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Photosynthetic characteristics and protective mechanisms against oxidative stress during chilling and subsequent recovery in two maize varieties differing in chilling sensitivity

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Abstract

Plants of two maize varieties differing in chilling sensitivity (Z7, tolerant and Penjalinan, sensitive) were subjected to 5 °C, 300 μmol m^{-2} s^{-1} PPFD and 12-h photoperiod during 5 days and recovered during 3 days at 25 °C at the same light regime. Gas exchange and chlorophyll fluorescence analyses showed the same degree of photoinhibition during chilling period in both varieties. However, chlorophyll fluorescence analysis showed an increase in the process of energy dissipation mainly in Z7 leaves. Besides, Z7 leaves showed higher carotenoids/total chlorophyll ratio than Penjalinan. On the other hand, Z7 leaves showed a marked increase in antioxidant enzyme activities (superoxide dismutase, SOD; ascorbate peroxidase, APX; glutathione reductase, GR), while in Penjalinan decreased the cytoplasmatic and chloroplastic CuZn and Fe SODs activities and increased the APX and GR activities in less extent than in Z7. Such antioxidant enzyme behaviour could be responsible for chloroplast damage caused by superoxide anion (O_2^-) during chilling in Penjalinan. When plants were returned to 25 °C, Z7 leaves quickly recovered photosynthetic activity thus diminishing the potential power for reactive oxygen species generation. On the contrary, Penjalinan leaves only recovered photosynthetic activity after 3 days at 25 °C. Moreover, during chilling recovery, Penjalinan leaves increased SOD and GR activity, while APX activity decreased. As a consequence, oxidation damage due to H_2O_2 accumulation could occur in Penjalinan leaves.

Keywords: Antioxidant enzymes; Carotenoids; Chilling stress; Chlorophyll fluorescence; Maize; *Zea mays* L.

1. Introduction

Many tropical crop species including maize are sensitive to chilling stress [1,2]. One of the main causes of chilling injury is the production of reactive oxygen species (ROS) during chilling which are highly stimulated in light inducing photooxidation. ROS are produced during chilling conditions because enzyme activities of the Calvin–Benson cycle are slowed down and the $NADP^+$ supplement to accept electrons from chain electron transport is restricted leading to excess energy absorption by oxygen. Thus, there are three main mechanisms to diminish photooxidation during chilling: avoiding production of ROS by diminishing electron transport chain, dissipating excess energy as heat via violoxanthin de-epoxidation and scavenging ROS formed by antioxidant compounds and enzymes (for review see Ref. [3]). Also, the chloroplastic water–water cycle, that is referred to the electron flow from water in photosystem II to O_2 reduction in PSI without a net change of O_2, has been proposed as an effective mechanism to dissipate the excess excitation energy under environmental stress [4]. This cycle is also composed by some antioxidant enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) [4].

Maize is especially sensitive to chilling stress at early stages of development although the degree of chilling injury is different among maize varieties [5]. The different sensitivity to chilling stress among maize varieties is due in part to a differing antioxidant system. Thus, when maize plants are grown at low temperature, chill-

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ing-tolerant varieties increase antioxidant enzyme activities while chilling-sensitive ones do not [6,7]. Furthermore, in previous studies conducted for short periods of time in different maize varieties under chilling conditions no differences were found in the amount of carotenoids [8]. On the other hand, chilling-tolerant varieties developed at low temperatures for longer periods of time have shown a greater amount of carotenoids than chilling-sensitive ones [6,9]. However, such results correspond to plants grown at sub-optimal temperatures (10–15 °C) and almost no information is available about differences in antioxidant systems among maize varieties during more severe chilling conditions (e.g. 5 °C) as well as during subsequent recovery [10]. In fact, during chilling recovery, the CO_2 exchange is also limited, in part because the conductance is diminished [6,11] and as a consequence, production of ROS may increase in the same way as during drought stress when the stomata are closed [12]. Thus, although differences in chilling tolerance among maize varieties can be correlated with the capacity to develop antioxidant systems during chilling recovery, there is still not much information about this topic [8,10].

In this study, we used two maize varieties that have been previously used by other researchers working on chilling tolerance [8,9,13–16]: Z7, a chilling-tolerant variety and Penjalinan, a chilling-sensitive one. Kocsy and co-workers [15,16] have studied the role of glutathione synthesis during low temperature stress (3 days at 12 °C), but information about other antioxidant enzymes or compounds in these two varieties is not available. Therefore, the aim of this research was to study antioxidant system, including antioxidant enzymes, pigment composition and dissipation capacity of Z7 and Penjalinan varieties during severe chilling stress and subsequent recovery.

2. Materials and methods

2.1. Plant material

Seeds of chilling-tolerant Z7 and chilling-sensitive Penjalinan were surface disinfected with 0.02% (w/v) of $HgCl_2$ and germinated in a growth chamber at 25 °C, 70% RH, 12-h photoperiod and 300 μmol photons m^{-2} s^{-1} on wet perlite. When third leaf was fully developed, plants were transferred to a growth chamber at 5 °C (and identical the rest of growth conditions) during 5 days. After this period, plants were returned again to 25 °C for 3 days. At the end of recovery period, percentage of necrotic leaf area was measured as described by Irigoyen et al. [17].

2.2. Gas exchange analysis

CO_2 and H_2O exchange rates of control and chilled plants were measured on the central part of enclosed third leaf in a gas-exchange cuvette (1010-M, Walz, Effeltrich, Germany) by using a portable photosynthesis system (HCM-1000, Walz). The conditions inside the cuvette were the same as in growth chambers: 25 or 5 °C, 70% RH, 300 μmol photons m^{-2} s^{-1} and CO_2 concentration of 400 mg kg^{-1}. Net photosynthesis (NP) and leaf conductance (g) was calculated as described by von Caemmerer and Farquhar [18].

2.3. Chlorophyll fluorescence analysis

Chlorophyll fluorescence was measured using a photosynthesis yield analyser (MINI-PAM, Walz). Measurements were made in the same leaf as the gas exchange measurements at 25 or 5 °C and 300 μmol photons m^{-2} s^{-1}. Also, measurements were carried out in dark-adapted leaves after 15 min of dark period. F_0, F_m, F_s and F'_m were recorded (for nomenclature see Ref. [19]) and optimal quantum yield of photosystem II (F_v/F_m, where F_v is $F_m - F_0$) and effective quantum yield of photosystem II ($\Delta F/F'_m$, where ΔF is $F'_m - F_s$) were calculated as described by Genty et al. [20]. Also, the rate constant of non-photochemistry process (fluorescence, heat emission, and energy transfer to PSI, $1/F'_m$) was calculated according to Havaux et al. [21].

2.4. Pigment analysis

0.25 cm^2 of the third leaf were immersed in 5 ml of ethanol (95% v/v) at 80 °C during 10 min to extract the pigments. The absorbance of extracts was measured at 470, 648.6 and 664.2 nm with a spectrophotometer. The extinction coefficients and the equations reported by Lichtenthaler [22] were used to calculate pigment concentrations.

2.5. Antioxidant enzyme assay

One hundred and twenty-five milligrams of the third leaf were homogenized in a mortar with 5 ml of 100 mM phosphate buffer (pH 7.0) containing 0.1 mM DTPA and 50 mg PVPP. The homogenate was filtered and centrifuged at 38 000 × g for 10 min. The supernatant was separated to determine the activity of antioxidant enzymes. SOD was assayed as described by Becana et al. [23] and one unit of SOD was defined as the amount of enzyme which produced a 50% inhibition of nitroblue tetrazolium reduction. In order to determine the different types of SOD, some leaf extracts were pre-incubated during 30 min in 5 mM KCN or 5 mM KCN plus 5 mM H_2O_2 and the same KCN and H_2O_2 concentrations were used during activity measure-

ments. Mn SOD is resistant to KCN and H_2O_2, Fe SOD is resistant to KCN and sensitive to H_2O_2 and CuZn SOD is sensitive to both KCN and H_2O_2 [24]. APX was assayed as described by Nakano and Asada [25] with slight modifications as a 2-ml reaction mixture containing 80 mM phosphate (pH 7.0), 0.5 mM ascorbate, and 0.25 mM H_2O_2. The oxidation of ascorbate was recorded at 290 nm for 3 min after adding 100 μl of leaf extract. GR was assayed as described by Schaedle and Bassham [26] with slight modifications as 2 ml reaction mixture containing 50 mM Tris–HCl (pH 7.5), 0.15 mM $NADPH_2$, 0.5 mM oxidized glutathione (GSSG), and 3 mM $MgCl_2$. The oxidation of $NADPH_2$ was recorded at 340 nm for 3 min after adding 200 μl of leaf extract. Total protein was measured by the method of Bradford [27]. All enzymatic activities were measured at 25 °C.

2.6. Statistical analysis

Means of all treatments of each parameter were compared by ANOVA and Fisher's LSD tests.

3. Results

After 3 days recovery, Penjalinan plants showed about three times more necrotic leaf area than Z7 plants (Fig. 1). All the parameters measured during chilling recovery were done only in green tissues.

Penjalinan control leaves (day 0) showed higher NP rate and leaf conductance (g) than Z7 (Fig. 2(A) and (B)); however, no differences were observed between the two varieties in their intrinsic water use efficiency values (NP/g; Fig. 2(C)). On the first day of chilling treatment (5 °C) the leaves of both maize varieties decreased their NP and g to the same values (Fig. 2(A) and (B)), although at this moment Z7 leaves showed

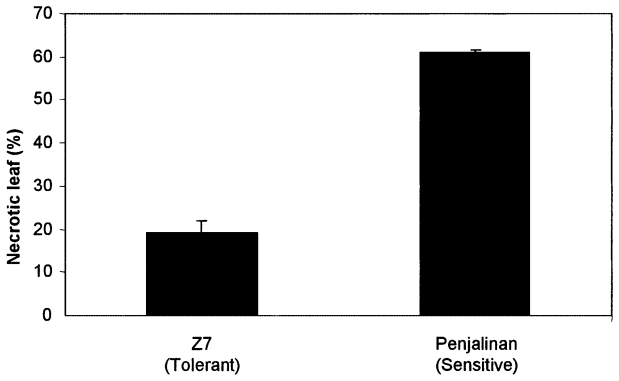

Fig. 1. Percentage of necrotic leaf on dry weight basis of two maize varieties differing in chilling sensitivity: Z7 (tolerant) and Penjalinan (sensitive). Measurements were made at the end of the experiment (5 days at 5 °C plus 3 days at 25 °C). Bars represent mean ± SE of three plants.

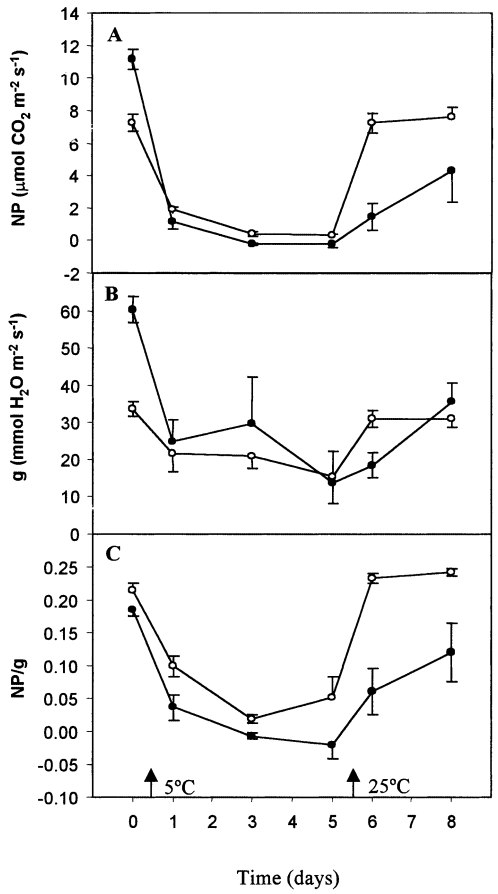

Fig. 2. NP rate (A), leaf conductance (B) and intrinsic water use efficiency (C) of two maize varieties differing in chilling sensitivity: Z7 (tolerant, white symbols) and Penjalinan (sensitive, black symbols) subjected to 5 days at 5 °C plus 3 days of recovery at 25 °C. Arrows indicate the start and end of chilling treatment. Circles represent the mean ± SE of five plants.

higher NP/g values than Penjalinan ones (Fig. 2(C)). On third and fifth days of chilling treatment, NP and g values remained almost unchanged with respect to the first day in both varieties (Fig. 2(A) and (B)). Nevertheless, in the first day of recovery (day 6), Z7 leaves recovered totally their NP, g and NP/g values, while Penjalinan leaves showed the same values as during chilling treatment (Fig. 2). At the end of recovery period, Penjalinan leaves recovered totally their NP/g, but the NP and g values reached only 40 and 60% of the control values, respectively (Fig. 2). Z7 leaves showed higher NP/g values than Penjalinan at the end of recovery period (Fig. 2(C)).

The behaviour of optimum (F_v/F_m) and effective ($\Delta F/F'_m$) quantum yield of photosystem II was the same as that observed in NP (Figs. 2(A), 3(A) and (E)), except at the end of recovery period when Penjalinan leaves showed the same F_v/F_m and $\Delta F/F'_m$ values than Z7. Although during chilling treatment no differences in maximum (F_m) and variable (F_v) fluorescence in dark-adapted leaves were observed between Z7 and

Penjalinan, during recovery F_m and F_v values were higher in Z7 than in Penjalinan. Moreover, Penjalinan leaves were incapable of fully recover their control F_m and F_v values (day 0; Fig. 3(B) and (C)). Penjalinan leaves did not change minimum fluorescence in dark-adapted leaves (F_0) neither during chilling treatment nor during recovery period (Fig. 3(D)). On the contrary, Z7 leaves showed a progressive reduction in their F_0 value during chilling treatment and a significant increase during recovery (Fig. 3(D)). Both maize varieties increased their rate constant for non-photochemistry process ($1/F'_m$) during chilling treatment, being gradual in Z7 and very fast in Penjalinan (Fig. 3(F)). Also, Z7 leaves recovered the control values of $1/F'_m$ on the first day of recovery (day 6), while in Penjalinan it took three days (day 8; Fig. 3(F)).

Total chlorophyll content did not suffer any significant change during chilling and subsequent recovery in Z7 leaves (Fig. 4(A)). On the contrary, Penjalinan leaves increased it on the third day of chilling treatment approaching Z7 values, then decreased significantly but after 3-days recovery reached control values (Fig. 4(A)). Fig. 4(B) shows the ratio carotenoid/total chlorophyll. Such ratio increased in Z7 leaves only the first day of recovery period. However, in Penjalinan the ratio increased the first day of chilling and during recovery. In general, Z7 leaves showed higher ratio carotenoid/total chlorophyll than in Penjalinan, except for the last day of recovery.

APX activity was always higher in Z7 leaves than in Penjalinan (Fig. 5(A)). Moreover, on the first day of chilling treatment, Z7 leaves showed a drastic increase in APX activity (around three times greater than control), although immediately recovered control values. Penjalinan leaves showed a transitory APX activity increase on the first day of chilling treatment (about 35%), but on the fifth day of chilling treatment there was a reduction in about 50%. During recovery, Penjalinan leaves showed an initial increase of APX activity, followed by a slight decrease on the last day (Fig. 5(A)).

In control leaves, no differences were found between the two varieties in GR activity (Fig. 5(B)). Chilling treatment caused an increase in GR activity in both varieties, although such increase was about 150 and 70% in Z7 and Penjalinan, respectively (Fig. 5(B)). Both varieties maintained high values of GR activity until the first day of chilling recovery (day 6), with Z7 leaves always showing higher values than Penjalinan (Fig. 5(B)). Nevertheless, on the last day of chilling recovery,

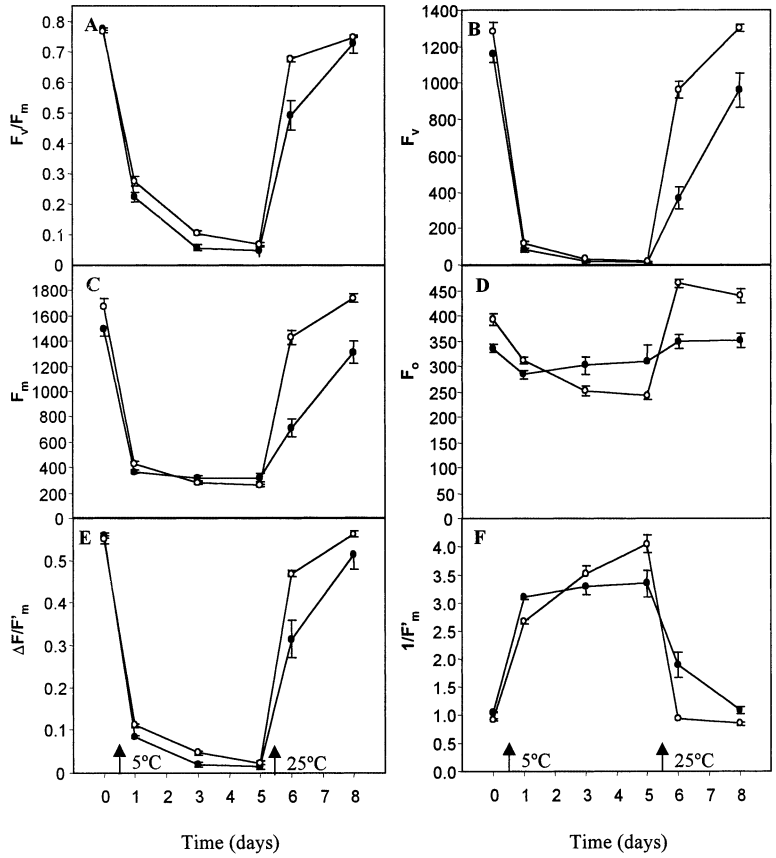

Fig. 3. Optimal quantum yield (A), variable fluorescence (B), maximal (C) and minimal (D) fluorescence in dark adapted leaves, effective quantum yield (E) and rate constant for non-photochemistry (F) of two maize varieties differing in chilling sensitivity. Otherwise as for Fig. 2.

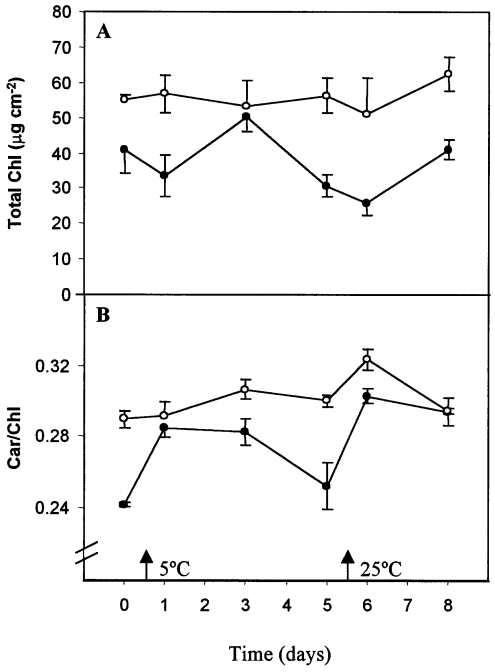

Fig. 4. Total chlorophyll content (A) and carotenoid/total chlorophyll ratio (B) of two maize varieties differing in chilling sensitivity. Otherwise as for Fig. 2.

Penjalinan showed higher GR activity than Z7, which reached control values.

Z7 leaves increased their total SOD activity on the first day of chilling treatment, but then no significant changes were found during the rest of the experiment (Fig. 6(A)). On the contrary, Penjalinan leaves decreased their total SOD activity on the first day of chilling and remained at low values during the rest of chilling period (Fig. 6(A)). During recovery, the first day (day 6) Penjalinan increased their total SOD activity but on the last day reached control values (Fig. 6(A)). Z7 CuZn and Mn SODs activities showed the same behaviour than total SOD activity along the whole experiment (Fig. 6(A)–(C)). Which respect to Z7 Fe SOD activity, although during chilling treatment had the same behaviour than total SOD activity, during recovery the activity was higher than in control (Fig. 6(D)). Moreover, in Z7 no Fe SOD activity was detected neither in control leaves nor at the end of chilling treatment (day 5; Fig. 6(D)). Penjalinan CuZn and Fe SODs activities showed the same behaviour than total SOD activity (Fig. 6(A), (B) and (D)). On the contrary, Penjalinan Mn SOD activity did not change significantly during chilling treatment but increased on the first day of recovery period (Fig. 6(C)).

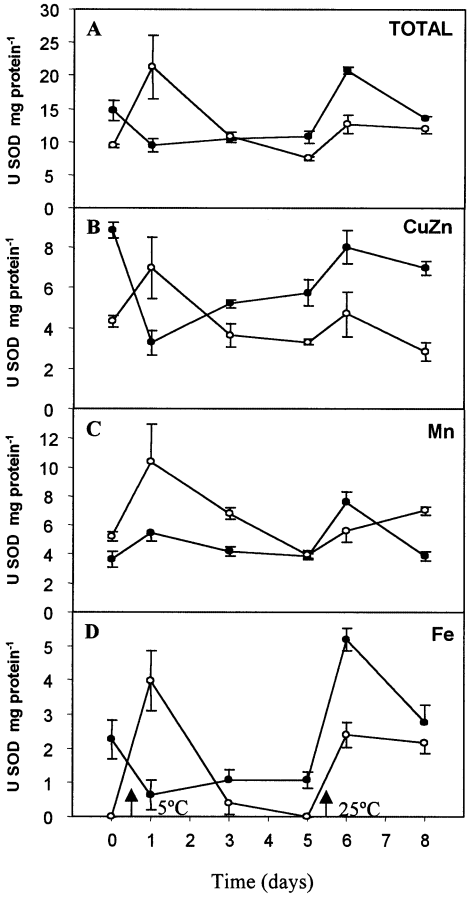

Fig. 6. Total (A), CuZn (B), Mn (C) and Fe (D) SODs activities of two maize varieties differing in chilling sensitivity. Otherwise as for Fig. 2.

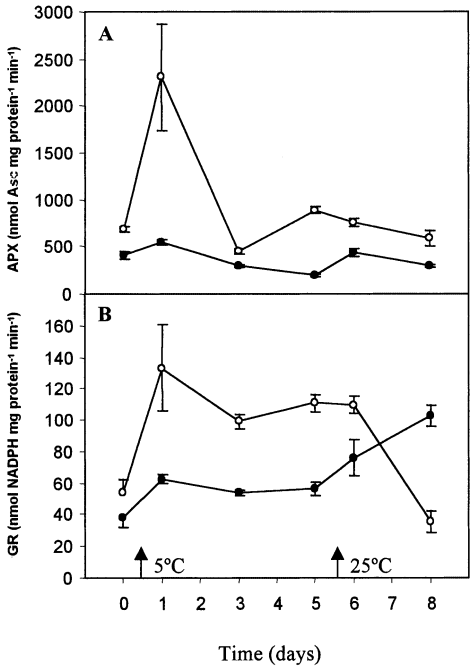

Fig. 5. APX (A) and GR (B) activities of two maize varieties differing in chilling sensitivity. Otherwise as for Fig. 2.

4. Discussion

Percentage of necrotic leaf area has been used as indicator of chilling injury in several chilling studies [14,17]. In the present study, such percentage indicated that Penjalinan plants suffered more from chilling damage than Z7 (Fig. 1). As it was mentioned previously, the main cause of chilling damage in light conditions is the photooxidation caused by photoinhibition associated with low temperatures [3]. During chilling treatment, both maize varieties showed practically the same CO_2 assimilation rate (NP; Fig. 2(A)) and quantum yield of non-cyclic electron transport estimated as $\Delta F/F'_m$ [20] (Fig. 3(E)). Therefore, both maize varieties had the same potential power for generating ROS during chilling treatment, since in recent studies [28] it has been suggested that the donation of electrons from electron transport chain to oxygen may increase during periods of low temperature. One mechanism to avoid ROS generation is to increase non-radiative energy dissipation in the pigment bed [29]. During chilling treatment, Z7 leaves decreased their F_0 level while no changes were observed in Penjalinan (Fig. 3(D)). Such decrease in F_0 may be correlated with energy dissipation processes in the pigment bed of Z7 leaves [30,31]. Moreover, Fig. 3(F) shows that during chilling treatment Z7 leaves had the capacity to increase the rate constant of non-photochemistry process ($1/F'_m$) while Penjalinan maintained it at constant value. Similar increase in $1/F'_m$ has been previously observed in a chilling-tolerant maize variety subjected to short chilling stress [32]. We found differences in $1/F'_m$ between the two varieties only on the fifth day of chilling (Fig. 3(F)).

Carotenoids act as photoprotective pigments by avoiding the generation of singlet oxygen by quenching the triplet-state chlorophyll molecules and by scavenging any singlet oxygen produced thus avoiding chlorophyll photooxidation [33]. Although on the first day of chilling, Penjalinan leaves showed an increase in the carotenoids/total chlorophyll ratio (Fig. 4(B)), Z7 leaves maintained almost always higher ratio throughout the chilling and the recovery period (Fig. 4(B)). Therefore, Z7 leaves appeared to be more capable of avoiding the production of singlet oxygen and to scavenge them than Penjalinan ones.

Chilling also causes an increase of superoxide (O_2^-) production in chloroplast and mitochondria, especially in chilling-sensitive species [34,35]. Higher plants have three different types of SODs to dismutate O_2^- to O_2 and H_2O_2. The three SOD forms differ in the metal co-factor and the sub-cellular localization. They are located in the cytosol and chloroplast (CuZn SOD), in the mitochondria (Mn SOD) and in the chloroplast (Fe SOD) [36]. On the first day of chilling, Z7 leaves increased the activity of all forms of SOD while Penjalinan decreased total SOD activity due to a decrease in CuZn and Fe SODs (Fig. 6) which will probably lead to an accumulation of O_2^- in Penjalinan chloroplasts. On the subsequent days of chilling, Penjalinan leaves showed greater activity of CuZn and Fe SODs than Z7 ones (Fig. 6). Overexpression of different types of SOD in leaves of some species lead to the increase of other antioxidant enzymes, such as APX or GR [37,38] thus increasing chilling tolerance. APX reduces H_2O_2 formed by SOD to H_2O and GR reduces glutathione oxidized implicated in the regeneration of ascorbate [39]. We found a parallel increase in SOD, APX and GR activities in Z7 leaves, while in Penjalinan leaves such behaviour was not observed (Figs. 5 and 6). That means that during chilling Penjalinan leaves may accumulate O_2^- and H_2O_2, caused by lower activities and co-ordination between the three antioxidant enzymes. Also, greater APX and GR activities observed in Z7 than in Penjalinan leaves, may be a consequence of an increase of water–water cycle in Z7 as proposed by Fryer et al. [28], that may act as a mechanism of dissipation energy [4].

CO_2 assimilation rate was totally recovered on the first day of recovery in Z7 while in Penjalinan leaves it did not occur even after 3-day recovery (Fig. 2). Also, Penjalinan showed lower values of intrinsic water use efficiency (NP/g) than Z7 (Fig. 2(C)). These lower NP/g values may indicate that CO_2 assimilation rate was not totally inhibited by leaf conductance in Penjalinan leaves and suggests that photosystem II function or C_4 metabolism enzymes are also restricted. Chlorophyll fluorescence analysis showed faster recovery of F_v/F_m and $\Delta F/F'_m$ in Z7 than in Penjalinan, indicating that Penjalinan leaves had more potential for ROS generation than Z7. However, after 3 days recovery, Z7 and Penjalinan leaves showed the same F_v/F_m and $\Delta F/F'_m$ values, thus suggesting that the difference in NP/g may be rather caused by a reduction in C_4 enzyme activity metabolism [13]. Moreover, during recovery Penjalinan showed lower F_v and F_m values than Z7 (Fig. 3(B) and (C)), which may indicate higher photooxidative damage and senescence process in such variety [40,41]. In fact, Penjalinan leaves showed higher $1/F'_m$ values than Z7 (Fig. 3(F)), being also related to higher senescence and necrotic area in such variety [41] (Fig. 1). On the other hand, during chilling recovery, Z7 leaves increased their F_0 values (Fig. 3(D)) which may be correlated with a decrease in the transfer of excitation energy from the light-harvesting complex of photosystem II to the photosystem II reaction centre, thus preventing the overexcitation of the photosystem II reaction centre [29,42].

Also, during the first day of recovery, Penjalinan leaves showed a decrease in total chlorophyll content (Fig. 4(A)) possibly caused by ROS generated during chilling treatment [43]. Therefore, the increased carotenoid/total chlorophyll ratio in Penjalinan (Fig.

4(B)) could be due to a decrease in chlorophyll content rather than to an increase in carotenoid content. On the other hand, the increased carotenoid/total chlorophyll ratio (Fig. 4(B)) without any change in total chlorophyll content (Fig. 4(A)) during the first day of recovery in Z7 leaves, may suggest a photoprotective mechanism [33] in Z7.

Recently, it has been found that during low temperatures, bundle sheath cells of maize suffered more oxidative damage than mesophyll ones [44] and that CuZn SOD and APX enzymes are localized exclusively in bundle sheath cells [45]. The first day of recovery period, Penjalinan leaves showed higher CuZn and Fe SODs activities (Fig. 6(B) and (D)), but lower GR and APX activities than Z7 (Fig. 5). Therefore, Penjalinan leaves may suffer an accumulation of H_2O_2 formed by high activity of SOD. Also, since on the third day of recovery, Penjalinan leaves increased their GR activity coupled with a slight decrease in APX activity and high CuZn SOD activity; more H_2O_2 may accumulate in their bundle sheath cells. On the contrary, Z7 leaves, which increased their Fe SOD activity during chilling recovery might have sufficient APX activity (Fig. 5) since symptoms of photooxidation were not observed (Fig. 1).

In summary, we found that in order to avoid photooxidative damage during chilling and subsequent recovery, Z7 leaves co-ordinated SOD, APX and GR activities. On the contrary, in Penjalinan the increase in SOD during chilling and subsequent recovery was not co-ordinated with an increase in APX activity. Such behaviour of Penjalinan may cause accumulation of H_2O_2 and subsequent oxidative damage. Besides, Z7 recovered faster photosynthetic activity than Penjalinan, thus diminishing the potential for ROS generation during chilling recovery.

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