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Uniconazole confers chilling stress tolerance in soybean (*Glycine max* L.) by modulating photosynthesis, photoinhibition, and activating oxygen metabolism system

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Abstract

The current study investigates a possible mediatory role of uniconazole in improving chilling stress tolerance in chilling-sensitive (Hefeng 50) and chilling-tolerant (Kenfeng 16) soybean varieties. Chilling stress decreased photosynthetic pigments and inhibited net photosynthetic rate which was ascribed to stomatal limitation. The maximum quantum efficiency of PSII primary photochemistry and electron transport rate were lower in uniconazole-treated plants compared with the control. The increased contents of superoxide ($O_2^{\bullet-}$) and malondialdehyde during chilling stress indicated oxidative stress. Chilling stress reduced glutathione and ascorbic acid contents, and promoted peroxidase, glutathione reductase, and ascorbate peroxidase activities. Uniconazole improved the tolerance of chilled plants. This might be due to better antioxidant defense mechanisms including higher contents of antioxidants and activities of antioxidant enzymes, which retard lipid peroxidation. Thus, uniconazole has a positive effect and improves the chilling resistance of soybean.

Additional key words: carotenoid; catalase; chlorophyll; reactive oxygen species; superoxide dismutase.

Introduction

Chilling stress is one of the destructive abiotic stresses that restricts photosynthesis and eventually limits agricultural production and quality of tropical and subtropical plants (Allen and Ort 2001, Ikeda *et al*. 2009, Liu *et al.* 2012, Nahar *et al*. 2015). Reduction in electron transport (Zhao *et al*. 2016) and photosynthetic efficiency, and changes in protein structure and enzyme activities are some of the most common and primary chilling injury symptoms within plants. Inhibition of the carbon reduction cycle (Allen and Ort 2001), PSII activity (Strauss *et al*. 2006), and photosynthesis (Savitch *et al*. 1997, Allen and Ort 2001) under chilling stress increases generation of reactive oxygen species (ROS) (Gill and Tuteja 2010, Zhao *et al*. 2016). To overcome such conditions and neutralize or counteract the deleterious effects of ROS and protect plants from oxidation damage, the antioxidant defense system with different antioxidant components maintains a delicate balance between ROS generation and scavenging (Nahar *et al*. 2015). The antioxidant defense system consists of nonenzymatic antioxidants, such as glutathione (GSH), ascorbic acid (AsA), and enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and peroxidase (POD). These enzymes scavenge ROS with AsA and GSH as electron acceptors (Gill and Tuteja 2010, Liu *et al*. 2015, Nahar *et al*. 2015). Various strategies are being employed to alleviate the adverse effects of chilling stress in plants. Foliage spray of plant growth regulators (PGRs) is an effective, facile, and practical technique to enhance tolerance of plants, which has been used widely in recent years (Zhang *et al*. 2007, Manafi *et al.* 2015, Zhao *et al*. 2016).

S-(+)-uniconazole has been used as a plant growth retardant, inhibiting biosynthesis of gibberellins (GA) and abscisic acid (ABA) (Saito *et al*. 2006), and for

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Abbreviations: ABA – abscisic acid; APX – ascorbate peroxidase; AsA – reduced ascorbic acid; Car – carotenoids; CAT – catalase; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DAB – 3,3-diaminobenzidine; DHA – oxidized ascorbic acid; DHAR – dehydroascorbate reductase; E – transpiration rate; ETR – electron transport rate; FM – fresh mass; F_v/F_0 – size and number of active reaction centers; F_v/F_m – maximum photochemical efficiency of PSII; GA – gibberellins; GR – glutathione reductase; g_s – stomatal conductance; GSH – reduced glutathione; GSSG – oxidized glutathione; MDA – malondialdehyde; MDHAR – monodehydroascorbate reductase; NBT – nitroblue tetrazolium chloride; PGRs – plant growth regulators; P_N – net photosynthetic rate; POD – peroxidase; ROS – reactive oxygen species; SOD – superoxide dismutase.
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enhancement of plant tolerance to environmental stresses (Todoroki *et al*. 2009), such as water deficit (Zhang *et al*. 2007, Duan *et al*. 2008), heat (Wan and Kothare 1999), waterlogging (Qiu *et al*. 2005), chilling (Chucheep *et al*. 2005), metal (He *et al*. 2017), and salt stress (Al-Rumaih and Al-Rumaih 2015). All these stresses lead to a decline of the net photosynthetic rate (P_N) and to increases in ROS contents. Recent studies have identified that uniconazole possessed antioxidant activity modulated by augmenting contents of antioxidants and stimulating antioxidant enzymes to scavenge excess ROS (Wan and Kothare 1999, Qiu *et al*. 2005, Zhang *et al*. 2007). Moreover, in soybean, uniconazole pretreatment can effectively suppress excessive vegetative growth during the flowering stage, delay senescence of leaves at pod-setting stage, and improve final yields (Yan *et al*. 2015).

Soybean (*Glycine max* L.) is native to subtropical regions and is regarded as chilling-sensitive species. When temperature drops below 20°C, its growth and development significantly retards (Balestrasse *et al*. 2010). Due to expansion of soybean growing areas toward cooler climates, the whole growth and development of soybean is exposed to chilling stress (Balestrasse *et al*. 2010). Therefore, the tolerance to chilling stress at the beginning of the flowering stage is one of the important challenges under a cold climate. Several studies exist on soybean chilling-stress tolerance in the seedling (Balestrasse *et al*. 2010, Tian *et al*. 2015), flowering (Ohnishi *et al*. 2010), pod-formation (Takahashi *et al*. 2005), and seed-filling stages (Funatsuki *et al*. 2004, Ikeda *et al*. 2009). Ohnishi *et al*. (2010) reported decreased pollen grain number on stigma, suggesting that insufficient pollination leads to low pod setting. Hall (2000) and Singh *et al*. (2010) showed that the response of vegetative growth is more sensitive than reproductive growth under chilling stress. Photosynthesis is the major determinant of a pod and seed number (Egli 2005). To ensure stable production of soybean under temperate climates, a greater understanding of chilling stress effects is required, especially at the flowering stage.

There are few studies in soybean leaves concerning the effect of uniconazole on the photosynthesis, photoinhibition, and the balance of ROS generated under chilling stress, especially at the beginning of the flowering stage. Therefore, the present investigation was conducted to determine whether uniconazole confers chilling tolerance to soybean plants and if such tolerance is correlated with changes in photosynthesis, photoinhibition, and oxygen metabolism.

Materials and methods

Plant material, growth conditions, and chilling-stress treatment: The experiment was conducted at Heilongjiang Academy of Agricultural Sciences located in Northeast China (34°30'N, 119°32'E) from May to October 2017. The seeds of popular soybean varieties, Kenfeng 16 and Hefeng 50, were sown at a rate of ten seeds per plastic pot (lower inside diameter of 20.0 cm, upper inner diameter of 28.0 cm, and height of 23.0 cm) filled with premixed air-dried soil [150.0 g $(NH_4)_2 \cdot SO_4$, 130.0 g K_2SO_4, and 50 g $(NH_4)_2HPO_4$ per pot], and no additional fertilization. Subsequently, five seedlings were retained after thinning at the second trifoliate leaf stage (V2 growth stage). All pots were placed on greenhouse benches under the natural environment. Standard practices suiting pot experiments were followed, and no pest or disease problems were found during the experimental period. At the beginning of the flowering stage (R1), one third of the plants (60 plants) were sprayed with 50 mg L^{-1} uniconazole solution (Zhang *et al*. 2007) and the rest (120 plants) were sprayed with distilled water. About 36 h after uniconazole spray, 60 of uniconazole-treated and 60 of water-sprayed plants were placed in a growth chamber under low temperature conditions [15/15°C – day/night temperature, relative humidity (RH) of 75%, and a natural light photoperiod] (Funatsuki *et al*. 2004, Ikeda *et al*. 2009). The remaining water-treated plants were placed under natural environments as controls. This experiment was set up in a completely randomized design with four replications. Fully expanded third trifoliate leaves were sampled from the main apex of each treatment on 1, 2, 3, and 4 d after spraying. The collected samples were allowed to recover for 1, 2, 3, and 4 d, then frozen in liquid nitrogen, and stored at −80°C until physiological and biochemical analyses were done.

Chlorophyll (Chl) content: Photosynthetic pigments, such as Chl *a*, Chl *b*, Chl (*a*+*b*), and carotenoids (Car), were determined following the method described by Arnon (1949) with a minor modification. Fresh leaf tissue (100 mg) was soaked in 10 mL of absolute ethanol until the pellets became colorless. The optical density of the solution was measured spectrophotometrically at 663, 645, and 470 nm (*UV-3600 Plus*, *Shimadzu*, Japan).

Gas-exchange parameters: Net photosynthetic rate (P_N), transpiration rate (E), intercellular CO_2 concentration (C_i), and stomatal conductance (g_s) were measured on the third fully expanded trifoliate leaves from the main apex using a portable photosynthesis system (*Li-Cor 6400*, *Li-Cor Inc*., Nebraska, USA). Plants were measured under PPFD of 1,000 μmol(photon) m^{-2} s^{-1}, CO_2 concentration of 500 μmol mol^{-1}, 25 ± 3°C, and 80% humidity.

Chl *a* fluorescence parameters: Maximum quantum efficiency of PSII primary photochemistry of dark-adapted plants (F_v/F_m), size and number of active reaction centers of PSII (F_v/F_0), and electron transport rate (ETR) were measured by a modulated Chl fluorescence spectrometer system (*OS5p*, *OPTI-Sciences*, USA) on control and chilling-stress-treated fully expanded leaves that were used for fluorescence measurements.

Histochemical detection of hydrogen peroxide and superoxide anion: Sampling for histochemical detection analysis was carried out 4 d after low temperature treatment. Accumulation of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) were localized histochemically by staining with 1% 3,3-diaminobenzidine (DAB) and 0.1% nitroblue tetrazolium chloride (NBT) solution following

the method of Chen *et al*. (2010) and Wei *et al*. (2015), respectively. After soaking for 6 h, the dyed leaves were immersed in 50 mL of mixed solution of absolute ethanol and glacial acetic acid (3:1, v/v) and placed in a hot water bath for 30 min. Photos were taken when the leaves turned colorless.

Superoxide anion and lipid peroxidation: Leaves were homogenized in a K-P buffer solution (pH 7.8) and centrifuged at 5,000 × *g* to monitor the formation of nitrite from hydroxylamine, following Wang and Luo (1990). Supernatant was mixed with extraction buffer and hydroxylamine hydrochloride. After 20 min of incubation, sulfanilamide and naphthylamine were added. The production rate of superoxide anion was measured spectrophotometrically at 530 nm (*UV-3600 Plus*, *Shimadzu*, Japan) and expressed as $nM(O_2^{\bullet-})\ min^{-1}g^{-1}$(protein).

The level of lipid peroxidation in leaves was measured by the thiobarbituric acid (TBA) test with modifications from Dhindsa *et al*. (1981) and Liu *et al*. (2015). TBA test determined content of malondialdehyde (MDA), an end product of lipid peroxidation. Leaf samples of 0.1 g were homogenized in 5 mL of 0.05 M phosphate buffer, pH 7.8, and then centrifuged at 12,000 × *g* for 20 min. Supernatant (1 mL), 1 mL of PBS buffer (pH 7.8), and 2 mL of 0.5% TBA were incubated in boiling water for 15 min. Then the mixture returned to room temperature and was centrifuged at 1,800 × *g* for 10 min. Samples were measured using a spectrophotometer (*UV-3600 Plus*, *Shimadzu*, Japan) at 532 and 600 nm. The MDA content was calculated using the extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

Extraction and measurement of ascorbate and glutathione: The content of nonenzymatic antioxidants, such as reduced (AsA) and oxidized (DHA) ascorbate, was determined following the method given by Zhang and Kirkham (1996). The supernatant was extracted with 5% phosphoric acid, and centrifuged at 22,000 × *g* for 15 min at 4°C. The supernatant was collected and used for the analysis of AsA and DHA content according to Nahar *et al.* (2016). The mixture contained 0.5 mL of enzyme extract, 1.5 mL of 5% phosphoric acid, 1.0 mL of absolute ethanol, 0.5 mL of 0.4% H_3PO_4-ethanol, 1.0 mL of 0.5% 4,7-diphenyl-1,10-phenanthroline-ethanol, and 0.5 mL of 0.03% $FeCl_3$-ethanol, and then was placed under a constant temperature to a water bath at 30°C for 90 min. Samples were measured using a spectrophotometer (*UV-3600 Plus*, *Shimadzu*, Japan) at 525 nm.

The supernatant was used for assays of reduced (GSH) and oxidized glutathione (GSSG) contents, following the method described by Grace and Logan (1996). A 0.1-g leaf sample was homogenized in 5 mL of 5 μmol(EDTA-TCA) L^{-1} and centrifuged at 11,500 × g min^{-1} for 15 min. The reaction mixture consisted of 1.0 mol(NaOH) mL^{-1}, dithionitrobenzoic acid (DTNB), and the enzyme extract. The reaction was initiated by the addition of 0.2 unit of glutathione reductase (GR) (from yeast, *Boehringer Mannheim*) in a total volume of 2 mL. Samples were measured using a spectrophotometer (*UV-3600 Plus*, *Shimadzu*, Japan) at 412 nm.

Antioxidant enzyme extraction and assays: Using a precooled mortar and pestle, soybean leaves (0.5 g) were homogenized in 10 mL of 50 mM ice cold PBS buffer (pH 7.0) and centrifuged at 11,500 × *g* for 15 min at 4°C. The supernatant was used for enzyme activity assay.

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured following the method of Cakmak and Marschner (1992). The reaction mixture contained 100 mM H_2O_2, 50 mM PBS (pH 7.0), 15 mM AsA, 0.1 mM EDTA, and enzyme extract. The activity was measured by observing the decrease in absorbance at 290 nm (*UV-3600 Plus*, *Shimadzu*, Japan) for 1 min using an extinction coefficient of 2.8 $mM^{-1}\ cm^{-1}$.

Catalase (CAT, EC 1.11.1.6) activity assessment was made according to the method of Fu and Huang (2001) by monitoring the change of absorbance (by decomposition of H_2O_2) and recording its value at 240 nm (*UV-3600 Plus*, *Shimadzu*, Japan) for 1 min. The activity was calculated using the extinction coefficient of 39.4 $M^{-1}\ cm^{-1}$.

The activity of glutathione reductase (GR, EC 1.6.4.2) was determined according to Esterbauer and Grill (1978) based on the rate of NADPH oxidation at 340 nm. The reaction mixture consisted of 0.3 mL of 20 mM disodium ethylenediaminetetraacetate (EDTA-Na_2), 0.4 mL of 5 mM oxidized glutathione (GSSG), and 1.1 mL of 50 mmol L^{-1} (pH 7.8) PBS buffer, and placed in a water bath at 25°C for 5 min. Reaction was initiated with 0.2 mL of 10 mmol L^{-1} NADPH and decrease in absorbance at 340 nm (*UV-3600 Plus*, *Shimadzu*, Japan) was recorded for 1 min (extinction coefficient of 6.2 $mM^{-1}\ cm^{-1}$).

Peroxidase (POD, EC 1.11.1.7) activity was determined by the method of Polle *et al*. (1994). One unit of POD was defined as the amount of enzyme that oxidated guaiacol at 470 nm (*UV-3600 Plus*, *Shimadzu*, Japan) upon incubation at 30°C during the linear phase of the reaction. The activity of POD was expressed as μmol(product) $min^{-1}\ g^{-1}$(FM). For the POD assay, a 3.0 mL reaction mixture contained 20 mmol L^{-1} guaiacol and 0.1 M H_2O_2 in 50 mmol L^{-1} (pH 7.8) PBS buffer. The reaction was initiated by adding 0.2 mL crude enzyme preparations.

Statistical analysis: The differences between treatment and control are presented in figures and tables and were performed on four replicates. All data were subjected to analysis of variance (*ANOVA*) and *Duncan*'s multiple range test at $P<0.05$ significance level between treatment and control with *SPSS* (21.0) software. The figures were drawn by *OriginPro 9.1* software (*OriginLab*, Northampton, MA, USA).

Results

Photosynthetic pigments: The Chl content is an important index to assess the damage caused by stress factors for photosynthetic organs (Erdal 2012). Chl *a*, Chl *b*, Chl (*a*+*b*), and Car contents in both soybean varieties declined with the prolongation of the low temperature treatment when compared to their controls (Fig. 1). However, the decline in Chl *a*, Chl *b*, Chl (*a*+*b*), and Car content was lower in chilling-sensitive Hefeng 50 as compared to chilling-

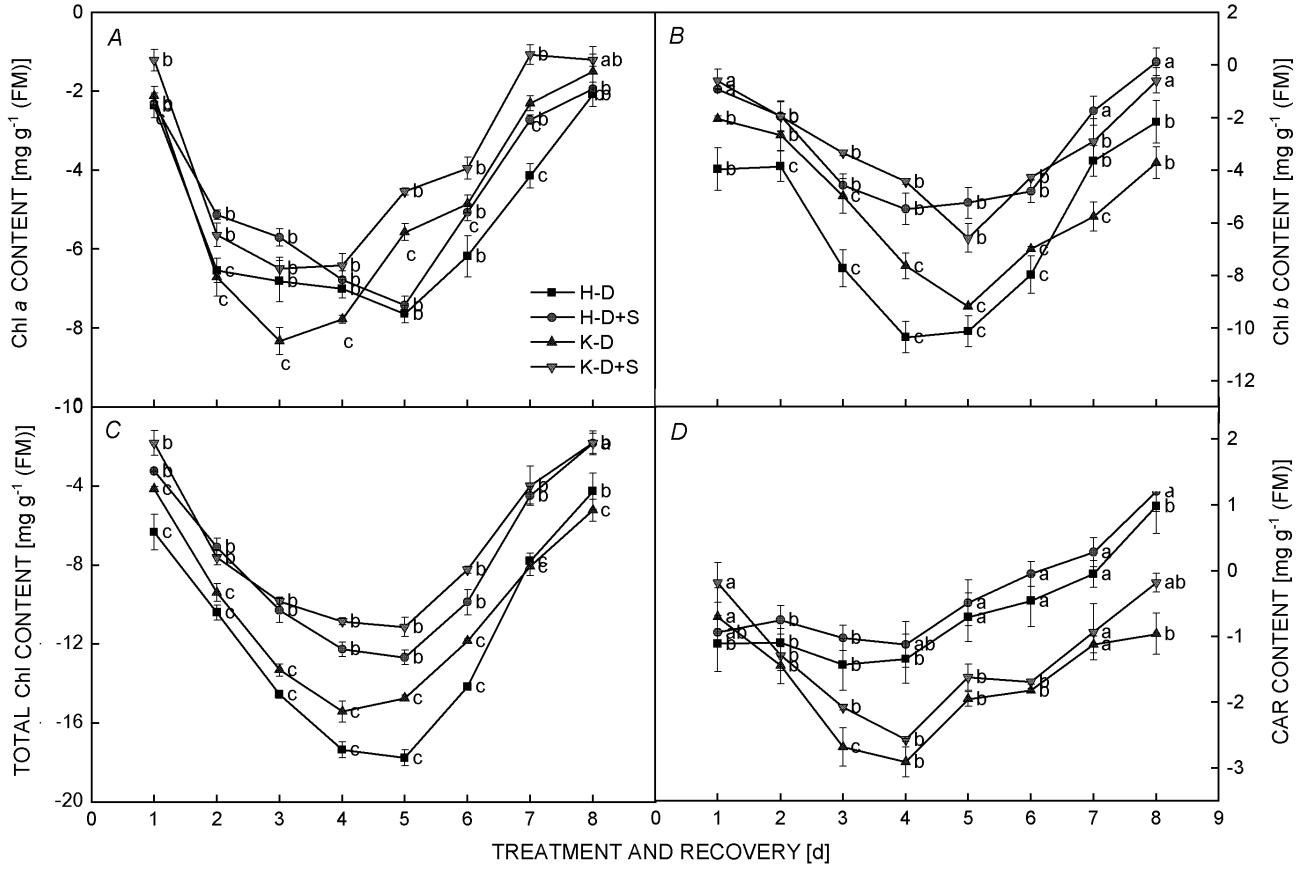

Fig. 1. Effect of uniconazole on chlorophyll *a* (*A*), chlorophyll *b* (*B*), chlorophyll (*a*+*b*) (*C*), and carotenoid (*D*) contents in soybean leaves. From day 1 to day 4, the plants were under chilling stress, and from day 5 to day 8, the plants returned to their natural environment (recovery period). Data (means ± SE, $n = 4$) are the difference between treatments. *Different letters* above horizontal lines indicate significant differences between treatments. D – chilling stress, D + S – chilling stress + uniconazole, H – Hefeng 50, K – Kenfeng 16.

tolerant Kenfeng 16. From day 1 to day 4, the above indicators decreased by 9.5–28.0, 22.6–65.9, 14.9–42.6, and 44.3–56.0%, respectively, in Hefeng 50, while they decreased by 7.0–26.8, 12.4–52.4, 8.9–35.3, and 36.4–49.5%, respectively, in Kenfeng 16. It is worth noting that the degradation rate of Chl *a* was slower than that of Chl *b*. The application of uniconazole effectively relieved the degradation of the above contents in chilling-stressed plants.

Chl *a*, Chl *b*, Chl (*a*+*b*), and Car contents in soybean leaves gradually increased with the prolongation of recovery time (from day 5 to day 8), and the increasing Chl content was significantly promoted by uniconazole treatment (Fig. 1). Altogether, uniconazole treatment in chilling-stressed plants alleviated the toxic effect on Chl but the above contents were lesser than that of control during chilling stress and recovery periods.

Photosynthesis: The decline of P_N was accompanied by a reduction in g_s and E, which increased C_i under chilling stress (Fig. 2). Protective effects of uniconazole were reflected by reduction of photosynthesis parameters, such as P_N, g_s, and E. In Hefeng 50, uniconazole treatment reduced P_N by 34.9% compared with control, while in Kenfeng 16, it was by 26.7% after 4 d of chilling stress. P_N of the control was 10.81 μmol m^{-2} s^{-1} and 11.24 μmol m^{-2} s^{-1}, respectively (Fig. 2*A*). In this study, photosynthesis recovery proceeded very slowly. In chilling-sensitive Hefeng 50, chilling stress caused a 9.5% decrease in P_N, while in Kenfeng 16, it was 16.1% after 4 d of recovery, compared with the control (Fig. 2*A*). However, when chilling-stressed plants were supplemented with uniconazole, P_N was enhanced compared to that under the chilling stress only.

Chl fluorescence parameters: The study of Chl *a* fluorescence is helpful for identification of the stress-induced damage in photosynthetic apparatus (Erdal 2012). F_v/F_0 (reflecting the potential PSII activity) was affected by chilling stress, manifesting a partial damage of the PSII reaction centers (Table 1). The large decline of P_N followed by the decrease of F_v/F_m confirmed that PSII is the major target site of chilling stress, particularly during the recovery process (Fig. 3*B*). Thus, reduced P_N could result in the accumulation of excessive photon energy and lead to photoinhibition. The ETR of PSII was gradually reduced with the prolongation of low-temperature treatment (Fig. 3*A*). Chilling stress induced a reduction in the ETR of PSII, which was attributed to both the decrease in the number of open PSII reaction centers and the efficiency of

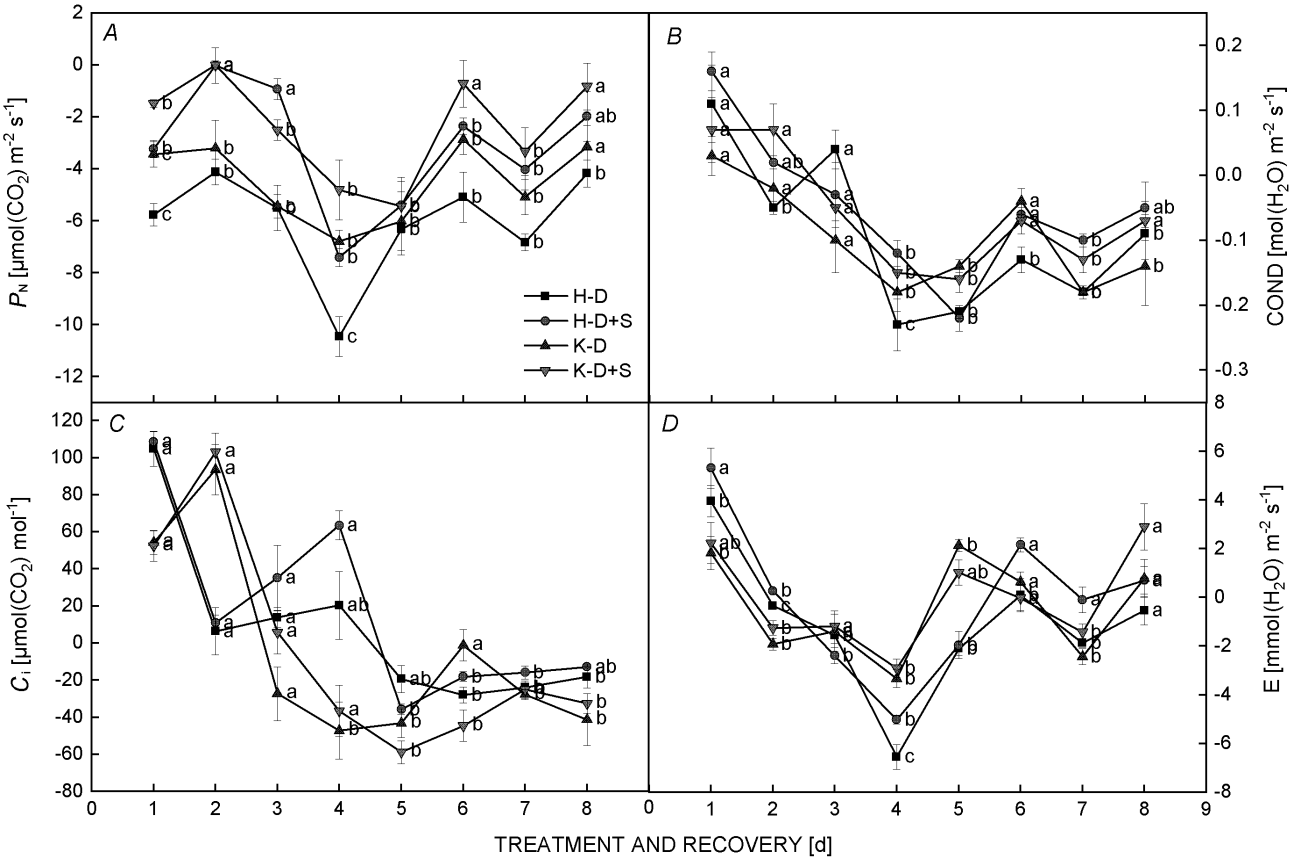

Fig. 2. Effect of uniconazole on net photosynthetic rate (P_N) (*A*), stomatal conductance (g_s) (*B*), intercellular CO_2 concentration (C_i) (*C*), and transpiration rate (*E*) (*D*) in soybean leaves. From day 1 to day 4, the plants were under chilling stress, and from day 5 to day 8, the plants returned to their natural environment (recovery period). Data (means ± SE, n = 4) are the difference between treatments. *Different letters* above horizontal lines indicate significant differences between treatments. D – chilling stress, D + S – chilling stress + uniconazole, H – Hefeng 50, K – Kenfeng 16.

Table 1. Effects of uniconazole on F_v/F_0 in soybean leaves exposed to chilling stress and after recovery to natural environment. Data in the table are the differences between treatment and control. All data are means ± SE of four repetitions (n = 4). *Different letters* within the same column represent significant differences (P<0.05). D – chilling stress, D + S – chilling stress + uniconazole, H – Hefeng 50, K – Kenfeng 16.

Treatment	Time of treatment [d]	H-D	H-D+S	K-D	K-D+S
Chilling stress	1	–0.85 ± 0.13^b	–0.04 ± 0.13^a	–0.97 ± 0.04^c	–0.36 ± 0.08^b
	2	–0.99 ± 0.03^c	–0.53 ± 0.13^b	–1.10 ± 0.02^c	–0.70 ± 0.05^b
	3	–0.43 ± 0.01^b	–0.13 ± 0.06^a	–1.11 ± 0.07^c	–0.94 ± 0.05^b
	4	–1.11 ± 0.05^b	–0.75 ± 0.34^b	–0.89 ± 0.07^c	–0.42 ± 0.08^b
Recovery	1	–1.34 ± 0.08^c	–0.23 ± 0.01^b	–1.39 ± 0.08^c	–0.47 ± 0.03^b
	2	–1.18 ± 0.08^c	–0.39 ± 0.02^b	–1.09 ± 0.08^b	–0.29 ± 0.02^a
	3	–0.36 ± 0.01^b	–0.03 ± 0.01^a	–0.33 ± 0.01^c	–0.28 ± 0.01^b
	4	–0.39 ± 0.04^b	–0.01 ± 0.01^a	–0.51 ± 0.02^b	–0.11 ± 0.03^a

energy capture by these open centers.

Reactive oxygen species (ROS): Leaves were soaked in DAB and NBT solution to localize the spots of $O_2^{\bullet-}$ and H_2O_2 at the subcellular level in soybean plants affected by chilling stress. A significant increase of deep blue spots and dark brown patches indicated the accumulation of $O_2^{\bullet-}$ and H_2O_2 in the leaves of soybean plants under chilling stress. However, uniconazole addition reduced the spots of $O_2^{\bullet-}$ and H_2O_2 under chilling stress (Fig. 4).

Our data confirmed that chilling stress induced dramatic accumulation of $O_2^{\bullet-}$ and malondialdehyde (MDA, indicator

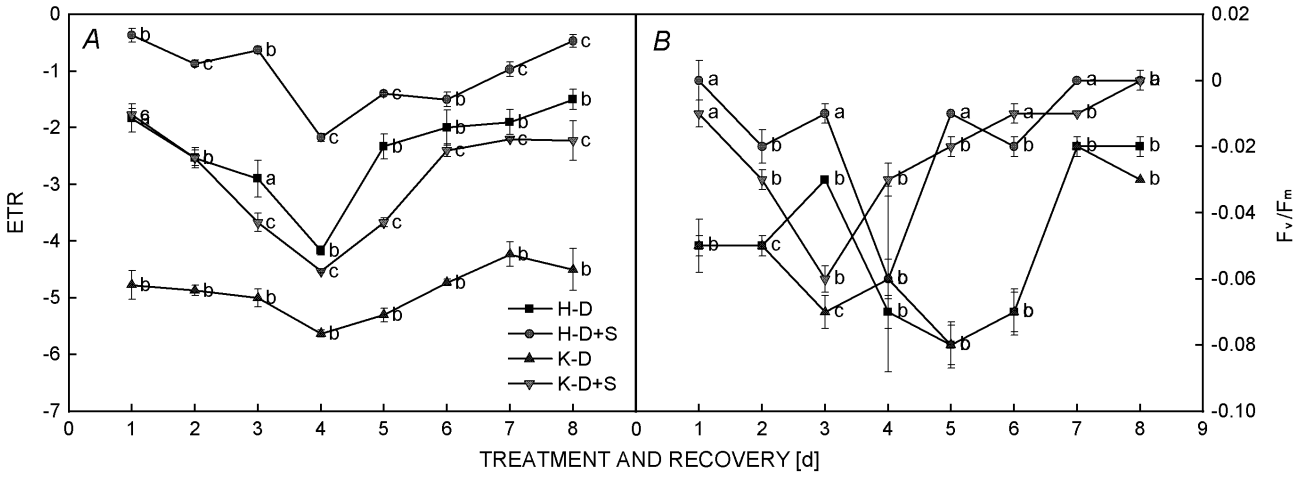

Fig. 3. Effect of uniconazole on electron transport rate (ETR) (*A*) and maximum photochemical efficiency of PSII (F_v/F_m) (*B*) in soybean leaves. From day 1 to day 4, the plants were under chilling stress, and from day 5 to day 8, the plants returned to their natural environment (recovery period). Data (means ± SE, n = 4) are the difference between treatments. *Different letters* above horizontal lines indicate significant differences between treatments. D – chilling stress, D + S – chilling stress + uniconazole, H – Hefeng 50, K – Kenfeng 16.

Fig. 4. Histochemical localization of $O_2^{\bullet-}$ (*upper row*) and H_2O_2 (*lower row*) in soybean leaves. K, H, D4, and D4 + S indicate Kenfeng 16, Hefeng 50, chilling stress for 4 d and chilling stress + uniconazole for 4 d, respectively. CK – plants were placed under natural environments.

of lipid peroxidation) in both soybean varieties (Fig. 5). The above contents reached their maximum value when plants were exposed to chilling stress for 4 d. However, the rate of $O_2^{\bullet-}$ was higher in chilling-sensitive Hefeng 50 as compared to chilling-tolerant Kenfeng 16. Exogenous application of uniconazole significantly reduced the $O_2^{\bullet-}$ and MDA contents in both varieties. Our results confirmed that the uniconazole treatment could effectively alleviate the accumulation of ROS in soybean leaves and improve the tolerance to chilling stress.

Accumulation of $O_2^{\bullet-}$ and MDA gradually decreased with the prolonged recovery time. In Hefeng 50, uniconazole treatment caused 18.0 and 4.0% decrease in the $O_2^{\bullet-}$ content, while in Kenfeng 16, it was 28.0 and 11.0% at 5 and 8 d, respectively, compared with the control (Fig. 5). Moreover, in Hefeng 50 and Kenfeng 16, the uniconazole treatment obviously decreased the MDA content during the recovery process, compared to chilling-treated plants

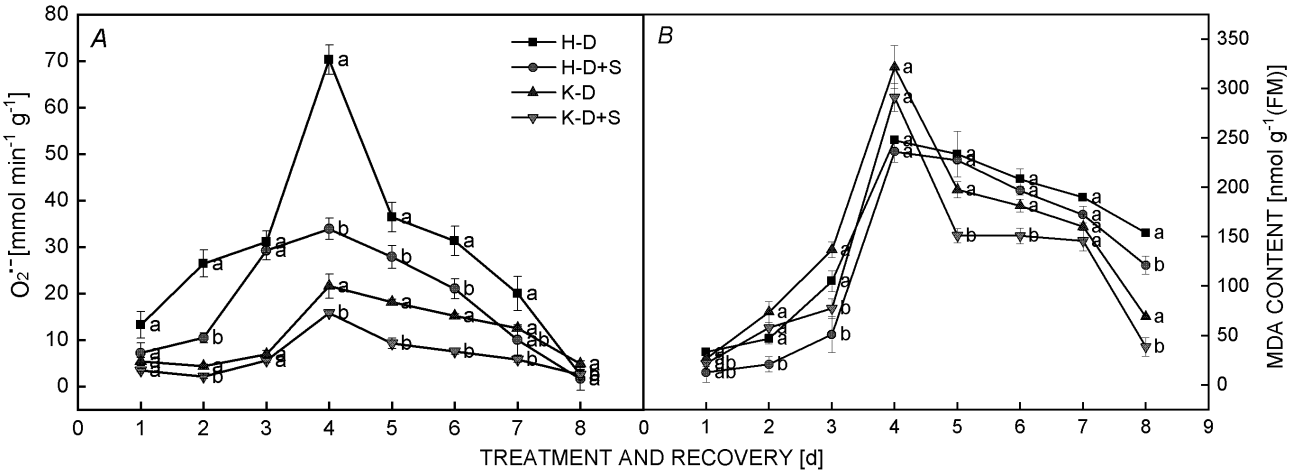

Fig. 5. Effects of uniconazole on superoxide anion ($O_2^{\bullet-}$) (*A*) and lipid peroxidation (expressed as malondialdehyde content) (*B*) in soybean leaves. From day 1 to day 4, the plants were under chilling stress, and from day 5 to day 8, the plants returned to their natural environment (recovery period). Data (means ± SE, n = 4) are the difference between treatments. *Different letters* above horizontal lines indicate significant differences between treatments. D – chilling stress, D + S – chilling stress + uniconazole, H – Hefeng 50, K – Kenfeng 16.

(Fig. 5). In short, the plants supplemented with uniconazole could maintain lower $O_2^{\bullet-}$ and MDA content compared to the plants exposed to chilling stress without supplementation during low-temperature treatment and recovery process (Fig. 5). Uniconazole treatments in chilling-stressed plants alleviated the toxic effect of $O_2^{\bullet-}$ and MDA but the values were still lower than that of the control during chilling stress and recovery periods.

Nonenzymatic antioxidants: The contents of GSH, GSSG, and GSH + GSSG dramatically increased during low-temperature stress (compared with the control), which was similar to trends of changes in AsA, DHA, and AsA + DHA contents (Fig. 6). Exogenous uniconazole pretreatment significantly increased these parameters in chilling-stressed plants, compared to the plants exposed to chilling stress only. Leaf contents of GSH, GSSG, and GSH + GSSG showed opposite trends when recovering in the natural environment. In chilling-sensitive Hefeng 50, chilling stress caused increases in GSH, GSSG, and GSH + GSSG contents, which were 5.1, 0.2, and 2.7%, respectively, higher than that of the control when plants went back to their natural environment for 4 d (Fig. 6*A–C*). Meanwhile, in chilling-tolerant Kenfeng 16, chilling stress caused increases in GSH, GSSG, and GSH + GSSG contents, which were 2.4, 2.3, and 2.37% higher, respectively, than that of the control for 4 d (Fig. 6*A–C*). The plants supplemented with exogenous uniconazole further increased their GSH, GSSG and GSH + GSSG contents when plants recovered under natural environment.

Leaf contents of AsA + DHA, AsA, and DHA also showed opposite trends when they recovered under the natural environment. In chilling-sensitive Hefeng 50, chilling stress caused a marked increase in AsA + DHA, AsA, and DHA contents which were 39.3, 57.5, and 23.5% higher, respectively, than that of the control when plants recovered under the natural environment for 4 d compared with the control (Fig. 6*E–G*). However, in chilling-tolerant Kenfeng 16, chilling stress caused a marked increase in AsA + DHA, AsA, and DHA contents, which were 6.8, 3.3, and 9.3% higher, respectively, than that of the control when plants recovered under natural environment for 4 d compared with the control (Fig. 6*E–G*). When plants returned back to natural conditions, exogenous uniconazole pretreatment could effectively delay the reduction of AsA + DHA, AsA, and DHA contents. The content of AsA + GSH was increased during the low temperature treatment and then declined during the recovery process. This pattern was similar but slightly lower in the uniconazole-treated plants (Fig. 6*H*).

Enzymatic antioxidants under chilling stress and recovery: CAT, POD, APX, and GR are the protective enzymes of the ROS scavenging system in plants. Under chilling stress, the activity of CAT dramatically decreased in Hefeng 50 and Kenfeng 16. In both Hefeng 50 and Kenfeng 16, the activity of CAT was 79.5 and 45.9% lower, respectively, than that of the control after 4 d of low temperature treatment. The uniconazole treatment under chilling stress maintained higher CAT activities, compared to chilling stress alone (Fig. 7*A*). After 1–4 d of low temperature stress, POD activity was sharply enhanced in both soybean varieties, compared with control, whereas in Kenfeng 16, POD activity was higher than that of Hefeng 50. Further, POD activity increased in uniconazole-pretreated, chilling-stressed plants, compared with plants under chilling stress alone (Fig. 7*B*). In Hefeng 50 and Kenfeng 16, the activity of APX was 128.9 and 71.4% higher, respectively, than that of the control after low temperature stress for 4 d. The uniconazole treatment under chilling stress maintained higher APX activities, compared to chilling stress alone, whereas in chilling-tolerant Kenfeng 16, the activity was lower than that of Hefeng 50 (Fig. 7*C*). The treatment by uniconazole

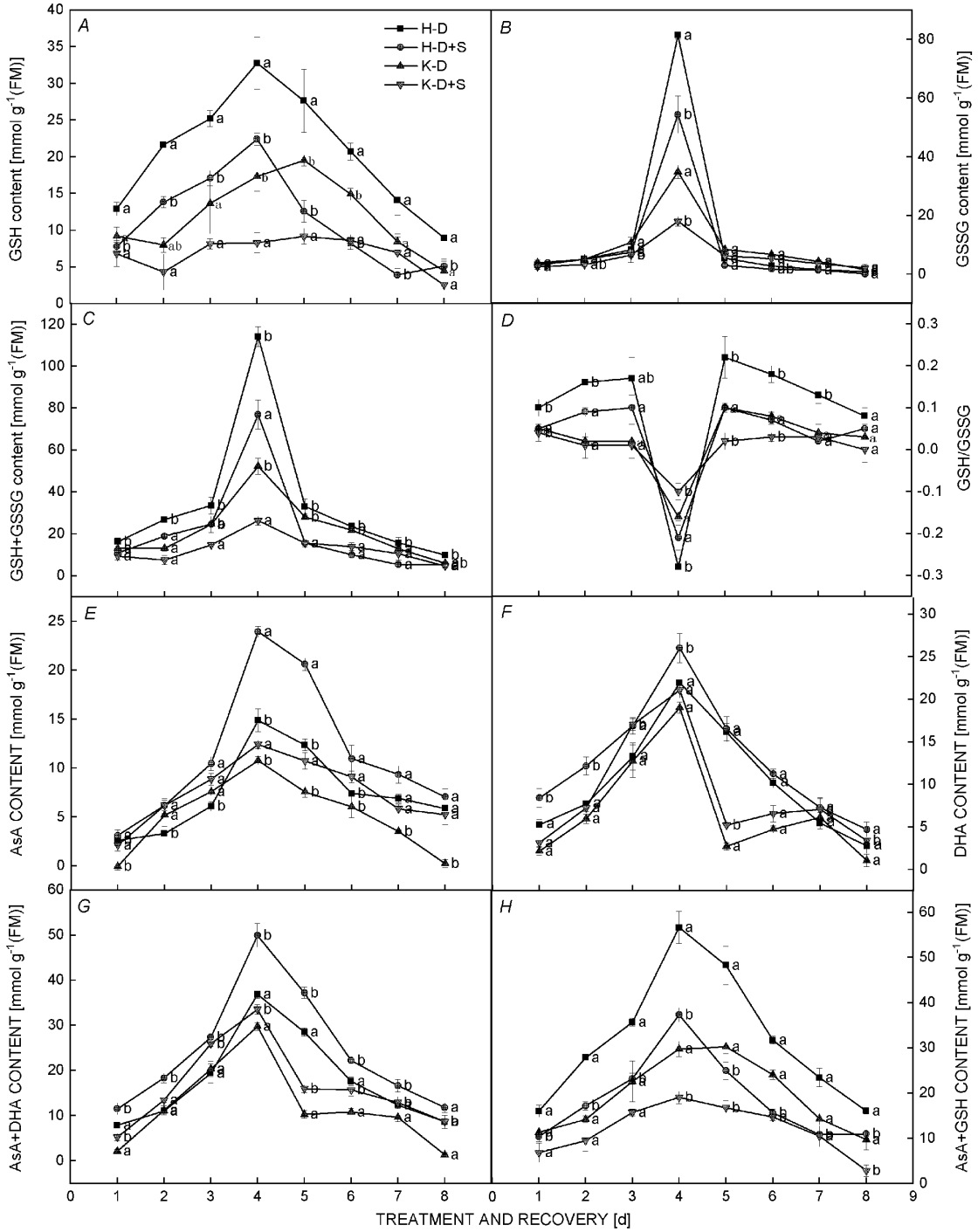

Fig. 6. Effects of uniconazole on the content of nonenzymatic antioxidants in soybean leaves. (*A*) reduced glutathione (GSH), (*B*) oxidized glutathione (GSSG), (*C*) GSH + GSSG, (*D*) GSH/GSSG, (*E*) ascorbate (AsA), (*F*) dehydroascorbate (DHA), (*G*) AsA + DHA, and (*H*) AsA + GSH. From day 1 to day 4, the plants were under chilling stress, and from day 5 to day 8, the plants returned to their natural environment (recovery period). Data (means ± SE, $n = 4$) are the difference between treatments. *Different letters* above horizontal lines indicate significant differences between treatments. D – chilling stress, D + S – chilling stress + uniconazole, H – Hefeng 50, K – Kenfeng 16.

under chilling stress increased APX activities in Hefeng 50 by 91.9, 66.7, 11.6, and 0.2%, respectively, during the plant recovery to natural environment for 1–4 d, while in Kenfeng 16, it was by 53.9, 37.3, 13.9, and 11.2%, respectively. The significant enhancement in GR activity was observed under chilling stress. Soybean plants pretreated with uniconazole showed higher GR activity compared to chilling stress-treated plants alone (Fig. 7*D*).

Discussion

Chl and Car are critical for photosynthesis; they allow plant to absorb, transmit, and transform light energy used to provide energy and synthesize photosynthetic products for plant growth and development (Erdal 2012). Kreslavski *et al*. (2012) reported that chilling stress lead to a decrease in the Chl content. Chl synthesis was inhibited because of pigment synthase activity decreased; and chloroplast structure was damaged. In our experiment, chilling stress caused the reduction in Chl *a*, Chl *b*, Chl (*a*+*b*), and Car content. In Kenfeng 16, the Chl was slightly higher than that of Hefeng 50 which was due to its better tolerance. Previous studies have revealed that Chl could be bleached under chilling stress, and foliage spray PGRs could improve the Chl content in leaves under low-temperature treatment, which was consistent with the results of this study (Balestrasse *et al*. 2010, Manafi *et al.* 2015). The contents of Chl and Car increased when the plants subjected to 15°C stress were transferred to their natural environment. The rate of enhancement was higher after the uniconazole treatment.

Damage to membrane components and proteins in thylakoid membrane is commonly the first visible ultrastructural symptom of chilling stress. Therefore, the absorption and transmission of light energy are further affected, which results in a decrease in photosynthetic rate (Allen and Ort 2001). This may be due to chloroplast structure damage and the lower Chl content. In our study, P_N and g_s of the two cultivars significantly decreased under chilling stress, while C_i significantly increased in both soybean varieties. However, the reduction was higher in the chilling-sensitive Hefeng 50 (Fig. 2). It indicated that chilling stress-induced P_N decrease occurred mainly due to nonstomatal limitations. The changes in plant physiological activities and morphological characteristics caused by uniconazole were closely related to alterations in hormone contents in leaves following treatment (Leul and Zhou 1998). Uniconazole was used as a plant growth retarder, which affected the endogenous hormonal balance in leaves after plants were exposed to abiotic stress. Under abiotic stress, uniconazole pretreatment could enhance the ability of chloroplast to absorb and utilize light energy, and alleviate the chloroplast degradation, which improves the ability of plants to resist abiotic stress (Leul and Zhou 1998, Zhang *et al*. 2007, Duan *et al*. 2008, Al-Rumaih and Al-Rumaih 2015). Uniconazole treatment significantly increased P_N, g_s, and E in soybean leaves under chilling stress compared to chilling treatment alone. The present results indicated that the effect of uniconazole on the photosynthetic characteristics of soybean plants under chilling stress was consistent with that of Zhang *et al.*

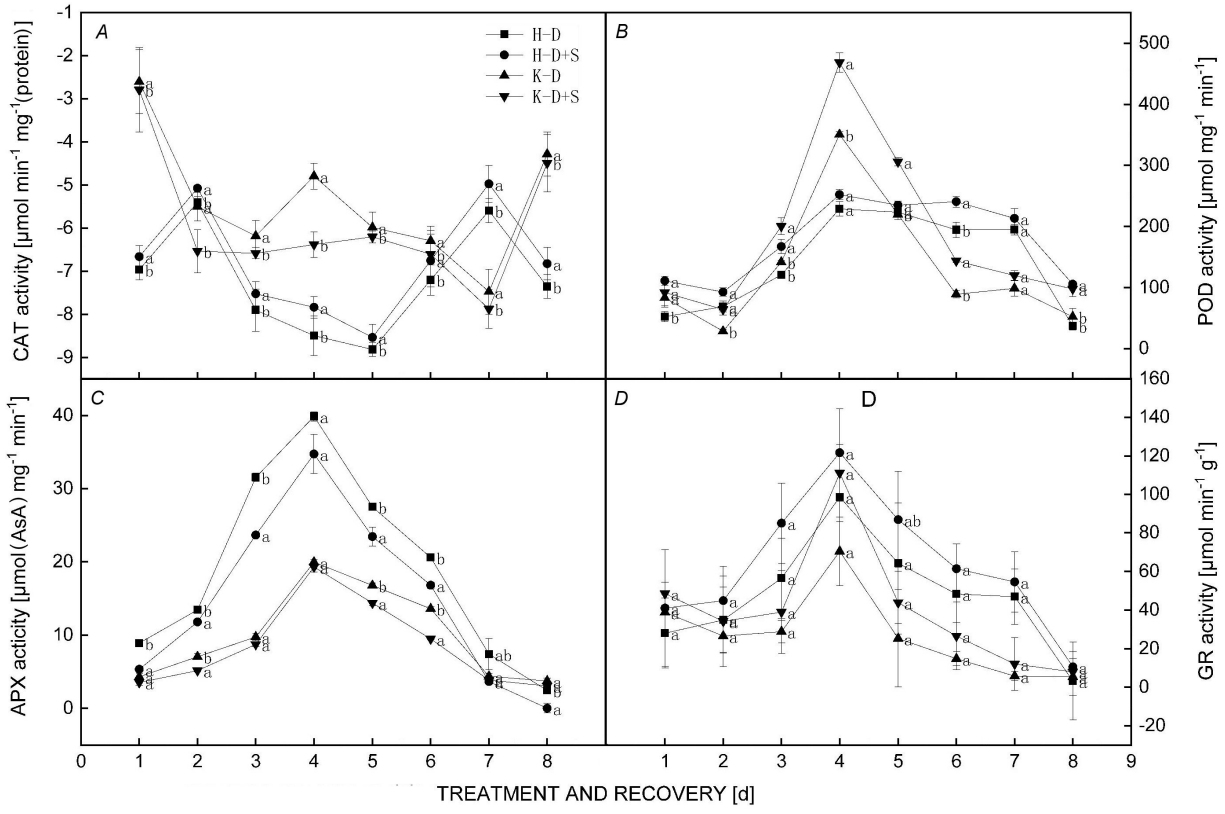

Fig. 7. Effect of uniconazole on the activity of antioxidant enzymes in soybean leaves. (*A*) catalase (CAT), (*B*) peroxidase (POD), (*C*) ascorbate peroxidase (APX), and (*D*) glutathione reductase (GR). From day 1 to day 4, the plants were under chilling stress, and from day 5 to day 8, the plants returned to their natural environment (recovery period). Data (means ± SE, n = 4) are the difference between treatments. *Different letters* above horizontal lines indicate significant differences between treatments. D – chilling stress, D + S – chilling stress + uniconazole, H – Hefeng 50, K – Kenfeng 16.

(2007). However, the photosynthetic parameters of the plants showed the opposite trend during recovery (Fig. 2). When the plants were transferred to their natural environment, the recovery of P_N occurred more quickly after the uniconazole treatment compared to chilling stress only. In chilling-sensitive Hefeng 50 and chilling-tolerant Kenfeng 16, the P_N decreased by 9.5 and 4.2%, respectively, when the plants subjected to 15°C stress were transferred to natural environment for 4 d.

Compared to the gas-exchange parameters, Chl fluorescence parameters can directly reflect the absorption and utilization of light energy in PSII, which can assist to analyze the location and extent of photosynthetic organ damage by stress (Demmig and Björkman 1987, Aro *et al*. 1993, Erdal 2012). Chilling stress induced a decrease of F_v/F_m, indicating that chilling stress can cause partial inactivation or injury to PSII (Demmig and Björkman 1987). Photosynthetic organs were damaged and the photosynthetic electron transport process was blocked which ultimately resulted in a decrease of ETR (Ögren 1991). The results revealed that the fluorescence parameters of the two varieties of soybean showed the same trend. Under chilling stress, F_v/F_m and ETR in soybean leaves significantly decreased, and the reduction was higher in the chilling-sensitive Hefeng 50 (Table 1, Fig. 3). Under chilling stress, uniconazole pretreatment could further decrease F_v/F_m and ETR, which indicates that the mechanism of plant thermal dissipation was inefficient. Chilling stress caused irreversible inactivation or destruction of PSII reaction centers. Under chilling stress, exogenous uniconazole treatment significantly increased P_N, g_s, E, F_v/F_m, and ETR, avoided excessive light damage, maintained high PSII photochemical activity, and relieved the inhibition of soybean growth. However, the photosynthetic parameters of the plant showed the opposite trend during recovery days (Table 1, Fig. 3).

Chilling stress is a major factor limiting the growth, development, survival, and yield of many temperature-sensitive plants (Allen and Ort 2001). Chilling stress accelerates the production of ROS and induction of oxidative stress, which eventually results in cell death (Gill and Tuteia 2010, Kreslavski *et al*. 2012, Nahar *et al*. 2015). In the present study, histochemical staining localized $O_2^{\bullet-}$ and H_2O_2 at the tissue level in soybean leaves. Chilling stress induced the accumulation of $O_2^{\bullet-}$ and H_2O_2 (Fig. 4). $O_2^{\bullet-}$ is a toxic compound which is injurious to the cell and excessive accumulation of $O_2^{\bullet-}$ is one of the indicators of oxidative stress (Gill and Tuteja 2010). Lipid peroxidation is a better known indicator for determining the extent of oxidative stress. In our study, both $O_2^{\bullet-}$ and MDA were found to be increased under chilling stress which was in agreement with several previous reports (Liu *et al*. 2009, 2011, 2012; Balestrasse *et al*. 2010, Erdal 2012, Nahar *et al*. 2015). On the contrary, chilling-treated plants supplemented with exogenous uniconazole showed lower $O_2^{\bullet-}$ and MDA contents (Figs. 4, 5) which was due to their more active nonenzymatic and enzymatic antioxidant defense system. Exogenous uniconazole-induced upregulation of nonenzymatic and enzymatic antioxidant defense and concomitant decrease in $O_2^{\bullet-}$ and MDA contents were observed in many plant species including soybean (Balestrasse *et al*. 2010). This is consistent with the study reported by Zhang *et al*. (2007) in soybean under water deficit stress. Chucheep *et al*. (2005) reported that exogenous uniconazole increased a tolerance of mung bean seedlings to chilling stress. Therefore, it is clear that uniconazole can alleviate membrane damage caused by overaccumulation of ROS. Other researches have confirmed that pretreatment with uniconazole can decrease ROS in plants under different abiotic stresses (Leul and Zhou 1998, Qiu *et al*. 2005, Zhang *et al*. 2007, Duan *et al*. 2008).

Plants in a long process of evolution have formed a variety of mechanisms to resist oxidative stress and to remove excess ROS, so that oxygen free radicals maintain at a low concentration (Gill and Tuteja 2010, Nahar *et al*. 2015). AsA and GSH contents are important parameters to measure the ability of plants to resist low temperature stress. Two molecules of AsA as substrate are utilized by APX to scavenge H_2O_2 to water, and with the concomitant generation of MDHA, which can disproportionate into DHA and AsA. NADPH acts as electron donor and the reaction is catalyzed by ferredoxin in a water-water cycle or MDHAR (Hasanuzzaman *et al*. 2012). Two molecules of GSH are utilized by DHAR to reduce DHA to AsA, and during this reaction, GSSG is generated (Gill and Tuteja 2010). Previous studies have shown that exogenous PGRs increased the content of AsA and GSH under low temperature stress, reduced the oxidative damage in plant cells, and improved the tolerance to stress (Liu *et al*. 2009, 2011, Balestrasse *et al*. 2010, Manafi *et al.* 2015, Nahar *et al*. 2015, Zhao *et al*. 2016). Our results revealed that chilling stress led to the increase in AsA, DHA, and total AsA contents in both soybean varieties (Fig. 6*E–G*). Moreover, uniconazole treatment dramatically increased AsA, DHA, and total AsA contents, but the increase of AsA content was higher than that of DHA. This result indicated uniconazole could improve the tolerance of soybean plants to low temperature mainly by increasing the content of AsA as an antioxidant. GSH acts as an antioxidant to scavenge different ROS by preventing the oxidation of protein thiol groups and eventually preventing denaturation of proteins (Nahar *et al*. 2015). Nahar *et al*. (2015) reported that chilling stress slightly increased GSH content and reduced GSH/GSSG ratio, while pretreatment with spermidine increased GSH content and GSH/GSSH ratio, compared to seedlings under chilling stress alone. It was found that GSH, GSSG, GSH + GSSG contents and GSH/GSSG ratio of both varieties increased. The similar results were observed after the uniconazole pretreatment of both soybean cultivars. The enhancement of the GSH/GSSG ratio was beneficial to maintain a balance state of AsA-GSH cycle and scavenging ROS (Fig. 6). In addition, APX activity was enhanced in both soybean varieties subjected to chilling stress; exogenous uniconazole increased the activity further which indicated the H_2O_2 scavenging role of uniconazole (Fig. 7*C*). In short, exogenous uniconazole increased the activity of APX and the contents of AsA and GSH, which indicated that uniconazole may promote the AsA-GSH cycle under chilling stress.

SOD, POD, and CAT are ROS scavengers. The main function of SOD is to clear the generation of H_2O_2 and oxygen molecules, and POD and CAT can remove H_2O_2 in cells and prevent H_2O_2 and $O_2^{\bullet-}$ interaction, so as to maintain the balance of active oxygen metabolism (Gill and Tuteja 2010, Hasanuzzaman *et al*. 2012). Previous studies have shown that the activities of SOD, POD, and CAT in different plants are different under low temperature stress. Upadhyaya *et al*. (1990) elucidated that uniconazole-induced stress tolerance is due to enhanced antioxidant activity which reduced stress-related oxidative damage to cell membranes. Manafi *et al*. (2015) reported that in soybean seedlings treated by 10°C temperature stress, SOD and POD activity increased. Nahar *et al*. (2015) found that CAT activity decreased under low temperature stress. In this study, it was found that chilling-stress treatment increased POD activity and no significant difference of SOD activity was found (the data not shown) in soybean leaves (Fig. 7*A*). Zhang *et al*. (2007) found that uniconazole could increase the activity of SOD and POD under water deficit stress, and decrease the content of MDA. Foliar sprays of uniconazole under waterlogging stress significantly increased the activities of POD and CAT enzymes and significantly reduced the MDA content at the seedling and flowering stage, while there was no significant difference in SOD activity between uniconazole and waterlogging stress at both stages (Leul and Zhou 1998). The results of this study also showed that uniconazole treatment significantly improved the activity of POD and CAT in the leaves of soybean plants (Fig. 7*A*,*B*), and reduced the degree of plasma membrane peroxidation ($O_2^{\bullet-}$ and MDA content) and maintained the integrity of cell membrane. In both soybean varieties from our experiment, POD and CAT activities obviously increased under low temperature stress, higher activities were observed in chilling-sensitive Hefeng 50. Exogenous uniconazole resulted in further enhancement of the above indicators.

However, the ROS, nonenzymatic, and enzymatic antioxidants showed the opposite trend during recovery days (Figs. 5, 6). When the plants were transferred to natural environment, the recovery of above indicators occurred more quickly after the uniconazole treatment compared to chilling stress only. In chilling-tolerant Kenfeng 16, the above indicators mostly reached normal levels when the plants were transferred to natural environment for 4 d. In summary, we infer that uniconazole could improve the low temperature tolerance of crops, because the uniconazole treatment could reduce the production of ROS and promote nonenzymatic and enzymatic antioxidant defense system to eliminate excessive ROS in plants under low temperature stress. When the plants were transferred to natural environment, the recovery of photosynthetic pigment, gas-exchange parameters, ROS, nonenzymatic and enzymatic antioxidants occurred more quickly and mostly reached normal levels.

Conclusion: The results of our study indicated that chilling stress decreased photosynthetic pigments and P_N, and increased the $O_2^{\bullet-}$ and MDA content in two soybean varieties. Considering the above results, we conclude that foliar application of 50 mg(uniconazole) L^{-1} to plants together with chilling stress improved the growth, photosynthesis, and ROS metabolism (ROS production and removal) compared to plants treated with chilling stress alone. In all cases, Kenfeng 16 performed better than Hefeng 50 under low temperature stress. Thus, we suggest that the application of uniconazole has practical importance in agricultural systems.

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