Photoinhibition of photosynthesis and possible role of xanthophyll cycle in protection against photodamage in sorghum seedlings.

A thesis submitted to Goa University for the degree of

Doctor of Philosophy
in
Botany

by

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April 2000

STATEMENT

As required under the university ordinance 0.413, I state that the present thesis entitled "Photoinhibition of photosynthesis and possible role of xanthophyll cycle in protection against photodamage in sorghum seedlings" is my original contribution and that the same has not been submitted on any previous occasion to the best of my knowledge, the present study is the first comprehensive study of its kind from the area mentioned.

The literature conceiving the problem investigated has been cited. Due acknowledgements have been made wherever facilities have been availed.

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CERTIFICATE

This is to certify that the thesis entitled "Photoinhibition of photosynthesis and possible role of xanthophyll cycle in protection against photodamage in sorghum seedlings" submitted by Ms Sangeeta G. Sankhalkar for the award of the degree of Doctor of Philosophy in Botany is based on the results of experiments carried out by her under my supervision. The thesis or any part thereof has not previously been submitted for any other degree or diploma.

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Guide

Dr. Prabhat K. Sharma

DEDICATED TO MY LOVING

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ACKNOWLEDGEMENT

I would like to express my sincere gratitude to Dr. Prabhat Kumar Sharma, Department of Botany, Goa University, Goa, for his critical guidance and scientific support throughout my Ph.D. work. His practical guidance, encouragement and kindness throughout the course helped me in successfully completing of the thesis. I am indebted to him.

I am grateful to Prof. Dr. D. J. Bhat, Head, Department of Botany, Prof. Dr. S. Mavinkurve, Head, Department of Microbiology and Prof. Dr. U M. X. Sangoadkar, Dean, Faculty of Life Sciences and Environment, Goa University, for allowing me to use their Departmental facilities. I am also grateful to Mahyco Hybrid Seed Company Ltd., Jalna, Maharastra, for providing continuos supply of sorghum seeds for carrying out the research work.

I would like to thank Dr. Saroj Bhosle, Reader, Department of Microbiology, for helping me with the HPLC and Dr. Sanjeev Ghadi, Senior Lecturer, Department of Marine Science and Biotechnology for his assistance in scanning the protein gel on densitometer. I would also like to thank all the teaching and non-teaching staff of Department of Botany, for their support throughout the work.

Thanks are also due to the Liberian for providing the library facilities and assisting in procurement of necessary books and scientific reprints etc.

I sincerely acknowledge my friend Ms. Shanti Desai for being there whenever I needed her and all friends I have made in the laboratory particularly Mrs. Preetha Anand and Ms. Shandra Sequira.

Finally I am indebted to my parents for their support, understanding and encouragement throughout my research work.

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ABBREVATIONS

A	Antheraxanthin
ABA	Abscisic acid
APX	Ascorbate peroxide
ASC	Ascorbic acid (Sodium salt)
CAM	Crassulacean acid metabolism
CAP	D-threochloromphenicol
CAT	Catalase
Car	Carotenoid
CFo	Intrinsic part of ATP synthase
CF1	Extrinsic part of ATP synthase
Chl a	Chlorophyll a
Chl b	Chlorophyll b
$Cytb_6/f$	Cytochrome b_6/f complex
D1	Protein containing heterodimeric centre of photosystem II
DCMU	3-(3,4-dichlophenyl)-1,1-dimethylurea
DCIP	2,6-dichlorophenolindophenol
DPC	Diphenylcarbazide
DTT	Dithiothreitol
ESR	Electron spin resonance
Fd	Ferrodoxin
Fm	Maximum chlorophyll fluorescence
F'm	Maximum fluorescence at steady state
F_o	Initial fluorescence
Fs	Steady state fluorescence
Fv	variable fluorescence
F'v	Variable fluorescence yield in a light adapted state, where F'v = F'm- F'o
GR	Glutathione reductase
GSH	Reduced glutathione
K_D	Non radiative dissipation constant
L	Lutene
LHC I	Light harvesting chlorophyll a/b proteins associated with photosystem I
LHC II	Light harvesting chlorophyll a/b proteins associated with photosystem II
LHCP	Light harvesting chlorophyll protein
Fe-SOD	Iron containing form of Superoxide dismutase
Mn-SOD	Manganese containing form of Superoxide dismutase
MV	Methyl viologen
MDA	Monodehydroascorbate
N	Neoxanthin
OEC	Oxygen evolution complex
$^1O_2$	Singlet oxygen
O_2^-	Superoxide radicals

OEC	Oxygen evolving complex
PS I	Photosystem I
PS II	Photosystem II
PAR	Photosynthetic active radiation (400-700 nm)
PFD	Photon flux density
P680	Chlorophyll a dimer which absorb at 680 nm (PS II)
P700	Chlorophyll a dimer which absorb at 700 nm (PS I)
PAM	Pulse amplitude modulation fluorometer
Pheo	pheophytin
ΔpH	Trans thylakoid pH gradient
PC	Plastocyanin
PQ	Plastoquinone
PQH_2	Plastoquinol (fully reduced form of plastoquinone)
Q	Quencher
Q_A	Primary electron acceptor of PS II
Q_B	Secondary electron acceptor of PS II
qE	High energy quenching
qN	Non photochemical quenching
qP	Photochemical quenching
ROH	Lipid radicals
ROOH	lipid peroxide radicals
SOD	Superoxidde dismutase
SM	Silicomolybdate
UV-B	Ultraviolet-β radiation
V	Violaxanthin
Z	Zeaxanthin
KDa	Kilodalton

ABSTRACT

The effect of photoinhibition at 5-50°C in plants fed with ascorbate and DTT on photosynthetic electron transport, chlorophyll fluorescence and oxidative stress, antioxidant enzymes and Xanthophyll cycle was investigated in leaves and isolated chloroplasts of the chilling sensitive *sorghum bicolor* L. The objective of the study was to understand the mechanism of photoinhibitory damage in the leaves and relate it to the level of chloroplasts and to study the protective mechanism (enzymatic and non-enzymatic) against photodamage and to elucidate the possible role of Xanthophyll cycle in energy dissipation. Photosynthetic electron transport, chlorophyll fluorescence and peroxidation of thylakoid lipids was studied as an indicator of damage to photosynthesis. The photoinhibition treatment of intact leaves resulted in inhibition of PS II activity; the extent of inhibition was directly proportional to the duration of the treatment. Chilling and high temperature in combination with light exacerbated the damage. The mechanism of damage at the two temperature appears to be different. PS II minus oxygen evolution and PS I activity remained largely unaffected in photoinhibited leaves, however, photoinhibition of isolated chloroplasts resulted in decrease in PS II minus oxygen evolution and PS I activity. Likewise Fv/Fm ratio, an indicator of utilisation of light energy and qP, an indicator of utilisation of harvested light in ATP synthesis declined due to the photoinhibition treatment. Non-photochemical quenching, however, increased as a result of photoinhibition treatment. The leaves were also fed with ascorbate and DTT, which stimulates and inhibit the Xanthophyll cycle respectively, to correlate the changes in qN with the change in Xanthophyll cycle. It was demonstrated that the increase in qN was much greater in leaves fed with ascorbate than seen in leaves fed with DTT, irrespective of temperature. Level of de-epoxidation of V to Z was much greater in leaves fed with ascorbate and photoinhibited than in leaves fed with DTT prior to the photoinhibition treatment. The photoinhibition treatment also resulted in significant changes in β-carotene and Neoxanthin.

The results obtained confirmed that level of de-epoxidation of V to Z is directly related to qN, indicative of dissipation of excess radiation in the form of heat, thereby suggesting role of Xanthophyll cycle in energy dissipation.

Photoinhibition treatment also resulted in the increased in MDA a product formed as a result of lipid peroxidation. The level of peroxidation could be brought down if chloroplasts were supplemented with β-carotene, ascorbate and glutathione before the photoinhibition treatment. Further confirmation that photoinhibition caused oxidative damage in sorghum under our experimental conditions was seen as a enhanced activity of superoxide dismutase and ascorbate peroxidase due to photoinhibition.

Plants were also grown with exogenously supplied ABA to understand the relationship between ABA and Xanthophyll cycle as V is a precursor for the ABA synthesis. Our results, as a first report, show that plants grown on ABA supplemented medium showed better energy dissipation (higher level of qN) and higher level of V+A+Z and greater de-epoxidation of V to Z, indicative of a possible role of ABA in protection against photodamage by a unknown mechanism, probably by stimulating the Xanthophyll cycle..

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

Photosynthesis is the process by which chlorophyll containing plants convert solar energy into photochemical energy. This energy is stored in the form of carbohydrates providing food for humans and all other heterotrophic organisms. In addition it provide us with fuel as well as fibre. The productive potential of this process has, until recent years, seem endless. However, the burgeoning human population with its constantly increasing demand on both present and past products of photosynthetic activity threatens our future survival. An understanding of this process under changing environmental conditions which put plants under environmental stress resulting in an aberrant change in physiological processes brought about by either one or a combination of environmental factors such as light, UV & visual (radiation), temperature and drought etc. An understanding of the process of photosynthesis under these environmentally unfavourable conditions and understanding of the process(es) involved in protection of photosynthesis against these stresses may help us improving the efficiency of photosynthesis and engineer plants to withstand unfavourable environmental conditions without significant loss to productivity.

In this work attempts are made to study the effect of high light (Photoinhibition) at various level of temperature (5-50°C) on photosynthetic electron transport, photochemical reactions, photosynthetic efficiency,

photochemical and non-photochemical quenching, to determine the extent of light energy being utilised photochemically towards generation of ATP and $NADP^+$ and extent of light energy being dissipated as heat. To study the role of xanthophyll cycle in protection against photodamage of photosynthesis by stimulating or inhibiting the xanthophyll cycle in the process, to study the role of antioxidant like β-carotene, ascorbate and glutathione on protection against photodamage under *in vitro* conditions and also to study the effect of high light on the response of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase and catalase etc. and also to study the effect of protein profile of thylakoid membrane and to look into the role of ABA in photo protection by influencing the xanthophyll cycle.

Because plants are rooted in one place they have limited capacity to avoid unfavourable changes in their environment such as insufficient or excessive light, extremes of temperature water shortage, and shortage of mineral nutrients. Plants growth and developmental patterns can be altered to deal with such problem for example increased location of biomass to roots occurs in dry and mineral nutrient deficiency conditions. Plant stature, leaf angle, hairiness and waxiness influence light absorption and heat balance thereby affecting leaf temperature and transpiration rate. Many of the developmental responses to the environment are mediated by plant hormones. All these feature are of importance in the survival of plants native to extreme environment. Fast growing crop plants on the other hand are less

well able to survive the extreme. Nevertheless, there is interest in the possibility that certain biochemical and metabolic features of plants could contribute to their growth and survival under sub optimal conditions. These biochemical features can be manipulated more by recombinant DNA techniques than mere complex processes such as development. A better understanding of how metabolism responds to the environment and the extent to which changes in the pathways and the production of particular end products improve performance against extreme environment is needed.

1.1 RADIANT ENERGY AND PHOTOSYNTHETIC EFFICIENCY:

Light is a form of electromagnetic radiation. All electromagnetic radiation has same characteristics and travel at the same speed of $3x10^8$ m s^{-1}. Sun radiates energy representing the entire electromagnetic spectrum but the earth's atmosphere is transparent only to part of the infra-red and ultraviolet light and all the visible light. Wave length of visible light are conveniently expressed as nanometer. Historically light intensity was measured in terms of lumens, a lumen being defined as the luminous flux on a unit surface all points of which are at unit distance from a uniform point source of one candle. Intensity of illumination was expressed either as foot candle (1 lumen h^{-1} ft^{-1}) or lux (one lumen m^{-2}). Now a days preferred term is radiant flux density or irradiance. Since photochemical reactions in photosynthesis depend more on the number of photons incident on a surface

rather than on the energy content of these photons, it is more logical to express photosynthetic irradiance in terms of the number of quantas (photons) falling on unit surface in unit time, i.e. as the photon flux density. The photon (or quantum) flux density in a particular wave length region is measured in units of mol $m^{-2}s^{-1}$ In this work we have used term PPFD density to indicate number of quanta (photon) falling on the surface in photosynthetic active radiation (400-700 nm).

Photosynthetic efficiency on global basis may be defined as the fraction of photosynthetically active radiation (PAR) that falls on the earth's surface that is converted to stored energy by photosynthesis in the biosphere. The solar energy striking the earth's atmosphere every year is equivalent to about $56x10^{23}$ J of heat. Of this roughly half is reflected back by the cloud and by the gases in the upper atmosphere. Of the remaining radiation that reaches the earth's surface only 50% is in the spectral region of light that could bring about photosynthesis, the other half being weak infra-red radiation. Thus the annual influx of energy of photosynthetically active radiation, that is from violet to red light, to the earth surface is equivalent to about $15x10^{23}$J. However, some 40% of this is reflected by ocean surface, deserts etc. and only the rest can be absorbed by the plant life on land and sea. Recent estimate of total annual amount of biomass (plant matter produced by photosynthesis) are about $2x10^{11}$ tonnes of organic matter which is equivalent to $3x10^{21}$ J of energy. Above 40% of this

organic matter is synthesise by phytoplankton, minute plants living near the surface of the ocean.

1.2 ABSORPTION OF LIGHT:

The photosynthetic apparatus is that part of the leaf or a algal cell which contains the ingredients for absorbing light and for channelling the energy of the excited pigment molecules into a series of chemical and enzymatic reactions. Chlorophyll's and carotenoids are the pigment responsible for capturing high quanta in higher plants. Chlorophyll is contained in chloroplasts, a saucer-shaped bodies of 4-10 mm in diameter and 1 mm in thickness. The number of chloroplasts per cell in higher plants varies from one to more than 100 depending upon the particular plants and on the growth conditions.

Internally the chloroplasts is comprised of a systems of lamellae or flattened thylakoid which are arranged in stacks as granas. The grana are embedded in a colourless matrix called the stroma and the whole chloroplast is surrounded by a double membrane, the chloroplast envelope. Within the chloroplasts the grana are inter connected by a system of loosely arranged membrane called stroma lamellae. The presence of lipids in the thylakoid membrane facilitate energy storage and selective permeability of sugars, salts, substrates etc. Chloroplast lipid play an important role in maintaining membrane structure and function and one of the causes for the thermal and

photo-decay of chloroplasts is the release of lipids from the membranes and their oxidation.

Chlorophyll molecule is consisting of various atoms. When such molecule absorb light by ground state atoms, they are lifted to an energy rich excited state. This electronically excited state can revert to the ground state in a number of ways. It can transfer the electronic excitation to another acceptor molecule as is the case in the photosynthesis, energy of which is utilised to synthesise ATP and reductant NADPH through the process of the photosynthetic electron transport. It can also dissipate part of the acquired energy as heat and emit photon of longer wave length than the absorbed wave length back to the space by the process called fluorescence. A third route by which an excited molecule can lose its energy is by transfer from its original excited singlet state into metastable triplet state with a much longer life time by a mechanism called inter system crossing. From the metastable triplet state the molecule can revert to the natural ground state by emitting a photon at a longer wavelength. This weak emission is known as phosphorescence.

1.3 MOLECULAR ORGANIZATION OF THYLAKOID MEMBRANE:

The thylakoid membrane net work of most higher plants and green algae have characteristic interconnected regions of stacked and unstacked membranes. The appressed membranes of the granal thylakoid stacks are in

close contact and are not directly exposed to stroma. In contrast the non appressed membranes, whose outer surface are in direct contact with the chloroplasts stroma, include the stroma lamellae and the end membranes and margins of the grana stacks. The inner surface of both appressed and non appressed membranes enclosed a narrow aqueous space called lumen.

Freeze-fracture electron microscopy has shown distinct differences in both the size and number of particles in the four fracture faces of appressed and non-appressed membranes (Staehelin and Arntzen 1983). The variation in particle distribution in membrane implies differences in the location of the multiprotein, spanning complexes of thylakoid membranes. Biochemical fractionation studies of thylakoid fragmented with detergents or mechanical shearing showed that the photosystems were indeed structural entities as they could be physically separated from one another. Stacked granal membrane fractions were enriched in PS II compared to the non-appressed stromal thylakoid fraction, which contain mainly PS I complex. Sane et al., (1970) had proposed that both PS II and some PS I were located close together in the appressed grana regions to participate in linear electron transport, while other PSI involved in cyclic photophosphorylation was distributed in the non-appressed stroma thylakoids.

Ackerlund *et al.* (1976) showed that appressed membrane fractions were enriched in PSII complex and PSII activity. Following analysis of the

chlorophyll proteins in appressed and non-appressed membranes fractions, Anderson and Anderson (1980) presented a new model for thylakoid membrane organisation (fig 1.1) which proposed an extreme lateral heterogenity in the distribution of photosystems, consequently most PS II complexes and their associated chlorophyll a/b protein (LHC II) are located in the appressed membrane region and are largely segregated from the PS I complex, ATP synthase is found exclusively in the non appressed regions (Miller and Staehelin 1976). It has been accepted that most of the light harvesting pigments of PS II and PS I were in contact with each other with the regulation of excitation energy distribution between the photosystems being controlled by spill over.

The model (Fig. 1.1) also explains heterogeneity of PS II. Melis (1984) showed biophysical measurements that PS II was heterogeneous. The major PS II pool, with larger light harvesting antenna, is located in the appressed membrane fraction and the minor PS II pool, with a smaller light harvesting antenna is present in stroma thylakoid vesicles. Immunogold electron microscopy has allowed the direct detection of antigens in appressed and non-appressed membranes (Goodchild *et al.* 1985; Anderson and Goodchild 1987). The studies have shown that there is a total extension of PS I complex from appressed granal thylakoid, and cytochrome b_6/f complex is present in both membrane regions. However, molecular organisation of thylakoid membranes should not be regarded as fixed and static. Certain

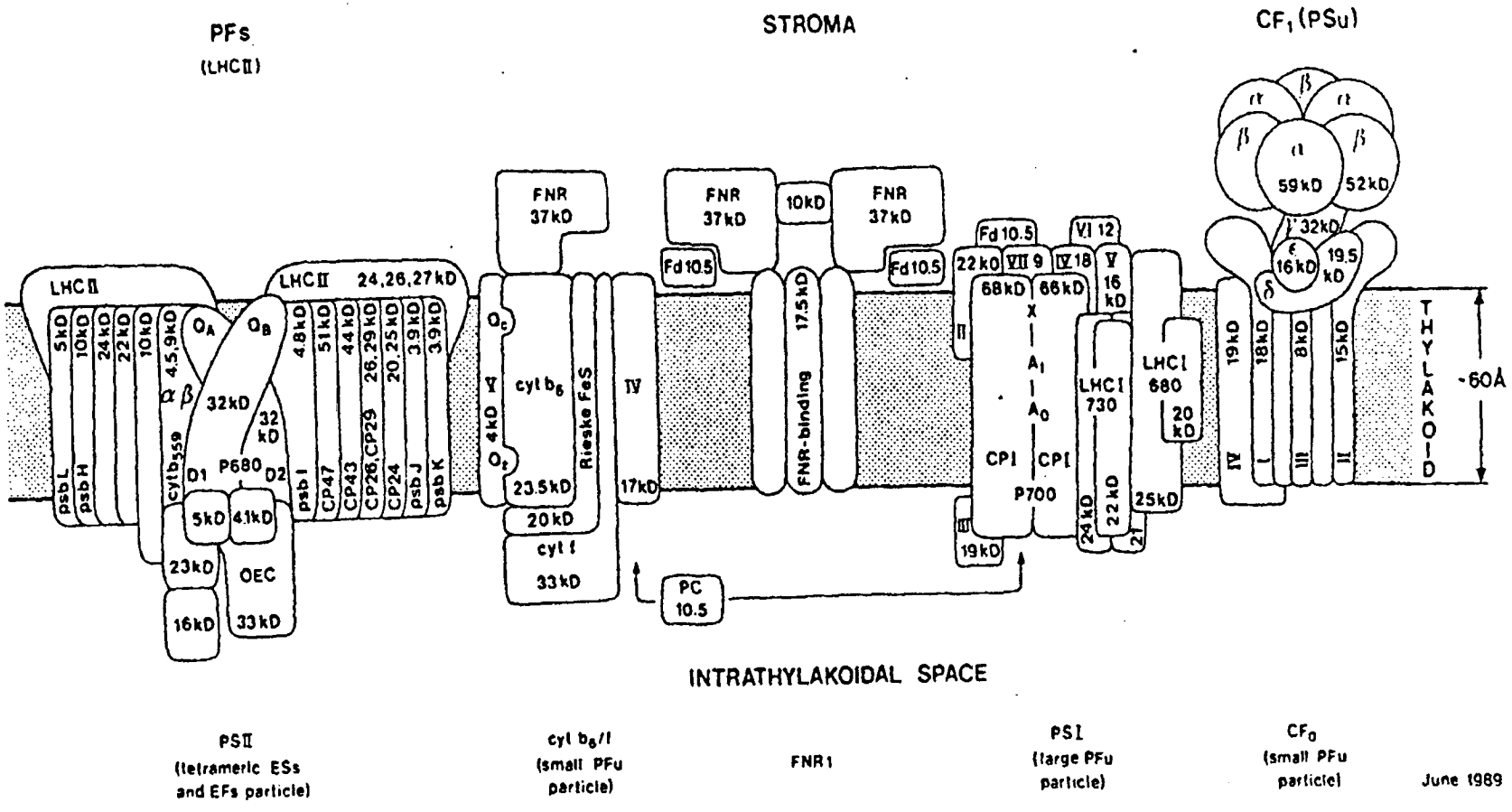


Fig 1.1 Model of thylakoid membrane showing the known polypeptide components of the major complexes.

environmental stimuli can evoke controlled reversible lateral movement of some protein complexes between the appressed and non-appressed regions, often in a matter of minutes.

1.3.1 THYLAKOID MEMBRANE PROTEINS: (ORGANIZATION OF ELECTRON TRANSPORT CHAIN):

Proteins of chloroplast are organised into a number of integral membrane- spanning complexes within the thylakoid membrane (Fig 1.1). These complexes together in co-ordination with each other function to conserve the absorbed solar energy as biologically important chemical forms, ATP and NADPH.

1.3.1.1 PHOTOSYSTEM II (PS II):

Photosystem II of higher plants is a multiprotein complex consisting of at least 20 polypeptides (Table 1.1). Marder and Barber (1989) have shown that the reaction centre chlorophyll (P680) and the intermediate acceptor pheophytin are present at the inter junction of the two 32 KDa polypeptides which are also known as D1 and D2 proteins respectively. PS II acts as a water plastoquinone oxidoreductase which has a four electron gate on its oxidising side and a two electron gate on its reducing side. Despite the fact that PS II is a multiprotein complex the above redox reaction are restricted to only two polypeptides known as D1 and D2 proteins (Fig. 1.2). A

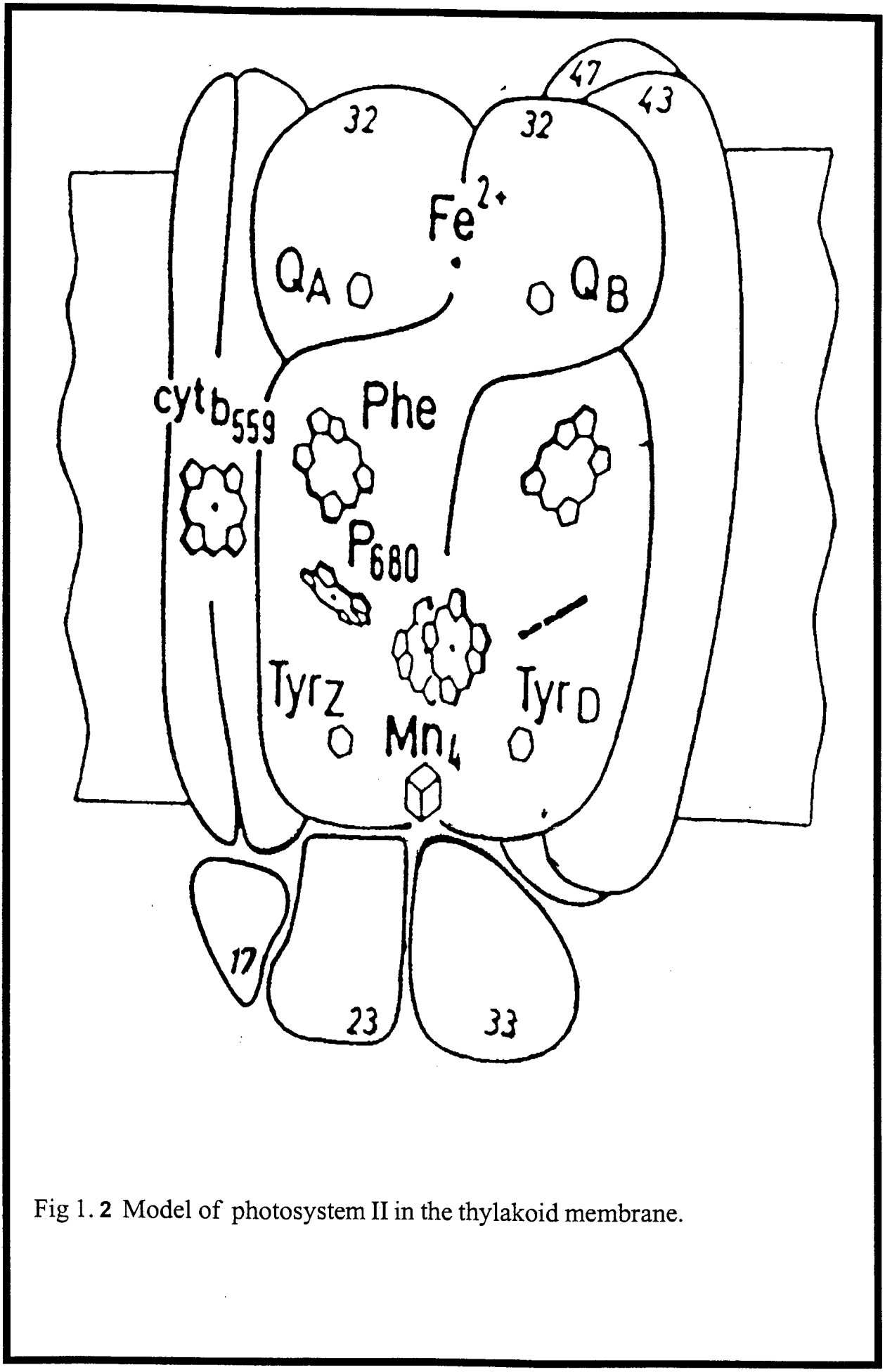
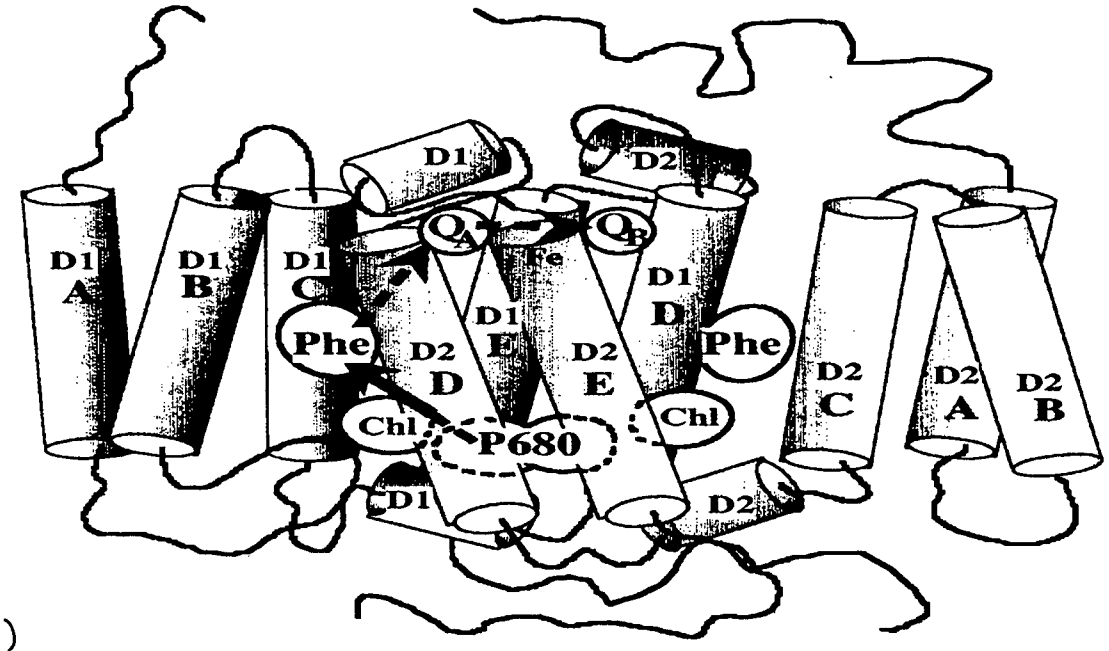


Fig 1. **2** Model of photosystem II in the thylakoid membrane.

considerable homology in amino acid sequences between D1 and D2 and between D1, D2 and the L and M subunits of the reaction centres of photosynthetic bacteria is reported. Biochemical confirmation of this relationship came from the isolation of reaction centre complex of PS II containing D1/D2 proteins (Nanba and Satoh 1987; Barber et al 1987). The D1 protein is also known as the herbicide binding protein and has been proposed to participate in Mn binding (Metz *et al.* 1986; Colemsn and Govindjee 1987; Dismukesh 1988; Virgin *et al.* 1988). The spatial organisation of the polypeptides of PS II complex is shown in Fig 1.3a

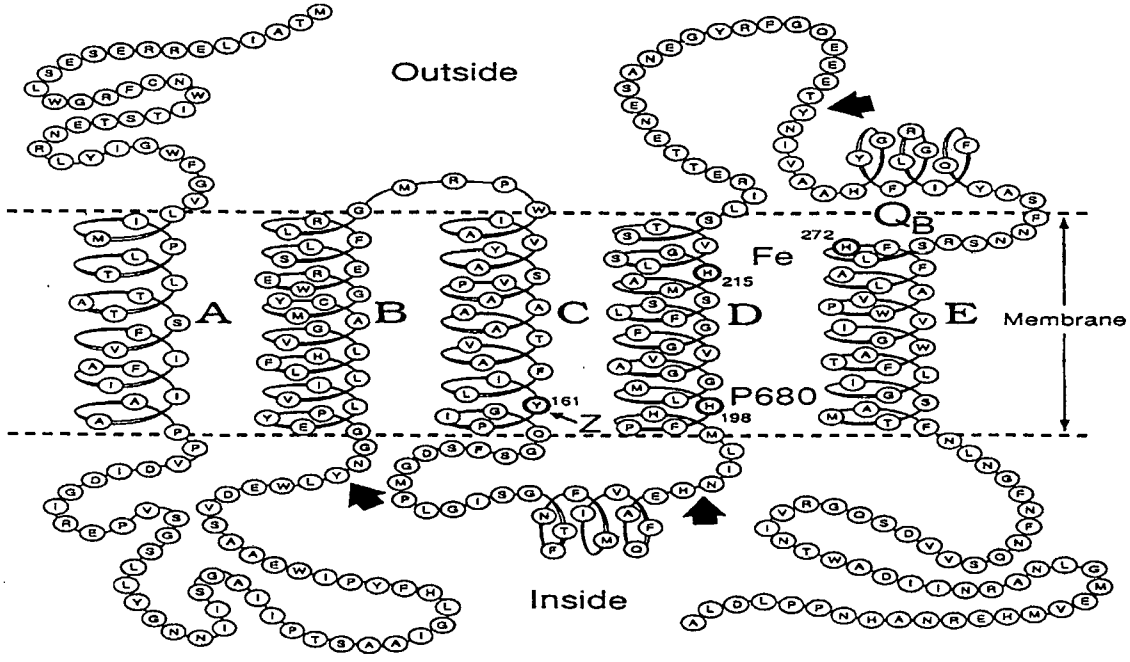
On receiving light energy from LHCII through the antenna, the reaction centre chlorophyll P680 reduces a bond quinone (QA) through a pheophytin molecule. The oxygen-evolving complex reduces P680 by splitting water and evolves oxygen. The species of Z, which transfers electrons from the oxygen evolution complex (OEC) to $P680^+$, is an amino acid residue tyrosine 161, in the D1 protein (Debus *et al.* 1988a). The dark stable radical known as D^+ (which gives rise to ESR signal II slow) has been identified as tyrosine 160 in the D2 protein (Barry and Babcock 1987; Debus *et al.* 1988b).

The process of photosynthetic oxygen evolution is partially understood but it is known to involve the accumulation of four oxidising equivalents on Mn leading to the four electron oxidation. In addition to the catalytic role as water plastoquinone oxidoreductase. PS II also plays a central role in light



(A)

Fig 1.3 Folding model for D1 and D2 protein to emphasize how they interact to form the reaction center of PS II. Each is believed to contain five trans membrane alpha-helical.



(B)

Fig 1.3 A model of the folding of the D1-polypeptide based on the amino acid sequence of spinach. The arrows identify approximate location of cleavage sites due to donor and acceptor side photodamage.

acclimation of the photosynthetic apparatus (Anderson and Anderson, 1988) and is also recognised as the target of photoinhibition of photosynthesis.

The isolated PS II reaction centre (Barber *et al.* 1987; Gounaris *et al.* 1988; Nanba and Satoh 1987) contains the D1 and D2 polypeptides, two subunits of cyt b559 (Webber *et al.* 1989) and 4.8 KDa protein (Webber *et al.* 1989). The D1-D2, Cytochrome b559 reaction centre complex also contains six Chlorophyll a, two pheophytin and two β-carotene molecules.

1.3.1.2 PHOTOSYSTEM I (PS I):

The photosystem I complex mediate electron transport from reduced plastoquinone in the thylakoid lumen to oxidised ferrodoxin in the stroma. It consists of an inner core surrounded by outer layers of light harvesting polypeptides. The major components of the reaction centre are two highly homologous chlorophyll binding polypeptide of about 65 KDa, subunit Ia and Ib. They are thought to bind P700, the reaction centre chlorophyll and the intermediary electron carrier complex (Bengis and Nelson 1977; Golbeck *et al.* 1988). There is no homology between these polypeptides and L and M and H subunits of the purple bacterial reaction centres (Fish *et al.* 1985). The structure and function of the PS I complex has been studied in detail by several workers (Rutherford and Herthcote 1985; Malkin and Webber 1990; Bassi and Simpson 1987; Golbeck 1988; Boekema *et al.* 1990).

Table - 1 . 1

Protein mass and redox potential of components of photosynthetic electron transport chain.

Component of electron transport chain	REDOX POTENTIAL (Volts)	MASS (kDa)	Gene
Water oxidising protein	+0.82	34	
Z (tyrosine 161)	+1.0	-	D1 - psbA
D (tyrosine 160)	+1.0	-	D2 - psbD
P680 (Chlorophyll a dimer)	+1.0	2 x 8.92	
Pheophytin (Chlorophyll a minus Mg)	-0.614	8.68	
QA (Quinone) Fe-S centre (non heme)	0 to -0.2	7.40	
Cyt b559	+0.08 (LP)	2 x 10	psbE
	+0.38 (HP)	4	psbF
Cyt b6	-0.5 (HP)	23.4	petB
	-0.17 (LP)		
Cyt f	+0.35	34	petA
Rieske Centre	+0.29	20	petD
P700 (Chlorophyll a dimer)	+0.48	2 x 890	
Ao (Chlorophyll a monomer)	-	-	psaA
A1 (Vitamin K)	-	-	psaB
X (4 Fe-s cluster)	-0.71	-	-
[Fe-S] A	-0.550	-	psaC
[Fe-S] B	-0.590	-	
Fd-FNR	-0.380	40	
Fd	-0.42	11	
Pc	+0.360	10.5	

1.3.1.3 CYTOCHROME b_6/f COMPLEX:

Cytochrome b_6/f complex in higher plants and green algae catalyses the electron/proton exchange between PS II and PS I and is required for cyclic electron flow supported by PS I (Cramer *et al.* 1987). The cytochrome b_6/f complex contains two plastoquinone-binding sites known as site E and site Z, which are located near the two surfaces of the membrane. Cytochrome b_6 is a dimer of 23.5 KDa protein, cytochrome f is 33 kDa protein which interact with plastocyanin. The Rieske Fe-S centre is 19.5 KDa protein. One more protein of 17 kDa is there which is known as unit IV.

1.3.2 ATP SYNTHASE COMPLEX:

The CFo-CF1 complex is a membrane bound enzyme which can couple a transmembrane H^+ transport with ATP synthesis/hydrolysis. This complex has a hydrophobic, membrane-integrated part CFo, which acts as a proton channel through the membrane and a hydrophilic part CF1, which contains the nucleotide-binding sites. CF1 contains five different subunits with the stoichiometry $\alpha 3$, $\beta 3$, $\gamma 3$, $\delta 1$, $\varepsilon 1$, while CFo consists of four different subunits with the stoichiometry I, II, III, IV. The structure and function of this complex have been studied extensively (McCarty and Moroney 1985; Boekema *et al.* 1990).

1.4 PHOTOINHIBITION:

Plants absorb solar radiation through chlorophyll molecules in light harvesting complexes. The absorbed excitation is transferred to a specialised pair of chlorophyll molecules known as the PS II reaction centre where charge separation takes place. In this process electrons, ultimately derived from water, are energised. This generates the potential for them to pass through a series of electron carriers to acceptors such as ferrodoxin & $NADP^+$. During the process ATP is formed. ATP & NADPH are utilised for the synthesis of organic molecules through fixation of CO_2 via the Calvin cycle. If synthesis of ATP/NADPH or the enzymes to Calvin's cycle are affected due to various environmental stresses the quantum requirement for CO_2 assimilation decreases. Under these conditions available quantum flux becomes excess to requirement. This excess radiation, if not dissipated away from the PS II reaction centre, decreases the efficiency of photosynthesis (Sharma and Hall 1992a).

Photoinhibition occurs when photosynthetic organisms are exposed to light level in excess to that which can be dissipated by their normal photochemical processes resulting in decreased photosynthetic capacity and occurs without the photooxidation of pigments (Sharma and Hall 1990; 1996; Powles 1984). Plants acclimated to low light are specially liable to photoinhibition and it is not surprising that the phenomena of photoinhibition

has been extensively studied in such plants. Environmental stress factor such as drought, low temperature (Bjorkman and Powles 1984; Sharma and Singhal 1993; Richards and Hall 1987; Mishra *et al.* 1993; Anderson *et al.* 1995; Sharma *et al.* 1994) and high temperature (Lodlow & Bjorkman 1984) often amplify the photoinhibition. Powles & Critchley (1980) reported that either low temperature and high irradiance or high temperature and low irradiance produced similar effects suggesting that the inhibition process is fundamentally similar in both cases.

1.4.1 TEMPERATURE:

The process of photosynthesis is sensitive to temperature low and high. Low temperature have been reported to have effect on regulation of photosynthetic carbon assimilation is *Zea mays* and *Hordeum vulgare* (Labate *et al.* 1990). At temperature lower than 4°C the main injury was loss of osmotic responsiveness (Webb *et al.* 1994; Bergevin *et al.* 1993), which was associated with the formation of the hexagonal II phase in the plasma membrane. A reduction in leaf chlorophyll and carotenoid content due to low temperature but an increase in the activity of oxygen detoxifying enzymes such as SOD and Ascorbate peroxidase (Marsacci *et al.* 1995). Integrity of thylakoid membrane was also affected by low temperature. Chilling resistant plants did not show any difference in the integrity of

thylakoid membrane but chilling sensitive plants showed an uncoupling of thylakoids (Peeler and Naylor 1998).

Photosynthetic membranes appear to be sensitive to high temperature. The sensitivity to higher temperatures varies from plant to plant and is applied to intact tissue (Mishra and Singhal 1993) or to isolated organelles. Plants exhibit the structural response to heat stress such as:

- A decrease in the amount of stacked membrane regions coupled with structural alteration of such regions (Armond et al. 1980),
- Release of manganese (Thompson et al. 1989; Nash et al., 1988),
- Heat denaturation of certain thylakoid polypeptides (Thompson 1989) &
- Formation of phase separated aggregates of non-bilayer forming lipids (Gounaris et al. 1983).

In general PS II mediated electron transport, photophosphorylation and certain soluble enzymes involved in CO_2 fixation are particularly affected at higher temperature.

Photochemical reactions are not temperature dependant as long as the lipid bilayer membrane remains stable so the production of ATP and NADP may continue at a wide range of low to high temperature. However enzymatic process of carbon dioxide fixation are reduced or ceased at very low and high temperature resulting in over energization if plants are exposed

to high light. This accumulation of energy in the low/absence of CO_2 fixation causes the formation of free radicals (superoxide and singlet oxygen) which if not removed from the system will oxidise unsaturated fatty acids and proteins in the surrounding membrane and exacerbating the damage.

Biological membranes are dynamic systems and are constantly in a state of motion. The fluidity of the membrane depends on the chemical structure of lipids and proteins organised in a bilayer composition. The percentage of lipids and proteins varies in the different membranes. Environmental factors such as temperature, light and hormonal effects can influence membrane fluidity thereby affecting its physiological functions.

Light through its action on phytochromes may also have an effect on membrane structure and permeability though actual proof of phytochrome occurring in membrane is lacking. Temperature is one of the most critical parameters known to affect membrane structure and function. The phospholipids are particularly sensitive to temperature change. Low temperatures cause a more structural configuration making the membrane more rigid. At high temperature the acyl groups are less organised and more randomly configured and the membrane takes a more fluid nature. The carbon length of fatty acid groups and the number of double bonds in the chain also affect the nature of the membrane.

1.4.2 PHOTOINHIBITION AT CHILLING TEMPERATURES:

Plants may be particularly prone to photoinhibition at low temperature (Oquist *et al.* 1987). Not only may low temperature impose rate limitations on photosynthetic apparatus but such temperature makes it venerable to over energization even at moderate light levels. Low temperature may also inhibit the alternative means of energy dissipation through systems such as photorespiration and Mehler reaction which also provide protection against photoinhibition. The repair mechanism responsible for recovery from photoinhibition may also be inhibited by low temperature (Oquist *et al.* 1987). Differences in response to chilling stress among plants might be explained by the fact that plants can be chilling sensitive, chilling tolerant or freezing tolerant. In chilling resistant plants, low temperature as such apparently causes no detectable dysfunction in photosynthesis but the synergies between light and chilling temperature may still inhibit photosynthesis (Ogran and Oquist 1984; Levitt 1980). A complete understanding of light as a stress factor at low temperature would require a consideration of irradiance in relation to

- The extent of temperature lowering.
- Duration of photoinhibition exposure.
- The low temperature tolerance of plants.
- The temperature effects on alternative mechanism of dissipating excess

excitation energy and

- Temperature effects on repair process.

The exposure of chilling sensitive plants to low temperature (0-12°C) is much more damaging to photosynthesis, when exposure occurs in the light compared to the dark (Sharma and Hall 1992; Long *et al.* 1983; Greer et al. 1988; Smillie *et al.* 1988). Exposing chilling sensitive C4 plants like *Zea mays*, *Sorghum bicolor* (Sharma and Hall 1992; Sharma *et al.* 1996) and some other chilling sensitive species, like *Pennisetum typhoides* to a temperature of 10°C and irradiance of 170 Wm^{-2} for a few days caused a strong inhibition of photosynthesis. It was also seen that the degree of inhibition increased with increased irradiance and duration of exposure. Chilling sensitive C3 species of higher plants like Cucumis sativus (Van-Hasselt and Van-Berlo 1980), *Phaseolus vulgaris* (Powles *et al.* 1983), *Lycopersicum esculantum* (Martin et al. 1981; Yakir *et al.* 1986), *Oryza sativa* (Moll and Steinback 1986), *Hordeum valgare* (Richards and Hall 1987; Sharma *et al.* 1996), *Spinacea oleracea* and the green algae *Chalamimydomonas reinhardii* (Kyle *et al.* 1984) showed photoinhibition of photosynthesis when expose to chilling temperature. Decrease in the stomatal conductance of higher plants are not to account for photoinhibition as observed decrease in the CO_2 assimilation only when plants were exposed to light at chilling temperature and not exposed to the dark. It is a common feature of all chilling sensitive plants that their photosynthesis is more

sensitive to chilling temperature in light than in darkness. There is evidence that chilling in darkness causes some inhibition as well (Martin et al. 1981).

It is suggested that oxygen radicals are significant factors in the induction of photoinhibition at low temperature (Sharma and Singhal 1992 a, b). Chilling of *Cucumus sativus* (Van-Hastt and Van-Haslt-Berlo 1980) and *Lemna gibba* (Lindeman 1979) in light did not cause photoinhibition of photosynthesis unless oxygen was present.

1.4.3 PHOTOINHIBITION AT HIGH TEMPERATURE:

As with other environmental stresses, high temperature has the potential to predispose chlorophyllus tissue to injury by excess light. Four types of injury is caused by high temperature.

The photosynthesis is one of the most heat sensitive process effecting growth. Plant differ greatly in their potential for photosynthetic acclimation to temperature. Plants from the cold adopted species have much higher photosynthetic capacity at low temperature than the plants from heat adopted species of deserts and vice versa. A decrease in photosynthesis is attributed to an irreversible inactivation of photosynthesis at different temperatures for the two types of plants (cold adopted and heat adopted). The effect is on the photosynthetic machinery directly and not on other

secondary processes that result in membrane permeability and breakdown (Berry and Bjorkman 1980).

1.4.3.1 HIGH TEMPERATURE EXACERBATED PHOTOINHIBITION:

Photoinhibition in leaf experiencing some other stress (example water stress) is exacerbated by high temperature. The exacerbation increases with temperature between 30-42°C. No such injury however occurs in the absence of light. This type of photoinhibition is partially or completely reversible in leaf temperature or light level falls even though water stress continues.

1.4.3.2 HIGH TEMPERATURE INDUCED PHOTOINHIBITION:

The interaction of light and high temperature (42-48°C) induces photoinhibition. If leaf temperature or light level falls plants can show partial or complete recovery depending on the severity of the treatment. High temperature induced photoinhibition has something in common with low temperature induced photoinhibition, in that both are associated with dysfunction of photosynthetic membrane. However, it is not apparently known whether like low temperature induced photoinhibition, high temperature induced photoinhibition is dependant upon the presence of oxygen (Powles 1984).

1.4.3.3. REVERSIBLE HIGH TEMPERATURE INJURIES:

Temperature between 42-48°C causes damage to the photosystems of leaves in the absence of light. Partial or complete recovery occurs if leaf temperature falls.

1.4.3.4 IRREVERSIBLE HIGH TEMPERATURE INJURY:

Leaves are killed by short less than (30 min) exposure to these temperature (Bilger et al. 1989) irrespective of light or other stresses the higher the temperature, the shorter the exposure required to cause the given level of injury/(light stress at high temperature) is that the lowest temperature to initiate such irreversible injuries delineates the maximum temperature or high temperature induced photoinhibition and irreversible high temperature injury.

1.4.4 PHOTOINHIBITION IN INTACT LEAVES:

The effect of photoinhibition in intact leaves of higher plants has been studied using CO_2 gas exchange and chlorophyll fluorescence techniques. Photoinhibitory treatment in *Kalanchoe daigremontiana* (a CAM plant) showed progressively greater reduction in the quantum yield, maximum photosynthetic rate and Fv/Fm ratio (Adams *et al.* 1987). Sharma and Hall (1992 a, b) have studied the effect of high irradiance on primary photochemical events and light regulated enzymes of photosynthetic carbon

metabolism. They showed that PS II was site of damage while PS I was comparatively unaffected. The activities of NADP malate dehydrogenase, PEP carboxylase and phosphoribulokinase in Sorghum leaves increased upon exposure to higher irradiance. Sharma and Singhal (1992a) also studied the effect of high light under aerobic and anaerobic conditions on thylakoid lipids and primary photochemistry of photosynthesis in intact leaves of wheat and reported that the photoinhibitory damage to photosynthesis was directly proportional to peroxidation of thylakoid lipids which occur more in plants photoinhibited under aerobic conditions than in anaerobic conditions.

Effect of photoinhibition in intact leaves of chilling sensitive species (cucumber) and chilling resistant species (pea) were studied by Peelar and Nayler (1988) They observed that pea photosynthesis was largely unaffected by chilling in the dark or chilling during 15 h light and 9 h dark cycle. However, Cucumber showed reduced CO_2 fixation when chilling in light but chilling in the dark showed decreases in CO_2 gas exchange but quantum yield and variable fluorescence were unaffected.

Similar results were observed by other workers on various species (Demmig et al. 1989) in *Populas balsamifera*, Arbutus unedo and *Helianthun annus*, (Greer and Laing 1989) in *Actinidia deliciosa*; (Strand and Oquist 1985) in pine needles, (Hodgson *et al.* 1987) in *Spinacea oleracea*, *Cucumus sativus* and *Nerium oleander*, (Stamp 1987) in *Zea mays*,.

1.4.5 PHOTOINHIBITION OF ISOLATED CHLOROPLASTS:

Photoinhibition has also been studied by exposing isolated chloroplasts, suspended in a suitable incubation medium to high photosynthetic photon flux densities. The advantage of this system is that the cellular mechanisms that protect against or repair photoinhibitory damage do not operate thus allowing the primary sites of damage to be more easily determined, Also the effects of photoinhibitory treatment are not lost during the subsequent chloroplast isolation procedure from the leaves. Photoinhibitory effects may, however be confused with the symptoms of chloroplast ageing, but with good chloroplast preparations these latter effects will only occur very slowly.

Bradbury and Baker (1986) studied the kinetics of a range of chlorophyll fluorescence parameter, non-cyclic electron transport and the capacity of the thylakoid to bind atrazine during photoinhibition treatment of intact pea chloroplasts. The contribution of the photochemical quenching processes to the loss of fluorescence during the photoinhibitory treatment showed two distinct phases of damage. During the initial 5 min period of exposure to light the minimal fluorescence level (Fo) increased while the maximum fluorescence level (Fm) decreased both coupled and uncoupled non-cyclic electron transport to methyl viologen decreased and the ability to bind Atrazine to the thylakoid decreased. Fluorescence analysis

demonstrated that during this period thylakoids became less efficient at generating and maintaining a transmembrane proton electrochemical gradient.

Photoinhibitory damage that occurred at later times between 5-20 min was of very different nature. Both Fo and Fm level declined and loss of coupled and uncoupled non-cyclic electron transport was observed together with a loss of the capacity to photooxidise water. However, no further loss of atrazine binding was associated with such changes. They also reported a decrease in the quantum yield throughout the photoinhibitory treatment. Barenyi and Krause (1985) showed severe inhibition of photosynthetic activities in Spinach chloroplasts with red light (850 Wm^{-2}). They observed that photoinactivation was prevented by addition of bicarbonate which allowed normal carbon metabolism to proceed during illumination. Chlorophyll fluorescence data obtained at room temperature and 77K indicated that photosystem II reaction centre were altered. Addition of superoxide dismutase, catalase and 1,4-dizabicyclo octane to isolated thylakoids prior to preillumination subsequently diminished photoinhibition. They concluded that reactive O_2 species were involved in the damage.

1.5 MOLECULAR EVENTS OF PHOTOINHIBITORY INACTIVATION:

The interaction between light and photosynthetic membrane appears more and more complex to understand the process of capture and conversion

of the light energy to a chemical form. An understanding of the phenomenon of photoinhibition will contribute to a better appreciation of its role in photosynthesis and its productivity. It is suggested that primary photochemistry of photosynthesis is involved in the photoinhibitory inactivation (Cleland 1988; Critchley 1988, Krause 1994). This may include oxidising side of the reaction centre (Sharma and Hall 1992, Barber and Andersson 1992, Wang *et al.* 1992, Vass et al. 1992), reducing side of the reaction centre (Kyle 1987; Mattoo et al. 1984, Trebst et al. 1988) or the reaction centre itself (Vass *et al.* 1992; Andersson and Barber, 1996). The evidence show that the inhibition of photochemistry proceeds from an initial damaging event that under stress condition could ramify other components including the oxygen evolving system and D1 protein. There are two possibilities conserving the nature of the primary event that; it is caused by an aberrant chemical species such oxygen radical or there is some change in the geometry of the reaction centre that result in the inhibition.

There are observations indicating that the initial damage to PSII involves photochemical processes that inactivates its function, trigger the D1 and D2 proteins for enzymatic degradation. There is no evidence to suggest that proteins are directly cleaved by photochemical reactions. Studies using *in vivo* and *in vitro* systems have revealed two distinct pathways by which photodamage may occur, one induced from the acceptor

side of PS II and another from the donor side of PSII (Eckert *et al.*1991, Anderson and Styring 1991; Prasil *et al.* 1992).

1.5.1 ACCEPTOR SIDE - INDUCED PHOTODAMAGE:

The support that photoinhibition involved the acceptor side of PSII system from the work of Kyle *et al.* (1984) who suggested the turnover of D1 protein under high light and suggested that damaging mechanism may involve a reactive form of plastoquinone bound to the QB site (Kyle 1987). Further it was confirmed by the work of Mattoo *et al.* (1984) ,Trebst *et al.* (1988), Jonsen *et al.* (1993) who showed that certain herbicides such as DCMU and atrazine, which bind to the D1 protein and displace plastoquinone from QB pocket are protective and prevent its rapid turnover, subsequently it was postulated that photoinhibition was due to the interaction of molecular oxygen with the plastoquinone anion in the QB site and that the resulting oxygen radicals would in turn lead to protein damage there by inhibiting electron transport (Kyle et al 1984). No degradation of D1 protein was observed when chloroplasts were photoinhibited under *in vitro* condition in the absence of oxygen (Arntz and Trebst 1986; Hundel *et al* . 1990; Kunn and Boger 1990; Nedbal *et al.* 1992; Sharma and Singhal 1992) indicating the role of oxygen radical in the photoinactivation. It was suggested that this type of damage occur when the photoquinone pool is fully reduced by strong illumination (Vass *et al.* 1992; Crofts and Wraight 1983). It has been

shown that exposure of isolated thylakoids to photoinhibitory illumination is accompanied by singlet oxygen production (Hideg *et al.* 1994) moreover, generation of singlet oxygen by isolated PS II reaction centre has been shown by detection of luminescence at 1270 nm (Macpherson *et al.* 1993, Telfer *et al.* 1994a) and by an indirect chemical trapping method (Telfer and Barber 1994; Telfer *et al.* 1994b). Oxygen radical could be generated from the triplet state of chlorophyll. Chlorophyll molecule absorbs quantum of light and is excited to the single state and could also be converted to the triplet form by intersystem crossing which then interacts within the oxygen molecule to form oxygen radical. The radical subsequently attacks P680 and there by inactivating it. Alternatively P680 itself forms a triplet, which subsequently interact with oxygen forming a radical which again attack the closest molecule. A triplet state is formed more often when Q is reduced (Moore and Smith 1984). In other case oxygen directly alters P680 itself by absorbing second quanta by oxidising P680. Many of these modifications of P680 would affect its electrical properties. There is a evidence that O_2 is involved in photoinhibition (Wise and Naylor 1987). In high light excessive excitation might generate (^{3}Chl) in the antenna which can transfer its excitation energy to ground state $^3O_2$ forming $^1O_2$ as long as triplet chlorophyll is not quenched by carotenoids (Asada and Takahashi 1987; Siefermenn-Harms 1987).

$$^1Chl^* \rightarrow {}^3Chl$$
$$^3Chl + {}^3O_2 \rightarrow {}^1Chl + {}^1O_2$$

superoxide ($O_2^{\cdot-}$) and hydroxide ($OH^{\cdot}$) radical is formed by reducing the oxygen under conditions where reductant generated in the photochemical reactions are not utilised by biochemical reactions. Though superoxide ($O2^{\cdot-}$) radical are rather unreactive but can give rise to the formation of hydroxyl radicals in the Haber-Weis reaction (Youngman 1984).

$$O_2 + Fe^{3+} \rightarrow Fe^{2+}\ O_2^{-\cdot}$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^{\cdot}$$

Further study of isolated system has also indicated that the target of singlet oxygen attack in the P680 chlorophyll which may be at its conjugated ring system. Alternatively it could ligate with histidine, which is known to react with singlet oxygen (Halliwell and Gutteridge 1989). Another possibility is the formation of bityrosine cross links (Davies 1987; Prasil *et al.* 1992).

The concept that acceptor side photoinhibition is due to damage by singlet oxygen generated when radical pair recombination occurs is recognised with various observations from a number of laboratories (Setlik *et al.* 1990; Kirilovsky *et al.* 1990, Vass *et al.* 1992; Aro *et al.* 1993). The conclusion is that the situation is not fully clear since the detailed role of primary (QA) and secondary (QB) plastoquinone acceptors during photoinactivation remains to be conclusively established particularly with

respect to the situation *in vivo*. Despite the compelling arguments for singlet oxygen as a main damaging species of PSII, other toxic oxygen and hydroxyl radicals may be involved (Ritcher *et al.* 1990, Greenberg *et al* . 1990). Several experiments using various oxygen radical scavengers have been shown to partially protect the D1 proteins from degradation (Barenyi and Krause 1985, Ritchter *et al.* 1990, Sopory *et al* . 1990).

1.5.2 DONOR SIDE - INDUCED PHOTODAMAGE:

Some of the complications experienced in the interpretation of data in terms of the acceptor side/singlet oxygen mechanism are due to the fact that there seems to be a second oxygen independent route by which PSII can be photoinactivated (Barber and Anderson 1992). The photoinactivation occur when the rate of electron donation to PS II does not match the rate of electron removal to the acceptor side. This situation may occur during normal steady state electron flow even at low light intensities and most certainly under conditions which destabilise the water splitting system such as low temperature (Wang *et al.* 1992a & b). Such stabilisation result in an increased lifetime for the $P680^+$ state, which is a high oxidising potential and has the capacity to extract electrons from its surroundings and thus cause irreversible oxidative damage.

Experimental evidence for the donor side inactivation has come mainly from *in vivo* studies. Susceptibility of PSII to photoinduce inactivation and

protein degradation has been shown in hydroxylamine treated leaf segment (Callanhan *et al* . 1986) in leaves subjected to cold stress (Wang *et al.* 1992a, Sharma and Hall 1992b, in Tris- (Wang *et al* . 1992b) and hydroxylamine treated thylakoid (Blubaugh *et al.* 1991) and in Cl^- depleted thylakoids (Critchley *et al.* 1984; Jagerschold *et al* . 1990). The increased susceptibility of the LF-1 mutant of Scenedesmus (Gong and Ohad 1991) and the psb O- less mutant of Synechocystis 6803 (Philbrick *et al.* 1991) compared to their wild types is also probably due to donor side effects. This is because the LF-1 mutant does not have water splitting activity due to its inability to process the carboxy- terminus of the D1-protein (Bowyer *et al* . 1992), while the absence of the 33 kDa extrinsic proteins of the PSII in the psbO-less Synechocystis mutant perturbs its donar side activity (Vass *et al* . 1992).

It is generally agreed that the site of donor side inactivation is between the manganese cluster and P680 (Eckert *et al.* 1991; Jagerschold *et al.* 1990; Blubaugh and Cheniae 1990). Blubaugh *et al* . (1991) ressolved three kinetically different phases of inactivation. The first phase was to be a decrease in the rate of electron transfer between y_z and $P680^+$, second was a loss of y_z^+ formation. The third phase was very slow and was observed as a loss of y_D^+ formation. They also identified the formation of carotenoids and chlorophyll radicals under conditions of donor side induced photoinhibition.

Blaubaugh and Cheniae (1990) have proposed that in addition to cation radical mechanism ($P680^+$ and y_z^+) for explaining donor side photoinhibition, there is also a rapid process which involves superoxide radicals. But there are greater evidence that donor side photoinhibition can occur under anaerobic condition (Jagerschold et al 1990; Shipton and Barber 1992), then the involvement of superoxide may not present a primary route of damage.

In brief it is proposed that acceptor side mechanism involves recombination of radical pair $P680^+$ Pheophytin$^-$. The recombination lead to the production of the $P680^+$ triplet which is not quenched by carotenoids but instead lead to the formation of highly toxic singlet O_2.

$$^3P680^+ + ^3O_2 \rightarrow P680 + ^1O_2.$$

As a consequence, the D1 protein is modified by triggering of proteolytic degradation. The degradation process involves an initial cleavage in the loop joining transmembrane segments D and E near to the QB binding site (C-terminal side of residue 238).

The donor side mechanism, however, is not dependent on the presence of oxygen and result from damage due to long lived oxidation states $P680^+$. Extensive oxidation of this type destabilises the D1 and D2 protein and pattern of degradation products observed is different from that generated by the acceptor side mechanism. The primary cleavage occurs on

the D1 protein in the loop transmembrane segment A and B. There is no evidence that either side induced degradation of protein is due to direct photochemical cleavage. Rather it seems that the detrimental photochemical processes give rise to confirmational changes in D1 and D2 proteins that signal proteolytic reaction (Fig. 1.3b).

1.6 CHLOROPHYLL FLUORESCENCE:

When a substance absorb radiation its energy content increases. Conversely, when it emits radiation its energy content decreases. In the quantum theory, it is found that the energy content of atoms and molecules can not assume any arbitrary value, but must be confined to certain fixed values, called energy levels. The energy leaves are characteristic of the particular atom or molecule under study. When energy is absorbed, and an electron undergoes a transition from a lower energy level to a higher one the atom becomes excited. The wavelengths of light that an atom can absorb by the exact gap between the ground and excited states. Once in excited state, the electron tends to return to the stable, ground state. In atoms this can be by radiationless decay (heat) to the surroundings, or can be accompanied by re-emission of light. Once a molecule is in a metastable excited state there are several ways in which the excitation energy may be lost, each of which leads to the attainment of a stable, lower energy state. Fluorescence is one of the ways in which an excited molecule can regain the ground state. It

involves the emission of radiation, which can be detected fairly readily. Room temperature fluorescence emission spectrum of intact chloroplasts. It exhibits a peak at 682 nm and a broad shoulder at about 740 nm. There is general agreement that at room temperature, contrary to the situation at low temperature, chlorophyll a fluorescence is largely emitted by PS II and by the attached LHC. However, PS I emission may contribute to the long wavelength band.

The fluorescence induction exhibits characteristic phases with fluorescence levels usually termed O, I, D, P, S, M, T. The level O (Fo) denotes the constant fluorescence, seen when after dark period all reaction centre of PS II are open. The fluorescence level above the Fo level is termed variable fluorescence, Fv. From Fo the induction rises rapidly to the peak, P. This rise shows two phases. The maximum of the first phase has been termed I (Inflection), followed by a dip D, of fluorescence emission. From P the fluorescence a decline, usually with slower kinetics, to stationary level S. This may be followed by another maximum , M, and terminal level T. This general course of induction can, however, vary substantially depending on experimental conditions and the subject under study.

The Fo level of fluorescence is thought to represent emission by excited antennae chlorophyll a molecules occurring before the exciton have migrated to the reaction centre. Thus the quantum yield of Fo is independent

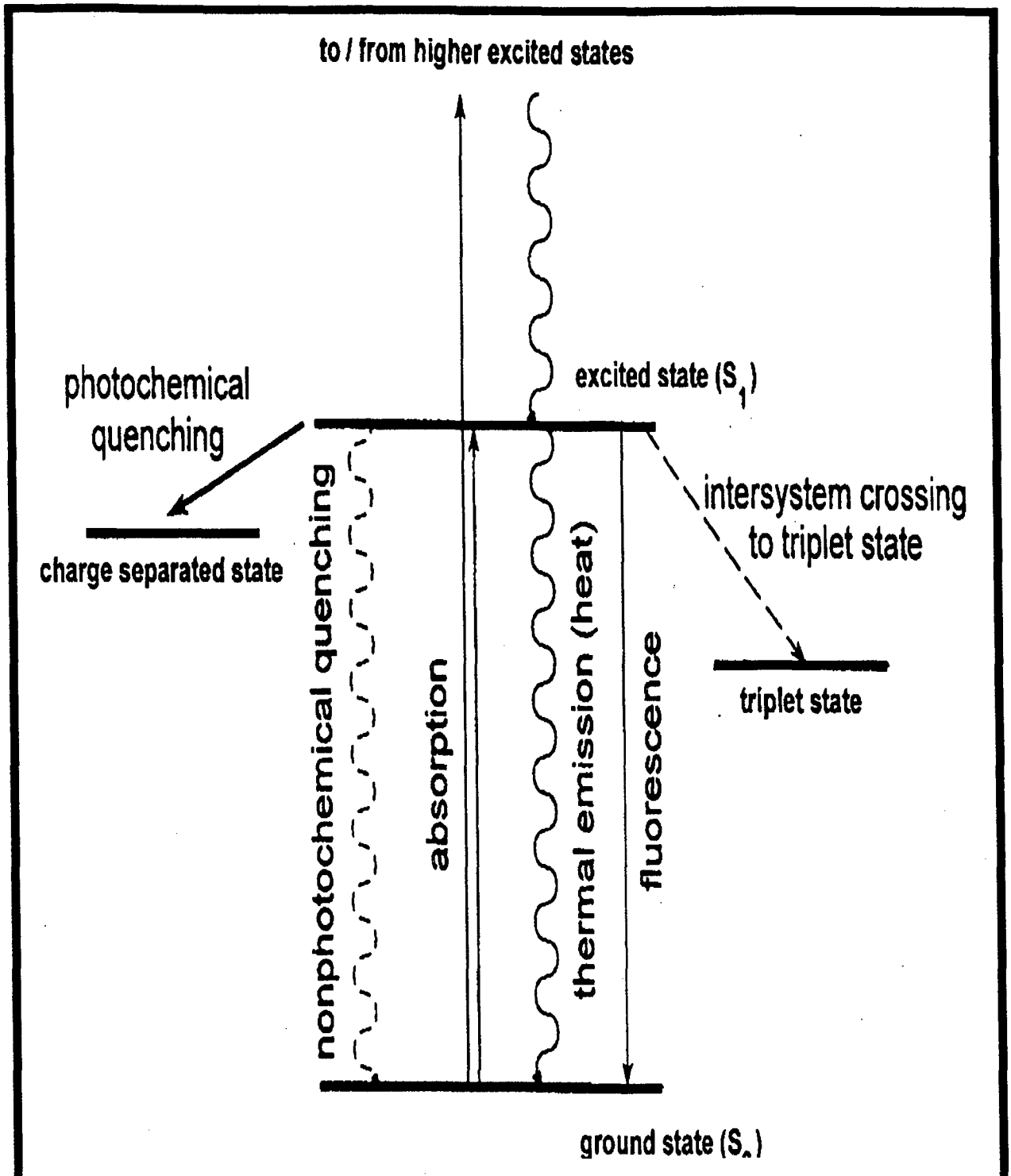


Fig 1.4 Processes competing for decay of chlorophyll excited states back to the ground state. Processes to the left of the absorption lines occur *in vivo* only, processes to the right of the absorption lines occur both *in vivo* and *in vitro*.

of photochemical events. Emission from PS I antennae may contribute to the Fo level of long wavelength fluorescence. Fo can be achieved by dark incubation for several minutes. The Fo level is known to be affected by environmental stress that causes structural alterations at the PS II pigment level. The fluorescence rise from Fo is usually considered to reflect reduction of the electron acceptor Q_A and is known as variable fluorescence (Fv = Fm-Fo).

1.6.1 PHOTOCHEMICAL QUENCHING:

This is due to oxidation of the excited state P680 by the PS II electron acceptor Q_A. The extent of qP depend upon the redox state of Q_A and so upon the rate of oxidation of Q_A by PS I.

1.6.2 NON-PHOTOCHEMICAL QUENCHING AND REGULATION OF LIGHT ENERGY UTILIZATION:

Regulation of energy utilisation in photosynthesis is accomplished by a class of reactions that are collectively called non-photochemical quenching, or qN. these reactions dissipate absorbed light energy by competing with photochemical quenching (qP) in the reaction centres for Chlorophyll excited states in the antenna (Fig. 1.4). The distinction between qP and qN is not strictly defined by either the site at which the quenching occurs (reaction centre versus antenna pigments) or by the participation of photochemical

charge separation, because at least two components of qN may utilise some from of charge separation in the PSII reaction centre as a part of the quenching mechanism. Rather, the distinction should be made at the level of whether or not the quenching reaction leads to stable storage of the excited state energy in biological oxidants and reductants (qP) or dissipation of the energy as heat (qN).

The phenomenology of qN and qP is closely tied with measurement of Chlorophyll fluorescence yield because the reactions of qN and qP compete with the 'internal' decay processes of fluorescence, thermal emission and triplet formation. The identification and quantitation of qN and qP is most easily accomplished by measurements of room temperature fluorescence emission (Schreiber *et al.* 1986; Krause and Weis, 1991). As a result, qN is commonly referred to as 'non-photochemical quenching of Chlorophyll fluorescence'. This term is misleading because the organism is not regulating the availability of excited states for photosynthesis by introducing new processes which compete with all other processes, including photochemistry and fluorescence, for excited states (fig. 1.4). In addition, because room temperature fluorescence emission is dominated by PS II antenna pigments, the phenomenology of qN and qP is largely restricted to processes in PS II.

It is now well established that the competition between fluorescence and photochemistry in PS II is the origin of variable fluorescence and qP

(Krause and Weis,1991; Govindjee, 1995). Fluorescence quenching that is independent of photochemistry (qN) was first reported by Murata and Sugahara (1969). Subsequent work by Wraight and Crofts (1970) demonstrated that this quenching on the extent of the pH gradient across the thylakoid membrane. Bradbury and Baker (1981) and Krause *et al.* (1982) introduced the first techniques to separate and quantify qN and qP. Most recently, the availability of commercial modulated Chlorophyll fluorometers (Schreiber *et al.* 1986; Bolhar-Nordenkampf *et al.* 1989) has greatly simplified the measurement of qN and qP and their correlation with other physiological phenomena. Using these techniques, the complexity of processes that contribute to qN is slowly being revealed. These processes range between those that serve to protect the photosynthetic apparatus against the effects of excess light and to those that are consequences of damage induced by excess light. The common feature of these processes is that they represent new pathways for decay of Chlorophyll excited states that compete with fluorescence and with stable charge separation in the PS II reaction centre.

1.6.2.1 COMPONENTS OF NON PHOTOCHEMICAL QUENCHING:

The result of numerous studies indicate that the physiological processes that contribute to total non-photochemical quenching are both

complex and heterogeneous. In general, these processes fall into three categories:

Energy-dependent quenching (qE) which is regulated to a large extent by the pH of the thylakoid lumen (Demming-Adams, 1990; Horton *et al.* 1994). Photoinhibitory quenching (qI) which is related to the slowly reversible, light-dependent depression in the light-saturated rate of photosynthesis (Krause, 1988; Ruban and Horton, 1995), and light-state transitions which quench PS II fluorescence by physically altering the antenna size of PS II units and/or spillover of energy to PS II (Williams and Allen, 1987; McCormac *et al.* 1994). While each of these categories contain processes that contribute to protection of the photosynthetic apparatus against the effects of excess light, qI also contains contributions from the damage to PS II that results from excess light.

Under normal physiological growth conditions, qE is though to be the major component of total qN, and is thus the dominant process regulating light energy utilisation in PS II (Horton *et al.* 1994). The term energy-dependent quenching arises from the observation that the extent of qE is regulated by the size of the (pH across the thylakoid membrane; that is, by the extent that the membrane is energised for ATP synthesis). Increases in qE correlate with decreases in the quantum yield of photochemistry in open PS II reaction centres (Weis and Berry, 1987) clearly indicating its primary

role in regulating the utilisation of absorbed light energy. The amount of qE exceeds the capacity of the dark reactions to use the products of electron transport either at saturating light or when environmental stress has depressed the capacity of reactions that limit the rate of photosynthesis. As a result, qE has been widely used as an indicator of the responses of plants to environmental stress. Simultaneous measurements of qN and qP may be sufficient to estimate the rate of linear election transport (Weis and Berry, 1987) although it is important to note that the relationship between qP, qN and electron transport is empirical and will likely be dependent on the species and previous growth conditions.

Under more severe stress (excess light) conditions, qI, the photoinhibitory component of qN, may become dominant due to increase in both the range of photoprotective processes that the plant calls upon to deal with excess light and to the accumulation of damaged PS II reaction centre complexes resulting from excess light absorption. The photoprotective component of qI, like qE, serves to reduce excess light-induced damage in PS II by dissipating excess absorbed light energy in competition with photochemistry and fluorescence in PS II. Unlike qE, qI does not readily relax in the dark, nor is it sensitive to the action of uncouplers (Ruban and Horton, 1995). Thus, although the two quenching components may utilise common constituents of the thylakoid membrane, the regulation of the underlying process must be distinct. The photodamage component of qI is the result of

the well characterised loss of variable fluorescence that occurs upon photoinhibitory damage to the PS II reaction centre complex (Krause, 1988). The damaged reaction centre remains an efficient quencher of excited states from the PS II antenna, accounting for loss of variable fluorescence and the observation that the reaction centre appears to be 'stuck' at Fo. The mechanism of this quenching is poorly understood, however, it does not result in stable charge separation at PS II reaction centres. Distinguishing between the protective and damage-related components of ql is essential because the principle role of the protective component is to limit the extent of damage to PS II (Ting and Owens, 1994).

Light state transitions were originally observed in algae exposed to light that was preferentially absorbed by PS I (State I) or PS II (State II). More recently, state transitions in higher plants and green algae have been attributed to a phosphorylation-induced redistribution of a portion of the LHC II antenna between the appressed and stroma-exposed regions of the thylakoid membrane (Williams and Allen, 1987). The extent of LHC II phosphorylation is under such that over excitation of PS II leads to a reduction of the plastoquinone pool, phosphorylation of LHC II and subsequent movement of the phospho LHC II to the stroma-exposed membranes (Allen,1992). The fate of excited states in the phospho-LHC II remains controversial, but the net result is a decrease in the antenna size of PS II in state II with a corresponding decrease in fluorescence yield

(McCormac *et al.* 1994). Thus the effect of LHC II phosphorylation associated with light state transitions is to reduce the excitation density in PS II at any incident light intensity. Although it is generally believed that ql is a minor component of qN (Krause and Weis, 1991; Andrews et al. 1993) the overall significance of light state transitions on the regulation of light energy utilisation remains poorly understood.

1.7 OXIDATION STRESS/ACTIVATION OF OXYGEN & DEFENSE MECHANISM:

Atmospheric oxygen in its ground state is distinctive among the gaseous elements because it has two unpaired electrons. This makes oxygen very unlikely to participate in reactions with organic molecules unless it is activated. The requirement for activation occurs because the two unpaired electrons in oxygen have parallel spins. According to Pauli's exclusion principle, this precludes reactions with a divalent reductant, unless this reductant also has two unpaired electrons with parallel spin opposite to that of the oxygen, which is a very rare occurrence. Hence, oxygen is usually non-reactive to organic molecules (Youngmen 1984; Halliwell & Gurtteridge 1989).

Activation of oxygen may occur by two different mechanisms. Absorption of sufficient energy to reverse the spin on one of the unpaired electrons, or monovalent reduction. If triples oxygen absorbs sufficient

energy to reverse the spin of one of its unpaired electrons, it will form the singlet state. This activation overcomes the spin restriction & singlet oxygen can consequently participate in reactions involving the simultaneous transfer of two electrons. The second mechanism of activation is by the stepwise monovalent reduction of oxygen to form superoxide ($O_2^{\cdot}$) hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) & finally water (Krause 1994; Robinson 1988; Krause and Cornie 1987) (Fig. 1.5). The first step in the raduction of oxygen forming superoxide is endothermic but subsequent reductions are exothermic.

A dismutation reaction leading to the formation of hydrogen peroxide and oxygen can occur spontaneously or is catalyzed by the enzymes superoxide dismutase (SOD). H_2O_2 is not a free radical because all of its electrons are paired. H_2O_2 readily permeates membranes & it is therefore, non compart-metalized in the cell. Reactivity of hydrogen peroxide is not due to its reactivity perse, but requires the presence of a metal reducatant to from the highly reactive hydroxyl radical ($^{\cdot}OH$) which is the strongest oxidizing agent known & reacts with organic molecules (lipid, proteins & DNA) at diffusion limited rates. In biological system ferrous ions limits the rate of reaction.

$$O2^{\cdot} + H_2O_2 \rightarrow O_2 + OH + OH^{\cdot}$$

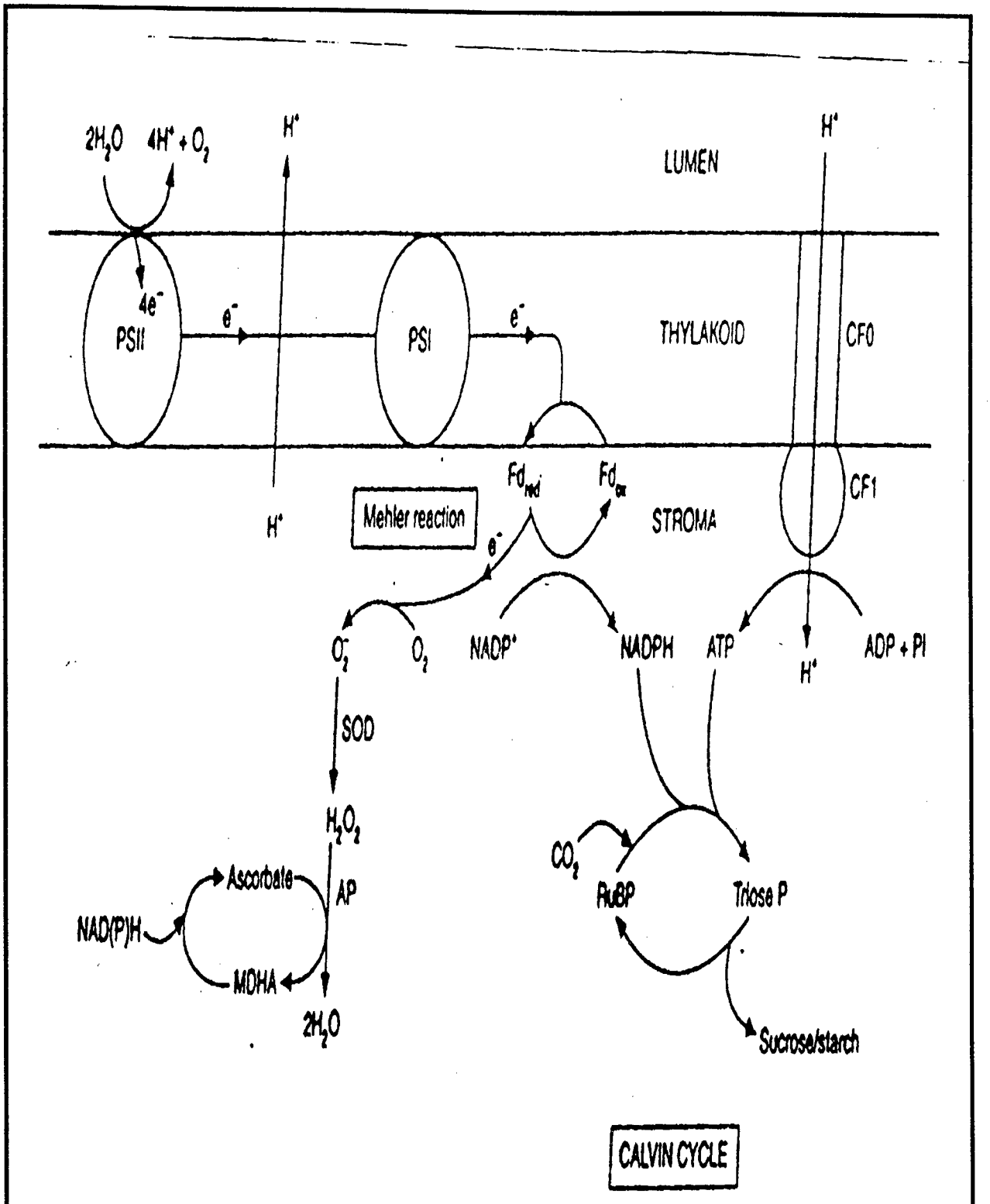


Fig 1.5 Mehler reaction

The oxidation of organic substances may proceed by two possible reactions- addition of ·OH to the organic molecule, or abstraction of a hydrogen atom from it. In the addition reaction, the hydroxyl radical adds to an organic substrate forming a hydroxylated product that is further oxidised by ferrous ions, or oxygen to a stable oxidised product.

$$\cdot OH + R \rightarrow \cdot ROH$$
$$\cdot ROH + Fe^{3+} \rightarrow ROH + Fe^{2+} + H^{+}$$
$$\cdot ROH + O_2 \rightarrow ROH + O_2^{\cdot} + H^{+}$$

In the abstraction reaction, the hydroxyl radical oxidises the organic substrate forming water & an organic radical. The organic radicals can react with O_2 in the triplet ground state, which can lead to the formation of a peroxyl radical which can readily abstract hydrogen from another organic molecule leading to the formation of a second Carbon radical. This chain reaction is why oxygen free radicals cause damage far in excess of their initial concentration

$$\cdot OH + RH \rightarrow R\cdot + H_2O$$
$$\cdot R + O_2 \rightarrow ROO\cdot$$
$$ROO\cdot + RH \rightarrow R\cdot + ROOH\cdot$$

Elstner (1991) have described four sites within the chloroplasts that can activate oxygen.

- Reducing side of PSI is thought to contribute significantly to the monovalent reduction of oxygen under conditions where NADP is

- limiting. This would occur, for example, if the Calvin cycle did not oxidise NADPH as rapidly as PSI supplied electrons.

- Photo activated chlorophyll normally transfers its excitation energy to PS reaction centre, but under conditions that prevents the captured light energy from being utilised in the electron transport systems, these conditions include environmental stress, etc.

- The oxidising side of PS II facilitates four single electron transfers from water to the PS II reaction centre releasing triplet or ground state oxygen.

- Photorespiration is the most obvious oxygenation pathway in the Chloroplast. Rubisco catalyses the addition of O_2 to Carbon 2 of RUBP forming phosphoglycolate & phosphoglycerate. Although this does not generate activated oxygen in the chloroplasts, the subsequent metabolism of glycolate in the peroxisomes does.

1.7.1 DUAL ROLE OF OXYGEN IN PHOTOINHIBITION:

Oxygen play dual role in photoinhibition on as a protective role against photoinhibition and another as a enhancement of photoinhibition.

1.7.1.1 PROTECTIVE EFFECT OF OXYGEN:

(A) Photorespiration:

Studies have shown that levels of oxygen present in the atmosphere which promote photorespiration diminish the extent of photoinhibition (Krause and Cornic, 1987) The protective effect of photorespiration has been attributed to the consumption of photosynthetic energy (NADPH and ATP) (Wu *et al.* 1991; Scheuramann *et al.* 1991; Stuhlfauth *et al.* 1990).

(B) Mehler reaction (reduction of oxygen):

Oxygen serves as an alternative electron acceptor of PSI and other acceptor such as $NADP^+$ or NO_2 are insufficient to reoxidise to reduce ferrodoxin (Mehler 1951; Badger 1985). This predominant pathway of oxygen reduction proceeds from reduced ferrodoxin and generate superoxide radical.

$$2O_2 + 2Fd\ red \rightarrow 2O_2^{\cdot} + 2Fd\ ox$$
$$2O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

In excess light oxygen reduction contributes to the electron drain from PSI there by preventing photodamage (Heber *et al.* 1990).

1.7.1.2 ENHANCEMENT OF PHOTOINHIBITION BY OXYGEN:

Increasing the level of oxygen from 1 or 2% to 21% or higher causes promotion of photoinhibition under excess light conditions. This damaging effect of oxygen can also be observed under conditions that over

compensate, diminish or eliminate the protection provided by photorespiration which generally occur under *in vitro* condition (Barengi and Krause 1985). The exacerbation of photoinhibition by oxygen can also be seen under *in vivo* conditions by irradiating leaves at low temperature when carbon metabolism occur at much reduced rate (Krause 1994; Rowley and Taylor 1972; Powles *et al.* 1983; Hetherington *et al.* 1989).

1.7.2 BIOLOGICAL REACTIONS OF OXYGEN RADICAL:

The reactions of activated oxygen with biological systems there are more complex due to the surface properties of membrane, electrical charges, binding properties of macromolecules, and compartmentelization of enzymes, substrates & catalysts. Thus various sites even within a single cell differ in the nature & extent of reactions with oxygen (1,2,3). The mechanisms by which oxygen radicals damage membrane lipid are well accepted and associated with there peroxidation reactions in membrane lipid, but activated forms of oxygen also degrade proteins & nucleic Acids.

1.7.2.1 OXIDATIVE DAMAGE TO LIPID:

The peroxidation of lipid involves three distinct steps: initiation, propagation & termination. The initiation reaction between an unsaturated fatly acids (such as linoleate, which is common in plant cell membranes) & the hydroxyl radical involves the abstraction of an H atom from the methyl

vinyl group on the fatty acids, which results in molecular rearrangement. In the propagation reactions, this resonance structure reacts with triplet oxygen, most abundant from of O_2 in the cell. This reaction forms a peroxy radical. The peroxy radical than abstracts an H atom from a second fatty acid forming a lipid hydroperoxide & leaving another carbon centred free radical that can participate in a second H abstraction. Therefore, once one hydroxyl radical initiates the peroxidation reaction by abstracting a single H atom, it creates a carbon radical product (R·) that is capable of reacting with ground state oxygen in a Chain reaction. The role of the hydroxyl radical is analogous to a "spark" that starts a fire. The lipid hydroperoxide (ROOH) is unstable in the presence of Fe or other metal catalysts. Therefore, in the presence of Fe, the chain reactions are not only propagated but amplified (Girotti 1990; Khajuria 1997). The whole process is depicted in figure 1.6.

The peroxidation reactions in membrane lipids are terminated when the carbon or peroxy radicals cross-link to form conjugated products that are not radicals.

$$R\cdot + R\cdot \rightarrow R\text{-}R$$
$$R\cdot + ROO\cdot \rightarrow ROOR$$
$$ROO\cdot + ROO\cdot \rightarrow ROOR + O_2$$

1.7.2.2 OXIDATIVE DAMAGE TO PROTEINS:

Oxidative attack on proteins results in site specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross linked

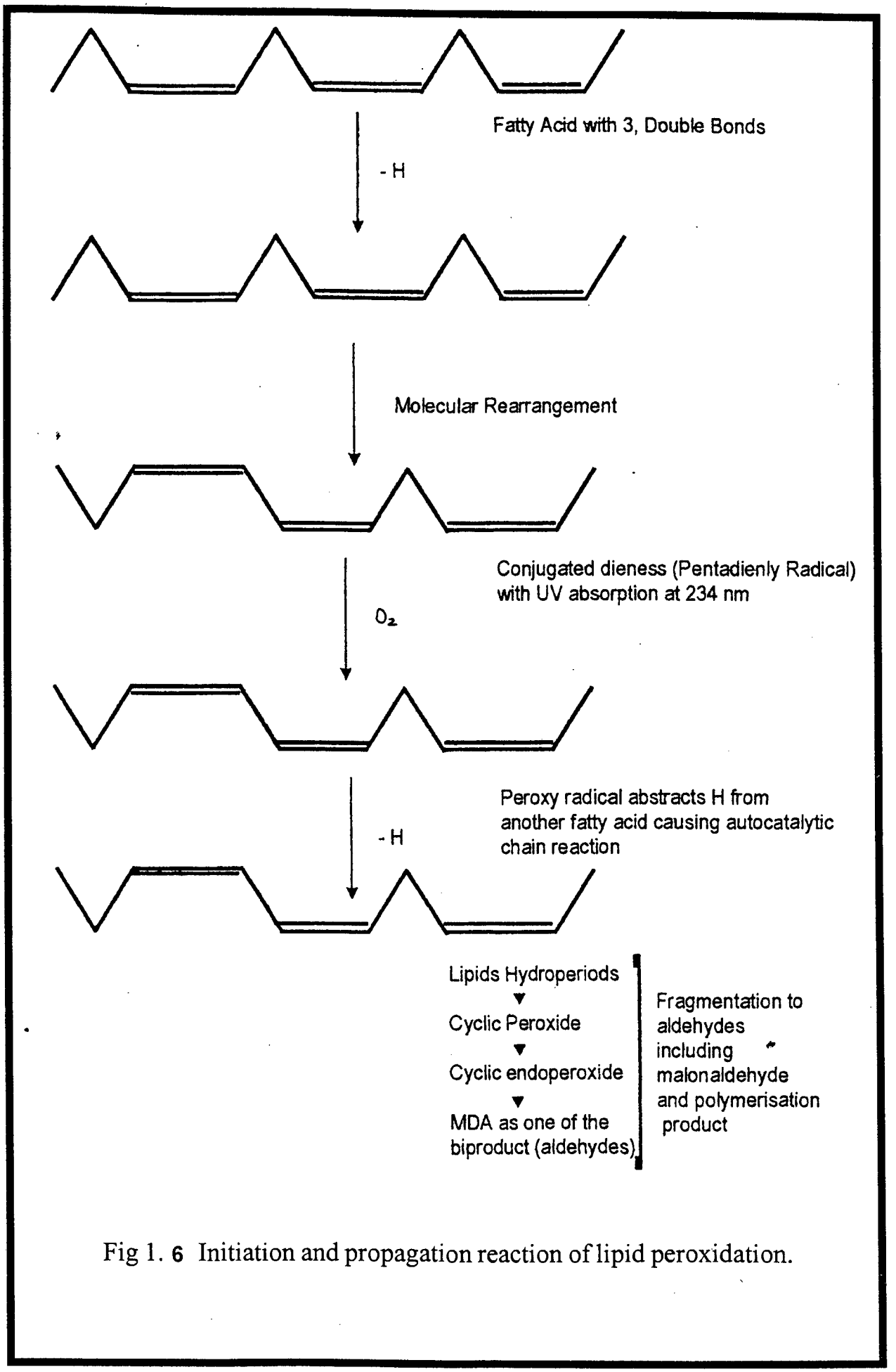


Fig 1. 6 Initiation and propagation reaction of lipid peroxidation.

reaction products, altered electrical charge and increased susceptibility to proteolysis. The amino acids in a peptide differ in their susceptibility to attack and the various forms of activated oxygen differ in their potential reactivity. Sulphur containing amino acids & thiol groups specifically, are very susceptible sites. Activated oxygen can abstract H atoms from cystine residue to form thiyl radicals that will crosslink to form disulphide bridges. Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives. Reduction of both of these may be accomplished in microbial systems by thioredoxin & thioredoxin reductase (Farr & Kogoma 1991). A protein- methionine oxide reductase has been measured in pre chloroplasts (Ferguson & Burke 1992). This enzyme reduces the methionyl sulfoxide back to methionyl residues in the presence of thioredoxin.

The oxidation of iron-sulphure centres by superoxide destroys enzymatic function (Gardner & Fridirich 1991). Many amino acids undergo specific irreversible modifications when a protein in oxidised, for example, tryptophan in readily (non-linked) to form bityosine products (Davies 1987). Histidine, lysine, proline, arginine and serine form carbonyl groups on oxidation. The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of redox cycling, such as Fe. In these cases, the metal binds to a divalent cation binding site on the protein. The metal then react with hydrogen peroxide in a Fenton reaction to form a hydroxyl radical that rapidly oxidizes an amino acid residue at or near the

cation binding site of the. This site specific alteration of an amino acid usually inactivates the enzyme by destruction of the cation binding site.

Oxidative modification of specific amino acid is one mechanism of marking a protein for proteolysis. There are specific proteases that degrade oxidized proteins (Farr & Kogome 1991). It is well documented that the various peptide components of PS II turnover at different frequencies; the D1 protein specifically is noted for its high rate of turnover, and it is assumed that this is a consequence of oxidative attack at specific site on the protein (Barber & Andersson 1992).

1.7.3 DEFENSE MECHENISM AGAINST OXIDATIVE DAMAGE:

1.7.3.1 SUPEROXIDE DISMUTASE:

The enzyme was identified by a number of names, erythrocuprein, indophenol oxidase & terazolium oxidase until its catalytic function was discovered by McCord & Fridovitch (1969). SOD is now known to catalyze the dismutation of superoxide to hydrogen peroxide & water (Fig. 1.7).

$$2H^{+} + 2O_2^{\cdot-} \rightarrow H_2O_2 + O_2$$

Since SOD is present in all aerobic organism & most subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defense against oxidative stress (Beyer *et al.* 1991; Bowler *et al.* 1992; Scandalias 1993). There are three distinct types

of SOD classified on the basis of the metal Cofactor: Copper-Zink (Cu/Zn-SOD), manganese (Mn-SOD) & iron (Fe-SOD) isozymes (Bannister *et al.* 1987). The Mn-SOD is found in the mitochondria of eukaryotic cells. Some Cu/Zn SOD isozyme is found in the cytosol, others in the Chloroplast of higher plants. Fe-SOD is usually associated with the Chloroplast compartment (Bowler et al., 1992). The prokaryotic Mn-SOD & Fe-SOD & the eukaryotic Cu/Zn-SOD enzymes are dimers, whereas the Mn-SOD of mitochondria are tetramers (Scandalias 1993). All forms of SOD are nuclear encoded and are targeted to their respective subcellular compartments by an amino terminal targeting sequence (Bowler *et al.* 1992; Scandalias 1990).

Each of the SOD isozymes are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments, but how this is communicated at the molecular level is unknown. Bowler *et al.* (1992) have suggested that this role may be served by unique lipid peroxidation products from each organelle that diffuse from the site of oxidative damage to the nucleus where they would enhance transcription of specific SOD genes.

1.7.3.2 CATALASE:

Catalase is a heme-containing enzymes that catalyzes the dismutation of hydrogen peroxide into water (Fig. 1.7). The enzyme is found in all aerobic eukaryotes & is important in the removal of hydrogen peroxide

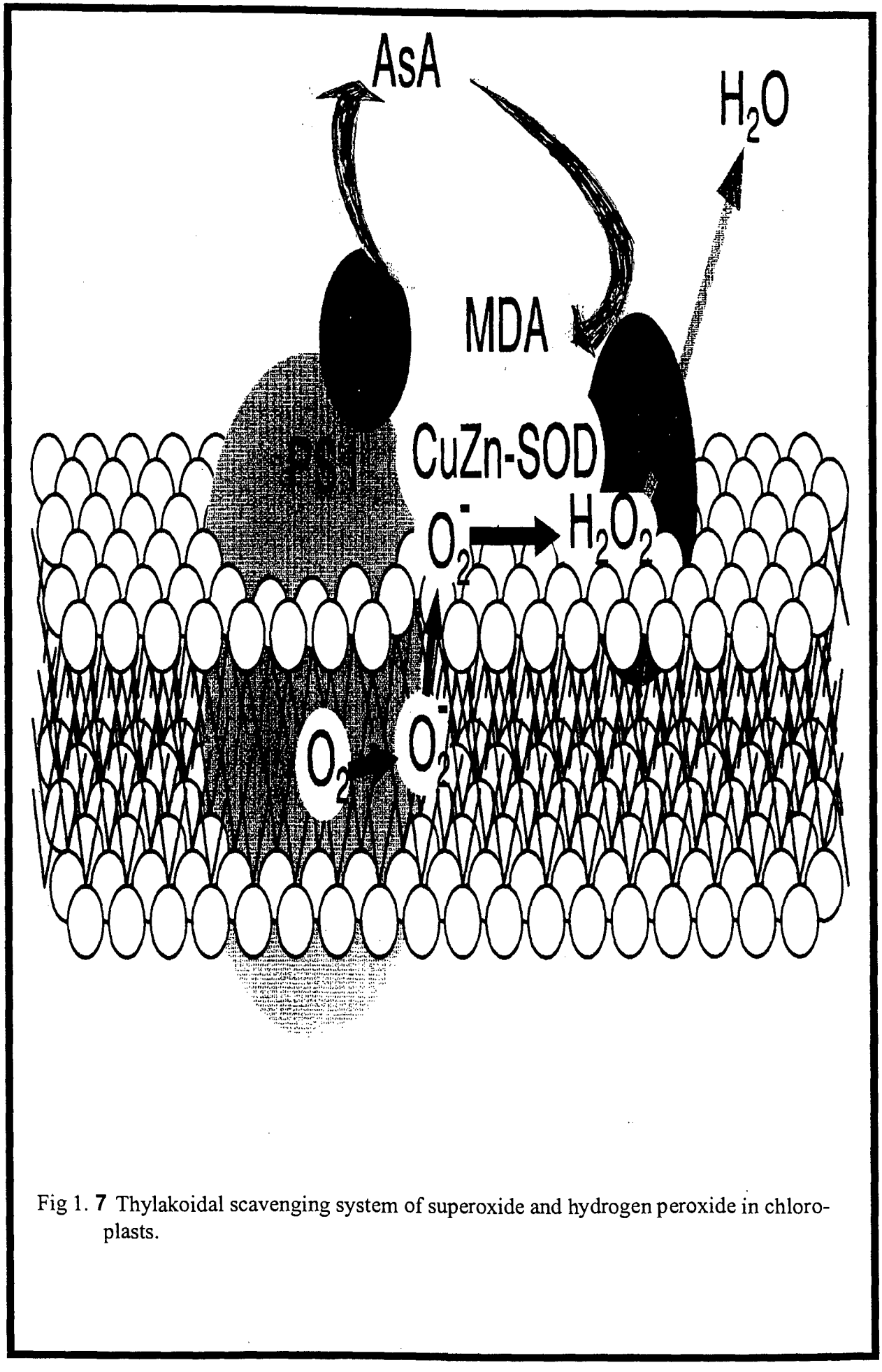


Fig 1. 7 Thylakoidal scavenging system of superoxide and hydrogen peroxide in chloroplasts.

generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, the glyoxylate cycle (photorespiration) & purine catabolism. All form of catalase are tetramers in excess of 220 KDa molecular weight. Multiple forms of catalase have been described in many plants. Catalase is very sensitive to light and has a rapid turn over similar to that of D1 protein of PS II (Hertwig *et al.* 1992). Regardless, the stress conditions which reduce the rate of protein turnover, such as salinity, heat shock or cold, cause the depletion of catalase activity. This may have significance in the plant's ability to tolerate the oxidative component's of there environmental stresses (Hertwig *et al.* 1992; Feierabend *et al.* 1992).

1.7.3.3 PEROXIDASE:

Catalase is limited in effectiveness to metabolize H_2O_2 by its relatively poor affinity for H_2O_2 & its subcellular location in the peroxisomes & also susceptible to photo inactivation & degradation (Feierabend & Enger 1986; Streb *et al.* 1993). As ascorbate peroxidase also scevenges H_2O_2 in chloroplasts (which do not contain catalase) simultaneous oxidation & reduction of the ascorbate & glutathione pools when H_2O_2 was added suggested that enzymes of as ascorbate-glutathione cycle were involved in coupling electron transport to H_2O_2 destruction. Observation of direct photoreduction of the MDHA radical by the thylakoid membranes suggested that this is probably the preferred path way of ascorbate regeneration (Foyer

& Libendias 1993). H_2O_2 generated during the Mehler reaction is considered to be scavenged primarily at the thylakoid level while the stromal ascorbate peroxidase represents a second level of defence against H_2O_2 escaping the thylakoid.

1.8 CAROTENOIDS:

The carotenoides are yellow or orange pigments found in all photosynthesizing cells. Carotenoids contain a conjugated double bond system of the polyene type. They are usually either hydrocarbons (carotene) or oxygenated hydrocarbons (carotenols or xanthophyll) of 40 carbon chains built up from addition of isoprene subunits. They have triple-bonded absorption spectra in the region from about 400-530 nm. The energy absorbed by the carotenoids may be transferred to chlorophyll lamellae in the close proximity to the chlorophyll. In addition to functioning as accessory light harvesting pigments (Davidson and Cogdell 1981). Carotenoids also play an important role in protection of photosynthetic apparatus against oxidative damage (Krause 1994). The cartenoides of chloroplasts include β-carotene (25-45%) lutein (40-57%) Violaxanthin (8-12%) and neoxanthin (5-13%) as major and regular components of thylakoids of higher plants, their amount varies within the range depending upon the growth conditions and stress factors (Britton, 1990).

In addition to working as antioxidant, carotenoids through the Xanthophyll cycle can also dissipate excess radiation in the form of heat away from the reaction centre thereby preventing the over energization of the reaction centre chlorophyll. In other terms they are able to work in a reverse gear thereby preventing the absorbed energy in light harvesting complex to be channeled to reaction centre (Sharma and Hall 1991,1993,1996; Deming *et al.* 1987; Deming-Adam-1990).

Carotenoids pigments, which are abundant in leaves have been studied for a number of years because of their diverse role in photobiology & photo chemistry. Carotenoids have the ability to act as chain breaking anti-oxidant & thus protect cells & organisms again photodamage. The ability of carotenoids to quench singlet molecular oxygen is well known. (Oliveros *et al.* 1994; Bohm *et al.* 1995; Hill *et al.* 1995). Burton & Ingold (1984) suggested that β-Carotene is not a conventional antioxidant & that at high O_2 partial pressure it may even act as a pro- oxidant.

As noted by Britton (1995) Carotenoids are not just another group of natural pigments they are substance with special & remarkable properties that form the basis of their varied functions & actions in all kinds of living organism. As well as the ability to quench excited states, carotenoids can also react with free radicals. However, unlike the quenching of singlet oxygen which mainly lead to energy dissipation as heat, the reaction of

carotenoids (or any antioxidant) with a free radical will lead to electron transfer.

This free radicals (R.) reacting with Carotenoid (car), reaction such as

$$R^{\cdot} + car(H) \rightarrow RH + Car^{\cdot} \quad \& \quad R^{\cdot} + Car \rightarrow R^{-} + Car^{\cdot +}$$

There are two proven photoprotective reactions of carotenoids, one is to dissipate the excessive energy by transferring energy from the triplet to ground state and the other is to scavenge the O2 radicals generated under photoinhibitory conditions

$$\begin{aligned} \text{Chlorophyll} &\rightarrow {}^3chl^* \\ {}^3chl^* + {}^1car &\rightarrow {}^1chl + {}^3car^* \\ {}^3car^* &\rightarrow {}^1car + heat \end{aligned}$$

The quenching of 3chl* by carotenoids to prevent the generation of singlet O2 through triple sensitization is a protection mechanism. Equation (1), (2), (3) indicates that the relative energies of components which make the above reactions possible. The another is to remove singlet O_2 generated during photoinhibition by mechanism of epoxidation as seen in the equation below.

$$\text{Carotenoids} \underset{\text{dark}}{\overset{h\nu, O_2}{\rightleftharpoons}} \text{Epoxy- Caroterioids}$$

Carotenoids are biosynthesised and accumulated in the photosynthetic tissue of all higher plants. The cyclic carotenes and xanthophylls found in leaf tissues can have two different ring types, namely

β and ε. Only dicyclic xanthophylls and particularly those with hydroxyl substituents at C-3 or C3' have been found in the photosynthetic tissue of higher plants.

1.8.1 XANTHOPHYLL CYCLE:

Xanthophylls are O_2 containing carotenoids. During photoinhibitory treatment violaxanthin is de-epoxidised to zeaxanthin via antheraxanthin, which is reversed in the dark (figure 1.8). Violaxanthin is a functional component of the xanthophyll cycle of thylakoids and is phototransformed via antheraxanthin (A) to its de-epoxidised form zeaxanthin (Z). The conditions for the operation of xanthophyll cycle have been established (Hager 1980, Yamamoto 1979). The forward reaction i.e. the photoconversion of V to Z is catalysed by the membrane bound enzyme de-epoxidase and requires an acid pH in the thylakoid lumen and only proceeds when the irradiance is sufficiently high to provide the proper acid PH In the dark or low light conditions which do not maintain delta-pH in the thylakoid lumen, the back reaction takes place, i.e. the epoxidation of Z into, which also requires O_2 and NADPH and another membrane bound enzyme epoxidase. In recent years a photoprotective role has been ascribed to this cycle (Demmig *et al* . 1987, Demmig-Adams 1990, Sharma and Hall, 1993; Choudury *et al.* 1993, Adams *et al* . 1990, Pfundel and Bilger 1994 , Bjorkman and Demmig - 1987, Thayer and Bjorkman 1990). It has been

proposed that the dissipation of excess energy may occur at several sites within or around PSII and PSI reaction centre (Fig. 1.9). The dissipation process within the pigment complex involves xanthophyll cycle (Demmig *et al.* 1987 , 1989). A correlation was found between the calculated activity of radiation less energy dissipation represented by qN and the content of xanthophyll cycle (Synthesis of Z) in the leaves of Nerium oleander (Demmig *et al* . 1987). The observation of such a correlation led to the proposal that Zeaxanthin may be involved in this dissipation. Further evidence to the hypothesis came from the knowledge that Z acts as a competitor for the excitation energy under excess light, since increase in radiationless energy dissipation, result in a decrease in photochemical efficiency (Bjorkman and Demmig 1987). The capacity for a rapid removal of Z facilitated a rapid increase in the qP upon return to conditions under which light is no longer excessive. When leaves were treated with dithiothreitol (DTT; which was administered through cut petiole completely inhibited de-epoxidation of V to Z without affecting photosynthetic O_2 evolution) it was observed that such leaves has an approximately 30 % greater reduction state of PS II compared to that of leaves containing Z (Bilger *et al.* 1989) indicating an increased amount of energy reaches the PS II reaction centre. Work with certain lichens (algae in association of fungi naturally lacking the xanthophyll cycle and compared with green algae which possess the xanthophyll cycle (Demmig-Adam *et al.* 1990) showed that upon exposure to excess light the

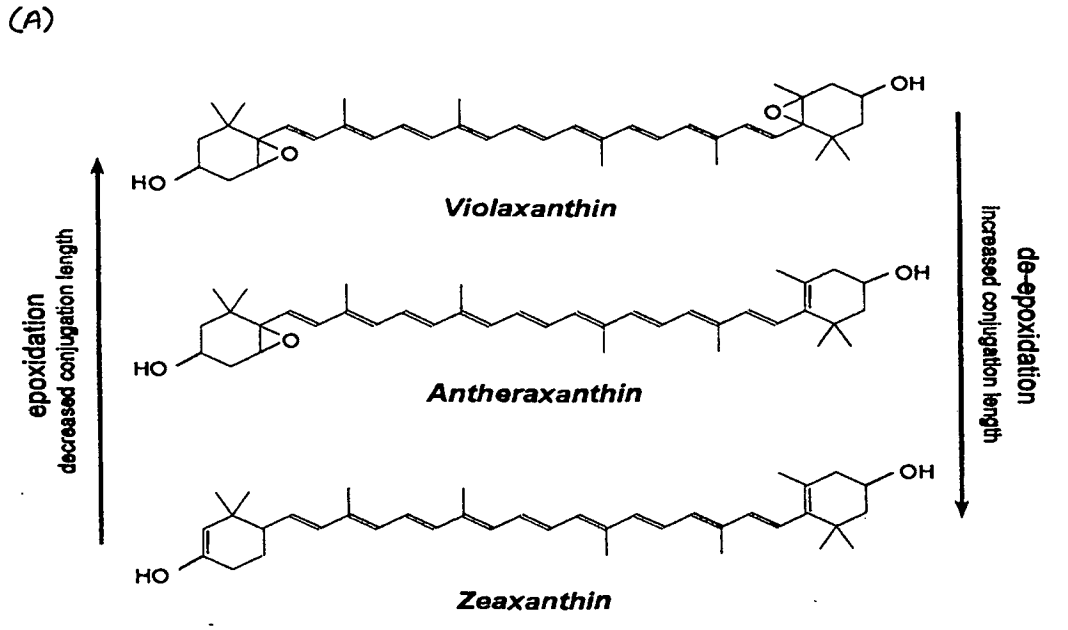


Fig 1.8 The xanthophyll cycle in higher plants.

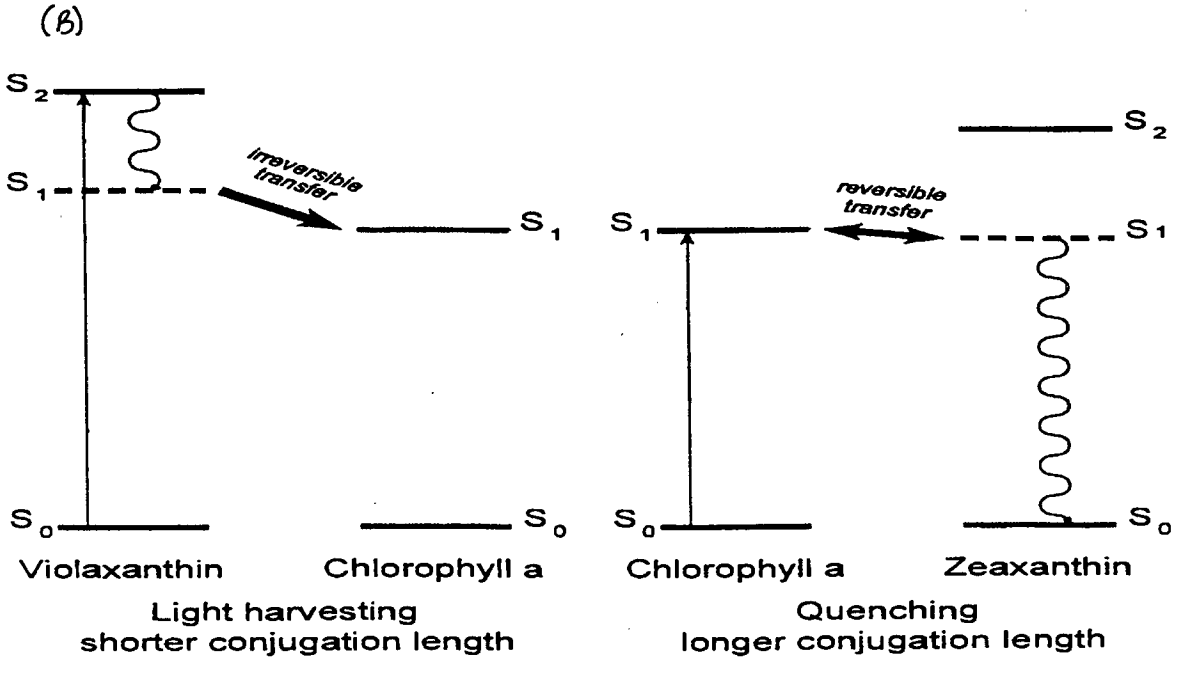


Fig 1.8 Comparision of the predicted energy transfer pathways between carotenoids of the xanthophyll cycle and Chl a. Violaxanthin has 9 conjugated C-C double bonds and zeaxanthin has 11 conjugated double bonds.

cyanobacteria associated with lichens which lack Zeaxanthin, did not form any Zeaxanthin and also did not exhibit rapidly developing and relaxing non-photochemical quenching and the reduction state of PSII centre remain high under excess irradiance whereas Zeaxanthin-containing green algae showed greater tolerance to larger exposure at high irradiance by forming zeaxanthin rapidly from de-epoxidation of v and also exhibited greater non-quenching and the reduced state of PS II RC was maintained at low level. Work by Chowdary *et al.* (1993) also indicated a relationship between xanthophyll cycle and photoprotection through better dissipation of excess radiation under *in vitro* condition. When involved chloroplasts were treated with DTT it resulted in a greater damage to as compared to chloroplast which were treated with ascorbate (a stimulator of de-epoxidation of V to Z). There may also be limitation to the role of the Xanthophyll cycle as a protection mechanism against photoinhibition (Sharma and Hall 1990, 1993). Since Z content after a photoinhibition treatment at 1600 μmol m^{-2} s^{-1} at 5°C was not further increased when plants were photoinhibited at a higher PPFD (2500 μmol m^{-2} s^{-1}). It was also seen that changes in xanthophyll cycle were temperature dependent showing better rate of de-epoxidation at 20°C than at 5°C (Sharma and Hall 1990).

Despite the mentioned above work, Z accumulation as well as a direct dissipation of light energy by Z has been, however, not been accepted by everyone (Havaux *et al.* 1991; Lichtenthaler and Schindler 1992; Richter *et*

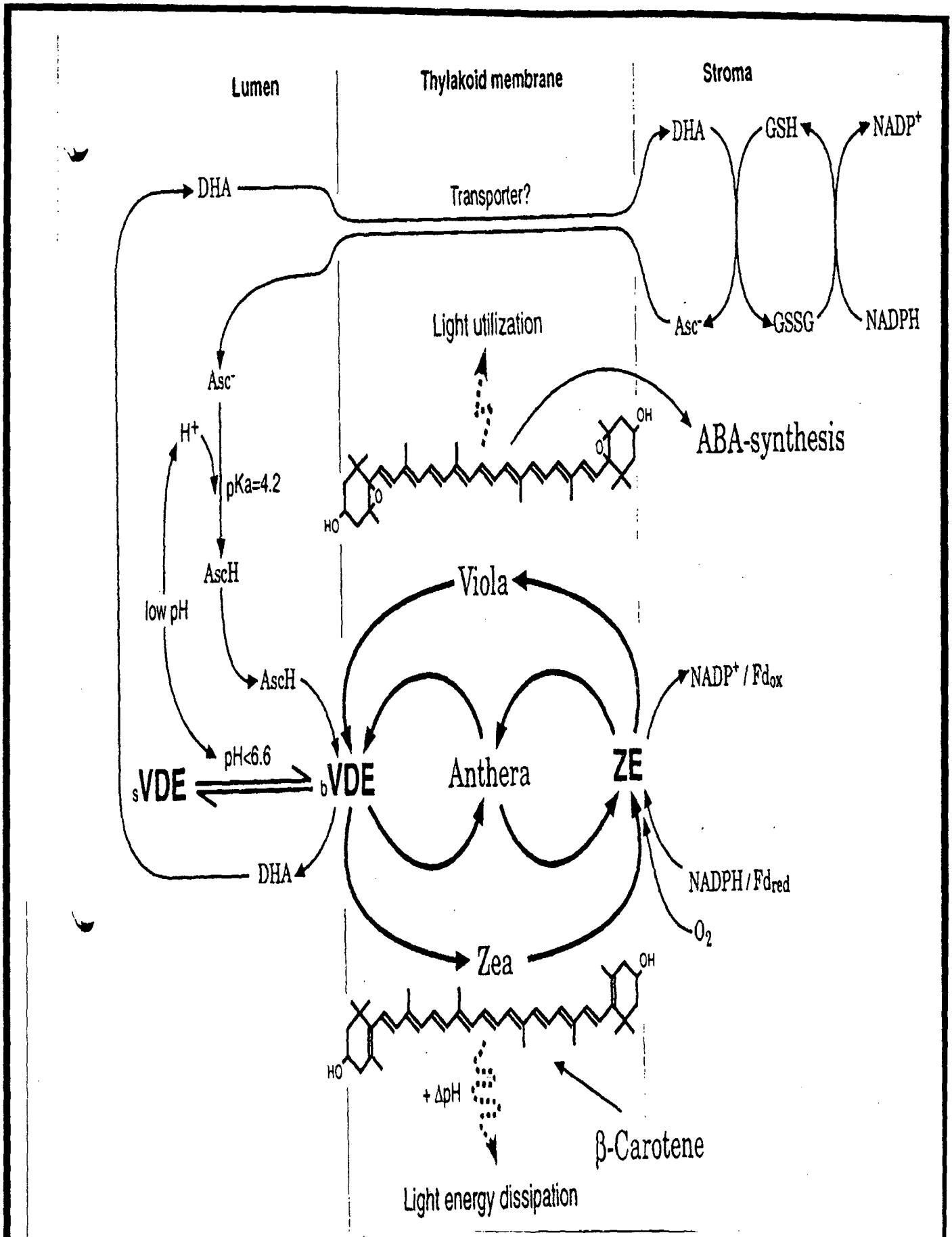


Figure 1.9 Xanthophyll cycle and its relationship with other scavenging system.

al. 1994). In order to obtain more information on the possible relationship of high energy chlorophyll a fluorescence quenching and zeaxanthin accumulation, more studies are required more specifically to quantitate the extent of protection provided by the xanthophyll cycle under photoinhibiting conditions. Beside direct role of Z in the energy dissipation there are reports which indicate that the zeaxanthin may influence the fluidity of membrane (Havaux *et al.* 1991) there by influencing the process of photochemistry. Also now it has been experimentally proved that violaxanthin acts as a precursor for ABA (Fig. 1.10) , a plant hormone which involved is various stress stimulated process.

1.9 ABSCISIC ACID:

Plants are subjected to a wide variety of environmental and biological stresses throughout their life cycle requiring adaptation to survive conditions which are frequently harsh and variable. A large number of plant stress responses have been described which include both abiotic stress imposed by temperature, salinity, water, draught, light etc. Different stresses conditions also may induce common responses such as the enhancement of phytohormones (Liu *et al.* 1993). Studies have demonstrated that ABA plays a important role in modulation of adaptive responses of plants under adverse environmental conditions (Chen *et al.* 1983; Orr *et al,* 1986; Ramgopal 1987; Singh *et al.* 1989; Pena-Cortes *et al* . 1989). In addition to

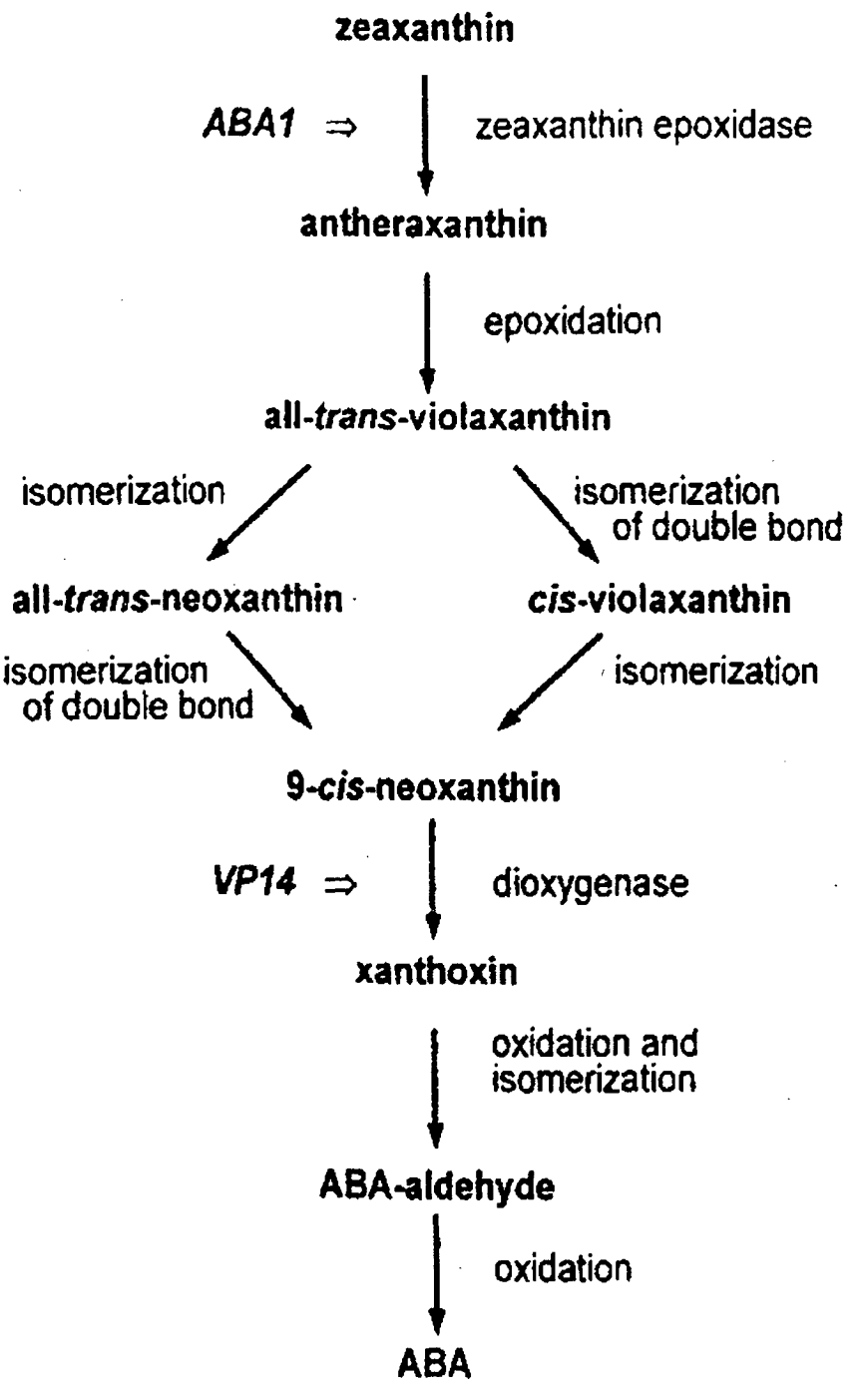


Fig 1.10 The ABA Biosynthetic Pathway in Higher Plants.

mediating the adaptive response of plants, ABA is also involved in regulation of a number of other related physiological processes which include.

- embryo morphogenesis and the development of seeds (Quatrsno 1987; Dure *et al* . 1989)
- Seed dormancy and germination (Koornelf *et al* . 1989) and
- Plants defense from invading pathogens.

It has been suggested that ABA acts as a common mediator controlling adaptive plant responses to environmental stresses (Daie and Campbell 1981) because of the numerous instances in which ABA has been implicated. As a result of its central position, the precise role of ABA under various stress conditions is currently the subject of intense investigation in many laboratories.

The details of the ABA biosynthetic pathway in higher plants have remained obscure, even though MVA2 is known to be a precursor (Li and Welton 1987 ; Nodddle and Robinson 1969) and the stereochemistry of ABA formation has been shown to be identical to that of the carotenoids (Milborrow and Rubery 1985) It is still not clear whether ABA is derived directly from a cleavage product of xanthophyll such as violaxanthin or whether it is derived directly from a C-15 precursor such as farnesyl pyrophosphate. The initial suggestion that ABA may be derived from a

xanthophyll came from observations that violaxanthin can be converted to naturally occurring C-15 composed xanthoxin by photooxidation (Li and Welton 1987; Taylor and Burden 1973) or by lipoxygenase (Firn and Friend 1972).

Recently there has been a renewed interest in the possibility that xanthophylls may act as precursor for ABA in higher plants. Various corn mutants have been described which lack the ability to synthesize carotenoids and which accumulate little or no ABA. In addition, inhibitors of carotenoids biosynthesis such as norflurazon and fluridone, also inhibit the accumulation of ABA under some conditions (Crumble and Mullet 1986; Moore and Smith 1984 ; Quarrie and Lister 1984). A recent experiment reported by Schwartz *et al.* (1997b) suggest very convincingly a precursor role for xanthophylls.

Since the elucidation of the structure of the ABA its biosynthetic derivation from carotenoids has been proposed in higher plants (Taylor and Smith 1967) However, the enzymatic cleavage of carotenoids has been difficult to demonstrate in vitro , for this reason the cleavage reaction remain controversial. Direct evidence for a cleavage enzyme in ABA biosynthesis is lacking because of the apparent low abundance and lability of the enzyme. Labelling experiments with $^{18}O_2$ (Zeevaart and Creelman 1988) suggest that ABA is synthesized from large precursor pool that contains two of the four

oxygens found in the molecule (Creelman and Zeevaart 1984). Oxygen derived from the hydroxygen and epoxide of neoxanthin or violaxanthin was suggested to account for the observed $^{18}O_2$ labelling pattern from the work of the (Marin *et al.* 1996 Schwart *et al.* 1997; McCarty 1995 Sindhu and Walton 1988; Parry *et al.* 1992). A biosynthetic pathway of ABA in higher plants is shown in (fig. 1.10).

Since the role of xanthophyll cycle in the protection of photosynthetic apparatus from photoinhibition is well documented and since the strong evidence for the pathway ABA biosynthesis from epoxy-carotenoids has been reported (Parry et al 1990; Parry and Horzen 1991a , Rock and Zeevaart 1991) it is of interest to investigate the influence of exogenously applied ABA on the light - dependent Zeaxanthin formation and try to look for relationship between endogenous xanthophylls and ABA levels under photoinhibitory conditions. Figure 1.11 shows the net work of interconnected reaction coupled with photosynthesis. Any change in one part of the network ultimately leads to the effect on the photosynthesis.

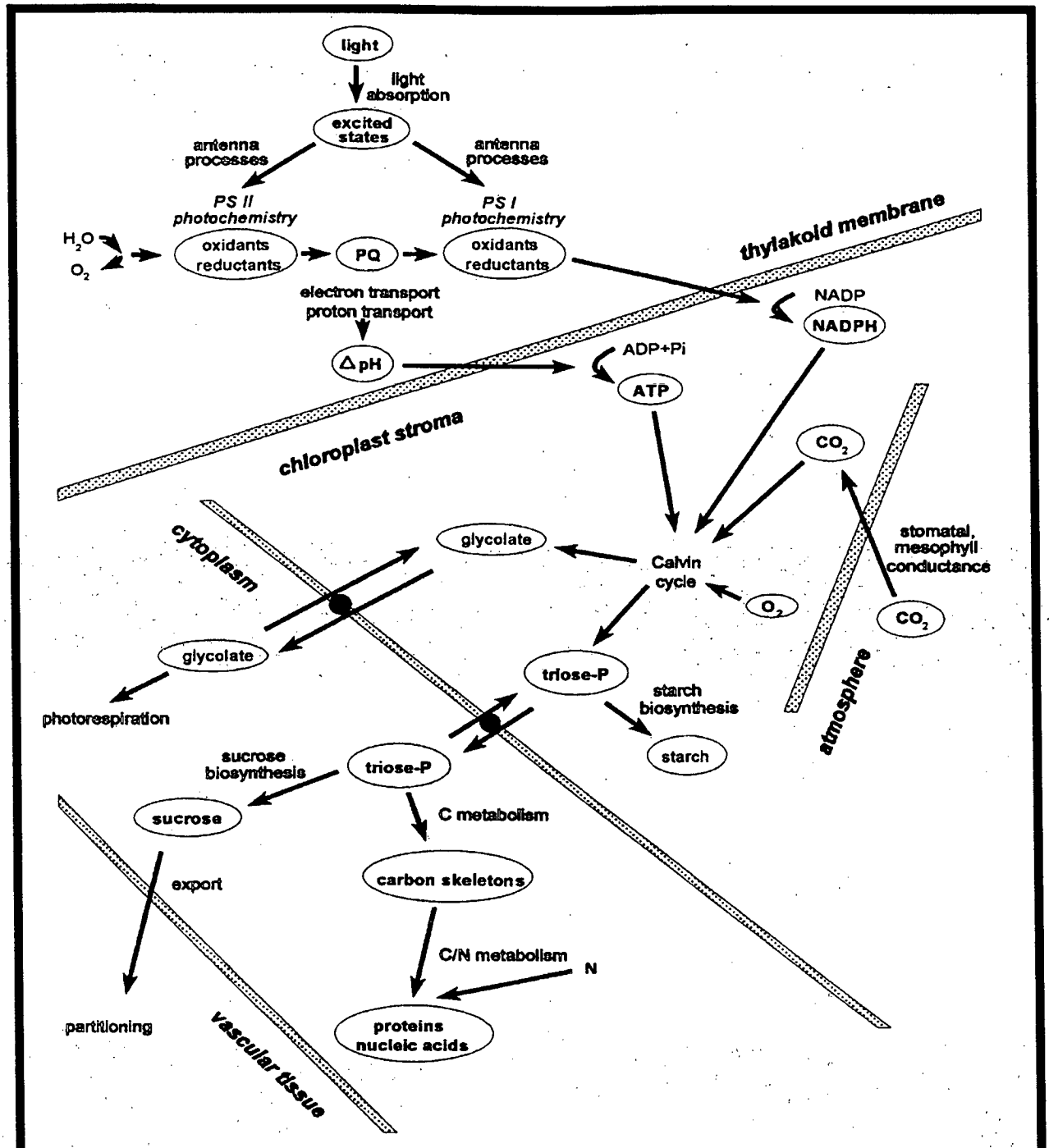
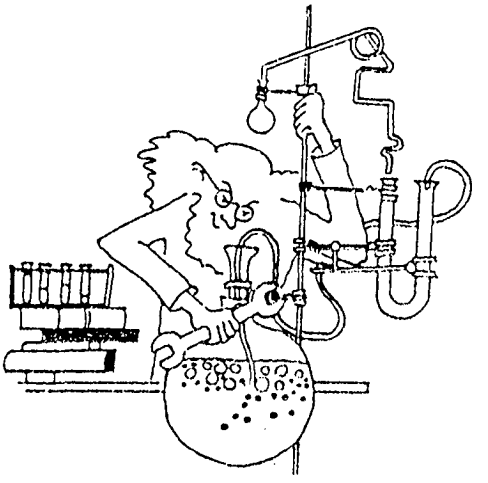


Fig 1.11The network of interconnected reactions that couple photosynthesis with other cellular processes in a typical plant cell. Feedback reactions among these coupled processes are capable of affecting photosythesis to the level of utilization of light energy in the antenna.

CHAPTER 2



MATERIAL & METHODS

2.0 MATERIAL AND METHODS

2.1 PLANT MATERIAL: Seeds of *Sorghum bicolor* L. cultivar MSH-51 were obtained from Mahyco Hybrid Seeds Company, Jalna, Maharastra, India and were stored in a dessicator at room temperature.

2.2 GROWTH CONDITIONS: Prior to sowing the seeds were soaked in tap water for an hour and were surface sterilised with $HgCl_2$ (0.1%) for 2 min. The seeds were grown densely in plastic pots (diameter 10 cm) containing vermiculite and routinely bottom irrigated with Hoagland solution. The plants were grown in a controlled environmental chamber with 14 hour photoperiod provided with incandescent bulbs and fluorescent tubes (visible spectrum of light in growth chamber is given in figure 2.1 a) having a non photoinhibitory level of 250 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic active radiation (PAR), measured using a radiometer (Licor, model LI-189, USA). The day/night temperature was maintained at 30°C ± 2°C , and relative humidity at 50%. Plants of 8 day old were used for the experiments.

2.2.1 SEEDLINGS GROWN WITH ABSCISIC ACID (ABA): The seeds were also germinated in the petri-plates (9.6 cm diameter) containing moist cotton pad and blotting paper soaked in Hoagland solution, for studying the effect of exogenously applied ABA on the protection against photodamage. The control plants were irrigated with Hoagland solution everyday, while ABA

treated plants were irrigated with ABA solution (10^{-5} M), prepared in the dilute KOH solution after 4 days of normal growth. The growth conditions were same as described in section 2.2.

2.3 TREATMENT CONDITIONS: Intact leaves and isolated chloroplasts were exposed to irradiance of 1000 (low) 2200 (medium) and 3600 (high) μmol m^{-2} s^{-1} at a range of temperature (5, 30 and 50°C) for various duration of 0-6 h in case of intact leaves and 0-120 min in case of isolated chloroplasts, using tungston-halogen lamp (visible spectra of the light source is shown in figure 2.1. b) at a range of temperature (5-50°C) for various duration (0-6 h).

2.3.1 PHOTOINHIBITION TREATMENT UNDER *IN VIVO* CONDITIONS: Experiments were carried out with intact leaves of 8 days old seedlings. Leaves were detached along with their roots and were exposed to irradiance of 3600 and 2200 μmol $m^{-2}s^{-1}$ PAR. In case of electron transport study leaves were also exposed to 1000 μmol m^{-2} s^{-1} PAR. A metallic box (27 cm Lx15 cm Wx13 cm H) covered with thermal insulation on all side except the top was fabricated which had an outlet and an inlet for water circulation. This box was connected to cryogenic water circulator to maintain the temperature of the surface of the box as per our experimental requirement. Temperature was maintained in the range of 5-50°C during the exposure of leaf to the high light. Prior to the photoinhibitory treatment the leaves

were placed on the water circulating metallic box for 10 minutes so as to adapt to the desired experimental temperature in the diffused light of 80 μmol m^{-2} s^{-1} PAR. Subsequently leaves were exposed to required light intensity (3600 or 2200 μmol m^{-2} s^{-1} PAR as the case may be) through a fibre optic tube exposing leaf area of 1cm^2 (middle portion of the leaf) using Schott KL 1500 electronic projector. The exposed (1cm^2) part of the leaf was used for chlorophyll fluorescence measurements as well as for extraction of xanthophylls and ABA. The leaves for electron transport study were exposed using 250 W and 15 V tungsten halogen bulbs as amount of leaves required for isolation of chloroplasts for measurements of various assays of electron transport chain was greater. The leaves were placed on aluminium foil which was floated on water surface in a chiller with temperature of water set as per the experimental requirement.

2.3.2 PHOTOINHIBITION OF LEAVES FED WITH DITHIOTHREITOL (DTT): Plants grown for 8 days were used for feeding dithiothreitol. After 8 days of growth leaves were cut from their petioles and were fed with DTT (4mM) solution through their cut petioles for 3 hours in a growth chamber maintained at 30°C ± 2°C. The solution was kept stirred at low speed continuously through out the duration of feeding DTT, using a magnetic stirrer. DTT fed leaves were exposed to high irradiance of 3600 and 2200 μmol m^{-2} s^{-1} PAR using instrument and procedure described in section

2.3.1. Exposed leaf area (1 cm^2) was used for chlorophyll fluorescence measurements as well as for the extraction of xanthophylls for their HPLC analysis. The leaves were fed with DTT in order to inhibit the de-epoxidation of V to Z and to study the impact of this inhibition (Z formation) on photoprotection against high light.

2.3.3 PHOTOINHIBITORY TREATMENT OF LEAVES FED WITH ASCORBATE: Experiment were also conducted with feeding ascorbic acid (5 mM, pH 6.8) to 8 day old Sorghum plants grown in growth chamber. The plants were cut at their bases and were fed with ascorbate solution for 3 hours through the cut petioles in the growth chamber maintained at 30°C±2°C. The solution was kept stirred during the feeding of ascorbate. The leaves were subsequently photoinhibited by exposing 1 cm^2 of leaf portion to a irradiance of 3600 and 2200 $\mu mol\ m^{-2}\ s^{-1}$ PAR using instruments and procedure described in section 2.3.1. Chlorophyll fluorescence measurements were taken from exposed $1cm^2$ leaf area and the same portion of the leaf was used for extraction of carotenoids and ABA for their quantitative and qualitative estimation using HPLC.

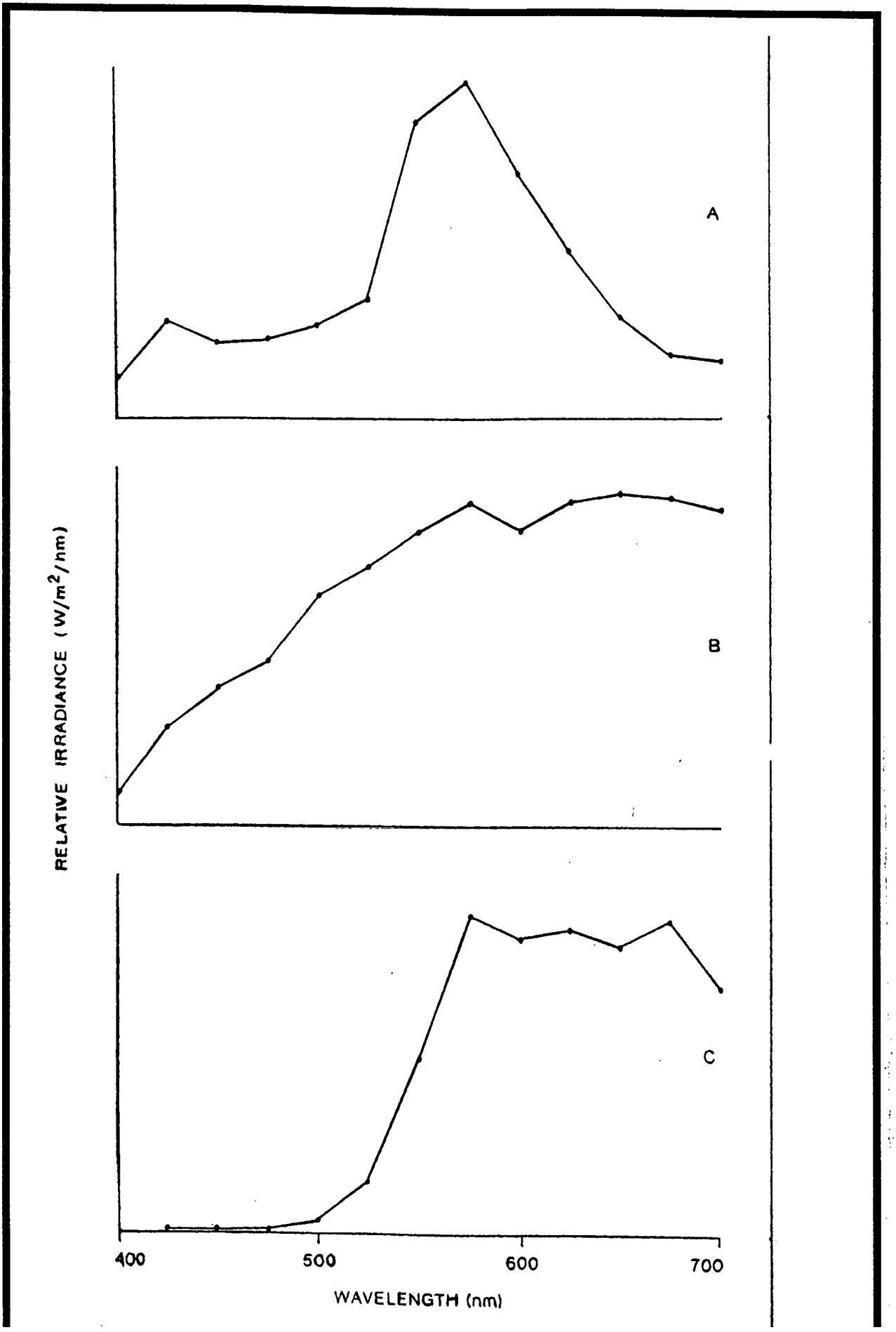
2.3.4 PHOTOINHIBITION OF LEAVES FED WITH ABA: Plants grown for four days under conditions described in section 2.2. were allowed to grow under exogenously applied ABA (10^{-5}; for detail see section 2.2.1) up to 8 days

Fig.2.1 Visible spectra of the light sources used in this study; measured using a spectroradiometer (Model SR, Instrumentation Specialities Co., Lincoln, Nebraska, USA).

(A) Combination of incandescent and fluorescent lamps used for the growth of plants.

(B) Tungston-halogen lamps used for the photoinhibition of intact leaves and isolated chloroplasts

(C) Tungston-halogen lamps used for the illumination of isolated chloroplasts in the oxygen electrode.


A
B
C
RELATIVE IRRADIANCE (W/m²/nm)
400
500
600
700
WAVELENGTH (nm)

of growth (for 4 day after ABA application). The plants were then exposed to an irradiance of 3600 and 2200 μmol m^{-2} s^{-1} PAR using the instrument and procedure described in section 2.3.1. The exposed leaf area (1cm^2) was used for chlorophyll fluorescence measurements. After the chlorophyll fluorescence measurements, the exposed area of leaf (1cm^2) was cut out, weighed and used for extraction of pigments for their qualitative and quantitative estimation.

2.4 PHOTOINHIBITION UNDER *IN VITRO* CONDITIONS: Plants grown for 8 days in growth chamber under controlled conditions according to the method described in the section 2.2 were used for isolation of chloroplasts. The isolated chloroplasts were then treated to photoinhibitory treatment of 2200 and 3600 μmol m^{-2} s^{-1} at 5, 30 and 50°C according to the method described in section 2.4.4.

2.4.1 CHLOROPLAST ISOLATION: Isolation of chloroplast from 8 day old sorghum leaves was carried out according to Sharma and Singhal (1993). Sorghum leaves, 25 g (top to middle portion) were cut into small (2-5 mm) pieces and placed in a domestic electric blender with 100 ml pre cooled grinding medium.

Composition of Grinding Medium:

S. No.	Reagents	Required Concentration	Molecular Weight	g L^{-1}
1	Sorbitol	0.33 M	182.17	60.116
2	Tricine-Cl (pH 7.8)	30 mM	121.14	3.63
3	EDTA	1 mM	372.24	0.372
4	$MgCl_2$	1 mM	203.3	0.203
5	$MnCl_2$	1 mM	197.91	0.198

The tissue was ground for 30 sec at full speed. The slurry was filtered through 2 layers and then through 8 layers of muslin cloth into pre cooled plastic centrifuge tubes. Chloroplasts were sedimented by rapid centrifugation at 7000 x g for 5 min using centrifuge (Remi, RC-24). The pellet was washed and re-suspended with 2-3 drops of re-suspending buffer.

Composition of Resuspending Medium:

S. No.	Reagents	Required Concentration	Molecular Weight	g l^{-1}
1	Sorbitol	0.33 M	182.17	60.116
2	Tricine (pH 7.8)	50 mM	121.14	6.057
3	EDTA	1 mM	372.24	0.372
4	$MgCl_2$	1 mM	203.3	0.203
5	$MnCl_2$	2 mM	197.91	0.396
6	NaCl	10 mM	58.44	0.584
7	DTT	2 mM	154.24	
8	BSA	0.5%	---	5.0

2.4.2 DETERMINATION OF CHLOROPHYLL CONCENTRATION: Chlorophyll concentration was calculated according to Arnon (1949). Chloroplasts equivalent (100 μl) were added to 20 ml of 80% acetone and centrifuged at 1000 x g for 5 min. The absorbance was taken at 652 nm. Final concentration of chlorophyll used in all the studies were maintained at 1 mg chlorophyll ml^{-1} chloroplasts.

2.4.3 DETERMINATION OF INTACTNESS OF CHLOROPLASTS: The degree of intactness of isolated chloroplasts was determined by comparing the rate of oxygen evolution during the photoreduction of potassium ferricyanide (FeCN) by chloroplasts as isolated and chloroplast broken by osmotic shock (Lilley *et al.* 1975). Oxygen evolution was measured polarographically as described in section 2.5 for the assay of chloroplast as isolated, 100 µl of chloroplasts suspension (equivalent to 0.1 mg chlorophyll) was added to 1.9 ml of reaction medium. For the assay of osmotically shoked chloroplasts, 100 µl of chloroplasts

Composition of Reaction Medium:

S. No.	Reagents	Required Concnetration	Molecular Weight	g l^{-1}
1	Sorbitol	0.33 M	182.17	60.116
2	Tricine-Cl (pH 7.8)	50 mM	121.14	6.057
3	EDTA	1 mM	372.24	0.372
4	$MgCl_2$	1 mM	203.3	0.203
5	$MnCl_2$	2 mM	197.91	0.396
6	NaCl	10 mM	58.44	0.584

suspension was added to 0.9 ml of distilled water (pH adjusted to 7.0). The chloroplasts were then stirred for 1 min in the oxygen electrode to allow rupture of the chloroplasts envelope before adding 1.0 ml of double concentrated reaction medium. Oxygen evolution was followed in the light in the presence of 5 mM of FeCN as electron acceptor. After a minute or so 5 mM of NH_4Cl was added to uncouple the reaction. The percentage of intactness was determined from the rate of uncoupled oxygen evolution for 'as isolated' (B) and 'osmotically shoked' (A) chloroplasts as followes:

$$\text{Percentage intactness} = \frac{A-B}{A} \times 100$$

2.4.4 PHOTOINHIBITARY TREATMENT OF ISOLATED CHLOROPLASTS:

Photoinhibitory treatment to isolated chloroplasts was given in a metal cuvette used for measuring chlorophyll fluorescence under *in vitro* conditions supplied along with PAM Chlorophyll fluorometer (Walz, Germany). The cuvette was placed on a dedicated magnetic stirrer (Ikamay Reo, Drehghal electronics, Germany). Isolated chloroplasts (200 μl

of chloroplast of chlorophyll concentration of 1 mg chlorophyll ml^{-1} chloroplasts) were placed in the cuvette and fitted with a fibre optic cable (101F Walz, Germany) for irradiating the chloroplasts (using Schott KL 1500 electronics light source), as well as with the sensor to measure the chlorophyll fluorescence at the top of the cuvette. The chloroplasts were kept constantly stirred (900 rpm min^{-1}) throughout the duration of photoinhibitory treatment (1 min to 120 min) and irradiated with high light intensity of 1000, 2200 and 3600 μmol m^{-2} s^{-1} PAR. Chloroplasts isolated from ascorbate or DTT fed intact 8 day old leaves were also exposed to photoinhibitory treatment. The photoinhibition to isolated chloroplasts was given in order to exclude the protection provided against photodamage by cellular processes other than the xanthophyll cycle and prevent the recovery of the photodamage and to compare the photoinhibition of photosynthesis under *in vitro* conditions as well as under *in vivo* condition in plants fed with DTT and ASC (plants were also fed with distilled water as control), to elucidate the extent of recovery of photodamage under these two sets of experimental conditions and to find out extent of protection provided by the xanthophyll cycle.

2.5 POLAROGRAPHIC MEASUREMENT OF OXYGEN EVOLUTION:

Measurement of oxygen evolution by isolated chloroplasts was carried out with the Rank-type Clark electrode (Hansatech, UK) which can detect

changes in the order of 0.01 μmol in the oxygen content of a suspension. The electrode was connected to a polarising circuit with a power supply of 4 x 1.5 V batteries and the electrode was polarised to a voltage of 700 mV. The membrane, separating the electrode from reaction vessel, was changed and the electrode disk cleaned at the start of each day of the experiment to ensure reproducible electrode response. The electrode was polished with polishing grade aluminium oxide (0.075 μM) particle size and ringed with distilled water. After assembling the electrode the reaction vessel was filled with distilled water and the membrane was allowed to equilibrate for 1 h before use. The temperature of the reaction mixture was maintained at 25°C using a chillier thermo-circulator through the water jacket of the reaction vessel. The reaction was illuminated using a projector with 300 W tungsten halogen lamp (visible spectra of the light source is shown in figure 2.1.C) the light was filtered through a orange cinemoid filter (No. 105, Lee, Andover, UK) with a cut off wave length of 500 nm and 4 cm of water in a flat glass bottle. The photosynthetically photon flux density (PPFD) at the reaction centre was 1200 μmol m^{-2} s^{-1}.

2.5.1 CALIBRATION OF OXYGEN ELECTRODE: The electrode was routinely calibrated to give 90-100% of full scale deflection with air saturated distilled water containing 0.257 μmol oxygen ml^{-1} at 25°C, subsequently addition of a few crystals of sodium dithionite giving a zero baseline. The absolute

calibration of the electrode and the linearity of its response was checked by measuring the extent of oxygen evolution on sequential addition of 0.25 μmol hydrogen-peroxide 4 ml volume of chloroplasts reaction medium containing 4000 U of catalase which catalises the dismutation of the hydrogen-peroxide in to water by following reaction.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Theoretical yield of oxygen for each 0.25 μmol aliquot of hydrogen-peroxide added was shown to have an accurate, linear response (Fig. 2.2). The amount of oxygen evolved when the reaction was carried out in the distilled water was the same as when the reaction was carried out in the reaction medium indicating that the solute present in the reaction medium did not affect the affinity of the dissolved oxygen.

The electrode was also calibrated with sequential addition of 0.25 mM FeCN in a reaction medium containing 0.05 μg chlorophyll ml^{-1}. The reaction showed a linear response followed as oxygen evolution.

2.5.2 ASSAY OF ELECTRON TRANSPORT: Assay of electron transport from chloroplasts isolated from photoinhibited leaves (photoinhibition under *in vivo* condition) or isolated from control leaves and subsequently photoinhibited (photoinhibition under *in vitro* condition) were used for the assay of electron transport chain. Chloroplast equivalent of 50 μg

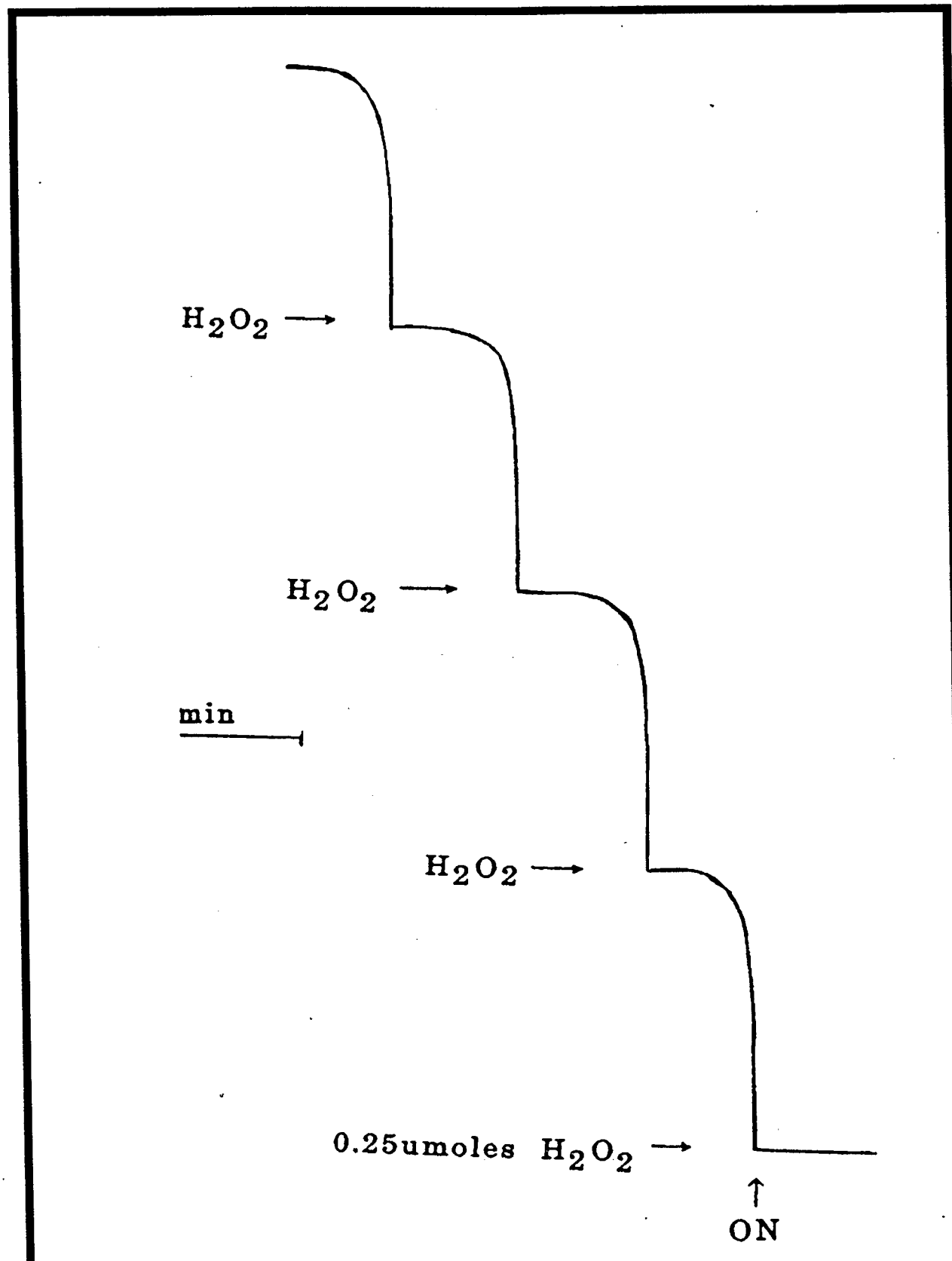


Fig 2.2 Absolute calibration of oxygen electrode by introducing 0.25 μM hydrogen peroxide in the reaction mixture in the oxygen electrode.

chlorophyll was used for electron transport assay using various electron donors and acceptors in a Clarcks type oxygen electrode (Hansatech U.K) at 25°C. The chloroplasts were illuminated using 300 W projector (Rondetle 1500 R.F). The light was passed through a orange cinemoid filter (No 105, Lee co. Ltd, Andover, U.K) with a cut of wavelength of 500 nm. Following reactions were assayed according to (Sharma and Singhal 1992) in a 2 ml reaction mixture containing Sorbitol (0.33M), Tricine (50 mM, pH 7.8), Ethyldiamine tetraacetic acid (EDTA; disodium salt; 1 mM), Magnesium Chloride (4mM), Sodium Chloride (10mM) and chloroplasts equivalent to 0.05 mg chlorophyll.

2.5.2.1 ASSAY of PS II + PS I ACTIVITY (WATER TO FeCN): FeCN 100 µl at a final concentration of 50 mM was added to the reaction chamber containing the reaction mixture. Light was switched on to measure the rate of coupled electron transport. The reaction was then uncoupled by adding 5 mM NH_4Cl and the evolution of oxygen was followed.

2.5.2.2 ASSAY of PS II + PS I ACTIVITY (WATER TO METHYL VIOLOGEN; MV): Methyl viologen at a final concentration of 50 µM was added to the reaction vessel immediately before turning on the light and the rate of coupled electron transport was measured. The reaction was then uncoupled by adding 5 mM NH_4Cl. The reaction rate was followed as

oxygen uptake from MV auto-oxidation. 1 mM NaN_3 was included in the reaction mixture to inhibit the endogenous catalase activity.

2.5.2.3 ASSAY OF PS II ONLY ACTIVITY (WATER TO PHENYLENEDIAMINE; PD): Phenylenediamine (100 µl) at a final concentration of 15 µM was added to a reaction mixture containing 50 mM FeCN and 5 mM NH_4Cl. The reaction rate was followed as oxygen evolution.

2.5.2.4 ASSAY of PS II + PS I MINUS OXYGEN EVOLUTION ACTIVITY (DIPHENYLCARABAZIDE; DPC TO METHYL VIOLOGEN; MV): Before assay, the chloroplasts were heated at 50°C for 6 min in a water bath to inhibit the water splitting reaction. DPC at a final concentration of a 300 µM was added in the light to a reaction mixture containing 50 µM MV, 1 mM NaN_3 and 5 mM NH_4Cl. The reaction was followed as oxygen uptake.

2.5.2.5 ASSAY OF PS I ACTIVITY ONLY (REDUCED 2,6 DICHLOROPHENOL INDOPHENOL; DCIP TO MV): DCIP at a final concentration of 1 µM and ascorbic acid (sodium salt) at a final concentration of 400 mM were added to a reaction mixture containing 1 µM MV, 1 mM NaN_3, 5 mM NH_4Cl and 5 µM DCMU. The reaction was followed as oxygen uptake.

2.5.2.6 ASSAY OF PS I ACTIVITY ONLY (REDUCED N,N,N',N' TETRAMETHYL-p-PHENYLENEDIAMINE; TMPD TO MV): TMPD at a final concentration of 300 μM and sodium ascorbate at a final concentration of 2 mM was added simultaneously in light to a reaction mixture containing 50 μM MV, 1 mM NaN_3, 5 mM NH_4Cl and 1 mM DCMU. The reaction was followed as oxygen uptake.

2.6 FLUORESCENCE MEASUREMENTS: The room temperature chlorophyll fluorescence was measured using a pulse amplitude modulation fluorometer (PAM 101 and 102, Walz Effelrich, Germany) as described by Schreiber *et al.* (1986).

2.6.1 FLUORESCENCE MEASUREMENTS FROM INTACT LEAVES: Leaves after the photoinhibition treatment were dark adapted for 30 min prior to measurements at same temperature as was used for the photoinhibition treatment. The dark adapted leaf was exposed to a modulated light with an intensity of 4 μmol m^{-2} s^{-1} to measure initial fluorescence (Fo). This was followed by an exposure to a saturating pulse of white light of 4000 μmol m^{-2} s^{-1} which was strong enough to reduce all the PSII reaction centre to provide the maximum fluorescence (Fm). After measurement of Fm the leaf was allowed to reach a steady state fluorescence (Fs) while exposed to actinic light intensity of 800 μmol m^{-2} s^{-1}. Another burst of saturating light at Fs state was used to measure F'm. After reaching to the

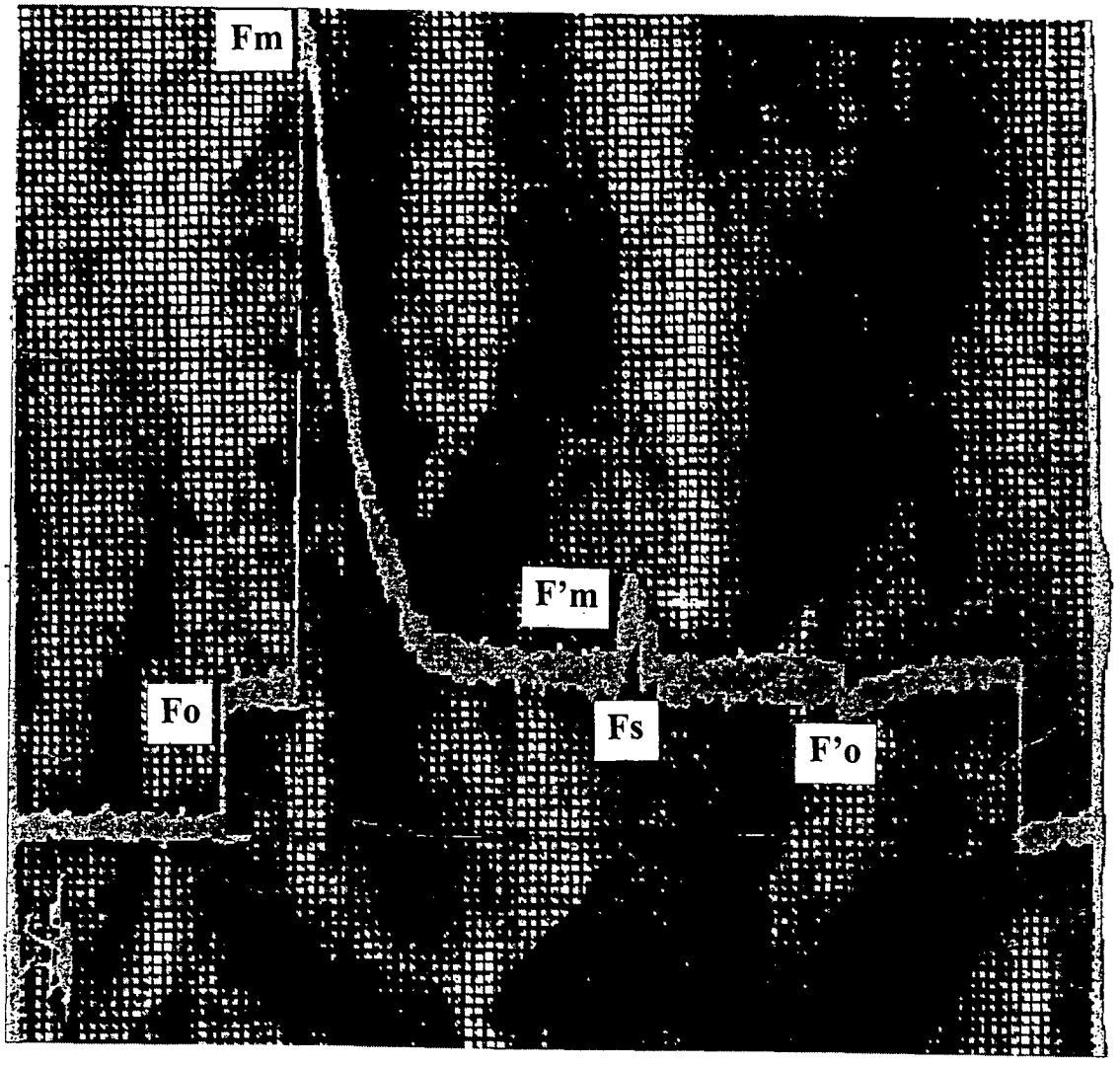


Figure 2.3: A typical chlorophyll fluorescence induction curve.

steady state again, leaf was exposed to far-red radiation light of 12 μmol m^{-2} s^{-1} to measure F'o. The coefficient of photochemical quenching (qP) defined as (F'm- Fs)/ (F'm-F's) and non photochemical quenching (qN) defined as 1- (F'm-F'o)/ (Fm-Fo) were calculated according to Sharma *et al* . (1998). A typical fluorescence curve from intact leaf at 30°C is shown in figure 2.3.

2.6.2 FLUORESCENCE MEASUREMENTS FROM ISOLATED CHLOROPLATS: Chlorophyll fluorescence from isolated chloroplasts was also taken. Isolated chloroplasts, 200 μl of 1 mg chlorophyll chloroplasts^{-1} concentration were placed in a cuvette (Walz, Germany) supplied for measurements of chlorophyll fluorescence from isolated chloroplasts. The chloroplasts were kept stirred at 900 rpm by placing the cuvette on a magnetic stirrer (Ikamy Reo, Drehghal Electronics, Germany). The top transparent window of the cuvette was fixed with the fibre optic cable (101F Walz, Germany) carrying modulated and actinic light to the chloroplasts in the cuvette and fluorescence from the chloroplasts in the cuvette to detector. The fluorescence measurements were taken according to the procedure described in the section 2.6.1.

2.7 ANALYSIS OF PHOTOSYNTHETIC PIGMENTS: The photosynthetic pigments, specially pigments of the xanthophyll cycle were analysed to compare with the changes in qN to elucidate their role in protection against photodamage.

2.7.1 CAROTENOID SAMPLE PREPARATION: Pigment extraction was carried out according to Sharma and Hall (1996). After the desired treatment the exposed portion of the leaf (1 cm^2) was cut and weighed to 4 digit of the decimal on precision balance (Sortorius). The weighed leaf was ground in 100% acetone (containing 0.1% BHT) to a final volume of 500 μl extract. The extract was centrifuged at 6000 x g for 5 min at 4°C and supernatant was filtered through a 0.42 μm filter and stored in -70°C freezer for HPLC analysis of photosynthetic pigments. Same extract was also used for quantitative analysis of ABA using HPLC.

2.7.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC): Identification and separation of pigments was carried out according to Sharma and Hall (1996) using HPLC (Spectraphysics, U.K) with a C_{18} reverse phase column (ET 250/4 Nucleosil 100-5 C_{18} ODS), Spectraphysics SP ternary HPLC pump with SP 4270 integrator and spectra 100 variable wavelength detector. Filtered extract 10 μl of the sample was injected on to the HPLC column and separation was carried out using linear gradient of 0-100% ethyl acetate in acetonitrile water (9:1 v/v) over 25 minutes at a flow rate of 1.2 ml min^{-1} and peaks were detected at 445 nm. Peaks were identified by using retention times of their standards and their spectrum (Figure 3.17A & B). Peaks were quantitated using β-carotene as an external standard.

2.7.3. PREPARATION OF STANDARD FOR CAROTENOIDS: Fresh leaf tissue (10 g) was homogenized in 50 ml of acetone containing 0.1% BHT and extracted for 2 h on ice. The extract was centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was collected and concentrated to 1 ml by flushing with N_2 gas. The extract was stored at -70°C until used for TLC. Ready made TLC plates of 30% silica gel (Merck) were used. 200 µl extract was loaded and plates were developed in a saturated chamber with solvent system containing Hexane, Ethyl acetate and Triethylamine in 58:32:12 ratio. The different pigments were identified on the plates by their Rf value as well as spectral characteristics. The separated pigments were scrapped off from the TLC plate and dissolved in acetone, centrifuged & spectrum was taken to identify individual pigment through their characteristics spectrum (Fig 3.17A & B).

2.8 ABA ANALYSIS: Extraction of ABA from leaf tissue was carried out by the same process as described in section 2.7.1. The ABA was qualitatively separated and quantitatively determined using HPLC at a 254 nm with a linear gradient of methanol and water at flow rate of 1.2 ml min^{-1}. The ABA was quantitatively estimated using an external standard ABA (from sigma) according to Afilthile *et al.* (1993).

2.9 LIPID PEROXIDATION : Peroxidation of thylakoid lipids was assayed according to Halliwell and Gutturidge (1989) by 2-thiobarbituric acid-malondialdehyde (TBA-MDA) adduct formation (2 molecules of TBA react with one molecule of MDA). Malondialdehyde formation was followed by adding equal amounts of 0.5% TBA in 20% TCA to an aliquot of the incubation buffer containing 50 mM Tris (pH 8.0), 175 mM NaCl and chloroplast equivalent to 50 μg of chlorophyll. The solution was incubated at 95°C for 25 min and centrifuged at 250 g for 2 min to pellet out the protein precipitated by TCA. The clear supernatant was used for determination of lipid peroxidation. Absorbance at 530 minus 600 nm was measured. The absorbance at 600 nm was subtracted from absorbance at 530 nm in order to correct for the non specific turbidity. The amount of MDA formation was calculated using an extinction coefficient of 155 mM cm^{-1}.

2.10 EFFECT OF ANTIOXIDANTS ON PHOTOSYNTHESIS AND LIPID PEROXIDATION: Effect of various antioxidants like β-carotene, Ascorbate and Glutathione on photodamage to photosynthesis through chlorophyll fluorescence measurements and lipid peroxidation was studied. Chloroplasts were isolated according to the method described in section 2.4.1 from 8 day old plants grown in growth chamber. Chloroplasts equivalent to 200 μg of chlorophyll were used for the measurements. β-Carotene, Ascorbate and

Glutathione in final concentration of 0.5 mM was added to the chloroplasts and incubated for 30 min prior to high light treatment. The high light (3600 and 2200 μmol m^{-2} s^{-1} PAR) at 5, 30 and 50°C was given according to method described in section 2.4.4 for various duration. Chlorophyll fluorescence measurements were taken according to method described in section 2.6.2 and lipid peroxidation was measured according to method described in section 2.9.

2.11 PROTEIN ESTIMATION: Tissue extract (0.1 ml) was taken in 5 ml of solution prepared by mixing 100 ml of 2% Na_2CO_3 in 0.1 N NaOH with 2 ml of 0.5% $CuSO_4.5H_2O$ in 1% Sodium potassium tartarate. Folin-Ciocalteau's phenol reagent (0.5 ml) diluted to (1:1 v/v) with distilled water was added to this mixture and was incubated at room temperature for 30 min. Absorbance was taken at 750 nm. The protein content of extract was determined using standard protein curve obtained using known concentration of Bovine Albumine Serum.

2.12 ASSAY OF ANTI OXIDANT ENZYMES:

2.12.1 EXTRACTION: Leaves (0.5 g) were homogenized in chilled extraction buffer. The homogenate was filtered and centrifuged at 12,000 x g for 15 min at 4°C. Protein content of the Supernatant was measured according to method as described in section 2.11 (Lowry *et al* . 1951).

Supernatant equivalent to 50 μg was used for the estimation of enzyme activity.

Extraction Buffer

S. No.	Reagents	Required Concentration
1	Potassium Phosphate (pH 7.5)	50 mM
2	EDTA (di sodium salt)	1 mM
3	Triton x 100	0.1%
4	Soluble polyvinyl pyrophosphate	1.0%

2.12.2 SUPEROXIDE OXIDOREDUCTASE (EC 1.15.1.1) SUPEROXIDE DISMUTASE: Superoxide dismutase (SOD) is absolutely specific to superoxide radicals ($O_2^{\cdot-}$). SOD has a molecular weight of 32.6 KDa and is a dimer (subunit molecular weight 16.3 KDa). The enzyme contains two atoms of Zn^{2+} which is necessary for structural stability and 2 atoms of Cu^{2+} which is necessary for enzymatic activity. SOD activity is essentially independent of pH between 5.5 and 9.5. Assay of SOD, however, was performed at pH 7.5.

$$2\,O_2^{\cdot-} + 2\,H+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

SOD was assayed at 25°C according to Badiani *et al.* (1993). The assay medium contained 50 mM potassium phosphate (pH 7.8), 0.1mM EDTA, 10 mM cytochrome c, 0.1mM xanthine and the leaf

homogenate equivalent to 50 μg of protein . The reaction was followed spectro-photometrically as reduction in the rate of cytochrome c at 550 nm. One unit of SOD activity is defined as the amount of enzymes required to inhibit the rate of cytocrome c reduction by 50% under the specified conditions.

2.12.3 HYDROGEN PEROXIDE: HYDROGEN PEROXIDE OXIDOREDUCTASE (EC 1.11.1.6): CATALASE: Catalase reacts with hydrogen peroxide (relative rate = 1) or with alkyl hydrogen peroxide such as methyl peroxide (relative rate = 0.14) or ethyl peroxide (relative rate 0.003). Catalase has molecular weight 240-250 KDa and is a tetramer with subunit having molecular weight 60-65 KDa. Each subunit contain iron bound inner protoheam IX group.

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2$$

Activity of catalase was determined spectrophotometrically by following the decomposition of H_2O_2 at 240 nm at 25°C in a reaction mixture containing 50 mM potassium phosphate, 11 mM H_2O_2 and the leaf extract equivalent of 50 μg of protein, according to Mishra *et al.* (1993).

2.12.4 DONOR:HYDROGEN PEROXIDE OXIDOREDUCTASE (EC .1.11.1.7): PEROXIDASE:

Peroxidase (POD) is very specific to hydrogen acceptor which must be hydrogen peroxide (relative rate = 100). Peroxidase is a monomeric glyco-protein with the molecular weight of 44 KDa which contain 1 molecule of protoheam IX as prosthetic group and two molecules of Ca^{2+}. POD exist in greater than 10 isomeric forms and has a pH optimum of 6 to 6.5.

Peroxidase

$$H_2O_2 + DH_2 \text{ ----------} \rightarrow 2\ H_2O + D\ (D = \text{guaicol})$$

Peroxidase activity was monitored according to Afilthile *et al* . (1993). Activity was assayed in a reaction mixture containing 3.4 mM guaicol, 0.9 mM H_2O_2 & 50 mM potassium phosphate (pH 6.0) and leaf extract equivalent of 50 μg of protein. The mixture was incubated for 5 min at 25°C, and the reaction was stopped by adding 0.5 ml of 5% (v/v) sulphuric acid. Enzyme activity was calculated using an extinction Coefficient of 25.5 $mM^{-1}\ cm^{-1}$ at 470 nm for tetra-guaicol.

2.13 GEL ELECTROPHORESIS: Electrophoresis was performed in slab gel using Hoefer gel system for rapid electrophoresis.

2.13.1 SAMPLE PREPARATION: Chloroplasts were isolated according to the method described in section 2.4.1. and treated *in vitro* according to method described in section 2.4.4. Thylakoid membrane 100 µl (1mg chlorophyll ml^{-1} chloroplast) were washed twice with buffer containing 0.065 M Tris-HCl buffer pH 7.8 and centrifuged in an eppendurf tube at 8000 x g for 5 min at 4°C. The pellet was washed twice with distilled water and then dissolved in sample buffer.

Sample buffer

S. No.	Reagent	Required Concentration	Molecular Weight	g L^{-1}
1	Tris-HCl (pH 6.8)	0.125 M	121.14	15.143
2	Sodium Dodecyl Sulphate	4 % (w/v)	--	40.0
3	Glycerol	20% (v/v)	--	200
4	2-Merceptoethanol	10% (v/v)	--	100
5	Bromophenol blue	0.05% (w/v)	--	0.5

The sample was heated at 80°C for 3min and immediately transferred to ice. Protein content was assayed according to Lowry's method described in section 2 .11.

2.13.2 SLAB GEL: Each solution for the slab gel was prepared separately and then mixed at the desired concentration of acrylamide in the resolving gel. The final concentration of resolving gel and stacking gel are as follows.

Concentration of Resolving and Stacking Gel

S.No.	Reagents	Resolving	Stacking	Tank Buffe
1	Acrylamide and bisacrylamide	10-20% T and 2.7 % C	5 % 2.7 % C	-----
2	Tris-HCl	0.375 M	0.12 M	0.02 M
3	Glycine	--------	---------	0.192 M
4	pH	8.8	6.8	8.3
5	SDS	0.1%	0.1%	0.1%
6	Ammonnium per sulphate	0.1% (w/v)	0.05% (w/v)	-----
7	TEMED	0.07% (v/v)	0.05% (v/v)	-------

Solutions were degassed prior to adding ammonium persulphate and TEMED. Slab gels of 1.0 mm thickness was prepared with a linear gradient of 10-20% top to bottom on the top of the resolving gel a layer of butanol (water saturated) was overlaid and allowed to polymerize for 30-45 min. After polymerization of the resolving gel the butanol was decanted and the gel was washed with distilled water twice. The stacking gel solution (containing ammonium persulfate and TEMED) was poured on top of resolving gel to make a 2 cm long and 1.0 mm thick stacking gel. A gel comb was inserted in the stacking gel and allowed to polymerize for 30-45 min.

2.13.3 ELECTROPHORESIS: After polymerisation the gel was transferred to the tank buffer. Sample equivalent of 15 μg of protein was loaded along with one sample of standard protein mixture from sigma (SDS-7). Electrophoresis was performed using electrophoretic system from Hoefer (PS 500 x T.D.C power supply) at constant voltage of 80 V till the protein entered the resolving gel and then at voltage of 120 V.

2.13.4 STAINING AND DESTAINING: After electrophoresis gel was immediately transferred to a container filled with stain and left for staining for 90 min at room temperature on a rocking platform.

Staining Solution

S.No.	Regeant	Concentration
1	Coomassie blue R-250	0.125%
2	Metahnol	50%
3	Glacial acetic acid	10%

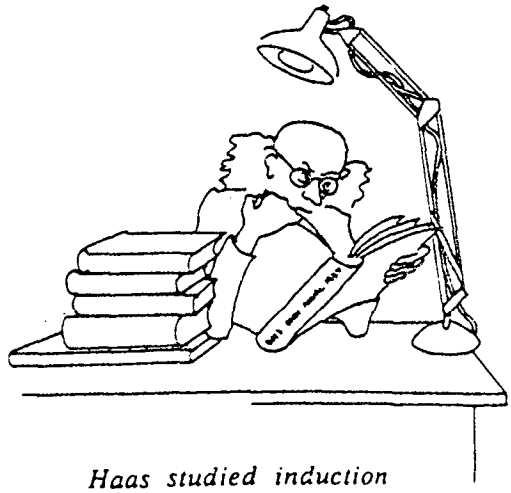
Finally the stain solution was decanted and the gel was washed with distilled water and left on rocking table for 30min in a destaining solution.

Destaning Solution

S. No.	Reagents	Concentration
1	Methanol	50%
2	Glacial acetic acid	10%

CHAPTER 3

"there is correlation



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RESULTS

3.0 RESULTS

3.1 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES ON PHTOSYNTHETIC ELECTRON TRANSPORT:

Effect of light at different temperature were studied in order to determine the effect of photoinhibition on photochemical reaction and to find out where, if any, specific site of photo-damage is located in the photosynthetic electron transport chain. The leaves were photoinhibited at 1000 and 2200 μmol m^{-2} s^{-1} PAR and 10, 30 and 50°C temperature up to 6 hour and PS I+PS II activity assayed as H_2O to FeCN or H_2O to MV (Fig. 3.1), PS II only activity assay as H_2O to PD and PS II minus water oxidation activity assayed as DPC to MV (Fig. 3.2) and PS I only activity assayed as DCIP to MV or TMPD to MV (Fig. 3.3.) were assayed according to the method described in section 2.5.2. It was observed that light treatment at 1000 μmol m^{-2} s^{-1} at 10 and 50°C resulted in decrease activity of PS I+PS II and PS II only activity. With the increase in the duration of photoinhibitory treatment at 10 and 50°C the activity decreased linearly while at 30°C the activity increased. However, PS II minus water oxidation and PS I only activity did not show any significant changes. It was seen that light treatment at 1000 μmol m^{-2} s^{-1} at 10°C showed a linear decrease in uncoupled PS I+ PS II and PS II only activity as compared to control (controls were growth chamber grown plants). It was seen that 6 hour photoinhibition treatment at 10°C and 1000 μmol m^{-2} s^{-1} resulted in a decrease of 26% in PS I + PS II activity assayed as H_2O to FeCN and 34% when assayed as H_2O to MV as compared to the control (Fig. 3.1A). The

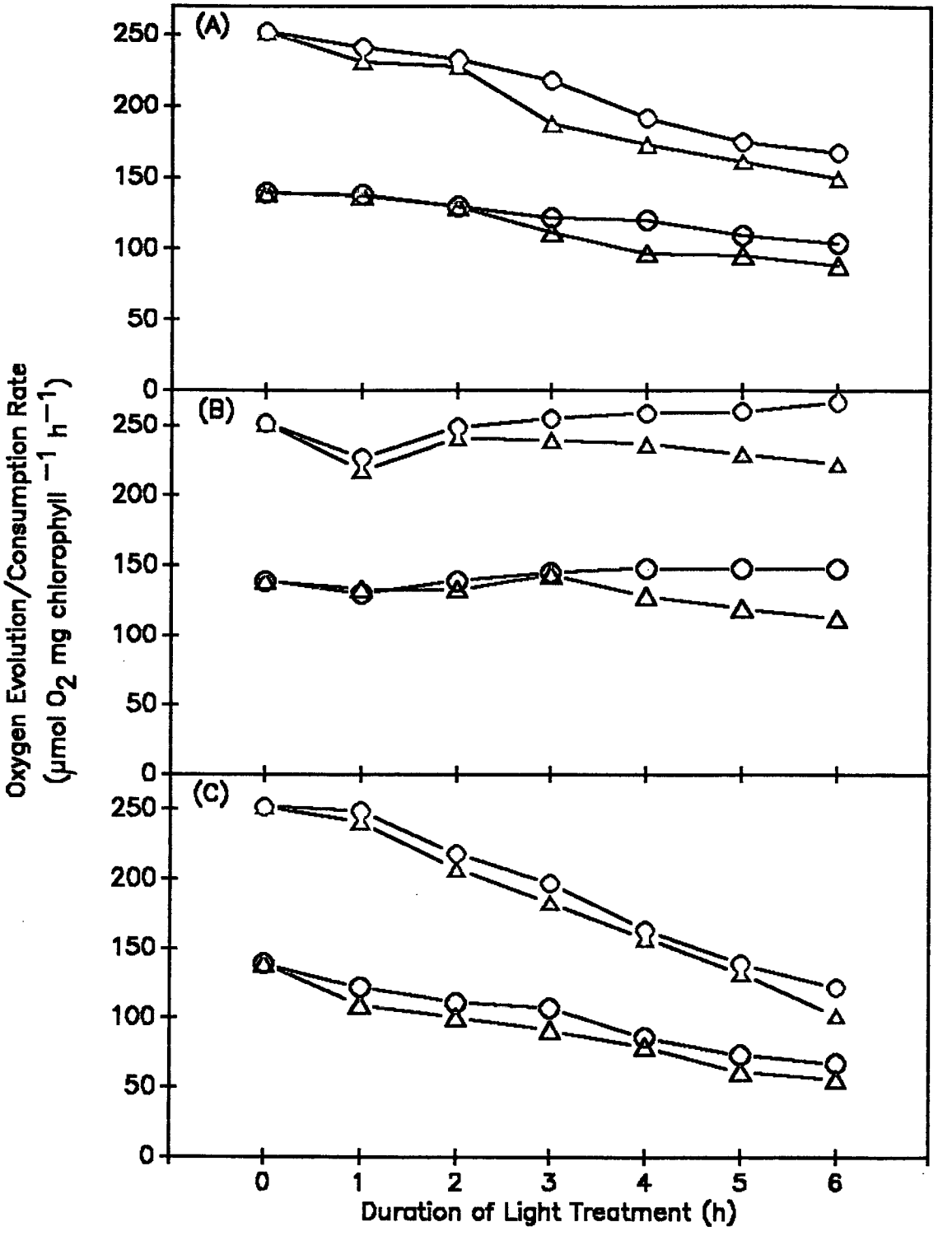


Fig. 3.1: Effect of photoinhibition at 10°C (A), 30°C (B) and 50°C (C) and 1000 μmol m^{-2} s^{-1} PAR on uncoupled PS I+PS II activity assayed as H_2O to FeCN (-o-) and H_2O to MV (-o-) and at 2200 μmol m^{-2} s^{-1} on uncoupled PS I+PS II activity assayed as H_2O to FeCN (-Δ-) and H_2O to MV (-Δ-) in chloroplasts isolated from photoinhibited leaves. The PPFD during assay was 1200 μmol m^{-2} s^{-1}. The points represent mean value of 4-6 replicates. SE bars are not seen on the scale.

photoinhibitory treatment at 30°C and 1000 μmol m^{-2} s^{-1} resulted in a linear increase in PS I+PS II activity with increase in the duration of the exposure. The observed increase in the electron transport after 6 hour of treatment was 7% in PS I+PS II activity assayed as H_2O to FeCN and 6% when assayed as H_2O to MV (Fig. 3.1B). However, leaves treated at 50°C at 1000 μmol m^{-2} s^{-1} PAR showed greater decrease in PS I+PS II activity as compared to seen in plants photoinhibited at 10°C or 30°C. The 6 hour light treatment at 50°C caused significantly more damage to PS I+PS II activity (52% decrease when assayed as H_2O to FeCN or as H_2O to MV; Fig. 3.1C; Table 3.1

Uncoupled PS II activity assayed as H_2O to PD also decreased by 43% when leaves were photoinhibited at 1000 μmol m^{-2} s^{-1} PAR at 10°C for 6 hour (Fig. 3.2A). The same light treatment at 30°C, however, did not show any change in the PS II activity (Fig. 3.2B), while the light treatment at 50°C resulted in 57% loss of the PS II activity (Fig. 3.2C) as compared to control.

When intact leaves were photoinhibited at 2200 μmol m^{-2} s^{-1} PAR and 10, 30 and 50°C, it was seen that the extent of decrease in PS I+PS II activity (Fig. 3.1) and PS II only activity (Fig. 3.2) was much greater than seen in plants treated at 1000 μmol m^{-2} s^{-1} at respective temperatures (Table 3.2). Six hour light treatment at 10°C and 2200 μmol m^{-2} s^{-1} PAR showed a decrease of 37% in PS I+PS II activity assayed as H_2O to FeCN and 41% decrease when assayed as H_2O to MV (Fig. 3.1.A). The PS II only activity assayed as H_2O to PD decreased by 48% as compared to control (Fig. 3.2A).

The decrease observed in PS I+PS II activity assayed as H_2O to FeCN or H_2O to MV after 6 hour of the treatment of leaves at 10°C and 2200 μmol m^{-2} s^{-1} PAR was

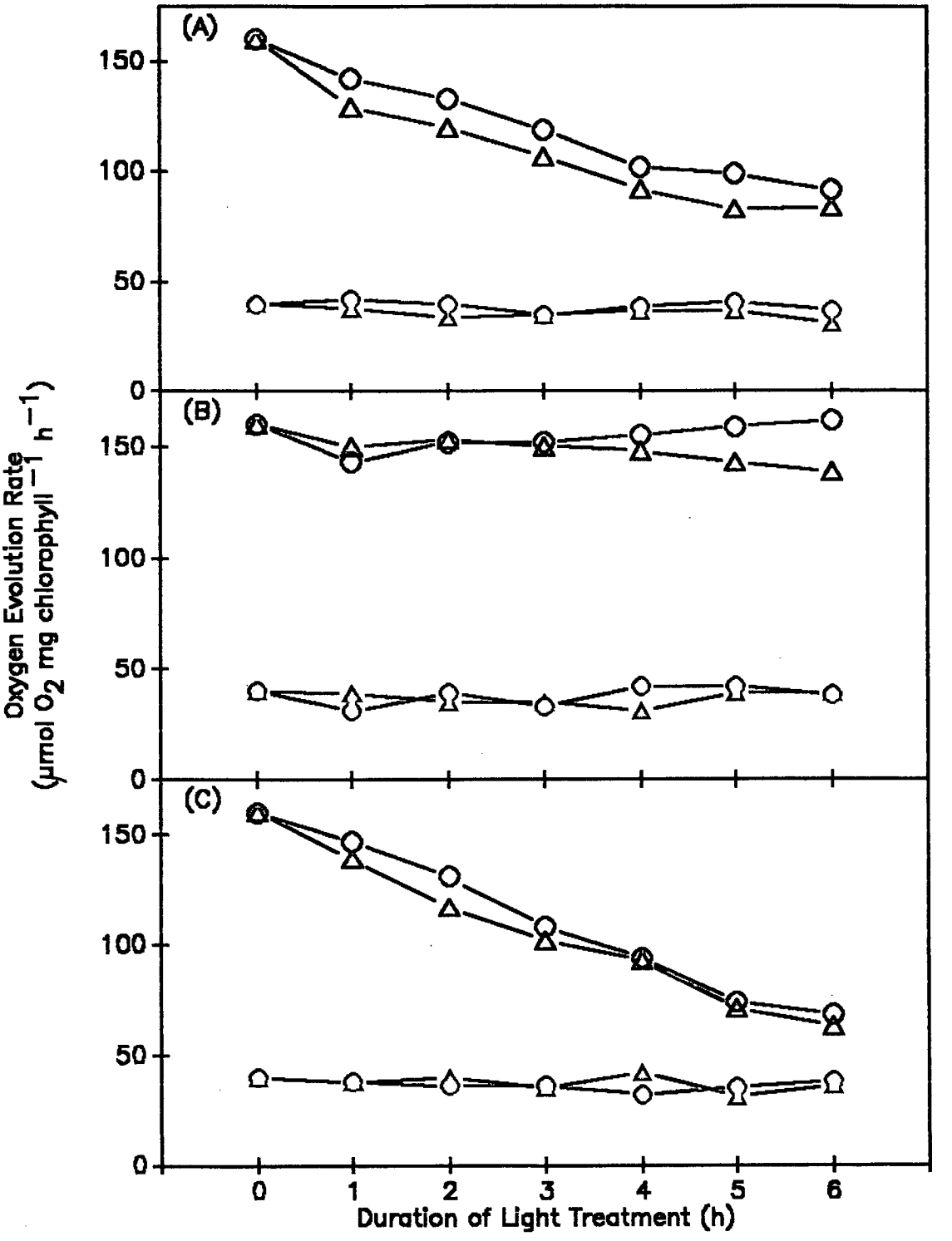


Fig. 3.2: Effect of photoinhibition at 10°C (A), 30°C (B) and 50°C (C) and 1000 μmol m^{-2} s^{-1} PAR on uncoupled PS II activity assayed as H_2O to PD (-o-) and DPC to MV (-o-) and at 2200 μmol m^{-2} s^{-1} on uncoupled PS II activity assayed as H_2O to PD (-Δ-) and DPC to MV (-Δ-) in chloroplasts isolated from photoinhibited leaves. The PPFD during assay was 1200 μmol m^{-2} s^{-1}. The points represent mean value of 4-6 replicates. SE bars are not seen on the scale.

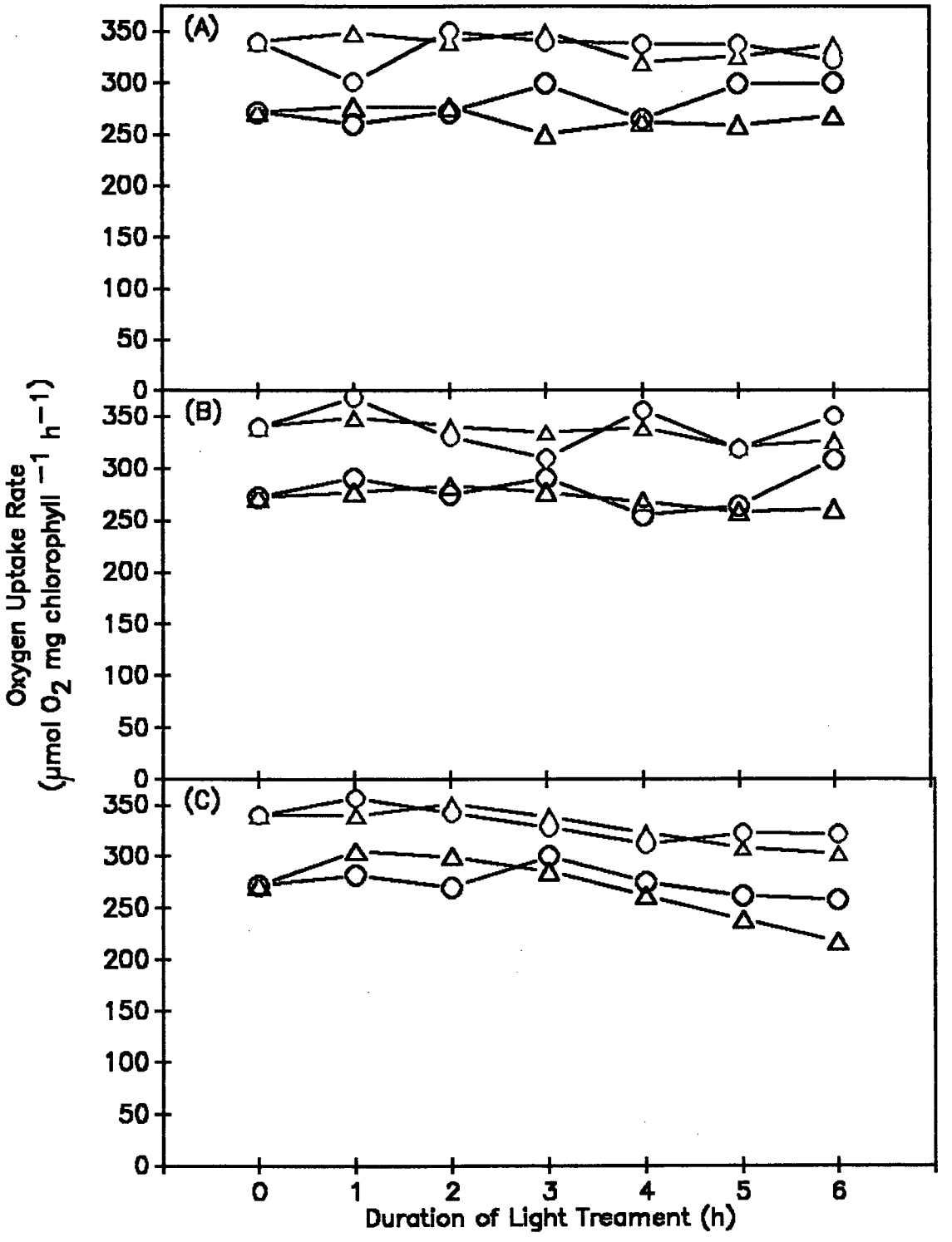


Fig. 3.3: Effect of photoinhibition at 10°C (A), 30°C (B) and 50°C (C) and 1000 μmol m^{-2} s^{-1} PAR on uncoupled PS I activity assayed as DCIP to MV (-o-) and TMPD to MV (-o-) and at 2200 μmol m^{-2} s^{-1} on uncoupled PS II activity assayed as DCIP to MV (-Δ-) and TMPD to MV (-Δ-) in chloroplasts isolated from photoinhibited leaves. The PPFD during assay was 1200 μmol m^{-2} s^{-1}. The points represent mean value of 4-6 replicates. SE bars are not seen on the scale.

11% and 7% higher respectively, as compared to photoinhibition of leaves at 10°C and 1000 μmol m^{-2} s^{-1} for the same duration. PS II only activity assayed as H_2O to PD in leaves treated as 10°C and 2200 μmol m^{-2} s^{-1} for 6 hour also showed 5% greater damage than seen in leaves treated at 1000 μmol m^{-2} s^{-1} PAR at same temperature for the same duration (Table 3.1 & 3.2)..

The light treatment of 2200 μmol m^{-2} s^{-1} at 30°C resulted in relatively less decrease in PS I+PS II activity (19% decrease when assayed as H_2O to FeCN and 12% decrease when assayed as H_2O to MV (Fig. 3.1B) and PS II activity (13% decrease) assayed as H_2O to PD compared to decrease in plants photoinhibited at 10°C. The light treatment at 50°C and 2200 μmol m^{-2} s^{-1} caused greater damage to PS I+PS II and PS II only activity compared to the same light treatment (2200 μmol m^{-2} s^{-1} PAR) at 10°C and 30°C. It was observed that 6 hour light treatment at 50°C resulted in a decrease of 40% and 60% in PS I+PS II activity assayed as H_2O to FeCN and H_2O to MV respectively, as compared to control (Fig. 3.1C). The decrease in PS II activity assayed as H_2O to PD was 61% as compared to control (Fig. 3.2C). The decrease observed in PS II activity in leaves photoinhibited at 2200 μmol m^{-2} s^{-1} at 50°C was 48% greater than seen in leaves treated at 30°C and 13% greater than seen in leaves treated at 10°C for the same light regime (2000 μmol m^{-2} s^{-1}) and duration (Table 3.2).

PS II minus water oxidation activity assayed as DPC to MV (Fig. 3.2) and PS I activity assayed as DCIP to MV or TMPD to MV (Fig. 3.3) at 10, 30 and 50°C did not change significantly.

Table 3.1: Effect of high light treatment (1000 μmol m^{-2} s^{-1}), of intact leaves, at different temperature for 6 hour on their light saturated photosynthetic electron transport activity. The photosynthetic photon flux density during assay was 1200 μmol m^{-2} s^{-1}. The reaction rates are expressed as μmol Oxygen consumed or evolved mg chlorophyll ml^{-1} $hour^{-1}$. The chlorophyll concentration in the reaction mixture was 0.05 mg. Mean±SD values were calculated from 4-6 experiments.

	10°C							30°C						50°C					
Assays	0 h	1 h	2 h	3 h	4 h	5 h	6 h	1 h	2 h	3 h	4 h	5 h	6 h	1 h	2 h	3 h	4 h	5 h	6 h
PSI+PSII																			
H_2O to FeCN	60	50	65	58	53	44	42	52	59	65	71	72	74	54	44	40	33	30	27
(Coupled)	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	10	7	8	10	6	8	7	9	8	7	10	9	12	10	10	12	6	7	6
H_2O to FeCN	139	138	130	122	120	109	103	130	139	145	148	148	148	122	111	107	86	73	67
(Uncoupled)	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	18	15	12	13	17	12	12	13	12	14	12	12	15	12	10	10	11	10	8
H_2O to MV	108	103	100	92	83	76	70	107	108	112	114	117	120	102	91	80	66	57	50
(Coupled)	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	13	10	10	8	9	8	6	9	6	8	10	9	12	8	8	6	8	5	6
H_2O to MV	252	241	233	218	192	175	167	227	249	255	259	260	267	249	218	197	163	139	122
(Uncoupled)	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	22	25	18	11	12	9	10	8	8	10	11	11	13	10	10	12	11	12	8
PS II only																			
H_2O to PD	160	142	133	119	102	99	92	143	152	152	155	159	162	147	131	108	94	74	68
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	12	17	14	8	7	9	8	9	9	12	10	9	9	10	9	8	9	9	6
DPC to MV	40	42	40	38	39	41	37	31	39	33	42	42	38	38	36	36	32	35	38
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	4	6	6	5	6	8	5	5	6	6	5	7	6	5	8	6	5	7	5
PS I only																			
DCIP to MV	272	260	272	300	265	300	301	291	275	291	255	264	309	282	270	300	275	262	258
Asc	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
Asc	25	31	20	18	15	18	21	17	19	20	18	20	21	23	17	19	20	16	21
TMPD to	340	301	351	341	339	339	323	368	330	310	356	319	351	357	342	328	312	323	322
MV	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	33	30	28	29	32	36	21	28	23	20	19	23	28	28	25	30	21	27	29

0 h represent controls. Control plants were kept at growth conditions.

Table 3.2: Effect of high light treatment (2200 μmol m^{-2} s^{-1}), of intact leaves, at different temperature for 6 hour on their light saturated photosynthetic electron transport activity. The photosynthetic photon flux density during assay was 1200 μmol m^{-2} s^{-1}. The reaction rates are expressed as μmol Oxygen consumed or evolved mg chlorophyll ml^{-1} $hour^{-1}$. The chlorophyll concentration in the reaction mixture was 0.05 mg. Mean±SD values were calculated from 4-6 experiments.

Assays	10°C							30°C						50°C					
	0 h	1 h	2 h	3 h	4 h	5 h	6 h	1 h	2 h	3 h	4 h	5 h	6 h	1 h	2 h	3 h	4 h	5 h	6 h
PSI+PSII																			
H_2O to FeCN	60.2	52	60	55	43	34	30	50	52	62	62	58	58	50	40	37	30	26	22
(Coupled)	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	10	6	6	5	5	4	5	5	6	5	7	5	5	6	7	5	5	4	4
H_2O to FeCN	139	137	130±	111	97	97	88	133±	143	128	119	112±	109	110	91	79	61	56	56
(Uncoupled)	±	±	12	±	±	±	±	10	±	±	±	10	±	±	±	±	±	±	±
	18	15		15	10	8	7		9	10	13		10	8	7	7	6	6	7
H_2O to MV	108	100	100	93	78	69	61	103	109	115	107	103	98	102	88	71	62	49	43
(Coupled)	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	13	8	7	8	6	7	6	9	9	7	8	8	7	8	9	8	6	6	8
H_2O to MV	252	231	228	188	173	161	149	218	241	240	237	230	223	241	207	183	157	132	102
(Uncoupled)	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	22	17	15	18	12	11	12	12	13	17	13	15	12	14	12	10	10	8	7
PS II only																			
H2O to PD	160	129	120±	107	92	83	84	150	153	150	148	143	139	139	117	102	93	71	63
	±	±	10	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	12	9		10	8	8	7	9	10	10	11	12	8	9	10	8	9	8	7
DPC to MV	40	38	34	35	37	37	31	39	35	35	31	39	39	38	40	35	42	31	36
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	4	4	5	5	5	5	6	5	4	5	4	6	7	5	5	6	5	3	4
PS I only																			
DCIP to MV	272	277	277	251	263	260	269	277	283	277	268	259	261	305	300	285	262	239	217
ASC	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	25	25	23	25	30	27	29	23	22	25	23	29	23	21	20	24	22	27	21
TMPD to MV	340	349	341	350	321	327	338	349	341	335	339	321	327	340	451	339	323	308	303
ASC	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	32	28	30	32	28	27	32	33	31	27	29	30	32	32	33	30	29	31	28

0 h represent controls. Control plants were kept at growth conditions.

3.2 EFFECT OF PHOTOINHIBITION OF ISOLATED CHLOROPLASTS ON PHOTOSYNTHETIC ELECTRON TRANSPORT ACTIVITY:

The extent of photoinhibitory damage to electron transport was much greater when photoinhibitory treatment was given to the chloroplasts, isolated from leaves. Chloroplasts, isolated from normal leaves, were exposed to high light 1000 μmol m^{-2} s^{-1} (Table 3.3), 2200 μmol m^{-2} s^{-1} (Table 3.4) and 3600 μmol m^{-2} s^{-1} (Table 3.5) at 10, 30 and 50°C for a duration of 0-120 min. It was seen that extent of inhibition of electron transport was directly proportional to the PPFD and duration of light. High temperature (50°C) caused greater damage to electron transport activity than low temperature (10°C). As was seen in case of photoinhibition of intact leaves, photoinhibition of chloroplast isolated from normal leaves also showed significant decrease in PS I+PS II (Fig. 3.4) and PS II (Fig. 3.5) activity. PS II minus oxygen evolution activity (Fig. 3.5) and PS I only activity (Fig. 3.6) decreased slightly when chloroplasts were exposed to longer 60-120 min duration.

It was seen that uncoupled PS I+PS II activity assayed as H_2O to FeCN and H_2O to MV in chloroplasts isolated from control leaves and exposed to 1000 μmol m^{-2} s^{-1} PAR at 10°C for 120 min, decreased by 34% and 40% respectively as compared to control (Fig. 3.4A). However, chloroplast exposed to higher PPFD of 2200 and 3600 μmol m^{-2} s^{-1} PAR resulted in further decrease in the extent of damage to PS I and PS II activity. Chloroplasts exposed to 2200 μmol m^{-2} s^{-1} at 10°C temperature for 6 hour duration resulted in 92% decrease in PS I+PS II activity assayed as H_2O to FeCN and 89% when assayed as H_2O to MV (Fig. 3.4A). Approximately 98% decrease in PS

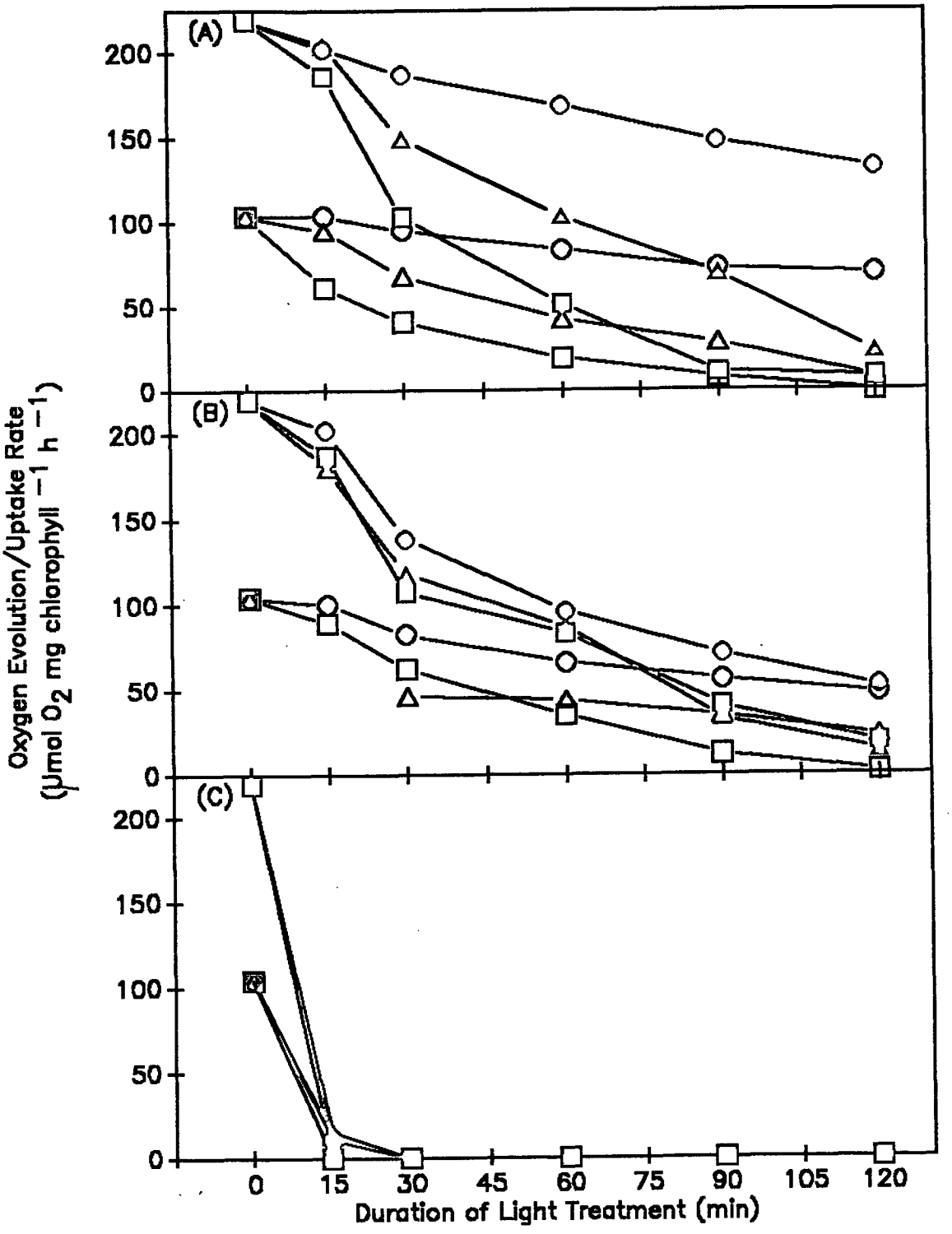


Fig. 3.4: Effect of photoinhibition of isolated chloroplasts (photoinhibition under *in vitro* conditions) at 10°C (A), 30°C (B) and 50°C (C) and 1000 μmol m^{-2} s^{-1} PAR on uncoupled PS I+PS II activity assayed as H_2O to FeCN (-o-) and H_2O to MV (-o-) and at 2200 μmol m^{-2} s^{-1} on uncoupled PS I+PS II activity assayed as H_2O to FeCN (-Δ-) and H_2O to MV (-Δ-) and photoinhibition at 3600 μmol m^{-2} s^{-1} PAR on uncoupled PS I+PS II activity assayed as H_2O to FeCN (-□-) and H_2O to MV (-□-). The PPFD during assay was 1200 μmol m^{-2} s^{-1}. The points represent mean value of 4-6 replicates. SE bars are not seen on the scale.

I+PS II activity, assayed as H_2O to FeCN or as H_2O to MV, was observed in chloroplasts treated at 3600 μmol m^{-2} s^{-1} for the same temperature (10°C) and duration (120 min; Fig. 3.4A). The PS II activity also decreased due to the photoinhibition of isolated chloroplasts. photoinhibition treatment at 10°C and 1000, 2000 and 3600 μmol m^{-2} s^{-1} PAR for 120 min resulted in 41, 88 and 100% decrease respectively in PS II activity as compared to control (Fig. 3.5A). Control assays were carried out from chloroplasts kept for 120 min at respective temperature in the dark.

PS II minus oxygen evolution activity assayed as DPC to MV, unlike seen in photoinhibition of intact leaves, showed a decrease. It was seen that photoinhibition of isolated chloroplasts at 10°C and 1000 μmol for 120 min showed decrease of 26% as compared to the control which was further damaged to 33% and 45% as Chloroplast were exposed to 2200 and 3600 μmol m^{-2} s^{-1} PAR respectively at 10°C for 120 min (Fig. 3.5A).

Photosystem I activity assayed as DCIP to MV or TMPD to MV, however, did not show linear changes and did not decrease to significant level when photoinhibited at 1000, 2200 or 3600 μmol m^{-2} s^{-1} PAR at 10, 30 and 50°C (Fig. 3.6).

The temperature in combination with light also influenced the extent of damage to photosynthetic electron transport chain. It was observed that when isolated chloroplast were photoinhibited at 1000 μmol m^{-2} s^{-1} and at 10°C, the damage to PS I+PS II activity assayed as H_2O To MV was 8% after 15 min of exposure followed by 15% after 30 min, 32% after 90 min and 40% after 120 min, as compared to control (Fig. 3.4A). However, photoinhibition of PS I+PS II activity assayed as H_2O to MV at 30°C

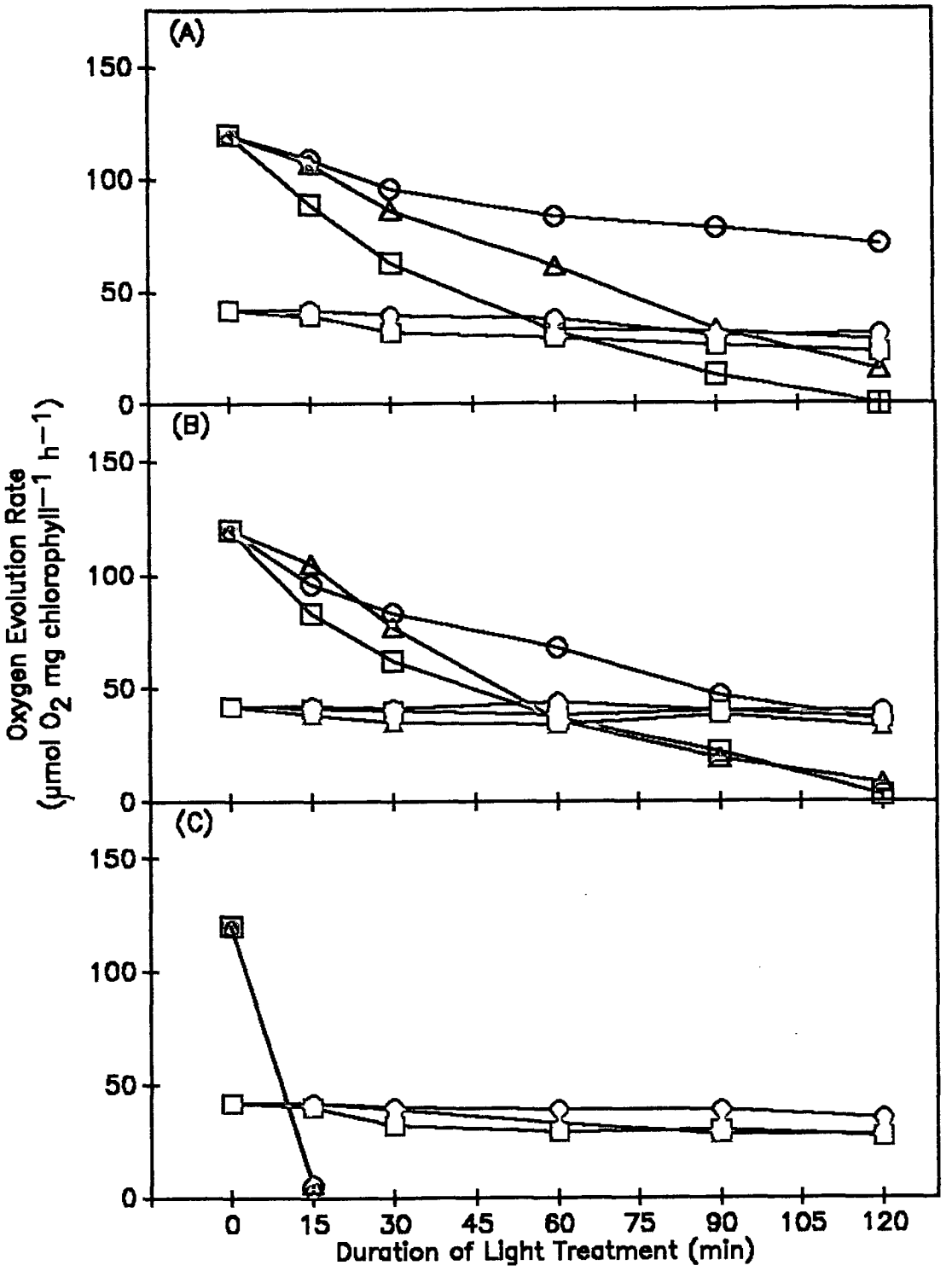


Fig. 3.5: Effect of photoinhibition of isolated chloroplasts (photoinhibition under *in vitro* conditions) at 10°C (A), 30°C (B) and 50°C (C) and 1000 μmol m^{-2} s^{-1} PAR on uncoupled PS II activity assayed as H_2O to PD (-o-) and DPC to MV (-o-) and at 2200 μmol m^{-2} s^{-1} on uncoupled PS II activity assayed as H_2O to PD (-Δ-) and DPC to MV (-Δ-) and photoinhibition at 3600 μmol m^{-2} s^{-1} PAR on uncoupled PS II activity assayed as H_2O to PD (-□-) and DPC to MV (-□-). The PPFD during assay was 1200 μmol m^{-2} s^{-1}. The points represent mean value of 4-6 replicates. SE bars are not seen on the scale.

(Fig. 3.4B) for 120 min resulted in a decrease of 76% which was 36% greater than observed in leaves photoinhibited at same level of light but at 10°C (Fig. 3.4A). The photoinhibition at 50°C (Fig. 3.4C) resulted in complect inhibition of the activity. PS II activity was also influenced by the exposure to low and high temperature at same PPFD (1000 μmol m^{-2} s^{-1}). It was seen that PS II activity, assayed as H_2O to PD, showed a decrease of 41% when photoinhibited at 10°C (Fig. 3.5A), 70% when photoinhibited at 30°C (Fig. 3.5B) and 100% when photoinhibited at 50°C (Fig. 3.5C) at 1000 μmol m^{-2} s^{-1} for 120 min.

The effect of temperature become more damaging to photosynthetic electron transport when given along with higher light intensities of 2200 μmol m^{-2} s^{-1} (Table 3.4) and 3600 μmol m^{-2} s^{-1} (Table 3.5). The treatment of isolated chloroplast at 2200 μmol m^{-2} s^{-1} and 10, 30 and 50°C on PS I+PS II activity assayed as H_2O to MV resulted in a decrease of 89%, 94% and 100% respectively, as compared to the control. The photoinhibition treatment at 3600 μmol m^{-2} s^{-1} PAR and at 10, 30 and 50°C temperature caused 96%, 91% and 100% decrease in the PS I+PS II activity (Fig. 3.4).

PS II activity decreased to greater extent than PS I+PS II activity. The photoinhibition treatment of isolated chloroplasts at 3600 μmol m^{-2} s^{-1} PAR and at 10, 30 and 50°C temperature for 120 min resulted in complete inhibition of the activity, as compared to the control. Where as, photoinhibition at 2200 μmol m^{-2} s^{-1} PPFD at 10°C temperature resulted in decrease of 88%, 93% at 30°C and complete inhibition at 50°C compared to control (Fig. 3.5). Results show that photoinhibition at 50°C was highly inhibitory than at 10 and 30°C.

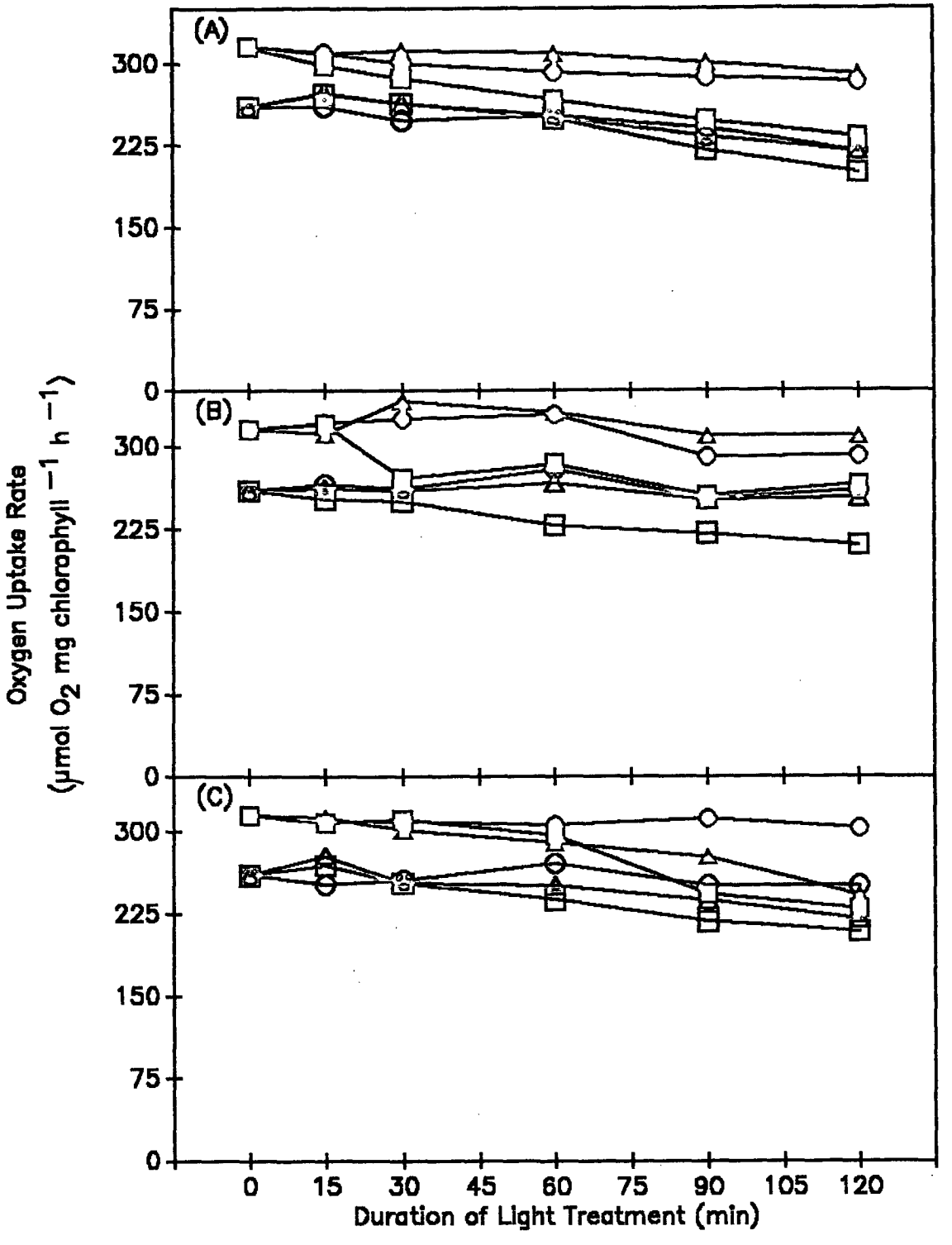


Fig. 3.6: Effect of photoinhibition of isolated chloroplasts (photoinhibition under *in vitro* conditions) at 10°C (A), 30°C (B) and 50°C (C) and 1000 μmol m^{-2} s^{-1} PAR on uncoupled PS I activity assayed as DCIP to MV (-o-) and TMPD to MV (-o-) and at 2200 μmol m^{-2} s^{-1} on uncoupled PS I activity assayed as DCIP to MV (-Δ-) and TMPD to MV (-Δ-) and photoinhibition at 3600 μmol m^{-2} s^{-1} PAR on uncoupled PS I activity assayed as DCIP to MV (-□-) and TMPD to MV (-□-). The PPFD during assay was 1200 μmol m^{-2} s^{-1}. The points represent mean value of 4-6 replicates. SE bars are not seen on the scale.

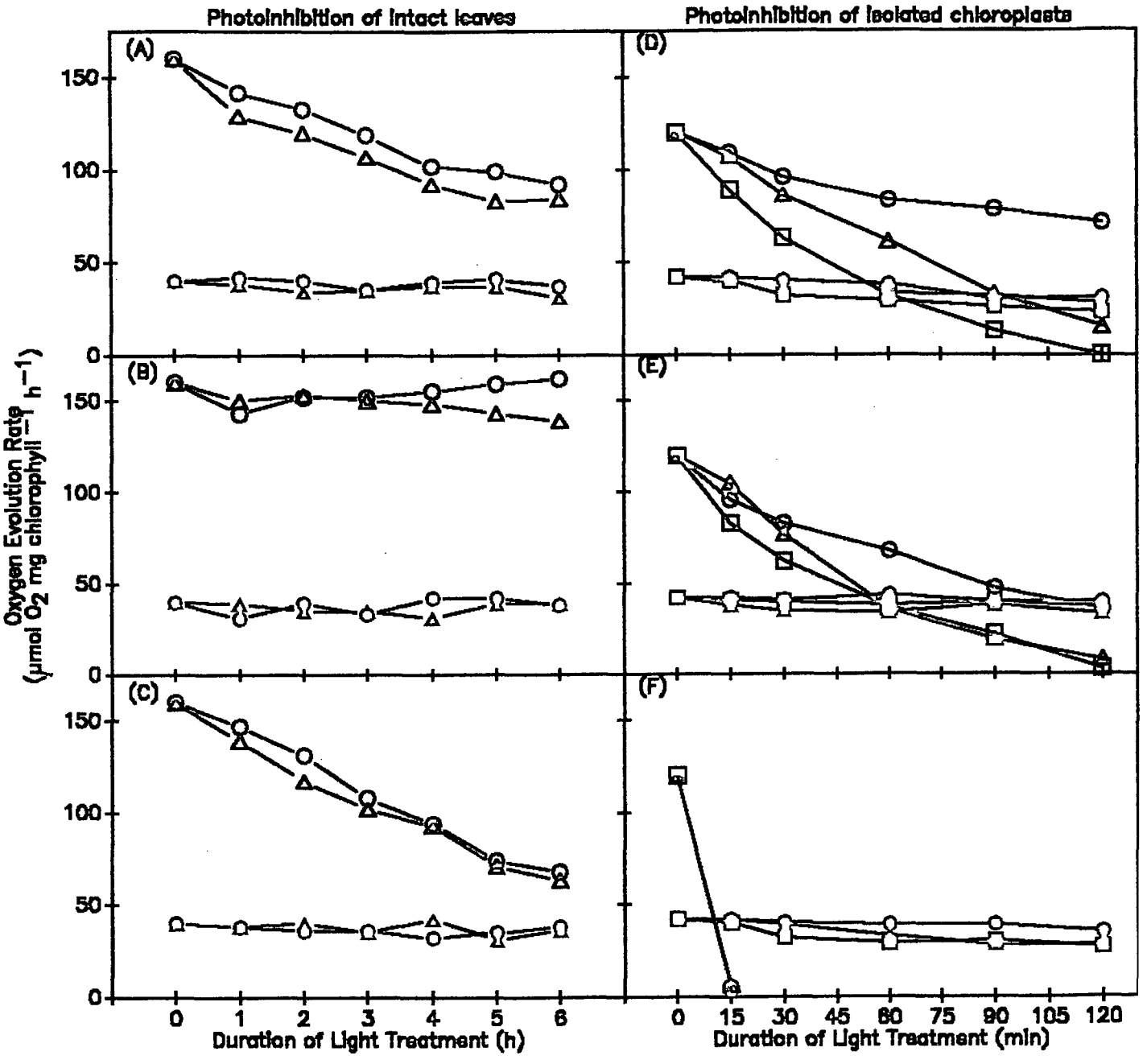


Fig. 3.7: Effect of photoinhibition under *in vivo* (A, B & C) and *in vitro* (D, E & F) conditions at 10°C (A & D), 30°C (B & E) and 50°C (C & F) on uncoupled PS II activity assayed as H_2O to PD (open symbols; -o-, -Δ-, -□-) and DPC to MV (filled symbols; -o-, -Δ-, -□-). The photoinhibitory treatment was given at 1000 μmol m^{-2} s^{-1} (as H_2O to PD -o-; and DPC to MV -o-), 2200 μmol m^{-2} s^{-1} (as H_2O to PD -Δ-; and DPC to MV -Δ-), and 3600 μmol m^{-2} s^{-1} (as H_2O to PD -□-; and DPC to MV -□-) PAR. The PPFD during assay was 1200 μmol m^{-2} s^{-1}. The points represent mean value of 4-6 replicates. SE bars are not seen on the scale.

Table 3.3: Effect of high light treatment (1000 μmol m^{-2} s^{-1}), of chloroplasts isolated from leaves, at different temperature for 6 hour on their light saturated photosynthetic electron transport activity. The photosynthetic photon flux density during assay was 1200 μmol m^{-2} s^{-1}. The reaction rates are expressed as μmol Oxygen consumed or evolved mg chlorophyll ml^{-1} $hour^{-1}$. The chlorophyll concentration in the reaction mixture was 0.05 mg. Mean±SD values were calculated from 4-6 experiments.

Assays	10°C						30°C					50°C				
	0 min	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min
PSI+PSII																
H_2O to FeCN	55	58	49	43	38	35	68	72	59	48	34	7	0	0	0	0
(Coupled)	±	±	±	±	±	±	±	±	±	±	±	±				
	7	7	6	5	3	2	8	6	6	5	3	1				
H_2O to FeCN	104	104	95	83	72	69	100	82	66	56	48	11	0	0	0	0
(Uncoupled)	±	±	±	±	±	±	±	±	±	±	±	±				
	10	8	9	7	8	8	8	9	7	5	5	1				
H_2O to MV	85	91	82	67	53	50	78	46	32	29	21	3	0	0	0	0
(Coupled)	±	±	±	±	±	±	±	±	±	±	±	±				
	8	8	8	6	6	5	6	5	3	3	3	1				
H_2O to MV	219	202	187	169	148	132	202	139	96	71	52	12	0	0	0	0
(Uncoupled)	±	±	±	±	±	±	±	±	±	±	±	±				
	17	12	13	15	12	11	12	13	8	6	6	2				
PS II only																
H_2O to PD	120	109	96	83	78	71	96	83	68	47	36	5	0	0	0	0
	±	±	±	±	±	±	±	±	±	±	±	±				
	8	6	7	5	6	6	8	6	7	6	5	1				
	42	42	40	35	30	31	42	41	44	40	40	42	40	39	39	35
DPC to MV	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	5	6	5	5	3	3	4	3	5	4	4	4	5	3	4	3
PS I only																
DCIP to MV	260	260	248	252	233	220	265	262	279	252	260	252	233	271	251	251
Asc	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	18	23	20	17	18	15	19	20	18	17	15	14	14	12	13	18
TMPD to MV	315	309	300	292	287	283	321	351	328	290	291	309	309	306	312	303
Asc	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	22	21	22	17	19	23	20	28	13	14	12	14	17	17	19	16

0 h represent controls. Isolated chloroplasts kept at respective temperature for 120 min were used as control

Table 3.4: Effect of high light treatment (2200 μmol m^{-2} s^{-1}), of chloroplasts isolated from leaves, at different temperature for 6 hour on their light saturated photosynthetic electron transport activity. The photosynthetic photon flux density during assay was 1200 μmol m^{-2} s^{-1}. The reaction rates are expressed as μmol Oxygen consumed or evolved mg chlorophyll ml^{-1} $hour^{-1}$. The chlorophyll concentration in the reaction mixture was 0.05 mg. Mean±SD values were calculated from 4-6 experiments.

Assays	10°C						30°C					50°C				
	0 min	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min
PSI+PSII																
H_2O to FeCN (Coupled)	55 ± 7	55 ± 5	42 ± 4	29 ± 3	12 ± 1	0	60 ± 5	53 ± 6	48 ± 4	41 ± 3	29 ± 2	9 ± 1	0	0	0	0
H_2O to FeCN (Uncoupled)	104 ± 10	95 ± 8	68 ± 4	42 ± 3	28 ± 2	8 ± 1	104 ± 7	46 ± 4	44 ± 5	35 ± 4	23 ± 2	13 ± 1	0	0	0	0
H_2O to MV (Coupled)	85 ± 8	80 ± 5	62 ± 6	40 ± 4	17 ± 2	0	65 ± 7	47 ± 5	37 ± 4	35 ± 4	14 ± 5	7 ± 2	0	0	0	0
H_2O to MV (Uncoupled)	219 ± 17	204 ± 15	149 ± 8	103 ± 8	69 ± 4	23 ± 2	180 ± 11	118 ± 9	87 ± 6	33 ± 4	14 ± 2	15 ± 2	0	0	0	0
PS II only																
H_2O to PD	120 ± 8	107 ± 10	86 ± 6	61 ± 5	33 ± 2	15 ± 2	105 ± 7	77 ± 6	36 ± 3	19 ± 2	8 ± 1	6 ± 1	0	0	0	0
DPC to MV	42 ± 5	39 ± 4	38 ± 5	33 ± 4	32 ± 3	28 ± 3	38 ± 4	35 ± 4	34 ± 3	38 ± 4	33 ± 3	42 ± 5	39 ± 4	33 ± 4	28 ± 5	20 ± 3
PS I only																
DCIP to MV / Asc	260 ± 18	273 ± 19	263 ± 18	252 ± 17	241 ± 20	220 ± 22	262 ± 18	260 ± 19	266 ± 17	251 ± 16	253 ± 18	276 ± 15	253 ± 21	251 ± 18	237 ± 17	220 ± 17
TMPD to MV / Asc	315 ± 22	308 ± 23	311 ± 22	309 ± 27	301 ± 28	290 ± 23	311 ± 25	351 ± 28	330 ± 23	309 ± 31	309 ± 29	313 ± 26	301 ± 29	290 ± 28	277 ± 23	242 ± 18

0 h represent controls. Isolated chloroplasts kept at respective temperature for 120 min were used as control

Table 3.5: Effect of high light treatment (3600 μmol m^{-2} s^{-1}), of chloroplasts isolated from leaves, at different temperature for 6 hour on their light saturated photosynthetic electron transport activity. The photosynthetic photon flux density during assay was 1200 μmol m^{-2} s^{-1}. The reaction rates are expressed as μmol Oxygen consumed or evolved mg chlorophyll ml^{-1} $hour^{-1}$. The chlorophyll concentration in the reaction mixture was 0.05 mg. Mean±SD values were calculated from 4-6 experiments.

ASSAY	10°C						30°C					50°C				
	0 min	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min	15 min	30 min	60 min	90 min	120 min
PSI+PSII																
H_2O to FeCN (Coupled)	55 ± 7	64 ± 8	34 ± 5	13 ± 3	0	0	50 ± 7	41 ± 5	24 ± 3	9 ± 1	0	0	0	0	0	0
H_2O to FeCN (Uncoupled)	104 ± 10	61 ± 8	41 ± 5	19 ± 3	8 ± 1	0	89 ± 8	62 ± 5	35 ± 4	12 ± 1	2 ± 1	0	0	0	0	0
H_2O to MV (Coupled)	85 ± 8	68 ± 7	28 ± 1	17 ± 2	5 ± 1	0	61 ± 4	30 ± 2	17 ± 1	7 ± 1	0	0	0	0	0	0
H_2O to MV (Uncoupled)	219 ± 17	186 ± 11	103 ± 13	51 ± 4	11 ± 1	8 ± 1	187 ± 10	107 ± 6	83 ± 8	41 ± 5	19 ± 2	0	0	0	0	0
PS II only																
H_2O to PD	120 ± 8	89 ± 7	63 ± 5	32 ± 3	13 ± 1	0	83 ± 5	62 ± 5	37 ± 4	22 ± 2	3 ± 1	0	0	0	0	0
DPC to MV	42 ± 5	40 ± 5	32 ± 4	29 ± 5	26 ± 4	23 ± 3	41 ± 4	40 ± 5	38 ± 3	40 ± 4	37 ± 3	40 ± 4	32 ± 5	29 ± 4	30 ± 3	27 ± 3
PS I only																
DCIP to MV ASC	260 ± 18	272 ± 20	263 ± 15	250 ± 18	221 ± 13	200 ± 13	25 2± 12	250 ± 18	228 ± 15	221 ± 17	210 ± 17	269 ± 15	253 ± 12	238 ± 20	218± 18	209 ± 12
TMPD to MV ASC	315 ± 22	298 ± 29	286 ± 21	267 ± 27	248 ± 17	233 ± 25	320 ± 28	271 ± 21	284 ± 23	255 ± 21	265 ± 22	308 ± 23	311 ± 22	297 ± 20	243 ± 18	230 ± 19

0 h represent controls. Isolated chloroplasts kept at respective temperature for 120 min were used as control

Figure 3.7 show comparative changes in PS II activity, assayed as H_2O to PD and PS II minus water oxidation, assayed as DPC to MV, in photoinhibited leaves (Fig. 3.7A, B & C) and photoinhibited chloroplasts (Fig. 3.7D, E & F). The figure show a much greater decrease in PS II activity in photoinhibited chloroplasts than seen in photoinhibited leaves.

3.3 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES ON CHLOROPHYLL FLUORESCENCE:

Chlorophyll fluorescence, at room temperature, was used as a tool to study the effect of photoinhibition on primary reaction in PS II in intact leaves and isolated chloroplasts. Effect of high light (3600 μmol m^{-2} s^{-1}) at different temperature 5, 30 and 50°C was studied on Fv/Fm ratio, photochemical quenching (qP) and non photochemical quenching (qN) to find out the extent of absorbed energy being utilised for photochemical reaction and dissipated as a non radaitive dissipation under our experimental conditions, with a view to correlate the extent of qN with Xanthophyll Cycle. To be able to correlate the qN with Xanthophyll Cycle we stimulated the Xanthophyll Cycle by feeding the leaves with ascorbate (5 mM; pH 6.8) and inhibited the Xanthophyll Cycle by feeding Dithiothreitol (4 mM) and observed the changes in qN under these conditions. Plants fed with distilled water were used as control.

3.3.1 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES FED WITH DISTILLED WATER ON CHLOROPHYLL FLUORESCENCE:

Room temperature chlorophyll fluorescence measurements (Fv/Fm ratio, qP and qN) of leaves photoinhibited at 3600 μmol m^{-2} s^{-1} at 5, 30 and 50°C temperature for a duration of 0 to 6 hours is shown in table 3.6. It was observed that with the increase in duration of photoinhibitory treatment the Fv/Fm ratio decreased linearly. The decrease in the Fv/Fm ratio was greater in the leaves photoinhibited at 5°C than at 30°C or 50°C. Photoinhibition for 1 hour at 5°C resulted in a 17% decrease in Fv/Fm ratio followed by a decrease of 30% afetr 2 hour, 38% after 3 hour, 45 % after 4 hour, 52% after 5 hour and 61% after 6 hour of the photoinhibitory treatment, as compared to the control (Fig. 3.8A). However, same light treatment at 30°C resulted in a decrease of only 5% in Fv/Fm ratio after 1 hour of photoinhibition treatment and 46% after 6 hour of the treatment (Fig. 3.8B). The photoinhibition of leaves at 50°C resulted in greater decrease than observed at 30°C in Fv/Fm ratio. The 6 hour photoinhibition treatment caused 56% decrease in the Fv/Fm ratio (Fig. 3.8C) compared to control. Control leaves were kept at growth conditions.

Photochemical quenching (qP) showed a linear decrease when leaves fed with distil water and photoinhibited at 5°C (Fig. 3.8A). The six hour treatment caused a decrease of 47%, as compared to the control. However, photoinhibitory treatment at 30°C (Fig. 3.8B) and 50°C (Fig. 3.8C) resulted in an initial increase in qP. A one hour of photoinhibition treatment caused a increase of 1% in leaves treated at 30°C 18% increase in leaves treated at 50°C compared to the control. However, the initial increase

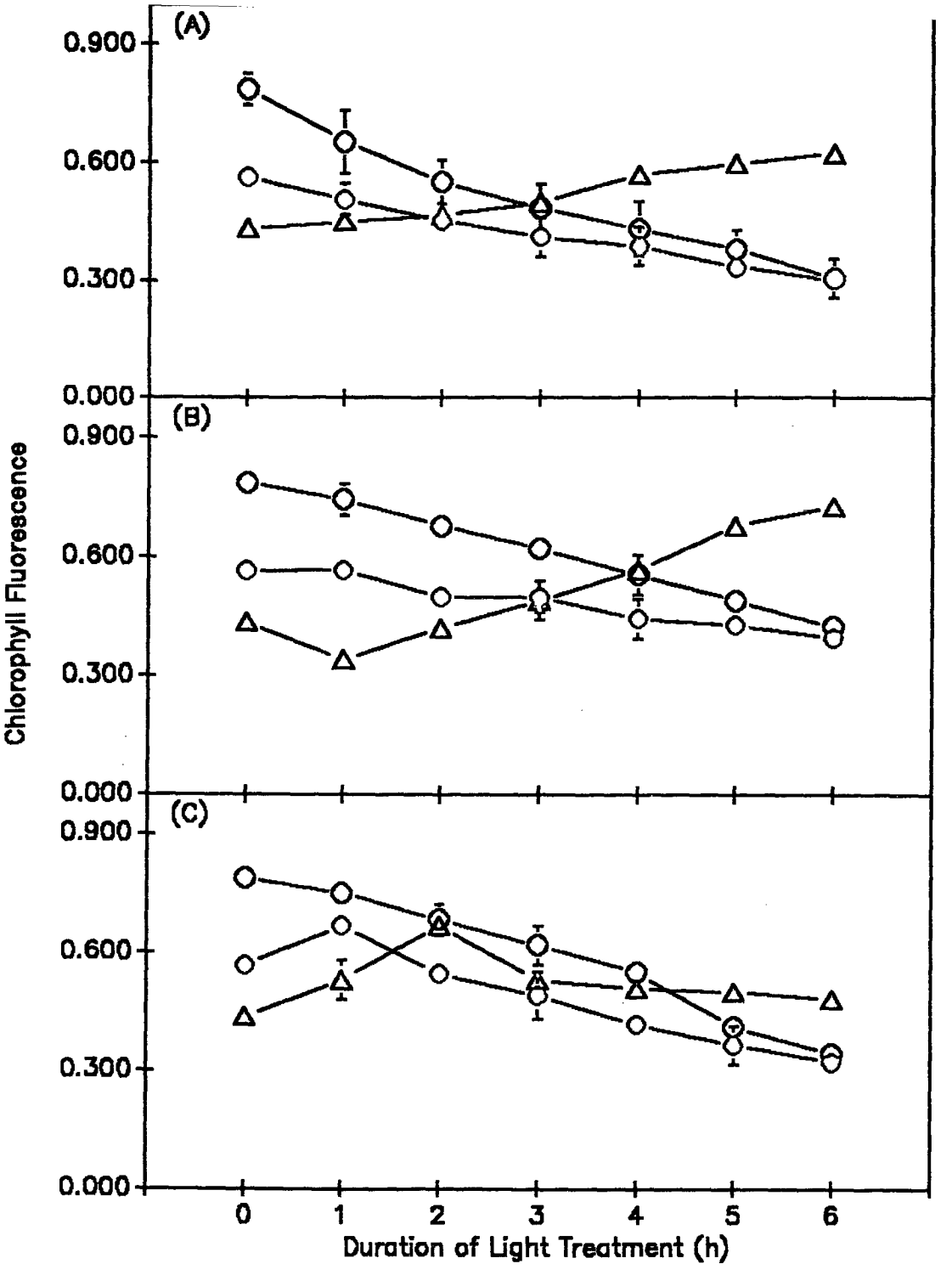


Fig. 3.8: Effect of photoinhibition of intact leaves fed with distilled water at 5°C (A), 30°C (B) and 50°C (C) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR on Fv/Fm ratio (-o-), photochemical quenching (-o-) and non-photochemical quenching (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

in qP declined with increase in the duration of photoinhibition treatment. Six hour photoinhibitory treatment of intact leaves at 30°C resulted in a decrease of 30% while, six hour treatment at 50°C resulted in a decrease of 43%, compared to the control.

Non-photochemical quenching showed variable changes due to the photoinhibitory treatment. Leaves fed with distilled water and photoinhibited at 3600 μmol m^{-2} s^{-1} at 5°C showed a linear increase in the qN (Fig. 3.8A). Six hour photoinhibition treatment at 5°C caused a 44% increase in qN as compared to control. However, leaves photoinhibited at 30°C showed an initial decrease in qN for first 2 hour of the treatment followed by an increase (Fig. 3.8B). The qN decreased by 21 % after one hour and 3% after 2 hour of the of photoinhibition treatment at 30°C followed by an increase of 13% after 3 hour and 68% after 6 hour of the treatment, as compared to the control. The photoinhibition at 50°C also resulted in a initial increase in qN as was also seen when leaves were photoinhibited at 30°C. It was observed that one hour of photoinhibition treatment caused an increase of 22% which was further increased to 53% after 2 hour of the photoinhibition treatment. Further increase in the duration of photoinhibitory treatment caused a slight decrease in the qN as compared to the increase observed in qN after 2 hour of the photoinhibition treatment but remain higher than control. Six hour of the photoinhibitory treatment resulted in 11% increase in qN, as compared to control (Fig. 3.8C).

3.3.2 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES FED WITH ASCORBIC ACID ON CHLOROPHYLL FLUORESCENCE:

The leaves were fed with ascorbic acid (5 mM, pH 6.8) with an objective to stimulate the Xanthophyll Cycle since ascorbic acid facilitates the de-epoxidation of Violaxanthin to Zeaxanthin, which in turn provide protection against photodamage by regulating light energy to the photosynthetic reaction centre and to correlate the changes in Xanthophylls with chlorophyll fluorescence, specially the qN.

Leaves were also fed with ascorbic acid and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 5, 30 and 50°C for duration of 6 hour. Results obtained are shown in table 3.6. The Fv/Fm ratio declined linearly in leaves photoinhibited at 5, 30 or 50°C. Six hour light treatment at 5°C resulted in 61% decrease in the Fv/Fm ratio, as compared to control (Fig. 3.9A), while the light treatment at 30°C (Fig. 3.9B) and 50°C (Fig. 3.9C) for the same duration resulted in a decrease of 42% and 59% in the Fv/Fm ratio.

Photochemical quenching declined in leaves treated with high light 3600 μmol m^{-2} s^{-1} PAR at 5°C (Fig. 3.9A). One hour treatment caused a decrease of 3% in qP, as compared to the control, followed by a decrease of 22% after 3 hour of the treatment and 40% decrease after 6 hour the of treatment. The 1-2 hour light treatment at 30°C however, caused an initial increase in qP upto 9%. Longer duration of treatment, however, resulted in a decline in qP. Six hours light treatment at 30°C decreased the qP by 27%, as compared to the control (Fig. 3.9B). High light treatment at 50°C also resulted in a slight increase in qP after one hour of treatment (2%) followed by a linear decline. The decrease in qP after 6 hour of the photoinhibition treatment was 41% as

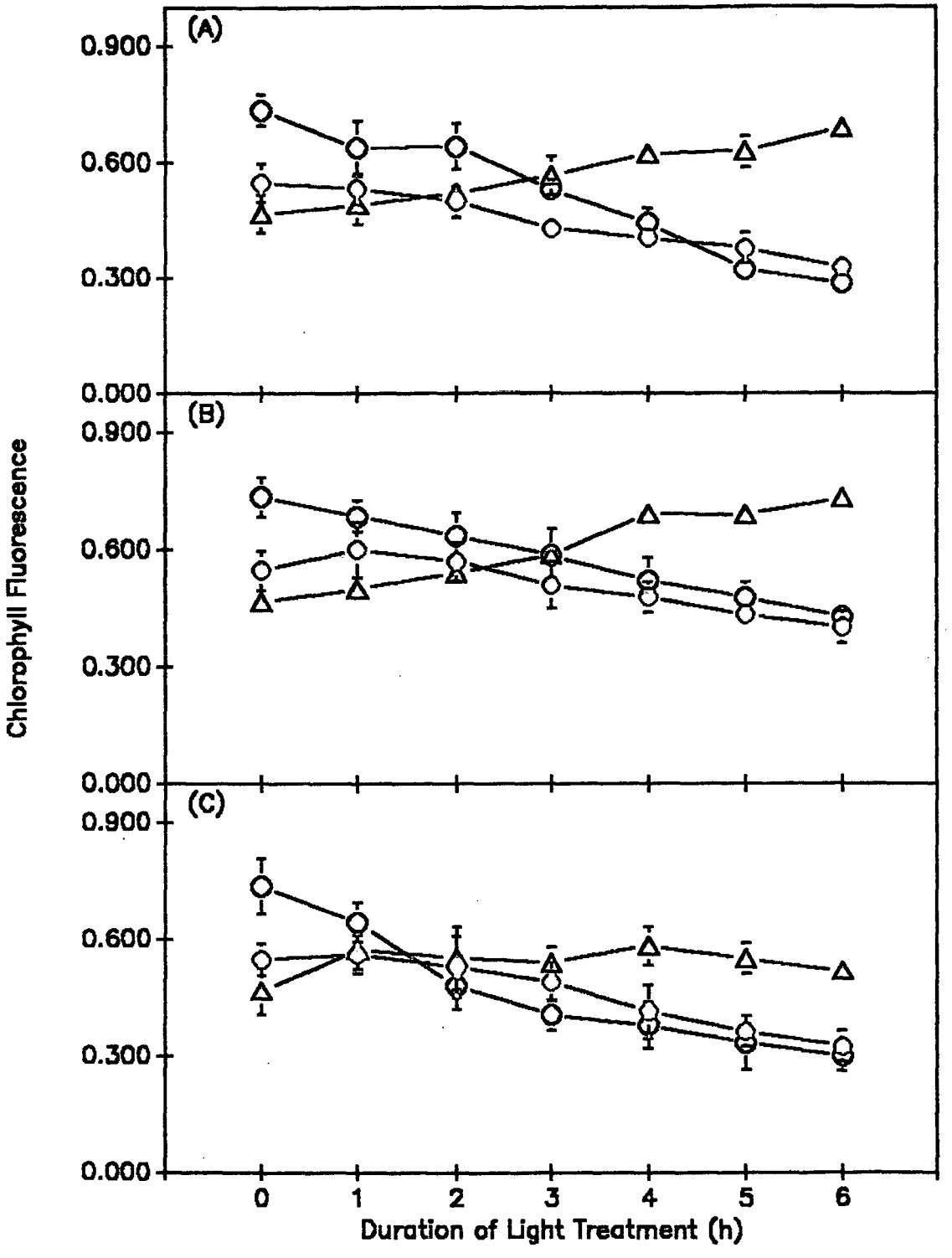


Fig. 3.9: Effect of photoinhibition of intact leaves fed with 5 mM ascorbic acid at 5°C (A), 30°C (B) and 50°C (C) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR on Fv/Fm ratio (-o-), photochemical quenching (-o-) and non-photochemical quenching (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

compared to the control. This was greater than observed in plants treated at 30°C but more or less same when compared to the light treatment at 5°C for the same duration (Table 3.6).

Non-photochemical quenching (qN) increased in plants fed with ascorbate and subsequently photoinhibited at 3600 μmol m^{-2} s^{-1} irrespective of the temperature of the treatment (Table 3.6). Maximum increase in qN was seen in leaves photoinhibited at 30°C for 6 hour (57% increase as compared to control; Fig. 3.9B) followed by photoinhibitory treatment for the same duration at 5°C (47% increase compared to the control; Fig. 3.9A). The photoinhibitory treatment at 50°C initially caused an increase in the qN (22% after 1 hour and 14% after 2 hour of the treatment, as compared to control) but the increase in the qN was relatively less with increase in the duration of the photoinhibition (Fig. 3.9C).

3.3.3 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES FED WITH DITHIOTHREITOL ON CHLOROPHYLL FLUORESCENCE:

Dithiothreitol affect the de-epoxidation of V to Z without having an effect on photosynthesis. The study was carried out with an objective to see the effect of switching off the Xanthophyll Cycle on qN. The leaves were fed with dithiothreitol (4 mM) and photoinhibited at 3600 μmol m^{-2} s^{-1} and 5, 30 and 50°C temperature for 6 hours (Table 7.6).

The Fv/Fm ratio decreased linearly in leaves photoinhibited at either 5, 30 or 50°C compared to control. However, the decrease in Fv/Fm ratio at 50°C was greater

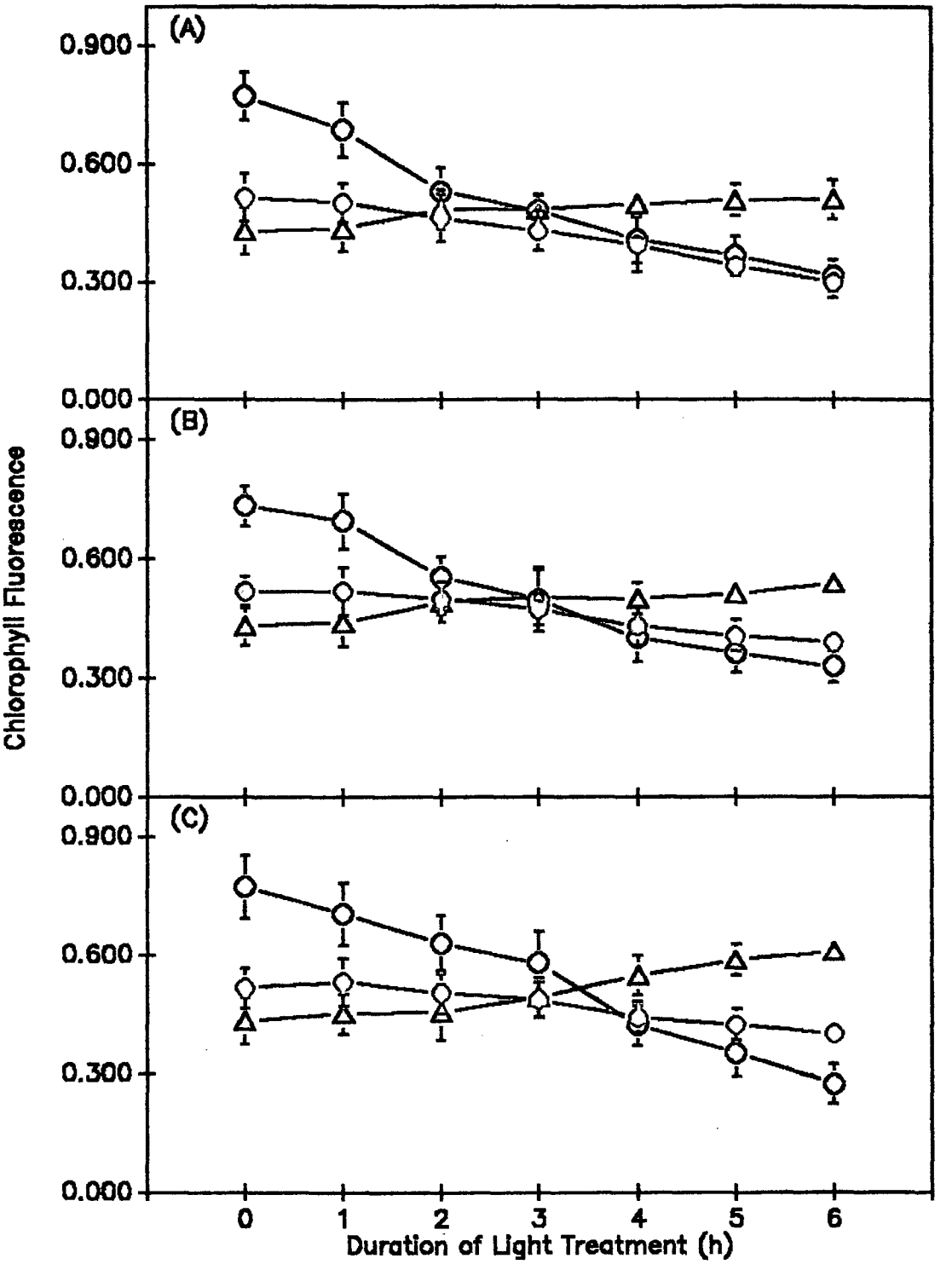


Fig. 3.10: Effect of photoinhibition of intact leaves fed with 5 mM DTT at 5°C (A), 30°C (B) and 50°C (C) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR on Fv/Fm ratio (-o-), photochemical quenching (-o-) and non-photochemical quenching (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

than observed at 5°C or 30°C (Table 3.6). Treatment at 50°C for 6 hour resulted in 65% decrease in the Fv/Fm ratio compared to control (Fig. 3.10C). This decrease in Fv/Fm was 8% greater (57% compared to the control) than observed at 30°C (Fig. 3.10B) and 6% more (59% compared to control) than seen in leaves photoinhibited at 5°C for the same duration (Fig. 3.10A).

Photochemical quenching declined linearly in leaves photoinhibited at 3600 μmol m^{-2} s^{-1} at 5°C (Fig. 3.10A). Six hour light treatment resulted in a decrease of 42% as compared to control. Photoinhibition at 30°C also declined linearly to a level of 24% after 6 hour of the treatment, as compared to the control (Fig. 3.10B). However, photoinhibition at 50°C caused slight increase in qP after one hour of the treatment (3% compared to control) followed by a linear decrease to the level of 23% after 6 hour of the treatment (Fig. 3.10C). The results indicate maximum decrease in qP when leaves were treated at 5°C compared to 30°C and 50°C.

Non-photochemical quenching increased linearly when leaves photoinhibited at 3600 μmol m^{-2} s^{-1} PAR irrespective of the temperature of the photoinhibitory treatment. It was seen that photoinhibition for 6 hours at 50°C (Fig. 3.10C) caused increase in qN (4%) compared to the control, while increase of 25% and 18% was seen in leaves photoinhibited at 30°C (Fig. 3.10B) and 5°C (Fig. 3.10A) respectively, for the same duration.

If we compare the result of leaves fed with H_2O, ascorbate and dithiothreitol at different temperature on non-photochemical quenching it was seen that leaves fed with DTT and photoinhibited at different temperature showed minimum increase in the qN

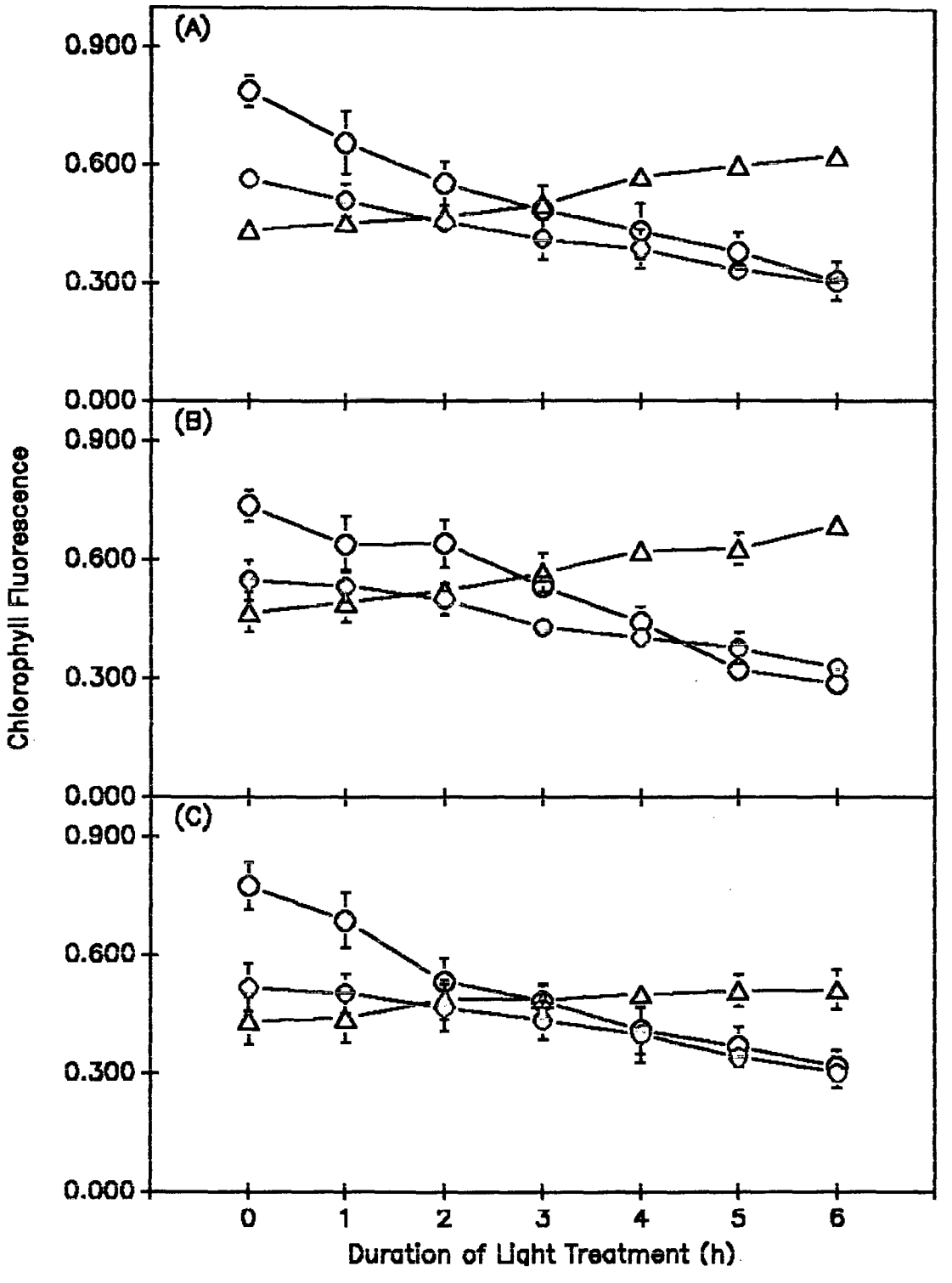


Fig. 3.11: Effect of photoinhibition of intact leaves at 5°C and 3600 μmol m^{-2} s^{-1} PAR on Fv/Fm ratio (-o-), photochemical quenching (-o-) and non-photochemical quenching (-Δ-) in leaves pretreated with H_2O (A), 5 mM Ascorbic acid (B) and 4 mM DTT (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

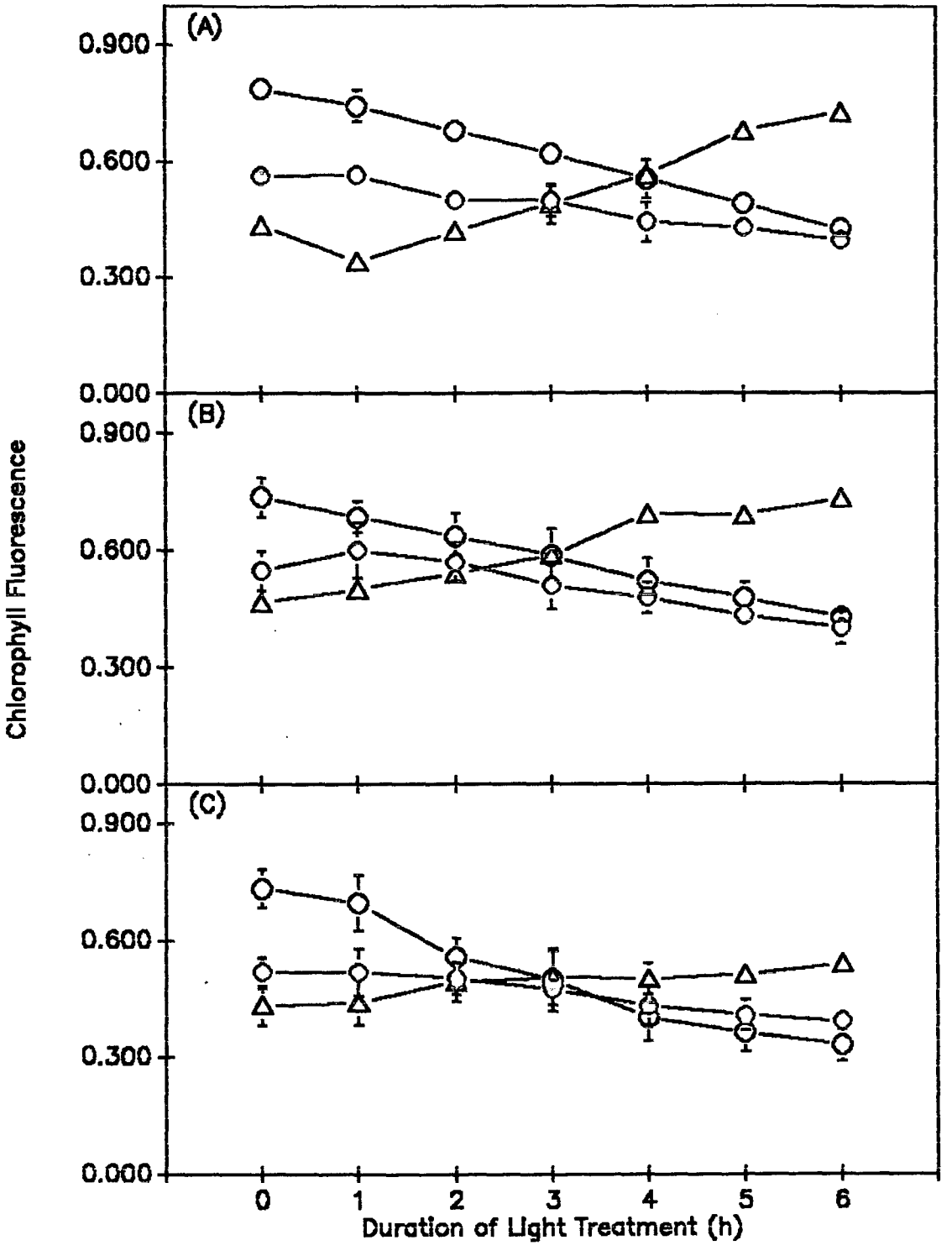


Fig. 3.12: Effect of photoinhibition of intact leaves at 30°C and 3600 μmol m^{-2} s^{-1} PAR on Fv/Fm ratio (-o-), photochemical quenching (-o-) and non-photochemical quenching (-Δ-) in leaves pretreated with H_2O (A), 5 mM Ascorbic acid (B) and 4 mM DTT (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

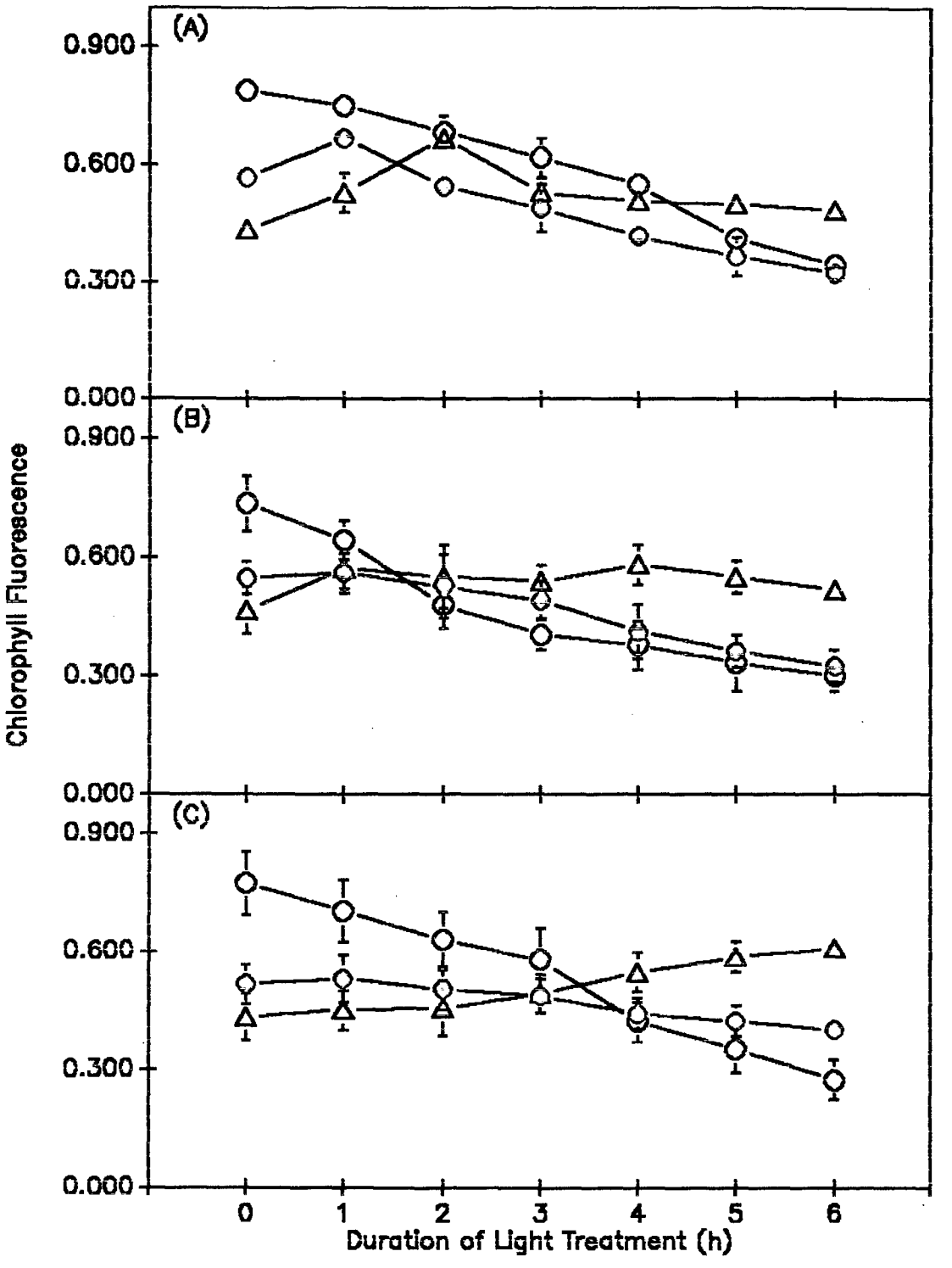


Fig. 3.13: Effect of photoinhibition of intact leaves at 50°C and 3600 μmol m^{-2} s^{-1} PAR on Fv/Fm ratio (-o-), photochemical quenching (-o-) and non-photochemical quenching (-Δ-) in leaves pretreated with H_2O (A), 5 mM Ascorbic acid (B) and 5 mM DTT (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

le 3.6: Room temperature steady state chlorophyll fluorescence in leaves fed with distilled water, ascorbic acid and DTT photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 5, 30 and 50°C. The Fv/Fm ratio, qP and qN were calculated according to eiber (1986). Mean±SD values were calculated from 4-6 experiments.

	H_2O								
	5°C			30°C			50°C		
Duration of teatment (h	Fv/Fm	qP	qN	Fv/Fm	qP	qN	Fv/Fm	qP	qN
0	0.787 ±0.04	0.564 ±0.04	0.435 ±0.02	0.787 ±0.02	0.564 ±0.015	0.434 ±0.04	0.787 ±0.035	0.564± 0.02	0.435 ±0.035
1	0.656 ±0.08	0.509 ±0.04	0.452 ±0.02	0.744 ±0.04	0.567 ±0.035	0.341 ±0.02	0.75 ±0.02	0.667 0.035	0.529 ±0.05
2	0.553 ±0.06	0.455 ±0.02	0.467 ±0.04	0.679 ±0.02	0.5 ±0.02	0.42 ±0.02	0.683 ±0.04	0.544± 0.02	0.667 ±0.03
3	0.486 ±0.06	0.411 ±0.05	0.5 ±0.02	0.622 ±0.03	0.5 ±0.04	0.491 ±0.05	0.618 ±0.05	0.489± 0.06	0.53 ±0.04
4	0.432 ±0.07	0.387 ±0.05	0.571 ±0.03	0.556 ±0.05	0.444 ±0.05	0.567 ±0.02	0.55 ±0.02	0.417± 0.03	0.508 ±0.04
5	0.380 ±0.05	0.333 ±0.02	0.60 ±0.01	0.491 ±0.03	0.429 ±0.02	0.68 ±0.03	0.412 ±0.035	0.365± 0.05	0.5 ±0.02
6	0.304 ±0.05	0.301 ±0.02	0.625 ±0.02	0.425 ±0.01	0.396 ±0.02	0.727 ±0.02	0.344 ±0.02	0.322± 0.02	0.483 ±0.03

	ASC									DTT								
	5°C			30°C			50°C			5oC			30oC			50°C		
Fv/Fm	qP	qN	Fv/Fm	qP	qN	Fv/Fm	qP	qN	Fv/Fm	qP	qN	Fv/Fm	qP	qN	Fv/Fm	qP	qN
0.736 ±0.04	0.548 ±0.05	0.467 ±0.05	0.736 ±0.05	0.548 ±0.05	0.467 ±0.05	0.736 ±0.07	0.548 ±0.04	0.467 ±0.06	0.773 ±0.06	0.517 ±0.06	0.432 ±0.06	0.773 ±0.05	0.517 ±0.04	0.432 ± 0.05	0.773 ±0.08	0.517 ±0.05	0.432 ±0.06
0.638 ±0.07	0.523 ±0.04	0.491 ±0.05	0.686 ±0.04	0.60 ±0.07	0.5 ±0.06	0.644 ±0.05	0.561 ±0.05	0.572 ±0.05	0.688 ±0.07	0.503 ±0.05	0.438 ±0.06	0.695 ±0.07	0.518 ±0.06	0.44 ±0.06	0.703 ±0.08	0.532 ±0.06	0.451 ±0.05
0.642 ±0.06	0.5 ±0.04	0.522 ±0.04	0.636 ±0.06	0.57 ±0.05	0.54 ±0.08	0.479 ±0.06	0.528 ±0.08	0.553 ±0.08	0.532 ±0.06	0.465 ±0.06	0.487 ±0.05	0.533 ±0.05	0.50 ±0.04	0.492 ±0.05	0.63 ±0.07	0.503 ±0.05	0.455 ±0.07
0.531 ±0.04	0.429 ±0.02	0.567 ±0.05	0.586 ±0.07	0.509 ±0.06	0.588 ±0.05	0.405 ±0.04	0.491 ±0.05	0.540 ±0.04	0.483 ±0.04	0.433 ±0.03	0.486 ±0.04	0.498 ±0.08	0.473 ±0.04	0.503 ±0.07	0.579 ±0.08	0.487 ±0.05	0.493 ±0.05
0.442 ±0.04	0.403 ±0.04	0.622 ±0.03	0.522 ±0.06	0.479 ±0.04	0.693 ±0.04	0.378 ±0.06	0.413 ±0.07	0.583 ±0.05	0.409 ±0.06	0.397 ±0.07	0.5 ±0.03	0.401 ±0.06	0.431 ±0.05	0.50 ±0.04	0.421 ±0.05	0.442 ±0.04	0.548 ±0.05
0.322 ±0.02	0.377 ±0.04	0.627 ±0.04	0.478 ±0.04	0.433 ±0.03	0.691 ±0.05	0.333 ±0.07	0.363 ±0.04	0.551 ±0.04	0.367 ±0.03	0.341 ±0.04	0.509 ± 0.04	0.363 ±0.05	0.407 ±0.04	0.512 ±0.03	0.351 ±0.06	0.423 ±0.04	0.587 ±0.04
0.286 ±0.03	0.327 ±0.04	0.687 ± 0.04	0.429 ±0.03	0.401 ±0.04	0.733 ±0.04	0.30 ±0.04	0.325 ±0.04	0.519 ± 0.04	0.317 ±0.04	0.300± 0.40	0.511 ±0.05	0.329 ±0.04	0.391 ±0.02	0.539 ±0.04	0.273 ±0.05	0.400 ±0.02	0.609 ±0.04

as a result of photoinhibitory treatment compared to plants fed with ascorbate or H_2O. Leaves fed with ascorbate and subsequently photoinhibited at different temperatures showed largest increase in qN as compared to leaves fed with dithiothreitol or H_2O (Fig. 3.11, 3.12 & 3.13).

3.4 EFFECT OF PHOTOINHIBITION OF CHLOROPLASTS ISOLATED FROM NORMAL LEAVES ON CHLOROPHYLL FLUORESCENCE:

Isolated chloroplasts were photoinhibited at 3600 μmol m^{-2} s^{-1} photoinhibitory light at different temperature of 5, 30 and 50°C to study the effect when no cellular or recovery mechanism was functional or functional at a low level. This was carried out to observe the extent of photoinhibitoty damage on Fv/Fm ratio, photochemical quenching and non-photochemical quenching. It was seen that the extent of damage to various parameters of chlorophyll fluorescence was much rapid and greater in photoinhibition of isolated chloroplasts, as compared to seen in photoinhibition of intact leaves. Photoinhibitory treatment at 5°C was more damaging than seen for the same photoinhibitory treatment at 30°C or 50°C (Table 3.7).

Fv/Fm ratio decreased linearly when chloroplasts were photoinhibited at all three temperature (5, 30 and 50°C). Six hour photoinhibition treatment at 5°C for 60 min resulted in a 83% decrease in the Fv/Fm ratio (Fig. 3.14A) 48% decrease observed in chloroplasts photoinhibited at 30°C (Fig. 3.14B) and 73% decrease observed when chloroplasts were photoinhibited at 50°C (Fig. 3.14C) compared to control. This decrease in Fv/Fm ratio was much greater than observed when intact leaves were photoinhibited at respective temperature (Fig. 3.15).

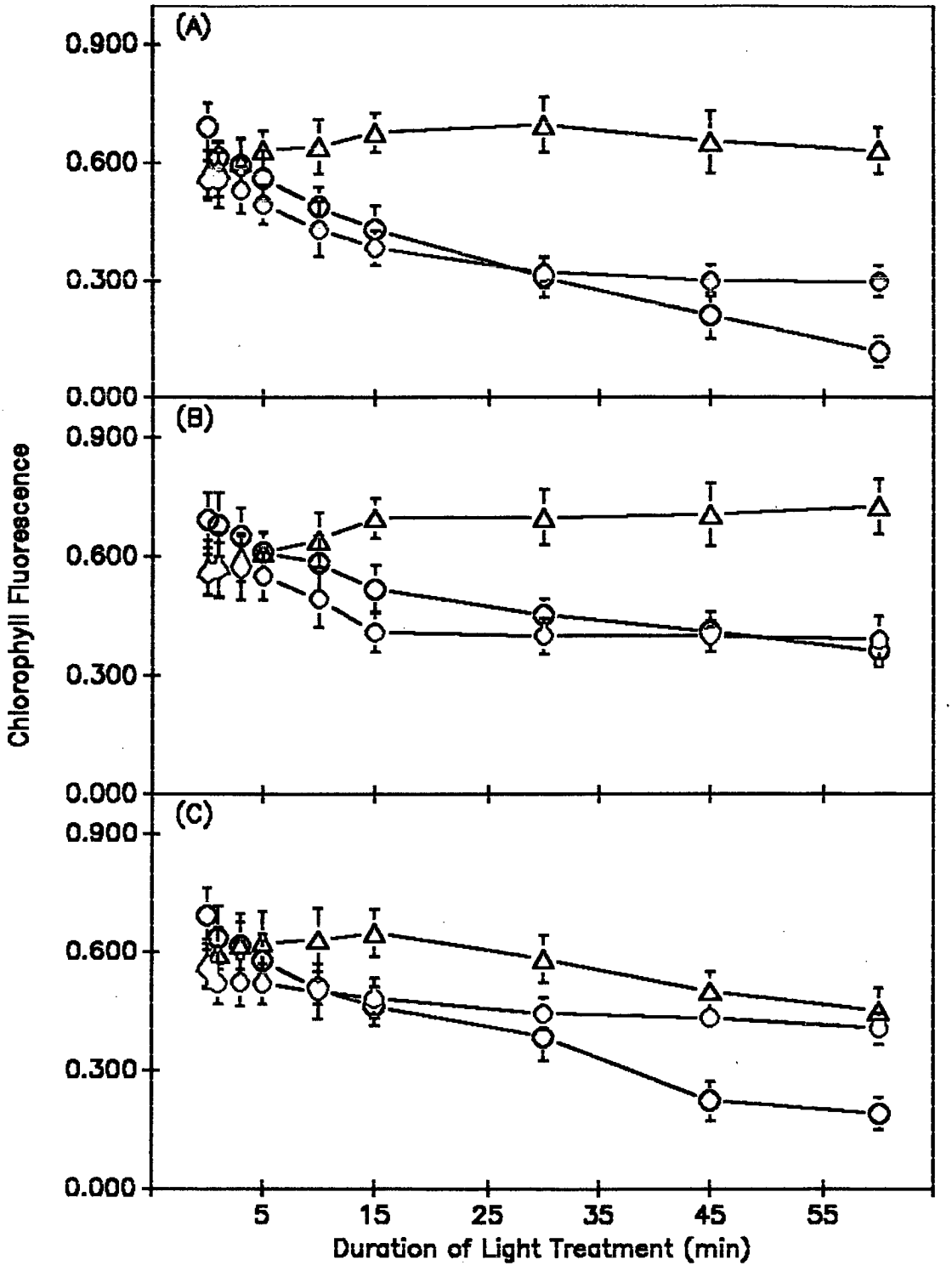


Fig. 3.14: Effect of photoinhibition of isolated chloroplasts at 5°C (A), 30°C (B) and 50°C (C) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR on Fv/Fm ratio (-o-), photochemical quenching (-o-) and non-photochemical quenching (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

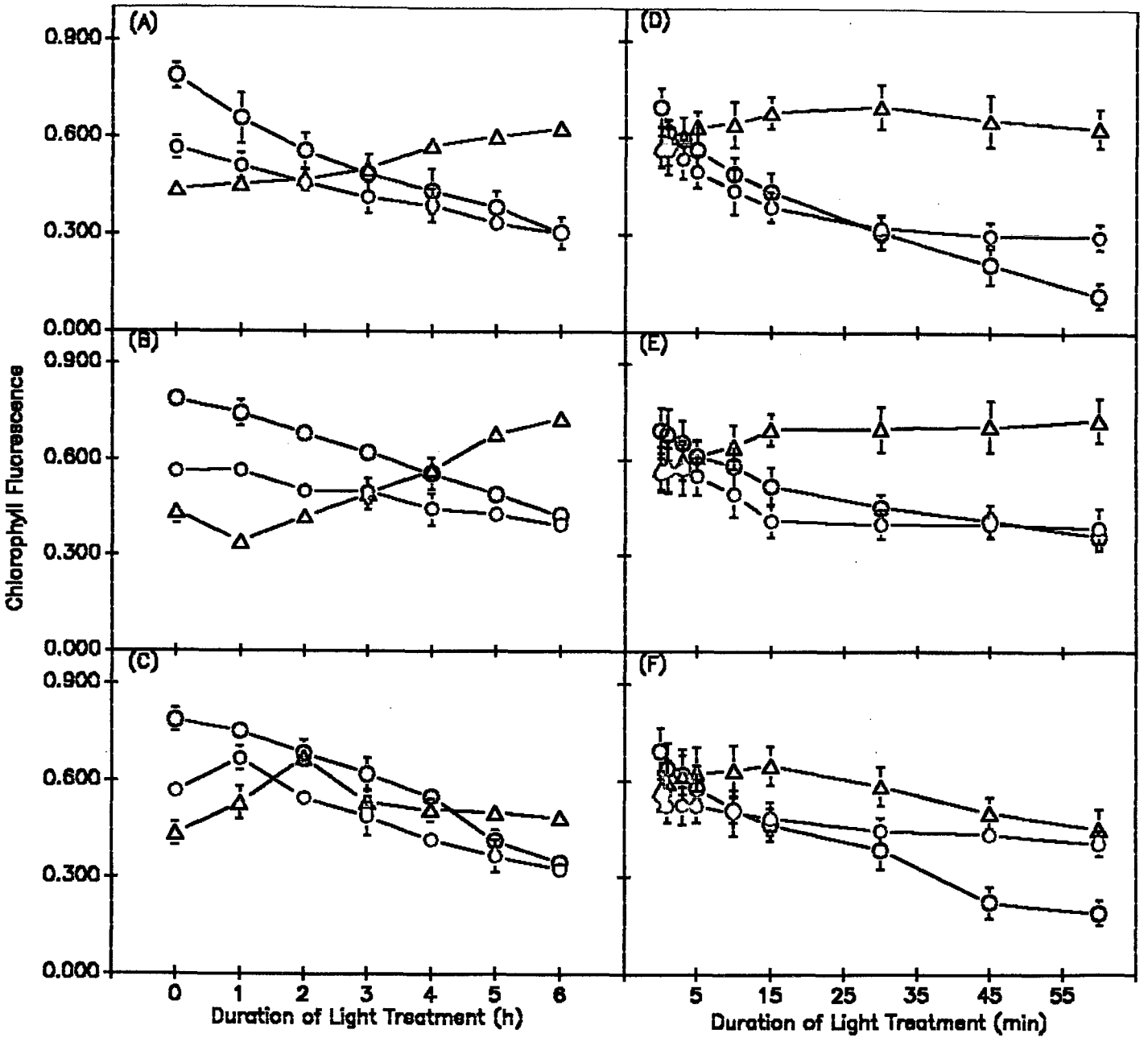


Fig. 3.15: Effect of photoinhibition of intact leaves (A, B & C) and isolated chloroplasts (D,E & F) at 5°C (A & D), 30°C (B & E) and 50°C (C & F) on Fv/Fm ratio (-o-), photochemical quenching (-o-) an non-photochemical quenching (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

Table 3.7: Room temperature steady state chlorophyll fluorescence in chloroplasts isolated from leaves fed with distilled water, ascorbic acid and DTT and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 5, 30 and 50°C. The Fv/Fm ratio, qP and qN were calculated according to Schreiber (1986). Mean±SD values were calculated from 4-6 experiments.

Duration of treatment (h)	5°C			30°C			50°C		
	Fv/Fm Ratio	qP	qN	Fv/Fm Ratio	qP	qN	Fv/Fm Ratio	qP	qN
0	0.692± 0.06	0.556± 0.05	0.571± 0.06	0.692± 0.07	0.556± 0.05	0.571± 0.07	0.692± 0.07	0.556± 0.05	0.571± 0.06
1	0.615± 0.04	0.556± 0.07	0.583± 0.07	0.680± 0.08	0.567± 0.07	0.579± 0.08	0.636± 0.08	0.519± 0.05	0.593± 0.07
3	0.593± 0.07	0.532± 0.06	0.601± 0.06	0.653± 0.07	0.571± 0.08	0.598± 0.06	0.617± 0.06	0.522± 0.06	0.617± 0.08
5	0.562± 0.07	0.495± 0.05	0.632± 0.05	0.612± 0.05	0.550± 0.06	0.611± 0.05	0.577± 0.07	0.519± 0.05	0.623± 0.08
10	0.489± 0.05	0.433± 0.07	0.641± 0.07	0.583± 0.07	0.493± 0.07	0.642± 0.07	0.509± 0.04	0.500± 0.07	0.631± 0.08
15	0.432± 0.06	0.385± 0.05	0.678± 0.05	0.519± 0.06	0.410± 0.05	0.697± 0.05	0.463± 0.05	0.483± 0.05	0.648± 0.06
30	0.309± 0.05	0.322± 0.04	0.697± 0.07	0.453± 0.04	0.400 ±0.05	0.699± 0.07	0.385± 0.06	0.444± 0.04	0.573± 0.06
45	0.210± 0.06	0.300± 0.04	0.653± 0.08	0.411± 0.05	0.400± 0.04	0.707± 0.08	0.222± 0.05	0.433± 0.03	0.500± 0.05
60	0.117± 0.04	0.297± 0.04	0.632± 0.06	0.362± 0.04	0.391± 0.06	0.728± 0.07	0.190± 0.04	0.405± 0.04	0.450± 0.06

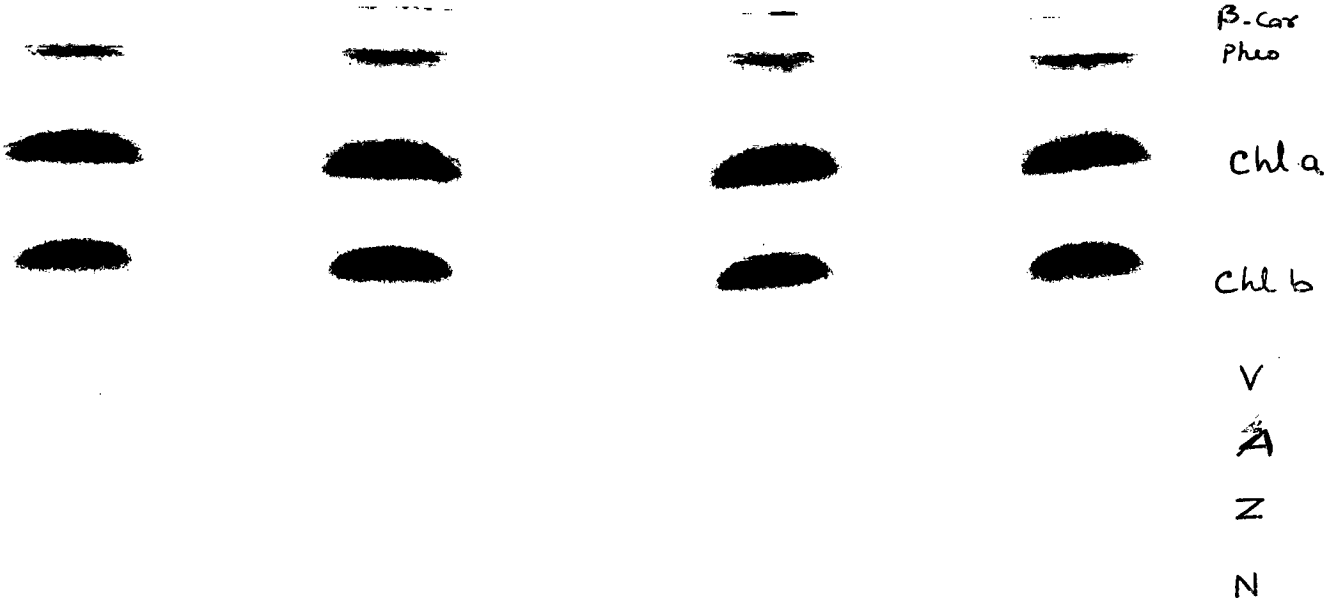
0 h represent control. Chloroplasts kept for 6 h at respective temperature in the dark were used as control

Photochemical quenching also declined when chloroplasts photoinhibited at 3600 μmol m^{-2} s^{-1} PAR irrespective of the temperature. However, maximum decrease in qP was seen in chloroplasts photoinhibited at 5°C. A 60 min photoinhibitory treatment at 5°C and 3600 μmol m^{-2} s^{-1} PAR resulted in a 47% decrease in qP compared to that of the control (Fig. 3.14A). The decrease in qP in chloroplast photoinhibition at 5°C was much greater than observed in chloroplasts photoinhibited at 30°C (3% decrease as compared to control; Fig. 3.14B) or at 50°C 27% decrease compared to control when photoinhibited for the same duration (Fig. 3.14C).

Non-photochemical quenching increased in chloroplasts photoinhibited at 3600 μmol m^{-2} s^{-1} at 5°C or 30°C. However, photoinhibitory treatment at 50°C resulted in 13% increase in qN when photoinhibited for 15 min followed by decrease of 21% after 60 min of treatment, as compared to control (Fig. 3.14C). The photoinhibitory treatment at 5°C caused maximum 22% increase in qN after 30 min of the treatment followed by a slight decrease when chloroplasts were treated for longer duration (Fig. 3.14A). Only 11% increase in qN was observed when chloroplasts were photoinhibited at 30°C for 3 hours compared to the control, however, qN increased to 27% after 60 min of the photoinhibition treatment compared to that of control (Fig. 3.14B).

3.5 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES PRE TREATED WITH ASCORBATE AND DITHIOTHREITOL ON CAROTENOID CONTENT.

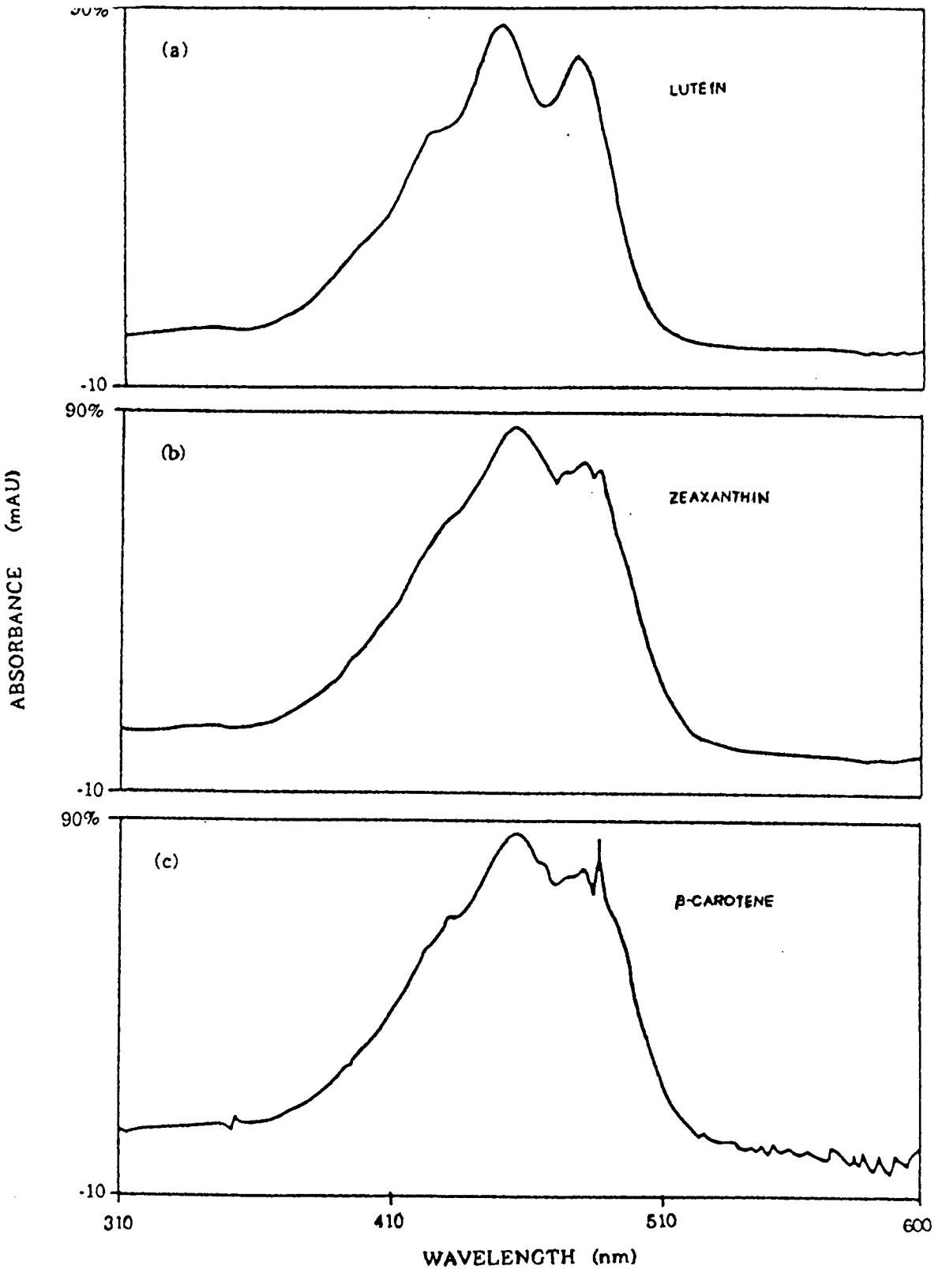
Changes in carotenoid content were studied in order to find out the influence of high light on Xanthophyll Cycle under conditions, which stimulate and inhibit the


β-Car
Pheo
Chl a
Chl b
V
A
Z
N

Xanthophyll Cycle in order to elucidate its role in protection of plants against excess light through energy dissipation. Results are shown in table 3.8 and 3.9. It was seen that leaves pre treated with ascorbate and subsequently photoinhibited at 3600 μmol m^{-2} s^{-1} significantly promoted oxidation of V to Z (table 3.9). However, leaves treated with DTT did not promote de-epoxidation of V to Z. Leaves fed with distilled water also promoted de-epoxidation of V to Z. The results indicate that V, A, Z, Neoxanthin and β-carotene responded significantly to the high light treatment at 5, 30 and 50°C, where as lutein and chlorophyll content did not change significantly in response to the photoinhibition treatment. An HPLC profile of analysis of plant pigments is shown in Fig. 3.16. he pigments were identified based on their spectrum (Fig. 3.17A & B) after separating them using TLC (Fig. 3.18).

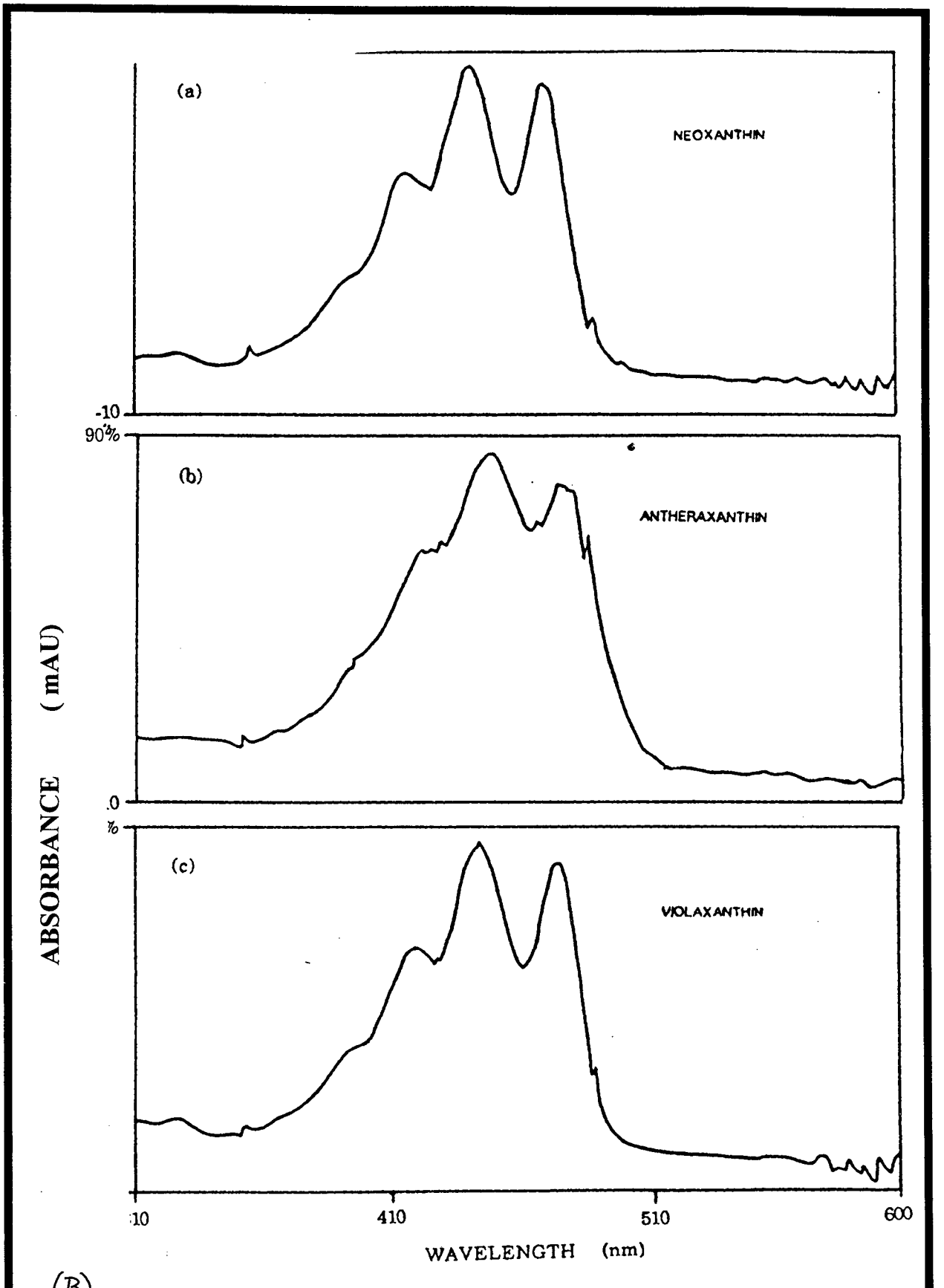
Leaves fed with distilled water and photoinhibited for 6 hours at 5°C showed increase in Neoxanthin content to 22% compared to control. The increase in Neoxanthin content was only 19% when leaves were photoinhibited at 30°C. Photoinhibition treatment at 50°C resulted in 11% increase in the Neoxanthin content as compared to the control (Table 3.8).

Leaves fed with ascorbate and photoinhibited at 3600 μmol m^{-2} s^{-1} also showed change in Neoxanthin content. It was seen that 6 hour light treatment resulted in 28% increase in Neoxanthin in leaves photoinhibited at 30°C with respect to control. The photoinhibition treatment at 50°C, however resulted in a slight (4%) decrease in the Neoxanthin content (Table 3.8). Leaves fed with DTT and subsequently photoinhibited at 3600 μmol m^{-2} s^{-1} 5, 30 and 50°C did not show increase in the Neoxanthin content.



(A)

Fig 3.17 Spectrum of chromatographically recorded individual cartoeniod peaks.



(B)

Fig 3.17 Spectrum of chromatographically recorded individual cartoeniod peaks.

Six hour treatment at 5, 30 and 50°C resulted in a increase of 2%, 0% and 3% respectively (Table 3.8).

Violaxanthin and Zeaxanthin content responded to a greater extent as a result of photoinhibition treatment. Leaves fed with water and photoinhibited at 3600 μmol m^{-2} s^{-1} at 5, 30 and 50°C resulted in significant increase in Zeaxanthin and decrease in Violaxanthin. The increase in Zeaxanthin was greater than the decrease in Violaxanthin. Six hour photoinhibition treatment at 5°C caused 101% decrease in the level of Violaxanthin and 441% increase in the Zeaxanthin content. On the actual content basis the decrease in Violaxanthin content was of 2.08 μg . g FW^{-1} while increase in Zeaxanthin content was 3.18 μg . g FW^{-1}. Antheraxanthin content showed slight decrease after 6 hour of the photoinhibition treatment (Table 3.9). The photoinhibition treatment at 30°C resulted in 190% decrease in Violaxanthin (decrease of 4.22 μg . g FW^{-1}) while Zeaxanthin content rose to 518% (increase of 5.28 μg. g FW^{-1}) as compared to control. Similarly the photoinhibition treatment at 50°C also resulted in de-epoxidation to Violaxanthin to Zeaxanthin. Violaxanthin content decrease by 32% (a decrease of 6.42 μg . g FW^{-1}) and Zeaxanthin content increased by 420% (an increase of 4.18 μg . g FW^{-1}) as compared to control (Table 3.9).

Leaves fed with ascorbate and photoinhibited at 3600 μmol m^{-2} s^{-1} at 5, 30 and 50°C showed greater level of de-epoxidation of V to Z. Even the control leaves (leaves fed with ascorbate but not photoinhibited) showed greater V content than seen in control leaves fed with distilled water or DTT (Table 3.9). Six hour photoinhibition treatment of leaves fed with ascorbate at 5°C resulted in 13% decrease in Violaxanthin content and

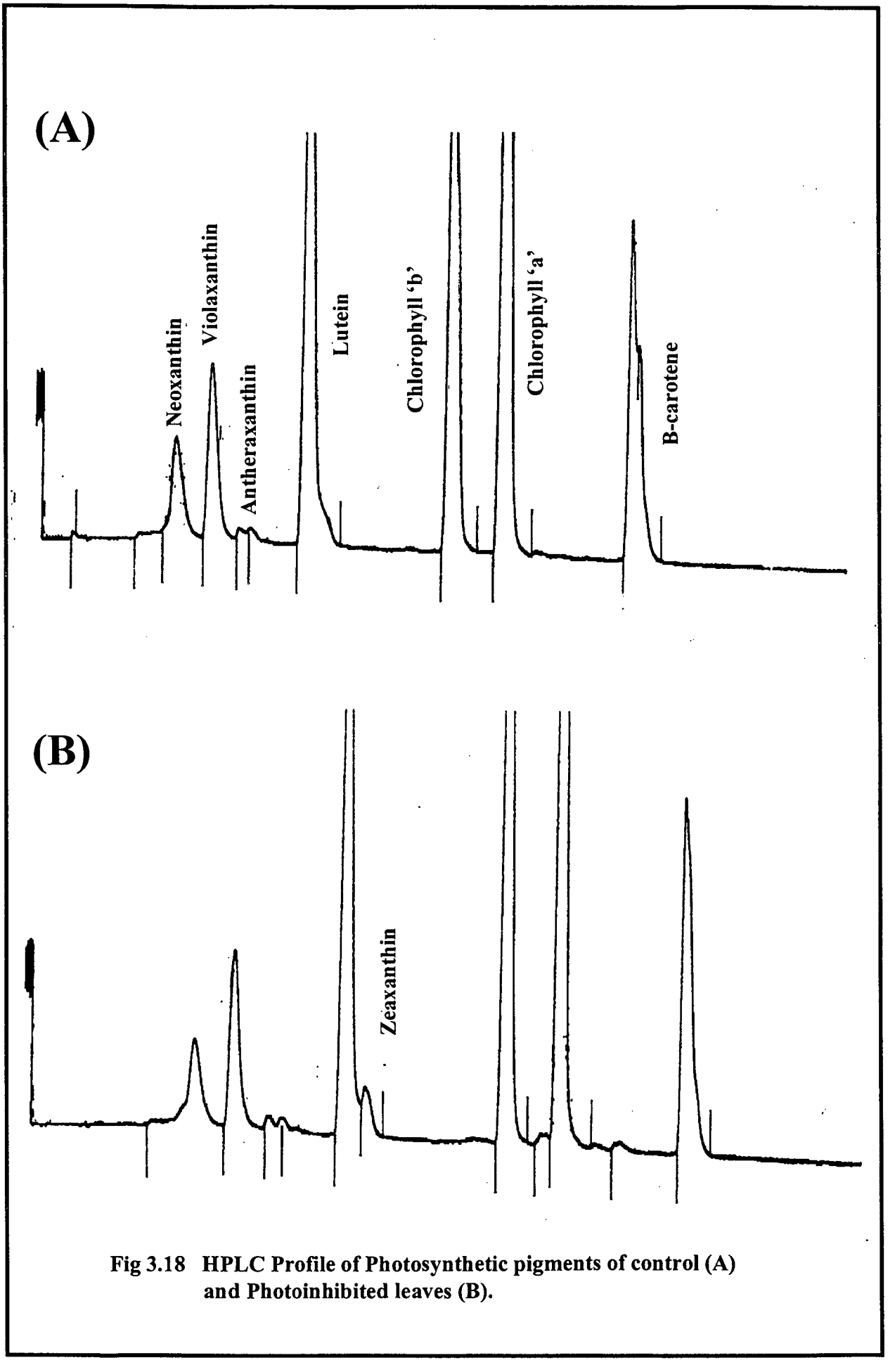


Fig 3.18 HPLC Profile of Photosynthetic pigments of control (A) and Photoinhibited leaves (B).

302% increase in Zeaxanthin as compared to control. On the actual content basis the decrease in V was 3.22 μg . g FW^{-1} while increase in Z content was 3.08 μg . g FW^{-1}. The Photoinhibition treatment for 6 hour at 30°C resulted in 37% decrease in V and 379% increase in Z. When the level of V and Z were compared with plants fed with water and given same photoinhibition treatment it was seen that percent increase in the Z, in ascorbate fed photoinhibited leaves, at 30°C was 139% less than leaves fed with water and photoinhibited at same light and temperature. However, actual content of Z rose from 1.67 μg. g FW^{-1} in control leaves (no photoinhibition) fed with ascorbate, to 8.0 μg . g FW^{-1} in leaves fed with ascorbate and given photoinhibition for 6 hours, compared to increase in Z from 1.02 μg . g FW^{-1} (0 hour) to 6.30 μg. g FW^{-1} observed in leaves fed with water and photoinhibited for 6 hour (Table 3.9). Leaves photoinhibited at 50°C showed greater decrease in V content (51% after 6 hour of treatment) compared to decrease in V seen at 5 and 30°C, but Z did not show comparatively higher level as seen in leaves photoinhibited at 5 and 30°C. The photoinhibitory treatment for 6 hour resulted in 191% increase in Z content which was considerably less than observed when leaves were photoinhibited at 5 and 30°C (Table 3.9).

Leaves fed with DTT and photoinhibited at 3600 at 5, 30 and 50°C showed considerably less de-epoxidation of V to Z compare to de-epoxidation seen in leaves fed with distilled water and ascorbate and then photoinhibited. Six hour photoinhibition treatment at 5°C resulted in 10% decrease in V (decrease of 2.17 μg . g FW^{-1}) while Z content rose to 120% (an increase of 1.2 μg . g FW^{-1}). The photoinhibition treatment at 30°C showed a slight increase in de-epoxidation of V to Z compare to seen at 5°C. However, the de-epoxidation level was significantly less than observed in plants fed

ıble 3.8: Carotenoid composition of sorghum leaves fed with distilled water, ascorbate and DTT and photoinhibited at 3600 μmol s^{-1} at 5, 30 and 50°C. The results are presented as μg pigments . g FW^{-1} using β-carotene as an external standard. Mean±SD values : calculated from 3 experiments.

ments	H_2O						ASC						DTT					
	5°C		30°C		50°C		5°C		30°C		50°C		5°C		30°C		50°C	
	0 h	6 h	o h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h
ıxanthin	3.39	4.12	4.05	4.83	4.25	4.70	3.01	3.85	4.29	5.07	4.25	4.08	3.17	3.23	3.40	3.40	3.09	3.19
	±0.5	±0.7	±0.4	±0.5	±0.5	±0.8	±0.3	0.8±	±0.6	±0.5	±0.7	±0.5	±0.5	±0.5	±0.8	±0.7	±0.9	±0.2
laxanthin	21.20	19.12	22.0	17.78	20.25	13.80	24.0	20.78	21.92	13.83	23.9	11.65	20.67	18.5	19.6	14.3	19.6	12.10
	±1.1	±1.3	±0.9	±1.1	±1.0	±0.9	±1.3	±1.7	±1.2	±0.8	±1.0	±0.7	±1.0	±1.0	±1.3	±1.5	±1.8	±1.2
theraxanthin	1.40	1.13	1.21	0.92	1.72	1.30	1.73	0.72	1.37	1.65	1.21	2.02	0.3	0.35	1.10	0.72	0.92	1.30
	±0.2	±0.1	±0.3	±0.2	±0.3	±0.5	±0.3	±0.1	±0.2	±0.5	±0.1	±0.3	±0.1	±0.1	±0.5	±0.3	±0.1	±0.3
ein	44.6	38.3	42.9	43.6	44.5	39.4	43.2	45.6	41.7	38.0	45.1	40.08	40.8	47.5	45.3	40.1	43.7	43.0
	±2.6	±3.1	±2.8	±2.5	±3.0	±3.3	±3.1	±2.8	±2.9	±3.0	±2.3	±3.0	±3.1	±2.8	±2.7	±3.2	±3.0	±2.3
ıxanthin	0.72	3.90	1.02	6.30	0.0	4.20	1.02	4.10	1.67	8.0	1.75	5.10	0.0	1.2	0.0	1.6	0.0	0.89
	±0.1	±0.3	±0.5	±0.2		±0.8	±0.1	±0.3	±0.3	±0.5	±0.3	±0.4		±0.4		±0.4		±0.4
lorophyll a	30.0	37.5	31.7	43.3	42.0	33.3	41.9	37.0	32.5	39.7	35.2	30.9	37.0	31.3	40.7	41.2	38.5	38.3
	±2.0	±2.8	±3.1	±3.2	±2.3	±2.8	±2.8	±2.5	±2.5	±1.8	±1.9	±1.6	±2.7	±2.3	±3.0	±2.6	±2.1	±2.6
lorophyll b	49.7	53.3	48.9	52.1	53.6	47.1	55.3	50.1	49.3	53.2	48.5	48.4	50.3	51.1	53.6	57.0	55.0	59.6
	±1.2	±2.9	±1.8	±1.7	±1.2	±1.6	±2.0	±1.3	±2.1	±1.9	±1.5	±1.1	±1.6	±2.5	±2.6	±1.9	±2.1	±2.3
arotene	17.3	13.5	18.9	12.3	13.5	12.0	20.2	18.3	19.85	18.32	20.0	17.9	18.5	15.9	17.3	17.5	15.6	13.0
	±0.9	±1.2	±1.9	±0.9	±1.1	±0.8	±0.9	±0.9	±2.5	±0.9	±1.1	±0.8	±0.6	±0.7	±1.8	±1.1	±1.6	±1.3
A+Z	23.12	24.03	24.23	25.0	21.94	19.3	26.75	25.6	24.96	23.48	26.88	18.72	20.97	20.2	20.7	16.6	20.5	14.29
	±2.1	±1.7	±2.5	±2.1	±2.3	±1.3	±2.0	±1.9	±2.2	±1.3	±1.5	±1.4	±1.7	±1.8	±2.3	±1.6	±1.1	±1.4
otenoids/ lorophylls	1.1	0.88	1.12	0.90	0.88	0.94	0.68	1.08	0.80	0.91	1.14	1.02	0.96	1.05	0.70	0.79	0.89	0.75
S= 0.5A/V+A+Z	0.950	0.819	0.933	0.730	0.962	0.749	0.930	0.826	0.913	0.624	0.912	0.676	0.990	0.925	0.97	0.883	0.99	0.899

represent controls. Control leaves were fed with respective solution and kept at growth conditions.

Table 3.9:

Xanthophyll cycle (V+A+Z) content and non-photochemical quenching of chlorophyll fluorescence in sorghum leaves fed with distilled water, ascorbate and DTT and photoinhibited at 3600 μmol m^{-2} s^{-1} at 5, 30 and 50°C. The results are presented as μg pigments . g FW^{-1} using β-carotene as an external standard. Mean±SD values are calculated from 3 experiments.

	3600 μmol m^{-2} s^{-1}					
+H_2O	5° C		30° C		50° C	
	0 h	6 h	0 h	6 h	0 h	6 h
Violaxanthin	21.20±1.1	19.12±1.3	22.0± 0.9	17.78±	20.22±1.0	13.80±0.9
Antheraxanthin	1.40±0.2	1.13±0.1	1.21±0.3	0.92±0.2	1.72±0.3	1.30±0.5
Zeaxanthin	0.72±0.1	3.90±0.3	1.02±0.5	6.30±0.2	00±0.0	4.20±0.8
qN	0.435±0.02	0.625±0.02	0.434±0.04	0.727±0.02	0.435±0.04	0.483±0.03
+ASC						
	0 h	6 h	0 h	6 h	0 h	6 h
Violaxanthin	24.0±1.3	20.78±1.7	21.92±1.2	13.83±0.8	23.92±1.0	11.65±0.7
Antheraxanthin	1.73±0.3	0.72±0.1	1.37±0.2	1.65±0.5	1.21±0.2	2.02±0.4
Zeaxanthin	1.02±0.1	4.10±0.3	1.67±0.3	8.0±0.5	1.75±0.3	5.10±0.4
qN	0.467±0.05	0.687±0.04	0.467±0.05	0.733±0.04	0.467±0.06	0.619±0.04
+DTT						
	0 h	6 h	0 h	6 h	0 h	6 h
Violaxanthin	20.67±1.0	18.50±1.0	19.60±1.3	14.30±1.5	19.61±1.9	12.10±1.2
Antheraxanthin	0.30±0.2	0.35±0.1	1.10±0.5	0.72±0.3	0.92±0.1	0.13±0.3
Zeaxanthin	00±0.0	1.2±0.4	00±0.0	1.60±0.5	00±0.0	0.89±0.9
qN	0.432±0.06	0.511±0.05	0.432±0.05	0.539±0.04	0.432±0.06	0.609±0.04

0 h represent controls. Control leaves were fed with respective solution and kept at growth conditions.

with water or ascorbate and photoinhibited. The photoinhibition treatment at 50°C caused 38% decrease in V and 89% increase in Z as compare to control.

When the changes in V and Z are compared to non-photochemical quenching (Table 3.9) it was observed that there was direct relationship in the level of de-epoxidation of V to Z and the level of qN. It was seen that maximum increase in qN was seen in plants fed with ascorbate which also correspond to the higher level of de-epoxidation and least increase in qN was observed in leaves fed with DTT which also inhibited the de-epoxidation of V to Z to a large extent.

β-carotene content also responded to photoinhibition treatment. It was observed that photoinhibition of leave fed with distilled water showed a slight increase in the level of β-carotene as compared to control (Table 3.8) where as, photoinhibition of ascorbate and DTT fed leaves showed a decrease in the content of β-carotene. This decrease in the β-carotene was greater in leaves fed with DTT than leaves fed with ascorbate (Table 3.8). Lutein and Chlorophyll a and b content did not show much changes (Table 3.8).

3.6 EFFECT OF ADDITION OF ANTIOXIDANTS DURING THE PHOTOINHIBITION OF CHLOROPLASTS ON CHLOROPHYLL FLUORESCENCE MEASUREMENTS:

This experiment was carried out to see if any protection to photoinhibitory damage to photosynthesis (measured as Fv/Fm ratio, qP and qN) is provided by addition of known antioxidant such as β-carotene, Ascorbic acid and Glutathione under

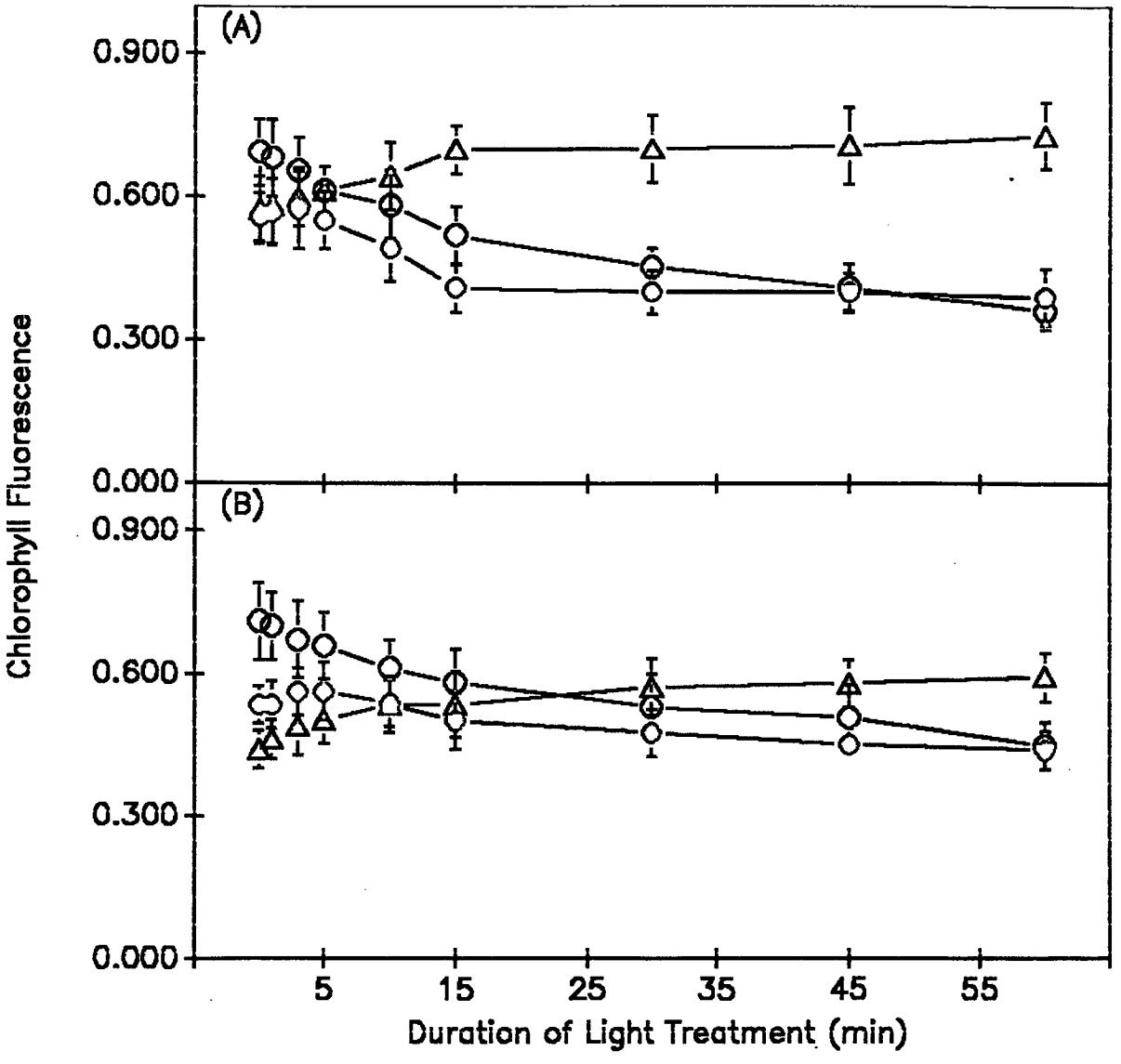


Fig. 3.19: Effect of photoinhibition of isolated chloroplasts treated with none i.e. control (A) and β-carotene (B) as an antioxidant during photoinhibitory treatment at 30°C and 3600 μmol m^{-2} s^{-1} PAR on FV/Fm ratio (-o-), photochemical quenching (-o-) and non-photochemical quenching (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

in vitro conditions, where inherent protection as well as cellular recovery processes are either not functioning or functioning at minimal level. The photoinhibitory treatment was given at 3600 μmol m^{-2} s^{-1} at 30°C for 0-60 min duration. The results were compared with the chloroplasts photoinhibited without adding any anti-oxidant (control) to find out relative extent of protection provided by addition of the antioxidants.

The results (Table 3.10) indicate changes in Fv/Fm ratio qP and qN in chloroplasts added with antioxidants during the photoinhibitory treatment. It was seen that Fv/Fm ratio showed less damage when supplemented with anti-oxidants during the photoinhibition treatment (Fig. 3.19B and 3.20A and B) than chloroplasts photoinhibited without having any anti-oxidants. (Fig.3.19A). The Fv/Fm ratio declined 30% after 60 min of the photoinhibitory treatment to chloroplasts mixed with β-carotene as compared to the control (Fig 3.19B). This decrease in Fv/Fm ratio was 18% less than observed in chloroplasts photoinhibited without any anti-oxidants (48 % decrease in the Fv/Fm after 60 min of the treatment; Fig. 3.19A and B).

Photochemical quenching remained relatively higher in chloroplast added with anti-oxidants and then photoinhibited compared to chloroplasts photoinhibited without anti-oxidant. The qP was 30% higher, as compared to 0 hour in chloroplasts photoinhibited without any anti-oxidant for 60 min (Fig. 3.19A). While chloroplasts added with β-carotene showed only 18% decrease in qP after 60 min of the treatment as compared to their control (chloroplasts kept for 60 min at 30°C were used as control; Fig. 3.19B). Similarly chloroplasts added with ascorbic acid (Fig. 3.20A) and

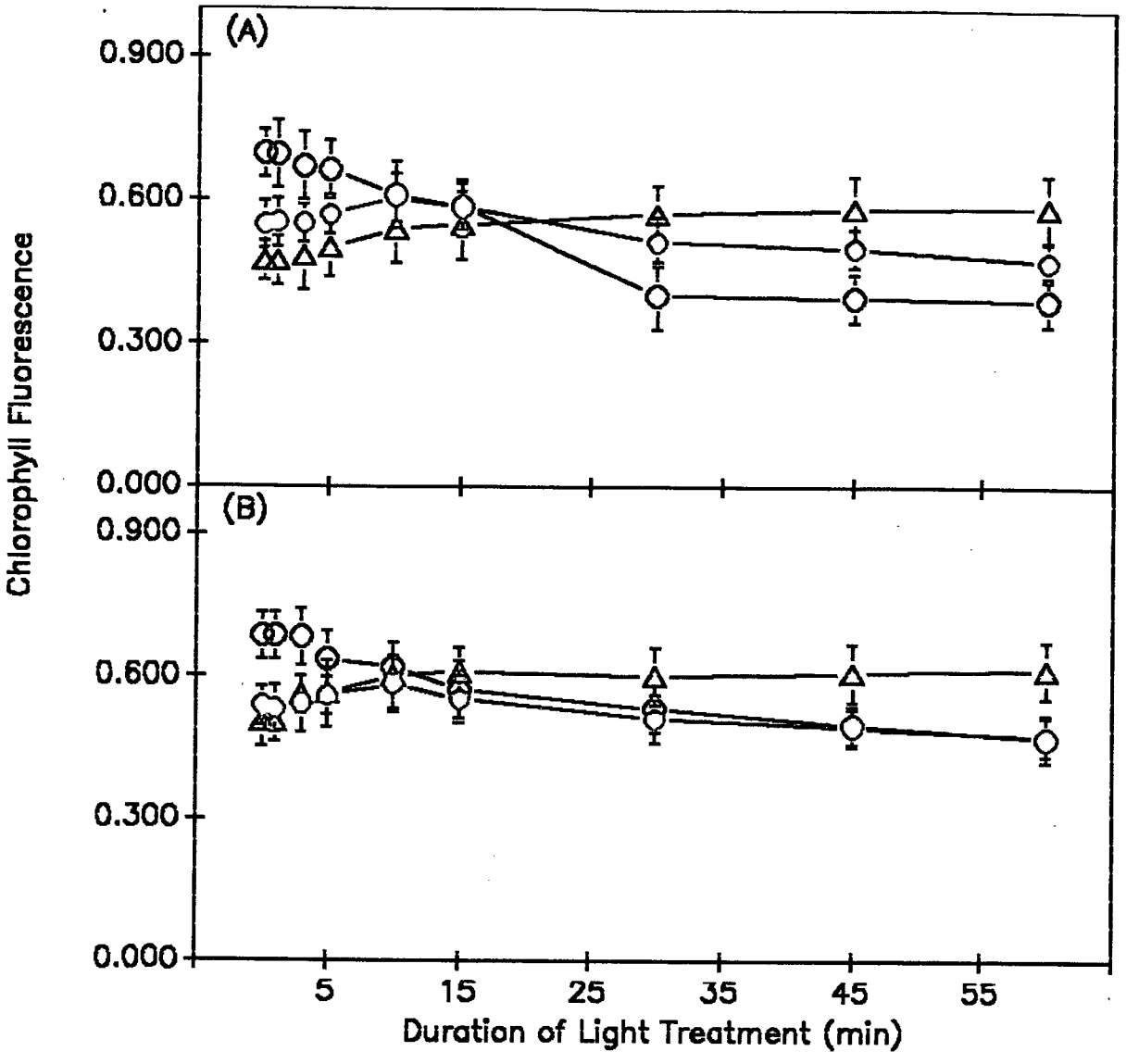


Fig. 3.20: Effect of photoinhibition of isolated chloroplasts treated with 5 mM ascorbic acid (A) and 5 mM glutathione (B) as antioxidants during photoinhibitory treatment at 30°C and 3600 μmol m^{-2} s^{-1} PAR on FV/Fm ratio (-o-), photochemical quenching (-o-) and non-photochemical quenching (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

glutathione (Fig. 3.20B) showed a decrease of only 14% and 12% in qP respectively after 60 min of the photoinhibitory treatment.

Non-photoinhibitory treatment in all cases (with or without anti-oxidant), however, showed increase in qN. The increase in chloroplasts added with β-carotene and photoinhibited for 60 min was much greater (34 % compared to 27% qN observed in chloroplast photoinhibited without anti-oxidant for 60 min). Chloroplasts added with ascorbate and photoinhibited for 60 min resulted in 23% increase while chloroplasts added with glutathione and then photoinhibited resulted in 22% increase in qN compared to their respective control (0 hour; Fig.3.20A and B).

3.6.1 EFFECT OF PHOTOINHIBITION OF CHLOROPLASTS WITH ANTIOXIDANTS ON LIPID PEROXIDATION OF THYLAKOID MEMBRANES.

The addition of antioxidant such as β-carotene, ascorbic acid and glutathione during the photoinhibition treatment (3600 μmol m^{-2} s^{-1} at 30°C of isolated chloroplasts was carried out. It was seen that addition of antioxidants during the photoinhibition treatment of isolated chloroplast decreased the extent of peroxidation of thylakoid lipids when compared to the level of peroxidation of thylakoid lipids observed when isolated chloroplasts were photoinhibited without addition of anti-oxidant (Table 3.11). Results indicate that six hour photoinhibition treatment of isolated chloroplasts supplemented with β-carotene, ascorbic acid or glutathione resulted in 6%, 18% and 27% less damage respectively to thylakoid lipids compared to the photoinhibition of isolated chloroplasts without addition of anti-oxidants (Table 3.11).

Table 3.10: Effect of photoinhibition of chloroplasts, isolated from normal leaves, added with antioxidants β-carotene, ascorbate and glutathione on room temperature chlorophyll fluorescence (Fv/Fm ratio, qP and qN). The photoinhibition treatment was given at 3600 μmol m^{-2} s^{-1} PAR at 30C. Mean±SD values were calculated from 3 experiments.

Duration of Treatment (min)	Control			β- carotene			Ascorbate			Glutathione		
	Fv/Fm ratio	qP	qN	Fv/Fm ratio	qP	qN	Fv/Fm ratio	qP	qN	Fv/Fm ratio	qP	qN
0	0.692 ±0.07	0.556 ±0.05	0.571 ±0.07	0.710 ±0.08	0.535 ±0.04	0.441 ±0.08	0.697 ±0.05	0.549 ±0.05	0.473 ±0.04	0.685 ±0.05	0.538 ±0.04	0.503 ±0.05
1	0.680 ±0.08	0.567 ±0.07	0.579 ±0.08	0.70± 0.070	0.535 ±0.05	0.462 ±0.04	0.695 ±0.07	0.552 ±0.05	0.473 ±0.05	0.685 ±0.05	0.531 ±0.05	0.503 ±0.04
3	0.653 ±0.07	0.571 ±0.08	0.598 ±0.06	0.672 ±0.08	0.562 ±0.05	0.489 ±0.06	0.670 ±0.07	0.550 ±0.04	0.483 ±0.07	0.683 ±0.06	0.542 ±0.06	0.561 ±0.04
5	0.612 ±0.05	0.550 ±0.06	0.611 ±0.05	0.659 ±0.07	0.563 ±0.06	0.503 ±0.05	0.663 ±0.06	0.569 ±0.04	0.500 ±0.06	0.635 ±0.06	0.559 ±0.04	0.563 ±0.07
10	0.583 ±0.07	0.493 ±0.07	0.642 ±0.07	0.611 ±0.06	0.538 ±0.05	0.535 ±0.00	0.610 ±0.07	0.604 ±0.05	0.539 ±0.07	0.619 ±0.05	0.583 ±0.06	0.601 ±0.05
15	0.519 ±0.06	0.410 ±0.05	0.697 ±0.05	0.581 ±0.07	0.500 ±0.06	0.535 ±0.07	0.585 ±0.05	0.583 ±0.06	0.547 ±0.07	0.572 ±0.06	0.551 ±0.05	0.609 ±0.06
30	0.453 ±0.04	0.400 ±0.05	0.699 ±0.07	0.530 ±0.07	0.475 ±0.05	0.572 ±0.07	0.401 ±0.07	0.512 ±0.05	0.569 ±0.06	0.531 ±0.05	0.510 ±0.05	0.600 ±0.06
45	0.411 ±0.05	0.400 ±0.04	0.707 ±0.08	0.508 ±0.07	0.452 ±0.03	0.580 ±0.06	0.395 ±0.05	0.499 ±0.04	0.580 ±0.07	0.496 ±0.04	0.492 ±0.04	0.607 ±0.06
60	0.362 ±0.04	0.391 ±0.06	0.728 ±0.07	0.499 ±0.05	0.439 ±0.04	0.593 ±0.05	0.389 ±0.05	0.471 ±0.04	0.583 ±0.07	0.469 ±0.05	0.473 ±0.04	0.613 ±0.06

0 h represent control. Chloroplasts added with respective antioxidant and kept for 6 h at room temperature in dark were used as control

Table 3.11: Effect of photoinhibition of chloroplasts isolated from normal leaves and supplemented with antioxidants β-carotene, ascorbate and glutathione on peroxidation of thylakoid lipids. The chloroplasts were photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 30°C up to 6 hour. The results are presented as μmol MDA formed. Mean±SD values were calculated from 3 experiments.

Duration of treatment (h)	H_2O	β-carotene	Ascorbate	GLUTATHIONE
0	0.45±0.05	0.42±0.08	0.42±0.12	0.38±0.11
3	0.95±0.08	0.78±0.18	0.73±0.1	0.89±0.2
6	1.45±0.18	1.37±0.23	1.19±0.18	1.06±0.18

0 h represent control. Chloroplasts added with respective antioxidant and kept for 6 h at room temperature in dark were used as control

3.7 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES FED WITH DISTILLED WATER, ASCORBATE AND DITHIOTHREITOL ON PEROXIDATION OF THYLAKOID LIPIDS:

Effect of light (3600 and 2200 μmol m^{-2} s^{-1}) at different temperature (5, 30 and 50°C) for 0-6 hour in leaves pre treated with either distilled water, ascorbic acid or dithiothreitol were studied on MDA formation to ascertain the oxidative damage caused to the thylakoid lipids under our experimental conditions. The results are shown in Table 3.12.

3.7.1 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES FED WITH DISTILLED WATER ON PEROXIDATION OF THYLAKOID LIPIDS:

One hour photoinhibition treatment at 3600 μmol m^{-2} s^{-1} PAR at 5°C in leaves fed with distilled water resulted in an increase of 40% in MDA formation as compared to the control, followed by an increase of 85% after 2 hour, 145% after 3 hour, 190% after 4 hour, 225% after 5 hour and 265% after 6 hour of the treatment (Fig. 3.21A). The photoinhibition treatment at 30°C, however, resulted in a lower level of damage. The six hour treatment at 30°C caused a increase of 128%, as compared to 265% seen in leaves treated at 5°C. The photoinhibition treatment at 50°C was most damaging. A 3 hour treatment caused 176% increase in the peroxidation level as compared to control and six hour treatment at 50°C resulted in a 533% increase in the MDA formation (indicative of thylakoid lipid peroxidation) as compared to control (Fig. 3.21A). This increase in MDA level at 50°C temperature was 4 times greater than observed in leaves

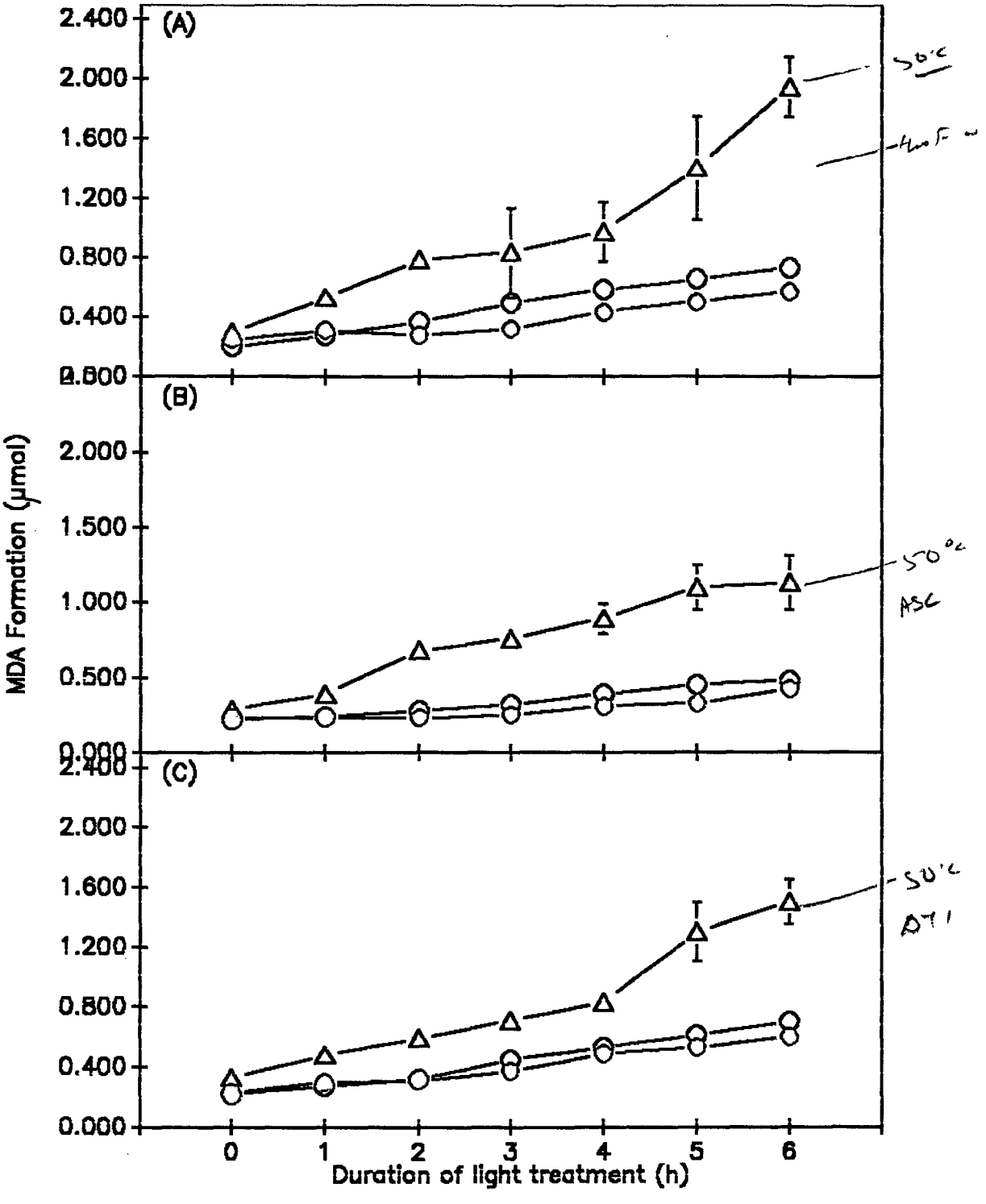


Fig. 3.21: Effect of photoinhibition of intact leaves pre treated with H_2O (A), 5 mM ascorbic acid (B) and 4 mM DTT (C) and photoinhibited at 3600 µmol m^{-2} s^{-1} PAR on the peroxidation of thylakoid lipids measured as MDA formation in chloroplasts isolated from treated leaves at 5°C (-o-), 30°C (-o-) and 50°C (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

photoinhibited at 30°C and 2.5 times greater than observed in leaves photoinhibited at 5°C for the same duration (Fig. 3.23).

The photoinhibition treatment at 2200 μmol m^{-2} s^{-1} PAR at 5, 30, 50°C also caused the damage to thylakoid membrane seen as increase in the MDA formation (Table 3.12). The increase in the peroxidation of the thylakoid lipids was relatively less in plants exposed to light level of 2200 μmol m^{-2} s^{-1} at 5 and 30°C (Fig. 3.22A) than seen in leaves exposed to 3600 μmol m^{-2} s^{-1} at respective temperature for the same duration (Fig. 3.21A). However, the peroxidation level in leaves photoinhibited at 2200 μmol m^{-2} s^{-1} at 50°C was more or less same as seen in the leaves exposed to 3600 μmol m^{-2} s^{-1} PAR at 50°C for the same duration. One hour photoinhibition treatment of leaves at 2200 μmol m^{-2} s^{-1} light and 5°C temperature did not cause any change in the level of MDA formation compared to control, but 2 hour treatment resulted in an increase of 25% followed by 55% increase in MDA after 3 hour of the treatment, 115% after 4 hour, 160% after 5 hour and 195% increase after 6 hour of the treatment, as compared to control (Fig. 3.22A). The increase in MDA formation (damage to thylakoid lipids) in leaves treated to 2200 μmol m^{-2} s^{-1} at 30°C was considerably less compared to the same light treatment at 5°C and 50°C. Three hour and six hour photoinhibitory treatment at 30°C resulted in an increase in MDA formation by 12% and 44% respectively, as compared to control. This increase in MDA formation at 30°C was considerably less (151%) than increase observed in leaves treated at the same light level but at 5°C (55% increase after 3 hour and 195% increase after 6 hour of the treatment, as compared to control; Table 3.12). The 2200 μmol m^{-2} s^{-1} PAR treatment at 50°C, however, showed

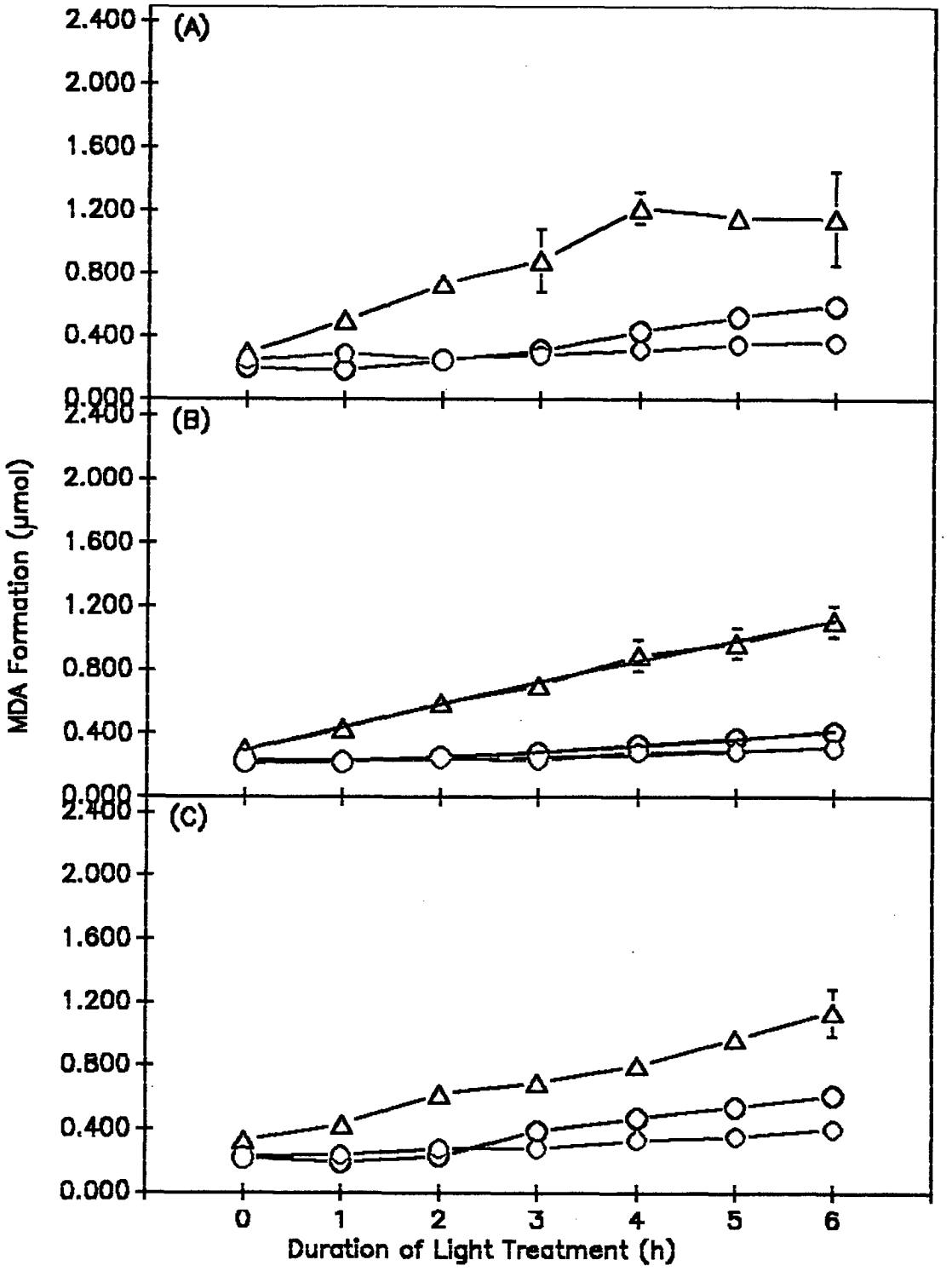


Fig. 3.22: Effect of photoinhibition of intact leaves pre treated with H_2O (A), 5 mM ascorbic acid (B) and 4 mM DTT (C) and photoinhibited at 2200 µmol m^{-2} s^{-1} PAR on the peroxidation of thylakoid lipids measured as MDA formation in chloroplasts isolated from leaves at 5°C (-o-), 30°C (-o-) and 50°C (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

much greater increase in MDA formation (166% increase after 3 hour of the treatment and 563% increase after 6 hour of the treatment) as compared to the control (Fig. 3.22A). This increase in the level of MDA at 50°C at 2200 μmol m^{-2} s^{-1} light level was more or less same as observed at 3600 μmol m^{-2} s^{-1} light at 50°C (Fig. 3.21A).

3.7.2 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES FED WITH ASCORBIC ACID ON PEROXIDATION OF THYLAKOID LIPIDS:

Photoinhibition of ascorbate fed leaves at 3600 μmol m^{-2} s^{-1} at 5, 30 and 50°C resulted in a linear increase in the peroxidation level of the thylakoid lipids (Fig. 3.21B). However, this increase was considerably less than observed in the plants fed with distilled water and treated with the same light level (3600 μmol m^{-2} s^{-1}) at the respective temperature. The high light treatment at 5°C for 6 hour duration of leaves fed with 5 mM ascorbic acid caused an increase of 118% as compared to control (Fig. 3.21B), which was 147% less than seen in plants fed with distilled water for the same photoinhibitory treatment (Fig. 3.21A). The high light treatment of ascorbate fed leaves at 30°C also caused linear increase in the MDA formation. Six hour treatment resulted in 83% increase in MDA formation, as compared to 118% observed in leaves fed with ascorbate and treated to same photoinhibitory treatment at 5°C (Fig. 3.21B). The increase of 83% in MDA formation in leaves fed with ascorbate and treated at high light at 30°C was 45% less than seen in leaves fed with distilled water and treated to the same photoinhibitory treatment (Fig. 3.21A).

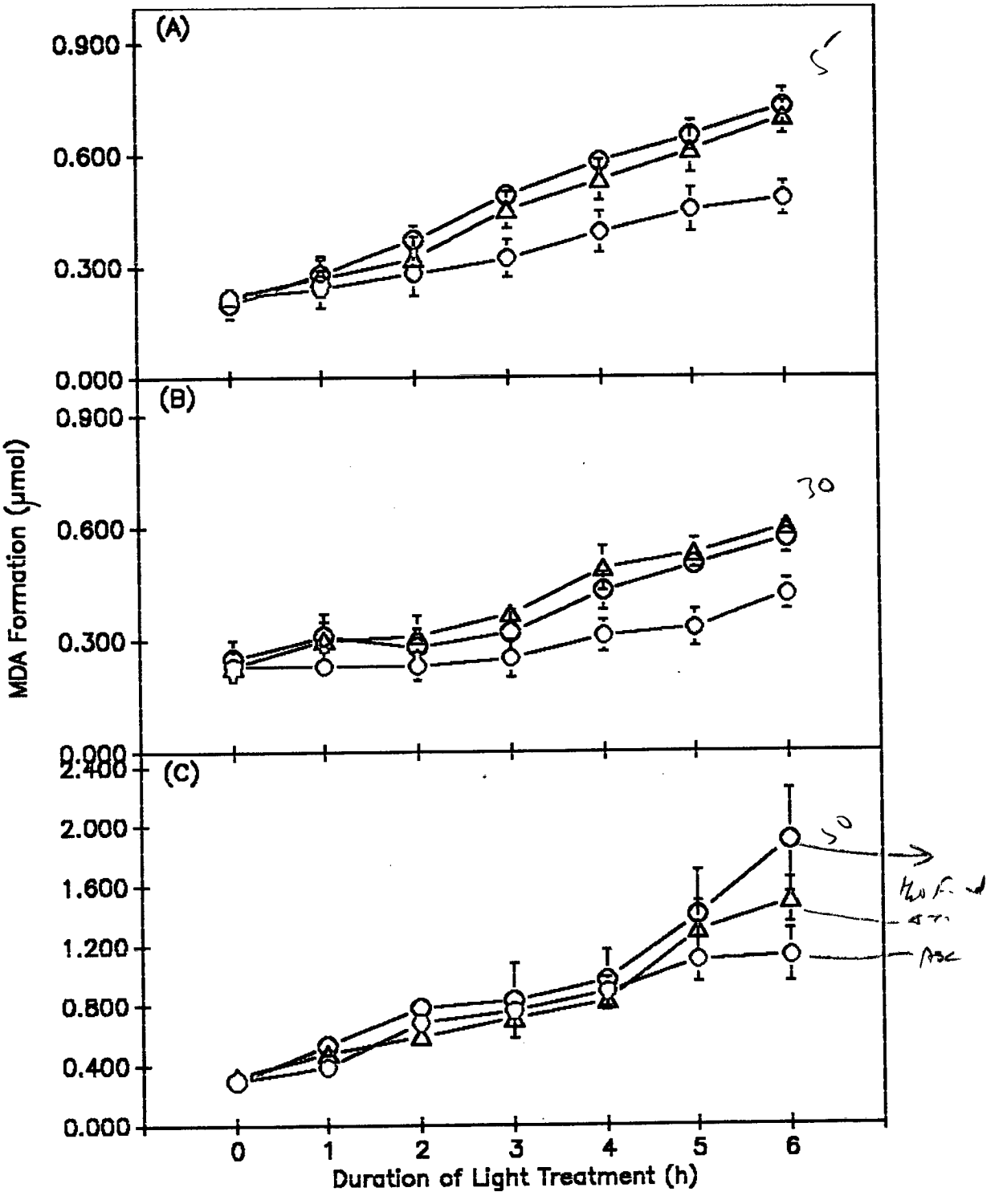


Fig. 3.23: Effect of photoinhibition of intact leaves fed with H_2O (-o-), 5 mM ascorbic acid (-o-) and 4 mM DTT (-Δ-) and photoinhibited at 3600 µmol m^{-2} s^{-1} PAR on the peroxidation of thylakoid lipids measured as MDA formation in chloroplasts isolated from leaves at 5°C (A), 30°C (B) and 50°C (C). The leaves were fed The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

3.21 (3.23

Photoinhibition treatment of ascorbate fed leaves at 2200 μmol m^{-2} s^{-1} and all three temperature 5, 30 and 50°C caused increased in MDA as compared to control, but the increase was considerably less than observed in leaves (ascorbate fed) photoinhibited at 3600 μmol m^{-2} s^{-1} at respective temperatures (Table 3.12). Six hour light treatment at 2200 μmol m^{-2} s^{-1} at 5°C for 6 hours caused 86% increase in peroxidation level of thylakoid lipids, as compared to control (Fig. 3.22B). The increase (86%) in MDA formation was 32% less than seen in ascorbate fed leaves exposed to 3600 μmol m^{-2} s^{-1} PAR at 5°C for the same duration (Fig. 3.21B). Similarly the medium light treatment (2200 μmol m^{-2} s^{-1}) at 30°C for 6 hour increased the level of peroxidation of thylakoid lipids only 34% as compared to the 86% seen at 5°C (Fig. 3.22B). The increase (34%) seen at photoinhibitory treatment at 30°C was 49% less than observed for ascorbate fed leaves exposed to high light (3600 μmol m^{-2} s^{-1}) at the same temperature for the same duration. The light treatment (2200 μmol m^{-2} s^{-1}) at 50°C for 6 hour caused a 370% increase in the MDA formation as compared to only 34% and 86% observed at 30°C and 5°C respectively for the same photoinhibitory treatment (Fig. 3.22B; Table 3.12).

3.7.3 EFFECT OF PHOTOINHIBITION OF INTACT LEAVES FED WITH DITHIOTHREITOL ON PEROXIDATION OF THYLAKOID LIPIDS:

Photoinhibition of dithiothreitol fed leaves at 3600 and 2200 μmol m^{-2} s^{-1} at all three temperature resulted in a linear increase in the peroxidation of thylakoid lipids. The extent of photoinhibitory damage was greater in leaves exposed to 3600 μmol m^{-2} s^{-1} PAR (Fig. 3.21C) than at 2200 μmol m^{-2} s^{-1} PAR (Fig. 3.22C) at 5, 30 and 50°C.

Also the level of peroxidation of thylakoid lipids at all photoinhibitory treatments was greater in leaves fed with dithiothreitol than leaves fed with ascorbate or distilled water (Table 3.12).

Photoinhibition treatment of dithiothreitol fed leaves at 3600 μmol m^{-2} s^{-1} at 50°C for 6 hour caused an 354% increase in MDA formation, as compared to 161% and 218% seen for the same photoinhibition treatment at 30°C and 5°C respectively (Fig. 3.23). However, leaves treated at 2200 μmol m^{-2} s^{-1} for 6 hour resulted in 177% increase in MDA formation when treated at 5°C, 74% when treated at 30°C and 245% when treated at 50°C as compared to controls (Fig. 3.22C).

Figure 3.23 show the changes in the level of MDA formation in leaves fed with distilled water, ascorbate and DTT at 3600 μmol m^{-2} s^{-1} at 5°C (Fig. 3.23A), 30°C (Fig. 3.23B) and 50°C (Fig. 3.23C).

3.8 EFFECT OF PHOTOINHIBITION OF CHLOROPLASTS ISOLATED FROM LEAVES FED WITH DISTILLED WATER, ASCORBIC ACID AND DITHIOTHREITOL ON PEROXIDATION OF THYLAKOID LIPIDS:

Photoinhibition of isolated chloroplasts resulted in greater level of peroxidation of thylakoid lipids than seen when intact leaves were given the photoinhibitory treatment. Also the difference in the extent of damage (MDA formation), whether a photoinhibitory treatment was given at light level of 3600 μmol m^{-2} s^{-2} or 2200 μmol m^{-2} s^{-1} was relatively less in isolated chloroplasts in comparison to the difference in the

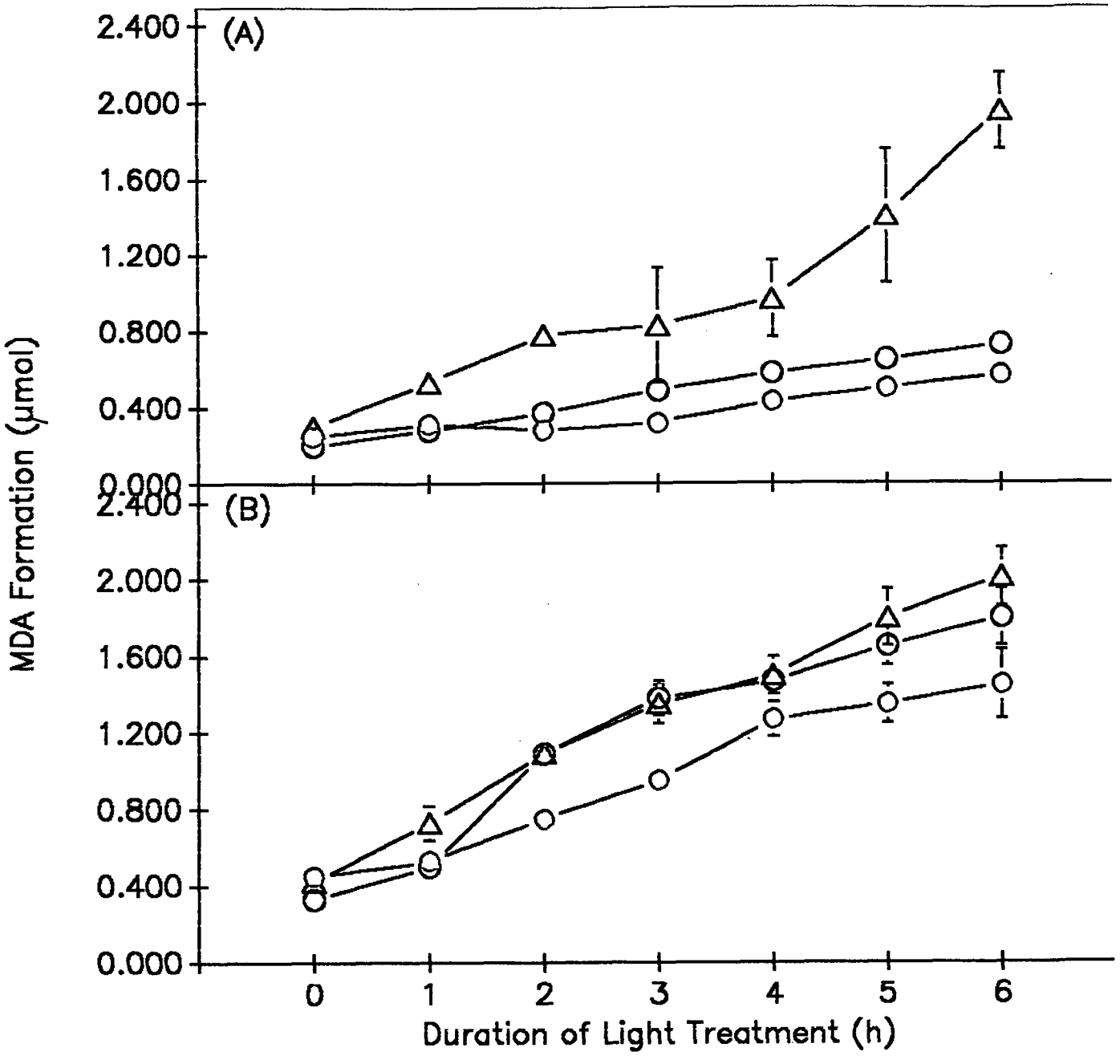


Fig. 3.24: Effect of photoinhibition of intact leaves fed with H_2O (-o-), 5 mM ascorbic acid (-o-) and 4 mM DTT (-Δ-) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 30°C (A) and isolated chloroplasts photoinhibited at 30°C and 3600 μmol m^{-2} s^{-1} PAR (B) on the peroxidation of thylakoid lipids measured as MDA formation in chloroplasts isolated from leaves. The leaves were fed The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

Table 3.12: Effect of Photoinhibition of intact leaves on the peroxidation of thylakoid lipids (MDA formation). The sorghum leaves fed with distilled water, ascorbate and DTT were photoinhibited at 3600 and 2200 μmol m^{-2} s^{-1} for 0-6 h. The results are presented as μmol MDA formed. Mean±SD values were calculated from 4-6 experiments.

Duration of treatment (h)	H_2O			3600 μmol m^{-2} s^{-1} ASC			DTT		
	5°C	30°C	50°C	5°C	30°C	50°C	50°C	30°C	50°C
0	0.20±0.04	0.25±0.05	0.30±0.05	0.22±0.03	0.23±0.03	0.30±0.05	0.22±0.03	0.23±0.04	0.33±0.06
1	0.28±0.05	0.31±0.04	0.53±0.08	0.24±0.05	0.23±0.04	0.39±0.05	0.27±0.05	0.30±0.07	0.48±0.04
2	0.37±0.04	0.28±0.05	0.78±0.06	0.28±0.06	0.23±0.04	0.68±0.09	0.32±0.06	0.31±0.06	0.59±0.07
3	0.49±0.03	0.32±0.06	0.83±0.03	0.32±0.05	0.25±0.05	0.76±0.08	0.45±0.05	0.37±0.04	0.71±0.05
4	0.58±0.04	0.43±0.05	0.97±0.02	0.39±0.05	0.31±0.04	0.89±0.10	0.53±0.05	0.49±0.06	0.83±0.05
5	0.65±0.04	0.50±0.04	1.40±0.04	0.45±0.06	0.33±0.05	1.16±0.15	0.61±0.06	0.53±0.04	1.30±0.20
6	0.73±0.05	0.57±0.04	1.90±0.05	0.48±0.04	0.42±0.04	1.13±0.18	0.70±0.05	0.60±0.04	1.50±0.15
	5°C	30°C	50°C	2200 μmol m^{-2} s^{-1} 5°C	30°C	50°C	50°C	30°C	50°C
0	0.20±0 .06	0.25±0.05	0.30±0.05	0.22± 0.03	0.23±0.03	0.30±0.05	0.22±0.03	0.23±0.04	0.33±0.06
1	0.19±0.04	0.29±0.05	0.50±0.09	0.22± 0.04	0.23±0.04	0.43±0.06	0.19±0.06	0.24±0.05	0.43±0.08
2	0.25±0.05	0.26±0.05	0.88±0.08	0.25± 0.05	0.24±0.04	0.59±0.09	0.23±0.04	0.28±0.06	0.62±0.05
3	0.31±0.04	0.28±0.04	1.21±0.02	0.28± 0.05	0.23±0.04	0.71±0.09	0.39±0.07	0.28±0.07	0.69±0.07
4	0.43±0.04	0.31±0.06	1.53±0.01	0.33± 0.06	0.28±0.04	0.89±0.10	0.47±0.06	0.33±0.04	0.80±0.07
5	0.52±0.07	0.35±0.05	1.78±0.06	0.37± 0.05	0.29±0.05	0.97±0.10	0.54±0.05	0.35±0.06	0.97±0.09
6	0.59±0.08	0.36±0.06	1.99± 0.03	0.41± 0.05	0.31±0.04	1.42±0.10	0.61±0.05	0.40±0.07	1.14±0.15

0 h represent controls. Control leaves were fed with respective solution and kept at growth conditions.

extent of damage observed when intact leaves were photoinhibited at 3600 μmol m^{-2} s^{-2} or 2200 μmol m^{-2} s^{-1} light intensity. Results of effect of photoinhibition of isolated chloroplasts on MDA formation is shown in table 3.13).

Photoinhibition of chloroplasts, isolated from leaves fed with distilled water, at 3600 μ mol m^{-2} s^{-1} at 5, 30 and 50°C for 6 hour duration resulted in an increase of 445%, 222% and 379% respectively, in MDA formation as compared to the control (Fig. 3.24B). The extent of peroxidation of thylakoid lipids in chloroplasts photoinhibited at 2200 μmol m^{-2} s^{-1} at 5, 30, 50°C for 6 hour was, however, 384, 217% and 376% as compared to the control (Table 3.13).

Photoinhibition of chloroplasts isolated from ascorbate fed leaves at 3600 μmol m^{-2} s^{-1} at 5, 30 and 50°C for 6 hour duration resulted in an increase in MDA formation of 259%, 214% and 384% respectively, as compared to the control (Table 3.24B). The extent of peroxidation of thylakoid lipids in chloroplasts photoinhibited at 2200 μmol m^{-2} s^{-1} at 5, 30, 50°C for 6 hour was however 197%, 160% and 364% respectively as compared to the control (Table 3.13).

Photoinhibition of chloroplasts isolated from dithiothreitol fed leaves at 3600 μmol m^{-2} s^{-1} at 5, 30 and 50°C for 6 hour duration resulted in an increase in MDA formation of 438%, 328% and 359% respectively, as compared to the control. The extent of peroxidation of thylakoid lipids in chloroplasts photoinhibited at 2200 μmol m^{-2} s^{-1} at 5, 30 and 50°C for 6 hour was however 368%, 205% and 335%, as compared to the control (Fig. 3.24B; Table 3.13).

Table 3.13: Effect of Photoinhibition of chloroplasts isolated from normal leaves, on the peroxidation of thylakoid lipids (MDA formation). The chloroplasts were isolated from sorghum leaves fed with distilled water, ascorbate and DTT and photoinhibited at 3600 and 2200 μmol m^{-2} s^{-1} for 0-6 h. The results are presented as μmol MDA formed. Mean±SD values were calculated from 4-6 experiments.

Duration of treatment (h)	H_2O			3600 μmol m^{-2} s^{-1}					
				ASC			DTT		
	5°C	30°C	50°C	5°C	30°C	50°C	5°C	30°C	50°C
0	0.33±0.04	0.45±0.05	0.42±0.06	0.37± 0.06	0.35±0.07	0.45±0.07	0.37±0.06	0.40±0.09	0.45±0.07
1	0.50±0.07	0.53±0.09	0.73±0.09	0.48± 0.07	0.35±0.07	1.00±0.09	0.69±0.09	0.57±0.09	1.00±0.09
2	1.09±0.06	0.75±0.05	1.09±0.07	0.61 ± 0.05	0.43±0.09	1.87±0.13	0.93±0.10	0.95±0.10	1.87±0.13
3	1.38±0.09	0.95±0.08	1.35±0.10	0.83 ± 0.1	0.48±0.10	2.03±0.15	1.38±0.10	1.37±0.08	2.03±0.15
4	1.47±0.08	1.27±0.10	1.50±0.10	1.03 ± 0.1	0.57±0.08	2.11±0.14	1.71±0.09	1.42±0.09	2.11±0.15
5	1.65±0.10	1.35±0.10	1.80±0.15	1.21 ±0.13	0.98±0.09	2.20±0.19	1.89±0.13	1.59±0.14	2.20±0.19
6	1.80±0.15	1.45±0.18	2.01±0.15	1.33 ± 0.14	1.10±0.14	2.18±0.23	1.99±0.12	1.71±0.13	2.18±0.23
				2200 μmol m^{-2} s^{-1}					
	5°C	30°C	50°C	5°C	30°C	50°C	5°C	30°C	
0	0.33±0.08	0.45±0.07	0.42± 0.09	0.37±0.07	0.35±0.07	0.45±0.08	0.37±0.07	0.40±0.09	0.45±0.08
1	0.48±0.09	0.50±0.10	0.80± 0.07	0.48±0.08	0.36±0.08	0.90±0.09	0.60±0.15	0.43±0.10	0.93±0.09
2	0.60±0.08	0.88±0.09	1.59±0.09	0.57±0.10	0.39±0.07	1.23±0.13	0.93±0.10	0.54±0.08	1.23±0.13
3	0.87±0.10	1.03±0.12	1.78±0.11	0.71±0.10	0.41±0.09	1.35±0.11	1.42±0.10	0.63±0.09	1.35±0.11
4	1.11±0.10	1.30±0.09	1.97± 0.12	0.92±0.11	0.45±0.10	1.08±0.12	1.59±0.11	0.87±0.13	1.45±0.17
5	1.47±0.10	1.38±0.10	2.00±0.14	1.00±0.13	0.86±0.09	1.83±0.16	1.64±0.14	1.01±0.10	1.83±0.16
6	1.60±0.14	1.43±0.12	2.00±0.23	1.10±0.13	0.91±0.12	2.09±0.23	1.73±0.13	1.22±0.12	2.09±0.23

0 h represent controls. Isolated chloroplasts kept for 6 h at 30°C were used as control.

3.9 EFFECT OF PHOTOINHIBITION ON ACTIVITY OF ANTIOXIDANT ENZYMES:

The effect of photoinhibition on activity of various antioxidant enzymes was studied in order to find out the changes taking place in the activity of antioxidant enzymes in response to oxidative stress under our experimental conditions.

3.9.1 EFFECT OF PHOTOINHIBITION ON SUPEROXIDE DISMUTASE:

Intact leaves pre-treated with distilled water, ascorbate or dithiothreitol were photoinhibited at 3600 μmol m^{-2} s^{-1} or 2200 μmol m^{-2} s^{-1} at all three temperatures (5, 30 and 50°C) upto 6 hour and changes in the activity were followed. It was seen that leaves fed with distilled water and photoinhibited at 3600 μmol m^{-2} s^{-1} at 5°C showed a linear increase in the activity of superoxide dismutase. One hour photoinhibition treatment caused an increase of 29%, as compared to the control, followed by an increase of 55% after 2 hour of the treatment, 112% after 3 hour, 115% after 4 hour, 213% after 5 hour and 244% after 6 hour of the treatment (Table 3.14). Photoinhibition at 30°C also resulted in the linear increase of the activity of superoxide dismutase, however, the increase at 30°C was relatively less than observed at 5°C. Six hour photoinhibitory treatment at 30°C caused only 216% increase in the superoxide dismutase activity, as compared to control, which was 28% less than seen in leaves photoinhibited at 5°C for the same duration (Fig. 3.25A). The photoinhibitory treatment at 50°C caused an initial increase in the superoxide dismutase activity followed by a decrease in longer treatment. The treatment at 50°C caused 124% increase in the activity after 3 hour of the photoinhibition which was declined to 25% after 4 hour of the treatment and 54%

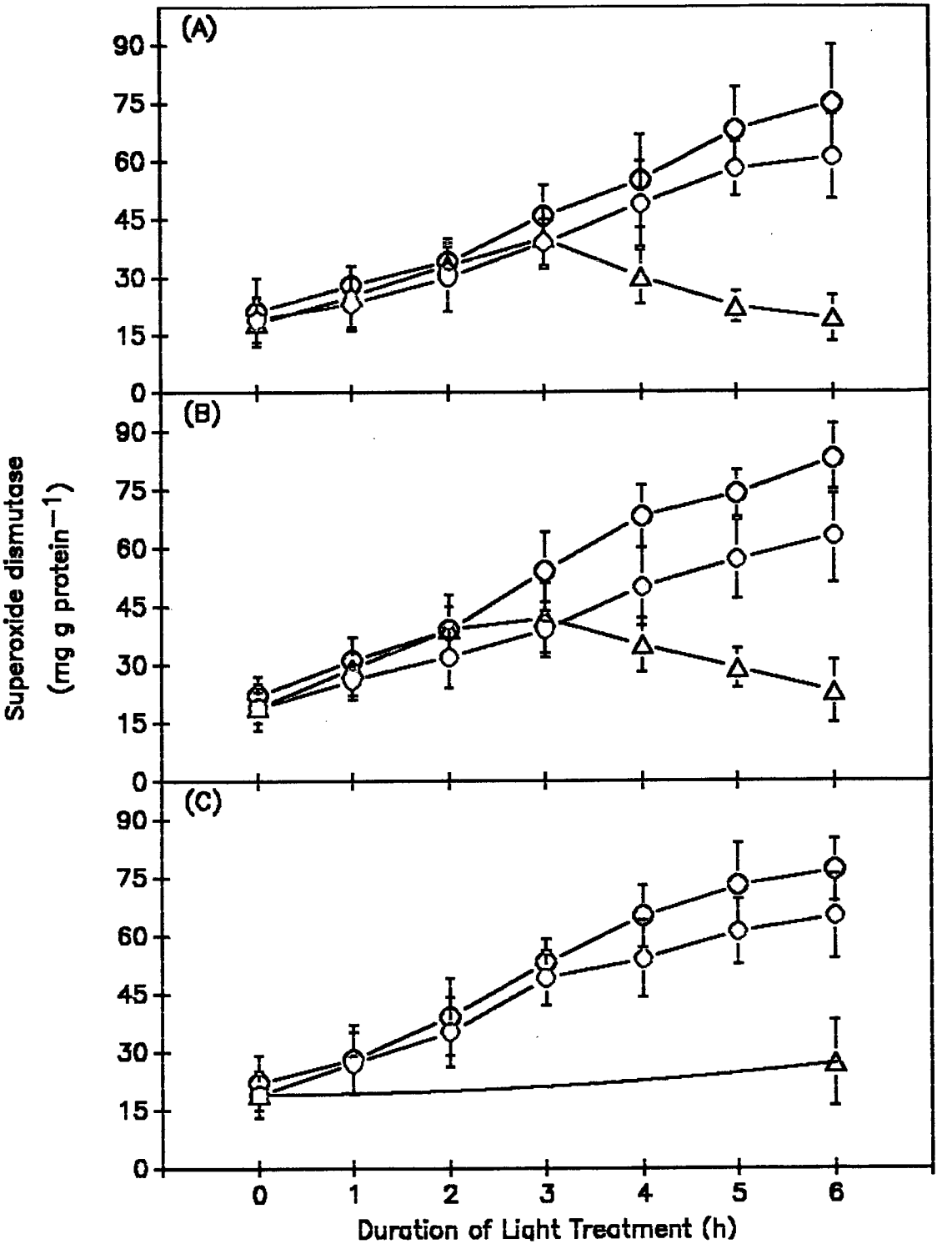


Fig. 3.25: Effect of photoinhibition on superoxide dismutase activity in leaves pre treated with H_2O (A), 5 mM ascorbic acid (B) and 4 mM DTT (C) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 5°C (-o-), 30°C (-o-) and 50°C (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

after 6 hour, as compared to the increase observed in superoxide dismutase after 3 hour of the photoinhibition treatment. However, when compared to the SOD activity in control leaves the increase in the superoxide dismutase activity after six hour of the treatment was only 4% (Fig. 3.25A).

Photoinhibition of distilled water fed leaves at 2200 μmol m^{-2} s^{-1} at 5, 30 and 50°C temperature also showed similar trends in the changes in the activity of superoxide dismutase (Table 3.14). However, extent of the increase in the activity of superoxide dismutase was relatively less than seen in leaves photoinhibited at 3600 μmol m^{-2} s^{-1} at the respective temperature (Fig. 3.25 and 3.26). The photoinhibition at 2200 μmol m^{-2} s^{-1} at 5°C showed a linear increase to 224% in superoxide dismutase activity after 6 hour of the treatment as compared to control (Fig. 3.26A). This increase in superoxide dismutase was 20% less than seen in leaves photoinhibition at 3600 μmol m^{-2} s^{-1} at the same temperature for same duration (Fig. 3.25A). The photoinhibitory treatment for 6 hour at 30°C resulted in the 180% increase in superoxide dismutase activity as compared to control. The treatment at 50°C, however, showed a linear increase to 143% as compared to control after 3 hour of the treatment, following which the activity showed a linear decline to 38% after 6 hour of the treatment, as compared to the increase observed after 3 hour of the treatment. However, the superoxide dismutase activity still remained greater (50%) than seen in control (0 hour photoinhibition) leaves (Fig. 3.26A; Table 3.14).

Photoinhibition of ascorbate fed 8 day old sorghum leaves at 3600 μmol m^{-2} s^{-1} PAR and 30°C also resulted in a linear increase in the superoxide dismutase activity.

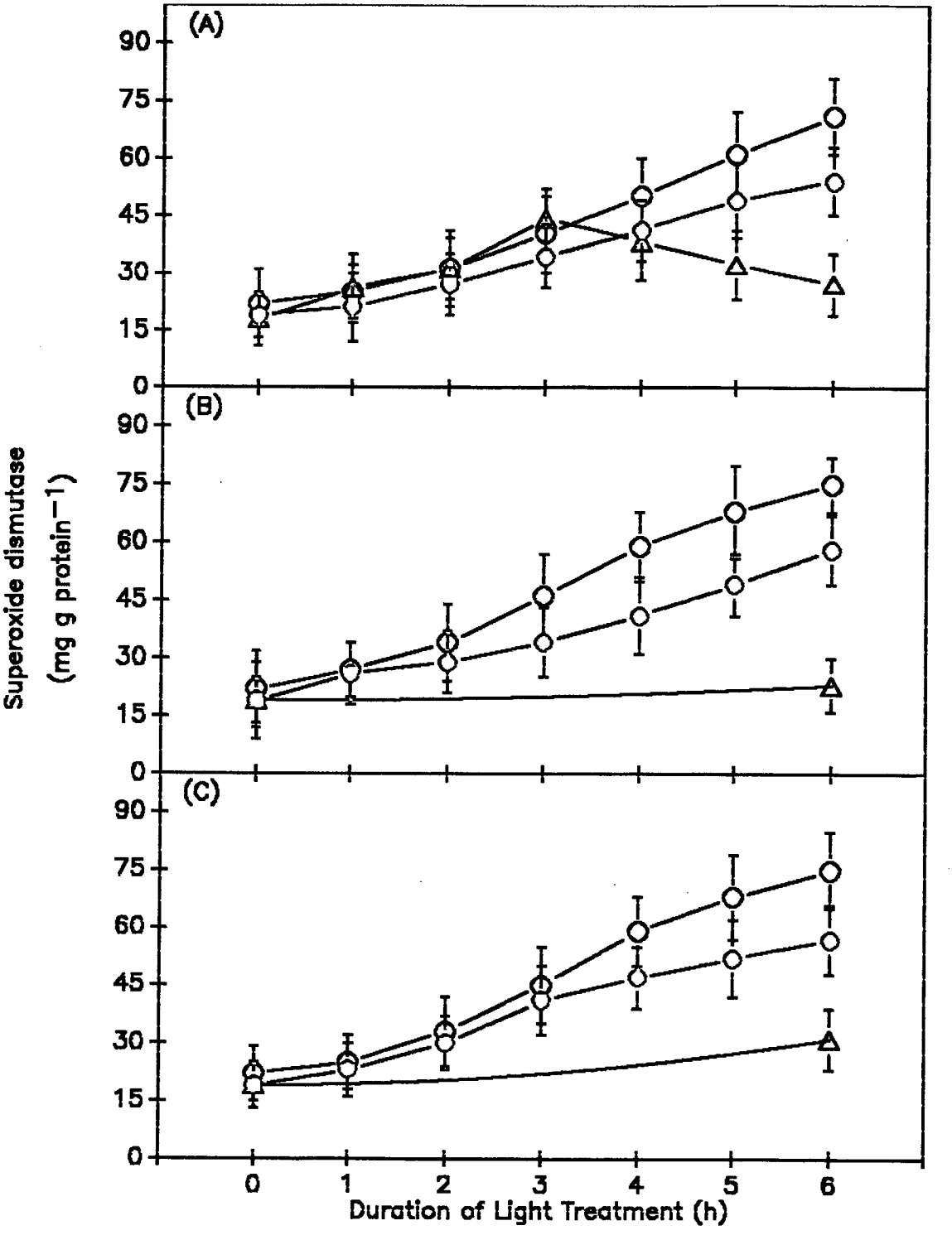


Fig. 3.26: Effect of photoinhibition on superoxide dismutase activity in leaves pre treated with H_2O (A), 5 mM ascorbic acid (B) and 4 mM DTT (C) and photoinhibited at 2200 μmol m^{-2} s^{-1} PAR at 5°C (-o-), 30°C (-o-) and 50°C (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

The treatment at 50°C, however, caused initial increase followed by a decrease (Table 3.14). The treatment at 5°C for 3 hour increased the superoxide dismutase activity by 114% as compared to the control and 274% after 6 hour of the treatment (Fig. 3.25B). This increase in superoxide dismutase activity was 47% greater than seen in leaves photoinhibited at 30°C (227 %) for the same duration of the treatment. The Photoinhibition of leaves at 50°C showed an initial increase in the activity. One hour Photoinhibition treatment caused 51% increase followed by 101% increase after 2 hour, 117% increase after 3 hour as compared to control. Further increase in the duration of the photoinhibition treatment resulted in a slight decrease in the activity. The decrease in the activity was 27% after 4 hour, 31% after 5 hour and 45% after 6 hour of the photoinhibitory treatment as compared to activity observed after 3 hour of the treatment. However, the superoxide dismutase activity still remained higher after 6 hour of the treatment (18%) than observed in control leaves (Fig. 3.25B).

Photoinhibition of ascorbate fed leaves at 2200 μmol m^{-2} s^{-1} PAR at 5, 30 and 50°C also showed increase in the activity of superoxide dismutase as compared to their control (Table 3.14). The photoinhibition treatment at 5°C for 6 hour caused 238% increase in the superoxide dismutase activity as compared to control (Fig. 3.26B). This increase (238%) was 36% less than seen when leaves were photoinhibited at 3600 μmol m^{-2} s^{-1} at same temperature for the same duration (274%) as compared to their control (Fig. 3.25B). Similarly photoinhibition at 30°C for 6 hour resulted in an increase of 202%, as compared to control, which was again less (25%) as compared to seen in leaves photoinhibited at 3600 μmol m^{-2} s^{-1} at 30°C for the same duration. The photoinhibition at 50°C for 6 hour, however, showed only 20% increase in the

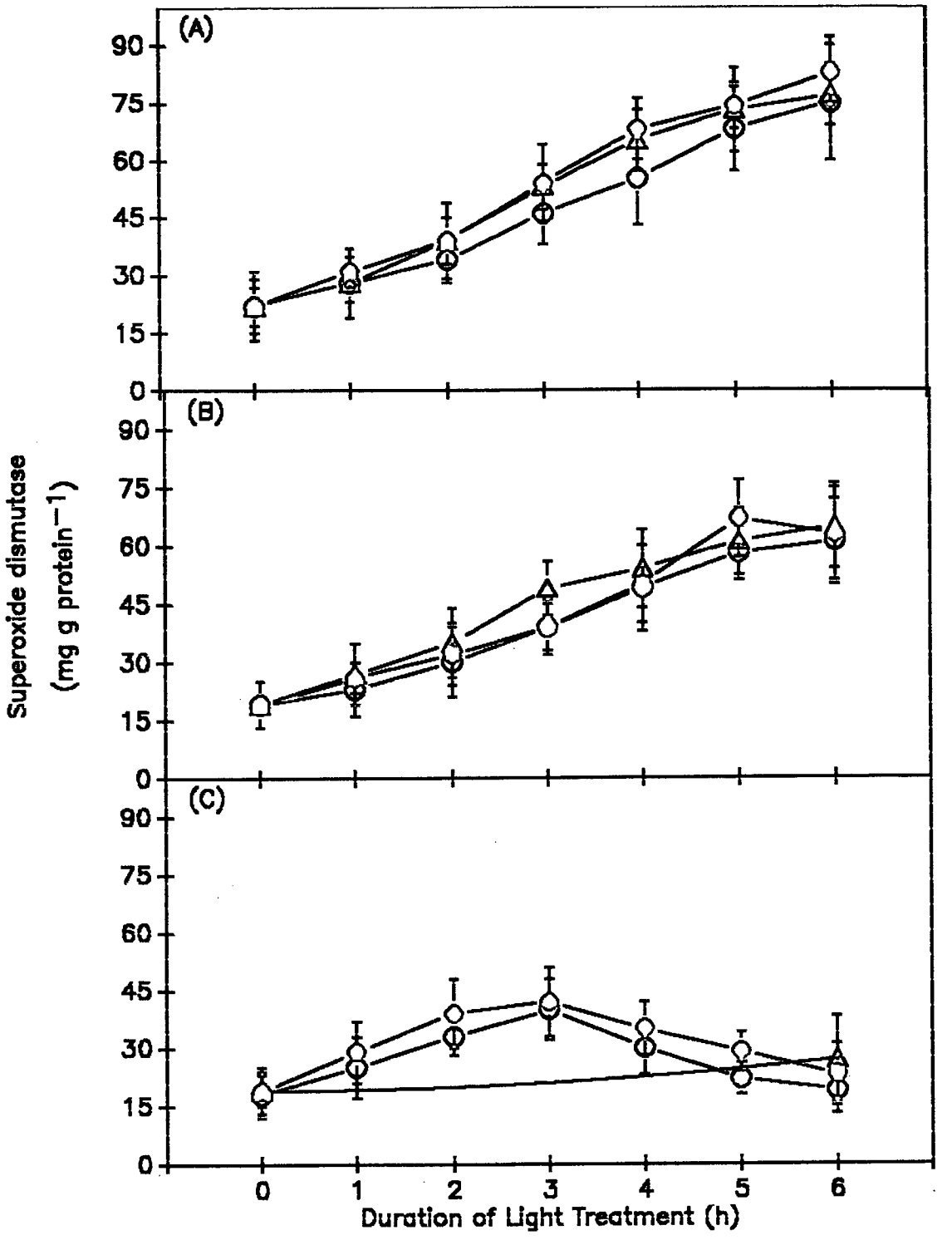


Fig. 3.27: Effect of photoinhibition on superoxide dismutase activity assayed in leaves fed with H_2O (-o-), 5 mM ascorbic acid (-o-) and 4 mM DTT (-Δ-) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 5°C (A), 30°C (B) and 50°C (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

superoxide dismutase activity, as compared to control, which was more or less same as seen in the plants photoinhibited at 3600 μmol m^{-2} s^{-1} and 50°C for the same duration (Fig. 3.26B and 3.25B).

The photoinhibition treatment of the leaves fed with dithiothreitol at 3600 μmol m^{-2} s^{-1} at 5 and 30°C showed a linear increase in the activity. Six hour photoinhibitory treatment at 5°C resulted in an increase of 248% as compared to the control. The extent of increase in the superoxide dismutase activity in case of plants photoinhibited at 50°C for 6 hour was considerably less (40%) compared to the increase seen in leaves photoinhibited at 5°C or 30°C (Fig. 3.25C).

The photoinhibition of dithiothreitol fed leaves at 2200 μmol m^{-2} s^{-1} PAR at 5 and 30°C also showed a linear increase. Six hour photoinhibitory treatment at 5°C and 30°C showed an increase in superoxide dismutase activity by 238% and 196% respectively, as compared to the control (Fig 3.26C). The photoinhibition at 50°C resulted in much less increase (55%) in the superoxide dismutase activity as compared to treatment at 5°C or 30°C (Fig. 3.26C).

In this study it is observed that increase in activity of superoxide dismutase at a particular photoinhibitory temperature was more or less same irrespective of whether leaves were pre treated with distilled water, ascorbate or DTT (Fig. 3.27).

Table 3.14: Effect of Photoinhibition of intact leaves on superoxide dismutase activity assayed from leaves fed with distilled water, ascorbate and DTT and photoinhibited at 3600 & 2200 μmol m^{-2} s^{-1} PAR at 5, 30 and 50°C temperature up to 6 hour. The results are expressed as mg g $protein^{-1}$. Mean±SD values were calculated from 4-6 experiments.

Duration of treatment	H_20			3600 μmol m^{-2} s^{-1} ASC			DTT		
(h)	5°C	30°C	50°C	5°C	30°C	50°C	5°C	30°C	50°C
0	21.83±08	19.32±13	18.03±06	22.19±05	19.32±06	19.32±06	22.19±07	19.32±06	19.32±06
1	28.19±05	23.09±07	25.17±08	30.70±08	26.19±04	29.23±08	28.03±09	27.12±08	-
2	33.87±06	29.71±09	32.83±05	39.46±06	31.92±08	38.75±09	39.12±10	35.30±09	-
3	46.22±08	39.36±06	40.34±08	53.54±10	38.78±07	41.83±09	53.42±06	48.64±07	39.17±07
4	54.90±10	48.77±11	30.27±07	68.3 ± 08	50.36±10	35.11±07	64.81±08	54.05±10	-
5	68.31±11	58.29±07	22.15±04	74.08±06	57.10±10	28.75±05	72.81±11	61.17±09	-
6	75.16±05	61.08±11	18.70±06	83.00±09	63.11±12	22.85±08	77.20±08	65.00±11	27.00±11
	5°C	30°C	50°C	2200 μmol m^{-2} s^{-1} 5°C	30°C	50°C	5°C	30°C	50°C
0	21.83±09	19.32±06	18.03±06	22.19±05	19.32±06	19.32±06	22.19±07	19.32±06	19.32±06
1	24.68±07	21.32±09	26.08±09	27.38±07	25.80±08	-	25.16±07	23.42±07	-
2	30.68±08	26.84±08	31.00±10	33.81±10	29.14±08	-	33.05±09	30.35±07	-
3	40.14±10	33.51±08	43.80±08	45.64±11	33.66±09	36.30±08	44.85±10	40.73±09	35.90±09
4	49.18±11	41.16±08	38.19±10	58.78±09	41.47±10	-	58.93±09	47.26±08	
5	61.18±11	48.92±10	31.87±09	68.04±12	48.56±08	-	67.73±11	52.38±10	
6	70.81±10	54.19±09	27.10±08	75.0 ±07	58.32±09	23.18±07	75.00±10	57.20±09	30.00±08

0 h represent controls. Control leaves were fed with respective solution and kept at growth conditions.

3.9.2 EFFECT OF PHOTOINHIBITION ON ASCORBATE PEROXIDASE:

Intact leaves, pre-treated with distilled water, ascorbate or dithiothreitol were photoinhibited at 3600 μmol m^{-2} s^{-1} or 2200 μmol m^{-2} s^{-1} PAR at all three temperatures (5, 30 and 50°C) upto 6 hour and changes in the ascorbate peroxidase the activity were followed. It was seen that leaves fed with distilled water and photoinhibited at 3600 μmol m^{-2} s^{-1} at 5°C showed a linear increase in the activity of ascorbate peroxidase. One hour photoinhibition treatment caused an increase of 9%, as compared to the control, followed by an increase of 25% after 2 hour of the treatment, 50% after 3 hour, 65% after 4 hour, 75% after 5 hour and 244% after 6 hour of the treatment, as compared to control (Table 3.15). Photoinhibition at 30°C also resulted in the linear increase of the activity of ascorbate peroxidase, however, the increase at 30°C was relatively less than observed at 5°C. Six hour photoinhibitory treatment at 30°C caused only 46% increase in the ascorbate peroxidase activity, as compared to control, which was 39% less than seen in leaves photoinhibited at 5°C for the same duration (Fig. 3.28A). The photoinhibitory treatment at 50°C caused an initial increase in the ascorbate peroxidase activity followed by a decrease in longer treatment. The treatment at 50°C caused 30% increase in the activity after 4 hour of the photoinhibition which was declined to 6% after 5 and 6 hour of the treatment, as compared to ascorbate peroxidase activity observed after 4 hour of the photoinhibition treatment. However, when compared to the ascorbate peroxidase activity in control leaves the increase in the APX activity after six hour of the treatment was only 21% (Fig. 3.28A).

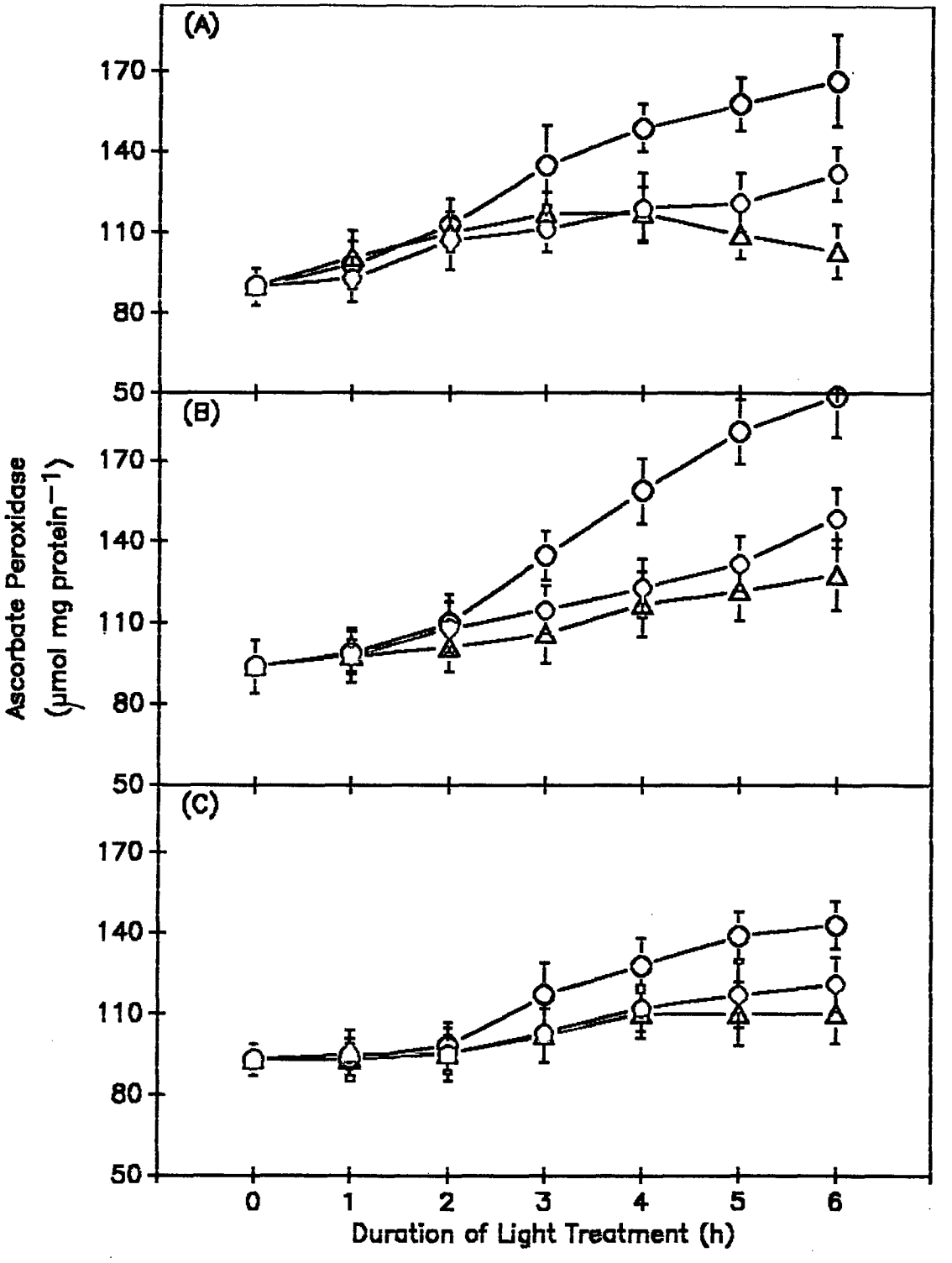


Fig. 3.28: Effect of photoinhibition on ascorbate peroxidase activity in leaves pre treated with H_2O (A), 5 mM ascorbic acid (B) and 4 mM DTT (C) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 5°C (-o-), 30°C (-o-) and 50°C (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

Photoinhibition of distilled water fed leaves at 2200 μmol m^{-2} s^{-1} at 5, 30 and 50°C temperature also showed similar trends in the changes in the activity of ascorbate peroxidase (Table 3.15). The photoinhibition at 2200 μmol m^{-2} s^{-1} at 5°C showed a linear increase of 53% in ascorbate peroxidase activity after 6 hour of the treatment as compared to control (Fig. 3.29A). This increase in the ascorbate peroxidase was 32% less than seen in leaves photoinhibited at 3600 μmol m^{-2} s^{-1} at the same temperature for the same duration (Fig. 3.28A). The photoinhibition treatment for 6 hour at 30°C resulted in the increase in ascorbate peroxidase activity to 30%, as compared to control. The treatment at 50°C, however, showed a linear increase to 26% after 6 hour of the treatment (Fig. 3.29A).

Photoinhibition of ascorbate fed 8 day old sorghum leaves at 3600 μmol m^{-2} s^{-1} PAR and 30°C also resulted in a linear increase in ascorbate peroxidase activity (Table 3.14). The treatment at 5°C for 3 hour linearly increased the ascorbate peroxidase activity by 44%, as compared to the control and 106% after 6 hour of the treatment (Fig. 3.28B). This increase in ascorbate peroxidase activity was 47% greater than seen in leaves photoinhibited at 30°C (59% increase as compared to control) for the 6 hour duration of the treatment. The Photoinhibition of leaves at 50°C showed an linear increase in the activity to 36% increase after six hour of the photoinhibition in the activity.

Photoinhibition of ascorbate fed leaves at 2200 μmol m^{-2} s^{-1} PAR at 5, 30 and 50°C also showed increase in the activity of ascorbate peroxidase as compared to their control (Table 3.15). The photoinhibition treatment at 5°C for 6 hour caused 57%

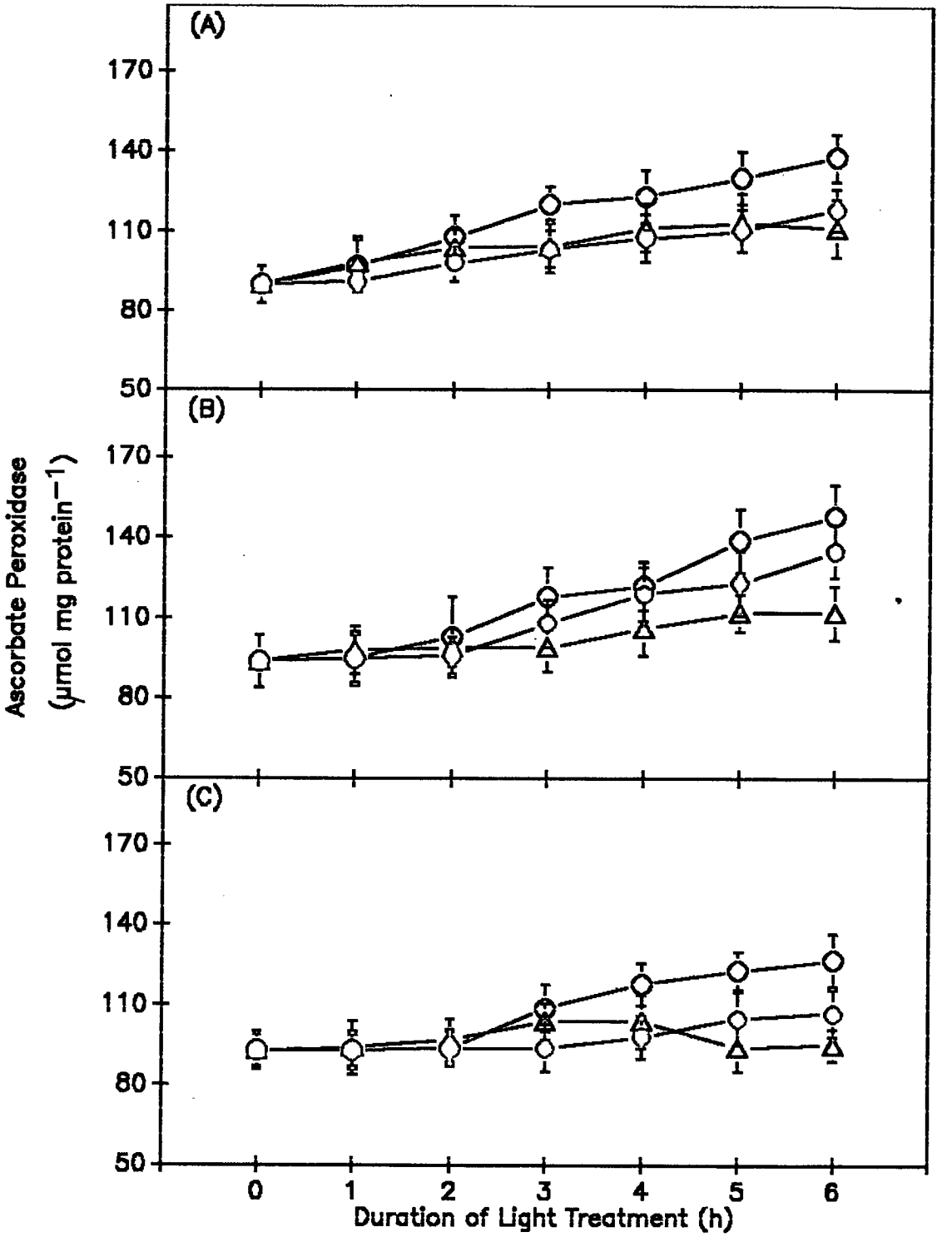


Fig. 3.29: Effect of photoinhibition on ascorbate peroxidase activity in leaves pre treated with H_2O (A), 5 mM ascorbic acid (B) and 4 mM DTT (C) and photoinhibited at 2200 μmol m^{-2} s^{-1} PAR at 5°C (-o-), 30°C (-o-) and 50°C (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

increase in the ascorbate peroxidase activity, as compared to control (Fig. 3.29B). This increase (57%) was 49% less than seen when leaves were photoinhibited at 3600 μmol m^{-2} s^{-1} at the same temperature for the same duration (106%) as compared to their control (Fig. 3.29B). Similarly photoinhibition at 30°C for 6 hour resulted in an increase of 43%, as compared to control which was again less (16%) as compared to seen in leaves photoinhibited at 3600 μmol m^{-2} s^{-1} at 30°C for the same duration. The photoinhibition at 50°C for 6 hour showed only 28% increase in ascorbate peroxidase activity, as compared to control, which was 8% less than seen in the plants photoinhibited at 3600 μmol m^{-2} s^{-1} and 50°C for the same duration (Fig. 3.28B and 3.29B).

The photoinhibition treatment of the leaves fed with dithiothreitol at 3600 μmol m^{-2} s^{-1} at 5 and 30°C showed a linear increase in the activity. Six hour photoinhibitory treatment at 5 and 30°C resulted in an increase of 54% and 30% respectively, as compared to the control (Fig. 3.28C). The extent of increase in the of ascorbate peroxidase activity in case of plants photoinhibited at 50°C for 6 hour was considerably less (18%) compared to increase seen in leaves photoinhibited at 5°C or 30°C (Fig. 3.28C).

The photoinhibition of dithiothreitol fed leaves at 2200 μmol m^{-2} s^{-1} PAR at 5 and 30°C also showed a linear increase. Six hour photoinhibitory treatment at 5°C and 30°C showed an increase in ascorbate peroxidase activity by 36% and 15% respectively as compared to the control (Fig 3.29C). The photoinhibition at 50°C resulted in much less

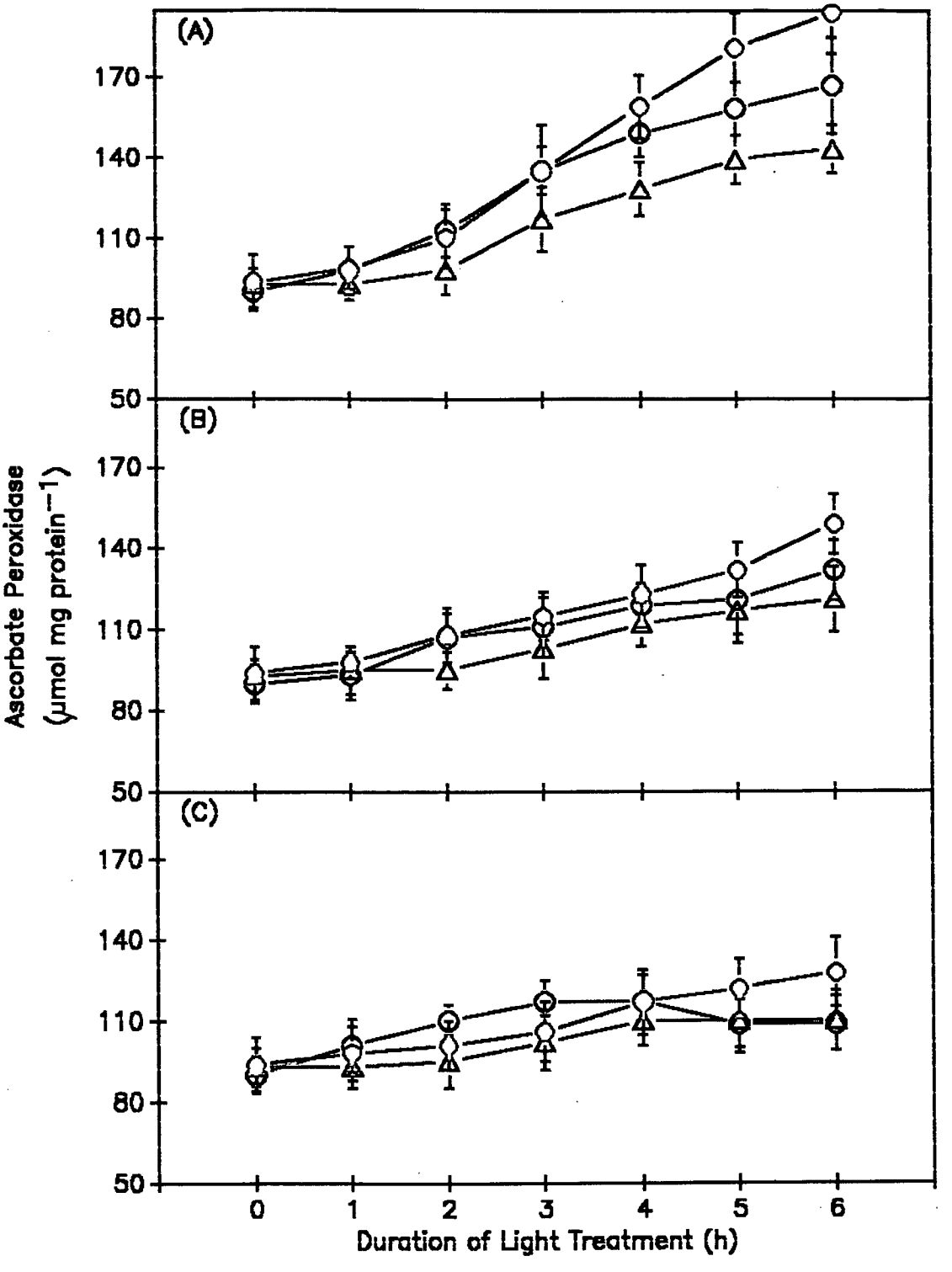


Fig. 3.30: Effect of photoinhibition on ascorbate peroxidase activity assayed in leaves fed with H_2O (-o-), 5 mM ascorbic acid (-o-) and 4 mM DTT (-Δ-) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 5°C (A), 30°C (B) and 50°C (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

Table 3.15: Effect of Photoinhibition of intact leaves on ascorbate peroxidase activity assayed from leaves fed with distilled water, ascorbate and DTT and photoinhibited at 3600 & 2200 μmol m^{-2} s^{-1} PAR at 5, 30 and 50C temperature up to 6 hour. The results are expressed as μmol mg $protein^{-1}$. Mean±SD values were calculated from 4-6 experiments.

Duration of treatment	H_20			3600 μmol m^{-2} s^{-1} ASC			DTT		
(h)	5°C	30°C	50°C	5°C	30°C	50°C	5°C	30°C	50°C
0	90.19 ±07	90.19 ±07	90.19 ±07	94.0 ±10	94.0 ±10	94.00 ±10	93.23 ±06	93.23 ±06	93.23 ±06
1	98.2 ±09	92.85 ±09	101.30±10	99.15 ±08	97.77 ±06	98.36 ±09	93.0 ±08	95.36 ±09	93.0 ±08
2	112.73±10	107.35±09	109.75±06	119.59±10	108.3 ±10	100.78±09	98.2 8±09	95.42 ±07	95.7 ±10
3	135.6 ±17	110.78±11	117.0 ±08	138.18± 09	114.40±09	106.80±11	116.89±02	102.73±11	102.11±10
4	149.18±09	118.50±08	117.35±10	158.36±12	122.88±11	177.68±12	128.14±10	112.00±08	110.00±09
5	157.78±10	120.9 ±13	109.82±09	180.63±13	132.70±10	122.35±11	130.29±09	116.84±12	110.87±12
6	167.3 ±18	132.0 ±11	109.53±10	194.2 ±15	149.10±11	128.07±13	144.73±09	121.38±10	110.13±11
				2200 μmol m^{-2} s^{-1}					
	5°C	30°C	50°C	5°C	30°C	50°C	5°C	30°C	50°C
0	90.19 ±07	90.19 ±07	90.19 ±07	94.0 ±10	94.0 ±10	94.0 ±10	93.23 ±06	93.23 ±06	93.23 ±06
1	97.85 ±10	91.0 ±04	98.2 ±10	95.13 ±10	95.17 ±09	98.06 ±10	93.2 ±07	93.53 ±06	94.0 ±10
2	108 ±08	97.62 ±07	104.15±08	103.40±15	95.95 ±07	99.38 ±09	93.79 ±06	94.0 ±07	96.73 ±08
3	120.28±07	103.13±07	104.80±07	117.82±11	108.00±09	99.52 ±11	109.03±09	94.3 ±09	104.42 ±07
4	122.93±10	107.35±09	111.00±10	122.30±09	119.00±10	106.81±12	118.38±08	97.83 ±08	104.00 ±10
5	130.18±10	110.20±07	113.27±10	139.00±12	123.42±12	112.22±11	123.71±07	104.53±10	95.7 ±09
6	138.3 ±07	117.6 ±08	114.0 ±09	147.87±12	134.67±10	120.17±10	127.08±10	107.46±09	95.7 ±06

0 h represent controls. Control leaves were fed with respective solution and kept at growth conditions.

increase (3%) in the of ascorbate peroxidase activity as compared to the treatment at 5°C or 30°C (Fig. 3.29C).

In this study it is observed that increase in activity of ascorbate peroxidase at a 30°C and 50°C was more or less same irrespective of whether leaves were pre treated with distilled water, ascorbate or DTT. However, the treatment at 5°C of leaves pre treated with distilled water, ascorbic acid and DTT showed changes (Fig. 3.30). It was seen that leaves pre treated with ascorbic acid and photoinhibited at 5°C showed greater increase in ascorbate peroxidase activity than seen in leaves pre treated with distilled water and DTT (Fig. 3.30).

3.9.3 EFFECT OF PHOTOINHIBITION ON CATALASE ACTIVITY

Intact leaves pre treated with distilled water, ascorbate or dithiothreitol were photoinhibited at 3600 or 2200 μmol m^{-2} s^{-1} PAR at all the three temperature (5, 30 and 50°C) for 6 hour. Unlike results seen in superoxide dismutase and ascorbate peroxide activity, catalase activity declined in all the treatment (Table. 3.16). The photoinhibitory treatment at 50°C was highly inhibitory. The photoinhibitory treatment at 50°C for longer duration (greater than 4 hour) resulted in complete inhibition of catalase activity irrespective of light intensity (3600 or 2200 μmol m^{-2} s^{-1}; Fig. 3.31A). The photoinhibition of leaves fed with distilled water at 5°C resulted in a linear decrease. A six hour photoinhibition treatment decreased the activity to 76%, as compared to control (Fig. 3.31A). The same light treatment at 30°C, however caused less decrease in the activity of catalase. Only 55% decrease in catalase activity was seen after 6 hour of the photoinhibition treatment at 30°C. The photoinhibition at 50°C

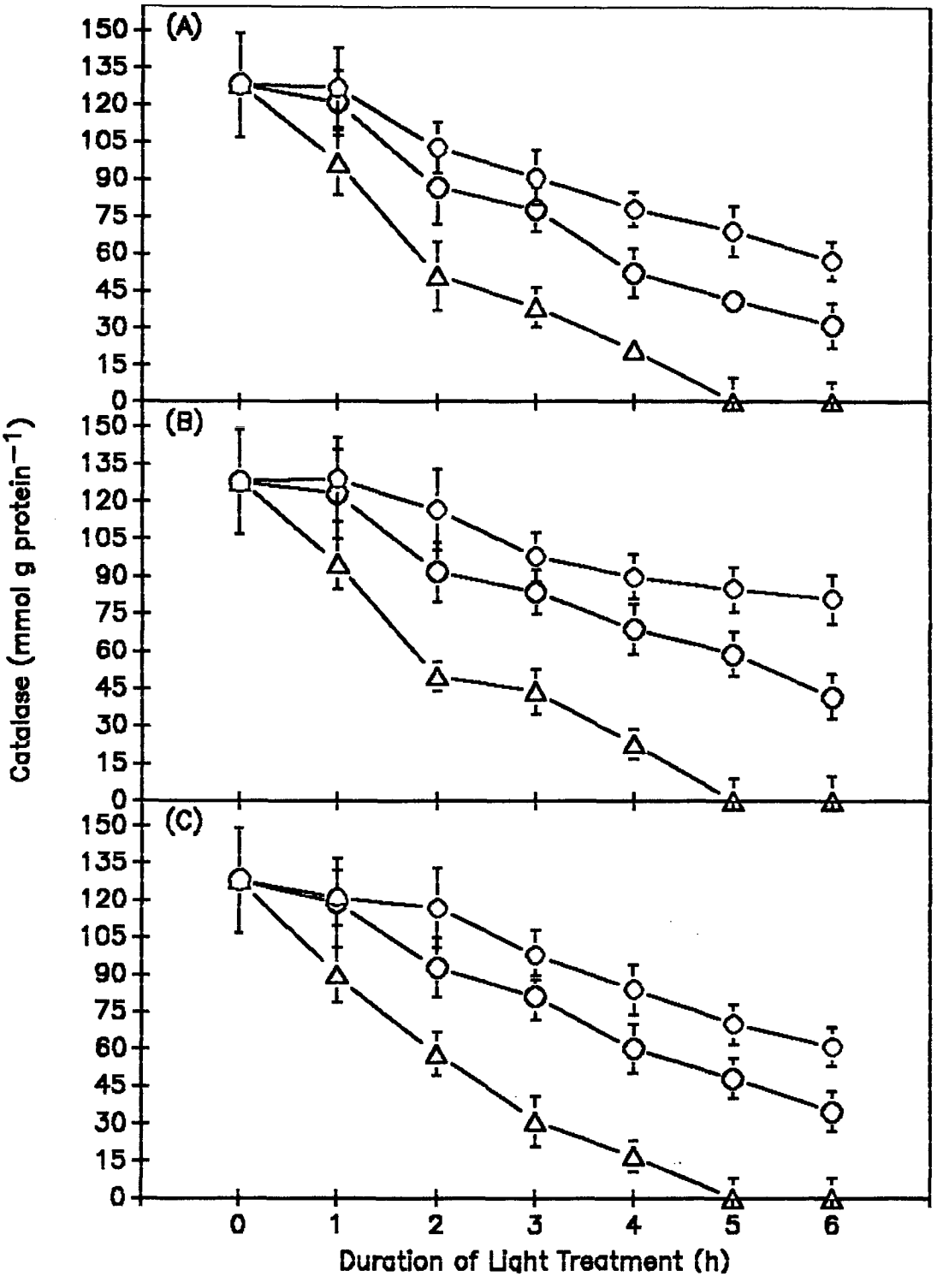


Fig. 3.31: Effect of photoinhibition on catalase activity in leaves pre treated with H_2O (A), 5 mM ascorbic acid (B) and 4 mM DTT (C) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 5°C (-o-), 30°C (-o-) and 50°C (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

resulted in a much rapid decrease in the catalase activity. The photoinhibition at 50°C for 4 hour brought down the activity to 83%, as compared to control. Further increase in the duration of the treatment completely inhibited the activity (Fig. 3.31A).

Photoinhibition of leaves pre treated with ascorbate and photoinhibited at 3600 μmol m^{-2} s^{-1} at 5°C again decreased the catalase activity by 67% after 6 hour of the treatment, as compared to control (Fig. 3.31B). However, the decrease in the catalase activity was comparatively much less in leaves photoinhibited at 30°C (37% decrease after 6 hour of the treatment; Fig. 3.31B). The photoinhibition at 50°C resulted in complete inhibition after 5 hour of the treatment (Fig. 3.31B).

Photoinhibition of dithiothreitol fed leaves at 3600 μmol m^{-2} s^{-1} at 5°C and 30°C after 6 hour of the treatment resulted in decrease catalase activity to 73% and 52% respectively, as compared to control (Fig. 3.31C). The photoinhibition treatment at 50°C declined the catalase activity to 86% after 4 hour of the treatment, longer duration of the treatment caused complete inhibition of the activity in leaves photoinhibited at 50°C (Fig. 3.31C).

Similar results were obtained when leaves were photoinhibited at 2200 μmol m^{-2} s^{-1} at 5, 30, and 50°C except that extent of inhibition was relatively less than seen in leaves treated at 3600 μmol m^{-2} s^{-1} at respective temperatures (Table 3.16). The results indicate that photoinhibition at 50°C was more inhibitory as compared to the light treatment at 30°C (Fig. 3.32A). Leaves fed with ascorbate prior to photoinhibition treatment also showed comparatively less inhibition in catalase activity compared to leaves fed with distilled water or dithiothreitol for the same duration (Fig. 3.32).

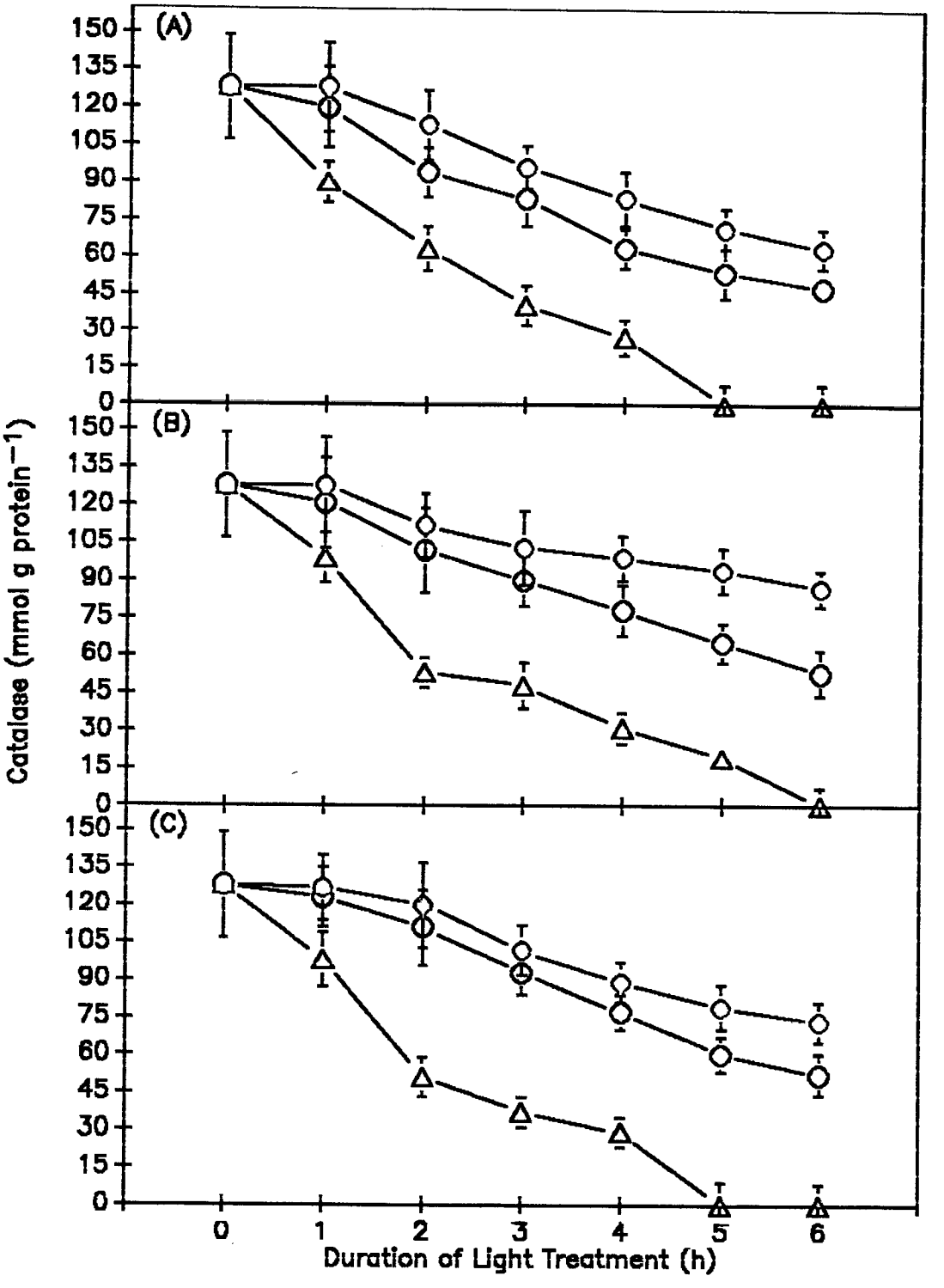


Fig. 3.32: Effect of photoinhibition on catalase activity in leaves pre treated with H_2O (A), 5 mM ascorbic acid (B) and 4 mM DTT (C) and photoinhibited at 2200 μmol m^{-2} s^{-1} PAR at 5°C (-o-), 30°C (-o-) and 50°C (-Δ-). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

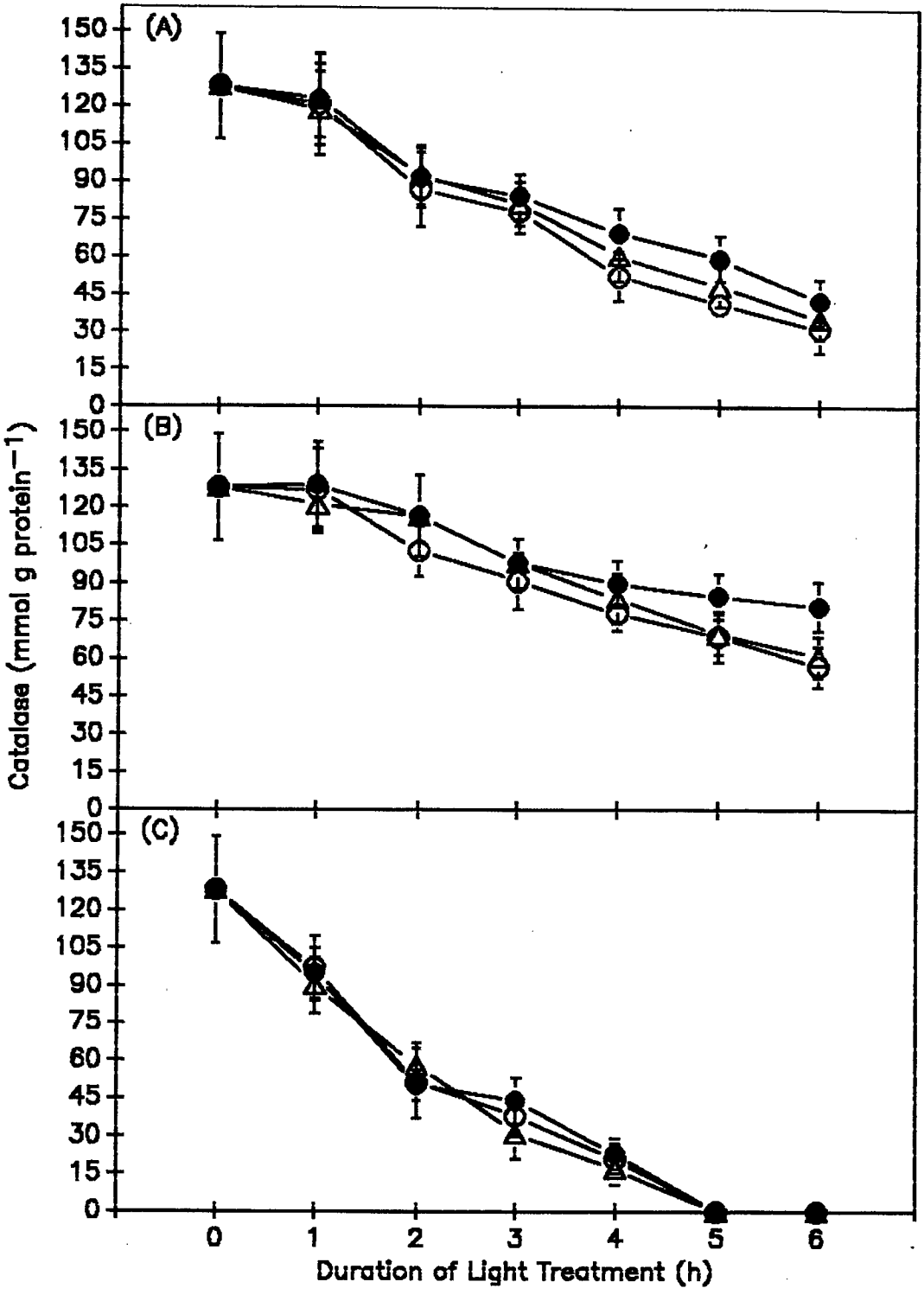


Fig. 3.33: Effect of photoinhibition on catalase activity assayed in leaves fed with H_2O (-o-), 5 mM ascorbic acid (-●-) and 4 mM DTT (-Δ-) and photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 5°C (A), 30°C (B) and 50°C (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

Table 3.16: Effect of Photoinhibition of intact leaves on catalase activity assayed from leaves fed with distilled water, ascorbate and DTT and photoinhibited at 3600 & 2200 μmol m^{-2} s^{-1} PAR at 5, 30 and 50°C temperature up to 6 hour. The results are expressed as mmol mg $protein^{-1}$. Mean±SD values were calculated from 4-6 experiments.

Duration of treatment	H_20			3600 μmol m^{-2} s^{-1} ASC			DTT		
(h)	5°C	30°C	50°C	5°C	30°C	50°C	5°C	30°C	50°C
0	128.19±21	128.19±21	128.19±21	128.19±21	128.19±21	128.19±21	128.19±21	128.19±21	128.19±21
1	121.27±13	127.10±16	97.4 ±13	123.3 ±18	129.39±17	95.00 ±10	119.00±18	121.00±11	0
2	87.23 ±15	103.31±10	51.07 ±14	92.05 ±12	117.35±16	50.26 ±06	93.27 ±12	117.36±16	58.12 ±09
3	78.3 ±09	91.37 ±11	38.24 ±08	84.25 ±09	98.47 ±10	44.90 ±09	81.2 ±09	97.60 ±10	30.73 ±10
4	52.11 ±10	78.08 ±07	21.37 ±06	68.98 ±10	90.22 ±09	23.14 ±06	59.73 ±10	84.30 ±10	17.41 ±06
5	41.07 ±06	69.14 ±10	0	59.42 ±09	84.63 ±09	0	47.61 ±08	70.41 ±08	0
6	30.92 ±09	57.12 ±08	0	42.31 ±09	80.75 ±10	0	35.1 ±08	60.93 ±08	0
	5°C	30°C	50°C	2200 μmol m^{-2} s^{-1} 5°C	30°C	50°C	5°C	30°C	50°C
0	128.19±21	128.19±21	128.19±21	128.19±21	128.19±21	128.19±21	128.19±21	128.19±21	128.19±12
1	120.0 ±16	128.30±18	90.08 ±08	121.21±18	128.00±19	99.27 ±10	123.35±12	127.10±13	98.0 ±11
2	94.14 ±10	112.51±14	63.14 ±09	101.73±17	111.83±13	53.41 ±06	111.07±15	120.82±17	50.79 ±08
3	82.77 ±11	96.2 ±09	40.22 ±08	90.18 ±10	103.41±15	48.71 ±09	92.81 ±09	101.11±10	37.20 ±06
4	63.35 ±08	83.07 ±11	27.10 ±07	78.51 ±09	98.51 ±09	31.05 ±06	76.51 ±07	88.8 ±08	29.49 ±06
5	53.46 ±10	71.37 ±08	0	65.17 ±08	94.13 ±09	19.80 ±05	60.4 ±07	79.3 ±09	0
6	47.35 ±06	62.78 ±08	0	53.19 ±09	87.78 ±07	0	51.83 ±08	73.2 ±08	0

0 h represent controls. Control leaves were fed with respective solution and kept at growth conditions.

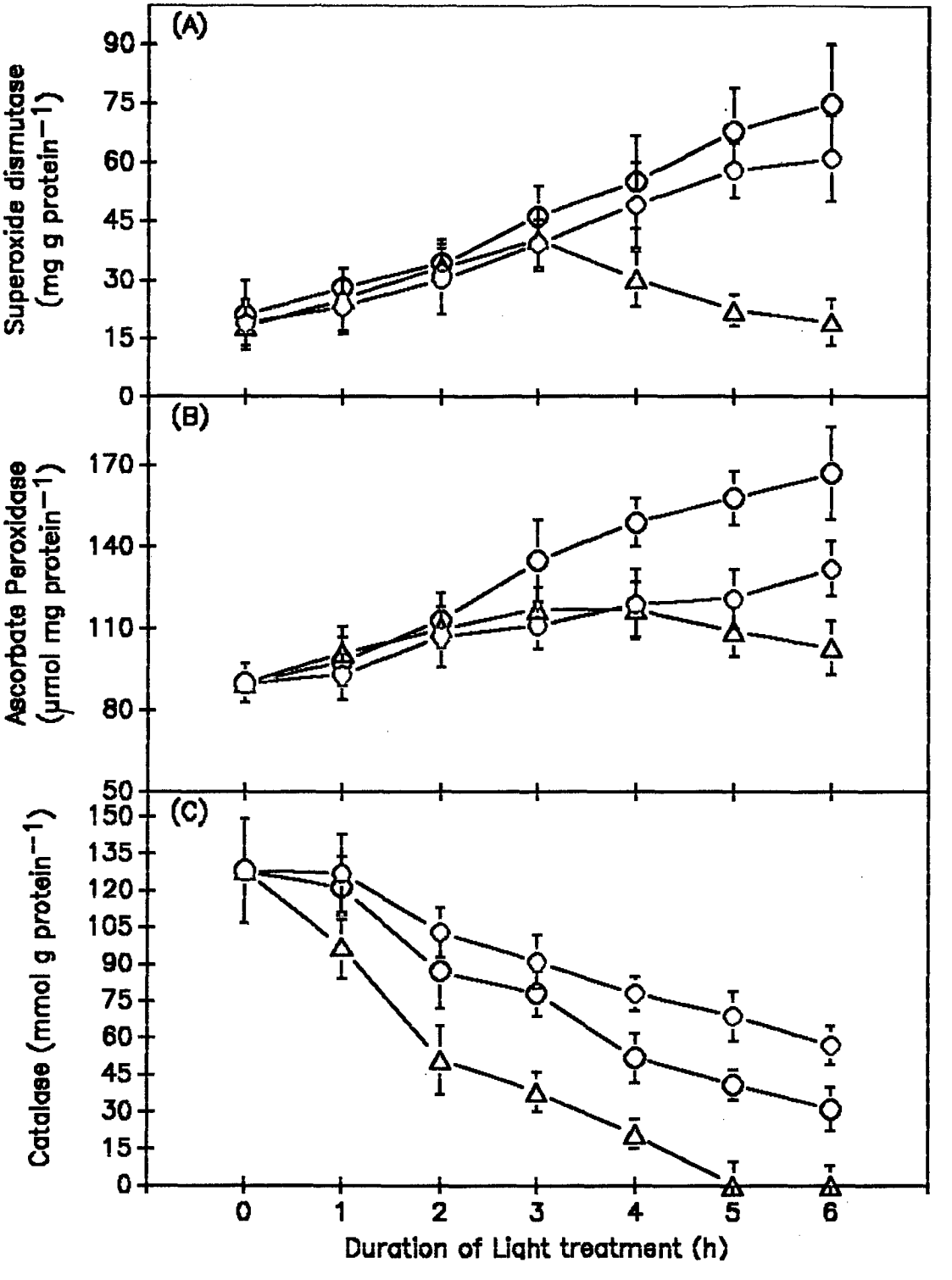


Fig. 3.34: Effect of photoinhibition of intact leaves at 3600 µmol m^{-2} s^{-1} PAR at 5°C (-o-), 30°C (-o-) and 50°C (-Δ-) temperature on activity of superoxide dismutase (A), ascorbate peroxidase (B) and catalase (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

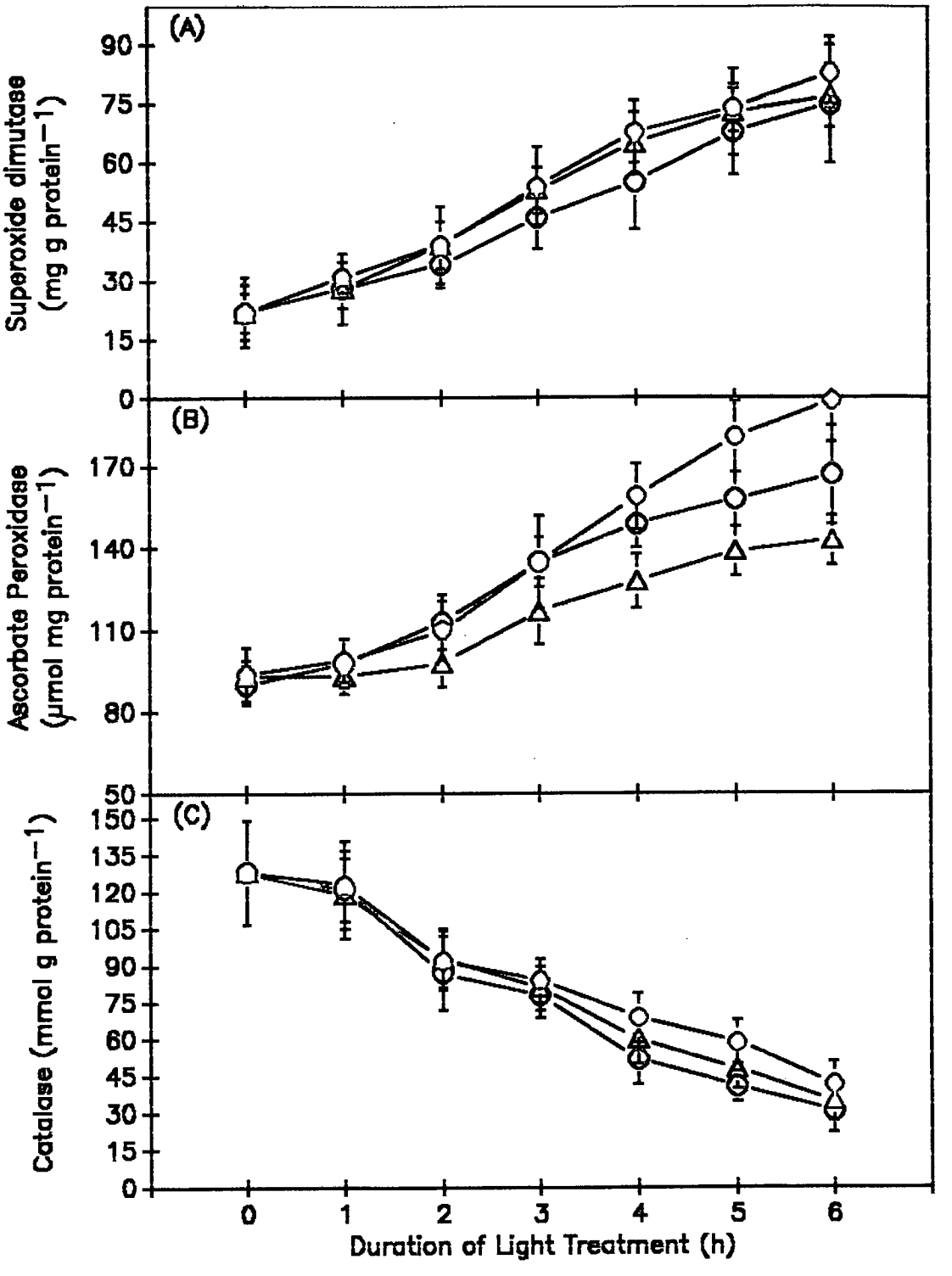


Fig. 3.35: Effect of photoinhibition of intact leaves at 3600 µmol m^{-2} s^{-1} PAR at 5°C in leaves pre treated with H_2O (-o-), 5 mM ascorbic acid (-o-) and 4 mM DTT (-Δ-) on activities of superoxide dismutase (A), ascorbate peroxidase (B) and catalase (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

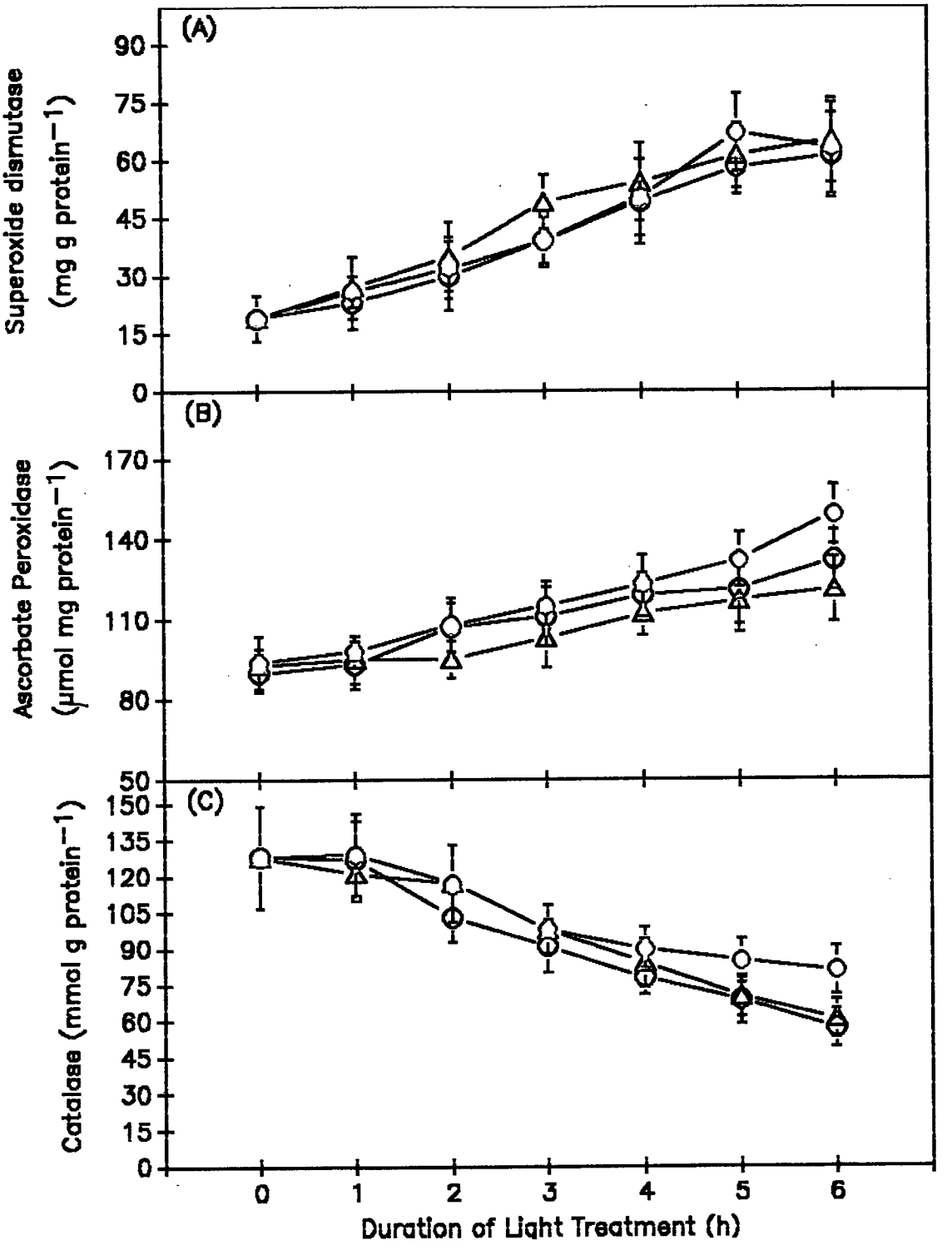


Fig. 3.36: Effect of photoinhibition of intact leaves at 3600 µmol m^{-2} s^{-1} PAR at 30°C in leaves pre treated with H_2O (-o-), 5 mM ascorbic acid (-o-) and 4 mM DTT (-Δ-) on activities of superoxide dismutase (A), ascorbate peroxidase (B) and catalase (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

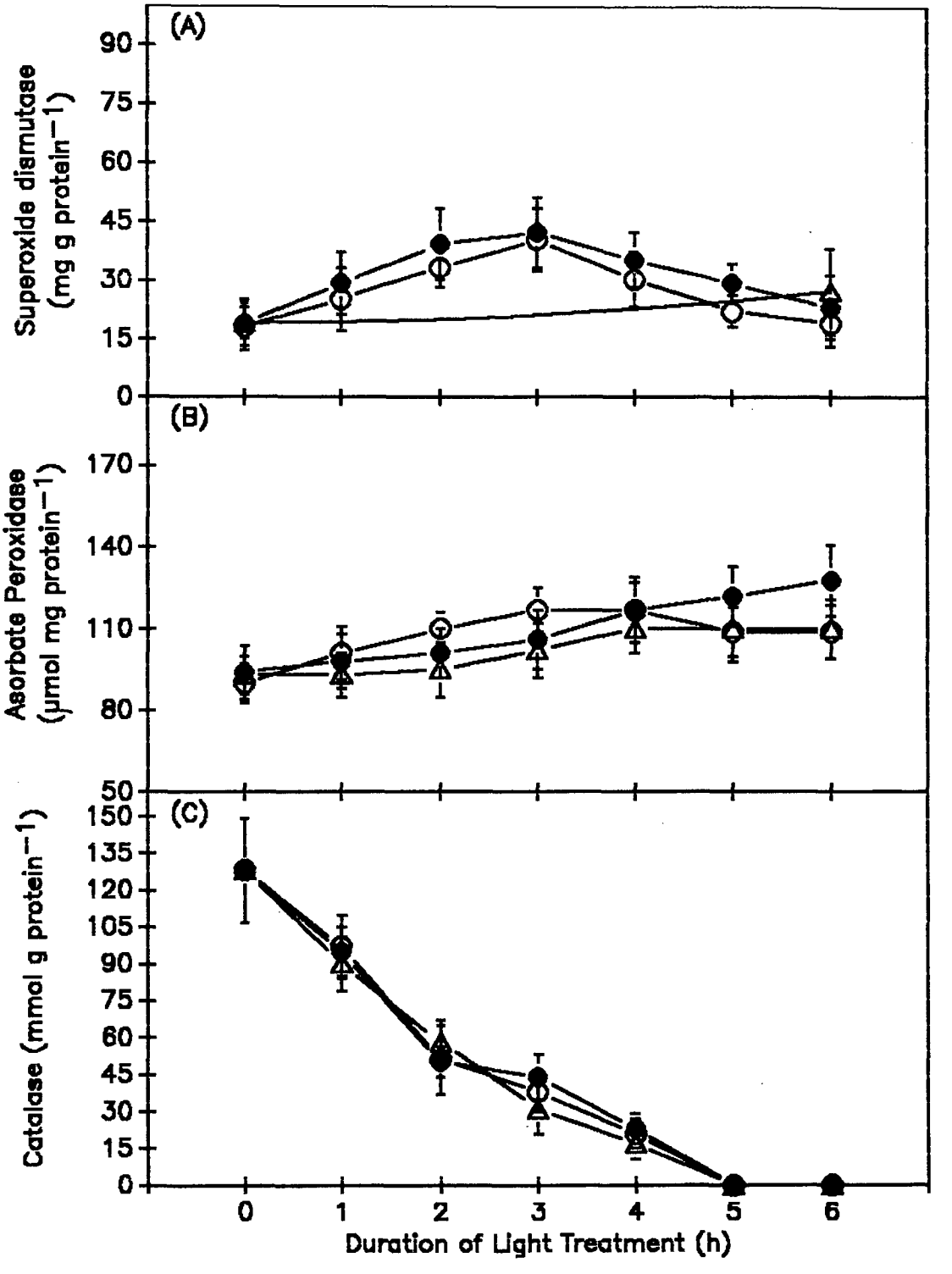


Fig. 3.37: Effect of photoinhibition of intact leaves at 3600 µmol m^{-2} s^{-1} PAR at 50°C in leaves pre treated with H_2O (-o-), 5 mM ascorbic acid (-●-) and 4 mM DTT (-Δ-) on activities of superoxide dismutase (A), ascorbate peroxidase (B) and catalase (C). The points represent 5-6 separate sets of experiments. Vertical bars show standard deviation.

Figure 3.34 show the effect of photoinhibition at different temperature on SOD, APX and catalase activity. Fig. 3.35, 3.36 and 3.37 show the effect of photoinhibition at 5, 30 and 50°C respectively at light intensity of 3600 on the activation of superoxide dismutase, ascrbate peroxidase and catalase in distileld water, ascorbate and DTT fed leaves. It was seen that temperature played a greater role on the activites of the antioxidant enzymes studied than the pre treated leaves with distilled water, ascorbic acid and DTT.

3.10 EFFECT OF PHOTOINHIBITION OF LEAVES TREATED WITH EXOGENOUS ABSCISIC ACID DURING GROWTH:

Sorghum plants treated for four days (for 5-8 day of growth) with exogenous supply of abscisic acid in order to elucidate the relationship between abscisic acid and Xanthophyll Cycle and qN. The treated leaves were photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 30°C up to 6 hours. It was seen that sorghum leaves treated with abscisic acid showed a much lesser level of decrease in Fv/Fm ratio as a result pf photoinhibition treatment compared to leaves which were not fed with abscisic acid (Table 3.17). The decrease in Fv/Fm ratio in plants grown in ABA supplemented medium and photoinhibited for 3 hours was 36% as compared 64% decrease observed in Fv/Fm ratio in plants grown without any abscisic acid. The decrease in Fv/Fm ratio was further increased with increase in the duration of the treatment. Six hour of the photoinhibition treatment of plants grown in abscisic acid supplemented medium resulted in 56% decrease in the Fv/Fm ratio compared to 73% decrease seen in plants grown without any abscisic acid for the same duration of the photoinhibition.

Table 3.17: Effect of photoinhibition of intact leaves grown with or without exogenous abscisic acid (10^{-5} M) supplemented Hoagland solution on room temperature chlorophyll fluorescence (Fv/Fm ratio, qP and qN) and xanthophyll contents. The photoinhibition treatment was given at 3600 μmol m^{-2} s^{-1} PAR at 30°C up to 6 hour. Mean±SD values were calculated from 5 experiments. Values of pigments are presented as μg . g FW^{-1}.

Duration of treatment (h)	Without ABA Fv/Fm	qP	qN	With ABA Fv/Fm	qP	qN
0	0.784±0.02	0.505±0.05	0.366±0.03	0.786±0.02	0.610±0.03	0.609±0.03
1	0.485±0.02	0.523±0.04	0.384±0.02	0.776±0.02	0.592±0.03	0.644±0.02
2	0.328±0.03	0.531±0.04	0.443±0.03	0.620±0.03	0.607±0.05	0.649±0.06
3	0.280±0.02	0.531±0.04	0.589±0.02	0.501±0.05	0.621±0.03	0.663±0.07
4	0.203±0.03	0.487±0.03	0.595±0.05	0.426±0.04	0.597±0.03	0.639±0.04
5	0.211±0.01	0.479±0.04	0.538±0.04	0.351±0.04	0.600±0.02	0.639±0.05
6	0.208±0.02	0.433±0.03	0.548±0.02	0.343±0.04	0.583±0.03	0.633±0.07
Xanthophylls		**0 hour**	**6 hour**		**0 hour**	**6 hour**
Violaxanthin		19.33±1.6	15.68±1.3		24.28±2.3	20.80±1.7
Antheraxanthin		1.72 ±0.4	0.98 ±0.4		1.12 ±0.6	1.33 ±0.5
Zeaxanthin		00 ±0.0	4.60 ±0.5		00 ±0.0	6.32 ±0.5
V+A+Z		21.05±1.1	21.26±1.3		25.40±1.0	28.45±1.5

0 h represent controls. Control leaves were kept at growth conditions.

The plants grown in abscisic acid supplemented Hoagland solution and photoinhibited also showed higher values of qP and qN, as compared to plants which were grown without abascisic acid. The abscisic acid treated plants without any photoinhibitory treatment showed a 66% higher value of qN compared to plants grown without ABA. Subsequent photoinhibition of abscisic acid grown plants showed a slight increase in the qN value (3-9% for various duration of photoinhibitory treatment). However, photoinhibitory treatment of plants grown without abscisic acid showed a much greater increase (up to 65%) in the qN value as compared to control but the actual qN value (0.548 after 6 hour of the treatment) remained lower than seen in the plants grown with abscisic acid (0.633 after 6 hour of the photoinhibition treatment).

When these observation of chlorophyll fluorescence were compared with the changes in Xanthophyll Cycle it was seen that pool of V+A+Z was higher (25.4 μg. g FW^{-1}) in leaves grown with abscisic acid than without abscisic acid (19.13 μg . g FW^{-1}). No Zeaxanthin was observed in either type of leaves without photoinhibitory treatment. Violaxanthin content was much greater in leaves grown with abscisic acid (24.28 μg. g FW^{-1}) than was observed in leaves grown without abscisic acid (19.33 μg. g FW^{-1}). However, when leaves grown with abscisic acid were photoinhibited at 3600 μmol m^{-2} s^{-1} PAR at 30°C, the V de-epoxidised to Z to a greater extent (6.32 μg. g FW) compared to the Zeaxanthin formation observed in leaves grown without abscisic acid (4.6 μg. g FW^{-1}). Antheraxanthin also showed variable changes in both with and without abscisic acid grown plants (Table 3.17).

Table 3.18: Effect of phtoinhibition on ABA content measured using HPLC in 8 day old sorghum leaves grown with or without exogenous abscisic acid (10^{-5} M) supplemented Hoagland solution. The photoinhibition treatment was given at 3600 μmol m^{-2} s^{-1} PAR at 30°C up to 6 hour. Mean±SD values were calculated from 3 experiments. Values of ABA are presented as μg . mg FW^{-1} were calculated using +ABA as an external standard.

Duration of treatment (h)	Without ABA		With ABA	
	3600μmol m^{-2} s^{-1}	2200 μmol m^{-2} s^{-1}	3600 μmol m^{-2} s^{-1}	2200 μmol m^{-2} s^{-1}
0	0.104±0.02	0.104±0.02	0.120±0.03	0.120±0.03
1	0.219±0.05	0.202±0.05	0.217±0.05	0.303±0.04
2	-	-	0.258±0.03	0.314±0.04
3	0.283±0.05	0.289±0.06	0.263±0.04	0.265±0.05
4	-	-	-	-
5	-	-	0.236±0.06	0.303±0.06
6	0.210±0.04	0.278±0.05	0.216±0.04	0.256±0.05

0 h represent controls. Control leaves were kept at growth conditions.

3.10.1 ABSCISIC ACID CONTENT:

ABA content of leaves grown on Hoagland medium supplemented with or without exogenous ABA (10^{-5} M) is shown in table 3.18. The ABA content in plants grown with ABA supplemented Hoagland medium and not photoinhibited was 15% higher compared to plants grown without ABA supplemented medium. Six hour photoinhibition treatment at either 2200 or 3600 μmol m^{-2} s^{-1} PAR at 30°C resulted in slightly higher level of endogenous level of ABA content in plants grown without ABA supplemented medium (Table 3.18).

3.11 EFFECT OF PHOTOINHIBITIOIN OF INTACT LEAVES ON PROTEIN PROFILE OF THYKLAKOID MEMBRANE:

Effect of light 3600 μmol m^{-2} s^{-1} PAR at 5, 30 and 50°C was studied on protein profile of thylakoid membrane in order to study the effect of photoinhibition treatment on changes in the polypeptides. The figure 3.38 show the changes in the polypeptides of thylakoid membrane. It was seen that photoinhibition at 5, 30 and 50°C resulted in degradation of 45 KDa protein which bind to chlorophyll pigments. Slight degradation of 32 KDa protein was also seen. Low molecular weight protein were also affected due to the photoinhibition treatment (Fig. 3.39).

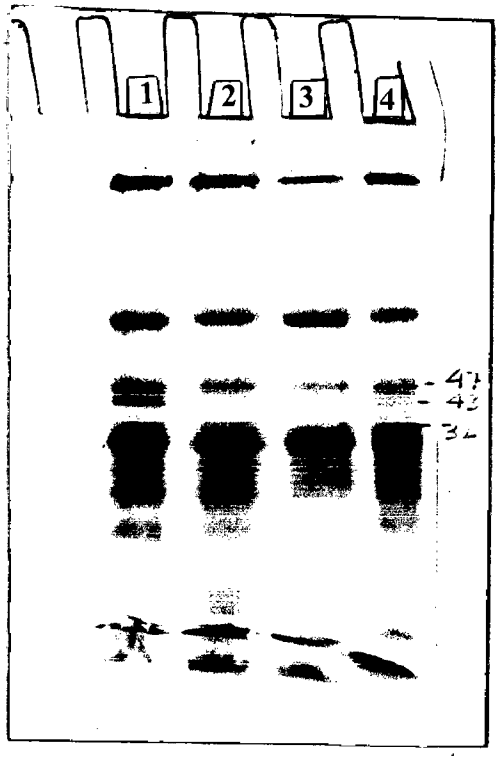
Fig. 3.38 Pigment profile of thylakoid isolated from photoinhibited sorghum leaves.

1. Control

2. Photoinhibition of leaves at5°C and 3600 μmol m^{-2} s^{-1}

3. Photoinhibition of leaves at30°C and 3600 μmol m^{-2} s^{-1}

4. Photoinhibition of leaves at50°C and 3600 μmol m^{-2} s^{-1}


1
2
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47
43
34

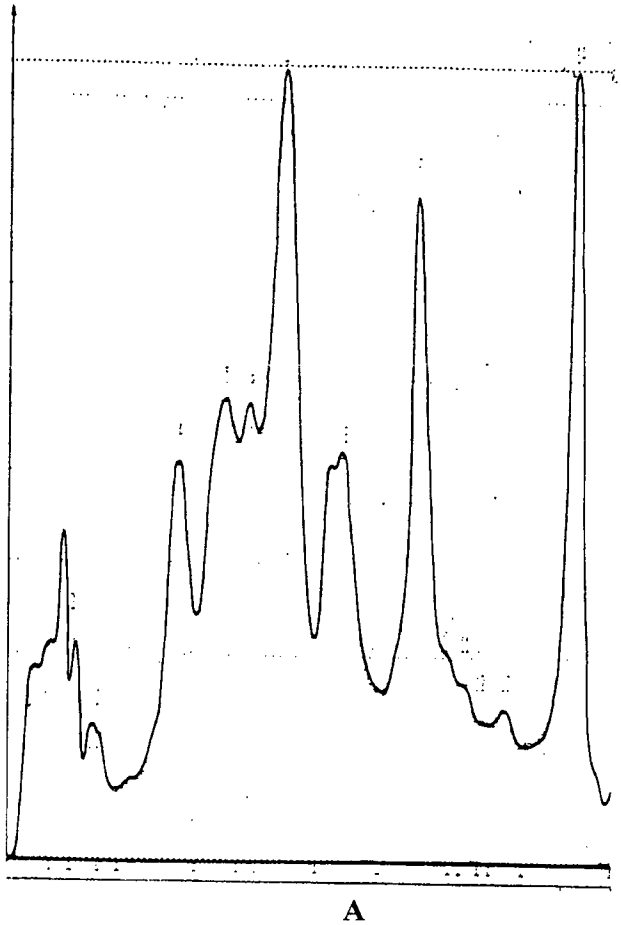
Fig. 3.39 Densitometer tracing of SDS_PAGE gel of thylakoid membranes showing qualitative and quantitative changes due to photoinhibition of leaves. MW are indicated in KD.

(A) Control leaves.

(B) Photoinhibition at 5°C and 3600 μmol m^{-2} s^{-1} for 6 h.

(C) Photoinhibition at 30°C and 3600 μmol m^{-2} s^{-1} for 6 h.

(D) Photoinhibition at 50°C and 3600 μmol m^{-2} s^{-1} for 6 h.



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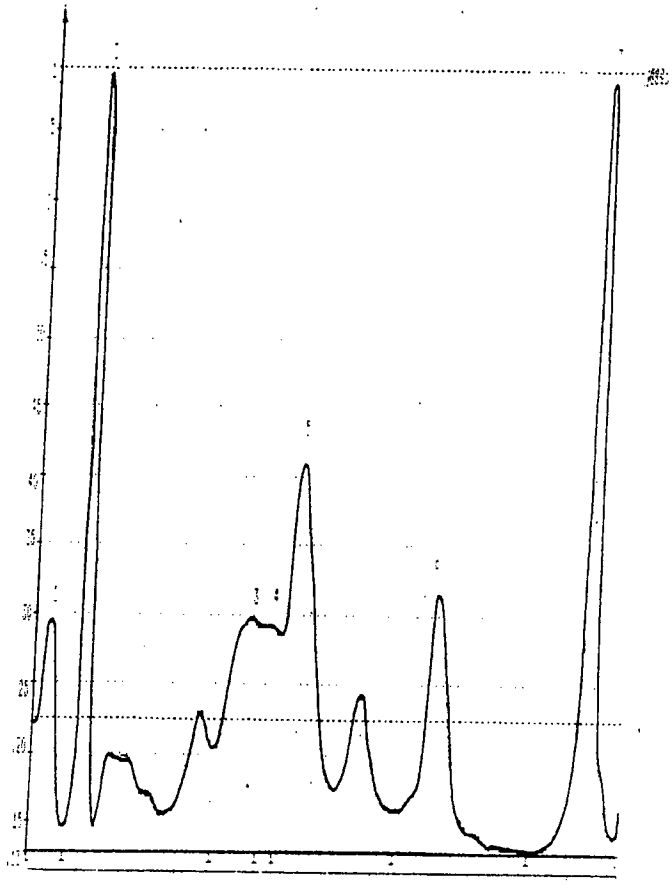

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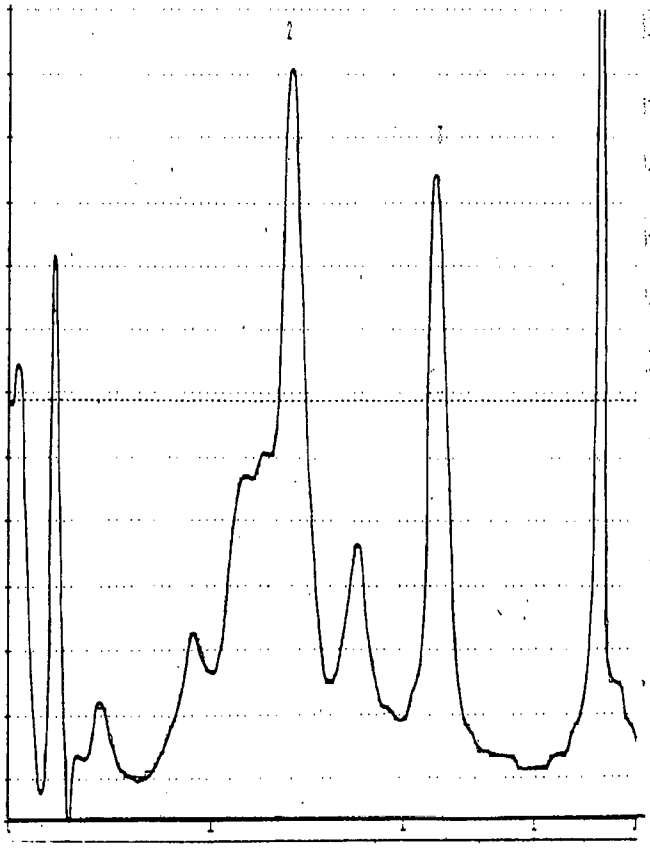

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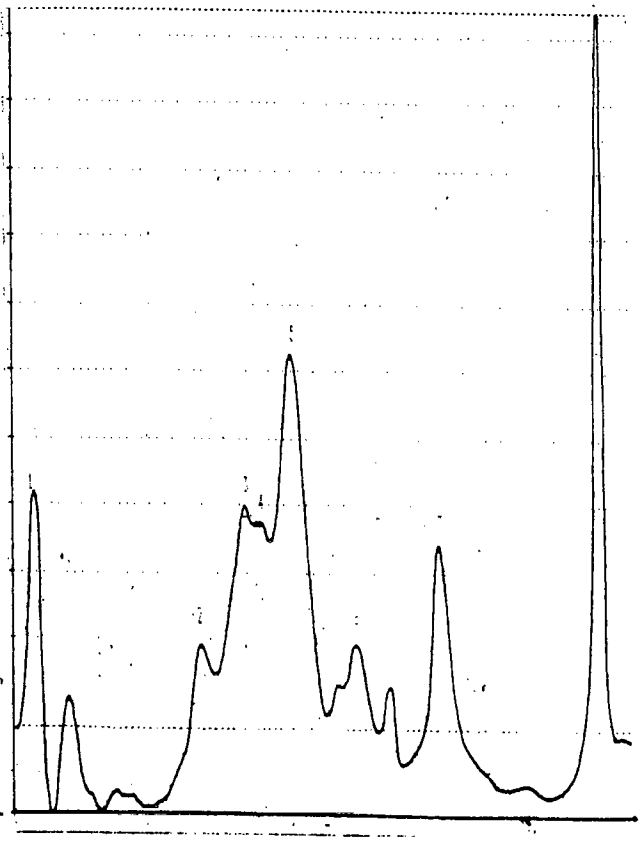
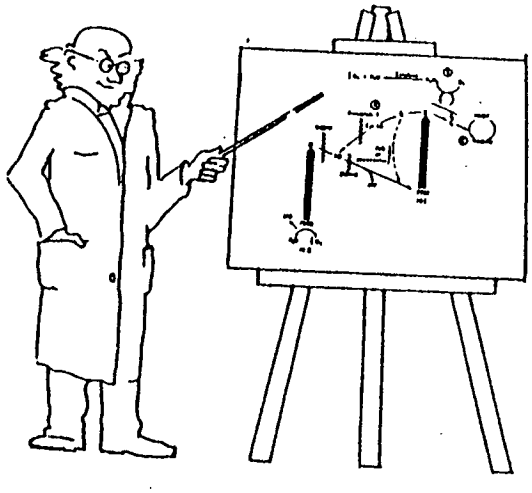


D

CHAPTER 4



DISCUSSION

4.0 DISCUSSION

The results of this study show that sorghum plants illuminated with light higher than that received during growth, experienced inhibition of photosynthetic electron transport, Fv/Fm ratio and qP while qN increased. The photoinhibition was enhanced when photoinhibition treatment was given at chilling or high temperature. Photoinhibition of leaves which were pre treated with ascorbate or DTT did not show significant changes in Fv/Fm ratio, and qP as compared to control. However, qN increased considerably in leaves pre treated with ASC and then photoinhibited while qN decreased in leaves which were treated with DTT prior to the photoinhibition treatment. Carotenoid contents also responded to the treatment. Neoxanthin, Violaxanthin, Zeaxanthin and β-carotene contents changed due to the photoinhibition treatment. The leaves treated with ascorbate prior to photoinhibition treatment resulted in higher de-epoxidation of Violaxanthin to Zeaxanthin, while leaves treated with DTT prior to the photoinhibition treatment resulted in minimum de-epoxidation level. When the de-epoxidation state was compared with chlorophyll fluorescence, it was observed that there was a linear relationship between de-epoxidation state of Violaxanthin and qN. The results also indicate oxidative damage to plants by the photoinhibition treatment, which is indicated by peroxidation of thylakoid lipids and proteins and by the significant increase observed in the activity of superoxide dismutase and ascorbate peroxidase. In this work we also tried to study the possible role of ABA on protection against photodamage and it was demonstrated that qN was

relatively higher in plants grown with ABA supplemented medium. Also the de-epoxidation state was greater in plants grown with ABA supplemented medium than in plants grown without ABA.

PHOTOSYNTHETIC ELECTRON TRANSPORT :

Our results indicate that photoinhibition of leaves resulted in damage mainly to PS II activity while photoinhibition of isolated chloroplasts also caused slight damage to PS I in addition to PS II (Table 3.1-3.5). The photoinhibition of intact leaves resulted in decrease in PS II activity assayed as H_2O to PD. PS I+PS II activity also declined but this decreases in PS I+PS II activity was mainly due to the decrease in PS II only activity assayed as H_2O to PD as PS I only activity assayed as DCIP or TMPD to MV_2 remained unchanged in the photoinhibition of intact leaves. PS II activity measured using DPC as an electron donor and MV as an electron acceptor did not show any inhibition of the activity. This suggest that the major photoinhibitory damage occurs to the oxidising side of the PS II, i.e. prior to the DPC binding site and probably involves the water splitting reaction i.e. OEC. However, photoinhibition of isolated chloroplasts showed damage to PS II activity measured as DPC to MV and PS I only activity in addition to PS II activit. The extent of damage to PS II activity was greater in photoinhibited chloroplast then in photoinhibited leaves, which could be because of non-operation of protection and recovery processes or being functional at low level in isolated system.

The temperature played an important role in the photoinhibition of photosynthesis. The light treatment as 5°C and 50°C was far greater damaging then light treatment given at growth temperature (30°C). The results obtained indicate a separate mode of damage to photosynthetic electron transport activity during photoinhibition at 5°C and 50°C. The reason for exacerbated damage to photosynthetic electron transport at 5°C compared to at 30°C might be that at low temperature protective mechanism, such as scavenging of active oxygen species formed due to light stress in our study which is indicated by increased level of MDA formation and greater activities of antioxidants enzymes, by enzymes such as APX, SOD, which are temperature dependent and may be due to the damage to process of recovery or inactivation (operating at low level) by the low temperature. The result indicate that net photoinhibitory damage to photosynthesis result from balance between the photoinhibition process and reaction process. The greater extent of damage to isolated chloroplasts further substantiate the role of protection and recovery during and after photoinhibition, since no cytoplasm protective mechanism or restoration mechanism will be operational in isolated system.

The photoinhibition at 50°C also resulted in greater damage to photosynthetic electron transport. PS II reaction was completely inhibited indicating probably a different mechanism of damage then seen during photoinhibition at 5°C. Our results show that photoinhibition at 50°C resulted in complete inhibition of PS II activity, this may be due to dis-organisation of photosynthetic membrane (thylakoid) at high temperature, as high temperature

would influence the membrane fluidity and unsaturation level of thylakoid lipids thereby influencing the electron transport, in addition to the effect of high temperature on activity of enzymes involved in the scavenging oxygen radicals generated due to the photoinhibition treatment.

There are reports suggesting damage to oxidising side of PS II as well as reducing side of the PS II. Ottander et al. (1993) studied the effect of photoinhibition at 5 and 20°C and also reported that PS II was the affected site. They suggested that it may be because of photoinhibition at low temperature may predispose the PS II by either generation of excessive excitation or inhibition of repair process of PS II. They concluded that initial decrease in photosynthesis under photoinhibitory condition may be because of over excitation of the primary electron acceptor QA, however, the damage to photosynthesis during longer duration of photoinhibition may be due to the inhibited repair cycle. In our study we observed that photoinhibition was far greater at 5°C than at 30°C (Fig. 3.1-3.3; Table 3.1-3.2). This could be due to the temperature limitation on the recovery process. It has been reported (Greer et al. 1991, Ottander et al. 1993) that low temperature causes limitation of the recovery processes. They carried out experiments using D-threochloromphenicol (CAP) as an inhibitor of chloroplast encoded protein synthesis, including D1 protein at 5 and 20°C. They observed that with repair of PS II going on, the susceptibility of photosynthesis to photoinhibition was controlled by the reduction state of QA. Blocking the repair of D1 protein resulted in a severe inhibition of

photosynthesis at 20°C but at 5°C, almost no increase in sensitivity of PS II to photoinhibition occurred. This showed that the repair cycle is largely inactivated at 5°C, thus CAP treatment had a very limited protective role at low temperature. Our results of which resulted in greater inhibition of photosynthesis at 10°C compared to 30°C could be explained by suggesting that at 10°C the processes which scavenge the oxygen radicals and processes which dissipate the excessive radiation may be limited by the low temperature, thus making plants more susceptible to the photoinhibition treatment. It can be suggested that PS II repair cycle is important in protecting sorghum from photoinhibition. The increased susceptibility to photoinhibition at low and high temperature may be explained both by an increase excitation pressure on PS II at low and high temperature and also by inhibition of repair cycle of PS II. Greer et al. (1991), Chow et al. (1989), Ottander et al. (1993) have suggested that photochemically inactive reaction centres which are accumulated during photoinhibition exposure at low temperature (which may also occur at high temperature) are not degraded but act as potential quenchers of exciting energy, thus may confer protection to remaining photochemically active centres. High temperature in addition to the above mentioned effect may also cause disorganization of thylakoid membrane resulting in increase susceptibility of the photosynthesis to photoinhibition damage.

Vermaas et al. (1995) suggested that one of the possible factors causing the photoinhibition of photosynthesis at high light is the oxidizing primary donor $p680^+$ which may be harmful to PS II because of its highly oxidising nature. They measured the turnover of D1 protein in a mutant Synechocystis species PC6803 which lack tyrosine Z (physiological donor to $p680^+$). They found that in the mutant, D1 degradation was an order of magnitude faster than seen in wild type. Similar results were also suggested by Krieger et al. (1992), Krieger and Weis (1993). Campbell et al. (1996) also reported that photoinhibition of photosynthesis is observed under condition where D1 degradation exceedes the synthesis of D1 protein. Van Wijk and Hasselt (1993) suggested that photoinhibition was preceded by down regulation through light induced acidification of lumen. They proposed that photoinhibition in spinach takes place when the PS II reaction centre are down regulated by energy dependent quenching. They concluded that photoinhibition depend on the energy state of the photosynthetic membrane in combination with redox balance of PS II.

Sharma et al. (1994) also studied the effect of photoinhibition in various plant system viz, wheat, sorghum and barley and reported that there may be different sites of primary damage to photosynthesis under photoinhibitory condition in different plants, depending on their being chilling sensitive or chilling resistant.

Demeter et al. (1987) studied the effect of photoinhibition in spinach and *Chlamydomonas reinhardii* cells in order to locate the primary site of photoinhibition using the light induced absorbance change of pheophytin. They reported that photoinhibition of spinach thylakoid membranes resulted in a parallel decrease in the amplitude of the pheophytin and QA photoreduction signals. In intact Chlamydomonas cells the pheophytin photoreduction and oxygen evolution activity exhibited similar decreases during photoinhibition. Complete recovery of both activities was observed within 60 min of incubation under normal growth conditions. They concluded that the primary site of photoinhibition involve the components (p680 and/or Pheophytin) of the primary charge separation.

However, in our study we observed the photoinhibition site to be prior to DPC binding i.e. prior to primary electron donor Z as activity assayed from DPC to MV did not show change due to photoinhibition (Fig. 3.2) but PS II activity assayed as water to PD (Fig. 3.2) showed a great decrease suggesting the site of photoinhibition to be in between electron donation by water i.e. oxygen evolving complex and electron donation by DPC i.e. Z.

The photoinhibition of isolated chloroplasts (*in vitro* condition) resulted in damage to PS I also. This may be due to that under *in vitro* conditions where other photoprotective mechanisms (cellular) are not operating, PS I might have over excited in order to cycle the electron from PS II and experience photodamage when such state prolong. The greater damage to

photosynthesis under *in vitro* condition (Table 3.3-3.5) then under *in vivo* conditions (table 3.1-3.2) may be due to non functional of recovery and restoration and protective process in the isolated system. There are reports by various workers indicating damage to PS I reaction centre under photoinhibitory conditions. Havaux and Davard (1994), Terashima et al. (1994) have reported that photoinhibition can be main target of light stress if it occurs in combination with chilling stress under *in vivo* conditions. Herrmann et al. (1997) observed light stress related changes in the properties of PS I in photoautotrophic cultured cells of *Marchantri polymorpha* L. They observed that light treatment of 1300 μmol m^{-2} s^{-1} for 3 hours reduced the PS I activity by 50% while the PS II activity decreased by 35%. They suggested that linear lectron transport is a cause of the loss of PS I activity and the PS I inactivation caused the changes in PS I organization. Work by Satoh (1970), Sonoike (1995), Baba et al. (1996) have shown inhibition of PS I activity due to high light in the absence of chilling.

sonoike (1996) also reported degradation of psaB gene product during photoinhibition possibly due to involvement of active oxygen species in the process. He reported that degradation of psaB gene product give rise to 51 KDa and 45 KDa fragments. He also reported that addition of MV, an artificial electron acceptor from PS I, completely protected PS I from photoinhibition, which lead to the conclusion that they concluded that superoxide

anion is not directly responsible for the photoinhibition of PS I activity, since the superoxide anion increases by the addition of MV.

Styring et al. (1990) have also shown damage to oxygen evolution due to strong light photoinhibition. They applied EPR spectroscopy and found that photoinhibition resulted in inhibition of the oxygen evolution and measurements of S2 state multilines EPR signal. They suggested three possible reason for the inhibition of multilines signal formation. (1) The Mn complex is destroyed but the primary photochemistry including charge separation and charge stabilization is left intact. (2) The electron transfer from pheophytin to QA is inhibited and the primary charge separation reaction is inhibited.

Ivoun et al. (1989) have also studied the effect of high light on PS I and have reported that the site of photoinhibition in PS I under extremely reducing condition is probably in between Ao and Fe-sx.

CHLOROPHYLL FLUORESCENCE AND XANTHOPHYLL CYCLE:

Chlorophyll fluorescence give indication about the health of the photosynthetic system in leaves. In our study we have observed that photoinhibition of intact leaves at 3600 μmol m^{-2} s^{-1} PAR at 5, 30 and 50°C resulted in decrease in Fv/Fm ratio which suggest that the efficiency of transfer of excitation energy from LHC to reaction centre was declined, more so in

leaves photoinhibited at 5 and 50°C than at 30°C indicating greater damage to the efficiency of energy transfer. Leaves fed with ascorbate prior to the photoinhibition treatment showed relatively less decrease in Fv/Fm ratio than seen in leaves treated with DTT (Table 3.6) indicating that ascorbate may prevent potodamage probably by mechanism such as direct scavenging the oxygen radicals or facilitating the process by ascorbate peroxidase. Also ascorbate could help in better energy dissipation by stimulating Xanthophyll cycle. In this study we also reported decrease in photochemical quenching an indicator of utilization of harvested light by potochemical reaction i.e. ATP synthesis and NADPH reduction (Fig. 3.8-3.13; Table 3.6). We also observed the decrease in qP was relatively less in ascorbate pre treated leaves then in leaves pre treated with DTT (Table 3.6). This may again suggest the role of ascorbate in protection against photodamage not only through ascorbate peroxidase but by dissipating excess energy through de-epoxidation of V.

We have also reported the increase in qN due to the photoinhibition treatment (Table 3.6; Fig. 3.8-3.13). The qN indicate dissipation of excessive energy in the pigment bed, thereby, preventing over excitation of reaction centre under high light conditions. This may be through Xanthophyll cycle as ascorbate is one of the regulating component in the de-epoxidation of V to Z. It is suggested that Xanthophyll cycle is involved in energy dissipation as in this study a direct correlation has been observed between qN and Xanthophyll cycle under all three conditions, i.e. leaves fed with distilled water, ascorbate or DTT (Table 3.9). The results indicate that ascorbate treated

leaves showed better protection against photoinhibition seen as less decrease in Fv/Fm ratio, and qP and higher increase in qN; Table 3.9). This may be due to two reasons (1) Ascorbate would stimulate the activity of ascorbate peroxidase enzymes, since ascorbate act as one of the substrate for the enzyme, thereby, preventing as well as scavenging the active oxygen species and (2) Ascorbate may also stimulate Xanthophyll cycle which may protect plants against photodamage, by dissipating the excess radiation away from the reaction centre through stimulating Xanthophyll cycle. Our results with Xanthophyll cycle also indicate that ascorbate had an stimulating effect on Xanthophyll cycle while DTT had inhibitory effect and thus DTT treated leaves suffered greater photodamage seen as greater decrease in qP and Fv/Fm ratio while little increase in qN (Table 3.9).

In the present investigation, photoinhibition of PS II activity on the oxidising side of the electron acceptor QA is also suggested by the decrease in room temperature fluorescence (where most of the fluorescence arise from PS II associated chlorophyll) The decrease in the maximum fluorescence (Fm) yield was largely due to the decrease in variable fluorescence (Fv) indicating an altered redox state of QA. This may result from photoinhibitory damage to oxidising side of PS II reaction centre or damage to the reaction centre itself leading to a lower reducing capacity. The decrease in variable fluorescence can also be due to non-photochemical quenching (Baker and Horton 1987).

The Fv/Fm ratio measured in photoinhibited leaves or isolated chloroplasts also decreased suggesting that photoinhibition resulted in a decrease of efficiency of photon capture. Decrease in the Fv/Fm ratio due to photoinhibition has been reported by various workers in different plants (Baker et al. 1989; Genty et al. 1989; Groom et al. 1991).

Photoinhibition of has been studied in natural tropical plants by Krause and Winter (1996). They observed that shade leaves (100 μmol m^{-2} s^{-1} PAR) of various plant species when got exposure to 1800 μmol m^{-2} s^{-1} PAR, they exhibited substantial photoinhibition *in situ* as manifested by decrease in Fv/Fm ratio.

Herrmann et al. (1995) also reported similar results. They observed that when microalgae, *Ulva laetervirens*, was exposed to solar radiation the ratio of Fv/Fm as well as oxygen production declined rapidly with increasing duration of the exposure.

Mohanty and Yamamoto (1996) have showed two types of qN in carbon assimilatory chloroplasts distinguishable by effects of ascorbate and de-epoxidation. They reported that in the presence of ascorbate, fluorescence quenching is antenna type and obligatory on violaxanthin de-epoxidation while in the absence of ascorbate fluorescence quenching is reaction centre type and unrelated to violaxanthin de-epoxidation. our results also support this as

we saw high qN value in ascorbate treated plants compared to DTT treated plants (Table 3.9) suggesting fluorescence quenching in the photosynthetic antenna. These results (that ascorbate dependent fluorescence quenching is from photosynthetic antenna system) were further substantiated by our work on Xanthophyll cycle which also showed a greater level of de-epoxidation of V to Z in leaves treated with ascorbate and accompanied by high qN.

The correlation between energy dependent qN and de-epoxidation is well established (Demmig-Adams 1990; Caletaynd et al. 1997; Sharma and Hall 1996; Deltro et al. 1998) though the mechanistic aspect behind Z-mediated dissipation of excess energy are still controversial (Schindler and Lichtenthaler 1994 and 1996). Whether de-epoxidation is obligatory (Gilmore and Yamamoto 1993; Mohanty and Yamamoto 1995) or non-obligatory (Noctor et al. 1991; Rees et al. 1992) is debatable. Our results support the view that de-epoxidation is obligatory in as much as antenna quenching was concerned as we reported a direct correlation between ascorbate treated leaves which stimulate de-epoxidation state of V to Z and showed greater qN values and in DTT treated leaves, which inhibit the de-epoxidation of V to Z which showed low qN since there was no de-epoxidation of V to Z (Table 3.8 & 3.9). A slight increase in qN in DTT treated leaves was also observed which could be due to reaction centre regulated qN (Table 3.9). Mohanty and Yamamoto (1996) suggested that ascorbate is not directly required for Xanthophyll dependent fluorescence quenching but they also suggested that conditions could exist where ascorbate

synthesis in cell or its diffusion into the chloroplasts may be insufficient to support de-epoxidation and to prevent donor side inhibition, under such condition ascorbate mediated de-epoxidation may show direct relationship with qN. The chloroplast membrane and the Mehler-ascorbate peroxidase reaction can limit ascorbate diffusion in to the lumen Neubauer and Yamamoto 1993). This is consistent with our results observed with the activity of ascorbate peroxidase due to photoinhibition. The activity of APX was increased many fold, which uses ascorbate as a substrate and thus under such condition ascorbate may cause substrate limitation for de-epoxidation of V to Z which would overcome by treating leaves to exogenous ascorbate resulting in direct relationship between qN and de-epoxidation of V to Z in our study.

Our results with Xanthophyll cycle clearly showrelationship with qN and thereby, providing protection to plants against over energization by dissipating the excess light energy in the pigment bed and may also do so in reaction centre (Telfer et al. 1994). Our results showed that there was a greater V+A+Z pool in leaves pre treated with ascorbate than with distilled water or DTT (Table 3.8). Similarly the de-epoxidation level of V to Z was also greater in leaves treated with ascorbate prior to photoinhibition except at 50°C (Table 3.9) which could be due to that high temperature may cause inhibition/degradation of enzyme de-epoxidase responsible for conversion of V to Z. Our results also indicate direct relationship between Violaxanthin de-epoxidation and qN (Table 3.9). We observed that leaves pre treated with

ascorbate prior to the photoinhibition treatment showed greater V to Z conversion and this was also accompanied by higher qN value in ascorbate treated leaves than observed in leaves treated with DTT which inhibited de-epoxidation of V to Z and this was accompanied by low qN value. A slight increase in qN value seen in plants treated with DTT may be due to de-epoxidation independent component of the qN as qN represent qE and qI as well. Zeiger and Zhu (1998) reported that capacity of non radiative dissipation (qN)was correlated with amount of photoconvertible V across a wide range of species with different photosynthetic capacity and pathways. Anderson and Aro (1994) and Anderson et al, (1988) have shown that amount of photoconvertible V always appears to be correlated with Chlorophyll a/b ratio. It is known that Chlorophyll a/b ratio correlated with degree of thylakoid stacking.

The evidence presented with respect to qN and Xanthophyll cycle here fits well with reports on correlation between Zeaxanthin content and qN formation during photoinhibition treatment. High light may inactivate or denature enzymes that catalyze the back conversion of Z to V. It seems from our results that higher capacity to dissipate excess energy during our experiments was related to higher level of de-epoxidation of V to Z.

Phillips et al. (1999) reported that de-epoxidation of V to Z in light was accompanied by eight fold increase in *trans* to 13-*cis* isomerisation of V. On recovery in dark the original isomeric content and composition was observed. They suggested that *trans* to *cis* isomerisation may develop separate pool of V and this process may cause ceiling on the extent of V available for de-epoxidation to Z on exposure to light, at least in part, because of the formation of a pool of *cis*-isomers that is not available to violaxanthin de-epoxidation. Our results also support this view as we discuss later that V act as a precursor for Z and ABA which is also enhanced due to photoinhibition since there is a common precursor for Z and ABA the isomeric forms help in the regulation of synthesis of Z and ABA under excess excitation conditions.

Chowdhery et al. (1993) have also shown that ascorbate treatment to isolated chloroplasts induced Z formation in wheat leaves upon illumination. They observed that Z level increased three times when the leaves were incubated with ascorbate for 3 hours. The increase in V was accompanied with relatively less decrease in photochemical activity and pigments, whereas, treatment with ascorbate and DTT resulted in nullifying the protection to photosynthesis under high light condition. These results are in line with the results observed by us in this study. DTT infiltration of leaves has shown to be an effective procedure preventing Zeaxanthin accumulation (Havaux et al. 1991) since it is known to be inhibitor of de-epoxidase enzyme (Yamamoto 1972). DTT itself does not influence photosynthesis and also not much

evidence is available that it interacts directly with membrane lipids affecting the fluidity. It also does not affect the electrophoretic pattern of thylakoid proteins (Yamamoto 1972).

Venema et al. (1999) have shown changes in Xanthophyll content due to chilling at low light levels (75 μmol m^{-2} s^{-1} PAR) in tomato, however, we did not observe accumulation of Z due to chilling only.

Haldimann (1997) also examined carotenoid composition and photosynthetic activity induced by combination of light and chilling in chilling sensitive and chilling resistant cultivars of maize. They reported increase in Xanthophyll content during the stress which was greater in chilling sensitive genotype. However, short term exposure to stress caused changes in violaxanthin de-epoxidation which were similar in both type of genotype suggesting that chilling resistant is not due to the de-epoxidation of V to Z.

Schindler and lichtenthaler (1994) observed in young tobacco mutant, that accumulates Z very efficiently, showed better protection against photoinhibition indicating a possible photoprotective function of Z. However, kinetic studies on light induced Z accumulation and degree of variable chlorophyll fluorescence (Fv/Fm ratio) showed that they proceed fairly independent of each other with different kinetics though both processes are irradiance dependent. They reported that Z formation was completed before a significant decrease in chlorophyll `a' fluorescence was detected.

They also suggested that Zeaxanthin does not play a major or specific role in direct energy dissipation of absorbed light energy but, may possess a different mode of action. They concluded that mechanism of possible photoprotective function of Z is still open and requires new investigation. We have not studied the kinetics of qN and Xanthophyll cycle in this study.

Gruszecki et al. (1994) also reported that Xanthophylls present in light harvesting complex and their possible involvement in the regulation of light driven excitation quenching in LHC II.

Winter and Koniger (1989) carried out the effect of light in DTT fed *Nerium oleander* leaves and observed results similar to ours. They observed that DTT fed leaves failed to accumulate Zeaxanthin but total carbon gain was not reduced by DTT during the photoinhibition treatment. This again suggest that DTT treatment does not affect photosynthesis. They also observed that incubation of leaves to DTT led to large sustained decrease in the photon use efficiency of photosynthetic oxygen evolution. Pfundel et al. (1994) reported that Zeaxanthin dependent quenching is pH dependent triggered by the pH of localized membrane domain. Goss et al. (1995) showed a pronounced Zeaxanthin dependent amplification of qN due to build up of an artificial ΔpH. They proposed that ΔpH during the synthesis of Z that activates the mechanism of Z-dependent amplification of qE (a component of qN).

The relationship between susceptibility to photoinhibition, Zeaxanthin formation and chlorophyll fluorescence quenching at suboptimal temperature was also studied by Koroleva et al. (1995). They concluded that Xanthophyll cycle was strongly influenced by cold acclimation. Results suggesting correlation between Xanthophyll cycle and non-radiative dissipation of energy has been suggested by several workers. Adams et al. (1994), Richter et al. (1994), Falbel et al. (1994), Phillips et al. (1995), Bilger et al. (1995), Fisahn et al. (1995).

Sharma and Hall (1993) observed that there may be a certain limitation to the role of Xanthophyll cycle in protection against photoinhibition. This was based on their observation that Zeaxanthin content after a photoinhibition treatment at 1600 μmol m^{-2} s^{-1} at 5°C did not increase further when plants were photoinhibited at higher PFD of 2500 μmol m^{-2} s^{-1} increased though photoinhibition damage increased. In addition greater increase in Z and decrease in V content were observed at 20°C where the photoinhibition observed was not as great as at 5°C. They concluded that (1) Changes in Xanthophyll cycle are temperature dependent. Under chilling conditions the dissipation of excessive radiation energy by carotenoids is less than 20°C resulting in greater damage to photosynthesis at 5°C. With higher irradiance greater than 1600 μmol m^{-2} s^{-1} no further increase in the Z content suggests a limitation in dissipation of excessive energy by the Xanthophylls.

Sharma and Hall (1996) also studied changes in carotenoid content and photoinhibition in young and old sorghum leaves and observed that older leaves had a higher pool of V+A+Z and on photoinhibition did not cause significant level of increase in Z in older leaves but better protection than seen in younger leaves. In younger leaves the V+A+Z pool was doubled following the photoinhibition but greater photodamage. Re-photoinhibition of the leaves provided better protection to younger leaves suggesting that increased level of V+A+Z during the first photoinhibitory treatment provided better protection against photoinhibition due to accumulation of xanthophyll pool during first photoinhibition phase.

Schindler and Lichtenthaler (1996) studied the effect of sunny and cloudy day on qN and Xanthophyll in maple leaves. They reported that there was a correlation between qN and Xanthophyll in plants grown under cloudy conditions but in plants on sunny day showed no clear correlation between the changes in chlorophyll fluorescence parameters and the rise and fall of Z in the course of the sunny day. They reported that in the afternoon the Z level decreased much earlier than the decline in qN and the rise and regeneration of the initial values of qP and Fv/Fm ratio.

Sandman et al. (1993) studied the role of carotenoids in photoprotection of D1 degradation under high light conditions in mutants of Anacystis which form different amount of carotenoids. They observed a highly significant correlation between photosynthetic oxygen evolution and the amount of

synthesized carotenoids. They studied the influence of carotenoids with Scenedesmus culture grown in dark in the presence of norflurazon, an inhibitor of carotenoid biosynthesis. They observed that illumination of these cells resulted in decrease of photosynthetic activity that accompanied by loss of the D1 protein. The loss of the D1 protein was greater when carotenoid content was lowered during previous growth of the norflurazon treated cultures.

Logan et al. (1996) studied the influence of high light on size and composition of carotenoid pool vis-a-vis ascorbate pool in nineteen plants species collected from multiple sites representing a broad taxonomic range. They observed that plants consistently possessed larger total carotenoids pool when found growing in more exposed sites. They also observed a significant positive correlation between β-carotene content and growth PFD. Neoxanthin content exhibited no significant trend. The pigments of Xanthophyll cycle exhibited the most pronounced response to growth light and comprised a much greater portion of the total carotenoid pool in high light acclimated plants. the pool of reduced ascorbate was also several fold greater in high light acclimated plants. These changes in carotenoid and ascorbate content are consistent with a need for a greater capacity to dissipate excessive absorbed light energy in high light acclimated plants as ascorbate is required for de-epoxidation of V to Z and showed a link between the function of ascorbate and the Xanthophyll cycle in photoprotection.

Arvidsson et al. (1997) reported that temperature had a strong influence on the rate and degree of maximal V to Z conversion. They reported that low temperature (4°C) decreased the conversion of V to Z which was increased with increasing temperature (25-37°C). However, in our study we gave photoinhibition treatment at 50°C which may also be inhibiting.

Darko et al. (1996) also suggested a correlation between chlorophyll fluorescence quenching and Xanthophyll cycle in atrazine resistant and sensitive biotypes of *Conyza candensis*. They observed that qP, qN and qE were lowered due to actinic light in atrazine resistant biotypes. The conversion of Xanthophyll cycle component V to Z was also limited in atrazine resistant biotypes. The higher photosensitivity of atrazine resistant plants and the lowered Z formation in these biotypes suggest that beside D1 protein mutation, the limited conversion of Xanthophyll cycle component may contribute to the higher susceptibility of atrazine resistant plants to photoinhibition.

Logan et al. (1997) showed the response of Xanthophyll cycle in non-radiative dissipation in Alocasis to sunflecks in rain forest. They reported that plants which experienced sunflecks showed increase in the size of Xanthophyll cycle pool in comparison to understory plants that never received sunflecks. They reported that subsequent to the first sunflecks, plants tended to retain their pool of Xanthophyll cycle carotenoids in the de-epoxidized forms throughout the day. They concluded that retention of these de-epoxidation

forms allowed the trance thylakoid membrane proton gradient to engage and disengage dissipation rapidly in response to a sunflecks, thereby, mitigating photooxidative damage and ensuring a rapid return to efficient light utilization via photosynthesis in limiting light. Our results also agree with these studies which show a relationship between Xanthophyll cycle and non radiative dissipation, thereby, providing photoprotection against photodamage.

Demmig-Adams has done a considerable work on the Xanthophyll cycle and she has also shown a direct relationship between qN and dissipation of V to Z. Dammig-Adams et al. (1990) using certain cyanobacteria found in symbiotic association with lichens, which lack Xanthophyll cycle and green algae, which contain Xanthophyll cycle, carried out a comparative study of chlorophyll fluorescence characteristics as well as the tolerance of photosynthesis to high irradiance. They observed that upon exposure to excess light, the green algae associated with lichens formed the Z rapidly from V and also exhibited the type of rapid fluorescence quenching associated with quenching of Fo. In contrast, the cyanobacteria associated with lichens which lack Xanthophyll cycle did not form any Z and also did not exhibited rapidly developing and relaxing non-photochemical quenching, indicative of radiation less dissipation and the reduction state (indicative of light stress) of PS II centre was high under excess irradiance in these cyanobacteria. They observed tolerance to longer exposure at high irradiance was considerably

greater in Z containing green algae than in the Z free cyanobacteria. These results observed by Demmig-Adams et al. (1990) resembles our results with ASC and DTT treated leaves and strengthen the argument that there is a relationship between radiationless dissipation and Xanthophyll cycle and there by protection of photosynthesis against photodamage.

ADDITION OF ANTIOXIDANTS DURING PHOTOSYNTHESIS OF CHLOROPLASTS:

Our results with chloroplasts supplemented with antioxidant like ascorbate, β-carotene and glutathione clearly show that addition of these antioxidant has effect on protection against photodamage, as seen in the form of less decrease in Fv/Fm ratio and qP and also less formation of MDA, indicative of lesser oxidative damage to membrane lipids. This could be due to the action of antioxidants. The photoinhibition causes oxidative damage seen in our study (Table 3.11; Fig. 3.19B) as formation of MDA and also reported by several workers. These antioxidants quench/scavenge the active oxygen radical and thereby, protecting the sensitive unsaturated thylakoid lipids which though do not have any catalytic function but may be important for the efficient functioning of proteins. The protection to thylakoid lipid peroxidation was also observed in our study (Table 3.11) concluding that addition of antioxidant during photoinhibition under *in vitro* condition protect the photosynthesis probably by scavenging oxygen radicals during the

photoinhibition. Antioxidants may also prevent the damage to oxidation of proteins as well but this was not study in detail in our study (Fig. 3.38 & 3.39).

Edge et al. (1997) have studied the role of carotenoids in photobiology and reactivity of carotenoids with singlet oxygen and the interaction of carotenoids with range of free radicals. They also suggested that β-carotene may not only act as antioxidant but under certain conditions β-carotene may also act as prooxidant.

Foote and Denny (1968) demonstrated that β-carotene could inhibit photosensitized oxidation and was therefore, an efficient quencher of $^1O_2$.

Burton and Ingold (1984) reported that as well as the ability to quench excited states, carotenoids can also react with free radicals. However, unlike the quenching of singlet oxygen which mainly lead to energy dissipation as heat, the reaction of β-carotene or ascorbate or glutathione (any antioxidant) with a free radicals will lead to electron transfer or possibly addition reactions i.e. the `odd' electron which characterizes a free radical is not lost.

Liebler (1991) studied the ability of β-carotene on radical scavenging in a liposome system, they observed that with β-carotene incorporated into the liposome, lipid peroxidation was suppressed. They found β-carotene acting as a better inhibitor of peroxidation at low oxygen pressure than at high O_2 pressure.

Palozza and Krinsky (1992) have shown that β-carotene and α-tocopherol (Vitamin E) can act synergisticaly as radical scavengers in rat liver. Niki et al. (1995) also reported that addition of ascorbate with β-carotene also provided an overall synergistic protective role against oxygen radicals.

The role of carotenoids as radical trapping antioxidant and prooxidant species is of much current interest and many aspects of this are still not understood. In this study we show that addition of β-carotene as well as ASC and glutathione to chloroplasts photoinhibited under *in vitro* condition protect them against photodamage and thisprobably is by preventing the oxidative damage to membrane lipids by scavenging the active oxygen species during the treatment (Fig. 3.19; Table 3.11). However, functional role of β-carotene *in vivo* remains to be elucidated (Logan et al. 1996).

Telfer et al. (1991) also studied the role of β-carotene within the isolated PS II reaction centre. They reported that photodamage was observed only when β-carotene was photobleached by $p860^{+}$ and not when Mn II was added as an electron donor to $p680^{+}$ again indicating protective function for β-carotente against photodamage.

In our study we also observed that addition of ascorbate during photoinhibition to isolated chloroplasts also provided protection to photosynthesis against photodamage (Table 3.11; Fig. 3.20). This may be due to

three possible factors. One is possible due to ascorbate being a excellent scavenger of free radicals and act as reductant. Second is that ascorbate could also facilitate the activity of ascorbate peroxidase an enzyme which catalyzes H_2O_2 and thereby, prevent oxidative damage. Third, ascorbate could also facilitate the de-epoxidation of V to Z. Since the same ascorbate pool is used for APX activity and de-epoxidation of V to Z under these conditions, limitation of ascorbate would result in damage to photosynthesis, but external addition of ascorbate could overcome this limitation and provide protection against oxidative damage as is also seen in our study.

Logan et al. (1996) has suggested that 20-40% of the cellular ascorbate is localized in the chloroplasts. Since ascorbate is the endogenous reductants for V de-epoxidation, sun acclimated plants may require more ascorbate to rapidly convert their large Xanthophyll cycle pool to A and Z under conditions of excessive light absorption. Additional, high concentration of ascorbate in sun acclimated tissue may be necessary for the de-toxification of singlet oxygen, which could potentially form as a result of photosensitization of reaction in the pigment bed, or $O_2^{\cdot}$ formed via direct reduction of O_2 by the electron transport chain. In our study plants were grown under low light level and when exposed to high light level the extra ascorbate pool provided by the exogenous addition may have prevented ascorbate limitation under photoinhibitory conditions and facilitate operation of its role as reductant, substrate for APX and substrate for de-epoxidation of Xanthophyll, thereby,

providing better protection against photodamage to leaves exposed to high light as was seen in this study in the form of less decrease in Fv/Fm ratio and qP and also low level of peroxidation of thylakoid lipids (Fig. 3.20A; Table 3.11).

Glutathione may also provide such protection against photodamage, as is seen in our study (Fig. 3.20B). Glutathione is a substrate which is reduced by NADPH dependent glutathione reductase. educed glutathione in turn is used to maintain a pool of reduced ascorbate. Glutathione and ascorbate are major reductant of plant cells and offer protection against the adverse effects of several active oxygen species (Noctor et al. 1997; Noctor and Foyer 1998). In our study we have also observed that addition of glutathione resulted in protection of photosynthesis against photoinhibitory damage seen as low decrease in Fv/Fm ratio, qP and MDA formation indicative of protection to photosynthesis. This may be because glutathione is important to maintain the pool of reduced ascorbate, which is essential for the removal of H_2O_2 from chloroplasts. Glutathione facilitate their rapid regeneration of reduced ascorbate pool, thereby, better scavenging of oxygen radicals through the activity of APX. Glutathione may also have effect on the rapid de-epoxidation of V to Z by maintaining the ascorbate pool essential for the activity of membrane bound V de-epoxidase.

Loggini et al, (1999) also suggested that Z formation may be relevant in avoiding irreversible damage to PS II in drought sensitive wheat cultiver.

Steenvoorden et al. (1997) reported induction of endogenous antioxidants in improving photoprotection of skin. They reported that skin possesses elaborate antioxidant defense system to deal with UV induced oxidative stress. They induced the transdermal delivery of various antioxidant, such as glutathione peroxidase, catalase and SOD. They reported that supplementation of non-enzymatic antioxidant such as glutathione, α-tocopherol, ascorbate and β-carotene was also found to be very effective in photoprotection. Although treatment with single components of the antioxidant system were successful against a wide varieties of photodamage, the balance between different antioxidant was very important. Our results also indicate similar type of improved photoprotection to photosynthesis when chloroplasts were supplemented with antioxidants were photoinhibited.

Mortensen et al. (1997) studied the mechanism and rates of free radicals scavenging by carotenoids (lycopene, lutein, Z etc.) by pulse radiolysis. They reported that chloroplasts react with active oxygen species via electron transfer to generate the carotenoids radical cation. Rate constant for radical scavenging were in the order of 10^7-10^9 m^{-2} s^{-1}. They reported that mechanism and rate of scavenging is strongly dependent on the nature of the oxidising radical species but much less dependent on the carotenoid structure. Mortensen and Skibsted (1997) established the hierarchy of antioxidant studied as α-tocopherol > lycopene~β-tocopherol > γ-tocopherol > β-carotene> Z~lebda tocopherol > lutein > echinenone.

Hartel et al. (1998) using transgenic chlorophyll deficient tobacco plants under different light levels studied the relationship between Xanthophyll cycle and energy dependent fluorescence quenching (qE). They reported that qE did not vary among transformants in comparison to wild type when grown under limited light conditions. However, a high qE developed which was high in low light growing plants, and correlated with the increase of the pool size of xanthophyll cycle pigments in the wild type only. In high light grown transformants plants qE was insensitive to larger pool size (on chlorophyll basis) and the higher fraction of photo convertible V than found in wild type. They suggested that qE is largely independent of the extent of chlorophyll reduction in the transgenic plants.

Hurry et al. (1997) suggested that accumulation of Z in ABA deficient mutants did not affect qN or sensitivity to photoinhibition *in vivo*. These results are in contrast to results obtained in our study. Reason could be that a transgenic system provide a good system to study various physiological and biochemical processes, however, they may also affect normal processes.

ANTIOXIDANT ENZYMES:

In our study we also observed increase in the activity of antioxidant enzymes SOD and APX while catalase activity decreased (Fig. 3.25-3.30; Table 3.14-3.16). The increase in activity was influenced by temperature. Chilling temperature caused increase in APX and SOD but increase was

relatively less than observed at 30°C. Photoinhibition at 50°C caused initial increase followed by decrease. The treatment of leaves with ASC or DTT prior to photoinhibition did not cause significant difference in the activities of antioxidant enzymes. These results indicate that due to photoinhibition there was a generation of oxygen free radicals and in response to free radicals, enzymatic antioxidants system increased its activity to scavenge the oxygen free radical in order to prevent oxidative damage to plant system. There are several workers who have also reported similar results of induction of APX and SOD under environmental stress conditions. Biehler and Fock (1996) have reported a role of Mehler-peroxidase reaction in oxygen consumption in drought stressed wheat. Though we have not studied the effect of drought and under our experimental conditions no loss of relative water content (RWC) of leaf was observed (data not presented). Light stress also leads to osmotic stress, thus our results can also be compared with studies of other stress factor which also cause osmotic stress. The author (Biehler and Fock 1996) reported that from measurements of the rate of carbon flux through the photorespiration, estimated by the analysis of the specific radioactivities of glycolate, the rate of photorespiration is decreased with drought stress, therefore, the oxygen taken up in the light appears to be preferentially used by the Mehler peroxidase reaction. In stressed leaves 29.1% of the photosynthetic electron were consumed by mehler-peroxidase reaction and 18.4% used by the photorespiration, which would not be present in our experiments as sorghum is a C4 plant which does not experience photorespiration. They concluded

that over-reduction of photosynthetic electron transport chain is avoided preferably by the mehler epoxidase reaction, when drought stress restricts CO_2 reduction.

Tyystjarvi et al. (1999) have reported over expression of glutathione reductase and SOD due to photoinhibition of photosynthesis. They reported 5-8 times higher level of Fe-SOD and glutathione reductase. We have also observed 5-6 times increase in the level of SOD activity under our experimental conditions.

Several plant lines have been screened to elucidate the effect of SOD on photoinhibition of photosynthesis. In some cases over production of SOD in the chloroplast or in the cytosol resulted in no protection against photoinhibition (Tepperman and Dunsmuir 1990; slooten et al. 1995; Van camp et al. 1996) However, evidence for higher tolerance against high light stress in plants over producing SOD are provided by Jansen et al. (1989) and Sen Gupta et al. (1993a). In their studies APX activity was also higher in treated leaves (Sen Gupta et al. 1993b).

Jansen et al. (1989) also reported that photoinhibition caused overexpression of several other antioxidant enzymes. Our results also show similar results of elevated level of SOD and APX under photoinhibitory conditions (Fig. 3.25-3.30; Table 3.14-3.15) and also support the view that susceptibility of PS II to photoinhibition under *in vivo* is decreased due to the

Thomas et al. (1999), Bruggemann et al. (1999) have also reported the increase in SOD, APX and GR due to chilling. Thomas et al. (1999) reported that SOD at very low temperature 0-10°C does not provide protection against photodamage. In our study we also observed that extent of increase in SOD activity was less in plants treated at 5°C than at 30°C resulting in better protection of photosystem against photoinhibition in plants treated at 30°C

Tarisz et al. (1998, Scebba et al. (1998) and Garcie-Plezaola et al. (1999) have also studied the antioxidative defense system in various plant material at chilling temperature and have found results similar to ours.

Morita et al. (1999) have even suggested that induction of cytosolic ascorbate peroxides mRNA in rice is a kind of indicator of oxidative stress in the plants.

Brambille et al. (1997) reported over expression of antioxidant enzymes due to deficiency in mitochondrial respiratory chain. They down regulated the respiratory chain by treating it with ethedium bromide or chloramphenicol, this resulted in increase of Se-dependent and independent glutathione peroxidase as well as glutathione peroxidase and heme oxygenase mRNA. Catalase activity and catalase mRNA did not show appreciable changes. They suggested that deficiency of respiratory chain resulted in H_2O_2 formation and this led to induction of certain antioxidant enzymes. However, catalase, an enzyme which metabolizes H_2O_2 to H_2O, did not

increase. In our study we also did not observed in crease in catalase due to photoinhibition rather we observed a decrease (Fig. 3.31-3.33; Table 3.16). Sharma et al. (1998) have also reported that due to UV-B treatment oxidative damage was observed but catalase activity did not increase.

MDA FORMATION:

In our study we observed MDA formation due to the photoinhibition treatment. Photoinhibition at chilling temperature caused maximum photoperoxidation of thylakoid lipids. Treatment of leaves with ASC and DTT prior to photoinhibition did not result in significant protection of peroxidation of thylakoid lipids. Molecular oxygen is a sensitizing agent for lipid peroxidation. Increase in the MDA formation, a substrate formed as a result of photooxidation of thylakoid lipids in our study, indicate that photoinhibition caused oxidative stress by producing free radicals. These free radicals if not quenched by the various processes such as carotenoids and enzymatic and non enzymatic process causes peroxidation of thylakoid unsaturated lipids, indicated in our study as increase in MDA formation (Fig. 3.21-3.24; Table 3.12-3.13) and can also cause oxidation of proteins, also seen in our study as degradation of CP47 and CP43 along with low molecular weight protein (Fig. 3.38 & 3.39). The lipid peroxidation has been linked to effect such as (i) Increased ion permeability (ii) Loss of fluidity (iii) Cross linking of amino lipids and polypeptides (iv) Inactivation of membrane enzymes and receptors

resulting in a decrease in photosynthesis (Girotti 1990; Logani and Davis 1980; Kappus 1986).

Sharma and Singhal (1992a and b; 1993) have suggested that damage to photosynthetic electron transport involves at least in part, the peroxidation of thylakoid lipids. They showed a relationship between photosynthetic electron transport and peroxidation of thylakoid lipids suggesting the involvement of lipids in the functioning of chloroplasts. Thylakoid lipids in addition to fulfilling a structural role in membrane organisation, play a functional role in the activity of several integral membrane protein complex (Howarth et al. 1987). Since lipids do not have catalytic properties similar to proteins the peroxidation of chloroplast lipids may not be directly related to the functioning of the phtosynthetic apparatus, however, lipids may play a vital role in maintaining the optimal environment necessary for the functioning of various pigment-protein complexes. Wismer et al. (1998) have also reported lipid peroxidation due to low temperature in Solanum tuberosum.

Blokhime et al. (1999) have shown a relationship between lipid peroxidation and anoxia tolerance in range of species during post-anoxic reaeration. They showed that oxidative stress caused by re-exposure of plants to oxygen led to an increase of conjugated diene/triene formation, another indicator of peroxidation of membrane lipids. They also showed that rate of lipid peroxidation correlated negatively with anoxic stress tolerance.

The composition of membrane lipids and the degree of their unsaturation appear to be important for stress protection. Chirkova et al. (1989) in rice mitochondrial and chloroplast membranes, reported that degree of fatty acids unsaturation was higher and stayed unchanged for a longer time in comparison with that of the unresistant wheat. Evidence has been accumulated on the importance of lipid metabolism, specially on unsaturated fatty acids in the induction of defense reaction under abiotic and biotic stresses (Shamala et al. 1998). The degree of fatty acid unsaturation have also been observed under abiotic stress conditions; a 4-fold increase in the ratio of unsaturated to saturated fatty acids in a cold tolerant cultivars of bermudagrass was observed under low temperature (Samala et al. 1998).

ABSCISIC ACID:

Our results with leaves grown with ABA clearly indicate a protection against photodamage when compared to leaves grown without ABA. This was seen as less decrease in Fv/Fm ratio and higher values of qN (Table 3.17). The ABA grown leaves also showed greater V+A+Z pool, which may play a significant role in protection of photosynthesis in ABA grown plants compared to without ABA grown plants. Endogenous ABA content were also slightly higher in ABA grown leaves. ABA is a plant hormone which is induced under most of the abiotic stresses which results in osmotic/oxidative stress. As we seen in our study we have observed oxidative stress to sorghum leaves exposed to high light at various temperatures. The mechanism

of ABA response to plant protection is largely believed to be due to control on stomatal opening/closing, thereby, preventing water losses. However, in this study we have observed that ABA grown plants showed higher Xanthophyll pool which may facilitate the protection of photosynthesis against photodamage by dissipating excess energy in the form of heat. Thus ABA may have indirect role in better energy dissipation.

Since the report of Schwartz et al. (1997) that V act as a precursor for ABA synthesis it was important to study that how the two processes, that synthesis ABA and Zeaxanthin from Violaxanthin is regulated. Other reports about synthesis of ABA from V also been published (Cowan and Richardson 1993; 1997; Li and Watlon 1987; Lee and Milborrow 1997). As reported by Phillips et al. (1999) about the isomerisation of V from *trans* to *cis* form under high light and also by Schwartz et al. (1997) in the biosynthetic pathway of ABA from V it seems that *trans* form of Violaxanthin is responsible for conversion to Z while *cis* form is converted to ABA. V exist in pool of these isomers. Since plant experiencing photoinhibition in natural environment would most likely to face water stress also which may result in rate limitation for synthesis of Z or ABA due to limitation of V pool as reported by Phillips et al. (1999) since the V has to convert either to ABA or Z. If the greater amount of V pool is used for ABA synthesis then it may result in limitation of Z synthesis and thereby less efficient energy dissipation under excess light conditions which may result in photodamage to photosynthesis. Similarly if

Kuwabara et al. (1999) have reported that ABA treatment resulted in atleast 12 secretory proteins in winter wheat, which they suggested may be related to plant defense system against abiotic and biotic stress in winter wheat. Lee et al. (1997) have reported role of ABA in chilling tolerance of rice seedlings by modulation of polyamines. They reported that levels of free putrescine and activity of arginine decarboxylase in both shoot and roots and levels of free spermidine/spermine in shoot increased after exposure to chilling in chilling tolerant cultivars; which may be responsible for the chilling tolerance.

Dallaire et al (1994) have also studied the role of ABA in the induction of freezing tolerance in wheat. They found no tolerance due to exogenous application of ABA nor they reported increase in the endogenous levels of ABA, but they reported that ABA caused accumulation of 32 KDa polypeptide which in turn induces freezing tolerance. Cowan and Myemene (1991) reported that light causes catabolism of ABA in to phaseic acid. In our study we have also seen that ABA grown leaves did not show higher levels of endogenous ABA (Table 3.18) which could be because of it being metabolised in to phaseic acid. Phaseic acid is also reported to carry out ABA mediated physiological functions. In our study we have, however, not studied the phaseic acid content.

Campos and Thi (1997) have observed that ABA pre treatment prevent membrane damage in Vigna. They treated leaves with 0.1 mM ABA and exposed them to osmotic stress. They found that ABA treatment prevented

membrane lipids degradation specially of galactolipids and preserved their linolenic acid content. They concluded that ABA had a protection effect on membrane lipids and suggested enhanced capacity to maintain membrane integrity. This could also explain the better protection in plants grown with ABA against photodamage in our study. As de-epoxidation of V to Z and epoxidation of Z to V is regulated by membrane bound de-epoxidase and epoxidase enzymes respectively, ABA treatment may prevent damage to membrane thereby, facilitating efficient operation of Xanthophyll cycle which shall provide better protection against photodamage.

It is of interest to note that in the work of Hurry et al. (1997) ABA deficiency resulted in nullifying the effect of three times higher amount of Zeaxanthin and conversion of V to Z, which may indicate a significant role for ABA in the Xanthophyll cycle as is seen in our study with plants grown with exogenous ABA and subsequently photoinhibited. These aspects needed further study to conclusively established relationship between each other.

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