Pak. J. Bot., 46(4): 1159-1164, 2014.

FOLIAR-APPLIED UREA MODULATES NITRIC OXIDE SYNTHESIS METABOLISM AND GLYCINEBETAINE ACCUMULATION IN DROUGHT-STRESSED MAIZE

LIXIN ZHANG[1*], ZHI RUAN[1], LI TIAN[1], JINGHUAN LAI[1], PENG ZHENG[1], ZONGSUO LIANG[1] AND ASHOK K. ALVA[2]

[1]*College of Life Sciences, Northwest A&F University, Yangling 712100, P. R. China*
[2]*USDA-ARS-Vegetable and Forage Crops Research Unit, 99350, Prosser, WA, USA*
**Correspondence author e-mail address: zhanglixin@nwsuaf.edu.cn Tel.: +0086-29-87092262.*

Abstract

Foliar urea has been proved to play a better positive role in enhancing accumulation of nitric oxide (NO) and glycinebetaine (GB) in maize (*Zea mays* L.) under drought stress (DS). However, it is unclear how foliar urea affects biosynthetic metabolism of NO and its relationship with GB accumulation. This study was on investigating the effect of foliar- applied urea on seedlings of maize cultivar Zhengdan 958 grown in a hydroponic medium under DS or No DS. Contents of NO and GB and nitric oxide synthase (NOS) activity increased and peaked 12 h after the treatment. Nitrate reductase activity (NRA) followed the similar pattern 6h after the treatment. Under DS foliar urea application increased NR and NOS activity and, thereby, increased NO formation. Therefore, enhancement in activities of both NRA and NOS resulted in an increase of NO accumulation. Foliar- applied urea could induce an increased NO burst by enhanced NO synthesis metabolism as a nitrogen signal, possibly resulting in GB accumulation under DS.

Introduction

Maize (*Zea mays* L.) is an important cereal crop in the world, which suffers great loss in yield due to drought stress (DS) (Li, 2007). Plants undergo extensive biochemical and physiological responses to DS, resulting in a turgor loss in plant cells and eventually death (Ashraf & Mehmood, 1990; Taiz & Zeiger, 2002).

To ensure the optimal plant growth condition of crops, osmolytes accumulate sufficiently to adjust osmotic potential of plants under DS (Zhang *et al*., 2009). For some glycinebetaine (GB) accumulators, they produce GB to reduce depletion of cellular water (Ashraf, 2010). GB plays multi-roles in stress resistance such as better osmotic modulation function, the protection of oxidative damage of biological macromolecules as a quaternary ammonium compound (Ashraf & Foolad, 2007). A high level of GB in the plant is necessary for holding optimal water status and growth under water deficit conditions (Sakamoto & Murata, 2002; Sithtisarn *et al.*, 2009; Ashraf, 2010).

Nitric oxide (NO) employs some key physiological responses to adapt to abiotic stresses such as drought, including osmotic adjustment to control water homeostasis as the most characterized stress signal molecule (Misra *et al.*, 2011; Habib *et al.*, 2013). NO plays a role in plant defense mechanisms affecting growth and development functions under stress conditions like a hormone (Arasimowicz & Floryszak-Wieczorek, 2007). Many investigations have demonstrated that NO, being a signal molecule, could activate antioxidant enzyme system and regulate osmolyte metabolism under environmental stress (Liu & Zhang, 2009; Misra *et al.*, 2011). There are at least three pathways of NO synthesis in plants, i.e nitric oxide synthase (NOS), nitrate reductase (NR) and non-enzymatic NO production pathways. NR has long been known as a main source for NO. Regulation of NR activity by reversible serine phosphorylation has been shown to modulate NO production (Planchet & Kaiser, 2006). Another pathway for NO production is by NOS, which catalyzes NO and L-citrulline formation from O_2 and L-arginine (Ribeiro *et al.*, 1999). Although the modulating effects of NO have been greatly documented (Siddiqui *et al.*, 2011), it is still unclear how this unstable molecule regulates GB metabolism induced by foliar urea application under drought (Ashraf, 2010).

Nitrogen (N) deficiency can disorder some physiological responses and even inhibit plant growth in drought conditions (Saneoka *et al.*, 2004; Li, 2007; Zhang & Li, 2007; Zhang *et al*., 2009). Thus, foliar-applied urea may improve N absorption by the leaves and in turn enhance plant N status which results in an enhancement of drought tolerance (Hu *et al.*, 2008). Our previous study has shown a considerable response to foliar urea in a drought-sensitive maize cultivar than that in a tolerant one under DS. It is likely that GB plays a vital role in osmotic adjustment of the maize cultivars under drought stress (Zhang *et al*., 2009; Zhang *et al*., 2012).

The aim of this study was to clarify the role of foliar-applied urea to maize seedlings subject to DS on the NO synthesis and GB accumulation in response to DS.

Materials and Methods

A solution culture experiment was conducted in a growth chamber using maize cultivar ZD958. Seed germination experiment followed our previous method (Zhang *et al*., 2010). At the three-leaf growth stage, DS was imposed by putting 15% (w/v) polyethylene glycol (PEG-6000) into the nutrient solution to attain -0.72 MPa osmotic potentials (ψs) (Wang & Li, 2002). Complete nutrient solution without PEG-6000 was included as control (C). Sub treatments were (i) no foliar urea (NFN) or (ii) foliar spray prior to DS treatment (0 h) using 15g urea/L (N) in 0.10% neutral soap solution. The seedlings in NFN treatment were sprayed with similar solution without urea. A random block design was followed with 4 treatments, and 4 replicates (6 plants per replicate) on 0, 6, 12, 24, 48 h of PEG treatment.

Sampling: The second or third leaf from top of three randomly selected plants per replicate was sampled for analyses of nitrate reductase activity, nitric oxidesynthase, contents of nitric oxide and glycinebetaine. The samples were stored in liquid N at -40°C prior to assay. The experiment was proceeded repeatedly and data were shown as mean ± S.E. of eight replicates for each treatment (n=8).

GB analyses: Glycinebetaine (GB) was isolated and the content measured according to the method described by Grieve & Grattan (1983) and our paper by Zhang & Li (2007) with some modifications. The GB content was expressed as nmol g^{-1} DW.

NO analyses: Determination of NO concentration was conducted according to the instructions laid down in the NO assay kit (Beyotime Institute of Biotechnology, Shanghai, China) using Griess Reagent II solution referring to Griess (1897) with some modifications. The OD was read at 540 nm. For quantification of NO a standard curve using $NaNO_2$ was developed.

NRA assay: NRA was determined *In vitro* followed the procedure of Wray & Filner (1970) and our paper by Zhang and Li (2007). Enzyme activity was calculated in terms of nitrite released per gram DW leaf per hour (NO_2^-µmol mg^{-1} protein h^{-1}).

NOS Activity: NOS was determined following the procedure of Guo *et al*. (2003)., NOS activity was determined by the citrulline assay using the NOS assay kit (Cayman Chemical). The protein contents in the supernatant were measured referring to the method of Bradford (1976) with bovine serum albumin as a standard.

Statistical analysis: All data were subjected to analysis of variance (ANOVA) using SAS software (SAS Institute Inc., Cary, NC, USA, 1996). Standard errors of the means (SE) were calculated. The significance of the treatment effect was determined using *F*-test, and the significance difference between the means was determined by the LSD test at 0.05 probability level.

Results

Effects of foliar-applied urea on NO content in leaves under drought stress (DS): NO content increased with time of sampling in the plants with or without the foliar application of urea under DS (Fig. 1). NO content was significantly greater in the plants which received foliar urea as compared to that of the plants without urea application 12 h after the treatment. Under DS, the NO contents attained peak 12 and 24 h following treatment in plants without and with foliar urea application, respectively. These peak values were 4.9 to 8-fold greater than the corresponding values for the plants without DS.

Effects of foliar-applied urea on NRA trends in leaves under drought stress (DS): Urea spray stimulated increased NRA only 12 h following the treatment in plants under both no DS or with DS (Fig. 2). Nitrate Reductase Activity (NRA) reached their peak after 12 h of foliar urea treatment in both DS no DS plants. Subsequently the NRA values slightly declined and stabilized in 24 and 48 h sampling.

Effects of foliar-applied urea on NOS in leaves of two maize cultivars under DS: Foliar-applied urea has no significant effects on NOS activity in plants without the DS (Fig. 3). In the plants subjected DS, however, NOS activity increased significantly with foliar urea application as compared to that of the plants with foliar urea only 12 h following the treatment. This trend was consistent in the 24 and 48 h sampling. The DS significantly increased NOS activity, regardless of foliar urea treatment, 6 h following the treatment. The NOS activity foliar increased in 12 to 48 h only in foliar urea applied plants. In the case of the plants without foliar urea, NOS activity slightly decreased by 48 h following the treatment.

Effects of foliar-applied urea on GB in leaves of two maize cultivars under DS: The accumulations of GB increased under DS, 6 h following the treatment (Fig. 4). The urea application had no significant effects on GB content of plants under no DS. The GB content further increased from 6 to 12 h in DS treatment only. Under DS, the foliar urea applied plants as compared to that of the plants without foliar urea in 12-48 h.

Correlations between different response: In the plants without the DS, only significant corrolation was between NO content and NOS activity (Table 1). In contract, all four response parameters were significantly correlated among each other in the plants subjected to DS.

Table 1. Correlation coefficients of NO content (NOC, nmol g^{-1} DW); NR Activity (NRA, NO_2^-µmol mg^{-1} protein h^{-1}; NOS activity (NOSA, U mg^{-1} protein); glycinebetaine content (GBC, nmol g^{-1} DW) of maize seedling leaves under drought stress (DS) (above diagonal) or with no DS (below diagonal).

Character	NO	NRA	NOS	GB
NO		0.649**	0.984**	0.969**
NRA	0.259		0.748**	0.781**
NOS	0.685**	0.276		0.992**
GB	0.325	0.008	0.270	

*, **significant at 5 %, 1 % level of significance respectively

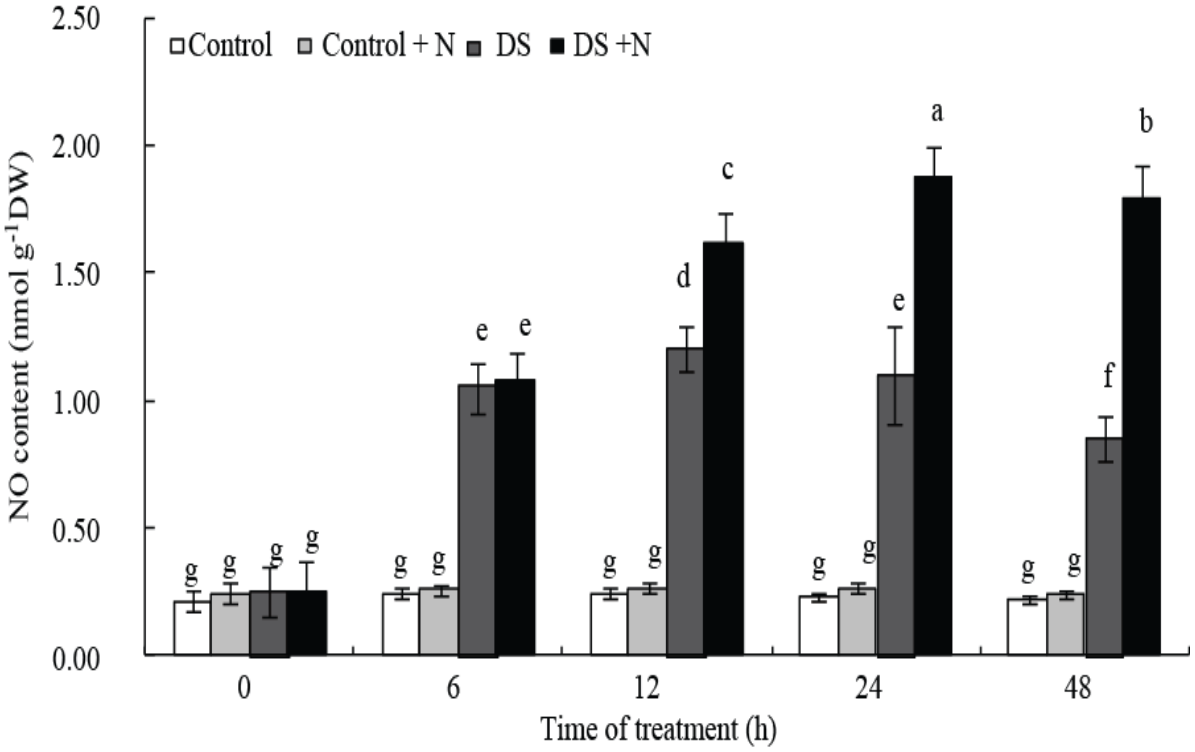

Fig. 1. Effects of foliar-applied urea on nitric oxide content in leaves under drought stress.
Each value is the mean ± S.E. of eight replicates for each treatment (n=8).
N represents foliar urea treatment. DS and Control represent drought stress and no DS, respectively.
Mean values with the same letter are not significantly different at the 0.05 level, across all treatment and sampling time.

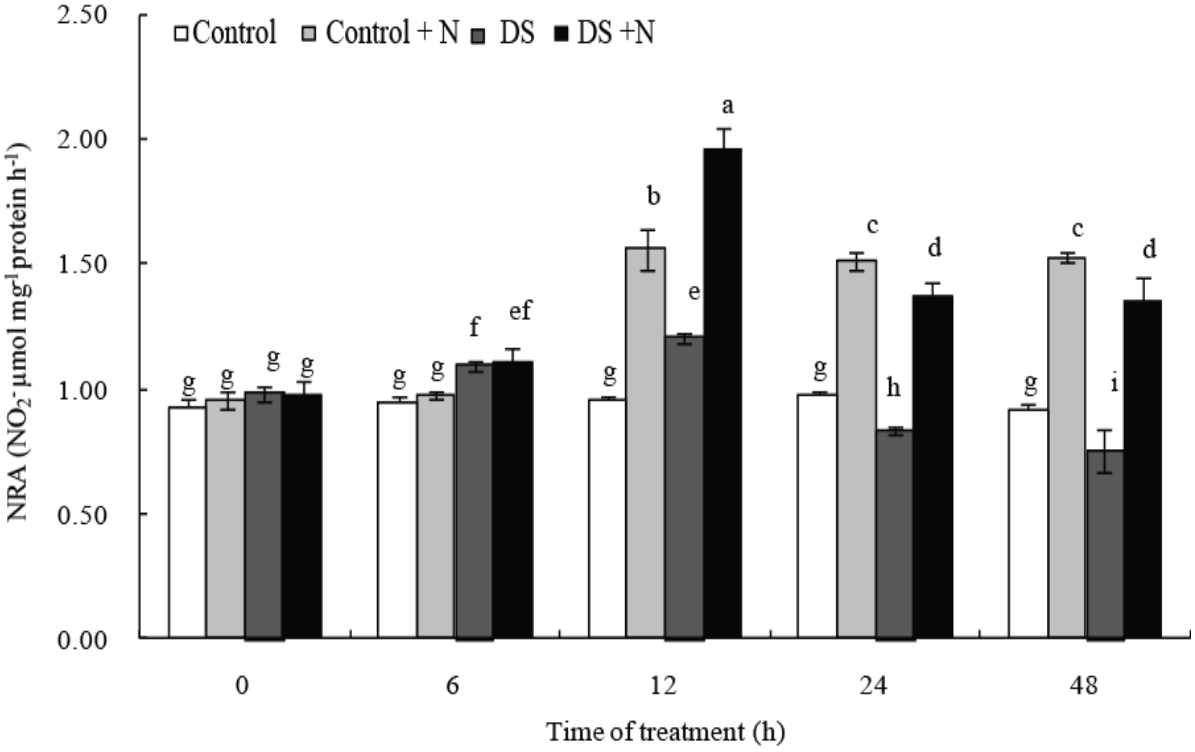

Fig. 2. Effects of foliar-applied urea on Nitrate Reductase activity in leaves under DS.
Each value is the mean ± S.E. of eight replicates for each treatment (n=8).
N represents foliar urea treatment. DS and Control represent drought stress and no DS, respectively.
Mean values with the same letter are not significantly different at the 0.05 level, across all treatment and sampling time.

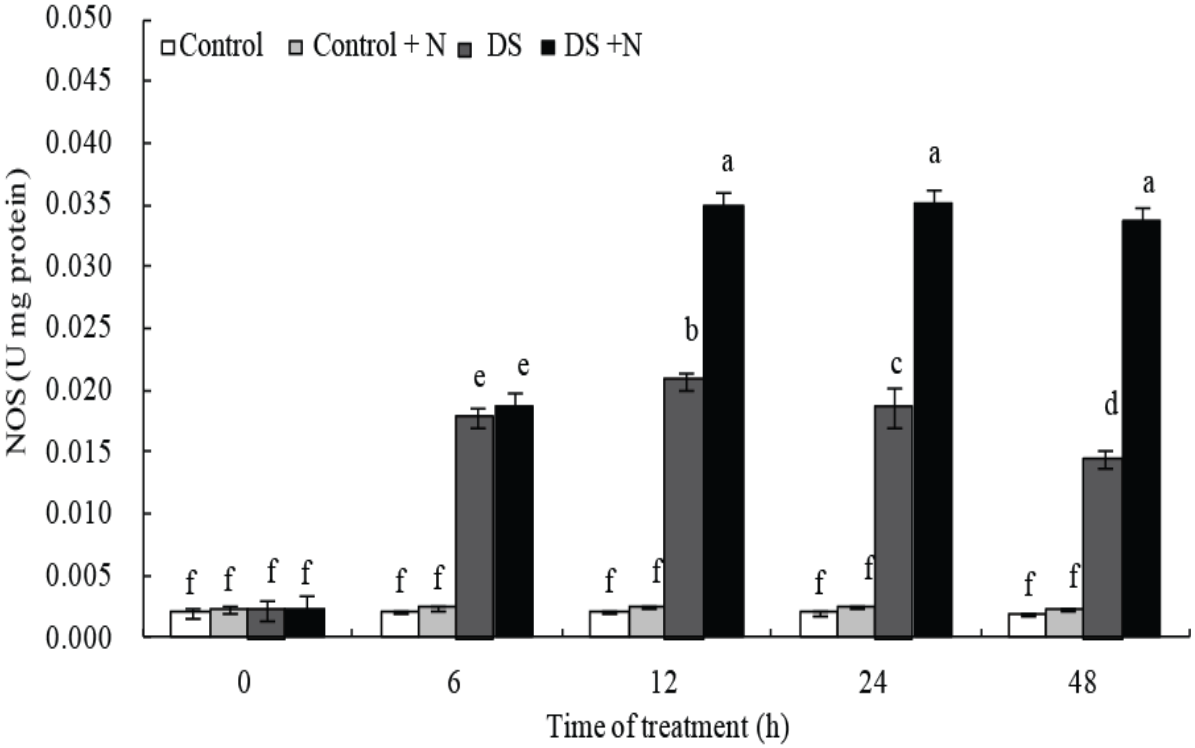

Fig. 3. Effects of foliar-applied urea on nitric oxidesynthase in leaves of two maize cultivars under drought stress.
Each value is the mean ± S.E. of eight replicates for each treatment (*n*=8).
N represents foliar urea treatment. DS and Control represent drought stress and no DS, respectively.
Mean values with the same letter are not significantly different at the 0.05 level, across all treatment and sampling time.

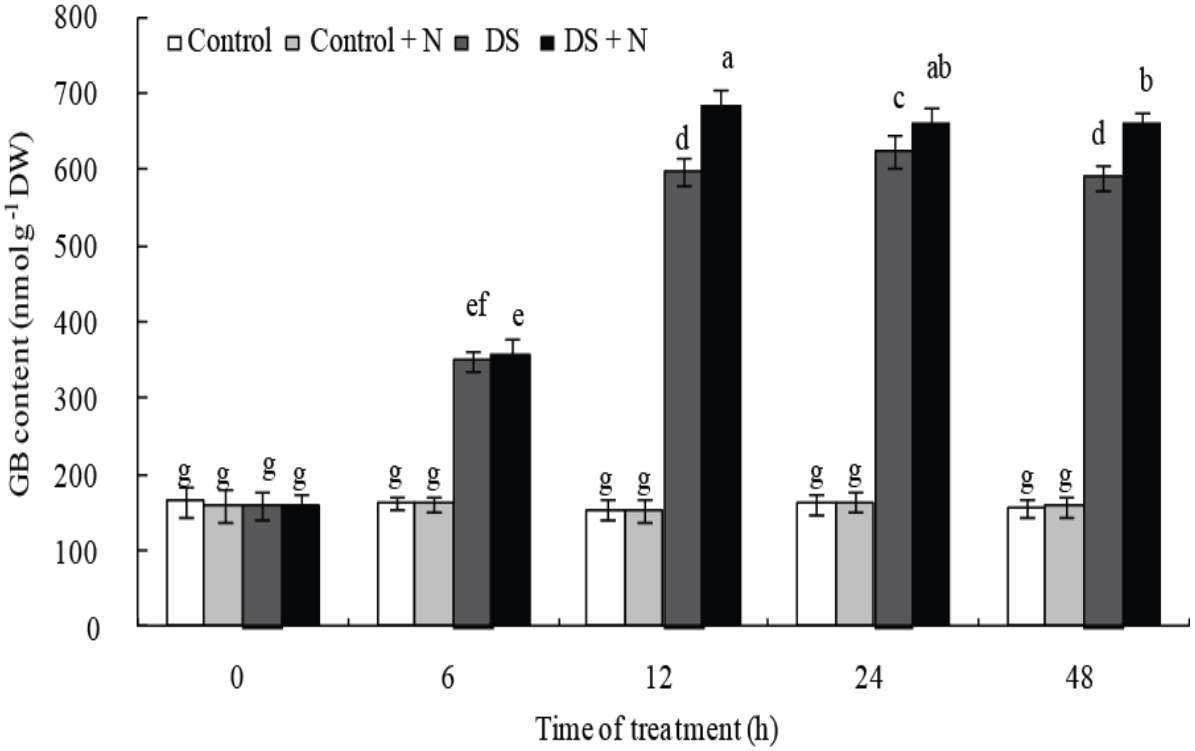

Fig. 4. Effects of foliar-applied urea on glycinebetaine in leaves of two maize cultivars under drought stress.
Each value is the mean ± S.E. of eight replicates each treatment (*n*=8).
N represents foliar urea treatment. DS and Control represent drought stress and no DS, respectively.
Mean values with the same letter are not significantly different at the 0.05 level, across all treatment and sampling time.

Discussion

It has been demonstrated that foliar application of urea is an effective approach to provide nitrogen (N) for crops since 1950's (Finney *et al*., 1957). Plants can reduce NO^{3-} in both roots and shoots (Guo & Marschner, 1995). However, nitrate reduction is significantly greater in leaves than that in root. Further, the role of NO in promoting GB accumulation in maize under DS was not well understood (Siddiqui *et al.*, 2011). This study showed a significant increase in NO in the plants under DS as compared to that of the plants with no DS. Plants which received foliar significant in increase NO occurred after 12 h (Fig. 1). Pagnussat *et al*., (2003) reported of an important role of NO in plants under stress by improving cyclic guanosine monophosphate levels and regulating the antioxidant enzyme system.

NRA is an indicator of N assimilation potential (Srivastava, 1992; Saroop *et al.*, 1998; Singh *et al.*, 2002). Nitrate reductase can synthesize nitric oxide by utilizing NO^{2-} and NADH as substrates (Yamasaki *et al.*, 1999). This mechanism is highly correlated to some specific response reactions to DS, such as stomatal closure (Neill *et al.*, 2003). Reductive formation of NO is assumed be depend at NR activity. Our results (Fig. 2) revealed that NRA was different between treatments 12 h after the treatment. The NRA activity of the plants under DS was greater in a drought-tolerant cultivar as compares to a sensitive (Zhang *et al.*, 2009). This was reflected by the greater reduction nitrate assimilation in crop plants in a drought sensitive cultivar as compared to that of a drought cultivar. Our results on maize are in agreement with the findings of Li et al. in wheat (Li *et al.*, 1990). Foliar application increased the level of NRA regardless of DS treatment (Fig. 2). Urea is regarded as an intermediate participated in N remobilization from source tissues. In higher plants, the degree of drought tolerance is often related to increased level of NRA and a greater volume of osmotic solute, which are beneficial mitigate stress-induced damage and change N metabolism at the cellular level (Zur *et al.*, 1981; Zhang *et al*., 2012).

The NOS activity was induced in cytosolic and microsomal fractions under DS. The activity of antioxidant enzymes, such as SOD and APX, are promoted subsequent to change in NOS activity (Sang *et al.*, 2008; Corpas *et al.*, 2009). Results of this study have shown significantly greater NOS activity in DS plants 12 h after foliar urea application as compared to that of the plants with no foliar urea (Fig. 3). Although plant NOS (gene, cDNA, or protein) has not been characterized yet, the relationship between NOS activity and NO accumulation has been reported in different studies (Ninneman & Maier, 1996; Barroso *et al.*, 1999; Table 1).

Nitrogen nutrition is fundamental for biosynthesis of free amino acid derivative such as GB in plants (Hsiao *et al*., 1984; Li, 2007). If the GB accumulators' plants, such as maize and beet, subjected to N deficient condition, their growth is likely to be delirious under DS. Thus, the role of N on osmotic nitrides accumulation under DS has been an activity response area for a number of years. Adequate N supply is indispensable to maintain high osmotic adjustment and overcome yield losses under DS (Zhang *et al.*, 2009). Studies on GB metabolism affected by N in drought-stressed plants can help to figure out the mechanism of N modulation in abiotic condition (Saneoka *et al.*, 2004; Zhang *et al*., 2012). Our study also showed that the GB content of plants under DS improved significantly by urea application (Fig. 4). Among the response parameters, GB was highly correlated to NO content. This might owe to its mediating signal substrate NO, a signal molecular in stress-induced condition. Although urea foliar application can influence the activity of NR and NOS in plants with no DS, this effect was significantly greater under DS. Furthermore, urea foliar application increased the GB content in the plants subject to DS as compared to that of plants under no DS. GB can be degraded to carbon, nitrogen and energy in normal growth condition (Taiz & Zeiger, 2002; Misra *et al.*, 2011).

Conclusion

The NO and GB content and nitric NOS activity peaked 12 h after DS treatment and reached summits after 12h of DS treatment. The peak of NRA was observed after 6 h of treatment. Foliar urea application increased NR and NOS activity and, promoted NO level under DS. Foliar urea application could induce an increased NO accumulation by modulation of NO synthesis metabolism and improved GB accumulation. These findings suggest foliar urea might invoke NO burst as the nitrogen signal not only its nutritive function due to its slight amount of absorption by leaves of plant under DS. This statement need to confirm further. A better understanding of urea signal in plants involving uptake, transduction and induce responses such as GB synthesis and accumulation to adapt to stress condition will be necessary to assess and possibly improve direct usage of urea by crops.

References

Arasimowicz, M. and J.F. Wieczorek. 2007. Nitric oxide as a bioactive signaling molecule in plant stress responses. *Plant Sci.,* 172(5): 876-887.

Ashraf, M. and M.R. Foolad. 2007. Roles of glycinebetaine and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot*., 59: 206-216.

Ashraf, M. 2010. Inducing drought tolerance in plants: some recent advances. *Biotechnol. Adv*., 28: 169-183.

Ashraf, M. and S. Mehmood. 1990. Responses of four *Brassica* species to drought stress. *Env. Exp. Bot*., 30(1): 93-100.

Barroso, J.B., F.J. Corpas, A. Carreras, L.M. Sandalio, R. Valderrama, J.M. Palma, J.A. Lupiáñez and L.A. del Río. 1999. Localization of nitric-oxide synthase in plant peroxisomes. *J. Biol. Chem.,* 274(51): 36729-36733.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochemi.*, 72(1): 248-254.

Corpas, F.J., J.M. Palma, L.A. Del Río and J.B. Barroso. 2009. Evidence supporting the existence of l-arginine-dependent nitric oxide synthase activity in plants. *New Phytol.,* 184(1): 9-14.

Finney, K.F., J.W. Meyer, F.W. Smith and H.C. Fryer. 1957. Effect of foliar spraying of Pawnee wheat with urea solutions on yield, protein content, and protein quality. *J. Agron.,* 49(7): 341-347.

Griess, P. 1897. Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt "Ueber einige azoverbindungen." *Chem. Ber.,* 12: 426-428.

Grieve, C.M. and S.R. Grattan. 1983. Rapid assay for determination of water soluble quaternary ammonium compounds. *Plant Soil,* 70: 303-307.

Guo, F.Q., M. Okamoto and N.M. Crawford. 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science,* 302: 100-103.

Guo, Y. and H. Marschner. 1995. Uptake, distribution, and binding of cadmium and nickel in different plant species. *J. Plant Nutr.,* 18(12): 2691-2706.

Habib, N., M. Ashraf and M. Shahbaz. 2013. Effect of exogenously applied nitric oxide on some key physiological attributes of rice (*Oryza sativa* L.) plants under salt stress. *Pak. J. Bot.*, 45(5): 1563-1569.

Hsiao, T.C., J.C. O'Toole, E.B. Yambao and N.C. Turner. 1984. Influence of osmotic adjustment on leaf rolling and tissue death in rice (*Oryza sativa* L.). *Plant Physiol.*, 75(2): 338-341.

Hu, Y.C., B. Zoltan and S. Urs. 2008. Effect of foliar fertilization application on the growth and mineral nutrient content of maize seedlings under drought and salinity. *Soil Sci. Plant Nutr.*, 54: 133-141.

Li, S.X. 2007. *Dry land Agriculture in China*. Science Press, Beijing, China.

Li, Y.Y., Y.Q. Wang, M. Cao and H.Z. Wu. 1990. A comprehensive evaluation on drought resistance of winter wheat. *Plant Physiol. Commun.*, 2: 5.

Liu, Y.G. and J.H. Zhang. 2009. Rapid accumulation of NO regulates ABA catabolism and seed dormancy during imbibition in *Arabidopsis*. *Plant Signal. Behav.*, 4: 905-907.

Misra, A.N., M. Misra and R. Singh. 2011. Nitric oxide ameliorates stress responses in plants. *Plant Soil Environ.,* 57: 95-100.

Neill, S.J., R. Desikan and J.T. Hancock. 2003. Nitric oxide signalling in plants. *New Phytol.*, 159(1): 11-35.

Ninnemann, H. and J. Maier. 1996. Indications for the occurrence of nitric oxide synthases in fungi and plants and the involvement in photoconidiation of *Neurospora crassa*. *Photochem. Photobiol.*, 64(2): 393-398.

Pagnussat, G.C, M.L. Lanteri and L. Lamattina. 2003. Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. *Plant Physiol.*, 132(3): 1241-1248.

Planchet, E. and W.M. Kaiser. 2006. Nitric oxide production in plants. *Plant Signal. Behav*., 1(2): 46-51.

Ribeiro, Jr E.A., F.Q. Cunha, W.M.S.C. Tamashiro and I.S. Martins. 1999. Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. *FEBS Letters*, 445: 283-286.

Sakamoto, A. and N. Murata. 2002. The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. *Plant Cell Environ.*, 25: 163-171.

Saneoka, H., R.E.A. Moghaieb, G.S. Premachandra and K. Fujita. 2004. Nitrogen nutrition and water stress effects on cell membrane stability and leaf water relation in *Agrostis palustris Hud*. *Environ. Exp. Bot.*, 52: 131-138.

Sang, J., M. Jiang, F. Lin, S. Xu, A. Zhang and M. Tan. 2008. Nitric oxide reduces hydrogen peroxide accumulation involved in water stress-induced subcellular anti-oxidant defense in maize plants. *J. Integr. Plant Boil.*, 50(2): 231-243.

Saroop, S., V.S. Thaker, S.V. Chanda and Y.D. Singh. 1998. Light and nitrate induction of nitrate reductase in kinetin- and gibberellic acid-treated mustard cotyledons. *Acta physiol. Plant.*, 20(4): 359-362.

Siddiqui, M.H., M.H. Al-Whaibi and M.O. Basalah. 2011. Role of nitric oxide in tolerance of plants to abiotic stress. *Protoplasma*, 248: 447-455.

Singh, D.B., S. Varma and S.N. Mishra. 2002. Putrescine effect on nitrate reductase activity, organic nitrogen, protein, and growth in heavy metal and salinity stressed mustard seedlings. *Biol. Plantarum,* 45(4): 605-608.

Sithtisarn, S., P. Harinasut, S. Pornbunlualap and S. Cha-um. 2009. Accumulation of glycinebetaine and betaine aldehyde dehydrogenase activity in *Eucalyptus camaldulensis* clone T5 under *In vitro* salt stress. *Kasetsart J. (Nat. Sci.)*, 43: 146-152.

Srivastava, H.S. 1992. Multiple functions and forms of higher plant nitrate reductase. *Phytochem.*, 31(9): 2941-2947.

Taiz, L. and E. Zeiger. 2002. *Plant Physiology*. (3rd Ed) Sinauer Associates Inc. Publishers, Massachusetts.

Wang, X. and Y.D. Li. 2002. Synthesis and characterization of lanthanide hydroxide single-crystal nanowires. *Angew. Chem. Int. Ed.*, 41(24): 4791-4793.

Wray, J.L. and P. Filner. 1970. Structural and functional relationships of enzyme activities induced by nitrate in barley. *J. Biochem.*, 119: 715-725.

Yamasaki, H., Y. Sakihama and S. Takahashi. 1999. An alternative pathway for nitric oxide production in plants: new features of an old enzyme. *Trends Plant Sci.*, 4(4): 128-129.

Zhang, L.X., M. Gao, J.J. Hu, X.F. Zhang, K. Wang and M. Ashraf. 2012. Modulation role of abscisic acid (ABA) on growth, water relations and glycinebetaine metabolism in two maize (*Zea mays* L.) cultivars under drought stress. *Int. J. Mol. Sci.*, 13(3): 3189-3202.

Zhang, L.X., S.X. Li, Z.S. Liang and S.Q. Li. 2009. Effect of foliar nitrogen application on nitrogen metabolism, water status and plant growth in two maize (*Zea mays* L.) cultivars under short-term moderate stress. *J. Plant Nutr.*, 32: 1-21.

Zhang, L.X. and S.X. Li. 2007. Effects of application of N and K on nitrogen metabolism of two genotype varieties of maize under water-stressed condition. *Plant Nutr. Fert. Sci.*, 13: 554-560.

Zur, B., K.J. Boote and J.W. Jones. 1981. Changes in internal water relations and osmotic properties of leaves in maturing soybean plants. *J. Exp. Bot.,* 32(6): 1181-1191.

(Received for publication 20 June 2013)