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).

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 25ºC 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 0-4ºC. 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 10000 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 12000 g for 10 min. The supernatant was neutralised with 5 M K_2CO_3 to pH 5.5–6. The homogenate was centrifuged at 12000 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 4ºC. The extract was kept at -20ºC 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 x 2.1 mm i.d.; ThermoFisher Scientific) with a 5 to 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.

2.6. Statistical Analysis

The data were analysed by two-way ANOVA using the SPSS Statistic 23 software. Treatment means were separated with Tukey's Multiple Range Test ($p \leq 0.05$). Also, a correlation matrix was performed, using Statgraphics Centurion Software, which provides a very fast and simple way to confirm whether the variables studied are correlated or not.

3. Results

In a preliminary experiment, we studied the effect of different KNO_3 concentrations on the germination rate and early seedling growth in pea seeds. The incubation of water-imbibed pea seeds in the presence of KNO_3 levels higher than 40 mM reduced plant growth (data not shown). As a result, we assayed lower KNO_3 levels. Concentrations of 0.5-1 mM do not have a significant effect on seedling growth. However, 10 mM KNO_3 significantly increased the seedling fresh weight (FW), whereas no effect was produced in the presence of 30 mM KNO_3 (Fig. 1). The increase in FW induced by 10 mM ranged from 14 to 35%, depending on the experiment. In contrast, 10 mM KNO_3 produced no significant effect on seedling length, but 30 mM KNO_3 produced a 27% decrease in this parameter (Fig. 1). On the other hand, the above-mentioned KNO3 concentrations did not have an effect on the germination rate.

In further experiments, we also studied the effect of SNP, a NO donor, on germination rate and seedling growth. The SNP experiments were carried out in the presence or absence of 200 μM cPTIO, a NO-scavenger. Both 10 mM KNO_3 and 50 μM SNP significantly increased seedling FW (Fig. 2A), as also occurred in control and KNO_3-treated seeds in the presence of 200 μM cPTIO. However, although the FW of seedlings treated with 50 μM SNP in the presence of cPTIO increased by up to 11%, the

plants later partially recovered control values (Fig. 2A). Moreover, 100 µM SNP did not affect seedling growth either in the presence or absence of cPTIO.

Both in the presence and absence of cPTIO, 10 mM KNO_3, caused a significant increase in seedling length. Moreover, incubation with 50 µM SNP plus cPTIO had the same effect, but the 50 µM SNP treatment alone did not (Fig. 2B).

3.1. Antioxidative metabolism

According to ANOVA analysis, all of the ASC-GSH cycle enzymes were significantly affected by the treatment with cPTIO but not by the treatments with KNO_3 or SNP alone, except MDHAR. Furthermore, no interaction between both factors (Treatments*cPTIO) was observed. AOX activity was only significantly affected by the KNO_3 and SNP treatments. In contrast, SOD and POX activities were significantly affected by the treatments with the priming agents KNO_3 and SNP and by the treatments with the NO-scavenger (cPTIO). Furthermore, an interaction between both factors was observed (Table 1).

The antioxidant enzymes that KNO_3 had the greatest effect on were POX and AOX, whose levels increased 4.5-fold and 1.5-fold, respectively (Table 1). SNP treatment also produced significant increases in SOD activity and, especially, in POX and AOX activity (nearly 3-fold), although a significant decrease (up to 38%) was observed in MDHAR activity (Table 1). The treatment with the NO-scavenger produced an increase in APX activity in controls (66%) as well as in KNO_3-and SNP-treated seeds (96% and 85%, respectively) (Table 1). A similar pattern occurred in MDHAR activity, which increased between 37% and 49% in controls and KNO_3-treated seeds and 2-fold in seeds treated with SNP (Table 1). The treatment with cPTIO also increased GR activity in KNO_3-treated seeds and AOX activity in SNP-primed seeds. Finally, cPTIO induced a dramatic decrease in POX activity in both seeds primed with SNP and, especially, KNO_3 (Table 1).

3.2. Ascorbate and Glutathione contents

The treatments with the priming agents produced a significant effect on ASC, DHA and GSSG levels. The treatment with the NO-scavenger cPTIO produced significant effects on ASC, DHA, GSH and total glutathione contents, and an interaction between both factors occurred for ASC, DHA, GSH and total glutathione levels (Table 2).

The treatment with KNO_3 strongly increased the reduced (ASC) and the oxidized (DHA) forms of ascorbate as well as the GSSG levels, leading to a decrease in the redox state of ascorbate and the glutathione (Table 2). Similarly, SNP also enhanced the ASC and DHA levels but to a lesser extent, whereas no significant effects on either form of glutathione occurred (Table 2). The use of cPTIO restored the control levels of ASC in KNO_3- and SNP-treated seeds. However, its effect on DHA was different depending on the treatment. In control seeds, cPTIO strongly increased DHA levels, leading to a decrease in the redox state of ascorbate. In KNO_3-treated seeds, cPTIO restored the control levels of DHA, but no change was observed in SNP-primed seeds (Table 2). Regarding glutathione levels, in general the application of cPTIO produced a decrease in the GSH levels in SNP-treated seeds, whereas the the NO-scavenger induced an accumulation of GSSG in KNO_3-primed seeds, leading in both cases to a decline in the redox state of glutathione (Table 2).

3.3. GA and ABA contents

Both priming treatments and cPTIO application significantly affected the levels of GA4 and ABA. However, an interaction between both factors only took place for GA4 (Fig. 3).

Regarding the effect of KNO_3 and SNP on GAs, we were not able to detect the active gibberellin GA1 and could only detect the active GA4 in pea seedlings under our experimental conditions. Both the 10 mM KNO_3 and 50 μM SNP treatments produced a 3.5-fold increase in GA4 concentrations (Fig. 3A). Furthermore, the presence of the NO-scavenger (cPTIO) significantly reduced the GA4 levels in pea seedlings, particularly in the combined treatments with KNO_3 or SNP.

Both KNO_3 and SNP significantly reduced the ABA concentrations in pea seedlings (Fig. 3B). This effect led to a decrease in the ABA/GA ratio. In contrast, in the presence of the NO-scavenger, an increase in the ABA levels took place, leading to an increase in the ABA/GA ratio.

4. Discussion

The treatment of seeds with nitrogen-containing compounds, such as KNO_3, has been linked to a higher germination rate in different plant species (Cárdenas et al., 2013; Shim et al., 2008). This effect can be dependent on the KNO_3 concentration used as well

as the mode of application. The direct application of KNO_3 in amounts ranging from 10-50 mM to seeds in petri dishes increased the germination rate in *Paspalum* sp and *Passiflora* sp (Cárdenas et al., 2013; Shim et al., 2008). In our work, KNO_3 was applied in two different ways. In preliminary experiments, pea seeds were directly imbibed in the presence of different KNO_3 concentrations for 24 h, and the seeds were then placed in petri dishes with dH_2O. Under these conditions, 40 mM KNO_3 increased the seedlings' FW, although it did not improve the germination rate (data not shown). These results were somewhat different when the seeds previously imbibed with dH_2O were incubated in the presence of KNO_3 in petri dishes. In this case, 40 mM strongly decreased the germination rate and the seedling FW, whereas 10 mM KNO_3 stimulated early seedling growth.

NO has also been found to induce a positive effect on the seed germination process in different plant species. SNP is the most commonly used treatment as a NO source. Imbibing *Lupinus luteus* L. seeds with concentrations of SNP ranging from 0.1-800 µM increased the germination rate by about 30% as well as root length (Kopyra and Gwóźdź, 2003). In tomato, 10 µM SNP improved the germination rate and increased the root and shoot length (Hayat et al., 2012). These data contrast with our results, because no increases in the germination rate or in the seedling length were produced by 50 µM SNP in pea. We only observed a significant effect on seedling FW. As described for KNO_3, the mode of SNP application could influence its effect. The authors mentioned here applied the SNP treatment directly from imbibition to the end of the experiment (up to 48 h post-imbibition) as described by Kopyra and Gwózdz (2003). In the experiment performed by Hayat et al. (2014), seeds were soaked for 8 h in the presence of the different SNP concentrations and then germinated in the presence of dH_2O. In our case, however, we imbibed seeds with dH_2O, and the seeds were then incubated in the presence of the different treatments. We therefore consider that the way the priming treatment is applied is a variable to take into consideration. The results can thus vary depending on many factors, including the treatment application method (during the imbibition or post-imbibition period), the plant species, the germination conditions, the temperature and the number of seeds per unit area.

4.1. Effect of KNO_3 and SNP on antioxidative metabolism

Information about the effect of KNO_3 and SNP priming on the antioxidative metabolism of seedlings is very scarce. Only a few works provide some data, which are

mainly related to POX, CAT, SOD and APX activities, but there is no information on the effect on the ASC-GSH cycle. It is interesting to mention that in pea seedling cv. Lincoln, CAT activity was not detected at 72 h post-imbibition as previously observed in other experiments carried out in our laboratory (Barba-Espin et al., 2012). In adult pea plants (30 d old) from the same cultivar, researchers have observed that most of the CAT activity was present in leaves, fruits and flowers (Corpas et al., 1999). Seeds and roots, however, presented very low CAT activity. In addition, in the pea cultivar Alaska, CAT activity was detected in seedlings at 48 h and 72 h post-imbibition (Barba-Espin et al., 2010; Diaz-Vivancos et al., 2010).

It is also worth mentioning that the control pea seedling cv. Lincoln showed MDHAR activity values about 7-fold greater than the DHAR activity values, suggesting that ascorbate is recycled mainly by MDHAR "via NADH" in these plants.

In the current study, we observed a clear effect of both treatments on the increase of POX and AOX activities, and SNP stimulated SOD activity and reduced MDHAR activity. The SNP treatment did not have a significant effect on the other ASC-GSH cycle enzymes (APX, DHAR and GR). Our results contrast with those of by Shan et al. (2012) in tomato plants. These authors reported that NO increased all of the ASC-GSH cycle enzymes, and that the effect was suppressed by pre-treatment with cPTIO (Shan et al., 2012). Moreover, in salt-treated tomato plants, SNP application was found to induce a general stimulation of the antioxidative system, including SOD, APX, GR and POX activities (Manai et al., 2014).

In tomato seedlings, improvements in the germination rate induced by KNO_3 paralleled increases in SOD and CAT activities (Lara et al., 2014), but no effect was detected on APX, as occurred in our work. Both KNO_3 and SNP strongly increased POX and AOX activities, which correlated with seedling growth stimulation. The dramatic increase in POX observed in KNO_3-treated seedlings is likely mediated by NO, due the fact that KNO_3 can partially produce NO (Nonogaki 2017). The fact that incubation with CPTIO produced a decrease in POX activity supports this conclusion.
It appears that AOX may be involved in plant cell growth, especially during in the early phases of development; furthermore, POXs are also involved in cell wall metabolism and development (Kärkönen and Kuchitsu, 2015; Kato and Esaka, 1999).

In tomato and lupine seedlings, SNP also increased POX activity (Hayat et al., 2012; Kopyra and Gwóźdź, 2003). Moreover, SNP has also been found to increase SOD activity (Hayat al., 2014; this work). This response is dependent on the NO source,

however, because nitrosoglutathione was not found to produce any changes in SOD, and peroxinitrite was found to inhibit SOD (Holzmeister et al., 2015).

On the other hand, the *in vivo* effect of SNP on POX activity described in this work and by other authors (Hayat et al., 2012; Kopyra and Gwóźdź, 2003) contrasts to that observed by Ros-Barceló et al. (2002) under *in vitro* conditions. The incubation of samples of intercellular washing fluid from *Zinnia elegans* with SNP (5-10 mM) reduced the POX activity (Ros-Barceló et al., 2002), whereas under our conditions, a 3-fold increase in POX activity was reported. However, in both cases, recovery of the POX activity to control values occurred in the presence of the NO-scavenger cPTIO.

Regarding the ASC-GSH cycle enzymes, the presence of the NO-scavenger cPTIO stimulated APX, MDHAR and, to a lesser extent, GR activity. The ASC-GSH cycle is an essential pathway controlling H_2O_2 in different cell compartments (Jimenez et al., 1997; Noctor et al., 2012), and it has been reported that these enzymes are subjected to NO-regulation by nitration and S-nitrosilation reactions (Begara-Morales et al., 2014). Specifically, it has been described that cytosolic APX activity is regulated by NO by the nitration of a Tyr residue, producing the inactivation of the enzyme, whereas the S-nitrosilation of a Cys residue has been found to activate the enzyme (Begara-Morales et al., 2014). Moreover, it has been reported in pea leaves that MDHAR can be nitrated into three different Tyr residues by peroxinitrite (Begara-Morales et al., 2015). These authors reported MDHAR inhibition in the range 30-65% depending on the NO donors used ($ONOO^-$ or GSNO). These data agree with the results obtained in this work, because 50 μM SNP decreased MDHAR activity by up to 38%. Furthermore, no significant changes in the GR activity were reported due to $ONOO^-$ or GSNO (Begara-Morales et al., 2015), as reported in the present work. The lack of effect of KNO_3 or SNP on GR activity could be explained by the fact that the pea seedling would need to maintain a certain GSH level and the redox state to support the antioxidant activity of the ASC-GSH cycle during the early seedling growth process, as suggested by Begara-Morales et al., (2015). Under *in vivo* conditions, the ASC-GSH cycle enzymes can therefore undergo post-translational modification by NO in order to modulate their enzymatic activity, mainly under environmental stress conditions and by playing a role in signalling processes (Begara-Morales et al., 2014, 2015). In the present study, the presence of the NO-scavenger cPTIO impaired the action of NO and, under our experimental conditions, activated APX, MDHAR, and, to a lesser extent GR, activities.

According to our results, we can suggest that KNO_3 and, especially, NO, can modulate the metabolism of ascorbate and glutathione in pea plants. Both treatments (KNO_3 and SNP) caused a significant increase in ASC. NO appears to be directly involved in this effect because the presence of the NO-scavenger cPTIO decreased the ASC content. Decreases in ASC levels due to NO-scavengers have also been reported in other plant species. In *Agropyron cristatum* plants, for instance, water stress increased both NO and ASC levels as well as the activity of L-galactono-1,4-lactone dehydrogenase (GalLDH), the last enzyme of the ascorbate biosynthetic pathway (Shan et al., 2012). This increase in ASC and GalLDH was significantly reduced by the effect of NO inhibitors (L-NAME) or NO scavengers (cPTIO) (Shan et al., 2012). Moreover, in salt-treated tomato plants, SNP increased the ASC levels in leaves and roots (Manai et al., 2014).

The treatments had no significant effect on GSH in pea seedlings, although the values were statistically higher in SNP-treated seeds than in KNO_3-treated seeds. In addition, the KNO_3 treatment produced an accumulation of GSSG, especially in the presence of cPTIO, leading to a decrease in the redox state of glutathione. The SNP-primed seeds contained the highest levels of GSH, GSSG and therefore of total glutathione. The effect of SNP on GSH and total glutathione levels was reverted by cPTIO, suggesting that NO had a direct effect on the glutathione increase. A similar effect was reported in salt-treated tomato plants, in which cPTIO treatment reduced the GSH and total glutathione levels (Shan et al., 2012).

The fact that GR activity did not increase under the same conditions (a slight decrease was even recorded) leads us to suggest that glutathione synthesis could be induced by NO. It is known that NO can also regulate GSH biosynthesis through the regulation of □-*ecs* (encoding Gamma-glutamylcysteine synthetase) and *gshs* (encoding Glutathione synthetase) gene expression (Innocenti et al., 2007). This effect was also reported in Arabidopsis subjected to NO fumigation (100 ppm NO gas) for one hour (Kovacs et al., 2015). Two hours after the treatment, total glutathione increased, especially the reduced form (GSH); furthermore, these authors observed that the NO-induced GSH accumulation was dependent on *de novo* biosynthesis and not on the recycling of the oxidised form. Similar to results in the present work, these authors did not observe significant changes in GR activity (Kovacs et al., 2015). Therefore, our data also suggest that NO can induce an increase in GSH biosynthesis.

4.2. Effect of KNO_3 and NO on the ABA/GA balance

Both treatments increased GA4 levels and decreased ABA levels, thus leading to a reduction in the ABA/GA ratio, so it is clear that KNO_3 and NO may influence the metabolism of both hormones. Several authors have described that either NO accumulation or exogenous nitrate incubation can reduce ABA levels (Matakiadis et al., 2009; Sanz et al., 2015). This decrease in ABA concentrations has been found to correlate with an increase in the *CYP707A2* (coding abscisic acid 8'-hydroxylase 2) expression level and protein, which is involved in ABA catabolism (Liu et al., 2010; Matakiadis et al., 2009). Moreover, a correlation between NO and GAs has been established. It is known, for instance, that NO is required for the transcription of *GA3ox1* and *GA3ox2*, which are involved in the biosynthesis of active GAs (Sanz et al., 2015). The effect of KCN on seed dormancy breaking in Arabidopsis correlated with an increase in GA biosynthesis and with increased expression of *GA3ox1* and *GA3ox2*; furthermore, the expression of both genes decreased by 50% in the presence of cPTIO (Bethke et al., 2007).

It has been also reported that H_2O_2 up-regulates genes involved in ABA catabolism (Liu et al., 2010), resulting in a decrease in ABA levels in both seeds and seedlings (Barba-Espin et al., 2010; Liu et al., 2010). This response seems to also be mediated by NO (Liu et al., 2010). Similar to H_2O_2, KNO_3 and/or NO can also be involved in the modulation of ABA and GA contents in pea seedlings. Treatments with cPTIO or diphenyleneiodonium (DPI, inhibitor of ROS production) have been found to decrease *CYP707A* gene expression and seed germination in Arabidopsis, suggesting that both H_2O_2 and NO may be involved in the regulation of ABA metabolism during germination. This could explain the effect of cPTIO in control seeds, in which an increase in ABA levels was observed. In pea, however, H_2O_2 stimulated seed germination and seedling growth (Barba-Espín et al., 2010, 2011), whereas NO (SNP) and KNO_3 only had a significant effect on seedling growth and not on seed germination. It therefore seems that the effects of KNO_3 and NO on pea-seed germination stimulation are less important than those mediated by H_2O_2. Nevertheless, all of the treatments described (H_2O_2, KNO_3 and SNP) decreased the ABA levels in seedlings (Barba-Espin et al., 2010; Liu et al., 2010) and increased the GA levels and/or the expression of the genes involved in GA biosynthesis (the present work, Liu et al., 2010). In Arabidopsis, SNP did not up-regulate genes involved in GA biosynthesis, suggesting that SNP can

modulate GA biosynthesis via regulation of ABA catabolism during seed imbibition (Liu et al., 2010).

Given that the effects of KNO_3 and SNP were overcome by the NO-scavenger cPTIO, however, it is possible that some of the effects could be due to the partial production of NO from KNO_3.

5. Conclusions

In conclusion, a relationship between antioxidant metabolism and the ABA/GA ratio during early seedling growth occur in pea. This suggests a role for KNO_3 and NO in the modulation of GA4 and ABA levels and antioxidant metabolism in pea seedlings. Furthermore, this response correlates with an increase in the biomass of pea seedlings. According to a correlation matrix performed (Supplementary Table 1), there is a direct relationship between ABA and GA4 and antioxidant metabolism. Moreover, we found a positive correlation between GA4 and fresh plant growth (Supplementary Table 1). Finally, besides stimulating plant growth, the decrease in the ABA/GA ratio in treated (KNO_3 or SNP) seedlings also increased certain antioxidant defences, including POX, DHAR, AOX, ASC and glutathione levels.

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References

Anosheh, H.P., Sadeghi, H., Emam, Y., 2011. Chemical priming with urea and KNO_3 enhances maize hybrids (*Zea mays* L.) seed viability under abiotic stress. J. Crop Sci. Biotechnol. 14(4), 289-295.

Bailly, C., El-Maarouf-Bouteau, H., Corbineau, F., 2008. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. Comptes Rendus Biologies 331(10), 806-814.

Barba-Espin, G., Diaz-Vivancos, P., Clemente-Moreno, M.J., Albacete, A., Faize, L., Faize, M., Perez-Alfocea, F., Hernandez, J.A., 2010. Interaction between

hydrogen peroxide and plant hormones during germination and the early growth of pea seedlings. Plant Cell Environ. 33(6), 981-994.

Barba-Espin, G., Diaz-Vivancos, P., Job, D., Belghazi, M., Job, C., Antonio Hernandez, J., 2011. Understanding the role of H_2O_2 during pea seed germination: a combined proteomic and hormone profiling approach. Plant Cell Environ. 34(11), 1907-1919.

Barba-Espin, G., Nicolas, E., Almansa, M.S., Cantero-Navarro, E., Albacete, A., Hernandez, J.A., Diaz-Vivancos, P., 2012. Role of thioproline on seed germination: Interaction ROS-ABA and effects on antioxidative metabolism. Plant Physiol. Biochem. 59, 30-36.

Begara-Morales, J.C., Sánchez-Calvo, B., Chaki, M., Valderrama, R., Mata-Pérez, C., López-Jaramillo, J., Padilla, M.N., Carreras, A., Corpas, F.J., Barroso, J.B., 2014. Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation. J. Exp. Bot. 65(2), 527-538.

Begara-Morales, J.C., Sánchez-Calvo, B., Chaki, M., Mata-Pérez, C., Valderrama, R., Padilla, M.N., López-Jaramillo, J., Luque, F., Corpas, F.J., Barroso, J.B., 2015. Differential molecular response of monodehydroascorbate reductase and glutathione reductase by nitration and S-nitrosylation. J. Exp. Bot. 66(19), 5983-5996.

Beligni, M.V., Lamattina, L., 2000. Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta 210(2), 215-221.

Bethke, P.C., Libourel, I.G., Jones, R.L., 2006. Nitric oxide reduces seed dormancy in Arabidopsis. J. Exp. Bot. 57(3), 517-526.

Bethke, P.C., Libourel, I.G.L., Aoyama, N., Chung, Y.-Y., Still, D.W., Jones, R.L., 2007. The Arabidopsis Aleurone Layer Responds to Nitric Oxide, Gibberellin, and Abscisic Acid and Is Sufficient and Necessary for Seed Dormancy. Plant Physiol. 143(3), 1173-1188.

Calvo, P., Nelson, L., Kloepper, J.W., 2014. Agricultural uses of plant biostimulants. Plant Soil 383(1), 3-41.

Cárdenas, J., Carranza, C., Miranda, D., Magnitskiy, S., 2013. Effect of GA_3, KNO_3, and removing of basal point of seeds on germination of sweet granadilla (*Passiflora ligularis* Juss) and yellow passion fruit (*Passiflora edulis* f. flavicarpa). Revista Brasileira de Fruticultura 35, 853-859.

Corpas, F.J., Palma, J.M., Sandalio, L.M., López-Huertas, E., Romero-Puertas, M.C., Barroso, J.B., Del Río, L.A., 1999. Purification of Catalase from Pea Leaf

Peroxisomes: Identification of Five Different Isoforms. Free Rad. Res. 31(sup1), 235-241.

de Gara, L., Pinto, M., Arrigoni, O., 1997. Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination. Physiol. Plant. 100(4), 894-900.

Diaz-Vivancos, P., Barba-Espin, G., Clemente-Moreno, M.J., Hernandez, J.A., 2010. Characterization of the antioxidant system during the vegetative development of pea plants. Biol. Plant. 54(1), 76-82.

Diaz-Vivancos, P., Barba-Espin, G., Hernandez, J.A., 2013a. Elucidating hormonal/ROS networks during seed germination: insights and perspectives. Plant Cell Rep. 32(10), 1491-1502.

Diaz-Vivancos, P., Faize, M., Barba-Espin, G., Faize, L., Petri, C., Hernández, J.A., Burgos, L., 2013b. Ectopic expression of cytosolic superoxide dismutase and ascorbate peroxidase leads to salt stress tolerance in transgenic plums. Plant Biotechnol. J. 11 976-985.

El-Maarouf-Bouteau, H., Bailly, C., 2008. Oxidative signaling in seed germination and dormancy. Plant Signal. Behav. 3(3), 175-182.

Feelisch, M., 1998. The use of nitric oxide donors in pharmacological studies. Naunyn-Schmiedeberg's Archives of Pharmacology 358(1), 113-122.

Gomes, M.P., Garcia, Q.S., 2013. Reactive oxygen species and seed germination. Biologia 68(3), 351-357.

Hayat, S., Yadav, S., Wani, A.S., Irfan, M., Alyemini, M.N., Ahmad, A., 2012. Impact of sodium nitroprusside on nitrate reductase, proline content, and antioxidant system in tomato under salinity stress. Hort. Environ. Biotechnol. 53(5), 362-367.

Hayat S., Yadav, S., Alyemeni, M.N., Ahmad, A. 2014 Effect of sodium nitroprusside on the germination and antioxidant activities of tomato (*Lycopersicon esculentum* L.). Bulgarian Journal of Agricultural Science, 20 (1), 140-144.

Holzmeister, C., Gaupels, F., Geerlof, A., Sarioglu, H., Sattler, M., Durner, J., Lindermayr, C., 2015. Differential inhibition of Arabidopsis superoxide dismutases by peroxynitrite-mediated tyrosine nitration. J. Exp. Bot. 66(3), 989-999.

Innocenti, G., Pucciariello, C., Le Gleuher, M., Hopkins, J., de Stefano, M., Delledonne, M., Puppo, A., Baudouin, E., Frendo, P., 2007. Glutathione synthesis is regulated by nitric oxide in *Medicago truncatula* roots. Planta 225(6), 1597-1602.

Ishibashi, Y., Kasa, S., Sakamoto, M., Aoki, N., Kai, K., Yuasa, T., Hanada, A., Yamaguchi, S., Iwaya-Inoue, M., 2015. A Role for Reactive Oxygen Species Produced by NADPH Oxidases in the Embryo and Aleurone Cells in Barley Seed Germination. PLOS ONE 10(11), e0143173.

Jimenez, A., Hernandez, J.A., Del Rio, L.A., Sevilla, F., 1997. Evidence for the Presence of the Ascorbate-Glutathione Cycle in Mitochondria and Peroxisomes of Pea Leaves. Plant Physiol. 114(1), 275-284.

Kärkönen, A., Kuchitsu, K., 2015. Reactive oxygen species in cell wall metabolism and development in plants. Phytochem. 112, 22-32.

Kato, N., Esaka, M., 1999. Changes in ascorbate oxidase gene expression and ascorbate levels in cell division and cell elongation in tobacco cells. Physiol. Plant. 105(2), 321-329.

Kopyra, M., Gwóźdź, E.A., 2003. Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. Plant Physiol. Biochem. 41(11), 1011-1017.

Kovacs, I., Durner, J., Lindermayr, C., 2015. Crosstalk between nitric oxide and glutathione is required for NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1)-dependent defense signaling in Arabidopsis thaliana. New Phytol. 208(3), 860-872.

Lara, T.S., Lira, J.M.S., Rodrigues, A.C., Rakocevic, M., Alvarenga, A.A., 2014. Potassium Nitrate Priming Affects the Activity of Nitrate Reductase and Antioxidant Enzymes in Tomato Germination. J. Agric. Sci. 6(2), p72.

Liu, Y., Ye, N., Liu, R., Chen, M., Zhang, J., 2010. H(2)O(2) mediates the regulation of ABA catabolism and GA biosynthesis in Arabidopsis seed dormancy and germination. J. Exp. Bot. 61(11), 2979-2990.

Manai, J., Kalai, T., Gouia, H., Corpas, F.J., 2014. Exogenous nitric oxide (NO) ameliorates salinity-induced oxidative stress in tomato (*Solanum lycopersicum*) plants. J. Soil Sci. Plant Nut. 14, 433-446.

Matakiadis, T., Alboresi, A., Jikumaru, Y., Tatematsu, K., Pichon, O., Renou, J.P., Kamiya, Y., Nambara, E., Truong, H.N., 2009. The Arabidopsis abscisic acid catabolic gene CYP707A2 plays a key role in nitrate control of seed dormancy. Plant Physiol. 149(2), 949-960.

Noctor, G., Mhamdi, A., Chaouch, S., Han, Y., Neukermans, J., Marquez-Garcia, B., Queval, G., Foyer, C.H., 2012. Glutathione in plants: an integrated overview. Plant Cell Environ. 35(2), 454-484.

Nonogaki, H. 2017. Seed biology updates – highlights and new discoveries in seed dormancy and germination research. Front. Plant Sci. 8, 524.

Paparella, S., Araújo, S.S., Rossi, G., Wijayasinghe, M., Carbonera, D., Balestrazzi, A., 2015. Seed priming: state of the art and new perspectives. Plant cell Rep. 34(8), 1281-1293.

Pellny, T.K., Locato, V., Vivancos, P.D., Markovic, J., De Gara, L., Pallardo, F.V., Foyer, C.H., 2009. Pyridine Nucleotide Cycling and Control of Intracellular Redox State in Relation to Poly (ADP-Ribose) Polymerase Activity and Nuclear Localization of Glutathione during Exponential Growth of Arabidopsis Cells in Culture. Mol. Plant 2(3), 442-456.

Ros-Barceló, A., Pomar, F., Ferrer, M.A., Martínez, P., Ballesta, M.C., Pedreño, M.A., 2002. In situ characterization of a NO-sensitive peroxidase in the lignifying xylem of *Zinnia elegans*. Physiol. Plant. 114(1), 33-40.

Sanz, L., Albertos, P., Mateos, I., Sánchez-Vicente, I., Lechón, T., Fernández-Marcos, M., Lorenzo, O., 2015. Nitric oxide (NO) and phytohormones crosstalk during early plant development. J. Exp. Bot. 66(10), 2857-2868.

Shan, C., He, F., Xu, G., Han, R., Liang, Z., 2012. Nitric oxide is involved in the regulation of ascorbate and glutathione metabolism in *Agropyron cristatum* leaves under water stress. Biol. Plant. 56(1), 187-191.

Shim, S.I., Moon, J.-C., Jang, C.S., Raymer, P., Kim, W., 2008. Effect of Potassium Nitrate Priming on Seed Germination of *Seashore Paspalum*. HortScience 43(7), 2259-2262.

Terasaki, S., Sakurai, N., Yamamoto, R., Wada, N., Nevins, D.J., 2001. Changes in Cell Wall Polysaccharides of Kiwifruit and the Visco-elastic Properties Detected by a Laser Doppler Method. J. Jap. Soc. Hort. Sci. 70(5), 572-580.

Wojtyla, L., Lechowska, K., Kubala, S., Garnczarska, M., 2016. Different Modes of Hydrogen Peroxide Action During Seed Germination. Front. Plant Sci. 7, 66.

Table 1 .- The effects of 10 mM KNO_3 and 50 µM SNP, in the absence and presence of 200 µM cPTIO, on the activity of different antioxidant pea seedling enzymes. APX, MDHAR, DHAR, GR, POX and AOX are expressed in nmol x min^{-1} x mg^{-1} prot. SOD is expressed in U x mg^{-1} prot. Data represent the mean ± SE from at least six measurements. Different letters indicate significant differences according to Tukey's test ($P<0.05$).

Treatment	APX	MDHAR	DHAR	GR	SOD	POX	AOX
Control							
- cPTiO	94.0 ± 4.8a	262.3 ± 16.8bc	37.4 ± 3.3c	121.9 ± 7.2abc	54.1 ± 4.7a	280.4±25.0a	68.0 ± 6.0a
+ cPTiO	156.4 ± 5.2bc	358.2 ± 22.0d	15.4 ± 1.0a	130.9 ± 6.7bc	68.7 ± 1.9ab	169.4 ±18.9a	71.5 ± 9.6a
KNO_3 10 mM							
- cPTiO	95.8 ± 6.3a	216.2 ± 24.8ab	31.5 ± 4.0bc	115.3 ± 6.8ab	60.9 ± 3.3ab	1247.5 ± 108.7d	104.8 ± 5.5b
+ cPTiO	187.8 ± 19.9c	321.1 ± 9.9cd	25.0 ± 3.2abc	141.4 ± 7.7c	100.4 ± 16.4d	470.5 ± 21.3b	99.8 ± 2.0b
SNP 50 µM							
- cPTiO	80.6 ± 3.7a	160.3 ± 8.6a	33.8 ± 0.1bc	101.9 ± 3.1a	89.4 ± 2.4cd	814.3 ± 41.0c	181.1 ± 10.7c
+ cPTiO	149.4 ± 17.6b	339.1 ± 38.4d	22.3 ± 2.5ab	123.4 ± 7.5abc	73.3 ± 5.2bc	220.5 ± 46.5a	152.8 ±8.2d

Source of variation	[a]F APX	MDHAR	DHAR	GR	SOD	POX	AOX
[b]Treatments (A)	2.74ns	3.60*	0.12ns	2.71ns	8.65**	45.74***	78.78***
cPTIO (B)	57.08***	40.57***	14.34**	10.26*	7.54*	92.24***	2.33ns
A * B	1.05ns	1.78ns	1.81ns	0.77ns	11.43***	13.43***	2.17ns

. [a] F values from two-way ANOVA for the different antioxidant enzymes analysed. [b]Treatments with KNO_3 or SNP. F values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability. ns. not significant.

Table 2: The effects of treatment with 10 mM KNO_3 and 50 µM SNP, in the presence or absence of 200 µM cPTIO, on ascorbate and glutathione concentrations in pea seedlings. Reduced (ASC) and oxidised (DHA) ascorbate are expressed as µmol g^{-1} FW, and reduced (GSH) oxidised (GSSG) and total glutathione are expressed in nmol g^{-1} FW. Data represent the mean ± SE from at least six measurements. Different letters indicate significant differences according to Tukey's test ($P<0.05$).

Treatment	ASC	DHA	ASC/(ASC+DHA)	GSH	GSSG	GSH/(GSH+ GSSG)	Total Glutathione
Control							
- cPTIO	10.05 ± 1.22a	1.01 ± 0.07a	0.908	826.07 ± 52.34bc	68.76 ± 5.27a	0.923	892.86±48.25ab
+ cPTIO	18.93 ± 5.18ab	12.65 ± 0.49d	0.599	748.71 ± 33.86ab	66.64 ± 6.78a	0.918	815.14 ± 26.76ab
KNO_3 10 mM							
- cPTIO	33.71 ± 4.38d	4.23 ± 0.42c	0.894	748.95 ± 70.10ab	99.87 ± 8.32bc	0.882	835.20 ± 63.75ab
+ cPTIO	21.57 ± 0.24bc	0.95 ± 0.09a	0.958	694.10 ± 39.72ab	108.25 ± 6.43c	0.865	799.16 ± 41.54b
SNP 50 µM							
- cPTIO	29.65 ± 1.49cd	1.97 ±0.14 b	0.938	932.49 ± 43.62c	86.02 ± 4.88ab	0.915	1018.20 ± 42.05a
+ cPTIO	14.36 ± 2.27ab	2.31 ± 0.30b	0.861	633.20 ± 47.38a	77.36 ± 4.90a	0.891	708.88 ± 52.50b

	[a]F				
Source of variation	**ASC**	**DHA**	**GSH**	**GSSG**	**Total Glutathione**
[b]Treatments (A)	8.23*	149.31***	1.10ns	16.16***	0.530ns
cPTIO (B)	5.99*	151.61**	12.80**	0.025ns	13.23**
A * B	9.50*	340.47***	3.86*	0.86ns	4.93*

[a] F values from two-way ANOVA for the different non-enzymatic antioxidants analysed. [b]Treatments with KNO3 or SNP. F values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability. ns. not significant.

Legend to Figures

Fig. 1.- Effects of different KNO_3 concentrations on the length and the fresh weight (FW) of pea seedlings. Data represent the mean ± SE from at least 30 measurements. Different letters indicate significant differences according to Tukey's test ($P<0.05$). Significant differences for length are represented in capital letters. The differences observed for FW are indicated in lower-case letters.

Fig. 2.- Effects of 10 mM KNO_3 and SNP (50-100 μM) in the presence (**+i**) or absence of 200 μM cPTIO on pea seedling growth. A) Effects on seedling fresh weight (FW). B) Effects on seedling length. Data represent the mean ± SE from at least 30 measurements. Different letters indicate significant differences according to Tukey's test ($P<0.05$).

Fig. 3.- Effects of 10 mM KNO3 and SNP (50-100 μM) in the presence (**+i**) or absence of 200 μM cPTIO on the GA and ABA levels in pea seedlings (without cotyledons). [a]F values from two-way ANOVA for the GA and ABA levels. [b]Treatments with KNO_3 or SNP. F values significant at 99.9% (***) or 99% (**) levels of probability. ns. not significant. Data represent the mean ± SE from at least four measurements. Different letters indicate significant differences according to Tukey's test ($P<0.05$).

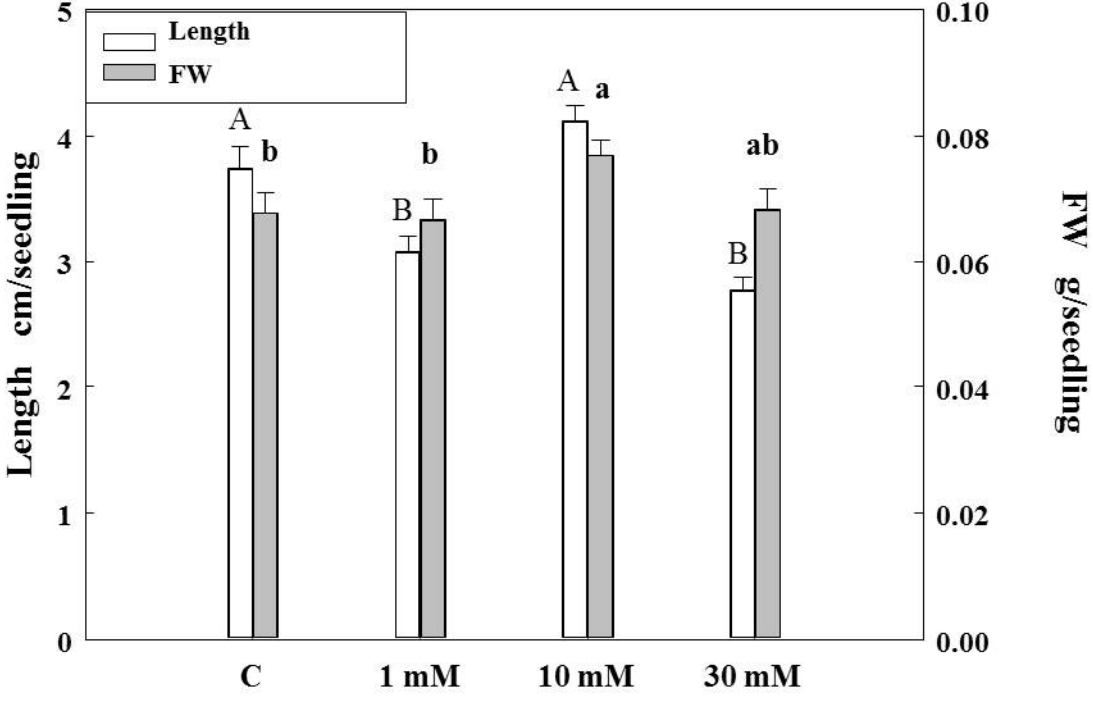
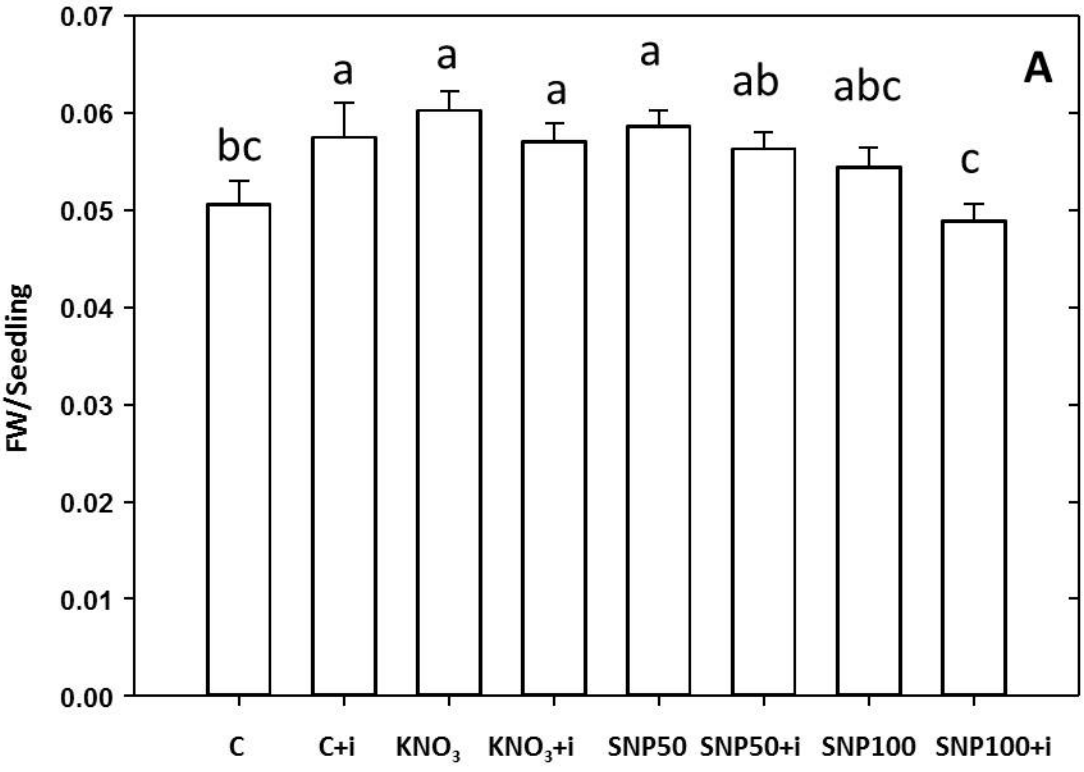

Length
FW
Length cm/seedling
FW g/seedling
0
1
2
3
4
5
0.00
0.02
0.04
0.06
0.08
0.10
A
b
B
b
A
a
B
ab
C
1 mM
10 mM
30 mM

Fig. 1


A
FW/Seedling
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
bc
a
a
a
a
ab
abc
c
C
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KNO3
KNO3+i
SNP50
SNP50+i
SNP100
SNP100+i

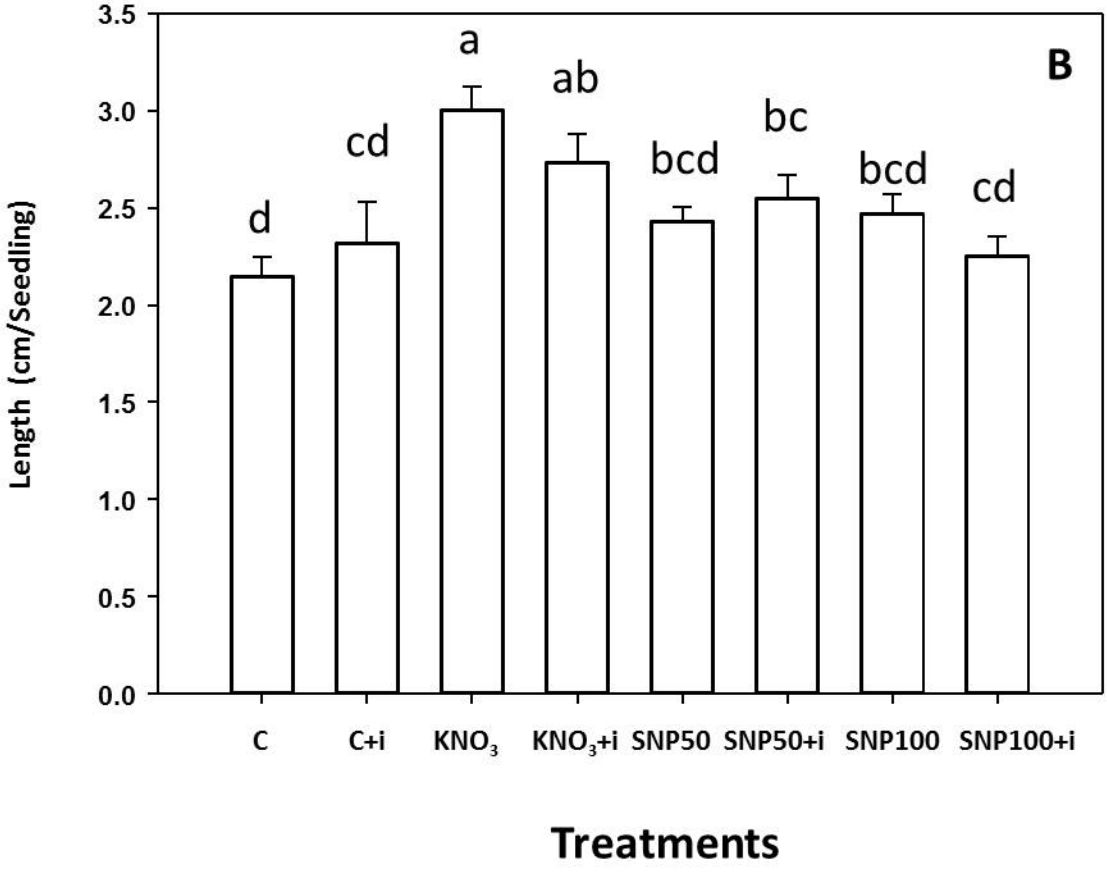
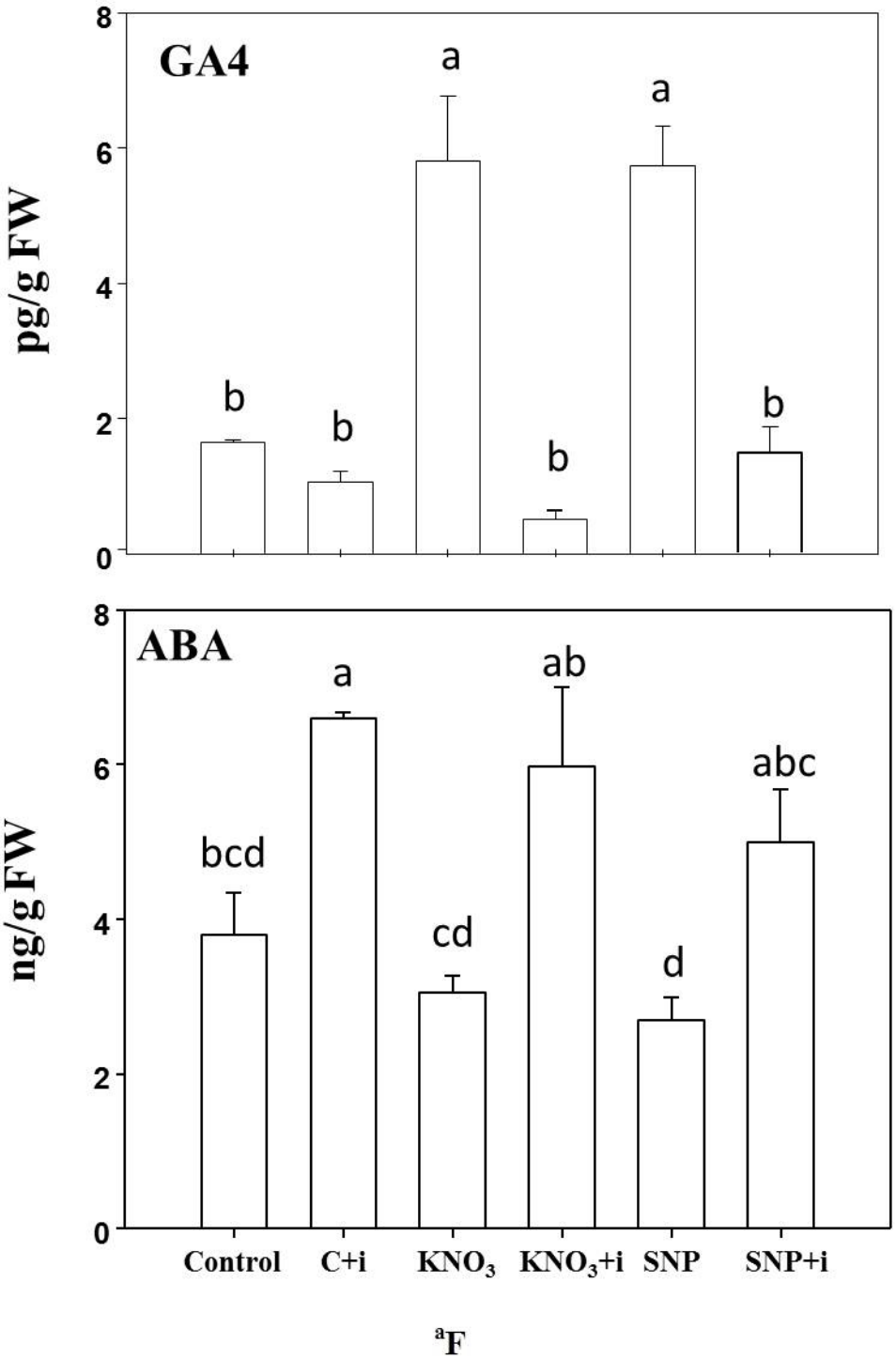

B
Length (cm/Seedling)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
d
cd
a
ab
bcd
bc
bcd
cd
C
C+i
KNO3
KNO3+i
SNP50
SNP50+i
SNP100
SNP100+i
Treatments

Fig. 2



[a]F

Source of variation	GA4	ABA
[b]Treatments (A)	13,25***	16,45***
cPTIO (B)	86,36***	71,35***
A * B	13,74***	0,27ns

Fig. 3

Supplementary Table 1.- Correlation matrix of all variables measured in relation to the effect of 10 mM KNO3 and 50 µM SNP, in the presence or the absence of cPTIO, in plant growth, antioxidant metabolism and ABA and GA levels in pea seedlings. The values of these correlation coefficients ranges from -1 (negative correlation) to +1 (positive correlation), and measure the strength of the linear relationship between the variables. The second number in each block of the table is a P-value that tests the statistical significance of the estimated correlations (P). P-Values below 0.05 indicate correlations significantly different with a confidence level of 95.0% (These significant values are marked in red). Asterisks indicate the level of probability: * P <0.05. ** P <0.01 and *** P <0.001. P >0.05 Non-significant differences are indicated by "ns".

	Length	Weight	ABA	GAs	POX	DHAR	MDHAR	GR	APX	SOD	AOX	ASC	DHA	Total Glutathione	GSH
Weight	0.73 0.00***														
ABA	-0.1268 0.11 ns	-0.1083 0.17 ns													
GAs	0.1506 0.06ns	0.1680 0.036*	-0.8832 0.00***												
POX	0.2989 0.00***	0.2104 0.008**	-0.7899 0.00***	0.8646 0.00***											
DHAR	-0.1253 0.12 ns	-0.1708 0.033*	-0.5889 0.00***	0.3768 0.00***	0.2345 0.003**										
MDHAR	-0.1020 0.20 ns	-0.1223 0.13 ns	0.8687 0.00***	-0.8613 0.00***	-0.7089 0.00***	-0.5709 0.00***									
GR	0.2278 0.004**	-0.0051 0.95 ns	-0.4031 0.00***	0.0919 0.25 ns	0.3194 0.00**	0.3657 0.00**	-0.2344 0.003**								
APX	0.0850 0.29 ns	0.0118 0.88 ns	0.7181 0.00***	-0.7452 0.00***	-0.4631 0.00***	-0.6456 0.00***	0.7685 0.00***	-0.0076 0.93 ns							
SOD	0.0993 0.23 ns	0.1225 0.13 ns	0.0288 0.72 ns	-0.1362 0.09 ns	-0.0265 0.74 ns	-0.3256 0.00***	0.0965 0.23 ns	0.0558 0.49 ns	0.4879 0.00***						
AOX	0.0616 0.44 ns	0.1478 0.065 ns	-0.4851 0.00***	0.4680 0.00***	0.2406 0.002**	-0.0631 0.43 ns	-0.3541 0.00***	0.0281 0.73 ns	-0.2048 0.01*	0.4316 0.00***					
ASC	0.2875 0.00***	0.2386 0.003**	-0.6339 0.00***	0.7768 0.00***	0.8858 0.00***	-0.0105 0.89 ns	-0.5979 0.00***	0.0878 0.27 ns	-0.2996 0.00***	0.2551 0.001**	0.3659 0.00***				
DHA	-0.0027 0.97 ns	0.0903 0.26 ns	0.4320 0.00***	0.0015 0.98 ns	-0.0369 0.65 ns	-0.5109 0.00***	0.2295 0.004**	-0.7741 0.00***	0.0815 0.31 ns	-0.2315 0.004**	-0.2540 0.001**	0.1405 0.08 ns			
Total Glutathione	-0.1241 0.12 ns	-0.0058 0.94 ns	-0.5897 0.00***	0.5615 0.00***	0.3110 0.00***	0.4865 0.00***	-0.6822 0.00***	-0.1511 0.06 ns	-0.6468 0.00***	0.0420 0.60 ns	0.2892 0.00***	0.3202 0.00***	-0.1308 0.10 ns		
GSH	-0.1577 0.049*	-0.0238 0.77 ns	-0.5600 0.00***	0.5483 0.00***	0.2614 0.00***	0.4935 0.00***	-0.6571 0.00***	-0.2020 0.011*	-0.6905 0.00***	-0.0483 0.55 ns	0.2629 0.00***	0.2560 0.001**	-0.0829 0.30 ns	0.9916 0.00***	
GSSG	0.3535 0.00***	0.1729 0.03*	-0.2853 0.00***	0.1863 0.02*	0.5457 0.00***	-0.0849 0.29 ns	-0.1871 0.02*	0.4944 0.00***	0.3095 0.00***	0.5367 0.00***	0.1185 0.14 ns	0.5791 0.00***	-0.2952 0.00***	-0.0832 0.30 ns	-0.1938 0.015*

Author Statement

The contribution of each author is listed below:

Antonia Vidal: Investigation; Methodology

Daniel Cantabella: Investigation; Methodology

Agustina Bernal-Vicente: Investigation; Methodology

Pedro Díaz-Vivancos: Investigation; Methodology, Software, Supervision; Validation; Writing - review & editing.

José A. Hernández: Investigation; Methodology¸ Project administration; Resources; upervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.