Effect of Polyamine on Pigmentation, Reactive Oxidative Species and Antioxidant under Drought in Maize (*Zea mays* L.)

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ARTICLE INFO	ABSTRACT
	To examine polyamines (PAs) effect in modulating the drought induced by polyethylene glycol (PEG) in maize (*Zea mays* L.) seedlings (variety Khoibhutta, 8 day seedlings grown in petri dish in incubator) were subjected to 20% PEG (polyethylene glycol) followed by 20 μmol PAs, Putrescine (Put), Spermidine (Spd) and Spermine (Spm) with PEG solution for 48 hours. Sharp decrease in Relative Water Content (RWC), Chl *a*, Chl *b*, carotenoid (Car) and total pigment content was observed under drought compared to control condition, while PAs application reversed their decreasing trends. PEG significantly increased Reactive Oxidative Species (ROS) [superoxide ($O_2^{\bullet-}$) and H_2O_2], Methyl Glyoxal (MG), Melondialdehyde (MDA) and Lipoxigenase (LOX) activity, while Pas decreased the contents considerably (except MG) as compared to those under drought. Drought increased proline content, which was further augmented in PA treatments. PAs failed to incline glyoxalase's (Gly-I and Gly-II) activities, reduced under PEG. The activity and western blot confirmed the accumulation of Glutathione *S*-Transferase (GST) under drought, but PAs failed to augment the activity. Ascorbic Acid (AsA) and Glutathione (GSH) got oxidized into Dehydroascorbate (DHA) and oxidized Glutathione (GSSG) under drought but PAs effectively maintained homeostasis. Superoxide Dismutase (SOD), Peroxidase (POD), Ascorbate Peroxidase (APX), Glutathione Peroxidase (GPX), Monodehydroascorbatereductase (MDHAR), Dehydroascorbatereductase (DHAR), and Glutathione Reductase (GR) inclined in drought stressed seedlings, while Catalase (CAT) activity decreased under drought. PAs addition increased SOD, POD, GPX, CAT, MDHAR, and GR activities, but declined DHAR activity. These findings suggested important role of PAs in increasing tolerance under short term drought by modulating antioxidant effect.

Introduction

Drought changes plant metabolic activity, which leads to enhanced production of ROS (reactive oxygen species) comprising free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), as well as non-radical molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and so forth which genesis follows a sequential reduction of molecular oxygen (O_2) by high-energy exposure or, electron-transfer reactions (Sharma et al., 2012). ROS functions are lipid peroxidation, protein oxidation, nucleic acid damage, enzyme inhibition, damage in photosynthetic pathway, and activation of programmed cell death (PCD) pathway, and ultimate cell death (Sharma and Dubey, 2005). There are two different mechanisms by which the plants regulate their intercellular ROS concentrations. One is by enabling the fine modulation of low levels of ROS for signaling purpose, and another one is by proceeding the detoxification of excess ROS, especially during stress (Mitter, 2002). ROS scavenging or, detoxification is accomplished by an efficient antioxidative system comprising of the non-enzymic as well as enzymic antioxidants (Noctor and Foyer, 1998). The enzymic antioxidants include SOD, CAT, GPX, enzymes of ascorbate-glutathione (AsA-GSH cycle) like APX, MDHAR, DHAR, and GR (Noctor and Foyer, 1998). AsA, GSH (reduced glutathione), tocopherols, and phenolics serve as potent non-enzymic antioxidants. However, many advanced research have found positive

influence of PAs, aliphatic nitrogen compounds, in stress tolerance mechanism through increased ROS scavenging via increase in antioxidative activities, as they are involved in the regulation of physical and chemical properties of membranes, nucleic acids structure and functions, and modulation of enzyme activities (Galston and Kaur Sawhney, 1990). Three major PAs, found in all living cells are diamineputrescine (Put), the triaminespermidine (Spd), and the tetraminespermine (Spm). It has been reported that chloroplasts and photosynthetic sub-complexes including thylakoids and PSII membranes are enriched with three major PAs, while PSII (photosystem II) core and the reaction center of PSII are exclusively rich in Spm (Kotzabasis et al., 1993; Navakoudis et al., 2003) and detoxify effectively cyto-compartments by influencing the effectiveness of enzymic and non-enzymic antioxidants. Furthermore, genetic transformation with PAs biosynthetic genes encoding arginine Decarboxylase (ADC), Ornithine Decarboxylase (ODC), *S*-Adenosylmethionine Decarboxylase (SAMDC) or, Spd Synthase (SPDS) improved stress tolerance mechanism in different environmental and pathogenic adversity (Liu et al., 2007). That is why, to know the role of exogenous application of PAs in improving stress tolerance through regulating enzymic and non-enzymic antioxidants is gaining importance to understand the underlying stress tolerance mechanism under abiotic stress including drought.

Materials and Methods

Plant Materials and Stress Treatments

Maize variety "Khiovutta" seedlings were grown in incubator. Eight days seedlings were subjected to 5 treatments namely Control, 20% of PEG, 20% PEG + 20 μmol Put, 20% PEG + 20 μmol Spd and 20% PEG + 20 μmol Spm for 48 hours (Plate 1). The experiments were repeated three times each containting three replications. After 48 hours exposure of maize seedling, data were recorded on different parameters.

Relative Water Content (RWC) Estimation

RWC was estimated according to Barrs and Weatherley (1962). Fresh weight (FW), turgid weight (TW) and dry weight (DW) of leaves were recorded. The RWC was calculated by the following formula: RWC (%) = (FW − DW) × 100 / (TW − DW).

Chlorophyll (Chl) and Carotenoid (Car) Determination

Extraction and measurement of Chl and Car were performed according to the method of Arnon (1949). Chl and Car were calculated using the formulas below and expressed in mg g^{-1} fresh weight (FW).

Chl*a* (mg g^{-1} l) $= (0.0127) \times (A_{663}) - (0.00269) \times (A_{645})$
Chl*b* (mg g^{-1}) $= (0.0229) \times (A_{.645}) - (0.00468) \times (A_{663})$
Car (mg g^{-1}) $= A_{470} + (0.114 \times A_{663} - 0.638 \times A_{645})$

Assay of Proline

Prolines' colorimetric determination proceeded according to Bates et al. (1973) based on prolines' reaction with ninhydrin.

Estimation of ROS

Superoxide radical was determined according to the method of Elstner and Heupel (1976) with modifications. Leaves (0.3 g) were homogenized in 3 ml of 65 mM (K-P) buffer (pH 7.8) and then centrifuged at 4°C and 5,000 $\times g$ for 10 min. 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 placed 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 placed 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 $NaNO_2.H_2O_2$ which was assayed according to Yu et al. (2003). LOX activity was measured following Doderer et al. (1992). Lipid peroxidation was measured by a decomposed product of the peroxidized poly-unsaturated fatty acid component of the membrane lipid, using TBA (thiobarbituric acid) as the reactive material following the method of Heath and Packer (1968).

Determination of Enzymes of Glyoxalase System (Gly-I and Gly-II)

Gly-I assay was carried out according to Yadav et al. (2005). Briefly, the assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulfate, 1.7 mM GSH, and 3.5 mM MG in a final volume of 0.7 ml. The reaction was started by the addition of MG, and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of 3.37 mM^{-1} cm^{-1}. Gly-II activity was determined according to 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 {5,5′-dithio-bis (2-nitrobenzoic acid)}, and 1mM SLG (S-D-lactoylglutathione) 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}. MG estimation was done following Wild et al. (2012).

Extraction of Soluble Protein and Assay of Enzymatic Antioxidant Activities

Fresh leaves of maize seedlings were extracted by homogenizing in an equal volume of 25 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA (ethylenediaminetetraacetic acid), 1% (w/v) AsA, and 10% (w/v) glycerol with mortar pestle. The homogenate was centrifuged at 11,500 $\times g$ for 15 min, and the supernatant was used as a soluble protein solution for enzyme assay. SOD activity was determined using the method of Spitz and Oberley (1989). CAT activity was measured according to the method of Csiszar et al. (2007) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. APX activity was assayed following the method of Nakano and Asada (1981). GPX activity was measured as described by Elia et al. (2003) using H_2O_2 as a substrate. MDHAR activity was determined by the method of Hossain et al. (2010). DHAR activity was determined by the procedure of Nakano and Asada (1981). POD activity was estimated according to Hemeda and Klein (1990). GR activity was

measured by the method of Hossain et al. (2010). GSTs separation, purification, and polyclonal antibody preparation were done following the method of Rohman et al. (2010).

Extraction and Assay of Non-Enzymic Antioxidant

Maize leaves (0.5 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer (5% meta-phosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at $11,500 \times g$ for 15 min at 4°C, and the supernatant was collected for analysis of AsA and GSH. AsA content was assayed following the method of Huang et al. (2005). The glutathione pool was assayed according to Yu et al. (2003), utilizing 0.40 ml of aliquots of supernatant neutralized with 0.6 ml of 0.5 M K-P buffer (pH 7.0). Oxidized glutathione (GSSG) was determined after removal of GSH by 2-vinylpyridine derivatization. Reduced glutathione (GSH) was found by deduction of GSSG from total glutathione. Specific standard curves with GSH and GSSG were used.

Determination of Protein

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

SDS-PAGE and Western Blotting

SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) was done by the method of Laemmli (1970). The western blot was done following the protocol of Perkin-Elmer Life Science Inc. For protein detection, silver staining was done for detecting as little as 5 mg protein.

Statistical Analysis

All data obtained was analyzed by SAS (Version 9.1) program following complete randomized design (CRD) and the mean differences were compared by least significant (LSD) tests. P values <0.05 were considered to be significant.

Results

Relative water content decreased when seedlings are subjected to PEG induced drought, but it augmented in PA (PEG + Put, PEG + Spd, and PEG + Spm) treated plants by 15%, 17%, and 13%, respectively when compared to drought condition (Figure 1A). Drought decreased Chl*a* content by 34% compared to normal growing environment (Figure 1B), while Put, Spd, and Spm increased Chl*a* content by the level of 27%, 21%, and 23%, respectively over that in drought. Chl*b* content also decreased significantly (about 39%) under PEG treatment (Figure 1C). However, when plants were subjected to Put, Spd, and Spm in PEG application, they reversed the decreasing tendency showing 39%, 34%, and 32%, respective increase of Chl*b* content. Like Chl, Car content decreased (39%) during drought exposure (Figure 1D). PAs (Put, Spd, and Spm) supplementation were associated to induce Car by15%, 11%, and 11%, respectively as compared to drought. In case of total pigment, significant loss was observed in drought stressed seedlings (Figure 1E). Through exogenous PAs application, total pigment was inclined (27% by put, 25% by spd, and 24% by spm). Proline was accumulated in drought stressed seedlings by 34% as compared to control, while Put, Spd, and Spm further inclined the content by 12%, 22% and 17%, respectively (Figure 1F). Formation rate of $O_2^{•-}$ became sharply higher (73.9%) in seedlings subjected to drought while Put, Spd, and Spm treatments decreased the $O_2^{•-}$ content by 33%, 35%, and 27%, respectively (Figure 2A). Drought induced the content of H_2O_2 by 133% over control but Put, Spd, and Spm application decreased H_2O_2 content to a substantial level by 31%, 35%, and 24%, respectively (Figure 2B). Put showed higher effectiveness among the three PAs in decreasing H_2O_2 value through maintaining proper homeostatic balance. Drought augmented MDA contentas 111% higher as compared to that in control (Figure 2C). Spd treatment significantly decreased MDA content by 35% while Put and Spm decreased MDA level by 23% and 14%, respectively. Substantial increase (90%) in LOX activity was observed under drought as compared to control (Figure 2D). Put, Spd, and Spm treatment decreased the LOX activity by 29%, 40% and 36%, respectively. 112% substantial increment in MG content was observed under drought condition (Figure 3A). However, MG content was decreased in presence of Put, Spd, and Spm to a negligible level by 10%, 5% and 6%, respectively when compared to drought stress. Gly-I remained almost similar to those in normal growth condition (Figure 3B). Addition of Put, Spd, and Spm treatment in PEG also failed to increase Gly-I activity. Drought increased the Gly-II activity by 11% which was statistically similar to normal growth condition. Presence of PAs in PEG also failed to increase the activity (Figure 3C).
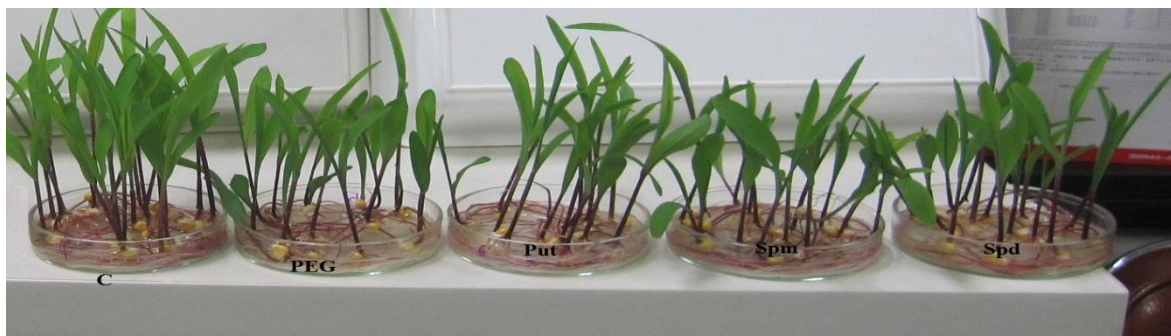

Plate 1 Different treatments: (a) Control, (b) PEG induced drought, (c) Put supplementation, (d) Spm supplementation, and (e) Spd application
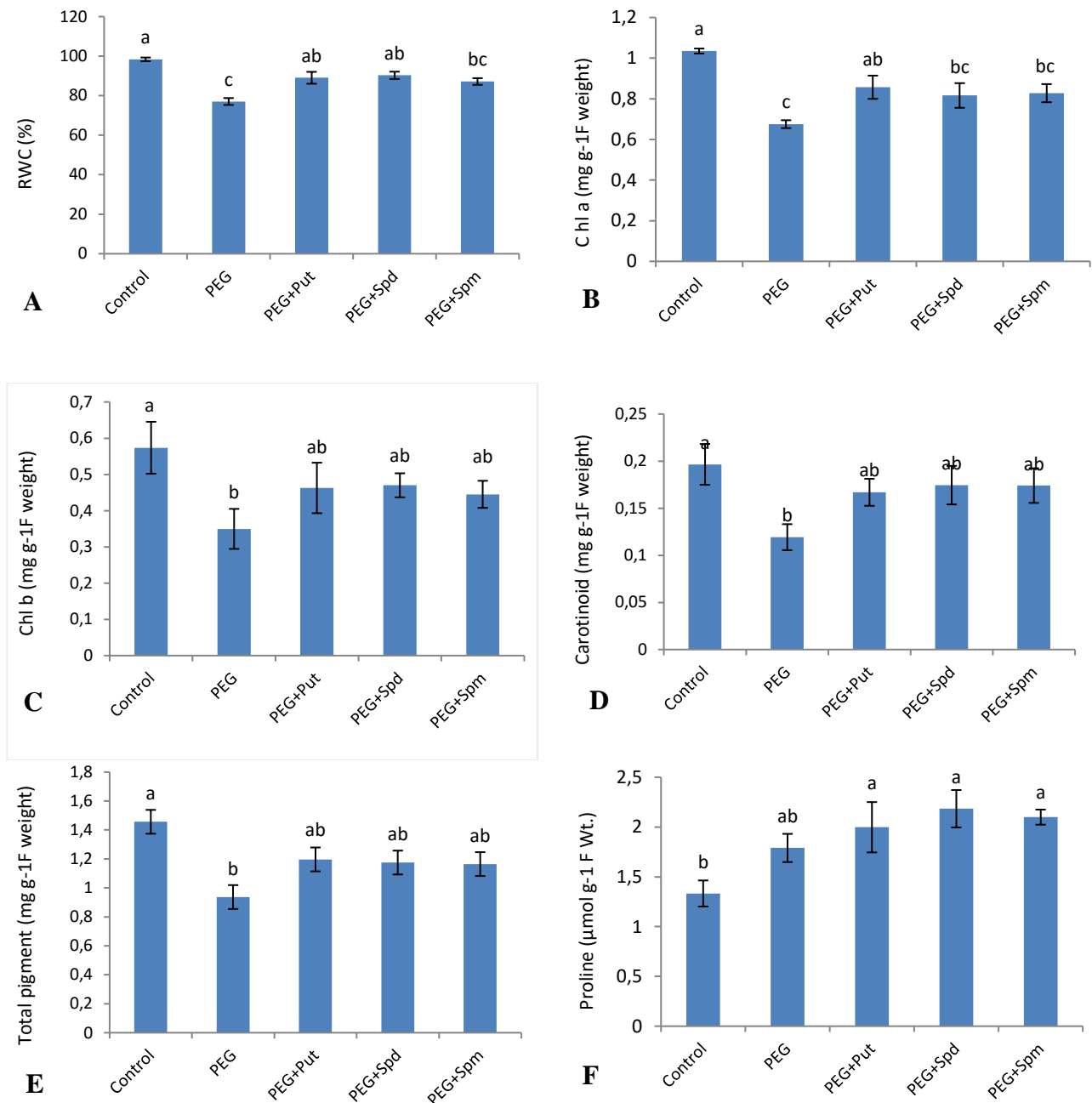
Figure 1 PAs Effect on RWC, Photosynthesis and Proline Content
(A) Relative Water Content, B) Chl*a* content, (C) Chl*b* content, (D) Carotenoid content, (E) Total pigment,
(F) Proline content in maize seedlings under control, PEG, PEG + Put, PEG + Spd, and PEG + Spm treatment

Drought decreased AsA content by 14% (Figure 4A) and simultaneously, 29% increase was found in DHA content (Figure 4B). Put, Spd, and Spm treatment augmented AsA content by 12%, 11% and 10%, respectively. Drought decreased the GSH content by 27% (Figure 4D). On the contrary, GSSG content increased by 63% (Figure 4E). Put, Spd, and Spm maintained higher GSH content by 15%, 12%, and 11%, respectively over the contents under drought. The redox state reported the activity of PAs in reducing the oxidation of GSH (Figure 4B, 4F). SOD activity was increasedby 38% when exposed to PEG (Figure 5A). Put, Spd, and Spm supplementation increased the activity by 12%, 14%, and

4%, respectively. But, treatment effects on the basis of actuarial data were somewhat similar. POD activity increased under drought, but the level was statistically similar to that in control seedlings (Figure 5B). In Put, Spd, and Spm treated seedlings, the activity was higher by 38%, 41%, and 32% respectively, compared to drought. Seedlings under drought showed decreased CAT activity (18%) compared to normal growth condition (Figure 5C). In PA treated seedlings, 22%, 18%, and 27% higher activities were found by Put, Spd, and Spm compared to drought stressed seedlings, respectively. Significant increase in APX activity occurred under water stress over controlled condition (Figure 5D). However, remarkable

changes were not found in the activity treated with PAs. GPX activity increased considerably (144%) in drought stressed seedlings (Figure 5E). PAs treatments (Put and Spd) showed 12% and 17% higher activity than that in drought treated seedlings. 27% GR activity incremented was observed in drought than that in normal ambience (Figure 5F). Put, Spd, and Spm supplementation induced the activity by 19%, 21%, and 10%, respectively. No significant change in MDHAR activity occurred under drought (8% decrease) compared to control (Figure 5G).

In PA treated seedlings, minute increase (15%, 12%, and 7%) was observed compared to drought by Put, Spd, and Spm, respectively. DHAR activity was increased by 68% under drought (Figure 5H). Put, Spd, and Spm lowered the activity by 23%, 30%, and 25%, respectively than drought. In control seedlings, GST activity was 77.6 nmol min^{-1} mg^{-1} protein (Figure 6A). Under drought condition, GST showed 60% increment over controlled condition. However, all of PAs did not show remarkable changes in GST activities.
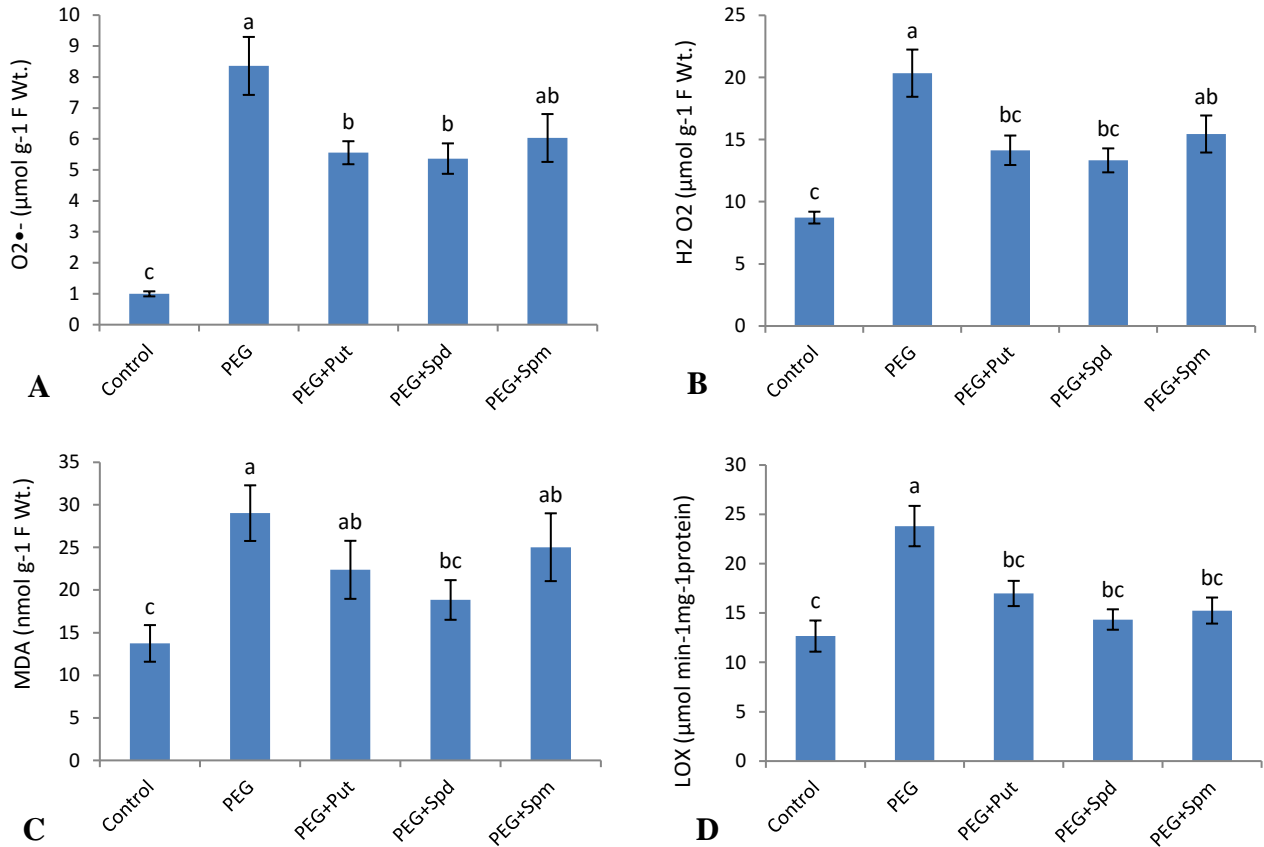

Figure 2 PAs Effect on ROS Activity
(A) Generation rate of $O_2^{\cdot-}$, (B) Content of H_2O_2, (C) Content of MDA, and (D) LOX activity in maize seedlings under PEG induced drought in presence of PAs

Discussion

PAs Effect on RWC, Photosynthesis and Proline Content
Present study observed sharp increase in RWC under drought (Figure 1A). D'Souza et al. (2014) and Rohman et al. (2016) also reported decrease in RWC during drought exposure. Water deficit inhibits root growth leading to decrease in its activity, associated with stomatal closure due to accumulation of abscisic acid in roots and guard cell, which may attribute to shrinking in RWC content (D'Souza et al., 2014). PAs delayed the loss of water under drought (Figure 1A). Similarly, D'Souza et al. (2014) found effective contribution of PAs, implying promising activity of Pas in enriching cytoplasmic Ca^{2+} concentration, which in turn activates the K^+ inward rectifier, thereby, evoke stomatal closure, attenuate transpiration rates and help maintaining turgor through balanced RWC content. Sharp decrease in photosynthetic activities was observed under drought (Figure 1B-E).

Under abiotic stress including drought, photosynthetic apparatus are the major components to be damaged (Gill and Tujeta, 2010). Several researches showed decreased amount of Chl*a*, Chl*b*, Car, and total pigment (Paul and Paneerselvam, 2014; Anjum et al., 2011). Increased activity of different Chl degrading enzymes like chlorophyllase and peroxidase may act as key reason behind the decreased content of Chl (Paul and Paneerselvam, 2014; Abaaszadeh et al., 2007). Besides, under stress, SOD and AsA reduce H_2O_2 through "iron-catalyzed" reaction and induce $OH^{\cdot}$ production in thylakoids, which poses a great threat to Chl (Sharma et al., 2012). Car, which belongs to the lipophilic antioxidants, scavenge $^1O_2$, a cursor of oxidative degradation and evokes Chl molecule to prevent $^1O_2$ synthesis and protect photosynthetic apparatus.
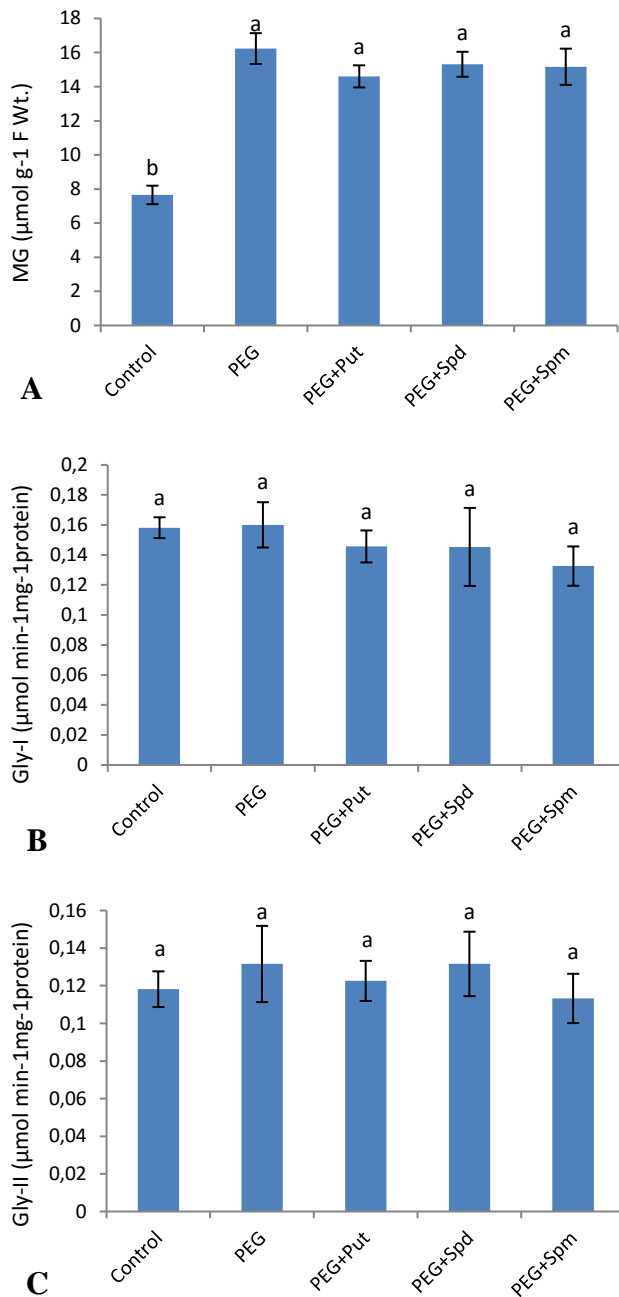
Figure 3 Effect of PAs on Glyoxalase System
(A) MG content, (B) Gly-I activity, and (C) Gly-II activity in maize seedlings under PEG induced drought in presence of Pas

The scavenging capability of Car is due to a chain of isoprene residues which permits easy energy uptake from excited molecules and release of excess energy as heat (Mittler, 2002). This observation having decreased Car content showed consistency with several previous reports (Logini et al., 1999; Agastian et al., 2000) under different stresses as reported in a number of plant species. However, PA application increased Chl (Figure 1B, 1C) and Car content (Figure 1D). Verma and Mishra (2005) also reported increased Car content under Put supplementation. Radhakrishnan and Lee (2014) also reported positive influence of Spd on pigment content in salt stressed cucumber plants. Proline is considered as an important parameter for evaluating stress tolerance capacity of plants (Delauney and Verma, 1993), as,

during drought stress its accumulation serves as the early indicator (Sairam and Tyagi, 2004). Proline acts as an effective osmoprotectant, which can accumulate in a huge amount without insisting any kind of detrimental increase in ionic diffusion effect (Yancey et al., 1982). This experiment showed stiff increase of proline content during scarce water exposure regardless the PA application, which showed strong consistence with previous studies conducted by Parida et al., 2007 and Ramanjulu and Sudhakar, 2000. Increased proline accumulation may be due to the decreased proline dehydrogenase function, a catabolic enzyme of proline (Sundaresan and Sudhakaran, 1995; Lee and Liu, 1999). PAs application increased proline content implying effective PA impact on proline activity in alleviating ROS toxicity. This supposed impact may have a concern with the concept that metabolic pathways involved in proline and PA biosynthesis all require glutamate, arginine and ornithine as precursor (Aziz et al., 1995). Furthermore, taking comparative level of Put metabolism and Proline accumulation under consideration, a precursor-product relationship between Put and proline can be assumed (Aziz et al., 1995). Higher Spd effectiveness in proline synthesis among the three Pas collaborated with the findings of Roy Choudhry et al. (2011) reporting that exogenous Spd strongly incremented reducing sugar and proline level in salinity stress.

PAs Effect on ROS

Present observation reported increased content of ROS ($O_2^{\cdot-}$ and H_2O_2) under stressed condition (Figure 2A, 2B). Reason behind the enhanced production of ROS may be decreased assimilation of CO_2, associated with altered photosynthetic activity via the Mehler reaction (Asada, 1999). PAs supplementation considerably decreased ROS content. The antioxidative effect of PAs is due to their anion and cation binding properties involving a radical scavenging function (Bors et al., 1998) and its ability to enervate both lipid peroxidation (Kitada et al., 1979) and metal catalyzed oxidative reactions (Tadolini, 1988). Supportively, Shen et al. (2000) reported that, Spd pretreatment of cucumber seedlings growing under chilling condition lowered ROS production. MDA, produced from unsaturated fatty acid peroxidation is responsible for cell membrane damage (Halliwell and Gutteridge, 1989). MDA serves as an indicator of membrane damage expressed as increased electrolyte leakage under stressed condition (Tiburcio et al., 1994). Present study showed increment in MDA content under drought (Figure 2C), which is in accordance with the investigation of Saleethong et al., 2011). Present study reported PAs effect to alleviate MDA content significantly (Figure 2C) and consisted with Zhang et al. (1996) and Xie et al. (1999). In this study, Spd seemed to be more effective in lowering MDA content (Figure 2C). Higher effectiveness of Spd in lowering MDA may be due to having highly propionate amino and imino group, which undergo conjugation with different negatively charged molecules like nucleic acids, proteins and phospholipids, and stabilize thylakoid membranes (Ahmad et al., 2012).
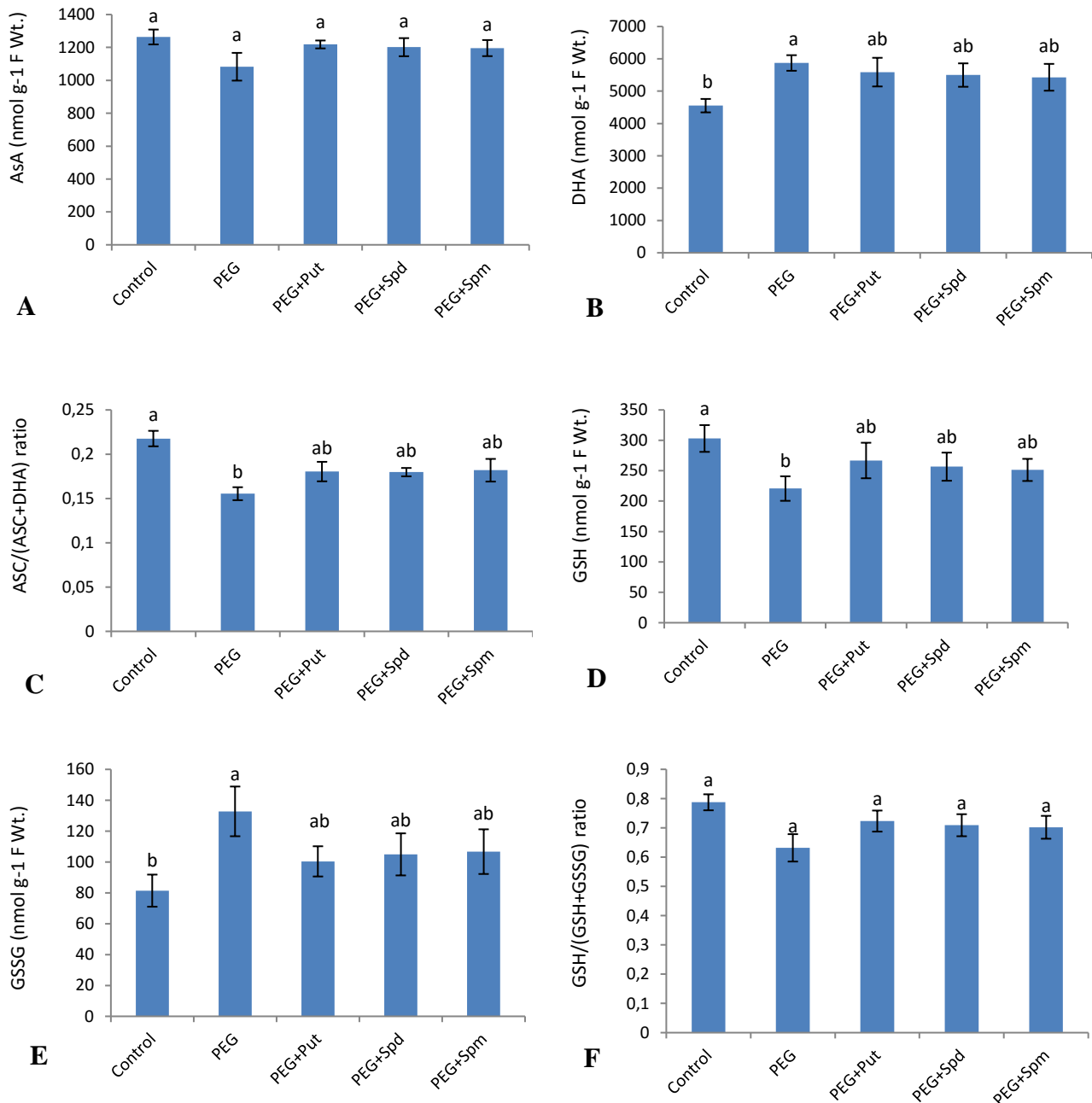
Figure 4 PAs Effect on Non-enzymic antioxidants
(A) Content of AsA, (B) DHA and (C) AsA redox (D) Content of GSH, (E) GSSG and (F) GSH redox in maize seedlings under PEG induced drought in presence of Pas

LOX activity was also correlated with increased MDA content in maize seedlings (Figure 2D). LOX is considered as causal agent to produce MDA which catalyzes the peroxidation of polyunsaturated fatty acids to their corresponding hydrogen peroxides (Doderer et al., 1992). Increased LOX activity was also reported in water stressed barley leaves (Kubis, 2006) and in senescing musk melon tissues (Lester, 2000). Stress induces membrane destabilization, causing increased membrane permeability, which in turn leads to accelerated LOX activation. Present experience showed higher activity of PAs specially Spd providing highest performance among all, in decreasing LOX activity. This observation consisted with the investigation of Chattopadhayay et al. (2002) and Lester (2000), which proposed effectiveness of exogenous application of Spd and Spm protecting cells

against degradation by suppressing protease and RNAse functioning and thus maintaining cell membrane integrity.

PAs Effect on Glyoxalase System

The glyoxalase system, major pathway for cytotoxic MG metabolism includes the activity of two enzymes: glyoxalase 1 (Gly-I) and glyoxalase 2 (Gly-II). These enzymes use GSH as a co-factor to detoxify MG through a two-step reaction (Hossain et al., 2012; Thornalley, 1990). GSH and MG reaction forms hemithioacetal, which is then converted to S-D-lactoylglutahione (SLG) by Gly II and then Gly-I hydrolyzed SLG to D-lactate (Hossain et al., 2012). This coordinated function of Gly-I and Gly-II serves as an activator of stress tolerance mechanism (Hoque et al., 2007; Singla-Pareek et al., 2008).
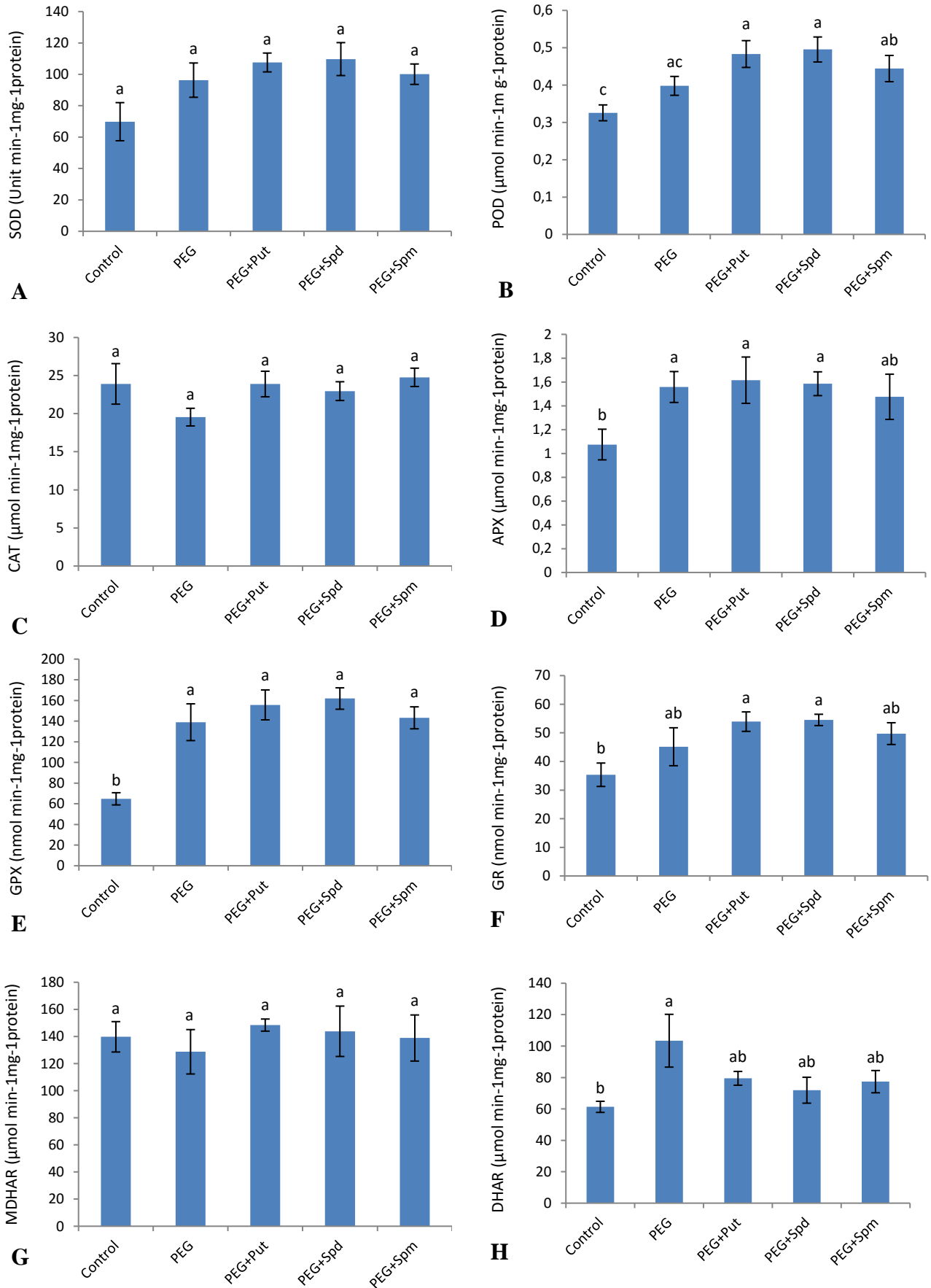
Figure 5 PAs Effect on Enzymic Antioxidants
(A) Activity of SOD, (B) Activity of POD, (C) Activity of CAT, (D) Activity of APX, (E) Activity of GPX, (F) Activity of GR, (G) Activity of MDHAR, and (H) Activity of DHAR in maize seedlings under PEG induced drought in presence of Pas

A

B

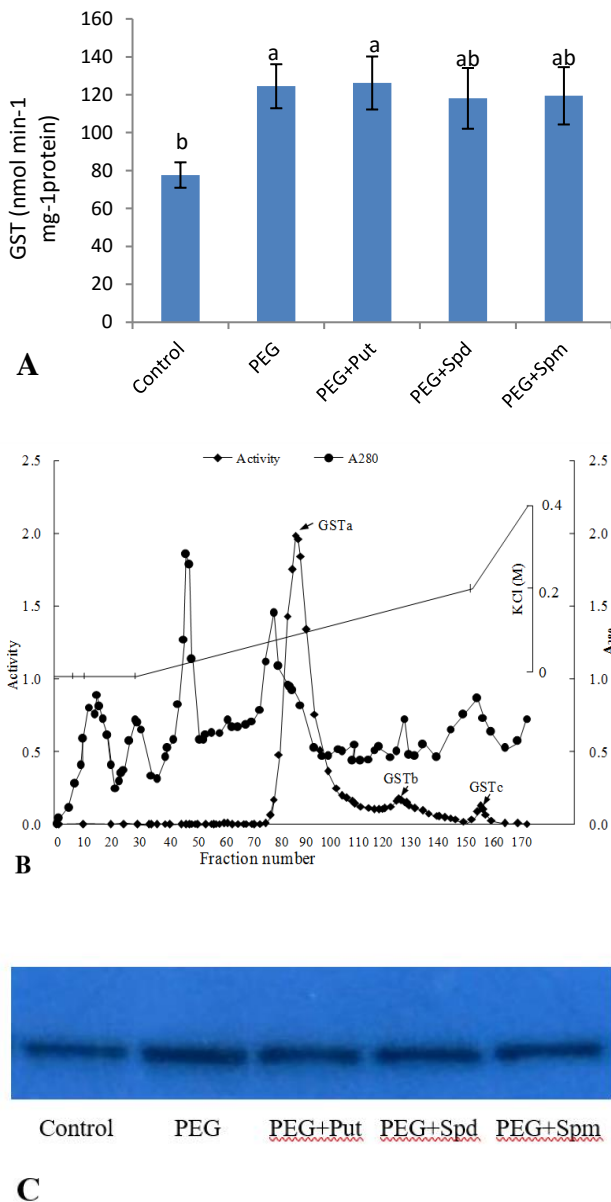Control   PEG   PEG+Put   PEG+Spd   PEG+Spm

C

Figure 6
(A) Activity of GST in maize seedlings under PEG induced drought in presence of Pas, (B) Separation of GST by DEAE column chromatography, and (C) Western blotting of GSTa

Decreasing tendency of Gly-I and Gly-II and increased MG under drought (Figure 3B, 3C) ultimately suggested interaction with MG content under water stress, which showed consistency with many previous studies (Banu et al., 2010; Ghosh et al., 2014). As reasons behind this phenomenon it could be suggested that, increased rates of glycolysis under stress (Kaur et al., 2015) and/or deficiency in GSH limiting the production of hemithioacetal (Hossain et al., 2012) may act. Even PA supplementation did not show notable efficiency in decreasing MG concentration. Nahar et al. (2015) also documented failure of spd to increase Gly-I activity in mungbean significantly under low temperature stress.

PAs Effect on Non-enzymic Antioxidant

Present study observed decrease in AsA level under drought (Figure 4A). Several other studies (Sharma and Dubey, 2007 and Srivastava and Dubey, 2011) also

observed altered AsA level in response to the level and type of environmental stress. The AsA level under environmental stresses depends on the balance between the rates and capacity of AsA biosynthesis and turnover related to antioxidant demand (Chaves et al., 2002). In this study, PAs implied positive influence on AsA concentration over that in drought. In stressed cells, DHA is produced in the second step of AsA oxidation or, from spontaneous mutation of transitory radical, MDHA by NADPH dependent enzyme, MDHAR activity (Asada, 1994). As being a short lived radical, DHA is either hydrolyzed irreversibly to 2, 3-diketogulonic acid or, reduced to AsA through DHAR enzyme catalyzed GSH recycling via the AsA-GSH cycle (Sharma et al., 2012). This study demonstrated inclined content of DHA under PEG induction (Figure 4B). Shalata et al. (2001) and Mittova et al. (2004) also showed consistency to our observation in regard to augmented amount of DHA in stress. However, PAs did not show significant activity in enervating AsA oxidation. GSH is one of the crucial antioxidants, for it protects macromolecules (i.e., proteins, lipids, DNA) either by the forming adducts directly with reactive electrophiles (glutathiolation) or, by donating proton in the presence of ROS or, organic free radicals, yielding GSSG (Asada, 1994). In present observation, GSH activity showed declining trend under drought (Figure 4D). This result showed consistency with (Hefny and Kader, 2009 and Tausz et al., 2004) as found in several crop species. In case of PA impact, GSH content increased. Verma and Mishra (2005) are in agreement with this observation on positive impact of PA (put) on GSH content. Foyer and Halliwell (1976) reported Put to maintain a high GSH/GSSG ratio, essential for the modulation AsA threshold level and activation of different CO_2 fixing enzymes.

PAs Effect on Enzymic Activities

SOD provides the first line protection from cytotoxic $O_2^{\bullet-}$ by converting it into H_2O_2. The H_2O_2 is then converted to H_2O in presence of POD, CAT, APX, and GPX. SOD and POD activities were higher under drought (Figure 6A, 6B). Sharma and Dubey (2005) and Mishra et al. (2011) also reported increased SOD and POD activities under diversified stresses including drought. PAs application increased the SOD and POD activities (Figure 6A, 6B) suggesting the protective role of PAs from oxidative damage. Verma and Mishra (2005) and Sheokand et al. (2008) conferred increased SOD activity due to Put protecting the biomolecules from $O_2^{\bullet-}$ attack. Besides, put could bind different antioxidant enzymes like SOD producing a covalent Put-SOD complex, which was 20 fold more membrane permeable than SOD alone and facilitate oxygenic protection in mammalian cells (Poduslo and Curran, 1996; Wengenack et al., 1997). However, in this study, Spd seemed to be more effective in SOD activity augmentation. Supportively, Chang et al. (2010) reported increased SOD activity for Spd exposure in cucumber seedling under salinity stress suggesting Spd to be mostly associated with stress tolerance among three major PAs (Put, Spd, and Spm). Higher POD activity in presence of PAs was in agreement with Moussa and Aziz (2008) and Wang et al. (2012). Verma and Mishra (2005)

observed upsurge in POD activity with Put under high salinity. CAT could be the principal enzymic H_2O_2 scavenger in plants under abiotic stresses including drought, where the cellular H_2O_2 level becomes several fold higher than in plants grown under normal conditions (Nor'aini et al., 1997; Cheeseman, 2006). Present observation showed decreased CAT activity under drought induction (Figure 5C). Several previous studies (Corpas et al., 1993; Sharma and Dubey, 2005) reported decreased CAT activity in diversified stress condition as shown in different crop species. Declined CAT activity indicates that, under prospective state, CAT may not act as an effective H_2O_2 scavenger (Sharma and Dubey, 2005). Besides, this enzyme, although, has so a fast turnover rate but poses a relatively poor affinity for H_2O_2 and in light it undergoes photo-inactivation followed by degradation (Hertwig et al., 1992). However, in presence of PAs, CAT activity inclined sharply (Figure 5C). Similarly, Shi et al. (2013) also observed that, after salt and drought treatments, PAs treated seedlings exhibited significantly augmented CAT activity than those of non-treated seedlings. Verma and Mishra (2005) also reported increased CAT activity due to Put treatment. Besides, APX and GPX along with MDHAR and DHAR are important antioxidants in ascorbate-glutathione cycle in maintaining H_2O_2 as well as GSH and AsA (Apel and Hirt, 2004). Under drought, 50% higher APX activity along with AsA oxidation (Figure 5D, 4A) suggested that AsA has participated in the degradation of H_2O_2 catalyzed by APX. Many researchers (Srarma and Dubey, 2005 and Han et al., 2009) also documented increased APX activity in different stress conditions. However, remarkable alteration in APX activity was not found under PA supplementation (Figure 5D). In GSH-dependent metabolism, H_2O_2 is metabolized by GPX where GSH is converted to GSSG. In this study, higher GPX activity under drought (Figure 5E) indicated a role of this enzyme in H_2O_2 metabolism using GSH (Figure 4D). Increased GPX activity in wheat under drought condition has been reported by Sayfzadeh and Rashidi (2010) while involvement of two GPX genes in H_2O_2 tolerance was reported by Zhai et al. (2013). GPX induction by PAs (Figure 5E) Figureured PAs role in H_2O_2 scavenging. GR, an NADPH-dependent enzyme, acts as catalyst for the reduction of GSSG to GSH and thus maintains high cellular GSH. In this study, GR activity increased under drought (Figure 5F) implying strong impact of GR on maintaining of GSH. Several studies also reported increased GR activity under stressed condition in different plant species (Sharma and Dubey, 2005; Maheshwari and Dubey, 2009). Present study showed induced GR activity in PAs application (Figure 5F). Verma and Mishra (2005) reported Put induced GR activity with increased ratio of NADP+ and NADPH and thus ensuring the availability of NADP+ to accept electron resulting into lower flow of electron to O_2 for ROS generation. Nahar et al. (2015) also reported higher GR activity in presence of Spd. Enzyme like MDHAR and DHAR have important role in in maintaining AsA contents in cell. In this study, the negative changes in MDHAR under drought (Figure 5G)

suggested the possibility of maintaining AsA in maize seedlings, essential for maintenance of a balanced redox status or inactivation of MDHAR activity due to scarce NADPH (Hossain et al., 2011). This result consisted with findings reported by many workers such as Hasanuzzaman et al. (2012) and Fan et al. (2014) as conducted in many stressful conditions. However, MDHAR content increased under PAs treatment, particularly under Put supplementation. Spd effect was also appreciable which is in agreement with the investigation of Li et al. (2015), who reported increased tomato tolerance for increase in MDHAR activity in salinity- alkalinity stressed Spd supplementation compared to that in salinity- alkalinity stress. DHAR, a monomeric thiol enzyme, catalyzes the reduction of DHA to AsA using the electron provided by GSH, a reducing substrate and thus plays a key role in recycling AsA (Ushimaru et al., 1997). In this study, DHAR activity increased remarkably under drought (Figure 5H). These findings suggested that, DHAR has played a significant role in reducing oxidized DHA to AsA by oxidizing GSH to GSSG. Sharma and Dubey (2005) and Boo and Jung (1999) also found increase in DHAR content in response to level of dose as tested for cadmium tolerance. However, respect to PA effect, DHAR content decreased significantly in compared to stress (Figure 5H). Lower DHAR activity in presence of PAs is not clear. GST uses GSH for participating in detoxification of ROS, xenobiotics, and membrane lipid peroxidation. Maize GST was reported to have three component GST (Rohman et al., 2016), among which GST (GSTa) contained more than 90% of total activity (Figure 14C). The antibody of GSTa was used to examine the accumulation of the protein by Western blotting. This finding also confirmed the accumulation of GSTa in PEG induction (Figure 14A) and it seemed not to have positive effect of PAs in accumulation of the GST. Several other studies (Jogeswar et al., 2006; George et al., 2010) reported increased activity of GST in many stressful conditions, which might be due to regulation of flavonoid (Rohman et al., 2010).

Conclusion

Respect to our present study some conclusions are drawn, 1) Strong correlation between polyamine and stress concerned parameters has been evidenced, 2) Specially, Putrescine and Spermidine has been observed to be highly effective in mitigating stress severity, and 3) Though, in most of the cases, strong effectiveness of polyamine has been demonstrated, they have yet showed inconsistency with expected result.

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