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# Lipid peroxidation and superoxide dismutase activity in relation to photoinhibition induced by chilling in moderate light

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**Abstract.** The effect of a chilling stress, at a moderate photon flux density for a few hours, on the peroxidation of membrane lipids and on superoxide dismutase (SOD) activity was compared in leaf slices of chilling-sensitive and chilling-insensitive plants. The aim was to determine if susceptibility to chill-temperature photoinhibition could be related to either damage to membrane lipids by superoxide and-or a decrease in activity of chloroplast SOD. Plants used were *Nerium oleander* L., grown at 45° C, and *Cucumis sativus* L., both susceptible to chill-temperature photoinhibition, and *N. oleander*, grown at 20° C and *Spinacia oleracea* L., both insensitive to chill-temperature photoinhibition. Lipid peroxidation was assessed by measuring the concentration of malondialdehyde (MDA). Leaf slices from all plants showed a basal level of MDA which decreased by about 15% when the leaf slices were chilled in the light. The level of MDA was not increased by the addition of either $KHCO_3$ or methyl viologen during chilling but it was increased, up to threefold, by the addition of Rose Bengal, which produces singlet oxygen. Chloroplast SOD activity was assessed in leaf extracts as the cyanide-sensitive production of $H_2O_2$ in a system which produced superoxide. Activity of SOD was similar in all the plants and was altered little by chilling. The results show that for the plants tested, chilling at a moderate photon flux density for 5 h does not increase the susceptibility of cell membranes to peroxidative damage nor does it decrease the activity of SOD. It was concluded that the susceptibility of chilling-sensitive plants to chill-temperature photoinhibition cannot be explained on the basis of differences in the vulnerability of membrane lipids to damage by superoxide or differences in SOD activity.

**Key words:** Chilling – *Cucumis* – Lipid peroxidation – *Nerium* – Photoinhibition, chill-temperature – Superoxide dismutase – *Spinacia* – Temperature (chilling)

## Introduction

The photosynthetic activity of chilling-sensitive plants, but not chilling-insensitive plants, is severely inhibited when the leaves are exposed to chilling temperatures for a few hours at a moderate photon flux density (PFD), i.e. a PFD of less than about 550 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Garber 1977; Hodgson et al. 1987, 1989; Peeler and Naylor 1988; Ichiro et al. 1989). This inhibition is dependent on oxygen and has been termed chill-temperature photoinhibition (Hodgson and Raison 1989). Since superoxide ($O_2^-$) production by chloroplasts increases in chilling-sensitive plants during the chilling treatment, it has been suggested that damage to chloroplasts, mediated by $O_2^-$, promotes the chill-temperature photoinhibition (Hodgson and Raison 1991).

Superoxide is produced on the surface and-or within thylakoid membranes during illumination (for a review, see Elstner 1982; Asada et al. 1983). Therefore, since the membrane lipids of thylakoids contain a high proportion of polyunsaturated acyl chains they would be particularly vulnerable to peroxidation so that damage to thylakoid structure might be the mechanism potentiating chill-temperature photoinhibition. If this is so there should be a marked difference in the susceptibility of chilling-sensitive and chilling-insensitive plants to peroxidation since chilling, at a moderate PFD, does not initiate photoinhibition in the latter plant group. However, the damaging effects of $O_2^-$ can be modulated if the radical is scavenged by reacting with superoxide dismutase (SOD) (Takahama and Nishimura 1976; Clare et al. 1984; Furusawa et al. 1984; Rabinowitch and Fridovich 1985; Fridovich 1986; Robinson 1988). Thus,

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*Abbreviations:* Chl = chlorophyll; MDA = malondialdehyde; MV = methyl viologen; $O_2^-$ = superoxide; 20°-oleander = *Nerium oleander* grown at 20° C; 45°-oleander = *N. oleander* grown at 45° C; PFD = photon flux density; SOD = superoxide dismutase

in investigations aimed at studying the relationship between photoinhibition, induced by chilling at a moderate PFD and $O_2^-$-mediated peroxidation of membrane lipids, it is essential to know if the conditions imposed altered SOD activity.

In this paper we examine the extent of lipid peroxidation and the level of SOD in plants which show differing sensitivity to chill-temperature photoinhibition.

## Materials and methods

*Sources of chemicals.* Aminotriazole, 3-amino-1,2,4-triazole (Aldrich, Milwaukee, Wis., USA); bovine serum albumin (Boehringer Mannheim, North Ryde, Australia); catalase, beef liver (Boehringer Mannheim); diethylene triaminepentaacetic acid, N,N-bis-[2-bis([carboxymethyl]amino)ethyl]glycine (Sigma, St. Louis, Mo., U.S.A.); methyl viologen (MV), 1,1′-dimethyl-4,4′-bipyridinium dichloride (Sigma); nitro blue tetrazolium, 2,2′-di-*p*-nitro-phenyl-5,5′-diphenyl-3,3′-[3,3′-dimethyl-oxy-4,4′-diphenylene] ditetrazolium chloride (Sigma); potassium cyanide (Sigma); riboflavin (Aldrich); Rose Bengal, 4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein (Sigma); sodium dodecylsulphate (Sigma); tetramethylethylenediamine, 1,2-bis[dimethylamino]ethane (Sigma); thiobarbituric acid, 4,6-dihydroxy-2-mercaptopyrimidine (Aldrich); Tris, 3-amino-2-(hydroxymethyl)-1,3-propanediol (Sigma). All other chemicals were analytical grade obtained from local suppliers.

*Plant material.* The conditions for growing spinach (*Spinacia oleracea* L.), cucumber (*Cucumis sativus* L.) and oleander (*Nerium oleander* L.) plants and the method of preparation of leaf slices were as described (Hodgson et al. 1987). Oleander plants acclimated to growth at 20° C or 45° C are referred to as 20°-oleander and 45°-oleander, respectively. Treatment of leaf slices consisted of incubating in a buffer containing 75 mM $KHCO_3$, pH 7.5, at either 4° or 25° C at a PFD of 450 μmol · $m^{-2}$ · $s^{-1}$ for 5 h (Hodgson et al. 1987). Rose bengal (1 mM) and MV (2 mM) were added to the buffer solution where indicated.

*Determination of peroxidation.* About 0.5 g FW of leaf slices was removed from the incubation mixture, blotted dry, weighed, and quickly ground in a pre-cooled mortar and pestle with 3 ml of $N_2$-saturated, 10 mM sodium-phosphate buffer, pH 7.4. The slurry was made to 10 ml with the same buffer and mixed thoroughly. Samples of the slurry were used to determine the chlorophyll (Chl) content as described (Hodgson and Raison 1989), and lipid peroxidation by measuring malondialdehyde (MDA) concentration according to Ohkawa et al. (1979). A 400-μl sample of tissue slurry was mixed with 200 μl of 8.1% (w/v) sodium dodecyl sulphate, 1.5 ml of 20% (v/v) acetic acid adjusted to pH 3.5 with NaOH, 1.5 ml of 0.8% (w/v) aqueous thiobarbituric acid and 400 μl of $H_2O$. The mixture was heated at 98° C for 60 min, cooled to room temperature, and clarified by centrifuging at 1000 · *g* for 10 min. The MDA concentration in the supernatant was determined from the absorption at 535 minus 600 nm using a molar extinction coefficient of $1.56 \cdot 10^{-5}$ $M^{-1} \cdot cm^{-1}$.

*Determination of SOD.* A sample of the slurry, obtained as described above and equivalent to about 1 g FW, was centrifuged at 10000 · *g* for 30 min at 4° C. The SOD activity in the supernatant was determined at 25° C as the rate of oxygen uptake (Marshall and Worsfold 1978) in a reaction mixture which produced $O_2^-$ from riboflavin and tetramethylethylenediamine. Oxygen uptake was measured polarographically using a Clarke-type oxygen electrode (Rank Bros., Cambridge, UK), in a reaction mixture which contained 66 mM Tris, pH 8.9, 9 mM tetramethylethylenediamine, 110 mM diethylene triaminepentaacetic acid and 33 μg · $ml^{-1}$ of bovine serum albumin as well as freshly prepared 100 μM nitro blue tetrazolium, 20 μM riboflavin and 5 mM aminotriazole. Catalase was inhibited by aminotriazole (Allen and Whatley 1978). For each assay the buffer mixture was added to the oxygen electrode and, after equilibration with air in the dark, 80 μl of tissue supernatant was added and the vessel sealed. After establishing a stable trace in darkness the sample was illuminated at a PFD of 1000 μmol · $m^{-2}$ · $s^{-1}$ white light from a 150-W tungsten lamp. The steady-state rate of oxygen uptake was recorded before KCN (final concentration 0.5 mM) was added to obtain a trace for the rate of cyanide-insensitive activity. The cyanide-sensitive activity was obtained by difference.

## Results

The MDA concentration in tissue was initially determined by a general procedure for plant tissue (Heath and Packer 1968) and compared with a procedure devised for animal tissue (Ohkawa et al. 1979). Both procedures gave identical trends in matched-sample comparisons (data not shown). However, the procedure of Ohkawa et al. (1979) was more sensitive and thus was adopted in subsequent experiments.

The concentration of MDA in extracts of leaf slices is shown in Table 1. Leaf tissue of both cucumber and spinach contain a relatively high concentration of material which reacts with thiobarbituric acid and adsorbs at 535 nm. Ascorbate and simple sugars are known to react with thiobarbituric acid (Halliwell and Gutteridge 1985) so the concentration determined by this method cannot be considered an accurate estimate of the absolute amount of MDA. The apparent reduction in the concentration of MDA when the slices were incubated at 4° C for 5 h at a moderate PFD most likely reflects a reduction in the level of endogenous components which react with thiobarbituric acid rather than a loss of MDA. When the leaf slices were incubated in the presence of

**Table 1.** An estimate of membrane lipid peroxidation by measuring malondialdehyde (MDA) concentration in leaf slices. The values for MDA (μmol · (g FW)$^{-1}$) are means ± SD, n > 8

| Plant | Chilling duration (h) | Chilled in the light[a] | | | Chilled in the light then dark incubated[b] |
|---|---|---|---|---|---|
| | | No additives | + Rose Bengal (1 mM) | + MV (2 mM) | |
| Spinach | 0 | 38 ± 3 | 46 ± 1 | 35 ± 1 | 38 ± 3 |
| | 5 | 35 ± 1 | 110 ± 2 | 39 ± 1 | 32 ± 1 |
| Cucumber | 0 | 49 ± 1 | 53 ± 1 | 40 ± 2 | 40 ± 1 |
| | 5 | 35 ± 1 | 124 ± 1 | 19 ± 1 | 88 ± 1 |

[a] Incubated at 4° C at 450 μmol photon · $m^{-2}$ · $s^{-1}$

[b] Incubated at 4° C at 450 μmol photon · $m^{-2}$ · $s^{-1}$ then at 25° C in darkness for 12 h

**Table 2.** A comparison of the effect of chilling on the peroxidation of lipids in leaf slices of chilling-sensitive and chilling-insensitive plants. Leaf slices were incubated for 5 h at a PFD of 450 μmol · $m^{-2}$ · $s^{-1}$ at the temperatures indicated. The values for MDA (μmol · (g FW)$^{-1}$) are means ± SD, n > 8

| Incubation temperature | MDA formation | | | |
|---|---|---|---|---|
| | Spinach | Cucumber | 20°-Oleander | 45°-Oleander |
| 25° C | 31 ± 4 | 37 ± 1 | 18 ± 2 | 15 ± 1 |
| 4° C | 25 ± 1 | 33 ± 1 | 15 ± 1 | 13 ± 1 |
| % of 25° C value | 81 ± 7 | 89 ± 3 | 87 ± 6 | 90 ± 5 |

Rose Bengal, conditions which produce singlet oxygen (Percival and Dodge 1983), MDA production was greatly stimulated. However, for slices incubated with MV, which promotes $O_2^-$ production (Farrington et al. 1973), MDA levels were reduced (Table 1). Singlet oxygen is highly reactive and readily oxidizes membrane lipids (see Elstner 1982) so the increase in the level of MDA after treatment with Rose Bengal shows the method detects the products of lipid peroxidation. An increase in MDA production was also apparent after leaf slices of cucumber were incubated at 4° C for 5 h and subsequently maintained in darkness at 25° C for 12 h.

Table 2 shows a comparison of the effect of chilling in the light on MDA production in leaf slices from two chilling-insensitive plants, spinach and 20°-oleander, with that of two chilling-sensitive plants, cucumber and 45°-oleander. Leaves of spinach and cucumber contain similar basal concentrations of MDA which were about twice that of the leaves from the two oleanders. These differences were also apparent when the concentration of MDA was expressed relative to Chl with values of 30 ± 1, 21 ± 3, 13 ± 1 and 8 ± 1 μmol · mg Chl$^{-1}$, in the order given, for spinach, cucumber, 20°-oleander and 45°-oleander. As shown in Table 2, incubation at 4° C for 5 h, reduced the MDA concentration by about 15% for slices from all plants compared with that for slices incubated for the same time at 25° C.

The activity of SOD in the extracts from leaf slices was estimated from measurements of the rate of oxygen uptake in a basic reaction mixture containing riboflavin and tetramethylethylenediamine which, when illuminated, produced the the substrate $O_2^-$ (Marshall and Warsfold 1978). As shown in Fig. 1a, the rate of oxygen uptake in the basic reaction mixture depended on the addition of SOD and was inhibited by KCN. Extracts of leaf tissue used for the estimation of SOD activity contained small amounts of Chl so it was necessary to determine that the Chl did not produce $O_2^-$ and thus contribute to the measured rate of oxygen uptake. As shown in Fig. 1b, oxygen uptake in the reaction mixtures containing leaf extract was dependent on the addition of the riboflavin and tetramethylethylenediamine to produce the substrate. This figure also shows that oxygen uptake, and hence dismutation of $O_2^-$ catalyzed by the leaf extract, was not completely inhibited by KCN. The residual activity represents the presence of some cyanide-insensitive

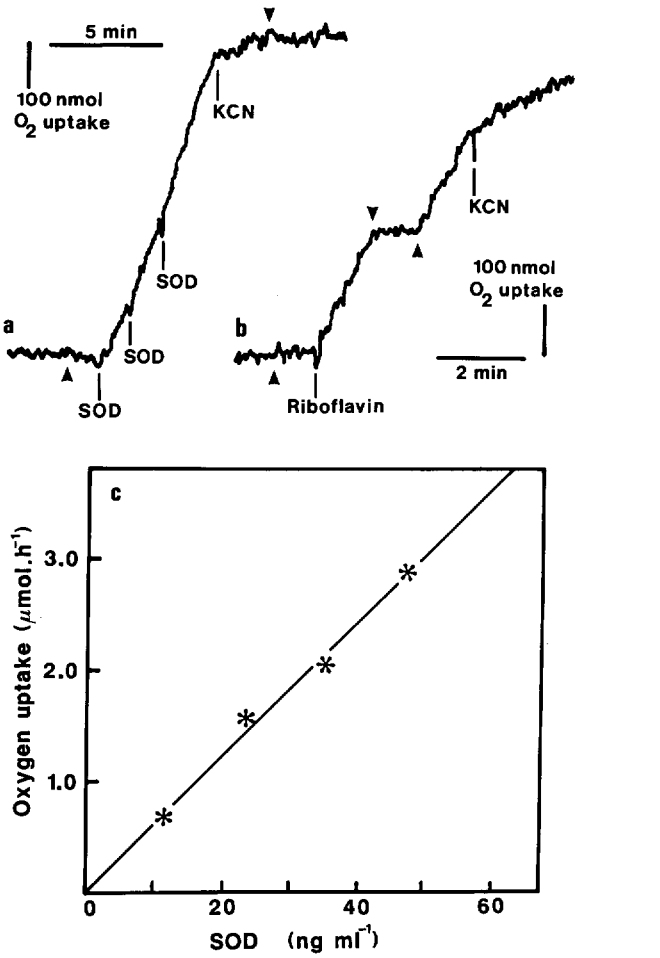


**Fig. 1a–c.** Factors affecting the rate of oxygen uptake in relation to the determination of SOD activity. **a** Trace shows oxygen uptake for a solution containing riboflavin, tetramethylethylenediamine and nitro blue tetrazolium when SOD was added. **b** Traces show activity after the addition of riboflavin to a solution containing leaf extract, tetramethylethylenediamine and nitro blue tetrazolium. The light was switched on at the up (↑) arrow and off at the down (↓) arrow. **c** Increase in oxygen uptake at 25° C with increasing amounts of bovine-erythrocyte SOD. Each value is the mean of three determinations

SOD. Estimation of the activity of the cyanide-sensitive SOD of chloroplasts was therefore determined as the difference in the rate of oxygen uptake before and after the addition of KCN. The suitability of this method for measuring SOD activity in extracts of leaf tissue was tested by measuring activity after adding SOD obtained from bovine erythrocytes. As shown in Fig. 1c the rate of oxygen uptake was proportional to the amount of added SOD. These results show that the method is suitable for comparative studies aimed at determining differences in the capacity of leaf tissue to dismutate $O_2^-$ and hence it provides information on the potential of chloroplasts to scavenge $O_2^-$.

Using the method described above the activity of cyanide-sensitive SOD was determined in extracts obtained from leaf slices after incubation at eithet 25° or 40° C for 5 h. As shown in Table 3, the cyanide-sensitive SOD activity was similar for all the plants studied and

**Table 3.** The effect of temperature on the activity of SOD in extracts from leaf slices of chilling-sensitive and chilling-insensitive plants. Leaf slices were incubated at a PFD of 450 μmol · $m^{-2}$ · $s^{-1}$ at the temperatures and times indicated. Values are the means ± SD, n > 6

| Incubation conditions | Spinach | Cucumber | 20°-Oleander | 45°-Oleander |
|---|---|---|---|---|
| *Activity of cyanide-sensitive SOD as % of total SOD* | | | | |
| Untreated (0 h) | 67 ± 11 | 68 ± 8 | 57 ± 11 | 59 ± 10 |
| 5 h at 25° C | 69 ± 12 | 69 ± 11 | 58 ± 10 | 59 ± 15 |
| 5 h at 4° C | 68 ± 8 | 68 ± 10 | 59 ± 9 | 61 ± 14 |
| *SOD activity (μmol $O_2$ uptake · $(gFW)^{-1}$ · $s^{-1}$)* | | | | |
| Untreated (0 h) | 6.1 ± 1.0 | 5.4 ± 3.2 | 3.2 ± 0.3 | 3.4 ± 0.5 |
| 5 h at 25° C | 6.3 ± 1.2 | 5.6 ± 0.5 | 3.3 ± 1.0 | 3.4 ± 0.4 |
| 5 h at 4° C | 5.6 ± 0.7 | 5.6 ± 0.7 | 3.6 ± 1.5 | 3.1 ± 1.6 |

was about 60–70% of the total SOD activity. Incubation for 5 h at either 25° or 4° C had no significant effect on the subsequent activity of SOD in any of the plants.

## Discussion

The results reported here on the vulnerability of the membrane lipids of leaf tissue to peroxidation during chilling at a moderate PFD (Table 2) and the level of SOD activity (Table 3) were obtained using leaves from the same plants and the same chilling conditions as those used previously to determine $O_2^-$ production by chloroplasts (Hodgson and Raison 1991) and the susceptibility of plants to chill-temperature photoinhibition (Hodgson and Raison 1989). The results are therefore pertinent to the question of whether $O_2^-$ potentiates chill-temperature photoinhibition.

Although chilling leaf slices of cucumber and 45°-oleander for 5 h, at a moderate PFD, stimulated the production of $O_2^-$ in chloroplasts (Hodgson and Raison 1991) it did not result in an increase in the peroxidation of membrane lipids of these plants (Table 2). This is consistent with results reported by Wise and Naylor (1987a) who have also shown that chilling leaves of cucumber, at a moderate PFD, does not induce lipid peroxidation. Based on these data it is concluded that while chilling for a few hours at a moderate PFD increases $O_2^-$ production in chilling-sensitive plants, the inhibition of photosynthesis (Hodgson and Raison 1989) and the loss (Wise and Naylor 1987b) and disruption to some thylakoid components (for a review, see Powles 1984), observed under these conditions, are not directly related to the peroxidation of membrane lipids.

Chilling cucumber for a few hours at a moderate PFD followed by rewarming in the dark does appear to cause changes which subsequently lead to peroxidation of membrane lipids (Table 1). Omran (1980) has also noted accumulation of peroxide after 48 h of chilling. The lack of an equivalent peroxidation within leaf slices of spinach under the same conditions indicates that chilling does differentially effect cucumber and spinach membranes, and that the adverse effect(s) in cucumber does ultimately lead to lipid peroxidation.

An alternative explanation for differences in the susceptibility of plants to chill-temperature photoinhibition is that for chilling-insensitive plants the $O_2^-$ produced in chloroplasts is more efficiently dismutated to $H_2O_2$ and oxygen by SOD. This hypothesis was tested by measuring SOD activity before and after chilling. For all plants tested about 70% of the SOD activity was cyanide-sensitive (Table 3), indicating it was the Cu-Zn SOD associated with chloroplasts (Rabinowitch and Fridovich 1983). However, there was no significant difference in the level of SOD activity in any of the plants tested nor was there any significant change in SOD activity when the leaf slices were incubated at a chilling temperature for 5 h (Table 3). Thus, a correlation between low SOD activity in chloroplasts and loss of SOD during the development of injury as a consequence of chilling (Michalski and Kaniuga 1981; Clare et al. 1984) was not observed for the leaf slices and chilling conditions used here.

These results (Table 3) indicate that susceptibility of leaf slices of chilling-sensitive plants to chill-temperature photoinhibition at a moderate PFD does not result from a damage to thylakoids because of an inability to effectively dismutate $O_2^-$. The possibility should not be excluded that the increase in $O_2^-$, observed in chilling-sensitive plants during incubation at 4° C (Hodgson and Raison 1991), leads to an increase in $H_2O_2$ after dismutation by SOD. Hydrogen peroxide regulates the efficiency of $CO_2$ fixation and the activity of some enzymes of the Calvin cycle (Kaiser 1979; Robinson 1988) and in the presence of $Fe^{3+}$ can react with $O_2^-$ to produce the highly reactive and randomly destructive hydroxyl radical (Elstner 1987; Halliwell 1982). Thus, while susceptibility to chill-temperature photoinhibition by chilling-sensitive plants cannot be directly attributed to an increase in $O_2^-$ production, its exclusion as a factor is difficult because of an inability to measure the production of hydroxyl radicals accurately.

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