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Acetylcholine mechanism of action to enhance tolerance to salt stress in *Nicotiana benthamiana*

C. QIN[*], Y.Y. SU[*], B.S. LI[*], Y.Q. CHENG[*], C.C. WEI[**], S. YUAN[***], N. AHMED[#], M. ASHRAF[##], and L.X. ZHANG[*,+]

College of Life Sciences, Northwest A&F University, 712100 Yangling, China[*]
Tobacco Science Institution of Shaanxi Province, 710061 Xi'an, China[**]
Technology Center of China Tobacco Shaanxi Industrial Co., Ltd., 710065 Xi'an, China[***]
Department of Botany, Mohi-Ud-Din Islamic University, 12080 Tarar Khal, Pakistan[#]
Institute of Molecular Biology and Biotechnology, The University of Lahore, 54590 Lahore, Pakistan[#]

Abstract

Acetylcholine (ACh) is one of the important neurotransmitters, involved in signal transduction function in human and animal brain. However, the influence of ACh treatment on salt-stress tolerance in plants is yet unknown. Salt stress caused a reduction in gas-exchange parameters, chlorophyll content, antioxidant enzyme activities, and leaf relative water content of *Nicotiana benthamiana* plants. However, the above inhibitions could be significantly alleviated by application of leaf spray or root application of ACh. Exogenous ACh reduced the accumulation of malondialdehyde by enhancing activities of antioxidant enzymes such as peroxidase and superoxide dismutase. In addition, enhanced accumulation of organic osmolytes including soluble sugars and proline possibly regulated the signal mechanisms related to stress. Application of ACh could also improve gas-exchange parameters and photosynthetic pigment accumulation in leaves of salt-stressed plants. These effects of ACh were beneficial for maintaining better water status in plants, the concentration of 10 μM ACh applied both in the form of leaf spray or root application was the most effective. Therefore, our findings provided a stronger evidence for a physiological role of ACh and its potential use at optimal concentration by leaf or root application to alleviate damage caused by salt-stress in plants.

Additional key words: antioxidant system; leaf gas exchange; reactive oxygen species; salinity stress.

Introduction

Salinity is a premier environmental cue that restricts growth and quality of agricultural crops (Zhu 2003, Ismail and Horie 2017), because it causes multiple effects in plants such as ionic stress, osmotic stress, nutritional disorder occurring individually or jointly (Ashraf and Harris 2004, Munns and Gilliham 2015). Several reports exist in the literature that show the differences in photosynthetic characteristics of salinity-tolerant *vs.* sensitive plants (Chaves *et al*. 2009, Sarkar *et al*. 2013, Feng *et al*. 2014). A variety of physio-biochemical processes including primarily photosynthesis are drastically impaired by unfavorable conditions (Lahive *et al*. 2018, Zlobin *et al*. 2019). These include modification of characteristics of the photosynthetic apparatus, and changes in chlorophyll (Chl) contents (Bae *et al*. 2012). Chl is an essential component of the process of photosynthesis and it plays a critical role in plant development (Zhen *et al*. 2014). Membrane lipid peroxidation occurs as a consequence of the stress-induced cellular damage produced by the generation of reactive oxygen species (García *et al*. 2016). Therefore, antioxidant defense enzyme activities and contents of compatible osmolytes, such as proline and soluble sugars, undergo changes in plants exposed to saline environment (Ashraf and Foolad 2007, Miller *et al*. 2010, Parvaiz *et al*. 2016).

To achieve improved plant tolerance to salinity and hence enhanced crop productivity, application of exogenous substances has been used widely (Ahmad *et al*. 2016, Li *et al*. 2016, 2017a). In the past few years, research on function of animal neurotransmitter in stress tolerance has obtained much interest. For example, melatonin plays an important role in plant defense against stressful cues, such as drought, salinity, radiation, and chemical

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[+]Corresponding author; e-mail: zhanglixin@nwsuaf.edu.cn
Abbreviations: ACh – acetylcholine; C_i – intercellular CO_2 concentration; Chl – chlorophyll; DM – dry mass; E – transpiration rate; FM – fresh mass; g_s – stomatal conductance; LRWC – leaf relative water content; MDA – malondialdehyde content; P_N – net photosynthetic rate; POD – peroxidase; ROS – reactive oxygen species; SOD – superoxide dismutase; SS – soluble sugars; TBA – thiobarbituric acid; TCA – trichloroacetic acid.
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stresses (Zhang *et al*. 2015). Acetylcholine (ACh) is one of the most exemplary neurotransmitters (Wessler *et al*. 2001). It was first revealed in non-animal organisms (Ewins 1914). Since then, it has been found in all types of plants as well as in bacteria and fungi (Fluck and Jaffe 1976). It has also been detected in non-neural tissues, *e.g.*, sperms, erythrocytes, and placental cells (Sastry and Sadavongvivad 1978). Sagane *et al.* (2005) demonstrated that the acetylcholinesterase (AChE) family cloned from maize entails a unique AChE family that is widely found in the plant kingdom. Therefore, the presence of ACh and its related molecules might play a role in plant response to environmental stimuli. After that, Yamamoto *et al.* (2011) reported a beneficial role of AChE in maize for resistance to heat stress mediated by overexpression of maize AChE in transformed tobacco (*Nicotiana benthamiana*) plants relative to that in nontransformed ones. Research on the effects of ACh on several growth and developmental phenomena in plants have been conducted (Tretyn and Kendrick 1991). Sugiyama and Tezuka (2011) revealed that the emergence and elongation of lateral roots of *Raphanus sativus* could be regulated by 1 nM ACh. Braga *et al.* (2017) showed that the growth and dry matter accumulation were promoted by ACh under osmotic stress in soybean seedlings. However, relevant information about the possible regulatory effect of ACh on tolerance to salt stress in plants is still lacking.

Nicotiana benthamiana, with a short growth cycle, is an important model plant and has special significance in the field of genetics, development, and transgenesis (Wang *et al.* 2015). The aim of the study is to understand if ACh is involved in salt tolerance of *Nicotiana benthamiana*. We determined whether ACh treatment could alleviate salt stress by regulating main physiological metabolism of *Nicotiana benthamiana* seedlings. For this purpose, regulation of gas-exchange characteristics, antioxidant defense system and contents of some key organic osmolytes in salt-stressed *Nicotiana benthamiana* plants fed with ACh was appraised.

Materials and methods

Plant material: *Nicotiana benthamiana* plants at the six-leaf stage were used in this study. Seeds were germinated in vermiculite and nutritional soil. The plants of uniform size were transplanted into plastic culture pots (245 × 170 × 75 mm; four plants per pot) containing 1.5 L of 1/2 strength Hoagland's nutrient solution. The experiment was conducted in a growth chamber (*RXZ-380C-LED*, *ZheJiang*, *China*) at the Northwest A&F University, Yangling, Shaanxi, P. R. China. The day/night air temperatures were 28/25°C with 16-h day, and relative humidity of 60–70%.

Pre-cultivation: Seedlings were treated with 1, 10, 50, and 100 µM ACh solution in the form of leaf spray or root application. Acetylcholine chloride was procured from *Sigma-Aldrich* (≥ 99%, St. Louis, MO, USA). Foliar spraying ensured that both sides of the leaves remained wet. This process occurred in the morning and in the afternoon. Root application was performed by applying different concentrations of ACh into solution.

Leaf discs and *in vitro* test: Leaf discs of 15 mm in diameter were cut from healthy *Nicotiana benthamiana* seedlings. They were immediately placed in Petri dishes each filled with 20 mL of either distilled water (C), or 150 mM of NaCl solution (S), or 150 mM NaCl + 10 µM ACh (S-A 10), or 150 mM NaCl + 100 µM ACh (S-A 100). All the treatments were subjected to 25°C room temperature. Photographs were taken after 72 h of the treatment.

Salt-stress treatment was started by adding an amount of NaCl equivalent to 150 mM to the nutrient medium in the absence or presence of ACh. Finally, in the main experiment, salt stress treatment and ACh applications were performed as follows:

Treatment	Leaf spray	Root application
C	H_2O	H_2O
S	H_2O	150 mM NaCl
R-1	H_2O	1 µM ACh +150 mM NaCl
R-10	H_2O	10 µM ACh + 150 mM NaCl
R-50	H_2O	50 µM ACh + 150 mM NaCl
R-100	H_2O	100 µM ACh + 150 mM NaCl
L-1	1 µM Ach	150 mM NaCl
L-10	10 µM ACh	150 mM NaCl
L-50	50 µM ACh	150 mM NaCl
L-100	100 µM ACh	150 mM NaCl

Both the application of NaCl and ACh were done at the same time (Fig. 1). Then, after 15 d of treatments, the second upper fully developed leaves of *Nicotiana benthamiana* plants were taken, instantly placed in liquid

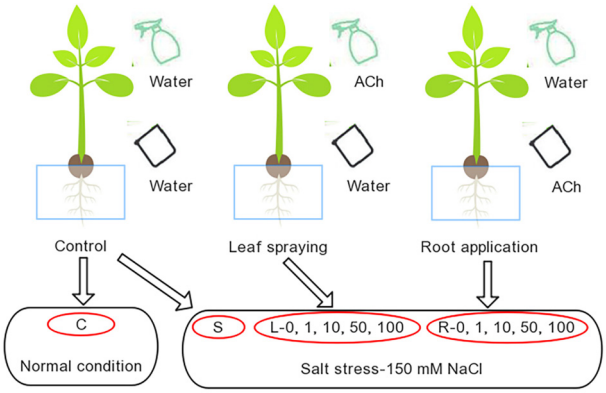

Fig. 1. A schematic diagram of leaf spraying or root application with acetylcholine (ACh) and salt-stress treatments. C – plants grown under normal conditions for 15 d after supplying water through roots or as foliar spray; S – plants grown at 150 mM NaCl for 15 d after supplying water through roots or as foliar spray. L – plants grown at 150 mM NaCl for 15 d after supplying ACh (1, 10, 50, and 100 µM) to leaves; R – plants grown at 150 mM NaCl for 15 d after supplying ACh (1, 10, 50, and 100 µM) to roots.

N_2, and then transferred to a freezer at –80°C until further analytical procedures. Within each treatment there were three biological replicates.

Chl content: Leaf samples (each 0.1 g) were extracted in 15 mL of 80% acetone using a shaker overnight at room temperature. After centrifugation at 5,000 × *g* for 5 min, the supernatant obtained was checked for absorbance at two wavelengths, *i.e.*, 645 and 663 nm, using a spectrophotometer (*UV-2800*, *Shimadzu*, Kyoto, Japan). Total Chl concentration was estimated following the formula developed by Gao *et al.* (2016): Chl (a+b) = 20.29 × A_{645} + 8.05 × A_{663}. Simultaneously, the Chl content of seedlings was measured with a chlorophyll meter (*SPAD-502*, *Minolta*, Japan).

Leaf gas-exchange parameters: Fully expanded leaves from each plant were used to estimate net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) using a *LI-6400* photosynthesis system (*LI-COR Inc*., Lincoln, NE, USA) during the period of 9:00–11:00 h. The instrument was set at a light intensity of 1,000 μmol(photon) m^{-2} s^{-1} and airflow rate of 500 μmol s^{-1}. The cuvette CO_2 was adjusted at 400 μmol(CO_2) mol^{-1}(air) (Gao 2006).

Proline content: Fresh leaf sample was used for the following procedure described by Bates *et al.* (1973). To a 0.5-g leaf sample, 5 mL of 3% sulfosalicylic acid were added; the mixture was kept in boiling water for 10 min. The supernatant (each 2.0 mL) was mixed with 2 mL of acetic acid and 3 mL of 2.5% ninhydrin solution. The mixture was placed in hot water for 40 min, and then 4 mL of methyl benzene were added. The optical density (OD) of the reacted mixture was noted at 520 nm (*UV-2800*, *Shimadzu*, Kyoto, Japan). Proline content was expressed as μg g^{-1}(FM).

Soluble sugar content: Dried leaf was used for assay following Spiro (1966). Dry leaf sample (each 0.5 g) was transferred to a glass vial containing 10 mL of 80% (v/v) ethanol and subjected to 80°C for 30 min. The mixture was centrifuged at 3,500 × *g* after cooling. The extract was filtered and diluted to 20 mL using 80% (v/v) ethanol. The supernatant was added to 3 mL (final volume) of assay media containing 1.08 M H_2SO_4, 1.09 mM thiourea, and 2.1 mM anthrone. The sample was subjected to 100°C for 10 min and OD was recorded at 620 nm (*UV-2800*, *Shimadzu*, Kyoto, Japan). A calibration curve drawn using pure D-glucose was prepared to calculate the concentrations of soluble sugars in all samples.

Enzyme extracts and assays: Fresh leaf sample (each 0.2 g) was homogenized using a pestle and mortar with 0.05 M sodium phosphate buffer (pH 7.5). The mixture was subjected to centrifugation for 20 min at 10,000 × *g*. The supernatant was used for estimating the activities of superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POD, EC 1.11.1.7). The above steps were carried out at 4°C. The SOD activity was appraised as described by Gao (2006). The reaction mixture contained 50 mM Na_2HPO_4-NaH_2PO_4 buffer, methionine, nitroblue tetrazolium salt (NBT), EDTA-Na_2, riboflavin, and 0.3 mL of the enzyme extract. All treated samples were stirred under darkness, and then exposed to fluorescent lamps [160 μmol(photon) m^{-2} s^{-1}] for 5 min; the reaction temperature was controlled between 25 and 35°C. The reacted mixtures were read at 560 nm using a spectrophotometer (*UV-2800*, *Shimadzu*, Kyoto, Japan) against a blank. One unit of SOD was considered as the amount of enzyme inhibiting 50% of NBT reduction. The SOD activity was expessed as U g^{-1}(FM) h^{-1}.

The POD activity was assayed following Gao (2006). Reaction solution (4 mL) comprised 0.1 mol L^{-1} sodium phosphate buffer (pH 7.8), 28 μL of guaiacol (10 mM), 30% H_2O_2, and 1 mL of the enzyme extract. Changes in the OD of the reaction solution at 470 nm were recorded using a spectrophotometer (*UV-2800*, *Shimadzu*, Kyoto, Japan) every 1 min. The POD activity was expressed as U g^{-1}(FM) min^{-1}.

Lipid peroxidation was assayed by quantifying the malondialdehyde (MDA) contents using the method of Hodges *et al.* (1999). The leaf sample (0.5 g) was triturated in 8 mL of 0.1% (w/v) trichloroacetic acid (TCA) and subjected to centrifugation for 20 min at 1,000 × *g*. To the suspension, 1.5 mL of reaction solution (0.5% thiobarbituric acid solution (TBA) with 5% TCA) was added and then placed in a water bath. The mixture was centrifuged for 10 min at 7,888 × *g*. Absorbance measurements were obtained at 532 and 660 nm (*UV-2800*, *Shimadzu*, Kyoto, Japan). Following formula was employed to calculate the MDA content: 6.45 × (A_{532} – A_{600}) – 0.56 × A_{450}.

Leaf relative water content (LRWC) was assessed following Gao *et al.* (2016). The leaves were detached from the seedlings and their fresh mass (FM) measured instantly. Thereafter, the turgid mass (TM) was appraised after placing the leaves in deionized distilled water overnight at 4°C. Finally, the leaves were oven-dried at 80°C for 48 h and the dry mass (DM) was recorded. LRWC [%] was calculated according to the following formula: LRWC = [(FM – DM)/(TM – DM)] × 100.

Statistical analysis: Statistical significance of the treatments was evaluated by analysis of variance (*ANOVA*) followed by the mean separation by *Duncan*'s multiple range test (DMRT) using *SPSS 17.0*. The results represent mean ± standard error (SD) of three replicates for each treatment (P≤0.05).

Results

Chl content: An obvious chlorosis was observed in salt-stressed *Nicotiana benthamiana* caused by reduced accumulation of Chl (Fig. 2). The ACh application was found to be effective in alleviating this salt-stress-induced leaf chlorosis by increasing the Chl content in leaf discs. However, the most promising effect was found at the lower concentration of ACh (10 μM). In order to

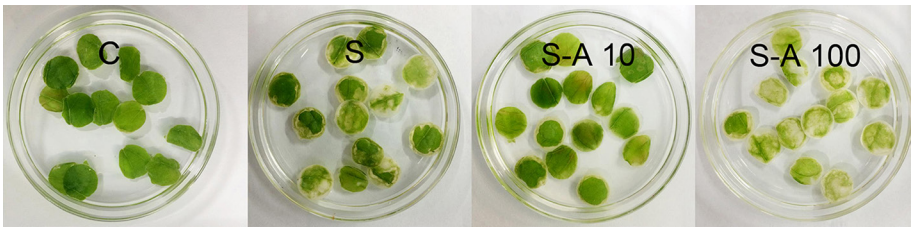

Fig. 2. Changes in green color intensity in leaf discs of *Nicotiana benthamiana* plants. C – water, S – salt stress (150 mM NaCl), S-A 10 – 150 mM NaCl + 10 µM acetylcholine (ACh), S-A 100 – 150 mM NaCl + 100 µM ACh. Photographs were taken after 72 h of discs incubation in different solutions.

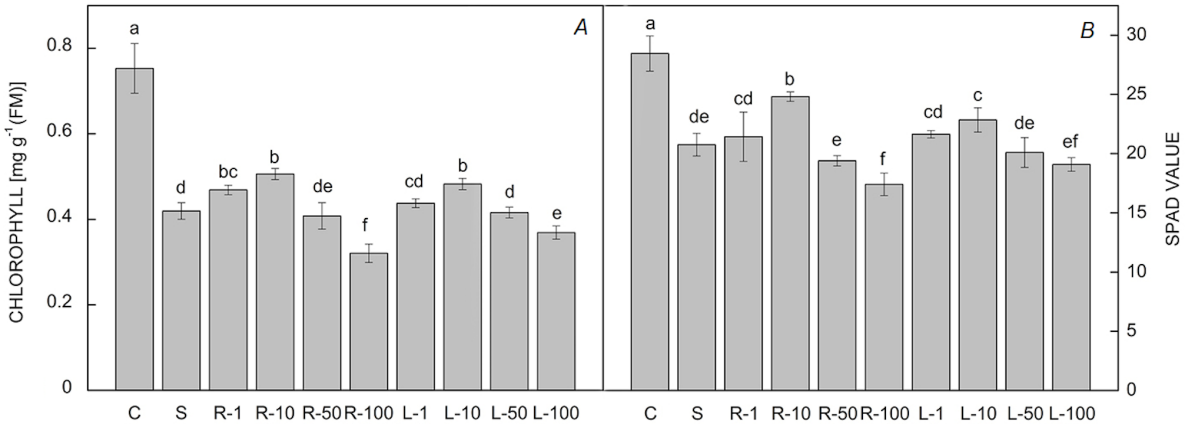

Fig. 3. Effects of the root application (R) or leaf spraying (L) with 1, 10, 50, and 100 µM acetylcholine (ACh) on the chlorophyll content (*A*) and SPAD value (*B*) of 15-d-old *Nicotiana benthamiana* plants grown in 1/2 Hoagland solution with or without 150 mM NaCl. Data are the means ± SD, $n = 3$. *Different letters* in the same column indicate significant difference between treatments at $p \leq 0.05$ using DMRT.

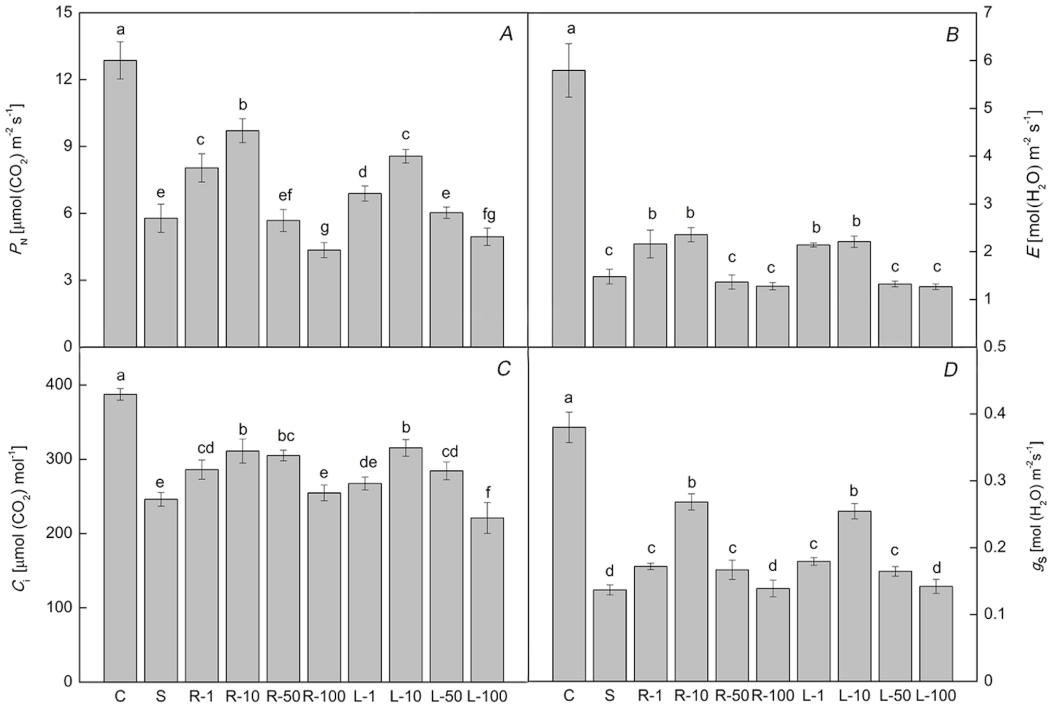

Fig. 4. Effects of the root application (R) or leaf spraying (L) with 1, 10, 50, and 100 µM acetylcholine (ACh) on gas-exchange parameters of 15-d-old *Nicotiana benthamiana* plants grown in 1/2 Hoagland solution with or without 150 mM NaCl. (*A*) P_N – net photosynthesis rate, (*B*) E – transpiration rate, (*C*) C_i – intercellular CO_2 concentration, and (*D*) g_s – stomatal conductance. Data are the means ± SD, $n = 3$. *Different letters* in the same column indicate significant difference between treatments at $p \leq 0.05$ using DMRT.

examine whether ACh could alleviate the salinity-induced impairment in Chl anabolism, measurement of the Chl content either spectrophotometrically or SPAD was carried out. The results showed that Chl contents of leaves decreased markedly under salt stress. (Fig. 3). However, the Chl content was enhanced significantly by 15 and 20.6% in L-10 and R-10, respectively, compared with that under the S treatment. The Chl content decreased when the ACh concentration was higher than 50 μM. The lowest Chl content was found in R-100 and L-100. A similar trend was observed in SPAD values.

Photosynthetic gas-exchange parameters: P_N, g_s, C_i, and E decreased significantly by S in comparison with C treatment (Fig. 4). With the increase in the concentration, either root application (R) or foliar spraying treatments (L) with ACh showed first an increasing and then a decreasing effect. The P_N increased significantly by 48.1 and 67.9% in L-10 and R-10, respectively, compared with that in S (Fig. 4*A*). The highest concentration of ACh (100 μM) did not show an alleviating effect of on photosynthetic rate of salt-stressed plants. P_N was the lowest at L-100 and R-100. A similar effect of ACh was observed on E with the most effective concentration being 10 μM (Fig. 4*B*). C_i was high when the exogenous ACh concentration was lower than 50 μM, but it consistently decreased when the ACh concentration was over 50 μM. C_i was reduced by 22.7% in L-100 and no reduction was found in R-100 compared with that under S treatment (Fig. 4*C*). Thus, exogenous application of 10 μM of ACh markedly improved g_s of the seedlings 1.96 and 1.86 times in R-10 and L-10, respectively, compared with that at S treatment (Fig. 4*D*).

MDA accumulation: Salt stress (S) resulted in a significant increase of the MDA accumulation as compared to that in the C treatment. However, both root application and leaf spraying with 1 and 10 μM ACh decreased the MDA content significantly (Fig. 5*A*). The MDA content at R-10 and L-10 decreased by 44.6 and 44.5%, respectively, compared with that at S (Fig. 5*A*).

Activities of SOD and POD: In this study, the salt-stressed seedlings (S) had higher SOD and POD activities compared with control (C) (Fig. 5*B*,*C*). However, an increase in SOD and POD activities was observed in salt-stressed plants after the application of ACh (both root application and leaf spray). R-10 and L-10 treatments further enhanced the SOD activities by 23.7 and 37.2%, respectively, compared to S plants. Similarly, R-10 and L-10 treatments also further enhanced the POD activities by 27.2 and 54.2%, respectively, compared to plants exposed to salt stress alone (Fig. 5*B*,*C*).

Proline accumulation: Proline content was significantly higher (approximately 3.43 fold) in S than that in C treatment (Fig. 6*A*). Varying exogenous concentrations of ACh applied both by leaf spraying or root application could enhance proline accumulation under salt stress. The highest content of proline was observed in R-10 and L-50,

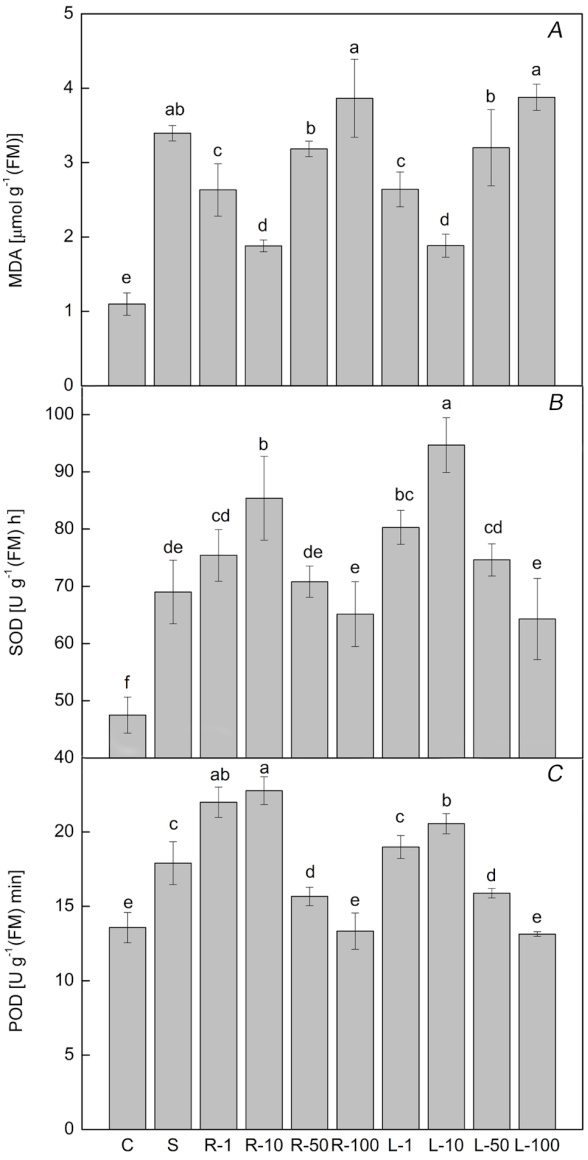

Fig. 5. Effects of the root application (R) or leaf spraying (L) with 1, 10, 50, and 100 μM acetylcholine (ACh) on malondialdehyde (MDA) content (*A*), superoxide dismutase (SOD) activity (*B*), and peroxidase (POD) activity (*C*) of 15-d-old *Nicotiana benthamiana* plants grown in 1/2 Hoagland solution with or without 150 mM NaCl. Data are the means ± SD, $n = 3$. *Different letters* in the same column indicate significant difference between treatments at $p \leq 0.05$ using DMRT.

which showed 68.3 and 51.3% increase compared to that in S treatment, respectively.

Soluble sugar accumulation: The S treatment significantly increased soluble sugar accumulation in leaves compared with C treatment (Fig. 6*B*). The maximum content of soluble sugars was recorded in R-10 and L-10, it was 33.9 and 32.4% higher compared to S treatment, respectively. However, the high concentration of ACh (R-100 and L-100) resulted in a reduction in the soluble sugar content compared to S plants.

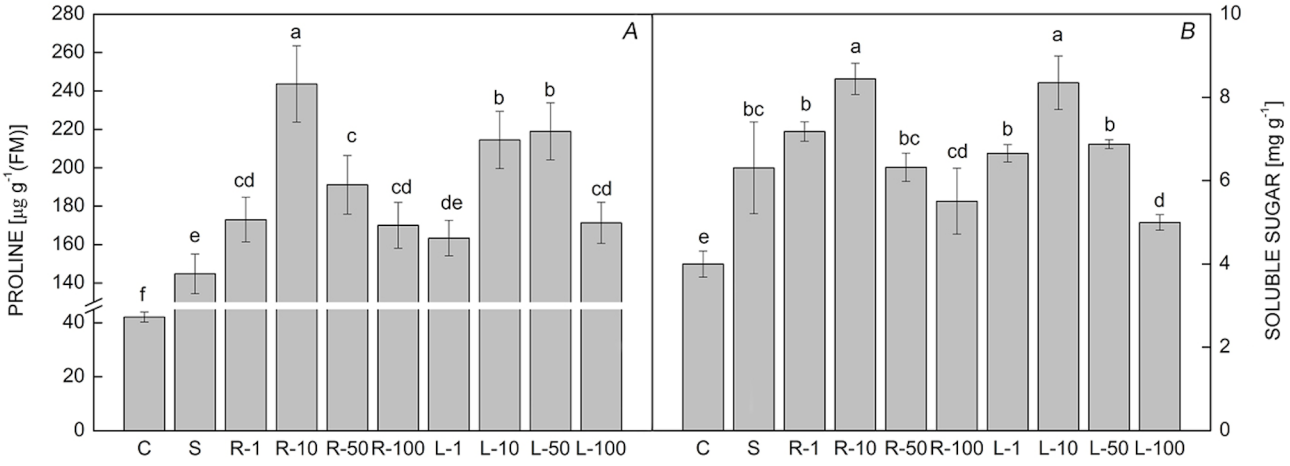

Fig. 6. Effects of the root application (R) or leaf spraying (L) with 1, 10, 50, and 100 μM acetylcholine (ACh) on the proline (*A*) and soluble sugar (*B*) contents of 15-d-old *Nicotiana benthamiana* plants grown in 1/2 Hoagland solution with or without 150 mM NaCl. Data are the means ± SD, $n = 3$. *Different letters* in the same column indicate significant difference between treatments at $p \leq 0.05$ using DMRT.

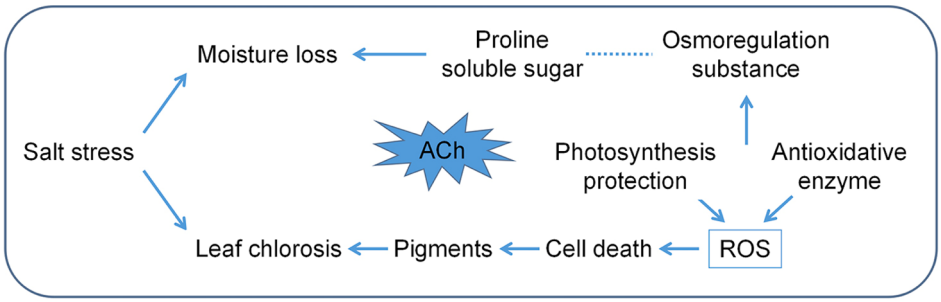

Fig. 7. A model depicting how acetylcholine (ACh) improves tolerance to salt stress. Under salinity stress, the increased photosynthetic ability contributes to suppression of ROS production, activates antioxidant enzymes which leads to reduced membrane lipid peroxidation, promotes the accumulation of key osmolytes, maintains the water status, and reduces chlorosis, thereby improving salt-stress tolerance.

Leaf relative water content (LRWC): There was a significant difference between the S and C treatments with respect to RWC of leaves (Table 1). Salinity stress significantly decreased LRWC by 26.7% but it was alleviated by ACh. The addition of 10 μM ACh (R-10 and L-10) showed the greatest reversal of salt-induced decline in LRWC.

Discussion

The lack of studies on regulatory role of ACh in salt-stressed plants: Acetylcholine (ACh) has a vital regulatory role in animals, but its role in the regulation of metabolic processes in plants has not been adequately studied (Braga *et al.* 2017). Numerous studies have illustrated that exogenous application of this substance could reduce the adverse effects of salt stress, which is manifested as improved gas exchange as well as biochemical responses of plants (Abbas *et al.* 2015, Li *et al.* 2017b, Matuszak-Slamani *et al.* 2017). Therefore, the main aim of our work was to investigate whether the presence of ACh could ameliorate salt stress-induced adverse effects on *Nicotiana benthamiana* plants.

Effect of Chl accumulation by exogenous ACh under salt stress: The *in vitro* test on leaf discs was conducted to assess the extent of chlorosis in salt-stressed *Nicotiana benthamiana* seedlings. It is clear from the results that 10-μM ACh solution was effective in alleviating the salt-stress-induced chlorosis on leaves (Fig. 2). It is widely known that chlorosis can destruct Chl pigments, hinder Chl synthesis, and perturb pigment protein complex (Rasool *et al.* 2013, Zhao *et al.* 2016). To confirm such a visual observation, we assayed the total Chl and used SPAD instrument to verify biochemical response of *Nicotiana benthamiana* leaf resulting in chlorosis. In our experiment, total Chl and SPAD values were significantly depressed under salt stress and the adverse effect was caused by the 100 μM ACh (both leaf and root application) (Fig. 3). Taken together, the exogenous ACh was beneficial for the stabilization of Chl and improvement of the photosynthetic attributes in response to salt stress. These findings would be important to further uncover physiological functions in stressed plants triggered by ACh.

Regulation of photosynthetic capacity by exogenous ACh under salt stress: Photosynthetic parameters are

Table 1. Effects of the root application (R) or leaf spraying (L) with 1, 10, 50, and 100 μM acetylcholine (ACh) on the leaf relative water content (LRWC) of 15 d tobacco plants grown in 1/2 Hoagland solution with or without 150 mM NaCl. Data are the means ± SD of three replicates. *Different letters* in the same column indicate significant difference between treatments at $p \leq 0.05$ using DMRT. C – plants grown under normal conditions for 15 d after supplying water through roots or as foliar spray; S – plants grown at 150 mM NaCl for 15 d after supplying water through roots or as foliar spray. L – plants grown at 150 mM NaCl for 15 d after supplying ACh (1, 10, 50 and 100 μM ACh) to leaves; R - plants grown at 150 mM NaCl for 15 d after supplying ACh (1, 10, 50 and 100 μM ACh) to roots.

Treatment	LRWC [%]
C	89.22± 1.62^{a}
S	65.40± 1.02^{d}
R-1	73.43± 0.77^{b}
R-10	71.14±2.08^{bc}
R-50	62.98± 0.71^{d}
R-100	61.97 ±1.42^{d}
L-1	71.77± 0.39^{b}
L-10	74.75± 3.00^{b}
L-50	65.98±8.40^{cd}
L-100	63.39± 2.79^{d}

widely used to assess the relative impact of environmental stresses (Singh *et al*. 2017). Nelson *et al*. (2007) have reported that P_N and g_s were sensitive to saline stress. Our results showed that gas-exchange indices (P_N, g_s, C_i, and E) of *Nicotiana benthamiana* seedlings were inhibited by salinity stress. Similar reports also show that stress markedly reduced gas-exchange characteristics in many plant species, such as barley (*Hordeum vulgar*e L.) (Mahlooji *et al*. 2018), sunflower (*Helianthus annuus* L.) (Shahbaz *et al*. 2011), and wheat (*Triticum aestivum* L.) (Sikder *et al*. 2015). In addition, 10 μM ACh solution applied to roots (R-10) or leaves (L-10), both alleviated the salt-induced damage to *Nicotiana benthamiana* seedlings and significantly enhanced leaf photosynthetic performance (Fig. 4). Many studies have shown that C_i decreased under different stresses, *e.g.*, cadmium toxicity (Ali *et al*. 2015), water deficiency (Radwan and Fayez 2016), salinity stress (Moradi and Ismail 2007, Naeem *et al.* 2010), and high temperature (Wu *et al.* 2012). Our current results showed that there was a high linear correlation between the P_N and g_s in the leaves and both P_N and g_s significantly decreased, indicating that some stomatal closure under salt stress restricted the external CO_2 from entering the leaves, thereby resulting in the reduction of C_i value.

Effect of the exogenous ACh on membrane lipid peroxidation and antioxidant enzymes under salinity stress: It is widely believed that MDA is a potential indicator of oxidative stress-induced lipid peroxidation (Hernandez *et al.* 2010, Xi *et al.* 2013, Yang and Guo 2018). In the current study, the MDA content increased dramatically under salt stress (Fig. 5*A*). However, the exogenously applied ACh reduced the negative effect of salt stress on MDA and the most effective concentration was 10 μM (R-10 or L-10). However, oxidative damage is controlled by a defensive system composed of various antioxidant enzymes, such as SOD and POD (Hayat *et al*. 2012). Our results revealed that application of 10 μM ACh (R-10 or L-10) increased the activities of SOD and POD in leaves under salt stress, which indicated that it could strengthen the cell membranes by enhancing the activities of antioxidant enzymes under salinity stress (Fig. 5 *B*,*C*). The above ameliorative effects of the exogenous ACh on MDA accumulation could significantly improve water status in salt-stressed *Nicotiana benthamiana* seedlings (Table 1).

Effect of exogenous ACh on osmoregulation under salt stress: Osmotic stress is the main cause of salt injury due to obstruction of water uptake by roots and internal dehydration (Khan *et al*. 2012, Iqbal *et al*. 2016). In order to resist to salt stress, plant cells can effectively and quickly trigger the synthesis of osmolytes, such as proline, glycine betaine, and soluble sugars to improve the ability of plant cells to acquire water (Hu *et al.* 2012, Lei *et al.* 2016). Our results showed that proline and soluble sugar contents were enhanced by salt stress (Fig. 6 *A*,*B*). Salinity stress dramatically increased the contents of proline and soluble sugars in many plants, such as wheat (Li *et al*. 2017b) and okra (*Hibiscus esulentus* L.) (Abbas *et al*. 2015), *etc*. Additionally, the higher content of proline and soluble sugars was recorded in R-10 and L-50, suggesting that these concentrations of ACh are more effective in promoting the accumulation of both key osmolytes. Such, the better osmoregulation imposed by optimal ACh concentration could maintain reduction of water loss in plant under salt stress (Table 1).

Conclusions: ACh is the established neurotransmitter widely used in human and animals, which is now being investigated as a novel inducer of plant tolerance to salt stress. This study demonstrated the alleviation of negative effects of salinity stress on *Nicotiana benthamiana* plants by either leaf spraying or root application of ACh. It is important to note that the most effective concentration of ACh was 10 μM. Under salinity stress, ACh-induced increased photosynthetic ability contributed to suppression of ROS production. It also activated antioxidant enzymes and decreased the extent of membrane lipid peroxidation. ACh was also effective in the upregulation of osmotic adjustment phenomenon in the *Nicotiana benthamiana* plants grown under saline stress. Maintenance of leaf water status and reduction of chlorosis were also the indicators of alleviating effects of ACh on salt-stressed *Nicotiana benthamiana* plants.

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