

Removal of high lead concentration by hydroponic cultures of normal and transformed plants of *Scirpus americanus* Pers

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Abstract

Lead is a very toxic metal which affects human health. An alternative to remove it from contaminated water is the use of macrophytes, as *Scirpus americanus*. This species is tolerant to salt and metals, and has high biomass. The present research analyzed the capacity of hydroponic cultures of normal and transgenic plants (line T12) from *S. americanus* to remove high concentrations of lead (100, 250 and 400 mg kg⁻¹ Pb). The MINTEQ3.1 program was used to define the media composition in order to have the metal available to the plants. The presence of metal did not affect the growth of roots and stems at all concentration tested. The normal and T12 plants accumulated 69389 and 45297 mg lead kg⁻¹, respectively, and could be considered as hyperaccumulators. The bioconcentration factor indicated that plant bioconcentrated between 192-300 times the metal. The activation of the antioxidant mechanisms seems to participate in lead tolerance. Then, *S. americanus* could be used for phytoremediation of water contaminated with high concentration of lead.

Introduction

Lead is widely use for the manufacture of batteries, ammunitions, pigments, glass, metals supplies, and devices to avoid irradiation, among others. These anthropogenic activities have polluted soil and aquatic environments with high levels of this metal (Kushwaha et al. 2018).

Lead is very toxic and one of the more persistent metals in the environment. It affects almost every organ, but the nervous system is the most altered. The children present learning deficits, lower IQ and behavior problems, the adults exhibited ischemic heart disease and strokes, and the pregnant women affections in fetus developing. At cellular level, it promotes an excessive accumulation of reactive oxygen species (ROS), causing an oxidative stress, which in turns, induced lipid peroxidation, DNA damage and protein oxidation (Kumar et al. 2018).

The World Health Organization estimate that Pb exposure is responsible of 21.7 million years lost to disability and death worldwide due to long-term effects on health. These effects represent 30% of the global burden of idiopathic intellectual disability, 4.6% of the global burden of cardiovascular disease and 3% of the global burden of chronic kidney diseases (WHO 2021).

As the lead cannot be degraded must be removed from contaminated areas. A biotechnological approach for removal of a variety of contaminants, including lead, is the phytoremediation. This methodology is defined as the use of plants to contain, remove or render toxic environmental contaminants harmless. A variety of tolerance mechanism to lead in plants has been described, including the liberation of organic acids, the increase in carotenoids, anthocyanin, proline, sugar and proteins, the induction of phytochelatins, and the activation of antioxidant mechanisms (Osmolovskaya et al. 2018; Pirzadah et al. 2020; Pasricha et al. 2021).

The antioxidant mechanisms of tolerance to lead involved enzymatic and non-enzymatic processes. The first include the activation of the enzymes, such as, superoxide dismutase, peroxidase, catalase, and

ascorbate peroxidase, while the non-enzymatic mechanisms are related to the synthesis of glutathione and ascorbate. These antioxidants reduce and control the formation of reactive oxygen species, which inhibits normal cell functions (Dalyan et al. 2020).

A group of plants widely used for the phytoextraction of diverse pollutants from water, are the macrophytes (Manorama Thampatti et al. 2020). These plants are low cost, easy handling, exhibit fast growth and have a great ability to uptake nutrients and contaminants. *Scripus americanus* is an emergent macrophyte with well-developed rhizomes, that easily exceeds 2 m in height. It is considered as a model plant to evaluate marsh ecology and the responses to climate change (Blum et al. 2021). *S. americanus* can grow in water with high levels of salt and in lagoons contaminated with organic compounds and heavy metals (Montante-Montelongo et al. 1995; Janousek et al. 2020). Previous reports showed that also the *in vitro* root cultures of *S. americanus* were able to remove lead ($148.3 \text{ mg Pb kg}^{-1}$) in absence of rhizospheric bacteria (Santos-Díaz et al. 2007). The lead removal capacity increased 3.8 times in hairy roots obtained after genetic transformation of *in vitro* roots cultures with *Agrobacterium rhizogenes*, in comparison to non-transformed roots. Additionally, it was possible to regenerated transformed plants (line T12) from transgenic roots (Alfaro-Saldaña et al. 2016). However, the ability of normal and transformed plants to remove high levels of lead has not been explored.

Based on the above, the objective of this work was to analyzed the capacity of normal and transgenic plants of *S. americanus* to remove high lead concentrations from water, and to determine if the antioxidant mechanisms are participating on metal tolerance.

Materials And Methods

Establishment of *S. americanus* hydroponic cultures

The normal and transgenic plants, regenerated as described Alfaro-Saldaña et al. (2016), were propagated in Erlenmeyer flasks containing 75 mL liquid Murashige Skoog basal medium, MS (Murashige and Skoog 1962), supplemented with $116 \text{ }\mu\text{M}$ myo-inositol, $1.2 \text{ }\mu\text{M}$ thiamine-HCl, 30 g L^{-1} sucrose, pH 5.7. When the plants reached 12 cm were collected, the roots were washed with sterile deionized water to eliminate the medium, and were placed in 1 L vessels with 750 mL of hydroponic medium, pH 5.7 (Hydroenvironment® S.A. de C.V., México). The plants were maintained in greenhouse conditions, and the medium was changed weekly until they reached 20 cm height. Five plants were transferred to plastic basins (15 L), containing 12 L of the same commercial hydroponic medium, pH 5.7. Plants from 50 cm tall were employed for lead removal experiments (Esquivel-Ramos et al. 2019).

Chemical modeling

Before exposing the plants to lead, a chemical modeling was performed. The MINTEQ3.1 program was selected because it calculates metal speciation, solubility equilibria, sorption effects, and percentage of chemical species in solution or precipitated (<https://vminteq.lwr.kth.se>). The Hoagland (Hoagland and

Arnold 1950), MS and Steiner (Steiner 1966) media, in presence of different theoretical lead concentrations, were analyzed using this program.

Exposure of *S. americanus* plants to lead

Five normal and T12 plants (about 50 cm tall) were placed in basins with 12 L of modified Hoagland medium (without phosphate, sulfate and molybdenum) containing 0, 100, 250 and 400 mg L⁻¹ Pb, as Pb(NO₃)₂, pH 5.7. Stem and root length, and medium pH were measured each 2 days, during 12 days. To calculate fresh weight, the plants were dried on absorbent paper to eliminate superficial water, transferred to a previously weighed test tube (1 L) and weighed. Dry weight was calculated after dried the tissue at 80°C during 48 h.

Quantification of lead in the medium and *S. americanus* tissues

Each 2 days during 12 days, 10 mL of medium were collected. The samples were acidified with 30 µL of concentrated HNO₃, filtered through HDPE membranes (0.22 µ pore diameter, Millipore), and stored in HDPE flasks until analysis.

To desorb the metal from the root surface, the tissue was treated with 3 mL 0.042% HNO₃ under constant agitation during 3 h, and the metal released was quantified by as described below. To measure the lead accumulated on roots and stems, 1 mL of concentrated HNO₃ was added to 20 mg of dried tissue, and the metal concentration was assayed according to Santos-Díaz and Barrón-Cruz (2011) by atomic absorption (Varian-SpectrAA 3110, Palo Alto, USA). The reference plant material (peach leaves 1547, NIST, USA), and blanks (reaction mixture with no metals) were included to validate the analysis. The bioconcentration factor (BCF) and translocation factor (TF) were calculated according to Espinoza-Quinones et al. (2005).

Lead removal kinetics

Five normal and T12 plants were placed in five basins with 12 L modified Hoagland medium, pH 5.7, containing 400 mg L⁻¹ Pb. The plants were collected at 0, 2, 4, 6 and 8 days. The 50% of roots and stems were weight, divided in packages of 500 mg (fresh weight) each, and were frozen immediately on liquid nitrogen for the enzymatic analysis. The remaining 50% tissue was frozen at -20°C and stored for lead quantification.

Obtainment of the cellular extracts and protein assay

The frozen roots and stems (0.5 g) were homogenized with 3 mL cold extraction buffer (1 M phosphate buffer, 0.66 M EDTA and 2% polyvinylpyrrolidone, pH 7). The samples were sonicated three times during 1 min, and the volume was adjusted to 3 mL with the same cold buffer. The tubes were centrifuged at 8000 rpm during 15 min at 4°C, the supernatant was collected and used for protein and enzymatic quantifications.

The protein content was measured per triplicate using the Bradford reagent, and the standard curve was performed using bovine serum albumin (Bradford 1976).

Quantification of enzymatic and non-enzymatic antioxidants

For the enzymatic analysis, the protein concentration was adjusted to $50 \mu\text{g mL}^{-1}$. The SOD activity was determined as described Giannopolitis and Ries (1977), the CAT activity according to Hodges et al. (2001), and the POX activity as reported by Guarachi et al. (2008).

The glutathione (GSH) content was determined using a commercial kit (Invitrogen No. EIAGSHC) by adjusting the protein concentration to $15 \mu\text{g}$ in $50 \mu\text{L}$ of 1% sulfosalicylic acid.

Statistical analysis

A random design was used and data were expressed as mean values ($n = 5$). An analysis of variance (ANOVA) was performed, with significant differences between means determined by the Tukey's test at 95% significance level, using the GraphPad Instat 3 Program (GraphPad Software Inc., Version 3.10 for Windows)

Results And Discussion

The adaptation process of the *in vitro* plants to *ex vitro* conditions was successful since 95% of plants survived. After 4 months, healthy and vigorous normal and T12 plants, from ≥ 50 cm height, were obtained in the hydroponic medium.

Chemical modeling

Initially, the Hoagland, Steiner and MS media were selected to study the capacity of normal and T12 plants to remove lead. Before to exposure the plants to the metal, the chemical MINTEQ3.1 program was used to predict the chemical lead species formed, and the percentage of metal precipitation. This information was relevant since the metal can precipitate at the tested concentrations ($100\text{--}400 \text{ mg L}^{-1}$). The results showed that about 90% of metal precipitated at all concentrations and media tested (Table 1). According to the program more than 20 species can formed including sulfate complexes $[\text{PbSO}_4, \text{Pb}_2(\text{OH})_2\text{SO}_4]$, phosphate complexes $[\text{Pb}_5(\text{PO}_4)_3\text{Cl}, \text{Pb}_5(\text{PO}_4)_3\text{OH}, \text{Pb}_3(\text{PO}_4)_2, \text{PbHPO}_4]$, molybdenum complexes (PbMoO_4), ferrite, ferrhydrite, and $\text{Pb}(\text{OH})$. Since the MINTEQ3.1 program attributed the precipitation to the presence of phosphate, sulfate and molybdenum, these compounds were eliminated from media composition. These new media were designed as modified media.

Table 1
Lead precipitated according to the MINTEQ3.1 program

Medium	Pb precipitated (%)		
	100 mg L ⁻¹	250 mg L ⁻¹	400 mg L ⁻¹
Hoagland	99.9	99.9	99.9
Hoagland modified ^a	0	0	44
Steiner	99.7	99.9	99.9
Steiner modified ^a	0	13.9	57.15
MS	90.2	92.14	99.6
MS modified ^a	0	12.93	55.67
^a Media without phosphate, sulphate and molybdenum			

With these changes, the program predicted a significant reduction in lead precipitation, with the lowest precipitation in modified Hoagland's medium (Table 1). Therefore, this medium was selected to calculate the chemical species present according to pH, and to perform the experiments of metal removal. The MINTEQ3.1 program indicated that lead precipitated at pH higher than 6, and it was mainly due to the PbOH⁺ formation (Fig. 1). The soluble lead species predicted, at pH 5.7, were Pb²⁺, Pb₂OH³⁺, PbNO₃⁺, Pb(NO₃)₂. These data confirmed that the use of the MINTEQ3.1 program was a very useful tool to assure that a high level of lead was bioavailable to plants.

Effect of lead on *S. americanus* growth

The normal and T12 plants of *S. americanus* were transferred to Hoagland modified medium, pH 5.7, containing 0, 100, 250 and 400 mg L⁻¹ Pb. The last concentration was also included because no metal precipitation was observed when the solution was prepared. Data were reported as the increase (Δ) in root and stem length, since it is difficult to have all the plants with the same size. The results showed that root length of normal plants in presence of lead was higher than in control without metal (Fig. 2a). The root length at 250 mg L⁻¹ was three folds longer than in control plants at the end of the experiment.

In the T12 plants, the increase in root length was similar between control and 250 and 400 mg L⁻¹ treatments, but lower at 100 mg L⁻¹ (Fig. 2b). In general, the root length in normal plants was higher than in T12 plants. This difference could be attributed to the growth pattern, since T12 plants developed more branched roots, while normal plants have a vigorous principal root.

On the other hand, the stem length increased progressively in normal plants at all lead concentrations until day 6, kept constant until day 8, and decreased rapidly from day 10 (Fig. 2c). In the T12 plants a similar behavior was observed, however, the plants maintained in 400 mg L⁻¹ Pb exhibited a higher

increase in stem length compared with control plants at day 6 and 8. After this time, a rapid decrease in stem growth was observed in all treatments (Fig. 2d).

The reduction on stem growth of normal and T12 plants was observed in plants exposed or not to lead. Therefore, this effect cannot be attributed to metal toxicity, but to a nutritional deficiency possibly caused by the absence of phosphate, sulfate and molybdenum in the media. It is known that phosphate deficiency decreases Rubisco activity, rate of photosynthesis, oxidative phosphorylation, and synthesis of amino acids. The sulfate is a source of sulfur, which reduced forms incorporate to cysteine and methionine. Thus, a reduction in sulfate would affect synthesis of proteins, and antioxidants like GSH. The molybdenum is a cofactor of nitrate reductase and nitrogenase and therefore is involved in nitrogen metabolism (George et al. 2008). Deficiency of these compounds would contribute to reduce the stem growth in *S. americanus* plants.

At the end of the experiment all plants survived, and had a good appearance even at the highest concentration tested indicating that *S. americanus* plants were able to tolerate high concentration of lead. In contrast, other species like *Medicago sativa*, *Triticum aestivum*, *Brassica napus*, *Ricinus communis*, and *Acalypha indica* presented a shoot and root growth inhibition after lead exposure (Dalyan et al. 2020). Also, the biomass and height of several wetland plants decreased in the presence of 900 mg Pb kg⁻¹ in soil (Yang and Ye 2015).

Lead removal by *S. americanus* plants

To perform the lead removal kinetics the selected metal concentration was 400 mg L⁻¹. The lead content at time 0 in the negative control (medium without plants) was around 240 mg L⁻¹, and in medium with plants was 215 mg L⁻¹, indicating that about 41.6% of the metal was precipitated, probably due to lead-hydroxide complexes in the liquid medium (Fig. 3a).

In presence of normal and T12 plants, the lead concentration tends to decrease progressively from day 4 until the end of the experiment. The normal plants removed 63 mg L⁻¹, while T12 plants removed 49 mg L⁻¹ from medium at day 8 (Fig. 3a).

To know the metal accumulation pattern in the tissues, the lead content in roots and stems was analyzed. The metal rapidly accumulated in roots of normal (64000 mg kg⁻¹ DW) and T12 plants (36500 mg kg⁻¹ DW) until 4 day, and subsequently remained without significant changes ($p > 0.05$). The roots of normal plants accumulated 1.75 times more lead than those of T12 plants (Fig. 3b). The lead content in stems, of both normal and T12 line, was about 7.5–10% of the value detected in roots, ranged between 4000 and 4800 mg kg⁻¹ and did not improve with time (Fig. 3b). The lead adsorbed into the root's surface corresponded to 435 and 317 mg kg⁻¹ (Fig. 3c). Our results are in accordance with those reported for several wetland plants, which accumulated three to four times more lead in roots than in the aerial parts (Yang and Ye 2015). On the contrary, in *Zea mays*, the accumulation of lead was higher in shoots than in roots (Jagetiya and Kumar 2020).

It has reported that lead can be transported to the root cell through voltage gated cation channels present in plasma membrane, and by the transporters LCT1, ATM3 and AtPDR12 (Song et al. 2014). The metal introduced to roots could interact with carboxyl groups of galacturonic acid and pectin from the cell wall, or be sequestered in the vacuole. Deposits of chloropyromorphite have been identified in membranal structures and vacuoles of different species (Kopittke et al. 2008). Additional research must be done to know if the metal in *S. americanus* roots is deposited in cell wall or in vacuoles.

On the other hand, the lead translocated to aerial parts was very low indicating a poor mobility of metal through vascular bundles. Lead translocation from root to shoot was generally low in most species (Yang and Ye 2015).

The measurement of pH of the medium was also performed. Data showed a rapid pH reduction at day 2 in normal and T12 plants not exposed to lead, which increased until day 6. The reduction on pH, at 400 mg L⁻¹ was slower but higher, than in control treatment. The pH decreased 1 and 1.2 units in the presence of normal and T12 plants, respectively, at day 4 in relation to time 0 (Fig. 4). Thus, the maximum metal removal from media at day 4, corresponded to the maximum accumulation in the roots, and to the maximum decrease in medium pH. It is possible that in the first days, the roots of normal and T12 plants exudated organic acids, or activated the proton pump causing a reduction in the medium pH, promoting the complexation and precipitation of lead on the rhizosphere.

The T12 plants would be more efficient to reduce the metal internalization by a higher reduction in the pH medium, and in consequence the metal accumulation in tissues would be lower. In the root exudates of *S. triqueter* a variety of organic acid were identified (hexadecanoic, pentadecanoic, vanillic, octadecanoic, citric, succinic and glutaric acids) after exposure to lead (Hou et al. 2015). It remains to identify the chemical nature of *S. americanus* roots exudates to verify if they correspond to organic acids.

The data presented suggest that during the first 4 days, the metal is immobilized by a phytostabilization process due to the formation of complexes with the root exudates, and after this time the *S. americanus* plants removed lead mainly by a rhizofiltration mechanism.

Subsequently, the BCF and TF were calculated to determine the lead accumulation capacity by *S. americanus* plants. The normal and T12 plants accumulated 69389 and 41063 mg Pb kg⁻¹ DW, respectively (Table 2). These concentrations were around 300–400 times higher than in *S. americanus* plants growing in water polluted by municipal and industrial wastewater (Carranza-Álvarez et al. 2008). Considering that an hyperaccumulator of lead accumulated 1000 mg kg⁻¹ DW (Luo et al. 2016), the normal and T12 plants of *S. americanus* can be considered as hyperaccumulators of Pb. The lead accumulated in *S. americanus* plants, also, exceed the concentration reported in *Noccaea* (23,000 mg kg⁻¹), *Arabis alpine* (1484 mg kg⁻¹), and *Phyllostachys pubescens* (1048 mg kg⁻¹) (Dinh et al. 2018; Li et al. 2019; Liu et al. 2015).

Table 2
Bioaccumulation factor (BCF) and translocation factor (TF) of normal and T12 plants
exposed to 400 mg kg⁻¹ Pb at day 8

Plant	Absorbed (mg kg ⁻¹)		Adsorbed (mg kg ⁻¹) Root	Total (mg kg ⁻¹)	BCF ¹	TF
	Root	Stem				
N	64000	4800	589	69389	325.76	0.075
T12	36500	4000	563	41063	192.78	0.10
¹ BCF was calculated according to lead bioavailable in medium (213 mg L ⁻¹)						

On the other hand, the BCF indicated that normal and T12 plants bioconcentrated between 192 and 300 times the metal. The TF was lower than 1 in both plants but the normal plants translocated 33% less lead than T12 plants (Table 2).

Antioxidant activities of *S. americanus* plants

The activity of POX, SOD, CAT, and the content of GSH were determined in normal and T12 plants of *S. americanus* exposed to 400 mg L⁻¹ Pb, to know if the tolerance involved the activation of antioxidant mechanisms.

The POX activity in roots and stem of normal plants not exposed to lead increased significantly at day 4 and 6, respectively, but did not improved in presence of the metal (Fig. 5a). In the T12 plants, the POX activity was similar between root and stem at 0 and 400 mg L⁻¹ Pb (Fig. 5b).

The quantification of SOD activity showed a significant increase ($p < 0.05$) in roots from normal plants exposed to lead in relation to control plants, which in general, improved with time. In the stems the SOD activity was very low and did not increased at 400 mg L⁻¹ (Fig. 5c). The roots of T12 plants also exhibited higher activity of SOD but only at 6th and 12th day. The activity in stems or both normal and transgenic plants was similar in control and in presence of lead (Fig. 5d).

The CAT activity improved in roots and stems of normal plants exposed to metal only at day 4 (Fig. 5e). In the T12 line there was a significant difference in the stems of plants exposed to metal at day 8 (Fig. 5f).

The analysis of GSH in normal plants showed an increase in the roots at day 4 and 6, and in the stems at days 6 and 8 (Fig. 5g). In T12 plants the GSH concentration was higher in the roots at day 4 and in the stems at day 2 (Fig. 5h).

Thus, roots of normal plants exposed to lead mainly improved the SOD activity and the levels of GSH at day 4, period at which the maximum metal uptake was observed. GSH increased at day 6 and 8 in the

stem suggesting that this response could be due to translocation of metal to this tissue.

In the T12 plants, the activation of SOD was lower but GSH accumulation was higher at day 4 in relation to normal plants. The higher levels of GSH in stems could be related to the higher TF observed in T12 plants. Variations in the antioxidative response to lead, were also reported in *Phyllostachy pubescens*, where SOD activity decreased, and increased POX activity and GSH levels (Wang et al. 2019), while in *Arachis hypogaea* (Nareshkumar et al. 2014) and *Acalypha indica* (Venkatachalam et al. 2017) increased SOD, APX, GPX, GR and GST.

It is reported that lead improved ROS production, including superoxide. This compound promotes hydroxyl-radical formation and DNA damage. The increase of SOD activity in *S. americanus* plants could be the first defense mechanism by converting superoxide to hydrogen peroxide and oxygen. Subsequently, the hydrogen peroxide could be transformed to water and oxygen by the catalase or by glutathione peroxidase, this last using GSH as an electron donor (Dalyan et al. 2020). These antioxidant mechanisms would be contributing to lead tolerance in *S. americanus* plants.

Conclusions

The use of the MINTEQ3.1 program was a very useful tool to define media formulations and to assure that lead is available to plants. The normal and T12 plants of *S. americanus* accumulated 69389 and 41063 mg Pb kg⁻¹, respectively and can be considered as new hyperaccumulators of lead. The tolerance of plants mainly involved the activation of the SOD activity and GSH accumulation. The T12 plants could be used to treat industrial waters for recycling, cooling or to reduce metal concentration to permissible levels. Beside these applications, the normal plants also could be used to establish artificial wetlands and contribute to improve the quality of water, for agricultural or industrial purposes.

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors contribution

Esquivel-Ramos E realized the experimental work, and participated on elaboration of figures and revision of manuscript. Alfaro-de la Torre MA participated in media formulation using the MINTEQ3.1 program, revision and discussion of results. Santos-Díaz MS is the leader of the group, designed the project and experimental work, participates in revision, discussion, and wrote the paper.

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Ethical Approval

No animals or persons were used in this work.

Consent to participate

Esquivel-Ramos E, Alfaro-de la Torre MA, and Santos-Díaz MS give their consent to participate in this paper.

Consent to publish

Esquivel-Ramos E, Alfaro-de la Torre MA, and Santos-Díaz MS give their consent for the publication of this paper.

Availability of data and material (data transparency)

Data and material are available at the Faculty of Chemistry of the “Universidad Autónoma de San Luis Potosí”.

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Figures

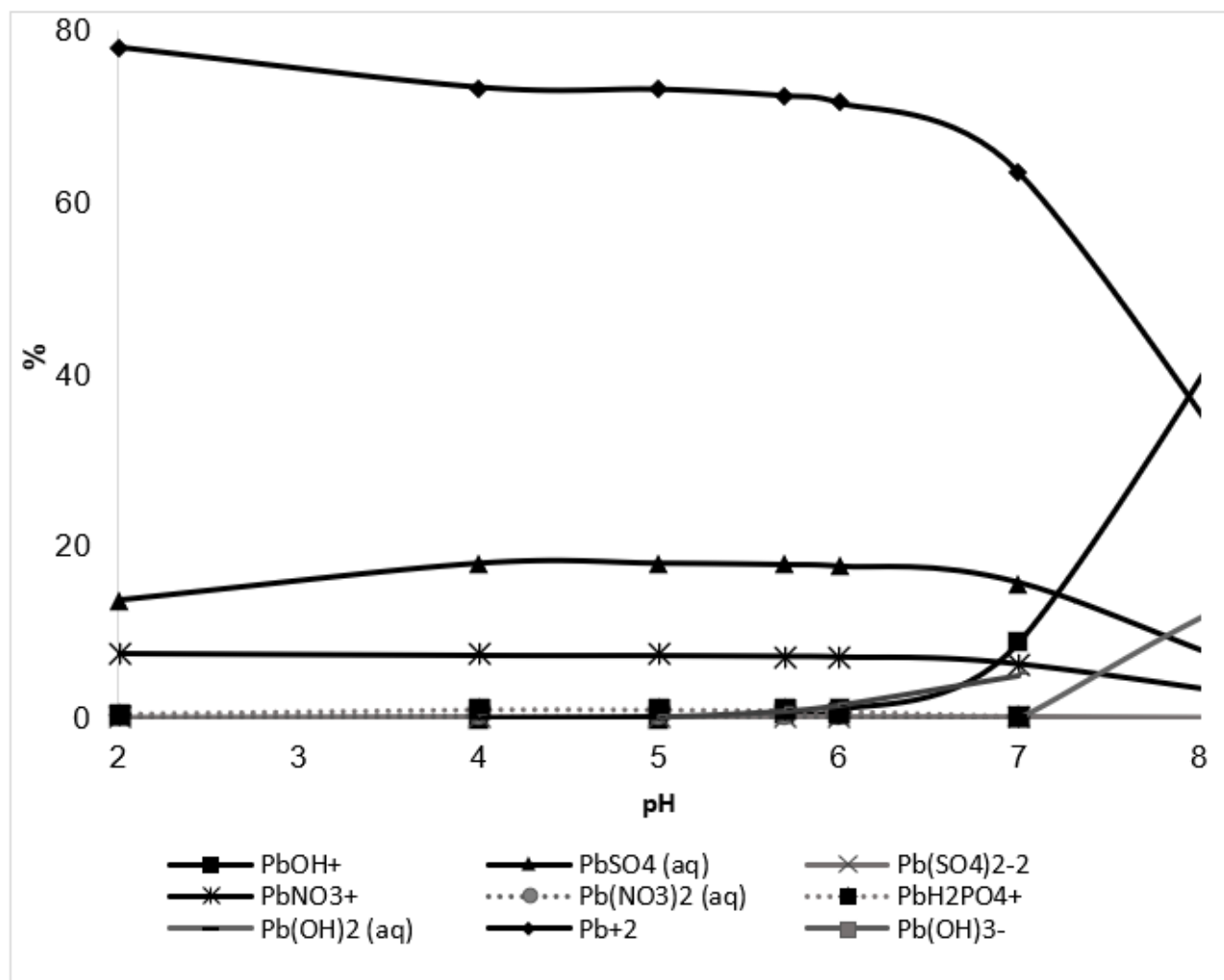


Figure 1

Chemical species of lead calculated with the MINTEQ3.1 program in Hoagland modified medium at different pH

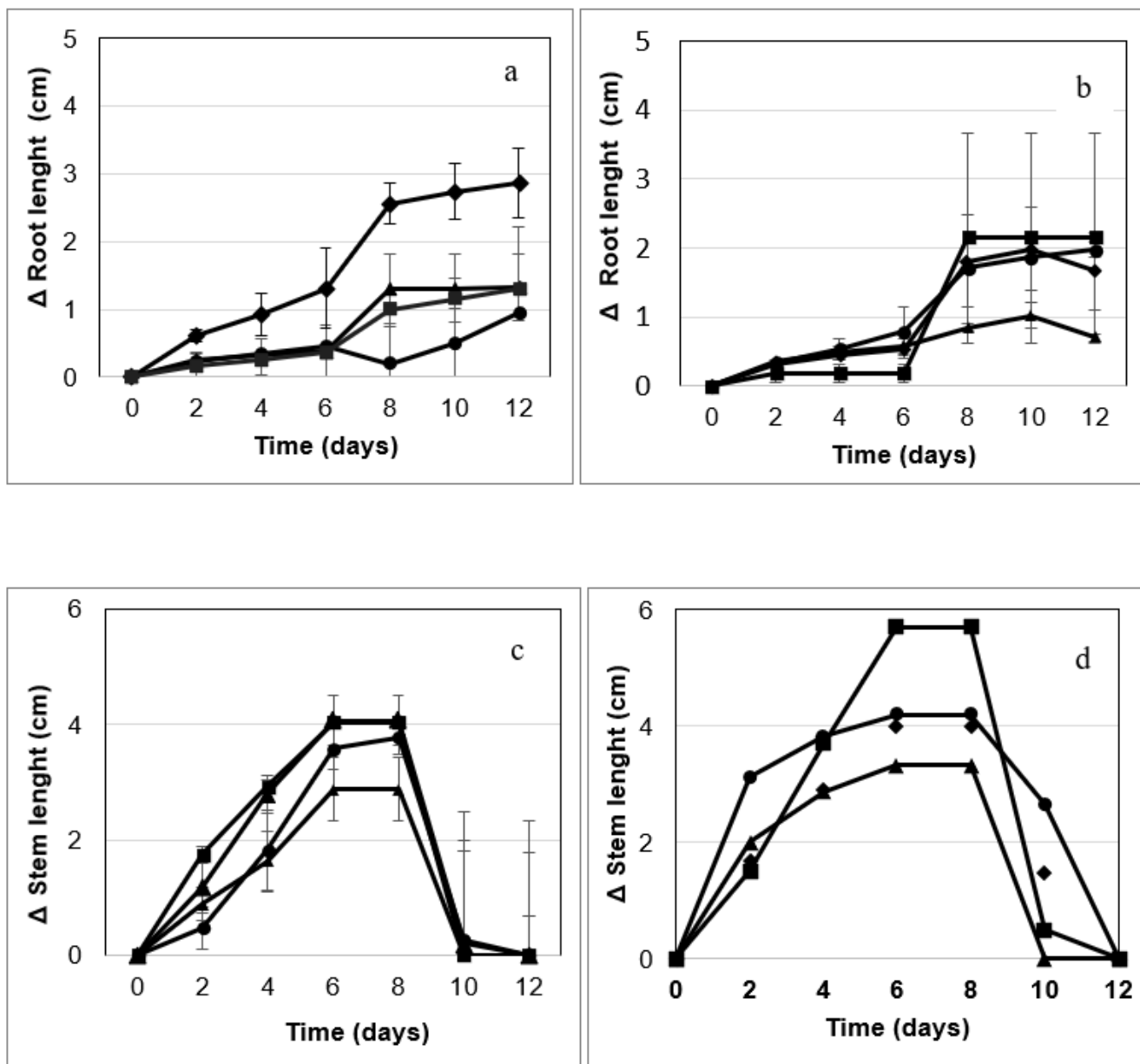


Figure 2

Effect of lead concentration on *S. americanus* development. Increase in root length in normal (a) and T12 plants (b). Increase in stem length in normal (c) and T12 (d) plants. Plants were maintained in Hoagland's modified medium containing 0 (●), 100 (▲), 250 (◆) or 400 (■) mg L⁻¹ Pb. Bars represent ± SD (n=10)

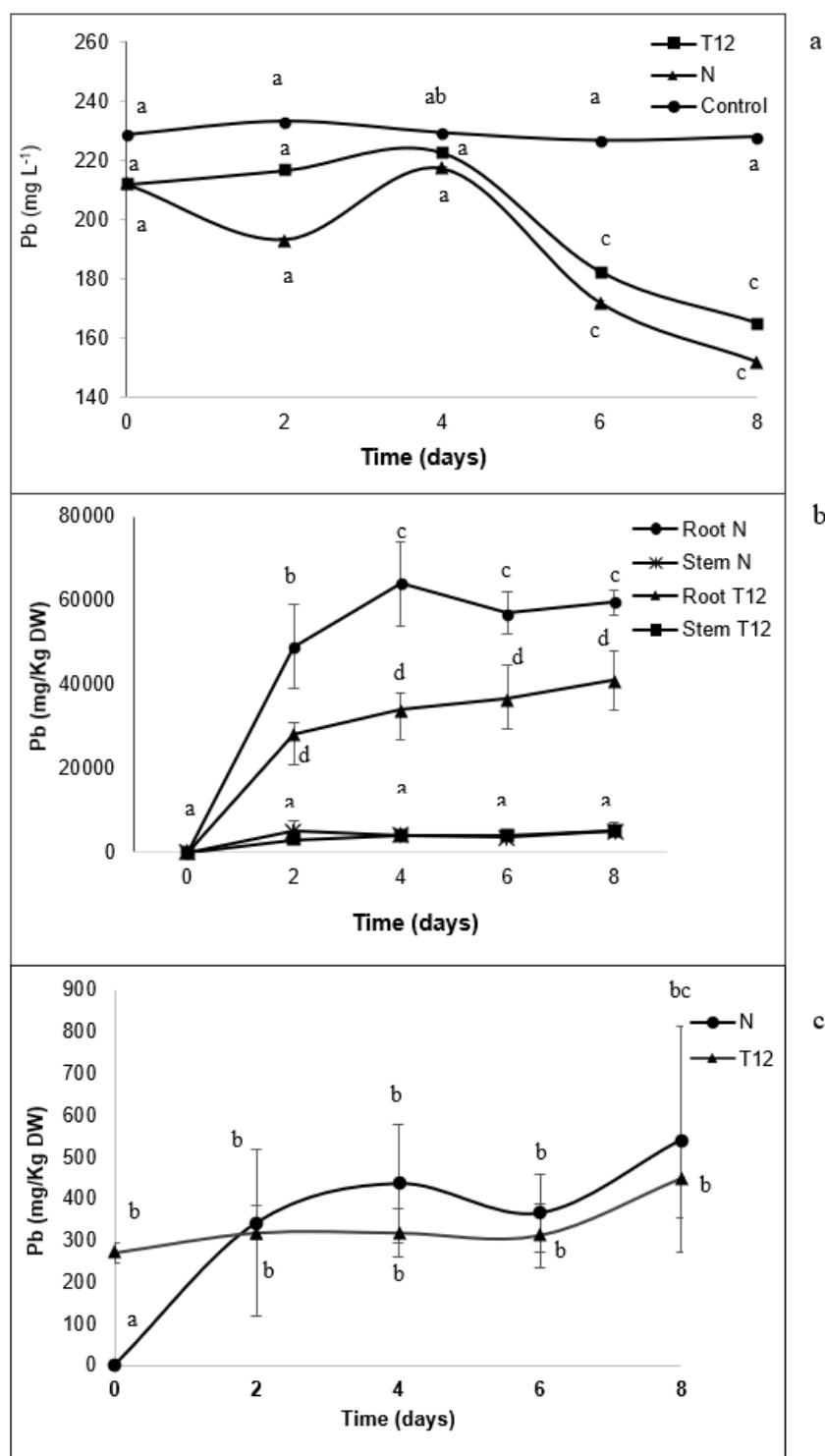


Figure 3

Lead removal kinetics by *S. americanus* plants. a) lead removed from medium, b) lead accumulated in root and stem, and c) lead adsorbed into the root's surface. Plants were exposed to 400 mg L⁻¹. Bars correspond to $\pm$ SD (n=5). Different letters represent statistical differences ($p \leq 0.05$, Tukey test)

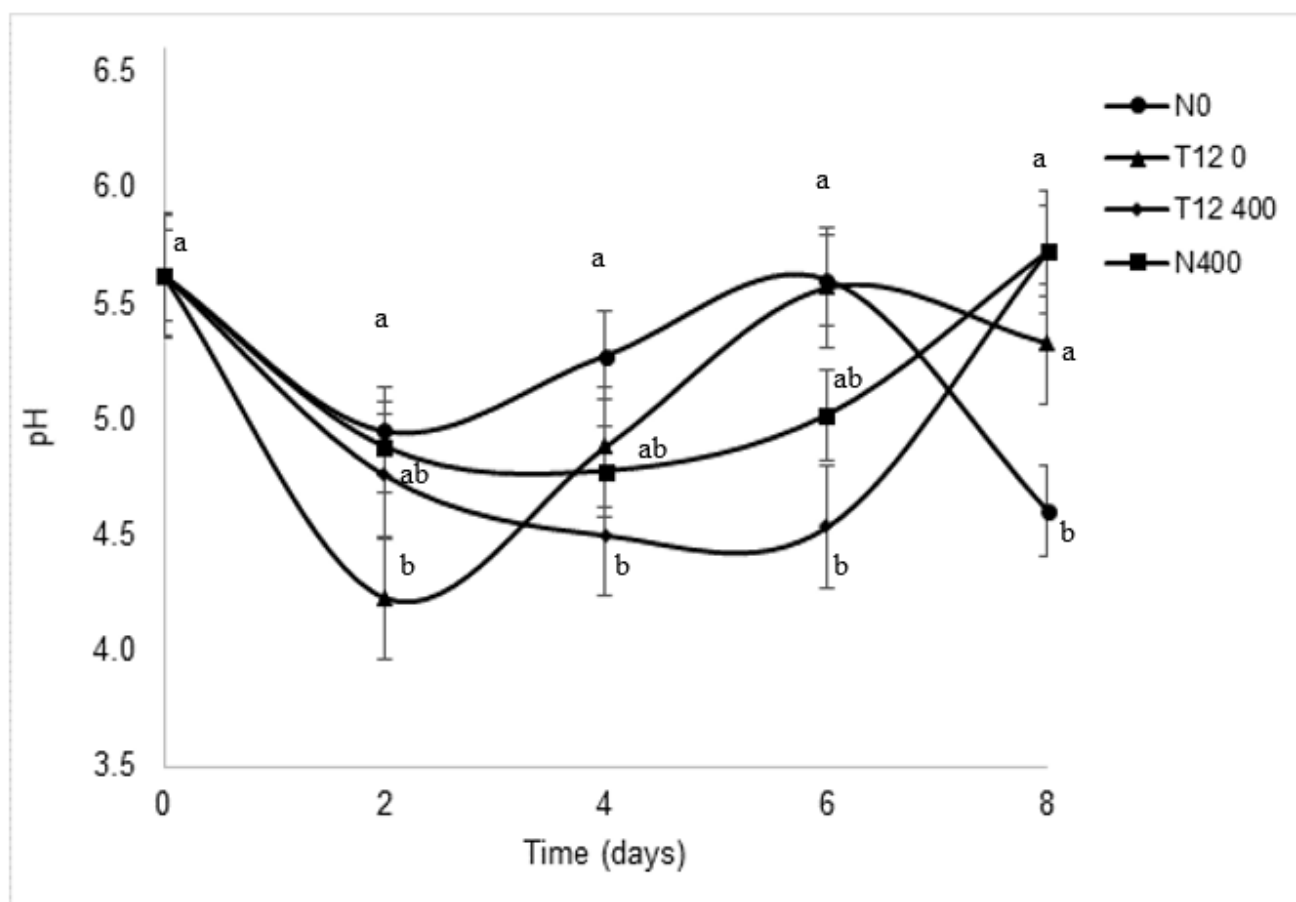


Figure 4

Changes in the medium pH in presence of normal and T12 *S. americanus* plants. Plants were exposed to 400 mg L⁻¹ Pb. Bars correspond to $\pm$ SD (n=5). Different letters represent statistical differences ($p \leq 0.05$, Tukey test)

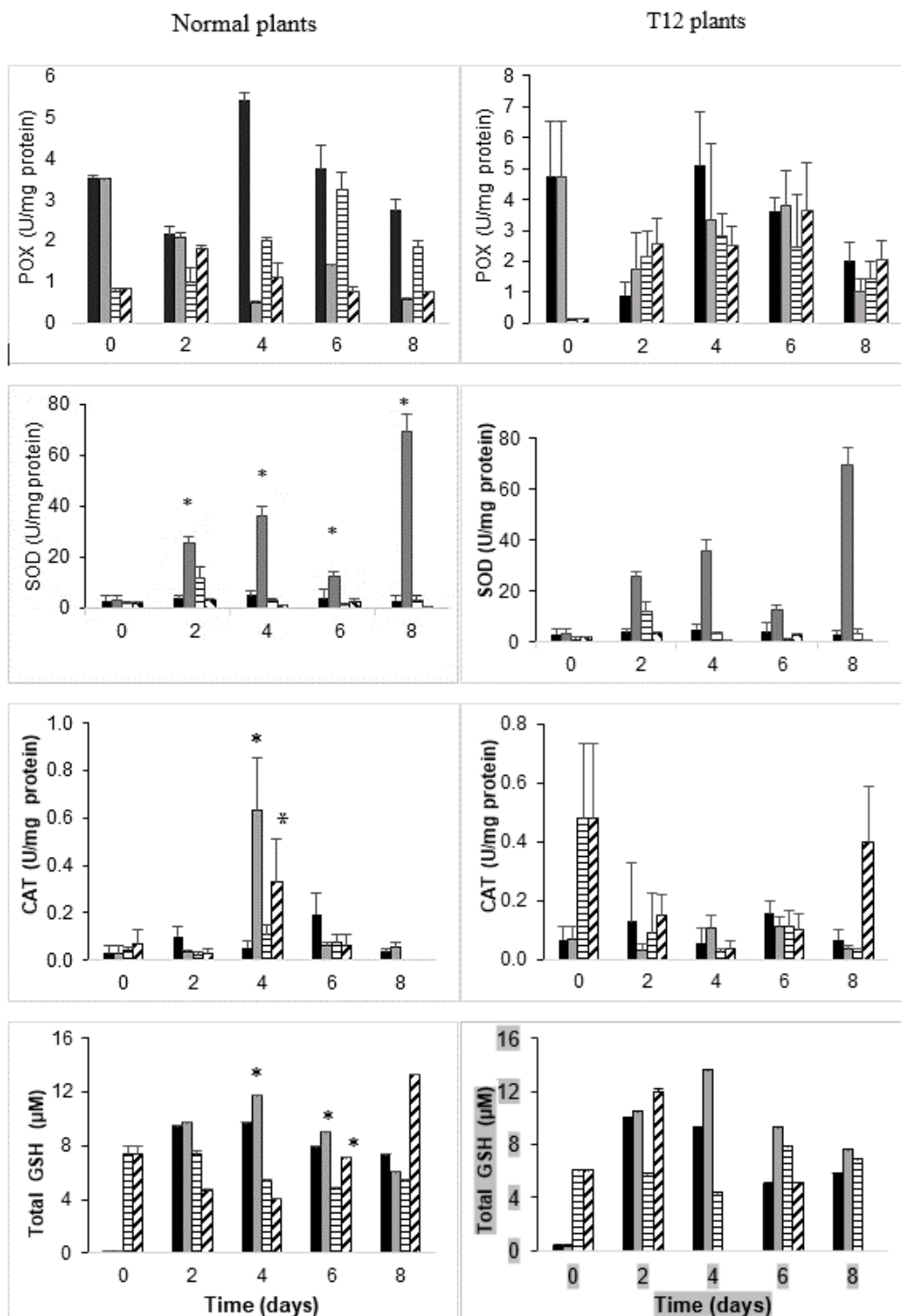


Figure 5

Antioxidant enzymatic activities and glutathione levels in normal and transformed plants of *S. americanus* at 400 mg L⁻¹ Pb. a-b) peroxidase activity, c-d) superoxide dismutase activity, e-f) catalase activity, g-h) total glutathione content. Bars correspond to $\pm$ SD. Asterisk represent statistical difference ($p < 0.05$, Tukey test)