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Drought enhances maize chilling tolerance. II. Photosynthetic traits and protective mechanisms against oxidative stress

Ricardo Aroca[1], Juan José Irigoyen and Manuel Sánchez-Díaz*

Departamento de Fisiología Vegetal, Universidad de Navarra, C/Irunlarrea s/n 31008, Pamplona, Spain
[1]Present address: Division of Biology, University of California at San Diego, La Jolla CA 92093–0116, USA
**Corresponding author, e-mail: msanchez@unav.es*

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In the present research we studied the photosynthetic traits and protective mechanisms against oxidative stress in two maize (*Zea mays* L.) genotypes differing in chilling sensitivity (Z7, tolerant and Penjalinan, sensitive) subjected to 5°C for 5 days, with or without pretreatment by drought. The drought pretreatment decreased the symptoms of chilling injury in Penjalinan plants estimated as necrotic leaf area and maximum quantum yield of photosystem II. Furthermore, drought pretreatment diminished the level of lipid peroxidation caused by chilling in Penjalinan plants. After one day of recovery from chilling the Z7 and drought-pretreated Penjalinan plants showed higher net photosynthesis rates than the non-drought-pretreated Penjalinan plants, thereby decreasing the probability of generating reactive oxygen species. The greater net photosynthesis was correlated with the greater NADP-malate dehydrogenase activity. No differences in either the de-epoxidation state of the xanthophyll cycle or the antioxidant enzyme activities were found among the chilled groups of plants. However, a drastic decrease in ascorbate content was observed in chilled Penjalinan plants without drought pretreatment. As we found an increase of H_2O_2 content after drought pretreatment, we suggest its involvement as a signal in the drought-enhanced chilling tolerance of maize.

Introduction

Many tropical crop species including maize are sensitive to chilling stress, especially at an early stage of development (Stamp 1984). During and after chilling, the rate of CO_2 fixation slows down and the $NADP^+$ supplement available to accept electrons from the electron transport chain is restricted, leading to excessive reduction of oxygen and thereby increasing the generation of reactive oxygen species (ROS) (Wise 1995). Therefore, the different degrees of chilling tolerance among maize varieties are linked to the capacity to remove ROS during and after low temperature events (Hodges et al. 1996, Aroca et al. 2001).

To remove ROS, the plants have antioxidant enzymes and compounds. The ascorbate–glutathione cycle is the most important antioxidant cycle in plants (Alscher et al. 1997). The first ROS produced in the plant cells is the superoxide radical anion (O_2^-), that is dismutated to H_2O_2 by superoxide dismutase enzyme (SOD). The H_2O_2 is reduced to H_2O by the ascorbate peroxidase enzyme (APX). The ascorbate oxidized by APX is reduced by the reduced form of glutathione, and the glutathione is again reduced by the glutathione reductase enzyme (GR) (see Alscher et al. 1997). At the same time, the plants can diminish the formation of ROS during chilling by increasing the de-epoxidation state of the xanthophylls pool and dissipating the excess of energy as heat (Gilmore 1997).

In earlier work in our laboratory, it was shown that drought pretreatment enhanced the chilling tolerance of

Abbreviations – APX, ascorbate peroxidase; AsAT, aspartate aminotransferase; Ch, chilled plants without drought pretreatment; D, drought-treated plants; D-Ch, chilled plants with drought pretreatment; DHA, dehydroascorbate; DTPA, diethylenetriaminepentaacetic acid; F_v/F_m, maximum quantum yield of photosystem II; g, leaf conductance; GR, glutathione reductase; NADP-MDH, NADP-malate dehydrogenase; NADP-ME, NADP-malic enzyme; NP, net photosynthesis; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PVPP, polyvinyl polypirrolydone; ROS, reactive oxygen species; RWC, leaf relative water content; SOD, superoxide dismutase; TBARS, thiobarbituric acid reacting substances; TCA, trichloracetic acid; WW, well-watered plants.

maize (Irigoyen et al. 1996). Thus, drought-acclimated maize plants showed higher CO_2 exchange rates and less necrotic leaf area than non-acclimated plants during the recovery period. Such enhancement of photosynthetic activity by drought pretreatment might be explained by the improvement of the water status (Irigoyen et al. 1996, Aguilera et al. 1999). Drought pretreatment reduces water deficit induced by chilling by the fast closure of the stomata and by raising the abscisic acid content (Irigoyen et al. 1996, Pérez de Juan et al. 1997). However, better defence against oxidative stress and/or higher activity of CO_2 fixation enzymes may also be implicated. In fact, plants that are subjected to drought are more tolerant to oxidative stress than well-watered plants (Burke et al. 1985).

In the present research we analysed the protective mechanisms against oxidative stress (including antioxidant enzymes and compounds, and pigments of the xanthophyll cycle) and the activity of CO_2 fixation enzymes after chilling in drought-acclimated or non-acclimated maize plants of two genotypes differing in chilling sensitivity. In addition, as oxidative damage during chilling increases at higher light intensity (Wise 1995), we elevated the light intensity from 150 $\mu mol\,m^{-2}\,s^{-1}$, as used in the previous work (Irigoyen et al. 1996) to 400 $\mu mol\,m^{-2}\,s^{-1}$. To separate photooxidative damage from injury associated with water deficit suffered during chilling, some experiments were performed at high relative humidity (90% RH) to avoid water stress by diminishing leaf transpiration (Janowiak and Dörffling 1996). To determine the level of chilling injury we measured the percentage of necrotic leaf area and the maximum quantum yield of photosystem II (Ribas-Carbo et al. 2000, Aroca et al. 2001). At the same time, the oxidative damage was estimated by measuring the thiobarbituric acid reacting substances (TBARS) (Dhindsa et al. 1981).

Materials and methods

Plant material and experimental design

In the present work, we used two maize (*Zea mays* L) genotypes differing in chilling sensitivity: Z7, which is tolerant, and Penjalinan, which is sensitive (Haldimann 1997, Pérez de Juan et al. 1997, Ribas-Carbo et al. 2000, Aroca et al. 2001). Seeds of both genotypes were surface disinfected by a solution of 0.02% (w/v) of $HgCl_2$ and sown in wet perlite at 25°C. Five days after sowing, the plants were transferred to 250 ml pots filled with perlite (1 plant/pot) and placed in a growth chamber at 25°C, 12 h of photoperiod, 60% RH and 400 $\mu mol\,m^{-2}\,s^{-1}$ of photosynthetic photon flux density (PPFD) at the leaf level. When the second leaves were fully developed, we suppressed the watering to half of the plants of each genotype for a period of 4 days. At the end of this period we had two groups of plants: well-watered plants (WW) and plants subjected to drought (D). The D plants were re-watered and together with the WW were transferred to a cold chamber at 5°C for a period of 5 days, at 60% RH (plants chilled at 60% RH) or 90% RH (plants chilled at 90% RH) (the other growth conditions were the same as in the 25°C growth chamber). After the chilling period, the plants were returned to the 25°C growth chamber for 1 day. At the end of this period, we had four groups of plants: plants subjected to chilling at 60% RH with (D-Ch) or without (Ch) drought acclimation, and plants subjected to chilling at 90% RH with (D-Ch90) or without (Ch90) drought acclimation. The experimental design is summarized in Fig. 1.

Necrotic leaf area

The percentage of necrotic leaf area was measured in Ch, D-Ch, Ch90 and D-Ch90 plants 24 h following the chilling treatment as previously described Irigoyen et al. (1996). Obviously, all the other measurements were made in green tissues.

Leaf water status

Leaf water status was assayed in the youngest fully developed leaves of each plant group of both genotypes by measuring the relative water content (RWC) by the Weatherley's method (Weatherley 1950) with slight modifications. Leaf samples were weighed [fresh weight (FW)] immediately after harvesting, then placed in a water-saturated vial at 5°C for 48 h and weighed [turgid weight (TW)]. The samples were dried in an oven at 80°C for a period of 48 h and their dry weights (DW) were obtained. Then the relative water content was calculated using the following equation: $(FW - DW)/(TW - DW) \times 100$.

Chlorophyll fluorescence analysis

Chlorophyll fluorescence emission was recorded by a photosynthesis yield analyser (MINI-PAM; Heinz Walz, Effeltrich, Germany) in the youngest fully developed leaves of each plant group of both genotypes. The measurements were made in the same conditions as those in which the plants were grown (25°C, 60% RH). Measurements were made on dark-adapted leaves. Minimal (F_o) and maximal (F_m) fluorescence emission in dark-adapted leaves were recorded. Then the maximum quantum yield of photosystem II (F_v/F_m, where F_v is $F_m - F_o$) were calculated (for details see Ribas-Carbo et al. 2000, Aroca et al. 2001).

Hydrogen peroxide determination

The hydrogen peroxide (H_2O_2) content was measured as described in Patterson et al. (1984) with slight modifications. Five hundred milligrams of youngest fully developed leaves of each plant group were homogenized in a cold mortar with 5 ml 5% TCA containing 0.1 g activated charcoal and 0.1% PVPP. The homogenate was filtered and centrifuged at 18 000 *g* for 10 min. The supernatant was filtered through a Millipore filter (0.45 μm) and used for the assay. A 200-μl aliquot was brought to 2 ml with 100 m*M* K-phosphate buffer (pH 8.4) and 1 ml

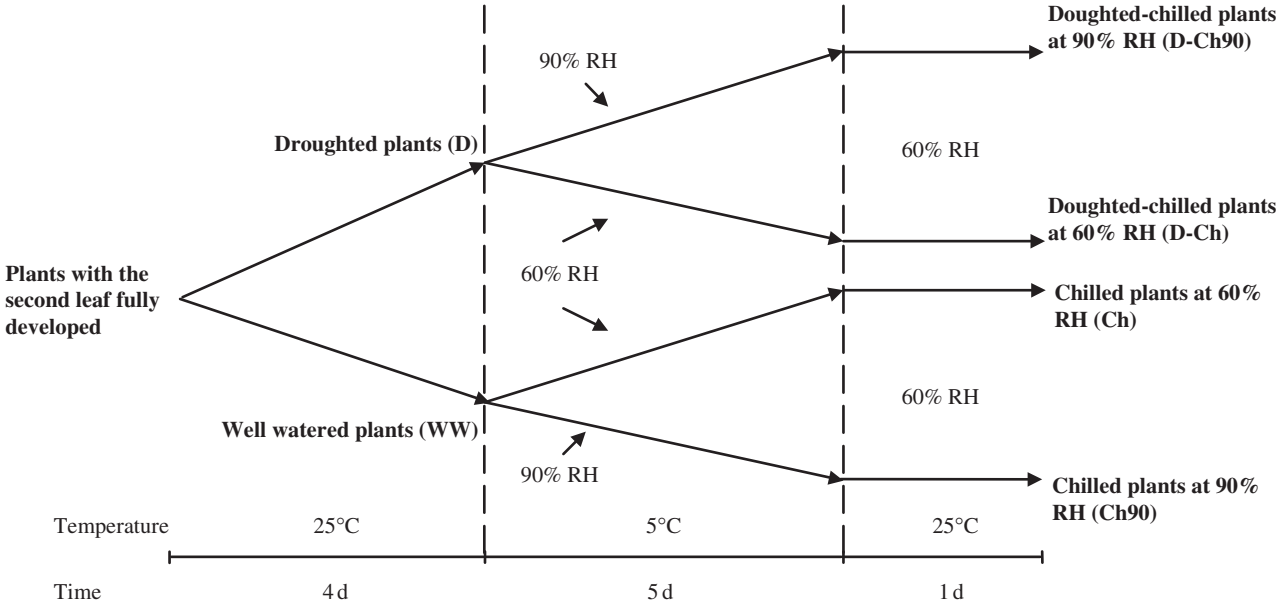

Fig. 1. Diagram of the experimental design. When the plants had the second leaf fully developed, half of the plants of each genotype were droughted by withholding water for 4 days, to produce droughted plants (D) or well-watered plants (WW). Later, the D plants were re-watered and put together with the WW plants at 5°C for a period of 5 days at 60 or 90% RH. All the plants were allowed to recover for 1 day at 25°C. Thus we had droughted-chilled plants (D-Ch) and chilled plants (Ch) without drought pretreatment at 60% (Ch) or 90% (Ch90) RH. For more details see Materials and methods.

colorimetric reagent was added. This reagent was made daily by mixing 1 : 1 (v/v) 0.6 m*M* potassium titanium oxalate and 0.6 m*M* 4–2 (2-pyridylazo)resorcinol (disodium salt). The samples were incubated at 45°C for 60 min and the absorbance at 508 nm was recorded. The blanks were made by replacing leaf extract by 5% TCA.

Lipid peroxidation

The level of lipid peroxidation in the leaves was estimated by measuring the content of TBARS as described Dhindsa et al. (1981).

Gas exchange analysis

CO_2 and H_2O exchange rates were measured in the same leaves used to measure chlorophyll fluorescence using a portable photosynthesis system (HCM-1000; Heinz Walz). The central part of each leaf was enclosed in a gas-exchange cuvette (1010-M; Heinz Walz), and the conditions inside the cuvette were the same as those in which plants were grown (25°C, 60% RH, 400 $\mu mol\, m^{-2}\, s^{-1}$ PPFD and CO_2 concentration 400 $mg\, kg^{-1}$). Net photosynthesis (NP) and leaf conductance (*g*) were calculated as described by von Caemmerer and Farquhar (1981).

C_4 metabolism enzymes activities

Two hundred and fifty milligrams FW of the youngest fully developed leaves of each plant group were homogenized in a cold mortar with 4 ml 50 m*M* phosphate buffer (pH 7.8) containing 1 m*M* EDTA, 8 m*M* $MgCl_2$, 5 m*M* DTT and 1% (w/v) PVPP. The homogenate was centrifuged at 27 000 *g* for 5 min at 4°C, and the supernatant was used for enzyme activity determinations. Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) and NADP malic enzyme (NADP-ME, EC 1.1.1.40) activities were assayed as described by Usuda et al. (1984). The NADP-malate dehydrogenase (NADP-MDH, EC 1.1.1.82) was assayed according to Nakamoto and Edwards (1983). Aspartate aminotransferase (AsAT, EC 2.6.1.11) was assayed according to Hatch and Mau (1973). Phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.49) was assayed according to Walker et al. (1995). Rubisco (EC 4.1.1.39) activity was measured following the method of Lilley and Walker (1974). We measured the initial activities of each enzyme.

Leaf pigment composition

Pigment composition was measured in 0.60 cm^2 discs of youngest fully developed leaves of each plant group. The leaf discs were ground in a mortar with pure acetone and a pinch of sodium ascorbate as described by Abadía et al. (1999). Pigment extracts were analysed by HPLC (De las Rivas et al. 1989).

Antioxidant enzymes activities

One hundred and twenty-five milligrams of youngest fully developed leaves of each plant group were homogenized

in a mortar with 5 ml 100 m*M* phosphate buffer (pH 7.0) containing 0.1 m*M* 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. Superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.7) and glutathione reductase (GR, EC 1.6.4.2), activities were determined as previously described Aroca et al. (2001).

Ascorbate determination

Ascorbate and dehydroascorbate (DHA) were assayed photometrically by the reduction of 2,6-dichlorophenolindophenol (DCPIP) as described by Leipner et al. (1997). Two hundred milligrams of youngest fully developed leaves of each plant group were homogenized in 5 ml ice-cold 2% (w/v) metaphosphoric acid in the presence of 1 g NaCl. The homogenate was filtered through a paper filter. An aliquot of 300 µl was mixed with 200 µl 45% (w/v) K_2HPO_4 and 100 µl 0.1% (w/v) homocysteine to reduce DHA to ascorbate for the determination of the total ascorbate pool. For the determination of ascorbate, the homocysteine solution was replaced by the same volume of distilled water. After 15 min incubation at 25°C, 1 ml 2 *M* citrate-phosphate buffer (pH 2.3) and 1 ml 0.003% (w/v) DCPIP were added. The absorbance at 524 nm was measured immediately. The content of ascorbate was calculated by reference to a standard curve. The amount of DHA was obtained as the difference between the total ascorbate pool and the ascorbate.

Glutathione determination

Glutathione content was measured as described Smith (1985). Five hundred milligrams of youngest fully developed leaves of each plant group were homogenized in a cold mortar with 5 ml 5% (w/v) sulfosalicylic acid, the homogenate was filtered and centrifuged at 1000 *g* for 10 min. One millilitre of supernatant was neutralized by 1.5 ml 0.5 *M* K-phosphate buffer (pH 7.5). The standard incubation medium was a mixture of: 0.5 ml 0.1 *M* sodium phosphate buffer (pH 7.5) containing 5 m*M* EDTA, 0.2 ml 6 m*M* 5,5′-dithiobis-(-2-nitrobenzoic acid), 0.1 ml 2 m*M* NADPH, and 0.1 ml (1 unit) GR. The reaction was initiated by the addition of 0.1 ml glutathione standard or extract. The change in absorbance at 412 nm was recorded for 9 min.

Statistical analysis

The experiments were performed three times and the mean of the three experiments and standard error are shown. The mean of all treatments of each parameter were compared using ANOVA and Fisher LSD tests.

Results

We followed the water status of plants during the drought period by measuring the RWC. After 4 days of withholding water both genotypes decreased their RWC by about 30% compared to WW plants (Table 1). We used the same water status parameter (RWC) to determine the water deficit caused by chilling. After 5 days of chilling treatment all plants had similar RWC (82–96%), except the plants of the chilling sensitive genotype (Penjalinan) without predrought treatment chilled at 60% RH (RWC = 61%, Table 1).

To determine the degree of chilling injury we measured the necrotic leaf area and the maximum quantum yield of photosystem II of the chilling sensitive (Penjalinan) and chilling tolerant (Z7) plants after 1 day of recovery at 25°C as in our previous studies (Ribas-Carbo et al. 2000, Aroca et al. 2001). After 1 day of recovery, the chilling-sensitive plants without drought pretreatment, whether chilled at 60 or at 90% RH, showed a much larger degree of leaf necrosis (28–39% by surface) than all other chilled plants (2–10% by surface) (see Table 1).

Data for the maximum quantum yield of photosystem II are expressed as the ratio of F_v/F_m (see Material and methods). After 1 day of recovery from chilling, the lowest values of F_v/F_m were found in chilling-sensitive plants without drought pretreatment (Ch and Ch90 plants). When these plants were first droughted they had higher maximum quantum yields, similar to those of the chilling-tolerant genotype (see Table 1). As the plants of the sensitive genotype chilled at both humidities had the same symptoms of chilling injury, the rest of parameters were only measured in 60% RH-chilled plants.

Table 1. Percentage of necrotic leaf area (NLA), maximum quantum yield of photosystem II (F_v/F_m) and leaf relative water content (RWC, %) in two maize genotypes differing in chilling sensitivity (Z7, tolerant and Penjalinan, sensitive) grown at 25°C well-watered (WW) or withholding water for 4 days (D) or grown at 5°C for 5 d. at 60% RH (Ch-non-predroughted or D-Ch predroughted) or at 90% RH (Ch90, non-predroughted and D-Ch90, predroughted). Means ± SE are shown. Means followed by the same letter do not differ significantly ($P > 0.05$). n.d, not determined.

	NLA		F_v/F_m		RWC	
Treatments	Z7	Penjalinan	Z7	Penjalinan	Z7	Penjalinan
WW	n.d	n.d	0.710 ± 0.010ab	0.728 ± 0.011b	94.9 ± 0.8a	97.1 ± 0.5a
D	n.d	n.d	0.656 ± 0.009a	0.668 ± 0.013ab	69.3 ± 3.7b	68.4 ± 4.7bc
Ch	4.2 ± 1.4ad	38.7 ± 4.1b	0.468 ± 0.034c	0.152 ± 0.021d	91.5 ± 2.0ad	60.5 ± 6.2c
D-Ch	1.6 ± 1.6a	8.0 ± 2.4ad	0.441 ± 0.037ce	0.387 ± 0.050ef	95.9 ± 0.5a	91.2 ± 1.7ad
Ch90	2.4 ± 0.1ad	27.7 ± 4.8c	0.309 ± 0.041fg	0.147 ± 0.024d	94.2 ± 2.3ad	81.6 ± 4.6d
D-Ch90	5.2 ± 2.4ad	10.0 ± 1.5d	0.277 ± 0.040 g	0.302 ± 0.055fg	94.1 ± 2.3ad	93.3 ± 1.6ad

The levels of H_2O_2 increased two fold by drought treatment in both genotypes. On the other hand, chilling treatment did not change the H_2O_2 control levels (WW plants) in any of the plant groups (Fig. 2A). Lipid peroxidation was estimated by measuring TBARS. The TBARS contents were generally slightly higher in Penjalinan than in Z7 in all the treatments; however, the most remarkable change was observed in chilling treatment (Ch), in which Penjalinan showed the highest values, whereas Z7 showed the lowest ones (Fig. 2B).

The photosynthetic apparatus was characterized by gas exchange measurements. The NP was decreased significantly by the drought treatment in both genotypes, and no differences were observed between them (Fig. 3A). The same behaviour was found for the leaf conductance (*g*) (Fig. 3B). All chilled plants showed a decrease in their NP, and the greater decrease was found in chilled plants of the sensitive genotype (Penjalinan) without drought pretreatment. These plants showed the same leaf conductance (*g*) as drought-acclimated plants of both genotypes (Fig. 3B).

The effects of drought and chilling on the initial activities of some CO_2 fixation enzymes are shown in Fig. 4. In Z7 (tolerant genotype), drought treatment caused an increase of NADP malate dehydrogenase (NADP-MDH) and phosphoenolpyruvate carboxykinase (PEPCK), although it did not have an effect on any of the other enzymes. In Penjalinan (sensitive genotype),

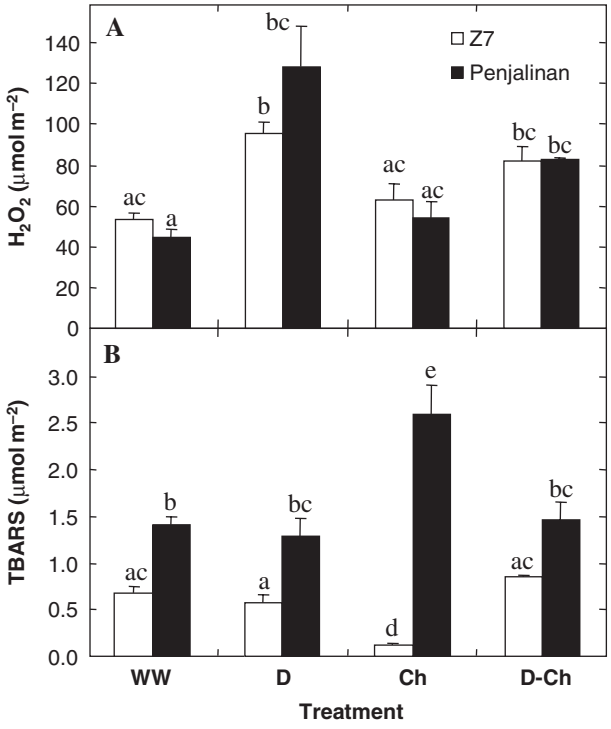

Fig. 2. Leaf hydrogen peroxide (A) and thiobarbituric acid reacting substance (TBARS) contents of two maize genotypes differing in chilling sensitivity: Z7 (tolerant, white bars) and Penjalinan (sensitive, black bars). Otherwise as for Table 1.

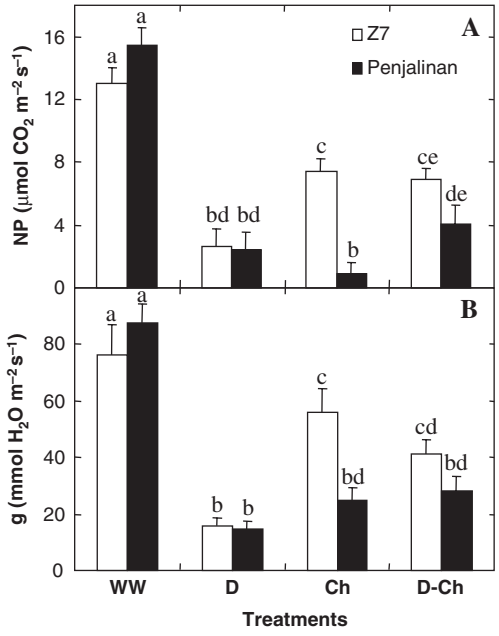

Fig. 3. Net photosynthesis (NP, A) and leaf conductance (*g*, B) of two maize genotypes differing in chilling sensitivity. Otherwise as for Table 1.

drought caused a decrease in NADP-MDH and aspartate aminotransferase (AsAT) activities but the activities of the other enzymes were unchanged. With regard to chilled plants, PEPC activity was unaffected by chilling treatment in Z7 plants, whereas it decreased in Penjalinan ones, such a decrease being greater in-non-predroughted plants. The NADP-MDH activity decreased after chilling treatment in all plant groups except the Z7-non-predroughted plants, with the smallest decrease in Z7 predrought plants and the largest in Penjalinan non-predroughted plants. AsAT decreased in Penjalinan after the chilling treatment, there being no difference between drought or-non-drought-pretreated plants. In chilled Z7 plants, the AsAT activity decreased in predroughted plants, whereas it increased in non-predrought plants. Malic enzyme (ME) activity was decreased by chilling in all plant groups to the same level. PEPCK activity increased in Z7 non-predrought plants and decreased in Penjalinan predroughted plants. Finally, Rubisco activity decreased to the same level in Penjalinan chilled plants and to a lesser extent in Z7 plants.

The Z7 plants had inherently more chlorophyll content than Penjalinan plants (Fig. 5A). Chlorophyll content decreased only in chilled predroughted plants of both genotypes (Fig. 5A). As one of the main roles of xanthophyll pigments is to protect chlorophyll molecules from photooxidative stress (Gilmore 1997) and we observed differences in chlorophyll content among plant groups, we expressed xantophyll contents on a chlorophyll basis. Drought treatment decreased violaxanthin (V) and increased zeaxanthin (Z) content in both genotypes, whereas antheraxanthin (A) content only

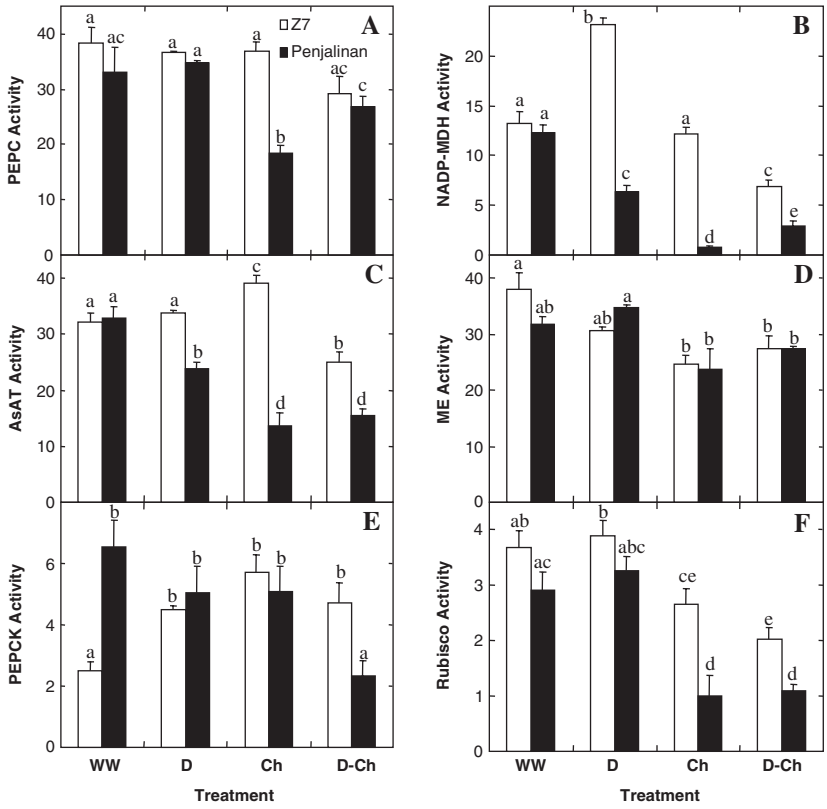

Fig. 4. Phosphoenolpyruvate carboxylase (PEPC, A), NADP-malate dehydrogenase (NADP-MDH, B), aspartate aminotransferase (AsAT, C), NADP-malic enzyme (NADP-ME, D), phosphoenolpyruvate carboxykinase (PEPCK, E) and Rubisco (F) initial activities expressed as $\mu mol\, m^{-2}\, s^{-1}$, of two maize genotypes differing in chilling sensitivity. Otherwise as for Table 1.

increased in Penjalinan plants. As a result, the plants of both genotypes increased their de-epoxidation state (A + Z)/(V + A + Z) after drought treatment (Fig. 5E). Chilling treatment had no significant effect on V and A contents in any of the chilled plant groups (Fig. 5B and C). However, chilled Penjalinan plants, predroughted or not, increased their Z content, whereas chilled Z7 plants showed the Z control values (Fig. 5D). Thus, the de-epoxidation state of the xanthophyll cycle components only increased in chilled Penjalinan plants (Fig. 5E).

We analysed the leaf activities of three antioxidant enzymes: SOD, APX and GR. The SOD and APX enzymes did not change their activities after drought treatment, whereas GR activity increased in both genotypes (Fig. 6). In control conditions (WW plants), the activities of the three antioxidant enzymes were greater in Z7 than in Penjalinan plants (Fig. 6). No significant effect of chilling treatment on the APX activity was observed in any of the chilled plant groups (Fig. 6B). The SOD activity was only decreased by chilling treatment in the Z7 non-predroughted plants, while maintaining the control values in the others (Fig. 6A). GR activity was not changed by chilling in drought-pretreated plants of both genotypes. On the contrary, in-non-predroughted plants it increased in Penjalinan and decreased in Z7 (Fig. 6C).

We measured the leaf ascorbate and glutathione contents. Drought treatment did not change the leaf levels of ascorbate, total ascorbate or dehydroascorbate (DHA) in any of both genotypes, but it increased glutathione content in Z7 plants (Fig. 7). In Z7 plants, chilling did not change the levels of total ascorbate in any of the two forms (ascorbate or DHA) (Fig. 7). On the contrary, the contents of total ascorbate were diminished in chilled Penjalinan plants, as a consequence of a reduction in ascorbate. However, the diminution was smaller in drought-pretreated (D-Ch) than in non-pretreated (Ch) plants, where ascorbate was undetectable by our method (Fig. 7B). The glutathione content increased in chilled plants of both genotypes, although the lowest increase was observed in Z7 plants without drought pretreatment (Fig. 7D).

Discussion

In a previous study, we found that during our chilling conditions (5 days at 5°C) there was almost no net growth in maize plants (Pérez de Juan et al. 1997). For this reason, we can compare the data from plants before and after chilling treatment, because they were in the same stage of development. Moreover, we used the youngest fully developed leaf for all the measurements, which was normally the second or third leaf.

Two parameters commonly used to determine the degree of chilling injury in maize are the percentage of necrotic leaf area and the maximum quantum yield of PSII (F_v/F_m) (Irigoyen et al. 1996, Ribas-Carbo et al. 2000, Aroca et al. 2001). In our study, these two

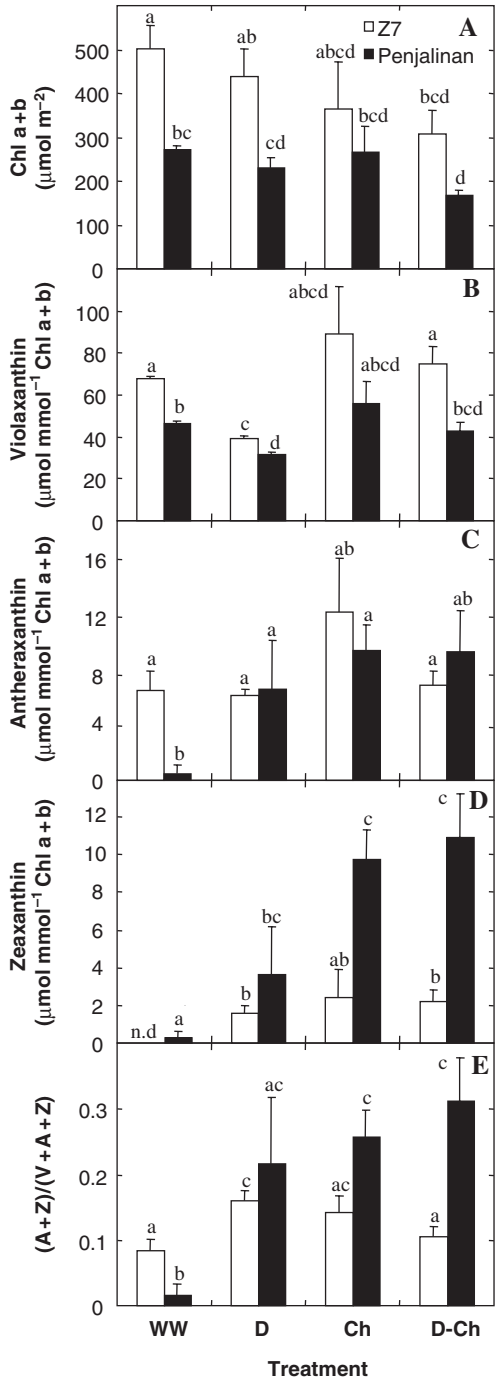

Fig. 5. Total chlorophyll (A), violaxanthin (B), antheraxanthin (C), zeaxanthin (D) contents and de-epoxidation state of xanthophyll pool, calculated as the ratio between antheraxanthin plus zeaxanthin contents/antheraxanthin plus zeaxanthin plus violaxanthin contents, of two maize genotypes differing in chilling sensitivity. n.d, not detected. Otherwise as for Table 1.

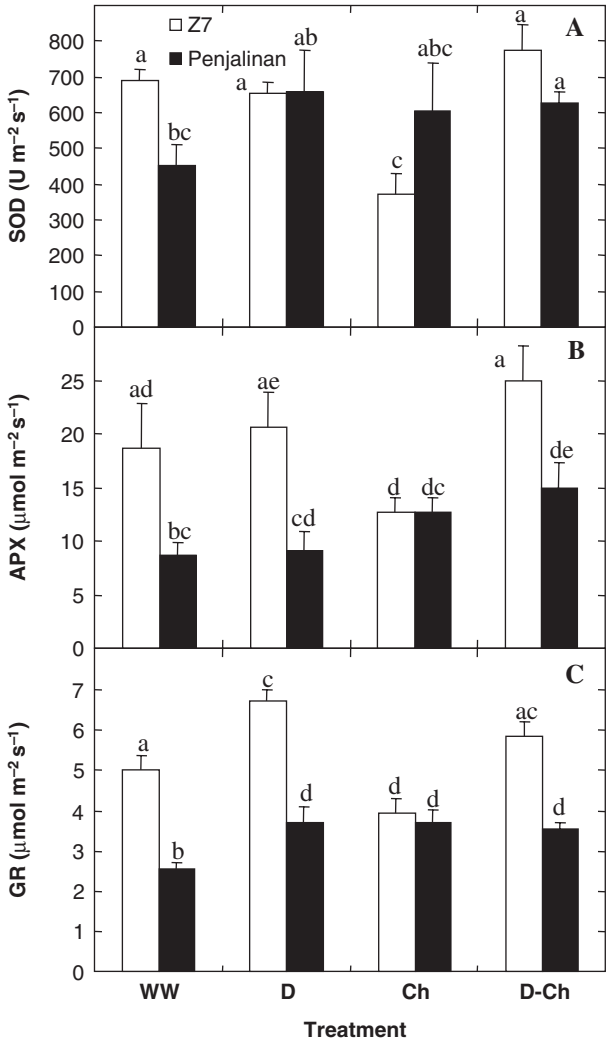

Fig. 6. Leaf superoxide dismutase (SOD, A), ascorbate peroxidase (APX, B) and glutathione reductase (GR, C) activities of two maize genotypes differing in chilling sensitivity. Otherwise as for Table 1.

parameters indicated that after 1 day of chilling recovery, chilled Penjalinan (sensitive genotype) plants without drought pretreatment were in the worst state, at either humidity regime. These plants showed the highest values of necrotic leaf area and lowest of F_v/F_m (Table 1). Therefore we confirm that Penjalinan is more chilling-sensitive than Z7 (Pérez de Juan et al. 1997, Ribas-Carbo et al. 2000, Aroca et al. 2001). At the same time, drought-pretreated Penjalinan (D-Ch) plants showed similar values for these two parameters as those of the chilling-tolerant Z7 plants (Table 1), confirming that drought pretreatment is an effective method to acclimate maize plants against chilling stress (Irigoyen et al. 1996, Pérez de Juan et al. 1997).

One of the most evident effects of drought pretreatment was the water status improvement of chilled Penjalinan plants (Table 1), which is associated mainly with ABA production and stomatal closure (Irigoyen et al. 1996, Pérez de Juan et al. 1997). However, to understand if drought pretreatment enhanced the chilling tolerance of maize is not only linked to the improvement of water status, we avoided in a group of plants (Ch90) the

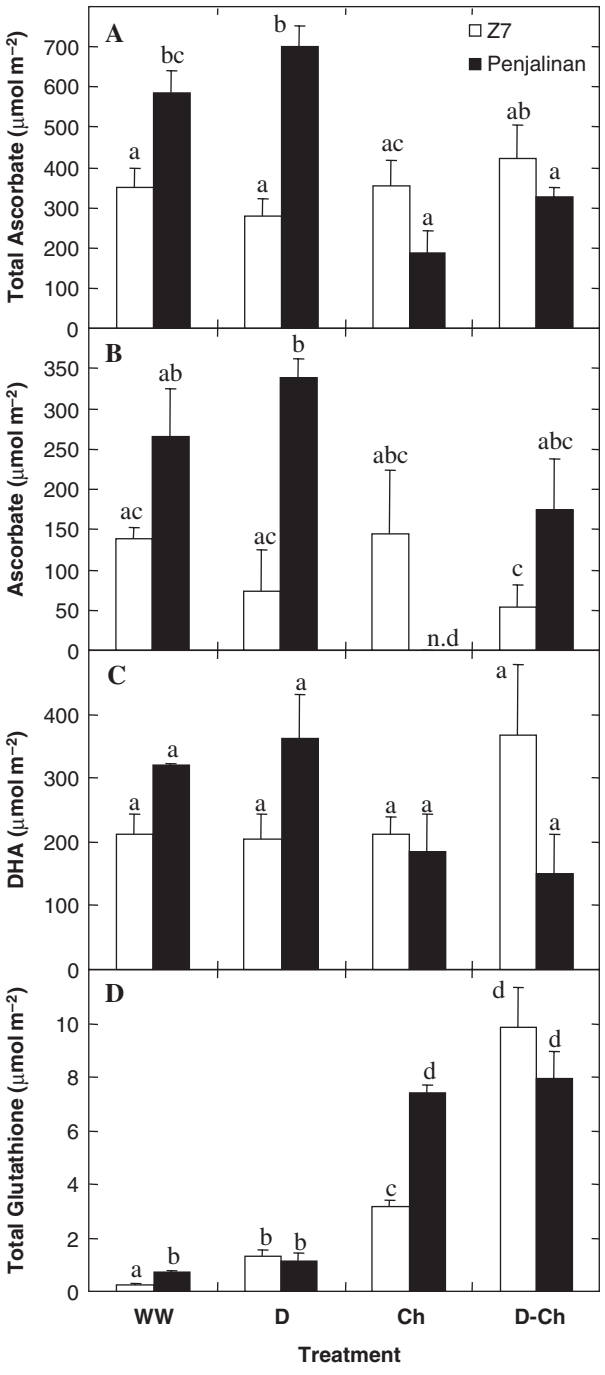

Fig. 7. Leaf total ascorbate (A), ascorbate (B), dehydroascorbate (DHA, C) and total glutathione (D) contents of two maize genotypes differing in chilling sensitivity. n.d, not detected. Otherwise as for Table 1.

chilling-induced water stress by increasing the RH (Janowiak and Dörffling 1996). Thus, Penjalinan plants chilled at 90% RH (Ch90) showed a higher RWC than Penjalinan plants chilled at 60% RH (Ch) and similar to chilled Z7 plants (Table 1). However, as discussed above, these two groups of plants (Ch and Ch90 of Penjalinan) showed a similar degree of chilling injury (Table 1). Therefore, we can conclude that in our experimental conditions of light intensity and temperature, the main cause of chilling injury was not the chilling-induced water deficit but quite likely the photooxidative stress caused by chilling (Wise 1995). In fact, 400 μmol m^{-2} s^{-1} of PPFD was enough to cause oxidative stress in others studies (Schöner and Krause 1990, Hodgson and Raison 1991). As we did not find differences in the level of chilling injury under both humidities in Penjalinan plants without drought pretreatment, the rest of the measurements were made only in plants chilled at 60% of RH.

One common parameter used to estimated the degree of oxidative stress is the measurement of lipid peroxidation (Dat et al. 2000). We estimated lipid peroxidation by measuring the TBARS (Dhindsa et al. 1981). After chilling treatment, the only plants that increased their TBARS content were the Ch Penjalinan plants (Fig. 2B). These results confirm that drought pretreatment diminishes photooxidative stress induced by chilling in Penjalinan plants.

In the past few years it has becoming more apparent that H_2O_2 can act as an acclimatory stress signal (Dat et al. 2000, Pastori and Foyer 2002). Thus, the exogenous application of H_2O_2 induced maize chilling tolerance (Prasad et al. 1994, Gong et al. 2001). Moreover, the induction of chilling tolerance in maize by heat-shock treatment is also mediated by H_2O_2 (Gong et al. 2001). We found an increase of H_2O_2 content during drought pretreatment in both genotypes (Fig. 2A), that could act as an acclimatory signal.

Oxidative stress during chilling is caused mainly because light energy absorbed by leaves exceeds energy utilization by CO_2 fixation, since CO_2 fixation enzymes are less active (Long 1983). This energy excess can be absorbed by dioxygen molecules (Wise 1995) generating ROS thereby inducing oxidative stress. The CO_2 fixation (net photosynthesis) decreased in all chilled plants, although such decrease was greatest in Ch (non-pretreated with drought) Penjalinan plants (Fig. 3A); therefore in those plants the generation of ROS is more likely. The differences observed in NP among chilled plants could not be due to differences in leaf conductance (*g*), since Ch Penjalinan plants had the same leaf conductance as D-Ch (drought pretreated) Penjalinan and Z7 plants (Fig. 3B).

Then, we assayed the activity of CO_2 fixation enzymes to understand their involvement on the reduction of photosynthesis observed in chilled maize plants. The most evident difference between the four chilled maize groups were that Ch Penjalinan plants showed lower NADP-MDH and PEPC activities than D-Ch Penjalinan and chilled Z7 plants (Fig. 4). Kingston-Smith et al. (1997) suggested that the PEPC activity would be the most temperature-sensitive step of photosynthesis in maize leaves. Furthermore, the NADP-MDH activity probably is the most limiting step in C_4 metabolism (Furbank et al. 2000). We observed a strong reduction

of NADP-MDH activity in Ch Penjalinan plants (about 95%). Therefore, as in sugarcane (Du et al. 1999), NADP-MDH may be one of the most limiting enzymes of CO_2 fixation during chilling in maize.

It is known that MDH activity is sensitive to oxidative stress, and that such sensitivity is different among plant species (Guerrier et al. 2000). Penjalinan drought-pretreated plants had less oxidative damage estimated as TBARS content (Fig. 2B). In this way, the NADP-MDH enzyme could be less injured. Nevertheless, the better status of photosynthetic apparatus of D-Ch Penjalinan plants after chilling treatment, could also be correlated with their better water status during chilling (Table 1), as proposed by Aguilera et al. (1999).

One of the mechanisms that helps the plants to avoid photooxidative stress is the dissipation of the excess of energy as heat via violaxanthin de-epoxidation (Gilmore 1997). We did not find differences in the de-epoxidation state of xanthophyll cycle between Ch and D-Ch Penjalinan plants, and even the most chilling-injured Penjalinan plants showed a de-epoxidation state higher than chilled Z7 plants (Fig. 5E). These results agree with those found by Haldimann (1997) in the same genotypes after 5 days at 10°C. However, it is possible that drought enhancement of chilling tolerance of maize may be correlated with an increase in the de-epoxidation state of the xanthophyll pool before chilling treatment as occurs in maize plants adapted to chilling stress by growing at suboptimal temperatures (Leipner et al. 1997) or by increasing ascorbate contents (Leipner et al. 2000). In fact, droughted Penjalinan plants showed a 19-times higher de-epoxidation state in comparison with WW plants (Fig. 5E).

Another mechanism to avoid photooxidative damage is to increase the activity of the ascorbate–glutathione cycle to remove generated ROS (Wise 1995). Thus, the superoxide radical (O_2^-) generated in photosystem I is converted to H_2O_2 by SOD. Then, the H_2O_2 is reduced to water by APX, which oxidized ascorbic acid. This ascorbic acid is regenerated by the oxidation of glutathione, which is also reduced by GR (Alscher et al. 1997). We did not find differences in the total activity of the three antioxidant enzymes tested (SOD, APX and GR) between Ch and D-Ch Penjalinan plants (Fig. 6). Thus, the activity of these antioxidant enzymes is not linked to the enhanced maize chilling tolerance by drought. However, the induction of new isoenzymes could be implicated as in placobutrazol-induced maize chilling tolerance (Pinhero et al. 1997).

After chilling treatment, the Penjalinan plants without drought pretreatment had no ascorbate in its reduced form (Fig. 7B). As we did not find differences in the GR activity and glutathione content between Ch and D-Ch Penjalinan plants (Figs 6C and 7D), the absence of ascorbate in Ch Penjalinan plants could be correlated with the higher ROS generation during chilling in those plants. Another possibility is that Ch Penjalinan plants increased the oxidation of ascorbate to tartaric and oxalic acids during chilling (Saito et al. 1997). Clearly the oxidation of ascorbate during chilling should be studied in greater depth.

We observed the same amount of glutathione content in Ch and D-Ch Penjalinan plants, and even more than in Ch Z7 plants (Fig. 7D). This fact is not strange, since Ch Penjalinan plants had a lower photosynthetic rate and therefore more possibilities to generate ROS. Apparently, Ch Penjalinan plants would need more antioxidant capacity than the other chilled plant groups. Furthermore, Creissen et al. (1999) found that by increasing the glutathione content in the chloroplasts of transgenic tobacco plants, the oxidative stress is also increased.

In summary, we conclude that the chilling tolerance induced by drought pretreatment in the sensitive maize genotype was caused mainly by the faster recovery of the net photosynthetic rate after chilling, that could be correlated with the improvement of water status and the diminution of oxidative stress. The improvement of NADP-MDH activity by drought pretreatment can be correlated with the recovery of photosynthesis. We found that the ascorbate content and its metabolism is critical in the ascorbate–glutathione cycle during chilling in maize. Finally, we suggest that H_2O_2 is involved as a signal in the processes of maize chilling acclimation by drought.

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