South African Journal of Botany 109 (2017) 42–49

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Enzymatic reduction of hydrogen peroxide on *Polypogon australis* plants grown in a copper mining liquid waste

D. Jara-Hermosilla [a], D. Barros-Vásquez [a], A. Muñoz-Rojas [a], S. Castro-Morales [b], C. Ortiz-Calderón [a,*]

[a] Laboratorio de Bioquímica Vegetal y Fitorremediación, Facultad de Química y Biología, Universidad de Santiago de Chile, Av. Libertador Bernardo O'Higgins 3362, Estación Central, Santiago, Chile
[b] Cedenna, Avda. Ecuador 3493, Estación Central, Santiago, Chile

ARTICLE INFO

Article history:
Received 24 March 2016
Received in revised form 6 July 2016
Accepted 13 December 2016
Available online xxxx

Edited by M Vaculik

Keywords:
Detoxifying enzymes
Heavy metals
Mining liquid waste
Hydrogen peroxide
Polypogon australis

ABSTRACT

This study was undertaken to characterize the status of H_2O_2-reducing enzyme activity in the metallophyte plant species *Polypogon australis* when treated with a mining liquid waste (MLW) derived from a copper mine. In order to determine the effect of the solubility of some metals present in the MLW the accumulation of the elements, the variation of H_2O_2 and lipoperoxidation levels, and their relationship with the H_2O_2 reduction activity was studied in *P. australis* plants at pH 5.1 (acidic MLW) and at pH 6.7 (neutral MLW) during two weeks. Plants treated with the acidic MLW showed an elevated copper and zinc accumulation in their tissues. Oxidation of lipids determined by the malondialdehyde (MDA) content increased during the first half hour in the roots of the treated plants, and remained constant to the end of the treatment. In leaves of the treated plants, MDA levels steadily increased throughout the treatment. H_2O_2 levels increased during the first 2 h of the treatment, then decreased and reached values close to that observed in untreated control plants. The increase of copper in the roots of the plants treated with the acidic MLW and the H_2O_2 levels detected in the tissues was correlated with the activity of H_2O_2-reducing enzymes. The results show that the treatment of the plants with the MLW but not the solubility of metals provokes an increase of the H_2O_2 content in the tissues and triggers the enzymatic control of H_2O_2 levels.

1. Introduction

Mining liquid wastes (MLW) contain high heavy metal concentrations and their pH ranges from 2.0 to 8.0. When not properly disposed of, these MLW can pollute soils and waterways causing environmental impact and human health problems (Fediuc and Erdei, 2002; Lopez Pamo et al., 2002). This effect is more dramatic when the solubility of the metal elements present in the residues increases, which normally occurs below pH 5.0 (Ginocchio et al., 2009). There are some plant species that are able to tolerate and even to remove metals from soil and water, accumulating the elements in their leaves and roots (Bonanno and Lo Giudice, 2010). However, a high intracellular concentration of metals in the tissues of the plants can cause cellular damage due to the increase of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) (Verma and Dubey, 2003; Dietz et al., 2006; Khatun et al., 2008; Quan et al., 2008; Posmyk et al., 2009; Adrees et al., 2015). The Fenton reaction triggered by Cu^{++} and Fe^{++} present in MLWs involves the decomposition of H_2O_2 and production of hydroxyl radicals, that turns out to be highly toxic, because it quickly oxidizes biomolecules such as proteins, lipids and nucleic acids (Turrens, 2003; Ricci et al., 2008). Even some cellular mechanisms to avoid the toxic effect of ROS, such as the activity of superoxide dismutase for $O_2^{\bullet -}$, leads to the generation and increase of cellular H_2O_2. Therefore, the accurate control of intracellular content of H_2O_2 in plant tissues that accumulate metals is vital for the tolerance and survival of the individuals.

Hydrogen peroxide is a molecule relatively stable under physiological conditions, and can be reduced into water and oxygen by enzymatic processes led by catalase (CAT) (Bienert et al., 2007). H_2O_2 plays an important role in of the response to various abiotic and biotic stresses (Matamoros et al., 2010). In maize, Tewari et al. (2004) reported H_2O_2 concentrations of 75 μmol/g FW with N deficiency. In response to ozone exposure, Chen and Gallie (2005) reported tobacco leaf H_2O_2 levels increased four times. Karpinski et al. (1997) reported an oxidative burst in *Arabidopsis* with exposure to excess irradiance that increased the leaf content of H_2O_2 from 5 μmol/g FW to about 7 μmol/g FW. Lately, Habiba et al. (2015) reported that H_2O_2 content could be increased with heavy metal toxicity in *Brassica napus* L. Additionally, cellular membranes have high permeability to H_2O_2, conferring the molecule a

Abbreviations: MLW, mining liquid waste; ROS, reactive oxygen species; CAT, catalases; APX, ascorbate peroxidases; GPX, glutathione peroxidases; G-POD, guaiacol peroxidases; PRX, peroxiredoxins; TRX-dp PRX, thioredoxin-dependent peroxiredoxins; GR, glutathione reductase; NADPH, nicotinamide adenine dinucleotide phosphate; MDA, malondialdeahyde; H_2O_2, hydrogen peroxide; $O_2^{\bullet -}$, superoxide radicals; $OH^{\bullet}$, hydroxyl radical; ICP-OES, inductively coupled plasma-optical emission spectrometry.

* Corresponding author at: Postal address Avenida Bernardo OHiggins 3363, Estacion Central, Santiago 9170022, Chile.
E-mail address: claudia.ortiz@usach.cl (C. Ortiz-Calderón).

http://dx.doi.org/10.1016/j.sajb.2016.12.017
0254-6299/

signaling function in cell communication (Dietz et al., 2006; Quan et al., 2008). The dual role of H_2O_2, as a reactive oxygen species and a messenger molecule, requires an efficient system to keep an adequate intracellular concentration of it to control its accumulation (Dietz et al., 2006; Quan et al., 2008). Plant cells possess a series of H_2O_2-reducing enzymes such as catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), guaiacol peroxidase (G-POD) and peroxiredoxins (PRX) (Dietz et al., 2006). The efficiency and specificity of the enzymatic system for H_2O_2 reduction can minimize the occurrence of the Fenton reaction and the subsequent ROS formation, allowing the cell to tolerate the effect of metal accumulation (Mittler, 2002; Dietz et al., 2006; Giorgio et al., 2007; Khatun et al., 2008).

Polypogon australis Brongn. (*Poaceae*) is a Chilean native and non-endemic grass. *P. australis* plants collected in a copper tailing dam in northern Chile showed a copper content of 670 mg kg^{-1} in leaves, and 223 mg kg^{-1} in roots (Ortiz et al., 2008). *P. australis* plants cultivated *in vitro* can grow in up to 626 μM of $CuSO_4$ and the plants are easily reproduced in hydroponics (Ortiz, personal observation). The plant's ability to grow naturally in sites polluted with copper might be related to an efficient H_2O_2-detoxifying enzymatic system. In this work, we studied the enzymatic system for H_2O_2 reduction in *P. australis* plants that accumulate copper and zinc in leaves and roots when treated with a mining liquid waste (MLW) adjusted to pH 6.7 (neutral MLW), and adjusted to pH 5.1 (acidic MLW).

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *Polypogon australis* plants collected from a copper processing mine located in the Third Region of Chile (27.3°S, 70.3°W), were used in this study. Seeds were soaked in distilled water for 24 h and then germinated for 3 d at 25 °C before being transferred at hydroponic trays for 3 months. Plants were grown in a nutrient solution (Phostrogen® 0.3 g/L) at pH 5.1 with a photoperiod of 14 h light and 10 h darkness, at 25 °C.

2.2. Plant treatments

Mine liquid wastes (MLW) were obtained from Codelco-El Teniente Mine, located 80 km south from Santiago de Chile. Ninety day old plants were transferred to a tray containing the MLW at pH 6.7 (neutral MLW) or at pH 5.1 (acidic MLW). Each hydroponic system contained 50 plants. Plant sampling was carried out at 30 min, 2 h, 12 h, 48 h and 336 h during the treatment. Roots and leaves were separated, frozen in liquid nitrogen immediately after harvesting, and were stored at −80 °C for further analysis. Control plants were maintained in the nutrient solution (0.3 g/L of Phostrogen®) at pH 5,1 and samples were taken at the same times than for the treatment.

2.3. Metal determinations

Metals in the MLW were determined by the method Agency, U.S.-E.P.A (1996a). The MLW was treated with *aqua regia* ($1HNO_3$:3HCl high purity) and the solution was kept at 85 °C for 1 h and then filtered through nitrocellulose membrane (0.45 μm). The most representative metals for the MLW (Cd, Cu, Pb and Zn) were detected by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, OPTIMA 2000 DV, Perkin Elmer). The sediment recovered after the filtration of the MLW was dried at 50 °C and then digested in *aqua regia* at 60 °C for 16 h. Two percent of HNO_3 was added to each sample and the solution was kept at 60 °C for 2 h and then filtered through nitrocellulose membrane before analysis.

Metals (Cd, Cu, Pb and Zn) were determined by ICP-OES in leaves and roots of treated and control plants. For the detection, 100 mg of the sample was dried at 45 °C until complete dryness, determined by a constant weight. Each sample was digested with HNO_3:H_2O_2:MilliQ water (5:1:5) in a microwave (MILESTONE© mls 1200 mega) for 15 min in a four-stage program (5 min at 250 Watts (W); 1 min at 0 W; 4 min at 400 W; 5 min at 570 W). The digested samples were diluted with MilliQ Water and filtered through a nitrocellulose membrane (0.45 μm) before analysis (Agency, U.S.-E.P.A, 1996b).

2.4. Hydrogen peroxide (H_2O_2) content

The H_2O_2 content was determined in shoots and roots with a reflectometer (RQflex®, Merck). 100 mg of root shoot tissues was weighed and then was dried and grinded separately with 1 mL of extraction buffer [50 mM sodium phosphate, 40 mM 3-amino triazole, at pH 7.0]. The samples were centrifuged at 13,000 rpm for 1 min at 4 °C and the supernatant was used for the determination of H_2O_2.

2.5. Malondialdehyde (MDA) content

The MDA content was determined in shoots and roots, using a spectrophotometer (SHIMADZU UV mini-1240) at $\lambda = 532$ nm and $\varepsilon = 155\ mM^{-1}\ cm^{-1}$ (Hodges et al., 1999). 100 mg of ground tissue was mixed with 1 mL of 1% trichloroacetic acid (TCA) and centrifuged at 12,500 rpm for 5 min at 4 °C. The supernatant was mixed with 1 mL of 0.5% thiobarbituric acid solution (20% in 1% TCA) and incubated at 95 °C for 30 min.

2.6. Protein extraction and quantification

100 mg of *P. australis* leaves and roots samples was homogenized in a protein extraction buffer (100 mM sodium phosphate, 1 mM EDTA, 2% PVP, 1 mM DTT, pH 7.0). The mix was centrifuged at 13,000 rpm for 20 min at 4 °C. Protein content was detected according to Bradford (1976) adding 25 μl of protein extract to a 1 ml of Bradford reagent. The mix was incubated at room temperature for 10 min previous absorbance measurement in a UV-spectrophotometer at $\lambda = 595$ nm.

2.7. H_2O_2-reducing enzyme activities

All reducing enzyme activities were determined using a UV-spectrophotometer (SHIMADZU UV mini-1240) at room temperature. Catalase (CAT) activity was determined at $\lambda = 240$ nm and for the activity calculation, the extinction molar coefficient $\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$ was used (Cakmak and Marschner, 1992). Ascorbate peroxidase (APX) activity was determined at $\lambda = 290$ nm and $\varepsilon = 2.8\ mM^{-1}$ cm (Nakano and Asada, 1981). Gluthathione peroxidase (GPX) activity was determined at $\lambda = 340$ nm and $\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$ (Posmyk et al., 2009). Guaiacol proxidase (G-POD) activity was measured at $\lambda = 470$ nm and $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$ according to Cakmak and Marschner (1992). Thioredoxins-dependent peroxiredoxin (TRX-dp-PRX) activity was determined at $\lambda = 340$ nm and $\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$ (Köning et al., 2002).

2.8. Statistical analysis

All data were processed by two-way repeated measures ANOVA. Significant differences were obtained through comparison with Tukey test using the two statistical packages Prism-GraphPad Software v. 6.0 and XLSTAT 2014.

3. Results

3.1. Metal content in the mining liquid waste (MLW)

The total content of metals in the MLW with pH 6.7 and pH 5.1 was analyzed in the soluble phase and in the sediment obtained after filtration. The results showed that both zinc and copper were the metals