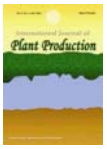

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Drought-induced chilling tolerance in cucumber involves membrane stabilisation improved by antioxidant system

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Abstract

We assessed changes in ultrastructure, membrane lipid peroxidation and antioxidant systems for cucumbner seedlings subjected to low temperature stress (day/night temperature of 8 ^{o}C /5 ^{o}C) that had been either pre-treated with 10% PEG for 2 days or not. We found extensive cell structure damage in the non-treated seedlings, whereas the seedlings pretreated with PEG to simulate drought remained essentially undamaged, except for slight damage to plasma membrane lipids and alveolation in the mitochondria. Low temperature stress increased electrolyte leakage, MDA levels and H_2O_2; decreased the activities of SOD, CAT and APX, and AsA and GSH content. An increase in POD activity was observed in the PEG-pretreated seedlings during the chilling period, while non-treated seedlings showed an increase in POD activity only in the early days of chilling stress. PEG pre-treatment diminished the level of lipid peroxidation caused by chilling compared to the non-treated seedlings, possibly due to a decrease in electrolyte leakage and MDA content. Furthermore, PEG pre-treatment increased the activities of SOD, POD, CAT and APX and AsA and GSH content in the chilling-stressed seedlings. These results suggest that PEG pre-treatment stimulates the adaptation of cucumber to low temperature. This could be due to stabilisation of the cell structure, alleviation of lipid peroxidation as a result of the increased activity of antioxidant enzymes and contents of antioxidant metabolites.

Keywords: Chilling tolerance; Drought-stress induction; Cell ultrastructure; Antioxidant system; Cucumber.

Abbreviations

polyethylene glycol	PEG
malonaldehyde	MDA
hydrogen peroxide	H_2O_2
superoxide dismutase	SOD
peroxidase	POD
catalase	CAT
ascorbate peroxidase	APX
ascorbate	AsA
glutathione	GSH
reactive oxygen species	ROS

Introduction

Cucumbers (*Cucumis sativus* L.) are sensitive to chilling stress; however, in the north of China, where they are mainly cultivated through the winter in solar-greenhouses, an abiotic stress due to low temperatures frequently occurs. Cold temperature slow the energy-consuming Calvin cycle enzymes more than the energy-transducing light reactions, thus causing leakage of energy to oxygen (Wise, 1995). To remove the ROS, plants utilise antioxidant enzymes such as CAT, POD, SOD, APX, etc. and non-enzymatic antioxidants such as AsA, GSH and α-tocopherol (Arca et al., 2003; Del Río et al., 1998; Ahmad et al., 2008). Altered activities of these antioxidant enzymes and changes in antioxidant content have been commonly reported in plants and are frequently used as indicators of stress (Koricheva et al., 1997).

An increasing number of studies have revealed the existence of cross-tolerance in plants, exposure of tissues to moderate stress induces resistance to other stresses. This cross-adaptation has been shown for different kinds of stresses. For example, heat acclimation not only enhances the plant's tolerance to heat but also to cold (Wang et al., 2004; Gong et al., 2001), salinity (Gong et al., 2001; Kuznetsov et al., 1993), and drought (Kuznetsov et al., 1999). Hincha (1994) reported that pretreatment of spinach with salt induced cold hardiness. Cold acclimation increased the heat tolerance of winter rye (Fu et al., 1998). Drought-hardening increased the cold tolerance of maize seedlings (Irigoyen et al., 1996; Aroca et al., 2003). The value of frost tolerance of winter after exposure to low temperatures, i.e. cold-acclimated wheat, is considerably higher, as compared to non-acclimated

wheat (Gholipoor, 2008). In our earlier work, we showed that PEG 6000 (PEG, simulate drought) pretreatment enhanced the chilling tolerance of cucumber (Dong et al., 2011), and suggest that may be related to the improvement of osmoregulation. Previous researchers considered that drought pretreatment reduced the water deficit induced by chilling, which contributed to a fast closure of the stomata and an increase in the abscisic acid content (Irigoyen et al., 1996; Pe'rez de Juan et al., 1997). However, better defence against oxidative stress may also be implicated (Arca et al., 2003). The different degrees of chilling tolerance among maize varieties are linked to their capacity to remove ROS during and after low temperature events (Arca et al., 2001). Oxidative stress induced by a moderate stress can improve the plant's ability to scavenge active oxygen, thus improving the plant's tolerance to other stresses (Foyer et al., 1994). This paper focuses on the influences of PEG pretreatment on the chilling tolerance of cucumbers. The objective of this study is to investigate if the chilling tolerance induced by PEG involves the regulation of lipid peroxidation in the cell membrane and the antioxidant system in cucumber seedlings.

Materials and Methods

Plant material

Cucumber (cv 'Jinyou 3') seeds were germinated on moist filter paper in the dark at 28 ^{o}C for 24 h, then grown in nutrition pots containing a mixture of 3 sand: 1 perlite: 1 vermiculite (v/v) in a solar greenhouse (air temperature was 18-30 ^{o}C, and RH 75-90%) for 15 days. The seedlings were transferred to plastic pots (35×28×13 cm) filled with aerated full-nutrient solution containing 4.0 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 1.0 mM $MgSO_4$, 0.66 mM $NH_4H_2PO_4$, 0.057 mM Fe-EDTA, 0.048 mM H_3BO_3, 0.009 mM $MnSO_4$, 0.76 μM $CuSO_4$ $5H_2O$, 0.96 μM $ZnSO_4$ $7H_2O$ and 0.38 μM $(NH_4)_6$ Mo_7O_{24} $4H_2O$. Then they were placed in a growth chamber with a photon flux density (PFD) of 480±20 μmol m^{-2} s^{-1}, a 26 ^{o}C /18 ^{o}C thermo-period, 80% RH and a 12-h photoperiod (control growth conditions). The pH and electric conductivity (EC) of solution were maintained at 6.5-7.2 and 2.1-2.5 ms/cm, respectively. When three leaves fully expanded, half of the seedlings were pretreated by adding 10% PEG 6000 (-0.8 MPa) to the nutrient solution (PEG treatment) and the others were used as the control. 2 days later, the PEG-treated seedlings were recovered in normal nutrient solution for 2 days and together

with the control seedlings, were transferred to a growth chamber at 8 ^{o}C /5 ^{o}C (day/night) air temperature, 100 μmol m^{-2} s^{-1} PFD, 70-80% RH and a 12-h photoperiod for 7 days. There were 3 replicates per treatment and 30 seedlings per replicate. Young, fully-expanded leaves were sampled for analyses before and after chilling stress for 1 d, 3 d, 5 d and 7 d.

Observation of cell ultra-structure

Rectangular segments (1-2 mm square) were cut from the both sides of leaf midvein. The segments were fixed for 2 h in 3.5% glutaraldehyde and 1% osmic acid, respectively. After fixation and dehydration with graded ethanol, leaf samples were embedded in s Epon 812 resin. The samples were sliced using an LKB-5 ultrathin slicer and the ultrathin sections were stained with uranyl acetate followed by lead citrate and examined in a JEM-1200 EX transmission electron microscope.

Measurement of electrolyte leakage

0.2 g of each sample were incubated at 25 ^{o}C in 25 ml test tubes containing 20 ml deionized water. Electrical conductivity of the bathing solution was measured at 3 h (EC_1) of incubation using a E-201-C conductivity meter (Shang, China). The samples were then autoclaved (100 ^{o}C) for 10 min and electrical conductivity (EC_2) of bathing solution was then measured after cooling. Electroly teleakage (E) was calculated from the following equation: $E=EC_1/EC_2\times100$.

Measurement of hydrogen peroxide content

The hydrogen peroxide (H_2O_2) content in cucumber leaves was measured as described by Liu et al. (2000)

Measurement of malonaldehyde content and antioxidant enzymatic activities

All samples were prepared for MDA and enzyme analyses by homogenisation of the fresh tissue with a mortar and pestle in a solution (4 ml g^{-1} fresh weight) containing 50 mM KH_2PO_4/K_2HPO_4 (pH 7.8), 1% PVP, 0.2 mM EDTA and 1% Triton X-100. After the homogenate was centrifuged at 12,000×g for 20 min at 4 ^{o}C, the supernatant was used to

determine the enzymatic activities (Cho and Park, 2000). All the spectrophotometric analyses were conducted using a UV-visible spectrophotometer (UV-2450, Shimadzu, Japan). The MDA content was measured using the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). The SOD activity was assayed by the method of Beyer and Fridovich (1987). The POD activity was determined according to the method of Omran (1980). The CAT activity was measured using the method of Chance and Maehly (1995). The ascorbate peroxidase (APX) activity was measured by the decrease in absorbance at 290 nm as the ascorbate was oxidised (Nakano and Asada, 1981).

Measurement of Ascorbic acid and Glutathione contents

The ascorbic acid (AsA) and glutathione contents (GSH) were determined as described by Wang et al. (2004).

Statistical analysis

The values presented are the means ± standard deviation (SD) of three replicates. Statistical analyses were carried out by analysis of variance (ANOVA) using DPS software. The Duncan's multiple range test (DMRT) was applied to compare significant differences among treatments.

Results

Mesophyll cell structure

In chilling without PEG-pretreated leaves (control), the membranes, chloroplasts and mitochondria of mesophyll cells were damaged (Figure 1). The chloroplasts membrane became incomplete. The number of grana lamella, the size and number of starch grains all decreased, while many osmiophilic droplet (grains which combined with osmicacid in mesenchyme) accumulated in chloroplasts. The mitochondria showed markedly dilatated, the membrane was invisible, and the internal structure becme irregular. However, the PEG-pretreated seedlings showed mainly normal cell ultrastructure except for some plasma membranes' injury and vesicles occurrence in mitochondria. This reveal that drought pretreatment plays an important role in the stabilisation of cell structure in cucumber leaves.

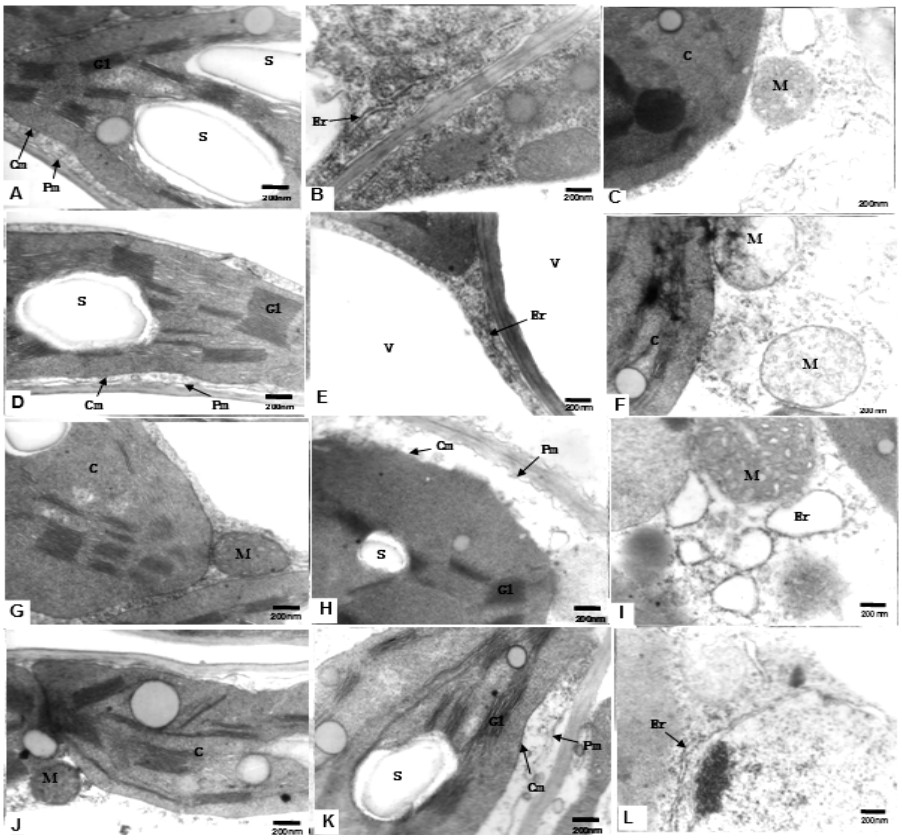

Figure 1. Cell ultrastructure of chilling stressed (day/night temperature 8 °C / 5 °C) cucumber seedlings with or without PEG pretreatment.
A, B, C: Untreated; D, E, F: Treated with 10% PEG 6000 for 2 days and then recovery under normal condition for 2 days; G, H, I: 7 days after chilling stress without PEG 6000 pretreatment; J, K, L: 7 days after chilling stress with 10% PEG 6000 pretreatment.
S: starch grain; Cm: chloroplast membrane; Pm: cell membrane; Er: endoplasmic reticulum; C: chloroplast; M: mitochondria; Gl: grana lamella; V: vacuole.

Electrolyte leakage, H_2O_2 and MDA content

Chilling stress led to a significant increase ($P<0.05$) in electrolyte leakage in the cucumber seedlings (Figure 2A). After 7 days of chilling treatment, the electrolyte leakage of the control seedlings increased by 59.5%, while that of the PEG-treated seedlings only increased by 24.2%.

Figure 2B shows that the H_2O_2 content increased gradually during chilling days, but the extent of the increase varied. Compared to the control, the increase in H_2O_2 content was remarkably less in the PEG-treated seedlings ($P<0.05$).

Chilling stress caused a significant increase in the MDA content (Figure 2C). Compared to the control, the PEG-treated seedlings showed a marked decrease in MDA content ($P<0.05$). These data suggest that chilling stress lead to lipid peroxidation, which can be alleviated by drought acclimation in cucumber seedlings.

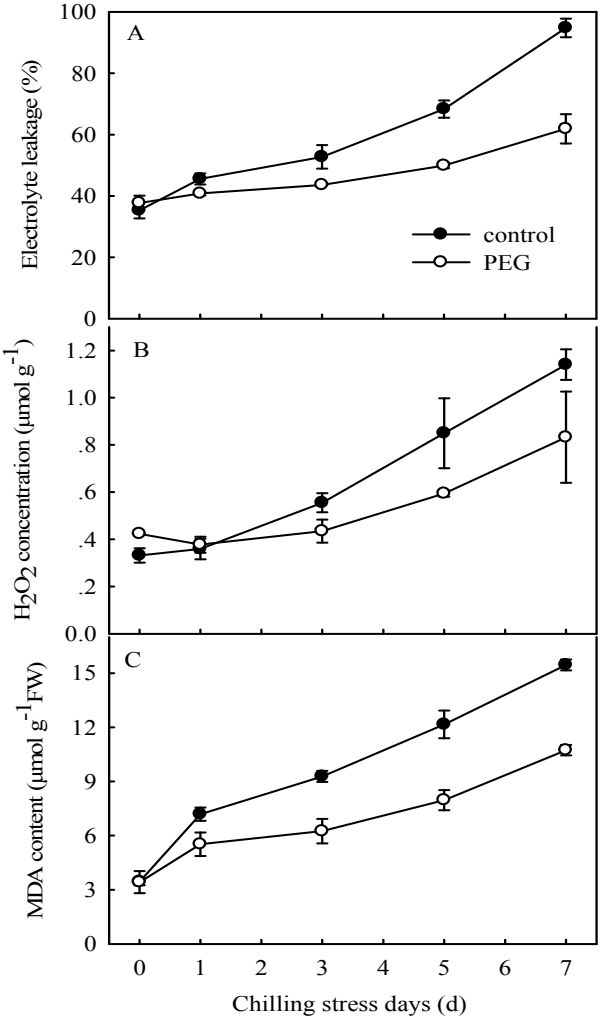

Figure 2. Electrolyte leakage (A), contents of H_2O_2 (B) and MDA (C) of chilling stressed (day/night temperature 8 °C/5 °C) cucumber seedlings with or without PEG pretreatment. Control: Exposed to low temperature (8 °C /5 °C) without PEG pretreatment; PEG: Pretreated with 10% PEG 6000 before low temperature stress. All values shown are mean±SD (n=3).

Activities of antioxidant enzymes

Figure 3A reveals that the SOD activity increased at the first day of chilling treatment but subsequently decreased. The PEG-treated seedlings showed significantly higher SOD activity than the control ($P<0.05$).

Chilling stress led to a rapid elevation in POD activity during the first 3 days in all the treatments (Figure 3B). Afterwards, the POD activity in PEG-treated seedlings continuously increased, whereas that in the control seedlings declined gradually. At the end of the chilling stress (7 d), the POD activity in PEG-treated seedlings increased by 59.5% compared to the control.

The CAT activity decreased gradually in the control seedlings during the chilling days (Figure 3C). However, the PEG-treated seedlings showed an increase in CAT activity at the first day of chilling stress and then decreased gradually during the subsequent chilling days. After 7 days of chilling stress, the CAT activity in the PEG-treated seedlings decreased by 39.7% and that of the control decreased by 49.7%.

The APX activity showed the same trend as the CAT activity during chilling days (Figure 3D). After 7 days of chilling stress, the APX activity of the PEG-treated seedlings was 35.9% higher than that of the control. This data indicate that PEG pretreatment increase the activities of antioxidant enzymes, and this may contribute to higher cold tolerance in the PEG-treated seedlings.

AsA and GSH contents

From figure 4A, we found that the AsA content in the cucumber leaves increased at the first day of chilling stress and showed no significant difference between the PEG treatment and the control. In the subsequent chilling days, the AsA contents decreased gradually in all the treatments, but the extent of reduction varied. Compared to the control, the decrease in AsA content was remarkably less in the PEG treatment ($P<0.05$).

The change tendency of the GSH contents in the two treatments was similar to that of the AsA contents during the chilling days (Figure 4B). At the end of the experiment, the GSH content of the PEG treatment increased by 20.4% compared to the control.

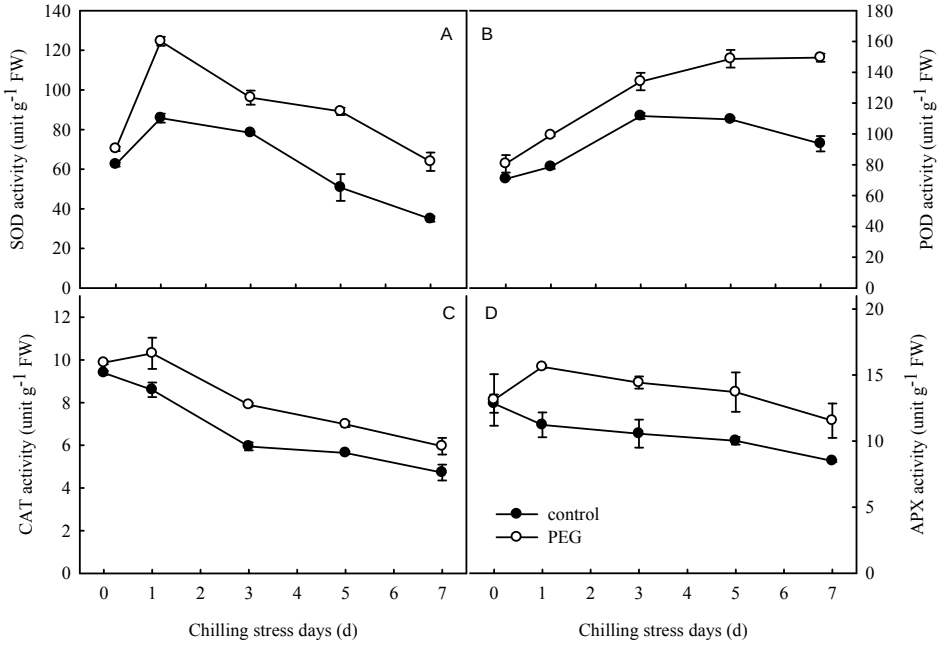

Figure 3. Activities of antioxidant enzymes in chilling stressed (day/night temperature 8 °C / 5 °C) cucumber seedlings with or without PEG pretreatment. Control: Exposed to low temperature (8 °C /5 °C) without PEG pretreatment; PEG: Pretreated with 10% PEG 6000 before low temperature stress. All values shown are mean±SD (n=3).

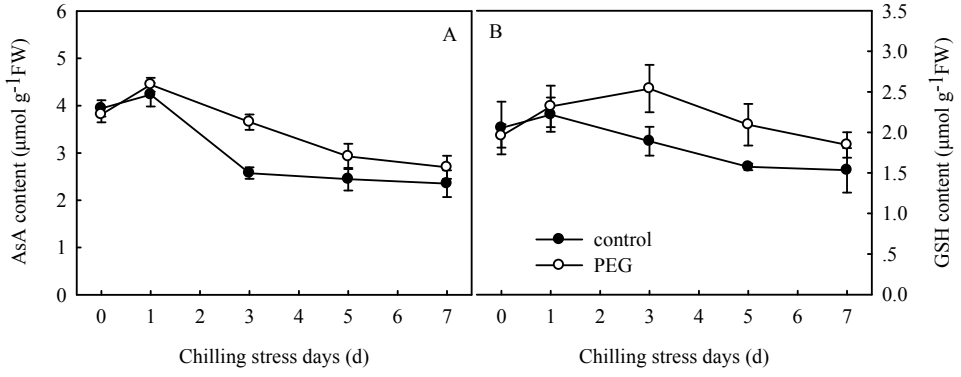

Figure 4. AsA and GSH contents in chilling stressed (day/night temperature 8 °C / 5 °C) cucumber seedlings with or without PEG pretreatment. Control: Exposed to low temperature (8 °C /5 °C) without PEG pretreatment; PEG: Pretreated with 10% PEG-6000 before low temperature stress. All values shown are mean±SD (n=3).

Discussion

We have documented that PEG pretreatment induces an adaptation to low temperature in cucumber seedlings by improving the osmoregulatory capability. Pretreated seedlings showed a higher relative water content, water potential and osmotic potential than non-treated seedlings (Dong et al., 2011). Low temperature stress first damages the cell membrane of plants and results in an increase in permeability and cell leakage. Lipid peroxidation and an increase in MDA content can also be caused by chilling stress (Liu et al., 2009). Therefore, electrolyte leakage and MDA content are commonly used to determine the degree of chilling injury to plant cell membranes (Ribas-Carbo et al., 2000; Aroca and Irigoyen, 2001). In our study, the two parameters indicated that chilling-stressed seedlings pretreated with PEG were less damaged. These seedlings showed lower electrolyte leakage and MDA content (Figure 1). This suggests that drought pretreatment is an effective method to protect cucumber plants against chilling stress.

H_2O_2 is hypothesized to act as a signal molecule initiating several protective resistance mechanisms against pathogens, chilling and heat stresses (Pastori and Foyer, 2002; Shi et al., 2006). Previous work has shown that exogenous application of H_2O_2 induced chilling tolerance in plants (Prasad et al., 1994; Gong et al., 2001). The induction of chilling tolerance in maize by heat-shock treatment is also hypothesized to be mediated by H_2O_2 (Gong et al., 2001). Aroca et al. (2003) found that H_2O_2 content increased under drought conditions in maize and suggested that H_2O_2 could act as an acclimating signal. However, H_2O_2 is also an important ROS and excessive accumulation of H_2O_2 will cause damage to cell membranes and structures. Our results showed that PEG pre-treatment decreased the H_2O_2 content during chilling stress, and suggests that drought pretreatment alleviated the ROS-mediated damage to cell membrane induced by chilling stress.

Antioxidant enzymes provide one of the most important ROS detoxification systems in plant cells. In order to avoid peroxidation damage, plants usually increase the antioxidant enxymes activity to remove generated ROS. Therefore, an induced increase in antioxidant enzyme activity is usually considered as an important mechanism in the cellular defence strategy against oxidative stress (Shi et al., 2006). As the first line of defence against ROS, SOD converts the superoxide radical (O_2^-) to H_2O_2. The H_2O_2 is then reduced to water by the POD, CAT, APX, etc., thus preventing further injury to the cell membrane (Kornyeyev et al., 2001). In our study, PEG treatment increased the activities of SOD, POD, CAT and

APX in cucumber seedlings under chilling conditions (Figure 3), providing evidence that higher activities of antioxidant enzymes is one of the important mechanisms of adaptation to low temperature induced by drought pretreatment in cucumber seedlings.

AsA and GSH are the key metabolites of the AsA-GSH cycle (Noctor and Foyer, 1998) and are generally considered to be one of the main ROS detoxification systems in plants (Xu et al., 2006). In the AsA-GSH cycle, the APX reduces H_2O_2 to water using AsA as the electron donor, the resulting dehydro-ascorbate is recycled to AsA using the reduced GSH as the electron donor and the oxidized glutathione (GSSG) that is formed is converted back to GSH by NAD (P) H-dependent GR (Foyer and Halliwell, 1976). It has been reported that the GSH content in the chilling acclimation of strawberries was higher than that found in non-acclimation plants (Zhang et al., 2008). High or low temperature stress led to a decrease in the AsA and GSH contents in grape leaves. However, pretreated plants at low or high temperature (cross treatment) showed higher contents of AsA and GSH than untreated plants (Wang et al., 2004). From our results, we found that the AsA and GSH contents maintained higher levels in PEG-pretreated seedlings than in the control seedlings (Figure 3). This suggested that the damage to cucumber cells by ROS was minimised or prevented by the increased metabolite content of the antioxidant systems.

In summary, PEG pretreatment induced chilling tolerance in cucumber seedlings, as shown by the decrease in stress-induced electrolyte leakage, decreased the contents of H_2O_2 and MDA, which were correlated with the diminution of oxidative stress.

Acknowledgments

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