Cu in the tissues, thus alleviating the effects of Cu toxicity.

In the second growth period, photosynthetic parameters of the grass increased with Cu rates. A likely explanation for this pattern is that the Cu toxicity that the plants were exposed to during second growth period was lower than in the first growth period. Thus, these plants were able to accelerate their metabolism and better deal with the disorders caused by escess Cu. This metabolism acceleration is denominated eustress, a stress that does not cause permanent damage but instead promotes plant health and productivity (Hideg et al. 2013). Plants exposed to residual Cu of 750 μmol L^{-1} were not able to accelerate their metabolism because the high rate of Cu that the grass was exposed to in the first growth period. This high Cu exposure weakened the plant in a way that the grass could not positively respond to the stress in the second growth period. This caused a distress event, also called destructive stress, a strong stress event with subsequent negative modifications in environmental conditions leading to metabolic damage that can advance to death of cells or to the whole plant (Hideg et al. 2013). Plants supplied with Si did not perform as well as those plants that did not receive Si, in relation to the photosynthetic parameters. This might be due the fact that plants supplied with Si did not experience the same level of stress as those grown in the absence of Si. In other words, these plants did not accelerate the metabolism in the same level than the ones with no added Si because the stress level was considerably lower.

The main compound formed when the membrane is attacked by ROS is MDA, a product of the fragmentation of polyunsaturated fatty acids present in the membrane and used as an indicator for oxidative damages in these organelles (Du et al. 2014). Malondialdehyde serves as a signal for the plant, but its formation also implies damage to DNA and proteins. The increment in Cu rates increased MDA concentration in tanzania guinea grass in the first growth period. Gilabel et al. (2014) exposed tanzania guinea grass plants to Cu rates up to 1000 μmol L^{-1} and found a drastic decrease in biomass production due to oxidative stress, identified by the increase in the concentrations of MDA and H_2O_2. Similar results were reported in rice plants exposed to Cu of 100 μmol L^{-1} (Kim et al. 2014). In the second growth period of the present study, MDA concentration was lower in plants exposed to Cu of 250 and 500 μmol L^{-1} compared to other Cu rates. This may be a result of the positive effect that the eustress induced by Cu toxicity promoted on photosynthetic parameters, leading to a reduction in lipid peroxidation.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species and therefore its accumulation is a direct indicator of the imbalance between ROS production and detoxification by antioxidant enzymes. Hydrogen peroxide increased with the increment in Si rates and decreased with the increment in Cu rates in the second growth. Gilabel et al. (2014) also reported a reduction of H_2O_2 concentration with the increment in Cu rates in plants of tanzania guinea grass. This reduction shown only in plants in the second growth period can also be justified by the positive effect of the eustress in photosynthetic parameters induced by Cu toxicity.

The amino acid proline, an organic osmoprotective antioxidant accumulated at high concentrations under abiotic stress, is widely used to evaluate the stress intensity caused by metals (Foyer & Noctor 2000). Proline acts on the stabilization of proteins and protein complexes in the chloroplast and cytosol, protection of the photosynthetic apparatus and enzymes involved in the detoxification of ROS (Szabados & Savoure 2010). In addition, proline modulates respond to biotic and abiotic stress, acting as a metabolic marker that regulates metabolites and redox balance, and controls the expression of innumerable genes, thus influencing plant growth and development (Szabados & Savoure 2010). In the first growth, the proline concentration of tanzania guinea grass increased with Cu rates and with the decrease in Si supply. This might be a result of an alleviation in Cu stress provided by Si supply. Since the Si supplied grass was under less stress than the grass that did not receive Si, it produced less proline. In the second growth period, plants supplied with Si produced more proline but the proline concentrations in general were drastically reduced when compared with values from plants of the first growth period. Proline concentrations in roots increased with Cu rates and decreased with Si supply. This suggests a relationship between increasing proline concentrations and decreasing MDA concentrations in roots.

In order to decrease the synthesis of ROS, plants under stress activate enzymes that convert the superoxide anion and radical peroxides into less and non-toxic forms, thus reducing damage to plant cells (Heldt & Piechulla 2005). Superoxide dismutase activity in tanzania guinea grass, which is considered as the first line of defense during a stress event, increased with Si rates. This positive Si effect on SOD activity was also reported in *Arabidopsis* and cotton plants exposed to Cu rates of up to 30 and 50 μmol L^{-1}, respectively (Khandekar & Leisner 2011, Ali et al. 2016). On the other hand, CAT, GPX and GR did not increase with Si supply.

The ability of plants to increase antioxidant protection to fight the negative consequences of heavy metal stress appears to be limited, since many studies show that when exposed to high rates of redox reactive metals, the activity of antioxidant enzymes is reduced (Schutzendubel & Polle 2002). As copper rates increased, the activities of CAT and GR decreased. However, GPX activity did not show the same pattern and increased with the raising of Cu rates.

3.5.Conclusion

Although copper is an essential element to plant life, when present in excessive rates, it can cause irreversible damage, even in plants with metal tolerance such as *Panicum maximum* cv. Tanzania. Silicon supply improved photosynthetic parameters in the first growth period. As a consequence of this, stress indicators were considerably lower in plants supplied with Si. In the second growth, Cu stress increased gas exchange parameters and decreased stress indicators, sugesting an eustress event. Besides the fact that the activities of antioxidant system enzymes were reduced by Cu stress, SOD activity was increased by Si supply.

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