



Heat-shock positively modulates oxidative protection of salt and drought-stressed mustard (*Brassica campestris* L.) seedlings

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Abstract

A large number of studies have shown the existence of cross-tolerance in plants, but the exact physiological and biochemical mechanism(s) is poorly understood. In this study, heat-shock (42 °C, 5 h) induced salinity and drought tolerance and possible involvement of antioxidative and glyoxalase systems were investigated in mustard (*Brassica campestris* L.) seedlings. Seven-day-old seedlings were subjected to salt (150 mM NaCl, 48 h) and drought stress (induced by 20% PEG, 48 h) with or without heat pre-treatment. Both salt and drought stresses led to a severe oxidative stress as indicated by profound increases in hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) levels. A significant increase in ascorbate (AsA) content was observed in response to drought stress but the glutathione (GSH) and glutathione disulfide (GSSG) contents increased in response to both salt and drought stress. The GSH/GSSG ratio decreased significantly in response to drought stress. Salt stress led to a significant increases of ascorbate peroxidase (APX), glutathione reductase (GR), glutathione S-transferases (GST) activities; whereas, catalase (CAT) and glyoxalase II (Gly II) activities decreased. Drought stress resulted in a significant increase in monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX) and glyoxalase I (Gly I) activities; whereas, CAT and Gly II activities decreased. Seedlings primed with heat-shock positively modulates the activities of APX, DHAR, GR, GST, GPX, CAT, Gly I and Gly II, and maintained lower levels of GSSG, H₂O₂ and MDA as compared to the control mostly also salt and drought-stressed seedlings. Our results showed that a retention of the imprint of previous stress exposure (heat-shock) protects the plants from salt and drought-induced oxidative stress by stimulation of antioxidative and glyoxalase defense systems.

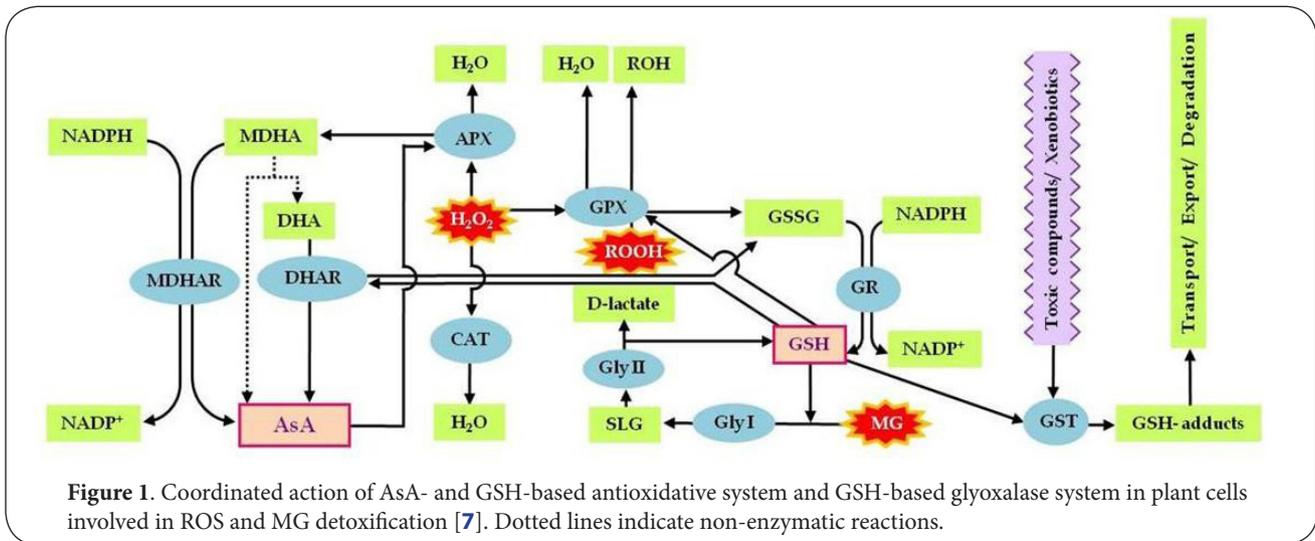
Keywords: cross-tolerance, heat-shock, salt and drought stress, antioxidative and glyoxalase system, *Brassica campestris* L.

Introduction

Soil salinity and drought are the two most common abiotic stresses constraining crop growth and productivity [1]. Currently, more than 800 million ha of land is affected by salinity [2], and about 1/3 of the world's arable land has experienced yield reduction due to cyclical or unpredictable drought [3]. Problems of salinity and drought get aggravated due to climate change [4]. As a result, the development of improved levels of tolerance to these stresses has become an urgent concern for many crop breeding programs to ensure global food security to an increasing world population. In parallel, much research effort is being applied to gain a better understanding of the complex adaptive mechanisms used by plants to combat abiotic stress [5], although we are far from complete understanding of this complexity [1]. Identification of key metabolic pathways, genes and proteins underlying abiotic stresses has thus become a priority in the research for improved crop stress tolerance [6-10]. A understanding of the regulation of these pathways and genes and their response to stress would provide opportunities for the manipulation of gene expression in crop plants [6].

Salt and drought stresses lead to oxidative stress in plant

cell due to higher leakage of electrons towards O₂ during photosynthetic and respiratory processes leading to enhancement of reactive oxygen species (ROS) and free radicals such as singlet oxygen (¹O₂), superoxide radicals (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals ([•]OH) [11-12]. Methylglyoxal (MG), a cytotoxic compound, was also found to increase in response to various abiotic stresses [13-16]. An increase in MG level in plant cells further intensifies the production of ROS by inactivating the antioxidant enzymes [16-18] and interfering with photosynthesis [19]. This increase in ROS and MG exposes cells to oxidative stress leading to lipid peroxidation, chlorophyll destruction, biological macromolecule deterioration, membrane dismantling, ion leakage, and DNA-strand cleavage and finally death of plants [7,9,16,20]. To protect against oxidative stress, plant cells possess an extensive ROS scavenging network, which involves non-enzymatic antioxidants, including vitamin C, vitamin E, glutathione, carotenoids and flavonoids, as well as numerous enzymatic mechanisms such as multiple superoxide dismutases (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), catalase (CAT),



glutathione peroxidase (GPX), glutathione S-transferase (GST) [21]. Likewise, the glyoxalase system is an integral component and major pathway of cellular metabolism of cytotoxic MG, consists of two enzymes: glyoxalase I (Gly I) and glyoxalase II (Gly II). These enzymes act coordinately to convert MG and other 2-oxoaldehydes to their 2-hydroxyacids using GSH as a cofactor in a two-step reaction [16,22]. The overexpression of the glyoxalase pathway enzymes in transgenic tobacco and rice plants has been found to lower the levels of ROS and MG under stress conditions by maintaining GSH homeostasis [23-25]. Transgenic tobacco plants overexpressing both Gly I and Gly II genes also showed higher salinity tolerance by additional increase of GSH metabolizing enzyme (GST, GPX and GR) activities further denoting the close interaction between the antioxidant system and the glyoxalase system in imparting stress tolerance in plants [7,16,23]. The results of numerous recent studies have shown that the reduction of oxidative damage and increased resistance to abiotic stresses are often correlated with the more efficient regulation of both antioxidative and glyoxalase systems [Figure 1; 7,15,16,20, 26-29].

Heat stress - high temperature- affects the metabolism and structure of plants, especially cell membrane and many basic physiological processes, such as photosynthesis, respiration, and water-relations [31-32]. Plant must cope with heat stress for survival, so they developed different mechanisms including the maintenance of cell membrane stability, capturing the ROS, synthesis of antioxidant, accumulation and osmoregulation of osmoticum, induction of transcription factor, accumulation of heat shock proteins (HSPs) [31-32]. The mechanism(s) of cross-adaptation is little known although a few hypotheses have been proposed to try to elucidate it; most are based on roles of H₂O₂, GSH, AsA and HSPs [33-40]. Involvement of glutathione in heat shock- and hydrogen peroxide-induced cadmium (Cd) tolerance of rice (*Oryza sativa* L.) seedlings

was reported by Chao *et al.*, [36]. Later on, Chao and Kao [38] showed that heat-shock (45 °C, 3 h) induced AsA accumulation in leaves induces Cd-induced oxidative stress tolerance of rice (*Oryza sativa* L.) seedlings. Heat-shock induced Cd-tolerance rice seedlings were also found to be associated with higher APX and GR activities [41]. Recently, Ferreira-Silva *et al.*, [39] showed that high temperature positively modulates oxidative protection in salt-stressed cashew (*Anacardium occidentale*) plant by the activation of antioxidant enzymes such as SOD, APX, CAT as well as favorable changes in the ascorbate redox state. Additionally, few recent studies showed an increase in Gly I and II activities, Gly I gene and protein expression by heat stress [13,42-43] and efficient regulation both AsA and GSH contents and their utilizing and regenerating enzymes is an important predominant factor controlling ROS and MG levels to ensure abiotic stress tolerance [15,26-29]. However, it is unclear whether there is a cross-adaptation between heat, salt and drought stresses in Brassica and if there any involvement of antioxidative and glyoxalase defense systems. Additionally, there is no evidence showing that heat-shock induced salinity and drought tolerance is due to an efficient regulation of glyoxalase system and antioxidant defense system of plants. Considering the total aspect, the present study was therefore undertaken to explore the possible biochemical mechanisms of heat-shock induced salinity and drought tolerance. To our knowledge, these data represent the first evidence that heat pre-treatment enhances salt and drought stress-induced oxidative stress tolerance in mustard seedlings by stimulating the antioxidative and glyoxalase defense systems.

Materials and methods

Plant materials and growth conditions

Uniform size seeds of the selected mustard (*Brassica campestris* cv. Shampad) variety was surface-sterilized with 70% ethanol then washed several times with distilled water. The seeds were

then soaked with distilled water for 15 min and sown in Petri dishes (9 cm) lined with 4 layers of filter paper moistened with 10 ml of distilled water for germination under dark conditions for 3 days following the method of Hossain *et al.*, [29]. Germinated seedlings were then grown in Petri dishes that contained 10,000-fold diluted Hyponex solution (Hyponex, Japan) under controlled conditions (light, 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$; temp, 25 \pm 2 $^{\circ}\text{C}$; RH, 65–70%).

Heat-shock pre-treatment and salt and drought stress treatments

Seven-day-old seedlings of approximately equal sizes were employed for heat shock pre-treatment (42 $^{\circ}\text{C}$ for 5 h) under dark conditions. After this heat shock pre-treatment, the seedlings were kept at 25 $^{\circ}\text{C}$ in the dark for 6 h for recovery. The control seedlings were kept at 25 $^{\circ}\text{C}$. Afterwards, the seedlings with and without heat-shock were subjected to salt stress (150 mM NaCl) and drought stress (20% PEG-6000) in Hyponex solution and grown under the above conditions [44] for 48 h to test their cross-adaptation. Control plants were grown in Hyponex solution only. After treatment data were taken from the leaf samples and immediately used. The experiment was replicated three times under the same conditions.

Extraction and analysis of ascorbate and glutathione

After stress treatments, mustard leaves (0.5 g fresh weight) were homogenized in 1.5 ml ice-cold acidic extraction buffer (6% metaphosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at 11,500 \times g for 15 min at 4 $^{\circ}\text{C}$ and the supernatant was collected for analysis of ascorbate and glutathione as described by Hossain *et al.*, [28].

The amount of ascorbate content in the sample was determined following the method of Huang *et al.*, [45] with some modifications [27,28]. The supernatant was neutralized with 0.5 M K-phosphate buffer (pH 7.0). The AsA was assayed spectrophotometrically at 265 nm in 100 mM K-phosphate buffer (pH 5.6) with 0.5 unit of ascorbate oxidase (AO). A specific standard curve with AsA was used for quantification. The glutathione pool was assayed according to previously described methods [46] with modifications [47] utilizing 0.4 ml of aliquots of supernatant neutralized with 0.6 ml of 0.5 M K-phosphate buffer (pH 7.5). Based on enzymatic recycling, glutathione is oxidized by 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of GR, and glutathione content was evaluated by the rate of absorption changes at 412 nm of 2-nitro-5-thiobenzoic acid (NTB) generated from the reduction of DTNB. GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves were generated with reduced and oxidized glutathione.

Enzyme extraction and assays

Extraction of enzyme from the leaf tissues was done following our previous established methods [27,28]. Using a pre-cooled

mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 ml of 50 mM ice-cold K-phosphate 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 \times g for 10 min and the supernatants were used for determination of enzyme activity. All procedures were performed at 0–4 $^{\circ}\text{C}$.

APX (EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada [48]. The reaction buffer solution contained 50 mM K-phosphate 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 0.7 ml. The reaction was initiated by the addition of H_2O_2 and activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 $\text{mM}^{-1} \text{cm}^{-1}$.

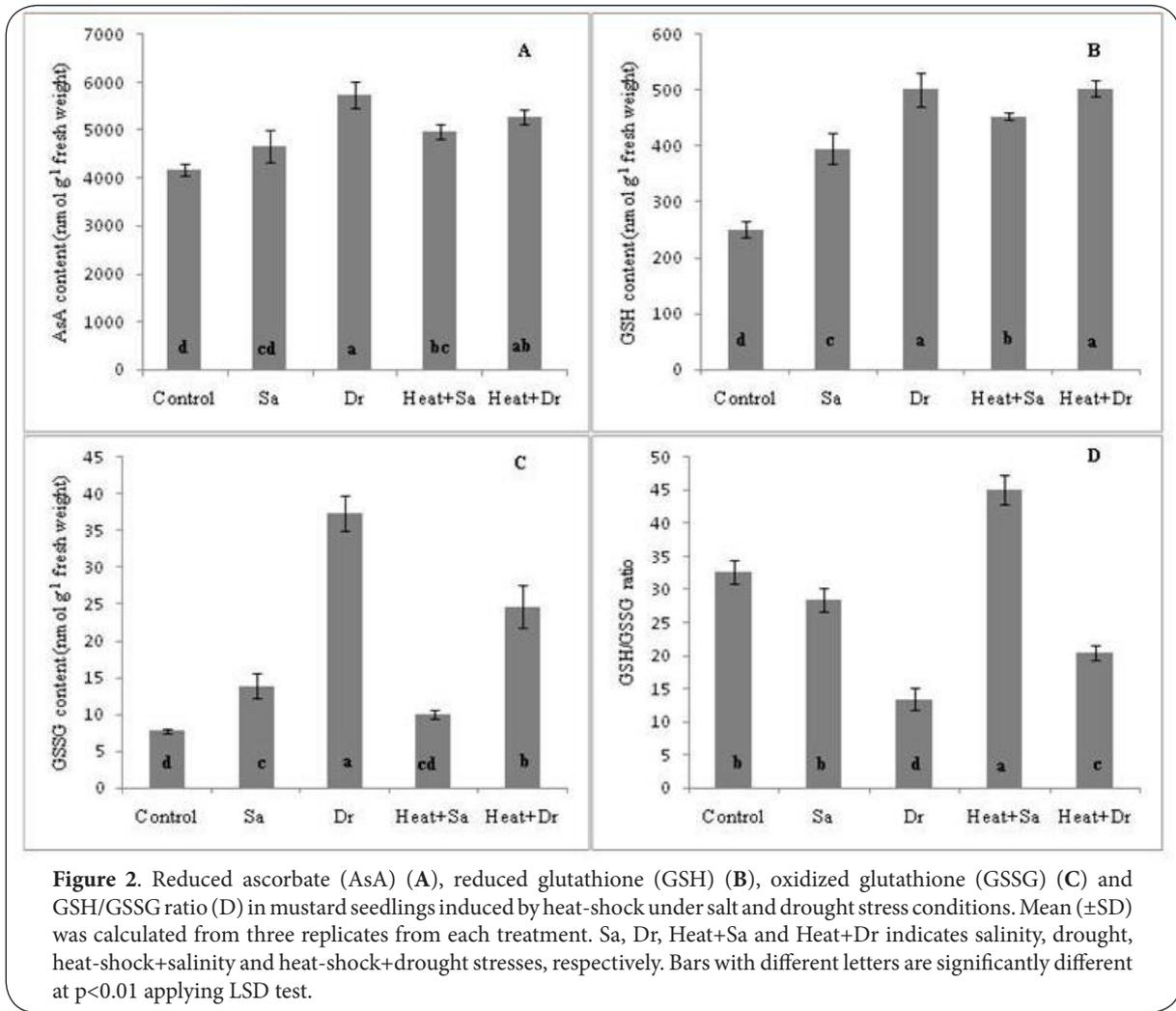
MDHAR (EC: 1.6.5.4) activity was determined by the method of Hossain *et al.*, [49]. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 units of AO and enzyme solution in a final volume of 0.7 ml. The reaction was started by the addition of AO. The activity was calculated from the change in ascorbate at 340 nm for 1 min using an extinction co-efficient of 6.2 $\text{mM}^{-1} \text{cm}^{-1}$.

DHAR (EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada [48]. The reaction buffer contained 50 mM K-phosphate 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 an extinction co-efficient of 14 $\text{mM}^{-1} \text{cm}^{-1}$.

GR (EC: 1.6.4.2) activity was measured by the method of Hossain *et al.*, [28]. The reaction mixture contained 0.1 M K-phosphate buffer (pH 7.8), 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 due to NADPH oxidation was recorded for 1 min. The activity was calculated using an extinction co-efficient of 6.2 $\text{mM}^{-1} \text{cm}^{-1}$.

GPX (EC: 1.11.1.9) activity was measured as described by Hossain *et al.*, [28] using H_2O_2 as a substrate. The reaction mixture consisted of 100 mM Na-phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM NaN_3 , 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2 and 20 μl of sample solution. The reaction was started by the addition of H_2O_2 . The oxidation of NADPH was recorded at 340 nm for 1 min and the activity was calculated using the extinction co-efficient of 6.62 $\text{mM}^{-1} \text{cm}^{-1}$.

GST (EC: 2.5.1.18) activity was measured as described by Hossain *et al.*, [28] with some modifications (Hossain *et al.*, 2009). 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 0.7 ml. 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 an extinction co-efficient of 9.6 $\text{mM}^{-1} \text{cm}^{-1}$.



CAT (EC: 1.11.1.6) activity was measured according to the method of Hossain *et al.*, [13] by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2 . The reaction mixture contained 50 mM K-phosphate buffer (pH 7.0), 15 mM H_2O_2 and enzyme solution in a final volume of 0.7 ml. The reaction was initiated with enzyme extract and the activity was calculated using an extinction co-efficient of $39.4 M^{-1} cm^{-1}$.

Gly I (EC: 4.4.1.5) assay was carried out according to the method of Hossain *et al.*, [13]. Briefly, the assay mixture contained 100 mM K-phosphate buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM GSH and 3.5 mM MG in a final volume of 0.7 ml. 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 an extinction coefficient of $3.37 mM^{-1} cm^{-1}$.

Gly II (EC: 3.1.2.6) activity was determined according to the method of Hossain *et al.*, [28] 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

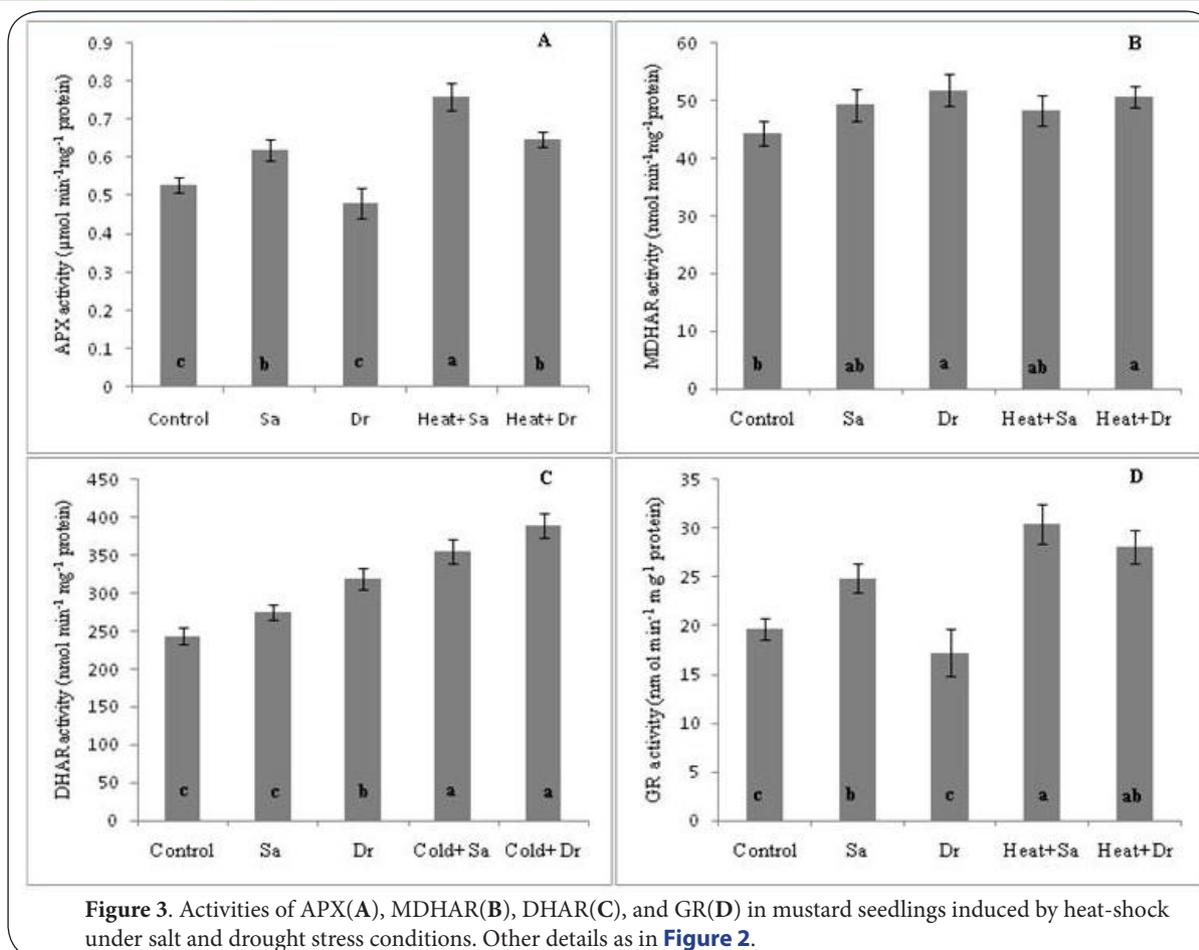
S-D-lactoylglutathione (SLG) in a final volume of 1 ml. The reaction was started by the addition of SLG and the activity was calculated using an extinction co-efficient of $13.6 mM^{-1} cm^{-1}$.

Measurement of hydrogen peroxide (H_2O_2)

The level of H_2O_2 was measured in leaf tissue according to the method described by Yu *et al.*, [50]. H_2O_2 was extracted by homogenizing 0.5 g of leaf tissue with 3 ml of 50 mM K-phosphate buffer pH (6.5) at 4°C. The homogenate was centrifuged at $11,500 \times g$ for 15 min. A 3-ml sample of supernatant was mixed with 1 ml of 0.1% $TiCl_4$ in 20% H_2SO_4 (v/v), and the mixture was then centrifuged at $11,500 \times g$ for 15 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content ($\epsilon = 0.28 \mu M^{-1} cm^{-1}$) and expressed as $\mu mol g^{-1}$ fresh weight [28].

Measurement of lipid peroxidation (MDA)

The level of lipid peroxidation was measured in leaf tissue by estimating 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 [51] with slight modifications [52]. The concentration of MDA was calculated by using an extinction co-efficient of 155 $\text{mM}^{-1} \text{cm}^{-1}$ and expressed as $\text{nmol of MDA g}^{-1}$ fresh weight [28].

Determination of protein

The protein concentration of each sample was determined by the method of Bradford [53] using BSA as a protein standard.

Statistical analysis

The data obtained were analyzed by one-way analysis of variance (ANOVA) and mean differences were compared by a least significant difference (LSD) test by using MSTATC. Differences at $P < 0.01$ were considered to be significant.

Results

Non-enzymatic antioxidant contents

Drought stress showed a significant increase (37%) in AsA content when compared with control (**Figure 2A**). Heat pre-treated salt and drought-stressed seedlings also showed a

significant increase (19% and 26% by salt and drought stress, respectively) in AsA content when compared with control.

Either salt or drought stress showed a significant increase in GSH content (57% and 99% by salt and drought stress, respectively) when compared with control (**Figure 2B**). Heat pre-treated salt and drought-stressed seedlings also showed a 80% and 101% increase in GSH content when compared with control. Importantly, heat pre-treated salt-stressed seedlings showed a significant increase (15%) in GSH content when compared with the seedlings subjected to salt stress without heat pre-treatment.

Salt stress showed a significant increase (80%) in GSSG content but a robust increase (385%) was observed in response to drought stress when compared with control (**Figure 2C**). Heat pre-treated drought-stressed seedlings showed a sharp increase (220%) in GSSG content when compared with control. Surprisingly, heat pre-treated drought-stressed seedlings maintained significantly lower level of GSSG content (35%) when compared with the seedlings subjected to drought stress without heat pre-treatment.

Drought stress showed a significant decrease (59%) in GSH/GSSG ratio when compared with control (**Figure 2D**).

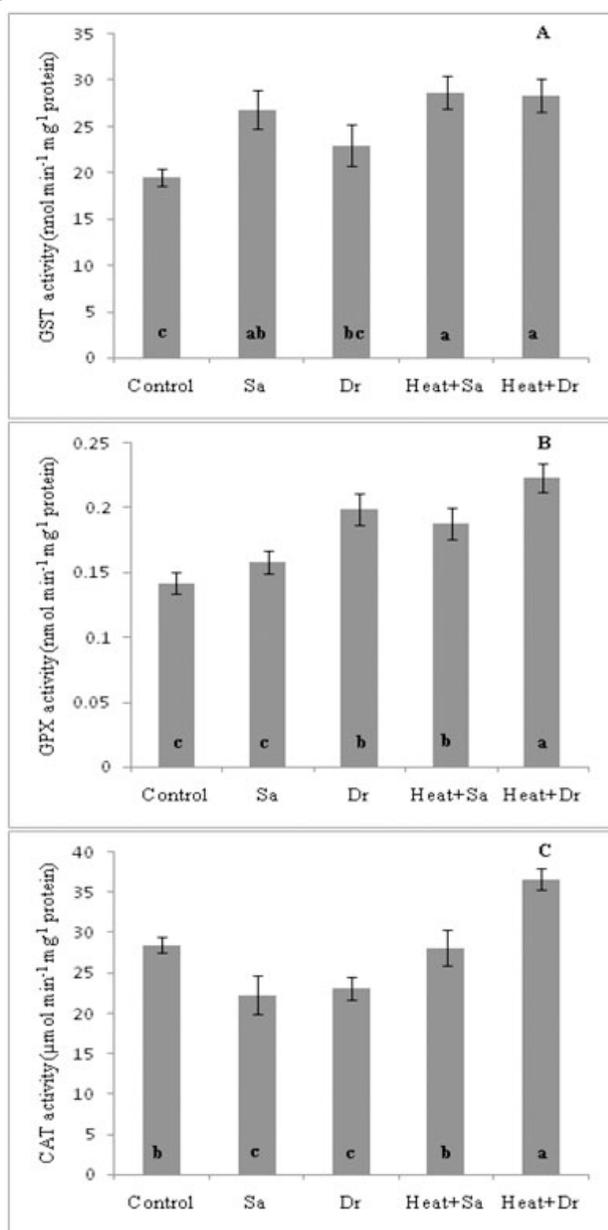


Figure 4. Activities of GST (A), GPX (B) and CAT(C) in mustard seedlings induced by heat-shock under salt and drought stress conditions. Other details as in **Figure 2**.

Importantly, heat pre-treated salt and drought-stressed seedlings showed a significant increase (58% and 52% by salt and drought stress, respectively) in GSH/GSSG ratio when compared with the seedlings subjected to salt and drought stress without heat pre-treatment.

Activities of antioxidant enzymes

Salt stress showed a significant increase (17%) in APX activity when compared with control (**Figure 3A**). Heat pre-treated salt and drought-stressed seedlings showed a significant increase (43% and 23% by salt and drought stress, respectively) in

APX activity when compared with control. Importantly, heat pre-treated salt and drought-stressed seedlings showed a significant increase (23% and 35% by salt and drought stress, respectively) in APX activity when compared with the seedling subjected to salt and drought stress without heat pre-treatment.

Drought stress showed a significant increase (17%) in MDHAR activity when compared with control (**Figure 3B**). Heat pre-treated drought stressed seedlings also showed a significant increase (14%) in MDHAR activity when compared with control.

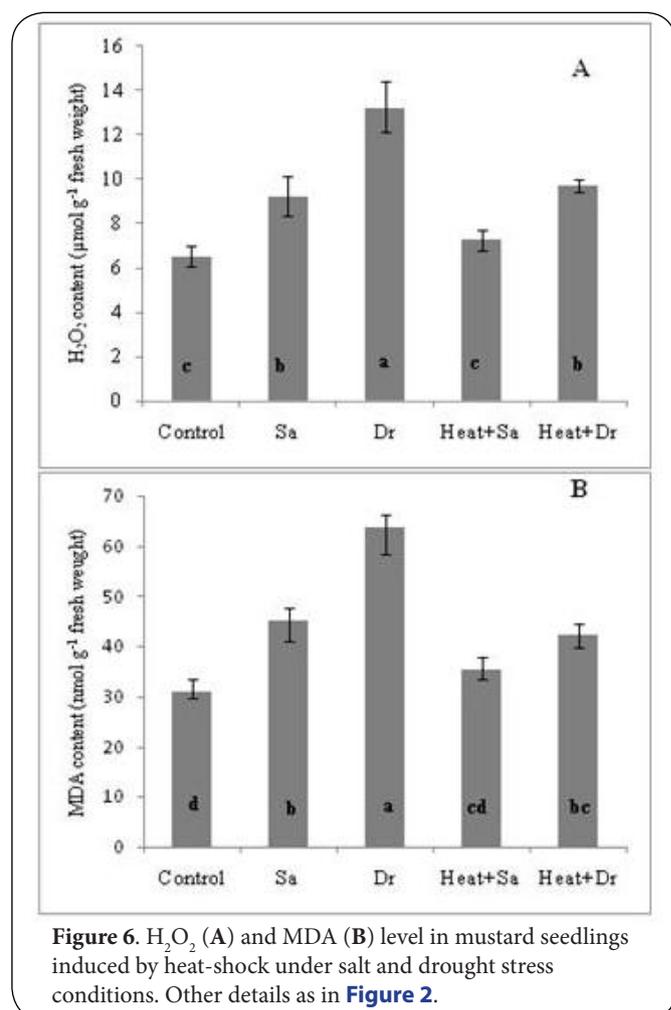
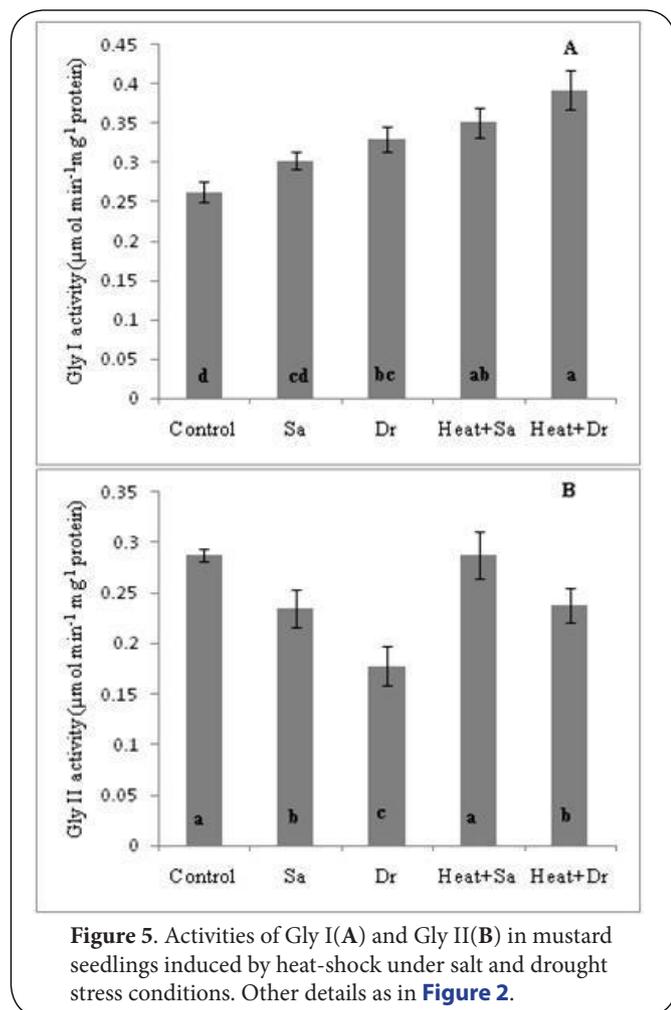
Drought stress showed a significant increase (31%) in DHAR activity when compared with control (**Figure 3C**). Importantly, heat pre-treated salt and drought-stressed seedlings showed a significant increase in DHAR activity (46% and 59% by salt and drought stress, respectively) when compared with control. Heat pre-treated salt and drought-stressed seedlings maintained significantly higher DHAR activity (29% and 22% by salt and drought stress, respectively) when compared with the seedling subjected to salt and drought stress without heat pre-treatment.

Salt stress showed a significant increase (27%) in GR activity when compared with control (**Figure 3D**). A sharp increase in GR activity (55% and 43% by salt and drought stress, respectively) was also observed in heat pre-treated salt and drought-stressed seedlings when compared with control. Importantly, heat pre-treated salt and drought stress seedlings showed a significant increase (23% and 63% by salt and drought stress, respectively) when compared with the seedlings subjected to salt and drought stress without heat pre-treatment.

Salt stress showed a significant increase (38%) in GST activity when compared with control (**Figure 4A**). Heat pre-treated salt and drought-stressed seedlings showed a significant increase in GST activity (47% and 46% by salt and drought stress, respectively) when compared with control. Importantly, heat pre-treated drought-stressed seedlings maintained a significantly higher GST activity (23%) when compared with the seedlings subjected to salt and drought stress without heat pre-treatment.

Drought stress showed a significant increase (40%) in GPX activity when compared with control (**Figure 4B**). Importantly, heat pre-treated salt and drought-stressed seedlings showed a significant increase (32% and 62% by salt and drought stress, respectively) in GPX activity when compared with control. Heat pre-treated salt and drought stress seedlings maintain significantly higher GPX activity (19% and 16% percent by salt and drought stresses, respectively) when compared with the seedlings subjected to salt and drought without heat pre-treatment.

Either salt or drought stress showed a significant decrease (22% and 19% by salt and drought stresses, respectively) in CAT activity when compared with control (**Figure 4C**). Heat pre-treated drought-stressed seedlings showed a significant



increase (29%) in CAT activity when compared with control. Importantly, heat pre-treated salt and drought-stressed seedlings showed a significant increase in CAT activity (26% and 59% by salt and drought stresses, respectively) when compared with the seedlings subjected to salt and drought stresses without heat pre-treatment.

Activities of glyoxalase pathway enzymes

Drought stress showed a significant increase (26%) in Gly I activity when compared with control (**Figure 5A**). Heat pre-treated salt and drought-stressed seedlings showed a 34% and 50% increase in Gly I activity when compared with control. Importantly, heat pre-treated salt and drought-stressed seedlings showed a significant increase (16% and 19% by salt and drought stresses, respectively) when compared with the seedlings subjected to salt and drought stresses without heat pre-treatment.

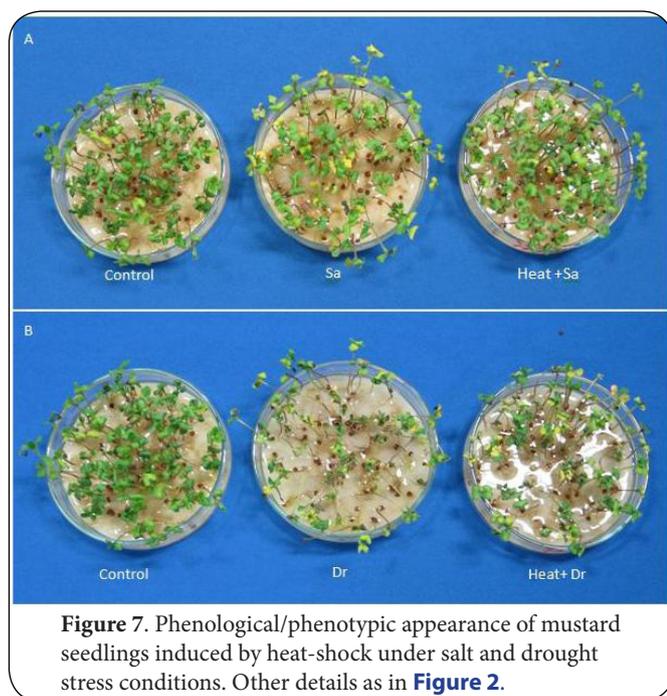
Either salt or drought stress showed a significant decrease (18% and 38% by salt and drought stresses, respectively) in Gly II when compared with control (**Figure 5B**). Importantly, heat pre-treated salt and drought-stressed seedlings showed significantly higher Gly II activity (22% and 38% by salt and

drought stresses, respectively) when compared with the seedlings subjected to salt and drought stress without heat pre-treatment.

Hydrogen peroxide (H₂O₂) and lipid peroxidation (MDA) contents

Either salt or drought stress showed a significant increase (42% and 103% by salt and drought stress, respectively) in H₂O₂ accumulation when compared with control (**Figure 6A**). Heat pre-treated drought-stressed seedlings also showed a 49% increase in H₂O₂ content when compared with control. Importantly, heat pre-treated salt and drought-stressed seedlings maintained significantly lower level (21% and 27% by salt and drought stress, respectively) of H₂O₂ content when compared with the seedlings subjected to salt and drought stresses without heat pre-treatment.

Either salt or drought stress showed a significant increase (46% and 105% by salt and drought stresses, respectively) in MDA content when compared with control (**Figure 6B**). Heat-pretreated drought-stressed seedlings also showed a significant increase (36%) in MDA content when compared



with control. Surprisingly, heat pre-treated salt and drought stressed seedlings showed a significant decrease in MDA content (21% and 38% by salt and drought stress, respectively) than those of the seedlings subjected to salt and drought stress without heat pre-treatment.

Phenotypic observations also showed that heat pre-treated salt and drought-stressed seedlings had better visual appearance (less chlorosis/yellowing and wilting) when compared with the seedlings subjected to salt and drought stress without heat pre-treatment (**Figure 7A,B**).

Discussion

Acquired stress tolerance in plants is often a result of various stress-response mechanisms that act coordinately or synergistically to prevent cellular damage and to re-establish cellular homeostasis [54]. The phenomenon of cross-tolerance is extremely important for agriculture because plants can be selectively bred that are tolerant to more than one stress. Additionally, cross-tolerance allows us to compare and contrast individual responses and to examine the roles of common signal transducing molecules [55]. Cross-tolerance involves the synergistic co-activation of specific stress responsive pathways that enable a general increase in stress resistant. Numerous studies have shown that ROS exhibit important signaling functions in responses to both biotic and abiotic stresses, implying that they might be central components controlling cross-tolerance, at least at the cellular level [33,38]. To survive under stress conditions, it is very important that the plant antioxidant system is able to work in harmony with glyoxalase system, thus providing better defense and regeneration of the active defense forms [7,20,56]. Plants are capable of

adapting to a wide range of temperatures by reprogramming their transcriptome, proteome and metabolome [57]. Stress adaptation through retention of imprints of previous stress exposure has recently been described in plants. Drought and salinity are the two most important abiotic stresses affecting crop yield, improving our understanding of the plant response and tolerance to these environmental challenges is a major research priority. To elucidate the biochemical mechanisms of abiotic stress tolerance in plants with special reference to ROS and MG metabolism, we previously reported osmoprotectants (proline, glycinebetaine), signalling molecule (nitric oxide) and selenium-induced abiotic stress tolerance in mung bean, rapeseed and wheat seedlings [26-29,58]. Here, we extended our understanding on biochemical mechanisms of heat-shock induced salinity and drought tolerance in mustard seedlings. We hypothesized that heat pre-treatment induces salinity and drought tolerance by balance interaction between the ROS and MG metabolism and by modulating antioxidative and glyoxalase systems.

In plants cells, ascorbic acid (AsA) is the most abundant water-soluble antioxidant and redox buffer. It serves as a cofactor for enzymes, as a regulator of cell division and growth, as well as in signal transduction [59]. AsA can directly quench 1O_2 , $O_2^{\cdot-}$ and $\cdot OH$ and regenerate α -tocopherol from α -chromanoxyl radical thereby providing protection to membranes [60]. It is the substrate of APX, which is a critical component of the AsA-GSH cycle for H_2O_2 detoxification. An increased content of AsA protects protein and lipids against oxidative damage in plants and stress tolerant varieties have higher AsA levels and AsA utilizing antioxidant enzyme activity as compared to stress-sensitive varieties [61,62]. The results of the present study reveal that salt stress slightly increased AsA content whereas a significant increase was observed in the seedlings imposed to drought stress. Recently, we reported an increase in AsA content in rapeseed seedlings in response to drought stress [44] whereas a decrease in AsA content was observed in response to salt stress in mung bean, rapeseed and wheat seedlings [26,27,29]. AsA level increased in a drought-tolerant rice genotype (N22) while in the susceptible genotype (N118) its level decreased [63]. However, decrease in AsA content in response to drought stresses was also reported [28,44,64-65]. Plants feeding with exogenous AsA also found to induce abiotic oxidative stress tolerance by enhancing photosynthetic capacity and protein stability and by reducing lipid peroxidation through synergic functions with other antioxidants [66-68]. In the present study, heat pre-treated salt-stressed seedlings showed a non-significant increase in AsA content, whereas, heat pre-treated drought-stressed seedlings showed a non-significant decrease as compared to the seedlings subjected to salt and drought stress without pre-treatment. The phenomenon of lower level of AsA is probably due to higher APX activity in heat pre-treated salt and drought-stressed seedlings because the level of AsA under environmental stress depends on the

balance between the rates and capacity of AsA biosynthesis and turnover related to antioxidant demand [69-70]. Our results are not consistent with the results of few recent findings where cold- and heat-shock induced chilling and heavy metal tolerance was found to be associated with higher biosynthetic capacity of AsA [38,40].

Parallel to AsA, the tripeptide glutathione (GSH) is the main low molecular weight thiol in most plant tissues and plays diverse roles (Figure 1) to protect against ROS induced oxidative damage [71]. Elevated levels of GSH appear to be correlated to active plant responses to environmental stress and responses of GSH synthesis, GSH redox status, and GSH related enzyme activities (GST, GPX, Gly I, DHAR and GR) have been found repeatedly in plants under stress [7,72,73]. GSH accumulates in response to increased ROS or to compensate for decrease in the defense capability of other antioxidants and GSH levels are constitutively higher in plants adapted to stress conditions [74-76]. The regeneration from GSSG to GSH is catalyzed by GR and NADPH is used as the reducing power. GSH may function as a cellular sensor to ensure maintenance of the NADPH pool [77]. The property of GSH is of great biological importance since it allows fine-tuning of the cellular redox environment under normal conditions and upon onset of stress. In the present experiment, upon imposition of salt and drought stress a profuse increase in GSH content was observed in mustard seedlings (Figure 2B). Similar to our results, rapid increase in GSH content in response to salt and drought stress were also reported in mustard and rapeseed seedlings [26,27,44,78]. This marked increase in GSH content might be due to higher GSH biosynthesis or stimulation of GR activity [40,62,78]. In the present experiment the significant increases in GSSG content was observed in response to both salt and drought stresses. The formation of GSSG in salt and drought-stressed seedlings might be due to the reaction of GSH with oxyradicals generated due to oxidative stress or due to enhancement of DHAR, GPX and GST activity that decompose H_2O_2 and organic hydroperoxide or insufficient increase of GR activity [7,26,44,52]. Heat pre-treated salt-stressed seedlings maintained higher GSH level and GSH/GSSG ratio as compared to the untreated control and seedlings subjected to salt stress without heat pre-treatment, whereas, heat pre-treated drought stressed seedlings maintain significantly lower level of GSSG content and higher GSH/GSSG ratio. Therefore, heat pre-treatment play a crucial role in maintaining higher GSH level either through efficient functioning of GR or by modulating higher GSH synthesis [26-28,40,44,79]. Similar to our results heat-shock induced higher GSH biosynthesis was also reported in rice seedlings [36]. Importantly, higher accumulation of GSH is not the only factor for oxidative stress tolerance [46].

Ascorbate peroxidase is a central component of AsA-GSH cycle, and plays a pivotal role in the control of intracellular ROS levels. APX uses two molecules of AsA to reduce H_2O_2 with a concomitant generation of two molecules of MDHA. In the present study, upon imposition of salt stress APX activity

increased significantly whereas a non-significant decrease was observed in response to drought stress. Increase in APX activity in response to salt stress was also reported in *Brassica juncea* [80-81] and *Brassica napus* [26]. Similar to our results decrease in APX activity in response to drought stress was also reported in other crop species [44,82]. The decrease in APX activity in non pre-treated drought-stressed seedlings was probably due to inhibition of APX enzyme by MG [18] although the AsA content was higher. However, heat pre-treated salt and drought-stressed seedlings had higher APX activity as compared to the seedlings subjected to salt and drought stress without heat pre-treatment. Our results supported well by the few recent findings where high temperature induced modulation of APX activity induces salt and Cd tolerance of plants [38,39]. These results suggest that heat pre-treatment could contribute to detoxifying H_2O_2 by enhancing APX activity under salt and drought stress.

The MDHAR and DHAR are two important enzymes of AsA-GSH cycle responsible recycling of MDHA and DHA to AsA and to control its redox state under oxidative stress condition [29,83-84]. In the present experiment, both salt and drought stress increased the MDHAR and DHAR activities however greater increase in DHAR activity was observed. This indicates when one antioxidant system is inhibited then the plant exhibits another antioxidant defense system to face the adverse challenges by oxidative stress [85]. Increase in MDHAR and DHAR activities in response to salt and drought stresses were also reported [44,83,86-88]. Importantly, heat pre-treatment had no significant influence on MDHAR activity. However, the DHAR activity increased significantly in response to both salinity and drought stresses. Similar to our results crofton weed subjected to heat and drought stresses, the up regulation of DHAR activity was observed rather than MDHAR, suggesting that under these stress conditions, AsA is regenerated via GSH dependent DHAR [87]. It may be that DHAR activity could participate in AsA regeneration under conditions of severe stress when MDHAR activity is limited by the availability of NADPH [89].

Glutathione reductase which converts GSSG to GSH using NADPH is ubiquitous in living systems. It is necessary for maintaining the high ratio of GSH/GSSG ratio in the plant cells and accelerating the H_2O_2 scavenging pathway in plants particularly under stress conditions. GR plays an essential role in cell defense against reactive oxygen metabolites by sustaining the reduced status of glutathione and ascorbate pools which in turn maintain cellular redox state under stress. The adaptive behaviors of tolerant and -sensitive genotypes suggest that GR plays a significant role in maintaining the glutathione redox state under oxidative stress. Importantly, the increase of GR activity increases the ratio of $NADP^+/NADPH$ and ensures to availability of $NADP^+$ to accept electrons from the photosynthetic electron transport chain thus reducing the formation of $O_2^{\cdot-}$ which reduces the facilitation of metal-catalyzed formation of the $\cdot OH$ through the Haber-Weiss

reaction [90-91]. Generally, stress-tolerant plants tend to have high activities of GR as compared to the sensitive plants [63,83,92-93]. In the current study, a significant increase in GR activity was observed in response to salt stress whereas the activity decreased under drought stress. Therefore, the decrease in GR activity was the main reason for the over accumulation of GSSG in drought-stressed seedlings which was attributed to significant decrease in GSH/GSSG ratio (Figures 2C,D and 3C). Decrease in GR activity in response to drought stress was also reported in sensitive cultivar [83]. By contrast, heat pre-treated salt and drought-stressed seedlings had higher GR activity (Figure 4C). Increased GR activity in the heat pre-treated salt and drought-stressed seedlings contributes to the maintenance of higher GSH/GSSG ratio and GSH level which is used by DHAR and other GSH-dependent enzymes involved in the ROS and MG detoxification systems [7,26-28,52].

The potential role of GST isozymes has been extensively studied in plants due to its well-defined role on plant detoxification reactions [94-95]. Various abiotic stress effects are powerful inducers of GST activity in plants [96-101]. GST catalyse the binding of various xenobiotics with GSH to produce less toxic and more water-soluble conjugates [94]. Besides catalyzing the conjugation of electrophilic metabolites to GSH, GST isozymes also have peroxidase activity. A recent comprehensive study of rice GST gene expression showed its pivotal role in plant stress tolerance. Many GST genes were found to be significantly up-regulated in response to various abiotic (desiccation, salt, cold and arsenate) and biotic stresses. Surprisingly, many of the GST genes were commonly regulated by developmental processes, hormones, abiotic and biotic stresses [101]. Additionally, glutathione peroxidases are a family of isoenzymes, which catalyzes the reduction of H_2O_2 and organic peroxides, including lipid- and phospholipid hydroperoxides by using GSH [73]. Transgenic plants over-expressing GST and GPX genes also showed salt and drought stress induced oxidative stress tolerance in different crop species [102-105]. Our results showed that salt stress resulted in a significant increase in GST activity and a non-significant increase in GPX activity, whereas drought stress caused a slight increase in GST activity and a significant increase in GPX activity. Salt and drought stress-induced increase in GST and GPX activities were also reported in different plant species [20,29,44,97]. The modulation of GST and GPX activities in response to salt and drought stress was not sufficient to protect cells from salt and drought-induced oxidative damage. Importantly, heat pre-treatment favorably modulates the GST and GPX activities and suppressed the production of H_2O_2 and MDA level denoting that GPX and GST play important role in reducing salt and drought-induced oxidative damage.

Catalase is one of the H_2O_2 detoxifying enzymes and mostly associated with peroxisomes, where it removes H_2O_2 formed during photorespiration. Increase in CAT activity is supposed to be an adaptive trait possibly helping to overcome the damage to tissue metabolism by reducing toxic levels of H_2O_2 [92]. Our

results indicated a sharp decline in CAT activity in response to both salinity and drought stresses which suggests that CAT appears not to be an effective scavenger of H_2O_2 in mustard and the increase in H_2O_2 is possibly due to the decreased activity of CAT [26-27,29]. A decline in CAT activity due to salt and drought stresses was also reported in different crop species [44,93,106]. Such a decrease in CAT activity could indicate its inactivation by the accumulated H_2O_2 induced by drought and salt stresses and could be partly explained by photoinactivation of enzymes [107-108]. Importantly, heat pre-treated salt and drought-stressed seedlings maintained higher CAT activity as compared to the seedlings subjected to salt and drought stresses without heat pre-treatment. Similar modulation of CAT activity by heat-shock under salt stress was also reported [39].

Glyoxalase system is an integral component of cellular detoxification of MG. The accumulation of MG in plants is rapidly increased in response to various stresses and its detoxification might be a strategy for conferring tolerance against various abiotic stresses [13,15,17,20,28,29,52,109,110]. A high level of MG accumulation is toxic to cells, since it inhibits cell proliferation [111] and results in a number of adverse effects such as increasing the degradation of proteins and inactivating the antioxidant defense system [17-18]. Apart from MG, pathway intermediates SLG (substrate for Gly II) has also been found to be cytotoxic at high concentrations [112]. Besides detoxification of MG, the glyoxalase system could also play a role in providing tolerance under stress by recycling GSH that would be "trapped" spontaneously by MG to form hemithioacetal, thereby maintaining glutathione homeostasis [113]. Higher Gly I and Gly II activities might protect plants against MG that is formed during abiotic stresses and confers tolerance by increasing the GSH based detoxification system and decreasing lipid peroxidation [23,25]. Our results showed an increase in Gly I activity while the Gly II activity decreased. Salt and drought stress induced increase in Gly I activity was also found in our previous study [13,44,110]. By contrast, decrease in Gly II activity in response to salt and drought stresses was corroborated well with previous study [15,20,44]. A decrease in Gly II activity might be due to inactivation or proteolytic degradation of enzyme. Based on the results of the present study and supporting evidences from literature, we surmise that the detoxification of MG via the glyoxalase system is not sufficient under salt and drought stress conditions. In contrast, heat pre-treated seedlings could significantly alleviate the salinity and drought induced oxidative damage by maintaining higher Gly I and II activities, suggesting that both of them were able to enhance GSH regeneration via the glyoxalase system. As GSH is recycled by the glyoxalase system, it was assumed that an increased level of MG was detoxified efficiently in the heat pre-treated seedlings, thus creating the possibility of the up-regulation of GSH/GSSG ratio via the glyoxalase cycle and AsA-GSH cycle. Recently, Upadhyaya *et al.*, [15] showed that transgenic plants overexpressing GalUR (AsA

biosynthetic gene) induces salinity tolerance by maintaining higher activity of the ROS removing antioxidant enzymes (APX, DHAR, GR, GST, GPX, Gly I and Gly II) as well as enhanced GSH/GSSG ratio. Transgenic plants resulted in enhanced accumulation of AsA and resisted an overall increase in MG level under salinity stress, thus, reducing the MG toxicity. Concomitantly, a relatively higher GSH/GSSG ratio is also maintained in the transgenics which protects them from salinity induced oxidative stress. These parameters along with an enhanced antioxidative capacity of transgenic plants seem to confer enhanced salt stress tolerance and metabolic interaction of ROS and MG detoxification systems.

The peroxidation of lipid membrane represented by MDA is both a reflection and measure of stress induced damage at the cellular level. Its overall effects on plant cells are to decrease membrane fluidity, to increase the leakiness of membrane protein, enzymes, and ion channels. H₂O₂ at low concentrations is suggested to be involved in the signaling of many processes, however, excessive accumulation of H₂O₂ can lead to oxidative stress. It is thus the mechanism in plant to regulate the levels of H₂O₂ under salt stress rather than to remove it completely. In the current experiment salt and drought stresses significantly increased MDA and H₂O₂ levels and control of the levels of H₂O₂ and MDA is thought to be a mechanism by which plants tolerate these stresses [92]. These results corroborate well with previous reports where salinity and drought caused a sharp increase in H₂O₂ and MDA levels in different plant species including rapeseed and mustard [28-29,44,52,114]. A sharp increase in the levels of H₂O₂ and MDA in response to salt and drought stresses was due to insufficient antioxidant defense and MG detoxification system. Importantly, we found an active H₂O₂ peak in the leaf tissues of mustard seedlings after 5 h of heat-shock treatment (data not shown). However, heat-shock priming favorably modulated the MDA and H₂O₂ levels as compared to the seedling subjected to salt and drought stresses without heat pre-treatment. The biochemical results of our present experiments were well correlated with the phenotypic appearance of the seedlings (Figures 7A, B).

The results allow us to conclude that both salt and drought stresses induce a severe oxidative stress due to poor induction of H₂O₂ and MG-detoxification systems. Importantly, seedling primed with heat-shock positively modulates the activities of APX, DHAR, GR, GST, GPX, Gly I and Gly II, and maintained lower levels of GSSG, H₂O₂ and MDA as compared to the control as well as in most cases seedlings subjected to salt and drought stresses without heat-shock which indicated that heat-shock priming played a pivotal role in reducing oxidative damage. The results of this study, together with our previous results [26-29,52] and available results found in the literature [15,20,109] we, therefore, concluded that synergistic co-activation of antioxidative and glyoxalase systems is essential for controlling ROS and MG levels and the damage caused by them under stress. Our results showed that a retention

of the imprint of previous stress exposure (heat-shock) induces salt and drought induced oxidative stress tolerance through sustained activation of antioxidative and glyoxalase defense systems which implying that they might be the vital components inducing cross-tolerance, at least at the cellular level. However, the precise mechanisms underlying heat shock-mediated salinity and drought tolerance need to be further clarified by combining molecular, physiological and metabolic studies.

Abbreviations

AO- ascorbate oxidase; APX- ascorbate peroxidase; AsA- ascorbic acid; CAT- catalase; CDNB- 1- chloro-2, 4-dinitrobenzene; 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 adenine dinucleotide phosphate; NTB- 2-nitro-5-thiobenzoic acid; PEG- polyethylene glycol; ROS- reactive oxygen species; SLG- S-D-lactoylglutathione; TBA- thiobarbituric acid; TCA- trichloroacetic acid

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Mohammad Anwar Hossain participated in the design of the experiment, data collection, data analysis and manuscript preparation. Mohammad Golam Mostofa participated in the data collection and data analysis. Masayuki Fujita monitored the experimental work and critically read the manuscript. All authors read and approved the final manuscript.

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