

LC-MS/MS shotgun proteomics reveals biochemical mechanisms of *Paspalum fasciculatum* tolerance to Pb-stress

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Abstract

Paspalum fasciculatum Willd. ex Flügge grows in mining soils which are Cd- and Pb-contaminated where it exhibits tolerance to Pb and the ability to extract Pb from these soils. To elucidate tolerance mechanisms to Pb-stress, liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to quantify changes in the accumulation of proteins in leaves. We identified 323 proteins involved in primary metabolism and response to biotic or abiotic stresses. Although proteins involved in the processes of photosynthesis and saccharide and energy metabolism presented the greatest amount of down-regulated proteins, the plant was able to maintain photosynthetic functions and obtain energy to sustain the vital balance. *P. fasciculatum* based their tolerance on increased antioxidant defenses, improving the protection and repair of proteins and transduction signals to coordinate physiological response to Pb-stress. Our results provide important information to understand the tolerance mechanisms in *P. fasciculatum* and could be important in future molecular studies on the resistance and accumulation of Pb in plants.

Keywords: *Paspalum fasciculatum*, Pb-stress, shotgun proteomics, protein accumulation, tolerance mechanism.

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Abbreviations: ATPase α , β - ATP synthase subunit alpha, beta; cyt *c* - cytochrome *c*; DG-3P-D - glyceraldehyde 3-phosphate; CDPK - Ca²⁺-dependent protein kinase; CPN60 - chaperonin CPN60; FtsH - ATP-dependent zinc metalloprotease; H⁺ATPase - plasma membrane ATPase; HSE - heat shock elements; HSF - heat shock factors; HSPs - heat shock proteins; HSP17 - 17.0 kDa class II heat shock protein; HSP70 - heat shock cognate 70 kDa protein 1; HSP70-2 - heat shock cognate 70 kDa protein 2; HSP70S - stromal 70 kDa heat shock-related protein; HSR - heat shock response; LC-MS/MS - liquid chromatography with tandem mass spectrometry; 14-3-3 - 14-3-3-like protein; 14-3-3-A - 14-3-3-like protein GF14-A; 14-3-3-C - 14-3-3-like protein GF14-C; GPALSU1 - glucose-1-phosphate adenylyltransferase large subunit 1; GPASSU - glucose-1-phosphate adenylyltransferase small subunit; LHCP-1 - chlorophyll *a/b* binding protein of LHC II type I; LHCP-2 - chlorophyll *a/b* binding protein type 2 member 1B; LHCP-25 - chlorophyll *a/b* binding protein 25; MDHC - malate dehydrogenase; NADH-D8 - NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial; NADH subunit I - NAD(P)H - quinone oxidoreductase subunit I; NADP-G3PDH - NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; PE pyruvate - phosphoenolpyruvate; 3PG - 3-phosphoglycerate; PCKA2 - phosphoenolpyruvate carboxykinase (ATP) 2; PE pyruvate - phosphoenolpyruvate; PSI-B - photosystem I P700 chlorophyll *a* apoprotein A2; PSII D1 - photosystem II protein D1; Prx-1 - peroxidase 1; Prx-Q - peroxiredoxin Q; Rab7 - Ras-related protein Rab7; RABA1e - Ras-related protein RABA1e; Rab-2-B: Ras-related protein Rab-2-B; RABB1c - Ras-related protein RABB1c; ROS - reactive oxygen species; RuBisCO LSU - ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; RuBisCO SSU - ribulose bisphosphate carboxylase small subunit; RuBisCO activase - ribulose bisphosphate carboxylase/oxygenase activase; SCS-alpha - succinate-CoA ligase [ADP-forming] subunit alpha; SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis; O₂⁻ - superoxide; TC - treatment control, doping-free soil; TFs - transcription factors; TP30 - treatment with 30 mg kg⁻¹ Pb; TP50 - treatment with 50 mg kg⁻¹ Pb; Trx-M - thioredoxin M-type; V-ATPase SUB-V-type - proton ATPase subunit B 2.

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Introduction

Lead is an amphoteric trace metal and belongs to the most dangerous heavy metals as it is a threat to human health, agricultural production, and ecological safety (Xia *et al.* 2019). It is naturally present in the environment due to processes such as the erosion of rocks, soil erosion, volcanic eruptions, forest fires, and the decay products of radioactive elements (Kumar *et al.* 2018). The rapid industrial growth with the widespread use in storage batteries, gasoline, paints and dyes, ammunition, glasses, cosmetic products, and other anthropogenic activities such as mining and Pb mineral smelting have turned this element into an important environmental pollutant (Obiora *et al.* 2016, Wang *et al.* 2016). Soil contamination with Pb is considered one of the most serious threats to humans and other living organisms (Arshad *et al.* 2008, Ma *et al.* 2016).

The Pb enters the soil by the deposition of particles dragged by the wind, contact with industrial wastewater, the irrigation of crops with waters containing small fractions of this metal, and runoff water from mineral stacks (Acosta *et al.* 2011). High concentrations of Pb in the soil can cause a root absorption of this element by plants; thus Pb enters the trophic chain and affects herbivores. The phytotoxic effects of Pb on plants include altering growth parameters such as root elongation and rooting efficiency, leaf area, number, and length of leaves, shoots multiplication rate, plant height, and fresh and dry masses of underground and aboveground parts (Khan *et al.* 2018, López-Orenes *et al.* 2018).

To reduce heavy metals in soil *in-situ* and *ex-situ* technologies are available for remediation (Kuppusamy *et al.* 2016, Peng *et al.* 2018). Phytoremediation, an *in-situ* plant-based method, is a cost-effective and eco-friendly technique for the remediation of Pb and other trace metals from contaminated soil (Kuppusamy *et al.* 2016, Ma *et al.* 2016, Taiwo *et al.* 2016, Mahdavian *et al.* 2017). Phytoremediation includes processes such as phytostabilization, where plants have the ability to reduce the mobility of the heavy metals in soils and phytoextraction, in which plants can extract metals from the soil and accumulate them in their shoots (Wuana and Okieimen 2011, Singh and Singh 2016). The plants used for phytoremediation must have mechanisms, which enable them to survive in soils contaminated with heavy metals. Thus, understanding their responses to heavy metal stress might help to develop effective detoxification measures and identify stress-tolerant genes or proteins (Benyó *et al.* 2016).

In the ecophysiological adaptations of plants to

soils contaminated with heavy metals proteins are very important because they have a direct and fundamental role in the adaptive response to these stress conditions, such as changes in the plasma membrane, the cell cytoplasm, and the intracellular compartment (Kosová *et al.* 2011). Mass spectrometry (MS)-based proteomic technologies are powerful tools used for large-scale protein identification and quantitation (Cravatt *et al.* 2007). Label-free shotgun proteomics is a very effective technique for the identification of peptides, determining the abundance of the proteins, and subsequently, obtaining a global protein profile of a sample, which is important for applications in complex biological systems (Pang *et al.* 2016, Han *et al.* 2021, Shen *et al.* 2021, Szuba *et al.* 2021).

Consequently, proteomics is a very useful technique to study plant responses to heavy metal stress. It has been used to identify, describe, and interpret the biological functions of proteins in response to different biotic or abiotic stresses (Chen *et al.* 2016, Jiang *et al.* 2017). Many studies describe the molecular mechanisms of tolerance to Pb based on the differential proteins accumulation in different plant species such as *Populus trichocarpa* (Shen *et al.* 2021), *Arabidopsis thaliana* (Jiang *et al.* 2017, Simiele *et al.* 2021), *Triticum aestivum* (Han *et al.* 2021), *Populus × canescens* (Szuba *et al.* 2021), *Glycine max* (Baig *et al.* 2018), and *Acalypha indica* (Venkatchalam *et al.* 2017).

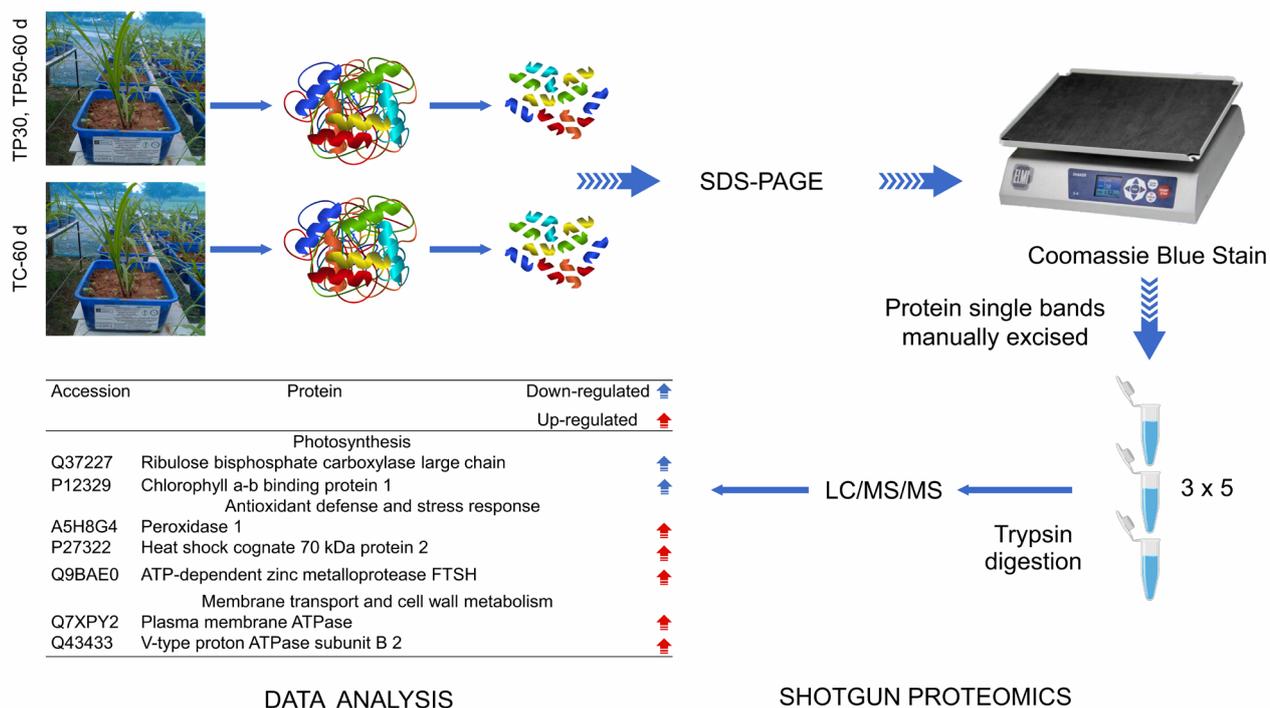
In our case, we focused on the *Paspalum fasciculatum* (*Poaceae*) because it can grow in mining soils with high Pb contamination. Furthermore, it seems to limit the effects of lead on a small group of proteins involved in photosynthesis and energy metabolism (Salas-Moreno and Marrugo-Negrete 2019, Salas-Moreno *et al.* 2019). Nevertheless, protein components of the Pb-tolerance mechanisms in *Paspalum fasciculatum* remain unknown yet. In order to elucidate some components of these tolerant mechanisms, we used a proteomic approach based on LC-MS/MS shotgun techniques.

Materials and methods

Sampling and preparation of soils and plant growth conditions: Soil samples were collected to a depth of 30 cm from the surface (Smolińska and Cedzyńska 2007), in the “El Alacrán” gold mine, located in northwestern Colombia, between coordinates 7°44'29.01" North and 75°44'10.8" West, in the municipality of Puerto Libertador (Córdoba), following the protocol of Salas-Moreno and Marrugo-Negrete (2019). Briefly, mine soil samples were characterized before they were doped with PbSO₄ solutions

Table 1. Physical and chemical characteristics of the soil from “El Alacrán” gold mine. Means \pm SE, $n = 3$ [OM (%) - organic matter percentage; CEC - cation exchange capacity].

Properties of bioavailability		Texture		Metals [mg kg ⁻¹]	
pH	3.7 \pm 0.03	Sand [%]	27.5 \pm 0.03	Cd	7.3 \pm 0.10
OM [%]	1.5 \pm 0.09	Clay [%]	4.4 \pm 0.09	Pb	2.7 \pm 0.40
CEC	13.1 \pm 0.01	Silt [%]	68.1 \pm 0.06	Hg	0.5 \pm 0.01
Ca/Mg	0.6 \pm 0.02	-	-	Ni	25.2 \pm 0.50
Ca/K	2.5 \pm 0.30	-	-	Fe	13.1 \pm 0.20
Mg/K	3.9 \pm 0.03	-	-	Mn	19.1 \pm 0.70



DATA ANALYSIS

SHOTGUN PROTEOMICS

Fig. 1. Workflow used for the shotgun proteomics methodology in the *P. fasciculatum* leaves exposed to Pb-stress. To determine quantitative differences, normalized abundance ratio between treatments (TP30/TC, TP50/TC) ≥ 1.5 (up-accumulated) or ratio ≤ 0.67 (down-accumulated) and a P -value less than 0.05 were considered significant. Biological significance [$\log_2(\text{fold-change})$], was used to describe the changes induced by Pb-treatments on the *P. fasciculatum* leaves proteome (proteins with more/less than 1.5 fold-change on abundance). Differentially accumulated proteins identified by LC-MS/MS analysis were classified based on their gene ontology terms annotated in the *UniProtKB* protein database for their molecular functions and biological processes.

to prepare individual samples with two concentrations of Pb: 30 and 50 mg Pb per kg of soil, designated as TP30 and TP50, respectively. As a control (TC) we used a sample of the same mining soil described above, but without doping; due to the low concentrations of Pb, specifically 2.72 mg kg⁻¹. The initial Cd and Pb concentrations in the soil samples are shown in Table 1.

Paspalum fasciculatum Willd. ex Flügge plants from soils free of Pb were cut, and its taxonomic determination was realized in the herbarium of the University of Cordoba, Colombia. For the processing in the laboratory, establishment, growth conditions, and monitoring of plants in doped soils and control in greenhouse conditions, we rely on what is reported by Salas-Moreno and Marrugo-Negrete (2019). The plant growth, the amount of biomass

generated, and metal content in the soil and plant tissues (leaf, stem, and root) were measured during 90 d. The test was performed in triplicate with three biological replicates. Soil and plant tissue samples were collected monthly following the methodology proposed by Salas-Moreno and Marrugo-Negrete (2019). After digestion of the samples (Jedrzejczak 2002), the total content of Pb was determined by graphite furnace atomic absorption spectroscopy (GF-AAS) using a Thermo Scientific iCE 3000 (Waltham, MA, USA) analyzer.

Protein extraction and digestion: Protein extraction was performed on the leaf samples taken after 60 d of exposure to the treatments TP30 and TP50, and the control (three biological replicates per treatment). The leaves were

immediately inserted into a tank with liquid nitrogen, transported to the laboratory, and stored at -80°C . Samples were weighed (1 g) and ground to a fine powder in liquid nitrogen using a pestle and mortar. Proteins were extracted (three independent extractions per treatment) from the frozen homogenized pool tissue by using the trichloroacetic acid (TCA)-acetone-phenol protocol as reported in Wang *et al.* (2006) and quantified with Bradford assay (Bradford 1976). Next, protein samples were concentrated in a single band using an SDS-PAGE (12 %, m/v) and revealed with Coomassie stain (Pascual *et al.* 2017). Protein single bands were manually excised, destained, and digested with trypsin sequencing grade (Roche, Mannheim, Germany) as is described in Castillejo *et al.* (2015) with minor modifications. Briefly, gel plugs were incubated (twice for 30 min) with a solution containing 100 mM ammonium bicarbonate (AmBic)/50 % (m/v) acetonitrile (AcN) at 37°C . Then, they were dehydrated with AcN and incubated in 100 mM AmBic containing the first 20 mM dithiothreitol (DTT) for 30 min (second hydration with AcN and 100 mM AmBic containing 55 mM iodoacetamide for 30 min). Next, they were washed with 25 mM AmBic and 25 mM AmBic/50 % AcN two times each. After dehydration in AcN, trypsin at a concentration of 12.5 ng mm^{-3} was added to a buffer containing 25 mM NH_4HCO_3 , 10 % AcN and 5 mM CaCl_2 , and the digestion proceeded at 37°C for 12 h. Digestion was stopped, and peptides were extracted from gel plugs by adding 10 mm^3 of 1 % (v/v) trifluoroacetic acid (TFA) and incubating for 15 min.

Shotgun LC-MS/MS analysis: Nano-LC was performed in a *Dionex Ultimate 3000 nano UPLC* (Thermo Scientific) with a C_{18} $75\text{ }\mu\text{m} \times 50$ *Acclaim PepMam* column (Thermo Scientific). The peptide mix was previously loaded onto a $300\text{ }\mu\text{m} \times 5$ mm *Acclaim PepMap* precolumn (Thermo Scientific) in 2 % (m/v) AcN/0.05 % (m/v) trifluoroacetic acid (TFA) for 5 min at $5\text{ mm}^3\text{ min}^{-1}$. Peptide separation was performed at 40°C for all runs. Mobile phase buffer A was composed of water, and 0.1 % (m/v) formic acid. Mobile phase B was composed of 80 % AcN, and 0.1 % formic acid. Samples were separated during a 60-min gradient ranging from 96 % solvent A to 90 % solvent B and a flow rate of $300\text{ mm}^3\text{ min}^{-1}$. Eluted peptides were converted into gas-phase ions by nanoelectrospray ionization and analyzed on a *Thermo Orbitrap Fusion (Q-OT-qIT)*, (Thermo Scientific) mass spectrometer operated in positive mode. Survey scans of peptide precursors from 400 to 1500 m/z were performed at 120 K resolution (at 200 m/z) with a 4×10^5 ion count target. Tandem MS was performed by isolation at 1.2 Da with the quadrupole, CID fragmentation with a normalized collision energy of 35, and rapid scan MS analysis in the ion trap. The automatic gain control was set to 2×10^3 and the maximum injection time was 300 ms. Only those precursors with charge states 2 - 5 were sampled for MS2. The dynamic exclusion duration was set to 0.25 min with a tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top 30 mode with 3 s cycles, meaning that the instrument

would continuously perform MS2 events until a maximum of top 30 non-excluded precursors or 3 s, whichever was shorter. Fig. 1 shows the workflow used for the shotgun proteomics methodology.

Protein identification and functional classification: The raw data derived from MS experiments were processed using *Proteome Discoverer v.1.4* software (Thermo Scientific). MS2 spectra were searched with the *SEQUEST* engine against the protein-fasta files created by a combination of the following databases: *UniProtKB Swiss-Prot* database with taxonomy restrictions to *Viridiplantae*. Precursor mass tolerance was set to 10 ppm and fragment ion mass tolerance fixed to 0.1 Da. Only charge states +2 or greater were used. Identification confidence was set to a 5 % false discovery rate (FDR) and acetylation of N terminus, oxidation of methionine, and carbamidomethyl cysteine formation were set as variable modifications. No fixed modifications were set. Trypsin was set as a proteolytic enzyme and a maximum of two misscleavages were set for all searches. A minimum XCorr of 2 and proteins with 2 or more peptides matched were considered. For relative quantification, the peak area of each identified peptide was used.

The protein peak areas were normalized by the total sum of the peak area values per sample, and missing values were corrected. Mean values and standard deviation (SD) of the peak areas of protein species were determined for three independent analyses. The remaining sequences were used as a database for the protein identifications and their functions were identified using *Mercator v.3.6-PlabiPD* (<https://www.plabipd.de/portal/Mercator-sequence-annotation>). Protein function classification was based on the annotation from *AgBase v. 2.00* (McCarthy *et al.* 2010).

Statistical analysis: For the experiments (physical and chemical characteristics, proteins yield, biomass, Pb content, and protein peak areas) with *P. fasciculatum* under Pb stress, the results are presented as means \pm standard deviations of triplicate determinations. After assessing normality using the Shapiro-Wilk test and homogeneity of variance using the Bartlett test, the data were subjected to *ANOVA*, and when necessary, a comparison of means was performed using the Bonferroni test. The statistical software *GraphPad PRISM v. 8.0.1* was used for all analyses. Results were considered to be significant at the $P \leq 0.05$.

Results

The Pb content in plant tissue was as in our previous work (Salas-Moreno and Marrugo-Negrete 2019). Pb content in *P. fasciculatum* leaves at 30, 60, and 90 d was in the range of 0.49 to 4.84 mg kg^{-1} (f.m.) The highest content was observed at 30 d (Fig. 1 Suppl.). Also, these plants showed important bioaccumulation of Pb in their roots, with content between 4.99 and 36.70 mg kg^{-1} (f.m.). Nevertheless, the growth of *P. fasciculatum* was not

Table 2. Protein yield from *Paspalum fasciculatum* leaves exposed to Pb-stress for 60 d. Means \pm SE, $n = 3$ [TC - control, TP30 - 30 mg(Pb) kg⁻¹(soil), TP50 - 50 mg(Pb) kg⁻¹(soil)].

Treatments	Protein [mg(protein) g ⁻¹ (f.m.)]
TC	2.03 \pm 0.17
TP30	2.33 \pm 0.40
TP50	1.54 \pm 0.14

inhibited demonstrating its tolerance to Pb presence. Only at the end of the incubation period (90 d), they exhibited toxic effects mainly in form of biomass reduction in all tissues, accompanied by slight symptoms of chlorosis and necrosis (Salas-Moreno and Marrugo-Negrete 2019).

Protein samples obtained from the Pb treated (TP30 and TP50) leaves for 60 d and control (TC) leaves (Table 2) were subjected to quantitative proteomic analysis using label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) shotgun proteomics. The resulting mass spectra were searched with the *SEQUEST* engine as mentioned above. A total of 1 227 proteins were

identified and 323 of them accomplished the parameters and inclusion criteria for the quantitative analysis (high confidence for peptide identifications, XCorr \geq 2, and at least two distinct peptides per protein). In the case of plants grown in TP30 treatment, 225 proteins were differentially accumulated in comparison with control, 89 were up-regulated and 136 down-regulated; whereas 218 proteins were found in TP50 treatment, 90 were up-regulated and 128 down-regulated (Table 3). However, only 80 of these proteins showed differences statistically significant ($P \leq 0.05$) among treatments/control and 48 of them showed the same behaviour in both treatments. Therefore, to agile the functional analysis, the data are grouped into only two groups, control, and treatment.

It was found that the amount of proteins concerning photosynthesis and energy metabolism was decreased by Pb-stress, while the amount of those proteins with molecular functions such as heat shock, antioxidant activity, stress response, and signal transduction was increased.

Functional classification of all significantly variable proteins is represented in Fig. 2. The responsive proteins

Table 3. Summary of the behaviour of the proteins identified in *P. fasciculatum* leaves in response to the Pb-treatments during 60 d.

Treatments	Proteins identified in each treatment	Common proteins with respect to the control	Proteins only detected in one treatment	Proteins up-regulated	Proteins down-regulated
TP30	323	16	10	89	136
TP50	323	19	6	90	128

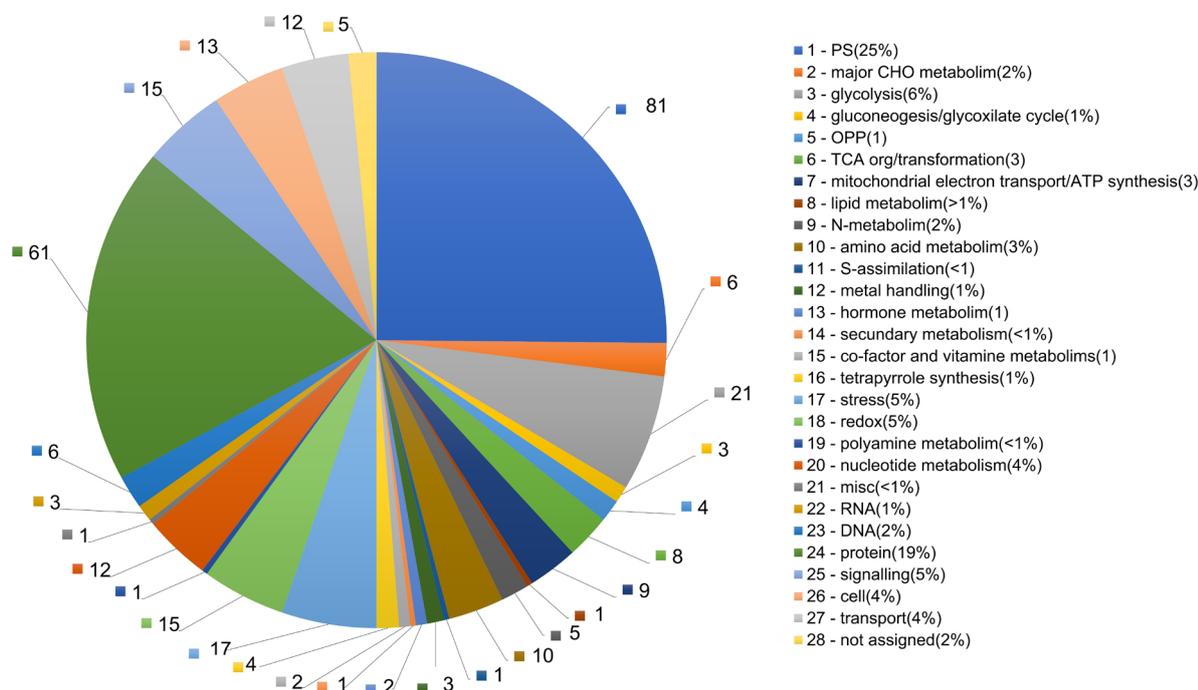


Fig. 2. Functional classification of the identified proteins in *P. fasciculatum* leaves exposed Pb-stress for 60 d. (CHO - carbohydrate, TCA - tricarboxylic acid, misc - mitochondrial solute carrier). The numbers around the cycle indicate the amount of proteins identified into each functional group.

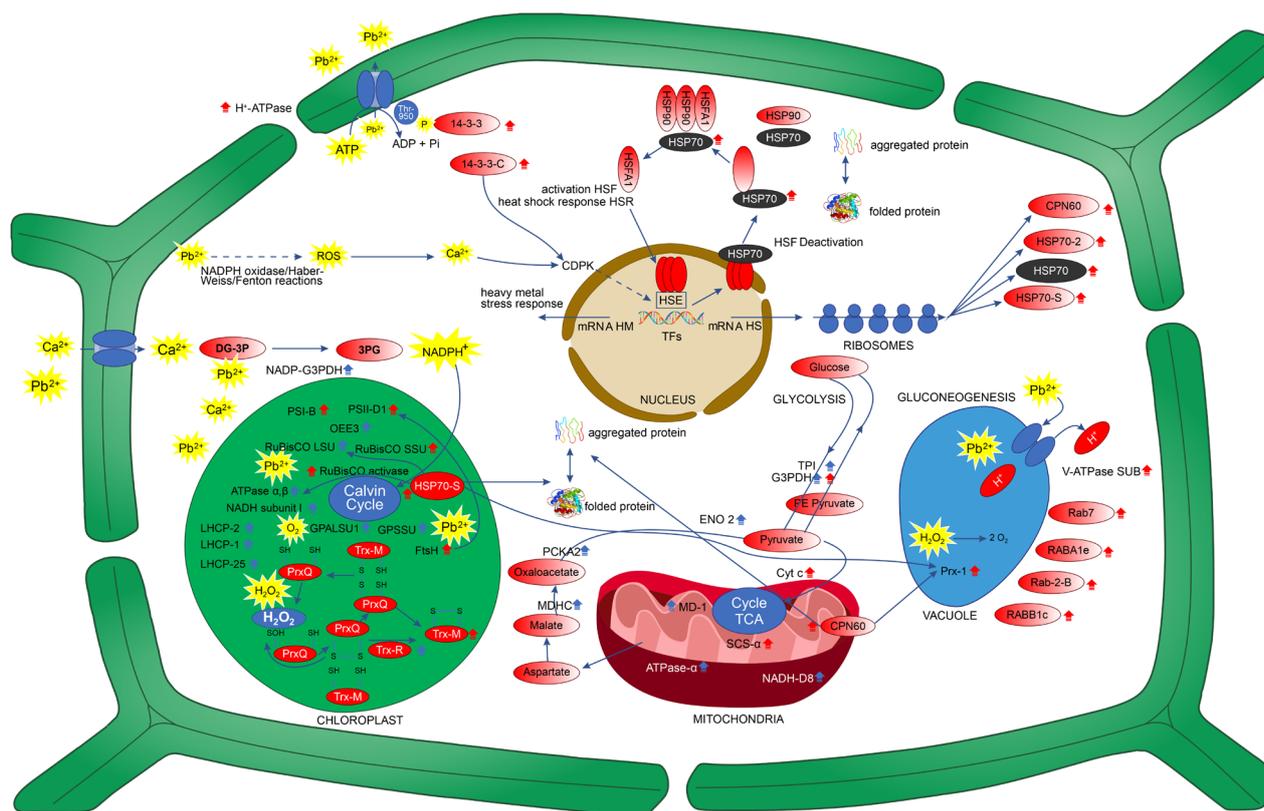


Fig. 3. Possible metabolic pathways involved in tolerance mechanisms in the response to Pb-stress in of *Paspalum fasciculatum* leaves. ATPase α,β - ATP synthase subunit alpha, beta; Cyt c - cytochrome c; DG-3P-D - glyceraldehyde 3-phosphate; CDPK - Ca^{2+} -dependent protein kinase; CPN60 - chaperonin CPN60; FtsH - ATP-dependent zinc metalloprotease; HSE - heat shock elements; HSF - heat shock factors; HSPs - heat shock proteins; HSR - heat shock response; HSP17 - 17.0 kDa class II heat shock protein; HSP70 - heat shock cognate 70 kDa protein 1; HSP70-2 - heat shock cognate 70 kDa protein 2; HSP70S - stromal 70 kDa heat shock-related protein; H^+ ATPase - plasma membrane ATPase; 14-3-3 - 14-3-3-like protein; 14-3-3-A - 14-3-3-like protein GF14-A; 14-3-3-C - 14-3-3-like protein GF14-C; GPALSU1 - glucose-1-phosphate adenylyltransferase large subunit 1; GPASSU - glucose-1-phosphate adenylyltransferase small subunit; LHCP-1 - chlorophyll *a+b* binding protein of LHCII type I; LHCP-2 - chlorophyll *a+b* binding protein type 2 member 1B; LHCP-25 - chlorophyll *a+b* binding protein 25; MDHC - malate dehydrogenase; NADH-D8 - NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial; NADH subunit I-NAD(P)H - quinone oxidoreductase subunit I; NADP-G3PDH - NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; PE pyruvate - phosphoenolpyruvate; 3PG-3 - phosphoglycerate; PCKA2 - phosphoenolpyruvate carboxykinase (ATP) 2; PSI-B - photosystem I P700 chlorophyll a apoprotein A2; PSII D1 - photosystem II protein D1; Prx-I - peroxidase I; Prx-Q - peroxidase Q; Rab7 - ras-related protein Rab7; RABA1e - ras-related protein RABA1e; Rab-2-B - ras-related protein Rab-2-B; RABB1c - ras-related protein RABB1c; RuBisCO LSU - ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; RuBisCO SSU - ribulose bisphosphate carboxylase small subunit; RuBisCO activase - ribulose bisphosphate carboxylase/oxygenase activase; SCS- α - succinate-CoA ligase [ADP-forming] subunit alpha; TFs - transcription factors; Trx-M - thioredoxin M-type; V-ATase SUB-V-type - proton ATPase subunit B2; (red arrow - up-regulation; blue arrow - down-regulation).

of *P. fasciculatum* mainly fell into six different functional classes: 1) proteins involved in primary and energy metabolism, including photosynthesis (PS, 26.11 %), glycolysis (6.39 %), tricarboxylic acid cycle (TCA)/org transformations (2.50 %), saccharide metabolism (1.94 %), gluconeogenesis/glyoxylate cycle (0.83 %), oxidative pentose phosphate pathway (OPP; 1.11 %); 2) proteins involved in protein metabolism (18.89 %), including protein biosynthesis, structure and transport (3.61 %); 3) proteins involved in cellular metabolism including secondary metabolism (0.28 %), lipid metabolism (0.28 %), Co-factor and vitamin metabolism (0.56 %),

ATP synthesis (2.78 %), amino acid metabolism (3.06 %), tetrapyrrole synthesis (1.11 %), hormone metabolism (0.56 %), N-metabolism (1.67 %), nucleotide metabolism (3.61 %), polyamine metabolism (0.20 %), cellular development (3.89 %) and signaling pathways (4.72 %); 4) proteins involved in antioxidant defense (4.72 %) and stress response (5.28 %); 5) proteins involved in RNA and DNA processing (2.77 %), and 6) other proteins with unknown functions (1.67 %). The most important detailed functional categories are discussed in the following sections.

Discussion

Mass spectrometry is a classic technique of proteomic analysis widely used in many investigations on the tolerance mechanisms associated with metal stress in phytoremediation or tolerant plants (Farooq *et al.* 2018, Lan *et al.* 2018, Xia *et al.* 2019).

P. fasciculatum is a Cd- and Pb-tolerant plant with a good phytoremediation capacity that makes it a promising species for bioremediation of areas degraded by mining. In order to further study, we performed label-free LC-MS/MS shotgun proteomics experiments in *P. fasciculatum* leaves to identify the metabolic pathways most affected by the presence of Pb and thus provide the first evidence of the tolerance mechanisms in this species in response to Pb-stress.

Saccharide and energy metabolism: Proteins involved in photosynthesis were highly affected by Pb treatment, with 23 of them showing significant changes, followed by saccharide and energy metabolism, and protein metabolism with 14 and 9 proteins changed, respectively.

Photosynthesis and saccharides and energy metabolism pathways showed the highest amount of proteins with reduced accumulation. Photosynthesis is the main biological process in the leaves, and it is important for carbon fixation and energy metabolism in plants (Lan *et al.* 2018). By treatments, 15 proteins were down-regulated and 6 up-regulated under TP50; while 15 and 4 in TP30 one. The major photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) was reduced in all treatments. RuBisCO is an enzyme that catalyzes the CO₂ assimilation in plants, it is involved in the first step of the Calvin cycle and also catalyzes the reaction that regenerates the CO₂ acceptor ribulose-1,5-bisphosphate (Fig. 3) (Hossain *et al.* 2012). Similar studies conducted in pea (Rodriguez *et al.* 2015), showed that Pb decreases the activity of RuBisCO. However, in *Typha latifolia* species, Pb-induced the accumulation of RuBisCO large subunit (LSU) and RuBisCO activase (Bah *et al.* 2010). These results suggest that the efficiency of carbon fixation in our plant under Pb stress might be significantly decreased, which could be associated with some damage to the photosynthetic apparatus (Lan *et al.* 2018). In fact, chlorophyll *a,b* binding protein (fragment), chlorophyll *a,b* binding protein 1, chlorophyll *a,b* binding protein 25, chlorophyll *a,b* binding protein type 2 member 1B, chlorophyll *a,b* binding protein of LHClI type I, and chlorophyll *a,b* binding protein P4 were also strongly down-regulated by Pb-stress in both treatments. These are the apoproteins of the light-harvesting complex of photosystem I and II (PS I and PS II), which are normally complexes with chlorophyll and xanthophylls and serve as the antenna complex (Wientjes *et al.* 2017).

Our results also reinforce those studies that attribute an inhibitory role of Pb-stress to the activity of PS I and II (Prasad *et al.* 2015); and were similar to the studies conducted by Kumar *et al.* (2011) in *Catharanthus roseus* under Pb-stress, which showed a down-regulation in two chlorophyll-binding proteins (CP26 in PS II and type

III in PS I). In PS I and PS II chlorophyll *a/b*-binding proteins have an essential role in oxygenic photosynthesis; therefore, its down-regulation likely would affect oxygen production (Wang *et al.* 2015). Besides, it is well known that heavy metals Cd and Pb can replace Ca²⁺ and Mn²⁺ ions in the reaction center of PS II, affecting the oxidizing system of water of PS II, decoupling the electron transport in chlorophyll, and generating high amounts of ROS as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) (Siedlecka and Baszyński 1993, Faller *et al.* 2005, Takahashi and Murata 2008). Hence, the observed effects of Pb-stress also agree with the irreversible oxidative damage previously described for RuBisCO LSU proteins and chlorophyll *a/b* binding protein CP26 in leaves of *P. fasciculatum* and that proposes protein carbonyl index as a potential biomarker of the heavy metal toxicity in leaves (Salas-Moreno *et al.* 2019).

As a consequence of the down-regulation suffered by photosynthetic proteins, seven other proteins involved in glycolysis and gluconeogenesis were also significantly affected. Two of them correspond to triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase 1. Comparing quantitatively these proteins with those reported in the controls, we observed that these proteins presented a down-regulation of 2.1- (TP30), 10.7- (both treatments), and 8.7-fold (both treatments), respectively; which is significantly sensitive in energy terms. These proteins are important to energy production and the synthesis of triose phosphates (Bohler *et al.* 2013). Another important less accumulated enzyme was enolase, which is a key glycolytic enzyme for the synthesis of phosphoenolpyruvate (PE pyruvate). Likewise, the fundamental gluconeogenesis proteins malate dehydrogenase and phosphoenolpyruvate carboxykinase (ATP) 2 were found down-regulated. A similar effect on glycolysis and gluconeogenesis proteins was observed in the studies conducted by Xia *et al.* (2019) on *Cannabis sativa* tolerant to Pb, where a significant down-regulation was recorded for these proteins without effects on plants survival. Like photosynthetic proteins, the glycolysis proteins were previously identified as sensible to irreversible oxidative damage induced by Pb-stress (Salas-Moreno *et al.* 2019).

Into the last group of down-accumulated proteins belonging to those involved in energy metabolism. NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 and ATP synthase subunit alpha of the electron transport respiratory chain were identified under both treatments along with mitochondrial and cytoplasmic malate dehydrogenase, which also show an effect of the Pb-stress on the tricarboxylic acid (TCA) cycle. This cycle consists of a series of chemical reactions aimed at storing energy by the oxidation of acetyl-CoA derived from sugars, fats, and proteins into carbon dioxide and the formation of adenosine triphosphate (ATP), the increase in the accumulation of TCA proteins is related to the resistance to Cd damage in *Microsorium pteropus* plants (Lan *et al.* 2018).

Despite the adverse changes in the energetic metabolic pathways of *P. fasciculatum*, no lethal effects were evidenced during plant growth and, by contrast, its

development accounts for a good tolerance of this species to the presence of Pb, in our assay conditions. This could be due to the activation of other important pathways for protein repair and adaptation to Pb stress; which could promote a metabolic standby state to survive the produced energy drop. Similar behaviour was observed in maize plants, which can enter a standby mode to cope with cold stress (Riva-Roveda *et al.* 2016). Notwithstanding down-regulation of photosynthesis, saccharides, and energy metabolism proteins of *P. fasciculatum* seedlings well-grown during its 60 d of exposition to Pb without exhibition of evident toxicity signs on leaves, for example, chlorosis and necrosis (Salas-Moreno and Marrugo-Negrete 2019). This good tolerance, in terms of primary metabolism, could be related to the up-regulation of eight photosynthesis proteins, a glyceraldehyde-3-phosphate dehydrogenase cytosolic isoform, and the mitochondrial succinate-CoA ligase.

These proteins could help to overcome a metabolic standby state, help maintain photosynthesis and provide the ATP necessary for growth. Thus, for example, the overexpression of the ATP-dependent zinc metalloprotease FtsH protein (FtsH) presented 7.8- and 8.2-folds in TP50 and TP30, respectively, which are important in the formation of thylakoid membranes during early chloroplast development and protein quality control during photosynthesis (Kato and Sakamoto 2018). Therefore, its activity plays a vital role in the development and maintenance of the functioning of chloroplasts, as well as in the repair or turnover of photosynthesis proteins affected by Pb-stress. According to our results, FtsH was fundamental to improving photosynthesis in our plants because it also participates in the PS II repair cycle through the degradation of photosystem II protein D1 and its subsequent *de novo* synthesis (Kato and Sakamoto 2018), which is reinforced by the high up-regulation of D1 protein (13.9-fold changes in TP30). Along with FtsH, the M-type thioredoxin (1.7-folds in TP50) protein was another important protein up-accumulated in this study, as it performs a key antioxidant mechanism to maintain redox homeostasis and functionality of photosynthetic activities (Rey *et al.* 2013).

Other accumulated proteins that might contribute to good tolerance to Pb stress were those identified in the TP30 treatment: photosystem II reaction center protein H, phosphoglycerate kinase 2, ribulose biphosphate carboxylase/oxygenase activase; along with those identified in the Pb50 treatment which includes oxygen-evolving enhancer protein 3-1, photosystem I reaction center subunit psaK, pyruvate, phosphate dikinase 1, and cytochrome *f*. The observed behaviour in this group of proteins was similar to that described for *Brassica napus* (Farooq *et al.* 2018) and *Microsorium pteropus* (Lan *et al.* 2018), providing evidence that some proteins of the photosynthetic machinery are up-regulated when the plants face metal stress conditions.

The mitochondrial ADP/ATP carrier protein, which showed a great accumulation (6,9-fold changes) in the TP30 treatment, and the cytochrome *c* (in both treatments) could mitigate the energy imbalance during the

phytoremediation process and the cytoplasmic content of ATP; while cytochrome *c* enhances respiration, allowing the efficient transportation of electrons from NADH through the respiratory chain (Brandt 2006). Besides, cytochrome *c* also plays an important role as an antioxidant enzyme in the mitochondria by removing O_2^- and H_2O_2 (Bowman and Bren 2008). Therefore, we propose both as important proteins in the maintenance of the machinery and production of vital energy for the *P. fasciculatum* leaf cells during exposition to Pb-stress.

In this way, the plant might be able to tolerate the inhibitory effects involved in these important primary biosynthetic pathway and demonstrate that *P. fasciculatum* is able to maintain the efficiency of photosynthesis and the energy supply necessary to allow it to grow even in the presence of Pb. A protective response, reinforced by the up-accumulation of proteins involved in antioxidant defense, metal sequestration and compartmentalization, protein defense and repair, stress response, and protein regulation, as seen below.

Ribosomal proteins: Into the first steps of a plant's tolerance or adaptation to an adverse environment, increased synthesis of ribosomal proteins is essential. In the case of heavy metal stress, protein regulation plays an important role in the adaptive response and regulates the balance between synthesis and degradation to achieve a balanced cellular response (Lan *et al.* 2018).

In this study, the differential accumulation of nine ribosomal proteins in response to Pb-induced stress was also identified, five of them were specifically up- and four down-regulated in *P. fasciculatum* leaves. These proteins participate in the assembly, regulation, and functioning of ribosomes and in the transcription and protein synthesis processes, which are crucial for adaptation to Pb-stress.

This type of change in the accumulation of ribosomal proteins has been previously described under heavy metal stress conditions. Thus, for example, the up-regulation of type L and S ribosomal proteins along with 32 others was observed by Xia *et al.* (2019) in two *Cannabis sativa* cultivars, Pb-tolerant, and Pb-accumulating plants. Since L and S proteins are mainly involved in the biosynthesis of multiple stress resistance proteins in plants (Yao *et al.* 2006), we propose them as vital components of the activation of metabolic pathways involved in the defense and response to Pb-stress in *P. fasciculatum*.

Antioxidant defense proteins: Several proteins related to the antioxidant defense and the stress response were also identified, which support the presence of other important protector mechanisms in the leaves of *P. fasciculatum* exposed to Pb-stress. One involves proteins related to the generation and direct effects of reactive oxygen species (ROS). ROS generation is one of the main adverse effects of plants when exposed to heavy metals stress (Apel and Hirt 2004). Heavy metals such as Pb have the ability to displace essential cations from specific enzymatic binding sites such as Fe and Cu that catalyze the Haber-Weiss/Fenton reactions ($Cu^+ \leftrightarrow Cu^{2+} + e^-$ and $Fe^{2+} \leftrightarrow Fe^{3+} + e^-$), in which H_2O_2 decomposes into hydroxyl radical (OH^\bullet)

(Fig. 3) (Sahi and Sharma 2005, Valko *et al.* 2006, Shahid *et al.* 2014). Heavy metals can also activate NADPH oxidase, this enzyme can use cytosolic NADPH to generate $O_2^{\cdot -}$, which is rapidly transformed into H_2O_2 by the action of SOD (Fig. 3) (Potocký *et al.* 2007, Pourrut *et al.* 2008, 2013, Weyemi and Dupuy 2012). Pb-induced ROS generation by NADPH oxidase was reported in treated *Vicia faba* (Pourrut *et al.* 2008). Plants have a ROS scavenging system under heavy metal stress conditions; this system includes antioxidant enzymes and amino acid-derived compounds such as glutathione, among others (Kosová *et al.* 2013).

In this study, seven proteins involved in the oxidative stress response were differentially up-regulated, some such as glutamine synthetase (TP30), Trx-M (TP50), and peroxidase 1 (Prx-1) were identified under both treatments. These enzymes were up-regulated 7.4-, 2.2-, and 1.9- (TP30), and 1.7-folds (TP50), respectively. Glutamine synthetase and Trx-M are important proteins in glutamine synthesis, important in protein biosynthesis and ROS removal; both located in the chloroplasts, the organelle with the greatest adverse effects due to Pb-stress in this study. Similarly, Prx-1 is important in the removal of H_2O_2 in the vacuoles, which was important in the process of detoxification and compartmentalization of Pb in *P. fasciculatum*. Probably one of the keys to the tolerance of *P. fasciculatum* to Pb stress was based on the differential accumulation of proteins that inhibited the massive generation of ROS, avoiding generalized damage of biomolecules and cell membranes, thus allowing plant survival.

Trx-M is an enzyme present in chloroplasts, this protein, together with Prx-Q, is fundamental in the detoxification of H_2O_2 chloroplasts, experimental data shows that Prx-Q can be oxidized by coupling with thiol group electron donors such as Trx-M in plants and *Corynebacterium glutamicum* (Dietz 2007, Su *et al.* 2018, Nikkanen and Rintamäki 2019). Studies on metal stress-tolerant plants, such as *Eichhornia crassipes*, *Pistia stratiotes*, *Microsorium pteropus*, *Brassica napus*, and *Cannabis sativa* observed this up-regulation of proteins involved in oxidative stress (Li *et al.* 2015, Farooq *et al.* 2018, Lan *et al.* 2018, Xia *et al.* 2019).

Protein folding, heat shock proteins (HSPs): Another defense mechanism was related to heat shock proteins (HSPs). HSPs are an important group of proteins that have the function of protecting other proteins from damage, repairing damaged proteins, or protecting nascent proteins during their transport. They have a specific role in the folding and degradation of proteins, acting on the misfolded proteins that have lost their normal assembly and function under abiotic stress conditions (Chen *et al.* 2018, Ge *et al.* 2012). HSPs can accumulate when plants are exposed to various abiotic stresses (Li *et al.* 2015). We know that the induction of HSP proteins is a form of protection, ecophysiological and genetic adaptation of the organism to adverse environments (Hasan *et al.* 2017). In the present study, four HSPs were identified in *P. fasciculatum* in response to Pb-stress: chaperonin CPN60

highly increased in TP30; while heat shock cognate 70 kDa protein 1 (HSP70), stromal 70 kDa heat shock-related protein (HSP70S), and heat shock cognate 70 kDa protein 2 (HSP70-2) were found more represented in the TP50 treatment (Table 1 Suppl.). Studies have shown that HSP gene expression can positively regulate the activity of antioxidant enzymes (Haq *et al.* 2019). In tobacco plants it has been observed that heat shock protein 16.9 (HSP16.9) increased catalase, superoxide dismutase, and peroxidase activities; likewise, 17.8 kDa class I heat shock protein (HSP17.8) in *Arabidopsis* increased superoxide dismutase activity (Driedonks *et al.* 2015). These investigations are similar to our results concerning the up-regulation of antioxidant proteins explained above. The HSPs in *P. fasciculatum* could play an important role in the up-regulation of Trx-M and Prx-1; in the repair of proteins such as RuBisCO LSU and the different chlorophyll *a/b* binding proteins (LHCPs) and other proteins involved in energy metabolism in mitochondria and cytosol (Fig. 3).

Proteins of detoxification and vacuolar compartmentalization:

Detoxification processes are essential for heavy metal stress tolerance in plants. In our case, the V-type H^+ ATPase subunit B2 and Ras-related proteins were found up-regulated in TP50 treatment. The first one is an important membrane transporter in eukaryotic cells that is involved in the detoxification of metals from the cytosol (Kabala *et al.* 2014, Baig *et al.* 2018). For this, the trans-tonoplast proton gradient generated by V-ATPase can be used as a driving force (cation diffusion facilitators and cation/proton exchangers) to remove harmful heavy metals from the cytoplasm (Singh *et al.* 2016). Similar studies in *Arabidopsis* seedlings and *Glycine max* have confirmed the significant role of V-ATPase in adaptation to Cd and Pb stress, respectively (Yang *et al.* 2016, Baig *et al.* 2018). Therefore, this protein could be involved in the sequestration and vacuolar compartmentalization of Pb in *P. fasciculatum* leaves, helping either the detoxification in the cytosol and minimizing its adverse effects on important biomolecules or cell organelles; hence, improving tolerance to this metal.

Other proteins that are also important in the detoxification processes, transport, and compartmentalization of heavy metals in vacuoles are Ras-related proteins (Goodwin and Sutter 2009), which demonstrated a great accumulation in all treatments in *P. fasciculatum*, especially in Ras-related protein RABA1e, Ras-related protein Rab7, and Ras-related protein Rab-2-B. This could explain the process of detoxification of Pb in the vacuoles of the *P. fasciculatum* leaves, in a similar way to those observed in *Arabidopsis thaliana* exposed to aluminium (Wang *et al.* 2013).

Within this group, it is also important to mention the plasma membrane ATPase (H^+ ATPase) protein. It is a proton pump that translocates positive charges out of the cytosol using ATP energy, participating in the transport of anions, protons, organic compounds, and nutrients (Zhang *et al.* 2017). Under stress conditions, an up-regulation of this H^+ ATPase produces an electrochemical gradient through the membrane that would be used for the passive transport of nutrients and to release ions and toxic

substances (Janicka-Russak 2016). In this way, H⁺ ATPase could contribute to the elimination of Pb⁺² from the cytosol and increase the assimilation of organic compounds such as glucose, improving the energy homeostasis of the cell (Fig. 3). Therefore, this protein could have also played a fundamental role in the growth and development of these plants, specifically the increase in biomass at 60 d of the treatments.

Proteins involved in the signal transduction:

Another protein group in the tolerance mechanisms of *P. fasciculatum* includes proteins related to signal transduction, which surely played an important role in the response to Pb-stress, due to their implications in the regulation of environmental adaptation and heavy metal detoxification processes.

These proteins are important in diverse plant developmental processes and tolerance to environmental stresses (Agarwal *et al.* 2009). Our data suggest that signal transduction pathways are activated to provide a series of tolerance mechanisms that are vital in *P. fasciculatum*, through specific proteins involved in the regulation of development and response to Pb-stress. In particular, two proteins of the 14-3-3 like family were up-accumulated: 14-3-3-like protein and 14-3-3-like protein GF14-C. Protein family 14-3-3 is activated under stress conditions at the cellular level, which allows the cells to modify cellular processes and metabolic pathways, such as defense, photosynthesis, and redox homeostasis (Cheng *et al.* 2010).

Proteins 14-3-3 are important in the activation of Ca²⁺-dependent protein kinases (CDPK) (in the same way as H₂O₂, which increases cytosolic Ca²⁺ by activating CDPK), which activate transcription factors that induce gene expression in response to metal stress (Fig. 3) (Price *et al.* 1994, Ludwig *et al.* 2004, Weckwerth *et al.* 2014). The 14-3-3 proteins can also activate ROS-mediated mitogen-activated protein kinase (MAPK), which are important in the removal of ROS by Cd-stress; they are also essential in the activation of H⁺ ATPase *via* phosphorylation (Fig. 3) (Cao *et al.* 2016, Falhof *et al.* 2016).

14-3-3 represents an important factor in the network of molecular mechanisms of tolerance in *P. fasciculatum*, these proteins interact with other cellular signalling molecules, proteins involved in protein folding and repair, energy metabolism, and antioxidant defense, which demonstrates its central role in the response of these plants to Pb-stress.

In conclusion, *P. fasciculatum* might be a Pb-phytoextractor and showed a capacity for Pb accumulation. Our proteomic results indicate resistance mechanisms in *P. fasciculatum* under Pb exposure. The proteins differentially expressed in leaves were mainly involved in photosynthesis, protein metabolism, saccharide and energy metabolism, and antioxidant and stress defense. Although a large number of proteins decreased, this plant was able to reach a physiological and biochemical balance to tolerate Pb stress and grow without exhibiting severe toxic effects. Our proteomics data support that some proteins, such as 14-3-3 proteins, HSPs, and oxidative stress defense

proteins such as peroxidase 1 and Trx-M, play a key role in Pb tolerance mechanisms. Proteins related to the transport and signal transduction were also crucial in the adaptation of *P. fasciculatum* to mining soils contaminated with Pb. Overall, our results demonstrate that this plant used several biosynthetic routes for sequestration, detoxification, and tolerance to Pb.

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