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Chilling photoinhibition in Zea mays L and Zea diploperennis Iltis, Doebely and Guzman: The role of oxygen and antioxidants

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CHILLING PHOTOINHIBITION IN *Zea mays* L. and *Z. diploperennis*
Iltis, Doebley & Guzman: THE ROLE OF OXYGEN AND
ANTIOXIDANTS

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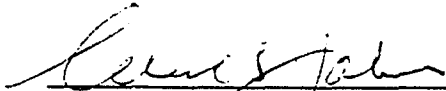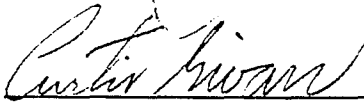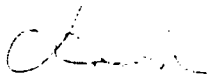
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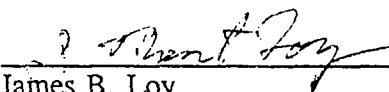
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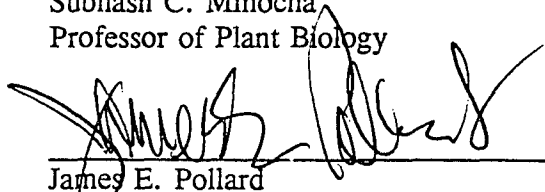
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ABSTRACT

CHILLING PHOTOINHIBITION IN *Zea mays* and *Z. diploperennis* : THE ROLE OF OXYGEN AND ANTIOXIDANTS

By

Kajal Ghoshroy

University of New Hampshire, September, 1995

Light absorbed by photosynthetic pigments must be distributed either for chemical work, reemitted as fluorescence or safely dissipated as heat. Adverse environmental conditions reduce the dissipation capacity of plants and the excess energy leads to damage to the photosynthetic mechanism, termed photoinhibition. Low, non-freezing temperatures cause such photoinhibition, especially in tropical plants grown in the temperate zone. This damage occurs in the photosystem II and is triggered by highly reactive radicals or reactive forms of dioxygen. Numerous studies point to the involvement of oxygen and antioxidant enzymes and substrates in amelioration of these damages. In C_3 plants, dioxygen is thought to offer some protection against photoinhibition by functioning as energy sink in the process of photorespiration and Mehler reaction. In C_4 plants this has not been previously investigated.

The goal of this research was to investigate the role of oxygen and antioxidants in low temperature photoinhibition by comparing two C_4 plants: one chilling sensitive corn (*Zea mays*) and the other its chilling-tolerant relative *Z. diploperennis*. Attached leaves

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of these plants were exposed to chilling (5°C) and ambient (25°C) temperatures at different concentrations of oxygen and either darkness or varying light intensities, and the extent of photoinhibition and concentration of antioxidants were then measured. The rates of recovery under non-stressful conditions were also monitored.

Our results show that oxygen imparted a significant protection to corn, but not *Z. diploperennis*, at low temperature. Nevertheless, *Z. diploperennis* sustained less photoinhibitory damage than corn. Photoinhibition in corn was accompanied by lower antioxidant concentrations. Both photosynthesis and antioxidants recovered by 2 days, suggesting that slow recycling of the latter induced photoinhibition at low temperature and retarded recovery. Maximum oxidation of the antioxidants took place in the presence of high light and low temperature. Chilling induced photoinhibition also required the presence of light.

ABBREVIATIONS

ADP	Adenosine di phosphate
APX	Ascorbate peroxidase
Asat	Light-saturated rates of photosynthesis
ATP	Adenosine tri phosphate
BHA	Butylated hydroxy anisol
CFo	Cofactor O of thylakoid membrane-bound ATPase
CF1	Cofactor 1 of thylakoid membrane-bound ATPase
DCMU	3-(3', 4'-dichlorophenyl)-1, 1-dimethyl urea
DHA	Dehydro ascorbic acid
DHAR	Dehydro ascorbate reductase
DTPA	Diethylene triamine pentaacetic acid
DTT	Di thio threitol
ϕ_{CO_2}	Quantum yield of photosynthesis
Fd	Ferredoxin
Fm	Maximum fluorescence level of Kautsky curve
ϕ_{PSII}	Quantum yield of photosystem II
Fv	Variable fluorescence level of Kautsky curve
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GTR	Glutathione reductase
HPLC	High performance liquid chromatography
LHC	Light harvesting chlorophyll complex
LHC I	Light harvesting chlorophyll complex of photosystem I
LHC II	Light harvesting chlorophyll complex of photosystem II
MDHAR	Monodehydro ascorbate reductase
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NPQ	Non photochemical quenching
OEC	Oxygen evolving complex
P_{680}	The photosystem II reaction center chlorophyll
PEP	Phospho enol pyruvate
PFD	Photon flux density
Ph	Phaeophytin
PQ	Plastoquinol
PQH_2	Reduced plastoquinol
PS I	Photosystem I
PS II	Photosystem II
Q_A	Quinone A
Q_B	Quinone B
q_I	Photoinhibitory quenching

RuBisCO   RuBP carboxylase oxygenase
RuBP   Ribulose 1,5-bisphosphate
SOD   Superoxide dismutase

INTRODUCTION

Green plants carry out photosynthetic reactions in specialized organelles called chloroplasts. Photosynthetic reactions consist of the so-called `light' and `dark' reactions. The light reactions are involved with absorption and conversion of light energy into chemical energy. The dark reactions fix the carbon of carbon dioxide into carbohydrates. This is carried out enzymatically by utilizing the chemical energy produced by the light reactions. The chloroplasts contain an intricate network of membranes, termed the thylakoid membranes and stroma lamellae. These membranes consist of pigments and proteins, embedded in the lipid-bilayer; they are necessary to carry out the light reaction. The dark reactions are carried out by soluble enzymes present in the aqueous matrix of the chloroplast, called stroma.

The thylakoid membrane (Fig. 1) has two different photosystems: Photosystem I (PS I) and Photosystem II (PS II). Each photosystem is made up of proteins that bind pigments and electron carriers. Light-harvesting complexes (LHC), containing a number of pigments, surround these photosystems and function in absorbing light energy and transferring it to the reaction center of the photosystems. The reaction center is the site where conversion of light to chemical energy takes place. The photosystem II reaction center consists of two protein subunits, namely D1 and D2. The reaction center chlorophyll of PS II is a chlorophyll a molecule, termed P_{680}. The antenna complexes absorb light and transfer the absorbed energy to P_{680}. When P_{680} absorbs light, or is excited by energy transfer from the antenna system, an electron is given up initially by the energized pigment to phaeophytin (Ph), then to quinone A (Q_A), and finally to quinone B (Q_B). The reduced quinone (Q_B^-) then detaches from PS II and is converted

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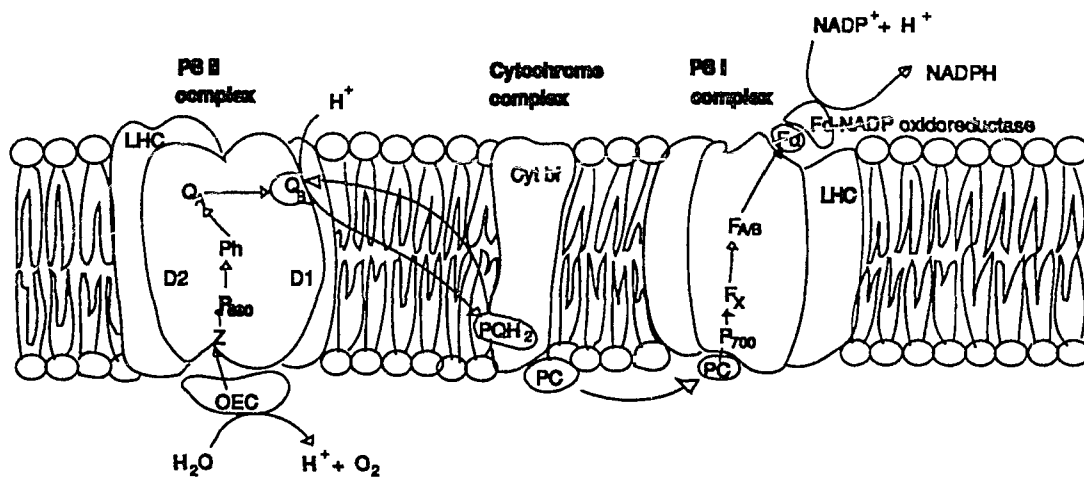
Fig. 1. Electron transport in the thylakoid membranes of chloroplasts.
Adapted from Raven & Johnson (1986).

to plastoquinol (PQH_2) by protons from the stroma. This PQH_2 then diffuses into the lipid bilayer and is oxidized and recycled back to PS II as Q_B by the cytochrome (cyt b/f) complex. An oxygen evolving complex (OEC) is also part of PS II. The electron lost to Ph by P_{680} is replenished by dissociation of water molecules by OEC. Dioxygen (O_2) is generated as a byproduct of this dissociation. The electrons which have reduced the cytochrome complex are transported to PS I. Light is also absorbed directly by LHC of PS I, in which case the PS I reaction center pigment P700 continues the transport of electrons, which are ultimately accepted by Ferredoxin (Fd), on the stromal side of PS I. This electron is used to reduce $NADP^+$ to NADPH by the enzyme Fd-NADP oxidoreductase. This photosynthetic reductant is utilized by reductive step in carbon assimilation and other reducing reactions in green plants.

During this light-induced electron transport, protons are pumped into the lumen of thylakoid membranes by the plastoquinol system as well as during disassociation of water by OEC. This results in acidification of the lumen, and generation of a proton gradient across the membranes. This gradient is then used to generate ATP, the unit of cellular energy, from ADP by thylakoid-membrane-bound ATPase called Cofactor O and 1 (CF_o and CF_1). The major metabolic pathways that utilize this chemical energy are the reactions of photosynthetic carbon assimilation and other ATP-dependent reactions.

When chloroplasts absorb light, they can utilize this energy in one of the following ways: a) for photochemistry, b) to convert it to heat, c) for fluorescence, or d) to transfer the energy to some other compound. Plants maintained under optimum light and temperature conditions are able to funnel most of the light intercepted by the chloroplasts to the electron transport system, generating NADPH and ATP in turn. It has been observed that absorption of excessive light during active electron transport causes damage to the photosystems. This damage ultimately results in the inhibition of photosynthesis, which is known as photoinhibition.

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When plants are exposed to high light intensities, the electron transport system becomes saturated even at ambient temperature. At low temperature, the leaves attain saturation at light intensities much below those at ambient temperature. Hence at chilling temperatures a larger percentage of the absorbed light energy is not 'used' for photosynthesis and must be dissipated by other means. Chilling may further limit the other energy dissipation mechanisms thus allowing excess energy to damage the photosynthetic process.

The photoinhibitory damage is thought to be initiated in PS II by absorption of excess light and it eventually leads to formation of highly reactive compounds, like active-oxygen radicals. These active-oxygen compounds can bring about damage to photosynthetic membranes or enzymes by causing oxidation of lipids and proteins. However, plants have inherent protection mechanisms against some of these radicals. Antioxidants are a group of such compounds that destroy active-oxygen radicals. There are also certain enzymes that help scavenge these radicals.

The goal of this research was to quantitate the extent of photoinhibition in corn (*Zea mays* L.) plants under varying temperature, light and oxygen concentrations. A comparison of chilling photoinhibition was also made between chilling sensitive corn and chilling tolerant *Zea diploperennis* Iltis, Doebley & Guzman, leaves. Correlation between the extent of photoinhibition and the levels of antioxidants and scavenging-enzyme activities were also made in these two species.

Photoinhibition:

Photoinhibition is attributable to oxidative stress in plants. Photoinhibition is defined as inhibition of the PS II reaction center, resulting from absorption of light in excess to what can be efficiently utilized by the photosynthetic electron transport system (Krause, 1994). In this inhibited state, the light trapped by the reaction center is

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dissipated as heat and is prevented from carrying out photochemical reactions (Krause, 1994). PS I, on the other hand has been shown to be inhibited in vitro under high light (Barenyi and Krause, 1985; Krause and Laasch, 1987; Krause et al., 1988; Satoh, 1970), however little or no in vivo effect is observed (Krause, 1988).

The primary location of photoinhibition is thought to be at the reaction center of PS II (Baker, 1991; Krause, 1994), specifically, at the D1 protein. D1 degradation is thought to be not the cause, but rather a consequence of photoinhibition (Krause, 1994). The decline in PS II electron transport takes place not as a result of decrease in the total number of D1 proteins, but due to the accumulation of inactive reaction centers containing damaged D1 protein (Krause, 1994). D1 protein has a very rapid turnover rate, with half-time of about 60 min (Barber and Andersson, 1992). In order for repair mechanisms to occur, it is speculated that the damaged PS II complexes migrate to a non-appressed thylakoid region, where the D1 protein is first degraded by a membrane protease (Barbato et al., 1993). These damaged PS II continue absorbing photons, but convert them into heat instead of chemical energy (Krause, 1988). A newly synthesized D1 protein is soon incorporated into this damaged PS II complex, upon which the repaired complex migrates back to the appressed region (Barbato et al., 1993; Krause, 1994). The extent of photoinhibition thus not only depends on the amount of damage, but also on the mobility of PS II complexes within the membrane, the rate of degradation by protease, and resynthesis and incorporation of D1 proteins into PS II complexes (Baker, 1994; Krause, 1994). Any or all of these factors could be affected by stress conditions. Hence the extent and duration of photoinhibition under stress conditions would be controlled by these factors (Baker, 1994). Recovery from photoinhibition has been shown to involve protein synthesis (Baker, 1994; Greer et al., 1986), specifically involving de novo synthesis of D1 protein, encoded by the chloroplast genome (Krause, 1994). However, photoinhibition is far from being a uniform phenomenon (Krause,

1988). Various mechanisms contribute to this process in different plants depending on the genetic makeup, growth conditions, acclimation etc.

The amount of damage imparted to PS II depends on its cross-sectional area (Krause, 1988). This area is proportional to the amount of light that the system intercepts. Hence shade plants, having larger antenna size than sun plants, exhibit a higher photoinhibitory damage at the same light intensity (Anderson and Osmond, 1987; Bjorkman, 1981). The damage imparted to PS II can be evaluated either by measuring the quantum yield of photosynthesis (ϕ_{CO2}) or by monitoring chlorophyll a fluorescence. Quantum yield of photosynthesis is defined as the moles of CO_2 fixed per mole of photon absorbed by the leaf. ϕ_{CO2} is governed both by the efficiency with which photons reach the reaction center and the proportion of "open" reaction centers, i.e., centers that have oxidized acceptors (i.e. acceptors capable of accepting electrons) (Baker, 1991). ϕ_{CO2} is thought to be correlated with the rate of ATP and NADPH synthesis and allocation to photosynthetic reduction cycle rather than other metabolic pathways like photorespiration (Baker, 1994; Baker and Nie, 1994). As it is difficult to measure the number of photons absorbed, it is customary to measure the *apparent quantum yield*. The apparent quantum yield is the rate of carbon dioxide fixed (or oxygen-evolved) per quanta of incident light. The actual quantum yield is measured with the help of standard photosynthetic curve (Fig. 2) of *absorbed* light vs. rate of CO_2 assimilation. The slope of the line at light limited regions of photosynthesis is equivalent to the actual quantum yield (ϕ_{CO2}). The apparent quantum yield is measured from a similar curve of *incident* light vs. rate of photosynthesis, and is directly proportional to the actual quantum yield.

Measurement of the light-saturated rates of photosynthesis (Asat), on the other hand, is an indicator of the turnover capacity of the carbon fixation pathway rather than light reactions. Asat of photosynthesis is also measured from the standard photosynthetic curve of light vs. rate of photosynthesis (Fig. 2). Asat is the maximum

rate of photosynthesis at light-saturated regions of the curve. Asat is partly affected by carbon dioxide diffusion rates and partly by carboxylase activity of RuBisCO and the rate of generation of RuBP (Baker, 1994). However, a majority of crop plants function most of the time at a rate much below the Asat (Baker, 1994). This is because not only are most leaves on a plant partly shaded by others, but also because the PFD (photon flux densities) fluctuate diurnally. Typically, the light intensities during a day are below the saturation levels at most times, especially at early mornings and late afternoons. During the mid-day, however, light saturating light intensities anywhere between 1000 to 3000 $\mu mol\ m^{-2}\ s^{-1}$. A mid-morning depression of Asat is seen in majority of plants exposed to such high light intensities, mostly due to stomatal closure.

When PS II absorbs light under normal conditions, the excited chlorophyll molecule is deactivated by transfer of the absorbed energy into either : (a) fluorescence, (b) heat (termed thermal deactivation), (c) PS I, or (d) by photochemical reaction. About 1% of absorbed energy is converted to fluorescence under low light and optimum conditions (Krause, 1988). Chlorophyll fluorescence serves as an intrinsic indicator of photosynthetic reaction in the chloroplasts of green plants. Chlorophyll fluorescence originates primarily from chlorophyll a in PS II. Changes in fluorescence yield thus reflect the properties of excitation and energy conversion at PS II. However, as PS II is connected to other components of the photosynthetic apparatus, fluorescence yield can be an indirect indicator of the whole photosynthetic process. When a leaf is illuminated with continuous light following a period of darkness, fluorescence rises from an original level (Fo) to a maximum level (Fm) (Fig. 3). This is known as the Kautsky effect and can be measured with a modulated fluorometer. The Fo-level is monitored by weak modulated measuring beam and is a measure of initial energy distribution to PS II and of the efficiency of excitation trapping at P_{680}. Following a period of darkness, all reaction centers are "open" at Fo. This indicates that all primary electron acceptors, especially

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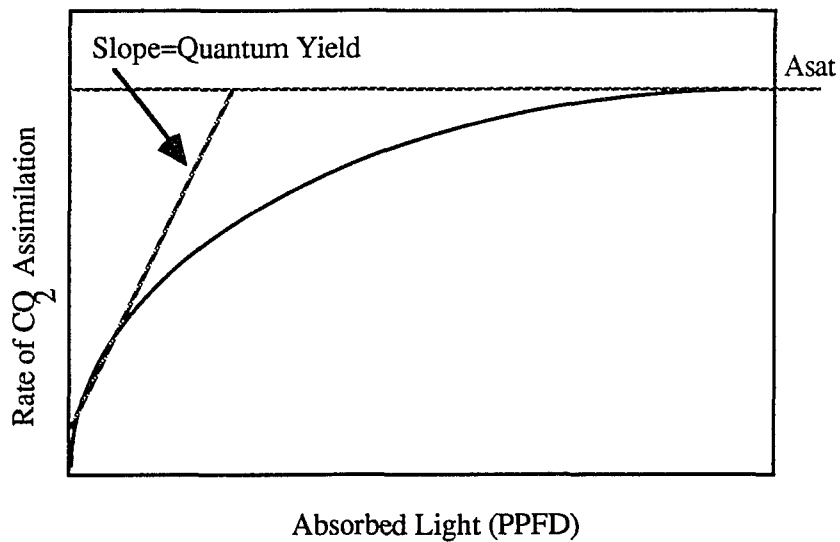
Fig. 2. Standard photosynthetic curve of absorbed light (PPFD = photosynthetic photon flux densities) vs. rate of carbondioxide assimilation. Asat = light-saturated rates of photosynthesis, and quantum yield = slope of the line at light-limited regions. Adapted from Long et al., (1994).

Q_A, are fully oxidized and therefore are able to accept electrons and quench fluorescence (Baker, 1991). Upon the onset of strong illumination the fluorescence rises to Fm, which reflects reduction of Q_A (i.e., reduced Q_A cannot accept electrons). A modulated fluorometer detects the fluorescence in the 710-760 nm range. Fluorescence excitation is carried out by white light filtered to block any radiation that is longer than 700 nm. To measure Fo, a weak beam of light, typically of 0.1 $\mu mol\ m^{-2}\ s^{-1}$ intensity is used. Fm is measured by a higher intensity of light, typically around 5000-10,000 $\mu mol\ m^{-2}\ s^{-1}$. The intensity of chlorophyll fluorescence is, thus, a strong function of the redox state of the reaction center of photosystem II. The fluorescence intensity is low when the centers are open (Fo) and high when they are closed (Fm). Chlorophyll fluorescence can thus be used to measure the extent of damage to PS II, denoted by quantum efficiency of PS II (ϕ_{PSII}). This is done by monitoring the Fv/Fm ratio, where Fv, the variable fluorescence, is the difference between Fm and Fo (Fig. 3). Fv/Fm ratio is influenced by the efficiency of transfer of an absorbed photon to PS II reaction center, as well as the number of centers that are capable of transferring an electron to a quinone acceptor (Baker, 1994). Fv/Fm ratio thus indicates the efficiency of energy capture by "open" PS II reaction centers. Non-stressed plants have an Fv/Fm of about 0.83 (Bjorkman and Demmig, 1987). A direct correlation between ϕ_{CO2} and ϕ_{PSII} exists under the conditions where photorespiration is minimal (Baker, 1994). As plants are exposed to photoinhibitory conditions, increasing number of centers become fluorescence quenchers as they convert the absorbed energy into heat (Krause, 1988). Photoinhibition has hence been shown to lower the Fv/Fm ratio of plants mostly due to a decrease in Fm and only partially due to a rise in Fo (Krause, 1988). This lowering, termed photoinhibitory quenching (q_I), mainly occurs in the antenna of PS II (Baker, 1994). Photoinhibitory quenching at low temperature has been shown to be partly due to the increase in the rate constant of thermal dissipation (Asada, 1994; Bjorkman, 1987). The xanthophyll cycle (discussed

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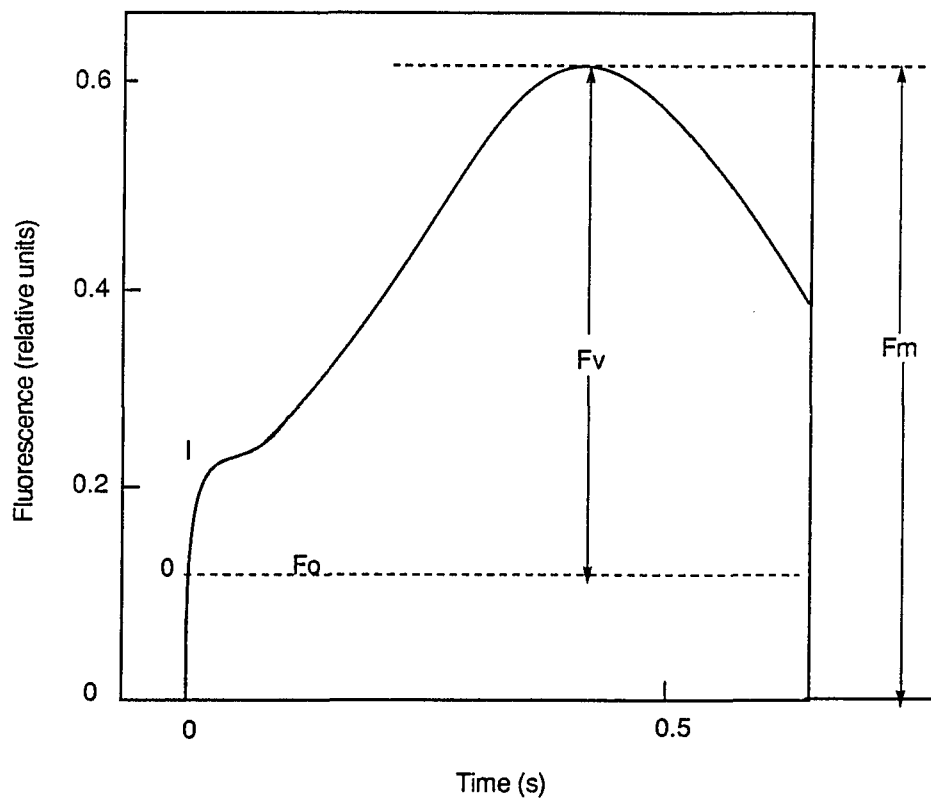
Fig. 3. Typical fluorescence induction kinetics (Kautsky effect).
Adapted from Schreiber & Bilger (1987).

below) has been thought to be involved with this type of non photochemical quenching. LHC II (light harvesting chlorophyll a/b protein complex of PS II) aggregation occurring as a result of lumen acidification, has been linked with this light-induced enhancement of non-photochemical quenching in the antenna of PS II (Horton et al., 1991).

Damage to D1 protein

Oxygen is thought to be a major cause of D1 damage. Photoinhibition causes synthesis of highly reactive molecules that cleave D1 proteins at different sites (Barbato et al., 93). There are different hypotheses as to which reactive species of molecules are responsible for the inactivation of photosynthesis.

In one of the proposed explanations, over-energization of the thylakoid membranes causes over-reduction of the quinones in PS II (Barbato et al., 1993; Krause, 1994). This is often called the "receptor side" cause of photoinhibition. Under normal conditions, stabilization of charge separation in PS II occurs by transfer of electrons from P_{680}^+ to Ph⁻ (reduced phaeophytin), and then to Q_A and finally Q_B (Krause, 1988). But when the Q_A is over reduced (by absorption of excess light by PS II) it dissociates from the D2 protein and therefore prevents transfer of electrons to Q_B. This is because the affinity of the quinol (Q_B^{2-}) for the binding site is lower than that of the semiquinone (Q_B^-) (Baker, 1994). This loss of Q_B binding capacity, detected by Atrazine binding properties of PS II, is now thought to initiate photoinhibition (Barber and Andersson, 1992; Kyle, 1987). Atrazine is an herbicide that competes for the same binding site as Q_B. The binding of Atrazine thus indicates loss of Q_B binding to D1 protein (Baker, 1994). Atrazine binding is measured by incubating ^{14}C-Atrazine with broken chloroplasts. The number of binding sites are then calculated by using control reaction mixtures that are devoid of chloroplasts. This loss of binding capacity is soon followed by D1 degradation (Kyle, 1987). The lack of Q_B on D1, and further energization of the

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reaction center during stress, causes reduction of the Q_A to Q_A^{2-} (Barber and Andersson, 1992). This results in charge separation between P_{680}^+ and Ph^- (Barbato et al., 1993; Barber and Andersson, 1992; Krause, 1988), leading to charge recombination by production of triplet state ($^3P_{680}$) (Barbato et al., 1993; Barber and Andersson, 1992; Krause, 1994).

$$P_{680} \, Ph \quad \xrightarrow{\; h\upsilon \;} \quad P_{680}^* \, Ph \quad \longrightarrow \quad P_{680}^+ \, Ph^- \quad \longrightarrow \quad ^3P_{680} \, Ph$$
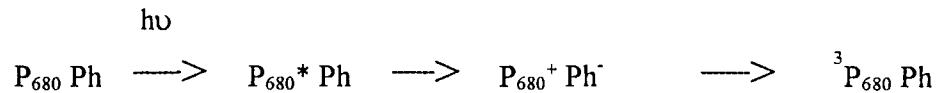
The triplet state of P_{680} will readily generate active-oxygen species (Barber and Andersson, 1992), such as $^1O_2$ (Krause, 1994). Thereafter, the $^1O_2$ causes damage to the membranes or specifically to D1 protein.

$$^3P_{680} + \, ^3O_2 \longrightarrow \, ^1P_{680} + \, ^1O_2$$
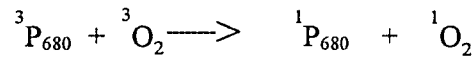
The P_{680}^+ is also a strong oxidant and can cause damage to D1 directly (Krause, 1988). Semiquinone radical (Q_A^-) accumulating in PS II under high light (due to lack of oxidized Q_B), may also be able to promote photoinhibition (Cleland and Melis, 1987; Cleland et al., 1986; Krause, 1994). $^3O_2$ (ground state dioxygen), upon reaction with this radical, has also been thought to either directly cause inactivation or dissociation of the quinone from PS II; or indirectly give rise to active-oxygen species (Krause, 1988; Krause, 1994). The quenching of the variable fluorescence (Fv) has been assumed to be a direct result of such reduction/oxidation (Krause, 1988; Krause, 1994). Vass et al. (1992) have suggested singlet oxygen (the first excited state of dioxygen) to be the specific type of active-oxygen species that triggers D1 damage. This hypothesis is supported by the evidence of D1 damage enhancement in absence of β-carotene, the

singlet oxygen quencher, *in vivo*. A direct evidence for $^3O_2$ interaction in photoinhibition, however, is yet to be seen. Most evidence, on the other hand, points to the active-oxygen species induced damage as being the primary cause of photoinhibition of the reaction centers.

In another scheme of photoinhibition, water splitting complex is thought to be dysfunctional during photoinhibition (Barbato et al., 1993). Under these conditions, an increase in the lifetime of highly reactive species like P_{680}^+ and Z^+ takes place as they cannot be reduced by the Mn complex of the water splitting complex (Barbato et al., 1993). These highly reactive molecules are thought to cleave oxidized pigment molecules and damage D1 proteins (Barbato et al., 1993). This is referred to as the "donor-side" photoinhibition.

It is yet unresolved as to whether the inhibition of electron transport between P_{680}^* and Q_A^- or the loss of Q_B binding capacity occurs first. But as all factors are located on the D1/D2 heterodimer, it is possible that various changes affect structural organization of the proteins (Krause, 1988). Moreover, the various theories of photoinhibition may rise from the fact that different changes take place under different situations in different plants (Krause, 1988).

Active-oxygen

Among the active-oxygen species, most damaging are superoxide radical (O_2^-), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). The Mehler reaction (Mehler, 1951) is a process by which O_2^- is produced in the chloroplasts. During this reaction, O_2 acts as an electron acceptor at the reducing side of PS I when acceptors like $NADP^+$ are not sufficient to reduce ferredoxin (Fd_{red}) or Fd:NADP oxidoreductase. O_2^- is then converted to hydrogen peroxide either spontaneously or by

superoxide dismutase. Mehler reaction functions to some extent in plants even under normal conditions (Badger, 1985).

$$2O_2 + 2(\text{Fe-S center})_{red} \longrightarrow 2O_2^{\cdot -} + (\text{Fe-S center})_{ox}$$

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$$
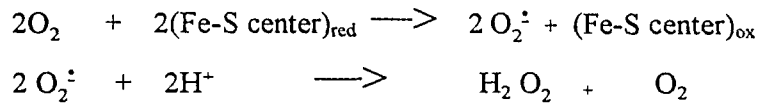
Prolonged exposure to high oxidative stress conditions, like winter stress, causes bleaching of pigments (like chlorophyll a and b, β-carotene and xanthophylls) and lipid peroxidation in plants (Krause, 1994). These reactions are commonly called photooxidation. Photoinhibition, triggered at a much more rapid rate than pigment bleaching, might actually be protecting antenna pigments against destruction by light (Krause, 1988).

Lipid peroxidation in plants is often monitored by ethane production. Atrazine, an herbicide that reduces the Mehler reaction by blocking electron transfer between Q_B site of PS II and PS I, is able to inhibit ethane production during oxidative stress in cucumber (Wise and Naylor, 1987a; Wise and Naylor, 1987b). The authors speculate that superoxide radicals, generated during oxidative stress, are the primary cause of lipid peroxidation. Dark incubation of thylakoid membranes with xanthine oxidase, a $O_2^{\cdot -}$ generator, has been shown to inhibit electron transport (Takahama and Nishimura, 1975). However, $O_2^{\cdot -}$ itself is not a very strong oxidant. But O_2^- gives rise to $^\cdot OH$ radicals by Haber-Weiss reaction with iron cations, H_2O_2 being supplied by superoxide dismutase reaction (Upham and Jahnke, 1986; Krause, 1994):

$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^\cdot OH$$
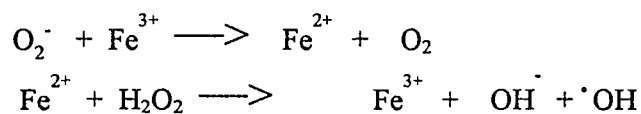
$^{\bullet}$ OH, a strong oxidant, starts a radical chain reaction by first oxidizing an unsaturated membrane fatty acid into a lipid radical (L$^{\bullet}$), and finally into lipid peroxy radical (LOO$^{\bullet}$) with oxygen (Krause, 1994). (Kyle, 1987) has postulated that $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}$OH may be generated at the Q_B site of D1 protein by electron transfer from Q_A^- or Q_B^- to oxygen.

$^1O_2$ is thought to be the active-oxygen species with key role in photoinhibition. $^1O_2$ is produced by photoactivated chlorophyll when transfer of electrons to NADP become limited due to stress (discussed above). Part of the $^1O_2$ may also be contributed by lipid hydroperoxides (LOOH) and peroxy radicals (Krause, 1994).

$$2LOO^{\bullet} \longrightarrow LOOL + {}^1O_2$$
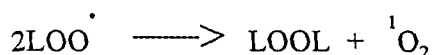
Lipid peroxidation, in turn, is also enhanced by $^1O_2$. Rose Bengal, a dye which generates $^1O_2$, and D_2O, which prolongs the lifetime of $^1O_2$, were shown to enhance lipid peroxidation (Hodgson and Raison, 1991; Wise and Naylor, 1987a) in plants. $^1O_2$ can directly convert membrane unsaturated acyl residues into LOOH, which in turn can degrade lipids by radical chain reactions (Krause, 1994). Blocking electron transport between PS II and PS I by inhibitors like 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea (DCMU), results in decreased lipid peroxidation, but causes increased energization of antenna pigments. This overenergization increases ^{3}Chl production and may finally lead to synthesis of $^1O_2$ (Krause, 1994). The lifespan of $^1O_2$ is increased when it is present in the lipophilic phase of the membranes (Asada and Takahashi, 1987; Krause, 1994). Thus, when generated at a critical site in the reaction center, $^1O_2$ has the potential to cause degradation of compounds like quinones and D1 proteins. The primary damage inflicted by $^1O_2$, has been shown to be on the pigment systems. This results in bleaching of leaf pigments, primarily of β-carotene, followed by chlorophyll (Krause, 1994). It has

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been suggested that $^1O_2$ can directly oxidize P_{680}, the primary electron donor of PS II. Artificially generated $^1O_2$ has been shown to inhibit electron transport and quench variable fluorescence and $^3P_{680}$ (Krause, 1994). This $^1O_2$ induced photoinhibition closely resembled actual photoinhibition of untreated plants, thereby pointing to $^1O_2$ as being an important cause of photoinhibition.

The Dual Role of Oxygen in Photoinhibition

Protective role

In C_3 plants, photorespiration plays a protective role against photoinhibition (Krause, 1994; Krause and Cornic, 1987). This protection is imparted by consumption of photosynthetically generated ATP by the oxygenation pathway of RuBP carboxylase oxygenase. Illuminated bean plants (*Phaseolus vulgaris*) exposed to increasing concentrations of oxygen, showed 80% photoinhibition at 1% oxygen, in the absence of CO_2 (Powles and Osmond, 1978), i.e. when carbon fixation is halted. However, the same plant indicated no photoinhibition between 7-21% oxygen under the above CO_2 concentration. Wu et al. (1991) have also demonstrated that photorespiration is able to support linear electron transport in spinach leaves in the absence of CO_2. In the steady state with an high irradiation of 600 W m^{-2}, these leaves showed photochemical quenching of chlorophyll fluorescence, indicating significant reoxidation of Q_A. The amount of oxidized Q_A increased with increasing oxygen concentrations, suggesting that photorespiration might prevent the over-reduction of Q_A, which is the primary step leading to photoinhibition (Krause, 1994).

Similar results were observed in water-stressed sunflower, bean and *Digitalis lanata* (Krause, 1994). C_4 plants like corn (*Zea mays*) on the other hand, showed only slight decrease of photoinhibition when exposed to oxygen concentrations of 1-50% under water stress (Powles et al., 1980). The latter phenomenon is to be expected, since

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minimal photorespiration occurs in C_4 plants (Baker, 1991), where CO_2 concentrating mechanisms in the bundle sheath chloroplasts prevent oxygenation reactions of RuBisCO. Corn plants, in particular are NADP-malic enzyme type species. Krause (1994) points out that under water stress and high illumination, the stomata of leaves remain closed, thus forcing the carbon reduction-oxidation cycles to operate at the CO_2 compensation point. These cycles together are able to reduce photoinhibition significantly, in spite of low electron transport rates, by maintaining Q_A in oxidized state and by high proton gradient across thylakoid membranes (Krause, 1994; Wu et al., 1991). Hence, during concomitant water stress and high light, conditions frequently encountered by plants, photorespiration imparts considerable protection against photoinhibition at normal oxygen levels in C_3 plants.

Coupled cyclic electron transport (i.e. cyclic photophosphorylation) may be another process of deactivation of excitons in the chloroplasts, specially in case of C_4 plants. In this process electrons are cycled between PS I and the plastoquinone components, thus generating enough H^+ in the lumen to synthesize ATP. This not only provides protection from photoinhibition (Heber and Walker, 1992) but also generates extra ATP in order to meet the increased demands of C_4 photosynthesis (Furbank et al., 1990). The photoinhibitory protection is imparted in two ways. One, by using energy, and two, by acidifying the lumen (which is also essential for the xanthophyll cycle). But Baker (1994) argues that cyclic electron transport, being always a constant percentage of the non-cyclic transport cannot be regulated to meet increased ATP demands. Hence according to him, pseudo-cyclic electron transport (involving Mehler reaction, discussed below) becomes an important candidate for increased ATP generation.

The Mehler reaction is another process by which chloroplasts dissipate excess energy. Under light stress, significant number of electrons are drained from PS I to H_2O_2 via this process when other energy-consuming mechanisms, like photorespiration, are

active (Baker, 1994; Krause, 1994; Krause and Laasch, 1987; Wu et al., 1991). This is thus another process of preventing overenergization of PS II, provided active-oxygen species generated as a result of this reaction can be efficiently scavenged (Baker, 1994). However, the Mehler reaction alone, in absence of photorespiration and carbon fixation (which occurs during chilling stress), is too small to prevent Q_A over-reduction, and therefore prevent photoinhibition (Krause, 1994; Wu et al., 1991). Energy is also dissipated by the scavenging enzymes involved in the ascorbate-glutathione cycling (Fig. 4). This pathway utilizes the reductants NADPH and NADH to scavenge Mehler reaction products, thereby acting as a sink for the photosynthetic products.

Destructive role of oxygen

Photoinhibition has been shown, by many groups, to increase with the increase of oxygen concentrations from 1 to 20% (Powles, et al., 1983; Rowley and Taylor, 1972; Krause, 1994). This is especially true under conditions that reduce the protection provided by photorespiration (Krause, 1994). The gradual increase of photoinhibition with increase in oxygen concentration is thought to be due to its slow accessibility to the reaction center, where most of the damage takes place (Krause, 1994). This increase also seems to preclude the involvement of Mehler reaction in photoinhibition, which is fully saturated much below the normal oxygen concentration of 21%.

Isolated chloroplasts, which lack photorespiratory cycles, are good candidates for testing oxygen effects on photoinhibition. In a CO_2 free medium, spinach (a C_3 plant) chloroplasts showed oxygen effects on photoinhibition (Krause, 1994). Under CO_2 saturated medium, however, photoinhibition was completely eliminated as C-metabolism promoted high rates of electron transport (Krause, 1994). Similar results were observed when phosphate deficient chloroplasts were used (Wu et al., 1991). The site of inhibition was diagnosed to be in PS II of thylakoid membranes isolated from these

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photoinhibited chloroplasts (Wu et al., 1991). Irradiation of isolated chloroplasts also inhibits PS I, though to a lesser degree (Krause, 1988). This inhibition of PS I is oxygen dependent (Satoh, 1970) and of little significance *in vivo* (Krause, 1994).

In intact C_3 leaves, the inhibitory affects of oxygen on photoinhibition is compensated by the protective actions of photorespiration (Wu et al., 1991; Powles et al., 1984; Krause, 1994). Under chilling stress, however, when the photorespiratory cycle itself is suppressed, the effect of oxygen on photoinhibition is significant (discussed below). At normal temperatures, this phenomenon is evident only when photorespiration can be down-regulated. *Elatostema repens*, a tropical shade plant, has shown oxygen related photoinhibition at 24°C (Le Gouallec and Cornic, 1988). This is probably due to its low inherent antioxidant capacities (Krause, 1994).

In the complete absence of oxygen, greater photoinhibition is observed compared to low oxygen (1-2%) (Krause, 1994). This is thought to be due to extreme overreduction of the electron transport system due to lack of electron acceptors (Krause, 1994). This anaerobic photoinhibition is relevant to the protective mechanisms of the Mehler reaction (Krause, 1994). Primary stages of photoinhibition are thought to be produced under these conditions, which lead to complete inactivation of PS II under oxygen (Hundal et al., 1990; Krause, 1994; Vass et al., 1992). According to Vass et al. (1992) these primary stages also occur under aerobic conditions, but are temporary because of interference from oxygen (Vass et al., 1992). This primary stage of photoinhibition can be recovered in darkness without *de novo* protein synthesis, as there is no degradation of the D1 protein (Hundal et al., 1990; Vass et al., 1992). In contrast, low light is needed for recovery from aerobic photoinhibition (Krause, 1994). On introduction of oxygen, enhanced degradation is seen among the PS II subunits that are undergoing anaerobic photoinhibition (Vass et al., 1992), suggesting that oxygen induces a secondary stage of photoinhibition.

The defense system

The photoinhibitory reactions start after a lag phase (Wise and Naylor, 1987a), during which the defense system of the plant is actively involved (Krause, 1994). This defense system, during stress conditions, is active for different lengths of time depending on the tolerance of the plant (Krause, 1994; Wise and Naylor, 1987a).

Three of the important defense systems in the chloroplast are antioxidants, energy dissipaters and active-oxygen scavenging enzymes. The antioxidants include ascorbic acid, reduced glutathione (GSH), and α-tocopherol. The carotenoids act as efficient energy dissipaters in the thylakoid membranes. Superoxide dismutase (SOD), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydro ascorbate reductase (MDHAR) and glutathione reductase (GTR) are the five important scavenging enzymes of chloroplasts (See Fig. 4 for reactions) that are involved with the ascorbate-glutathione cycle. Ascorbate and glutathione are water soluble antioxidants that scavenge soluble active-oxygen species like O_2^- and H_2O_2. Vitamin E and β-carotene, (located within the thylakoid membranes), will quench lipid peroxy radicals, like ^3Chl (triplet excited chlorophyll), and $^1O_2$ in the membranes (Wise and Naylor, 1987a). Within the leaf, 30-40% of ascorbate, 10-50% of glutathione and all the of α-tocopherol and β-carotene are located in the chloroplast (Gilham and Dodge, 1987; Wise and Naylor, 1987a).

The ascorbate-glutathione system of the chloroplasts is a very efficient system for scavenging active-oxygen species of the chloroplasts (see Fig. 4, and Foyer, 1993). O_2^- is primarily converted by SOD to H_2O_2 (Foyer, 1993) which subsequently is reduced by APX to water with help of ascorbate, catalase being completely absent from the chloroplast (Asada, 1991; Foyer, 1993; Halliwell, 1987). Ascorbic acid is also essential for recycling of α-tocopherol, that scavenges active-oxygen species from membranes, and in the xanthophyll cycle (Fig. 6, discussed below) (Foyer, 1993). Hence ascorbate is

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the key antioxidant of both the aqueous and lipid phase of the chloroplast (Foyer, 1993). Chloroplastic APX and SOD are present as soluble, as well as thylakoid-bound, forms (Foyer, 1993). Ascorbate is first oxidized into monodehydro ascorbate radical by APX. This monodehydro ascorbate radical is the first product of H_2O_2 destruction in chloroplasts (Foyer, 1993). Monodehydro ascorbate radical is also formed during oxidation of ascorbate in the reduction of hydroxyl radicals and regeneration of α-tocopherol (Fig. 5) (Hess 1993; Foyer, 1993). This radical can then undergo a disproportionation reaction into ascorbate and DHA, if not re-reduced rapidly into ascorbate by MDHAR (Foyer, 1993). DHA is also reduced to ascorbate by DHAR with the help of GSH (Foyer, 1993). Hence these two different enzymes, MDHAR and DHAR, responsible for the vital regeneration of the ascorbate, maintain the ascorbate pool in its reduced state. GSH, by the above reaction of DHAR, is converted to its oxidized form (GSSG). The regeneration of GSH is done by the fifth enzyme of the cycle, namely GTR, with help of the reductant NADPH. The ascorbate-glutathione cycle thus consumes reductants from the chloroplastic electron transport pathway, thereby generating H^+ gradients and subsequently ATP (Foyer, 1993).

Whether this system is efficient enough under stress conditions to detoxify the chloroplast from active-oxygen species has not been resolved yet. *In vitro* photoinhibition of thylakoids was reduced by SOD plus catalase, and GSH plus ascorbate (Barenyi and Krause, 1985; Richter et al., 1990). These treatments were able to protect fully D1 protein but only partially protected PS II activity (ca. 80% of control), as measured by Fv/Fm fluorescence quenching. They also only partially prevented loss of α-tocopherol from the membranes. This indicated an involvement of another factor in photoinhibition, that was not scavenged by either the antioxidants or scavenging enzymes. Krause (1994) suggested that this factor might be the singlet oxygen.

$$2H^+ + O_2^- \cdot + O_2^- \cdot \xrightarrow{\text{Superoxide} \atop \text{Dismutase}} H_2O_2 + O_2$$
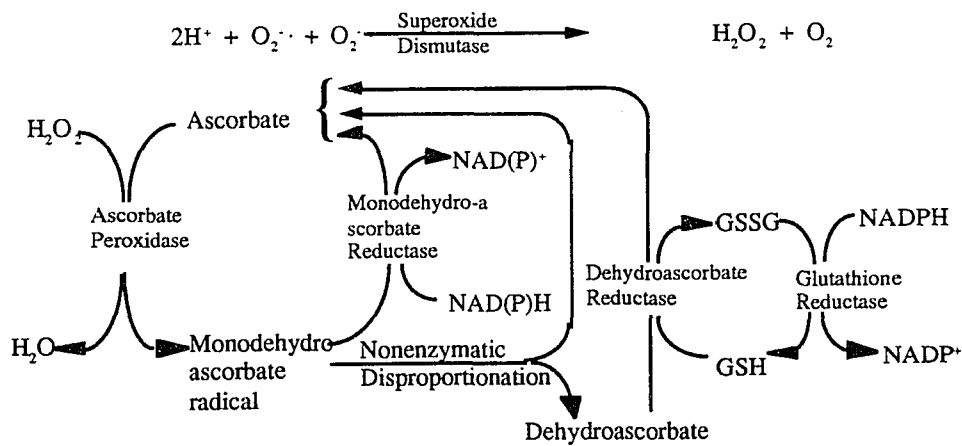
Fig. 4. The active oxygen scavenging pathway of the chloroplast. Adapted from Jahnke et al., (1991).

Acclimation to stress conditions has been shown to improve the tolerance levels of plants. Acclimation is mostly accompanied by an increase in the antioxidant and scavenging systems of plants. Spinach plants that were cold-acclimated under high light showed tolerance against photoinhibition at chilling temperatures (Somersalo and Krause, 1989; Somersalo and Krause, 1990). Acclimation to low temperatures resulted in increased activities of SOD, APX, MDHAR, GTR, along with increased production of ascorbate in various C_3 plants (deKok and Oosterhuis, 1983; Gillham and Dodge, 1987; Guy and Carter, 1984; Schoner et al., 1990; Schoner and Krause, 1990)

Singlet oxygen ($^1O_2$) on the other hand, can be scavenged by carotenoids and tocopherols (Krause, 1994) which protect the hydrophobic environments of the membranes from oxidative damage. α-Tocopherol, also known as vitamin E, is a lipid soluble antioxidant that is found in the thylakoid membranes. This molecule can scavenge O_2^{-}, $^1O_2$, triplet state of chlorophyll (^{3}Chl) and lipid peroxide radicals (LOO$^\bullet$) (Hess, 1993; Krause, 1994) and thus protect pigments and membranes from active-oxygen species that partition into the lipid phase. Vitamin E is thought to function near the surface of the membrane (Hess, 1993). α-Tocopherol is converted to α-tocopheroxy radical during free radical trapping reactions, which in turn is reduced back to the alcohol by ascorbate at the membrane-aqueous interphase (Fig. 5) (Hess, 1993). Hence vitamin E and C (ascorbate) function in a co-operative manner during radical scavenging reactions. The monodehydroascorbate radical generated in the process is reduced back to ascorbate by MDHAR and NAD(P)H (Hess, 1993). It is also postulated by some authors that glutathione too can regenerate α-tocopherol from its oxidized form (Goin et al., 1991). Plants show variation in the amounts of α-tocopherol concentration under different situations and in different plant parts (Hess, 1993). Corn leaves showed an increase in their α-tocopherol content with age and variation between leaves on the same plant (Jahnke, 1994, unpublished). Increased lipid peroxidation has

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been shown to be correlated with increased α-tocopherol content and decreased chlorophyll concentrations of leaves (Hess, 1993).

Carotenoids, especially β-carotene in the antenna system, are thought to prevent chlorophyll bleaching by quenching ^{3}Chl and ^{1}O$_2$ (Baker, 1994; Krause, 1994):

$$^3\text{Chl} + {}^1\text{Car} \longrightarrow {}^1\text{Chl} + {}^3\text{Car}$$

$$^1\text{O}_2 + {}^1\text{Car} \longrightarrow {}^3\text{O}_2 + {}^3\text{Car}$$
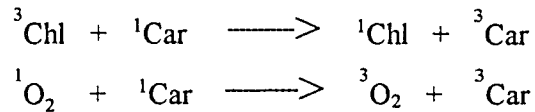
Most of the ^{3}Car reaches ground state by radiationless transition, however a few may be destroyed during the scavenging process (Krause, 1994). Chlorophyll bleaching probably starts after a substantial loss of carotenoids. The role of the reaction center β-carotene in triplet state quenching is however not yet explicit (Krause, 1994).

Xanthophylls are carotenoids with at least one oxygen atom. Some are antenna pigments (like lutein), while others are involved with thermal dissipation of excess light energy absorbed by light harvesting chlorophyll pigment bed (Demmig-Adams and Adams, 1994). This type of energy dissipation is known as either NPQ (non photochemical quenching (Demmig-Adams and Adams, 1994) or pH-dependent NPQ (Krause et al., 1982). Plants respond to excess light either by increasing the xanthophyll pool size or by converting the existing violaxanthin (Fig. 6) to zeaxanthin (Demmig-Adams and Adams, 1994). There are three xanthophylls involved with this cycle, namely zeaxanthin, antheraxanthin and violaxanthin, and all three are found on the LHCP of PS I and II (Thayer and Bjorkman, 1992). All three xanthophylls are synthesized from the common precursor, β-carotene (Jones and Porter, 1986). On increasing irradiance, de-epoxidation of violaxanthin to antheraxanthin and then to zeaxanthin takes place (Demmig-Adams and Adams, 1994). The reverse reaction takes place on return to low light (Demmig-Adams and Adams, 1994). Antheraxanthin does not exhibit pronounced

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ROOH ← α-tocopherol• Ascorbate → NAD[P]+

Monodehydro Ascorbate Reductase

ROO• α-tocopherol Monodehydro Ascorbate Radical NAD[P]H
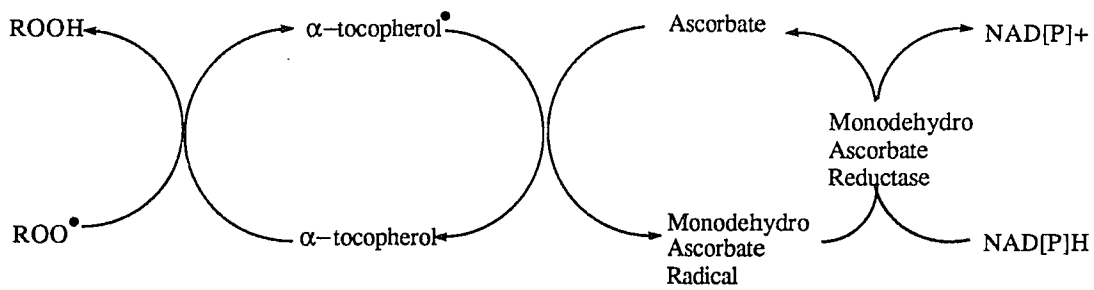
Fig. 5. α-Tocopherol recycling scheme. Adapted from Hess (1993).

concentration changes during these conversions (Demmig-Adams and Adams, 1994). The absolute amounts of zeaxanthin formed has negative correlation with the photosynthetic capacity of a leaf (Demmig-Adams and Adams, 1994). Hence leaves which can dissipate energy via photosynthetic reactions have a lower xanthophyll pool, as their need to utilize xanthophylls for energy dissipation is also lower.

The xanthophyll cycle is controlled by regulation of the enzymes responsible for the conversions of the xanthophylls (Hager, 1980; Siefermann-Harms, 1977; Yamamoto, 1979). The enzyme violaxanthin de-epoxidase, which converts violaxanthin to zeaxanthin, is regulated by acidic lumen pH and reduced ascorbate (Yamamoto, 1979). The reverse reaction, catalyzed by the enzyme epoxidase requires oxygen and NADPH, besides having an optimum pH of 7.5 (Hager, 1980; Siefermann-Harms, 1977; Yamamoto, 1979). There is substantial evidence (Demmig-Adams and Adams, 1994) that energy dissipation, as measured by lowering of Fm, is greater in cells with higher levels of zeaxanthin. Inhibition of the de-epoxidase reaction has been shown to result in loss of energy dissipation in the pigment bed (Bilger et al., 1989; Demmig-Adams and Adams, 1994; Demmig-Adams et al., 1989; Gilmore and Yamamoto, 1992). With increase in light energy in the pigment bed, the energy dissipation activity of the xanthophyll cycle increases and the photosynthetic capacity of PS II decreases (as light is prevented from reaching the reaction center) (Demmig-Adams and Adams, 1994). Sun-leaves have a larger xanthophyll pool size and thereby a greater capacity to dissipate energy (Demmig-Adams and Adams, 1994). Presence of low lumen pH is essential both for NPQ as well as the conversion of violaxanthin to zeaxanthin (Gilmore and Yamamoto, 1992). Chloroplasts that contained zeaxanthin were shown to carry out NPQ even in darkness (Gilmore and Yamamoto, 1992). This was shown in a remarkable study by Gilmore and Yamamoto (1992) by adding ATP to generate transthylakoid pH gradient via ATP synthase working in reverse. Although a major portion of the NPQ
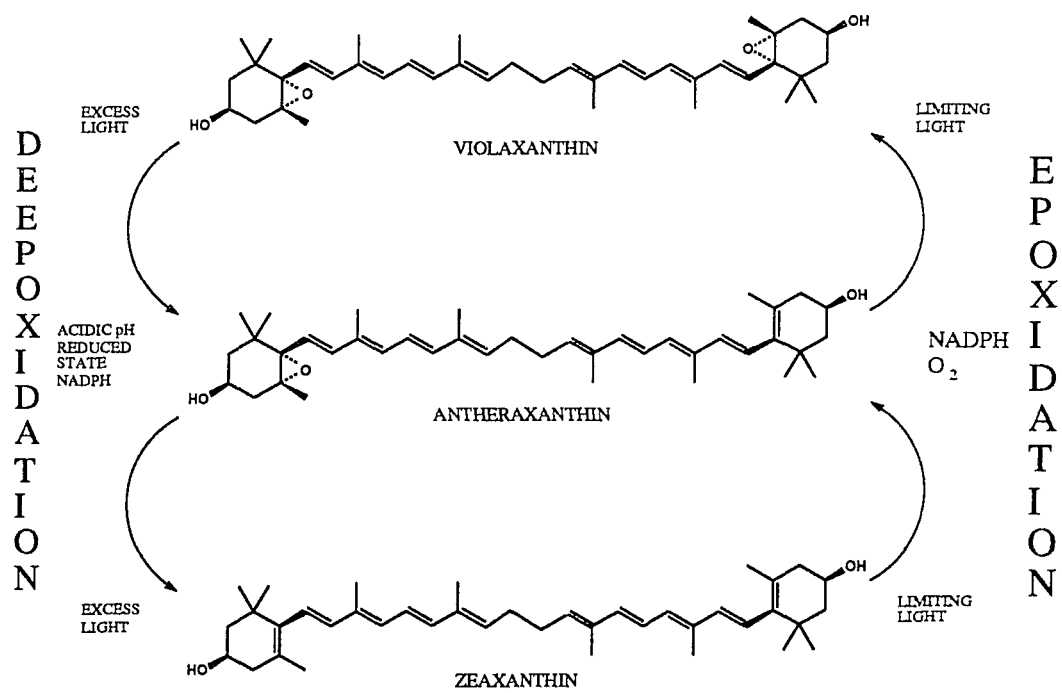
Fig. 6. The xanthophyll cycle. Adapted from Demmig-Adams et al., (1989).

under high light is associated with the xanthophyll cycle, there could be other mechanisms associated with this type of quenching. Zeaxanthin is thought to carry out NPQ by deactivating singlet excited state of chlorophyll under excessive light in the pigment bed (Demmig-Adams and Adams, 1994), although this hypothesis is not universally accepted.

Chilling stress

Chilling temperatures ($0-12°$) reduce the metabolic activities of plants. The average temperature for more than 70% of the world's land mass is below $5°C$ (Baker, 1994). When plants are exposed to low temperatures, they may undergo disruption of many metabolic processes (Baker, 1994). This is known as chilling stress. This is responsible for reducing yields of many crops, particularly in sub-tropical plants like corn, introduced to and being cultivated in the temperate regions (Baker, 1994). One of the first processes to be affected during chilling stress is photosynthesis (Powles, 1984). Low temperature reduces the possibility of deactivation of excitons via controlled photochemical processes, thereby increasing the likelihood of photoinhibition (Baker and Nie, 1994; Taylor and Craig, 1971; Taylor and Rowley, 1971). Photoinhibition is of important concern in sub-tropical plants, like corn, that are major agricultural plants in temperate climates. Chilling temperatures lower the CO_2 assimilation rates in these tropical plants (Baker, 1991). Low temperature exposure has been shown to lower both the light-limited (ϕ_{CO2}) and light-saturated (Asat) rates of CO_2 assimilation in light (Baker, 1991; Long et al., 1983). Although, in leaves grown under optimum conditions, Asat is certainly lowered in response to lowering of temperature, ϕ_{CO2} is less affected (Baker, 1994; Long et al., 1983). In C_4 plants, however, though ϕ_{CO2} is tolerant of immediate fluctuations in temperature, there is a stress-related decrease after prolonged exposure to low temperature (Baker, 1994). In C_3 plants, on the other hand, ϕ_{CO2}

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increases on lowering temperature due to decreasing affinity of RuBisCO for oxygen (Baker, 1994). Chilling injury to plants is significantly higher in light than darkness (Long et al., 1983; Ortiz-Lopez et al., 1990; Powles et al., 1984). Even moderate amounts of light may lead to photoinhibition at chilling temperatures (Krause, 1988). Chilling stress, according to some authors, results in lower stomatal conductance (Harris, 1978). However, the chilling photoinhibition in presence of light has been shown to be either unaffected by this lowering of stomatal conductance (Long et al., 1983; Powles et al., 1983) or not to increase the stomatal restriction (Bongi and Long, 1987; Martin and Ort, 1985; Strand and Oquist, 1985). In the case of corn chilled at $5^{\circ}C$, stomatal resistance has been shown not to affect photoinhibition (Ortiz-Lopez et al., 1990). When corn plants were chilled at $5^{\circ}C$ for 6h in light (1000 μmol m^{-2} s^{-1}) a 50% decrease in ϕ_{CO2} was observed, which was accompanied by lowering of the Fv/Fm ratio and Atrazine-binding properties (Baker and Nie, 1994; Butler and Katijima, 1975). This indicates that chilling in light induces photoinhibitory damage to the PS II complex. When corn plants were exposed to the same temperature but only 50 μmol m^{-2} s^{-1} of light, ϕ_{CO2} was reduced by only 20% (Ortiz-Lopez et al., 1990). Drastic photoinhibition was also seen in unshaded leaves of corn plants growing in the field, when dawn temperatures fell below $10^{\circ}C$ in presence of high early morning light (Baker et al., 1988). However, below $5^{\circ}C$, all developmental stages of leaves showed significant photoinhibition (Baker and Nie, 1994).

Protection of C_3 plants from photoinhibition is unlikely to be provided by photorespiration at low temperature (Baker, 1994). Decrease in temperature increases the solubility of CO_2 at the active site of RuBisCO compared to O_2, thereby decreasing the oxygenase reaction (Long and Drake, 1992). However, oxygen has been shown to be a major part of the photoinhibitory damage induced in light at low temperature (Powles et al., 1983; Rowley and Taylor, 1972; Van Hasselt, 1972; Van Hasselt and Van

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Berlo, 1980). Chilling sensitive plants have increased production of superoxide in light at chilling temperatures (Hodgson and Raison, 1989).

Wise and Naylor (1987a; 1987b) have demonstrated a continuous loss of antioxidants, namely β-carotene, the three xanthophylls, ascorbate, and glutathione, during 12 hours of chilling at 5°C under high light in cucumber, a chilling sensitive C_3 plant. This was also accompanied by rapid loss of α-tocopherol within the first three hours, followed by pigment bleaching. In contrast, chilling-resistant pea plants showed no significant damage under similar conditions. This indicated either a more efficient defense system and/or a rapid antioxidant-replenishing pathway in pea. α-tocopherol was concluded by them to be an important antioxidant involved with long term protection of photosynthetic pigments. Chilling at 5°C showed much slower rates of zeaxanthin synthesis and NPQ development compared to 20°C (Demmig-Adams et al., 1989). This slowing down has been attributed to the chilling induced retardation of de-epoxidase activity (Demmig-Adams et al., 1989). Leaves containing high zeaxanthin prior to onset of chilling stress showed less sensitivity of PS II to photoinhibition than leaves with low zeaxanthin (Demmig-Adams et al., 1989). In corn, zeaxanthin formation was strongly inhibited by low temperature (Koroleva et al., 1994).

Chilling stress also lowers activities of enzymes, like nitrate reductase (Baker and Van Hasselt, 1982), NADP-malate dehydrogenase and some of the important enzymes of photosynthetic reduction cycle like fructose 1,6-bisphosphatase, PEP carboxylase , NADP-dependent malate dehydrogenase, RuBisCO, and pyruvate Pi dikinase (Baker, 1994; Gilmore and Yamamoto, 1992; Long et al., 1983; Hull et al., 1995). In corn, more than 70% decrease of RuBisCO took place when they were exposed to 5°C for 1-2 days (Baker, 1994). Lowering the assay temperature from 19°C to 5°C resulted in reduction of the specific activity of SOD and APX by up to 40%, MDHAR and GTR reduced by more than 65% and DHAR by 100% in corn (Jahnke et al., 1991). Clearly

then, the active-oxygen scavenging system of plants like corn declines severely at low temperature. This would result in increased concentration of active-oxygen species in the chloroplast, which thereby might cause D1 protein damage in PS II or other types of damage.

Low-temperature-induced phase transition of membranes (i.e., liquid crystal to gel) have been thought to be the cause of chilling damage and photoinhibition in the past. But Baker (1994) argues that this cannot be the only cause of photoinhibition, as plants take hours to recover from chilling stress while membrane lipids recover their physical state shortly on return to ambient temperature. Furthermore, the majority of the membrane lipids in chilling-sensitive plants do not undergo phase transition changes, however a small percentage of lipids undergoing transition could alter membrane function in many ways (Baker, 1994).

The photoinhibitory effect of low temperatures at least partially result from the slowing down of both the rate of protein degradation by proteases, as well as the de novo protein synthesis of D1 proteins (Baker, 1991; Baker and Nie, 1994). Low temperatures inhibit corn thylakoid protease and prevent incorporation of chloroplast encoded proteins into thylakoid membranes (Hayden and Covello, 1983). Even though low temperatures affect all metabolic rates, chloroplasts are specially handicapped under these conditions as their rate of incorporation of chloroplast encoded proteins is much slower than those encoded by the nucleus (Nie and Baker, 1991). This reduction is thought to be more as a result of disruption of integration and stabilization of proteins in the thylakoid membranes, and not due to inhibition of chloroplast protein synthesis (Baker, 1991).

Various attempts have been made to develop plants with increased chilling resistance, especially where crop plants are concerned. But development of successful chilling resistant plants have mostly been handicapped by the lack of detailed knowledge

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of chilling induced damage (Baker, 1994). More detailed information regarding chilling photoinhibition will be beneficial not only in developing chilling resistance in crop plants by altering the specific sites of damage, but also in screening of genotypes for potential source of tolerant features at the molecular level.

The harmless dissipation of excess energy (from sunlight) becomes an increasing problem at low temperatures for all plants since the normal energy sinks have reduced rates of enzyme turnover and the protective enzymes and antioxidant systems are likely to turnover slower as well.

Corn is one of the most chilling sensitive plants in American agriculture. In Northern USA and Canada, the growing season is considerably shortened due to this sensitivity. This shorter season thus limits production of corn. Corn plants, when grown at chilling temperatures, show small chlorotic leaves, reduction in chlorophyll and thylakoid contents per leaf area, which inevitably reduces Asat of photosynthesis (Baker, 1994).

The ability of corn to repair its chilling induced damage when returned to optimum temperature is of critical consideration especially for plants that grow in the temperate regions (Baker and Nie, 1994). Recovery from photoinhibition consists in part of the repair of the damaged reaction center (Krause, 1988). Mature corn leaves have been shown to recover by about 2 days when returned to optimum temperatures (Baker and Nie, 1994). But, when developing leaves are exposed to chilling stress, the leaves do not recover even after 6 days of optimum temperature, mainly due to impairment of the chloroplast developmental processes (Baker and Nie, 1994). This is thought to have major implications on crop yields in the field, since plants will have to wait for new leaf development under optimum temperatures to be photosynthetically restored again (Baker and Nie, 1994). Z. diploperennis, Iltis, Doebley & Guzman, on the other hand, is a chilling tolerant close relative of corn (Long et al., 1987). It has been

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shown to have approximately twice the specific activity of oxygen scavenging enzymes as corn (Jahnke et al., 1991). Hence in this study, a comparison of these two C_4 species, differing in chilling tolerance, was made. Both *Zea* species are C_4 plants. Since the vast majority of published studies of chilling photoinhibition have used C_3 plants, the different physiology and biochemistry of C_4 photosynthesis invites comparison. It is clear that C_3 plants have mechanisms to dissipate excess energy harmlessly (including photorespiration and the Mehler reaction). These mechanisms are known to be much less active in C_4 plants like corn. Both photorespiration and the Mehler Reaction require molecular oxygen as an energy safety-valve in C_3 plants. The possible ways in which oxygen may interact with C_4 plants and their anti-oxidants to either help dissipate excess energy or cause damage to the photosynthetic system is not understood. This is the general subject of this thesis.

OBJECTIVES

My main aim was to answer the following questions

1. What are the recovery rates, photosynthetic parameters and antioxidants of corn leaves exposed to chilling stress under light and normal oxygen levels?

2. What is the extent of photoinhibition in the two *Zea* species : corn (*Z. mays*) and *Z. diploperennis*, in presence and absence of light at ambient vs. low temperature?

3. What is the extent of photoinhibitory damage imparted to the two species under increasing and decreasing oxygen concentration?

4. What are the effects of low-temperature and light induced damage to antioxidants, energy dissipating and active-oxygen scavenging systems of the leaves from the above experiments?

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In order to quantitate chilling photoinhibition in these two species, Fv/Fm ratio, Asat and ϕ_{O2} were measured in leaves treated at 5° or 25^oC, 0 or 1500 $\mu mol\ m^{-2}\ s^{-1}$ of light. In order to assess the effect of oxygen, the two species were exposed to 2, 20 or 40% oxygen during photoinhibitory treatments at low and high temperature. The antioxidant, energy dissipating and scavenging system of leaves, in the above experiments, were then measured by assaying the following:

ascorbic acid, dehydroascorbic acid and total ascorbic acid.

α-Tocopherol

zeaxanthin, violaxanthin and antheraxanthin

enzyme activities of APX, MDHAR and GTR.

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MATERIALS AND METHODS

Zea mays L. cv LG11 and *Z. Diploperennis* Iltis, Doebley & Guzman, were grown in the greenhouse under approximately 12 h daylength conditions. The greenhouse temp was about $25\pm3°C$. *Z. mays* was grown from seeds while *Z. diploperennis* was grown by planting tillers of older plants. Mid-regions of fully expanded attached leaves, from mid-section of 2-3 week old plants, were sealed into air-tight Plexiglas treatment chambers. These chambers were maintained at the desired light intensity (i.e. 1500 μmol m^{-2} s^{-1} or darkness), leaf temperature (either 5°C or 25°C), and gas mixture (2, 20 or 40% O_2, with 0.04% CO_2, balance nitrogen), for the 6 h duration of each incubation. Light was obtained from banks of twelve 300 W quartz-halogen lamps (type ELH) filtered through 6 inches of circulating water to remove excess heat. Light intensities at the leaf surface were measured with a Li-Cor LI-185D Quantum Radiometer. Untreated attached leaves of plants served as controls. Control and treated leaves were harvested and used to measure Fv/m, quantum yield (ϕ_{O2}) and Asat of photosynthesis; concentrations of ascorbic acid, dehydroascorbic acid (DHA) total ascorbate, α-tocopherol and xanthophylls; and the activities of scavenging enzymes. Control leaves were harvested, at random, at various times of the day. Each experiment was repeated at least three times, using leaves from three individual plants for each replicate. The mean and standard error of these three replicates were then calculated and plotted.

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Recovery experiments of *Zea mays*

Attached leaves of *Z. mays* were exposed to treatment chambers maintained at 5°C, 20% oxygen and 1500 μmol m^{-2}s^{-1} of light for 6 h. The plants were then allowed to recover at room temperature, in air, under standard white fluorescent lamps at about 25 μmol m^{-2}s^{-1} of light. Untreated and treated leaves were then harvested after 0, 3, 22 and 49 h of recovery and were used to measure Fv/m, quantum yield and Asat of photosynthesis; ascorbic acid, DHA, total ascorbate and α-tocopherol levels; and the activities of scavenging enzymes. Each experiment was repeated at least three times, using individual plants for each replicate.

Measurement of Fv/Fm of Photosynthesis

Treated leaves were first held in darkness for 10 min, using leaf clamps, in order to allow complete oxidation of photosystem II (Schoner et al., 1989). Fo, Fm and Fv/m values were then measured using automated modular fluorometer by Opti Science 500 with a modulation pulse of 90 units. Fo was measured using a weak light with an intensity of 0.1 μmol m^{-2} s^{-1}, while Fm was measured with a strong intensity of 5-10 mmol m^{-2}s^{-1}. The excitation light was filtered to remove radiations longer than 700 nm. Detection was done at 710-760 nm. Three separate measurements were taken for each treated leaf and their mean value was used as one replicate. The mean and standard error of three such replicates from three different experiments were then plotted.

Measurement of Quantum yield and Asat of Photosynthesis

Photosynthetic oxygen evolution, as indicated by net oxygen evolution, was measured using Hansatech oxygen electrode maintained at 25°C as described by Walker (1987). Approximately 10 cm^{2} leaf discs were excised from the control and treated leaves and introduced into the electrode chamber. The discs were first exposed to 10 min

of darkness while its dark oxygen evolution, due to respiration, was plotted. They were then exposed to increasing light intensities up to 1500 μmol m^{-2} s^{-1} while the resulting oxygen evolution rates were plotted. The electrodes were standardized by injecting known volumes of air, and the conversion factor for volts to μmol of oxygen ratio was calculated as described by Walker (1987). Photosynthetic rates of the leaf discs from slopes of the plotted lines were then expressed as μmol m^{-2} s^{-1} of oxygen and plotted further against light intensities. Asat values were then obtained from the maximum light-saturated rates of photosynthesis, while the quantum yield (ϕ_{O2})was calculated from slope of the line at light-limited regions of the curve (refer to Fig. 2 for a standard curve). The mean and standard error of three replicates from three different experiments were then plotted.

Extraction and Measurement of Ascorbic acid and Dehydroascorbic acid.

Extraction: One half gram of control or treated deribbed leaves were frozen in liquid nitrogen and stored at -80°C. They were then ground in liquid nitrogen, extracted with 5 ml of 3% meta-phosphoric acid containing 0.01% DTPA (diethylene-triamine pentaacetic acid), using a tissuemizer for 30 s and centrifuging at 20,000 g for 10 min at 4°C. The supernatant was then used for measuring ascorbic acid and dehydroascorbic acid by isocratic HPLC separation and electrochemical detection (based on the method of Behrens and Madere, 1987). Ascorbate was measured directly, while total ascorbate was measured by reducing DHA to ascorbate with DTT. The amount of dehydroascorbate was calculated from the difference between the total and reduced ascorbate per sample.

Assay: 100 μl of 1% DTT were added to 500 μl of the supernatant, incubated for 30 min in dark at room temperature and volume then made up to 2 ml with 0.85% meta-phosphoric acid. 50 μl of this solution were diluted to 2 ml with mobile phase, filtered

through a 0.2 μm filter and injected into the HPLC system to measure the amount of total ascorbate. Reduced ascorbate was assayed similarly using 100 μl of 0.85% meta-phosphoric acid instead of DTT.

Standards were prepared from a frozen stock of 5 mg ml^{-1} of ascorbic acid in 3% meta-phosphoric acid containing 0.01% DTPA. A series of dilutions (0-80 μg ml^{-1}) were made prior to injection by using the above solution. 500 μl of each standard was then treated with DTT, incubated for 30 min in dark at room temperature and volume then made up to 2 ml with 0.85% meta-phosphoric acid. 50 μl of this solution was diluted to 2 ml with mobile phase, filtered through a 0.2 μm filter and injected into the HPLC system. A representative chromatograph of a known quantity of acsorbate standard is shown in Fig. 7. The retention time of ascorbate was 3.3 min.

Separation: Ascorbic acid was separated on a 25 cm long C18 Spherisorb column, using a Rheodyne 7025 injector containing 20 μl sample loop and Waters M45 pump with a flow rate of 0.8 ml min^{-1}. The mobile phase consisted of 15% MeOH, 0.015% meta-phosphoric acid and 2 mM n-octylamine (as ion pair reagent) in 80 mM sodium acetate buffer (pH 4.8). A representative chromatograph of a treated sample is shown in Fig. 8. Retention time for ascorbate was 3.4 min.

Detection: Electrochemical detection of ascorbate was performed using LC-4B amperometric detector from Bioanalytical Systems, set at 20 nA range and glassy carbon electrode TL-5 at +0.7 V potential against a Ag/AgCl reference electrode RF-1. The data were analyzed using Gilson 712 integration software and expressed as μg ascorbate g^{-1} fresh weight. Integration was performed at 10 mV full scale, with a peak width of 0.4 min and a peak sensitivity of 2.0%. Three separate measurements were taken for each treated leaf and their mean value was used as one replicate. The mean and standard error of three such replicates from three different experiments were then plotted.

Fig. 7. Scaled plot of ascorbate standard. Integrated at 10.0 mV full scale, for 15 min, EC detection, peak width 0.40 min, peak sensitivity 2.0%. Retention time of ascorbate = 3.3 min

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Fig. 8. Scaled plot of ascorbate extracted from corn leaves Treated to 25°C, 40% oxygen, in light. Integrated at 10.0 mV full scale, for 15 min, EC detection, peak width 0.40 min, peak sensitivity 2.0%. Retention time of ascorbate = 3.4 min.

Extraction and Measurement of α-Tocopherol

Extraction : Fifty to one hundred milligrams treated deribbed leaves were frozen in liquid nitrogen and stored at -80°C. They were then ground in liquid nitrogen in a ground-glass tube with a glass pestle and extracted by regrinding at least twice with 1 ml acetone containing 25 μl of 0.5 mg ml^{-1} butylated hydroxyanisol (BHA) until the tissue fragments were completely bleached. The pooled acetone extracts were transferred to a glass tube. One milliliter petroleum ether (30-50°C fraction) was added to the pooled extract, mixed and washed with 8 ml water. The petroleum ether layer was carefully removed into a fresh tube and the process repeated again with 1 ml more of petroleum ether. The pooled petroleum ether fraction was evaporated by bubbling with nitrogen. The residue was redissolved in 500 μl of methanol, its volume was measured, and filtered through 0.2 μm filter and injected into the HPLC system.

Standards: α-tocopherol (Sigma Chemical Co., St. Louis, Missouri) standards were prepared each day in MeOH and diluted until their absorbance at 290 nm was between 0.10-0.2 absorbance units. The concentration of α-tocopherol in the standards was then determined using a molar extinction coefficient of 3,185 (290 nm). The standard samples were then filtered through a 0.2 μm filter and injected as above. A representative chromatograph of a known concentration of α-tocopherol standard is shown in Fig. 9. The retention time of α-tocopherol peak was at 6.99 min

Separation: This method was based on that of Chou et al., (1985). Vitamin E was separated on a 4.6 mm ID x 25 cm long Rainin Microsorb-MV C8, 5 μm column, using the hardware as above. A mobile phase of 2% of 1.25 M sodium acetate buffer (pH 5.0) in MeOH was used with a flow rate of 1 ml min^{-1}. A representative chromatograph of treated leaf sample is shown in Fig. 10. The retention time of α-tocopherol was 6.99 min.
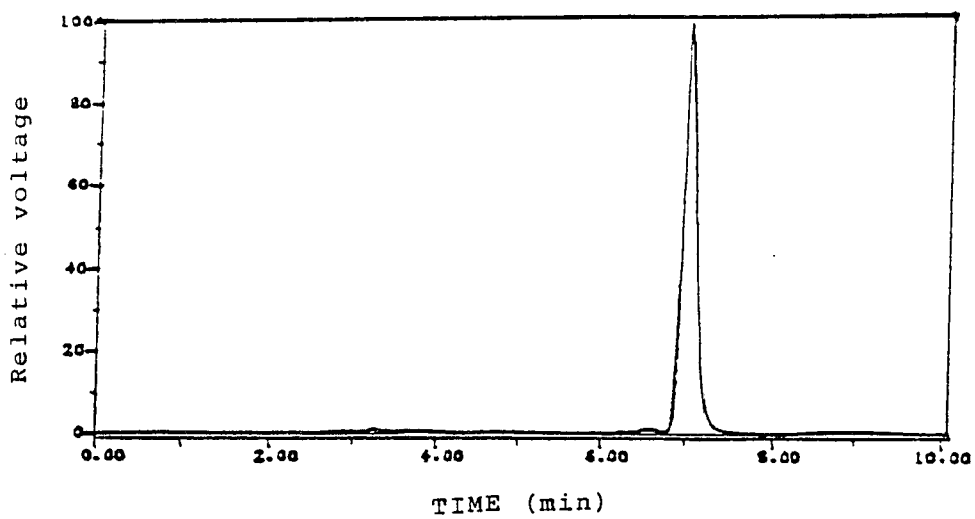
Fig. 9. Scaled plot of α-tocopherol standard. Integrated at 26.8 mV
full scale, for 10 min, EC detection, peak width 0.40 min, peak
sensitivity 3.5%. Retention time of α-tocopherol = 6.99 min

<u>Detection</u>: Electrochemical detection of vitamin E was performed using the above detector at 50 or 100 nA range and +0.6 V potential. The data were analyzed as above at 10-30 mV full scale, a peak width of 0.4 min and a peak sensitivity of 3.5% and expressed as μmol α-tocopherol g^{-1} fresh weight. At least two separate measurements were taken for each vitamin E extraction of treated leaf and their mean value was used as one replicate. The mean and standard error of three such replicates from three different experiments were then plotted.
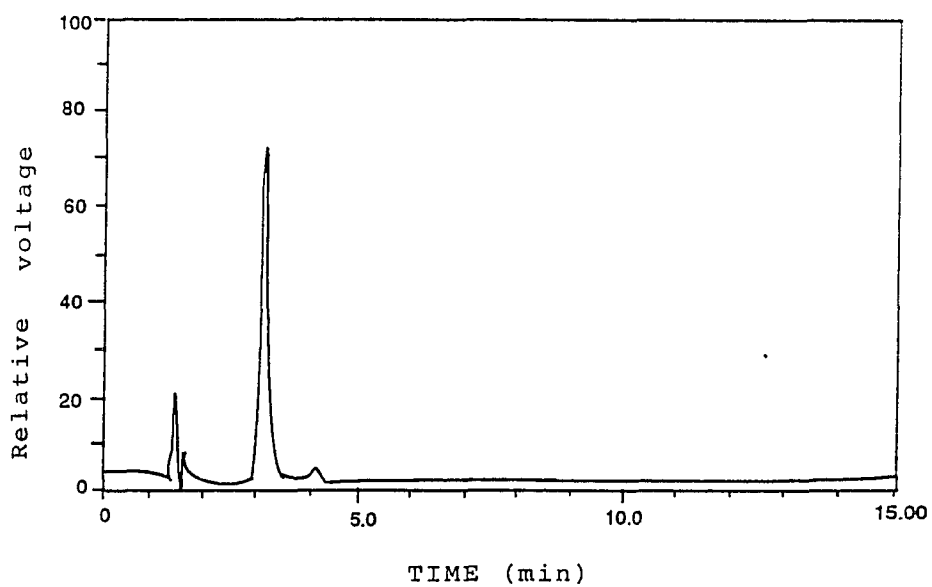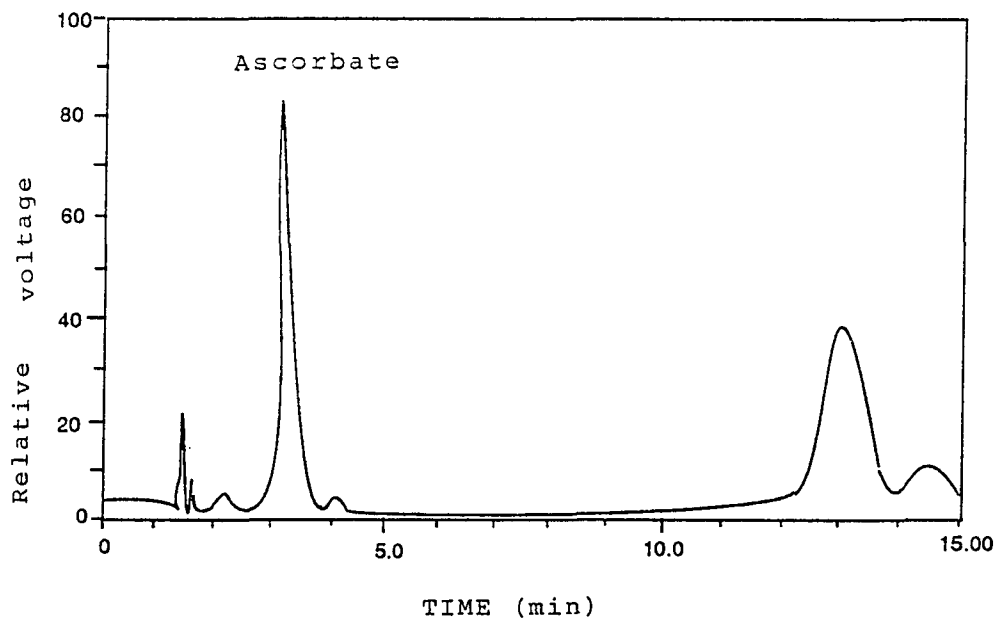
Extraction and Measurement of Xanthophylls

<u>Extraction</u>: One gram treated deribbed leaves were frozen in liquid nitrogen and stored at -80°C. They were first ground in liquid nitrogen followed by 3 ml of 85% acetone in a mortar and pestle. The extract was transferred to a tube and the mortar was rinsed with 100% acetone and the wash collected. The extract was then kept on ice in dark for 15 min, centrifuged at 20,000g for 10 min at 4°C and the pellet reextracted twice with 3 ml of 85% acetone. The pooled supernatant was filtered through a 0.2 μm filter, stored under nitrogen gas at -20°C and injected into the HPLC system.

<u>Separation</u>: Xanthophylls were separated on a 25 cm long C18 Bakerbond column, using the above hardware and a flow rate of 1.0 ml min^{-1}. The column was first equilibrated with 1% water in MeOH for at least 10 min and then changed to 100% acetone at the time of sample injection using a 4-way switching valve.

<u>Standard</u> : The concentration of a pure zeaxanthin standard was determined using a dual beam spectrophotometer (Perkin-Elmer Lambda-3B) and the extinction coefficient of zeaxanthin (Thayer and Bjorkman, 1992). This was then injected into the HPLC system and a conversion factor for calculating μg of xanthophyll per peak area was obtained. A representative chromatograph of a known concentration of zeaxanthin is shown in Fig. 11. The retention time of zeaxanthin was 6.4 min.
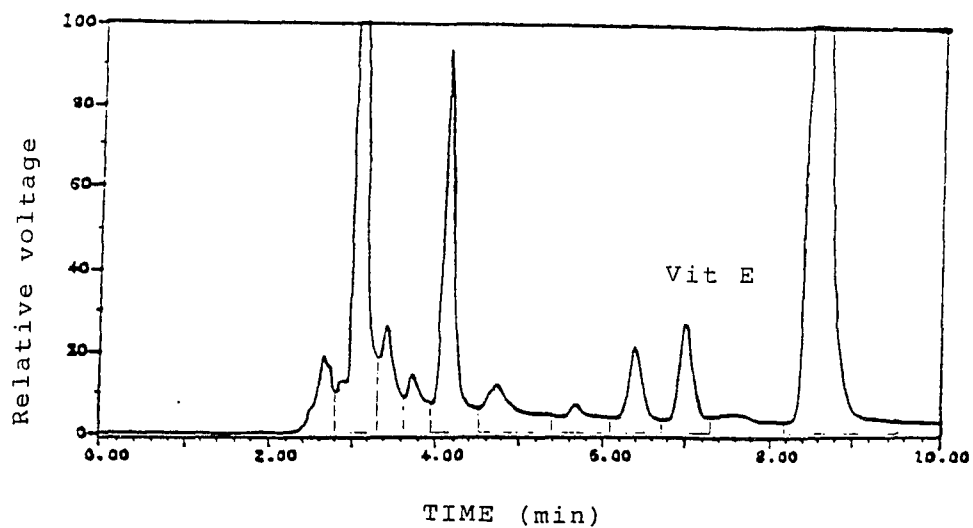
Fig. 10. Scaled plot of α-tocopherol extracted from *Zea diploperennis* leaves treated to 25°C, 2% oxygen in light. Integrated at 10.0 mV full scale, for 10 min, EC detection, peak width 0.40 min, peak sensitivity 3.5%. Retention time of α-tocopherol = 6.99 min

Detection: Peak detection was performed using a Beckman 164 variable wavelength detector set at 450 nm with a full-scale range of 0.05 absorbance units. The data were analyzed using the above software and expressed as $\mu g \ g^{-1}$ F. W.. A representative chromatograph of an untreated control sample is shown in Fig. 12. The retention time of violaxanthin was 4.2 min, of antheraxanthin 5.1 min and that of zeaxanthin was 6.4 min. At least two analyses per sample of xanthophyll extracts were done and their mean was used to represent one replicate. The mean and standard error of three such replicates from three different experiments were then plotted.

Analysis of scavenging enzymes

Enzyme extraction: One-half gram of treated leaf was ground in liquid nitrogen and extracted with 3.3 ml of chilled extraction solution consisting of 50 mM phosphate (pH 7.0), 10 mM ascorbate, 5 mM DTPA and 1.0 ml of PVPP slurry by homogenizing in a Polytron blender for 5 s, and centrifuging at 24,000 g for 10 min at 4°C, as described by Jahnke et al., (1991). The supernatant was desalted on a Sephadex G-25 column pre-equilibrated with 100 mM phosphate buffer (pH 7.0) containing 0.2 mM DTPA and 40 μM ascorbate. The extract was then used to assay GTR, MDHAR, APX and SOD activities and to measure its soluble protein content. At least two separate measurements were taken from each extraction of treated leaf and their mean value was used as one replicate. The mean and standard error of three such replicates from three different experiments were then plotted.

Determination of soluble protein concentration: Protein concentrations of the extracts were determined according to Bradford (1976). 3.0 ml of Bradford reagent (from Bio-Rad) was added to 50 μl of the extract, obtained after desalting on Sephadex G-25 column, incubated for 2-5 min and its absorbance was measured at 600 nm. Protein was calculated using a standard curve obtained with 5-40 μg bovine serum albumin.
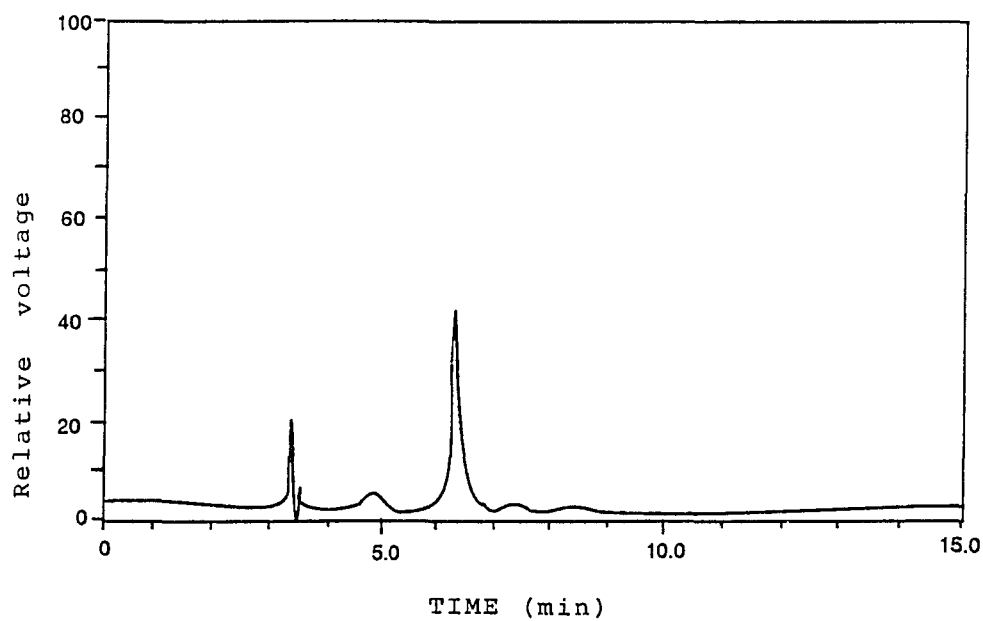
Fig. 11. Scaled plot of zeaxanthin standard. Integrated at 10.0 mV full scale, for 15 min, UV detection at 450 nm, peak width 0.90 min, peak sensitivity 1.0%. Retention time of xeaxanthin = 6.4 min.
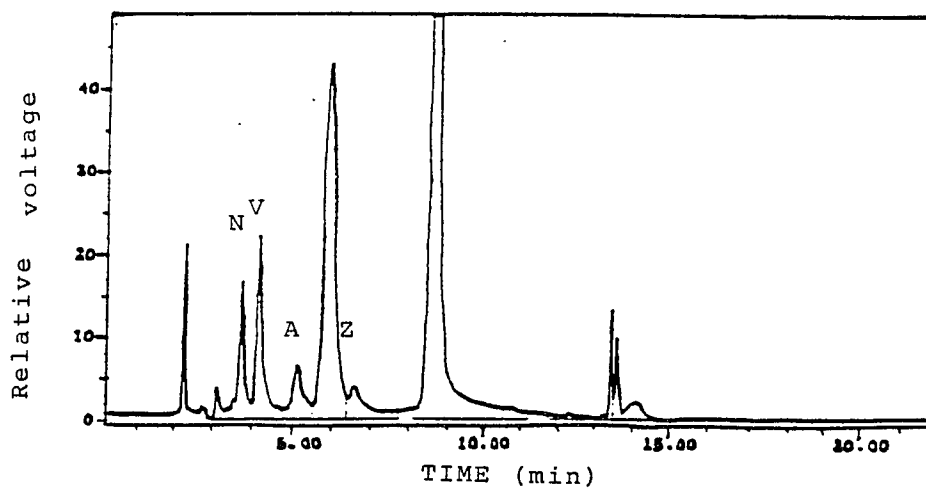
Fig. 12. Scaled plot of xanthophylls extracted from untreated control leaves of corn. Integrated at 19.0 mV full scale, for 22 min, UV detection at 450nm, peak width 0.30 min, peak sensitivity 1.0%. Retention time of violaxanthin = 4.2 min, antheraxanthin = 5.1 min and of zeaxanthin = 6.4 min.

<u>Enzyme assays:</u>

Ascorbate peroxidase (EC 1.11.1.11) (APX) activity was determined by the procedure of Jahnke et al., (1991). A 1.0 ml reaction mixture contained 50 mM phosphate (pH 7.0), 0.2 mM DTPA, 0.5 mM ascorbate, 50 µl extract and 0.25 mM hydrogen peroxide. Oxidation of ascorbate was monitored directly in the above mixture at 290 nm using a dual-beam spectrophotometer (Perkin-Elmer Lambda-3B) equipped with a 1 nm slit in quartz semi-micro cuvets.

Monodehydroascorbate reductase (EC 1.6.5.4) (MDHAR) activity was measured by the procedures of Jahnke et al., (1991). A 1.0 ml reaction mixture contained 25 mM phosphate (pH 7.8), 0.2 mM DTPA, 0.5 mM ascorbate, 0.15 mM NADH, 0.2 Units of ascorbate oxidase and 50 µl extract. Oxidation of NADH was monitored directly in the above mixture at 340 nm in semi-micro cuvets.

Glutathione reductase (EC 1.6.4.2) (GTR) activity was determined according to Schaedle and Bassham (1978). A 1.0 ml reaction mixture contained 25 mM phosphate (pH 7.8), 0.2 mM DTPA, 0.5 mM oxidized glutathione, 0.15 mM NADPH and 50 µl extract. Oxidation of NADPH was monitored directly in the above mixture at 340 nm in glass cuvets.

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RESULTS

Part A: RECOVERY EXPERIMENTS of *Zea mays*

These experiments follow various photosynthetic parameters before and after a 6-h low-temperature, high-light stress exposure, and pursue the changes in the photosynthetic activity and antioxidant concentrations during a 2-day non-stress recovery period.

Photoinhibition

Fluorescence induction changes: The F_v/F_m ratio of corn plants during the 6 h of chilling at $5^\circ C$ and 1500 μmol m^{-2} s^{-1} of light in air (Fig. 13 and 14) declined by about 63% from a control value of 0.76. The sharp drop in F_v/F_m ratio with chilling was partly due to an increase of F_o (increased by 102.5% of control) and partly due to the decrease of F_m (deceased by 74% of control). When plants were allowed to recover at room temperature, in air, at 25 μmol m^{-2} s^{-1} light, the F_v/F_m ratio increased sharply to 0.55 by 2 h and had recovered to 93% of pre-stress control within 49 h. This recovery was mostly due an increase in the F_m value, which superseded the control value of 524 fluorescent units by 3 h, and had increased by 98% over control values within 49h.

Quantum yield and light-saturated rates of photosynthesis: The Asat of photosynthesis decreased by ca. 32% during chilling (Fig. 15) from a control value of 17.9 μmol m^{-2} s^{-1} of oxygen. In contrast to fluorescence data, however, the rates did not recover on return to ambient temperature. After 3 h of recovery, the leaves continued to show inhibition, and the Asat was repressed further by ca. 77.5% at 49h.

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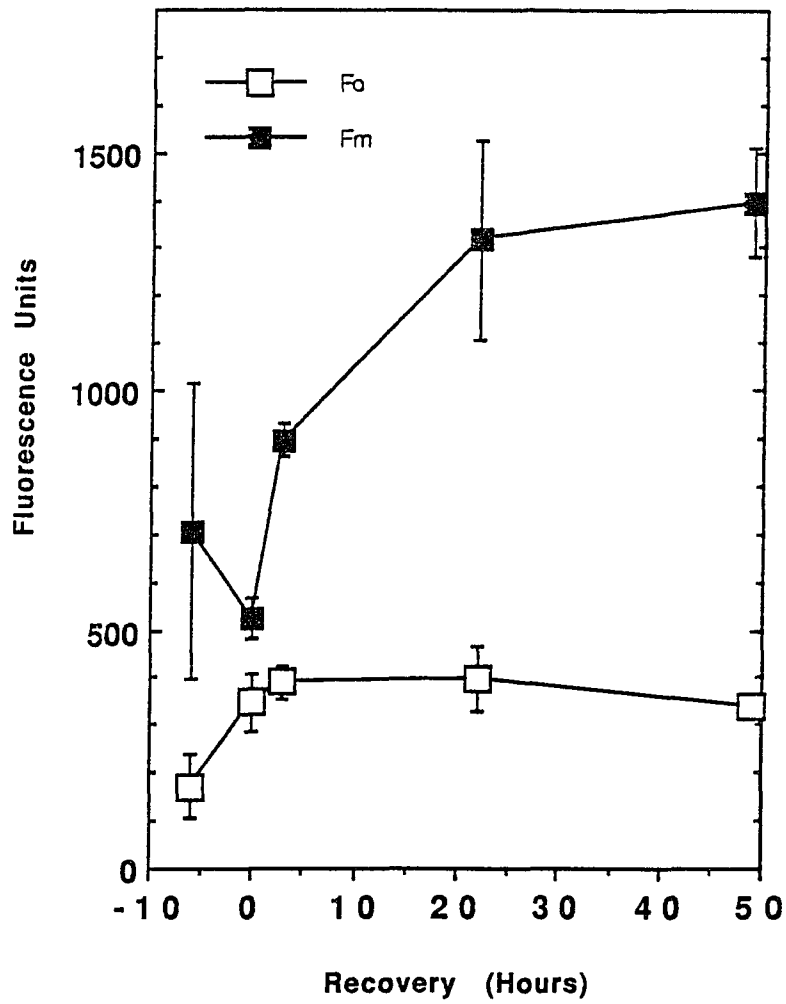
Fig 13. Fo and Fm values of corn leaves during recovery from chilling stress at -6h to 0h at 1500 μmoles/m2/s at 5°C in air.
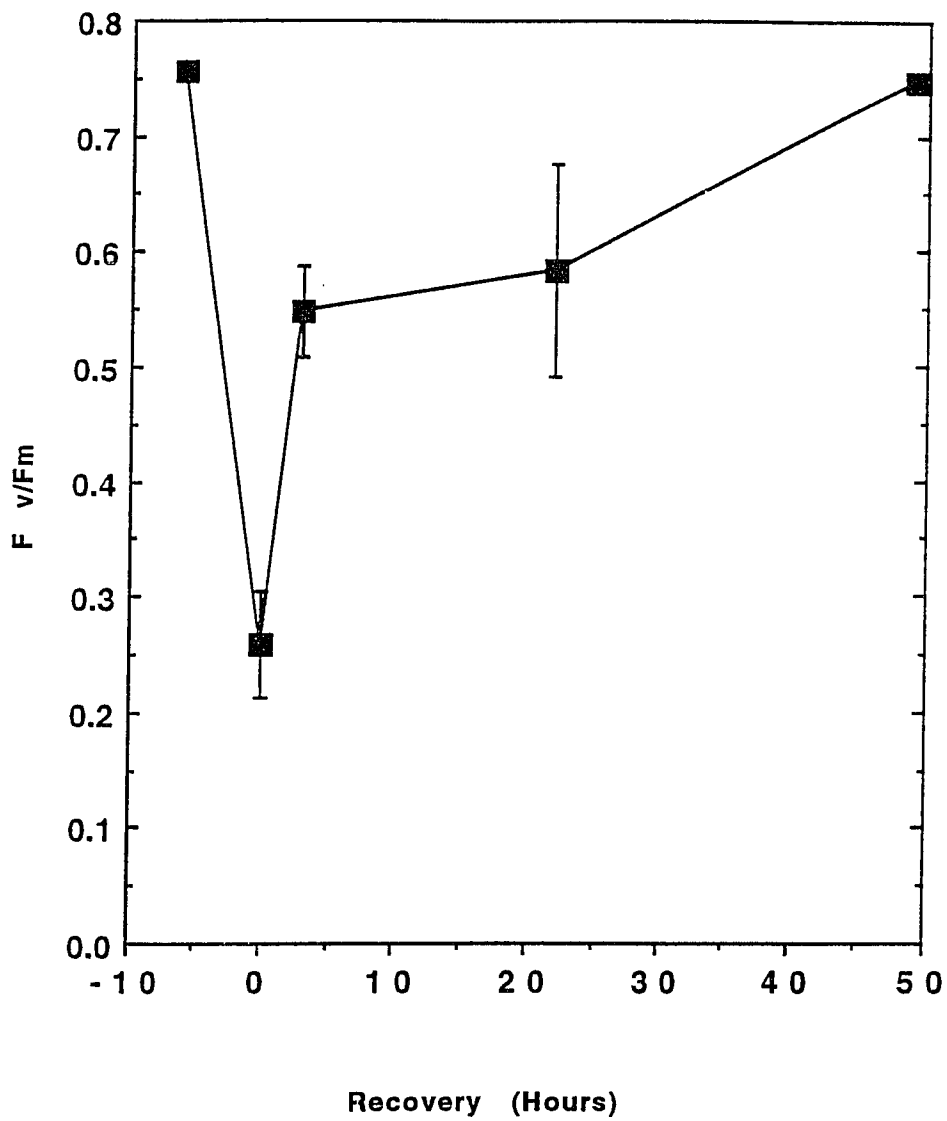
Fig 14. Fv/Fm ratios of corn leaves during recovery
from chilling stress at -6h to 0h at 1500 μmoles/m2/s
at 5°C in air.

The effects of chilling on quantum yield (ϕ_{O2}) were less drastic than on Asat. There was a decrease in ϕ_{O2} (Fig. 15) from 0.048 to 0.018 (ca. 38% of control). On return to ambient temperature the ϕ_{O2} recovered to ca. 44% of control within first 3 h, and then continued to increase to 0.044 by 49h (ca. 92% of control).

Antioxidants

Vitamin E: The vitamin E (α-tocopherol) content of corn leaves decreased (by ca. 57%) from 77.3 mg g^{-1} F. W. to 33.3 mg g^{-1} F. W. (Fig. 16) during chilling treatments. On return to room temperature, the α-tocopherol levels recovered from 2 to 22 h to 4.9 mg g^{-1} F. W.. Thereafter, the α-tocopherol level increased to ca. 92% of controls within 49 h.

Ascorbate, dehydroascorbate and total ascorbate: Ascorbate decreased (by ca. 36%) from 466.0 mg g^{-1} F. W. to 165.5 mg g^{-1} F. W. during chilling (Fig. 17). On return to ambient temperature, no recovery of reduced ascorbate levels was seen up to 22 h. The levels increased to ca. 90% of control by 49 h.

Dehydroascorbate decreased (by ca. 30%) from 115.4 mg g^{-1} F. W. to 34.5 mg g^{-1} F. W. during chilling (Fig. 17). On return to ambient temperature, DHA levels were recovered completely within 3 h and maintained up to 49 h.

There was a ca. 34% decrease in the total ascorbate during chilling (Fig. 17). On return to ambient temperature, the ascorbate pool size increased to ca. 54% of control within 3 h, and then to 85% by 49h.

The ratio of mean reduced ascorbate: mean DHA (Fig. 18) increased slightly to 4.8 from an untreated control value of 4.04 during chilling. On return to ambient temperature, this ratio decreased to 1.01 (a decrease of ca. 7%) within 3 h, and was maintained at this ratio until 22 h. By 49 h the ratio had increased to 5.3.

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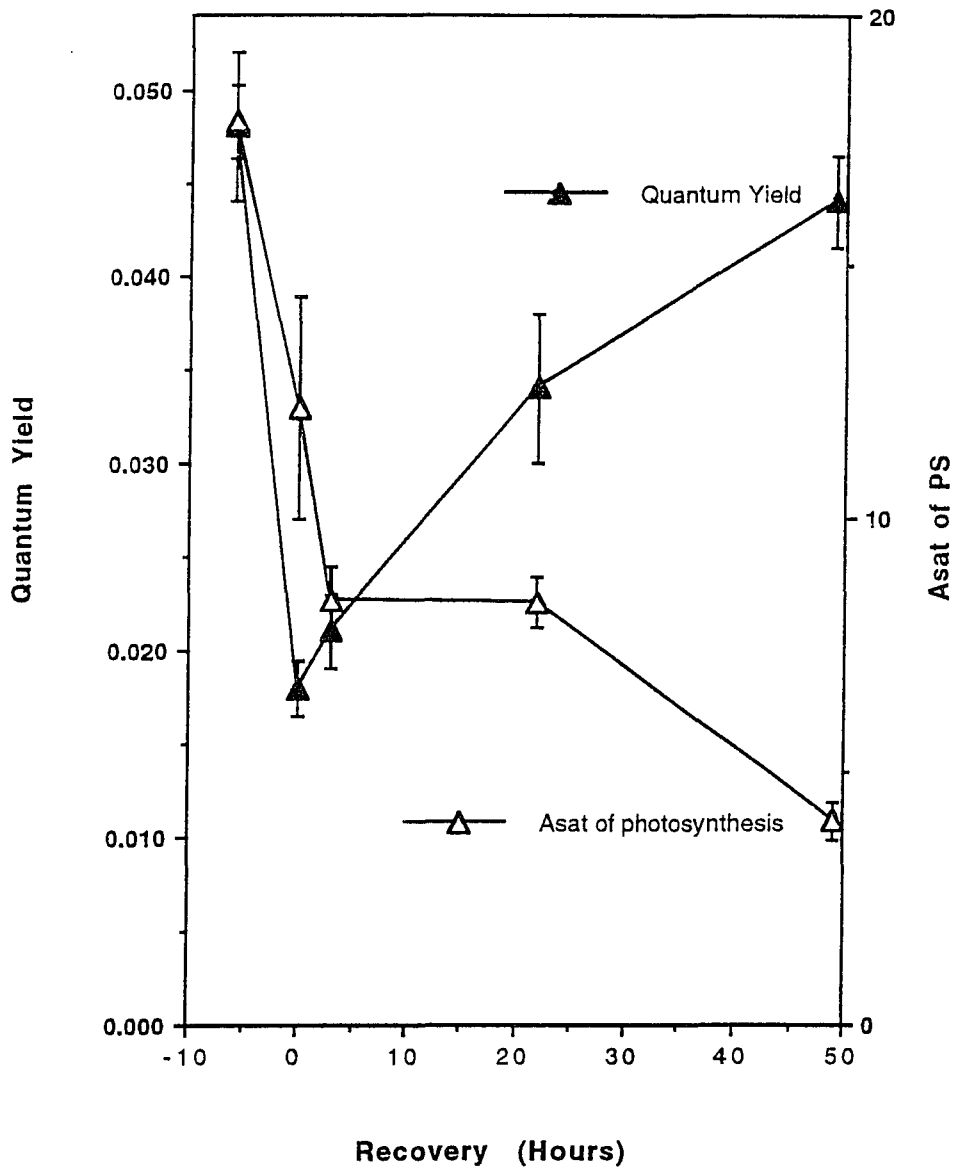
Fig 15. Quantum yield and Asat of photosynthesis during recovery of corn leaves from chilling stress at -6h to 0h at 1500 μmoles/m2/s at 5°C in air.
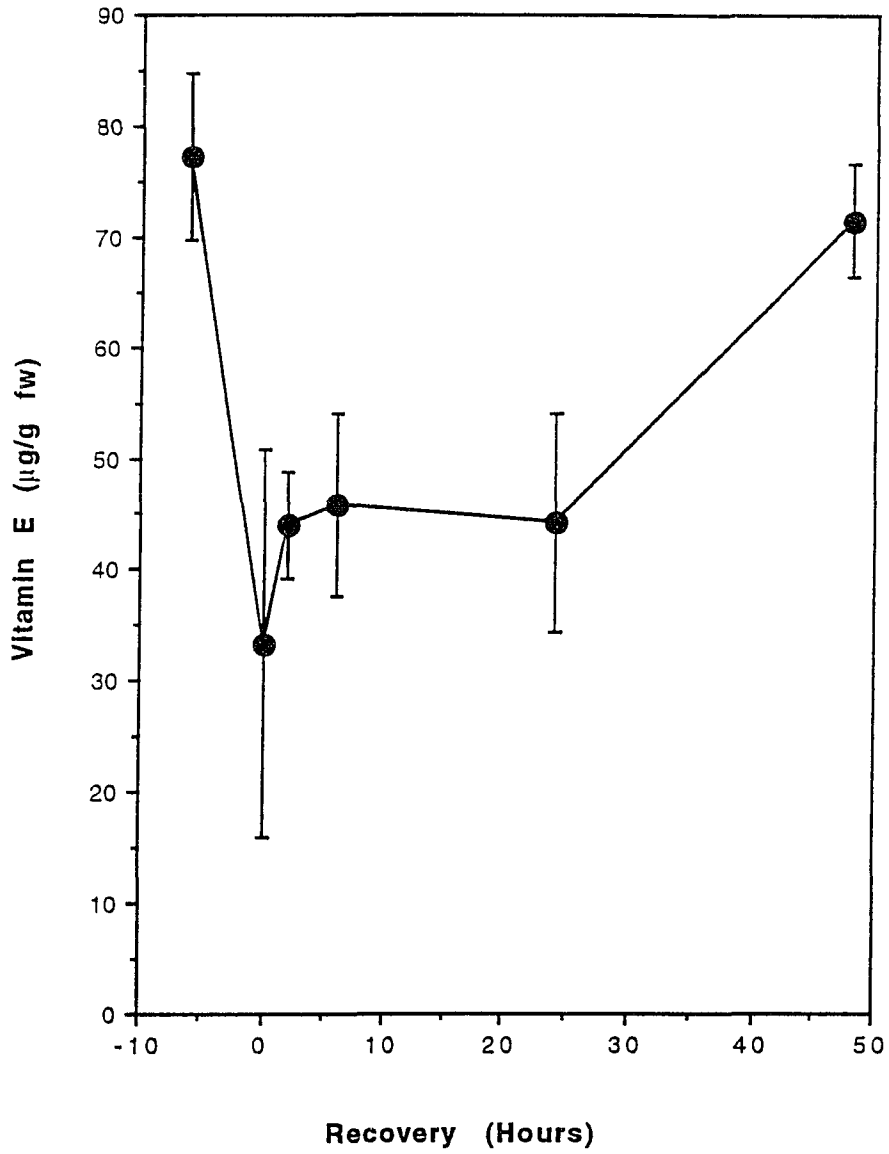
Fig 16. Vitamin E content of corn leaves during recovery
from chilling stress at -6h to 0h at1500 μmoles/m2/s
at 5°C in air.
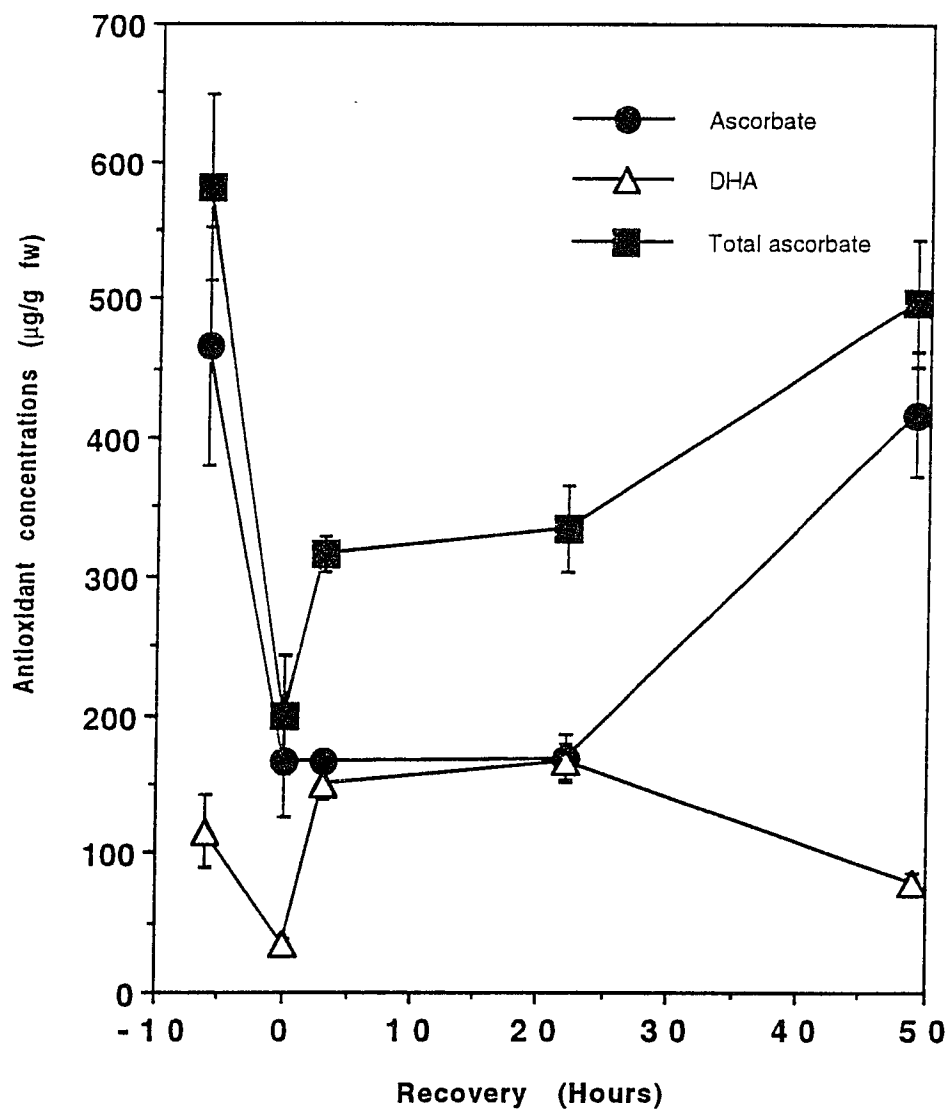
Fig 17. Ascorbate, DHA and total ascorbate levels in corn leaves during recovery from chilling stress at -6h to 0h at 1500 μmoles/m2/s at 5°C in air.
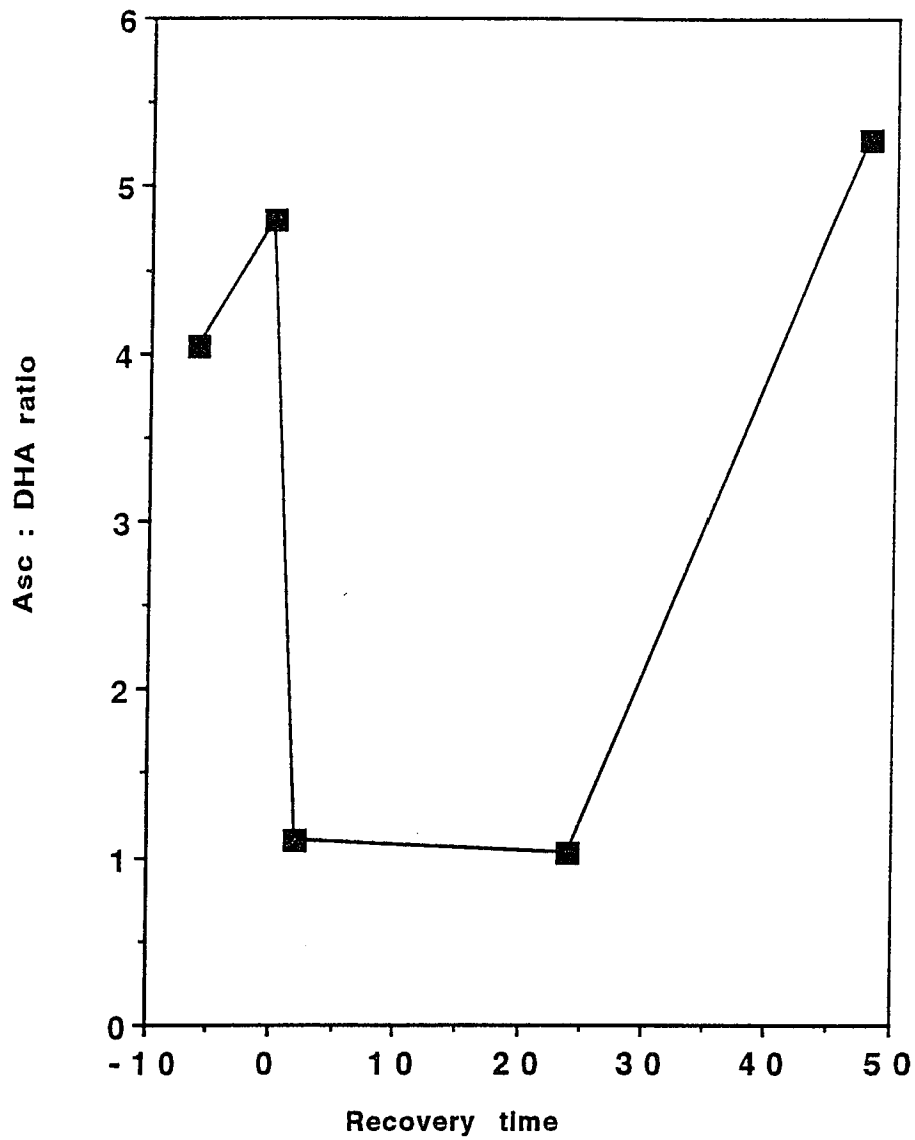
Fig 18. Asc/DHA ratio of corn leaves during recovery from chilling stress at -6h to 0h at 1500 μmoles/m2/s at 5°C in air.

Activities of Scavenging Enzymes

Ascorbate peroxidase: The specific activity of ascorbate peroxidase decreased by ca. 49% within 6h of lowering the temperature in presence of light (Fig. 19). But the activity had recovered to 100% by 3 h of exposure to ambient temperature and low light. Thereafter, a slow decline in the activity was observed, followed by a stable activity between 22h and 49h. Thus the final activity at the end of this recovery experiment was significantly lower (by ca. 33%) than the control levels.

Monodehydroascorbate reductase: A ca. 62% decrease in the activity of monodehydroascorbate reductase was also observed on exposure to chilling stress (Fig. 20). This inhibition too had recovered completely within 3 h of exposure to normal temperature and low light. By 6 h of recovery treatment, the activity was reduced to ca. 30% of control and maintained as such until 49 h.

Glutathione reductase: The activity of glutathione reductase declined only by ca. 28% on exposure to chilling stress (Fig. 20). On return to ambient temperature and low light, the activity recovered completely within 3 h. Thereafter, the activity returned to control levels by 6 h and was maintained as such up to 49 h of recovery treatment.
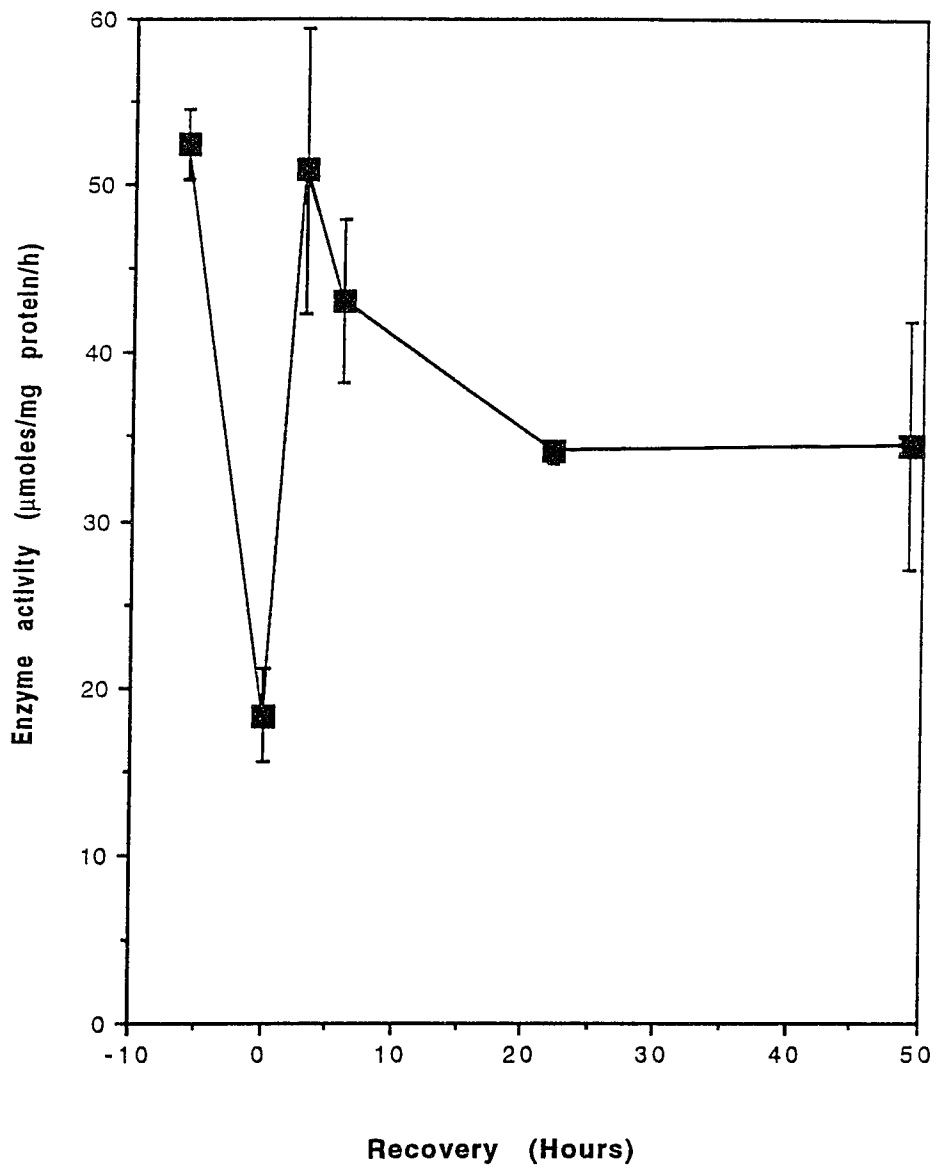
Fig 19. Activity of APX during recovery of corn leaves
from chilling stress at -6h to 0h at 1500 μmoles/m2/s
at 5°C in air. Enzymes were extracted and assayed at 25°C.
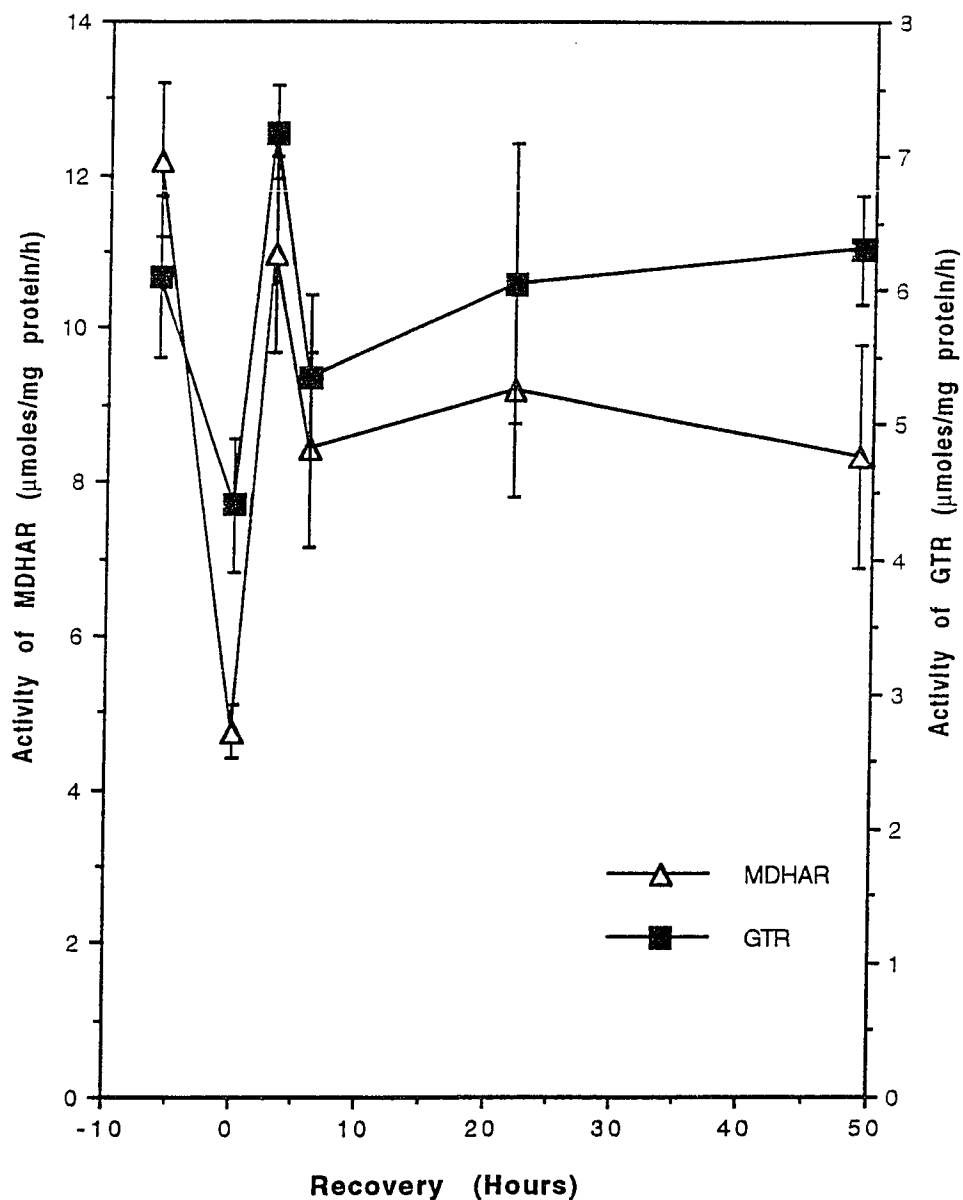
Fig 20. Activities of MDHAR and GTR during recovery of corn leaves from chilling stress at -6h to 0h at 1500 μmoles/m2/s at 5°C in air. Enzymes were extracted and assayed at 25°C.

Part B: OXYGEN LEVELS AND CHILLING PHOTOINHIBITORY EXPERIMENTS WITH *Zea mays* and *Z. diploperennis*

These experiments follow the effects of three oxygen concentrations on various photosynthetic parameters and antioxidants before and after a 6 h exposure with and without light at two temperatures, in both *Zea* species.

Photoinhibition

Fluorescence induction changes: The untreated control leaves of corn had an Fv/Fm ratio of 0.756 (Table 1a) and *Z. diploperennis* of 0.75 (Table 1b). In dark, there was no change in the Fv/Fm ratio between 5°C and 25°C nor under various oxygen concentrations in either species. The Fv/Fm ratios under all of the above conditions were close to the control values in both species. Hence, dark treatments did not show any quenching of fluorescence, even following low temperature incubation.

There was slight quenching of fluorescence at 25°C in light (reduced by ca. 16% compared to control) at both 20% and 40% oxygen in corn (Fig. 21). However, at 25°C and 2% oxygen in light, more than 32% quenching was observed. *Z. diploperennis* showed similar quenching of fluorescence (ca. 25%) at 2% and 20% oxygen at 25°C as corn. However, at 40% oxygen there were distinct differences between the two species. Maximum quenching was observed with lowering of temperature to 5°C, in the presence of light. Higher oxygen levels seemed to impart some protection to corn from photoinhibition at this temperature and in presence of light, where only a ca. 68% reduction in quenching was observed at 40% oxygen compared to ca. 83% reduction at 2% oxygen. A 63% decrease in Fv/Fm was seen in *Z. diploperennis* compared to control values. No significant difference was, however, observed between the oxygen concentrations in *Z. diploperennis* at this temperature. However, much less quenching in light at low temperature took place in *Z. diploperennis* in comparison to corn.

Table 1a: Photosynthetic data from corn treated to 6h of dark under varying oxygen concentrations and temperature (data showing mean $\pm$ S.E.). Untreated corn had a Fv/Fm ratio of 0.756 ± 0.008; a ϕ_{O2} value of 0.048 ± 0.004; and an A_{sat} value of 17.89 ± 0.72 μmoles of $O_2.m^{-2}.s^{-1}$.

Temperature :	Fv/Fm		ϕ_{O2}		A_{sat}	
	5°C	25°C	5°C	25°C	5°C	25°C
2% oxygen	0.738 ±0.03	0.757 ±0.004	0.034 ±0.004	0.038 ±0.004	10,9 ±1.4	10.8 ±1.9
20% oxygen	0.752 ±0.01	0.707 ±0.001	0.032 ±0.001	0.04 ±0.002	7.9 ±0.9	6.4 ±0.7
40% oxygen	0.756 ±0.01	0.799 ±.006	0.048 ±0.006	0.042 ±0.002	8.2 ±0.6	4.9 ±0.2

Table 1b: Photosynthetic data from Z. *diploperennis* treated to 6h of dark under varying oxygen concentrations and temperature (data showing mean $\pm$ S.E.). Untreated leaves had a Fv/Fm ratio of 0.747; a ϕ_{O2} value of 0.046 ± 0.007; and an A_{sat} value of 7.72 ± 0.132 μmoles of $O_2.m^{-2}.s^{-1}$.

Temperature :	Fv/Fm		ϕ_{O2}		A_{sat}	
	5°C	25°C	5°C	25°C	5°C	25°C
2% oxygen	0.766	0.760	0.058 ±0.0	0.054 ±0.002	11.4 ±0.61	13.8 ±0.60
20% oxygen	0.756 ±0.0	0.757 ±0.02	0.056 ±0.004	0.06 ±0.002	10.3 ±0.77	4.8 ±0.38
40% oxygen	0.776 ±0.02	0.783	0.054 ±0.003	0.062 ±0.003	8.4 ±0.59	4.4 ±0.60
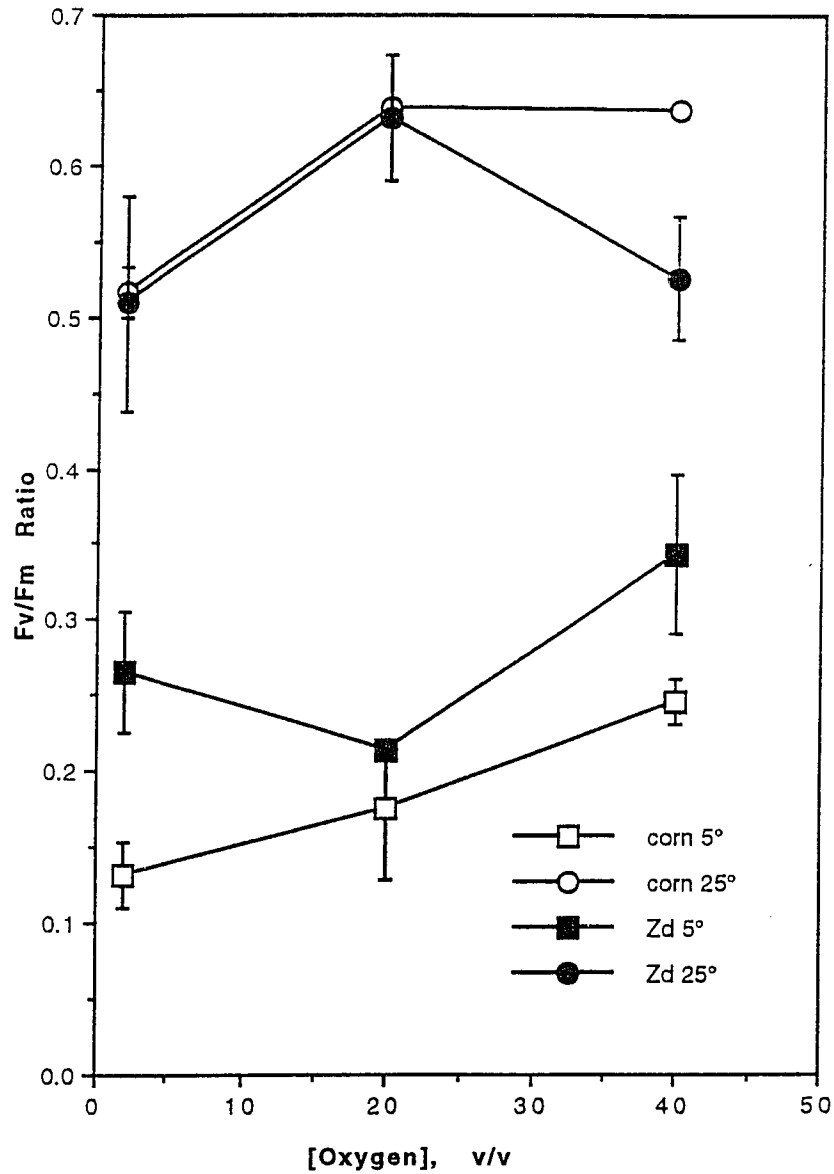
Fig 21. Fv/Fm ratios of corn and Z. diploperennis (Zd) after 6h light treatments at 1500 μmoles/m2/s at either 5° or 25°C, and various oxygen concentrations. The untreated control value at 20% oxygen was 0.76 for corn and 0.75 for Zd.

Light-saturated rates of photosynthesis and quantum yield: The light-saturated rate of photosynthesis (i.e. Asat) were measured by oxygen electrode. The untreated control leaves of corn had an Asat rate of 17.9 μmoles of oxygen $m^{-2} s^{-1}$ (Table 1a). There was a significant decrease in all light-saturated rates following the six hour dark treatment in corn. At both 25°C and 5°C, the rates decreased with increase of oxygen concentrations. Asat values (Table 1b) of Z. diploperennis did not show a decrease from the control values after the dark treatment at 25°C. The rates were actually higher at 2% oxygen (by ca. 78%) in comparison to the control value of 7.72 μmol $m^{-2} s^{-1}$. At 20% and 40% oxygen, however, the Asat values were similar to the control in Z. diploperennis. Following 5°C in dark, the Asat values decreased with an increase in oxygen concentrations in this species.

In light and at normal temperatures, the Asat rates at 20% and 40% oxygen showed small differences from the control value of 17.9 μmoles of oxygen $m^{-2} s^{-1}$ in corn (Fig 22). But a ca. 50% decrease was observed at 2% oxygen at this temperature. Chilling temperatures in presence of light again showed the oxygen effect in corn, where higher oxygen concentrations seemed to impart partial protection from photoinhibition. Hence at 2% oxygen, 5°C temperature, and 1500 μmol $m^{-2} s^{-1}$ of light, maximum inhibition of Asat (decreased by ca. 80% from control) was observed. On exposure to 25°C and light, no significant difference was observed in Z. diploperennis in the light-saturated rates between oxygen concentrations. On exposure to light at low temperature, a definite oxygen effect was observed, with 2% oxygen having a much higher Asat value than control. Hence, the higher oxygen concentrations were similar to the untreated control values of Z. diploperennis at both temperatures.

Quantum yield (ϕ_{O2}) of untreated control leaves, as measured by oxygen evolution, was 0.048 (Table 1a) for corn leaves and 0.046 (Table 1b) for Z
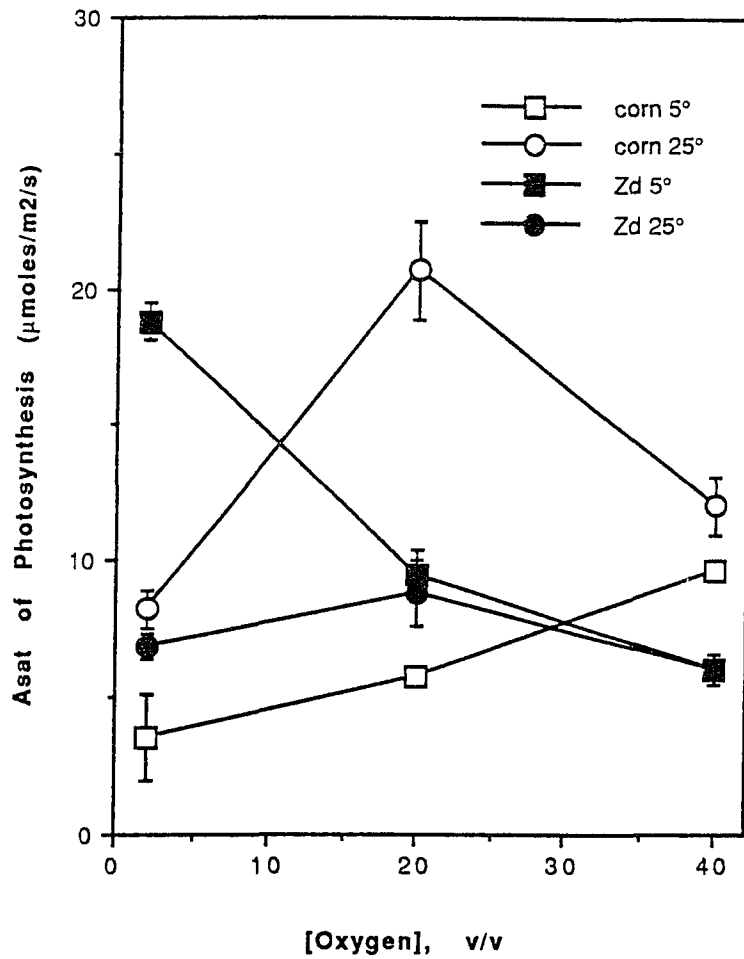
Fig 22. Asat rates of corn and Z. diploperennis (Zd) after 6h light treatments at 1500 μmoles/m2/s at either 5° or 25°C, and various oxygen concentrations. The untreated control value at 20% oxygen was 17.9 for corn and 7.7 for Zd.

. *diploperennis*. In the dark treated leaves, no significant changes in the quantum yield were observed in relation to either temperature or oxygen concentration in either species.

Under high light at 25°C, a ca. 40% reduction in quantum yield was seen in corn at 20% or 40% oxygen when compared to control (Fig. 23). But a decrease of more than 65% was observed at 2% oxygen. The ϕ_{O2} values at 5°C in light was significantly lower than the other treatments. Low temperature exposure in presence of light also showed comparable oxygen effects in corn as seen above in the Fv/Fm data. Where 40% oxygen produced a 65% inhibition from control compared to 88% at 2% oxygen. In the light treated leaves of *Z. diploperennis*, a decrease in the quantum yield (of ca. 10% of control) was observed at 25°C but no significant difference between the various oxygen concentrations was noticed. With lowering of temperature in presence of light, a further decrease (ca, 65% of control) was observed, in this species. No oxygen effects were observed even at this low temperature. Note that at 2% oxygen at 5°C in light, *Z. diploperennis* was more cold tolerant than corn (i.e., suffered a smaller reduction in quantum yield). Also the oxygen effect (i.e. increased protection against photoinhibition with increasing oxygen concentration) at low temperature in light was absent in *Z. diploperennis*.

Antioxidants

Ascorbate: There were no significant differences in the reduced ascorbate levels in the dark between low and high temperatures and at varying oxygen concentrations in corn (Table 2a) or *Z. diploperennis* (Table 2b). However, a decrease in the ascorbate levels, compared to untreated control values of 466 $\mu g\ g^{-1}$ F. W., was noted in all the above treatments.

When exposed to high light at 25°C, the reduced ascorbate contents did not vary significantly from the dark treatments in corn (Fig. 24). However, a significant decrease
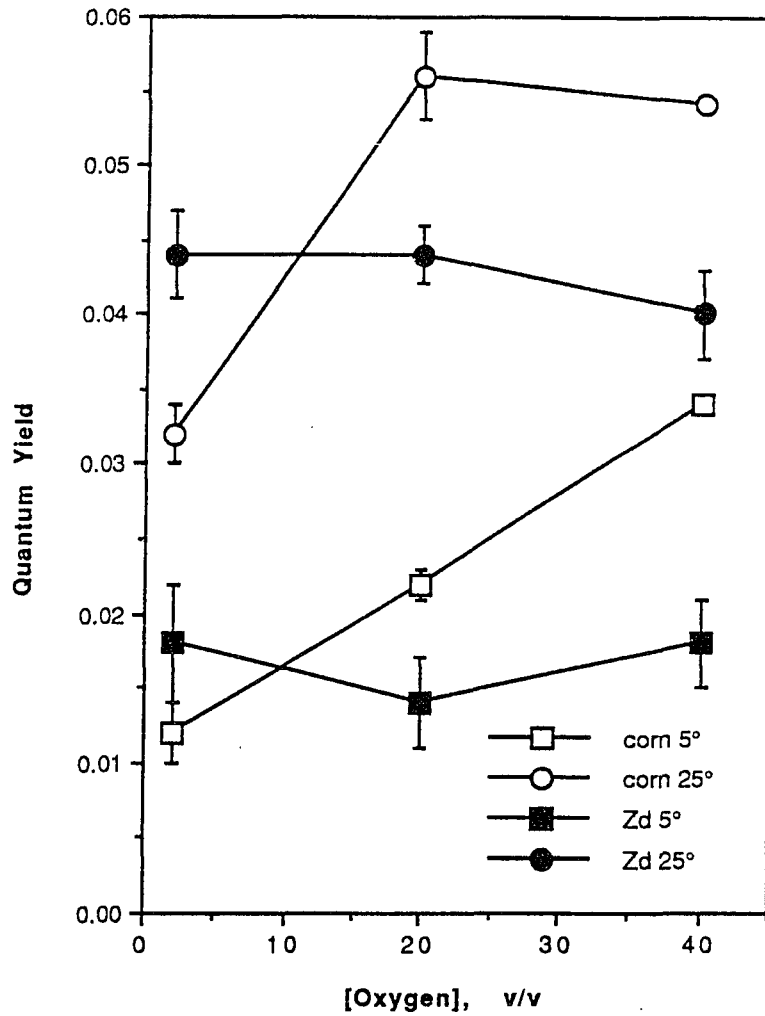
Fig 23. Quantum yield of corn and Z. diploperennis (Zd) after 6h light treatments at 1500 µmoles/m2/s at either 5°C or 25°C, and various oxygen concentrations. The untreated control value at 20% oxygen was 0.048 for corn and 0.046 for Zd.

Table 2a: Ascorbate data from corn treated to 6h of dark under varying oxygen concentrations and temperature (data showing mean ± S.E.). Untreated corn had an ascorbate concentration of 466.04±41.04;   a DHA concentration of 115.44±25.44; and total ascorbate concentration of 581.48±67.48 µg.g fw⁻¹.

Temperature :	Ascorbate		DHA		Total ascorbate	
	5°C	25°C	5°C	25°C	5°C	25°C
2% oxygen	154.52 ±13.83	98.14 ±8.80	41.77 ±0.68	51.75 ±34.75	196.28 ±13.15	149.88 ±43.54
20% oxygen	148.66 ±13.72	106.17 ±33.29	80.96 ±23.58	43.00 ±7.58	229.62 ±9.86	149.17 ±25.71
40% oxygen	124.61 ±36.86	126.55 ±42.91	15.05 ±19.85	52.51 ±11.54	238.51 ±35.35	179.06 ±31.38

Table 2b: Ascorbate data from *Z. diploperennis* treated to 6h of dark under varying oxygen concentrations and temperature (data showing mean ± S.E.). Untreated leaves had an ascorbate concentration of 147.82±33.19;   a DHA concentration of 211.79±107.62; and total ascorbate concentration of 359.61±93.47 µg.g fw⁻¹.

| Temperature :	Ascorbate		DHA		Total ascorbate	
	5°C	25°C	5°C	25°C	5°C	25°C
2% oxygen	105.55 ±8.15	156.41 ±32.94	188.50 ±76.92	151.04 ±6.20	294.05 ±85.07	307.5 ±39.14
20% oxygen	167.06 ±31.60	164.09 ±82.70	349.05 ±16.19	201.38 ±16.01	516.1 ±47.79	365.5 ±66.69
40% oxygen	110.12 ±1.27	139.73 ±0.26	240.01 ±116.77	274.80 ±44.53	350.1 ±115.50	414.5 ±44.79
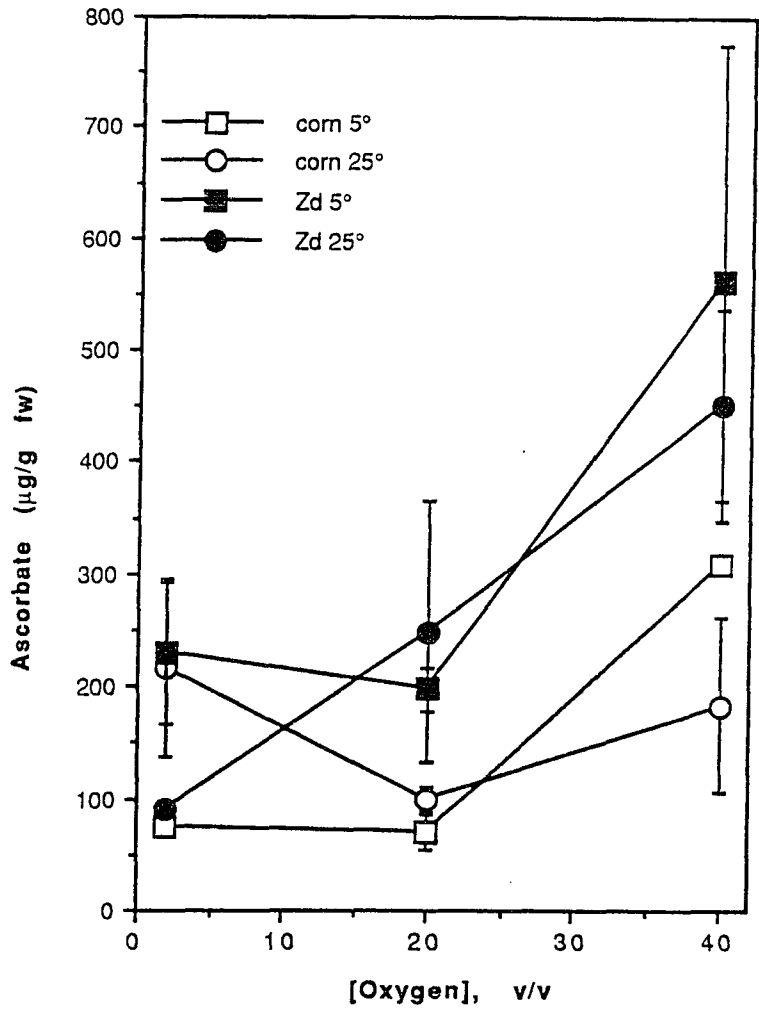
Fig 24. Ascorbate levels of corn and Z. diploperennis (Zd) after 6h light treatments at 1500 μmoles/m2/s at either 5°C or 25°C, and various oxygen concentrations. The untreated control value at 20% oxygen was 466.0 for corn and 147.82 for Zd.

of ascorbate levels from control values were observed under all oxygen concentrations. At 5°C in light, the ascorbate concentration, in this species, did not vary significantly from dark values. A higher ascorbate concentration was observed at 40% oxygen at this temperature. However, marked reductions, at all oxygen levels at low temperature were observed in comparison with the corn control leaves. With exposure to light at both 5°C and 25°C, higher ascorbate concentrations were observed in Z. diploperennis with increasing oxygen concentrations. There was very little difference between the two temperatures in this species.

Dehydroascorbate: Untreated control leaves of corn had DHA concentration of 115 μg g⁻¹ F. W.. Only slight differences were observed in the DHA levels between oxygen concentrations at 25°C in dark (Table 2a). However, a ca. 44% decrease from control levels was observed in all dark treatments. In Z. diploperennis (Table 2b), however, no significant difference in DHA levels at any oxygen concentrations and temperatures were observed.

At 25°C in presence of light, an increase of ca. 73% was seen at 2% oxygen (Fig. 25) in corn. DHA concentrations at other oxygen concentrations were similar to dark treatments at this temperature. At 5°C in light, the DHA concentration decreased with increasing oxygen concentrations. The DHA levels of Z. diploperennis at 25°C in light was higher than at 5°C at all oxygen concentrations. There was also no oxygen effect observed at either temperature. Note, however, that at normal temperature, Z. diploperennis had almost twice the amount of DHA as corn.

Total Ascorbate : There was no significant difference in total ascorbate of corn (Table 2a) or Z. diploperennis (Table 2b) in dark between low and high temperatures and at varying oxygen concentrations There was, however, a decrease of ca. 67%, compared to untreated control values of 581.5 μg g⁻¹ F. W. in corn, in all the above treatments. No such decrease was observed in the Z. diploperennis values.
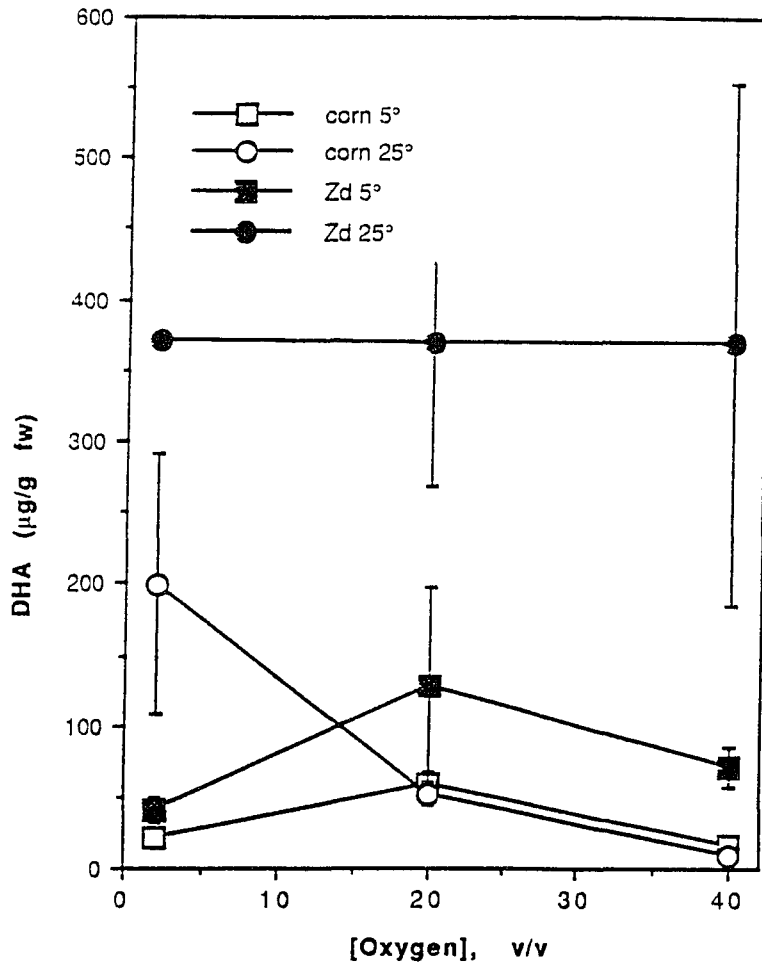
Fig 25. DHA concentrations of corn and Z.
diploperennis (Zd) after 6h light treatments
at 1500 μmoles/m2/s at either 5°C or 25°C, and
various oxygen concentrations. The untreated
control value at 20% oxygen was 115.4 for corn
and 21.79 for Zd.

When exposed to high light at 25°C at 2% oxygen, no significant difference in total ascorbate was observed versus the control corn leaves (Fig. 26). An increase in oxygen concentrations at 25°C resulted in a decrease of total ascorbate of corn. At 5°C in light, total ascorbate levels decreased at 2% and 20% oxygen. While at 40% oxygen, there was no significant difference from dark controls. Nevertheless, total ascorbate concentrations were significantly less than corn control leaves at 20% and 40% oxygen. The total ascorbate contents of Z. diploperennis leaves were higher at 25°C than at 5°C in light. An increase in total ascorbate was observed, at both temperatures, with an increase in oxygen concentration. Note that Z. diploperennis had higher total ascorbate concentrations at both temperatures, except 2% oxygen at low temperature, than corn.

Reduced ascorbate : DHA ratio: The ratio of reduced to oxidized ascorbate of corn remained around or slightly below (Fig. 27) an untreated control value of 4.0 (data not shown) under most conditions except 40% oxygen in presence of light. Under the latter condition, at both 5°C and 25°C, the ratio increased by more than 400%. The ratio in untreated Z. diploperennis control leaves was 0.7 (data not shown). There was no significant change in the ratios of dark treatments as well as light treatment at 25°C (Fig. 28). An increase was, however, observed at 2% and 40% oxygen at low temperature in presence of light. There was no increase observed at 20% oxygen in comparison to either dark or control treatments. Note, however, that at 5°C, in light at 40% oxygen, the increase in the ratio was much more enhanced in corn.

Activities of scavenging enzymes

Ascorbate peroxidase: There was no significant difference between APX activities in dark (Table 3) and light (Fig. 29), or as a function of temperature. Under most of the above conditions, however, the activities were significantly lower than those of untreated control leaves.
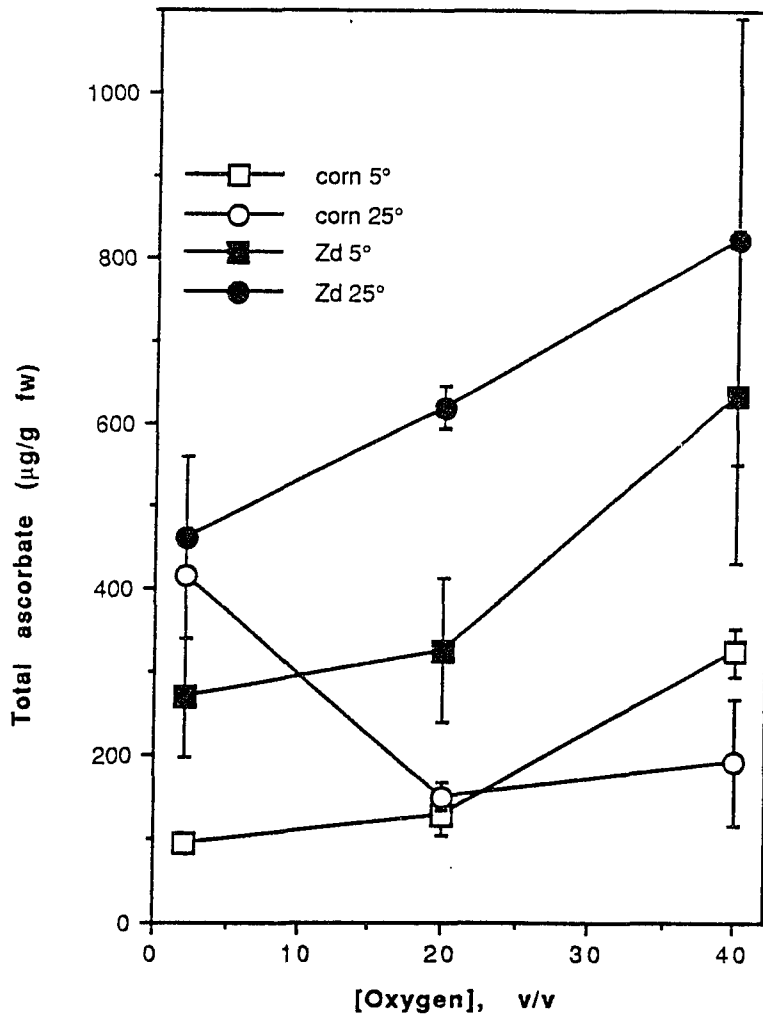
Fig 26. Total ascorbate content of corn and Z. diploperennis (Zd) after light treatments at 1500 μmoles/m2/s at either 5°C or 25°C, and various oxygen concentrations. The untreated control value at 20% oxygen was 581.5 for corn and 359.61 for Zd.
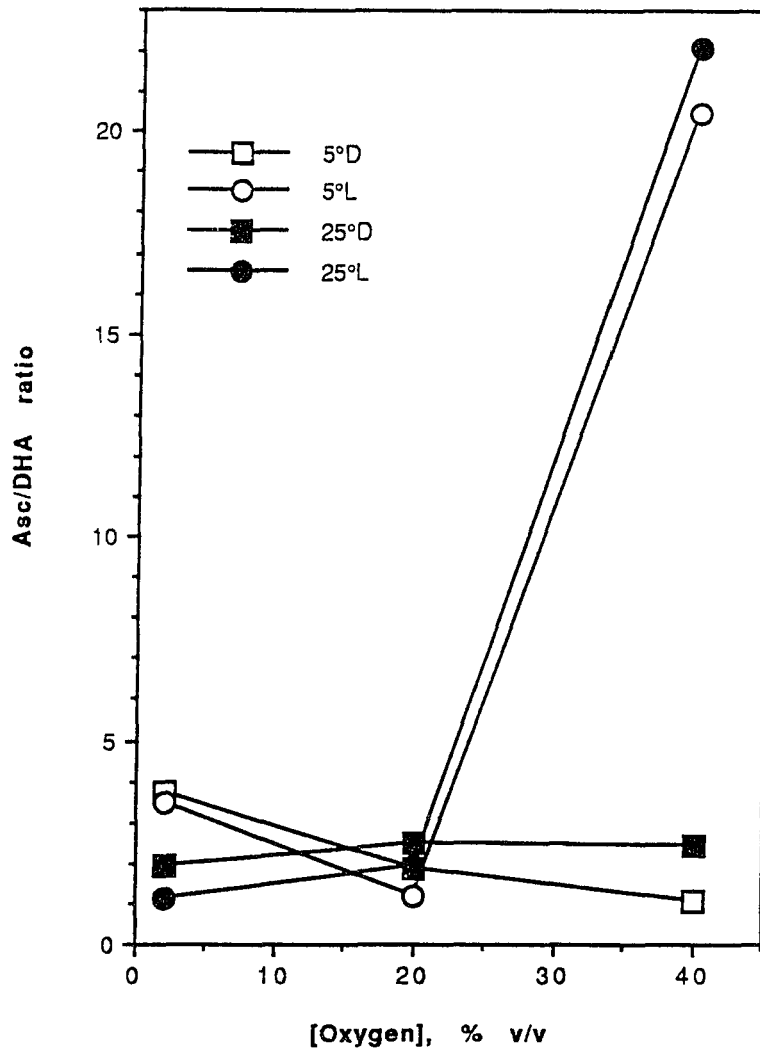
Fig 27. Ascorbate:DHA ratios of corn after 6h
light and dark treatments at either 5° or 25°C,
and various oxygen concentrations. The
untreated control value at 20% oxygen was 4.0.
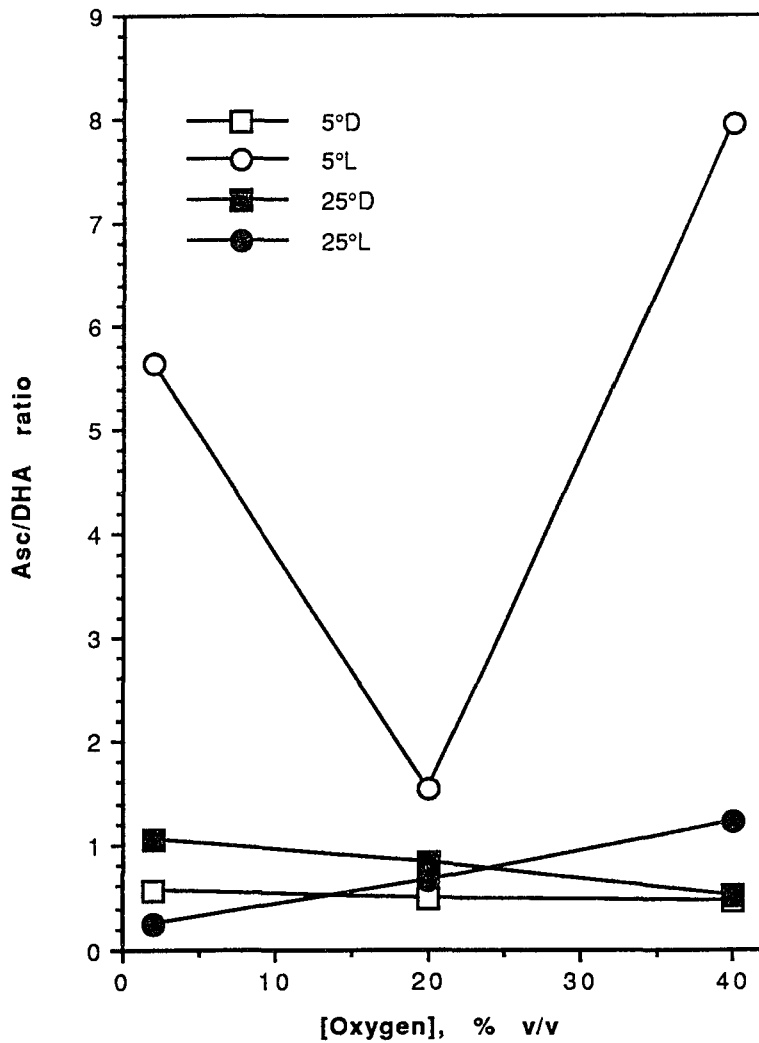
Fig 28. Ascorbate/DHA ratios of Z. diploperennis after 6h light treatments at 1500 μmoles/m2/s at either 5° or 25°C, and various oxygen concentrations. The untreated control ratio at 20% oxygen was 0.70.

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Table 3:   Activities of scavenging enzymes in corn treated to 6h of dark under varying oxygen concentrations and temperature (data showing mean $\pm$ S.E.). Untreated corn had an APX activity of 52.5$\pm$2.1; MDHAR activity of 12.2$\pm$1.0; and GTR activity of 5.5$\pm$0.4 μmoles.mg protein^{-1}.h^{-1}.

| | APX | | MDHAR | | GTR | |
Temperature :	5°C	25°C	5°C	25°C	5°C	25°C
2% oxygen		28.97 ±18.49		2.44 ±1.56		2.6
20% oxygen	28.7	16.85 ±7.25	6.1	1.65 ±0.15	5.1	3.3
40% oxygen		40.8 ±28.3		4.35 ±2.05		4.8

Fig 29. APX activities of corn after 6h light treatments at 1500μmoles/m2/s at either 5° or 25°C, and various oxygen concentrations. The untreated control activity at 20% oxygen was 52.5 μmoles/mg protein/h.

Monodehydroascorbate reductase: There was no significant difference in the MDHAR activities in dark between the three oxygen concentrations (Table 3) or light (Fig. 30) at various oxygen concentrations.

Glutathione reductase: In dark at 25°C, GTR activity increased with increase in oxygen concentrations (Table 3). In light, no oxygen effect was observed at 25°C (Fig. 31). However, a decrease in activity with increase in oxygen concentration was observed at low temperature. Therefore, the difference between the two temperatures was negligible at 2% and 20% oxygen, but was wider at 40% oxygen.

Xanthophyll cycle

Under 5°C and 25°C in dark, more than 80% of the xanthophyll cycle components were present as violaxanthin at all three oxygen concentrations in corn (Table 4a) and Z. diploperennis (Table 4b). There was, however, a slight increase in the zeaxanthin concentration at 40% oxygen at 5°C in corn compared to other dark treatments. Note that the control Z. diploperennis plants had a smaller pool size than the corn control plants.

With exposure to high light at 25°C (Fig. 32), there was an increase in zeaxanthin levels, at the expense of violaxanthin, at all three oxygen concentrations. The zeaxanthin concentration was higher at 20% oxygen than at the other two oxygen concentrations. This increase was mainly due to a larger xanthophyll pool size in comparison to the other two. No significant difference between the antheraxanthin concentrations were observed under these conditions. With lowering of temperature to 5°C, in presence of light (Fig. 33), no significant differences were observed between the three oxygen concentrations, either in the total pool size or in each of the xanthophyll components. Although, on comparing the zeaxanthin levels between high and low temperatures, a decrease was observed at 5°C. With exposure to high light at 25°C Z. diploperennis leaves showed
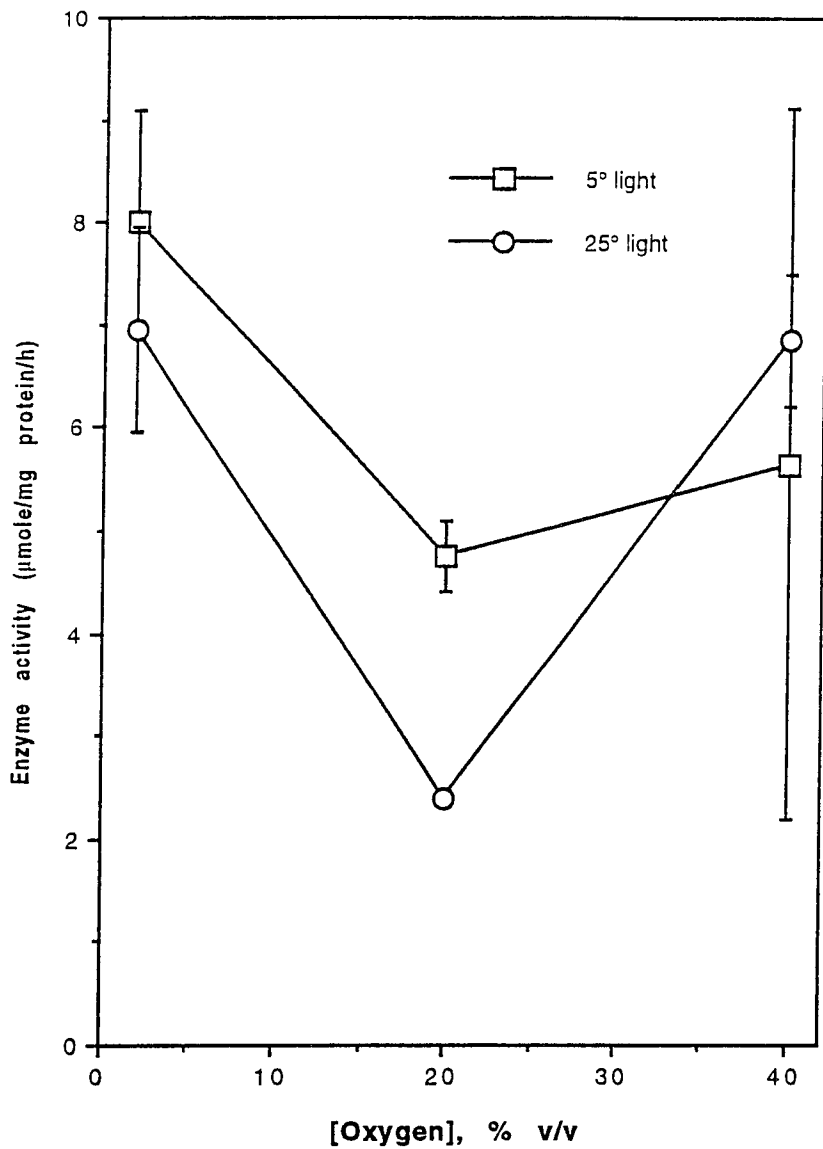
Fig 30. MDHAR activity in corn after 6h light treatments at 1500μmoles/m2/s at either 5° or 25°C, and various oxygen concentrations. The untreated control activity at 20% oxygen was 12.2 μmoles/mg protein/h.
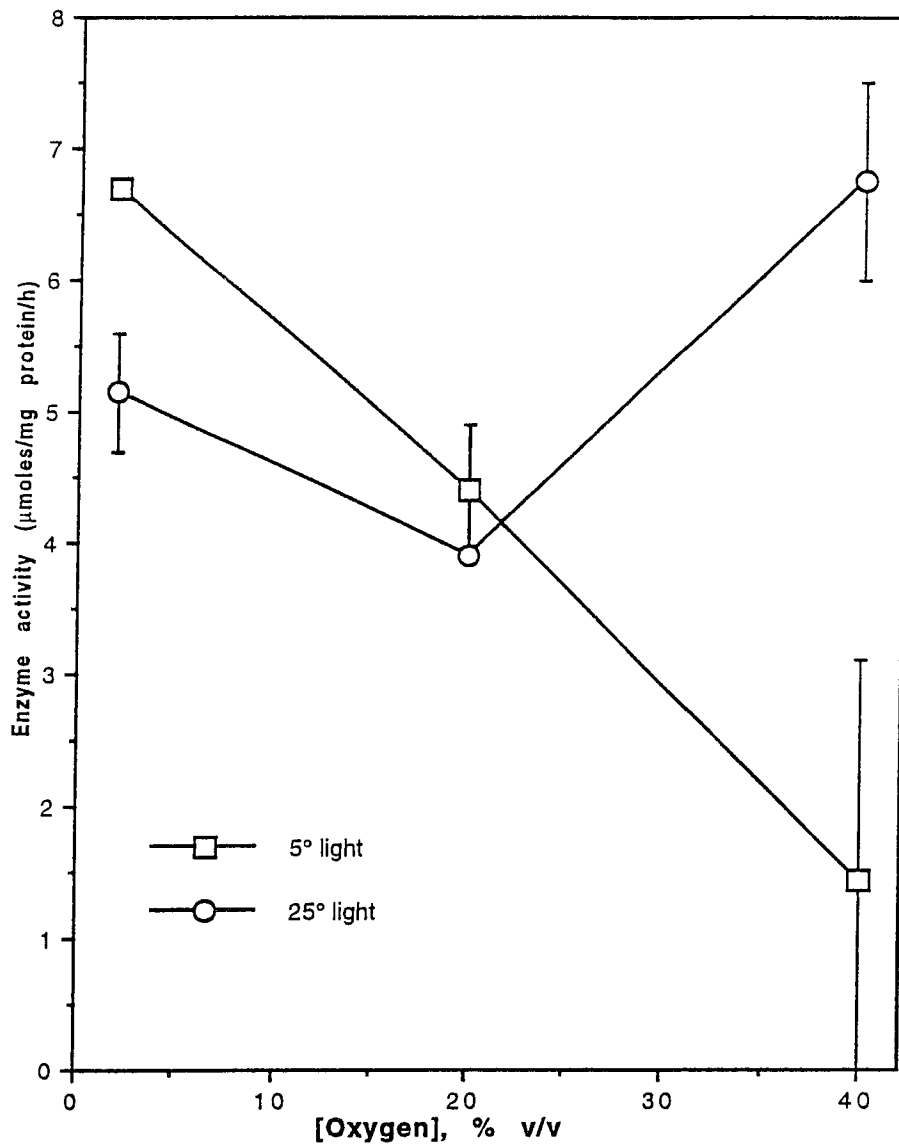
Fig 31. GTR activity in corn after 6h light treatments at 1500μmoles/m2/s at either 5° or 25°C, and various oxygen concentrations. The untreated control activity at 20% oxygen was 5.5 μmoles/mg protein/h.

Table 4a: Xanthophyll concentrations of corn treated to 6h of dark under varying oxygen concentrations and temperature (data showing mean ± S.E.). Untreated corn had a violaxanthin content of 5.02±0.71; antheraxanthin content of 1.45±0.05; and zeaxanthin content of 1.50±0.31 μg.g fw^{-1}.

Temperature :	Viola		Anthera		Zea	
	5°C	25°C	5°C	25°C	5°C	25°C
2% oxygen	7.56		0.63		0	
20% oxygen		5.76 ±0.48		0.34 ±0.03		0.33 0.04
40% oxygen	5.75 ±0.01		0.47 ±0.006		0.61 ±0.58	

Table 4b: Xanthophyll concentrations of *Z. diploperennis* treated to 6h of dark under varying oxygen concentrations and temperature (data showing mean ± S.E.). Untreated leaves had a violaxanthin content of 3.56±0.005; antheraxanthin content of 0.144±0.0; and zeaxanthin content of 0.05±0.005 μg.g fw^{-1}.

Temperature :	Viola		Anthera		Zea	
	5°C	25°C	5°C	25°C	5°C	25°C
2% oxygen	2.56		0.20		0	
20% oxygen		3.36		0.11		0.21
40% oxygen		4.28		0.40		0.38
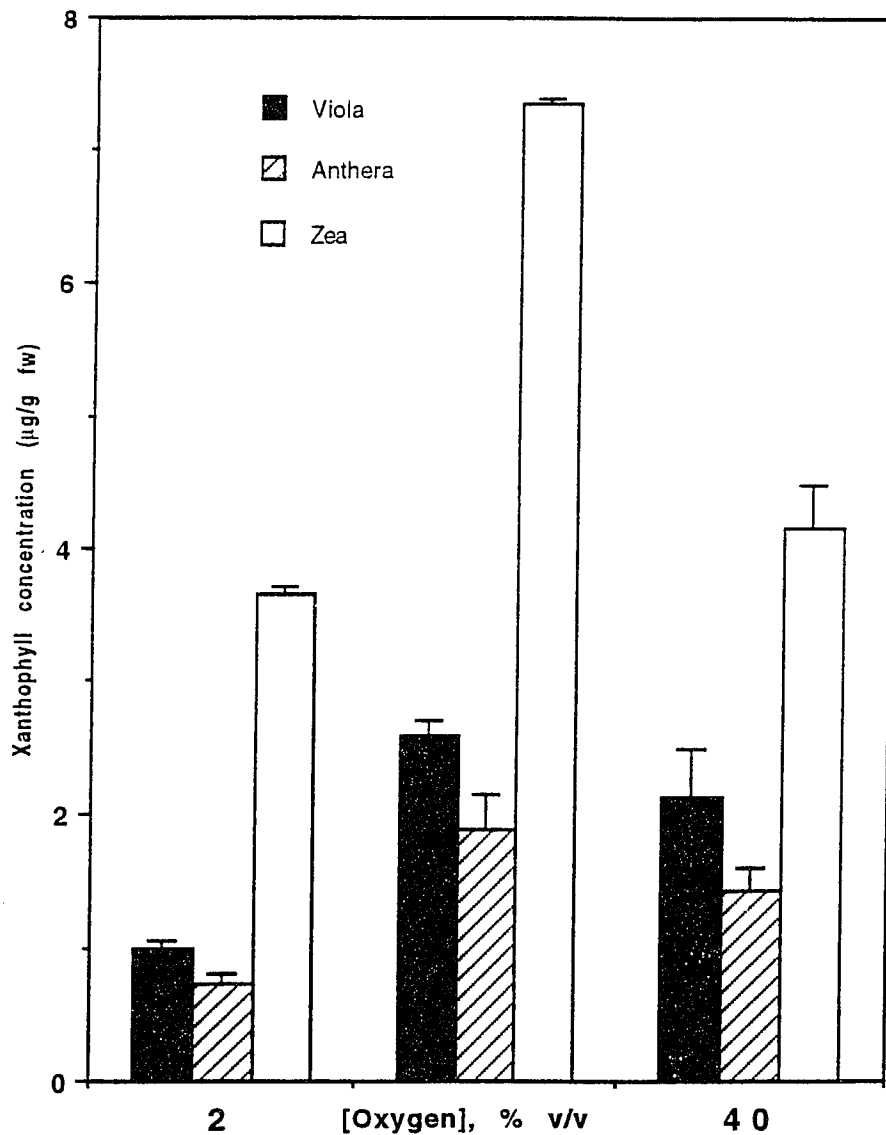
Fig 32. Xanthophyll concentrations of corn after 6h light treatments at 1500μmoles/m2/s at 25°C, and various oxygen concentrations. The untreated control leaves at 20% oxygen and ambient temperature had a violaxanthin content of 5.0, antheraxanthin content of 1.5 and zeaxanthin content of 1.5 μg/g fw.

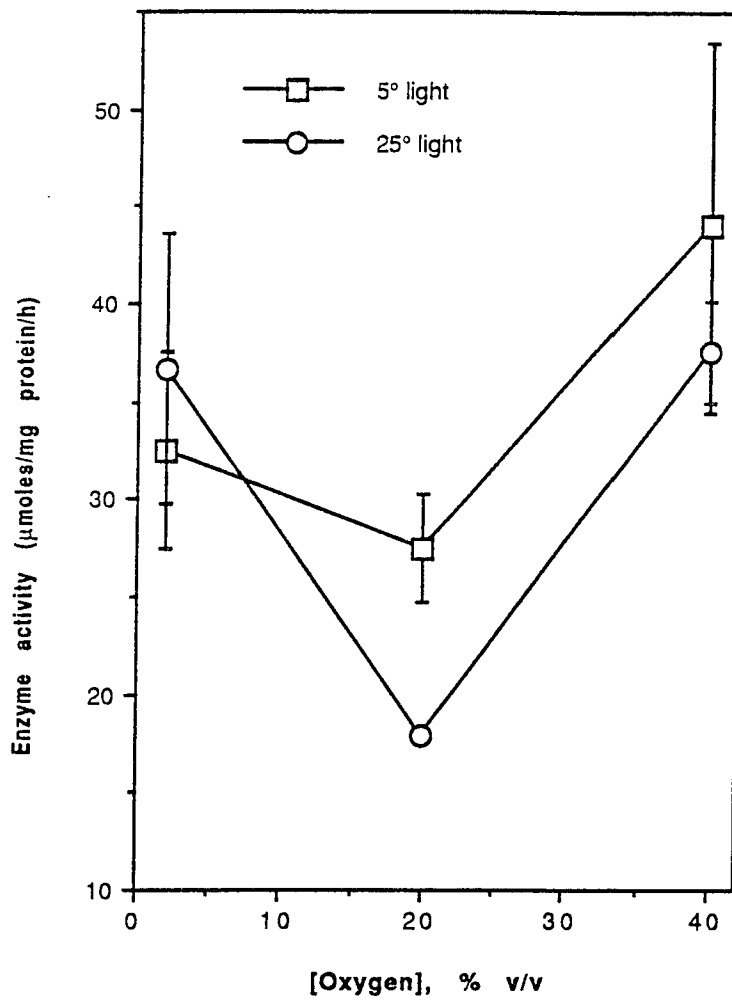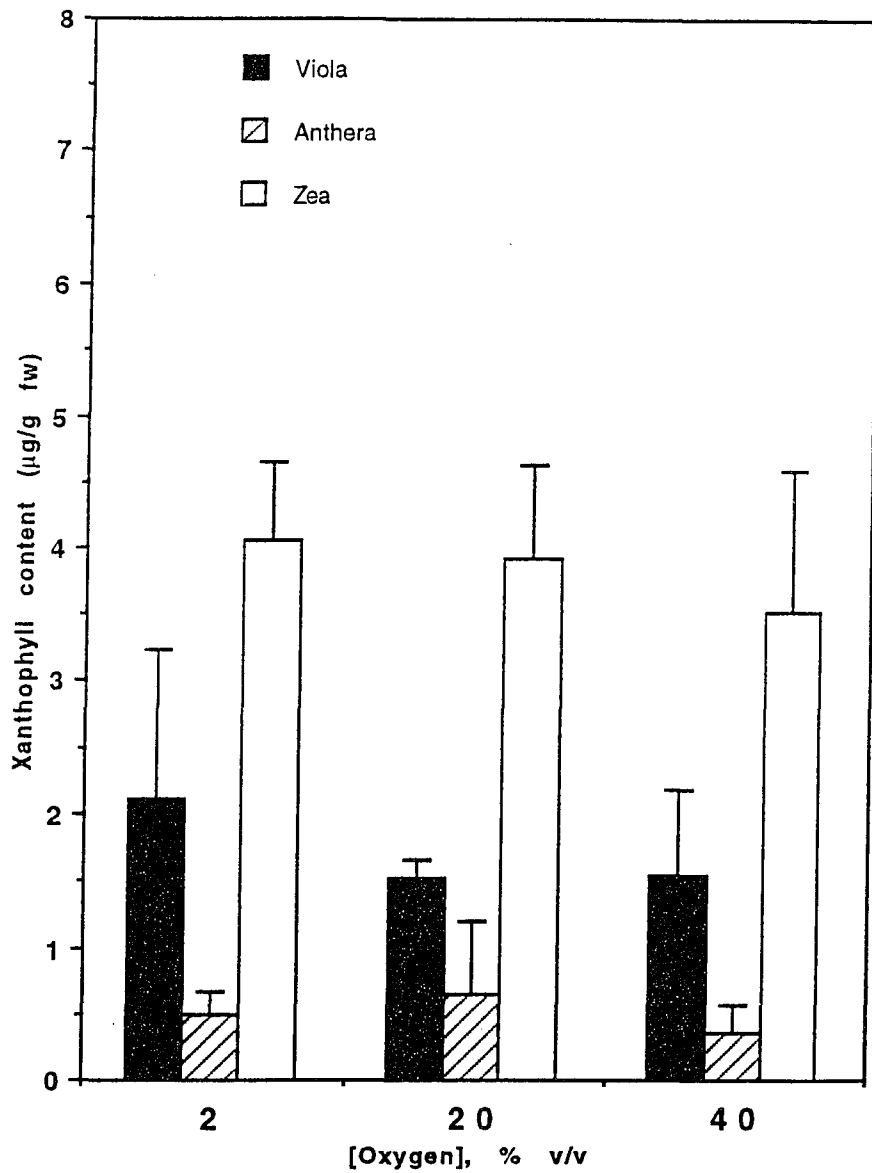Fig 33. Xanthophyll concentrations of corn after 6h light treatments at 1500μmoles/m2/s at 5°C, and various oxygen concentrations. The untreated control leaves at 20% oxygen and ambient temperature had a violaxanthin content of 5.0, antheraxanthin content of 1.5 and zeaxanthin content of 1.5 μg/g fw.

an increased zeaxanthin level (Fig. 34). This increase was at the expense of violaxanthin at all oxygen concentrations in this species. No significant difference in the zeaxanthin, antheraxanthin, or violaxanthin levels, however, was noted between the oxygen concentrations at this temperature. With lowering of temperature to $5^{\circ}C$ in presence of light, lower antheraxanthin and zeaxanthin concentrations were observed at 2% and 20% oxygen in *Z. diploperennis* (Fig. 35). There was a simultaneous decrease in the total xanthophyll cycle pool size under these two oxygen concentrations. Note that under all situations, *Z. diploperennis* had lower xanthophyll pool size than corn.
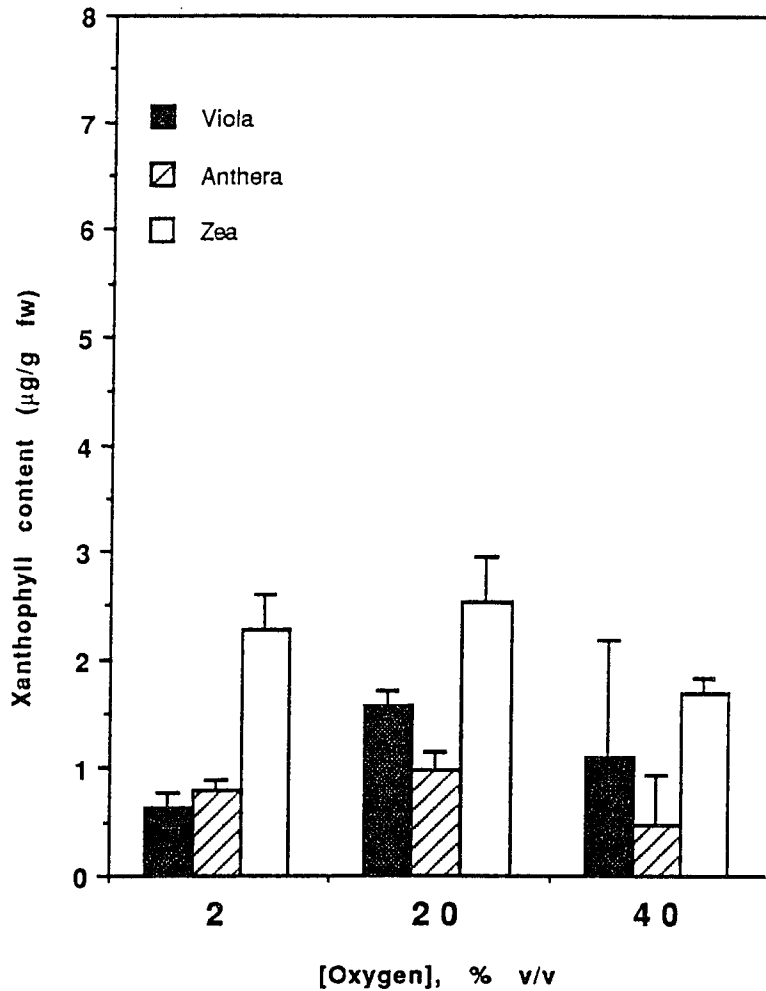
Fig 34. Xanthophyll concentrations of Z.
diploperennis after 6h light treatments at 1500
µmoles/m2/s at 25°C and various
oxygen concentrations. The untreated control
zeaxanthin concentration at 20% oxygen was
3.56, of antheraxanthin was 0.144 and of
violaxanthin was 0.05 µg/g fw.
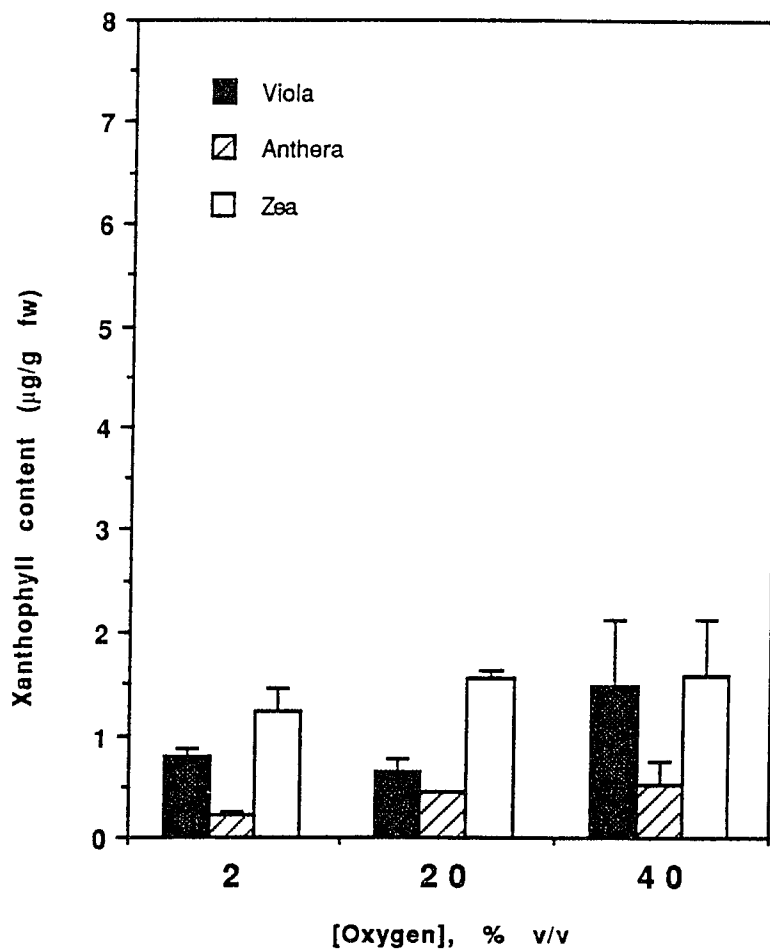
Fig 35. Xanthophyll concentrations of Z. diploperennis after 6h light treatments at 1500 μmoles/m2/s at 5°C and various oxygen concentrations. The untreated control zeaxanthin concentration at 20% oxygen was 3.56, of antheraxanthin was 0.144 and of violaxanthin was 0.05 μg/g fw.

DISCUSSION

The main objectives of this study were to quantitate the low-temperature inhibition of photosynthesis in the two *Zea* species, one chilling susceptible (*Z. mays*) and the other chilling tolerant (*Z. diploperennis*). Both *Zea* species are C_4 types. Besides quantifying chilling effects on the active-oxygen defense system, recovery rates from photoinhibitory damages were also monitored in corn. Most studies involving chilling photoinhibition have been done with C_3 (i.e., photorespiratory) plants at ambient oxygen concentrations, so information about photoinhibition under other oxygen concentrations is generally lacking. Low-temperature photoinhibition is a type of photooxidative stress, and the role of oxygen and antioxidants in this stress response is not well understood. We thus undertook to not only measure the extent of chilling photoinhibition under varying oxygen concentrations (both high and low) but also to assess the antioxidant, energy dissipation and active-oxygen scavenging capacities of these two genotypes under varying oxygen concentrations.

Part A: LOW TEMPERATURE PHOTOINHIBITION AND THE RECOVERY PROCESS IN *Zea mays*

Corn plants exhibited severe chilling photoinhibition when exposed to 6h of chilling at 5°C under 1500 μmol m^{-2} s^{-1} of light and in air. The Fv/Fm ratio decreased by ca. 63%, while the ϕ_{O2} decreased by ca. 38% of control (Figs. 14 and 15) while the Asat decreased only by ca. 32%. A similar decrease in ϕ_{CO2} was observed after chilling plants under light, which is also accompanied by lowering of Fv/Fm ratio (Peeler and Naylor, 1988) and Atrazine binding properties (Baker et al., 1983; Baker et al., 1988; Baker and

Nie, 1994; Butler and Katijima, 1975; Greer et al., 1986). The latter (i.e., Atrazine binding) indicates chilling induced damage to the Q_B binding site in PS II (discussed above). Increased reduction of Q_A is observed at low temperature (Ottander et al., 1993). This indicates slower re-oxidation of the Q_A site in PS II. The fluorescence quenching, in our data, indicated an impairment of either the transfer mechanism of energy to the reaction centers or a chilling-induced decrease in the number of "open" reaction centers. The decrease in Fv/Fm ratio was mostly due to a decrease in the Fm and partly due to an increase in the Fo values (Fig. 13), as is also observed by Krause (1988), Baker et al., (1983), and Greer and Hardacre (1989). Sustained increase of Fo indicates photoinhibitory damage of PS II. The increase in Fo is thought to result from either a decrease in the rate constant of photochemistry, or of radiationless energy dissipation in the chlorophyll pigment bed, or of energy transfer to the PS II reaction center (Demmig-Adams, 1990). Dissociation of the antenna complex increases the Fo levels (Demmig-Adams and Adams, 1994). Simultaneous decrease in Fm with an increase in Fo is thought to indicate a chilling induced damage to the water splitting system of the chloroplasts (Demmig-Adams, 1990). Our results suggest that there was not only an impairment of the efficiency of energy transfer to the PS II reaction centers, but that a number of reaction centers were also "closed", as denoted by the increase in Fo. The decrease in number of "open" reaction centers could be due to damage to the D1 protein. It should be pointed out that the Fv/Fm decrease during chilling stress of corn leaves indicates photoinhibition of mesophyll cells, as corn, an NADP-malic enzyme type C_4 plant, lacks PS II in the bundle sheath cells (Koroleva et al., 1994). The damaged D1 proteins cannot be degraded at low temperature (Aro et al., 1990). Hence D1 damage rates exceed the D1 repair rates at chilling temperatures, resulting in net photoinhibition (Kyle and Ohad, 1986). Such damaged reaction centers confer some protection to the photosynthetic membranes by dissipating excess energy non-radiatively

(Greer et al., 1991). Plants are known to become photoinhibited when only 40% of the traps become closed, even though majority of the reaction centers remain functional (Anderson, 1993).

Recovery from Photoinhibition

Following the 6h incubation, the corn plants were allowed to recover from photoinhibitory damages under low light (25 μmol m^{-2} s^{-1}) and ambient air temperature (ca. 23°C) for 2 days. Moderate temperatures are needed for recovery from photoinhibition, as damage repair require metabolic activity (Greer et al., 1986). Maximum rates of recovery have been observed at 25-30°C (Greer and Laing, 1988). One of the crucial events during recovery is a complete degradation of the damaged D1 protein, followed by resynthesis, and assembly into D1 depleted reaction centers. All these above steps are inhibited during low temperature exposure of leaves (Ottander et al., 1993). It has also been shown that low light (10-20 μmol m^{-2} s^{-1}) is essential for recovery of chloroplasts from chilling photoinhibition (Greer, 1990; Greer et al., 1986). Low light is thought to induce protein synthesis (Greer, 1990) required for repair mechanisms. The Fv/Fm ratio of corn showed ca. 57% recovery within two hours of return to ambient temperature which then increased to 93% of controls by 49h (Fig. 14). Ortiz-Lopez et al., (1990) also found that corn plants take about 48h to recover from photoinhibitory damage. The recovery of Fv/Fm in our case, was due to an increase in the Fm values (the latter having surpassed control values) as the high Fo values had only slightly tapered off by 49h. This suggested that permanent damages were sustained by some of the PS II centers.

The ϕ_{O2} (Fig. 15) recovered to 44% of control by 2h, followed by an increase up to 92% of control by 49h. This indicated an increased efficiency of energy transfer to the PS II reaction center and/or an increase in the number of open reaction centers during

recovery of corn plants. Baker et al., (1983) have suggested that initial decreases in quantum yield are due to increased energy distribution to PS I and including cyclic electron transport. Hence our results showed reversal of energy distribution back to the PS II during recovery and increase in cyclic electron transport system. Our results showed a close correlation between Fv/Fm values and quantum yields. Photoinhibited corn has consistently shown this correlation (Baker et al., 1988; Ortiz-Lopez et al., 1990).

Asat of photosynthesis, as measured by light-saturated rates of oxygen evolution, decreased due to chilling photoinhibition (Fig. 15). However the damage to the Asat rates (which reflects impairment of dark reactions) was more pronounced in corn than the light reactions (as indicated by fluorescence and quantum yield data). This decrease of Asat persisted even after 2 h at room temperature and low light incubation. No recovery was observed up to at least 49 h at ambient temperature. Long et al. (1983), noted impairment of physiological functions in corn when returned to ambient temperature after chilling. This decrease in Asat is an indication of chilling induced damage to the carbon-fixation system of the chloroplasts. According to Baker et al. (1988) many metabolic processes affect the Asat of C_4 plants during chilling stress. Chilling temperature (in the dark) also lowers CO_2 assimilation rates in other plants (Baker, 1991; Martin and Ort, 1985; Long et al., 1983). Low temperature not only affects the rate of diffusion of CO_2 through the stomata, but also the regeneration capacities of RuBP (Baker et al., 1988). Low-temperature-induced inactivation of carbon-fixation enzymes like fructose 1,6-bisphosphatase, PEP carboxylase, NADP-dependent malate dehydrogenase, RuBisCO and pyruvate Pi dikinase (Baker, 1994; Baker et al., 1988; Gilmore and Yamamoto, 1993; Hull et al., 1995; Long et al., 1983; Taylor et al., 1974) have been seen in many plants. The damage to the dark reactions in our study, thus appeared to be more severe than those imparted to the electron transport

system of corn during chilling in light. Corn plants exposed to similar conditions show quick recovery of Asat while the quantum yield takes longer to return to normal rates (Long et al., 1987). However, their rate of recovery of quantum yield was similar to ours. The difference in the Asat rates could have been due to a difference in temperature (which was not described in the above paper) or due to techniques of Asat measurement. While we measured Asat under saturating CO_2 concentrations using oxygen electrode, Long et al. (1987) used ambient CO_2 levels in an IRGA (Infrared gas analyzer). However, since most photosynthetic leaves normally function much below the light-saturated levels, photoinhibition of Asat does not have a major impact on the overall photosynthetic production of a crop plant in the field. Any changes in the quantum yield, however, renders a greater impact on crop yields. This effect has been confirmed in field studies (Long et al., 1987; Baker, 1994).

Chilling Induced changes of Antioxidants

The antioxidant contents of leaves were also measured before and during recovery of corn plants. The α-tocopherol contents of leaves decreased significantly (by ca. 50% from untreated control leaves) during chilling (Fig. 16). Ascorbate contents of corn leaves also dropped (by ca. 36% of untreated control leaves) on exposure to chilling stress (Fig. 17). This drop in ascorbate level during chilling was accompanied by concomitant decrease in DHA, giving rise to ca. 34% decrease in total ascorbate contents. Our results suggest that there was either a degradation or increased metabolism of total ascorbate contents during chilling of corn leaves. A chilling induced inactivation of the ascorbate synthetic pathway takes place in plants (Dai et al., 1993; Demmig-Adams and Adams, 1994), especially if lack of reducing power during photoinhibition leads to inactivation of some specific reaction(s) in this pathway. Regeneration of the ascorbate pool has been thought to be impeded at low temperature

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(Heber and Walker, 1992). This loss of ascorbate pool is thought to be an indicator of severe stress and failure of the defense system against oxidative stress (Foyer, 1993; Heber and Walker, 1992). DHA is known to breakdown into products like tartarate and oxalate (Foyer, 1993). Hence the antioxidant systems of corn leaves, both those involved with lipophilic (α-tocopherol) as well as hydrophilic (ascorbate system) active-oxygen scavenging, were depleted by 6h of chilling stress, thereby making corn leaves highly susceptible to damage from increased accumulation of active-oxygen radicals and their products. Since ascorbate is responsible for reduction of the α-tocopheroxy radical back to α-tocopherol (reviewed by Hess, 1993; Foyer, 1993), depletion of ascorbate during chilling stress could very well hamper vitamin E regeneration, as seen in our results at the end of the chilling exposure. In sharp contrast, a six-hour low temperature photoinhibitory incubation of Z. diploperennis produced a 16% reduction in vitamin E levels (Jahnke, personal communication). Ascorbate, also being involved as a cofactor in the de-epoxidation of violaxanthin to zeaxanthin, could affect the xanthophyll cycle of leaves. As seen below (Fig. 33), a decrease in ascorbate levels does correlate with decreased amounts of zeaxanthin in corn leaves treated to low temperature in presence of light.

Following two hours at ambient temperature, the corn leaves had begun showing increased total ascorbate concentrations (Fig. 17). However, full recovery of reduced ascorbate content was only observed after 49 h. The increased DHA content contributed to increase in the total ascorbate levels of leaves, which persisted until 22h of recovery. By 49h of recovery, total ascorbate content had returned to control levels resulting in complete restoration of the reduced ascorbate concentration of corn leaves. The increase in total ascorbate during recovery may be due to slower metabolism of ascorbate under low light (Demmig-Adams and Adams, 1994) thus leading to accumulation, or activation of the ascorbate synthetic pathway (as discussed above).

Even though there was partial recovery of vitamin E contents within 2h of return to ambient temperature, complete recovery did not take place until after 22h (Fig. 16). Thus during the initial 22 h recovery period the ascorbate : DHA ratio (Fig. 18) decreased sharply to almost 1:1 and was restored to control values of ca. 5 by 49h. The decrease in the ascorbate : DHA ratio, at the onset of recovery, suggested ascorbate-scavenging system being active. This is to be expected as the depletion of ascorbate-system and α-tocopherol at the end of chilling stress leads to decreased scavenging of active-oxygen species (Hess, 1993; Foyer, 1993). The recovery of total ascorbate as well as ascorbate : DHA ratio by 49h correlated with the restoration of α-tocopherol contents of leaves. Therefore, this suggested that complete recovery of ascorbate system, in particular reduced ascorbate, was necessary for regeneration of α-tocopherol to pre-stress levels. Increase in total ascorbate levels during recovery could be either due to higher rates of synthesis or slower rates of degradation. In sharp contrast to corn, the chilling tolerant *Z. diploperennis* showed complete recovery of prestress vitamin E levels within two hours at ambient temperature (Jahnke, personal communication).

Chilling Induced Changes to Scavenging Enzymes

Activities of three crucial scavenging enzymes, APX, MDHAR, and GTR were monitored before and during recovery. The enzyme APX catalyzes reduction of H_2O_2 with help of ascorbate, thereby oxidizing the latter to monodehydroascorbate (MDHA) radical (Fig. 4). The ascorbate pool is converted to its free-radical oxidized state (MDHA) during oxidation of α-tocopheroxy radial and by APX.. The enzyme MDHAR, on the other hand, carries out reduction of MDA radical back to reduced ascorbate. Nonenzymatic disproportionation of MDA radical also leads to synthesis of DHA and reduced ascorbate. DHA in turn is reduced back to ascorbate with help of the enzyme DHAR and the antioxidant glutathione. GTR is responsible for regeneration of

GSH from its oxidized state. The specific activities of all three enzymes (assayed at room temperature), namely APX, MDHAR and GTR, were reduced after exposure to low temperature (Fig. 19 and 20). The specific activity of MDHAR was reduced by ca. 62%, APX by about 50%, while GTR was only inhibited by ca. 18%. Many enzymes have been shown to be susceptible to low temperature in plants. A decrease in enzyme activities is due to cold lability or H_2O_2 mediated oxidation (Demmig-Adams and Adams, 1994). In the presence of accumulated superoxide and H_2O_2 in the chloroplast, OH can be produced by a Haber-Weiss reaction (Asada, 1994), which in turn can further inhibit certain enzymes. Hull et al. (1995) have shown that APX undergoes an 85% reduction in the relative specificity constant for H_2O_2 (which directly affects its scavenging activity) at low temperature. The relative specificity constant includes both the low temperature effect on Vmax and Km of the enzyme. A chilling induced impairment of APX activity, on the other hand, suggested a decline of H_2O_2 scavenging capacity of corn leaves. This would lead to not only increased active-oxygen related damage to chloroplasts, but also to changes in the redox state of ascorbate. This was observed both in the decline of the photosynthetic capacities and DHA contents of corn leaves in our study. Hull et al. (1995), have further discovered that the relative specificity constant for APX in Z. *diploperennis* decreases by only 40% of $5°C$ suggesting better peroxide scavenging capabilities than in corn at low temperature.

Reduction in the MDHAR activity also suggested lack of ascorbate regeneration capabilities under low temperature, which would lead to decreased reduced state of ascorbate as well as in α-tocopherol contents, as seen in our data. Ascorbate is essential for the activation of APX, as well as being its substrate. Depletion of this antioxidant during chilling could lead to irreversible inactivation of APX. Ascorbate can be completely oxidized in the chloroplasts within 80 s if the regeneration mechanisms are inoperative (Asada, 1994). Jahnke et al. (1991) have shown corn MDHAR to be a cold

labile enzyme. They observed that when corn grown at 25°C is placed in a temperature stressed environment (14°C), a 60% increase in MDHAR specific activity takes place within 24h. Reduced GTR activity could also impair the glutathione cycle, thereby ultimately affecting the redox state of ascorbate. Although Km of GTR for GSH is found to be unaffected by cold in corn leaves by Hull et al. (1995), the relative specificity constant of GTR decreases by 70% at low temperature. However, in our case GTR was the least affected of the three enzymes. The glutathione cycle does not seem to be as susceptible to chilling stress as the ascorbate cycle. Furthermore, Asada (1994) has argued that the flux through the DHAR and GTR phases of the cycle is small compared to the MDHAR and APX fluxes. However, a simultaneous decline in activity of all three enzymes definitely suggested a chilling-induced inactivation of the ascorbate-glutathione cycle, which could lead to an increase in active-oxygen species.

During recovery from chilling stress, complete and rapid restoration of the enzyme activities were seen within the first two hours following warming. At this point, corn leaves seemed to resume their normal scavenging activities. This recovery correlated with increase in the Fv/Fm ratio, ϕ_{O2}, and improvement of the ascorbate redox state (as observed by the recovery of DHA contents) and α-tocopherol contents. Net recovery during exposure to ambient temperature and low light takes place as the rate of repair exceeds the rate of damage under these conditions (Greer et al., 1986). The reverse of this is true under low temperature and high light exposure. Thus, under conditions where dissipation of absorbed energy is optimum and rate of recovery is maximal, minimal photoinhibition takes place (Greer et al., 1986). However, corn leaves did not fully recover from chilling injury until 49h of recovery at ambient temperature and under low light. Decline in the activities of APX and MDHAR (to ca. 30% of control) were observed to be taking place at 6h and did not recover from this second inactivation even by 49h. The recovery of some other parameters was much faster than

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that of the scavenging enzymes. This suggested that some inhibitory factor(s) was present in the leaves even after 22 h. This factor could very well have been an active-oxygen species or ascorbate, especially in case of APX (since ascorbate concentrations had not recovered before 49h). The lack of complete recovery by 2h was also observed by the continued inhibition of Asat of photosynthesis and α-tocopherol contents, which suggested that long term damage might have been sustained. It has been suggested (Long et al., 1983) that corn leaves are particularly susceptible to chilling photoinhibition due to its long C_4-pathway with additional cold-labile enzymes and intra-cellular and inter-cellular transport systems. Thus there seemed to be a rapid recovery involving partial restoration of Fv/Fm, ϕ_{O2}, α-tocopherol contents, DHA and ascorbate pool size in corn by 2h. But complete recovery of Fv/Fm, ϕ_{O2}, α-tocopherol and ascorbate contents and redox state took place only by 49h. Even after 49h, APX, MDHAR, Asat of photosynthesis and total ascorbate levels were still below control values. A rapid recovery from chilling photoinhibition is observed in other plants, followed by a slower phase of recovery that lasts over 2 days (Nie et al., 1992; Ortiz-Lopez et al., 1990).

By comparing the overall recovery pattern, it seemed probable that PS II reaction centers are affected in a manner different from other processes in chloroplasts. Light in presence of low temperature seemed to inactivate PS II, thereby lowering the Fv/Fm and quantum yield. This inhibition initially appeared to be from Q_A over-reduction and D1 damage, rather than damage from active-oxygen species. Low temperature also reduced enzyme activities, thereby affecting dark reactions (as observed by the decrease in Asat), scavenging enzymes and ascorbate biosynthetic pathways (as observed by the decrease in the ascorbate pool size). This in turn should result in increased concentrations of active-oxygen species. On return to ambient temperature, a rapid and considerable recovery of PS II activity (as seen by Fv/Fm and quantum yield data), activities of scavenging enzymes, α-tocopherol, DHA contents and ascorbate pool size took place within 2h.

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But Asat of photosynthesis showed long-term damage, possibly due to oxidation of some key enzymes by increased accumulation of active-oxygen species during photoinhibition. The activities of APX and MDHAR declined again after 2h. This second decline of the enzymes could be due to either active-oxygen species or from sustained decrease in the ascorbate levels. By 49h, it is apparent that excepting Asat and MDHAR, all other parameters show complete recovery. Thus enzyme activities, including those of the dark reaction, MDHAR and ascorbate biosynthetic pathways are most susceptible to chilling stress and show longest time-period for recovery.

Part B: CHILLING PHOTOINHIBITORY EXPERIMENTS IN *Z. mays* and *Z. diploperennis* UNDER VARIOUS OXYGEN CONCENTRATIONS

Photoinhibition

Corn plants were subjected to 5°C and 25°C with 2%, 20% or 40% oxygen either in presence of high light (1500 μmol m^{-2} s^{-1}) or in complete darkness. In darkness, there was no change in the Fv/Fm ratio at either 5°C or 25°C even under varying oxygen concentrations (Table 1a). The Fv/Fm ratios subsequent to dark incubation conditions were unchanged from the control values of 0.76, close to unstressed value of 0.8 in most plants (Bjorkman, 1987). This clearly showed that change in oxygen concentrations at normal temperature was not important for fluorescence quenching. Also, low temperature did not affect the fluorescence parameters of corn chloroplasts in absence of light, even when the oxygen concentration was raised to 40%. Quantum yield (ϕ_{O2}) of corn leaves in dark at 25°C and 5°C, like the Fv/Fm values, neither differed significantly from control nor showed any oxygen effects. When the light-saturated rates of photosynthesis were observed, untreated controls showed an Asat value of 17.9 μmoles oxygen m^{-2} s^{-1}. Corn leaves showed significant decrease in Asat in dark treated leaves at 5°C or 25°C, from control. No oxygen effect was observed at 25°C (i.e. oxygen concentration related changes in light-saturated rates). Increase in oxygen concentrations resulted in decrease of Asat rates only at 5°C. The low Asat following 6h dark incubation was not due to damage to the thylakoid reactions. The unchanged Fv/Fm ratio indicated no changes in energy transfer efficiency in PS II and the slight decrease in the quantum yield implied that electron transport was proceeding without any damage under light-limited intensities. The decrease in the Asat rates could be due to lack of adequate activation of Benson-Calvin cycle enzymes (Taylor et al., 1974). Likewise, low temperature incubation did not change Asat from the 25°C treatments in

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absence of light. Thus it may be concluded that no inhibition of the reaction centers occurred in absence of light in corn, even after lowering the temperature to $5^{\circ}C$ and varying the oxygen concentration. However, the dark reactions, as indicated by light-saturated rates of photosynthesis (i.e., Asat), decreased significantly in dark and further show a decrease at $5^{\circ}C$ with increase of oxygen concentrations.

As with Z. mays no significant differences in Fv/Fm data between $5^{\circ}C$ and $25^{\circ}C$ were seen in Z. diploperennis at any oxygen concentration after dark incubation (Table 1b). The ϕ_{O2} of all dark treatments, at both temperatures and oxygen concentrations, were similar to control values, as was in Z. mays. The Asat values at $25^{\circ}C$, were higher at 2% oxygen in comparison to those at 20% and 40% oxygen when incubated in darkness. At $5^{\circ}C$, the Asat values were higher than control, however, no oxygen effect was observed at this temperature. Thus in dark Z. diploperennis behaved quite similar to Z. mays except for the Asat values. It may also be noted that the Asat values in Z. diploperennis were higher than Z. mays following low temperature in darkness.

In the presence of light , however, the Fv/Fm of corn was reduced by ca. 16% at $25^{\circ}C$ when compared to control, at both 20% and 40% oxygen (Fig. 21). This indicates that partial high-light induced photoinhibition took place at room temperature. Similar decrease in Fv/Fm was noted on transfer of plants to high light (Foyer et al., 1989). This inhibition was not enhanced by higher oxygen concentrations. However, at 2% oxygen in light, more than 32% quenching was observed. From these results it was clear that oxygen, above 20%, imparted a protective role against photoinhibition at normal temperature. Maximum quenching, however, was observed with lowering of temperature in presence of light. Thus low temperature photoinhibition was due to an interaction of high light and low temperatures. Similar chilling induced decrease in Fv/Fm is observed in corn (Baker et al., 1988) and pumpkin under high light (Aro et al., 1990). At low temperature again, we saw the protective effect of higher oxygen

concentration. At 5°C, photoinhibitory damage to PS II (as Fv/Fm) was inversely related to oxygen concentration (Fig. 21).

Under high light at 25°C, the quantum yield was also reduced by a third at 2% oxygen, while no inhibition was seen at 20% or 40% oxygen, when compared to the control (Fig. 23). As with Fv/Fm values, oxygen concentrations above 20% imparted photoinhibitory protection to ϕ_{O2} at 25°C in light. The corn plants showed significant photoinhibition of ϕ_{O2} under low temperature in presence of light. Similar decreases are observed in corn (Long et al., 1989; Baker et al., 1988; Ortiz-Lopez et al., 1990) and other plants chilled under high light (Foyer et al., 1989). Chilled leaves show a decrease in quantum yield due to increase in energy distribution to PS I relative to PS II (Baker et al., 1983). Again, like Fv/Fm data, low temperature exposure of corn in presence of light showed an oxygen effect on quantum yield, with 40% oxygen imparting the maximum and 2% the minimum protection against photoinhibition. When exposed to 25°C and high light, the Asat values at 20% and 40% oxygen were comparable to dark treatments (Fig. 22). However, a significant photoinhibition was observed at 2% oxygen. Chilling temperatures in presence of light again showed the maximum photoinhibition. A similar decrease was observed in corn at chilling temperatures (Baker et al., 1988). Also, at 5°C, higher oxygen concentrations seemed to impart protection from photoinhibition.

Hence, it is clear from the photosynthetic parameters that little or no photoinhibition took place in the absence of light, in spite of lowering the temperature to 5°C. Ortiz-Lopez (1990) also found that chilling induced depressions of quantum yield are light dependent. The various oxygen concentrations had no effect on photosynthetic parameters in absence of light, both at 5°C and 25°C. At normal temperature in presence of high light, partial photoinhibition of corn leaves was observed (as evident in the partial

quenching of PS II fluorescence). Severe photoinhibition of corn leaves was observed at chilling temperature in presence of light.

Photoinhibition primarily results from damage to the D1 protein of PS II (Aro et al., 1990; Baker et al., 1988). It has been recognized by many that at ambient temperature, both photoinhibition (i.e. damage to D1 protein) as well as repair and recovery of D1 protein take place simultaneously (Aro et al., 1990; Kyle and Ohad, 1986). However, at low temperature the rate of D1 protein recovery is significantly retarded. This is mainly because of the slowing down of the proteolytic degradation of the damaged D1 protein at low temperature, which is a prerequisite to D1 repair and recovery. This difference in increased damage but decreased recovery at chilling temperature causes a net increase of photoinhibition (Ottander et al., 1993). Increase in photoinhibition at low temperature is also due to decrease in dissipation in excitation energy through carbon assimilation (resulting in decrease of Asat), as well as due to partial phosphorylation of peripheral LHC II (Aro et al., 1990). The rate of carbon metabolism decreases under low temperature, which in turn decreases effective sink size for the photosynthetic reductants and ATP (Baker, 1994). This results in a decrease in non-cyclic electron transport, thereby decreasing quantum yield and inducing non-photochemical quenching of fluorescence (Baker, 1994). Thus, low temperature alone is not sufficient to induce damage, but contributes as a concerted effect with light (Greer, 1990). Oxygen was shown to impart protection against photoinhibition of corn in our study. The protection was partly observed at and above normal oxygen concentrations at 25°C in presence of light, but it became more evident at low temperature, where an increase in oxygen concentrations always correlated with a decrease in photoinhibition. A higher and irreversible photoinhibition has been observed under anaerobic conditions (Asada, 1994; Hundal et al., 1990) compared to an aerobic environment. This indicated that photoinhibition at 2% oxygen might have been quite different from those at higher

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oxygen concentrations. The degradation of D1 protein is independent of oxygen and occurs within 30 min under anaerobic conditions (Jagershold and Stenbjorn, 1991). This explained the higher inhibition under low oxygen, which was not induced by active-oxygen species. This inhibition is thought to be due to highly oxidizing radicals in PS II like P_{680}^+ or Z^+. Typically, C_3 plants show a decrease in quantum yield with increase in temperature, as the oxygen affinity of RuBisCO increases under higher temperature (Baker et al., 1988; Osmond, 1981), thereby increasing its photorespiratory reactions. These plants would also show reduced Asat rates under high oxygen concentrations for the same reason. But C_4 plants like corn, do not have this problem due to their Kranz anatomy, which largely precludes oxidation of RuBP, by dioxygen.

Oxygen can also have a protective function. When PS I electron acceptors are absent, oxygen reduction by the Mehler reaction (also termed pseudocyclic electron flow, Heber and Walker, 1992), H_2O_2 scavenging and cyclic electron transport (Foyer, 1993) all generate H^+ gradients and help protect against D1 degradation (Asada, 1994). Increased pseudocyclic electron flow can be induced by high light (Foyer, 1993). This protective mechanism, however, is possible only if a functional scavenging system exists to detoxify the resultant oxygen radicals (Baker, 1994). Superoxide, produced at the Fe-S centers of PS I (Asada, 1994), also initiates putative cyclic electron flow around PS I and simultaneously reduces superoxide concentrations in membrane. Also, an ascorbate-dependent H_2O_2 scavenging system is capable of supporting high rates of electron transport (Heber and Walker, 1992), thereby protecting D1 protein against damage. Hence, either all or one of these mechanisms could be effective in imparting protection to corn chloroplasts against photoinhibitory damage under increasing oxygen concentrations. It should be borne in mind that this protection is not complete, and that some photoinhibition will take place in spite of these mechanisms.

When leaves of *Z. diploperennis* were exposed to high light at 25°C, a measurable inhibition was observed at all oxygen concentrations (Fig. 21), which again was comparable to *Z. mays*. However, no protective effects of oxygen were observed in this genotype. At 5°C in light, increased quenching of fluorescence took place at all oxygen levels. The degree of inhibition at low temperature, however, was less than those of *Z. mays*, thus indicating the chilling tolerance of *Z. diploperennis*. No protective effects of oxygen concentrations were observed here. The ϕ_{O2} was partially inhibited at 25°C in light (Fig. 23) like *Z. mays*, however, it did not show any oxygen affect. Under low temperature and light, the ϕ_{O2} was further inhibited like *Z. mays*, again without any difference between the oxygen concentrations. The Asat rates at 25°C were similar to control under all oxygen concentrations, in light. (Fig. 22). Hence, the oxygen effect, unlike *Z. mays* was not observed here. On exposure low temperature, an increase in Asat rates was observed at 2% oxygen. The higher oxygen concentrations had Asat rates similar to control. Thus only the light reactions were affected by chilling temperature in *Z. diploperennis*, while the dark reactions seemed quite resistant. Hence: (1) *Z. diploperennis* had a much higher resistance to photoinhibition at 5°C in light in comparison to *Z. mays*, and (2) the oxygen effect at low temperature in light was abolished. Thus the photoinhibition in *Z. diploperennis* was mostly due to the effect of low temperature. Higher oxygen concentrations protect *Z. mays* from photoinhibition, but in no way affect those of *Z. diploperennis*. It may be possible that oxygen induced electron transport may not be active in this genotype.

Antioxidants

In dark treated leaves no significant difference in the reduced ascorbate levels was observed between low and normal temperatures and at varying oxygen concentrations in corn (Table 2a). However, a decrease of ca. 73% was observed in all

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the above treatments when compared to untreated control values. The DHA levels also did not vary between the three oxygen concentrations at 25°C in dark. The 5°C treatments showed a slight decrease in DHA content with decrease in oxygen concentrations in the dark. There were no significant differences in the total ascorbate levels in dark between low and high temperatures and at varying oxygen concentrations. There was, however, a decrease of ca. 67%, compared to untreated control values in all the above treatments. Hence it was clear that no difference in either reduced or total ascorbate took place in dark by lowering of temperature or by changing oxygen concentrations. On comparing this with the photoinhibition data, it was apparent that no oxidative stress is induced in corn leaves in darkness, even by lowering temperature or increasing oxygen concentrations. However, a decrease in the total ascorbate was observed in all dark treatments. This very likely was due to the lack of photosynthetic reductants and ATP in dark, which are the major cofactors in the recycling of oxidized ascorbate (Asada, 1994; Foyer, 1993).

When exposed to high light at 25°C, the reduced ascorbate contents did not vary significantly from the dark treatments in corn (Fig. 24), although there was a marked reduction from untreated control values. This decrease was due to the decrease in total ascorbate levels (Fig. 26), which could result from decrease in photosynthetic reductants and ATP due to high light induced photoinhibition in corn. Although there was no significant difference between the ascorbate contents at varying oxygen concentrations at 25°C, a significant reduction in DHA levels (Fig. 25) was observed with increasing oxygen concentrations. The total ascorbate concentrations (Fig. 26) were lower at 2% and 20% oxygen while those at 40% were similar to dark treatments. The redox state of ascorbate was markedly affected at 40% oxygen (Fig. 27). It is apparent that the chloroplast antioxidant systems were more efficient at increasing oxygen concentrations. This increase could be due to increase in the photosynthetic reductant NADPH and ATP

(which affects both recycling of ascorbate and other synthetic reactions) resulting from the pseudocyclic and cyclic electron transport (discussed above) under high oxygen concentrations. This would effectively protect the photosynthetic membranes from active-oxygen damage, but show changes in redox state of ascorbate, as observed here. At 2% oxygen, however, the DHA contents of corn remained practically unchanged, indicating that photoinhibition at 2% oxygen may not have been due to oxidative stress (discussed above). At 5°C in light, the reduced and total ascorbate concentration at 2% and 20% oxygen were significantly lower than at 40% oxygen (Figs. 24 and 26); so were the redox states of ascorbate (Fig. 27). However, the DHA levels did not vary with changes in oxygen levels. Impairment of the regeneration of ascorbate pool has been observed under low temperature in other plants (Heber and Walker, 1992) but the effects of oxygen concentration on the ascorbate pool have not been reported before. The decrease in concentrations of reduced ascorbate and total ascorbate at 2% oxygen from those at 25°C, indicated the presence of high stress levels under these conditions. The difference between 25°C and 5°C suggested that photoinhibition at 2% oxygen might have been due to different causes at different temperatures. The difference between 25°C and 5°C, in terms of ascorbate redox states and concentrations, were negligible at normal oxygen concentrations. Thus, the difference in photoinhibition between the two seemed to be more due to temperature effects than oxygen effects. At high oxygen concentrations, again a marked difference between the redox state of ascorbate and total ascorbate concentrations were observed between the two temperatures, suggesting that low temperature and high oxygen concentrations together imposed a greater stress on the antioxidant capacities of corn. When the different oxygen concentrations are compared at 5°C, a decrease in redox state and total concentration of ascorbate was observed with decrease in oxygen concentrations, which again correlated with the increase in photoinhibition under decreasing oxygen concentrations.

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There were no significant differences in the levels of reduced ascorbate, DHA and total ascorbate between the dark treatments in *Z. diploperennis* at both 25°C and 5°C and all oxygen concentrations (Table. 2b). There were also no significant differences between the ascorbate levels at normal vs. low temperature with exposure to light (Fig. 24). A higher ascorbate concentration was observed with increasing oxygen concentration, especially at 40% oxygen, under both temperatures. Similar oxygen induced increase was observed in *Z. mays*. However, at increased oxygen concentrations, a higher ascorbate concentration was observed in *Z. diploperennis* than *Z. mays*. This might account for greater chilling tolerance in *Z. diploperennis*, as noted by the photoinhibitory results. The DHA concentrations at 5°C were lower than those at 25°C in *Z. diploperennis* under all oxygen concentrations (Fig. 25). This again indicated an increased reduced state and thus a higher scavenging capacity of the antioxidant at low temperature in *Z. diploperennis*. There were no differences between the varying oxygen concentrations under these conditions. This was consistent with fluorescence and quantum yield results, which showed increased inhibition with temperature but no oxygen effect. DHA contents of corn were also lower than those of *Z. diploperennis*. The total ascorbate levels at 25°C in light was higher than those at 5°C in corn (Fig. 26), again indicating cold lability of the antioxidant system. An increase in total ascorbate content of leaves were observed with increasing oxygen concentrations at both temperatures. This indicated that oxygen-induced synthesis of ascorbate might be required to prevent photoinhibition at higher oxygen concentrations. Again, the total ascorbate concentrations of *Z. diploperennis* were much higher than those of *Z. mays*, especially at 20% and 40% oxygen, indicating a greater antioxidant capacity of the former genotype. No significant change in the redox state of ascorbate was noted in light or dark at 25°C or at 5°C in dark (Fig. 28). An increase was, however, observed at

2% and 40% oxygen at 5°C in light. Note, however, that at 5°C, in light at 40% oxygen, the redox state of *Z. mays* was much more reduced than *Z. diploperennis*.

Scavenging enzymes

In the dark, there were no significant differences between the activities of APX and MDHAR in corn at various oxygen concentrations, although they were substantially lower than untreated control values (Table 3). The GTR activities in dark were lower at 25°C, but a slight increase in the activity with increase in oxygen concentrations were observed. This oxygen-induced increase in GTR may be related to the proposed increase in photosynthetic reductants at higher oxygen concentrations (discussed above).

In the light, no significant difference between the activities of APX or MDHAR were observed at various oxygen concentrations and temperature (Figs. 29 and 30), although the majority of the values were lower than those of control leaves. Hull et al. (1995) observed no difference in the Km of corn APX for ascorbate at low vs. high temperature, however the relative specificity constant for H_2O_2 was drastically reduced under low temperature. The GTR activities, however, were closer to control values in light (Fig. 31). No difference between the two temperatures was observed at 2% or 20% oxygen. However, at 5°C, a consistent decrease in the GTR activity was observed with an increase in oxygen concentration. Under 40% oxygen, there were marked difference between the activities at 5°C vs. 25°C. Hence it may be concluded that corn APX and MDHAR are unaffected by our variations of light, oxygen and temperature experiments. However, activities of both enzymes under all above conditions were much below that of control. GTR, however, is affected by the low temperature treatments in presence of high oxygen. Also the effect on GTR activity may not be crucial to the active-oxygen scavenging system, as evidenced by the antioxidant concentrations above.

Xanthophyll cycle components

Under 5°C and 25°C in dark, more than 80% of the xanthophyll cycle components were present as violaxanthin at all three oxygen concentrations in corn (Table 4a). There was, however, a slight increase in the zeaxanthin concentration at 40% oxygen at 5°C compared to other dark treatments. This indicated that in the absence of light, de-epoxidation of xanthophylls does not take place either by lowering of temperature, or raising oxygen concentrations. Unless, of course, the two take place together, when a small increase in de-epoxidation of violaxanthin was observed.

With exposure to high light at 25°C (Fig. 32), there was an increase in zeaxanthin levels, compared to dark values, at all three oxygen concentrations. This increase was at the expense of violaxanthin. Similar results are found in other plants (Bjorkman, 1987). Light has been shown to induce de-epoxidation of violaxanthin to zeaxanthin within 10 min (Foyer et al., 1989) and also increase the xanthophyll pool size (Baker and Nie, 1994). A direct correlation between fluorescence quenching at 25°C and zeaxanthin concentrations thus was observed in the present study in corn. However, the zeaxanthin concentration at 20% oxygen was higher compared to the other two oxygen concentrations. This increase was mainly due to a larger xanthophyll pool size. It is probable that the enzyme violaxanthin de-epoxidase was affected by changes in the oxygen concentrations. No significant difference between the antheraxanthin concentrations were observed under these conditions. Zeaxanthin associated dissipation of excess energy is thought to prevent formation of active-oxygen species (Demmig-Adams and Adams, 1994). This could account for lack of increased photoinhibition under high oxygen at 25°C, where the scavenging enzymes remained unaffected. With lowering of temperature to 5°C, in presence of light (Fig. 33), no significant difference was observed between the three oxygen concentrations, either in the total xanthophyll pool size or in each of the xanthophyll components. Even though the zeaxanthin level at

5°C was substantially lower than that at 25°C. This indicated that de-epoxidation of violaxanthin or epoxidation of zeaxanthin was affected by low temperature. This may be due to cold temperature inhibition of enzyme activity. It has been reported that zeaxanthin concentrations below 10°C is kinetically controlled by a temperature-dependent decrease in de-epoxidation (Demmig-Adams et al., 1989; Demmig-Adams, 1990). The low concentrations of zeaxanthin at 5°C thus does not protect against photoinhibition. Koroleva et al. (1994) noted that zeaxanthin facilitates energy dependent quenching in corn, but does not protect against photoinhibition. This indicated that an inefficient scavenging system at low temperature, as also noted by Jahnke et al. (1991), may be responsible for the increased photoinhibition at low temperature.

More than 80% of the xanthophyll pool in *Z. diploperennis* was present as violaxanthin under all dark treatments (Table 4b) and very little zeaxanthin was observed under these conditions. Note that the control *Z. diploperennis* plants had a smaller pool size than control *Z. mays* plants. With exposure to high light at 25°C (Fig. 34) the zeaxanthin levels increased at the expense of violaxanthin under all oxygen concentrations. No significant difference in the zeaxanthin, antheraxanthin, or violaxanthin levels, however, were noted between the oxygen concentrations under these conditions. This then, for the first time, indicated a presence of the xanthophyll cycle in *Z. diploperennis*, which de-epoxies violaxanthin to zeaxanthin under light. With lowering of temperature to 5°C in presence of light, lower violaxanthin concentrations were observed in *z. diploperennis* at 2% and 20% oxygen compared to 40%, while the antheraxanthin and zeaxanthin concentrations were similar in all cases (Fig. 35). The antheraxanthin and zeaxanthin concentrations at 2% and 20% oxygen were also lower compared to those at 25°C and same oxygen concentrations. There was a simultaneous decrease in the total xanthophyll cycle pool size under these situations. Similar cold

lability of the de-epoxidation and total xanthophyll pool size was observed in *Z. mays*. At 40% oxygen, no differences in xanthophyll contents of *Z. diploperennis* between the two temperatures were noted. Under all situations, *Z. diploperennis* had a lower xanthophyll pool size than corn.

Conclusions

1. Oxygen (O_2) offers significant protection to corn at 5°C either when measured as quantum yield or as Fv/Fm ratios.

2. At room temperature, the protective effect of oxygen is negligible in corn. In C_3 plants the opposite effect of oxygen at low temperatures has been observed repeatedly by numerous workers (i.e. that oxygen contributes significantly to photoinhbition at low temperatures).

3. With the low-temperature tolerant *Z. diploperennis*, photoinhibition of photosynthesis in light, is not affected by oxygen concentrations at either low or ambient temperatures. Nevertheless, *Z. diploperennis* suffered less photoinhibition than corn under the similar conditions.

4. The recycling of ascorbate and hence α-tocopherol is not only severely limited at low temperature in corn, but takes at least 48 h to recover at ambient temperature. This reduction of antioxidant concentrations during and after chilling stress seems to induce severe photoinhibition as well as limit the recovery of photosynthesis. The depletion of α-tocopherol and ascorbate in corn may explain the significant increase of MDHAR observed by Jahnke et al. (1991) on transfer of corn grown at 25°C to 14°C.

5.   Increased oxidation of ascorbate (as dehydroascorbate) is observed with increase in oxygen in corn, particularly at 25°C. Low temperature and high light together impose a greater stress on the antioxidants than any one of these by itself.

6.   Low temperature damage to the photosystem II (PS II) occurs only in the presence of light, whereas the reduction of Asat is observed even during dark chilling. This indicates that low-temperature alone is sufficient to down-regulate the dark reaction of photosynthesis. Low-temperature induced damage to photosystems occurs only in the presence of light. Furthermore, a decrease in ascorbate concentrations during dark treatments elucidate the crucial role light produced reductants play in recycling oxidized ascorbate back to ascorbate.

7.   Violoxanthin is de-epoxidized to zeaxanthin in the presence of light in corn. Low temperatures cause a reduction of this de-epoxidation, irrespective of oxygen concentration. The presence of the xanthophyll cycle is observed in *Zea diploperennis* for the first time. The de-epoxidation reaction of *Z. diploperennis* is also chilling-sensitive, as in corn.

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