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Physiological and Biochemical Mechanisms of Improving Salt and Drought Tolerance in Okra Plants Based on Applied Attapulgite Clay

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Abstract: Attapulgite clay (AC), which is rich in good adsorption, catalysis, rheology and heat resistance, is an important mineral resource. However, the roles of AC in regulating stress tolerance of plants have not been investigated. In this study, culture pot experiment was used to analyze the effects of AC applied into the soil on growth and physiological metabolism of okra plants. The applied AC significantly enhanced salt and drought tolerance of okra plants. Component analyses showed that the significant increases of ABA, proline, soluble protein, soluble sugar and photosynthetic pigment content, as well as the significant decreases of hydrogen peroxide, superoxide anion radical and malondialdehyde content were observed in okra plants grown in the soil with applied 30 g/kg AC under salt and drought stresses. Enzymatic analyses indicated the activities of 9-cis-epoxycarotenoid dioxygenase, pyrroline-5-carboxylate synthase, superoxide dismutase and peroxidase were also significantly increased under salt and drought stresses. These results demonstrate that the applied AC can alleviate damage caused by salt and drought stresses, leading to the enhanced salt tolerance and drought tolerance of okra plants. The AC has the potential to be used to develop plant growth regulators to enhance the tolerance to abiotic stresses in plants.

Keywords: ABA, Attapulgite Clay, Okra, Proline, Salt and Drought Tolerance

1. Introduction

Okra (*Abelmoschus esculentus* L.), as a new type of health vegetable with the reputation of "plant ginseng", scientific name "coffee abelmosk", is one of the most important vegetables widely grown for its tender fruits and young leaves in worldwide, which contains many effective constituents, such as flavonoids, polysaccharide, pectin, trace elements, vitamins, proteins, etc [1]. With high nutritional value and low planting cost, okra has a broad economic market. Global warming, freshwater resources reduction, unreasonable irrigation and coastal seawater back-irrigation result in inducing land salinization and accelerating the drying rates of soil under the conditions of insignificant precipitation increase, which poses great challenges to okra cultivation. Therefore, it is of great importance to investigate the tolerance to abiotic stresses of okra plants.

Abiotic stresses, such as salt and drought, are most severe problems in agriculture worldwide and NaCl is the predominant salt in most saline environments, especially in arid and semiarid regions [2, 3]. Plant adaptation to high concentration of salts or absence of water environment of soil, is dependent on the activation of cascades of molecular networks involved in stress perception, signal transduction, the expression of specific stress related genes, physiological and metabolic activities [4-6]. Osmotic adjustment is an effective example in response to abiotic stresses, as the accumulated more levels of osmoprotectants or compatible solutes after stress treatments are common responses observed in plant systems [5, 6]. In particular, salt and drought stresses accelerate the production of reactive oxygen species (ROS) and cause lipid peroxidation of plants [7, 8]. The roles of compatible solutes might protect plants from stresses in these ways of detoxifying radical oxygen species (ROS), reducing lipid peroxidation and stabilizing the quaternary structures of proteins to maintain their function [5, 9].

Attapulgite clay (AC), also known as palygorskite, which has a fibrous reticular structure with many nanoscale channels giving it unique physical and chemical properties, such as a large specific surface area, adsorption, suspension, slow releasing, dispersion, ion-exchanging, water adsorption and retention, and low specific gravity, is a natural nonmetal clay mineral [10]. AC is sticky and plastic when wet and when drying shows little shrinkage [11, 12]. Also, research findings reveal that AC is rich in a small amount of elements including Si, Al, Mg, Fe, K, Ca and Mn, which is regarded as a source of many microelements [13]. There are some studies on the relationship between AC and quality improvement of plant Yang et al. [14] reported that AC combined with compound fertilizer increased dry matter accumulation and polysaccharide content of *Radix Hedysari*. The study of Guan et al. [10] found that the maize yield was increased based on using slow-release AC-coated fertilizers. To the best of our knowledge, there have not been any studies about the effect of AC on the responses of physiological and biochemical mechanisms of inducing salt and drought tolerance of okra plants. Therefore, it is worth investigating the possible regulatory roles of applied AC inducing abiotic stress tolerance of okra plants.

In this study, to our knowledge for the first time, we investigated the effects of applied AC on hormone metabolism, osmotic adjustment substances, photosynthesis pigment and ROS metabolism in inducing salt and drought tolerance of okra plants, which may provide more information to enhancement of okra salt and drought tolerance for sustainable agricultural development in stress areas.

2. Materials and Methods

2.1. Plant Materials and Experimental Design

Okra seedlings cultivar Dongjingwujiao were transplanted to plastic pots (diameter, 19 cm) containing a mixture of turf, humus and vermiculite (1:1:1, v/v/v) with applied attapulgite clay (AC) with different concentrations (0, 15, 30, 45 and 60 g/kg), respectively, for a total of 6 treatments, named CK, AC_{15}, AC_{30}, AC_{45} and AC_{60}, respectively. All seedlings were watered sufficiently with a half-Hoagland solution for 4 weeks until the seedlings formed new leaves. Subsequently, salt and drought stresses were performed, the treatments include: (i) Normal, each plant was irrigated with distilled water only for 4 weeks; (ii) 150 mM NaCl, each plant was irrigated with 200 mL of a 150 mM NaCl solution once every 2 d for 4 weeks; (iii) drought stress without water for 6 weeks. After treatments, the growth was observed and fresh weight (FW) and dry weight (DW) were measured immediately.

2.2. Measurements of ABA, Proline, Soluble Protein and Soluble Sugar Content

Endogenous ABA levels were performed by indirect enzyme-linked immuno sorbent assay (ELISA) in the leaves of okra plants treated for 2 weeks with 150 mM NaCl or for 3 weeks by drought stress based on in vivo assay as described by Wang et al. [15]. Proline, soluble protein and soluble sugar content of the above materials were measured according to the methods of Zhai et al. [16], respectively.

2.3. Assays of Photosynthetic Pigments

Chlorophyll *a* and *b* and total carotenoids content of the above materials were determined by Lichtenthaler and Buschmann [17], respectively.

2.4. Analyses of MDA, H_2O_2 and O^{2-} Content and Electrolyte Leakage

Malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and superoxide anion radical (O^{2-}) content and electrolyte leakage of the above materials were analyzed according to the methods of Gao et al. [18], Wang et al. [1], Zhang et al. [3] and Hatami al. [19], respectively.

2.5. DAB Staining

3,3′-Diaminobenzidine (DAB) staining was performed as described by Jiang et al. [20] Leaves from okra plants in pots and incubated for 4 week under optimum growth condition, for 2 weeks with 150 mM NaCl or for 3 weeks by drought stress based on in vivo assay, were cut and soaked in a 1% solution of DAB in 50 cm Tris-HCl buffer and in a 0.1% solution of NBT in 10 cm potassium phosphate buffer for hydrogen peroxide (H_2O_2) detection, respectively.

2.6. Assays of NCED, P5CS, SOD and POD Activities

The 9-cis-epoxycarotenoid dioxygenase (NCED) activity of the above materials was measured with Plant NCED ELISA Kit (Uscn Life Science Inc., Shanghai, China). The activities of pyrroline-5-carboxylate synthase (P5CS), superoxide dismutase (SOD) and peroxidase (POD) of the above materials were measured according to the methods of Hayzer et al. [21], Gao et al. [18] and Wang et al. [15], respectively.

2.7. Statistical Analysis

The experiments were repeated three times and the data presented as the mean ± standard error (SE). Where applicable, data were analyzed by Student's *t*-test in a two-tailed analysis. Values of $P < 0.05$ or < 0.01 was considered to be statistically significant.

3. Results

3.1. Induced Salt and Drought Tolerance in Okra Plants

The experiment of pot-planting was used to study the effects of salt tolerance and drought tolerance of okra plants by applied attapulgite clay (AC) with adding amounts (0, 15, 30, 45 and 60 g/kg). The results were shown in Figure 1, applied AC can significantly improve salt tolerance and drought resistance of okra plants. The okra plants with applied AC showed good growth and increased physical size and their FW and DW were increased by 185%-323% and 74%-166%, respectively, under 150 mM NaCl stress, and 242%-369% and 113%-187%, respectively, under drought stress, while okra plants without using AC died (Figure 1). Thus, phenotype identification results showed that AC_{30}-treated okra plants had the optimal phenotype of salt and drought tolerance, which were used to analyze the physiological and biochemical mechanisms of inducing salt and drought tolerance.

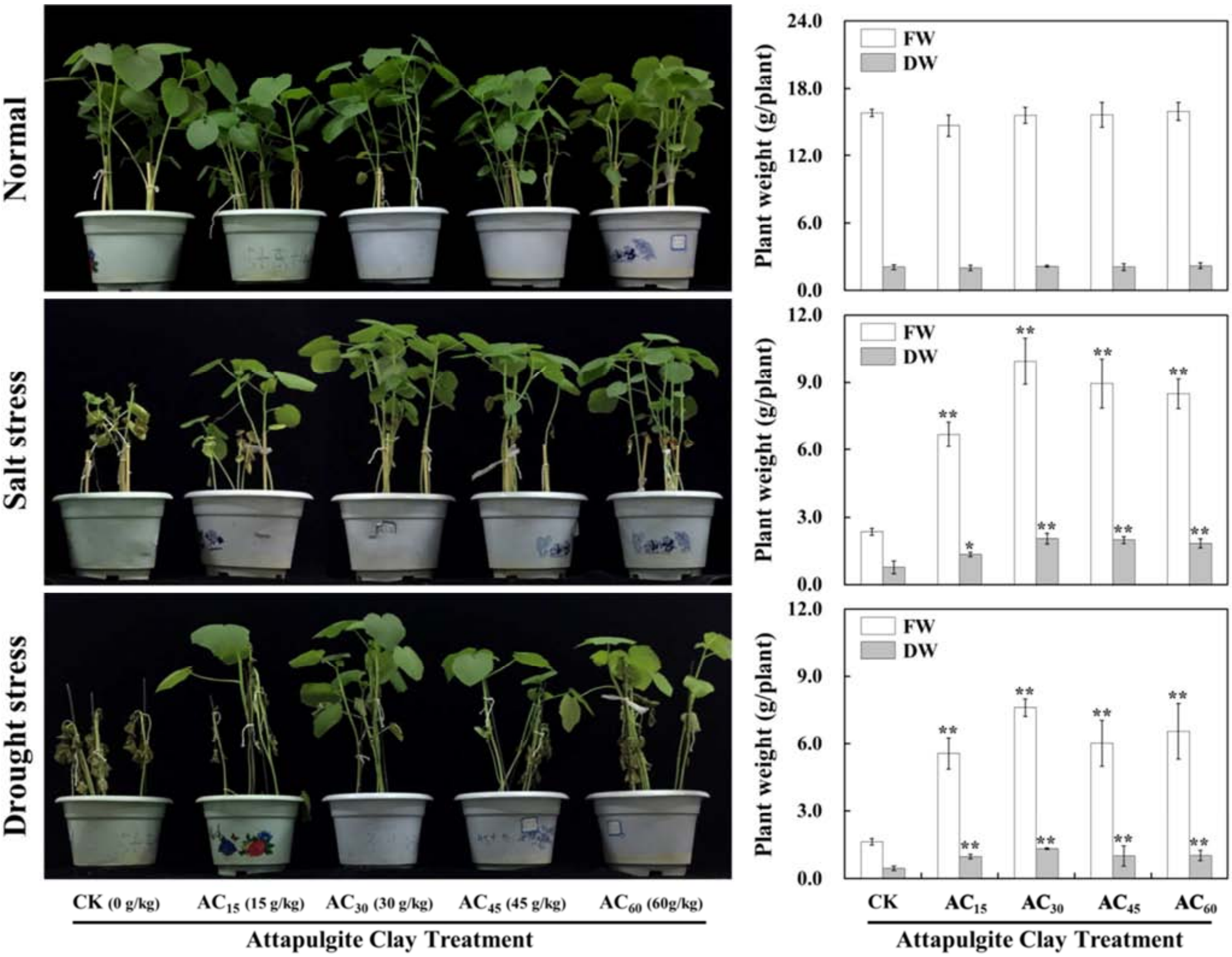

*Figure 1. Responses of okra plants grown in the soil with applied attapulgite clay (AC) with different concentrations (0, 15, 30, 45 and 60 g/kg) under salt and drought stresses. * and ** indicate significant differences at P < 0.05 and < 0.01, respectively, by Student's t-test.*

3.2. Increased ABA and Proline Accumulation Levels in Okra Plants

The content of ABA and proline in the leaves of AC-treated okra plants were measured under salt and drought stresses. Our works showed that the AC-treated okra plants showed increased ABA and proline content under salt and drought stresses (Figures 2 and 3). Further analyses found that the activities of NCED and P5CS, as a key rate limiting enzyme of ABA biosynthesis and proline biosynthesis, respectively, were significantly enhanced in the AC-treated okra plants compared with control okra plants under salt and drought stresses (Figures 2 and 3). Thus, the application of AC might increase NCED and P5CS activities, which accumulated more levels of ABA and proline, leading to the enhanced salt and drought of okra plants.

3.3. Improved Soluble Protein and Soluble Sugar Content in Okra Plants

The content of soluble protein and soluble sugar in the leaves of AC-treated okra plants were measured under salt and drought stresses. In this study, more accumulation levels of soluble protein and soluble sugar were observed in the AC-treated okra plants under salt and drought stresses (Figure 4). Thus, the application of AC might accumulate more levels of soluble protein and soluble sugar to regulate osmotic balance, leading to the enhanced salt and drought of okra plants.

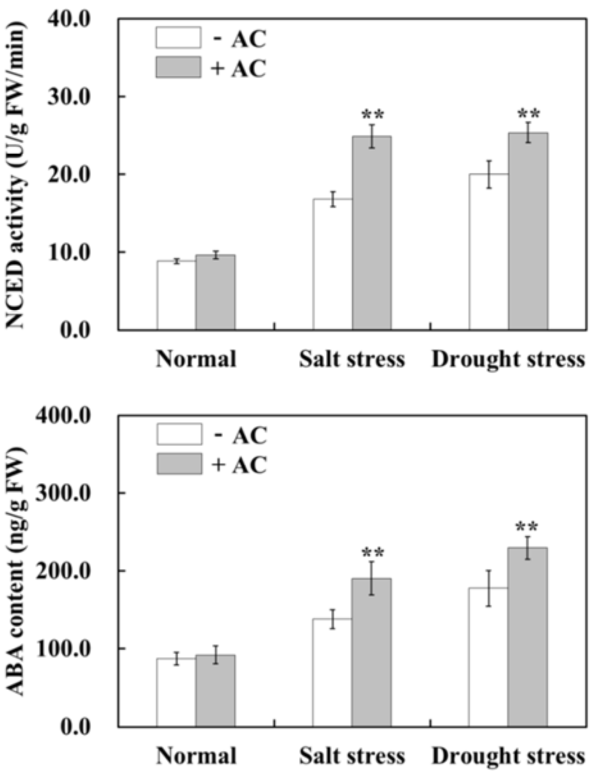

*__Figure 2.__ Effects of exogenous AC on nine-cis-epoxycarotenoid dioxygenase (NCED) activity and ABA content of okra plants under salt and drought stresses. * and ** indicate significant differences at P < 0.05 and < 0.01, respectively, by Student's t-test.*

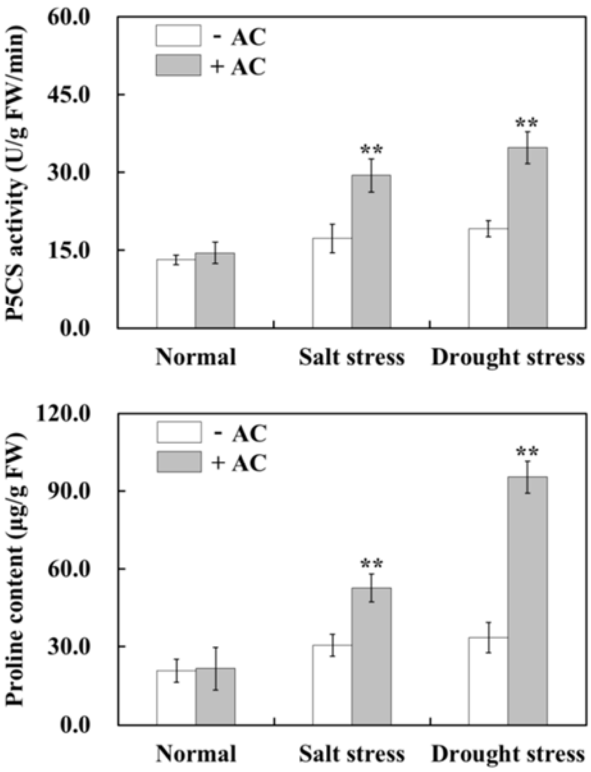

*__Figure 3.__ Effects of exogenous AC on pyrroline-5-carboxylate synthase (P5CS) activity and proline content of okra plants under salt and drought stresses. * and ** indicate significant differences at P < 0.05 and < 0.01, respectively, by Student's t-test.*

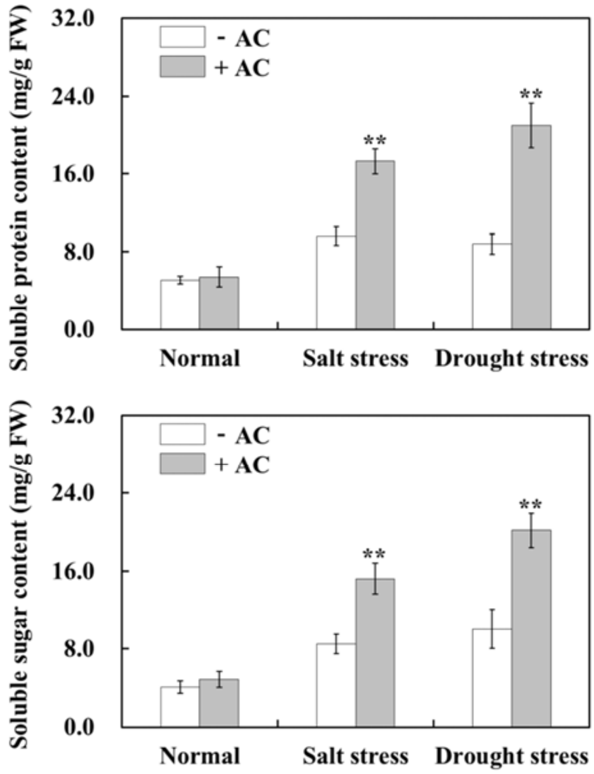

*__Figure 4.__ Effects of exogenous AC on soluble protein and soluble sugar content of okra plants under salt and drought stresses. * and ** indicate significant differences at P < 0.05 and < 0.01, respectively, by Student's t-test.*

3.4. Enhanced Photosynthesis Capacity in Okra Plants

Salt and drought stresses affects photosynthesis in plants and increase the number of free radicals in chloroplasts, thereby destroy the chlorophyll, and affect the photosynthesis of plants [22, 23]. The photosynthetic pigment (chlorophyll and carotenoids) content in the leaves of okra plants were significantly decreased after salt and drought stresses treatment (Figure 5). However, after treatment with exogenous 30 g/kg AC, the chlorophyll *a* and *b*, and total carotenoids content were remarkably increased in the leaves of AC-treated okra plants under salt and drought stresses (Figure 5). As a consequence, it is thought that the application of AC can increase photosynthetic pigment content, which enhances photosynthesis capacity, resulting in improved salt and drought tolerance in okra plants.

3.5. Reduced H_2O_2 and O^{2-} Accumulation and in Okra Plants

In plants, H_2O_2 and O^{2-}, important physiological indices, have been widely used to evaluate the plant stress response [15, 20, 24]. The levels of H_2O_2 accumulation were evaluated by means of DAB staining and the content of H_2O_2 and O^{2-} was analyzed in the leaves of okra plants with or without using exogenous 30 g/kg AC under salt and drought stresses. These results showed that the significantly less H_2O_2 and O^{2-} accumulation were observed in the AC-treated okra plants under salt and drought stresses, compared with control okra plants (Figures 6 and 7). Further analyses found that the activities of SOD and POD were significantly enhanced in the AC-treated okra plants compared with control okra plants under salt and drought stresses (Figure 8). These results suggested that the application of AC inhibited ROS damage by decreasing H_2O_2 and O^{2-} levels and enhancing antioxidant enzyme activities under salt and drought stresses.

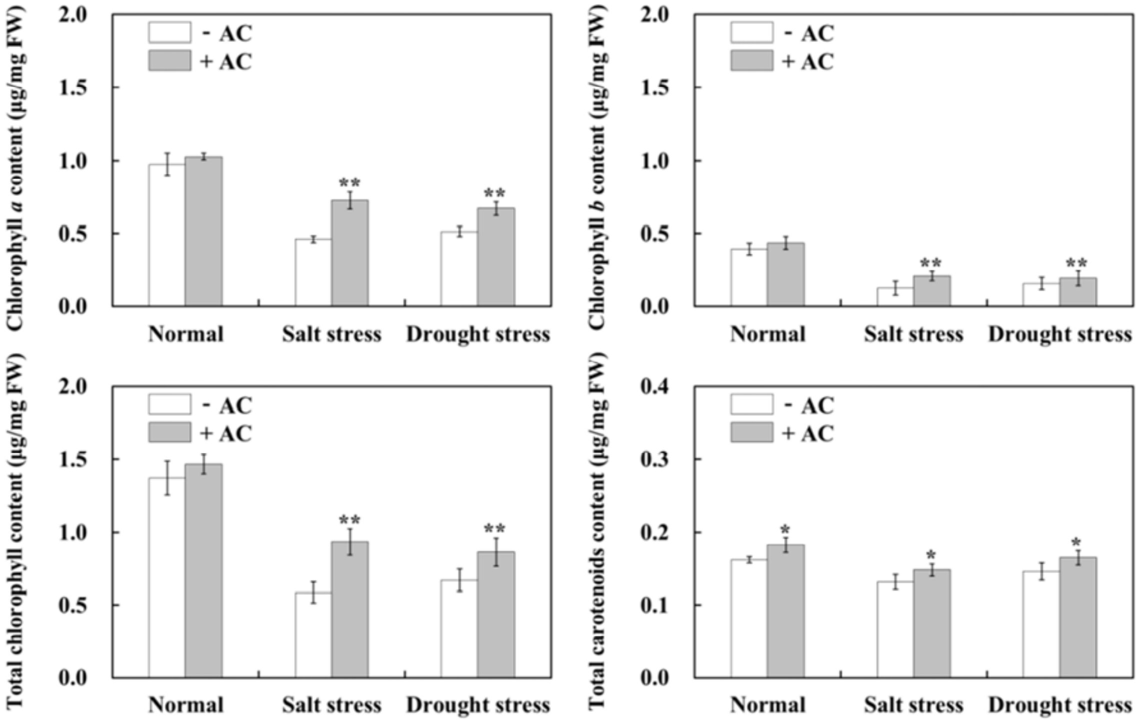

*Figure 5. Effects of exogenous AC on photosynthetic pigment content of okra plants under salt and drought stresses. * and ** indicate significant differences at P < 0.05 and < 0.01, respectively, by Student's t-test.*

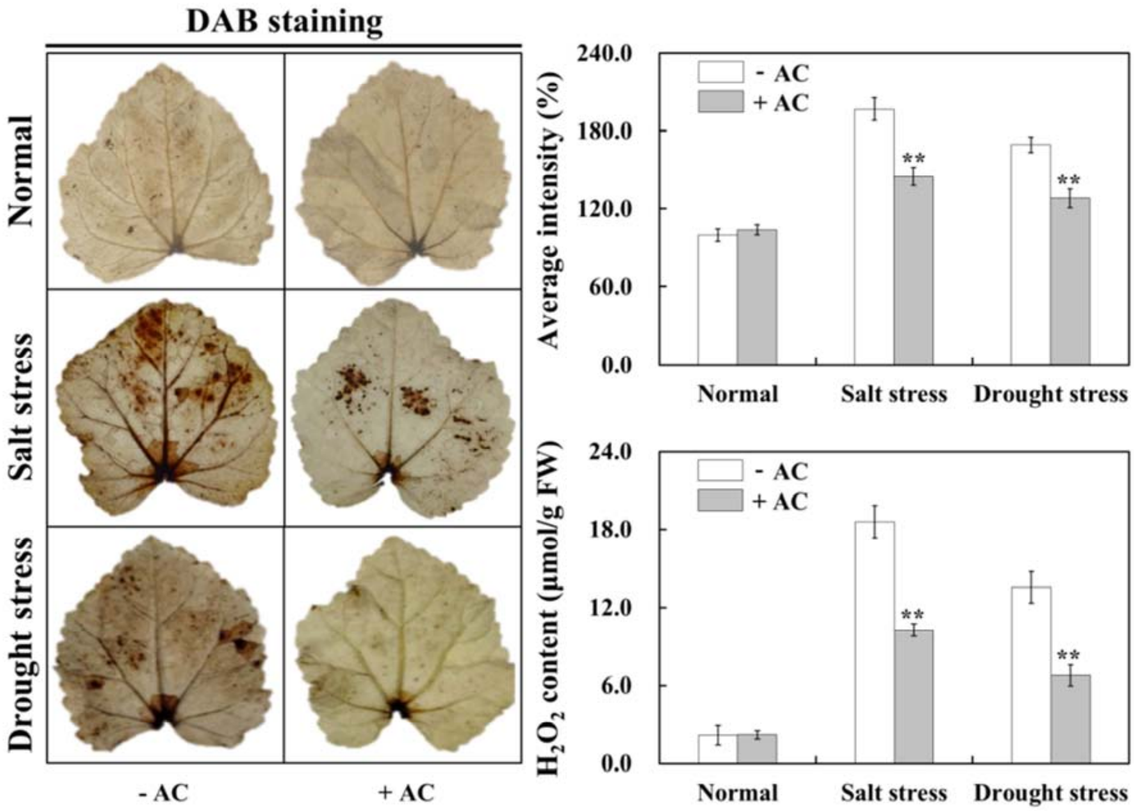

*Figure 6. Effects of exogenous AC on H_2O_2 content of okra plants under salt and drought stresses. * and ** indicate significant differences at P < 0.05 and < 0.01, respectively, by Student's t-test.*

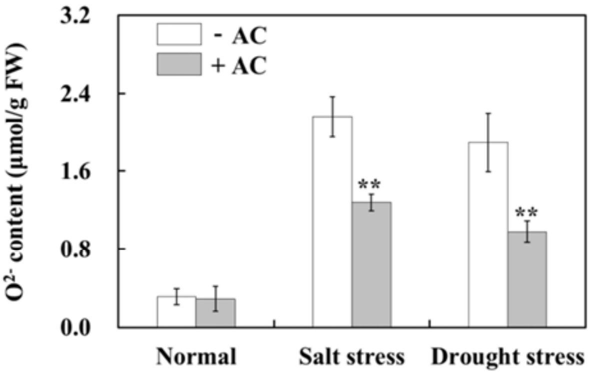

Figure 7. *Effects of exogenous AC on O^{2-} content of okra plants under salt and drought stresses. * and ** indicate significant differences at P < 0.05 and < 0.01, respectively, by Student's t-test.*

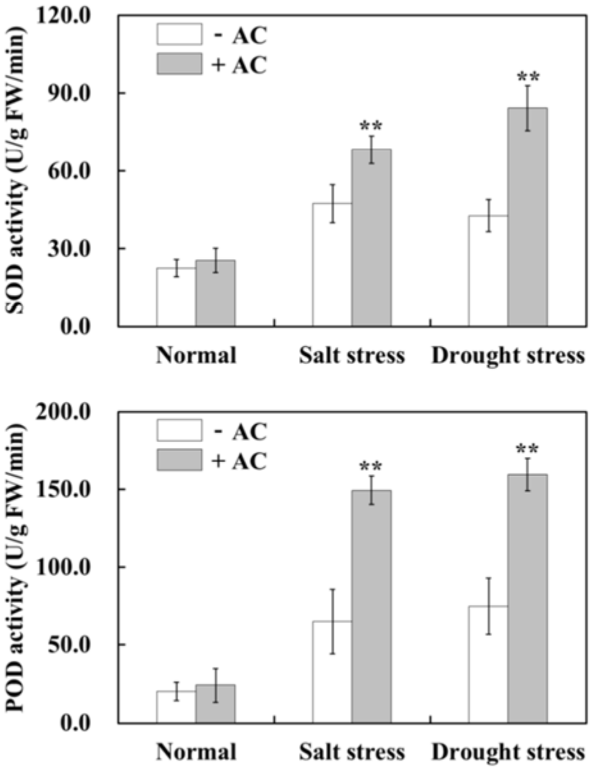

Figure 8. *Effects of exogenous AC on SOD and POD activities of okra plants under salt and drought stresses. * and ** indicate significant differences at P < 0.05 and < 0.01, respectively, by Student's t-test.*

3.6. Alleviated Membrane Lipid Peroxidation in Okra Plants

MDA is often considered a reflection of cellular membrane degradation, and its accumulation increases with production of superoxide radicals and hydrogen peroxide [26]. The levels of MDA accumulation were evaluated in the leaves of okra plants with or without using exogenous 30 g/kg AC under salt and drought stresses. Our data showed that after salt and drought stresses, MDA content was clearly increased in okra plants; however, accumulated lower MDA levels were observed in the AC-treated okra plants than that in control okra plants, while no obvious difference was found under normal conditions (Figure 9). Thus, it is hypothesized that the enhanced salt and drought tolerance of okra plants, at least in part, duo to the accumulated lower MDA levels.

3.7. Decreased Cell Death in Okra Plants

Electrolyte leakage measurements were performed to monitor cell death [25]. The electrolyte leakage rates were determined in the leaves of okra plants with or without using exogenous 30 g/kg AC under salt and drought stresses. There was no significant difference in electrolyte leakage rates among these plants before stress treatment (Figure 9). However, after salt and drought stress treatments, their electrolyte leakage rates were significantly different. The control okra plants exhibited the higher electrolyte leakage rates, whereas the AC-treated okra plants had the lower electrolyte leakage rates (Figure 9). It is thought that the application of AC can alleviate cell damage caused by salt and drought stresses, resulting in the enhanced salt and drought tolerance of okra plants.

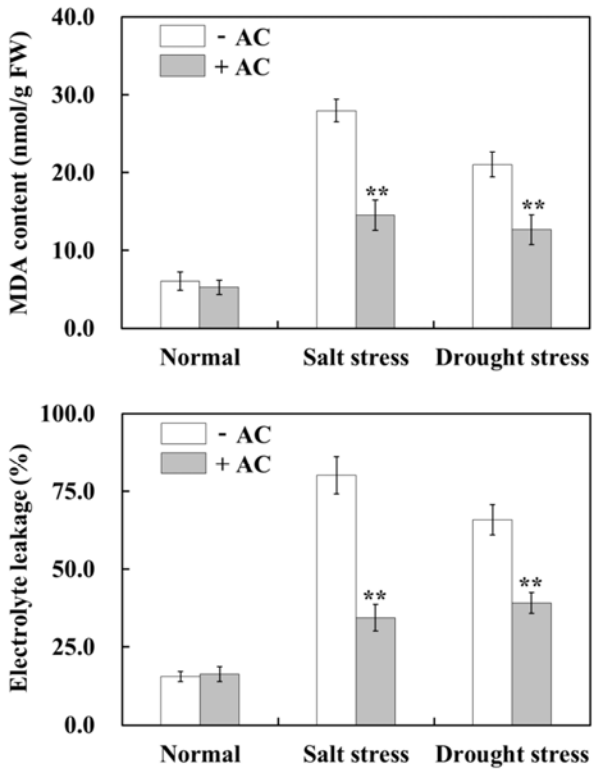

Figure 9. *Effects of exogenous AC on MDA content and electrolyte leakage of okra plants under salt and drought stresses. * and ** indicate significant differences at P < 0.05 and < 0.01, respectively, by Student's t-test.*

4. Discussion

Osmotic and oxidative stresses resulting from high salinity and drought stress environment represent the primary and secondary phases of toxicity, respectively, where plants respond to each component at different times. Under salt and drought conditions, many plants produce and accumulate osmoprotectants such as proline, soluble protein to soluble sugar defend against hyperosmotic stress [27-29]. In this study, for the first time, the application of AC can significantly improve salt and drought tolerance of okra plants based on the experiment of pot-planting (Figure 1). Furthermore, we investigated the possible regulatory roles of applied AC on hormone metabolism, osmotic adjustment substances, photosynthesis pigment and ROS metabolism in inducing salt and drought tolerance in okra plants.

4.1. The Roles of AC in Enhancing Salt and Drought Tolerance by Accumulating More ABA Levels

An important feature of drought and salt stresses is that the hyperosmotic signal causes the accumulation of the endogenous phytohormone ABA [6, 30]. Endogenous ABA can participate in the regulation of a variety of physiological responses of plants to abiotic stresses, such as drought, salt and chilling stress [16, 30-33]. In this study, the enhanced activity of NCED and the significant increase of endogenous ABA content were observed in the AC-treated okra plants compared with the control okra plants under salt and drought stresses (Figure 2). Thus, it is thought that the application of AC might increase the production of ABA as a signaling molecule by enhancing the activity of ABA biosynthesis key enzyme NCED, which further regulate physiological responses to salt and drought stresses of okra plants (Figure 10).

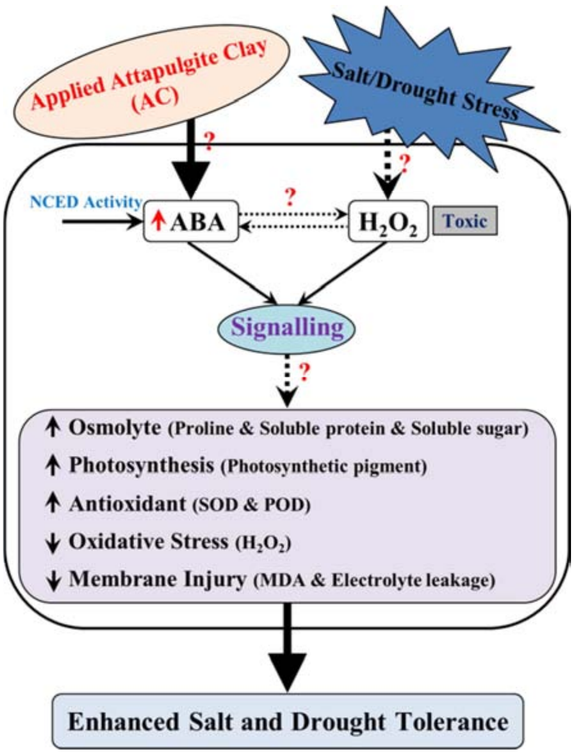

Figure 10. *Model of physiological mechanism of exogenous AC in enhancing salt and drought tolerance of okra plants.*

4.2. The Roles of AC in Increasing Salt and Drought Tolerance by Reguating Osmotic Adjustment Substances

Proline accumulation can enhance salt and drought tolerance in many plant species [24]. The increased proline accumulation can enhance salt and drought tolerance of rice plants [35-37]. The sweetpotato plants exhibiting more proline accumulation had significantly improved salt or drought tolerance [16, 35, 38, 39]. More proline accumulation levels were related to the enhanced salt and drought tolerance in *Arabidopsis* [1, 15, 25, 32]. In the present study, the AC-treated okra plants had significantly higher proline content compared with the control okra plants (Figure 3). More proline accumulation in the AC-treated okra plants might maintain the osmotic balance between the intracellular and extracellular environment and protect membrane integrity, which result in the enhanced salt and drought tolerance (Figure 10). The similar results were also reported in several studies [3, 16, 25, 38-40].

Soluble sugars and soluble proteins are primarily used as osmotic agents to prevent plant cells from dehydration and death and to maintain the internal stability of the cells under abiotic stresses [22, 41, 42]. As an important osmotic regulator, soluble sugars can significantly increase the osmotic potential of plant cells and regulate ion balance after stress treatment [22]. In this study, when treated with salt and drought stresses, the content of total soluble sugar and total soluble protein in the okra plants were significantly increased, while the AC-treated okra plants showed the increased content of these substances compared with the control okra plants (Figure 4). Thus, it is thought that the application of AC might increase total soluble sugar and total soluble protein content to maintain the osmotic balance between the intracellular and extracellular environment, leading to the enhanced salt and drought tolerance of okra plants (Figure 10).

4.3. The Roles of AC in Improving Salt and Drought Tolerance by Enhancing Photosynthesis Capacity

Salt and drought stresses perturb plant water uptake in leaves, and disrupt the osmotic, ionic and nutrient balances, and increase the number of free radicals in chloroplasts to thereby destroy the chlorophyll, leading to quick response in stomatal conductance to affect the photosynthesis of plants [22, 23]. A decrease in relative water content results in the disturbance of a multitude of physiological processes including stomatal conductance, CO_2 assimilation, and photosynthesis [29, 43, 44]. These stresses affect photosynthetic electron transport and the activities of enzymes for carbon fixation [39, 45, 46]. The damaging effects of singlet oxygen and hydroxyl radicals on PSII can be reduced by proline in isolated thylakoid membranes [3, 16, 39, 47]. Proline protects PSII photofunctions against photodamage which gets accelerated in plants under salt and drought stresses [16, 39, 48]. In this study, we observed a decrease in the levels of photosynthetic pigment (chlorophyll and carotenoids) content of okra plants under salt and drought stresses (Figure 5). Whereas, photosynthetic pigment content in the AC-treated okra plants were higher than in those of the control okra plants under salt and drought stresses (Figure 5). A reduced amount of chlorophyll can be attributed to stomatal closure leading to less CO_2 uptake, ion toxicities in the chloroplasts [49]. Chlorophyll degradation by ROS [50]. activation of the chlorophyllase enzyme, or instability of pigment-protein complex by salt ions [51]. Similar to our results, Qin et al. [52] and Mostofa et al. [29] also showed a decrease in total chlorophyll content in response to salt stress. Maintenance of plant water status and chlorophyll content has been considered one of the crucial components of plant tolerance to salt and drought stresses [53]. The less affected photosynthesis of okra plants could be explained by that the accumulated proline in the AC-treated okra plants provides protection against photoinhibition under salt and drought stresses [16, 39]. Our data indicates that the application of AC might enhance photosynthesis capacity by increasing the content of photosynthetic pigment, resulting in the improved salt and drought tolerance of okra plants under salt and drought stresses (Figure 10).

4.4. The Roles of AC in Conferring Salt and Drought Tolerance by Reducing ROS Accumulatio

Salt and drought stresses generally lead to oxidative stress through the production of ROS [34, 54]. Increased ROS levels provoke lipid peroxidation and cause electrolyte leakage, loss of membrane permeability, and malfunctioning of membrane proteins and ion channels [54-56]. MDA, as a reflection of cellular membrane degradation or dysfunction, is an important intermediary agent in ROS-scavenging [34]. It is important to maintain a stronger ROS-scavenging ability under salt and drought stresses to alleviate the induced oxidative damage, especially in plant leaves where photosynthesis is dramatically impacted [57]. Higher ability of ROS-scavenging enzymes decreased over-accumulated ROS levels, led to the enhanced salt and drought tolerance [20, 26]. SOD and POD play important roles in scavenging ROS by detoxifying H_2O_2 into water and stable oxygen, leading to enhanced stress tolerance [1, 34]. In this study, salt and drought stress treatment resulted in an increase accumulation of H_2O_2 and O^{2-} with a concomitant increase in MDA content (Figures 6, 7 and 9), indicating an evident oxidative burst in the leaf tissues of okra plants. Consistent with this phenomenon, the significantly increase of ROS scavenging key enzyme (SOD and POD) activities were observed in the AC-treated okra plants under salt and drought stresses (Figure 8), suggesting that the improved salt and drought tolerance of AC-treated okra plants is also due to the enhanced ROS scavenging. Similar to our results, Zhai et al. [16] and Zhang et al. [3] also provided a clear link between stress-induced ROS production and lipid peroxidation in sweetpotato plants. Thus, it is thought that AC pretreatment of okra plants to salt and drought stresses resulted in lower ROS production and lower MDA content, which might be achieved through AC-mediated direct ROS scavenging, membrane stabilizing, or modulating the antioxidative mechanism involved in eliminating ROS (Figure 10). Our results support that more proline accumulation activates ROS scavenging system, leading to the enhanced salt and osmotic tolerance in the AC-treated okra plants [1, 3, 16, 32].

5. Conclusions

In this study, the applied AC significantly enhanced salt and drought tolerance of okra under salt and drought stresses. Enzymatic assays indicated the activities of SOD and POD were significantly increased in okra plants grown in the soil with applied AC under salt and drought stresses. Further analyses under salt and drought stresses showed significant increases of ABA, proline, soluble sugar, soluble protein and photosynthetic pigment content, as well as significant decreases of MDA, H_2O_2 and O^{2-} content. The AC has the potential to be used to develop plant growth regulators to enhance the tolerance to abiotic stresses in plants.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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