Role of nitric acid or H$_2$O$_2$ in antioxidant defense system of Pisum sativum L. under drought stress

Helal Ragab Moussa and Mohamed Abd El-Fattah Hassan Mohamed

Abstract: Water shortage is likely to be one of the major global environmental stresses of the 21st century. Drought is an important environmental constraint limiting the productivity of many crops worldwide. Experiments were conducted to investigate the effects of seed pretreatment by hydrogen peroxide at 70 mM or sodium nitroprusside (SNP; nitric oxide donor) at 10 μM on drought tolerance in pea seedlings. Osmotic stress was provoked by addition of polyethylene glycol to the nutrient solution at the flowering stage. H$_2$O$_2$ or SNP pretreatment alleviated oxidative damage, accelerate proline accumulation and enhance total chlorophyll, carotenoid, photosynthetic activity ($^{14}$CO$_2$ fixation), and total yield/plant in pea seedlings subjected to osmotic stress. The results showed that osmotic stress induced decrease in the enzyme activities of ascorbate peroxidase, glutathione peroxidase, catalase and overproduction of O$_2$•− in pea leaves, which in turn caused exacerbation of lipid peroxidation and depression of photosynthesis. Application of H$_2$O$_2$ or SNP significantly increased the enzyme activities and decrease O$_2$•− production and hence inhibited lipid peroxidation. Level of H$_2$O$_2$, proline and Evan blue uptake in seedlings pretreated with H$_2$O$_2$ or SNP were markedly lower than under drought stress, indicating the operation of antioxidant system in them. Moreover, seedlings arising from H$_2$O$_2$ or SNP pretreatment enhanced the membrane stability, as revealed from greatly reduced malondialdehyde content. The present data suggest that pea seed pretreatment with H$_2$O$_2$ or SNP, a stress signal, could trigger the activation of antioxidants in seeds, which persists in the seedlings to alleviate the oxidative damage, leading to improvements in physiological attributes for the seedling growth under drought.

Keywords: Antioxidative enzymes, Drought stress, Photosynthesis, Proline, Pea

1. Introduction

Drought stress is one of the main causes for crop yield reduction in the majority of agricultural regions of the world (Moussa, 2011). Reactive oxygen species (ROS) are enhanced during drought stress through the disruption of electron transport system and oxidizing metabolic activities occurring in chloroplast, mitochondria and microbodies (Sofo et al., 2005). Plants eliminate ROS produced in non-stressful conditions through production of non-enzymatic and enzymatic antioxidants (Inze and Montagu, 2000), whereas during severe drought conditions the production of ROS exceeds the capacity of the antioxidative systems to remove them, causing oxidative stress (Sofo et al., 2005). In these conditions cells could be protected either by the endogenous molecular systems or exogenously applied compounds that mitigate the stress (Ingram and Bartels, 1996). ROS are highly reactive and in the absence of effective protective mechanism, can seriously damage plants by lipid peroxidation, protein degradation, breakage of DNA and cell death (Beligni and Lamattina, 1999). Among various technique strategies, pre-sowing treatment and priming of plant seeds are easy, low cost, low risk and effective approaches to enhance plant tolerance to the stressful environments (Ashraf and Foolad, 2007). Hydrogen peroxide is a major kind of ROS in plant tissues. It acts as an important signal molecule involved in acclamatory signaling, triggering higher tolerance against various biotic and abiotic stresses at low concentrations, whereas at high concentrations, it is toxic to plant tissues and can trigger programmed cell death (Quan et al., 2008). H$_2$O$_2$ pretreatment could improve salt stress (Fedina et al., 2009), oxidative stress (Chen et al., 2009), and multiple stresses (Uchida et al., 2002). Many previous studies have reported presence of NO in the plant kingdom as an important endogenous plant signaling molecule and its involvement in growth, development and defense responses (Lamotte et al., 2005). As a relatively stable free-radical molecule, and due to its highly lipophilic nature, NO diffuses through membranes and may act as a synchronizing chemical messenger that is involved in many physiological processes in plants (Franciele et al., 2010). Nitric oxide is an important signal molecule involved in plant response to biotic and abiotic stresses (Jinfang, 2007).
et al., 2008). However, there is very little information about the effect of exogenously applied H$_2$O$_2$ or SNP and drought stress in pea seedlings.

The aim was to provide experimental basis for understanding the mechanism of drought tolerance in pea induced by H$_2$O$_2$ or SNP pretreatment.

2. Material and Methods

Plant material and growth conditions.

A homogenous lot of pea seeds (Pisum sativum L.), cv. Master, was obtained from the Crop Institute, Agricultural Research Center, Giza, Egypt. The caryopsis was kept at 4°C. They were surface sterilized in 0.1 % (w/v) sodium dodecyl sulphate solution and then thoroughly rinsed with sterile deionized water. Clean-healthy dry seeds were soaked for 20 h in solutions of H$_2$O$_2$ (70 mM) or sodium nitroprusside (10 µM) and with deionized water as the control. The H$_2$O$_2$ and SNP–treated and water-soaked seeds were sown in black polyethylene pots (40 cm diameter, 45 cm height) filled with silicon sands. Ten seeds were sown per pot. After the seedlings reached the first true leaf stage, they were thinned to four plants per pot. The sand-filled pots irrigated with half strength Hoagland’s solution (Rafi et al., 1994). The seedlings from each treatment were placed under a Bell jar, which was used as a photosynthetic chamber. Radioactive 14CO$_2$ was generated inside the chamber by a reaction between 10% HCl and 50 µCi (1.87×10$^6$ Bq) NaH$^{14}$CO$_3$ + 100 mg Na$_2$CO$_3$ as a carrier. Then the samples were illuminated with a tungsten lamp. After 30 min exposure time, the leaves were quickly detached from the stem, weighed and frozen for 5 min to stop the biochemical reactions, then subjected to extraction by 80% hot ethanol. The $^{14}$C was assayed from the ethanolic extracts in soluble compounds using a Bray Cocktail (Bray, 1960) and a liquid scintillation counter (LSC2-Scaler Ratemeter SR7, Nuclear Enterprises, Edinburgh, UK).

Biosynthetic activity ($^{14}$CO$_2$-fixation).

Photosynthetic activity was measured in the Atomic Energy Authority, Radioisotope Department, Cairo, Egypt, with the method of Moussa (2011). The seedlings from each treatment were placed under a Bell jar, which was used as a photosynthetic chamber. Radioactive $^{14}$CO$_2$ was generated inside the chamber by a reaction between 10% HCl and 50 µCi (1.87×10$^6$ Bq) NaH$^{14}$CO$_3$ + 100 mg Na$_2$CO$_3$ as a carrier. Then the samples were illuminated with a tungsten lamp. After 30 min exposure time, the leaves were quickly detached from the stem, weighed and frozen for 5 min to stop the biochemical reactions, then subjected to extraction by 80% hot ethanol. The $^{14}$C was assayed from the ethanolic extracts in soluble compounds using a Bray Cocktail (Bray, 1960) and a liquid scintillation counter (LSC2-Scaler Ratemeter SR7, Nuclear Enterprises, Edinburgh, UK).

Statistical analysis.

All data were subjected to ANOVA and the means were compared using Duncan’s multiple range tests (P<0.05).

3. Results:

Water deficit decreased significantly the total chlorophyll (a+b), carotenoid content, photosynthetic efficiency ($^{14}$CO$_2$-fixation) and total yield/plant (Table 1). However, pretreatment with H$_2$O$_2$ or SNP increased significantly the above parameters as compared to water deficit stressed plants (Table 1).
Table (1). Effects of H$_2$O$_2$ or SNP pretreatment on total chlorophyll $a+b$ (mg/gFW), carotenoids (mg/gFW), photosynthetic efficiency (*KBq/mgFW) and total yield/plant (g) in pea seedlings under drought stress. Data presented are the means of four separate experiments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total chlorophyll ($a+b$)</th>
<th>Carotenoids</th>
<th>Photosynthetic activity</th>
<th>Total yield/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.98$^c$</td>
<td>6.3$^c$</td>
<td>19829$^a$</td>
<td>58$^a$</td>
</tr>
<tr>
<td>Drought stress</td>
<td>2.57$^c$</td>
<td>3.9$^c$</td>
<td>15422$^d$</td>
<td>40$^c$</td>
</tr>
<tr>
<td>SNP</td>
<td>4.18$^d$</td>
<td>7.4$^d$</td>
<td>21498$^e$</td>
<td>63$^d$</td>
</tr>
<tr>
<td>Drought stress+ SNP</td>
<td>3.74$^b$</td>
<td>5.8$^c$</td>
<td>19032$^b$</td>
<td>55$^c$</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>4.05$^d$</td>
<td>6.8$^b$</td>
<td>20602$^e$</td>
<td>60$^b$</td>
</tr>
<tr>
<td>Drought stress+ H$_2$O$_2$</td>
<td>3.51$^a$</td>
<td>5.5$^b$</td>
<td>18837$^f$</td>
<td>53$^b$</td>
</tr>
</tbody>
</table>

Values followed by the same letter within columns are not significantly different according to Duncan’s multiple range tests ($P<0.05$). *kilo Becquerel (10$^3$ Bq).

H$_2$O$_2$ content increased significantly under drought stress as compared with the control (Table 2). Exogenous application of H$_2$O$_2$ or SNP in drought stressed plants decreased significantly H$_2$O$_2$ content under both normal and stress conditions (Table 2). Exposure to water stress increased malondialdehyde, proline concentration, Evans blue uptake and O$^{−}$ concentrations more significantly as compared with controls. Pretreatment with H$_2$O$_2$ or SNP alleviated Evans blue uptake, reduced the overproduction of O$^{−}$, proline and MDA accumulation as compared with water deficit plants (Table 2).

Table (2). Effects of H$_2$O$_2$ or SNP pretreatment on H$_2$O$_2$ ($\mu$M/gFW), MDA content ($\mu$M/gFW), O$^{−}$ concentration ($\mu$M/gFW), Evan blue uptake (OD$_{600nm}$/gFW), and free proline level ($\mu$M/gFW), in pea seedlings under drought stress. Data presented are the means of four separate experiments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>H$_2$O$_2$</th>
<th>MDA</th>
<th>O$^{−}$</th>
<th>Evans blue uptake</th>
<th>Free proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.8$^e$</td>
<td>2.2$^b$</td>
<td>1.1$^b$</td>
<td>0.128$^a$</td>
<td>8.7$^a$</td>
</tr>
<tr>
<td>Drought stress</td>
<td>7.3$^c$</td>
<td>5.8$^c$</td>
<td>3.7$^d$</td>
<td>0.346$^e$</td>
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</tr>
<tr>
<td>SNP</td>
<td>1.1$^b$</td>
<td>1.7$^a$</td>
<td>0.8$^c$</td>
<td>0.080$^b$</td>
<td>18.8$^b$</td>
</tr>
<tr>
<td>Drought stress+ SNP</td>
<td>2.6$^c$</td>
<td>3.0$^a$</td>
<td>1.7$^b$</td>
<td>0.204$^d$</td>
<td>14.3$^c$</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>1.3$^a$</td>
<td>1.9$^a$</td>
<td>0.7$^c$</td>
<td>0.075$^b$</td>
<td>17.1$^c$</td>
</tr>
<tr>
<td>Drought stress+ H$_2$O$_2$</td>
<td>3.0$^d$</td>
<td>2.8$^c$</td>
<td>1.6$^b$</td>
<td>0.251$^a$</td>
<td>19.3$^d$</td>
</tr>
</tbody>
</table>

Values followed by the same letter within columns are not significantly different according to Duncan’s multiple range tests ($P<0.05$).

Water deficit markedly increased proline content and nearly tripled. Drought stress decreased significantly the activity of CAT, APX and GPX as compared with the control (Table 3). However pretreatment H$_2$O$_2$ or SNP application to drought stressed plants enhanced the activity of these enzymes (Table 3).

Table (3). Effects of H$_2$O$_2$ or SNP pretreatment on enzyme activities of APX ($\mu$M ascorbate/min.gFW), CAT ($\mu$M H$_2$O$_2$/min.gFW) and GPX ($\mu$MNADPH/min.gFW) in pea seedlings under drought stress. Data presented are the means of four separate experiments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>APX</th>
<th>CAT</th>
<th>GPX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.6$^b$</td>
<td>1.8$^a$</td>
<td>5.1$^b$</td>
</tr>
<tr>
<td>Drought stress</td>
<td>1.7$^a$</td>
<td>0.8$^c$</td>
<td>3.5$^c$</td>
</tr>
<tr>
<td>SNP</td>
<td>2.4$^b$</td>
<td>2.3$^b$</td>
<td>8.4$^e$</td>
</tr>
<tr>
<td>Drought stress+ SNP</td>
<td>2.1$^c$</td>
<td>1.9$^a$</td>
<td>4.6$^a$</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>2.8$^e$</td>
<td>2.5$^b$</td>
<td>7.3$^d$</td>
</tr>
<tr>
<td>Drought stress+ H$_2$O$_2$</td>
<td>2.2$^d$</td>
<td>1.7$^a$</td>
<td>4.6$^a$</td>
</tr>
</tbody>
</table>

Values followed by the same letter within columns are not significantly different according to Duncan’s multiple range tests ($P<0.05$).
4. Discussion

Various environmental stresses such as drought can inhibit plant growth and development, leading to crop reduction. For food security in the world and agricultural production, more efforts are needed to develop multiple effective strategies to improve crop stress tolerance (Moussa, 2011). In this study, we investigated the effects of seed treatment with H$_2$O$_2$ or SNP on pea growth characteristics and physiological- biochemical response to drought stress. Overproduction of ROS, especially H$_2$O$_2$, is believed to be primarily responsible for drought triggered oxidative damage and cell death (Moussa, 2011). H$_2$O$_2$ acts as signal molecules in plant response to stress in low concentrations (Desikan et al., 2004; Liheng et al., 2009). H$_2$O$_2$ can effectively modulate the related gene expression and subsequently lead to the enhancement of plant tolerance to the stress (Hung et al., 2005; Liheng et al., 2009). Proline is such an antioxidant which accumulates in response to biotic and abiotic stresses, including water stress (Zhang et al., 1995). The change in stability of biological membranes is a key indicator of cellular damage. Drought and other stresses always results in cellular membrane injuries including the increase of membrane permeability and MDA content due to membrane lipid peroxidation (Farooq and Azam, 2006; Agrawal and Rathore, 2007). Like other physiological/biochemical processes affected by drought, the photosynthetic activity ($^{14}$CO$_2$-fixation), total chlorophyll and carotenoid content is also adversely affected by drought stress (Akram et al., 2007; Moussa, 2011). H$_2$O$_2$ treatment enhanced photosynthetic efficiency in wheat under water stress condition (Liheng et al., 2009). Paralleling with the improvement of photosynthesis upon drought, the total yield/plant production largely increased. SNP promotes a significant increase in chlorophyll content and chloroplast membrane density in maize plants (Graziano et al., 2002). Also, SNP application stimulating the photosynthetic efficiency and inhibiting the degradation of chlorophyll in wheat (Tue et al., 2003). Opposite to this result, exposure to NO reduced photosynthesis in leaves of oats and alfalfa (Hill and Bennett, 1970). Also, Franciele et al. (2010), reported that SNP treatment in soybean seedlings decreased H$_2$O$_2$ and Evans blue uptake concentration. Also, Tian and Lai (2006) investigated that pretreatment with SNP in wheat under drought stress increase the enzyme activities of CAT and GPX and decrease MDA content. Liheng et al. (2009) reported that H$_2$O$_2$ pretreatment enhanced membrane stability by greatly reduced MDA content and increasing the antioxidant enzyme activity of CAT and APX in drought stressed wheat seedlings (Liheng et al., 2009). The increased stress tolerance is attributed partly to an enhanced level of antioxidant defense system induced by H$_2$O$_2$ or SNP pretreatment, which alleviates ROS accumulation and oxidative damage during the subsequent stress conditions (Hu et al., 2005). As is now commonly accepted NO as a second messenger in plants, it is supposed that low concentration of NO might be a signal molecule to induce/stabilize the expression of many antioxidant enzymes including SOD, CAT (Frank et al., 2000). The protective effect of NO may also be related to its ability to react with some ROS, such as O$_2^-$, making NO act as a chain breaker and show its proposed antioxidant properties (Conner and Grisham, 1996). Moreover it has been reported that NO can react with lipid alcoxyl (LO$'$) and peroxy (LOO$'$) radicals, leading to the expectation that NO could stop the propagation of radical-mediated lipid oxidation in a direct fashion (Lamotte et al., 2004), which is in well agreement with our result in the decrease of MDA content. Thus NO may help plants to survive stressful conditions through its action as signaling molecule to activate antioxidative enzymes and reaction with active oxygen and lipid radicals directly. Tolerance to drought is enhanced in wheat treated with SNP (García-Mata and Lamattina 2001, 2002). Incubation of soybean roots in solutions of SNP at very low concentrations (1–10 µM) promotes growth, whereas greater concentrations inhibit it (Hu et al., 2005). Proline as a cytosolic osmoticum and a scavenger of OH$^-$ radical can interact with cellular macromolecules such as DNA, protein and membranes and stabilize the structure and function of such macromolecules (Kavir-Kishor et al., 2005). The data are of considerable value in understanding the mechanisms of plant stress tolerances and in developing effective methods for crop protection against environmental stresses. Thus, it may be a useful management tool in afforestation projects in arid and semiarid areas.

5. Conclusion

Applying H$_2$O$_2$ or SNP prior to water deficit stress could partially alleviate the detrimental effect of water stress on growth of pea through increasing fixation of $^{14}$CO$_2$, and improving antioxidant system and reduce the oxidative damage of cellular membranes. It was concluded that H$_2$O$_2$ or SNP minimized the pea yield loss caused by water deficits.

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