Pak. J. Bot., 43(3): 1587-1594, 2011.

MODULATION OF PLANT GROWTH, WATER STATUS AND ANTIOXIDANTIVE SYSTEM OF TWO MAIZE (*ZEA MAY* L.) CULTIVARS INDUCED BY EXOGENOUS GLYCINEBETAINE UNDER LONG TERM MILD DROUGHT STRESS

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Abstract

Modulation of lipid per-oxidation, antioxidant enzyme activity, water status and plant growth induced by glycinebetaine (GB) applied foliarly was investigated in the plants of two maize (*Zea mays* L.) cultivar i.e. drought-tolerant Shaandan 9 (S_9) and -sensitive Shaandan 911 (S_{911}) under long-term mild drought stress (LMDS). Long-term mild drought stress was found to decrease dry matter (DM), grain yield (GY) and leaf relative water content (RWC), but to increase malondialdehyde (MDA) accumulationin in leaves of both cultivars. The patterns of rises initially and declines afterward in activities of superoxide dismutase (SOD: EC 1.15.1.1), peroxidase (POD: EC 1.11.1.7), and catalase (CAT: EC 1.11.1.6) were closely dependant on cultivar and growth stage. Dry matter, GR, RWC and these antioxidative enzymes activities were greater but MDA concentration was lower for S_9 than those for S_{911} under LMDS. Additionally, exogenous GB application increased DM, GR, RWC and antioxidant enzymes activities measured, but reduced MDA accumulation in both cultivars under LMDS unlike well-watered control, which exhibited no such obvious effect with GB. The modulation induced by GB applying was more pronounced in S_{911} than that in S_9 under LMDS. The greatest positive role of GB seemed to be found in the plants subjected to the largest MDA accumulation at mature stage. It is, therefore, concluded that GB may protect cells against oxidative damage and alleviate the negative effect of DS on water status and plant growth, particularly in this drought sensitive cultivar and imposed to more serious damage from DS environment.

Introduction

Drought stress, one of the most important constraints to crop production, has become more prevalent especially in arid and semi-arid regions of the world (Boyer, 1982). Maize (*Zea mays* L.) is the second (following wheat) most important cereal crop in the north of China, which is frequently subjected to delay in irrigation or drought stress (DS) resulting in a significant reduction in crop yield (Li, 2007). The DS tolerance to crops is largely dependent on the crop cultivar. The more sensitive to drought the cultivar is, the more serious the reduction of yield will be (Chandrasekar *et al*., 2000). Drought stress creates a wide array of biochemical and physiological changes, beginning from a variable decline in leaf relative water content (RWC) as a better indicator of plant water status (Taiz & Zeiger, 2002; Seghatoleslami *et al.*, 2008). Crucial changes in water homeostasis can lead to osmotic stress, which are primary effects of DS. Such, free radicals and other active derivatives of oxygen may produce inevitably as by-products of physiological redox reactions. It has been suggested that DS caused elevated levels of active oxygen species (AOS) (Arora *et al*., 2002), and the production of active oxygen exceeded the capacity of the scavenging systems, resulting in oxidative damage (Taiz & Zeiger, 2002). The increased levels of AOS can inactivate enzymes, damage important cellular components, which induced plant growth arrest, and even death finally (Arora *et al*., 2002). To mitigate the deleterious effect of DS on regular metabolism and ensure crops under optimal growth conditions, plants have evolved various strategies to contend with this problem. By necessity, plants possess a number of antioxidants such as superoxide dismutase (SOD: EC 1.15.1.1), peroxidase (POD: EC 1.11.1.7), and catalase (CAT: EC 1.11.1.6) in order to protect cellular membranes and organelles from the damaging effects of toxic concentrations of AOS and maintain their integrity and stability under drought-stressed conditions (Arora *et al.*, 2002). As cell membranes are the first targets of many plant stresses, AOS may destroy normal metabolism through peroxidation of membrane lipids (Arora *et al*., 2002). Lipid peroxidation of biological membranes might lead to structural alterations such as denaturalization of proteins and nucleic acids in drought-stressed plants. Experimental evidences suggest that lipid peroxidation reactions of cellular membranes may play an important role in radical mediated cell injury in view of malondialdehyde (MDA) accumulation (Zhang *et al.* 2007). Therefore, activity of antioxidant enzymes and MDA content may act efficient determinant criteria in the toxic degree to drought stressed plants (Arora *et al*., 2002; Aslam *et al.*, 2006). The cascade of studies on physiological adjustment to crops revealed that the change patterns of production, RWC and activities of SOD, POD, CAT as well as MDA content were associated with cultivar and development stage (Sairam & Srivastava, 2001; Dhanda *et al.*, 2004; Ramachandra *et al.*, 2004; Ma *et al.*, 2006; Hamidou *et al*., 2007; Jabeen *et al.*, 2008; Seghatoleslami *et al.*, 2008; Chutipajit *et al.*, 2009).

Among the many quaternary ammonium compounds known in plants, glycinebetaine (GB) occurs most abundantly in response to dehydration stress in many crop plants (Zhang & Li, 2004). In addition to its osmoprotectant roles (Ashraf & Foolad, 2007), GB may also protect cells from environmental stresses indirectly through positive effects on enzyme and membrane integrity by stabilizing the structure of proteins (Zhang *et al.,* 2004), protecting cytoplasm and chloroplasts (Ma et al., 2006), protecting photosynthetic apparatus (Sakamoto and Murata, 2002) and by functioning as oxygen radical scavengers (Arora *et al*., 2002). However, in many crop plants, the natural accumulation of GB is lower than sufficient to ameliorate the adverse effects of dehydration caused by various environmental stresses such as drought stress (Rhodes & Hanson, 1993; Subbarao *et al.*, 2001; Yang *et al.*, 2003). Moreover, because naturally produced GB does not normally break down in plants, it can easily be collected as a relatively inexpensive by-product from high-producing plants such as sugar beets (*Beta vulgris* L.) (Rhodes & Hanson, 1993). Applications of exogenous GB on crop plants unable to synthesis GB in sufficient amounts has been suggested as a

possible approach to overcome the environmental limitations of crop production (Zhang & Li, 2004). Externally-applied GB can rapidly penetrate through leaves and be transported to other organs, where it would contribute to improved stress tolerance, which could result in improving growth, survival and tolerance of a wide variety of GB-accumulator/nonaccumulator plants under drought (Mäkelä *et al*. 1996). There are many reports on the ability of exogenous GB to increase plant growth and stress tolerance of many plants including tobacco (*Nicotianatabacum* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum uhulgare* L.), maize under different stress conditions. The data presented in these paper confirm the well-established protective effect of GB from salinity- or drought stress after exogenous application as a foliar spray to cultivars differing in their tolerance to stress (Mäkelä *et al*., 1996; Agboma *et al*., 1997; Sakamoto & Murata, 2000; Yang & Lu, 2005, Ma *et al*., 2007).

However, the vast majority of relative studies have been reported in plant growth, water status and anti-oxidative effects during short-term (a single growth stage) only following exposure of plants to abrupt artificial DS or salinity stress (Demiral & Türkan, 2004; Ashrafa & Foolad, 2006; Demiral & Türkan, 2006; Ma *et al*., 2007; Raza *et al*., 2007; Chutipajit *et al*., 2009). Hence, to our present knowledge, there is little information available in literature on GB-induced antioxidant modulation in maize cultivars differing in drought tolerance subjected to long-term mild and gradual DS during an entire growth period and interaction effects amongst cultivar, water regime and GB on these biological and physiological traits (Gupta *et al*., 2001; Ma *et al*., 2006; Ma *et al*., 2007; Hamidou *et al*., 2007). In addition, effectiveness of GB has not been clarified, which is mainly due to its nutritive role (as a micro-molecular nitride) or physiological protective function in the same trial. Keeping in view the above facts, we hypothesize that higher constitutive or induced activity of antioxidant enzymes in leaves of maize plants provides a mechanism of tolerance to different cultivars under long-term mind drought stress (LMDS) in terms of GB applying. With this aim, we designed a pot experiment used soil to clarify the responses of two maize cultivars with respect to anti-oxidative effects, water status and plant growth imposed to a LMDS and GB spray. The interaction effects of cultivar, water regime and GB amongst all parameters were also examined.

Materials and Methods

Plant material and experimental design: Two contrasting cultivars of maize (*Zea mays* L.), drought-tolerant Shaandan 9 (S_9) and -sensitive Shaandan 911(S_{911}), were obtained from Agronomy College of Northwest A & F University, Yangling (34º20¹N, 108º24¹E), China (Zhang *et al*., 2007). Five seeds of each cultivar were planted in June in each pot (28 cm diameter and 35 cm height) in glasshouse. The plants were thinned to one per pot at the three-leaf stage and harvested in late September.

Each pot was filled with 12 kg air-dried soil ground and sieved (0.5 mm). The soil used was a typical manural loessial field sample with clay-loam texture from 0-20 cm layer in Yangling, China. Selected properties of the soil used included: total N=0.96 g/kg; available N=42.3 mg/kg; Olsen-P=23.2 mg/kg; exchangeable K=216 mg/kg (NH_4Oac Extraction, Flame Spectrometry); field capacity water content = 22.6% (weight basis). The measured soil water content before planting was 13% (weight basis), equivalent to 10.6 kg oven-dry soil. The soil was amended with 0.10 g N/kg and 0.15 g P/kg soil and mulched with wheat straw.

The main treatments included two soil water regimes: (i) well-watered (CK), i.e. soil water content maintained at 19±1% of water content (weight basis) equivalent to 85±5% of field capacity; (ii) drought stress (DS), maintained at 15±1% of water content (weight basis), equivalent to 65±5% of field capacity. Sub-treatments were: (i) exogenous glycinebetaine (GB) application (T) (produced in Shiying Chemical Plant, Changping, Beijing), i.e., 20, 40, 60 and 60 ml GB solution per plant at a concentration of 50 mg/L was applied at seedling, elongation, heading and maturation stages on day 13 and 15, 29 and 31, 54 and 56 as well as 83 and 85 after planting, respectively; (ii) no GB spray (T_0), i.e., sprayed with distilled water in the same manner as the GB-treated plants. A randomized complete block design was used with a total of 8 treatments, each cultivar including 4 treatments: CK (T_0), CK (T); DS (T_0), DS (T). Each treatment had 17 replicates and one pot without any seedlings for using in gravimetric soil water content calibration by time domain reflectometry (TDR). Pots were hand weeded as required.

Soil water status was measured every 1-3 days before application of irrigation water. The volumetric soil water content was estimated in each pot using a TDR probe, installed at a depth of 35 cm midway between plant and pot rim. A water meter was used to measure the quantity of irrigation water required. The amount of water (W) applied at each irrigation was calculated using the following equation:

$$W = Y \times H \times A \times (W_i - W_0)$$

Y, bulk density; H, soil depth; A, area of each pot; W_i, target soil water content; W_0, measured soil water content before irrigation.

Sampling and experimental observations: Leaf samples were taken from four plants at several key developmental stages i.e. seedling, elongation, heading, and maturity on day 25, 38, 65, and 95 after planting, respectively. Completely developed third or fourth leaf from the top of the plant was used for all measurements. Leaves were collected on ice between 10:30-11:00 h and brought to the laboratory, washed with distilled water, and the excess water removed.

Determination of dry matter and leaf relative water content: The plant samples for dry matter (DM) determination were dried in a forced-ventilation oven at 65 °C until constant dry weight. Leaf relative water content (RWC) was estimated by recording the turgid weight of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in hot air oven till constant weight using the following relationships (Gao, 2000).

$$RWC\ (\%) = [(W-DW) / (TW-DW)] \times 100$$

W represents sample fresh weight; TW represents sample turgid weight; DW represents sample dry weight.

Antioxidant enzymes assays: The leaves blades (1.0g) were cut, mixed and homogenized in ice cold 4 ml 50 mmol/L phosphate buffer (pH 7.8) containing 1% PVP (V/V) and a little quartz sand with pre-chilled pestle and mortar. The homogenate was transferred to centrifuge tubes and was centrifuged at 4 °C in refrigerated centrifuge for 20 minutes at $10000\times g$. The supernatant was used as an enzyme extract. SOD (EC 1.15.1.1) activity was estimated by recording the

decrease in absorbance (560nm) of superoxide-nitro blue tetrazolium complex by enzyme. One unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzyme (Dhindsa *et al.*, 1981). POD (EC 1.11.1.7) activity was determined specifically with guaiacol at 470 nm and one unit of enzyme activity was taken as the rate of guaiacol which was oxidized in three minutes (Puter, 1974). CAT (EC 1.11.1.6) was assayed by measuring the residual H_2O_2 by Tris-HCl reagent. Absorbance was recorded immediately at 240nm every one minute in four minutes and one unit of enzyme activity was taken as the enzyme which reduced the absorbance (A_{240}) reading of samples to 10% in comparison with tubes dead enzyme in one minute (Taranishi *et al*., 1974). The activity of each enzyme was expressed on protein basis (U/mg protein). Protein concentration of the crude extract was measured by the method of Gao, (2000). 0.5 g fresh leaves were ground in 2.5 mL extraction buffer (30 mmol/L Tris-HC1 pH 8.7, l mmol/L DTT, 1 mmol/L Vitamin C, 1 mmol/L EDTA, 5 mmol/L $MgC1_2$) with a glass rod and a small amount of quartz sand over an ice-bath. The combined syrup and rinsing liquid was transferred into a 10 mL-tube and then centrifuged at 5000 rpm for 10 min. The supernatant was protein solution. After measuring the volume of solution, 0.1 mL of the protein solution, 0.9 mL distilled water and 5 mL Coomassie Brilliant Blue G-250 were transferred into a cuvette. Absorbance was measured using a spectrophotometer at 595 nm. Concentration of soluble protein was estimated by referring to a standard curve obtained by using absorbance readings of different concentrations of standard protein solution (100μg/mL Bovine Serum Albumin solution).

MDA concentration measurement: The level of lipid peroxidation in leaf samples was determined in terms of MDA concentration according to the method of Gao, (2000). MDA concentration was determined by the thiobarbituric acid (TBA) reaction. Absorbance of supernatant was read at 532nm, 450nm, 600nm.The MDA concentration was calculated using the following relationship:

$$\text{MDA } (\mu\text{mol /g FW}) = [6.452 \times (D_{532}-D_{600}) - 0.559 \times D_{450}] \times V_t/V_1 \times \text{FW}$$

V_t represents total volume of extraction (ml); V_1 represents the extracted liquid volume for detesting (ml); FW represents fresh weight of samples (g).

Data statistical analysis: All data were subjected to analysis of variance (ANOVA) with SAS software package (Anon.., 1996). Appropriate standard errors of the means (S.E.) were calculated for presentation with table and figures. The significance of the treatment effect was determined using F-test, and to determine the significance of the means differences, least significant differences (LSD) were estimated at 5% probability level.

Results

Plant growth performance: Plant growth was reflected by measuring dry matter (DM) and grain yield (GY). Dry matter and GY of two maize cultivars were significantly inhibited by long-term mild drought stress (LMDS). Under LMDS condition, DM of Shaandan 9 (S_9) and Shaandan 911 (S_{911}) decreased by 15-24 and 30-37%, respectively. Grain yield of them declined by 8 and 20% correspondingly. Such, S_9 maintained greater DM production and GY than S_{911} under LMDS. However, control treatments resulted in no significant difference in DM production between both cultivars at seedling and elongation stages. Whereas, a markedly higher DM at heading and mature stage and GY were recorded for S_{911} as compared to those for S_9 (Table 1).

Inhibition in plant growth was significantly alleviated by exogenous glycinebetaine (GB) under LMDS (Table 2). Fifty mg/L GB spray markedly increased DM accumulation and GY of two cultivars under LMDS unlike controls, which produced no such significant effect. Exogenously applied GB increased DM of S_{911} by 12% and that of S_9 by 8%. Grain yield showed the similar pattern, whose increase rates was 9% and 7% for S_{911} and S_9, respectively (Table 2).

Plant water status: Leaf relative water content (RWC) is known as an alternative measure of plant water status. By comparison with control conditions, leaf RWC in each cultivar was declined under LMDS. RWC of both cultivars reduced at a higher decrease rate in S_{911} than those in S_9. As a result, S_{911} documented higher RWC under LMDS. In contrast, control plants followed no significant difference between the two cultivars (except mature stage) (Table 3).

Treatment with exogenous GB significantly increased RWC of the two cultivars under LMDS. The greater effects of exogenous GB on raising RWC were apparent with S911 compared with S9 when imposed to the same DS treatment. However, no significant impacts of exogenous GB spaying occurred on well-watered plants of either cultivars (Table 4).

Antioxidant enzymes activities: The changes of SOD, POD and CAT enzyme activities in leaves suggest that oxidative stress may be an influential component of LMDS on maize growth. By comparison with controls, SOD activity in S_9 increased at higher rate than that in S_{911} at both seedling and elongation stags, whereas decreased at lower rate at both heading and mature stage under LMDS (Fig. 1A). CAT activity followed the similar pattern as SOD for the two cultivars at each stage (Fig. 1B). With respect to POD activity, S_9 were found to more increase as compared with S_{911} at seedling and elongation stages. At heading stage, POD activity of S_9 increased only. However, POD activity of S_9 decreased at lower rate than that of S_{911} at mature stage (Fig. 1C). Of these protective enzymes, activities of SOD and POD reached their peak later than that of CAT during LMDS. However, with the control treatments, no substantial difference was recorded in antioxidant enzymes activities (SOD, POD and CAT) between the two cultivars (except SOD, POD at mature stage).

We depicted quantitative estimation of antioxidant enzymes activities in leaves by means of exogenous GB application in Table 5. Addition of GB significantly increased activities of SOD and POD in the two cultivars together with CAT activity in S_{911} only under LMDS. The greater effects of exogenous GB on raising activities of SOD, POD and CAT were obtained in S_{911} than those in S_9 with the same DS (data shown in Table 5). However, no marked impact occurred in the control plants of the two cultivars.

Lipid peroxidition: The MDA concentration, an important indicator, was assayed in the present study (Fig. 1D and Fig. 2). In comparison to controls, LMWS caused greater accumulations of MDA in S_{911} than those in S_9. Regardless of cultivar, the maximum MDA concentration occurred at mature stage. However, with controls, no striking difference was

recorded in MAD concentration between the two cultivars except mature stage.

Table 1. Differential effects of long-term mild drought stress (LMDS) and two maize cultivars on dry matter (DM) at different growth stages and grain yield (GY).

Treatment		DM (g/plant)				
		Seedling	Elongation	Heading	Mature	GY (g/plant)
LMDS	S_9	8.63±0.08 b (d)	52.04±1.53 b (c)	79.34±2.81 c (b)	92.37±2.06 c (a)	49.38±1.49 c
	S_{911}	7.39±0.30 c (d)	44.94±1.20 c (c)	75.98±2.84 d (b)	89.16±1.26 d (a)	46.84±1.80 d
$Control_1$	S_9	10.11±0.75 a (d)	68.65±1.89 a (c)	100.14±2.37b(b)	118.66±2.09b (a)	53.22±1.67 b
	S_{911}	10.51±0.31a (d)	71.10±2.17 a (c)	107.05±2.73a (b)	129.71±1.99a (a)	58.58±1.76 a

Each value is mean ± S.E. based on average data of T_0 (no GB spray) and T (50mg/L GB spray) treatments for four replicates (n=4). Mean values in the same column by the same letters within variables are not significantly different among four treatments at the same growth date at the 0.05 level by the LSD (t) range test. Mean values in the same row followed by the same letters in parentheses within variables are not significantly different among four growth dates with the same treatment at the 0.05 level by the LSD (t) range test

* S_9: cv. Shaandan 9; S_{911}: cv. Shaandan 911.

Table 2. Differential effects of exogenous glycinebetaine (GB) on dry matter (DM) and grain yield (GY) in two maize cultivar under long-term mild drought stress (LMDS) and control.

Treatment		DM (g/plant)		GY (g/plant)	
		S_9	S_{911}	S_9	S_{911}
LMDS	T_0	56.02 ± 1.49 c (a)	51.31 ± 1.45 c (b)	47.74 ± 1.33 c (a)	44.76 ± 1.69 c (b)
	T	60.17 ± 1.75 b (a)	57.42 ± 1.35 b (b)	51.12 ± 1.65 b (a)	48.92 ± 1.90 b (b)
Control	T_0	74.58 ± 2.06 a (b)	79.56 ± 1.54 a (a)	53.10 ± 1.43 a (b)	58.92 ± 1.45 a (a)
	T	72.20 ± 1.49 a (b)	79.62 ± 2.06 a (a)	53.33 ± 1.90 a (b)	58.23 ± 2.06 a (a)

Each value is mean ± S.E. based on average data of four growth date for four replicates (n=4). Mean values in the same column by the same letters within variables are not significantly different among four treatments with the same cultivar at the 0.05 level by the LSD (t) range test. Mean values in the same row followed by the same letters in parentheses within variables are not significantly different between two cultivars at the 0.05 level by the LSD (t) range test

* S_9: cv. Shaandan 9; S_{911}: cv. Shaandan 911; T_0: no GB spray; T: 50mg/L GB spray

Table 3. Differential effects of long-term mild drought stress (LMDS) and two maize cultivars on leaf relative water content (RWC) (%) at different growth stages.

Treatment		Seedling stage	Elongation stage	Heading stage	Mature stage
LMDW	S_9	77.39 ± 1.24 b (b)	74.79 ± 1.16 b (c)	80.95 ± 1.08 b (a)	66.85 ± 1.41 c (d)
	S_{911}	73.64 ± 1.12 c (b)	69.89 ± 1.19 c (c)	78.9 3 ± 0.92 c (a)	63.86 ± 0.90 d (d)
Control CK	S_9	84.53 ± 1.30 a (b)	83.64 ± 0.91 a (b)	86.19 ± 1.34 a (a)	78.30 ± 1.10 b (c)
	S_{911}	85.96 ± 0.37 a (b)	84.30 ± 1.64 a (b)	87.22 ± 0.83 a (a)	80.49 ± 1.09 a (c)

Each value is mean ± S.E. based on average data of T_0 (no GB spray) and T (50mg/L GB spray) treatments for four replicates (n=4). Mean values in the same column by the same letters within variables are not significantly different among four treatments at the same growth date at the 0.05 level by the LSD (t) range test. Mean values in the same row followed by the same letters in parentheses within variables are not significantly different among four growth dates with the same treatment at the 0.05 level by the LSD (t) range test.

* S_9: cv. Shaandan 9; S_{911}: cv. Shaandan 911

Table 4. Differential effects of exogenous glycinebetaine (GB) on leaf relative water content (RWC) (%) in two maize cultivar under long-term mild drought stress (LMDS) and control.

Treatment	LMDS		Control	
	S_9	S_{911}	S_9	S_{911}
T_0	72.54 ± 1.61 c (a)	68.64 ± 1.29 c (b)	83.21 ± 1.05 a (a)	84.52 ± 0.92 a (a)
T	77.45 ± 0.96 b (a)	74.52 ± 1.17 b (b)	83.12 ± 1.16 a (a)	84.47 ± 1.46 a (a)

Each value is mean ± S.E. based on average data of four growth date for four replicates (n=4). Mean values in the same column by the same letters within variables are not significantly different among four treatments with the same cultivar at the 0.05 level by the LSD (t) range test. Mean values in the same row followed by the same letters in parentheses within variables are not significantly different between two cultivars at the 0.05 level by the LSD (t) range test

* S_9: cv. Shaandan 9; S_{911}: cv. Shaandan 911; T_0: no GB spray; T: 50mg/L GB spray

GB spray significantly decreased MDA concentration in the two cultivars under LMDS and thus alleviated adverse DS effects by reducing lipid peroxidation (data shown in Table 5). In contrast, no obvious effect with GB contributed to the well-watered plants. The alleviation level of lipid per-oxidation with GB was determined using decrease rate of MDA concentration compared to no GB spray under LMDS. With the increase of MDA accumulation, the decrease rates of MDA concentration were correspondingly rised by means of GB spray for the two cultivars under LMDS. Shaandan 911 recorded higher decrease rate of MDA concentration than S_9 under the same DS condition, which resulted in greater effects of GB on up-regulation lipid per-oxidation. The better positive roles of GB seemed to be found in the plants subjected to more serious damage from DS due to greater MDA accumulation at elongation and mature stages as compared to those at seedling and heading stages, respectively (Fig. 2).

Interaction of maize cultivar, water regime and exogenous GB for all parameters: Analysis of variation indicated the presence of a considerable amount of genetic variability, water supply and exogenous GB variability for the parameters of plant growth, water status and anti-oxidative system under both

LMDS and control (Table 6). The magnitudes of mean square for DM, GR, RWC, activities of SOD,CAT and MDA content in GB treatment (Gb) were, in general, less than water regime (W) while higher than cultivar (Cv). However, for POD activity, the mean square was in the order of Gb >Gv >W. Moreover, mean square due to W-Cv, W-Gb and Cv-Gb were significant both for most of parameters (except for POD activity and CAT activity due to Gn-Gb). Significant mean square due to Gn-W-GB was also obtained for DM, GR, RWC, SOD activity, CAT activity and MDA concentration (except for POD activity).

Discussion

Effects of drought stress and maize cultivar on water status and plant growth as well as lipid peroxidation and antioxidant enzymes activities: Drought stress (DS) is one of the most important abiotic stresses and seriously affects water relation and productivity of a crop. Relative water content (RWC) in leaves is known as an important and efficient alternative measure of plant water status, reflecting the metabolic activity in tissues (Taiz & Zeiger, 2002; Seghatoleslami *et al.*, 2008). Decrease in RWC indicates a loss of turgor that results in limited water availability for the cell extension process in crop plants (Taiz & Zeiger, 2002). Drought stress can disrupt homeostasis in water status in plants. Crucial changes in water status lead to molecular damage, growth inhibition and even death. It is already known that different crop cultivars hold different responses to different levels of DS in view of water status and plant growth. The present studies have elucidated that LMDS could stimulate a more serious reduction in RWC and production in a drought sensitive cultivar Shaadan 911 (S_{911}) than those in a drought tolerance cultivar Shaadan 9 (S_9) (Table1 & Table 3), which have been proved in wheat (*Triticum aestivum* L.) by Chandrasekar *et al.,* (2001) and proso millet (*Panicum Miliaceum* L.) by Seghatoleslami *et al.*, (2008).

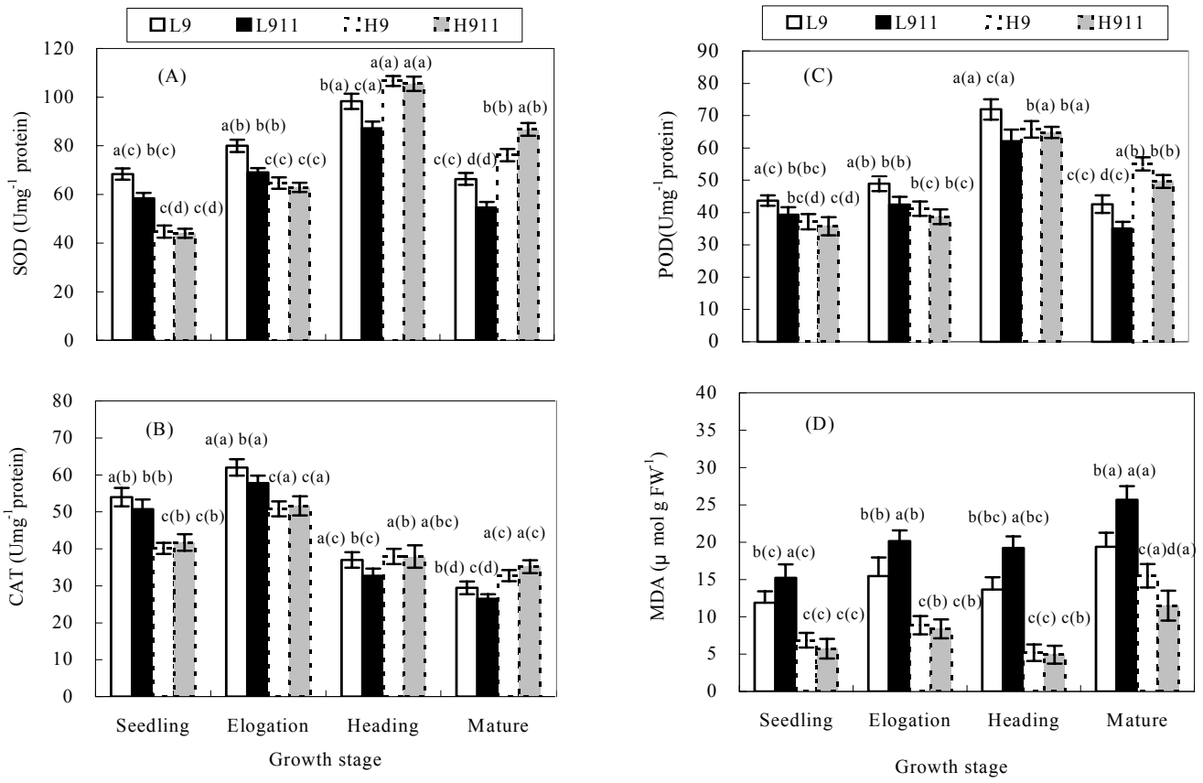

Fig. 1. Differential effects of long-term mild drought stress (LMDS) and two maize cultivars on activities (U/mg protein) of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) at different growth stages.
Each value is mean±S.E. based on average data of T_0 (no GB spray) and T (50mg/L GB spray) treatments for four replicates (n=4): (A) SOD activity; (B) CAT activity; (C) POD activity; (D) MDA concentration.
At the top of each column by the same letter out and in parentheses within variables are not significantly different among four treatments at the same date and four growth dates with the same treatment at the 0.05 level by the LSD (t) range test, respectively.
L9 and L911: cv. Shaandan 9 and Shaandan 911 exposure to long-term mild water stress, respectively; H9 and H911: cv. Shaandan 9 and Shaandan 911 exposure to control treatments, respectively.

It is clear that the capacity of the antioxidative defence system and level of lipid peroxidation are important in limiting oxidative damage and destroying excessive active oxygen species (AOS) for normal metabolism under DS (Arora *et al*., 2002). As a widespread response, the elevated production of antioxidants is being thought to counteract the dehydration effect resulting in reducing lipid peroxidation and maintaining macromolecular structure or function under drought. Considerable evidences suggest that the change pattern of antioxidant enzymes activities and MDA accumulation in water-stressed plants might be closely marked differently depending on drought resistance of a cultivar, level of DS as well as growth stage (Mencon *et al*., 1995; Arora *et al.*, 2002; Ge *et al*., 2004; Fig. 1; Fig. 2). In this work, the comparison of lipid peroxidation and antioxidant enzymes activities in two maize cultivar exposure to LMDS may be helpful in developing a better understanding of tolerance mechanisms to drought. In general, with the development of plant growth,

antioxidant enzymes activities initially rised and declined afterward to varied levels under DS, but the date of change varied with different drought-resistant cultivars. Activities of SOD and POD reached their summit later than that of CAT. Plants consenescence might deepen the lipid peroxidation in leaves due to the largest accumulation of MDA at mature stage (Fig. 1 & Fig. 2). It is now well concluded that the oxidative enzymes activities in a drought sensitive cultivar may usually decline earlier at greater decrement than those in a drought tolerance cultivar under the same strength of DS, which is in agreement with results obtained by Ge et al., (2006). As previous studies (Zhang & Kirkham, 1994; Zhang *et al.*, 2007), a drought-tolerance cultivar can employ great activities of antioxidant enzymes than a drought-sensitive cultivar under drought. The increased activities of anti-oxidative enzymes induced by moderate DS can protect cell membranes, proteins and metabolic machinery, which would preserve sub-cellular structure from damage as a result of cell dehydration (Arora *et al*., 2002). Consequently, a drought tolerance cultivar can possess a stronger ability to eliminate excessive AOS and reduce lipid peroxidation (Mencon *et al*., 1995; Arora *et al*., 2002; Ge *et al.*, 2006), resulting in higher crop production finally. These findings have further proved the results reported in an earlier paper by the authors in terms of nitrogen supply under drought (Zhang *et al.,* 2007). It is also can be primarily demonstrated that responses of plants to DS appeared to be slower in SOD and POD than CAT in maize leaves. An aged-dependent MDA accumulation appeared to be related to different growth stage (Ge *et al.*, 2006; Zhang *et al.,* 2007; Fig. 1; Fig. 2).

Table 5. Differential effects of exogenous glycinebetaine (GB) on activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) (U/mgprotein) and malondialdehyde (MDA) concentration (μmol/gFW) in two maize cultivars under long-term mild drought stress (LMDS).

Treatment			SOD activity	POD activity	CAT activity	MDA concentration
LMDS	S_9	T_0	73.33 ± 2.76 b	48.84 ± 2.18 b	44.84 ± 2.21 ab	17.22 ± 1.91 b
		T	83.14 ± 2.94 a (13)	54.74 ± 2.26 a (12)	46.35 ± 2.41 a	12.96 ± 2.22c (24)
	S_{911}	T_0	61.78 ± 2.28 c	41.31 ± 3.81 c	40.53 ± 2.59 c	23.92 ± 1.72 a
		T	72.80 ± 2.27 b(18)	48.19 ± 2.43 b (17)	42.43 ± 1.50 bc	16.26 ± 1.69bc(31)
Control	S_9	T_0	73.10 ± 2.02 b	49.78 ± 3.18 ab	40.45 ± 2.44 c	9.29 ± 1.79 d
		T	73.02 ± 2.68 b	49.81 ± 3.19 ab	40.37 ± 2.00 c	9.42 ± 1.81 d
	S_{911}	T_0	74.67 ± 2.69 b	46.78 ± 2.89 b	40.62 ± 1.55 c	8.05 ± 2.12 d
		T	74.90 ± 2.07 b	47.68 ± 3.78 b	41.05 ± 1.69 c	7.74 ± 1.97 d

Each value is mean ± S.E. based on average data of four growth date for four replicates (n=4). Mean values in the same column by the same letters within variables are not significantly different among eight treatments at the 0.05 level by the LSD (t) range test. Data in parentheses are increase rates (%) for activities of SOD, POD and CAT or decrease rate (%) for MDA concentration with GB compared with no GB spray
* S_9: cv. Shaandan 9; S_{911}: cv. Shaandan 911; T_0: no GB spray; T: 50mg/L GB spray

Table 6. Mean square of exogenous glycinebetaine (GB) with two maize cultivars in normal (Control) and long-term mild drought stress (LMDS) environment for dry matter (DM) , grain yield (GY), relative water content (RWC), activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) and malondialdehyde (MDA) concentration.

Source of variation	d.f.	DM	GY	RWC	SOD activity	POD activity	CAT activity	MDA concentration
W	1	2000.56***	296.94***	490.510***	168.911***	7.492***	71.139***	436.139***
Cv	1	3.24***	42.45***	8.166***	17.561***	11.165***	9.804***	17.459***
Gb	1	23.60***	49.30***	33.796***	21.869***	69.054***	16.105***	58.625***
W× Cv	1	106.76***	132.54***	6.531***	406.644***	131.461***	8.260***	70.144***
W×Gb	1	59.41***	23.40***	9.126***	156.519***	51.480***	7.304***	55.176***
Cv×Gb	1	7.23***	3.44***	0.331*	1.638**	0.0001^{ns}	0.163^{ns}	4.463***
Cv×W×Gb	1	0.48*	3.21*	0.380*	1.042*	1.105^{ns}	2.996**	2.476***
Error	21	0.07	0.62	0.067	0.118	0.411	0.184	0.036

*p<0.05, **p<0.01, ***p<0.001, ns: not significant
W, Cv and Gb represent water regime, cultivar and glycinebetaine treatment, respectively

Effects of exogenous glycinebetaine (GB) on water status and plant growth as well as lipid peroxidation and antioxidant enzymes activities in two maize cultivars under drought stress (DS): Crop losses caused by environmental stress might be reduced by applying osmoprotectans to crop canopies (Demiral & Türkan, 2004; Ashraf & Foolad, 2007). Since the GB level in the plant tissue serves as an index of the internal water status of plants, an increased level of leaf RWC in crop plants under DS suggests that GB may play an protective role in preventing cell injury from stress-induced dehydration (Ashraf & Foolad, 2007). There are many reports demonstrating positive effects of exogenous GB on water relation, plant growth and final crop yield under drought: examples include those in wheat, maize, sorghum (*Sorghum Bicolor* L.), common beans (*Phaseolus vulgris* L.) and barley (*Hovdeum vulgare* L.) (Agboma *et al.*, 1997; Wei Bing & Rajashekar, 1999; Tian, 2001; Table 2; Table 4). However, differential effects of GB on modulation crop production and water relation might be largely associated with drought resistance of a cultivar, level of DS. In this experiment, the authors found that the sensitive cultivar S_{911} registered greater increment of production and RWC than the tolerant cultivar S_9 (Table 2; Table 4).

Drought tolerance is often correlated with a more efficient antioxidative system. Peroxidation of lipid membrane of higher plant usually reflects free radical-induced oxidative damage at the cellular level under DS (Mencon *et al.*, 1995). Hence, studies on exogenous GB expressing increased activities of antioxidative enzymes and alleviated lipid peroxidation in environment-stressed plants have employed

lots of plant scientist to fall within these scopes to confer the possible physiological mechanism underlying a degree of tolerance to stress by this means. Some studies have mainly focused on anti-oxidative system responses with GB under salinity stress (Demiral & Türkan, 2004; Zhang & Li, 2004; Ashrafa & Foolad, 2006; Raza *et al.*, 2007), however, with little relative literature under long-term mild drought stress (LMDS). Fewer researchers found the positive effectiveness of GB spray on raising some antioxidant enzymes activities whereas reducing MDA concentration under short duration of DS (Tian, 2001; Ma. *et al.*, 2006), which is in accordance with the findings of authors (Table 5; Table 6). In spite of these, in the present study, we have further enlightened than the varied positive effects of GB on lipid peroxidation and antioxidant enzymes activities might be closely dependent on drought resistance of cultivar as well as level of DS (Table 5). Prominent increases in activities of antioxidant enzymes whereas marked declines in MDA accumulation with GB were more pronounced in a sensitive cultivar (S_{911}) than those in a tolerant one (S_9). Various effects to relief lipid peroxidation with GB appeared to be determined by the detrimental degree from DS (MDA concentration). As a preliminary result, an aged-dependent MDA concentration decrement with GB appeared to be related to differences of MDA accumulation at different growth stages. The greatest positive function of GB seemed occur in the maize plants yield to the most severe damage from dehydration due to the most MDA accumulation at mature stage (Fig. 2).

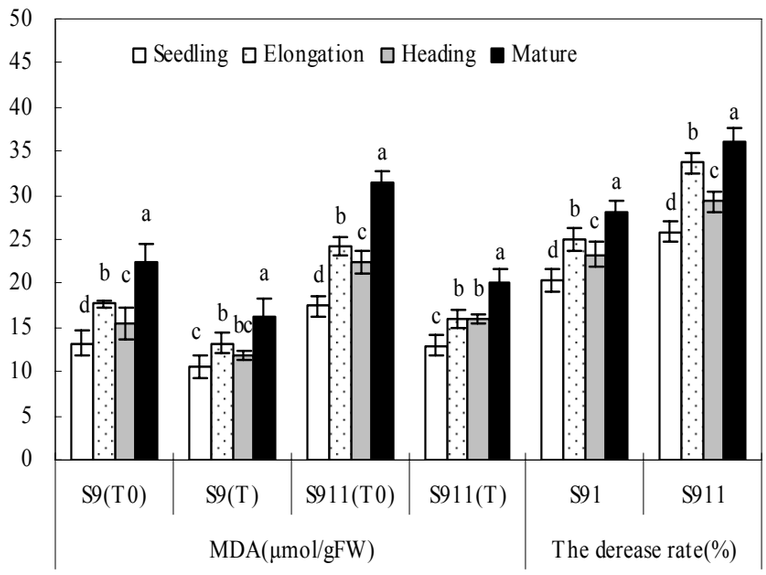

Fig. 2. Differential effects of exogenous glycinebetaine (GB) on the alleviation of lipid per-oxidation at different growth stages under long-term mild drought stress (LMDS).
At the top of each column, different letters indicate significant differences amongst four growth dates with the same cultivar *(p<0.05)*.
S9 (T0) and S9 (T) represent cv. Shaandan 9 with and without GB application, respectively; S911 (T0) and S911 (T) represent cv. Shaandan 911 with and without GB application, respectively. Decrease rate (%) of MDA concentration (%) indicates the effect of GB on the alleviation of lipid per-oxidation in comparison to controls.

Thus, it is well established that exogenous GB obviously reduced the impact of DS on plant growth, water relation and antioxidant system unlike the well-watered conditions, which exhibited its marked anti-drought roles whereas denied its nutritive function. The optimal rate of GB might greatly protect plant cells from environmental stresses indirectly through positive effects on the marker enzymes related to anti-oxidative system (such as SOD, POD and CAT) and membrane integrity (Taiz & Zeiger, 2002; Ashrafa & Foolad, 2006; Table 5; Table 6). Crops production were corresponding raised finally with GB under drought (Díaz-Zorita *et al.*, 2001; Zhang & Li, 2004; Table 2). However, an opposite response of crop plants with GB to DS was also observed by Sun et al., (2001). They found that external GB (1mmol) decreased the activities of SOD and POD and RWC whereas increased the content of MDA in barley leaves under osmotic stress (PEG4000, -0.86Mpa). It seems that effective and efficient effects of GB may vary with plant species, developmental stage, the doses and time of application, and environmental conditions under which plants are grown (Ashraf & Foolad, 2007).

Interaction of maize cultivar, water supply and exogenous GB for all parameters: Different crop cultivars maintain different plant growth, water status and antioxidant system parameters, which are significantly affected by exogenous GB (Agboma *et al.*, 1997; Zhang & Li, 2004). Significant mean square values for interaction among cultivar, water regime and exogenous GB, suggested that the choice of environments and cultivar were appropriate (Table 6). Thus, the variation of environments (water regime and exogenous GB) over cultivar for production, RWC and most of anti-oxidative traits could provide scope for exogenous GB applying by virtue of crop yield, water use efficiency and antioxidant level under drought (Chandrasekar *et al.,* 2000; Sakamoto *et al.*, 2000; Ashraf & Foolad, 2007).

Conclusions

In summary, we have confirmed the difference in biological and physiological characteristics and GB-modulated ability differing in drought tolerance crops under long-term mild drought stress (LMDS). Significant interaction among cultivar, water regime and exogenous GB for all parameters measured have been also elucidated. Hence, the results obtained in the previous and present studies suggest that LMDS could induce substantial growth retardance and water states non-homeostasis by hastening lipid peroxidation and unbalancing antioxidant system. Greater SOD, POD and CAT enzyme activities as well as lower MDA accumulation in a drought-tolerant cultivar could produce better water status and higher DM and GY under drought rather than in a drought-sensitive cultivar. Greater GB-induced modulation of lipid peroxidation, antioxidant enzymes activities as well as plant growth and water status were observed in the maize plants exposure to LMDS unlike well-watered conditions. Such, the outstanding function of GB in view of drought resistance have been conferred based on denying its nutritive function in the same trial. Beneficial effects of GB spray were attributed to enhanced SOD, POD and CAT enzyme activities as well as decreased MDA accumulation, leading to increased drought tolerance and water status as well

as higher DM and GY production in DS plants. These favorable responses to GB spray were greater in a drought-sensitive cultivar as compared to those in a drought-tolerant cultivar. The greatest positive function of GB seemed occur in the maize plants yield to the most severe damage from dehydration due to the largest MDA accumulation at mature stage. Hereby, we propose that GB should be firstly applied to a drought-sensitive cultivar plants submitted to stronger DS damage to bring out its potential to ameliorate plants growth fully. Nevertheless, there is a need for optimizing the dose-response relationship in different plant species, at different developmental stages and growth conditions before the use of GB as a "stress–tolerance stimulator" may become routine and commercially profitable.

Acknowledgements

This work was jointly supported by China Postdoctoral Science Foundation, Chinese Universities Scientific Fund (QN2009069), and the Foundation of State Key Laboratory of Soil Erosion and Dryland Farming (10501-J-2).

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(Received for publication 10 June 2010)