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Favouring NO over H_2O_2 production will increase Pb tolerance in *Prosopis farcta* via altered primary metabolism

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ABSTRACT

Reactive oxygen species (ROS) and nitric oxide (NO) are known in triggering defense functions to detoxify heavy metal stresses. To investigate the relevance of ROS production, Pb treatment (400 μM) alone and in combination with 400 μM sodium ascorbate (Asc: as H_2O_2 scavenger) were given to hydroponically grown *Prosopis farcta* seedlings over a time course of 72 h. Data presented here indicate that, the low extent of H_2O_2 due to scavenging by ascorbate, together with high level of NO improved Pb + Asc- treated *Prosopis* growth. Following the evoked potential of both the signals, significant increases in phenolic acids; caffeic, ferulic and salicylic acid were observed with Pb treatment; which are consistent with observed increase in lignin content and consequently with growth inhibition. In contrast, Pb + Asc treatment induced more flavonoids (quercetin, kaempferol, luteolin), diminished phenolic acids contents and also lignin. Elicited expression rate of phenylalanine ammonia-lyase gene (*PAL*) and also its enzymatic activity verified the induced phenylpropanoid metabolism by Pb and Pb + Asc treatments. In comparison with Pb stress, Asc + Pb application induced the high expression of arginine decarboxylase gene (*ADC*), in polyamines biosynthesis pathway, and conducted the N flow towards polyamines and γ-amino butyric acid (GABA). Examining the impact on enzyme activities, catalase, and guaiacol peroxidase; Pb + Asc reduced activity but this increased ascorbate peroxidase, and aconitase activity. Our observations are consistent with conditions favouring NO production and reduced H_2O_2 can improve Pb tolerance via wide-ranging effects on a primary metabolic network.

1. Introduction

Lead (Pb), one of the most abundant globally distributed toxic elements, posing a significant risk to the health of humans, animals, and plants. At the whole-plant level, high concentration of Pb causes the disruption of physiological and biochemical processes like a decrease in photosynthesis, altered uptake of essential elements, inhibition of growth, lower biomass and yields (Ali et al., 2014; Arias et al., 2010). At the molecular level, Pb changes cell membrane permeability, reacts with active groups of different enzymes (for example, haem groups), reacts with phosphate groups of ADP or ATP (Pourrut et al., 2011). These results in negative effects are associated with oxidative damage to plant cell due to a compromised antioxidant defense machinery and the production of reactive oxygen species (ROS) (Verma and Dubey, 2003). However, despite their potential for causing harmful oxidations, it is now well established that ROS; and most often H_2O_2, may also function as signaling molecule (Zafari et al., 2016; Ali et al., 2014).

To cope with oxidative stress, plants have evolved two protective enzymatic and non-enzymatic mechanisms to detoxify ROS species. The former includes catalase (CAT: E.C.1.11.1.6.), ascorbate peroxidase (APX: E.C.1.11.1.11), guaiacol peroxidase (GPX: E.C.1.11.1.7) and the latter involves ascorbate and glutathione (Gill and Tuteja, 2010); which work in concert to detoxify ROS. The low-molecular-weight antioxidant ascorbate functions as redox buffer that reduces ROS. It is the well-known molecule in the detoxification of H_2O_2, particularly as a substrate of APX, and is fundamental component of the ascorbate-glutathione cycle, which is present in most cellular compartments (Smirnoff and Wheeler, 2000). Crucially, ascorbate can also acts as a metabolic interface to modulate the appropriate induction of acclimation responses (Foyer and Noctor, 2005).

Abbreviations: NO, nitric oxide; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; PAL, phenylalanine ammonia-lyase; Put, putrescine; Spd, spermidine; Spm, spermine; Asc, ascorbate; SL, Shoot length; RDIR, Relative dry weight increase rate; GR, growth rate; PCA, perchloric acid; DW, dry weight; FW, fresh weight; GABA, γ-Amino butyric acid; CAT, catalase; GPX, guaiacol peroxidase; APX, ascorbate peroxidase

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ROS and potentially ascorbate contribute to mechanisms that allow plants to withstand abiotic stresses that could affect their vigour and survival (Mittler, 2017; Huang et al., 2010). In response to metals exposure, plants accumulate different metabolites to concentrations in the millimolar range, particularly phenolic and nitrogenous compounds such as amino acids and polyamines (Sharma and Dietz, 2006). Plant phenolics contribute to ROS quenching and are considered as parts of defensive mechanism (Dǔcić et al., 2008). Crucially phenylalanine ammonia-lyase (*PAL*) gene expression is inducible by ROS (Lin et al., 2005), and in heavy metal-treated plants leads to phenolic acid accumulation (Kováčik et al., 2009). Also, the accumulation of some amino acids such as proline may help in ameliorating of negative consequences of metal toxicity (Kováčik et al., 2010). These solutes are also sources of carbon and nitrogen during environmental challenges (Dubay and Pessarakli, 1995).

Polyamines including putrescine (Put), spermidine (Spd) and spermine (Spm) influence various processes in controlling plant growth and development. Due to the presence of positively charged groups, the interaction of polyamines with proteins, nucleic acids, membrane phospholipids, and cell wall constituents can activate or stabilize these molecules. As such polyamines contribute significantly in enhancing plant defense strategies in response to abiotic stresses including heavy metal (Groppa and Benavides, 2008).

Along with H_2O_2, the free radical NO has gained special interest in plant signaling pathways controlling processes that range from biotic and abiotic stress responses to growth and development (Mur et al., 2012). Numerous studies have reported NO and H_2O_2 induce profound changes in the expression, enzymes activities and metabolite levels in phenylpropanoid and nitrogen pathways employed as tolerance mechanisms (Gao et al., 2009; Iqbal et al., 2014). Similarly, we have recently shown that NO acts as a signal molecule mediating Pb-induced stress tolerance in *Prosopis farcta*; a perennial trees/shrub species well known for their resistance to heavy metals (Zafari et al., 2016). However, it is likely that the *Prosopis* response is not triggered by NO alone but is the result of a cross-talk between different signals and metabolic pathways, especially ROS-dependent ones. This current study applied ascorbic acid as a H_2O_2 scavenger to establish that NO and H_2O_2 interact to regulate metabolome changes in *Prosopis* conducting to Pb tolerance strategies.

2. Material and method

2.1. Plant culture and treatments

Prosopis farcta L. seeds were scarified with 98% sulphuric acid and sterilized in a solution of sodium hypochlorite (2% w/v), and then thoroughly rinsed in distilled water. Seeds were incubated at 25 °C for 3 days to germinate. Germinated seeds were then transferred into plastic containers with 2.5 dm^3 of half strength Hoagland nutrient solution (pH 6). Plants were kept at 27 (light) /22 °C (dark) with a 16 h light photoperiod (200 μmol m^{-2} s^{-1}) and 60–80% air humidity. After 21 days, uniform seedlings were selected and exposed to various treatment solutions: (1) Hoagland solution (Control); (2) Hoagland solution +400 μM lead (Pb in the form of Pb $(C_2H_3CO_2)_2$); (3) Hoagland solution +400 μM Pb +400 μM Sodium ascorbate (Pb + Asc); (4) Hoagland solution +400 μM Sodium ascorbate (Asc). After that, the shoots at selected time points: 0 (i.e. start treatment), 6, 12, 24, 48, 72 h after treatment were rinsed with distilled water and used to assay the following parameters.

2.2. Determination of Pb content

The dried samples were burnt to ash at 500 °C for 6 h and then dissolved with 0.1 M HCl. Pb was analyzed in this acid extract according to the method described by Camacho- Cristóbal and González-Fontes (2002). Pb was measured using an atomic absorption spectrometer (Shimadzu AA-6709).

2.3. Ascorbate assay

One gram of fresh leaves was ground in liquid nitrogen and extracted with 4 mL of 6% ice-cold metaphosphoric acid containing 1 mM EDTA, centrifuged at 20 000 × *g* for 15 min, and the supernatant was used for the assay. Total ascorbate and reduced ascorbate were estimated according to De Pinto et al. (1999). Total ascorbate was determined by incubating samples with 1 mM dithiothreitol. A standard curve for Asc was used.

2.4. Assays of enzyme activities

Liquid N frozen shoots (0.2g) were extracted in 50 mM potassium phosphate buffer (pH 7.0) containing EDTA and polyvinylpyrrolidone. The homogenate was centrifuged at 12 000 × *g* for 20 min at 4 °C and then the supernatant was used for measuring the following enzymes activities.

CAT activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm for 1 min by Cakmak and Marschner (1992). GPX was based on the determination of guaiacol oxidation at 470 nm by H_2O_2 following the method of Lin and Wang (2002). APX was measured at 290 nm according to Nakano and Asada (1981), based on the decrease in absorbance of ascorbic acid within 1 min. The enzymatic activity of aconitase (E.C.4.2.1.3) was monitored photometrically at 240 nm using the protocol reported by Racker (1950). PAL was assayed by the method described by the phenolic acids determination in this paper. One unit of PAL activity was defined as the amount of cinnamic acid (CA) produced at 300 nm in 1 h under the specified conditions (Wakabayashi et al., 1997). Protein concentration was estimated by the method of Bradford (1976), with bovine serum as the standard.

2.5. Determination of NO content and H_2O_2

NO generation quantified by determination of nitrite concentration in vivo using Griess reagent. Shoot tissue (0.6g) was ground in a mortar and pestle in 3 mL of 100 mM cool PO_4^{3-} buffer (pH). The homogenates were centrifuged at 10 000 × *g* for 15 min at 4 °C. Samples (0.2 mL) were then incubated with 1.8 mL of PO_4^{3-} buffer and 0.2 mL of Griess reagent (1% sulfanilamide and 0.1% N-1- naphthylethylenediamine dihydrochloride in 5% phosphoric acid solution) at room temperature for 10 min (Kaur et al., 2015). Absorbance of the reaction mixture was read at 540 nm and concentration of NO determined from a calibration curve prepared using sodium nitrite as standard.

To determine H_2O_2 concentration, shoot tissue (100 mg) was extracted with 5 mL trichloroacetic acid (TCA; 0.1%, w/v) in an ice bath and centrifuged at 12 000 × *g* for 15 min (Velikova et al., 2000). An aliquot (0.5 mL) of supernatant was added to 0.5 mL of phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The absorbance of the mixture was read at 390 nm·H_2O_2 content was determined using the extinction coefficient 0.28 M^{-1} cm^{-1} and amount expressed as nmol g^{-1} fresh weight (FW).

2.6. Chromatographic separation of nitrogenous metabolites (polyamines and amino acids)

To extract polyamines, 200 mg FW of *Prosopis* shoots were homogenized with 5% (v/v) perchloric acid (PCA) and centrifuged at 20 000 × *g* for 30 min at 4 °C (Sharma and Rajam, 1995). Aliquots (0.2 mL) of supernatant was dansylated, toluene-extracted and analyzed by HPLC as described by Torrigiani et al. (1995) with a reverse phase C18 column (Perfectsil Target ODS-3 (5 mm), 250 × 4.6 mm; MZ Analysentechnik, Mainz, Germany). The elution was composed of acetonitrile (A) and water (B) and facilitated by gradient system as