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Responses of the antioxidant systems and xanthophyll cycle in *Phaseolus vulgaris* to the combined stress of high irradiance and high temperature

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Abstract

Changes in the activities of enzymes involved in scavenging active oxygen species were followed after exposing bean seedling leaves (*Phaseolus vulgaris* L.) to various cross stresses of irradiance and temperature. The activities of superoxide dismutase (SOD, EC 1.15.1.1) and ascorbate peroxidase (AsAPOD, EC 1.11.1.11) increased to different extent with prolonged irradiation of the leaves, and were stimulated by high temperature (HT). The activity of catalase (CAT, 1.11.1.6) decreased when exposed to strong *irradiance* (HI), and the decrease was further exacerbated when HI was combined with HT. CAT activity was more sensitive to HT than to HI. Ascorbate (AsA) content slightly decreased and then increased during the treatment of HI, but decreased under the cross stress of HI and HT. On the contrary, glutathione (GSH) content increased all the time during various treatments of irradiance and temperature. The increase in the combined stress was even more pronounced. Irradiance is the major reason in triggering the operation of xanthophyll cycle, which was difficult to be started by HT. The antioxidant systems tended to be inactivated with prolonged exposure to the cross stress of HI and HT. The de-expoxidated state of xanthophyll cycle, however, was increasing all the time, which indicated that the zeaxanthin-dependent thermal dissipation was one major energy dissipation pathway during the cross stress of HI and HT.

Additional key words: antheraxanthin; ascorbate peroxidase; catalase; dithiothreitol; glutathione; superoxide dismutase; violaxanthin; zeaxanthin.

Introduction

Up to a certain level, increases in photon flux density (PFD) result in further increases in photosynthetic CO_2 fixation, and trigger the operation of several photo-protection mechanisms, which can prevent the potential damage from the accumulation of excess excitation energy in the photochemical apparatus. One is the thermal dissipation of excess excitation energy, especially the thermal dissipation involving the xanthophyll cycle presumably in the chlorophyll pigment bed (Bilger and Björkman 1990, Demmig-Adams and Adams 1992). The xanthophyll cycle consists of light-dependent conversion of three xanthophylls in a cyclic reaction, *i.e.*, violaxanthin, antheraxanthin, and zeaxanthin (Yamamoto 1979, Demmig-Adams 1990). There are enough evidences that zeaxanthin is involved in thermal energy dissipation and it is the major dissipation process that occurs in leaves over a range of conditions normally encountered by plants (Niyogi *et al.* 1998, Bilger and Björkman 1990, Demmig-Adams and Adams 1992).

Above a certain PFD or under stress (salt, cold, heat, *etc.*), photosynthesis will be incapable of utilizing all the energy absorbed by chlorophyll. Thermal energy dissipation is insufficient for scavenging the absorbed radiant energy, then the photosynthesis apparatus may be damaged and oxidative stress follows. During evolution, higher plants have developed an efficient defence system that helps them to survive under various adverse conditions (Bowler *et al.* 1992). Thus, the antioxidant systems composed of SOD, catalase, ascorbate peroxidase, MDHAR, and several antioxidants such as ascorbate, GSH, α-tocopherol, carotenoids, flavonoids, *etc.*, form another energy dissipation pathway by scavenging the active oxygen species, $O_2^{\cdot-}$, ·OH, and H_2O_2. For example, Mehler reaction represents a

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Abbreviations: A, antheraxanthin; AsA, ascorbate; AsAPOD, ascorbate peroxidase; CAT, catalase; GSH, glutathione; HI, high irradiance; HT, high temperature; PFD, photon flux density; PS, photosystem; SOD, superoxide dismutase; V, violaxanthin; Z, zeaxanthin.
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dissipation pathway (Radmer and Kok 1976) when coupled to the ascorbate peroxidase reaction (Schreiber *et al.* 1991, Foyer *et al.* 1994). Further more, photorespiration contributes to sustain the electron transport and discharges some extra energy, even photosystem (PS) 2 repair cycle could be considered an energy dissipation pathway nowadays.

The direct reduction of O_2 by PS1 in the Mehler reaction results in the formation of the superoxide anion radical, $O_2^{\cdot-}$. SOD catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2 (McCord and Fridovich 1969, Monk *et al.* 1989). Superoxide radicals and hydrogen peroxide are relatively unreactive by themselves, but they can form hydroxyl radical, ·OH, in the presence of metal ions by Haber-Weiss reaction (Bowler *et al.* 1992). Hydroxyl radicals and their derivatives can react indiscriminately to cause lipid peroxidation, the denaturation of protein, and the mutation of DNA. Therefore, H_2O_2 must be eliminated before damages occur. H_2O_2 can react with ascorbate *via* ascorbate peroxidase to form water and O_2 by the Halliwell-Asada pathway, involving the oxidization of GSH. H_2O_2 can also be scavenged by CAT in the peroxisomes (Bowler *et al.* 1992).

More research was concentrated on the effects of irradiance stress combined with chilling (Demmig-Adams *et al.* 1989, Greer *et al.* 1991, Adams *et al.* 1995, Adams and Demmig-Adams 1995), drought (Demmig *et al.* 1988, Björkman and Schäfer 1989, Corlett *et al.* 1994, Brestic *et al.* 1995, Loggini *et al.* 1999), and nutrient limitation (Khamis *et al.* 1990, Morales *et al.* 1990, Demmig-Adams and Adams 1992) on plant growth. Less effort was exerted on photoinhibition at high temperature. Cross stress of light and temperature always happens in summer, especially during the 3~5 h in the noon. The enzymes involved in photoprotection pathways may be inactivated. The photochemical apparatus may be impaired when the photoprotection systems can not dissipate the excess of excitation energy efficiently. However, various plants experience the summer season and appear to be less affected by the cross stress.

In this paper, we report the changes in activities of the enzymes, especially, SOD, catalase, and ascorbate peroxidase, and in the contents of ascorbate and GSH involved in the scavenging of active oxygen species. These species are generated during various combinations of irradiance and temperature treatment on *Phaseolus vulgaris* seedlings, especially during short period stresses. The operation of xanthophyll cycle was studied under the cross stress to illuminate the individual or combined effects of irradiance and temperature on the xanthophyll cycle, which is not studied thoroughly till now.

Materials and methods

Plants and treatments: The bean plants (*Phaseolus vulgaris* L.) were grown in an exposed position on the campus of Shandong Agricultural University in summer season. The grown leaves of seedlings were used as materials in about two weeks. Leaf discs (diameter 1.5 cm) were cut from the seedlings under weak irradiance in the morning, and exposed to low (50 μmol m^{-2} s^{-1}), moderate (600 μmol m^{-2} s^{-1}), or high (1500-1600 μmol m^{-2} s^{-1}) irradiance provided by a 1000 W tungsten-halogen lamp. Water was continuously circulated between the leaf discs and lamps. Temperature was controlled by super thermostated water bath, and leaf discs were placed on humid filter paper to keep the turgor.

Enzyme activities: Samples [0.2 g(FM)] were homogenized on an ice bath and extracted with 1 cm^3 50 mM potassium phosphate buffer, pH 7.0, 1 % polyvinyl pyrrolidone. The supernatant was used for soluble protein content determination (Bradford 1976) and enzyme activities assay after centrifugation (167 rps, 10 min).

SOD (EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977). One unit of enzyme activity was the amount of enzyme bringing about 50 % inhibition of the photochemical reduction of NBT. CAT (EC 1.11.1.6) activity was measured as the rate of decrease in absorbance of hydrogen peroxide at 240 nm according to Havir and Machale (1987). AsAPOD (EC 1.11.1.7) activity was determined by following the oxidation of ascorbate by H_2O_2 as a decrease in A_{290} (Arrigoni 1992).

AsA, GSH, and pigment content determinations: Leaf tissue was homogenized in cold 6 % (M/V) trichloroacetic acid and centrifuged at 17 000×*g* for 5 min. AsA content was measured according to Mukherjee and Choudhuri (1985), GSH according to Ellman (1959). The leaf tissue was homogenized in 3 % metaphosphoric acid, and centrifuged (67 rps, 10 min). Pigment analyses were performed by HPLC on 0.25 cm^2 samples according to Thayer and Björkman (1990). Statistical significance among means was analyzed by Tukey's test, at $\alpha = 0.05$.

Results and discussion

Antioxidant enzyme system: Plants are resistant to single HI, and many evidences showed that photochemical apparatus could keep their integrity and the recovery from photoinhibition is fast. SOD activity showed non-significant difference in 3-h treatment of HI (1500~1600 μmol m^{-2} s^{-1}), and during the 2-h recovery

period in low irradiance of 50 μmol m^{-2} s^{-1} at 25 °C (Fig. 1*A*). SOD activities tend to increase at a lower level in a short period treatment of the combined stress. High SOD activity has also been reported during the treatment of low temperature and HI stress, drought (Dhindsa and Matowe 1981, Burke *et al.* 1985), and chilling (Clare *et al.* 1984). The increases in SOD activity provided another pathway for the removal of excess electrochemical energy. In sum, the changes in SOD activity (Fig. 1) are very small. Elongating the stress time may get more information concerning superoxide production or other changes in metabolism.

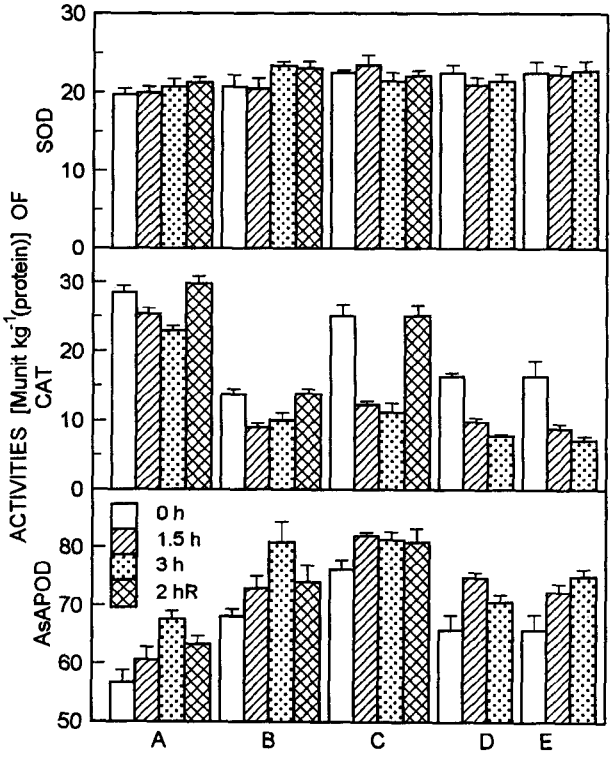

Fig. 1. Effects of cross stress of high irradiance (HI) and high temperature (HT) on the enzyme activities of SOD, CAT, and AsAPOD. The enzyme activities were measured at 0, 1.5, and 3 h of the stress treatment and after 2 h of recovery (2 hR, 25 °C, 50 μmol m^{-2} s^{-1}). *A*, HI (1500-1600 μmol m^{-2} s^{-1}) treatment at 25 °C; *B*, HI treatment at 35 °C; *C*, HI treatment at 38 °C; *D*, HT treatment at low irradiance (50 μmol m^{-2} s^{-1}); *E*, HT treatment at moderate irradiance (600 μmol m^{-2} s^{-1}).

AsAPOD activity showed the same increasing trend at 25 and 35 °C under HI, but decreased after 3-h HI treatment at 38 °C (Fig. 1). There was sustained increase in AsAPOD activity at moderate irradiance while it decreased after 3-h exposure to low irradiance in HT stress, indicating even moderate irradiance could produce more H_2O_2 under HT. The AsAPOD activity increase at HI under HT was also observed after irradiating at chilling conditions (Öquist *et al.* 1987, Mishra *et al.* 1993), and during O_3 fumigation, which indicates the increased rates of active oxygen species generation.

CAT is irradiance-sensitive and easy to be inactivated by irradiating (Feierabend and Engel 1986). There exists a rapid turnover of CAT-like D1 protein of PS2, which is generally sensitive to stress conditions combined with irradiance. Photoinhibition of CAT and of PS2 represent specific and widely occurring early symptoms of incipient photodamage indicating that the repair capacity is not sufficient (Feierabend *et al.* 1992, Streb *et al.* 1993). Despite its restricted localization and photolability, CAT may play a significant role in scavenging H_2O_2, which can readily diffuse across the membrane (Bowler *et al.* 1992). Under various irradiances at 38 °C, CAT activity decreased by 38.78 (1.5 h) and 51.98 (3 h) % at low irradiance, 44.84 (1.5 h) and 55.50 (3 h) % at moderate irradiance, 51.40 and 54.99 % at HI (Fig. 1). Apparently, there was no significant difference in the CAT activity decrease at 38 °C under different irradiances. Moreover, only 19.58 % of CAT activity were decreased at single HI treatment in 3 h, far less than HT treatment at low irradiance. The CAT activity was more sensitive to temperature than to irradiance. Under the cross effect of irradiance and temperature, most decrease of CAT activity was caused by temperature, and irradiation exacerbated the inactivation of CAT. CAT activity, however, was soon recovered in 2 h of low irradiance at 25 °C after various stress conditions. This further convinced that concomitant resynthesis of catalase compensates for the loss of catalase and maintains a constant level under irradiation when plants are not exposed to stress (Feierabend and Engel 1986).

Antioxidant non-enzyme system: AsA-GSH cycle could scavenge H_2O_2, and support the electron chain when excess excitation energy accumulated, avoiding the potential damage to photosynthetic apparatus (Foyer *et al.* 1994). AsA could also help the conversion of the xanthophyll cycle contents as reducer. AsA is one of the most important antioxidants in plants (Demmig-Adams 1992). At single HI treatment, AsA content increased slightly, and tended to recover in 2 h of low irradiance at 25 °C. When HI stress was combined with HT, AsA content decreased to the same extent at 1.5 and 3 h, and there was even a sustained decrease in the recovery period (Fig. 2). Ascorbate synthesis could also be stimulated in these conditions but it may also be oxidized rapidly.

GSH plays a pivotal role in stress tolerance and adaptation to environmental stress. It protects sulfhydryl of enzymes by reacting with $^1O_2$ and ·OH. Meantime, dehydro-ascorbate was reduced to AsA, combined with GSH being oxidized to GSSH. GR may be a rate-limiting enzyme for defense against active oxygen toxicity (Tanaka 1994). We found an up-rising of GSH content

during HI, or combined with HT, even in the recovery period (Fig. 2), which indicated the sustained high-demand of GSH in plants exposed to stress.

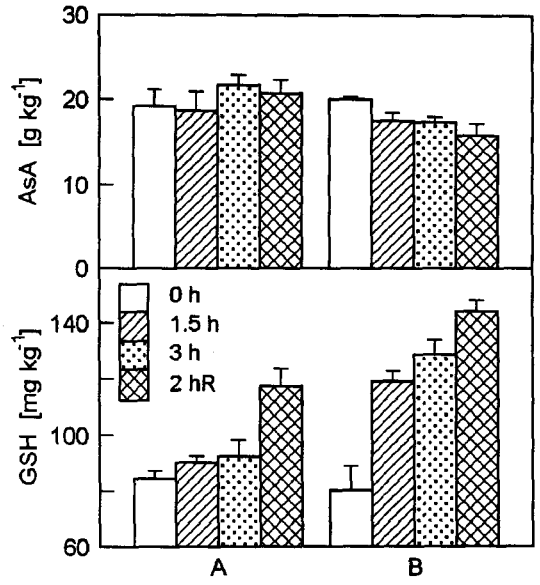

Fig. 2. Effects of cross stress of high irradiance (HI) and high temperature (HT) on the contents of AsA and GSH. AsA and GSH contents were measured at 0, 1.5, and 3 h of the stress and after 2 h of recovery (2 hR). *A*, HI (1500-1600 μmol m^{-2} s^{-1}) treatment at 25 °C; *B*, HI treatment at 38 °C.

Xanthophyll cycle: Leaf discs were exposed to a series of temperature separately at low (50 μmol m^{-2} s^{-1}) or moderate (600 μmol m^{-2} s^{-1}) irradiance for 1 h (Table 1). At low irradiance, zeaxanthin formation was difficult to be triggered by elevating temperature, and only formed a little at 42 °C. The de-epoxidized state did increase with the temperature increasing, and reached 0.571 at 42 °C, but prevalent form of the de-epoxidized state was antheraxanthin instead of zeaxanthin. When irradiance was up to 600 μmol m^{-2} s^{-1}, zeaxanthin appeared and increased with the increasing temperature except at 33 °C. The de-epoxidized state increased gradually, and major state was zeaxanthin instead of antheraxanthin. The results of the temperature treatment under two irradiances showed that irradiation was the major element in the formation of zeaxanthin, and temperature could promote its formation. The formation of zeaxanthin indicated that the excess excitation energy was accumulated and the dissipation mechanism was in operation.

Violaxanthin content was higher (except at 25 °C, when violaxanthin was highest at low irradiance) at 33 °C under two irradiances (Table 1). This indicated the most appropriate temperature for excited energy utilization and needed the least operation of xanthophyll cycle to dissipate excess energy.

Sustained HT (38 °C) at low irradiance (50 μmol m^{-2} s^{-1}) (Table 2) was difficult to trigger the operation of

Table 1. Effects of elevating temperature on the xanthophyll cycle contents [mmol kg^{-1}(DM)] under low (50 μmol m^{-2} s^{-1}) and moderate (600 μmol m^{-2} s^{-1}) irradiance. Leaf discs were treated at each temperature for 1 h under low and moderate irradiances. The xanthophyll cycle contents were measured by HPLC. Means followed by the same letter in the column do not differ statistically by the Tukey's test at α = 0.05. A, antheraxanthin; V, violaxanthin; Z, zeaxanthin; -, not detected.

Irradiance	T [°C]	V	A	Z	(Z+A)/(Z+A+V)
50	25	0.685a	0.010bc	-	0.014
	29	0.495a	0.020bc	-	0.040
	33	0.548a	0.055b	-	0.100
	37	0.350b	0.118a	-	0.337
	42	0.149c	0.123a	0.075	0.571
600	29	0.205a	0.218a	0.116b	0.620
	33	0.247ab	0.220a	0.066b	0.542
	37	0.179a	0.215a	0.123b	0.654
	42	0.086c	0.097b	0.400a	0.852

xanthophyll cycle, and only formed a little antheraxanthin and zeaxanthin in 3-h treatment. Thus the xanthophyll cycle was insensitive to temperature. Georgieva and Yordanov (1994) consider that HT may trigger some protective mechanisms, for example, increasing of non-photochemical fluorescence quenching and electron transfer of PS1 cycle. The formation of zeaxanthin indicated the operation of zeaxanthin-dependent thermal dissipation, which resulted in the increase in non-photochemical fluorescence quenching. However, the extent of thermal dissipation was low without relatively stronger irradiance. Thus PS2 could maintain its physiological activity in a wide temperature range.

Dithiothreitol (DTT), inhibitor of the formation of zeaxanthin (Yamamoto 1979, Demmig-Adams *et al.* 1990) was introduced into leaves under low irradiance (Table 3). In control plants, violaxanthin was deexpoxidized to zeaxanthin gradually, indicating the efficient thermal dissipation of xanthophyll cycle. On the contrary, no zeaxanthin formed in DTT-treated leaves under HI, but some violaxanthin was still de-epoxidized to antheraxanthin, which contributed to the increase of deepoxidized state. After DTT treatment, the photoinhibition was exacerbated and lot of D1 protein degraded as shown during previous study. Thus, antheraxanthin showed little effects in thermal dissipation.

HT exacerbated the irradiance damage to plant, producing more excess excitation energy. SOD, CAT, and AsAPOD were affected to different extent after 3-h treatment of HI and HT. However, the rate of violaxanthin de-expoxidization was accelerated (Table 4), decreasing by 77 % in 1 h, and the violaxanthin concentration decreased by 85.7 % after 3 h. The xanthophyll cycle operated to its largest extent and more zeaxanthin was formed, which proved that the energy dissipation dependent on xanthophyll cycle was the major

dissipation way under the cross stress of HI and HT.

Plants are relatively resistant to HI, which just caused reversible decrease of photochemical efficiency (values not shown) with no photochemical system destroyed,

Table 2. Time-dependent effects of high temperature (38 °C) on the xanthophyll cycle pigment contents [mmol kg^{-1}(DM)] under low irradiance (50 μmol m^{-2} s^{-1}). Pigments were measured at 0, 1.5, and 3.0 h of the treatment of high temperature under low irradiance. See legend of Table 1 for further details.

Stress time [h]	V	A	Z	(Z+A)/(Z+A+V)
0	0.613a	-	-	-
1.5	0.322b	0.117a	-	0.363
3.0	0.406b	0.098a	0.015	0.218

Table 3. Effects of strong irradiance on the xanthophyll cycle contents [mmol kg^{-1}(DM)] after treatment with DTT. DTT (3 mM) was introduced into leaves from petioles under low irradiance (50 μmol m^{-2} s^{-1}) for 3 h. Pigments were then measured after the treatment of leaves with strong irradiance (1500-1600 μmol m^{-2} s^{-1}). See legend of Table 1 for further details.

	Stress time [h]	V	A	Z	(Z+A)/(Z+A+V)
-DTT	0	0.638a	-	-	-
	1.5	0.289b	0.095b	0.325b	0.592
	3.0	0.197c	0.137a	0.459a	0.752
+DTT	0	0.640a	0.005b	-	0.009
	1.5	0.453b	0.137a	-	0.302
	3.0	0.360c	0.142a	-	0.394

accompanied by the active responses of antioxidant systems (Fig. 1) and low operation of the xanthophyll cycle (Table 3). At the earlier period of the treatment of HT and HI, the antioxidant system continued to dissipate excess excited energy, but tended to be inactivated at later period (Figs. 1 and 2). On the contrary, the operation of xanthophyll cycle was increasing all the time with the exacerbation of the cross stress of HT and HI (Table 4),

Table 4. Time dependent effects of cross stress by high temperature (38 °C) and high irradiance (1500-1600 μmol m^{-2} s^{-1}) on the contents of pigments [mmol kg^{-1}(DM)] of the xanthophyll cycle. See legend of Table 1 for further details.

Stress time [h]	V	A	Z	(Z+A)/(Z+A+V)
0	0.749a	0.011c	0.020c	0.040
1	0.172b	0.131a	0.564b	0.802
3.0	0.124c	0.089b	0.654a	0.857

which is an unusual biochemical phenomenon in plants. Therefore, it is very difficult to judge at what level of the operation of xanthophyll cycle the other protective systems began to be inactivated. Moreover, the violaxanthin de-expoxidization rate increased quickly in 1 h, near the highest point at 3 h. In our additional experiment, a series of leaf discs were pretreated for 1 h at irradiances of 100, 300, 500, 700, and 900 μmol m^{-2} s^{-1} at room temperature. They produced an increasing zeaxanthin content gradient with the increasing irradiances according to Demmig-Adams and Adams (1992). When these leaf discs were then exposed to HI and HT for 1.5 h, the photochemical efficiency, F_v/F_m, showed no difference, which indicated the higher zeaxanthin content before stress condition brought no advantage during the later stress treatment. One possible reason is that the zeaxanthin formation rate is too fast and soon makes up the zeaxanthin content difference. Irradiance is one major reason in triggering the operation of xanthophyll cycle. Single HT stress is difficult to start the de-expoxidiaztion of violaxanthin. We conclude that the zeaxanthin-dependent thermal dissipation is important in protecting the photosynthetic apparatus and avoiding damages, especially when the concomitant syntheses of various antioxidant enzymes and low-molecular antioxidants are insufficient to compensate the loss of them under HI combined with other stresses.

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