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BRIEF COMMUNICATION

The effect of exogenously applied nitric oxide on photosynthesis and antioxidant activity in heat stressed chrysanthemum

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Abstract

The effect of exogenously applied nitric oxide on the heat tolerance of *Chrysanthemum morifolium* was investigated by applying the NO donor sodium nitroprusside (SNP). We found that the SNP partially alleviated the heat stress by slowing down the reduction of photosynthetic pigment content and net photosynthetic rate. SNP treatment also lowered the increase in the non-photochemical quenching of fluorescence and malondialdehyde content and maintained higher activities of superoxide dismutase, peroxidase, catalase and ascorbate peroxidase.

Additional key words: ascorbate peroxidase, catalase, chlorophyll fluorescence, *Chrysanthemum morifolium*, malondialdehyde, net photosynthetic rate, peroxidase, superoxide dismutase.

Heat stress can deleteriously affect many of the physiological processes including the inhibition of photosynthesis, sugar accumulation, enzyme activities, and disruption of cell membranes integrity. In addition, high temperature stress often induce the overproduction of reactive oxygen species (ROS), which leads to membrane lipid peroxidation, protein denaturation, nucleic acid damage, *etc.* (Mittler 2002). Nitric oxide, a highly reactive, membrane-permeating free radical is associated with a broad spectrum of regulatory functions in plant growth and development (Simontacchi *et al.* 2004). Exogenously supplied NO has been observed to improve the heat tolerance in many plants, but little is known about its action on chrysanthemum. It has also been involved in alleviating iron deficiency-induced stress in *Solanum nigrum* (Xu *et al.* 2009). The chrysanthemum, an important world-wide cut and pot flower, grows optimally when the temperature remains in the range 18 - 21 °C. High temperatures during the summer months severely affect its year around production. Therefore, the aim of this paper was to determine the effect of NO treatment on changes in photosynthesis and antioxidant enzymes induced by heat stress.

The *Chrysanthemum morifolium* L. cv. Zaohuang was obtained from the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. Rhizogenic cuttings were transplanted into 8 × 10 cm pots containing a mixture of garden soil:*Vermiculite*: plant ash (1:1:2) in a greenhouse, at relative humidity of 60 - 70 %, day/night temperature of 25/20 °C and 150 - 160 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) during 12-h photoperiod. Plants at the 8 - 10 leaf stage were sprayed with 200 μM sodium nitroprusside (SNP) in the morning and evening over three days (the SNP treatment referred as SNPtr). The plants sprayed with distilled water were used as control (CK2). The SNPtr and CK2 plants were subjected to 45 °C in a growth chamber with PAR 300 μmol m^{-2} s^{-1} and relative humidity 60 - 70 % for 3, 6, 12 or 24 h. The water sprayed plants without heat stress were referred as un-heated control (CK1).

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; c_i - intercellular CO_2 concentration; E - transpiration rate; F_m, F_0, F_v - maximum, basic and variable fluorescence; g_s - stomatal conductance; MDA - malondialdehyde; NBT - nitroblue tetrazolium; NPQ - non-photochemical quenching of fluorescence; PAR - photosynthetically active radiation; P_N - net photosynthetic rate; POD - peroxidase; PS 2 - photosystem 2; ROS - reactive oxygen species; SNP - sodium nitroprusside ; SOD - superoxide dismutase.

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Net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s) and intercellular CO_2 concentration (c_i) of the fourth and fifth mature fully expanded leaves (5 leaves at each time) were determined with a *LI-COR 6400* portable photosynthesis system (*LI-COR*, Lincoln, NE, USA) at 30 ºC. The CO_2 concentration in the chamber was 380 ± 10 μmol mol^{-1} and photosynthetic photon flux density of 600 μmol m^{-2} s^{-1} provided by an LED (*LI-COR 6400-02*). Chlorophyll fluorescence was determined in the same intact leaves using a pulse-amplitude modulated chlorophyll fluorometer (*PAM*, *Walz*, Effeltrich, Germany) at 30 °C. Leaves were kept in the dark for at least 30 min before the potential maximal efficiency of photosystem (PS) 2 (F_v/F_m) was estimated from the ratio $(F_m - F_0)/F_m$, and the actual PS 2 efficiency (Φ_{PS2}) in the light-adapted state from $(F_m' - F_s)/F_m'$. Non-photochemical quenching of fluorescence (NPQ) (the non-radiative dissipation of energy) was estimated by $(F_m - F_m')/F_m'$. The nomenclature used for the various fluorescence parameters followed Van Kooten and Snel (1990). Leaf pigments were extracted with 95 % ethanol and absorbance was determined with *New century T6* spectrophotometer (*New century*, Beijing, China) at wavelengths of 470, 649, and 665 nm, respectively (Li 2000).

Leaf samples were ground to a fine powder in liquid nitrogen, and extracted in ten volumes of 50 mM potassium phosphate buffer containing 1 mM EDTA, 1 % (m/v) soluble polyvinylpyrrolidone, pH 7. The homogenate was centrifuged at 12 000 *g* for 15 min at 4 °C. SOD activity was determined by a nitroblue tetrazolium (NBT) test. One unit of SOD was defined as the amount of enzyme able to inhibit NBT reduction by 50 %. Peroxidase (POD) activity was estimated from the change in absorbance at 470 nm caused by the oxidation of guaiacol, following Yin *et al*. (2008). CAT assay was based on method of Aebi (1984) and APX activity was assayed by monitoring the decline in absorbance in the presence of ascorbic acid at 290 nm, according to Nakano and Asada (1981). One unit of POD, CAT and APX was defined as the amount of enzyme required to degrade 0.01 μM of substrate per min per g leaf fresh mass. The MDA content was used as an indicator of membrane lipid peroxidation, following Li (2000). The NO content was determined with an NO kit following the manual (Research Institute of Nanjing Jiancheng Bioengineering, Jiangsu, China).

All the experiments were repeated three times and means were compared using *DPS v3.01* (Tang and Feng 2002) software, along with Duncan's multiple range test.

In CK1, P_N, E and g_s were higher but c_i was lower than in SNPtr and CK2. Though P_N, E and g_s of the SNPtr and CK2 both showed a decreasing trend with heat duration, the SNP alleviated the decline. After 24 h treatment, P_N, E and g_s of the SNPtr plants were significantly different from those of CK1 and CK2 plants (Table 1). The c_i was higher in both SNPtr and CK2 than in CK1 except for the 12 h treatment (Table 1).

An increase in F_0 and a decrease in F_v/F_m and Φ_{PS2} with heat stress duration was observed in both SNPtr and CK2. F_v/F_m and Φ_{PS2} of CK1 were higher than those of SNPtr and CK2 (Table 1), while F_0 was lower in CK1 than in CK2 and especially SNPtr (Table 1). NPQ remained stable in CK1, but significantly increased in CK2. NPQ in SNPtr decreased at 3 and 6 h compared to CK1 (Table 1).

The contents of both chlorophylls and carotenoids in CK1 did not change obviously. Due to 24-h heat treatment, the content of chlorophyll declined by 12.7 % in SNPtr, but by 26.3 % in CK2; while the carotenoid content decreased by 39.2 % (SNPtr) and by 52.2 % (CK2) in comparison with CK1, respectively (Table 1).

SNPtr plants maintained a higher activity of SOD, POD, CAT and APX than CK2 did (Table 1). MDA content did not significantly change in CK1, however, the SNPtr inhibited the accumulation of MDA, especially at 12-h and 24-h heat treatment (Table 1).

The NO content of the SNPtr and CK2 were higher than that of CK1. NO content rose faster over the first 12 h in the SNP treated plants (especially at 6 h) than in CK2. The NO contents of the SNPtr and CK2 by 24 h were 3.68 fold and 5.28 fold higher than at 0 h, respectively (Table 1).

Photosynthesis is one of the most heat sensitive processes (Wang *et al.* 2009). The decreasing of P_N in the chrysanthemum under the heat stress was likely due to a reduction in chloroplast activity, rather than reflecting a change in stomatal behaviour, since c_i increased in response to the heat treatment, similarly as was found in oak (Haldimann and Feller 2004). The SNP treatment alleviated the fall in P_N (Table. 1), which is consistent with the ability of NO to maintain the activity of PS 2 (Shao *et al*. 2008). Correspondingly, SNP treatment helped retain the level of F_v/F_m and retarded the rise in F_0 (Table 1). NPQ has been closely associated with the triggering of excess energy dissipation by non-radiative processes, which gives some protection to the photosynthetic apparatus (Gilmore 1997). At the same time, however, it lowers the delivery of the excitation energy that is necessary for PS 2 photochemistry (Genty *et al*. 1990). Here, NPQ increased faster in CK2 than in SNPtr plants (Table 1), similarly as was observed in wheat (Shao and Shang 2008).

The chloroplast is not only the site of photosynthesis, but also of the production of reactive oxygen species (ROS). Here, heat stress reduced the chlorophyll content in SNPtr plants less severely than that in CK2, which is consistent with the observations in potato leaves treated with the herbicide diquat (Beligni and Lamattina 1999) and in bean seedlings irradiated with UV-B (Shi *et al*. 2005). The carotenoids not only absorb and transmit electrons, but they are involved in the neutralization of singlet oxygen, superoxide anions and other free radicals, and, thereby helping to protect the plant against photo-

Table 1. Changes in net photosynthetic rate, transpiration rate, stomatal conductance, internal CO_2 concentration, parameters of chlorophyll fluorescence, contents of chlorophylls and carotenoids, activities of antioxidant enzymes and contents of MDA and NO in chrysanthemum leaves after 0, 3, 6, 12 and 24 h of heat stress. Means ± SE, $n = 3$ (activities of SOD, POD, CAT and APX and contents of MDA and NO) or 5 (P_N, E, g_s, ci, F_0, F_v/F_m, Φ_{PS2}, NPQ, Chl and Car). Means significantly different at $P = 5$ % level are marked by different letters in each time point of each parameter. SNP - SNP treated plants under heat stress, CK2 - plants under heat stress sprayed with distilled water, CK1 - control, non-heat stressed plants.

Parameters	Treatments	0 h	3 h	6 h	12 h	24 h
P_N	CK1	5.823±0.077^a	9.533±0.093^a	7.355±0.314^a	4.016±0.080^a	5.729±0.223^a
[μmol(CO_2) $m^{-2}s^{-1}$]	SNP	5.540±0.166^a	5.477±0.613^b	4.609±0.399^b	3.622±0.293^a	1.204±0.056^b
	CK2	5.758±0.315^a	4.332±0.067^c	2.805±0.140^c	1.062±0.051^b	0.332±0.021^c
E	CK1	0.825±0.038^a	3.195±0.153^a	1.632±0.039^a	0.431±0.032^b	1.121±0.033^a
[mmol(H_2O) $m^{-2}s^{-1}$]	SNP	0.792±0.027^a	1.062±0.062^b	0.909±0.038^b	0.761±0.049^a	0.243±0.022^b
	CK2	0.871±0.052^a	0.879±0.014^b	0.654±0.025^c	0.223±0.028^c	0.127±0.035^c
g_s	CK1	36.903±0.573^a	127.32±1.127^a	70.473±2.685^a	8.724±0.886^c	80.848±1.364^a
[mmol(H_2O) $m^{-2}s^{-1}$]	SNP	34.629±0.637^a	50.645±3.364^b	43.237±0.365^b	34.711±2.288^a	11.799±0.496^b
	CK2	37.843±2.338^a	39.999±0.131^b	29.925±1.248^c	10.155±0.783^b	6.301±0.884^c
ci	CK1	121.5±2.1^a	113.7±3.3^b	108.2±3.2^b	206.5±8.5^a	123.2±2.1^c
[μmol mol^{-1}]	SNP	113.4±1.1^a	197.9±11.5^a	191.0±14.6^a	196.1±6.1^a	201.1±3.2^b
	CK2	124.2±7.8^a	190.5±4.9^a	215.2±6.7^a	195.8±3.8^a	284.8±8.0^a
F_0	CK1	0.293±0.008^a	0.294±0.011^b	0.285±0.014^b	0.288±0.005^b	0.281±0.009^b
	SNP	0.307±0.008^a	0.311±0.013^{ab}	0.319±0.021^{ab}	0.337±0.019^a	0.366±0.023^a
	CK2	0.292±0.009^a	0.353±0.007^a	0.357±0.004^a	0.362±0.006^a	0.380±0.013^a
F_v/F_m	CK1	0.837±0.006^a	0.841±0.008^a	0.848±0.012^a	0.832±0.005^a	0.837±0.009^a
	SNP	0.841±0.012^a	0.819±0.008^{ab}	0.820±0.004^{ab}	0.813±0.003^b	0.778±0.009^b
	CK2	0.841±0.002^a	0.775±0.008^b	0.753±0.011^b	0.734±0.004^c	0.728±0.010^b
Φ_{PS2}	CK1	0.615±0.023^a	0.658±0.015^a	0.651±0.011^a	0.630±0.008^a	0.674±0.013^a
	SNP	0.603±0.012^a	0.598±0.011^a	0.578±0.017^b	0.555±0.009^b	0.399±0.015^b
	CK2	0.575±0.010^a	0.408±0.036^b	0.316±0.007^c	0.269±0.012^c	0.293±0.014^c
NPQ	CK1	0.735±0.011^a	0.727±0.024^b	0.736±0.020^b	0.727±0.017^a	0.720±0.014^c
	SNP	0.740±0.034^a	0.620±0.091^b	0.442±0.020^c	0.789±0.076^a	1.050±0.079^b
	CK2	0.785±0.014^a	1.397±0.094^a	1.820±0.017^a	1.201±0.045^a	1.391±0.046^a
Chl	CK1	1.835±0.07^a	1.970±0.04^a	2.058±0.04^a	1.960±0.04^a	1.861±0.02^a
[g m^{-2}]	SNP	1.816±0.01^a	1.703±0.02^b	1.689±0.07^b	1.628±0.01^b	1.585±0.09^b
	CK2	1.820±0.05^a	1.643±0.07^b	1.584±0.01^b	1.409±0.05^c	1.342±0.07^c
Car	CK1	0.310±0.01^a	0.329±0.01^a	0.299±0.01^a	0.285±0.01^a	0.279±0.01^a
[g m^{-2}]	SNP	0.309±0.01^a	0.280±0.01^b	0.242±0.02^b	0.209±0.01^b	0.188±0.01^b
	CK2	0.311±0.02^a	0.231±0.01^c	0.188±0.01^c	0.156±0.01^c	0.148±0.01^b
SOD	CK1	751±13.2^a	802±42.3^b	738±52.1^b	688±7.4^b	847±58.2^b
[U g^{-1}(f.m.) min^{-1}]	SNP	759±30.1^a	959±25.3^a	1076±37.1^a	1100±74.2^a	1040±29.1^a
	CK2	759±30.1^a	845±26.2^b	775±40.2^b	985±21.1^a	854±17.3^b
POD	CK1	1046±45.1^a	1541±89.2^a	1545±12.5^a	1441±22.9^a	1201±75.6^a
[U g^{-1}(f.m.) min^{-1}]	SNP	946±22.2^a	1448±100.4^a	1491±52.4^b	1096±4.1^b	1069±81.7^b
	CK2	946±22.2^a	1239±77.5^b	939±56.4^c	547±34.6^c	486±32.2^c
CAT	CK1	538±25.4^a	526±28.7^c	448±36.4^c	491±23.3^b	506±20.8^a
[U g^{-1}(f.m.) min^{-1}]	SNP	524±16.5^a	913±15.1^a	776±20.8^a	715±60.0^a	392±19.8^b
	CK2	524±16.5^a	711±48.1^b	646±3.8^b	293±18.9^c	339±8.5^b
APX	CK1	379±17.3^a	422±5.7^b	383±6.0^c	451±4.6^b	495±32.6^c
[U g^{-1}(f.m.) min^{-1}]	SNP	369±13.1^a	529±33.6^a	749±39.5^a	570±43.2^a	808±43.2^a
	CK2	369±13.1^a	531±6.4^a	451±3.8^b	405±46.5^b	610±32.2^b
MDA	CK1	5.26±0.37^a	5.50±0.15^a	4.66±0.06^b	5.31±0.35^b	5.24±0.24^b
[nmol.g^{-1}(f.m.)]	SNP	5.37±0.06^a	5.51±0.14^a	5.77±0.24^{ab}	6.23±0.26^{ab}	6.83±0.25^a
	CK2	5.37±0.06^a	5.90±0.23^a	6.44±0.14^a	7.15±0.15^a	8.22±0.29^a
NO	CK1	1.03±0.03^a	0.99±0.01^b	1.14±0.18^b	1.20±0.06^b	2.11±0.06^c
[μmol.g^{-1}(f.m.)]	SNP	1.07±0.03^a	1.31±0.01^a	2.86±0.11^a	1.82±0.16^a	3.94±0.17^b
	CK2	1.07±0.03^a	1.160.10^{ab}	1.43±0.08^b	1.48±0.04^{ab}	5.65±0.25^a

oxidative damage. The carotenoid content of SNPtr plants was higher than that in CK2 plants and help their protection against oxidative stress.

The role of NO in the oxidative stress responses depends on its ability to maintain cellular redox homoeostasis and to neutralize the toxicity of ROS. Here, we observed a transient increase in SOD, POD, CAT and APX activity during first 6 h of the heat stress both in SNPtr and CK2 (Table 1). This promotion of antioxidant activity is probably a defence response. Despite this, the exogenous supply of NO further increased antioxidant enzymes activity up to 6 h after the onset of stress, and retarded its later decrease. The protective effect of NO in heat stressed chrysanthemum may therefore be mediated by increased activities of these enzymes.

Membrane injury is a consequence of lipid peroxidation (Scandalios 1993). Here, SNP treatment moderated the accumulation of MDA in the leaves of heat-stressed chrysanthemum plants (Table 1). The protective role of NO may reflect, in part, its interaction with lipid hydroperoxyl or superoxide radical, which promote lipid peroxidation (Wink and Mitchell 1998).

NO is an important signalling molecule for abiotic stresses, and its production can be triggered by osmotic stress, and UV-B irradiation (Gould *et al*. 2003). It is also known to be involved in the heat stress response, since this stress increased synthesis of NO in alfalfa (Neill *et al*. 2003). Here, the endogenous NO production was accelerated under heat stress and especially in SNPtr (Table 1). We speculate that the precocious generation of NO induced by the SNP treatment in heat stressed chrysanthemum acts as a signal for the adaptive response to heat injury. NO has both antioxidant and pro-oxidant effects on plants, depending on its concentration (Tewari *et al*. 2007). In conclusion, the NO treatment plays an important protective role in the heat-stressed chrysanthemum plants.

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