Relationship Between Non-Photochemical Quenching (qN) and Xanthophyll Cycle In Rice Seedlings Grown Under Different Light Intensities

A THESIS SUBMITTED TO GOA UNIVERSITY
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IN BOTANY

By

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STATEMENT

As required under the University Ordinance 0-413, I state that the present thesis entitled “**Relationship Between Non-Photochemical Quenching (qN) and Xanthophyll Cycle In Rice Seedlings Grown Under Different Light Intensities**” is my original contribution and that the same has not been submitted on any previous occassion 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 acknowledgement have been made wherever facilities have been availed.

J.V.

Janet Vaz

Place: Goa University

Date: 23/08/2008

CERTIFICATE

This is to certify that the thesis entitled "**Relationship Between Non-Photochemical Quenching (qN) and Xanthophyll Cycle In Rice Seedlings Grown Under Different Light Intensities**" submitted by Ms Janet Vaz 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.

Guide

Dr. P. K. Sharma

Place: Goa University

Date: 23/08/2008

Dedicated with love to my
Mother…

CONTENT

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Janet Vaz

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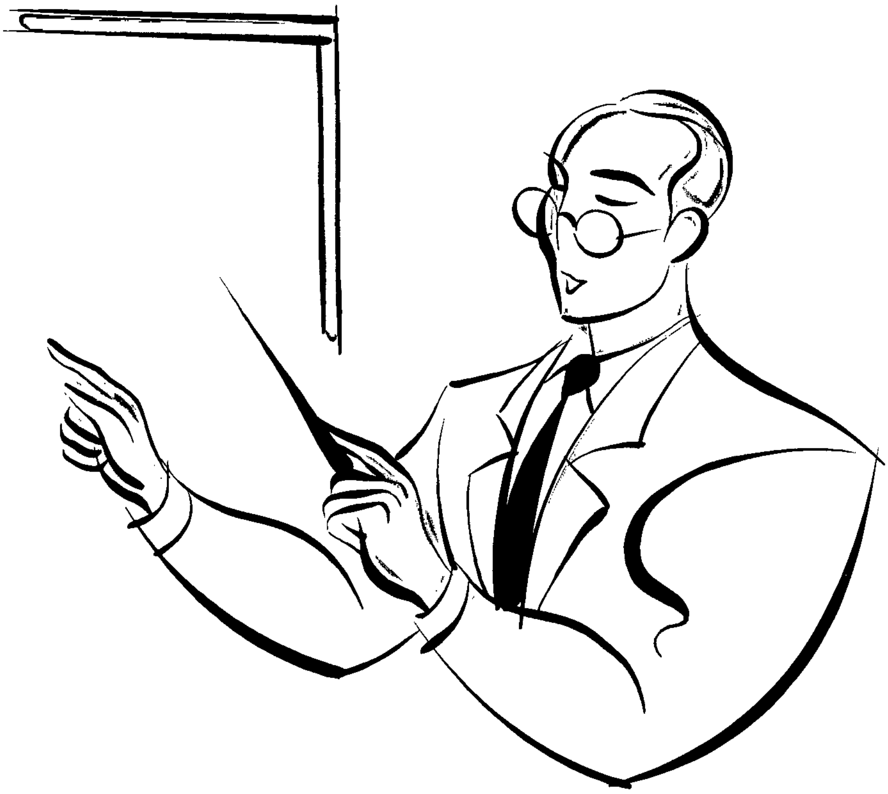
ABBREVIATIONS

α-car	α-carotene
β-car	β-carotene
^{1}Chl*	Singlet excited state of chlorophyll
$^1O_2$*	Singlet oxygen
^{3}Chl*	Triplet excited state of Chl
^{3}P680*	Triplet state of P680
A	Antheraxanthin
ABA	Abscissic acid
APX	Ascorbate peroxidase
AsA	Ascorbate
ATP	Adenosine triphosphate
ATPase	ATP synthase
BHT	Butylated hydroxytoluene
CAT	Catalase
CF	Coupling factor
Chl	Chlorophyll
CP	Chl *a/b* light harvesting proteins
Cyt	Cytochrome
DEI	De-epoxidation index
DGDG	Digalactosyl diacylglycerol
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
Fm	Maximum fluorescence
Fo	Initial fluorescence
FQR	Ferredoxin-plastoquinone oxidoreductase
Fs	Steady state fluorescence
Fv	Variable fluorescence
Fv/Fm	Maximal quantum efficiency of PS II
GSH	Glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
LHC I	Light harvesting Chl *a/b* associated with PS I
LHC II	Light harvesting Chl *a/b* associated with PS II
MGDG	Monogalactosyl diacylyglycerol
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
Nx	Neoxanthin
$O_2^{\cdot}$	Superoxide anion radical
$^{\cdot}$OH	Hydroxyl radical
P680	Chl *a* dimmer which absorb at 680 nm (PS I)
PAR	Photosynthetically active radiation
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PFD	Photon flux density
PG	Phosphatidylglycerol
Pheo	Pheophytin
PPFD	Photosynthetic photon flux density
PS I	Photosystem I

PS II	Photosystem II
qE	Energy-dependent quenching
qI	Photoinhibitory quenching
qN	Non-photochemical quenching
qP	Photochemical quenching
qT	State-transition quenching
RC	Reaction centers
RO'	Alkoxy radicals
ROO^-	Peroxy radicals
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SQDG	Sulfoquinovosyl diacylglycerol
V	Violaxanthin
VDE	Violaxanthin de-epoxidase
XCP	Xanthophyll cycle pigments
Y_Z^+	Oxidized secondary electron donor of PS II
Z	Zeaxanthin
ZE	Zeaxanthin epoxidase

ABSTRACT

The effect of direct sunlight on the photosynthetic efficiency (Fv/Fm), non-photochemical quenching (qN) and photosynthetic pigments (xanthophyll cycle) and relationship between the two (qN and xanthophyll cycle), along with oxidative damage to membrane lipids and changes in total soluble and insoluble carbohydrates, lipids and fatty acids were studied in rice (*Oryza sativa* L. cv. Jyothi) plants grown in the glasshouse (150-200 µmol $m^{-2}s^{-1}$) or shade (600-800 µmol $m^{-2}s^{-1}$), and results were compared with rice plants grown under full sunlight (1200-2200 µmol $m^{-2}s^{-1}$). Thirty days old glasshouse and shade grown plants were transferred to direct sunlight to determine the extent of photoinhibitory damage and capability of rice plants grown at lower light intensities to acclimate to full sunlight over a period of seven days. Transfer of glasshouse and shade grown plants to full sunlight for a day resulted in a reversible decline in their Fv/Fm and an increase in qN. The decline in Fv/Fm was much greater in transferred glasshouse grown plants as compared to transferred shade grown plants. While in sun grown plants Fv/Fm throughout the day did not show much variation from their predawn measurements. The lower susceptibility to photoinhibition of PS II in sun grown plants compared to glasshouse and shade grown plants was also related to an increased pool size of xanthophyll cycle pigments. Transfer of glasshouse and shade grown plants to full sunlight resulted in an increase in antheraxanthin (A), zeaxanthin (Z), lutein and Chl *a/b* ratio. This increase in the A and Z content was largely due to increase in the activity of violaxanthin de-epoxidase in treated plants. Increased levels of lutein and Chl *a/b* ratios may provide additional photoprotection to the rice plants and contribute, alongside the xanthophyll cycle. The increase in A and Z was correlated with the increase in the qN in glasshouse and shade grown plants transferred to direct sunlight for a day, indicating that thermal dissipation of excitation energy is dependent on the xanthophyll cycle activity. However, when sun exposure had been extended up to seven days, the increase in qN was less proportional to the A+Z pool, since A+Z was seen to increase while qN decreased, indicating that, part of the qN may not be xanthophyll cycle dependent under long-term photoinhibitory conditions. Plants fed with ascorbate showed higher Fv/Fm and qN than plants fed with dithiothreitol (DTT). Ascorbate fed leaves had efficient thermal dissipation of excess energy as well as de-epoxidation of V, contrary to DTT which inhibited de-epoxidation of the xanthophyll cycle as well as energy dissipation process in the pigment bed. Hence, greater damage in the DTT-fed rice leaves. Plants fed with exogenous ABA and subsequently exposed to sunlight resulted in most of the V being made available for conversion to Z and this was correlated with higher level of qN and better photoprotection. While plants subjected to salinity and exposed to sunlight exhibited an increase in the endogenous ABA level while Z was absent indicating that the V was converted into ABA and not Z, thereby undermining energy dissipation in NaCl treated plants. The results suggest that V act as a common precursor for Z, under high light conditions, and ABA under salt stress. In transferred glasshouse and shade grown plants an increase in the extent of lipid peroxidation was observed, while prolonged exposure up to seven days resulted in a decline in the peroxidation level. The decrease in the peroxidation level could be result of acclimation of plants to direct sunlight as a result of Z, which also acts as an antioxidant and may also involve enzymatic and non-enzymatic antioxidants. The concentration of total soluble carbohydrates was higher than the concentration of total insoluble carbohydrates in the plants of all the three different growth conditions, which on transfer of plants to direct sunlight resulted in an increase in the concentration of both total soluble and insoluble carbohydrates indicating no limitation to carbohydrate metabolism under high light conditions. Quantitative as well as slight qualitative changes observed in the lipids and fatty acid composition of cell and thylakoid membrane of glasshouse, shade and sun grown rice plants in response to high light, are probably the result of physiological reactions in the rice plants, in order to sustain optimum structure and function of the cell and thylakoid membrane to maintain active photosynthesis even under stress conditions. Although, low light grown rice plants are initially highly susceptible to high light stress; they substantially acclimate to full sunlight conditions by employing physiological mechanisms, such as non-radiative energy dissipation through the xanthophyll cycle, increase in lutein content, changes in Chl *a/b* ratio and qualitative as well as quantitative changes in lipids and fatty acid composition as in high light grown plants.



CHAPTER-1

INTRODUCTION

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INTRODUCTION

1.1. Plants in relation to their environment

The term environment etymologically means surroundings. Thus, environment is a complex of many factors which surround an organism and affect the life of an organism in many ways. These environmental factors can be living (biotic) or non-living (abiotic). These environmental factors exhibit diurnal, seasonal, annual and cyclic variations which the plants are subjected to, and therefore they have to develop strategies to cope with these changes in the environment (Sharma 1997).

1.2. Light as an environmental factor

Light is well known for its effects on the basic physiological processes of plants, such as photosynthesis, transpiration, seed germination, flowering, etc. Sun is the main source of light energy for photosynthesis. The sun radiates energy representing the entire electromagnetic spectrum (ranging from the cosmic rays to radio waves) (Fig. 1.1), but the earth's atmosphere is transparent only to part of the infra-red and ultraviolet light and all the visible light. The visible portion of the electromagnetic spectrum ranges from the violet at about 380 nm to the far red at 750 nm. Radiation from about 400 to 700 nm is used in higher plant photosynthesis and is called photosynthetically active radiation or PAR and is measured by quantum sensors (Lawlor 1987).

1.3. Photosynthesis

The term photosynthesis literally means building up or assembly by light. In this process green plants in the presence of sunlight synthesise carbohydrates using carbon dioxide and water from the environment with the subsequent release of molecular oxygen, in the process the energy-poor compounds carbon dioxide and water are

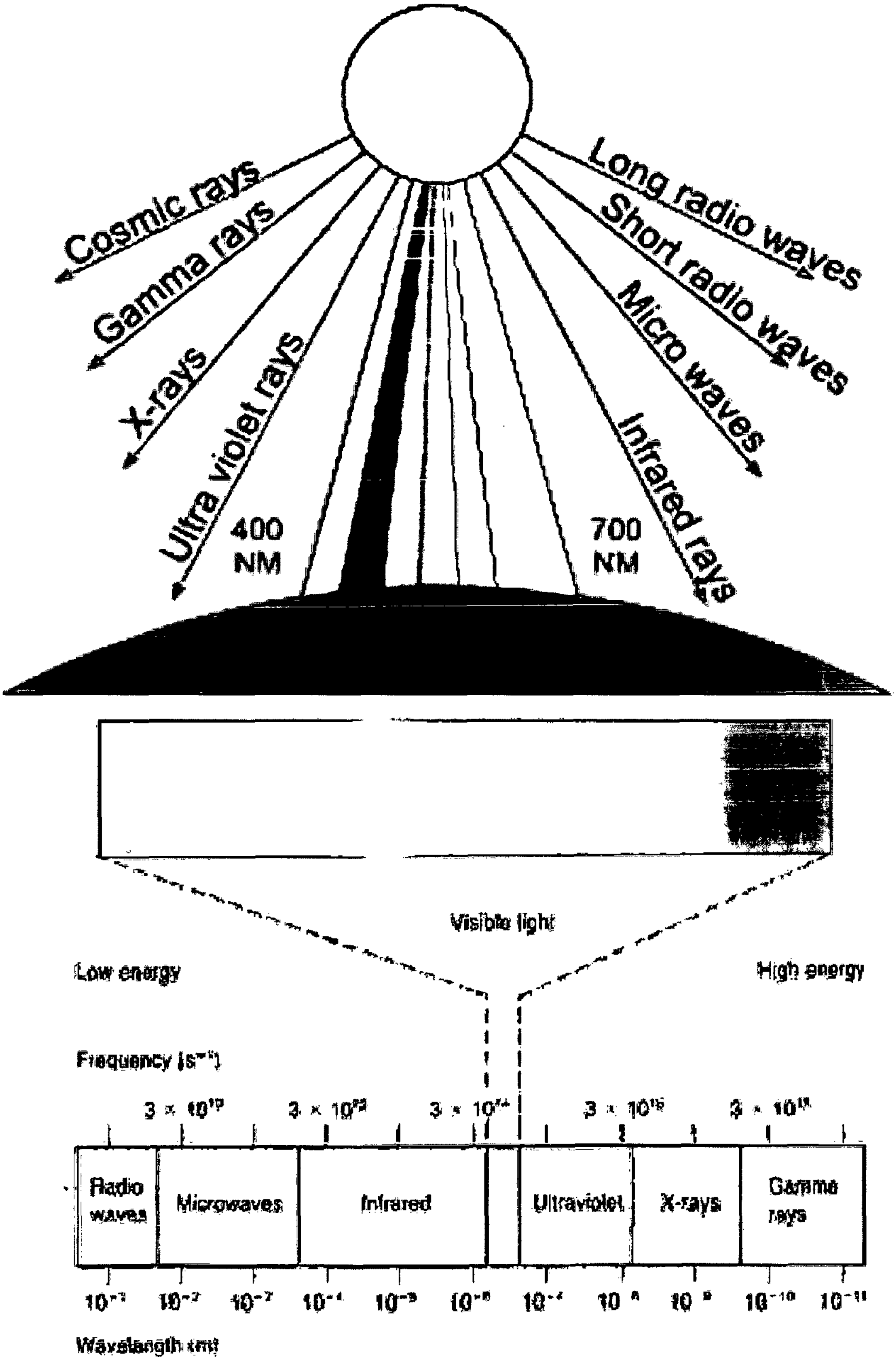


Figure 1.1: Electromagnetic spectrum of solar radiation

converted to the energy-rich compound carbohydrates, and oxygen is given out as a by product.

1.4. Chloroplast structure and organization

Photosynthetic process occurs in chloroplasts, which are found in mesophyll and palisade cells of the leaves (Fig. 1.2). Chloroplasts are semiautonomous organelles having a circular genome with a size of 134,525 bp (Hiratsuka et al., 1989). Electron microscope pictures showed that chloroplasts in higher plants are saucer shaped bodies 4 to 10 µm in diameter and 1 µm in thickness (1 µm = 10^{-6} m) with an outer double membrane or envelope separating them from the rest of the cytoplasm. The inner envelope membrane of the chloroplast acts as a barrier controlling the flux of organic and charged molecules in and out of the chloroplast. Water passes freely through the envelope membranes, as do other small neutral molecules like carbon dioxide and oxygen (Whitmarsh and Govindjee 1999).

Internally the chloroplast is comprised of a complicated membrane system of lamellae or flattened thylakoids, known as the photosynthetic membrane (or thylakoid membrane), which are arranged in stacks in dense green regions known as grana. The grana are embedded in a colourless matrix called the stroma, and are interconnected by a system of loosely arranged membranes called the stroma lamellae. Four supra-molecular protein complexes are embedded in the thylakoids: photosystem II (PS II), cytochrome b_6f (Cyt b_6f), photosystem I (PS I) and ATP synthase (ATPase) (Nelson and Ben-Shem 2004). Figure 1.3 shows the organization of these protein complexes in the thylakoid membrane. The first three complexes participate in electron transport from water to $NADP^+$. During photosynthetic electron transport, protons from the stroma are transferred to the luminal space through the thylakoid membrane,

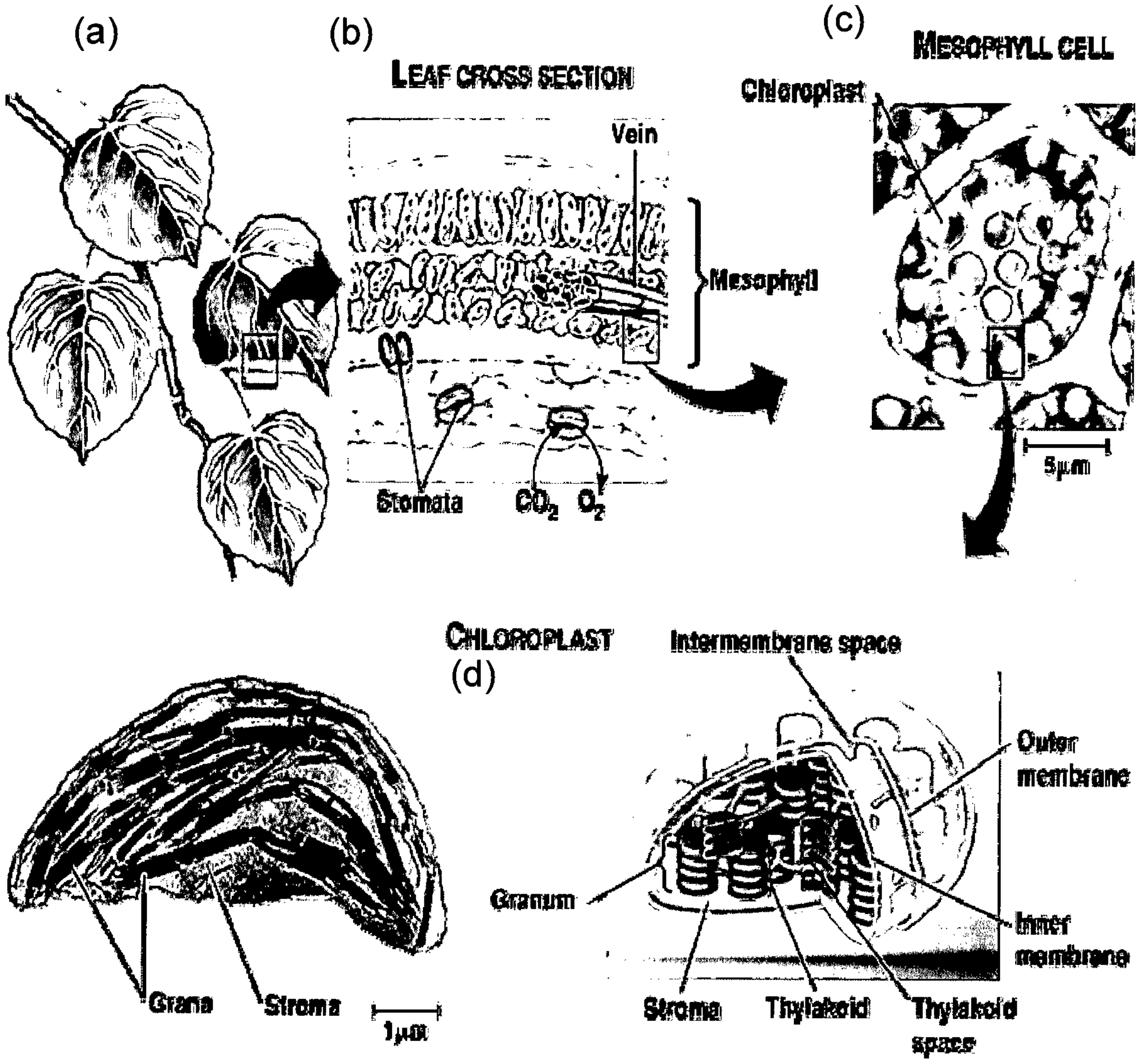


Figure 1.2: Structure of chloroplast
(a) Intact leaf
(b) Leaf cross section
(c) Mesophyll cell
(d) Chloroplast

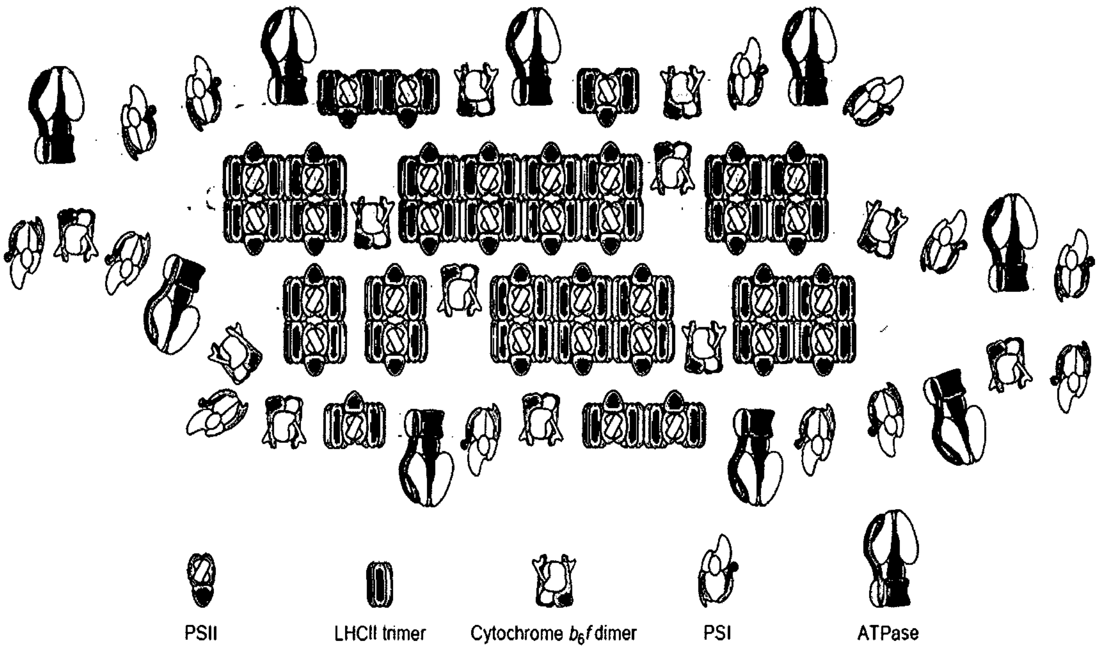


Figure 1.3: Organization of the protein complexes of the thylakoid membrane. Photosystem II is located predominantly in the stacked regions of the thylakoid membrane; photosystem I and ATP synthase are found in the unstacked regions protruding into the stroma. Cytochrome b_6f complexes are evenly distributed. This lateral separation of the two photosystems requires that electrons and protons produced by photosystem II be transported a considerable distance before they can be acted on by photosystem I and ATP-coupling enzyme.

developing a trans-thylakoid pH gradient essential for ATP synthesis by the ATP synthase.

1.4.1. Lipids and fatty acids in the photosynthetic membranes

The photosynthetic membrane is composed mainly of lipids and proteins (Kirk and Tilney-Basset 1978). The major lipids are polar glycerolipids, such as glycolipids and phospholipids. In plants and eukaryotic algae, glycerolipids and fatty acids are synthesized in chloroplasts (Murata and Siegenthaler 1998). The thylakoid membranes of the chloroplast of higher plants contain three glycolipids, namely, monogalactosyl diacylyglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG), and one phospholipid, phosphatidylglycerol (PG) (Joyard et al., 1998).

The structural lipids play a role in maintaining the appropriate fluidity of the membrane, which is a consequence of the high degree of unsaturation of these lipids. Furthermore, MGDG can form, under certain conditions, non-bilayer configurations (Williams 1998) and therefore, can influence photosynthetic functions. By contrast, the functional lipids are bound to proteins and tend to be less unsaturated. These molecules are involved in specific interactions with proteins that ensure an adequate maintenance of the confirmation and/or orientation of the proteins in the membrane.

1.4.2. Photosynthetic pigments

The photosynthetic membrane contains one or more organic pigments capable of absorbing visible radiation which will initiate the photochemical reactions of photosynthesis. The major classes of pigments found in plants are the chlorophylls (Chls) and the carotenoids. Chlorophylls are the primary light-capturing pigments which drive the photochemical reactions. Chlorophyll *a* is the major photosynthetic pigment in all organisms capable of oxygenic photosynthesis. However, Chl *a* has only moderate absorptivity in the green spectral region, causing the green colour of vegetation. It is therefore, supplemented by additional light-harvesting pigments. In green plants Chl *a* is accompanied by Chl *b,* which extends the absorption of light from either side into the 'green hole'. The presence of Chl *b,* therefore, narrows somewhat the "green gap" in the absorption spectrum of leaves (Sandmann and Scheer 2000).

The carotenoids are yellow or orange pigments found in all photosynthetic cells. They have triple banded absorption spectra in the region from about 400 to 550 nm. The carotenoids of higher plants include α-carotene (α-car), β-carotene (β-car), lutein, neoxanthin (Nx), violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z); their amount varies within the cell depending upon the growth conditions and stress factors (Britton 1990). Carotenoids act as accessory light harvesting pigments (Davidson and Cogdell 1981) and also protect against photooxidative damage (Krinsky 1979). In higher plants Z and A formed by the xanthophyll cycle (Yamamoto et al., 1962), increase non-radiative dissipation of absorbed energy (Demmig et al., 1987a; Sharma and Hall 1991).

1.5. Organisation and composition of the photosynthetic complex

Oxygenic organisms have two photosystems namely PS I and PS II. Each photosystem consists of a reaction centre core complex which carries out the primary charge separation, and a light-harvesting complex, which serve to increase the cross-sectional area utilised for light absorption. In higher plants LHC I and LHC II are associated with PS I and PS II respectively. Photon capture by the photosystem antennae and excitation transfer to the two photosystems, provide the energy for oxidation of water and electron movement to acceptors, which donate electrons to biochemical processes, and for passage of protons into the thylakoid lumen, for synthesis of ATP.

1.5.1. Light-harvesting complexes

Light-harvesting complexes are pigment-proteins whose function is to absorb light energy and to transfer it rapidly and efficiently to the photosynthetic reaction centres (RCs). They bind most of the photosynthetic pigments found in higher plants acting as antennae in trapping and transferring excitation energy.

Light harvesting complex II protein has a trimeric arrangement of monomers in a radially symmetric complex with internal symmetry within each monomer (Fig. 1.4) (Kühlbrandt and Wang 1991). In each monomer there are three membrane-spanning helices, helices A and B showing a high intramolecular similarity, inserted into the membrane with an angle of 30-32° form a cross with each other, while a third helix C lies perpendicular to the membrane plane. Cross-braced to helices A and B, xanthophylls are located in two symmetrical sites called L1 and L2, respectively, connecting the stromal loop with the amphyphilic helix D and the N-terminal domain with the luminal loop. LHC II contains about 8 Chl *a* and 6 Chl *b* together with 2 xanthophylls (Kühlbrandt and Wang 1991). The 14 Chls have their chlorine rings roughly perpendicular to the membrane plane and are arranged in two layers near the surfaces of the membrane. Helices A and B serve as a scaffold for packing the pigments into a small volume so that the light-harvesting proteins have an unusually large number of pigment molecules, i.e. one Chl per 15 amino acid residues, bound to a single polypeptide (Kühlbrandt et al., 1994).

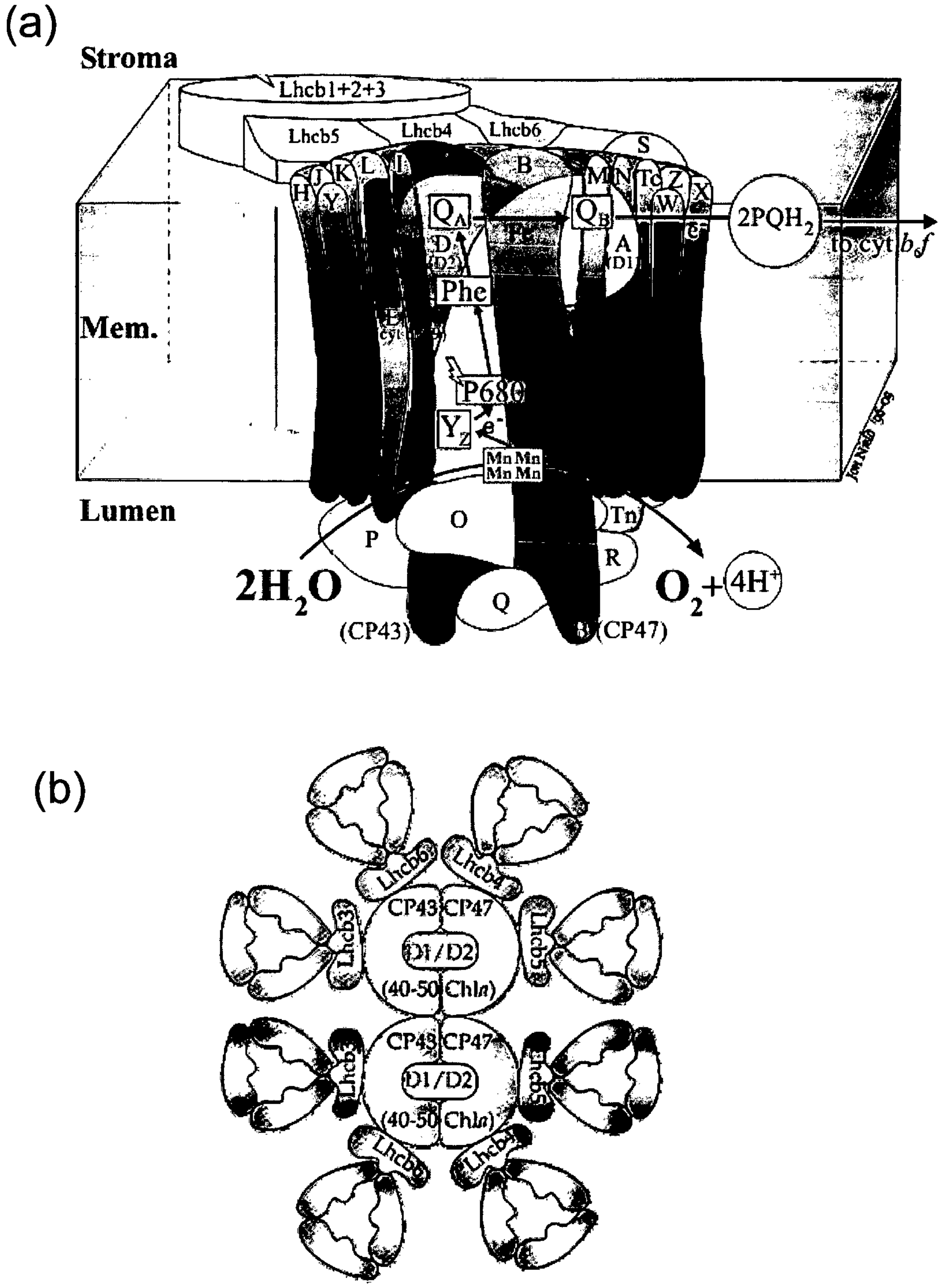


Figure 1.4:

(a) Organisation of light harvesting complex II, showing the association of specific Chl-binding complexes with PS II.

(b) In PS II an integral core of Chl *a*-binding proteins, designated CP43 and CP47 are closely associated with the D1/D2 RC complex, several peripheral Chl *a/b* binding proteins are also present.

Different proteins of LHC II have been identified as closely related pigment-proteins encoded by a family of nuclear genes (Green et al., 1992). All proteins are encoded by nuclear DNA, synthesized in the cytoplasm as precursor proteins with transit sequences which direct the protein into the chloroplast. LHC II preparations yield LhcbI and Lhcb2 proteins with various amounts of Lhcb3 and others. Lhcb1 is very homologous to the Lhcb2 protein, and is part of the most peripheral antenna of PS II (Spangfort and Anderson 1989), where it exists with Lhcb1 in mixed trimers (Simpson and Knoetzel 1996). Lhcb3 is the smallest pigment-protein found in LHC II, and in contrast to Lhcb1 and 2 is tightly bound to the reaction centre of PS II. *Lhcb4* codes for CP29, the largest of the LHC proteins, and is part of the inner antenna which connects the LHC II to the PS II reaction centre. *Lhcb5* and *Lhcb6* code for the apoproteins of CP26 and CP24, respectively. The LHC II includes 40-50 Chl *a* molecules bound to CP43 and CP47 (*psbC* and *psbB*); a consensus antenna size value for the Chls organised in the Lhcb proteins is 230-250 Chl (Melis 1991).

The antenna of PS I (LHC I) contains three pigment-protein complexes: a heterodimeric LHCI-730 complex composed of a 21 and 22 kDa polypeptide and two LHCI-680 complexes with single apoproteins of 25 kDa (LHCI-680A) and 23 kDa (LHCI-680B) (Knoetzel et al., 1998; Fig. 1.5). The PS I core is surrounded by a monolayer of 8 LHC polypeptides (Boekema et al., 1990). In PS I, about half of the light-harvesting Chl is associated with the two large polypeptides that contain the RC pigment P700 and form the CPI complex. This consists of two homologous 82 kDa proteins which bind about 90 molecules of Chl *a* and 12-16 molecules of β-carotene.

Although the primary function of photosynthetic light-harvesting complexes is the absorption of light and the transfer of the excitation energy to the photochemical

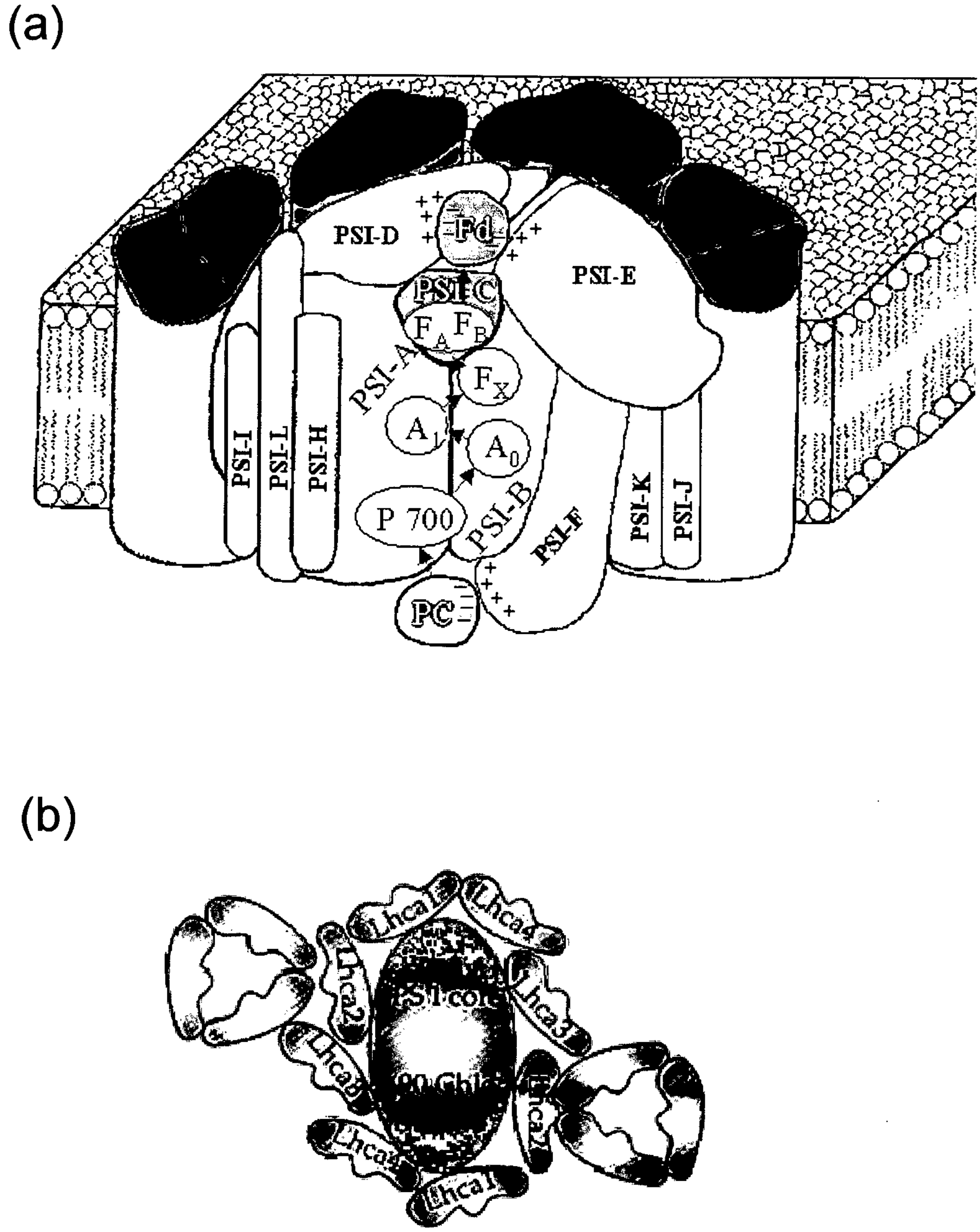


Figure 1.5:

(a) Organisation of light harvesting complex I, showing the association of specific Chl-binding complexes with PS I.

(b) In PS I the core complex contain approximately 90 Chl *a* molecules, additional Chl is present in LHC-I complexes, which contain both Chl *a* and *b*.

reaction centres, they are also essential for regulation and distribution of excitation energy within the photosynthetic apparatus, and respond to both short- and long-term fluctuations in light intensity and quality. The LHC II proteins carry out these regulatory functions through different mechanisms: phosphorylation, dissociation into monomers, migration from grana to stroma, binding of xanthophylls, etc. (reviewed by Bassi et al., 1997).

1.5.2. Photosystem II (PS II)

Photosystem II is located in the appressed granal regions of the chloroplast (Anderson 2002), and consists of more than 20 protein subunits (Table 1.1), composed of the reaction centre core complex with a molecule of P680 joined to the oxygen evolving complex and the peripheral light-harvesting antenna assembly (Rhee 2001; Fig. 1.6a). Photosystem II uses light energy to drive two chemical reactions - the oxidation of water and the reduction of plastoquinone. The basic structural unit of PS II is the so-called LHC II-PS II supercomplex (Hankamer et al., 1997). At the heart of PS II, the reaction centre D1/D2 protein heterodimer binds all of the redox factors necessary for stable light-induced charge separation across the thylakoid membrane (Diner and Babcock 1996). These factors are (from the oxidizing side to the reducing side) a tetra manganese cluster with a calcium- and a chloride-ion, two tyrosine residues as referred to Y_Z and Y_D, four to six Chl *a* molecules, two pheophytins, and two plastoquinones. Compared to these, cofactors, such as non-heme iron and bicarbonate anions (Govindjee and Rensen 1993), do not function as a direct electron carrier but are thought to be crucial for this process. Two β-car molecules are present in the RC and are involved in the secondary photochemical reactions (Rhee 2001). The oxygen-evolving core complex comprises other proteins closely associated with the D1/D2 heterodimer. These are the Chl *a* proteins, CP43 and CP47, α- and β-subunits of

Table 1.1: Photosystem II subunits. The size of each protein is based on its amino acid sequence. The name of the genes and their location, chloroplast (C) or nuclear (N) genome is given. Structural features of proteins are indicated.

Components	**Gene**	**Molecular weight (kDa)**	**Structural features**
D1	*psbA* (C)	32	Reaction center (RC)
D2	*psbD* (C)	34	Reaction center
Cyt b559α	*psbE*	9	Photoprotection
Cyt b559β	*psbF*	4	Photoprotection
CP47	*psbB* (C)	47	*Chl a*-binding RC antenna
CP43	*psbC* (C)	43	*Chl a*-binding RC antenna
PS II-H	*psbH* (C)	10	Light-dependent phosphorylation
PS II-I	*PsbI* (C)	4.8	Reaction centre
PS II-J	*psbJ* (C)	4	Stabilization of assembly
PS II-K	*psbK* (C)	3.5	Absent in purified oxygen-evolving core
PS II-L	*psbL* (C)	5	Regulation of the P680$^+$ reduction
PS II-M	*psbM* (C)	3.8	In oxygen evolving core
PS II-N	*psbN*	4.7	In oxygen evolving core
PS II-R	*PsbR* (N)	10	Membrane anchored
OEC1	*PsbO* (N)	33	Regulate oxygen-evolution
OEC2	*PsbP* (N)	23	Regulate oxygen-evolution
OEC3	*PsbQ* (N)	17	Regulate oxygen-evolution
CP29	*Lhcb4* (N)	28	Binds Chl *a* and *b*, lutein, Nx, V
CP26	*Lhcb5* (N)	27	Binds Chl *a* and *b*, lutein, Nx, V
CP24	*Lhcb6* (N)	23	Binds Chl *a* and *b*, lutein, Nx, V
CP22	*PsbS* (N)	22	Binds Chl *a* and *b*

cytochrome b559, several small membrane-spanning helical proteins, a cluster of four manganese atoms probably all attached to D1 protein and the 33 kDa, 23 kDa and 17 kDa extrinsic luminal proteins (Barber 2002). In higher plants, and some green algae, the central core is flanked by a family of Chl *a/b* proteins, monomeric CP29, CP26 and CP24, and the outer antenna trimeric LHC II that regulate light-harvesting (Horton et al., 1996).

1.5.3. Photosystem I (PS I)

Photosystem I is distributed in non-appressed stromal lamellae and peripheral regions of the grana (Anderson 2002) and catalyses the oxidation of plastocyanin, a small soluble copper-protein, and the reduction of ferredoxin, a small iron-sulphur protein. It is made up of the P700 RC and is composed of a heterodimer of proteins that act as ligands for most of the electron carriers (Nelson and Yocum 2006; Fig. 1.6b). The PS I monomer consists of 15 protein subunits (Table 1.2), of these, only PsaA, PsaB, and PsaC bind the cofactors of the electron transfer system. PsaA and PsaB form the core complex around which other subunits are organized. The PsaC, PsaD, PsaH, and PsaE proteins form the stromal peripheral domain that contains the terminal electron donors and the ferredoxin-docking site. PsaN is a lumenal peripheral protein (Chitnis 2001). PsaN and the large luminal domain of PsaF form the plastocyanin docking site of PS I. The remaining proteins of PS I are integral membrane proteins with 1–3 transmembrane helices. The PsaA and PsaB proteins of the PS I core are homologous in their primary sequences and transmembrane topography. They contain 11 transmembrane helices each with their N termini in the stroma. Two domains in these proteins can be defined based on evolutionary and functional considerations. The C-terminal domains of PsaA and PsaB contain five transmembrane helices each and contain residues whose side chains provide coordinating groups for the redox centers.

Table 1.2: Photosystem I subunits. The size of each protein is based on its amino acid sequence. The name of the genes and their location, chloroplast (C) or nuclear (N) genome is given. Structural features of proteins are indicated.

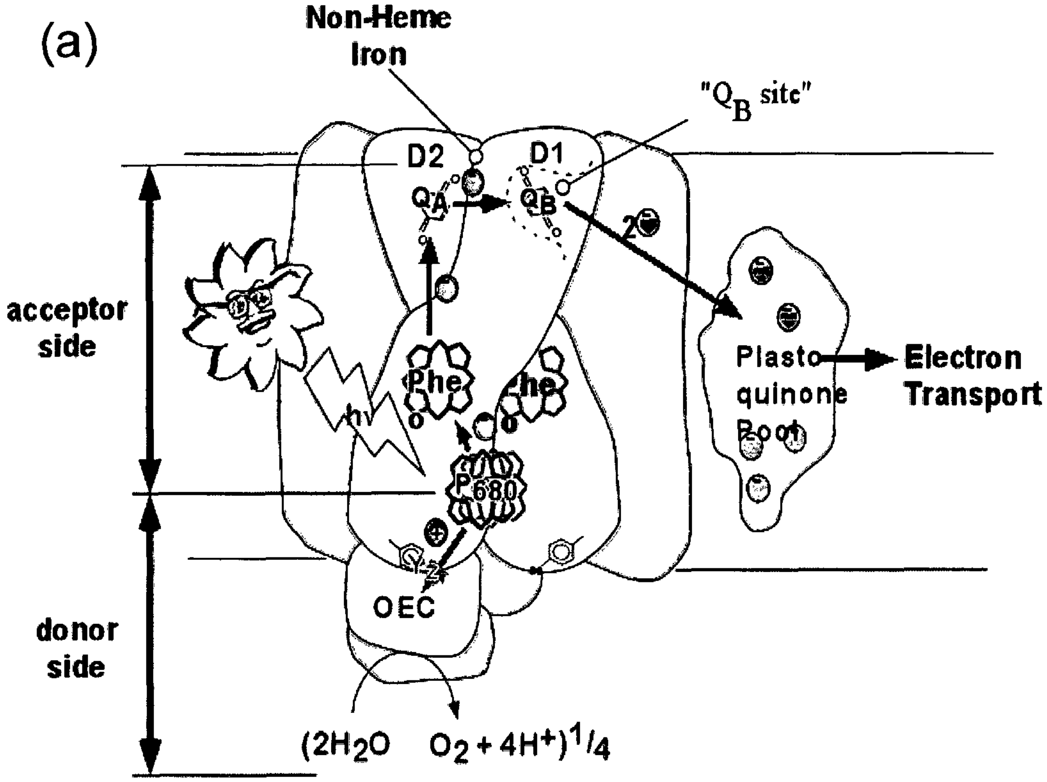
Components	**Gene**	**Molecular mass (kDa)**	**Structural features**
PS I-A	*psaA* (C)	83	Heterodimer binds about 100 Chl a, 12-15 β-carotene
PS I-B	*psaB* (C)	82	
PS I-C	*psaC* (C)	9	Terminal electron acceptors
PS I-D	*PsaD* (N)	17.9	Ferredoxin docking
PS I-E	*PsaE* (N)	9,7	Peripheral on stromal side
PS I-F	*PsaF* (N)	17.3	Plastocyanin docking
PS I-G	*PsaG* (N)	10.8	Found in higher plants only
PS I-H	*PsaH* (N)	10.2	Found in higher plants only
PS I-I	*psaI* (C)	4.6	One transmembrane α-helix
PS I-J	*psaJ* (C)	3.3	One transmembrane α-helix
PS I-K	*PsaK* (N)	5.6	Two transmembrane α-helices
PS I-L	*PsaL* (N)	15.4	Two transmembrane α-helices
PS I-M	*psaM*	3.5	One transmembrane α-helix
PS I-N	*psaN*	4.8	Peripheral on the luminal side
PS I-O	*PsaO* (N)	9	One transmembrane α-helix
Ferredoxin	*PetG* (N)		Fe-S centre
FNR	PetH (N)	35.4	Ferredoxin-NADP reductase

Figure 1.6:

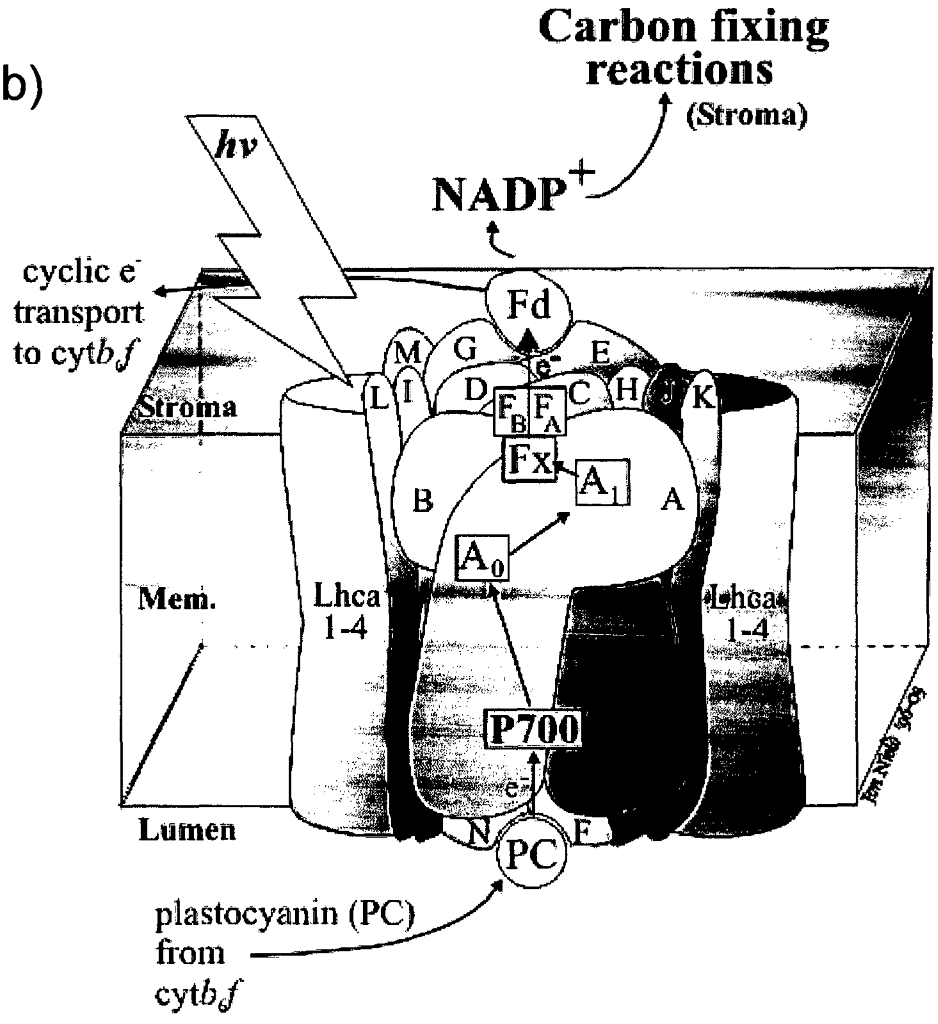
(a) Structural model of photosystem II reaction center, a schematic representation showing the structure dominated by the two PS II RC proteins D1 and D2.

(b) Structural model of photosystem I reaction center, a schematic representation showing the organisation of the two major portions in this complex the psaA and psaB subunits, designated here as A and B.

(a)


Non-Heme Iron
"QB site"
D2
D1
QA
QB
2e
acceptor side
donor side
Pheo
Pheo
hν
P680
Plastoquinone Pool
Electron Transport
Yz
OEC
(2H2O O2 + 4H+)1/4

(b)


Carbon fixing reactions
(Stroma)
hν
NADP+
cyclic e- transport to cytb6f
Fd
M
G
E
e-
L
I
D
FB
FA
C
H
J
K
Stroma
Fx
A1
B
A
A0
Mem.
Lhca 1-4
Lhca 1-4
P700
e-
Lumen
N
F
PC
plastocyanin (PC) from cytb6f

Interactions among the helices in the C-terminal domains of PsaA and PsaB are primarily responsible for forming the heterodimer. The tilted ten transmembrane helices, along with four parallel helices of the C-terminal domain, make a cage for coordination and protection of the electron transfer chain. The N-terminal domain contains the remaining six transmembrane helices, which resemble the transmembrane topology of the CP43 and CP47 proteins of PS II. These helices, along with the transmembrane segments of other PS I proteins, coordinate the chlorophyll molecules of the internal antenna of PS I (Chitnis 2001).

1.5.5. Electron transport chain

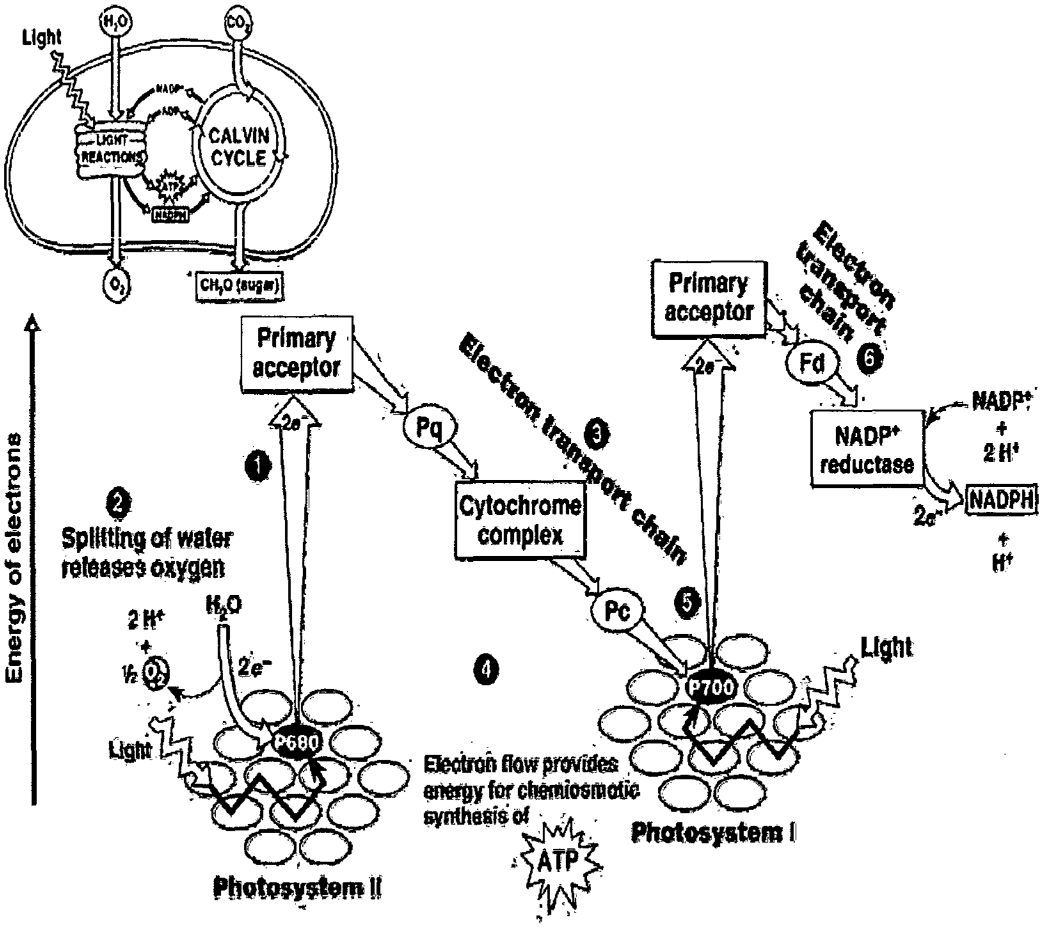
In oxygenic photosynthetic organisms, the two different photosystems, PS II and PS I, operate in series in the non-cyclic electron transport chain to oxidise water, reduce $NADP^+$ and generate ATP (Fig. 1.7). Photosystem I can also function independently in the cyclic electron transport pathway to generate ATP. In the light, PS II feeds electrons to PS I by intermediate carriers. The intermediates of the electron transport chain can be divided into several classes: cytochromes (cyt f, b6 and b559), non-haeme iron-sulphur centres (e.g. Ferredoxin and Rieske centre), quinones (eg. plastoquinone), proteins with transition metals as the prosthetic group (plastocyanin, Mn-containing proteins), Chl and pheophytin. The Rieske FeS-protein, functions as the primary oxidant of quinol in cytochrome b_6 complexes (Rieske 1976). It contains a 2Fe-2S-centre with a positive redox potential. The Rieske FeS-proteins of chloroplast are encoded in nuclear genes (Salter et al., 1992). An additional complex in the thylakoid membrane is the ATP synthase, found only in non-appressed region. The ATP synthase may be separated into two parts: coupling factor 1 (CF_1) and CF_o. CF_1 is an oligomeric hydrophilic protein that contains the catalytic sites of the

Figure 1.7:

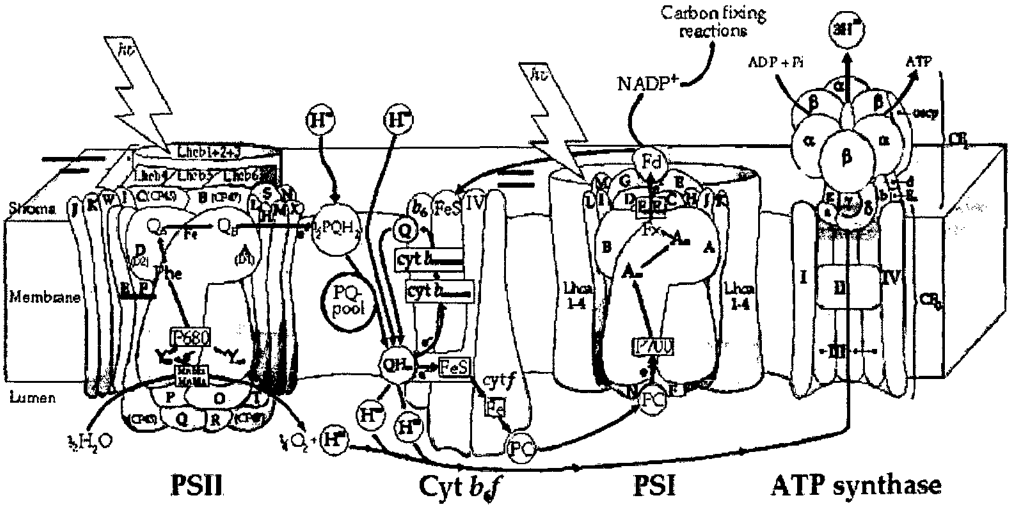
(a) Schematic representation of the Z-scheme of photosynthesis.

(b)Membrane organisation of the Z-scheme. The various components of the chloroplast electron transport chain and ATP-synthesizing apparatus are illustrated in the thylakoid membrane. Four membrane complexes – PS II, PS I, the cyt b6f complex and the ATP synthase are shown. Electrons are transferred from water to NAD+; accompanying this electron transfer, a proton gradient is ultimately utilised for the synthesis of ATP by the ATP synthase.

(a)


Light
H₂O
CO₂
NADP⁺
ADP
LIGHT REACTIONS
CALVIN CYCLE
ATP
NADPH
O₂
CH₂O (sugar)
Energy of electrons
Primary acceptor
2e⁻
1
2
Splitting of water releases oxygen
H₂O
2 H⁺
+
½ O₂
2e⁻
Light
P680
Photosystem II
Pq
Cytochrome complex
3
Electron transport chain
Pc
4
Electron flow provides energy for chemiosmotic synthesis of
ATP
5
P700
Light
Photosystem I
Primary acceptor
2e⁻
Electron transport chain
Fd
6
NADP⁺ reductase
NADP⁺
+
2 H⁺
2e⁻
NADPH
+
H⁺

(b)


hν
Lhcb1+2+3
Lhcb4
Lhcb5
Lhcb6
Stroma
Membrane
Lumen
J
K
W
I
C(CP43)
B(CP47)
S
N
L
H
M
X
Q_A
Fe
Q_B
D (D2)
A (D1)
Phe
E
F
P680
Y_Z
Y_D
MnMn MnMn
P
O
T
Q
R
(CP43)
(CP47)
½H₂O
½O₂+H⁺
H⁺
H⁺
PQH₂
PQ-pool
b₆
FeS
IV
Q
cyt b
cyt b
QH₂
FeS
cyt f
Fe
H⁺
H⁺
PC
hν
Carbon fixing reactions
NADP⁺
Fd
M
G
E
L
I
D
C
H
J
K
B
Fx
A
A
A
Lhca 1-4
Lhca 1-4
P700
N
F
PC
ADP + Pi
ATP
3H⁺
α
β
β
α
α
β
ε
γ
δ
b
I
II
III
IV
CF₁
CF₀
PSII
Cyt b₆f
PSI
ATP synthase

synthase. It consists of five different polypeptide subunits and a total of nine individual subunits. The subunits are labelled α to ε in order of decreasing molecular weight. CF_o binds CF_l and functions to conduct protons across the thylakoid membrane. It contains four different polypeptides, labelled in Roman numerals, I-IV (Fromme et al., 1987). Subunits I, II and III are numbered in order of decreasing molecular weight. Subunit IV is the largest CF_o protein. The net reaction is the transfer of electrons from a water molecule to $NADP^+$, producing the reduced form, NADPH. Driven by the H^+ gradient built up across the membrane by electron transfer reactions, the ATP synthase converts ADP into ATP.

1.6. Photosynthesis and photoinhibition

All oxygenic photosynthetic organisms exist in an environment in which many factors affecting the rate and capacity for photosynthesis vary on time scales from as short as a few seconds to periods of days or longer (Owens 1994). Principal among these is light intensity. The short-term response of photosynthetic organisms to light intensity is typically described by the photosynthesis-irradiance (P-I) curve (Fig. 1.8a) in which the steady-state rate of photosynthesis is plotted against incident light intensity. At low light intensities (>100 µmol $m^{-2}s^{-1}$) more than 80% of the absorbed quanta would be utilized, in accordance with the maximum measured quantum yields of oxygen evolution (Björkman and Demmig 1987). When the irradiance approaches half of full sunlight (- 1000 µmol $m^{-2}s^{-1}$) as little as 25% of the absorbed quanta are utilized, and at full sunlight, utilization drops to approximately 10%. At the same time, the rate of light absorption by the plants is essentially linear in incident intensity to well beyond the physiological range. The difference between the two curves (shaded area, Fig. 1.8b) represents the absorbed light energy which is in 'excess' of the capacity of photosynthesis to utilize the absorbed energy to drive photosynthesis.

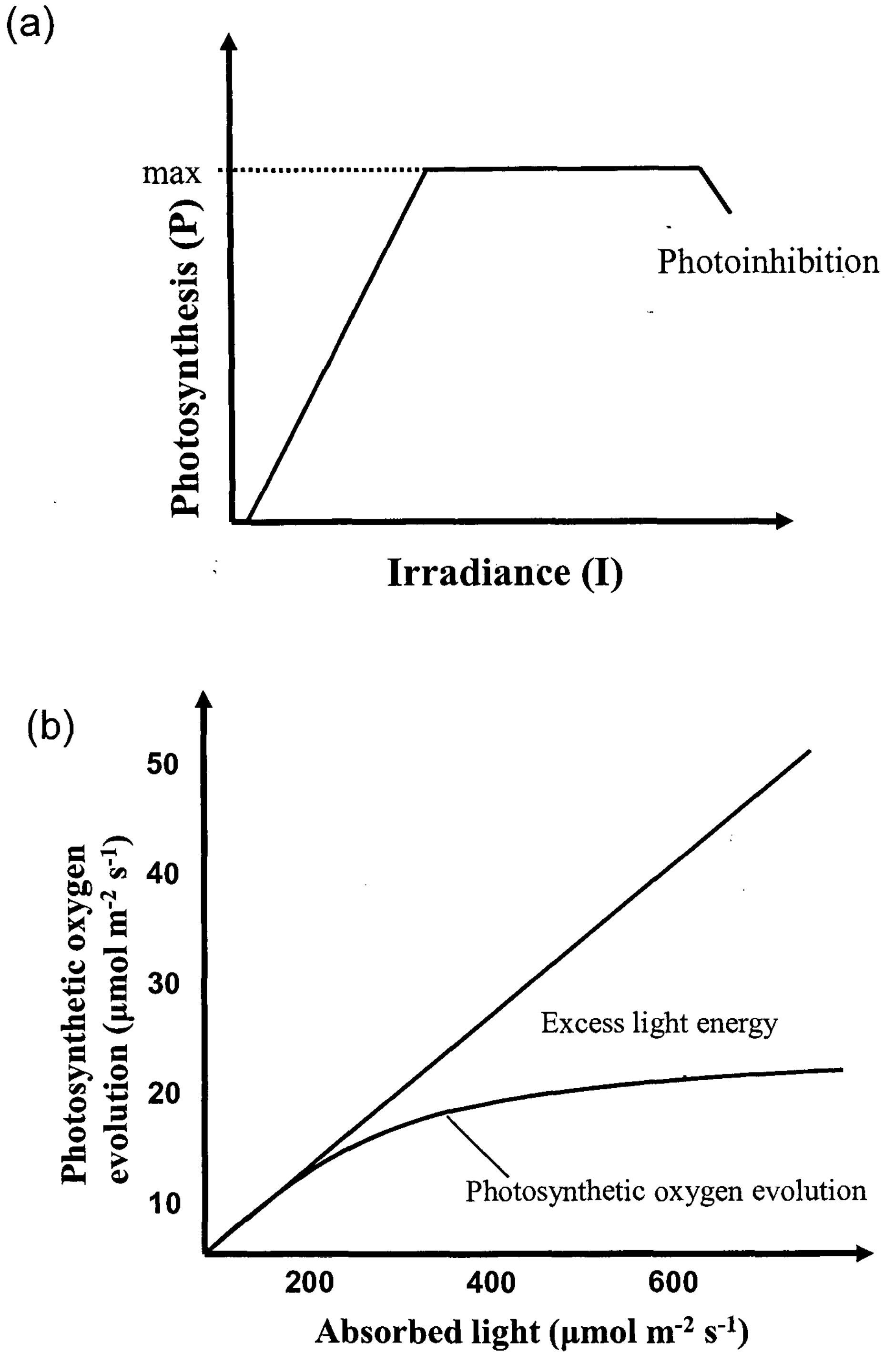


Figure 1.8:

(a) A graph showing the rate of photosynthesis with increasing irradiance.

(b) The predicted response of absorption, utilization and excess of quanta to incident photon flux.

The principal consequences of excess light absorption are photoinhibitory damage to the PS II reaction centre (Krause 1988) and generalized damage to membranes and proteins by singlet oxygen, superoxide and other free radicals (Pell and Steffen 1991). Kok (1956) have defined photoinhibition as the light-dependent reduction in photosynthetic efficiency. Furthermore, photoinhibition of the photosynthetic capacity can become more severe in the presence of an additional stress. Under natural conditions in the field, additional environmental stress factors (other than light) such as low or high temperature, drought, etc., are often present which increase the gap between light absorption and utilization, and further increases the potential for over-excitation of the photosynthetic apparatus whenever environmental conditions suppress photosynthetic carbon metabolism. For instance, heat stress results in the loss of grana stacking due the dissociation of peripheral light-harvesting complexes from the core complex (Gounaris et al., 1984). Reduction of PS II activity by heat stress primarily results from an inactivation of the oxygen evolving complex (Enami et al., 1994), but also perturbations of electron transport within the PS II reaction centers have been documented (Pospišil and Tyystjärvi 1999). Low temperatures sensitize photosynthesis to photoinhibition, so that even low light may induce photoinhibition. This is thought to occur primarily through temperature-imposed thermodynamic constraints on carbon metabolism which prevent excitation energy from being effectively utilized for carbon dioxide assimilation and thus promote photoinhibition (Krause 1994; Huner et al., 1998). Photosynthetic activity is also reduced by water stress. One of the earliest responses to drought is stomatal closure, which limits carbon dioxide diffusion to chloroplasts (Muller and Whitsitt 1996). With short periods of drought, or with relatively mild water stress, stomatal limitations account for most of the decrease in photosynthesis (Cornic and Masacci

1996). However, when drought is prolonged and/or more severe, the breakdown of the photosynthetic apparatus may contribute to the inhibition of carbon dioxide gain in water-stressed plants.

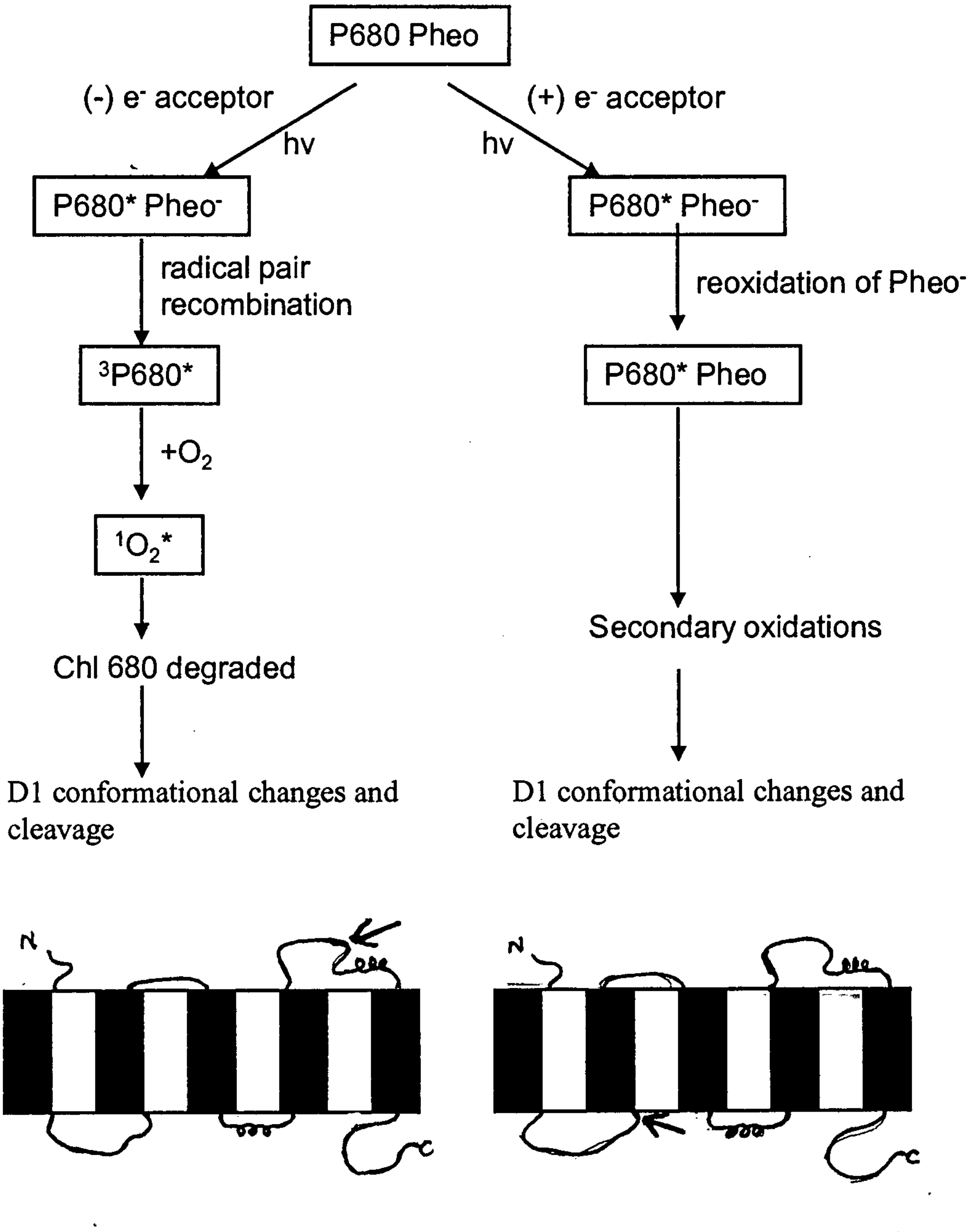
There are two main classes of photoinhibition, largely on the basis of their relaxation times (Osmond 1994). The first is dynamic photoinhibition which occurs most rapidly in sun-grown healthy plants as they are exposed to variations in photon flux that drive their photosynthetic capacity up and down the light response curve without significant damage. Chronic photoinhibition usually predominates in shade grown plants and is more slowly reversible. It occurs following sustained exposure of the photosynthetic apparatus to photon fluxes in excess of those encountered during growth, or to photon fluxes within the growth experienced, but under environmental conditions which themselves impair photosynthetic function. During chronic photoinhibition, the light response curve of photosynthesis is dispersed downwards.

1.6.1. Photoinhibition at PS II

The primary target of high irradiance causing photoinhibition of photosynthesis is PS II (Powles 1984; Krause 1994; Sharma 2002). In this process, two mechanisms are involved affecting either the acceptor side or the donor side of PS II (Fig. 1.9). The two mechanisms are distinguished on the basis of differences in the primary site of electron transport malfunctioning, the subsequent D1 protein degradation, and the oxygen requirement of the process.

Acceptor side-induced photoinhibition of PS II occurs under high irradiance when it exceeds the saturation of photosynthetic electron transport (Barber and Andersson 1992). Excess exposure causes non-physiological over-reduction of the first quinone electron acceptor in PS II. This brings about sequential modifications at the level of the Q_A and Q_B acceptors (Keren et al., 1997). These conditions lead to the recombination of the radical pair, $P680^+$ $Pheo^-$ (Vass et al., 1992) and the production of the triplet state of P680 ($^3P680^*$). This $^3P680^*$ is quenched by oxygen and singlet oxygen ($^1O_2^*$) is thus produced. The $^1O_2^*$ initiates and also triggers degradation of the reaction centre protein D1, probably by promoting a special conformational change which makes the protein susceptible to proteolytic cleavage (Fig. 1.9). The acceptor-side damage generates a 23.5 kDa N-terminal D1 fragment *in vivo* (Virgin et al., 1990). Two specific membrane-bound proteases, namely, endoprotease DegP2 and metalloprotease FtsH, degrade the D1 protein in the photodamaged PS II (Haussuhl et al., 2001). Rapid degradation of D1 under strong illumination can destabilize the PS II reaction center and expose D2 to the protease(s) (Ohad et al., 1990). Stability of D2 is necessary to stabilize the PS II complex in the membrane (Erickson et al., 1986). Therefore, whenever D1 and D2 are destabilized, PS II reaction centers may disassemble. The internal antennae, CP43 and CP47, also dissociate from PS II core and appear to move out to the stroma lamellae (Giardi 1993). The specific loss of PS II components is assumed to lead to the destabilization and degradation of LHC II, causing chlorosis (Kilb et al., 1996).

Donor side-induced photoinhibition of PS II occurs when the capacity of water oxidising complex to donate electrons to the RC P680 is inactivated by high irradiance (Minkov et al., 1999) (Fig. 1.9). Under such conditions the water oxidising complex is unable to keep up the rate at which electrons are transferred from P680 towards acceptor side components. This leads to an increase in the lifetime of $P680^+$ with a high oxidising potential. The $P680^+$ extracts electrons from the surrounding environment (amino acids, primarily histidines) which subsequently leads to



ACCEPTOR-SIDE PHOTOINHIBITION

DONOR-SIDE PHOTOINHIBITION

Figure 1.9: Scheme showing the two routes of damage due to acceptor and donor side photoinhibition of PS II.

destruction of D1, Chl and β–car associated with the reaction centre of PS II (Barber 1994).

D1 degradation *in vivo*, occurs at light fluencies much below the saturation threshold for photosynthesis (Mattoo et al., 1984; Jansen 1993). Photodamage to D1 occurs whenever a plastosemiquinone anion radical is generated in an oxygen-rich environment (Greenberg et al., 1990). The anion may be transiently produced by electron transfer to Q_B (either during cyclic or non-cyclic electron flow) or by direct interaction of UV light with plastoquinone molecules. The anion radical could then interact with oxygen to oxidize D1, possibly through the formation of peroxyl or hydroxyl radicals that can react with amino acid residues or metal ligands in D1. *In vitro,* a 24 kDa C-terminal D1 fragment can be generated by a cleavage in the loop between helices A and B on the luminal side of the membrane (Shipton and Barber 1992). This fragmentation has been attributed to the donor-side damage.

1.6.2. Photoinhibition at PS I

Photosystem I potential activity is remarkably stable in excessive light as compared to PS II (Aro et al., 1993; Barth et al., 2001). However, many authors have provided evidence that in certain circumstances PS I can be photoinhibited as much as or even faster than PS II (Havaux and Davaud 1994; Barth and Krause 1999; Sonoike 1996). Potential activity of PS I *in vivo* can be assessed by measuring the P700 absorbance change around 810-830 nm (Klughammer and Schreiber 1994). A preferential photo-inactivation of PS I was observed at chilling temperature in potato leaves (Havaux and Davaud 1994) and in cold-sensitive *Cucumin sativus* L., when leaves were chilled under low light (Sonoike 1996) or both under low and high light (Barth and Krause 1999). Mechanism of photodamage to PS I involve the destruction of iron-sulphur centres (F_A, F_B, F_X) (Sonoike et al., 1995), which triggers proteolysis of the PS I-A/B reaction centre proteins and of extrinsic polypeptides of the PS I complex, due to the generation of reactive oxygen species (Sonoike 1996).

1.7. Reactive oxygen species in photosynthesis

In plants the production of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and singlet oxygen ($^1O_2^*$), is an unavoidable consequence when the absorption of light energy exceeds the capacity for photosynthesis (Foyer et el., 1994; Cheeseman 2006). In the light-harvesting antenna of PS II, unused light energy increases the lifetime of the excited state of chlorophyll (^{1}Chl*), which can be converted to the triplet excited state (^{3}Chl*) through the photo physical process of intersystem crossing. ^{3}Chl* itself is not harmful, but it is long-lived and can transfer energy to ground-state oxygen to generate highly reactive singlet oxygen as shown below

$$^1\text{Chl} + h\nu \longrightarrow {}^1\text{Chl}^*$$

$$^1\text{Chl}^* \longrightarrow {}^3\text{Chl}^*$$

$$^3\text{Chl}^* + {}^3\text{O}^* \longrightarrow {}^1\text{Chl} + {}^1\text{O}_2^*$$

In addition to the deleterious formation of singlet oxygen, in the Mehler reaction the direct reduction of oxygen by PS I, when the rate of carbon dioxide fixation slows down and the $NADP^+$ supplement available to accept electrons from the electron transport chain is restricted, results in the formation of superoxide anion radical. Superoxide radical can then undergo a dismutation reaction forming hydrogen peroxide. Superoxide radical is unique in sense that it may act as both an oxidant and reductant. Hydrogen peroxide can readily diffuse across biological membranes and may cause oxidative stress far from site of formation. Both O_2^- and H_2O_2 are highly reactive, oxidizing cations such as Fe^{2+}, form the hydroxyl radical in the Haber-Weiss reaction (Halliwell and Gutteridge 1985). Hydroxyl radical is the most reactive of the reactive oxygen species described having capacity to oxidize and react with organic molecules. It is thought that much of the damage that occurs via the production of O_2^- and H_2O_2 results from their subsequent conversion to ^{-}OH. Singlet oxygen, superoxide, and other reactive forms of oxygen irreversibly oxidize DNA and membrane-bound lipids, proteins and pigments in their immediate vicinity.

1.7.1. Damage to organic molecules by ROS

1.7.1.1. Lipids

The reaction of ROS, especially of hydroxyl radiacl, with lipids is one of the most prevalent mechanisms of cellular injury and is dependent on the degree of membrane fluidity, which in turn is a function of the saturation state of the lipid bilayer (Halliwell and Gutteridge 1999). The degradation products of lipid peroxidation are aldehydes, such as malonaldehyde, and hydrocarbons, such as ethane and ethylene (Gutteridge and Halliwell 1990). Thylakoid lipids are especially susceptible to oxidative damage because of the abundance of unsaturated fatty acid side chains. Reactive oxygen attack of these lipids initiates peroxyl-radical chain reactions, which eventually can destroy the thylakoid membrane (Knox and Dodge 1985). The mechanism involving lipid peroxidation is that oxygen radicals catalyze oxidative modification of lipids, as shown in Figure 1.10. Presence of double bond adjacent to methylene group makes methylene C-H bonds of PUFA weaker and therefore hydrogen becomes more prone to abstraction. While lipid peroxidation is not initiated by O_2^- and H_2O_2, $^{\cdot}OH$, alkoxy radicals (RO') and peroxy radicals ($ROO^{\cdot}$) result in initiating lipid peroxidation (Halliwell 2006). This lead to self perpetuating process since peroxy radicals arc both reaction initiators as well as end products of lipid peroxidation. Lipid peroxy radicals react with other lipids, proteins and nucleic acids,

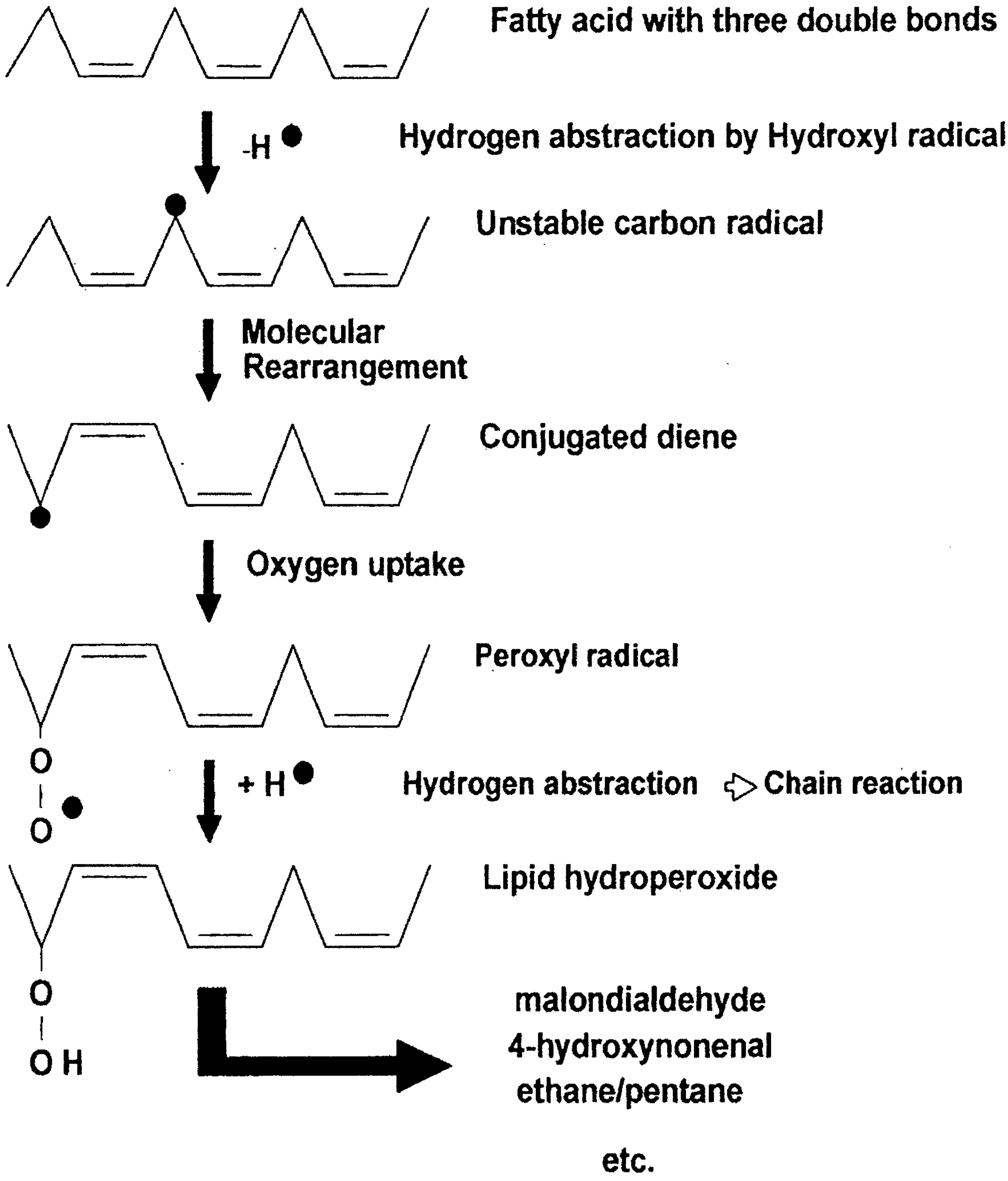


Figure 1.10: Initiation and propagation reaction of lipid peroxidation.

propagating thereby transfer of electrons and bringing about oxidation of substrates. Cell membranes, which are structurally made up of large amounts of poly unsaturated fatty acids, are highly susceptible to oxidative attack, and consequently, bring about changes in membrane fluidity, permeability and cellular metabolic functions (Halliwell 2006).

1.7.1.2. Proteins

In the PS II RC, the formation of $^1O_2*$ under excess light is thought to be the cause of direct damage to structural protein components (D1 and D2), which require *de novo* protein synthesis to be repaired (Melis 1999). Oxidative attack on proteins results in site specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge, and increased susceptibility to removal and degradation. The amino acids in a peptide differ in their susceptibility to attack, and the various forms of ROS also differ in their potential reactivity. The primary, secondary, and tertiary structure of a protein determines the susceptibility of each amino acid to attack by ROS (Halliwell and Gutteridge 1999). For many enzymes, the oxidation by O_2^- of iron-sulphur centres inactivates enzymatic function and other amino acids, such as histidine, lysine, proline, arginine, and serine, and form carbonyl groups when oxidized. A wide range of proteins and their amino acid building blocks are damaged or degraded by ROS, and the accumulation of these proteins in cells has been hypothesized to be part of the aging process (Lesser 2006). Under high irradiance $P680^+$ with a high oxidizing potential is capable of extracting electrons from the surrounding amino acids, primarily histidines, which subsequently leads to the destruction of D1 protein of PS II (Barber 1994).

1.7.1.3. DNA

The generation of ROS can induce numerous lesions in DNA that cause deletions, mutations, and other lethal genetic effects. Both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single-strand breakage, and cross-linking to proteins (Imlay 2003). In vitro, H_2O_2 or O_2^- cannot by themselves cause strand breaks under normal physiological conditions, and therefore, their toxicity *in vivo* is most likely the result of Fenton reactions in the presence of a transition metal (Imlay 2003). Both prokaryotic and eukaryotic cells have DNA repair enzymes; for a cell with DNA damage, it is the balance between damage and repair that determines the fate of that cell (Beyer et al., 1991).

1.8. Protective processes

To achieve high efficiency of photosynthesis, plants have developed several adaptive and protective mechanisms to adapt their photosynthetic apparatus to variable light conditions, which can rapidly change both in duration and intensity during the day. These include adjustment of leaf orientation (Öquist and Huner 1991), chloroplast movement as a light-avoidance response (Park et al., 1996), adjustment to a smaller antenna (Park et al., 1997), photorespiration as a safety valve (Osmond and Grace 1995), the Mehler reaction (Park et al., 1996), *de novo* synthesis of D1 protein (Greer 1986), scavenging of reactive oxygen species by antioxidants (Asada 1996) and dissipation via zeaxanthin in the xanthophyll cycle (Demmig et al., 1987a; Gilmore 1997). In the following sections, few of these photoprotective strategies are singled out for discussion.

1.8.1. D1 protein repair cycle

To avoid total disassembly and inactivation of PS II, plants have evolved a PS II (or D1) repair cycle to rapidly replace the damaged D1 protein (Barber and Andersson 1992). In the D1 protein repair cycle, non-functional dimeric PS II complexes with damaged D1 protein are phosphorylated, phosphorylated peripheral LHC II Chl *a/b* proteins are detached (Rintamäki et al., 1999) and then the phosphorylated PS II core dimer is monomerized in the appressed granal domain (Baena-González and Aro 2002). The phosphorylated PS II monomers laterally migrate to non-appressed stroma thylakoids where CP43 is first dephosphorylated and then detached from the damaged PS II cores. The D1 and D2 proteins are initially dephosphorylated, damaged D1 protein is degraded and new D1 protein is simultaneously synthesized on chloroplast ribosomes bound to the stroma lamella membrane, then processed and assembled with its heterodimeric partner, D2 protein. Following photoinactivation the LHC II dissociates from the photoinactivated PS II; cleavage of the D1 protein induces also the dissociation of the oxygen evolving complex proteins that are not degraded and may reassociate with a newly assembled PS II. The PS II complex further dissociates into sub complexes that may diffuse laterally toward the stroma lamellae where either the processed D1 protein may reassemble with PS II subunits; the reassembled complex diffuses within the membrane plane, and upon reaching the grana domain, reassociates with LHC II (Adir et al., 2003). Then CP43 is re-attached and PS II monomers migrate back to appressed granal domains (Baena-González and Aro 2002). Thus, the highly light-regulated cycle between functional PS II dimers and non-functional PS II containing damaged D1 protein located in the appressed granal membranes, and the replacement of damaged D1 protein by D1 protein degradation and *de novo* synthesis in non-appressed membrane domains, involve marked dynamic structural and compositional heterogeneity of PS II.

Both synthesis and degradation of the D1 protein are light dependent. Under normal conditions, a balance is maintained between the rates of D1 synthesis and degradation, which allows intact and functional PS II. Under photoinhibitory conditions, D1 degradation rates increase. This increment is perceived as either a prime cause for PS II inactivation (Kyle et al., 1984) or a part of the repair cycle (Kettunen et al., 1996).

1.8.2. Antioxidant system

To overcome photooxidative damage mediated by reactive oxygen species, plants have developed an elaborate defense system to scavenge reactive oxygen species and other free radicals that can potentially form in the chloroplast of higher plants. This system of scavengers includes enzymatic and non-enzymatic antioxidants that act in an integrated manner to safely remove these dangerously reactive compounds before they damage cellular constituents. There are mainly three enzyme systems in plant cell to scavenge the free radicals; these are superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase. The non-enzymatic antioxidants are carotenoids, ascorbic acid, reduced glutathione (GSH) and α-tocopherol (vitamin E) which help in the dissipation of excess energy directly where the energy is absorbed, i.e. in the light-collecting chlorophyll/carotenoid-binding complexes.

1.8.2.1. Superoxide dismutase (SOD)

SOD catalyses the dismutation of superoxide to hydrogen peroxide and water.

$$2H^+ + 2O_2 \longrightarrow H_2O_2 + O_2$$

Since SOD is present in all aerobic organisms and most sub cellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defense against stress (Bowler et al., 1992). There are three distinct types of SOD classified on the basis of the metal cofactor: Copper-zinc, Manganese and iron isozymes. The Manganese-SOD is found in the cytosol, others in the chloroplast of higher plants. Iron-SOD is usually associated with the chloroplast compartment (Bowler et al., 1992).

1.8.2.2. Catalase (CAT)

Catalase is a heme-containing enzyme that carries out the dismutation of hydrogen peroxide into water.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes by oxidase involved in β-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism. The stress conditions which reduce the rate of protein turn over cause the depletion of catalase activity and thus catalase is not very effective enzyme in removing the free radicals under such conditions (Sharma and Singhal 1992).

1.8.2.3. Ascorbate peroxidase (APX)

As catalase is limited in effectiveness to metabolize H_2O_2 by its relatively poor affinity for H_2O_2 and its sub cellular location in the peroxisomes and also susceptible to photo-inactivation and degradation (Streb et al., 1993), ascorbate peroxidase is the main enzyme to scavenge the H_2O_2 in chloroplasts. Simultaneous oxidation and reduction of the ascorbate and glutathione pools when H_2O_2 was added suggested that enzymes of ascorbate-glutathione cycle were involved in coupling electron transport to H_2O_2 destruction. Observation of direct photodestruction of MDHA radical by the thylakoid membranes suggested that this probably is the preferred pathway of ascorbate regeneration. 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 defense against H_2O_2 escaping thylakoid (Sharma 2002).

1.8.2.4. Ascorbic acid

L-ascorbic acid, or vitamin C, is an essential vitamin in animals and is abundant in plant tissues. All plants and animals, except humans, can synthesize ascorbate *de novo*; animals also can obtain vitamin C through their diet. Ascorbate functions as a reductant source for many ROS, thereby minimizing the damage caused by oxidative stress. Ascorbate scavenges not only H_2O_2 but also O_2^-, $^{\cdot}HO$, and lipid hydroperoxides without enzyme catalysts (Fridovich 1998), and it can indirectly scavenge ROS by recycling α-tocopherol to its reduced form. Ascorbate has been found in plant cell chloroplasts and cytosol, where it also acts as a substrate for ascorbate peroxidase.

1.8.2.5. Glutathione

Glutathione (GSH) is a tripeptide (Glutamine-Cysteine-Glycine) found in animals and plants. It forms a thiyl radical that reacts with a second oxidized glutathione, forming a disulphide bond when oxidized (GSSG; Halliwell and Gutteridge 1999). The ratio of GSH/GSSG is often used as an indicator of oxidative stress in cells, and glutathione functions as an antioxidant in many ways by reacting with $^1O_2$, O_2^-, and $HO^{\cdot}$. Glutathione can also act as a chain-breaker of free radical reactions and is an essential substrate for glutathione peroxidase (Halliwell and Gutteridge 1999). The maintenance of GSH levels, and therefore the reducing environment of cells, is crucial in preventing damage to cells exposed to conditions that promote oxidative stress.

1.8.2.6. Tocopherol

The tocopherols, specifically α-tocopherol (vitamin E), are lipid soluble antioxidants that scavenge ROS (Halliwell and Gutteridge 1999). This phenolic antioxidant is found in both animals and plants. α-tocopherol, due to its hydrophobic nature, is located exclusively within the bilayers of cell membranes. α-tocopherol is generally considered to be the most active form of the tocols. Plants synthesize α-tocopherol in chloroplasts, with the aromatic ring formed by the shikimic acid pathway - the same pathway that produces ultraviolet-absorbing compounds, the mycosporine-like amino acids, in many marine algae. By contrast, animals must acquire tocopherol through their diet. The antioxidant properties of tocopherol are the result of its ability to quench both singlet oxygen and peroxides (Halliwell and Gutteridge 1999). A marine-derived tocopherol known as α-tocomonoenol has been isolated from salmon eggs and provides enhanced antioxidant protection because of its ability to diffuse in viscous lipids and prevent lipid peroxidation (Yamamoto et al., 2001).

1.8.2.7. Carotenoids

In photosynthetic organisms, some carotenoids function as accessory pigments in light harvesting, whereas others quench ROS produced as a result of overexcitation of the photosynthetic apparatus by light (Demmig and Adams 1993). Two β–Car molecules are present in the RC of PS II, which absorb light and transfer the excitation energy to Chl *a*. These β–Car molecules also protect the RC Chl from high light damage (Telfer et al., 1994). β–Car protects the PS II RC against photo-oxidative damage via quenching of $^1O_2*$ or $^3Chl*$ but it is unable to quench the triplet excited state of P680 (Yamamoto and Bassi 1996; Telfer 2002).

Chlorophyll triplet quenching

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

$$^3\beta\text{–Car} \longrightarrow \beta\text{–Car} + \textit{heat}$$

Singlet oxygen scavenging

$$^1\text{O}_2^* + \beta\text{–Car} \longrightarrow \text{O}_2 + {}^3\beta\text{–Car}^*$$

$$^3\beta\text{–Car} \longrightarrow \beta\text{–Car} + \textit{heat}$$

As shown in the above equations, energy is transferred to ground sate carotenoids and dissipated by radiationless decay from the resulting triplet carotenoids as heat to the medium (Baroli and Niyogi 2000).

Lutein (Chow 1994; Niyogi et al., 1997), zeaxanthin and violaxanthin (in decreasing order of effectiveness) are also involved in protecting against Chl photo-bleaching by quenching both $^3Chl*$ and $^1O_2*$, whereas neoxanthin functions mainly in $^1O_2*$ quenching (Croce et al., 1999). Carotenoids thus are an important ‘safety valve’ dissipating the excess energy of the excited pigment and the products of the reaction with oxygen. Carotenoids can also dissipate excess excitation energy through the xanthophyll cycle (Krinsky 1989) that prevents the overexcitation of the photosynthetic apparatus.

1.9. Xanthophyll cycle-dependent energy dissipation

Xanthophylls are oxygen containing carotenoids. The harmless dissipation of excessive absorbed light energy in the light-collecting pigment bed involves the carotenoids of the xanthophyll cycle, consisting of violaxanthin (V), antheraxanthin

(A) and zeaxanthin (Z) (Fig. 1.11), which was discovered by Sapozhnikov in 1957. He described a decrease in the content of V in plants after high light treatment and its subsequent increase in low light or darkness. Yamamoto et al., (1962) showed that decrease in the V concentration in plants in intensive light was connected to the V transformation into a different xanthophyll pigment Z. No function was coupled to the cycle until Demmig et al., (1987a) and Sharma and Hall (1991) found a correlation between Z formation and the decrease in re-emission of light absorbed by chlorophyll, i.e. non-photochemical quenching (qN), whereby excessive excitation energy is siphoned away from chlorophyll and harmlessly dissipated as heat in the light-collecting pigment bed.

The components of the xanthophyll cycle make up from 10 to 40% of the total carotenoids in the leaves, depending on species and growth conditions. Growth in high light results in a larger pool of xanthophyll-cycle pigments (Thayer and Björkman 1990). The cycle is also termed the V cycle to distinguish it from another xanthophyll cycle, which comprises only one de-epoxidation step, the diadinoxanthin cycle of the algae. The pigments of the xanthophyll cycle have been localized to the light-harvesting complexes of both photosystems (Thayer and Björkman 1992; Lee and Thornber 1995). Their location within the antennae presumably allows for effective dissipation of excessive light energy thereby minimizing the formation of singlet oxygen and preventing the over-reduction of the electron transport chain.

During exposure to excess light, violaxanthin (a diepoxide) is enzymatically de-epoxidised to antheraxanthin (a monoepoxide) and further to zeaxanthin which is epoxide free. The conversion of V to Z takes place when higher plants are illuminated with high light intensities, while low light intensities or darkness stimulates the

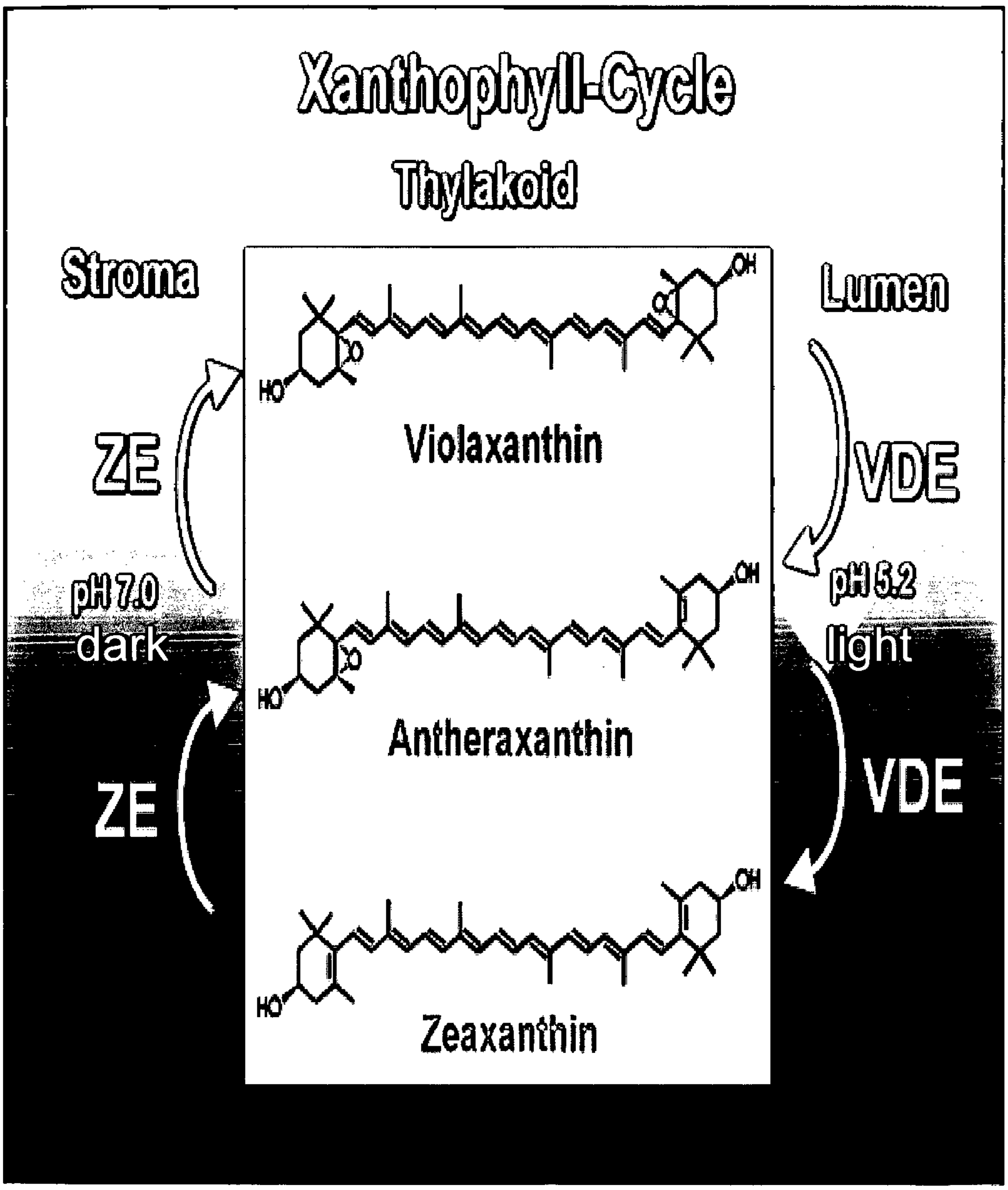


Figure 1.11: The xanthopphyll cycle. Violaxanthin is converted to zeaxanthin through an intermediate antheraxanthin by the enzyme violaxanthin de-epoxidase (VDE). The reverse reaction is catalysed by the enzyme zeaxanthin epoxidase (ZE). Violaxanthin is converted to zeaxanthin in high light and reverts back in the dark.

reverse reaction which leads to the conversion of Z back to V. Two enzymes localized on opposite sides of the thylakoid membrane are engaged in this process: violaxanthin de-epoxidase (VDE) present on the thylakoid lumen side of the membrane, catalyses the de-epoxidation of V to Z and zeaxanthin epoxidase (ZE), carrying out the reverse reaction of epoxidation of Z to V, is localized on the stromal side of the thylakoid membrane (Hager 1966).

Violaxanthin de-epoxidase is a 43 KDa nuclear-DNA encoded protein (Rockholm and Yamamoto 1996) and has a pH optimum of 5.2 (Hager and Holocher 1994). The enzyme requires ascorbate as a co-substrate to reduce the epoxy group, which is then eliminated as water (Neubauer and Yamamoto 1994; Bratt et al., 1995). Violaxanthin de-epoxidase is a water soluble enzyme at neutral or alkaline pH (Hager and Holocher 1994). Under high light conditions, the enzyme is activated by a decrease in the pH of the thylakoid lumen, whereas, it is inactive in the dark, when the pH in the thylakoid lumen is neutral or alkaline (Hager and Holocher 1994). Violaxanthin de-epoxidase can be either unbound or bound to the thylakoid membrane depending on the luminal pH. Connection to the membrane is important for enzymatic activity (Hager and Holocher 1994). At pH lower than 6.0, all VDE molecules are associated with the membrane. If pH increases to 7.0 or more, the VDE exists in an unbound form. ΔpH is caused by proton migration during light-induced electron transport once irradiance exceeds that required to saturate photosynthesis. Therefore once irradiance is excessive and a pH gradient is generated, this in turn triggers the de-epoxidation of V to A and Z, which reduces the efficiency of PS II and protects the RC from photooxidation (Demmig-Adams and Adams 1994). Changes in the de-epoxidation state of the xanthophyll cycle and/or the magnitude of the ΔpH allow plants to alter the level of thermal energy dissipation and closely track changing light conditions in their natural environment such that only excess absorbed light energy is dissipated and carbon fixation is presumably uncompromised.

Besides the decrease in the lumenal pH, VDE activity also requires the presence of the galactolipid monogalactosyldiacylglycerol (MGDG), which has been proposed to facilitate the solubilization of the substrate V and/or to represent a functional component of the enzyme VDE itself (Yamamoto and Higashi 1978). Zeaxanthin epoxidase requires oxygen and ferredoxin/NADPH as a reductant, and is optimal near pH 7.5 (Siefermann and Yamamoto 1975; Hager 1975). With the build up of protons gradient within the thylakoid lumen, the cycle shifts towards Z and A, and energy dissipation occurs (Gilmore et al., 1996). Both A and Z protect the plants from photoinhibition by dissipating the excess light energy as heat (Gilmore 1997; Goss et al., 1998). This protective function has been demonstrated in plants subjected to various conditions of high light (Demmig-Adams and Adams 1992; Sharma et al., 2002) and light in combination with various other stress factors such as restricted carbon dioxide (Demmig et al., 1987a), chilling (Verhoeven et al., 1996; Havaux and Niyogi 1999), drought or nutrient deficiency (Demmig-Adams and Adams 1992), and high salinity (Björkman et al., 1988). A range of factors influences the degree of conversions of V to Z such as temperature, water stress and light quality, as well as plant species (Thayer and Björkman 1990).

Most of the xanthophylls are bound to the Chl *a/b* binding antenna proteins of both photosystems, while only a fraction of the pool of xanthophyll cycle pigments V, A and Z may also be present in the lipid phase of the membrane (Havaux 1998; Macko et al., 2002). In higher plants, ten different antenna proteins constitute the family of Chl *a/b* binding proteins: Lhcb1-6 in PS II and Lhca1-4 in PS I (Jansson

1994). Analysis of X-ray structure of the trimeric LHC II from spinach (Liu et al., 2004), which consists of Lhcb1-3 proteins, identified four different xanthophyll-binding sites in antenna proteins: two lutein are bound to the central L1 and L2 sites associated with the two central transmembrane helices A and B, neoxanthin is bound to the more peripheral helix B and protrudes into the lipid phase, while V is located at the monomer interface (Standfuss et al., 2005). The convertibility of V to Z depends on both the binding site and protein-specific properties: V bound to the V1 site seems to be easily accessible for de-epoxidation in all antenna proteins analysed so far, while V bound to L2 is only slowly or not at all convertible to Z (Jahns et al., 2001). These observations indicate the existence of different pools of antenna-bound V, a convertible fraction involved in photoprotective mechanisms and a non-convertible pool that should serve other functions, either related to light-harvesting and/or the stabilization of antenna proteins.

1.9.1. Molecular mechanism of the xanthophyll cycle

Even though intensive research is being carried out on the molecular mechanism of the xanthophyll cycle, it is still far from being completely understood. To convert V into Z, VDE has to remove two epoxy groups attached to two rings of the V molecule. In accordance with requirement of the MGDG for the xanthophyll cycle, it has been proposed that V is located in the micelles of this galactolipid (Yamamoto et al., 1974; Latowski et al., 2002). In such structures, believed to consist on average of 28 MGDG molecules, V can oscillate and finally come in contact with substrate binding site of the VDE. Then, one of the two epoxy groups of the ionone rings can be de-epoxidated and V changed into A. For the transformation of A to Z, it has been suggested that A undergoes a flip-flop mechanism. This flip-flop mechanism involves the change in orientation of A molecules within the thylakoid membrane (Gilmore 1997; Fig.

1.12a). Flip-flop of A is necessary for its de-epoxidation because it requires the epoxide group of A to face the VDE enzyme (Yamamoto and Higashi 1978). Researchers (Thayer and Björkman 1992) suggested that *in vivo* V de-epoxidation occurs within thylakoid pigment-protein complexes, but a great number of experiments demonstrated that the reaction takes place in the lipid part of the membrane. The xanthophyll cycle pigments are not freely located in thylakoid membranes but are bound by proteins of light harvesting complexes, however, these pigments can easily dissociate from their loci (Ruban et al., 2002). Perpendicular location of V in the thylakoid membrane was confirmed by experiments with VDE added from the stromal side of the thylakoids (Akerlund et al., 1995).

For its activity ZE requires NADPH, flavin adenine dinucleotide (FAD), ferridoxin and oxygen (Hager 1975; Bouvier et al., 1996). It has been suggested that electrons from NADPH are transferred through ferridoxin:NADP$^+$ oxidoreductase to ferredoxin, then to FAD (Bouvier et al., 1996). Reduced FAD is able to bind oxygen molecule and to form hydroperoxyl moiety. Part of this moiety is transferred to Z, as a hydroxyl radical. Zeaxanthin forms unstable carbocation and then proton from Z is transferred through FAD to oxygen and finally water molecule is formed. After loss of proton Z becomes A in the membrane protein complexes. Similarly to de-epoxidation reaction, A has to perform a flip-flop type movement to be further epoxidated to V. However, until now there is no knowledge how second ionone ring can be exposed to ZE. Two mechanisms are possible: (i) A molecule turns in precincts of protein complex, (ii) it leaves the complex and translocates to lipid domains, containing reversed hexagonal structures where the flip-flop takes placc. It is also possible, that both proteins and lipids are engaged in the A flip-flop (Latowski et al., 2004).

1.9.2. PsbS protein and its role in the xanthophyll cycle

A light harvesting protein, PsbS, was shown to have a key role in the dissipation of excess energy in higher plants (Li et al., 2000; Külheim et al., 2002). PsbS is a 22-kDa PS II subunit that belongs to the light harvesting complex protein superfamily (Kim et al., 1992). The schematic model for qE in PS II of plants is depicted in Fig. 1.12b. In limiting light, the steady-state thylakoid lumen pH is greater than 6 (Kramer et al., 1999). Violaxanthin is bound mainly to the V1 site in LHC II and the L2 site in other LHC proteins (such as CP29 and CP26) (Caffari et al., 2001; Morosinotto et al., 2002). In excess light, the thylakoid lumen pH drops below 6, driving protonation of carboxylate side chains in VDE and PsbS. Protonation of VDE activates the enzyme and allows for its association with the membrane (Hager and Holocher 1994), where it converts multiple V molecules to Z. Protonation of glutamate residues E122 and E226 in PsbS activates symmetrical binding sites for xanthophylls with a de-epoxidised β-ring end group (i.e. Z).Binding of Z to protonated sites in PsbS results in the qE state in which de-excitation of ^{1}Chl* occurs. The binding of Z to PsbS gives rise to a strong red shift in the absorption spectrum, providing an explanation of the ΔA_{535} which is strongly correlated with qN and which has been shown to at least partially arise from a sub-pool of red-shifted zeaxanthin (Ruban et al., 2002).

There are two main thoughts concerning the nature and mechanism by which a simple alteration in the xanthophylls composition of LHC affects the balance between photon capture and energy dissipation at different irradiances (Young et al., 1997; Horton et al., 1999).

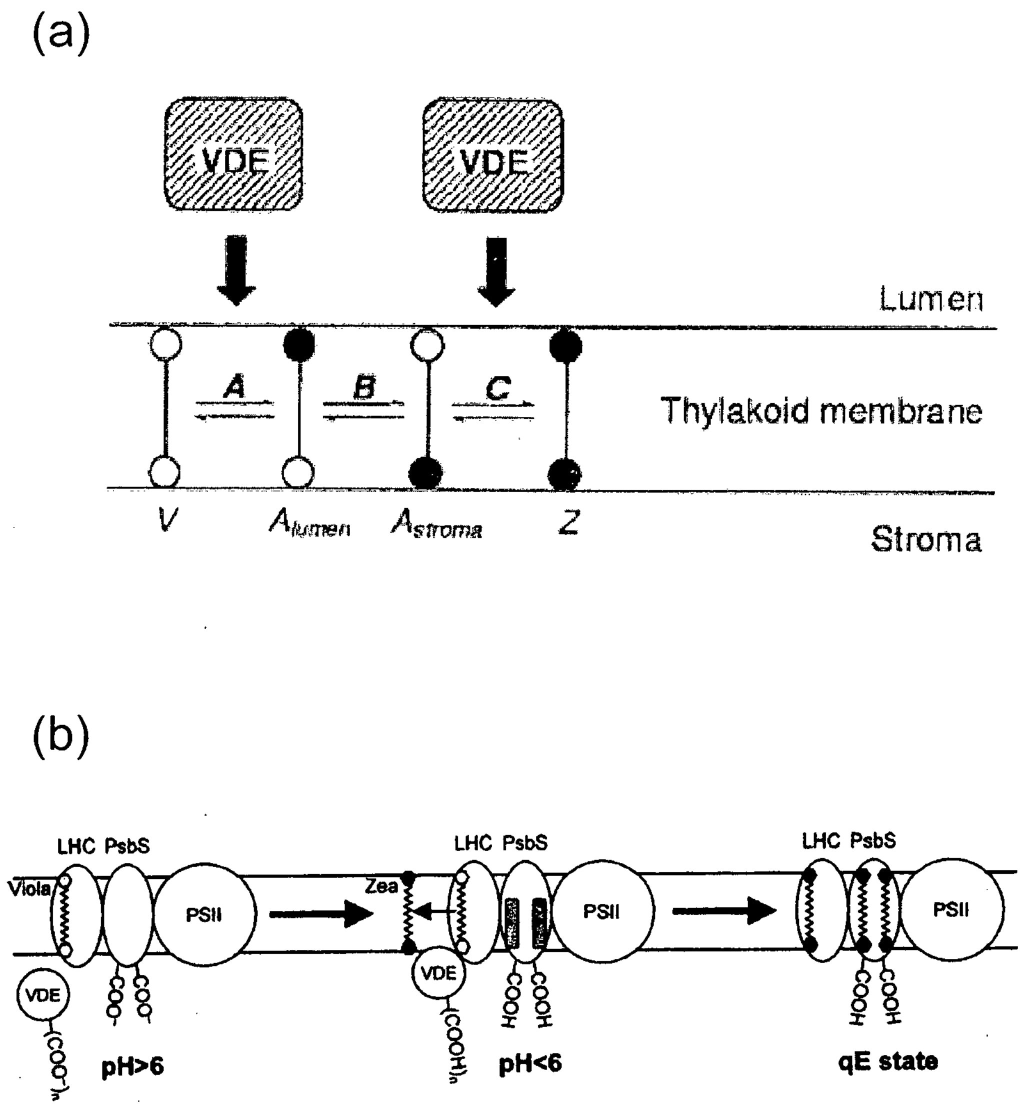


Figure 1.12:

(a) Schematic model showing the flip-flop movement of Antheraxanthin in the thylakoid membrane.

(b) Schematic model showing the role of PsbS in xanthophyll cycle and qE. In excess light, a low thylakoid lumen pH leads to binding of H to the carboxylates of two glutamate residues in PsbS and zeaxanthin synthesis from violaxanthin is induced. Binding of Z to sites in PsbS results in the qE state in which the de-excitation of excited Chl occurs.

1.9.3. Direct quenching

The possibility that differences in the S_1 energy levels of V and Z might account for the operation of the xanthophyll cycle was first proposed by Demmig-Adams (1990) and later elucidated by Owens et al., (1992). They suggested that Z has a S_1 energy identical to that of β-Car as these molecules are essentially iso-electronic. The S_1 state of Z lies just below that of Chl *a* and allowing the Z molecule to act as a sink for excitation energy of Chl *a* (Fig. 1.13a). The energy content of Chl *a* is lower than the value for the S_1 state of V but higher than that of Z. This would suggest that it is energetically possible for the S_1 state of Z to quench Chl fluorescence by deactivation of ^{1}Chl*. In contrast the higher S_1 level of V would only permit it to function as a light-harvesting pigment, transferring its excitation energy to Chl *a*. Thus, at high PFD, when dissipation of excess excitation energy is required, Z is formed within LHC II and its formation serves to deactivate the ^{1}Chl* *a* and dissipate excitation energy harmlessly as heat.

1.9.4. Indirect quenching

The structural differences between V and Z may provide an explanation as to how the xanthophyll cycle pigments could indirectly control energy-dependent quenching (qE). It is suggested that Z induces structural changes of the light-harvesting system of thylakoid membrane (Fig. 1.13b) that favour the radiation-less dissipation process (Ruban et al., 1996). The changes in structure of LHC probably favour their aggregation. This aggregation is possible because of the interactions between the LHC trimers. This mechanism couples the changes in the aggregation of LHC II with the increase in qN. In the light-harvesting Chl *a/b* binding protein, the Chl molecules are situated at close proximity, but are separated from each other by xanthophyll molecules. These strong anti-quenchers (xanthophylls) prevent close

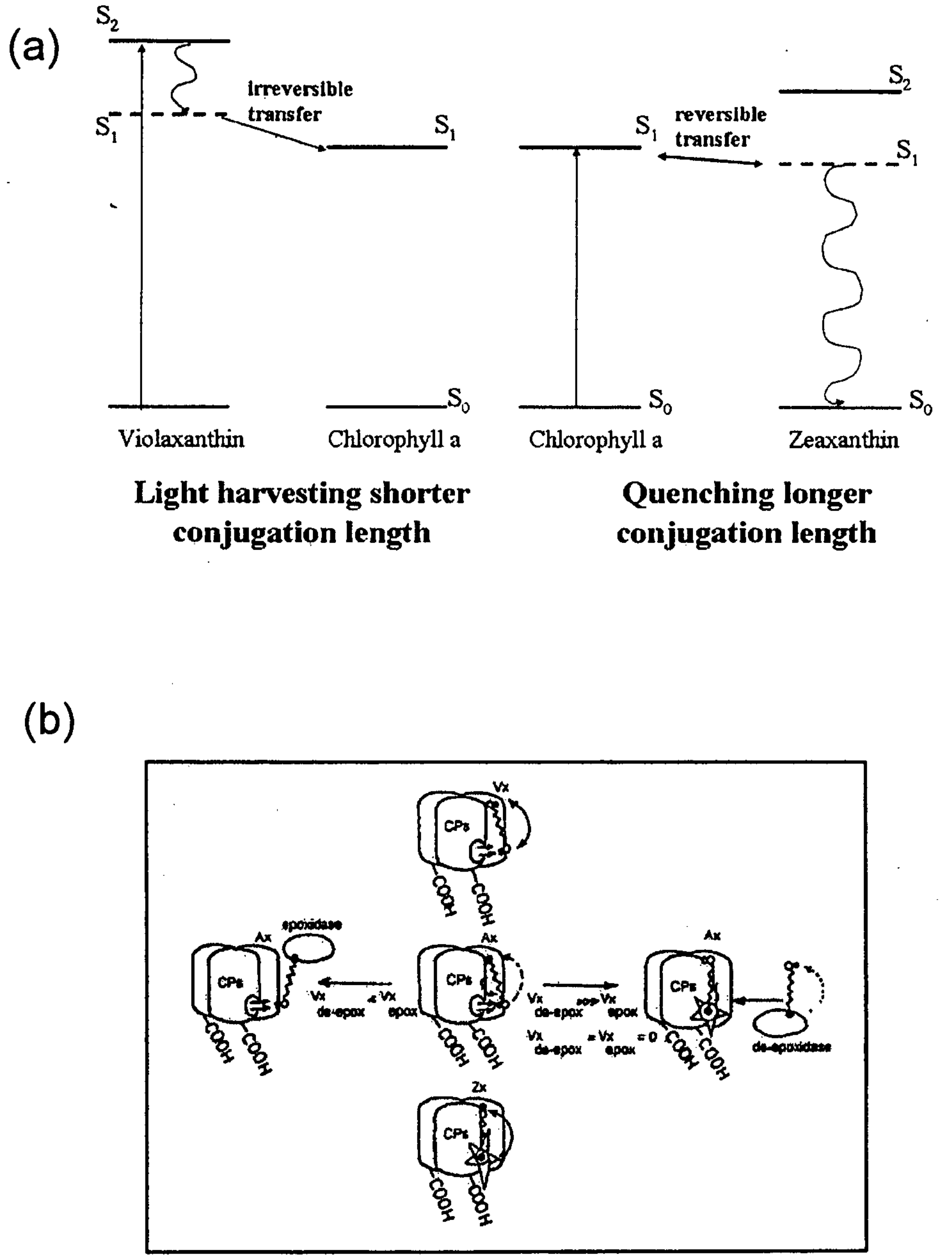


Figure 1.13:
(a) Comparison of the predicted energy transfer pathways between carotenoids of the xanthophyll cycle and chlorophyll *a*
(b) The model shows how the structures of xanthophyll cycle pigments (Z) binding to protonated chlorophyll proteins of the PS II inner antennae cause non-photochemical quenching of PS II Chl *a* fluorescence.

Chl-Chl interaction and quenching (Searle et al., 1991) but do not interfere with optimal energy transfer. The key process of energy dissipation by xanthophyll cycle is protonation-promoted changes in the protein structure leading to a Chl/xanthophyll aggregation and thereby allowing direct quenching of ^{1}Chl* by Z and energy dissipation (Eskling et al., 1997; Gilmore 1997).

1.9.5. Other functions of Z

Other than energy dissipation Z is proposed to involve in various other functions.

1.9.5.1. As an antioxidant

Antioxidant roles for Z has been reported during long-term high irradiance stress (time-scale of days) (Li et. al., 2002). Using *Arabidopsis npq1* mutants lacking Z, Havaux et al., (2000) showed that the xanthophyll cycle specifically protects thylakoid membranes against lipid peroxidation. The *Arabidopsis npq4* mutants that were defective in qE but possessed a normal xanthophyll cycle activity showed tolerance to lipid peroxidation, demonstrating that photo-protection mediated by the xanthophyll cycle is not solely a result of the involvement of Z in qE (Havaux et al., 2000).

1.9.5.2. Thylakoid membrane fluidity

Modulatory effects of carotenoids on physical properties of model and natural membranes have been known since the seventies. Soon after xanthophyll cycle characterisation, there was a suggestion that this process may regulate physical properties of thylakoid membranes (Siefermann and Yamamoto 1975; Yamamoto 1979). This hypothesis has been confirmed by other studies (Gruszecki and Strzalka 1991; Tardy and Havaux 1997). In natural and model membranes, Z appears to have the strongest influence on such membrane properties as temperature of phase transition, molecular dynamics, permeability and polarity gradient (Havaux and Gruszecki 1993; Strzalka and Gruszecki 1997).

1.9.5.3. Blue light receptor

Another postulated function of the xanthophyll cycle is blue light reception. It is suggested that Z is responsible for the blue-light-dependent stomata opening (Quinones et al., 1996), chloroplast movement (Tlalka et al., 1999) and phototropism (Quinones and Zeiger 1994). HPLC measurement of Z level as function of chloroplast movement in strong and weak light in *Lemna trisulca* proved a good correlation between these two variables (Tlalka et al., 1999). Because of this finding, Z is supposed to be a photoreceptor in blue-light stimulated chloroplast movement. The degree of increase in Z level (regulated by red light, darkness periods, and use of DTT) correlated well with the blue-light stimulated phototropism of maize coleoptiles (Quinones and Zeiger 1994). The DTT-related inhibition of Z synthesis consequently inhibited blue-light dependent stomata opening in *Vicia faba* epidermis, which is considered to be a proof of the photoreceptor function of Z (Srivastava and Zeiger 1995). However, it has also been shown in experiments correlating the level of protein phosphorylation and blue-light induced phototropism, that there is no connection between Z, or any other carotenoid, and phototropism (Palmer et al., 1996).

1.10. Chlorophyll fluorescence

Light absorption by a Chl molecule transforms it into an energy-rich excited state. An excited Chl molecule is not stable and electrons return rapidly to their ground level releasing absorbed photon energy in a number of ways:

1. Electronic excitation energy can be transferred to another acceptor molecule, this results in photosynthetic electron transport. This part can be measured as photochemical quenching (qP).
2. Excitation energy can be released as heat (thermal dissipation), which can be measured as non-photochemical quenching (qN).
3. It can be released by emitting energy as photon of lower energy content (i.e. higher wavelength), which is known as fluorescence.
4. Another way by which an excited Chl molecule can lose its energy is by transfer from its original excited singlet state (total electronic spin is s=0) into a metastable triplet state (s=1) with a much longer lifetime by a mechanism called intersystem crossing. From the metastable triplet state the chlorophyll molecule can revert to the natural ground state by emitting a photon at a longer wavelength. This weak emission is known as delayed fluorescence or phosphorescence.

The emission maximum of the fluorescence spectrum of a molecule is always at longer wavelength than the wavelength of the corresponding absorption spectrum. The most common physical observable used to assess photosynthetic function and its subsequent down regulation in excess-light conditions is Chl fluorescence, because it is sensitive to a wide range of changes in the overall apparatus (Holt 2004). Photochemistry, heat and fluorescence de-excitation pathways compete for excitation energy. When all PS II RC are capable of a stable charge separation, fluorescence yield is minimal (Fo) and these centers are referred to as open reaction centers. When all reaction centers are in the closed state, i.e., a state in a stable charge separation is not possible, the fluorescence yield is maximal (Fm). The quenching of fluorescence caused by photochemistry in PS II results in variable fluorescence (Fv). The maximum variable fluorescence Fv=(Fm-Fo) is thus a measure for photochemistry in open PS II centers. The quantum yield of photochemistry in open PS II centres is defined, as the probability of photochemistry after absorption of a photon, and is determined by the efficiency of excitation energy transfer from the antennae to the reaction centre and equals (Fm-Fo)/Fm (Genty et al., 1989).

Processes that decrease the overall Chl fluorescence quantum yield are generally divided into two categories, photochemical quenching (qP), which is exclusively associated with photochemical charge separation in the PS II reaction center, and non-photochemical quenching (qN), which is broadly defined as all fluorescence quenching that is not directly related to charge separation. Factors contributing to qN are generation of strong pH gradient in thylakoid membrane, phosphorylation and dephosphorylation of light harvesting protein components and associated energy redistribution, concentration of cations (Mg^{2+}), photoinhibition, development of xanthophyll cycle, etc.

Different processes that contribute to qN relax at different rates in darkness following a period of irradiation. The kinetics of relaxation of these different processes can be therefore used to distinguish them. On basis of the kinetics,qN is divided into three types:

1. Energy-dependent quenching (qE)
2. State-transition quenching (qT)
3. Photoinhibitory quenching (qI)

1.10.1. Energy-dependent quenching (qE) and the role of xanthophyll cycle

The fast component of qN is called energy-dependent quenching (qE) due to its dependence on the organization of thylakoid membrane (Horton et al., 1996; Müller et al., 2001). qE is a process of heat dissipation (or non-radiative dissipation) which is important in the protection of the photosynthetic apparatus against photo-oxidative damage under excess irradiation. For induction of maximal qE, both low thylakoid lumen pH and the presence of xanthophylls are necessary (Krause and Jahns 2003). When the absorption of photon energy exceeds the capacity of the dark reactions to utilise ATP and NADPH produced by a photosynthetic electron transport, synthesis of ATP and NADPH are limited. This subsequently leads to a decrease in pH within the thylakoid lumen and the feedback regulation of light-harvesting by triggering the dissipation of excess absorbed energy as heat. The control by lumen pH allows induction and reversal of the energy dissipation within second to minutes.

qE is associated with the inter-conversion of xanthophyll pigments in the xanthophyll cycle. Low lumen pH activates the enzyme violaxanthin de-epoxidase which converts violaxanthin to zeaxanthin via the intermediate antheraxanthin (Eskling et al., 1997). V de-epoxidation can be blocked by an inhibitor of VDE, dithiothreitol (DTT). Blocking this de-epoxidation reaction results in inhibition of qE (Bilger and Björkman 1990). In addition to the inhibitor studies, the requirement for the de-epoxidized xanthophylls in qE has been proved by using xanthophyll cycle mutants (Niyogi et al., 1998). The involvement of Z and A formation via the xanthophyll cycle in qE was also confirmed in studies using VDE antisense tobacco plants which exhibit suppressed ability to form Z and A (Chang et al., 2000). qE relates better with the amount of Z and A than with the amount of Z alone (Gilmore and Yamamoto 1993). These findings support the idea that both A and Z are involved in qE. In addition to A and Z, also a third xanthophyll, lutein, because of its structural similarity to A, has recently been implicated in qE (Pogson et al., 1998; Niyogi et al., 2001; Lokstein et al. 2002). Direct evidence for the involvement of these pigments in qE has come from studies of mutants of the green alga *Chlamydomonas reinhardtii*

(Niyogi et al., 1997) and the laboratory weed *Arabidopsis thaliana* (Niyogi et al., 2001) and from experiments with transgenic tobacco plants with reduced levels of VDE (Chang et al., 2000). The *Arabidopsis npq1* mutant, which is defective in the gene encoding VDE, has very low levels of qE (Niyogi et al., 1998). The *Arabidopsis lut2* mutant is defective in the lycopene ε-cyclase, is unable to synthesize β-carotene and lutein, and exhibits slower induction and a lower extent of qE (Pogson et al., 1998). Essentially all qE is abolished in the *npq1 lut2* double mutant (Niyogi et al., 2001).

In addition, qE requires the presence of a functional version of the PsbS protein (Li et al., 2000; refer to Fig. 1.12). The PsbS protein, presumably acting as a pH sensor (Li et al. 2004), links thermal dissipation to the trans-thylakoid pH gradient as a measure of excess light. Using PsbS-deficient Arabidopsis mutants, PsbS was found to play an important role in plant fitness in a fluctuating light environment, but not in constant high light (Külheim et al. 2002). Furthermore, over expression of PsbS in Arabidopsis resulted in greater levels of flexible qN (qE) as well as prevention of sustained depressions in maximal PS II efficiency or photoinhibition (Li et al. 2002).

qE is important for photo-protection by ^{1}Chl* quenching, which results in harmless dissipation of excess excitation energy as heat. Up to 80% of the Chl excited states can be dissipated into heat, thereby protecting PS II RCs from over-excitation (Bassi and Caffari 2000). Intersystem crossing is one of possible pathways of ^{1}Chl* de-excitaion, by which the ^{3}Chl* is formed. ^{3}Chl* can interact with ground-state triplet oxygen to produce $^1O_2^*$, an extremely damaging reactive oxygen species (Baroli and Niyogi 2000). Thus, qE decreases the lifetime of ^{1}Chl* and consequently the probability of $^1O_2^*$ generation in LHC II. Further, by decreasing the efficiency of energy transfer to PS II RCs, qE may also prevent the over-reduction of the electron transport chain resulting in the recombination of the primary radical pair, $P680^+Pheo^-$, which can generate the excited triplet states of both the accessory Chl molecule located in the D1 protein and P680, the latter as a minor population (Aro et al., 1993). As in LHC II, interaction between $^3Chl^*$ and oxygen within PS II RCs results in formation of $^1O_2^*$. qE may also prevent the over-acidification of the thylakoid lumen that can inhibit the electron transport at PS II donor side resulting in the generation of long-lived $P680^+$ and/or Y_Z^+ (oxidized secondary electron donor of PS II; Niyogi 1999). $P680^+$ and Y_Z^+ are themselves capable of oxidizing nearby pigments and proteins, causing the damage to PS II RCs (Aro et al., 1993).

1.10.2. State-transition quenching (qT)

The middle component qT, is associated with the phenomenon of state transitions and is therefore called state-transition quenching (Allen and Forsberg 2001). State-transitions are short-term processes that change the antenna sizes of PS II and PS I. qT induction and reversal caused by these alterations occur over time-scales ranging from minutes to tens of minutes. The basis of state-transitions lies in the reversible phosphorylation of light-harvesting complex of PS II (LHC II) and the movement of phosphorylated LHC II from PS II to PS I. The decrease of the PS II antenna size is accompanied by a reduction of the amount of excitation energy in PS II fluorescence intensity. Unlike qE, an involvement of qT in photo-protection is unclear. It was suggested that LHC II phosphorylation protects the photosynthetic apparatus against photoinhibition. However, some studies show that LHC II phosphorylation is inhibited upon short-term exposure to high irradiance. This effect was observed in higher plants both *in vivo* (Demmig et al., 1987b) and in intact chloroplasts (Ebbert and Godde 1994). A contribution to qT to the overall qN is rather negligible under high irradiance stress in most plants (Müller et al., 2001). qT seems to be important only in low irradiances, when it regulates the distribution of excitation energy between PS I and PS II and thereby optimises the photosynthetic reactions.

1.10.3. Photoinhibitory quenching (qI)

The fast component of qN saturates at high irradiance, whereas the slow component increases as the irradiance is increased (Walters and Horton 1991). The slow component is caused by photoinhibition and is therefore called photoinhibitory quenching denoted as qI (Ruban and Horton 1995). Under high irradiance the protective capacity of qE is saturated, under such conditions qI becomes significant. This quenching shows the relaxation in the range of hours to days and reflects both slowly relaxing photo-protective processes and damage to PS II RCs under stress conditions.

The photo-protective component of qI, like qE, reduces high irradiance induced damage in PS II by dissipating excess absorbed photon energy and it appears to possess some characteristics of qE. Some part of qI may be associated with ΔpH, which is necessary for qE induction. For efficient qE, the inter-conversion of xanthophylls in the xanthophyll cycle together with the formation of ΔpH is necessary. The involvement of the xanthophyll cycle activity in qI was also reported such that relaxation of qI has been correlated with the conversion of Z and A back to V (Jahns and Miehe 1996).

1.11. Thermal energy dissipation within PS I

At high irradiance, when photo-protective thermal dissipation of excess absorbed photon energy within PS II (qE) is engaged, PS I absorbs more photons compared to the electron flux from PS II. This excess photon energy absorbed by PS I can be dissipated via cyclic electron transport around PS I, which is also suggested to have an important role in photoprotection (Niyogi 1999). In addition to dissipating energy absorbed by PS I, cyclic electron transport may be involved in generating or maintaining the ΔpH that is necessary for down regulation of PS II by thermal dissipation of excess absorbed light energy (Heber and Walker 1992). Biochemical approaches have led to the conclusion that there are at least two pathways of PS I cyclic electron transport, one involving a ferredoxin-plastoquinone oxidoreductase (FQR) and the other involving an NADPH/NADH dehydrogenase (NDH) complex (Bendall and Manasse 1995). The FQR has not yet been identified, although the PsaE subunit of PS I is possibly involved. The NDH pathway involves a protein complex bound to the thylakoid membrane that is homologous to the NADH dehydrogenase complex I of mitochondria. Several subunits of this complex are encoded by genes on the chloroplast genome of many plants (Friedrich et al., 1995). Mutants affecting the NDH complex have been generated by disrupting *ndh* genes in the chloroplast genome of tobacco by homologous recombination (Burrows et al., 1998). These mutants have no obvious phenotype under normal growth conditions. However, measurements of Chl fluorescence and PS I reduction kinetics revealed that cyclic electron transport is partially impaired. Induction of thermal dissipation upon sudden illumination was slightly delayed in mutants subjected to water stress, consistent with the idea that PS I cyclic electron transport is involved in maintaining a ΔpH that is necessary for down-regulation of PS II activity by qE (Burrows et al., 1998; Heber and Walker 1992).

1.12. ABA and the xanthophyll cycle

ABA is a plant hormone involved in many physiological and developmental processes such as transpiration, germination, dormancy and also with the adaptation of plants to

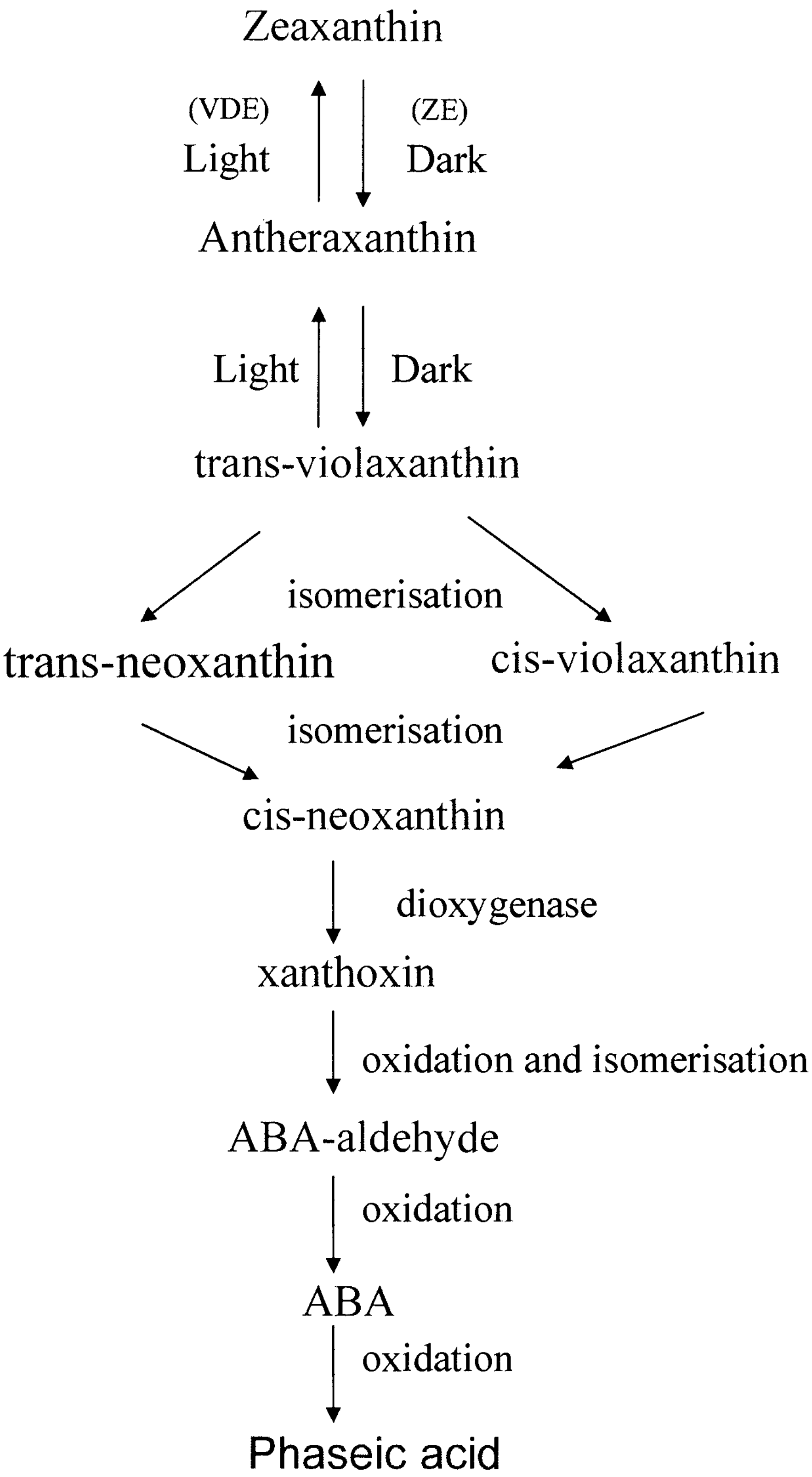


Figure 1.14: The abscissic acid biosynthetic pathway in higher plants, showing relationship with xanthophyll cycle.

environmental stresses (e.g. drought, chilling, salinity and pathogen attack; Cramer and Quarrie 2002). Early on the similarity in structure between ABA and the end groups of certain carotenoids led to the proposal that ABA may be a breakdown product of carotenoids, with xanthoxin as an intermediate (Hirschberg 2001; Fig. 1.14). This idea was supported by the finding that plants that do not accumulate carotenoids either because of mutation or treatment with inhibitors also lack ABA. Furthermore labelling studies with $^{18}O_2$ established that one $^{18}O_2$ atom is rapidly incorporated into the carboxyl group of ABA, indicating that there is a large precursor pool (i.e. carotenoids) that already contains the oxygen on the ring of the ABA molecule (Zeevaart et al. 1989). In etiolated leaves and roots, which have low levels of carotenoids, a 1:1 stoichiometry was found between the disappearing of V and Nx and the appearance of ABA and its catabolites (Li and Walton 1990; Parry et al., 1992). Direct evidence for carotenoids being ABA precursors was provided by isolation and characterization of the carotenoid cleavage enzyme, nine-cis-epoxycarotenoid dioxygenase, which converts nine-cis-epoxycarotenoid (9-cis-violaxanthin and/or 9-cis-neoxanthin) to xanthoxin, the committed step in ABA synthesis (Schwartz et al. 1997, Chernys and Zeevaart 2000; Fig. 1.14).

1.13. Acclimation of photosynthesis to the environment

Terrestrial higher plants grow under conditions that vary widely in environmental parameters such as irradiance, temperature, water availability, and soil quality. For instance, with respect to the light environment, plants can be found growing at maximal irradiances that differ by over two orders of magnitude (greater than 2000 μmol photons $m^{-2}s^{-1}$ for midday sunlight versus less than 20 μmol photons $m^{-2}s^{-1}$ for some deeply shaded environments; Logan et al., 1999). While the basic mechanism of photosynthetic carbon assimilation is invariant, the relationship between its component processes and the relative emphasis placed upon each is subject to acclamatory alterations and varies widely across differing environments. Photosynthetic carbon assimilation can be separated into the three component processes of light absorption, transduction of absorbed light energy into chemical energy (ATP) and reducing power (NADPH), and the use of ATP and NADPH to fix atmospheric carbon dioxide. Depending upon growth conditions, plants may emphasize light absorption or energy transduction and carbon assimilation. These acclamatory adjustments enhance a plant's ability to grow and compete in its environment, however, no plant, including rapidly growing crops, is known to utilize all of the excitation energy absorbed during exposure to full sunlight for carbon assimilation (Long et al., 1994). In all but deeply shaded environments, plants regularly have to contend with excitation energy that more than saturates their photosynthetic apparatus and therefore is excessive. To cope up with such excess light plants have developed various adaptive processes (Niyogi 1999).

1.13.1. Acclimation to sun versus shade

Leaves are the site of higher plant photosynthesis and provide the necessary conditions to maintain it. Leaf structure, shape and cell distribution are genetically determined by change, within limits with growth conditions, allowing adjustment to environment. The rate of photosynthesis by the whole leaf and its response to environmental conditions differs greatly between species of plant and is correlated with habitat.

Distinction may be made between plants with high relation of photosynthesis and growth in very intense light, so called sun plants, which are inefficient, with poor photosynthesis and survival in dim light, and shade plants which photosynthesize and survive only in dim light but are unable to function efficiently in bright light (low maximum rates of photosynthesis and photochemical damage; Lawlor 1987). However many species show flexibility in response to light intensity. They are facultative sun/shade species and may grow in high and low intensity illumination but lack the ability to adapt to the extremes.

It has long been known that plants acclimated to high light environments are capable of higher maximal photosynthetic rates than plants acclimated to low light environments (Björkman 1981). Sun plants which include many crops and plants of tropical regions achieve maximum rates of photosynthesis greater than 30 μmol CO_2 $m^{-2}s^{-1}$ and respiration rates in darkness of 2 μmol CO_2 $m^{-2}s^{-1}$. Not only are maximal rates of photosynthesis higher in high light-acclimated plants, a higher light intensity is required to saturate the photosynthetic apparatus. In order to support their higher metabolic rate, high light-acclimated plants also have a greater respiration rate. Because of this higher respiration rate, high light-acclimated plants have a higher light compensation point (the light intensity where photosynthesis exactly balances leaf respiration), and, as a result have a lower net rate of carbon assimilation in the light-limiting region when compared to low light-acclimated plants. Shade plants may have photosynthesis rates less than 10 μmol CO_2 $m^{-2}s^{-1}$ at light intensity perhaps 1/10th of sun species and maybe damaged by light intensities above half that of sunlight. Shade and sun plants and leaves of the same plant from different illumination differ in chloroplast membrane and light-harvesting and electron transport mechanism.

1.13.1.1. Plant architecture

The productivity of a shade-acclimated plant is often limited by its ability to gather light energy for carbon assimilation. Therefore shade-acclimated plants tend to allocate more resources to photosynthetic tissues and have a relatively low root to shoot biomass ratio (Givnish 1988). Carbon acquisition in plants growing in exposed environments is likely not limited by light energy absorption. Exposed environments can often be characterized by high temperatures and large leaf-atmosphere vapour pressure deficits, which lead to high transpiration rates in these plants. High growth rates exhibited by sun-acclimated plants require greater amounts of nutrients as well. In order to meet these needs, sun-acclimated plants allocate more resources to root growth and have higher root to shoot biomass ratios than shade-acclimated plants (Poorter and Remkes 1990).

1.13.1.2. Leaf architecture

Sun-acclimated leaves tend to be thicker, due to more highly developed palisade and spongy mesophyll layers (Nobel et al., 1975). They also have a lower internal resistance to carbon dioxide diffusion, allowing for greater carbon dioxide availability necessary to support their high rates of carbon assimilation.

1.13.1.3. Chloroplast movements and morphology

Chloroplasts in the palisade cells of sun-acclimated leaves tend to be located adjacent to the anticlinal cell walls, parallel to the incident light beam, thereby shading each other during exposure to high PFD. They also have a greater fraction of stromal, unstacked thylakoid membrane, providing more membrane surface area for $NADP^+$ reduction and ATPase activity needed to support high rates of carbon fixation.

Chloroplasts are usually more numerous in mesophyll cells of shade plants and arranged near the upper leaf surface while leaves of the lower leaf mesophyll have few chloroplasts. However, as the number of cell layers is smaller, shade plants often have less chlorophyll per unit leaf area. Under exposure to low PFD, chloroplast of palisade cells of shade-acclimated leaves tend to be located adjacent to the periclinal cell walls, perpendicular to the incident light beam, and are therefore in an optimal position to intercept light energy (Chow et al., 1987). Chloroplasts from shade-acclimated leaves have a greater proportion of granal stacked thylakoid membrane (Chow et al., 1987), associated with high concentrations of peripheral light-harvesting complexes of PS II thus increasing the area of light-capturing membrane. Grana are often irregularly orientated which may increase capture of diffuse or variably orientated light.

These chloroplast positions are not static; chloroplast in both shade-and sun-acclimated plants can move in response to changing light conditions in order to maximize light interception when light is limiting and to minimize light interception when it is saturating (Haupt and Scheuerlein 1990).

1.13.1.4. Quantum yields

At low PFD, the photosynthetic apparatus appears remarkably capable of using the vast majority of absorbed photons for photochemistry, independent of the light environment in which the plants were grown or any genetic adaptation to sun and shade environments. It can be concluded that in the absence of stress the maximum quantum yield of sun and shade adapted species, or of plants of a species acclimated to different light environment, do not differ significantly. This conclusion is supported by observation of similar values of the fluorescence ratio Fv/Fm indicating similar maximum quantum yield of PS II photochemistry in both (Björkman and Demmig 1987).

The nearly maximum possible quantum yield indicates that several potential constraints on the efficiency of light use are overcome under the differing light qualities of sun and shade environments and compositions of the photosynthetic apparatus. These high quantum yields indicate that photons are partitioned equally between PS II and PS I despite the apparent excess of PS II in shade plants and despite differences in light quality. Any other partitioning would reduce the quantum yield. Both light quality and quantity are known to regulate photosynthetic stoichiometry. Comparison of photosynthetic stoichiometries in sun and shade plants from natural environment reverse generally lower PSII/PSI reaction centre ratios but also considerable overlap in values (Chow et al., 1990). Thus potential differences in excitation must be balanced by adjustments in antenna sizes associated with each photosystem. Stress effects such as low nitrogen supply, drought, either too high or low temperature may also reduce quantum yields in high light because of photoinhibiton. These factors may account for much of the differences in quantum yields reported for sun and shade acclimated plants (Pearcy 2000).

1.13.1.5. Light harvesting and electron transport

In light-limited environments a greater emphasis is placed upon light gathering. At the level of the thylakoid membrane this is reflected in a greater concentration of light-harvesting complexes associated with the reaction centres. Shade plants may contain 4 to 5 times more Chl *a* and *b* per unit volume of chloroplast and have a higher *b/a* ratio than sun plants because the LHC increases (Anderson 1986). Both PS I and PS II decrease with shade but the ratio of antenna chlorophyll to reaction centre is slightly larger than in high light grown plants of sun species. The electron transport chain in shade plants is not increased, as there is relatively much less (1/5th) cytochrome f, plastoquinone, ferredoxin and carotenoids per unit of chlorophyll than in sun plants. Shade plants have therefore more light-collecting apparatus, but a smaller complement of electron carriers than sun plants. In dim light the rate of electron transport is limited by the number of photons falling on the leaf, it would be no advantage for shade plants to produce a large capacity electron transport chain. With a pool of plastoquinone receiving electrons from PS II reaction centres, this rate limiting step is minimized in the shade plants except when the plant is exposed to light of brightness outside the normal range.

Sun plants have less developed thylakoid system, fewer granal stacks and partitions and less LHC so they are less efficient at absorbing light energy at low photon flux than shade plants and so have lower quantum yield. Electron transport in sun plants maybe 15-30 times faster than in shade species (uncoupled rate). Clearly the main differences between the two groups of plants are the capacity of the light-harvesting system and of electron transport. Increases in the concentrations of various electron transport constituents in plants acclimated to higher PFDs partially account for the two-fold increase in photosynthetic rate of these plants (Chow and Anderson 1987). The light absorbing system in shade plants makes them very effective at gathering the light available and passing it to the reaction centre, especially in dim light, but they are limited in bright light by the rate of electron transfer, sun plants in contrast are very efficient at transporting electrons but not at gathering weak light.

1.13.1.6. Rubisco

Plants grown at higher PFDs had Rubisco activities over two-times greater than plants acclimated to lower PFDs (Chow and Andersson 1987).

1.13.1.7. Photosynthetic capacity in sun and shade acclimated leaves

It is now well established that growth in high versus low light results in increased rubisco activity, PS II electron capacity, capacity of cytochrome f and chloroplast coupling factor (CF_1) per unit leaf area (Anderson and Osmond 1987). Dynamic measurements of acclimation following transfer of plants between light environments have revealed a range of responses of light-saturated photosynthetic capacity depending on the species. For some species mature leaves exhibit number or at least only a modest acclimation response (Pearcy and Sims 1994).

The photosynthetic system of strongly illuminated shade plants maybe irreversibly damaged by very intense light, whereas sun plants are apparently insensitive. Slow movement of electron through plastoquinone at high rates of electron flow in bright illumination causes backing up of electrons and reaction centre cannot use excitation energy so that the high energy state of Chl accumulate and damage increases. PS II appears more sensitive to photoinhibition than PS I. The structure of shade plants thylakoid makes them more easily damaged. Possibly the structure of the PS or carotenoid complement, which reduces the energy load and provides a safety valve is inadequate in shade plants. Sun plants have relatively smaller light-harvesting system so are inefficient in weak light, but the electron transport chain for electron flow and excess energy dissipating system contribute to their greater efficiency and capacity to assimilate carbon dioxide in intense illumination.

1.13.1.8. Response of energy dissipation

The total pool size of xanthophyll cycle pigments (V+A+Z) changes with the growth light environment as part of the acclamatory response that occurs over a time-scale of days (Björkman and Demmig-Adams 1994). Plants growing in more exposed locations, where they might be expected to have a greater need to dissipate excessive absorbed light energy, tend to have larger pools of xanthophyll cycle pigments (Thayer and Björkman 1990; Logan et al., 1996). In general, plants acclimated to sun-exposed sites are capable of rapidly converting a greater fraction of their xanthophyll to A and Z in full exposure than are shade-acclimated plants (Brugnoli et al., 1994). These acclamatory adjustments presumably provide sun-acclimated plants with the means to dissipate the excessive absorbed light energy which they experience for hours every day.

1.14. Physiological-ecological response range of PS II to excess light

1.14.1. Short-term response

The first order of defense for a typical PS II unit, of a plant growing in a shade environment when exposed to rapid intermittent doses of excess light, is the xanthophyll cycle-dependent energy dissipation. Typically, otherwise non-stressed, healthy plants show a diurnal time course where the level of xanthophyll cycle-dependent energy dissipation tracks the solar light intensity, peaking at midday when it becomes most excessive. Indeed, it is now clear that xanthophyll cycle-dependent energy dissipation is part of the daily and day-to-day life of almost all terrestrial higher plants and many algae and diatoms (Demmig-Adams et al., 1996).

1.14.2. Long-term responses

As an extension to the highly choreographed short-term (seconds to hours) photoprotective responses of the PS II unit to excess light levels described above, there is also a well defined long-term (hours to seasons) pattern of events involving changes in the overall pigment-protein content and structural composition of the PS II unit. When the duration and or extent of excess light exposure increases, for example, in a fully sun exposed environment, or during suboptimal temperatures, it is possible that the molecular content of the PS II unit is altered such that there is a decrease in the size of the peripheral PS II antenna. This is mostly attributed to a decrease in the

LHC II *b* and subsequent decrease in its component pigments, namely Chl *a*+*b*, lutein and neoxanthin. In most cases, long term acclimation of PS II is probably accompanied by no change or a significant increase in the number of xanthophyll cycle pigments, V, A and Z; thus the ratio of V+A+Z to total Chl *a*+*b* increases (Demmig-Adams et al., 1996; Gilmore 1997).

1.14.3. Extreme responses - damage and repair of PS II reaction centers

A natural follow up of the above concepts concerning the limitations of the photoprotective avoidance and dissipation mechanisms is to understand what happens when these photoprotective strategies fail under prolonged and/or extreme stresses. It is important to consider that the degree of stress required to inflict failure of PS II function is usually associated with an environmental condition so severe that the primary biochemical functions are almost completely inhibited. The observed degradation of the PS II core (D1, D2, CP43, CP47, etc.) proteins under extremely high-light exposure, at freezing-low temperatures or with limiting electron acceptors (CO_2, O_2) can be viewed as symptomatic, as opposed to a causal effect, of the inhibited photosynthesis. However, the sustained inhibition of photosynthetic productivity upon return to favorable environmental conditions is a significant and genuine concern and is the primary reason for the intense interest in the photoprotection and photodamage/repair phenomena. It has been demonstrated in many studies that when the rate of reaction center damage exceeds that of repair (reaction center protein synthesis and assembly) accumulation of inactivated PS II units ensues (Chow 1994). Thus, optimizing the balance of PS II damage and damage-repair mechanisms is clearly an important photoprotective strategy in plants exposed to extreme light stress. Further, because inactive or photoinhibited PS II centers have increased rate constants of heat dissipation, it has been proposed that inactive centers may serve a photoprotective purpose by dissipating excess light that may otherwise cause further damage to intact-functional PS II centers that share excitation energy (Chow 1994).

1.15. Objectives

Most of the work on photoinhibition has been carried out by growing plants under artificial low light conditions and exposing them to artificial constant high light under laboratory conditions. However, very little is known about the extent of photoinhibition and protection under field conditions. In order to obtain a detailed characterization of the extent of damage and protection of the photosynthetic apparatus under field conditions, a study of rice plants grown at three different growth conditions ranging from extremely low to extremely high was carried out. In this study we use natural sunlight as photoinhibitory treatment in order to consider the fluctuations in the light intensities during the course of the treatment.

Based on the measurements of pigment composition and chlorophyll *a* fluorescence parameters at room temperature we attempted to asses the capacity of acclimation of rice plants to high light and to obtain a more detailed view of the relation between the xanthophyll cycle associated energy dissipation and non-photochemical quenching of chlorophyll *a* fluorescence in preventing adverse effects of full sunlight on the photochemical system of low light and high light acclimated leaves. More specific objectives were as follows:

- To study the rate of photosynthesis, photoinhibition under conditions of excess excitation to determine extent of damage to plants grown under different light intensities

- To measure changes in xanthophyll cycle components and other carotenoids under different growth conditions and treatment conditions and to study the extent of protection
- To study relationship between ABA and xanthophyll cycle with respect to protection against over-energization
- To study changes in lipids of cell membrane and thylakoid membrane, in relation to damage and protection of photosynthesis, under various conditions of over-energization
- To study rate of VDE and ZE under different growth conditions and to relate it to the efficiency of the xanthophyll cycle

CHAPTER-2

MATERIALS & METHODS

MATERIALS AND METHODS – CONTENTS

MATERIALS AND METHODS

2.1 Plant material: The study was carried out using rice (*Oryza sativa* L. c.v. Jyothi) plants. This is the most widely cultivated variety in Goa. The seeds were obtained from ICAR Complex Old Goa, Goa, and were stored in plastic bags at 4°C.

2.2 Germination of seeds and growth conditions: Seeds were treated with 0.1% sodium hypochlorite for 5 min and washed thoroughly before soaking in tap water for three days to allow the seeds to germinate. The germinated seeds were then transferred to earthen pots having a diameter of 20 cm, containing common garden soil and vermiculite in the ratio 3:1. The plants were grown for thirty days under three different growth environments.

One set of plants was grown in a naturally lit glasshouse where the photon flux density (PFD) received by the plants ranged from 150-200 μmol $m^{-2}s^{-1}$ and the relative humidity was 85-90%.

The second set of plants was grown in the shade by placing the pots in the shade of a building, where the peak PFD varied between 600-800 μmol $m^{-2}s^{-1}$ and 70-75% RH.

The third set of plants was grown outdoors in the university campus, under full sunlight in a non-shaded area, where the plants received a maximum PFD of 2200 μmol $m^{-2}s^{-1}$ with 70-75% RH (Fig. 2.1). All the plants were irrigated daily.

Figure 2.1: Thirty days old rice plants grown in direct sunlight.

2.3. Treatment conditions

2.3.1. Photoinhibition of plants (*in vivo*): After thirty days of growth the plants were about 20 cm in height and had three leaves in all the three different growth conditions. Leaf three was used for all measurements. This was the third leaf to emerge on the primary tiller, ignoring the seed leaf. The potted plants were transferred from growth condition to direct sunlight from 1030 h to 1630 h (6 h of treatment) and leaf samples where collected at 1130 h, 1330 h and 1630 h on day one of transfer. The plants were not shifted back to the growth conditions for the night, and the treatment was followed for a period of seven days and leaf samples were collected at 1630 h of the third, fifth and seventh day after transfer to sunlight, to study various parameters. In the case of glasshouse and shade plants control measurements were taken prior to transfer to direct sunlight (control plants), while in the case of plants grown in full sunlight the control leaf samples were collected at predawn (0630 h) and further leaf samples were collected at 1130 h, 1330 h and 1630 h. In sun plants measurements were not followed for a period of seven days since these plants were growing in full sunlight for last thirty days, they would show more or less same reading as seen for the first day of measurement. In the present study the time of the day was taken into consideration and not the duration of treatment since the light intensity varied in the course of the day (Table 2.1).

2.3.2. Photoinhibition of plants fed with dithiothreitol (DTT) and ascorbate (AsA): Violaxanthin de-epoxidase (VDE) requires ascorbate as a reductant for the de-epoxidation of violaxanthin (V) to zeaxanthin (Z; Bratt et al., 1995), while the de-epoxidation reaction is inhibited by DTT (Bilger et al., 1989). In the present study rice leaves were fed with ascorbate to stimulate the de-epoxidation of V to Z and with DTT in order to inhibit de-epoxidation of V to Z to study the impact of stimulation

Table 2.1: Photon flux density (PFD; μmol $m^{-2}s^{-1}$) during the day

Time of day (h)	**Photon flux density (PFD) μmol $m^{-2}s^{-1}$**
0630 (predawn)	150
0730	615
0830	946
0930	1401
1030	1823
1130	2050
1230	2184
1330	2156
1430	1997
1530	1707
1630	1326
1730	1137
1830	706

and inhibition of xanthophyll cycle on energy dissipation (measured as non-photochemical quenching) under high light conditions.

Rice leaves were cut from their petioles and placed in vials containing AsA (5 mM pH 6.8) or DTT (30 mM pH 6.4) or water for the control. Solution was kept stirred at low speed continuously throughout the duration of feeding AsA or DTT, using magnetic stirrer. Prior to the light treatment rice leaves were allowed to be fed with AsA/DTT for duration of 1 h under growth conditions and subsequently exposed to direct sunlight for duration of up to 6 hours i.e. 1030 h to 1630 h. Control plants were fed with distilled water for 6 h under growth conditions. Both control as well as leaves fed with AsA or DTT was used for chlorophyll fluorescence measurements as well as for the extraction of xanthophylls for their HPLC analysis.

2.3.3. Photoinhibition of plants exogenously supplied with abscisic acid (ABA) and sodium chloride (NaCl): Since violaxanthin serves as a common precursor for Z and ABA (Parry and Horgan 1991; Hirschberg 2001), the influence of NaCl stress and exogenously supplied ABA on the light-dependent Z formation, under high light stress, was studied, to ascertain if exogenously ABA supplied plants would show a better protection under high light treatment, since V pool shall be available for the formation of Z, while in plants subjected to salt stress the increase in ABA levels would presumably be from V, which may limit the V pool for Z formation, undermining energy dissipation function.

Rice plants were grown in small plastic pots having diameter of 8 cm, containing vermiculite under shade and watered with Hoagland solution. Seven days old seedlings were watered with Hoaglands solution supplemented with 10 µM ABA (pH 6.6) for next fifteen days (+ABA). Control plants were watered with only

Hoagland solution without adding ABA (-ABA). Plants (both –ABA and +ABA) were then treated with high light by exposing them to direct sunlight (+PI). Leaf samples were collected for chlorophyll fluorescence measurements, pigment and ABA analysis at 1130 h, 1330 h and 1630 h. Control readings were taken before the plants (-/+ABA) were exposed to direct sunlight (-PI). Plants were also fed with ABA (5 µm pH 6.6) through cut petioles and photoinhibited, in addition of giving ABA through roots to be able to compare data with AsA and DTT fed plants.

After twenty five days some rice plants were subjected to salt stress (100 mM and 250 mM) for duration of six days. The salt treated rice plants were then transferred from growth conditions to direct sunlight at 1030 h and leaf material was harvested for chlorophyll fluorescence measurements, pigment and ABA analysis at 1130 h, 1330 h and 1630 h. Control readings were taken before the plants (-/+NaCl) were exposed to direct sunlight (-PI).

2.4. Photoinhibition of chloroplasts (*in vitro*): This experiment was conducted with an objective to compare the extent of damage and recovery under *in vivo*, where cellular recovery processes will be accompanying the photoinhibitory damage, and *in vitro* conditions, where no cellular recovery will be observed.

2.4.1. **Isolation of chloroplasts:** Chloroplast isolation was carried out with thirty days old rice plants grown in the different growth conditions according to Sharma and Hall (1992). 20 g of rice leaves were cut into 0.5 – 1 cm long pieces and placed in a domestic blender with 100 mL of the grinding medium (Table 2.2). The mixture was blended for 45 s, with 3 bursts of 15 s at high speed. The homogenate was filtered through 8 layers of muslin cloth into centrifuge tubes and was centrifuged in a cooling centrifuge for 8 min at 5800 g The supernatant was discarded and the pellet was

Table 2.2: Composition of grinding and reaction medium

Sr. No.	Reagent	Grinding medium	Reaction/ RE-SUSPENDING Medium
1.	Sorbitol	0.4 M	0.4 M
2.	Tricine	30 mM (pH 7.2)	50 mM (pH 7.8)
3.	Ethylene Diamine Tetra Acetate	1 mM	2 mM
4.	Magnesium Chloride	1 mM	4 mM
5.	Manganese Chloride	1 mM	2mM
6.	Ascorbate	2 mM	----
7.	Bovine Serum Albumine	0.5 %	0.5%
8	Sodium Chloride	----	10 mM

washed with re-suspending medium and then dissolved in 1-2 drops of re-suspending medium (Table 2.2).

2.4.2. Chlorophyll estimation: Chlorophyll estimation of the isolated chloroplasts was done according to Arnon (1949). 50 μL of the isolated chloroplast preparation was dissolved in 10 mL of 80% acetone and centrifuged at 250 g for 2 min. The supernatant was used for chlorophyll estimation. The absorbance was read at 652 nm wavelength using a spectrophotometer (UV-2450 UV-visible spectrophotometer, Schimadzu) and the optical density was multiplied by 5.8 to get the value in mg of chlorophyll per mL of chloroplasts.

2.4.3. Photoinhibition of isolated chloroplasts: Isolated chloroplasts, having a concentration of 1 mg of cholorophyll/mL of chloroplasts, were treated for 3 and 6 h with a light intensity of 1200 μmol m^{-2}s^{-1}, using a halogen cold light source. 6 mL of the chloroplast suspension was treated in a double-layered glass cuvette through which temperature-controlled water was circulated to maintain a constant temperature of 30°C during the treatment. The chloroplast suspension was kept stirred at low speed throughout the duration of treatment using a small magnetic flea in order to avoid self-shading. The stirring speed was low enough so as not to cause any mechanical damage.

2.5. Estimation of violaxanthin de-epoxidase (VDE) activity

2.5.1. Preparation of photosystem II (PS II) particles: The PS II particles having LHC II, from the control and treated (one day sun exposed) leaves were prepared according to Kuwabara et al., (1998) with some modifications.

20 g of leaves were macerated in a buffer containing 10 mL of 50 mM Na-K phosphate buffer (pH 6.4), 0.3 M sucrose and 100 mM NaCl (1:1:1,v/v), to which 30 mL of Triton-X 100 (2%, w/v) was added. The mixture was filtered through 8 layers of muslin cloth and the filtrate was centrifuged at 5800 g for 60 min at -4°C. The pellet was suspended in 5 mL of 40 mM Na-K phosphate buffer (pH 6.4) and centrifuged at 100 g for 1 min. The supernatant was then centrifuged at 8000 rpm for 60 min at -4°C. The resulting pellet was washed twice with 6 mL of buffer containing 25 mM MES-NaOH (pH 6.5), 0.3 M sucrose and 10 mM NaCl (1:1:1 v/v), and was suspended in 1 mL of the same medium.

2.5.2. Assay of VDE activity: The assay of VDE activity was carried out as described by Rivas et al., (1989) with some modifications. To 2.4 mL of 50 mM sodium acetate-HCl buffer (pH 4.9) were added 0.1 mL of water and 0.1 mL of the suspension of PS II particles. The reaction was started by the addition of 0.08 mL of 0.8 M sodium ascorbate. The mixture was incubated at 30°C for 3 h. The reaction was stopped by the addition of 0.09 mL of 0.2 M dithiothreitol to quench VDE activity. The particles were collected by centrifugation at 5800 g for 60 min and analyzed for the contents of xanthophyll cycle pigments by HPLC. The de-epoxidation state of PS II particles were described according to Pandey et al., (2005) in terms of the de-epoxidation index (DEI), i.e., (A+2Z)/(V+A+Z).

2.5.3. Extraction and determination of xanthophyll cycle pigments (XCP): After reaction the XCP from the PS II particles were extracted according to Pandey et al., (2005), with 1 mL of acetone:methanol (7:2, v/v) by vortexing, followed by centrifugation at 5800 g for 30 min. To the supernatant 0.08 mL of dichloromethane was added. Dichloromethane had been mixed with 1/100 vol of 1 M

Tris-HCl buffer (pH 8.0) to prevent the acidification that causes the rearrangement of 5,6-epoxide of V to the furanoid-5,8-epoxide. The colorless insoluble materials were precipitated by centrifugation at 5800 g for 30 min. The supernatant was used for analysis of XCP by HPLC (Waters) according to Sharma and Hall (1996) as described in the section 2.7.

2.6. Chlorophyll fluorescence: Chlorophyll fluorescence measurements were performed on intact leaves or isolated chloroplasts, which were dark adapted for 10 minutes prior to measurements at room temperature, using a pulse amplitude modulation fluorometer (PAM 101, Walz, Effelrich, Germany) as described by Sharma et al., (1998), to measure the extent of photoinhibition and non-radiative dissipation of excess energy (Fig. 2.2). The dark adapted leaves were 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 $m^{-2}s^{-1}$ to provide the maximum fluorescence (Fm). After measurement of Fm leaves were allowed to reach steady state fluorescence (Fs) while exposed to actinic light intensity of 330 μmol $m^{-2}s^{-1}$. Another burst of saturating light at Fs state was used to measure F'm. After reaching the steady state again, leaves were exposed to far-red radiation light to measure F'o. Calculations were carried out according to Schreiber et al., (1986). Maximal quantum efficiency of PS II (Fv/Fm) was estimated from the variable to maximum fluorescence ratio, Fv/Fm = (Fm-Fo)/Fm. Photochemical quenching coefficients were calculated as (qP) = (Fm′-Fs)/(Fm′-Fo′) and non-photochemical quenching coefficients were calculated as (qN) = 1- (F'm-F'o)/(Fm-Fo). While in isolated chloroplasts Fv/Fm was the only parameter measured.

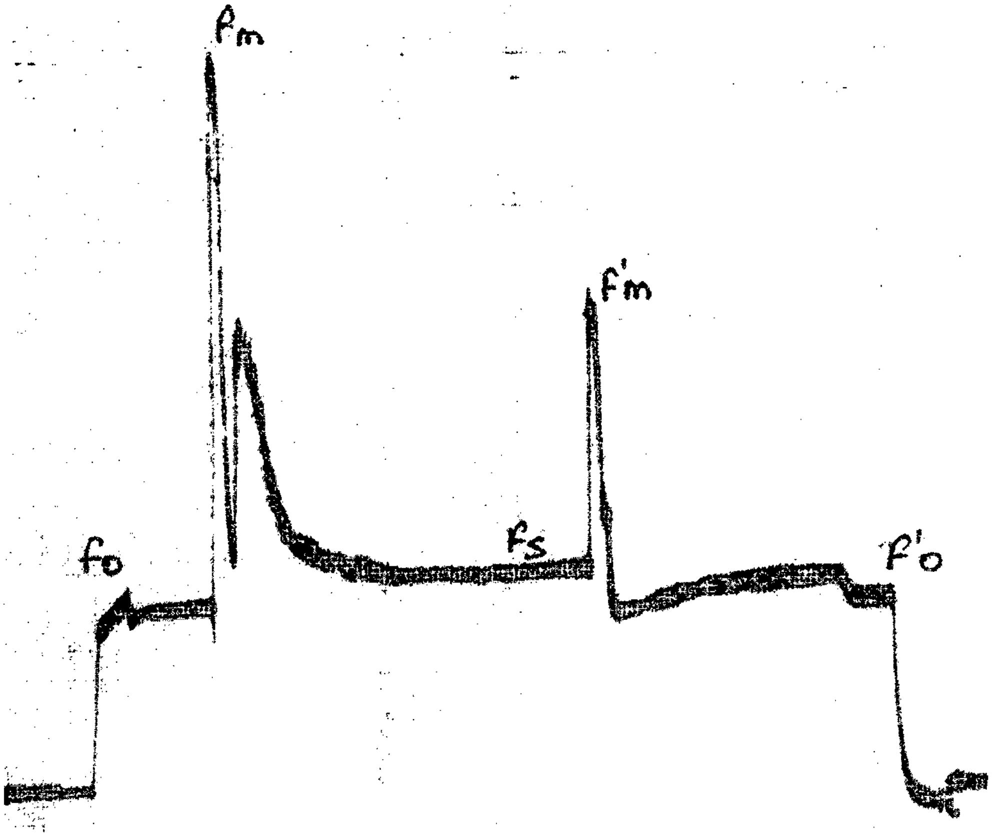


Figure 2.2: A typical fluorescence induction curve.

2.7. Extraction and analysis of photosynthetic pigments: Extraction and estimation of pigments was carried out according to Sharma and Hall (1996) using HPLC (Waters), to study the extent of xanthophyll cycle-dependent energy dissipation in plants exposed to excess light. 0.25 g of the leaf sample was homogenized in 100% acetone to a final volume of 5 mL extract using a mortar and pestle. The homogenate was then incubated overnight at 4°C in the dark. Extract was centrifuged at 1800 g for 10 minutes and 1 mL of the supernatant was stored in eppendorf tubes, at -70°C freezer for subsequent HPLC analysis of pigments. The sample was filtered through 0.2-µm-nylon filter (Pall Pharmalabs) prior to loading on HPLC. Identification and separation of pigments was done using a reverse phase column (Waters Spherisorb ODS 2.5 µm, 4.6mm x 250 mm) using a phase diode array detector (Waters 2996). 20 µl of the pigment extract was injected into the HPLC. The gradient for separation was 0 – 100% ethyl acetate in acetonitrile/water (9:1) over 34 min with flow rate of 1.2 mL/min. The pigments were detected at 450 nm and were quantitated on a fresh weight basis from peak area value using β-carotene as external standard. Zeaxanthin was well seperated from lutein.

2.8. Analysis of ABA by HPLC: Part of the leaf extract (section 2.7) was also used for the ABA analysis. The separation and quantitative estimation of ABA was carried out according to Cowan et al. (1997) and Sharma et al., (2002) using HPLC (for details of the machine and specifications, see section 2.7). The separation was carried out using a linear gradient of 0 – 100% methanol in 0.5% aqueous glacial acetic acid over 32 min at a flow rate of 1 mL/min, and quantified with peak integration after calibration with external standard ABA.

2.9. Peroxidation of cell and thylakoid membrane lipids: Lipid peroxidation which is an indicator of oxidative stress in cells and tissues was studied by determining thiobarbituric-malonaldehyde (TBA-MDA) adduct formed, which signify the formation of polyunsaturated fatty acid peroxides in biological systems.

0.5 g of leaf tissue was ground in 0.5% trichloroacetic acid (TCA). The homogenate was made up to 5 mL and centrifuged at 4000 g for 15 min. The supernatant was collected and used for measuring the peroxidation of membrane lipids. 1 mL of supernatant was added to the test tube containing 2.5 mL of freshly prepared (0.5%) TBA in (20%) TCA and 2.5 mL of incubation buffer (50 mM Tris HCl and 175 mM NaCl, pH 8.0). A glass marble was placed on the test tube and was incubated for 30 min at 90°C in a water bath. After incubation, it was allowed to cool and centrifuged for 2 min at 650 g to settle the debris and non-specific precipitation. The optical density was taken on spectrophotometer at 532 nm and 600 nm (UV-2450 UV-visible spectrophotometer, Schimadzu). 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-TBA adduct formed was quantitated according to Halliwell and Gutteridge (1989) and Sharma et al., (1998) by using extinction coefficient of MDA 155 μmol cm^{-1}m^{-1}.

2.10. Estimation of total soluble and insoluble carbohydrates: Measuring carbohydrate pools gives information about the amount of stored carbohydrates and the form in which they are stored. Plants that are stressed typically accumulate higher amounts of soluble carbohydrates; therefore, the ratio of soluble carbohydrates to starch is indicative of the level of stress in the leaf. Quantitative estimation of total soluble and insoluble carbohydrates was carried out by colorometric method according to Buysse and Merckx (1993), briefly 0.15 g of leaf tissue was ground in 5 mL of 80% ethanol and centrifuged at 450 g for 10 min. The pellet was re-suspended in a further 5 mL of 80% ethanol and re-centrifuged. Supernatants were pooled. Starch contained in the pellets was hydrolyzed by suspending the pellet in 5 mL of 3% hydrochloric acid solution for 3 h, following which solutions were centrifuged and supernatants were made upto 10 mL with 80% ethanol.

1 mL sample of ethanol extracted soluble carbohydrates or hydrolysed starch were added to 1 mL of 28% phenol:water (v/v), 10 mL of concentrated sulphuric acid and the tubes were vortex–mixed. Colour reaction was allowed to develop for 15 min and absorption was measured at 490 nm on a spectrophotometer (UV-2450 UV-visible spectrophotometer, Schimadzu). Standard curve was constructed using known concentration of glucose.

2.11. Lipid analysis: The study was carried out with an objective of studying the changes in the different cell and thylakoid membrane lipids in response to high light stress.

2.11.1. Extraction of total lipids: Total lipids were extracted according to the method described by Turnham and Northcote (1984) and Bhandari and Sharma (2006). 2 g of leaf tissue was boiled in 5 mL of isopropanol for 2 min using spirit lamp to inhibit the lipase activity. Isopropanol was decanted and leaf tissue was homogenized in chloroform:methanol (1:2 v/v) to make the final volume to 15 mL and few crystals of BHT were added as an antioxidant in the lipid extraction solvent system. The homogenate was incubated overnight at 4°C. Lipid extract was centrifuged for 5 min at 1000 g. to get rid of cell debris. To the supernatant, 4 mL of double distilled water was added to make the ratio of chloroform:methanol:water to

1:2:0.8 in monophasic system. Biphasic system was attained by further addition of 5 mL of chloroform and 5 mL of 0.88% potassium chloride in a separating funnel to make the ratio of chloroform:methanol:water to 1:1:0.9 respectively. The mixture was shaken vigorously for 5 min and was kept for separation for 30 min. Only the lower phase of chloroform, containing appreciable amounts of extracted lipids was collected for lipid analysis. This step was repeated once more with 1 mL of chloroform and the chloroform phase was pooled in 10 mL screw capped vials fitted with teflon lining. The sample was completely dried under the nitrogen gas and redissolved in small volume of chloroform (1 mL) and stored at -20°C until further use. The entire extraction and purification process was done in diffused light to protect the lipids from photo-oxidation.

2.11.2. Separation of total lipids by thin layer chromatography: Separation of total lipids into polar and neutral lipids was carried out by thin layer chromatography (TLC) on silica gel H according to Liljenberg and Von Arnold (1987). Uniform slurry of 40 g of silica gel H was prepared in 100 mL of distilled water and stirred for 1-2 h on a magnetic stirrer. Glass plates of 0.4x20x20 cm was washed with acetone and fixed to the surface using single strip of leucoplast. The slurry was pulled at a steady rate across the plate, from top to bottom using glass rod. The plates were placed in the drying rack, left at room temperature for an hour and dried at 120°C for 2 h. They were then stored in the storage cabinet, but were heated again at 120°C for 1 h before use. After cooling the plates, 50 μL of the sample was applied as a discrete spot, 1.5-2 cm from the bottom of the plate, by means of a syringe and the plate was then placed in chromatographic chamber containing developing solvents, i.e. chloroform:methanol:glacial acetic acid:water (85:15:10:3.5) for polar lipids and petroleum:diethyl ether: glacial acetic acid (90:10:1) for neutral lipids. The plates were air dried and spots were visualized as bands by using iodine vapors. The different bands of lipids were identified by their R_f values against known standards (Kates 1986).

2.11.3. Quantitative estimation of glycolipids: Amount of glycolipids present in total lipids was determined by the phenol-sulfuric acid method according to Kushawa and Kates (1981). 100 µL of lipid extract was pipetted out into a test tube and the solvent was evaporated to dryness under steam of nitrogen. To the residue 2 mL of distilled water and 1 mL of 5% phenol solution was added, and mixed gently by vortexing, making sure that the film of lipid at the bottom of tube is undisturbed. To this 5 mL of concentrated sulphuric acid was added with vortexing and heated for 5 min in a boiling sand bath, resulting in developing of orange colour. The mixture was allowed to cool and the absorbance was read at 490 nm using a spectrophotometer (UV-2450 UV-visible spectrophotometer, Schimadzu). The amount of sugars in the unknown sample was calculated from a calibration curve, prepared at the same time by performing the reaction on known amount of standard sugar solution using glucose.

2.11.4. Quantitative estimation of phospholipids: Phospholipids were estimated by determining the amount of phosphorus in lipid fraction according to Bartlett method (Christie 1982). An aliquot of lipid sample (25 µL) was dried with nitrogen gas and 0.4 mL of 70% perchloric acid was added to it and the liquid was digested by gently refluxing for 2 h on sand bath until the solution become colorless. The mixture was cooled and 2.4 mL of ammonium molybdate reagent was added, mixed properly, followed by the addition of 2.4 mL of reducing reagent. (Ammonium molybdate reagent was prepared by dissolving 4 mM of the compound in 3.5 mL of concentrated sulphuric acid and making up the final volume to 250 mL with distilled water. The reducing reagent consisted of 52 mM sodium bisulphite, 15 mM sodium sulphite and 0.7 mM 1-amino-2-naphthol-4-sulphonic acid).

The solutions were mixed thoroughly and were heated on a boiling sand bath for 10 min for color development. The solution was allowed to cool and absorbance measured at 830 nm (UV-2450 UV-visible spectrophotometer, Schimadzu). The amount of phosphorus in the unknown sample was read from a calibration curve, prepared at the same time by performing the reaction on known amount of standard phosphate solution using sodium dihydrogen orthophosphate.

2.11.5. Analysis of fatty acids: Before the fatty acid composition was determined by gas chromatography, volatile methyl ester derivatives of the fatty acid components were prepared.

2.11.5.1. Preparation of methanoic hydrochloric acid (HCL): Methanoic HCL was prepared according to the method described by Christie (1982) and Bhandari and Sharma (2006). 5 g of ammonium chloride was taken in a conical flask (with side arm), which was connected to another receiver, which was fitted to a side arm conical flask containing 25 mL of methanol. Concentrated sulphuric acid (10 mL) was added to ammonium chloride using buchner funnel. This reaction liberates HCL, which was bubbled in methanol (until entire ammonium chloride dissolves) with continuous mixing using magnetic stirrer for about 15 min. 5 mL of methanoic-HCL was titrated against 1N sodium hydroxide and the concentration of hydrochloric acid in methanol was calculated using formula, $N_1V_1=N_2V_2$.

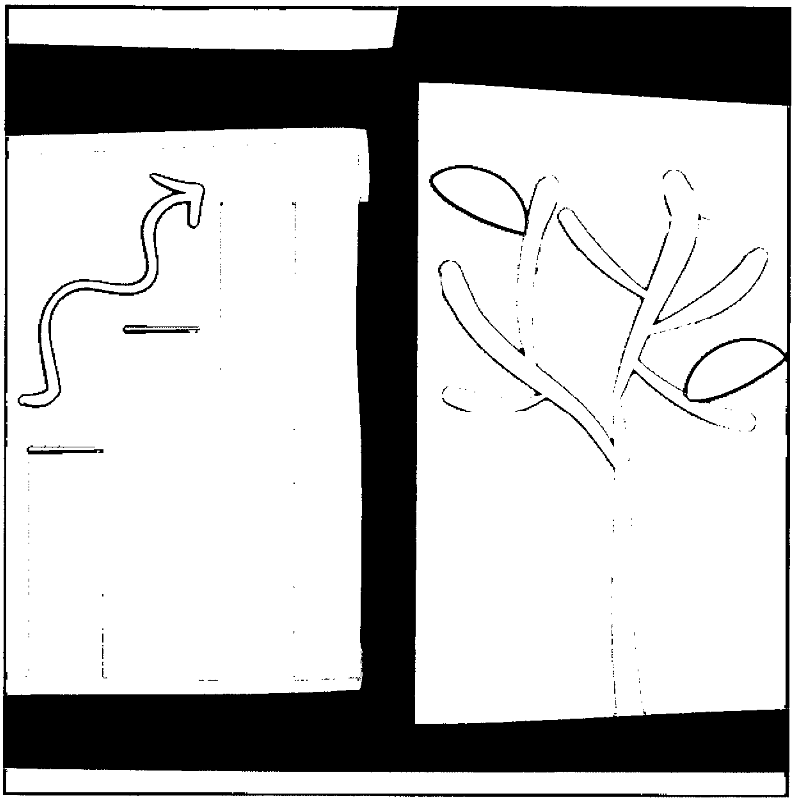
Where N_1= Normality of Methanol-HCl needed for esterification (1.66N), V_1= Volume of methanol needed for esterification (to be calculated), N_2= Normality of HCl in methanol (2.26N) and V_2= Volume of the methanol taken (25 mL).

2.11.5.2. Preparation of methyl esters: The fatty acid methyl esters were prepared according to the method described by Christie (1982) and Bhandari and Sharma (2006). The total lipid sample (1 mL) was evaporated to dryness with nitrogen gas after adding known amount (50 µL) of internal standard (1 mM heptadeconoic acid) and 5 mL of methanoic-HCl was added to 10 mL screw capped vials with teflon lining. The vials were loosely closed with a teflon-lined cap and placed in an oven at 70°C for 10 min before the caps were securely tightened. After 2 h in the oven, the vials were cooled at room temperature, 5 mL of distilled water was added (light yellow color develops), the two phases were mixed by vortexing and methyl esters were extracted with three successive 2 mL portions of distilled hexane, vortexing for 90 s. Pasteur pipette were used to separate the hexane extract, which were pooled in a test tube. The hexane solution of methyl esters was treated with 5 mL of saturated solution of sodium bicarbonate and neutralized by shaking (vortexing) for 15 s. Then washed with 5 mL of distilled water, vortexed for 15 s and upper (hexane) solution was transferred to another test tube and dried with anhydrous calcium chloride (2-3 crystals). The hexane solution was taken in vials and evaporated to dryness in a water bath at 35-40°C with the help of nitrogen gas. The methyl esters were taken in small volume (100 µL) of fresh hexane and vials were capped (teflon lined) and stored in a freezer at -20°C until needed for gas chromatography, which should be done as soon as possible to minimize danger of loss of unsaturated esters.

2.11.5.3. Analysis of fatty acids using gas chromatography: Methy esters of fatty acids (1 μL) were run on a Shimadzu gas chromatograph (GC - 2014) equipped with hydrogen flame ionization detector and chromatopack data processor. The column (6 mm x 2 mm i.d, stainless steel) was packed with DEGS 10% on 80-100 mesh chromosorb W-HP (Chemlabs, Bangalore). Column temperature was 180°C and injector temperature was 220°C and nitrogen was used as carrier gas (flow rate 30 mL/min). 1 μL of sample was injected to the injector port of gas chromatograph using 10 μL fixed-needle syringe. Fatty acid methyl esters peaks were identified by comparing their retention times with methyl esters of pure fatty acid standards and were quantified by using the peak areas of individual fatty acids calculated using the following formulas.

1. Response Correction Factor: RCF = Amount of compound 1/Peak area of 1 x Peak area of reference/Amount of reference
2. Correction of Peak Area of fatty acid methyl esters for varying detector sensitivity: CPA = Peak area of the component 1 x RCF
3. Conversion of corrected peak area (Wt.% to Moles): Mole Corrected peak area = Corrected peak area/Molecular weight of each fatty acid
4. Mole Corrected peak area to Mole % fatty acid methyl esters: Mole % = Mole Corrected peak area/Total of Mole Corrected peak areas of all fatty acid methyl esters x 100

2.12. Statistical analysis: The experimental data were tested for significance by using a Students *t*-test for 2 samples assuming either equal variances or unequal variances. All statistical tests were performed with analysis tools from Microsoft® office excel 2003. The probability of error ($P<0.05$) is noted in figure legends wherever appropriate.



CHAPTER-3

RESULTS

RESULTS - CONTENTS

RESULTS

This chapter deals with the changes in chlorophyll fluorescence, xanthophyll pigments, lipid peroxidation, total soluble and insoluble carbohydrates, lipids and fatty acids, in glasshouse, shade and sun grown plants in response to high light stress.

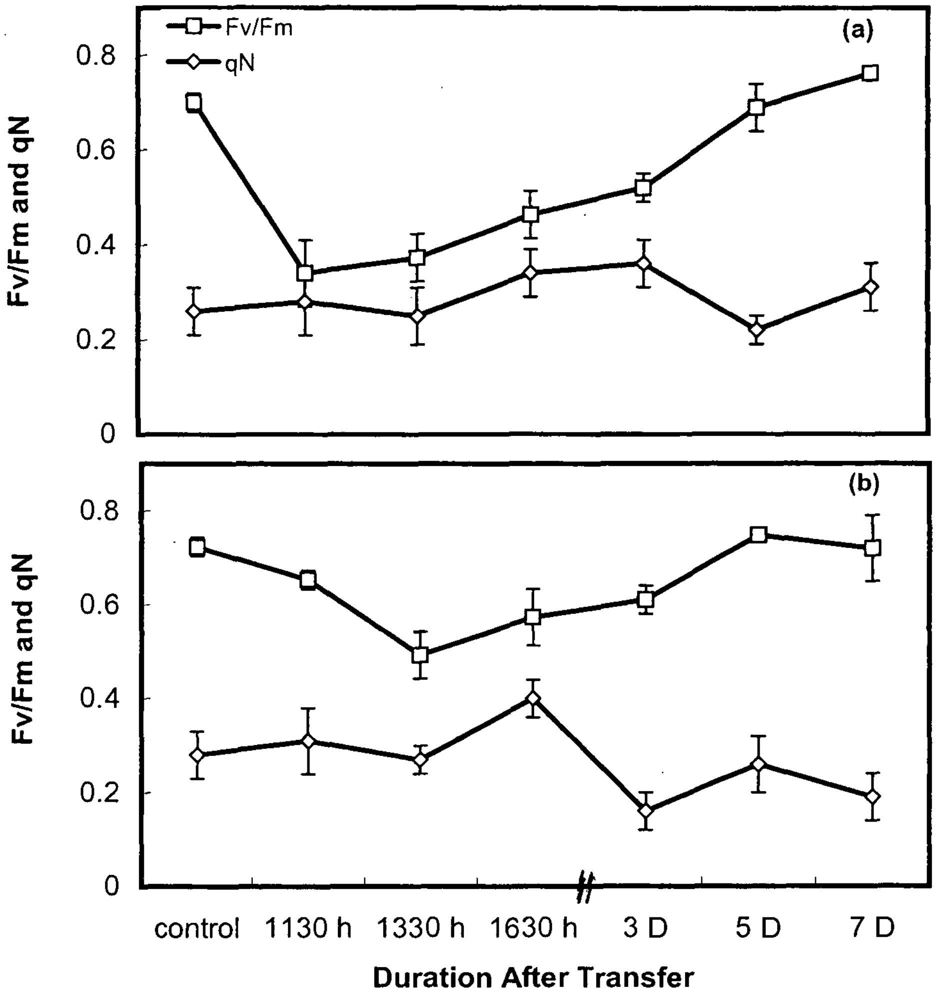
3.1. Effect of sunlight on chlorophyll fluorescence under *in vivo* conditions

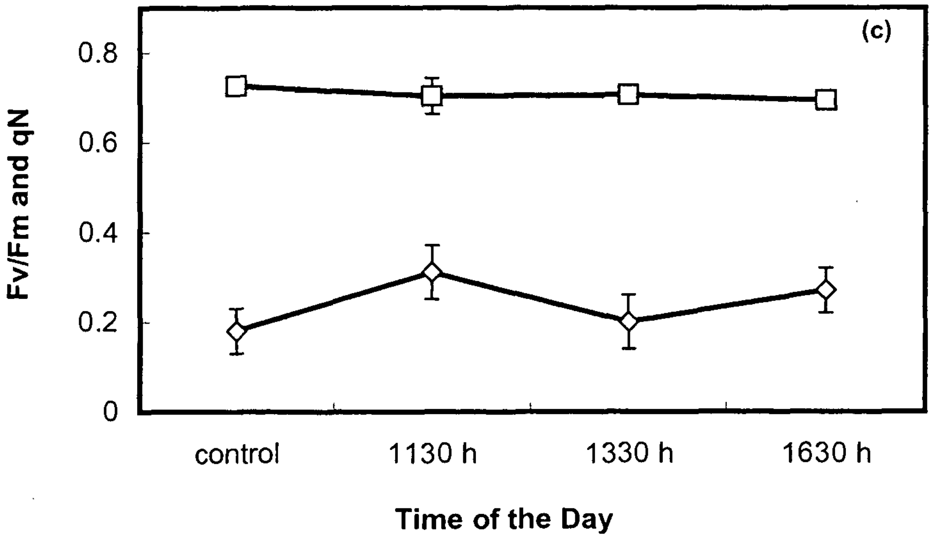
Chlorophyll fluorescence studies were carried out in thirty days old glasshouse and shade grown rice plants exposed to direct sunlight for a period of seven days, to measure the extent of photoinhibition and photosynthetic acclimation of these plants under high light conditions and to compare it with plants grown in full sunlight (sun grown plants).

3.1.1. Glasshouse grown plants

Figure 3.1a shows the changes in the maximum photochemical efficiency of PS II of glasshouse grown plants, in the dark-adapted state, measured as Fv/Fm ratio in response to exposure to direct sunlight. Transfer of glasshouse grown plants to direct sunlight for 1 h (up to 1130 h) resulted in an initial decline of 51% in the Fv/Fm ratio as compared to the glasshouse plants prior to transfer to direct sunlight (control plants). Following the 1 h sun treatment there was a linear recovery in the Fv/Fm ratio with the increase in the duration of the sunlight treatment. Three hours of photoinhibition (up to 1330 h) resulted in a 9% recovery of Fv/Fm, which further increased to 24% by 1630 h (6 h of treatment), as compared to the Fv/Fm observed at 1130 h (1 h of treatment). Prolonged exposure to three days led to a further recovery of 52% in the Fv/Fm ratio as compared to 1130 h. On day five after transfer to direct

Figure 3.1: Effect of sunlight on the photochemical efficiency of PS II (Fv/Fm) and non-photochemical quenching (qN) in thirty days old rice plants grown in the (a) glasshouse, (b) shade and (c) direct sunlight. In glasshouse and shade grown plants control refers to plants prior to transfer to direct sunlight and in sun plants control refers to measurements taken at predawn (0630 h). (n=3, ± SD).


Fv/Fm
qN
(a)
(b)
Fv/Fm and qN
0
0.2
0.4
0.6
0.8
control
1130 h
1330 h
1630 h
3 D
5 D
7 D
Duration After Transfer


(c)
Fv/Fm and qN
0
0.2
0.4
0.6
0.8
control
1130 h
1330 h
1630 h
Time of the Day

sunlight Fv/Fm ratio recovered fully and was similar to control plants. While at the end of the seven days period Fv/Fm was 8% higher than seen in control plants.

Effect of direct sunlight on photochemical quenching (qP) in glasshouse, shade and sun grown plants is shown in Table 3.1. Exposure of glasshouse grown plants to direct sunlight till 1130 h (1 h of treatment) resulted in 7% increase in the qP, however, high light exposure till 1630 h (6 h of treatment) resulted in 18% increase in the qP as compared to control plants. Prolonged exposure to direct sunlight up to seven days resulted in higher qP than seen in control plants (ranging from 0.90 - 0.98).

Non-photochemical quenching (qN) did not change significantly after 3 h of exposure to direct sunlight (up to 1330 h), to glasshouse grown plants but resulted in a linear increase of 38% after three days of exposure to direct sunlight (Fig. 3.1a). Prolonged exposure till seven days resulted in only 19% increase in the qN as compared to control plants.

3.1.2. Shade grown plants

Shade grown plants photoinhibited for 3 h (up to 1330 h) exhibited a decline of 31% in the Fv/Fm ratio, as compared to control plants (i.e. prior to transfer to direct sunlight; Fig. 3.1b). However, further exposure to sunlight resulted in a linear recovery of Fv/Fm as compared to the decline seen after 3 h of treatment. At the end of the seventh day after transfer to direct sunlight, Fv/Fm had recovered to its control level.

Photochemical quenching increased slightly (up to 12%) on exposure to direct sunlight as compared to control plants (Table 3.1). Prolonged exposure to sunlight till seven days did not show any increase in the qP value of shade grown plants as compared to control.

Table 3.1: Effect of sunlight on the photosynthetic efficiency (Fv/Fm), photochemical quenching (qP) and non-photochemical quenching (qN) in glasshouse, shade and sun grown plants. In glasshouse and shade plants control refers to plants prior to transfer to direct sunlight while in sun plants control refers to measurements taken at predawn (0630 h). In glasshouse and shade plants data has been shown till seven days while in sun plants data during the course of a day has been shown. (n=3, ± SD).

Treatment	**Photosynthetic Efficiency (Fv/Fm)**	**Photochemical Quenching (qP)**	**Non-photochemical Quenching (qN)**
Glasshouse			
Control	0.700 ± 0.02	0.79 ± 0.06	0.26 ± 0.05
1130 h	0.340 ± 0.07	0.85 ± 0.05	0.28 ± 0.07
1330 h	0.373 ± 0.05	0.69 ± 0.02	0.25 ± 0.06
1630 h	0.463 ± 0.05	0.94 ± 0.06	0.34 ± 0.05
3 D	0.520 ± 0.03	0.98 ± 0.03	0.36 ± 0.05
5 D	0.690 ± 0.05	0.84 ± 0.05	0.22 ± 0.03
7 D	0.762 ± 0.01	0.90 ± 0.04	0.31 ± 0.05
Shade			
Control	0.723 ± 0.02	0.80 ± 0.04	0.28 ± 0.05
1130 h	0.653 ± 0.02	0.92 ± 0.04	0.31 ± 0.07
1330 h	0.493 ± 0.05	0.90 ± 0.06	0.27 ± 0.03
1630 h	0.573 ± 0.06	0.85 ± 0.04	0.40 ± 0.04
3 D	0.610 ± 0.03	0.85 ± 0.02	0.16 ± 0.04
5 D	0.748 ± 0.009	0.86 ± 0.04	0.26 ± 0.06
7 D	0.720 ± 0.07	0.83 ± 0.05	0.19 ± 0.05
Sun			
Control	0.726 ± 0.02	0.90 ± 0.005	0.18 ± 0.05
1130 h	0.703 ± 0.04	0.95 ± 0.02	0.31 ± 0.06
1330 h	0.706 ± 0.005	0.97 ± 0.02	0.20 ± 0.06
1630 h	0.693 ± 0.02	0.85 ± 0.03	0.27 ± 0.05

Non-photochemical quenching in shade grown plants increased by 10% on transfer to direct sunlight for 1 h (up to 1130 h). Six hours (up to 1630 h) of direct sunlight treatment led to a 42% increase as compared to the control plants (Fig. 3.1b). Further exposure to high light resulted in a reduction in the qN and was 42% and 32% lower after third and seventh day of treatment respectively as compared to control plants.

3.1.3. Sun grown plants

For sun grown plants (plants grown for thirty days under full sunlight), the measurements were observed only for one day till 1630 h (6 h of treatment). The Fv/Fm and qP showed no significant change during the course of the day as compared to control plants (i.e. predawn; Fig. 3.1c and Table 3.1). A 72% increase in the non-photochemical quenching was observed in the morning till 1130 h, which declined in the evening but still remained slightly higher than observed in control plants (Fig. 3.1c).

Comparison showed that, the Fv/Fm ratio in the leaves of glasshouse and shade grown plants prior to transfer to direct sunlight (control plants) and control leaves of sun grown plants (i.e. predawn), remained more or less same, but varied after exposure to sunlight (Fig. 3.2a). Fv/Fm ratio declined initially after transfer to direct sunlight in glasshouse and shade grown plants and recovered slowly thereafter to more or less control value after five to seven days of exposure to direct sunlight. While in sun plants Fv/Fm did not change significantly.

Prior to transfer to direct sunlight the qP was almost similar in glasshouse and shade grown plants (ranging from 0.79-0.8) while it was slightly higher in predawn sun grown plants (0.9; Table 3.1). In response to exposure to direct sunlight, qP

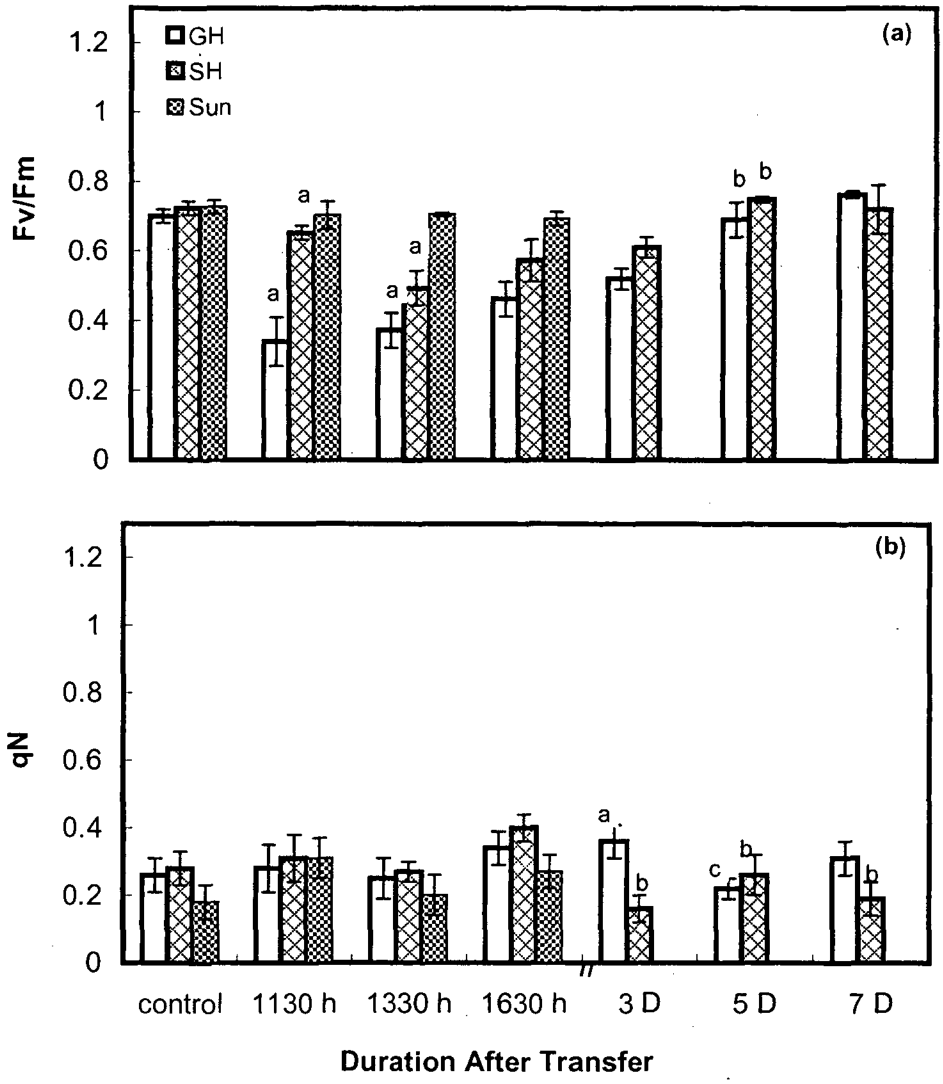


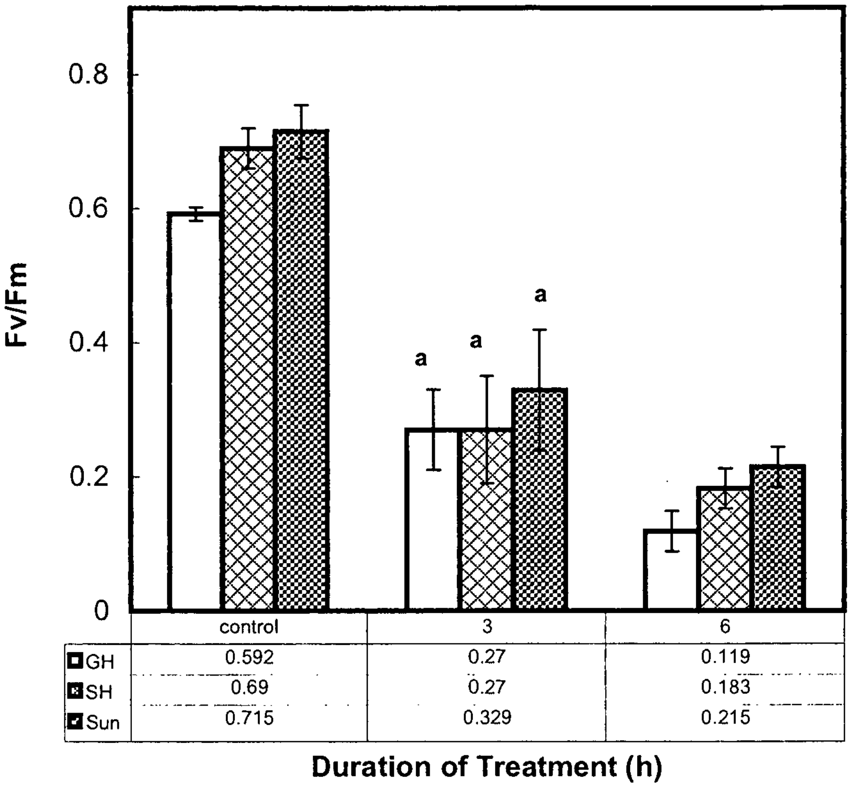
Figure 3.2: Comparison between the effect of sunlight on the (a) photochemical efficiency of PS II (Fv/Fm) and (b) non-photochemical quenching (qN) in thirty days old rice plants grown in the glasshouse (GH), shade (SH) and direct sunlight (Sun). In glasshouse and shade grown plants control refers to plants prior to transfer to direct sunlight and in sun plants control refers to maesurements taken at predawn (0630 h). (n=3, ± SD). a - significantly different from control; b - significantly different from 1630 h; c - significantly different from 3 D ($p < 0.05$).

increased in both glasshouse and shade grown plants, but increase was greater in glasshouse grown plants than seen in shade grown plants. While in sun grown plants qP showed a small increase in the morning and a slight decline at the end of the day. Non-photochemical quenching of glasshouse and shade grown plants prior to exposure to sunlight exceeded that of predawn sun grown plants (Fig. 3.2b). qN increased in glasshouse and shade grown plants after one day of exposure to direct sunlight, however, further exposure up to seven days resulted in a decline in qN. Predawn sun grown plants exhibited lower (0.18) qN than glasshouse (0.26) and shade (0.28) grown plants prior to transfer to direct sunlight. However, at 1630 h qN was lower in sun grown plants (0.27) as compared to glasshouse (0.34) and shade (0.40) grown plants (3.2b).

3.2. Effect of artificial high light on Fv/Fm under *in vitro* conditions

This experiment was conducted with an objective to compare the extent of damage and recovery under *in vivo*, where cellular recovery processes will be accompanying the photoinhibitory damage, and *in vitro* conditions, where no cellular recovery will be observed.

Figure 3.3 shows the effect of artificial high light (1200 μmol $m^{-2}s^{-2}$) at 30°C, on the Fv/Fm ratio of chloroplasts isolated from plants grown under the different growth conditions. Chloroplasts isolated from glasshouse grown plants exhibited lowest Fv/Fm ratio (0.592) while chloroplasts isolated from sun grown plants exhibited highest Fv/Fm ratio (0.715). High light treatment of isolated chloroplasts from all the three different growth conditions resulted in a considerable decline in the Fv/Fm ratio. Three hours of photoinhibition resulted in a decline in Fv/Fm ratio of 54%, 60% and 53% in chloroplasts isolated from glasshouse, shade and sun grown



	control	3	6
☐GH	0.592	0.27	0.119
▩SH	0.69	0.27	0.183
▨Sun	0.715	0.329	0.215

Figure 3.3: Effect of artificial high light treatment (1200 µmol $m^{-2}s^{-1}$) on photochemical efficiency (Fv/Fm) of chloroplasts isolated from thirty days old rice plants grown in the glasshouse (GH), shade (SH) and direct sunlight (Sun). Control refers to chloroplasts not treated with high light. (n=3, ± SD). a - significantly different from control.

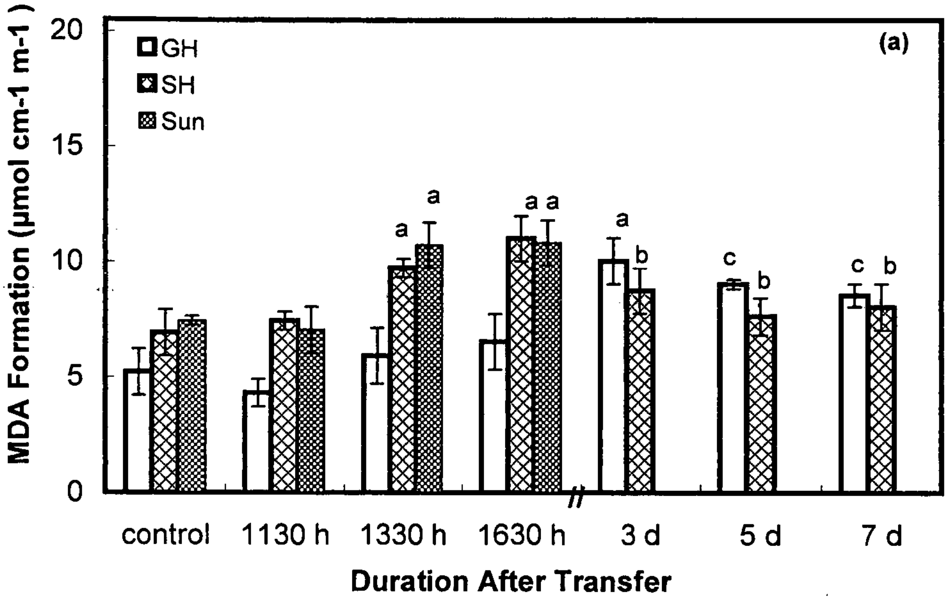
plants respectively, as compared to their respective control. Six hours of high light treatment led to a further decline in the Fv/Fm ratio, which was greater in chloroplasts isolated from glasshouse grown plants (80%), than seen in chloroplasts isolated from shade (73%) and sun (70%) grown plants, as compared to their respective control.

3.3.1. Effect of sunlight on lipid peroxidation of cell membrane under *in vivo* conditions

Lipid peroxidation which is an indicator of oxidative stress in cells was studied by determining thiobarbituric-malonaldehyde (TBA-MDA) adduct formation, which signify the formation of polyunsaturated fatty acid peroxides in biological systems.

Figure 3.4a shows the relative increase in lipid peroxidation when plants were transferred from low light to high light. Transfer of glasshouse grown plants to direct sunlight for 1 h (up to 1130 h) resulted in an initial decline of 17% in the MDA content and further exposure to sunlight up to 1630 h resulted in a linear increase. Three days of exposure to direct sunlight resulted in a further increase in the MDA content and was 92% higher than that of control plants (i.e. prior to transfer to direct sunlight). Prolonged exposure to sunlight till seven days led to a small decline in the amount of lipid peroxidation as compared to that observed at the end of the third day, but peroxidation level was still 63% higher than the control plants.

Shade grown plants also exhibited a linear increase in the MDA content on transferring the plants to direct sunlight. Peroxidation level increased from 7% seen at 1130 h to 59% observed at 1630 h as compared to control (i.e. prior to transfer to direct sunlight; Fig. 3.4a). Three days of exposure to direct sunlight resulted in 20% decline as compared to day one after exposure. Prolonged exposure to sunlight up to seven days did not show further increase in the level of peroxidation but resulted in



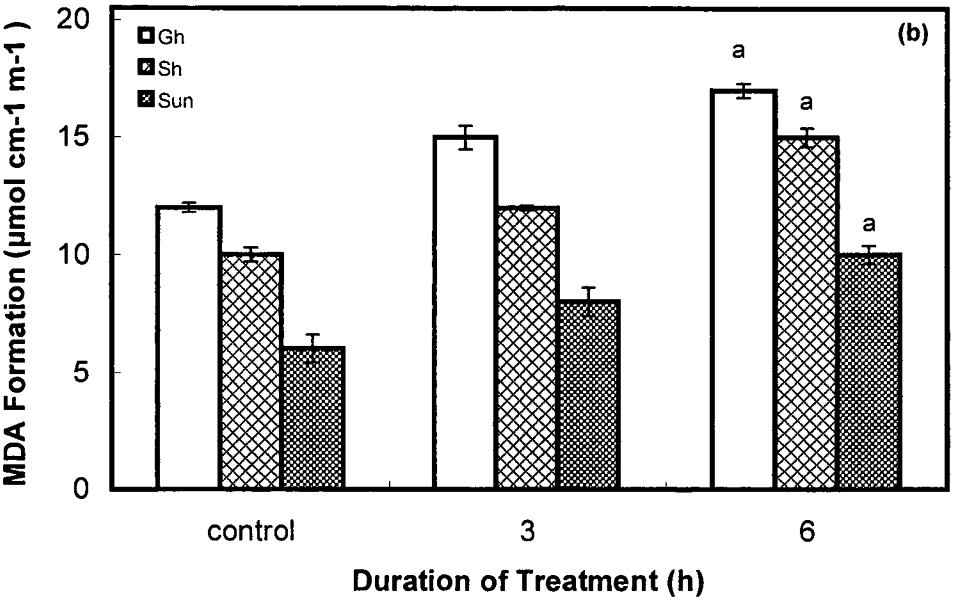


Figure 3.4:

(a) Effect of sunlight on MDA-TBA adduct formation (µmol $m^{-2}s^{-1}$) in thirty days old rice plants grown in glasshouse (GH), shade (SH) and direct sunlight (Sun). In glasshouse and shade grown plants control refers to plants prior to transfer to direct sunlight and in sun grown plants control refers to measurements made at predawn (0630 h).

(b) Effect of artificial high light treatment (1200 µmol m^{-2} s^{-1}) on the MDA-TBA adduct formation (µmol m^{-2} s^{-1}) in chloroplasts isolated from glasshouse (GH), shade (SH) and sun grown plants. Control refers to chloroplasts not treated with high light. (n=3, ± SD). a - significantly different from control; b - significantly different from 1630 h; c - significantly different from 3 D ($p < 0.05$).

Table 3.2: Effect of sunlight on MDA-TBA adduct formation (μmol $cm^{-1}m^{-1}$) in glasshouse, shade and sun grown plants. In glasshouse and shade plants control refers to plants prior to transfer to direct sunlight while in sun plants control refers to measurements taken at predawn (0630 h). In glasshouse and shade plants data has been shown till seven days while in sun plants data during the course of a day has been shown. (n=3, ± SD)

MDA-TBA adduct formation (μmol $cm^{-1}m^{-1}$)			
Treatment	**Glasshouse**	**Shade**	**Sun**
Control	5.2 ± 1.0	6.9 ± 1.0	7.4 ± 0.2
1130 h	4.3 ± 0.6	7.4 ± 0.4	7.0 ± 1.0
1330 h	5.9 ± 1.2	9.7 ± 0.4	10 ± 1.0
1630 h	6.5 ± 1.2	11 ± 1.0	10 ± 1.0
3 D	10 ± 1.0	8.7 ± 1.0	-
5 D	9.0 ± 0.2	7.6 ± 0.8	-
7 D	8.5 ± 0.5	7.9 ± 1.0	-

Table 3.3: Effect of artificial high light (1200 μmol $m^{-2}s^{-1}$; 30°C) treatment on MDA-TBA adduct formation (μmol $cm^{-2}s^{-1}$) in chloroplasts isolated from glasshouse, shade (prior to transfer to direct sunlight) and sun (predawn) grown plants. Control refers to chloroplasts not treated with high light. (n=3, ± SD)

MDA-TBA adduct formation (μmol $cm^{-1}m^{-1}$)			
Treatment	**Glasshouse**	**Shade**	**Sun**
Control	12 ± 0.2	10 ± 0.3	6 ± 0.6
3 h	15 ± 0.5	12± 0.1	8 ± 0.6
6 h	17 ± 0.3	15 ± 0.4	10 ± 0.4

8% decline as compared to day three after exposure. However, the level of lipid peroxidation remained comparatively higher (15%) than the control plants. Sun grown plants showed higher level of MDA content even in control plants (i.e. predawn), and the level of peroxidation linearly increased during the course of the day (Fig. 3.4a).

Comparison showed that, glasshouse plants prior to transfer to direct sunlight (control plants) had lower MDA content (5.2 μmol $cm^{-1}m^{-1}$) than control shade (6.9 μmol $cm^{-1}m^{-1}$) and control sun (i.e. predawn; 7.4 μmol $cm^{-1}m^{-1}$) grown plants (Table 3.2). On day one after transfer to direct sunlight glasshouse grown plants exhibited lower level of lipid peroxidation as compared to shade and sun grown plants. However, prolonged exposure up to seven days resulted in higher level of peroxidation in glasshouse grown plants than shade grown plants. In shade and sun grown plants the increase in the lipid peroxidation level after one day was more or less similar.

3.3.2. Effect of artificial high light on peroxidation of thylakoid membrane lipids under *in vitro* conditions

Figure 3.4b shows the effect of artificial high light (1200 μmol $m^{-2}s^{-2}$) at 30°C, on the peroxidation of thylakoid membrane lipids of chloroplasts isolated from plants grown under the different growth conditions. Level of lipid peroxidation was highest in chloroplasts isolated from glasshouse and lowest in chloroplasts isolated from sun grown plants. Three hours of high light treatment of chloroplast isolated from glasshouse grown plants resulted in 25% increase in the amount of MDA formation, while longer duration of high light treatment till 6 h increased the peroxidation level to 41% increase, as compared to control. In chloroplasts isolated from shade grown plants, 3 h of high light treatment resulted in 20% higher MDA formation as compared to control and further high light treatment for 6 h resulted in an increase in MDA formation by 50%. While in chloroplasts isolated from sun grown plants, the level of lipid peroxidation increased by 33% and 66% after 3 and 6 h of high light treatment respectively, as compared to their control.

3.4. Effect of sunlight on total soluble and insoluble carbohydrates

Transfer of glasshouse grown plants to direct sunlight led to a linear increase in the amount of total soluble carbohydrates (Fig. 3.5a). After one day of exposure to direct sunlight (up to 1630 h) the total soluble carbohydrates increased by 47% and at the end of the seven day period it was 376% higher than control plants (i.e. prior to transfer to direct sunlight). The amount of total insoluble carbohydrates also increased on exposure to sunlight. The sunlight treatment of glasshouse grown plants up to 1630 h caused an increase of 180% in the amount of total insoluble carbohydrates as compared to control plants. However, three days of exposure to sunlight resulted in 14% decrease in the amount of total insoluble carbohydrates as compared to that of first day. While, prolonged exposure for seven days resulted in 25% increase in the concentration of total insoluble carbohydrates, as compared to that of third day.

In response to exposure to direct sunlight, shade grown plants exhibited a linear increase in the concentration of total soluble carbohydrates and after five days of the treatment it increased by 71% as compared to the control plants. Further exposure to sunlight till seven days resulted in a decline of 20% as compared to that of fifth day (Fig. 3.5b). The concentration of total insoluble carbohydrates also increased (166%) on exposure to sunlight till 1630 h (6 h of treatment). Further exposure to sunlight till three days resulted in a 37% decline as compared to that of one day and was more or less constant up to seven days of treatment.

Figure 3.5: Effect of sunlight on the concentration (mg/g) of total soluble and insoluble carbohydrates in thirty days old rice plants grown in the (a) glasshouse, (b) shade and (c) direct sunlight. In glasshouse and shade grown plants control refers to plants prior to transfer to direct sunlight and in sun grown plants control refers to measurements made at predawn (0630 h). (n=3, ± SD). a - significantly different from control ($p < 0.05$).

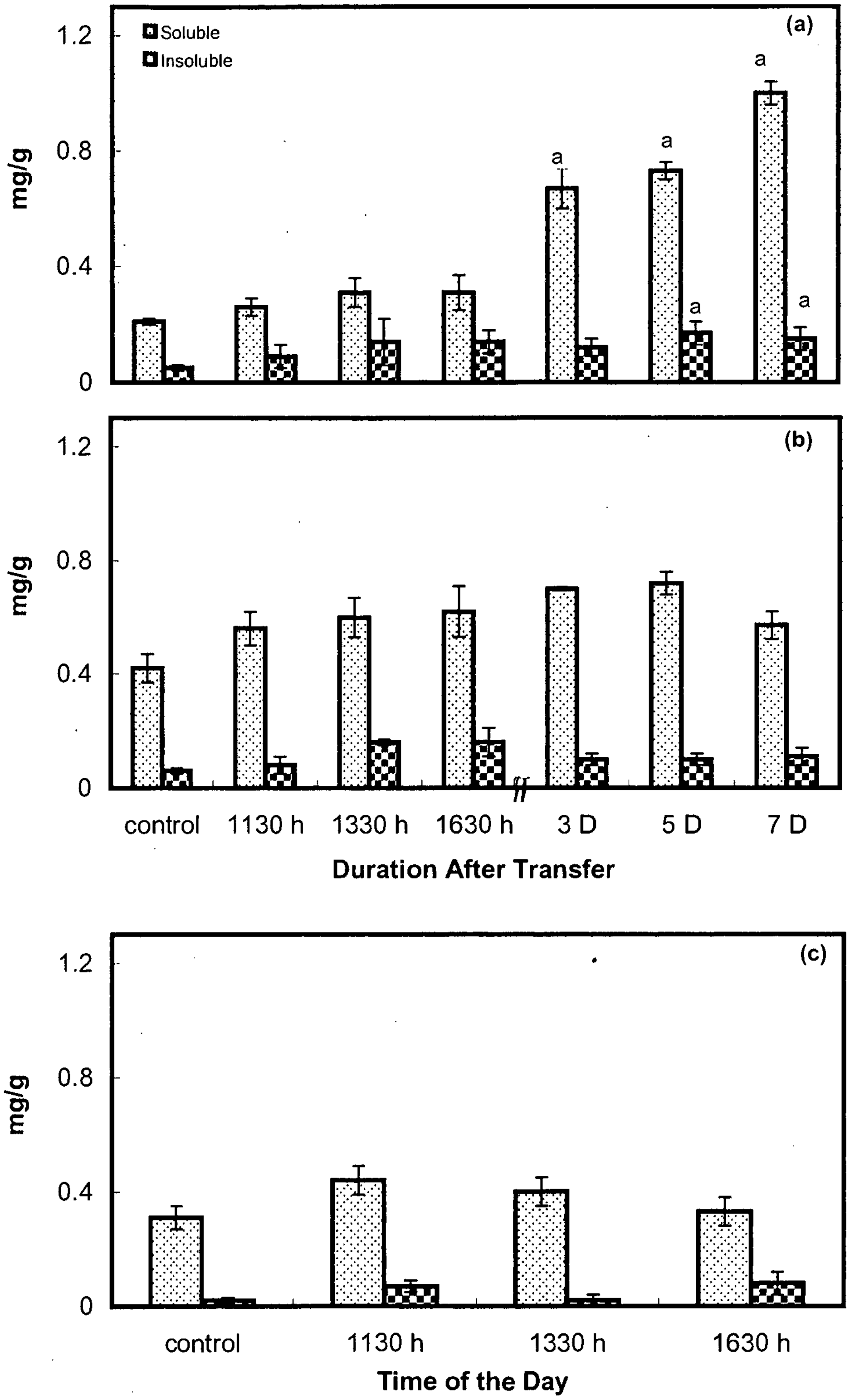

(a)
Soluble
Insoluble
mg/g
1.2
0.8
0.4
0
a
a
a
a
a
(b)
mg/g
1.2
0.8
0.4
0
control
1130 h
1330 h
1630 h
3 D
5 D
7 D
Duration After Transfer
(c)
mg/g
1.2
0.8
0.4
0
control
1130 h
1330 h
1630 h
Time of the Day

Table 3.4: Effect of sunlight on the concentration and ratio of total soluble and insoluble carbohydrates in glasshouse, shade and sun grown plants. In glasshouse and shade plants control refers to plants prior to transfer to direct sunlight while in sun plants control refers to measurements taken at predawn (0630 h). In glasshouse and shade plants data has been shown till seven days while in sun plants data during the course of a day has been shown. (n=3, ± SD)

Treatment	**Soluble (mg/g FW)**	**Insoluble (mg/g FW)**	**Ratio**
Glasshouse			
Control	0.21 ± 0.01	0.05 ± 0.01	4.2
1130 h	0.26 ± 0.03	0.09 ± 0.04	2.8
1330 h	0.31 ± 0.05	0.14 ± 0.08	2.2
1630 h	0.31 ± 0.06	0.14 ± 0.04	2.2
3 D	0.67 ± 0.07	0.12 ± 0.03	2.2
5 D	0.73 ± 0.03	0.17 ± 0.04	4.3
7 D	1.00 ± 0.04	0.15 ± 0.04	6.6
Shade			
Control	0.42 ± 0.05	0.06 ± 0.01	7
1130 h	0.56 ± 0.06	0.08 ± 0.03	7
1330 h	0.60 ± 0.07	0.16 ± 0.01	3.7
1630 h	0.62 ± 0.09	0.16 ± 0.06	3.8
3 D	0.70 ± 0.05	0.10 ± 0.02	7
5 D	0.72 ± 0.04	0.10 ± 0.02	7.2
7 D	0.57 ± 0.05	0.11 ± 0.03	5.2
Sun			
Control	0.31 ± 0.04	0.02 ± 0.01	15
1130 h	0.44 ± 0.05	0.07 ± 0.02	6.2
1330 h	0.40 ± 0.05	0.02 ± 0.01	20
1630 h	0.33 ± 0.05	0.08 ± 0.04	4

In sun grown plants the concentration of total soluble carbohydrates increased initially by 41% at 1130 h as compared to control plants (i.e. predawn), however, at 1630 h sun grown plants exhibited a small decrease as compared to 1130 h and was almost similar to that of control plants (Fig. 3.5c). The concentration of total insoluble carbohydrates was very low in control plants (0.02 mg/g) and it increased at 1630 h by 4 times (0.08 mg/g).

In comparison, glasshouse plants prior to transfer to direct sunlight (control plants) had the least (0.21 mg/g) concentration of total soluble carbohydrates followed by sun grown plants (0.31 mg/g), while shade grown plants had the highest concentration (0.42 mg/g) of total soluble carbohydrates (Table 3.4). Transfer of glasshouse, shade and sun grown plants to direct sunlight caused an increase in the concentration of soluble carbohydrates and highest increase was exhibited by glasshouse grown plants (376%) after seven days, than 35% increase in shade and 6% increase in sun grown plants as compared to their respective control plants.

In control glasshouse and shade grown plants the concentration of total insoluble carbohydrates was more or less same (0.05-0.06 mg/g). Exposure of glasshouse and shade grown plants to direct sunlight for a period of seven days resulted in an increase in total insoluble sugars. The increase was slightly higher in glasshouse grown plants than observed in shade grown plants (Table 3.4). Sun grown plants exhibited the least amount of total insoluble carbohydrates as compared to glasshouse and shade grown plants.

3.5.1. Effect of sunlight on the xanthophyll cycle

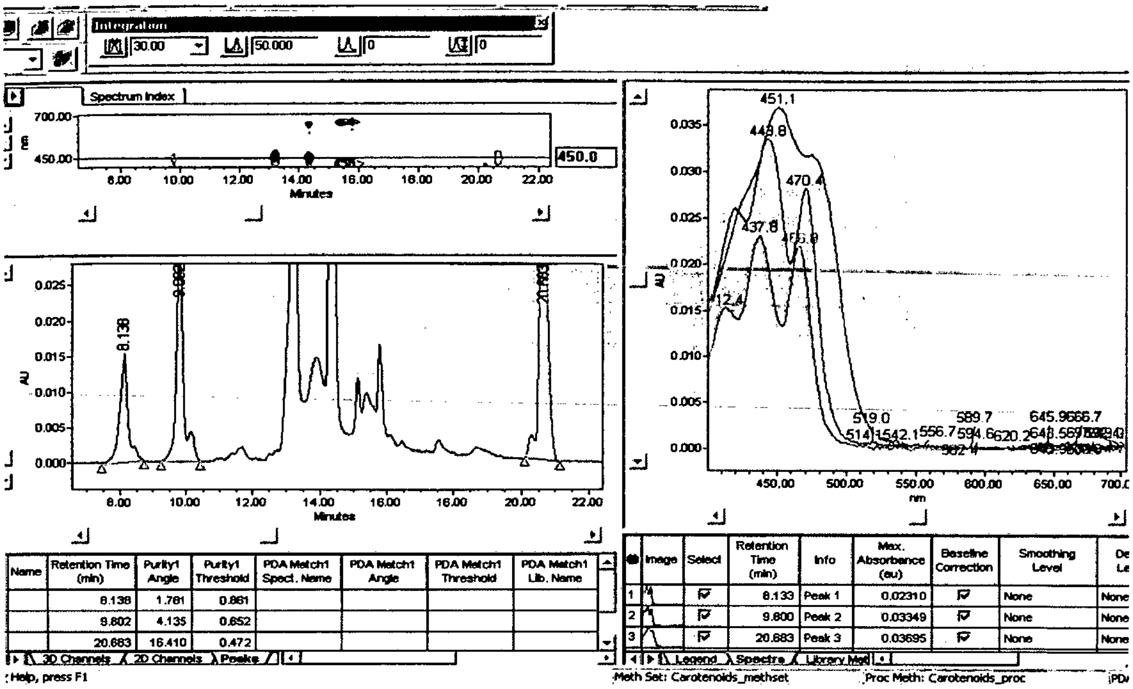
Changes in the xanthophyll pigments were studied to find out the extent of xanthophyll cycle-dependent energy dissipation in response to high light stress.

Table 3.5 shows pigment content in glasshouse, shade and sun grown plants in response to exposure to direct sunlight. Analysis of photosynthetic pigments showed higher level of violaxanthin (V) and lower level of antheraxanthin (A) and absence of zeaxanthin (Z) in glasshouse and shade grown plants prior to transfer to direct sunlight and predawn sun grown plants. Transfer of all the three types of plants to sunlight led to a reduction in V and accumulation of A and Z (Table 3.5). In glasshouse and shade grown plants Z was seen only after 1330 h and was present throughout the duration of treatment (up to seven days), while in sun grown plants Z was observed at 1130 h (1 h of treatment) and continued to increase linearly till 1630 h (6 h of treatment; Table 3.5). In glasshouse grown plants highest concentration of Z was observed after five days of treatment (Table 3.5), while in shade grown plants it was seen after seven days (Table 3.5). The sum increase in A and Z was not proportional to the decrease observed in V. Glasshouse (8.09 mg/g) and shade (3.66 mg/g) grown plants prior to transfer to direct sunlight possessed a lower pool of xanthophyll pigments than predawn sun plants (11.58 mg/g; Table 3.5).

The analysis of other pigments indicated that glasshouse and shade grown plants prior to transfer to direct sunlight had lower Chl *a/b* ratios and low levels of lutein compared to predawn sun grown plants (Table 3.5). There was relatively a small increase in Chl *a/b* ratio in glasshouse and shade grown plants on the fifth and seventh day after transfer to direct sunlight respectively. Similarly sun grown plants exposed to sunlight till 1630 h (6 h of treatment) exhibited higher Chl *a/b* ratios than that observed at predawn. Exposure of shade grown plants to sunlight for a day caused an increase in the lutein levels, however, no such increase in lutein was observed in glasshouse and sun grown plants (Table 3.5).

Table 3.5: Effect of sunlight on pigment content (mg/g FW) in glasshouse, shade and sun grown plants. In glasshouse and shade plants control refers to plants prior to transfer to direct sunlight while in sun plants control refers to measurements taken at predawn (0630 h). In glasshouse and shade plants data has been shown till seven days while in sun plants data during the course of a day has been shown. Chl *a/b* ratios were calculated based on the HPLC data. V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; L, lutein; Chl, chlorophyll (n=3, ± SD)

Treatment	V	A	Z	V+A+Z	**Lutein**	**Chl *a/b***
Glasshouse						
control	7.44 ± 2	0.65 ± 0.2	0	8.09 ± 1	20 ± 4	0.18 ± 0.03
1130h	0.45 ± 0.1	0.71 ± 0.4	0	1.16 ± 0.2	18 ± 2	0.12 ± 0.01
1330h	3.00 ± 0.5	1.67 ± 0.4	0.42 ± 0.07	5.09 ± 0.3	15 ± 3	0.20 ± 0.02
1630h	4.50 ± 1	2.00 ± 0.5	0.37 ± 0.01	6.87 ± 0.5	16 ± 2	0.18 ± 0.01
3 D	7.70 ± 2	1.06 ± 0.2	0.15 ± 0.02	8.91 ± 0.7	12 ± 1	0.19 ± 0.01
5 D	3.40 ± 0.5	2.34 ± 0.5	1.97 ± 0.09	7.71 ± 3	16 ± 2	0.26 ± 0.06
7 D	3.11 ± 1	1.91 ± 0.3	0.58 ± 0.2	5.60 ± 0.4	22 ± 3	0.16 ± 0.01
Shade						
control	3.00 ± 0.5	1.00 ± 0.1	0	4.00 ± 0.2	15 ± 1	0.20 ± 0.01
1130h	6.00 ± 0.7	1.20 ± 0.3	0	7.20 ± 0.5	34 ± 4	0.16 ± 0.01
1330h	2.40 ± 0.4	1.40 ± 0.2	0.33 ± 0.01	4.13 ± 0.2	20 ± 4	0.20 ± 0.02
1630h	3.00 ± 0.3	2.00 ± 0.4	1.30 ± 0.09	6.30 ± 0.2	32 ± 3	0.19 ± 0.01
3 D	3.60 ± 0.2	3.60 ± 0.4	1.17 ± 0.03	8.37 ± 0.2	24 ± 2	0.21 ± 0.03
5 D	2.16 ± 0.1	2.60 ± 0.1	0.87 ± 0.01	5.63 ± 0.7	22 ± 3	0.16 ± 0.01
7 D	0.50 ± 0.1	1.20 ± 0.2	1.56 ± 0.02	3.26 ± 0.1	12 ± 1	0.44 ± 0.03
Sun						
control	10.6 ± 1	0.98 ± 0.2	0	11.5 ± 0.6	32 ± 2	0.75 ± 0.03
1130h	6.21 ± 0.5	2.61 ± 0.1	0.2 ± 0.01	9.02 ± 0.2	26 ± 3	0.70 ± 0.02
1330h	6.27 ± 0.4	1.91 ± 0.1	0.6 ± 0.03	8.78 ± 0.1	19 ± 1	0.78 ± 0.02
1630h	4.97 ± 0.2	1.50 ± 0.1	1.0 ± 0.01	7.47 ± 0.1	14 ± 1	0.87 ± 0.03



Name	Retention Time (min)	Purity1 Angle	Purity1 Threshold	PDA Match1 Spect. Name	PDA Match1 Angle	PDA Match1 Threshold	PDA Match1 Lib. Name
	8.138	1.761	0.861				
	9.802	4.135	0.652				
	20.683	16.410	0.472				

	Image	Select	Retention Time (min)	Info	Max. Absorbance (au)	Baseline Correction	Smoothing Level	De Le
1		☑	8.133	Peak 1	0.02310	☑	None	None
2		☑	9.800	Peak 2	0.03349	☑	None	None
3		☑	20.683	Peak 3	0.03695	☑	None	None

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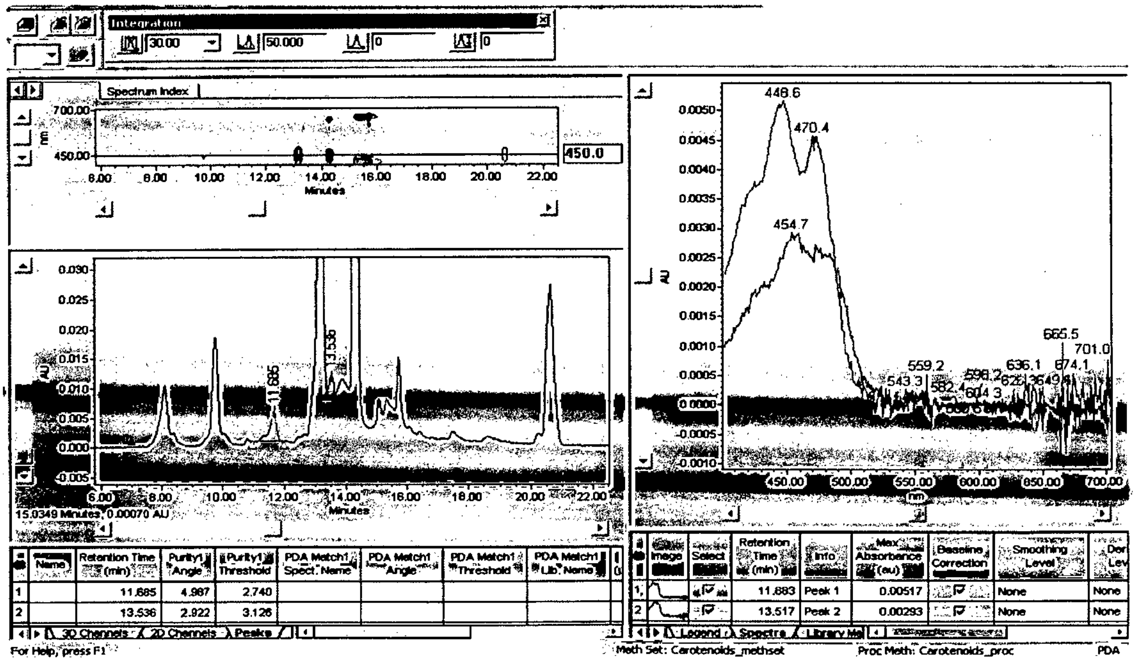


15.0349 Minutes, 0.00070 AU

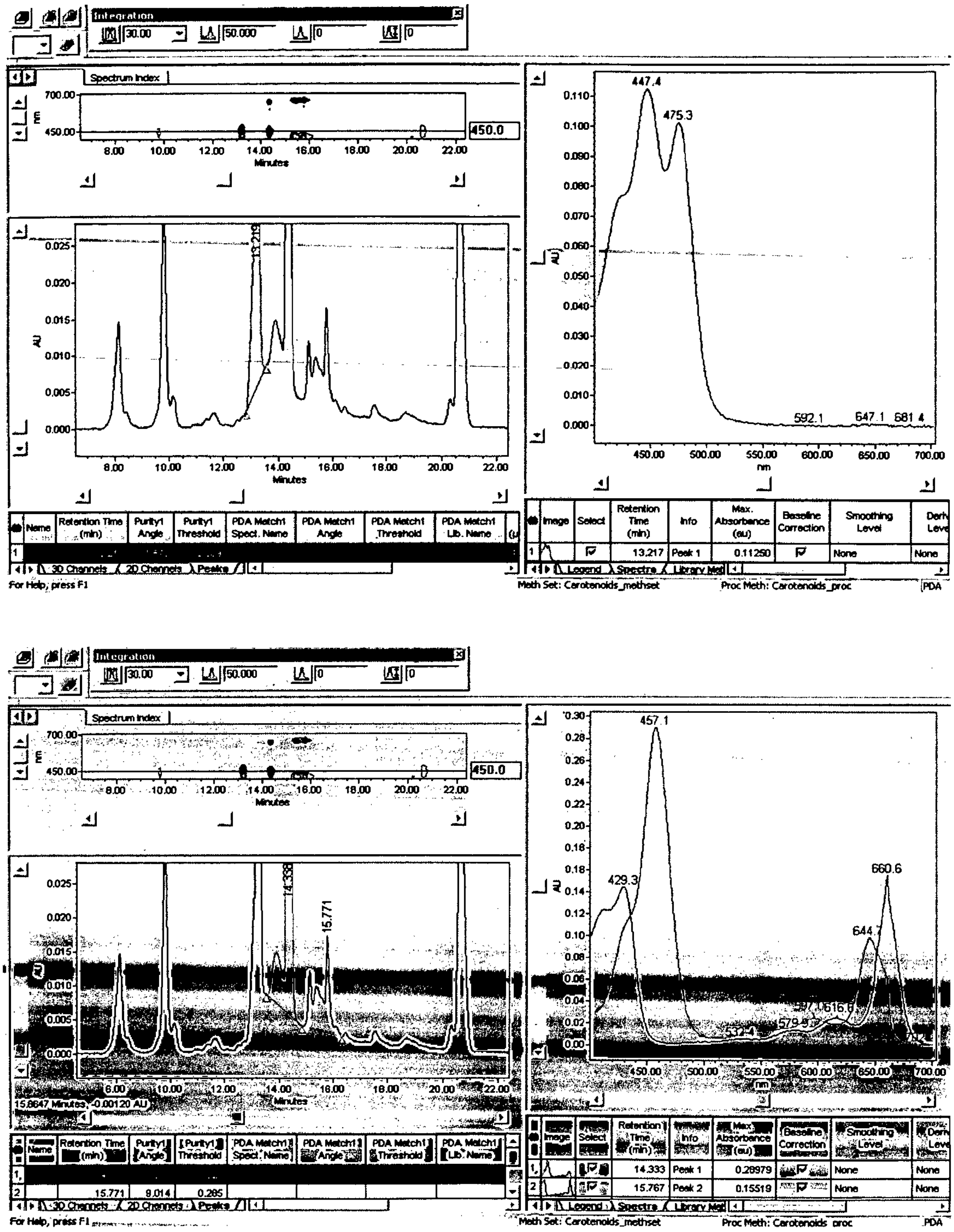
	Name	Retention Time (min)	Purity1 Angle	Purity1 Threshold	PDA Match1 Spect. Name	PDA Match1 Angle	PDA Match1 Threshold	PDA Match1 Lib. Name
1		11.685	4.967	2.740				
2		13.536	2.922	3.126				

	Image	Select	Retention Time (min)	Info	Max. Absorbance (au)	Baseline Correction	Smoothing Level	Der Lev
1		☑	11.683	Peak 1	0.00517	☑	None	None
2		☑	13.517	Peak 2	0.00293	☑	None	None

For Help, press F1

Meth Set: Carotenoids_methset Proc Meth: Carotenoids_proc PDA

Figure 3.6: HPLC profile of photosynthetic pigments at 450 nm and absorption spectra of peaks with retention time of 8.138 (neoxanthin), 9.802 (violaxanthin), 11.685 (antheraxanthin), 13.536 (zeaxanthin) and 20.683 (β-carotene) min.



	Name	Retention Time (min)	Purity1 Angle	Purity1 Threshold	PDA Match1 Spect. Name	PDA Match1 Angle	PDA Match1 Threshold	PDA Match1 Lib. Name
1								

	Image	Select	Retention Time (min)	Info	Max. Absorbance (au)	Baseline Correction	Smoothing Level	Deri Leve
1		☑	13.217	Peak 1	0.11250	☑	None	None

	Name	Retention Time (min)	Purity1 Angle	Purity1 Threshold	PDA Match1 Spect. Name	PDA Match1 Angle	PDA Match1 Threshold	PDA Match1 Lib. Name
1								
2		15.771	9.014	0.285				

	Image	Select	Retention Time (min)	Info	Max. Absorbance (au)	Baseline Correction	Smoothing Level	Deri Leve
1		☑	14.333	Peak 1	0.28979	☑	None	None
2		☑	15.767	Peak 2	0.15519	☑	None	None

Figure 3.7: HPLC profile of photosynthetic pigments at 450 nm and absorption spectra of peaks with retention time of 13.219 (lutein), 14.336 (Chl *b*) and 15.771 (Chl *a*) min.

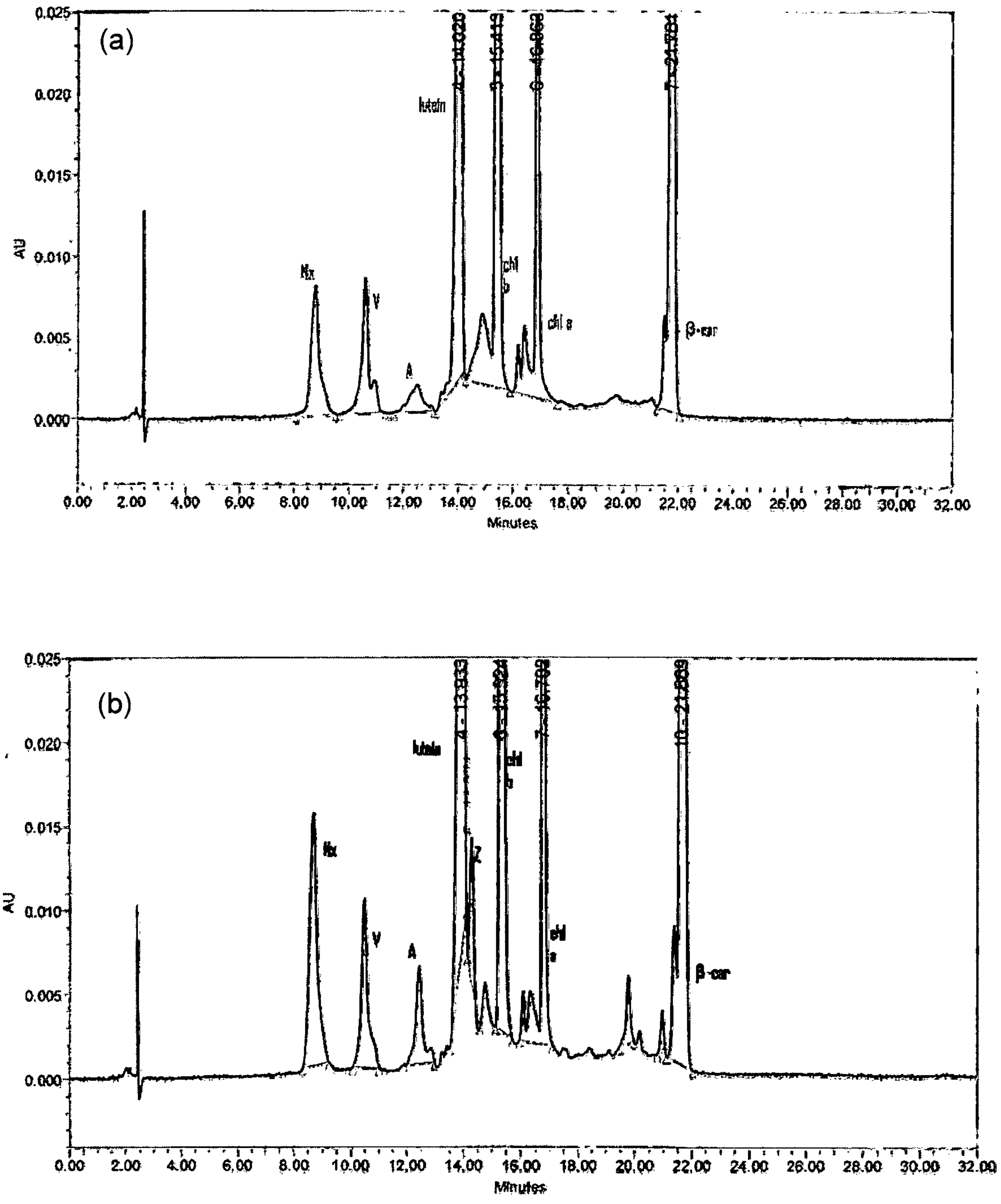
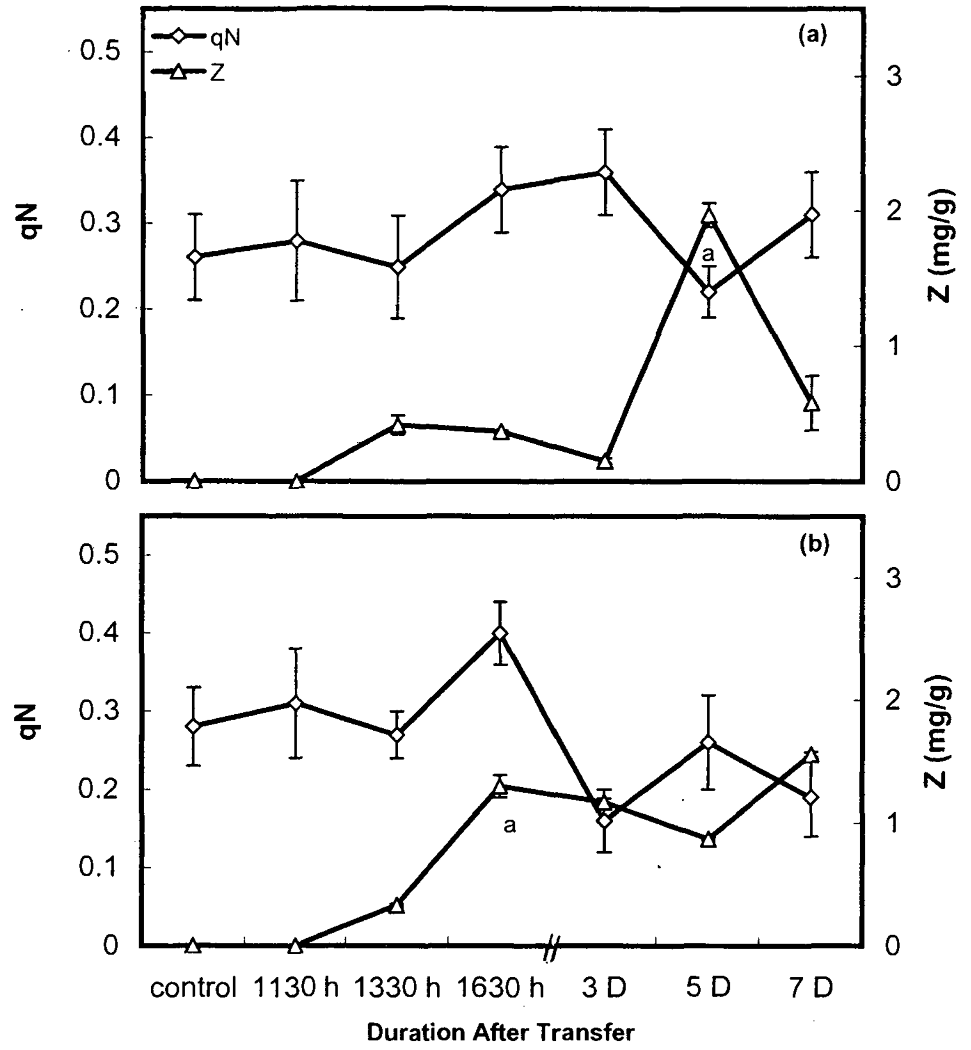
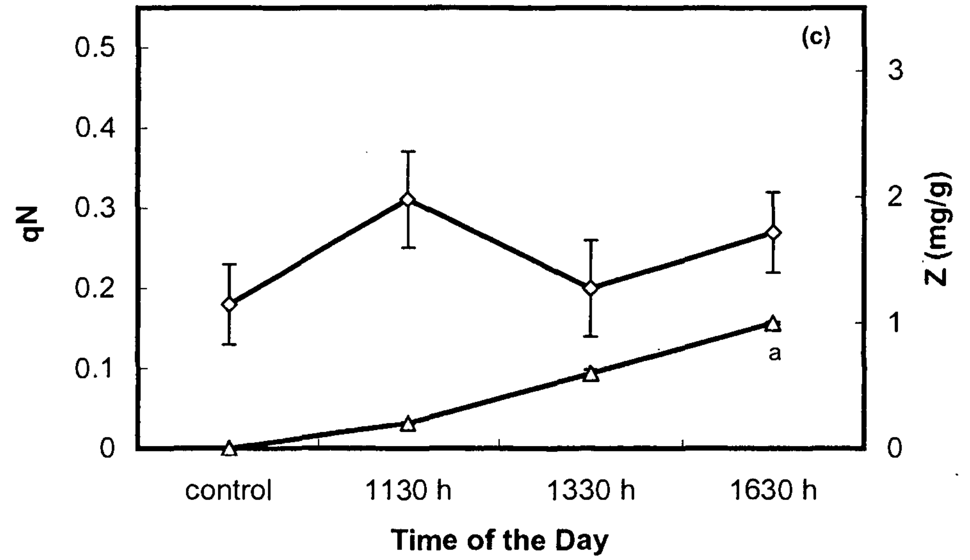


Figure 3.8: HPLC profile of photosynthetic pigments at 450 nm extracted from thirty days old glasshouse grown plants before (a) and after 6 h of exposure to direct sunlight (b).

Figure 3.9: Relationship between the non-photochemical quenching (qN) and zeaxanthin (Z) (mg/g) in thirty days old rice plants grown in the glasshouse (a), shade (b) and direct sunlight (c) and exposed to direct sunlight. In glasshouse and shade grown plants control refers to plants prior to transfer to direct sunlight and in sun grown plants control refers to measurements taken at predawn (0630 h). (n=3, ± SD). a - significantly different from control ($p < 0.05$).


qN
Z
(a)
(b)
qN
Z (mg/g)
0.5
0.4
0.3
0.2
0.1
0
3
2
1
0
a
a
control
1130 h
1330 h
1630 h
3 D
5 D
7 D
Duration After Transfer


(c)
qN
Z (mg/g)
0.5
0.4
0.3
0.2
0.1
0
3
2
1
0
a
control
1130 h
1330 h
1630 h
Time of the Day

3.5.2. Effect of sunlight on the VDE activity in isolated PS II particles

The present study was carried out to study the role of violaxanthin de-epoxidase (VDE) activity in the xanthophyll cycle of rice plants exposed to high light stress using isolated PS II particles containing LHC II.

The VDE activity increased in glasshouse, shade and sun grown plants after one day of sun exposure as compared to prior to transfer to direct sunlight. Results indicate highest activity of VDE refereed as de-epoxidation index (DEI), under both control and treated conditions in the glasshouse grown plants followed by the shade grown plants and least activity was observed in sun grown plants (Fig. 3.10). Transfer of glasshouse and shade grown plants to direct sunlight for a day resulted in a significant increase in the VDE activity as compared to their respective control plants (i.e. prior to transfer to direct sunlight).

3.6. Effect of sunlight on the chlorophyll fluorescence and xanthophyll cycle in plants fed with ascorbate (AsA) and dithiothreitol (DTT) through cut petioles

In the present study rice leaves were fed either with DTT, in order to inhibit de-epoxidation of V to Z, or with ascorbate, to stimulate the de-epoxidation of V to Z, to study the impact of inhibition and stimulation of xanthophyll cycle and its relation with energy dissipation (measured as qN) under high light conditions. Plants fed with AsA and DTT were compared with plants fed with water as control under growth condition.

3.6.1. Glasshouse grown plants

Figure 3.11a depicts the changes in the Fv/Fm ratio, qN and sum of A and Z of glasshouse grown rice plants fed with AsA in response to exposure to direct sunlight.

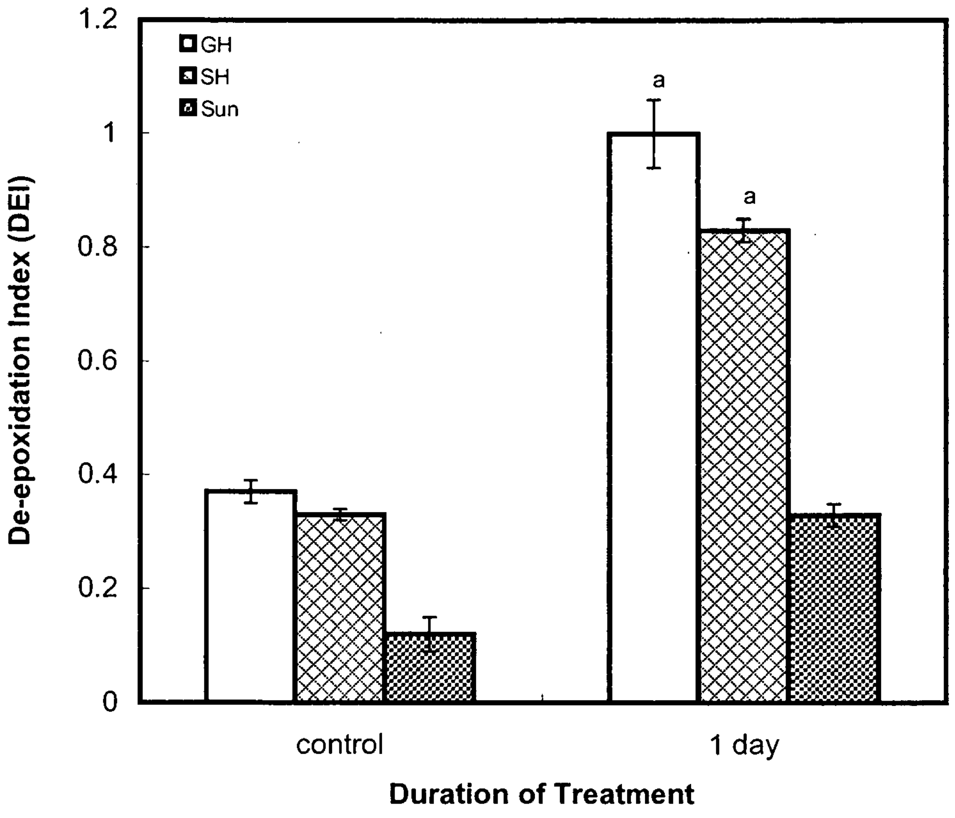


Figure 3.10: Effect of sunlight on the violaxanthin de-epoxidase activity measured as DEI, in PS II particles isolated from thirty days old control and one day sun exposed rice plants grown in glasshouse (GH), shade (SH) and direct sunlight (Sun). In glasshouse and shade grown plants control refers to plants prior to transfer to direct sunlight and in sun grown plants control refers to predawn (0630 h). (n=3, ± SD). a - significantly different from control ($p < 0.05$)

Table 3.6: Effect of sunlight on the xanthophyll cycle pigment content (%) and de-epoxidation index (DEI) in reactions carried out with PS II particles isolated from glasshouse, shade and sun grown plants. In glasshouse and shade plants control refers to plants prior to transfer to direct sunlight while in sun plants control refers to measurements taken at predawn (0630 h). V, violaxanthin; A, antheraxanthin; Z, zeaxanthin. (n=3, ± SD)

	V	**A**	**Z**	**V+A+Z**	**DEI**
Glasshouse					
Control	5 ± 0.2	3 ± 0.4	0	8 ± 0.5	0.37 ± 0.02
1 day	4 ± 0.1	2 ± 0.1	4 ± 0.2	10 ± 1	1.0 ± 0.06
Shade					
Control	3 ± 0.3	0.25 ± 0.03	0.5 ± 0.01	3.75 ± 0.4	0.33 ± 0.01
1 day	3 ± 0.1	1.00 ± 0.05	2 ± 0.2	6 ± 0.7	0.83 ± 0.02
Sun					
Control	7 ± 0.3	1 ± 0.02	0	8 ± 1	0.12 ± 0.03
1 day	6 ± 0.3	3 ± 0.1	0	9 ± 0.9	0.33 ± 0.02

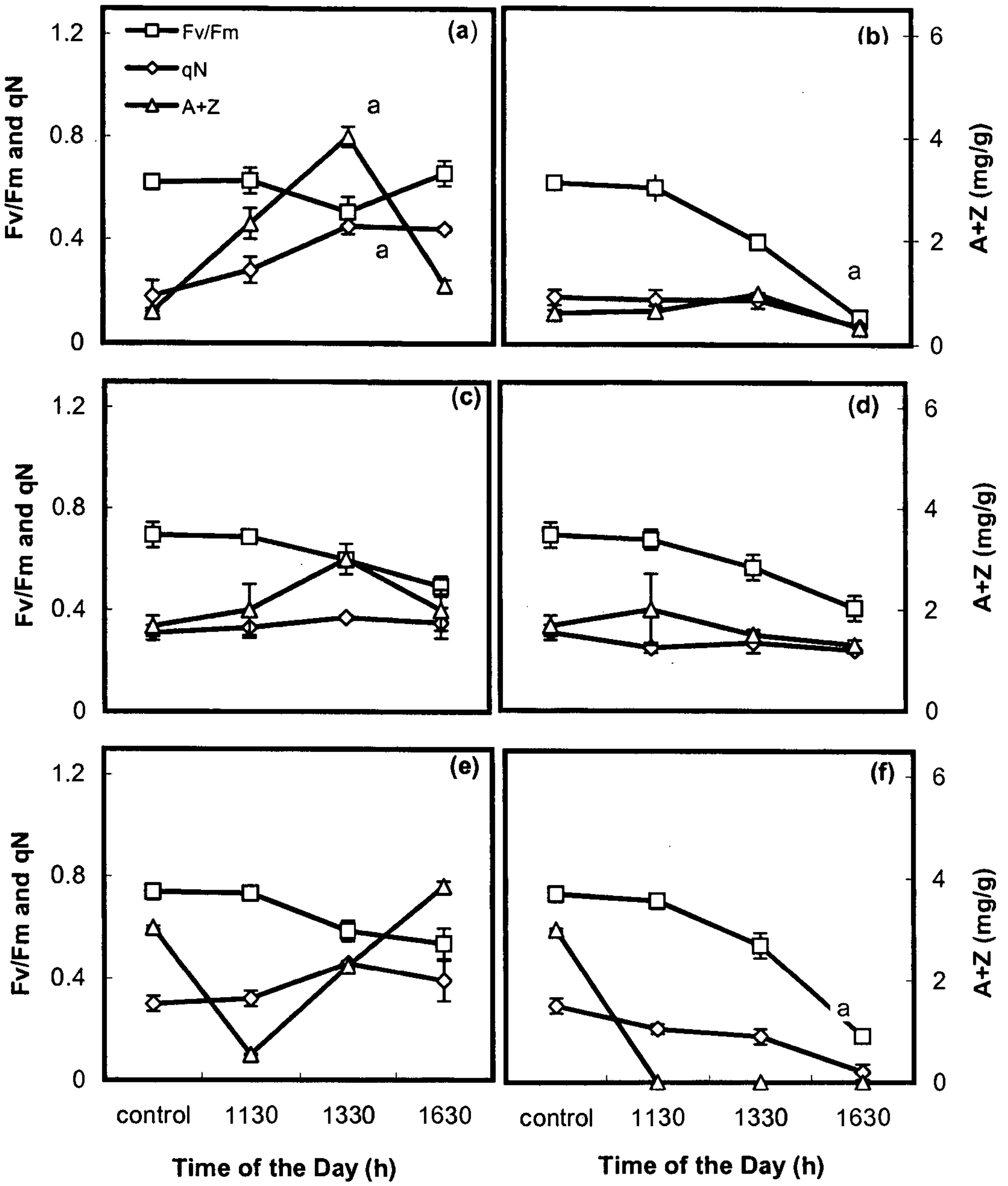


Figure 3.11: Effect of direct sunlight on the photochemical efficiency of PS II (Fv/Fm) and non-photochemical quenching (qN) and the sum of antheraxantnin and zeaxanthin (A+Z) in thirty days old rice plants grown in the glasshouse fed with ascorbic acid (a), DTT (b); shade (c and d) and direct sunlight (e and f). Control refers to plants fed with water for 6 h under growth conditions. (n=3, ± SD). a - significantly different from control ($p < 0.05$).

Figure 3.12: Comparison between the effect of sunlight on the photochemical efficiency of PS II (Fv/Fm), non-photochemical quenching (qN) and sum of A and Z in glasshouse (a, b, c), shade (d, e, f,) and sun (g, h, i) grown plants fed with ascorbic acid and DTT through cut petioles. (n=3, ± SD). a - significantly different from ascorbic acid fed plants ($p < 0.05$).

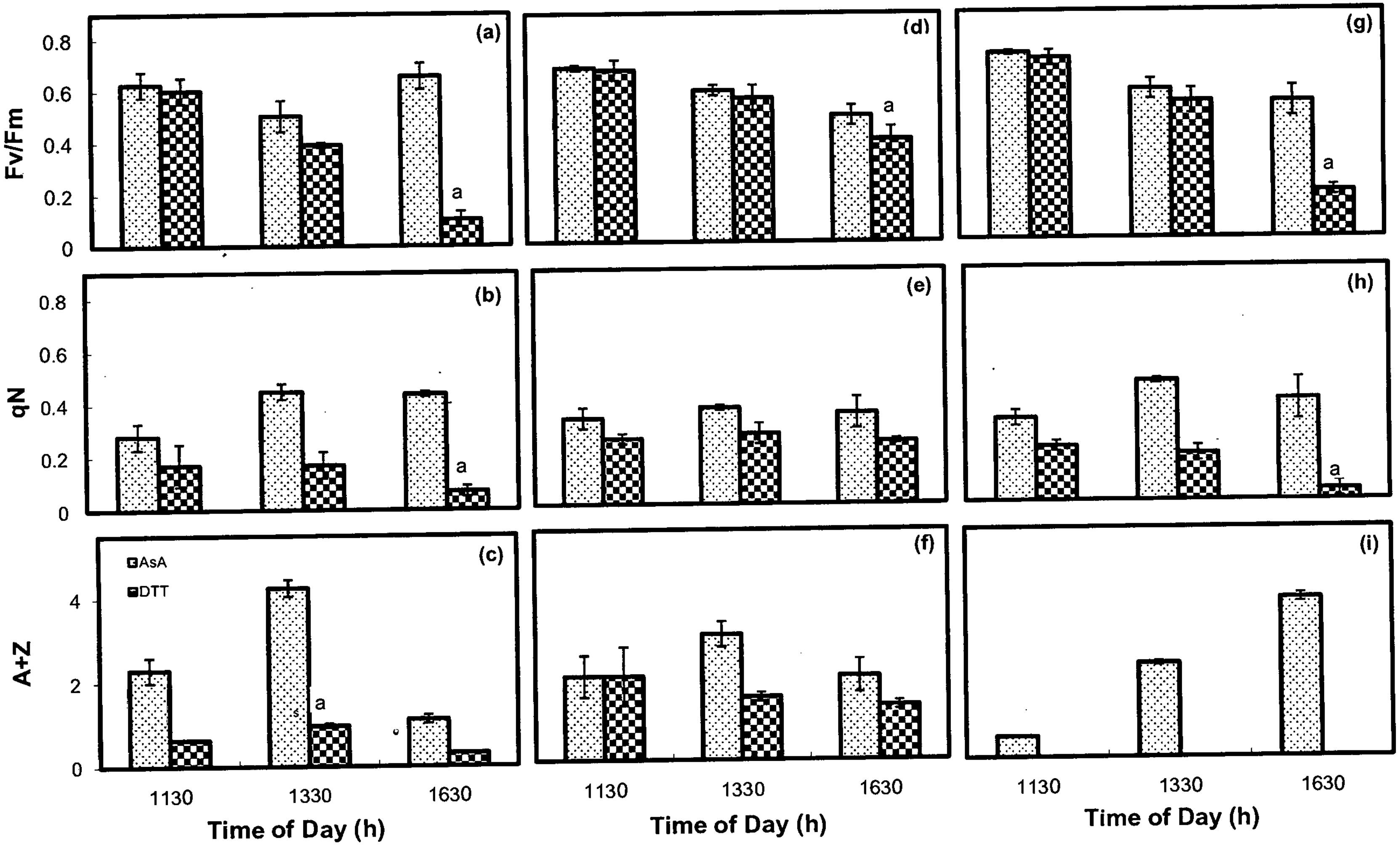

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Fv/Fm
qN
A+Z
0.8
0.6
0.4
0.2
0
4
2
AsA
DTT
a
1130
1330
1630
Time of Day (h)

Table 3.7: Effect of sunlight on the photochemical efficiency (Fv/Fm), photochemical quenching (qP) and non-photochemical quenching (qN) in glasshouse, shade and sun grown plants fed with ascorbic acid and DTT through cut petioles. Control refers to plants fed with water for 6 h under growth conditions. (n=3, ± SD)

Treatment	**Photosynthetic Efficiency (Fv/Fm)**	**Photochemical Quenching (qP)**	**Non-photochemical Quenching (qN)**
Glasshouse			
Control	0.621 ± 0.03	0.85 ± 0.04	0.18 ± 0.03
Ascorbic acid			
1130 h	0.627 ± 0.05	0.87 ± 0.07	0.28 ± 0.05
1330 h	0.505 ± 0.06	0.58 ± 0.07	0.45 ± 0.03
1630 h	0.658 ± 0.05	0.87 ± 0.01	0.44 ± 0.01
DTT			
1130 h	0.603 ± 0.05	0.83 ± 0.07	0.17 ± 0.08
1330 h	0.393 ± 0.01	0.50 ± 0.02	0.22 ± 0.05
1630 h	0.136 ± 0.03	0.43 ± 0.05	0.21 ± 0.02
Shade			
Control	0.694 ± 0.05	0.77 ± 0.04	0.31 ± 0.03
Ascorbic acid			
1130 h	0.686 ± 0.01	0.79 ± 0.03	0.33 ± 0.04
1330 h	0.596 ± 0.02	0.56 ± 0.03	0.37 ± 0.01
1630 h	0.496 ± 0.04	0.78 ± 0.02	0.35 ± 0.06
DTT			
1130 h	0.676 ± 0.04	0.98 ± 0.04	0.25 ± 0.02
1330 h	0.566 ± 0.05	0.58 ± 0.03	0.27 ± 0.04
1630 h	0.402 ± 0.05	0.57 ± 0.01	0.24 ± 0.01
Sun			
Control	0.739 ± 0.03	0.91 ± 0.04	0.30 ± 0.03
Ascorbic acid			
1130 h	0.734 ± 0.01	0.87 ± 0.05	0.32 ± 0.03
1330 h	0.587 ± 0.04	0.77 ± 0.05	0.46 ± 0.01
1630 h	0.537 ± 0.06	0.90 ± 0.04	0.39 ± 0.08
DTT			
1130 h	0.713 ± 0.03	0.95 ± 0.04	0.21 ± 0.02
1330 h	0.539 ± 0.05	0.76 ± 0.02	0.18 ± 0.03
1630 h	0.328 ± 0.02	0.54 ± 0.06	0.12 ± 0.03

Table 3.8: Effect of sunlight on the pigment content (mg/g FW) in glasshouse, shade and sun grown plants fed with ascorbic acid and DTT through cut petioles. Control refers to plants fed with water for 6 h under growth conditions. V, violaxanthin; A, antheraxanthin; Z, zeaxanthin. (n=3, ± SD)

Treatment	**V**	**A**	**Z**	**A+Z**
Glasshouse				
Control	0.92 ± 0.01	0.60 ± 0.06	0	0.60 ± 0.06
Ascorbic acid				
1130 h	4.6 ± 0.1	1.54 ± 0.04	0.76 ± 0.02	2.30 ± 0.3
1330 h	3.5 ± 0.3	1.56 ± 0.02	2.70 ± 0.06	4.26 ± 0.2
1630 h	1.5 ± 0.05	1.12 ± 0.01	0	1.12 ± 0.1
DTT				
1130 h	3.4 ± 0.2	0.64 ± 0.01	0	0.64 ± 0.01
1330 h	5.8 ± 0.5	0.97 ± 0.03	0	0.97 ± 0.05
1630 h	3.7 ± 0.4	0.32 ± 0.01	0	0.32 ± 0.01
Shade				
Control	2.0 ± 0.1	1.68 ± 0.02	0	1.68 ± 0.2
Ascorbic acid				
1130 h	4.0 ± 1	2.0 ± 0.05	0	2.0 ± 0.5
1330 h	3.0 ± 0.3	3.0 ± 0.03	0	3.0 ± 0.3
1630 h	2.0 ± 0.1	2.0 ± 0.04	0	2.0 ± 0.4
DTT				
1130 h	7.0 ± 2	2.0 ± 0.07	0	2.0 ± 0.7
1330 h	6.0 ± 1	1.5 ± 0.01	0	1.5 ± 0.1
1630 h	6.4 ± 2	1.3 ± 0.01	0	1.3 ± 0.1
Sun				
Control	2.1 ± 0.5	3.0 ± 0.03	0	3.0 ± 0.3
Ascorbic acid				
1130 h	1.0 ± 0.05	0.5 ± 0.01	0	0.5 ± 0.01
1330 h	2.0 ± 0.08	1.1 ± 0.05	0	1.1 ± 0.05
1630 h	5.0 ± 0.03	3.8 ± 0.1	0	3.8 ± 0.1
DTT				
1130 h	1.0 ± 0.02	0	0	0
1330 h	1.0 ± 0.01	0	0	0
1630 h	1.0 ± 0.03	0	0	0

One hour of exposure to sunlight did not result in any significant difference in the Fv/Fm ratio as compared to control (plants fed with water for 6 h under growth conditions). When AsA fed plants were exposed to direct sunlight up to 1330 h, it resulted in 19% decline in Fv/Fm ratio as compared to 1130 h (1 h of treatment). Prolonged sun exposure, up to 1630 h, resulted in 30% recovery in the Fv/Fm ratio as compared to 1330 h.

Non-photochemical quenching increased in glasshouse grown plants fed with AsA and exposed to direct sunlight. Six hours of high light treatment led to 144% higher qN than seen in the control. Exposure of glasshouse grown plants fed with AsA to direct sunlight for 1 h (up to 1130 h) resulted in a significant increase (283%) in the sum of A and Z as compared to control. When the AsA fed plants were exposed to sunlight till 1330 h (3 h of treatment) they exhibited a further increase in the sum of A and Z which was almost seven times more than the control. However, longer sun exposure till 1630 h (6 h of treatment) resulted in a decrease (73%) in the sum of A and Z, as compared to 1330 h.

In DTT fed glasshouse grown plants the Fv/Fm ratio declined linearly on exposure to direct sunlight (Fig. 3.11b). At 1630 h Fv/Fm ratio declined by 78% as compared to control. qN increased by only 16% as compared to control, as a result of high light treatment for 6 h. At 1130 h (1 h of treatment) DTT fed plants exhibited similar sum of A and Z as control (0.63-0.64 mg/g; Fig. 3.11b). After 3 h of sun exposure A+Z increased by 51%, however, 6 h of sun exposure resulted in 64% decline in A+Z, as compared to 3 h.

Comparative analysis of AsA and DTT fed glasshouse grown plants indicated 383% higher Fv/Fm ratio and 109% higher qN in AsA fed plants than DTT fed plants at the end of the 6 h photoinhibitory treatment (Fig. 3.12a and b). Similarly, the sum of A and Z was higher in AsA fed plants as compared to DTT fed plants throughout the treatment (Fig. 3.12c).

3.6.2. Shade grown plants

Shade grown plants fed with AsA did not exhibit any significant difference in the Fv/Fm ratio after 1 h of exposure to direct sunlight (up to 1130 h), as compared to control (Fig. 3.11c). Six hours of photoinhibitory treatment led to a linear decline in the Fv/Fm ratio (28%). Non-photochemical quenching (qN) did not differ much after 1 h of sun exposure compared to control, however, at 1630 h qN was approximately 13% higher in AsA fed plants. The sum of A and Z also increased in response to 6 h of high light treatment in shade grown plants fed with AsA. Highest increase (78%) in the A+Z was observed at midday (at 1330 h).

DTT fed shade grown plants did not exhibit any significant decline in the Fv/Fm ratio after 1 h of exposure to direct sunlight (up to 1130 h), as compared to control (Fig. 3.11d). Further exposure caused a significant decline in the Fv/Fm ratio and at the end of the 6 h high light treatment (up to 1630 h) it was approximately 42% lower than control. qN decreased by 20% within 1 h of high light treatment than observed in control. Exposure to 3 h of sunlight resulted in 8% increase in qN as compared to 1130 h. However, further exposure up to 1630 h resulted in 11% increase in qN as compared to 1330 h. The sum of A and Z declined linearly in DTT fed plants as a result of sun exposure. Six hours of sunlight treatment resulted in a decrease of 35% in the A+Z content as compared to 1130 h.

In comparison to AsA fed shade grown plants, DTT fed shade grown plants exhibited lower Fv/Fm ratio and qN throughout the measurement period (Fig. 3.12d and e). Similarly shade grown plants fed with DTT exhibited lower sum of A and Z compared to AsA fed plants (Fig. 3.12f).

3.6.3. Sun grown plants

Sun grown plants when fed with AsA for 1 h (up to 1130 h) showed no significant changes in Fv/Fm ratio as compared to control (Fig. 3.11e). In the evening a decline of 27% in the Fv/Fm ratio was observed as compared to control. qN declined by 6% at 1130 h in AsA fed plants as compared to control. However, at 1330 h qN increased by 53% as compared to control and declined by 15% at 1630 h as compared to 1330 h. The sum of A and Z was initially low at 1130 h in AsA fed plants as compared to control. However, at 1630 h the sum of A and Z increased by 26%.

DTT fed sun grown plants exhibited a slight decrease of 3% in the Fv/Fm ratio at 1130 h as compared to control plants (Fig. 3.11f). Six hours of sun exposure resulted in 53% decline in the Fv/Fm ratio. Non-photochemical quenching declined by 30% at 1130 h as compared to control which declined further to 60% at 1630 h as compared to control. Both A and Z were absent in sun grown plants fed with AsA.

Sun grown plants fed with AsA had higher Fv/Fm ratio (0.734), qN (0.32) and sum of A and Z (3.8 mg/g) as compared to DTT fed sun grown plants (Fig. 3.12g, h and i).

Comparative analysis showed that glasshouse grown plants fed with AsA exhibited highest Fv/Fm ratio (0.658) and qN (0.44) than AsA fed shade (0.496; 0.35) and sun grown plants (0.537; 0.39) at 1630 h (Table 3.7). The sum of A and Z was also higher in AsA fed glasshouse grown plants (4.36 mg/g) than AsA fed shade (3 mg/g) and sun grown plants (2.24 mg/g) at 1330 h (Table 3.8). However, at 1630 h the sum of A and Z was higher in sun grown plants fed with AsA (3.8 mg/g) than glasshouse (0.32 mg/g) and shade grown plants (1.3 mg/g) (Table 3.8).

Glasshouse, shade and sun grown plants fed with DTT exhibited a linear decline in the Fv/Fm ratio from 1130 h to 1630 h (Table 3.7). Highest decline was observed in DTT fed glasshouse grown plants (78%). qN also declined in shade and sun grown plants fed with DTT for 6 h up to (1630 h). The decline was higher in sun grown plants (60%) as compared to shade grown plants (22%). Unlike shade and sun grown plants, glasshouse grown plants fed with DTT exhibited an increase in qN in response to sun exposure. The sum of A and Z during the light treatment was higher in DTT fed shade grown plants (2 mg/g) as compared to DTT fed glasshouse grown plants (0.94 mg/g) (Table 3.8). In sun grown plants A and Z both were absent throughout the measurement period (Table 3.8).

3.7. Effect of sunlight on chlorophyll fluorescence, xanthophyll cycle and endogenous ABA levels in plants exogenously supplied with ABA and NaCl

Since violaxanthin serves as a common precursor for Z and ABA (Parry and Horgan 1991; Rock and Zeevart 1991), the influence of exogenously supplied ABA and NaCl stress on the light-dependent Z formation, under high light condition was studied to ascertain if exogenously ABA supplied plants would show a better protection under high light treatment, since V pool shall be available for the formation of Z, while in plants subjected to salt stress the increase in ABA levels would presumably be from V, which may limit the V pool for Z formation, undermining energy dissipation function.

3.7.1. Shade grown plants fed with ABA through roots

Seven days old rice seedlings grown in the shade were fed with ABA (10 μM) through roots for fifteen days and subsequently exposed to sunlight, referred to as +ABA+PI plants. Plants fed with exogenous ABA and not exposed to sunlight are referred to as +ABA–PI plants. Control plants not fed with ABA and not exposed to sunlight are referred to as -ABA-PI plants, while control plants not fed with ABA and exposed to sunlight are referred to as –ABA+PI plants.

Exposure of -ABA plants to direct sunlight up to 1630 h (6 h of treatment) caused a relatively small decline of 6% in the Fv/Fm ratio as compared to –ABA-PI plants (Fig. 3.13a). Photochemical as well as non-photochemical quenching also declined (7% and 35% respectively) after 6 h of photoinhibition (Table 3.9). Measurements made after 6 h of exposure to direct sunlight (up to 1630 h) demonstrated a 60% reduction in neoxanthin (Nx) content and 53% reduction in V content in –ABA plants (Table 3.10). However, the content of A increased on exposure to sunlight till 1630 h. Zeaxanthin also showed a similar pattern of increase on exposure to sunlight up to 1330 h (3 h of treatment) but declined at the end of the high light treatment (up to 1630 h), but was comparatively higher than that is the –ABA-PI plants. In –ABA plants the level of endogenous ABA increased by 90% after 1 h of photoinhibition (up to 1130 h) and on continuous exposure till 1630 h (6 h of treatment) it declined, but was 19% higher than control plants.

The Fv/Fm ratio in +ABA plants declined linearly after 3 h of high light treatment (14%), and longer exposure to sunlight up to 1630 h resulted in small recovery of 9% as compared to 1330 h (Fig. 3.13b). qP showed a linear decrease on exposure to sunlight in +ABA plants (Table 3.9). While qN was also reduced by 56%

Figure 3.13: Effect of direct sunlight on the photochemical efficiency of PS II (Fv/Fm), non-photochemical quenching (qN), zeaxanthin (Z; mg/g) and endogenous ABA (μg/g) in shade grown plants fed without (a) and with exogenous ABA through roots (b). Comparison between the Fv/Fm (c), qN (d), Z (e) and endogenous ABA (f) of shade grown plants fed with or without ABA. Control refers to plants not exposed to direct sunlight. (n=3, ± SD). a - significantly different from control plants; b - significantly different from –ABA plants ($p < 0.05$).

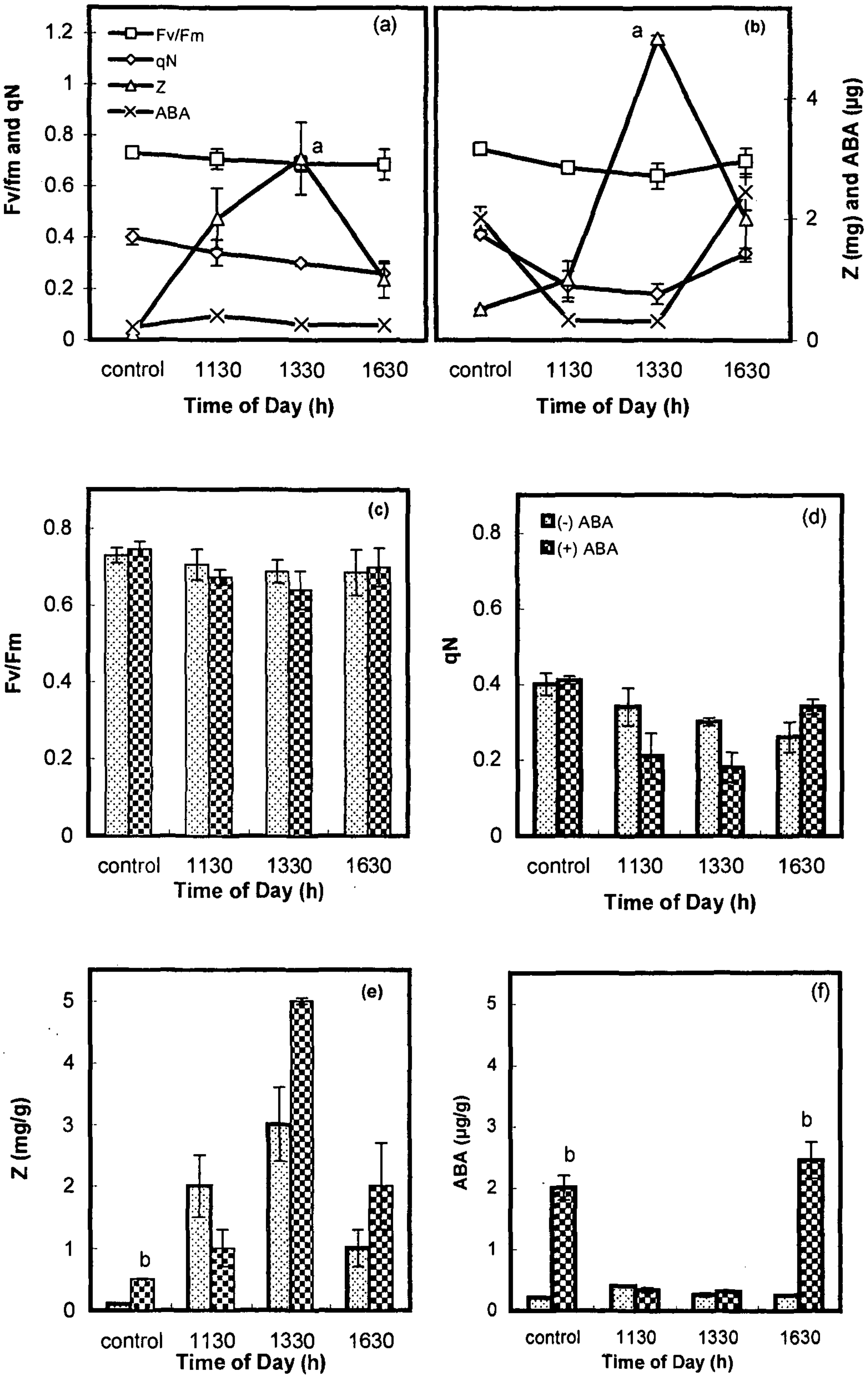

(a)
Fv/Fm
qN
Z
ABA
a
Fv/fm and qN
1.2
1
0.8
0.6
0.4
0.2
0
control
1130
1330
1630
Time of Day (h)
(b)
a
Z (mg) and ABA (µg)
4
2
0
control
1130
1330
1630
Time of Day (h)
(c)
Fv/Fm
0.8
0.6
0.4
0.2
0
control
1130
1330
1630
Time of Day (h)
(d)
(-) ABA
(+) ABA
qN
0.8
0.6
0.4
0.2
0
control
1130
1330
1630
Time of Day (h)
(e)
Z (mg/g)
5
4
3
2
1
0
b
control
1130
1330
1630
Time of Day (h)
(f)
ABA (µg/g)
5
4
3
2
1
0
b
b
control
1130
1330
1630
Time of Day (h)

Table 3.9: Effect of sunlight on the photosynthetic efficiency (Fv/Fm), photochemical quenching (qP) and non-photochemical quenching (qN) in shade grown plants fed with or without ABA through roots. Control refers to plants not exposed to sunlight (-PI). (n=3, ± SD)

Treatment	**Photosynthetic Efficiency (Fv/Fm)**	**Photochemical Quenching (qP)**	**Non-photochemical Quenching (qN)**
-ABA			
Control	0.730 ± 0.02	0.95 ± 0.04	0.40 ± 0.03
1130 h	0.706 ± 0.04	0.94 ± 0.02	0.34 ± 0.05
1330 h	0.688 ± 0.03	0.92 ± 0.01	0.30 ± 0.01
1630 h	0.686 ± 0.06	0.88 ± 0.03	0.26 ± 0.04
+ABA			
Control	0.746 ± 0.02	0.95 ± 0.05	0.41 ± 0.01
1130 h	0.673 ± 0.02	0.94 ± 0.05	0.21 ± 0.06
1330 h	0.640 ± 0.05	0.93 ± 0.04	0.18 ± 0.04
1630 h	0.700 ± 0.05	0.92 ± 0.05	0.34 ± 0.02

Table 3.10: Effect of sunlight on pigment content (mg/g FW) and endogenous ABA level (µg/g FW) in shade grown plants fed with or without ABA through roots. Control refers to plants not exposed to sunlight (-PI). Nx. Neoxanthin; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; ABA, abscisic acid. (n=3, ± SD)

Treatment	Nx	V	A	Z	V+A+Z	ABA
-ABA						
Control	10 ± 2	15 ± 3	1.5 ± 0.04	0.1 ± 0.01	16.6 ± 2	0.21 ± 0.02
1130 h	7 ± 1	5 ± 0.6	2.5 ± 0.08	2 ± 0.5	9.5 ± 0.3	0.4 ± 0.01
1330 h	6 ± 0.5	4 ± 0.3	2.1 ± 0.03	3 ± 0.6	9.1 ± 0.4	0.26 ± 0.03
1630 h	4 ± 0.4	7 ± 1	3.6 ± 0.05	1 ± 0.3	11.6 ± 0.4	0.25 ± 0.01
+ABA						
Control	9 ± 1	13 ± 2	1.2 ± 0.05	0.5 ± 0.01	14.7 ± 0.7	2 ± 0.2
1130 h	8 ± 0.5	7 ± 0.8	1.5 ± 0.01	1 ± 0.3	9.5 ± 0.3	0.33 ± 0.04
1330 h	8 ± 0.3	4 ± 0.1	1.8 ± 0.03	5 ± 0.05	10.8 ± 0.4	0.31 ± 0.02
1630 h	7 ± 0.2	7 ± 0.5	1.7 ± 0.02	2 ± 0.7	10.7 ± 0.2	2.45 ± 0.3

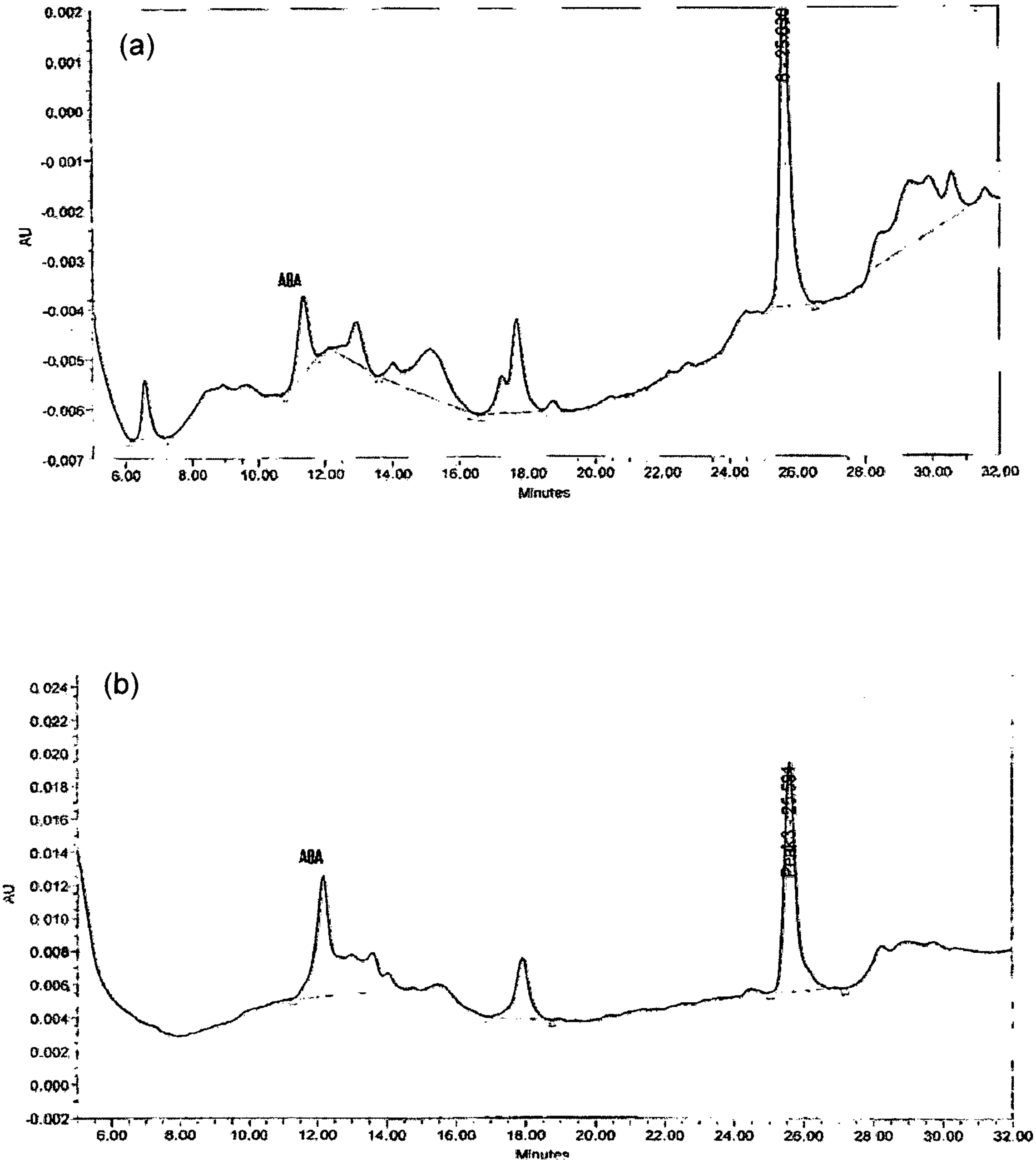


Figure 3.14: HPLC profile of endogenous ABA at 254 nm extracted from shade grown plants fed without (a) and with exogenous ABA through roots (b).

on exposure to 3 h of sunlight, however, after 6 h of exposure qN increased by 88% as compared to 1330 h (Fig. 3.13b). Nx and V content declined by 22% and 46% respectively after 6 h of exposure to direct sunlight, while the content of A increased by 40% as compared to +ABA-PI plants (Table 3.10). +ABA-PI plants had 90% lower Z than +ABA plants exposed to 3 h of sunlight, however exposure till 6 h resulted in a decline of 60% as compared to 1330 h (Fig. 3.13b). The level of endogenous ABA dropped (84%) in +ABA plants after 3 h of exposure to direct sunlight, while longer duration of exposure to sunlight for 6 h caused a 22% increase in the ABA level than +ABA-PI plants.

+ABA-PI plants exhibited slightly higher Fv/Fm ratio than –ABA-PI plants (Fig. 3.13c). Photochemical quenching was similar in -/+ABA-PI plants (0.95), and exposure to sunlight till 1630 h (6 h of treatment) led to a linear decline in qP in both -/+ABA plants (Table 3.9). However, at 1630 h the decrease in qP was higher in –ABA plants (7%) than in +ABA plants (3%). Non-photochemical quenching was also similar in both -/+ ABA-PI plants (0.4), however, at 1630 h qN was higher in +ABA plants (30%) than –ABA plants (Fig. 3.13d). –ABA-PI plants had approximately 11% higher Nx than +ABA-PI plants, and exposure to 6 h of sunlight resulted in a linear decline in the Nx content in both -/+ABA plants (Table 3.10). However at 1630 h Nx content was higher (75%) in +ABA plants than –ABA plants. Similarly, +ABA-PI plants had higher V content (13%) than –ABA-PI plants, and exposure to 6 h of sunlight resulted in reduction of V in both -/+ ABA plants. The content of A was higher in –ABA plants while Z was higher in +ABA plants, throughout the measurement period (Table 3.10). Rice plants grown without exogenously supplemented ABA showed a lower level of endogenous ABA in comparison to that of plants grown with ABA supplemented medium (Fig. 3.13f).

When transferred to direct sunlight the ABA levels increased in both types of plants but increase was more in +ABA than −ABA plants (Fig. 3.13f).

3.7.2. Plants fed with ABA through cut petioles

Plants were also fed with ABA through cut petioles in addition of giving ABA through roots to be able to compare data with AsA and DTT fed plants.

3.7.2.1. Glasshouse grown plants

Glasshouse plants fed with ABA exhibited 14% higher Fv/Fm ratio than control after 1 h of exposure to direct sunlight (Fig. 3.15a). After 3 h of high light treatment the Fv/Fm ratio showed a slight decline (2%) as compared to 1130 h, but was 12% higher than control plants. Longer exposure up to 1630 h did not result in any changes in the Fv/Fm ratio as compared to 1330 h. Non-photochemical quenching also increased linearly on exposure to direct sunlight and at 1630 h qN was 83% higher than observed in control.

ABA fed glasshouse plants exhibited higher Nx, V and A after 6 h of high light exposure as compared to control (Table 3.12). Zeaxanthin was absent in control as well as ABA fed plants exposed to sunlight up to 1330 h, and was seen only at 1630 h (Fig. 3.15a). Endogenous ABA level decreased by 44% after 1 h of exposure to direct sunlight, however after 6 h of high light treatment ABA increased by approximately 15% as compared to control (Fig. 3.15a).

3.7.2.2. Shade grown plants

Effect of ABA in shade grown plants fed through petioles was also studied on xanthophyll cycle and qN.

Figure 3.15: Effect of sunlight on the photochemical efficiency of PS II (Fv/Fm), non-photochemical quenching (qN), zeaxanthin (Z; mg/g) and endogenous ABA (µg/g) in glasshouse (a), shade (b) and sun (c) grown plants fed with exogenous ABA through cut petioles. Control refers to plants fed with water for 6 h under growth conditions. (n=3, ± SD). a - significantly different from control plants ($p < 0.05$).

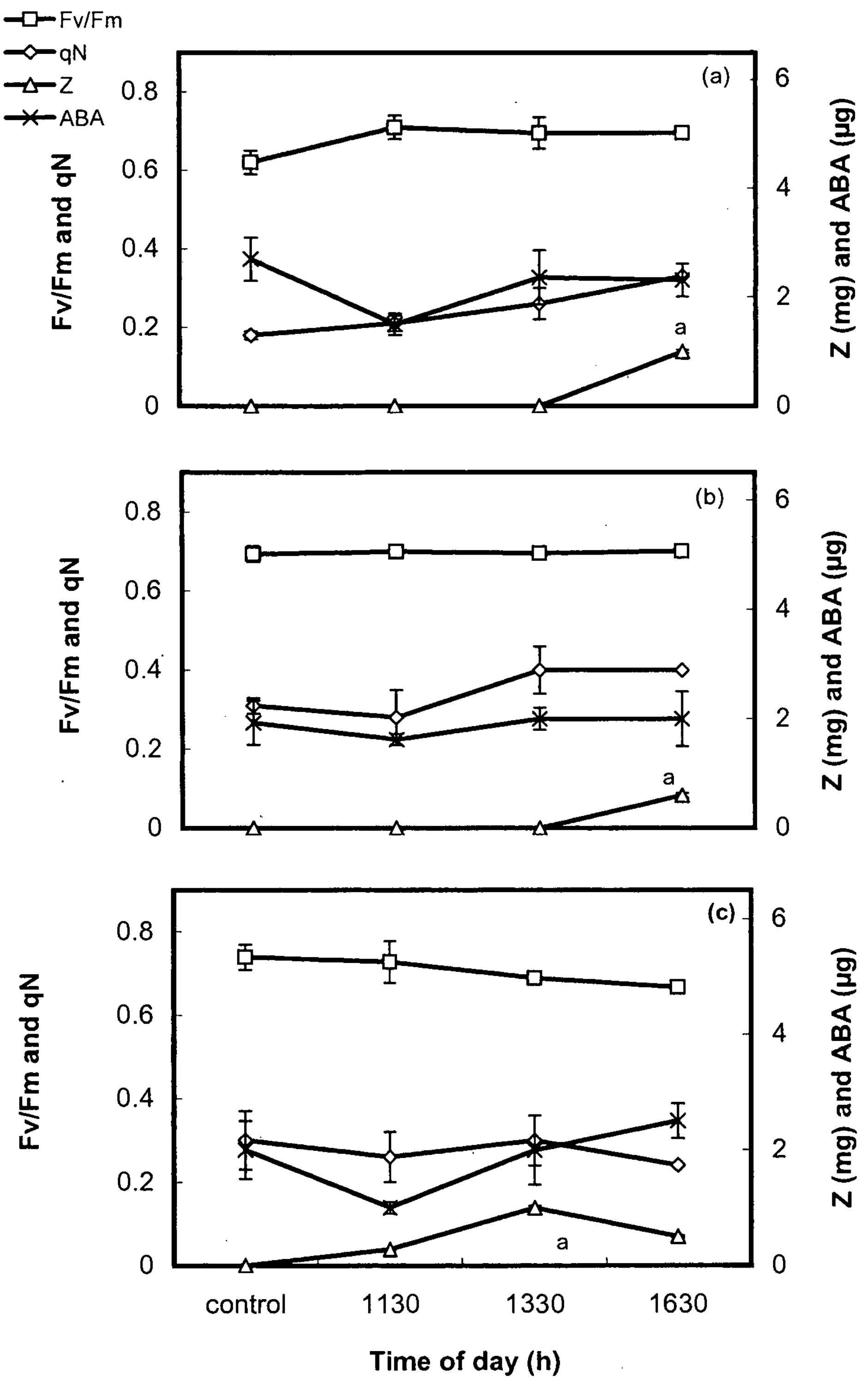

Fv/Fm
qN
Z
ABA
(a)
(b)
(c)
a
Fv/Fm and qN
Z (mg) and ABA (µg)
0
0.2
0.4
0.6
0.8
2
4
6
control
1130
1330
1630
Time of day (h)

Figure 3.16: Comparison between the effect of sunlight on the photochemical efficiency of PS II (Fv/Fm; a), non-photochemical quenching (qN; b), and endogenous ABA (µg/g; c) in glasshouse (GH), shade (SH) and sun grown plants fed with or without exogenous ABA through cut petioles. Control refers to plants fed with water for 6 h under growth conditions. (n=3, ± SD).

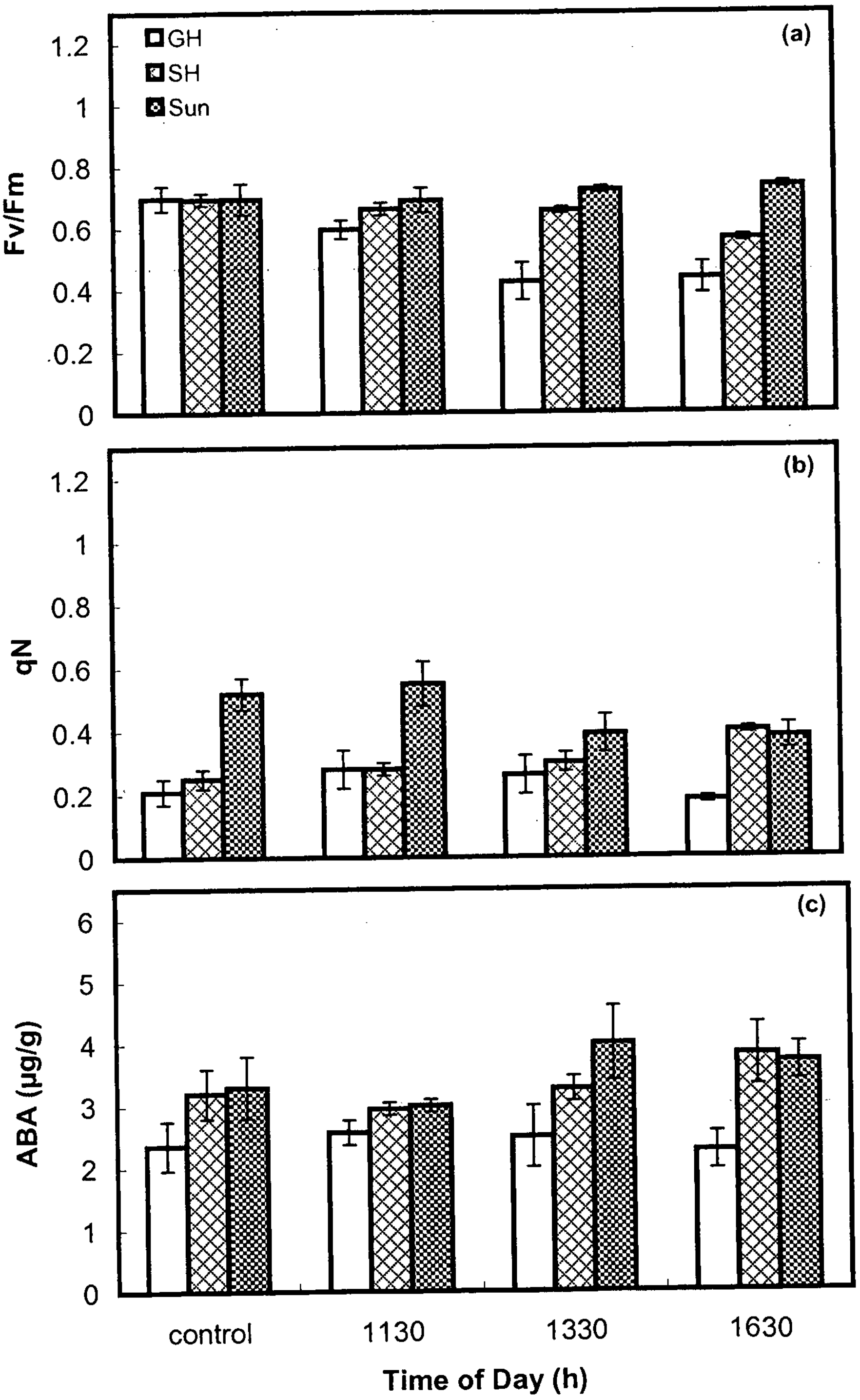

(a)
GH
SH
Sun
Fv/Fm
1.2
1
0.8
0.6
0.4
0.2
0
(b)
qN
1.2
1
0.8
0.6
0.4
0.2
0
(c)
ABA (µg/g)
6
5
4
3
2
1
0
control
1130
1330
1630
Time of Day (h)

Table 3.11: Effect of sunlight on the photosynthetic efficiency (Fv/Fm), photochemical quenching (qP) and non-photochemical quenching (qN) in glasshouse, shade and sun grown plants fed with ABA through cut petioles. Control refers to plants fed with water for 6 h under growth conditions. (n=3, ± SD)

Treatment	**Photosynthetic Efficiency (Fv/Fm)**	**Photochemical Quenching (qP)**	**Non-photochemical Quenching (qN)**
Glasshouse			
Control	0.621 ± 0.03	0.85 ± 0.04	0.18 ± 0.03
1130 h	0.710 ± 0.03	0.97 ± 0.02	0.21 ± 0.01
1330 h	0.696 ± 0.03	0.95 ± 0.01	0.26 ± 0.02
1630 h	0.696 ± 0.04	0.89 ± 0.05	0.33 ± 0.04
Shade			
Control	0.694 ± 0.05	0.77 ± 0.04	0.31 ± 0.04
1130 h	0.700 ± 0.02	0.97 ± 0.02	0.28 ± 0.02
1330 h	0.701 ± 0.08	0.88 ± 0.01	0.40 ± 0.07
1630 h	0.739 ± 0.03	0.90 ± 0.05	0.49 ± 0.06
Sun			
Control	0.739 ± 0.03	0.91 ± 0.04	0.30 ± 0.03
1130 h	0.727 ± 0.03	0.94 ± 0.04	0.26 ± 0.07
1330 h	0.689 ± 0.05	0.93 ± 0.05	0.30 ± 0.06
1630 h	0.667 ± 0.01	0.94 ± 0.02	0.24 ± 0.06

Table 3.12: Effect of sunlight on pigment content (mg/g FW) and endogenous ABA (μg/g FW) in glasshouse, shade and sun grown plants fed with ABA through cut petioles. Control refers to plants fed with water for 6 h under growth conditions. Nx. Neoxanthin; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; ABA, abscisic acid. (n=3, ± SD)

	Nx	**V**	**A**	**Z**	**V+A+Z**	**ABA**
Glasshouse						
C	1.3 ± 0.04	0.9 ± 0.02	0.6 ± 0.01	0	1.5 ± 0.1	2.7 ± 0.4
1130 h	1.6 ± 0.06	3.5 ± 0.09	0.8 ± 0.03	0	4.3 ± 0.6	1.5 ± 0.2
1330 h	1.9 ± 0.01	1.2 ± 0.01	0.5 ± 0.02	0	1.7 ± 0.1	2.3 ± 0.5
1630 h	3.8 ± 0.4	4.4 ± 1	2 ± 0.5	1.5 ± 0.03	7.9 ± 0.7	2.3 ± 0.3
Shade						
C	5.5 ± 0.8	2 ± 0.05	1.6 ± 0.03	0	2.6 ± 0.05	1.9 ± 0. 4
1130 h	2.0 ± 0.05	2 ± 0.03	1 ± 0.01	0	3 ± 0.07	1.6 ± 0.1
1330 h	2.0 ± 0.06	1 ± 0.02	2 ± 0.04	0	3 ± 0.08	2 ± 0.2
1630 h	4.0 ± 0.6	1 ± 0.04	2 ± 0.06	0.6 ± 0.04	3.6 ± 0.09	2 ± 0.5
Sun						
C	6.3 ± 1	5 ± 0.07	3 ± 0.09	0	8 ± 0.8	2 ± 0.5
1130 h	6 ± 0.9	3 ± 0.02	2 ± 0.05	0.3 ± 0.01	5.3 ± 0.2	1 ± 0.1
1330 h	4 ± 0.07	3 ± 0.02	1 ± 0.03	1.0 ± 0.04	5 ± 0.4	2 ± 0.6
1630 h	8 ± 2	4 ± 0.01	1 ± 0.02	0.5 ± 0.02	5.5 ± 0.6	2.5 ± 0.3

In ABA fed shade grown plants the Fv/Fm ratio increased linearly on exposure to direct sunlight and at 1630 h it was 6% higher than control (Fig. 3.15b). Non-photochemical quenching also increased on exposure to sunlight and after 6 h of high light treatment it was 58% higher than control.

Shade grown plants fed with ABA exhibited lower neoxanthin than control (Table 3.12). Six hours of high light treatment resulted in 27% lower Nx as compared to control. Violaxanthin declined on exposure to sunlight and at 1630 h it was 50% lower than control. A 25% increase in A was observed after 6 h of high light treatment. Zeaxanthin was absent in control as well as ABA fed plants exposed to sunlight up to 1330 h and was seen only at 1630 h. Endogenous ABA level were almost similar in ABA fed and control plants (Fig. 3.15b).

3.7.2.3. Sun grown plants

As seen in Fig. 3.15c, at 1130 h the Fv/Fm ratio did not differ significantly in ABA fed plants as compared to control. However, longer exposure up to 1630 h resulted in a decline of 9% in the Fv/Fm ratio. qN did not differ after 3 h of high light treatment in ABA fed and control plants. However, after 6 h of high light treatment qN declined by 20% as compared to control (Fig. 3.15c).

Nx did not differ significantly after 1 h of exposure to direct sunlight as compared to control (Table 3.12). However, after 6 h of high light treatment Nx increased by 26% as compared to control. ABA fed plants exhibited lower V and A as compared to control. Z was absent in control plants but was seen in ABA fed plants, which increased on exposure to 6 h of sunlight. ABA fed plants exhibited 25% higher endogenous ABA than control after 6 h of exposure to direct sunlight (Fig. 3.15c).

Comparative analysis showed that glasshouse and shade grown plants fed with ABA and photoinhibited for 6 h exhibited an increase in the Fv/Fm ratio and qN (Fig. 3.16a and b). While sun grown plants fed with ABA exhibited a slight decline in the Fv/Fm ratio and qN at 1630 h. Photochemical quenching was relatively higher in ABA fed plants of all the three different growth conditions as compared to their respective control (Table 3.11).

Zeaxanthin was seen only on longer duration of treatment up to 1630 h (6 h of treatment) in ABA fed glasshouse and shade grown plants and not in control (Table 3.12). While in sun grown plants fed with ABA, Z was present throughout the measurement period. In glasshouse and shade grown plants the VAZ pool was higher in ABA fed plants, while in sun grown plants the VAZ pool was higher in control (Table 3.12). Endogenous ABA levels were slightly less in ABA fed plants than seen in control (Table 3.12). Exposure of ABA fed glasshouse, shade and sun plants to direct sunlight resulted in an increase in the endogenous ABA levels, at 1630 h (6 h of treatment) the ABA levels were almost similar in glasshouse and sun plants, while in shade plants ABA levels were approximately 25% less than sun plants (Fig. 3.16c).

3.7.3. Plants treated with 100 and 250 mM NaCl

Thirty days old rice plants grown in the three different growth conditions were irrigated with 100 mM NaCl for the next six days, to study the effect of NaCl with reference to ABA and VAZ pool. Data were obtained from plants treated with salt without photoinhibition (+NaCl-PI) and salt treated plants exposed to 6 h of sunlight (up to 1630 h) (+NaCl+PI).

Fig. 3.17b depicts the changes in the Fv/Fm ratio of glasshouse grown plants treated with 100 mM NaCl in response to exposure to direct sunlight. Glasshouse

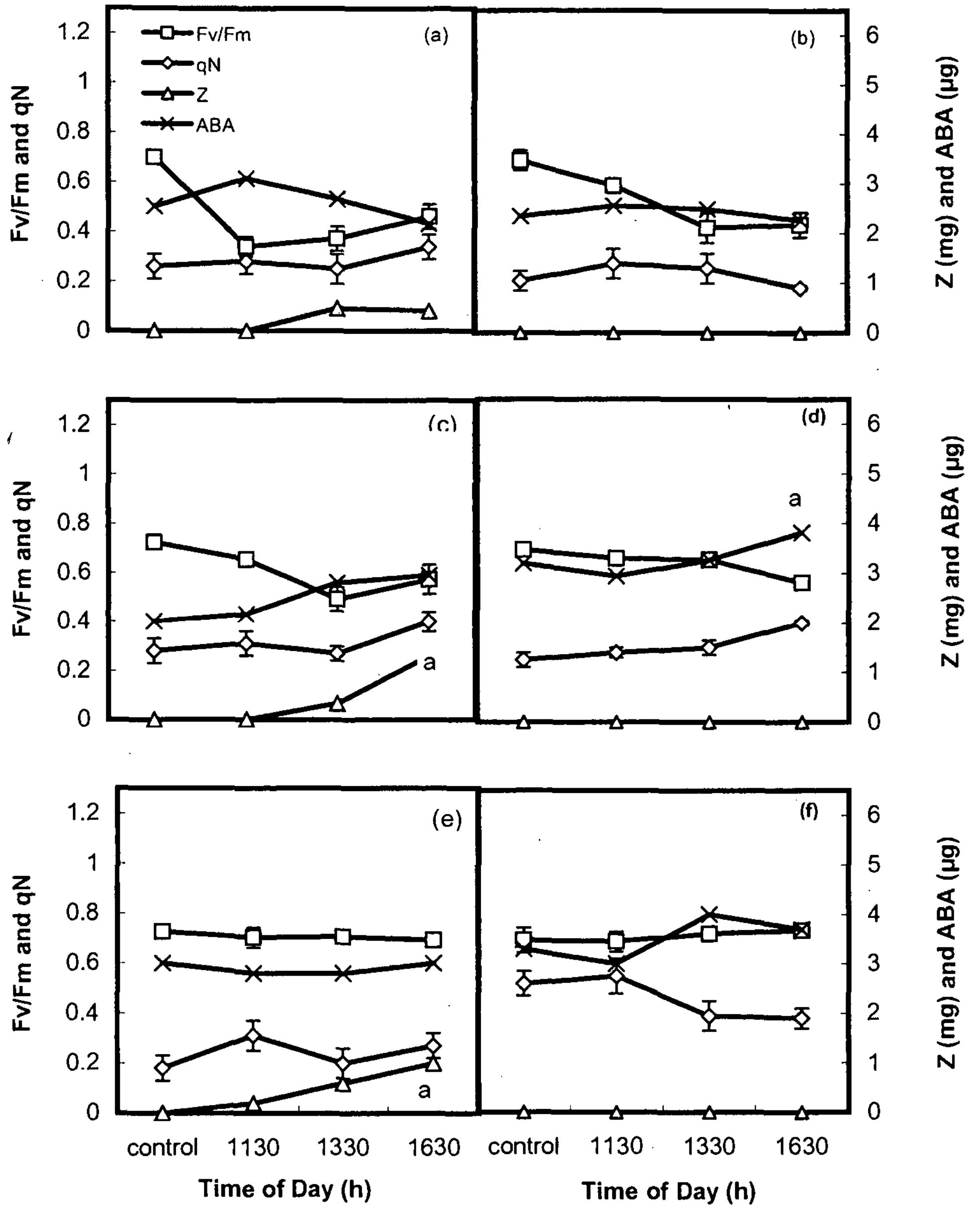


Figure 3.17: Effect of sunlight on the photochemical efficiency (Fv/Fm), non-photochemical quenching (qN), Zeaxanthin (Z; mg/g) and endogenous ABA (µg/g) in glasshouse(a and b), shade (c and d) and sun grown plants (e and f) treated without or with NaCl. Control refers to plants not exposed to sunlight. (n=3, ± SD). a - significantly different from control ($p<0.05$).

Figure 3.18: Comparison between the effect of sunlight on the photochemical efficiency of PS II (Fv/Fm), non-photochemical quenching (qN), and endogenous ABA (μg/g) in glasshouse (a, b, c), shade (d, e, f) and sun (g, h, i) grown plants treated with or without NaCl. Control refers to plants not exposed to sunlight. (n=3, ± SD).

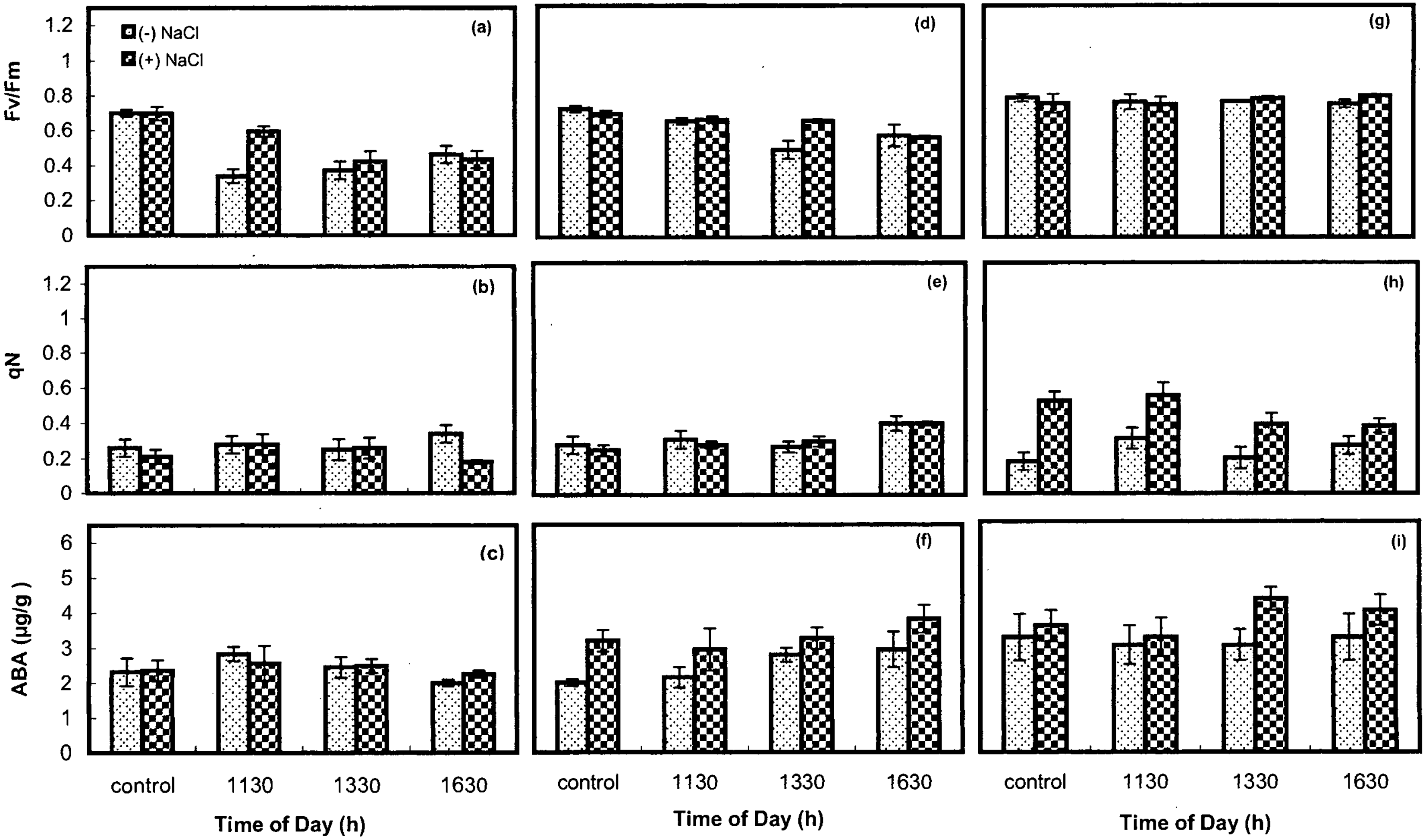

(-) NaCl
(+) NaCl
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Fv/Fm
qN
ABA (µg/g)
1.2
1
0.8
0.6
0.4
0.2
0
6
5
4
3
2
1
0
control
1130
1330
1630
Time of Day (h)
Time of Day (h)
Time of Day (h)

Table 3.13: Effect of sunlight on the photosynthetic efficiency (Fv/Fm), photochemical quenching (qP) and non-photochemical quenching (qN) in glasshouse, shade and sun grown plants fed with or without NaCl. (n=3, ± SD)

Treatment	**Photosynthetic Efficiency (Fv/Fm)**	**Photochemical Quenching (qP)**	**Non-photochemical Quenching (qN)**
Glasshouse			
-NaCl-PI (control)	0.700 ± 0.02	0.79 ± 0.06	0.26 ± 0.05
1130 h	0.340 ± 0.07	0.85 ± 0.05	0.28 ± 0.07
1330 h	0.373 ± 0.05	0.69 ± 0.02	0.25 ± 0.06
1630 h	0.463 ± 0.05	0.94 ± 0.06	0.34 ± 0.05
+NaCl-PI	0.698 ± 0.04	0.81 ± 0.05	0.21 ± 0.04
1130 h	0.596 ± 0.03	0.78 ± 0.03	0.28 ± 0.06
1330 h	0.424 ± 0.05	0.89 ± 0.03	0.26 ± 0.06
1630 h	0.435 ± 0.05	0.89 ± 0.05	0.18 ± 0.01
Shade			
-NaCl-PI	0.723 ± 0.02	0.80 ± 0.04	0.28 ± 0.05
1130 h	0.653 ± 0.02	0.92 ± 0.04	0.31 ± 0.07
1330 h	0.493 ± 0.05	0.90 ± 0.06	0.27 ± 0.03
1630 h	0.573 ± 0.06	0.85 ± 0.04	0.40 ± 0.04
+NaCl-PI	0.695 ± 0.02	0.94 ± 0.03	0.35 ± 0.03
1130 h	0.662 ± 0.02	0.96 ± 0.01	0.28 ± 0.02
1330 h	0.655 ± 0.01	0.98 ±0.02	0.30 ± 0.03
1630 h	0.563 ± 0.01	0.98 ± 0.03	0.40 ±0.01
Sun			
-NaCl-PI	0.726 ± 0.02	0.90 ± 0.005	0.18 ± 0.05
1130 h	0.703 ± 0.04	0.95 ± 0.02	0.31 ± 0.06
1330 h	0.706 ± 0.005	0.97 ± 0.02	0.20 ± 0.06
1630 h	0.693 ± 0.02	0.85 ± 0.03	0.27 ± 0.05
+NaCl-PI	0.696 ± 0.05	0.77 ± 0.01	0.52 ± 0.05
1130 h	0.690 ± 0.04	0.82 ± 0.06	0.55 ± 0.07
1330 h	0.722 ± 0.01	0.89 ± 0.04	0.39 ± 0.06
1630 h	0.735 ± 0.01	0.86 ± 0.03	0.38 ± 0.04

Table 3.14: Effect of sunlight on pigment content (mg/g FW) and endogenous ABA (μg/g FW) in glasshouse, shade and sun grown plants fed with or without NaCl. Nx. Neoxanthin; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; ABA, abscisic acid. (n=3, ± SD)

	Nx	**V**	**A**	**Z**	**V+A+Z**	**ABA**
Glasshouse						
-NaCl-PI	4.3 ± 0.3	7.44 ± 2	0.65 ± 0.2	0	8.09 ± 1	2.3 ± 0.4
1130 h	4.4 ± 0.4	0.45 ± 0.1	0.71 ± 0.4	0	1.16 ± 0.2	2.8 ± 0.2
1330 h	3.5 ± 0.2	3.00 ± 0.5	1.67 ± 0.4	0.4 ± 0.03	5.07 ± 0.3	2.4 ± 0.3
1630 h	3.5 ± 0.1	4.50 ± 1	2.00 ± 0.5	0.3 ± 0.01	6.80 ± 0.5	2.0 ± 0.1
+NaCl-PI	0.2 ± 0.02	0.4 ± 0.04	0.1 ± 0.02	0	0.5 ± 0.02	2.3 ± 0.3
1130 h	0.1 ± 0.01	0.1 ± 0.01	0.3 ± 0.02	0	0.4 ± 0.01	2.5 ± 0.5
1330 h	0.2 ± 0.01	0.1 ± 0.02	0.5 ± 0.01	0	0.6 ± 0.03	2.5 ± 0.2
1630 h	0.2 ± 0.02	0.3 ± 0.03	0.2 ± 0.03	0	0.5 ± 0.02	2.2 ± 0.1
Shade						
-NaCl-PI	3 ± 0.3	3.00 ± 0.5	1.00 ± 0.1	0	4.00 ± 0.2	2.0 ± 0.1
1130 h	6 ± 0.3	6.00 ± 0.7	1.20 ± 0.3	0	7.20 ± 0.5	2.1 ± 0.3
1330 h	4 ± 0.2	2.40 ± 0.4	1.40 ± 0.2	0.3 ± 0.01	4.10 ± 0.2	2.8 ± 0.2
1630 h	5 ± 0.5	3.00 ± 0.3	2.00 ± 0.4	1.3 ± 0.05	6.30 ± 0.2	3.0 ± 0.5
+NaCl-PI	6 ± 0.7	8 ± 0.9	0.6 ± 0.03	0	8.6 ± 0.6	3.2 ± 0.3
1130 h	5 ± 0.5	3 ± 0.2	1.0 ± 0.01	0	4.0 ± 0.3	2.9 ± 0.6
1330 h	4 ± 0.6	3 ± 0.4	2.0 ± 0.03	0	5.0 ± 0.4	3.2 ± 0.3
1630 h	8 ± 0.7	7 ± 0.7	3.0 ± 0.04	0	10 ± 1	3.8 ± 0.4
Sun						
-NaCl-PI	6 ± 0.7	10.6 ± 1	0.98 ± 0.2	0	11.5 ± 0.6	3.0 ± 0.6
1130 h	5 ± 0.3	6.21 ± 0.5	2.61 ± 0.1	0.2 ± 0.01	9.02 ± 0.2	2.8 ± 0.5
1330 h	4 ± 0.4	6.27 ± 0.4	1.91 ± 0.1	0.6 ± 0.03	8.78 ± 0.1	2.8 ± 0.4
1630 h	2 ± 0.3	4.97 ± 0.2	1.50 ± 0.1	1.0 ± 0.01	7.47 ± 0.1	3.0 ± 0.6
+NaCl-PI	4 ± 0.01	4 ± 0.06	3 ± 0.05	0	7 ± 0.8	3.2 ± 0.4
1130 h	4 ± 0.01	5 ± 0.02	1 ± 0.04	0	6 ± 0.4	3.5 ± 0.5
1330 h	3 ± 0.03	2 ± 0.01	0.7 ± 0.03	0	2.7 ± 0.07	3.5 ± 0.3
1630 h	3 ± 0.05	3 ± 0.01	1 ± 0.04	0	4 ± 0.8	3.0 ± 0.4

grown plants treated with salt without photoinhibition (+NaCl-PI) did not show any difference in the Fv/Fm ratio as compared to control (-NaCl-PI). The salt treated plants when exposed to sunlight (+NaCl+PI) exhibited a 14% decline in the Fv/Fm ratio within an hour of exposure as compared to +NaCl-PI plants. The Fv/Fm ratio further declined to 37% after 6 h of high light treatment (up to 1630 h) as compared to –PI plants. The decrease in the Fv/Fm ratio in +NaCl+PI was relatively less than observed in –NaCl+PI plants in shorter duration of treatment up to 3 h (up to 1330 h). However, 6 h of exposure to sunlight resulted in less decrease in the Fv/Fm ratio in –NaCl+PI plants compared to +NaCl+PI plants (Fig. 3.18a).

Photochemical quenching increased slightly (9%) as a result of high light treatment in salt treated glasshouse grown plants as compared to +NaCl-PI plants (Table 3.13). Non-photochemical quenching in +NaCl+PI plants did not differ significantly from control plants (-NaCL-PI) (Fig. 3.18b). Salt treated plants exhibited initial increase of 33% in qN at the end of 1 h of treatment (up to 1130 h), but it declined by 14% at the end of 6 h, as compared to +NaCl-PI plants.

Salt treated glasshouse grown plants (+NaCl-PI) exhibited a decrease of 95% in Nx, decrease of 94% in V and decrease of 76% in A as compared to control (-NaCl-PI) plants (Table 3.14). Salt stressed plants on exposure to sunlight exhibited a decline in V and an increase in A but no Z formation was observed. Nx also did not change in salt treated plants as a result of sun treatment compared to salt stressed plants –PI. VAZ pool also remained constant in +NaCl+PI plants compared to +NaCl-PI plants, except for the 3 h of the sunlight treatment where VAZ pool increased by 34% as compared to +NaCl-PI plants.

Endogenous ABA content did not show significant changes in glasshouse grown plants treated with or without NaCl and photoinhibited (3.18c).

Shade grown plants when treated with 100 mM salt (+NaCl) exhibited more or less same photosynthetic efficiency as seen in glasshouse grown salt treated plants (Table 3.13). However, PI treatment of shade grown +NaCl plants showed relatively less decrease in the Fv/Fm ratio than glasshouse grown salt treated plants exposed to sunlight. Six hours of sunlight treatment in shade grown salt stressed plants resulted in 19% decline in the Fv/Fm ratio as compared to their control (+NaCl-PI plants).

qP was also higher in the case of shade grown salt treated and photoinhibited plants as compared to same treatment for glasshouse grown plants (Table 3.13). qN was relatively higher (19%) in shade grown +NaCl+PI plants as compared to glasshouse grown +NaCl+PI plants (Table 3.13). Six hours of sun treatment resulted in an increase of 122% in qN value as compared to glasshouse grown +NaCl+PI plants.

Xanthophyll cycle content showed considerable increase in shade grown salt treated with or without photoinhibition as compared to glasshouse grown salt treated -/+PI plants (Table 3.14). Neoxanthin declined (33%) initially as a result of light treatment for 3 h, as compared to +NaCl-PI plants. However, sunlight treatment for 6 h resulted in an increase in Nx content. Violaxanthin content declined (63%) initially as a result of light treatment for 3 h, as compared to +NaCl-PI plants. However, V content increased (150%) as a result of 6 h of light treatment as compared to 3 h. Antheraxanthin content showed a linear increase as a result of sunlight treatment. No Z content was observed as a result of sunlight treatment in shade grown salt treated plants. VAZ pool also declined (44%) initially as a result of 3 h of light treatment; however, it increased on longer duration of sun exposure.

Endogenous ABA content was higher in shade grown +NaCl+PI plants as compared to glasshouse grown +NaCl+PI plants (Table 3.14). ABA content of shade grown salt treated plants remained more or less same as a result of 3 h of PI treatment. However, ABA content increased (19%) as a result of 6 h of PI treatment as compared to +NaCl-PI shade grown plants. Shade grown salt treated plants had higher ABA levels than non-salt treated plants throughout the measurement period (Fig. 3.18f).

Sun grown plants treated with 100 mM NaCl did not show any decrease in the Fv/Fm ratio in the course of the day (Fig. 3.17f). qN in sun grown salt treated plants was much higher than seen in non-salt treated sun plants as well as shade and glasshouse grown salt treated plants (Table 3.13). However, qP was relatively less in sun grown salt treated plants than non-salt treated sun plants as well as glasshouse and shade grown salt treated plants (Table 3.13).

VAZ pool in sun grown salt treated plants was relatively less as compared to their counterparts for shade grown plants, but higher than their counterparts for glasshouse grown plants (Table 3.14). Same results were observed with reference to V and Nx content. No Z was observed in sun grown salt treated plants. ABA content in sun grown salt treated plants and exposed to sunlight were more or less same as seen for the shade grown salt treated plants and exposed to sunlight but higher than observed in non-salt treated sun plants as well as glasshouse grown salt treated and PI plants (Table 3.14).

Plants were also treated with higher NaCl concentration (250 mM) to see if there is further decline in the V and ABA content. However, the results were similar as seen in 100 mM salt stressed plants.

3.8. Effect of sunlight on cell and thylakoid membrane lipids

3.8.1.1. Effect of sunlight on qualitative changes in phosphoglycolipids of cell membrane in glasshouse, shade and sun grown plants

Glasshouse, shade and sun grown plants treated with sunlight were analysed for the qualitative changes in phosphoglycolipids (PGL) and neutral lipids in cell membrane.

In glasshouse grown plants prior to transfer to direct sunlight (control plants) 8 different PGL spots with Rf values 25, 41, 46, 72, 76, 87, 94 and 99 were seen (Fig. 3.19a). Exposure to sunlight for a period of seven days resulted in some qualitative changes in PGL of glasshouse grown plants. After one to three day of sun exposure, two new lipids having Rf values of 37 and 67 were observed in addition to the lipids observed in control plants. However, seven days of sun exposure to glasshouse grown plants exhibited similar lipid profile as control plants.

In shade grown plants only three different PGL spots with the Rf values of 46, 52 and 94 were observed, which remained unchanged after transfer to direct sunlight over a period of seven days (Fig. 3.19b). The lipids with the Rf value of 46 and 94 were common in glasshouse and shade grown plants, while the lipid with the Rf value 52 was present only in shade grown plants (Table 3.15).

Sun grown plants exhibited six different PGL spots with Rf values of 37, 44, 70, 84, 94 and 97 (Fig. 3.19c), of these, PGL with Rf value 94 was common to glasshouse, and shade grown plants as well, while the PGL with Rf values 37 was common to glasshouse and sun grown plants (Table 3.15).

Figure 3.19: TLC profile of phosphoglycolipids in the cell membrane of glasshouse (a), shade (b) and sun (c) grown plants exposed to direct sunlight for a period of seven days. Control refers to plants not exposed to sunlight.

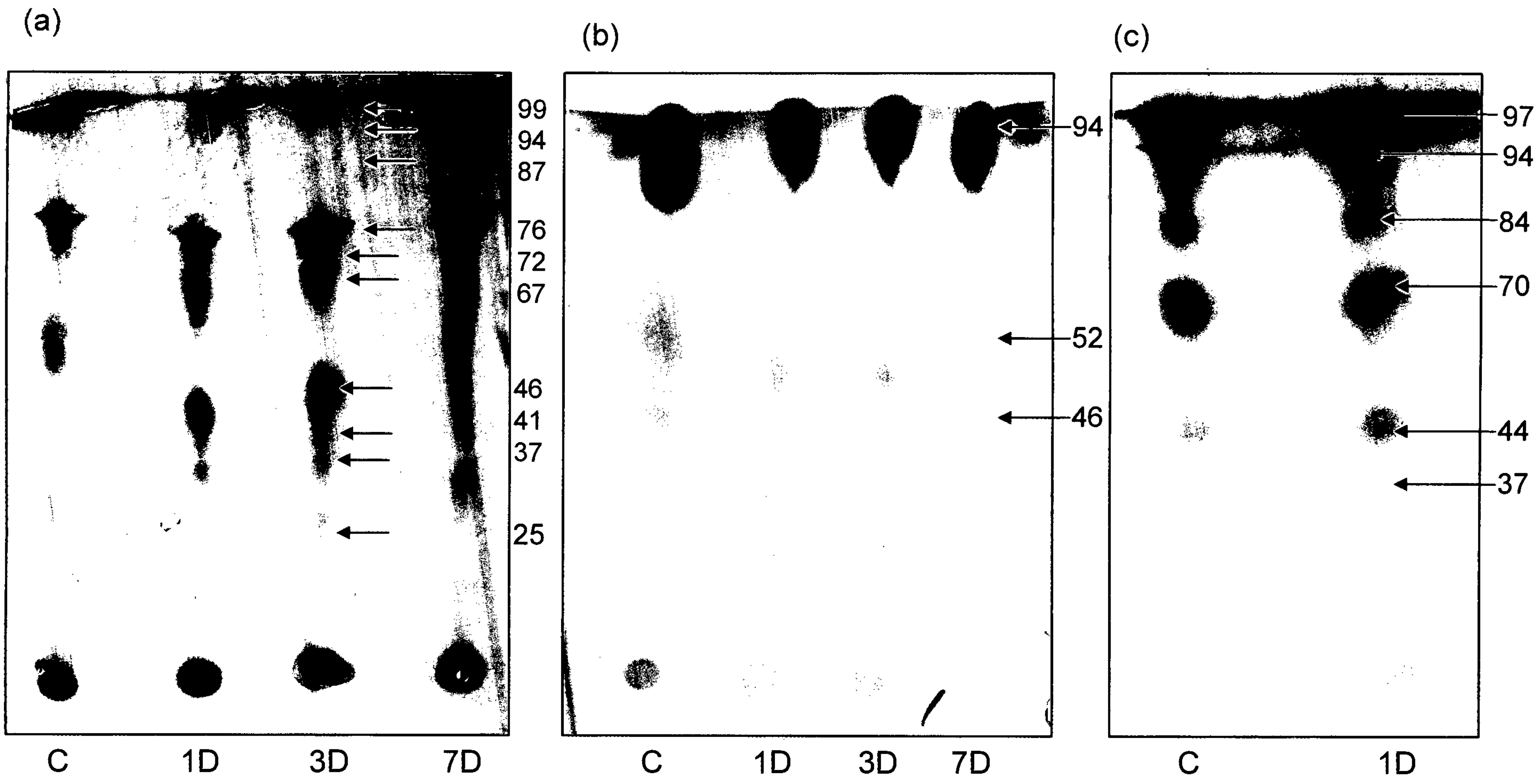

(a)
99
94
87
76
72
67
46
41
37
25
C
1D
3D
7D
(b)
94
52
46
C
1D
3D
7D
(c)
97
94
84
70
44
37
C
1D

Figure 3.20: TLC profile phosphoglycolipids of thylakoid membrane of chloroplasts isolated from glasshouse (a), shade (b) and sun (c) grown plants treated with artificial high light treatment (1500 µmol $m^{-2}s^{-1}$) for 6 h. Control refers to chloroplasts not treated with high light.

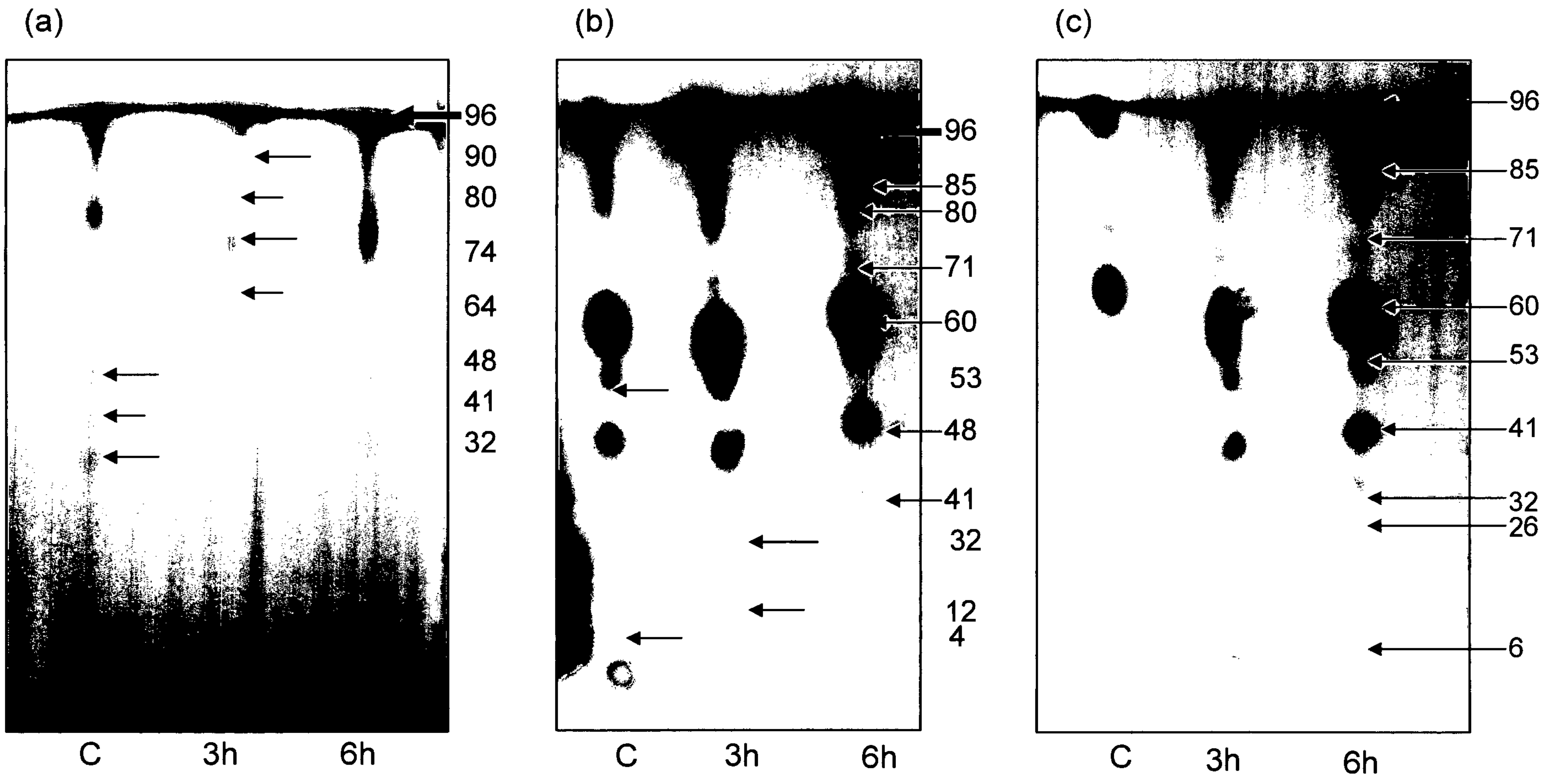

(a)
96
90
80
74
64
48
41
32
C
3h
6h
(b)
96
85
80
71
60
53
48
41
32
12
4
C
3h
6h
(c)
96
85
71
60
53
41
32
26
6
C
3h
6h

Table 3.15: Effect of sunlight on the qualitative composition of phosphoglycolipids in the cell membrane of glasshouse, shade and sun grown plants. In glasshouse and shade grown plants control refers to plants prior to transfer to direct sunlight while in sun grown plants control refers to predawn (0630 h). In glasshouse and shade grown plants data has been shown till seven days while in sun grown plants data at the end of a day has been shown. + indicates presence of spot and – indicates absence of spot.

Rf x 100	Glasshouse				Shade				Sun		Tentative identification
	C	1 D	3 D	7 D	C	1 D	3 D	7 D	C	1 D	
25	+	+	+	++	-	-	-	-	-	-	Digalactosydiglyceride
37	-	+	+	-	-	-	-	-	+	+	?
41	+	++	++	++	-	-	-	-	-	-	Phosphatidylglycerol
44	-	-	-	-	-	-	-	-	++	++	?
46	++	+++	+++	++	++	+	+	+	-	-	Phosphatidyl inositol
52	-	-	-	-	++	+	+	+	-	-	Phosphatidylethanolamine
67	-	+++	+++	-	-	-	-	-	-	-	Phosphatidyl N,N-dimethylethanolamine
70	-	-	-	-	-	-	-	-	+++	+++	?
72	++	+++	+++	+++	-	-	-	-	-	-	?
76	+++	+++	++++	++++	-	-	-	-	-	-	?
84	-	-	-	-	-	-	-	-	++	++	phosphatidylethanolamine
87	+	+	+	+	-	-	-	-	-	-	?
94	+	+	+	+	++++	+++	+++	++++	++++	++++	Monogalactosyl diglyceride
97	-	-	-	-	-	-	-	-	++++	++++	?
99	++++	++++	++++	++++	-	-	-	-	-	-	?

Table 3.16: Effect of artificial high light (1200 µmol $m^{-2}s^{-1}$; 30°C) treatment on the qualitative composition of phosphoglycolipids in the thylakoid membrane of chloroplasts isolated from glasshouse, shade (prior to transfer to direct sunlight) and sun (predawn) grown plants. Control refers to chloroplasts not treated with high light. + indicates presence of spot and – indicates absence of spot.

Rf x 100	Glasshouse			Shade			Sun			Tentative identification
	C	3 h	6 h	C	3 h	6 h	C	3 h	6 h	
4	-	-	-	+	+	+	-	-	-	?
6	-	-	-	-	-	-	+	+	+	?
12	-	-	-	+	+	+	-	-	-	Sulpholipid
26	-	-	-	-	-	-	+	+	+	Digalactosyldiglyceride
32	+	+	+	+	+	+	+	+	+	Lecithin
41	+	+	+	++	++	++	+	++	++	?
48	+	+	++	+++	+++	+++	-	-	-	Phosphatidyl inositol
53	-	-	-	++	++	++	+	++	++	Phosphatidyl serine
60	-	-	-	++++	++++	++++	+++	+++	++++	Phosphatidyl N-methylethanolamine
64	+	+	+	-	-	-	-	-	-	?
71	-	-	-	+	+	+	+	+	+	?
74	++	++	++	-	-	-	-	-	-	?
80	++	+	++	++	++	++	-	-	-	?
85	-	-	-	++	++	++	+	++	++	Phosphatidylethanolamine
90	++	++	++	-	-	-	-	-	-	?
96	+++	+++	+++	++++	++++	++++	+++	++++	++++	Monogalactosyl diglyceride

3.8.1.2. Effect of artificial high light on qualitative changes in phosphoglycolipids of thylakoid membrane in chloroplasts isolated from glasshouse, shade and sun grown plants

Chloroplasts isolated from glasshouse grown plants (control) had eight different PGL spots with Rf values of 32, 41, 48, 64, 74, 80, 90 and 96 (Fig. 3.20a) while chloroplasts isolated from shade grown plants (control) had eleven different PGL spots with Rf values of 4, 12, 32, 41, 48, 53, 60, 71, 80, 85 and 96 (Fig. 3.20b). PGL with Rf values of 32, 41, 48, 80 and 96 were common to chloroplasts isolated from both glasshouse and shade grown plants (Table 3.16).

Chloroplasts isolated from sun grown plants had nine different PGL spots with Rf values of 6, 26, 32, 41, 53, 60, 71, 85 and 96 (Fig. 3.20c). PGL with Rf values of 32, 41 and 96 were common in chloroplasts isolated from glasshouse, shade and sun grown plants (Table 3.16). Phosphoglycolipids with Rf values of 53, 60, 71 and 85 were present in chloroplasts isolated from both shade and sun grown plants. Six hours of artificial high light treatment did not result in any qualitative changes in PGL of chloroplasts isolated from the three different growth conditions (Table 3.16).

3.8.2.1. Effect of sunlight on qualitative changes in neutral lipids of cell membrane in glasshouse, shade and sun grown plants

Glasshouse grown plants prior to transfer to direct sunlight (control plants) showed the presence of five different neutral lipid spots with Rf value of 5, 17, 22, 87 and 94 (Fig. 3.21a). No qualitative changes were observed in neutral lipids even after seven days of sun exposure to glasshouse grown plants. Shade grown plants prior to transfer to direct sunlight had nine different neutral lipid spots with Rf values of 8, 17, 22, 24, 49, 61, 90, 96 and 98 (Fig. 3.21b). Shade grown plants also did not exhibit any

Figure 3.21: TLC profile of neutral lipids in the cell membrane of glasshouse (a), shade (b) and sun (c) grown plants exposed to sunlight for a period of seven days. Control refers to plants not exposed to sunlight.

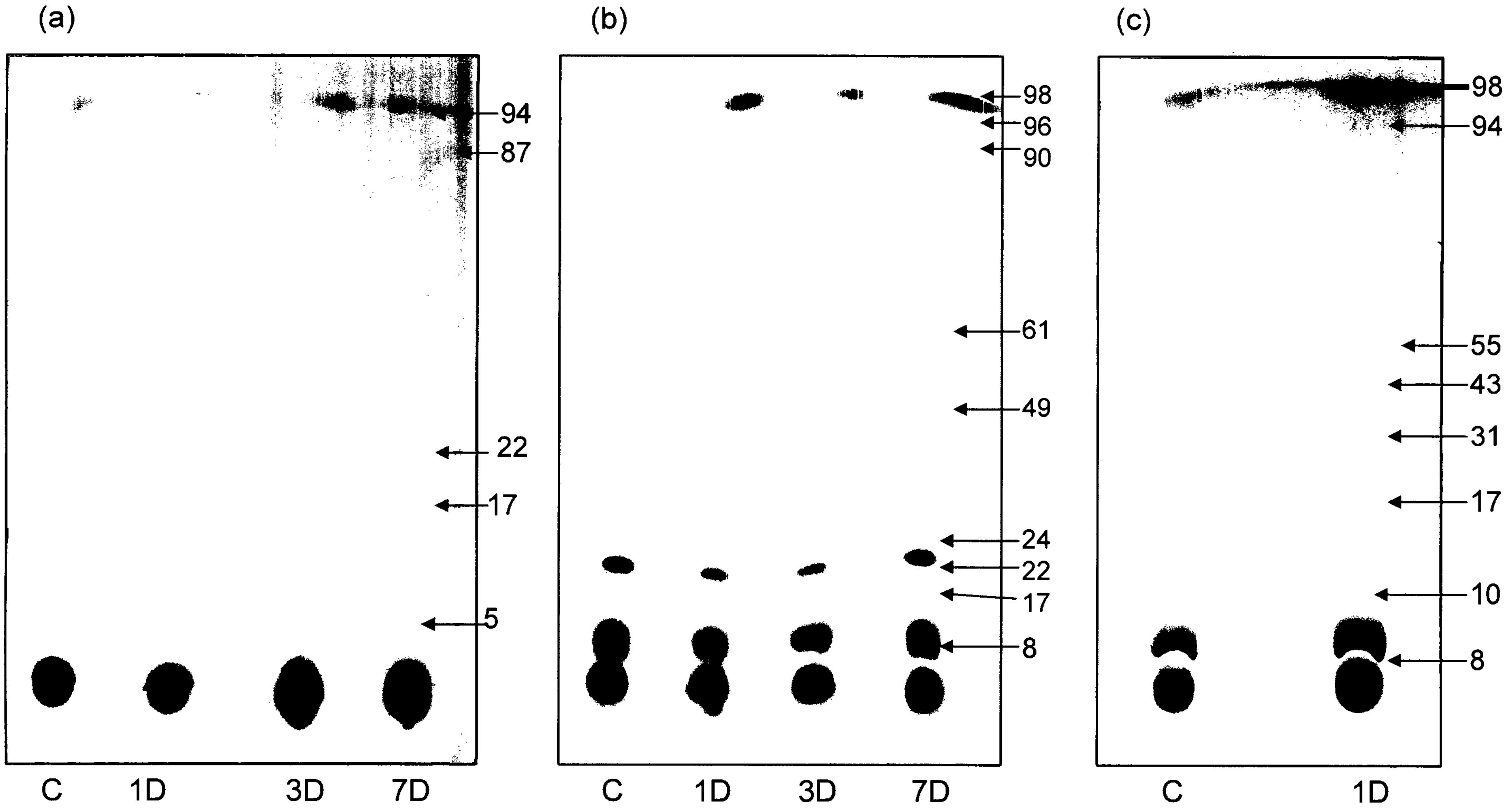

(a)
94
87
22
17
5
C
1D
3D
7D
(b)
98
96
90
61
49
24
22
17
8
C
1D
3D
7D
(c)
98
94
55
43
31
17
10
8
C
1D

Figure 3.22: TLC profile of neutral lipids in the thylakoid membrane of chloroplasts isolated from glasshouse (a), shade (b) and sun (c) grown plants treated with artificial high light treatment (1500 μmol $m^{-2}s^{-1}$) for 6 h. Control refers to chloroplasts not treated with high light.

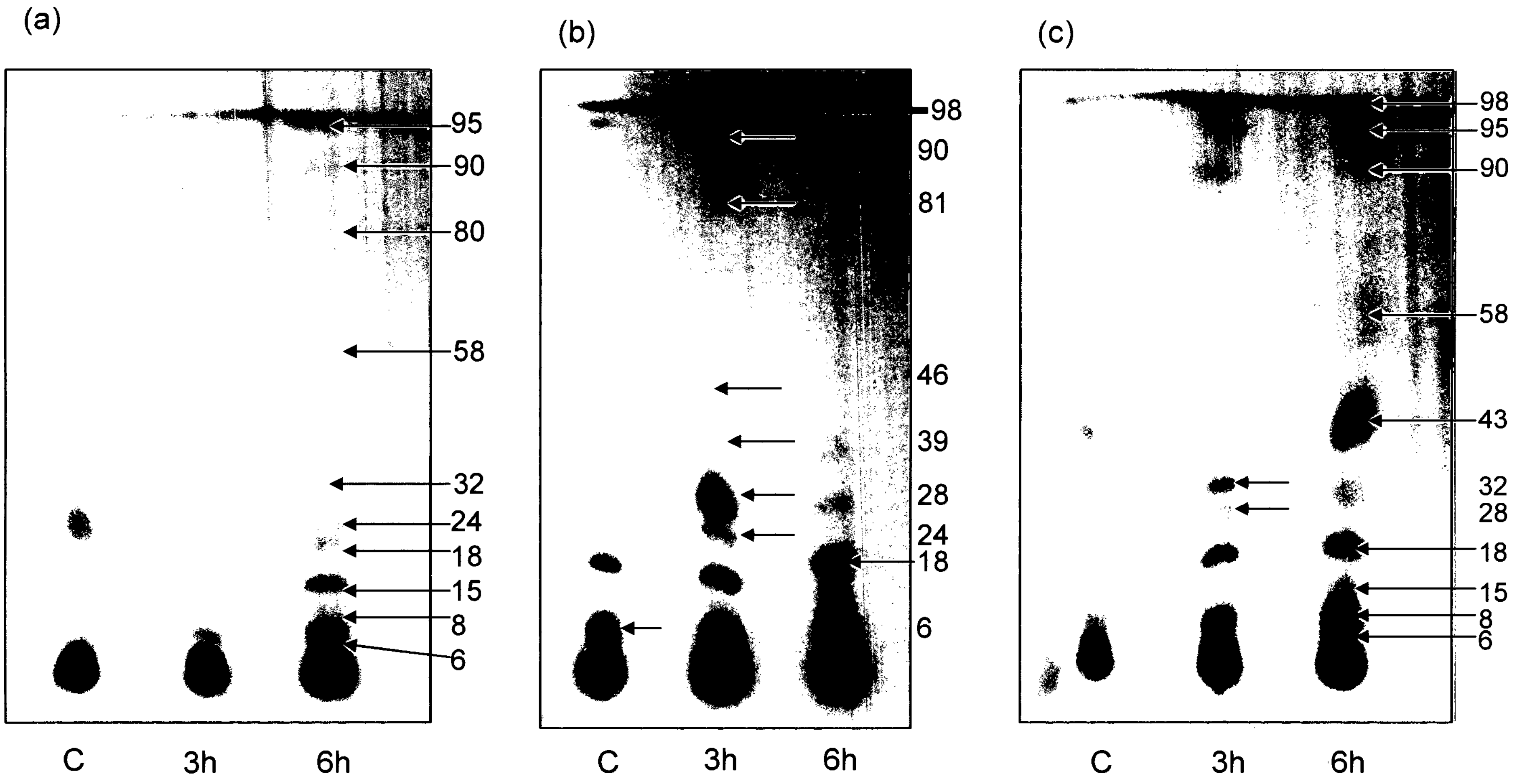

(a)
95
90
80
58
32
24
18
15
8
6
C
3h
6h
(b)
98
90
81
46
39
28
24
18
6
C
3h
6h
(c)
98
95
90
58
43
32
28
18
15
8
6
C
3h
6h

Table 3.17: Effect of sunlight on the qualitative composition of neutral lipids in the cell membrane of glasshouse, shade and sun grown plants. In glasshouse and shade grown plants control refers to prior to transfer to direct sunlight and in sun grown plants control refers to predawn (0630 h). In glasshouse and shade grown plants data has been shown till seven days while in sun grown plants data at the end of the day has been shown. + indicates presence of spot and – indicates absence of spot.

Rf	**Glasshouse**				**Shade**				**Sun**		**Tentative identification**
x 100	**C**	**1 D**	**3 D**	**7 D**	**C**	**1 D**	**3 D**	**7 D**	**C**	**1 D**	
5	+	+	+	++	-	-	-	-	-	-	?
8	-	-	-	-	++++	++++	++++	++++	++++	++++	1.3 diacyl glycerols
10	-	-	-	-	-	-	-	-	++	++	?
17	+	+	+	+	+	+	+	+	+	+	Monoglyceride
22	+	+	+	+	++++	++	++	++++	-	-	?
24	-	-	-	-	+	+	+	+	-	-	Co-enzyme Q
31	-	-	-	-	-	-	-	-	+	+	Diglyceride
43	-	-	-	-	-	-	-	-	+	+	Diglyceride
49	-	-	-	-	++	+	+	++	-	-	?
55	-	-	-	-	-	-	-	-	+	+	?
61	-	-	-	-	+	+	+	++	-	-	Fatty acid methyl esters
87	+	+	+	++	-	-	-	-	-	-	?
90	-	-	-	-	+	+	+	+	-	-	Trialkyl glycerol ethers
94	++	+	++	++	-	-	-	-	+	+	Esters
96	-	-	-	-	+	+	+	++	-	-	?
98	-	-	-	-	++	++++	++	++++	++	+++	?

Table 3.18: Effect of artificial high light (1200 µmol $m^{-2}s^{-1}$; 30°C) treatment on the qualitative composition of neutral lipids in the thylakoid membrane of chloroplasts isolated from glasshouse, shade (prior to transfer to direct sunlight) and sun (predawn) grown plants. Control signifies chloroplasts not treated with high light. + indicates presence of spot and – indicates absence of spot.

Rf	**Glasshouse**			**Shade**			**Sun**			**Tentative identification**
x 100	**C**	**3 h**	**6 h**	**C**	**3 h**	**6 h**	**C**	**3 h**	**6 h**	
6	+++	+++	++++	+++	++++	++++	+++	+++	++++	?
8	++	+	++	-	-	-	++	++	+++	1.3 diacyl glycerols
15	++	++	++	-	-	-	+	+	+	Long chain fatty acid
18	++	+	++	+++	+++	+++	++	+++	+++	Fatty acids
24	++	+	++	+	++	+	-	-	-	Co-enzyme Q
28	-	-	-	++	+++	++	+	++	++	?
32	+	+	+	-	-	-	+	++	+	Triglycerols
39	-	-	-	++	++	++	-	-	-	Diglyceride
43	-	-	-	-	-	-	++	+	++	?
46	-	-	-	+	+	+	-	-	-	?
58	+	+	++	-	-	-	+	+	+	Alkyl diacyl glycerols
80	+	+	+	-	-	-	-	-	-	?
81	-	-	-	+	+	+	-	-	-	?
90	+	+	+	+	++	+	+	++	++	Trialkyl glycerol ethers
95	+	+	++	-	-	-	+	++	++	Esters
98	-	-	-	++	++	++	++	++	++	?

qualitative changes in the neutral lipids on exposure to direct sunlight. Sun grown plants had eight neutral lipids with Rf values of 8, 10, 17, 31, 43, 55, 94 and 98 (Fig. 3.21c).

The neutral lipid with Rf value of 17 was common to glasshouse, shade and sun grown plants, while the neutral lipid with Rf value 8 and 98 were common to shade and sun grown plants. Neutral lipid with Rf value 94 were present in both glasshouse and sun grown plants (Table 3.17).

3.8.2.2. Effect of artificial light on qualitative changes in neutral lipids of thylakoid membrane in chloroplasts isolated from glasshouse, shade and sun grown plants

Chloroplasts isolated from glasshouse grown plants showed the presence of ten different neutral lipid spots with Rf values of 6, 8, 15, 18, 24, 32, 58, 80, 90 and 95 (Fig. 3.22a). Chloroplasts isolated from shade grown plants, however, had nine neutral lipids with the Rf values of 6, 18, 24, 28, 39, 46, 81, 90 and 98 (Fig. 3.22b). Chloroplasts isolated from sun grown plants showed the presence of 11 neutral lipids with the Rf values of 6, 8, 15, 18, 28, 32, 43, 58, 90, 95 and 98 (Fig. 3.22c). No qualitative changes were observed in the neutral lipids in chloroplasts isolated from plants grown in the three different growth conditions.

Neutral lipids having Rf values 6, 18 and 90 were common in chloroplasts isolated from glasshouse, shade and sun grown plants (Table 3.18). While neutral lipid with Rf value of 24 was found only in glasshouse and shade grown plants. Lipids with Rf value of 28 and 98 were common in shade and sun grown plants, while lipids with Rf value 8, 15, 32, 58 and 95 were present in both glasshouse and sun grown plants.

3.8.3.1. Quantitative estimation of glycolipids of cell membrane in glasshouse, shade and sun grown plants

Glycolipid content in glasshouse grown plants increased as a result of exposure to sunlight (Fig. 3.23a). It was seen that exposure to sunlight for a day increased the glycolipid content by 22%, which was further increased to 108% at the end of three days of sunlight treatment, as compared to control plants. However, longer exposure to sunlight for seven days resulted in decline of 31% as compared to the plants exposed to three day of sun exposure.

Glycolipid content of shade grown plants showed an increase of 40% as a result of one day of sun treatment, which remained constant during the sun exposure for three days (Fig. 3.23a). Seven days of sun exposure resulted in an increase of only 9% in glycolipid content as compared to control. Though shade grown plants had a higher level of content of glycolipids (0.55 mg/g) as compared to glasshouse grown plants (0.53 mg/g), transfer to direct sunlight resulted in greater accumulation of glycolipid content in glasshouse grown plants (Table 3.19).

Sun grown plants showed highest (0.87 mg/g) glycolipid content as compared to glasshouse (0.35 mg/g) and shade grown plants (0.55mg/g), which declined slightly (13%) at the end of one day of the exposure. However, the glycolipid content remained still higher than glasshouse and shade grown plants (Fig. 3.23a)

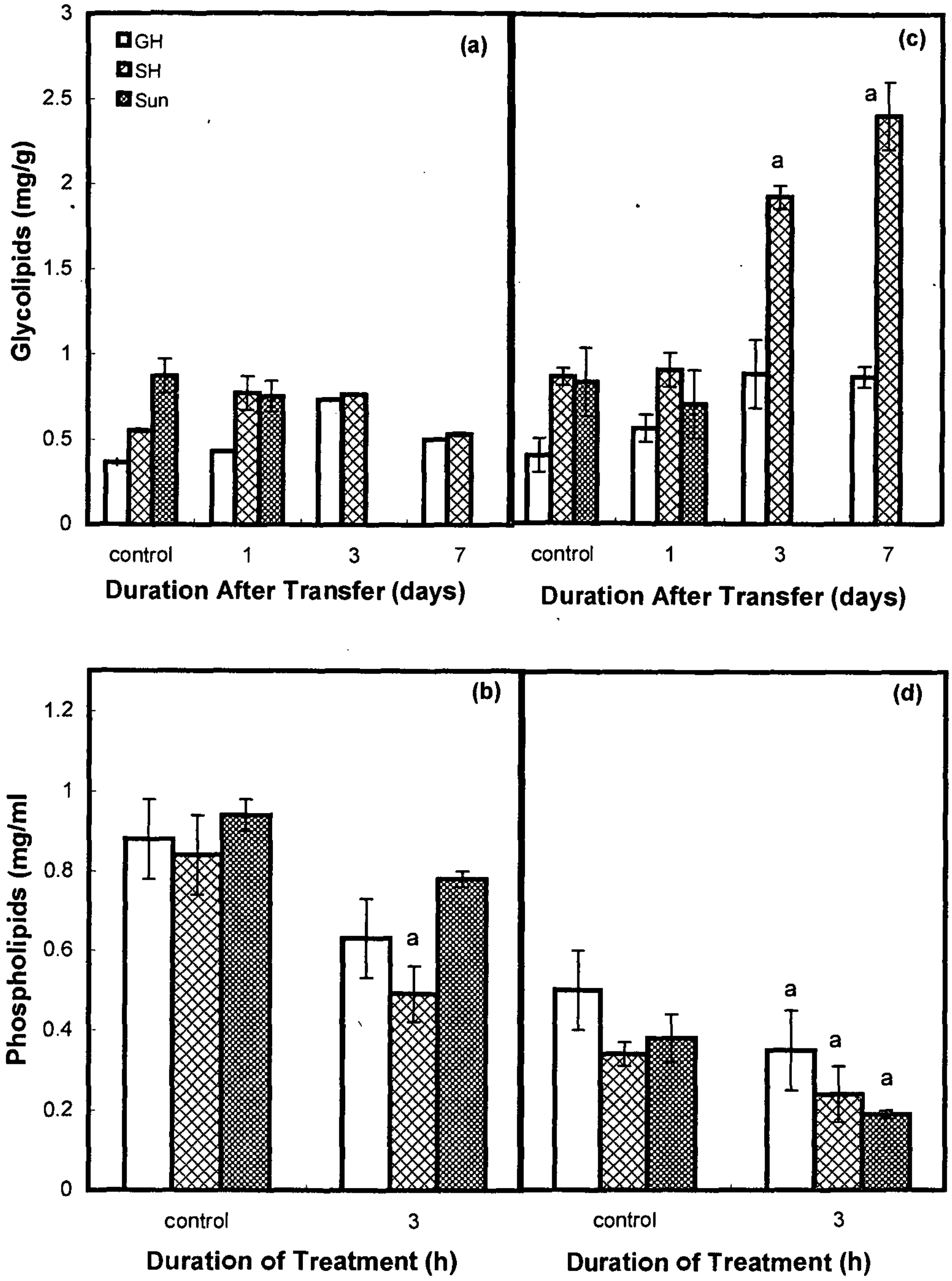


Figure 3.23: Effect of sunlight on the concentration (mg/g) of (a) glycolipids and (c) phospholipids in the cell membrane of glasshouse (GH), shade (SH) and sun grown plants.

Effect of artificial high light (1200 µmol m^{-2} s^{-1}) treatment on the concentration (mg/g) of (b) glycolipids and (d) phospholipids in the thylakoid membrane of chloroplasts isolated from glasshouse, shade and sun grown plants. (n=3, ± SD). a - significantly different from control plants ($p < 0.05$).

Table 3.19: Effect of sunlight on the quantitative composition of glycolipids and phospholipids (mg/g FW) in the cell membrane of glasshouse, shade and sun grown plants. In glasshouse and shade plants control refers to prior to transfer to direct sunlight and in sun plants control refers to measurements taken at predawn (0630 h). In glasshouse and shade plants data has been shown till seven days while in sun plants data during the course of a day has been shown. (n=3, ± SD)

Treatment	**Glycolipids**	**Phospholipids**
Glasshouse		
Control	0.35 ± 0.02	0.40 ± 0.1
1 D	0.43 ± 0.01	0.56 ± 0.08
3 D	0.73 ± 0.01	0.88 ± 0.2
7 D	0.50 ± 0.05	0.86 ± 0.06
Shade		
Control	0.55 ± 0.01	0.86 ± 0.05
1 D	0.77 ± 0.1	0.90 ± 0.1
3 D	0.76 ± 0.05	1.92 ± 0.07
7 D	0.53 ± 0.01	2.40 ± 0.2
Sun		
Control	0.87 ± 0.1	0.83 ± 0.2
1 D	0.53 ± 0.01	0.70 ± 0.2

Table 3.20: Effect of high light (1200 μmol $m^{-2}s^{-1}$; 30°C) treatment on the quantitative composition of glycolipids and phospholipids (mg/ml) in the thylakoid membrane of chloroplasts isolated from glasshouse, shade (prior to transfer to direct sunlight) and sun (predawn) grown plants. Control refers to chloroplasts not treated with high light. (n=3, ± SD)

Treatment	**Glycolipids**	**Phospholipids**
Glasshouse		
Control	0.88 ± 0.1	0.50 ± 0.1
3 h	0.63 ± 0.1	0.35 ± 0.1
Shade		
Control	0.84 ± 0.1	0.34 ± 0.03
3 h	0.49 ± 0.07	0.24 ± 0.07
Sun		
Control	0.94 ± 0.04	0.38 ± 0.06
3 h	0.78 ± 0.02	0.19 ± 0.01

3.8.3.2. Quantitative estimation of glycolipids of thylakoid membrane in chloroplasts isolated from glasshouse, shade and sun grown plants

Glycolipid content of isolated chloroplasts from glasshouse, shade and sun grown plants were more or less same (Fig. 3.23b). Treatment of chloroplasts isolated from glasshouse grown plants with artificial light (1200 μmol $m^{-2}s^{-2}$) resulted in 28% decline in the glycolipid content (Fig. 3.23b). Decrease in the glycolipids content for the same treatment for chloroplasts isolated from shade grown plants was 41% and for the sun grown plants was 17% as compared to their control.

3.8.4.1. Quantitative estimation of phospholipids of cell membrane in glasshouse, shade and sun grown plants

Phospholipid content of glasshouse grown plants prior to transfer to direct sunlight (control plants) was considerably less (0.4 mg/g) than seen in shade grown plants prior to transfer to direct sunlight (0.85 mg/g) and sun grown predawn plants (0.83 mg/g; Fig. 3.23c). Glasshouse grown plants on exposure to sunlight exhibited an increase of 120% in phospholipids content after three days as compared to control plants. A longer exposure to sunlight (up to seven days) did not further increase the phospholipids content. Shade grown plants on exposure to sunlight resulted in a linear increase in the phospholipids content over a period of seven days. The seven days exposure to sunlight resulted in an increase of 179%. The increase in the phospholipids content in shade grown plants was much higher (2.4 mg/g) than the increase observed in glasshouse grown plants (0.86 mg/g) as a result of sunlight treatment. Sun grown plants did not show any increase in the phospholipids content as compared to its predawn plants.

Plants grown in the three different growth conditions exhibited lower glycolipid content than phospholipids (Table 3.19). The extent of increase in the phospholipids content was much higher as compared to the increase in the glycolipid content in the plants of all the three different growth conditions as a result of exposure to direct sunlight for a period of seven days.

3.8.4.2. Quantitative estimation of phospholipids of thylakoid membrane in chloroplasts isolated from glasshouse, shade and sun grown plants

The phospholipid content of isolated chloroplasts from plants grown under the three different growth conditions resulted in a decline as a result of artificial light treatment (Fig. 3.23d). Chloroplasts isolated from sun plants on treatment of high light 1200 umol $m^{-2}s^{-2}$ for 3 h showed the maximum decline 50% than seen in chloroplasts isolated from glasshouse 30% and shade grown plants 30% for the same treatment, as compared to their respective control.

Chloroplasts isolated from the plants grown in the three different growth conditions exhibited higher glycolipid content than phospholipids (Table 3.20). Artificial high light treatment to isolated chloroplasts from the three different growth conditions resulted in greater decrease in glycolipids than phospholipids.

3.8.5.1. Analysis of fatty acids of cell membrane in glasshouse, shade and sun grown plants

In rice plants the fatty acid groups identified were Lauric acid C_{12}, Myristic acid C_{14}, Palmatic acid C_{16}, Stearic acid C_{18}, Oleic acid $C_{18:1}$, Linoleic acid $C_{18:2}$ and Linolenic acid $C_{18:3}$. Only quantitative changes were observed in the concentration of the different saturated and unsaturated fatty acid groups of total lipids in glasshouse, shade and sun grown rice plants, as a result of high light treatment (Table 3.21).

Table 3.21: Effect of sunlight on the fatty acid composition in mole % in cell membrane of glasshouse, shade and sun grown plants. In glasshouse and shade plants control refers to prior to transfer to direct sunlight and in sun plants control refers to predawn (0630 h). In glasshouse and shade plants data has been shown till seven days while in sun plants data during the course of a day has been shown.

	Lauric Acid	**Myristic Acid**	**Palmatic Acid**	**Stearic Acid**	**Oleic Acid**	**Linoleic Acid**	**Linolenic Acid**
Glasshouse							
C	13.81	14.52	13.81	13.60	14.67	14.78	14.78
1 D	13.84	14.48	13.61	13.64	14.74	14.69	14.97
3 D	13.90	14.56	13.68	13.69	14.76	14.82	14.58
7 D	13.94	14.68	13.72	13.71	14.78	14.91	14.22
Shade							
C	14.48	14.92	12.94	14.22	15.36	14.98	13.06
1 D	13.95	14.60	13.94	13.74	14.82	14.80	14.12
3 D	13.90	14.63	13.89	13.73	14.81	14.66	14.34
7 D	13.96	14.73	13.37	13.74	15.01	14.80	14.35
Sun							
C	14.25	14.97	13.15	14.11	15.21	15.08	13.20
1 D	14.19	14.91	13.66	13.92	14.91	14.14	14.24

Table 3.22: Effect of artificial high light (1200 µmol $m^{-2}s^{-1}$; 30°C) treatment on the fatty acid composition in mole % in thylakoid membrane of chloroplasts isolated from glasshouse, shade (prior to transfer to direct sunlight) and sun (predawn) grown plants. Control refers to chloroplasts not treated with high light.

	Lauric Acid	**Myristic Acid**	**Palmatic Acid**	**Stearic Acid**	**Oleic Acid**	**Linoleic Acid**	**Linolenic Acid**
Glasshouse							
C	13.94	14.60	13.51	13.72	14.84	14.81	14.55
3 h	13.89	14.53	13.68	13.68	14.77	14.84	14.58
6 h	13.58	14.33	13.54	13.41	14.48	14.51	15.12
Shade							
C	13.95	14.68	13.93	13.76	14.83	14.76	14.06
3 h	13.93	14.64	13.76	13.71	14.81	14.83	14.29
6 h	13.83	14.53	13.83	13.61	14.70	14.70	14.77
Sun							
C	13.85	14.54	13.81	13.65	14.74	14.76	14.60
3 h	13.81	14.52	13.81	13.59	14.69	14.80	14.74
6 h	13.82	14.55	13.78	13.62	14.71	14.78	14.71

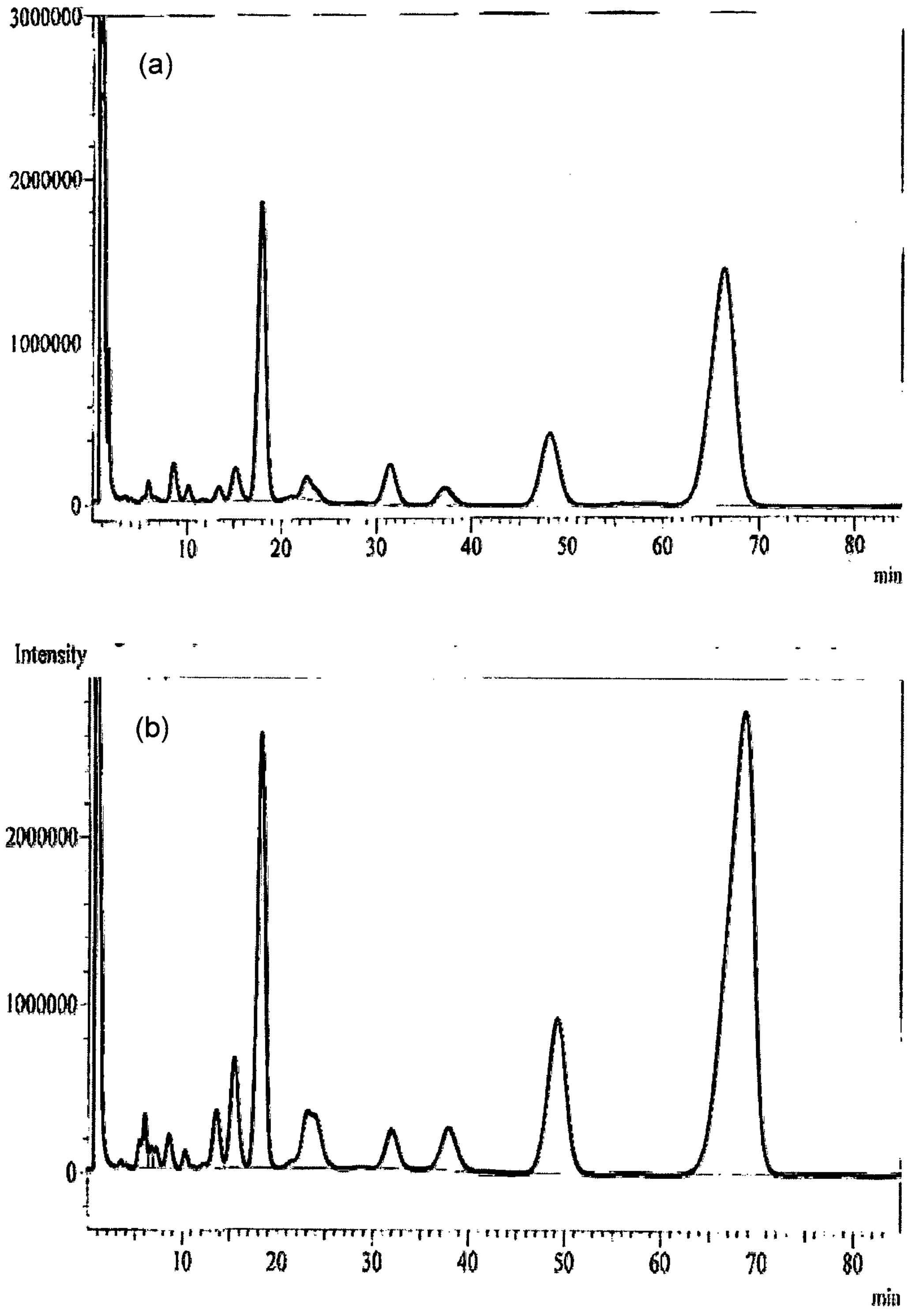


Figure 3.24: GC profile of fatty acids of sun grown plants. (a) predawn (b) at 1630 h

Lauric acid content was highest (14.48) in shade grown plants prior to transfer to direct sunlight (control) and least (13.81) in glasshouse grown plants prior to transfer to direct sunlight (control; Table 3.21). The content of Lauric acid increased as a result of seven days exposure to sunlight in glasshouse plants, while it declined in shade and sun plants, as compared to their respective control. However, even after decline in Lauric acid content in shade and sun plants, as a result of exposure to direct sunlight, the Lauric acid content remained higher in shade and sun plants as compared to glasshouse plants. Myristic acid content was highest in control sun plants (predawn; 14.97) and least in control glasshouse plants (14.52). Seven days of exposure to sunlight of glasshouse plants resulted in a slight increase (14.68) in Myristic acid, while it resulted in a decrease (14.73) in shade plants as compared to their respective controls. In the course of the day sun plants exhibited slightly lower Myristic acid than seen at predawn. Palmatic acid content was highest in control glasshouse plants (13.81) while it was the least in control shade plants (12.94). Transfer of glasshouse plants to direct sunlight for a period of seven days resulted in a slight decline of the palmatic acid content. However, in shade and sun plants Palmatic acid content increased (3%) as compared to their respective control plants. Stearic acid content was highest in control shade plants (14.22) and least in control glasshouse plants (13.60). Seven days of high light treatment resulted in an increase in the Stearic acid content in glasshouse plants, while it declined in shade and sun plants.

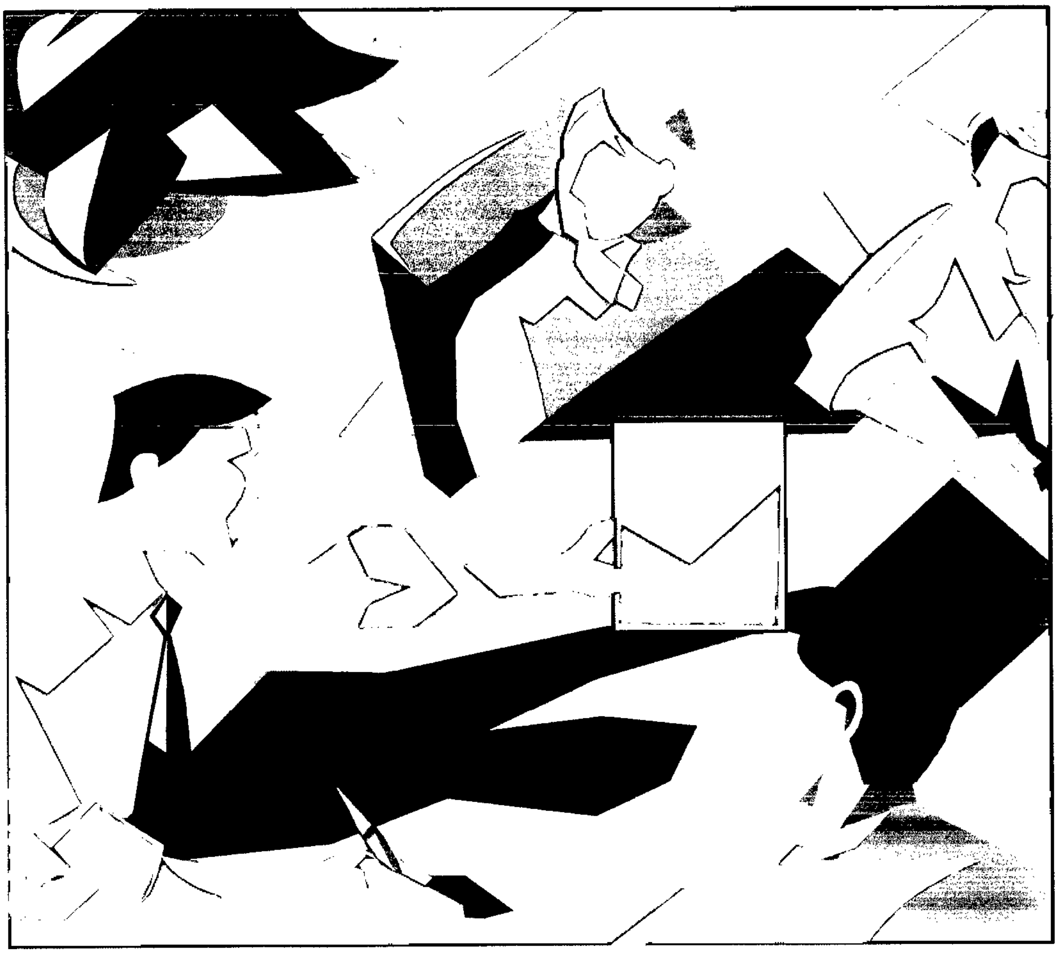
Oleic acid, a single double bond unsaturated fatty acid, was highest in control shade grown plants (15.36) and least (14.67) in control glasshouse grown plants. Transfer of glasshouse plants to direct sunlight for seven days resulted in an increase in oleic acid content, while it declined for the treatment of same duration, in shade and sun plants. Linoleic acid was observed to be highest in control sun grown plants

(15.08) and least (14.78) in control glasshouse grown plants. Linoleic acid content increased as a result of sun exposure in glasshouse plants while it declined in shade and sun plants. Linolenic acid was observed to be highest in control glasshouse plants (14.78) and least in control shade plants (13.06). Glasshouse plants exhibited a decline in the linolenic acid content on transfer to direct sunlight, while it increased in shade plants and sun plants as compared to their respective control.

3.8.5.2. Analysis of fatty acids of thylakoid membrane in chloroplasts isolated from glasshouse, shade and sun grown plants

Lauric acid content was slightly lower (13.85) in chloroplasts isolated from sun grown plants (-PI), while it was similar in chloroplasts isolated from shade and glasshouse plants (13.95; Table 3.22). Six hours of artificial high light treatment resulted in decline in the lauric acid content in isolated chloroplasts from the plants of all the three different growth conditions. Higher myristic acid content was observed in chloroplasts isolated from shade plants (14.68), while chloroplasts isolated from sun plants had the least (14.54) myristic acid content. High light treatment for 6 h resulted in a decline in the myristic acid content of chloroplasts isolated from glasshouse and shade plants as compared to their respective control plants. However, high light treatment did not result in any changes in the myristic acid content of chloroplasts isolated from sun plants. Palmatic acid content was highest in chloroplasts isolated from shade plants (13.93) and least in chloroplasts isolated from glasshouse plants (13.51). Six hours of high light treatment resulted in increase in palmatic acid content of chloroplasts isolated from glasshouse plants and decline in palmatic acid content of chloroplasts isolated from shade and sun plants. Stearic acid was also highest in chloroplasts isolated from shade plants (13.76) and least in chloroplasts isolated from sun plants (13.65). Six hours of high light treatment resulted in a decline in the stearic acid content of chloroplasts isolated from the plants of all the three different growth conditions.

Oleic acid content was observed to be higher in chloroplasts isolated from glasshouse plants and shade grown plants (14.84) and least in chloroplasts isolated from sun plants (14.74). Oleic acid content declined in chloroplasts isolated from the plants of all the three different growth conditions as a result of 6 h of high light treatment. Linoleic acid content was higher in chloroplasts isolated from glasshouse plants (14.81) and was similar in chloroplasts isolated from shade and sun plants (14.76). The content of linoleic acid declined in chloroplasts isolated from glasshouse and shade plants as a result of high light treatment. While in chloroplasts isolated from sun plants it increased at the end of 6 h of high light treatment. Linolenic acid was observed to be highest in chloroplasts isolated from sun plants (14.60) and least in chloroplasts isolated from shade plants (14.06). The content of linolenic acid increased as a result of high light treatment in plants of all the three different growth conditions.



CHAPTER-4

DISCUSSION

DISCUSSION – CONTENTS

DISCUSSION

This study examined photoinhibition and photosynthetic acclimation in rice plants transferred from glasshouse and shade (low light) conditions to direct sunlight (high light) in comparison to rice plants grown under full sunlight conditions. The data presented here reveal characteristic differences in chlorophyll flourescence parameters, photosynthetic pigments, xanthophyll cycle activity, lipid peroxidation, and lipids between rice plants grown at low and high light intensities. Certain acclimative alterations such as xanthophyll cycle meditaed energy dissipation, induced by the high light stress in low light grown plants were similar in both low and high light grown plants, however, there were slight differences in acclimitization process in glasshouse and shade grown plants over a period of seven days, mainly due to the role of Chl *a/b* and lutein in shade grown plants.

4.1. Chlorophyll fluorescence

Glasshouse and shade grown rice plants prior to transfer to direct sunlight (control) and predawn sun grown plants (control) exhibited similar Fv/Fm ratio (Fig. 3.2a. and Table 3.1), indicating similar photosynthetic efficiency in control plants of all the three different growth conditions. On the onset of high light, low light grown plants exhibit photoinhibition for a short period which could be recovered to a large extent over a period of seven days, as plants showed acclimatization to the high light conditions. These results indicate an adaptive mechanism in the low light grown rice plants in response to high light stress.

The higher degree of photoinhibition (decrease in the Fv/Fm ratio) observed in glasshouse grown plants than shade grown plants after 1 h of high light treatment (Fig. 3.2a), indicates that the former experienced higher light stress under comparable light conditions, because of its inability to handle the excess radiation, due to its acclimitization to low light, as photochemical (qP) and non-photochemical quenching (qN) were lower in glasshouse grown plants than observed in shade grown plants (Table 3.1). Higher qN values in shade grown plants indicates higher capacity of non-photochemical quenching in these plants compared to glasshouse grown plants. Acclimation of plants to different light conditions has profound influences on the structure and function of the photosynthetic apparatus. The adaptation of shade grown plants to high light was reflected by the low degree of photoinhibition of PS II occurring in shade grown plants exposed to direct sunlight. Thus indicating that glasshouse grown plants were more sensitive to high light stress and had less capacity for photosynthetic acclimation following transfer to direct sunlight than shade grown plants as demonstrated by an inability to attain Fv/Fm values similar to that of plants grown at higher light intensities (shade grown plants) within the same durations.

Unlike glasshouse and shade grown plants, plants grown in full sunlight did not show significant changes in their photosynthetic efficiency during the course of the day as compared to their predawn values (Fig. 3.2a). Sun grown plants could manage the excess light energy absorbed through increased photochemical as well as non-photochemical quenching processes, in addition to the xanthophyll cycle mediated energy dissipation, as seen by higher qP, qN and xanthophyll cycle pigments in sun grown plants as compared to low light grown plants (Table 3.1 and 3.5).

Prolonged exposure to full sunlight till seven days resulted in photosynthetic acclimation of low light grown rice plants to high light as indicated by the recovery of the Fv/Fm ratio (Fig. 3.2a). Low light grown plants subjected to high light stress initially undergo photoinhibitory damage, but are able to recover from this damage in the course of time. Recovery was possible since the damage was temporary in nature and could be overcome by the various protective mechanisms such as the xanthophyll cycle mediated energy dissipation, which helped the low light grown plants to acclimatize under high light conditions. Since rice plants are genetically capable to grow under high light conditions, glasshouse and shade grown plants exposed to full sunlight for a period of seven days adapt to the high light conditions through better dissipation of excess energy as seen by the increase in the non-photochemical quenching (qN) and recovery of Fv/Fm ratio in our results (Table 3.1). In addition to increase in qN, the increase in qP observed in sun transferred glasshouse and shade grown plants (Table 3.1), shows that the low light grown plants improved their photosynthetic performance in response to increased light flux. Chlorophyll *a/b* ratio also changed in order to deal with the changing light regime (Table 3.5).

In our study with isolated chloroplasts, high light treatment for 6 h did not result in any recovery in the photosynthetic efficiency measured as Fv/Fm ratio (Fig. 3.3). The reason for this being that in intact leaves the photoinhibitory damage was accompanied by adaptive processes. While under *in vitro* conditions, such adaptive processes are absent, therefore no recovery was observed in high light treated chloroplasts.

Inhibition of photosynthesis under high light has been reported earlier by several groups such as Sharma and Hall (1991), Aro et al., (1993), Pearcy and Sims (1994), Demmig-Adams et al., (1997), Krause et al., (1999), Estelle (2001), Zhou et al., (2007) and Ragni et al., (2008). Pearcy and Sims (1994) observed that many plant species grown at low light undergo physiological shock upon exposure to more open high light environment. They reported that sudden exposure of vegetation structurally and functionally attuned to a low irradiance to high light can induce dysfunction in photosynthesis and other physiological activities. In our study, the decrease in the photochemical efficiency (Fv/Fm ratio) observed in glasshouse and shade grown plants after 1 h of sun exposure (Fig. 3.2a) could be a result of physiological shock upon exposure to direct sunlight. Zhou et al., (2007) reported a decline of photosynthetic efficiency in rice seedlings when they were subjected to the combined stress of water and high light. They concluded that the drought-induced inhibition of photosynthesis under different irradiance levels was due to both diffusive and metabolic limitation: diffusive limitations that occurred due to stomatal closure and low mesophyll conductance to carbon dioxide upon moderate drought conditions; and metabolic limitation of photosynthesis which may be related to the adverse effects of the oxidative damage to the chloroplast.

The lower susceptibility to photoinhibition of PS II in high light grown rice plants (shade and sun grown plants) compared to low light grown plants (glasshouse grown plants) observed in the present study (Fig. 3.2a), could be related to faster kinetics and higher degree of V de-epoxidation and an increased pool size of xanthophyll cycle pigments. Under natural conditions, the intensity of full sunlight can be excessive and potentially damaging to the photosynthetic apparatus, causing reduction in the photochemical yield. However, plants acclimated to high light conditions grow well under full sunlight and show little to no sign of damage. This in part could be due to higher Chl *a/b* ratio and also related to an increased capacity of sun leaves to utilize absorbed light in photosynthesis (Björkman 1981), which is indicated by higher Fv/Fm ratios and qP in sun grown plants than in transferred glasshouse and shade grown plants in our study (Table 3.1). Sun grown plants are thought to have protective system that safely dissipate excess light energy, neutralise potentially damaging products or repair damage to the photosynthetic apparatus, thus preventing or minimizing photoinhibition. Photoinhibition and photoprotection are related aspects of plant responses to high light as plants adapt to adverse light environment. The various protective mechanisms include increased photosynthetic capacity (Demmig and Björkman 1987), active PS II repair cycle (Oquist et al., 1992), xanthophylls associated energy dissipation (Gilmore et al., 1996; Sharma and Hall 1991) and antioxidant activity (Havaux and Niyogi 1999; Sankhalkar and Sharma 2002).

Increase in qN in response to high light has been observed by Spunda et al., (1993), Xu et al., (2004) and Guo et al., (2006). Spunda et al., (1993) found that the photochemical apparatus of *Picea abies* was able to acclimate within two days after transfer to high light conditions by an increase in photochemical and non-photochemical quenching processes. Gray et al., (1996) and Huner et al., (1998) reported that cereals such as wheat and winter rye grown under high irradiance are resistant to photoinhibition due to an increased photosynthetic capacity (qP) rather an increased efficiency of non-radiative dissipation. Śpundová et al., (2005), reported that the steady-state values of qN were lower in high light treated barley plants. Similar results were reported by Kurasová et al., (2002), in high-light-adapted barley (1000 μmol $m^{-2}s^{-1}$) as compared with low-light-grown barley (50 μmol $m^{-2}s^{-1}$). They suggested a specific high irradiance-acclimation of barley without a pronounced increase in non-radiative dissipation through thermal and photochemical de-excitation within PS II. However, in our study, rice plants grown at low light intensities (glasshouse and shade) showed an increased efficiency of non-radiative dissipation as well as an increased photosynthetic capacity (Table 3.1).

Similar to our results, photosynthetic acclimation of low light grown plants to high irradiance has been reported earlier by Demmig-Adams et al., (1997), Mohammed and Parker (1999), Krause et al., (2001) and Murchie et al., (2002). Demmig-Adams et al., (1997) noted that in the short term adaptation of low light plants to high light stress, pre-existing leaves rely largely on biochemical and physiological adaptations such as active PS II repair cycle, xanthophylls associated energy dissipation, etc. While in the much longer term adaptation; new folliage is produced that is in structural and functional equilibrium with the new environment. In the present study the adaptation of low light grown rice plants to direct sunlight to seven days is probably a result of energy dissipation through the xanthophyll cycle and modification of Chl *a/b* ratio suitably in order to dissipate the excess light energy. Krause et al., (2001) have shown photosynthetic acclimation of tropical tree seedlings to excessive light in simulated tree-fall gaps. They suggested that high β-carotene levels and increased Chl *a/b* ratios may contribute to photoprotection in these plant species.

4.2. Xanthophyll pigments

Pigments in rice leaves were quantitated by HPLC to observe how their levels change in response to high light stress and to relate it to non-photochemical quenching. The depressions in PS II efficiency were accompanied by retention of Z and A in sun transferred glasshouse and shade grown plants (Table 3.5). The formation of Z indicated the operation of Z-dependent thermal dissipation in sun transferred plants. Glasshouse and shade grown plants exposed to direct sunlight underwent a greater degree of conversion of xanthophyll cycle (V) into its de-epoxidised form Z and A, similar to sun grown plants, than non-sun transferred plants (Table 3.5), and are indicative of an increased allocation of absorbed light towards energy dissipation during high light treatment. An increase in xanthophyll-cycle mediated thermal dissipation observed in this study is potentially beneficial for the rice plants as it can protect the PS II reaction centers from overexcitation and subsequent photoinhibition and it also can reduce the probability of triplet Chl and singlet oxygen formation in the LHCs. This is consistent with a number of studies demonstrating increases in the engagement and capacity for xanthophyll cycle-dependent energy dissipation in both short-term and long-term responses of plants growing at higher light environments (Gilmore and Ball 2000; Verhoeven et al., 2001; Sharma et al., 2002; Adams et al., 2002; Lu et al., 2003, Krause et al., 2004). Verhoeven et al., (2001) reported that the transgenic tobacco with suppressed Z formation on exposure to high light for longer duration resulted in photoinhibition, indicating a photoprotective function for the xanthophyll cycle through energy dissipation.

Thiele et al., (1996; 1998) observed both in the laboratory and in natural tree-fall gaps that a fast phase of recovery from photoinhibition (approximately 1 h) is probably related to the light-dependent turnover of the xanthophyll cycle pigments indicating a protective regulatory response to high light stress. Leitsch et al., (1994) observed that a slower recovery phase (requiring several h) appears to be associated with the turnover of the D1 protein in the PS II reaction center. This suggested that the photoinhibition of PS II in nature is to a large extent caused by a persistent binding of Z to LHC Chl *a* and *b* binding protein complexes and represents a photoprotective down-regulation of PS II that facilitates thermal dissipation of excitation energy in addition to the fast-relaxing ΔpH-dependent quenching (qE mechanism). In our study the relationship between energy dissipation and xanthophyll cycle conversion state was similar for sun grown plants and transferred glasshouse and shade grown plants. In plants the xanthophyll cycle and the ability for energy dissipation thus appear to adjust continuously to increases in light stress encountered (Sharma and Hall 1996; Demmig-Adams 1998; Guo et al., 2006).

4.3. Violaxanthin de-epoxidase (VDE) activity

The xanthophyll cycle is mediated by the enzyme VDE. The increase in the DEI observed in glasshouse, shade and sun grown plants transferred to direct sunlight as compared to their respective control (i.e. plants prior to transfer to direct sunlight) (Fig. 3.10), indicates an increase in the VDE activity on exposure to sunlight. Under high light conditions, the enzyme is activated by a decrease in the pH of the thylakoid lumen, whereas, it is inactive in low light, when the pH in the thylakoid lumen is neutral or alkaline. Therefore, on exposure of glasshouse and shade grown plants to excessive sunlight a pH gradient is generated, which activates the VDE enzyme. The activated enzyme in turn triggers the de-epoxidation of V to A and Z, as seen by a decrease in V and an increase in A and Z in our study (Table 3.6). However, the decrease in V was not proportional to the increase in A and Z, which implies that along with the de-epoxidation of V to A and Z a continuous *de novo* synthesis of Z was also taking place probably via β-carotene. Synthesis of Z from β-carotene has been proposed by Demmig-Adams (1990).

Zeaxanthin was seen in glasshouse and shade grown plants on exposure to sun light but was absent in the sun grown plants (Table 3.6). The absence of Z in sun grown plants could be the reason for the lower DEI (VDE activity) compared to the VDE activity seen in glasshouse and shade grown plants (Fig. 3.10). In sun grown plants, although V was de-epoxidised to A by the activated VDE enzyme under high light conditions, no Z formation was seen probably due to loss of pigments during preparation of PS II particles as V to Z formation was seen in sun plants on analysis of their pigments (Tab.3.5).

Similar to our results, an increase in the VDE activity under high light conditions has been reported in tobacco and spinach by Bugos et al., (1999) and Pandey et al., (2005) respectively. However, Eskling and Akerlund (1998) reported a decrease in the quantity and activity of VDE when spinach plants were shifted from low (100 μmol $m^{-2}s^{-1}$) to high light (950 μmol $m^{-2}s^{-1}$). The decrease found for the amount of VDE indicated the presence of a rapidly responding control system, influencing the synthesis or degradation of VDE. In our study, the higher VDE activity observed in sun exposed glasshouse, shade and sun grown plants could also be due to active synthesis of the VDE enzyme under high light conditions.

Although numerous reports support the hypothesis that A and Z offer a photoprotective function through a process of thermal dissipation measured as non-photochemical quenching in plants, Sun et al., (2001), reported that photosynthesis and plant productivity under the field conditions examined were not strictly dependent on the VDE activity and in turn on the levels of A and Z. Their results suggested that the xanthophyll cycle-mediated non-photochemical quenching does not serve a critical factor in photoprotecting plants against high light under the field conditions examined and they concluded that xanthophyll cycle-related non-photochemical quenching may not be the only protective mechanism against excess light, but it may serve a photoprotective function under conditions of sudden excess light or when the rate of photodamage exceeds the capacity for D1 repairing. They have proposed that the xanthophyll cycle is a member of a protective system that works in parallel or series with other protective processes such as D1 repair cycle, antioxidants, etc. to protect plants against excess light.

4.4. Lutein

In the present study, higher amounts of lutein seen in sun grown plants and the increase in lutein observed after sun exposure in shade grown plants, and to a lesser extent in glasshouse grown plants may indicate a role for lutein in protection against high light stress (Table 3.5). In our study, sun exposed shade grown plants synthesized more lutein than glasshouse and sun grown plants, which may indicate role of lutein under specific conditions such by slowly reversible conversion of lutein epoxide to lutein, which may provide improved photo-protection against triplet chlorophyll and reactive oxygen species under high light stress in addition to the xanthophyll cycle. In glasshouse and sun grown plants there was no increase in the lutein content due to the photoinhibition. Increase in lutein levels occurring in response to high light stress have been reported by Chow (1994), Demmig-Adams et al., (1996), Barker et al., (1998), Koroleva et al., (2000), Niyogi et al., (2001) and Krause et al. (2004). Chow (1994) suggested that since α-carotene derived xanthophylls, lutein and loroxanthin are predicted to be energetically similar to A, they might function in the direct de-excitation of singlet chlorophyll. Niyogi et al., (2001) suggested that lutein plays a role in qE mechanism of energy dissipation in PS II in addition to its structural functions in Chl *a/b* binding LHC. According to them lutein may have a direct role in the quenching of excited singlet Chl *a*. This is energetically feasible, since lutein has the energy level of the lowest singlet excited state below that of Chl *a*. Matsubara et al., (2008) reported that slow lutein epoxide accumulation in *Inga* leaves may improve light harvesting under limiting light, while quick de-epoxidation of lutein epoxide to lutein in response to excess light may enhance photoprotection. A general antioxidative function of lutein also appears feasible (Niyogi et al., 1997).

However, there are reports which suggest that lutein is not essential for light harvesting and photoprotection when Z is present. Pogson et al., (1996) observed that lutein can be replaced in the LHC by other xanthophylls like Z, such that the size of the xanthophyll pool with respect to Chl remains unchanged. They also reported that lutein-deficient mutants show no apparent defect in light harvesting under laboratory conditions. However, lutein deficiency has been shown to alter the molecular organisation of the LHC in *Arabidopsis* (Lokstein et al., 2002).

4.5. Chlorophyll a/b ratio

In the present study the low Chl *a/b* ratio in glasshouse and shade grown rice plants prior to transfer to direct sunlight (Table 3.5), indicates a higher content of Chl *b* compared to Chl *a* in the LHC. Increased amount of Chl *b* in glasshouse and shade grown plants would increase the light absorption cross-section of PS II LHC under low light conditions, which would result in higher amount of light energy transfer to Chl *a* molecules thus facilitating higher rate of photosynthesis also seen as greater Fv/Fm in controls of these plants. However, sun grown plants had a higher Chl *a/b* ratio, indicating a lower amount of Chl *b* compared to Chl *a* in these plants. Lower amount of Chl *b* in sun grown plants decreases the light absorption cross-section of PS II LHC and consequently minimises the light stress under high light conditions by preventing excess excitation of Chl *a* molecules. These differences in Chl *a/b* ratio of rice plants grown in the glasshouse, shade and direct sunlight are indicative of a strong photosynthetic acclimation of rice plants to different irradiances. Murchie et al., (2005) reported similar sun/shade differences in Chl *a/b* of rice plants grown in high irradiance compared to low irradiance.

At a given light flux density, the susceptibility of PS II to photoinhibition depends on the light absorption cross section of the antenna pigments. Therefore, in the present study low light adapted plants having a higher amount of Chl *b* than sun grown plants exhibit stronger photoinhibition initially on transfer to direct sunlight (as seen by a decline in the Fv/Fm ratio; Fig. 3.1), due to an increased absorption of light which could not be utilised in photosynthesis. The relatively small increase observed in the Chl *a/b* ratio occurring in glasshouse and to a higher extent in shade grown plants on the seventh and fifth day respectively after transfer to direct sunlight (Table 3.5), could be due to a selective degradation of the Chl *a+b* binding LHC to diminish excessive photon absorption under high light conditions. This in turn would increase the quantum efficiency of photosynthesis at high light. Štroch et al. (2004) reported that the higher resistance of Chl *b*-less barley mutant chlorine *f2* to high growth irradiances is related particularly to the reduction of functional LHC size and the xanthophyll cycle pigments that prevent the excess excitation of Chl *a* molecules.

4.6. VAZ pool and non-photochemical quenching

In our study characteristic differences between glasshouse, shade and sun grown plants in xanthophyll cycle activity and pools of the pigments involved were observed. Sun grown plants possessed higher pool of VAZ pigments than glasshouse and shade grown plants (Table 3.5). However, transfer of shade grown plants to direct sunlight caused an increase in the pool size of the xanthophyll cycle pigments (V+A+Z) (Table 3.5). The increase in A+Z pool was not entirely due to the de-epoxidation of V but due to the *de novo* synthesis of Z. The increase in VAZ pool was correlated with greater qN. This rise in the xanthophyll cycle pool indicates that the relative concentration of xanthophylls in the LHC of PS II was increased to cope with high irradiance. The results indicate that the xanthophyll cycle, played a major role in energy dissipation and photosynthetic acclimation in transferred glasshouse and shade grown plants as indicated by an increase in A and Z and qN in these plants (Table 3.5 and Fig. 3.9).

The mechanism to explain the increase of VAZ pool under stress conditions is not clearly understood, but two possibilities have been pointed out. Demmig-Adams (1990) suggested formation of Z at the expense of the existing β-carotene pool through the enzyme β-carotene hydroxylase. Schindler and Lichtenthaler (1996) also reported formation of Z from hydroxylation of β-carotene under stress conditions, which also contributes towards the maintenance of the VAZ pool. While, Björkman and Demmig-Adams (1994) suggested *de novo* synthesis of the VAZ pigments through carotenogenesis. Garcia-Plazaola et al., (2002) using a carotenogenesis inhibitor norfluazon demonstrated that the increase of the VAZ pool was mainly due to the *de novo* synthesis of new molecules. In the present study the increase observed in the VAZ pool in transferred shade plants could possibly be a result of *de novo* synthesis of the VAZ pigments.

However, in glasshouse grown plants in our study, the VAZ pool did not increase in response to exposure to direct sunlight, while the contents of de-epoxidised components Z and A increased and this was correlated with the increase in the qN (Table 3.5 and Fig. 3.9a), suggesting that not the total VAZ pool, but the accumulation of its Z and A are effective in photoprotection of glasshouse grown rice plants under high light conditions through a process of thermal dissipation measured as qN. Wang et al., (2005) reported that two super-rice hybrids, with 15 to 20% higher yields than the traditional rice cultivars, were better photoprotected under natural high irradiance stress, by the more efficiently working of their xanthophyll cycle (i.e. accumulation of A and Z) rather than an increase in their VAZ pool.

Increase in VAZ pool in response to high light exposure have been reported before by Bugos et al., (1999), Garcia-Plazaola and Becerril (2000), Ye et al., (2000), Kurasová (2002) and Guo et al., (2006). Bugos et al., (1999) reported an increased pool size of VAZ pigments throughout leaf development in field-grown tobacco plants. Studies conducted by Ye et al., (2000) on *Phaseolus vulgaris* subjected to the combined stress of high irradiance and high temperature resulted in increase in the de-epoxidated state of xanthophyll cycle, indicating that Z-dependent thermal dissipation was the major energy dissipation pathway during the combined stress of high irradiance and high temperature.

Bilger and Björkman (1990) demonstrated that VAZ pool size is not influenced by PPFD as such, but by the amount of PPFD in excess for photosynthesis. A daily cycle with increases in the VAZ pool at midday has been described in a wide diversity of species and habitats such as the alpine plants (Streb et al., 1997) or the subtropical shrub *Schefflera arboricola* (Schiefthaler et al., 1999). Leaves are also able to adjust (increase) their VAZ pool size after transfer to stressful conditions, as has been shown in response to drought treatments by Demmig et al., (1988), or chilling by Koroleva et al., (2000), or salinity stress by Sharma and Hall (1992). The reverse pattern (decrease in VAZ pool size) has been also described when plants are shifted to non-stressful conditions by Björkman and Demmig-Adams (1994). Niinemits et al., (1998) suggested that VAZ pool size adjustments would provide a mechanism for leaf acclimation in the time scale of days, as was also seen in our study in glasshouse and shade grown plants exposed to sunlight for a period of seven days (Table 3.5).

4.7. Xanthophyll cycle and non-photochemical quenching

In the present study, the increase in Z was accompanied by an increase in qN in sun exposed plants. A correlation was observed between the levels of Z+A and levels of qN in glasshouse and shade grown plants transferred to direct sunlight for a day (Fig. 3. 9a and b), indicating that thermal dissipation of excitation energy is dependent on the xanthophyll cycle activity. However, when sun exposure had been extended up to seven days, the increase in qN was less proportional to the A+Z pool, since A+Z was seen to increase while qN decreased, indicating that, part of the qN may not be xanthophyll cycle dependent under long-term photoinhibitory conditions.

Correlation between levels of Z+A and qN has been reported by Demmig-Adams (1998), Niyogi et al., (1998), Chang et al., (2000), Ruban et al., (2004) and García-Mendoza and Colombo-Pallotta (2007). A central role of Z in non-radiative dissipation of excess light energy was confirmed by Niyogi et al., (1998) by using *npq1* mutants of *Chlamydomonas reinhardtii* and *Arabidopsis thaliana* defective in violaxanthin de-epoxidase activity. Similarly Chang et al., (2000) observed that qN was greatly reduced in the antisense VDE in tobacco plants. They demonstrated that a significant level of the qN requires de-epoxidation of V.

However, some authors have reported no correlation between Z and qN (Johnson et al., 1993, Havaux and Niyogi 1999, Davison et al., 2002 and Eisenstadt et al., 2008). Experiments by Davison et al., (2002) on *Arabidopsis* indicated that doubling of amount of xanthophyll cycle carotenoids in PS II antenna had no visible effect on the maximum non-photochemical quenching. Their results demonstrated that photoprotection mediated by the xanthophyll cycle is not solely a result of the involvement of Z in non-photochemical quenching.

Rice plants fed with ascorbate, a stimulator of the de-epoxidation state of violaxanthin to zeaxanthin, showed higher Fv/Fm ratios and qN values than in DTT fed rice leaves (Fig. 3.12). In ascorbate fed leaves V was de-epoxidised to Z, indicating the efficient thermal dissipation of xanthophyll cycle (Table 3.8). On the contrary DTT inhibited de-epoxidation of the xanthophyll cycle as well as the xanthophyll cycle associated energy dissipation process in the pigment bed. Hence, a decrease in the Fv/Fm ratios and qN values are observed in the DTT fed rice leaves, indicating that an increased amount of energy reaches the PS II reaction centre, due to the absence of Z formation, thereby causing greater damage in the DTT-fed rice leaves. Allthough an increase in A and qN was observed in glasshouse grown plants fed with DTT after 3 h of sun exposure (Table 3.7 and 3.8), it did not prevent the decline in the Fv/Fm ratio as a result of high light exposure. Thus A showed little effects in thermal dissipation under these conditions. The presence of A in DTT fed plants indicates partial inhibition of the xanthophyll cycle in these plants (Fig. 3.11b). Absence of Z under high irradinace in DTT treated leaves has also been reported by Ye et al., (2000).

DTT fed rice leaves also showed more damage to qP in our study, than ascorbate fed rice leaves (Table 3.7). Since ascorbate fed rice leaves could dissipate the excess energy absorbed more efficiently through the de-epoxidation of V to A and Z, than the DTT fed rice leaves, the AsA fed plants had better photosynthetic ability. Similar to our results, a decline in Fv/Fm, qN and VAZ were observed by Guo et al.,

(2006) in DTT fed leaves compared to control ones under high irradiance. A decrease in the Fv/Fm ratio in DTT fed leaves has also been reported by Li et al., (2007) in *Ipomoea setosa* plants treated with 30 min of high irradiance. The obvious decrease in the Fv/Fm ratio in DTT fed leaves under high irradiance implies that the xanthophyll cycle could protect PS II efficiently under short-term high irradiance.

Interestingly, Z was absent in shade and sun grown plants fed with ascorbic acid, indicating that in shade and sun grown plants the concentration of AsA used did not stimulate the xanthophyll cycle, hence, compared to AsA fed glasshosue grown plants no recovery of photochemical efficiency was observed in these plants on exposure to sunlight (Table 3.7 and 3.8).

4.8. Lipid peroxidation and zeaxanthin

In our study, in addition to the decrease in the photosynthetic efficiency, an increase in MDA production was also observed as a result of high light treatment (Fig. 3.4). Increases in MDA are indicative of increased lipid peroxyl radical formation in the cell and thylakoid membrane as a result of increased production of singlet oxygen within the LHCs during sun exposure. This indicates ROS related oxidative damage to plants under our experimental conditions.

The decrease observed in the amount of MDA production after three days of transfer to sunlight in glasshouse and shade grown plants as compared to day one after transfer (Fig. 3.4a), in our study, could be due to the activity of both enzymatic and non-enzymatic antioxidants, as discussed by Ye et al., (2000) and Verhoeven et al., (2005). This could also be due to Z, which also acts as an antioxidant in addition to its role in energy dissipation. It is widely thought that the main function of Z is as a quencher of the excited chlorophyll state through the process of non-photochemical quenching (Müller et al., 2001). Comparative analysis of different *npq* mutants demonstrated that the xanthophyll cycle, in addition to their dissipative function in the antenna may also play a crucial role in membrane fluidity, thermostability and in the protection of the membrane against lipid peroxidation (Havaux et al., 2000; Davison et al., 2002; Baroli et al., 2003). They suggested that Z may protect from light stress by directly quenching singlet oxygen and free radicals, thus preventing the accumulation of lipid peroxides. Havaux and Niyogi (1999) reported that when lipid contents were measured in response to high illumination in *npq1* mutant, without VDE, in comparison to the wild *Arabidopsis*, *npq1* mutant had significantly higher level of lipid photooxidation, indicating that photoprotection mediated by the xanthophyll cycle is not solely a result of the involvement of Z in qE, but seems to be more complex. Consistent with these observations, Davison et al., (2002) found that *Arabidopsis* plants over-expressing β-carotene hydroxylase, which catalyses the conversion of β-carotene to Z, showed reduced lipid peroxidation compared to the wild type under stress conditions. Thus, it seems that Z may be important in the termination of lipid peroxidation chain reactions by decreasing the thylakoid membrane fluidity (Tardy and Havaux 1997). They suggested that the decrease in fluidity could be important by lowering the penetration of reactive oxygen species inside the thylakoid.

Antioxidant roles of Z become evident only during longer-term high irradiance (on the time scale of days; Li et al., 2002). Jin et al., (2003) reported that Z participates in the protection of photo-damaged and disassembled PS II core complexes during PS II repair cycle. In the present study the decrease in MDA production observed in glasshouse and shade grown plants on longer duration of exposure to direct sunlight (Fig. 3.4a), could possibly be due to the antioxidant role of

Z, in addition to other non-enzymatic and enzymatic antioxidants (which have been studied in detail in our laboratory; Sankhalkar and Sharma 2002; Bhandari and Sharma 2006). However, the increase in MDA production observed in cell membrane lipids of sun grown plants in our study (Fig. 3.4a), indicates limitation of protection by the xanthophyll cycle and other antioxidative processes against oxidative damage in the sun grown rice plants.

In our study with isolated chloroplasts, high light treatment for 6 h resulted in a continuous increase in the amount of lipid peroxidation (Fig. 3.4b). The reason for this could be that unlike intact leaves, isolated chloroplasts did not have any enzymatic or non-enzymatic antioxidants, which could protect it against lipid peroxidation. Even though sun grown plants exhibited higher lipid peroxidation than glasshouse and shade grown plants (Fig. 3.4a), chloroplasts isolated from sun grown plants had lower level of lipid peroxidation compared to chloroplasts isolated from glasshouse and shade grown plants (Fig. 3.4b), indicating that sun grown plants had greater level of protection of thylakoid membrane lipids against ROS.

4.9. Xanthophyll cycle and ABA

When rice plants were fed exogenously with ABA the V pool need not be diverted towards the ABA synthesis as it was made available exogenously. Therefore, in the present study, in sun exposed plants most of the V could be available for conversion to Z as indicated by higher amount of Z in ABA treated plants (Table 3.10), thus resulting in higher qN (efficient energy dissipation) (Fig. 3.13d) and provided better protection to PS II against photoinhibition as indicated by higher Fv/Fm ratios in +ABA plants at 1630 h as compared to –ABA plants (Fig. 3.13c). This suggests that exogenous application of ABA enhanced the potential activity of energy dissipation and reduced the high light induced photoinhibition of PS II probably by enhanced V pool for de-epoxidation to Z. Zhou et al., (2006) reported that ABA-treated plants had higher Fv/Fm and qN than water treated plants during chilling. Even though in our study the content of Z and qN was higher in shade plants fed with ABA through cut petioles, it did not enhance the photosynthetic efficiency of these plants (Fig. 3.15b).

Since V has been proved as a precursor in the ABA biosynthetic pathway (Parry and Horgan 1991; Rock and Zeevart 1991; Marin et al., 1996; Pogson et al., 1996; Audran et al., 1998; North et al., 2007), it can be said that exogenously applied ABA reduces the requirement of V for the ABA biosynthesis resulting in an increased pool of V to be converted to Z during exposure to full sunlight, in rice plants grown with medium supplemented with ABA in comparison to rice plants grown under –ABA conditions in the present study. The increase in Z in +ABA rice plants is also reflected with increase in qN as compared to –ABA rice plants (Fig. 3.13b). Ivanov et al., (1995) reported that exogenous application of ABA to barley seedlings enhances their resistance to photoinhibition at low temperature and co-related it with enhanced activity of xanthophyll cycle. In their study the activity of the xanthophyll cycle measured by the epoxidation state of xanthophyll under high light treatment was higher in ABA treated plants compared with non-ABA treated plants. Pasqualini et al., (1999) in studies conducted on *Nicotiana tabacum* suggested the existence of a relationship between xanthophyll cycle pigments and ABA synthesis. They reported that after ozone treatment, V level in leaves strongly decreased which was matched by the increase in the ABA content as A and Z contents did not change. Sharma et al., (2002) also observed that *Sorghum* seedlings grown on ABA supplemented medium had better energy dissipation (greater qN) and much greater level of de-epoxidation (high level of Z) than non-ABA seedlings.

In plants violaxanthin exists in a pool of cis and trans isomers. Phillip et al., (1999) reported that the isomerization of V from trans to cis form takes place under high light stress. Schwartz et al., (1997) concluded that the trans form of V is responsible for conversion to Z while the cis form is converted to ABA. Plants experiencing high light stress are also likely to face water stress which may result in rate limitation for synthesis of Z or ABA due to limitation of V pool as discussed by Phillip et al., (1999), since the V has to convert either to ABA or Z. When greater amount of V pool is used for ABA synthesis, then it may result in limitation of Z synthesis and thereby affecting energy dissipation under excess light condition which may result in photodamage to PS II. Similarly if the V pool is used more for Z formation it may result in limitation of ABA which may affect various physiological functions such as stomatal limitation and plants may suffer water loss, which could predispose plants to photoinhibitory damage even at low light intensities. Thus plants must have a very efficient system to balance ABA and Z formation and to provide the best protection.

In the present study the lower Fv/Fm ratios in control (non-sun exposed) salt stressed plants (+NaCl-PI) in comparison to control (non-sun exposed) non-salt stressed plants (-NaCl-PI) indicate that NaCl causes damage to the photochemistry of PS II thus lowering the photosynthetic efficiency in salt stressed plants (Fig. 3.17). Salt stress causes decrease in plant growth and productivity by disrupting physiological processes, especially photosynthesis. It may cause a rate limitation in the photosynthetic system, thereby, disposing the photosynthetic system to overenergization even at low light (Sharma and Hall 1992). Similarly, the photosynthetic efficiency of PS II was affected upon high light exposure in both

-/+NaCl plants but on longer exposure recovery was observed (Fig. 3.17). However, in –NaCl plants the recovery of Fv/Fm was to a higher extent than +NaCl plants, due to better dissipation of the excess energy as seen by the increase in qN and Z (Table 3.13 and 3.14).

Although Z was absent in control non-salt treated plants in the present study, exposure to sunlight resulted in formation of Z (Table 3.14). The observed increase in Z content is consistent with increase in non-photochemical quenching on exposure to direct sunlight in -NaCl plants. As the V pool is used for ABA synthesis it limits the formation of Z in plants treated with NaCl and exposed to sunlight. This probably is the reason why plants subjected to salinity stress showed an increase in the endogenous ABA level while Z was absent (Table 3.14).

It is generally believed that ABA plays a key role in the process of acclimation of the photosynthetic apparatus of plants to various environmental stresses. Similar increase in endogenous ABA levels under stress has been reported by Li and Walton (1990), Parry et al., (1992), Popova et al., (1996), Bravo et al., (1998), Cramer and Quarrie (2002) and Makela et al., (2003). Li and Walton (1990) and Parry et al., (1992) using etiolated leaves and roots have reported correlations between reductions in V and Nx levels and increase in ABA and its metabolites following stress. A similar reduction in V and Nx levels and an increase in ABA content were observed in glasshouse grown plants subjected to salt stress in the present study (Table 3.14).

Chandler and Robertson (1994) reported that ABA is involved in the expression of numerous stress-induced genes as well as expression of genes that are independent of stress. Andersson et al., (1994) observed that exogenous ABA treatment enhances the chilling resistance of maize seedlings along with the induction of antioxidant systems. Further, it has been reported by Campos and Thi (1997) that ABA had a protective effect on membrane lipids and has suggested an enhanced capacity to maintain membrane integrity. This could 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 enzyme respectively, ABA treatment may prevent damage to membrane thereby facilitating efficient operation of the xanthophyll cycle, which provides 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 Z 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. Since V pool was made available for formation of Z, while in plants subjected to salt stress the increase in ABA was probably from V which limited the V pool for Z formation undermining energy dissipation in high light treated plants. These results indicate a possible role of ABA in energy dissipation, probably by further stimulating the xanthophyll cycle.

4.10. Carbohydrate pool

Measuring carbohydrate pools does not provide direct information about photosynthetic rates, but it does give information about their use, amount of stored carbohydrates and the form in which they are stored. Furthermore, it has been reported that plants that are stressed typically accumulate higher amounts of carbohydrates in the soluble form (sucrose and other competent small sugars such as glucose and fructose). Therefore, the ratio of soluble sugars to starch is indicative of the level of stress in the leaf (Stitt and Hurry 2002; Lutts et al., 2004).

In the present study shade and sun grown plants prior to transfer to direct sunlight stored higher amounts of carbohydrates as compared to glasshouse grown plants, as indicated by higher concentration of soluble and insoluble carbohydrates in shade and sun grown plants (Table 3.4), which may be due to higher photosynthetic rates in these plants as compared to glasshouse grown plants as seen by their higher qP value (Table 3.1). On transfer to direct sunlight plants grown in all the three different growth conditions increased the amount of both soluble and insoluble carbohydrates, indicating no limitation to carbohydrate metabolism under high light conditions. However, an increase in the ratio of soluble to insoluble carbihydrates was observed in sun grown plants at midday, indicating some amount of limitation to carbohydrate metabolism under high light conditions in plants grown under full sunlight. (Fig. 3.5). Külheim and Jansson (2005) reported increased soluble to insoluble sugar ratio in *npq* mutants under field condition, indicating higher levels of stress in these mutants under high light. The accumulation of carbohydrates is proposed to be part of adaptive mechanisms that help to stabilize proteins and membranes under stress conditions.

4.11. Lipids and fatty acids

The slight qualitative changes observed in the phosphoglycolipids in the glasshouse grown plants after one day of sun exposure, indicate a role of these lipids in protecting the plants which were grown at very low light and then transferred to high sun light as such qualitative changes were not seen in shade and sun grown plants (Fig. 3.19). Very little work has been done with reference to high light and changes in lipids profile in higher plants. A few studies have been done on algae but indicate more of a quantitative change in the lipid profile than qualitative (Nishida and Murata 1996; Walsh et al., 1997). No qualitative changes were observed in the neutral lipids of glasshouse and shade grown plants on exposure to direct sunlight (Fig. 3.21), indicating that the neutral lipids did not play any significant role in the acclimation of low light grown plants to high light. Although, some of the PGL and neutral lipids were common to the plants grown in all the three different growth conditions, a few of the PGL and neutral lipids were present exclusively only in glasshouse or shade or sun grown plants (Table 3.15 and 3.17). Thus, indicating requirement of different type of lipid composition of rice plants in response to their different growth irradiances which on transfer to high light change qualitatively (more so in glasshouse grown plants) and quantitatively on exposing to high sun light as was seen in this study (Fig. 23a and c). These qualitative and quantitative changes in the lipids may be required for better functioning of the biochemical reactions under the changed conditions in order to provide protection or acclimation.

The increase in the glyco and phospholipids may be due to an increase in the enzymes involved in lipid synthesis in order to change the lipid composition of the membrane (Nishida and Murata 1996). However, we have not studied enzymes involved in the lipid biosynthesis.

Plants are known to change their membrane lipid composition in response to the alteration of major environmental factors. The changes in the lipid composition and content observed in the present study may be related to adaptation and acclimation of the rice plants under the changing environmental conditions, in order to sustain optimum structure and function of the cell and thylakoid membrane to maintain active photosynthesis even under stress conditions. The thylakoid membrane has to be stabilized in a bilayer structure by increasing the concentration of the bilayer forming lipids such as DGDG and phosholipids (Mock and Kroon 2002). This probably could be the reason for the increase observed in the concentration of glycolipids and phospholipids in glasshouse and shade grown plants on transfer to direct sunlight (Fig. 3.23a and c). In higher plants it is well known that light largely stimulates "*de novo*" synthesis of lipids and fatty acids. Klyachko-Gurvich et al., (1999) observed changes in lipid or fatty acid quality during light acclimation of plants in addition to quantitative changes in chloroplast lipids. Tasaka et al., (1996) reported that this change is more distinct in combination with low temperatures. However, in our study with isolated chloroplasts, high light treatment did not result in any increase in the glyco and phospholipid content (Fig. 3.23b and d), since chloroplasts being an isolated system "*de novo*" synthesis of lipids was not possible.

The present data shows that high light treatment resulted in a slight decline in the saturated fatty acids content of total lipids (Table 3.21), thus changing the ratio of unsaturation to saturation level of fatty acids in favour of unsaturation, which may be required for the adaptation of low light grown plants to direct sunlight. The slight increase observed in unsaturated fatty acids would also change the ratio of fatty acid in the membrane towards the unsaturation level, leading to increased membrane fluidity and adaptation to stress conditions. These regulating mechanisms are probably required to establish membrane integrity and consequently active photosynthesis in plants under the altered light conditions.

Guerfel et al., (2008) also reported an increase in the degree of unsaturation in leaf lipids of olive trees in response to water stress. Walsh et al., (1997) reported that the changes in the fatty acid composition due to high level of light intensity may occur as a cellular response to reduce the susceptibility of the membrane to photooxidation. Gombos et al., (1997) reported that the unsaturation of the fatty acids in membrane lipids did not affect the extent of light-induced damage but accelerated the recovery from damage. Further, it has been pointed out by Siegenthaler and Murata (1998) that the degree of fatty acid desaturation and the lipid composition in thylakoid membranes is critical in maintaining optimal photosynthesis.

The study indicates that the primary photoprotective mechanism utilised by the rice plants transferred from low light to high light conditions, is increased xanthophyll cycle mediated energy dissipation in addition to changes in Chl *a/b* ratio, lutein and lipids and fatty acids. The investigation shows that the glasshouse and shade grown rice plants adjusted physiologically to direct solar irradiation in a similar fashion over a period of few days, as in high light grown plants.

CONCLUSIONS

- Rice plants possess a highly flexible photosynthetic apparatus that not only allows them to grow under different PPFD regimes, but also to adapt to changes in the light environment.

- Transfer of low light grown plants to direct sunlight caused damaging effects on parameters such as photochemical efficiency of PS II and lipid peroxidation in short term.

- Changes in the parameters such as higher qN, increased VDE activity, higher A and Z content, higher VAZ pool, increased Chl *a/b* ratio and higher lutein levels under high light conditions maybe responsible for providing the adaptation to low light grown plants, as seen by acclimatization of these plants to high light conditions within a period of seven days.

- A correlation was observed between the levels of Z+A and levels of qN and photoprotection in glasshouse grown plants, which was not so strong in shade grown plants exposed to sunlight for longer than one day, indicating that part of the qN may not be xanthophyll cycle dependent under long-term photoinhibitory conditions.

- Plants fed with exogenous ABA showed better photoprotection than plants treated with NaCl, indicating that the V was converted into Z in ABA fed plants, thereby protecting the plants under high light stress. While in NaCl treated plants V was converted into ABA and not Z, thereby undermining energy dissipation in NaCl treated plants.

- Qualitative as well as quantitative changes observed in lipids and fatty acid composition may also play an important role in acclimatization of low light grown rice plants to high light conditions.

- The data presented here reveals acclimative alterations in the photosynthesis, xanthophyll cycle mediated energy dissipation and lipids in glasshouse, shade and sun grown plants in their responses to natural sunlight under field conditions, where the plants are subjected to fluctuations in the intensity of the solar radiation during the course of the day.

- The acclimation allows the rice plants to maintain or even improve photosynthetic performance before new leaves develop under the altered light regime.

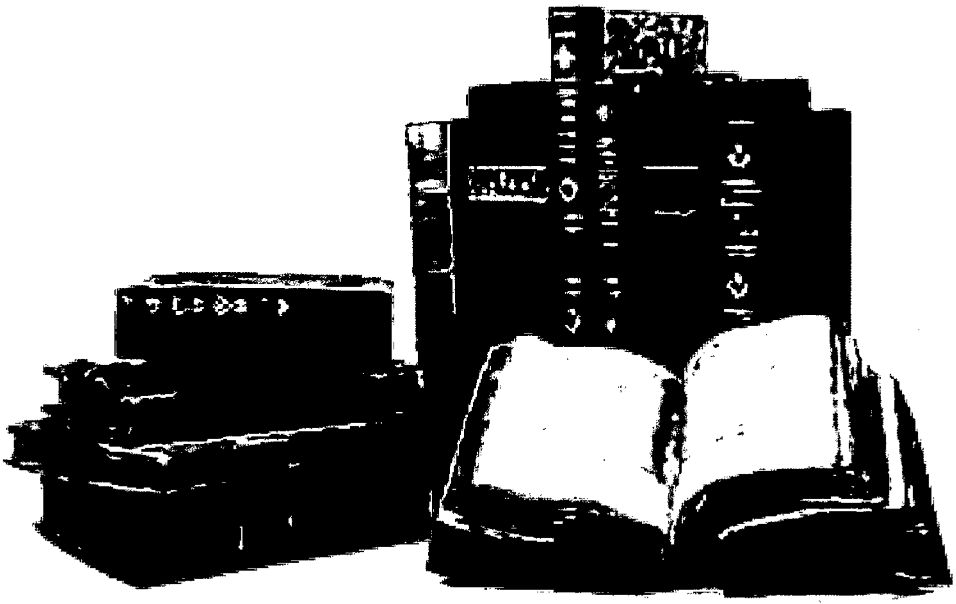
Future Plan of Work:

We plan to carry out further research work on the following aspects:

1. We would like to follow up this study till the reproductive stage to see the effect of high light stress on the grain yield of low light grown plants and compare it with that of high light grown plants.

2. In the present study we have subjected the plants to high light stress at the vegetative stage. We would also like to study the effect of high light on the yield of low light grown plants when they are subjected to high light stress at the flowering stage and compare it to that of sun grown plants.

3. Understanding physiological and biochemical processes under high light stress in the present work, we would like to relate it to the molecular level by studying expression of various genes related to photoinhibition and protection/recovery such as violaxanthin de-epoxidase and enzymes of antioxidant activity such as SOD, APX, GSH and enzymes of ascorbate biosynthetic pathway such as VTC2 and VTC5.



CHAPTER-5

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Publications

CHAPTER THIRTY FIVE

Effect of NaCl and Exogenously Supplied ABA on Xanthophyll Cycle Pigments and Energy Dissipation in Rice Plants Under High Light Stress

Prabhat Kumar Sharma and Janet Vaz

Abstract In the present study rice (*Oryza sativa* L. cv. Jyothi) plants grown in the shade and direct sunlight were subjected to salt stress (100 mM) or fed with ABA (10 mM) through roots to investigate their influence on the light-dependent Z formation and energy dissipation. Plants (both −NaCl/+NaCl and −ABA/+ABA) were then treated with high light by exposing them to direct sunlight (1,200–2,200 μmol m^{-2} s^{-1} PFD). Leaf samples were collected for chlorophyll fluorescence measurements, pigment and ABA analysis at different intervals during the exposure. Results show plants subjected to salinity and high light stress showed an increase in the endogenous ABA level while Z was absent. Fv/Fm and qN remained more or less same in plants grown with NaCl and photoinhibited and plants grown without NaCl and photoinhibited. Neoxanthin (Nx) content were much higher in salt grown plants but declined initially after onset of sun light treatment but increased after longer duration of the exposure. When plants were grown with exogenously supplied ABA and subsequently exposed to sunlight most of the V was made available for conversion to Z and this was correlated with higher level of qN and better photoprotection.

Keywords ABA, high light stress, NaCl, non-photochemical quenching, xanthophyll cycle

Introduction

To avoid photoinhibitory damage under excess light conditions the plants have developed several adaptive and protective mechanisms, one such process is ΔpH dependent xanthophyll cycle which play an important role in the heat dissipation under excessive light. This cycle consists of light dependent conversion of three xanthophylls in a cyclic reaction, i.e., violaxanthin (V) is de-epoxidised to zeaxanthin (Z) via an intermediate antheraxanthin (A).

Violaxanthin also act as a precursor to biosynthesis of abscisic acid (ABA), a stress-related hormone, by a dioxygenase via the synthesis of xanthoxin (Hirschberg 2001). Endogenous levels of ABA increases in response to a variety of stresses, including salinity (Cramer and Quarrie 2002). Since V serves as a common precursor for

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Photoinhibition and photosynthetic acclimation of rice (*Oryza sativa* L. cv Jyothi) plants grown under different light intensities and photoinhibited under field conditions

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Thirty-days old rice (*Oryza sativa* L. cv. Jyothi) plants grown under the greenhouse (150-200 µmol m^{-2} s^{-1}) or shade (600-800 µmol $m^{-2}s^{-1}$) were exposed to 7 days of full sunlight and compared with plants grown under direct sunlight (1200-2200 µmol $m^{-2}s^{-1}$).Transfer of greenhouse and shade plants to full sunlight for a day resulted in a decline in their photosynthetic efficiency (F_v/F_m) and an increase in non-photochemical quenching (qN). The decline in F_v/F_m was much greater in transferred greenhouse plants (33%) as compared to transferred shade-plants (20%). Sun-plants did not show much variation in the F_v/F_m ratio (4%) from their predawn measurements (control). The sun-grown plants showed a higher pool of xanthophyll pigments (violaxanthin + antheraxanthin + zeaxanthin). Transfer of greenhouse and shade-plants to full sunlight resulted in an increase in lutein, Chl *a/b* ratio, antheraxanthin (A) and zeaxanthin (Z) content. Increase in A and Z was correlated with the increase in the qN. The increase in the A and Z content was due to increase in the activity of violaxanthin de-epoxidase. Greenhouse and shade plants on exposure to sunlight showed an increase in lipid peroxidation (LPO). Prolonged exposure of greenhouse and shade plants up to 7 days resulted in recovery of the F_v/F_m, an increase in Z and A and a decline in the LPO. The study demonstrated that rice plants grown at lower light intensities initially underwent photoinhibitory damage on exposure to full sunlight, but were able to acclimate to the high irradiance by dissipating the excess light through various mechanisms such as an increase in lutein, high Chl *a/b* ratio and xanthophyll cycle, suggesting use of energy dissipation as a mechanism of protection against high irradiance, but to different extent and to some extent by different processes. The study was unique, as plants were grown and photoinhibited under natural conditions rather than the artificial light, as was the case in most of the studies so far. Results showed better adaptation of high-light grown plants and suggested role for chl a/b ratio and lutein, in addition to xanthophylls cycle in shade plants. Low-light grown plants could also completely adapt to full level of sunlight within 3 days of the treatment and xanthophylls cycle (measured as V, A and Z) and activity of de-epoxidase seemed to be important in this adaptation.

Keywords: Energy dissipation, Lipid peroxidation, Non-photochemical quenching, Photosynthetic acclimation, Photosynthetic efficiency, Rice plants, Sunlight, Xanthophyll cycle, Chlorophyll a/b ratio, Violaxanthin de-epoxidase

Photoinhibition occurs when light energy absorbed by plants exceeds the capacity of light utilization in photosynthesis[1,2]. The excess of absorbed light energy leads to production of reactive oxygen species (ROS) and damage the photosynthetic apparatus, if not dissipated safely[3]. Due to its specific physiochemical properties, photosystem II (PSII) is more susceptible to photoinhibition and/or photo-oxidative damage than photosystem I (PSI)[4]. Photoinhibition of PSII can be easily detected *in vivo* by a decrease in the 'dark-adapted' ratio of variable to maximum chlorophyll *a* fluorescence[5]. Photo-oxidative damage to cellular lipids can be used to indicate oxidative damage to biological membranes[6]. The susceptibility of plants to photoinhibition depends on the species and growth light environments. In general, the plants grown at low-light intensities are more susceptible to photoinhibition than at high-light intensities[3]. Since photoinhibition lowers plant growth and productivity, its avoidance is essential for the survival of plants in natural habitats for which plants have developed various processes[7].

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Abbreviations: A, antheraxanthin; Chl, chlorophyll; DPS, de-epoxidation statc; F_v/F_m, photosynthetic efficiency of photosystem II; LHCII, light harvesting complex II; L, lutein; LPO, lipid peroxidation; MDA, malonaldehyde; PFD, photon flux density; PSI, photosystem I; PSII, photosystem II; qN, non-photochemical quenching; qP, photochemical quenching; TBA, thiobarbutaric acid; TCA, trichloroacetic acid; V, violaxanthin; VDE, violaxanthin de-epoxidase; XCP, xanthophyll cycle pigments; Z, zeaxanthin.

Communicated manuscripts:

Quantitative and qualitative changes in the lipids and fatty acid profile of the cell and thylakoid membrane of rice plants grown at low and moderate light conditions and subsequently exposed to full sunlight

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Abstract

The effect of high light on the quantitative and qualitative changes in the lipids and fatty acid profile of the cell and the thylakoid membrane along with oxidative damage to membrane lipids was studied in thirty days old rice (*Oryza sativa* L. cv. Jyothi) plants grown under low (150-200 µmol $m^{-2}s^{-1}$) or moderate (600-800 µmol $m^{-2}s^{-1}$) light conditions and results were compared with rice plants grown in high (1200-2200 µmol $m^{-2}s^{-1}$) light conditions. High light treatment of plants and isolated chloroplast resulted in an increase in the amount of MDA formation, indicating oxidative damage to lipids. Qualitative and quantitative changes in the phosphoglycolipids and quantitative changes in neutral lipids were observed in rice plants grown under the different growth conditions. A few of the phosphoglycolipids and neutral lipids were present exclusively only in low or moderate or high light grown plants, indicating requirement of different type of lipid composition of rice plants in response to their different growth irradiances. Quantitative changes were also observed in the concentration of the different saturated and unsaturated fatty acid groups of total lipids in low, moderate and high light grown rice plants, as a result of high light treatment. No qualitative changes in the fatty acid composition due to difference in growth irradiance or high light treatment were seen. The changes observed in the lipids and fatty acid composition of cell and thylakoid membrane of low, moderate and high light grown rice plants in response to high light, are probably the result of physiological changes in the rice plants, in order to sustain optimum

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Relationship between xanthophyll cycle and non-photochemical quenching in rice plants grown at low and moderate light conditions and subjected to high light stress

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Abstract

Thirty days old rice plants grown under low and moderate light conditions were transferred to full sunlight for one day, to observe the extent of photoinhibitory damage and protective mechanism with reference to study the adaptation and the relationship between xanthophyll cycle and non-photochemical quenching (qN) under changing light environment. Results were compared to rice plants grown in high light conditions. Low and moderate light grown plants prior to transfer to direct sunlight and predawn high light grown plants exhibited similar Fv/Fm ratio, indicating similar photosynthetic efficiency in control plants. Low light grown plants exhibited higher degree of photoinhibition than moderate light grown plants after 1 h of high light treatment, indicating that the former experienced higher light stress under comparable light conditions, because of its inability to handle the excess radiation, as qN was lower in low light grown plants than moderate light grown plants. Higher qN values in moderate light grown plants indicates better capacity of energy dissipation in these plants compared to low light grown plants. The increase in qN was accompanied by retention of A and Z in sun exposed plants. The formation of Z indicated the operation of Z-dependent thermal dissipation in sun transferred plants. Rice plants fed with ascorbate, a stimulator of the de-epoxidation state of violaxanthin to zeaxanthin, showed higher Fv/Fm ratios and qN values than in DTT (an inhibitor of xanthophyll cycle) fed rice leaves. This indicates that an increased amount of energy reaches the PS II reaction centre, due to the absence of Z formation, thereby causing greater damage to photosynthesis in the DTT-fed rice leaves. The study highlights the importance of the xanthophyll cycle in protecting the rice plants grown under low and moderate light conditions and subsequently transferred to high light, by dissipating the excess light energy through non-photochemical quenching.

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