Role of nitric acid or H_2O_2 in antioxidant defense system of *Pisum sativum* L. under drought stress

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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_2O_2 or SNP are active molecules involved in mediation of various biotic and abiotic stress induced physiological responses in plants. H_2O_2 or SNP pretreatment alleviate 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 $O_2^{\bullet-}$ in pea leaves, which in turn caused exacerbation of lipid peroxidation and depression of photosynthesis. Application of H_2O_2 or SNP significantly increased the enzyme activities and decrease $O_2^{\bullet-}$ production and hence inhibited lipid peroxidation. Level of H_2O_2, proline and Evan blue uptake in seedlings pretreated with H_2O_2 or SNP were markedly lower than under drought stress, indicating the operation of antioxidant system in them. Moreover, seedlings arising from H_2O_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_2O_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.

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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_2O_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

et al., 2008). However, there is very little information about the effect of exogenously applied H_2O_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_2O_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_2O_2 (70 mM) or sodium nitroprusside (10 µM) and with deionized water as the control. The H_2O_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 and Epstein, 1999) as well-watered conditions. Pots were kept in a controlled-growth chamber at photo flux density of 240 µmole $M^{-2}S^{-1}$ (12/12 h day/night period) at relative humidity of 55-60%, and 25±2 °C temperature. Cultural practices, such as weed control and irrigation, were performed as needed. Drought stress was achieved by adding polyethylene glycol (PEG-6000, –0.5MPa) solution at the flowering stage (40 day after planting). The experiments conducted four times in complete randomization replicated design. Samples were collected 10 days after the water treatment was applied, between 9:30 and 10:30 a.m., and kept in liquid nitrogen until analyzed. At harvest, the effects of treatments on the total seed yield/plant were recorded.

Biochemical assays.

Free proline was determined according to the method described by Bates *et al.* (1973). The amount of total chlorophyll (*a*+*b*) and carotenoids were determined according to the method of Lichtenthaler and Wellburn (1983).

Determination of $O_2^{\bullet-}$ and H_2O_2 concentrations.

The superoxide radical ($O_2^{\bullet-}$) was determined by 2, 3-bis (2-methoxy-4-nitro-5-sulphenyl) (2H) tetrazolium-5-carboxanilide (XTT) assay (Frahry and Schopfer, 2001). To determine H_2O_2 concentration, the root extract was mixed with 0.1% titanium chloride in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6 000 *g* for 15 min. The absorbance was measured at 410 nm (Hsu and Kao, 2007).

Determination of lipid peroxidation and Evans blue uptake.

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content using the thiobarbituric acid reaction as described by Madhava Rao and Sresty (2000). The loss of plasma membrane integrity was detected by the non-permeable dye (Evans blue) uptake in the root cells, which has also been used as an indicator of cell death (Baker and Mock, 1994).

Determination of antioxidant enzyme activities.

The catalase (CAT, EC 1.11.1.6) activity was assayed from the rate of H_2O_2 decomposition following the method of Aebi (1983). The ascorbate peroxidase enzyme activity (APX, EC 1.11.1.1) was determined as the decrease in absorbance at 290 nm due to ascorbate oxidation (Nakano and Asada, 1981). The glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined as the decrease in absorbance at 340 nm due to the oxidation of NADPH (Navrot *et al.*, 2007).

Photosynthetic 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_2CO_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_2O_2 or SNP increased significantly the above parameters as compared to water deficit stressed plants (Table 1).

Table (1). Effects of H_2O_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.

Treatments	Total chlorophyll (*a+b*)	Carotenoids	Photosynthetic activity	Total yield/plant
Control	3.98[c]	6.3[b]	19829[a]	58[a]
Drought stress	2.57[e]	3.9[c]	15422[d]	40[e]
SNP	4.18[d]	7.4[d]	21498[e]	63[d]
Drought stress+ SNP	3.74[b]	5.8[a]	19032[b]	55[c]
H_2O_2	4.05[d]	6.8[e]	20602[c]	60[a]
Drought stress+ H_2O_2	3.51[a]	5.5[a]	18837[f]	53[b]

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_2O_2 content increased significantly under drought stress as compared with the control (Table 2). Exogenous application of H_2O_2 or SNP in drought stressed plants decreased significantly H_2O_2 content under both normal and stress conditions (Table 2). Exposure to water stress increased malondialdehyde, proline concentration, Evans blue uptake and $O^{\bullet-}$ concentrations more significantly as compared with controls. Pretreatment with H_2O_2 or SNP alleviated Evans blue uptake, reduced the overproduction of $O^{\bullet-}$, proline and MDA accumulation as compared with water deficit plants (Table 2).

Table (2). Effects of H_2O_2 or SNP pretreatment on H_2O_2 (µM/gFW), MDA content (µM/gFW), $O^{\bullet-}$ concentration (µM/gFW), Evan blue uptake (OD_{600nm}/gFW), and free proline level (µM/gFW), in pea seedlings under drought stress. Data presented are the means of four separate experiments.

Treatments	H_2O_2	MDA	$O^{\bullet-}$	Evans blue uptake	Free proline
Control	1.8[a]	2.2[b]	1.1[a]	0.128[a]	8.7[a]
Drought stress	7.3[e]	5.8[e]	3.7[d]	0.346[e]	23.9[e]
SNP	1.1[b]	1.7[a]	0.8[c]	0.080[b]	18.8[b]
Drought stress+ SNP	2.6[c]	3.0[d]	1.7[b]	0.204[d]	14.3[c]
H_2O_2	1.3[b]	1.9[a]	0.7[c]	0.075[b]	17.1[b]
Drought stress+ H_2O_2	3.0[d]	2.8[c]	1.6[b]	0.251[c]	19.3[d]

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_2O_2 or SNP application to drought stressed plants enhanced the activity of theses enzymes (Table 3).

Table (3). Effects of H_2O_2 or SNP pretreatment on enzyme activities of APX (µM ascorbate/min.gFW), CAT (µMH_2O_2/min.gFW) and GPX (µMNADPH/min.gFW) in pea seedlings under drought stress. Data presented are the means of four separate experiments.

Treatments	APX	CAT	GPX
Control	2.6[b]	1.8[a]	5.1[b]
Drought stress	1.7[a]	0.8[c]	3.5[c]
SNP	2.4[b]	2.3[b]	8.4[e]
Drought stress+ SNP	2.1[c]	1.9[a]	4.6[a]
H_2O_2	2.8[b]	2.5[b]	7.3[d]
Drought stress+ H_2O_2	2.2[d]	1.7[a]	4.6[a]

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_2O_2 or SNP on pea growth characteristics and physiological- biochemical response to drought stress. Overproduction of ROS, especially H_2O_2, is believed to be primarily responsible for drought triggered oxidative damage and cell death (Moussa, 2011). H_2O_2 acts as signal molecules in plant response to stress in low concentrations (Desikan *et al.*, 2004; Liheng *et al.*, 2009). H_2O_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 injures 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_2O_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_2O_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_2O_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_2O_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 antioxidative 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 $O_2^{\bullet-}$, 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 ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) 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 $OH^{\bullet}$ 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_2O_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_2O_2 or SNP minimized the pea yield loss caused by water deficits.

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