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Exogenous salicylic acid ameliorates short-term drought stress in mustard (*Brassica juncea* L.) seedlings by up-regulating the antioxidant defense and glyoxalase system

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Abstract

In this study, the protective role of salicylic acid (SA) in relation to the water status, chlorophyll content, antioxidant defense and glyoxalase system was investigated in drought stressed mustard (*Brassica juncea* L. cv. BARI Sharisha 11) seedlings. Two sets of 10-d-old seedlings were subjected to two different levels of drought (10% and 20% PEG, 48h), where one set of seedlings was supplemented with 50 μM SA. The relative water content (RWC), Chl *b* and Chl (*a*+*b*) decreased at any level of drought, while Chl *a* content decreased only at severe drought (20% PEG). Drought stress caused a sharp increase in proline (Pro) content. A sharp increase in malondialdehyde (MDA) and H_2O_2 was observed in both levels of stresses. Decline in AsA content and increase in GSH and GSSG content was observed at both levels of drought. Compared to control the activities of catalase (CAT) and monodehydroascorbate reductase (MDHAR) did not change due to drought stress. The activity of glutathione reductase (GR) slightly increased only at 10% PEG, while ascorbate peroxidase (APX) and glutathione *S*-transferase (GST) activity increased at any level of stress. The activities of glutathione peroxidase (GPX) and glyoxalase II (Gly II) decreased only at severe stress (20% PEG), while dehydroascorbate reductase (DHAR) and glyoxalase I (Gly I) activities decreased at any level of stress. Spraying with SA alone had little influence on the non-enzymatic antioxidants and the activities of antioxidant enzymes. However, supplementation of SA in drought stressed seedlings increased the RWC and Chl content, increased the AsA and GSH, decreased the GSSG content and maintained a higher ration of GSH/GSSG. Salicylic acid supplemented drought stressed seedlings also enhanced the activities of MDHAR, DHAR, GR, GPX, CAT, Gly I, and Gly II as compared to the drought-stressed plants without SA supplementation, with a concomitant decrease in H_2O_2, and lipid peroxidation level. These results suggest that the exogenous application of SA assisted the plants to become more tolerant to drought stress-induced oxidative damage by enhancing their antioxidant defense and glyoxalase systems.

Keywords: Abiotic stress tolerance; AsA-GSH cycle; Methylglyoxal; Oxidative stress; Polyethylene glycol.
Abbreviations: AO- ascorbate oxidase; APX- ascorbate peroxidase; AsA- ascorbic acid; BSA- bovine serum albumin; CAT- catalase; CDNB- 1- chloro-2, 4-dinitrobenzene; Chl- chlorophyll; DHA- dehydroascorbate; DHAR- dehydroascorbate reductase; DTNB- 5,5′-dithio-bis (2-nitrobenzoic acid); EDTA- ethylenediaminetetraacetic acid; Gly I- glyoxalase I; Gly II- glyoxalase II; GR- glutathione reductase; GSH- reduced glutathione; GSSG- oxidized glutathione; GPX- glutathione peroxidase; GST- glutathione *S*-transferase; MDA- malondialdehyde; MDHA- monodehydroascorbate; MDHAR- monodehydroascorbate reductase; MG- methylglyoxal; NADPH- nicotinamide adenosine dinucleotide phosphate; NTB- 2-nitro-5-thiobenzoic acid; PEG- polyethylene glycol; Pro- Proline, ROS- reactive oxygen species; RWC- relative water content; SA- salicylic acid; SLG- *S*-D-lactoylglutathione; TBA- thiobarbituric acid; TCA- trichloroacetic acid.

Introduction

Drought is one of the major abiotic stresses which adversely affects crop growth and yield and thus a constraint for plant productivity worldwide (Jaleel et al. 2009; Hasanuzzaman et al. 2010; Hasanuzzaman and Fujita 2011; Hasanuzzaman et al. 2012a). The effects of drought stress are expected to increase more with climate change and a growing water crisis. Drought stress adversely affects a variety of vital physiological and biochemical processes in plants (Hasanuzzaman et al. 2012a). A common phenomenon of drought is increased production of reactive oxygen species (ROS) like superoxide radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•) (Hasanuzzaman et al. 2012a; 2013). Loss of water, decay of photosynthetic pigments and lipid peroxidation are also significantly accelerated due to drought stress (Jaleel et al. 2009; Hasanuzzaman and Fujita 2011; Kadioglu et al. 2011). If drought stress is delayed for long time, damage of the antioxidant system occurs due to excessive ROS production which results in cellular damage and death (Reddy et al. 2004; de Carvalho 2008). These ROS are extremely reactive in nature because they can interact with a number of cellular molecules and metabolites, thereby leading to irreparable

metabolic dysfunction and death. The enhancement of antioxidant defense mechanisms is considered to be an adaptive mechanism of plants to drought stress and the strengthening of these defense mechanisms, through the enhanced functions of antioxidant components, may reduce or prevent oxidative damage and improve the drought resistance of plants (Hasanuzzaman and Fujita 2011; Hasanuzzaman et al. 2012a). To counteract the toxicity of ROS a highly efficient antioxidative defense system, composed of both non-enzymatic and enzymatic constituents, is present in all plant cells. The enzymatic antioxidant defense mechanisms are represented by the enzymes, superoxide dismutase (SOD); 4 enzymes of the ascorbate-glutathione cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR); catalase (CAT); glutathione peroxidase (GPX); and glutathione *S*-transferase (GST). Non-enzymatic antioxidants include carotenoids, ascorbte (AsA), glutathione (GSH), flavanones and anthocyanins. (Noctor and Foyer 1998; Gill and Tuteja 2010). Besides ROS, Methylglyoxal (MG) is another highly toxic compound for plant the concentration of which must be kept under control. However, it is generally detoxified by the glyoxalase system consisting two enzymes viz. Glyoxalase I (Gly I) and Glyoxalase II (Gly II) (Yadav et al. 2005a, b). The Gly I converts MG to S-D-lactoylglutathione by using GSH and while Gly II converts S-D-lactoylglutathione to D-lactic acid and during this reaction GSH is regenerated. Several reports indicated that the upregulation or overexpression of Gly I and Gly II alleviates the abiotic stresses by detoxifying MG in plants and enhanced tolerance to plants (Hoque et al. 2007; Hasanuzzaman and Fujita 2011). Several research results support the notion that coordinated induction and regulation of the antioxidant and glyoxalase pathway enzymes are necessary to obtain substantial tolerance against oxidative stress and detoxification of both ROS and MG that might be a strategy for tolerance against various abiotic stresses (Singla-Pareek et al. 2008; Hasanuzzaman et al. 2011a, b; Hasanuzzaman et al. 2012b). Salicylic acid (SA) is an endogenous growth regulator of phenolic nature and also an important signaling molecule which regulates physiological processes in plants such as growth, photosynthesis and some other metabolic processes (Syeed et al. 2011; Nazar et al. 2011; Khan et al. 2012). Several studies support a major role of SA in modulating the plant response to various abiotic stresses (Hayat et al. 2008; 2010 Kadioglu et al. 2011). In addition, it has been found that plants treated with SA generally exhibited better resistance to drought stress (Al-Hakimi and Hamada 2001; Hayat et al. 2008; Kadioglu et al. 2011). It is a well-found fact that SA potentially generates a wide array of metabolic responses in plants and also affects plant water relations (Hayat et al. 2010). Treatment of drought stressed plants with exogenous SA was found to be effective in modulating both enzymatic and non-enzymatic components of antioxidant defense system (Wang et al. 2004; Kadioglu et al. 2011). To the best of our knowledge, there are not enough data about the effect of SA treatment on antioxidant defense as well as glyoxalase enzymes. Therefore, the objective of our study was to observe the beneficial role of SA in enhancing the activities of antioxidant enzymes and glyoxalase pathway enzymes as well as relative water content (RWC), chlorophyll (Chl) and proline (Pro) content under drought stress condition in mustard seedlings.

Results

Relative water content

Leaf relative water content (RWC) of mustard seedlings was decreased significantly upon exposure to drought stress. In the media containing 10% and 20% of PEG, RWC decreased by 46% and 53%, respectively compared to control (Fig. 1A). Non-stressed seedlings which were sprayed with SA did not show any differences in RWC compared to control. The drought-stressed seedlings sprayed with SA showed 40 and 50% increase in RWC at 10 and 20% of PEG, respectively compared to the drought imposed seedlings which were not sprayed with SA (Fig. 1A).

Chlorophyll and proline content

Chlorophyll *a* content of the leaves did not changed under 10% PEG, however, it decreased significantly under 20% PEG (39% lower than control) (Fig. 1B). Salicylic acid treated control seedlings also showed decrease in Chl *a* content. Compared to drought stress alone, the seedlings exposed to 20% PEG supplemented with SA spray showed 31% higher Chl *a* content. The Chl *b* content also significantly decreased under any level of drought stress (47 and 59% lower at 10 and 20% of PEG, respectively). However, the seedlings sprayed with SA showed significantly higher amount of Chl *b* content (79% higher) compared to stress (20% PEG) alone (Fig. 1C). Consequently, Chl (*a*+*b*) content also markedly decreased under drought stress which was measured as 30 and 48% lower at 10 and 20% PEG, respectively compared to control (Fig. 1D). However, the drought stress seedlings when supplemented with SA, showed significantly higher amount of Chl (*a*+*b*) compared to stress alone. Drought stress caused a profound increase in Pro content in mustard seedlings. Upon exposure to 10 and 20% PEG, Pro contents increased by 501 and 896%, respectively compared to control (Fig. 2). On the other hand, SA supplemented drought stressed (20% PEG only) seedlings maintained the Pro content significantly lower than the seedlings treated with PEG only.

Levels of MDA and H_2O_2

A sharp increase in MDA content (the product of lipid peroxidation) was observed under drought stress. The seedlings sprayed with 10 and 20% PEG caused 58 and 179% increase in MDA content compared to control (Fig. 3A). On the other hand, SA supplemented drought-stressed seedlings showed significantly lower MDA content compared to drought exposed seedlings without SA (22 and 32% lower compared to the seedlings exposed to 10 and 20% PEG only). Compared to control, the levels of H_2O_2 increased by 41 and 95% with 10 and 20% PEG, respectively. However, SA sprayed seedlings maintained same level of H_2O_2 as control (Fig. 3B). Importantly, a significant reduction of H_2O_2 was observed in SA supplemented drought-stressed seedlings compared to the seedlings expose to drought only.

Contents of ascorbate and glutathione

A significantly decline in AsA content was observed in drought stressed mustard seedlings (14 and 34% lower at 10 and 20% PEG, respectively) compared to untreated control (Fig. 4A).

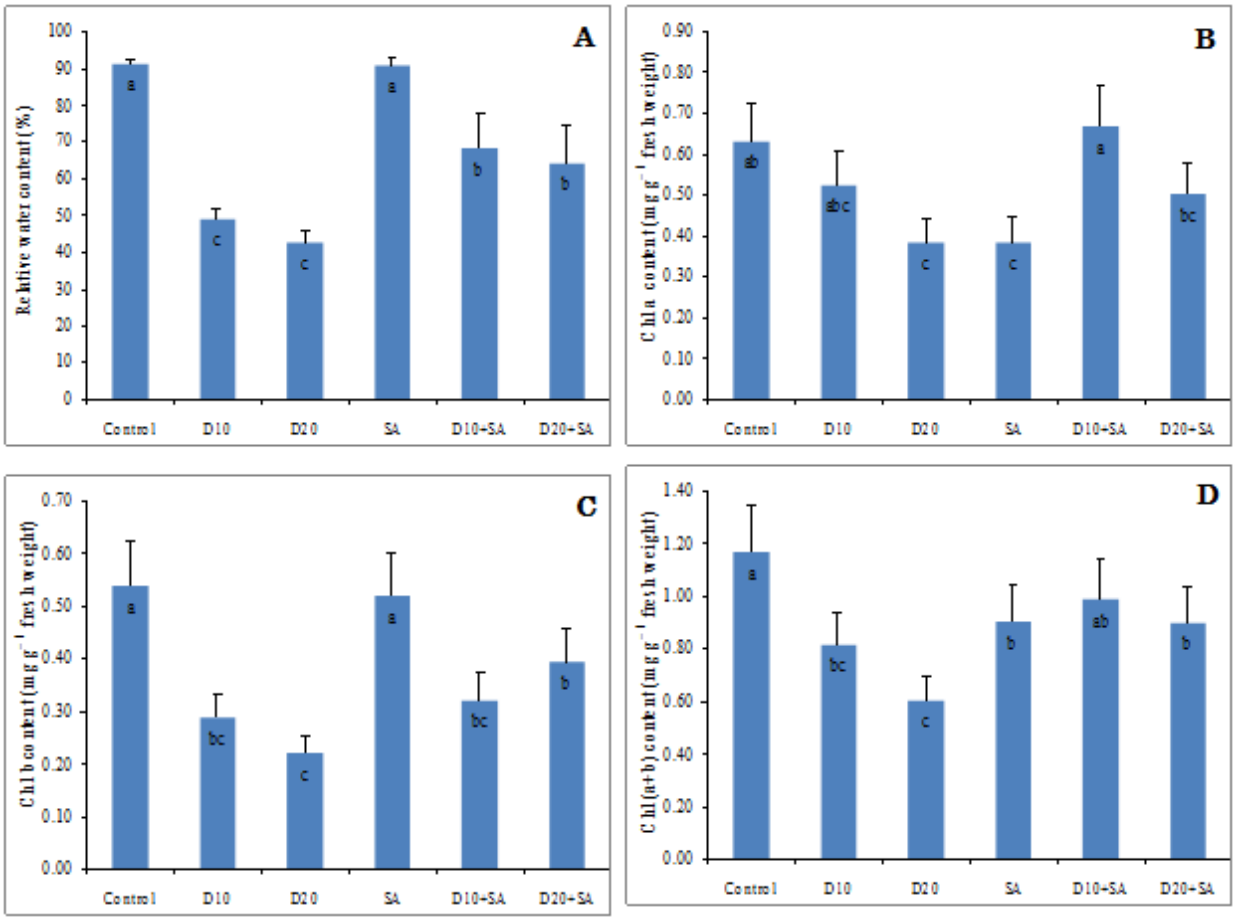

Fig 1. A: Leaf relative water content (RWC), B: chlorophyll a (Chl *a*), C: chlorophyll b (Chl *b*) and D: chl (*a*+*b*) content in mustard seedlings induced by salicylic acid under drought stress conditions. D_{10}, D_{20}, SA, D_{10}+SA and D_{20}+SA indicates 10% PEG, 20% PEG, 50 µM salicylic acid, 10% PEG+50 µM SA, 20% PEG+50 µM SA, respectively. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying LSD test.

The SA supplemented drought-stressed seedlings significantly restored the AsA content (17 and 28% at 10 and 20% PEG, respectively) compared to the drought stressed seedlings without SA. Compared to control GSH contents were increased by 32 and 25% under 10 and 20% PEG, respectively (Fig. 4B). Moreover, SA supplemented drought-stressed seedling showed further increase in GSH content compared to drought stress alone and control as well. GSSG content markedly increase with increasing levels of drought and it was observed that compared to control the GSSG contents were 48 and 101% higher at 10 and 20% PEG, respectively (Fig. 4C). In contrary, the seedlings which were sprayed with SA showed lower GSSG levels similar to control. GSH/GSSG ratio showed no change under mild stress (10% PEG), while it significantly decreased (34% lower than control) upon exposure to severe drought stress (20% PEG). However, SA supplemented drought stressed seedlings showed significantly improved GSH/GSSG ratio (68 and 90% higher with 10 and 20% PEG) compared to drought stress alone (Fig. 4D).

Activities of antioxidant enzymes

Compared to control 24 and 33% increase in APX activities were observed at 10 and 20% PEG, respectively (Fig. 5A). Spraying of drought stressed seedlings with SA could not improve the APX activities at any level. MDHAR activity was not changed upon exposure to drought stress (Fig. 5B). However, the seedlings supplemented with SA spray showed significantly higher activities of MDHAR at any levels of drought compared to the seedlings which was grown under drought stress without SA. Significant decreases in DHAR activity was observed under drought stress. Treatment with 10 and 20% PEG resulted in 33 and 30% decrease in DHAR activities compared to control. However, spraying of SA in drought seedlings restored the DHAR activities similar to control (Fig. 5C). Under the mild drought stress (10% PEG), a slight increase (31%) of GR was observed compared to control, while under severe stress (20% PEG) it remained unchanged (Fig. 5D). Importantly, SA spray in drought stressed seedlings resulted a considerable increase in GR activities which were 29 and 49% higher at 10 and 20% PEG, respectively. The activities of GST increased by 30 and 47% at 10 and 20% PEG, respectively (Fig. 6A). However, in this case SA spray could not contribute in increasing the activities of GST at any level of stress. Compared with the control, GPX activity did not changed at 10% PEG, while it decreased by 28% at 20% PEG (Fig. 6B). However, the SA supplemented seedlings under drought stress caused a profound increases in GPX activities (63 and 82% higher at 10 and 20% PEG, respectively) as compared to stressed seedlings without SA spray. Drought stress caused no significant changes in the activities of CAT of mustard seedlings. However, compared to drought stress alone, the drought imposed seedlings sprayed with SA showed significant increased of CAT activity at any level of stress (Fig. 6C).

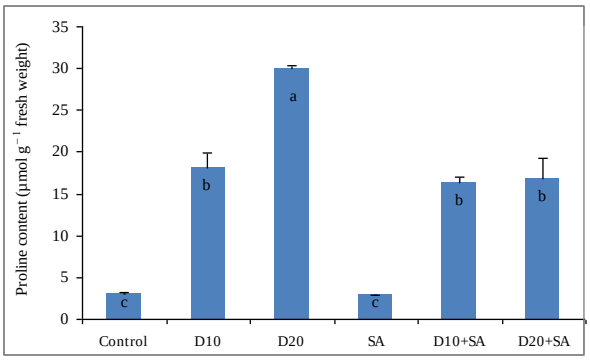

Fig 2. Proline (Pro) content in mustard seedlings induced by salicylic acid under drought stress conditions. D_{10}, D_{20}, SA, D_{10}+SA and D_{20}+SA indicates 10% PEG, 20% PEG, 50 µM salicylic acid, 10% PEG+50 µM SA, 20% PEG+50 µM SA, respectively. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying LSD test

Activities of glyoxalase enzymes

The activity of Gly I decreased by 34 and 44% upon exposure to 10 and 20% PEG, respectively (Fig. 7A). On the other hand, drought stressed seedlings sprayed with SA enhanced the activities as compared to drought stress alone. The activity of Gly II did not show any change upon exposure to 10% PEG, while it significantly decreased (by 29%) at 20% PEG (Fig. 7B). The drought stressed seedlings sprayed with SA showed significant increase in the Gly II activity at any level of stress (57 and 83% higher than the seedlings treated with 10 and 20% PEG, respectively).

Discussion

The present investigation revealed how exogenous application of SA may help to reduce adverse effects of drought in *Brassica juncea* by increasing water status, photosynthetic pigment content and antioxidant defense. The most obvious effect of physiological drought is the loss of water from tissue. Thus leaf RWC is considered as an efficient factor for evaluating plants for tolerance to drought stress (Gonzales and Gonzales-Vilar, 2001). Although at any levels of PEG, RWC decreased significantly, SA spray in those seedlings helped in maintaining water status in the tissue. Salicylic acid induced enhancement of RWC was also observed by Kadioglu et al. (2011). In our experiment Chl content in leaves decreased under drought stress (Fig. 1B-D). A decrease in chlorophyll concentration in case of drought stress might be due to changes in chloroplast structure, inhibition of biosynthesis of precursors of Chl (Plesnicár et al. 1997). However, this effect was mostly reverted when plants were supplemented with SA. A number of plant studies had indicated the role of SA in enhancing the photosynthetic pigment content and photosynthesis in different crops (Khan et al. 2003; Khodary 2004). However, the protective effect of SA was more prominent at severe stress (20% PEG) (Figs 1B-D). Proline is the most common compatible solute that occurs in a wide variety of plants. Proline has been suggested to function as a mediator of osmotic adjustment, as a stabilizer of macromolecule, as a compatible solute to protect enzymes and also as a store for carbon and nitrogen for using during water deficit and other stress regimes (Ashraf and Foolad 2007). The accumulation of Pro is also a sign of stress induction (Rampino et al. 2006). Although Pro content profoundly increased under drought stress condition, SA treatment in combination with drought reduced the content (Fig. 2). This was due to reduction of osmotic stress after SA application. In our study, drought stress caused a marked increase in lipid peroxidation (MDA content), while exogenous SA treatment prevented lipid peroxidation at any level of stress (Fig. 3A). This was due to the prevention of membrane damage and induction of antioxidant responses by SA, which protects the plant from oxidative damage. Similar protective effect of SA was also observed by Senaratna et al. (2000) and Kadioglu et al. (2011). At higher concentration, H_2O_2 leads to oxidative stress (Quan et al. 2008) and it may inactivate enzymes by oxidizing their thiol groups. In our study drought stress caused a remarkable increase of H_2O_2 content which is a clear indication of oxidative stress. Spraying with SA on the other hand, prevented the accumulation of H_2O_2 in drought stressed plants which was due to the upregulation of H_2O_2 scavenging enzymes such as GPX, GST and CAT (Fig. 3B, 6A-C). This result is well agreed with Kadioglu et al. (2011). The antioxidant defenses appear to provide crucial protection against oxidative damage in cellular membranes and organelles in plants grown under drought stress (Hasanuzzaman and Fujita 2011). Thus, plants are equipped with complex and a highly efficient antioxidative defense system which can respond and adapt to drought stress. Under stressful condition, acceleration of antioxidant defense system is necessary to detoxify excess ROS. In our study exogenous SA played significant role in enhancing both non-enzymatic and enzymatic components. Our study indicated that AsA content significantly decreased (Fig. 4A) under drought stress, This decrease was mainly due to the reduction of DHAR activity (Fig. 5C) and enhanced APX (Fig. 5A) activity. Glutathione is a very important soluble antioxidant, because it protects many cellular components under oxidative stress. Glutathione plays a vital role in the antioxidant defense system as well as the glyoxalase system by acting as a substrate or cofactor for certain enzymes. In our experiment, the GSH content (Fig. 4B) also increased with increased drought stress, however, the rate of increase was smaller under severe stress. The increased GSH content might be due to the increase in GR activities as well as higher GSH biosynthesis (Mittova et al. 2003). However, supplementation with SA under drought stress showed significant increase of both AsA and GSH (Fig. 4A, B) which indicated a clear role of SA in producing non-enzymatic antioxidant. SA might took part in the regeneration of AsA by up-regulating the related enzymes i.e. MDHAR and DHAR; SA also accelerated efficient recycling of GSH is also ensured by GR activity. In our experiment, the GSSG content in drought stress was astonishingly higher (Fig. 4C) than control ones. This increase might be partly attributed to a decrease in the rate of GSH recycling or to an increase in the rate of degradation of GSH (Noctor and Foyer 1998). However, SA treated drought-stressed seedlings showed significantly lower GSSG. The GSH/GSSG ratio also markedly enhanced by SA application under drought stress condition (Fig. 4D). It has been suggested that the GSH/GSSG ratio, indicative of the cellular redox balance, may be involved in ROS perception (Shao et al. 2005). Similar observations were reported by several researchers (Kadioglu et al. 2011; Hasanuzzaman and Fujita 2011). The AsA–GSH cycle is the major defense system against ROS in chloroplasts, cytosol, mitochondria, peroxisomes and apoplasts. The AsA–GSH cycle involves 4 enzymes (APX, MDHAR, DHAR and GR) as well as AsA, GSH and NADPH which work together to detoxify H_2O_2 in a series of cyclic reactions and further regenerate AsA and GSH

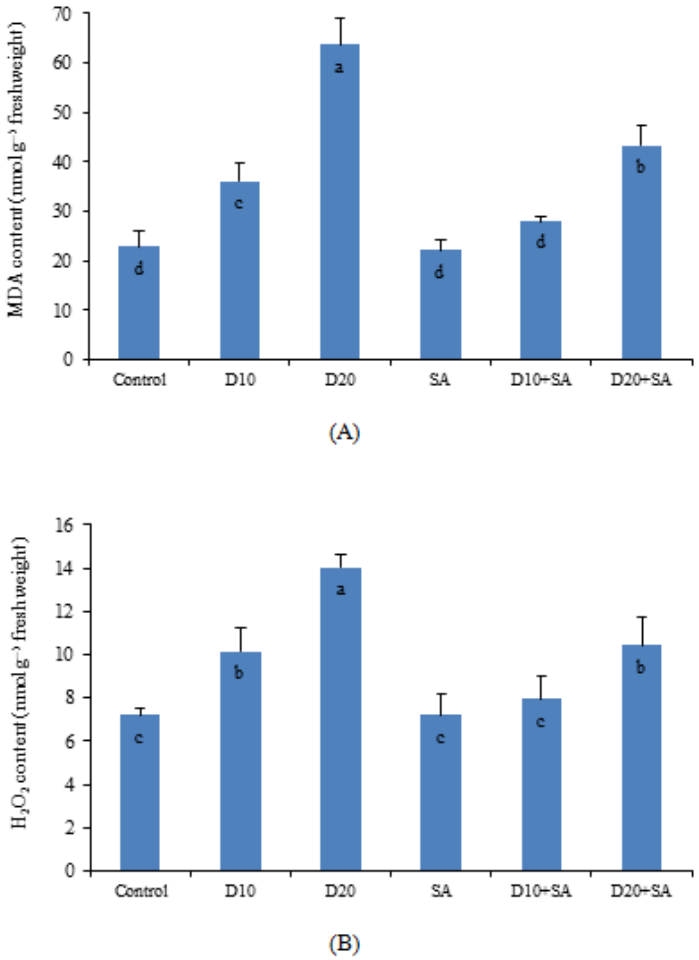

Fig 3. A: MDA (A) and H_2O_2 (B) level in mustard seedlings induced by salicylic acid under drought stress conditions. D_{10}, D_{20}, SA, D_{10}+SA and D_{20}+SA indicates 10% PEG, 20% PEG, 50 µM salicylic acid, 10% PEG+50 µM SA, 20% PEG+50 µM SA, respectively. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying LSD test.

(Hasanuzzaman et al., 2012a). In this cycle APX catalyses the reduction of H_2O_2 to H_2O with the simultaneous generation of monodehydroascorbate (MDHA), which is further converted to AsA by the action of NADPH-dependent MDHAR or disproportionates nonenzymatically to AsA and dehydroascorbate (DHA). The DHA undergoes irreversible hydrolysis to 2, 3-diketogulonic acid or is recycled to AsA by DHAR, which uses GSH as the reductant (Chen et al. 2003). This results in the generation of GSSG, which is regenerated to GSH by GR (Hasanuzzaman et al. 2012a). In this study, APX and GR slightly increased by drought, while DHAR and MDHAR activities decreased (Fig. 5A-D). This suggests that under drought stress condition the activities of AsA-GSH cycle enzymes were not efficient to regenerate AsA and GSH. However, SA treated seedlings significantly upregulated the activities which helped in increasing the AsA and GSH content under drought stress.Plant GSTs are a superfamily of multifunctional enzymes which catalyze the conjugation of electrophilic xenobiotic substrates with GSH (Dixon et al. 2010). Glutathione peroxidases (GPXs) are a large family of diverse isozymes that use GSH to reduce H_2O_2 and lipid hydroperoxides (LOOHs), and therefore protect plant cells from oxidative stress (Noctor et al. 2002). GPX is also a principal cellular enzyme capable of repairing membrane lipid peroxidation and is an important protectant against oxidative membrane damage (Kühn and Borchert 2002). In this experiment, GST activities increased under drought stress condition, but no further enhancement of the activities were observed upon SA supplementation (Fig. 6A). GPX activity (Fig. 6B) on the other hand, significantly decreased under severe drought (20% PEG). However, exogenous SA enhanced the activity of this enzyme which helped in detoxifying H_2O_2 and preventing lipid peroxidation (Kadioglu et al. 2011; Hasanuzzaman and Fujita 2011). Catalases (CATs) are tetrameric heme-containing enzymes that use H_2O_2 as a substrate and convert it to H_2O and O_2, thus preventing cells from oxidative damage (Sanchez-Casas and Klesseg 1994). In this study, CAT activity was not affected by drought, however, when SA was sprayed in those seedlings, it enhanced the CAT activity significantly (Fig. 6C). It indicated a protective role of SA in scavenging H_2O_2 in mustard seedlings under drought stress condition. The glyoxalase enzymes viz. Gly I (lactoylglutathione lyase) and Gly II (hydroxyacylglutathione hydrolase), together convert a variety of α-keto aldehydes into hydroxyacids in presence of GSH (Quan et al. 2010). They have important roles in detoxification of MG by the sequential action of two thiol-dependent enzymes; firstly Gly I, which catalyses the isomerisation of the spontaneously formed hemithioacetal adduct between GSH and 2-oxoaldehydes (such as methylglyoxal) into *S*-2-hydroxyacylglutathione (Singla-Pareek et al. 2006, 2008). The efficient manipulation of glyoxalase pathway enzymes in different plants inhibits an increase in MG level under oxidative stress and confers tolerance (Yadav et al. 2005a, b; Singla-Pareek et al. 2006, 2008). Our results indicated that drought stress decreased both Gly I and Gly II activities (Fig. 7A, B). Decrease in the activities of glyoxalase enzymes were also reported in recent studies (Hasanuzzaman et al. 2011a, b; Hasanuzzaman and Fujita 2011). Importantly, in both of the cases, SA treatment enhanced the activities which indicate an efficient detoxification of MG under drought stress condition.

Materials and methods

Plant materials and stress treatments

Mustard (*Brassica juncea* L. cv. BARI Sharisha 11) seeds of uniform size were selected and surface-sterilized with 70% ethanol for 10 minutes followed by washing several times with sterilized distilled water. The seeds were then sown in petri plates (9 cm) lined with 6 layers of filter paper moistened with 10 ml of distilled water for germination for two days. Germinated seedlings were then allowed to grow under controlled condition (light, 100 µmol photon m^{-2} s^{-1}; temperature, 25±2°C; RH, 65–70%) that contained 10,000-fold diluted Hyponex solution (Hyponex, Japan). After 10 days, two sets of seedlings were subjected to two different levels of drought stress viz. 10% and 20% PEG in Hyponex solution. Another two sets of seedlings were sprayed with 50 µM salicylic acid (Wako, Japan) containing 0.02% Tween 20 (Polyoxyethylenesorbitan monolaurate, Wako, Japan). Each set of seedlings was sprinkled twice a day. Control plants were grown in Hyponex solution only. Data were taken after 48 hours of treatment. The experiment was repeated three times under the same conditions.

Measurement of relative water content

Relative water content (RWC) was measured according to

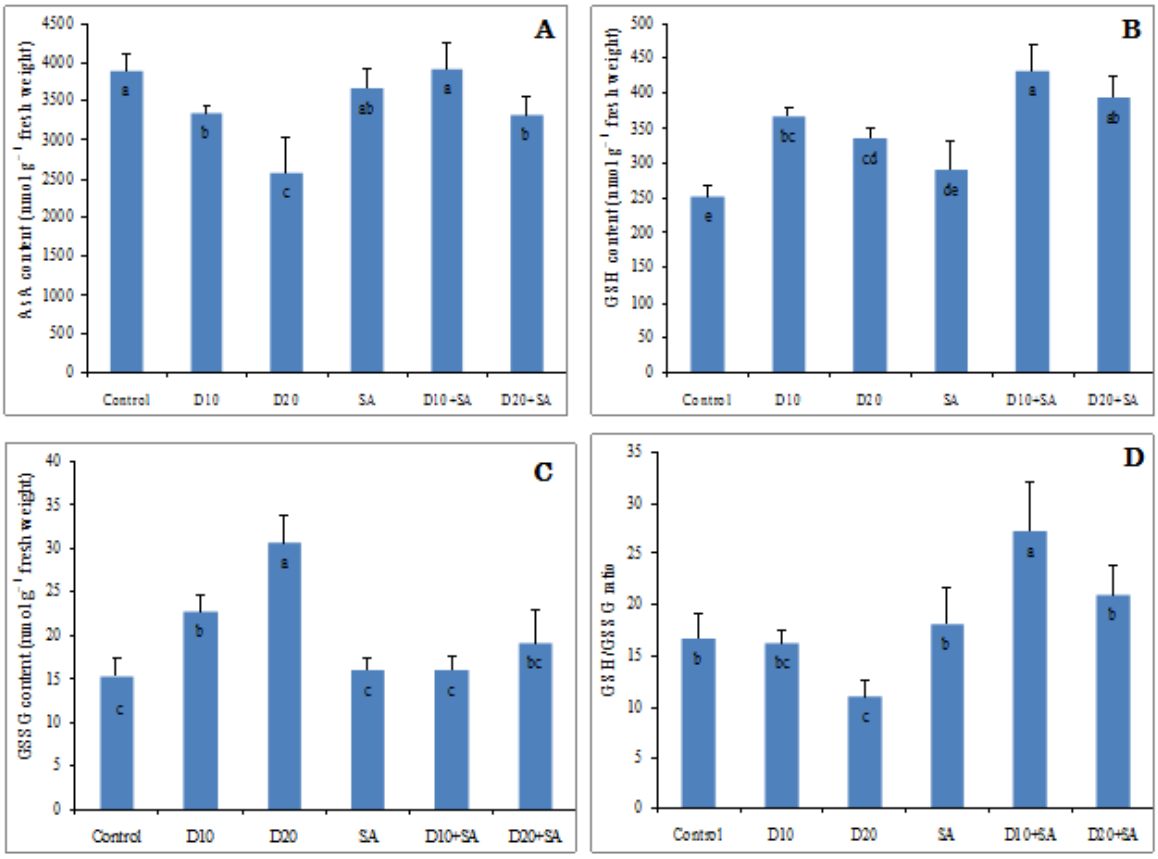

Fig 4. A: Reduced ascorbate (AsA), B: Reduced glutathione (GSH), C: Oxidized glutathione (GSSG), and D: GSH/GSSG ratio in mustard seedlings induced by salicylic acid under drought stress conditions. D_{10}, D_{20}, SA, D_{10}+SA and D_{20}+SA indicates 10% PEG, 20% PEG, 50 µM salicylic acid, 10% PEG+50 µM SA, 20% PEG+50 µM SA, respectively. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying LSD test

Barrs and Weatherly (1962). Leaf discs from randomly chosen plants were taken from the fully developed leaves. Discs were weighed (fresh wt, FW) and then immediately floated on distilled water in a petri dish for 8 h in the dark. Turgid weights (TW) of leaves were obtained after drying excess surface water with paper towels. Dry weights (DW) of leaves were measured after drying at 70°C for 48 h. Relative water content was calculated using the following formula:

$$\text{RWC (\%)} = \frac{FW-DW}{TW-DW} \times 100$$

Determination of chlorophyll content

Chlorophyll (Chl) content was determined by taking fresh leaf samples (0.5 g) from randomly selected seedlings. The samples were homogenized with 10 ml of acetone (80% v/v) using pre-cooled pestle and mortar and the homogenate was centrifuged at 5,000× *g* for 10 min. The absorbance was measured with a UV-visible spectrophotometer at 663 and 645 nm and Chl contents were calculated using the equations proposed by Arnon (1949).

Determination of proline content

Free proline in leaf tissues was appraised following the protocol of Bates et al. (1973). Fresh leaf tissue (0.5 g) was homogenized well in 10 ml of 3% sulfo-salicylic acid in ice. The homogenate was centrifuged at 11,500×*g* for 15 min. Two ml of the filtrate was mixed with 2 ml of acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) and 2 ml of glacial acetic acid. The mixture was placed at 100°C in water bath for 1 h, then transferred in to test tube and kept in ice to be cooled, after a while when it was cooled, 4 ml of toluene was added and mixed thoroughly by vortex mixture. After sometimes by transferring the upper aqueous layer the optical density of the chromophore containing toluene was read spectrophotometrically at 520 nm using toluene as a blank. The amount of Pro was determined by comparison with a standard curve.

Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating malondialdehyde (MDA), a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Heath and Packer (1968) with slight modifications as described by Hasanuzzaman et al. (2011a).

Measurement of H_2O_2

H_2O_2 was assayed according to the method described by Yu et al. (2003). H_2O_2 was extracted by homogenizing 0.5 g of leaf samples with 3 ml of 50 mM potassium-phosphate (K-P) buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 11,500× *g* for 15 min. Three ml of supernatant was mixed

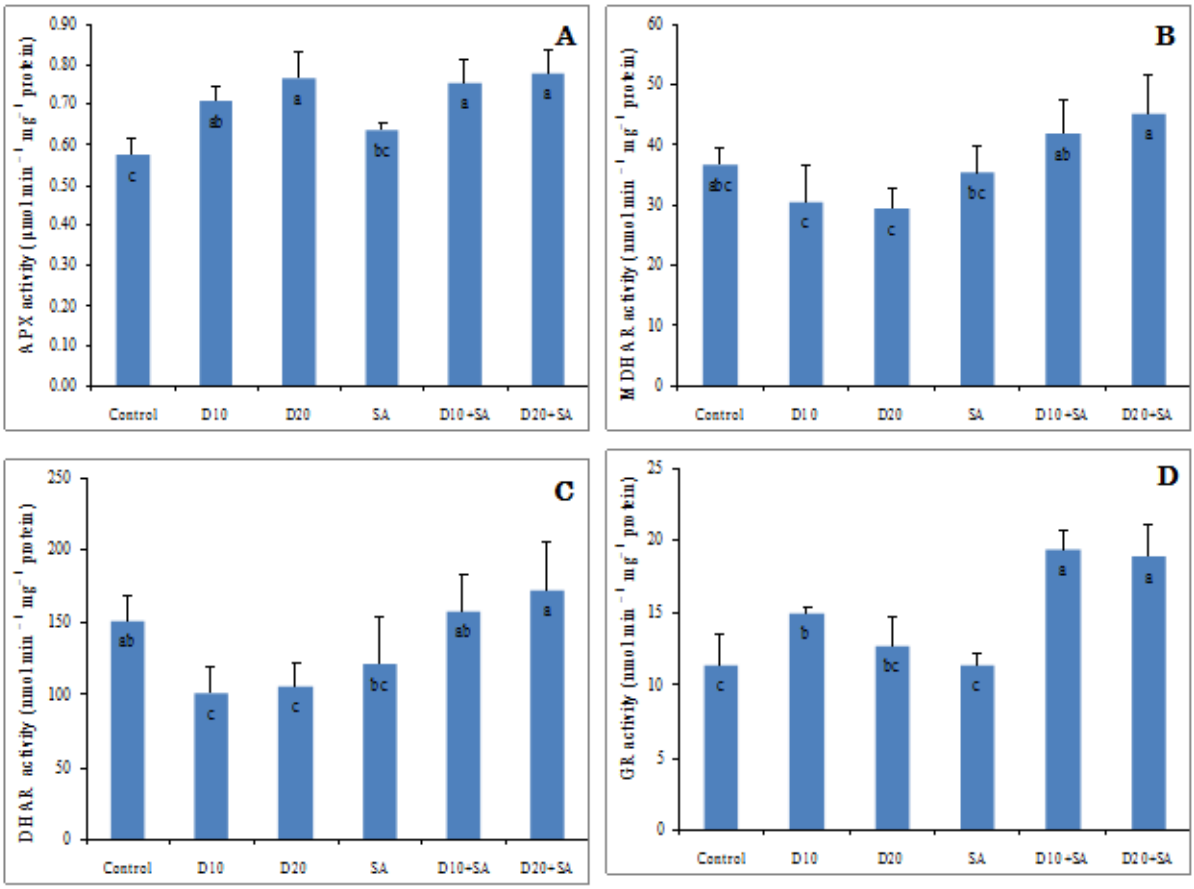

Fig 5. Activities of APX (A), MDHAR (B), DHAR (C), and GR (D) in mustard seedlings induced by salicylic acid under drought stress conditions. D_{10}, D_{20}, SA, D_{10}+SA and D_{20}+SA indicates 10% PEG, 20% PEG, 50 µM salicylic acid, 10% PEG+50 µM SA, 20% PEG+50 µM SA, respectively. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying LSD test

with 1 ml of 0.1% $TiCl_4$ in 20% H_2SO_4 (v/v) and kept in room temperature for 10 min. After that the mixture was again centrifuged at 11,500× *g* for 12 min. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content (ϵ=0.28 $\mu M^{-1}cm^{-1}$) and expressed as µnmol g^{-1} fresh weight.

Extraction and measurement of ascorbate and glutathione

Mustard leaves (0.5 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer (5% meta-phosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at 11,500× *g* for 15 min at 4°C and the supernatant was collected for analysis of ascorbate and glutathione. Ascorbate content was determined following the method of Huang et al. (2005) with some modifications as described by Hasanuzzaman et al. (2011a). The glutathione pool was assayed according to previously described methods (Yu et al. 2003) with modifications as described by Paradiso et al. (2008) and Hasanuzzaman et al. (2011a). Standard curves with known concentrations of GSH and GSSG were used. The content of GSH was calculated by subtracting GSSG from total GSH.

Determination of protein

The protein concentration of each sample was determined following the method of Bradford (1976) using BSA as a protein standard.

Enzyme extraction and assays

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 ml of 50 mM ice-cold K-P buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM *β*-mercaptoethanol and 10% (w/v) glycerol. The homogenates were centrifuged at 11,500× *g* for 15 min and the supernatants were used for determination of enzyme activity. All procedures were performed at 0−4°C. APX (EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract in a final volume of 700 µl. The reaction was started by the addition of H_2O_2 and the activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. MDHAR (EC: 1.6.5.4) activity was determined by the method of Hossain et al. (1984). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 unit of AO and enzyme solution in a final volume of 700 µl. The reaction was started by the addition of AO. The activity was calculated from the change in absorbance at 340 nm for 1 min using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. DHAR (EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada

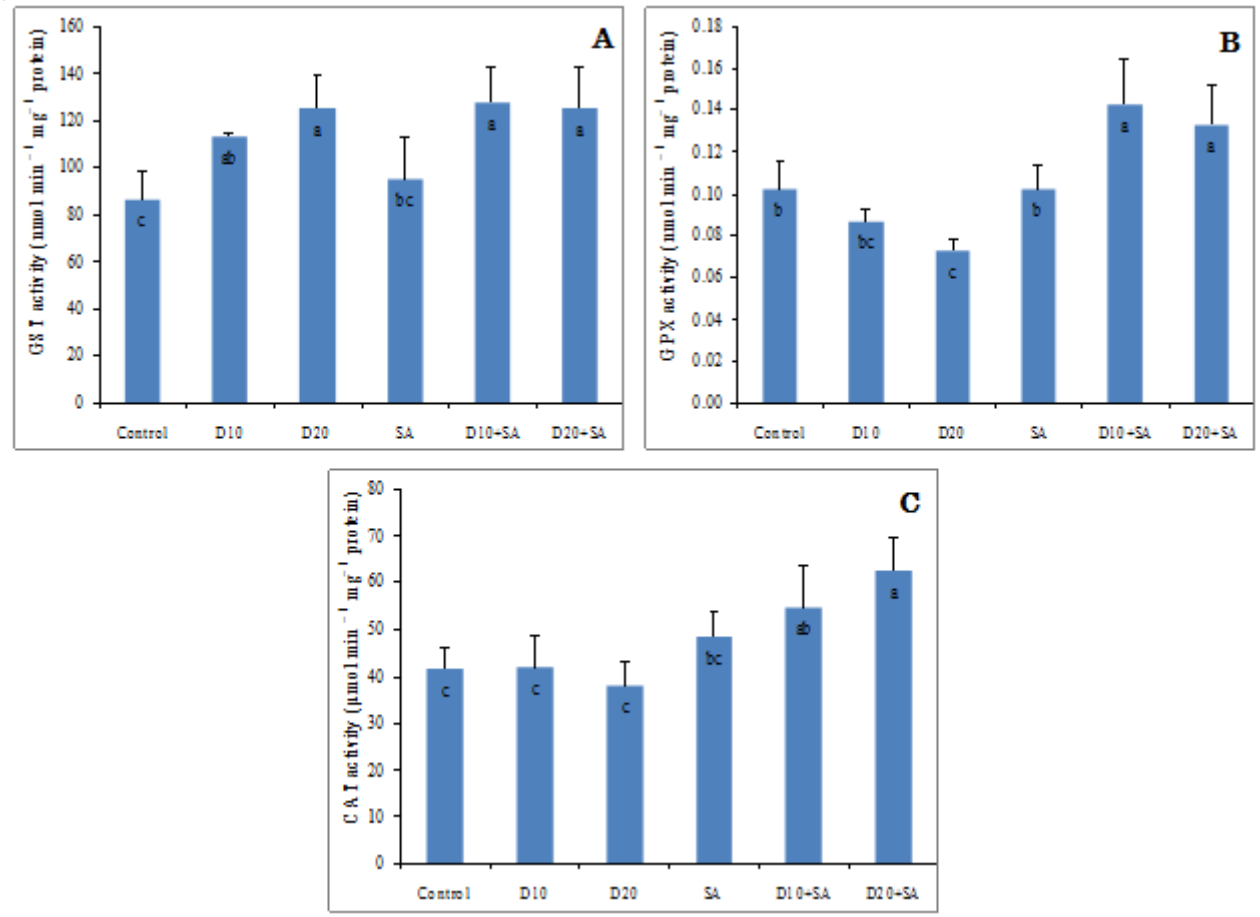

Fig 6. Activities of GST (A), GPX (B), and CAT (C) in mustard seedlings induced by salicylic acid under drought stress conditions. D_{10}, D_{20}, SA, D_{10}+SA and D_{20}+SA indicates 10% PEG, 20% PEG, 50 µM salicylic acid, 10% PEG+50 µM SA, 20% PEG+50 µM SA, respectively. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying LSD test

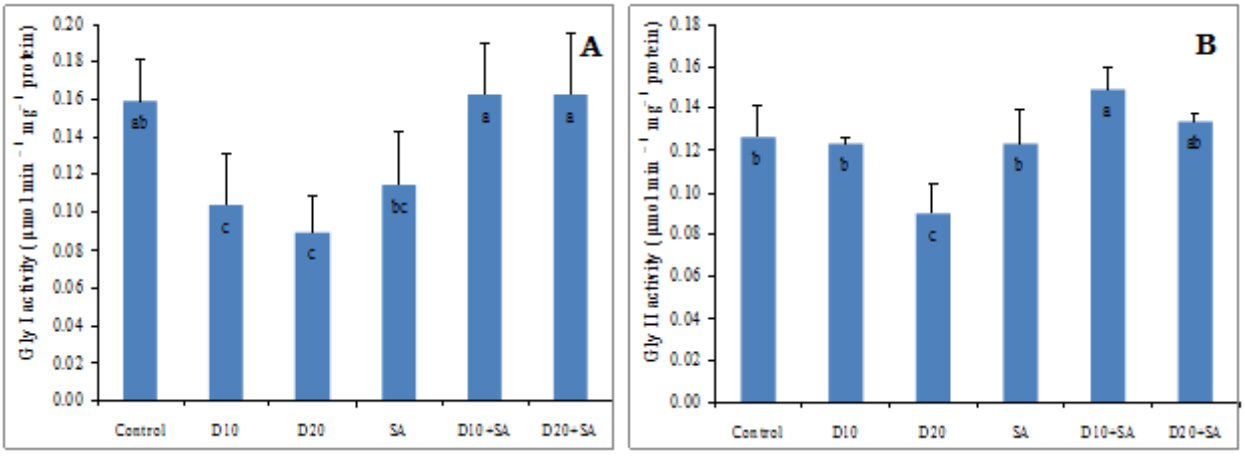

Fig 7. Activities of Gly I (A) and Gly II (B) in mustard seedlings induced by salicylic acid under drought stress conditions. D_{10}, D_{20}, SA, D_{10}+SA and D_{20}+SA indicates 10% PEG, 20% PEG, 50 µM salicylic acid, 10% PEG+50 µM SA, 20% PEG+50 µM SA, respectively. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying LSD test

(1981). The reaction buffer contained 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. The reaction was started by adding the sample solution to the reaction buffer solution. The activity was calculated from the change in absorbance at 265 nm for 1 min using extinction coefficient of 14 $mM^{-1}cm^{-1}$. GR (EC: 1.6.4.2) activity was measured by the method of Hasanuzzaman et al. (2011b). The reaction mixture contained 0.1 M K-P buffer (pH 7.0), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution in a final volume of 1 ml. The reaction was initiated with GSSG and the decrease in absorbance at 340 nm was recorded for 1 min. The activity was calculated using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. GST (EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Hossain

et al. (2006) with some modifications. The reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4- dinitrobenzene (CDNB), and enzyme solution in a final volume of 700 µl. The enzyme reaction was initiated by the addition of CDNB and the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction coefficient of 9.6 $mM^{-1}cm^{-1}$. GPX (EC: 1.11.1.9) activity was measured as described by Elia et al. (2003) with slight modification as described by Hasanuzzaman et al. (2011a). CAT (EC: 1.11.1.6) activity was measured according to the method of Hasanuzzaman et al. (2011a) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. The reaction was initiated with enzyme extract and the activity was calculated using the extinction coefficient of 39.4 $M^{-1}cm^{-1}$. Glyoxalase I (EC: 4.4.1.5) assay was carried out according to Hasanuzzaman et al. (2011a). Briefly, the assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM GSH and 3.5 mM MG in a final volume of 700 µl. The reaction was started by the addition of MG and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of 3.37 $mM^{-1}cm^{-1}$. Glyoxalase II (EC: 3.1.2.6) activity was determined according to the method of Principato et al. (1987) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.2), 0.2 mM DTNB and 1 mM *S*-D -lactoylglutathione (SLG) in a final volume of 1 ml. The reaction was started by the addition of SLG and the activity was calculated using the extinction coefficient of 13.6 $mM^{-1}cm^{-1}$.

Statistical analysis

All data obtained were subjected to analysis of variance (ANOVA) and the mean differences were compared by a Duncan's multiple range test (DMRT) using XLSTAT v.2010 software (Addinsoft 2010). Differences at $P < 0.05$ were considered significant.

Conclusion

Based on the above results, together with results found in the available literature, we therefore conclude that exogenous SA spray is an effective way to improve short-term drought tolerance, which could be partially attributed to the increase in non-enzymatic and enzymatic antioxidants as well as the activities of glyoxalase enzymes. Although a number of reports indicated the protective role of SA under abiotic stress conditions, to date the research conducted on the physiological roles of SA under stressful conditions is scarce. Therefore, further studies are required to elucidate the molecular mechanism and signaling pathways underlying the role of SA in the stress tolerance of plants. Complete elucidation of the physiological roles of SA with its detailed protective mechanisms would be helpful for developing stress tolerance in plants.

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