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Foliar-application of α-tocopherol enhanced salt tolerance of *Carex leucochlora*

Y.R. YE, W.L. WANG*, C.S. ZHENG, D.J. FU, H.W. LIU, and X. SHEN

College of Horticulture Science and Engineering, State Key Laboratory of Crop Biology, Shandong Agricultural University, Taian 271018, P.R. China

Abstract

Several different concentrations of α-tocopherol were applied to *Carex leucochlora* after plants had been treated with high salinity (0.8 % NaCl) in a greenhouse for one month. The results revealed that 0.8 mM α-tocopherol treatment showed the greatest alleviation of growth inhibition and cell membrane damage induced by salt stress. In comparison with NaCl alone, the 0.8 mM α-tocopherol application significantly decreased the content of hydrogen peroxide and the rate of superoxide radical generation, and increased the content of chlorophyll *b*, carotenoids, free proline, and soluble protein, but had no effect on the content of chlorophyll *a* and soluble sugar. These results suggest that α-tocopherol could effectively protect *C. leucochlora* plants from salt stress damage presumably by quenching the excessive reactive oxygen species to protect the photosynthetic pigments and by enhancing the osmotic adjustment.

Additional key words: carotenoids, chlorophylls, hydrogen peroxide, malondialdehyde, reactive oxygen species, salinity.

Introduction

Carex leucochlora Bunge, with short rhizome and dense cluster, is one of the most ecologically important genera of perennial herbs. The genus is evergreen in subtropical regions and it is adapted to a wide range of ecological condition (Ning *et al.* 2014). Salinity stress is one of the most common abiotic stresses, which negatively affect plant growth in different regions. Therefore, it is important to develop and exploit the salt-tolerant plants (Alig *et al.* 2004). Efforts to control salinity by various means, such as foliar application of antioxidants, have been made for developing the sustainable agriculture and landscape gardening (Azooz *et al.* 2002).

Tocopherols are members of the vitamin E compounds and α-tocopherol is localized in chloroplasts, and usually accounts for more than 90 % of the leaf content of vitamin E (Espinoza *et al.* 2013). It is widely believed that this soluble antioxidant may protect the photosynthesis by quenching singlet oxygen generated mostly by chlorophyll in photosystem II (PS II), and prevents the membrane lipid peroxidation by scavenging harmful radicals in thylakoid membranes (Krieger-Liszkay 2005, Munne-Bosch 2005). The content of α-tocopherol in plant leaves changes in response to environmental constraints, depending on the magnitude of the stress and species sensitivity to stress (Semchuk *et al.* 2009, Espinoza *et al.* 2013). The accumulation of α-tocopherol correlates with higher water content, elevated photosynthesis, and decreased damages induced by oxidative stress (Espinoza *et al.* 2013). In turn, plants with lower total tocopherol content are more susceptible to abiotic stresses (Abbasi *et al.* 2007).

To date, few studies have been published regarding the function of α-tocopherol in ground cover plants with high ornamental value under salt stress. The foliar application of α-tocopherol may be a useful method to reduce salt stress. Therefore, the aim of this study was to investigate the effects of different concentrations of α-tocopherol on growth characteristics and physiological attributes of *C. leucochlora* under salinity stress.

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Abbreviations: Car - carotenoids; Chl - chlorophyll; MDA - malondialdehyde; $O_2^{\cdot-}$ - superoxide radical; PPFD - photosynthetic photon flux density; PS II - photosystem II; ROS - reactive oxygen species; TBA - thiobarbituric acid; TCA - tricarboxylic acid.

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* Corresponding author, e-mail: wangwenli169@163.com

Materials and methods

Plants, cultivation, and treatments: The experiment was conducted in the greenhouse of the Horticulture Experimental Site, Shandong Agricultural University, northern China, from November 2015 to March 2016. Temperature, humidity, and photosynthetic photon flux density (PPFD) in the greenhouse was measured with a data logger *HOBO U12-012* (*Onset Computer Corporation*, MA, USA). During the test, daily temperatures averaged 18.8 °C, relative humidity 69.3 %, and PPFD 362 μmol m^{-2} s^{-1}.

Uniform perennial plants of *Carex leucochlora* Bunge were selected and divided into similar ramets, which were obtained from Mount Tai, Shandong province of China. Each pot was filled with *Vermiculite* that had been disinfected with potassium permanganate. Each treatment group contained 10 plants and each treatment was repeated three times. All pots were arranged completely randomized in the greenhouse. After 30 d, the plants were added with the mixture of half-strength Hoagland solution with 0.8 % (m/v) NaCl. The NaCl solution (1 dm^3) was administered once a week and the water was applied twice a week throughout the experiment. The selection of NaCl concentration was based on our preliminary study. The concentrations of NaCl above 0.8 % were proved to be lethal for these plants. Plants in each plot were sprayed to run-off with 0, 0.25, 0.5, 0.8, and 1.0 mM α-tocopherol (*Yuanye Bio-Technology Co*., Shanghai, China) after salt stress treatment, and then the sprays were repeated at the third, fifth, and seventh day after salt stress treatment. The concentrations, the number and the timing of sprays were based on results from a preliminary study. Because α-tocopherol has low solubility in water, to ensure optimal penetration into leaf tissues, 0.1 % (v/v) *Tween-20* was added to the foliar sprays as a surfactant.

Samples were collected at the end of the spraying to screen for optimum foliar-applied concentration of α-tocopherol according to the changes of plant height, dry biomass, relative membrane permeability, and MDA content of plant leaves. The other parameters were determined at the end of experiment.

Growth, membrane permeability, and malondialdehyde content: At the end of each experiment, the height of plants was measured. Plants were moved smoothly to remove the adhering sand particles and divided into leaves and roots. Dry masses of the roots and shoots were measured after placing the samples in an oven run at 80 °C to a constant mass. Relative membrane permeability was determined by measuring electrical conductivity according to Zhang *et al.* (2013) with some modifications. One sample of each duplicate was soaked in 20 cm^3 of deionized water, using 0.5 g samples of fully-expanded leaf tissue, and maintained in the incubator at 25 °C in a water bath for 24 h. The initial electrical conductivity of the solution was measured using a conductivity meter (*FG3-ELK*, *Mettler-Toledo Co.*, Zurich, Switzerland). The sample was then boiled at 100 °C for 10 min, and conductivity was measured again.

Lipid peroxidation was determined by estimating malondialdehyde (MDA) content by the method described by Heath and Packer (1968) with some modifications. Leaf tissue (0.2 g) was homogenized with 50 mM phosphate buffer (pH 7.8), and then centrifuged at 8 000 *g* for 15 min. Then, 1 cm^3 of supernatant was mixed with 2.5 cm^3 of thiobarbituric acid (TBA) solution and heated at 100 °C for 30 min. After cooled on ice, the mixture was centrifuged at 10 000 *g* for 5 min and absorbances of supernatant were measured at 532 and 600 nm using a spectrophotometer *UV-2450* (*Shimadzu*, Kyoto, Japan).

Hydrogen peroxide (H_2O_2) content and superoxide radical ($O_2^{\cdot-}$) production rate: The H_2O_2 content was estimated according to Shi *et al.* (2005). Fresh leaf samples (0.5 g) were homogenized in an ice bath with 5 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA) and centrifuged at 12 000 *g* for 15 min. Then, 1 cm^3 of supernatant was added to 1 cm^3 of 100 mM potassium phosphate buffer (pH 7.0) and 2 cm^3 of 1 M potassium iodide. Absorbance was measured at 390 nm and the content of H_2O_2 was calculated using a standard curve plotted with known concentrations of H_2O_2.

The production rate of $O_2^{\cdot-}$ was measured as follows: fresh leaves (0.1 g) were cut into small fragments and immersed in 1 cm^3 of 50 mM phosphate buffer (pH 7.8), and then the solution was centrifuged at 10 000 *g* for 10 min. Afterwards, 0.5 cm^3 of the supernatant was added to 0.5 cm^3 of 65 mM phosphate buffer (pH 7.8) and 0.1 cm^3 of 10 mM hydroxylamine chlorhydrate and then the mixture was incubated at room temperature for 1 h. After then, 17 mM sulphanilamide (1 cm^3) and 7 mM α-naphthylamine (1 cm^3) were added to the mixture. After 20 min, absorbance was read at 530 nm and the production rate of $O_2^{\cdot-}$ was calculated from a standard curve generated with known concentrations of $NaNO_2$ (Elstner and Heupel 1976).

Chlorophyll and carotenoid content: The photosynthetic pigments analysis was carried out as described by Liu *et al.* (2012) based on Lichtenthaler and Wellburn (1983). Fresh leaves (0.2 g) were homogenized with ice-cold 96 % (v/v) ethanol. The homogenates were centrifuged at 8 000 *g* and 4 °C for 10 min. Supernatants were repeatedly extracted twice with 1 cm^3 ice-cold 96 % ethanol. The content of chlorophyll (Chl) *a*, Chl *b*, and carotenoids (Car) was quantified by measuring absorbances at 665, 649, and 470 nm, respectively.

Free proline, total soluble sugar and protein content:

Osmotic adjustment was assessed by determining free proline, total soluble sugar, and protein content. Proline content in leaves was measured using the rapid colorimetric method outlined by Bates *et al.* (1973). Fresh leaf samples (0.5 g) were ground to powder with 10 cm^3 of 3 % (m/v) sulfosalicylic acid and then the mixture was centrifuged at 10 000 *g* for 10 min. In a test tube, supernatant (2 cm^3) was added to 2 cm^3 of freshly prepared acid-ninhydrin reactive solution and 1 cm^3 of glacial acetic acid. Samples were incubated in a water bath at 100 °C for 30 min and the reaction was terminated in an ice-bath. The mixture was centrifuged at 2 000 *g* for 10 min and absorbance of supernatant was read at 520 nm. Proline content was determined from a standard curve prepared using analytical grade proline. Total soluble sugars were assessed by the method of Robyt and White (1987). A fresh samples of 0.5 g were homogenized in 5 cm^3 of 80 % (v/v) methanol, boiled at 70 °C for 30 min, and 1 cm^3 of phenol and 5 cm^3 of concentrated sulfuric acid were added to 1 cm^3 of the extract after cooling. Then, absorbance of the mixtures was read at 640 nm with pure methanol as a blank. Soluble protein of the extract was determined using Coomassie brilliant blue G-250 reagent according to the method of Bradford (1976) with bovine serum albumin as a standard.

Statistical analysis: All data were subjected to a one-way analysis of variance (*ANOVA*) using the statistical software *SPSS v. 17.0* (*SPSS Inc.*, Chicago, IL, USA), and statistical significance of the means were compared by Duncan's multiple range test using Least Significant Difference (*LSD*) test (α = 0.05 or 0.01). Each data point represents the mean of three replicates (n = 3), and was expressed as the mean ± standard error (SE).

Results

Under salt stress, significant reduction in plant height, shoot dry mass, and root dry mass (by 23.0, 41.0, and 25.3 %, respectively) was observed in comparison with the control (Table 1). In contrast, α-tocopherol ranging from 0.2 to 0.8 mM significantly (P < 0.05) ameliorated these reductions. The 0.8 mM α-tocopherol was found to be most effective, increasing the above growth characteristics by 18.9, 54.2, and 14.1 %, respectively.

Table 1. Effect of exogenous α-tocopherol on growth characteristics of *Carex leucochlora* under salt stress (0.8 % NaCl). Control was sprayed with distilled water. Means ± SEs, n = 3. Means with different letters within the same columns indicate significant differences among treatments. The growth characteristics were determined after 7 d of the treatment.

Treatments	Plant height [cm]	Shoot dry mass [g $plant^{-1}$]	Root dry mass [g $plant^{-1}$]
Control	13.73 ± 0.33 a	2.00 ± 0.22 a	0.237 ± 0.028 a
NaCl	10.57 ± 0.58 b	1.18 ± 0.15 b	0.177 ± 0.012 b
NaCl+0.20 mM α-tocopherol	10.70 ± 0.42 b	1.59 ± 0.23 ab	0.193 ± 0.003 b
NaCl+0.50 mM α-tocopherol	11.90 ± 0.81 ab	1.72 ± 0.04 a	0.187 ± 0.003 b
NaCl+0.80 mM α-tocopherol	12.57 ± 0.71 ab	1.82 ± 0.06 a	0.202 ± 0.006 ab
NaCl+1.00 mM α-tocopherol	11.33 ± 0.88 b	1.58 ± 0.17 ab	0.183 ± 0.003 b

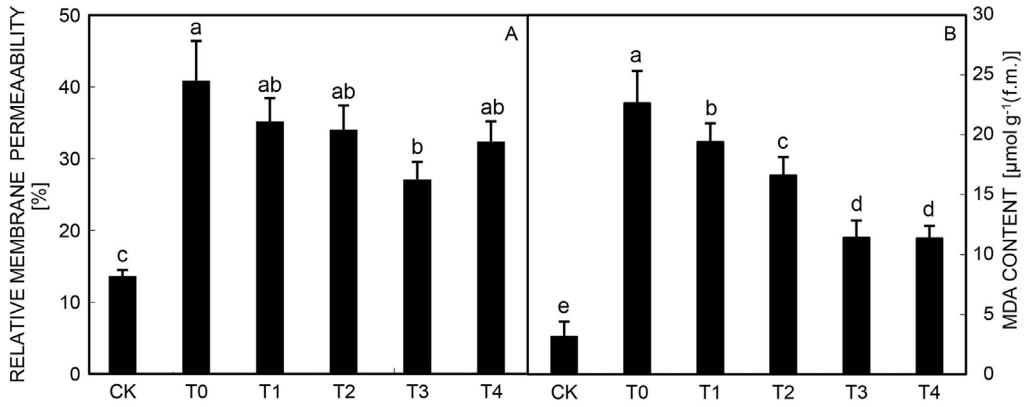

Fig. 1. Effect of exogenous α-tocopherol on the relative membrane permeability (*A*) and MDA content (*B*) in leaves under NaCl stress. CK - control, T0 - 0.8 % NaCl, T1 to T4 - NaCl + 0.2, 0.5, 0.8 and 1.0 mM α-tocopherol, respectively. Mean ± SE (n = 3). Different letters indicate significant differences at P < 0.05.

However, when the α-tocopherol concentrations were higher than 0.8 mM, the rate of increase declined.

Salt stress alone caused a significant increase ($P < 0.05$) in relative membrane permeability (Fig. 1*A*) and MDA content (Fig. 1*B*). After spraying 0.2 - 1.0 mM α-tocopherol on leaves, the content of MDA and relative membrane permeability were less increased, and again, 0.80 mM α-tocopherol was found to be most effective, decreasing the content of MDA and relative membrane permeability by 49.7 and 34 %, respectively, compared to NaCl treatment alone.

The increase in H_2O_2 content under NaCl stress treatments was delayed with respect to the production rate of $O_2^{\cdot-}$, and the peak values of both parameters were 2.6 and 2.3 times higher than in the control, respectively. In contrast, α-tocopherol foliar application caused a significant decrease ($P < 0.05$) in the content of H_2O_2 and the rate of $O_2^{\cdot-}$ generation by 13.3 and 30.6 % in peak, respectively, with respect to the plants treated with NaCl treatment alone (Fig. 2*A*,*B*).

Salt stress caused a significant decrease ($P < 0.05$) in the content of Chl *a* and Chl *b* by 29.2 and 36.8 %, respectively (Table 2). The content of Chl *b* of α-tocopherol treated plants was less decreased but Chl *a* content was not affected by exogenous α-tocopherol. In contrast, the content of carotenoids (Car) was increased under the salt stress as well as under NaCl and α-tocopherol treatment (Table 2).

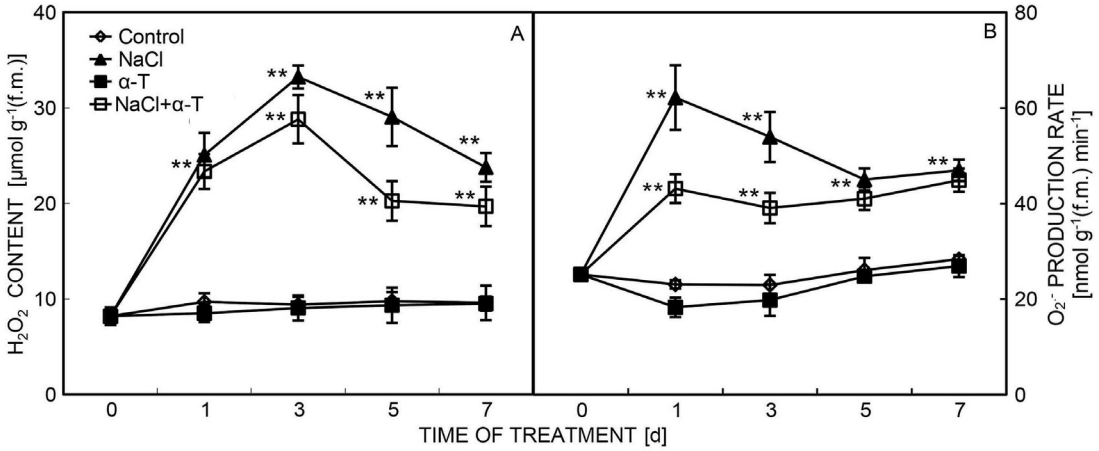

Fig. 2. Effect of exogenous α-tocopherol (0.8 mM α-T) on H_2O_2 content (*A*) and the rate of $O_2^{\cdot-}$ generation (*B*) in leaves under salt stress (0.8 % NaCl). Control was sprayed with distilled water. Means ± SEs, $n = 3$, ** and * - significant differences at $P < 0.01$ and $P < 0.05$, respectively.

Table 2. Effect of exogenous α-tocopherol on content of photosynthetic pigments in leaves under salt stress (0.8 % NaCl). Control was sprayed with distilled water. Means ± SEs, $n = 3$. Means with different letters within the same columns indicate significant differences between the treatments. The pigment content was determined after 7 d of the treatment.

Treatments	Chl *a* [mg g^{-1} (f.m.)]	Chl *b* [mg g^{-1} (f.m.)]	Total Chl [mg g^{-1} (f.m.)]	Car [mg g^{-1} (f.m.)]
Control	1.16 ± 0.02 a	0.39 ± 0.03 a	1.56 ± 0.12 a	0.410 ± 0.022 b
α-Tocopherol	1.22 ± 0.03 a	0.41 ± 0.02 a	1.63 ± 0.35 a	0.414 ± 0.034 b
NaCl	0.82 ± 0.01 b	0.25 ± 0.01 b	1.07 ± 0.27 c	0.443 ± 0.097 a
NaCl+α-tocopherol	0.84 ± 0.03 b	0.46 ± 0.03 a	1.30 ± 0.31 b	0.453 ± 0.051 a

Free proline content was significantly ($P < 0.05$) affected by NaCl and α-tocopherol as well (Fig. 3*A*). The content of free proline in NaCl treated plants was higher than that in control and spraying α-tocopherol further increased free proline content. The soluble sugar content also increased under NaCl stress, but it did not differ between exogenous α-tocopherol treatment or NaCl treatment alone (Fig. 3*B*). In contrast, the content of soluble protein in α-tocopherol treatment was always higher than that of NaCl alone treatment. On the first and third day of the NaCl treatment, the applied α-tocopherol strongly increased ($P < 0.05$) soluble protein content by 52.8 and 38.1 %, respectively, compared with the controls. The soluble protein content was reduced and stabilized after the third day (Fig. 3*C*).

Discussion

Non-halophytes are very sensitive to salt, which leads to the inhibition of plant growth under the salt stress. Results of the current study further confirmed the negative effect of NaCl treatments on plant growth of *C. leucochlora*. However, we observed that exogenous α-tocopherol partially rescued the deleterious effect of salt stress on the growth of this species. A significant increase in plant height, shoot dry mass and root dry mass was observed, which was concomitant with the decreased relative membrane permeability and MDA content in comparison with NaCl stress alone. We observed that 0.8 mM α-tocopherol treatment could effectively ameliorate NaCl induced oxidative stress suggesting that

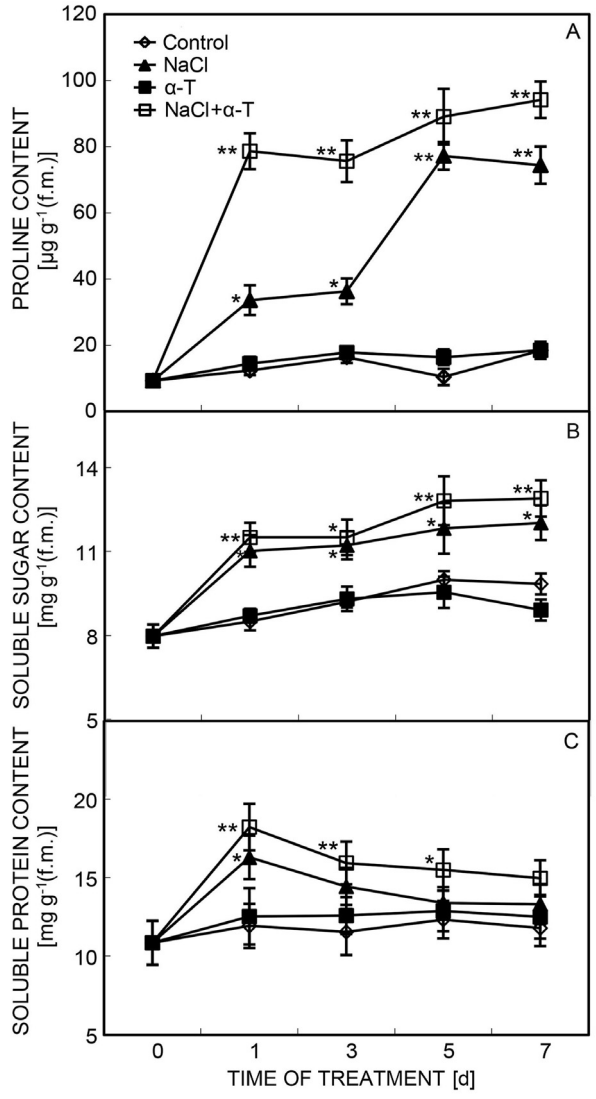

Fig. 3. Effect of exogenous α-tocopherol (0.8 mM α-T) on free proline (*A*), soluble sugars (*B*), and proteins (*C*) in leaves under salt stress (0.8 % NaCl). Control was sprayed with distilled water. Means ± SEs, $n = 3$, ** and * - significant differences at $P < 0.01$ and $P < 0.05$, respectively.

α-tocopherol might fulfill crucial roles in scavenging radicals and thus reduce excess salt-stress induced ROS, and, consequently, promote healthy plant growth under moderate salinity as previously reported by Bosch (1995).

α-Tocopherol is located in the chloroplast envelope, thylakoid membranes, and plastoglobuli. This antioxidant deactivates photosynthesis-derived ROS, and prevents the propagation of lipid peroxidation by scavenging lipid peroxyl radicals in thylakoid membranes. It affects many physiological processes (Azooz *et al.* 2002, Barakat 2003) and directly neutralizes superoxide radicals or singlet oxygen in plant cells (Foyer and Noctor 2005) that confirm a significant decrease in the content of H_2O_2 and the production rate of $O_2^{\cdot-}$ by the application of α-tocopherol under salt stress (Fig. 2). α-Tocopherol is an important part of the plant defence machinery involved in maintaining the integrity and normal function of the photosynthetic apparatus (Liu *et al.* 2008). Chlorophyll content is widely used as an index to indicate the abiotic tolerance in plants. It is well documented that the stressful environments such as salinity result in decreased Chl content. The results presented here show that foliar application of 0.8 mM α-tocopherol to *C. leucochlora* plants led to a significant increase in Chl *b* and Car content under NaCl stress.

Osmotic adjustment plays an important role in plants under salt stress. Free proline acts as an osmoprotectant, a membrane stabilizer, and a ROS scavenger in plants (Song *et al*. 2016). Thus, the change of proline content is an indication of a critical point for plant growth under stress (Watanabe *et al.* 2001). Little is known about how exogenous α-tocopherol regulates osmotically active substances in *C. leucochlora* plants. We have shown that the content of free proline and soluble protein increased, and soluble sugar did not show any significant change due to application of 0.8 mM α-tocopherol compared with plants treated with NaCl alone. These results are not completely consistent with the previous results reported by Azooz *et al.* (2011), Abd El-Samad *et al.* (2011), El-Lethy *et al.* (2013), and Semida *et al.* (2014) from different plant species. This may depend on the magnitude of the stress and species-sensitivity to stress.

In conclusion, these results suggested that exogenous α-tocopherol treatment could alleviate the negative effect of salt stress, and therefore allows plants to increase their tolerance to unfavorable conditions. Our results extend the available knowledge of the mechanisms underlying salt tolerance induced by α-tocopherol. The potential mechanisms include: *1*) elimination of ROS and protection of membranes from lipid peroxidation; *2*) increase in the content of photosynthetic pigments and osmotically active substances. As a result, growth of the plants is promoted. Therefore, the exogenous application of α-tocopherol may be a useful way to improve the salt tolerance of ground cover plants.

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