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BRIEF COMMUNICATION

Photosynthesis, respiration and antioxidant enzymes in pepper leaves under drought and heat stresses

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Abstract

Leaf gas exchange, chlorophyll fluorescence and activities of antioxidant enzymes were studied in two pepper (*Capsicum annuum* L.) cultivars grown under drought (D) and heat (H), as well as under drought and heat in combination (HD). The drought-tolerant cv. Zhengjiao 13 exhibited greater net photosynthetic rate (P_N) and cytochrome respiratory pathway activity (R_{SHAM}), and lower contents of superoxide radical and hydrogen peroxide, as compared to the drought-sensitive cv. Longkouzaojiao. The P_N and R_{SHAM} decreased and ROS production increased under D and HD in both cultivars. As compared to the Longkouzaojiao, Zhengjiao 13 retained higher non-photochemical quenching (NPQ), photorespiration rate (R_L), and alternative respiratory pathways (R_{KCN}) under D and HD. Drought increased the superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities in the cytosol, chloroplasts and mitochondria in the two cultivars. Although SOD and APX activities decreased in Longkouzaojiao under HD, SOD activity increased in Zhengjiao 13. There was no H-induced reactive oxygen species production due to increase of R_L, NPQ, R_{SHAM}, R_{KCN} and activities of SOD and APX. However, H slightly decreased the P_N. The results indicated that HD was more detrimental than either stress alone.

Additional key words: alternative oxidase, ascorbate peroxidase, *Capsicum annuum*, chlorophyll fluorescence, drought tolerance, net photosynthetic rate, photorespiration rate, superoxide dismutase.

It is generally accepted that biotic and abiotic stresses including drought and heat may increase the formation of ROS and lead to oxidative stress through the disturbance of redox homeostasis in plant cells (Noctor and Foyer 1998, Reddy *et al.* 2004, Ali *et al.* 2005). Meanwhile, plants also evolved avoidance and scavenging mechanisms to protect them from destructive oxidative stress (Mittler 2002). The chloroplasts and mitochondria are two potentially important sources of ROS production in plant cells (Mittler *et al*. 2004). In chloroplasts, excited pigments in the thylakoid membranes may interact with O_2 to form ROS under conditions limiting CO_2 fixation (Asada and Takahashi 1987). The interaction of O_2 with the reduced components of the over-reduction electron transport chain in the mitochondria may also lead to ROS formation under stress conditions (Møller 2001). In long-term evolution, plants have developed several energy dissipation pathways to protect chloroplasts: photo-respiration (Osmond *et al.* 1997), cyclic flow of electron (Miyake and Yokota 2001), water-water cycle (Asada 1999, Zhou *et al.* 2004), and xanthophyll cycle-dependent energy dissipation as heat from antenna in PS 2 (Xu *et al.* 1999, Hu *et al*. 2008b). Similarly, the mitochondria also contain three so-called "first line" defenses that act to limit the production of ROS *via* the electron transport chain: the alternative oxidase, the internal NADH dehydrogenase, and the uncoupling protein (Møller 2001, Mittova *et al.* 2004). These avoidance mechanisms may prevent excess electron flux to oxygen in the chloroplasts and mitochondria, as well as decrease ROS production (Schöner and Krause, 1990, Møller 2001, Ort and Baker 2002).

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Abbreviations: AOX - alternative oxidase; APX - ascorbate peroxidase; F_v/F_m - maximum photochemical efficiency of PS 2; MDA - malondialdehyde; NPQ - non-photochemical quenching; P_N - net photosynthetic rate; PPFD - photosynthetic photon flux density; R_L - photorespiration rate; ROS - reactive oxygen species; RWC - relative water content; SHAM - salicylhydroxamic acid; SOD - superoxide dismutase; R_{KCN} - alternative pathway activity; R_{SHAM} - cytochrome pathway activity; Φ_{PS2} - quantum efficiency of PS 2.

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The major ROS-scavenging enzymes of plants include superoxide dismutase (SOD) and ascorbate peroxidase (APX) (Mittler 2002). In plants, SOD and APX are found in the chloroplasts, mitochondria, peroxisomes, and cytosol (Mittler *et al.* 2004). Information about ROS scavenging in chloroplasts and mitochondria under combined stresses is still scanty (Møller 2001, Mittler *et al*. 2004). Drought and heat stresses often occur simultaneously (Wollenweber *et al.* 2003, Griffin *et al.* 2004) and can induce cellular damage due to accumulation of ROS (Ali *et al.* 2005, Lei *et al.* 2006, Bhattacharjee 2008, Wang *et al*. 2009). They also increase certain avoidance mechanisms such as photorespiration, xanthophyll-mediated thermal dissipation, and AOX to alleviate ROS production (Noctor *et al.* 2002, Zhou *et al.* 2007, Bartoli *et al.* 2005, Zhao *et al*. 2008). Moreover, they increase the activities of antioxidant enzymes. To characterize the relationship of ROS production, avoidance, and scavenging mechanisms involved in the response of plants to a combination of drought and heat stresses, we studied the effects of the combination stresses on two cultivars of pepper.

Capsicum annuum L. drought-tolerant cv. Zhengjiao 13 and drought-sensitive cv. Longkouzaojiao were used. The seeds were sown in a mixture of grass peat and *Perlite* (8:2, v/v) in a tray placed in a greenhouse. Plants at the 4-leaf stage were transferred into pots (30 × 30 cm) containing grass peat and *Perlite*, placed in a growth chamber (12-h photoperiod, photosynthetic photon flux density, PPFD, of 300 - 500 μmol m^{-2} s^{-1}, day/night temperature of 28/18 °C). Plants at the 20 to 25-leaf stage were fully watered (water content of the soil above 80 %) and then placed under four treatments for 5 d: *1*) control: the plants watered daily and grown under 28/18 °C; *2*) heat stress (H): the plants watered daily and grown under 45/35 °C; *3*) drought stress (D): irrigation was withheld in plants for 5 d (soil water content was 48 - 52 % at the 5th day) and grown under 28/18 °C; *4*) combined stress (HD): irrigation was withheld for 5 d (soil water content was 37 - 40 % at the 5th day) and grown under 45/35 °C. At the end of the experiment, all measurements were performed on fully expanded leaves, using five replicates.

The relative water content (RWC) was determined gravimetrically as described by Zhou *et al.* (2007) The electrolyte leakage was determined following the method of Hu *et al.* (2006). Net photosynthetic rate (P_N) and photorespiration rate (R_L) were measured using a portable photosynthetic system (*Ciras-1, PP-systems*, Hitchin, UK) on the fully developed leaves of each plant. PPFD, CO_2 concentration, air relative humidity, and leaf chamber temperature were maintained at 600 μmol m^{-2} s^{-1}, 360 μmol mol^{-1}, 80 - 90 % and 28 °C, respectively, by an automatic control. The P_N measured under two O_2 concentrations (21 and 2 %) and PPFD 600 μmol m^{-2} s^{-1} was used to calculate R_L (Jiang *et al*. 2006). Chlorophyll fluorescence was measured with a portable pulse modulated fluorometer (*FMS-2*, *Hansatech Instruments*, Norfolk, UK) on the same leaves previously used for gas exchange measurements. The leaves were dark-adapted for at least 30 min prior to measurement of the variable to maximum fluorescence ratio (F_v/F_m) characterizing maximum quantum efficiency of PS 2. An actinic light source (600 μmol m^{-2} s^{-1}) was applied to achieve a steady state of the fluorescence yield, namely, the quantum efficiency of PS 2 (Φ_{PS2}) and the non-photochemical quenching (NPQ) (Zhou *et al*. 2009).

The respiration rate was measured as a decrease in oxygen concentration in a 2-cm^3 closed cuvette using a Clark-type oxygen electrode (*Oxygraph-lab*, *Hansatech*) at 28 °C. The samples (0.1 g fresh mass) were kept in the dark for 30 min before respiration measurements were taken. To assess alternative pathway activity, the cytochrome pathway was inhibited with 10 mM KCN, whereas the alternative pathway was inhibited with 20 mM salicylhydroxamic acid (SHAM). The temperature was controlled by a water bath set (*2219 Multitemp II Thermostatic Circulator*, Germany).

Malondialdehyde (MDA) content was measured as described previously (Hu *et al*. 2006). The $O_2^{\bullet-}$ producing rate and the H_2O_2 content were analyzed as described by Zhou *et al*. (2004). In general, $O_2^{\bullet-}$ was measured by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\bullet-}$. H_2O_2 content was assayed diluting extract 2.5-fold with acetone and measured by monitoring the A_{410} of the titanium-peroxidase complex.

The chloroplasts and mitochondria were isolated from the leaves as described by Mittova *et al*. (2000), with a modification. Briefly, the leaves (10 g each) were chopped using a blender (*HR-2826*, *PHILIPS*, China) with 5 volumes of medium containing 50 mM HEPES [*N*-(2-hydroxyethyl) piperazine-*N'*- (2-ethanesulfonic acid); pH 7.5], 5 mM γ-caproic acid, 0.3 % bovine serum albumin (m/v), 0.4 M sucrose, 10 mM NaCl, 10 mM mercaptoethanol, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1 % (m/v) polyvinylpyrrolidine (PVP). The homogenates were filtered through four layers of gauze. The crude chloroplasts fraction from the leaves was sedimented by centrifugation at 1 000 *g* for 5 min. The chloroplasts in the residues were then purified by a 10, 40, 70, and 90 % *Percoll* discontinuous gradient in the presence of 2 mM ascorbate, and centrifugation at 4 700 *g* for 15 min. An intact chloroplast layer was obtained in between the 40 and 70 % *Percoll* fractions. The ferricyanide method was used to measure the intactness of the chloroplasts (Takeda *et al*. 1995). About 83 - 91 % of the chloroplasts were intact. The 1 000 *g* supernatant was re-centrifuged at 12 000 *g* for 15 min, and the pellet was collected and re-suspended in the following: 20 mM HEPES-KOH (pH 7.5), 330 mM sorbitol, 10 mM NaCl, and 2 mM EDTA. The pellets were fractionated on a 25, 37, 45, and 57 % (m/m) sucrose gradient at 68 000 *g* for 3.5 h, and the intact mitochondria layer between the 37 and 45 % sucrose fractions was removed. The integrity of the mitochondria estimated from cytochrome *c* oxidase activity (Millenaar *et al.* 2002) was 78 - 86 %. The activities of SOD and APX were assayed in intact chloroplasts and mitochondria diluted 2.5-fold with

25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbic acid (AsA), and 2 % (m/v) PVP. The activity was determined according to Zhou *et al.* (2004). An aliquot of the extract was used to determine the protein content following Bradford (1976), using bovine serum albumin as standard. SOD was measured by the photochemical method. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the rate of *p*-nitroblue tetrazolium chloride reduction at 560 nm. APX activity was determined by monitoring the rate of ascorbate oxidation at 290 nm (coefficient of absorbance 2.8 mM cm^{-1}). The reaction mixture contained 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 100 mM H_2O_2, 0.25 mM AsA, and the enzyme aliquot.

The results were tested with *SPSS11.5* for *Windows* (*SPSS Inc.*, Chicago, IL, USA) *via* one-way analysis of variance (*ANOVA*). Significant differences between the treatment means were separated by using the least-significant difference (LSD) test at $P < 0.05$.

Table 1. Effects of drought and heat stresses on relative water content (RWC), electrolyte leakage (EL), maximum photochemical efficiency of PS 2 (F_v/F_m), $O_2^{\bullet-}$ producing rate, H_2O_2 content, MDA content, net photosynthetic rate (P_N), photorespiration rate (R_L), quantum efficiency of PS 2 (Φ_{PS2}), non-photochemical quenching (NPQ), total respiration (R_{TOTAL}), SHAM-resistant respiration (R_{SHAM}), KCN-resistant respiration (R_{KCN}), SOD activity [U mg^{-1}(protein)] and APX activity in leaves of two pepper cultivars. Each value represents the mean of at least three replicates ± SE. Different letters indicate statistically significant differences between treatments at the 5 % level.

Parameters	Cultivar	Control	H	D	HD
RWC [%]	Zhengjiao 13	95.47 ± 0.83a	96.13 ± 0.72a	84.94 ± 1.53b	74.95 ± 1.98d
	Longkouzaojiao	96.19 ± 0.93a	97.03 ± 1.23a	79.41 ± 1.75c	64.47 ± 2.85e
EL [%]	Zhengjiao 13	21.32 ± 1.77d	24.50 ± 1.97d	30.79 ± 2.17c	39.75 ± 2.68b
	Longkouzaojiao	20.94 ± 1.35d	23.65 ± 1.85d	36.84 ± 2.71b	46.74 ± 2.86a
F_v/F_m	Zhengjiao 13	0.87 ± 0.008a	0.85 ± 0.007a	0.80 ± 0.006b	0.56 ± 0.011d
	Longkouzaojiao	0.87 ± 0.004a	0.85 ± 0.006a	0.68 ± 0.004c	0.47 ± 0.031e
$O_2^{\bullet-}$	Zhengjiao 13	38.70 ± 7.50d	39.50 ± 5.30d	63.90 ± 6.40c	87.60 ± 7.80b
[nmol g^{-1}(f.m.) min^{-1}]	Longkouzaojiao	38.70 ± 6.10d	47.90 ± 6.40d	84.80 ± 5.70b	106.90 ± 8.10a
H_2O_2	Zhengjiao 13	224.50 ± 19.7f	241.30 ± 27.2ef	332.10 ± 37.0d	477.60 ± 33.3b
[nmol g^{-1}(f.m.)]	Longkouzaojiao	241.80 ± 27.6ef	284.80 ± 29.7e	425.60 ± 18.6c	563.80 ± 14.6a
MDA	Zhengjiao 13	9.80 ± 0.60f	12.90 ± 0.70e	21.50 ± 0.60d	42.10 ± 1.10b
[nmol g^{-1}(f.m.)]	Longkouzaojiao	9.80 ± 0.70f	11.70 ± 0.90e	34.30 ± 0.90c	53.10 ± 1.70a
P_N	Zhengjiao 13	15.20 ± 1.10a	12.60 ± 0.90b	8.70 ± 1.10c	4.50 ± 0.70d
[μmol(CO_2) m^{-2} s^{-1}]	Longkouzaojiao	14.80 ± 1.30a	12.10 ± 0.70b	5.60 ± 0.30d	2.70 ± 0.60e
R_L	Zhengjiao 13	5.20 ± 0.40b	7.70 ± 0.80a	4.80 ± 0.40b	2.50 ± 0.30d
[μmol(CO_2) m^{-2} s^{-1}]	Longkouzaojiao	5.30 ± 0.30b	7.20 ± 0.60a	3.60 ± 0.30c	1.70 ± 0.20e
Φ_{PS2}	Zhengjiao 13	0.47 ± 0.04a	0.32 ± 0.01b	0.19 ± 0.02c	0.13 ± 0.02d
	Longkouzaojiao	0.46 ± 0.01a	0.33 ± 0.18b	0.15 ± 0.01d	0.09 ± 0.01e
NPQ	Zhengjiao 13	1.24 ± 0.13cd	1.56 ± 0.14b	2.05 ± 0.15a	1.40 ± 0.08bc
	Longkouzaojiao	1.20 ± 0.05d	1.49 ± 0.11b	1.37 ± 0.12bcd	0.52 ± 0.08e
R_{TOTAL}	Zhengjiao 13	8.56 ± 0.40b	11.03 ± 0.35a	8.73 ± 0.75b	5.55 ± 0.30c
[nmol(O_2) g^{-1}(f.m.) s^{-1}]	Longkouzaojiao	8.89 ± 0.38b	10.38 ± 0.34a	6.15 ± 0.50c	4.66 ± 0.38d
R_{SHAM}	Zhengjiao 13	6.75 ± 0.32b	7.55 ± 0.16a	5.50 ± 0.38c	2.97 ± 0.16d
[nmol(O_2) g^{-1}(f.m.) s^{-1}]	Longkouzaojiao	7.10 ± 0.31b	7.64 ± 0.19a	3.06 ± 0.01d	2.01 ± 0.21e
R_{KCN}	Zhengjiao 13	4.88 ± 0.22bc	6.47 ± 0.49a	6.10 ± 0.37a	4.35 ± 0.15cd
[nmol(O_2) g^{-1}(f.m.) s^{-1}]	Longkouzaojiao	4.11 ± 0.22d	5.30 ± 0.21b	5.19 ± 0.29b	3.93 ± 0.53d
SOD in cytosol	Zhengjiao 13	3.47 ± 0.42ef	7.50 ± 0.56c	9.29 ± 0.42a	5.11 ± 0.26d
[U mg^{-1}(protein)]	Longkouzaojiao	4.07 ± 0.52e	7.09 ± 0.54c	8.33 ± 0.18b	3.16 ± 0.52f
APX in cytosol	Zhengjiao 13	2.42 ± 0.17de	2.94 ± 0.36c	4.96 ± 0.25a	2.54 ± 0.15cd
[μmol mg^{-1}(protein) min^{-1}]	Longkouzaojiao	2.02 ± 0.14e	2.95 ± 0.27c	4.33 ± 0.36b	1.39 ± 0.12f
SOD in chloroplasts	Zhengjiao 13	2.29 ± 0.20f	3.57 ± 0.13d	6.31 ± 0.26a	2.99 ± 0.33e
[U mg^{-1}(protein)]	Longkouzaojiao	2.83 ± 0.12e	4.66 ± 0.14c	5.83 ± 0.39b	2.65 ± 0.36ef
APX in chloroplasts	Zhengjiao 13	0.59 ± 0.05f	0.89 ± 0.03d	1.91 ± 0.07a	0.69 ± 0.08ef
[μmol mg^{-1}(protein) min^{-1}]	Longkouzaojiao	0.73 ± 0.03e	1.16 ± 0.04c	1.62 ± 0.10b	0.42 ± 0.09g
SOD in mitochondria	Zhengjiao 13	1.85 ± 0.17e	2.73 ± 0.21d	5.97 ± 0.24a	2.59 ± 0.25d
[U mg^{-1}(protein)]	Longkouzaojiao	2.47 ± 0.19d	3.66 ± 0.23c	5.03 ± 0.27b	2.57 ± 0.28d
APX in mitochondria	Zhengjiao 13	0.47 ± 0.04f	0.79 ± 0.05d	1.70 ± 0.06a	0.49 ± 0.07f
[μmol mg^{-1}(protein) min^{-1}]	Longkouzaojiao	0.63 ± 0.05e	1.14 ± 0.06c	1.53 ± 0.07b	0.40 ± 0.07f

In both the drought-tolerant and drought-sensitive pepper cultivars grown at control and heat stress (H), there were no significant differences in RWC, electrolyte leakage (EL) and F_v/F_m. However, the combination of H and drought (D) caused higher decreases in RWC and F_v/F_m and increases in EL as compared to D alone. In agreement with our early observation (Hu *et al.* 2008a), the results showed that H aggravated the injury caused by D.

As compared to D alone, HD caused a greater decrease of P_N, Φ_{PS2} and R_{SHAM}, while simultaneously causing a greater increase of $O_2^{\bullet-}$ producing, H_2O_2, and MDA contents, especially in the sensitive cv. Longkouzaojiao. In our study, we found that the different drought tolerance of pepper cultivar were associated with the different responses of energy dissipation pathways to D. As compared to Longkouzaojiao, Zhengjiao 13 retained a higher R_L, NPQ, and R_{KCN} under D and HD treatment, with NPQ and R_{KCN} significantly increased under D. These results likely demonstrate that higher abilities of energy dissipation in the chloroplasts and mitochondria in Zhengjiao 13 under D and HD stresses might be involved in its greater drought tolerance as compared to Longkouzaojiao.

We also observed the marked production of $O_2^{\bullet-}$ and the accumulation of H_2O_2 and MDA in pepper leaves under D and HD stresses. Fortunately, plants also possess efficient scavenging systems for ROS to protect themselves from destructive oxidative stress. Drought stimulation induced an upregulation of ROS-scavenging enzymes SOD and APX in cytosol, chloroplasts and mitochondria of the two pepper cultivars, especially in Zhengjiao 13. Under HD, SOD activity was slightly increased, and APX activity was not affected in Zhengjiao 13; however, SOD and APX activities were deceased in Longkouzaojiao. In agreement with previous studies, these results showed that higher activities of antioxidant enzymes are associated with lower ROS accumulation and membrane injury during D, which are in turn linked to drought tolerance (Bowler *et al*. 1992, Türkan *et al*. 2005, Gao *et al*. 2008). In Zhengjiao 13 treated by D or HD, SOD activity increased by 175.1 and 30.4 % in the chloroplasts, and by 223.3 and 40.1 % in the mitochondria, respectively. SOD activity also increased by 106.0 and 103.5 % in the chloroplasts and mitochondria of Longkouzaojiao treated by D alone. However, SOD activity in the chloroplasts and mitochondria of Longkouzaojiao was not affected under HD. Over-productions of SOD and APX have been shown to improve oxidative stress tolerance in transgenic plants (Allen 1995, Van Breusegem *et al*. 1999). Accordingly, the higher ROS-scavenging capability of Zhengjiao 13 might be another major reason for its higher drought tolerance as compared to Longkouzaojiao.

As compared to the control plants, there was no ROS accumulation in the two pepper cultivars treated by H alone. We also observed that H increased R_L, NPQ, and R_{KCN} and SOD and APX activities. These facts suggested that pepper is a heat-tolerant plant, as it is usually grown in summer, and that this tolerance is perhaps due to its high-energy dissipation pathways and ROS-scavenging capabilities in the chloroplasts and mitochondria.

In conclusion, we have shown that drought resulted in a decrease in P_N and the cytochrome respiration pathway, which then resulted in ROS accumulation in the two pepper cultivars. Meanwhile, as compared to the drought-sensitive cultivar, the drought-tolerant cultivar showed a higher capability for protective mechanisms such as thermal dissipation, photorespiration, alternative respiratory pathway, and higher ROS scavenging capacity in the chloroplasts and mitochondria. Heat combined to drought aggravated the decrease of P_N and cytochrome respiration, and also decreased the energy dissipation capabilities and ROS scavenging enzymes activities (SOD and APX). These results show that energy dissipation and ROS-scavenging mechanisms are important processes to minimize the adverse effects of drought and heat.

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