

Physiological response of the protective enzymatic mechanism of *Pontederia parviflora* Alexander caused by chromium absorption stress

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ABSTRACT. The use of plants to eliminate organic or inorganic compounds is one of the alternatives for environmental treatment, but to guarantee the efficiency of process is necessary to know the effects in plant metabolism. This study sought to evaluate the enzymatic mechanism involved in the protection of the metabolism of *Pontederia parviflora* Alexander caused chromium absorption stress. The experiment was carried with solution of chromium (III) in three concentrations (10, 50 and 100 ppm) in the period of four days and evaluations daily. The concentration of chromium in the solution influenced the form of storage of metal in the tissues of plants. In 24 hours, there was increase of glutathione in the root in 10ppm solution and in 50 and 100 ppm the accumulation of glutathione occurs in the stem, coinciding with the peak absorption metal (48 hours). The activity of the catalase was higher in the roots, the peroxidase showed higher activity at the 50 ppm concentration, and the guaiacol had similar activity to catalase. The tolerance of *P. parviflora* in response to chromium stress occur for complex mechanisms, phytohormone-producing as significantly regulated under Cr stress and the formation of phytoquelatins was in sync with the absorption of metal. Due to the tolerance shown by *P. parviflora*, it becomes important for the environmental treatment of contaminated water.

Keywords: Phytochelatins; reactive species oxygen; heavy metal; chlorophyll; phytotreatments.

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Introduction

The contamination of water bodies in urban area is constant and harmful for every species of the environment. The origin of these pollutants is varied: of industries, agriculture, or sewers, studies indicated: traces of the presence of metals in water and the route of mobilization of some metals, such as chromium, is spatially associated with the location of leather industries (Nahar, Zhang, Ueda, & Yoshihisa, 2014). Agricultural activities change the levels of chemicals with excessive parameters of chlorides, phosphates, and nitrates (Sasakova et al., 2018).

The Chromium stands out among the heavy metals for has diverse industrial applications due to its corrosion resistant quality and hardness. It is used at a large scale in various industries including metallurgical, electroplating, tanning, wood preservation, manufacturing of stainless steel, production of paints, pigments, pulp, and paper (Lu, Ouyang, Zhang, & Lu, 2013). Remediation of Cr is possible through various physical, chemical, and biological method.

The use of plants to eliminate organic or inorganic compounds appears as an alternative in many cases, but to guarantee the efficiency of process is necessary to know the effects in plant metabolism. Metal tolerance in plants is usually energy consuming resulting in low biomass accumulation. Research were development to identify hyperaccumulator plants, those able to tolerate and accumulate high concentrations of chromium in their tissues (Mant, Costa, Williams, & Tambourgi, 2006; Thayaparan, Iqbal, & Iqbal, 2015; Bilal et al., 2018). To date, Cr uptake and transport pathway in plants are not defined. Being a strong oxidizer, Cr (VI) is extremely toxic and more mobile in soil/water systems than Cr (III) (Dubey, Shri, Gupta, Rani, & Chakrabarty, 2018).

The principal bioindicators in plants of the exposition heavy metals are lipidic peroxidation, variation in the relation chlorophyll/carotenoids, high in concentration of jasmonic acid, nicotiana, glutathione (GSH) and thiols, emergence of peptides chelating and phytochelatins and high of activity of antioxidant enzymes (Hernández et al., 2015).

The formation of reactive oxygen species (ROS) is the answer of the metabolism, this process is the lipidic peroxidation through which the ROS attack the polyunsaturated fatty acids of phospholipids from cell membranes, disintegrating them and allowing the entry of these species into the structures intracellular (Rodrigues, Santos, Santos, Pereira, & Sobrinho, 2016). The environmental stress and the disrupted of the balance between production and antioxidant activity cause it to occur oxidative damage in the cellular structures (Kim & Kwak, 2010; Sytar et al., 2013).

The ability to trigger antioxidant defense mechanisms can prevent the accumulation of ROS and extreme oxidative stress (Bhattacharjee, 2010). They stand out among the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX, EC 1.11.1.1), glutathione reductase (GR, EC 1.6.4.2), peroxidases (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6) and polyphenoloxidase (PPO, EC 1.14.18.1). Among the main antioxidant metabolites are ascorbic acid (AsA), glutathione (GSH), α -tocopherol and carotenoids (Dinakar, Djilianov, & Bartels, 2012). The CAT is one of the main enzymes in the elimination of H_2O_2 generated during photorespiration and β -oxidation of fatty acids. In chloroplasts to photoreduction of oxygen to water can generate H_2O_2 , which is eliminated by action APX, even at low concentrations (Barbosa, Silva, Willadino, Ulisses, & Camara, 2014).

The Glutathione (GSH) is a marker of cellular health, the levels of glutathione are decreased by the synthesis of phytochelatins and by their chelating action of these elements (Rodrigues et al., 2016; Mylona & Polidoros, 2011).

In the environmental area, one of the major applications of phytochelatins determination is the selection of species capable of hyperaccumulating metals for use in the phytoremediation process, which enables the decontamination of environments by the sorption of metals by plants (Hernández et al., 2015). Studies with *Pontederia parviflora* Alexander in the absorption of metals have been developed and this species has proved to be resistant to heavy metals (Balassa, Souza, & Lima, 2010) and in different environmental conditions (Souza, Locastro, Lima, & Iwakura, 2015).

The understanding of those mechanisms, that allow these species to be hyperaccumulators, will lead to the improvement of phytoremediation techniques for their successful application in real situations. Thus, this study sought to evaluate the enzymatic mechanism involved in the protection of the metabolism of *Pontederia parviflora* Alexander caused chromium absorption stress.

Material and methods

The experiment was carried out at the bench level in containers of 500 mL capacity, with solution of chromium (III) in three concentrations (10, 50 and 100 ppm) in triplicates. The period of contact with the metals was four days and the evaluations were daily.

The plants were observed for 96 hours to verify changes in the leaves and stems that indicate the beginning of the process of senescence. As these are qualitative characteristics, these data were analysed only to establish resilience. Daily the pH was determined with potentiometer and the ambient temperature variations was controlled with maximum and minimum thermometer.

The retention time was four days and the sampling interval of 0h, 24h, 48h and 96h with the withdrawal random of the plants of system. The plants had roots and stems removed and frozen for the enzymatic study, and portions of roots and stems too was dehydrated and packed in kraft paper to determine the concentrations of metals.

Determination of chlorophyll

Leaf samples were collected daily, frozen and protected of light. The chlorophyll was determined by the colorimetric method. The leaves (200 mg) were macerated in 2 mL of aqueous solution of acetone 90 % and saturated solution of carbonate of magnesium 10 %. After the samples was centrifuged 3000 rpm at 20 min. and analysed in spectrophotometer in 664 nm chlorophyll a (Eaton, Clesceri, Rice, Greenberg, & Franson, 2005).

Analysis of metals

The chemical analysis of the metal was performed with plasma atomic emission spectrometry coupled inductively, separately in solution, of portions of metal and vegetation (roots and stems) before and after the experiment according to the methodology reported in APHA (Eaton et al., 2005).

Protein extraction

Samples of leaves and root (each treatment) was collected in a mortar, added liquid nitrogen, and macerated until flour form. Add the extraction buffer (1: 3 w v⁻¹ - tissue ratio: buffer). The extraction buffer is constituted by a 100 mM potassium phosphate solution (pH = 7.5) containing 1 mM EDTA (0.372 g L⁻¹ buffer), 3 mM DTT (dithiothreitol) (0.462 g L⁻¹) and 4% of PVPP (polyvinyl pyrrolidone). The homogenate was centrifuged at 1000 rpm for 30 min. at 4°C. Separate the supernatant, divide into 3.5 mL aliquots, transfer to Eppendorf, identify and store in freezer (-20°C) until analysis.

Catalase activity – CAT

Catalase activity was determined spectrophotometrically at 25°C, as described by Kraus, McKersie, and Fletcher (1995) with some modifications according to Azevedo, Alas, Smith, and Lea (1998). In reaction mixture containing: 1.5 mL of potassium phosphate buffer (100 mM) pH 7.5; 150 µL of extract and 50 µL H₂O₂ (30% solution). The activity followed by the decomposition of H₂O₂ for 2 min. through changes in absorbance at 240 nm. The results were expressed as µmol min. mg protein⁻¹.

Ascorbate peroxidase activity - APX

The activity of the APX enzyme was determined spectrophotometrically at 25°C in according to Zhu et al. (2004). The total volume of the reaction mixture is 3 mL, containing: 2.5mL of 25 mM (pH 7.0) sodium phosphate buffer; 250 µL of 0.1 mM EDTA; 50 µL of 0.25 mM ascorbate, 50 µL of 1.0 mM H₂O₂ and 150 µL extract enzymatic. The oxidation of ascorbate was accompanied by decrease in absorbance at 290 nm. The results were expressed as µmol min. mg protein⁻¹.

Activity of guaiacol peroxidase - GPX

Peroxidase activity was determined according to the technique described by Matsuno and Uritani (1972) for spectrophotometrically at 25°C. The reaction mixture containing: 2.5 mL of phosphate-citrate buffer (0.2 M sodium dibasic phosphate solution and 0.1 M citric acid); 300 µL of enzyme extract and 250 µL of 0.5% guaiacol. These solutions must be mixed by vortex. Then add 25 µL H₂O₂ and mix in vortex again. Incubate the mixture at 30°C for 15 min. After the mixture is incubated take to an ice bath and add 250 µL of 2% sodium metabisulfite solution. After mix in vortex, allow the mixture to stand for 10 min. and read the absorbance at 450 nm. The results were expressed as µmol min. mg protein⁻¹.

Separation of Glutathione (GSH) and Tiois PCs into column chromatography

Glutathione and thiols are quantified in liquid chromatography-HPLC with a reverse phase column (Octadecylsilane, 4.6 mm x 150 mm). A sample of 20 µL of the protein extract was injected using as a mobile phase a 5% solution of acetonitrile/0.1% trifluoroacetic acid (TFA) at the rate of 1 mL min⁻¹. The eluent solution of the column is separated according to the retention time of each component (GSH-3.30 min. and PCs-6.98 min.) (Jacques-Silva et al. (2001).

Analyze of dates

All experiments were performed in completely random design. Data from figures represent established linear regressions, showing the trends of the measured parameters during the experimental period in Cr-treated 10 ppm, 50 ppm, 100 ppm and control plants, whereas the time was considered as independent variable. The enzyme activity was evaluated by Principal Component Analysis (PCA) seeking which enzyme and metal concentration was highlighted in the protection of the plant. The analyzes were performed by RStudio Team (2015), and the graphics by Microsoft Excel®.

Results

The ambient temperature ranged from 24°C to 30°C, and the initial pH was 4.74, increasing to 6.1 in the solutions of 10 ppm and 50 ppm and in the solution of 100 ppm it varies up to 4.88, below the ideal level of development of these plants.

The chromium in the solutions reduced to below 20 ppm in the first day in the solution of the 100 ppm concentration and zeroed in other solutions. The concentration of chlorophyll reflected how chromium was absorbed by the plant, occur reduction on the concentration at the beginning (Table 1). Highlights the variation occurred in 100 ppm solution, that after 48 hours the concentration of chlorophyll returns to the initial values (Table 1).

Table 1. Chlorophyll, Glutathione, Phytochelatyns in *P. parviflora* and pH in the solutions 10 ppm, 50 ppm and 100 ppm of chromium.

Time (hour)	Chromium reduction of the solution (%)	Chlorophyll (mg Kg ⁻¹)	Glutathione (µg g ⁻¹)		Phytochellatyns (µmol mg ⁻¹)	
			Root	Stem	Root	Stem
10 ppm						
0		17.3	3564		23164	34210.4
24	99.9	10.59	8380	2197	23643	24539
48	99.9	7.09		4690	18818.2	71047.3
96	99.9	10.19	6582		11000.6	72901
50 ppm						
0		17.3	3564		23164	34210.4
24	99.9	6.62		1156	19335.9	88301
48	99.9	6.18		4638	26220	18936
96	99.9	8.32	1023	2230	20126.4	38897.8
100 ppm						
0		17.30	3564		23164	34210.4
24	76.5	9.33		4086	8186.3	53151
48	95.9	4.62		3055.3	22107.1	40325.2
96	99.7	16.58		9661	19395.8	11945

The concentration of chromium in the solution influenced the form of storage of metal in the tissues of plants. In lower concentration no transfers for stem in the first 24 hours, the allocation occurs only in the roots (Figure 1a). In the solution with 50 ppm the transfer begins after 24 hours of accumulate in the roots (Figure 1b). In the high concentration solution (100 ppm), the metal was transfer from the beginning, with the greatest accumulation in the stems after the 96 hours of experiment (Figure 1c).

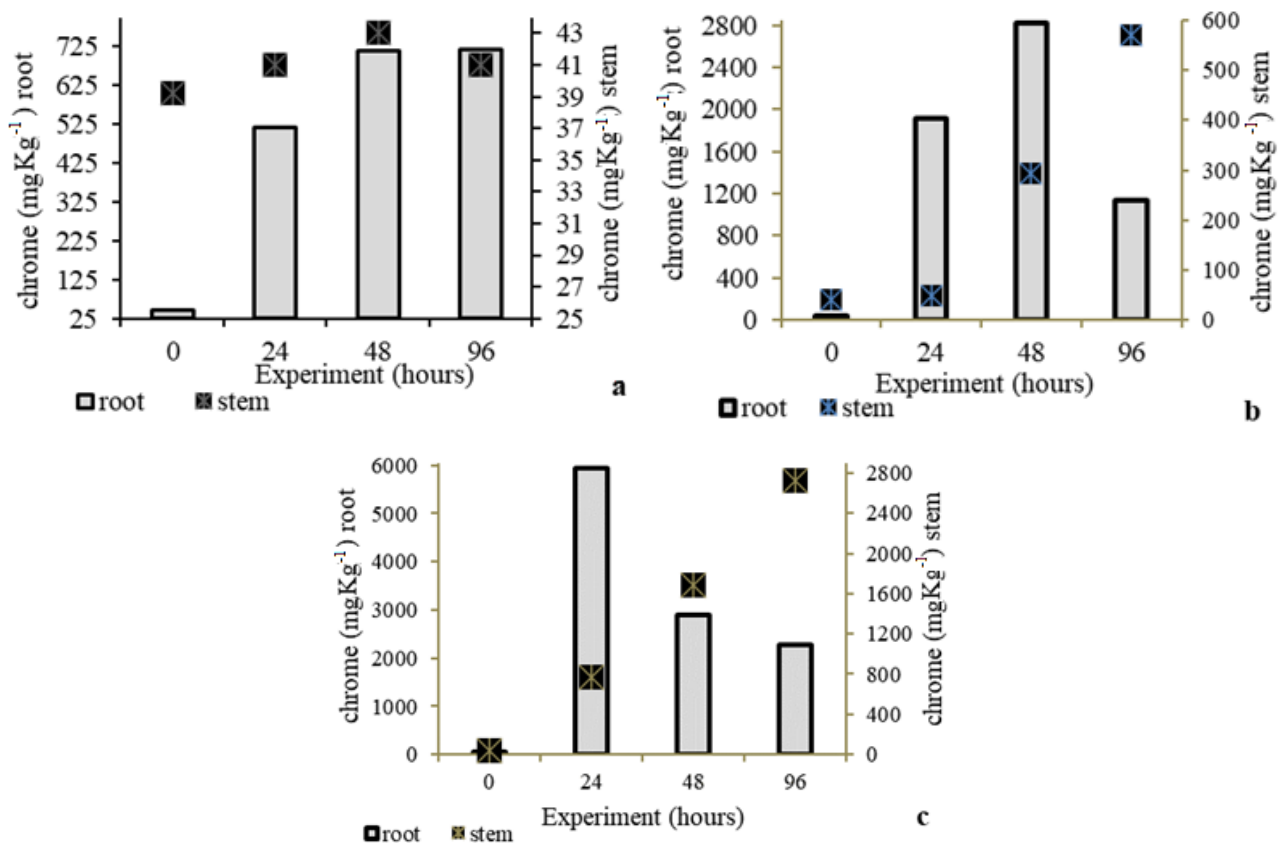


Figure 1. Concentration of chromium by plants tissues in the solutions of a) 10ppm, b) 50ppm and c) 100ppm.

In the first 24 hours there was a marked increase of glutathione mainly in the root in 10ppm solution (Table1). In solutions 50 and 100 ppm the accumulation of glutathione occurs in the stem, followed by great consumption for the formation of (γ glutamyl-cysteine)n glycine coinciding with the peak absorption metal (48hours). The formation of phytochelatyns was accentuated in the first hours, reducing in the end of the experiment (Table 1). These data made it possible to calculate the prediction equation of the phytochelatyn by accumulation of chromium $y = -2,1029x + 24765$, $R^2 = 0.5$ and $p < 0.001$. The 50% R2 reflects the consumption of phytochelatyns in metal immobilization.

The activity of the CAT was higher in the roots (Figure 2b) than in the stems (Figure 2a), being the highest activity observed at 10 ppm after 48 hours of accumulation of metal, coinciding with the maximum metal accumulated in this tissue, followed by reduction of the enzymatic activity with the translocation of the metal to the stem.

APX showed higher activity at the 50ppm concentration, highlighting in activity at the root in the first 24 hours (Figure 2d) and increasing its activity in the stems in the following hours (Figure 2c), when the chromium concentration increases. The activity of the GPX was more pronounced in the stems after the accumulation of metals (Figure 2e), the only exception was for the root in the solution 50ppm with 48 hours (Figure 2f).

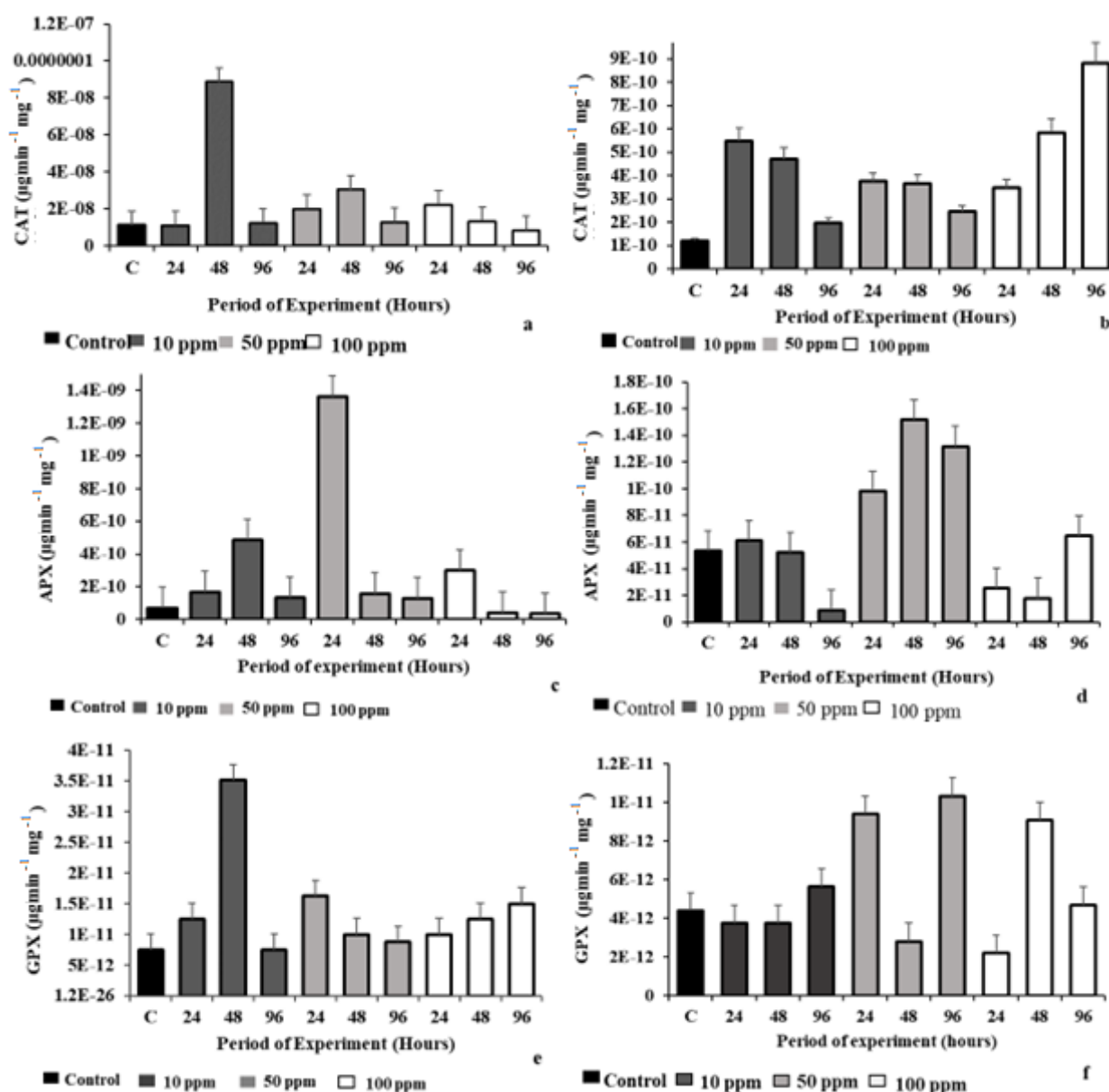


Figure 2. Enzyme activity in response to exposure to chromium a) Catalase in leaf; b) Catalase in root; c) Ascorbate Peroxidase in leaf; d) Ascorbate Peroxidase in root; e) Guaiacol Peroxidase in leaf; and f) Guaiacol Peroxidase in root.

The activities of enzymes with high influence in the axis of principal component analysis, with 92,4% of explication was the GPX. The activity of CAT was similar in the three concentration, being larger in roots, in PCA these points away from the other (Figure 3a). The CAT remains active for 48 hours, returning at level initial in the last 24 hours. The activity of GPX was high in solution of 50 ppm, separating this concentration from the others in a grouping near the first axis of the PCA (Figure 3b). The activity of GPX in the roots was crescent at the end of experiment. The Guaiacol had similar activity to CAT, separated the roots of the stem and of the control (Figure 3c).

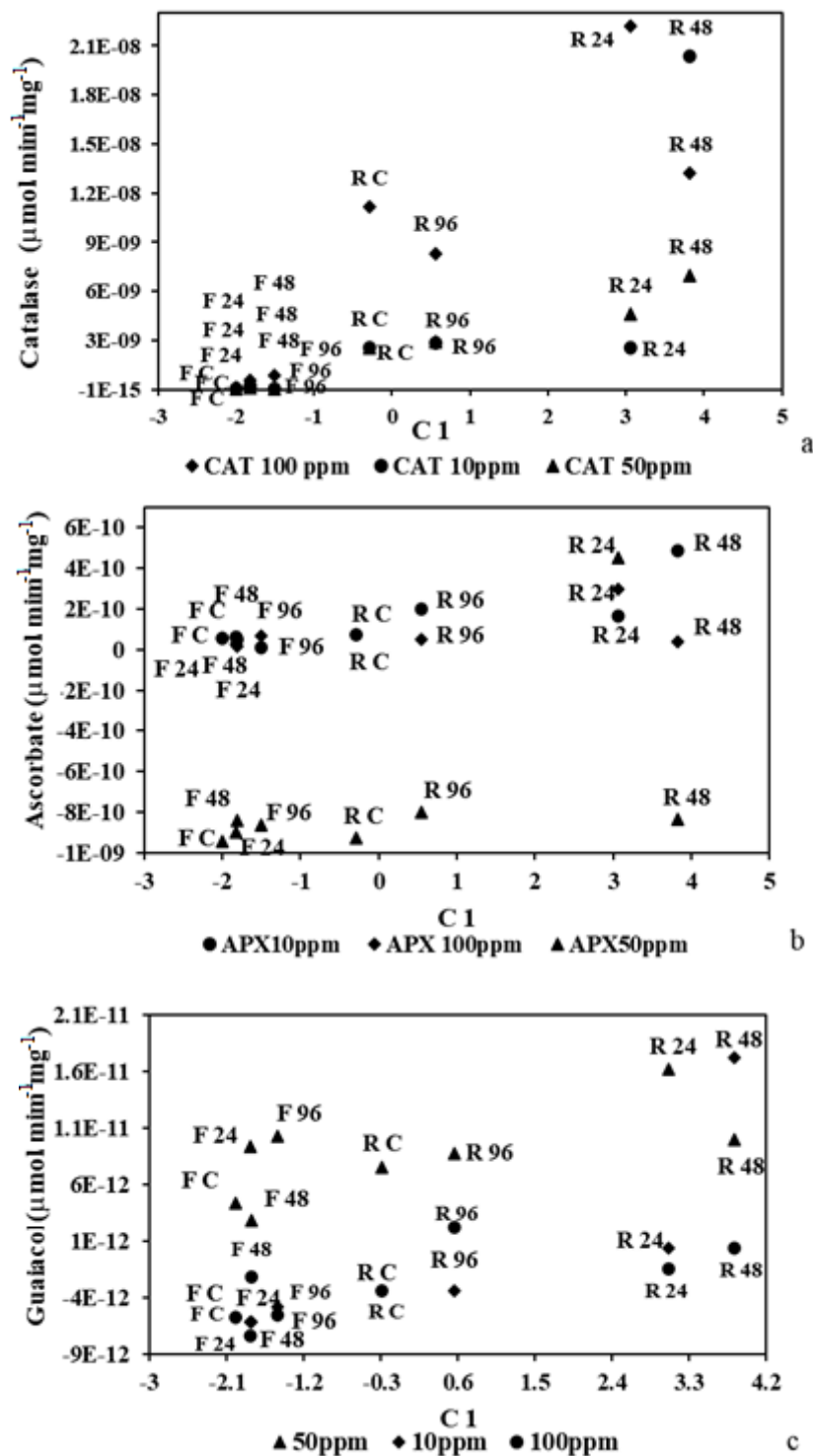


Figure 3. Principal Components Analyze with enzymes a) catalase peroxidase; b) ascorbate peroxidase and c) guaiacol peroxidase. R= roots, F= leaf, RC=roots control, RF leaf control, and numbers indicate the intervals of experiment 24, 48 and 96 hours.

Discussion

The chromium was slightly transported from the root to the stem in high concentrations, totally different behavior observed in the low concentrations, previous studies also showed this tendency of metal accumulation in stem (Balassa et al., 2010; Souza et al., 2015), in that experiment *P. parviflora* showed ability to absorb 99% of the chromium of solutions in 48 hours. A study with *Lemna minor* showed an absorption capacity of 10% in 120 hours (Thayaparan et al., 2015), and the authors considered the species to be a hyperaccumulator to the bioconcentration factor. *Penisetum purpureum* achieved removal efficiencies of 97–99.6% within 24h of solutions containing 10 and 20 mg Cr dm⁻³ (Mant et al., 2006)

The first defense mechanism of the plant started with the release of root exudate that caused the pH change, which went from close to 4 to 6. This change is due to the speciation of metals, favoring the binding of ions on the walls of the cells (Rodrigues et al., 2016). The release of exudates was best succeeded at concentrations 10 and 50ppm, showing that this strategy can help in some situations, but needs a metabolic complement to ensure the efficiency of the process.

Alterations observed in the levels of chlorophylls, which already at the beginning of the experiment reduce sharply, are associated with the effect of the stress caused by the metal that reduce the concentration of enzymes responsible to produce chlorophyll (Santos et al., 2011). These alterations of Cr-induced abnormalities in the chlorophyll content, structural and enzymatic activities, and electron transport chain would deteriorate the net photosynthesis and transpiration rates (Chow, Lee, Zakaria, & Foo, 2018). The consumed of the O₂ by plant in photorespiration is diverted to produce species reactive of oxygenic (ROS) in various subcellular loci such as chloroplasts, mitochondria, and peroxisomes (Sharma, Jha, Dubey, & Pessarakli, 2012; Matsuo et al., 2015). The photorespiration is the principal formation of the H₂O₂ in photosynthetic cells (Barbosa et al., 2014).

The response in the chlorophyll levels highlights the ability of this species not only to accumulate the metal, but to restore its metabolism, an important characteristic to establish the potential use in treatment systems. Mechanisms of the accumulation of Cr ions in vacuoles, their binding with organic ligands, scavenging of Cr generated reactive oxygen species (ROS) via antioxidative enzymes have been discovered in plants to provide them resistance against hexavalent chromium (Shahid et al., 2017).

The oxidative stress that is the reduction in the antioxidant capacity of the plant occurs due to the reduction in the levels of GSH, which is an abundant compound in plants that directly scavenges ROS and may protect enzyme thiol groups (Borges et al. 2018). GSH was diverted from the production of chlorophyll precursor enzymes and probably acted directly in the metal chelation process, this high relationship between the variations in the GSH and phytochelutins levels and the metal accumulation. As GSH is consumed only phytochelutins have been expressed by the regression equation. GSH and PC mediated metal sequestration are subsequently deported to the vacuole, reducing the arbitrary metal ions in the cell cytosol (Bari, Akther, Reza, & Kabir, 2019).

At the 96 hours of the experiment with the end of chromo in the solution there was also the reduction in the levels of phytochelutins and the return of GSH. There were gaps in the performance of the GSH in relation to the stress caused by chromium, earlier studies showed that Cr is unable to induce phytochelutins (Dubey, et al. 2018). However, Tripathi et al. (2014) that studies *Oryza sativa* glutathione s-transferases (OsGSTs) play a major function in Cr (VI) detoxification, perhaps via chelation and sequestration of glutathione Cr (VI) complexes into vacuoles.

In response to the formation of ROS occurring by the absorption and chelation of metal, the enzymes CAT, APX and GPX increased their activities. CAT and APX activities are primarily responsible for H₂O₂ scavenging and redox homeostasis regulation (Cuyppers et al., 2012). Bilal et al. (2018) in experiment with *Glycine max* observed that high Cr concentrations (5.0 mM) slightly growth, while significantly enhancing upregulated its antioxidant system, enhancing reduced glutathione (GSH), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities to counteract Cr-induced oxidative stress.

CAT presented the highest activity on the second day, highlighting the root in the solution of 10ppm coinciding with the greatest accumulation of metal. In the stems the highest activity was observed in 100ppm solution, together with the higher metal accumulation. The high activity of CAT in the stems of 100ppm on the last day associated with the return of metabolism in these plants and with the return to the initial levels of chlorophyll concentration in these plants. This is because CAT is one of the main enzymes in the elimination of H₂O₂ generated during photorespiration and β -oxidation of fatty acids (Barbosa et al., 2014).

The highest activity of APX in relation to control was in the stems of 50ppm solution, this is a key enzyme of the ascorbate-GSH pathway, which has been reported to be involved in the transformation of H_2O_2 into H_2O that utilizing ascorbate as electron donor (Locato et al., 2015; Cuypers et al., 2012). APX can also directly detoxify ROS via its sulfhydryl group (Noctor et al., 2012). APX was the enzyme that stood out as the most important in the defense system, directing the distribution of the schedules of treatment in the first axis of PCA with 92%. APX has been reported to modulate secondary cell wall-related gene expression (Bilska, Wojciechowska, Alipour, & Kalemba, 2019) APX is mainly involved in the alteration of H_2O_2 detoxification, while CAT is dedicated to the bulk of high H_2O_2 induced by abiotic stress (Mittler, 2002).

The GPX initially activates in the roots and returns close to the initial levels after 48 hours, at concentrations of 10 ppm and 50 ppm, equating to the behavior presented by the GSH and responding to the translocation of the metal. Thus, in the stems this enzyme is activated after the initial 24 hours with inconstant behavior throughout the experiment. This behavior of the guaiacol enzyme is very important because it indicates that the plant can defend itself from the metal while inactive in the interior of cellular vacuoles. The accumulation of these substances in plant cells can be toxic, and this species triggers molecules for the induction of genes and the polymerization of proteins that make up the cell wall, besides stimulating the production of antioxidative enzymes or cleaning (Lukasik & Golawska, 2012).

PCA confirms the difference in activity between stems and roots for all enzymes and highlights the proximity between the enzymatic activity in the roots without metal addition and after 96 hours when the metal had already been absorbed by the plants. This shows not only the plant's ability to absorb large concentrations of metal as its resilience, showing that after metal chelation the plant resumes its metabolism to normal levels, an important characteristic for a plant to be used in phytotreatment.

Conclusion

This paper presents advance knowledge on the mechanistic basis of Cr uptake and tolerance in *P. parviflora*. The tolerance in response to chromium stress is associated with complex mechanisms, and the phytohormone-producing was significantly regulated under Cr stress. *P. parviflora* activated the formation of phytoquelatins and this was responsible for the chelation of metal and its inactivation, allowing the return of plant metabolism evaluated by recovery in chlorophyll levels. The system of protection of ROS was triggered by enzymes CAT, APX, and highlight GPX enzyme. Thus, can be indicated *Pontederia parviflora* as a hyperaccumulating and its use in environmental recovery.

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