Exogenous Spermidine Inhibits Ethylene Production in Leaves of Cucumber Seedlings under NaCl Stress

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ABSTRACT. To examine whether 1 mM of spermidine (Spd) modifies plant ethylene production in response to short-term salt stress, cucumber (Cucumis sativus) seedlings were grown in nutrient solution with or without 75 mM NaCl stress for 3 days, and the leaves were sprayed with 1 mM Spd or water (control). We investigate the effects of the treatments on ethylene production, 1-aminocyclopropane-1-carboxylate (ACC) content, 1-(malonylaminomalononitrile) cyclop propane-1-carboxylic acid (MACC) content, activities of 1-aminocyclopropane-1-carboxylate synthase (ACS), and 1-aminocyclopropane-1-carboxylate oxidase (ACO) and gene expression of aco1, aco2, and acs2 in the cucumber leaves. The results indicate that ethylene production was increased significantly under salt stress as did ACC and MACC content, the activities of ACS and ACO, and the transcriptional level of acs2, whereas the gene expression of aco1 and aco2 was somewhat decreased. However, exogenous Spd treatment depressed the content of ACC and MACC, ACS activity, and the level of acs2 transcripts in the leaves of salt-stressed cucumber. Although the activity of ACO and gene expressions of aco1 and aco2 increased by Spd, ethylene emission was inhibited. Our results suggest that application of exogenous Spd could reverse salinity-induced ethylene production by inhibiting the transcription and activity of ACS under salt stress. We conclude that exogenous Spd could modify the biosynthesis of ethylene to enhance the tolerance of cucumber seedlings to salt stress.

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Salt stress poses a major environmental threat to agricultural production. At high concentrations of salts in the soil, plants experience a physiological drought because of the inability of roots to extract water, and high concentrations of salts within the plant can be toxic (Munns and Tester, 2008). When plants are exposed to environmental stresses, plants typically synthesize increased levels of the phytohormone ethylene and are often unable to grow and proliferate to any great extent, at least until the stress is removed and the ethylene level is lowered (Gamalero and Glick, 2012). Various biotic and abiotic stresses can cause an imbalance in ethylene production, and increased ethylene levels can cause leaf senescence and inhibit root elongation and overall plant growth (Bacilio et al., 2004; Cheng et al., 2007; Klasson, 2002; Mayak et al., 2004; Zahir et al., 2009).

There are several steps in ethylene biosynthesis. Two of the most important biosynthetic enzymes are 1-aminocyclopropane-1-carboxylate synthase, which controls production of 1-aminocyclopropane-1-carboxylate, and 1-aminocyclopropane-1-carboxylate oxidase, controlling oxidative degradation of ACC. ACC is the immediate precursor of ethylene, and it is converted by S-adenosylmethionine (SAM). Some ACC is conjugated with malonate to form 1-(malonylaminomalononitrile) cyclop propane-1-carboxylic acid, which cannot be converted back to ACC. The formation of ACC is often cited as the rate-limiting step in ethylene biosynthesis (Kende, 1993), and lower accumulation of ACC diminishes the negative effects of stress-induced ethylene, resulting in better plant growth (Penrose and Glick, 2001).

Polymamines (PAs) are low-molecular-weight aliphatic amines that are ubiquitous in all organisms. Common natural PAs include the higher PAs, spermine (Spm) and spermidine, and their diamine obligate precursor putrescine. In plants, PAs have been implicated in many physiological processes such as organogenesis, embryogenesis, floral initiation and development, leaf senescence, fruit development and ripening, and abiotic and biotic plant stress responses (Alcazar et al., 2006; Bagh et al., 2001; Bouchereau et al., 1999; Galston and Sawhney, 1990; Kumar et al., 1997; Kusano et al., 2008; Malmberg et al., 1998; Walden et al., 1997). PAs and ethylene biosynthetic pathways share the same precursor, SAM (Slocum et al., 1984). Therefore, there may be an antagonistic relationship between biosyntheses of these two types of compounds. PAs can inhibit biosynthesis of ethylene in higher plant tissues and fruit protoplasts (Apelbaum et al., 1981; Roberts et al., 1984). However, in another study, there was no competition between PAs and ethylene biosyntheses (Mathooko et al., 1995).

Cucumber is one of the most important vegetables worldwide. It is highly sensitive to salinity, especially its germination and early growth phases (Baysal and Tipirdamaz, 2004). It has been showed that exogenous Spd can improve salt tolerance in cucumber (Du et al., 2010; Duan et al., 2008). Little is known about the effects of exogenous PAs on ethylene production in cucumber seedlings under salt stress. Therefore, we investigated whether an exogenous PA, Spd, could modify ethylene production and enhance growth and salt tolerance of cucumber seedlings.

Materials and Methods

PLANT MATERIAL AND STRESS TREATMENTS. Cucumber (cv. Jinchun No. 2) seeds were germinated on moist filter paper in...
the dark at 28 °C for 30 h. The germinated seedlings were transferred to plastic trays (41 × 41 × 5 cm) containing quartz sand and were grown in a greenhouse at Nanjing Agricultural University at 25 to 30 °C (day) and 15 to 18 °C (night) under natural light with relative humidity of 60% to 75%. When the cotyledons had expanded, seedlings were supplied with water containing half-strength Hoagland’s nutrient solution, and the solution was renewed every 2 d. The nutrient solutions were kept at 20 to 25 °C and were continuously aerated using an air pump at an interval of 20 min to maintain the dissolved oxygen at 8.0 ± 0.2 mg L⁻¹.

After 3 d of pre-culture, the cucumber seedlings were treated as follows: 1) control plants were grown in Hoagland’s solution and the leaves were sprayed with H₂O (C); 2) plants were grown in Hoagland’s solution and the leaves were sprayed with 1 mM Spd (CS); 3) plants were grown in Hoagland’s solution containing 75 mM NaCl and the leaves were sprayed with H₂O (S); and 4) plants were grown in Hoagland’s solution containing 75 mM NaCl and the leaves were sprayed with 1 mM Spd (SS). The leaves of the control and salt-treated plants were transferred to plastic trays (41 cm × 17.6 cm) and were continuously aerated with 50% relative humidity of 20 to 25 °C (day) and 15 to 18 °C (night) under natural light with relative humidity of 60% to 75%. When the University at 25 to 30 °C (night) under natural light with relative humidity of 60% to 75%. When the cotyledons had expanded, seedlings were supplied with water containing half-strength Hoagland’s nutrient solution, and the solution was renewed every 2 d. The nutrient solutions were kept at 20 to 25 °C and were continuously aerated using an air pump at an interval of 20 min to maintain the dissolved oxygen at 8.0 ± 0.2 mg L⁻¹.

After 3 d of pre-culture, the cucumber seedlings were treated as follows: 1) control plants were grown in Hoagland’s solution and the leaves were sprayed with H₂O (C); 2) plants were grown in Hoagland’s solution and the leaves were sprayed with 1 mM Spd (CS); 3) plants were grown in Hoagland’s solution containing 75 mM NaCl and the leaves were sprayed with H₂O (S); and 4) plants were grown in Hoagland’s solution containing 75 mM NaCl and the leaves were sprayed with 1 mM Spd (SS). The leaves of the control and salt-treated plants were transferred to plastic trays (41 cm × 17.6 cm) and were continuously aerated with 50% relative humidity of 20 to 25 °C (day) and 15 to 18 °C (night) under natural light with relative humidity of 60% to 75%. When the cotyledons had expanded, seedlings were supplied with water containing half-strength Hoagland’s nutrient solution, and the solution was renewed every 2 d. The nutrient solutions were kept at 20 to 25 °C and were continuously aerated using an air pump at an interval of 20 min to maintain the dissolved oxygen at 8.0 ± 0.2 mg L⁻¹. After the addition of water-insoluble polyvinylpolypyrrolidone (2%, w/v) and vortexing for 10 s, the extract was centrifuged at 20,000 g for 10 min at 4 °C. The supernatant was gel-filtered on a Sephadex G-25 (Shoude, Nanjing, China) column equilibrated with 5 mM EPPS (pH 8.5), 1 mM DL-dithiothreitol, 5 μM pyridoxal phosphate, and protease inhibitors. The protein content of the extracts was determined according to the method of Bradford (1976), and then all extracts were adjusted to the same protein concentration. Activity of ACS was assayed in glass flasks containing 0.4 mL protein extract and final concentrations of 80 mM EPPS (pH 8.5), 20 μM pyridoxal phosphate, and 100 μM SAM (Shenggang, Shanghai, China) in a total volume of 0.5 mL at 30 °C for 2 h. Blanks omitting SAM were incubated in parallel. The reaction was stopped by adding 100 μL 10 mM HgCl₂ on ice. ACC was converted to ethylene as described previously, and 1 mL of the gas was analyzed by gas chromatography. Activity of ACS is expressed as nanomoles ACC per gram protein per hour.

**ASSAY OF ACO ACTIVITY.** ACO enzyme activity was assayed according to Kato et al. (2000). Leaves (0.5 g) were homogenized in 1 mL extraction buffer consisting of 0.1 M Tris-HCl, pH 7.5, 5 mM dithiothreitol, 30 mM Na-ascorbate, and 10% glycerol (v/w) at 4 °C. The homogenate was centrifuged at 14,000 g for 20 min at 4 °C. The protein content of supernatants was determined, and then all extracts were adjusted to the same protein concentration. ACO activity was measured as described previously (Kato and Hyodo, 1999) and is expressed as micromole ethylene per gram protein per hour.

**RT-PCR ASSAY.** Total RNA was extracted from leaves using the Trizol reagent protocol (Takara Bio, Shiga, Japan). For all samples, total RNA (1 μg) was converted to cDNA using the Superscript first-strand synthesis system for reverse transcription–polymerase chain reaction (PCR) according to the manufacturer’s instructions (Takara Bio). Primers were designed according to sequences obtained from National Center for Biotechnology Information and a cucumber database (Huang et al., 2009). The following primers were used for amplifications: for *acs2*, F: 5'-ATGGGAAAAATGTGAGGGA-3' and A: 5'-AAGACAGTGCCAGGCTAGA-3'; for *aco1*, F: 5'-TGCGTCTTCTTCTATCA-3' and A: 5'-CGTCCAGTTCCACCTTGT-3'; for *aco2*, F: 5'-CACCTTGCTCAACCGG3'- and A: 5'-TTTATTTCCTTCCGCCG-3'; and for *actin*, F: 5'-CTGTGTTGGAAGGGTCA-3' and A: 5'-GGGATCTTATTTTGAGACG-3'. The PCR conditions were optimized for each primer set. PCR amplification was carried out after denaturing cDNA at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing temperature for 30 s, and extension at 72 °C for 35 s. The final PCR extension step was at 72 °C for 7 min. The amplified cDNA fragments were detected using agarose gel electrophoresis.

**RESULTS**

**ETHYLENE PRODUCTION.** Ethylene production increased by 142.67% in leaves of salt-stressed plants compared with that of the control. Exogenous Spd reduced salinity-induced ethylene production by 29.78%, but it did not significantly affect ethylene production in plants grown in Hoagland’s solution (Fig. 1).

**ACC AND MACC CONTENT.** Compared with the control, ACC and MACC content in the cucumber leaves increased significantly by 108.18% (Fig. 2A) and 42.6% (Fig. 2B), respectively.
under salt stress. Exogenous Spd reduced salinity-induced ACC and MACC content by 34.93% (Fig. 2A) and 48.01% (Fig. 2B), respectively, but it did not significantly affect ACC content in leaves of plants grown in normoxic nutrient solution (Fig. 2A). Exogenous Spd decreased the MACC content by 39.82% in normoxic nutrient solution compared with the control (Fig. 2B).

**ACS AND ACO ACTIVITIES.** The activities of ACS and ACO in the leaves were markedly increased under salt stress compared with the control (Fig. 3). Exogenous Spd reduced ACS activity by 43.03% under salt stress, whereas it did not significantly affect its activity in plants grown in normoxic nutrient solution (Fig. 3A). However, exogenous Spd increased ACO activity by 165.8% in plants grown in normoxic nutrient solution and by 59.2% in plants under salt stress (Fig. 3B).

**EXPRESSIONS OF GENES ENCODING ACS AND ACO.** Expression of *acs2* was up-regulated in salt-stressed cucumber leaves, whereas exogenous Spd decreased its transcription in plants in
Hoagland’s solution and under salt stress. Expressions of aco1 and aco2 were somewhat downregulated by salt stress, whereas after application of exogenous Spd, their mRNA levels were increased (Fig. 4).

**Discussion**

Growth inhibition is one of the most common symptoms observed in plants subjected to environmental stress factors. According to our previous studies, exposure of cucumber seedlings to NaCl stress led to considerable declines in growth, whereas exogenous Spd alleviated the inhibition of plant biomass (Du et al., 2010; Duan et al., 2008).

Ethylene is a stress hormone involved in many stress responses. It regulates many aspects of the plant life cycle, including seed germination, root initiation, root hair development, flower development, sex determination, fruit ripening, senescence, and responses to biotic and abiotic stresses (Lin et al., 2009). Ethylene is produced by all cells during plant development, but the rates of production vary, with the highest rates being associated with meristematic, stressed, or ripening tissues. Although ethylene was believed to be the signaling molecule regulating the salt tolerance response activated by certain receptors such as ETR1, ETR2, ERS1, ERS2, and EIN4 (Cao et al., 2008), excessive ethylene production induced by salt stress can aggravate and inhibit plant growth. It has been suggested that many steps in ethylene biosynthesis are activated by abiotic stresses. For example, the rate of ethylene production dramatically increased under water deficit stress in excised wheat (*Triticum aestivum*) and was accompanied by increased levels of ACC and MACC (Hoffman et al., 1983). Hypoxia stress stimulated ACS activity and increased ACC levels and ethylene production rates (Wang and Arteca, 1992). The changes in ACC content, ACS and ACO activities, level of ACS gene transcripts, and ethylene production were consistent in response to ultraviolet-B irradiation in tomato (*Solanum lycopersicum*) leaves (An et al., 2006). Our results also showed that ethylene production significantly increased in leaves of salt-stressed cucumber seedlings (Fig. 1). We found that under salt stress, ACC synthesis and consumption were activated, together with increased activities of ACS and ACO, and there were higher levels of aco2 transcripts (Fig. 4). In contrast, the expressions of the genes encoding ACO were slightly downregulated. These results indicate that ACO is not regulated at the transcriptional level in cucumber under salt stress but may be regulated at the post-translational level.

When exposed to various types of abiotic and biotic stresses, increased ethylene levels correspond to increased damage in plants, implying that stress-induced ethylene is deleterious to plants. Thus, many researchers have attempted to reduce ethylene production by various approaches. For example, coinoculation with rhizobacteria containing ACC deaminase improved growth and nodulation in mung bean (*Vigna radiata*) under salt stress (Ahmad et al., 2011). Siddikee et al. (2011) found that rhizobacteria with ACC deaminase activity enhanced the growth and salt tolerance of red pepper (*Capsicum annuum*) seedlings by reducing salt stress-induced ethylene production. Wi et al. (2010) used transgenic tobacco (*Nicotiana tabacum*) expressing an antisense transcript of the ACC synthase gene, which inhibited ethylene production. This resulted in enhanced tolerance to abiotic stress and reduced accumulation of reactive oxygen species. Fuhrer et al. (1982) showed that exogenous spermidine inhibited ethylene biosynthesis and delayed senescence of oat (*Avena sativa*) leaves. The results of our study showed that application of exogenous Spd to salt-stressed cucumber seedlings resulted in decreased activity of ACS, decreased expression of aco2, and reduced levels of ACC. These results indicate that exogenous Spd significantly inhibited ACC synthesis under salt stress, which is consistent with the findings of Apelbaum et al. (1981) and Even-Chen et al. (1982), who also reported that polyamines inhibited ACC synthesis to decrease ethylene production. In addition, we observed that exogenous Spd downregulated expression of aco2 under non-stress conditions (Fig. 4). This might be ascribed to regulation in transcription of some genes encoding ethylene biosynthetic enzymes by polyamines. In other studies, transgenic tomato overexpressing the spermidine

![Fig. 4. Effect of salt stress and/or exogenous application of 1 mM spermidine (Spd) on expression of aco2, aco1, and aco2 in leaves of cucumber seedlings after 3-d treatments. C = control; CS = control + Spd foliar spray; S = 75 mM NaCl; SS = 75 mM NaCl + Spd foliar spray. Transcript abundance was determined (A), and the relative abundance ratio of genes was analyzed (B) using actin as the internal standard. Different letters indicate significant differences (Duncan’s multiple range test at P < 0.05).](image-url)
synthase gene accumulated higher levels of Spd and showed downregulation of ACS gene transcripts (Handa et al., 2011; Nambeesan et al., 2012). According to our findings and the results of other studies, we conclude that exogenous Spd inhibits ACC synthesis, which is a crucial step in ethylene production.

Another point of regulation of ethylene production is the consumption of ACC. Some is converted into ethylene by ACO, and some is conjugated to form MACC. In the present study, ACO activity and aco1 and aco2 gene transcripts increased in response to Spd treatments of salt-stressed cucumber seedlings. Meanwhile, exogenous Spd reduced the MACC content in salt-stressed cucumber seedlings, indicating that it inhibited the conversion of ACC into MACC. We also found that exogenous Spd enhanced ACO activity and inhibited MACC accumulation under non-stress conditions. The ACO enzyme activity and gene activation were possibly regulated by Ca²⁺ and phosphoinositides in signal transduction pathway (Jung et al., 2000). In recent years, Spd-derived H₂O₂ has been identified as an important second messenger in signal transduction networks. Wu et al. (2010) found Spd induced an increase in the cytosolic Ca²⁺ concentration, which was exerted through a second messenger. Therefore, the increase of ACO activity and gene by Spd might be ascribed to Spd signal regulation. Although the conversion of ACO activity was enhanced by Spd under salt stress, ethylene emission was still lower than that under salt stress alone. We suggest that in the salt-stressed cucumber system, the inhibition of ethylene production by Spd was mainly through regulating ACS, the enzyme that catalyzes the rate-limiting step in ethylene production.

Higher PAs Spd and Spm biosynthesis and ethylene production used the same substrate SAM. Duan et al. (2008) found the activity of S-adenosylmethionine decarboxylase (SAMDC) was induced by NaCl stress in cucumber, indicating that the conversion of SAM to Spd and Spm was increased. Meanwhile, our results showed that the conversion of SAM to ACC was also increased under salt stress by the induction of ACS activity. However, the syntheses of PAs and ethylene are not competitive with each other in cucumber (Wang, 1987), so the conversion of SAM to PAs and ethylene might be independent under salt stress. Together with the result of Duan et al. (2008), exogenous Spd increased the level of endogenous PA biosynthesis and inhibited the conversion of SAM to ACC under salt stress, which might be the protective roles of exogenous PAs against the salt stress.

In conclusion, our results suggest that salt stress significantly increased the rate of ethylene production, which aggravated damage to plant growth. Application of exogenous Spd reversed the salt-induced ethylene production by inhibiting the transcription and activity of ACS. This indicated that exogenous Spd could modify ethylene biosynthesis to enhance the salt tolerance of cucumber seedlings.

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