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Supplementation of Acetylcholine Mediates Physiological and Biochemical Changes in Tobacco Lead to Alleviation of Damaging Effects of Drought Stress on Growth and Photosynthesis

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Abstract

Drought is a global problem limiting plant growth and productivity by hampering the physiological and biochemical processes. Acetylcholine (ACh), a potential neurotransmitter found in lower and higher plants, has the potential to promote growth. However, little is known about ACh-mediated physiological and biochemical changes that promote plants' growth under drought stress conditions. Current experiments were undertaken to assess the effect of ACh (0.01 and 0.1 mM ACh) supplementation on the growth performance of tobacco under drought stress (10%, PEG 6000). The current findings exhibit that drought stress substantially reduced the physiological and biochemical parameters. However, the ACh application enhanced the growth and biomass of tobacco plants. Moreover, ACh application significantly enhanced the activity of PSII and chlorophyll fluorescence under normal and drought stress treatment, respectively. Furthermore, PEG treatment increases reactive oxygen species and oxidative damage; however, ACh application reduced H_2O_2, O_2^- and lipid peroxidation. In addition, exogenous application of ACh improved plant water status by improving the accumulation of proline and regulating the stomatal opening and closing. Besides, ACh-induced amelioration of oxidative stress was related to the up-regulation of antioxidant enzyme activities like SOD, POD, CAT, and APX. Hence, it can be concluded that ACh treatment improved photosynthesis in tobacco by regulating the stomatal and non-stomatal factors and up-regulation of the antioxidant system.

Keywords *Nicotiana benthamiana* · Quantum yield of PSII · Rubisco activity · ATP content · Drought stress · Antioxidant enzymes · Photosynthesis

Introduction

Water deficit stress is a serious environmental issue which restricts agricultural production, and global climate change plays an important role in persuading this production to constrain globally (Mathur et al. 2019). Water deficit inhibits the performance of vital biochemical mechanisms such as the photosynthetic process, nitrogen absorption, and secondary metabolite accumulation by influencing the function of essential enzymes (Chastain et al. 2014). Water scarcity reduces the water possibility in cells, resulting in plant water loss and wilting (Koffler et al. 2014). Water deficiency for an extended period of time destroys the membrane structure, removing the membrane's permeability and causing other serious damage. The most apparent symptoms of drought stress in soybean are reduction in germination percentage, cell elongation and expansion, hypocotyl length, shoot, and root fresh weight, increased root length, leaf flipping, leaf

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clamping, and initiation of early flowering (Sadeghipour and Abbasi, 2013). Drought stress also disturbs carbon assimilation and plant phenology (Du et al. 2020). Tobacco plants contain a variety of bioactive components that are used in a variety of pharmaceuticals (Ruiz-Rodriguez et al. 2008). Previous research showed that water stress has a detrimental effect on synthesizing secondary metabolism and bioactive compounds in plants (Mohasseli and Sadeghi, 2019). Under these conditions, the addition of fertilizers may not guarantee an increase in such compounds and crop growth (Sbrana et al. 2014). Therefore, plants require specific additives to protect them from osmotic stress and increase crop growth. Improving yield in unfavourable conditions in the current climate changes is a great challenge for growers. Several techniques have been used to reduce the constraints of the water deficit. Acetylcholine (ACh), a crucial cholinergic neurotransmitter, is considered very useful for promoting various eco-physiological processes that lead plants to overcome drought stress.

ACh, a crucial cholinergic neurotransmitter in animals, has been reported in different plant species, e.g., *Spinacea oleracea*, *Avena sativa*, *Phaseolus aureus,* etc. (Horiuchi et al. 2003). There is strong evidence that ACh plays important role in regulating various physiological and biochemical processes that mediate growth and development in plants under normal or stress conditions (Evans 1972; Roshchina and Mukhin 1985; Tretyn and Kendrick 1991; Braga et al. 2017; Qin et al. 2019). For instance, Evans (1972) demonstrated that ACh promoted the cell elongation ability by changing the cell wall properties of *Avena sativa* L. Furthermore, ACh along with auxin enhanced the expression of expansins substantially, thereby changing the cell wall properties (Di Sansebastiano et al. 2014). Likewise, Roshchina and Mukhin (1985) found that ACh in chloroplasts regulated membrane ion permeability and electron transport along with photophosphorylation, in *Pisum sativum* L. cv. Prevoskhodnii. In another study with etiolated wheat seedling mesophyll protoplasts, Tretyn et al. (1990) demonstrated that ACh caused a rapid swelling of protoplasts by regulating membrane permeability and the influx of Ca^{2+}, K^{+}, and Na^{+}. Similarly, drought stress-associated changes in leaf wilting and leaf recovery were reported to be associated with changes in ACh level in the leaves of *Macroptilium atropurpureum* (Siratro) (Momonoki and Momonoki 1993). Moreover, it was found that exogenous application of ACh caused the recovery from wilting of drought-stressed detached leaves (Momonoki and Momonoki 1993). In addition, several reports have demonstrated that ACh, receptor of ACh and its metabolic enzymes are distributed in various plant tissues and organs, signified its role in key physio-biochemical processes. Several research findings have shown that ACh has various physiological roles such as water and ion homeostasis, stomatal movement, communication between root and shoot. However, fewer studies are present on the role of ACh in improving plant growth under abiotic stresses (Yamamoto et al. 2011; Braga et al. 2017; Qin et al. 2019). For example, transgenic rice plants over-expressing acetylcholinesterase (AChE) from maize had greater ACh content and heat stress tolerance than those in non-transgenic rice plants (Yamamoto et al. 2011). Similarly, Braga et al. (2017) found that exogenous application of 1 mM ACh improved soybean seed germination and seedling growth under PEG-induced osmotic stress. In addition, a recent study by Qin et al. (2019) reported that the exogenous application of 10 µM ACh improved the growth of tobacco plants by improving photosynthetic activity and reducing oxidative damage under salinity stress. However, this information is insufficient to understand the role of ACh in alleviating the adverse effects of abiotic stresses, including drought stress, in plants. Therefore, it needs to be further explored.

The yield and quality of flue-cured tobacco are vulnerable to drought stress (FAO 2003; Guang et al. 2018). Therefore, it is necessary to devise strategies to improve drought tolerance in tobacco plants. Exogenous growth regulators, osmotic regulators, signal molecules, or antioxidants used through foliar spray or application through rooting medium were reported to improve stress tolerance of plants (Ashraf and Foolad 2007; Braga et al. 2017; Noreen et al. 2017; Elkeilsh et al. 2019). The aim of the study is to understand if ACh is involved in drought tolerance of tobacco. We determined whether ACh treatment could alleviate drought stress by regulating the main physiological metabolism of tobacco. For this purpose, regulation of gas-exchange characteristics, photosynthesis, contents of some key organic osmolyte and antioxidant defense system in drought-stressed tobacco plants fed with ACh was appraised.

Materials and Methods

Plant Material and Treatments

Seeds of the tobacco (*Nicotiana benthamiana* Domin) were surface sterilized in 5% sodium hypochlorite solution for 20 min and then washed three times with sterile water. Afterward, the seeds were germinated in sterilized moist vermiculite in a growth chamber maintained at 16 h light/8 h dark, 25 °C/18 °C day/night temperature, 200 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR). After three weeks seedlings were transferred to plastic pots (7 × 7 cm) filled with sand. Tobacco plants were irrigated with half-strength Hoagland's nutrient solution. At a four-leaf stage, plants were transplanted to hydroponics and were supplied with half-strength Hoagland's nutrient solution or 10% polyethylene glycol 6000 (PEG 6000) supplemented with or without ACh (0.01 and 0.1 mM). PEG and ACh were prepared by dissolving

in Hoagland nutrient solution. Therefore experimental setup constituted four treatments as: (a) Hoagland nutrient solution (CK), (b) 10% PEG, (c) 10% PEG + 0.01 mM ACh, and (d) 10% PEG + 0.1 mM ACh. Each treatment has three replicates. After 24, 36, and 48 h of drought stress and ACh treatment plants were analyzed for physiological and biochemical attributes described below.

Determination of Photosynthesis and Chlorophyll Fluorescence Parameters

Plant photosynthetic capacity was measured using an *LI*-6400-XT photosynthesis system (LI-COR, USA). Gas exchange characteristics were measured on fully matured but youngest leaves of tobacco plants by setting photosynthetically active radiation (PAR) at 1000 µmol m^{-2} s^{-1}. The gas flow rate was set at 500 µmol s^{-1} while the temperature of the leaf chamber was set at 30 ± 2 °C. Following gas exchange parameters were recorded: net photosynthetic rate (P_N), transpiration rate (T_r), intercellular CO_2 concentration (C_i) and stomatal conductance (g_s). All measurements were recorded between 10:00 and 11:00 a.m.

Chlorophyll fluorescence of the tobacco plant leaves was measured using a fluor-imaging system (FC 800-C/1010, PSI, Germany). The leaves were dark-adapted for 20 min prior before recording the measurements. The images of chlorophyll fluorescence of the leaves were processed using the FluorCam software and calculated non-photochemical quenching (NPQ), photochemical quenching coefficient (qP), the maximum quantum yield of PSII (F_v/F_m), and PSII potential activity (F_v/F_0).

The second youngest leaf from the top was cut into 0.5 cm × 0.5 cm squares. The stomatal aperture of the leaf epidermis of tobacco plants from each treatment was determined by FESEM (Thermo Fisher Scientific).

Assay of Leaf and Root Activity

Cell viability in the leaves was measured by staining leaf segments with the Evans blue method (Baker and Mock 1994; Nv et al. 2017). Image-j software was used to calculate the area of the whole leaf (M) and the area of the unstained area (m). Relative cell activity (V) = m/M × 100%. Root activity was determined by staining with 2,3,5-triphenyl tetrazolium chloride (TTC) following Towill and Mazur (1975). Mitochondrial dehydrogenase reduced the colourless TTC to red tri-phenyl-formazan, indicating the extent of cellular respiration activity.

Determination of Relative Water Content, Proline, Soluble Proteins and Soluble Sugars

Relative water content (RWC) was measured by following the protocol of Barrs and Weatherley (1962). Fully developed but young leaves from the top were harvested after 24, 36 and 72 h, and their fresh weights were measured. The leaves were immersed in distilled water for 12 h, and their weight as turgid leaf weight was measured. The leaves were then oven-dried at 70 °C, and their dry weights were recorded. Leaf RWC was calculated using the formulae given elsewhere.

$$\mathrm{RWC}(\%) = \frac{(\mathrm{FW} - \mathrm{DW})}{(\mathrm{TW} - \mathrm{DW})} \times 100.$$

For the determination of proline content, 0.2 g fresh leaf was ground in 5 mL of 3% sulfosalicylic acid and then centrifuged. In the supernatant, proline was measured following the method of Gao (2006). Soluble proteins were extracted by grinding 0.1 g fresh leaf material in 5 mL of ice-cooled 50 mM phosphate buffer (pH 7.6) and then centrifuged at 6000 × g for 15 min. The supernatant (1 mL) was reacted with 4 mL of Bradford Reagent and the absorbance was determined at 595 nm. The soluble proteins in the extract solution were measured using specific formulae (Bradford 1976). Soluble sugars in the leaves were measured as described by Gao (2006). Soluble sugars were extracted from 0.1 g dry leaf with 5 mL of 80% ethanol and then centrifuged at 3000×*g*. The dry leaf material was washed with ethanol thrice. The collected supernatant was diluted to 100 mL and then 5 mL of leaf extract in ethanol was reacted with the anthrone reagent. The green colour complex so formed was read on a spectrophotometer.

Measurement of Rubisco Activity and ATP Content

The ATP content and rubisco activity were measured using commercial kits (Nanjing Jiancheng, China). The leaves were ground into a powder with liquid nitrogen, homogenized in 1.8 mL of double-distilled water. The leaf homogenized material was heated in a boiling water bath for 10 min, and centrifuged at 3000×*g* for 10 min. For the measurement of rubisco activity, 0.2 g leaf powder was homogenized in 2 mL of phosphate buffer (50 mM, pH 7.8) and the sample was centrifuged at 5000×*g* for 10 min. The supernatants from both groups were used for determining the concentration of ATP and the activity of rubisco.

Determination of Hydrogen Peroxide (H_2O_2), Superoxide Anion (O_2^-) and Malonaldehyde (MDA) Content

Drought stress induced oxidative stress was measured as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) content by the tissue staining method, while the oxidative damage to membranes was measured as malondialdehyde (MDA) contents spectrophotometrically. The measurements

of (H_2O_2) and (O_2^-) were carried out using 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively (Thordal-Christensen et al. 1997; Kumar et al. 2014). Following Gao (2006), membrane damage was evaluated by measuring the absorbance of thiobarbituric acid (TBA) colouration at 532 and 600 nm. The difference in absorbance at the two wavelengths was multiplied by the extinction coefficient (155 mM^{-1} cm^{-1}).

Assay of Antioxidant Capacity

To extract antioxidant enzymes in the leaves, 0.2 g fresh leaf was ground into a powder with liquid nitrogen and then homogenized in 5 mL phosphate buffer (50 mM, pH 7.8). The leaf homogenate was centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant was used for the assay of antioxidant enzymes. The activities of SOD, CAT, and POD were assayed by the nitroblue tetrazolium (NBT) method, ultraviolet absorption method, and guaiacol method (Gao 2006), respectively. The activity of APX was determined following Nakano and Asada (1981).

Statistical Analysis

The physiological and biochemical attributes date were computed and expressed as mean ± standard deviation of three replicates. The data of all attributes were subjected to one-way analysis of variance (ANOVA) using the SPSS-20 software (SPSS, Chicago). Mean values of each attribute were compared using LSD at $P < 0.05$.

Results

Effects of Exogenous ACh on Photosynthetic Efficiency of Tobacco Plants Under PEG-Induced Drought Stress

Leaf P_N decreased in the drought stressed tobacco plants (Fig. 1a). Reduction in P_N was maximal after 48 h of drought stress. Exogenous application of 0.01 mM ACh significantly enhanced P_N of tobacco plants, and this effect was reflected in each period of drought stress (24, 36 and 48 h). Similarly, drought stress caused a significant reduction in g_s and T_r at all time intervals (Fig. 1). Both g_s and T_r did not further decrease at 36 and 48 h of drought stress. Exogenous application of 0.01 mM ACh enhanced both g_s and T_r. After 24 h of drought stress, C_i remained unchanged. However, after 36 and 48 h of drought stress, C_i decreased substantially. Application of 0.01 mM ACh increased C_i in plants experiencing 24, 36 or 48 h of drought stress, whereas 0.1 mM ACh caused an increase in C_i after 36 h of drought stress (Fig. 1d). Values of water use efficiency (WUE, P_N/T_r) and intrinsic water use efficiency (P_N/g_s) were substantially increased after 36 h of PEG-induced drought stress and 0.01 mM ACh application.

Drought stress reduced the g_s as the guard cells of tobacco leaves lost water and were shrunk (Fig. 2). Drought stress decreased the stomatal pore circumference, area, length, and width of the tobacco plants. The supply of ACh improved the size and shape of the guard cells and promoted stomatal opening under drought stress. The stomatal opening of plants treated with 0.01 mM ACh (69%) was higher than that of plants treated with 0.1 mM ACh (61%) after 36 h of drought stress.

qP, F_v/F_m and F_v/F_0 of PSII photochemistry decreased due to drought stress, whereas NPQ increased in tobacco leaves under drought stress conditions (Fig. 3). Reduction in qP, F_v/F_m and F_v/F_0 in tobacco plants was higher after 48 h of drought stress. Likewise, drought stress caused a maximal increase in NPQ in tobacco plants after 48 h of stress. Application of 0.01 mM ACh significantly improved qP, F_v/F_m and F_v/F_0 of tobacco plants under drought stress, while NPQ increased only at 24 h. Moreover, 0.1 mM ACh caused a further decrease in F_v/F_m and F_v/F_0 values, whereas NPQ and qP of the drought stressed plants supplied with 0.1 mM ACh were like to those in the 10% PEG stressed plants.

Effects of Exogenous ACh on Leaf and Root Activity of Tobacco Plants Under PEG-Induced Drought Stress

Histochemical staining was used to determine the activity of tobacco leaf cells. The results showed that the leaves of non-stressed tobacco plants had ~ 99% cell viability (Table 1) and only petioles of the leaves had blue coloration, which might have been due to mechanical injury (Fig. S1). Drought stress decreased the cell viability of the leaves of tobacco plants, and it decreased consistently with an increase in time from 24 to 48 h of drought stress (the tissue staining area extended from the vein to 84.30% of the total leaf area). Application of ACh reduced cell death and improved cell viability of the drought stressed plants. The effect of ACh on cell death was more significant at 24 h and 36 h under drought stress. Moreover, exogenous application of 0.01 mM ACh could improve the cell activity of tobacco leaves more effectively. The leaf cell activity of tobacco plants treated with 0.01 mM ACh for 24 h, 36 h and 48 h were 1.1, 1.7 and 4.9 times of that treated with 10% PEG, respectively.

Tobacco root cell activity was also significantly reduced due to drought stress (Table 1). Drought stress increased tawny coloration of tobacco roots, and tawny coloration increased with the extension of drought time. In general, darker colors indicate greater root damage and lower root cell activity. Under drought stress, the root color of tobacco became lighter with the application of 0.01 mM ACh, which

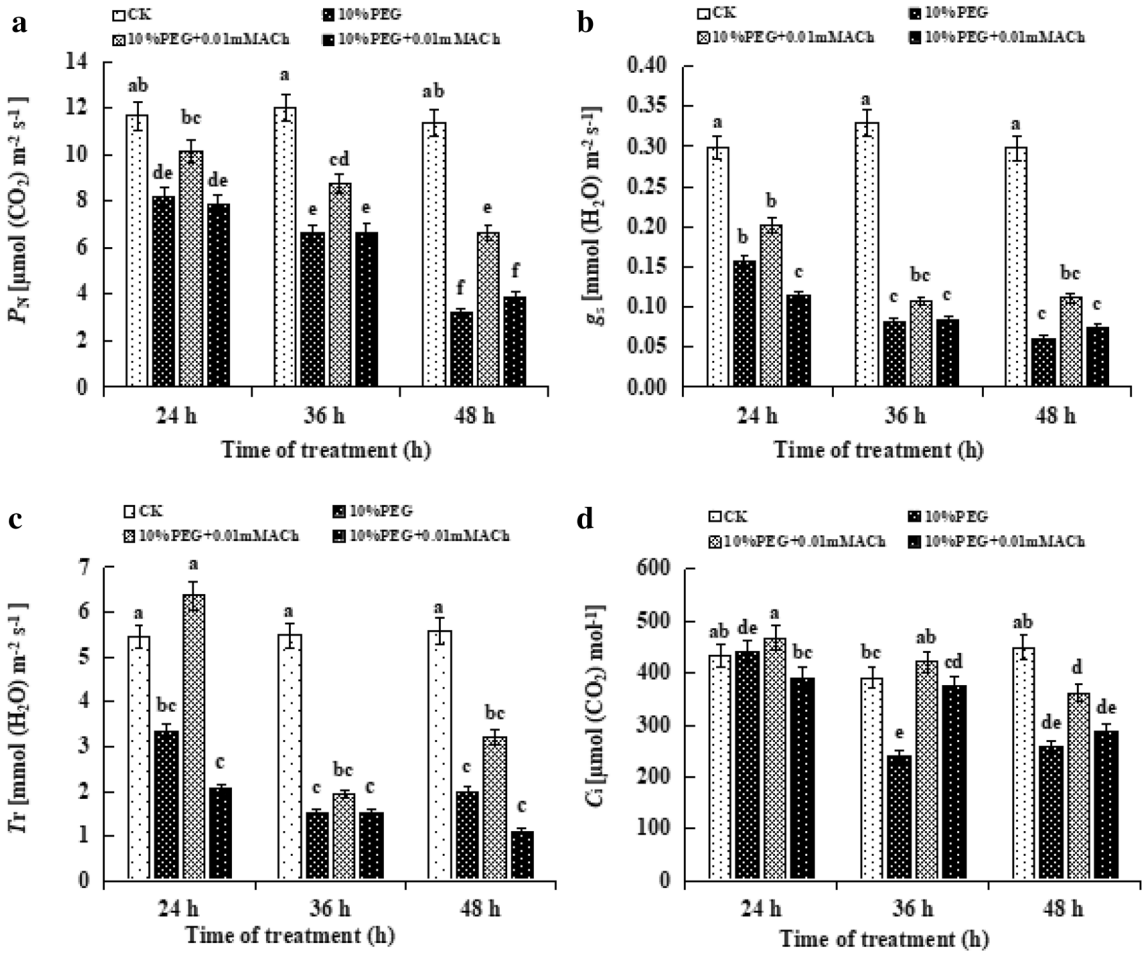

Fig. 1 Effects of ACh application on gas exchange parameters of tobacco leaves under drought stress. **a** P_N; **b** g_s; **c** T_r; **d** C_i. Data are means of three replicates (± SD). Means denoted by different letters show significant differences ($P < 0.05$)

indicated that the root activity increased (Fig. S2). The root activity of tobacco plants treated with 0.01 mM ACh for 24 h, 36 h and 48 h were 1.2, 1.5 and 1.5 times of that treated with 10% PEG, respectively. However, 0.1 mM ACh treatment was less effective in improving the root activity of drought stressed tobacco plants. These results indicated that the application of 0.01 mM ACh could effectively relieve the damage of tobacco leaf cells and roots under drought stress.

Effects of Exogenous ACh on Osmoregulation of Tobacco Plants Under PEG-Induced Drought Stress

Leaf RWC of the tobacco plants was significantly reduced by 10% PEG-induced drought stress (Fig. 4a). The reducing effect of the PEG-induced drought stress on the leaf RWC was more prominent after 48 h of drought stress. Exogenous application of 0.01 mM ACh improved the RWC of the tobacco plants after 24, 36 and 48 h of drought stress. Moreover, the treatment of 0.1 mM ACh was not effective in improving the leaf RWC.

Leaf soluble proteins, soluble sugars and proline content of the tobacco plants were significantly increased due to PEG-induced drought stress. Moreover, all these biochemical attributes increased substantially with an increase in the duration of drought stress. Exogenous application of ACh caused a further increase in soluble proteins in the drought stressed plants, particularly in the plants treated with 0.01 mM ACh. Soluble proteins were the highest in 0.01 mM ACh treated tobacco plants exposed to PEG-induced drought stress for 36 h. In contrast, soluble sugars and proline contents were maximal in the tobacco plants treated with 0.01 mM ACh and exposed to 48 h of drought stress. These results indicated that the application of low concentration (0.01 mM) of ACh under drought stress promoted the accumulation of osmotic substances and increased the RWC of tobacco leaves.

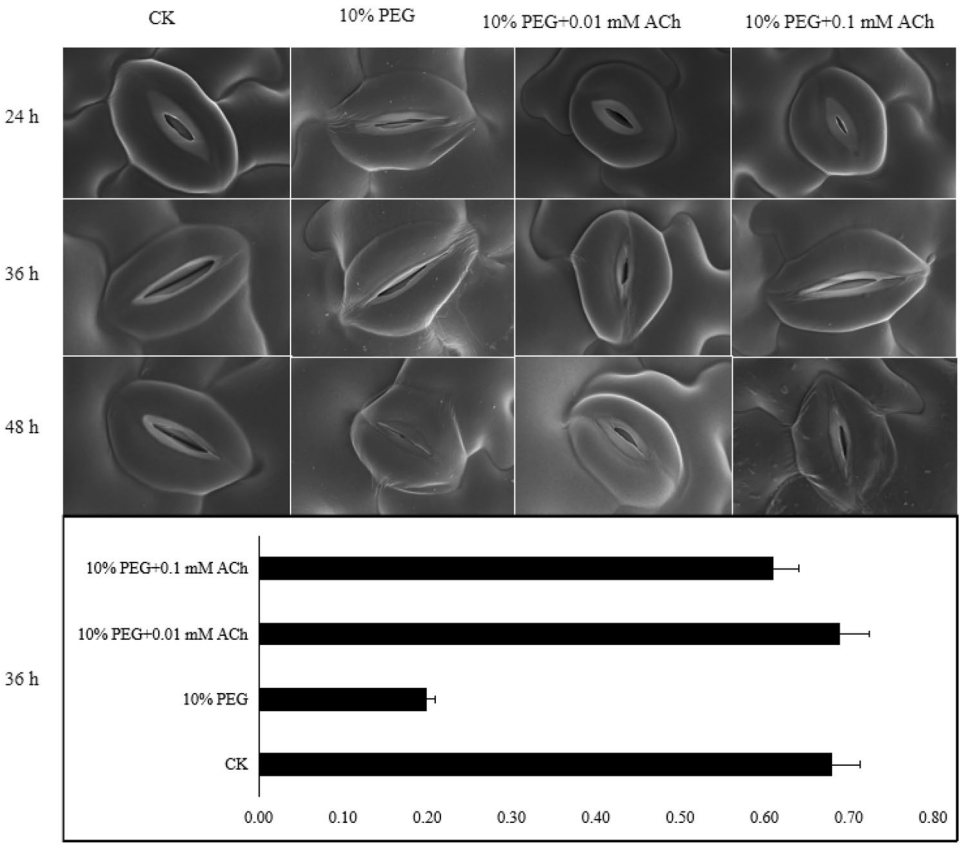

Fig. 2 Effect of ACh application on stomata of tobacco leaves under drought stress Stomatal aperture in the third leaves of tobacco pre-treated with ACh or untreated in watered conditions (control) and under drought stress. In the picture with stomatal magnification of × 8000, the column showed the stomatal opening rate under drought stress for 36 h. The ratio of the number of open stomata in the same unit area of each treatment to the total number of stomata was obtained by statistics

Effects of Exogenous ACh on Carbon Assimilation Potential of Tobacco Plants Under PEG-Induced Drought Stress

As a bifunctional enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) is one of the determinants of P_N. ATP provides energy for carbon assimilation and plays an important role in regulating the activity of rubisco. Therefore, the content of ATP can reflect the carbon assimilation ability of tobacco. Rubisco activity decreased continuously with the prolongation of drought stress, especially after 36 h (Fig. 5a). The results showed that the damage caused by drought stress began to aggravate after 36 h. Application of 0.01 mM ACh can not only alleviate the decrease of rubisco activity caused by drought, but also increase the rubisco activity of tobacco plants at the normal level. At the initial stage of drought stress (24 h), ATP content in tobacco was induced by self-protection mechanism to adapt to stress. However, ATP content decreased significantly with prolonged drought stress and aggravation of injury. Application of 0.01 mM ACh could significantly increase ATP content in tobacco leaves under stress. In contrast, application of 0.1 mM ACh further reduced the ATP content of tobacco plants under drought stress.

Effects of Exogenous ACh on Antioxidant Capacity of Tobacco Plants Under PEG-Induced Drought Stress

Drought stress is believed to generate reactive oxygen species (ROS) and increase the degree of lipid peroxidation. In this study, H_2O_2 and O_2^- in tobacco leaves increased significantly under drought stress, and H_2O_2 increased continuously with the extension of stress time. O_2^- remained at a stable level and did not increase continuously. However, 0.01 mM ACh significantly reduced the H_2O_2 and O_2^- contents in tobacco plants under drought stress (Fig. 6a, b). A maximal reduction in H_2O_2 and O_2^- contents (26.58% and 22.90%) by 0.01 mM ACh was observed in the tobacco plants after 24 h of drought stress. The membrane lipid peroxidation of tobacco plants increased under drought stress (evaluated by MDA content) (Fig. 6c). Exogenous application of ACh decreased the content of MDA in tobacco leaves under drought stress, especially when the concentration of ACh was 0.01 mM. These results clearly indicate that 0.01 mM ACh can reduce ROS accumulation in tobacco plants under drought stress.

The activities of four antioxidant enzymes were compared between two different concentrations of ACh and untreated tobacco plants under PEG-induced drought

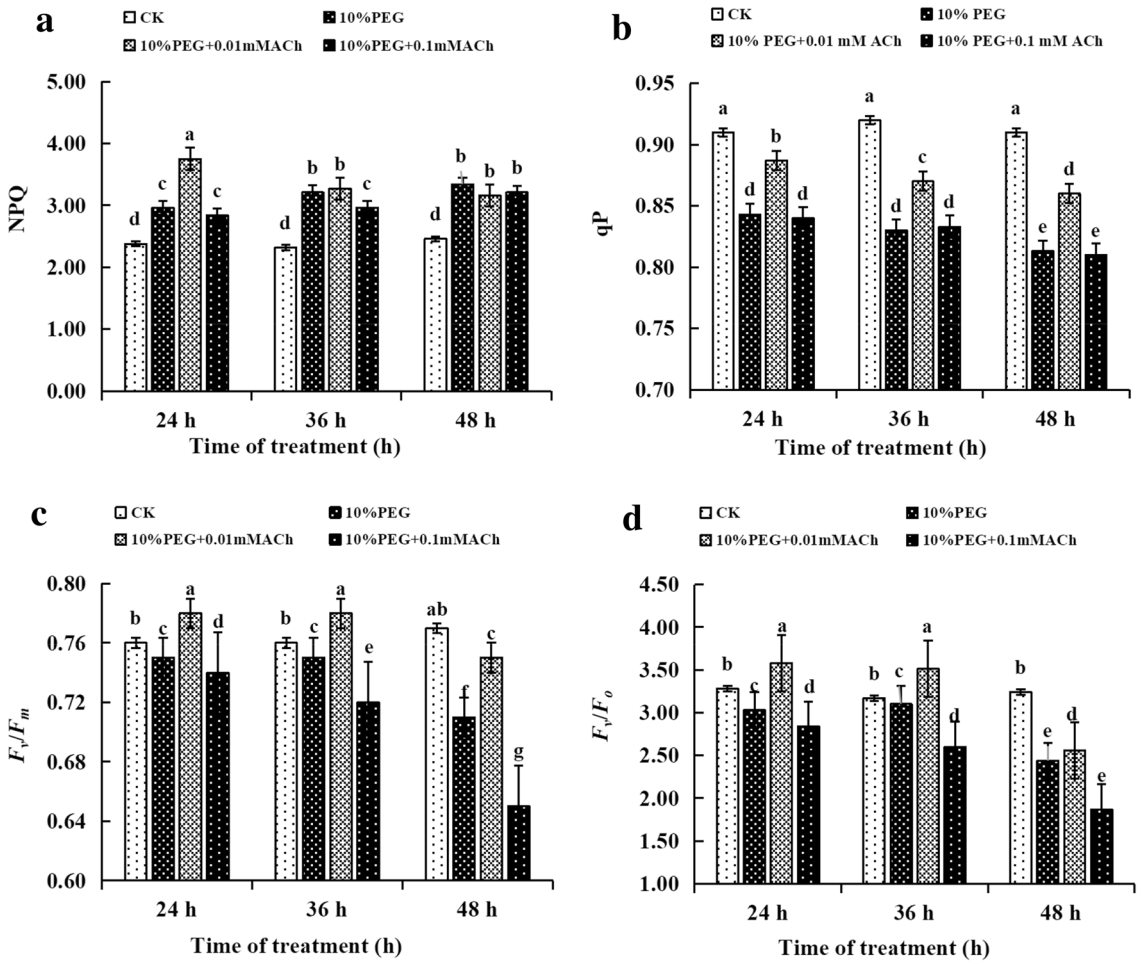

Fig. 3 Effects of ACh application on photosynthetic characteristics of tobacco leaves under drought stress. **a** NPQ; **b** qP; **c** F_v/F_m; **d** F_v/F_0. These parameters of leaves were measured under dark adaptation (20 min). Data are means of three replicates (±SD). Means denoted by different letters show significant differences ($P < 0.05$)

Table 1 Cell viability in leaves and roots of tobacco under drought stress

	CK	10% PEG	10% PEG + 0.01 mM ACh	10% PEG + 0.1 mM ACh
Leaves				
24 h	99.347 ± 0.28a	87.149 ± 0.77d	95.832 ± 1.50b	93.590 ± 1.25bc
36 h	99.423 ± 0.15a	54.900 ± 1.82f	93.176 ± 1.75c	86.857 ± 1.90d
48 h	99.476 ± 0.16a	15.700 ± 1.29g	76.960 ± 1.80e	54.865 ± 2.38f
Roots				
24 h	0.90 ± 0.03a	0.58 ± 0.01c	0.69 ± 0.14b	0.62 ± 0.02bc
36 h	0.94 ± 0.01a	0.41 ± 0.02f	0.61 ± 0.03c	0.54 ± 0.06cde
48 h	0.93 ± 0.03a	0.40 ± 0.01f	0.58 ± 0.01cd	0.50 ± 0.05def

stress. The activities of SOD, CAT and APX increased significantly due to PEG-induced drought stress (Fig. 7). In contrast, POD activity increased after 36 h of drought stress. Both concentrations of ACh increased the activities of SOD, CAT and APX in tobacco plants under drought stress. However, the enhancement effect of low concentration ACh (0.01 mM) was more significant. POD activity

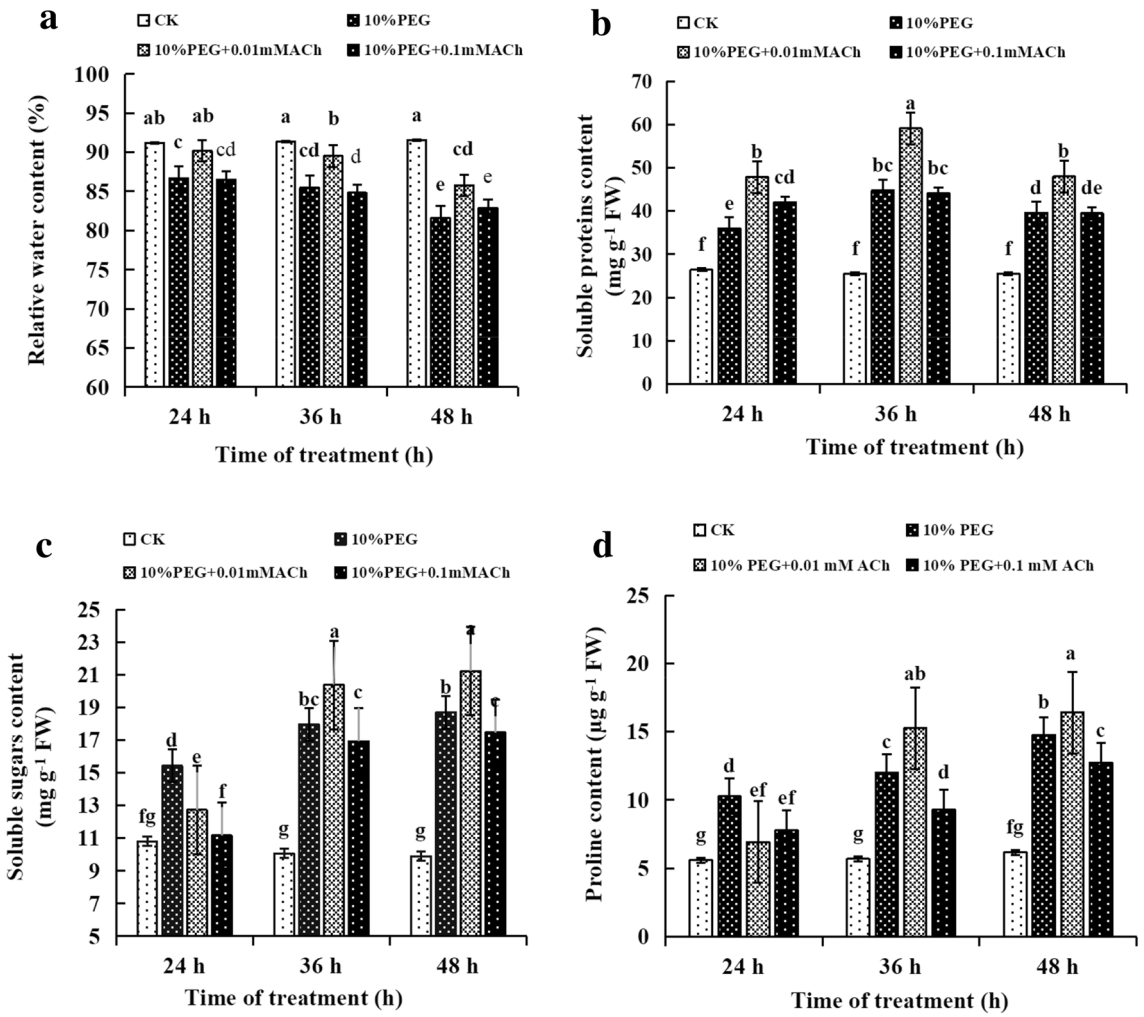

Fig. 4 Effects of ACh application on osmoregulatory substance content of tobacco leaves under drought stress. **a** RWC; **b** soluble proteins; **c** soluble sugar; **d** proline. The contents of these parameters were measured in the third leaves of tobacco treated with different concentrations of ACh or without ACh in watered conditions (control) and under drought stress. Data are the means of three replicates (±SD). Means denoted by different letters show significant differences ($P < 0.05$)

increased and reached the highest after adding 0.01 mM ACh 36 h (Fig. 7b).

Discussion

Some studies have shown that exogenous application of ACh can maintain plant homeostasis under stress (Qin et al. 2019, 2020, 2021; Su et al. 2020a, b). This study showed that exogenous 0.01 mM ACh could effectively alleviate the damage of drought to tobacco plants. On the contrary, a higher dose of ACh (0.1 mM) proved to be the least effective or partially inhibitory. These results can be explained by the view of Tretyn and Kendrick (1991) and Di Sansebastiano et al.(2014) that although ACh is present in different plant species, the optimal physiological concentration of ACh varies from species to species, ranging from nM to mM, e.g., 23 pmol g^{-1} in Arabidopsis (Horiuchi et al. 2003), and 2.94 μmol g^{-1} in bamboo shoots (Kawashima et al. 2007). Similarly, an optimum dose for exogenous application of ACh was also different in different studies such as 0.01 mM ACh for oat seedlings (Evans 1972), 0.01–1 mM for pea (Roshchina and Mukhin 1985), 10 μM for *Vicia faba* leaves (Wang et al. 2000), and 1 nM for germinating radish (Sugiyama and Tezuka 2011). Moreover, the application of ACh in an mM range or higher than this concentration proved to be inhibitory on seedling growth (Lawson et al. 1978). Variation in the endogenous level of ACh in different plant species or differences in optimum dose for exogenous application of ACh might have been due to differences in ACh requirement in each species to maintain the growth rate. For example, upper rapidly growing shoots of bamboo (*Phyllostachys*

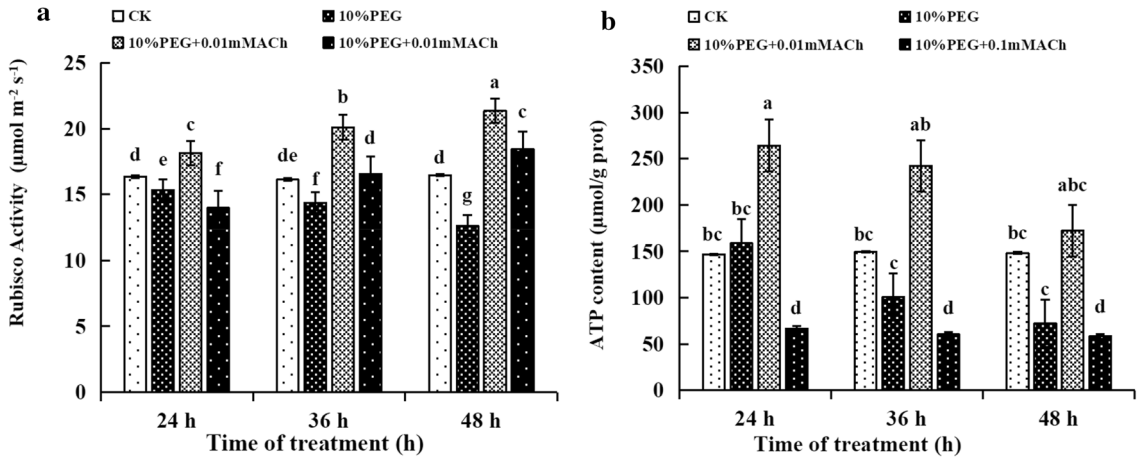

Fig. 5 Effects of ACh application on Rubisco activity and ATP content of tobacco leaves under drought stress. **a** Rubisco activity; **b** ATP content. These parameters were measured in the third leaves of tobacco. Data are means of three replicates (±SD). Means denoted by different letters show significant differences ($P < 0.05$)

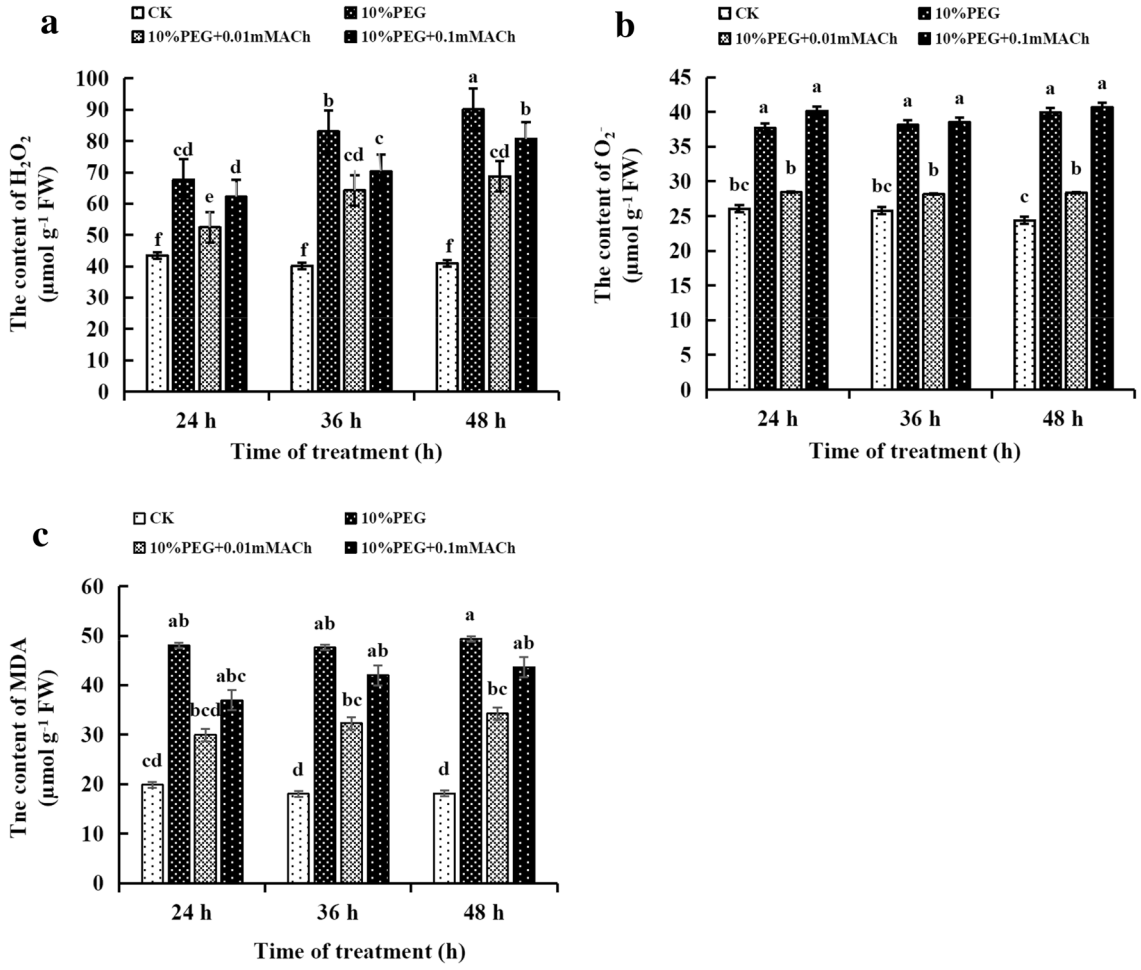

Fig. 6 Effects of ACh application on oxidative stress (H_2O_2 and O_2^-) and oxidative damage (MDA content) of tobacco leaves under drought stress. **a** H_2O_2 content; **b** O_2^- content; **c** MDA content. Data are the means of three replicates (±SD). Means denoted by different letters show significant differences ($P < 0.05$)

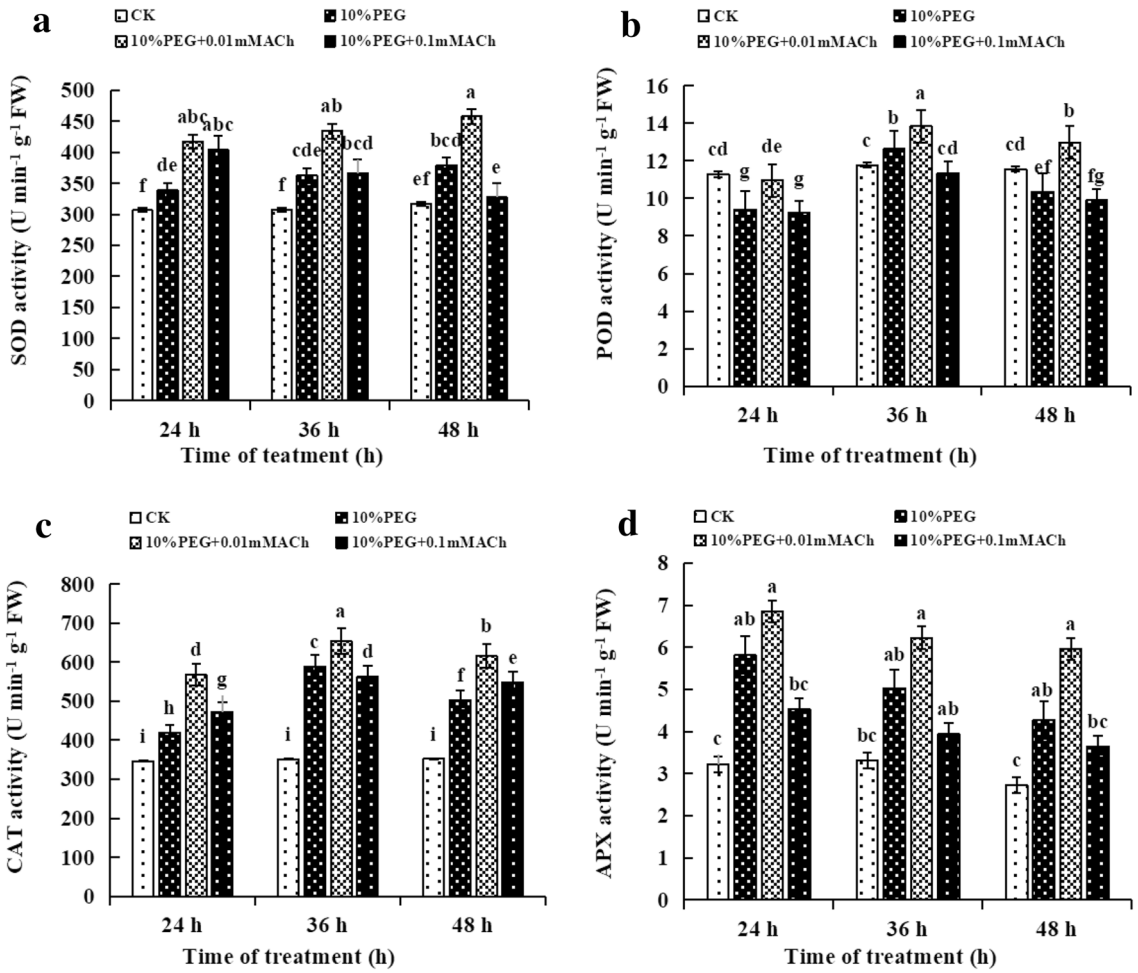

Fig. 7 Effects of ACh application on antioxidant capacity of tobacco leaves under drought stress. **a** SOD activity; **b** POD activity; **c** CAT activity; **d** APX activity. These parameters were measured in the third leaves of tobacco treated with different concentrations of ACh or without ACh in watered conditions (control) and under drought stress. Data are the means of three replicates (± SD). Means denoted by different letters show significant differences ($P < 0.05$)

bambusoides) had the highest endogenous ACh concentration (2.94 μmol g^{-1}) as compared to that in the slowly growing lower part of the bamboo shoot (Horiuchi et al. 2003).

Effect of Exogenous ACh on Water Status and Osmoregulation of Tobacco Under Drought Stress

Maintenance of plant water status under drought stress is an important physiological attribute that governs cell division and cell elongation in meristematic regions of shoots and roots, thereby regulating plant growth and development (Taiz et al. 2015). In this study, drought stress inhibited the water state of tobacco leaves, and exogenous application of ACh was helpful to maintain the tissue water state of tobacco plants under drought stress. These results are consistent with the previously observed results that exogenous ACh has a restoring effect on the wilting of detached leaves of *Macroptilium atropurpureum* under drought stress (Momonoki and Momonoki 1993). Recently, it has been widely accepted that the accumulation of organic osmotica (such as soluble sugars, soluble proteins and proline) plays a more important role than inorganic osmotica (such as K^+ and Cl^-) in maintaining tissue water status under drought conditions (Blum and Tuberosa 2018). Although osmotic regulation was not evaluated in this study, the decrease of plant water status caused by drought stress (measured as RWC) induced the accumulation of soluble protein, soluble sugar and proline, and exogenous application of ACh further promoted their accumulation (Fig. 4). These results are similar to those of several analogous studies in which exogenous application of plant growth regulators enhanced the accumulation of proline, improved plant water status and enhanced stress tolerance, as observed in soybean (Braga et al. 2017), tomato (Di Sansebastiano et al. 2014), canola (Athar and Ashraf 2009), and wheat (Noreen et al. 2017). However, the

contribution of soluble proteins to drought stress adaptation seems to be less as compared to that of proline because the accumulation of soluble proteins increased after 24 h of drought stress, while in contrast, it decreased after 36 h of drought stress (Fig. 4b). Moreover, application of 0.01 mM ACh was more effective in improving proline accumulation and maintaining water status, both being pre-requisites for enhanced drought stress tolerance.

Effect of Exogenous ACh on Membrane Lipid Peroxidation and Antioxidant Enzymes of Tobacco Under Drought Stress

In normal, the production and scavenging of ROS are in dynamic equilibrium. When the plants are subjected to drought stress, ROS is accumulated in plant cells, resulting in membrane-lipid peroxidation (Foyer 2018). Plants activate antioxidant enzymes to scavenge these ROS species (Taiz et al. 2015). In this study, drought stress increased the accumulation of hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-), and increased MDA content, which indicated that drought stress caused oxidative stress-induced membrane damage (Fig. 6). Four antioxidant enzymes: SOD, POD, APX and CAT, were activated by tobacco plants to lower the level of ROS and reduce the damage caused by drought. After the application of 0.01 mM ACh, the activities of antioxidant enzymes were further increased, which led to the improvement of drought resistance of tobacco plants (Fig. 7).

Effect of Exogenous ACh on Gas-Exchange Characteristics and Photosynthesis of Tobacco Under Drought Stress

Photosynthesis, which are influenced by gas exchange characteristics, are widely used to assess the relative impact of environmental stresses (Singh et al. 2017). The results from the present study showed that a decrease in P_N in tobacco plants occurred at the early stage of drought stress due to stomatal closure and the non-availability of CO_2. But after 48 h of drought stress, a decrease in P_N in the tobacco plants occurred without a further decrease in g_s and C_i, indicating some metabolic limitations (Fig. 1). Values of water use efficiency (WUE, P_N/T_r) and intrinsic water use efficiency (P_N/g_s) were substantially increased after 36 h of PEG-induced drought stress with the application of 0.01 mM ACh. The major reason for an increase in values of these two attributes was the decline in T_r and g_s, which led us to suggest stomatal limitation was a major limiting factor for reduction in photosynthetic activity during early 36 h of PEG-induced drought stress. Application of 0.01 mM ACh could effectively improve the P_N by improving stomatal factors (g_s, C_i) under drought stress. From the results of microscopic analysis of leaf for stomatal density and stomatal area, it is obvious that drought stress did not affect the stomatal density, but stomata became elongated, and their width decreased. Application of ACh increased stomatal opening in the tobacco leaves under drought stress (Fig. 2). Moreover, stomatal aperture decreased with an increase in ACh concentration. These results can be explained in the light of earlier findings of Wang (2000), who reported that the application of ACh increased the stomatal aperture in the leaves of *Vicia faba*. They also found that ACh receptors (nicotinic and muscarinic ACh receptors) are involved in ACh-mediated stomatal movement by activating ion channels and initiating secondary messengers and G-proteins. Therefore, it can be inferred from the results that changes in photosynthetic rate due to the application of ACh were mainly associated with stomatal factors.

A balance between solar energy capture as electron transport from PSII to PSI and its consumption as ATP and NADPH in the Calvin cycle under normal or stress conditions is central to avoid ROS generation and photoinhibition. This can be achieved by appropriately distributing the captured energy in photochemical and non-photochemical reactions (Foyer 2018). In this study, with the increase of drought stress time, F_V/F_m and F_V/F_0 were inhibited (Fig. 3), and P_N decreased significantly (Fig. 1a). However, NPQ increased with the increase in drought degrees. This indicates that with the increase of drought stress, the reaction center is destroyed and even reversibly inactivated, while tobacco consumes excess light energy through heat dissipation, relieving the inhibition of excessive photoelectron production during the primary reaction of photosynthesis, thus protecting the photosynthetic apparatus, which reflects the adaptability of tobacco to drought stress. Application of 0.01 mM ACh significantly increased qP and NPQ and decreased F_0 under drought stress. These results indicated that ACh could enhance the ability of tobacco leaves to capture light energy by antenna pigments under drought stress and protect photosynthetic reaction centers from damage by increasing the proportion of light energy consumed by the heat dissipation in energy distribution. ATP is the only source of energy for the activation of rubisco. The high activity of Rubisco and high concentration of ATP are important conditions for carbon assimilation. Application of 0.01 mM ACh may improve the carbon assimilation ability of plants through this pathway and then improve the P_N of tobacco under drought stress (Fig. 5).

The damage of drought to plants can be measured by evaluating the degree of cell damage. In this study, we measured the degree of cell injury by measuring the cell activity of leaves and roots. In this study, cell activity in both leaves and roots decreased consistently with a gradual increase in PEG-induced drought stress, while application of ACh counteracted the harmful effects of drought stress and enhanced

the cell activity of leaf and root through osmoregulation, reducing the membrane lipid peroxidation and improved photosynthesis, particularly with 0.01 mM ACh.

In this study, cell activity in both leaves and roots decreased consistently with a gradual increase in PEG-induced drought stress, while application of ACh counteracted the harmful effects of drought stress and enhanced the cell activity of leaf and root through osmoregulation, reducing the membrane lipid peroxidation and improve photosynthesis, particularly with 0.01 mM ACh.

Conclusions

According to the findings of this study, the ACh application effectively stimulated tobacco plant growth and photosynthetic capacity under drought stress conditions. The ACh application significantly improved the plant water status and osmolyte, which positively impacted the regulation of stomatal and non-stomatal attributes and antioxidant systems. Furthermore, findings demonstrate that ACh application dramatically reduced MDA formation via modulating ROS production during drought stress. As a result, ACh application could be a viable option for recovering drought-affected land.

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Author Contributions MQ, XZ and GN performed the experiments. LZ designed and supervised the project. MQ, XZ, AY and PW analyzed the data. MQ and XZ prepared first draft. NA, NSM, SAR, MHS, RK and HA prepared final draft preparation. All authors discussed the results, revised the manuscript and approved submission of this work.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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