Environmental and Experimental Botany 141 (2017) 41–49

Contents lists available at ScienceDirect

Environmental and Experimental Botany

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

Nitric oxide production shifts metabolic pathways toward lignification to alleviate Pb stress in *Prosopis farcta*

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ARTICLE INFO

Keywords:
Nitric oxide
Phenolic compounds
Polyamines
NO synthase
Nitrate reductase
Prosopis farcta

ABSTRACT

The mechanisms through which nitric oxide (NO) can influence plant tolerance to Pb are unknown, but may be based on the accumulation of secondary metabolites such as phenolic compounds and polyamines (PAs). This hypothesis was tested by exposing hydroponically-cultured *Prosopis farcta* to Pb alone and in combination with sodium nitroprusside (SNP; NO donor), L-NAME (NO synthase inhibitor) and tungstate (TUN; nitrate reductase inhibitor) for a time course of 72 h. The inhibitors strongly repressed NO generation, verifying that Pb and Pb + SNP-induced NO is probably mediated by nitrite and arginine-dependent pathways. The results show that the Pb-induced inhibition of growth was alleviated through application of NO donor, which decreased Pb accumulation in the plant samples, but deteriorated with the decrease in NO, which indicates a higher level of Pb absorbed.

The elevated contents of phenolic acids under Pb and Pb + SNP treatments was reduced by the NOS and NR inhibitors and was significantly associated with phenylalanine ammonia-lyase (*PAL*) gene expression patterns. The arginine decarboxylase (*ADC*) gene in the PAs biosynthesis pathway remained unchanged. Considerable alteration was observed in the conjugated PAs in response to the Pb and Pb + SNP applications when compared with the NR and NOS inhibitors that exhibited an increase in free PAs. The results suggest that Pb-evoked NO promotes homeostasis of metabolic pathways to phenolic acids and conjugated PAs that enhance lignification to strengthen *P. farcta* against stress.

1. Introduction

Lead (Pb) is one of the most abundant globally-distributed toxic elements. It poses a significant risk to the health of humans, animals and plants (Adhikari et al., 2001). At the whole-plant level, high concentrations of Pb disrupt physiological and biochemical processes and can decrease photosynthesis, alter uptake of essential elements, inhibit growth and lower biomass and yield (Ali et al., 2014; Arias et al., 2010). At the molecular level, Pb alters cell membrane permeability, reacts with active groups of enzymes (for example, haem groups), reacts with phosphate groups of ADP or ATP and replaces important ions such as Mg and Fe (Pourrut et al., 2011). These changes result in the negative effect associated with oxidative damage to plant cells from compromised antioxidant defense mechanisms and the production and accumulation of reactive oxygen species (ROS) (Verma and Dubey, 2003), in particular hydrogen peroxide (H_2O_2) (Ali et al., 2014; Zafari et al., 2016). The mechanism of Pb tolerance is still not well understood.

Plants have developed exquisite mechanisms to cope with a vast array of abiotic stresses that affect their vigor and survival. Upon exposure to metals, plants often synthesize a diverse set of metabolites that accumulate in concentrations in the millimolar range, particularly phenolic compounds and polyamines (PAs) (Sharma and Dietz, 2006; Groppa and Benavides, 2008). Plant phenolics contribute to ROS quenching and are part of the plant defense mechanism (Dŭcić et al., 2008). The authors have previously reported that the application of 400 μM Pb elicited increased phenylalanine ammonia-lyase (PAL) activity; a rate-limiting step on the phenylpropanoid pathway leading to biosynthesis of phenolic acids and flavonoids (Zafari et al., 2016).

PAs, including putrescine (Put), spermidine (Spd) and spermine (Spm) play integral roles in plant response to biotic and abiotic stress (Zeier, 2013). PAs can be covalently conjugated to small molecules like phenolic acids to form conjugated PAs (Mutlu and Bozcuk, 2007). As

Abbreviations: NO, nitric oxide; PA, polyamine; SNP, sodium nitroprusside; NOS, NO synthase; NR, nitrate reductase; PAL, phenylalanine ammonia-lyase; ADC, arginine decarboxylase; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; Put, putrescine; Spd, spermidine; Spm, spermine; L-NAME, NG-nitro-L-arginine-methyl ester; TUN, tungstate; SL, shoot length; RDIR, relative dry weight increase rate; GR, growth rate; PCA, perchloric acid; RT-qPCR, quantitative real-time polymerase chain reaction; DW, dry weight; FW, fresh weight

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http://dx.doi.org/10.1016/j.envexpbot.2017.06.011
Received 23 April 2017; Received in revised form 26 June 2017; Accepted 27 June 2017
Available online 29 June 2017
0098-8472/

PAs are involved in multiple metabolic pathways, their homeostasis must be regulated. Put can be synthesized directly from ornithine by ornithine decarboxylase or from arginine (Arg) through several biosynthetic steps that involve arginine decarboxylase (ADC) (Takahashi and Kakehi, 2010). Data to date indicates that *ADC* is an important stress-responsive gene (Wang et al., 2007). The polyamine metabolic pathway is deeply linked to other metabolic routes involved in the formation of several signaling molecules, such as NO (Gao et al., 2009) and it may be through NO that PAs confer Pb tolerance.

The free radical NO has been increasingly shown to be involved in a number of signaling pathways controlling processes that range from biotic and abiotic stress responses to growth and development (Mur et al., 2012). In plants, endogenous NO can be generated along two arginine-dependent and nitrate/nitrite-dependent pathways which are catalyzed by nitric oxide synthase (NOS) and nitrate reductase (NR) enzymes, respectively. NO generation by NOS has been reported primarily in animals and NOS-like activity has been detected in plants (Crawford, 2006). Inhibitor experiments using NG-nitro-L-arginine-methyl ester (L-NAME; an NOS inhibitor) and tungstate (TUN; an NR inhibitor) have also been shown to inhibit NO generation in plants, suggesting the existence of NOS and NR enzymes for NO production in plant species (Neill et al., 2003).

Indirect approaches such as the exogenous application of NO provide efficient protection for plants against the toxicity of heavy metals like copper (Yu et al., 2005) and cadmium (Singh et al., 2008). Several studies have reported that NO induces major changes on the phenylpropanoid pathway to confer tolerance mechanisms against heavy metals. NO production has been observed in response to exogenously-applied PAs (Tun et al., 2006) and the latter may directly converted to NO through arginine, a common precursor in their proposed biosynthetic routes (Filippou et al., 2013). Alteration in the homeostasis of PA could affect NO bioavailability and vice versa. Few studies have focused on the interaction of PAs and NO, so their many potential links must be verified (Yamasaki and Cohen, 2006).

The authors have recently shown that NO acts as a signal molecule mediating Pb-induced stress tolerance (Zafari et al., 2016) in *Prosopis farcta*, a deep rooting plant species that can tolerate severe heavy metal contamination. This species is a good model for elucidating Pb tolerance mechanisms; however the origins of the NO responsible and its role in triggering other defense mechanisms in response to Pb remain unclear. To further elucidate the role of NO as an upstream regulator of metabolic responses to Pb, the current study used a NO donor (SNP), NOS inhibitor (L-NAME) and NR inhibitor (TUN) to determine the cross-talk between the Pb-induced NO generation and metabolite responses in *P. farcta*.

2. Materials and method

2.1. Plant culture and treatment

Prosopis farcta L. seeds were scarified with 98% sulfuric acid and sterilized in a sodium hypochlorite solution (2% w/v), then thoroughly rinsed with distilled water. The seeds were incubated at 25 °C for 3 days to germinate. The germinated seeds were then transferred into containers with 2.5 l of half strength Hoagland nutrient solution (pH 6). The plants were kept at 22/27 °C (day/night) 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 the various treatment solutions: (1) Hoagland solution (control; Ctr); (2) Hoagland solution + 400 μM lead (Pb in the form of $Pb(C_2H_3CO_2)_2$); (3) Hoagland solution + 400 μM Pb + 200 μM SNP; (4) Hoagland solution + 400 μM Pb + 200 μM L-NAME; (5) Hoagland solution + 400 μM Pb + 200 μM sodium tungstate (TUN). The shoots were removed at selected time-points (i.e. 0 (onset of treatment) and 12, 24, 48 and 72 h of treatment), 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. The ashes were then dissolved in 0.1 M HCl. Pb was analyzed in this acid extract according to the method described by Camacho-Cristóbal and Gonzólez-Fontes (2002). Pb was measured using an atomic absorption spectrometer (Shimadzu AA-6709).

2.3. Determination of lignin

Lignin content was measured via acetyl bromide procedure adapted from Iiyama and Wallis (1988). In brief, 8 mg of fine-powdered, air-dried cell wall was treated with a mixture of 25% acetyl bromide (v/v in glacial acetic acid) and 0.1 ml of 70% HClO4 and incubated at 70 °C for 30 min. Samples were rapidly cooled in ice, and mixed with NaOH and glacial acetic acid. The lignin content was determined by measuring absorbance at 280 nm.

2.4. Growth parameters

2.4.1. Shoot length (SL)

All samples were randomly taken from individual plants with a comparable growth rate. At least 6 replicates per treatment were used to obtain shoot length after 72 h of treatment (Liu et al., 2004).

2.4.2. Relative dry weight increase rate (RDIR)

The growth rate (GR) was measured using the following equation:

$GR(\%) = \frac{Wa - Wb}{Wb} \times 100$, Where Wa and Wb are the shoot dry weight at 72 h and 0 h of treatment, respectively. The calculation of RDIR under different treatments was determined using the following equation (Liu et al., 2004):

$RDIR\ (\%) = \frac{GRoftreatment}{GRofcontrol} \times 100.$

2.5. Determination of NO content

NO generation quantified by determination of nitrite concentration *in vivo* using Griess reagent. Shoot tissue (0.6 g) was ground in a mortar and pestle in 3 ml of 100 mM cool PO_4^{3-} buffer (pH 7.0). 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- napthylethylenediamine 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.

2.6. Determination of phenolic acid

For phenolic acid quantification, samples (0.2 g) were homogenized in methanol (three times). The extracts were pooled and evaporated to dryness. The residues were suspended in acetonitrile and washed with hexane twice, then were evaporated again (Owen et al., 2003). The final extract was dissolved in methanol for phenolic acid separation by HPLC (Agilent Technologies; 1260 Infinity, USA) with a C18 column (250 × 4.6 mm ID; 5 μm; Alltech). The elution solvent was composed of 2% acetic acid aqueous solution (A%) and methanol (B%) with a gradient program as followed: 0–2 min: A (95); 2–10 min: A (75); 10–20 min: A (60); 20–30 min: A (50); 30–40 min: A (0); 40–50.0 min: A (95) (Owen et al., 2003). Standard phenolic acids (cinnamic, ferulic and caffeic acids) were purchased from Sigma-Aldrich (Germany). The detection wavelengths of the phenolic acids were set at 278–300 nm using a UV dual-array detector. The flow rate was 1 ml/min.