

Response of iron-enriched palisadegrass seedlings to toxic Cr³⁺ revealed by physiological indicators and protein profiles using microfluidic electrophoresis

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Abstract

Most studies on crop responses to Cr toxicity lack using acidic growing medium in order to maximize Cr uptake and taking measures to prevent Cr-induced Fe deficiency. Also, the evaluation of protein profiles is still to be carried out in association with physiological responses to Cr stress. The purpose of this study was to investigate the effects of Cr³⁺ on the physiology and protein profiles of Fe-enriched palisadegrass seedlings in an acidic nutrient solution under controlled conditions. Following 30 days of continuous stress, a sharp reduction in leaf area was the most evident alteration caused by Cr³⁺ stress, while a tendency of depressing effects was observed in the other physiological variables. Nutrient solution acidity had no significant effect, which could be associated with the high tolerance of palisadegrass to acidic soils. Symptoms of Fe deficiency in Cr³⁺-treated seedlings were minimal, suggesting Fe-enrichment was efficient in isolating such a stress from Cr³⁺ effects. Proteins were differentially expressed in Cr³⁺-treated leaf samples, as revealed by microfluidic electrophoresis and SDS-PAGE profiles. In both cases, protein spots were less noticeable under Cr³⁺ stress. It is concluded that a depressed leaf area is the most rapid and detectable response of the species to Cr³⁺ toxicity, along with an overall down-regulation of protein spots. Other variables, such as number of leaves, number of dead leaves, shoot length and chlorophyll level are putative indicators for a prolonged exposure to Cr³⁺.

Keywords: *Brachiaria brizantha*, Cr³⁺, iron supply, medium pH, microfluidic electrophoresis, palisadegrass, physiological indicators, toxicity.

Abbreviations: AEBSF_4-(2-aminoethyl) benzenesulfonyl fluoride; C4H_cinnamate 4-hydroxylase; Chl_Chlorophyll content; DTT_dithiothreitol; GAPDH_glyceraldehyde-3-phosphate dehydrogenase; E-64_N-(trans-Epoxy succinyl)-L-leucine-4-guanidinobutylamide, trans-Epoxy succinyl-L-leucylamido-(4-guanidino)butane; HCl_hydrochloric acid; LA_leaf area; LA/SL_Leaf area/Shoot length; MM_molecular mass, NaOH_sodium hydroxide; NL_Number of leaves; PAL_phenylalanine ammonia-lyase; RL_root length; SL_shoot length.

Introduction

The contamination by toxic metals in soils and water sources has reached alarming levels, especially due to the increasing impact of agronomic and industrial activities (Mirlean et al., 2007). In this regard, chromium (Cr) has been reported as an important contaminant with severe negative effects on many crops (Volland et al., 2014). As extensively reviewed (Singh et al., 2013), toxicity of Cr to plants is influenced by its valence state, in which Cr⁶⁺ is highly toxic and mobile, whereas Cr³⁺ is less toxic but more intensively bound to soil organic matter. Because plants are devoid of corresponding transporting systems, Cr is taken up by carriers of essential ions such as sulfate or iron (Fe). Toxic effects of Cr on plant growth and development include impairment of germination, expansion of roots, stems and leaves and yield reduction. Cr also induces harmful effects on physiological processes such as photosynthesis, water relations and mineral nutrition (Subrahmanyam, 2008). Toxic Cr-induced metabolic alterations include direct effects on enzymes and other metabolites, and the generation of reactive oxygen species which may cause oxidative stress (Shanker et al., 2005). The Cr contamination issues have been intensively approached through the use of plants for soil phytoremediation. In this

case, most studied Cr hyper-accumulator species are either low in biomass or slow in growth; thus, limiting their sustainable application (Diwan et al., 2008). Species with agronomic importance tend to have limited phytoremediation potential because Cr accumulates in the root system and if a high Cr content occurs in the shoots, toxicity will be extreme (Shahandeh and Hossner, 2000).

Nevertheless, efforts remain towards examining high biomass forage grasses capable of providing useful data to further ameliorate plant tolerance to Cr toxicity and alleviation of Cr pollution in soils. Recently, guinea grass (*Panicum maximum*) was found as a potentially moderate Cr³⁺ accumulator (Olatunji et al., 2014). In Brazil, pastures of palisadegrass (*Brachiaria brizantha* Hochst. Ex A. Rich) Stapf. (syn. *Uruchloa brizantha* (Hochst. Ex. A. Rich) R.D. Webster) cover about 30 million ha of mostly acidic soils (Boddey et al., 2004) including areas with long-reported incidence of Cr contamination (Jordão et al., 2005).

Palisadegrass might have a role in bioremediation since closely related *Brachiaria* species are Cr hyperaccumulators (Pires et al., 2007). The known genetic variability of the species is small but the possibility of generating crossbreds

with other species of the same genus (Fuzinatto et al., 2012) is likely to favor genetic breeding for desirable Cr tolerance traits. In this particular case, knowledge on protein profiles and their alterations in response to stress could be potentially useful for supporting early selection strategies.

Protein profiles enable distinctive insights into biological systems that cannot be acquired from genomic or transcriptomic approaches (Sharmin et al., 2012). The application of such a strategy to verify biomolecular responses to heavy metal stress has been useful for identifying putative metal-specific protein markers of susceptibility (Takac et al., 2011). The microfluidic analysis systems (Wu et al., 2008) provide additional advantages in the small sample volumes required, reduced reagent consumption, enhanced mass detection, and yielding analytical figures of merit superior to traditional slab gel electrophoresis.

In spite of such prospects, most investigations on Cr toxicity in plants lack studying physiological alterations as associated with protein profiles. Additionally, the strategies are short of approaching two important methodological details, which may lead to further advances in the matter. One is the use of acidic growing media, since Cr uptake depends upon an acidic aqueous fraction with a pH near 4.00 (Singh et al., 2013). Such a measure could be additionally advantageous for matching the soil acidity commonly verified in the wet subtropics (Joris et al., 2013); thus, improving possibilities of practical application of the findings. The other is the Cr-induced sharp decrease in Fe contents in both the growing medium and the plant tissues along with the appearance of Fe deficiency symptoms (Mallick et al., 2010). Therefore, the conduction of studies under acidic conditions could optimize Cr absorption and the inclusion of a Fe supply step prior to inducing Cr stress could help preventing Fe deficiency to provide a better isolation of Cr effects.

To date, there is no report on the effects of toxic Cr on the behavior of palisadegrass. The currently available information for other species strongly suggests that the assessment of the effects on physiological variables along with protein profiles has the potential to reveal new behavioral patterns, which may be useful for progress in knowledge with direct use in the genetic improvement of the species. Therefore, this study was aimed at studying such a species regarding the effects of Cr³⁺ on physiological indicators and protein profiles measured by microfluidic electrophoresis technology. Seedlings were grown in acidic nutrient solution and received Fe supply prior to treatment exposure, as an attempt to optimize Cr uptake and alleviate possible interferences of Fe deficiency.

Results

Visual symptoms of the effects of low pH and Cr³⁺ exposure on Fe-enriched palisadegrass

The typical visual aspects of plants grown under each studied treatment are shown in Fig. 1. The more conspicuous alterations in relation to the control were a depressed volume of the root system in response to pH 4.0, a reduction in LA as caused by Cr³⁺ and a light weakening of the green pigmentation in leaves as observed in pH 4.0 and Cr³⁺. The inhibition of leaf growth appears to be a direct response to Cr³⁺ toxicity, as verified in other species (Sundaramoorthy et al., 2010). The loss of pigmentation suggests a decrease in chlorophyll concentration as caused by both the acidic pH

and Cr³⁺ stress, but also a Cr³⁺-effected Fe deprivation, as previously verified elsewhere (Ali et al., 2011). Finally, plants under Cr³⁺ treatment were the only ones to show incidence of dead leaves, a behavior which could be further verified as an additional indicator of response to Cr³⁺ toxicity in palisadegrass. The root system, in turn, showed no other apparent alteration than an evident decline in the volume of adventitious roots in plants exposed to Cr³⁺.

Effects of low pH and Cr³⁺ exposure on growth and physiology of Fe-enriched palisadegrass

The results obtained with growth and physiological indicators are exhibited in Table 1. The only significant effect ($p \leq 0.05$) was observed with LA, for which exposure to toxic Cr³⁺ was substantially depressing. Most variables (i.e. RL, SL, NL, LA/SL ratio, transpiration and Chl), albeit with no significant variation, showed a tendency of decreasing responses as Cr³⁺ was added to the nutrient solution. FW means, in turn, were not altered. Under the pH 5.5 treatment, there was a tendency of enhanced transpiration rates and RL and SL, while pH 4.0 tended to promote LA and LA/SL ratio.

The reported tendencies appear to signal a rapid response of palisadegrass to Cr toxicity as reported in other species (Chigbo and Batty, 2013). Such an absence of statistically significant differences for most variables might be explained by the relatively short duration of the assays (Pandey et al., 2005). The tendency of increased FW after addition of Cr³⁺ would need further investigation. On the other hand, the sharp reduction in LA clearly indicates that the effects of Cr³⁺ toxicity in the species are practically immediate. Given longer evaluation times, other variables might show depressing responses.

Effects of low pH and Cr³⁺ exposure on protein profiles of Fe-enriched palisadegrass

The linear regression equation for standard protein data was $y = 0.0401x + 0.0057$, and the corresponding coefficient of determination was $R^2 = 0.9372$. The experiment was performed using $1 \mu\text{g } \mu\text{L}^{-1}$ of sample.

The gel-like images obtained in the microfluidic electrophoresis system using this protein extraction are shown in Fig. 2. The proteins expressed in each treatment were separated according to their respective molecular mass and retention times. Protein profiles revealed eight protein bands. The most conspicuous protein bands were observed in the sizes of nearly 20.7 and 25.7 kDa in all treatments, and also of approximately 51.4 and 53.6 kDa. Those profiles indicate that proteins were differentially expressed in treatments. Also, the intensity of the protein bands was greater with pH 4.0 rather than pH 5.0 or Cr³⁺ (Fig. 2).

Comparisons focused on the plotted areas of these detected protein mass values revealed even more distinct contrasts (Fig. 3). The electropherograms showed that the areas relative to the 20.7 and 25.7 kDa were greatly reduced in Cr³⁺ when compared to those obtained with the other treatments. It should be noted that the seedlings exposed to Cr³⁺ did not express proteins with 51.4 or 53.6 kDa (Fig. 3). Moreover, other molecular mass peaks, which coincided with the treatments lacking exposure to Cr³⁺ were observed but not considered for the evaluations because they represented low level (below 5%), which is below the precision threshold of the technique. The same criterion was applied to several

Table 1. Effects of varying pH and Cr⁺³ exposures of the growing medium on growth and physiological responses of palisadegrass seedlings.

Treatment	SL (cm)	RL (cm)	NL	Fresh wt. (g)
Control	48.6± 3.77 ^a	15.23± 1.98 ^a	25.2± 3.54 ^a	0.95± 0.21 ^a
pH 5.5	53.0± 3.58 ^a	18.32± 1.64 ^a	22.4± 1.85 ^a	0.99± 0.14 ^a
pH 4.0	48.6± 4.50 ^a	16.07± 2.55 ^a	26.6± 7.55 ^a	0.95± 0.13 ^a
Cr ⁺³	40.4± 9.73 ^a	14.45± 1.09 ^a	17.8± 5.53 ^a	1.02± 0.29 ^a

Treatment	LA (cm ²)	LA/SL Ratio (cm)	Transpiration (cm ² .s ⁻¹)	Chl (SPAD)
Control	82.074± 5.40 ^{ab}	1.6909± 0.0545 ^a	28.80± 22.71 ^a	48.24± 12.75 ^a
pH 5.5	81.366± 12.60 ^{ab}	1.5458± 0.2818 ^a	36.10± 22.34 ^a	46.72± 11.77 ^a
pH 4.0	85.806± 14.05 ^a	1.7867± 0.3652 ^a	30.84± 11.38 ^a	49.50± 11.76 ^a
Cr ⁺³	53.404± 20.55 ^b	1.2738± 0.2124 ^a	25.60± 12.91 ^a	38.90± 10.71 ^a

For each variable, means followed by the same letter are not significantly different (P>0.05) by the Tukey test.



Fig 1. Palisadegrass depressed root volume in response to lowered pH and diminished leaf area in response to the addition of Cr⁺³ to the growing medium.

Table 2. Treatment-effects on palisadegrass leaf protein molecular masses (kDa). Estimations were carried out with the microfluidic technique and data reliability was verified against profiles obtained with SDS-PAGE technique.

Peaks	Control		pH 5.5		pH 4.0		Cr ⁺³	
	SDS-PAGE	Microfluidic	SDS-PAGE	Microfluidic	SDS-PAGE	Microfluidic	SDS-PAGE	Microfluidic
1	109.66	---	108.51	---	104.79	---	106.55	---
3	66.75	66.20	65.17	66.30	63.75	62.50	---	---
4	56.57	53.70	55.55	53.60	54.35	52.90	55.68	---
5	51.28	51.80	51.29	51.40	---	50.96	---	---
6	41.13	39.10	---	38.90	---	37.80	---	---
7	35.72	35.00	34.83	35.30	34.51	34.80	---	---
8	27.61	25.40	26.91	25.50	27.00	25.31	27.04	25.75
9	21.61	20.70	21.18	20.80	21.92	20.53	22.64	20.30
10	14.24	---	13.77	---	13.66	---	13.62	---
11	11.42	12.50	11.04	12.50	11.37	12.56	11.06	---

Absence of values indicates lack of band detection.

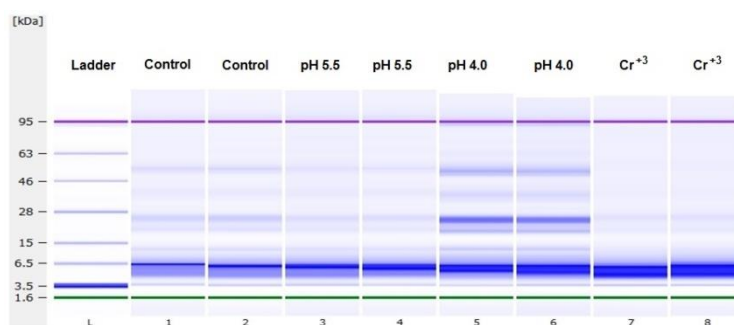


Fig 2. Gel-like images obtained in the microfluidic chip electrophoresis system with the proteins extracted from leaf samples of palisadegrass in response to the studied nutrient solution treatments. Proteins were separated according to molecular mass and retention time. Critical protein bands were detected in sizes close to 20.7 and 25.7 KDa. Band intensity was superior in pH 4.0 and gradually decreased in the order of pH 5.0, Control and Cr⁺³.

Table 3. Protein molecular mass, concentration, total percentage and plot area for palisadegrass leaf samples in response to the nutrient solution treatments.

Treatment	Protein spot	MM (KDa)	Protein concentration (ng.µL ⁻¹)	Protein total percentage	Protein plot area
Control	1	20.80±0.07	54.50±13.70	2.8±1.80	56.7±30.90
	2	25.60±0.20	156.80±60.80	7.4±2.30	165.8±48.80
	3	51.30±0.20	23.50±14.70	0.7±0.40	20.3±13.00
	4	53.50±0.04	70.60±22.50	3.1±0.60	90.6±29.04
pH 5.5	1	20.78±0.03	36.80±8.01	1.5±0.40	36.1±7.04
	2	25.62±0.14	144.04±27.50	4.3±0.70	110.8±15.10
	3	51.25±0.13	12.97±1.00	0.5±0.04	15.6±1.40
	4	53.54±0.18	63.40±11.50	2.2±0.40	76.5±18.40
pH 4.0	1	20.50±0.11	117.30±26.60	6.4±4.15	85.9±46.70
	2	25.28±0.30	410.30±13.40	22.1±15.34	338.7±104.90
	3	51.00±0.60	133.90±111.10	5.9±4.7	93.2±54.30
	4	53.04±0.70	106.10±47.50	3.8±1.4	136.1±84.70
Cr ⁺³	1	20.70±0.20	30.90±1.80	1.7±1.00	22.2±4.60
	2	25.80±0.10	95.00±9.80	7.4±4.70	61.0±17.00

Protein plot areas refer to graphical representations of detected protein mass values as performed with the software Molecular Analyst.

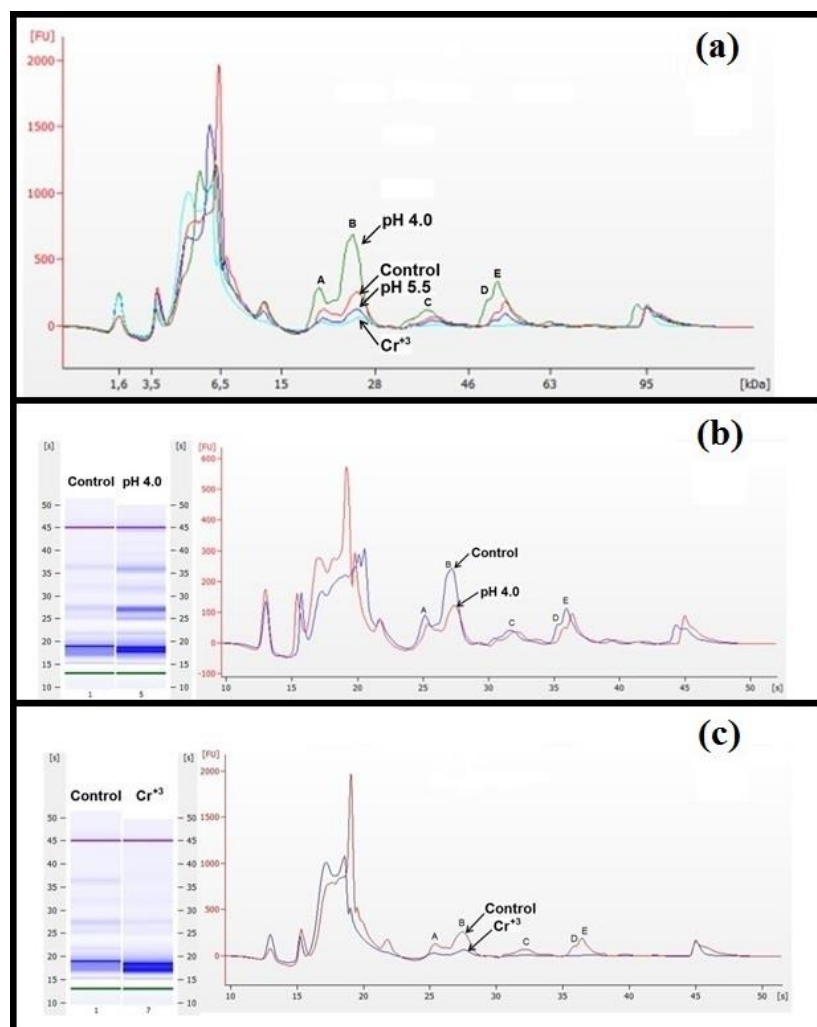


Fig 3. Lab-on-a-chip capillary electrophoresis analysis of palisadegrass leaves shown as (a) elution profiles in response to pH 4.0, pH 5.5 and Cr⁺³ with critical peaks detected from nearly 20.7 to 25.7 KDa, (b) electropherogram and respective simulated gel pattern (on the left) comparing Control and pH 4.0, and (c) electropherogram and respective simulated gel pattern (on the left) comparing Control and Cr⁺³.

Table 4. Proteins currently identified in palisadegrass according to the database of the National Center for Biotechnology Information (2014) and respective molecular masses as determined elsewhere under varying conditions.

Protein	Molecular Mass (kDa)	Reference
Ribosomal protein L16	19.0	Klein et al. (2004)
NADH dehydrogenase subunit F	30.0	Lamattina et al. (1993)
GAPDH	36.0	Russell and Sachs (1989)
Cinnamoyl-CoA reductase	37.5	Hu et al. (2011)
Caffeic acid <i>O</i> -methyltransferase	39.7	Kim et al. (2006)
C ₄ H	57.8	Pina et al. (2012)
PAL	80.0	Kawamata et al. (1992)

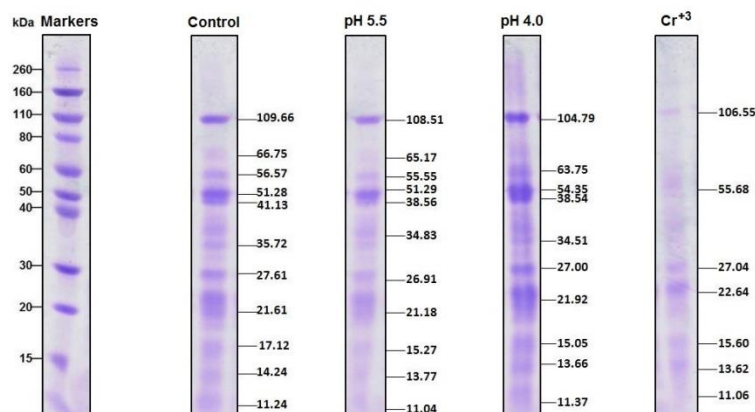


Fig 4. SDS-PAGE electropherograms of proteins extracted from palisadegrass leaf samples showing the band profiles obtained with Control, pH 5.5, pH 4.0 and Cr³⁺.

other small peaks, which apparently indicate the occurrence of proteolysis during protein extraction.

Further comparisons revealed a greater graph area of pH 4.0 as compared to the one of the control, relative to protein masses of 20.7 and 25.7 KDa, and 51.4 and 53.6 KDa (Fig. 3). Thus, lowered pH in the growing medium is likely to have contributed to a higher expression of those protein fragments. The separation achieved with the microfluidic electrophoresis technique was compared with the corresponding one obtained using SDS-PAGE (Fig. 4, Table 2). Both methodologies yielded similar protein profiles, although SDS-PAGE revealed 11 protein fragments and characterized two of them with molecular masses of, respectively, around 14 kDa and 108 kDa. The latter could not be detected on the microfluidic electrophoresis, since the utilized kit was specific for the molecular mass range of 5-80 kDa. The SDS-PAGE gel image showed that proteins were differentially expressed in treatments by means of the intensity of the protein bands (Fig. 4).

Table 3 shows data on molecular mass, protein concentrations, peak area (electropherograms) in response to the studied treatments, which were estimated by the Agilent 2100 Expert Software. The highest concentration was found for proteins of 20.8, 25.6 51.3 and 53.5 kDa. The control condition yielded proteins of 20.8 and 25.6 kDa, with relative concentrations of respectively 54.5 and 156.8 ng μL^{-1} , and of 51.3 and 53.5 kDa, with relative concentrations of respectively 23.5 and 70.6 ng μL^{-1} . The pH 5.5 treatment yielded concentrations similar to those obtained under control condition. The pH 4.0 treatment had the highest relative concentrations. Proteins with sizes of 51.3 and 53.5 kDa which were not expressed with Cr³⁺ and proteins with sizes

of 20.5 and 25.3 kDa were found with the lowest relative concentrations.

Discussion

The concomitant evaluation of physiological responses and protein profiles has seldom been conducted in studies of Cr toxicity in plants, leaving a wide range of questions unanswered that could lead to advancements in the knowledge on the matter which may favor breeding programs. On the other hand, the use of acidic media and the prevention of Fe-deficiency have not been carried out so far in Cr-stress studies. Both aspects were approached in our investigation, leading to the discovery of tendencies which are likely to improve the way Cr toxicity is studied. Finally, the evaluation of such stress on palisadegrass with attempts to solve the above limitations is unprecedented, with the remarkable potential of contributing to progress in future related research work.

It is worth clarifying some details of the procedure utilized in this study. Cr³⁺ was chosen over Cr⁶⁺ because it is the oxidation state that prevails on the root surface (Zayed et al., 1998) and promotes much greater Cr accumulation in plant tissues (Zhang et al., 2007). Secondly, the applied level of toxic Cr³⁺, although much lower than other contents evaluated elsewhere (Sharmin et al., 2012), has been long-reported as the initial toxic concentration in studies carried out in nutrient solution (Vernay et al., 2007) and corresponds to the lowest contamination level registered for natural soils (Oze et al., 2007). In the present work, the Cr³⁺ proved to cause rapid physiological damage and protein profile alterations; thus, eliminating the need for evaluating more toxic concentrations. Finally, the step of a pre-treatment with

Fe-enriched solution followed by the suppression of that micronutrient during exposure to Cr³⁺ was carried out in order to prevent Fe deficiency during the studies, since Cr³⁺ impairs Fe uptake (Volland et al., 2012). Since Fe deficiency symptoms are very mild in the Cr³⁺-treated plants, the carrying out of Fe pre-supply apparently minimized such an interaction.

Under those novel experimental conditions, results revealed a series of relevant plant responses. The acidic pH in the nutrient solution caused only a slight decrease in the leaf green pigmentation and no other physiological alteration. Although this mild response differs from more depressing pH effects verified with other grasses (Passos et al., 1999), such a behavior would not be surprising, since palisadegrass is long-regarded as tolerant to low fertility acid soils (Adamowski et al., 2008). With reference to Cr³⁺ toxicity, the most rapid and evident influence on physiological indicators was the decrease in LA and the tendencies of depressed NL and SL. Such an overall behavior is in agreement with previous reports (Sharmin et al., 2012). The observed Cr³⁺-induced incidence of dead leaves has not been verified in similar approaches (Arduini et al., 2006).

The observed tendency of a decrease in Chl is consistent with the reduction in the green pigmentation of plants exposed to Cr, resembling the onset of Fe deficiency. From these observations it seems that a prolonged exposure to Cr³⁺ would cause more pronounced reductions in those variables, as verified elsewhere (Ali et al., 2011). The results apparently confirm that Cr³⁺ impairs or greatly reduces Fe uptake (Sundaramoorthy et al., 2010) and chlorophyll biosynthesis (Pandey and Sharma, 2003), even with the care of providing substantial Fe supply prior to Cr³⁺-stressing the plants. As observed by Sharma and Sharma (1999), leaf growth variables are likely to serve as suitable indicators of Cr pollution and as well of tolerant genotypes in selection programs.

Other important findings were achieved with the protein profiling approach. The protein profiles obtained with palisadegrass leaf samples as analyzed through the traditional SDS-PAGE technique revealed 12 protein bands which varied in size from approximately 8 to 109 kDa. This result is not in agreement with data reported by Machado Neto et al. (2002), who determined the protein profiles in husked seeds of five palisadegrass genotypes and observed 17 different SDS-PAGE polypeptide bands with molecular mass ranging from 18.7 to 100.6 kDa. Such a contrast in the number of bands may have been caused by genetic differences between the populations evaluated in each case. So, new studies considering a greater extent of the genetic basis of the species are still needed to provide a more detailed assessment.

Regarding characterization, seven proteins are reported for palisadegrass in the database of the National Center for Biotechnology Information (2014), and their respective molecular masses have been determined in different species (Table 4). The polypeptide molecular masses detected in this study (Table 2) are consistent with those proteins. Once treatments were applied, such a profile experienced only a fading of band intensities in Cr³⁺-treated samples as revealed by the SDS-PAGE technique. This indicates that possible alterations are likely to appear in a more extended time frame than the one covered in this study. Studies on Cr toxicity in other species (Sharmin et al., 2012) have reported Cr-responsive proteins, such as inositol monophosphatase, nitrate reductase, adenine phosphoribosyl transferase, formate dehydrogenase and a putative dihydrolipoamide dehydrogenase. In the present study, no protein profile matched the molecular masses of those proteins; thus,

indicating that the occurrence of such a behavior in palisadegrass merits further research.

Taking the mass profiles obtained from SDS-PAGE approach into account, observing a variation margin of 3 kDa, it is reasonable to suggest the following as possibly detected proteins and respective primary functions: ribosomal protein L16 (19 kDa), which is required for early stages of ribosome assembly (Wolfe et al., 1992), NADH dehydrogenase subunit F (30 kDa), which catalyzes the transfer of electrons from NADH to plastoquinone (Mus et al., 2005), GAPDH (36 kDa), which catalyzes the oxidation of triose phosphates during glycolysis (Holtgreffe et al., 2008), caffeic acid O-methyltransferase (39.7 kDa), which catalyzes key steps in the pathway of lignin monomer biosynthesis (Parvathi et al., 2001), and C4H (57.8 kDa), which participates in the phenylpropanoid pathway giving rise to an array of metabolites (Achnine et al., 2004). Hence, further advancement in the knowledge of this subject must consider the definition of the structure of each detected protein to confirm whether the respective amino acid sequences are homologous to the present identifications, and the evaluation of the responses of the regulated processes to Cr³⁺ toxicity.

To the best of our knowledge, the current study can be considered as the first report of Cr³⁺ toxic effects on palisadegrass and also the first inclusion of a Fe pre-treatment to minimize Cr³⁺-induced Fe-deficiency. Our results indicate that there is room for seeking additional understanding about the mechanisms of Cr³⁺ toxicity and also physiological sources for tolerance in palisadegrass. Besides evaluating longer times of exposure, putative niches for progress in this area would be monitoring Cr³⁺ accumulation in tissues (Vajpayee et al., 1999), its relation to the uptake of essential elements (Sundaramoorthy et al. 2010) and the evaluation of enzyme-effected oxidative changes in tissues (Subrahmanyam, 2008).

With reference to Cr³⁺ translocation, much work is still needed. Although recent evidence indicated that Cr accumulation is much greater in roots than in shoots (Sundaramoorthy et al., 2010), the opposite situation has also been reported (Ramachandran et al., 1981). Moreover, the observation of a consistent translocation of Cr to dead leaves (Arduini et al., 2006) indicates this aspect should be considered in verifications of Cr toxicity in plants.

Very few data are available about plant genetic variability for tolerance to Cr toxicity. Diwan et al. (2008) carried out such an evaluation in Indian mustard (*Brassica juncea* (L.)) and observed that all the genotypes responded to Cr-induced oxidative stress by modulating nonenzymatic (glutathione and ascorbate) and enzymatic antioxidants (superoxide dismutase, ascorbate peroxidase, and glutathione reductase). Since the level of induction differed among the genotypes, there is room for genetic selection towards tolerance to Cr. Therefore, enzymatic evaluations along with detailed proteomic approaches have a high potential to serve as a basis for genotypic selection of palisadegrass for tolerance to Cr³⁺.

The behavioral profiles detected in this study are potentially useful for breeding purposes, particularly when the extreme demand for improving *Brachiaria* under acidic soil conditions are taken into account (Fuzinatto et al., 2012). Further progress is to be achieved with approaches including the measurement of Cr³⁺ levels in the plant tissues, and more detailed analyses in the rhizosphere. Also, the exam of the genetic variability of palisadegrass regarding tolerance to Cr³⁺ toxicity aiming at increased forage production in acidic soils is of paramount importance.

Briefly, the present study demonstrated that palisadegrass seedlings exhibit detectable visual symptoms and significant physiological responses to Cr^{3+} toxicity such as growth and leaf area inhibition and decrease in leaf chlorophyll content. Those responses are readily detectable when plants are grown under acidic conditions and without the interference of Cr-induced Fe deficiency. The initial examinations of Cr^{3+} toxicity effects on protein profiles also detected changes; thus, leading to the possibility of further progress with more detailed proteomic approaches.

Materials and Methods

The study was conducted in the Laboratories of Biotechnology and Plant Physiology and Molecular Genetics of Embrapa Dairy Cattle, Juiz de Fora, Brazil. All chemicals used were of analytical grade.

Plant growth conditions

Seedlings were obtained through *in vitro* micropropagation according to the protocol of Cabral et al. (2011) with minor modifications. Acclimated plants were subsequently transferred to pots containing 200 mL of aerated half-strength Clark's nutrient solution (Clark, 1975) with 8-fold the recommended iron supply to mitigate the Cr^{3+} -effected Fe deficiency (Pandey and Sharma, 2003), except for the seedlings to be evaluated in the control. Such a magnitude of Fe-enrichment was estimated to be sufficient for plant growth during the total length of each essay, based upon reported Fe contents in Cr-stressed and Cr-unstressed plant tissues in the same or similar periods of time (Vernay et al., 2007). Plants were kept in this condition for seven days.

Treatment description

Seedlings were then transferred to pots containing 200 mL of aerated half-strength Clark's nutrient solution, with one of the following treatments: Control: complete half-strength nutrient solution, pH 5.5-6.5; pH 5.5-half-strength nutrient solution without Fe, pH 5.5-6.5; pH 4.0-half-strength nutrient solution without Fe, pH 4.0; and Cr^{3+} -half-strength nutrient solution without Fe added $5 \text{ mg L}^{-1} \text{Cr}^{3+}$ supplied as $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$, pH 4.0 (i.e. $96.1 \mu\text{M Cr}$). The pH level of the nutrient solution was altered to 4.0 or 5.5 or kept in the original range of 5.5-6.5 according to the corresponding experimental treatment. Subsequently, pH levels were checked twice a day and adjusted with 1 N HCl or 1 N NaOH when needed. The pH measurements were performed with a Metrohm 827 pH-meter. Nutrient solution volumes were completed with distilled water whenever needed and solutions replaced every seven days. Plants were grown for 30 days under controlled conditions ($28 \pm 2^\circ\text{C}$, $60 \pm 4\%$ RH, 16h photoperiod and $280 \mu\text{mol.s}^{-1}.\text{m}^{-2}$ radiation). Experiments were repeated four times, totaling 800 seedlings.

Experimental design

The stage of growing seedlings in nutrient solution, conducting growth and physiological evaluations, and extracting protein considered a complete randomized design, with four treatments, five replications and 10 plants per plot. The analysis through microfluidic electrophoresis considered a complete block design, with four treatments, 12 blocks (i.e. the number of microfluidic chips) and one observation per plot.

SDS-PAGE evaluations considered a complete randomized design, with four treatments, six replications (i.e. the number of wells) and one observation per plot.

Growth and physiological evaluations

Plant physiological indicators were determined at harvest, which was performed at 10:00 AM of the thirtieth day of continuous growth. While plants were still undisturbed, chlorophyll levels were measured with a portable chlorophyll meter (Minolta, Model SPAD-502) in the midpoint of the last fully developed leaf (Hoel, 1998). Also, transpiration rates were determined in the base of fully expanded leaf blades using a diffusion porometer (Delta-T devices, Model AP4). Plants were then harvested and immediately evaluated regarding RL and SL (with a precision ruler, Trident, Model RM-TR Molegata), number of leaves, LA (measured with a portable leaf area meter, LI-COR Model LI-3000A), fresh wt. (determined with an analytical balance, Shimadzu Model AUX-220), and LA/SL ratio. Subsequently, leaf samples were frozen with liquid N and stored at -80°C until further processing.

Protein extraction

Protein extraction was performed with a commercial kit (Sigma-Aldrich) with minor alterations in the established protocol (Tastet et al., 2003). The kit includes a chaotropic reagent with enhanced solubilizing power (protein extraction reagent type 4 - *PER-4*) and a protease inhibitor cocktail (*PIC*). *PER-4* consists of 7.0 M urea, 2.0 M thiourea, 40 mM Tris-(hydroxymethyl) aminomethane, 1.0% C7BzO, pH 10.4. *PIC* is a mixture of protease inhibitors with broad specificity for inhibiting serine, cysteine, metalloproteases, aspartic and aminopeptidases. It includes pepstatin A, AEBSF, E-64, leupeptin, 1,10-phenanthroline, and bestatin. *PIC* was diluted in methanol ($10 \mu\text{L}.\text{mL}^{-1}$) and this solution (*MSol*) was used in the same day.

In short, for preparing each experimental sample, frozen (-80°C) leaf tissue was ground to a fine powder with mortar and pestles with the aid of liquid N_2 and immediately processed or otherwise kept at -20°C . The sample was added 1.5 mL *MSol*, briefly vortexed and incubated for 5 min at -20°C with periodic vortexing. The suspension was then centrifuged at $28,000 \times g$ for 5 min at 4°C (Hermle refrigerated centrifuge model Z323K, Hermle Labortechnik, Wehingen, Germany). The supernatant was discarded and the pellet processed as above for two more times. All visible residues of *MSol* were removed from the final pellet, followed by addition of 1.5 mL acetone, brief vortexing, and incubation for 5 min at -20°C . Such a suspension was then centrifuged at $28,000 \times g$ for 5 min at 4°C . The supernatant was removed and the undisturbed pellet was allowed to dry for 10 min at room temperature (RT) in order to completely remove the acetone. *PER-4* was then applied to the dried sample ($4 \text{ mg } \mu\text{L}^{-1}$) and the pellet was completely broken up by vortexing. The suspension was incubated for 15 min at RT with intermittent vortexing and subsequently centrifuged at $28,000 \times g$ for 30 min to pellet debris. The supernatant (total protein extract) was removed and transferred to a 2 mL v-bottom labeled freezing vial and stored at -20°C until processing.

Protein level determination

Determination of sample microgram protein levels was carried out through the Bradford Coomassie brilliant blue

(CBB) assay (Bio-Rad), adapted from Bearden (1978). CBB reagent was diluted five-fold with ultrapure water, according to the manufacturer. Bovine serum albumin (BSA) solutions (Sigma-Aldrich) were prepared with ultrapure water in order to obtain increasing concentrations in the range 200-2,000 $\mu\text{g mL}^{-1}$ which were used to construct the standard curves. For the routine determination of protein levels, a minimum volume of 4 μL of either standards or samples was dissolved into 200 μL of diluted CBB reagent. The microtubes with their respective mixtures were homogenized and their absorbance was measured after 5 min incubation at RT. Ultraviolet spectrophotometry was measured at 595 nm in NanoDrop spectrophotometer ® ND-1000. Protein contents were obtained from the calculated linear regression equation.

Microfluidic electrophoresis (Lab-on-a-Chip technology)

The microfluidic electrophoresis was conducted using an Agilent 2100 Bioanalyzer system and the associated Protein 80 kit (Agilent Technologies, Waldbronn, Germany). The kit includes the chips on which electrophoresis is performed, corresponding reagents such as the gel matrix solution, protein dye concentrate solution, marker protein buffer solution and a protein molecular mass ladder solution (Anema, 2009). The gel matrix and destain solutions were prepared according to the protocols supplied with the chips.

SDS-PAGE

SDS-PAGE was performed using Amersham ECL Gel Box system (GE Healthcare Bio-Sciences AB, Björkgatan, Sweden). The samples were prepared by adding 10 μL of sample and 10 μL of sample buffer (0.125 M Tris-HCl, pH 6.8, 4% (v/v) SDS, 20% glycerol, 0.2 M DTT and 0.02% (v/v) bromophenol blue) and heated at 95 °C for 5 min.

Initially, a pre-run gel was conducted for 12 min at 160 V. Then, samples and pre-stained protein standards (Invitrogen) were loaded in the 15-well precast 8-16% gradient gels (Amersham ECL) and dispersed in the Amersham ECL Gel Running Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 - GE Healthcare). The gels were run at 140 V and 45 mA for approximately 90 min. After each run, the gels were stained overnight using 0.1% (w/v) Coomassie Brilliant blue R-250, 50% (v/v) ethanol and 12% (w/v) trichloroacetic acid. Next, gels were destained using an 8% (v/v) acetic acid solution and 25% (v/v) ethanol until a clear background was achieved. Subsequently, the gels were digitally documented with a densitometer (Bio-Rad) and the integrated intensities of the protein bands were analyzed with the Molecular Analyst program (Bio-Rad). The calibration was based upon standard protein spots with known molecular masses. Parameters such as volume and area were used to compare the quantity of the protein spots from the gels.

Statistical analysis

Data were processed by analysis of variance (Statistical Analysis System program, Version 9.2, SAS Institute, 2008). Comparisons of means were made by the Tukey test. A significant level was defined as a probability of $p \leq 0.05$.

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References

- Achnine L, Blancaflor EB, Rasmussen S, Dixon R (2004) Colocalization of l-phenylalanine ammonia-lyase and cinnamate 4-hydroxylase for metabolic channeling in phenylpropanoid biosynthesis. *Plant Cell*. 16: 3098-3109.
- Adamowski EV, Pagliarini MS, Do Valle CB (2008) Meiotic behaviour in three interspecific three-way hybrids between *Brachiaria ruziziensis* and *B. brizantha* (Poaceae: Paniceae). *J Genet*. 87: 33-38.
- Ali S, Zeng F, Qiu L, Zhang G (2011) The effect of chromium and aluminum on growth, root morphology, photosynthetic parameters and transpiration of two barley cultivars. *Biol Plantarum*. 55: 291-296.
- Anema SG (2009) The use of “lab-on-a-chip” microfluidic SDS electrophoresis technology for the separation and quantification of milk proteins. *Int Dairy J*. 19: 198-204.
- Arduini I, Masoni A, Ercoli L (2006) Effects of high chromium applications on miscanthus during the period of maximum growth. *Environ Exp Bot*. 58: 234-243.
- Bearden J (1978) Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay. *Biochim Biophys Acta*. 533: 525-529.
- Boddey RM, Macedo R, Tarré RM, Ferreira E, De Oliveira OC, Rezende CP, Cantarutti RB, Pereira JM, Alves BJR, Urquiaga S (2004) Nitrogen cycling in *Brachiaria* pastures: the key to understanding the process of pasture decline. *Agr Ecosyst Environ*. 103: 389-403.
- Cabral GB, Carneiro VTC, Lacerda AL, Do Valle CB, Martinelli AP, Dusi DMA (2011) Somatic embryogenesis and organogenesis in apomictic and sexual *Brachiaria brizantha*. *Plant Cell Tiss Org*. 107: 271-282.
- Chigbo C, Batty L (2013) Effect of combined pollution of chromium and benzo (a) pyrene on seed growth of *Lolium perenne*. *Chemosphere*. 90: 64-169.
- Clark RB (1975) Characterization of phosphatase of intact maize roots. *J Agr Food Chem*. 23: 458-460.
- Diwan H, Ahmand A, Iqbal M (2008) Genotypic variation in the phytoremediation potential of Indian mustard for chromium. *Environ Manage*. 41: 734-741.
- Fuzinato VA, Pagliarini MS, Do Valle CB (2012) Meiotic behavior in apomictic *Brachiaria ruziziensis* x *B. brizantha* (Poaceae) progenies. *Sci Agr*. 89: 380-385.
- Hoel BO (1998) Use of a hand-held chlorophyll meter in winter wheat: evaluation of different measuring positions on the leaves. *Acta Agr Scand Sect. B-S P*. 48: 222-228.
- Holtgrefe S, Gohlke J, Starmann J, Druce S, Klocke S, Altmann B, Wojtera J, Lindermayr C, Scheibe R (2008) Regulation of plant cytosolic glyceraldehyde 3-phosphate dehydrogenase isoforms by thiol modifications. *Physiol Plantarum*. 133: 211-228.
- Hu Y, Di P, Chen J, Xiao Y, Zhang L, Chen W (2011) Isolation and characterization of a gene encoding cinnamoyl-CoA reductase from *Isatis indigotica* Fort. *Mol Biol Rep*. 38: 2075-2083.
- Jordão CP, Pereira MG, Matos AT, Pereira J (2005) Influence of domestic and industrial waste discharges on

- water quality at Minas Gerais State, Brazil. *J Brazil Chem Soc.* 16: 241-250.
- Joris HAW, Caires EF, Bini AR, Scharr DA (2013) Effects of soil acidity and water stress on corn and soybean performance under a no-till system. *Plant Soil.* 365: 409-424.
- Kawamata S, Yamada T, Tanaka Y, Sriprasertsak P, Kato H, Ichinose Y, Kato H, Shiraishi T, Oku H (1992) Molecular cloning of phenylalanine ammonia-lyase cDNA from *Pisum sativum*. *Plant Mol Biol.* 20: 167-170.
- Kim BG, Lee YJ, Park Y, Lim Y, Ahn J-H (2006) Caffeic acid *o*-methyltransferase from *Populus deltoids*: functional expression and characterization. *J Plant Biol.* 49: 55-60.
- Klein DJ, Moore PB, Steitz TA (2004) The roles of ribosomal proteins in the structure assembly, and evolution of the large ribosomal subunit. *J Mol Biol.* 340: 141-177.
- Lamattina L, Gonzalez D, Gualberto J, Grienemberger JM (1993) Higher plant mitochondria encode an homologue of the nuclear-encoded 30-kDa subunit of bovine mitochondrial complex I. *Eur J Biochem.* 217: 831-838.
- Machado Neto NB, Custódio CC, Peniche AGPF (2002) *Brachiaria* access germplasm distinction using SDS-PAGE. *Acta Sci-Agron.* 24: 1439-1445.
- Mallick S, Sinan G, Mishra RK, Sinha S (2010) Interactive effects of Cr and Fe treatments on plants growth, nutrition and oxidative status in *Zea mays* L. *Ecotox Environ Safe.* 73: 987-995.
- Mirlean N, Roisenberg A, Chies JO (2007) Metal contamination of vineyard soils in wet subtropics (southern Brazil). *Environ Pollut.* 149: 10-17.
- Mus F, Cournac L, Cardellini V, Caruana A, Peltier G (2005) Inhibitor studies on non-photochemical plastoquinone reduction and H₂ photoproduction in *Chlamydomonas reinhardtii*. *Biochim Biophys Acta.* 1708: 322-332.
- National Center for Biotechnology Information (2014) *Brachiaria brizantha*. Bethesda: U.S. National Library of Medicine. <http://www.ncbi.nlm.nih.gov/protein/?term=Brachiaria%20brizantha> (accessed in Nov 7, 2014).
- Olatunji OS, Ximba BJ, Fatoki OS, Opeolu BO (2014) Assessment of the phytoremediation potential of *Panicum maximum* (guinea grass) for selected heavy metal removal from contaminated soils. *Afr J Biotechnol.* 13: 1979-1984.
- Oze C, Bird DK, Fendorf S (2007) Genesis of hexavalent chromium from natural sources in soil and groundwater. *P Natl Acad Sci USA.* 104: 6544-6549.
- Pandey N, Sharma CP (2003) Chromium interference in iron nutrition and water relations of cabbage. *Environ Exp Bot.* 49: 195-200.
- Pandey V, Dixit V, Shyam R (2005) Antioxidative responses in relation to growth of mustard (*Brassica juncea* cv. Pusa Jaikisan) plants exposed to hexavalent chromium. *Chemosphere.* 61: 40-47.
- Parvathi K, Chen F, Guo D, Blount JW, Dixon A (2001) Substrate preferences of *O*-methyltransferases in alfalfa suggest new pathways for *O*-methylation of monolignols. *Plant J.* 25: 193-202.
- Passos LP, Vidigal MC, Pereira AV, Ferreira RP, Verneque RS (1999) Responses of elephantgrass to nutrient solution pH levels. *J Plant Nutr.* 22: 701-708.
- Pina A, Zhebentyayeva T, Errea P, Abbott A (2012) Isolation and molecular characterization of cinnamate 4-hydroxylase from apricot and plum. *Biol Plantarum.* 56: 441-450.
- Pires AMM, Marchi G, Mattiazzi ME, Guilherme LRG (2007) Organic acids in the rhizosphere and phytoavailability of sewage sludge-borne trace elements. *Pesqui Agropecu Bras.* 42: 917-924.
- Ramachandran V, D'Souza TJ, Mistry KB (1981) Distribution of chromium in red kidney beans (*Phaseolus vulgaris* L.). *Experientia.* 37: 718-720.
- Russell DA, Sachs MM (1989) Differential expression and sequence analysis of the maize glyceraldehyde-3-phosphate dehydrogenase gene family. *Plant Cell.* 1: 793-803.
- Shahandeh H, Hossner LR (2000) Plant screening for chromium phytoremediation. *Int J Phytoremediat.* 2: 31-51.
- Shanker AK, Cervantes C, Loza-Tavera H, Avudainayagam S (2005) Chromium toxicity in plants. *Environ Int.* 31: 739-753.
- Sharma DC, Sharma PC (1999) Chromium uptake and its effects on growth and biological yield of wheat. *Cereal Res Commun.* 21: 317-321.
- Sharmin SA, Alam I, Kim KH, Kim YG, Kim PJ, Bahk JD. (2012) Chromium-induced physiological and proteomic alterations in roots of *Miscanthus sinensis*. *Plant Sci.* 187: 113-126.
- Singh HP, Mahajan P, Kaur S, Batish DR, Kohli RK (2013) Chromium toxicity and tolerance in plants. *Environ Chem Lett.* 11: 229-254.
- Subrahmanyam D (2008) Effects of chromium toxicity on leaf photosynthetic characteristics and oxidative changes in wheat (*Triticum aestivum* L.). *Photosynthetica.* 46: 339-345.
- Sundaramoorthy P, Chidambaram A, Ganesh KS, Unnikannan P, Baskaran L (2010) Chromium uptake in paddy: (i) Nutrient status of paddy under chromium stress; (ii) Phytoremediation of chromium by aquatic and terrestrial weeds. *C R Biol.* 333: 597-607.
- Takac T, Pechan T, Samaj J (2011) Differential proteomics of plant development. *J Proteomics.* 74: 577-588.
- Tastet C, Charmont S, Chevallet M, Luche S, Rabilloud T (2003) Structure-efficiency relationships of zwitterionic detergents as protein solubilizers in two-dimensional electrophoresis. *Proteomics.* 3: 111-121.
- Vajpayee P, Sharma SC, Tripathi RD, Rai UN, Yunus M (1999) Bioaccumulation of chromium and toxicity to photosynthetic pigments, nitrate reductase activity and protein content of *Nelumbo nucifera* Gaertn. *Chemosphere.* 39: 2159-2169.
- Vernay P, Gauthier-Moussard C, Hitmi A (2007) Interaction of bioaccumulation of heavy metal chromium with water relation, mineral nutrition and photosynthesis in developed leaves of *Lolium perenne* L. *Chemosphere.* 68: 1563-1575.
- Volland S, Bayer E, Baumgartner V, Andosch A, Lütz C, Sima E, Lütz-Meindl U (2014) Rescue of heavy metal effects on cell physiology of the algal model system *Micrasterias* by divalent ions. *J Plant Physiol.* 171: 154-163.
- Volland S, Lütz C, Michalke B, Lütz-Meindl U (2012) Intracellular chromium localization and cell physiological response in the unicellular alga *Micrasterias*. *Aquat Toxicol.* 109: 59-69.
- Wolfe KH, Morden CW, Plamer JD (1992) Function and evolution of a mineral plastid genome from a nonphotosynthetic parasitic plant. *Proc Natl Acad Sci USA.* 89: 10648-10652.
- Wu D, Qin J, Lin B (2008) Electrophoretic separations on microfluidic chips. *J Chromatogr A.* 1184: 542-559.
- Zayed A, Lytle CM, Qian JH, Terry N (1998) Chromium accumulation, translocation and chemical speciation in vegetable crops. *Planta.* 206: 293-299.
- Zhang X-H, Liu J, Huang H-T, Chen J, Zhu Y-N, Wang D-Q (2007) Chromium accumulation by the hyperaccumulator plant *Leersia hexandra* Swartz. *Chemosphere.* 67: 1138-1143.