Chiang Mai J. Sci. 2018; 45(3) : 1296-1306
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Ascorbic Acid Application Improves Salinity Stress Tolerance in Wheat

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Received: 31 March 2017
Accepted: 31 July 2017

ABSTRACT

Salinization converts fertile land into infertile land, and is a severe threat to food security and crop productivity worldwide. In this study, we treated wheat plants (*Triticum aestivum* L. var. Yecora Rojo) with or without ascorbic acid (ASA), both under salt stress and non-stress conditions, to understand the effect of ASA on plant response to salinity stress. We monitored the amounts of photosynthetic pigments, osmoprotectants, and antioxidant enzymes. Application of ASA enhanced growth characteristics, such as shoot and root length, shoot and root fresh weight, shoot and root dry weight. It also improved the physiological and biochemical parameters, such as carbonic anhydrase (CA) and Rubisco activities, photosynthetic pigments (*Chl a, b,* and Total *Chl*), and compatible solutes (proline [Pro] and glycinebetaine [GB]) contents, under non-stress conditions. Under NaCl stress, the ASA addition improved growth attributes, levels of pigments, accumulation of Pro and GB, and antioxidant enzymes activities, such as superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase. Also, ASA decreased *Chl* degradation, electrolyte leakage, and concentration of malondialdehyde and hydrogen peroxide in wheat plants. We conclude that supplementing ASA can benefit wheat plants by improving their tolerance to salinity stress.

Keywords: *Triticum aestivum,* ascorbic acid, salinity, reactive oxygen species, chlorophyll degradation, rubisco

1. INTRODUCTION

Nowadays, salinity is one of the major abiotic stresses that is causing risks to food security for escalating world population. Salinization causes a severe problem for agricultural crops, as increasing salinization reduces the land available for crop cultivation. According to the FAO report [1], salinity affects more than 800 million hectares of agricultural land worldwide. The presence of high amounts of salts in the soil, especially NaCl, has extreme effects on plant growth and physiological processes [2-3]. Among species and genotypes, there is great genetic variation in tolerance to salinity [4]. The majority of crops is unable to survive or complete their lifecycle under salt stress

conditions. High salt in the soil causes a series of changes in plants, such as impairing ion homeostasis, photosynthesis, enzymes activity, proteins and compatible solutes synthesis, and energy and lipid metabolism [5]. Plants respond to salinity through complex mechanisms, including osmotic adjustment, which requires the accumulation of inorganic ions and compatible solutes, such as proline, glycinebetaine, and soluble carbohydrates. These organic solutes also regulate enzymes activity. However, the role of both organic and inorganic solutes in osmotic adjustment varies among different plant species, and even within the different parts of the same plant [2, 4]. Osmoprotectants help plants to develop a plethora of molecular mechanisms to overcome salt stress by regulating gene expression of osmoregulation [5]. Thus, it may be possible to enhance plant salt-tolerance by modulating physiological and biochemical processes.

High concentrations of salts in soil impedes water uptake by plants and leads to excessive production of reactive oxygen species (ROS) that cause oxidative damage. Due to intrinsic antioxidant properties, ascorbic acid (ASA) reduces ROS production in plants by providing electron donors. Additionally, ASA has emerged as a potent antioxidant and plays a key role in plant stress signaling and also in growth, physiological, and biochemical processes [6-7]. ASA helps plants to grow and function by regulating a series of cellular processes, such as cell division, cell differentiation, and senescence [6]. ASA is a co-factor of several phytohormones, as well as many enzymes. Therefore, this study investigated the role of ASA in mitigating salt stress in wheat plants by regulating the physiological and biochemical attributes of plants. In addition, exogenous application of ASA mitigates water deficit stress and improves nutrient uptake, root and shoot growth, and also reduces oxidative damage induced by different environmental stresses in plants [7]. Therefore, it is essential to study the effect of ASA on plants growth and development under salt stress to understand the role of ASA in mitigating oxidative damage by improving physiological and biochemical mechanisms.

Wheat (*Triticum aestivum* L.) is an important cereal crop that is cultivated worldwide, which provides 85% of basic calories and 82% of protein to the world population. In addition, it contains some minerals and is used in various bakery products. Similar to other cereals, wheat is very sensitive to salt stress and its yield is severely affected [8]. It may be necessary to exploit suitable management practices to improve the tolerance of wheat plants to salt stress. This study, in addition to exploring the role of ASA in plant growth under salt stress and also investigated the changes in organic solutes and photosynthetic pigments, and enzymatic activity.

2. MATERIALS AND METHODS

2.1 Seeds and Growth Conditions

The experiment was conducted in a growth chamber under controlled conditions (temperature 25 ± 2 °C with 16/8-h light/dark cycle (450 μmol of photons m^{-2} s^{-1}), relative humidity 60 ± 2%). Seeds of wheat (*Triticum aestivum* L. var. Yecora Rojo) were obtained from the local market. Plastic pots (6 in. diameter) were filled with acid-washed sand and arranged in a randomized sample design. The disinfectant, sodium hypochlorite (1%) was used to sterilize seeds of wheat. The seeds were pre-germinated in tap water for 24 h until the radical emerged to 0.5 mm from the testa. Six seeds (pre-germinated in tap water) were sown in pots with Raukura's nutrient solution [9]. When 2-3 leaves appeared in

the seedlings after 10 days of sowing, the application of NaCl was gradually applied from 0 mM to reach a final concentration of 90 mM. Treatments comprised: (1) 0.0 mM ASA + 0.0 mM NaCl; (2) 0.2 mM ASA + 0.0 mM NaCl; (3) 0.8 mM ASA + 0.0 mM NaCl; (4) 0.0 mM ASA + 90 mM NaCl; (5) 0.2 mM ASA + 90 mM NaCl; and (6) 0.8 mM ASA + 90 mM NaCl. Application of ASA and NaCl was repeated three times during 35 days of cultivation. After 35 days of sowing, wheat seedlings were harvested to determine morphological, physiological, and biochemical attributes.

2.2 Growth Parameters

Wheat plant performance was assessed by measuring growth characteristics, such as shoot length (SL), root length (RL), shoot fresh (SFW), root fresh weight (RFW), shoot dry weight (SDW), and root dry weight (RDW), all on a per plant basis SL and RL were measured using a scale. After measuring SFW and RFW, samples were oven-dried at 70 °C for at least 48 h to measure SDW and RDW.

2.3 Physiological and Biochemical Attributes

Pre-chilled mortar and pestle was used to extract chlorophylls from fresh leaves of wheat plants using dimethyl sulfoxide (DMSO). The absorbance of pigments was measured using a UV-vis Spectrophotometer (SPEKOL 1500; Analytik Jena AG, Jena, Germany). Pigment content was determined according to the formula of Barnes *et al.* [10]. After extraction of pigments, *Chl* degradation was determined according to Ronen and Galun [11].

Fresh leaf samples were collected and washed, and the leaves were chopped using a sharp sterile scalpel and placed in Petri dishes containing 0.2 M cysteine hydrochloride solution for 20 min at 4 °C, after which they were transferred to test tubes with 4 mL of 0.2 M sodium bicarbonate solution and 0.2 mL of 0.022% bromothymol blue. Titration was done using 0.05 N HCl with methyl red as an indicator. The activity of CA was expressed as CO_2 kg^{-1} leaf FW s^{-1} [12].

Rubisco activity was determined by measuring NADH oxidation at 340 nm using a UV-vis spectrophotometer (SPEKOL 1500; Analytik Jena AG, Jena, Germany) [13]. The enzyme was extracted by homogenizing leaves using a chilled mortar and pestle in ice-cold extraction buffer solution (100 mM Hepes- KOH, pH 7.5, 0.5 mM EDTA, pH 8.0, 10 mM potassium acetate, 5 mM DTT, 20 mM β-mercaptoethanol, 5% [v/v] glycerol, 1% [w/vL PVP, 0.05% [w/v] Triton X-100, and 0.5 mM PMSF). The homogenate was centrifuged at 10,000 × *g* for 10 min at 4 °C to remove debris and the supernatant was used for the enzyme assay. The reaction mixture comprised 100 mM Tris-HCl (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.2 mM NADH, 1 mM ATP, 1 mM EDTA, 5 mM DTT, 4 units of glyceraldehyde-3-phosphate dehydrogenase, and 4 units of 3-phosphoglycerate phosphokinase. NADH oxidation was initiated by adding the enzyme extract to 1 mM ribulose-1,5-bisphosphate (RuBP). The absorbance of the reaction was measured for 1 min after the reaction was terminated. Enzyme activity was expressed as μmol CO_2 fixed min^{-1} mg^{-1} protein. Protein content was quantified according to the method of Bradford [14] using bovine serum albumin as a standard.

The ninhydrin method of Bates *et al.* [15] was adopted to determine Pro content in leaves. Leaf samples were washed and macerated in 3% aqueous sulfosalicylic acid and centrifuged at 10,000 × g to remove debris and the supernatant was used to estimate Pro content. Acid ninhydrin and

glacial acetic acid were added to the supernatant, after which the reaction mixture was boiled at 100 °C for 1 h. After cooling in an ice bath, the reaction mixture was separated by adding toluene and absorbance was measured at 520 nm.

Glycinebetaine (GB) content was measured according to the method of Grieve and Grattan [16]. Dried leaves were ground to a fine powder and suspended in deionized water at 100 °C for 60 min. The content of GB was measured spectrophotometrically at 365 nm, using aqueous extracts of dry-ground leaf material after reacting with KI_2-I_2.

To measure membrane permeability, electrolyte leakage (EL) was estimated following the method of Lutts *et al.* [17]. Samples were washed three times with double distilled water (DDW) to remove surface contamination. The leaf discs were placed in a closed vial with 10 mL of DDW and incubated for 24 h on a rotatory shaker. The electrical conductivity of the solution was then determined. Next, samples were boiled at 120 °C for 20 min and electrical conductivity was measured.

Lipid peroxidation was determined by measuring malondialdehyde (MDA) content in leaf samples in accordance with the method given by Heath and Packer [18].

Hydrogen peroxide (H_2O_2) was measured as described by Velikova *et al.* [19]. Fresh leaf samples were crushed in 0.1% (w/v) TCA and centrifuged at 12,000 rpm for 15 min. The supernatant was added to a reaction mixture containing 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide. The absorbance was measured at 390 nm.

The activities of the antioxidant enzymes were assayed by homogenizing fresh leaves in an extraction buffer (0.5% Triton X-100 and 1% polyvinylpyrrolidone in 100 mM potassium phosphate buffer, pH 7.0). The homogenate was filtrated using a muslin cloth and centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatant was stored at -20 °C for enzymatic assays. The activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) was determined using the method of Giannopolitis and Ries [20], Aebi [21], Chance and Maehly [22], and Nakano and Asada [23], respectively. Enzyme activities are presented as units of enzyme activity mg^{-1} protein.

2.4 Statistical Analysis

Each experimental pot represented one replicate, and each treatment had four replicates. The data were analyzed using ANOVA (complete randomized) with SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). The Duncan's multiple range test was used to assess the significant ($P < 0.05$) differences between individual treatments.

3. RESULTS AND DISCUSSION

Results of the present study indicate that salt stress substantially impaired the growth, physiological and biochemical parameters of wheat plants (Figures 1-4). Application of ASA, however, improved these parameters by suppressing the oxidative damage in plants under salinity.

In this study, the efficacy of ASA in improving growth performance was assessed by measuring SL, RL, SFW, RFW, SDW, and RDW of plants under salt stress (Figure 1A-F). These plant attributes were significantly suppressed when plants were subjected to NaCl. A decrease in growth parameters may have been due to changes in metabolic processes, nutrient assimilation, and photosynthesis under salt stress [2]. However, the addition of ASA to the growth medium under salt stress was found to be efficient in restoring suppressed growth attributes.

Application of ASA also enhanced growth parameters under the non-stress condition. Improved growth performance of wheat plants might be due to the beneficial role of ASA in plants, as it regulates various physiological and biochemical processes and provides electrons to various biosynthetic pathways, and induces cell elongation and cell division [24]. We clearly observed that ASA has direct and indirect effects on these attributes. This, in turn, could have reversed the suppressed SL and RL, resulting in better orientation of leaves, which helped the plants to harvest solar energy leading to higher biomass production. The application of 0.8 mM of ASA had the highest effect by giving the highest values for almost all growth parameters under stress and non-stress conditions, compared to 0.2 mM of ASA. Thus, it can be postulated that addition of ASA to the growth medium improved growth performance by restoring the growth characteristics of wheat plants.

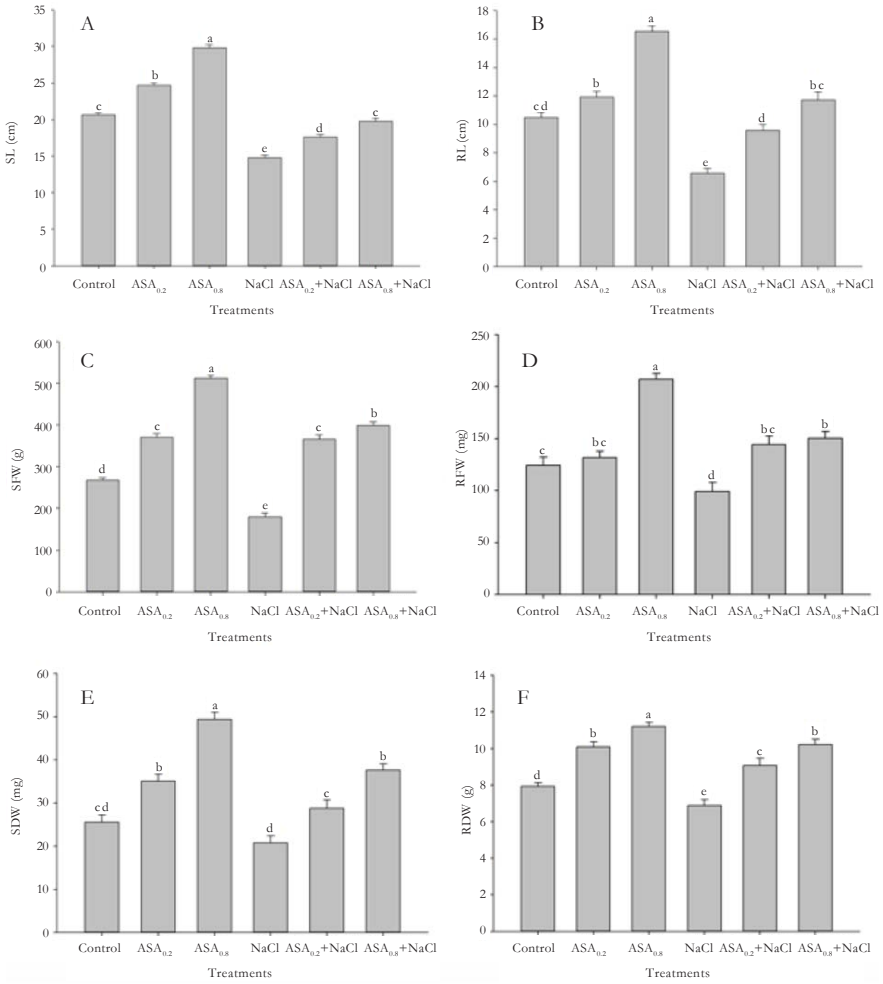

Figure 1. Effect of ASA on (A) shoot length (SL) plant^{-1}, (B) root length (RL) plant^{-1}, (C) shoot fresh weight (SFW) plant^{-1}, (D) root fresh weight (RFW) plant^{-1}, (E) shoot dry weight (SDW) plant^{-1} and (F) root dry weight (RDW) plant^{-1} of wheat plants under salinity. Bars followed by the same letters show no statistical difference at $P<0.05$ (Duncan's multiple range test. Values are given as bars showing mean ± standard error for four determinations.

Photosynthesis is a vital process for plant growth and development, and is regulated by photosynthesis pigments. As revealed in Figure 2 A-D, the addition of NaCl to the growth medium significantly inhibited the biosynthesis of chloroplast pigments, such as *Chl a, b*, and Total *Chl*, and enhanced *Chl* degradation. The suppression of these pigments in NaCl-stressed plants might have been due to *Chl* degradation (Figure 2D), partly caused by excess accumulation of Na^+ ions in the chloroplasts [2]. In addition, the accumulation of Na^+ ions alters the fine structure of photosynthetic apparatus, such as chloroplasts, the plastid envelope, and thylakoids, and causes changes in pigment protein complexes and chlorophyll destruction. Furthermore, a decrease in pigment content in NaCl-stressed plants could also be due to the formation of chlorophyll-degrading enzymes, such as chlorophyllase [25]. Both levels of ASA (0.2 and 0.8 mM) significantly enhanced the accumulation of *Chl a, b*, and Total *Chl* in wheat plants under non-stress conditions. In addition, both levels of ASA were effective in enabling the wheat plants to synthesize pigments under stress conditions. We observed that addition of 0.8 mM of ASA had a greater alleviating effect compared to 0.2 mM ASA supplement. However, the effect of 0.2 mM of ASA was at a par with 0.8 mM of ASA application when considering the synthesis of *Chl a* and *b* under salt stress. Application of both levels of ASA significantly inhibited *Chl* degradation under salt stress. According to Gul *et al.* [26], ASA is involved in protecting the photosynthetic apparatus from oxidative damage induced by salt stress and induces chlorophyll synthesis. It was also reported that ASA stimulates the synthesis of IAA and GA_3 and depresses ABA formation, which shields the chloroplast, resulting in increased production of photosynthetic pigments.

Photosynthesis is a primary process that underpins almost all life forms on the planet by converting solar energy into chemical energy. The enzyme Rubisco plays a central role in photosynthesis by catalyzing the fixation of atmospheric CO_2. In addition, the enzyme CA plays a pivotal role in catalyzing reversible conversion of CO_2 to bicarbonate and maintaining the continuous supply of CO_2 to Rubisco. We observed the activity of both photosynthetic enzymes (CA and Rubisco) to be was significantly enhanced by the addition of ASA to the growth medium under non-stress conditions (Figure 2E & F). However, the activity of CA and Rubisco was inhibited in plants exposed to NaCl. Interestingly, addition of ASA to the growth medium led to increase in the activities of both enzymes. Application of 0.8 mM of ASA was found to be more effective than the application of 0.2 mM in alleviating the adverse effects caused by salinity, perhaps by improving both enzymes. The increased activity of both enzymes may be correlated, as CA activates Rubisco activity, which subsequently enhances photosynthetic carbon fixation and pigment content in plants [27]. Therefore, the finding from this study reveal that the application of ASA can improve the tolerance of wheat plants, possibly by modulating the activity of CA and Rubisco.

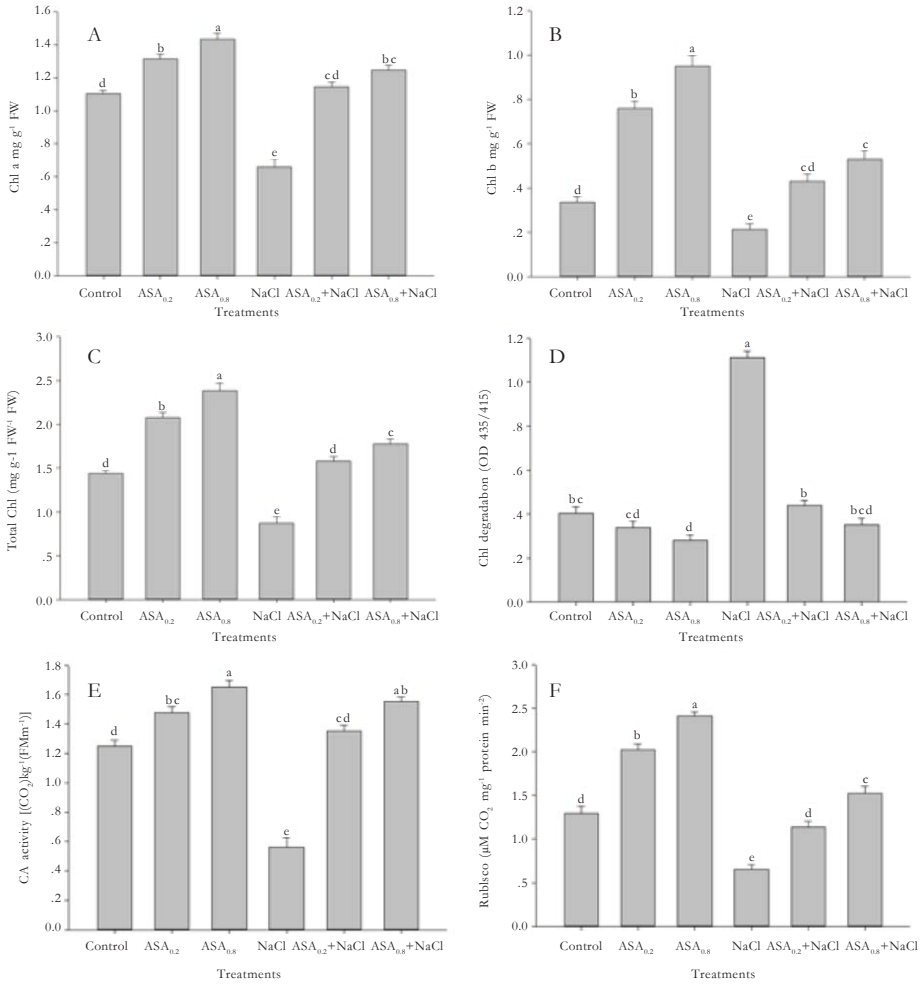

Figure 2. Effect of ASA on (A) chlorophyll (*Chl) a*, (B) *Chl b*, (C) Total *Chl,* (D) *Chl* degradation, and (E) carbonic anhydrase (CA) and (F) Rubisco activities in wheat plants under salinity. Bars followed by the same letters show no statistical difference at $P < 0.05$ (Duncan's multiple range test). Values are given as bars showing mean ± standard error for four determinations.

When exposed to harsher environments, plants adapt by synthesizing compatible solutes in cytosol and chloroplasts to regulate osmotic adjustments. These organic solutes play key roles in the stabilization of proteins, enzymes and minimizing oxidative damage caused by ROS, thereby maintaining plant cell membrane integrity at the cellular level [30]. In the present study, the inclusion of NaCl in the growth medium stimulated the accumulation of both compatible solutes (Pro and GB) (Figure 3 A & B). Meanwhile, the maximum values for Pro and GB were recorded at both levels of ASA under stress and non-stress conditions. Both levels of ASA (0.2 and 0.8 mM) appeared to enhanced Pro and GB further under salt stress, although 0.8 mM of ASA had the greatest influence on both the osmoprotectants under salt stress compared to 0.2 mM.

Application of ASA improved the tolerance of wheat plant to salt stress because Pro plays an important roles in scavenging of ROS, in providing organic carbon, nitrogen, and energy, and in balancing the redox status by maintaining the cytosolic pH [2, 28]. GB regulates the signal transduction cascade, and maintains the intracellular osmotic equilibrium by increasing water flow into cells and protecting membrane stability to helps channels and ion carriers to function properly during stress recovery [30]. Therefore, it is clear that ASA induced Pro and GB accumulation and enhanced the tolerance of plants against salt stress by offsetting the detrimental effects of salinity.

Metabolic activities in plant cells, particularly those experiencing abiotic stress lead to the generation of ROS. In our studies using wheat plants that were subjected to salt treatment, we observed enhanced levels of EL, MDA and H_2O_2 compared to the control plants and those supplemented with ASA (Figure 3C-E). Therefore, plant defense mechanisms were triggered to limit or detoxify ROS production and repair oxidative damages caused by free radicals. NaCl supplemented to the growth medium probably led to increased levels of EL and MDA and H_2O_2, compared to their respective controls. EL, MDA and H_2O_2 were found to be at low levels in plants that received 0.2 and 0.8 mM of ASA sipplements under non-stress conditions. Under NaCl stress, we found that ASA at both levels tested were found to be effective in alleviating the adverse effects of salinity in plants by suppressing MDA and H_2O_2 accumulation and EL. Application of 0.8 mM of ASA resulted in lower values for EL, MDA, and H_2O_2, in comparison to 0.2 mM of ASA under salt stress. Detoxification of ROS might have been possible due to the accumulation of compatible solutes (Figure 3 A & B) and enhanced activity of antioxidant enzymes (SOD, POD, CAT, and APX) in plants under salt stress (Figure 4 A-C). In addition, lipid peroxidation associated membrane deterioration could be inhibited by the accumulation of Pro and GB, and antioxidant enzymes. ASA regulates the ascorbate-glutathione cycle and directly reduces MDA formation, as it stabilizes membrane integrity by scavenging $^1O_2$, $O2^{-\bullet}$, and $^\bullet OH$ [29].

Data of the present study reveal that maximum activity of antioxidant enzymes (SOD, POD, CAT, and APX) was recorded in plants exposed to NaCl compared to control and ASA-fed plants (Figure 4 A-C). Clearly, under NaCl stress, either of the two doses of ASA tested enhanced the activity of antioxidant enzymes. We found that the effect of of 0.8 mM ASA was generally more pronounced than that of 0.2 mM ASA in alleviating the adverse effects of salt stress on the activity of SOD, POD, and APX, but its effect on CAT was comparable. Interestingly, these enzymes activities were increased further when ASA was applied to plants under salt stress. According to Pourcel *et al.* [30], ASA plays a key role in the regulation of many enzymes and in the suppression of oxidative stress by synergistic action with other antioxidants, and our observations are similar.

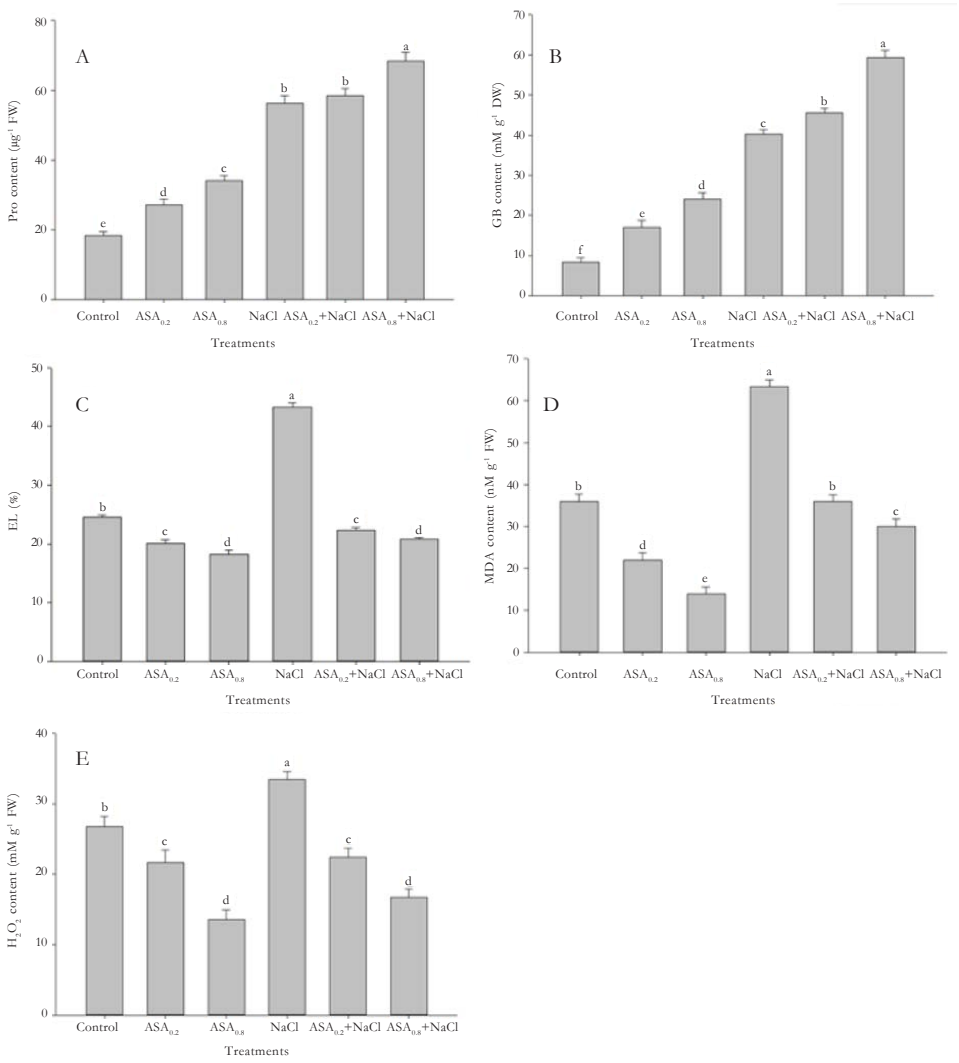

Figure 3. Effect of ASA on the content of (A) proline (Pro), (B) glycinebetaine (GB), (C) electrolyte leakage (EL), (D) malondialdehyde (MDA) content and (E) H_2O_2 content in wheat plants under salinity. Bars followed by the same letters show no statistical difference at $P<0.05$ (Duncan's multiple range test). Values are given as bars showing mean ± standard error for four determinations.

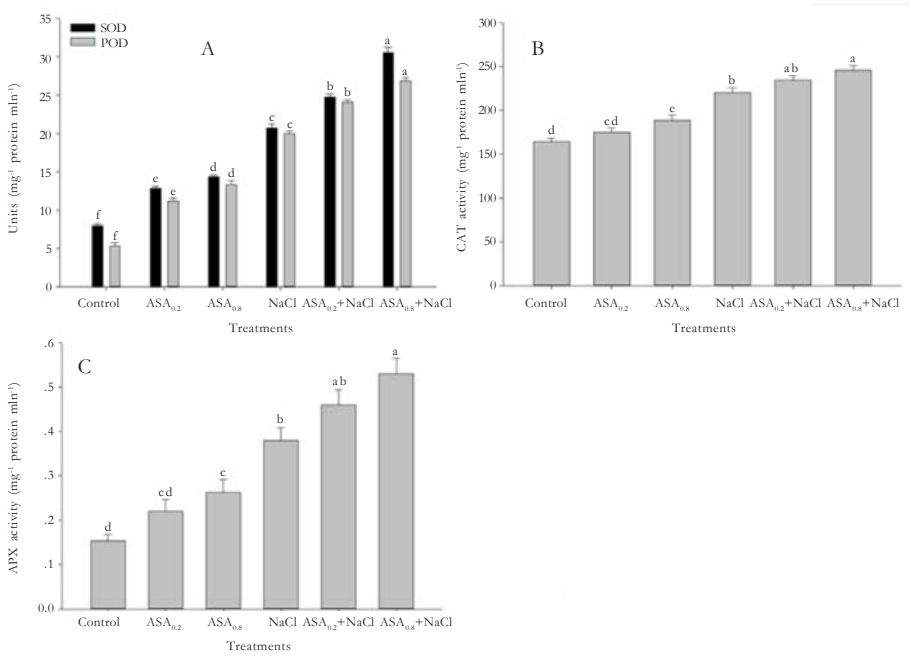

Figure 4. Effect of ASA on the activities of (A) SOD and POD, (B) CAT and (C) APX in wheat plants under salinity. Bars followed by the same letters show no statistical difference at $P < 0.05$ (Duncan's multiple range test). Values are given as bars showing mean ± standard error for four determinations.

4. CONCLUSION

This study concluded that the application of ASA enhanced growth characteristics of plants, such as SL, RL, SFW, and RFW, by increasing the activity of CA and Rubisco due to the accumulation of photosynthetic pigments (*Chl a, b,* and Total *Chl*) under non-stress conditions. However, under NaCl stress, the application of ASA improved the tolerance of wheat plants by increasing the levels of pigments, and the accumulation of compatible solutes (Pro and GB), resulting in increased growth characteristics. These results also showed that addition of ASA to the growth medium enhanced the activity of antioxidant enzymes (SOD, POD, CAT, and APX) and organic solutes under salt stress, which may have caused inhibition of lipid peroxidation and membrane deterioration by decreasing the accumulation of MDA and H_2O_2, and EL; including minimizing degradation of *Chl* and other photosynthetic pigments. Therefore, this study provides evidence that the addition of ASA to the growth medium could be an effective approach in agriculture to mitigate salinity stress challenges.

ACKNOWLEDGMENTS

Financial support was provided by the Deanship of Scientific Research of King Saud University, Riyadh, Saudi Arabia, to Research Group No. RGPVPP-153.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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