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ORIGINAL ARTICLE

Insights into spermine-induced combined high temperature and drought tolerance in mung bean: osmoregulation and roles of antioxidant and glyoxalase system

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Abstract High temperature and drought stress often occur simultaneously, and due to global climate change, this kind of phenomenon occurs more frequently and severely, which exerts devastating effects on plants. Polyamines (PAs) play crucial roles in conferring abiotic stress tolerance in plants. Present study investigated how exogenous pretreatment of spermine (Spm, 0.2 mM) enhances mung bean (*Vigna radiata* L. cv. BARI Mung-2) seedlings tolerance to high temperature (HT, 40 °C) and drought [induced by 5 % polyethyleneglycol (PEG)] stress individually and in combination. Spm pretreatment reduced reactive oxygen species (ROS) production including H_2O_2 and $O_2^{\bullet-}$, lipoxygenase (LOX) activity, and membrane lipid peroxidation (indicated by malondialdehyde, MDA) under HT and/or drought stress. Histochemical staining of leaves with diaminobenzidine and nitro blue tetrazolium chloride also confirmed that Spm-pretreated seedlings accumulated less H_2O_2 and $O_2^{\bullet-}$ under HT and/or drought stress. Spermine pretreatment maintained the ascorbate (AsA) and glutathione (GSH) levels high, and upregulated the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) which were vital for imparting ROS-induced oxidative stress tolerance under HT and/or drought stress. The cytotoxic compound methylglyoxal (MG) was overproduced due to HT and/or drought, but exogenous Spm pretreatment reduced MG toxicity enhancing the glyoxalase system. Spermine pretreatment modulated endogenous PA levels. Osmoregulation and restoration of plant water status were other major contributions of Spm under HT and/or drought stress. Preventing photosynthetic pigments and improving seedling growth parameters, Spm further confirmed its influential roles in HT and/or drought tolerance.

Keywords Abiotic stress · Antioxidant system · Global climate change · Methylglyoxal · Osmoregulation · Plant growth regulator

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Abbreviations

AO	Ascorbate oxidase
APX	Ascorbate peroxidase
AsA	Ascorbic acid (ascorbate)
BSA	Bovine serum albumin
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase

DTNB	5,5′-dithio-bis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
Gly	Glyoxalase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GPX	Glutathione peroxidase
GST	Glutathione *S*-transferase
LOX	Lipoxygenase
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
MG	Methylglyoxal
NADPH	Nicotinamide adenine dinucleotide phosphate
NTB	2-nitro-5-thiobenzoic acid
SLG	*S*-D-lactoylglutathione
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

Introduction

Plants growing under field condition are exposed to diversified environmental stresses. Most of the time, plants are exposed to multiple stresses under field condition which adversely affect plant physiological processes. Increased temperature is in the center of concern among different components of environment. It has been predicted that average world temperature will increase 2.23–6.63 °C by the year 2100 (EPA 2011). Extreme high temperature (HT) causing cellular death within minutes and moderate HT may cause cell injury or death with long-term exposure. Increased temperature may change plant adaptation or may cause species extinction (Howarth 2005). Drought stress is also one of the most complex and devastating threats having multiple damaging effects. Drought damage within the plants begins primarily with disruption of osmotic balance, then perpetuate to metabolic and physiological disorders (Hasanuzzaman and Fujita 2011). High temperature and drought stress often occur simultaneously which exert severe devastating effects on plants, compared to single HT or drought stress. The combination of drought and HT stress modifies physiological and molecular processes such as photosynthesis, accumulation of lipids, and transcript expression (Rizhsky et al. 2002, 2004).

High temperature and drought are also accompanied by an increased production of reactive oxygen species (ROS) like other stress condition. Reactive oxygen species (such as singlet oxygen, $^1O_2$; superoxide, $O_2^{\bullet-}$; hydrogen peroxide, H_2O_2; and hydroxyl radical, $OH^{\bullet}$) damage biomolecules and biomembrane (Miller et al. 2008; Choudhury et al. 2013). Peroxidation of lipids and oxidation of amino acids, protein carbonylation, are the results of ROS-induced primary stress and damage effects which then reduce and collapse cellular structural integrity, physiological and biochemical processes. Reactive oxygen species can cause substantial cellular damage, oxidation of nucleic acids, and even programmed cell death (Moller et al. 2007; Gill and Tuteja 2010). Plants prevent ROS production and oxidative stress by its antioxidant defense system in a complex manner involving multiple enzymes and antioxidants. The chief non-enzymatic antioxidant components are ascorbic acid (AsA), glutathione (GSH), phenolic compounds, alkaloids, non-protein amino acids, and α-tocopherol. Antioxidant enzymes may include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione *S*-transferase (GST) (Gill and Tuteja 2010). Methylglyoxal (MG) is a toxic compound that develops through the glycolysis pathway and through some other biochemical reactions. The production of MG is limited under normal growing condition. But, MG can be overproduced many folds due to adverse effects of different abiotic stresses. Methylglyoxal, due to its cytotoxic nature, can also cause oxidative damage to a cell and its ultrastructural components. Methylglyoxal can cause DNA damage and mutation (Yadav et al. 2008; Wang et al. 2009). Methylglyoxal detoxification system which is also known as glyoxalase system proficiently detoxifies overproduced MG by the activity of glyoxalase I (Gly I) and glyoxalase II (Gly II) using GSH as a substrate (Yadav et al. 2008; Hasanuzzaman and Fujita 2011).

Polyamines (PAs) [diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm) being the most common PAs] are low-molecular-weight aliphatic polycations ubiquitously distributed in all living organisms. Polyamines have cationic nature which allows them to interact with various macromolecules including nucleic acids, proteins, and phospholipids (Tun et al. 2006). Polyamine is often regarded as a class of growth hormone. Interacting with other hormones, signaling molecules, PAs regulate physiological and developmental processes and events (Tun et al. 2006; Arasimowicz-Jelonek et al. 2009). Diverge nature of PAs in response to environmental stress tolerance has been proved (Arasimowicz-Jelonek et al. 2009; Wang et al. 2013; Li et al. 2014).

The present study was attempted to elucidate ROS formation and antioxidant response to alleviate ROS-induced oxidative stress, MG detoxification system in mung bean which have been grown under HT, drought, and under the combined HT and drought stress. We also investigate the roles of exogenously applied Spm in alleviating those stress effects under HT and/or drought stress, because previous studies have investigated the role of polyamines in plants affected by a single type of stress. Protective functions of PAs in presence of multiple and simultaneous stresses are rare. To determine the overall tolerance response, we observed the growth and physiological attributes of mung bean seedlings under HT and/or drought stress.

Materials and methods

Plant materials and stress treatments

Healthy and uniform seeds of mung bean (*Vigna radiata* L. cv. BARI Mung-2) were immersed into 70 % ethanol for 5 min and then washed thoroughly with distilled water. Seeds were sown in Petri dish containing six layers of blotting paper with 10 mL of distilled water and placed in the dark germinator for 3 days. Germinated seedlings were then grown in new Petri dishes under controlled conditions (light, 350 μmol photon m^{-2} s^{-1}; temperature, 25 ± 2 °C; and relative humidity, 65–70 %), where 10,000-fold diluted Hyponex solution (Hyponex, Japan) was applied as nutrient. Before starting the main experiment, we have conducted several trial experiments where different concentrations of Spm were used against HT and/or drought stress. Based on preliminary test, 0.2 mM Spm was selected among different concentrations of Spm as using that concentration we got the best result considering the protective effect against HT and/or drought stress. A set of 5-day-old seedlings were pretreated with 0.2 mM Spm and grown for 24 h. These pretreated seedlings and another set of six-day-old seedlings were either grown without stress or exposed to HT (40 °C) and drought [induced by 5 % polyethyleneglycol 6000 (PEG)] stress both individually and in combination. Control seedlings were grown only with Hyponex solution. Another set of seedlings were grown with Spm without any stress. The performance of seedlings was observed and data were recorded after 48 h. The experiment was repeated three times under the same condition.

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

The H_2O_2 and $O_2^{\bullet-}$ were localized histochemically (Chen et al. 2010) by staining leaves with 0.1 % 3,3-diaminobenzidine (DAB) and 0.1 % nitroblue tetrazolium chloride (NBT) solution, respectively. After that, leaves were blanched in boiling ethanol to see the spots.

Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) was assayed according to Yu et al. (2003) by extracting leaves in potassium phosphate buffer (pH 6.5; centrifugation at 11,500×*g*), then adding it to a mixture of $TiCl_4$ in 20 % H_2SO_4 (*v*/*v*), and the resulting solution was measured spectrophotometrically at 410 nm.

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

The rate of $O_2^{\bullet-}$ generation was determined following Yang et al. (2011) with some modifications. Fresh leaves were homogenized in 65 mM potassium phosphate buffer solution (pH 7.8) and centrifuged at 5000×*g* for 10 min. Supernatant was mixed with extraction buffer and 10 mM hydroxylamine hydrochloride for incubation at 25 °C for 20 min. The mixture was again mixed with 17 mM sulfanilamide and 7 mM naphthylamine, which were again incubated at 25 °C for 20 min. The absorbance was measured at 530 nm (Elstner and Heupel 1976).

Lipid peroxidation

The level of lipid peroxidation was measured by estimating malondialdehyde (MDA, a product of lipid peroxidation) using thiobarbituric acid (TBA) (Heath and Packer 1968; Hasanuzzaman et al. 2011).

Extraction and measurement of ascorbate and glutathione

Leaves (0.5 g) were homogenized in 5 % meta-phosphoric acid containing 1 mM ethylenediaminetetraacetic acid (EDTA; centrifuged at 11,500×*g*) for 15 min at 4 °C, and the supernatant was collected for analysis of AsA and GSH. Ascorbate content was determined following the method of Huang et al. (2005) with some modifications (Hasanuzzaman et al. 2011). To determine total ascorbate, the oxidized fraction was reduced by adding 0.1 M dithiothreitol for 1 h at room temperature and then read at 265 nm using 1.0 u ascorbate oxidase (AO). Oxidized ascorbate (dehydroascorbate (DHA)) content was determined by subtracting reduced AsA from total AsA. The GSH pool was assayed according to previously described methods (Yu et al. 2003) with modifications as described by Paradiso et al. (2008) and Hasanuzzaman et al. (2011).

Protein determination

The protein concentration of each sample was determined following the method of Bradford (1976) using Bovine Serum Albumin (BSA) as a protein standard.

Enzyme extraction and assays

Leaves were homogenized with 50 mM K-P (potassium phosphate) buffer (pH 7.0) containing 100 mM KCl, 1 mM AsA, 5 mM β-mercaptoethanol, and 10 % (*w*/*v*) glycerol and centrifuged at 11,500×*g*. Supernatants were used for enzyme activity assay.

SOD (EC 1.15.1.1) activity (El-Shabrawi et al. 2010). It was assayed using a xanthine-xanthine oxidase system; K-P buffer (50 mM), NBT (2.24 mM), catalase (0.1 u), xanthine oxidase (0.1 u), xanthine (2.36 mM, pH 7.0), and enzyme extract were used in a reaction mixture. Change in absorbance was read at 560 nm.

CAT (EC 1.11.1.6) activity (Hasanuzzaman et al. 2011). Decrease in absorbance (by decomposition of H_2O_2) was recorded at 240 nm (activity was calculated using an extinction coefficient of 39.4 M^{-1} cm^{-1}).

APX (EC 1.11.1.11) activity (Nakano and Asada 1981). The reaction buffer solution contained 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract. The reaction was started by adding H_2O_2. Activity was measured at 290 nm (using an extinction coefficient of 2.8 mM^{-1} cm^{-1}).

MDHAR (EC 1.6.5.4) activity (Hossain et al. 1984). The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 2.5 mM AsA, 0.5 u of AO, and enzyme solution. Absorbance was taken at 340 nm (using an extinction coefficient of 6.2 mM^{-1} cm^{-1}).

DHAR (EC 1.8.5.1) activity (Nakano and Asada 1981). The reaction buffer contained 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. Activity was calculated from the change in absorbance at 265 nm (using an extinction coefficient of 14 mM^{-1} cm^{-1}).

GR (EC 1.6.4.2) activity (Hasanuzzaman et al. 2011). The reaction mixture contained 0.1 M K-P buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM oxidized glutathione (GSSG), 0.2 mM NADPH, and enzyme solution. Decrease in absorbance was recorded at 340 nm (activity was calculated using an extinction coefficient of 6.2 mM^{-1} cm^{-1}).

GST (EC 2.5.1.18) activity (Hossain et al. 2006). The reaction mixture contained 100 mM Tris–HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and enzyme solution. Increase in absorbance was measured at 340 nm (activity was calculated using an extinction coefficient of 9.6 mM^{-1} cm^{-1}).

GPX (EC 1.11.1.9) activity (Elia et al. 2003; Hasanuzzaman et al. 2011). The reaction mixture consisted of 100 mM K-P buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 0.12 mM NADPH, 2 mM GSH, 1 u GR, 0.6 mM H_2O_2 (as a substrate), and 20 μL of sample solution. The oxidation of NADPH was recorded at 340 nm (activity was calculated using an extinction coefficient of 6.62 mM^{-1} cm^{-1}).

Glyoxalase I (EC 4.4.1.5) (Hasanuzzaman et al. 2011). 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. Increase in absorbance was recorded at 240 nm (activity was calculated using an extinction coefficient of 3.37 mM^{-1} cm^{-1}).

Glyoxalase II (EC 3.1.2.6) (Principato et al. 1987). Formation of GSH at 412 nm was monitored for 1 min. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.2), 0.2 mM DTNB, and 1 mM *S*-D-lactoylglutathione (SLG; activity was calculated using an extinction coefficient of 13.6 mM^{-1} cm^{-1}).

LOX (EC 1.13.11.12) activity (Doderer et al. 1992). Absorbance was recorded at 234 nm using linoleic acid as a substrate. The activity was calculated using an extinction coefficient of 25 mM^{-1} cm^{-1} and expressed as units (1 nmol of substrate oxidized per minute) mg^{-1} protein.

Methylglyoxal level

Leaves were homogenized in 5 % perchloric acid (PCA) and centrifuged at 11,000×*g*. The supernatant was decolorized and neutralized with charcoal and saturated solution of potassium carbonate. The supernatant was used for MG estimation by adding sodium dihydrogen phosphate and *N*-acetyl-L-cysteine. Formation of the product *N*-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl) cysteine was recorded after 10 min at a wavelength of 288 nm according to Wild et al. (2012).

Measurement of free polyamine content

Leaf tissue (0.1 g) was homogenized in 1 mL of 5 % (*v*/*v*) cold PCA. The homogenates were kept at 2 °C for 2 h and centrifuged at 15,000×*g* for 20 min. The supernatant was collected and stored at 2 °C. Aliquots (200 μL) of supernatant was mixed 1:1 (*v*/*v*) with 12 N HCl and hydrolyzed for 16 h at 110 °C in flame-sealed ampoules. The hydrolyzed products were centrifuged at 3000×*g* to remove carbonized material and then evaporated at 70 °C. The dried pellet was redissolved in 5 % PCA. The non-hydrolyzed PCA supernatant containing free PAs was subjected to benzoylation in alkaline medium. The benzoyl-PAs were extracted with diethyl ether and then evaporated to dryness in a water bath. The benzoyl-PAs were redissolved in methanol, and free PAs were analyzed by HPLC (at 254 nm) (Kotzabasis 1993).

Proline content

For assessing proline (Pro) content, leaves were homogenized in 3 % sulfosalicyclic acid and centrifuged at 11,500×*g*. Supernatant was mixed with acid ninhydrin, glacial acetic acid, and phosphoric acid. After incubating the mixture at 100 °C for 1 h and cooling, toluene was added; after several minutes, chromophore containing toluene was read spectrophotometrically at 520 nm (Bates et al. 1973).

Measurement of plant water status

Relative water content (RWC) of leaves and whole plant was measured according to Barrs and Weatherly (1962). Fresh weight (FW), turgid weight (TW), and dry weight (DW) of leaves and seedlings were measured, and RWC was calculated using the following formula:

$$\mathrm{RWC}(\%) = [(\mathrm{FW}-\mathrm{DW})/(\mathrm{TW}-\mathrm{DW})] \times 100$$

According to the methods of Sangakkara et al. (1996), the following parameters were measured:

Water saturation deficit (WSD) = (100 − RWC)
Water retention capacity (WRC) = TW/DW
Water uptake capacity (WUC) = (TW − FW)/DW

Chlorophyll and carotenoid contents

Leaf supernatant was extracted with 80 % *v*/*v* acetone (centrifugation at 5000×*g*); absorbances were taken with a UV-visible spectrophotometer at 663, 645, and 470 nm for chlorophyll (chl) *a*, chl *b*, and carotenoid (Car), respectively, and were calculated according to Arnon (1949).

Determination of growth parameters

Plant height and root length were measured and expressed as centimeter (cm). Seedlings were dried at 80 °C (for 48 h) which was considered as DW and expressed as gram (g). Leaf area was determined and expressed in square centimeter (cm^2).

Statistical analysis

All obtained data were subjected to analysis of variance (ANOVA), and the mean differences were compared by Fisher's least significant difference (LSD) test using XLSTAT v. 2015.1.01 software (Addinsoft 2015). Differences at $P \leq 0.05$ were considered significant.

Results

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

The leaves of mung bean were subjected to DAB staining to visualize the brown patch of H_2O_2, whereas dark blue spots of $O_2^{\bullet-}$ were visualized by NBT staining. Increase of brown patch and dark blue spot in leaves of HT and drought affected seedlings or in combined stress treatment indicate accumulation of H_2O_2 and $O_2^{\bullet-}$, respectively. Seedlings pretreated with Spm showed reduction of those patches and spots which indicates reduction of H_2O_2 and $O_2^{\bullet-}$ accumulation (Fig. 1a, b).

Reactive oxygen species, LOX activity, and membrane integrity

Like other stresses, HT and/or drought stress enhanced ROS generation; free radicals and non-radical compounds such as $O_2^{\bullet-}$ (expressed as $O_2^{\bullet-}$ generation rate) and H_2O_2 level rose extremely under those stresses (Fig. 2a, b). The activity of oxidative enzyme, LOX, increased under those stress conditions (Fig. 2c). Consequently, lipid peroxidation (indicated by MDA) level increased (Fig. 2d). Combined stresses resulted in higher oxidative stress, compared to individual. The increase of H_2O_2 level, $O_2^{\bullet-}$ generation rate, LOX activity, and MDA contents under combined HT and drought stress were 122, 146, 108, and 120 %, respectively, in contrast to control. Spermine pretreatment reduced $O_2^{\bullet-}$ generation rate, H_2O_2 level, LOX activity, and MDA content in mung bean seedlings under HT stress, compared to stress treatments without Spm (Fig. 2a–d).

Antioxidant defense against oxidative stress

AsA and GSH homeostasis and their redox status

High temperature and/or drought stress decreased the AsA content and increased DHA content which decreased the ratio of AsA/DHA. The ratio of AsA/DHA decreased by 64, 54, and 76 % due to HT, drought, and HT + drought stress, compared to control. Spermine pretreatment decreased DHA content, increased AsA content, and the ratio of AsA/DHA in HT and/or drought affected seedlings (compared to HT and/or drought stress only; Fig. 3a–c). High temperature and/or drought did not affect GSH pool, in contrast to control treatment. But, the GSSG pool increased highly under HT and/or drought stresses (compared to control). As a result, the ratio of GSH/GSSG decreased. Mung bean seedlings pretreated with Spm showed 84, 56, and 96 % higher values for GSH/GSSG ratio under HT, drought, and combined HT and drought stress, respectively (compared to stress-affected seedlings without Spm; Fig. 3d–f).

Antioxidant enzymes

The activity of SOD was not altered when drought stress was applied alone, but increased slightly under HT stress and combined HT and drought stress, in contrast to control. Spermine pretreatment increased SOD activity significantly by 23, 28, and 20 %, respectively, in HT, drought, and in combined HT and drought stress treatment (compared to stress treatments without Spm pretreatment; Fig. 4a). High temperature and/or drought stress decreased the activity of CAT than the control. Increased CAT activity were 22, 13, and 32 % in Spm-pretreated mung bean seedlings affected by HT, drought, and combined HT and drought stress (compared to stress treatments without Spm pretreatment; Fig. 4b). GPX activity increased under all stresses over the control treatment. In contrast to stress treatments, GPX activity increased further in Spm-pretreated HT, drought, and combined HT and drought treatment by 20, 35, and 34 %, respectively (Fig. 4c). The activity of GST increased under stress conditions of all kinds of stress application over control. Spermine pretreatment

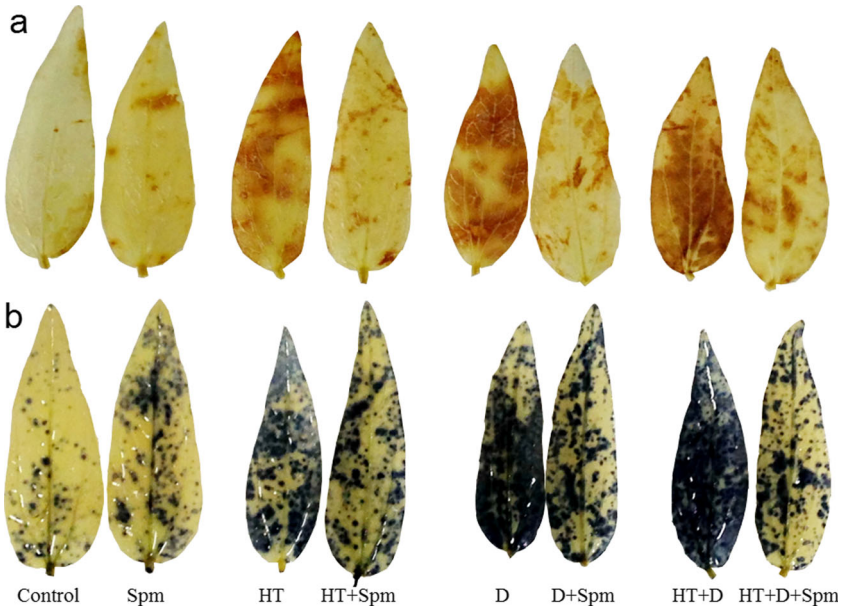

Fig. 1 Histochemical localization of H_2O_2 through DAB staining (**a**) and $O_2^{\bullet-}$ through NBT staining (**b**) in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (D, 5 % PEG), and combined HT and drought stress

failed to increase its activity in stress-affected seedlings (Fig. 4d).

Activity of APX increased by 42, 20, and 31 % in mung bean seedlings subjected to HT, drought, and combined HT and drought stress, respectively, compared to control. Spermine supplementation did not affect the APX activity under stress condition (Fig. 5a). High temperature, drought, and combined HT and drought stress reduced the MDHAR

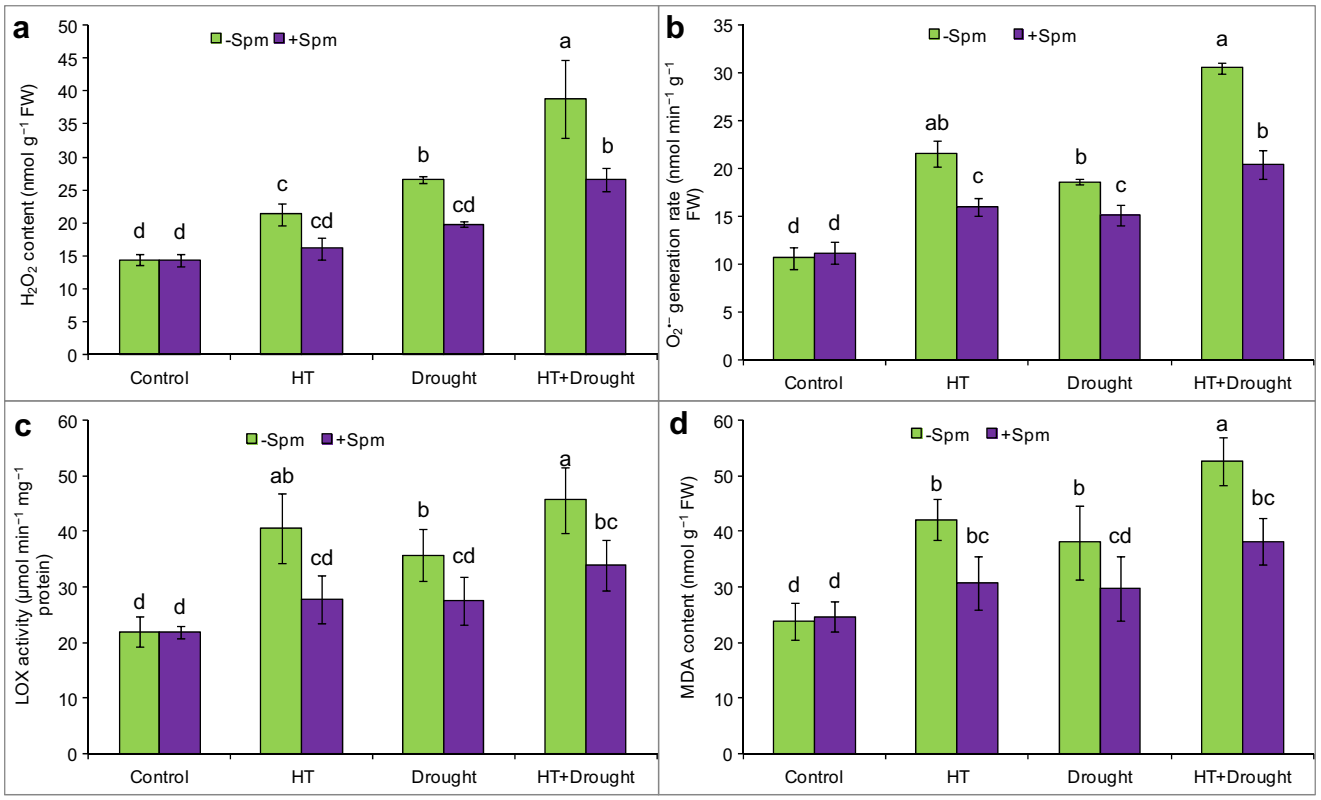

Fig. 2 H_2O_2 content (**a**), $O_2^{\bullet-}$ generation rate (**b**), LOX activity (**c**), and MDA (malondialdehyde) content (**d**) in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (5 % PEG), and combined HT and drought stress. Mean (±SD) was calculated from three replicates for each treatment. *Bars with different letters* are significantly different at $P \leq 0.05$ applying Fisher's LSD test

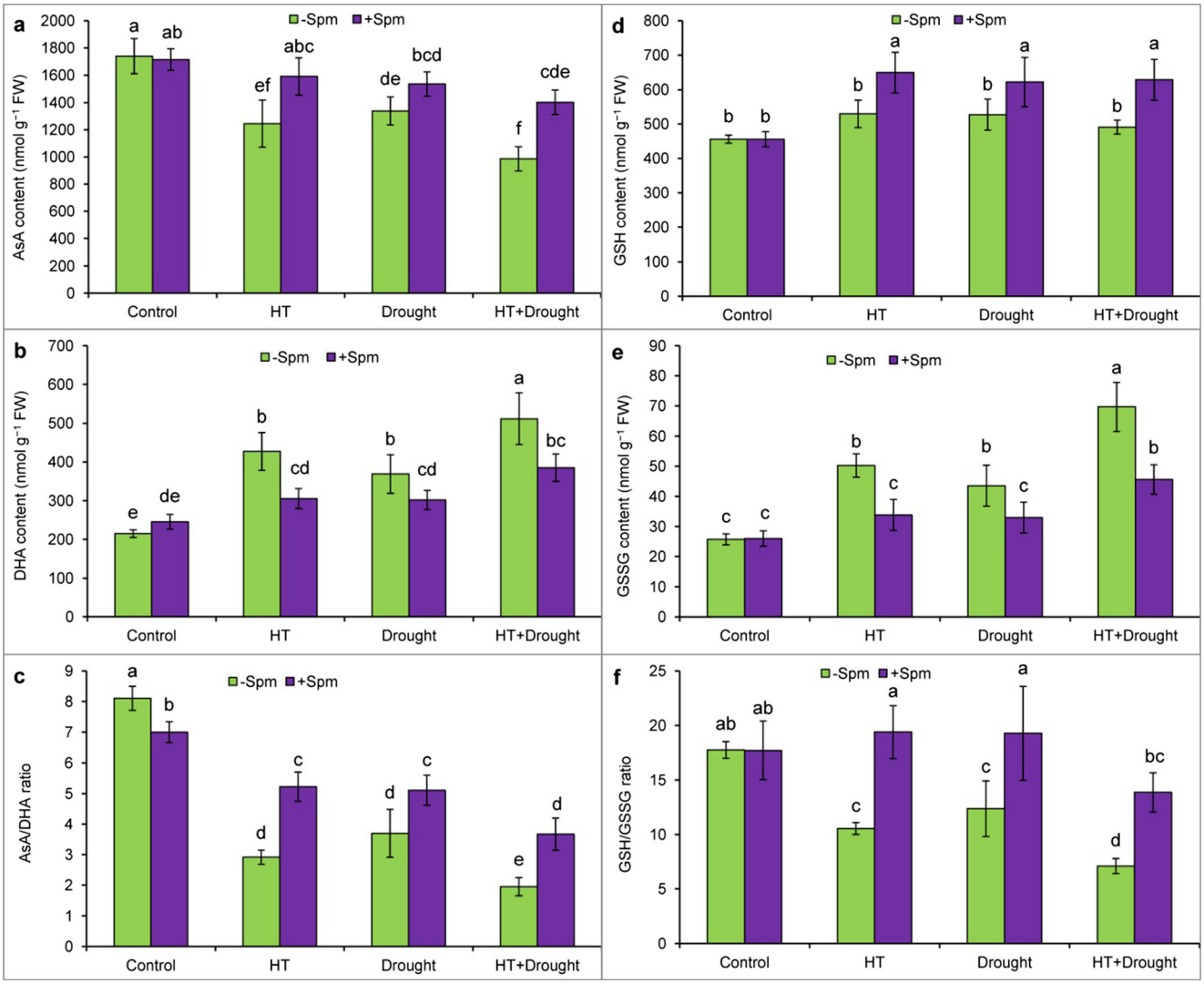

Fig. 3 AsA (**a**) and DHA (**b**) contents, AsA/DHA ratio (**c**), GSH (**d**) and GSSG (**e**) contents, and GSH/GSSG ratio (**f**) in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (5 % PEG), and combined HT and drought stress. Mean (±SD) was calculated from three replicates for each treatment. *Bars with different letters* are significantly different at $P \leq 0.05$ applying Fisher's LSD test

activity by 17, 26, and 22 %, respectively, in contrast to the control treatment. There was no significant change in MDHAR activity due to Spm application in stress-affected mung bean seedlings (Fig. 5b). The activity of DHAR decreased due to HT and/or drought stress. On the other hand, in contrast to stress treatments without any Spm, the exogenous Spm pretreatment with HT, drought, and combined HT and drought increased DHAR activity by 23, 19, and 35 %, respectively (Fig. 5c). High temperature increased GR activity by 50 %, drought increased its activity by 42 %, and combined HT and drought stress increased its activity by 60 %, compared to control. Exogenous Spm treatment showed positive effects on GR activity improving its activity by 42, 47, and 36 % in mung bean seedlings imposed by HT, drought, and combined HT and drought stress, respectively (compared to stress treatment only; Fig. 5d).

Methylglyoxal detoxification mechanism

High temperature and/or drought increased MG toxicity in mung bean plants. The toxic MG level amplified by 102, 69, and 199 % in mung bean seedlings subjected to HT, drought, and combined HT and drought stress, respectively (in contrast to control seedlings; Fig. 6c). Enzymes of glyoxalase system differentially modulated under HT and/or drought stress. The activity of Gly I increased but the activity of Gly II decreased in HT and/or drought-affected mung bean seedlings, in contrast to the control. The increase of Gly I activity was not significant in Spm-supplemented stress-affected seedlings. In contrary, the

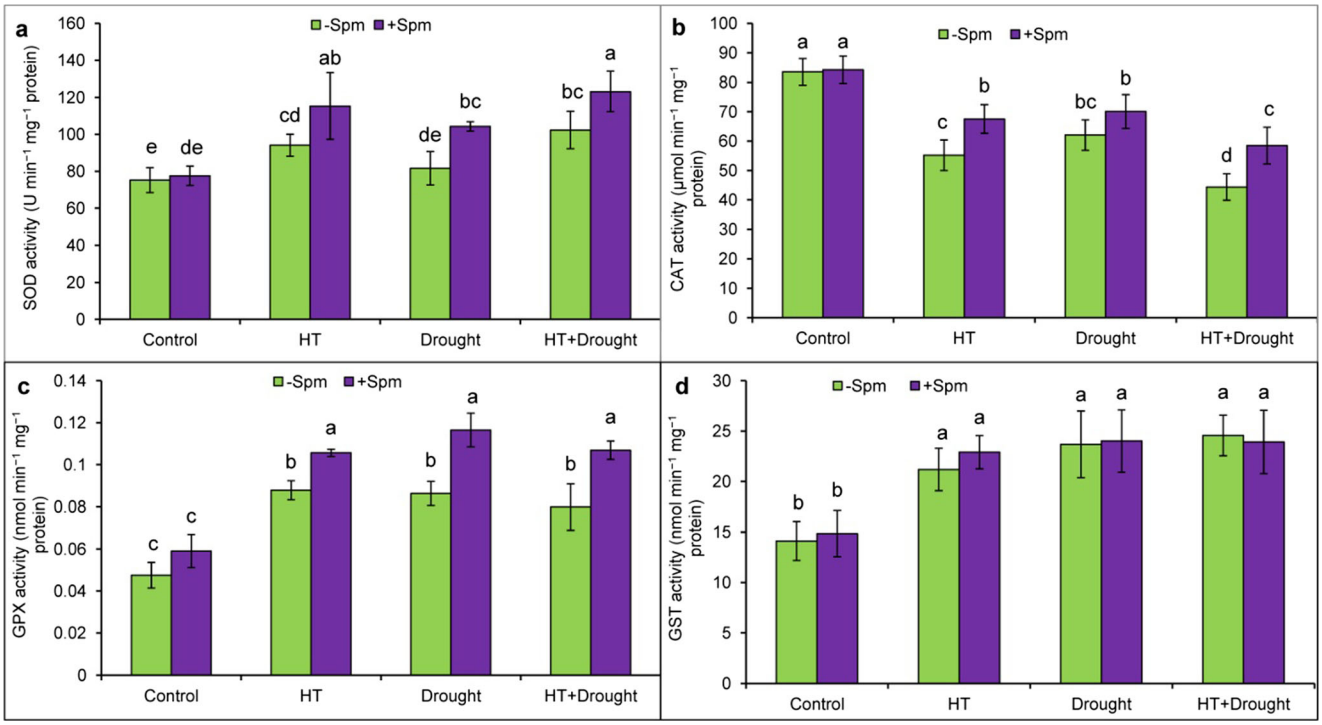

Fig. 4 Activities of SOD (**a**), CAT (**b**), GPX (**c**), and GST (**d**) in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (5 % PEG), and combined HT and drought stress. Mean (±SD) was calculated from three replicates for each treatment. *Bars with different letters* are significantly different at $P \leq 0.05$ applying Fisher's LSD test

Gly II activity was enhanced by exogenous Spm application with HT or drought treatment. Glyoxalase II activity increased by 24, 25, and 40 % after Spm pretreatment in HT, drought, and combined HT and drought stressed

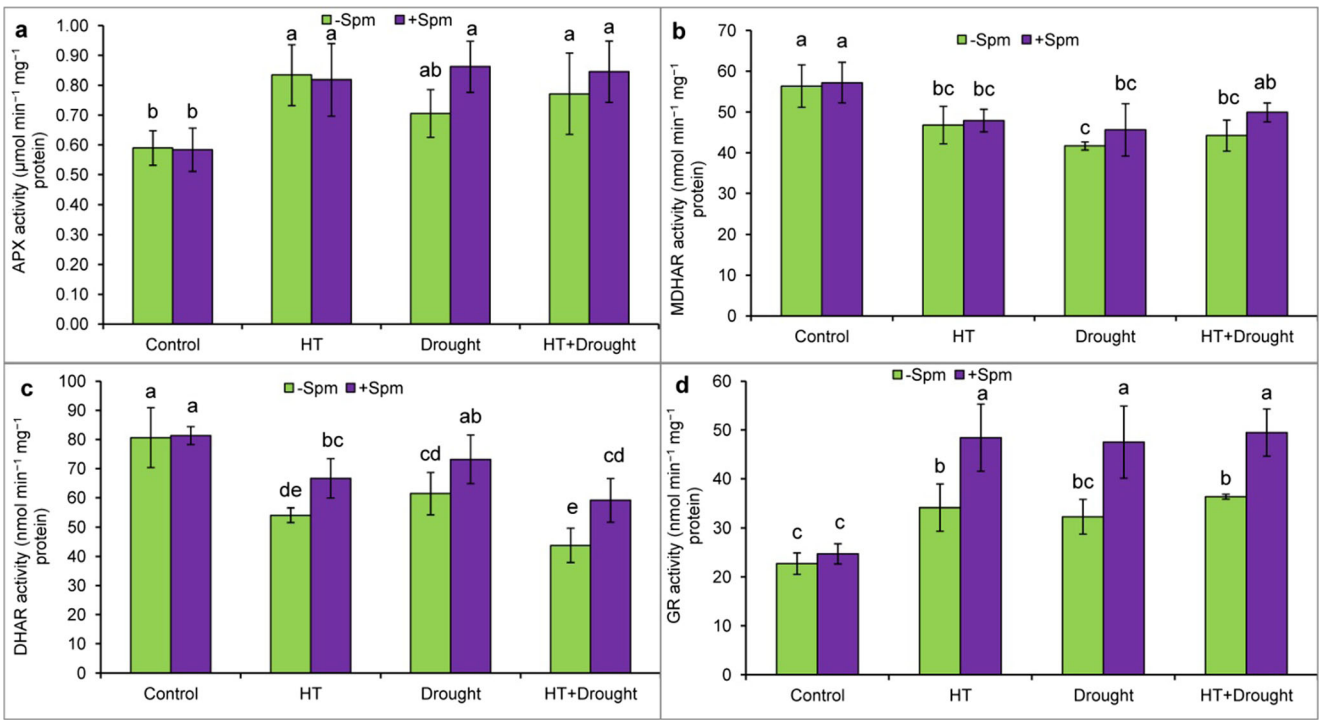

Fig. 5 Activities of APX (**a**), MDHAR (**b**), DHAR (**c**), and GR (**d**) in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (5 % PEG), and combined HT and drought stress. Mean (±SD) was calculated from three replicates for each treatment. *Bars with different letters* are significantly different at $P \leq 0.05$ applying Fisher's LSD test

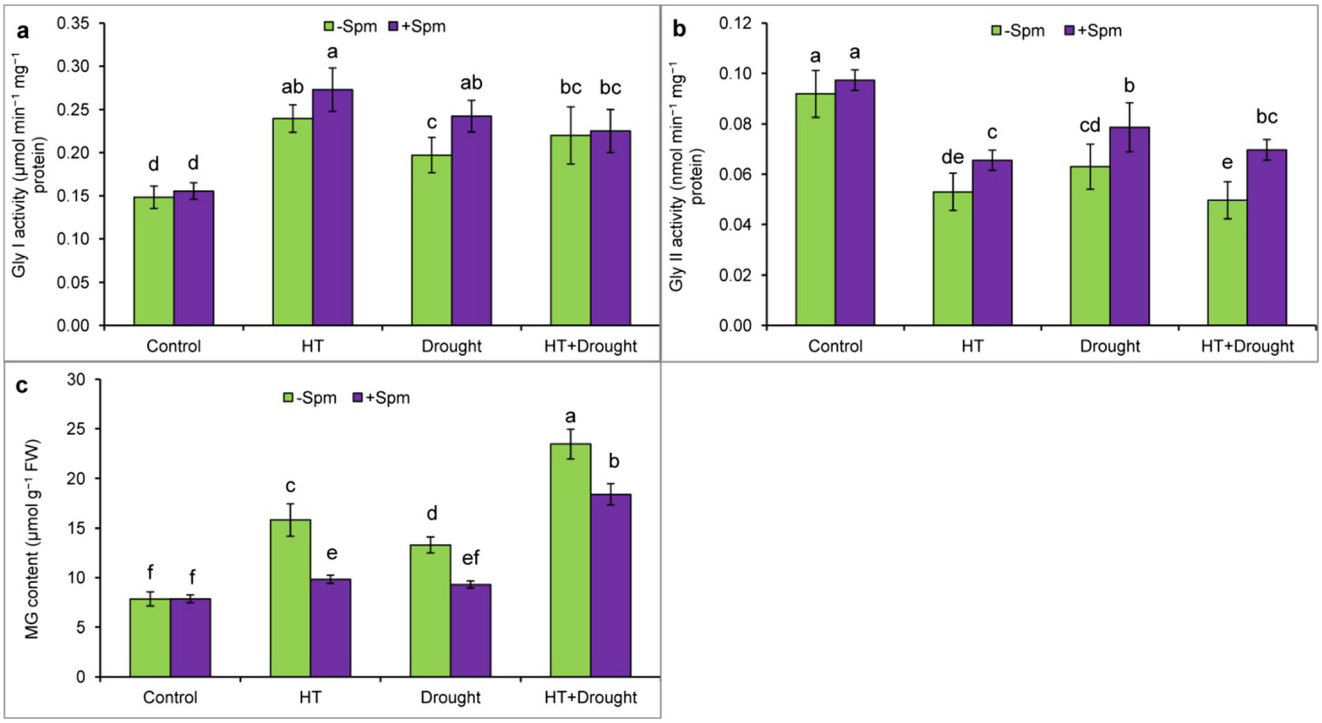

Fig. 6 Activities of Gly I (**a**) and Gly II (**b**) and MG contents (**c**) in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (5 % PEG), and combined HT and drought stress. Mean (±SD) was calculated from three replicates for each treatment. *Bars with different letters* are significantly different at $P \leq 0.05$ applying Fisher's LSD test

seedlings, respectively, compared to the stress treatment without Spm (Fig. 6a, b).

Endogenous polyamine contents

Putrescine content rose abruptly and highly in HT and drought-affected mung bean seedlings. The increases were 423, 354, and 680 % in HT, drought, and combined HT and drought treatments, respectively, compared to control. The Spd content was not affected significantly due to HT and/or drought imposition, compared to control. Spermine content increased only in combined HT and drought treatment, but in individual HT and drought treatment, Spm content did not change significantly, compared to the control treatment. These changes of Put, Spd, and Spm contents lead to decrease the [(Spd + Spm)/Put] ratio of mung bean seedlings affected by HT and/or drought, compared to control (Fig. 7a–d). Pretreatment with exogenous Spm decreased the endogenous Put content, in contrast increased the endogenous Spd content and Spm content in HT and/or drought-affected mung bean seedlings, compared to HT and/or drought-affected seedlings without exogenous Spm. Exogenous Spm addition with HT and/or drought also augmented the [(Spd + Spm)/Put] ratio. The [(Spd + Spm)/Put] ratio increased by 227, 127, and 206 %, respectively, due to exogenous Spm supplementation with HT, drought, and combined HT and drought stress, compared to the stress treatment only (Fig. 7).

Osmoregulation and plant water status

Leaf RWC decreased significantly by 19, 10, and 23 % under HT, drought, and combined HT and drought stress, respectively, compared to control. Leaf RWC was restored by exogenous Spm addition with HT and/or drought treatment, compared to HT and/or drought treatment. High temperature and/or drought decreased WRC but increased WSD and WUC. Whereas, exogenous Spm reversed this situation. The osmoprotectant, Pro level increased under HT, drought, and combined HT and drought stress by 21, 24, and 32 %, respectively, compared to control (Table 1). Exogenous Spm application with HT resulted in same Pro level in HT treatment only. Proline level increased significantly in Spm-supplemented drought and combined HT and drought treatment, compared to HT and/or drought treatment only.

Photosynthetic pigments and leaf area

Chlorophyll *a* and chl *b* contents decreased under HT and/or drought stress which attributed to decrease the total chl ($a + b$) content, compared to control. Total chl ($a + b$) content decreased by 24, 22, and 22 % due to HT, drought, and combined HT and drought imposition to the mung bean seedlings,

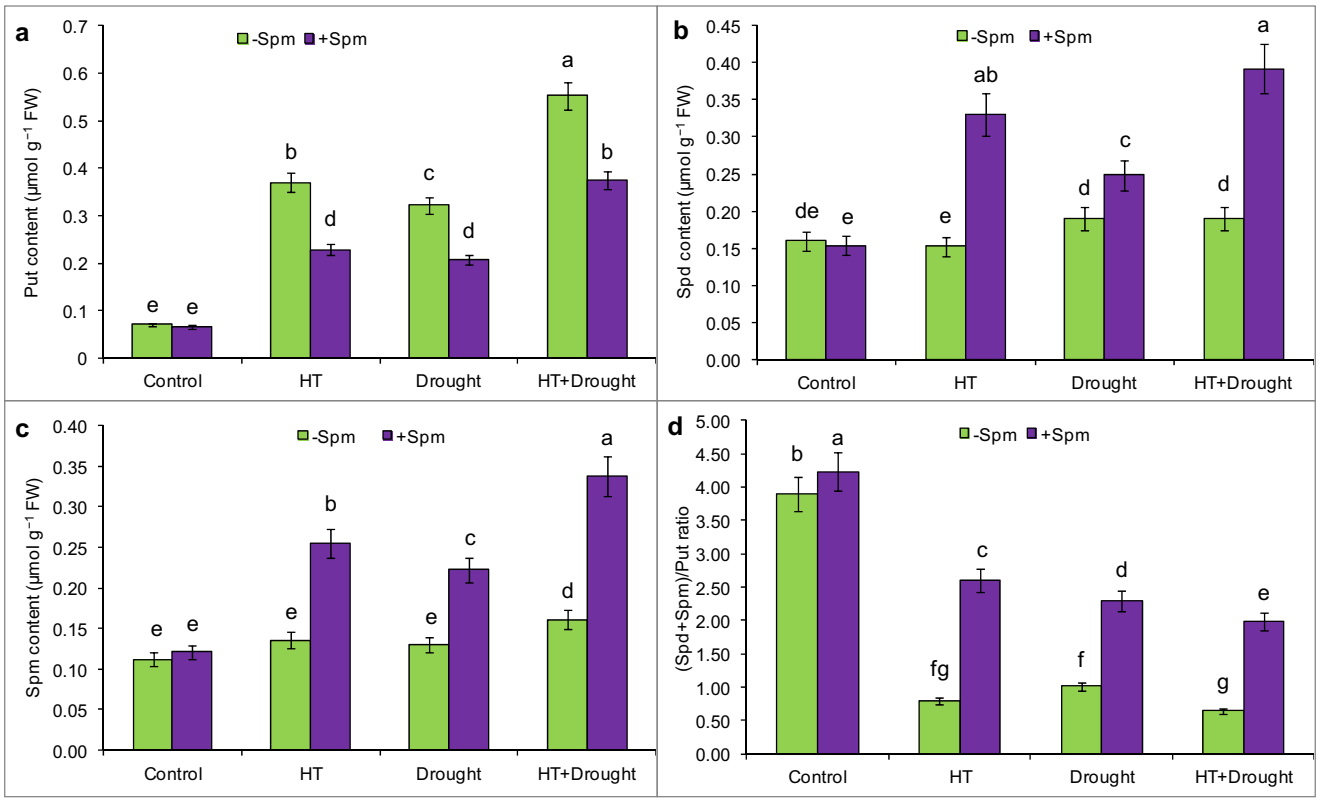

Fig. 7 Endogenous Put (**a**), Spd (**b**), and Spm (**c**) contents and (Spd + Spm)/Put ratio (**d**) in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (5 % PEG), and combined HT and drought stress. Mean (±SD) was calculated from three replicates for each treatment. *Bars with different letters* are significantly different at $P \leq 0.05$ applying Fisher's LSD test

respectively, compared to control (Fig. 8a–c). Decreases in Car content were 30, 31, and 37 % in HT, drought, and in combined HT and drought treatment, in contrast to control (Fig. 8d). However, exogenous Spm overwhelmed the stress induced by HT and/or drought and enhanced the levels of chl and Car, compared to HT and/or drought treatment alone (Fig. 8). High temperature and/or drought stress decreased leaf area, compared to control. Exogenous Spm increased leaf area of HT and/or drought-affected seedlings, compared to stress treatment alone (Table 2).

Seedling vigor and biomass accumulation

Seedling vigor was negatively affected by HT and/or drought. Growth of mung bean seedlings decreased under HT and/or drought. Plant height and root length decreased by 18 and 22 % under HT; these growth parameters were decreased by 16 and 18 % under drought stress. When these stresses were applied in combination, the reduction of plant height and root length privileged (compared to control). Reduction in leaf area and seedlings dry weight showed the similar pattern like plant

Table 1 Leaf RWC (%), water saturation deficit (WSD), water retention capacity (WRC), water uptake capacity (WUC), and Pro content in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (D, 5 % PEG), and combined HT and drought stress

Treatments	Leaf RWC (%)	WSD	WRC	WUC	Pro content (µmol g^{-1} FW)
Control	96.3 ± 0.6 a	4.7 ± 0.2 f	12.6 ± 0.9 a	0.5 ± 0.04 e	2.6 ± 0.2 d
Spm	95.7 ± 0.3 ab	6.0 ± 0.3 e	12.3 ± 0.9 ab	0.6 ± 0.05 de	2.6 ± 0.2 d
HT	77.9 ± 0.7 f	17.5 ± 0.9 b	10.7 ± 0.8 bc	1.6 ± 0.13 a	3.2 ± 0.2 cd
HT + Spm	91.7 ± 0.2 cd	9.4 ± 0.5 d	11.5 ± 0.8 abc	0.9 ± 0.08 c	3.7 ± 0.2 bc
D	86.5 ± 2.1 e	13.1 ± 0.7 c	10.2 ± 0.7 c	1.1 ± 0.10 b	3.3 ± 0.3 cd
D + Spm	93.2 ± 0.7 bc	9.0 ± 0.5 d	10.3 ± 0.8 c	0.8 ± 0.07 cd	4.3 ± 0.4 ab
HT + D	73.6 ± 1.1 g	19.1 ± 1.0 a	10.0 ± 0.7 c	1.6 ± 0.14 a	3.5 ± 0.3 bcd
HT + D + Spm	89.6 ± 1.1 de	8.7 ± 0.4 d	10.7 ± 0.8 bc	0.8 ± 0.07 cd	4.7 ± 0.4 a

Mean (±SD) was calculated from three replicates for each treatment. Different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

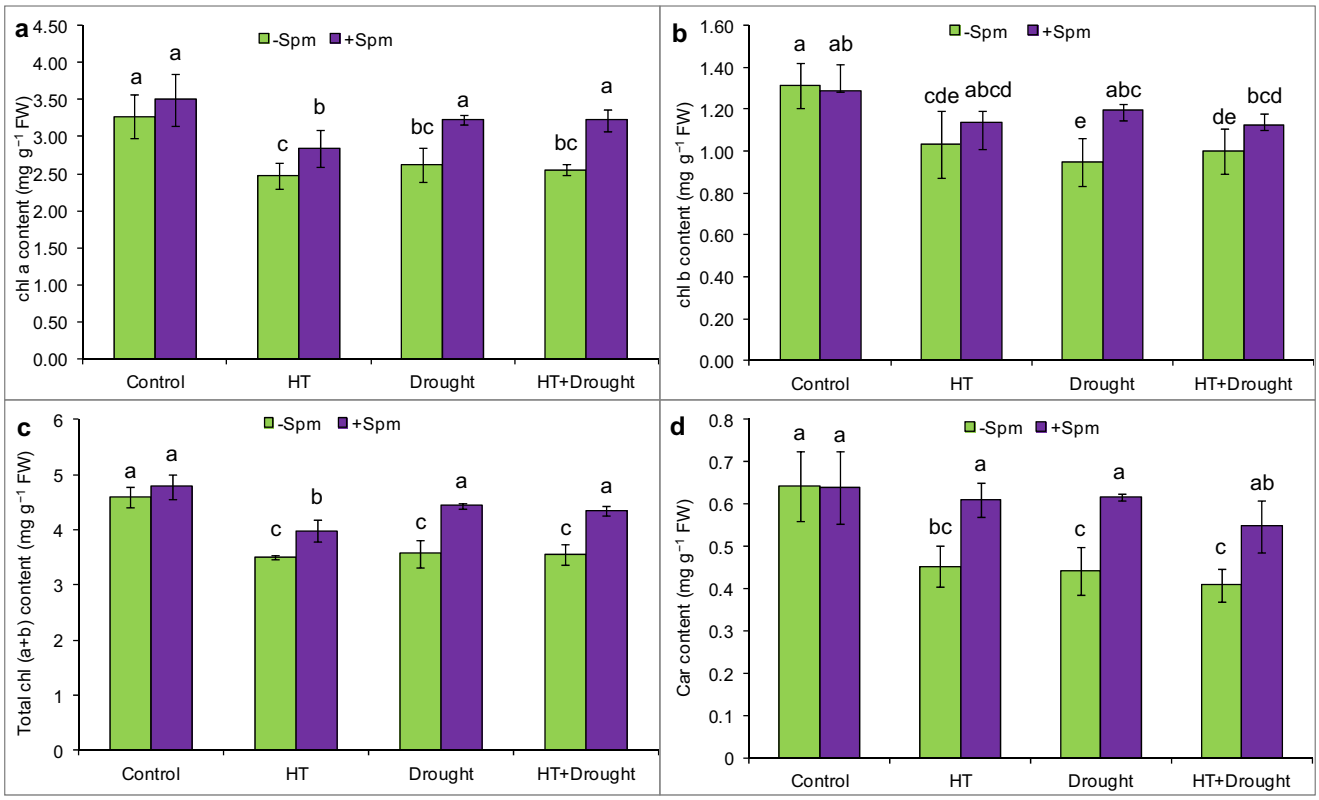

Fig. 8 Chlorophyll *a* (**a**), chl *b* (**b**), total chl (*a* + *b*) (**c**), and carotenoid (Car) (**d**) contents in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (5 % PEG), and combined HT and drought stress. Mean (±SD) was calculated from three replicates for each treatment. *Bars with different letters* are significantly different at $P \leq 0.05$ applying Fisher's LSD test

height and root length under HT and/or drought stress (compared to control). Exogenous Spm application alleviated the stress of HT and/or drought in mung bean seedlings and improved the growth parameters, compared to HT and/or drought stress alone (Table 2).

Discussion

High temperature increases ROS generation in chloroplasts and mitochondria in different ways. Disintegration of membrane stability and disturbance of biochemical reactions such as the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase result in photorespiration which increases ROS production. Disrupted activities of enzymes involved in respiration in the mitochondrial electron transport chain also enhance ROS production (Sharkey 2005; Jaspers and Kangasjarvi 2010). Drought stress decreases stomatal conductance; loss of balance between the light reactions and the Calvin–Benson cycle (Chaves et al. 2009) makes the electron carriers over reduced in chloroplasts and mitochondria, resulting in the production of ROS by the transfer of electrons

Table 2 Plant height, root length, leaf area, and dry weight in mung bean leaves induced by exogenous spermine (Spm, 0.2 mM) under high temperature (HT, 40 °C), drought (D, 5 % PEG), and combined HT and drought stress

Treatments	Plant height (cm)	Root length (cm)	Leaf area (cm^2)	Dry weight (g $seedling^{-1}$)
Control	9.5 ± 0.7 a	9.5 ± 0.8 a	2.2 ± 0.2 a	0.023 ± 0.003 a
Spm	9.4 ± 0.8 ab	9.4 ± 0.9 a	2.2 ± 0.2 a	0.024 ± 0.002 a
HT	7.8 ± 0.4 d	7.4 ± 0.7 de	1.4 ± 0.3 e	0.014 ± 0.003 e
HT + Spm	8.6 ± 0.6 bc	8.3 ± 0.9 c	1.9 ± 0.3 c	0.018 ± 0.003 d
D	8.0 ± 0.6 d	7.7 ± 0.7 d	1.6 ± 0.1 d	0.017 ± 0.001 d
D + Spm	9.0 ± 0.9 ab	9.1 ± 0.8 ab	2.0 ± 0.16 bc	0.022 ± 0.003 ab
HT + D	7.6 ± 0.7 d	7.2 ± 0.7 e	1.3 ± 0.2 e	0.017 ± 0.002 d
HT + D + Spm	8.6 ± 0.6 bc	8.9 ± 0.9 b	1.8 ± 0.2 d	0.020 ± 0.002 c

Mean (±SD) was calculated from three replicates for each treatment. Different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

to molecular oxygen (Foyer and Noctor 2012). The leaves of mung bean were subjected to DAB staining to visualize brown patch of H_2O_2, whereas dark blue spots of $O_2^{\bullet-}$ were visualized by NBT staining. Increases of brown patch and dark blue spot in leaves of HT and/or drought-affected leaves or in combined stress treatment indicate accumulation of H_2O_2 and $O_2^{\bullet-}$, respectively (Fig. 1a, b). Mung bean plants subjected to HT and/or drought stress enhanced ROS generation; free radicals such as superoxide $O_2^{\bullet-}$ (expressed as $O_2^{\bullet-}$ generation rate) and non-radical compounds like H_2O_2 level rose extremely under those stresses (Fig. 2a, b). The activity of oxidative and lipid-degrading enzyme, LOX, increased under those stress conditions (Fig. 2c). Consequently, the membrane integrity loosed under HT and/or drought stress, which was confirmed by higher level of lipid peroxidation (indicated by MDA) (Fig. 2d). Seedlings pretreated with Spm decreased patches and spots of H_2O_2 and $O_2^{\bullet-}$, reducing their contents and LOX activity which reduced subsequent oxidative damage (Figs. 1 and 2). Polyamines act as ROS scavenger. PAs compete with metal ions necessary for ROS formation, such as Fe^{2+} and Cu^{2+}, which are considered as indirect roles of PAs in reducing ROS production (Tadolini 1988). Activities of antioxidant enzymes can be improved by PAs (Shi et al. 2010; Tian et al. 2012; Fu et al. 2014). Similar advantageous roles are supposed to be obtained in present study by Spm under HT and/or drought stress to decrease oxidative stress.

Higher constitutive and induced activities of antioxidant enzymes are usually accompanied with improved stress tolerance (Ashraf 2009). Being the first line of the enzymatic defense system, SOD converts toxic $O_2^{\bullet-}$ to the more stable H_2O_2. Mung bean seedlings subjected to HT and/or drought stress showed higher SOD activity and higher $O_2^{\bullet-}$ generation rate (Figs. 2b and 4a). Sekmen et al. (2014) demonstrated a parallel increase in SOD activity with NADPH oxidase activity, which is one of the main producers of $O_2^{\bullet-}$ in plant cells. Spermine pretreatment increased the SOD activity further in HT and/or drought-treated seedlings which decreased $O_2^{\bullet-}$ generation (Figs. 1b, 2b, and 4a). CAT is the most efficient scavenger of H_2O_2 considering scavenging H_2O_2 in per unit time (in peroxisomes, chloroplasts, and cytosols) (Asada and Takashi 1987). Increased accumulation of H_2O_2 and lipid peroxidation in HT and/or drought-stressed mung bean seedlings might be caused by the decrease in CAT activity (Figs. 2a and 4b) (Sekmen et al. 2014). GPX reduces H_2O_2 and organic and lipid hydroperoxides using GSH (Noctor et al. 2002). The increased activity of GPX due to HT and/or drought stress in the mung bean seedlings (Fig. 4c) is supported by the results of previous reports (Hasanuzzaman and Fujita 2011). Adding Spm had influential roles in increasing CAT and GPX activities in HT and/or drought-affected mung bean seedlings, compared with HT and/or drought-affected seedlings (without Spm application; Fig. 4b, c). GST catalyzes conjugation of electrophilic xenobiotic substrates with the GSH and also associated with responses to abiotic stresses (Hossain et al. 2006; Dixon et al. 2010). Mung bean seedlings increased GST activity under HT and/or drought stress, compared to control which further increased after Spm supplementation (Fig. 4d). PAs have been reported to form complexes with SOD, GPX, and CAT for which these enzymes function more efficiently compared with isolated enzymes (Alcázar et al. 2010), and exogenous PAs also improved antioxidant enzyme activities including SOD, GPX, and CAT to improve abiotic stress tolerance (Wang et al. 2010; Fu et al. 2014; Li et al. 2014).

APX, MDHAR, DHAR, and GR are potential enzymes of the AsA-GSH cycle playing indispensable roles in reducing ROS and by maintaining the AsA and GSH levels (Noctor and Foyer 1998; Rao and Reddy 2008). The activity of APX increased but the activities of MDHAR and DHAR decreased under HT and/or stress which reduced the AsA content (Figs. 3a and 5a–c). Again using AsA, APX is engaged in ROS-scavenging process (Gill and Tuteja 2010), which is the reason for reduced AsA content (Figs. 3a and 5a). AsA is converted to DHA during scavenging of ROS (Gill and Tuteja 2010), which increased the DHA content (Fig. 3b) and decreased AsA/DHA ratio (Fig. 3c). The activities of MDHAR and DHAR spontaneously regenerate AsA (Asada 1992). After Spm addition with HT/or drought, DHAR activity increased significantly, which increased AsA content (Figs. 3a and 5c). In contrast to control, the GSH content was not altered under drought stress, but the GSSG content increased and GSH/GSSG ratio decreased, which increased ROS overproduction and oxidative damage (Figs. 1, 2, and 3d–f). Exogenous Spm decreased GSSG content and increased GSH content and the ratio of GSH/GSSG in HT and/or drought-affected mung bean seedlings, which is also corroborating with ROS reduction and overwhelm of oxidative damage (Figs. 1, 2, and 3d–f). The activity of GR is responsible for recycling of GSH (Gill and Tuteja 2010). Increased GR activity by exogenous Spm pretreatment supports the increased level of GSH under HT and/or drought treatment, compared to stress treatments alone (Figs. 3d and 5d). The ratios of AsA/DHA and GSH/GSSG have pivotal roles in signal transduction during development or stress adaptation (Gill and Tuteja 2010; Kumar et al. 2010). Similar to our results, in chill-affected ginger seedlings, the contents of AsA and GSH, and the ratios of AsA/DHA and GSH/GSSG, and activities of APX, DHAR, MDHAR, and GR, increased by exogenous Spd which reduced oxidative damage (Li et al. 2014). However, signaling or other roles of PAs in biosynthesis of antioxidant components are not clear and need further investigation.

Methylglyoxal levels markedly increased due to HT or drought treatment with higher increases in combined HT and drought stress (Fig. 6c), which is parallel to other research findings with HT and drought stress (Turóczy et al. 2011;

Nahar et al. 2015). The activity of Gly I increased, and the activity of Gly II decreased under HT and/or drought stress, compared with control (Fig. 6a, b), which is supported by the results of previous reports (Hasanuzzaman and Fujita 2011; Nahar et al. 2015). Increased Gly II activity (Fig. 6b) with a high GSH level (Fig. 3d) after Spm supplementation contributed to MG detoxification (Fig. 6c) (Nahar et al. 2015). Kong et al. (1998) demonstrated that modulation of the levels of PAs and MG in PEG affected white spruce. MG levels were studied mostly in animal systems, and so, it should be studied on plant system.

Reduction of leaf RWC (Table 1) and wilting of leaves designate HT and/or drought-induced water stress which corroborates with previous findings (Fu et al. 2014). Spm pretreatment increased the leaf RWC that means Spm reduced water loss. Water saturation deficit indicates the degree of water deficit in plants; WSD showed an opposite trend of RWC that increased under HT and/or drought stress. Water retention capacity/WRC indicates the capacity of cell to retain water which reduced due to HT and/or drought. Water uptake capacity illustrates the ability to absorb water per unit dry weight in relation to turgid weight. Stress condition is often associated with higher WUC because the plant would absorb more water to reach turgidity than a plant under control condition (Sangakkara et al. 1996). HT and/or drought increased the WUC compared to that of control. Application of Spm decreased WUC and WSD whereas increased WRC in HT and/or drought-affected mung bean seedlings, which refer a better plant water status (Sangakkara et al. 1996; Akhtar et al. 2013). Spm-pretreated seedlings suggested reducing or slowing water evaporation under HT and drought conditions (Fu et al. 2014). Exogenous application of PAs induces stomatal closure in *Arabidopsis* (Yamaguchi et al. 2007) and red tangerines (Shi et al. 2010), which prevented water loss. Proline is known as an osmoprotectant; its content increases under stress condition and imparts water deficit tolerance by osmotic adjustment. Proline also has ROS-scavenging function (Gill and Tuteja 2010). High temperature and/or drought stress increased the Pro level highly (compared to control), and application of Spm further increased the Pro content and restored the water, compared to stress treatment only (Table 1). Cvikrová et al. (2013) reported that transgenic tobacco plants overproducing Pro maintained better water status reducing the water loss under drought and combined drought and heat stress, compared to control where modulation of PAs was involved.

High temperature and drought stress increased the Put content of mung bean seedlings. Compared to the single stress, the combined HT and drought stress amplified Put level to a great extent. In contrast to the Put content, the contents of Spd and Spm did not change under single HT or drought stress, combined HT, and drought stress slightly increased the Spd and Spm levels (compared to control). As a result, the [(Spd + Spm)/Put] ratio decreased significantly (Fig. 7a–d). Increased Put content with decreased [(Spd + Spm)/Put] ratio indicates cellular toxicity, because Put causes depolarization of membranes and increases potassium leakage (Tiburcio et al. 1990). Excessive accumulation of free Put may lead to apoptotic cell death (Groppa et al. 2001; Wang et al. 2007). In present study, increase of free Put was accompanied by substantial generation of ROS under HT and/or drought stress (Figs. 2 and 7a). After exogenous Spm pretreatment, increases of free Spd and Spm, and the decrease of free Put, indicate reduction of toxicity of stress (Fig. 7a–c). Therefore, the elevation of the [(Spd + Spm)/Put] free ratio was critical in improving HT and/or drought tolerance of mung bean seedlings (Fig. 7d). This result is in agreement with other published results (Bouchereau et al. 1999; Wang et al. 2007; Yang et al. 2010) regarding salt, osmotic, copper, and cadmium stresses in different plant species, respectively. Plants can interconvert one type of PA to other. Put can be converted to Spd and Spm (by the activities of spermidine synthase and spermine synthase). Polyamine oxidase acts in the back-conversion pathway, converting Spm to Spd and Spd to Put (Moschou et al. 2008). Spd and Spm might act as signaling regulators in stress signaling pathways (Kasukabe et al. 2004; Sanchez et al. 2005). The modulation of PA levels in the present study was supposed to be due to the differential activity of enzymes involved in degradation or biosynthesis pathway, where signaling roles of PAs might be involved also.

High temperature and/or drought stress affect soil-plant water relation, photosynthesis, transpiration, respiration, and translocation of photosynthetic assimilate which adversely affect plant growth and productivity (Prasad et al. 2011). High temperature and drought stress decrease the chl content and photosynthetic rate. The chl may be destroyed due to ROS-induced oxidative stress, or its biosynthesis may be hindered under HT and drought (Prasad et al. 2011). Chlorophyll *a*, chl *b*, and total chl (*a* + *b*) and Car content of HT and/or drought-affected mung bean seedlings decreased, compared to control. Spermine pretreatment recovered the reduction of chl and Car by increasing the content in HT and/or drought-affected seedlings (Fig. 8a–d). Our results are in same line with the previous report where exogenous PAs restored the photosynthetic pigment content (Unal et al. 2008). Chloroplasts restrain high activities of PA biosynthetic enzymes and transglutaminase (TGase) catalyzing the covalent binding of PAs to proteins, and thus, PAs prevent chloroplast and chl from damage. Foliar spray with Spm treatment prevented degradation of chl *a* and chl *b* in senescing leaves, and increased TGase activity, producing more PA-protein conjugates (Serafini-Fracassini et al. 2010). In present study, exogenous Spm application with HT and/or drought also recovered the reduction of leaf area

and increased the leaf area (Table 2), which indicates the increased area to capture light to be utilized for photosynthesis which might be improved by photosynthetic performance (Serafini-Fracassini et al. 2010) and overall growth of mung bean seedlings. The overall tolerance of mung bean seedlings induced by exogenous Spm against HT, drought, and combined HT and drought stress was reflected through improved growth parameters including plant height, root length, and seedling dry weight of mung bean seedlings (Table 2). Polyamine-induced improved growth performance and abiotic stress tolerance was proved in several previous reports (Rajasekaran and Blake 1999; Cheng et al. 2009; Mostafa et al. 2010).

Conclusion

The present study revealed that Spm pretreatment enhanced the tolerance of mung bean plants to HT, drought, and combined HT and drought stress. Polyamines showed divergence function in imparting the HT and/or drought tolerance. At first, we observed that exogenous Spm had its vital roles in alleviating oxidative damage. Spermine significantly reduced the generation of ROS, LOX activity, and lipid peroxidation. The reason behind reducing oxidative stress was upholding of antioxidant compounds (AsA and GSH levels) and enhanced activities of antioxidant enzymes induced by PAs. Glyoxalase system of plants and its relation to stress tolerance are new areas of research. However, in present study, Spm showed influential roles in reducing MG toxicity upregulating the glyoxalase system. Exogenous Spm modulated the endogenous level of PAs in a positive way. Maintenance of plant water status is vital for HT and/or drought tolerance. Exogenous Spm regulated the level of osmoprotectant compound Pro and maintained a good water status of mung bean seedlings. The overall tolerance of plants was reflected from protection of photosynthetic pigments and prevention of reduction of leaf area which are vital for photosynthesis process. The tolerance of mung bean plant was further confirmed by the improved growth, biomass production, and phenotypic appearance. Thus, the current study provides the novel evidence supporting that PAs confer tolerance to multiple and simultaneous stresses.

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Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

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