UNIVERSITAT DE BARCELONA

FOTOSÍNTESIS, FOTOPROTECCIÓN, PRODUCTIVIDAD Y ESTRÉS ABIÓTICO: ALGUNOS CASOS DE ESTUDIO

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3. Oxidative stress and photodamage at low temperatures in soybean (*Glycine max* L. Merr.) leaves

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3.0. Abstract

Low temperatures severely limit photosynthesis and growth of chilling-sensitive species. The decrease in photosynthetic capacity may be partly due to chilling-associated oxidative damage to chloroplast components. Thus, we sought to determine the extent of lipid peroxidation and oxidative damage to thylakoid proteins in leaves of soybean (*Glycine max* L. Merr.) exposed to chilling stress under light. The ratio of variable to maximum fluorescence (F_v/F_m) decreased in plants exposed for 24 h to 7ºC and 500 μmol m^{-2} s^{-1} of photosynthetic photon flux density, but not in leaves chilled in darkness. The initial drop in F_v/F_m was exacerbated by treatment with the chloroplast protein synthesis inhibitor lincomycin, suggesting that concurrent repair ameliorated chilling-associated damage to photosystem II. The degree of oxidative damage to thylakoid proteins (i.e., carbonylation) and lipids (estimated as malondialdehyde content) did not change in response to chilling. Likewise, exogenous applications of the ascorbic acid precursor L-galactono-1,4-lactone or of the α-tocopherol analog 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) did not prevent the chilling-induced decrease of F_v/F_m, although they were effective in protecting thylakoids from oxidative damage caused by methyl viologen. Our data suggest that chilling stress does not cause rampant oxidative damage to thylakoid proteins and lipids, and that other cell compartments may be more susceptible than thylakoids to oxidative damage associated with low temperature stress.

3.1. Introduction

Low temperatures severely limit growth of plants of tropical or subtropical origin. The photosynthetic capacity declines in chilling-susceptible plants exposed to low temperatures, and this decline is related to a decrease in the quantum efficiency of photosystem II, and the activities of photosystem I, the *ATP* synthase and the stromal enzymes of the C_3 carbon reduction cycle (Sonoike et al. 1998; Allen and Ort 2001).

Some of the deteriorative effects of chilling may be due to increased production of reactive oxygen species (*ROS*) (Wise 1995). The formation of *ROS* in thylakoid membranes can be initiated through the univalent reduction of O_2 to form superoxide at the donor side of *PSI*, or through energy transfer from excited chlorophyll molecules to O_2 to yield singlet oxygen (Foyer et al. 1994). Production of *ROS* increases in response to stresses such as drought (Moran et al. 1994; Bartoli et al. 1999) and low temperature (Prassad et al. 1994a; Prassad 1996). *ROS* may cause a cascade of oxidative reactions of cellular components, and they have been implicated in photoinhibition (Hull et al. 1997) and cellular damage (Wise 1995) in chilling-susceptible plants exposed to low temperatures.

Photoprotective mechanisms, such as non-photochemical heat dissipation of energy (i.e., non-photochemical quenching), may contribute to the defence against photodamage associated with environmental stress. For example, non-photochemical quenching increases in chilling-susceptible plants exposed to low temperatures (e.g., Venema et al. 2000), but this may not be enough to prevent cellular damage. Several antioxidants remove *ROS* and, thereby, they prevent oxidative damage. Ascorbic acid and α-tocopherol are the main non-enzymatic antioxidants in the chloroplast and they work either by eliminating *ROS*, and, in the case of ascorbate, by also contributing to the regeneration of other antioxidants (Smirnoff et al. 2001). The contents of ascorbic acid and α-tocopherol increase at low temperatures in chilling-tolerant cereal leaves (Streb et al. 1999), whereas in chilling-sensitive maize ascorbate and α-tocopherol levels decrease at low temperatures (Leipner et al. 2000). Increases in glutathione content caused by pre-treatment with H_2O_2 improve chilling tolerance in mung bean (Yu et al. 2002). The activity a number of isoforms of *MnSOD*, ascorbate peroxidase and glutathione reductase increase in cucumber leaves at chilling temperatures (Lee et al. 2000). This suggests that reinforcing antioxidant defenses might improve the tolerance of the photosynthetic apparatus to chilling-induced damage. Indeed, overexpression of Cu/Zn-superoxide dismutase improved chilling

tolerance in chilling-sensitive tobacco (Sen Gupta 1993), and over-expression of chloroplast-targeted *MnSOD*, ascorbate peroxidase or glutathione reductase improves the recovery of photosynthesis after a short chilling treatment in cotton (Payton et al. 2001). Likewise, chilling tolerance increased in maize plants overproducing chloroplast-targeted *MnSOD* (Van Breusegem 1999), but not in tobacco plants overexpressing Fe-superoxide dismutase (Van Camp 1996).

If photoprotective and antioxidant mechanisms are overwhelmed, increased production of *ROS* lead to oxidative damage to lipids, proteins and other macromolecules (Foyer et al. 1994). Lipid peroxidation increases in plants under environmental stress, including drought (e.g., Moran et al. 1994) and low temperatures (Prassad 1996). Oxidative stress causes protein carbonylation, formation of disulfide and dityrosine bridges, and protein aggregation through hydrophobic interactions. Protein carbonylation increases in response to low temperatures (Prassad 1996) and water stress (Tambussi et al. 2000).

Determining the extent to which *ROS* and oxidative stress are involved in damage to the photosynthetic apparatus may help to understand the molecular basis of photosynthesis decrease in chilling-sensitive species. The aims of this work were to test whether chilling stress causes increased oxidative damage to thylakoid membranes of soybean (a chilling-sensitive species), and if oxidative stress is involved in the photodamage to thylakoid membranes during an episode of low temperature under light. To this end, we examined the changes in protein carbonylation and lipid peroxidation in thylakoids of chilled leaves, and we tested the involvement of oxidative damage by reinforcing water- and lipid-soluble non-enzymatic antioxidant defenses.

3.2. Material and methods

3.2.1. Plant material and growing conditions

Soybeans (*Glycine max* L. Merr.) cv. Clark were planted in pots with soil and grown at 25/15ºC day/night temperature, 500 μmol m^{-2} s^{-1} of photosynthetic photon flux density (*PPFD*) and a 10 h photoperiod. Three weeks-old plants were transferred to 7ºC for 24 h at 500 μmol m^{-2} s^{-1} *PPFD* (low temperature stress) or maintained at 25ºC and 500 μmol m^{-2} s^{-1} *PPFD* (non-chilled controls). After the chilling treatment, all plants were transferred to 25ºC and 80 μmol m^{-2} s^{-1} *PPFD* to allow leaves to recover from chilling/light stress (Kingston-Smith and Foyer 2000). All measurements were carried out on the first trifoliate

leaf. In some plants, the first trifoliate leaf was wrapped in aluminium foil to serve as a dark control.

3.2.2. Treatment with antioxidants

Antioxidants were sprayed on the first trifoliate leaf 5 h before the beginning of the chilling treatment. L-galactono-1,4-lactone (*Gal*, 50 mM) or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 0.5 mM, SIGMA-Aldrich, St. Louis, MO, USA) were dissolved in distilled water containing Tween 20 (0.05% v/v) as a surfactant. Control plants were sprayed with Tween 20 (0.05% v/v). The contents of oxidized and reduced ascorbate were measured as in Tambussi et al. (2000).

3.2.3. Inhibition of chloroplast protein synthesis

The synthesis of chloroplast-encoded proteins in detached first trifoliate leaves was inhibited by feeding lincomycin (0.1% w/v) through the cut end of the petiole starting 5 hours before the cold treatment. Pre-incubation with lincomycin before the chilling treatment was done under dim light (<10 μmol m^{-2} s^{-1} *PPFD*). Controls were immersed in distilled water. Care was taken to excise the leaves under water to prevent xylem embolism. Detached leaves were subjected to chilling and recovery treatments as described above for intact plants.

3.2.4. Induction of oxidative damage by methyl viologen, and protection by *Gal* and Trolox

In some experiments, oxidative stress was generated by spraying first trifoliate leaves with methyl viologen (5 μM). Leaves were subsequently incubated for 24 h at 200 μmol m^{-2} s^{-1} PPFD and 25ºC. Previous trials showed that, at this concentration, methyl viologen increased oxidative damage to proteins without causing visual symptoms of leaf necrosis or yellowing. A set of leaves was treated with *Gal* or Trolox as indicated above before application of methyl viologen.

3.2.5. Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured with a modulated fluorometer (Mini PAM Photosynthesis Yield Analyzer, Walz, Effeltrich, Germany or Fluorescence Monitoring System 2, Hansatech, Norfolk, UK). The initial (F_0) and maximum (F_m) fluorescence

emissions were measured after 30 minutes of dark adaptation, and the ratio of variable to maximum fluorescence (F_v/F_m) was calculated as (F_m - F_0)/ F_m. For the determination of non-photochemical quenching (Bolhár- Nordenkampf and Öquist 1993), F_m' (maximum fluorescence level under actinic light) was measured under the *PPFD* conditions of each treatment, and F_0' was determined in the same leaves after irradiation with far red light (6 μmol m^{-2} s^{-1}). *qNP* was calculated as (F_m - F_m') / (F_m - F_0') (Bolhár- Nordenkampf and Öquist 1993)

3.2.6. Determination of protein carbonylation and MDA content in thylakoids

Thylakoid isolation and carbonyl derivatization were done as in (Tambussi et al. 2000). Derivatized proteins were electrophoresed in 12% acrylamide mini-gels and electrotransferred to nitrocellulose membranes for carbonyl detection with a rabbit anti-DNP antibody (SIGMA, St. Louis, MO, USA) and a chemiluminescence detection kit (Rennaissance, DuPont, Boston, MA, USA). Developed films were scanned and band intensity was quantified with SIGMA Gel (SIGMA, St. Louis, MO, USA). To avoid the interferences normally experienced with colorimetric determination of *MDA* as thiobarbituric acid reactive substances, *MDA* was measured with an improved HPLC method as described in (Templar et al. 1999).

3.2.7. Chlorophyll content

Leaf disks and thylakoids were extracted with N,N dimethyl formamide, and chlorophyll content was calculated as in (Inskeep and Bloom 1985).

3.3. Results

3.3.1. Chilling stress and photodamage

To assess the extent of photodamage, we measured the ratio of variable to maximum fluorescence (F_v/F_m) of dark-adapted leaves, which is an estimate of potential quantum yield of *PSII*. F_v/F_m declined markedly in leaves subjected to chilling temperatures, in contrast to plants kept at normal growth temperature where F_v/F_m did not change much over the same time period (Fig. 1A). Leaves kept at chilling temperatures in darkness for 24 h did not show any decrease in F_v/F_m (Fig. 1A). The decrease in F_v/F_m was more pronounced during the first 12 h at low temperature; thereafter, chilling temperatures caused only a modest additional decline. Chilled leaves recovered completely after 24 h at 25ºC and low irradiance, with F_v/F_m returning to the initial values before the start of the chilling treatment. Leaf chlorophyll content did not change during the chilling treatment, but it decreased by 25% ($p<0.05$) during the ensuing recovery period (data not shown). Non-photochemical quenching (q_N) increased nearly 80% in leaves exposed to low temperatures (Fig. 1B). After the chilling episode, q_N decreased again to a level similar ($p < 0.05$) to that in control plants.

The initial decline of F_v/F_m during the first 6 h at chilling temperatures was exacerbated in leaves incubated with the chloroplast protein synthesis inhibitor lincomycin (Figs. 2A and B). The early effects of lincomycin aggravating the decline of F_v/F_m in chilled leaves (but not at 25ºC) indicates that concurrent repair of *PSII* mitigated against the loss of potential quantum yield, suggesting that chilling-associated decline in F_v/F_m was mostly due to damage to PSII. Beyond 6 h, lincomycin had nearly identical effects on leaves at 7 and 25ºC, which probably reflects the accumulation of photodamaged *PSII* centers in leaves where *PSII* repair is blocked (Guiamet et al. 2002).

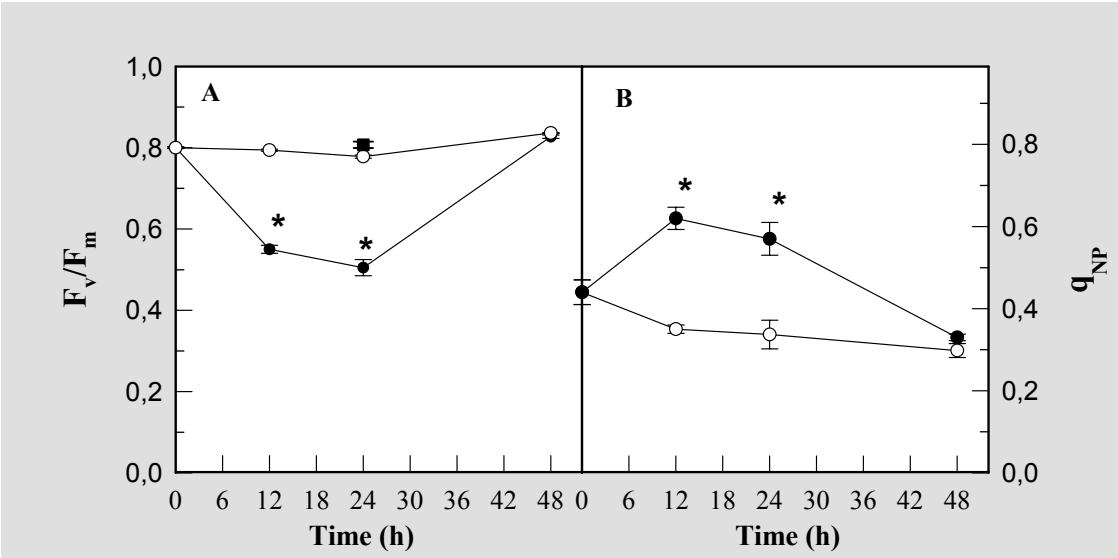

Figure 1. *The ratio of variable to maximum fluorescence of chlorophyll in dark adapted leaves (panel A) and non-photochemical quenching (panel B) of soybean leaves subjected to chilling under light (7ºC and 500 µmol m^{-2} s^{-1} PPFD, closed circles) or maintained under normal growth conditions (25ºC and 500 µmol m^{-2} s^{-1} PPFD, open circles) for 24 h. Thereafter, all plants were transferred to 25ºC and 80 µmol m^{-2} s^{-1} PPFD for another 24 h to allow recovery of chilled plants. The solid square at 24 h in panel A represents controls kept at chilling temperatures (7ºC) in darkness. Each value represents the mean ± SE of 5 replicates. Significant differences ($p \leq 0.05$) between treatments according to the LSD test are shown by an asterisk.*

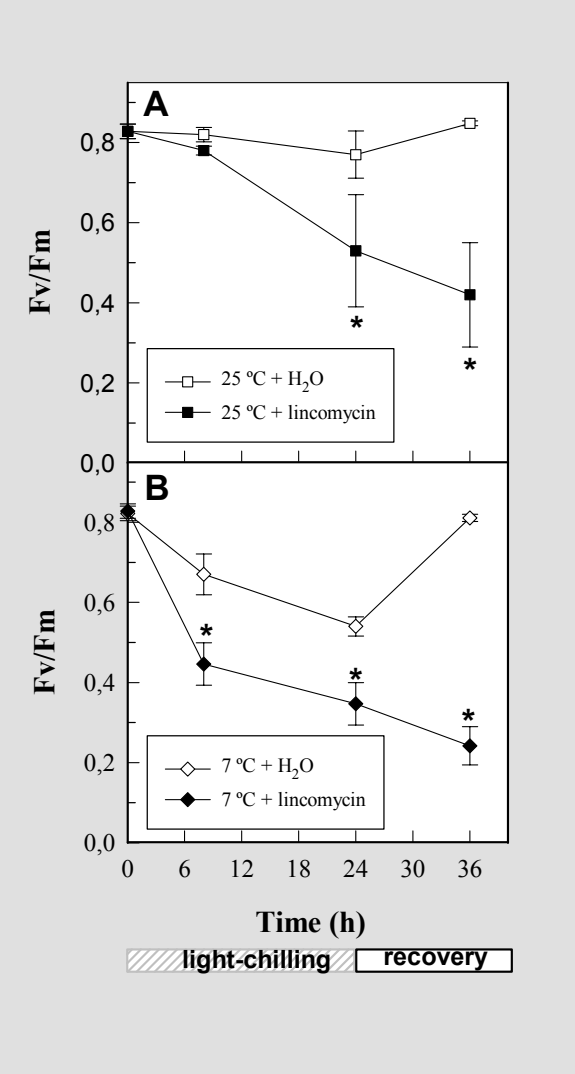

Figure 2. *Changes in the ratio of variable to maximum fluorescence (F_v/F_m) in leaves maintained at 25°C (control, panel A) or subjected to chilling (panel B) as described in Fig. 1. Leaves were supplied with either lincomycin (0.1% w/v, closed symbols) or water (open symbols) during all the experimental sequence. Each value represents the mean ± SE of 5 replicates. Significant differences ($p \leq 0.05$) between treatments according to the LSD test are shown by asterisks.*

3.3.2. Oxidation of thylakoid proteins and lipids

Since photodamage at low temperatures may be due to oxidative stress, we attempted to assess the extent of oxidative damage to the photosynthetic apparatus by determining the changes in protein carbonylation and lipid peroxidation in thylakoids of control and chilled leaves. Even in non-stressed (control) leaves maintained at 25ºC there was a basal level of oxidatively modified thylakoid proteins; for example, there were bands with detectable carbonylation signals at about 55, 46, 32 and 24 kDa (Fig. 3). Low temperatures under light did not cause any consistent and repeatable increase in overall carbonylation of thylakoid proteins. Besides, we could not detect consistent changes in carbonylation in any of the protein bands resolved with this system. Likewise, the level of oxidized proteins did not change much during the recovery period at normal growth temperature (Fig. 3).

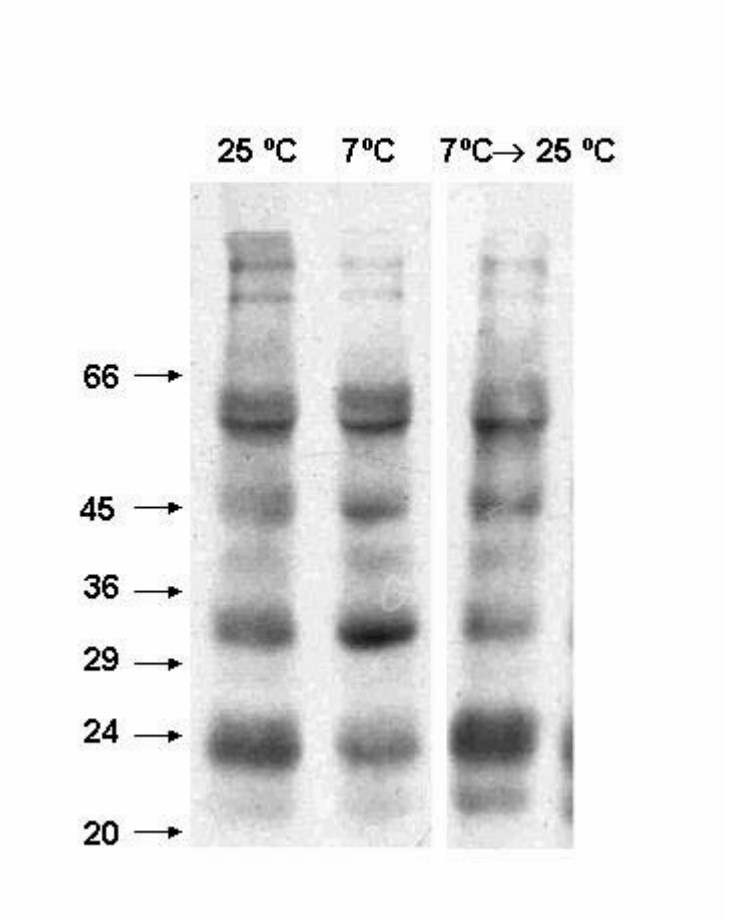

Figure 3. *Western blot of thylakoid proteins isolated from soybean leaves maintained at 25ºC and 500 μmol m^{-2} s^{-1} PPFD (controls, lane labelled 25°C), chilled for 24h at 7ºC and 500 μmol m^{-2} s^{-1} PPFD (labelled 7°C) or chilled for 24 h and then allowed to recover for 24 h at 25° C and 80 μmol m^{-2} s^{-1} PPFD (labelled 7°C → 25°C). Each well was loaded with solubilized thylakoids containing 2 μg of chlorophyll. Blots were incubated with an anti-DNP antibody to detect carbonylated proteins and developed with a chemiluminescent detection system. The position of molecular mass standards is indicated with arrows on the left margin of the blot.*

Because a substantial increase in protein oxidation was expected in cold-stressed plants, or after recovery, we checked the sensitivity of the carbonyl detection method

employed by subjecting another batch of leaves to oxidative stress generated with methyl viologen. Leaves sprayed with 5 μM methyl viologen at moderate irradiance (200 $\mu mol.m^{-2}.s^{-1}$) showed a strong increase in the overall level of carbonylated proteins (Fig. 4, Table 1), confirming the sensitivity of the immunoblot assay employed here.

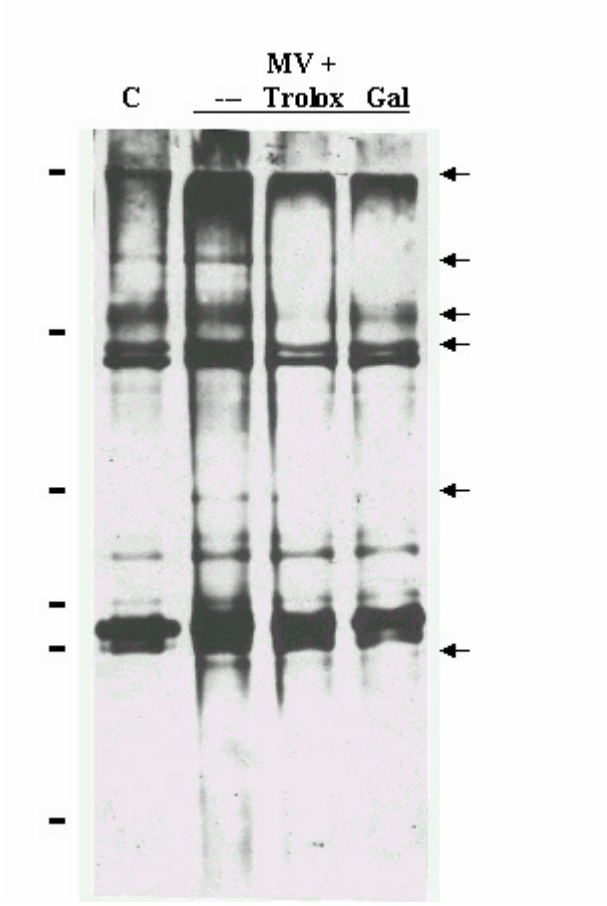

Figure 4. *Western blot of thylakoid proteins isolated from soybean leaves sprayed with methyl viologen (5 μM, MV) and subsequently incubated for 24 h at 200 μmol m^{-2} s^{-1} PPFD and 25ºC. C: control leaves without MV. Some leaves were sprayed with 0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) or 50 mM galactono-1,4-lactone (Gal) 5 h before MV treatment. Each well was loaded with solubilized thylakoids containing 2 μg of chlorophyll. Blots were incubated with an anti-DNP antibody to detect carbonylated proteins and developed with a chemiluminescent detection system. The position of molecular mass standards (66, 45, 36, 29, 24 and 20 kDa, from top to bottom) is indicated with dots on the left margin of the blot. Arrows on the right margin show some of the protein bands whose carbonylation signal decreased by addition of Trolox and Gal in MV-treated leaves.*

Table 1 – *Levels of carbonylated proteins in thylakoids of soybean leaves treated with water (controls) or methyl viologen (5μM), or sprayed with 50 mM galactono-1,4-lactone (Gal) or 0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) 5 h before application of methyl viologen.. Carbonylated proteins were analyzed by western blotting and the carbonylation signals of each band were added up to calculate overall thylakoid protein oxidation. Figures between parenthesis show the standard error of the mean (n = 4).*

	Levels of carbonylated thylakoid proteins (*rel. units μg chlorophyll*$^{-1}$)
Control	1.90 (0.20)
Methyl viologen	3.39 (0.53)
Methyl viologen + *Gal*	1.86 (0.51)
Methyl viologen + Trolox	1.58 (0.64)

Changes in the concentration of malondialdehyde were measured to estimate the extent of lipid peroxidation in thylakoid membranes of chilled leaves. *MDA* levels in leaves chilled for 24 h were similar to those in control leaves at 25ºC (Table 2), and recovery from low temperature stress at 25ºC and low irradiance was not accompanied by a decrease in *MDA* levels. Overall, this indicates that chilling did not cause a significant increase of lipid peroxidation of thylakoid membranes.

3.3.3. Effects of exogenously applied antioxidants

To further test the possible role of chilling-associated oxidative stress in damage to thylakoid membranes, we ascertained whether reinforcing antioxidant defences with the ascorbic acid precursor L-Galactono-1,4-lactone (*Gal*) or the α-tocopherol analog 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) might protect thylakoid function in chilled leaves. Spraying leaves with *Gal* 5 h before the start of the chilling treatment increased the ascorbic acid content of the leaves 3-fold, and ascorbate levels remained significantly higher in leaves sprayed with *Gal* even after 24 h of chilling (Table 3).

Significantly, treatment with *Gal* protected thylakoid membranes from methyl viologen induced oxidative damage, i.e., *Gal* prevented the increase in protein carbonylation brought about by application of methyl viologen (Table 1; Fig. 4). Thus, treatment with *Gal* was effective to increase the endogenous pool of ascorbate and mitigate against oxidative damage in thylakoids.

Table 2 – *The contents of malondialdehyde (MDA) in thylakoid membranes isolated from soybean leaves exposed to chilling temperatures under light for 24 h and then allowed to recover at 25ºC and low irradiance. Controls were kept at 25ºC for the duration of the experiment. Thylakoids were isolated from leaves held at 7 or 25ºC for 24 h, and after 24h of recovery at 25ºC following the chilling treatment. Figures between parenthesis show the standard error of the mean (n = 4).*

	Thylakoid MDA content, *pmol mg^{-1} chlorophyll*	
	Controls at 25ºC	Leaves chilled at 7ºC
24 h	35.4 (4.4)	27.7 (3.8)
48 h	37.3 (4.1)	
24 h at 7ºC followed by recovery at 25ºC for 24 h		40.0 (3.3)

However, under our experimental conditions *Gal* did not protect photosynthetic membranes from chilling-associated loss of *PSII* function. The decline in F_v/F_m was virtually identical in chilled leaves pre-treated with *Gal* or distilled water (Fig. 5). Similar results were obtained with Trolox, a water soluble analog of α-tocopherol. Trolox significantly reduced the
extent of protein oxidation caused by exogenous application of methyl viologen in the light (Table 1; Fig. 4). However, pre-treatment with Trolox before transferring the plants to low temperatures did not afford any significant ($p < 0.05$) protection of *PSII* function (Fig. 5).

Table 3 – The contents of reduced and oxidized ascorbic acid in soybean leaves sprayed with L-galactone-1,4-lactone (Gal) or distilled water (controls) 5 h before the chilling treatment. Leaves were assayed for ascorbate content 5 h after spraying (i.e., immediately before transfer to chilling temperature), after 24 h at 7 or 25ºC, and after 24h recovery at 25ºC following the chilling treatment. Figures between parenthesis show the standard error of the mean (n = 5).

	Ascorbic acid content μmol g^{-1} dry weight			
	Control		+ *Gal*	
	Reduced	Oxidized	Reduced	Oxidized
5 h after spraying	5.9 (1.0)	0.3 (0.1)	15.7 (0.7)	0.6 (0.1)
After 24 h at				
25ºC	5.0 (0.1)	0.25 (0.1)	20.1 (1.0)	0.2 (0.1)
7ºC	6.0 (0.4)	0.5 (0.2)	18.2 (0.5)	0.2 (0.1)
After 24 h at 7ºC and				
24 h recovery at 25ºC	4.1 (0.5)	0.3 (0.1)	9.8 (2.8)	0.5 (0.2)

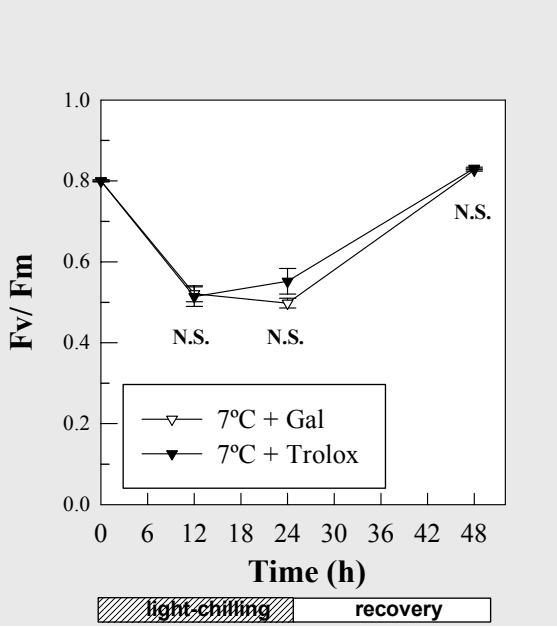

Figure 5. *Changes in chlorophyll fluorescence (F_v/F_m) of soybean leaves subjected to chilling under light (7ºC and 500 µmol m^{-2} s^{-1} PPFD) for 24 h, and then transferred to 25ºC and 80 µmol m^{-2} s^{-1} PPFD for another 24 h to allow recovery from chilling stress. In these experiments, leaves were treated with 50 mM L-galactone-1,4-lactone (Gal, open triangles) or 0.5 mM Trolox (0.5 mM, closed triangles) 5 h before the start of the low temperature treatment. The controls for these measurements (i.e., chilled leaves sprayed with distilled water) are the chilled leaves shown in Fig.1. Each value represents the mean ± SE of 5 replicates. N.S. indicates non-significant differences from chilled controls (shown in Fig. 1).*

3.4. Discussion

3.4.1. Photodamage at chilling temperatures

Soybean leaves subjected to low temperatures experience photodamage, which is manifested as a light-dependent decrease in the potential quantum efficiency of *PSII* photochemistry (i.e., F_v/F_m). Since F_v/F_m was measured after 30 minutes of dark adaptation, we minimized the contribution of energy-dependent non-photochemical quenching to F_v/F_m measurements, which suggests that the decline in F_v/F_m represents the accumulation of photodamaged *PSII* centers (Rosenqvist and van Kooten 2003). Thus, the drop in F_v/F_m in chilled leaves was due to a large extent to photodamage, rather than to reversible, photoprotective down-regulation of *PSII*.

3.4.2. Oxidative damage to thylakoids

Lipids and proteins are common targets for oxidative damage in tissues under environmental stress (e.g., Prassad 1996). Carbonyl content is a sensitive indicator of oxidative damage to proteins (Levine et al. 1994) and levels of carbonylated proteins increase in plants undergoing oxidative stress associated with drought (Boo and Jung 1999) and low temperatures (Prassad 1996, Kingston-Smith and Foyer 2000). As far as we know, this is the first work where the extent of oxidative damage associated with chilling stress has been directly determined in a specific cell compartment, i.e., the chloroplast membranes. The carbonylation level of thylakoid proteins did not increase in soybean leaves exposed to light-chilling stress. In addition to increased carbonyl content, the formation of high molecular mass aggregates, either by non-covalent crosslinking or through oxidative formation of disulphide or dityrosine bridges, is another hallmark of oxidative damage to proteins (Levine et al. 1994; Berlett and Stadtman 1997). For example, very high molecular mass aggregates containing the *D1* protein of *PSII* form in vitro after photooxidative treatment of thylakoids, presumably through the action of singlet oxygen (Roberts et al. 1991). However, in addition to the lack of increase in the overall levels of carbonylated proteins, we did not see any consistent increase in the amount of carbonylated bands of very high molecular mass in response to chilling. Failure to detect increased protein carbonylation in chilled leaves under light might suggest that thylakoid proteins are not suitable substrates for oxidation by *ROS*, but this can be ruled out because treatment with very low concentrations of methyl viologen led to increased oxidation of thylakoid proteins (Fig. 4, Table 1). Moreover, with the same immunoblot technique

employed here we detected a substantial increase in thylakoid protein carbonylation in leaves of wheat under drought (Tambussi et al. 2000).

Malondialdehyde is a common product of lipid peroxidation and a sensitive diagnostic index of oxidative injury (Janero et al. 1990). Thylakoid contents of *MDA* were virtually identical in chilled and control leaves, and thylakoid *MDA* levels did not decrease during recovery from chilling stress at 25 ºC and low irradiance (Table 2). Likewise, *MDA* content did not increase in leaves of chilling-sensitive cucumber and *Nerium oleander* kept at 5ºC for 5 h (Hodgson and Raison 1991). Overall, the lack of detectable increases in protein carbonylation and *MDA* content indicate that thylakoids did not experience substantial oxidative damage in chilled leaves. While thylakoid proteins were not subjected to increased oxidative damage in soybean leaves exposed to low temperatures in light, bulk protein oxidation increases at low temperatures in etiolated seedlings of maize (e.g., Prassad 1996). Chilling-associated damage in maize was related to a decrease in the activity of the cytochrome c-dependent respiration pathway (Prassad et al. 1994b). Interestingly, chilling-tolerance (i.e., retention of photosynthetic capacity) in a genotype of maize correlates with higher activity of ascorbate peroxidase and mitochondrial *MnSOD*, but the activity of chloroplastic *SODs* was similar in the tolerant and susceptible genotypes (Massacci et al. 1995; Iannelli et al. 1999). This suggests that cellular compartments other than the chloroplast (e.g., mitochondria) might be the primary targets for oxidative damage at low temperatures.

3.4.3. Antioxidants and photodamage

Exogenous applications of *Gal* and Trolox increased the antioxidant capacity of the leaves, and both were effective in protecting thylakoids against methyl viologen-induced oxidative damage. *Gal* is converted into ascorbic acid in vivo (Smirnoff et al. 2001) and although ascorbate is a water soluble antioxidant, ascorbate regenerates lipid-soluble α-tocopherol and quenches water soluble *ROS* before they start a lipid peroxidation chain (Buettner 1993). Trolox is a water soluble analog of α-tocopherol that partitions readily into lipid bilayers (Barclay et al. 1995) and protects membrane proteins from oxidative damage (Britt et al. 1992). Therefore, if oxidative stress associated with chilling were involved in photodamage, treatment with *Gal* or Trolox should have mitigated against these deleterious effects. Failure of *Gal* and Trolox to prevent or reduce the decline in F_v/F_m suggests that rampant production of *ROS* is not involved in photodamage at low

temperatures, which is consistent with the observation that thylakoid protein carbonylation and *MDA* levels did not increase in leaves chilled under light. Likewise, photoinhibition of maize leaves at low temperatures was not affected by application of *Gal*, in spite of a substantial increase in ascorbic acid content (Leipner et al. 2000). In general, there is no conclusive evidence showing that light-dependent damage of *PSII* competence is due to *ROS* (Tyystjärvi 2004). For example, overexpression of chloroplast targeted *MnSOD* decreases chilling-asociated plasma membrane damage in maize, but has no effect on the decrease of F_v/F_m (Van Breusegem et al. 1999). Our observations show that chilling temperatures do not cause rampant oxidative damage to thylakoids, and that oxidative damage may not play a primary role in the decrease of photochemical competence in leaves of a sensitive species subjected to chilling under light. However, oxidative damage to other cell compartments might play a major role in cell damage at low temperatures.

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3.5. References

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