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Mitigation of PEG-induced drought stress in rapeseed (*Brassica rapa* L.) by exogenous application of osmolytes

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ABSTRACT

Considering the adverse effect of drought on plants growth, development and physiology, we studied the protective role of three osmolytes, proline (Pro), glycine betaine (GB) and trehalose (Tre) for the mitigation the adversity of drought on rapeseed (*Brassica rapa* L. cv. BARI Sharisha-15) seedlings. The experiment consisted of eight treatments viz. control, Pro (0.5 mM), GB (0.5 mM), Tre (0.5 mM), Drought (20% Polyethylene glycol, D), Pro + D, GB + D and Tre + D. Reduced plant height, seedling fresh and dry biomass, relative water content (RWC) of leaf and chlorophyll (chl) contents were observed due to drought stress with increased levels of endogenous Pro, GB, and Tre. Drought stress also induced oxidative stress, manifested by the overgeneration of toxic superoxide ($O_2^{\bullet -}$) radicals, increased hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and methylglyoxal (MG) content, as well as impaired antioxidant defense and redox balance. However, pretreatment with Pro, GB and Tre upregulated the antioxidant defense mechanisms and reduced oxidative damage by reducing MDA, H_2O_2 and MG contents. Moreover, increased the glutathione (GSH), and decreased oxidized glutathione (GSSG) levels, finally improved the redox balance. In addition, exogenously applied osmolytes improved the chl contents and water balance, which improved the rapeseed seedlings development. Among the three osmolytes studied, Pro performed best in alleviating drought stress in rapeseed at the early seedling stage.

1. Introduction

Because of the multifarious damaging nature, drought is regarded as major environmental stress to plants (Cao et al., 2017). The recent worldwide crisis of both surface and groundwater is one of the reasons for increased drought stress day by day. Drought exerts detrimental effects on plant physiology; the ultimate result is growth reduction, and lower accumulation of biomass (Anjum et al., 2016; Hasanuzzaman et al., 2016). Water deficiency also results in lower respiration rate and stomatal conductance; consequently disrupt the energy balance (Hasanuzzaman et al., 2014a, b). Hence, the insufficient dissipation of energy directs to the over-generation of reactive oxygen species (ROS), for instance, singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) (Hasanuzzaman et al., 2012a). Reactive oxygen species are the principal toxic radicals that can further react potentially and can destruct the important biomolecules, like lipids, proteins, and DNA, which finally leads to irreversible metabolomic disorder and programmed cell death (Ahmad et al., 2016; Nahar et al., 2015a, b).

Fortunately, plants possess exceedingly well-organized antioxidant machinery to counter balance the toxic ROS, comprised of both enzymes and metabolites (Ahmad et al., 2010; Hasanuzzaman et al., 2018). The efficiency of the antioxidant defense pathway is a vital strategy to detoxify ROS effectively. Moreover, another toxic compound

Abbreviations: AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, Ascorbate; car, carotenoids; CAT, catalase; chl, chlorophyll; DHAR, dehydroascorbate reductase; Gly I, glyoxalase I; Gly II, glyoxalase II; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GB, glycine betaine; GST, glutathione *S*-transferase; LOX, lipoxygenase; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; MG, methylglyoxal; NADP, nicotinamide adenine dinucleotide phosphate; PEG, polyethylene glycol; Pro, proline; PS I, photosystem I; PS II, photosystem II; ROS, reactive oxygen species; RuBisCo, ribulose -1, 5- bisphosphate carboxylase or oxygenase; RWC, relative water content; SOD, superoxide dismutase; Tre, trehalose

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and glycolytic byproduct—methylglyoxal (MG), overproduced under dehydration stress condition and has the potentiality to damage large biomolecules like proteins, lipids, and carbohydrates. However, the plant possesses GSH dependent glyoxalase pathway, which can potentially detoxify MG in a cell by glyoxalase enzymes (glyoxalase I, Gly I and glyoxalase II, Gly II) under abiotic stress conditions, including drought (Hasanuzzaman et al., 2017).

Rapeseed (*Brassica rapa* L.) belongs to the family Brassicaceae are among the most important oilseed crops throughout the world. Reports on drought-stressed *Brassica* seedlings have shown a significant decrease in germination percentage, poor growth and vigor index with low biomass accumulation (Razaji et al., 2014); along with severe oxidative damages and impaired antioxidant defense (Alam et al., 2014; Hasanuzzaman et al., 2018).

Osmolytes or osmoprotectants are small, highly soluble, uncharged, and nontoxic organic molecules, for example, proline (Pro), glycine betaine (GB), trehalose (Tre), play significant roles in plant physiology under environmental adversities (Nahar et al., 2016a; Hasanuzzaman et al., 2019). Proline, GB and Tre take part in osmotic adjustment through stabilizing biological structures, macromolecules, photosynthetic pigments, and scavenging toxic ROS (Szabados and Savouré, 2010; Luo et al., 2010; Giri, 2011; Ahmad et al., 2017a, b).

Although osmolytes are biosynthesized and accumulated in plant cells naturally, their intrinsic accumulation, is nevertheless not high enough to give protection to plants from damages induced by drought. Under such condition, the exogenous application of various osmolytes can assist in reducing the negative impacts. However, the response of plants varies depending on genotypes, and the severity and extent of drought. Although hundreds of reports focused adversity of drought stress on *Brassica* spp., but there is hardly any study regarding the comparative efficacy of three osmolytes in mitigating drought stress. Therefore, we investigated the physiological mechanisms of drought stress tolerance mediated by exogenous Pro, GB and Tre on rapeseed (*B. rapa* L. cv. BARI Sharisha-15) focused on the growth enhancement, ROS metabolism and methylglyoxal detoxification. As per our knowledge, this is the first report to explicate the comparative physiological role of three osmoprotectants (Pro, GB and Tre) for conferring drought tolerance in rapeseed, where the amalgamated actions of antioxidant and glyoxalase pathway was simultaneously explored.

2. Materials and methods

2.1. Seedling growing and treatment condition

Uniform seeds of BARI Sharisha-15 (*B. rapa* L.) were sorted and sterilized using ethanol (70%) for 5 min, rinsed thoroughly in water, and were soaked for 10 min, and placed in Petri plates (9 cm diameter) on 6 layers of bloating paper saturated with 10 mL of distilled water, and placed for germination. The Petri plates were observed after 72 h, and deformed and abnormal seedlings were discarded. Finally, keeping 60 seedlings in each Petri plates, seedlings were grown in the hydroponic medium under controlled condition (light, 350 μmol photon m^{-2} s^{-1}; temperature, 25 ± 2 °C; RH, 65–70%), using 5000-fold diluted Hyponex solution (Hyponex, Japan). The nutrient solution contained 8% N, 6.43% P, 20.94% K, 11.8% Ca, 3.08% Mg, 0.07% B, 0.24% Fe, 0.03% Mn, 0.0014% Mo, 0.008% Zn, and 0.003% Cu. For Pro, GB and Tre pretreatment the seedlings were feed with nutrient solutions supplemented with 0.5 mM proline, 0.5 mM GB and 0.5 mM Tre solution and allowed to grow for 48 h. Afterward, the pretreated Petri dishes were washed several times and were grown with or without drought (induced by 20% polyethyleneglycol, PEG) individually or in combination. Data were recorded from the leaf samples, after 48 h of stress exposure, and the experiment was repeated three times maintaining the same conditions.

2.2. Growth and biomass accumulation

The average height of the randomly selected five plants was considered as the plant height for each treatment, whereas, the average weight of ten seedlings with primary root was considered as fresh weight (FW). After drying of the seedlings at 80 °C in an oven, seedling dry weight (DW) was obtained.

2.3. Measurement of relative water content

Previously standardized Barrs and Weatherley (1962) method was occupied for measuring the relative water content (RWC) using the following formula:

RWC (%) = [(FW-DW)/ (TW-DW)] × 100;

where TW stands for turgid weigght.

2.4. Determination of proline content

Proline content was measured according to the method stated by Bates et al. (1973). Immediately after plucking, leaf sample (0.25 g) was ground in 5 mL of sulfosalicylic acid and extract was collected after centrifugation (11,500 × *g* for 15 min at 4 °C). Afterward, an aliquot of 1000 μL of the supernatant, 1000 μL acid ninhydrin, and 1000 μL glacial acetic acid were mixed and heated at 100 °C for 60 mim. Afterward, 2 mL toluene was put into the mixture, vortexed thoroughly for separating the toluene chromophore containing Pro, and was optically measured at 520 nm keeping toluene as blank, and calculated from a Pro standard curve.

2.5. Determination of glycine betaine content

Glycine betaine content was measured using the protocol followed by Greive and Grattan (1983). Oven dried leaf sample (0.1 g) was homogenized with 4 mL deionized water followed by shaking for 24 h at 25 °C in a mechanical shaker and filtered. Aliquots of 0.25 mL filtrate was taken and mixed with 0.25 mL of 2N H_2SO_4, cooled (1 h), and 0.2 mL cold Iodine-potassium iodide (I_2–KI) reagent was mixed, gently and stored at 4 °C (16 h). Afterward, centrifugation (13500 × *g*, 15 min, 0 °C) was performed and the supernatant was aspired followed by 9 mL of 1, 2-dichloroethane was incorporated to the precipitate and thoroughly mixed to dissolve. After 2.5 h the absorbance was measured at 365 nm and calculated using a standard curve.

2.6. Determination of trehalose content

Trehalose amount was measured following Li et al. (2014). Fresh leaves (0.5 g) were ground in 5 mL 80% (v/v) ethanol and the supernatant was collected after centrifugation at 11,500 × *g* for 20 min. The solvent was evaporated and the dry sample was resuspended in 5 mL distilled water. An aliquot of the solution (100 μL) was mixed with 150 μL 0.2 N H_2SO_4 and 0.6 N NaOH was added to hydrolyze and destroy sucrose, glucose-1-phosphate and reducing sugars. Afterward, anthrone reagent (0.05 g anthrone per 100 mL of 72% H_2SO_4) was added, and the absorbance was recorded at 630 nm, and calculated using a standard curve.

2.7. Determination of chlorophyll content

For determining chl contents, fresh leaves (0.5 g) were homogenized in acetone (80% v/v) and centrifuged at 2000 × *g*, and supernatants were collected. The optical absorbance of the supernatants was observed at 663, and 645 nm for chl *a* and *b* content, respectively according to the method described by Arnon (1949). Chlorophyll (*a* + *b*) was calculated by adding the values of chl *a*, and chl *b*.

2.8. Measurement of lipid peroxidation and H_2O_2 generation

The lipid peroxidation level was measured following Hasanuzzaman et al. (2012b) by estimating malondialdehyde (MDA) content as thiobarbituric acid reactive substances (TBARS), which was optically read at 532 nm and deducted by the absorbance at 600 nm. The content was calculated using an extinction coefficient (155 mM^{-1} cm^{-1}). The procedure of Yu et al. (2003) was occupied for H_2O_2 determination, where 0.5 g leaves were extracted in potassium-phosphate (K-P) buffer (50 mM, pH 6.5), and centrifuged (11,500 × *g*, 15 min). In 3 mL of supernatant 1 mL of acidic $TiCl_4$ (0.1%) was mixed and kept in room temperature for 10 min, again centrifuged (11,500 × *g*, 12 min) and immediately the absorbance was recorded at 410 nm. The level of H_2O_2 was calculated employing an extinction coefficient (0.28 μM^{-1} cm^{-1}).

2.9. Histochemical confirmation of H_2O_2 and $O_2^{\bullet -}$

Stress-induced overproduction of H_2O_2 and $O_2^{\bullet -}$ in leaf tissue was confirmed following Chen et al. (2010) by dipping the leaves in acidic 0.01% 3-diaminobenzidine (DAB) and 0.01% nitrobluetetrazolium chloride (NBT) in dark condition (24 h) for H_2O_2 and $O_2^{\bullet -}$ confirmation, respectively. Afterward, leaves were bleached in hot ethanol, and the brown and blue spots appeared from H_2O_2 and $O_2^{\bullet -}$ with the reaction of DAB and NBT, respectively, were observed on the leaf surface and photographed were taken for documentation.

2.10. Extraction and measurement of ascorbate and glutathione

For measuring the contents of AsA and GSH pools, homogenization of 0.5 g freshly plucked leaves with 5% meta-phosphoric acid was performed followed by centrifugation (11,500 × *g*, 15 min), and the supernatants were collected for AsA and GSH pools analysis according to the procedure of Hasanuzzaman et al. (2011a). The optical absorbance of enzymatic oxidation of AsA was read in 265 nm and quantified with a standard curve. Afterward, GSH content was observed optically (412 nm) employing enzymatic method, where 5,5-dithio-bis(2-nitrobenzoic acid, DTNB) oxidized GSH, which is further reduced by NADPH dependent GR enzyme, and calculated using a standard curve.

2.11. Protein determination and enzyme activity assays

Extraction reagent (1 mL) containing 50 mM K-P buffer (pH 7.0), 100 mM KCl, 1 mM AsA, 5 mM β-mercaptoethanol, and glycerol (10%, w/v) was used to extract 0.5 g of fresh leaf tissue. The supernatants were preserved at (−60 °C) and further used for protein content determination and enzyme activaty assay.

Protein content was estimated following the protocol of Bradford (1976), where we prepared a bovine serum albumin (BSA) standard curve for calculating protein content.

Among the antioxidant enzymes, CAT (EC: 1.11.1.6) activity was assayed following Hasanuzzaman et al. (2012b) by monitoring the absorbance at 240 nm. Meanwhile, the activity of APX (EC: 1.11.1.11) was monitored at 290 nm following Nakano and Asada (1981). Afterward, the procedure of Hossain et al. (1984) was occupied for MDHAR (EC: 1.6.5.4) activity assay monitored at 340 nm. Further, DHAR (EC: 1.8.5.1) and GR activity was observed at 265 nm and 340 nm following Nakano and Asada (1981) and Hasanuzzaman et al. (2011b), respectively. Moreover, GSH dependent GPX (EC: 1.11.1.9) activity was recorded at 340 nm maintaining the procedure of Elia et al. (2003).

2.12. Determination of glyoxalase activity and methylglyoxal level

In the glyoxalase pathway, Gly I (EC: 4.4.1.5) activity was determined following Hasanuzzaman et al. (2011a), which was monitored at 240 nm. While, Gly II (Gly II; EC: 3.1.2.6) assay carried out following Principato et al. (1987), which was read optically at 412 nm. For MG determination, freshly plucked leaves were extracted using 5% perchloric acid. The supernatants were decolorized using activated charcoal and neutralized by saturated Na_2CO_3. The MG content was estimated from the production of N-α-acetyl-S-(1-hydroxy-2-oxoprop-1-yl) cysteine, monitored at 288 nm, and calculated from a MG standard curve (Wild et al., 2012).

2.13. Statistical analysis

The experiment was carried out in a completely randomized design (CRD) at three repetitions. The data obtained were statistically analyzed with XLSTAT 2016 (Addinsoft, 2016) following one-way ANOVA and mean separation was performed by Fisher's LSD test considering 5% level of probability.

3. Results

3.1. Growth and biomass accumulation

Significant variation in growth in terms of plant seedling was observed among stressed and non-stressed seedlings. Upon drought exposure, the seedling height decreased by 20%, compared to control, whereas exogenous Pro, GB, and Tre pretreated seedlings showed a significant increase in seedling height by 11, 8 and 7%, respectively, comparing non-treated seedlings. Moreover, drought condition resulted in a significant reduction of the FW and DW, but, pretreatment with Pro, GB and Tre improved the FW and DW of the rapeseed seedlings under drought stress (Table 1).

3.2. Osmolyte content and water balance

Proline content increased by 10-fold compared to the unstressed control. Exogenous Pro, GB and Tre pretreatment and subsequent drought exposure decreased Pro content that was 26, 33 and 28% lower respectively, than drought stress alone (Fig. 1A). A significant increase in the endogenous GB content by 192% in drought-exposed *B. rapa* seedlings over control was found. But, Pro, GB and Tre pre-treated drought-stressed seedlings restored GB content, compared to untreated seedlings (Fig. 1B).

Drought exposure again increased the endogenous Tre content by 59% comparing control. Trehalose pretreatment significantly increased endogenous Tre levels by 31%, compared to the control. Relative to drought stress Tre pre-treated stressed seedlings exhibited further augmentation of endogenous Tre (Fig. 1C).

Leaf relative water content lessened with the imposition of drought stress by 23%, compared with the control (Fig. 1D). But Pro, GB and Tre pretreated drought-stressed seedlings showed 22, 19 and 7% increase in RWC, correspondingly, comparing drought-stressed seedlings alone (Fig. 1D).

Table 1
Shoot length, FW, DW and RWC (%) of rapeseed seedlings induced by Pro, GB and Tre under drought stress condition.

Treatments	Seedling height (cm)	FW (g $seedling^{-1}$)	DW (g $seedling^{-1}$)
Control	2.65 ± 0.12a	0.43 ± 0.0029a	0.0510 ± 0.00012ab
Pro	2.55 ± 0.11 ab	0.45 ± 0.0017a	0.0506 ± 0.00012bc
GB	2.47 ± 0.08bc	0.42 ± 0.0003 ab	0.0496 ± 0.00007c
Tre	2.54 ± 0.11 ab	0.44 ± 0.0028a	0.0514 ± 0.00011a
D	2.12 ± 0.10e	0.35 ± 0.0031c	0.0433 ± 0.00019c
Pro + D	2.36 ± 0.08cd	0.38 ± 0.0012bc	0.0482 ± 0.00003c
GB + D	2.29 ± 0.07d	0.36 ± 0.0023c	0.0465 ± 0.000005c
Tre + D	2.28 ± 0.03de	0.36 ± 0.0034c	0.0478 ± 0.00004c

Pro-proline, GB-glycine betaine, Tre-trehalose.
Means (± SD) were calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

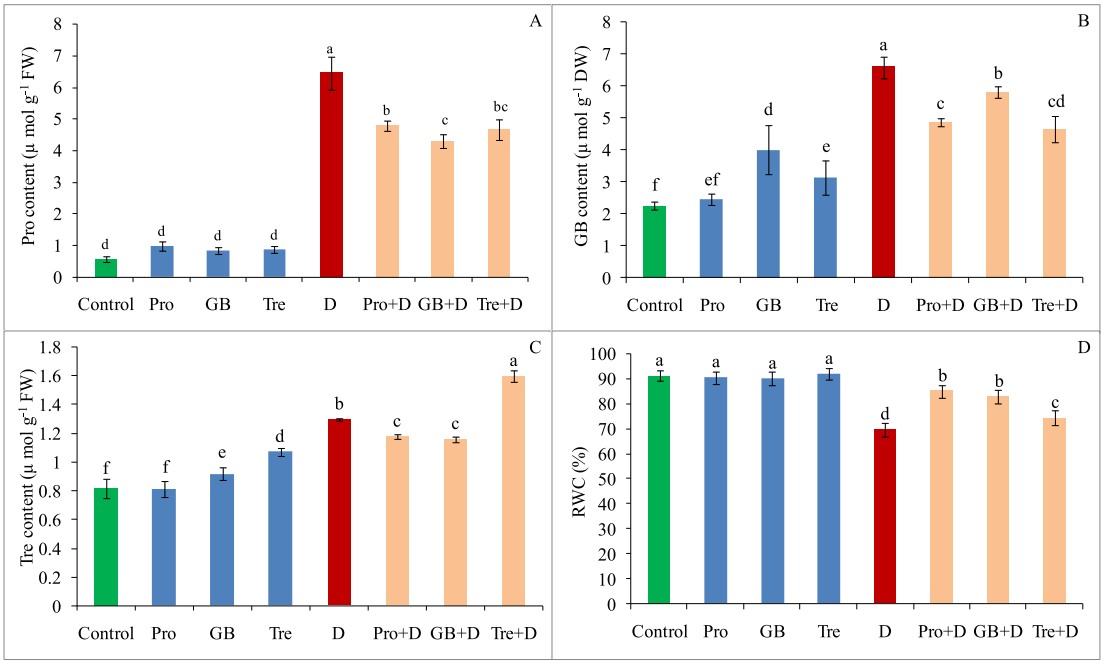

Fig. 1. Effect of exogenous Pro, GB and Tre on Pro (A), GB (B), Tre (C) and RWC (D) of rapeseed seedlings under drought stress. Means (± SD) were calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

3.3. Chlorophyll contents

Chlorophyll contents significantly declined in rapeseed seedlings under drought stress by 55 and 49% in case of chl *a* and chl *b* respectively, compared to control, which contributed to the reduction in chl (*a* + *b*) content by 50%. As compared to drought treatment alone increased chl (*a* + *b*) contents by 64 and 53%, respectively, was under Pro and GB pretreatment, whereas Tre pretreatment doesn't alter the contents of chl (*a* + *b*) (Fig. 2).

3.4. Levels of lipid peroxidation (MDA content), and H_2O_2 content

Sharply increased MDA content was visible under drought exposed seedlings by 82% comparing with the control (Fig. 3A). On the other hand, Pro, GB and Tre pretreated drought-exposed seedlings exhibited significantly reduced MDA levels comparing with drought exposed seedlings alone. On the other hand, the levels of H_2O_2 enhanced by 131% under drought condition compared with control. However, a significant reduction in H_2O_2 contents by 29, 17 and 10% were observed in Pro, GB and Tre pretreated drought-exposed seedlings

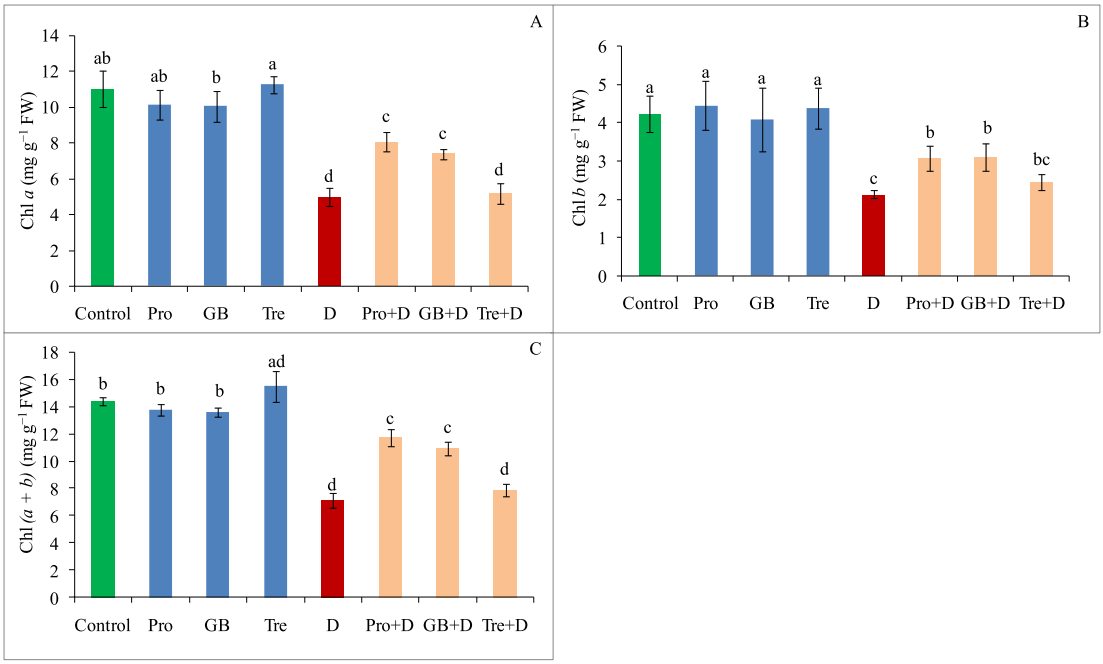

Fig. 2. Effect of exogenous Pro, GB and Tre on (A) Chlorophyll (chl) *a* (B) chl *b*, (C) and total chl (*a* + *b*) content of rapeseed seedlings under drought stress. Means (± SD) were calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

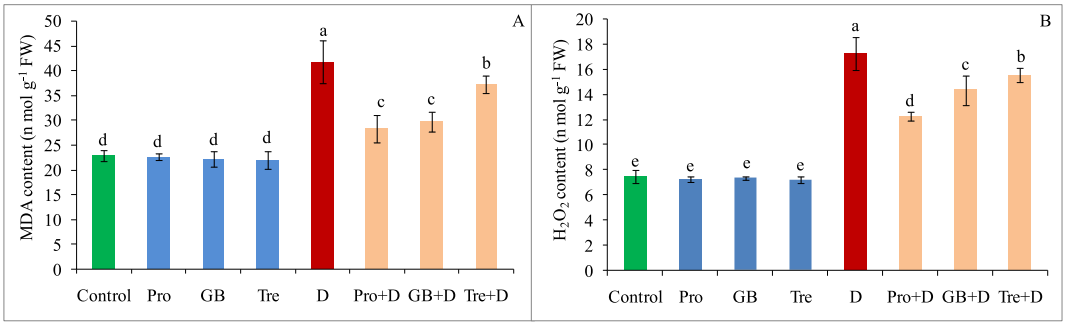

Fig. 3. Effect of exogenous Pro, GB and Tre on MDA (A) and H_2O_2 (B) content of rapeseed seedlings under drought stress. Means (± SD) were calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

comparing with drought-exposed seedlings alone (Fig. 3B).

3.5. Histochemical revelation of H_2O_2 and $O_2^{\bullet -}$ generation

Drought stress augmented H_2O_2 and $O_2^{\bullet -}$ generation in leaves in comparison with the control, which was evident with the intensity of brown and blue spots on the stained leaves. However, prior application of Pro, GB and Tre to drought-stressed seedlings decreased brown and blue spots on the leaves, compared to drought stress alone (Fig. 4).

3.6. Ascorbate – glutathione pool

Drought stress increased the endogenous AsA levels by 10% in rapeseed seedlings compared to control. Whereas, Pro pretreated drought exposed seedlings exhibited 3% increase in cellular AsA content except, GB and Tre pretreated seedlings, those showed 2 and 4% decrease regarding AsA content, respectively, as compared with drought stress alone (Fig. 5A).

The GSH content was enhanced by 72% in drought-stressed seedlings compared to unstressed seedlings. But, Pro pretreated drought stressed seedlings were found with further increased GSH level by 9%. However, GB and Tre pretreated drought stressed seedlings didn't show a significant difference in terms of GSH content compared to drought-exposed seedlings (Fig. 5B). Oxidized glutathione content was markedly increased (178%) under drought compared with the untreated control, in contrary, the seedlings that were pre-treated with Pro, GB and Tre showed lower GSSG levels by 47, 43 and 35%, respectively, in comparison with drought stress alone (Fig. 5C). Furthermore, GSH and GSSG ratio decreased by 38% compared to control upon drought stress. Contrary, in Pro, GB and Tre pretreated drought stressed seedlings, GSH/GSSG ratio increased by 106, 63 and 41%, respectively, compared to drought-stressed seedlings alone (Fig. 5D).

3.7. Activities of antioxidant enzymes

Drought stress resulted in a notable increase (23%) in APX activity in comparison with control. Whereas, Pro, GB and Tre pretreated drought exposed seedlings illustrate the non-significant variation in terms of APX activity in comparison with drought stress alone (Fig. 6A). As compared to the control, drought stress guided to a notable increase in MDHAR activity. While Pro pretreated drought-stressed seedlings were found with further increased MDHAR activity, but GB and Tre pretreatment couldn't alter MDHAR activity compared to drought stress alone (Fig. 6B). Dehydroascorbate reductase activity decreased significantly by 22% as compared to control. But Pro increased DHAR activity by 15%, while there were no differences among the drought-stressed and GB and Tre pretreated drought stressed seedling (Fig. 6C). The GR activity was noticeably enhanced by 81% in drought-exposed rapeseed seedlings over control. But, Pro and Tre treated drought-stressed seedlings reduced GR activities by 12%, respectively, in both cases compared to drought alone; while GR activity was indifferent among the drought-stressed and GB pre-treated drought stressed seedlings (Fig. 6D). Drought resulted in a citable increased (29%) in CAT activity in comparison with the control. Whereas, GB and Tre pretreated drought-exposed seedlings illustrated a decline in CAT activity compared to drought-stressed seedlings without pretreatment, but Pro pretreatment didn't show statistical difference among the drought-stressed and GB pre-treated drought stressed seedlings (Fig. 7A). However, drought stress led to a 26% increase in GPX activity but, Pro, GB and Tre treatment further augment the GPX activity by 19, 12 and

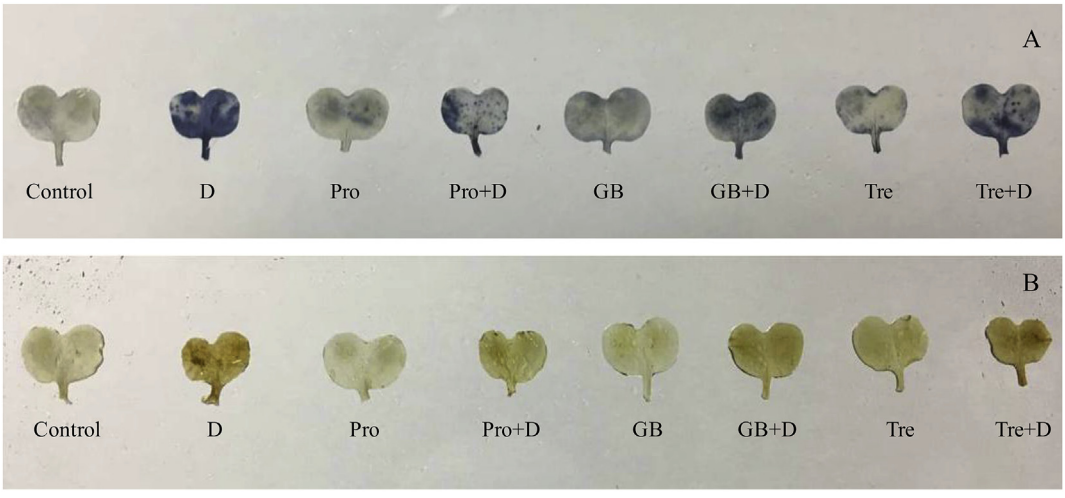

Fig. 4. Histochemical detection of (A) superoxide ($O_2^{\bullet -}$) and (B) hydrogen peroxide (H_2O_2) in the leaves of rapeseed seedlings under drought stress.

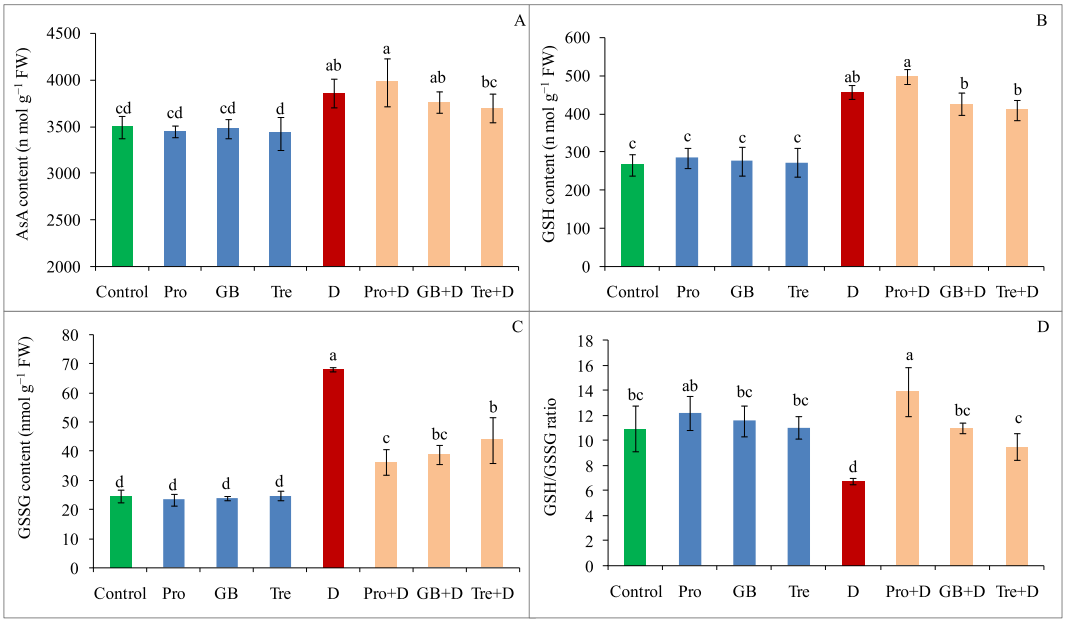

Fig. 5. Effect of exogenous Pro, GB and Tre on AsA (A), GSH (B), GSSG (C) content and GSH/GSSG ratio (D) of rapeseed seedlings under drought stress. Means (± SD) were calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

7%, respectively, as compared to drought treatment alone (Fig. 7B).

3.8. Methylglyoxal content and glyoxalase activity

Methylglyoxal content increased by 96% compared to control under drought stress. While, Pro, GB and Tre pretreatment decreased MG content by 34, 27 and 18%, respectively, as compared to drought stress alone (Fig. 8C). Drought stress didn't alter Gly I activity in both control and stressed seedlings. But Pro pre-treated seedlings under drought stress exhibited increased Gly I activity by 13%, whereas no variation was observed in GB and Tre pre-treated drought-stressed seedlings (Fig. 8A). Drought stress led to a significant decrease regarding Gly II activity by 29% in comparison with control. But, Pro, GB and Tre pretreated drought exposed seedlings illustrated significant increased Gly II activity of 53, 33 and 46%, respectively, as compared to drought-treated seedlings alone (Fig. 8B).

4. Discussion

Among the abiotic stresses, drought has an assortment of unpleasant effects upon plant growth, physiological as well as metabolomic processes (Hasanuzzaman et al., 2018). Dehydration due to drought stress alters water relations, causes osmotic stress, inhibit cell expansion and division, arrests photosynthesis and the overall growth of plants (Alam et al., 2013; Hasanuzzaman et al., 2018). We found drought exposure resulted in reduced plant height, biomass as well as the water content of

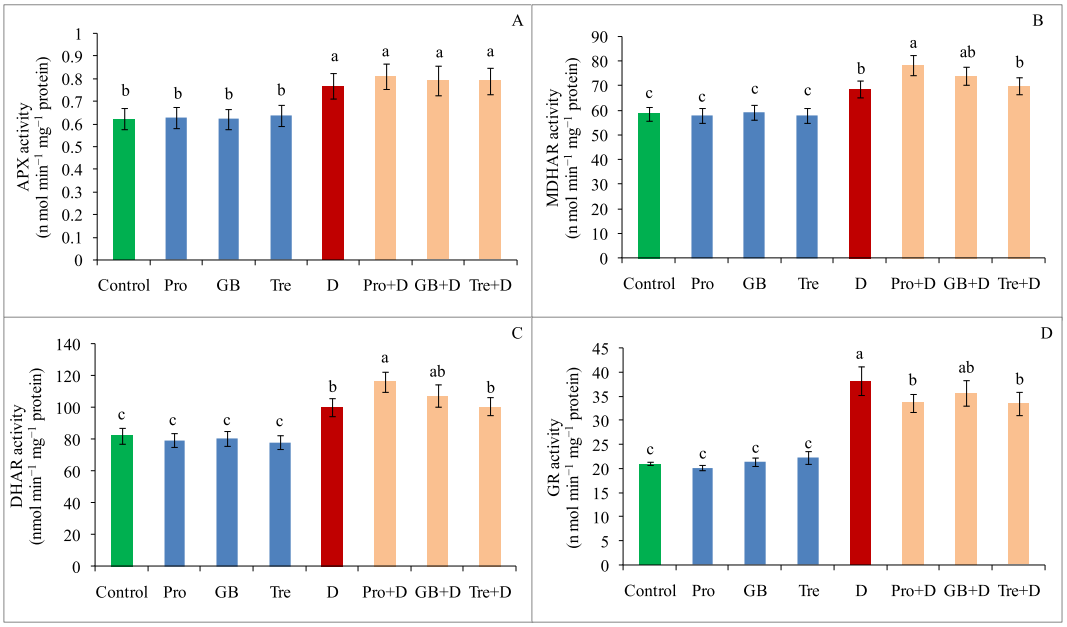

Fig. 6. Effect of exogenous Pro, GB and Tre on APX (A), MDHAR (B), DHAR (C) and GR (D) activity of rapeseed seedlings under drought stress. Means (± SD) were calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

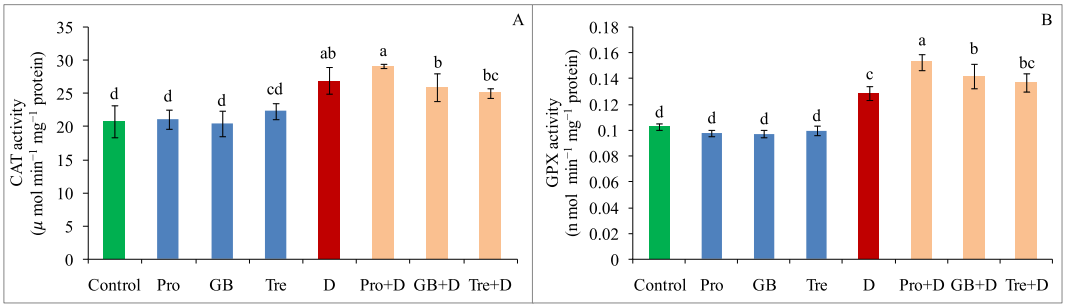

Fig. 7. Effect of exogenous Pro, GB and Tre on CAT (A) and GPX (B) activity of rapeseed seedlings under drought stress. Means (± SD) were calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

rapeseed seedlings, which was reported in many previous research reports (Alam et al., 2013; Nahar et al., 2015b; Hasanuzzaman et al., 2018). However, growth reduction and water balance were restored by exogenous Pro, GB and Tre pretreatment during drought exposure which was ascertained from exalted plant height, fresh and dry biomass in the studied seedlings. Exogenous osmolytes induced improved growth might be attributed to the positive role of these osmolytes in adjusting the osmotic balance, which might inhibit water loss and thus lifted the growth of the seedlings. Many previous research reports support our findings. Alam et al. (2014) working with three *Brassica* species found increased growth and biomass with exogenous Pro supplementation under drought condition. Exogenous Tre induced drought stress tolerance was also observed by Akram et al. (2016), where they observed increased growth, biomass and RWC of radish seedlings under drought condition.

Drought condition causes notable repression in the photosynthetic ability of plants, which is principally due to dehydration that causes stomatal closure, and limits CO_2 diffusion. Moreover, it inhibits the enzyme RuBisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase), the non-stomatal factor (Perdomo et al., 2017). Drought induced reduction of photosyntheric pigments might be due to the breakdown of pigments as well as their impaired biosynthesis. Pandey et al. (2012) reported that dehydration stress caused a considerable decline in light harvesting pigments viz. chl, car, and anthocynanin, which is attributed to oxidative damage of photosynthetic pigments, and impairment of their biosynthesis. In the present study, we also found lower chl *a*, chl *b* and total chl *(a + b)* content upon drought exposure, but increased upon Pro, GB and Tre pretreatment, which are in agreement with other research reports (Raza et al., 2007; Alam et al., 2014; Hasanuzzaman et al., 2014b; Ragab et al., 2015). On the other hand, Pro, GB and Tre induced increase of photosynthetic pigments levels under drought condition in studied species might be attributed to their higher biosynthesis or reduced damage, which further improved photosynthetic performance (Alam et al., 2014; Hasanuzzaman et al., 2014b; Khan et al., 2018). Furthermore, Pro, GB and Tre induced drought tolerance might be obtained from increased stomatal activity, meanwhile escape from desiccation through lower transpiration (Arfan et al., 2007; Saeed et al., 2016).

It has been widely reported that Pro, GB and Tre plays very important role under abiotic stress conditions including drought and maintainance of cellular osmotic balance, stabilizes enzymes, as well as protects biological structures of proteins and lipid membranes (Fernandez et al., 2010; Alam et al., 2014; Nahar et al., 2016a). We observed a noticeable increase in Pro, GB, and Tre contents under drought stresse in studied plant, where Pro and GB application reduced Pro, GB and Tre levels, while Tre application increased the endogenous Tre levels but decreased Pro and GB levels. Prevention of surplus Pro and GB biosynthesis as a result of Pro, GB and Tre pretreatment under

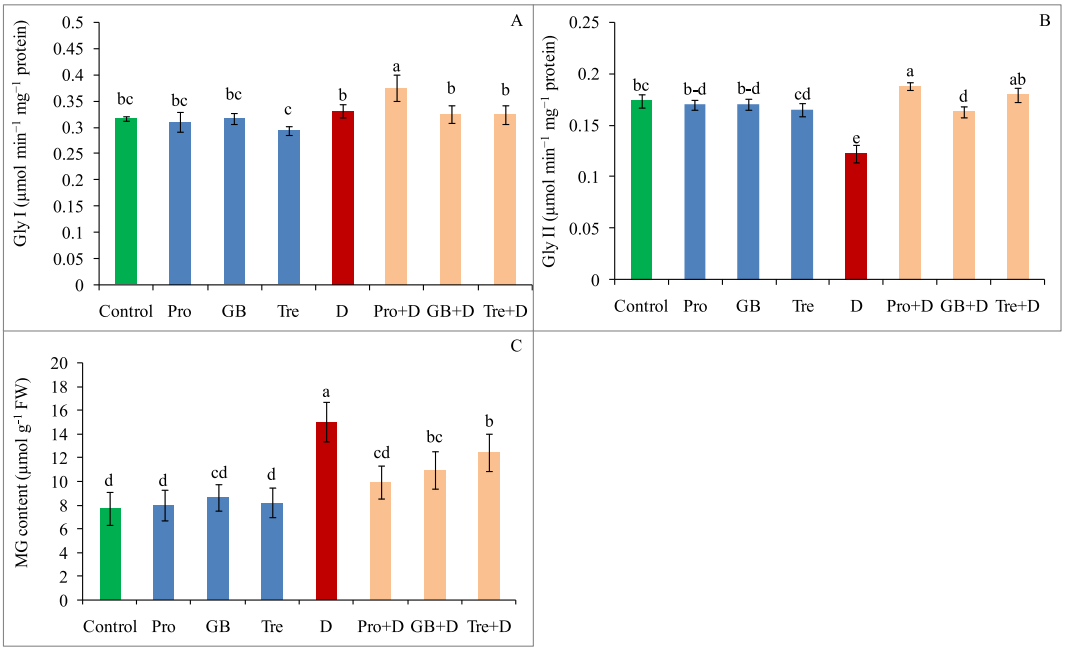

Fig. 8. Effect of exogenous Pro, GB, and Tre on Gly I (A), Gly II (B) and MG (C) content of rapeseed seedlings under drought stress. Means (± SD) were calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test.

drought exposure suggests that Pro, GB, and Tre might prevent rapeseed seedlings from the negative effects of dehydration by other processes rather than osmoprotection. These results are corroborated to the previous studies (Ali and Ashraf, 2011; Alam et al., 2014; Ragab et al., 2015). In this respect, the reduction of free Pro, GB and Tre due to the application of Pro and GB suggesting their interference in the process of osmotic adjustment (Ragab et al., 2015). On the other hand, Tre is readily uptaken with the roots as well as transported easily to the foliage, increases the Tre content, which is evident in our investigation, and corroborates previous findings (Nounjan et al., 2012; Ma et al., 2013). Hence, strengthen the capacity of plant potentially withstanding the destructive effects on drought stress.

Abiotic stresses like drought is known to enhance electrons leakage in the mitochondria and chloroplasts to excite the triplet oxygen $^3O_2$; thus, increases ROS viz. $^1O_2$, $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$, causes lipid peroxidation and increases the content of MDA (Hasanuzzaman et al., 2011a; Ahmad et al., 2018), which are the potential oxidative stress markers. In the present study, rapeseed seedlings exhibited enhanced production of MDA and $O_2^{\bullet -}$, H_2O_2 under drought stress condition, whereas, Exogenous application of Pro, GB, and Tre lessened the oxidative damages, evident from decreased MDA and H_2O_2 content. Reduction in $O_2^{\bullet -}$, H_2O_2 was also evident from the histochemical confirmation. This reduction in MDA contents might have attributed to the active role of Pro, GB and Tre as an osmolyte in protecting cell membranes and restoring enzymes activities, while reducing overgeneration of ROS (Fernandez et al., 2010). Protective effects of Pro, GB, and Tre in reducing the free radical damage also reported in other studies (Hasanuzaman et al., 2014a,b; Molla et al., 2014; Akram et al., 2016).

Among the nonenzymatic antioxidants, AsA has vital roles in the plant development as well as abiotic stress tolerance (Hasanuzzaman et al., 2012a, 2018). Increased AsA or GSH content can effectively reduce over-produced ROS, thus prevent oxidative stress (Nahar et al., 2016b). We found upscaled AsA content under drought, which further increased with the exogenous pretreatment of Pro, GB and Tre. We also observed increased APX activity in both drought-stressed and exogenous Pro, GB and Tre pretreated drought-stressed rapeseed seedlings, which assisted to scavenge the H_2O_2 efficiently. Another two enzymes related to AsA regeneration MDHAR and DHAR also upregulated under Pro and GB pretreatment, as a result the AsA level were increased and strongly maintained its redox balance during oxidative stress situation (Wang et al., 2010; Hasanuzzaman et al., 2014b). These findings are in agreement with previous research reports (Alam et al., 2013; Hasanuzzaman et al., 2014b, 2018). But no significant variation regarding both MDHAR and DHAR enzymes activities were observed in Tre pretreated and untreated drought stressed seedlings, which might be the reason behind reduced de novo regeneration of AsA in case of Tre supplementation.

Glutathione is a non-enzymatic antioxidant to keep the redox balance within plant tissue. We observed increased GSH content in rapeseed seedlings under drought stress condition, which incremented further with the supplementation of Pro, GB, Tre. In contrast, drought condition eventually show an abrupt increase in GSSG level, however, exogenous Pro, GB and Tre pretreatment reduced the GSSG level. The increment in GSSG level in drought condition might be attributed to increased DHAR activity. In contrast, a high GR activity is connected with increased GSH in the drought treated seedlings. However, supplementation of Pro, GB, and Tre in drought-stressed seedlings showed reduced activities of GR, which might be due to the contributions in regulating the H_2O_2 level under drought stress. The present result is corroborated with another study in rice (Hasanuzzaman et al., 2014b, 2018). Moreover, the GSH/GSSG ratio plays enormous tasks in cell redox balance and stress signaling. Exogenous supplementation of Pro, GB, and Tre in our experiment maintained higher GSH/GSSG ratio, which indicates that osmolyte pretreatments suppress GSSG accumulation probably due to higher Gly II activities. Therefore, other findings also supported our study (Alam et al., 2014). Catalase the potential enzyme antioxidant, has a high turnover speed has the capacity of converting two H_2O_2 molecules to water, is regarded as the efficient detoxifier of ROS (Hasanuzzaman et al., 2012a). Similarly, GPX also can scavenge H_2O_2 efficiently and thus provide protection against stress (Hasanuzzaman et al., 2011a). We found CAT and GPX activity increased significantly upon drought stress. Different stress-induced elevation of CAT and GPX activity was also reported in previous works (Liu et al., 2010; Dixit et al., 2015), which are corroborated with our findings (Ali and Ashraf, 2011; Duman et al., 2011; Nounjan et al., 2012), where exogenous Pro, GB and Tre induced upregulation of CAT and GPX activity further gave stress tolerance. Similarly, it was found that organic solutes increased the activity of CAT and GPX *Brassica* sp. under drought (Alam et al., 2014).

We observed increased Gly I activity and decreased Gly II activity under drought. During drought stress, along with the alteration of glyoxalase enzymes, MG level also increased significantly. Increases in MG levels under abiotic stresses impaired Gly I and Gly II activities were previously documented (Alam et al., 2013; Hasanuzzaman et al., 2016). But pretreatment with Pro, GB and Tre upregulated glyoxalase enzymes as well as further reduced MG content, this corroborated previous results (Alam et al., 2014; Hasanuzzaman et al., 2014b). Therefore, metabolomic modification of glyoxalase pathway in different plant positively altered MG content, which reduced oxidative stress. Therefore, higher GSH content with increased Gly I and Gly II function under Pro, GB and Tre pretreatment are evident for more efficiency of glyoxalase system, hence tolerate drought stress (Hasanuzzaman et al., 2014b; Alam et al., 2014). Thus, Pro, GB and Tre pretreatment made BARI Sharisha-15 more tolerant to drought. We tested three osmolytes in this study to confer drought stress tolerance in *Brassica* seedlings. Among the three osmolytes tested, Pro showed the best performance in improving growth and water balance, and upregulation of ROS and MG detoxification systems under drought in the present study.

5. Conclusion

Drought stress results in a significant reduction of growth, however, exogenous Pro, GB and Tre pretreatment improved the above-mentioned growth parameters. Leaf RWC and photosynthetic pigments content also improved by the exogenous supplementation of Pro, GB and Tre in stressed seedlings. Drought-induced oxidative damage resulted in drastically increased MDA and H_2O_2 contents, but the exogenous osmolytes attenuated the oxidative harm by reducing the MDA and H_2O_2 contents and also $O_2^{\bullet -}$ and MG generation. Drought stress also decreased GSH/GSSG ratio and alters the antioxidant defense system in rapeseed seedlings. In contrary, exogenous pretreatment of seedlings with Pro, GB and Tre modulated the activities of CAT, APX, MDHAR, DHAR, GR, GPX, Gly I and Gly II, and increased GSH/GSSG ratio associated decreased in oxidative stress parameters. Therefore, the present experimental results indicated the beneficial effects of exogenous Pro, GB and Tre in alleviating drought-induced oxidative damage. Therefore, we conclude that the coordinated function of antioxidants and glyoxalases is one of the important determinants for the acquisition of drought stress tolerance. The results also suggest that the performance of Pro was best as a protectant against drought stress because Pro showed better effects on all growth, physiological and antioxidative parameters, compared to GB and Tre. However, identification of additional key factors involved in Pro, GB and Tre induced-drought tolerance as well as the underlying signaling roles of Pro, GB and Tre required further research. Hence, further studies focusing on time- and dose-dependent experiment and exploring their suitability in field condition should be done.

Author contributions

T.F.B. with the help of M.H., and K.U.A. designed the experiment.

T.F.B., T.I.A. performed the experiment, with the active participation from K.N.; J.A.M. and M.H.M.B.B. M.H. did the statistical analysis. T.F.B. has written the manuscript. K.U.A.; M.H.; J.A.M.; K.N. and M.H.M.B.B. critically reviewed and edited the manuscript. K.U.A. and M.F. supported the laboratory and other technical facilities. All the authors read and approved the final manuscript.

Declarations of interest

None.

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