BIOLOGIA PLANTARUM 51 (2): 386-390, 2007

BRIEF COMMUNICATION

Effect of osmotic stress and sodium nitroprusside pretreatment on proline metabolism of wheat seedlings

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Abstract

Effect of osmotic stress and sodium nitroprusside (SNP, NO donor) pretreatment on growth and proline metabolism of wheat seedlings was investigated. Polyethylene glycol 6000 treatment for 2, 4 and 6 d could be termed as mild, moderate and severe stress, respectively, according to decrease in the relative water content. Severe osmotic stress significantly decreased the growth and photochemical efficiency, and increased proline content due to activation of its synthesis. 0.2 mM SNP pretreatment enhanced growth of wheat seedlings, increased variable to maximum fluorescence ratio (F_v/F_m) and fluorescence yield, while decreased proline content. However, 2 mM SNP retarded the seedlings growth and chlorophyll *a* fluorescence, and increased proline accumulation. Our results showed that NO might be involved in the regulation of osmotic stress in a concentration-dependent manner.

Additional key words: chlorophyll *a* fluorescence, exogenous nitric oxide, *Triticum aestivum* L.

Water stress is the major factor limiting plant productivity. One way many plants coped with water stress is to synthesize and accumulate compounds termed osmoprotectants (or compatible solutes) including polyols, sugars, amino acids, betaines, *etc* (Bohnert and Jensen 1996). Generally, in many plants free proline accumulates in response to a wide range of stresses, such as salinity stress (Delauney and Verma 1993), drought stress (Zhang *et al*. 1995, Chandra *et al*. 2004), extreme temperatures (Ruiz *et al*. 2002), heavy metal toxicity (Chen *et al*. 2001) and nutrient deficiency (Sanchez *et al*. 2002). However, the precise function of proline accumulation is still a controversial question (Hare *et al*. 1999).

Nitric oxide (NO) has emerged as a signalling molecule of ubiquitous importance nowadays (Durner *et al*. 1999). It has also proved that the effect of NO depends on its concentration, *e.g.*, 10 μM inhibited leaf growth, whereas 1 μM enhanced leaf growth in lettuce (Hufton *et al*. 1996) and pea (Leshem *et al*. 1998). Uchida *et al*. (2002) found that pre-treated rice seedlings with NO permitted the higher survival rate under salt and heat stresses than the non-treated controls. Similarly, Tu *et al*. (2003) reported that 0.1 mM sodium nitroprusside delayed the senescence of wheat leaves by inhibition of the degradation of chlorophyll and soluble proteins, especially Rubisco, while 0.5 mM would accelerate the process of senescence. NO interacts with reactive oxygen species (ROS) in various ways and might serve as an antioxidant during environmental stresses (Beligni and Lamattina 1999). Modulation of superoxide formation by NO (Conner and Grisham 1996) and inhibition of lipid peroxidation (Lamotte *et al*. 2004) also illustrated its potential antioxidant role. However, so far the mechanism of NO effect has not been fully understood.

In our study, we employed wheat seedlings as a model to elucidate whether NO mediates proline metabolism to ameliorate osmotic stress. The aims of this study are as followings: *1*) to determine the changes of proline metabolism under osmotic stress; *2*) to explore the effect of NO on osmotic stress tolerance.

Received 12 September 2005, *accepted* 21 April 2006.
Abbreviations: ABA - abscisic acid; P5C - Δ-pyrroline-5-carboxylate; P5CS - P5C synthetase; PEG - polyethylene glycol; RWC - relative water content; SNP - sodium nitroprusside.
Acknowledgements: The research was supported by the Program of "100 Distinguished Young Scientists" and "Knowledge Innovation Engineering" (No. KSCX2-SW-115) of the Chinese Academy of Sciences.
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Selected wheat (*Triticum aestivum* L. cv. W7) seeds, provided by Dr. Tao Wang, Chengdu Institute of Biology, China), were surface sterilized with 0.1 % $HgCl_2$ for 3 min, washed thoroughly under tap water and finally with distilled water. Then they were sown in silicon sands and after germination the seedlings were cultivated in phytotron with day/night temperature of 22/18 °C, relative humidity of 60 %, 14-h photoperiod and photon flux density of 120 µmol m^{-2} s^{-1}. SNP (0, 0.2 and 2 mM) resolved in half strength Hoagland's solution were applied to the seedlings every other day. The experiment was repeated three times and each treatment included 50 seedlings. After complete extension of the second leaf (14 d after sowing), the seedlings were treated with 15 % PEG (m/v, osmotic potential - 0.5 MPa, Smita and Nayyar 2005) for 0, 2, 4 and 6 d. Osmotic stress was termed as mild (RWC lowered by 8 to 10 %), moderate (RWC lowered by 10 to 20 %) and severe stress (RWC lowered by 20 to 50 %), respectively. After that, wheat shoots were harvested, immediately frozen in liquid N_2, and then stored at -20 °C for further analysis except for photochemical efficiency determination.

The relative water content (RWC) of shoots was calculated as: RWC = 100 × [(fresh mass - dry mass)/(saturated mass - dry mass)]. Saturated mass was determined after incubation of the shoots in water for 24 h at room temperature. Dry mass was measured following oven-drying at 105 °C to constant mass.

Chlorophyll *a* fluorescence was estimated using a modulated fluorometer (*PAM 2100*, *Walz*, Effeltrich, Germany) as described by Schreiber *et al*. (1994). Leaves were kept in clip curettes for dark adaptation for 30 min before measuring. Fluorescence was excited with a saturating beam of white light (PPFD 8 000 µmol m^{-2} s^{-1}, 1.0 s) to determine the maximum (F_m) and the minimum (F_0) fluorescence of the dark-adapted sample. Potential quantum yield was expressed as F_v/F_m, defined as $(F_m-F_0)/F_m$. The actual quantum yield is defined as F_v'/F_m' = $(F_m'-F_0')/F_m'$, where F_m' stands for maximal and F_0' for zero fluorescence of an illuminated sample. The actual quantum yield was measured under full light conditions (300 µmol m^{-2} s^{-1}).

Proline was extracted and determined as described by Bates *et al*. (1973). 0.5 g seedlings were homogenized in a mortar after the addition of a small amount of quartz sand and 10 cm^3 of a 3 % (m/v) aqueous sulfosalicylic acid solution. The homogenate was centrifuged at 3 000 *g* for 20 min. The supernatant was treated with acid ninhydrin (2.5 g ninhydrin per 100 cm^3 of a solution containing glacial acetic acid, distilled water and 85 % *ortho*-phosphoric acid at a ratio of 6:3:1) boiled for 1 h, and the reaction was terminated in a water bath of room temperature (25 °C) for 10 min. Then absorbance at 520 nm was determined using L-proline as standard.

For Δ-pyrroline-5-carboxylate synthetase (P5CS, EC 2.7.2.1/1.2.1.41) extraction (Chen *et al*. 2001), 0.5 g wheat seedlings were homogenized in pre-chilled mortar and pestle, with extraction medium at 4 °C. The extraction medium contained 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 10 mM mercaptoethanol, 1 % (m/v) PVP, 5 mM $MgCl_2$, and 0.6 M KCl. The homogenate was centrifuged at 12 000 *g* for 20 min at 4 °C. P5CS activity was determined in 0.7 cm^3 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$, 10 mM ATP, 1.0 mM NADH, 50 mM glutamate, and 0.1 cm^3 enzyme extract. The reaction medium was incubated at 37 °C for 30 min. Then the reaction was stopped by adding 0.3 cm^3 trichloroacetic acid (10 %, m/v) and the colour was developed by incubating the reaction mixture with 0.1 cm^3 *o*-aminobenzaldehyde (0.5 %, m/v) in ethanol (95 %, v/v) for 1 h. After centrifugation at 12 000 *g* for 10 min, the clear supernatant fraction was taken to measure the absorbance at 440 nm (Zhao and Liu 2000).

Statistical analyses were conducted using *SPSS for Windows* (*Version 11.0*). Difference (LSD) test was employed to determine differences among the treatments at $P < 0.05$ and $P < 0.01$ level.

Compared with 0 d, RWC 2, 4 and 6 d after PEG application decreased by 10.19, 22.40 and 37.35 %, respectively. At the first two days fresh mass of wheat shoots increased a little, whereas afterwards it decreased significantly. Low concentration (0.2 mM) of SNP enhanced water content and maintained growth of wheat seedlings, whereas high concentration (2 mM) inhibited wheat growth (Table 1).

Photochemical efficiency indicated by F_v/F_m and fluorescence yield decreased gradually during drought stress (Table 1). 0.2 mM SNP pre-treatment enhanced F_v/F_m and yield, and 2 mM SNP had the opposite effect especially after 4 and 6 d stress.

There was continuous accumulation of proline with the time of incubation under osmotic stress. For example, at the 6 d after stress, proline content was 1.51 times compared with 0 day. 2 mM SNP increased content of proline, while 0.2 mM SNP decreased its content compared with no SNP pre-treatment (Fig. 1*A*).

Drought stress increased the activity of P5CS progressively. Compared with the unstressed wheat seedlings, the activity of P5CS 2, 4 and 6 d after stress were increased by 33, 50 and 86 %, respectively. SNP pre-treatment also promoted the activity of P5CS, and the effect of 2 mM SNP was more obvious than 0.2 mM in activating P5CS activity (Fig. 1*B*).

In our study, water stress, especially severe stress, signi-ficantly inhibited growth of wheat seedlings (Table 1). On the other hand, chlorophyll *a* fluorescence, an important indicator of reversible and irreversible changes of photosystem 2 (PS 2), did not change much. However, when the stress became severe, there was an obvious decrease in F_v/F_m which might affect plant photosynthesis and inhibited seedling growth. Our results are in agreement with some previous studies suggested that under mild water stress PS 2 photochemistry was not

Table 1. Effect of osmotic stress (PEG treatment for 2 - 6 d) and SNP pre-treatment on the relative water content (RWC), fresh mass (FM), dry mass (DM) and chlorophyll *a* fluorescence of wheat seedlings. The significant differences among 0 (as control), 0.2 and 2 mM SNP were determined by one-way *ANOVA* in LSD test. * - $P < 0.05$, ** - $P < 0.01$.

	SNP [mM]	0 d	2 d	4 d	6 d
RWC [%]	0.0	81.34 ± 0.95	73.05 ± 1.54	62.99 ± 1.52	50.96 ± 2.66
	0.2	82.47 ± 0.88	76.39 ± 1.87*	67.04 ± 1.19**	56.55 ± 1.97**
	2.0	80.45 ± 0.71	72.14 ± 1.61	58.41 ± 2.01**	42.71 ± 2.07**
FM [mg plant^{-1}]	0.0	127.60 ± 8.12	146.93 ± 3.93	127.10 ± 1.82	111.80 ± 3.21
	0.2	167.67 ± 1.74**	173.73 ± 5.31**	155.10 ± 1.93**	125.70 ± 3.11**
	2.0	135.07 ± 5.11	150.40 ± 4.51	101.80 ± 4.20**	80.47 ± 5.03**
DM [mg plant^{-1}]	0.0	23.78 ± 5.32	30.33 ± 1.33	37.06 ± 2.22	40.97 ± 1.51
	0.2	29.28 ± 2.34**	35.71 ± 4.11**	40.97 ± 2.53**	52.08 ± 2.19**
	2.0	25.67 ± 3.21	29.49 ± 2.34	33.99 ± 4.20**	36.36 ± 2.61**
F_v/F_m	0.0	0.805 ± 0.006	0.795 ± 0.007	0.779 ± 0.027	0.701 ± 0.009
	0.2	0.806 ± 0.015	0.798 ± 0.004	0.793 ± 0.014	0.754 ± 0.011**
	2.0	0.808 ± 0.005	0.802 ± 0.011	0.757 ± 0.009**	0.723 ± 0.009*
Fluorescence yield	0.0	0.721 ± 0.021	0.601 ± 0.194	0.596 ± 0.022	0.591 ± 0.017
	0.2	0.727 ± 0.008	0.637 ± 0.057*	0.642 ± 0.117**	0.629 ± 0.013*
	2.0	0.728 ± 0.006	0.603 ± 0.027	0.571 ± 0.013	0.550 ± 0.029*

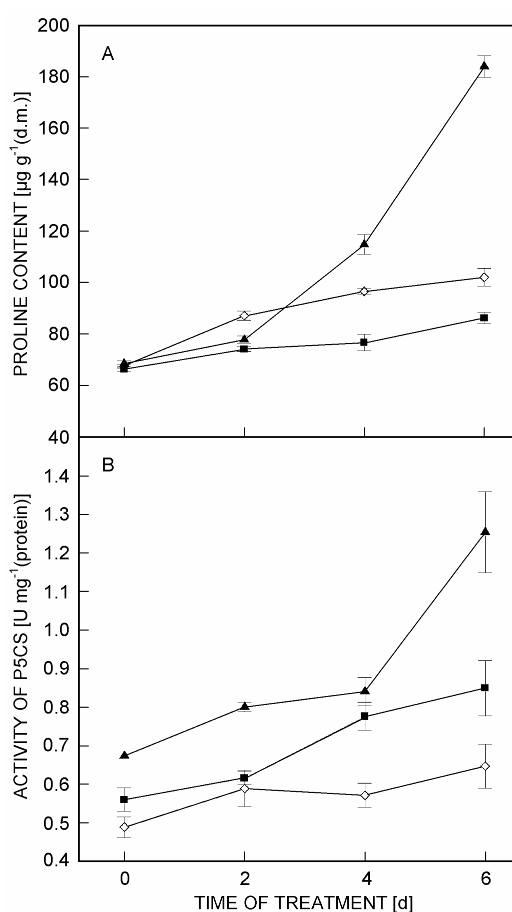

Fig. 1. Effect of osmotic stress and SNP pre-treatment (*open squares* - 0 mM SNP, *closed squares* - 0.2 mM SNP, *triangles* - 2 mM SNP) on proline content (*A*) and P5CS activity (*B*).

affected (Genty *et al.* 1987), whereas under severe stress damage occurs to both photosystems (Meyer and De Kouchkovsky 1993). It has been estimated that 30 % leaf water deficit is often the limit above which the photosynthetic biochemistry is significantly affected (Kocheva *et al.* 2005). 0.2 mM SNP pre-treatment promoted wheat growth and increased F_v/F_m and PS 2 quantum yield. One reason may be that NO could react with reactive oxygen species which were major damaging factors to the photosynthetic machinery under stress (Kim and Lee 2005). Moreover, it has also been reported that NO can react with lipid alcoxyl and peroxyl radicals, leading to the expectation that NO could stop the propagation of radical-mediated lipid oxidation (Lamotte *et al.* 2004). All these contributed to the protective function of SNP pre-treatment.

Free proline accumulation was observed in response to a wide range of abiotic and biotic stresses in plants, as manifested in our study (Fig. 1*A*). Proline accumulation under stressful conditions depended on both activation of biosynthesis and inhibition of degradation (Delauney and Verma 1993, Yoshiba *et al.* 1997). Our results showed that activity of P5CS, the key enzyme in proline synthesis, increased with the time of osmotic stress (Fig. 1*B*), suggesting that glutamic acid was converted to proline in wheat shoots. There is a general consensus that levels of transcripts encoding P5CS are rapidly induced to high levels upon dehydration and exposure to high NaCl concentrations (Peng *et al.* 1996, Igarashi *et al.* 1997). As the effect of different concentrations of SNP pre-treatment (0, 0.2 and 2 mM), we found that both 0.2 and 2 mM SNP increased the activity of P5CS (Fig. 1*B*). Uchida *et al.* (2002) also found that the

expression of P5CS was increased in response to NO in rice seedling leaves under salinity conditions, which could confer increased tolerance to salt stress. Moreover, many previous studies reported that the abscisic acid (ABA) was proposed to promote proline accumulation in response to a variety of environmental stresses (Dallier and Stewart 1992, Hare *et al*. 1999, Zhang *et al*. 2005). Ruan *et al*. (2004) showed that NO could activate the synthesis of endogenous ABA in wheat seedling leaves under 150 mM NaCl salt stress, which was in agreement with the results of Zhao *et al*. (2001) that the synthesis of ABA was inhibited by nitric oxide synthase (NOS) inhibitors in responding to drought stress. Therefore, NO might induce proline accumulation *via* ABA transduction cascade through activation of ABA synthesis.

In conclusion, osmotic stress decreased the relative water content and inhibited the growth of wheat seedlings. Severe stress induced significantly decreases in F_v/F_m and PS 2 quantum yield. Osmotic stress also resulted in proline accumulation due to activating its synthesis. 0.2 mM SNP pre-treatment enhanced wheat growth, maintained the relative water content and decreased proline content; while 2 mM SNP increased proline content and retarded wheat growth compared with no SNP treatment. The exact function of proline accumulation under stressful conditions as well as the relationship between nitric oxide and proline metabolism needs to be further elucidated.

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Teixeira da Silva, J.A. (ed.): **Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues.** Volumes I-IV. - Global Science Books, London 2006. 2532 pp. EUR 520.00 Print, eBook, CD-ROM. Print ISBN 978-4-903313-00-9, 978-4-903313-03-0, 978-4-903313-06-1, 978-4-903313-09-2.

Floriculture attracts attention of thousands of researchers and growers. Biotechnological methods can significantly help them in their hard effort to improve quality, widen the assortment, and decrease the price of cultivated flowering plants, as well as to preserve endangered naturally grown species.

The first volume comprises two sections “Structure, metabolism, development, physiology and genetics” and “Genes, genomes, genomics, and breeding” and each of them is divided into several parts. The contributions of the Parts 1 to 6 of the first section are devoted to flower growth, development and senescence, to cytology, to endogenous rhythms, to flower colour and scent, to phytohormones and to seeds. The Parts 1 to 3 of the second section deals with molecular techniques, with breeding and with mutagenesis.

The second volume consists of sections “Genetic engineering and transgenesis” and “Tissue culture and the *in vitro* environment”. After introduction, the main topics of contributions of the first section (Parts 1 to 6) are marker genes, transformation techniques, transgene expression, and applications and risks of genetically modified organisms. The second section incorporates papers concerning somaclonal variation, somatic embryogenesis, improved organogenesis, bioreactors, and practical applications of *in vitro* cultures.

The first section of the Volume III “The *ex vitro* environment, minerals, water stress, remediation” is addressed to abiotic stresses and contributions of this section discuss mainly effect of minerals, salinity and water stress (Parts 1, 2, 4). In addition, two methodological parts are included: hydroponic systems (Part 3) and phytoremediation (Part 5). The second section of this volume “Plant-organism interactions, disease, and control” deals with biotic stresses. After evaluation of general mechanisms, papers of following parts gives attention to plant-plant, plant-fungus, plant-bacteria, plant-virus and plant-insect interactions.

The fourth volume contains three sections “Flowering plants: the future”, “Ornamental plants and flowers in art and society” and “Novel ornamental gems and floricultural assets”. The contributions in this volume touch very broad spectrum of topics from nanotechnology in plant science and conservation of genes to plants for spaceships. Important seems to be that more than twenty contributions are focused on production of pharmaceutically important secondary metabolites and another twenty contributions to novel ornamentals.

This book is far from being a complete and systematic survey of this broad field, but attention is given to recent progress. Some contributions are reviews in nature, but others bring original data. Thus, in addition to new data, the readers find here useful surveys of up-to-date state of knowledge in some special fields or merits and pitfall of many biotechnological methods. Many illustrative figures, photographs and tables are included.

Ultimately, this comprehensive source of up-to-date information will both attract the new students to this field and stimulate further exploration of it by teachers, researchers and growers.

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