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Effect of exogenous application of ascorbic acid on antioxidant enzyme activities, proline contents, and growth parameters of *Saccharum* spp. hybrid cv. HSF-240 under salt stress

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Abstract: The present study was undertaken to examine the effects of exogenous application of ascorbic acid (AA) through different modes on growth and associated biochemical parameters in *Saccharum* spp. hybrid cv. HSF-240, under salt stress. In a pot experiment, AA was applied through irrigation or foliar-spray at the concentrations of 0.1, 0.5, and 1 mM with or without 100 mM NaCl concentration. Vegetative growth measurements, antioxidant enzyme activities (POD and SOD), and protein and proline contents of plants were recorded to study the effects of these treatments. The presence of salt reduced the growth of sugarcane plants. The AA application not only mitigated the inhibitory effects of salt stress but also induced a stimulatory effect on all the studied growth parameters. The activities of antioxidant enzymes (POD and SOD) as well as proline contents of plants were increased, although the protein contents were decreased after AA application. The exogenous application of AA through either way significantly alleviated the adverse effects of salinity on growth and biochemical parameters of sugarcane plants. However, in this study, the AA application through irrigation proved to be a better option in mitigating the adverse effects of salinity.

Key words: Antioxidant enzymes, ascorbic acid, proline, protein, salinity, sugarcane

Introduction

Salinity is an ever increasing environmental problem and is a substantial restraint to agriculture. The amount of salt-affected land worldwide is estimated to be 953 M ha, some 7% of the global total land mass and 20% of the world's irrigated land (1). High salt levels in soil result in hyperosmolarity, ion disequilibrium, nutrient imbalance, and production of reactive oxygen species (ROS), leading to plant growth retardation through molecular damage (2). The induction of salt tolerance in plants is crucial to maintain their economic yield. This can be achieved either through genetic modifications or chemical treatments (3). The strategies of plant breeding and genetic engineering are long-term and complex endeavors to develop salt tolerance that have had limited success (4). Alternatively, the exogenous application of plant growth regulating compounds is an efficient and technically simpler approach to cope with the deleterious effects of salinity on plants (5,6). During stress conditions the endogenous levels of growth regulators became low, which can be overcome by their exogenous application. Exogenous application of plant growth regulators, fertilizers, and nonenzymatic antioxidants has been successfully used to minimize the adverse effects of salinity on plant growth and yield (7,8).

Ascorbic acid (AA) is regarded as one of the most effective growth regulators against abiotic stresses (9). AA not only acts as an antioxidant but the cellular levels

of AA are correlated with the activation of complex biological defense mechanisms (10). It has also been used to counteract the adverse effects of salt stress in many crop plants (11-13). It has proposed functions in whole plant metabolism (14). Furthermore, experimental studies on different plants have shown that exogenous application of AA may reduce salt-induced adverse effects and results in a significant increment of growth and yield (12,13,15).

Sugarcane is a renewable, natural agricultural resource (16) that accounts for more than 60% of the world's sugar production (17) and provides numerous economically viable by-products (18). More recently, it has become a good source of ethanol biofuel, having the best CO_2 balance (19,20). Sugarcane is moderately sensitive to salinity with reduced crop yield and quality under saline conditions (21). In view of its economic value and low productivity due to increasingly saline conditions, there is an obvious need to explore newer approaches and to test the possible applicability of those that have shown promise in other similar crops towards better crop yield. The bio-saline studies on sugarcane are scanty. Therefore, the present study was designed with the main objective to appraise whether or not the adverse effects of salt stress on sugarcane plants could be mitigated by an exogenous application of AA. It also aimed to find out the relative efficacy of 2 treatment methods of AA, one involving foliar application and the other through irrigation, in terms of growth enhancement under saline conditions.

Materials and methods

Experimental conditions

The pot experiment was conducted at the Seed Centre, Department of Botany, University of the Punjab, Lahore, Pakistan, in a growth tunnel under natural sunlight conditions during March-May 2010. The prevailing average temperature highs and relative humidity during March to May were as given below (Table).

Table. Average temperature and relative humidity during the experimental period.

Months	Average temperature	Average humidity
March	25 °C	55%
April	31 °C	36%
May	34 °C	34%

Plant material and experimental layout

Twenty-day-old field-grown healthy (without any visual symptoms of disease) and equal-sized sugarcane plants (cultivar HSF-240) were procured from the Botanical Garden, University of the Punjab, Lahore, Pakistan, and were transferred to plastic pots (22 × 18 cm) containing 8 kg of sand as 1 plant/pot. Two hundred milliliters of quarter-strength Hoagland's nutrient solution was added to each pot twice a week in order to maintain the fertility status and water requirement of the plants. The treatments were started 3 weeks after the transfer of plants to pots. To study the role of AA (MW 176.2) alone and in combination with salt stress, 8 treatments including the control were tested for the sugarcane plants. Based on our previous experience with in vitro sugarcane cultures (22), 3 different concentrations of AA (0.1, 0.5, and 1.0 mM) and 2 levels of salt (0 and 100 mM NaCl) were selected; hence, the eight treatments were: 1) control (without salt and AA), 2) salt stress (100 mM NaCl), 3) 100 mM NaCl + 0.1 mM AA, 4) 100 mM NaCl + 0.5 mM AA, 5) 100 mM NaCl + 1.0 mM AA, 6) 0.1 mM AA, 7) 0.5 mM AA, 8) 1.0 mM AA. The statistical layout of the experiment was randomized complete block design involving 2 factors (salinity and AA treatments). The plants were divided into 2 groups, one for application of AA through irrigation and the other for application through foliar spray. The set-up consisted of 4 replicates for each treatment.

The nontreated plants (control) were provided with 200 mL of quarter-strength Hoagland's nutrient solution only. Salt stress was imposed by adding a solution of 100 mM NaCl (prepared in quarter-strength Hoagland's nutrient solution) to pots. In the case of treatment through irrigation, 200 mL of respective treatment solution (prepared in quarter-strength Hoagland's nutrient solution) was added to each pot. In the case of treatments by foliar spray, the plants were irrigated with or without 100 mM NaCl and sprayed with different concentrations of AA (0.1, 0.5, or 1.0 mM), prepared by directly adding the required amount of AA in distilled water containing

a few drops of Tween-20 (polyoxyethylene sorbitan monolaurate). Tween-20 was added as a surfactant to ensure penetration of AA into the leaf tissue. The control plants were sprayed with distilled water only. The spraying was done manually using a spraying bottle, on both sides of the leaves evenly. The treatments were given after an interval of 3 days throughout the course of the study (70 days). The pots were also flooded with tap water to flush down all the accumulated salts, as and when required. Termiticide (0.5% solution) was also applied at an interval of 20 days to avoid the attack of termites.

Physical characteristics of sand

The electrical conductivity (EC), total dissolved solids (TDS), and pH of the sand, in relation to the application of different treatments was also measured before and after the completion of the experiment. For this purpose, a sand sample (10 g) from a pot of each treatment was taken randomly. Sand was mixed in a small amount of water and allowed to set for 5 min. Then the water was separated and readings (Figure 1) for EC, TDS, and pH were recorded using EC and pH meters, respectively.

Growth parameters

Shoot lengths and diameters of the plants were recorded before and after 70-day treatments given to plants. Then plants from each pot were uprooted carefully, separated into shoots and roots, washed with tap water to remove any residue, and finely dried with blotting paper. Shoot and root fresh and dry weight, number of off-shoots, and leaf area were recorded. For recording dry biomass, plants were oven-dried at 65 °C for 5 days. Leaves were photographed and leaf area per plant was determined using the Image J program (Rosband, W.S., image J, US National Institute of Health, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/download.html).

Biochemical analysis

For protein and enzyme extraction, a sample of fresh leaves (0.5 g) was homogenized in 1 mL of 0.1 M phosphate buffer, 0.1 g of polyvinylpolypyrollidone (PVP), and 0.5% (v/v) Triton X-100. The resultant slurry was centrifuged at 14,000 rpm at 4 °C for 30 min. The supernatant was carefully separated and used further for quantitative estimation of protein, peroxidase, and superoxide dismutase.

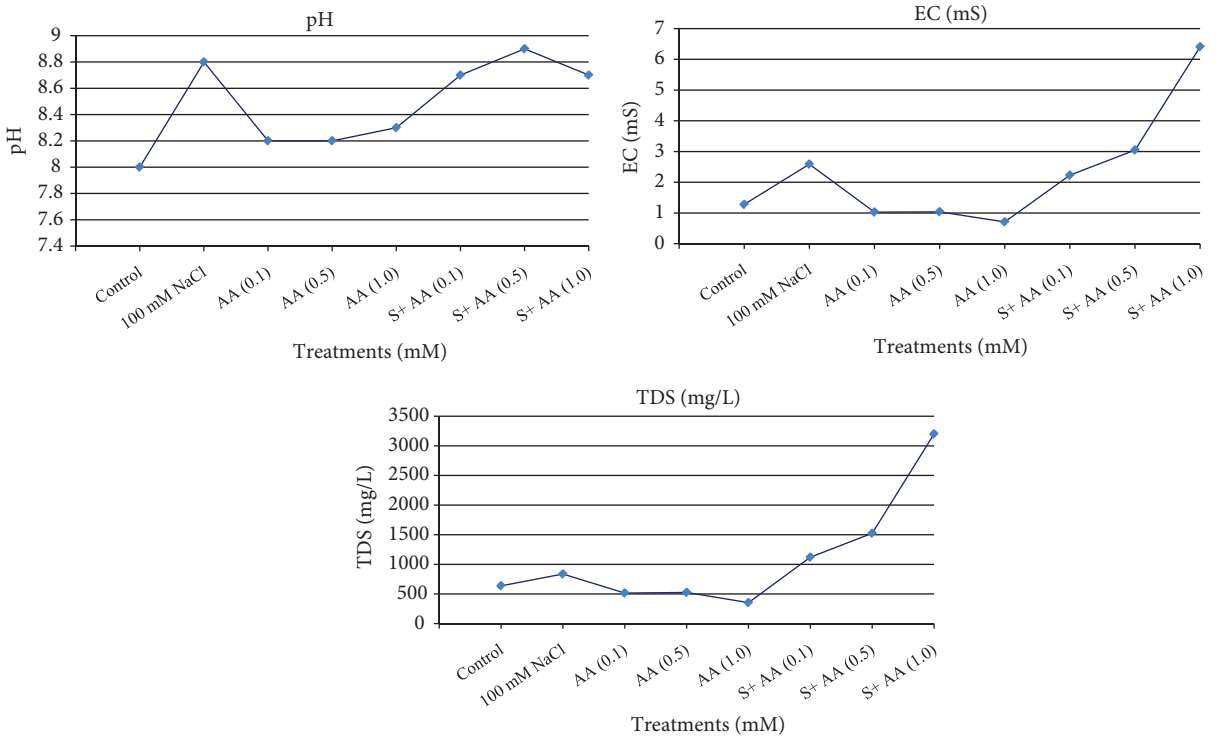

Figure 1. Physical characteristics of sand.

The Biuret method of Racusen and Johnstone (23) was employed for the estimation of soluble protein contents. The optical density was measured at 545 nm using a Hitachi U-1100 spectrophotometer. The protein concentration was determined from the standard curve, which was prepared by using bovine serum albumin.

Activity of peroxidase (E.C 1.11.1.7) was measured using the method proposed by Racusen and Foote (24). Superoxide dismutase (E.C 1.15.1.1) activity was assayed spectrophotometrically by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT), according to Maral et al. (25). SOD activity was expressed as U/mg of protein.

Proline was estimated according to the method of Bates et al. (26). Briefly, a sample of 0.5 g fresh leaf tissue was homogenized in 10 mL of 3% sulfosalicylic acid and the homogenate was centrifuged at 13000 rpm for 10 min at 4 °C. Then 2 mL of the supernatant was mixed with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid. This mixture was incubated at 100 °C for 1 h and then cooled at room temperature. Finally, 4 mL of toluene was added. Proline was extracted from the toluene layer and its absorbance was noted at 520 nm using toluene as a blank or reference. The proline concentration was determined from a standard curve.

Statistical analysis

The data were statistically analyzed with one way analysis of variance using SPSS (version 12.0.0). The mean values were recorded at 0.05% probability level. The experiment was repeated twice.

Results

Growth parameters

A positive response from exogenous application of AA was recorded for both shoot length as well as its diameter. Maximum increases in shoot length and diameter were recorded respectively at 0.5 mM AA applied through irrigation and at 1.0 mM AA applied as foliar spray, under both salt stressed and nonstressed conditions. Shoot length of salt-treated control plants was 16.21 cm, which increased to 33.37 cm when 1 mM AA was applied as foliar spray. The shoot diameter was 3.45, 3.65, and 3.85 mm when salt treatment was supplemented with foliar spray of AA at 0.1, 0.5, and 1.0 mM levels, respectively, in comparison with 1.87 mm when treated with 100 mM NaCl only (Figure 2a, b).

The number of off-shoots was generally higher in those plants treated with AA through irrigation as compared to foliar spray (Figure 2c). However, the overall effect of AA on number of off-shoots was not statistically significant.

The leaf area per plant was significantly reduced under salt stress, while AA applications markedly improved the inhibitory effects of salt on plants. The leaf area of salt-stressed plants was 90 cm^2, which showed an increasing trend in plants with AA supply through irrigation treatment. The maximum recorded value was 205 cm^2 at 1.0 mM AA level (Figure 2d). Likewise, in the case of foliar spray, a gradual increase in leaf area was observed with increasing AA levels. When salt treatments were supplemented with foliar spray of AA (1.0 mM) the leaf area increased from 96 cm^2 to 173 cm^2.

Salinity had a detrimental effect on shoot fresh/dry weights of sugarcane plants. The exogenous application of all 3 levels of AA (0.1, 0.5, and 1.0 mM) significantly increased the biomass production. The effect of AA treatments either through irrigation or foliar spray on shoot fresh weights was almost the same but in the case of dry weights the effect of irrigation was quite pronounced as compared to foliar spray at all the tested concentrations (Figure 3a, b).

Under salinity stress, a decrease in root biomass compared with the control was observed. In the case of irrigation of salt-treated plants with AA, only 0.5 and 1.0 mM AA improved the root biomass, while a lower concentration (0.1 mM) was not effective. However, this increase in root biomass by AA application was statistically nonsignificant as was the case with the foliar application of AA (Figure 3c, d).

Protein contents

Implementation of salt stress resulted in increased soluble protein contents, while AA application did not cause any further increase in this sugarcane cultivar. AA treatment with or without salt stress

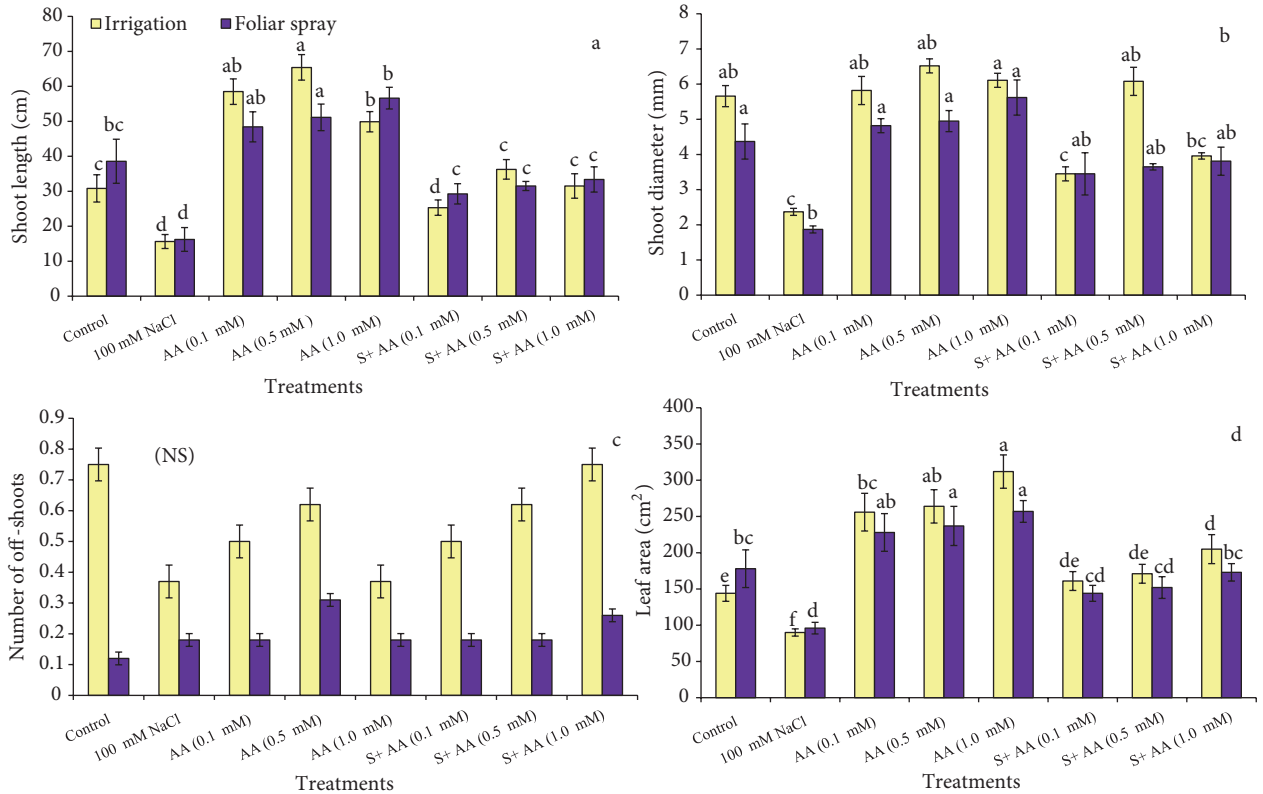

Figure 2. Comparative effect of irrigation and foliar spray of ascorbic acid (AA) on (a) shoot length, (b) shoot diameter, (c) number of off-shoots, and (d) leaf area of 100 mM NaCl-stressed (S) or nonstressed sugarcane plants. Different letters on bars indicate their relative significance at 0.05% probability level. NS indicates that values are statistically nonsignificant.

in both application modes in fact resulted in values always lower than those obtained with 100 mM NaCl although the difference was small in most cases. When salt-stressed plants were foliar-sprayed with AA at the concentrations of 0.1, 0.5, and 1.0 mM, the soluble protein contents were 0.33, 0.29, and 0.32 mg/g, respectively, in comparison with 0.37 mg/g when treated with NaCl only (Figure 4a). After irrigation with AA, these values were 0.30, 0.24, and 0.31 mg/g in comparison with 0.39 mg/g of salt stressed plants at 0.1, 0.5, and 1.0 mM AA.

Antioxidant enzyme activities

A considerable increase in the peroxidase activity of sugarcane plants was observed with AA application (Figure 4b). POD activity of plants maintained at 100 mM NaCl level was 0.16 mg/g, which slightly increased to 0.17 mg/g and 0.20 mg/g tissue after AA application through irrigation at the concentration of 0.1 mM or 0.5 mM, respectively. However, at 1.0 mM AA concentration, the POD activity decreased to 0.15 mg/g. A similar trend of POD activity was also quite apparent in the case of foliar application of AA to NaCl-treated plants.

The superoxide dismutase activity of sugarcane plants was also considerably affected by the application of various levels of AA. Under irrigation, AA at a concentration of 0.1 mM resulted in a minor change in the SOD activity of NaCl-treated plants. Afterwards, an increase in SOD activity up to 71.03 and 61.50 U/mg from 43.78 U/mg (SOD of salt-stressed plants) was observed at 0.5 and 1.0 mM AA, respectively (Figure 4c). In the case of foliar spray under salt stress, 0.1 mM AA resulted in a decrease in SOD activity compared with the saline control. However, an increase up to 98.84 and 77.69 U/mg from 65.23 U/mg (SOD of salt stressed plants) was obtained at 0.5 mM and 1.0 mM AA concentration, respectively.

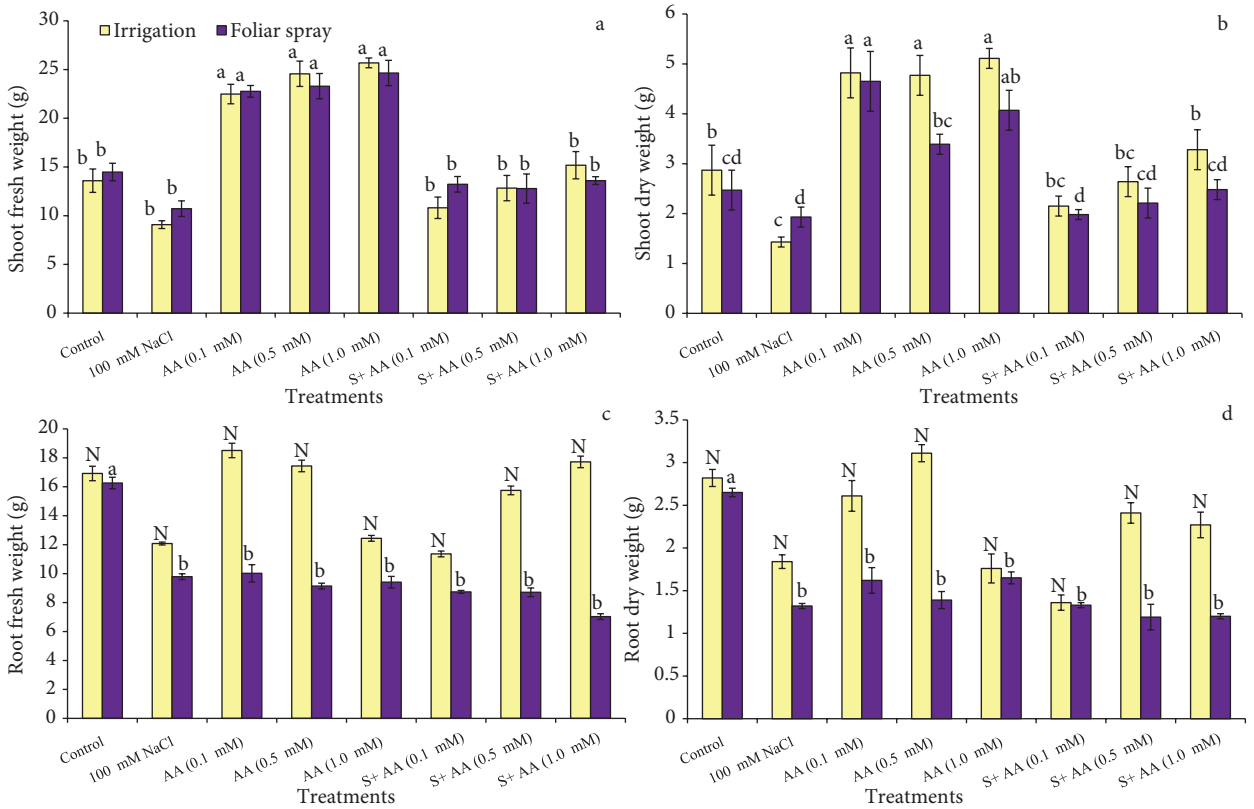

Figure 3. Comparative effect of irrigation and foliar spray of ascorbic acid (AA) on shoot and root fresh and dry weights of 100 mM NaCl-stressed (S) or nonstressed sugarcane plants. Different letters on bars indicate their relative significance at 0.05% probability level. N indicates the values that are statistically nonsignificant.

Proline contents

Proline contents increased under salinity stress and a further increase was observed after AA application. The proline contents of plants maintained at 100 mM NaCl level were 19.12 µmol/g, which increased to 21.25, 25.72, and 31.02 µmol/g after irrigation of AA at the concentrations of 0.1, 0.5, and 1.0 mM, respectively. The foliar spray of AA also considerably affected the proline contents of sugarcane plants. The highest proline contents (48.45 µmol/g) were recorded in plants maintained at 100 mM NaCl level after foliar spray with AA at the concentration of 1.0 mM, followed by 39.35 µmol/g in salinized plants foliar sprayed with 0.5 mM AA. It was observed that the plants that were subjected to foliar spray with different levels of AA generally had greater proline contents compared to those maintained under AA treatments through irrigation (Figure 4d).

Discussion

The present study explores the ameliorative effect of AA on pot-grown sugarcane plants (*Saccharum* spp. hybrid cv. HSF 240) under saline conditions. Results of our investigation showed that salt stress attenuated all the studied growth parameters (shoot length, shoot diameter, leaf area, number of off-shoots, fresh/dry weights). These inhibitory effects of salt stress on plant growth and biomass production are well known. Vasantha et al. (27) reported reductions in leaf area, dry weight, and juice quality of sugarcane cultivars under NaCl stress. Choudhary et al. (28) reported yield losses in sugarcane under saline conditions. Similarly, salinity adversely affected the leaf area in rice (29) and germination rate in wheat plants (30). Reduction in the rate of plant growth under salt stress is probably due to the accumulation of high amounts of toxic salts in the leaf and other tissues, which leads to

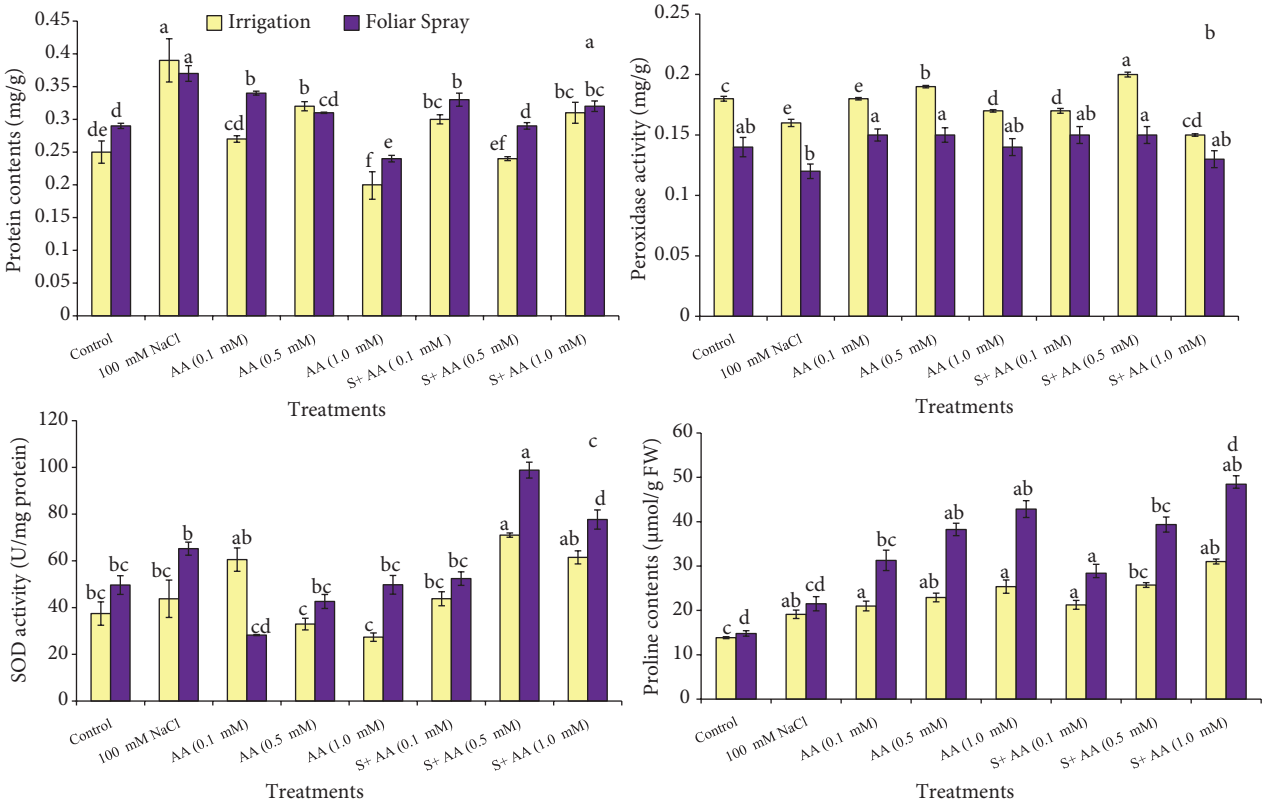

Figure 4. Comparative effect of irrigation and foliar spray of ascorbic acid (AA) on (a) soluble protein contents, (b) peroxidase, (c) superoxide dismutase activities, and (d) proline contents of 100 mM NaCl-stressed (S) or nonstressed sugarcane plants. Different letters on bars indicate their relative significance at 0.05% probability level.

dehydration and turgor loss, and eventually death of leaf cells and tissues (31).

In the present study, the exogenous application of AA appreciably increased the growth of sugarcane plants under both salt-stressed and nonstressed conditions as indicated by the studied growth parameters. These results are in agreement with some earlier findings in which external supply of AA resulted in a significant improvement in the salt tolerance of wheat (15,32) and sorghum plants (33). It was observed during the present study that shoot length, shoot diameter, and leaf area of sugarcane plants were considerably increased after AA application. In an earlier study, AA application greatly increased the leaf area, stem height, and diameter of *Khaya senegalensis* (34). The AA-induced growth improvement as observed for the sugarcane plants during the present investigation might be due to an AA-induced increase in leaf area, which is the photosynthesizing tissue of the plants. As a result, photosynthesis, which is a major controlling factor for plant growth (35), might have been increased. Another reason for increased growth may be the implication of AA in regulation of cell growth and cell division by influencing the cell cycle (36).

In the present study, external supply of AA to sugarcane plants appreciably enhanced the fresh and dry weights of plants. El-Tohamy et al. (37) found the same results under salinity stress in the case of foliar spray of AA on *Solanum melongena* L. The biomass production of *Brassica* plants was also improved after AA treatments (13). The results of our investigation for sugarcane plants are thus in agreement with these previous reports. However, no significant improvement in root biomass of sugarcane plants was observed after AA application. This might suggest a differential response of different plant organs to exogenous application of AA.

During the present investigation, 2 ways of AA application were tested and it was observed that

the AA application through irrigation caused better growth enhancement in sugarcane plants as compared to foliar spray, under both salt-stressed or control conditions. Foliar applications of growth regulators in sugarcane generally showed only minor absorption and translocation within the leaf as compared to additions through the rooting medium. These results are consistent with the findings published by Athar et al. (15). They reported that among the various ways of application the supply of AA through rooting medium gave the best results in induction of salinity tolerance in wheat plants. In contrast to our findings, Kaya et al. (8) reported that foliar spray was a better choice in minimizing the adverse effects of salinity in maize plants. Similarly, Arafa et al. (33) observed that seed pre-soaking plus foliar spraying proved to be helpful in improving growth of *Sorghum* seedlings. Besides these promising studies, it has also been shown by Khan et al. (5) that foliar spray of AA did not improve the growth of wheat plants under salt stress. Our results thus suggest focus on AA application through irrigation that has resulted in better growth parameters suggesting an efficient take-up of exogenous AA. Enhanced endogenous AA level due to root applied AA has earlier been suggested to protect wheat plants from salt-induced oxidative damage by controlling cellular redox state (32).

It was observed that among the various concentrations of AA used during the present experiment, 0.5 mM AA resulted in more growth enhancement under irrigation, while in the case of foliar spray 1.0 mM AA gave better results. Jayachandran et al. (38) found that 0.5 mM AA was the most effective treatment in reducing the effects of salinity in rice. In maize plants, it has been reported by Kaya et al. (8) that effect and efficiency of foliar spray of growth regulators (kinetin and indoleacetic acid) increased with increasing concentrations and 2 mM was the best. Comparison of the results from this study with earlier ones supports the viewpoint that effectiveness of growth regulators is also concentration dependent.

It was also observed during the present study that the protein contents of sugarcane plants were increased due to imposition of salt stress. Under salinity the plants produce stress-responsive proteins that are involved in detoxification of ROS and thus play a role in adaptation to stress (39,40). These stress responsive proteins may be synthesized de novo or may be present constitutively at low concentration and increase when plants are exposed to stress (41). Based on our results we were not able to demonstrate a clear association between exogenous AA supply and soluble protein contents in this sugarcane cultivar although considerable changes in other specific proteins and enzymes (as mentioned below for POD and SOD) were clearly observed. This observation perhaps indicates that changes in specific proteins and enzymes may not be quite reflective in terms of total soluble protein contents in this cultivar. It may also reflect no or a limited role of AA in the overall protein synthesizing ability in a particular plant species. One may not rule out the possibility of production of some inhibitors of protein synthesis under such circumstances (42). In contrast to this, it has been widely reported that the protein contents were increased after exogenous application of AA in potato (43) and chickpea (11) and after gibberellic acid application in *Sorghum* plants (44). It is well documented that the cellular protein contents of plants undergo variations under stress conditions (45,46). Wimmer et al. (47) reported that changes in endogenous levels of soluble protein contents are due to the structural modifications of the cells under stress conditions. These variations in protein contents may, however, depend on the plant species or cultivar (41).

In the present investigation, activities of antioxidant enzymes (POD and SOD) in sugarcane plants were increased under salt stress as well as after AA application. These higher levels of antioxidant enzymes might be attributed to their property to help develop the plant's resistance against oxidative damage. Athar et al. (15,32) reported an increase in antioxidant enzyme activities in wheat plants after AA application. Earlier work suggested that an increase in the activity of antioxidant enzymes helps the plants to maintain their growth under stress conditions and may be regarded as an indicator of salinity tolerance (48,49). Plants containing higher concentrations of antioxidants show more resistance to the oxidative damage caused by salt stress (50). Hence an increase in the activities of antioxidant enzymes of sugarcane plants as observed during the present work is in line with these earlier findings.

During the present work, the proline contents of sugarcane plants showed an increasing trend after AA application under salt stress. Similar results have been reported in sorghum (51), maize (52), rice (53), and tobacco (54) where exogenous application of various growth regulators increased the proline contents of plants. It has been suggested that plants accumulate higher amounts of proline as a protective strategy against stressful conditions (55,56). The accumulation of proline associated with stress might serve as a compatible solute in order to maintain the osmotic balance and allows the plants to acclimatize to unfavorable environments (57,58). Proline also acts as a component of the antioxidative defense system rather than merely as an osmotic adjustment mediator (59).

Conclusion

It is clear that adverse effects of salt stress on growth and biochemical attributes of pot-grown sugarcane plants were significantly improved by exogenous application of AA. The AA might overcome the destructive effects of salinity by increasing the endogenous levels of antioxidant enzymes and proline, which in turn was reflected in improved growth. The AA application by either way (foliar spray or irrigation) mostly led to a substantial increase in values of almost all the studied growth and biochemical parameters. However, the application of AA through irrigation was more effective than application through foliar spray. This study justifies further work on sugarcane plants under a broader range of field conditions to further evaluate the possibility of using AA for improving the growth and yield of sugarcane on a larger scale.

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