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BRIEF COMMUNICATION

Paraquat pretreatment alters antioxidant enzyme activity and protects chloroplast ultrastructure in heat-stressed cucumber leaves

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Abstract

Cucumis sativus L. seedlings were pretreated 1 h with 10 μM paraquat (PQ) and then were subjected to normal (25/18 °C) or elevated (42/38 °C) temperature to investigate whether PQ can protect plants against heat stress. Heat stress inhibited fresh and dry masses of the second leaf, root dry mass and shoot fresh mass. In leaves, the stress disintegrated membranes of 84.97 % chloroplasts and elevated contents of malondialdehyde, superoxide radical and hydrogen peroxide. In contrast, PQ pretreatment altered antioxidant activities in leaves, even after PQ was rinsed off before seedlings were exposed to different temperatures. Under heat stress, PQ pretreatment improved plant growth, decreased percentage of abnormal chloroplasts (53.03 %) and reduced contents of malonaldehyde, superoxide radical and hydrogen peroxide due to increased activities of antioxidant enzymes such as superoxide dismutase, catalase, guaiacol peroxidase, glutathione peroxidase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase.

Additional key words: antioxidant enzymes, *Cucumis sativus*, malondialdehyde, reactive oxygen species.

Heat stress damages photosynthetic apparatus and depresses plant growth (Ogweno *et al*. 2008). Stresses cause accumulation of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide, resulting in cell damage. To control ROS, plants evolve antioxidant enzymes (Wahid *et al*. 2007).

Paraquat (PQ), a bipyridylium herbicide, acts in irradiated chloroplasts by generating $O_2^{\bullet -}$ (Ananieva *et al*. 2004). However, it also stimulates antioxidant enzymes in leaves (Ekmekci and Terzioglu 2005), and thereby PQ pretreatment increased drought resistance of cucumbers (Liu *et al*. 2009). We hypothesize that PQ pretreatment at a certain concentration induces moderate stress and influences antioxidant activities in leaves. Then rinsing off PQ can eliminate PQ-induced stress, however, the increased antioxidant activities can protect chloroplasts and improve plant growth under subsequent heat stress. To date, no reports show that PQ pretreatment alleviates heat stress induced damage. In this study, PQ-pretreated cucumbers were exposed to day/night temperature of 42/38 °C as in Ma *et al*. (1998) to examine whether PQ could protect plants from heat stress and if this was associated with antioxidant activity regulation and chloroplast ultrastructural changes.

Each cucumber (*Cucumis sativus* L. cv. Chunguang No. 2) seedling was grown in one 10-cm pot filled with sand at 25 °C and 12-h photoperiod (600 μmol m^{-2} s^{-1}) and watered twice per day with Hoagland nutrient solution (Gao *et al*. 2010). At the two-leaf stage, 72 selected cucumber seedlings were randomly allocated into nine groups. The second leaves of one group were immediately harvested, four groups were watered with Hoagland nutrient solution containing 10 μM PQ, while the other four groups were watered with Hoagland nutrient solution alone. Seedlings were then kept at a weak irradiance (100 μmol m^{-2} s^{-1}) for 1 h to enable PQ to

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; DHAR - dehydroascorbate reductase; GPX - guaiacol peroxidase; GR - glutathione reductase; GSH-Px - glutathione peroxidase; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; PQ - paraquat; ROS - reactive oxygen species; SOD - superoxide dismutase.

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induce moderate stress. Subsequently, the second leaves were harvested from one group of PQ-pretreated and one group of PQ-untreated seedlings, and other plants were rinsed 12 times with Hoagland nutrient solution to remove PQ. Since PQ acts only under irradiance, the rinsed plantlets were kept in darkness for 24 h to eliminate the PQ stress. Then the second leaves were harvested from one group of PQ-pretreated and one group of PQ-untreated seedlings. Meanwhile, two separate groups of PQ-pretreated seedlings were exposed to 25/18 °C (PQ pretreatment) and 42/38 °C (PQ + heat treatment) in growth chambers. Two respective PQ-untreated groups were treated as above; the group at 25/18 °C was named 'control' and the group at 42/38 °C was named 'heat treatment'. The latter four groups were all exposed to 75 % air humidity and 12-h photoperiod (300 μmol m^{-2} s^{-1}) to enable seedlings to grow well in growth chambers, and the second leaves were harvested after keeping in heat stress for 72 h when the leaf edges dried in the heat treatment group. Three sets of plants grown at different time were used for each treatment.

Transmission electron microscopy of leaves was performed according to Helliot *et al*. (2003) with the modifications of Xu *et al*. (2008). Malondialdehyde (MDA) content was measured at 450, 532 and 600 nm according to Dhindsa *et al*. (1981) with the modifications of Zhang *et al*. (2005). $O_2^{\cdot-}$ formation rate and H_2O_2 content were determined according to Elstner and Heupel (1976) and Bernt and Bergmeyer (1974), respectively. The activity of superoxide dismutase (SOD) was assayed according to Hwang *et al*. (1999), the activity of catalase (CAT) according to Pereira *et al*. (2002), of guaiacol peroxidase (GPX) according to Ramiro *et al*. (2006), of glutathione peroxidase (GSH-Px) according to Xue *et al*. (2001), of ascorbate peroxidase (APX) according to Zhu *et al*. (2004), of monodehydroascorbate reductase (MDHAR) according to Hoque *et al*. (2007), of dehydro-ascorbate reductase (DHAR) according to Doulis *et al*. (1997) and glutathione reductase (GR) according to Foyer and Halliwell (1976). Protein contents of each enzyme extract were determined according to Bradford (1976). Data were collected from three replicates and were expressed as means ± standard deviations. Differences were analyzed with one-way *ANOVA* and the least significant difference (LSD). P values < 0.05 were considered to be significant.

In this study, heat treatment significantly decreased root dry mass, shoot fresh mass, and fresh and dry masses of the second leaf in comparison to control (Table 1). Wahid *et al*. (2007) also found inhibition of plant growth by heat. At 1 h after PQ pretreatment under irradiance or at 25 h after rinsing off PQ for 24 h, the growth parameters were not significantly different between PQ-pretreated and untreated plants, suggesting that PQ at a certain concentration does not influence plant growth at normal temperature. However, the growth parameters were significantly elevated in the PQ + heat treatment group when compared to the heat treatment group at 97 h, indicating that PQ improves plant growth under heat stress.

We found in the heat treatment group that 84.97 ± 2.53 % of chloroplasts were abnormal and swollen to varying degrees and had the disorganized non-parallel lamellae, whereas the control had normal chloroplasts and thylakoids (Fig. 1). Xu *et al*. (2006) also observed heat damages to chloroplasts and their thylakoids. Under heat stress, trehalose pretreatment protects chloroplast thylakoid membranes (Luo *et al*. 2010). Similarly, in the PQ + heat treatment group of our study, chloroplast thylakoids were well organized and only 53.03 ± 3.39 % of chloroplasts had abnormal shape, which was significantly lower than in the heat treatment group, indicating that PQ protected chloroplast ultrastructure in heat-stressed leaves. The altered ultrastructure is consistent with plant growth difference.

MDA contents in leaves were highest in heat treatment. Gao *et al*. (2010) also found that heat stress damaged cell membranes and increased MDA content. Also PQ treatment significantly increased MDA content in wild wheat (Ekmekci and Terzioglu 2005). Similarly, MDA content at 1 h was significantly higher in the PQ-pretreated seedlings than in control, suggesting that 1 h of PQ pretreatment under irradiance caused membrane lipid peroxidation. At 25 or 97 h, since PQ had been rinsed off for 24 or 96 h respectively, MDA contents in leaves were not significantly different between the PQ pretreatment and control groups. In contrast, MDA content in the PQ + heat treatment group was significantly lower than in the heat treatment group. So PQ decreased MDA content in heat-stressed leaves, which is similar to the report that salicylic acid alleviated heat stress-induced lipid peroxidation (Asthir *et al*. 2009). The decreased MDA in PQ-pretreated stressed seedlings is consistent with altered plant growth and chloroplast ultrastructure, suggesting that PQ reduced membrane lipid peroxidation and thereby alleviated heat-induced damage to chloroplast ultrastructure and so improved seedling growth.

The contents of $O_2^{\cdot-}$ and H_2O_2 were highest in heat treatment. Zhang *et al*. (2010) also found heat-induced ROS formation. PQ-induced $O_2^{\bullet-}$ in irradiated chloroplasts can be dismutated into H_2O_2 by SOD (Ananieva *et al*. 2004). Therefore, after PQ pretreatment for 1 h at irradiance of 100 μmol m^{-2} s^{-1}, $O_2^{\bullet-}$ and H_2O_2 contents were significantly higher than in control. However at 25 h, the contents of two ROS in PQ-pretreated leaves were not significantly different from those in control, because PQ had been rinsed off for 24 h and the PQ-induced stress was eliminated. PQ pretreatment can reduce $O_2^{\bullet-}$ and H_2O_2 contents under drought stress (Liu *et al*. 2009). Similarly, $O_2^{\cdot-}$ and H_2O_2 contents were significantly decreased in leaves of the PQ + heat treatment group compared to the heat treatment group. So PQ decreased ROS formation under heat stress,

which is consistent with the altered MDA content.

Some antioxidant enzymes alleviate ROS in chloroplasts (Gao *et al*. 2010, Liu *et al*. 2010). Therefore, we measured activities of eight enzymes to study the relationship between chloroplast ultrastructural changes and antioxidant activities.

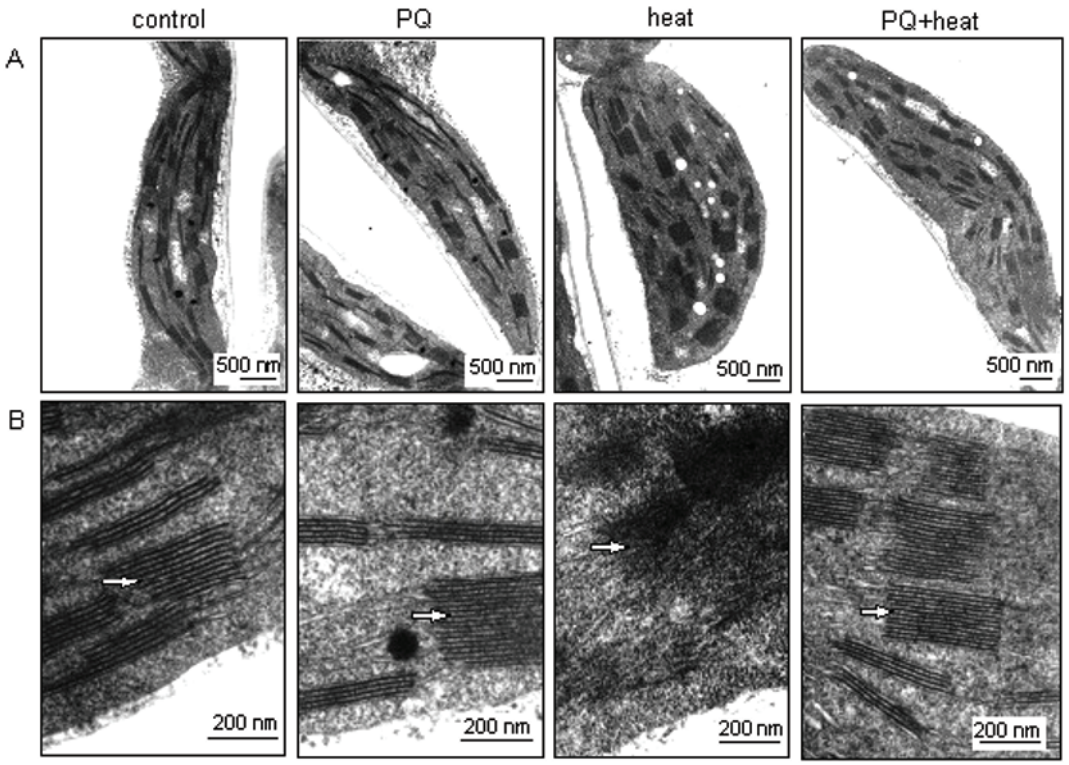

Fig. 1. Chloroplasts (*A*) and chloroplast thylakoids (*B*) in the first palisade parenchyma layer of PQ pretreated cucumber seedlings at 97 h. Control - 25/18 °C, PQ - PQ pretreatment and then 25/18 °C, heat - 42/38 °C, PQ + heat - PQ pretreatment and then 42/38 °C. The differences among the four treatments are marked with *white arrows*.

Table 1. Changes in plant growth, contents of MDA, $O_2^{\bullet-}$, H_2O_2 and activities of antioxidant enzymes in the second leaves after PQ pretreatment and heat stress. Control - 25/18 °C; PQ - PQ pretreatment and then 25/18 °C; heat - 42/38 °C; PQ + heat - PQ pretreatment and then 42/38 °C. Each value represents the mean of at least three replicates ± SD. Different letters indicate statistically significant differences between treatments at $P < 0.05$.

Parameters	0 h control	97 h control	PQ	heat	PQ + heat
Leaf fresh mass [mg]	628.37±109.49bc	891.53±29.86a	879.03±52.56a	478.80±16.13c	717.67±24.65b
Leaf dry mass [mg]	61.79±5.97c	91.27±6.25ab	91.60±1.19a	61.83±5.72c	80.27±3.20b
Root dry mass [mg]	26.37±2.99b	36.37±0.91a	38.27±1.91a	29.93±0.93b	39.60±2.05a
Shoot fresh mass [g]	1.71±0.07bc	1.97±0.05a	1.92±0.04ab	1.57±0.06c	1.91±0.04ab
MDA [nmol g^{-1}(f.m.)]	6.59±0.07bc	6.40±0.06cd	6.28±0.03d	7.60±0.14a	6.67±0.07b
$O_2^{\bullet-}$ [nmol g^{-1}(f.m)]	4.10±0.10b	4.16±0.09b	3.90±0.06bc	4.72±0.09a	3.97±0.09bc
H_2O_2 [nmol g^{-1}(f.m.)]	189.81±3.86d	167.52±2.23e	171.98±4.46e	285.65±2.23a	243.30±7.72b
SOD [U mg^{-1}(protein)]	58.91±0.94a	45.25±0.39c	46.82±0.34b	41.31±0.43e	43.19±0.47d
CAT [nmol mg^{-1}(protein) min^{-1}]	67.47±0.51e	92.17±1.16c	125.40±4.82ab	120.36±3.19b	134.13±2.62a
GPX [nmol mg^{-1}(protein) min^{-1}]	55.44±0.00e	121.88±5.86c	156.11±3.39b	161.89±6.23b	186.80±6.44a
GSH-Px [nmol mg^{-1}(protein) min^{-1}]	275.84±7.68d	218.29±12.36ef	352.37±10.25c	479.67±17.09b	544.76±11.76a
APX [nmol mg^{-1}(protein) min^{-1}]	701.80±12.67f	736.04±10.17ef	794.85±9.34cd	972.24±13.09b	1059.93±1.93a
MDHAR [nmol mg^{-1}(protein) min^{-1}]	114.37±5.26cd	134.00±5.83c	158.66±5.42b	161.62±1.79b	218.00±2.44a
DHAR [nmol mg^{-1}(protein) min^{-1}]	185.76±3.56e	192.35±0.81e	226.23±1.22c	345.89±3.57b	357.82±1.93a
GR [nmol mg^{-1}(protein) min^{-1}]	2.19±0.06g	2.88±0.08d	3.71±0.13c	4.70±0.03b	5.12±0.10a

Heat stress decreases SOD activity (Asthir *et al*. 2009) but increases GPX, CAT (Chaitanya *et al*. 2002) and APX (Hu *et al*. 2010) activities. Similarly, compared to control, our heat treatment obviously decreased SOD activity and significantly increased the activities of CAT, GPX, GSH-Px, APX, MDHAR, DHAR and GR. However, the heat treatment induced highest $O_2^{\bullet-}$ and H_2O_2 contents, indicating that generation of ROS exceeds the capacity of antioxidant enzymes. Compared to control, PQ-pretreated leaves at 1 h had the significantly increased CAT activity. Liu *et al*. (2009) also found that PQ activates CAT. In the PQ-pretreated seedlings at 25 h, activities of both CAT and APX were significantly higher than in control, showing that the effects of PQ pretreatment existed after PQ removal. Similarly, at 97 h, activities of SOD, GPX, GSH-Px, MDHAR, DHAR and GR were elevated. All 8 enzymes were significantly increased in the PQ pretreatment group compared to the control group, indicating that effects of PQ on antioxidant enzymes still existed at 97 h. Thus, when PQ-pretreated seedlings were transferred to heat conditions, activities of SOD, CAT, GPX, GSH-Px, APX, MDHAR, DHAR and GR in leaves of the PQ + heat treatment group were more increased compared to heat treatment alone. This is similar to a report that H_2O_2 pretreatment enhances the activities of SOD, CAT, GPX, GSH-Px, APX, MDHAR, DHAR and GR in cucumber leaves under osmotic stress (Liu *et al*. 2010), and we conclude that PQ increases antioxidant enzyme activities under heat stress. However, the irradiance at 1, 25, and 97 h was different, which might be another reason that antioxidant activities in the PQ pretreatment group varied during treatment time. However, all treatment groups at 97 h had the same experimental conditions except for temperature, and therefore the different antioxidant activities would be due to heat stress and PQ pretreatment. The higher activities of SOD, CAT, GPX, GSH-Px, APX, MDHAR, DHAR and GR in PQ-pretreated stressed leaves coincide with decreased $O_2^{\bullet-}$, H_2O_2 and MDA contents. Consistently with the ultrastructure and plant growth results, PQ increases cucumber's ability to eliminate $O_2^{\bullet-}$ and H_2O_2 *via* antioxidant enzymes. Therefore PQ application decreases lipid peroxidation, protects chloroplast ultrastructure in heat-stressed leaves and improves plant growth.

In conclusion, heat stress in cucumber leaves adversely affects growth, chloroplast shape and ultrastructure, due to increased contents of MDA, H_2O_2 and $O_2^{\bullet-}$. PQ pretreatment increases antioxidant enzyme activities and thereby eliminates accumulation of MDA, H_2O_2 and $O_2^{\bullet-}$ under heat stress, thus protecting chloroplast ultrastructure and improving plant growth.

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