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Short communication

Effect of exogenous application of nitric oxide on salt stress responses of soybean

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ABSTRACT

Salinity stress is one of the major factors that reduce annual agricultural produce. This has led to numerous studies investigating means to improve tolerance to salt stress. Nitric oxide (NO) is a gaseous signaling molecule involved in the regulation of diverse processes in plants. Certain studies have demonstrated the role of exogenous application of NO in mediating responses to abiotic stress. We investigated the role of exogenously applied NO 2,2′(hydroxynitrosohydrazono) bis-ethanimine (DETA/NO) in ameliorating long term salinity stress on soybean. Long term salinity stress in the form of a final concentration of 80 mM sodium chloride (NaCl) over a 16 day period drastically affected the plants as indicated by decreased biomass of shoots, roots and nodules of soybean plants. In contrast, supplementation with 10 μM DETA/NO improved growth of soybean plants under NaCl as evidenced by increased shoot, root and nodule weights and nodule number. Further analysis showed that long-term salinity stress led to increased cellular hydrogen peroxide (H_2O_2) content and high levels of cell death in the soybean. Treatments with NO, either as DETA/NO alone or in combination with NaCl, resulted in reversal of H_2O_2 to basal levels. This study showed that application of DETA/NO resulted in increased enzymatic activity of ascorbate peroxidase (APX). We propose that the role of NO in increasing tolerance to salinity stress in soybean may result from either its antioxidant capacity by direct scavenging of H_2O_2 or its role in activating APX activity that is crucial in scavenging H_2O_2.

1. Introduction

Crop plants are frequently exposed to various environmental stresses which generally result in decreased yield of agricultural produce. There have been several studies (Flowers, 2004; Mittler, 2006) aimed at reducing the decline of agricultural crop produce resulting from these abiotic stress conditions. Amongst the abiotic environmental factors such as drought, radiation temperature and heavy metal toxicity, soil salinity is regarded as one of the major factors that pose a great threat to agricultural yield (Zhu, 2003). Most saline soils are observed in regions where there is limited rainfall, high temperature and inadequate soil management. In South Africa, saline soils have been documented and there is a demand in increasing salt tolerance in crops cultivated in such regions (De Clercq and Van Meirvenne, 2005).

The deleterious effect of high salinity levels on plants can be attributed to several factors such as its capacity to disrupt the ability of roots to extract water hence inducing water stress, membrane disorganization, and inhibition of several physiological and biochemical processes (Hasegawa et al., 2000; Munns, 2002). A combination of these factors thus results in reduced plant growth, development and survival. Furthermore, it is possible that plants which are exposed to long term salinity could experience both ionic toxicity and osmotic stress which will subsequently lead to nutritional disorder and oxidative stress (Zhu, 2003). The plants could subsequently undergo oxidative damage due to increased rate of production of reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen ($^1O_2$) (Gueta, 1997; Grant and Loake, 2000). Excessive accumulation of ROS subsequently leads to inhibition of cell division, expansion and death (Zhu, 2001).

Soybean (*Glycine max*) is one of the most important leguminous plants used as a source of vegetable protein and oil because of its high protein and oil content (Moussa, 2004). Soybean is mostly referred to as a moderately salt sensitive plant and hence can be severely affected by highly saline soils (Katerji et al., 2000). Studies have shown the drastic effect of high salt levels in inhibiting soybean seed germination and seedling growth, reduction of nodulation and decrease in crop yield (Essa, 2002), reducing the number and weight of root nodules in soybean and other legumes (Lakstmi et al., 1974; Lauter et al., 1981; Tu, 1981) and in reducing leaf area and lower root/shoot ratios (Rahman et al., 2008). However, some studies have investigated the ability of plants to tolerate high salinity levels by assessing the percentage

Abbreviations: APX, ascorbate peroxidase; DETA, diethylenetriamine; DETA/NO, 2,2′(hydroxynitrosohydrazono) bis-ethanimine; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NO, nitric oxide; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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biomass production in saline against control conditions after a long term exposure (Greenway and Munns, 1980).

Dalton et al. (1986, 1993) intimated that in order to minimize the effects of oxidative stress induced by salinity, plants have developed different mechanisms to remove or reduce the accumulation of ROS. This involves the mobilization of an array of antioxidant enzymes and several antioxidant metabolites that function in neutralizing and detoxifying ROS. Amongst the antioxidant enzymes, ascorbate peroxidase (APX) plays a major role in regulating the level of H_2O_2, by catalyzing its conversion into H_2O through utilizing ascorbate as its electron donor (Dalton et al., 1986, 1993; Iturbe et al., 2001; Matamoros et al., 2006). There are studies showing increased enzymatic activities of several antioxidant enzymes in plants under salt stress (Lechno and Zamki, 1997; Hermandez et al., 1999; Meloni et al., 2003).

Nitric oxide (NO) is a signaling molecule that is involved in several physiological processes in plants. Apart from its role in promoting normal growth and development of plants at low concentrations (Beligni and Lamattina, 2001), there are several lines of evidence supporting its role in alleviating the oxidative damage of salinity in several plant species (Uchida et al., 2002; Zhang et al., 2006; Guo et al., 2009).

Although studies showing the effect of exogenous application of NO in inducing salinity tolerance in plants have been reported, there are only a few studies showing this protective role over a long-term salinity exposure in soybean. This study aimed at analyzing growth parameters (root nodule number and dry weight, shoot and root dry weights) of the plants grown under salinity-stress and exogenously applied NO donor — DETA/NO, 2,2′(hydroxynitrosohydrazono) bis-ethanimine. We evaluated cell viability, H_2O_2 content and the enzymatic activity of APX isoforms in soybean root nodules in relation to the role of NO in alleviating oxidative stress induced by high salinity.

2. Materials and methods

2.1. Plant growth

Plant growth and treatments were done by modifying a previously described method by Leach et al. (2010). Soybean (*G. max* L. Merr. cv. PAN 626) seeds were surface-sterilized in 0.35% sodium hypochlorite for 10 min, followed by five washes with sterile distilled water. The seeds were imbibed in sterile distilled water for 1 h and inoculated with *Bradyrhizobium japonicum* supplied as the commercial peat-based HiStick 2 Soybean Inoculant (Becker Underwood Ltd.). Seeds were sown in filtered silica sand that had been pre-soaked in distilled water, in 15 cm diameter plastic pots (one plant per pot) and were grouped into six experimental test sets: Untreated, 10 μM DETA/NO (a nitric oxide donor), 10 μM DETA (which lacks the NO moiety and serves as a control for NO treatments), 80 mM NaCl, 10 μM DETA/NO + 80 mM NaCl and 10 μM DETA + 80 mM NaCl. The germinated seedlings were grown on a 25/19 °C day/night temperature cycle under a 16/8 hour light/dark cycle, at a photosynthetic photon flux density of 300 μmol photons·m^{-2}·s^{-1} during the day phase, in a randomized design.

2.2. Treatment of plants

At VC stage (when unifoliolate leaves are fully expanded) the untreated were supplied with nutrient solution every third day. For the NO and control treatment, the nutrient solution was supplemented with a final concentration of 10 μM DETA/NO and 10 μM DETA respectively every third day. For salt treatments (salt alone, salt combined with either 10 μM DETA/NO or 10 μM DETA), the nutrient solution was supplemented with an incremental concentration of salt starting from a final concentration of 10 mM NaCl, 20 mM NaCl, 40 mM NaCl and 80 mM NaCl each at a three day interval respectively. The treatment with 80 mM NaCl was repeated twice (so that the total number of days of plant exposure to NaCl was 18 days). Plants were harvested one day (24 h) after this final treatment.

The freshly harvested plants were used for measurement of growth parameters and nodule cell viability whereas the snap-frozen (in liquid nitrogen) nodules were used for all other assays (in which case the tissue was stored at −80 °C until further use).

2.3. Measurement of growth parameters

Plants were carefully removed from the sand, avoiding any loss of roots or shoot during the process of harvesting. The root nodules from each set of the treatments were carefully detached from the roots and counted. The roots were cut off from the shoots and all fresh materials (roots, shoots and nodules) were weighed. The samples were oven-dried at −80 °C for three days and the dry weights were recorded using a Mettler digital analytical balance.

2.4. Evaluation of cell viability in soybean root nodules

In order to establish if application of NO (as 10 μM DETA/NO) could maintain root nodule cell viability after long term salinity stress, evaluation of root nodule cell viability was carried out. This cell viability assay was estimated in soybean root nodules by modifying a method previously described by Sanevas et al. (2007). The root nodules (100 mg per treatment) from three different plants of each of the six treatments were harvested and stained with 0.25% (w/v) Evans Blue for 15 min at room temperature. The root nodules were then washed for 30 min in distilled water, followed by extraction of the Evans Blue stain (taken up by dead nodule cells) from root nodule tissue using 1% (w/v) sodium dodecyl sulfate (SDS) after incubation for 1 h at 55 °C. Absorbance of the extract was measured using a spectrophotometer at 600 nm to determine the level of Evans Blue taken up by the root nodule tissue.

2.5. Measurement of H_2O_2 content

Hydrogen peroxide content was determined in the nodule extracts by modifying a previously described method by Velikova et al. (2000). *G. max* nodule tissue (100 mg) was ground to fine powder in liquid nitrogen and homogenized in 400 μl of cold 6% (w/v) trichloroacetic acid (TCA). The extracts were centrifuged at 12,000 ×*g* for 30 min at 4 °C and 50 μl of the supernatant was used to initiate the reaction in a mixture (total volume of 200 μl) containing 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI. The reaction was incubated at 25 °C for 20 min and absorbance readings were recorded at 390 nm. H_2O_2 content was calculated using a standard curve based on the absorbance ($A_{390\ nm}$) of H_2O_2 standards.

2.6. Determination of APX enzymatic activity

Plant APX activities were measured in nodule extracts by using a method previously described by Asada (1984). The nodule extracts which were supplemented with ascorbate to a final concentration of 2 mM were added to the assay buffer containing 50 mM K_2HPO_4, pH 7.0, 0.1 mM EDTA and 50 mM ascorbate. The reaction was initiated by adding 1.2 mM H_2O_2 in a final reaction volume of 200 μl and APX activity was calculated based on the change in absorbance at 290 nm using the extinction co-efficient of 2.8 mM^{-1} cm^{-1}.

2.7. Determination of protein concentration

Protein concentrations for all assays were measured in the extracts as instructed for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories).