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Research Article
Non-enzymatic Anti-oxidants Potential in Enhancing *Hibiscus sabdariffa* L. Tolerance to Oxidative Stress

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Abstract

Background and Objectives: In scope of roselle plant salinity tolerance, saline water irrigation is a principle cause of reduction in biomass and productivity. This study aimed to investigate the influence of three different non-enzymatic anti-oxidant protectants, i.e., ascorbic acid (AsA), citric acid and thiamin, applied as foliar spray, in ameliorating the adverse effects of salinity *on Hibiscus sabdariffa* L. var. Dark Red Calyces exposed to 0 and 75 mM NaCl. **Materials and Methods:** Pot experiment was conducted at the open field of the Experimental Farm of Agricultural Botany Department, Faculty of Agriculture, Ain Shams University, Qalyubia, Egypt, during the two successive seasons of 2016 and 2017. Growth parameters, yield components, leaf relative water content, membrane stability index and malondialdehyde and the activities of some anti-oxidants were determined. **Results:** Salt stress markedly decreased plant fresh and dry biomasses, leaf area, leaf fresh biomass, number of fruits, fruits dry biomass and calyces dry biomass/plant beside the loss of color in dry calyces while shoot length, root length and number of leaves relatively unaffected by exposure to salinity. Salinity also alleviated the anti-oxidant defense system in term of total soluble phenols, flavonoids, reduced glutathione (GSH) concentrations, superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and phenyl alanine ammonia lyase (PAL) activities and proline as osmoregulator. Exogenously applied AsA, citric acid and thiamin improved both growth and production in roselle plants under the two different concentrations of salinity; 0 and 75 mM NaCl. This improvement was found to be associated with increasing leaf relative water content, membrane stability index, total soluble phenols, flavonoids, proline, GSH concentrations in leaves and anthocyanins in dry calyces. On contrast, significant reduction was showed in malondialdehyde concentration when plants treated with foliar applications in compared with unsprayed plants and altered the activities of SOD, CAT, POD and PAL anti-oxidant enzymes due to balancing of the cell homeostasis under salt oxidative stress. **Conclusion:** The results of this study proved that AsA, citric acid and thiamin had antioxidant potential to improve roselle plant tolerance against salt stress.

Key words: *Hibiscus sabdariffa* L., roselle, salinity stress, ascorbic acid, citric acid, thiamin, anti-oxidant defense system

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Salinity considered one of the main abiotic stresses that seriously limit crop production in arid and semi-arid regions. Investigators reported that about 8% of total earth land and 20% of arable areas are suffered from high salinity[1]. The salt is engulfing the soil surface day by day. In Egypt, the poor drainage system of soil and using of underground water causes a big problem for agriculture in form of soil salinization to the cultivated areas[2]. Oxidative stress is a common effect of hazard environmental conditions including water and soil salinity[3]. Salinity presents two main problems: decrease in the osmotic potential of the soil solution and accumulate of Na^+ and Cl^- ions which cause toxicity at high concentrations and decrease the absorption availability of the essential elements[4] such as K^+ and Ca^{2+}. One of the consequences of salt stress is reduction in the stomatal conductance which leads to limit CO_2 availability and causes decrease in $NADP^+$ that is important for light reaction[3]. Tausz *et al.*[5] added that leakage of electron from ferredoxin occurs, resulting in over production of super oxide anion radicals (O_2^-). Then, the O_2^- may be protonated to hydroperoxyl radicals (HO_2) that converted automatically into hydrogen peroxide (H_2O_2) through the fentone reaction[4]. Excess reactive oxygen species (ROS) take place under stress by impairing the electron transport chain within cellular compartments as chloroplast and mitochondria[6]. Plants combat this osmotic stress through accumulation of osmolytes as proline in the cell cytosol in order to low osmotic potential and sustain absorption of water from saline soil solution[7] or induced salt tolerance by a complex antioxidant system which act as free-radical scavenger[8].

Cultivated *Hibiscus sabdariffa* L. (Roselle) has multiple purposes around the world. In tropical zone and north of Africa, roselle cultivated for its flowers calyces that have been used like tea in pharmaceutical and traditional medicine through its role as anti-cancer, hepatoprotective, anti-hyperlipidemic, anti-oxidant and has been used against hypertension[9]. Roselle calyces rich in anthocyanins, L-ascorbic acid, citric acid, β-carotene, malic acid, hibiscetin, cyanidin-3 rutinoside, quercetin, galactose, polysaccharide, stearic acid and wax[10]. Also, it has a high value of fiber yield[11].

In recent years, one of the main methods to resolve the problem of soil salinity is using exogenous protectants such as osmo-protectants, plant hormones, anti-oxidants, signaling molecules, polyamines and trace elements which have been found to be effective in mitigating the salt induced damage in plant[12]. Rasool *et al.*[1] stated that enzymatic and non-enzymatic anti-oxidative system such as SOD, CAT, POD, ascorbate peroxidase, glutathione reductase, phenolic compounds, ascorbate, glutathione, thiamin and citric acid can counteract the toxic effect of ROS.

Ascorbic acid (vitamin C) is naturally synthesizes in higher plants and plays a main role as a key product of D-glucose metabolism[13]. Zauberman *et al.*[8] reported that exogenous application of AsA through the rooting medium or foliar spray or as seed priming has been implicated in improving cell division and cell enlargement under salinity and control conditions. Furthermore, AsA concerns one of the main important anti-oxidant that protect plants from oxidative stress by regulating complex sequences of biochemical reactions such as activation or suppression of various key enzymatic reactions, induction of stress responsive protein synthesis and production of various chemical defense compounds[14] beside its role in ascorbate-glutathione cycle through donating or losing electrons to produce the reducing form and regulating of flowering and senescence[15].

Citric acid is one of the tri-carboxylic acid intermediate compounds which confirmed to be the source of carbon skeleton, cellular energy and utilized the respiratory cycle and some biochemical pathways[16]. Citric acid has been reported to be majorly served as an anti-oxidant organic acid that involved in defense pathway as response to salt stress tolerance[17].

Thiamin (vitamin B_1) plays essential roles in NADPH and ATP biosynthesis, formation of nucleic acids, carbohydrates metabolism, pentose phosphate pathway in calvin cycle and branched chain amino acid biosynthesis due to its role in intermediary metabolism in addition to its function as a cofactor for synthesis of some critical enzymes such as pyruvate and decarboxylase. Also, it has been well reported that thiamin was activated as a result of plant adaptation responses for salt stress to maintain the internal cell homeostasis[18].

Therefore, the current study was designed to investigate the adverse effects of salt stress on reducing roselle plants growth and calyces production and how to overcome these negative effects through treatment plants with three different non-enzymatic antioxidants, i.e., ascorbic acid (AsA), citric acid and thiamin which can be made effective management strategies to enhance plant tolerance under salt stress.

MATERIALS AND METHODS

Pot experiment was conducted at the open field of the Experimental Farm of Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima, Qalyubia Governorate, Egypt (30°06 42"N 31°14 46"E) during the two successive seasons of 2016 and 2017 to investigate the influence of foliar spraying

by some non-enzymatic anti-oxidant protectants i.e., ascorbic acid (AsA), citric acid and thiamin (vitamin B_1) on enhancing NaCl stress tolerance of roselle plants.

Plant material and culture condition: Seeds of roselle (*Hibiscus sabdariffa* L. var. Dark red calyces) were gently obtained from the Aromatic and Medicinal Plant Research Institute, ARC, Ministry of Agriculture, Egypt. Ten seeds were sown on 1st of May, 2016 and 2017 in plastic pots (35 cm deep and 25 cm diameter) filled with 15 kg air-dried sandy loamy soil; pH (1:2 water, v/v) was 7.15, EC = 0.98 dS m^{-1}. The sand was washed with HCl 1% (v/v) before mixing with clay loamy soil (2 sand:1loam soil) to displace any cations off and then rinsing with water to get rid of Cl^-. Ten seeds were sown in each pot, then pots were irrigated directly with tap water to keep seeds enough wetted until germination. After two weeks from planting, seedlings were thinned out to three uniform plants in each pot. Before the initiation of salinity treatment, plants were left under natural conditions for further one month from planting to make them established well.

Foliar sprays and salt stress applications: Pots were divided into 2 main plots, each plot divided also into 4 sub-plots. After a growing period of 21 days from planting, foliar sprays of ascorbic acid; AsA (10 mM)[19], citric acid (10 mM)[16] and thiamin (10 mM)[18] were estimated separately with a hand sprayer on grown plants four times, with one week interval in the sub-plots. Plants sprayed with distilled water were applied as control. Tween20 at 0.01% was added each time to the spraying solutions as a wetting agent. Solutions were sprayed to all plant surfaces until the point of runoff. At 30 days from planting, the two main plots of pots were subjected to 2 salt stress treatments via irrigation water as: control plants (without NaCl) and 75 mM of NaCl as a salt stress[20]. When plants needed water, the volume of irrigated saline water applied each time to the root zone in each pot ranged from 1-4 L depending on the size of plant with time. All treatments were laid out in a split plot design with three replicates for each treatment. Each replicate included 3 pots. Standard cultivation practices, i.e., culture conditions, soil type, fertilization, volume and time of irrigation, weeding and disease and pest control programs were followed during the entire plant development according to the Egyptian Ministry of Agriculture recommendations.

Growth parameters: Soon at 100 days from planting plant samples were uprooted carefully from pots, shoot and root length (cm), number of leaves/plant and plant fresh biomass (g) were measured immediately before any morphological changes occurred. Also, the 4th full expanded leaves were used to measure the leaf area (cm^2) and leaf fresh biomass (g). The ratio of leaf area/leaf fresh biomass was calculated. Another plant samples were dried in air-forced-draft oven at 70°C until achieving the constant biomass to record the plant dry biomass[21].

Yield components: Three plants per treatment were harvested randomly at the end of the two seasons (150 days old). Total fruits per plant were collected to estimate the number of fruits/plant, air dried fruits biomass (g)/plant and the yield of dry calyces biomass (g)/plant.

Leaf water status measurement: Leaf relative water content (LRWC) was proceeded at 50 days old according to the method of Kaya *et al.*[22]. The method was mentioned previously in Abdellatif[23].

Determination of membrane stability index (MSI): Membrane stability index (MSI) was determined at 50 days old to study salt stress injury on membrane stability by the method described by Sairam *et al.*[24]. MSI was calculated as:

$$\text{MSI (\%)} = \frac{\text{1- EC1}}{\text{EC2}} \times 100$$

where, EC1 is the initial electrical conductivity (EC) at 30°C and EC2 is the EC at 100°C, the tubes were leaved until the temperature brought down to 30°C. The EC was determined with Electrical Conductivity meter (HANNA, H199301).

Phytochemical constituents: At 50 days old, leaf samples were washed and weighted, then transferred directly in liquid nitrogen until stored under -80°C.

Determination of total soluble phenolic compounds: Phenols concentration was estimated by the method depends on the reduction of Folin-Ciocalteau reagent by the phenolic compounds due to a concomitant appearance of a blue complex as described by Singh *et al.*[25]. The absorbance was recorded at 765 nm. The records were compared by the standard curve prepared with gallic acid. The total soluble phenols concentration was expressed as mg of gallic acid g^{-1} f.wt.

Determination of total flavonoids: The total flavonoids were determined by the aluminium chloride colorimetric assay as

described by Marinova *et al.*[26]. The absorbance was determined at 510 nm. The total flavonoids were calculated from a calibration curve as mg rutin g^{-1} f.wt.

Determination of proline: Proline concentration was determined calorimetrically as an organic osmolyte at 520 nm using the method described by Bates *et al.*[27] based on the reaction between proline and ninhydrin reagent. The records were calibrated for each measurement with the standard proline solutions and expressed as µg g^{-1} f.wt.

Determination of reduced glutathione (GSH): The GSH concentration on reacted with 5,5'-dithiobis nitro benzoic acid was proceeded according to the method of Ellman[28]. The concentration of glutathione was calculated as µg GSH g^{-1} f.wt.

Determination of malondialdehyde (MDA) concentration: Status of membrane leakage due to lipid peroxidation under salt stress was appraised by determining the MDA concentration; a product of lipid peroxidation, according to the procedure of thio-barbituric acid[29]. The determination was measured at 532 nm using 0.5% TBA as a control. The MDA concentration was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol MDA g^{-1} f.wt.

Determination of anthocyanins: Total anthocyanins concentration in 1 g dry calyces, for each sample was determined using the procedure of Connor *et al.*[30] in which each extract was diluted (5:95, v/v) in mixture of HCl 1% (v/v) and methanol to obtain an absorbance between 0.500 and 1.000 at 530 nm. The values were expressed as mg cyanidin-3-glucoside equivalents per g dry wt. using a molar extinction coefficient of 27.900.

Anti-oxidant enzymes assay

Preparation of crude enzymes: About 1 g frozen leaf tissues, kept under -80°C were ground in mixture of 0.1 M cold potassium phosphate buffer (pH 7.0) and polyvinylpyrrolidone 1% (w/v). Then the mixtures were centrifuged under cooling at 15000×g for 15 min. The crude enzymes were carried out immediately at -80°C.

Superoxide dismutase (SOD); EC 1.15.1.1; activity assay was based on the method described by Beyer and Fridovich[31]. One unit of SOD enzyme activity was defined as the amount of enzyme required to suppress 50% of nitro blue tetrazolium reduction at 560 nm.

The activity of catalase (CAT); EC 1.11.1.6; was measured at 220 nm by an assay of hydrogen peroxide (H_2O_2) based on formation of its stable complex with ammonium molybdate[32].

Guaiacol peroxidase (POD); EC 1.11.1.7; activity was measured using the method described by Hammerschmidt *et al.*[33]. One unit of the enzyme was considered as the amount of the enzyme that was responsible for the increase in optical density value of 0.01 at 470 nm.

Phenylalanine ammonia lyase (PAL); EC 4.3.1.5; activity was assayed according to Lister *et al.*[20]. One unit of the enzyme was defined as increase in absorbance of 0.01 per h at 290 nm.

The specific activities of all previous enzymes were expressed as unit mg^{-1} of soluble protein min^{-1}. All biochemical measurements were performed with three replicates by using spectrophotometer UV 9100 B, LabTech.

Soluble protein estimation was determined in the crude enzyme extract according to Bradford[34] with Bovin Serum Albumin as a standard to calculate the specific activity of the anti-oxidant enzymes activity.

SDS-PAGE protein electrophoresis: Separation of leaf tissue protein was performed by using Sodium DodicylSulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to the method of Laemmli[35].

Statistical analysis: All data was analyzed by two-way ANOVA (salt x foliar sprays) using STATISTIX 8th ed. Software by Steel *et al.*[36]. The mean of each treatment was compared with its corresponding control by using the Duncan test at $p \leq 0.05$ where significant differences were given different asterisk for salinity, capital letters for foliar applications and lowercase letters for their interactions following to the method of Gomez and Gomez[37]. Standard deviation of each mean was calculated.

RESULTS

Interesting in this research, non-enzymatic anti-oxidative compounds may have an essential effect on *Hibiscus Sabdariffa* under salinity stress condition.

Effect of AsA, citric acid and thiamin on some vegetative growth parameters under salt stress: Data in Fig. 1-3 referred to significant reduction in the investigated growth parameters in term of shoot length, root length, number of leaves, plant fresh and dry biomasses, leaf area and leaf fresh biomass under salt stress. Foliar application with ascorbic acid

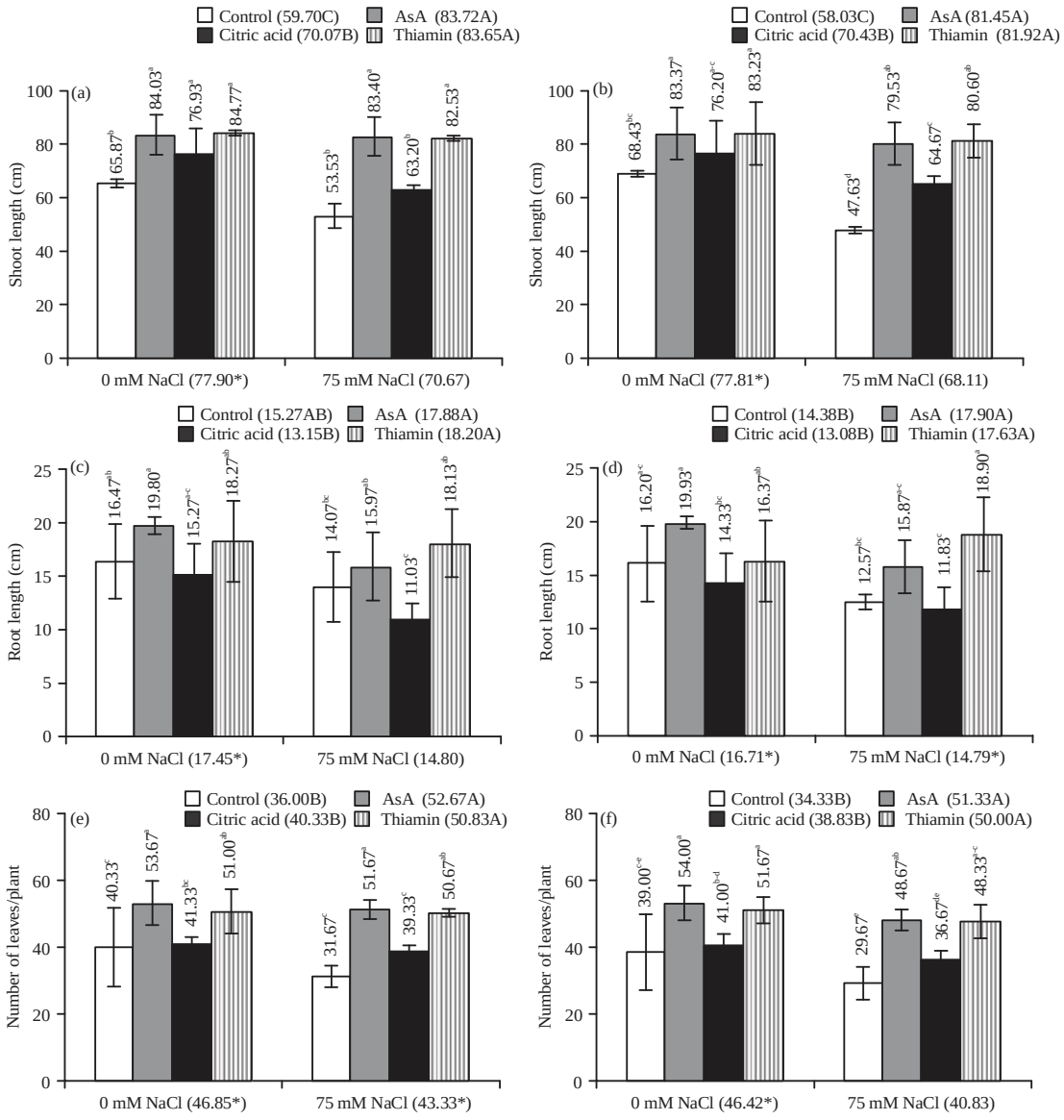

Fig. 1(a-f): Effect of salinity stress (0 and 75 mM NaCl), foliar applications (AsA, citric acid and thiamin at 10 mM) and their interactions on some growth parameters of roselle plants during the seasons of (a, c, e) 2016 and (b, d, f) 2017

Data revealed significant differences between treatments if means were marked with asterisk for salinity, capital letters for foliar applications and lowercase letters for their interaction according to Duncan's multiple range test at 5% level of probability

(AsA), citric acid and thiamin (vitamin B_1) improved these previous traits. AsA and thiamin showed better responses in improving growth more than citric acid. On the other hand, a significant increase was recorded in the ratio of leaf area/leaf fresh biomass under salt stress, at both seasons, in comparing to non-stressed plants. Whereas, spraying with non-enzymatic anti-oxidants reduced this ratio as a result of increasing the leaf fresh biomass. The interaction between salinity stress and foliar applications showed insignificant reduction in most parameters of growth between control plants (non-sprayed) exposed to saline or non-saline treatments. Foliar applications with the three tested antioxidant protectants improved growth characters under 0 and 75 mM NaCl.

Effect of AsA, citric acid and thiamin on plant yield and its components under salt stress: Significant reduction in number of fruits/plant and fruits dry biomass/plant were detected under saline stress. These reductions were positively stimulated by the different treatments of non-enzymatic antioxidants under both conditions of salt treatments, which consequently led to enhance yield of dry calyces/plant as shown in Fig. 4.

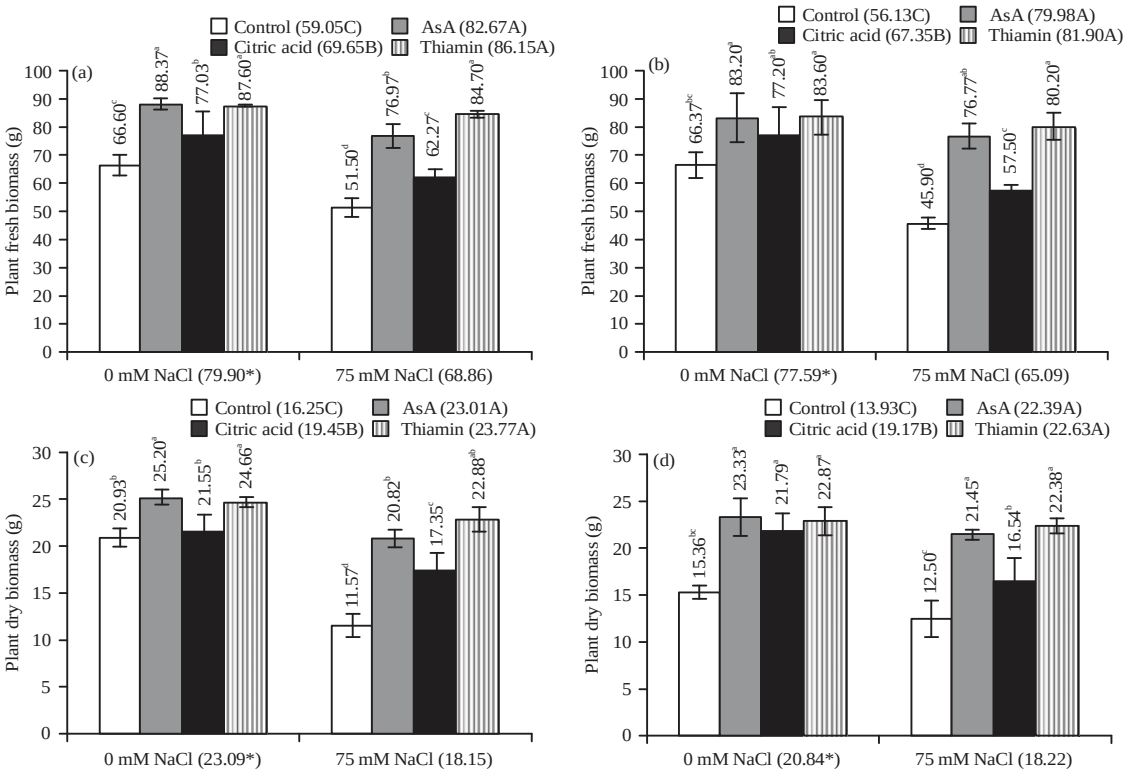

Fig. 2(a-d): Effect of salinity stress (0 and 75 mM NaCl), foliar applications (AsA, citric acid and thiamin at 10 mM) and their interactions on some growth parameters of roselle plants during the seasons of (a, c) 2016 and (b, d) 2017

Data revealed significant differences between treatments if means were marked with asterisk for salinity, capital letters for foliar applications and lowercase letters for their interaction according to Duncan's multiple range test at 5% level of probability

Effect of AsA, citric acid and thiamin on LRWC and MSI under salt stress: As shown in Fig. 5; 75 mM of NaCl induced a significant reduction on LRWC and MSI at both seasons. Foliar applications with the three different anti-oxidants improved both of LRWC and MSI and reduced the negative effect of salt stress to levels being significantly higher than those at non-stressed plants, especially with thiamin treatment.

Effect of AsA, citric acid and thiamin on soluble phenols, flavonoids and proline concentrations under salt stress: Data in Fig. 6 revealed that, except total soluble phenolic compounds at the first season, total soluble phenols, flavonoids and proline were significantly increased as a result of saline condition when compared with un-stressed plants in both seasons. The foliar applications with AsA, citric acid and thiamin resulted in more increasing in the previous measurements under both concentrations of NaCl; 0 and 75 mM. These increments were more pronounced in flavonoids concentration with thiamin foliar spraying under saline stress. In the same time, citric acid alleviated the maximum increment in total soluble phenolics and proline accumulation.

Effect of AsA, citric acid and thiamin on glutathione and MDA concentrations in leaves and anthocyanin in calyces under salt stress: Data in Fig. 7 showed that the mean values of reduced glutathione (GSH) significantly increased in salt treatment. Thiamin supplementation was noticed to confer a significant increase in GSH over un-sprayed plants, whereas GSH concentration was reduced when plants treated with AsA or citric acid. Also, it seemed that exogenous spray with AsA, citric acid and thiamin enhanced the concentration of GSH under salt stress relative to control plants at both seasons.

Lipid peroxidation in leaf tissues was expressed by MDA concentration, the final product of membrane lipids peroxidation as shown in Fig. 7. Significant increase was detected in the production of MDA in plants exposed to saline condition as a result of salt stress. It was noticed an inverse relationship between the increase in MDA concentration and the decrease in MSI (Fig. 5). Foliar spraying of all non-enzymatic anti-oxidant tested compounds showed a decreasing trend in MDA concentration. The maximum reduction was recorded with thiamin followed by AsA and citric acid.

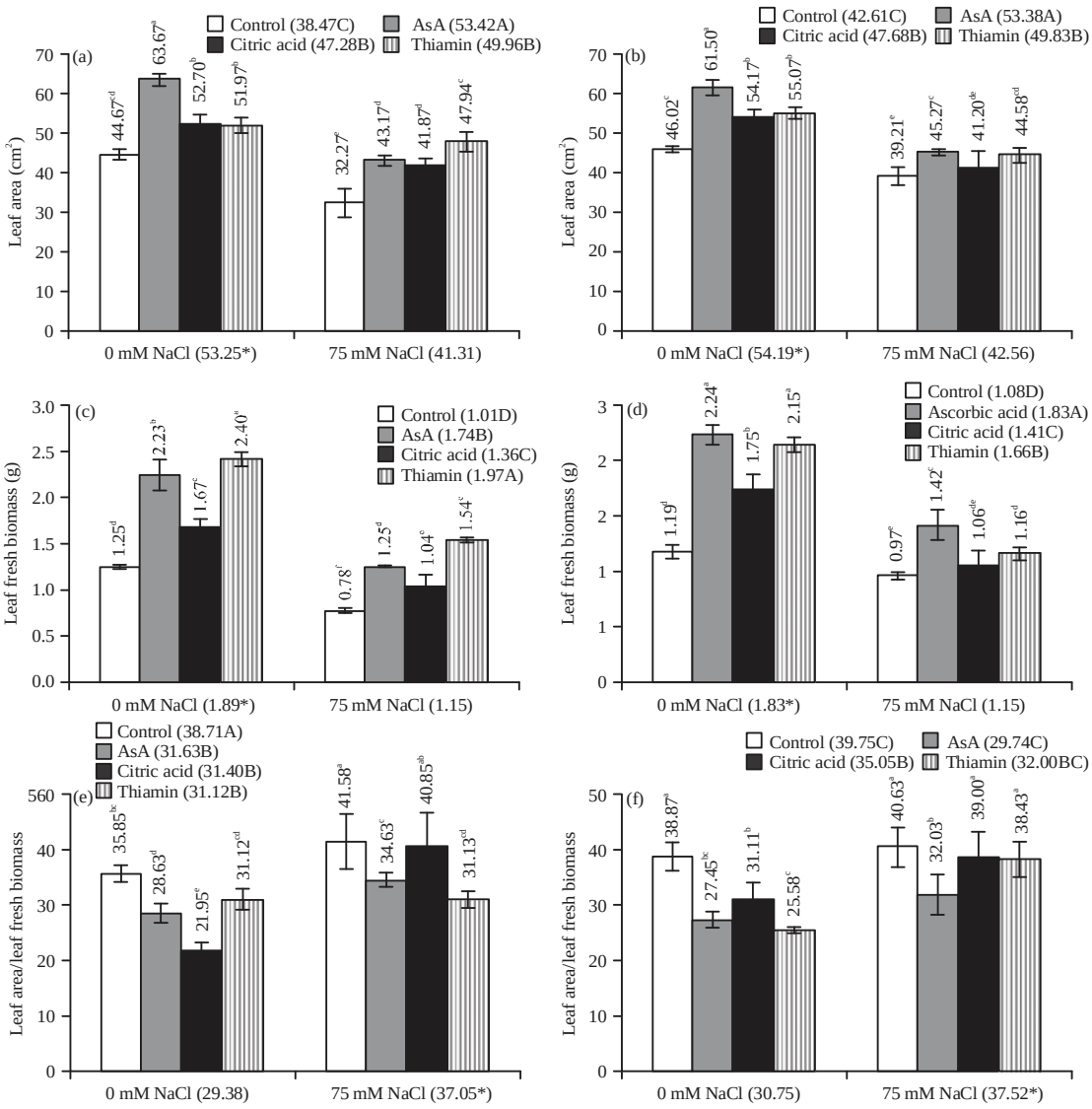

Fig. 3(a-f): Effect of salinity stress (0 and 75 mM NaCl), foliar applications (AsA, citric acid and thiamin at 10 mM) and their interactions on some growth parameters of roselle plants during the seasons of (a, c, e) 2016 and (b, d, f) 2017

Data revealed significant differences between treatments if means were marked with asterisk for salinity, capital letters for foliar applications and lowercase letters for their interaction according to Duncan's multiple range test at 5% level of probability

However, salt stress significantly reduced anthocyanin accumulation in areal dried calyces, at both seasons as shown in Fig. 7. Exogenous applications with anti-oxidants alleviated the anthocyanins accumulation in calyces which reached more the double concentration than non-sprayed plants under salt stress.

Effect of AsA, citric acid and thiamin on SOD, CAT, POD and PAL activities under salt stress: Except the activity of catalase in the first season, salt stress significantly elevated the activities of SOD, CAT, POD and PAL at both seasons in compared to control plants as shown in Fig. 8. In most cases application with AsA was noticed to be the most effective in enhancing the activities of SOD, CAT, POD and PAL anti-oxidant enzymes. Exogenous applied thiamin decreased SOD in the first season and POD in both seasons as well as increased CAT and PAL activities of both seasons. Citric acid treatment increased SOD activity, but the activities of CAT, POD and PAL were decreased. On contrast, the interaction between foliar spray with non-enzymatic anti-oxidants and

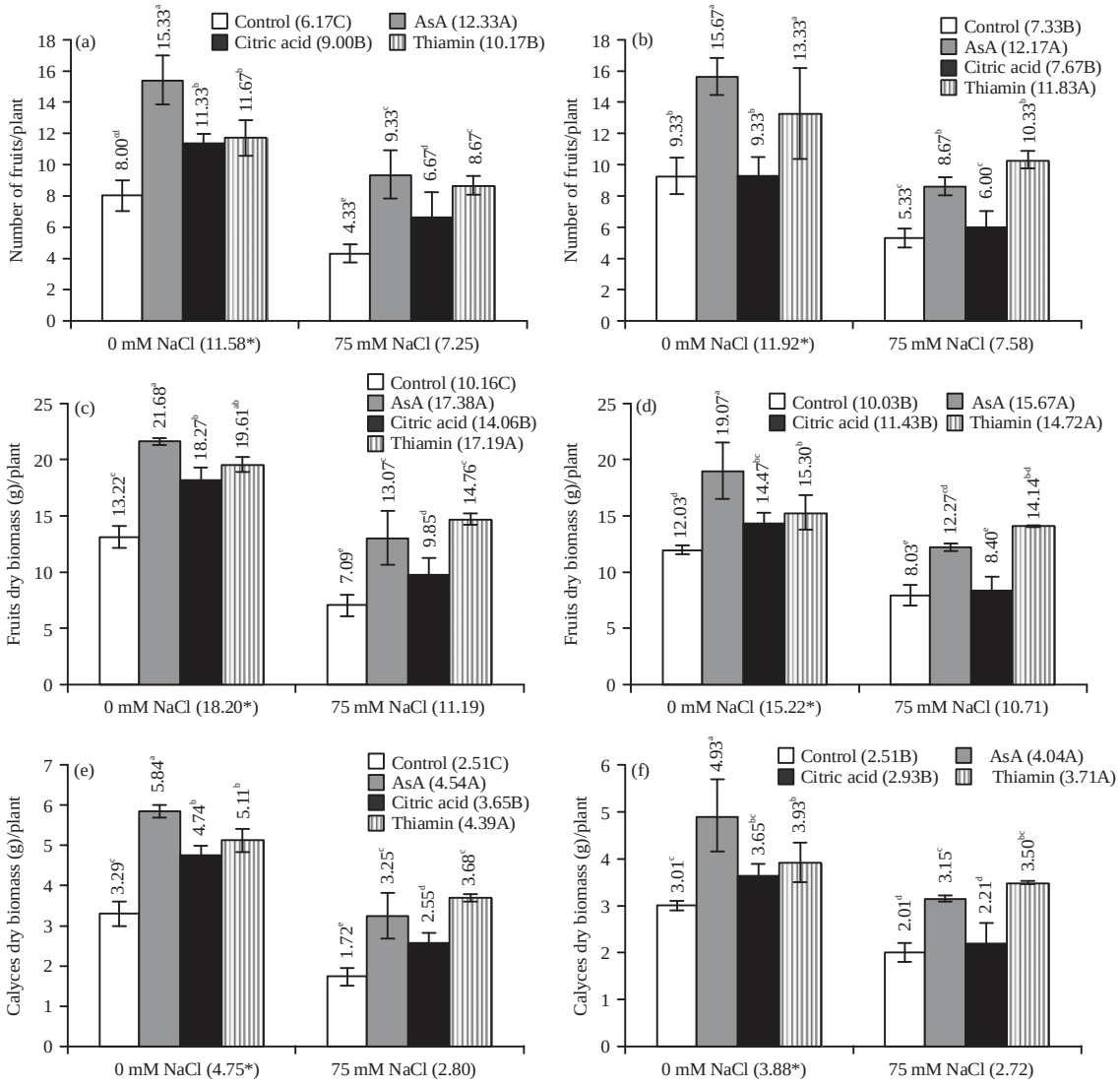

Fig. 4(a-f): Effect of salinity stress (0 and 75 mM NaCl), foliar applications (AsA, citric acid and thiamin at 10 mM) and their interactions on fruits number and calyces yield/plant in roselle plants during the seasons of (a, c, e) 2016 and (b, d, f) 2017

Data revealed significant differences between treatments if means were marked with asterisk for salinity, capital letters for foliar applications and lowercase letters for their interaction according to Duncan's multiple range test at 5% level of probability

saline water irrigation resulted in significant reduction in all tested anti-oxidant enzymes in compared to unsprayed plants at both seasons except with CAT at the first season.

Protein electrophoresis: Results of protein pattern with molecular biomass ranged from 20-150 kD were shown in Table 1 and Fig. 9.

Polymorphism percentage was 45.45%. Plants exposed to NaCl had an absent in band of 55.5, 42.78 and 36.38 kD compared to control (un stressed plants). NaCl-treated plants supplemented with AsA showed an absent in band of 42.78 and band of 36.38 kD was also absent in stressed plants treated with both AsA and citric acid. Except with thiamin under non-stressed condition, induction of new bands with MW 20.39 kD appeared with all foliar applications under both conditions of salinity in comparing with their corresponding controls. On the other hand, band of 15.81 kD was appeared with exogenous applied citric acid and thiamin on stressed plants instance it disappeared with all other different treatments.

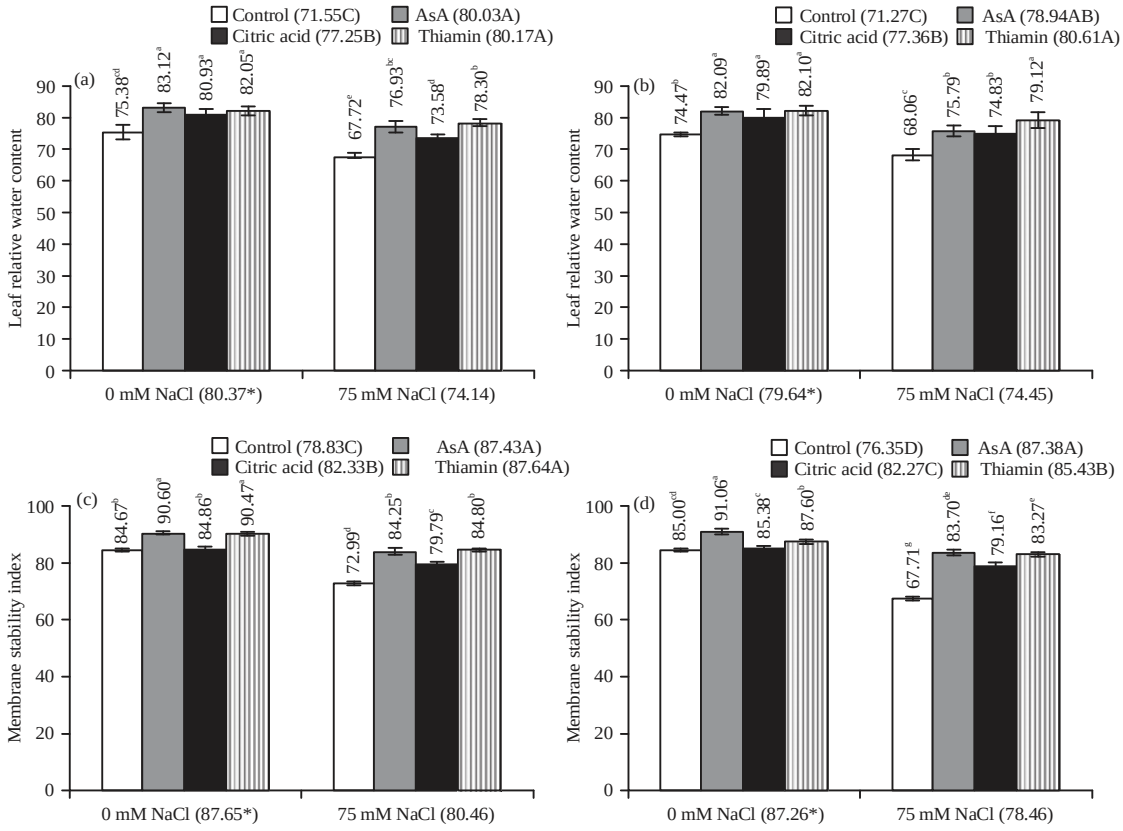

Fig. 5(a-d): Effect of salinity stress (0 and 75 mM NaCl), foliar applications (AsA, citric acid and thiamin at 10 mM) and their interactions on LRWC and MSI in roselle leaves during the seasons of (a, c) 2016 and (b, d) 2017

Data revealed significant differences between treatments if means were marked with different asterisk for salinity, capital letters for foliar applications whereas lowercase letters for their interaction according to Duncan's multiple range test at 5% level of probability

DISCUSSION

In the current study, the reduction in vegetative growth parameters and yield under salt stress indicated that saline water irrigation is a principle cause for reducing growth and productivity of roselle plants. These results were consistent with Rathnam[11] who found that roselle plants grown under saline condition revealed a high reduction in plant height, number of leaves, leaf area and flowers yield. In this context, FAO[38] reported that *Hibiscus sabdariffa* L. is a moderate salt tolerant plant but when salts accumulate within the 30 cm top of the soil surface inhibit growth by altering water potential, causing ions toxicity and inhibiting cell division and cell enlargement. A secondary aspect of salt stress is stress-induced production of ROS. AsA[19], citric acid[16] and thiamin[39] are the most water soluble antioxidant compounds that induce oxidative stress tolerance. Regarding the influence of non-enzymatic antioxidants on plant growth, El-Kobisy *et al.*[13] mentioned that ascorbic acid (AsA) has been responsible in cell division and cell elongation which stimulates vegetative growth and development. Moreover, AsA application increased the fresh and dry weights of *Viciafaba* stressed seedlings[40]. This stimulation effect is due to that AsA can detoxify and neutralize the ROS under stress condition that protects chloroplasts and membranes against NaCl toxicity[41]. Exogenous spraying of thiamin enhanced the anti-oxidative defense system in salt-stressed maize plants[18]. This better performance was attributed to the reaction of thiamin with adenosine triphosphate to form thiamin diphosphate which could serve as an active co-enzyme associated with the carbohydrate metabolism and transketolation reaction of pentose phosphate cycle in calvin cycle, where these pathways play an important role in converting carbohydrates and fats into energy necessary to stimulate growth and production[42].

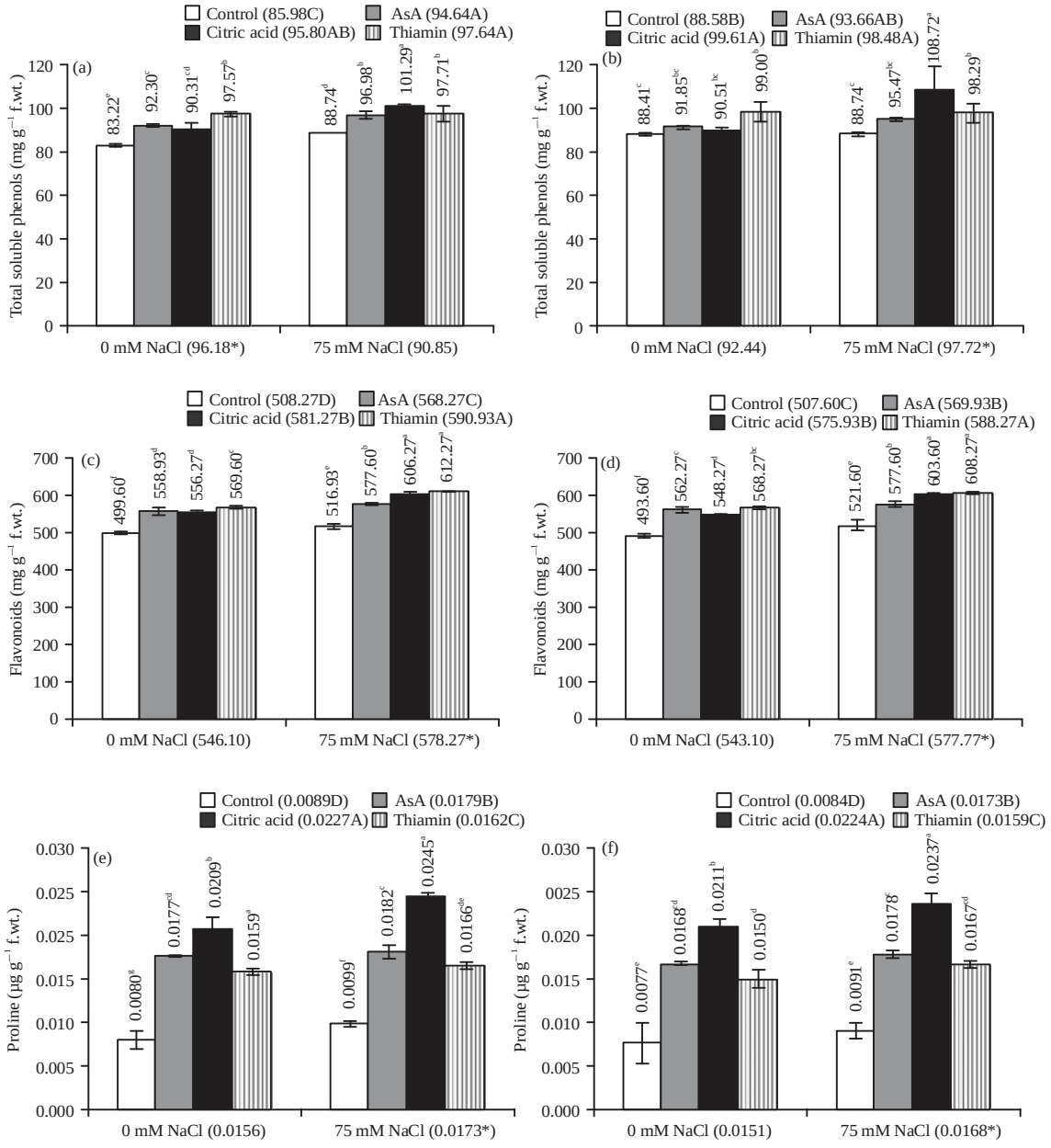

Fig. 6(a-f): Effect of salinity stress (0 and 75 mM NaCl), foliar applications (AsA, citric acid and thiamin at 10 mM) and their interactions on soluble phenols, flavonoids and proline concentrations in roselle leaves during the seasons of (a, c, e) 2016 and (b, d, f) 2017

Data revealed significant differences between treatments if means were marked with asterisk for salinity, capital letters for foliar applications and lowercase letters for their interaction according to Duncan's multiple range test at 5% level of probability

Similarly to the obtained data in decreasing LRWC and MSI under salt stress, Sairam *et al.*[43] reported that salinity decreased relative water content and membrane stability index. Possible explanation for this is that salt stress increases the osmotic strength of soil solution which disturbs plant's water relations due to decrease the availability of water from the soil as a result of lower osmotic potential[23]. On contrary, thiamin is a stress-response molecule that significantly elicited under salt stress which supply pentose phosphate for nucleotide synthesis to raise NADPH required for different synthetic pathways that alleviate LRWC and reduced membrane injury[44].

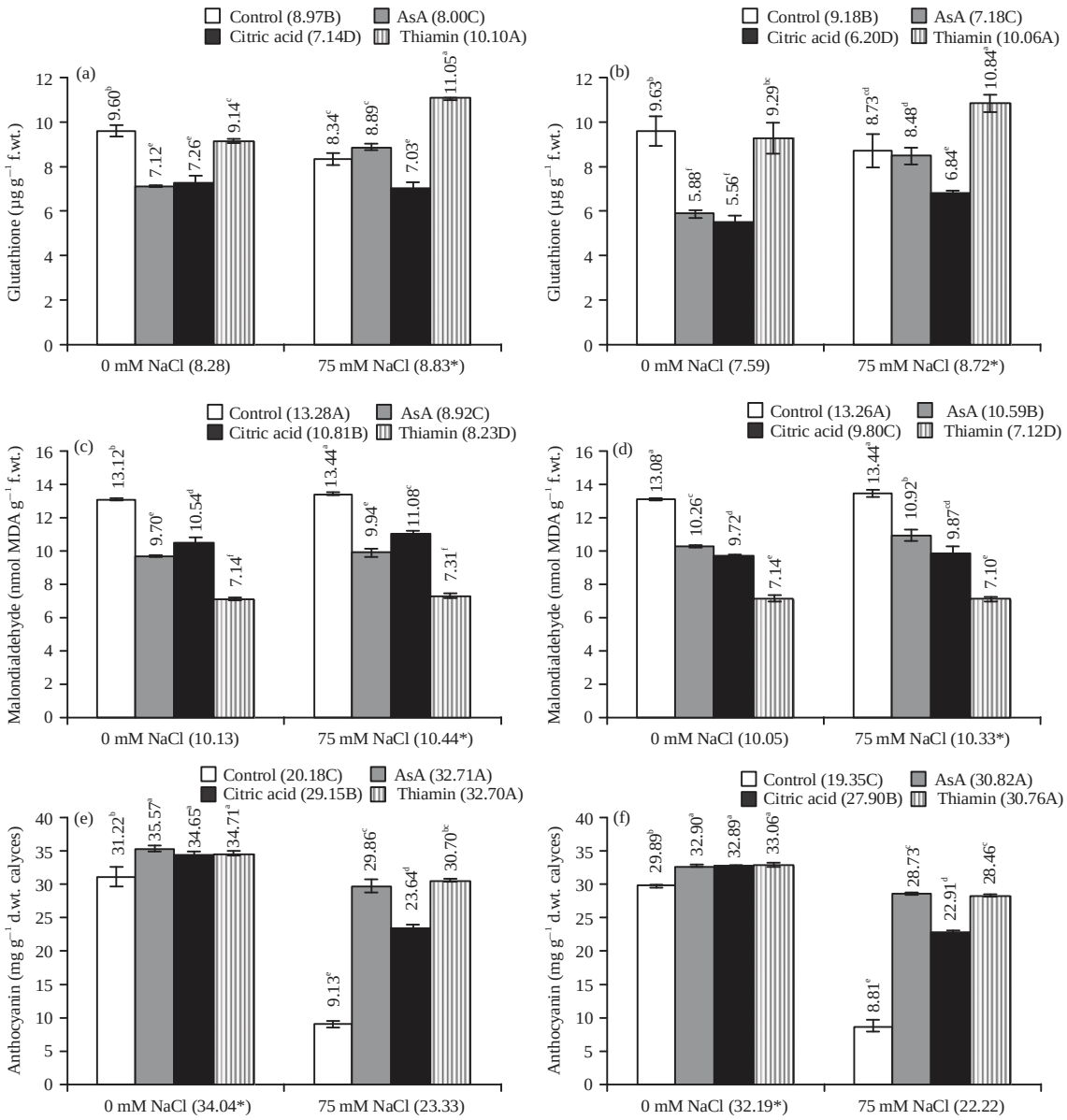

Fig. 7(a-f): Effect of salinity stress (0 and 75 mM NaCl), foliar applications (AsA, citric acid and thiamin at 10 mM) and their interactions on glutathione and MDA concentration in roselle leaves and anthocyanin concentration in calyces during the seasons of (a, c, e) 2016 and (b, d, f) 2017

Data revealed significant differences between treatments if means were marked with asterisk for salinity, capital letters for foliar applications and lowercase letters for their interaction according to Duncan's multiple range test at 5% level of probability

As a defense mechanism to detoxify the free radicals, plants increase the synthesis of anti-oxidants, especially phenolic compounds, in response to oxidative stress as recorded in the present study. In these connections, Abdellatif[23] illustrated more than 2-3 fold higher accumulation in flavonoids and total phenols in leaf tissues of wheat under salt stress. Phenolic compounds biosynthesis is not restricted to just their capacity in radical scavenging[45] but may also be attributed to donate a hydrogen atom to the ROS by oxidation reaction causing a relatively stable free radicals[46]. Flavonoids are plant secondary metabolites belong to low molecular weight phenolic compounds that have anti-oxidant potential against ROS[47]. Consequently, Boubakri *et al.*[48] approved that providing exogenous thiamin was the major reason for activating the expression of phenyl propanoid pathway genes which correlated with accumulation of phenols and flavonoids.

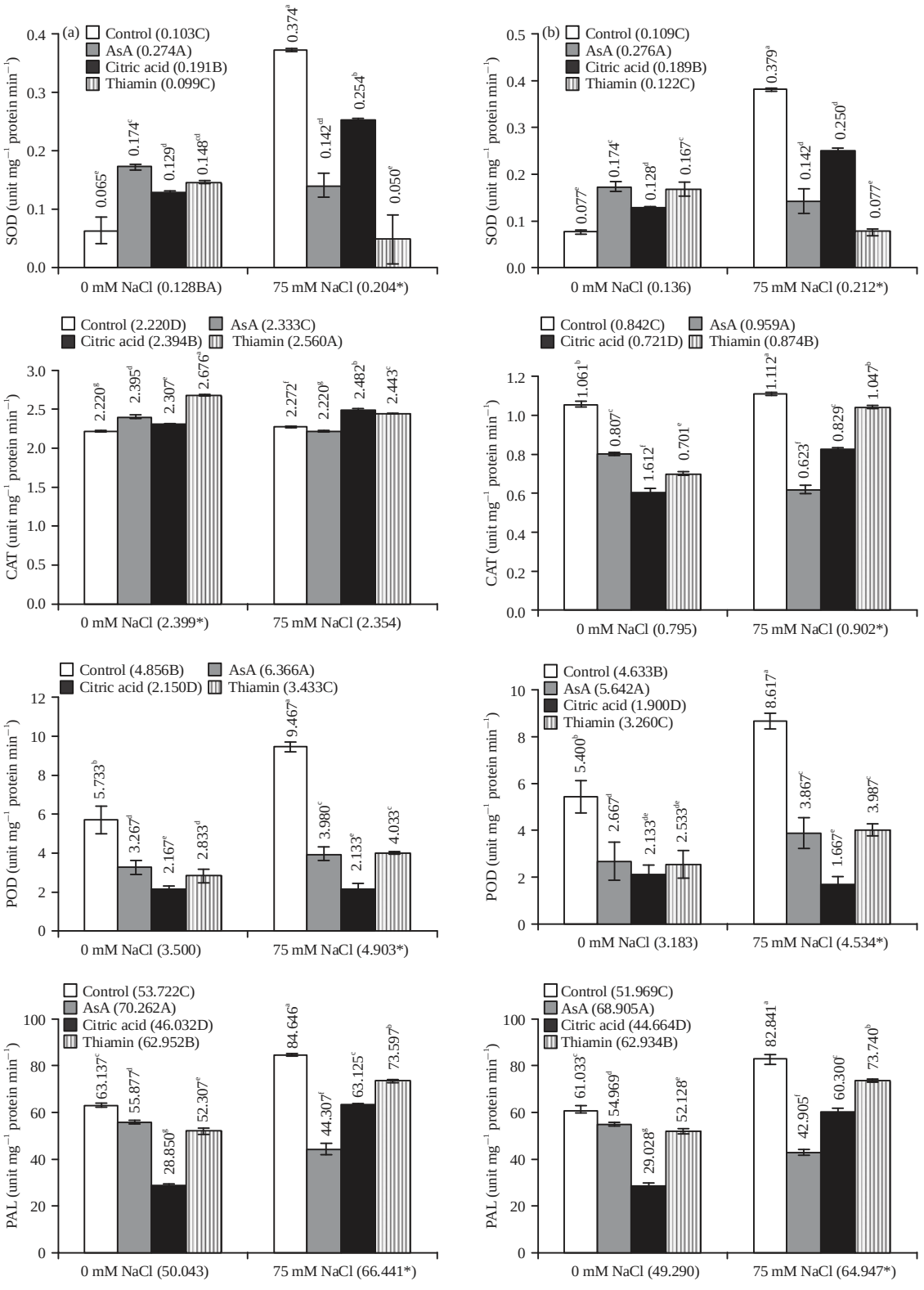

Fig. 8(a-b): Effect of salinity stress (0 and 75 mM NaCl), foliar applications (AsA, citric acid and thiamin at 10 mM) and their interactions on SOD, CAT, POD and PAL activities in roselle leaves during the seasons of (a) 2016 and (b) 2017

Data revealed significant differences between treatments if means were marked with asterisk for salinity, capital letters for foliar applications and lowercase letters for their interaction according to Duncan's multiple range test at 5% level of probability

Table 1: SDS-PAGE of *Hibiscus sabdariffa* leaves protein (polypeptides) as affected by exogenous application of AsA, citric acid and thiamin under salt stress

RF	MW	Control	AsA	Citric acid	Thiamin	NaCl 75 mM	NaCl+ AsA	NaCl+ citric acid	NaCl+ thiamin	Frequency	Polymorphism
0.402	55.507	1	1	1	1	0	1	1	1	0.875	Polymorphic
0.492	42.782	1	1	1	1	0	0	1	1	0.750	Polymorphic
0.548	36.383	1	1	1	0	0	0	0	1	0.500	Polymorphic
0.680	24.833	1	1	1	1	1	1	1	1	1.000	Monomorphic
0.708	22.901	1	1	1	1	1	1	1	1	1.000	Monomorphic
0.738	20.997	1	1	1	1	1	1	1	1	1.000	Monomorphic
0.748	20.398	0	1	1	0	0	1	1	1	0.625	Polymorphic
0.836	15.813	0	0	0	0	0	0	1	1	0.250	Polymorphic
0.955	11.207	1	1	1	1	1	1	1	1	1.000	Monomorphic
0.967	10.825	1	1	1	1	1	1	1	1	1.000	Monomorphic
0.978	10.485	1	1	1	1	1	1	1	1	1.000	Monomorphic
Total bands		9	10	10	8	6	8	10	11		

1 presence protein pattern and 0 absences protein patterns

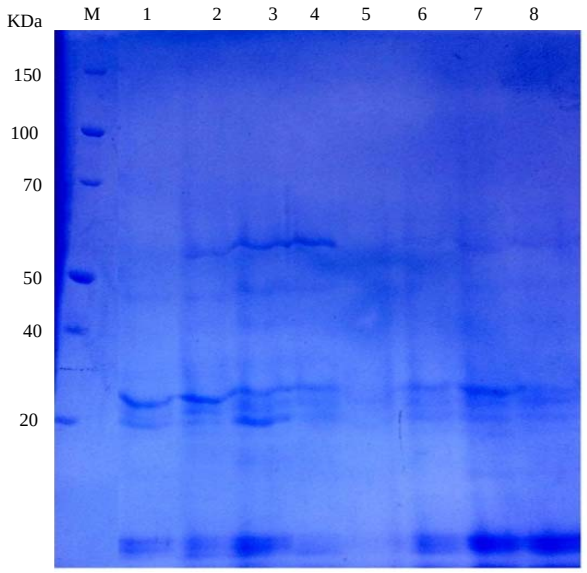

Fig. 9: SDS-PAGE profiles of soluble protein among the treatments

M: Maker, 1: Control, 2: AsA, 3: Citric acid, 4: Thiamin, 5: Salinity 75 mM NaCl, 6: Salinity+AsA, 7: Salinity+citric acid and 8: Salinity+thiamin

Significant increase in proline concentration in the present study was similar with Rathnam[11], who found that NaCl treatment enhanced the accumulation of proline in roselle shoots and roots. Free proline in high levels within cytosol and organelles under salt stress stimulates the maintenance of cell turgor for growth and protect membranes by supporting plant salt tolerance as osmoregulator[49]. Furthermore, proline participates in free radicals detoxification and cellular enzymes protection[7]. Citric acid plays an efficient role in mitochondrial citric acid cycle that involved in biosynthesis of several organic acids which act as a precursor for biosynthesis of various amino acids[50].

In response to increase GSH under salinity in the present investigation, the obtained results were agreed with Tausz *et al.*[5], who elucidated that endogenous glutathione in reduced form increased under salt stress to scavenge ROS, where GSH activates stress signaling molecules and biosynthesis of auxin that delay senescence and earliest flowering time in addition to its role in ascorbate-glutathione cycle. Free radicals induced by oxidative stress may oxidize the glutathione from its reduced form (GSH) into the oxidized form (GSSG). Thiamin supplementation reversed stress-induced reduction in GSH concentration[44] as shown in the present study.

A sharp increase in MDA concentration was followed by exposure to salt stress. In this connection,Trivellini *et al.*[51] stated that salt stress significantly elevated the electrolyte leakage in *Hibiscus rosasinensis*. This might be due to the increasing of ROS as a response of salinity that attacked membranes and induced peroxidation reaction that generated high levels of MDA[52]. In the present study, foliar spraying of all non-enzymatic anti-oxidant tested compounds showed a decreasing trend in MDA concentration. Previous studies reported that thiamin, AsA and citric acid treatments considerably reduced MDA level in salt-stressed plant tissues to reduce membrane permeability[18,19,16]. In addition, Potapovich and Kostyuk[53] found that the hydroxyl group presented in the structure of flavonoids can be modified into various powerful anti-oxidant properties that help in inhibition of lipid peroxidation in plants under stress.

The reduction in anthocyanins concentration under salt stress was also observed by Trivellini *et al.*[51], who indicated that salt stress negatively decreased the anthocyanin concentration in roselle flowers which resulted in a loss of color. Anthocyanins belong to flavonoid compounds that have an anti-oxidant capacity against superoxide radical anion and related ROS[54].

It is now noteworthy that activities of anti-oxidative enzymes increased in leaves under salt condition as found in the present study. Similar observation was also reported by Hassanein *et al.*[55], who observed that salt stress increased the activities of SOD, CAT and POD anti-oxidant enzymes in leaves of *Zea mays* plants. In these respect, Deeba *et al.*[56] reported that SOD is the first responsive enzyme in defense mechanism to detoxify oxidative stress that can scavenge super oxide anion (O_2^-) and dismutase the O^- to H_2O_2 and OH^-. In addition, De Azevedo-Neto *et al.*[57] mentioned that CAT and POD were anti-oxidant enzymes that up-regulated with the constitutively produced of H_2O_2 resulted from enhancing SOD activity. The PAL is the key anti-oxidant enzyme involved in phenylpropanoid pathway by which phenolic compounds are synthesized[58]. The reduction in the activities of anti-oxidant enzymes presented in this study with exogenous applied non-enzymatic anti-oxidants in comparison with control under salt stress was analogous to that observed by Tuna *et al.*[59], who explained the reason of these decreasing associated with the reduction in generating of cellular ROS. In the same manner, thiamin application induced plant tolerance to salt oxidative stress by reducing ROS accumulation and suppressing of protein oxidation[44]. The accumulation of phenols in sprayed plants under stress in the present study might be parallel with the decreasing in POD activity as revealed by Dolatabadian *et al.*[41], who reported that POD is a major enzyme which takes part in the phenolic compound oxidation. Tuna *et al.*[59] stated that exogenous spray with AsA improved plant growth under saline condition by enhancing their anti-oxidant capacity. AsA balanced water and ion homeostasis by protecting photosynthetic process of plant against salt-induced oxidative stress in addition to its role in ascorbate-glutathione cycle that protects plant from ROS[60].

The obtained results also cleared that there are remarkable changes in polypeptides in response to salt stress and foliar treatments. It appeared that a number of polypeptides disappeared and new polypeptides synthesized. In this connect, Davies[61] reported that radicals can react with amino acids, peptides and protein causing fragmentation and re-arrangement. Less protein damage was found in thiamin treated plants as shown from SDS-PAGE pattern. In the same trend, Arrigoni[62] detected that thiamin application exerted mostly positive effects on protein synthesis.

CONCLUSION

It is obvious from the previous results that salt stress negatively affected roselle plant growth and productivity. The concentration of 75 mM NaCl decreased LRWC and increased membrane leakage due to salt oxidative stress. This study has been shown that the activity of all assayed non-enzymatic anti-oxidants such as phenols, flavonoids and reduced glutathione and the activity of enzymatic anti-oxidants such as SOD, CAT, POD and PAL were significantly increased in plants subjected to salt condition. The three tested water soluble essential anti-oxidants alleviated plant salinity tolerance by up-regulating the non-enzymatic antioxidant defense system. Foliar application with AsA, citric acid and thiamin decreased all enzymatic antioxidants in applied plants due to maintenance of cellular redox state.

SIGNIFICANCE STATEMENT

This study confirmed that AsA, citric acid and thiamin are non-enzymatic antioxidants that can be beneficial to enhance roselle plant tolerance against free radicals generation which considered the critical area of the adverse effects of salt stress. AsA, citric acid and thiamin have anti-oxidant potential to scavenge the injuries of active oxygen. The current study recommended that non-enzymatic anti-oxidants specially AsA (vitamin C) and thiamin (vitamin B_1) could be used in ameliorating the defense system and improving flowers production of the pharmaceutical roselle plants under salinity condition in a safe way of human health.

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