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Nota Técnica

A FACTORIAL DESIGN APPLIED TO THE STUDY OF CHROMIUM TOXICITY ON THE GLUTATHIONE LEVELS OF *Brachiaria brizantha* AND *Brachiaria ruziziensis* SEEDLINGS

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Chromium toxicity affects redox reactions within plant cells, generating detrimental reactive oxygen species. Glutathione is an antioxidant peptide and also a substrate for the production of phytochelatins, which are chelating peptides reported to mitigate Cr^{3+} toxicity in plants. In this study, *Brachiaria brizantha* (*B. brizantha*) and *Brachiaria ruziziensis* (*B. ruziziensis*) seedlings were evaluated for physiological responses and glutathione production following the addition of zero or 5 mg L^{-1} Cr^{3+} to the nutrient solution. Glutathione levels were determined by colorimetric analysis at 412 nm using 5,5'-dithio-bis(2-nitrobenzoic acid) as a chromophore reagent and recovery with glutathione reductase (with evaluations at days 10 and 20 of continuous growth). The assessments were carried out in a completely randomized design with 2 authentic replications, and arranged in a 2^3 factorial. Cr^{3+} caused an average increase of 0.76 mg g^{-1} in the initial glutathione content. However, by day 20 there was an average reduction of 3.63 mg g^{-1}. Chromium-affected physiological detrimental responses, albeit detected in both species, were less-pronounced in *B. ruziziensis*, along with a much higher level of glutathione. This study indicates that *B. ruziziensis* has a greater tolerance for chromium toxicity than *B. brizantha*, and that glutathione is likely to be involved in the mitigation of chromium stress in *B. ruziziensis*.

Keywords: *Brachiaria ruziziensis*; *Brachiaria brizantha*; glutathione; chromium-toxicity; oxidative stress.

INTRODUCTION

Chromium toxicity in plants is observed at multiple levels, from reduced yield, through effects on leaf and root growth, to inhibition on enzymatic activities and mutagenesis.[1] The presence of chromium in the environment is mainly associated with volcanic activity and industries of steel, leather and textiles. In nature chromium is stable in two different oxidation states, trivalent (Cr^{3+}) and hexavalent (Cr^{6+}), both states being considered phytotoxic. The trivalent state is less phytotoxic than the hexavalent one, but such toxicity is verified even at low levels of concentration affecting plant growth, water balance, pigmentation and inhibition of enzyme activity, problems related to oxidative stress. The latter takes place due to the formation of reactive oxygen species (ROS), such as H_2O_2, $O_2^{\bullet-}$ and $HO^{\bullet}$, which damage DNA, proteins, lipids and pigments. An antioxidant metabolism is induced in order to mitigate the effects of ROS and involves both antioxidants both enzymatic (catalase (CAT), guaiacol peroxidase, glutathione reductase (GR), ascorbate peroxidase and superperoxidase dismutase) and non-enzymatic (ascorbate and glutathione (GSH)).[1–3]

GSH is a tripeptide consisting of residues of the amino acids cysteine, glycine and glutamic acid. Many of GSH reactions involve the thiol group (SH) of cysteine, which is highly polarizable, thus acting as a good nucleophile for reactions with electrophilic chemical compounds. This ability to donate electrons to other compounds also makes GSH a good reductant. The combination of its abundance in aerobic organisms and the chemical properties of the thiol group support the proposal that GSH appeared in biochemical evolution as a protection against ROS and electrophilic compounds generated by oxidative processes, both in the organism and in its living environment.[4] GSH occurs in cellular components such as chloroplasts, mitochondria, endoplasmic reticulum, vacuoles, and cytosol, and acts as a disulphide reductant to protect the thiol groups of enzymes, regenerate ascorbate, and react with $^1O_2$ and $OH^{\bullet}$.[5]

Despite the abundance of the high redox potential molecule ascorbate (AsA) in plants, a reduced form of GSH has long been known to occur in plant tissues at minute levels.[6,7] As critically reviewed, the ROS produced in response to biotic and abiotic stresses are cleansed by the AsA-GSH cycle, in which AsA is used for scavenging hydrogen peroxide as an electron donor.[8] The oxidation product monodehydroascorbate (MDA) is re-reduced to AsA by the flavin-containing enzyme MDA reductase using β-nicotinamide adenine dinucleotide phosphate (NADPH) or by reduced ferredoxin. Part of MDA is spontaneously disproportionated to dehydroascorbate (DHA) and AsA. Eventually, DHA is regenerated to AsA by DHA reductase using GSH as an electron donor, and the resulting oxidized glutathione (GSSG) is reduced to GSH by GR using NADPH. In the sequence, GSH is employed in the backup system for AsA regeneration. However, GSH plays significant roles in many other processes such as regulating growth and acting as electron donor for stress-associated enzymes so that its physiological significance as an antioxidant in plant metabolism merits further research.

The possible effect of exogenous GSH in mitigating chromium stress in rice was studied and it was verified that plant growth and chlorophyll content were dramatically reduced with exposure of plants to 100 μmol L^{-1} Cr.[9] Addition of GSH in the culture solution alleviated the reduction of plant growth and chlorophyll content. The activities of some antioxidant enzymes, including superoxide dismutase, CAT and GR in leaves, and CAT and glutathione peroxidase in roots increased under Cr stress, and such effect was diminished by GSH, along with a reduction in malondialdehyde accumulation. Since effects were detected in leaves and roots, the group concluded that GSH may enhance antioxidant capacity in Cr-stressed plants.

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Furthermore, exogenous GSH caused significant decrease of Cr uptake and root-to-shoot transport in the Cr-stressed rice plants.

Another important contribution of GSH is acting as substrate for the formation of larger peptides known as phytochelatins, whose presence in plants has been related to the ability to form chelates with potentially toxic metals. The formation of chelates would be favored by the presence of the thiol group in the cysteine residues which are present in GSH. It has been suggested that the metal ion activates the synthesis of the phytochelating agent, chelates itself to it and is then transported into the vacuole where it assumes a more complex aggregating shape.[10,11]

Brachiaria is the most widely used tropical grass in agriculture, with widespread pasture areas in Brazil, most of them consisting of *Brachiaria decumbens* and *B. brizantha*.[12] Despite their rusticity, those species lack desirable levels of nutritional quality to feed livestock, and *B. ruziziensis* has been increasingly utilized to partially fulfill the demand for feed quality, and also because it is the most important breeding stock in the genus *Brachiaria*.[13]

Few attempts have been conducted to examine phytochelatin production in the genus *Brachiaria.* Santos et al. studied such metabolic response in *B. decumbens* exposed to toxic levels of Cd and Zn.[14] They verified that Zn negatively affected chlorophyll and β-carotene levels, whereas Cd reduced VAZ cycle pigments (i.e. violaxanthin, antheraxanthin and zeaxanthin), and tocopherol content. Cd was the major inducer of the phytochelatin synthesis pathway in the species.

So far, plant production of GSH in response to chromium toxicity has not been studied in the genus *Brachiaria*. Taking into account the rusticity of *B. brizantha* and the relatively high nutritional quality of *B. ruziziensis*, the establishment of such a relationship in those species could add comparative advantages for the ongoing breeding programs of those species and related hybrids, with genetic modification of plants by introducing selected genes of GSH and GR, and also generate feasible conditions to further advance knowledge towards new bioremediation strategies against the increasing chromium contamination in soils.

The classical method for the determination of GSH is based on its reaction with acid 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). One product of this reaction, 2-nitro-5-mercapto-benzoic acid (TNB) may be determined spectrophotometrically at 412 nm. Initially, DTNB may react with other thiol groups present in other compounds. Therefore, after reacting with DTNB, GSH must be specifically recovered through the action of GR enzyme on the other reaction product. The recovered GSH reacts again with excess DTNB producing more TNB molecules. Consequently, the absorbance increase rate related to TNB production is expected to be proportional to GSH concentration. Since GR also reduces GSSG to GSH, the resulting value must be expressed in terms of total GSH concentration.[15–17] NADPH is used as catalyst for the reaction.

The purpose of this study was to evaluate physiological responses and GSH production in nutrient solution-grown seedlings of *B. brizantha* and *B. ruziziensis*, in response to root exposure to toxic levels of Cr^{3+}, as a means to further identify putative defense mechanisms against such toxicity. In order to study possible interactions among factors as well reduce the number of experiments, cost and time, a statistical approach taking into account a 2^3 factorial design was used. This study is the first report including a factorial approach for this class of comparisons, thus casting new enlightening on the subject.

EXPERIMENTAL

Chemicals and reagentes

Ammonium dihydrogenphosphate ($NH_4H_2PO_4$), β-Nicotinamide adenine dinucleotide phosphate (NADPH), Boric acid (H_3BO_3), Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$), Chromium(III) chloride hexahydrate ($CrCl_3.6H_2O$), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), Ferric-ethylenediaminetetraacetic acid complex (Fe-EDTA), Glutathione reductase (GR), L-Glutathione reduced, Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), Manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$), Potassium nitrate (KNO_3), Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$), Sodium phosphate tribasic (Na_3PO_4), 5-Sulfosalicylic acid dihydrate, and Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents used were of analytical grade.

Growth conditions

Seeds of *B. brizantha* and *B. ruziziensis* were sterilized and germinated in vermiculite at 26 ± 2 °C without artificial lighting.[18] Sixty days after germination, uniform seedlings were transferred to half-strength Clark's nutrient solution containing (in mmol L^{-1}): KNO_3, 5044; $Ca(NO_3)_2.4H_2O$, 1490; $NH_4H_2PO_4$, 10; $MgSO_4.7H_2O$, 98.8; H_3BO_3, 23.12; $MnCl_2.4H_2O$, 4.58; $CuSO_4.5H_2O$, 0.16; $ZnSO_4.7H_2O$, 0.04; $Na_2MoO_4.2H_2O$, 0.22; and Fe-EDTA, 10.[19] Seedling growth was carried out under controlled conditions (LAB-LINE model Biotronette Mark III environmental chamber) set at 28 ± 4 °C, 60% RH, 16h photoperiod and 280 mmol s^1 m^{-2} photosynthetically active radiation (PAR, measured with LI-190SA quantum sensor and LI-189 quantum meter (LI-COR). The pH was measured daily and adjusted whenever necessary (HI 221 ph-meter, Hanna) and the solution changed weekly. After a two-day-period for acclimation, plants were transferred to fresh nutrient solution with the treatments. The harvested samples were stored at -80 ºC until processing.

Experimental design

The study consisted of the evaluation of GSH concentrations in both plant species (*B. ruziziensis* and *B. brizantha*) considering two periods (10 and 20 days) of continuous growth in half-strength Clark's nutrient solution without Fe-EDTA and pH 4.0 with the addition of two chromium levels (0 and 5 mg L^{-1}). Chromium was applied as $CrCl_3.6H_2O$. A control with complete solution and pH 5.5-6.5 was added for the comparisons.

The 2^3 factorial design (two levels and three factors) was carried out as a complete randomized trial, considering as factors plant species, chromium levels and growing periods of the plants, with 2 authentic replications.

Physiological evaluations

Physiological evaluations were performed either in the starting or at the end of the experiment, prior to processing samples for GSH determinations. Lengths of root system and aerial part were measured with a ruler, respectively, at 0, 10 and 20 days of otherwise undisturbed growth. Total fresh weight of harvested seedlings was determined with an analytical balance (Shimadzu Model AUX220). Leaf chlorophyll levels were directly measured using a SPAD meter (Minolta model SPAD 502-DL).[20]

Glutathione determination

GSH levels were determined following the method reported by Griffith with minor modifications.[21] In short, plant samples were ground with mortar and pestles with the aid of liquid nitrogen into a fine powder to pass a 1 mm mash. Extractions were performed in Eppendorf microtubes by adding sulfosalicylic acid aqueous solution 5% (w/v). Samples were them centrifuged at 13,200 rpm during 5 min

(Micromax centrifuge, IEC). The supernatant was transferred to a new tube and maintained at 8 ºC until processing.

A GSH standard stock solution of 15 mg in 50 mL of 0.01 mol L^{-1} phosphate buffer pH 7 was prepared daily and kept at 8 ºC in order to limit hydrolysis and prevent oxidation. The calibration curves were constructed based on dilutions of this stock solution. An aliquot of 1.0 mL of the each diluted GSH standard solution (or 0.25 mL of the sample supernatant) was transferred to test tubes containing the following reaction mixture: 0.01 mL of GSH reductase suspension in 3.6 mol L^{-1} $(NH_4)_2SO_4$, pH 7.0, containing 0.1 mmol L^{-1} dithiothreitol (1 U mL^{-1} - one unit reduces 1.0 µmol of oxidized GSH per min at pH 7.0 at 216 ºC); 0.20 mL of 2.5 mmol L^{-1} DTNB in 0.01 mol L^{-1} phosphate buffer; 0.60 mL of 0.4 mmol L^{-1} NADPH; and 0.01 mol L^{-1} phosphate buffer pH 7, prepared in sufficient quantity to 3.31 mL.

The mixture was homogenized and transferred to a quartz cuvette with 1.0 cm optical path. At this moment, the timer was started. Immediately, each cuvette was measured in a spectrophotometer (CE 1010, CECIL) with readings performed at 412 nm. Readings in each sample were carried out every 30 s, totaling a time frame of 6 min.

Statistical analysis

A curve and respective linear regression were elaborated considering the time x absorbance data. The slope of this curve, i.e. the rate of increase of absorbance, was used to construct the calibration curve for rate x GSH mass through linear regression, following the analysis of variance of the data.[22] Regarding samples, aliquots of the supernatant were taken and the rate of increase of absorbance was properly replaced in the equation of the line obtained for the calibration curve. Then, a 2^3 full factorial design was performed on sample data.[23,24] Statistical analysis and experimental design were performed using Action 2.8 and Excel 2013 softwares.

RESULTS AND DISCUSSION

A typical image of the experimental assembly is shown in Figure 1. Following 20 days of continuous exposure to toxic chromium, *B. ruziziensis* exhibited a lesser decrease in total fresh weight than *B. brizantha* (Figure 2), suggesting it has a higher degree of tolerance to this stress. In turn, both species showed relatively similar and sharp drops in root and aerial part lengths, and in the variation of chlorophyll content (Table 1). Those detrimental effects tended to occur earlier with *B. ruziziensis* than with *B. brizantha*.

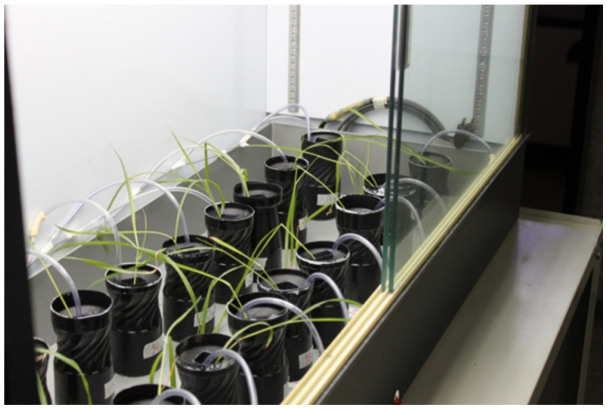

Figure 1. *Typical image of the experimental assembly. The picture refers to an essay carried out with B. ruziziensis*

The toxic effects of chromium on growth and physiology are well documented for many plant species,[25,26] and include inhibition

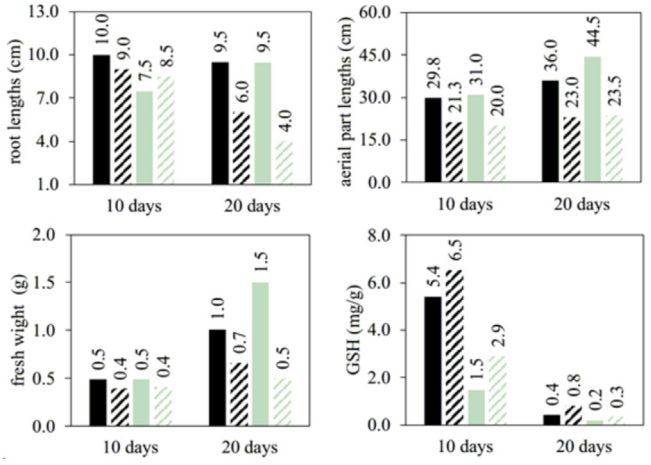

Figure 2. *Physiological values and glutathione amount at the end of the experiments; black column: B. ruziziensis; gray column: B. brizantha; full column: 0 mg L^{-1} Cr^{3+}; and striped column: 5 mg L^{-1} Cr^{3+}*

Table 1. Averaged variation of the chlorophyll during the extent of study

Species	Chromium level (mg L^{-1})	Clorophyll (%)	
		10 days	20 days
B. ruziziensis	0	31	-11
	5	15	-17
B. brizantha	0	-19	0
	5	33	-14

of seed germination, impairment of germination, expansion of roots, stems and leaves, and crop yield. Such stress is also harmful to photosynthesis, water relations and mineral nutrition, effects changes on enzymatic mechanisms, and induces the production of reactive oxygen species which may cause oxidative stress. The variables measured in the present study show that the same effects are likely to occur in the studied species, since all measured variables were consistently depressed throughout the exposure period. The reductions in the chlorophyll level, particularly with *B. brizantha*, suggest a possible Cr-induced sharp drop in Fe uptake, as reported elsewhere.[27]

As exhibited in Figure 3, there was a highly significant and linear relationship between the increment in absorbance and GSH mass, resulting in a calibration curve with a highly satisfactory fit. Thus, the concentration of GSH in plant samples was obtained from the equation $(y \pm 0.3) = (4.04 \pm 0.03).x + (43.9 \pm 0.2)$ for each level in the factorial design (Table 2). This result can be better visualized in Figure 4. In the shown cube, the ABCD, ACEG and ABEF sides

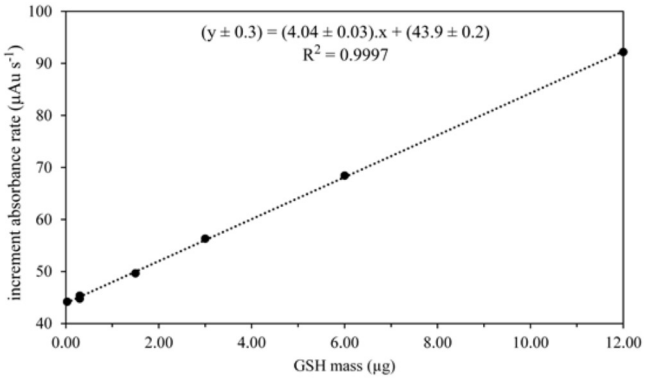

Figure 3. *Linear regression of the calibration curve. Regression significance: $F_{1;5;0.05} = 19502 > 6.61$; lack of fit: $F_{4;1;0.05} = 0.46 < 224.6$*

Table 2. 2^3 factorial design and average concentration of glutathione

Essay	Cr^{3+} / mg L^{-1}	Time / Day	Species	GSH / mg g^{-1} [a]
1	0	10	*B. ruziziensis*	5.41 ± 0.27
2	5	10	*B. ruziziensis*	6.53 ± 0.17
3	0	20	*B. ruziziensis*	0.43 ± 0.13
4	5	20	*B. ruziziensis*	0.82 ± 0.01
5	0	10	*B. brizantha*	1.49 ± 0.09
6	5	10	*B. brizantha*	2.91 ± 0.19
7	0	20	*B. brizantha*	0.21 ± 0.01
8	5	20	*B. brizantha*	0.34 ± 0.04

[a] average ± standard deviation.

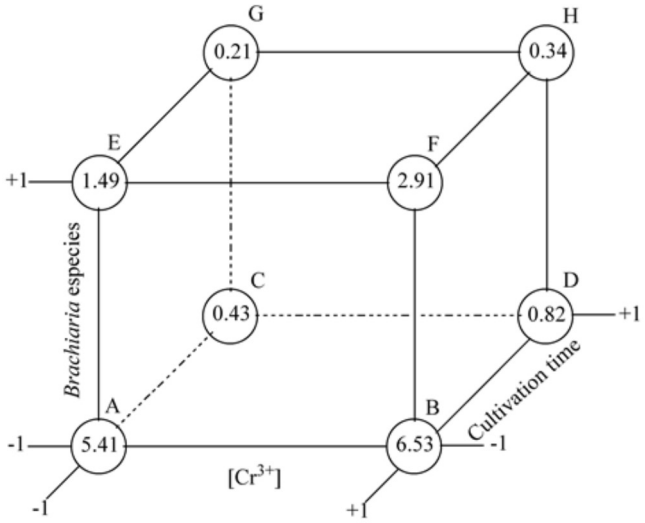

Figure 4. *2^3 factorial design geometric representation of glutathione levels. Values inside spheres correspond to glutathione concentration in mg g^{-1}*

represent the lowest levels for *Brachiaria* species, chromium concentration and cultivation time, respectively.

The statistical analysis (Table 3) shows that all main factors were significant in the alteration of the amount of GSH and it is possible to verify that when chromium content in the nutrient solution was augmented from 0 to 5 mg L^{-1}, there would be an averaged increase of 0.76 mg GSH per gram of plant material. Root secretion of organic acids in response to the addition of GSH to the nutrient solution have been reported to be responsible for alleviating the symptoms caused by chromium stress in rice plants.[28] In our studies, the fact that chromium addition to the growing medium induces in a first moment the GSH production in *Brachiaria* seedlings suggests there is room for studying this chemical as a tolerance factor to chromium toxicity in these and other grass species.

In general, *B. ruziziensis* seedlings are capable of producing GSH at higher quantities than the *B. brizantha* ones (2.06 mg g^{-1}, in average). However, the effect of the interaction between the variables chromium level and species was not significant. When the growing period of 20 days is considered alone, the data reveal that *B. ruziziensis* seedlings showed a smaller Cr-induced physiological damage and yielded higher production of GSH than the *B. brizantha* ones. That might indicate an inherent greater tolerance to chromium toxicity in *B. ruziziensis* as compared to *B. brizantha*.

The variables chromium and species showed a significant interaction effect with time. Although the presence of 5 mg L^{-1} chromium does induce an increased production of GSH, the duration of the growing period causes a drastic reduction in the quantity of this peptide, with an average reduction of 3.63 mg g^{-1} when the growing period is extended from 10 to 20 days. A probable cause for this behavior could be a reduced plant vigor as caused by severe structural and metabolic damage with a longer exposure to chromium. On the other hand, it has been demonstrated that chromium stress affects reductions in the level of GSH and increases the amount of cysteine in rice plants, suggesting there is an influence of chromium stress on the activity of glutamylcysteine synthetase and GSH biosynthesis.[28] However, the possibility of GSH being metabolized as an effect of such stress cannot be ruled out. For example, GSH-based synthesis of phytochelatins catalyzed by phytochelatin synthase, is commonly considered as an important mechanism for plants to tolerate heavy metal stress.[29,30]

Table 3. Calculated effects for the factorial designed and their significance

Effect	mg g^{-1}
Principal Effects	
1 (chromium concentration)	0.76 ± 0.07 [a]
2 (cultivation time)	-3.63 ± 0.07 [a]
3 (*Brachiaria* species)	-2.06 ± 0.07 [a]
Interaction of Two Factors	
12	-0.50 ± 0.07 [a]
13	0.01 ± 0.07 [b]
23	1.71 ± 0.07 [a]
Interaction of Three Factors	
123	-0.14 ± 0.07 [b]

Confidence interval (IC) = 0.16; [a] significant effect; |value of the effect | > IC; [b] non-significant effect; |value of the effect| < IC.

CONCLUSION

The presence of 5 mg L^{-1} of Cr^{3+} appears to induce the production of GSH as compared with the absence of such metal under the same growing conditions. This suggests that GSH may be involved in mitigating processes of stress caused by chromium in *Brachiaria*. There is a significant decrease in GSH levels with the extension of the growing period from 10 to 20 days. That might be caused by the metabolic utilization of GSH in the synthesis of other chemical species. *B. ruziziensis* seedlings showed a lesser Cr-induced physiological damage and yielded higher production of GSH than the *B. brizantha* ones. That might indicate an inherent greater tolerance to chromium toxicity in *B. ruziziensis* as compared to *B. brizantha*. The relevance of such findings to further improvement of the genus *Brachiaria* remains to be evaluated. It is possible that the evaluation of external factors beneficial in modulating GSH and GR pathways such as amino acids, plant steroids, phytohormones and essential elements result useful for a better understanding of the matter.

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