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Nitrate- and nitric oxide-induced plant growth in pea seedlings is linked to antioxidative metabolism and the ABA/GA balance

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ABSTRACT

This study looks at the effects of potassium nitrate (KNO_3) and sodium nitroprusside (SNP), a nitric oxide (NO)-donor, on the development, antioxidant defences and on the abscisic acid (ABA) and gibberellin (GA) levels in pea seedlings. Results show that 10 mM KNO_3 and 50 μM SNP stimulate seedling fresh weight (FW), although this effect is not reverted by the action of 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a NO-scavenger.

The KNO_3 treatment increased peroxidase (POX) and ascorbate oxidase (AOX) activities. SNP, on the other hand, reduced monodehydroascorbate reductase (MDHAR) activity and produced a significant increase in superoxide dismutase (SOD), POX and AOX activities. The "KNO_3 plus cPTIO" treatment increased ascorbate peroxidase (APX), MDHAR, glutathione reductase (GR) and SOD activities, but POX activity decreased in relation to the KNO_3 treatment. The "SNP plus cPTIO" treatment increased APX and MDHAR activities, whereas a huge decrease in POX activity occurred. Both the KNO_3 and the SNP treatments increased reduced ascorbate (ASC) concentrations, which reached control values in the presence of cPTIO. All treatments increased the dehydroascorbate (DHA) level in pea seedlings, leading to a decrease in the redox state of ascorbate. In the "KNO_3 plus cPTIO" treatment, an increase in the redox state of ascorbate was observed. Glutathione contents, however, were higher in the presence of SNP than in the presence of KNO_3. In addition, KNO_3 produced an accumulation of oxidised glutathione (GSSG), especially in the presence of cPTIO, leading to a decrease in the redox state of glutathione. The effect of SNP on reduced glutathione (GSH) levels was reverted by cPTIO, suggesting that NO has a direct effect on GSH biosynthesis or turnover.

Both the KNO_3 and SNP treatments produced an increase in GA4 and a decrease in ABA concentrations, and this effect was reverted in the presence of the NO-scavenger. Globally, the results suggest a relationship between antioxidant metabolism and the ABA/GA balance during early seedling growth in pea. The results also suggest a role for KNO_3 and NO in the modulation of GA4 and ABA levels and antioxidant metabolism in pea seedlings. Furthermore, this effect correlated with an increase in the biomass of the pea seedlings.

1. Introduction

Seed priming techniques are widely used to enhance seed vigour in order to increase germination rates and tolerance to environmental stresses (Paparella et al., 2015). Priming agents could act as signalling molecules that regulate plant development and induce plant defence responses (Calvo et al., 2014).

Different authors have reported the involvement of reactive oxygen species (ROS) in seed germination. The scientific literature contains a plethora of works regarding the positive effect of ROS [superoxide radicals ($O_2\cdot^-$), hydrogen peroxide (H_2O_2) hydroxyl radicals ($\cdot OH$)] on seed germination and early seedling growth in many plant species (Barba-Espin et al., 2011; Diaz-Vivancos et al., 2013a; Gomes and Garcia, 2013; Ishibashi et al., 2015; Wojtyla et al., 2016). As soon as the germination process begins, the activation of the metabolism can overproduce ROS, mainly in mitochondria, peroxisomes and the NADPH oxidases (El-Maarouf-Bouteau and Bailly, 2008). In order to regulate ROS production, antioxidant mechanisms are of pivotal importance for successful germination and optimal seed viability (Bailly et al., 2008; de Gara et al., 1997).

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Reactive nitrogen species, such as nitric oxide (NO), in addition to different nitrogen-containing compounds, including nitrite, nitrate and sodium nitroprusside (SNP, a NO-generating compound), have also been associated with seed dormancy breaking and with the germination process (Bethke et al., 2006; Diaz-Vivancos et al., 2013a). Potassium nitrate (KNO_3) has been used for seed priming and breaking seed dormancy in different plant species, such as tomato (Lara et al., 2014), maize (Anosheh et al., 2011) and Arabidopsis (Bethke et al., 2006; Matakiadis et al., 2009). In tomato seeds, KNO_3 treatment was found to reduce the germination time and increase the germination rate. Furthermore, this response paralleled increased protein concentrations and nitrate reductase, SOD and catalase activities (Lara et al., 2014). In Arabidopsis, the seed dormancy breaking induced by nitrate was correlated with the decrease in ABA levels (Matakiadis et al., 2009).

The ability of SNP, a NO-donor compound, to break seed dormancy in different plant species suggests a role for NO during seed germination and/or seed dormancy breaking (Beligni and Lamattina, 2000; Bethke et al., 2006). Low SNP concentrations (ranging from 10^{-4}–10^{-8} M) have been found to increase seed germination and plant growth in tomato. This effect has also been linked with increases in the activity of some antioxidant enzymes (POX, SOD, CAT) (Hayat et al., 2012). Furthermore, the effect of NO on the promotion of seedling growth has been linked to the activation of ABA catabolic enzymes (Bethke et al., 2006) and to the activation of exo- and endo-β-D-glucanase activity in the cell wall, favouring cell wall loosening and increased extensibility (Terasaki et al., 2001).

On the other hand, some works have shown that the effect of SNP on seed germination and/or seed dormancy breaking seems to be *via* cyanide (CN) production and not *via* NO generation (Bethke et al., 2006). This effect appears to be dependent on the presence or absence of light. Bethke et al. (2006), for instance, showed that SNP reduced dormancy in Arabidopsis when seeds were imbibed in the presence of light but not in the dark, because SNP can undergo photolysis in the presence of light, which triggers NO release (Feelisch, 1998). Nitrogen-containing compounds with a similar structure to SNP, such as potassium ferrocyanide and potassium ferricyanide, have been found to increase the germination rate of dormant Arabidopsis seeds in a similar way to SNP vapours, and these compounds were able to release volatile CN (Bethke et al., 2006). The effect of SNP could therefore also be due to the production of volatile CN. However, the loss of dormancy in Arabidopsis seeds induced by exogenous SNP, CN, nitrite or nitrate was inhibited by the effect of 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), an efficient NO-scavenger in biological systems. This fact suggests that in all cases NO must be produced after treatment with the above-mentioned compounds (Bethke et al., 2006).

In this work, we studied the effect of some nitrogen-containing compounds, such as KNO_3 and SNP, in the presence and absence of cPTIO, on the germination process and early seedling growth in pea seeds. In addition, we analysed the effect of these treatments on the antioxidant defences and the ABA and GA levels in pea seedlings in order to study a possible interplay among KNO_3/NO, antioxidant metabolism and plant hormones during the germination process and early seedling growth.

2. Material and methods

2.1. Plant material

We used pea seeds (*Pisum sativum* cv. Lincoln) purchased at Ramiro Arnedo S.A. (Murcia, Spain).

2.2. Germination

Pea seeds were first imbibed in dH_2O for 24 h. The seeds were then washed two times with dH_2O and placed in Petri dishes with a 15-cm diameter (2 Petri dishes per treatment, and 20 seeds/Petri dish) with two layers of filter paper moistened with dH2O (7 mL) or with different KNO_3 (0.5–30 mM) or SNP (10, 50, 100 μM) concentrations in the presence or absence of 200 μM cPTIO. Seeds were incubated at 25 °C for 72 h in darkness in a Cooled Incubator (MIR-153 Sanyo, Osaka, Japan). After this period, the length and weight of the seedlings was measured.

2.3. Enzyme extraction and assays

All operations were performed at 0–4 °C. After 72 h of growth, pea seedlings devoid of cotyledons were used for analyses. Samples (about 1 *g* fresh weight) were homogenised with an extraction medium (1/2, w/v) containing 50 mM Tris-acetate buffer (pH 6.0), 0.1 mM EDTA, 2 mM cysteine, and 0.2% (v/v) Triton X-100. For the APX activity, 20 mM sodium ascorbate was added to the extraction buffer. The extracts were centrifuged at 10,000 *g* for 15 min. The supernatant fraction was filtered on Sephadex G-25 NAP columns equilibrated with the same buffer used for the homogenisation. For the APX activity, 2 mM sodium ascorbate was added to the equilibration buffer.

The activities of the ASC-GSH cycle enzymes, POX, CAT, and SOD, were assayed as previously described (Diaz-Vivancos et al., 2013b). AAO was analysed by monitoring the oxidation of ASC at 290 nm (Barba-Espin et al., 2010).

2.4. Ascorbate and glutathione analyses

Pea seedlings devoid of cotyledons (four replicates per treatment) were snap-frozen in liquid nitrogen and then ground to a fine powder and extracted in 1 mL of 1 M $HClO_4$, in the presence of 1 mM EDTA and 1% PVPP (w/v). Homogenates were centrifuged at 12,000 *g* for 10 min. The supernatant was neutralised with 5 M K_2CO_3 to pH 5.5–6. The homogenate was centrifuged at 12,000 *g* for 1 min to remove the $KClO_4$ precipitate. The supernatant obtained was used to determine ascorbate and glutathione content (Diaz-Vivancos et al., 2013b). Reduced ascorbate was measured by the change in absorption at 265 nm, where ascorbate was determined *via* oxidation to DHA in the presence of ascorbate oxidase (Pellny et al., 2009). Glutathione concentrations (GSH, GSSG) were analysed using dithio-bis-2- nitrobenzoic acid and glutathione reductase in the presence of NADPH (Pellny et al., 2009).

2.5. Quantification of plant hormones

Hormone analysis was carried out in pea seedlings devoid of cotyledons. After 72 h of germination, pea seedling samples (about 100 mg/dry weight) were weighed, placed in a polypropylene tube and dipped in liquid nitrogen. The samples were then lyophilised. Samples were suspended in 80% methanol-1% acetic acid containing internal standards and mixed by shaking for one hour at 4 °C. The extract was kept at -20 °C overnight and then centrifuged, and the supernatant was dried in a vacuum evaporator. The dry residue was dissolved in 1% acetic acid and passed through an Oasis HLB (reverse phase) column as described in Seo et al. (2011). For GA and ABA quantification, the dried eluate was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an auto-sampler and reverse phase UHPLC chromatography (2.6 μm Accucore RP-MS column, 50 mm length × 2.1 mm i.d.; ThermoFisher Scientific) with a 5–50% acetonitrile gradient containing 0.05% acetic acid, at 400 μL/min over 14 min.

The hormones were analysed with a Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific) by targeted Selected Ion Monitoring (SIM). The concentrations of hormones in the extracts were determined using embedded calibration curves and the Xcalibur 2.2 SP1 build 48 and TraceFinder programs. The deuterium-labelled hormones were the internal standards for quantification of each of the different plant hormones.