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Insight into 5-aminolevulinic acid-induced modulation of cellular antioxidant metabolism to confer salinity and drought tolerance in maize

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Abstract: The current study investigated the comparative oxidative damage in two maize seedlings induced by saline, drought, and combined stress and the ameliorative role of two different doses (20 and 80 μM) of 5-aminolevulinic acid (ALA) against the above-mentioned stresses. Hydroponically grown 10-day-old maize (*Zea mays*, var. BARI Hybrid Maize-7 (BHM-7) and BARI Hybrid Maize-9 (BHM-9)) seedlings were exposed to 12 dS/m of saline solution, 200 mM mannitol-induced drought stress alone and their combined stress for 7 days. Result revealed that individual stresses retard the plant growth to some degrees; however, their combined stress has more detrimental effects, which might be correlated with lipid peroxidation (MDA)-induced oxidative stress in seedlings, enhanced Na^+/K^+ ratio, and augmented generation of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). In contrast, exogenous ALA supplementation at 20 μM concentration markedly recovered from chlorosis and growth inhibition, substantially scavenged reactive oxygen species (ROS) and MDA by preserving ion-homeostasis and relaxing oxidative stress; also, by boosting catalase (CAT) and glutathione *S*-transferase (GST), and exclusively via depressing the activity of lipoxygenase (LOX) antioxidant enzyme. On the contrary, 80 μM ALA made things worse; nevertheless, higher activities shown by other antioxidant enzymes; like, superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), and glutathione peroxidase (GPX), which were related to lessen the oxidative damage by highly produced $O_2^{\bullet-}$ and H_2O_2 under combined stress. Non-denaturing gel electrophoresis was done for further confirmation. However, ALA importantly increased the photosynthetic pigment contents in both genotypes irrespective of doses. Nevertheless, GST might have assisted the plants to escape from the herbicidal effect by detoxification. However, in the combined stress condition, high ALA concentration may have some positive role to play. Our findings also showed that BHM-9 performed better than BHM-7. Therefore, ALA at lower concentration was effective for single stress of saline and drought, while higher concentration can improve plant survival under combined stress.

Introduction

Ever-changing climatic conditions are intimidating agricultural sustainability in several planetary territories by restricting arable land and reducing the availability of water (Mickelbart *et al.*, 2015). These obstacles, fastened with a lot of abiotic stresses like excessive temperature, salinity, drought, and cold, cause significant losses in crop yields and their subsequent socio-economic repercussions. For instance, abiotic stresses (e.g., salinity, drought, etc.) are accountable for regression in the production of major crops up to a range of 50–70% (Mittler, 2006). Further, over 800 million hectares of arable land worldwide are contaminated one or the other by excess saline (397 million hectares) or sodium (434 million hectares) contents (Munns, 2005). Mostly, crops are being affected by drought than any other stresses and, by dint of global climate change, are rising even more acutely in the world. Statistically, from 1970 to 2000, drought stress turned out to double globally (Isendahl

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and Schmidt, 2006). In the light of the predicted climate change scenarios, an extensive increase in the production of agriculture would be obligatory to act in accordance with the world's food needs for the ongoing growing population in the upcoming half-century (Myers *et al*., 2017; Shaar-Moshe *et al*., 2017).

Changes in different physiological and metabolic processes, however, are the most common instances under excessive salt and water deficit stress, depends on the period and extent of stress and consequently impede agricultural production (Rozema and Flowers, 2008; Rahnama *et al*., 2010; James *et al*., 2011; Apel and Hirt, 2004; Gill and Tuteja, 2010; Hasanuzzaman *et al*., 2014a). Numerous changes have occurred in plants subjected to salinity and drought stress, including membrane destruction, nutrient disparity, capability impairment to detoxify reactive oxygen species (ROS), decreased photosynthetic function, variations in enzymatic activity of antioxidants, and decreased aperture of stomata (Rahnama *et al*., 2010; Apel and Hirt, 2004; Gill and Tuteja, 2010; Munns and Tester, 2008; HanumanthaRao *et al*., 2016). In addition, excessive salt and water deficit stress results in uncontrolled production of ROS products, like superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and radicals of hydroxyl ($OH^{\bullet}$) (Apel and Hirt, 2004; Gill and Tuteja, 2010; Rohman *et al*., 2016a; Rohman *et al*., 2016b). The formation of stress-induced ROS can leash to oxidative damage in several components of the cell include proteins, lipids, and DNA, and also disrupting essential plant cellular functions (Gupta and Huang, 2014). Conversely, plants possess a unique core mechanism to adapt to ROS toxicity and are assumed to counter water deficit stress by reinforcing these core mechanisms of defense unless removed or declined in the cell (Hasanuzzaman *et al*., 2014a; Rohman *et al*., 2016a; Rohman *et al*., 2016b).

In order to protect cells against ROS-induced cell injury, plants have formed a complex antioxidant mechanism (non-enzymatic and enzymatic) in plant tissue (Noctor *et al*., 2002; Choudhury *et al*., 2013; Hasanuzzaman *et al*., 2013). The antioxidant defense of plants includes enzymatic superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR); as well as non-enzymatic components, namely, ascorbic acid (ASA) and glutathione (GSH) (Apel and Hirt, 2004; Gill and Tuteja, 2010). Although extensive investigations have been done on the protective functions of antioxidants in various species of plants, yet the fundamental saline and drought-tolerant mechanisms is not completely understood.

For growing plants, the approach of using plant growth regulators (PGRs) is showing the potential to tolerate different abiotic stresses nowadays (Ali *et al*., 2013; Ali *et al*., 2014). 5-aminolevulinic acid (ALA) is one of them, and it is an important PGR engaged in the modulation of growth, development, and numerous physiological responses (Akram and Ashraf, 2013). ALA is a key component and precursor of porphyrin-derivatives biosynthesis as well as is involved in essential physiological processes of plants, including respiratory, photosynthetic, and other metabolomics activities (Wang *et al*., 2003; Zhang *et al*., 2015). Amassing evidence suggests that ALA can combat abiotic stresses (salinity, drought) by inducting plant resistance (Ali *et al*., 2013; Korkmaz *et al*., 2010; Naeem *et al*., 2011). Nonetheless, underlying mechanisms remain to be established as to how ALA stimulates antioxidant systems to modulate plant stress resistance under the combination of different stresses.

Moreover, plants must simultaneously cope with multifarious environmental stresses under both natural and agricultural systems and acclimatize with unrest environmental conditions. Under field conditions, salinity level increases with decreasing soil moisture. At the same time, salinity stress itself causes osmotic stress by raising the concentration of different ions in soil water. Therefore, plants encounter both saline and drought stress concurrently. However, the conventional method of working concentrates on short and severe single stresses of plant responses to abiotic stress in early vegetative phases, with plant endurance or retrieval levels as a magnitude for tolerance to plant stress. Plant acclimatization to multiple environmental stress combinations is more complex than to single stress and can generate certain physiological, molecular, and metabolic responses interacting and inhibiting each other (Suzuki *et al*., 2014; Prasch and Sonnewald, 2015). However, information on such responses under salinity and drought-mediated combined stress is still limited.

C_4 type maize (*Zea mays*) plants belong to the family Poaceae. By nature, due to being a cross-pollinated crop, maize is supposed to be more adaptable owing to superior photosynthesis, compared to C_3 plants, although at the expense of reduced photorespiration (Kanai *et al*., 1999). Maize was therefore found to be experiencing the least oxidative stress. Nevertheless, a few numbers of recent studies have shown that maize is suffering extensively under abiotic stresses resultant oxidative damage, especially excessive salt or water deficit stress (Rohman *et al*., 2016a; Rohman *et al*., 2016b; Rohman *et al*., 2019; Anjum *et al*., 2017). However, the effect of combined stress in maize also inadequate. Therefore, we designed the study to assess the potential protection of ALA supplementation on maize seedlings in two different concentrations under specific salinity and drought and their combined stress. In the current work, we investigated the following key attributes: (i) performance on plant growth, (ii) status of photosynthetic pigment, (iii) accumulation of proline, (iv) Na^+ and K^+ homeostasis, (v) oxidative damage in relation to elevated levels of ROS and lipid peroxidation, and (vi) improved antioxidant protection.

Materials and Methods

Plant materials

Two high-yield maize varieties, namely BARI Hybrid Maize-7 (BHM-7) (yield: 10.5–11.2 t/ha) (BARI, 2020) and BARI Hybrid Maize-9 (BHM-9) (yield: 10.2–12.0 t/ha) (BARI, 2020), were used to study the attenuating effects of 5-AminoLevulinic Acid (ALA, Wako, Japan) under salinity,

drought, and a combination of these stresses (drought and salinity). Seeds of both varieties were obtained from the Plant Breeding Division of Bangladesh Agriculture Research Institute (BARI), Joydebpur, Gazipur. After keeping the seeds in room temperature for 24 h, substantial healthy seeds were sown in a tray that was previously filled with a mixture of treated coarse sand and rock grinding and allowed to be kept in a greenhouse at 22°C for 7 days for germination. Afterward, the 7-day-old seedlings were subjected to grow in a controlled hydroponic condition (temperature: 22°C, relative humidity: 75–80%, light intensity: 370–555 μmol/m^2/s) using Hoagland solution as a nutrient, which renewed in every 2 days.

Seedlings treatments

Ten-day-old healthy maize seedlings of both varieties at V2 stage were then exposed to Hoagland nutrient solution (3½ liters per pot) containing 12 dS/m NaCl as saline stress only, 200 mM mannitol as drought stress only, and a combination of both stresses (12 dS/m NaCl + 200 mM mannitol). External supplementation of two unique concentrations of ALA (20 and 80 μM) was done by mixing them with the Hoagland nutrient solution. Thus, our experimental design composed of ten treatment composition as follows: (1) control (C), (2) 12 dS/m NaCl (S), (3) 12 dS/m NaCl + 20 μM ALA (S+20), (4) 12 dS/m NaCl + 80 μM ALA (S+80), (5) 200 mM mannitol (D), (6) 200 mM mannitol + 20 μM ALA (D+20), (7) 200 mM mannitol + 80 μM ALA (D+80), (8) 12 dS/m NaCl + 200 mM mannitol (S+D), (9) 12 dS/m NaCl + 200 mM mannitol + 20 μM ALA (S+D+20), (10) 12 dS/m NaCl + 200 mM mannitol + 80 μM ALA (S+D+80). The doses of ALA were selected based on the previous report (Hotta *et al.*, 1997). Both maize varieties exposed to the aforementioned ten different treatments were further subjected to grow for 7 days in the aforesaid growth conditions. Consequently, the fully opened second leaves from the top (penultimate leaves) of 17-day-old maize plants of both varieties were harvested to determine different morphological, physiological, and biochemical responses, revised by the imposition of NaCl, mannitol, and ALA. Two independent replications were used for each treatment for assessing individual parameters. Each replication consisted of 12 plants of each maize varieties under the same experimental conditions, which were repeated three times.

Growth parameter measurement

The assessment of shoot height, root length, and volume were performed, using the technique defined by Rohman *et al.* (2019).

Determination of photosynthetic pigments and proline content

The chlorophyll (Chl) *a*, *b*, and Chl (*a*+*b*), and carotenoid contents were estimated spectrophotometrically in the penultimate leaves and calculated following the method proposed by Lichtenthaler (1987) and Arnon (1949), consecutively. Proline (Pro) content in the penultimate leaves of maize plants was quantified according to the protocol of Bates *et al.* (1973).

Measurement of Na^+/K^+

To assess the sodium (Na^+) and potassium (K^+) contents, the sap was extracted from freshly harvested roots and shoots, using a tissue sap extractor (Horiba, Japan), and the contents were determined using the compact Na^+ ion (Horiba-731, Japan) and K^+ ion (Horiba-722, Japan) meters, respectively, following the methods stated in Rohman *et al.* (2019).

Assessment of the contents of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)

In the penultimate leaves of maize, radical superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) were determined following the procedures of Elstner and Heupel (1976) and Yu *et al.* (2003), correspondingly.

Histochemical ROS marker detection

To detect the localization of $O_2^{\bullet-}$ and H_2O_2 in the penultimate leaves, the procedures of Chen *et al.* (2010) and Thordal-Christensen *et al.* (1997), respectively, were followed.

Measurement of malondialdehyde (MDA) content and lipoxygenase (LOX) activity

The appraisement of lipid peroxidation product of leaves, namely malondialdehyde (MDA) contents and activity of LOX (EC: 1.13.11.12), was done using the method reported in Heath and Packer (1968) and Doderer *et al.* (1992), respectively.

Assay of enzymatic activities

The inclusive procedures mentioned by Rahman *et al.* (2019) have been used for preparing the enzyme extracts and determining the activities of SOD (EC: 1.15.1.1), CAT (EC 1.11.1.6), APX (EC: 1.11.1.11), POD (EC: 1.11.1.7), GPX (EC: 1.11.1.9) and GST (EC: 2.5.1.18).

Determination of the level of protein

The concentration of protein in the leaf extracts was quantified applying bovine serum albumin (BSA) as a protein standard following the Bradford method (Bradford, 1976).

Native PAGE and staining of activity

To visualize the activities of several enzymes protein changes these possesses isoenzymes activity, and scavenge reactive oxygen species (ROS), also participate in the ascorbate-glutathione cycle were studied in this study using PAGE (on a gel, 10% for SOD and 8% for others) maintaining non-denatured and non-reduced conditions at 4°C as described by Laemmli (1970). In each lane of the gel 50 μg of protein was applied. Procedures followed for gel running and staining are described below.

The method defined by Beauchamp and Friclorich (1971) was followed for detecting SOD activity. Prior to soaking the gel in 50000 μM Na_2PO_4 buffer (pH 7.8) at room temperature for 20 min in shady condition, electrophoresis was done. The buffer includes nitroblue tetrazolium (NBT) (240 μM) and riboflavin (28 μM). Afterward, TEMED (28000 μM) was included in the buffer, and then the gel was kept under an artificial light source until the band was visualized.

The procedure of Woodbury *et al.* (1971) was adopted to determine CAT isozymes. After electrophoresis, gels were soaked into 0.01% H_2O_2 for 5 min. After that, gels were rinsed with double distilled water. Finally, 1% $FeCl_3$

and 1% $K_3Fe(CN)_6$ were used to stain the gels and to detect the isozymes.

To find out the APX activity, the process of Mittler and Zilinskas (1993) was tracked. First, for 30 min, the gel was pre-run adding 2000 µM L-ascorbic acid without loading sample. Followed by immersing the gel in 50000 µM Na_2PO_4 buffer (pH 7) for 30 min; buffer contained 2000 µM L-ascorbic acid. Afterward, the gel was transferred to a solution composed of 50000 µM Na_2PO_4 buffer (pH 7), containing L-ascorbic acid (4000 µM) and H_2O_2 (2000 µM); the incubation period was 20 min. Subsequently, the gel was washed with 50000 µM Na_2PO_4 buffer (pH 7) for 1 min. Lastly, the gel was submerged in 50000 µM Na_2PO_4 buffer solution (pH 7.8), composed of TEMED (28000 µM) and NBT (2450 µM) for 15 min with gentle agitation.

POD isoenzyme activity was identified following the procedure of Fielding and Hall (1978). Potassium buffer (pH 7) (25000 µM) was used to soak the gel for 15 min. Then, the gel was infused into a newly prepared 25000 µM K-phosphate buffer (pH 7) solution until the POD activity band appeared, the buffer composed of guaiacol (18000 µM) and H_2O_2 (25000 µM).

The technique described by Das and Bagchi (2010) was adopted for showing GPX activity. Washing of gel was performed with 2.5% Triton X–100 and double distilled water; in each case, the washing duration was 15 min. Next, for 1 h gel was soaked in 10000 µM K-PO_4 buffer (pH 7.2), 2000 µM *o*-dianisidine dihydrochloride was mixed in the solution. In the last step, the gel was moved to 10000 µM K-PO_4 buffer (pH 7.2), containing 100 µM H_2O_2. The gel was kept in that solution until brown GPX bands appeared in contrast to the pale-yellow background.

Statistical analysis

Data reported in the figures and tables were expressed as means with standard deviations (SDs) of three independent replications (n = 3) of each treatment; each was repeated two times. Data obtained in this work were analyzed out using the two-way analysis of variance (ANOVA) using the software Statistix (version 10.0). Different letters represent significant differences among the treatments following the least significant difference test to compare the treatment means at $p \leq 0.05$.

Results

Phenotypic appearance

Exposure to salt, drought, and combined stress (salt+drought) condition severely damaged maize seedlings. By phenotype, resultant rolling of lower leaves, leaf tips burning, and, in certain cases, of the entire leaves, leaves drooping and yellowing of the whole plant. The most devastating result was found in the combined stress condition where maize seedling almost failed to survive. Supplementation of 5-aminolevulinic acid (ALA) at low concentration (e.g., 20 µM) notably overturned the stress-induced obliteration and amended the seedling's phenotypic appearance. More damaged seedlings were observed when seedlings were supplemented with a higher concentration of ALA (80 µM) compared to the low concentration (Fig. 1). However, the stress-induced damage was more visible in BARI Hybrid Maize-7 (BHM-7) than BARI Hybrid Maize-9 (BHM-9) variety.

Plant growth

The growth of plants considering shoots height, root length, and root volume was decreased due to stresses. Highly reduced growth was found under the combined stress

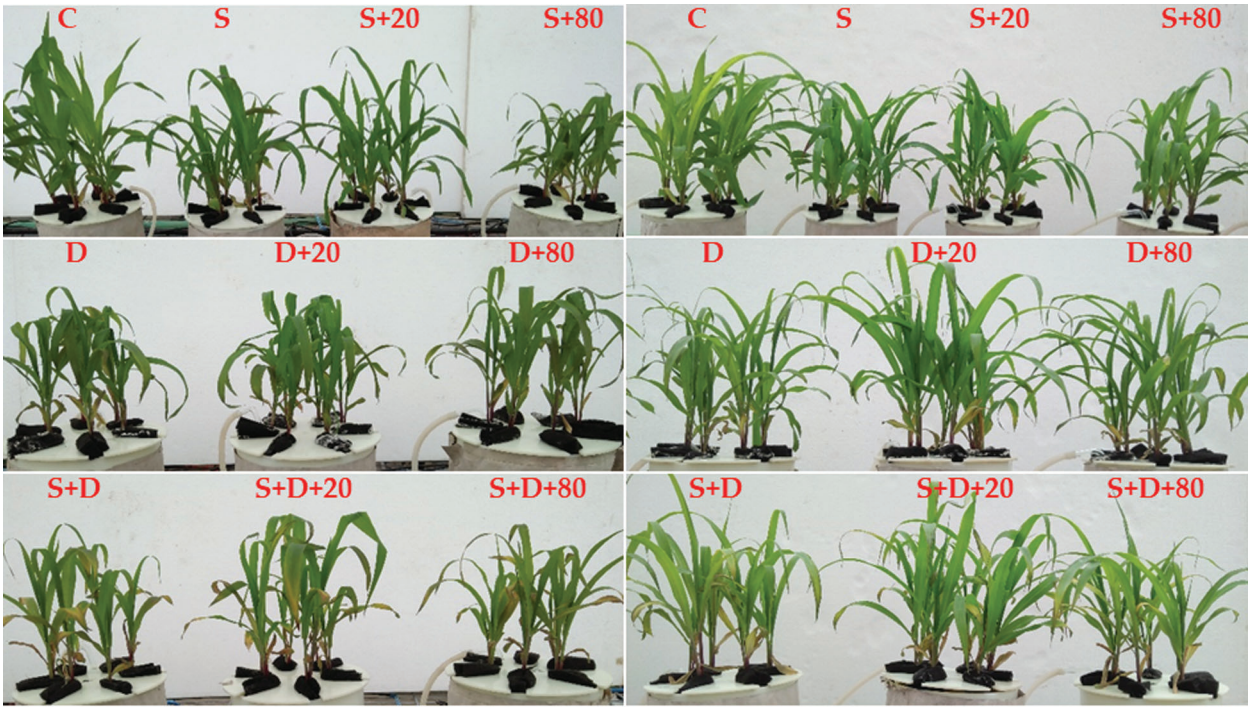

FIGURE 1. Effect of exogenous ALA on plant phenotype of BHM-7 and BHM-9 after exposing 7 days to salt, drought, and combined stress. C, control; S, salt stress (12 dS/m); D, drought stress (induced by 200 mM mannitol); 20, 20 µM ALA; 80, 80 µM ALA.

condition; where, shoot height, root length, and root volume, were diminished by 237.0, 23.0 and 296.7% in BHM-7; whereas, 152.5, 41.0 and 127.5% in BHM-9 compared to control seedlings (Tab. 1). Nevertheless, supplementation of 20 µM exogenous ALA to stresses-treated seedlings distinctly repaired the growth of plants. The restoration percentage of shoot height of BHM-7 was 11.1, 7.2 and 104.8%; root volume was 31.1, 7.0 and 151.7%; while, for BHM-9 shoot height refurbished by 48.2, 19.8 and 46.7%; root volume by 22.0, 48.8 and 23.5% in comparison with their respective stresses (Tab. 1). On the other hand, applying 80 µM ALA to the salt, drought and combined stress treatment reduced the growth of plants once again, which points out that a higher concentration of ALA acts as a suppressor to the plant growth. However, plant growth was highly affected in the BHM-7 variety than BHM-9 variety (Fig. 1). An exception was found in the case of root volume in BHM-7 under sole drought stress, where volume was higher than BHM-9; this because during estimation we observed more adventitious roots in drought-treated BHM-7 seedlings.

Photosynthetic pigments

Salt exposure of BHM-7 and BHM-9 maize seedlings resulted in decreased chlorophyll (chl) *a* (by 35.5 and 29.3%, accordingly), chl *b* (by 71.3 and 55.1%, accordingly) and chl (*a*+*b*) (by 50.3 and 39.6%, accordingly), compared to control seedlings. Further, drought stress reduced the contents of chl *a* (by 9.2 and 195.6%, individually), chl *b* (by 104.5 and 184.3%, individually), and chl (*a*+*b*) (by 39.9 and 190.5%, individually) in BHM-7 and BHM-9 seedlings, correspondingly, compared to that of control plants. However, in contrast to that of the control condition, maize seedlings treated with combined stress showed declination in the levels of chl *a* (by 54.7 and 87.2%, separately), chl *b* (by 144.0 and 130.5%, separately), and chl (*a*+*b*) (by 79.7 and 104.3%, separately) in BHM-7 and BHM-9 varieties, respectively (Tab. 2). Exogenous application of ALA to the salt, drought and combined stress-treated maize seedlings amended chl content compared to the seedlings treated with only salt or drought and in combined. Although, applying the ALA in higher concentration improved chl content in some treatment but decreased in others.

Carotenoid and proline (Pro) content

Under saline stress, BHM-7 and BHM-9 maize seedlings showed reduced carotenoid content (by 109.2 and 35.8%, accordingly), compared to control seedlings. Additionally, drought stress decreased the carotenoid contents (by 40.0 and 31.0%, individually) in BHM-7 and BHM-9 seedlings, correspondingly, compared to that of control plants. Nevertheless, in distinction to that of the control condition, combined stress treated maize seedlings exhibited a reduction in the level of carotenoid by (71.0 and 41.0%, separately) in BHM-7 and BHM-9 varieties, respectively (Tab. 3). Exogenous ALA application to the salt, drought, and combined stress-treated maize seedlings revised carotenoid content compared to sole salt or drought and in combined seedlings. Though, ALA supplementation in 80 µM concentration amended carotenoid content in some cases but diminished in other treatments. All the stresses markedly escalated in endogenous proline (Pro) content in maize seedlings of both varieties; however, the percent increase was higher in BHM-9 compared to BHM-7. In BHM-7 Pro content increased by 23.2, 18.7, and 20.5% under salt, water deficit, and combined stress, correspondingly, compared to that of control; in the same sequence of stress, BHM-9 showed 81.8, 625.0, and 359.0% increase (Tab. 3). Importantly, supplementation of ALA at 20 µM concentration decreased the endogenous Pro content under salt stress but increased in water deficit and combined stress than the control seedlings. Nevertheless, whenever ALA was applied at 80 µM concentration endogenous Pro

TABLE 1

Effect of exogenous ALA on shoot height, root length, and root volume of BHM-7 and BHM-9 after exposing 7 days to salt, drought, and combined stress

Treatment	Shoot height (cm)		Root length (cm)		Root volume (mL/plant)	
	BHM-7	BHM-9	BHM-7	BHM-9	BHM-7	BHM-9
Control (C)	38.69 ± 0.98^{a}	56.31 ± 0.46^{a}	$24.03 \pm 0.68^{a\text{-}d}$	32.45 ± 0.54^{a}	3.61 ± 0.08^{c}	5.71 ± 0.25^{a}
Salinity (S)	33.82 ± 0.37^{bc}	28.28 ± 0.34^{d}	23.51 ± 1.06^{cd}	$24.54 \pm 0.99^{a\text{-}d}$	2.89 ± 0.06^{d}	$3.69 \pm 0.08^{c\text{-}e}$
S+20 µM ALA	37.56 ± 0.32^{a}	41.90 ± 0.76^{b}	24.04 ± 0.61^{cd}	31.01 ± 0.57^{ab}	$3.79 \pm 0.10b^{c}$	4.50 ± 0.04^{bc}
S+80 µM ALA	36.03 ± 0.49^{ab}	32.52 ± 0.53^{c}	$18.46 \pm 0.60^{c\text{-}e}$	21.03 ± 0.82^{de}	2.30 ± 0.06^{e}	4.01 ± 0.04^{cd}
Drought (D)	30.31 ± 0.25^{cd}	27.11 ± 0.52^{d}	22.51 ± 0.71^{cd}	$28.04 \pm 1.06^{b\text{-}d}$	4.31 ± 0.08^{ab}	$3.30 \pm 0.12^{d\text{-}f}$
D+20 µM ALA	32.49 ± 0.29^{bc}	32.47 ± 0.52^{c}	25.03 ± 0.64^{cd}	32.02 ± 0.59^{a}	4.61 ± 0.12^{a}	4.91 ± 0.06^{ab}
D+80 µM ALA	28.89 ± 0.27^{d}	22.88 ± 0.65^{e}	18.49 ± 1.24^{cd}	$24.01 \pm 0.85^{a\text{-}c}$	3.31 ± 0.04^{cd}	$4.00 \pm 0.11^{b\text{-}d}$
S+D	11.48 ± 0.41^{f}	22.30 ± 0.36^{e}	19.54 ± 0.73^{cd}	23.01 ± 0.67^{cd}	0.91 ± 0.04^{g}	2.51 ± 0.10^{f}
S+D+20 µM ALA	23.51 ± 0.45^{e}	32.72 ± 0.33^{c}	21.52 ± 1.34^{cd}	$28.09 \pm 1.05^{a\text{-}d}$	2.29 ± 0.08^{e}	3.10 ± 0.11^{ef}
S+D+80 µM ALA	21.20 ± 0.78^{e}	21.91 ± 0.21^{e}	11.50 ± 0.86^{e}	18.18 ± 0.95^{cd}	1.70 ± 0.08^{f}	2.02 ± 0.17^{f}

*Values represent means and standard deviations (SDs) determined from three independent replications for each treatment ($n = 3$), each replicated two times. Different letters among the stresses within a variety are significantly different at $p \leq 0.05$, following a least significant difference (LSD) test.

TABLE 2

Effect of exogenous ALA on Chl *a*, Chl *b* and Chl (*a*+*b*) contents of BHM-7 and BHM-9 leaves after exposing 7 days to salt, drought, and combined stress. FW, fresh weight

Treatment	Chl *a* (mg/g FW)		Chl *b* (mg/g FW)		Chl (*a*+*b*) (mg/g FW)	
	BHM-7	BHM-9	BHM-7	BHM-9	BHM-7	BHM-9
Control (C)	0.710 ± 0.009^{bc}	0.745 ± 0.011^{bc}	0.632 ± 0.013^{a}	0.597 ± 0.010^{ab}	1.34 ± 0.011^{b}	1.34 ± 0.021^{b}
Salinity (S)	0.524 ± 0.006^{d}	0.576 ± 0.008^{d}	0.369 ± 0.017^{cd}	0.385 ± 0.007^{c}	0.893 ± 0.019^{e}	0.961 ± 0.015^{c}
S+20 μM ALA	0.756 ± 0.013^{b}	0.803 ± 0.010^{b}	0.635 ± 0.006^{a}	0.537 ± 0.006^{b}	1.39 ± 0.018^{b}	1.34 ± 0.016^{b}
S+80 μM ALA	0.829 ± 0.012^{a}	0.892 ± 0.003^{a}	0.668 ± 0.005^{a}	0.578 ± 0.006^{ab}	1.50 ± 0.015^{a}	1.47 ± 0.007^{a}
Drought (D)	0.650 ± 0.011^{c}	0.252 ± 0.014^{f}	0.309 ± 0.007^{de}	0.210 ± 0.007^{d}	0.959 ± 0.014^{e}	0.462 ± 0.020^{e}
D+20 μM ALA	0.749 ± 0.009^{b}	0.734 ± 0.010^{bc}	0.412 ± 0.007^{bc}	0.565 ± 0.011^{ab}	1.16 ± 0.016^{d}	1.30 ± 0.020^{b}
D+80 μM ALA	0.842 ± 0.011^{a}	0.750 ± 0.012^{bc}	0.458 ± 0.005^{b}	0.614 ± 0.008^{a}	1.30 ± 0.008^{bc}	1.36 ± 0.019^{ab}
S+D	0.459 ± 0.010^{d}	0.398 ± 0.007^{e}	0.287 ± 0.006^{e}	0.259 ± 0.007^{d}	0.747 ± 0.014^{f}	0.657 ± 0.012^{d}
S+D+20 μM ALA	0.752 ± 0.009^{b}	0.691 ± 0.010^{c}	0.384 ± 0.008^{c}	0.342 ± 0.010^{c}	1.14 ± 0.017^{d}	1.03 ± 0.019^{c}
S+D+80 μM ALA	0.775 ± 0.006^{ab}	0.701 ± 0.008^{c}	0.424 ± 0.010^{bc}	0.360 ± 0.012^{c}	1.20 ± 0.012^{cd}	1.06 ± 0.017^{c}

*Values represent means and standard deviations (SDs) determined from three independent replications for each treatment (n = 3), each replicated two times. Different letters among the stresses within a variety are significantly different at $p \leq 0.05$, following a least significant difference (LSD) test.

TABLE 3

Effect of exogenous ALA on Carotenoid and Pro contents of BHM-7 and BHM-9 after exposing 7 days to salt, drought, and combined stress. FW, fresh weight

Treatment	Carotenoid (mg/g FW)		Pro (μm/g FW)	
	BHM-7	BHM-9	BHM-7	BHM-9
Control (C)	0.272 ± 0.017^{d}	0.258 ± 0.012^{b-d}	0.654 ± 0.016^{de}	0.549 ± 0.004^{d}
Salinity (S)	0.130 ± 0.002^{a-c}	0.190 ± 0.007^{a-d}	0.806 ± 0.019^{c}	0.998 ± 0.011^{d}
S+20 μM ALA	0.181 ± 0.016^{cd}	0.233 ± 0.011^{b-d}	0.700 ± 0.024^{d}	0.519 ± 0.008^{d}
S+80 μM ALA	0.150 ± 0.012^{d}	0.233 ± 0.013^{d}	0.778 ± 0.009^{c}	0.903 ± 0.016^{d}
Drought (D)	0.203 ± 0.013^{ab}	0.197 ± 0.006^{a}	0.776 ± 0.015^{c}	3.98 ± 0.374^{a}
D+20 μM ALA	0.277 ± 0.012^{a-c}	0.328 ± 0.012^{cd}	0.604 ± 0.012^{e}	2.14 ± 0.376^{d}
D+80 μM ALA	0.267 ± 0.014^{b-d}	0.215 ± 0.005^{d}	1.28 ± 0.030^{a}	2.61 ± 0.427^{c}
S+D	0.159 ± 0.014^{a}	0.183 ± 0.010^{a}	0.788 ± 0.021^{c}	2.52 ± 0.068^{c}
S+D+20 μM ALA	0.296 ± 0.010^{a-c}	0.316 ± 0.009^{a-c}	0.617 ± 0.007^{e}	2.72 ± 0.047^{c}
S+D+80 μM ALA	0.268 ± 0.012^{a-c}	0.289 ± 0.013^{ab}	0.927 ± 0.008^{b}	3.68 ± 0.329^{b}

*Values represent means and standard deviations (SDs) determined from three independent replications for each treatment (n = 3), each replicated two times. Different letters among the stresses within a variety are significantly different at $p \leq 0.05$, following a least significant difference (LSD) test.

content slightly increased in salt, whereas a significant increase occurred under water deficit and combined stress than the control seedlings.

Na^+ and K^+ homeostasis

A markedly higher Na^+ uptake and Na^+/K^+ ratio was observed in the shoot and root of both varieties treated with saline, drought, and combined stress compared to that of control (Figs. 2A–2F). Conversely, K^+ reduced in both varieties. Exogenous ALA supplementation at 20 μM concentration to salt caused a significant decrease in Na^+ uptake in the shoot of maize seedlings by 853.9 and 836.5%, respectively (Fig. 2B). Accordingly, it decreased Na^+ in the root by 52.8 and 53.0% in BHM-7 and BHM-9, respectively (Fig. 2A). Conversely, it increased K^+ uptake in the shoot by 51.8 and 41.8% and in the root by 35.8 and 69.7% in BHM-7 and BHM-9, respectively. Similar results were found for drought and combined stress (Figs. 2C and 2D). However, ALA at 80 μM was not so effective in single stress (Figs. 2A–2F). Importantly, in combined stress, it significantly reduced the Na^+ and Na^+/K^+ ratio as well as increased K^+ in both varieties.

Reactive oxygen species (ROS) generation, lipid peroxidation (MDA) and lipoxygenase (LOX) activity

The salt, drought, and combined stress triggered an excessive generation of reactive oxygen species (ROS) in the maize seedlings compared to control, resultant boosted lipid

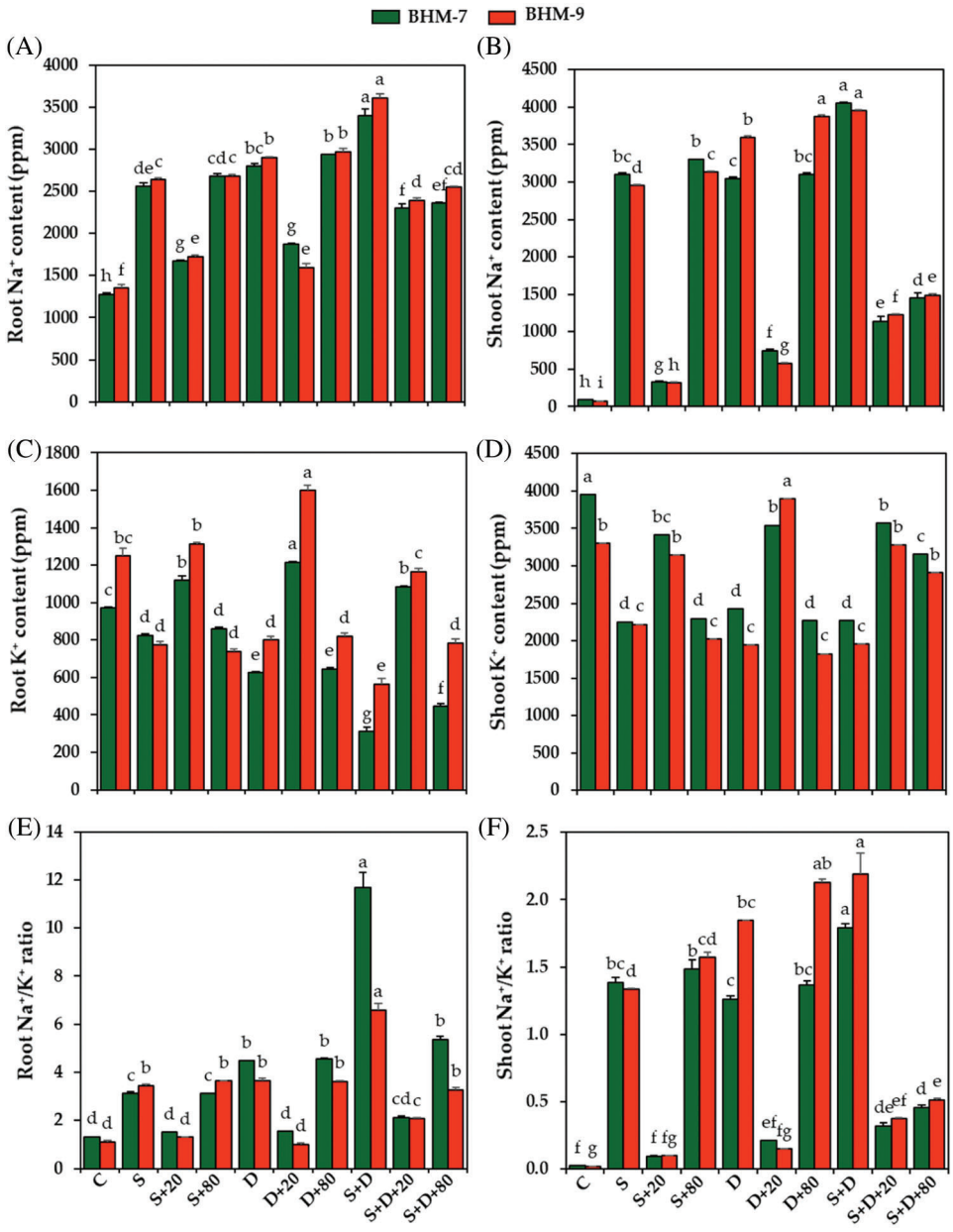

FIGURE 2. Influence of ALA supplementation on (A) root Na^+ content, (B) shoot Na^+ content, (C) root K^+ content, (D) shoot K^+ content, (E) root Na^+/K^+ ratio, and (F) shoot Na^+/K^+ ratio of BHM-7 and BHM-9 after exposing 7 days to salt, drought, and combined stress. Bars represent means and standard deviations (SDs) determined from three independent replications for each treatment (n = 3), each replicated two times. Different letters among the stresses within a variety are significantly different at $p \leq 0.05$. C, control; S, salinity; D, drought; 20, 20 μM ALA; 80, 80 μM ALA.

peroxidation (MDA) and lipoxygenase (LOX) activity (Figs. 3A–3F). Histochemical staining illustrates the excessive production of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) through dark blue spots (Fig. 3A) and brown spots (Fig. 3B), accordingly, in the stress-treated seedlings; spots were more evident in BHM-7 than BHM-9. Supplementation of ALA, especially 20 μM, significantly lessened the spots of $O_2^{\bullet-}$ and H_2O_2 in stress-treated seedlings.

Result also revealed increased $O_2^{\bullet-}$ and H_2O_2 content in seedlings subjected to stresses (Figs. 3C, 3D). Level of $O_2^{\bullet-}$ significantly increased by 206.6, 274.6 and 188.6% under salt, drought and combined stress condition, respectively, in BHM-7; and, in BHM-9 variety rose by 106.4, 38.3 and 197.7% than the control seedlings, respectively (Fig. 3C). In the same way, increment in H_2O_2 level was measured by 12.8, 27.8 and 49.3% in BHM-7; by 460.4, 314.6 and 241.7% in BHM-9 seedlings, exposed to salt, drought and combine stress, in contrast to control seedlings (Fig. 3D). However, supplementing ALA displayed an affirmative result in lessening $O_2^{\bullet-}$ and H_2O_2 level. Conversely, ALA supplementation at higher concentration raised the production of $O_2^{\bullet-}$ (Figs. 3A and 3C) and H_2O_2 (Figs. 3B and 3D) when compared with the lower concentration of ALA treatment.

Revealing to salt of BHM-7 and BHM-9 maize seedlings resulted in augmented MDA (by 16.3 and 76.9%, respectively), activity of LOX (by 238.8 and 9.0%, respectively); exposure to drought caused increased MDA (by 7.2 and 68.4%, respectively), LOX activity (by 129.8 and 117.7%, respectively); and, combined stress triggered MDA (by 67.1 and 96.1%, respectively), LOX activity (by 368.2 and 271.0%, respectively), compared to the control seedlings (Figs. 3E, 3F). Conversely, Exogenous application of ALA at 20 μM concentration reduced the membrane damage, indicated by 75.9, 5.9 and 43.2%, reduction in MDA content, likewise, 131.5, 82.3 and 171.5% decrease in LOX activity in BHM-7; similarly, in case of BHM-9 MDA content lessened by 86.5, 7.8 and 34.8%; and LOX activity diminished by 29.5, 195.0 and 135.1%, respectively, than the

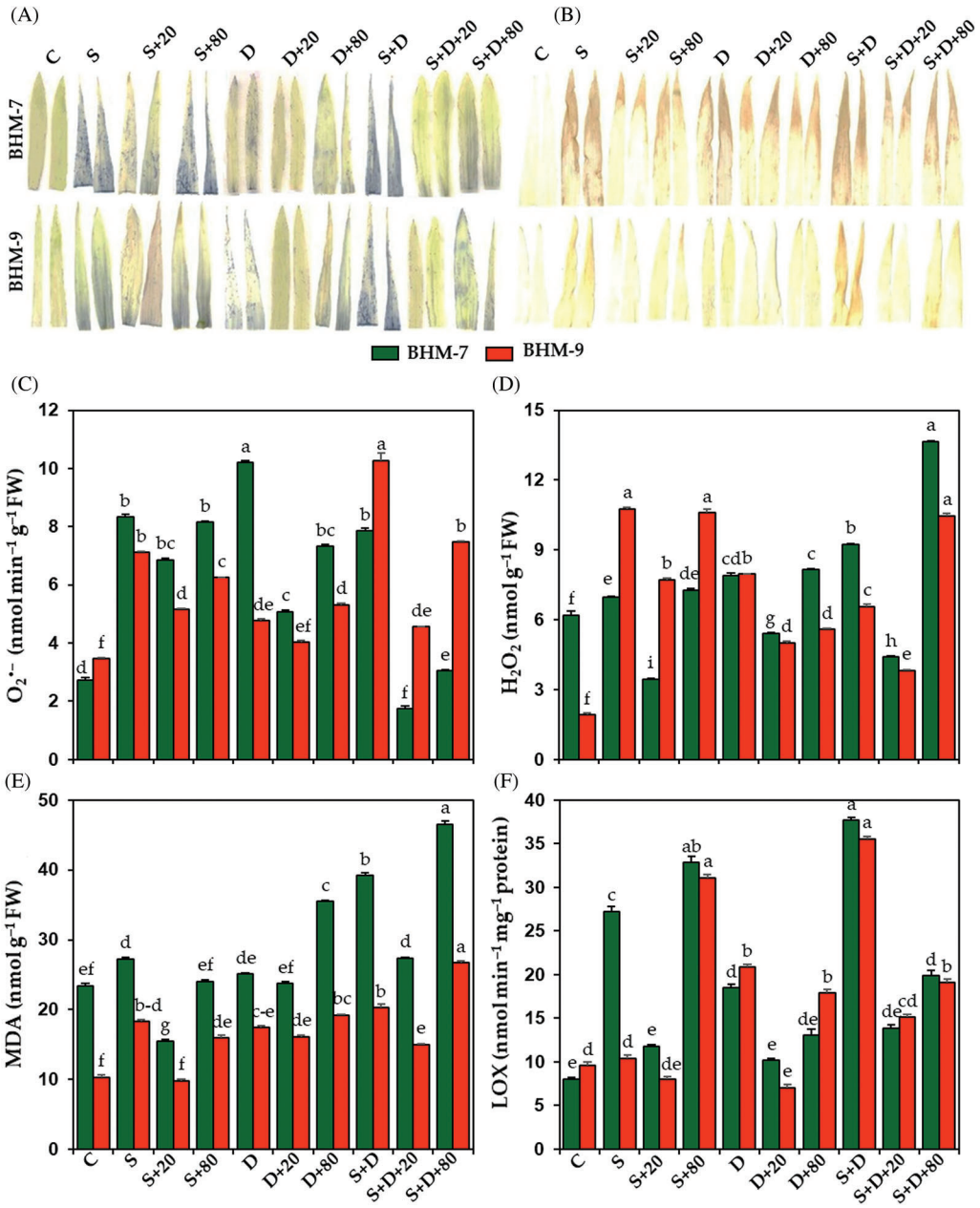

FIGURE 3. Effects of exogenous ALA on reactive oxygen species buildups and lipoxygenase activity in leaves of maize seedlings exposed to salt, drought, and combined stress for 7 days. (A) superoxide ($O_2^{\bullet-}$) tainted with nitro blue tetrazolium (NBT), (B) hydrogen peroxide (H_2O_2) tainted with diaminobenzidine (DAB). Estimation of (C) $O_2^{\bullet-}$, (D) H_2O_2, (E) malondialdehyde (MDA), and (F) Lipoxygenase (LOX) activity in leaves. Bars represent means and standard deviations (SDs) determined from three independent replications for each treatment (n = 3), each replicated two times. Different letters among the stresses within a variety are significantly different at $p \leq 0.05$. C, control; S, salinity; D, drought; 20, 20 µM ALA; 80, 80 µM ALA; FW, fresh weight.

respective solely stress-treated plant (Figs. 3E, 3F). However, applying ALA at 80 µM concentration tremendously triggered the membrane damage and LOX activity again, surprisingly, more than the individual stress treatments (Figs. 3E, 3F).

Activities of antioxidant enzymes

As compared to control, superoxide dismutase (SOD) activity increased in BHM-7 by 50.5, 34.3 and 67.1% in salinity, drought and combined stress, respectively; likewise, in BHM-9 by 47.2, 27.4 and 35.5% in seedlings exposed to salt, drought, and combined stress, respectively (Fig. 4A). Interestingly, exogenously 20 µM ALA application decreased SOD activity significantly in both varieties compared to salinity, but in drought, the activity decreased only in BHM-7. In combined stress, the activity decreased in both varieties, being significantly lower in BHM-9. In contrast, ALA at 80 µM concentration maintained higher activities in all stresses than those at 20 µM concentration (Fig. 4A). Importantly, at higher ALA concentration, BHM-9 showed significantly higher SOD activity than BHM-7 in both drought and combined stress. The changes in the activity levels were also supported by in-gel SOD activity (Fig. 5A).

The maize seedlings, exposed to different stresses had declined catalase (CAT) activity compared to that of control (Fig. 4B). In contrast, 20 µM ALA supplementation significantly stimulated CAT activity in salt-treated BHM-7

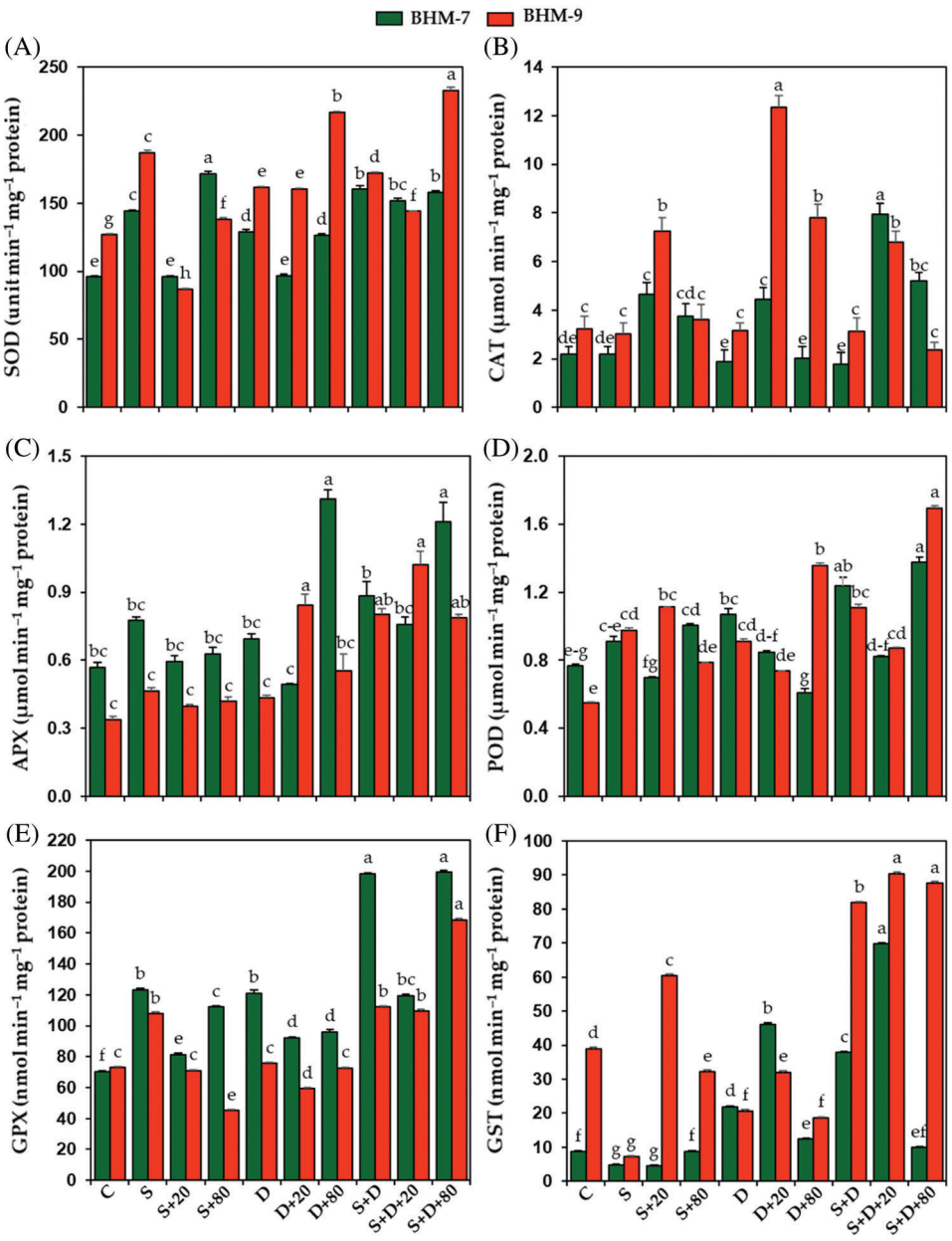

FIGURE 4. Effects of exogenous ALA on the activities of (A) SOD (superoxide dismutase), (B) CAT (catalase), (C) APX (ascorbate peroxidase), (D) POD (peroxidase), (E) GPX (glutathione peroxidase), and (F) GST (glutathione *S*-transferase), in leaves of maize seedlings subjected to 12 dS/m saline, drought (induced by 200 mM mannitol), and combined stress for 7 days. Bars represent means and standard deviations (SDs) determined from three independent replications for each treatment (n = 3), each replicated two times. Different letters among the stresses within a variety are significantly different at $p \leq 0.05$. C, control; S, salinity; D, drought; 20, 20 μM ALA; 80, 80 μM ALA.

and BHM-9 maize seedlings (by 112.3 and 140.9%, respectively), in drought (by 136.0 and 284.4%, respectively), and in combined stress (by 351.1 and 116.2%, respectively), while comparing with the seedlings treated with respective stress. On the contrary, exogenous supplementation of ALA at the rate of 80 μM in stress conditions decreased the CAT activity significantly in comparison with 20 μM ALA treated maize seedlings (Fig. 4B). The in-gel activity of CAT showed a similar banding pattern of the CAT isozymes (CAT*1* and CAT*2*) (Fig. 5B). Though CAT*1* was not so clear, CAT*2* intensity was greatly stimulated in leaves exposed to 20 μM ALA treatment.

Four bands of ascorbate peroxidase (APX) isoforms in the leaves of maize seedlings can be detected (Fig. 5C). In all isoforms band of stresses and 80 μM ALA treatment showed pronounced isozyme activity. The maize seedlings revealed to salt, drought, and combined-stress had escalated APX activity by 35.1, 21.1 and 54.4% in BHM-7, and, by 38.2, 29.1 and 135.3% in BHM-9, respectively, compared with control (Fig. 4C). Nevertheless, exogenous application of ALA at 20 μM decreased APX activity under salt stress in both varieties but again increased at 80 μM treatment, in contrast to solely salt-treated maize seedlings. However, in drought and combined stress conditions when ALA was applied at 20 μM concentration APX activity decreased in the BHM-7 variety but increased in the BHM-9 variety. On the other hand, vice versa result was found at 80 μM concentration. Isozymes (APX*1*, APX*2*, APX*3*, and APX*4*) of APX gave similar changes in the activity by changing band intensity in the gel (Fig. 5C).

Imposition of salt, drought and combined stress radically enhanced the peroxidase (POD) activity by 18.2, 39.0 and 61.0% in BHM-7, while, in BHM-9 it rose by 78.2, 65.5 and 101.8%, respectively, compared to control (Fig. 4D). Exogenous ALA supplementation at the rate of 20 μM in stressed seedlings reduced the POD activities drastically, compared to that of stress condition; whereas, 80 μM exogenous ALA application increased the POD activity

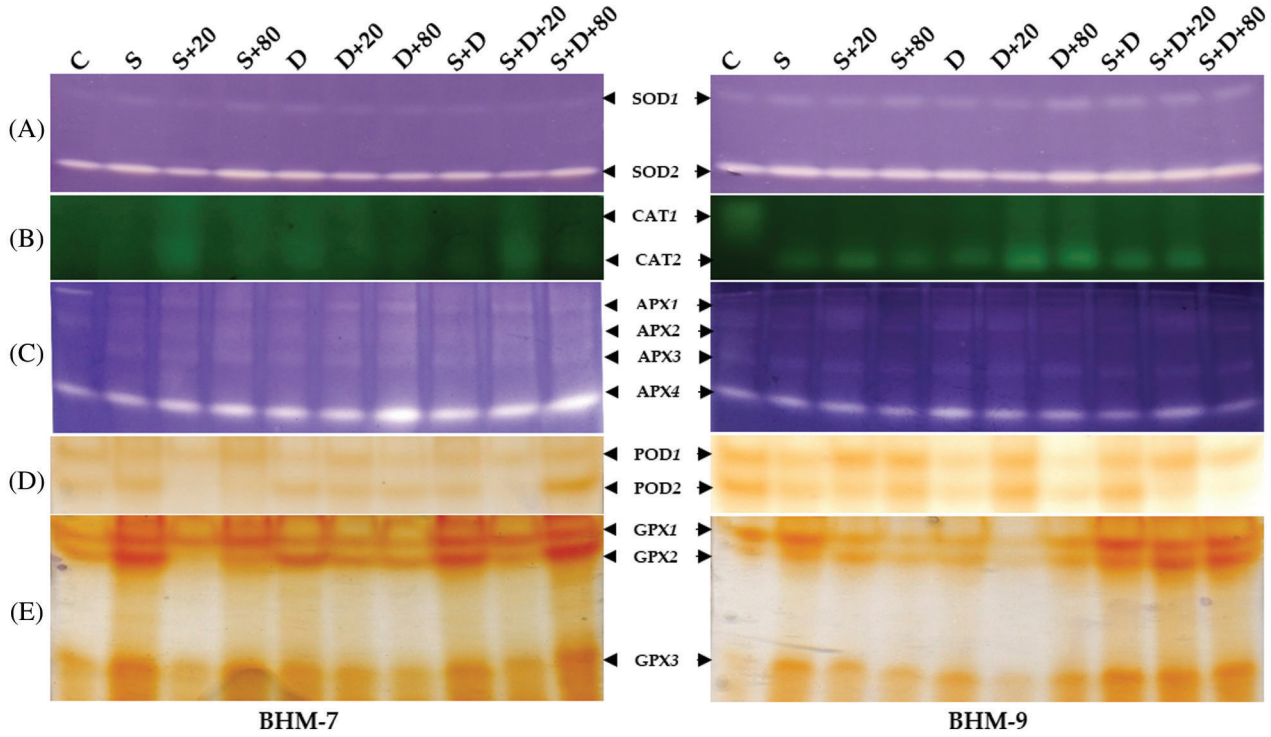

FIGURE 5. Visualization of non-reduced and non-denatured antioxidant enzymes activity in SDS-PAGE gel (10% for SOD, and 8% for others) for detecting proteins (50 µg in each lane) having isoenzymic activity. (A) SOD (superoxide dismutase), (B) CAT (catalase), (C) APX (ascorbate peroxidase), (D) POD (peroxidase), and (E) GPX (glutathione peroxidase), in leaves of maize seedlings subjected to 12 dS/m saline, drought (induced by 200 mM mannitol), and combined stress for 7 days. C, control; S, salinity; D, drought; 20, 20 µM ALA; 80, 80 µM ALA.

significantly even higher than the respective stress treatment (Fig. 4D). In SDS-PAGE, changes in expression of POD isozymes (POD*1* and POD*2*) were found in different treatments (Fig. 5D). However, the appearance of bands was diversified in BHM-7 and BHM-9 variety; it may be because of differences in varietal response.

In contrast to control seedlings, glutathione peroxidase (GPX) activity augmented significantly in BHM-7 variety under salt, drought and combined stress-treated seedlings by 74.7, 71.5 and 181.4%, respectively. Similarly, in BHM-9 variety by 47.8% in salinity and by 53.8% in combined stress. On the contrary, adding ALA at 20 µM concentration lessened the GPX activity in all treatments, but slightly boosted at 80 µM ALA concentration treatment in comparison to that of stresses alone (Fig. 4E). A similar banding pattern of GPX isozymes (GPX*1*, GPX*2* and GPX*3*) was found in gel electrophoresis (Fig. 5E). In addition to this, in combined stress treatments, the GPX activity band was found to be highly induced in salinity and combined stress, particularly with 80 µM ALA (Fig. 5E).

Glutathione *S*-transferase (GST) activity in maize seedlings subjected to stress conditions was not uniform for BHM-7 and BHM-9 variety. On the one hand, in BHM-7 salt stress resulted in decreased GST activity, then, increased in drought and combined stress; then again, the augmented activity of GST in BHM-9 was found only in combined stress condition (Fig. 4F). Outstandingly, compared with the respective stresses, GST activity increased in BHM-7 by 111.0% in drought, by 84.4% in combined stress; whereas, in BHM-9 by 740.1% under salinity, by 55.3% under drought stress, in exogenous 20 µM ALA supplementation treatment; nevertheless, drastically decreased in 80 µM ALA treatment except for salt stress in BHM-7 (Fig. 4F).

Discussion

Plants exposed to salinity, drought, and combined stress condition causes osmotic stress, and inhibits the growth, by instigating higher Na^+ accumulation and interrupting ion homeostasis (Tuncturk *et al.*, 2008; Munns, 2011). In the current research, stresses ensuing growth inhibition of maize seedlings in respect of shoot height, and root length and volume were noted, which were found to be renovated with a low concentration of ALA (20 µM) supplementation (Fig. 1; Tab. 1). The improvement of growth inhibition convincingly supported by our findings of amended ion homeostasis and suppressing water deficiency caused by osmotic stress, which is consistent with previous findings (Nunkaew *et al.*, 2014). Plant growth improvement by low concentration ALA treatment was also elucidated by Hotta *et al.* (1997), besides, in our study herbicidal response was found at 80 µM, and it was validated by the phenotypic outcomes of the current study.

Abiotic stresses affect the photosynthesis negatively by degrading the photosynthetic pigments, causing leakage in Chl molecules, and also reducing Chl contents (Kumar *et al.*, 2017; Mehta *et al.*, 2010). Certainly, in this study, maize seedlings subjected to stresses showed notable losses of Chls (Tab. 2) and carotenoid (Tab. 3). These results revealed that under stress conditions due to interrupted photosynthetic activity photosynthetic pigments supply was also disturbed, and consequently decreased the growth

and caused chlorosis (Fig. 1). Nevertheless, after the application of ALA, the content of Chls increased, even more than the control level. As we know, Chl is the successor of the ALA precursor (Schlicke *et al.*, 2014), and in this study, Chl biosynthesis was promoted by the supplementation of ALA. Ultimately, results showed that both 20 μM and 80 μM ALA supplementations improved the level of Chls of maize seedlings exposed to salt, drought, and combined stress (Tab. 2). Moreover, abiotic stresses promote chlorophyllase enzyme activity and/or boost ROS overproduction, and causes disruption of pigment-protein complex and lessen the photosynthetic pigments (Hasanuzzaman *et al.*, 2014b; Saha *et al.*, 2010), and in this study, supplementation of ALA restored and/or increases Chl contents of the leaf, it may by decreasing the activity of chlorophyllase enzyme.

Soluble proteins and Pro are vital osmotic protective elements in plants, they help to reduce osmotic potential under abiotic stresses (Arndt *et al.*, 2001). Stresses affected plants uptake different inorganic ions, produce and pile up numerous osmoprotective substances and trivial organic molecules (Qados, 2011). Pro is crucial to fight against abiotic stresses, as it possesses osmoprotective and antioxidative potential (Nahar *et al.*, 2016). Its concentration in cytosol increases with the exposure to abiotic stresses, as a result, water potential in cell decreases, and absorption of water increases (Kasuga *et al.*, 1999). Since, salt and drought stress causes physiological water shortage by means of interrupted water absorption and exosmosis, which results from osmotic stress and ion toxicity (Munns, 2011); in our study, maize seedlings exposed to stress showed elevated Pro content (Tab. 3), signifying stress-induced osmotic stress and malicious water content. This outcome is sturdily reinforced by Hasanuzzaman *et al.* (2014b). However, exogenous ALA application at 20 μM reduced Pro deposition in the cell might be due to relief to some extent from all types of stresses as phenotypic appearances of maize seedlings exposed to low ALA treatment had better phenotype than that in stresses without ALA (Fig. 1; Tab. 3). On the contrary, a higher dose of ALA showed the opposite result and proved its detrimental effect on plant growth. However, consistent result regarding the role of ALA on soluble proteins and Pro accumulation under salt and drought stress was also testified by Kosar *et al.* (2015) and Akram *et al.* (2012).

Na^+-induced K^+ efflux is the instant and prime reaction from plants subjected to an elevated level of salts (Anschuetz *et al.*, 2014; Bose *et al.*, 2014). Salt and drought-induced stress disrupt the ratio of Na^+/K^+ (Simaei *et al.*, 2012; Ali and Rab, 2017), and homeostasis of ions (Tuncturk *et al.*, 2008). In the study, salt, drought, and their combination-induced stress raised Na^+ content and declined K^+ content in maize seedlings' shoots and roots, which could result from the engross of greater levels of Na^+ into the plant (Fig. 2). Higher accumulation of Na^+ also causes a higher ratio of Na^+/K^+, which interferes with ion homeostasis by lowering the content of Mg, Mn, and Zn. The Na^+ influx and K^+ leakage possibly be also for higher ROS production, as our result showed. A similar disruption of ion homeostasis was demonstrated in previous studies under elevated salt and deficit water stress conditions (Ali and Rab, 2017; Demidchick and Maathuis, 2007; Wu and Wang, 2012). It was noted, nevertheless, that exogenous ALA at low concentrations decreased the Na^+ concentration in the cotton plant roots, and it was assumed that in growth media the manifestation of ALA could lead to a decrease in Na^+ uptake and could overcome water shortage due to osmotic stress induced by high Na^+ concentration nearby the roots (Watanabe *et al.*, 2000). Our results are also steady with the findings of Naeem *et al.* (2010); ALA applied to oilseed rape seedlings, grown under high saline stress drastically decreased Na^+ and K^+ accumulation, resulting in reduced Na^+/K^+ ratio in both roots and leaves than the control plants. Moreover, it has also been stated that elevated Na^+ buildup in the leaves in contrast to roots indicates that ALA does not stimulate Na^+ transportation from the roots to the shoots, but somewhat may withhold Na^+ ions uptake to the roots from the growth media. Youssef and Awad (2008) reported that decreased Na^+ accumulation in the leaves and lessened K^+ uptake by the roots, which resulted in a concurrent reduction in the K^+/Na^+ ratio. However, higher Na^+ and Na^+/K^+ ratio in single stresses at 80 μM ALA than 20 μM is not clear. One of the causes might be that higher ALA in single stress can encourage Na^+ uptake, and transportation of higher Na^+ can be arrested in xylem, another salt tolerance mechanism of the plant (James *et al.*, 2006), because those values in the leaf were comparatively lower at that concentration of ALA (Supplementary Fig. 1). In the present study, exogenous application of ALA at 20 μM concentration amended ion homeostasis by reducing Na^+ uptake and ROS generation, as verified by our results; or may be by relaxing the effects of salt and drought on maize seedlings (Fig. 2), as depicted by Ali and Rab (2017), and Watanabe *et al.* (2000). In contrast, external supplementation of ALA at higher concentration showed damaging effects on plants (Hara *et al.*, 2011), consistent result with this statement has been found in this study in the case of exogenous ALA application at 80 μM treatment (Fig. 2). Nevertheless, the role of ALA on Na^+/K^+ ratio and ion homeostasis on different plants exposed to abiotic stresses was reported previously in several studies (Ali *et al.*, 2014; Liu *et al.*, 2014; Akram and Ashraf, 2011); which are consistent with our present results.

Abiotic stresses alter plants with an adverse effect on the gas exchange, resultant in low CO_2 integration for photosynthesis and consequently a substantial drop in electron transport ensuing in increased ROS production (Gill and Tuteja, 2010; Møller *et al.*, 2007). In both cases, the generation of ROS is enhanced, which is extremely cytotoxic to cell organelles such as protein, DNA, lipid, and pigment (Gill and Tuteja, 2010). In this analysis, substantially elevated ROS ($O_2^{\bullet -}$, H_2O_2) was observed in salt, drought, and combined stressed seedlings than the control treatment (Figs. 3A–3D), indicated oxidative eruptions in maize leaf tissues. Elevated ROS causes lipid peroxidation, a latent biomarker of oxidative damage to the membranes, leading to electrolytes leakage, loss of membrane permeability, and degradation of membrane proteins and ion channels (Munns and Tester, 2008; Turan and Tripathy, 2013; Nedjimi, 2014). In the current study,

elevated ROS caused higher lipid peroxidation product (MDA) together with its associated enzyme, LOX (Fig. 3E, 3F). Thus, elevated ROS and MDA in maize seedlings can impair the root and shoot tissue. Our previous study also documented oxidative damage in maize resulted from salt stress (Rohman *et al.*, 2019). However, a number of researchers documented significantly higher rates of $O_2^{\bullet-}$, H_2O_2, and MDA than controls in mustard (Liu *et al.*, 2011; Ahmad *et al.*, 2012), cucumber (Li *et al.*, 2011), and maize (Rohman *et al.*, 2018), under salt and drought stress. Exogenous ALA was also reported to mitigate ionic imbalance, ROS bursts, and MDA content under salinity in beet seedlings (Liu *et al.*, 2014), and under drought in cucumber seedlings (Li *et al.*, 2011). Under abiotic stress, including salinity, ALA mediated changes in some of the main physiological attributes in plants and accumulates sugars and other corresponding solutes that assist as an osmoprotectant and, in certain cases, stabilized biomolecules under stress conditions (Akram *et al.*, 2012). Increased $O_2^{\bullet-}$ and H_2O_2 at higher ALA could be resulted from strongly inhibition of CAT (Figs. 4B and 5B); because, CAT possesses one of the highest turnover rates for all enzymes: a single molecule of CAT can convert ≈ 6 million molecules of H_2O_2 to H_2O and O_2 per minute (Gill and Tuteja, 2010). However, the higher concentration of ALA itself can also increase the density of growing media to create osmotic stress.

In the present study, the application of ALA at a concentration of 20 μM demoted salinity, drought, and combined stress-mediated elevated concentrations of $O_2^{\bullet-}$, H_2O_2, and MDA and activity of LOX in all stress treatments (Fig. 3). In all stress's MDA production was clearly linked to ROS generation and LOX activity. This is in line with other salinity and drought-imposed studies (Liu *et al.*, 2011; Ahmad *et al.*, 2012; Zhen *et al.*, 2012). Link amid these can be clarified by taking the antioxidative activities in mind. In addition, ALA mediated LOX activity inhibition is expected to diminish the generation of MDA in the maize leaf. On the contrary, ALA at higher concentration (80 μM) acted as the stress treatments alone, as a result, it increases ROS production ($O_2^{\bullet-}$ and H_2O_2), prompted MDA content and enhanced the LOX activity. Our findings are in harmony with the study of Liu *et al.* (2011). Definitively, in this study, low ALA treatment imposed to salt, drought, and combined stress, protected the plants by minimizing the ROS and MDA production, as well as, by reducing the LOX activity, which strongly supports our other findings, like, plant growth and lower Na^+/K^+ ratio; conversely, supports the herbicidal effect of ALA at higher concentration.

Among the antioxidant enzymes, SOD is thought to be a first-line enzymatic defense in regulating ROS, which alters $O_2^{\bullet-}$ to H_2O_2 (Gill *et al.*, 2015), and then CAT detoxifies H_2O_2 readily to H_2O and O_2 (Sanchez-Casas and Klesseg, 1994). The present work exhibited a substantial rise in SOD activity and a decline in CAT activity under conditions of salt, drought, and combined stress (Fig. 4A, 4B), these are consistent with earlier reports (Mishra *et al.*, 2013; Wutipraditkul *et al.*, 2015). This improved SOD activity may be attributed to increased production of $O_2^{\bullet-}$ and H_2O_2 and reduced CAT activity as a result of greater production of H_2O_2, which was caused by oxidative stress, this result is approvingly supported by former research works (Rohman *et al.*, 2016a; Rohman *et al.*, 2016b; Hasanuzzaman *et al.*, 2014b; Mishra *et al.*, 2013; Nahar *et al.*, 2015). Most interestingly, the exogenous application of ALA showed two different results in two different concentrations for SOD and CAT activity. Where, 20 μM ALA decreases SOD activity, but, increases CAT activity significantly; on the other hand, the supplication of ALA at 80 μM concentration enhanced SOD activity, while, reduces CAT activity under stresses. From this, it can be suggested that CAT is the most active antioxidant, which respond significantly in low concentration ALA treatment during this experiment, which greatly supports the morphological appearance of the stressed plant and minimized production of $O_2^{\bullet-}$ and H_2O_2. A parallel result was not reported yet. However, several results were found previously concluded that ALA supplementation increased both SOD and CAT activities and scavenged ROS simultaneously (Liu *et al.*, 2011; Zhang *et al.*, 2013; Niu *et al.*, 2017). As CATs are tetrameric heme-containing enzymes so exogenous ALA application might have some direct relation with CAT activity but the underlying process is yet to find. Further, in gel electrophoresis the expression of SODs and CATs were detected (Figs. 5A, 5B), which strengthened the present findings.

Ascorbate peroxidase is one of the essential enzymes of the AsA-GSH cycle and acts against oxidative stress along with AsA and GSH by reducing the generation of ROS and recycling of AsA and GSH (Mishra *et al.*, 2013). Ascorbate peroxidase converts H_2O_2 to produce H_2O by the oxidation of AsA to DHA. In the current study, the APX activity increased with salt, drought, and combined stress (Figs. 4C and 5C). The stimulation of this behavior of the antioxidant enzyme suggests its association with oxidative stress tolerance (Mishra *et al.*, 2013). The improved activity of APX may have been attributed to the higher content of H_2O_2. This observation is consistent with earlier studies (Hasanuzzaman *et al.*, 2014b; Mishra *et al.*, 2013; Ozfidan-Konakci *et al.*, 2015). However, the exogenous ALA application showed a confusing result. In both varieties, ALA at 20 μM decreased APX activity under salt stress but boosted at 80 μM treatment contrasted to only salt-treated maize seedlings. Conversely, in drought and combined stress conditions when ALA was applied at 20 μM concentration APX activity decreased in BHM-7 variety but increased in BHM-9 variety. On the other hand, vice versa result was found at 80 μM concentration. Maybe the reason behind this, the activity of different antioxidants varied in different varieties.

A heme-containing enzyme is POD, which is essential in stressed conditions for balancing of reactive intermediate products of O_2 and peroxide radicals (Vangronsveld and Clijsters, 1994). In this analysis, salinity, drought, and combined stress in both varieties significantly induced POD activity compared to control activity (Figs. 4D and 5D). POD, therefore, is crucial in the metabolism of ROS and thus conferred tolerance under all the stresses on maize seedlings. In our previous research, the induced behavior of POD under salinity and drought was also recorded in maize (Rohman *et al.*, 2019), mustard (Liu *et al.*, 2011; Ahmad

et al., 2012), and cucumber (Li *et al*., 2011; Zhen *et al*., 2012). In this study, in all stresses while ALA was applied at 20 µM concentration inhibited the activity, but salinity; on the other hand, at higher concentration ALA enhanced POD activity significantly, but in the drought condition of BHM-7. Although increased activity was documented in several studies due to ALA application in salinity and drought treated plants (Ahmad *et al*., 2012; Li *et al*., 2011); however, ALA mediated this form of POD activity in combined stressed maize or other plants has not been documented until now.

The GPX and GST enzymes function collectively to defend plants by creating comparatively less noxious and conjugates, which are water-soluble under oxidative stress through catalyzing the bonding of various xenobiotics and their electrophilic metabolites (Gill and Tuteja, 2010). In this study, the stress-persuaded oxidative stress redoubled GPX activity, nonetheless, declined GST activity (Figs. 4E, 4F and 5E). On the one hand, this increased GPX activity may be because of over-production of H_2O_2 and its' inadequate detoxification; on the other hand, reduced GST activity is due to increased GPX activity, because, as we know, GPX uses GSH for its activity, and GSTs works by GSH conjugation (Cummins *et al*., 2011). This result is partially consistent with Rohman *et al*. (2016b), and Hasanuzzaman *et al*. (2014b), who described the escalated activity of GPX and GST under saline-induced oxidative stress situations. However, exogenous ALA application at 20 µM in the stress-treated maize seedlings decreased GPX activity, but increased GST activity (Figs. 4E, 4F and 5E); increased GST might play a role in detoxifying H_2O_2 and in apoptosis to protect the plant. In addition, GST assisted in herbicide detoxification in high concentrations of ALA and this is why GPX activity increased. Dixon *et al*. (2010), and Mostofa *et al*. (2019) strongly support this result.

To sum up, not only we found that low concentration of ALA has significant optimistic role in plants but also its detrimental effect at higher concentration. An unswerving result was found by Liu *et al*. (2011), on mustard plant. They said that ALA treatment at higher concentrations killed the plants. Their finding importantly assists the result of the present study, where 80 µM ALA with salt and drought stress hampered the plant growth notably.

Conclusions

It may be deduced from this study that, salt, drought, and combined stress disrupt the antioxidant defense system of plants by increasing ROS production. Maize seedlings received the highest amount of damage under the combined stress condition; phenotypic appearance, morphological, physiological, and biochemical data confirmed that. ALA application exogenously at 20 µM concentration to stress-treated maize seedlings increased the detoxification of over-produced ROS by strengthening antioxidant protection specifically CAT function, excluding salt and drought stress-exerted oxidative stress; additionally, GST might have an imperative function in herbicidal detoxification generated by ALA application. However, under stress conditions, a lower dose of ALA showed a depressing response to most of the activity of antioxidant enzymes, except CAT and POD (in salt). Contrarily, a higher concentration of ALA improved the enzymatic activity of antioxidants (SOD, POD, APX, and GPX) but failed to ROS scavenging, which proved that exogenous ALA had a positive effect when applying at low concentration but demonstrate detrimental result when applying at higher concentration. Moreover, the gel activity of SOD, POD, CAT, APX, and GPX inveterate these findings. Importantly, high concentration application of ALA under combined stress has played a vital role in increasing antioxidant activity by destroying plant roots and preventing a greater accumulation of ALA in plants. So, a higher concentration of ALA application in the combined stress condition acted as a low concentrated ALA.

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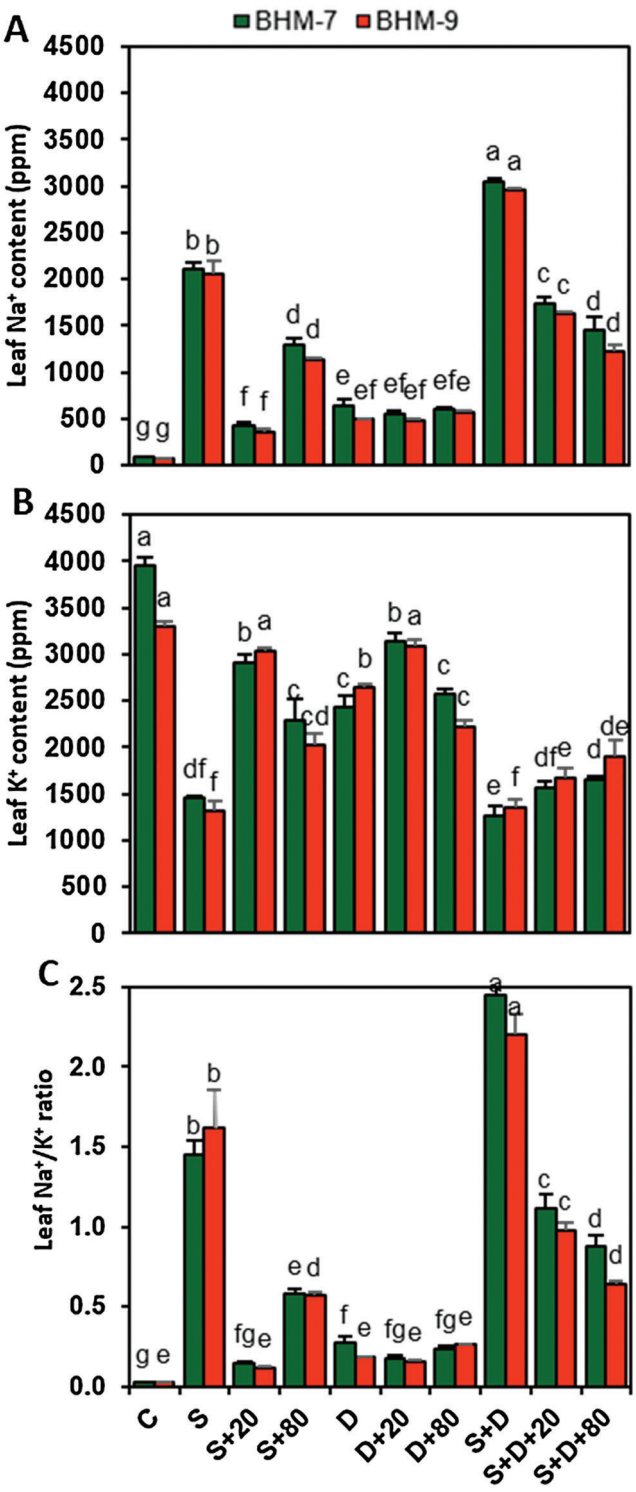

SUPPLEMENTARY FIGURE 1. Influence of ALA supplementation on (A) leaf Na^+ content, (B) leaf K^+ content, and (C) leaf Na^+/K^+ ratio of BHM-7 and BHM-9 after exposing 7 days to salt, drought, and combined stress. Bars represent means and standard deviations (SDs) determined from three independent replications for each treatment (n = 3), each replicated two times. Different letters among the stresses within a variety are significantly different at $p \leq 0.05$. C, control; S, salinity; D, drought; 20, 20 μM ALA; 80, 80 μM ALA.