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EXOGENOUS TREHALOSE BOOSTED DROUGHT TOLERANCE IN WHEAT SEEDLINGS (*TRITICUM AESTIVUM* L.) BY ENHANCING GLYOXALASE SYSTEM

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Abstract

The present experiment was conducted at the Molecular Breeding Laboratory of Bangladesh Agricultural Research Institute (BARI), Gazipur during 2016-2017, to investigate the potential of trehalose (Tre) as a protective agent against drought stress in wheat seedlings. Two genotypes, viz. CSISA DR 30 (drought-tolerant) and BAW 1163 (drought-sensitive), were subjected to varying levels of polyethylene glycol (PEG)-induced drought stress, with and without the addition of trehalose (Tre). Eight-day-old seedlings were subjected to drought stress for 2, 4, and 6 days. Results revealed that drought stress at various durations increased proline, lipid peroxidation (MDA) and methylglyoxal (MG) contents, but decreased relative water content (RWC) and reduced glutathione (GSH) compared to control in both genotypes at seedling stage. It was also observed that the activities of glyoxalase I (Gly I) and glyoxalase II (Gly II) enzymes were increased in CSISA DR 30 genotype, but decreased in BAW 1163 genotype under drought stress of different durations. However, the application of trehalose (10 mM) under drought stress led to a decrease in MG, MDA and proline levels. At the same time, this osmolyte increased RWC, activities of Gly I and Gly II enzymes, as well as GSH contents in both genotypes. Notable, the MDA and MG accumulation was found remarkably lower in CSISA DR 30 genotype than in BAW 1163 wheat genotype under control, drought (15% PEG) and drought with 10 mM trehalose condition. The results also suggested that the genotype CSISA DR 30 is drought tolerant and moreover, its capability is further enhanced by exogenous trehalose application.

Keywords: Trehalose, methylglyoxal, glyoxalase system, PEG, drought, wheat genotype.

Introduction

Wheat (*Triticum estivum* L.), a member of the family 'Poaceae' is one of the major cereal crops ranked second after rice mainly cultivated in the north and north-west region of Bangladesh. Wheat has an annual production of 778 million metric tons with a 10% value addition in agriculture (Kamal *et al*., 2020). It provides 1.8% fiber, 9.4% protein, 69% carbohydrates, and 2.5% fat (Ahmad *et al*., 2021). In recent years, due to climate change several periodic natural calamities of which drought is the most prominent and prevalent limiting factors of wheat production.

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Drought employs expressively adverse effects on production of wheat in northern and central part of Bangladesh and around 3.5 million ha land are vulnerable to crop production specially wheat due to drought (Alam, 2014). Therefore, the cultivation of wheat faces soil moisture stress due to inadequate and erratic rainfall and limited irrigation facilities. The area under wheat cultivation is gradually decreasing and its production has increased a little bit but not significantly due to temperature rise and changing in precipitation pattern after 2018-19.

Under water deficit conditions, plants experience oxidative stress, leading to increased accumulation of reactive oxygen species (ROS) (Rohman *et al*., 2016; Hasanuzzaman *et al*., 2014). These ROS act as major toxic radicals capable of damaging essential biomolecules such as proteins, lipids, and DNA (Rohman *et al*., 2016). Additionally, the generation of cytotoxic methylglyoxal (MG) under drought stress, induces similar damaging effects to those caused by ROS (Rohman *et al*., 2016; Alam *et al*., 2014). However, ROS and MG homeostasis in plant cells is very important to exert its physiological function. It is reported that 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).

Over the past few decades, the application of exogenous osmoprotectants, such as glycine betaine, proline, trehalose, and salicylic acid, has proven effective in mitigating the detrimental impacts of drought stress on plant growth (Ali, 2011; Alam *et al*., 2014). Trehalose, a non-reducing disaccharide of glucose, exists in plants in modest quantities, nearly at the detection limit. Yet it demonstrates the ability to reduce oxidative stress and enhance plant tolerance to abiotic stresses, including drought stress (Ali and Ashraf, 2011; Ma *et al*., 2013).

Extensive studies have explored the impact of trehalose on diverse crops facing drought conditions globally, encompassing growth, photosynthetic attributes, and ROS scavenging antioxidant defense (Ma *et al.*, 2013; Ali and Ashraf, 2011). Despite a number of research conducted on mitigation of drought stress and scavenging ROS, there is a notable scarcity of research on the effects of trehalose in MG detoxification system under drought stress. Specifically, the investigation into the strategies employed by wheat seedlings to endure drought conditions remains limited. This research aimed to fill this gap by examining the potential effects of trehalose in detoxifying MG with facilitating adaptive strategies in the leaves of wheat seedlings under drought stress induced by PEG. The present experiment was, therefore, conducted to elucidate how trehalose may contribute and influence the survival mechanisms adopted by wheat seedlings in the face of water scarcity.

Materials and Methods

Plant Materials and Stress Treatments: The study was carried out at the Molecular Breeding Laboratory of Bangladesh Agricultural Research Institute (BARI), Gazipur during 2016-2017. The experimental treatments comprised two wheat genotypes, namely CSISA DR 30 (relatively drought-tolerant) and BAW 1163 (drought-sensitive), which were obtained from the previous research (Ahsan, 2014) and three distinct drought stress conditions, viz. 0% PEG (used as a control), 15% PEG, and 15% PEG plus 10 mM trehalose (Tre). According to Bayomi *et al.* (2008), a solution of PEG-6000 was prepared using the weight-by-volume method by dissolving 150 g of PEG in 200 mL of distilled water, and the final volume was adjusted to one liter. The experiment was conducted following completely randomized design (CRD) with three replications. Twenty-five healthy and equal-sized seeds of both genotypes were selected andseeds were surface sterilized with 70% ethanol solution for 5 min followed by washing several times with sterile distilled water. Seeds were put in sterilized 9 cm petri-dishes containing germination paper moistened with 10 ml of the deionized distilled water to provide appropriate moisture for seed germination. The petri-dishes containing seeds of wheat were then kept in an incubator (VS-1203P3V, Japan) under controlled conditions (Temp, 25±2 °C; RH, 65–70%) for germination. After 3-days, germinated seedlings with petri dishes were transferred to growth chamber with 1,000-fold diluted 20 ml Hyponex nutrient solution (Type: 5-10-5, Hyponex, Japan) under control conditions (light intensity, 100 $molm^{-2}s^{-1}$; temperature, 23 ± 2 °C; relative humidity, 60–65%) during the growing period of 8 days. Eight-day old seedlings of approximately equal sizes of two wheat genotypes were imposed to drought stress induced by 15% polyethylene glycol (PEG) with or without 10 mM trehalose and grown under the above control condition in growth chamber. Control plants were grown with Hyponex solution only. Data were taken after 2, 4 and 6 days of treatment application.

Measuring relative water content

Relative water content of the leaves was determined following the procedure of Islam *et al.* (1998). The collected leaves of wheat seedlings were excised just prior to sun set and after taking fresh weight, their base immediately placed in glass jar containing 5 to 6 cm water height. The jars were enclosed with plastic cover to maintain 100% relative humidity and the leaves were allowed to watered 24 hours in dark. The following day, the saturated leaves were removed from jars and immediately the turgid weight of leaves was recorded with the help of a precise electronic balance. Finally oven dry weights of the leaves were recorded. Relative water content of leaves was measured by the following formula-

$$RWC = \frac{Fresh\ leaf\ weight - Dry\ leaf\ weight}{Turgid\ leaf\ weight - Dry\ leaf\ weight} \times 100$$

Determination of Proline

Proline colorimetric determination according to Bates *et al.* (1973) based on proline's reaction with ninhydrin. For proline colorimetric determinations, a 1:1:1 solution of proline, ninhydrin acid and glacial acetic acid was incubated at 100º C for 1 hour. The reaction was arrested in an iced bath and the cromophore was extracted with 4 ml toluene and its absorbance at 520 nm was determined in a UV-spectrophotometer (UV-1800, Shimadzu, Japan).

Measurement of Lipid Peroxidation (MDA)

The level of lipid peroxidation was measured as malondialdehyde (MDA), a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Heath and Packer (1968). Briefly, the leaf tissue (0.5 g) was homogenized in 3 mL 5% (w/v) trichloroacetic acid (TCA), and the homogenate was centrifuged at 11,500×g for 10 min. The supernatant (1 mL) 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} FW.

Measurement of Methylglyoxal (MG)

About 0.3 g leaf tissue was extracted in 3 mL of 0.5 M perchloric acid. After incubating for 15 min on ice, the mixture was centrifuged at 4 ℃ at 11,000 × g for 10 min. A colored supernatant was obtained in some plant extracts that was decolorized by adding charcoal (10 mg ml^{-1}), kept for 15 min at room temperature, and centrifuged at 11,000 × g for 10 min. Before using this supernatant for MG assay, it was neutralized by keeping for 15 min with saturated solution of potassium carbonate at room temperature and centrifuged again at 11,000 × g for 10 min. Neutralized supernatant was used for MG estimation following the method of Rohman *et al*. (2016). An aqueous 500mM N-acetyl-L-cysteine solution was freshly prepared. The reaction was carried out in 100 mM sodium dihydrogen phosphate buffer (adjusted to pH 7.0 with 10 M NaOH) at 25 °C. First, the MG solutions (5, 10, 15, 20 and 25 µL) equating to 0.5, 2 and 5 mM were added up to a volume of 980 µL with sodium dihydrogen phosphate buffer and the spectrophotometer was set to zero. The reaction was started by adding 20 µL of the N-acetyl-L-cysteine solution (final concentration up to 10 mM), and the formation of the product N-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl) cysteine was recorded for 10 min at a wave length of 288 nm.

Enzyme Extraction and Assays

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue of wheat seedlings 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 × g for 10 min, and the supernatants were used for determination of enzyme activity. All procedures were performed at 0 to 4°C.

Assay of enzymatic activities

Glyoxalase I (Gly-I, EC: 4.4.1.5) assay was carried out according to Yadav *et al*., 2005a). Briefly, the assay mixture contained 100 mM K-phosphate buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM reduced glutathione, and 3.5 mM methylglyoxal 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 the extinction coefficient of 3.37 mM^{-1} cm^{-1}.

Glyoxalase II (Gly-II, EC: 3.1.2.6) activity was determined according to the method of Principato *et al.* (1987) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.2), 0.2 mM DTNB, and 1 mM S-D-lactoyl glutathione (SLG) in a final volume of 1 ml. The reaction was started by the addition of SLG, and the activity was calculated using the extinction coefficient of 13.6 mM^{-1} cm^{-1}.

Extraction and Measurement of reduced Glutathione

Wheat seedling leaves (0.5 g fresh weight) were crushed using a mortar and pestle in 3 ml of ice-cold acidic extraction buffer comprising 5% metaphosphoric acid and 1 mM EDTA. The resulting homogenates were then centrifuged at 11,500 × g for 15 minutes at 4 °C, and obtained supernatant was utilized for the analysis of glutathione, following the procedures outlined by Hasanuzzaman *et al*. (2014).

Statistical Analysis

All data obtained were analyzed with the help of STATISTIX 10 program and mean separation was done by Least Significant Difference (LSD) test at 5% significance level of probability. Values presented in figures are mean of three independent experiments (each experiment consists of three replications).

Results and Discussion

Effect of trehalose on relative water content (RWC)

Drought stress induced a significant decline in leaf relative water content (RWC) in both wheat genotypes, with the drought-sensitive BAW 1163 experiencing more substantial reduction compared to the drought-tolerant CSISA DR 30 genotype (Fig. 2). CSISA DR 30 exhibited a remarkable resistance to RWC reduction,

showing declines of 10.34, 22.72, and 36.93%, while BAW 1163 recorded higher reductions at 17.08, 32.91, and 52.85% on days 2, 4, and 6 of drought stress. By the 6th day, CSISA DR 30 maintained a significantly higher RWC, emphasizing its superior drought tolerance. The addition of exogenous trehalose alleviated water loss during drought, resulting in a 13% increase in RWC for CSISA DR 30 and a 9.50% increase for BAW 1163 on the 6th day of drought stress. This indicates the significance of trehalose in mitigating water loss during drought stress. This positive impact of exogenous trehalose application on reducing the decline in RWC under drought stress, emphasizing its role in modulating leaf diffusive resistance and enhancing water retention in plant tissues was confirmed by earlier findings (Ali, 2011; Ali and Ashraf, 2011; Akram *et al*., 2016; Alam *et al*., 2014).

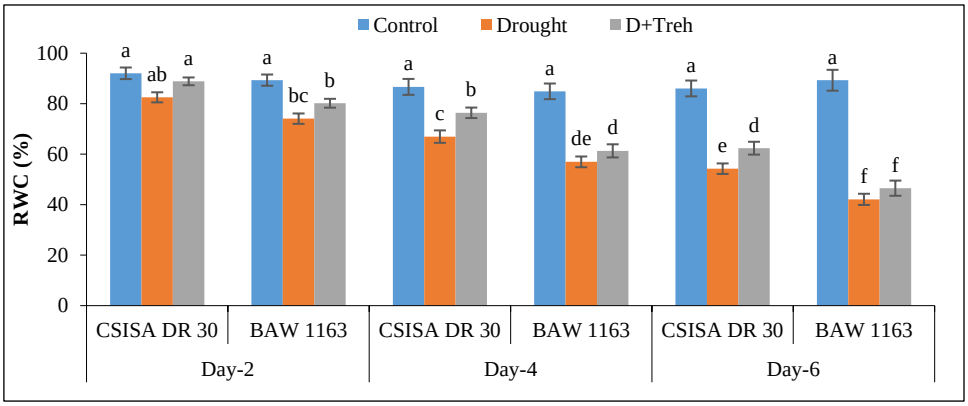

Fig. 2. Relative water content in drought tolerant (CSISA DR 30) and drought sensitive (BAW 1163) wheat genotypes induced by exogenous trehalose under drought stressed at different duration. Bars with the same letters are not significantly different at P≤0.05 by LSD test. Control, Drought (15% PEG 6000), D + Tre (15% PEG 6000 with 10 mM Tre).

Effect of trehalose on proline content

Drought stress caused significant increase in proline contents over time in both genotypes, with CSISA DR 30 displaying higher accumulation than BAW 1163 (Fig. 3). At 2, 4, and 6 days of drought stress, the proline content was significantly higher (84, 134 and 85%) in CSISA DR 30 than the BAW 1163 wheat genotypes. Nevertheless, the application of exogenous trehalose @ 10 mM (D + Treh) reduced proline accumulation in both the genotypes compared to their respective drought-stressed genotypes. The reduction in extra proline biosynthesis due to exogenous trehalose implies that trehalose protected wheat genotypes from the adverse effects of drought stress through alternative mechanisms, opposing the need for a further increase in proline levels. These findings corrugated the findings of Ali and Ashraf (2011), Nounjan *et al*. (2012) and Alam *et al*. (2014), contributing insights into proline dynamics in drought-tolerant and sensitive wheat genotypes while highlighting the regulatory role of trehalose in modulating proline accumulation.

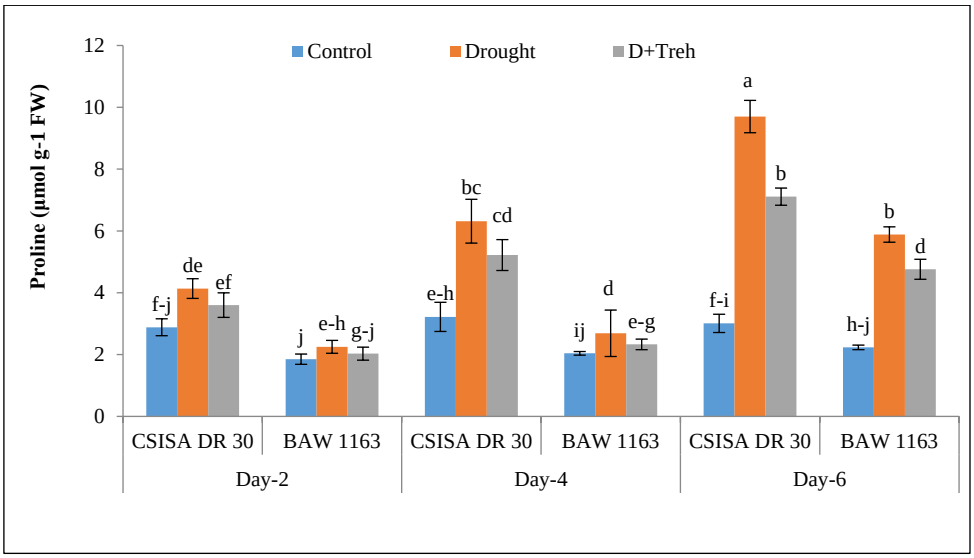

Fig. 3. Proline content in drought tolerant (CSISA DR 30) and drought sensitive (BAW 1163) wheat genotypes induced by exogenous trehalose under drought stressed at different duration. Bars with the same letters are not significantly different at P ≤0.05 by LSD test. Control, Drought (15% PEG 6000), D + Tre (15% PEG 6000 with 10 mM Tre).

Effect of trehalose on malondialdehyde (MDA) contents

The content of malondialdehyde (MDA) significantly increased with the duration of drought stress in both wheat genotypes. Notably, BAW 1163 exhibited higher MDA levels than CSISA DR 30 genotype (Fig. 4). Specifically, at 2, 4, and 6 days of drought stress, MDA content was 1.55, 1.71, and 1.93 times higher in CSISA DR 30 seedlings compared to their control, while BAW 1163 genotypes showed 1.73, 1.98, and 2.30 fold increases. Interestingly, treating both genotypes with 10 mM trehalose (D + Treh) reduced MDA levels compared to the content in drought stressed genotypes without trehalose. CSISA DR 30 genotypes demonstrated better MDA reduction than BAW 1163 genotypes when supplemented with exogenous trehalose during drought. Lower MDA content indicates higher antioxidative ability (Ahsan *et al.*, 2020; Ahmed *et al.*, 2021) and greater drought tolerance (Izabela *et al.*, 2013). In this study, MDA level was significantly and continuously increased in both wheat genotypes with increase drought stress intensity, while, CSISA DR 30 maintained significantly lower amount of MDA than BAW 1163 both under control and drought stress.

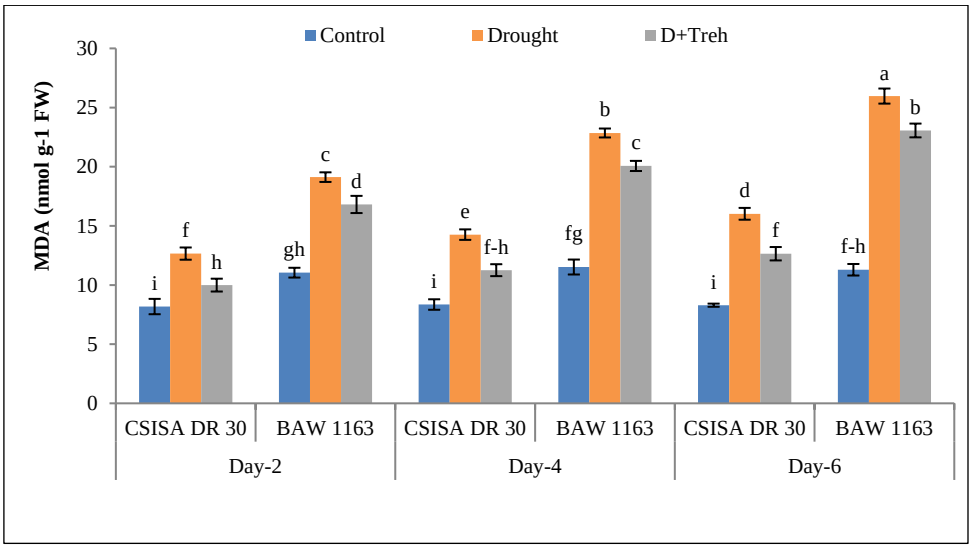

Fig. 4. MDA content in drought tolerant (CSISA DR 30) and drought sensitive (BAW 1163) wheat genotypes induced by exogenous trehalose under drought stressed at different duration. Bars with the same letters are not significantly different at P≤0.05 applying the LSD test. Control, Drought (15% PEG 6000), D + Tre (15% PEG 6000 with 10 mM Tre).

Effect of trehalose on methylglyoxal levels

In the presence of PEG-induced drought stress conditions, both the wheat genotypes exhibited an elevation in methylglyoxal (MG) accumulation compared to their corresponding control treatment (Fig. 5). The control conditions maintained relatively stable MG levels for both genotypes throughout the observation period. However, under drought conditions, a notable increase in MG accumulation occurred over time, with BAW 1163 consistently exhibiting higher levels (26, 29 and 32%) than CSISA DR 30 genotype. The introduction of exogenous trehalose @ 10 mM (D+Treh) demonstrated a mitigating effect on the drought-induced rise in MG levels throughout the observation periods. This indicates a potential protective role of trehalose in limiting MG accumulation under drought stress. The findings contribute valuable insights into the regulatory mechanisms by which trehalose influences methylglyoxal toxicity, enhancing our understanding of wheat responses to drought stress. Several researchers observed the reduction of MG levels under abiotic stress by exogenous osmotic protectant (Okuma *et al*., 2004; Rohman *et al*., 2016; Alam *et al*., 2014).

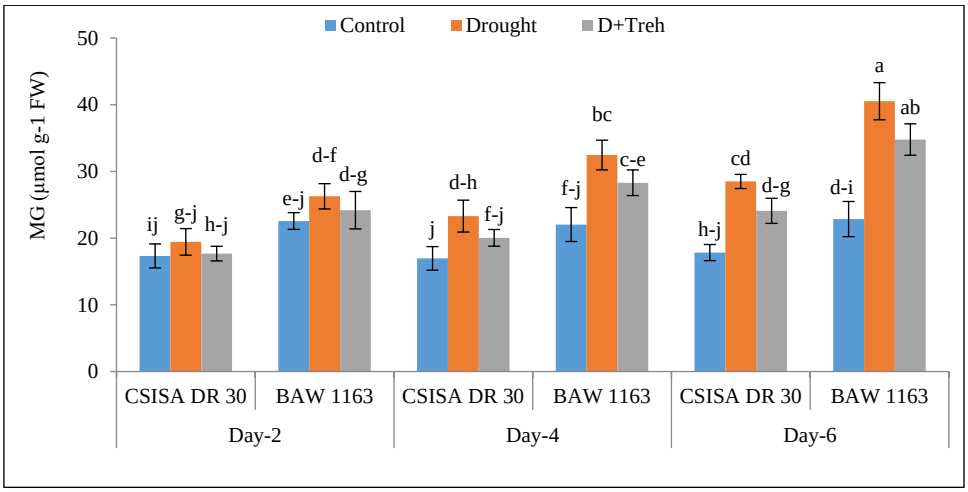

Fig. 5. Accumulation of MG in leaves of drought tolerant (CSISA DR 30) and drought sensitive (BAW 1163) wheat genotypes induced by exogenous trehalose under drought stress at different duration. Bars with the same letters are not significantly different at P≤0.05 applying the LSD test. Control, Drought (15% PEG 6000), D + Tre (15% PEG 6000 with 10 mM Tre).

Effect of trehalose on glyoxalase I and glyoxalase II activities

It was observed that drought stress increased Gly I and Gly II activities in drought tolerant CSISA DR 30 wheat genotypes (Fig. 6a and 6b). As compared to control, activities of Gly-I were increased by 27.87, 21.27 and 12.49% at 2, 4 and 6 days of drought stress, respectively, in CSISA DR 30 genotypes. Similarly, Gly-II activity slightly increased by 11.81, 7.14 and 2.85% at 2, 4 and 6 days of drought stress, respectively in this tolerant genotype. On the contrary in same conditions Gly I and Gly II activities were decreased in drought sensitive BAW 1163 wheat genotypes. The Gly I activity was decreased by 16.34, 34.02 and 42.03% in BAW 1163 genotypes under 2, 4 and 6 days of drought stress, respectively, compared to their control. The Corresponding values of Gly II for BAW 1163 genotypes were 10.87, 22.23 and 35.33% at 2, 4 and 6 days of drought stress, respectively. These results suggest that CSISA DR 30 wheat genotype efficiently protected the MG that was formed during oxidative stress due to its higher tolerance capacity. While, exogenous trehalose supplementation @ 10 mM in presence of PEG-induced drought stress improved Gly I and Gly II activities in two wheat genotypes in different stress duration that proved the enhanced tolerance to drought stress in wheat genotypes. Trehalose-induced upregulation of glyoxalase enzymes and subsequent abiotic stress including drought tolerance were also reported in other plants by other researchers (Hoque *et al*., 2008; Alam *et al*., 2014).

Effect of trehalose on GSH content

Although a gradual decrease was observed in GSH content with escalating drought stress severity in wheat genotypes, drought-tolerant CSISA DR 30 genotypes maintained higher GSH levels compared to the sensitive BAW 1163 genotype (Fig. 7). Under drought stress, CSISA DR 30 exhibited decreased GSH content by 4.59, 13.16, and 22.19% at 2, 4, and 6 days, respectively, while BAW 1163 experienced more substantial reductions (7.16, 37.10%, and 58.70%). Exogenous trehalose application @ 10 mM elevated GSH in both the genotypes, with CSISA DR 30 displaying higher increments (1.36, 6.33, and 10.74%) than BAW 1163 (0.74, 23.85, and 9.63%) at 2, 4, and 6 days of drought stress. The enhanced GSH content, facilitated by trehalose, may contribute to GPX-mediated ROS detoxification and GST-mediated leaf senescence, fostering stress tolerance in wheat genotypes, aligning with previous research findings (Rohman *et al*., 2016; Ma *et al*., 2013; Alam *et al*., 2014).

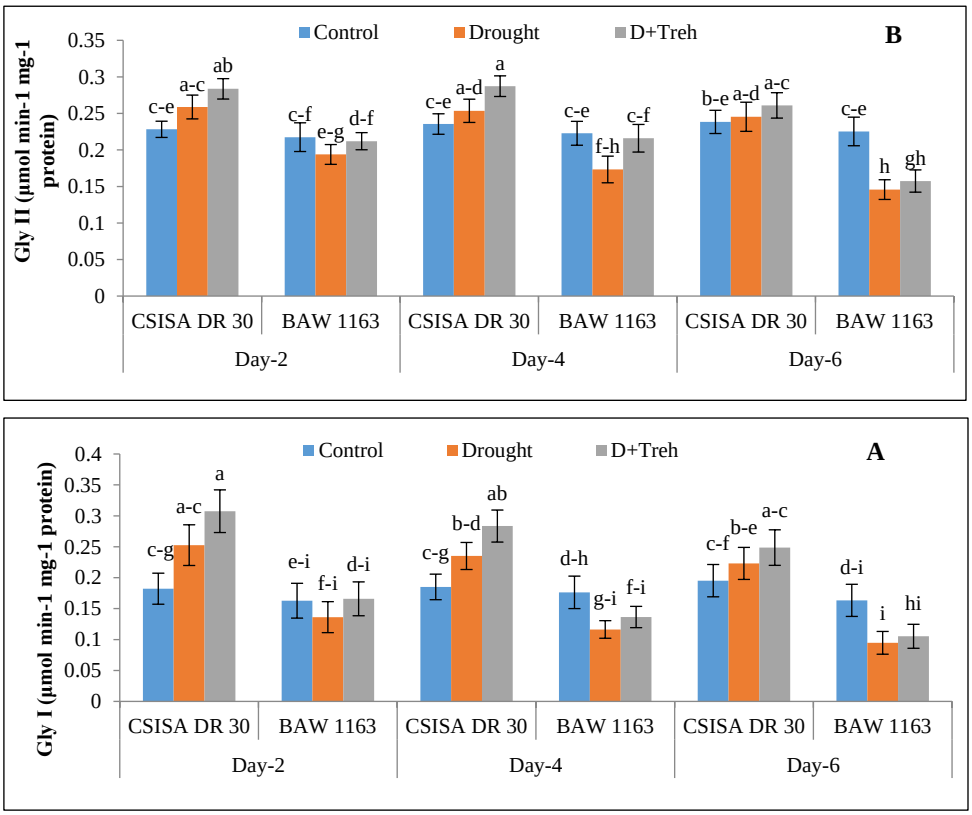

Fig. 6. Activities of Gly-I (A) and Gly-II (B) in leaves of drought tolerant (CSISA DR 30) and drought sensitive (BAW 1163) wheat genotypes induced by exogenous trehalose under drought stress at different duration. Bars with the same letters are not significantly different at P≤0.05 by LSD test. Control, Drought (15% PEG 6000), D + Tre (15% PEG 6000 with 10 mM Tre).

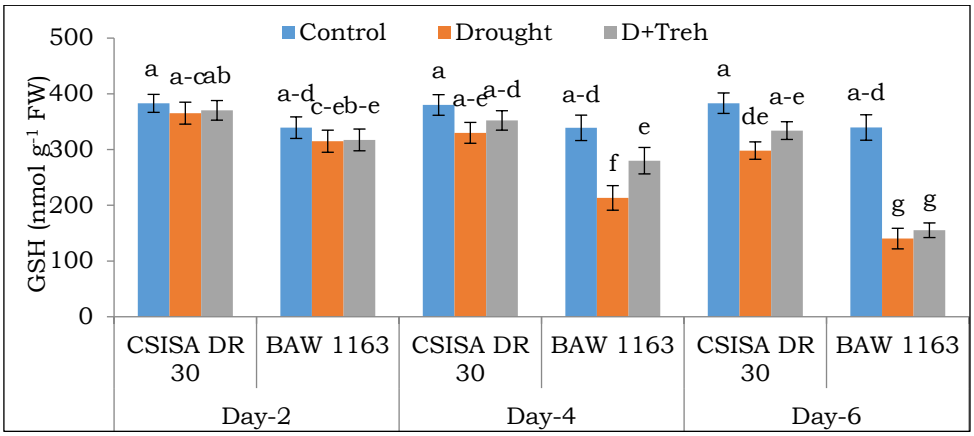

Fig. 7. Content of GSH in drought tolerant (CSISA DR 30) and drought sensitive (BAW 1163) wheat genotypes induced by exogenous trehalose under drought stress at different duration. Bars with the same letters are not significantly different at P ≤0.05 by LSD test. Control, Drought (15% PEG 6000), D + Tre (15% PEG 6000 with 10 mM Tre).

Fig. 8. Comparative tolerance to drought of two wheat genotypes (CSISA DR 30, drought tolerant and BAW 1163, drought susceptible) in presence or absence of 10 mM trehalose (Tre). Eight day-old seedlings were imposed to 15% PEG 6000 for 6 days.

Conclusion

Based on the above results under PEG-induced drought stress, the accumulation of lipid peroxidation (MDA), methylglyoxal (MG) was higher in susceptible BAW 1163 wheat genotype. Leaf relative water content (RWC%), proline, glutathione (GSH) and glyoxalase system enzymes (Gly I and Gly II) were also lower in this genotype compared to the tolerant CSISA DR 30 genotype. Trehalose (10 mM) played a significant role in MG detoxification through enhancing the glyoxalase system enzymes. Under drought stress, trehalose (10 mM) showed better maintenance of GSH as well as Gly I and Gly II activities and also regulate RWC% and proline accumulation at most favorable levels in CSISA DR 30 genotype than BAW 1163 genotype to protect cellular damage from higher MG. Trehalose maintenance of higher glyoxalase activities along with higher contents of GSH and its adjustment of the accumulated proline to an optimal level suggest the protective

role of trehalose in the seedlings of two wheat genotypes under PEG-induced drought stress. The tolerant CSISA DR 30 genotype can be used as a breeding material for future breeding program for developing drought tolerant wheat varieties.

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