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FOLIAR UREA APPLICATION AFFECTS NITRIC OXIDE BURST AND GLYCINEBETAINE METABOLISM IN TWO MAIZE CULTIVARS UNDER DROUGHT

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Abstract

Foliar urea has been proved to act a better role in alleviation of the negative effects of drought stress (DS). However, the modulation mechanism of foliar urea are not conclusive in view of nitric oxide (NO) burst and glycinebetaine metabolism and their relationship. Two maize (*Zea mays* L.) cultivars (Zhengdan 958, JD958, Jundan 20, ZD20) were grown in hydroponic medium, which were treated with spraying of urea concentration of 15 g L^{-1} and two water regimes (non-stress and DS simulated by the addition of polyethylene glycol (PEG, 15% w/v, MW 6000). The ten-day DS treatment increased betaine aldehyde dehydrogenase (BADH) activity, choline content and nitric oxide (NO) content acted as the key enzyme, initial substrate and a nitrogenous signal substance respectively in GB synthesis metabolism, thus, induced to great GB accumulation. The accumulation of NO reached the summit earlier than that of GB. The more positive/less negative responses were recorded in JD958 as compared with ZD20 to DS. Addition of foliar urea could increase accumulation of choline and BADH activity as well as NO content, thereby, increase GB accumulation under DS. These positive effects of urea applying foliarly on all parameters measured were more pronounced in cultivar JD20 than those in ZD958 under drought. It is, therefore, concluded that increases of both BADH activity and choline content possibly resulted in enhancement of GB accumulation. Foliar urea application could provoke better GB accumulation by modulation of GB metabolism, possibly mediating by NO burst as a signal molecule during drought, especially in the drought sensitive maize cultivar.

Introduction

The frequency of drought crisis has been increasing in many regions of the world, which is responsible for the greatest agriculture losses. Maize *(Zea mays* L.) is an important cereal crop in these areas, whose growth is highly sensitive to drought resulting in a significant yield reduction (Li, 2007). Drought stress (DS) creates a wide array of biochemical and physiological responses due to crucial changes in water homeostasis, which resulting in a decline in the chemical activity of water and a loss of turgor in plant cells, and inducing plant growth arrest, and even death finally (Taiz & Zeiger, 2002). On the other hand, to diminish the adverse effect of DS and ensure crops under optimal growth conditions, plants have evolved some defense mechanisms such as a large rapid accumulation of small molecular osmotic nitrides such as glycinebetaine (GB) to reduce depletion of cellular water and reestablishing cell turgor, which are derived from GB metabolism in plant cells (Ashraf, 2010). This efficient osmotic nitrides not hold better osmotic modulation function as a quaternary ammonium compound due to its zwitterionic and high hydrophilic characters, but protect biological macromoleculars from oxidative damage (Ashraf & Foolad, 2007). Such, high GB metabolism level in plant could be responsible for maintaining better water status and optimal growth under DS (Ashraf, 2010). The previous studies revealed that the pattern of GB accumulation was associated with crop cultivar, growth stage as well as DS treatment pattern (Ashraf & Foolad, 2007; Zhang *et al.*, 2009a). In plants, biological synthesis of GB contained two-step oxidation from its precursor choline. The first oxidation step is catalyzed by choline monooxygenase (CMO, EC 1.14.15.7), and the further oxidation to GB is catalyzed by betaine aldehyde dehydrogenase (BADH, EC 1.2.1.81), the enzymes in charge of GB biosynthesis (Sakamoto & Murata, 2002; Sithtisarn *et al.*, 2009).

The most characterized stress signal molecule is nitric oxide (NO), which posses pivotal physiological responses required for DS adaptation, including osmotic adjustment and water homeostasis (Misra *et al.*, 2011). Some reports showed NO in the plant kingdom can involve in growth, development and defense responses against oxidative stress under various adverse conditions (Misra *et al.*, 2011). In the recent years, the function of NO in plant has gained considerable attention of many researchers in stress physiology field. Much investigations indicated that NO not only has a vital antioxidant characteristic, but posses a signal function in activating antioxidant enzyme activities and some osmolytes metabolism under abiotic stress (Liu & Zhang, 2009; Misra, *et al.*, 2011). Although the modulation roles of NO have been well documented (Siddiqui *et al.*, 2011), it remains uncertain how this uniquely volatile molecule coordinately regulate stress adaptation in view GB metabolism submitted to foliar urea under drought (Ashraf, 2010).

One of the impact factors of plant growth under drought is mineral nutrition (Li, 2007). The positive of nitrogen (N) supply by roots has been well elucidated in relation to plant growth and its corresponding physiological process (Saneoka *et al.*, 2004; Li, 2007; Zhang & Li, 2007; Zhang *et al*., 2009a). However, its efficacy of uptake by roots become more restricted than foliar application under drought due to the negative effect of drought and less nutrient availability (Römheld & El-Fouly, 1999; Mosali, *et al.*, 2006), especially for early growth stages of crop plant (Mallarino *et al.*, 2001). Thus, foliar N application may be a more efficient measure to alleviate DS damage of crop plant under drought conditions (Hu *et al.*, 2008). In an earlier paper, the authors found better effectiveness with foliar urea might be obtained in a drought sensitive maize cultivar than a drought tolerance one in response to DS. It is likely that the main great contributor to osmotic adjustment might be GB in the two maize cultivars., which are vital factors known to be responsible for improving growth and production under DS (Zhang *et al*., 2009a & b).

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Keeping in view the above facts, we hypothesized that applications of foliar urea may compensate for the drought-induced deficiency in osmotic adjustment ability and consequently facilitate maize plant adaptation to a drought environment by employing higher constitutive GB metabolism mediating by NO burst. With this aim, we designed solution culture experiments to clarify the responses of foliar urea with respect to NO burst, GB metabolism and its relationship submitted to DS.

Materials and Methods

Plant material and trial location: Hydroponic experiments were carried out under a climatic growth chamber. Two maize (*Zea mays* L.) cultivars (drought-tolerant Zhengdan 958 and -sensitive Jundan 20) were used in the present experiments (Zhang *et al.*, 2007).

Plant growth and experiment design: After germination treatment of seeds, they were fixed into holes of styrofoam boards in deionized water in plastic boxes (26×18×12 cm) covered with black plastic in the growth chamber under the optimal environmental conditions (Zhang *et al.*, 2009a). One-half-strength and complete nutrient solution were supplied on 4th and 8th day after the seedlings growth in stead of deionized water (Hoagland & Arnon, 1938). The pH of the nutrient solution was maintained at the range of 6.3 (±0.1).

When seedlings grew at the stage of three-leaf, drought stress (DS) treatment was initiated by supplying 15% (w/v) polyethylene glycol (PEG-6000) dissolved in complete nutrient solution to achieve osmotic potentials (ψs) of -0.72 Mpa determined by a vapor pressure Wescor 5500 osmometer for 10 days (Zhang *et al.*, 2009a). Complete nutrient solution without PEG-6000 served as the control (C). For each water regime, the seedlings were sprayed with 15ml two levels of urea (reagent grade) concentration [0 (N_0) distilled water mixed with neutral soap (0.10%) and 15g urea L^{-1} (N) in 0.10% neutral soap solution] per plant each time. The spray was applied twice per day on the 1st, 3rd, and 6th day after stress treatment, respectively. Complete design was used with a total of 8 treatments, each cultivar including 4 treatments: DS (N_0), DS (N); C (N_0), C (N). Four replicates (6 plants per replicate, i.e. 24 plants each treatment) were maintained for each treatment in a random block design. A backpack sprayer was used to spray the nutrient solution or water. The foliar fertilization wet the whole leaf surface. All treatments were replicated 4 times. Desired PEG concentrations were maintained by irrigating sufficiently with new solution. The solution was aerated 12 hours a day.

To explore the relationship between NO and DS in promoting glycinebetaine accumulation in maize plants, a second experiment was carried out using both maize cultivars Zhengdan 958 and Jundan 20 under the two treatments, control and 15% (w/v) PEG-6000 in nutrient solution.

The experiment was carried out twice under the same environmental conditions. Data presented are the means of four replicates of the two experiments (n=8).

Sampling and recording of data: The maize plants were harvested on the 10th day after the onset of drought or foliar urea treatments. The second or third leaf from top of each plant was used for NO and GB metabolism parameters determinations.

NO concentration was assayed as described by Griess (1897) with some modifications. Fresh plant materials (1.0 g) were homogenizied in 1 mL 40 mmol $LHEPES^{-1}$ pH 7.2 buffer, washed with 4 mL buffer, filtered through 2 layers of gauze. The filtered solution was then centrifuged at 4440×g for 10 min at 4°C and supernatants were used for determination of NO concentration according to the indication on the NO assay kit (Produced by Beyotime Institute of Biotechnology, Shanghai, China). Aliquots of 1 ml were put in test tubes, cooled in ice water for 1 h, before 2 mL Griess Reagent I solution was added. The reaction was maintained 60 min at 37°C, then centrifuged at 710×g for 10 min at 25°C. Aliquots of 2 ml supernatant were added in 2 ml Griess Reagent II solution at 25°C. After 10 min, the absorbance was measured at 540 nm with UV-visible spectrophotometer. A standard curve using $NaNO_2$ was generated for quantification. The NO content was expressed as nmol g^{-1} DW.

Glycinebetaine (GB) content was determined following Grieve & Grattan (1983) with some modifications. Dried and finely powdered plant material (0.5 g) was shaken with 20 ml of deionized water for 24 h at 25°C. The extracts were diluted 1:1 with 2 N H_2SO_4. Aliquots of 0.5 ml were put in test tubes and cooled in ice water for 1 h, before a cold KI-I_2 reagent (200 µl) was added. The tubes were stored at 0-4°C for 16 h and then centrifuged at 12,000×g for 15 min at 4°C. The supernatant was aspirated. The periodite crystals were dissolved in 5 ml of 1, 2-dichloroethane. After 2-2.5 h, the absorbance was read at 365 nm with UV–visible spectrophotometer. Reference standards of GB (50–200 g m^{-1}l) were used for calibration and estimation of GB concentration in unknown samples. The GB content was expressed as nmol g^{-1} DW.

BADH activity was assayed as described by Daniell *et al.*, (2001) with some modifications. To obtain crude protein extracts, plant materials were homogenized in 250 µl homogenization buffer containing 50 mM HEPES-KOH, pH 8.0, 1 mM EDTA, 20 mM sodium metabisulfite, 10 mM sodium borate, 5 mM ascorbic acid, 5 mM dithiothreitol, and 2% (w/v) PVPP. The homogenates were then centrifuged at 12,000×g for 15 min at 4°C and the supernatants were used for determination of BADH activity. The BADH activity was assayed by monitoring the absorbance at 340 nm with 0.05 mM betaine aldehyde chloride as a substrate. The activity was calculated using the extinction coefficient of 6220/M cm for NADH. The BADH activity was expressed as µmol min^{-1} mg^{-1} DW.

Choline content was determined following Feng & Ren (2004) and Richard & Emily (1945) with some modifications. Dried and finely powdered plant material (0.5 g) was added with 70 ml of deionized water in a triangle vase (100 ml volume) and shacken up. The vase was bathed in hot water at 80°C for 15 min, shaken properly with an oscillator for 30 min, set to 100 ml volume scale, and then filtered through a filter paper. Aliquots of 10 ml filtered solution were poured into a triangle vase, cooled in ice water to -5°C before 15 ml Reinecke salt- methanol solution was added (4 g Reinecke salt was dissolved in 100 ml methanol). The mixed solution was stirred for 30 min and placed in a refrigerator

for 12 h. The red water insoluble substance was filtered out, washed with 10 ml propylalcohol three times, dissolved with acetone, and set to 25 ml volume scale. The choline content was assayed by monitoring the absorbance at 520 nm with UV–visible spectrophotometer using acetone as a control. Reference standards of choline (9-27 mg ml^{-1}) were used for calibration and estimation of choline concentration in the unknown samples. The choline content was expressed as nmol g^{-1} DW.

Statistical analysis

The data for all parameters were analyzed statistically using the SAS software package (SAS Institute Inc., Cary, NC, USA, 1996). The analysis of variance (ANOVA) was followed by the least significance test (LSD) to determine the significance of the means at the 0.05 level.

Results

Effects of drought stress (DS), foliar urea application on accumulation pattern of glycinebetaine (GB) in maize seedlings: Endogenous NO and GB in both cultivars accumulated with prolonged period of DS treatment. The more predominant response occurred in Zhengdan 958(ZD958) than those in Jundan 20 (JD20) (Fig. 1). Nitric oxide (NO) content in ZD958 and JD20 reached their maximum after 12 and 24 h of the start of DS treatment, respectively, being 6.1- and 5.2-fold of that of the control plants. In contrast, GB content in ZD958 and JD20 was maximum after 36 and 60 h of DS treatment, being 2.7- and 2.1-fold of that of the control plants, respectively. The maximum accumulation of NO occurred earlier than that of GB in the leaves of drought-stressed maize plants (Fig. 1).

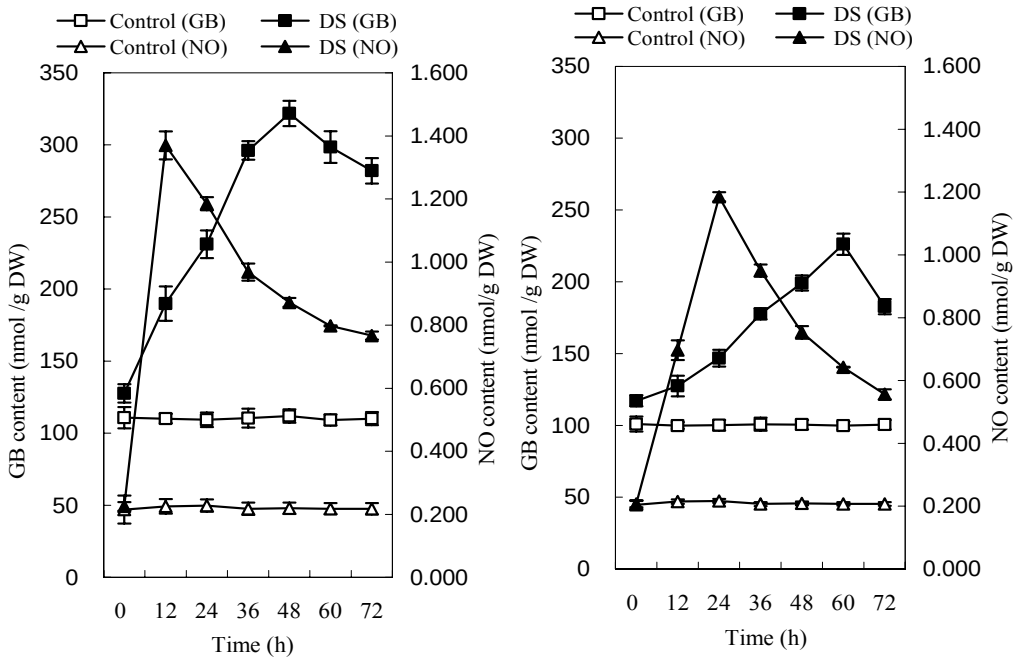

Fig. 1. Effects of drought treatment on contents of endogenous NO and glycinebetaine (GB) in the seedlings of Zhengdan 958 (left) and Jundan 20 (right) cultivars.

The accumulation of NO and GB was modulated by the foliar application of urea in maize plants subjected to DS. Addition of foliar urea increased endogenous NO and GB levels 1.1- and 1.8-fold in ZD958 and 0.8- and 1.4-fold in JD20, respectively under DS above control treatment. As for the control plants, application of foliar urea also increased endogenous NO levels, however, the increase rates became much less than those in drought-stresses plants. As for GB levels, the non-significant effects occurred in the controlled plants by foliar urea application (Fig. 2 A & B).

Modulation role of foliar urea application on GB metabolism in the leaves of maize plants under drought stress: Glycinebetaine metabolism determination can be done by assessing the amount of its precursor choline and the activity of the key enzyme BADH. The choline content and BADH activity in the leaves are shown in Fig. 2 C & D. The choline content and BADH activity both obviously increased by 1.1-fold and 2.7-fold in ZD958, and 0.8-fold and 2.0% in JD20 in plants not treated with foliar urea application, however, in contrast, 1.8-fold and 3.6-fold in ZD958, and 1.4-fold and 3.0-fold in JD20 in foliar urea-treated plants.

Under DS, plants of both cultivars treated with foliar urea application had higher BADH activity as compared with that in plants having no foliar urea application treatment. The beneficial effects of foliar urea application on BADH activity and choline content were superior in JD20 than those in ZD20 has compared with their counterparts under controls. In contrast, under non-stress conditions, the addition of foliar urea imposed no significant effects on both contributors (Fig. 2 C & D).

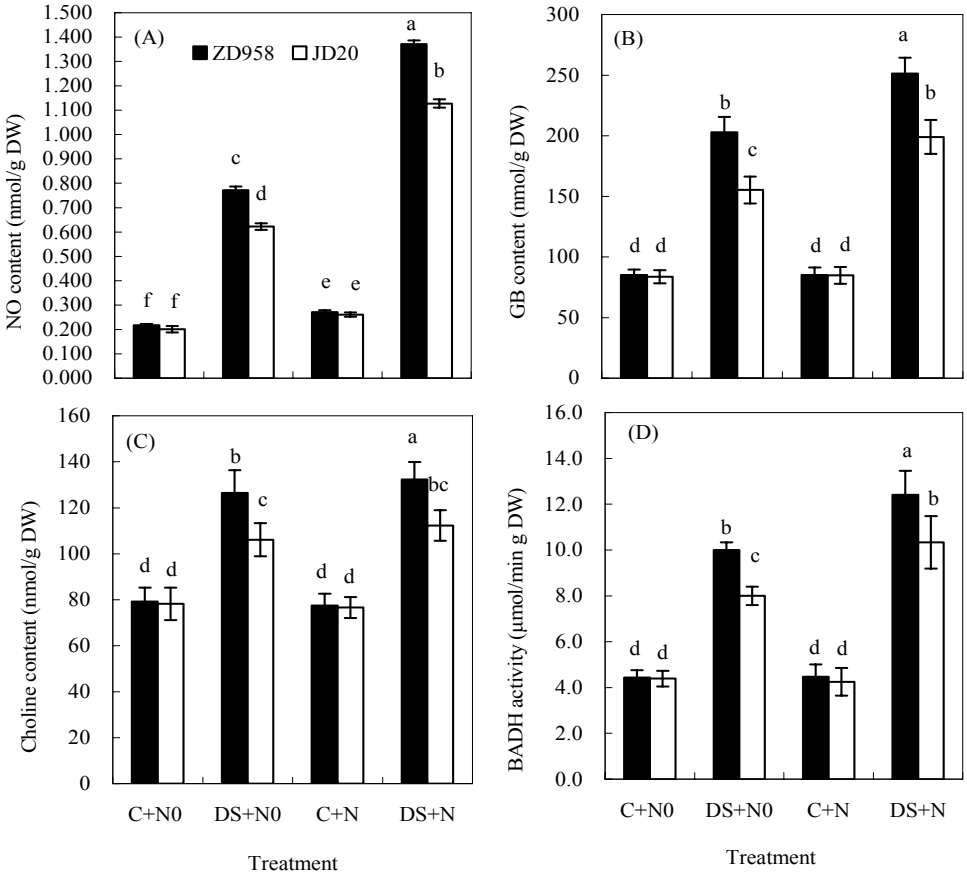

Fig. 2. Effects of foliar urea on endogenous NO content (A), glycinebetaine content (B), choline content (C) and BADH activity (D) in the seedlings of two maize cultivars under drought stress.

Each values is the mean±S.E. of eight replicates each treatment (n=8). JD20 and ZD958 represent Jundan 20 and Zhengdan 958 respectively. N0 and N represent without and with foliar urea treatment respectively. DS and C represent drought stress and non-DS respectively.

At the top of each column, different letters indicate significant differences for each parameter determined among treatments. Mean values with the same letter within variables are not significantly different at the 0.05 level.

Correlations among all parameters measured: Correlation coefficients among all traits evaluated were significantly higher under DS than those under control. Especially, correlations between content of NO and choline content/BADH activity were evident under DS but not for those under control treatment (Table 1).

Interaction of foliar urea treatment and water regimes as well as correlation coefficients for all parameters measured: Maize cultivars used in this study, water regimes and foliar urea treatments had significant effects on all parameters. The magnitudes of F values across the above parameters were in the order: water regime > foliar urea > cultivar. The interaction effects among the above treatments were also mostly significant for all response variables except Cv× FU and W×Cv×FU for choline content and BADH activity (Table 2).

Discussion

Drought stress (DS) is one of the important abiotic stresses and seriously affects many physiological and biochemical processes of a crop (Li, 2007). Glycinebetaine (GB) content in leaves is known as an important and efficient physiological index of osmotic adjust ability in plants (Ashraf, 2010). Considerable evidences suggest that different crop cultivars hold different responses to DS in view of GB accumulation (Ashraf & Foolad, 2007). The present studies and an earlier paper by the authors have elucidated that DS could stimulate more increase in GB content in a drought tolerant cultivar than that in a sensitive drought one (Zhang & Li 2007; Zhang *et al.*, 2009a& b). Glycinebetaine is synthesized from its precursor choline by a two-step oxidation via betaine aldehyde are choline monooxygenase (CMO), a ferredoxindependent soluble Rieske-type protein, betaine aldehyde dehydrogenase (BADH), and soluble NAD^+ dependent enzyme (Ashraf & Foolad, 2007). However, there are not sufficient data to prove the GB metabolism mechanism in drought-stressed maize plants (Ashraf, 2010). The current experiments have shown that the DS increased both BADH activity and choline content, as well as induced high accumulation of GB. Such increases in both BADH activity and choline content were correlated with enhanced accumulation of GB in the maize plants in response to DS (Figs. 1-2)

Table 1. Correlation coefficients of NO content (NOC, nmol g^{-1} DW); glycinebetaine content (GBC, nmol g^{-1} DW; choline content (CHC, µmol g^{-1} DW); BADH activity (BADHA, µmol min^{-1} mg^{-1} DW) of both cultivars under drought stress (DS) (above diagonal) and control condition (below diagonal).

Character	NOC	GBC	CHC	BADHA
NOC		0.995***	0.940***	0.902***
GBC	0.627*		0.918***	0.871***
CHC	0.538	0.747**		0.974***
BADHA	0.526	0.764**	0.799**	

*, **, *** Significance at 5%, 1% and 0.1 % level of significance, respectively

Table 2. *F* values of the effects of foliar urea treatment (FU), cultivar (Cv), water regime (W) and their interactions on NO content (NOC, nmol g^{-1} DW); glycinebetaine content (GBC, nmol g^{-1} DW; choline content (CHC, µmol g^{-1} DW); BADH activity (BADHA, µmol min^{-1} mg^{-1} DW).

Source of variation	Water regime (W)	Cultivar (Cv)	Foliar urea (FU)	W×Cv	W×FU	Cv×FU	Cv×W×FU
NOC	18311.5***	285.1***	5423.2***	274.8***	5395.7***	83.99***	88.14***
GBC	17893.8***	365.6***	3947.6***	282.3***	2783.1***	90.0***	61.7***
CHC	21699.8***	417.7***	1066.4***	364.2***	1234.1***	0.05	0.01
BADHA	10208.9***	201.2***	243.9***	171.3***	257.8***	0.37	0.01

*, **, *** Significance at 5%, 1% and 0.1 % level of significance, respectively

A widely occurring adaptation in plants to counteract abiotic stress is an accumulation of stress signal molecule (Taiz & Zeiger, 2002; Siddiqui *et al.*, 2011). Among these substances, NO is a commonly signal molecule in plants actively involved in the control of plant growth and development under drought. For example, the role of NO in closing stomata and proline accumulation of drought-stressed plants has been widely reported (Misra, *et al.*, 2011; Siddiqui *et al.*, 2011). However, a relationship between NO and DS in promoting GB accumulation in maize under drought is not well understood (Siddiqui *et al.*, 2011). In the current study, the authors found that the contents of both NO and GB increased in drought-stressed maize seedling, but the peak of NO accumulation occurred earlier than GB under the drought regime. These responses in a drought-resistant cultivar, Zhengdan 958(ZD958), were stronger than those in a sensitive Jundan 20 (JD20) (Fig. 1).

Nitrogen (N) nutrition is essential for biosynthesis of an amino acid derivative such as GB in crop plants , also for enzyme biosynthesis in plants such as nitrate reductase (NR) affecting NO biosynthesis (Li, 2007). Since level of GB in plant tissue serves as important indicators of the internal osmotic adjustment ability of plants, the function of N on accumulation of osmotic nitrides under DS have attracted many plant researchers to fall within these scopes (Zhang & Deng, 2001; Zhang *et al.*, 2009a). Hence, studies on N-modulated of GB metabolism in environment-stressed plants can help to confer the possible physiological mechanism underlying a degree of tolerance to stress by this means (Saneoka *et al.*, 2004). It is generally perceived that adequate N supplying is vital to maintain high osmotic adjustment ability and overcome crop losses under DS (Zhang & Li, 2007; Zhang *et al.*, 2009). Much investigations were conducted under the root N application. In recent year, the foliar fertilizer application has attracted much attentions of some scientist in stress physiology due to the higher efficacy of foliar fertilization than that of soil fertilizer application in these situations. The reasons for this are because of the supply of the required nutrient directly to the location of demand in the leaves and its relatively quick absorption (e.g. 0.5–2 h for N and 10–24 h for K), and the independence of root activity and soil water availability (Römheld & El-Fouly, 1999). Foliar fertilization could increase P and K supplies at a time when the root system is not well developed (Mallarino *et al.*, 2001; Mosali, *et al.*, 2006; Amanullaha *et al.*, 2009). In the present studies, differential effects of foliar urea have been further clarified on modulation of accumulation and metabolism of GB and its mediating signal substrate NO in maize under DS, which might be closely associated with the drought resistance ability of a cultivar. An effectiveness of foliar urea on NO content, GB content and its two metabolism parameters i.e. choline content and BADH activity was more pronounced in the sensitive JD20 than that in the tolerant ZD958 under DS (Fig. 2; Table 2). With respect for the control (C) plants of the two cultivars, the less and non-significant effects were found in NO accumulation and other contributors respectively. Much lower concentrations of GB might mainly provide carbon, nitrogen and energy by degradation in well-watered plant alone (Taiz & Zeiger, 2002; Misra, *et al.*, 2011). Moreover, a significant correlation among GB metabolism parameters and NO content were evident in maize plants under DS, but no or less significant under control treatments (Table 1). These results show that contents of NO, GB and choline as well as BADH activity could be used as potential selection criteria for drought tolerance in maize. Additionally, the effects of foliar urea applied on the above parameters were much significant. It is, therefore, suggested that optimal application of urea dose benefit plant growth under water deficit condition, whose response is associated with cultivar' drought tolerance ability (Table 2). Consequently, the moderate dose of foliar urea supplying could enhance accumulation of GB by regulating BADH activity and choline content mediating by NO burst in the maize plant under DS. The more dominant effects were obtained by a drought-sensitive cultivar as compared with a -tolerant one (Zhang *et al.*, 2009). However, foliar application of different N forms imposed different responses to DS. It's concluded that excessive NO_3^- or NH_4^+ might induced negative effects

water relation and plant growth under DS, but foliar urea seems to hold best effective and efficient alleviation effects of DS (Li, 2007; Amanullaha *et al*., 2009).

Conclusion

In summary, the results presented in this paper provide evidence that the peak of NO content reached earlier than that of GB in the leaves drought-stressed maize plants. Such, endogenous NO probably plays a positive role as a signal in the regulation of GB metabolism. Both BADH activity and choline content were involved for the enhanced accumulation of GB in maize plants under drought stress. The foliar urea seemed to be involved in modulating GB accumulation by enhancing BADH activity and choline content as well as NO accumulation, especially in the drought sensitive cultivar (JD20). The foliar urea provided some protection against the DS effects on maize plants by regulating NO burst and its mediated GB metabolism.

Acknowledgments

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