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Exogenous acetylcholine alleviates cadmium-induced phytotoxicity by modulating photosynthetic metabolism and antioxidant potential in tobacco (*Nicotiana benthamiana*)

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Abstract

Acetylcholine (ACh) plays a potential role in mediating various physiological processes in plants. However, the involvement of acetylcholine in cadmium (Cd) stress tolerance is unknown. Cd stress caused a decline in biomass, pigment, and photosynthetic capacity in *Nicotiana benthamiana* plants. A significant oxidative stress was evident through enhancing the accumulation of reactive oxygen species and lipid peroxidation. However, pretreatment with acetylcholine (50 and 100 μM) effectively decreased oxidative damage, especially by 50 μM ACh, which reduced H_2O_2 and $O_2^{\bullet-}$ contents by 50.4 and 45.3%, respectively, in plants treated with Cd. Our results indicated that the protection of photosynthesis by ACh was attributed to the improved photochemical activity of PSII and inhibited stomatal closure. Additionally, ACh-induced Cd toxicity tolerance was closely associated with improved activities of antioxidant enzymes and ascorbate and glutathione pools. Thus, our results suggest that acetylcholine may act as a positive regulator against Cd toxicity in tobacco.

Additional key words: antioxidant system; chlorophyll fluorescence; gas exchange; photoinhibition.

Introduction

Cadmium (Cd) is considered as a highly toxic element, and it is one of the main environmental limiting factors in physiological and biochemical processes (Clemens 2006). Cd induces the accumulation of superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), which cause oxidative damage, eventually disrupting normal metabolism (Apel and Hirt 2004). Cd also interferes with the photosynthetic efficiency, which is associated with the inhibition of PSII activity (Sharma *et al*. 2019). Particularly, it inhibits chlorophyll (Chl) biosynthesis, which results from the defects in the electron transport chain in chloroplasts due to the excessive generation of reactive oxygen species (ROS) for cellular redox homeostasis (Asada 2006, Kapoor *et al*. 2019). Furthermore, overaccumulation of ROS causes membrane damage by inducing lipid peroxidation (malondialdehyde, MDA), which damages to vital biomolecules (Gill and Tuteja 2010). In order to scavenge ROS, plants have formed diverse defense mechanisms, including the accumulation of osmolytes (*i.e*., proline, sugar), which maintains cellular homeostasis (Antoniou *et al*. 2017). Moreover, when exposed to severe prolonged Cd stress, plants have formed an antioxidant defense system that contains both enzymatic antioxidant [ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase (CAT)] and nonenzymatic antioxidants [glutathione (GSH) and ascorbate (AsA)] to protect cells against detrimental effects of ROS (Asada 1992). It has been indicated that application of some exogenous substances can enhance plants tolerance to Cd stress by increasing the antioxidant enzyme activities (Li *et al*. 2019). Besides, both enzymatic and nonenzymatic antioxidants coupled with AsA-GSH cycle have a vital role in the alleviation of various stresses (Ahmad *et al*. 2018). Thus, maintaining a moderate balance between ROS generation and free radical scavenging

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Abbreviations: ACh – acetylcholine; APX – ascorbate peroxidase; AsA – ascorbate; CAT – catalase; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DHA – dehydroascorbate; DM – dry mass; *E* – transpiration rate; FM – fresh mass; F_v/F_m – maximal quantum yield of PSII photochemistry; GSH – glutathione; g_s – stomatal conductance; GSSG – glutathione disulfide; MDA – malondialdehyde; NPQ – nonphotochemical quenching; P_N – net photosynthetic rate; q_P – photochemical quenching coefficient; ROS – reactive oxygen species; SOD – superoxide dismutase; TBA – thiobarbituric acid; TCA – trichloroacetic acid; $Y_{(II)}$ – actual photochemical efficiency of PSII.
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is essential for plant growth and development under Cd stress.

Acetylcholine (ACh) is widely distributed in animals and plants (Horiuchi *et al*. 2003). In animals, the role of acetylcholine as a neurotransmitter to propagate an electrical stimulus is well-established (Sagane *et al*. 2005). In plants, ACh regulates growth and development as well as several physiological functions (Tretyn and Kendrick 1991, Kawashima *et al*. 2007). Studies have shown that ACh, as a signal molecule, is closely related to regulating elongation of lateral roots, and pro-moting dry matter accumulation (Sugiyama and Tezuka 2011, Braga *et al*. 2017). Momonoki and Tokuhiro (1992) reported that the ACh is sensitive to gravity changes, Ca^{2+} concentration, and distribution of hormones under environmental stresses. It has been demonstrated that exogenously applied ACh can effectively ameliorate the adverse effects of salt stress in *Nicotiana benthamiana* (Qin *et al*. 2019). However, there is no relevant information about whether ACh can ameliorate Cd-induced damage in plants.

Nicotiana benthamiana, as a crucial model plant, has the characteristics of dwarf phenotype, short growth cycle, and can be successfully infected by the great majority of plant viruses, which has been widely used in transgenic experiments. To date, no investigation has been reported regarding the potential role of acetylcholine in heavy metal stress of tobacco plants. The hypothesis was that Cd-induced photoinhibition and oxidative stress were observed in tobacco plants and then such a process could be effectively alleviated by acetylcholine addition. In this study, the effects of acetylcholine on physiological processes, including growth attributes, photosynthetic performance, stomatal morphology, membrane lipid peroxidation, antioxidant and nonenzymatic antioxidants during Cd stress were studied. The potential mechanisms of the protections of acetylcholine were also explored and discussed. The results can provide further revealing of the potential roles of acetylcholine in regulating heavy metal stress.

Materials and methods

Plant treatments: Seeds of tobacco (*Nicotiana benthamiana*) were provided by Laboratory of Plant Physiology, Nanjing Agricultural University. Seeds were surface sterilized with 5% NaClO for 5 min and washed with distilled water two times, then soaked in sterile water for 24 h at 25°C in the darkness. The uniform seedlings were transplanted in the plastic pots (245 × 170 × 75 mm) and grown in a growth chamber. The day/night air temperature was maintained at 25/18°C with 16-h day length, relative humidity of 60–70%, and light intensity of 800 μmol(photon) m^{-2} s^{-1}. To ensure the tobacco seedlings grow normally, plants were cultured with 1/2 Hoagland nutrient solution, which was changed every 2 d to keep a constant solution concentration. When six leaves were expanded, the seedlings were treated with different concentrations of ACh [*Sigma-Aldrich* (≥ 99%, St. Louis, USA)] solution (0, 5, 10, 50, 100 or 150 μM). After 3 d of pretreatment with ACh, the seedlings were treated with 100 μM $CdCl_2$ (analytical grade, this concentration was selected by a previous trial, unpublished data). Briefly, the seedlings were separated into seven groups based on different treatments. Treatments were performed as reffered in the text table. The experiment was performed in triplicate and each replicate consisted of 12 plants, the plants were sampled after 15 d of Cd exposure then stored at –80°C until further analysis.

The growth parameters were measured after 15 d of Cd stress. Plant height and root length were obtained adopting a manual scale. The fresh mass of tobacco seedlings was taken after washing with distilled water. To determine the dry mass, the leaf tissues were dried to a constant mass at 60°C for 72 h.

SPAD index and leaf pigments: The relative chlorophyll (Chl) content of seedlings (fully expanded leaves from top) were measured with a Chl meter (*SPAD-502*, *Minolta*, Japan). The Chl content was also determined following the method of Wintermans and De Mots (1965). Leaves (0.1 g) were extracted using 10 mL of 95% ethanol after shaking at room temperature for 24 h in darkness. After centrifugation at 5,000 × *g* for 15 min, an absorbance of the supernatant was determined at 649 and 665 nm using a spectrophotometer (*UV-2800*, *Shimadzu*, Kyoto, Japan). The concentration was estimated according to the formula: Total Chl = $18.08 \times A_{649} + 6.63 \times A_{665}$.

Gas-exchange parameters: The second fully expanded leaves from the top were selected to measure net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) using a photosynthesis system (*Li-6400XT*, Lincoln, Nebraska, USA) during the morning (8:30–10:30 h). The measuring system was controlled to maintain leaf temperature at 25 ± 2°C, CO_2 concentration was set at 400 μmol(CO_2) mol^{-1}, and PAR was 1,000 μmol(photon) m^{-2} s^{-1}.

PSII activity: The second fully expanded leaves from the top were determined with *Open FluorCam* (*FC-800-C/1010*, Germany) after a 25-min dark adaptation. The maximum photochemical efficiency of PSII (F_v/F_m) and actual photochemical efficiency of PSII [$Y_{(II)}$] was calculated according to the method of Genty *et al*. (1989). The nonphotochemical quenching (NPQ) and photochemical quenching coefficient (q_P) were assayed using the method described by Tambussi *et al*. (2002).

Stomata: The fourth fully expanded leaves from the plant top were cut into 5 × 5 mm segments and fixed with FAA (formalin–acetic acid–alcohol) solution overnight, then washed two times with distilled water for 10 min at each step. Finally, the samples were placed on glass slides before observation. The samples were observed using a microscope (*Leica*, *Model DMi8*, Germany). The morphometric parameters of stomatal aperture and density were estimated following procedure of Snider *et al*. (2009).

***In vivo* visualization of ROS**: The $O_2^{\bullet-}$ staining was assayed as the procedure described by Kim *et al*. (2012).

Treatment	
Control	1/2 Hoagland nutrient solution
Cd	1/2 Hoagland nutrient solution + 100 μM Cd
5 ACh + Cd	5 μM ACh + 1/2 Hoagland nutrient solution + 100 μM Cd
10 ACh + Cd	10 μM ACh + 1/2 Hoagland nutrient solution + 100 μM Cd
50 ACh + Cd	50 μM ACh + 1/2 Hoagland nutrient solution + 100 μM Cd
100 ACh + Cd	100 μM ACh + 1/2 Hoagland nutrient solution + 100 μM Cd
150 ACh + Cd	150 μM ACh + 1/2 Hoagland nutrient solution + 100 μM Cd

After removal from plants, leaves were incubated in 0.5 mg mL^{-1} of nitroblue tetrazolium (NBT, pH 7.8) solution under darkness. H_2O_2 production was assayed by 3,3-diaminobenzidine (DAB) staining, adopting the procedure described by Christensen *et al.* (1997). Leaves were incubated in 1 mg mL^{-1} of DAB solution (pH 7.8) and in the absence of light for 5 h at 25°C. Then the leaves were washed in 95% (v/v) ethanol every 20 min at 80°C until complete removal of green background.

$O_2^{\bullet-}$ and H_2O_2 analysis: $O_2^{\bullet-}$ was measured as described by Schopfer *et al*. (2001). Fresh leaves were homogenized with 5 mL of 50 mM phosphate buffer (pH 7.8). The homogenized material was centrifuged at 12,000 × *g* for 20 min. Then, the supernatant was used for the determination of $O_2^{\bullet-}$ production, which was calculated using a standard curve based on sodium nitrite and expressed in mol g^{-1}(FM) min^{-1}.

To estimate H_2O_2 concentration, fresh leaves were homogenized with 5 mL of ice-cold 0.1% (w/v) trichloroacetic acid, followed by centrifugation at 10,000 × *g* for 20 min at 4°C. Supernatants were collected and combined with 0.5 mL 0.1 M potassium phosphate buffer (pH 7.0) and 1 mL of KI solution, and absorbance was recorded at 390 nm (*UV-2800*, *Shimadzu*, Kyoto, Japan). The concentration of H_2O_2 was based on a H_2O_2 standard curve and expressed as mmol g^{-1}(FM) (Patterson *et al*. 1984).

Determination of leaf malondialdehyde (MDA): The MDA assay was a modification of the procedure of Tonutti *et al*. (1997). Fresh leaf tissues (0.2 g) were homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 4,000 × *g* for 15 min at 4°C. Supernatant (1 mL) was mixed with 4 mL of 20% TCA, containing 0.65% (w/v) thiobarbituric acid (TBA). The mixture was heated in a water bath for 10 min, then immediately centrifuged for 15 min at 4,500 × *g* and its absorbance was recorded at 450, 532, and 660 nm (*UV-2800*, *Shimadzu*, Kyoto, Japan).

Proline accumulation: Briefly, leaf samples (0.2 g) were homogenized in 7.5 mL of 3% sulfosalicylic acid. The mixture solution containing 2 mL of filtrate, 2 mL of 2.5% ninhydrin reagent, and 2 mL of glacial acetic acid was kept in a boiling water for 1 h and then placed on ice for 30 min. Then, the reaction mixture was extracted using 4 mL of toluene. The absorbance of the extract was read at 520 nm (*UV-2800*, *Shimadzu*, Kyoto, Japan) (Bates *et al*. 1973).

Assay of antioxidant enzymes: For enzyme analyses, fresh leaf tissues (0.2 g) were homogenized in 3 mL of ice-cold phosphate buffer (50 mM, pH 7.0) consisting of 0.2 mM EDTA and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C, and the supernatant was used for the assay of antioxidant enzymes. Superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated by measuring the inhibition of photochemical reduction of NBT following the procedure of Giannopolitis and Ries (1977). The reaction mixture (3 mL) comprised of 50 mM Na_2HPO_4-NaH_2PO_4 buffer, 3 mM EDTA, 1 mM NBT, 14 mM methionine, 60 mM riboflavin, and 100 μL of enzyme extract. One unit of SOD was defined as the amount of enzyme, which inhibited the NBT reduction by 50% at 560 nm.

For ascorbate peroxidase (APX, EC 1.11.1.11) activity assay, 3 mL of reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 9 mM ascorbic acid, 12.5 mM H_2O_2, and 100 μL of enzyme extract (Nakano and Asada 1981). The activity was calculated by recording the decrease in absorbance at 290 nm per min (*UV-2800*, *Shimadzu*, Kyoto, Japan).

Catalase (CAT, EC 1.11.1.6) activity was assayed using the method of Cakmak and Marschner (1992). For the assay of CAT, the reaction mixture (3 mL) contained 12.5 mM H_2O_2, 50 mM sodium phosphate buffer (pH 7.0), and 200 μL of enzyme extract. The decomposition of H_2O_2 was determined by recording the absorbance at 240 nm.

Ascorbate and glutathione contents: The method of Masato (1980) was adopted for the measurement of ascorbate (AsA) and dehydroascorbate (DHA) contents. Fresh leaf tissues (0.1 g) were homogenized in 5% trichloroacetic acid and centrifuged at 4°C for 15 min at 15,000 × *g*. For total AsA, the reaction mixture comprised of 0.2 mL of the supernatant, 0.5 mL of 50 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.1 mL of 10 mM DTT. After incubation for 10 min at 25°C, the reaction mixture was added to 0.15 mL of 0.5% N-ethylmaleimide to remove excess DTT. For measurement of AsA, 200 μL of deionized H_2O was substituted for DTT and N-ethylmaleimide. Next, the reaction mixtures were added to 400 μL of 10% TCA, 400 μL of 44% phosphoric acid, 400 μL 70% α-dipyridyl in ethanol, and 200 μL of 0.3% (w/v) $FeCl_3$. The mixture was incubated at 40°C for 40 min in a water bath and the absorbance was recorded at 525 nm (*UV-2800*, *Shimadzu*, Kyoto, Japan).

The DHA concentration was detected by subtracting the AsA concentration from the total AsA concentration.

Glutathione (GSH) and glutathione disulfide (GSSG) were measured by the method of Kampfenkel *et al*. (1995) and Smith (1985). Fresh leaf tissues were ground in 2 mL of 5% sulfosalicylic acid containing 2 mM EDTA and centrifuged at 4°C for 10 min at 12,000 × *g*. For the total glutathione assay, 100 μL of the supernatant was added to 50 mM phosphate buffer (pH 7.5) containing 5 mM EDTA, 20 μL of 10 mM NADPH, and 80 μL of 12.5 mM DTNB. The reaction was started by adding 20 μL of GR (50 U mL^{-1}) and was monitored by measuring the changes in absorbance at 412 nm (*UV-2800*, *Shimadzu*, Kyoto, Japan).

GSH was masked by adding 20 μL of 2-vinylpyridine to the neutralized supernatant for the GSSG assay. The GSH concentration was obtained by subtracting the GSSG concentration from the total concentration.

Cd concentration: The root and leaf tissues were dried at 60°C until reaching the constant mass. Then the dried tissues (0.1 g) were ground to powder and digested with concentrated $HClO_4/HNO_3$ (1:4, v/v). Thereafter, the concentration of Cd was measured following the procedures of Wu *et al*. (2005) using an atomic absorption spectrophotometer (*Z-2000*, *Hitachi*, Japan).

Statistical analysis: The values are presented as mean ± standard deviation (SD) of independent replicates. Statistical significance of the treatments was by analysis of variance test (*ANOVA*) followed by mean separation by *Duncan*'s multiple range test at $P<0.05$.

Results

Plant growth: As shown in Fig. 1*A*, the leaf rolls and chlorosis were obviously found in Cd alone treatment. It was apparent that application of ACh (5–150 μM) improved the degree of leaf chlorosis and growth of tobacco seedlings, compared to control treatment (Fig. 1*A*). Meanwhile, Cd stress inhibited biomass accumulation in tobacco seedlings. However, application of ACh reduced Cd-induced inhibition in plant biomass. In the Cd alone treatment, values for FM and DM were reduced by 39.8 and 43.7%, respectively, in comparison with the control plants. Moreover, Cd stress treatment reduced the plant height of tobacco seedlings, while pretreated by ACh effectively alleviated this phenomenon (Fig. 1*B–D*). Furthermore, ACh with 5, 10, 100 or 150 μM concentration showed no obvious promotion in biomass accumulation and plant height under Cd toxicity, relative to the results of 50 μM acetylcholine.

Photosynthesis attributes: Our data demonstrated that Cd stress alone decreased total Chl content and SPAD by 38.5 and 47.9%, respectively, relative to the control treatment plants (Fig. 2*A*,*B*). It is noteworthy that application of ACh resulted in higher SPAD index and total Chl content than that of Cd alone treatment. Cd stress drastically decreased P_N, g_s, C_i, and E by 48.4, 70.2, 16.3, and 53.1%, respectively, in comparison to control treatment. However, pretreatment with various concentrations (5, 10, 100, and 150 μM) of ACh obviously alleviated Cd stress in leaves. Especially, application of 50 μM ACh was the most optimal concentration in alleviating photosynthetic inhibition (Fig. 2*C–F*).

PSII activity: To determine whether ACh was involved in Cd tolerance, we assayed Chl fluorescence attributes in leaves. Notably, Cd-induced reductions in F_v/F_m, $Y_{(II)}$, and q_P were markedly alleviated by the treatments with moderate concentration of ACh. Cd alone stress decreased F_v/F_m, $Y_{(II)}$, q_P by 35.2, 17.1, 25.3%, respectively, compared to the control treatment. Among the tested concentrations of ACh, the optimal concentration of 50 μM exhibited remarkable ameliorative effect as evident by 18.3, 14.9, and 11.8% increase in F_v/F_m, $Y_{(II)}$, and q_P, respectively, compared with the Cd alone treatment. When the

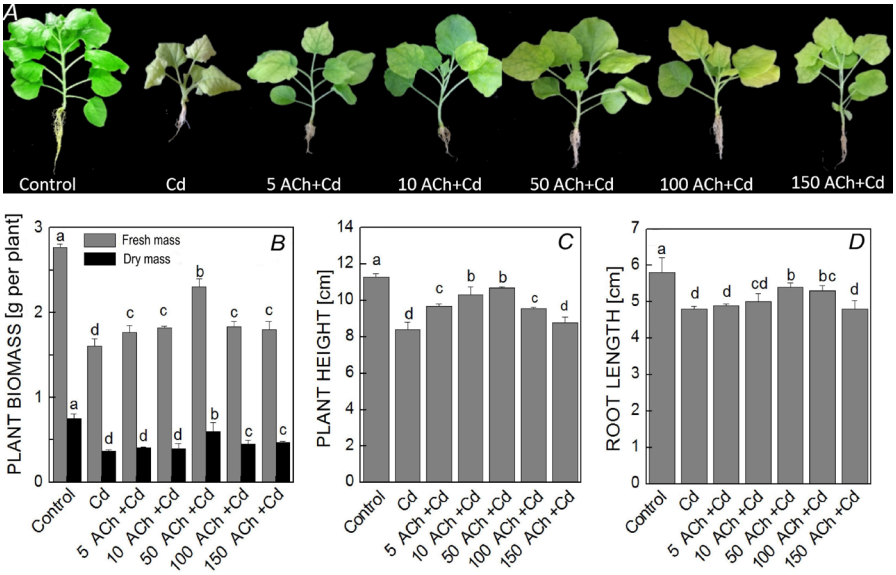

Fig. 1. Effects of exogenous acetylcholine on growth conditions after 15 d of Cd stress. (*A*) Phenotypic changes of tobacco seedlings, (*B*) fresh mass (FM) and dry mass (DM), (*C*) plant height, (*D*) root length. Data are means of three replicates ± SD. Bars with *different letters* indicate significant difference between treatments at $p\leq0.05$ by *Duncan*'s multiple range test. Control – 1/2 Hoagland nutrient solution. Cd – 1/2 Hoagland nutrient solution + 100 μM Cd. 5, 10, 50, 100 or 150 ACh + Cd – 1/2 Hoagland nutrient solution + 5, 10, 50, 100 or 150 μM ACh + 100 μM Cd.

Cd-stressed plants received different concentrations (5–150 μM) of ACh, especially 50 μM, a significant ameliorative decrease of NPQ (36.1%) was found, compared with Cd alone treatment (Fig. 3).

Stomata structure: To verify whether ACh-induced Cd tolerance is associated with stomatal behaviour, we analyzed stomatal density and aperture in leaves. As shown in Fig. 4, Cd led to decreases in the stomatal density and aperture, while pretreatment with ACh (10–50 μM) led to a significant increase in the stomatal aperture. Especially, pretreatment with 50 μM ACh markedly increased the stomatal density by 39.0%, compared to the treatment with Cd stress alone. Additionally, no significant differences were found in the stomatal aperture and stomata density between the 100 and 150 μM ACh-treated plants (Fig. 4).

ROS, MDA, and proline accumulation: Cd-exposure caused the generation of ROS, as evidenced by NBT and DAB staining in the leaves. $O_2^{\bullet-}$ is indicated by dark blue spots (Fig. 5*A*) and H_2O_2 is indicated by brown spots (Fig. 5*B*). Cd alone treatment was stained extensively in tobacco leaf, indicating that a severe cellular redox imbalance appeared. However, supplementation of ACh to

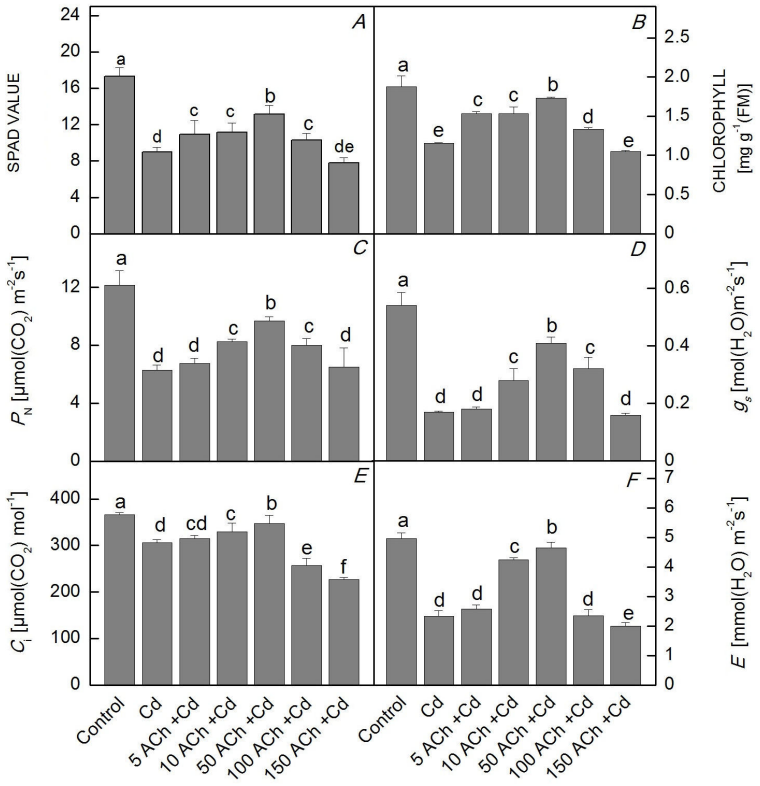

Fig. 2. Effects of exogenous acetylcholine on photosynthetic pigments and gas-exchange parameters in tobacco seedlings after 15 d of Cd stress. (*A*) SPAD index, (*B*) chlorophyll content, (*C*) net photosynthetic rate (P_N), (*D*) stomatal conductance (g_s), (*E*) intercellular CO_2 concentration (C_i), (*F*) transpiration rate (*E*). Data are means of three replicates ± SD. Bars with *different letters* indicate significant difference between treatments at $p \leq 0.05$ by *Duncan*'s multiple range test. Control – 1/2 Hoagland nutrient solution. Cd – 1/2 Hoagland nutrient solution + 100 μM Cd. 5, 10, 50, 100 or 150 ACh + Cd – 1/2 Hoagland nutrient solution + 5, 10, 50, 100 or 150 μM ACh + 100 μM Cd.

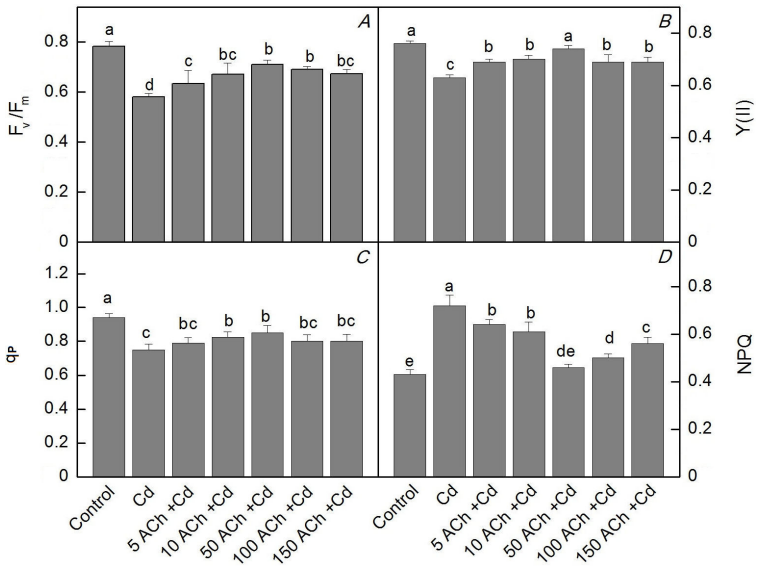

Fig. 3. Effects of exogenous acetylcholine on PSII in tobacco seedlings after 15 d of Cd stress. (*A*) maximum quantum yield of PSII (F_v/F_m), (*B*) actual photochemical efficiency of PSII [$Y_{(II)}$], (*C*) photochemical quenching coefficient (q_P), (*D*) nonphotochemical quenching (NPQ). Data are means of three replicates ± SD. Bars with *different letters* indicate significant difference between treatments at $p \leq 0.05$ by *Duncan*'s multiple range test. Control – 1/2 Hoagland nutrient solution. Cd – 1/2 Hoagland nutrient solution + 100 μM Cd. 5, 10, 50, 100 or 150 ACh + Cd – 1/2 Hoagland nutrient solution + 5, 10, 50, 100 or 150 μM ACh + 100 μM Cd.

Cd-stressed plants showed slight staining. In comparison with control plants, $O_2^{\bullet-}$ and H_2O_2 contents markedly increased after Cd alone treatment, whereas the exogenous application of ACh significantly reduced Cd-induced accumulation of $O_2^{\bullet-}$ and H_2O_2 (Fig. 5*C*,*D*). 50 μM ACh concentration was effective for alleviating Cd-induced accumulation of $O_2^{\bullet-}$ and H_2O_2. The application of 5 or 150 μM ACh showed to be less effective in decreasing ROS generation under Cd stress as compared to 50 μM concentration. Cd stress also caused severe oxidative damage to cell membranes. When tobacco seedlings were exposed to Cd alone stress, the contents of MDA dramatically increased 2.04-fold, as compared to control plants. However, pretreatment with ACh (50 μM) markedly reduced MDA content which was about 31.2% lower than that of the Cd alone treatment. Further, Cd stress also led to a remarkable accumulation of proline in leaves. Whereas, the treatments with different concentrations of ACh caused an increase in the proline in comparison with Cd stress alone. Application dose of 50 μM Ach along with Cd stress significantly increased the proline content by 44.5%, compared with Cd alone stress seedlings (Fig. 5*E*,*F*).

Activities of antioxidant enzymes: We also assessed the activities of vital antioxidant enzymes, such as SOD, APX, CAT, which significantly decreased by Cd alone treatment. Pretreatment with ACh led to much higher SOD activity than that measured in the control. Similar changes were observed in the APX and CAT activities. For instance, when treated with 50 μM ACh, the activity of antioxidant enzymes SOD, APX, and CAT increased 1.55, 0.78, and 2.13 times, respectively, in comparison with the control plants (Fig. 6).

Redox homeostasis: Compared with the control group, GSH and AsA values decreased, while glutathione disulfide (GSSG) and dehydroascorbate (DHA) contents were enhanced by Cd stress alone. However, pretreatment with ACh significantly increased GSH and AsA contents, whereas GSSG and DHA contents declined under Cd alone. When pretreated with lower concentration of ACh (5–50 μM), the content of GSH and AsA markedly increased. However, pretreatment with all concentrations of ACh (5–150 μM) caused no significant changes in GSSG and DHA (Fig. 7).

Cd accumulation: We investigated also the effect of ACh on Cd content in roots and leaves of tobacco seedlings. Table 1 showed that the maximum accumulation of Cd were observed in the leaves and roots, after exposure to Cd alone stress. Supplementation of 50 μM ACh obviously decreased the Cd uptake by 30.5% in root tissues. Application of various concentrations of ACh significantly decreased the Cd content in leaves, compared to Cd alone stress. However, there were no significant differences observed between all concentrations (10–150 μM) of ACh.

Discussion

Cadmium (Cd) is a widespread environmental pollutant because of its long half-life in soil, which alters the biochemical and physiological processes of plants. Cd toxicity induces lipid peroxidation and ROS accumulation, and then interferes with photosynthesis and growth (Cho and Seo 2005, Rahman *et al*. 2017). Therefore, it is necessary to reduce its phytotoxicity. Here, the potential feasibility of ACh application has been demonstrated in

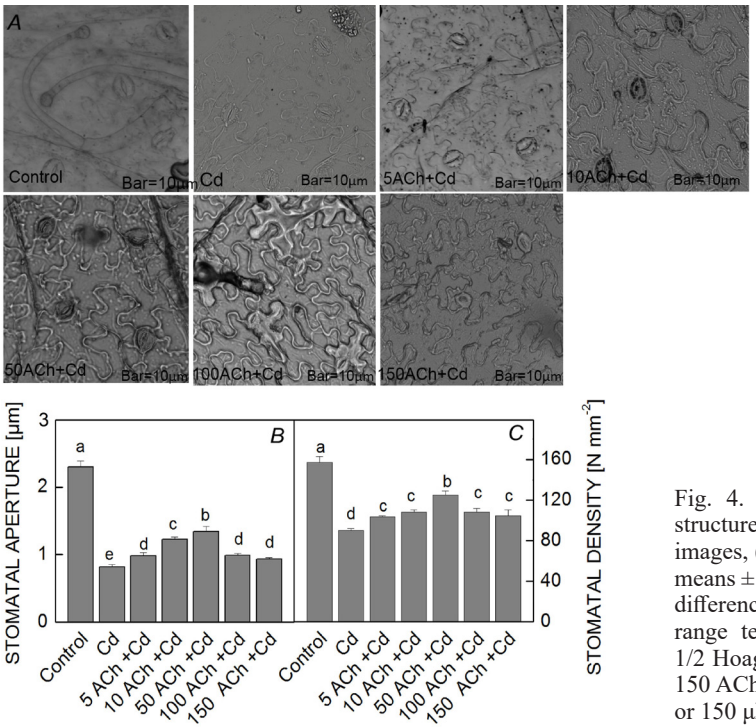

Fig. 4. Effects of exogenous acetylcholine on the stomatal structure of tobacco under Cd stress. (*A*) stomatal morphology images, (*B*) stomatal aperture, (*C*) stomatal density. Data are the means ± SD, $n = 5$. Bars with *different letters* indicate significant difference between treatments at $p \leq 0.05$ by *Duncan*'s multiple range test. Control – 1/2 Hoagland nutrient solution. Cd – 1/2 Hoagland nutrient solution + 100 μM Cd. 5, 10, 50, 100 or 150 ACh + Cd – 1/2 Hoagland nutrient solution + 5, 10, 50, 100 or 150 μM ACh + 100 μM Cd.

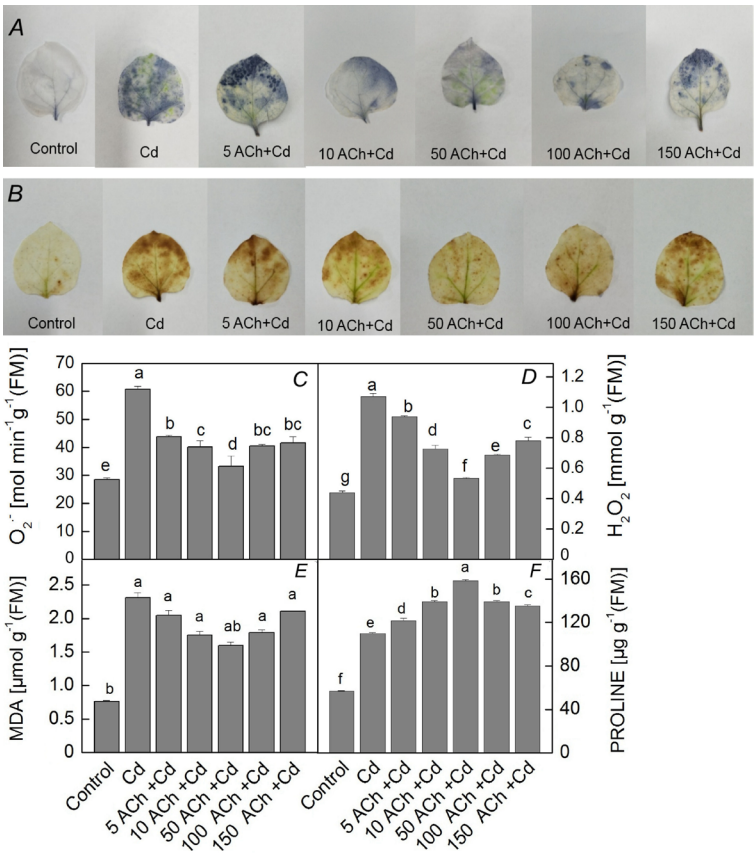

Fig. 5. Effects of exogenous acetylcholine on the production of ROS in tobacco seedlings after 15 d of Cd stress. (*A*) histochemical staining of $O_2^{\bullet-}$, (*B*) histochemical staining of H_2O_2, (*C*) $O_2^{\bullet-}$ content, (*D*) H_2O_2 content, (*E*) malondialdehyde (MDA) content, (*F*) proline content. Data are means of three replicates ± SD. Bars with *different letters* indicate significant difference between treatments at $p \leq 0.05$ by *Duncan*'s multiple range test. Control – 1/2 Hoagland nutrient solution. Cd – 1/2 Hoagland nutrient solution + 100 μM Cd. 5, 10, 50, 100 or 150 ACh + Cd – 1/2 Hoagland nutrient solution + 5, 10, 50, 100 or 150 μM ACh + 100 μM Cd.

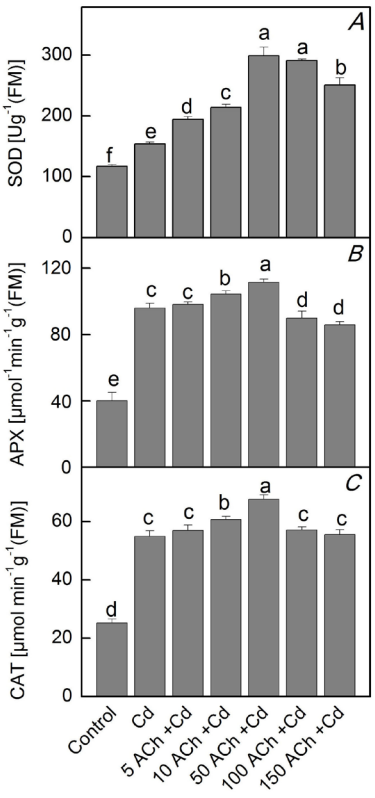

Fig. 6. Effects of exogenous acetylcholine on antioxidant enzyme activities in tobacco seedlings after 15 d of Cd stress. (*A*) superoxide dismutase (SOD), (*B*) ascorbate peroxidase (APX), (*C*) catalase (CAT). Data are means of three replicates ± SD. Bars with *different letters* indicate significant difference between treatments at $p \leq 0.05$ by *Duncan*'s multiple range test. Control – 1/2 Hoagland nutrient solution. Cd – 1/2 Hoagland nutrient solution + 100 μM Cd. 5, 10, 50, 100 or 150 ACh + Cd – 1/2 Hoagland nutrient solution + 5, 10, 50, 100 or 150 μM ACh + 100 μM Cd.

order to enhance the Cd tolerance of tobacco plants. Yi and Kao (2007) reported that leaf chlorosis is one of the common phenomena of Cd stress, which is well consistent with our current study where Cd caused leaf chlorosis or rolls (Fig. 1). Cd exposure also caused the decline in plant height, root length, and biomass accumulation, which has been attributed to hampered absorption of water and mineral nutrients (Lin *et al*. 2012). Furthermore, decreased photosynthetic pigment content directly affects photosynthesis and finally leads to growth inhibition. Thus, it is possible that Cd blocked Chl synthesis and further decreased P_N, thereby reducing plant biomass. However, exogenously applied ACh to plants treated with Cd alleviated growth inhibition, which might be attributed to its ability to mediate cell elongation and division by upregulating of endotransglycosylases/hydrolases (XTHs) (Yun *et al*. 2010).

Photosynthetic attributes are considered as sensitive indicators of abiotic stress, which can be negatively affected by heavy metal toxicity. Reduction in Chl content and gas-exchange parameters was consistent with the finding of Kaya *et al*. (2020), who revealed that Cd-induced a decrease of those indexes in wheat plants exposed to Cd stress. Parmar *et al*. (2013) reported that reduced Chl content in Cd-stressed plants is due to inhibited synthesis of Chl precursor (aminolevulinic acid), which is linked to the reduction of protochlorophyllide to chlorophyllide. However, application of ACh (50 μM) effectively promoted Chl synthesis and improved photosynthetic parameters (P_N, g_s, C_i, and E) (Fig. 2). This can be attributed to the fact that ACh promoted the synthesis of Chl-related precursors, reduced Chl degradation, and thus enhanced photosynthesis (Bajguz 2011). Furthermore, increased photosynthesis in ACh-treated plants may be attributed to increased CO_2 assimilation and stomatal conductance (Chen *et al*. 2011). Chl fluorescence analysis is one of the most useful procedures for obtaining the functional status of PSII (Baryla *et al*. 2001). Noctor *et al*. (2004)

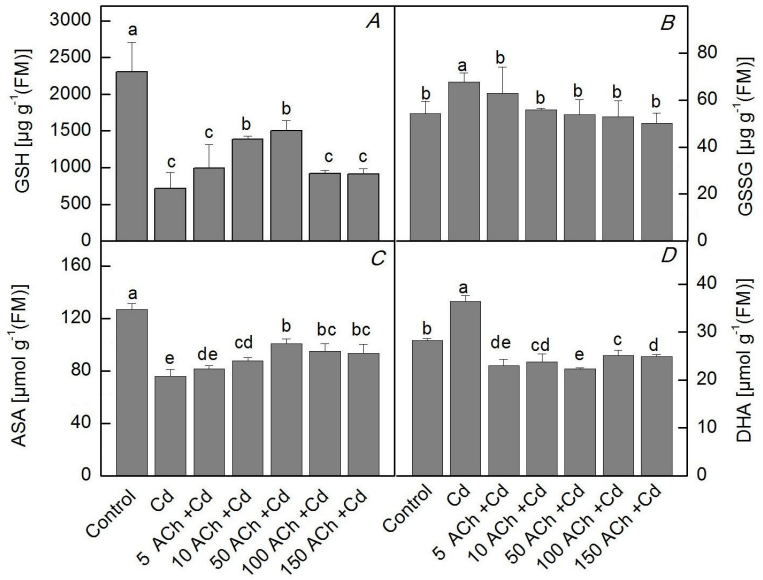

Fig. 7. Effects of exogenous acetylcholine on nonenzymatic antioxidants in tobacco seedlings after 15 d of Cd stress. (*A*) glutathione (GSH), (*B*) glutathione disulfide (GSSG), (*C*) ascorbate (AsA), (*D*) dehydroascorbate (DHA). Data are means of three replicates ± SD. Bars with *different letters* indicate significant difference between treatments at $p \leq 0.05$ by *Duncan*'s multiple range test. Control – 1/2 Hoagland nutrient solution. Cd – 1/2 Hoagland nutrient solution + 100 μM Cd. 5, 10, 50, 100 or 150 ACh + Cd – 1/2 Hoagland nutrient solution + 5, 10, 50, 100 or 150 μM ACh + 100 μM Cd.

Table 1. Effects of exogenous acetylcholine (Ach) on the content of Cd in root and leaf tissues after 15 d of Cd stress. Data are the means ± SD, $n = 3$. Means with *the same lowercase letters* are not significantly different at $p<0.05$ by *Duncan*'s multiple range test. Control – 1/2 Hoagland nutrient solution. Cd – 1/2 Hoagland nutrient solution + 100 μM Cd. 5, 10, 50, 100 or 150 ACh + Cd – 1/2 Hoagland nutrient solution + 5, 10, 50, 100 or 150 μM ACh + 100 μM Cd. nd – not detected.

Treatment	Cd content [μg g^{-1}(DM)]	
	Leaf	Root
Control	nd	nd
Cd	56.64 ± 3.24^{a}	100.76 ± 6.60^{a}
5 ACh + Cd	40.48 ± 4.43^{b}	92.86 ± 3.36^{b}
10 ACh + Cd	36.32 ± 3.37^{bc}	81.32 ± 2.52^{c}
50 ACh + Cd	30.07 ± 5.86^{c}	61.27 ± 0.95^{e}
100 ACh + Cd	35.56 ± 4.63^{bc}	67.49 ± 1.70^{d}
150 ACh + Cd	38.34 ± 1.67^{b}	72.49 ± 3.48^{d}

reported that decreased photosynthesis is due to reduced photosynthetic electron transport caused by Cd stress, which eventually resulted in an excessive energy generation and triggered photoinhibition. Interestingly, after exposure to Cd stress F_v/F_m, $Y_{(II)}$, and q_P remarkably decreased (Fig. 3). The reductions in those indexes might be caused by the restriction of light energy absorption and electron transport in PSI during Cd stress. Those results are supported by previous research of Vyas *et al*. (2007), who suggested that excessive ROS are the key cause of photosynthetic inhibition. Additionally, reduced F_v/F_m and q_P were linked to increased NPQ in Cd-stressed tobacco seedlings, indicating that the destructive energy dissipation was caused by Cd stress. Based on these observations, it is proposed that ACh can alleviate Cd-induced photosynthesis inhibition by regulating photosynthetic capacity and improving PSII activity.

The stomata occupy a central position in regulating the complex signal transduction pathways, such as maintenance of water vapor loss in leaves (Xu and Zhou 2008). When plants were subjected to Cd stress, closing stomata to reduce water evaporation is a crucial mechanism of plant tolerance, which ultimately leads to a significant decrease in g_s (Bindhu and Bera 2001). It is noted that stomatal aperture and stomatal density decreased under Cd alone stress, whereas ACh application reversed this phenomenon (Fig. 4). The reason might be that Ca^{2+}/CaM can act as a second messenger in ACh-induced stomatal opening and closing (Wang *et al*. 1999). Furthermore, ACh can mediate nicotinic and muscarinic receptors involved in this process (Wang *et al*. 1998, Wang *et al*. 2000). Therefore, this is due to the binding of ACh to its muscarinic ACh receptor, which triggers the changes of Ca^{2+} in guard cells. Afterwards, the influx of Ca^{2+} binds to CaM, which induces downstream pathway and ultimately leads to stomatal opening (Wang *et al*. 2003). Thus, application of ACh might improve the development of stomata, thereby altering g_s response to Cd and consequently alleviation of stomatal limitation of photosynthesis.

$O_2^{\bullet-}$ and H_2O_2 are the major ROS induced by Cd stress which can cause oxidative damage, directly inhibit or modify some proteins and ultimately lead to cell death (Pospíšil 2014). In the current study, exogenously applied ACh reduced the ROS accumulation, indicating that ACh application promoted the tolerance of plants to Cd stress by reducing ROS production. MDA and proline values are generally considered as vital indicators of membrane permeability or the loss of membrane integrity under metal stress (Fan *et al*. 2015). Previous study has confirmed that enhanced contents of H_2O_2 can lead to lipid peroxidation, which causes damage of the cell membrane (Sairam and Srivastava 2002). Interestingly, significant increases in MDA and proline were observed in Cd alone-treated plants (Fig. 5), which indicated that the free radical formation was stimulated by Cd toxicity, generating serious lipid peroxidation and leading to plasma membrane injury. Similar results were reported in *Triticum aestivum* L.

(Moussa and El-Gamal 2010) and *Brassica juncea* (Kaur *et al*. 2019) plants. However, application of ACh under Cd stress resulted in decrease of MDA content. Rahman *et al*. (2017) suggested that exogenous substances reduced free radical production by maintaining normal water and proline contents in cell. Hence, we hypothesized that ACh might maintain cell membrane stability by participating in the osmotic adjustment pathway responded to Cd stress.

Besides, the activity of antioxidant enzymes (SOD, CAT, and APX) plays an important role in Cd-induced oxidative stress. Hasan *et al*. (2015), who reported that Cd phytotoxicity could be mitigated by enhancing antioxidant potential after application of exogenous substance in *Solanum lycopersicum* L. Notably, Cd stress promoted the activities of SOD, CAT, and APX, whereas application of 50 μM ACh further increased these indexes in leaves during Cd exposure (Fig. 6), which indicated that exogenously applied ACh could activate the activity of antioxidant enzymes, thus alleviating Cd toxicity. GSH and AsA are major nonenzymatic antioxidants in the AsA-GSH cycle and play important roles in ROS scavenging (Schützendübel and Polle 2002). GSH acts as a redox buffer involved in antioxidant system, which is associated with the sequestration of Cd ions in the vacuoles. Interestingly, it was noticed that H_2O_2 and $O_2^{\bullet-}$ contents significantly decreased at lower concentration (5 μM) of ACh in treated plants, which indicated that the low concentration of ACh may promote the content of GSH and AsA, and consequently enhance ROS scavenging. These observations suggested that ACh-induced alleviation of oxidative stress is related to the enhancement of antioxidant enzymes activities and improvement of redox homeostasis in tobacco plants.

More Cd was accumulated in roots than that in leaves, which was associated with decreased transpiration rate and the decrease of Cd flux from roots to leaves due to the transport of Cd by the apoplastic pathway (Sterckeman *et al*. 2011). In our study, the higher concentration of Cd in roots than that in leaves may be related to the above reasons. The similar phenomenon was also found in tomato plants subjected to Cd exposure (Li *et al*. 2016). Previous study has shown that the reduction of Cd detoxification is also linked to cell wall binding, chelation with phytochelatins, and vacuolar sequestration (Lai 2015). In the current study, pretreatment with ACh obviously decreased the uptake of Cd in root and leaf tissues, compared to Cd stress alone (Table 1). These phenomena may be attributed to ACh, which can fix more Cd to the cell wall, sequester Cd in the vacuoles, then convert it into co-precipitation in the cytosol, thereby inhibiting the transport of Cd from the roots to shoots (Zhou *et al*. 2017).

Conclusions: It can be concluded that exogenous application of acetylcholine effectively improved the photosynthetic capacity and photochemistry of PSII as well as stomatal development. Moreover, acetylcholine induced ROS elimination as evidenced by upregulated antioxidant enzyme activity and maintenance of redox homeostasis. Thus, acetylcholine may act as a potential modulator of plant growth and play a critical role in plants tolerance to Cd. Further studies are needed to provide more molecular and genetic evidence to reveal the mechanism of acetylcholine involving Cd tolerance in plants.

References

Ahmad P., Ahanger M.A., Alyemeni M.N. *et al*.: Exogenous application of nitric oxide modulates osmolyte metabolism, antioxidants, enzymes of ascorbate-glutathione cycle and promotes growth under cadmium stress in tomato. – Protoplasma **255**: 79-93, 2018.

Antoniou C., Chatzimichail G., Xenofontos R. *et al.:* Melatonin systemically ameliorates drought stress-induced damage in *Medicago sativa* plants by modulating nitro-oxidative homeostasis and proline metabolism. – J. Pineal Res. **62**: e12401, 2017.

Apel K., Hirt H.: Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. – Annu. Rev. Plant Biol. **55**: 373-399, 2004.

Asada K.: Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. – Plant Physiol. **85**: 235-241, 1992.

Asada K.: Production and scavenging of reactive oxygen species in chloroplasts and their functions. – Plant Physiol. **141**: 391-396, 2006.

Bajguz A.: Suppression of *Chlorella vulgaris* growth by cadmium, lead and copper stress and its restoration by endogenous brassinolide. – Arch. Environ. Contam. Tox. **60**: 406-416, 2011.

Baryla A., Carrier P., Franck F. *et al*.: Leaf chlorosis in oilseed rape plants (*Brassica napus*) grown on cadmium-polluted soil: causes and consequences for photosynthesis and growth. – Planta **212**: 696-709, 2001.

Bates L.S., Waldren R.P., Teare I.D.: Rapid determination of free proline for water-stress studies. – Plant Soil **39**: 205-207, 1973.

Bindhu S.J., Bera A.K.: Impact of cadmium toxicity on leaf area, stomatal frequency, stomatal index and pigment content in mungbean seedlings. – J. Environ. Biol. **22**: 307-309, 2001.

Braga I., Pissolato M.D., Souza G.M.: Mitigating effects of acetylcholine supply on soybean seed germination under osmotic stress. – Braz. J. Bot. **40**: 617-624, 2017.

Cakmak I., Marschner H.: Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. – Plant Physiol. **98**: 1222-1227, 1992.

Chen X., Wang J., Shi Y. *et al*.: Effects of cadmium on growth and photosynthetic activities in pakchoi and mustard. – Bot. Stud. **52**: 41-46, 2011.

Cho U.H., Seo N.H.: Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. – Plant Sci. **168**: 113-120, 2005.

Christensen H.T., Zhang Z., Wei Y. *et al*.: H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. – Plant J. **11**: 1187-1194, 1997.

Clemens S.: Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. – Biochimie **88**: 1707-1719, 2006.

Fan J., Hu Z., Xie Y. *et al*.: Alleviation of cold damage to photosystem II and metabolisms by melatonin in Bermuda grass. – Front. Plant Sci. **6**: 925, 2015.

Genty B., Briantais J.M., Baker N.B.: The relationship between

the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – BBA-Gen. Subjects **990**: 87-92, 1989.

Giannopolitis C.N., Ries S.K.: Superoxide dismutases. 1. Occurrence in higher-plants. – Plant Physiol. **59**: 309-314, 1977.

Gill S.S., Tuteja N.: Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. – Plant Physiol. Bioch. **48**: 909-930, 2010.

Hasan M.K., Ahammed G.J., Yin L. *et al*.: Melatonin mitigates cadmium phytotoxicity through modulation of phytochelatins biosynthesis, vacuolar sequestration, and antioxidant potential in *Solanum lycopersicum* L. – Front. Plant Sci. **6**: 601, 2015.

Horiuchi Y., Kimura R., Kato N. *et al*.: Evolutional study on acetylcholine expression. – Life Sci. **72**: 1745-1756, 2003.

Kampfenkel K., Van Montaqu M., Inzé D.: Extraction and determination of ascorbate and dehydroascorbate from plant tissue. – Anal. Biochem. **225**: 165-167, 1995.

Kapoor D., Singh M.P., Kaur S. *et al*.: Modulation of the functional components of growth, photosynthesis, and anti-oxidant stress markers in cadmium exposed *Brassica juncea* L. – Plants-Basel **8**: 260, 2019.

Kaur P., Bali S., Sharma A. *et al*.: Cd induced generation of free radical species in *Brassica juncea* is regulated by supplementation of earthworms in the drilosphere. – Sci. Total Environ. **655**: 663-675, 2019.

Kawashima K., Misawa H., Moriwaki Y. *et al*.: Ubiquitous expression of acetylcholine and its biological functions in life forms without nervous systems. – Life Sci. **80**: 2206-2209, 2007.

Kaya C., Ashraf M., Alyemeni M.N., Ahmad P.: Responses of nitric oxide and hydrogen sulfide in regulating oxidative defence system in wheat plants grown under cadmium stress. – Physiol. Plantarum **168**: 345-360, 2020.

Kim J.M., Woo D.H., Kim S.H. *et al*.: *Arabidopsis* MKKK20 is involved in osmotic stress response *via* regulation of MPK6 activity. – Plant Cell Rep. **31**: 217-224, 2012.

Lai H.Y.: Subcellular distribution and chemical forms of cadmium in *Impatiens walleriana* in relation to its phytoextraction potential. – Chemosphere **138**: 370-376, 2015.

Li M.Q., Hasan M.K., Li C.X. *et al*.: Melatonin mediates selenium-induced tolerance to cadmium stress in tomato plants. – J. Pineal Res. **61**: 291-302, 2016.

Li Q., Wang G., Wang Y. *et al*.: Foliar application of salicylic acid alleviate the cadmium toxicity by modulation the reactive oxygen species in potato. – Ecotox. Environ. Safe. **172**: 317-325, 2019.

Lin L., Zhou W., Dai H. *et al*.: Selenium reduces cadmium uptake and mitigates cadmium toxicity in rice. – J. Hazard. Mater. **235-236**: 343-351, 2012.

Masato O.: An improved method for determination of L-ascorbic acid and L-dehydroascorbic acid in blood plasma. – Clin. Chim. Acta **103**: 259-268, 1980.

Momonoki Y.S., Tokuhiro M.: The influence of heat stress on acetylcholine content and its hydrolyzing activity in *Macroptilium atropurpureum* cv. Siratro. – Jpn. J. Crop Sci. **61**: 112-118, 1992.

Moussa H.R., El-Gamal S.M.: Role of salicylic acid in regulation of cadmium toxicity in wheat (*Triticum aestivum* L.). – J. Plant Nutr. **33**: 1460-1471, 2010.

Nakano Y., Asada K.: Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. – Plant Cell Physiol. **22**: 867-880, 1981.

Noctor G., Dutilleul C., De Paepe R., Foyer C.H.: Use of mitochondrial electron transport mutants to evaluate the effects of redox state on photosynthesis, stress tolerance and the integration of carbon/nitrogen metabolism. – J. Exp. Bot. **55**: 49-57, 2004.

Parmar P., Kumari N., Sharma V.: Structural and functional alterations in photosynthetic apparatus of plants under cadmium stress. – Bot. Stud. **54**: 45, 2013.

Patterson B.D., MacRae E.A., Ferguson I.B.: Estimation of hydrogen peroxide in plant extracts using titanium(IV). – Anal. Biochem. **139**: 487-492, 1984.

Pospíšil P.: The role of metals in production and scavenging of reactive oxygen species in photosystem II. – Plant Cell Physiol. **55**: 1224-1232, 2014.

Qin C., Su Y.Y., Li B.S. *et al*.: Acetylcholine mechanism of action to enhance tolerance to salt stress in *Nicotiana benthamiana*. – Photosynthetica **57**: 590-598, 2019.

Rahman M.F., Ghosal A., Alam M.F., Kabir A.H.: Remediation of cadmium toxicity in field peas (*Pisum sativum* L.) through exogenous silicon. – Ecotox. Environ. Safe. **135**: 165-172, 2017.

Sagane Y., Nakagawa T., Yamamoto K. *et al*.: Molecular characterization of maize acetylcholinesterase: a novel enzyme family in the plant kingdom. – Plant Physiol. **138**: 1359-1371, 2005.

Sairam P.K., Srivastava G.C.: Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. – Plant Sci. **162**: 897-904, 2002.

Schopfer P., Plachy C., Frahry G.: Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. – Plant Physiol. **125**: 1591-1602, 2001.

Schützendübel A., Polle A.: Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. – J. Exp. Bot. **53**: 1351-1365, 2002.

Sharma A., Kumar V., Shahzad B. *et al*.: Photosynthetic response of plants under different abiotic stresses: a review. – J. Plant Growth Regul. **38**: 1-23, 2019.

Smith I.K.: Stimulation of glutathione synthesis in photorespiring plants by catalase inhibitors. – Plant Physiol. **79**: 1044-1047, 1985.

Snider J.L., Choinski J.S., Wise R.R.: Juvenile *Rhus glabra* leaves have higher temperatures and lower gas exchange rates than mature leaves when compared in the field during periods of high irradiance. – J. Plant Physiol. **166**: 686-696, 2009.

Sterckeman T., Redjala T., Morel J.L.: Influence of exposure solution composition and of plant cadmium content on root cadmium short-term uptake. – Environ. Exp. Bot. **74**: 131-139, 2011.

Sugiyama K., Tezuka T.: Acetylcholine promotes the emergence and elongation of lateral roots of *Raphanus sativus*. – Plant Signal. Behav. **6**: 1545-1553, 2011.

Tambussi E.A., Casadesus J., Munné-Bosch S., Araus J.L.: Photoprotection in water-stressed plants of durum wheat (*Triticum turgidum* var. *durum*): changes in chlorophyll fluorescence, spectral signature and photosynthetic pigments. – Funct. Plant Biol. **29**: 35-44, 2002.

Tonutti P., Bonghi C., Ruperti B. *et al*.: Ethylene evolution and 1-aminocyclopropane-1-carboxylate oxidase gene expression during early development and ripening of peach fruit. – J. Am. Soc. Hortic. Sci. **122**: 642-647, 1997.

Tretyn A., Kendrick R.E.: Acetylcholine in plants: presence, metabolism and mechanism of action. – Bot. Rev. **57**: 33-73, 1991.

Vyas D., Kumar S., Ahuja P.S.: Tea (*Camellia sinensis*) clones with shorter periods of winter dormancy exhibit lower accumulation of reactive oxygen species. – Tree Physiol. **27**:

1253-1259, 2007.
Wang H.B., Wang X.C., Lou C.H.: Relationship between acetylcholine and stomatal movement in *Vicia faba*. – Acta Bot. Sin. **41**: 171-175, 1999.
Wang H.B., Wang X.C., Zhang S.Q. *et al*.: Nicotinic acetylcholine receptor is involved in acetylcholine regulating stomatal movement. – Sci. China Ser. C Life Sci. **41**: 650-656, 1998.
Wang H.B., Wang X.C., Zhang S.Q., Lou C.H.: Muscarinic acetylcholine receptor is involved in acetylcholine regulating stomatal movement. – Chinese Sci. Bull. **45**: 250-252, 2000.
Wang H.B., Zhang S.Q., Wang X.C., Lou C.H.: Involvement of Ca^{2+}/CaM in the signal transduction of acetylcholine regulating stomatal movement. – Chinese Sci. Bull. **48**: 351-354, 2003.
Wintermans J.F.G.M., De Mots A.: Spectrophotometric characteristics of chlorophylls *a* and *b* and their phenophytins in ethanol. – BBA-Biophysics **109**: 448-453, 1965.
Wu F.B., Dong J., Qian Q.Q., Zhang G.P.: Subcellular distribution and chemical form of Cd and Cd–Zn interaction in different barley genotypes. – Chemosphere **60**: 1437-1446, 2005.
Xu Z., Zhou G.: Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. – J. Exp. Bot. **59**: 3317-3325, 2008.
Yi T.H., Kao C.H.: Heat shock-mediated H_2O_2 accumulation and protection against Cd toxicity in rice seedlings. – Plant Soil **300**: 137-147, 2007.
Yun H.S., Kwon C., Kang B.G. *et al*.: A xyloglucan endotransglycosylase/hydrolase1, VrXTH1, is associated with cell elongation in mungbean hypocotyls. – Physiol. Plantarum **125**: 106-113, 2010.
Zhou J.T., Wan H.X., He J.L. *et al*.: Integration of cadmium accumulation, subcellular distribution, and physiological responses to understand cadmium tolerance in apple rootstocks. – Front. Plant Sci. **8**: 966, 2017.