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Trehalose-induced drought stress tolerance: A comparative study among different *Brassica* species

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Abstract

Comparative responses of three *Brassica* species including *B. napus*, *B. campestris* and *B. juncea* under polyethylene glycol induced drought stress and the protective effects of exogenous Trehalose were investigated. Although drought reduced fresh, dry weight, chlorophyll (chl) contents; increased proline (Pro) content and oxidative stresses (lipoxygenase, LOX activity; malondealdehyde, MDA; H_2O_2 contents) along with altered antioxidant and glyoxalase systems in all *Brassica* species, *B. juncea* seems to be the most drought tolerant species showing the least oxidative damage due to enhancement of some non-enzymatic and enzymatic antioxidants. Combination of Tre and drought improved performance of all species, but responses were different. In *B. juncea*, combination of Tre with drought improved seedlings' fresh weight, dry weight, leaf relative water content (RWC), chl *a*, chl *b*, ascorbate (AsA), glutathione (GSH) contents, AsA/DHA (ratio of AsA and dehydroascorbate) and GSH/GSSG (reduced to oxidized GSH) ratios; enhanced ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX) and glyoxalase II (Gly II) activities; reduced MDA, H_2O_2, Pro (proline), LOX activity. *Brassica napus* seedlings with Tre addition under drought showed improved seedlings' fresh weight, dry weight, GSH/GSSG ratio; upregulated catalase (CAT), glutathione *S*-transferase (GST), glyoxalase I (Gly I) activities; reduced MDA, H_2O_2 contents and LOX activity. In *B. campestris* Tre supplementation with drought improved fresh weight, RWC, chl *a*, chl *b*, chl (*a*+*b*) contents; AsA/DHA ratio, MDHAR activity. The results suggest that *B. juncea* is naturally drought tolerant species and moreover, its drought tolerance capability is further enhanced by exogenous Tre application.

Key words: Abiotic stress tolerance; antioxidants; oxidative stress; polyethylene glycol; reactive oxygen species; trehalose.
A**bbreviations:** AO- ascorbate oxidase; APX- ascorbate peroxidase; 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; JA- Jasmonic acid; LOX- Lipoxygenase; MDA- malondialdehyde; MDHA- monodehydroascorbate; MDHAR- monodehydroascorbate reductase; MG- methylglyoxal; NADPH- nicotinamide adenine dinucleotide phosphate; NTB- 2-nitro-5-thiobenzoic acid; PEG- polyethylene glycol; Pro- proline, ROS- reactive oxygen species; RWC- relative water content; SLG- *S*-D-lactoylglutathione; TBA- thiobarbituric acid; TCA- trichloroacetic acid; Tre- trehalose.

Introduction

Plants are continuously challenged by stressful environmental conditions like water deficit leading to changes in molecular, biochemical and physiological processes. Consequently, plant growth and development are adversely affected (Farooq et al., 2010). Water deficit conditions cause a reduction in plant photosynthetic efficiency and stomatal conductance, inhibit RuBisCo activity, and disrupt energy balance and distribution during photosynthesis (Demirevska et al., 2010; Rapacz et al., 2010) and these often result in increased accumulation of reactive oxygen species, ROS (superoxide, $O_2^{\cdot-}$; hydrogen peroxide, H_2O_2; hydroxyl radical, OH•) (Ashraf, 2009; Hasanuzzaman et al., 2012; Hasanuzzaman et al., 2014). The ROS are major toxic radicals which can damage biomolecules including proteins, lipids, and DNA (Vranova et al., 2002). Generation of cytotoxic methylglyoxal (MG) under different abiotic stresses including drought stress also causes similar damage effects like ROS (Alam et al., 2013). Under abiotic stresses, plants have evolved elaborate mechanisms to perceive and rapidly respond to diverse environmental cues (Demirevska et al., 2010). Enhancement of antioxidant defense system is an important strategyto proficiently scavenge ROS with non-enzymatic antioxidants such as ascorbate (AsA), glutathione (GSH), carotenoids, flavanones, and anthocyanins and by the

activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione *S*-transferase (GST) (Gupta et al., 2009; Hasanuzzaman et al., 2012). Detoxification of MG within plant is catalyzed by the glyoxalase system (glyoxalase I, Gly I and glyoxalase II, Gly II), which use glutathione (GSH) as a co-factor (Yadav et al., 2005). Enhanced antioxidant and glyoxalase systems improve drought tolerance in many crop plants (Hasanuzzaman et al., 2011a; Alam et al., 2013). Organic compatible solutes like proline (Pro), glycine betaine, trehalose (Tre) play important roles under multiple abiotic stresses (Ashraf and Foolad, 2007; Farooq et al., 2010; Nawaz and Ashraf, 2010). Among different compatible solutes, Tre is a non-reducing disaccharide of glucose that stabilizes biological structures and macromolecules such as proteins and membrane lipids during dehydration and other abiotic stresses (Aghdasi et al., 2008; Duman et al., 2010; Luo et al., 2010). Over production of osmoregulator, Tre in genetically engineered model plants or crop plants has proved to be better stress tolerance (Gouffi et al., 1999; Pellny et al., 2004; Wang et al., 2005). Exogenously applied Tre is readily accumulated and transported by leaf or roots tissues and displays significant roles as osmoprotectant (Smith and Smith 1973; Luo et al., 2010). Different plant species or genotypes within species respond differently to water deficit stress (Hong-Bo et al., 2006; Yildiz-Aktas et al., 2009). Similarly, there were large variations in drought tolerance among *Brassica* species (Pazoki et al., 2010; Din et al., 2011; Rad, 2012). The present study compared the performance of three *Brassica* species; *B. napus, B. campestris* and *B. juncea* under drought stress. This study also elucidate the roles of exogenously applied Tre in drought stress tolerance.

Results

Effect of Tre on fresh weight and dry weight of seedlings

Drought stress caused decreases in fresh and dry weights of seedlings of all *Brassica* spp. In *B. napus, B. campestris* and in *B. juncea* fresh weights were reduced by 23, 36 and 35%, respectively; while their dry weights were reduced by 25, 21 and 25%, respectively when compared to control seedlings. Exogenous application of Tre in combination with drought stress increased fresh weights and dry weights for *B. napus*, and in *B. juncea* but not for *B. campestris* compared to drought stress alone (Fig 1 A, B).

Leaf relative water content

Leaf relative water content (RWC) of *B. napus*, *B. campestris* and *B. juncea* seedlings were decreased by 13, 20 and 23%, respectively under drought stress, compared to unstressed control seedlings. Combination of trehalose and drought stress increased leaf RWC of all species except *B. campestris* (Table 1).

Proline content

Drought stress substantially increased Pro contents in leaves of of all *Brassica* spp. Under drought stress Pro contents of *B. napus*, *B. campestris* and in *B. juncea* were increased by 6.1, 6.5 and 6.7 fold 506, 546 and 517%, respectively, as compared to control seedlings. The Pro levels were significantly reduced in *B. campestris* and *B. juncea* seedlings by combination of Tre and drought stress (Table 1).

Chlorophyll content

Chlorophyll *a* and chl *b* contents decreased in all *Brassica* species under drought stress which contributed to reduction in total chl (*a*+*b*) by 14, 27 and 17%, respectively in *B. napus*, *B. campestris* and *B. juncea*. As compared to drought treatment alone, Tre combined with drought stress resulted in increased contents of chl *a*, chl *b* and chl (*a*+*b*) in *B. campestris* and *B. juncea* seedlings. However, contents of chl *a*, chl *b* and chl (*a*+*b*) in *B. napus* seedlings remained unchanged (Table 1)

Lipid peroxidation and hydrogen peroxide (H_2O_2) level and lipoxygenase (LOX) activity

The lipid peroxidations or MDA levels under drought stress increased by 193, 189 and 31%. Drought stress also resulted in increased accumulation of H_2O_2 by 57, 129 and 37% in *B. napus*, *B. campestris* and in *B. juncea* seedlings, respectively, compared to unstressed control seedlings. Trehalose in combination with drought stress significantly reduced MDA and H_2O_2 levels in all seedlings, compared to drought treated seedlings alone (Fig 2A, B). Lipoxygenase (LOX) activities in *B. napus*, *B. campestris* and in *B. juncea* seedlings were increased by 217, 51 and 135% respectively under drought stress, compared to non-stress control. As compared to drought treatment only LOX activities declined significantly in all species in Tre combined with drought treatment (Fig 2 C).

Ascorbate – glutathione pool

Drought stress caused a significant decrease in ascorbate (AsA) content of *B. campestris* seedlings but resulted in increased contents of AsA in *B. juncea* seedlings compared to control seedlings. Drought did not affect AsA contents of *B. napus* seedlings (Fig 3A). Under water stress dehydroascorbate (DHA) contents *B. juncea* remained unchanged whereas those of *B. napus* and *B. campestris* increased (Fig 3B). The ratio of AsA/DHA declined remarkably in *B. napus* and *B. campestris* but marginally increased in *B. juncea* under drought stress compared to control seedlings (Fig 3C). Compared to drought stress alone, exogenous Tre application in combination with drought stress increased AsA level in *B. campestris* and slightly in *B. juncea* . As compared to drought treatment alone, DHA level significantly decreased in all species except for *B. napus* (compared to control seedlings) (Fig 3A, B). Again, as compared to drought stress alone, exogenous Tre addition with drought treatment improved AsA/DHA ratio in *B. campestris* by 85% and in *B. juncea* by 43% and in *B. napus* it was decreased(Fig 3C). Drought stress significantly increased glutathione (GSH) levels of all *Brassica* spp seedlings, compared to control seedlings (Fig 3D). There were significant increases in oxidized glutathione, GSSG levels in all species. In *B. napus, B. campestris* and in *B. juncea* GSSG contents were increased by 70, 79 and 83%, respectively, compared to control seedlings (Fig 3E). Drought stress decreased the ratios of GSH/GSSG in seedlings of *Brassica napus* but remained unchanges in *B. campestris* and *B. juncea* seedlings. Addition of Tre with drought stress reduced GSSG contents and enhanced the

Table 1. Relative water content (RWC), chlorophyll and proline contents of *Brassica* seedlings under PEG-induced drought stress.

		RWC (%)	Proline (µmol g^{-1} fresh weight)	Chlorophyll contents (mg g^{-1} fresh weight)		
				Chl a	Chl b	Chl (a+b)
B. napus	Control	89.24±2.60ab	4.53±0.51e	0.63±0.028a	0.44±0.034a	1.08±0.006a
	Drought	77.62±0.79de	27.50±0.68a	0.58±0.016b	0.35±0.025c	0.93±0.041c
	Drought+Tre	81.51±7.18cd	24.94±0.67ab	0.58±0.007b	0.37±0.008bc	0.95±0.024bc
B. campestris	Control	91.23±1.33a	3.50±0.04e	0.63±0.031a	0.35±0.001c	0.99±0.030b
	Drought	73.03±4.20e	22.65±1.03b	0.50±0.031b	0.23±0.016e	0.72±0.047e
	Drought+Tre	85.54±1.92abc	11.21±0.95d	0.63±0.026a	0.29±0.015d	0.93±0.041bc
B. juncea	Control	84.98±1.10bc	4.08±0.15e	0.65±0.004a	0.40±0.04b	1.05±0.036a
	Drought	78.53±3.17d	27.39±0.87a	0.56±0.011b	0.31±0.014d	0.87±0.003d
	Drought+Tre	84.38±1.65bc	18.11±1.35c	0.62±0.028a	0.35±0.003c	0.98±0.024bc

Mean (±SE) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $P \leq 0.05$ applying DMRT

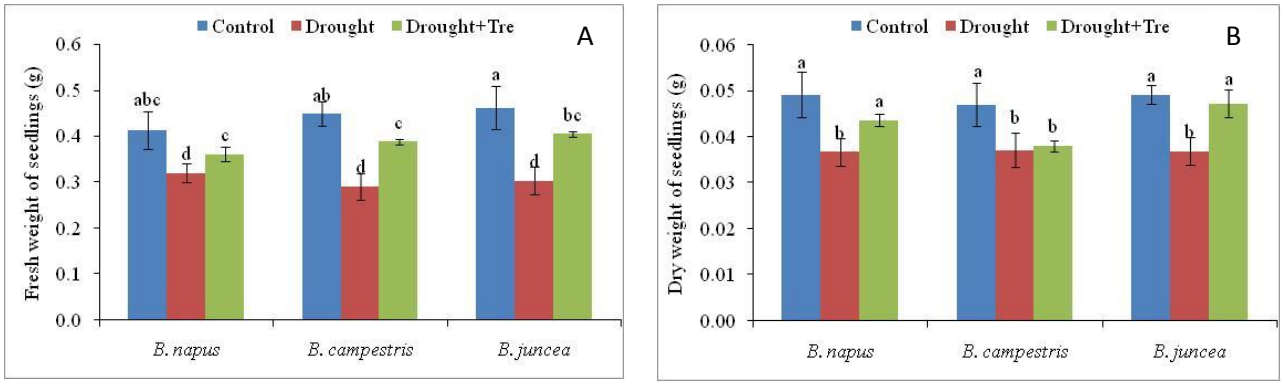

Fig 1. Fresh weight (A), dry weight (B) in *Brassica* seedlings induced by trehalose (Tre) under drought stress (15% PEG). Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying DMRT.

GSH/GSSG in seedlings of all species except that of in *B. campestris*, compared to drought treatment alone (Fig 3F).

Antioxidant enzymes

The APX activity increased in *B. campestris* and *B. juncea* by 26 and 22%, respectively under drought stress. Trehalose with drought stress significantly increased APX activity only in *B. juncea* (by 14%) (Fig 4A). Drought decreased MDHAR activity only in *B. campestris* as compared to control seedlings. Exogenous Tre addition in combination with drought stress increased MDHAR activity only in *B. campestris*, compared to drought stress alone (Fig 4B). Drought resulted in significant increase of DHAR activity (17%) in *B. napus* seedlings and significant decrease of DHAR activity (20%) in *B. campestris* seedlings, compared to control. Exogenous Tre in combination with drought stress *B. juncea* seedlings caused a significant increase in DHAR activity, compared to drought stressed seedlings alone (Fig 4C). As compared to control seedlings, increased GR activities were observed in all three species under water stress. In *B. napus*, *B. campestris* and *B. juncea* the GR activities were increased by 18, 74 and 35%, respectively. The GR activity was further increased only in *B. juncea* in Tre combined with water stress as compared to control or water stress treatments (Fig 4D). There were significant increases in GST activities in all *Brassica* spp seedlings under drought stress as compared to control seedlings Tre in combination with drought resulted in a significant decrease in GST activity in *B. compestris* and *B. juncea* as compared to the control or drought seedlings(Fig 5A). Drought resulted in increase of GPX activity in all *Brassica* spp seedlings, as compared to control seedlings. Addition of Tre with drought stress resulted in further increase (by 28%) of GPX activity only in *B. juncea* seedlings, compared to drought seedlings (Fig 5B). Drought stress increased SOD activity (by 16%) in *B. juncea* seedlings, while in the other two *Brassica* species SOD activities did not change, compared to control seedlings. Exogenous Tre maintained the same SOD activities all species except in *B. napus* where SOD activity decreased by 28% (Fig 5C). Drought stress decreased the CAT activity in *B. napus* (by 33%) and *B. campestris* (by 25%) whereas in *B. juncea* its activity increased (by 20%), compared to the control seedlings. Trehalose treatment increased CAT activity only in *B. napus* (Fig 5D).

Detoxification of MG through enhanced glyoxalase system

Drought stress increased Gly I activities of *B. napus*, *B. campestris* and *B. juncea* seedlings by 16, 42 and 22%, respectively, compared to control seedlings. On the other hand, Gly II activities decreased under drought stress (Fig 6A, B). Trehalose in combination with drought stress enhanced Gly I activity in *B. napus* only and in other species its activity was as high as drought treatment compared to drought stressed seedlings alone (Fig 6A). In contrary, Tre improved the Gly II activity only in *B. juncea* under drought stress (Fig 6B). An increase in MG contents under drought stress occurred in all species with the highest increase(by 122%) *in B. campestris*. In *B. napus* the MG content increased by 74% and in *B. juncea* MG content increased by 63%. As compared to water stress only, Trehalose addition with drought stress decreased MG contents in all species (Fig 6C).

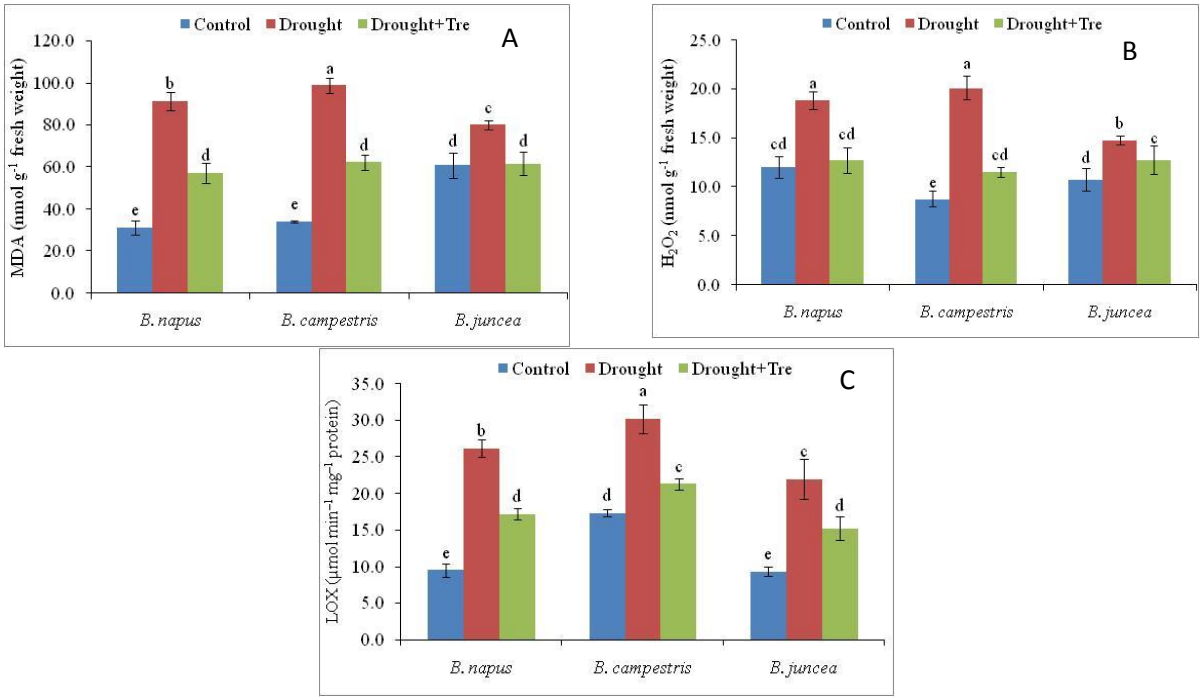

Fig 2. MDA content (A), H_2O_2 level (B), LOX activity (C) in *Brassica* seedlings induced by trehalose (Tre) under drought stress (15% PEG). Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying DMRT.

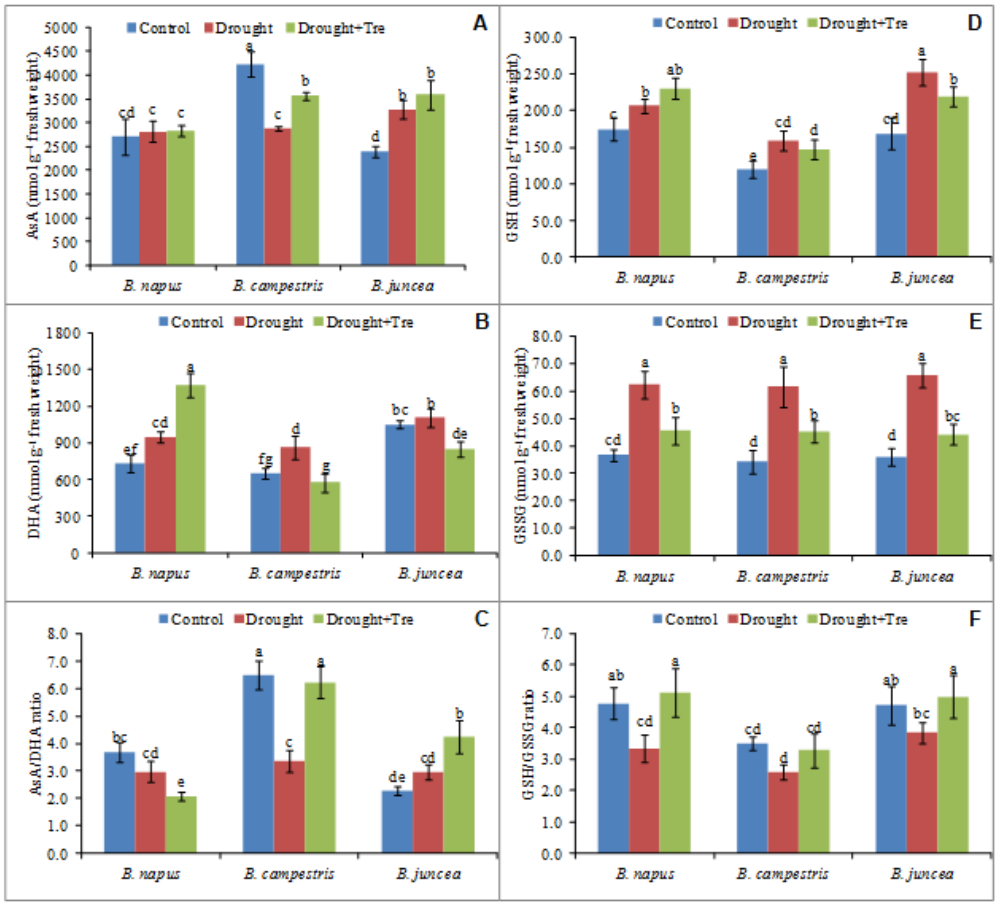

Fig 3. AsA content (A), DHA content (B), AsA/DHA ratio (C), GSH content (D), GSSG content (D), GSH/GSSG raio (E) in *Brassica* seedlings induced by trehalose (Tre) under drought stress (15% PEG). Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying DMRT.

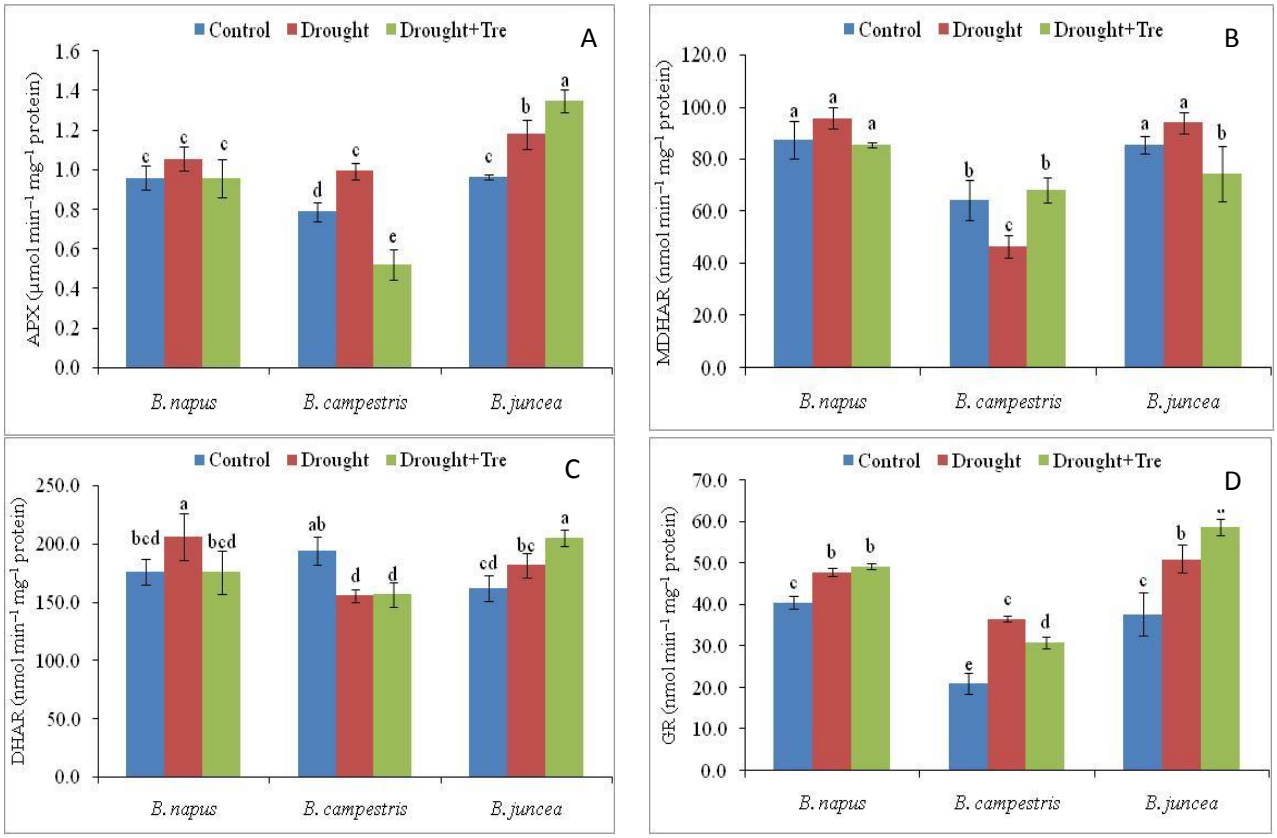

Fig 4. APX activity (A), MDHAR activity (B), DHAR activity (C), GR activity (D) in *Brassica* seedlings induced by trehalose (Tre) under drought stress (15% PEG). Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying DMRT.

Discussion

Trehalose as an osmoprotectant maintains cellular osmotic balance. It stabilizes dehydrated enzymes, proteins and lipid membranes efficiently. It protects biological structures from damage at descication (Garg et al., 2002). Previous studies proved Tre as efficient protectant against drought stress or under water deficit conditions (Gouffi et al., 1999; Wang et al., 2005; Ali and Ashraf, 2011). Our results seem to be corroborated with previous findings. Because Tre application alleviated adverse effects of drought stress in seedlings of different *Brassica* species by improving their growth and physiological attributes which will be discussed below. Drought stress has diversified adverse effects on plant physiological and metabolic processes (Hasanuzzaman et al., 2012). Drought affects plant–water relations, reduces water contents of leaf and plant, causes osmotic stress, inhibits cell expansion and cell division as well as growth of plants as a whole (Kirkham, 2005; Mahmood et al., 2012; Alam et al., 2013). In present study, drought stress reduced fresh and dry and leaf RWC of *Brassica* seedlings (Fig 1A, B). But as compared to other two species, B. juncea was more tolerant under drought stress. However, water content or growth reduction was restored by exogenous Tre supplementation under drought stress as evidenced by improved leaf RWC and fresh weight and dry weight of seedlings. Considering the restoration of leaf RWC, fresh and dry weight of seedlings induced by Tre under drought stress, the *B. juncea* was the best (Table 1; Fig 1A, B). Similar findings were documented previously by Tre addition with abiotic stress (Ali, 2011; Ali and Ashraf, 2011; Duman et al., 2011; Theerakulpisut and Gunnula, 2012). Important roles of Pro under abiotic stress conditions including drought stress are well recognized. Proline has vital roles in osmotic adjustment, stress signal transduction; it also acts as an antioxidant. Increase of Pro level under physiological stresses including drought stress conditions were documented previously (Bates et al., 1973; Nahar et al., 2013). Similarly profound increases of Pro levels under drought stresses were observed in all the species. Trehalose addition with drought stress reduced Pro levels in *B. campestris* and *B. juncea* seedlings (Table 1). Prevention of extra Pro biosynthesis due to exogenous Tre addition under drought stress suggests that Tre prevented *Brassica* seedlings from adverse effects of drought stress by other means so that the studied *Brassica* seedlings did not need to increase the Pro levels further. These results are corroborated to previous studies (Dolatabadian et al., 2009; Ali and Ashraf, 2011; Nounjan et al., 2012). Numerous studies reported that inhibition of photosynthesis were attributed by damages to photosynthetic pigments due to oxidation of pigments, impaired pigment biosynthesis (Anjum et al., 2011; Saraswathi and Paliwal, 2011; Pandey et al., 2012). In our study, reduced chl *a*, chl *b* and total chl *(a+b)* contents under drought stress were observed in all studies. Interaction of drought and Tre improved the photosynthetic pigment levels in studied species (Table.1) which is corraborated with the results of previous studies with *Lemmna gibba* L. where exogenous Tre improved photosynthetic pigment contents under cadmium stress (Duman et al., 2011). Induction of oxidative stress is a common effect of drought; significant increase of MDA and H_2O_2 levels in all *Brassica* species indicate the oxidative stress (Fig 2A, B). Similar oxidative stress induced by

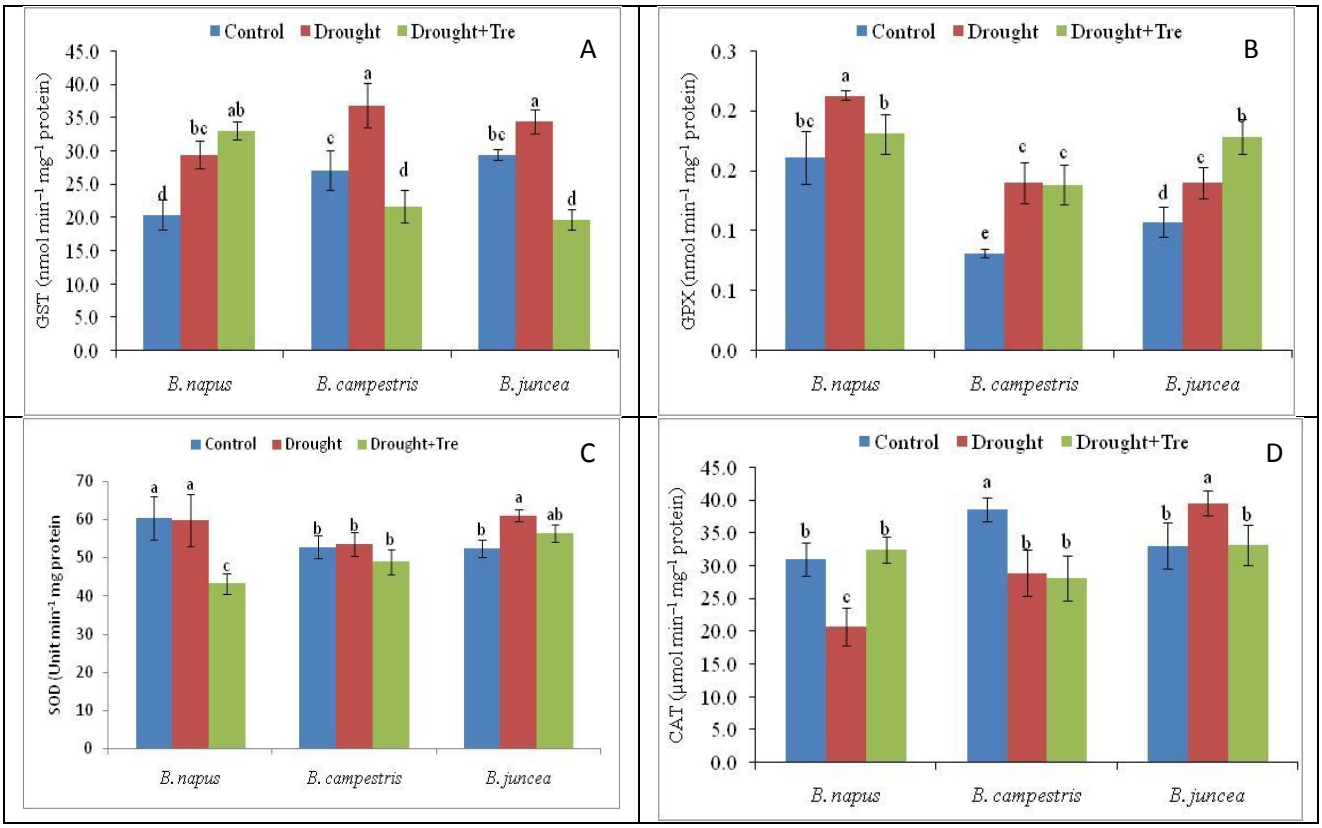

Fig 5. Activities of GST (A), GPX (B), SOD (C) and CAT (D) in *Brassica* seedlings induced by trehalose (Tre) under drought stress (15% PEG). Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying DMRT.

drought stress was documented in *B. juncea* in our previously (Alam et al., 2013) and in other studies also (Shan and Liang, 2010; Bideshki and Arvin, 2013). The beneficial roles of Tre in drought affected *Brassica* seedlings were observed as those treatments reduced oxidative stress (decreased H_2O_2 and MDA levels; Fig 2A, B), compared to Tre untreated drought affected seedlings. Exogenous Tre application was also effective in reducing oxidative stress of other plants in different abiotic stresses (Ali and Ashraf, 2011; Nounjan and Theerakulpisut, 2012; Ma et al., 2013). Increased LOX activity is responsible for oxidation of polyunsaturated fatty acids and thus enhances lipid peroxidation under stress conditions (Aziz and Larher, 1998, Sánchez-Rodríguez et al., 2012). Drought stress resulted in higher LOX activities in all *Brassica* seedlings (Fig. 2C) that partly contributed higher MDA levels (Fig 2A). Similar relationship of increased LOX activity and oxidative stress was observed in previous research findings (Kabiri et al., 2012; Sánchez-Rodríguez et al., 2012; Alam et al., 2013). Trehalose treatment with drought stress reduced the LOX activity and lipid peroxidation (Fig 2A, C) significantly in all the species that is in consistent with previous studies. Exogenous Tre significantly reduced LOX activity and H_2O_2, MDA contents which is also inconsistent with previous studies (Liu et al. 2013). Ascorbate, GSH with the enzymes APX, MDHAR, DHAR and GR predominantly constitute the AsA-GSH cycle (Hasanuzzaman et al., 2012). The roles of AsA-GSH cycle in antioxidant defense mechanisms are very important. Components of this cycle have vital roles in maintaining redox balance which prevent oxidative stress and have roles in transduction of signals towards abiotic stress tolerance. Ascorbic acid is considered as the most abundant, powerful and water soluble antioxidant that helps to reduce the oxidative damages caused by ROS in plants (Gill and Tuteja, 2010). Changes of AsA or oxidized AsA that is DHA pool and the ratio of AsA/DHA under drought stress is a part of antioxidant defense mechanism of plants (Alam et al., 2013; Nahar et al., 2013) and similarly in present study drought affected the AsA and DHA pool. Although the levels of AsA and DHA were affected differently in different *Brassica* species, their ratios were decreased under drought stress except in *B. juncea* (Fig 3C). Similar reductions of AsA or AsA/DHA ratio were found in previous findings (Sharma and Dubey, 2005; Shan and Liang, 2010). Ascorbate recycling is important for maintaining a balance state of cellular AsA (Chen et al., 2003). Trehalose treatment decreased DHA levels, increased AsA levels and AsA/DHA raios in *B. campestris* and *B. juncea* seedlings under drought stress (Fig 3A, B, C) which prove the beneficial roles of Tre. Similar enhancement of AsA pool was previously observed due to Tre addition under drought stress in wheat (Ma et al., 2013).

The GSH levels of all *Brassica* seedlings were increased by drought stress, compared to non-stressed control seedlings. Again, Tre addition with drought stress maintained the similar levels of GSH as in drought treated seedlings (Fig 3D) which are indications of improved antioxidant systems of *Brassica* seedlings both under drought stress and Tre supplemented drought stress conditions. From the Fig. 3D, it is clear that GSH level of *B. juncea* is the highest among all the species under drought stress which indicates its better antioxidant capacity. After Tre addition with drought, the GSH levels of *B. napus* and *B. juncea* were higher than that of *B. campestris* which also prove the better performance of first two species. The GSH is oxidized to GSSG when it is

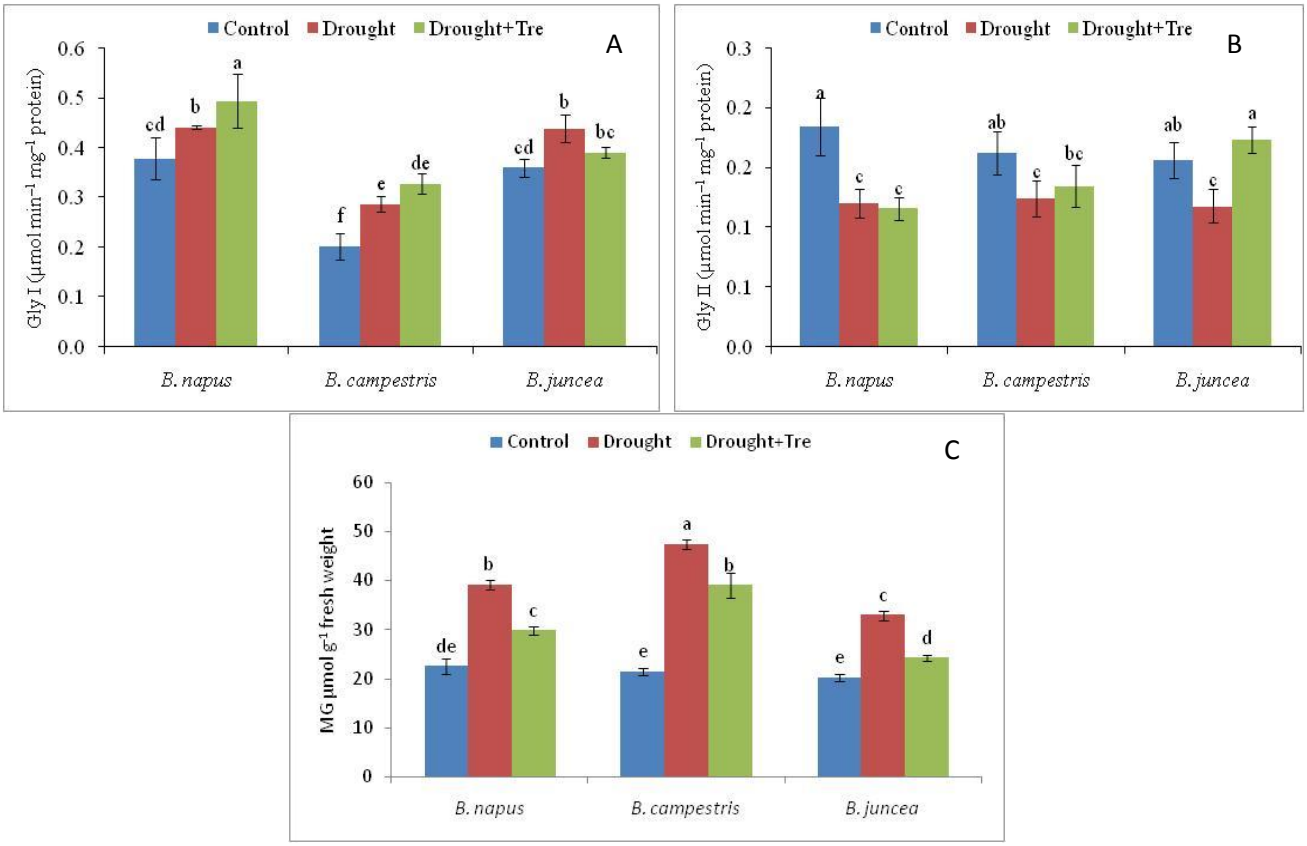

Fig 6. Gly I activity (A), Gly II activity (B), MG content (C) in *Brassica* seedlings induced by trehalose (Tre) under osmotic stress (15% PEG). Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying DMRT.

participated in ROS scavenging process that results in reduced GSH/ GSSG ratio (Hasanuzzaman et al., 2012). Increases in GSSG contents with decreases in GSH/GSSG were features of *Brassica* seedlings of present study. Our previous studies also showed increased GSH and GSSG level under drought stress (Alam et al., 2013; Nahar et al., 2013). Glutathione is often considered as the most important intracellular antioxidant, especially the GSH/GSSG ratio has immense functions in redox status and stress signaling processes. Thus higher GSH/GSSG is considered as supportive for improved abiotic stress tolerances including drought stress because GSH/GSSG ratio has vital roles in maintaining cellular redox balance and in transduction of stress signals (Gill and Tuteja 2010; Hasanuzzaman et al., 2012). Bringing this point in consideration, improvement of GSH/GSSG ratios of *B. campestris* and *B. juncea* by exogenous Tre application under drought stress (Fig 3F) (compared to single application of drought stress) is compassionate for improving their drought stress tolerance. In previous research findings, the augmentation of GSH by exogenous Tre application under drought stress improved plants' performances (Ma et al., 2013). The AsA and GSH or their redox balances are regulated and synchronized by enzymes of AsA-GSH cycles or by enzymes involved in their biosynthesis pathways. Reduced activity of MDHAR only in *B. campestris* seedlings was restored by Tre application with drought (Fig 4B); on the other hand, the DHAR activity was significantly enhanced in Tre sprayed *B. juncea* seedlings under drought stress, compared to drought treated seedlings alone (Fig 4C). These higher MDHAR or DHAR activities (Fig 4B, C) in *B. campestris* or in *B. juncea* might contribute to recycle AsA and thus their AsA levels were higher with Tre added drought treatment (Fig 3A). Increases in APX activities were documented in *B. campestris* and in B. *juncea*; whereas Tre application with drought stress resulted in further enhancement of its activity only in *B. juncea* (Fig 4A). In previous studies exogenous Tre under abiotic stresses enhanced the activities of enzymes involved in regeneration and recycling of AsA in different plant species which also improved the AsA levels (Nounjan and Theerakulpisut, 2012; Ma et al 2013). The GR activity is vital to recycle GSH (Shan and Liang, 2010; Hasanuzzaman et al., 2011a; Hasanuzzaman et al., 2012). Under drought stress GR activity significantly increased in all the *Brassica* species that might be due to enhanced antioxidative capacity under drought stress. Addition of Tre with drought stress only increased APX activity in *B. juncea* (Fig 4D). The role of exogenous Tre in enhancement of GSH level by increasing GR activity was previously studied (Ma et al., 2013). Exogenous application of Tre enhanced APX activity under Cd stress in *Lemna gibba* (Duman et al., 2011) and under salt stress in *Oryza sativa* (Nounjan et al., 2012). The GSTs are effective in reducing metal toxicity, herbicide detoxification, hormone homeostasis, vacuolar sequestration of anthocyanin, hydroxyperoxide detoxification, and detoxification of ROS. Thus, higher GST activity confers abiotic stress tolerance of plant species (Noctor et al., 2002; Dixon et al., 2010). Although GST activities of all *Brassica* species were increased under drought stress, Tre addition improved GST activity in *B. napus* under drought stress, compared to

drought stress alone (Fig 5A). The GPX reduces H_2O_2, organic and lipid hydroperoxides by using GSH (Noctor et al., 2002). Drought stress significantly increased GPX activities in all the studied species, compared to untreated control seedlings. But compared to drought stress alone, Tre supplemented drought treatment improved GPX activity further only in *B. juncea* (Fig 5B). Similarly, application of organic compatible solute increased GPX activity in *Nicotiana tabacum* under salt stress (Hoque et al., 2008). The SODs constitute frontline defense against ROS to dismutase $O_2^{\bullet-}$ to H_2O_2 and O_2 (Hasanuzzaman et al., 2012). Thus increased SOD activity under different abiotic stresses including water stress is influential to improve stress tolerance (Nounjan et al., 2012; Nounjan and Theerakulpisut, 2012; Ma et al., 2013). Similarly the results of our study are also supported by previous findings. The SOD activity was increased by exogenous Tre addition in Cd stress, salt stress in previous studies those prove the roles of exogenous Tre in modulating SOD activity under abiotic stress conditions (Ali and Ashraf 2011; Duman et al., 2011; Nounjan et al., 2012; Nounjan and Theerakulpisut 2012). However, in the present study, exogenous Tre addition with drought stress maintained the same levels of SOD activities as in drought stress conditions in different *Brassica* species except in *B. napus* (Fig 5C). Drought significantly reduced the CAT activities in all the species except in *B. juncea* (where its activity was increased) (Fig 5D). Similar result was observed in previous study (Sorial et al., 2010). Although CAT is one of the most important enzymes of antioxidant system having the highest turnover rates among all enzymes (Garg and Manchanda 2009), its activity under drought stress was restored by Tre supplementation only in *B. napus*. Several findings are corroborated to our study where CAT activity was increased by exogenous Tre under water stress in *Zea mays* (Ali and Ashraf, 2011) and under Cd stress in *Lemna gibba* (Duman et al., 2013). Enzymes of glyoxalase system, Gly I and Gly II effectively detoxify MG. In two step reaction, Gly I converts MG to *S*-D-lactoylglutathione (SLG) by using GSH as co-factor and second step converts SLG to D-lactate where GSH is recycled back (Yadav et al., 2005b; Mustafiz et al., 2010). In the present study, drought stress increased Gly I activities and decreased Gly II activities in all species (Fig 6A, B). With the alteration of glyoxalase enzymes in different *Brassica* species the increases of MG levels were highly significant under drought stress in case of all *Brassica* species (Fig 6C). Increases in MG levels under abiotic stresses (Yadav et al., 2005a; Singla-Pareek et al., 2006; Kumar and Yadav, 2009; Banu et al., 2010; El-Shabrawi et al., 2010; Turóczy et al., 2011) and altered Gly I and Gly II activities were also documented under drought stresses in previous studies (Alam et al., 2013). Comparing the plant species performance under drought condition and Tre combined drought stress condition, it is observed that Tre application boosted the Gly I activity only in *B. napus* and boosted the Gly II activity only in *B. juncea* species. However, Tre supplementation with drought stress detoxified the MG in *B. napus* and in *B. juncea* which corroborates with the activities of glyoxalase enzymes induced by Tre under drought stresses (Fig 6A, B, C). Similar augmentation of glyoxalase system conferred partly contributed drought stress tolerances in seedlings of different *Brassica* species in previous studies (Alam et al., 2013). Several researchers reported that the enhanced the Gly I and Gly II activities under abiotic stresses by exogenous organic compatible solute (Hoque et al., 2008; Kumar and Yadav 2009). In present study, we examined the comparative performance of different *Brassica* species under drought stress condition. Then we examined the effect of exogenous Tre in *Brassica* species under the same drought levels. Among the three *Brassica* species the *B. juncea* was the least prone to drought stress which is proved from the least oxidative damage, i.e. the lowest MDA, H_2O_2 and MG levels, LOX activity (Fig 2A, B, C; 6C) under drought which is supposed to due to the higher antioxidant and glyoxalase enzymes' activities (SOD, CAT, APX, MDHAR, GR, GST, Gly I) together with higher non-enzymatic antioxidant (AsA, GSH) levels compared to other two species. After *B. juncea*, the species *B. napus* performed better and the performance of *B. campestris* was the worst in the drought stress among the three species. In later two species (*B. napus* and *B. campestris*), the intensity of oxidative damage and state of their antioxidant and glyoxalase systems were interrelated as in *B. juncea*. Species differences exist because of genetic makeup those exhibit differences in growth habit and abiotic stress tolerance. Some findings of research studies also revealed that different *Brassica* species have different drought tolerance capacity (Pazoki et al., 2010; Din et al., 2011; Rad, 2012). Exogenous Tre application alleviated adverse effects of drought stress from seedlings of different *Brassica* species. But responses of different species were not similar. In *B. juncea*, Tre added drought treatment (as compared to drought stress alone) improved seedlings' fresh weight, dry weight, leaf RWC, chl *a*, chl *b*, AsA, GSH contents, AsA/DHA and GSH/GSSG ratios; enhanced activities of APX, DHAR, GR, GPX and Gly II; reduced levels of MDA, H_2O_2, Pro and LOX activity.*Brassica napus* seedlings with Tre addition under drought stress (compared to drought stress alone) showed improved seedlings' fresh weight, dry weight, GSH/GSSG ratio; upregulated CAT, GST, Gly I activities; reduced MDA, H_2O_2 contents and LOX activity. In *B. campestris* Tre supplementation with drought stress increased fresh weight, leaf RWC, chl *a*, chl *b*, chl *(a+b)* contents; improved AsA /DHA ratio, augmented MDHAR activity. So, the results suggest that *B. juncea* is a drought tolerant species by nature and moreover, its drought tolerance capability is further enhanced by exogenous Tre application.

Materials and Methods

Plant materials and stress treatments

Seeds of three *Brassica* spp. (*Brassica napus* L. cv. BARI Sharisha 13, *Brassica campestris* L. cv. BARI Sharisha 9 and *Brassica juncea* L. cv. BARI Sharisha 11) were surface-sterilized with 70% ethanol for 10 minutes and washed then placed in petri plates (9 cm) lined with 6 layers of filter paper moistened with 10 ml of distilled water and germinated in dark for two days. Each petri plates contained about fifty seedlings. Then seedlings were reared under controlled conditions (light, 350 µmol photon m^{-2} s^{-1}; temperature, 25±2°C; RH, 65–70%) which were maintained growing seedlings in growth chamber (IWAKI, Incubator, LIB-301, Japan). Seedlings were supplied with 10,000-fold diluted Hyponex (Hyponex, Japan) as nutrient. The experiment was conducted following CRD design with three replications. The experiment has nine treatments. After 10 days, one set of seedlings of each species of *Brassica* was subjected to drought stress by adding 15% PEG (PEG-6000, Wako, Japan) to the Hyponex solution. Another set of seedlings was supplied with 5 mM Trehalose (Trehalose dihydrate, Wako, Japan) in combination with drought or 15% PEG. Control plants were grown in Hyponex solution only. Each *Brassica*

species were grown with similar treatments. Data were collected after 48 hours.

Measurement of Fresh weight and dry weight of seedlings

Ten randomly selected fresh seedlings from each treatment were weighted, recorded and considered as fresh weight. Dry weight was determined after drying the seedlings at 80°C in oven (ADVANTEK, SP-450, Japan) for 48 h.

Measurement of Relative water content

Relative water content (RWC) was measured according to Barrs and Weatherly (1962). The whole leaf discs were weighed (fresh wt, FW), floated on distilled water in petri dish (8 h in dark). Turgid weights (TW) of leaves were taken drying excess surface water. Dry weights (DW) were measured after drying at 80 °C for 48 h. RWC was calculated by following formula:
RWC (%) = [(FW–DW)/(TW–DW)]×100

Measurement of Chlorophyll content

Leaves were extracted with 80% v/v acetone and supernatant were obtained by centrifuging at 5,000× *g*. Absorbances of the supernatants were taken with UV-visible spectrophotometer at 663 and 645 nm; chl contents were calculated according to Arnon (1949).

Determination of Proline content

Proline in leaf tissues was measured 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 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 some times chromophore containing toluene was read spectrophotometrically at 520 nm. Purified proline with different known concentrations was used to make a standard curve by comparing with which the proline content of plant samples were calculated.

Determination of Lipid peroxidation

The level of lipid peroxidation was measured by estimating malondialdehyde (MDA, a product of lipid peroxidation), using thiobarbituric acid (TBA) according to Heath and Packer (1968) with modifications (Hasanuzzaman et al. 2011a). The leaf samples (0.5 g) were homogenized in 3 ml 5% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 11,500× *g* for 10 min. One ml supernatant was mixed with 4 ml of TBA reagent (0.5% of TBA in 20% TCA). The reaction mixture was heated at 95°C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 11,500× *g* for 15 min. The absorbance of the colored supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as nmol of MDA g^{-1} fresh weight.

Determination of Hydrogen peroxide

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 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 (Є=0.28 $\mu M^{-1}cm^{-1}$) and expressed as µmol g^{-1} fresh weight.

Determination of Methylglyoxal level

Leaves were homogenized in 5% perchloric acid and centrifuged at 4ºC for 10 min at 11,000x g. The supernatant was decolorized by adding charcoal; then centrifuged at 11,000x g for 10 min. The supernatant was neutralized by saturated solution of potassium carbonate at room temperature. The neutralized supernatant was used for MG estimation by adding sodium dihydrogen phosphate and 20 µL of freshly prepared 0.5 M *N*-acetyl- L-cysteine to a final volume of 1 ml. Formation of the product *N*-*α*-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl)cysteine was recorded after 10 min at a wavelength of 288 nm according to Wild (2012). Using different known concentration of MG the standard curve was prepared by following the same procedure used for supernatant of plant sample. The MG content within the plant sample was calculated by using standard curve and expressed as µmol g^{-1} FW.

Extraction and measurement of ascorbate and glutathione

Leaves (0.5 g) were homogenized in 5% meta-phosphoric acid containing 1 mM EDTA, then 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 supernatant was neutralized with 0.5 M K-P buffer (pH 7.0). The reduced ascorbate was assayed spectrophotometrically at 265 nm in 100 mM K-P buffer (pH 7.0) with 0.5 unit of ascorbate oxidase (AO). 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) utilizing 200 µl of aliquots of supernatant neutralized with 300 µl of 0.5 M K-P buffer (pH 7.0). Based on enzymatic recycling, GSH is oxidized by 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of GR, and glutathione content is 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 with known concentrations of GSH and GSSG were used and same procedure was followed as in supernatant of plant sample. The content of GSH was calculated by subtracting GSSG from total GSH.

Protein determination

The protein concentration of each sample was determined following the method of Bradford (1976) using BSA as a protein standard where 5, 10, 15, 20, 25 µg μl^{-1} protein concentrations were used to prepare standard curve.

Enzyme extraction and assays

Leaves were homogenized in potassium phosphate (K-P) buffer (pH 7.0) containing KCl, ascorbate, *β*-mercaptoethanol and glycerol. Homogenates were centrifuged at 11,500× *g*, supernatants were assayed. SOD (EC 1.15.1.1) activity (El-Shabrawi et al. 2010) assay: Using xanthine–xanthine oxidase system SOD activity was assayed. The K-P buffer (50 mM), NBT (2.24 mM), catalase (0.1 units), xanthine oxidase (0.1 units), xanthine (2.36 mM) and enzyme extract were in reaction mixture. Catalase was added to avoid H_2O_2-mediated inactivation of CuZn-SOD. Change in absorbance was read at 560 nm. The SOD activity was expressed as units (amount of enzyme needed to inhibit NBT reduction by 50 %) min^{-1} mg^{-1} protein. APX (EC: 1.11.1.11) activity (Nakano and Asada 1981) assay: 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 (final volume 700 µL). Reaction was started by adding H_2O_2. Activity was measured at 290 nm for 1 min using an extinction coefficient 2.8 $mM^{-1}cm^{-1}$. MDHAR (EC: 1.6.5.4) activity (Hossain et al., 1984): 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 (final volume 700 µL). Reaction was started adding AO. Absorbance was taken at 340 nm; activity was calculated from change in for 1 min using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. DHAR (EC: 1.8.5.1) activity (Nakano and Asada 1981): Reaction buffer contained 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. Activity was calculated from 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 (Hasanuzzaman et al., 2011b): Reaction mixture contained 0.1 M K-P buffer (pH 7.0), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH; enzyme solution (final volume 1 mL). Reaction was initiated with GSSG; decrease in absorbance at 340 nm was recorded for 1 min (calculated using extinction coefficient 6.2 $mM^{-1}cm^{-1}$). GST (EC: 2.5.1.18) activity (Hossain et al., 2006): Reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4- dinitrobenzene (CDNB); enzyme solution (final volume 700 µL). Reaction was initiated by CDNB; increase in absorbance was measured at 340 nm, 1 min. Activity was calculated using extinction coefficient 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). The reaction mixture consisted of 100 mM K-P buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2 (as a substrate) and 20 µl of sample solution. The oxidation of NADPH was recorded at 340 nm for 1 min and the activity was calculated using the extinction coefficient of 6.62 $mM^{-1}cm^{-1}$. CAT (EC: 1.11.1.6) activity (Hasanuzzaman et al. 2011a): Decrease of absorbance (by decomposition of H_2O_2) at 240 nm was recorded for 1 min. Reaction was initiated with enzyme extract; activity was calculated from extinction coefficient 39.4 $M^{-1}cm^{-1}$. Glyoxalase I (EC: 4.4.1.5) (Hasanuzzaman et al. (2011a): Assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM GSH and 3.5 mM MG (final volume 700 µL). Reaction was started by adding MG; increase in absorbance was recorded at 240 nm, 1 min. Activity was calculated using extinction coefficient 3.37 $mM^{-1}cm^{-1}$. Glyoxalase II (EC: 3.1.2.6) (Principato et al. 1987): Formation of GSH at 412 nm was monitored in 1 min. Reaction mixture contained 100 mM Tris–HCl buffer (pH 7.2), 0.2 mM DTNB, 1 mM *S*-D - lactoylglutathione (SLG) (final volume of 1 mL). Reaction was started by SLG; activity was calculated using extinction coefficient 13.6 $mM^{-1}cm^{-1}$. LOX (EC 1.13.11.12) activity was estimated according to the method of Doderer et al. (1992) by monitoring the increase in absorbance at 234 nm using linoleic acid as a substrate. The activity was calculated using the extinction coefficient (25 mM^{-1} cm^{-1}) and express as units (1 nmol of substrate oxidized per minute) per mg protein.

Statistical analysis

The experiment was conducted with CRD design with three replications. 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 $P \leq 0.05$ were considered significant.

Conclusion

The present study and the available literature reviewed in the discussion suggest that exogenous Tre is an effective in improving short-term drought tolerance in different *Brassica* species where species difference is evident with respect to antioxidant and glyoxalase enzyme activities and physiological performances also. Performing diversified roles in all stages of development in plants, Tre acting as osmoprotectant, signaling molecule, still very little is known about the role of trehalose in plants. In plants, Tre accumulates in trace amounts but functions a lot (Hu et al., 2010; Sundaramurthi et al., 2010). Although some researchers have successfully manipulated trehalose accumulation in plants for crop improvement purposes; its roles in tolerances to diversified of stresses in relation to plant architecture, growth and metabolism still demand huge researches.

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