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Nitric oxide enhances salt tolerance in cucumber seedlings by regulating free polyamine content

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ABSTRACT

Nitric oxide (NO), an endogenous signaling molecule in plants and animals, mediates responses to abiotic and biotic stresses. This study was conducted in nutrient solution to investigate the effects of exogenous sodium nitroprusside (SNP), an NO donor, on plant growth and free polyamine content in cucumber leaves and roots under NaCl stress. The results showed that 100 μM SNP in solution significantly improved the growth of cucumber seedlings under NaCl stress for 8 days, as indicated by increased, plant height, stem thickness, fresh weight and increased dry matter accumulation. Further analysis demonstrated that the content of free polyamines and the activity of polyamine oxidase (PAO) in cucumber seedling leaves and roots initially increased dramatically under NaCl stress, although they decreased over a longer period of stress. Throughout the treatment period, the value of (spermine + spermidine)/putrescine [(Spd + Spm)/Put] also decreased under NaCl stress compared to the control. In contrast, the application of 100 μM SNP in the nutrient solution decreased the content of free Put, Spd, total free polyamines and PAO activity under NaCl stress. It also caused an increase in the content of Spm and the value of (Spd + Spm)/Put, adjusted the ratio of three kinds of free polyamines (Put, Spd, Spm) in cucumber seedlings. The high (Spd + Spm)/Put value and the accumulation of Spm were beneficial to improving the salt tolerance of plants. Therefore, NO alleviated the damage to cucumber seedlings caused by salt stress. NO enhanced the tolerance of cucumber seedlings to NaCl stress by regulating the content and proportions of the different types of free polyamines.

1. Introduction

Salinity is one of the major environmental factors limiting plant growth and yield (Parida and Das, 2005); especially in countries where irrigation is essential to agriculture. Several physiological processes in plants are affected by salinity. When plants are exposed to NaCl, salt stress disturbs the intracellular ion homeostasis, which leads to membrane dysfunction, metabolic disorder, and secondary effects that cause growth inhibition and lead to ultimately cell death (Hasegawa et al., 2000). Over the course of evolution, plants have developed a variety of molecular mechanisms to cope with salt stress. Mechanisms that enable plants to survive in high salt soils include (1) the selective accumulation or exclusion of ions, (2) control of ion uptake by roots and transport into leaves, (3) compartmentalization of ions at the cellular and whole-plant level, (4) synthesis of compatible solutes, (5) changes in the photosynthetic pathway, (6) alterations in membrane structures, (7) induction of anti-oxidative enzymes, and (8) induction of plant hormones. These salt tolerance mechanisms appear to involve changes in many biochemical pathways, and also involve changes that protect major processes such as photosynthesis and respiration (Parida and Das, 2005). Accordingly, an exogenous substance that could similarly regulate these biochemical pathways as well as protect the major processes may have the ability to mediate plant tolerance to salt stress.

Nitric oxide (NO) is a small, highly diffusible gaseous free radical and a ubiquitous bioactive molecule that plays a key role as an intra- and inter-cellular messenger that induces various processes via either redox or additive chemistry in plants. These processes include respiration (Millar and Day, 1996), photomorphogenic responses (Beligni and Lamattina, 2000), seed germination (Giba et al., 1998), senescence (Leshem et al., 1998), stress responses (Ruan et al., 2004; Qiao et al., 2009), programmed cell death (Beligni et al., 2002), and disease resistance (Durner et al., 1998; Van Camp et al., 1998; Chandok et al., 2003). Along with other plant growth regulators (such as abscisic acid or jasmonates) in different plants, NO may function to either mitigate or exacerbate the effects of stres-

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sors (Leshem and Kuiper, 1996). In recent years, there is increasing evidence demonstrating that NO serves as a signal in developmental, hormonal, and environmental responses in plants (Gouvêa et al., 1997; García-Mata and Lamattina, 2001; Beligni et al., 2002; Graziano et al., 2002; Guo et al., 2003; He et al., 2004; Pagnussat et al., 2004; Hu et al., 2005; Creus et al., 2005). It has been postulated that NO might function as an antioxidant by scavenging reactive oxygen species (ROS), such as superoxide radicals, and thereby limit cellular damage (Laspina et al., 2005). NO could also function as a signaling compound in molecular cascades leading to changes in gene expression (Lamattina et al., 2003).

Polyamines are ubiquitous low molecular weight aliphatic amines that exist widely in plants and are involved in the regulation of plant growth and development. Due to their polycationic nature at physiological pH levels, polyamines are able to interact with proteins, nucleic acids, membrane phospholipids and cell wall constituents; either activating or stabilizing these molecules. Recent studies have focused on the involvement of polyamines in the defense reactions of higher plants to various types of environmental stress (Bouchereau et al., 1999). Polyamine levels correlate with NO because L-arginine is a common precursor in their biosynthesis (Gao et al., 2009). A recent study presents evidence that polyamines induce the production of NO in *Arabidopsis thaliana* and reports that NO could be a link between the polyamine-mediated stress response and other stress mediators (Tun et al., 2006). Groppa et al. (2008) obtained similar results in wheat roots.

Cucumber, an important horticultural crop, was selected as the test plant for this study because it is highly sensitive to salinity, especially at germination and as a seedling (Baysal and Tipirdamaz, 2004). In previous studies (Fan et al., 2007), we demonstrated that the application of 100 μM exogenous SNP could alleviate the damage from salt stress via the regulation of the metabolism of ROS in cucumber leaves. Therefore, in this study, we investigated the effects of NO on the content of polyamines and in related enzyme activity in cucumber seedlings under NaCl stress. The goal of this study was to elucidate the physiological mechanism of the increased tolerance of cucumber plants to salt stress due to the application of exogenous NO.

2. Materials and methods

2.1. Materials and treatments

The experiments were carried out in a greenhouse at the Nanjing Agricultural University in China. Cucumber (*Cucumis sativus* L. cv. Jinchun 2) seeds were placed in sterile Petri plates on filter paper moistened with distilled water. They were allowed to germinate in the dark in a thermostatically controlled chamber at 29 ± 1 °C for approximately 30 h. The germinated seeds were sown in washed quartz sands. The average temperatures were 25–30 °C/16–20 °C (day/night) under natural light. Relative humidity fluctuated between 60% and 70%. At the second fully expanded-leaf stage, the cucumber leaves were numbered from the apical to basal nodes. Seedlings were taken out of the plastic plates, and the roots were rinsed with distilled water. Uniformly sized healthy seedlings were selected and transferred into troughs (51 cm × 33 cm × 20 cm) filled with 20 L of full-strength Hoagland's nutrient solution, which was aerated for 40 min each hour. After pre-culturing for 3 days, the seedlings were treated with one of the following methods: (1) control: full-strength Hoagland's nutrient solution; (2) NaCl treatment: full-strength Hoagland's nutrient solution containing 50 mM NaCl; (3) SNP treatment: full-strength Hoagland's nutrient solution containing 50 mM NaCl with 100 μM SNP; (4) $NaNO_2$ treatment: full-strength Hoagland's nutrient solution containing 50 mM NaCl with 1 μM $NaNO_2$. SNP was used as the NO donor. $NaNO_2$ treatment was carried out as a second control because the decomposition of 100 μM SNP generates a maximum of 1 μM NO_2^- as a by-product. Troughs were arranged in a completely randomized block design with three replicates for each treatment (for a total of 12 troughs). All the nutrient solutions were renewed every 2 days to maintain the identical concentrations.

Leaf and root samples from healthy cucumber seedling were harvested in triplicate at 0, 2, 4, 6, and 8 days after the treatment initiation and immediately analyzed. After 8 days of treatment, 15 plants per treatment were collected for the determination of plant height, stem thickness, fresh weight and dry weight.

2.2. Determination of growth

Plant height was measured by a ruler with an accuracy of 1 mm from cotyledons to apical point. Stem thickness was measured by a vernier caliper with an accuracy of 0.05 mm at a consistent point 1 cm below cotyledons. The cucumber seedlings were washed with tap water two to three times, rinsed twice with distilled water, gently blotted dry with a paper towel, and weighed for fresh weight. Samples were incubated at 105 °C for 15 min, then at 75 °C until they reached a constant weight, and then weighed for dry weight.

2.3. Polyamine analysis

Polyamines were extracted according to Sharma and Rajam (1995), with some modifications. Fresh leaf and root samples were homogenized in 3.2 mL of 5% (v/v) cold perchloric acid (PCA) and incubated at 4 °C for 1 h. 1,6-Hexanediamine was added to the homogenate as the internal standard. The homogenate was then centrifuged at 12,000 × *g* for 30 min at 4 °C and the supernatant used to quantify free polyamines.

Polyamines were assayed by high-performance liquid chromatography. Ten microliters of a methanol solution containing benzoyl polyamines was injected into a 20 μL loop, loaded onto a 4.6 mm × 250 mm, 5 μm particle size C18 reverse-phase column (Kromasil). The column temperature was maintained at 25 °C. Samples were eluted from the column with 64% methanol at a flow rate of 0.8 mL min^{-1} using a Dionex P680 pump. Polyamine peaks were detected at 254 nm using a UV detector.

The total content of free polyamines equals to the sum of free Put content, free Spd content and free Spm content. The value of (Spd + Spm)/Put equals to the sum of free Spd and free Spm content to free Put content.

2.4. Polyamine oxidase activity assay

PAO activity was determined by measuring the generation of H_2O_2, a product of polyamine oxidation, as described by Su et al. (2005), with some modifications. Fresh samples were homogenized in 100 mM potassium phosphate buffer (pH 6.5). The homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was used for the enzyme assay. The reaction mixture contained 2.5 mL potassium phosphate buffer (100 mM, pH 6.5), 0.2 mL 4-aminoantipyrine/*N*,*N*-dimethylaniline reaction solutions, 0.1 mL horseradish peroxidase (250 U mL^{-1}) and 0.2 mL of the enzyme extract. The reaction was initiated by the addition of 15 μL Spd + Spm (final concentrations of 20 mM each) for the PAO determination. 0.001 absorbance unit of the change in optical density at 555 nm min^{-1} was considered one unit of enzyme activity.

2.5. Statistical analysis

All data presented are the mean values. All experiments other than the growth were conducted using three replicates. Growth determinations were performed with 15 replicates. Statistical