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BRIEF COMMUNICATION

Effects of 5-aminolevulinic acid on the H_2O_2-content and antioxidative enzyme gene expression in NaCl-treated cucumber seedlings

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Abstract

The potential of 5-aminolevulenic acid (ALA) to enhance the salt tolerance of cucumber (*Cucumis sativus* L.) seedlings was investigated. ALA was applied at various concentrations (0, 1, 10, 25, 50, and 100 mg dm^{-3}) as foliar spray or root watering. Then the seedlings were exposed to 0 or 75 mM NaCl for 5 d. NaCl stress reduced the root and leaf dry masses, leaf area, and the leaf net CO_2 assimilation rate. These reductions were counteracted by exogenous ALA, and the most efficient was 50 mg dm^{-3} concentration *via* foliar spray. ALA decreased the H_2O_2 contents and increased the activities of ascorbate peroxidase (APX) and glutathione reductase (GR) in NaCl-treated cucumber roots and leaves and the activity of catalase (CAT) in leaves. The ALA application also up-regulated the expressions of *CAT* and *cAPX* genes in roots and leaves and the expression of *GR* gene in roots of the NaCl treated cucumber plants.

Additional key words: ascorbate peroxidase, catalase, *Cucumis sativus*, gene expression, glutathione reductase, salinity.

Salinity induces various biochemical and physiological responses in plants and affects almost all plant functions including photosynthesis, growth, and development (Zhu *et al.* 2008, Zuccarini 2008, Aghaleh *et al.* 2009). Of these effects, the production of reactive oxygen species (ROS) is a common phenomenon (Ashraf 2009). ROS can seriously disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids. Among these ROS, hydrogen peroxide as a more stable ROS, can diffuse across biological membranes and severely damages plant metabolism under stress conditions (Ashraf 2009, Kholová *et al.* 2009). To cope with ROS and maintain redox homeostasis, living organisms have developed a well-integrated antioxidant defense system that includes low molecular mass antioxidants (ascorbate, glutathione, *etc*.) and antioxidative enzymes, such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), and the ascorbate-glutathione cycle enzymes: ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), dehydroascorbate reductase (DHAR; EC 1.8.5.1), and monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) (Asada 2006).

Cucumber (*Cucumis sativus* L.) is one of the most popular vegetables under protected cultivation conditions. With the aggravation of soil secondary salinization, the plant growth and fruit yield of cucumber have also been reduced (Stępień and Kłobus 2006, Huang *et al.* 2009). In order to increase the stress tolerance, plant growth regulators (PGRs) are widely applied to agricultural cropst (Mulholland *et al.* 2003, He and Zhu 2008). 5-aminolevulenic acid (ALA), one of the PGRs, is a key precursor in the biosynthesis of all porphyrins such as chlorophyll, heme and phytochrome (Wang *et al.* 2005).

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Abbreviations: ALA - 5-aminolevulinic acid; APX - ascorbate peroxidase; AsA - reduced ascorbic acid; CAT - catalase; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; MDHAR - monodehydroascorbate reductase; PGRs - plant growth regulators; P_N - net photosynthetic rate; ROS - reactive oxygen species; SOD - superoxide dismutase.

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Recent research reported that exogenous applications of ALA can regulate plant growth and development (Watanabe *et al.* 2006). ALA in low concentrations can also improve tolerance to cold (Korkmaz *et al.* 2010) and salinity (Nishihara *et al*. 2003, Zhang *et al.* 2006, Naeem *et al*. 2010) and so has great application potential in agricultural production (Wang *et al.* 2003). However, data on the promotive effects of ALA on salt tolerance in cucumber seedlings are limited. Although ALA has been reported to be capable of enhancing the activity of antioxidant enzymes (Nishihara *et al.* 2003, Memon *et al.* 2009), its regulatory role in expression of their genes in plants under NaCl stress has not yet been studied.

Therefore, the objectives of this study were to evaluate the effects of ALA on the H_2O_2-scavenging system and antioxidant enzyme gene expression and to evaluate the effects of ALA concentration and method that would provide the best protection against NaCl stress in cucumber seedlings.

A salt-sensitive cucumber (*Cucumis sativus* L.) cultivar Jinchun No. 2 (Tianjin Kerun Cucumber Institute, China) was used (Zhu *et al.* 2008). Seeds were sown in 50 seedling plug trays filled with a 2:1 (v/v) mixture of peat and *Perlite*. 15 d after germination, seedlings of similar size with two true leaves were transplanted to 20 dm^3 plastic containers (8 plants per container) containing half-strength Hoagland solution (Hoagland and Arnon 1950) and continuously aerated by air pump. After 10 d pre-culture, half of the seedlings were sprayed with de-ionized water with 0.01 % surfactant *Tween-20* (control, CK) and 1, 10, 25, 50, or 100 mg dm^{-3} ALA (*Sigma Aldrich*, MO, USA) solutions (in 0.01 % surfactant) until both sides of the leaves were completely wet, whereas the other half were drenched in nutrient solutions with a volume of 15 cm^3 per plant ALA solutions between 17:00 and 18:00. On the following day between 7:00 and 8:00, all plants were subjected to 75 mM NaCl stress for 5 d. The electrical conductivity (EC) of the nutrient solutions with 0 and 75 mM NaCl were 2.15 and 8.83 dS m^{-1}, respectively. To avoid salt shock, NaCl was added to the nutrient solutions in increments of 37.5 mM per day until the desired concentrations were obtained. The optimum ALA application concentration and method for alleviating the inhibitory effect induced by NaCl stress were selected based on the data on plant growth and photosynthetic rate. Only 75 mM NaCl concentration was considered for the analysis of stress alleviation role of the chosen ALA concentrations. Treatments were replicated three times with 2 containers per replicate and all treatments were arranged in a randomized complete block design. The climate data in greenhouse under natural irradiance was collected by weather data collection system (*GIS-4-CE-3V*, *Nercita*, Beijing, China), which showed the day maximum photosynthetic photon flux density (PPFD) was 1051.2 μmol m^{-2} s^{-1} (mean 279.5 μmol m^{-2} s^{-1}), air temperature was between 21 and 30 °C (mean 25.5 °C), and relative humidity was between 55 and 85 % (mean 70 %) during the experimental period.

After 5 d NaCl stress, six randomly selected plants per treatment (2 plants per replicate) were separated into roots and shoots. The dry masses of the root and shoot were determined after oven-drying at 75 °C for 48 h. The leaf area was determined using a leaf area meter (*LiCOR 3100,* Lincoln, NB, USA). Leaf net photosynthetic rate (P_N) was measured using a *CIRAS-2* gas-exchange infrared analyzer system (*PP-Systems,* Hitchin, UK). H_2O_2 contents were calculated according to the method described by Brennan and Frenkel (1977). APX and DHAR assays were carried out according to Nakano and Asada (1981), whereas the activities of SOD, CAT, GR, and MDHAR were determined using the methods described by Dhindsa *et al.* (1981), Liu (2000), Foyer and Halliwell (1976) and Hossain *et al.* (1984), respectively. Reduced ascorbic acid (AsA) was determined using the method described by Chen and Wang (2002), whereas reduced glutathione (GSH) was determined as described by Castillo and Greppin (1988).

Total RNA was extracted from cucumber leaves and roots using *Trizol* (*Toyobo*, Osaka, Japan) according to the manufacturer's instructions and then treated with RNase-free DNase to remove contaminating DNA. First-strand cDNA of the total RNA from the leaves and roots was synthesised using *M-MuLV* reverse transcriptase and oligo-$(dT)_{18}$ as the primers following the supplier's recommendation (*Fermentas,* Shenzhen, China). Expression of the target genes in each of the tissues was measured by real-time polymerase chain reaction (RT-PCR). The specific primers (Table 1) used for RT-PCR were designed based on *Genbank* accession Nos. AAZ74666.1, AY274258, D88649 and EF530128 using *Primer express 3.0* software. The *actin* gene was used as an internal control. RT-PCR was performed using a *LightCycler 480 SYBR Green I* master kit (*Roche Diagnostics*, Mannheim, Germany) according to the supplier's protocols. PCR amplification included a 5-min preincubation step at 95 °C, followed by 40 cycles of 95 °C for 10 s, 58 °C for 15 s and 72 °C for 20 s. PCR products were quantified by the *LightCycler 480* RT-PCR

Table 1. Primers used for RT-PCR assays. F indicates forward and R indicates reverse.

Gene	Primer pairs
Actin	F: CCAAGCAGCATGAAGATCAA
	R: ATCTGCTGGAAGGTGCTGAG
CAT	F: GTTGGCTTTCTCTATGTCGTTGGT
	R: GCCTTTGTGTATCCGCATACG
APX	F: ATGGCACTCTGCTGGAACCT
	R: ATGGGCGAGCTCACTTTTGA
GR	F: TGGGATTTGAATGAGAAAGTGGAT
	R: CATTTAATCGGACTATCTCATCTGTCTT

detection system with the *SYBR Green I* master kit (*Roche Diagnostics*).

Statistical analyses were carried out through analysis of variance (*ANOVA*) using *SAS 8.1* software (SAS Institute, Cary, NC, USA), and the means were compared by Duncan's multiple range test at $P \leq 0.05$.

Salt stress often inhibits plant growth and photosynthetic capacity of plants (Stępień and Kłobus 2006, Zuccarini 2008). Similar results were obtained in cucumber seedlings in the present study, which showed significant NaCl-induced reductions in the dry masses of root and shoot, leaf area and P_N values when compared to control (Fig. 1). However, these reductions were counteracted by ALA application. This result is in accordance with the findings by Nishihara *et al.* (2003), Zhang *et al.* (2006) and Naeem *et al.* (2010). The present study also found that foliar application of 50 mg dm^{-3} ALA provided the best protection against NaCl-induced growth reduction. Therefore, the results suggest that application of ALA at the optimum concentration adapting a suitable methodology is necessary to get the desired significant protective effect of the compound

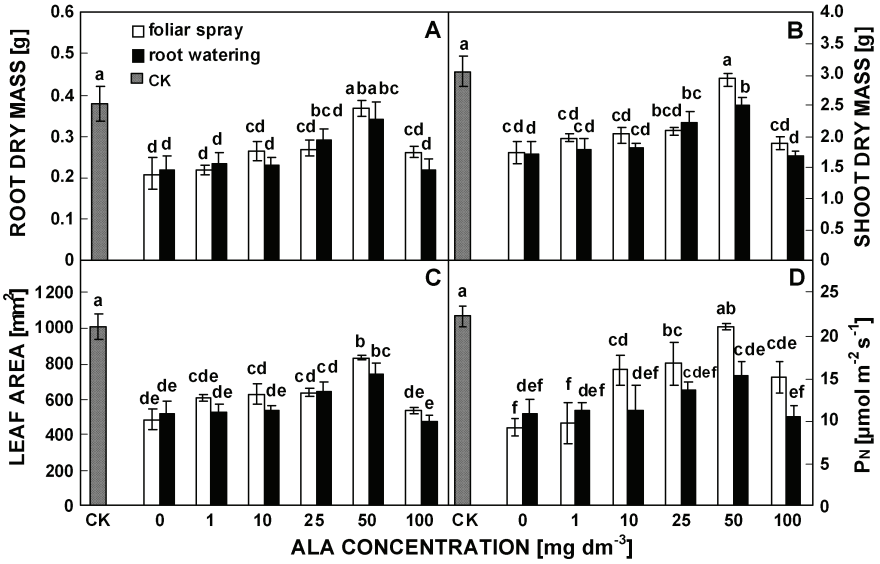

Fig. 1. Effects of ALA application on the root dry mass (*A*), shoot dry mass (*B*), leaf area (*C*) and P_N (*D*) of NaCl-treated cucumber seedlings. ALA was applied in various concentrations (0, 1, 10, 25, 50 and 100 mg dm^{-3}) as foliar spray or root watering. Plants only sprayed with the de-ionized water containing 0.01 % surfactant were taken as control (CK). Values are mean ± SE, $n = 6$. The *bars* marked with the same letters are not significantly different at $P \leq 0.05$, as determined by Duncan's multiple range test.

Table 2. Effects of exogenous ALA and NaCl on H_2O_2 content [µmol g^{-1} (f.m.)]; activities of SOD [U s^{-1} g^{-1}(f.m.)], CAT, APX, GR, MDHAR and DHAR [µmol g^{-1}(f.m.) min^{-1}]; relative transcript expressions of *CAT, cAPX,* and *GR*; and the AsA and GSH contents [µmol g^{-1} (f.m.)] in cucumber roots and leaves. Means ± SE, $n = 3$. Values marked with the same letter are not significantly different at $P \leq 0.05$ (DMRT test). CK - plants only sprayed with the de-ionized water containing 0.01 % surfactant; NaCl, plants exposed to only 75 mM NaCl stress; NaCl+ALA, plants exposed to 75 mM NaCl stress and sprayed with 50 mg dm^{-3} ALA.

Parameters	Roots			Leaves		
	CK	NaCl	NaCl + ALA	CK	NaCl	NaCl + ALA
H_2O_2	4.23±0.30c	8.83±0.79a	6.13±0.05b	12.1 ±0.25b	14.2 ±0.70a	12.1 ±0.62b
SOD	130.0 ±1.02a	129.0 ±1.04a	132.0 ±0.87a	132.0 ±2.22a	132.0 ±1.58a	131.0 ±5.35a
CAT	11.7 ±1.33a	12.0 ±0.58a	13.0 ±1.00a	370.0 ±77.7b	323.0 ±17.5c	463.0 ±5.51a
APX	457.0 ±27.3a	380.0 ±5.77b	433.0 ±24.0ab	520.0 ±20.0b	367.0 ±3.33c	630.0 ±15.3a
GR	250.0 ±10.0a	187.0 ±3.33b	270.0 ±11.6a	277.0 ±20.3a	187.0 ±3.33b	254.0 ±21.9a
MDHAR	14.7 ±1.20a	16.3 ±2.91a	18.3 ±0.88a	22.3 ±0.33a	23.0 ±2.08a	21.3 ±0.88a
DHAR	171.0 ±16.5a	107.0 ±15.0b	129.0 ±17.8ab	152.0 ±5.03a	116.0 ±12.8b	125.0 ±5.70ab
CAT	1.00±0.02	0.73±0.01	1.07±0.04	1.00±0.04	0.51±0.01	0.80±0.04
cAPX	1.00±0.05	0.37±0.01	0.59±0.02	1.00±0.01	0.36±0.02	0.97±0.09
GR	1.00±0.06	1.09±0.04	1.43±0.12	1.00±0.06	0.54±0.03	0.62±0.05
AsA	0.20±0.09b	0.62±0.08a	0.26±0.02b	0.27±0.03b	0.57±0.08a	0.59±0.04a
GSH	0.68±0.02b	0.88±0.02a	0.55±0.09b	1.23±0.18a	1.42±0.07a	1.43±0.30a

against NaCl stress, which is in agreement with the previous suggestion of Korkmaz *et al.* (2010).

NaCl stress increased the H_2O_2 content in the cucumber roots and leaves (Table 2), which is in agreement with the reported of Mandhania *et al.* (2006), Kholová *et al.* (2009), and Mallik *et al.* (2011). Increase in tissue H_2O_2 content is considered as a circumstantial evidence of oxidative stress induced by an abiotic stress (Shaw *et al.* 2004, Kim *et al.* 2005). Hence, it is necessary for the plants under stress to keep the cellular/tissue H_2O_2 accumulation to minimum to prevent the damaging Fenton reaction to occur (Shaw *et al.* 2004). In the present study, foliar application of 50 mg dm^{-3} ALA significantly decreased the H_2O_2 content in the roots and leaves of NaCl-treated cucumber seedlings. The results suggested that ALA treatment has the potential to protect cucumber seedlings against oxidative damages under NaCl stress, which is in agreement with the findings by Zhang *et al.* (2006).

The activity of SOD in cucumber roots and leaves and the activity of CAT in cucumber roots were not affected by either salinity or ALA. This is partially inconsistent with the findings in spinach (Nishihara *et al.* 2003). The differences in plant species may explain this discrepancy. NaCl stress significantly decreased the activities of APX, GR, and DHAR in cucumber roots and leaves and the activity of CAT in leaves, whereas foliar application of 50 mg dm^{-3} ALA mitigated these reductions as well as enhanced the activities of these enzymes in some cases (Table 2). Increase in the activity of antioxidant enzyme under salt stress is indicative of induction of a protective mechanism to reduce oxidative damage to cells (Meloni *et al.* 2003). The present study hence suggests that ALA application possibly protects cucumber seedlings from H_2O_2-induced oxidative damage under NaCl stress by increasing the activities of certain antioxidant enzyme. The stimulating effect of ALA on the activities of antioxidant enzymes under salt stress is in agreement with the reports of Nishihara *et al.* (2003). In addition, the activity of MDHAR in cucumber roots and leaves were unaltered by salinity. Similar result was observed in *Brassica oleracea* roots (Hernandez *et al.* 2010).

Treating plants with growth substances, such as abscisic acid and salicylic acid, can enhance chilling tolerance by inducing gene expression (Warren 2001, Kang *et al.* 2003). However, few studies have been carried out on the regulatory role of ALA in gene expression. The present study shows that the mRNA levels of *CAT* and *cAPX* in cucumber roots and leaves were down-regulated by NaCl stress, but these decreases were counteracted by the foliar application of 50 mg dm^{-3} ALA. The changes in the *cAPX* mRNA level in cucumber roots and leaves and the *CAT* mRNA level in leaves were consistent with the changes in their enzyme activity under NaCl or ALA treatment. These data suggest that exogenous ALA application regulates the activities of certain antioxidant enzymes by influencing expression of their genes. To the best of our knowledge, this is the first report providing evidence of ALA regulating the expression of genes of antioxidant enzymes in plant challenged by NaCl. However, the changes in the mRNA levels of *GR* in cucumber roots and leaves and that of *CAT* in roots did not reflect any changes in their enzyme activities. These discrepancies have been previously reported and may be attributed, among other factors, to post- transcriptional regulation of the enzymes (Attia *et al.* 2008).

Furthermore, AsA and GSH, as major redox compounds in ROS-scavenging system, are also crucial in preventing oxidative damage in plant cells (Ashraf 2009). AsA, as a reducing substrate, detoxifies H_2O_2 to H_2O and O_2 in reaction catalyzed by APX, and GSH is converted to oxidized glutathione (GSSG) in reaction catalyzed by DHAR (Asada 2006). The present study shows that NaCl stress increased the AsA and GSH contents in cucumber roots and leaves. Taking the changes in the antioxidant enzymes activities into account, we suggest that the decreased APX and DHAR activities might have restrained the conversion of AsA and GSH to dehydroascorbate (DHA) and GSSG, respectively, leading to increase in tissue/cellular levels of AsA and GSH. Arguably, ALA application did not lead to any increase in the AsA and GSH contents because of accelerating effect of the compound on the antioxidant enzyme activities.

In conclusion, this work demonstrates that foliar application of ALA at concentration 50 mg dm^{-3} is the optimum method for alleviating the NaCl-induced inhibition of plant growth and photosynthesis in cucumber seedlings. It enhances the H_2O_2-scavenging capacity of NaCl-treated cucumber seedlings by increasing the activities of the antioxidant enzymes, like CAT, GR, and APX and expressions of *CAT* and *cAPX*, thereby, counteracting NaCl-induced oxidative damage. This treatment is thus a useful strategy in improving resistance of cucumber seedlings to NaCl stress.

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