

BRIEF COMMUNICATION

Nitric oxide accumulation and glycinebetaine metabolism in two osmotically stressed maize cultivars supplied with different nitrogen formsL.X. ZHANG¹, P. ZHENG¹, Z. RUAN¹, L. TIAN¹, and M. ASHRAF^{2*}*College of Life Sciences, Northwest A&F University, Yangling 712100, P.R. China¹**University College of Agriculture, University of Sargodha, Sargodha, Pakistan²***Abstract**

Hydroponic experiments were conducted to investigate the effects of nitrogen forms on nitric oxide accumulation and glycinebetaine (GB) metabolism under osmotic stress (OS) simulated by the addition of polyethylene glycol (PEG-6000) to two maize (*Zea mays* L.) cultivars Zhengdan 958 (ZD958; drought-tolerant) and Jundan 20 (JD20; drought-sensitive). OS led to the accumulation of NO, GB, and choline, as well as to an increase of betaine aldehyde dehydrogenase (BADH) activity in both the cultivars. The responses of both the maize cultivars to OS were significantly higher under nitrate nutrition than under the mixed supply of ammonium and NO₃⁻ or sole NH₄⁺. Cultivar ZD958 was superior to JD20 under the osmotic stress. Furthermore, compared with sole NH₄⁺, sole NO₃⁻ and the mixed supply of NH₄⁺ and NO₃⁻ increased all the parameters in cv. ZD958 but only the GB and choline content in JD20. Therefore, it is concluded that ZD958 showed a higher accumulation of NO and enhanced GB metabolism under OS than JD20 regardless of N form.

Additional key words: betaine aldehyde dehydrogenase, choline, drought stress, *Zea mays*.

Drought stress is one of the major environmental limitations which adversely affect plant growth and yield of crops worldwide (Ashraf 2010). Plants have evolved various strategies including an enhanced accumulation of some organic osmolytes including glycinebetaine (GB) to alleviate detrimental effects of drought stress on plant metabolism and growth (Anjum *et al.* 2011). Nitric oxide (NO) is one of the stress signaling molecules (Misra *et al.* 2011). Zhang *et al.* (2013) provided a sound evidence of regulation of GB metabolism in drought stressed maize plants by exogenous application of NO. Foliar-applied urea causes a marked accumulation of NO especially in a drought sensitive maize cultivar (Zhang *et al.* 2012b). It is also very effective in enhancing a GB accumulation by modulating GB metabolism (Zhang *et al.* 2012a). Betaine aldehyde dehydrogenase (BADH, EC 1.2.1.81) is the key enzyme involved in the GB biosynthetic pathway (Rhodes and Hanson 1993). Nitrogen forms (NO₃⁻ versus NH₄⁺) influence plant responses to stress, including the pattern of accumulation of osmolytes proline and GB (Walch-Liu *et al.* 2000). Therefore, the aim of this study was to

investigate the interactive effects of N forms and osmotic stress (OS) on NO accumulation and GB metabolism in two maize cultivars with contrasting drought tolerance.

Seeds of maize (*Zea mays* L.) cvs. Jundan 20 (JD20; drought sensitive) and Zhengdan 958 (ZD958; drought tolerant) were surface-sterilized in a 1 % (m/v) sodium hypochlorite solution on a magnetic stirrer for 20 min and thoroughly rinsed with sterile deionized water. Then the seeds were germinated between two layers of blotter paper moistened with sterile water at a temperature of 28 ± 1 °C in the dark for 72 h. The seedlings were cultured in a hydroponic culture in a growth chamber with day/night temperatures of 25/18 °C, a relative humidity of 60 - 70 %, an irradiance of 350 µmol m⁻² s⁻¹, and a 16-h photoperiod.

In the first experiment, OS was implemented by adding 30, 90, and 120 g(PEG-6000) kg⁻¹ (distilled water) in a full strength nutrient solution (Hoagland and Arnon 1950) to gradually achieve osmotic potentials of -0.08, -0.13, and -0.23 MPa, respectively (Wang and Li 2002). In the second experiment, the seedlings were grown under

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Abbreviations: BADH - betaine aldehyde dehydrogenase; GB - glycine betaine; OS - osmotic stress; PEG - polyethylene glycol.

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control conditions or PEG (osmotic potential of -0.23 MPa) and subjected to the following N forms: NH_4^+ -N supplied as $(\text{NH}_4)_2\text{SO}_4$, NO_3^- -N supplied as $\text{Ca}(\text{NO}_3)_2$, and the mixture of NH_4^+ + NO_3^- in the ratio of 50:50. The maize plants were harvested after 0, 1, 2, 3, 5, 7, and 10 d after the onset of the osmotic stress in the first experiment, and after 10 d of the onset of the drought or N form treatments in the second experiment. The osmotic potential of the PEG solution was measured by a vapor pressure osmometer (Wescor model 5500, Logan, UT, USA).

The GB content in leaf was determined by the method of Grieve and Grattan (1983). The choline content was determined following Feng and Ren (2004) and Winzler and Meserv (1945). The NO content was assayed as described by Griess (1879) following the guidelines on the Griess reagent colorimetric kit (Beyotime Institute of Biotechnology, Shanghai, China). The NO content was determined through a standard curve using NaNO_2 and expressed as $\text{nmol g}^{-1}(\text{d.m.})$. For determination of BADH activity, plant material was homogenized in 250 mm^3 of a buffer containing 50 mM HEPES-KOH (pH 8.0), 1 mM Na_2EDTA , 20 mM sodium disulfite, 10 mM sodium borate, 5 mM ascorbic acid, 5 mM dithiothreitol, and 2 % (m/v) polyvinylpyrrolidone. Homogenates were then centri-

fuged at 10 000 g and 4 °C for 15 min and the supernatants were used for determination of BADH activity according to Daniell *et al.* (2001) by monitoring the absorbance at 340 nm with 0.05 mM betaine aldehyde chloride as substrate. The activity was calculated using the coefficient of absorbance of 6 220 $\text{M}^{-1} \text{cm}^{-1}$ for NADH. Protein content of the crude extract was measured by Coomassie Brilliant Blue G-250 method (Bradford 1976).

All data were subjected to the analysis of variance (ANOVA) using the SAS v. 9 software (SAS Institute, Chicago, USA). The significance of the treatment effect was determined using the *F*-test, and the significance of difference between means was determined by the LSD test at the 0.05 probability level.

Osmotic stress can induce some physiological mechanisms to adapt to it, including an accumulation of NO and organic osmolytes, such as GB (Bartels and Sunkar 2005, Ashraf *et al.* 2011, Zhang *et al.* 2013). In our experiments, OS caused a marked accumulation of NO and GB in two maize cultivars, and the accumulation of NO reached the maximum earlier than that of GB, which suggests that NO possibly acted as signal molecule to regulate other processes involved in drought tolerance in correspondence with our previous results (Zhang *et al.*

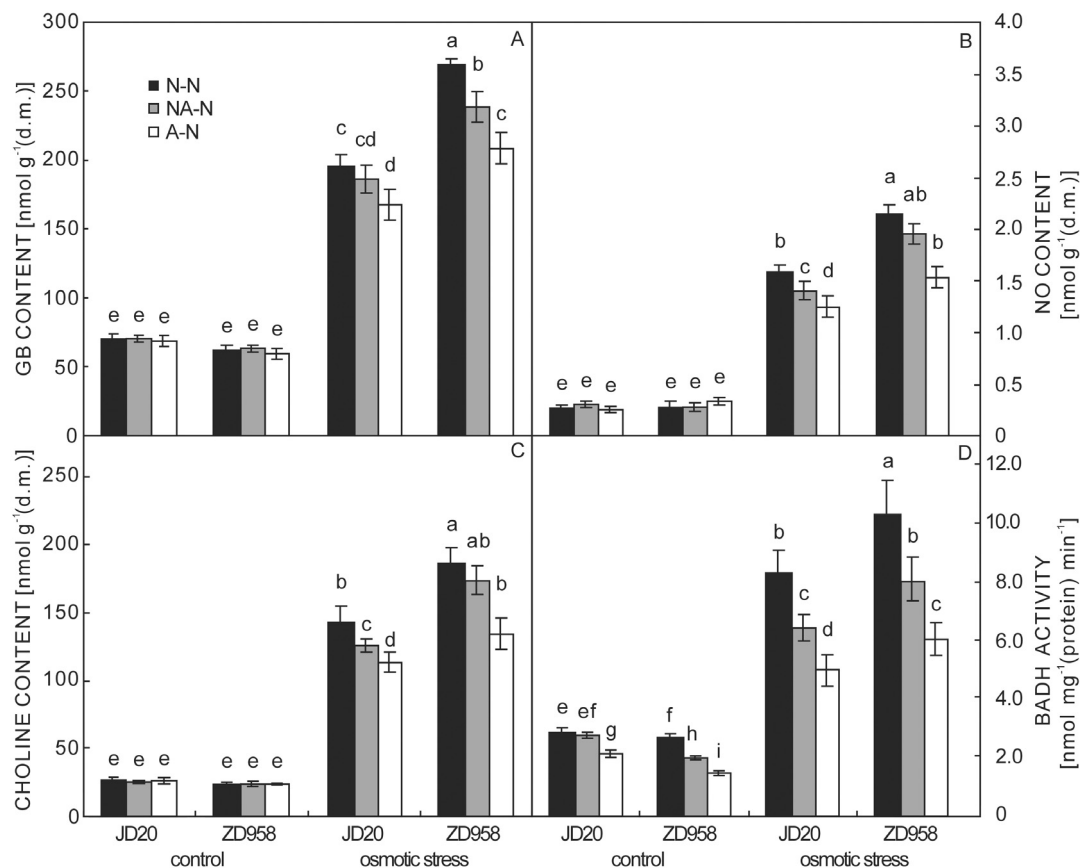


Fig. 1. Interactive effects of nitrogen forms and osmotic stress (OS) on the content of glycinebetaine (GB; A), NO (B), choline (C), and betaine aldehyde dehydrogenase (BADH) activity (D) in seedlings of two maize cultivars after 10 d of PEG treatment. JD20 and ZD958 represent cultivars Zhengdan 958 and Jundan 20, respectively. N-N, NA-N, and A-N represent plants supplied with NO_3^- , NH_4^+ + NO_3^- , and NH_4^+ , respectively. Means \pm SE, $n = 8$, different letters indicate significant differences among treatments at $P \leq 0.05$.

Table 1. The pattern of accumulation of endogenous glycinebetaine (GB) [nmol g⁻¹(d.m.)] and NO [nmol g⁻¹(d.m.)] in seedlings of maize cvs. ZD958 and JD20 after 0, 1, 2, 3, 5, 7, and 10 d of osmotic stress (PEG concentrations in parentheses). Means \pm SE, $n = 8$. Different letters in the columns indicate significant differences at $P < 0.05$ among the four treatments.

Cultivar			0 (0)	1 (60)	2 (90)	3 (120)	5 (120)	7 (120)	10 (120)
GB	control	JD20	55.24 \pm 2.23b	56.32 \pm 2.32c	53.85 \pm 3.04c	53.46 \pm 3.60c	53.94 \pm 2.38c	55.22 \pm 3.45c	54.22 \pm 3.19c
		ZD958	51.73 \pm 2.72c	51.74 \pm 2.72d	52.87 \pm 4.04c	52.48 \pm 3.35c	52.87 \pm 3.06c	52.48 \pm 4.66d	52.48 \pm 3.59d
	OS	JD20	55.55 \pm 2.15b	115.01 \pm 6.15b	153.02 \pm 6.04b	175.96 \pm 6.26b	193.02 \pm 5.68b	175.96 \pm 8.60b	174.02 \pm 6.02b
		ZD958	60.60 \pm 4.62a	148.46 \pm 4.62a	202.86 \pm 8.22a	229.60 \pm 9.03a	252.06 \pm 8.68a	229.60 \pm 7.00a	228.06 \pm 10.54a
NO	control	JD20	0.25 \pm 0.006c	0.24 \pm 0.008c	0.25 \pm 0.006d	0.26 \pm 0.008d	0.26 \pm 0.008d	0.29 \pm 0.008c	0.25 \pm 0.007d
		ZD958	0.25 \pm 0.005c	0.25 \pm 0.005c	0.31 \pm 0.005c	0.32 \pm 0.005c	0.34 \pm 0.006c	0.28 \pm 0.008c	0.31 \pm 0.007c
	OS	JD20	0.27 \pm 0.035b	0.71 \pm 0.098b	1.22 \pm 0.072b	1.76 \pm 0.092b	1.60 \pm 0.101b	1.44 \pm 0.108b	1.43 \pm 0.092b
		ZD958	0.30 \pm 0.048a	1.00 \pm 0.085a	1.52 \pm 0.105a	2.31 \pm 0.109a	1.98 \pm 0.091a	1.88 \pm 0.081a	1.87 \pm 0.095a

Table 2. Correlation coefficients between the glycinebetaine (GB) content [nmol g⁻¹(d.m.)] and the betaine aldehyde dehydrogenase (BADH) activity [nmol mg⁻¹(protein) min⁻¹], choline content [nmol g⁻¹(d.m.)], or NO content [nmol g⁻¹(d.m.)] in two maize cultivars under osmotic stress. Significant differences at * - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$.

Parameter	GB	BADH	Choline	NO
GB		0.683**	0.889***	0.024
BADH	0.853***		0.391	-0.258
Choline	0.966***	0.902***		0.303
NO	0.983***	0.897***	0.983***	

2012b). However, the relationship between NO and OS in promoting GB accumulation in maize under different time periods and intensities of OS is not well understood (Siddiqui *et al.* 2011, Zhang *et al.* 2012b). In our present study, the NO and GB accumulations in both the cultivars increased linearly with the duration and intensity of OS during 0 - 3 d of OS. Afterwards, the NO accumulation began to decrease earlier than that of the GB accumulation. The above responses were significantly greater in cv. ZD958 than in cv. JD20. A positive correlation of endogenous NO and GB in the leaves was observed under OS (Table 1). Moreover, the BADH activity and choline content in both the cultivars increased under OS regardless of N form, especially in the drought tolerant cultivar (ZD 958) (Fig. 1). Correlations between the NO content and

GB content or choline content or BADH activity were significant under OS (Table 2) but not under the control conditions.

Studies on N-induced modulation of NO and GB accumulations in stressed plants can be useful to elucidate the underlying phenomena involved in drought tolerance (Ashraf *et al.* 2011). It is generally believed that adequate N supply is necessary to maintain a high osmotic adjustment ability and to overcome crop losses under drought (Zhang *et al.* 2009). Many investigations were conducted under drought with single N form supply (Mihailovic, *et al.* 1992, Li *et al.* 2009, Zhang *et al.* 2009, 2012a,b, 2013). In our earlier study, NO, GB, as well as key GB metabolism parameters, *i.e.*, BADH activity and choline content, increased under drought when exposed to foliar-applied urea (Zhang *et al.* 2012 b). In the present studies, different effects of N forms on the pattern of accumulation and metabolism of GB and its mediating signal NO were further clarified in maize under OS. The changes in all the measured parameters in response to OS were greater under the NO₃⁻ nutrition as compared to the plants subjected to the NH₄⁺ nutrition. The cultivar ZD958 was more responsive than JD20 to the OS and N-nutrition. Furthermore, OS obviously increased the accumulations of NO, GB, and choline, and enhanced the BADH activity more under both sole NO₃⁻ and the mixed supply of NH₄⁺ and NO₃⁻ than under sole NH₄⁺, especially in cv. ZD958. Thus, the content of NO, GB, and choline, as well as the BADH activity could be used as potential selection

Table 3. *F*-values of leaf glycinebetaine (GB) content [nmol g⁻¹(d.m.)], betaine aldehyde dehydrogenase (BADH) activity [nmol mg⁻¹ (protein) min⁻¹], choline content [nmol g⁻¹(d.m.)], and NO content [nmol g⁻¹(d.m.)] in osmotic stressed maize cultivars supplied with different N forms. Significant differences at * - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$.

Source of variation	d.f.	GB content	BADH activity	Choline content	NO content
Water regime (W)	1	11968.50***	13234.90***	1835.59***	6396.68***
Cultivar (Cv)	1	323.44***	446.00***	17.69***	140.54***
N form (NF)	2	103.53***	121.85***	139.24***	59.36***
W×Cv	1	558.71***	334.31***	77.54***	179.85***
W×NF	2	83.47***	149.75***	50.48***	60.43***
Cv×NF	2	14.04***	8.36**	3.19	8.01**
Cv×W×NF	2	12.38***	27.53***	0.69	6.67**

criteria for drought tolerance in maize because an effectiveness of N form on all the measured parameters under OS was more pronounced in tolerant ZD958 than that in sensitive JD20. In the control plants of the two cultivars, the less and non-significant effects of different N forms on the NO accumulation and the other attributes measured were found (Fig. 1, Table 1).

The interactions among the OS, cultivars, and N forms on the above parameters were significant (Table 3). It is, therefore, suggested that the optimal application of N forms can promote NO accumulation and up-regulate GB metabolism under OS. Consequently, the optimal N forms could promote accumulation of NO thereby causing enhanced accumulation of GB by up-regulating BADH

activity and enhancing choline content.

In conclusion, an increased ratio of NO_3^- to NH_4^+ in nutrient solution (i.e. 0:100, 50:50, 100:0) under OS led to an enhanced NO accumulation and an up-regulation of GB metabolism in the two maize cultivars, thereby resulting in a greater GB accumulation, which alleviated drought-induced damage to the maize plants. The GB and NO accumulations increased consistently with the duration and intensity of OS. These effects were more pronounced in the drought tolerant cultivar (ZD958) than in the sensitive one (JD20). The results suggest a beneficial role of NO_3^- as compared to that of NH_4^+ in improving drought tolerance in the maize plants.

References

- Anjum, S.A., Xie, X.Y., Wang, L.C., Muhammad, F.S., Chen, M., Wang, L.: Morphological, physiological and biochemical responses of plants to drought stress. - *Afr. J. agr. Res.* **6**: 2026-2032, 2011.
- Ashraf, M.: Inducing drought tolerance in plants: some recent advances. - *Biotechnol. Adv.* **28**: 169-183, 2010.
- Ashraf, M., Akram, N.A., Al-Qurainy, F., Foolad, M.R.: Drought tolerance: roles of organic osmolytes, growth regulators, and mineral nutrients. - *Adv. Agron.* **111**: 249-296, 2011.
- Bartels, D., Sunkar, R.: Drought and salt tolerance in plants. - *Crit. Rev. Plant Sci.* **24**: 23-58, 2005.
- Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - *Anal. Biochem.* **72**: 248-254, 1976.
- Daniell, H., Muthukumar, B., Lee, S.B.: Marker free transgenic plants: the chloroplast genome without the use of antibiotic selection. - *Curr. Genet.* **39**: 109-116, 2001.
- Feng, Z.X., Ren, A.N.: Mensuration of the content of choline in diospyros leaves. - *Tianjin J. traditional chin. Med.* **21**: 248-249, 2004.
- Griess, P.: Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt "Ueber einige Azoverbindungen." - *Chem. Ber.* **12**: 426-428, 1879.
- Grieve, C.M., Grattan, S.R.: Rapid assay for determination of water soluble quaternary ammonium compounds. - *Plant Soil* **70**: 303-307, 1983.
- Hoagland, D.R., Arnon, D.I.: The water culture method for growing plants without soils. - *Calif. Agr. Exp. Sta. Circ.* **347**: 1-32, 1950.
- Li, Y., Gao, Y.X., Ding, L., Shen, Q.R., Guo, S.W.: Ammonium enhances the tolerance of rice seedlings (*Oryza sativa* L.) to drought condition. - *Agr. Water Manage.* **96**: 1746-1750, 2009.
- Mihailovic, N., Jelic, G., Filipovic, R., Djurdjevic, M., Dzeletovic, Z.: Effect of nitrogen form on maize response to drought stress. - *Plant Soil* **144**: 191-197, 1992.
- Misra, A.N., Misra, M., Singh, R.: Nitric oxide ameliorates stress responses in plants. - *Plant Soil Environ.* **57**: 95-100, 2011.
- Rhodes, D., Hanson, A.D.: Quaternary ammonium and tertiary sulfonium compounds in higher plants. - *Annu. Rev. Plant Physiol. Plant mol. Bio.* **44**: 357-384, 1993.
- Siddiqui, M.H., Al-Whaibi, M.H., Basalah, M.O.: Role of nitric oxide in tolerance of plants to abiotic stress. - *Protoplasma* **248**: 447-455, 2011.
- Walch-Liu, P., Neumann, G., Bangerth, F., Engels, C.: Rapid effects of nitrogen form on leaf morphogenesis in tobacco. - *J. exp. Bot.* **51**: 227-237, 2000.
- Wang, J., Li, D.Q.: Effects of water stress on AsA-GSH cycle and H_2O_2 content in maize root. - *Chin. J. Eco-Agr.* **2**: 94-96, 2002.
- Winzler, R.J., Meserve, E.R.: Spectrophotometric determination of small amounts of choline. - *J. biol. Chem.* **159**: 395-397, 1945.
- Zhang, L.X., Li, S.X., Liang, Z.S., Li, S.Q.: 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, 2009.
- Zhang, L.X., Zhai, Y.Y., Li, Y.Y., Zhao, Y.Y., Lv, L.X., Gao, M., Liu, J.C., Hu, J.J.: Effects of nitrogen forms and drought stress on growth, photosynthesis and some physico-chemical properties of stem juice of two maize cultivars (*Zea mays* L.) at elongation stage. - *Pak. J. Bot.* **44**: 2869-2874, 2012a.
- Zhang, L.X., Zhang, X.F., Wang, K., Zhao, Y.G., Zhai, Y.Y., Gao, M., An, Z.F., Liu, J.C., Hu, J.J.: Foliar urea application affects nitric oxide burst and glycinebetaine metabolism in two maize cultivars under drought. - *Pak. J. Bot.* **44**: 81-86, 2012b.
- Zhang, L.X., Zhao, Y.G., Zhai, Y.Y., Gao, M., Zhang, X.F., Wang, K., Nan, W.G., Liu, J.C.: Effects of exogenous nitric oxide on glycinebetaine metabolism in maize (*Zea mays* L.) seedlings under drought stress. - *Pak. J. Bot.* **44**: 1837-1844, 2013.