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Drought sensitive maize inbred shows more oxidative damage and higher ROS scavenging enzymes, but not glyoxalases than a tolerant one at seedling stage

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Abstract

This study was undertaken to unveil the oxidative stress tolerance mechanism in maize seedlings under drought. The level of oxidative stress and involvement of antioxidant and glyoxalase systems were investigated in seedlings of two maize inbreds: P134, a relatively drought tolerant, and P142, a drought susceptible inbred subjected to water deficit for 7 days and then rewatered to reveal the mechanism of oxidative stress tolerance under drought. Water content, chlorophyll (Chl), reactive oxygen species (ROS), lipid peroxidation, methylglyoxal (MG), lipoxygenase (LOX) activity, enzymatic and non-enzymatic antioxidants and glyoxalases status were investigated in the uppermost fully expanded leaves. The superoxide ($O_2^{\bullet-}$) generation rate, hydrogen peroxide (H_2O_2), lipid peroxidation and MG as well as LOX activity were higher in P142 throughout the drought period. Conversely, relative water content (RWC), Chl, carotenoid (Car) and proline contents were remarkably higher in P134. However, in rewatering, recovery of Chl and Car were higher in P142. The reduced glutathione (GSH), ascorbic acid (ASA) and their redox homeostasis indicated more oxidative damage in P142. The ROS scavenging enzymes like superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) activities were comparatively higher in P142 under drought, while catalase (CAT), monodehydroasacorbate reductase (MDHAR) and glutathione reductase (GR) activities were higher in P134. Though the activity of GST increased in both inbreds, activities of glyoxalase-I (Gly-I) and glyoxalse-II (Gly-II) increased only in P134. In rewatering, activities of most of the enzymes decreased in both inbreds. Taken together, the non-enzymatic antioxidant system was stronger in P134, but the higher SOD, POD, APX, GPX and DHAR activities in P142 suggesting that these enzymes might involve in cellular protection through reducing oxidative damage.

Keywords: Drought sensitivity, oxidative stress, antioxidants, glyoxalases, maize.
Abbreviations: APX_Ascorbate peroxidase; AO_Ascorbate oxidase; ASA_Ascorbic acid; BSA_ Bovine serum albumin; CAT_Catalase; Chl_Chlorophyll; CDNB_1-Chloro-2,4-dinitrobenzene; CRD_Complete randomized design; dSm^{-1}_Salinity measuring unit (decisimen per meter); DHA_Dehydroascorbate; DHAR_Dehydroascorbate reductase; DTNB_5,5′-dithio-bis (2-nitrobenzoicacid); DW_Dry weight; FW_Fresh weight; Gly-I_Glyoxalase-I; Gly-II_Glyoxalase-II; GPX_Glutathione peroxidase; GR_Glutathione reductase; GSH_Reduced glutathione; GSSG_Oxidized glutathione; GST_Glutathione *S*-transferase; LOX_Lipoxygenase; MDA_Melondialdehyde; MDHAR_Monodehydroascorbate reductase; MG_Methylglyoxal; NTB_2-nitro-5-thiobenzoicacid; NBT_Nitro blue tetrazolium; POD_Peroxidase; ROS_Reactive oxygen species; RWC_ Relative water content; SOD_Superoxide dismutase; SLG_*S*-D-lactoyl glutathione; TW_Turgid weight.

Introduction

Water availability is a major determinant of plant growth and a critical factor in agriculture. Drought causes the imbalance between energy capture and utilization and excessive energy generates reactive oxygen species (ROS) leading to substantial damages to photosynthetic machinery. Under drought condition, plants reduce water losses by closing stomata, which results in lower photosynthetic activities in leaves. As a consequence, when plants are exposed to an excess of light energy, it cannot convert light energy into biochemical energy and subsequently leads to the production of ROS (Demmig-Adams and Adams, 1992; Foyer and Noctor, 2005). ROS include free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), as well as non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a byproduct of various metabolic pathways localized in different cellular compartments (Gill and Tujeta, 2010; Noctor et al., 2012). The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to death of the cells (Mittler, 2002; Sharma et al., 2012). In addition, methylglyoxal (MG) is potential cytotoxic compound produced under abiotic stress which can react with and modify other molecules including DNA and proteins (Yadav et al., 2005a, b). Plants have evolved a complex array of antioxidant systems to prevent oxidative injury resulting from elevated levels of ROS. ROS scavenging enzymes include superoxide dismutase (SOD), peroxidase (POD), catalase

(CAT), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Apel and Hirt, 2004; Noctor et al., 2012). SOD deploys the primary protection to convert $O_2^{\bullet-}$ to H_2O_2, whereas POD, CAT, GPX and APX decompose H_2O_2 to water. In ascorbate-glutathione cycle, MDHAR and DHAR play essential role in defense system against ROS through maintaining ascorbic acid (ASA) while GR maintains the reduced status of glutathione (GSH). On the other hand, plant GSTs are known to function in herbicide detoxification, hormone homeostasis, vacuolar sequestration of anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification, regulation of apoptosis responses to biotic and abiotic stresses (Dixon et al., 2010). In addition, cytotoxic MG is detoxified via glyoxalase system (Yadav et al., 2005a) which consists of two enzymes: glyoxalase-I (Gly-I) and glyoxalase-II (Gly-II). Gly-I use GSH to convert MG to *S*-D-lactoyl glutathione (SLG), while Gly-II catalyzes the hydrolytic reaction to generate lactic acid and free GSH (Veena et al., 1999; Singla-Pareek et al., 2003). The coordinated inductions or regulations of both antioxidants and glyoxalase pathway enzymes are related to tolerance of plant against oxidative stress (Singla-Pareek et al., 2008; Saxena et al., 2011). Further, plants possess numerous low-molecular weight antioxidant compounds either water soluble like ascorbate and glutathione, or lipophiliclike carotenoids (Car) and tocopherols with osmoprotective and antioxidative roles under drought (Noctor and Foyer, 1998; Havaux and Niyogi, 1999; Havaux et al., 2005). Proline, the most common compatible solute, also accumulates under drought stress and contributes to osmotic adjustment, and stabilization and protection of membranes, proteins and enzymes from damaging effects of drought (Ashraf and Foolad, 2007). In addition, it can scavenge free radicals and ROS (Hasegawa et al., 2000; Hong et al., 2005). Although different research groups have reported the role of enzymatic and non-enzymatic antioxidants in drought tolerance in many plant species, most of the studies are limited with few antioxidants without considering glyoxalase system. Moreover, reports on the activities of antioxidant enzymes in relation to drought tolerance and sensitivity are limited. In maize, most of the studies are also limited to few antioxidants and glyoxalase system is not taken into consideration. Moussa and Abdel-Aziz (2008) studied only SOD, POD and CAT in two maize genotypes Giza 2 (drought tolerant) and Trihybrid 321 (drought sensitive). Ghahfarokhi et al. (2015) considered only POD, CAT and GR along with some physiological attributes in several drought sensitive and tolerant genotypes. Therefore, the objective of the study was to find out a relationship among enzymatic and non-enzymatic antioxidants, glyoxalases and their related metabolites in relation to drought tolerance in maize seedlings for better understanding of drought tolerance mechanism.

Results

Effect of drought and rewatering on relative water content in leaf

Upon exposure to drought stress, leaf RWC decreased significantly in both inbreds relative to respective controls (Fig. 1). However, reduction of RWC was lower in drought tolerant inbred P134 compared to drought sensitive inbred P142. At moderate stress, it was decreased by 22% and 28% in P134 and P142, respectively, while reduction was 47% and 55% in P134 and P142, respectively, under severe stress (Fig. 1). After rewatering, the RWC was increased in both inbreds and the level was almost similar in P134 and P142, but 22% and 21% lower than their respective control.

Effect of drought and rewatering on chlorophyll contents

Leaf Chl contents of both inbreds decreased gradually with the duration of stress (Table 1). In severe stress, the contents of Chl*a* and Chl*b* were significantly lower than their respective controls. However, the loss of Chl contents was considerably higher in P142 than that of P142. Like Chl, the content of Car was also decreased by drought in both inbreds and the loss was significantly higher under severe stress. In rewatering, Chl and Car contents were increased in both inbreds, but the recovery was higher in P142 (Table 1).

Effect of drought and rewatering on proline content

Drought stress caused a significant increase in proline content in both inbreds; however, the increment was higher in P134 compared to P142 (Fig. 2). Under moderate and severe stress, the content increased significantly compared to their respective controls (Fig. 2). Notably, under severe stress, the content was 19% higher in P134 than in P142. In rewatering, proline contents significantly decreased (55% and 42% in P134 and P142, respectively) as compared to those in severe stress (Fig. 2).

Effect of drought and rewatering on $O_2^{\bullet-}$ generation and H_2O_2 content

Drought stress increased the concentration of $O_2^{\bullet-}$ ion and H_2O_2 continuously in both inbreds (Fig. 3A, B). Under severe stress, the concentration of $O_2^{\bullet-}$ was 1.73 fold higher in P142 than in P134 while the concentration of H_2O_2 was 1.31 fold higher in P142 relative to P134. The histochemical detection of $O_2^{\bullet-}$ ion and H_2O_2 in leaves also showed higher concentration of $O_2^{\bullet-}$ ion and H_2O_2 in P142 (Fig. 3C, D). Upon rewatering, the $O_2^{\bullet-}$ and H_2O_2 decreased in both inbreds, although $O_2^{\bullet-}$ concentration was slightly higher in P142.

Effect of drought and rewatering on lipid peroxidation and LOX activity

The level of MDA and LOX activity also increased continuously in both inbreds with stress progression and being higher in P142 (Fig. 3E, F). Notably, in control seedlings, LOX activity was comparatively lower in P142. Under severe stress, the MDA and LOX activity were considerably higher by 37% and 11%, respectively, in P142. After rewatering, MDA and LOX activity reduced significantly in both inbreds compared to those in severe stress (Fig. 3E, F).

Effect of drought and rewatering on activities of SOD, POD, CAT and GPX

Increased SOD and POD activities were observed under drought in both inbreds (Fig. 4A, B). As compared to P134, the SOD activity was 43% and 28% higher in P142 under moderate and severe stress, respectively, while the POD activity was slightly higher in P142 under both moderate and drought stress. In rewatered seedlings, both SOD and POD activities decreased (Fig.4 A, B). However, significant difference was not found in catalase activity between control and drought or rewatering in both genotypes (Fig. 4C). In

Table 1. Chl and Car contents in maize seedlings subjected to drought stress and rewatering. Values represent the mean ± standard error (SE) from three independent experiments, each replicated 3 times. Similar letters among the treatments within a column are not significantly different at P≤0.05. Differences between P134 and P142 at a specific treatment are indicated by asterisk sign.

Stress condition	Chl*a*		Chl*b*		Car	
	P134	P142	P134	P142	P134	P142
Control	0.968^{a}±0.054	0.929^{a}±0.061	1.51^{a}±0.081	1.55^{a}±0.106	0.120^{a}±0.013	0.106^{a}±0.011
Moderate stress	0.630^{ab*}±0.074	0.447^{b}±0.041	1.12^{a}±0.091	1.03^{b}±0.110	0.094^{ab*}±0.007	0.074^{ab}±0.008
Severe stress	0.516^{b}±0.053	0.408^{b}±0.045	0.630^{b}±0.058	0.591^{c}±0.075	0.065^{b}±0.008	0.049^{b}±0.006
Rewatering	0.694^{ab}±0.067	0.652^{ab}±0.087	0.984^{ab}±0.067	1.01^{b}±0.099	0.069^{b}±0.007	0.063^{b}±0.006

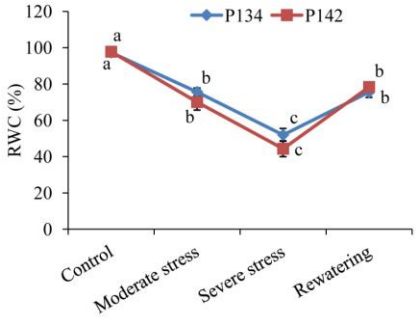

Fig 1. Relative leaf water content percentage [RWC (%)] in maize seedlings subjected to drought stress and rewatering. Values represent the mean ± SE from three independent experiments, each replicated 3 times. Similar letters among the treatments within a line are not significantly different at P≤0.05. Differences between P134 and P142 at a specific treatment are indicated by asterisk sign.

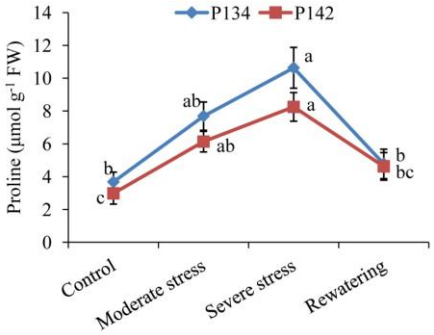

Fig 2. Content of proline in leaves of maize seedlings subjected to drought stress and rewatering. Values represent the mean ± SE from three independent experiments, each replicated 3 times. Similar letters among the treatments within a line are not significantly different at P≤0.05. Differences between P134 and P142 at a specific treatment are indicated by asterisk sign.

contrast, drought stress increased GPX activity in both inbreds, being higher in P142 (Fig. 4D). Rewatering of seedlings caused significant reduction in GPX activity in both inbreds (Fig. 4D).

Effect of drought and rewatering on ascorbate and glutathione levels

Contents of ASA and GSH were increased by drought in both inbreds, but P134 maintained higher levels of ASA and GSH both under control and drought stress (Fig. 5A, D). As compared to P142, the content of ASA in P134 was 49% and 68% higher under moderate and drought stress, respectively, while GSH was 27% and 74% higher under moderate and drought stress, respectively.

Drought stress caused higher oxidation of ASA and GSH in P142 resulting in higher DHA and GSSG contents (Fig. 5B, E). As compared to P134, the content of DHA was77% and 86% higher in P142 under moderate and severe stress, respectively, while GSSG content was 39% and 79% higher under moderate and severe stress, respectively. Higher oxidation of ASA and GSH altered the ASA- and GSH-redox states more in P142 (Fig. 5C, F). Rewatering of seedlings reduced the oxidation in both inbreds.

Drought stress increased APX activity in both inbreds, being higher in P142 (Fig. 6A). Under severe stress, the activity was significantly higher in P142 than in P134. Rewatering of seedlings caused significant reduction in APX activity in both inbreds (Fig. 6A).

On the other hand, drought stress increased the activities of MDHAR and DHAR remarkably in both inbreds (Fig. 6B,C). However, the activities under drought did not differ significantly between the inbreds. Interestingly, MDHAR activity was higher in P134 while DHAR activity was higher in P142. In rewatering, activities of both of the enzymes were decreased in both inbreds (Fig. 6B, C). In case of GR, drought stress caused comparatively higher induction in the activity in P134 compared to P142 and the activity level differed significantly under severe drought. After rewatering, activities of both of the enzymes were decreased in both inbreds, but the level was comparatively higher than respective control (Fig. 6D).

The CDNB conjugated GST activity increased in both inbreds by drought stress, where the level was higher in P142 (Fig. 7).

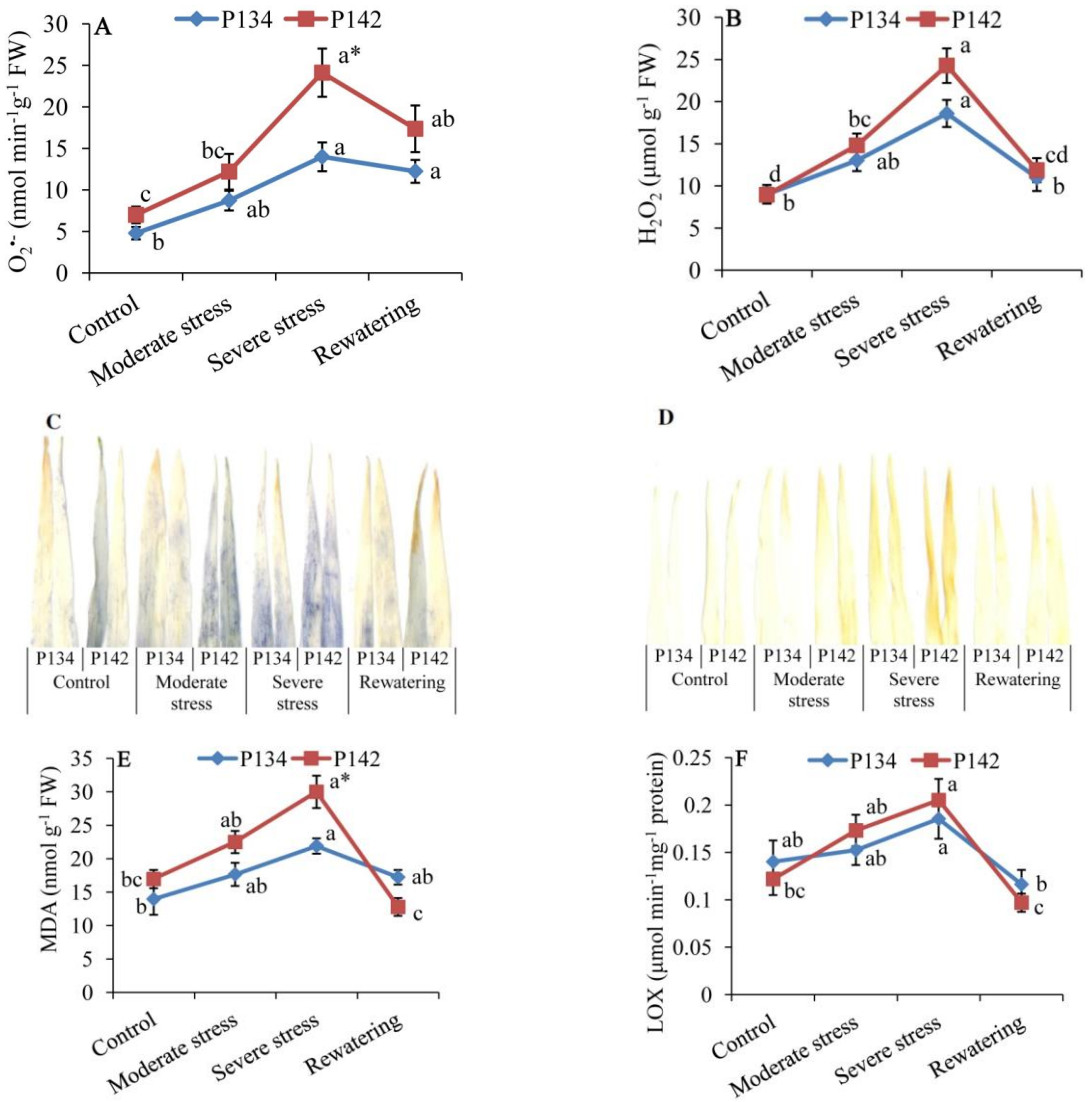

Fig 3. Rate of $O_2^{\bullet-}$ ion generation (A), H_2O_2 (B), histochemical detection of $O_2^{\bullet-}$ (bluish spot) (C) and H_2O_2 (deep drown) (D), MDA (E) and LOX activity (F) in leaves of maize seedlings subjected to drought stress and rewatering. In graphs, values represent the mean ± SE from three independent experiments, each replicated 3 times. Similar letters among the treatments within a line are not significantly different at P≤0.05. Differences between P134 and P142 at a specific treatment are indicated by asterisk sign.

However, significant difference was not found between the levels of activity of the inbreds. In severe drought, 19% higher activity was observed in P142. In rewatering, the activities decreased in both inbreds which was almost similar to respective control (Fig.7).

Effect of drought and rewatering on methylglyoxal and glyoxalases

Continuous and significant increase was observed in the content of MG in maize leaves under drought stress and the level was significantly higher in P142 (Fig.8A). In rewatering, the content decreased significantly in both inbreds. Drought stress increased the activities of Gly-I and Gly-II in P134 (Fig. 8B, C). However, in P142, Gly-I activity remained almost similar while Gly-II activity decreased under stress (Fig. 8B, C). As compared to P142, Gly-I activity was higher by 41% and 26% under moderate and severe drought, respectively, in P134 while Gly-II activity was higher by 59% and 78%, under moderate and severe drought, respectively. In rewatering, Gly-I activity decreased in both inbreds. On the other hand, Gly-II decreased in P134, but remained almost similar in P142.

Discussion

RWC is an important indicator for evaluating plants for tolerance to drought stress (Hu et al., 2010). In this study, RWC significantly reduced under drought conditions, but the level was comparatively higher in tolerant inbred P134 (Fig. 1). This result suggests that plants with higher RWC under water stress are able to maintain water turgor pressure within cells than those with lower RWC and thus more tolerant to water stress. As expected, RWC was increased on rewatering in both inbreds. Under drought stress, similar results were found in maize (Ghahfarokhi et al., 2015) and other crops (Hu et al., 2009; Ji et al., 2014).

Under drought, excessive production of ROS causes oxidative damage to cellular structures and macromolecules, which in turn causes photoinhibition of the photosynthetic apparatus resulting decreased Chl contents (Vranová et al., 2002; Ghane et al., 2011). In this study, as compare to

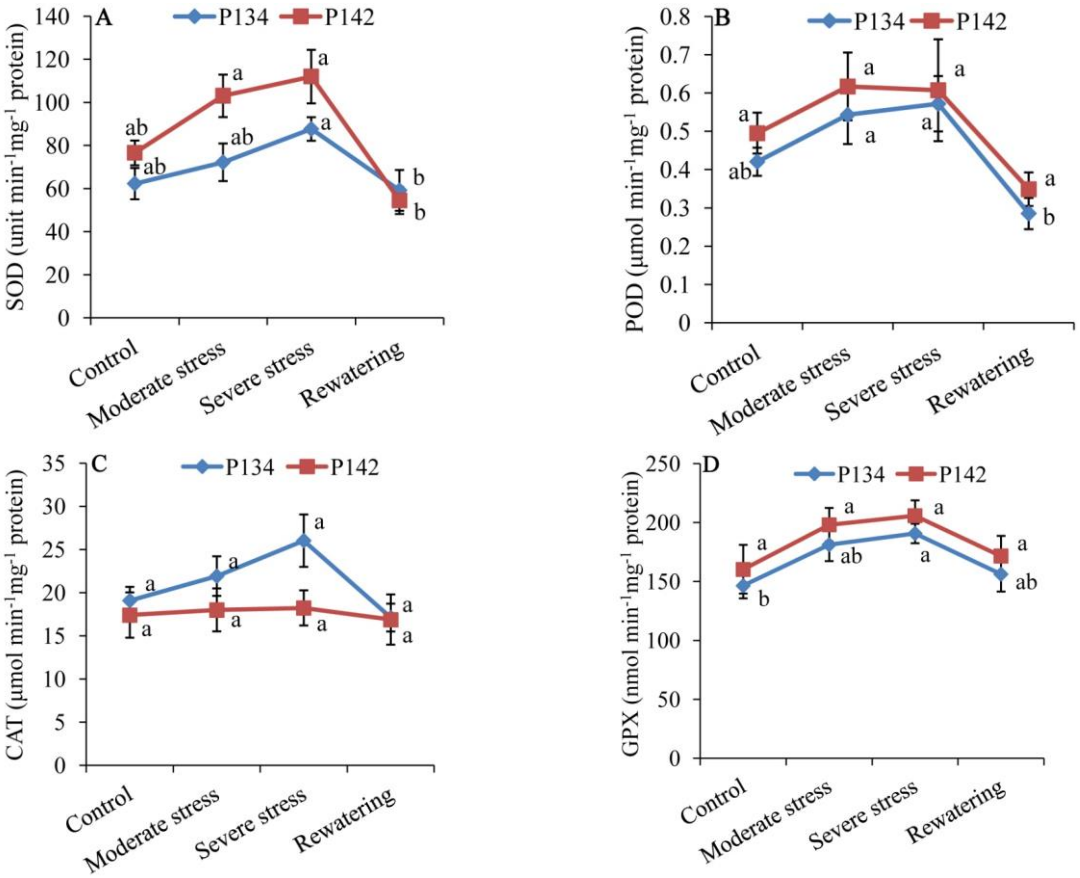

Fig 4. Activities of SOD (A), POD (B), CAT (C) and GPX (D) in leaves of maize seedlings subjected to drought stress and rewatering. Values represent the mean ± SE from three independent experiments, each replicated 3 times. Similar letters among the treatments within a line are not significantly different at P≤0.05. Differences between P134 and P142 at a specific treatment are indicated by asterisk sign.

respective control, the losses of Chl*a* and Chl*b* contents were comparatively higher in P142 than those in P134 (Table 1). Like Chl, Car content was also decreased in both inbreds and the tolerant inbred P134 maintained comparatively higher Car under drought (Table 1). These results suggest that tolerant of maize inbreds is associated with lower loss of Chl and Car. Higher Chl and Car contents in tolerant genotypes have also been reported earlier in maize and wheat (Pastori and Trippi, 1992; Sairam et al., 1998; Chandrasekar et al., 2000). In rewatering, the Chl*a*, Chl*b* and Car further increased in leaves of seedlings of both inbreds, but significant differences were not found in between the inbreds suggesting that susceptible inbred showed higher recovery in respect of Chl and Car contents. Ghahfarokhi et al. (2015) reported higher recovery in Chl components and carotenoid in both vegetative and reproductive stages in susceptible genotypes of maize.

Accumulation of compatible solutes like proline under abiotic stress including drought stress has been reported in a wide number of plant species. It maintains cellular water status and membrane stability, inhibits protein oxidation and scavenges free radicals through antioxidative action under osmotic stress (Ashraf and Foolad, 2007). Therefore, genotypes with higher proline content are considered as tolerant to drought and other abiotic stresses (Gilll and Tujeta, 2010). In this study, proline accumulation under drought significantly increased in both inbreds where accumulation was comparatively higher in P134 under drought (Fig. 2). Since P134 marginally had higher content of proline under both moderate and severe water stress, therefore, drought tolerance could be partly associated with proline content in maize. Moussa and Abdel-Aziz (2008) also reported increase of proline in both tolerant and susceptible maize genotypes. In rewateing, proline contents decreased in both inbreds suggesting partial recovery from drought stress. Elevated accumulation of proline in maize at vegetative and reproductive stages under drought stress was also reported by other group (Ghahfarokhi et al., 2015).

ROS generation is a common phenomenon in crop under abiotic stress including drought (Bartoli et al., 1999; Noctor et al., 2012). In this study, leaves of P142 had higher $O_2^{\bullet-}$ and H_2O_2 concentrations under both moderate and severe water stress suggesting more oxidative damage in P142. It might be due to limited capacity of scavenging of $O_2^{\bullet-}$ and H_2O_2 in P142. This capacity is explained by the activities of antioxidant enzymes. SOD is considered as the first defense against ROS, being responsible for the dismutation of $O_2^{\bullet-}$ to H_2O_2. On the other hand, enzymes like CAT, POD, GPX and APX catalyze the conversion of H_2O_2 to water and O_2 (Gratao et al., 2005). The balance between ROS production and activities of antioxidative enzymes determines whether oxidative signaling and/or damage will occur (Moller et al., 2007). Tolerance of a plant to drought depends on the capability of scavenging ROS and reducing their damaging effects (Tsugane et al., 1999). Tolerance level also depends upon intensity and duration of stress as well as plant species

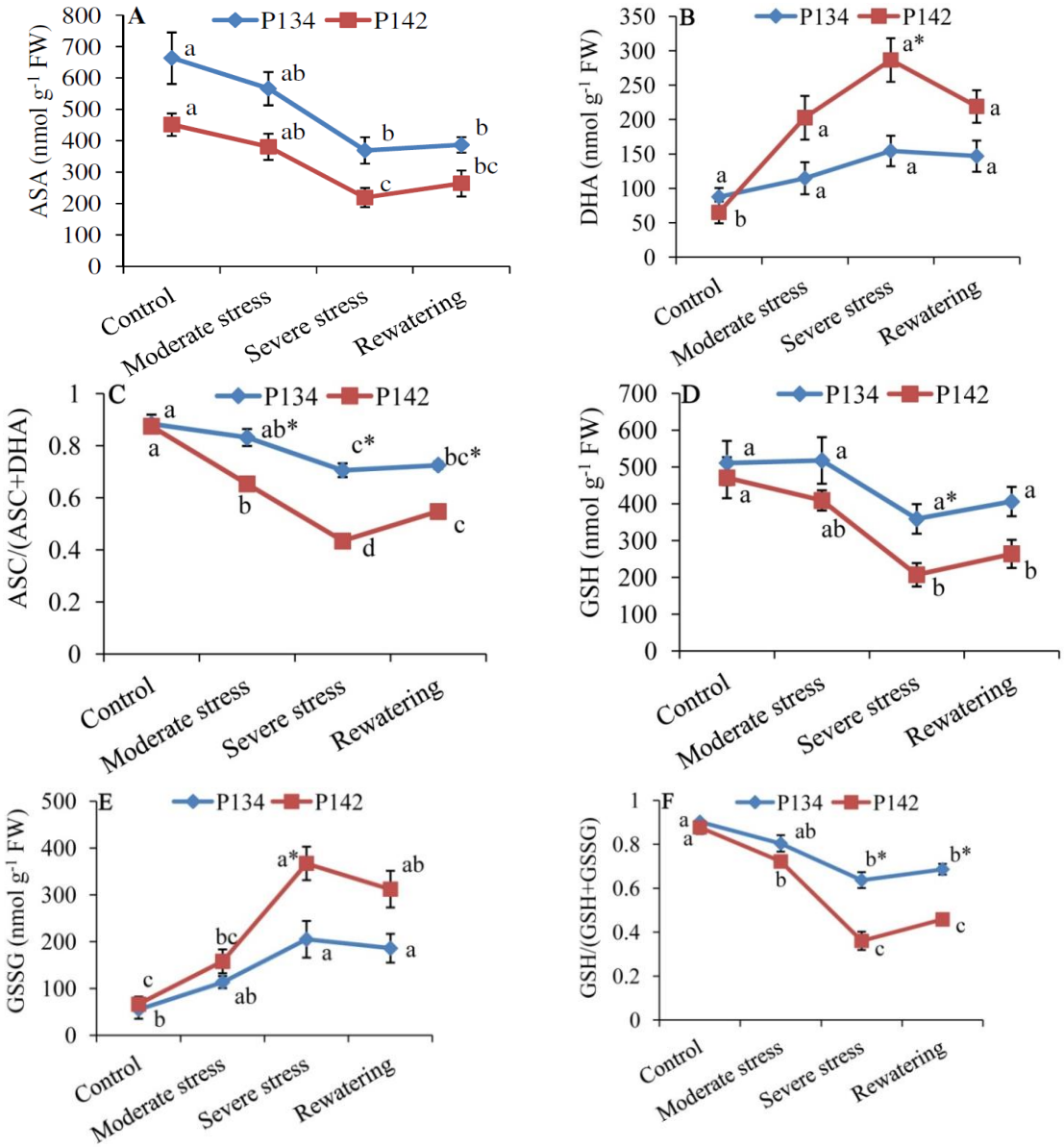

Fig 5. Contents of ASA (A), DHA (B), Ascorbate redox state (C), GSH (D), GSSG (E) and glutathione redox state (F) in leaves of maize seedlings subjected to drought stress and rewatering. Values represent the mean ± SE from three independent experiments, each replicated 3 times. Similar letters among the treatments within a line are not significantly different at P≤0.05. Differences between P134 and P142 at a specific treatment are indicated by asterisk sign.

and its developmental stage (Chaves et al., 2003; Jung, 2004; Dacosta and Huang, 2007). Like ROS, MDA is regarded as a marker for evaluation of lipid peroxidation or damage to plasmalemma and organelle membranes that increases with ROS accumulation under environmental stresses (Garg and Manchanda, 2009). In this study, $O_2^{\bullet-}$ generation rate and H_2O_2 and MDA contents were significantly lower in tolerant inbred (Fig. 3A, B, C, D, E). In review, Sharma et al. (2012) also reported comparatively higher ROS and MDA levels in a number of drought susceptible plant species. However, the increased ROS and MDA reversed following rewatering in both inbreds signifying a great elasticity in the plant's capacity to cope with free oxygen radicals. LOX enzyme catalyzes the peroxidation of polyunsaturated fatty acids to their corresponding hydroperoxides (Doderer et al., 1992). The increased LOX activity was assumed as a reason for increased lipid peroxidation of polyunsaturated fatty acids as reported in many plants (Demiral and Türkan, 2004; Azooz et al., 2009; Sánchez-Rodríguez et al., 2012). However, LOX activity was quite similar in two lines under the same water stress. LOX activity was also correlated with increased MDA content in susceptible inbred (Fig. 3E, F). Previously Moussa and Abdel-Aziz (2008) and Kellôs et al. (2008) also reported lower MDA and H_2O_2 contents in drought tolerant maize genotypes. The SOD and POD activities were higher in both inbreds under drought, but susceptible P142 showed comparatively higher level of activities (Fig. 4A, B).The major antioxidants, SOD and POD, can essentially reduce ROS accumulation, thus regulating the level of lipidperoxidation (Apel and Hirt, 2004). High SOD enzyme activity under drought may convert cytotoxic $O_2^{\bullet-}$ to H_2O_2, and POD catalyze the decomposition of H_2O_2 to water, thus alleviating the damaging effects of these products (Jin et al., 2011). Zhang et al. (2014) found concurrent increases in SOD and POD activities in two maize genotypes in vegetative stage under drought stress. The activity of

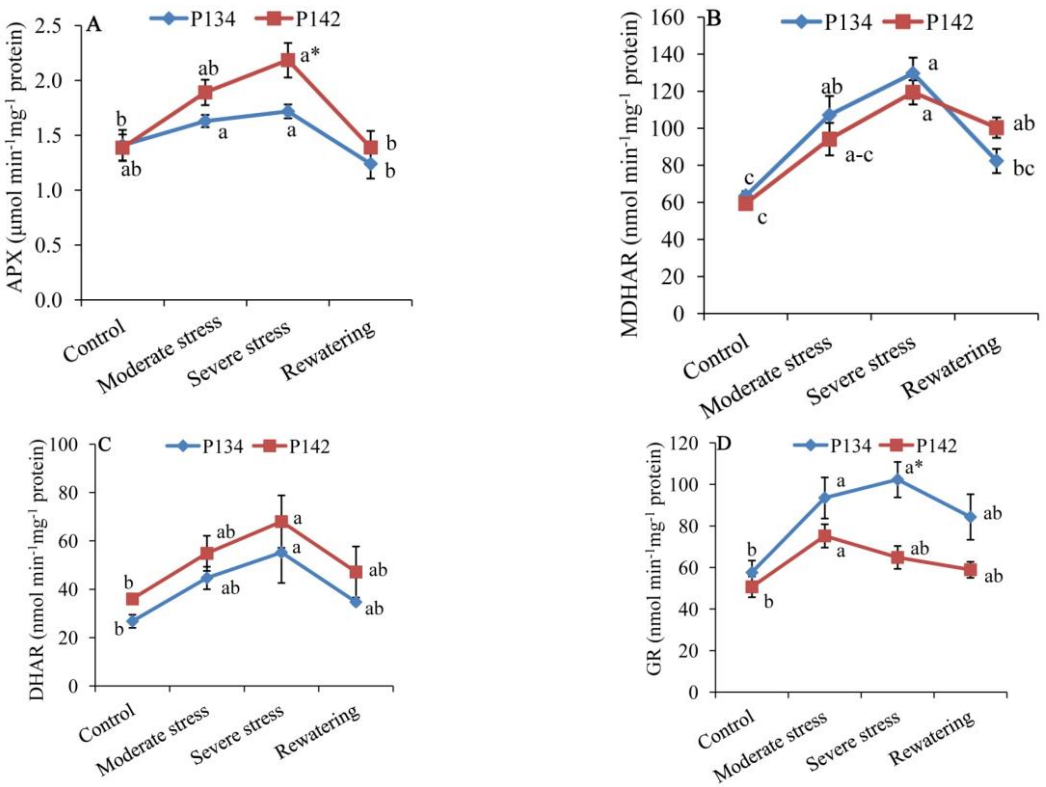

Fig 6. Activities of APX (A), MDHAR (B), DHAR (C) and GR (D) in leaves of maize seedlings subjected to drought stress and rewatering. Values represent the mean ± SE from three independent experiments, each replicated 3 times. Similar letters among the treatments within a line are not significantly different at P≤0.05. Differences between P134 and P142 at a specific treatment are indicated by asterisk sign.

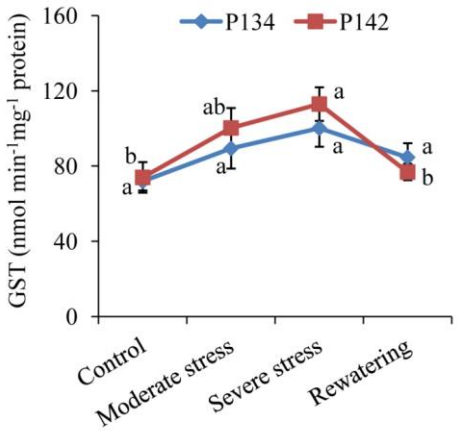

Fig 7. Activities of GST in leaves of maize seedlings subjected to drought stress and rewatering. Values represent the mean±SE from four independent experiments, each replicated 3 times. Values represent the mean ± SE from three independent experiments. Similar letters among the treatments within a line are not significantly different at P≤0.05. Differences between P134 and P142 at a specific treatment are indicated by asterisk sign.

antioxidant enzymes, including SOD and POD has also been reported to be correlated with drought tolerance in other crops (Türkan et al., 2005; Abedi and Pakniyat, 2010; Li et al., 2015; Ying et al., 2015). Besides, CAT, GPX and APX are associated with oxidative tolerance by scavenging of H_2O_2 (Miller et al., 2010; Gill and Tujeta, 2010). However, CAT, as compared to APX, GPX and POD, shows low affinity towards H_2O_2 but has a high processing rate (Scandalios, 2005). This is essentially because, unlike other H_2O_2 scavenging enzymes (APX, GPX and POD), enzymatic reaction of CAT is not saturated with increasing concentrations of peroxide and is independent of other cellular reductants for instituting its activity (Scandalios, 2005). In this study, CAT activity increased in tolerant inbred P134 under drought stress and being almost unchanged in P142 (Fig. 4C) suggesting important scavenger of H_2O_2 in this tolerant inbred. This result is supported by the findings of Zhang et al. (2014) at vegetative stage and Chugh et al. (2011) at seedling stage in maize. In GSH-dependent metabolism, H_2O_2 is removed by GPX where GSH is converted to GSSG. In this study, the increased GPX activities in both inbreds suggested a role of this enzyme in H_2O_2 metabolism in both drought tolerant and susceptible inbreds (Fig.4D). However, Sayfzadeh and Rashidi (2010)

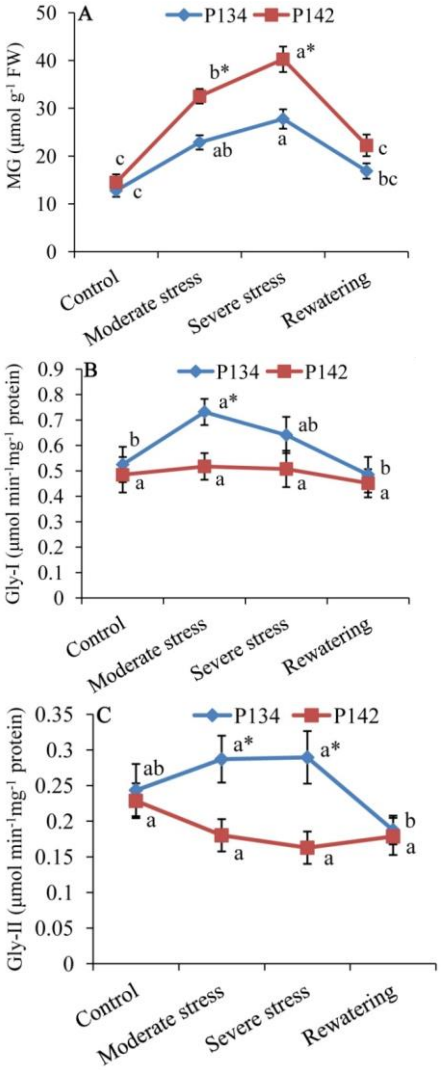

Fig 8. Contents of MG (A) and activities of Gly-I (B) and Gly-II (C) in leaves of maize seedlings subjected to drought stress and rewatering. For MG, values represent the mean ± SE from four independent experiments, each replicated 3 times while for Gly-I and Gly-II, values represent the mean ± SE from three independent experiments, each replicated 3 times. Similar letters among the treatments within a line are not significantly different at P≤0.05. Differences between P134 and P142 at a specific treatment are indicated by asterisk sign.

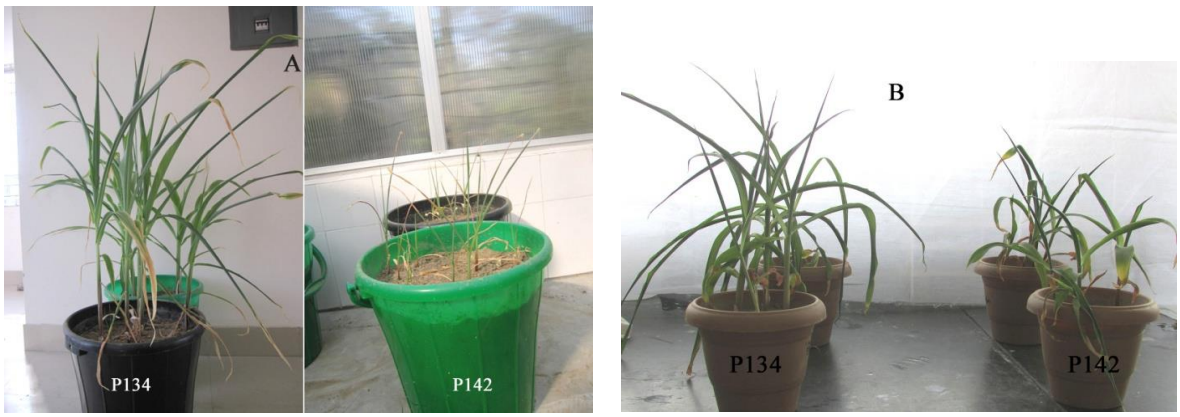

Fig 9. Comparative drought tolerance of P134 and P142 in Green house, A) 30 days old seedlings grown in bucket were subjected to stop watering for 2 months and B) 8 days old seedlings grown in small plastic pot were subjected to stop watering for 7 days.

reported increased GPX activity in wheat under drought condition. The other decomposers of H_2O_2, APX along with MDHAR, DHAR and GR are important antioxidants in ascorbate-glutathione cycle in maintaining GSH and ASA (Apel and Hirt, 2004, Noctor et al., 2012). Ascorbate and glutathione are found abundantly in plant cells (Noctor and Foyer, 1998; Rouhier et al., 2008). In our experiments, though both inbreds contained similar amounts of glutathione in control plants, the ASA content was much lower in susceptible inbred P142 (Fig. 5A, D). Upon water deficit, both GSH and ASA level has decreased in both inbreds. However, with increased stress severity, the oxidation of both of the compounds were much higher in P142 (Fig. 5B, E).These data along with 27% higher APX activity in P142 (Fig. 5D) suggests that ASA has participated in the degradation of H_2O_2 catalyzed by APX. This process might result in higher DHA contents in P134 under drought. These results are in agreement with the findings that ascorbate depletion occurs in susceptible plants under water stress together with an increased proportion of DHA (Jubany-Mari et al., 2010). However, the activities of most of the antioxidant enzymes reduced upon rewatering condition with a positive correlation between ROS and MDA. Similar results were found in leaves of *Medicago truncatula* where SOD, POD, CAT and APX activity reduced after rewatering (Filippou et al., 2011).

In ASA-GSH cycle, MDHAR and DHAR play significant role in maintaining ASA contents in cell, where ASA are utilized by APX to reduce H_2O_2 to water with concomitant generation of monodehydroascorbate (MDHA), a radical with short life time and can spontaneously dismutate into DHA and ASA or is reduced to ASA by NADPH dependent enzyme MDHAR (Miyake and Asada, 1994; Apel and Hirt, 2004). In this study, the changes in MDHAR suggests the possibility of maintaining ASA in both inbreds, as the increments of activities did not varied significantly between the inbreds (Fig. 6B). On the other hand, DHAR, which uses GSH in reducing DHA to ASA, was increased in both inbreds (Fig. 6C). These findings suggest that in both inbreds, DHAR has played a significant role in reducing oxidized DHA to ASA by oxidizing GSH to GSSG. In rewatering, the activities of MDHAR and DHAR reduced in both inbreds with higher proportion in P142 suggesting better ASA recovery after receiving water.

In maintenance of GSH in plant, GR plays a key role under abiotic stress including drought. GSH which is continuously oxidized to GSSG is recycled to GSH by NADPH-dependent GR (Noctor et al., 2012). Increased expression of GR has been detected and reported only in drought tolerant genotypes of wheat and maize (Jiang and Zhang, 2002; Sečenji et al., 2010). In the present study, 20% and 37% more GR activities have been observed in P134 under moderate and severe drought stress, respectively (Fig. 6D), which suggested a possible role of GR in maintaining higher GSH pool as well as GSH-redox in drought tolerant inbred line (Fig. 5F). Notably, the higher GR activity in P134 upon rewatering revealed its role in maintaining GSH pool. Ghahfarokhi et al. (2015) also reported higher GR activities in drought tolerant maize inbreds under drought at both vegetative and reproductive stages, where rewatering did not change the activity level. GST comprise an extensive family of proteins with a great variety of functions and are differentially induced by stress (Chi et al., 2011). The important roles of GST are conjugation and sequestration of xenobiotics, transport of flavonoids, detoxification of ROS and organic radicals, programmed cell death under conditions of elevated ROS levels, recycling of flavonoids and participation in fumarate synthesis during tyrosine catabolism (reviewed by Dixon et al., 2002). GST is also reported to have important role in leaf senescence during abiotic stress (Kunieda et al., 2005). In our study, GST activity has increased in both inbreds, being 19% higher in P142 (Fig. 7). Increased GST activity along with higher H_2O_2 and MDA in P142 indicated that GST might be involved in cellular protection through detoxification of hydroperoxides under drought stress. In rewatering, GST activity decreased in both inbreds. Similar results were reported by Filippou et al. (2011).

Methylglyoxal, a potential cytotoxic oxo-aldehyde produced as an intermediate under stress including drought, is detoxified by glyoxalase system (Maiti et al., 1997; Marasinghe et al., 2005). The present investigation demonstrated that drought stress caused significant accumulation of MG in both inbreds and under drought, the content was significantly higher in P142 (Fig. 8A). In rewatering, the MG contents were reduced in both inbreds, which suggested the elasticity of maize seedlings exposed to drought stress. Importantly, increased Gly-I and Gly-II activity in P134 suggesting a role of glyoxalase system in detoxifying excess amount of MG in drought tolerant plant under drought stress (Fig. 8B, C). Increased activities of glyoxalases under drought have also been reported in other plant species (Hasanuzzaman and Fujita, 2011; Alam et al., 2014; Nahar et al., 2015).

Materials and Methods

Plant materials and stress treatments

During screening of maize inbreds for drought tolerance, two maize inbreds: P134 (CML193-2-B), a relatively drought tolerant, and P142 (CML293-1-B), a susceptible inbred showed distinct phenotype (Fig. 9). Seedlings of the inbreds were grown in plastic pot and 8 day old seedlings were subjected to drought stress by withholding watering for 5 or 7 days and rewatered again for 2 days. Water withhold for 5 days was termed as moderate stress and for 7 days as severe stress following Marok et al. (2013). Data were recorded on different parameter in fully expanded upper leaves of stressed seedlings and 2 days after rewatering. Three independent experiments (except for MG, four experiments) following completely randomized (CRD) design were conducted, each containing three replications.

Measurement of Relative water content

Leaf relative water content (RWC) was measured according to Barrs and Weatherley (1962). Fresh weight (FW), turgid weight (TW) and dry weight (DW) of leaves were recorded. The RWC was calculated using the following formula: RWC (%)= (FW-DW) × 100/(TW-DW).

Chlorophyll and carotenoid content determination

Extraction and determination of chlorophyll (Chl) and carotenoid (Car) were performed according to the method of Arnon (1949). Briefly, five hundred milligrams (mg) of fresh leaf material (from each treatment) was ground with 10 ml of 80% acetone at 4°C and centrifuged at 5000 rpm for 10 minutes at 4°C. The absorbance of supernatant was read at 645, 663 and 470 nm for Chl*a*, Chl*b* and Car, respectively, with a spectrophotometer (UV-1800, Shimadzu, Japan)

against 80% acetone as blank. Chl and Car were calculated using following formulas and expressed in mg g^{-1} FW.

Chl*a* (mg g^{-1})= (0.0127) × (A_{663}) – (0.00269) × (A_{645})

Chl*b* (mg g^{-1})= (0.0229) × ($A_{.645}$) – (0.00468) × (A_{663})

Car (mg g^{-1})= A_{470} + (0.114 × A_{663} – 0.638 × A_{645})

Extraction and measurement of ascorbate and glutathione

Maize leaves (0.5 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer containing 5% meta-phosphoric acid and 1 mM EDTA using a mortar and pestle. Homogenates were centrifuged at 11,500×*g* for 15 min at 4°C, and the supernatant was collected for analysis of ascorbate and glutathione.

Ascorbate content was determined following the method of Huang et al. (2005). The supernatant was neutralized with 0.5 M potassium-phosphate (K-P) buffer (pH 7.0) and reduced ascorbate was assayed spectrophotometrically (UV-1800, Shimadzu, Japan) at 265 nm in 100 mM K-P buffer (pH 5.6) supplemented with 0.5 unit of ascorbate oxidase (AO). The ascorbate content in the leaf tissue was obtained from the standard curve generated from a series of known concentration of ascorbic acid (ASA).

The glutathione pool was assayed according to Yu et al. (2003) with modification, using 0.40 ml of supernatant neutralized with 0.6 ml of 0.5 M K-P buffer (pH 7.0). Based on enzymatic recycling, glutathione was oxidized by 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase (GR). Total glutathione content was estimated from the rate of absorption changes of 2-nitro-5-thiobenzoic acid (NTB) at 412 nm, which was generated from the reduction of DTNB. Oxidized glutathione (GSSG) was determined after removal of GSH by 2-vinylpyridine derivatization. Reduced glutathione (GSH) was measured by deduction of GSSG from total glutathione. Specific standard curve generated from either GSH or GSSG standard were used for the quantitation.

Extraction of soluble protein

Total soluble protein was extracted from fresh leaves of maize seedlings by homogenizing in an equal volume of 25 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1% (w/v) ascorbate and 10% (w/v) glycerol with a mortar and pestle. The homogenate was centrifuged at 11,500×*g* for 15 min at 4°C, and the supernatant was used for enzyme assay.

Assay of enzymatic activities

SOD (EC: 1.15.1.1): To determine SOD activity of whole cell homogenate, the reaction was prepared on ice in 50 mM potassium phosphate buffer (p^H 7.8, with 1.34 mM diethylenetriaminepentaacetic acid) and an indirect competitive inhibition assay was used for the measurement (Spitz and Oberley, 1989). One unit of activity was defined as the amount of protein required to inhibit nitroblue tetrazolium (NBT) reduction by 50%.

POD (EC: 1.11.1.7): POD activity was estimated according to Hemeda and Klein (1990). Activity was determined by the increase in absorbance at 470 nm due to guaiacol oxidation for 1 min using extinction coefficient of 26.6 mM^{-1} cm^{-1}.

APX (EC: 1.11.1.11): APX activity was assayed following the method of Nakano and Asada (1981). The activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

MDHAR (EC: 1.6.5.4): MDHAR activity was determined by the method of Hossain et al. (2010). The activity was calculated from the change in ascorbate at 340 nm for 1 min using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

DHAR (EC: 1.8.5.1): DHAR activity was determined by the procedure of Nakano and Asada (1981). The activity was calculated from the change in absorbance at 265 nm for 1 min using extinction coefficient of 14 mM^{-1} cm^{-1}.

GR (EC: 1.6.4.2): GR activity was measured by the method of Hossain et al. (2010). The activity was calculated using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

GST (EC: 2.5.1.18): GST activity was determined spectrophotometrically according to Rohman et al. (2010). The activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1}.

GPX (EC: 1.11.1.9): GPX activity was measured as described by Elia et al. (2003) using H_2O_2 as a substrate. 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): CAT activity was measured according to the method of Csiszár et al. (2007) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition (or degradation) of H_2O_2. The activity was calculated using the extinction coefficient of 39.4 M^{-1} cm^{-1}.

Gly-I (EC: 4.4.1.5): Gly-I assay was carried out according to Yadav et al. (2005a). The activity was calculated using the extinction coefficient of 3.37 mM^{-1} cm^{-1}.

Gly-II (EC: 3.1.2.6): Gly-II 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 activity was calculated using the extinction coefficient of 13.6 mM^{-1} cm^{-1}.

Lipoxygenase (LOX, EC: 1.13.11.12): LOX activity was measured following Doderer et al. (1992). The activity was expressed as μM hydroperoxide formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 25,000 M^{-1} cm^{-1}.

Measurement of the $O_2^{\bullet-}$ generation rate

Superoxide radical was determined according to the method of Elstner and Heupel (1976) with slight modifications. Leaves (0.3 g) were homogenized in 3 ml of 65 mM (K-P) buffer (pH 7.8) on an ice bath and were then centrifuged at 4°C for 10 min at 5,000 × *g*. The supernatants (0.75 ml) were mixed with 0.675 ml of 65 mM K-P buffer (pH 7.8) and 0.07 ml of 10 mM hydroxylamine chlorhydrate and the reaction was incubated at 25°C. After 20 min, 0.375 ml of 17 mM sulfanilamide and 0.375 ml of 7 mM α-naphthylamine were added, and the mixture was incubated at 25°C for another 20 min before it was mixed with 2.25 ml of ether. The absorbance was measured at 530 nm and the $O_2^{\bullet-}$ concentration was calculated from a standard curve of $NaNO_2$.

Measurement of H_2O_2

H_2O_2 was assayed according to the method described by Yu et al. (2003). The absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content (Є= 0.28 μM^{-1} cm^{-1}) and expressed as micromole g^{-1} FW.

Histochemical detections of $O_2^{\bullet-}$ and H_2O_2

Superoxide ($O_2^{\bullet-}$) and H_2O_2 were visualized in maize leaves according to the method of Wang et al. (2011) with modifications. Briefly, the second leaves were stained in 0.1 % NBT or 0.1% 3,3-diaminobenzidine (DAB) solution for 8 h under dark and light, respectively. Incubated leaves were

then decolorized by immersing in boiling ethanol which allowed visualization of bluish insoluble formazan (for $O_2^{\bullet-}$) or deep brown polymerization product (for H_2O_2). After cooling, glycerol was used to open the leaves and photographs were taken by placing the leaves between two transparent sheets.

Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating melondialdehyde (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). The concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nanomole of MDA g^{-1} FW.

Determination of proline

Proline colorimetric determination proceeded according to Bates et al. (Bates et al., 1973) based on proline's reaction with ninhydrin.

Measurement of MG

The content of MG was estimated following the method of Rohman et al. (2016) using N-acetyl-L-cysteine.

Determination of protein

The protein concentration in the leaf extracts was determined according to the method of Bradford (1976) using BSA as a protein standard.

Statistical analysis

All data obtained was analyzed by SAS (SAS Institute Inc. Cary, NC, USA, Version 9.3) program following complete randomized design (CRD) and the mean differences among the treatments within one inbred were compared by least significant (LSD) tests. Differences between P134 and P142 at a specific condition were analyzed simply by student t-test. P values ≤ 0.05 were considered to be significant.

Conclusion

In this study, we found higher ROS and MDA as well as lipid peroxidation under drought in susceptible inbred. Under drought, induction of ROS scavenging enzymes SOD, POD, DHAR and GPX suggests their involvement in scavenging ROS in both inbreds. However, comparatively higher activities of these enzymes in susceptible inbred lessened the oxidative damage by scavenging highly produced ROS. Contrary, enzymatic antioxidants like CAT and GR as well as non-enzymatic antioxidants ASA and GSH played important protective role in tolerant inbred. At the same time, higher glyoxalase activities in tolerant inbred also provided important role in tolerance in this inbred. We anticipate that inter-relation of oxidative damage with enzymatic- and non-enzymatic antioxidants and glyoxalases will be helpful to understand the drought mediated oxidative stress in maize.

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