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Dual role of superoxide radicals in the chilling-induced photoinhibition in maize seedlings

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Abstract

Maize (*Zea mays*) seedlings were exposed for 6 h to strong irradiance (1 000 μmol m^{-1} s^{-1} of PPFD) at 5, 12, 17, or 25 °C, followed by an exposure to the darkness for 6 h at 22 °C. Leaf chlorophyll fluorescence, net photosynthetic rate (P_N), and the amount of superoxide radicals ($O_2^{-\cdot}$) in relation to chilling-induced photoinhibition were investigated. During the photophase, a good correlation ($r = –0.879$) was observed between Φ_{PS2} (relative quantum efficiency of PS2 electron transport) and the amount of $O_2^{-\cdot}$. Treatment with exogenous $O_2^{-\cdot}$ reduced the P_N and Φ_{PS2} as the chilling stress did, that was inhibited by specific scavenger of $O_2^{-}\cdot$. Hence chilling-induced photoinhibition might be due to the production of $O_2^{-\cdot}$. In contrast, in the dark period, P_N and Φ_{PS2} of the seedlings treated with the exogenous $O_2^{-\cdot}$ were enhanced, but they were inhibited by the specific scavenger of $O_2^{-\cdot}$, showing the photoprotective role of $O_2^{-\cdot}$ in the recovery phase. Furthermore, in terms of the effect of exogenous $O_2^{-\cdot}$ on the xanthophyll cycle, the $O_2^{-\cdot}$ production suggested a promotion effect for the de-epoxidation of violaxanthin during the photophase, the epoxidation of zeaxanthin at the dark stage, and the increase of the xanthophyll pool both in the photophase and dark phase, resulting in an enhancement of the ability of non-photochemical quenching to avoid or alleviate the damage to photosynthetic apparatus.

Additional key words: chlorophyll fluorescence; net photosynthetic rate; photosystem 2; reactive oxygen species; SOD; violaxanthin; xanthophyll cycle; *Zea*; zeaxanthin.

Introduction

Excess photons photoinhibit photosynthesis and may lead to a photooxidative destruction of the photosynthetic apparatus (Long *et al.* 1994, Lavaud *et al.* 2002, Pätsikkä *et al.* 2002). The current research on photoinhibiton focuses mainly on photosystem 2 (PS2) in three aspects: (*a*) a cycle of PS2 reaction centre (RC) inactivation and its repair, particularly of the D1 polypeptide of PS2 RC (Fryer *et al.* 1995, Nie *et al.* 1995, Li *et al.* 2000); (*b*), the avoidance of over-excitation of the PS2 RC by decreased absorption or by internally increased thermal dissipation of excitation energy associated with the xanthophyll cycle activity (Gilmore 1997, Havaux and Niyogi 1999, Peterson and Havir 2000); and (*c*), the reactive oxygen species (ROS) generation and their action in photoinhibition (Fryer *et al.* 1998, Varadi *et al.* 2000, Veljovic-Jovanovic *et al.* 2001, Rodriguez *et al.* 2002). Excess photons may result in an increased production of ROS as by-products of photosynthesis, including superoxide ($O_2^{-\cdot}$), H_2O_2, hydroxyl radicals (·OH), and singlet oxygen ($^1O_2$), whereas the overproduction of ROS is extremely damaging to lipids, proteins, and pigments unless they are rapidly scavenged. The possible paths to produce ROS in chloroplasts include that (*a*) Q_A in PS2 is overexcited under excess of photons, which can lead to formation of the triplet state chlorophyll (Chl) (^{3}P680), and then the ^{3}P680 can transfer its energy to ground-state O_2 to generate singled oxygen ($^1O_2$) (Hideg *et al.* 1994, 1995); (*b*) the superoxide can be generated through Mehler reaction (Asada 1996); and (*c*) H_2O_2 is generated from the superoxide *via* the dismutation reaction catalyzed by superoxide dismutase (SOD) (Greenwald 1987). The ROS extremely damage chloroplasts and this might result in the photoinhibition by chilling stress. Fryer *et al.* (1998) observed photoinhibition in maize leaves under chilling stress and found a strong relationship between the photoinhibition and the generation of ROS at low temperature, but the photoprotective role of ROS in the photoinhibition has not been understood well till now. Recently, we have found that the ROS may act as a regulator on some normal metabolism processes in plants (Ke *et al.* 2002).

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The objective of this study was to investigate the dual-role of superoxide radical in chilling-induced photoinhibition.

Materials and methods

Plants and treatments: Maize (*Zea mays* L.) seeds were soaked for 24 h in distilled water, prior to being planted in pots (3 seeds per pot) supplied with a mixture of perlite and potting compost (1 : 1, v/v), N : P : K being 2 : 1 : 1. Seedlings grew in a controlled environment cabinet at 25 °C under a 16-h photoperiod (photosynthetic photon flux density, PPFD of 250 μmol m^{-1} s^{-1}), followed every day by 8 h in darkness at 22 °C. As the second leaves just reached full expansion (35–40 d from sowing), the plants were adapted first to darkness for 1 h at 25 °C, and then transferred to the controlled environment cabinet at 5, 12, 17, or 25 °C for a short 2-h adapting to the darkness. In the dark adapting period, 1 mM methyl viologen (MV), 0.5 mM xanthine-xanthine oxidase (X-XOD), 1 mM 1,4-diazabicyclo-2,2,2-octane (DABCO), and 1 unit/cm^3 superoxide dismutase (SOD) dissolved in distilled water was sprayed alone or in combination to the seedling leaves, 50 cm^3 for one seedling, twice for one hour. The same treatment with distilled water was used as the control. Then the seedlings were irradiated (PPFD of 1 000 μmol m^{-1} s^{-1}) and the second leaves that just reached full expansion were sampled for measurements of chlorophyll (Chl) fluorescence, net photosynthetic rate (P_N), content of superoxide radical, activity of SOD, and content of carotenoids.

Chl fluorescence: The minimal fluorescence yield, F_0 (dark adapted) and F_0' (light adapted), the fluorescence yield at steady-state photosynthesis, F_s, and the maximum yield induced by a 0.5 s saturating flash (10 000 μmol m^{-2} s^{-1}), F_m (dark adapted), and F_m' (light adapted) were determined at a PPFD of 200 to 1 500 μmol m^{-2} s^{-1} and 5, 12, 17, or 25 °C using a fluorometer (*Hansatech FMS2*, Norfolk, England). In addition, other fluorescence coefficients were calculated using the following equations: Φ_{PS2} (relative quantum efficiency of PS2 electron transport) = $(F_m' - F_s)/F_m'$, q_N (non-photochemical quenching coefficient) = $1 - (F_m' - F_0')/(F_m - F_0)$, and q_P (photochemical quenching coefficient) = $(F_m' - F_s)/(F_m` - F_0')$, as described by Genty *et al.* (1989).

***P*$_N$**: Oxygen evolution was measured with a leaf-disk oxygen electrode (*Hansatech*, Norfolk, UK) by the method of Brestic *et al.* (1995). Saturating concentration of CO_2 was provided from a cylinder by a controller system. The gas was bubbled through water at 25 °C before passing through the electrode compartment. The PPFD was in the range of 200–1 500 μmol m^{-2} s^{-1}.

Xanthophyll cycle: Twenty leaf discs (5 mm in diameter) were removed with a cork borer, frozen in liquid nitrogen, and stored at –70 °C. The samples were ground in liquid nitrogen, extracted overnight with acetone : water (85 : 15, v/v) at 0 °C, and then centrifuged for 30 min at 8 000×*g*. The pellet was re-extracted twice with pure acetone under similar conditions. Combined extracts were homogenized, re-centrifuged, and injected directly onto a reversed phase high performance liquid chromatography (HPLC) column. The xanthophyll composition and contents were measured using the HPLC method described by Thayer and Björkman (1990).

Superoxide radical ($O_2^{-}\cdot$): Three leaf discs (5 mm in diameter) were ground with a mortar in ice-cold distilled water, and then centrifuged for 4 min at 15 000×*g*. The supernatant was collected for the measurement of superoxide anion by the method of Hideg *et al.* (1995). The method is based on the reaction of tiron (1,2-dihydroxybenzene-3,5-disulphonate) with superoxide radical which yields a stable EPR detectable tiron (semiquinone) radical. Spin trapping EPR spectroscopy was performed in the presence of tiron as the following step. 0.5 cm^3 of 10 mM tiron was first added to 0.2 cm^3 of the supernatant, then immitted into a 100-mm^3 capillary whose one end had been sealed, and finally the capillary was inserted into a *ER-4103 TM* sample container of the *Bruker-ER-200 E/D SRG* spectrometer and the sample was irradiated with 400 μmol m^{-2} s^{-1}. X-band spectra were recorded at 30 °C with the 9.45 GHz microwave frequency, 16 mW microwave power, and 100 kHz modulation frequency. Free radical production was calculated from the area of EPR absorption.

SOD activity was assayed with a modification of the method of Fryer *et al.* (1998). The final reaction volume (1 cm^3) contained 30–800 mm^3 sample (prepared as for the superoxide radical analysis), to which 20 mm^3 of 0.025 % *Triton X-100* and 50 mm^3 of 1.2 mM nitro blue tetrazolium (NBT) were added, the balance being made by the SOD assay buffer (0.05 mM K_2PO_4 containing 200 μM EDTA). The reaction was started by adding 50 mm^3 of 0.2 M methionine and 50 mm^3 of 11.3 μM riboflavin. The reaction tube was irradiated with 800 μmol m^{-2} s^{-1} PPFD for about 8 min. The development of purple colour was then determined by measuring A_{560} in a spectrophotometer, the blank being SOD assay buffer. One unit of SOD activity was defined as the amount being contained in the volume of extract that caused a 50 % inhibition of the SOD fraction of NBT reduction.

Results and discussion

The effect of temperature on photoinhibition: P_N increased as PPFD was enhanced. However, as PPFD increased to a definite value, the P_N reached the maximum and remained constant, which indicated a saturation of PPFD for photosynthesis in maize seedlings (Fig. 1). Furthermore, the saturation PPFD decreased as temperature decreased. Since the saturation PPFD for photosynthesis at normal temperature (25 °C) is about 1 000 μmol m^{-2} s^{-1}, this irradiance was used for the study of the photoinhibition. As shown in Fig. 2*A*, under the excess photons, Φ_{PS2} decreased dramatically during the first 20 min of irradiation, reached a minimum value, but remained unchanged following a continuous irradiation. Moreover, the minimum of Φ_{PS2} declined as temperature further decreased. However, once the photon supply stopped, the Φ_{PS2} began to increase rapidly, but the recovery percentage of Φ_{PS2} declined as temperatures decreased. There was more than 90 % recovery at normal temperature, but only about 60 % recovery at 5 °C, suggesting that the chilling-induced photoinhibition was partially irreversible.

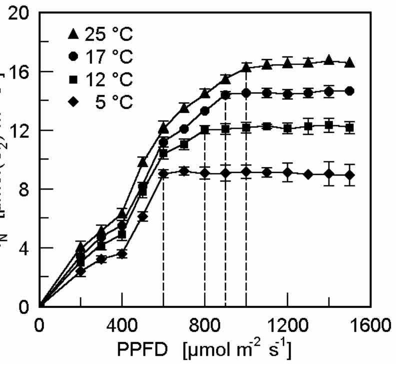

Fig. 1. The effect of irradiance (PPFD) on net photosynthetic rate (P_N) in maize seedlings at various temperatures. *Broken lines* show the saturation PPFD at various temperatures. *Vertical bars* indicate standard errors.

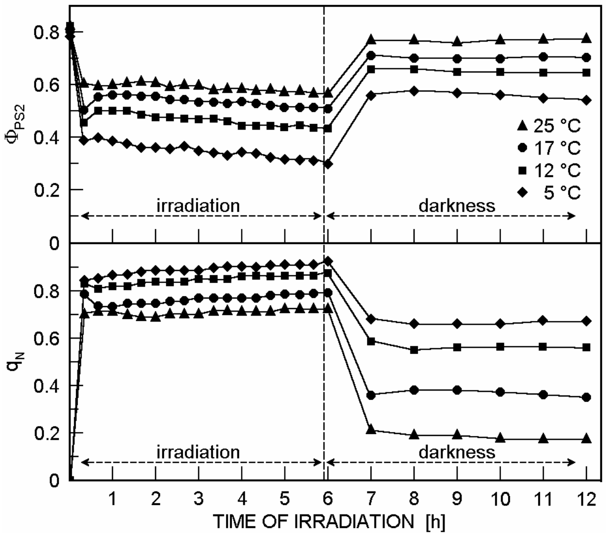

Fig. 2. Changes in Φ_{PS2} (*A*) and q_N (*B*) in maize seedlings exposed to strong irradiance (1 000 μmol m^{-2} s^{-1}), and then transferred to the dark at various temperatures.

In contrast to Φ_{PS2}, the q_N rose dramatically, then reached nearly a maximum during the first 20 min under strong irradiance, and finally remained almost unchanged following the six hours of irradiation. The q_N at chilling temperature was higher than that at normal temperature. As strong irradiance switched off, q_N declined remarkably, with a different level of q_N reversion at various temperatures (Fig. 2*B*). Because the non-photochemical quenching was generally regarded as a photoprotective factor to photoinhibition (Havaux and Niyogi 1999, Li *et al.* 2000), the values in Fig. 2*B* showed that the ability of NPQ for photoprotection could not be completely recovered from chilling-induced photoinhibition.

The detrimental role of superoxide radical to photosynthesis: The change of the $O_2^{-}\cdot$ level exhibited a tendency similar to that of Φ_{PS2} (Figs. 2*A*, 3*A*). A good negative coefficient of 0.879 was found between the $O_2^{-}\cdot$ level and Φ_{PS2}, implying that the increase of $O_2^{-}\cdot$ level may be a factor for the photoinhibition induced by chilling stress. The activity of SOD increased during the first 2–3 h of irradiation and then decreased slowly. As the photon supply stopped, the enzyme activity decreased constantly (Fig. 3*B*). Thus, the elevation of $O_2^{-}\cdot$ level may not be due to the falling in scavenging capability to

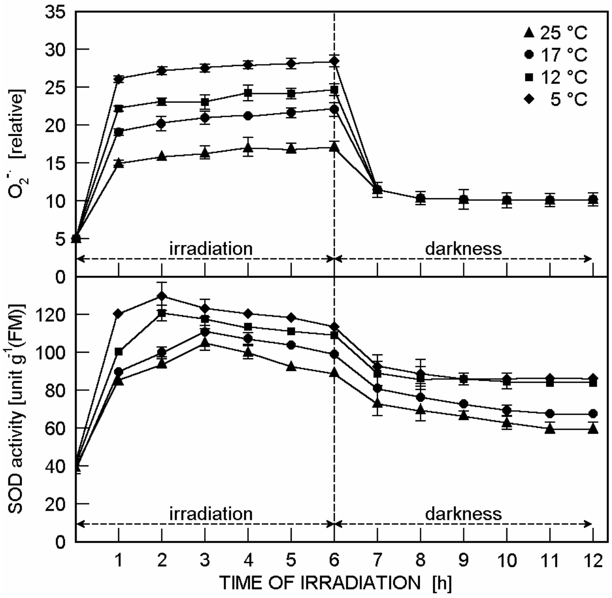

Fig. 3. Changes in $O_2^{-}\cdot$ (*A*) and SOD activity (*B*) in maize seedlings exposed to strong irradiance (1 000 μmol m^{-2} s^{-1}), and then transferred to the dark at various temperatures. *Vertical bars* indicate standard errors.

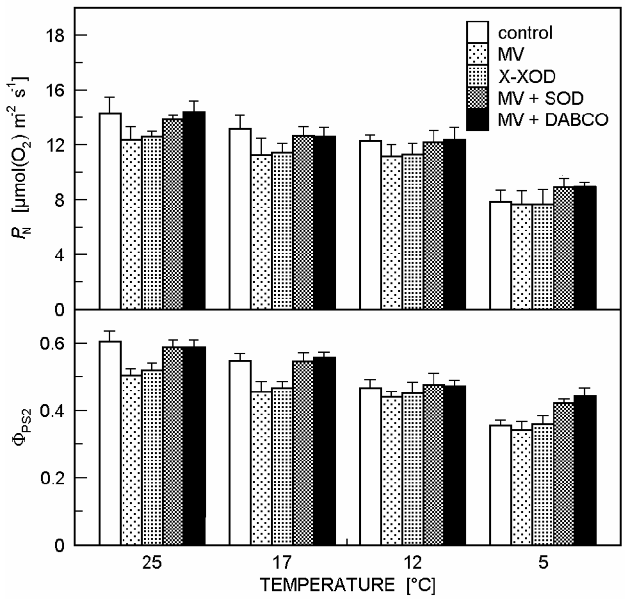

Fig. 4. Effects of MV, X-XOD, DABCO, and SOD on the net photosynthetic rate (P_N) and Φ_{PS2} in maize seedlings in the photoperiod (2 h of irradiation) at various temperatures. *Vertical bars* indicate standard errors.

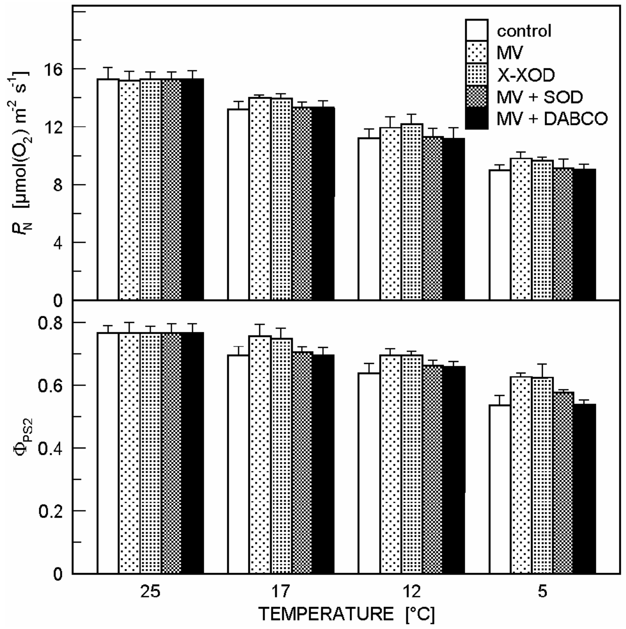

Fig. 5. Effects of MV, X-XOD, DABCO, and SOD on the recovery process in maize seedlings exposed to strong irradiance (1 000 μmol m^{-2} s^{-1}) and then transferred to the dark (2 h of darkness) at various temperatures. *Vertical bars* indicate standard errors.

$O_2^{-\cdot}$. In the dark period, the $O_2^{-\cdot}$ level decreased dramatically during the first 1 h, and then remained constant.

In this study, MV and X-XOD were used as the exogenous $O_2^{-\cdot}$ generation system, while DABCO and SOD were generally regarded as a scavenger for $O_2^{-\cdot}$ (Greenwald 1987). As shown in Fig. 4, the P_N and Φ_{PS2} remarkably diminished in maize seedlings treated with MV or X-XOD, but this decrease can be partially counteracted when SOD or DABCO was applied to the seedlings, further confirming that the photoinhibition was possibly caused by the elevation of chill-induced $O_2^{-\cdot}$ generation.

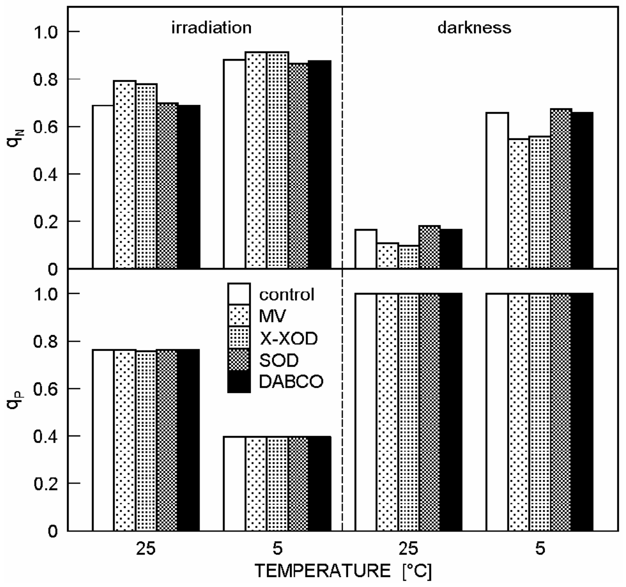

Fig. 6. Effects of MV, X-XOD, DABCO, and SOD on q_N and q_P in maize leaves in maize seedlings exposed to strong irradiance (2 h of 1 000 μmol m^{-2} s^{-1}) and then transferred to the dark (2 h of darkness) at various temperatures.

The protective role of superoxide radical to photosynthesis: The effect of exogenous $O_2^{-\cdot}$ on the recovery process is shown in Fig. 5. Apparently, treatment with MV or X-XOD promoted the recovery of P_N and Φ_{PS2} in the dark period, and the increase was more obvious as temperature decreased. In contrast, the addition of DABCO or SOD reduced markedly the recovery (Fig. 5*A,B*). This suggests that $O_2^{-\cdot}$ might play a protective role in the recovery under the darkness following irradiation.

To illustrate further the possible protective role of $O_2^{-\cdot}$, the changes in q_N and q_P of maize seedling leaves treated with $O_2^{-\cdot}$, SOD, or DABCO were determined under both strong irradiance and dark (Fig. 6). Both at normal temperature and under chilling stress, the q_N of seedlings treated with exogenous $O_2^{-\cdot}$ was higher under irradiation, but lower in darkness after strong irradiation, whereas the q_N in seedlings treated with SOD or DABCO exhibited no obvious change under both irradiation and darkness. This suggests that exogenous $O_2^{-\cdot}$ could promote the non-photochemical quenching to dissipate excess photon energy during irradiation and might be helpful for the recovery of the ability of the non-photochemical quenching in dark after irradiation. In addition, no change in q_P was observed either under irradiation or in dark after the above treatments, indicating that $O_2^{-\cdot}$ level had no effect on the photochemical process (Fig. 6).

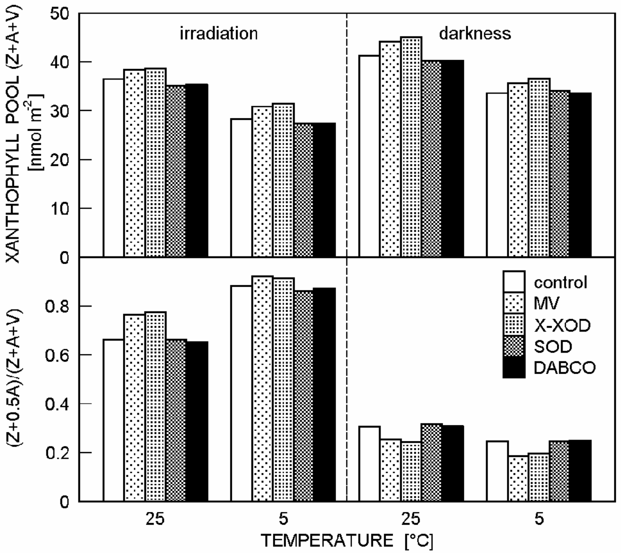

Fig. 7. Effects of MV, X-XOD, DABCO, and SOD on the xanthophyll cycle in maize seedlings exposed to strong irradiance (2 h of 1 000 μmol m^{-2} s^{-1}) and then transferred to the dark (2 h of darkness) at various temperatures.

The level of de-epoxidation of violaxanthin (V) in the xanthophyll cycle was characterized using the epoxidation index, $E_i = (Z + 0.5\ A)/(A + Z + V)$ (Vàradi *et al.* 2000), which was associated with the recovery of photosynthesis after chilling temperatures (Fryer *et al.* 1995). To evaluate whether the amount of superoxide radical might contribute to the non-photochemical quenching *via* the xanthophyll cycle, the changes in xanthophyll cycle pool and pigment content in maize seedlings treated with MV or X-XOD and DABCO or SOD were analyzed at various temperatures. In our study, there existed a positive correlation ($r = 0.987$) between the level of $O_2^{\cdot-}$ and E_i under irradiation but a negative correlation ($r = -0.977$) in darkness (Fig. 7). Moreover, the xanthophyll pool increased under both irradiation and darkness. Hence under strong irradiance, the $O_2^{\cdot-}$ might contribute to accumulation of zeaxanthin and maintain high xanthophyll pool, enhancing the ability of NPQ to reduce the damage to photosynthetic apparatus. In comparison to exposure to the strong irradiance, treatment with MV or X-XOD lead to accumulation of V and increase in the whole xanthophyll pool in darkness, and this might help in recovery of the ability of NPQ. Thus, the superoxide radical might protect from photoinhibition *via* the xanthophyll cycle.

As $O_2^{\cdot-}$ is generally a harmful factor to proteins, lipids, and DNA, it might be difficult to understand well the beneficial role of $O_2^{\cdot-}$ in the recovery process in the darkness after irradiation. In this study, the dual-role of $O_2^{\cdot-}$ in photosynthesis might be due to the double chemical characteristic of $O_2^{\cdot-}$. $O_2^{\cdot-}$ is an active reducer, easily loosing an electron and bringing a series of strong oxidation reaction with other oxidants; on the other hand, it can get an electron easily as an oxidant and bring a reductive reaction. As an oxidant, $O_2^{\cdot-}$ could enhance the NPQ *via* catalyzing the reaction of de-epoxidation of V to increase the accumulation of zeaxanthin (Z), whereas as a reducer, it might decrease the NPQ by catalyzing the reverse reaction from Z to V, which could promote the recovery of NPQ capability. Under strong irradiance, the photosynthetic electron transfer chain is in the state of excessive oxidation while $O_2^{\cdot-}$ mainly exhibits an oxidant characteristic, but in darkness the photosynthetic electron chain is in the reducing state while the $O_2^{\cdot-}$ shows reduced characteristic. Thus, superoxide radical might have a dual-role in the chilling-induced photoinhibition in maize seedlings.

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