

PHOTOSYNTHESIS DURING PROGRESSIVE WATER STRESS
IN INTERIOR SPRUCE (*PICEA GLAUCA* (MOENCH) VOSS):
PHYSIOLOGY AND PROTEIN COMPOSITION

by

Potter Ann Kathryn Eastman

B.Sc., University of Guelph, 1983

M.Sc., University of Guelph, 1989

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Botany & The Biotechnology Laboratory)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 1996

© Potter Ann Kathryn Eastman, 1996

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Botany

The University of British Columbia
Vancouver, Canada

Date 22 April 1996

Abstract

Although the stress responses of conifers have been extensively investigated in terms of growth and survival, the ramifications for the perennial photosynthetic apparatus are not well characterized. Coping with excess radiation is particularly important for conifers because the structurally expensive needles usually support multiple seasons of photosynthesis, surviving a range of conditions limiting carbon dioxide fixation. This thesis represents the first detailed characterization of a coordinated set of photosynthetic responses to progressive water stress by young interior spruce (*Picea glauca* (Moench) Voss X *P. engelmanni* Parry hybrid complex). Analysis of photosynthesis at the tissue and thylakoid membrane levels revealed a pattern of down-regulation that corresponded generally to that described for other species; however, it differed in that protective mechanisms were initiated during a moderate level of stress.

As water potentials declined, an integrated repertoire of mechanisms to cope with excess light energy unfolded. As mild water stress developed at water potentials below -1 MPa, gas exchange measurements indicated stomatal closure and minimal carboxylation. As moderate water stress developed below -1.6 MPa, chlorophyll fluorescence revealed decreases in photochemical efficiency. Nonphotochemical quenching was readily light-saturated in spruce seedlings at any water status; however, quenching increased during moderate water stress at low irradiances, revealing an effect on energy dissipation. Most significantly, electron flow through Photosystem II (PSII) decreased during moderate stress, in contrast to reports for angiosperm species.

Details of the thylakoid response to water stress were obtained by immunoquantification of proteins using antibodies to two of the oxygen-evolving enhancer proteins (OEE1 and OEE2), the D1 reaction centre protein, cytochrome *b*₅₅₉, chlorophyll *a/b* binding proteins, and

ATP synthetase at different stress phases defined by physiological criteria. This revealed that only D1 levels diminished early in the imposition of water stress; other protein levels were unaffected until the stress became severe. Synthesis of D1, calculated from ^{35}S -methionine labelling of needle proteins, decreased by 80 % in stressed trees whereas it was considerably enhanced in recovering trees, suggesting that D1 synthesis was limiting during water stress. A novel observation was that the membrane association of OEE2 with PSII, assessed by susceptibility to removal by detergent, decreased significantly during moderate stress. This occurred before photoinhibition suggesting that this nuclear-encoded protein is intimately involved in PSII down-regulation in spruce, possibly as a component of reversible inactivation.

Table of Contents

ABSTRACT	ii
TABLE OF CONTENTS.....	iv
LIST OF FIGURES	vi
LIST OF TABLES	ix
LIST OF ABBREVIATIONS	x
ACKNOWLEDGMENTS.....	xi
FOREWARD.....	xii
CHAPTER 1: OVERVIEW OF OXYGENIC PHOTOSYNTHESIS.....	1
1.1 CURRENT VIEW OF PHOTOSYSTEM II	1
1.2 REGULATION OF THE OEE PROTEINS	7
1.2.1 Transcription and Translation.....	7
1.2.2 Post-translational regulation.....	8
1.2.3 OEE Proteins and Stress	10
1.3 OXYGENIC PHOTOSYNTHESIS AND STRESS.....	12
1.3.1 Impact of Stress on PSII in Gymnosperms	12
1.3.2 Impact of Water Stress on PSII in Angiosperms.....	16
CHAPTER 2: REGULATION OF PHOTOSYNTHESIS IN SPRUCE DURING PROGRESSIVE WATER STRESS	19
2.0 SUMMARY	19
2.1 INTRODUCTION.....	20
2.2 MATERIALS AND METHODS	22
2.2.1 Experimental Rationale.....	22
2.2.2 Plant Material.....	23
2.2.3 Induction of Drought Stress.....	25
2.2.4 Measurement of Gas Exchange	25
2.2.5 Measurement of Chlorophyll Fluorescence.....	27
2.2.6 Data Analysis.....	28
2.3 RESULTS.....	29
2.3.1 Carbon Assimilation.....	29
2.3.2 Oxygen Evolution.....	34
2.3.3 Chlorophyll Fluorometry.....	39
2.4 DISCUSSION	59
CHAPTER 3: THYLAKOID PROTEIN CHANGES DURING PROGRESSIVE WATER STRESS.....	66
3.0 SUMMARY	66
3.2 MATERIALS AND METHODS	70
3.2.1 Antibody Production.....	70

3.2.1.1	<i>OEE Antibodies</i>	70
3.2.1.2	<i>D1 Antibody</i>	71
3.2.1.3	<i>Other Antibodies</i>	73
3.2.2	<i>Needle tissue</i>	73
3.2.3	<i>Membrane Isolation</i>	75
3.2.3.1	<i>Protocol Development</i>	75
3.2.3.2	<i>Preparation of Membranes</i>	76
3.2.3.3	<i>Effects of Triton X-100</i>	78
3.2.4	<i>Immunoquantification</i>	78
3.2.5	<i>Measurement of Electron Transport</i>	85
3.3	RESULTS	85
3.3.1	<i>Antibody Testing</i>	85
3.3.2	<i>Immunoquantification</i>	87
3.3.1.1	<i>Intrinsic Thylakoid Proteins</i>	87
3.3.1.2	<i>Extrinsic Thylakoid Proteins</i>	94
3.3.2	<i>Electron Transport</i>	97
3.4	DISCUSSION	99
CHAPTER 4: D1 PROTEIN SYNTHESIS BEFORE, DURING AND AFTER WATER STRESS		109
4.0	SUMMARY	109
4.1	INTRODUCTION	110
4.2	MATERIALS AND METHODS	111
4.2.1	<i>Experimental Rationale</i>	111
4.2.2	<i>Protocol Development</i>	112
4.2.3	<i>Plant Material</i>	114
4.2.4	<i>Radiolabelling of Needle Proteins</i>	115
4.2.5	<i>Preparation of Membranes</i>	116
4.2.6	<i>Fluorography and Immunoprobng</i>	118
4.2.7	<i>Direct Scintillation Counting</i>	121
4.3	RESULTS	124
4.3.1	<i>Immunoprobng</i>	124
4.3.2	<i>Fluorography</i>	129
4.3.3	<i>Direct Scintillation Counting</i>	132
4.4	DISCUSSION	140
CHAPTER 5: COMPREHENSIVE VIEW OF PHOTOSYNTHESIS IN SPRUCE DURING WATER STRESS		144
5.1	A HIERARCHY OF PHOTOSYNTHETIC REGULATION	144
5.2	FUTURE WORK...	150
LITERATURE CITED		152

List of Figures

Figure 1.1 Flow diagram of the hierarchy of photosynthetic change during progressive water stress summarizing information reported in the literature for angiosperm species.....	17
Figure 2.1 Relationships between predawn water potential and net CO ₂ fixation (a), stomatal conductance (b), and intercellular CO ₂ concentration (c) in interior spruce seedlings (Study I).....	30
Figure 2.2 Relationships between predawn water potential and net CO ₂ fixation (a), stomatal conductance (b), and intercellular CO ₂ concentration (c) in interior spruce seedlings and emblings (Study II).....	32
Figure 2.3 Light response curves for average oxygen evolution of freshly detached needles from interior spruce seedlings and emblings during Phases 1, 2 and 3 (Study II).....	35
Figure 2.4 Light response curves for average oxygen evolution of freshly detached needles from interior spruce seedlings during Phases 1, 2, 3, and 4 (Study III).....	37
Figure 2.5 Trends in photochemical efficiency in dark adapted needles (F_v/F_m) and photochemical efficiency of open PSII centres under a given actinic irradiance (Φ_P ; calculated as F_v'/F_m' (Study II).....	41
Figure 2.6 Trends in photochemical efficiency in dark adapted needles (F_v/F_m) and photochemical efficiency of open PSII centres under a given actinic irradiance (Φ_P ; calculated as F_v'/F_m' (Study III).....	43
Figure 2.7 Average photochemical efficiency of PSII (Φ_{II}) at increasing irradiance for interior spruce seedlings and emblings during Phases 1, 2 and 3 (Study II)	47
Figure 2.8 Average photochemical efficiency of PSII (Φ_{II}) at increasing irradiance for seedlings during Phases 1, 2, 3, and 4 (Study III).....	49
Figure 2.9 Average photochemical quenching (q_P) at different irradiances during Phases 1, 2, 3, and 4 (Studies II and Study III).....	51
Figure 2.10 Average nonphotochemical quenching (q_N) at different irradiances during Phases 1, 2, 3, and 4 (Studies II and Study III).....	53
Figure 2.11 Relationship between nonphotochemical quenching (q_N) at different irradiances and dark-adapted quantum yield (F_v/F_m) in response to predawn shoot water potentials in interior spruce trees (Studies II and Study III)	55
Figure 2.12 Relationship between average photochemical efficiency of PSII (Φ_{II}) and average oxygen quantum yield (Φ_{O_2}) during Phases 1, 2, 3, and 4 (Studies II and Study III).....	57

Figure 3.1 Immunoblots of interior spruce thylakoid proteins probed with the indicated antibodies showing the intensity of the antibody response with increasing amounts of protein.....	81
Figure 3.2 Relationship of average values of integrated optical density (IOD) of protein bands with increasing amounts of spruce thylakoids (sample set 'Y') probed with various antibodies.	83
Figure 3.3 Immunoprobings of interior spruce membrane protein preparations with antibodies for D1 and CP11: Representative protein immunoblot of thylakoids and Triton-treated membranes from needles harvested from seedlings during Phase 1, 2, 3 or 4 of water stress (Studies II and III).....	88
Figure 3.4 Immunoprobings of interior spruce needle protein blots with antibodies for OEE1 or OEE2, and CP11 from thylakoid and Triton-treated membrane preparations from spruce trees during Phase 1, 2, 3, and 4 of water stress.	95
Figure 4.1 Increases in the integrated optical density of ^{35}S -methionine labelled D1 bands in fluorography standards with increasing levels of label incorporated into total protein estimated by trichloroacetic acid precipitation.....	119
Figure 4.2 Relationship of values from direct scintillation counting of ^{35}S -methionine incorporation into D1 protein solubilized from acrylamide gels with increasing levels of label incorporated into total protein.	122
Figure 4.3 Immunoprobings of needle membrane samples from well-watered (W), stressed (S) and recovering R trees: Comparison of D1 levels in Triton-treated membranes and comparison of D1 levels in the 40,000 x g supernatant fraction of needle membrane samples treated with Triton X-100 at alkaline pH.....	125
Figure 4.4 Fluorography of ^{35}S -methionine incorporation into D1 protein in thylakoids prepared from well-watered, water-stressed and recovering spruce trees, (a) Coomassie Brilliant Blue stained SDS-polyacrylamide gel and (b) autoradiograms.....	127
Figure 4.5 Fluorographic estimation of ^{35}S -methionine incorporation into D1 protein in thylakoids prepared from well-watered, water-stressed and recovering spruce trees	130
Figure 4.6 Direct scintillation counting of ^{35}S -methionine incorporation into total protein precipitated by trichloroacetic acid from thylakoids prepared from well-watered, water-stressed and recovering spruce trees.	134
Figure 4.7 Comparison of ^{35}S -methionine incorporation in total protein, precipitated by trichloroacetic acid, and in D1 protein, determined by fluorography, in thylakoids prepared from well-watered, water-stressed and recovering spruce trees.	136

Figure 4.8 Direct scintillation counting of ^{35}S -methionine incorporation into D1 protein solubilized from acrylamide gels.....	138
Figure 5.1 Flow diagram of the hierarchy of photosynthetic change during progressive water stress for interior spruce seedlings.....	145

List of Tables

Table 1.1 Protein and gene names of the protein constituents of an eukaryotic oxygen-evolving PSII reaction centre.	2
Table 2.1 Average predawn water potential (Ψ) and dark-adapted quantum yield (F_v/F_m) for interior spruce seedlings and emblings in the different water stress phases (Studies II and III)	40
Table 3.1 Details of the protocol used for immunoblot development.	72
Table 3.2 The effects of Triton treatment on: average chlorophyll a/b ratios for thylakoids and Triton-treated membranes for the different sets of interior spruce plants analyzed, and on OEE2 levels in Triton-treated membranes relative to thylakoids (Studies II and III)..	79
Table 3.3 Protein quantification from immunoblots of thylakoids, before and after Triton X-100 treatment, isolated from interior spruce seedlings and emblings at different phases of water stress (Study II).	90
Table 3.4 Protein quantification from immunoblots of thylakoids, before and after Triton X-100 treatment, isolated from interior spruce seedlings at different phases of water stress (Study III)..	91
Table 3.5 Protein quantification from immunoblots of thylakoids, before and after treatment with Triton-X 100, isolated from spruce seedlings (Lethal Stress Study)	93
Table 3.6 Electron transport activities of thylakoid membranes and Triton X-100 treated thylakoids isolated from well-watered and lethally severely water-stressed interior spruce seedlings (Lethal Stress Study)	98
Table 4.1 Average predawn shoot water potential and dark-adapted maximal fluorescence quantum yield (F_v/F_m) of well-watered, water-stressed and recovering interior spruce seedlings.....	115
Table 4.2 Direct counting of ^{35}S present in thylakoid preparations from needles of well-watered (W), water-stressed (S), and recovering (R) spruce trees	118

List of Abbreviations*

Ci	= intercellular CO ₂ concentration (substomatal)
Φ_{II}	= average photochemical efficiency of PSII, calculated as $\Delta F/F_m'$, where ΔF is the difference between F_v and F_v'
Fm	= maximal chlorophyll fluorescence induced in dark-adapted tissue by a saturating flash
Fm'	= maximal chlorophyll fluorescence induced in light-adapted tissue by a saturating flash
Fo	= dark level chlorophyll fluorescence induced by application of a pulsed, low intensity (<1 $\mu\text{mol m}^{-2} \text{s}^{-1}$) red measuring light in dark-adapted tissue
Fo'	= dark level chlorophyll fluorescence induced by application of a pulsed, low intensity (<1 $\mu\text{mol m}^{-2} \text{s}^{-1}$) red measuring light in light-adapted tissue
ΦO_2	= apparent quantum yield using oxygen evolution rates corrected for dark respiration
ΦP	= average photochemical efficiency of open PSII centres under a given actinic irradiance calculated as F_v'/F_m'
Fv	= variable chlorophyll fluorescence induced in dark-adapted tissue by application of actinic light
Fv'	= steady state variable chlorophyll fluorescence induced by application of actinic light
Fv/Fm	= maximal photochemical efficiency of PSII in dark-adapted leaves
PSI	= photosystem I
PSII	= photosystem II
qN	= nonphotochemical quenching of chlorophyll fluorescence
qP	= photochemical quenching of chlorophyll fluorescence
Ψ	= shoot predawn water potential
Ψ_{TLP}	= shoot water potential at the point of turgour loss

* fluorescence definitions and abbreviations according to van Kooten and Snel (1990)

Acknowledgments

I am indebted to the members of my committee, Drs. Edith Camm, John Carlson, Beverley Green, and Rob Guy for their thoughtful advice and encouragement. I thank Dr. S.C. Grossnickle for extensive consultations and use of his research facilities and Ray Folk for his expertise, as well as Darren Goetze and Selma Rosenthal for interesting and helpful discussions. I feel especially privileged to have enjoyed thoughtful and witty inspiration from Dr. Edith Camm.

I am grateful for financial support from the Natural Sciences and Engineering Research Council of Canada, the Science Council of British Columbia and British Columbia Research Incorporated (BCRI), and Dr. Edith Camm.

I thank the organizations that contributed the plant material and the antibodies essential for my research. Spruce emblings were donated by BCRI. Seedlings were donated by BCRI, Pelton Reforestation and Surrey Nursery. Plasmid pPND1 was a gift from Dr. P.J. Nixon to Dr. B.R. Green. D1 and OEE2 antibodies were generous contributions from Dr. Roberto Barbato (Universita di Padova) and Dr. Abul Ekramadoullah (Forestry Canada, Victoria), respectively. The antibody to cytochrome *f* was a gift of Dr. R. Malkin (University of California at Berkeley). Antibodies to the chlorophyll *a/b* binding proteins of PSII (CPII), ATP synthetase, and cytochrome *b*₅₅₉ were provided by Dr. B.R. Green (University of British Columbia).

Foreward

Some of the work reported in Chapter 3 of this thesis was previously published in 1995 as a journal article in *Tree Physiology* (volume 15, pages 229 to 235) entitled, "Regulation of photosynthesis in interior spruce during water stress: changes in gas exchange and chlorophyll fluorescence" by P. Ann K. Eastman and Edith L. Camm. A. Eastman designed and conducted the experiments, interpreted the results in collaboration with E. Camm, and wrote the article with the scientific and editorial advice of E. Camm.

Chapter 1: Overview of Oxygenic Photosynthesis

1.1 Current View of Photosystem II

The supramolecular complexes that conduct the light reactions of photosynthesis have been intensively studied over the last decade and significant progress has been achieved in structural elucidation of the two photosystems. The photosystem I reaction centre is structurally similar to that of the green sulphur bacteria (Buttner *et al.*, 1992; Liebl *et al.*, 1993; Kuhn *et al.*, 1994) whereas the organization of the photosystem II (PSII) reaction centre is analogous to the purple bacterial reaction centre complex characterized by crystallography (Michel and Deisenhofer 1988). Three dimensional details of the supramolecular structure of PSII have been further characterized by electron microscopy (Haag *et al.*, 1990; Boekema *et al.*, 1995) and sedimentation analysis (Tanimura *et al.*, 1994). Nevertheless, the organization of the components that reduce the reaction centre in oxygenic photosynthesis is still controversial (Ford *et al.*, 1995; Frankel and Bricker 1995).

In eukaryotes, the minimal macromolecular assembly that is capable of light-driven oxygen evolution from water consists of the integral proteins of the reaction centre core (D1 and D2), cytochrome *b₅₅₉*, two chlorophyll *a* binding proteins (CPa-1 and CPa-2), the *psbI* gene product and the extrinsic oxygen-evolving enhancer (OEE) protein, OEE1, (reviewed in Xu and Bricker 1992), complexed with a tetranuclear manganese cluster, calcium and chloride cofactors, pigments and quinones (reviewed in Yachandra *et al.*, 1993). Table 1.1 indicates the protein and gene names for these major constituents of the PSII reaction centre. Recently, an additional low molecular weight protein, the *psbW* gene product, associated with the PSII

Table 1.1 Protein and gene names of the protein constituents of a eukaryotic oxygen-evolving PSII reaction centre, (a) all major proteins (Xu and Bricker 1992), and (b) alternative names for the oxygen enhancing extrinsic (OEE) proteins.

(a)

Protein Name	Molecular Weight (kDa)	Gene Name(s)	Coding Site
D1 (Q _B -protein)	32	<i>psbA</i>	chloroplast
D2	34	<i>psbD</i>	chloroplast
cytochrome <i>b₅₅₉</i>	9, 4.5	<i>psbE, psbF</i>	chloroplast
CPa-1 (CP47)	47	<i>psbB</i>	chloroplast
CPa-2 (CP43)	43	<i>psbC</i>	chloroplast
<i>psbI</i>	4.8	<i>psbI</i>	chloroplast
<i>psbW</i>	6.1	<i>psbW</i>	nucleus
OEE1	30-33	<i>psbO (oeel)</i>	nucleus
OEE2	21-24	<i>psbP (oeel2)</i>	nucleus
OEE3	16-18	<i>psbQ (oeel3)</i>	nucleus

(b)

Protein Name	Alternate Protein Names
OEE1	33-kDa, OEC33, <i>psbO</i> , OE33, P ₃₃ , PS II-O, oxygen-evolving enhancer 1, Mn-binding, Mn-stabilizing (MSP)
OEE2	23-kDa, OEC23, <i>psbP</i> , OE23, P ₂₃ , PS II-P, oxygen-evolving enhancer 2
OEE3	17-kDa, OEC17, <i>psbQ</i> , OE17, P ₁₇ , PS II-Q, oxygen-evolving enhancer 3

reaction centre core was described (Irrgang *et al.*, 1995). This discovery emphasizes that many structural and functional details of PSII are still undetermined.

In oxygenic photosynthesis, the eukaryotic PSII reaction centre is associated with nuclear-encoded OEE1, OEE2 and OEE3 (Westhoff *et al.*, 1985) whereas the prokaryotic PSII centre is associated with OEE1 plus two proteins that may be functionally analogous in some respects to OEE2 and OEE3 (Shen and Inoue 1993; Shen *et al.*, 1995). The function and stoichiometry of the OEE proteins are somewhat enigmatic (Xu and Bricker 1992). The OEE proteins were originally thought to be actively involved in the process of oxygen evolution itself (Barber 1984) and, although evidence for redox activity of OEE1 (Raval *et al.*, 1994) and OEE2 (Nievelstein *et al.*, 1995) has revived discussion of enzymatic roles, OEE proteins are currently of interest because there is growing evidence that they are associated with control of PSII organization and activity. The structural and functional relationships between the OEE proteins and the reaction centre have been extensively investigated using a variety of techniques.

Genetic analyses using mutants have provided interesting information on the role(s) of OEE1. In green algae, analysis of a *psbO* deletion mutant indicates that OEE1 is essential for stable assembly of photosystem II and oxygen evolution in developing chloroplasts (Mayfield *et al.*, 1987a); however, removal of OEE1 from assembled spinach thylakoids does not prevent oxygen evolution, provided high levels of calcium and chloride are present (Bricker 1992). Analyses of cyanobacterial deletion mutants have established that OEE1 is not essential for PSII assembly nor absolutely required for oxygen evolution (Burnap and Sherman 1991); however, PSII function is impaired and the mutants are more susceptible to photoinhibition and dark inactivation (Burnap *et al.*, 1992; Boichenko *et al.*, 1993; Engels *et al.*, 1994; Philbrick *et al.*, 1995; Komenda and Barber 1995). OEE1 is thought to have a primary role in protection of the

manganese cluster although OEE1 itself does not ligate manganese (Debus 1992). Recent work with double mutant cyanobacteria has shown that OEE1 has a definite effect on the activity of manganese in the reaction centre, somehow regulating manganese binding or redox activity (Chu *et al.*, 1994) and other workers have revived the claim that OEE1 is active in manganese oxidation (Raval *et al.*, 1994). Studies using recombinant OEE1 protein expressed in *E. coli* and used to reactivate OEE-depleted PSII membranes from a higher plant have just begun (Betts *et al.*, 1994). Hopefully, these investigations will clarify some of the distinctions between the prokaryotic and eukaryotic PSII centres, as well as the critical functions and features of OEE1.

The two smaller extrinsic thylakoid proteins, OEE2 (typically 23 kDa) and OEE3 (typically 16 kDa) are present in all green algae and vascular plants studied to date (Ko *et al.*, 1990) and functional equivalents that bind to OEE1 and affect oxygen evolution have been identified in photosynthetic prokaryotes (Shen and Inoue 1993; Shen *et al.*, 1995). In eukaryotes, OEE2 and OEE3 are implicated in maintaining the appropriate ionic environment for oxygen evolution (Debus 1992; Shen and Inoue 1993) and are necessary for physiological levels of oxygen production (Mayfield *et al.*, 1987b; Enami *et al.*, 1991; Rova *et al.*, 1994). Green algal mutants lacking OEE2 (*psbP* mutants) show increased requirements for Cl^- , and a related, increased sensitivity to photoinhibition which results in a lower PSII content in high light-grown cells; characteristics similar to membrane preparations chemically depleted of OEE2 (Rova *et al.*, 1994). OEE2 is also proposed to modulate the structural environment for binding of the Ca^{2+} associated with the water oxidation capacity of PSII (Homann 1987; Burda *et al.*, 1995). Some biophysical evidence indicates that OEE1 binds Ca^{2+} (e.g., Zhang *et al.*, 1995). Recent studies using $^{45}\text{Ca}^{2+}$ have clearly demonstrated that OEE2 and, possibly OEE1 and

OEE3 as well, affect Ca^{2+} binding by restricting the rate of ion exchange with the lumen (Adelroth *et al.*, 1995).

Structural analyses investigating the macromolecular associations among OEE and PSII reaction centre core proteins have revealed further details of the nature of these interactions. Nearest neighbour studies have suggested that the N-terminus of OEE1 is closely associated with the large extrinsic loop of an integral chlorophyll *a* binding protein, CPa-1 (Odom and Bricker 1992; Frankel and Bricker 1992). Biotin labelling of membrane bound and free OEE1 indicates that this interaction occurs via a salt bridge between OEE1 and CPa-1 (Frankel and Bricker 1995). A mutation in CPa-1 affecting a basic residue, thought to be involved in OEE1 binding, resulted in reduced oxygen evolution capacity, fewer functional PSII centres, and increased sensitivity to photoinhibition, although electron transport through PSII was unaffected (Putnam-Evans and Bricker 1992). This phenotype is similar to cyanobacterial mutants lacking OEE1 (Burnap *et al.*, 1992). Cross-linking studies have also shown that OEE1 is associated with cytochrome *b*₅₅₉, the *psbI* protein (Enami *et al.*, 1992), and the D1/D2 heterodimer (Mei *et al.*, 1989), and it may be important in stabilizing CPa-2 in assembling (Hiramatsu *et al.*, 1991) and disassembling (Yamamoto and Akasaka 1995) PSII centres. Other work has intimated that binding of OEE1 may stabilize the putative dimeric structure of the PSII reaction centre (Tanimura *et al.*, 1994).

Recent work has provided details of the intermolecular arrangement of the three OEE proteins (Ford *et al.*, 1995). There is some controversy as to whether OEE1 provides a binding site for OEE2 (de Vitry *et al.*, 1989); however, the two proteins can be crosslinked by reagents with a chain length of 11 angstroms or less (Enami *et al.*, 1990). Reconstitution studies indicate that OEE2 provides a binding site for OEE3 (Miyao and Murata 1989; Chapman *et al.*, 1989)

and may also bind directly with the PSII reaction centre core (Miyao and Murata 1983; Bernier and Carpentier 1995), possibly with CPa-2 (de Vitry *et al.*, 1989). This structural arrangement of the OEE proteins is supported by analyses with various *Chlamydomonas* mutants (de Vitry *et al.*, 1989), although many details of the interactions are still unknown (Frankel and Bricker 1995).

Biophysical analyses have shown correlations between PSII activity and membrane association of the OEE proteins. This has been demonstrated in isolated thylakoid preparations using *in vitro* measurements of oxygen evolution (e.g., Miyao *et al.* 1987; Homann 1988b, Burnap *et al.*, 1994), electrophoresis (Nedunchezian and Kulandaivelu 1993), chlorophyll fluorescence (e.g., Jursinic *et al.*, 1990; Krieger *et al.*, 1993; Burnap *et al.*, 1994), and EPR (e.g., Boussac *et al.*, 1989; Thompson *et al.*, 1989; Blubaugh *et al.*, 1991). Results from numerous recent studies using thermoluminescence (e.g., Ono *et al.*, 1992; Homann and Madabusi 1993; Krieger *et al.*, 1993; Burnap *et al.*, 1994) further emphasize the structural impact of the association of the OEE proteins on oxygen evolution. Information on the effects of the OEE proteins on membrane surface charge (Ivanov *et al.*, 1990), structural modeling of OEE1 (Beauregard 1992; Xu *et al.*, 1994), and immunocharacterization (Yamamoto and Akasaka 1995) allow further speculation on the biochemical interplay among the OEE proteins and the PSII centre. Although all of the current models of oxygenic photosynthesis suggest that these interactions may be regulatory, many details are still unknown.

1.2 Regulation of the OEE Proteins

1.2.1 Transcription and Translation

Transcript analyses show that there is an association, in some circumstances, between OEE gene expression and changes in PSII activity (Oswald *et al.*, 1990; Ko *et al.*, 1990). This was investigated during the establishment of the C₄ cycle in differentiating leaves of certain grass species; the low levels of OEE2 and OEE3 in bundle sheath cells correlate closely with the lower capacity for oxygen evolution relative to mesophyll cells, which suggests that these proteins have a regulatory role (Hofer *et al.*, 1992; Meierhoff and Westhoff 1993). In some instances, there are reduced OEE transcript levels concomitant with a loss of PSII activity (Oswald *et al.*, 1990). In other C₄ species, however, transcript levels are not limiting but OEE proteins show diminished accumulation which indicates that post-transcriptional control is involved (Hofer *et al.*, 1992).

Although transcript levels may be low, OEE proteins can accumulate in developing etioplasts, sometimes to significant levels (e.g., Hashimoto *et al.*, 1993). In developing chloroplasts that conduct C₃ photosynthesis, levels of OEE proteins initially increase as transcript levels of *psbO*, *psbP* and *psbQ* increase; however, in mature chloroplasts OEE protein levels remain constant although mRNA levels show a steady decline (Sheen *et al.*, 1987). Further evidence that transcript levels are not limiting for OEE1 and OEE2 production was demonstrated in tobacco plants transformed with antisense mRNA constructs for *psbP* and *psbO* in which transcript levels were suppressed but physiological levels of OEE1 and OEE2 were attained (Palomares *et al.*, 1993a,b).

It is not clear what controls transcription and translation of OEE proteins.

Overexpression of OEE1 in *Chlamydomonas reinhardtii* (Mayfield 1991) and the OEE2 protein in tobacco (Palomares *et al.*, 1993b) demonstrated the apparent lack of any homeostatic mechanisms to limit transcription or translation of OEE proteins. Phytohormone mediated control of OEE1 expression was demonstrated in etiolated lupine cotyledons; OEE1 level increased in response to cytokinin and was inhibited by abscisic acid treatment (Kusnetsov *et al.*, 1994). In cultured conifer tissue, cytokinin treatment transiently decreased production of chloroplast proteins, including OEE1 (Mazari and Camm 1993). In a cyanobacterium, site-directed mutations disrupting the highly conserved disulphide bridge (Kuwabara *et al.*, 1987; Borthakur and Haselkorn 1989), essential for OEE1 binding to PSII and oxygen evolution (Tanaka and Wada 1988; Irrgang *et al.*, 1992), had no effect on transcript levels but resulted in no detectable accumulation of the mutant OEE1 (Burnap *et al.*, 1994). A reversible chemically induced conformational change of the single conserved cysteine residue in OEE2 also affected rebinding and protease susceptibility (Kuwabara and Suzuki 1995). These observations emphasize a significant role for post-translational control of OEE protein levels.

1.2.2 Post-translational regulation

Post-translational regulation is perhaps the most important level of control for the OEE proteins. There are multiple processes involved, including organellar and luminal targeting, transit, and processing, as well as alterations in PSII association. The bipartite transit peptides for targeting luminal proteins have functional and structural similarities including a hydrophilic region that targets the nascent proteins into the chloroplast and a hydrophobic region that directs the polypeptides to the lumen (e.g., Clausmeyer *et al.*, 1993; Cline *et al.*, 1994). Yet

there are interesting distinctions between the mechanisms for translocation of the different OEE proteins into the lumen (e.g., Hulford *et al.*, 1994; Robinson *et al.*, 1994; Robinson and Klosgen 1994). In both prokaryotic and eukaryotic oxygen-evolving organisms, the luminal import of OEE1 involves a prokaryotic-type transport mechanism that requires stromal factors, probably a nucleotide binding protein, and ATP (Hoffman and Franklin 1994; Hulford *et al.*, 1994).

Association with a molecular chaperone in a transit complex, as established for chlorophyll *a/b* binding protein, was not demonstrated for OEE1 (Li *et al.*, 1995); however, recently a nuclear-encoded chloroplast SecA protein, a soluble chaperone, was identified and shown to stimulate the *in vitro* transport of OEE1 into the lumen, confirming that OEE1 import is very similar to the prokaryotic secretion mechanism (Nakai *et al.*, 1994; Nohara *et al.*, 1995). In contrast, import of OEE2 and OEE3 does not need stromal factors or nucleotides but does have an absolute requirement for a transmembrane proton electrochemical potential difference (Robinson and Klosgen 1994; Cline *et al.*, 1994). Competition studies have shown that the OEE proteins probably use the same ATP-requiring import process to enter the chloroplast, where they are processed by stromal-processing peptidases, and then are targeted for different translocation routes into the lumen, where thylakoid peptidases complete the processing (Clausmeyer *et al.*, 1993).

Once correctly imported and processed, OEE proteins are coupled with PSII by a light-mediated event, photoactivation, which is necessary for initiation of oxygen evolution by a nascent or regenerated PSII centre. In this process, photooxidized manganese ligates to the D1/D2 heterodimer as a tetranuclear complex and is stabilized by the association of OEE1 with the reaction centre (Tamura and Cheniae 1988; Hashimoto *et al.*, 1993). Absence of OEE1 in cyanobacterial mutants increases the susceptibility of Mn to dissociate reversibly from PSII and

reactivation requires religation of Mn (Engels *et al.*, 1994). With respect to the presence of the different OEE proteins within the developing chloroplasts, some differences have been reported among protist (Mizobuchi and Yamamoto 1989), angiosperm (Ryrie *et al.*, 1984; Tamura and Cheniae 1988; Hashimoto *et al.*, 1993) and gymnosperm (Mariani *et al.*, 1990; Shinohara *et al.*, 1992a,b; Kamachi *et al.*, 1994) species. In *Euglena*, accumulation of OEE1 appears to be the rate-limiting step for development of oxygen-evolving capacity, unlike higher plants and algae in which OEE1 and OEE2 accumulate in the dark (Mizobuchi and Yamamoto 1989; Hiramatsu *et al.*, 1991). The specific association of the OEE proteins with one another is dependent on binding to the PSII reaction centre; there is no apparent interaction among OEE proteins free in the lumen (Miyao and Murata 1989; Chapman *et al.*, 1989; de Vitry *et al.*, 1989).

1.2.3 OEE Proteins and Stress

Photosystem II is intrinsically susceptible to light damage (reviewed in Barber 1994) and any environmental stress that decreases carboxylation augments this damage (Aro *et al.*, 1993a). Frequently, stress-induced decreases in PSII activity have been correlated with changes in the levels of the OEE proteins. *In vivo*, photoinhibitory light exposure (e.g., Hundal *et al.*, 1990), freezing (Shen *et al.*, 1990), chilling (Wang *et al.*, 1992a), viral infection (Takahashi and Ehara 1992), exposure to acidic mist (Muthuchelian *et al.*, 1994), severe mineral deficiency (Godde and Hefer 1994), and leaf development at elevated CO₂ (Robertson and Leech 1995), have been reported to involve specific decreases in OEE protein levels. These were coincident with decreased oxygen evolution for all but the latter two studies in which oxygen production was not measured. Increased resistance to stress may also involve the OEE proteins. For example,

development of salt tolerance in photoautotrophic tobacco cell cultures was linked with increased binding affinity of OEE2 (Murota *et al.*, 1994). This observation provides support for the hypothesis that not only changes in OEE levels but also changes in the association with PSII can have important consequences for primary photochemistry during stress.

There is evidence for the involvement of the OEE proteins in the ongoing PSII repair cycle. OEE proteins that have detached from damaged PSII centres may reassociate with repaired centres reassembled with new D1 in the nonappressed lamellae (Hundal *et al.*, 1990; Virgin *et al.*, 1990). This model was developed from immunoprobings studies of everted thylakoid vesicles. Vesicles from appressed granal regions exposed to a high light treatment showed a reduction in levels of all three OEE proteins concomitant with loss of the D1 protein whereas nonappressed thylakoid vesicles exhibited increased OEE1 (Virgin *et al.*, 1990). Observations that detached OEE proteins remain capable of reassociation with PSII after chemical removal (e.g., Miyao and Murata 1989) and that heterologous OEE1 readily binds to mutant PSII lacking OEE1 (Betts *et al.*, 1994) indicate that a OEE recycling mechanism is conceivable. In the same vein, the presence of unassociated pools of all three OEE proteins observed in mature chloroplasts, unlike most other constituents of chloroplast protein complexes, was interpreted as evidence for a possible homeostatic role in maintaining PSII function, possibly permitting a rapid response to increased PSII turnover (Ettinger and Theg 1991). An additional protective role in ameliorating photoinhibitory damage has recently been ascribed to OEE1 binding to CPa-2 (Yamamoto and Akasaka 1995).

Other work reporting environmental effects on OEE accumulation or loss is more difficult to explain. For example, the accumulation of OEE1 to physiological levels in maize grown at suboptimal temperature, in contrast with reduced production of chloroplast-encoded

PSII constituents, was interpreted as a failing in nuclear-chloroplast communication (Robertson *et al.*, 1993). Alternatively, this could reflect an intrinsic protective/recovery mechanism. The most likely explanation is the absence of feedback inhibition on accumulation which might explain why OEE1 expression is not affected in PSII reaction centre core protein mutants (de Vitry *et al.*, 1989) and overexpression of OEE1 does not affect transport, processing or stable accumulation of the protein in the lumen nor oxygen evolution (Mayfield 1991). Accumulation of OEE proteins, however, is subject to control under some conditions as shown in the puzzling observation of an apparently specific decrease in OEE1 levels in some chloroplasts of wheat leaves developed at elevated CO₂ (Robertson and Leech 1995). These workers did not speculate on the basis of this phenomena but it does suggest there is some developmental regulation of OEE1 levels, as yet unidentified. Clearly, the significance of the OEE proteins in the stress response and recovery remains a relatively poorly understood aspect of PSII biochemistry.

1.3 Oxygenic Photosynthesis and Stress

1.3.1 Impact of Stress on PSII in Gymnosperms

The strategies engaged for the modulation of the light reactions of photosynthesis under different environmental conditions vary considerably among higher plant species (Oquist *et al.*, 1992a; Anderson *et al.*, 1993). In conifers, mechanisms to protect the perennial chloroplast from damage in a changing and frequently unfavourable environment are unquestionably significant, yet few details are known (reviewed in Gillies and Vidaver 1990). There is increasing evidence that there are some differences between angiosperm and gymnosperm species in terms of thylakoid membrane development (Kamachi *et al.*, 1994) and composition

(Jansson *et al.*, 1990), the expression of both nuclear- and chloroplast-encoded photosynthesis genes (Alosi *et al.*, 1990; Yamamoto *et al.*, 1991; Kojima *et al.*, 1992; Shinohara *et al.*, 1992a,b; Mazari and Camm 1993), and the chloroplast genome structure (Lidholm *et al.*, 1988; Lidholm and Gustafsson 1992). In addition to these developmental distinctions, the extended need in most conifers to maintain chloroplast function over multiple seasons of growth may result in patterns of photosynthetic regulation specific to gymnosperm species.

Investigation of photochemistry has revealed that there are some regulatory features that may be of particular importance to species with perennial leaves (Ball *et al.* 1994; Adams *et al.*, 1995). In various conifer species, a variety of responses to changes in light levels, occurring on timescales of less than a second to weeks, have been documented (reviewed in Gillies and Vidaver 1990). The long-term capacity of conifers to cope with light and avoid photooxidative stress is influenced by adaptations to environmental conditions, including temperature, nutrient status, pollution, and water relations. Several areas of conifer photosynthetic physiology and biochemistry are of ongoing interest: seasonal responses, changes induced by pollution damage, and the impact of water deficits.

In temperate forests, conifer species demonstrate significant changes in the photosynthetic electron transport capacity that are correlated with seasonal changes. During spring, transient declines in photochemistry observed in mature needles are coincident with nutrient translocation from the mature to developing photosynthetic tissue (reviewed in Camm 1993). During autumn, the onset of cold weather initiates a prolonged down-regulation of photosynthesis (e.g., Leverenz and Oquist 1987). Acclimation to low temperatures (frost hardening) may result in changes in chloroplast membrane composition (Oquist 1982), alterations in the light-harvesting complexes (Oquist and Strand 1986), and decreased

photochemical efficiency (Strand and Oquist 1988). Soluble and protein-associated thiols, thought to help preserve membrane structure, become maximal in winter (Grill *et al.*, 1988). As low temperature further inhibits carboxylation, management of excess light becomes increasingly important in evergreen tissue (Strand and Lundmark 1987; Gillies and Vidaver 1990; Orlander 1993). Conifers appear to resist cold temperature-associated photodamage primarily by increased nonradiative energy dispersion, nonphotochemical quenching, via a sustained high level of xanthophyll cycle-dependent dissipation which provides a long term down-regulation of photochemistry (Oquist and Huner 1991; Adams *et al.*, 1995; Strand and Lundmark 1995). Ultimately, freezing temperatures can result in the loss of EPR detectable PSII activity in pine and spruce needles (Tsel'niker and Chetverikov 1988). Recovery from this 'winter damage' is dependent on temperature and light levels, and may require protein and pigment synthesis (Lundmark *et al.*, 1988; Ottander and Oquist 1991; Adams and Demmig-Adams 1994; Adams *et al.*, 1995).

Anthropogenic oxidative stress can also have a significant effect on photochemistry in conifers (e.g., Lutz *et al.*, 1992; Byres *et al.*, 1992) and the contribution of various pollutants to novel forest decline is currently of intense interest (Foyer *et al.*, 1994). In response to air pollutants, increased chlorosis, decreased levels of redox components, and decreased capacity for photochemistry have been reported (Flammersfeld and Wild 1992; Evans *et al.*, 1992; Patterson and Rundel 1995); however, it is debatable as to whether PSII photochemistry is directly affected (reviewed in Wedler *et al.*, 1995). Superoxide dismutase and components of the ascorbate/glutathione cycle, which remove reactive oxygen species, may be upregulated in conifers during oxidative stress (Tandy *et al.*, 1989; Polle and Rennenberg 1992). An accelerated, abnormal needle senescence induced by oxidative stress was reported for several

conifer species, confounded by genetic sensitivity to oxidative damage and other environmental stresses (Patterson and Rundel 1995; Temple and Riechers 1995). There is some evidence from studies of pollution-damaged conifers that the gymnosperm photosynthetic responses to stress may differ in some respects from those described for angiosperm species (Flammersfeld and Wild 1992; Le Thiec *et al.*, 1994; Temple and Riechers 1995).

Another major environmental stress experienced by most conifer species is water deficit. Survival and productivity are significantly influenced by the capacity to withstand water stress (e.g., Brix 1979; Binder *et al.*, 1989). In needle tissue, water deficits can have long-term effects on protease composition (Pierre and Savoure 1990), affect secondary metabolism (Kainulainen *et al.*, 1992), alter nitrogen metabolism, and increase senescence rate (Manderscheid and Jager 1993). Physiological features that influence photosynthetic capacity such as stomatal regulation (e.g., Stewart *et al.*, 1995; Lu *et al.*, 1995; Edwards and Dixon 1995a), accumulation of osmolytes (e.g., Cyr *et al.*, 1990; Edwards and Dixon 1995b), and activation of active oxygen scavenging systems (e.g., Tandy *et al.*, 1989) have been described for different conifer species subjected to drought; however, relatively little is known about photosynthetic regulation at the membrane level. The dearth of information is underscored by the absence of a single reference to conifer species in a recent review of the effects of water stress on photosynthetic water oxidation (Eickmeier *et al.*, 1992). Investigation of the regulation of photosystem II activity during water stress in conifers has just begun (Toivonen and Vidaver 1988; Eastman and Camm 1995).

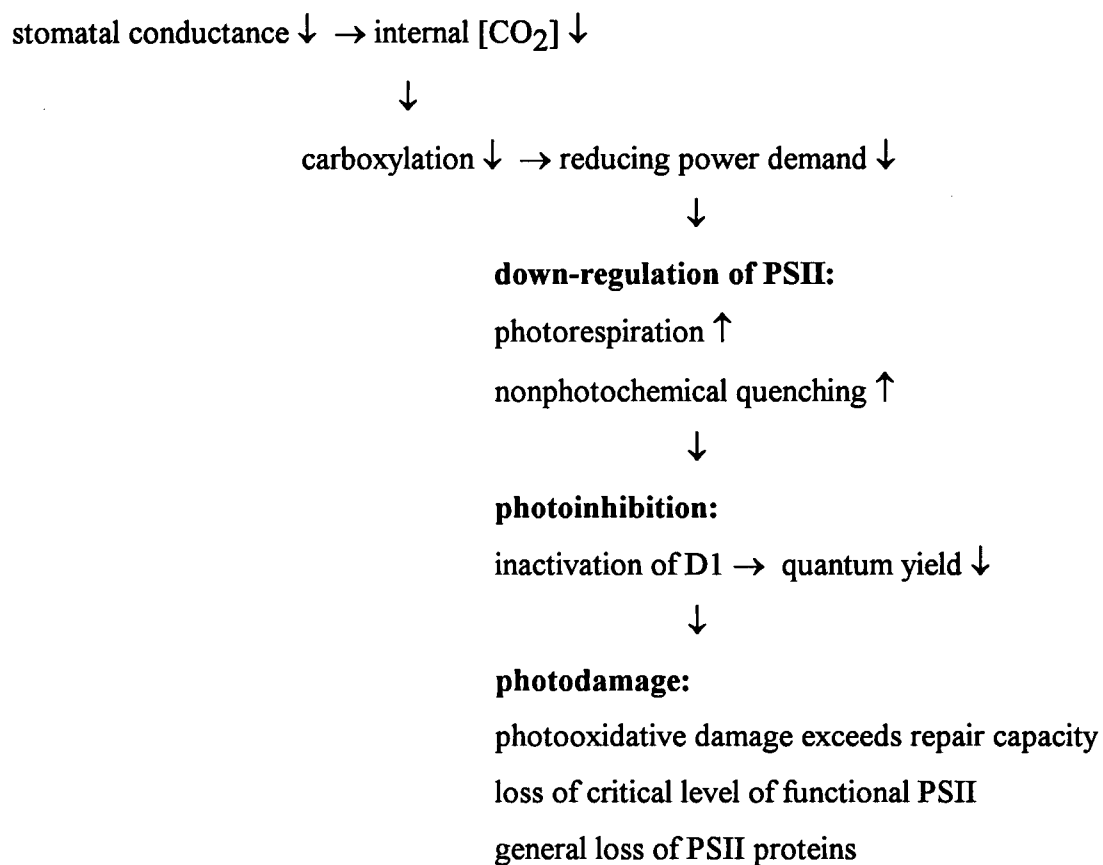
1.3.2 Impact of Water Stress on PSII in Angiosperms

The effect of water stress on PSII activity has been examined more extensively for angiosperm species including drought susceptible, resistant and highly resistant species (reviewed in Eickmeier *et al.*, 1992). In spite of the diversity of angiosperm species that have been studied, the response patterns are surprisingly similar with the major distinction being the point at which water deficit becomes damaging (Kaiser 1987; Chaves 1991; Eickmeier *et al.*, 1992). A flow diagram summarizes the reported water stress-induced changes in photosynthesis for angiosperm species (Figure 1.1) In general, PSII is reported to be quite resistant to the effects of water stress and significant changes in activity only develop in severe or prolonged water deficits (reviewed in Chaves 1991, and Cornic *et al.*, 1992).

Measurements of oxygen evolution, changes in chlorophyll fluorescence, and electron transport capacity have been extensively used in investigations of water stress. *In vivo* measurements of oxygen evolution as a function of CO₂ concentration have been used to demonstrate that dehydrated leaf tissue (30 % water deficit) can generate high rates of oxygen evolution, comparable to hydrated tissue, if CO₂ levels are elevated (Cornic *et al.*, 1992). Investigations using chlorophyll fluorescence demonstrate that as water stress develops there are increases in reversible thermal dissipation of energy, measured as nonphotochemical quenching and then, as PSII centres become inactivated, photoinhibition develops (reviewed in Eickmeier *et al.*, 1992). Whether photoinhibition¹ reflects a controlled down-regulation of PSII or the onset of damage is a controversial issue, although currently the former is favoured (e.g., Oquist *et al.*, 1992b; Critchley and Russell 1994). Many reports indicate that photoprotection through

¹For the purposes of this thesis, I use the term photoinhibition to refer to a decrease in maximal photochemical efficiency (Fv/Fm) not reversible by prolonged dark-adaption (Aro *et al.*, 1993a); however, this usage is not consistent in the literature.

Figure 1.1 Flow diagram of the hierarchy of photosynthetic change during progressive water stress summarizing information reported in the literature for angiosperm species.



nonphotochemical quenching is the most significant factor in maintenance of PSII efficiency during water stress (e.g., Stuhlfauth *et al.*, 1988; Grieu *et al.*, 1995; He *et al.*, 1995; Valentini *et al.*, 1995). In some species increased photorespiration during drought can maintain electron flow through PSII (Jefferies 1994) whereas in others water stress causes a significant decrease in activities of a key photorespiratory enzyme, glycolate oxidase (Moran *et al.*, 1994). Someworkers suggest that photorespiration is not as important as increased nonphotochemical quenching in avoidance of photodamage (Brestic 1995). Some loss of PSII activity was reported for water-stressed sorghum (Masojidek and Hall 1992). In general, however, the pattern reported for angiosperm species entails sustained PSII activity until water stress becomes extreme or is exacerbated by additional stresses such as photoinhibitory light or temperature (Havaux 1992; Eickmeier *et al.*, 1992; Grieu *et al.*, 1995; He *et al.*, 1995; Valentini *et al.*, 1995).

Electrophoretic and fluorographic analyses of changes in PSII proteins in response to water stress have been conducted for few angiosperm species, although there is some evidence for changes in water oxidation (Havaux *et al.*, 1986). Water or salinity stress caused some loss of the chlorophyll *a*-binding PSII core proteins, CPa-1 and CPa-2, in sorghum and this was exacerbated by photoinhibitory light treatment (Masojidek and Hall 1992). In wheat plants subjected to osmotic stress, D1 and D2 degradation were accelerated and a general decrease in the levels of other PSII constituents was observed; however, this was not observed until the stress was extreme, electron transport through PSII was impaired, and translation capacity decreased (He *et al.*, 1995).

Chapter 2: Regulation of Photosynthesis in Spruce during Progressive Water Stress

2.0 Summary

Photosynthetic response to water stress was analyzed in three experiments using one-year-old interior spruce (*Picea glauca* (Moench) Voss X *P. engelmanni* Parry hybrid complex) trees in which the buds had broken dormancy and new shoots were developing. In all three experiments, oxygen evolution and room temperature chlorophyll fluorescence were monitored during the decreases in predawn shoot water potential (Ψ). In two experiments, gas exchange was also measured. As Ψ decreased to -1 MPa, stomates closed and carbon assimilation declined rapidly. As Ψ decreased further to -1.6 MPa, oxygen evolution at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$ declined progressively. Photochemical efficiency of photosystem II (PSII) observed during actinic light exposure (Φ_{II} ; calculated as $\Delta F/F_m'$ where ΔF is the difference between F_v and F_v') decreased at irradiances above $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ as Ψ decreased. At the same light levels, photochemical quenching (qP) dropped with decreasing Ψ while nonphotochemical quenching (qN) increased steadily. At lower irradiances, major increases in qN were not observed until Ψ decreased below -1.6 MPa. Several phases of photosynthetic response to progressive water stress in spruce were identified - a pronounced decline in gas exchange mediated by stomatal closure, subsequent photoprotective changes in chlorophyll fluorescence as primary photochemistry was down-regulated, a decline in photochemical efficiency of dark-adapted needles (F_v/F_m), and the onset of photoinhibition.

2.1 Introduction

Spruce trees have been extensively studied with respect to changes in water relations during natural and imposed stresses. Efforts to identify the environmental and physiological basis for poor growth frequently reported for outplanted spruce seedlings have revealed that establishment and maintenance of turgor during water stress is critical (Day and Butson 1989; Grossnickle 1989; Margolis and Brand 1990). Diurnal and seasonal fluctuations in gas exchange (Watts and Neilson 1978; Binder *et al.*, 1989) and water status (Grossnickle 1989; Colombo and Teng 1992) have been well-characterized for field planted seedlings. In interior spruce (*Picea glauca* (Moench) Voss X *P. engelmanni* Parry hybrid complex), ontogenetic and phenological changes in water relations characteristics (Grossnickle 1989; Colombo and Teng 1992) and environmental history (Day and Butson 1989; Zwiazek and Blake 1989; Vance and Zaerr 1991) also significantly influence the response to water stress.

Low soil water potential or high vapour pressure deficits can have an immediate effect on gas exchange. In the field, spruce seedling carbon assimilation reportedly starts to decline in the predawn water potential (Ψ) range of -1 to -2 MPa and ceases in the range of -3 to -5 MPa (Day and Butson 1989). Early in the growing season, white spruce seedlings at Ψ of less than -1 MPa are capable of only limited gas exchange (Binder *et al.*, 1989). Drought stress is considered to be the most significant limitation to spruce seedling establishment and productivity (Binder *et al.*, 1989). Resolution of the physiological and biochemical mechanisms that permit conifer seedlings to tolerate water stress is needed to understand both the limits and the possibilities for maximizing survival and growth.

Adaptations that protect the perennial photosynthetic apparatus during drought have not been extensively investigated although, at the level of gas exchange, the capacity for

photosynthetic acclimation to water deficit is known to vary. For example, black spruce (*P. mariana* (Mill.) B.S.P.) seedlings subjected to repeated osmotic stress reportedly manifested acclimation as increased stomatal sensitivity to stress (Zwiazek and Blake 1989) although other studies contradict this (e.g., Stewart *et al.*, 1995). Red spruce (*P. rubens* Sarg.) seedlings subjected to eleven drying cycles did not show any changes in gas exchange characteristics in response to drought (Seiler and Cazell 1990). These observations and those of others (reviewed in Lamhamedi and Bernier 1994; Stewart *et al.*, 1995; Lu *et al.*, 1995) indicate that, in spruce species, changes in stomatal sensitivity may not be important in water stress acclimation and that changes in stomatal response is a minor component in drought resistance.

At the cellular level, osmotic adjustment by accumulation of amino acids and soluble carbohydrates in response to water stress is well-documented for spruce (Zwiazek and Blake 1989; Cyr *et al.*, 1990; Koppenaal *et al.*, 1991; Zwiazek 1991; Tan *et al.*, 1992; Lamhamedi and Bernier 1994). Although turgor maintenance can extend the Ψ range at which gas exchange can continue, it is noteworthy that stress resistance/tolerance by osmotic adjustment is minimal in white spruce seedlings during the vulnerable period of shoot elongation in spring and early summer (Grossnickle 1989; Colombo and Teng 1992). Thus, other mechanisms that permit drought tolerance, including sensitive control of photosynthetic activity at the chloroplast level, may be more important during this phase.

Within the chloroplast, excess light energy causes many of the injurious repercussions of drought on photosynthesis. Although light can reduce the consequences of water stress by affecting needle morphology, thereby providing long term protection from light energy damage and, indirectly, mechanical injury from cell collapse (Vance and Zaerr 1991; Zwiazek 1991), thermal and radiant energy damage to photosynthetic enzymes and membranes can be immediate

and extensive (Barber and Andersson 1992; Aro *et al.*, 1993a). Coping with excess radiation is particularly important to conifers because structurally expensive needle tissue must support multiple seasons of photosynthetic activity. Avoidance of photooxidative damage may be significant in long-term survival and productivity.

To identify strategies utilized by needle tissue to cope with stress, the following studies of young interior spruce were initiated. Physiological responses to water stress in terms of changes in gas exchange and regulation of stress-sensitive PSII (Barber 1994) were examined using three seedlots of interior spruce seedlings. In one study, a clonal lot of emblings generated by somatic embryogenesis (Webb *et al.*, 1989) was used. The goal of these investigations was to evaluate and physiologically characterize photosynthetic behaviour in response to drought. Four phases of sublethal water stress were defined and a repertoire of behaviour responses regulating photosystem activity in the perennial photosynthetic apparatus was identified.

2.2 *Materials and Methods*

2.2.1 *Experimental Rationale*

To characterize phases of water stress in young interior spruce trees, three studies of imposed, progressive drought were conducted. In all three studies, the plants had just broken quiescence; new shoots and needles were in the process of rapid development. This particular developmental stage was chosen for several reasons. Plants during this stage are particularly vulnerable to stress (Gillies and Vidaver 1990; reviewed in Lamhamedi and Bernier 1994), not capable of significant osmotic adjustment (Grossnickle 1989; Colombo and Teng 1992), readily generated at any time of the year by placing cold-stored plants that have met their chilling

requirement in a favourable environment, and well-characterized morphologically and developmentally.

Each water stress study was designed to address specific objectives. The information and questions arising from each experiment were used to plan subsequent work. The results from Study I defined the initial phases of stress and provided evidence that the trees could withstand a more extensive stress. Interpretation of the oxygen evolution and chlorophyll fluorescence data was limited by the use of a single, low irradiance level and sample size. In Study II, the use of more plants including two propagule types, a wider range of predawn shoot water potentials (Ψ), and, most importantly, light levels ranging from below the compensation point to above the levels used for growth, allowed the characterization of a third phase of stress using features of chlorophyll fluorescence. Study III was designed to confirm that the changes in chlorophyll fluorescence observed in Study II are characteristic of the interior spruce response to water stress. It was also of interest to determine at what point photoinhibition, as defined by a decrease in quantum yield not reversible by prolonged dark-adaption (Aro *et al.*, 1993a), occurred during progressive stress and whether there were other changes in photosynthetic behaviour as the stress increased.

2.2.2 Plant Material

For the first drought stress study conducted (Study I), one year old interior spruce trees (British Columbia Ministry of Forests Seedlot #4038, Pelton Reforestation Ltd., Maple Ridge, BC), that had been operationally grown and cold-stored, were removed from cold storage in late October 1991 and thawed overnight at 4°C. They were gently washed free of growth medium and planted in sand in 3-L pots. The seedlings were thoroughly watered and grown at 25°C

under white light irradiance of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured at shoot height (12 h-photoperiod). The 50 seedlings used had an average height of 27.4 cm (SE 0.5) and the average stem diameter, measured just above the root collar, was 3.55 mm (SE 0.07). Pressure-volume curves for well-watered seedlings, at the onset and conclusion of the experiment were generated from data obtained with a pressure chamber (Soil Moisture Corp. Model 3005) following the shoot transpiration method described by Grossnickle (1989). The curves were analyzed to define the osmotic potential at the turgor loss point (Schulte and Hinckley 1985).

In the second water stress experiment (Study II) the responses of two propagule types, seedlings and emblings derived from somatic embryogenesis, were compared, in part to further assess whether the emblings differed in their water stress response. Interior spruce seedlings were grown from seed from an open pollinated tree, EK20, and the emblings (Clone # W29) were derived from a seed embryo of open-pollinated tree, EK10 (Webb *et al.*, 1989). All plants were grown at Saanich Test Nursery (Ministry of Forests, Saanichton, BC) in styrofoam block containers and lifted following commercial nursery procedures (Grossnickle *et al.*, 1994) then stored at -2°C for one month. After removal from cold storage in late February 1992, 50 seedlings and 50 emblings were thawed overnight at 4°C , washed free of growth medium and planted in sand in 3-L pots and grown as described for Study I. The plants were watered regularly. At the onset of the experiment, the seedlings were slightly taller (mean height of 21.7 cm, SE 0.7) than the emblings (mean height 18.8 cm, SE 0.4) and average stem diameter, measured just above the root collar, was 3.95 mm for the seedlings and 3.99 mm for the emblings (SE 0.06, 0.07, respectively). Pressure-volume curves for five seedlings and five emblings were generated as described previously.

For the third stress experiment (Study III), one year old interior spruce trees (British Columbia Ministry of Forests Seedlot 3057, Surrey Nursery, Surrey, BC) were removed from cold storage in May 1994, thawed for several days at 4°C, then planted directly in sand in 1-L pots. The plants were grown in a growth cabinet at 20°C under white light at an irradiance of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at shoot height (16 h-photoperiod). A subset of plants was continuously watered. One week before watering was discontinued, all plants were watered with 200 mL of 1/10 strength Johnson's fertilizer solution (Epstein 1972).

2.2.3 Induction of Drought Stress

Watering was discontinued once new shoots had begun to flush. Predawn shoot water potential (Ψ) of lateral (Studies I and II) or terminal (Study III) branches from subsets of plants was determined with a pressure chamber (Ritchie and Hinckley 1975). The remaining plants were then rewatered and monitored for several days to assess recovery.

2.2.4 Measurement of Gas Exchange

In Studies I and II, gas exchange variables (net carbon assimilation, stomatal conductance, transpiration, and substomatal carbon dioxide concentrations) were determined with a LI-6200 gas exchange system (LI-COR Inc.). Measurements were made 1 h after the onset of the light cycle by enclosing a predefined portion of the terminal shoot in a 0.25 L measuring chamber (Model LI-6200-13). In Study I, measurements were made *in situ* at an average actinic level of 365 $\mu\text{mol m}^{-2} \text{s}^{-1}$. In Study II, measurements were conducted under a light source providing 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. In both experiments, new growth had been removed

from the sampled area of each plant one week previously to avoid complications associated with developing photosynthetic tissue. Total needle surface area was determined by multiplying the projected needle area, determined for each sample with a LI-3100 area meter, by four (LI-COR Inc.). Gas exchange measurements were then recalculated on the basis of total needle surface area of the sampled shoot.

Oxygen evolution was monitored in a Hansatech LD2 chamber equipped with an oxygen electrode maintained at 20°C by a circulating water bath (RM6 Lauda). In Studies I and II, this was carried out within 6 h after measurement of Ψ and gas exchange. In Study III, measurements were made on dark-adapted plants. For each sample, approximately 20 to 25 freshly detached first year needles were anchored on transparent tape and trimmed to form a 14 or 15 mm disc. Carbon dioxide concentration of approximately 10,000 $\mu\text{L L}^{-1}$ was maintained by application of 1 M sodium bicarbonate (pH 9) to capillary matting in the chamber (Walker 1987). In Study I, needle discs were dark adapted in a humid chamber for 15 min then transferred under a green safe light to the Hansatech chamber. In Study II, each needle disc was sealed in the chamber and exposed to a low irradiance ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 3 min, then oxygen consumption was monitored in the dark for 10 min to determine the rate of dark respiration and to dark-adapt the sample. In the third study, discs were prepared from dark-adapted (overnight) plants, transferred in darkness to the chamber and oxygen consumption measured in the dark for 10 min. Actinic light was provided by a Walz pulse-modulated fluorometer (Study I), a KL 1500 Schott lamp (Study II) or Dolan-Jenner lamp (Study III) a fitted with neutral density filters. Oxygen evolution was measured at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance in Study I. Oxygen evolution was recorded on exposure of the sample to light intensities of 10, 25, 50, 130, 250 and 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the second experiment and at intensities of 30, 125, 500 and 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the third

study. Irradiance was measured at the needle disc surface using a LI-189 Quantum Photometer (LI-COR Inc.). Light compensation points for plants above and below a Ψ of -1 MPa were determined using mean values of gross O_2 production at each light intensity. Apparent quantum yield (ΦO_2) using oxygen evolution rate corrected for dark respiration was calculated for each irradiance using incident light values.

2.2.5 Measurement of Chlorophyll Fluorescence

Chlorophyll fluorometry measurements were performed, simultaneously with oxygen consumption/evolution measurements, with a Walz pulse-modulated fluorometer (PAM 101,102,103) following the procedures of Schreiber *et al.* (1986). In Study I, a saturating pulse of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light produced by a KL 1500 Schott lamp was applied. Subsequent tests revealed that a saturating flash of at least $8000 \mu\text{mol m}^{-2} \text{s}^{-1}$ was required for optimal determination of quantum yield. Saturating pulses of $13,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $11,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ of white light were applied in Studies II and III, respectively. After the disc had equilibrated for 10 min in the dark, dark level fluorescence (F_o) was determined by activation of the measuring beam ($<1 \mu\text{mol m}^{-2} \text{s}^{-1}$, at 1.6 KHz), then a saturating flash (1 s) was activated and maximal, dark-adapted fluorescence (F_m) determined. The lowest actinic light exposure was then initiated, and the measuring pulse frequency switched to 100 KHz. These conditions were maintained until variable fluorescence (F_v) approached steady state (F_v'), usually within 15 min. Saturating flashes were fired every 60 s (Studies I and III) or every 30 s (Study II) to determine maximal fluorescence during actinic exposure (F_m'). The actinic light was then briefly turned off to monitor dark level fluorescence after actinic exposure (F_o'), and then, in Studies II and III, the next actinic light intensity applied. For this and subsequent light intensities, fluorescence

steady state was achieved within 10 min. Photochemical efficiency in dark adapted needles (F_v/F_m), photochemical quenching (q_P) and nonphotochemical quenching (q_N) were calculated (Schreiber *et al.*, 1986). For Studies II and III, photochemical efficiency of PSII under a given actinic irradiance (Φ_{II} ; calculated as $\Delta F/F_m'$, where ΔF is the difference between F_v and F_v') and photochemical efficiency of open PSII centres under a given actinic irradiance (Φ_P ; calculated as F_v'/F_m') were also calculated (Genty *et al.*, 1989). Estimates of Φ_{II} were compared to apparent quantum yield values obtained from oxygen evolution rates.

2.2.6 Data Analysis

Graphical analysis was performed on scatterplots with a smoothing function (LOWESS) that assigns equal value to all points (Wilkinson 1990). Although the collected data sets span a continuum of decreasing Ψ , plant samples could be separated into physiologically relevant groups defined by water status. In Studies I and II, whole plant gas exchange was minimal when Ψ was less than -1 MPa, whereas the largest changes in fluorescence quenching (q_P and q_N) occurred around -1.6 MPa. Thus, individual plants were categorized as Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa) or Phase 3 ($-1.6 < \Psi < -3$ MPa). In Study III, the stress was extended and a fourth phase defined. Phase 4 ($-3 \leq \Psi \leq -4$ MPa) included the range in which a decrease in F_v/F_m was not reversible by prolonged darkness, i.e. photoinhibition.

2.3 Results

2.3.1 Carbon Assimilation

In Study I, after watering was discontinued, the average shoot predawn water potential (Ψ) remained stable at -0.6 MPa for two weeks, then rapidly declined over the subsequent week. The water stress was continued until a subpopulation ($n=13$) had an average Ψ of -2.80 MPa. This was chosen as the endpoint of the water stress because pressure-volume curve analysis at the onset of the study established that the mean osmotic potential at turgor loss (Ψ_{TLP}) was -2.88 MPa (SE 0.08). The Ψ_{TLP} for an unstressed group of plants at the end of the experiment was -2.43 (SE 0.24). Concomitant with the decrease in Ψ , carbon fixation rapidly declined as intercellular CO_2 concentrations (C_i) initially declined in response to a decrease in stomatal conductance (Figures 2.1 and 2.2). The data scatter evident in Figure 2.1 (a) prevented extension of the smoothing curve to include the stomatal conductance values for well-watered plants; similar observations have been made for *Pinus strobus* at Ψ above -1 MPa (Maier and Teskey 1992). At about -1 MPa, stomatal conductance and C_i decreased to a minimum (Figures 2.1 and 2.2). Below this Ψ , calculated C_i increased.

The patterns of change in carbon assimilation observed in the second study (Figure 2.2) were very similar to those obtained in the first study (Figure 2.1). In Study II, plants were monitored and sampled until a subpopulation ($n=7$) of each propagule type had an average Ψ of -2.3 MPa. At the onset of this study the Ψ_{TLP} was -2.27 MPa (SE 0.10) and -2.31 MPa (SE 0.13) for seedlings and emblings, respectively. No differences were evident in the gas exchange characteristics of seedlings and emblings, so the pooled data sets are presented. As in Study I,

Figure 2.1 Relationships between predawn water potential and net CO₂ fixation (a), stomatal conductance (b), and intercellular CO₂ concentration (c) in interior spruce seedlings measured at ambient irradiance of 300 to 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and ambient CO₂ (Study I).

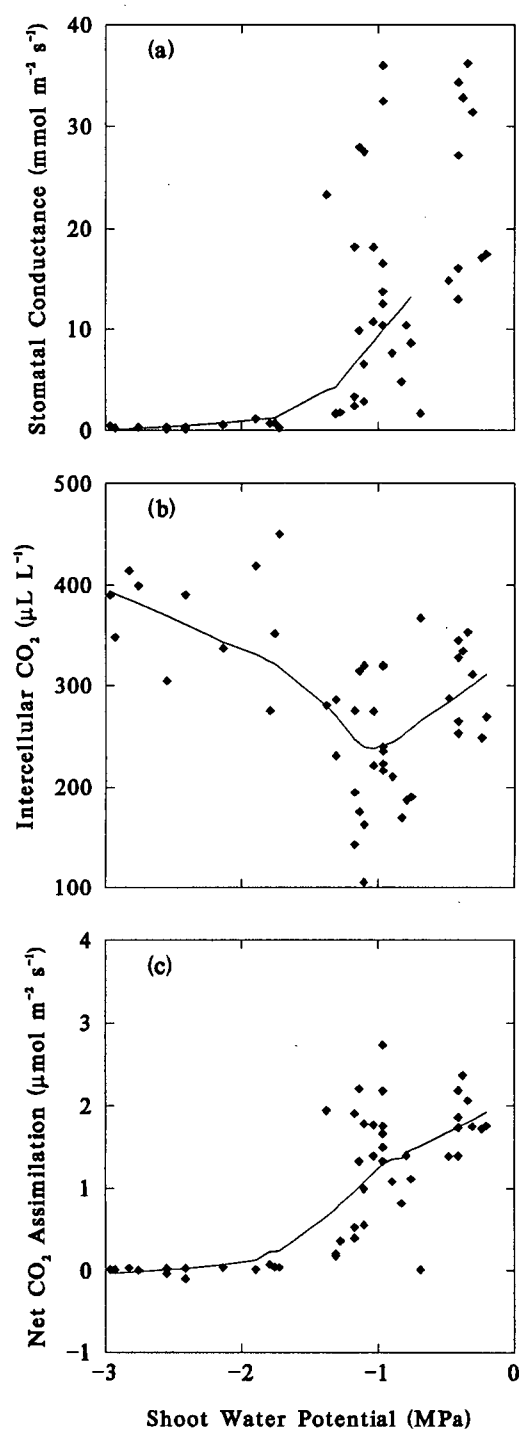
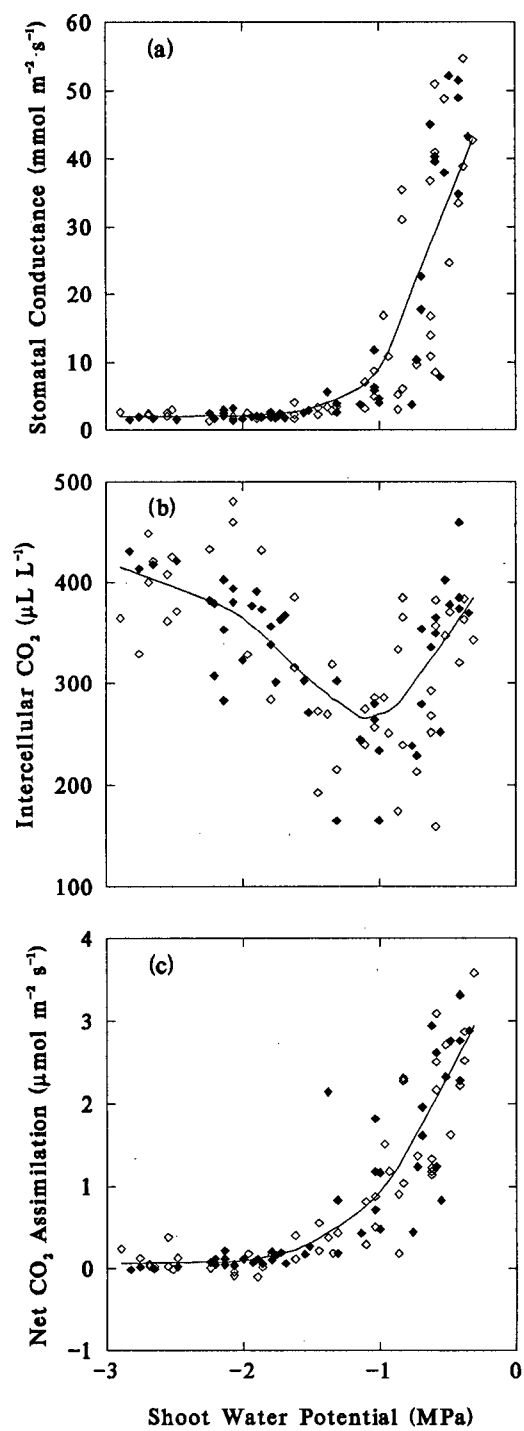


Figure 2.2 Relationships between predawn water potential and net CO₂ fixation (a), stomatal conductance (b), and intercellular CO₂ concentration (c) in interior spruce seedlings (open symbols) and emblings (closed symbols), measured at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and ambient CO₂ (Study II).



carbon fixation rapidly declined concurrent with stomatal closure as Ψ decreased below -1 MPa (Figure 2.2). Ambient CO_2 concentration ranged from 400 to 500 $\mu\text{L L}^{-1}$.

After rewatering, the majority of plants were capable of recovering photosynthetic activity. Two days after rewatering, average Ψ increased from -3.0 MPa to -0.66 MPa and carbon fixation increased from undetectable levels to 60 % of the average carbon fixation rate of Phase 1 plants. After 4 days of recovery, this rate increased to 84 % of the Phase 1 rate. In Study II, a subset of stressed plants (average Ψ = -2.5 MPa) was rewatered. After one day, the recovering plants (average Ψ = -0.7 MPa) were capable of 52 % of the mean carbon fixation rate of a well-watered subset ($n=10$) of plants (mean Ψ = -0.5 MPa). This increased to 78 % of the well-watered rate after 2 days.

2.3.2 Oxygen Evolution

At the elevated CO_2 concentrations used in the Hansatech chamber, CO_2 assimilation was not limited by stomatal closure; however, oxygen evolution decreased continuously at non-limiting irradiance, with no apparent threshold value of Ψ as the water deficit deepened. In Study I, in which oxygen evolution was measured at 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, close to the light compensation point, average oxygen evolution decreased in Phase 2 to half of the average Phase 1 rate. In Phase 3, this declined to 20 % of the average Phase 1 rate.

In Studies II and III, stress-induced decreases in mean oxygen evolution rate were greater at irradiances above 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figures 2.3 and 2.4). In the second study, the light compensation point was 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in Phase 1 and this increased to 88 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in Phase 3 (inset, Figure 2.3). In contrast, there was little apparent change in the compensation

Figure 2.3 Light response curves for average oxygen evolution of freshly detached needles from interior spruce seedlings and emblings (Study II) during Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa) and Phase 3 ($-1.6 < \Psi < -3$ MPa) measured at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$. Standard error is indicated only where it exceeds symbol size. The inset is an expansion of the plot of O_2 evolution at irradiances from 0 to $150 \mu\text{moles m}^{-2} \text{ s}^{-1}$.

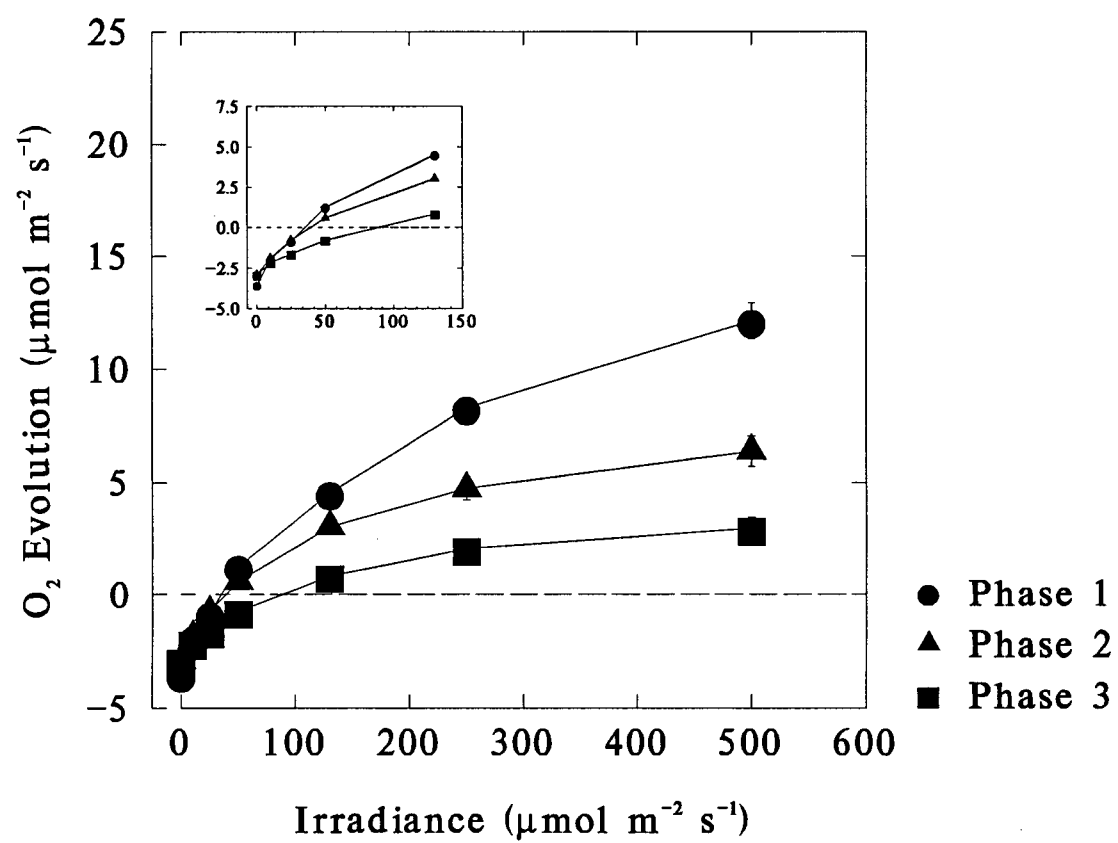
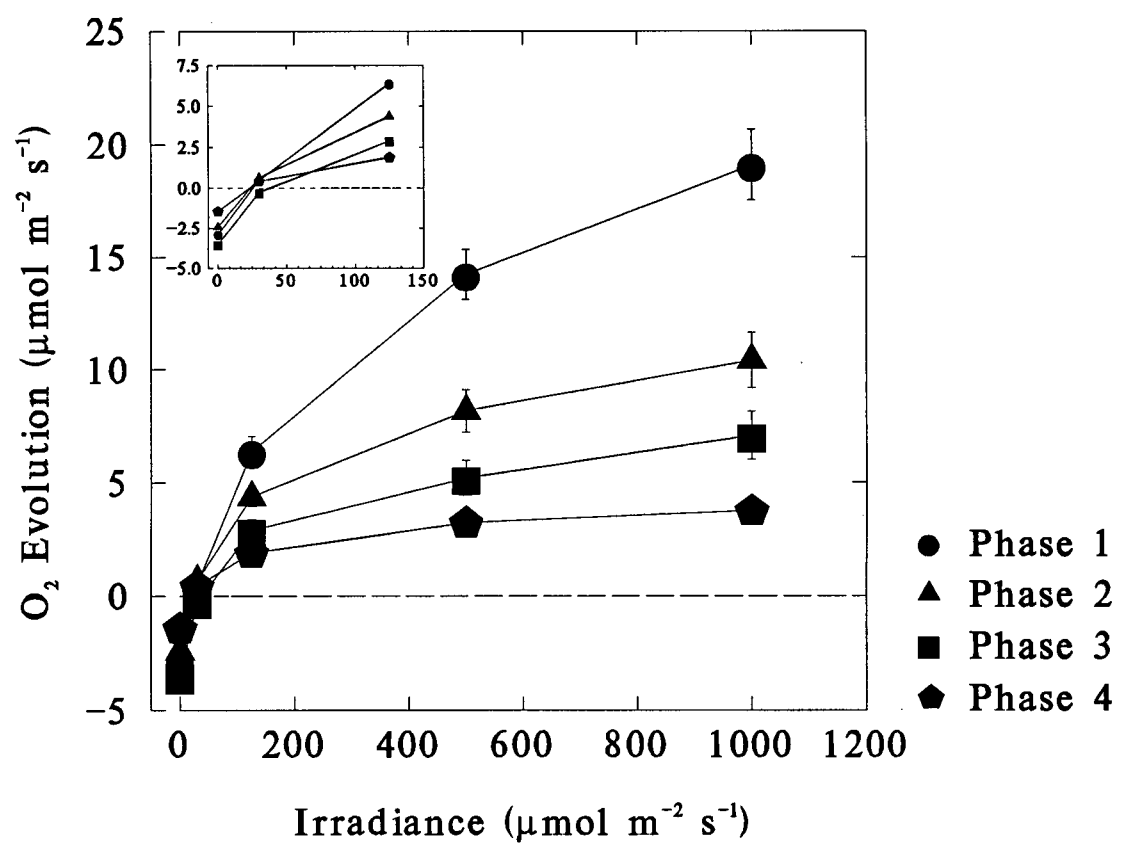


Figure 2.4 Light response curves for average oxygen evolution of freshly detached needles from interior spruce seedlings (Study III) during Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa), Phase 3 ($-1.6 < \Psi < -3$ MPa), and Phase 4 ($-3 \leq \Psi \leq -4$ MPa) measured at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$. Standard error is indicated only where it exceeds symbol size. The inset is an expansion of the plot of O_2 evolution at irradiances from 0 to $150 \mu\text{moles m}^{-2} \text{ s}^{-1}$.



point even in Phase 4 in the third study (inset, Figure 2.4). In Study II, dark respiration did not change with decreasing Ψ (Figure 2.3) whereas, in Study III, dark respiration was lower in the most stressed plants (inset, Figure 2.4).

2.3.3 Chlorophyll Fluorometry

In Study I, the ratio of variable fluorescence to maximal fluorescence after dark adaption (F_v/F_m) remained high throughout the drought stress. At the actinic irradiance used, $40 \mu\text{mol m}^{-2} \text{s}^{-1}$, there were no changes in photochemical quenching (q_P) or nonphotochemical quenching (q_N) at Ψ above -2 MPa. In Study II, F_v/F_m was high for both seedlings and emblings sampled at Ψ greater than -1.6 MPa; at lower Ψ , F_v/F_m values decreased (Figure 2.5). In the third study, F_v/F_m values remained high until the onset of Phase 4 (Figure 2.6). In Study II, a subset ($n=7$) of rewatered plants (average $\Psi_{\text{initial}} = -2.5$ MPa, average $\Psi_{\text{post-watering}} = -0.7$ MPa) had an average F_v/F_m value of 0.791 (SE 0.010). The average values of Ψ and F_v/F_m for Studies II and III are summarized for the various water stress phases in Table 2.1.

Quantum yield was higher in Study III than in Study I (not shown) and Study II (Figure 2.5) which could reflect a better nutrient status and/or the relaxation of slow-relaxing components of q_N effected by prolonged darkness. Additionally, because carbon fixation was not measured in this study, the plants were not exposed to exacerbation of the drought stress by light exposure (Masojidijek *et al.*, 1991). In a control experiment, changes in plant water potential in the light were assessed by comparing Ψ to shoot water potential measurements made after 6 h light exposure in 25 seedlings. Average water potential decreased by -0.26, -0.31 and -0.48 MPa in groups of plants classified as Phase 1, 2 and 3, respectively. Thus, differences

Table 2.1 Average predawn water potential (Ψ) and dark-adapted quantum yield (F_v/F_m) for the different water stress phases in Studies II (a) and III (b). The phases were defined as Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa), Phase 3 ($-1.6 < \Psi < -3$ MPa) and Phase 4 ($-3 \leq \Psi \leq -4$ MPa).

(a) Study II

	Phase 1	Phase 2	Phase 3
n	14	13	14
Ψ	-0.610 ± 0.049	-1.318 ± 0.068	-2.219 ± 0.114
F_v/F_m	0.817 ± 0.002	0.809 ± 0.002	0.761 ± 0.011

(b) Study III

	Phase 1	Phase 2	Phase 3	Phase 4
n	16	11	14	28
Ψ	-0.589 ± 0.033	-1.313 ± 0.054	-2.238 ± 0.073	-3.498 ± 0.070
F_v/F_m	0.826 ± 0.007	0.816 ± 0.009	0.809 ± 0.004	0.648 ± 0.031

Figure 2.5 Trends in photochemical efficiency in dark adapted needles (F_v/F_m) and photochemical efficiency of open PSII centres under a given actinic irradiance (Φ_P ; calculated as F_v'/F_m'), measured at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$, in response to changes in predawn shoot water potentials in interior spruce seedlings (open symbols) and emblings (closed symbols) in Study II.

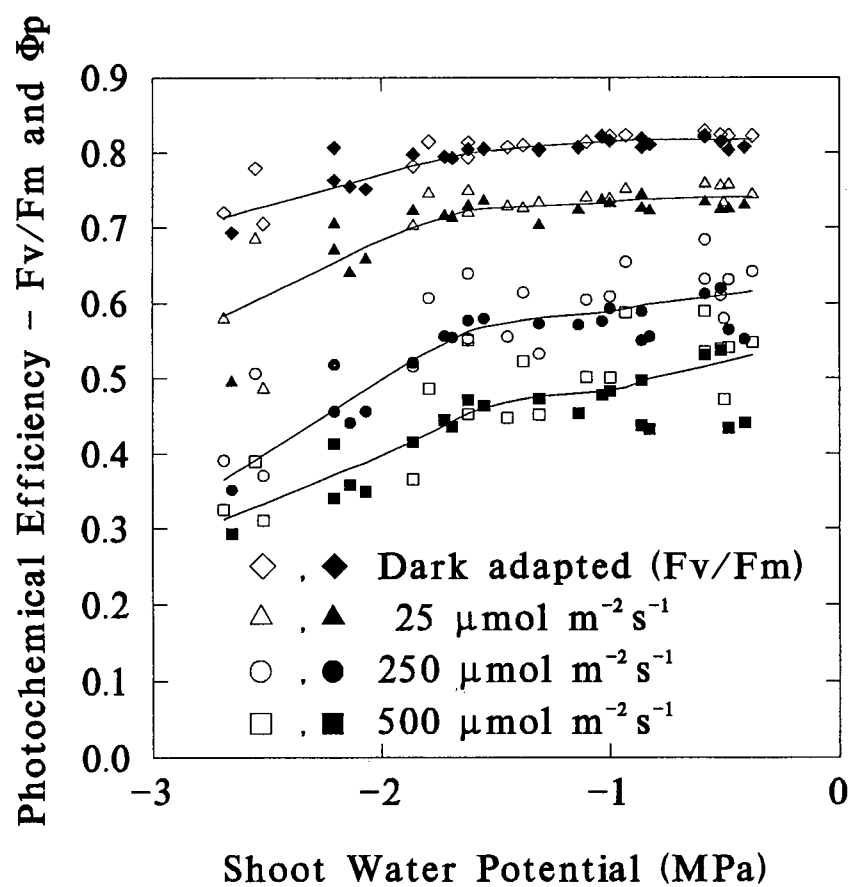
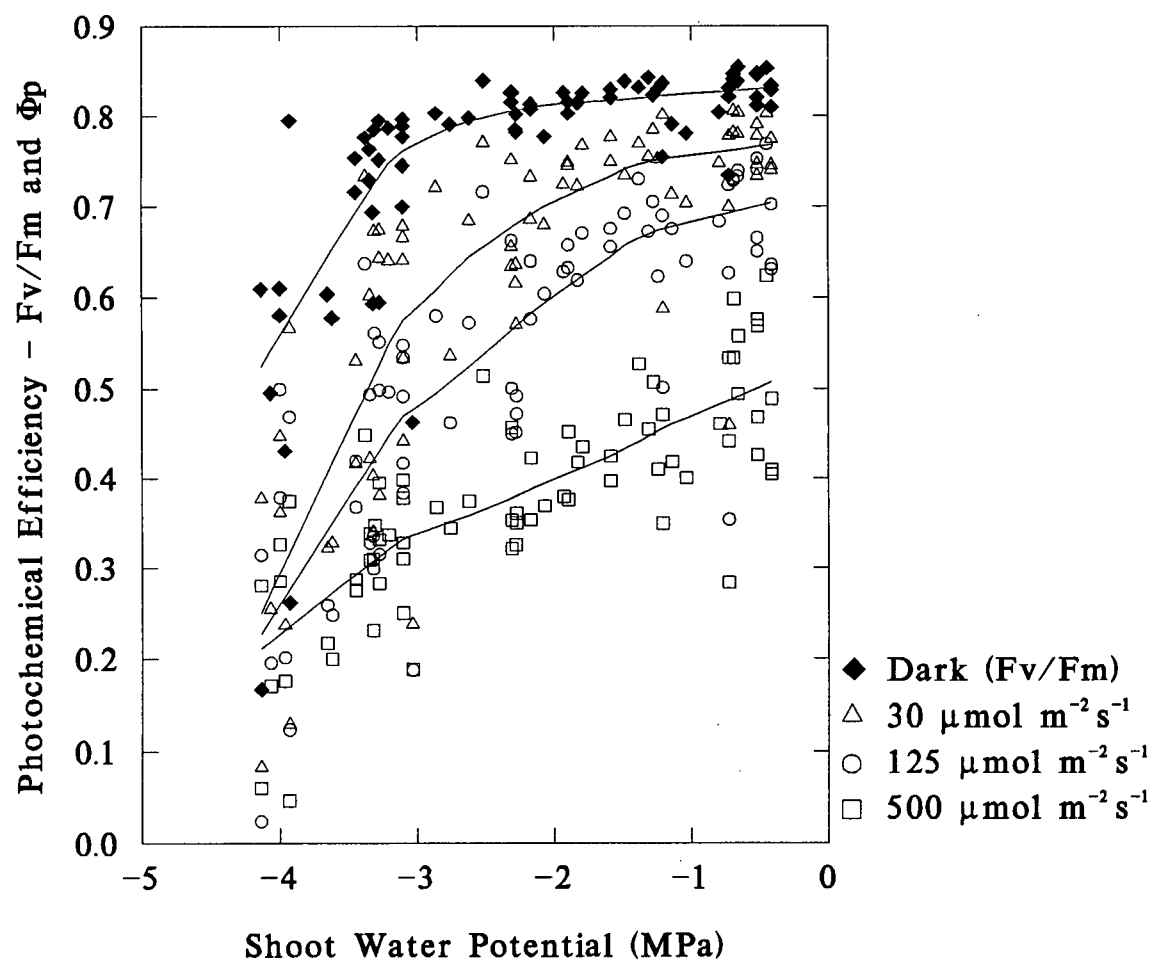


Figure 2.6 Trends in photochemical efficiency in dark adapted needles (F_v/F_m) and photochemical efficiency of open PSII centres under a given actinic irradiance (Φ_P ; calculated as F_v'/F_m'), measured at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$, in response to changes in predawn shoot water potentials in interior spruce seedlings in Study III.



between Studies II and III may also reflect changes in stress status in Study II plants *after* Ψ was measured.

In contrast to the small changes in F_v/F_m observed before Phase 4, photochemical efficiency of open centres (Φ_P), calculated as F_v'/F_m' significantly changed in response to moderate water stress in Studies II and III (Figures 2.5, 2.6). As seedlings became mildly stressed (Phase 2), mean estimates of the photochemical efficiency of PSII (Φ_{II}) decreased in an irradiance dependent manner (Figure 2.7). As the stress deepened to Phase 3 and 4, Φ_{II} decreased at all light levels (Figure 2.7). The Phase 1 emblings differed from the Phase 1 seedlings in that the Phase 1 emblings had similar Φ_{II} values to Phase 2 seedlings and emblings (Fig 2.7). There were decreases in Φ_{II} at all irradiances in Phase 3 for seedlings and emblings in Study II (Figure 2.7). The trends observed for seedlings in Study III were generally similar. At light levels below $1000 \mu\text{mol m}^{-2}\text{s}^{-1}$, values of Φ_{II} were lower in Phase 3 and 4. At $1000 \mu\text{mol m}^{-2}\text{s}^{-1}$, values were low in all phases (Figure 2.8).

In all studies, average photochemical quenching (qP) at limiting irradiance, that is $50 \mu\text{mol m}^{-2}\text{s}^{-1}$ and lower, remained high in Phases 2 and 3 (Figure 2.9). At irradiances of $125 \mu\text{mol m}^{-2}\text{s}^{-1}$ and higher, qP declined progressively in Phases 2 and 3 in Study II (Figure 2.9a). This was not apparent until higher irradiances ($500 \mu\text{mol m}^{-2}\text{s}^{-1}$ and above) in Study III (Figure 2.9b). At all irradiances, average qP values in Phase 4 were much lower than Phase 3 values.

At low irradiance, nonphotochemical quenching (qN) was low in Phases 1 and 2 in all three studies (Figure 2.10). Stress phases 1 and 2 could not be separated in Study II or III; however, qN increased with increasing irradiance (Figure 2.10). The seedlings in the two studies were not completely similar; when tested at $130 \mu\text{mol m}^{-2}\text{s}^{-1}$ and below during Phase 3, levels of qN were higher in Study III than in the second study (Figure 2.10). In Phases 3 and 4,

in both studies, qN was high at irradiances of $125 \mu\text{mol m}^{-2} \text{s}^{-1}$ and above (Figure 2.10).

Regardless of water status, qN approached saturation at $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 2.10). In Study III, it is clear that qN approached saturation at all irradiances as F_v/F_m decreased below 0.75 (Figure 2.11).

Figure 2.7 Average photochemical efficiency of PSII (Φ_{II}) at increasing irradiance for (a) seedlings and (b) emblings (Study II) during Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa) and Phase 3 ($-1.6 < \Psi < -3$ MPa) measured at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$. Standard error is indicated only where it exceeds symbol size.

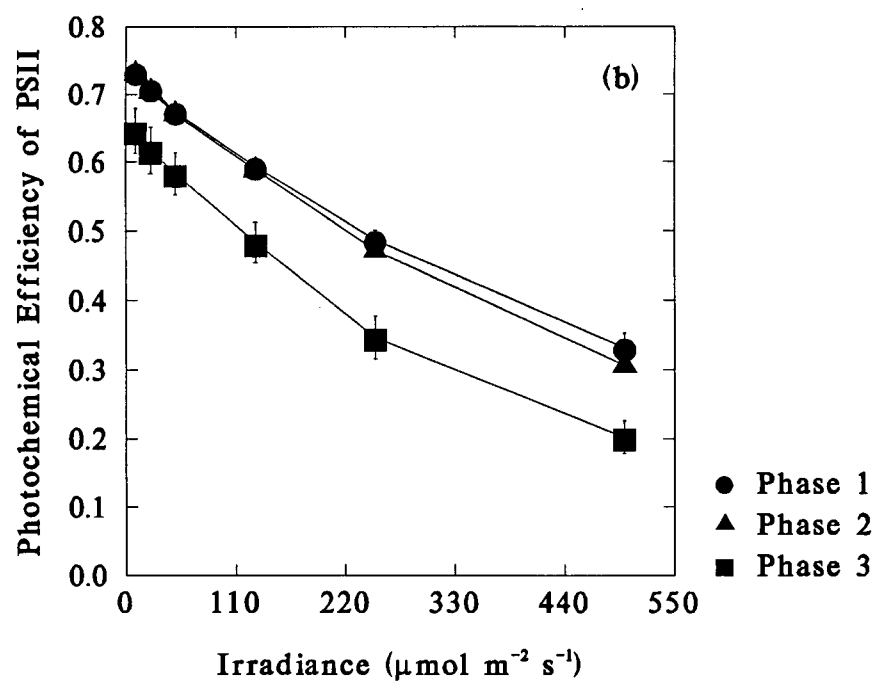
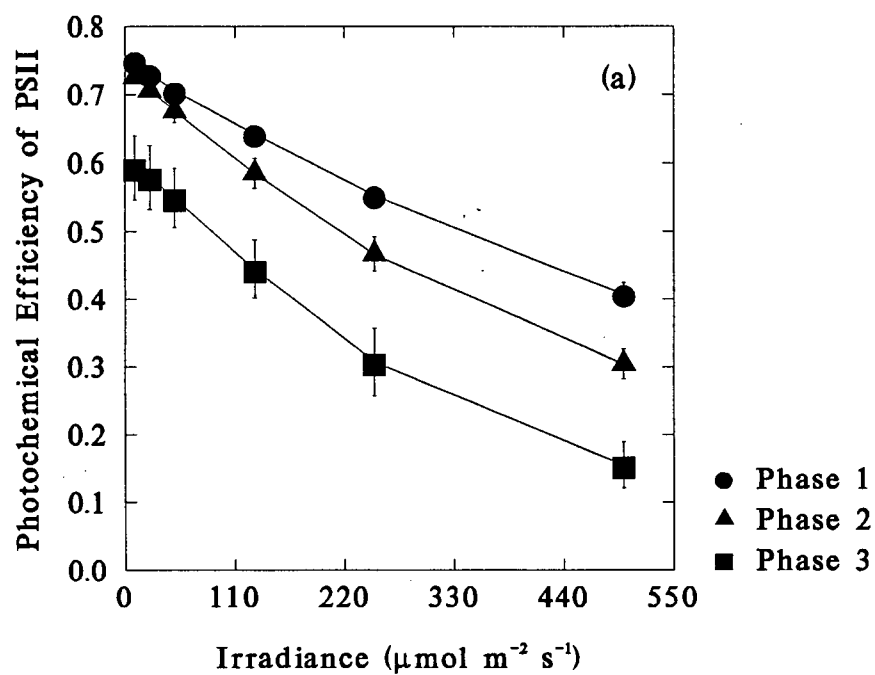


Figure 2.8 Average photochemical efficiency of PSII (Φ_{II}) at increasing irradiance for seedlings (Study III) during Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa), Phase 3 ($-1.6 < \Psi < -3$ MPa), and Phase 4 ($-3 \leq \Psi \leq -4$ MPa) measured at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$. Standard error is indicated only where it exceeds symbol size.

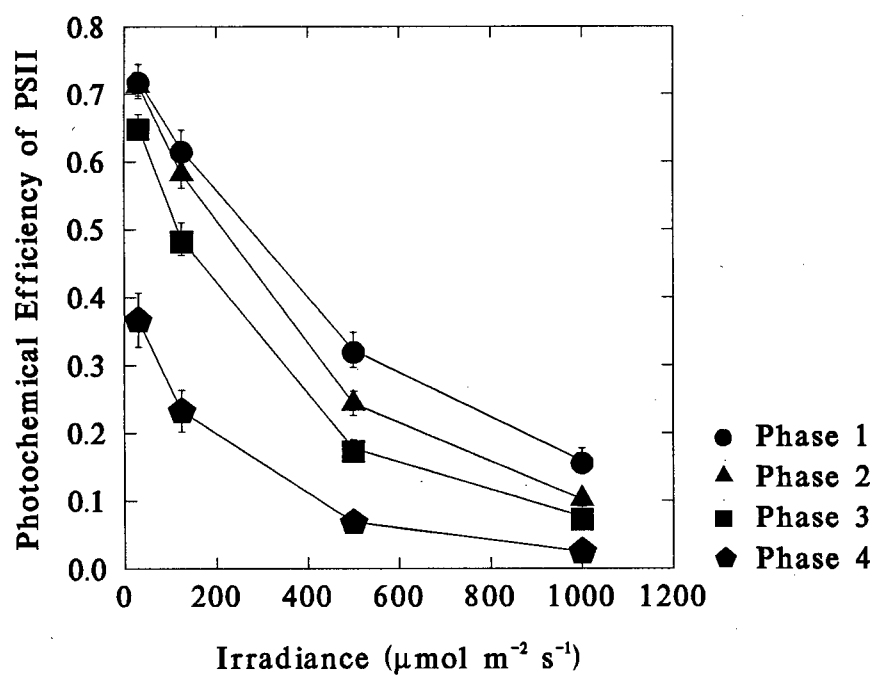


Figure 2.9 Average photochemical quenching (qP) at different irradiances during Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa), Phase 3 ($-1.6 < \Psi < -3$ MPa), and Phase 4 ($-3 \leq \Psi \leq -4$ MPa) measured at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$ in young interior spruce from (a) Study II and (b) Study III. Standard error is indicated only where it exceeds symbol size.

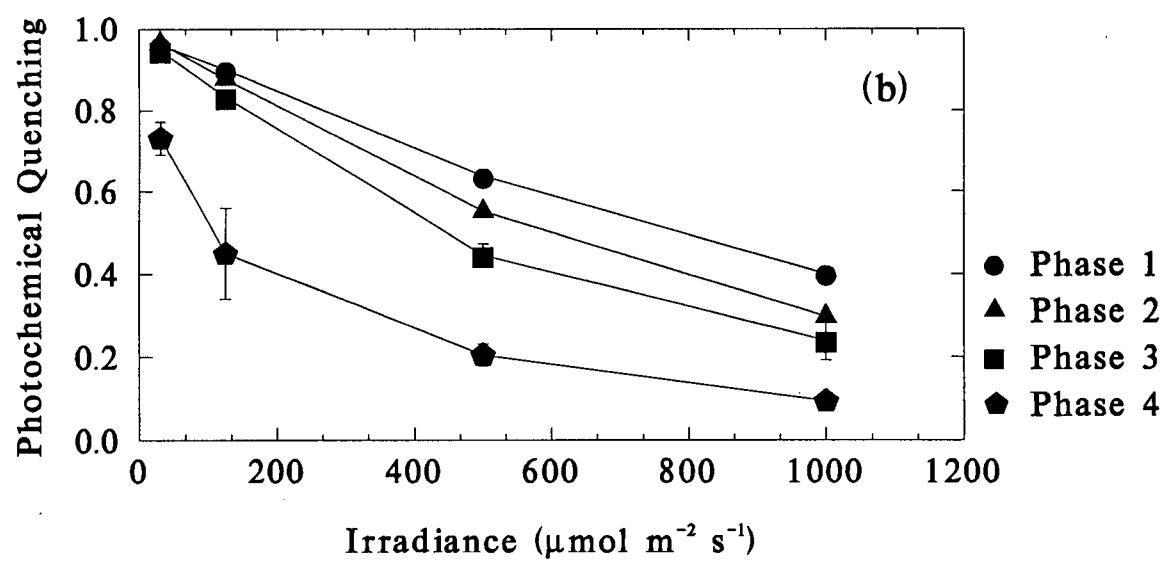
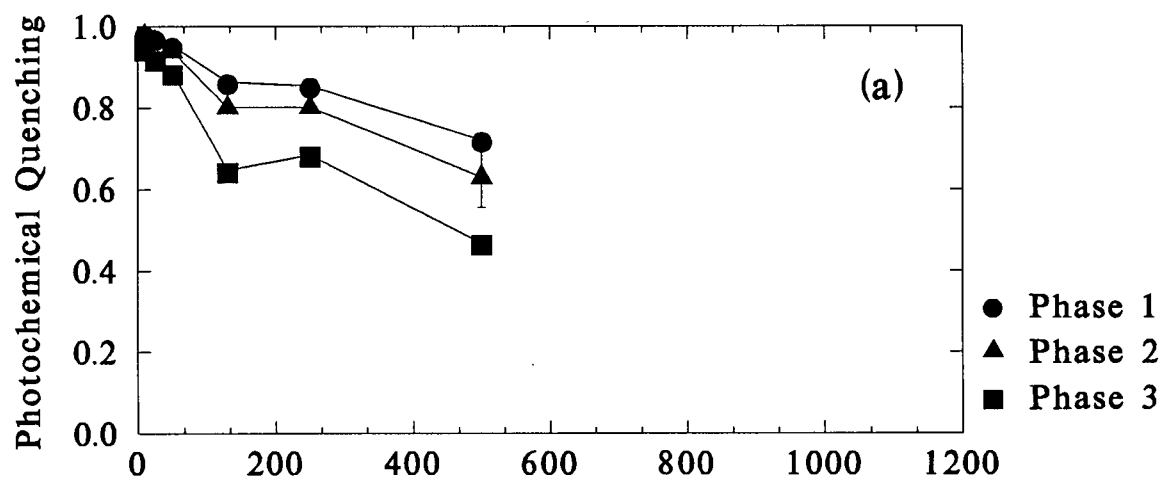


Figure 2.10 Average nonphotochemical quenching (qN) at different irradiances during Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa), Phase 3 ($-1.6 < \Psi < -3$ MPa), and Phase 4 ($-3 \leq \Psi \leq -4$ MPa) measured at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$ in interior spruce from (a) Study II and (b) Study III. Standard error is indicated only where it exceeds symbol size.

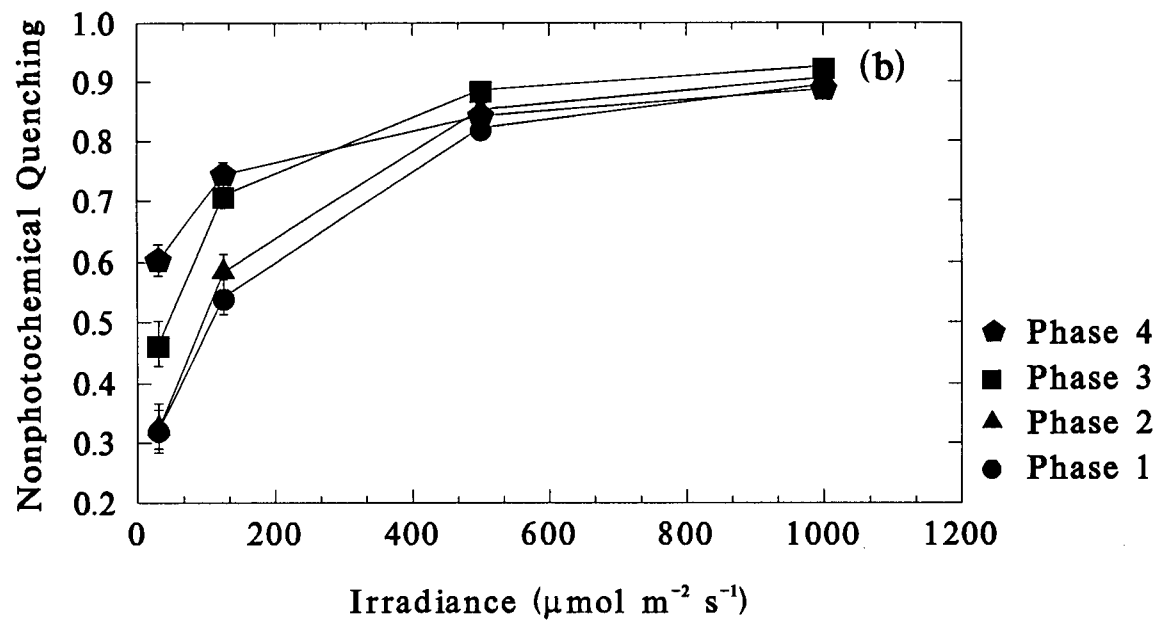
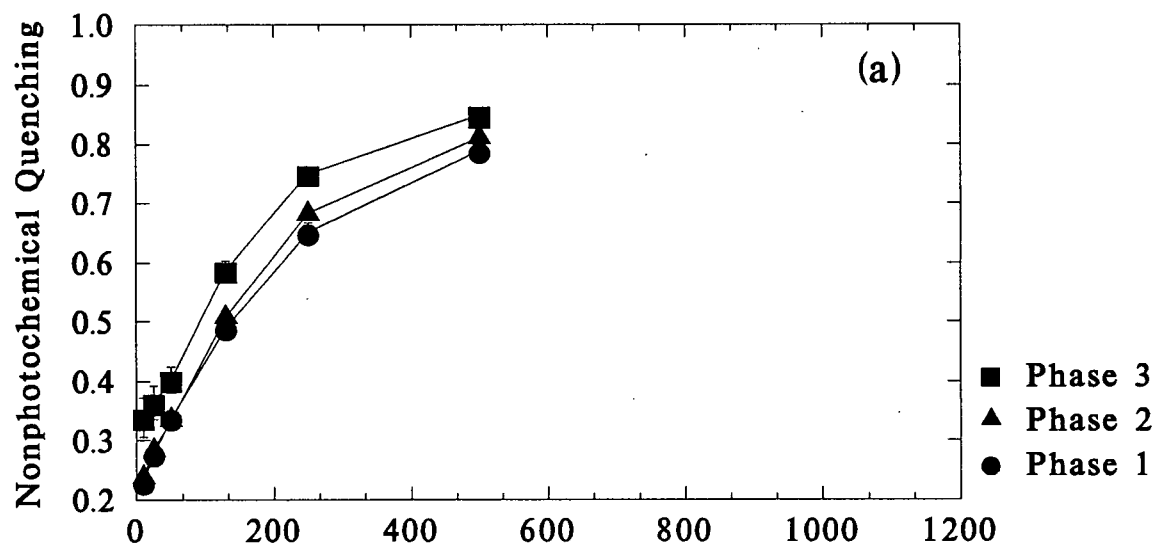


Figure 2.11 Relationship between nonphotochemical quenching (qN) at different irradiances and dark-adapted quantum yield (F_v/F_m) measured at $10,000 \mu\text{L CO}_2 \text{ L}^{-1}$, in response to predawn shoot water potentials in interior spruce trees in (a) Study II and (b) Study III.

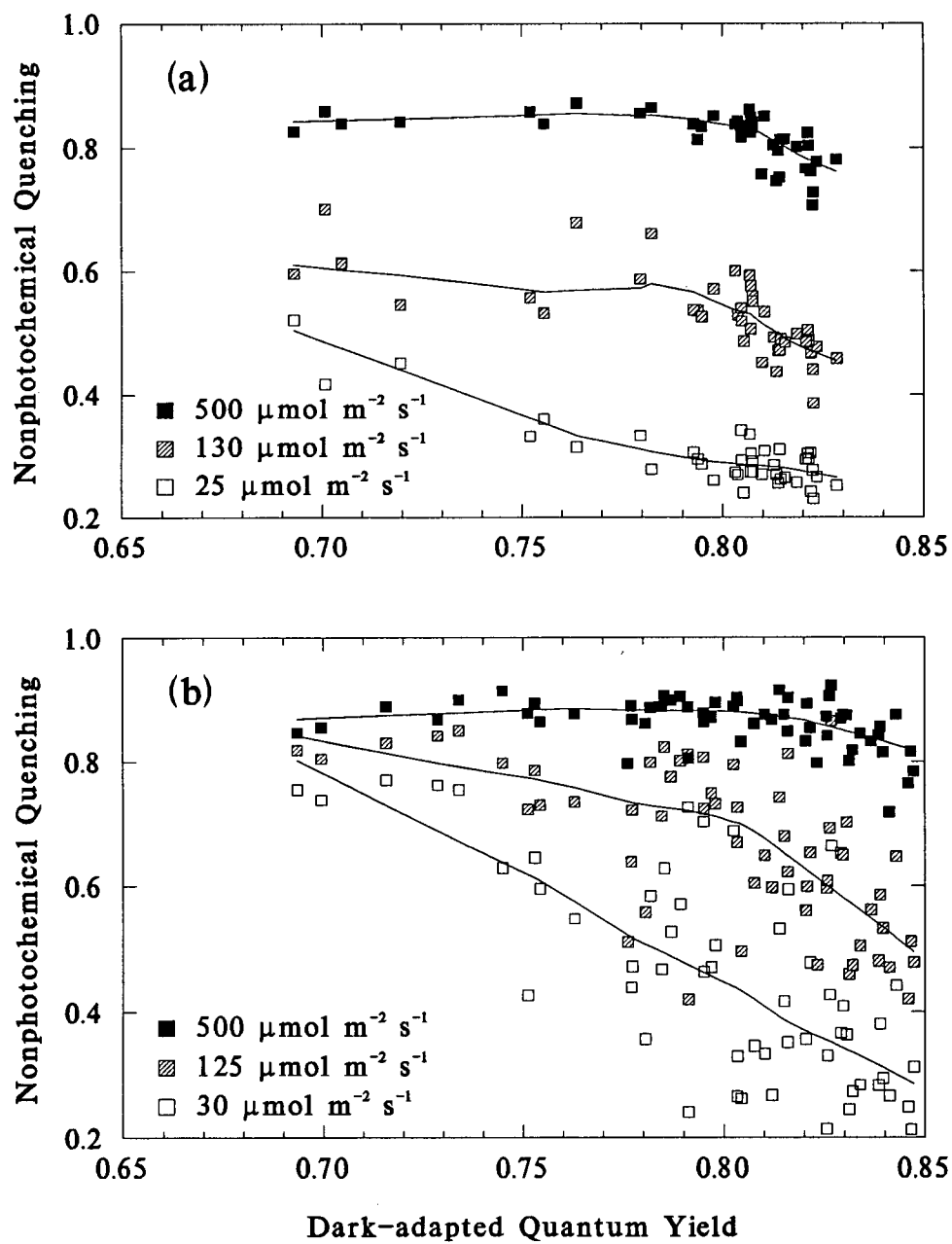
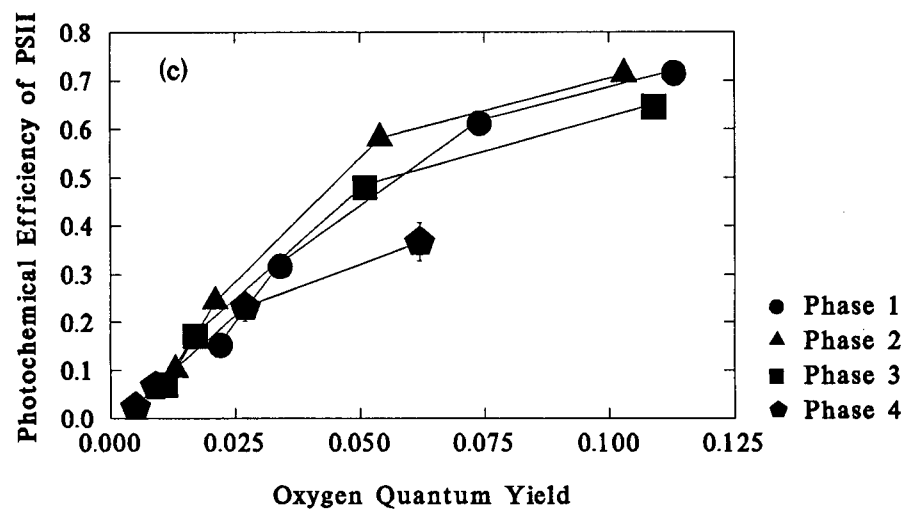
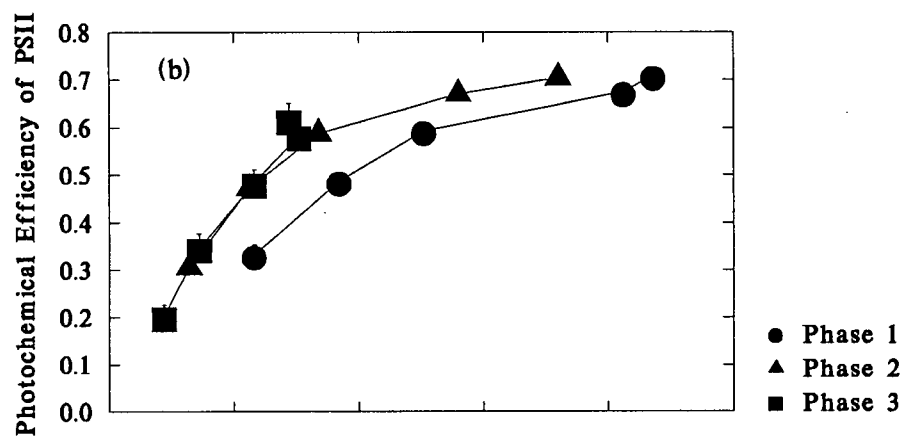
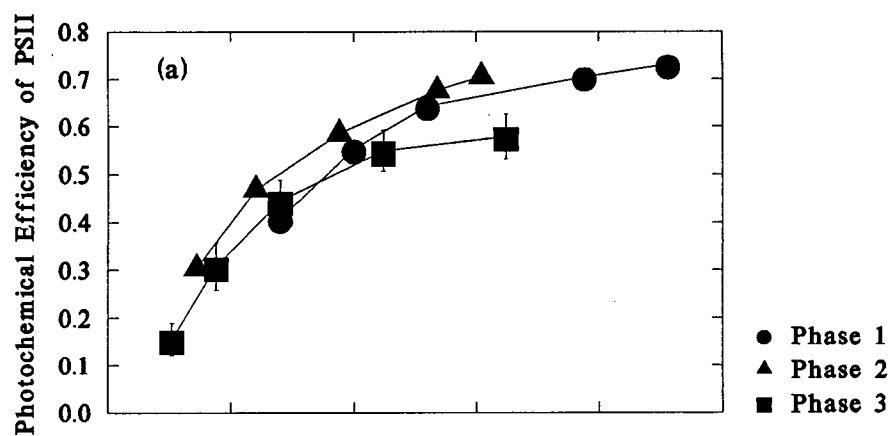


Figure 2.12 Relationship between average photochemical efficiency of PSII (Φ_{II}) and average oxygen quantum yield (Φ_{O_2}) during Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa), Phase 3 ($-1.6 < \Psi < -3$ MPa), and Phase 4 ($-3 \leq \Psi \leq -4$ MPa) measured at 10,000 mL $\text{CO}_2 \text{ L}^{-1}$ in (a) seedlings from Study II, (b) emblings from Study II and (c) seedlings from Study III. Standard error is indicated only where it exceeds symbol size.



Comparison of average values of Φ_{II} and apparent oxygen quantum yield (Φ_{O_2}) for irradiances above the compensation point revealed a curvilinear relationship that was only slightly affected by water status (Figure 2.12). The curve plotted for emblings in Phase 1 was slightly below the curves for Phases 2 and 3, as both Φ_{II} and Φ_{O_2} were low (Figure 2.12b). Values of Φ_{O_2} were also low at the lowest irradiance in Phase 3 emblings (Study II) and Phase 4 seedlings in Study III (Figure 2.12b,c).

2.4 Discussion

In young interior spruce plants undergoing shoot elongation, four phases in photosynthetic regulation during progressive water stress were identified. Phase 1 corresponded to plants subjected to little or no stress at Ψ above -1 MPa. Phase 2 ranged from the point of almost complete stomatal closure at Ψ of -1 MPa to -1.6 MPa when chlorophyll fluorescence changes started to become pronounced. Phase 3 extended from this point to Ψ of -3.0 MPa. A fourth phase was defined in Study III as extending from Ψ of -3 MPa, when photoinhibition became evident, to Ψ of -4 MPa. The most extreme level of stress attained in Studies II and III was nonlethal as, upon rewatering, most plants readily resumed photosynthetic activity.

In Phase 1, carbon assimilation declined rapidly. As Ψ dropped to -1 MPa, gas exchange of the mature needles fell to a minimal value at ambient CO_2 concentrations. A rapid response of gas exchange to mild drought under controlled conditions was previously reported for spruce seedlings and emblings (Grossnickle and Major 1994). In many C_3 species, including spruce species (Kaufmann 1976; Teskey *et al.*, 1986; Seiler and Cazell 1990; Grossnickle and Major

1994), the response to decreased water availability also appears to be a continuous decline in gas exchange linearly correlated with stomatal conductance.

The primary limitation on photosynthesis during water stress is currently believed to be stomatal restriction of CO₂ availability (Sharkey and Seemann 1989; Chaves 1991; Cornic *et al.*, 1992; Epron and Dreyer 1993); however, as Ψ decreases further, nonstomatal limitations including mesophyll resistance and other biochemical limitations become important (Graan and Boyer 1990; Renou *et al.*, 1990). The latter studies support the view that stomatal restriction of CO₂ is not the only determinant of reduced photosynthetic activity during the initial stages of drought. In agreement with this, an analysis of four woody angiosperm species with a range of drought stress sensitivities revealed that stomatal control of photosynthesis accounted for less than 50 % of changes in carbon fixation under all conditions, ranging from well-watered to severely stressed (Ni and Pallardy 1992).

In conifers, a variety of interpretations of the impact of stomatal closure on carboxylation are reported. Complete or partial restoration of carbon fixation in drought-stressed conifer species by application of elevated CO₂ concentrations (660 and 838 mL L⁻¹) have been interpreted to mean that stomatal conductance is the major limitation on photosynthesis during drought (Conroy *et al.*, 1986; Lange *et al.*, 1986; Seiler and Cazell 1990). Other groups support a different interpretation. For example, in *Pinus taeda* L. gas phase limitation by stomatal closure was not the major limitation to photosynthesis under a variety of environmental conditions including drought (Teskey *et al.*, 1986) and, in a recent study of cold storage and water stress in white spruce seedlings, mesophyll CO₂ conductance was considered to be more important than stomatal restrictions (Jiang *et al.*, 1995).

In this thesis work, it is unlikely measurements of O_2 production were limited by stomatal closure because CO_2 levels were maintained at $10,000 \text{ mL L}^{-1}$; an ambient concentration of $1,000 \text{ mL L}^{-1} CO_2$ was reported as saturating for spruce when the stomata were not tightly closed (Lange *et al.*, 1986). In my work, at irradiances above the compensation point, a rapid decline in oxygen production was observed in detached spruce needles from Phase 1 plants (Ψ above -1 MPa). It is important to emphasize that the stomata were not closed in Phase 1. Thus, the observed decline in oxygen evolution at elevated CO_2 reflects internal limitations, reflecting both a decrease in gaseous diffusion through the mesophyll and diminished carboxylation activity. Since down-regulation of carboxylation may be initiated as low CO_2 levels deactivate sucrose phosphatase (Sharkey 1990), stomatal and non-stomatal limitations on carbon fixation may not be readily separable.

A second phase of water stress was defined upon stomatal closure in young spruce trees. At Ψ between -1 and -1.6 MPa , the onset of regulatory processes was apparent from observations of oxygen production and chlorophyll fluorescence. Oxygen evolution measured at elevated CO_2 declined significantly; average rates were 40 % to 50 % of the rate measured in plants in Phase 1. Photochemical efficiency (F_v/F_m) of dark-adapted needles was unaffected; however, at irradiances of $125 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and above, decreases in the efficiency of photosystem II (Φ_{II}) revealed that linear electron transport was slowing. This was a consequence of decreases in both photochemical quenching (qP) and the efficiency of open PSII centres (Φ_P).

Despite the generally uniform response observed for young spruce trees, there were some distinctions between the seedlings and emblings. The observed differences in Φ_{II} between

Phase 1 seedlings and emblings in Study II could reflect a genetic difference. Yet, some individual seedlings demonstrated a pattern similar to the emblings, that is lower values of Φ_{II} and higher qN in Phase 1, which indicates that the embling response was a subset of the range possible for spruce plants in this developmental stage. The results of the third study, in which Phase 1 data were collected from a subset of well-watered plants throughout the stress, also support the suggestion that the response of the emblings was typical of this species. Most importantly, the curves for Phases 1 and 2 from the three data sets overlap.

Phases 1 and 2 were not readily separated using the chlorophyll fluorescence data. Although stomatal conductance was minimal in Phase 2, electron transport was minimally affected if CO_2 levels were elevated. It is important to acknowledge that the elevated CO_2 levels used during measurements of O_2 evolution and chlorophyll fluorescence may have extended the Phase 1 response pattern. Consequently, the decline in oxygen evolution may reflect an increase in photorespiration and/or dark respiration in the light rather than significant changes in the light reactions. Nonphotochemical quenching (qN) increased in this phase; however, average values of qN for Phase 1 and 2 were very close in all three studies again indicating that at this point any water stress effects on PS II were readily reversible.

In contrast, changes in chlorophyll fluorescence in young spruce trees during the third phase of water stress did reflect the down-regulation of primary photochemistry and the augmentation of photoprotective mechanisms that may avoid overreduction and photoinhibitory damage. The decline in oxygen evolution to less than 30 % of the Phase 1 rate paralleled a decrease in F_v/F_m in Study 2. This latter feature suggests that critical levels of PSII centres had become irreversibly damaged. At all irradiances, Φ_P decreased, resulting in both lowered qP and Φ_{II} . Although the greatest increases in qN were in response to increased irradiance at all stress

levels, qN increased concurrently with water stress in this phase. Increased qN , to dissipate the excess light energy, is a common drought response in many species (Stuhlfauth *et al.*, 1988, 1990; Massacci and Jones 1990; Epron and Dreyer 1992, 1993); however, in my work, the increases in qN at limiting irradiance demonstrate an enhanced need for dissipation of even low levels of light energy. The simultaneous increase in qN , decrease in F_v/F_m , and decreases in Φ_{II} in Phase 3 indicate that the system was significantly limited by this level of stress.

At $500 \mu\text{mol m}^{-2}\text{s}^{-1}$, qN appears to be almost maximal at all Ψ , suggesting that these plants have a limited capacity for thermal deexcitation. This could indicate limited development of qN mechanisms under the light conditions ($400\text{-}500 \mu\text{mol m}^{-2}\text{s}^{-1}$) used for growth since light acclimation has a significant effect on the qN capacity in many species (Ogren and Rosenqvist 1992; Brugnoli *et al.*, 1994; Demmig-Adams *et al.*, 1995). Nevertheless, in a seasonal study of 25 year-old plantation-grown Norway spruce, qN was saturated at $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Strand and Lundmark 1995). Spruce seedlings developing in the forest understory are typically exposed to low irradiance and *P. glauca* trees, in particular, demonstrate low physiological plasticity in response to changing light levels (Munson *et al.*, 1995). Thus, the apparent saturation of qN at $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ in my work may reflect a real limitation of the photoprotective capacity in young spruce tree needles. The authenticity of the observed qN saturation is further supported by the results of Study III in which dark adaption was overnight. Consequently, it seems likely that the observed pattern is not simply a consequence of limited recovery of the slow-relaxing components of qN (Demmig-Adams and Adams 1992; Horton *et al.*, 1994).

The most severely water-stressed plants investigated (Phase 4) included the Ψ range at which photoinhibition became pronounced and extended to the onset of lethal stress. The

average rate of oxygen evolution decreased further to approximately 20 % of the Phase 1 rate and appeared to approach saturation at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Quantum yield and Φ_{II} plunged. Photochemical quenching was low even at low light levels and qN was maximal. The limits of water stress tolerance were reached in this phase: photoprotective mechanisms had been utilized to capacity and the integrity of PS II centres was failing. With the techniques used, there was no evidence that other stress resistance/tolerance strategies were invoked.

There is a well-documented response to lowered carboxylation rates that was not directly evaluated in my studies - photorespiration. Although rapid synchronous control of the carbon reduction cycle and the activity of the two photosystems has been demonstrated (Foyer *et al.*, 1992), analysis of the coordinated down-regulation of the dark and light reactions in response to water stress is complicated by an increased diversion of electron flow to O_2 in photorespiration which can maintain a significant level of light energy consumption in C_3 species (Stuhlfauth *et al.*, 1990). However, in my work on young spruce trees, it is unlikely that photorespiration accounted for maintenance of PSII activity during chlorophyll fluorescence measurements because the relationship between Φ_{II} and Φ_{O_2} remained constant at non-limiting irradiance for all stress levels. The observed curvilinear relationship between Φ_{O_2} and Φ_{II} corresponds well with previous studies (Seaton and Walker 1990; Oquist and Chow 1992) for a variety of species, indicating that increased photorespiration (Cornic *et al.*, 1992) did not occur at the elevated CO_2 levels applied.

Although the relationship between Φ_{O_2} and Φ_{II} was consistent with other reports, the decreases observed in both of these measures of PSII activity, before drought stress led to photoinhibition, have not been previously documented. No comparable decrease in electron transport during *mild* water stress has been reported for an angiosperm species. In fact, even at

severe dehydration, no change in Φ_{II} was observed in tomato (Havaux 1992), white clover (Grieu *et al.*, 1995) or oak seedlings (Epron and Dreyer 1993). In some angiosperm species subjected to photoinhibitory light conditions (Oquist *et al.*, 1992b) or dehydration to the point of turgor loss (Cornic *et al.*, 1992), Φ_{II} does decrease; however, in young spruce trees decreases in Φ_{II} were observed before the onset of photoinhibition. A study of water-stressed *Helianthus annuus* plants under non-photoinhibitory conditions showed no drop in electron transport rate until the onset of senescence (Conroy *et al.*, 1988). In other angiosperm species the insensitivity of oxygen quantum yield to water deficits also implies that linear electron flow may be maintained until extensive photodamage occurs (Ben *et al.*, 1987; Cornic *et al.*, 1989; Epron and Dreyer 1993; Grieu *et al.*, 1995). The decreases in oxygen quantum yield and electron transport in mildly water-stressed young spruce trees indicate that the decreased stromal requirement for reducing power was translated into diminished electron flow, i.e. down-regulation of PSII, at an early stage of stress.

In conclusion, all three studies using four genetically distinct groups of 1 year old spruce trees consistently demonstrated the same physiological events during drought stress response. While the observed differences in the magnitude of dark-adapted quantum yield and oxygen evolution between the Study II and III may relate to differences in nutrient levels which clearly affect oxygen evolution and the capacity for electron transport in white spruce (Strand and Lundmark 1995), the overall pattern of change observed in the different studies was in accord. This implies that the reported hierarchy of photosynthetic down-regulation (stomatal closure → decreased carboxylation → down-regulation of linear electron transport) is an authentic description of water stress response for this gymnosperm species.

Chapter 3: Thylakoid Protein Changes during Progressive Water Stress

3.0 Summary

Changes in thylakoid membrane protein levels and membrane association were examined by immunoquantification of photosystem II (PSII) proteins, including the extrinsic oxygen-evolving enhancer (OEE) polypeptides, in thylakoids isolated from young interior spruce plants (*Picea glauca* (Moench) Voss X *P. engelmanni* Parry hybrid complex) of defined water status. After mild water stress, total D1 reaction centre protein content progressively decreased relative to levels in well-watered plants. Membrane association of extrinsic proteins, inferred from extractability in Triton X-100, showed progressive decreases for OEE2, but not OEE1, during nonlethal water stress. After a lethal water stress, D1, OEE1 and OEE2 levels decreased and further decreases in the membrane association of OEE2 which, along with losses of cytochrome *f*, suggested that thylakoid membrane integrity was affected.

Lethal water stress in spruce seedlings compromised oxygen evolution in isolated chloroplasts, measured using 2,6-dichloro-*p*-benzoquinone (DCBQ) as an electron acceptor from PSII. Addition of CaCl_2 partially restored oxygen evolution, suggesting that the stress response involved OEE2 protein loss or alteration. Severe water stress also diminished photoreduction of 2,6-dichlorophenol-indo-phenol (DCIP) in isolated chloroplasts. Added CaCl_2 or use of 1,5-diphenylcarbazine (DPC) as a direct electron donor to PSII augmented electron transport rate but did not restore it to levels measured for well-watered plants establishing that lethal water stress also involved loss of functional reaction centres.

Analysis of the immunoquantification results coupled with information from electron transport measurements was used to develop a hypothesis to explain resistance to photodamage of PSII during water stress in young spruce. Basically, down-regulation of PSII activity may be

initiated, before photoinhibitory damage occurs, by dissociation of the OEE2 protein concomitant with a decrease in oxygen evolution. This would result in diminished electron flow from PSII, possibly ameliorate production of reactive oxygen species, and, potentially, reduce the rate of D1 damage.

3.1 Introduction

Changes in electron transport and thylakoid protein dynamics during photoinhibition, evoked by light stress and exacerbated by other stresses including drought, have been modeled from studies using green algae and angiosperm species, mostly annuals (Aro *et al.*, 1993a). Photosystem II and, in particular, the D1 reaction centre protein have been identified as the primary targets of excess light. The cascade of events ranging from reduced electron transport to PSII inactivation and accelerated turnover is currently of intense research interest (Aro *et al.*, 1993a,b; Mishra *et al.*, 1994).

In the perennial photosynthetic apparatus, reductions in electron transport capacity in response to seasonal stress (Oquist and Strand 1986; Strand and Oquist 1988; Bolhar-Nordenkamp and Lechner 1988) and pollution damage (Dietz *et al.*, 1988; Amundson *et al.*, 1992; Evans *et al.*, 1992; Ruth and Weisel 1993) have been reported. Changes in protein synthetic capacity (Schmitz *et al.*, 1993) and in levels or activity of various thylakoid proteins (Godde 1992; Godde and Buchhold 1992; Lutz *et al.*, 1992; Flammersfeld and Wild 1992) have also been reported for pollution damaged Norway spruce. These studies show that levels of cytochrome *f* and, particularly, the D1 reaction centre protein, are significantly reduced in spruce by oxidative stress. Comparable investigations on the impact of water stress on photosystem II proteins in a conifer have not been published. Water stress, however, is a frequent environmental constraint on photosynthesis in spruce (Binder *et al.*, 1989) that affects PSII function as detailed in Chapter 2 of this thesis.

The biochemical processes leading to inhibition of water oxidizing ability during water stress have not been explored previously in a gymnosperm species although the impact of water stress on spruce seedling growth and vitality is well characterized at the whole plant level

(Binder *et al.*, 1989; Grossnickle 1989). Analysis of chlorophyll fluorescence induction kinetics has provided evidence that PSII activity is inhibited at the donor side in spruce (Toivonen and Vidaver 1988). In angiosperm species several mechanisms have been proposed for *in vitro* donor side photoinactivation; however, mechanisms *in vivo* are under debate (Aro *et al.*, 1993a; van Wijk and van Hasselt 1993).

Regulation of PSII activity during stress may involve changes in the extrinsic oxygen-evolving enhancer (OEE) polypeptides. There are numerous examples in which stress causes loss or dissociation of OEE polypeptides in angiosperm species. Changes in OEE protein levels and/or membrane association have been documented in response to freezing (Shen *et al.*, 1990), chilling (Wang *et al.*, 1992a), heat stress (Franzen and Andreasson 1984; Garstka and Kaniuga 1988; Enami *et al.*, 1994; Takeuchi and Thornber 1994; Becker and Brady 1995), leaf development at elevated CO₂ (Robertson and Leech 1995), and photoinhibitory light exposure (Hundal *et al.*, 1990). Acclimation to heat shock was linked with retention of OEE proteins (Scott A. Heckathorn, personal communication) and maintenance of electron transport during *in vivo* heat stress was clearly correlated with retention of the OEE1 protein (Enami *et al.*, 1994). Generation of a salt-tolerant photoautotrophic tobacco cell line was found to be related to increased affinity of OEE2 for thylakoid membranes (Murota *et al.*, 1994). This evidence suggests that the association of the OEE proteins is a fundamental, stress labile feature of PSII.

In this thesis work on the impact of water stress on the photosynthetic activity in young spruce trees, I examined the effect of progressive drought stress on changes in thylakoid protein levels and membrane association with particular focus on the OEE proteins. I include data obtained in collaboration with Dr. Abdur Rashid on electron transport through PSII in a study of lethally stressed spruce seedlings (referred to as the Lethal Stress Study). Although water stress

is known to increase the susceptibility of PSII to photodamage (Powles 1984), to the best of my knowledge, this work provides the first report of water stress-induced dissociation of an OEE protein. Additionally, the decreased membrane association of OEE2 occurred at stress levels *before* the onset of photoinhibition in dark-adapted plants indicating that this process may be a component of PSII down-regulation.

3.2 Materials and Methods

3.2.1 Antibody Production

3.2.1.1 OEE Antibodies

To generate antibodies to the oxygen-enhancing extrinsic proteins, OEE1, OEE2 and OEE3, PSII-enriched membranes were prepared from spinach using the MDT method of Dunahay *et al.* (1984). Buffer fractions containing released OEE1, OEE2 and OEE3 (Yamamoto and Kubota 1987) were concentrated using 3 kDa cutoff centrifugal microconcentrators (Microsep, Filtron Technology Corp.) and the proteins purified using preparative (16 cm x 16 cm, 1.5 mm thick) SDS (sodium dodecyl sulfate) gradient (T=10-15 %, C=2.7 %) polyacrylamide gels (Laemmli 1970). Gel pieces containing purified protein, stained with 0.05 % (w/v) Coomassie Brilliant Blue G-250 (CBB) in 40 % methanol/7 % acetic acid (v/v), were electroeluted (Micro-electroluter, Amicon) in 25 mM tris(hydroxymethyl)aminomethane (Tris)/ 193 mM glycine, pH 8.3, containing 0.1 % (w/v) SDS. The eluted proteins were concentrated in 10 kDa cutoff microconcentrators (Centriluters, Amicon). The approximate amount of purified protein was estimated by comparison of CBB-stained band intensities with a dilution series of BSA electrophoresed simultaneously.

Purified OEE1 (50 µg), OEE2 (20 µg), or OEE3 (20 µg) were mixed 1:1 with Freund's complete adjuvant (Sigma) and injected intramuscularly by technicians from the UBC Animal Care Centre (ACC) into New Zealand white rabbits. Samples (10 mL) of preimmune blood were collected from each rabbit by the staff, left overnight at 4°C to clot the red blood cells, spun at 10,000 x g for 10 min and stored at -15°C (Harlow and Lane 1988). Booster injections containing OEE1 (50 µg), OEE2 (55 µg), or OEE3 (30 µg) were mixed 1:1 with Freund's incomplete adjuvant (Sigma) and administered 1 month after the initial injection. The first set of test bleeds (25 mL) was performed two weeks later by ACC staff, the sera isolated as before, sterilized by filtration through 0.2 micron filter units (Nalgene), aliquoted and stored at -15°C. A second and third series of boosters were administered at six week intervals, test bleeds taken, and, finally, total body bleeds performed by ACC staff.

Sera were tested for antibody activity using chloroplast proteins electrophoresed on mini polyacrylamide gels (T=12 %, C=2.7 %) and electrophoretically transferred (Transblot, BioRad) at 4°C to nitrocellulose (0.45 micron, BioRad) using Towbin buffer (Towbin *et al.*, 1979). The protocol for development of immunoblots is outlined in Table 3.1.

3.2.1.2 D1 Antibody

For production of a D1 antibody, an *E. coli* expression vector carrying *lacI^q* (strain pUR292) was transformed (Sambrook *et al.*, 1989) with plasmid pPND1 which contains the entire *psbA* gene from *Poa annua* fused to the *B*-galactosidase gene, generously provided by Dr. P.J. Nixon (Nixon *et al.*, 1987). Transformation of colonies, selected on ampicillin (50 µg mL⁻¹)

Table 3.1 Details of the protocol used for immunoblot development.

Treatment	Reagent(s)	Incubation
Blocking of nitrocellulose	5 % (w/v) fish skin gelatin (FSG), Norland	60 min to overnight
Primary Antibody	preimmune or immune serum in phosphate-buffered saline (PBS) pH 7.0, 3 % FSG, 0.01 % sodium azide (Antibody Buffer)	60 min
Washes	0.05 % (w/v) Tween-20 (BDH) in PBS	3 x 10 min
Wash	PBS	10 min
Secondary Antibody	goat-anti rabbit IgG -alkaline phosphatase conjugate (BRL) diluted 1:3000 with Antibody Buffer	60 min
Washes	0.05 % Tween-20 in PBS	3 x 5 min
pH adjustment	50 mM Tris-HCl, pH 8.0	2 x 5 min
Colour Development	40 mg mL ⁻¹ 1,5-naphthalenedisulfonate salt, (Sigma) and 20 mg mL ⁻¹ sodium naphthal AS-MX phosphate (Sigma) in 50 mM Tris-HCl, pH 8.0	3 to 30 min
Stop	distilled water	3 brief rinses

medium, was verified by HindIII digestion of mini plasmid preparations (Zhou *et al.*, 1990) and products of the expected size were identified on 1 % (w/v) mini agarose gels. Transformants were amplified, fusion protein expression induced by 500 μ M isopropylthio- β -D-galactoside (Sigma), and the fusion protein purified from crude cell lysates on preparative SDS-PAGE (T=7.5 %, C=2.7 %). The purified protein was electroluted from gel slices and concentrated using 10 kDa Centrilters. An initial injection containing approximately 100 μ g was used to immunize a rabbit as described previously. Three booster injections, approximately 75 μ g each, were made over the next 3 months.

3.2.1.3 Other Antibodies

D1 and OEE2 antibodies were generous contributions from Dr. Roberto Barbato (Universita di Padova) and Dr. Abul Ekramadoullah (Forestry Canada, Victoria), respectively. The antibody to cytochrome *f* was a gift of Dr. R. Malkin (University of California at Berkeley). Antibodies to the chlorophyll *a/b* binding proteins of PSII (CPII), ATP synthetase, and cytochrome *b₅₅₉* were provided by Dr. B.R. Green (University of British Columbia).

3.2.2 Needle tissue

The production and physiological characterization of plants at four phases of progressive water stress studies was detailed in Chapter 2. Briefly, water stress was invoked in 1-year old interior spruce plants that were undergoing shoot elongation. Material from Studies II (seedlings and emblings) and III (two subsets of seedlings designated as 'X' and 'Y') were used for the immunoquantification analysis.

For the Lethal Stress Study, 1-year old interior spruce seedlings grown in styrofoam block containers (Pelton Reforestation Inc., Maple Ridge, BC) were maintained in a greenhouse at warm temperatures under natural photoperiod from September 1993 through to May 1994. Buds remained dormant throughout this period as indicated by the absence of new shoot growth. To induce water stress, water was withheld from subsets of the young trees. An adjacent, regularly watered group was used for comparison. Predawn shoot water potentials (Ψ) were measured with a pressure chamber (Soilmoisture Equipt. Corp., Model 3015). A Walz pulse-modulated fluorometer (PAM 101,103) was used to determine maximal fluorescence quantum yield (F_v/F_m) according to Schreiber *et al.* (1986). A portion of the intact shoot was dark-adapted for 10 min, dark level fluorescence measured and then a 1 s saturating flash ($11,000 \mu\text{mol m}^{-2} \text{s}^{-1}$) applied to determine maximal fluorescence. Plants were deprived of water until the average predawn shoot water potential was less than -3.5 MPa and the average maximal quantum yield (F_v/F_m) was between 0.3 and 0.5, indicating that these plants were significantly photoinhibited. Well-watered control seedlings had an average predawn shoot water potential of -0.5 MPa and an average F_v/F_m of 0.8. The reversibility of the stress was tested by rewatering a subset of stressed plants and assessing Ψ and quantum yield after 3 to 7 days.

For all studies, the needles were harvested from the plants by freezing the shoots in liquid nitrogen. The water stress phases were defined on the basis of the physiological data presented in Chapter 2. Briefly, individual plants were categorized as Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa), Phase 3 ($-1.6 < \Psi < -3$ MPa) or Phase 4 ($-3 \leq \Psi \leq -4$ MPa). For immunoquantification, the needles were stored at -80°C until used to prepare thylakoid samples. Needle samples from five or more plants from each phase were pooled.

3.2.3 Membrane Isolation

3.2.3.1 Protocol Development

To isolate spruce chloroplasts without disrupting thylakoid integrity, different features of the isolation, resuspension and storage buffers were considered. Levels of divalent magnesium ions were kept at 5 mM to maintain granal stacking (Staehelin 1976) and chloride levels were always above 20 mM to maintain OEE2 membrane association (Homann 1988a). Sucrose was determined to be better than sorbitol in maintaining O₂ evolution activity and stabilizing OEE proteins in spruce chloroplasts during storage; spruce membranes were determined to be more osmotically sensitive than those from spinach (Rashid and Camm 1994). The presence of protease inhibitors (phenylmethanesulfonyl fluoride, benzamidine, and leupeptin) was also important in maintaining OEE and D1 protein levels during storage of membrane preparations.

To determine whether any OEE proteins existed free in the lumen in spruce chloroplasts, I attempted to produce PSII-enriched membrane fragments washed free of lumenal content. Various combinations of chloroplast isolation and PSII preparation techniques (Dunahay *et al.*, 1984; White 1986; Van Leeuwen *et al.*, 1991) including a protocol used to generate PSII from spruce cotyledons (Kukidome *et al.*, 1986) were not successful with mature needle tissue. Modifications of the latter method did generate a membrane subfraction that appeared, in some respects, to be PSII-enriched; however, the yield was very low and could not be consistently reproduced.

Other workers have reported the effectiveness of a mild Triton X-100 treatment on the release of plastocyanin, a lumenal protein, plus a proportion of the OEE proteins (Ettinger and Theg 1991). A similar treatment with Triton X-100 (BDH) to simply disrupt the thylakoid membranes, allowing release of unbound or loosely-associated lumenal proteins to the isolation

medium, was used to distinguish bound and unbound OEE proteins in spruce membrane preparations. The efficacy of this protocol was verified by exploiting the well-established alkali-induced release of OEE proteins to the lumen (Cole *et al.*, 1986; Chapman *et al.*, 1989; Ebina and Yamashita 1992). After Triton treatment, OEE protein release was quantified by probing immunoblots of different membrane subfractions and supernatants using specific antibodies. This allowed comparison of total OEE protein levels (untreated thylakoid levels) with bound OEE protein levels (post Triton- treatment levels). Thus, changes in the proportion of OEE proteins associated with PSII centres could be quantified. The use of ionophores, 10 mM each of valinomycin and nigericin, with or without sonication treatment of the membranes, did not increase release of OEE proteins at high pH indicating the membranes were already disrupted; however, Triton treatment appeared necessary to detect significant and reproducible removal of the OEE proteins by the alkaline treatment. A Triton X-100 to chlorophyll ratio of 15:1 was effective in releasing lumenal populations of OEE proteins without solubilizing intrinsic proteins such as D1.

3.2.3.2 Preparation of Membranes

Initial analyses of the effects of water stress on thylakoid proteins were made using plant material from the Lethal Stress Study. For this work, the frozen needle tissue was crushed to a fine powder in liquid nitrogen and homogenized in 50 mM N-tris[hydroxymethyl]-methylglycine (tricine) -NaOH (pH 7.6) containing 500 mM sucrose, 10 mM NaCl, 5 mM MgCl₂, 2 % soluble polyvinylpyrrolidone 40 (PVP-40), 0.1 % bovine serum albumin (BSA), and 1 mM phenylmethanesulfonyl fluoride (PMSF). After filtering the resultant brei through cheesecloth, the filtrate was centrifuged at 2500 x g for less than 2 min (Sorvall RC-5B, DuPont). The

chloroplast pellets were washed 5 times by resuspension in the isolation buffer without PVP-40 or BSA, followed by 2 min centrifugations at 2500 x g. The final thylakoid pellet was resuspended in thylakoid storage buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) -NaOH pH 7.6, containing 1 M sucrose, 10 mM NaCl and 5 mM MgCl₂), and stored at -80°C. For immunoprobings, additional protease inhibitors (1 mM benzamidine and 1 µg mL⁻¹ leupeptin) were included in all solutions. Chlorophyll content was determined according to Arnon (1949) or Lichtenthaler and Wellburn (1983) using a Milton Roy Spectronic 3000.

To prepare detergent fractionated membranes from the Lethal Stress Study, thylakoids were thawed on ice and Triton X-100 added to a final detergent:chlorophyll ratio of 15:1. Samples were incubated in the dark for 20 min on ice with gentle agitation, then diluted with thylakoid storage buffer and briefly centrifuged at 2500 x g. The supernatant was centrifuged at 39,000 x g for 30 min and the final membrane pellet resuspended in 25 mM morpholinoethane sulfonic acid (Mes), pH 6, containing 2 M sucrose, 10 mM NaCl and 5 mM MgCl₂. For immunoprobings, additional protease inhibitors (1 mM benzamidine and 1 µg mL⁻¹ leupeptin) were included in the final storage buffer.

In Studies II and III, membrane preparations were made using modified buffers; BSA was omitted, but all buffers contained 2 mM PMSF added immediately before use from a stock solution freshly prepared in dimethyl sulfoxide. Other additions were 2 mM CaCl₂ and 10 mM ε-aminocaproic acid, a serine protease inhibitor (Gray 1982). Modified centrifugation protocols were used to prepare Triton-treated membranes in Study II and Study III. After Triton treatment, the membranes from Study II and Study III were spun at 10,000 x g for 30 min. In a

refinement of this procedure, the second seedling set ('Y') in Study III was briefly centrifuged at 2500 x *g* to pellet large membrane fragments before the higher speed centrifugation.

3.2.3.3 Effects of Triton X-100

Water status did not affect the measured chlorophyll *a/b* ratios in thylakoid or Triton-treated membrane preparations. With one notable exception, sample set 'Y' from Study III, the Triton treatment resulted in no change in chlorophyll *a/b* ratios, indicating that a selective depletion of PSI did not typically occur (Table 3.2a).

Triton treatment did have a significant effect on the levels of the OEE2 protein in all spruce membrane samples (Table 3.2b). There were no apparent changes in any of the other proteins quantified, including OEE1, after Triton treatment of the thylakoids (not shown).

3.2.4 Immunoquantification

Membrane preparations were solubilized at 90°C for 3 min in 70 mM Tris-HCl (pH 6.8) containing 20 % glycerol, 2 % SDS and freshly added 10 % β -mercaptoethanol. This high level of β -mercaptoethanol was essential for good resolution. Membrane polypeptides from the lethally stressed plants were loaded on the basis of chlorophyll (250 or 500 ng chlorophyll per lane) and resolved on mini polyacrylamide gels (T=12 % or 14 %, C=2.7 %) using the Laemmli (1970) buffer system. Electrophoresis of proteins (250 ng chlorophyll per lane) from Studies II and III was performed using mini high-Tris (0.75 M) gels (T=15 %, C=3.3 %) containing 10 % (w/v) glycerol (Fling and Gregerson 1986) which provided a significant increase in resolution. Gels were stained with CBB before or following electrophoretic transfer of the proteins to

Table 3.2 The effects of Triton treatment on (a) average chlorophyll *a/b* ratios for thylakoids and Triton-treated membranes for all stress phases, including well-watered plants for the different sets of young interior spruce plants analyzed in Study II, seedlings (S) and emblings (E), and Study III, seedling sets 'X' and 'Y', and (b) OEE2 levels in Triton-treated membranes expressed as relative percentages of the thylakoid levels for the four sets of young interior spruce plants analyzed in Studies II and III. The asterisk (*) signifies that a modified centrifugation protocol using a brief, initial centrifugation at 2500 x *g* was used after Triton treatment in these samples. Values are $\pm$ standard error.

(a) Average chlorophyll *a/b* ratios for thylakoids and Triton-treated membranes

	Thylakoids	Triton-treated Membranes
Study II (E)	3.294 $\pm$ 0.077	2.954 $\pm$ 0.082
Study II (S)	3.791 $\pm$ 0.158	3.583 $\pm$ 0.162
Study III (X)	3.322 $\pm$ 0.039	3.125 $\pm$ 0.094
Study III (Y)	3.857 $\pm$ 0.120	*2.846 $\pm$ 0.075

(b) Relative OEE2 levels in Triton-treated membranes

	Phase 1	Phase 2	Phase 3	Phase 4
Study II (E)	64.08 $\pm$ 3.6	80.21 $\pm$ 4.4	64.44 $\pm$ 5.6	—
Study II (S)	61.51 $\pm$ 4.4	62.25 $\pm$ 5.7	62.24 $\pm$ 3.6	—
Study III (X)	81.40 $\pm$ 7.1	63.23 $\pm$ 4.6	48.16 $\pm$ 5.7	47.59 $\pm$ 5.7
Study III (Y)	*52.28 $\pm$ 6.1	*17.99 $\pm$ 1.7	*16.34 $\pm$ 4.8	*23.02 $\pm$ 5.6

nitrocellulose. Protein blots were carefully cut into horizontal strips of defined molecular weight ranges using coloured high molecular weight markers (Amersham Rainbow) as guides. The strips were blocked, probed with the appropriate antibody, and processed (Table 3.1). The linearity of each antibody response was checked several times over the course of this work using a dilution series of spruce thylakoids (Figures 3.1 and 3.2).

In order to examine water stress related changes in levels of photosynthetic proteins, it was necessary to ensure that equivalent amounts of membrane were compared. In samples loaded on the basis of chlorophyll there was no change in the relative amount of protein identified by the antibody to the PSII chlorophyll *a/b* binding proteins (α -CP II) in thylakoids or Triton-treated membranes. Thereafter, for Studies II and III, normalization was performed on every immunoblot using the CP II antibody reaction. To accomplish this, the replicate protein blots were probed first with an antibody of interest (Figure 3.2), then each blot was treated with 0.1 M glycine-HCl, pH 2.2, overnight to remove the antibodies from the initial probing (Legocki and Verma 1981), rinsed with PBS and reprobed using α -CP II. In the Lethal Stress Study, in which there was a change in the amount of protein identified by α -CP II, correction factors were derived from scanning of the major *cab* protein zone (23 - 25 kDa) in Coomassie-stained gels to ensure consistent comparison of membrane samples.

Immunoblots were scanned with an XRS scanner using BioImage software (Visage version 4.6Q, Millipore Corp. 1993) for quantification. Defined scan areas were chosen and the integrated optical densities measured. Values corrected using either *cab* protein density or the CP II antibody reaction were expressed relative to the value for protein preparations from well-watered (Phase 1) plants.

Figure 3.1 Immunoblots of interior spruce thylakoid proteins probed with the indicated antibodies showing the intensity of the antibody response with increasing amounts of protein.

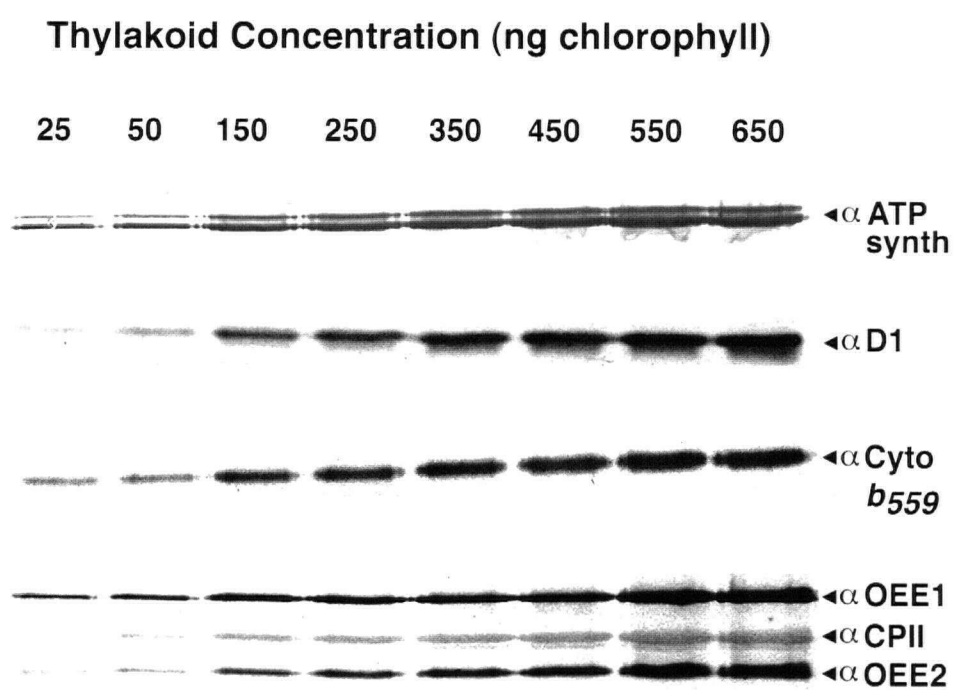
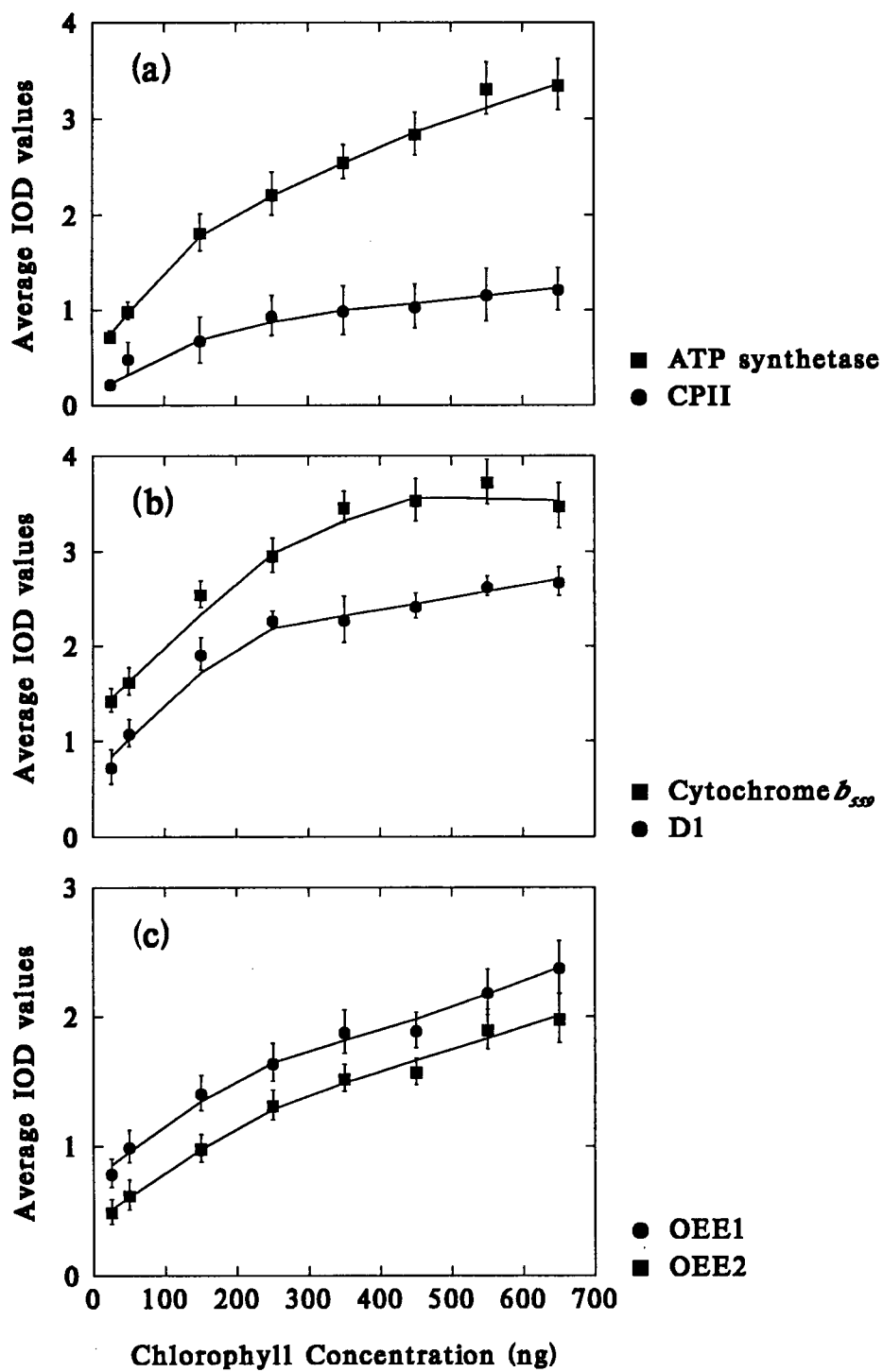


Figure 3.2 Relationship of average values of integrated optical density (IOD) of protein bands with increasing amounts of spruce thylakoids (sample set 'Y') probed with various antibodies: (a) ATP synthetase and C_{PII}, (b) cytochrome b₅₅₉ and D1, and (c) OEE1 and OEE2. Standard error is indicated. The arrow indicates the concentration (250 ng) used for immunoquantification.



3.2.5 Measurement of Electron Transport

Photosynthetic oxygen evolution by thylakoids or Triton-treated thylakoids equivalent to 40 μg chlorophyll was measured at 22°C using a Clark-type oxygen electrode (Hansatech Model DW1) upon exposure to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light (Kodak Ektagraphic III E projector equipped with a 300 W tungsten halogen lamp). The reaction mixture (2 mL) consisted of 25 mM Mes-NaOH (pH 6.0) containing 500 mM sucrose and 10 mM NaCl. As an electron acceptor from photosystem II, 500 μM 2,6-dichloro-*p*-benzoquinone (DCBQ) was used. Oxygen production was measured with and without the addition of 15 mM CaCl_2 . All measurements were performed in triplicate.

The capacity of thylakoids or Triton-treated membranes (equivalent to 30 μg chlorophyll) to photoreduce 2,6-dichlorophenol-indo-phenol (DCIP) was measured at 590 nm with a Cary 210 (Varian) spectrophotometer. The reaction mixture (2 mL) was the same as that used for oxygen evolution with the addition of 60 μM DCIP. Measurements made with or without the addition of 15 mM CaCl_2 and with or without 500 μM 1,5-diphenylcarbazide (DPC) were performed in triplicate.

3.3 Results

3.3.1 Antibody Testing

The preimmune sera from rabbits inoculated with spinach OEE1, OEE2, or OEE3 showed no detectable level of antibody reaction with leaf proteins from spinach or spruce. The OEE1 immune serum produced an intense, apparently monospecific reaction with proteins of 33 kDa in chloroplast preparations from spinach, lettuce, larch, pine and spruce. The OEE2 and

the OEE3 immune sera reacted with the OEE2 and OEE3 proteins of a dicot, spinach, and also those of a monocot, wheat (Scott Heckathorn, Syracuse University; personal communication); however, cross reactions with proteins from spruce chloroplast preparations were inconsistent. An OEE2 antibody raised to a fusion protein consisting of a 10 amino acid synthetic peptide derived from the N-terminal sequences of the OEE2 protein of *Pinus monticola* D. Don was generously provided by Dr. Ekramadoullah (Forestry Canada, Victoria). This antibody produced a monospecific reaction with OEE2 in spinach and a protein of slightly lower molecular weight in spruce membranes.

Preimmune sera and immune sera from the first test bleed from the rabbits injected with D1 fusion protein had high cross reactivity with many spinach proteins. Fortunately, sera from the third test bleed and the total body bleed showed a strong specific reaction with the D1 proteins in spinach and lettuce, confirmed by identification with a verified D1 antibody (gift of Dr. L. McIntosh, Michigan State University to Dr. B.R. Green). The sera also reacted with protein bands of slightly lower mobility in larch, pine, and spruce electrophoresed using the Laemmli buffer system; however, when the high Tris buffer gel system and higher percentage gels (T=15 %, C= 3.3 %) were used, the pattern changed. In this system, the antibody strongly reacted with a single band in spruce needle proteins co-migrating with the spinach D1 protein at approximately 34 kDa. The identity of this band as the spruce D1 protein was further confirmed by cross reaction with an antibody preparation raised to the 2-238 fragment of the wheat D1 protein (Barbato *et al.*, 1991), kindly provided by Dr. Roberto Barbato (Universita di Padova).

3.3.2 Immunoquantification

To evaluate changes in the thylakoid membrane composition in response to water stress, immunoblots of membrane samples from trees of defined water status, loaded on the basis of total chlorophyll, were probed with antibodies. The water stress phases, defined previously using the physiological data presented in Chapter 2, were Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa), Phase 3 ($-1.6 < \Psi < -3$ MPa) and Phase 4 ($-3 \leq \Psi \leq -4$ MPa). Protein levels were quantified relative to those found in Phase 1. Material from three separate water stress studies, Study II (seedlings and emblings), Study III (two subsets of seedlings designated as 'X' and 'Y'), and the Lethal Stress Study were used. Representative immunoblots probed with D1 and CII antibodies (Figure 3.3) or OEE1, OEE2 and CII antibodies (Figure 3.4) show the general patterns observed as water stress progressed in Study II and Study III. The results of immunoquantification are summarized in Tables 3.3 (Study II), 3.4 (Study III), and 3.5 (Lethal Stress Study).

3.3.1.1 Intrinsic Thylakoid Proteins

Overall, D1 levels decreased during drought stress, although the extent varied from experiment to experiment. In Study II, a minor decrease in D1 levels was evident in Triton-treated membranes from Phase 3 seedlings (Figure 3.3a, Table 3.3). No changes were detected in embling samples (Table 3.3). However, in Study III, significant, progressive decreases in D1 levels occurred in thylakoids prepared from both sets of seedlings examined (Figure 3.3b, Table 3.4). Low levels of D1 in Phase 4 thylakoid preparations were similar to those observed in the Lethal Stress Study (Table 3.5), about 68 % of the levels in well-watered (Phase 1 plants). Triton-treated membranes demonstrated similar trends.

Figure 3.3 Immunoprobings of interior spruce membrane protein preparations with antibodies for D1 and C_{PII}: (a) Representative protein immunoblot of thylakoids and Triton-treated membranes from needles harvested from seedlings during Phase 1, 2 or 3 of water stress as indicated (Study II), (b) Representative protein immunoblot of thylakoids and Triton-treated membranes from needles harvested during Phase 1, 2, 3, or 4 of water stress as indicated (Study III).

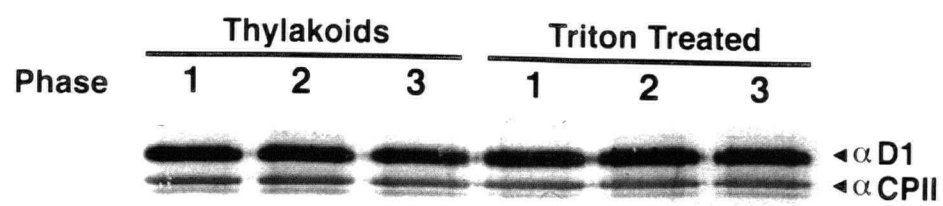
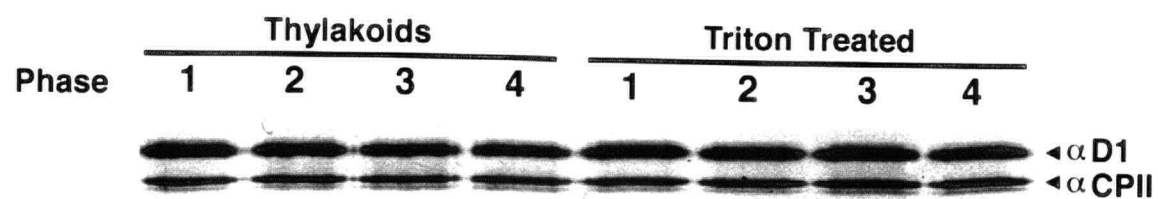
(a) Study II**(b) Study III**

Table 3.3 Protein quantification from immunoblots of thylakoids isolated from seedlings (S) and emblings (E) at different phases of progressive water stress during Study II. Values are means $\pm$ standard error of integrated optical density expressed relative to Phase 1 samples (number of immunoblots used for quantification in parentheses). Asterisks (* or **) indicate that the sample mean does not include 100 % (i.e. the well-watered level) at the 95 % or 99 % confidence level, respectively.

Protein	Thylakoids			Triton-treated Membranes	
	Sample	Phase 2	Phase 3	Phase 2	Phase 3
D1	S (8)	94.38 $\pm$ 2.95	90.64 $\pm$ 4.40	96.16 $\pm$ 4.34	90.68* $\pm$ 3.50
	E (5)	108.8 $\pm$ 7.16	96.52 $\pm$ 6.78	113.9 $\pm$ 8.95	104.0 $\pm$ 1.08
OEE1	S (7)	109.9 $\pm$ 1.49	106.77 $\pm$ 5.07	110.1 $\pm$ 3.68	121.2 $\pm$ 4.20
	E (5)	103.5 $\pm$ 5.13	101.8 $\pm$ 1.43	109.0 $\pm$ 7.28	96.82 $\pm$ 8.89
OEE2	S (6)	105.7 $\pm$ 3.72	100.9 $\pm$ 2.51	101.5 $\pm$ 3.62	49.20** $\pm$ 5.17
	E (5)	122.8 $\pm$ 2.57	113.0 $\pm$ 3.20	141.6 $\pm$ 7.43	118.0 $\pm$ 8.63
Cyt b₅₅₉	S (11)	105.9 $\pm$ 2.71	105.8 $\pm$ 2.75	104.6 $\pm$ 1.86	100.2 $\pm$ 3.29
	E (7)	114.6 $\pm$ 5.66	109.9 $\pm$ 5.88	104.6 $\pm$ 4.68	93.66 $\pm$ 6.87
ATP synthetase	S (13)	102.1 $\pm$ 3.20	106.7 $\pm$ 3.15	101.6 $\pm$ 3.05	112.4 $\pm$ 2.53
	E (8)	111.9 $\pm$ 5.59	113.9 $\pm$ 6.67	106.8 $\pm$ 3.69	98.02 $\pm$ 5.23

Table 3.4 Protein quantification from immunoblots of thylakoids, before and after Triton X-100 treatment, isolated from seedlings (sample sets 'X' and 'Y') at different phases of progressive water stress during Study III. Values are means $\pm$ standard error of integrated optical density expressed relative to Phase 1 samples (number of immunoblots used for quantification in parentheses). Asterisks (* or **) indicate that the sample mean does not include 100 % (i.e. the well-watered level) at the 95 % or 99 % confidence level, respectively.

Protein	Sample	Thylakoids				Triton-treated Membranes			
		Phase 2	Phase 3	Phase 4	Phase 2	Phase 3	Phase 4	Phase 2	Phase 3
D1	X (7)	89.26** $\pm$ 1.12	80.13** $\pm$ 1.94	73.38** $\pm$ 1.36	91.23** $\pm$ 1.85	81.73** $\pm$ 1.25	83.07** $\pm$ 2.48		
	Y (6)	85.72** $\pm$ 1.86	76.40** $\pm$ 3.88	61.42** $\pm$ 2.44	85.13** $\pm$ 3.32	78.92** $\pm$ 0.67	74.28** $\pm$ 2.00		
OEE1	X (7)	102.6 $\pm$ 3.98	98.00 $\pm$ 4.65	89.99* $\pm$ 3.52	90.77* $\pm$ 3.22	82.29** $\pm$ 4.41	81.56** $\pm$ 4.94		
	Y (6)	102.5 $\pm$ 4.80	91.68 $\pm$ 6.11	86.82 $\pm$ 5.98	91.02 $\pm$ 3.84	85.95** $\pm$ 2.93	82.72** $\pm$ 3.21		
OEE2	X (7)	100.2 $\pm$ 4.32	87.97* $\pm$ 3.35	83.68* $\pm$ 5.82	77.78** $\pm$ 2.04	52.47** $\pm$ 4.47	49.27** $\pm$ 5.51		
	Y (6)	94.40 $\pm$ 8.69	88.50 $\pm$ 7.74	77.80 $\pm$ 8.85	32.62** $\pm$ 2.55	26.95** $\pm$ 5.79	32.95** $\pm$ 6.90		
Cyt b ₅₅₉	X (11)	90.61 $\pm$ 4.32	92.62 $\pm$ 3.05	105.4 $\pm$ 4.91	93.14 $\pm$ 3.77	90.38 $\pm$ 4.34	86.11* $\pm$ 4.21		
	Y (12)	96.66 $\pm$ 1.98	95.36 $\pm$ 4.34	101.4 $\pm$ 6.12	95.17 $\pm$ 2.27	93.86 $\pm$ 4.28	83.41** $\pm$ 3.16		
ATP synthetase	X (11)	101.7 $\pm$ 4.02	98.33 $\pm$ 3.69	102.3 $\pm$ 3.45	104.1 $\pm$ 3.34	93.33 $\pm$ 4.86	99.67 $\pm$ 4.57		
	Y (9)	95.97 $\pm$ 3.23	95.46 $\pm$ 4.26	95.71 $\pm$ 5.76	95.71 $\pm$ 5.57	111.5 $\pm$ 8.35	97.43 $\pm$ 8.40		

Table 3.5 Protein quantification from immunoblots of thylakoids before and after treatment with Triton-X 100 after isolation from seedlings subjected to lethal water stress (Lethal Stress Study). Values are means $\pm$ standard error of integrated optical density expressed relative to well-watered samples (number of immunoblots used for quantification in parenthesis). Asterisks (* or **) indicate that the sample mean does not include 100 % (i.e. the well-watered level) at the 95 % or 99 % confidence level, respectively.

Protein	Thylakoids	Triton-treated Membranes
D1 (3)	69.43** $\pm$ 1.79	73.57 $\pm$ 6.60
OEE1 (6)	59.07** $\pm$ 3.58	58.57** $\pm$ 8.08
OEE2 (6)	59.82** $\pm$ 4.11	37.30** $\pm$ 5.23
Cyt <i>f</i> (3)	71.03 $\pm$ 9.82	40.00** $\pm$ 4.84
ATP synthetase (3)	71.25* $\pm$ 5.88	89.73 $\pm$ 12.33
CP II (3)	92.13 $\pm$ 5.12	84.23 $\pm$ 5.55

No changes in cytochrome b_{559} levels before or after Triton treatment were detected in Studies II and III. The electrophoresis protocol used when the Lethal Stress Study was conducted did not resolve this protein. A cytochrome f antibody preparation was used to probe membrane samples from this study and some samples from seedlings in Study II. Decreased levels of cytochrome f were detected after Triton treatment of membranes from plants from the Lethal Stress Study (Table 3.5). In Studies II and III, there were no significant changes in levels of cytochrome f in seedlings after Triton treatment in Phases 2 and 3 (not shown).

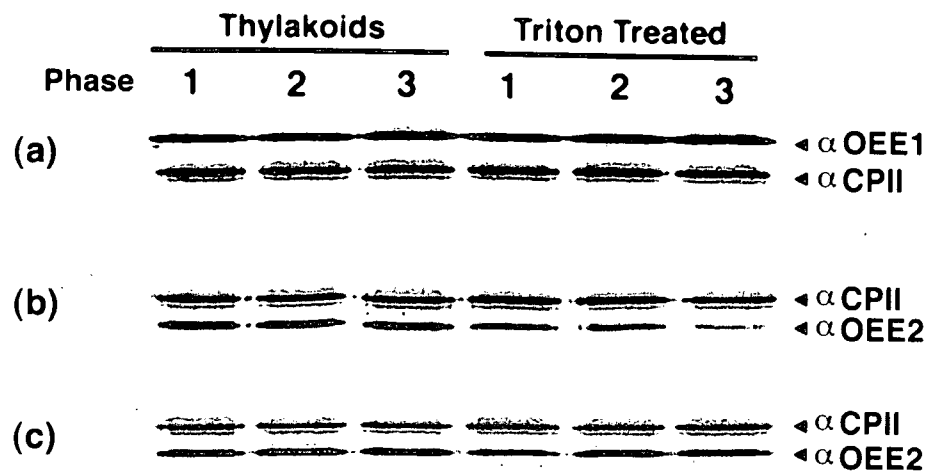
3.3.1.2 Extrinsic Thylakoid Proteins

The ATP synthetase antibody used in this work reacts mainly with the α and β subunits found on the stromal side of the thylakoid membrane. No changes in relative levels of these proteins before or after Triton treatment were detected in Studies II and III (Tables 3.3 and 3.4). Lower levels were noted in thylakoids from lethally stressed plants although levels were unchanged in the Triton-treated membranes (Table 3.5).

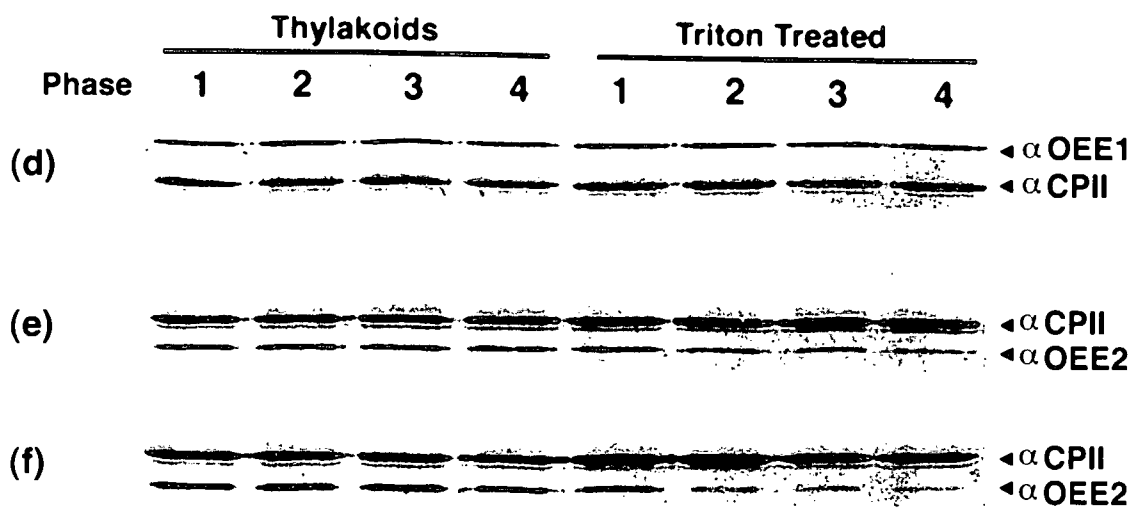
No changes in OEE1, before or after Triton treatment, were observed in Study II (Figure 3.4a, Table 3.3). Small decreases in thylakoid OEE1 levels in Phase 4 samples and small, progressive decreases in OEE1 levels in Triton-treated membranes were detected in Study III (Figure 3.4d, Table 3.4). The OEE1 levels in thylakoids from plants from the Lethal Stress Study were about 60 % of levels found in membranes from well-watered plants (Table 3.5), although little change in OEE1 levels was evident in a preliminary test conducted on the same seedlot when the plants were several months younger. This preliminary experiment was nonlethal, as gauged by the restoration of maximal quantum yield values several days after rewatering, and the lack of change in thylakoidal OEE1 levels in this case may reflect this.

Figure 3.4 Immunoprobings of interior spruce needle protein blots with antibodies for OEE1 or OEE2, and CPII. Panels (a, b, and c) are representative Study II immunoblots for seedling, (a) and (b), and embling (c) samples; each panel has thylakoid and Triton-treated membrane preparations from spruce trees during Phase 1, 2 and 3 of water stress. Panels (d, e, and f) are representative Study III immunoblots for seedling set 'X', (d) and (e), and set 'Y' (f) samples; each panel has thylakoid and Triton-treated membrane preparations from spruce trees during Phase 1, 2, 3, and 4 of water stress.

Study II



Study III



The results obtained with the OEE2 antiserum were the most striking. In particular, membrane association of OEE2, inferred from extractability in Triton X-100, decreased during nonlethal water stress. In Study II, there were no changes in OEE2 thylakoid levels; however, OEE2 was released after Triton treatment in Phase 3 seedlings (Figure 3.4b, Table 3.3). In Study III, the pattern was similar; however, after Triton treatment, the loss of OEE2 was even more pronounced (Figure 3.4e and f; Table 3.4). OEE2 levels decreased progressively in Triton-treated membranes from sample set 'X' (Figure 3.4e) whereas in sample set 'Y' use of the modified centrifugation protocol resulted in the observation that OEE2 membrane attachment was severely affected as early as Phase 2 samples (Figure 3.4f, Table 3.4). There were also slight decreases in thylakoid OEE2 levels in Phases 3 and 4 (Figure 3.4f, Table 3.4). In the Lethal Stress Study, levels of OEE2 in the thylakoid preparations were reduced and Triton treatment (Table 3.4) revealed levels comparable to those observed in Study III, sample set 'Y'.

3.3.2 Electron Transport

In lethally water-stressed spruce seedlings (Lethal Stress Study), measurements of oxygen evolution and DCIP photoreduction in spruce thylakoids showed that electron transport utilizing water as an electron donor was significantly reduced (Table 3.6). Using DCBQ as an electron acceptor from PSII ($\text{H}_2\text{O} \Rightarrow \text{DCBQ}$), the oxygen evolving rate of thylakoids from lethally water-stressed plants was 40 % of the rate measured for membranes isolated from well-watered plants (Table 3.6a). The rates obtained for water-stressed spruce thylakoids upon addition of 15 mM CaCl_2 were enhanced but still lower than those of non-stressed thylakoids (Table 3.6a). Photoreduction of DCIP with water as the electron donor ($\text{H}_2\text{O} \Rightarrow \text{DCIP}$) was

Table 3.6 Electron transport activities of thylakoid membranes and Triton X-100 treated thylakoids isolated from well-watered and lethally water-stressed seedlings (Lethal Stress Study), (a) Oxygen evolution measured using water as electron donor and DCBQ as electron acceptor, and (b) DCIP photoreduction measured using water or DPC as electron donor. Values are means $\pm$ standard error.

(a) Oxygen evolution

Measurement	Thylakoids		Triton-treated Membranes	
	Well-Watered	Stressed	Well-Watered	Stressed
Oxygen evolution ($\mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$)	411 $\pm$ 23	167 $\pm$ 5	225 $\pm$ 24	115 $\pm$ 2
plus 15 mM CaCl₂	-	221 $\pm$ 9	227 $\pm$ 23	162 $\pm$ 12

(b) DCIP photoreduction

Measurement	Thylakoids		Triton-treated Membranes	
	Well-Watered	Stressed	Well-Watered	Stressed
DCIP photoreduction ($\mu\text{mol DCIP mg chl}^{-1} \text{ h}^{-1}$)	228 $\pm$ 16	108 $\pm$ 8	253 $\pm$ 13	63 $\pm$ 2
plus 15 mM CaCl₂	229 $\pm$ 3	130 $\pm$ 2	254 $\pm$ 6	87 $\pm$ 1
plus 0.5 mM DPC	239 $\pm$ 20	150 $\pm$ 12	257 $\pm$ 10	161 $\pm$ 14

also diminished more than two fold in stressed thylakoids (Table 3.6b). Addition of CaCl_2 increased PSII activity in stressed membranes and use of DPC as a direct electron donor to PSII, in the absence of added CaCl_2 , also augmented electron transport capacity in thylakoids from stressed plants but in both cases the rates were still significantly less than the rates for membranes from well-watered plants (Table 3.6b).

To verify that exogenously added cofactors, electron donors and acceptors had access to the reaction centre of PSII, oxygen evolution and electron transport in Triton-treated membrane preparations were also measured (Table 3.6). Triton treatment resulted in the loss of some oxygen-evolving activity even in preparations from well-watered plants; however, in stressed plant membranes, oxygen evolution was significantly lower than in well-watered controls and little of the activity was restored by CaCl_2 (Table 3.6a). PSII activity using DCIP as electron acceptor was very low in Triton-treated membranes from stressed thylakoids (Table 3.6b). In this case, addition of CaCl_2 had a slight effect whereas DPC significantly enhanced DCIP photoreduction in membranes from stressed plants (Table 3.6b).

3.4 Discussion

A series of changes in thylakoid proteins as water stress progresses in young spruce trees is demonstrated in this work. The water stress phases were defined previously using the physiological data presented in Chapter 2. Individual plants were categorized as Phase 1 (Ψ above -1 MPa), Phase 2 ($-1 \leq \Psi \leq -1.6$ MPa), Phase 3 ($-1.6 < \Psi < -3$ MPa) or Phase 4 ($-3 \leq \Psi \leq -4$ MPa) and then the trees pooled into groups for immunoquantification of thylakoid proteins. Trees from three separate water stress studies were used. Trees in Study II (seedlings and emblings) were exposed to light before sample collection and trees in Study III (two subsets

of seedlings designated as 'X' and 'Y') were collected after overnight dark adaption. Trees from the Lethal Stress Study were also analyzed.

Some loss of the D1 protein and decreased binding of the OEE2 protein were observed in four of the five sets of plant samples. Exceptions to the general pattern were detected in the membrane samples prepared from emblings. Two Triton treatments were conducted on the thylakoids prepared from seedlings and emblings in Study II with equivalent results. Thus, the observations may reflect a legitimate difference in the embling material. The Phase 1 emblings, sampled at the onset of the imposed water stress, did demonstrate *lower* oxygen and fluorescence quantum yields than the seedlings in Study II. Accordingly, one hypothesis is that Phase 1 emblings were already exhibiting stress symptoms or were inadvertently sampled during a transient period of photosynthetic decline which may occur during new shoot elongation, as reported for other conifer species (Maier and Teskey 1992; Camm 1993). Values normalized to the low Phase 1 values would consequently appear higher than similar comparisons made for seedlings. It is also important to emphasize that OEE2 levels in Phase 3 were lower than in Phase 2 in emblings. The method of comparison used, i.e. expressing protein levels in stressed needle samples relative to the Phase 1 samples, obscures this observation which indicates that decreased OEE2 association did occur in Phase 3 in emblings.

In Study III, the analysis of the material by two separate rounds of thylakoid preparation and Triton treatment provided comparable results to observations made with the seedling samples in Study II, confirming that the extraction techniques were reproducible and that the observed changes were representative. The use of a modified centrifugation protocol in the Triton treatments of thylakoids from the second set ('Y') of plants, i.e. a preliminary low speed spin to remove large membrane fragments, further confirmed that the observations were

legitimate and revealed a progression of changes that may have been concealed using the other protocol.

Overall, nonlethal water stress caused little or no changes in ATP synthetase, cytochrome *b₅₅₉* and C_{PII} levels in Studies II and III, implying that a widespread loss of thylakoid proteins did not occur. Decreases in ATP synthetase activity, effected by a conformational change, occur in response to water stress but no change in protein levels has been observed (Meyer *et al.*, 1992). In contrast to nonlethal stress, lethal water stress caused decreases in D1, OEE1, OEE2, and cytochrome *f* levels. A comparable, general decline in PSII proteins was reported for wheat seedlings subjected to 48 h of osmotic stress (He *et al.*, 1995). In experiments using *Picea abies*, cytochrome *f* levels were sensitive to light levels, season, and pollution-damage (Flammersfeld and Wild 1992). In contrast, rapidly dehydrated tobacco leaves showed no changes in cytochrome *f* (Havaux *et al.*, 1986), suggesting that cytochrome *f* becomes affected only during prolonged stress. My observations of water-stressed spruce indicated that the susceptibility of cytochrome *f* to detergent extraction increased with stress, possibly reflecting alterations in the thylakoid lipid environment. Cytochrome *f* is selectively depleted from thylakoids during the initial phases of senescence (Roberts *et al.*, 1987). It is possible that the membranes in lethally water-stressed spruce were degenerating. In *P. abies*, drought stress caused a significant increase in monoterpenes (Kainulainen *et al.*, 1992), known to affect the distribution of fatty acids in polar and neutral lipids in spruce seedlings (Hideg *et al.*, 1994), which could contribute to reorganization or loss of thylakoid membrane proteins. Water stress also exacerbates photodamage (Powles 1984) and the resultant oxygen radicals generated in the thylakoid are implicated in a range of effects including protein degradation

(Hideg *et al.*, 1994; Mishra *et al.*, 1994) so a general loss of protein is not unexpected in extended or severe water stress.

In spruce trees subjected to moderate water stress, the D1 protein was the only protein quantified for which decreased levels were detected. Decreases in D1 levels have been reported for osmotically stressed *Secale cereale* L. (Hertwig *et al.*, 1992; Streb *et al.*, 1993) and *Triticum aestivum* L. (He *et al.*, 1995). The extent of D1 loss in water-stressed spruce varied somewhat between Studies II and III; levels of D1 declined slightly but significantly in Phase 3 seedling membrane samples in Study II whereas decreases were more pronounced and progressive in the material from Study III. Translation of D1 is light-regulated in many species including spruce (Godde *et al.*, 1991) and it can be preferentially synthesized even in extreme conditions (Roberts *et al.*, 1987; Droillard *et al.*, 1992). Thus, *de novo* synthesis of D1 may have confounded observations of more marked decreases in Study II seedlings which were exposed to actinic irradiance for a minimum of 2 h before the needles were collected. In the lethally stressed seedlings, reductions in D1 levels were comparable to those observed in Phases 3 and 4 of Study III.

Synthesis and degradation of D1 in spruce are known to be sensitive to other kinds of environmental stress. In severely pollution-damaged spruce needles, large decreases in D1 levels were reported (Wild *et al.*, 1992) and, in a growth chamber study, spruce trees exhibited increased D1 levels at low stress and decreased levels at higher pollution loads (Lutz *et al.*, 1992). Turnover of D1 was accelerated in pollution-damaged spruce; both synthesis and degradation increased (Godde 1992).

In water-stressed spruce trees, some loss of the D1 protein increased before the onset of photoinhibition and, in membrane preparations from lethally stressed spruce, D1 damage was

reflected in the incomplete restoration of DCIP photoreduction by the electron donor DPC. Yet, as detailed in Chapter 2 of this thesis, oxygen evolution in needles measured at elevated CO₂ was clearly affected early in the stress and decreased to 20 % of the well-watered rate in Phase 4. In cyanobacteria (reviewed in Kanervo *et al.*, 1993), green algae (Schuster *et al.*, 1988), and angiosperm species (reviewed in Aro *et al.*, 1993a; and Critchley and Russell 1994), researchers have commented on the disparity between D1 levels, variously determined, and the extent of inhibition of PSII activity. In Norway spruce, D1 content was reported to be very sensitive to stress and developmental stage, but not strongly correlated with electron transport (Lutz *et al.*, 1992, Godde 1992). In higher plants, photoinhibitory light levels can cause significant decreases in PSII activity with no detectable change in D1 levels until the stress is prolonged (Aro *et al.*, 1993a). Thus, although the D1 protein is damaged during photoinhibition above chilling temperatures and that recovery depends on *de novo* synthesis, the initial photoinactivation of D1 does not necessarily result in detectable protein loss (reviewed in Aro *et al.*, 1993a).

In addition to decreased levels of D1, the other significant effect of water stress in spruce thylakoid proteins was the decreased membrane association of the OEE2 protein determined by Triton treatment. Decreases were evident during nonlethal water stress and, in Study III, were detectable clearly before the onset of photoinhibition. This suggests that membrane binding of OEE2 is important in the water-stress induced modulation of PSII activity in spruce. Little change in total thylakoid levels of OEE2 were detected in nonlethal water stress. Other reports have shown no change in OEE2 levels during photoinhibition (Virgin *et al.*, 1988; Schuster *et al.*, 1988; Friso *et al.*, 1994). A study of osmotically stressed wheat revealed a general decrease in PSII proteins but no apparent decreases in OEE2 association in PSII-enriched membranes

isolated using Triton (He *et al.*, 1995). Interestingly, in PSII membrane preparations from angiosperm species observations of D1 degradation products generated by excess light exposure show progressive accumulation of several low molecular protein bands at 23 and 16 kDa (e.g., Ono *et al.*, 1995) which may obscure losses of OEE2 and OEE3 (approximately 23 kDa and 16 kDa, respectively). Alternatively, the common use of detergent fractionation to generate PSII-enriched membranes which usually selects for appressed grana may preclude observation of PSII complexes within or migrating to agranal regions for repair which could include complexes lacking OEE2.

Although decreases in levels of the PSII-associated OEE2 protein in spruce seedlings were specifically related to water stress, it is important to acknowledge that the Triton treatment released a significant amount of this protein in well-watered plants. Approximately 35 % of the total levels quantified for the thylakoids were released by Triton treatment of membranes from well-watered plants, suggesting that a pool of loosely-associated or unbound OEE2 exists in spruce thylakoids. Similar populations of free OEE proteins have been reported for pea and spinach (Ettinger and Theg 1991) and cross-linking studies have shown that not all OEE2 becomes immobilized by linking reagents (Enami *et al.*, 1994). The Triton treatment also affected *in vitro* oxygen evolution in spruce membranes. This is unlikely to be a consequence of loss of the requisite Ca^{2+} cofactor associated with the OEE2 polypeptide (Homann 1987) since supplementation with 15 mM CaCl_2 did not restore oxygen evolution to the levels observed in thylakoids. Furthermore, in membranes from well-watered plants the rates of electron transport through PSII measured by DCIP photoreduction using water as a donor were not affected by Triton suggesting that, in these plants, the observed Triton effect on oxygen evolution was not a consequence of solubilization of PSII-associated OEE2 and/or Ca^{2+} .

The increased release of OEE2 by Triton from thylakoids from water-stressed spruce seedlings suggests that decreases in oxygen evolution may be effected in part by a reversible dissociation of OEE2 from the reaction centre. This hypothesis is supported by the observation that Ca^{2+} partially reversed the loss of water-splitting ability seen in thylakoids from lethally stressed trees. In these membranes, electron transport through PSII using water as an electron donor and measured by oxygen evolution or DCIP photoreduction was diminished; however, although the rates obtained for water-stressed spruce thylakoids upon addition of CaCl_2 were still lower than those of non-stressed thylakoids, the enhancement by CaCl_2 indicated that some of the reduction in activity was caused by dissociation of OEE2, supporting the hypothesis based on immunoquantification. This differs from observations made in severely stressed wheat in which electron transport beyond P680 could not be restored (He *et al.*, 1995).

In spruce seedlings, the dissociation of OEE2 from the membrane was not directly related to the stress phase as defined by Ψ ; decreases in membrane association of OEE2 were evident at different stress phases in the two studies. This could be related to genotype or environmental history differences between the seedlots and, possibly, differences in the state of the thylakoid, energized by actinic exposure (Study II) or relaxed after prolonged darkness (Study III). Improvements in the membrane isolation techniques undoubtedly had a significant effect as well. The brief, low speed centrifugation after Triton treatment conducted for one subset of plants clearly yielded a PSII-enriched membrane population, reflected in the lower chlorophyll *a/b* ratio. This resulted in the observation of the lowest levels of OEE2 and detection of an apparent change in OEE2 association that was coincident with stomatal closure.

My hypothesis that OEE2 association is involved in the control of oxygen evolution has precedent in the literature. Control of water oxidation and thus PSII activity is evidently

affected by OEE proteins during photoactivation in protists (e.g., Hiramatsu *et al.*, 1991), angiosperms (e.g., Tamura and Cheniae 1988) and gymnosperms (e.g., Kamachi *et al.*, 1994). Mutant analysis and cross-linking experiments have demonstrated that OEE proteins are necessary for stabilization of PSII during assembly in eukaryotes (Mayfield *et al.*, 1987a; de Vitry *et al.*, 1989; Hashimoto *et al.*, 1993). Several lines of evidence indicate that the OEE proteins form a special microenvironment for oxygen evolution (Chu *et al.*, 1994; Adelroth *et al.*, 1995; Holzenburg *et al.*, 1994; Ford *et al.*, 1995). In contrast to other nuclear-encoded chloroplast proteins, there are pools of OEE polypeptides that are free in the lumen in mature chloroplasts, suggesting a persistent regulatory role, possibly as a component of the constitutive repair cycle of the labile PSII reaction centre (Hundal *et al.*, 1990; Ettinger and Theg 1991).

There are a few examples in which OEE2 binding has shown variation *in vivo*. Binding of OEE2 and OEE3 in a mangrove species, a halophyte, is sensitive to low osmotic strength such that these proteins are readily lost during membrane isolation whereas this response is not observed for spinach (Critchley *et al.*, 1984); a similar osmotic sensitivity was reported for spruce (Rashid and Camm 1994). Interestingly, a recent report demonstrated that increased tenacity in the membrane association of OEE2, induced by repeated subculture of tobacco cells in saline media, was correlated with increased salinity tolerance (Murota *et al.*, 1994). UV-B irradiation treatment was shown to increase the thermal stability of OEE1 and OEE2 binding and PSII activity (Nedunchezian and Kulandaivelu 1993). The latter two observations support the view that changes in the binding of OEE proteins with the PSII core may enhance PSII activity.

In this work, I report that decreased PSII activity in water-stressed plants is concurrent with decreases in D1 levels and OEE2 membrane association. Although, undoubtedly, damage

of the D1 protein is involved, an important component of the initial loss of electron transport activity may be a consequence of reduced association of OEE2 with PSII. Loss of OEE2 from the reaction centre is well-known to affect oxygen-evolving capacity (e.g., Miyao and Murata 1983) and mutants lacking OEE2 are more sensitive to photoinhibition (Rova *et al.*, 1994). Reversible inactivation of oxygen evolution *in vitro* increases the susceptibility to photoinhibition, although electron transfer within the reaction centre is not initially affected (Carpentier and Nakatani 1985; Theg *et al.*, 1986; Jegerschold *et al.*, 1990; Wang *et al.*, 1992a,b). Disassembly of photodamaged PSII centres and reassociation of the OEE proteins with newly assembling PSII in the stromal lamellae, are components of the current model for the repair mechanism involved in recovery from photoinhibition (Hundal *et al.*, 1990; Virgin *et al.*, 1990). These observations and interpretations support my hypothesis that reduced binding of OEE2 protein may be involved in the process of PSII down-regulation.

A major role of OEE2 is thought to be provision of a special environment for the Ca^{2+} cofactor (Homann 1987; Homann 1988b; Ono *et al.*, 1992; Homann and Madabusi 1993). Recently, a PSII regulatory mechanism was proposed based on the demonstration that low pH-induced release of Ca^{2+} reversibly modulates electron transport by effecting an increase of the midpoint redox potential of the primary quinone acceptor, Q_A , decreasing its ability to reduce plastoquinol (Krieger *et al.*, 1993). There is also evidence that the low pH effects on oxygen evolution involve redox modulation of the Mn cluster effected by OEE2 (Ono *et al.*, 1992). *In vitro* and *in vivo*, pH-dependent changes in PSII activity have been frequently observed. Loss of Ca^{2+} and/or changes in the Mn cluster effected by OEE2 dissociation at low pH may be part of this process. Measurements of lumenal pH in isolated thylakoids in which ATP synthesis is inhibited have yielded values as low as 4.2 (Krieger and Weis 1992). The reported pH for half

dissociation of OEE2 from PSII is 4.1 (Shen and Inoue 1991) which indicates that OEE2 dissociation could be significant at physiologically relevant conditions. This biochemical evidence indicates that dissociation of OEE2 under conditions of luminal acidification could regulate electron flow, thereby effecting down-regulation of PSII.

In vivo, light-induced acidification of the lumen is known to initiate down-regulation of PSII, preceding photoinhibition (van Wijk and van Hasselt 1993) and recently, it was suggested that low luminal pH may be sustained even in darkness when the photosystems are overreduced (Gilmore and Bjorkman 1994a,b). This was demonstrated to be a feature of the prolonged photoinhibition observed in the seasonal down-regulation of photosynthesis in conifers (Adams *et al.*, 1995). Water stress is another environmental restraint which results in luminal acidification as ATP synthetase activity decreases (Meyer *et al.*, 1992). To the best of my knowledge, this thesis is the first demonstration *in vivo* that water stress may affect binding of the OEE2 polypeptide concomitant with losses in electron transport activity. This provides important substantiation to the hypothesis that the OEE proteins may have a significant role in regulation of PSII activity and, in particular, that OEE2 membrane disassociation may be involved in PSII down-regulation in spruce.

Chapter 4: D1 Protein Synthesis Before, During and After Water Stress

4.0 Summary

Incorporation of ^{35}S -methionine into spruce needle proteins was used to examine synthesis of the D1 reaction centre protein of Photosystem II (PSII) in needles from well-watered, water-stressed and recovering spruce trees (*Picea glauca* (Moench) Voss X *P. engelmanni* Parry hybrid complex). After physiological characterization of trees by measurements of predawn shoot water potential (Ψ) and maximal dark-adapted quantum yield, needles were incubated in solutions of ^{35}S -methionine (1.85 MBq mL^{-1}). Needles from unstressed trees (mean $\Psi = -0.3 \text{ MPa}$) incorporated $10.09 \text{ kBq mg}^{-1} \text{ chlorophyll h}^{-1}$ into newly synthesized D1 protein. Needles from water-stressed trees (mean $\Psi = -3.4 \text{ MPa}$) showed an 80 % decrease in the D1 synthesis rate relative to needles from well-watered trees, incorporating label at a rate of $1.96 \text{ kBq mg}^{-1} \text{ chlorophyll h}^{-1}$. Yet, the rate of label incorporation into total thylakoidal protein in stressed tissue, relative to the rate for well-watered tissue, was less affected. The incorporation rate into total protein was 14.80 and $10.51 \text{ kBq mg}^{-1} \text{ chlorophyll h}^{-1}$ for well-watered and stressed trees, respectively. Needles from trees rewatered and allowed to recover for three days after an equivalent water stress (initial mean $\Psi = -3.3 \text{ MPa}$; after watering mean $\Psi = -0.5 \text{ MPa}$) had the highest rate of label incorporation into D1, $19.06 \text{ kBq mg}^{-1} \text{ chlorophyll h}^{-1}$, and into total protein, $26.91 \text{ kBq mg}^{-1} \text{ chlorophyll h}^{-1}$. The proportion of label in newly synthesized D1 in recovering trees was comparable to that for the well-watered trees suggesting that a general enhancement of translation capacity was involved in recovery from water stress. In contrast, D1 synthesis in stressed trees appeared to be specifically down-regulated.

4.1 Introduction

Multiple factors are involved in control of turnover of the D1 reaction centre protein, which demonstrates a relatively rapid turnover even under optimal growth conditions (Aro *et al.*, 1993a). These include chloroplast-encoded transcription factors (Tiller and Link 1993), nuclear-encoded translation activators (Danon and Mayfield 1991), stabilization of D1 translation intermediates and nascent apoprotein by chlorophyll (Kim *et al.*, 1994a), post-translational palmitoylation (Mattoo and Edelman 1987), phosphorylation of damaged D1 (Aro *et al.*, 1993a), and putative nuclear-encoded (Bracht and Trebst 1994) and chloroplast-encoded (Gong 1994) D1 cleavage systems. All of these regulatory features are reportedly regulated by light, directly or indirectly through phosphorylation. Analysis of mutants over-producing D1 demonstrated that the number of PSII centres, another light-regulated characteristic, regulates accumulation of D1 into the membranes of cyanobacteria (Soitamo *et al.*, 1994). Light acclimation further influences PSII turnover rate by dictating the conditions under which maximal D1 synthesis occurs (Sundby *et al.*, 1993). Light adaptation also affects the degree of granal stacking which in turn may affect the removal rate of damaged D1 (Aro *et al.*, 1993b). The rate of removal of damaged D1, which is distinct from the rate of D1 photodamage itself (Aro *et al.*, 1993a), dictates the availability of inactive modified centres in agranal regions which also can affect synthesis rate (Adir *et al.*, 1990; Aro *et al.*, 1993b; Soitamo *et al.*, 1994).

In addition to the complex regulation by light, the developmental, nutritional, and physiological status of the photosynthetic organism also affects turnover. For example, the rate of D1 synthesis is higher in newly developing needles than in mature needles of spruce (Godde *et al.*, 1991). In senescing tissue, D1 protein may be preferentially synthesized relative to other chloroplast-encoded proteins (Droillard *et al.*, 1992; Roberts *et al.*, 1987). Synthesis and

degradation are stimulated by magnesium and/or sulphur deficiency (Godde and Dannehl 1994; Godde and Hefer 1994) whereas degradation is decreased by phosphate deficiency (Bracht and Trebst 1994). Environmental conditions such as high light, sub- or supra-optimal temperature, pollution, or water stress affect utilization of light energy (Powles 1984) and, consequently, these factors interact to affect the PSII turnover cycle.

The decreases in D1 protein levels quantified during progressive water stress in young spruce trees (Chapter 3) could be a consequence of decreased synthesis and/or increased degradation. The observed reduction in absolute D1 levels occurred before the onset of photoinhibition which suggests that synchronization of synthesis and degradation was readily perturbed by stress in spruce. This is in contrast to the stress-induced acceleration of synthesis observed in some species, which completely compensates for increased degradation (Sundby *et al.*, 1993). In spruce, stress-enhanced synthesis may be delayed or inhibited by a general decrease in translation capacity during stress. In some plants, detection of net changes in functional D1 levels are confounded by stress-related accumulation of PSII centres with damaged D1 which is thought to be involved in stress acclimation (Vasilikiotis and Melis 1994; Critchley and Russell 1994; Russell *et al.*, 1995). This may occur differ in spruce. Thus, to distinguish changes in D1 synthesis and degradation rate in spruce trees of defined water status, incorporation of ^{35}S -methionine into spruce needle proteins was examined.

4.2 Materials and Methods

4.2.1 Experimental Rationale

Immunoquantification of D1 levels in thylakoids isolated from spruce trees of defined water status revealed that D1 levels decreased as water stress increased (Chapter 3). In plants

subjected to a moderate level of stress, D1 was the only protein for which a net loss was observed. To determine whether decreased D1 translation, increased D1 degradation, or both, occurred in water-stressed spruce plants, experiments to assess D1 turnover in well-watered, stressed and recovering plants were conducted under light and temperature levels comparable to the growth conditions. Plants recovering from water stress were included in this study to further confirm that recovery readily occurred and to determine whether changes in D1 turnover contributed to recovery.

4.2.2 Protocol Development

A published method for investigating D1 reaction centre protein turnover in spruce needles using radiolabelled amino acids (Godde *et al.*, 1991) was used as the basis of this study. Since this procedure involved vacuum infiltration of a solution into the needles and extended submersion, I felt it necessary to establish that this treatment itself did not affect D1 levels or membrane association of the OEE2 protein. Some workers have shown inactivation of oxygen evolution in protoplasts (Kaiser *et al.*, 1981; Sundari and Raghavendra 1990; Saradadevi and Raghavendra 1994) and leaf slices (Kaiser *et al.*, 1981) subjected to osmotic stress, although others report little effect on PSII activity unless the stress becomes extreme (He *et al.*, 1995).

To examine the effects of the osmotic strength of the incubation solution on D1 and OEE2 levels or membrane association, two experiments were conducted using needles from well-watered and water-stressed trees incubated in solutions of low or high osmotic strength. Predawn water potential and dark-adapted quantum yield were measured as described in Section 4.2.3. Then, two sorbitol solutions of osmotic potential (Nobel 1983) equal to the predawn shoot water potential of the well-watered trees (-0.44 or -0.49 MPa; 0.181 or 0.184 M sorbitol)

or the stressed trees (-2.93 or -2.57 MPa; 1.07 or 0.94 M sorbitol) were prepared. Needles were removed from well-watered and stressed trees with a razor blade, vacuum-infiltrated in either the low or high osmotic strength solution by application of vacuum to a sealed dessicator containing the samples, and then incubated on a shaking platform under white light for 20 or 90 min.

Thylakoids and Triton-treated membranes were prepared and immunoprobings conducted as described in Chapter 3. Needles from well-watered plants incubated in the high osmotic strength solution had lower D1 and OEE2 levels in Triton-treated membranes; this material had 75 % D1 and 60 % OEE2 relative to the levels in a replicate needle sample incubated in low osmotic strength solution. In low osmotic strength solution, Triton-treated membranes isolated from water-stressed plants contained 83 % D1 and 73 % OEE2 of the levels in membranes from well-watered plants. There was variation in the response of stressed tissue to the high osmotic strength solution; however, stressed plants overall had lower levels of D1 and OEE2, relative to samples from well-watered plants, in both young and mature needles. This was also true for membranes from control needles from stressed plants that had not been immersed. In summary, high osmotic strength of the incubation medium resulted in decreased protein levels in well-watered needles, whereas protein levels in stressed tissues appeared to be more affected by plant water status rather than short-term osmotic treatment. Thus the use of an osmoticum in the incubation medium was discontinued for subsequent experiments.

Another series of preliminary experiments was conducted to optimize incorporation of ^{35}S -methionine into needle proteins. From these it was determined that incubation of finely cut needles (approximately 1 to 4 mm in length) in a radiolabelled, carrier-free solution buffered at

pH 8, at 20°C yielded levels of label incorporation into D1 protein that were adequate for analysis.

4.2.3 Plant Material

One year old interior spruce trees (British Columbia Ministry of Forests Seedlot 28442, Surrey Nursery, Surrey, BC) were removed from cold storage in February 1995, thawed for several days at 4°C, then planted directly in sand in 1-L pots. The plants were grown in a growth cabinet at 20°C under white light at an irradiance of $350 \mu\text{mol m}^{-2} \text{s}^{-1}$ at shoot height (16 h-photoperiod). New shoots began to flush about 10 days later. Three treatment groups were established; one group to be continuously watered (W), one group to be water-stressed for 2 weeks (S), and one group to be stressed for 2 weeks then watered for 3 days (R) before radiolabelling. To ensure that all groups were simultaneously ready, watering was discontinued three days earlier for R trees than for S trees. Predawn water potential of terminal shoots was determined with a pressure chamber (Soilmoisture Equipt. Corp., Model 3015). To determine dark-adapted maximal fluorescence quantum yield, a portion of each intact shoot was dark-adapted for 10 min, dark level fluorescence measured and then maximal fluorescence determined with a 1 s saturating flash ($11000 \mu\text{mol m}^{-2} \text{s}^{-1}$) using a Walz pulse-modulated fluorometer (PAM 101,103). For R trees, water potential and quantum yield were determined two weeks after watering was discontinued and then again, three days after rewatering, just prior to radiolabelling. Measurements were made on S trees that had been water-stressed for two weeks and W trees that had been continuously watered prior to removing the needles for labelling. The physiological characteristics of the three sample groups are detailed in Table 4.1.

Table 4.1 Average predawn shoot water potential (Ψ) and dark-adapted maximal fluorescence quantum yield (F_v/F_m) of well-watered (W) and water-stressed spruce seedlings (S), and seedlings subjected to water stress, rewatered, then allowed to recover for three days (R).

	Ψ (MPa)	F_v/F_m
Well-watered (W)	-0.34 ± 0.06	0.8119 ± 0.003
Water-stressed (S)	-3.44 ± 0.36	0.6687 ± 0.038
Before rewatering	-3.27 ± 0.30	0.7187 ± 0.017
After rewatering (R)	-0.49 ± 0.06	0.7534 ± 0.015

4.2.4 Radiolabelling of Needle Proteins

Needles were harvested from the plants, then finely cut (less than 4 mm) with razor blades. Ten individual 1.0 g aliquots of W, S, or R needles were weighed into small plastic Petri dishes (100 x 15 mm) for a total of 30 samples. Six mL of incubation solution (25 mM tris(hydroxymethyl)aminomethane-HCl, pH 8), containing 1.85 MBq mL^{-1} of L- ^{35}S -methionine (*in vivo* cell labelling grade, Amersham) were added, and the samples were vacuum-infiltrated (3 x 20 s). Dishes were placed on aluminum foil covering a cooling platform and exposed to an irradiance of approximately $350 \mu\text{mol m}^{-2} \text{ s}^{-1}$ provided by a 500 watt tungsten halogen lamp (Iwasaki Electric Co.) in a fume hood. Irradiance was measured at the platform surface using a Quantum Photometer (LI-189). The platform was constructed of coiled copper tubing (6 mm OD) sandwiched between heavy aluminum mesh and plywood. Water maintained at 20°C by a

circulating water bath (RM6 Lauda) was cycled continuously through the tubing. To reduce the effects of the heat generated by the light source, a glass chromatography tank filled with water (7 cm thickness) was placed in front of the lamp. The tank was changed every half hour during the incubation. The temperature was monitored using a thermocouple thermometer (Digi-Sense, Cole Parmer) placed in a Petri dish of unlabelled buffer on the cooling platform. Two samples each of needles from W, S or R plants were incubated for 30, 60 or 90 min. For the remaining 12 samples, the labelled feeding solution was replaced with cold L-methionine (NBC; 100 mM in buffer) after 90 min of labelling. These were incubated for a further 45 or 90 min. For each sample, needles were removed from the incubation solutions, frozen immediately in liquid nitrogen and stored at -80°C .

4.2.5 Preparation of Membranes

Frozen needles were crushed to a fine powder in liquid nitrogen and homogenized in 50 mM N-tris[hydroxymethyl]-methylglycine-NaOH buffer (pH 7.6) containing 500 mM sucrose, 10 mM NaCl, 5 mM MgCl_2 , 2 mM CaCl_2 , 2 % soluble polyvinylpyrrolidone 40 (PVP-40), and 2 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was filtered through nylon mesh (102 micron, Nitex), then briefly (2 min) centrifuged at $5000 \times g$ (Sorvall RC-5B, DuPont). The chloroplast pellets were washed by resuspension in the isolation buffer (without PVP-40, plus 1 mM benzamidine and $1 \mu\text{g mL}^{-1}$ leupeptin), followed by a second centrifugation at $5000 \times g$. The thylakoid pellet was resuspended in thylakoid storage buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-NaOH pH 7.6, containing 1 M sucrose, 10 mM NaCl and 5 mM MgCl_2), and stored at -80°C . Chlorophyll concentration was determined using

a Milton Roy Spectronic 3000 (Lichtenthaler and Wellburn 1983). A quick test to monitor labelling was conducted: 15 μ L of each thylakoid sample was added to 10 mL of biodegradable scintillation cocktail (BCS, Amersham) and disintegrations per minute (dpm) averaged over 5 min, were determined with a scintillation counter (Beckman LS5000TA). Values were normalized to equivalent chlorophyll concentrations (Table 4.2).

Table 4.2 Direct counting of ^{35}S present in thylakoid preparations from needles of well-watered (W), water-stressed (S), and recovering (R) spruce trees. Needles were incubated in ^{35}S -methionine solution for 30, 60 or 90 min; or incubated in labelled solution for 90 min then incubated in a label free solution ('chase') for 45 or 90 min.

Status	DPM per μg chlorophyll				
	30 min	60 min	90 min	45 min (chase)	90 min (chase)
W	3313	4731	4947	2399	3052
S	3797	4069	4995	2987	2972
R	4258	4641	5054	3510	3979

To prepare Triton-treated membranes for immunoquantification, thylakoids were thawed on ice and Triton X-100 added to a final detergent to chlorophyll ratio of 15:1. Samples were incubated in the dark for 20 min on ice with gentle agitation, then diluted with thylakoid storage buffer and briefly centrifuged at 1,000 $\times$ g. The supernatant was centrifuged at 10,000 $\times$ g for 30 min and the final membrane pellet resuspended in 25 mM morpholinoethane sulfonic acid-NaOH buffer (pH 6), containing 2 M sucrose, 10 mM NaCl, 5 mM MgCl_2 , 2 mM CaCl_2 , and protease inhibitors (PMSF, leupeptin, benzamidine).

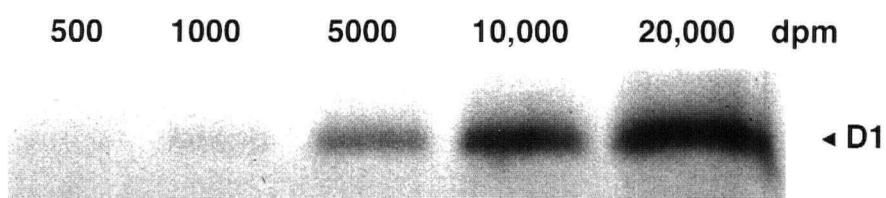
To determine whether any of the ^{35}S -methionine was incorporated into OEE1, which migrated closely with the D1 protein, or into OEE2, thylakoid samples were incubated with Triton X-100, as before, with the addition of 200 mM Tris-HCl, pH 9, to release the OEE proteins by alkaline treatment. Samples were spun at $40,000 \times g$ for 30 min, the pellets resuspended in sample buffer and the supernatants concentrated in 10 kDa cutoff microconcentrators (Centriluters, Amicon). Samples were then run on gels and fluorography performed as described in the next section.

4.2.6 Fluorography and Immunoprobng

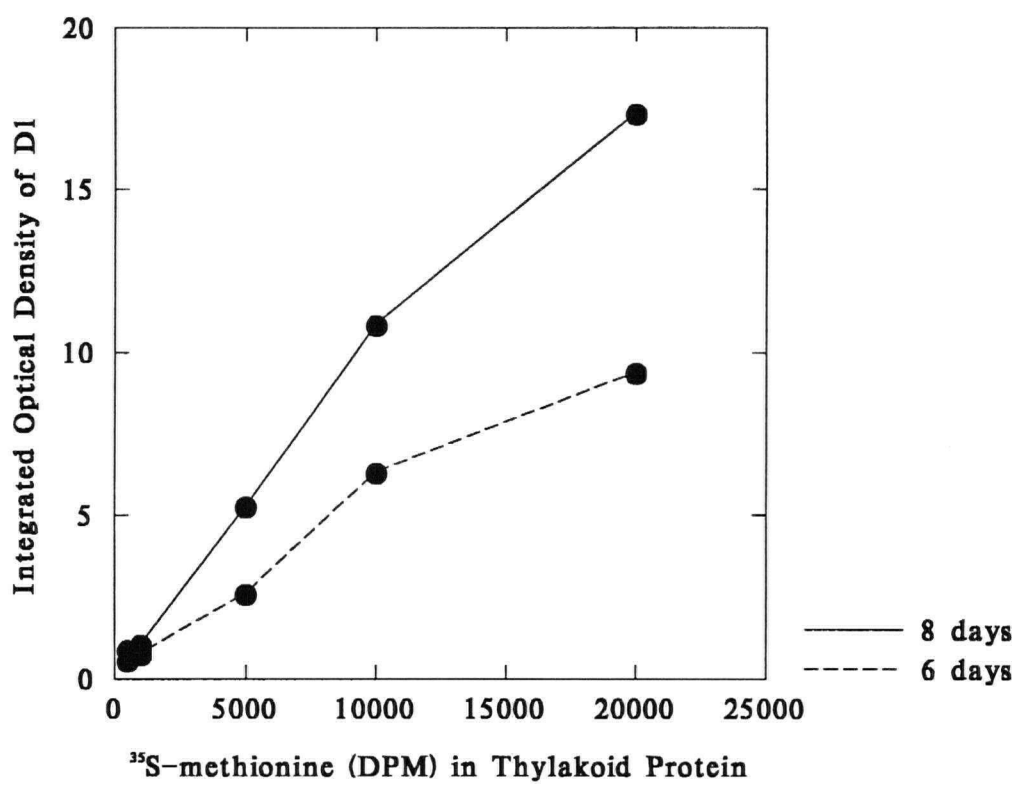
For fluorographic quantification, thylakoid samples (10 μg chlorophyll per lane) were solubilized at 90°C for 3 min in 70 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 6.8) containing 20 % glycerol, 2 % SDS and freshly added 10 % β -mercaptoethanol and resolved overnight at 30 mA on 16 mm high-Tris (0.75 M) gels (T=15 %, C=4 %) containing 10 % (w/v) glycerol (Fling and Gregerson 1986). Gels were stained with 0.05 % (w/v) Coomassie Brilliant Blue G-250 (CBB) in 40 % methanol/7 % acetic acid (v/v), destained in 40 % methanol/7 % acetic acid (v/v), incubated in scintillant (Amplify, Amersham) for 30 min, and dried onto chromatography paper (3MM, Whatman) at $70\text{--}80^\circ\text{C}$ for 2 h using a slab gel dryer (SGD4050, Savant). The dried gels were labelled with radioactive India ink and placed in close contact with film (BioMaxMR, Kodak). To quantify the amount of label incorporated into D1 and allow comparison between different gels, each film was simultaneously exposed to another dried acrylamide gel strip with five lanes representing an ascending series of standards of defined activity (disintegrations per min) measured by direct scintillation counting of trichloroacetic acid (TCA)- precipitated protein (Baszczynski *et al.*, 1983) and prepared using a spruce thylakoid

Figure 4.1 Increases in the integrated optical density of ^{35}S -methionine labelled D1 bands in fluorography standards with increasing levels of label incorporated into total protein estimated by trichloroacetic acid precipitation: (a) representative sample of fluorography standards developed for 6 days, and (b) relationship between the integrated optical density of ^{35}S -methionine labelled D1 bands in fluorography standards, developed for 6 or 8 days, with increasing levels of label incorporated into total protein. Values are means of optical density of D1 from three autoradiograms; symbol size is greater than standard error.

(a) Fluorography standard



(b)



preparation from the same experiment (Figure 4.1). For analysis, each film was exposed at -80°C for 6 days.

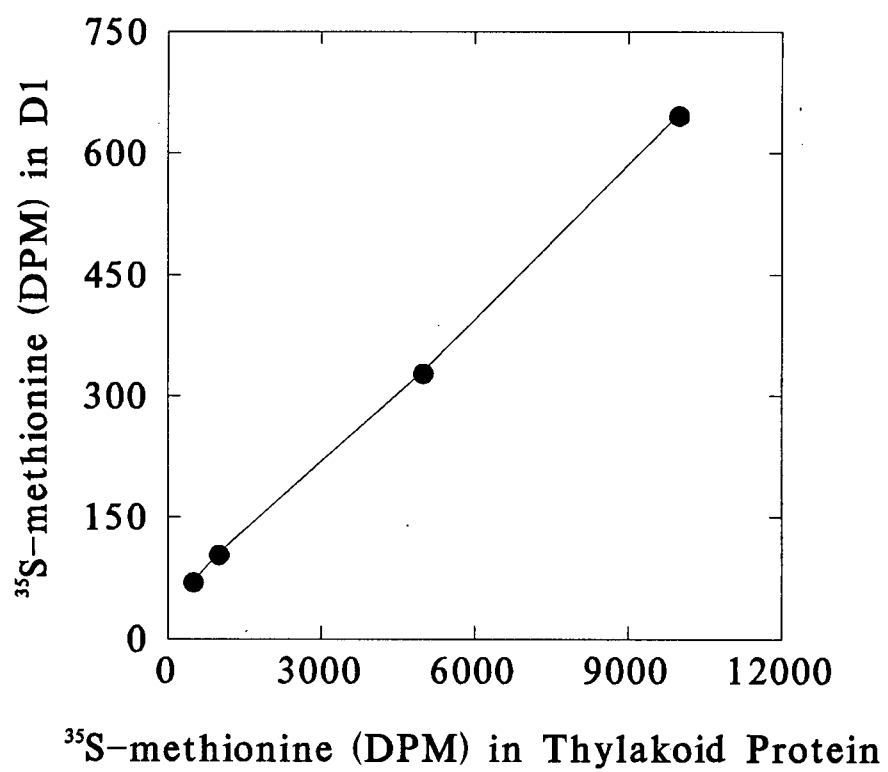
The amount of label incorporated into the D1 protein was quantified by scanning the autoradiograms using an XRS scanner using BioImage software (Visage version 4.6Q, Millipore Corp. 1993). Defined scan areas were chosen, the integrated optical densities measured, and the values converted to disintegrations per minute (dpm) using the 5000 dpm standards generated for each autoradiogram. Values were averaged from four autoradiograms representing two electrophoretic separations of membranes from the replicate needle samples.

For immunoprobng, thylakoids or Triton-treated membranes (250 ng or 10 µg chlorophyll) were resolved on high-Tris (mini or 16 mm) gels, electrophoretically transferred and immunoprobed as detailed in Chapter 3 (Section 3.2.3.1).

4.2.7 Direct Scintillation Counting

Total label incorporated into thylakoid proteins was determined by scintillation counting of TCA-precipitated protein (Baszczynski *et al.*, 1983). Aliquots (25 µL) of each membrane preparation were applied to 2.3 cm filter paper discs (3MM, Whatman), air-dried, heat-dried (Heat gun, Lab Apparatus Co.), placed into room temperature 10 % (w/v) TCA and brought to 80°C for 5 min. The discs were then washed once in room temperature 5 % (w/v) TCA, twice with 95 % (v/v) ethanol, once with diethyl ether, and then air-dried. The discs were placed, sample side up, into scintillation vials and 10 mL scintillant added. Activities (dpm) averaged over 5 min, were determined. Values were adjusted using chlorophyll concentrations. This procedure should precipitate most proteins onto the membrane while free label, labelled amino

Figure 4.2 Relationship of values from direct scintillation counting of ^{35}S -methionine incorporation into D1 protein solubilized from acrylamide gels with increasing levels of label incorporated into total protein. Values are means of disintegrations per min determined from samples from two gels.



acids, and lipids should be washed off by the solvent treatments, yielding a specific estimate of label incorporation into protein.

For comparison with fluorography estimates, a direct counting technique was used to measure label incorporation into D1. The zone known to include the D1 protein was excised from gels prepared as described above, neutralized by several rinses in phosphate-buffered saline (pH 7), treated overnight with 1.0 mL tissue solubilizer (NCS, Amersham), acidified with 30 μ L acetic acid, covered with 10 mL scintillant, and dpm measured over 5 min. As dpm initially increased over time with this method, samples were kept in the dark and sample counting was repeated until it appeared to stabilize. Values from three readings were averaged. Linearity tests for this technique were conducted by using slices from gels loaded with a thylakoid preparation to produce an ascending series of defined activity (Figure 4.2).

4.3 Results

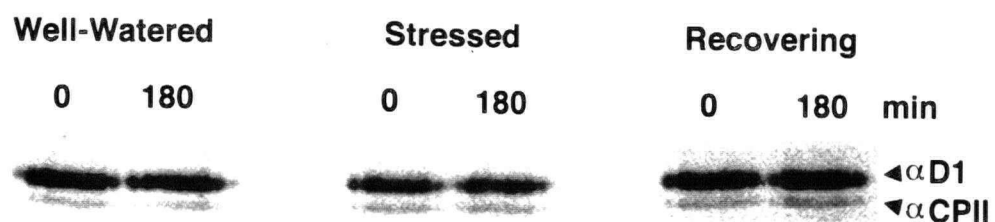
4.3.1 Immunoprobng

The major radiolabelled protein in the thylakoid preparations comigrated with the D1 protein band identified by immunoprobng.

As observed in preliminary experiments, the labelling technique itself did not appear to affect relative D1 levels (Figure 4.3a) or OEE2 association; levels of these proteins in membranes prepared from needles incubated for 180 min were indistinguishable from levels in control, unlabelled membranes prepared from needles not subjected to immersion (i.e. 0 min samples). Compared with membrane samples from well-watered trees, there were relatively lower levels of D1 in the membranes from stressed trees (Figure 4.3a). Membranes from recovering trees had an intermediate level of D1 (Figure 4.3a). Levels of PSII-associated OEE2

Figure 4.3 Immunoprobings of needle membrane samples from well-watered (W), stressed (S) and recovering R trees: (a) Comparison of D1 levels in control, unlabelled membranes prepared from needles not subjected to immersion (0 min) to those subjected to 90 min of labelling with ^{35}S -methionine, followed by 90 min incubation in a label free solution (total of 180 min immersion), 250 ng chl per lane; (b) Comparison of D1 levels in the 40,000 x g supernatant fraction of needle membrane samples treated with Triton X-100 at alkaline pH, equivalent to 1 μg chl per lane.

(a) Triton-treated membranes



(b) Supernatants

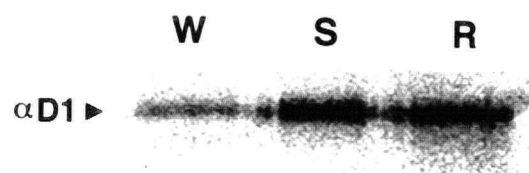
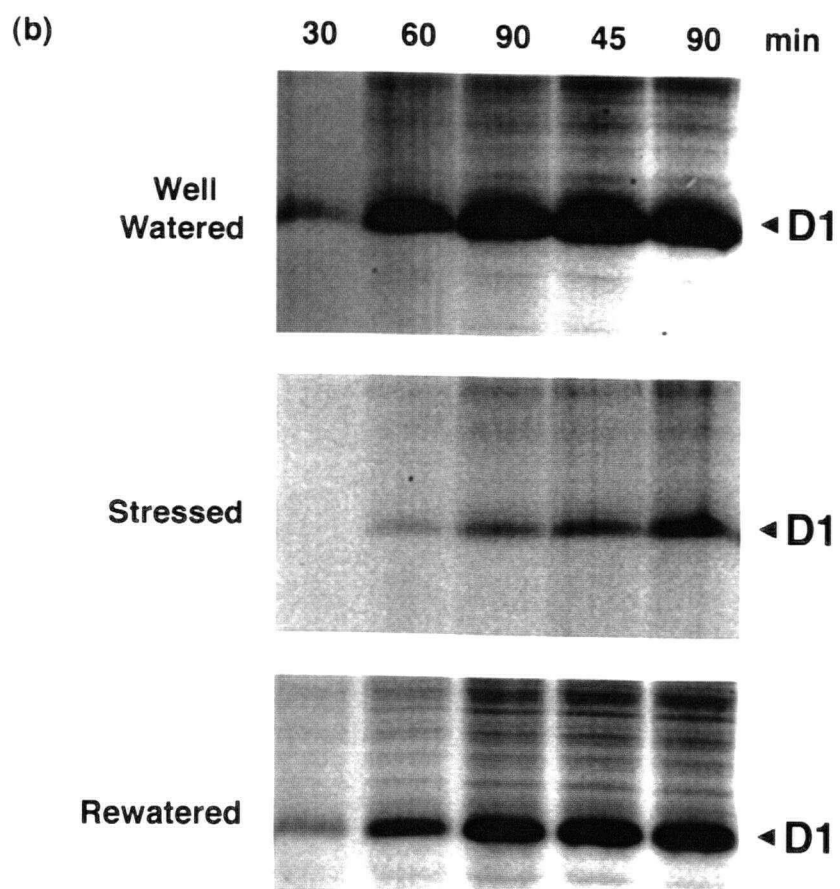
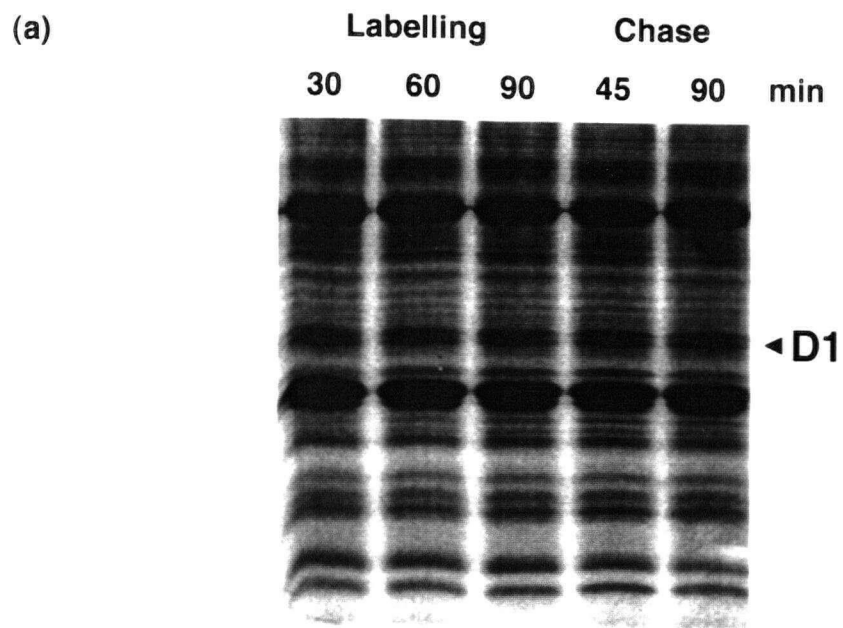


Figure 4.4 Fluorography of ^{35}S -methionine incorporation into D1 protein in thylakoids (10 μg chl per lane) prepared from well-watered, water-stressed and recovering spruce trees, (a) Coomassie Brilliant Blue stained SDS-polyacrylamide gel and (b) autoradiograms after 6 (W and S) or 4 (R) days of exposure. Needles were incubated in labelled solution for 30, 60 or 90 min; or incubated in labelled solution for 90 min then incubated in a label free solution ('chase') for 45 or 90 min.



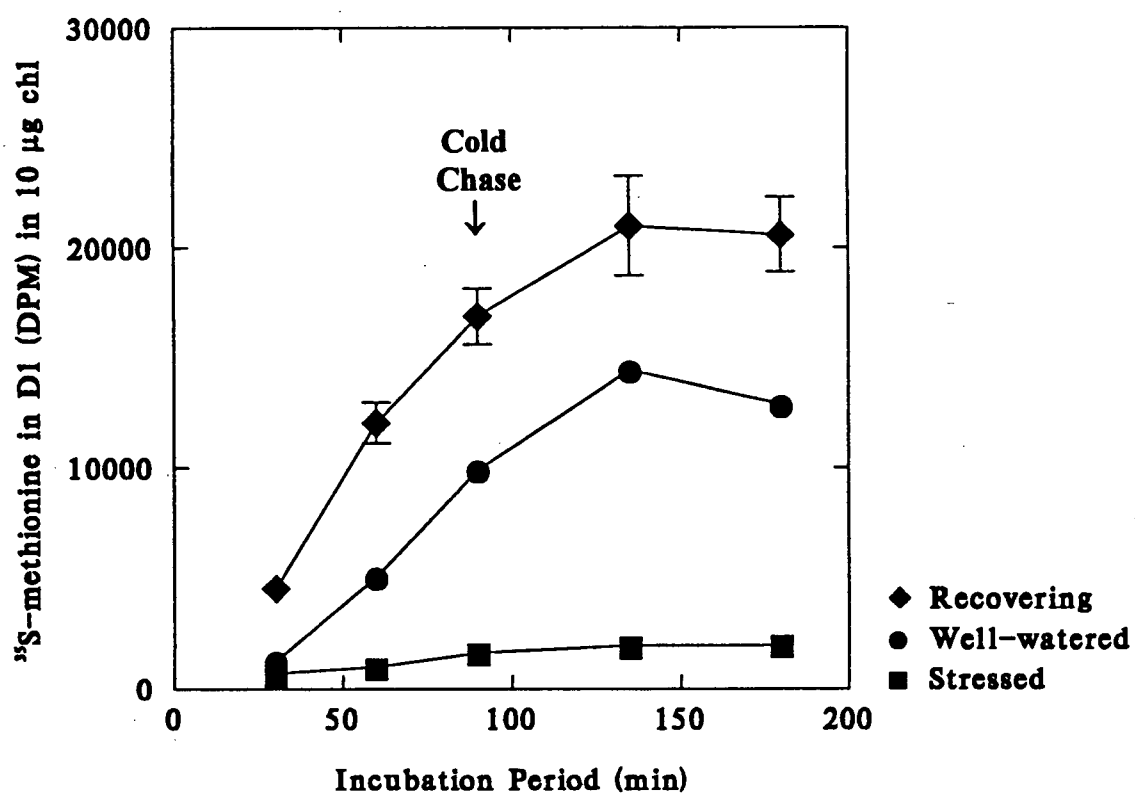
were lowest in the stressed trees, as previously observed (Chapter 3) and slightly lower in the recovering trees relative to the well-watered trees.

Immunoprobings of the 40,000 x g supernatants obtained after treating thylakoid samples with Triton X-100 at alkaline pH revealed the presence of D1 in all samples (Figure 4.3b). This differed from previous results obtained by treatment with Triton X-100 at neutral pH (Chapter 3).

4.3.2 Fluorography

In needle samples loaded on the basis of equal amounts of chlorophyll per lane (10 μg), the ^{35}S -methionine incorporated into the D1 protein represented approximately 75 % of the labelled protein resolved on the gel system used (Figure 4.4). Some of the labelled material did not enter the resolving gels. Needles from recovering plants had the greatest incorporation of label into D1 protein, needles from well-watered plants had less, and needles from the water-stressed plants had the least. The autoradiogram of rewatered samples shown in Figure 4.4 was developed for a shorter period of time than those for the well-watered and stressed samples; this example was chosen only for photographic clarity. For all analyses, all autoradiograms were developed for 6 days; this duration yielded a linear relationship between integrated optical density of the D1 band and the level of total label incorporation estimated by TCA precipitation (Figure 4.1b). The integrated optical density of the D1 bands was converted to dpm using a set of activity standards produced for each autoradiogram (Figure 4.1a). This confirmed that D1 synthesis was highest in recovering plants (R) at 19.06 $\text{kBq mg}^{-1} \text{ chlorophyll h}^{-1}$, moderate in well-watered plants (W) at 10.09 $\text{kBq mg}^{-1} \text{ chlorophyll h}^{-1}$ and very low in stressed plants (S) at 1.96 $\text{kBq mg}^{-1} \text{ chlorophyll h}^{-1}$ (Figure 4.5). This was consistent with preliminary observations.

Figure 4.5 Fluorographic estimation of ^{35}S -methionine incorporation into D1 protein in thylakoids (10 μg chl per lane) prepared from well-watered, water-stressed and recovering spruce trees. The arrow indicates the onset of the cold chase. Values are means $\pm$ standard error of disintegrations per min, calculated from the integrated optical density of the D1 band.



Label continued to be incorporated into protein 45 min after the labelled solution had been replaced with unlabelled methionine (at 90 min) in well-watered and recovering trees whereas little change could be resolved for the stressed trees (Figure 4.5). In W trees, there were decreased levels of label after 90 min of the cold chase, but this was variable for the R trees and could not be determined for the S trees.

Fluorography of the supernatants from the Triton/Tris treated thylakoid samples was unsuccessful in determining whether OEE proteins incorporated label as the amount of label present was undetectable in the exposure time used; as well, immunoquantification indicated that some D1 was present in these supernatants (Figure 4.3b).

4.3.3 Direct Scintillation Counting

The quick tests for the presence of label in the thylakoid samples indicated that comparable levels of label were present in all three tissue types and that there was a decrease in label level during the cold chase (Table 4.2). The estimates of label incorporated into TCA precipitable protein were variable but reflected the observations made with fluorography in that needles from the recovering trees incorporated the most label into protein and the stressed tissue the least (Figure 4.6). The amount of label incorporated into total protein, particularly in the stressed needles, was higher at all time points than the estimates of label incorporated specifically into the D1 protein. A comparison of the amount of label in D1 protein, estimated by fluorography, and in total TCA precipitable protein after 90 min of incubation indicates that, although the stressed needles were capable of translation, D1 translation was affected more (Figure 4.7). Incorporation of label into total protein in S samples, $10.51 \text{ kBq mg}^{-1} \text{ chlorophyll h}^{-1}$, was only slightly lower than the rate, $14.80 \text{ kBq mg}^{-1} \text{ chlorophyll h}^{-1}$, calculated for W

tissue. Needles from rewatered plants had the highest rate of $26.91 \text{ kBq mg}^{-1} \text{ chlorophyll h}^{-1}$. The proportion of D1 synthesis relative to total protein synthesis was very similar for W and R trees; approximately 70 % incorporated label was present in D1. In S trees, 19 % of the protein-associated label was incorporated into D1. These proportions were estimated from duplicate needle samples from one experiment.

Direct scintillation counting of label in D1-containing gel slices confirmed that D1 synthesis was rapid in W trees, more so in R trees, and reduced in S trees (Figure 4.8). Although standard curves were linear in the range tested (Figure 4.2) and the results for the two replicates very similar, the values obtained for label incorporation were about ten-fold lower than those from fluorographic quantification (Figure 4.5). Quenching and/or incomplete solubilization of the protein from the gel slices may have been significant. Other workers have reported lower estimates of label incorporation for spruce needles using this technique compared to levels estimated using fluorography (Godde *et al.*, 1991).

Direct counting of the supernatants from the Triton/Tris treated thylakoid samples indicated that this detergent/alkali treatment released label in increasing amounts from the thylakoids of W, S, and R needles, respectively. This could reflect label incorporated into the OEE proteins; however, immunoprobng demonstrated that this treatment released small quantities of D1 protein from the W, R, and S thylakoids in increasing amounts (Figure 4.3b), suggesting that D1 was the source of the detected label.

Figure 4.6 Direct scintillation counting of ^{35}S -methionine incorporation into total protein precipitated by trichloroacetic acid from thylakoids prepared from well-watered, water-stressed and recovering spruce trees. The arrow indicates the onset of the cold chase. Values are means $\pm$ standard error of disintegrations per min.

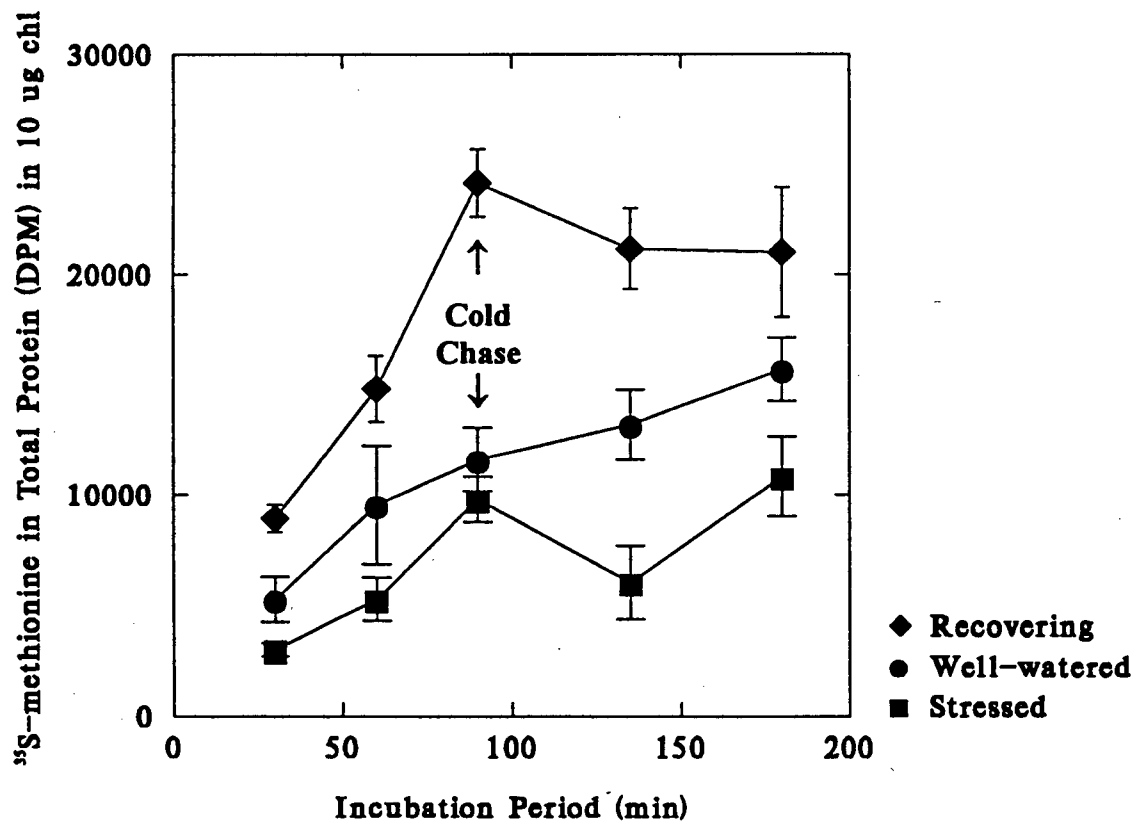


Figure 4.7 Comparison of ^{35}S -methionine incorporation in total protein, precipitated by trichloroacetic acid, and in D1 protein, determined by fluorography, in thylakoids prepared from well-watered, water-stressed and recovering spruce trees. Values are means $\pm$ standard error of disintegrations per min in 10 μg chlorophyll per h.

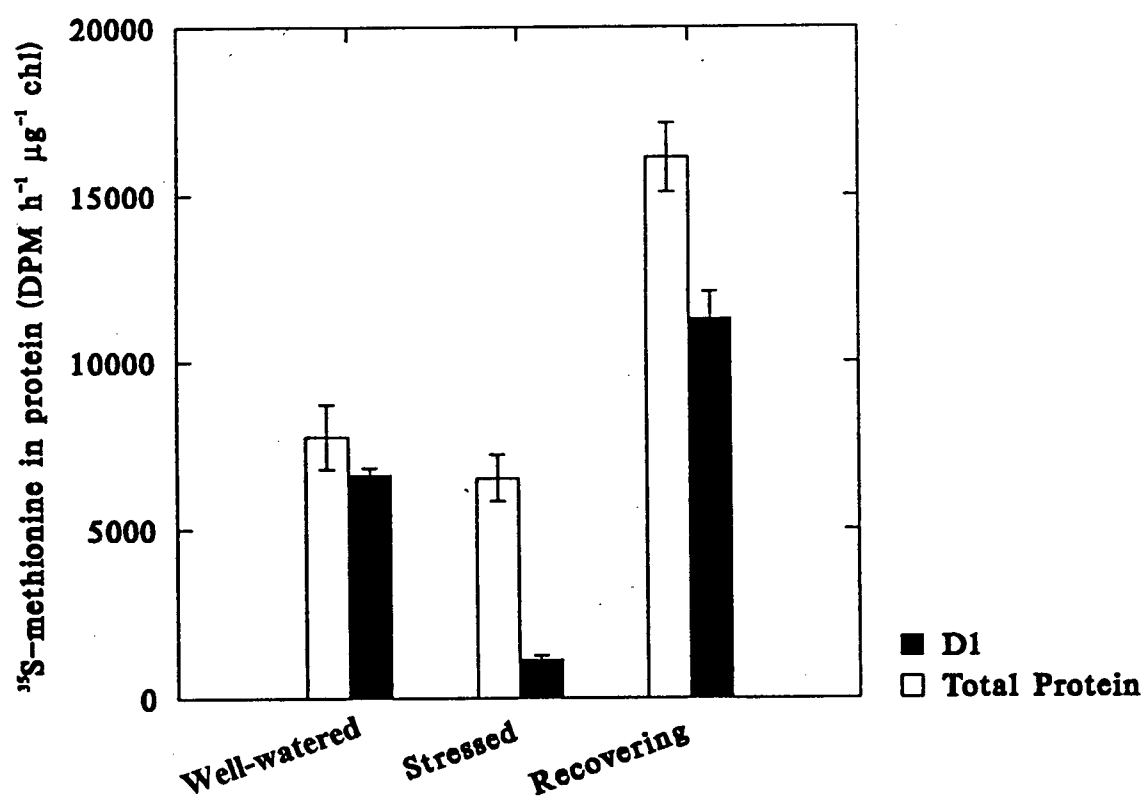
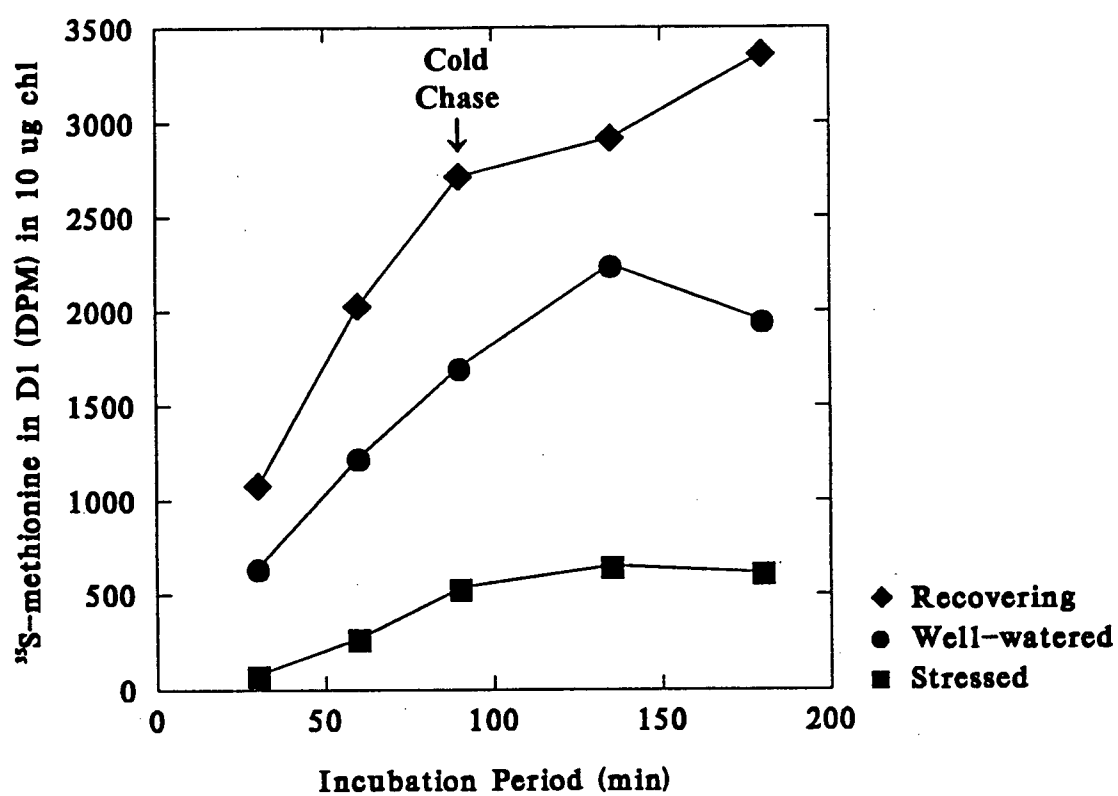


Figure 4.8 Direct scintillation counting of ^{35}S -methionine incorporation into D1 protein solubilized from acrylamide gels. Samples represent thylakoid preparations (10 μg chlorophyll) from well-watered, water-stressed and recovering spruce trees. The arrow indicates the onset of the cold chase. Values are means of disintegrations per min; symbol size is greater than standard error.



4.4 Discussion

The rate of incorporation of ^{35}S -methionine into newly synthesized D1 reaction centre protein was 80 % lower in water-stressed trees than in well-watered trees (1.96 and 10.09 kBq mg^{-1} chlorophyll h^{-1} , respectively); consistent with observations made in preliminary experiments. This may reflect decreased synthesis of the D1 protein or such significant turnover that label accumulation is precluded. In contrast, the overall translation capacity, estimated by label incorporation into protein precipitated from thylakoid preparations by trichloroacetic acid, was affected by water stress to a lesser extent. The apparently high levels of translation in the stressed needles may be a consequence of production of stress-induced proteins or co-precipitation of labelled non-proteinaceous compounds with protein by the trichloroacetic acid. Alternatively, this apparent discrepancy could reflect stress-induced accumulation of high molecular weight protein aggregates, known to be generated in photoinhibited thylakoids (Roberts *et al.*, 1991). It is possible such aggregates could not be solubilized in sample buffer or resolved on the gel system used; however, no differences were observed in the amount of label at the interface between the stacking and resolving gels.

Alternatively, the apparent decrease in D1 synthesis relative to overall translation in stressed needles may involve a selective, down-regulation of D1 translation. A similar observation, that is a specific decrease in D1 levels, was reported for a barley mutant carrying a nuclear-encoded gene apparently involved in stabilization of D1 translation intermediates (Kim *et al.*, 1994b). Significantly, although decreased protein synthesis was reported for conifer tissue (Hulbert *et al.*, 1988, Valluri *et al.*, 1988, 1989) and wheat seedlings (He *et al.*, 1995) stressed by transfer to high osmotic strength media, recent work with potato cultures shows

that, in contrast to abrupt exposure, a gradual imposition of water stress did not decrease overall protein synthesis (Leone *et al.*, 1994). It is well-established that oxidative stress of Norway spruce trees enhances needle protein synthesis and, in particular, cytoplasmic translation (Schmitz *et al.*, 1993). Additionally, the rate, nature, and developmental timing of an imposed stress may affect translation capacity differently. Thus, it is possible that the imposed water stress in my studies may have evoked some level of cellular adjustment/acclimation that maintained or enhanced overall translation capacity and repressed D1 translation in stressed tissues.

Another explanation for the observed lower level of incorporated label into D1 in needles from stressed trees entails decreased uptake of the label in stressed tissue, resulting in lower levels of label within the chloroplasts. The observations of apparently comparable levels of total label in the thylakoid preparations and label specifically present in TCA-precipitable protein suggests this is not the case.

The needle tissue from trees recovering from water stress demonstrated a different pattern of label incorporation. Although the synthesis rate was higher, the levels of D1 immunologically detected were still lower relative to levels for unstressed trees (Figure 4.3) suggesting that the turnover rate was still high. The proportion of ^{35}S -methionine in D1, relative to total protein synthesis was very similar for unstressed and recovering trees (68 % and 71 %, respectively) indicating that there was an enhancement of general translation capacity during recovery. Three days after rewatering, the predawn shoot water potential had recovered, yet dark-adapted, maximal fluorescence quantum yield was still lower than for unstressed controls. The slightly lower levels of D1 and membrane-associated OEE2 (75 and 85 %, respectively, of the well-watered levels) support the contention that PSII restoration was still in

progress in rewatered trees. Accelerated D1 synthesis in tissue recovering from photoinhibitory stress was previously reported in other species (Sundby *et al.*, 1993; Rintamaki *et al.*, 1994) and may be particularly important in spruce trees stressed to this degree.

During photoinhibition, the removal rate of photodamaged D1 is considered to be the limitation to stress tolerance in green algae (Adir *et al.*, 1990; Smith *et al.*, 1990; Kim *et al.*, 1993; Vasilikiotis and Melis 1994) and in higher plants (Aro *et al.*, 1993b; Sundby *et al.*, 1993), although limited synthesis of D1 was identified as the key factor in the moss *Ceratodon* (Rintamaki *et al.*, 1994). In cyanobacteria, complex transcriptional control of D1 involves two functionally distinct protein forms, encoded by a small gene family (reviewed by Kulkarni and Golden 1994); yet, D1 levels are not related to the extent of photoinhibition and reassembly/reactivation of PSII is considered to be the limitation to recovery in these organisms, as well (Kanervo *et al.*, 1993).

The rate of D1 degradation and, consequently, comparison of turnover rates, could not be determined in my study because label incorporation continued to increase in W and R needles 45 min after the onset of the cold chase and, after 90 min, results from different techniques (immunoquantification versus direct scintillation counting) were inconsistent. Continued incorporation of label into spruce needles during a cold chase was previously reported (Godde *et al.*, 1991). The same researchers have reported variable D1 content in pollution-damaged spruce, although, in spring, degradation consistently exceeded synthesis and photosynthesis declined markedly (Godde 1992, Godde and Buchhold 1992). In other species, researchers have reported no change in absolute D1 levels during reversible photoinhibition *in vivo*, implying controlled synchronization of synthesis and degradation (Sundby *et al.*, 1993; Schnettger *et al.*, 1994; Russell *et al.*, 1995). My results, and those of other workers (Godde 1992, Godde and

Buchhold 1992), indicate that this is not necessarily true for stressed spruce trees and, as observed for a moss species (Rintamaki *et al.*, 1994), limitations on D1 synthesis may occur readily and may reflect a different strategy for survival during photoinhibition.

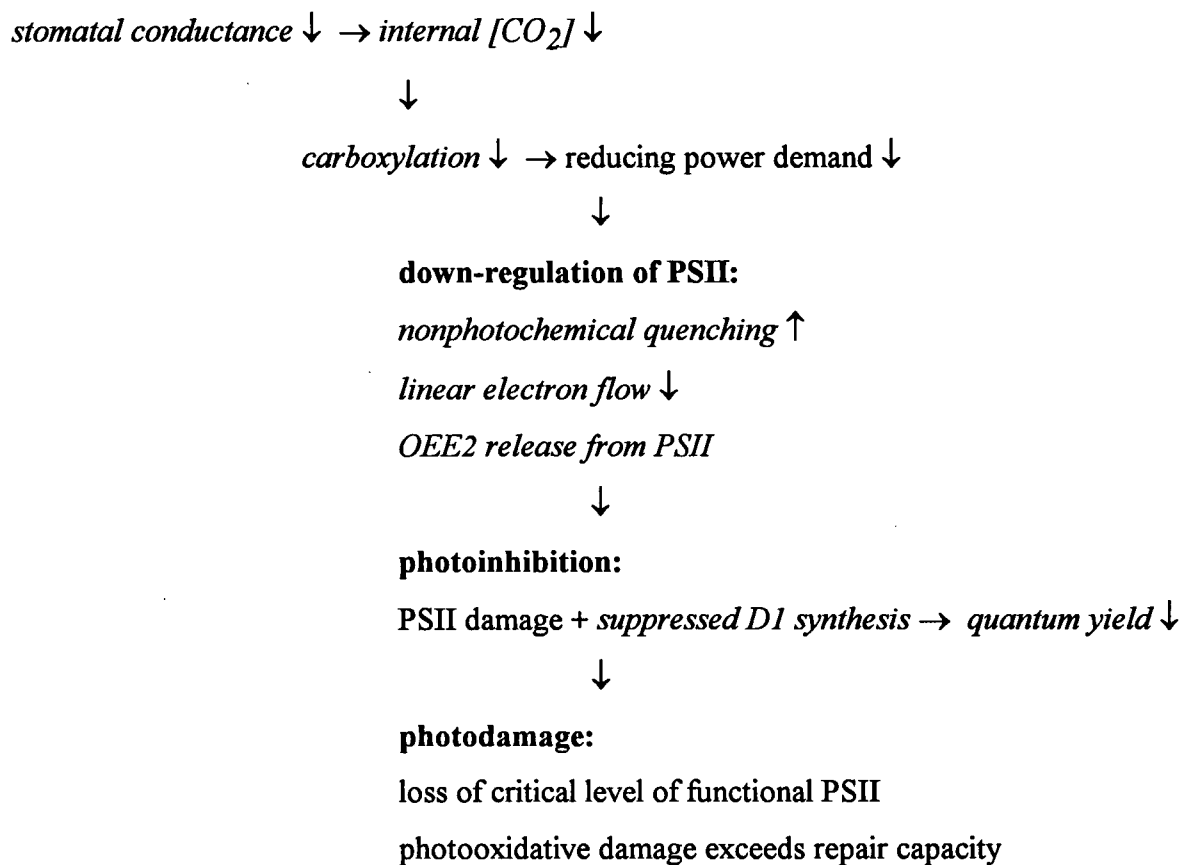
Chapter 5: Comprehensive View of Photosynthesis in Spruce during Water Stress

5.1 *A Hierarchy of Photosynthetic Regulation*

Interplay between the light and dark reactions of photosynthesis is highly coordinated (e.g., Campbell and Ogren 1990); however, the regulation of photosystem II (PSII) activity when carboxylation is minimal (e.g. during water stress) is not clearly understood. This is particularly true for the perennial photosynthetic apparatus of conifer species, although recent studies on seasonal inactivation have generated the hypothesis that evergreen species rely less on acclimation and more on tolerance through sustained photoinhibition during low temperature conditions (Adams *et al.*, 1995). My thesis work suggests that the response of spruce seedlings to progressive water stress shows another pattern that also differs from the angiosperm species response to water stress (Figure 5.1). Angiosperm species studied to date, both herbaceous (e.g., Grieu *et al.*, 1995; He *et al.*, 1995) and woody (e.g., Valentini *et al.*, 1995), appear to sustain PSII activity by increased nonphotochemical dissipation of excess energy until significant tissue water loss has occurred. In contrast, my results indicate that in spruce trees nonphotochemical quenching is readily light-saturated and that the down-regulation of PSII is already initiated at a moderate level of water stress.

In well-watered spruce trees in which predawn shoot water potentials (Ψ) were above -1 MPa (Phase 1), photosynthesis appeared to be primarily regulated by stomatal control of carbon dioxide levels, as has been widely reported for many plant species (e.g., Cornic *et al.*, 1992). Not surprisingly, decreased internal carbon dioxide levels were concomitant with decreases in carbon assimilation rate. At the membrane level other changes occurred; chlorophyll fluorescence analysis indicated modulation of PSII activity in response to light intensity. In particular, nonphotochemical quenching approached saturation at irradiances just

Figure 5.1 Flow diagram of the hierarchy of photosynthetic change during progressive water stress for interior spruce seedlings. Features investigated in this thesis are italicized.



above the light level used for growth of the young interior spruce trees. This leads to the question: if this photoprotective mechanism, often cited as the most significant factor in maintenance of PSII efficiency during water stress in angiosperm species (e.g., Grieu *et al.*, 1995), is so readily saturated in spruce, what does happen to PSII centres in spruce chloroplasts during progressive water stress?

At the level of water deficit that I have defined as a mild stress ($-1 \leq \Psi \leq -1.6$ MPa, Phase 2) for spruce, the stomata became fully closed and the consequences of restricted CO₂ supply began to be evident at the membrane level. In angiosperm species, changes in photosynthesis at this stage appear to be directly related to the lack of CO₂ and little change in PSII activity occurs (e.g., Chaves 1991). In spruce trees, the carboxylation rate approached a minimum, and, despite the elevated CO₂ levels in the measuring apparatus, oxygen evolution decreased and photochemical efficiency began to decline at saturating irradiance (Chapter 2). As carboxylation declines, other workers have reported increased photorespiration, which may be significant in maintaining electron flow (e.g., Jefferies 1994). Photorespiration probably plays a role in sustaining electron flow in spruce during the initial development of water-stress; however, my observations were conducted at elevated CO₂ which precluded analysis of this factor. As the stress develops, carbon metabolism may be diverted to accumulation of metabolites involved in osmotic responses (e.g., Chaves 1991). Osmotic changes in response to stress have been well-documented in spruce species (e.g., Cyr *et al.*, 1990; Lamhamedi and Bernier 1994) but this has not been evaluated specifically in terms of acclimation, tolerance or resistance of the perennial photosynthetic apparatus.

At the thylakoid level, changes in protein composition in response to water stress have rarely been investigated. A recent study of wheat seedlings subjected to a mild osmotic stress

showed no changes in PSII protein levels (He *et al.*, 1995). In contrast, my work with spruce seedlings did reveal changes in PSII proteins during mild water stress. Minor but significant decreases in D1 levels were detected, suggesting that D1 synthesis was insufficient to keep pace with increased D1 damage, and, although there were no changes in the total levels of OEE2, association of this potential regulatory protein with the PSII reaction centre decreased significantly as early as Phase 2 (Chapter 3).

In the third phase of water stress ($-1.6 < \Psi < -3$ MPa, Phase 3) no carboxylation was detectable and more pronounced changes in PSII activity became evident in spruce needles; oxygen evolution and photochemical efficiency of PSII decreased further (Chapter 2). Again this contrasts with observations made with angiosperm species in which oxygen evolution capacity may be sustained until the stress is severe and tissue water loss is significant (e.g., Epron and Dreyer 1993). In spruce, the changes in PSII proteins progressed; D1 levels and OEE2 association decreased further (Chapter 3). In this water stress phase (Phase 3), synthesis of D1 was restricted; the rate of methionine incorporation into D1 was significantly lower in stressed plants than in well-watered plants (Chapter 4). Significantly, overall protein translation was affected to a much lesser extent, possibly a consequence of accelerated cytoplasmic translation which was documented previously for stressed spruce seedlings (Schmitz *et al.*, 1993). Water stress may have had a specific effect on D1 translation or, possibly, on all chloroplastic translation. Although degradation rates could not be calculated from my labelling experiments, the information from immunoquantification and chlorophyll fluorometry, specifically, the decreases in the overall quantum efficiency of PII (Φ_{II}) and the quantum efficiency of open PSII centres (Φ_P), can be interpreted as evidence that a proportion of D1 may have been damaged but not degraded. Accumulation of inactivated PSII centers containing

photodamaged D1 has been clearly demonstrated during photoinhibition in a green algae, *Dunaliella salina* (Vasilikiotis and Melis 1995); spruce seedlings may utilize a similar strategy.

In the fourth phase of water stress ($-3 \leq \Psi \leq -4$ MPa, Phase 4) and in lethally stressed spruce seedlings, photoinhibition was evident. Specifically, maximal photochemical efficiency of PSII (Fv/Fm) showed decreases not readily reversible by prolonged (overnight) dark-adaption. Additionally, photochemical efficiency of PSII and open PSII centres plunged; photochemical quenching was low, even at low irradiance; and maximal *in vivo* oxygen evolution was 20 % of unstressed levels (Chapter 2). In thylakoids from lethally stressed plants, diminished electron transport through PSII could be partially restored *in vitro* by addition of calcium or a direct electron donor to P680 suggesting that loss or dissociation of OEE proteins was partly responsible for the loss of activity (Chapter 3). This is in contrast to a report for membranes from wheat undergoing severe osmotic stress in which no restoration was observed by addition of electron donors to PSII. In this case, researchers concluded that electron donation was not affected and that the lesion was beyond P680 (He *et al.*, 1995).

In lethally water-stressed spruce, decreases in thylakoid levels of D1, OEE1 and OEE2 were detected (Chapter 3), contrasting with the observations reported for angiosperm species. Photoinhibition-associated changes in PSII protein profiles, usually caused by excess light, have been investigated more intensively than changes associated with moderate stress. Typically, in photoinhibition, initial decreases in D1 and D2 levels are followed by a more general loss of PSII proteins, possibly reflecting a stress effect on translation (e.g., Ono *et al.*, 1995). No change in OEE2 levels was detected in photoinhibited material (Schuster *et al.*, 1988; Virgin *et al.*, 1990; Friso *et al.*, 1994) or in PSII membranes from osmotically stressed wheat seedlings (He *et al.*, 1995). These results may reflect a real difference in stress responses between angiosperm and

gymnosperm species or simply that technical reasons have prevented comparable observations in angiosperm species.

The most significant observation reported in this thesis is the change in OEE2 association with PSII before the onset of photoinhibition. Comparable observations have not been published. In addition to the processes previously reported to be involved in routine PSII reaction centre turnover, I suggest that OEE2 dissociation may be a component of the reversible mechanism for PSII down-regulation that was put forth recently by Krieger *et al.* (1993). On the basis of chlorophyll fluorescence and thermoluminescence measurements, this group proposed that a low, pH-dependent release of calcium from PSII raises the redox potential of the primary quinone acceptor Q_A which results in loss of electron transport through PSII. During water stress, ATP synthetase activity decreases and lumenal acidification results (Meyer *et al.*, 1992); thus, this could be the trigger for PSII down-regulation during drought. It has also been demonstrated that a redox shift of Q_A from a high to low potential form, occurs *in vivo* during photoactivation of PSII in a green alga (Johnson *et al.*, 1995), a process which includes the association of the OEE proteins with PSII centres. OEE2 is intimately associated with the Ca^{2+} cofactor of PSII (e.g., Homann and Madabusi 1993) and biochemical and structural information on PSII indicate that the OEE proteins comprise a protective microenvironment for the PSII reaction centre (e.g., Adelfroth *et al.*, 1995). It is conceivable that the difficulty in separating the effects of Ca^{2+} binding from the effects of OEE2 association simply reflects the mechanism by which OEE2 plays a critical role in modulating PSII activity.

5.2 Future Work...

This thesis work poses a number of questions that should be addressed to confirm unequivocally that water-stressed spruce seedlings restrict the rate of damaged D1 turnover or evoke a reversible mechanism for control of PSII activity involving OEE2 binding. These questions include: Are the reported observations related to osmotic changes within the chloroplast? If so, how is the signal transduced? Is overall chloroplast translation affected by water stress, or is there a specific effect on *psbA* translation? Do increased concentrations of ions directly affect protein behaviour? Does lumenal pH regulate OEE binding? Is there an interaction between osmotic potential and pH changes? Is chloroplast redox status involved?

It is also unclear how generally the described cascade of events within PSII may occur. Do the observations apply to this ontogenetic phase only or throughout the period of active growth, to seedlings only or also to mature trees? Do other conifer species demonstrate this pattern of responses? It would be interesting to test whether this is a mechanism specifically restricted to conifer species, i.e., species with perennial leaves, or is a more widespread phenomenon.

The analysis used in this thesis work could be extended to other stresses on the perennial photosynthetic apparatus, including investigating whether the observed phenomena are part of the constitutive PSII repair cycle or are regulatory features preceding photoinhibition. In particular, are the observed changes in OEE2 association causative, concurrent, or a consequence of PSII inactivation? Examination of changes in other nuclear- and chloroplast-encoded thylakoid proteins would be an obvious contribution to further understanding of PSII control. Comparison of PSII membrane responses to an array of environmental stresses could clarify the distinctions and limitations of coping mechanisms found in conifers.

A particularly interesting extension of this research would be analysis of the role of excitation pressure and/or redox status in initiating changes in chloroplast proteins. My observations could be extended readily to explore the recently proposed hypothesis that excitation pressure may be one component of a redox sensing/signaling pathway involved in communication between the nucleus and the chloroplast (Allen *et al.*, 1995). Such a pathway may explain how the highly-regulated coordination of nuclear and chloroplastic gene expression is coupled to electron transport activity and how photosynthetic acclimation is effected within the chloroplast protein complexes. In particular, it would be interesting to determine whether the OEE proteins are in fact sensitive to redox potential in a direct response to changes in luminal pH or indirectly to changes in Q_A redox status, and, ultimately, whether they play any role in photosynthetic acclimation.

Literature Cited

- Adams III, W.W., Demmig-Adams, B. (1994) Carotenoid composition and down-regulation of photosystem II in three conifer species during the winter. *Physiologia Plantarum* 92:451-458.
- Adams III, W.W., Demmig-Adams, B., Verhoeven, A.S., Barker, D.H. (1995) 'Photoinhibition' during winter stress: involvement of sustained xanthophyll cycle-dependent energy dissipation. *Australian Journal of Plant Physiology* 22:262-276.
- Adelroth, P., Lindberg, K., Andreasson, L.E. (1995) Studies of Ca^{2+} binding in spinach photosystem II using $^{45}\text{Ca}^{2+}$. *Biochemistry* 34:9021-9027.
- Adir, N., Shochat, S., Ohad, I. (1990) Light-dependent D1 protein synthesis and translocation in regulated by reaction center II. *The Journal of Biological Chemistry* 265:12563-12568.
- Allen, J.F., Alexciev, K., Hakansson, G. (1995) Regulation by redox signalling. *Current Biology* 5:869-872.
- Alosi, M.C., Neale, D.B., Kinlaw, C.S. (1990) Expression of *cab* genes in Douglas-fir is not strongly regulated by light. *Plant Physiology* 93:829-832.
- Amundson, R.G., Hadley, J.L., Fincher, J.F., Fellows, S., Alscher, R.G. (1992) Comparisons of seasonal changes in photosynthetic capacity, pigments, and carbohydrates of healthy sapling and mature red spruce and of declining and healthy red spruce. *Canadian Journal of Forest Research* 22:1605-1616.
- Anderson, J.M., Chow, W.S., Oquist, G. (1993) Dynamics of photosystem II: photoinhibition as a protective acclimation strategy. In *Photosynthetic Responses to the Environment*, Yamamoto, H.Y., Smith, C.M. editors, pp 14-26, Rockville, Maryland: The American Society of Plant Physiologists.
- Arnon, D.T. (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* 24:1-5.
- Aro, E.-M., Virgin, I., Andersson, B. (1993a) Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochimica Biophysica Acta* 1143:113-134.
- Aro, E.-M., McCaffery, S., Andersson, B. (1993b) Photoinhibition and D1 protein degradation in peas acclimated to different growth irradiances. *Plant Physiology* 103:835-843.
- Ball, M.C., Butterworth, J.A., Roden, J.S., Christian, R., Egerton, J.J.G. (1994) Applications of chlorophyll fluorescence to forest ecology. *Australian Journal of Plant Physiology* 22:311-319.

- Barbato, R., Shipton, C.A., Giacometti, G.M., Barber, J. (1991) New evidence suggests that the initial photoinduced cleavage of the D1-protein may not occur near the PEST sequence. *FEBS Letters* 290:162-166.
- Barber, J. (1984) Has the mangano-protein of the water splitting reaction of photosynthesis been isolated? *Trends in Biochemical Science* 9:79-80.
- Barber, J. (1994) Molecular basis of the vulnerability of photosystem II to damage by light. *Australian Journal of Plant Physiology* 22:201-208.
- Barber, J., Andersson, B. (1992) Too much of a good thing: light can be bad for photosynthesis. *Trends in Biochemical Sciences* 17:61-66.
- Baszczynski, C., Walden, D.B., Atkinson, B.G. (1983) Regulation of gene expression in corn (*Zea mays* L.) by heat shock. II. *In vitro* analysis of RNAs from heat-shocked seedlings. *Canadian Journal of Biochemistry and Cell Biology* 61:395-403.
- Beauregard, P. (1992) Modelling of the photosystem II 33 kDa protein: structure, functions and possible sulfate-sensitive sites derived from sequence-encoded information. *Environmental and Experimental Botany* 32:411-423.
- Becker, D. and Brady, S. (1995) Abstract # 512. Dissociation of extrinsic polypeptides from the OEC correlates with loss of oxygen evolution due to heat stress. *Plant Physiology* 108(S):104.
- Ben, G.-Y., Osmond, C.B., Sharkey, T.D (1987) Comparisons of photosynthetic responses of *Xanthium strumarium* and *Helianthus annuus* to chronic and acute water stress in sun and shade. *Plant Physiology* 84:476-482.
- Bernier, M., Carpentier, R. (1995) The action of mercury on the binding of the extrinsic polypeptides associated with the water oxidizing complex of photosystem II. *FEBS Letters* 360:251-254.
- Betts, S.D., Hachigian, T.M., Pichersky, E., Yocum, C.F. (1994) Reconstitution of the spinach oxygen-evolving complex with recombinant *Arabidopsis* manganese-stabilizing protein. *Plant Molecular Biology* 26:117-130.
- Binder, W.D., Spittlehouse, D.L., Draper D.A. (1989) Ecophysiology: post-planting physiological results and their implications for growing spruce. In *FRDA Report 030*. B.A Scrivener, J.A. MacKinnon, editors, pp. 33-35, Prince George, British Columbia: Northern Silviculture Committee.
- Blubaugh, D.J., Atamian, M., Babcock, G.T., Golbeck, J.H., Cheniae, G.M. (1991) Photoinhibition of hydroxylamine-extracted photosystem II membranes: identification of the sites of photodamage. *Biochemistry* 30:7586-7597.

- Boekema, E.J., Hankamer, B., Bald, D., Kruip, J., Nield, J., Boonstra, A.F., Barber, J., Rogner, M. (1995) Supramolecular structure of the photosystem II complex from green plants and cyanobacteria. *Proceedings of the National Academy of Science USA* 92:175-179.
- Boichenko, V.A., Limov, V.V., Mayes, S.R., Barber, J. (1993) Characterization of the light-induced oxygen gas exchanges from the IC2 deletion mutant of *Synechocystis* PCC 6803 lacking the photosystem II 33 kDa extrinsic protein. *Zeitschrift fur Naturforschung* 48c:224-233.
- Bolhar-Nordenkamp, H.R., Lechner, E.G. (1988) temperature and light dependent modifications of chlorophyll fluorescence kinetics in spruce needles during winter. *Photosynthesis Research* 18:287-298.
- Borthakur, D., Haselkorn, R. (1989) Nucleotide sequence of the gene encoding the 33 kDa water oxidizing polypeptide in *Anabaena* sp. strain PCC 7120 and its expression in *Escherichia coli*. *Plant Molecular Biology* 13:427-439.
- Boussac, A., Zimmermann, J.-L., Rutherford, A.W. (1989) EPR signals from modified charge accumulation states of the oxygen evolving enzyme in Ca^{2+} - deficient photosystem II. *Biochemistry* 28:8984-8989.
- Bracht, E., Trebst, A. (1994) Hypothesis on the control of D1 protein turnover by nuclear coded proteins in *Chlamydomonas reinhardtii*. *Zeitschrift fur Naturforschung* 49c:439-446.
- Brestic, M. (1995) Does photorespiration protect the photosynthetic apparatus in French bean from photoinhibition during drought? *Planta* 196:450-457.
- Bricker, T.M. (1992) Oxygen evolution in the absence of the 33-kilodalton manganese-stabilizing protein. *Biochemistry* 31:4623-4628.
- Brix, H. (1979) Effects of plant water stress on photosynthesis and survival of four conifers. *Canadian Journal of Forest Research* 9:160-165.
- Brugnoli, E., Cona, A., Lauteri, M. (1994) xanthophyll cycle components and capacity for non-radiative energy dissipation in sun and shade leaves of *Ligustrum ovalifolium* exposed to conditions limiting photosynthesis. *Photosynthesis Research* 41:451-463.
- Burda, K., Strzalka, K., Schmid, G.H. (1995) Europium- and dysprosium-ions as probes for the study of calcium binding sites in photosystem II. *Zeitschrift fur Naturforschung* 50c:220-230.
- Burnap, R.L., Qian, M., Shen, J.-R., Inoue, Y., Sherman, L. A., (1994) Role of disulfide linkage and putative intermolecular binding residues in the stability and binding of the extrinsic manganese-stabilizing protein to the photosystem II reaction center. *Biochemistry* 33:13712-13718.

- Burnap, R.L., Shen, J.-R., Jursinic, P.A., Inoue, Y., Sherman, L.A. (1992) Oxygen yield and thermoluminescence characteristics of a cyanobacterium lacking the manganese-stabilizing protein of photosystem II. *Biochemistry* 31:7404-7410.
- Burnap, R.L., Sherman, L.A. (1991) Deletion mutagenesis in *Synechocystis* sp. PCC6803 indicates that the Mn-stabilizing protein of photosystem II is not essential for O₂ evolution. *Biochemistry* 30:440-446.
- Buttner, M., Xie, D.-N., Nelson, H., Pinther, W., Hauska, G., Nelson, N. (1992) Photosynthetic reaction center genes in green sulfur bacteria and in photosystem 1 are related. *Proceedings of the National Academy of Science USA* 89:8135-8139.
- Byres, D.P., Johnson, J.D., Dean, T.J. (1992) Seasonal response of slash pine (*Pinus elliottii* var. *elliottii* Engelm.) photosynthesis to long-term exposure to ozone and acidic precipitation. *New Phytologist* 122:91-96.
- Camm, E. (1993) Photosynthetic responses in developing and year-old Douglas-fir needles during new shoot development. *Trees* 8:61-66.
- Campbell, W.J., Ogren, W.L. (1990) Electron transport through photosystem I stimulates light activation of ribulose biphosphate carboxylase/oxygenase (Rubisco) by rubisco activase. *Plant Physiology* 94:479-484.
- Carpentier, R., Nakatani, H.Y. (1985) Inhibitors affecting the oxidizing side of photosystem II at the Ca²⁺ - and Cl⁻ -sensitive sites. *Biochimica et Biophysica Acta* 808:288-292.
- Chapman, D.J., De Felice, J., Davis, K., Barber, J. (1989) Effect of alkaline pH on photosynthetic water oxidation and the association of extrinsic proteins with Photosystem Two. *Biochemical Journal* 258:357-362.
- Chaves, M.M. (1991) Effects of water deficits on carbon assimilation. *Journal of Experimental Botany* 42:1-16.
- Chu, H.-A., Nguyen, A.P., Debus, R.J. (1994) Site-directed photosystem II mutants with perturbed oxygen-evolving properties 2. Increased binding or photooxidation of manganese in the absence of the extrinsic 33 kDa polypeptide *in vivo*. *Biochemistry* 33:6150-6157.
- Clausmeyer, S., Klosgen, R.B., Herrmann, R., G. (1993) Protein import into chloroplasts - the hydrophilic luminal proteins exhibit unexpected import and sorting specificities in spite of structurally conserved transit peptides. *The Journal of Biological Chemistry* 268:13869-13876.
- Cline, K., Henry, R., Li, C., Yuan, J. (1994) Multiple pathways for protein transport into or across the thylakoid membrane. *The EMBO Journal* 12:4105-4114.

- Cole, J., Boska, M., Blough, N.V., Sauer, K. (1986) Reversible and irreversible effects of alkaline pH on Photosystem II electron-transfer reactions. *Biochimica et Biophysica Acta* 848:41-47.
- Colombo, S.J., Teng, Y. (1992) Seasonal variation in the tissue water relations of *Picea glauca*. *Oecologia* 92:410-415.
- Conroy, J.P., Smillie, R.M., Koppers, M., Bevege, D.I., Barlow, E.W. (1986) Chlorophyll *a* fluorescence and photosynthetic and growth responses of *Pinus radiata* to phosphorus deficiency, drought stress and high CO₂. *Plant Physiology* 81:423-429.
- Conroy, J.P., Virgona, J.M., Smillie, R.M., Barlow, E.W. (1988) Influence of drought acclimation and CO₂ enrichment on osmotic adjustment and chlorophyll *a* fluorescence of sunflower during drought. *Plant Physiology* 86:1108-1115.
- Cornic, G., J. Ghasghaie, B. Genty, J.-M. Briantais. (1992). Leaf photosynthesis is resistant to a mild drought stress. *Photosynthetica* 27:295-309.
- Cornic, G., Le Gouallec, J.-L., Briantais, J.M., Hodges, M. (1989) Effect of dehydration and high light on photosynthesis of two C₃ plants (*Phaseolus vulgaris* L. and *Elatostema repens* (Lour.) Hall f.). *Planta* 177:84-90.
- Critchley, C., Andersson, B., Ryrie, I.J., Anderson, J. M. (1984) Studies on oxygen evolution of inside-out thylakoid vesicles from mangroves: chloride requirement, pH dependence and polypeptide composition. *Biochimica et Biophysica Acta* 767:532-539.
- Critchley, C., Russell, A.W. (1994) Photoinhibition of photosynthesis *in vivo*: the role of protein turnover in photosystem II. *Physiologia Plantarum* 92:188-196.
- Cyr, D.R., Buxton, G.F., Webb, D.P., Dumbroff, E.B. (1990) Accumulation of free amino acids in the shoots and roots of three northern conifers during drought. *Tree Physiology* 6:293-303.
- Danon, A., Mayfield, S.P.Y. (1991) Light regulated translational activators: identification of chloroplast gene specific mRNA binding proteins. *The EMBO Journal* 10:3993-4001.
- Day, R.J., Butson, R.G. (1989) Seedling-water relationships after outplanting. In *Climate Applications in Forest Renewal and Forest Production*. MacIver, D.C., Street, R.B., Auclair, A.N., editors, pp. 55-62, Ottawa, Ontario: Canadian Government Publishing Centre.
- de Vitry, C., Olive, J., Drapier, D., Recouvreur, M., Wollman, F.-A. (1989) Posttranslational events leading to the assembly of photosystem II protein complex: a study using photosynthesis mutants from *Chlamydomonas reinhardtii*. *Journal of Cell Biology* 109:991-1006.
- Debus, R.J. (1992) The manganese and calcium ions of photosynthetic oxygen evolution. *Biochimica et Biophysica Acta* 1102:269-352.

- Demmig-Adams, B., Adams, W.W. (1992) Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Physiology and Plant Molecular Biology* 43:599-626.
- Demmig-Adams, B., Adams, W.W., Logan, B.A., Verhoeven, A.S. (1995) Xanthophyll cycle-dependent energy dissipation and flexible photosystem II efficiency in plants acclimated to light stress. *Australian Journal of Plant Physiology* 22:249-260.
- Dietz, B., Moors, I., Flammersfeld, U., Ruhle, W., Wild, A. (1988) Investigation on the photosynthetic membranes of spruce needles in relation to the occurrence of novel forest decline I. The photosynthetic electron transport. *Zeitschrift fur Naturforschung* 43c:581-588.
- Droillard, M.J., Bate, N.J., Rothstein, S.J., Thompson, J.E. (1992) Active translation of the D-1 protein of photosystem II in senescing leaves. *Plant Physiology* 99:589-594.
- Dunahay, T.G., Staehelin, L.A., Seibert, M., Ogilvie, P.D., Berg, S.P. (1984). Structural, biochemical and biophysical characteristics of four oxygen-evolving photosystem II preparations from spinach. *Biochimica et Biophysica Acta* 764:179-193.
- Eastman, P.A.K., Camm, E.L. (1995) Regulation of photosynthesis in interior spruce during water stress: changes in gas exchange and chlorophyll fluorescence. *Tree Physiology* 15:229-235.
- Ebina, M., and Yamashita, T. (1992) Evidence for strong relation between religation of Mn and reassembly of extrinsic polypeptides in reconstitution of O₂ evolving center of Tris-inactivated spinach thylakoids by photoreactivation. *Research in Photosynthesis* 2:389-392.
- Edwards, D.R., Dixon, M.A. (1995a) Mechanisms of drought response in *Thuja occidentalis* L. I. Water stress conditioning and osmotic adjustment. *Tree Physiology* 15:121-127.
- Edwards, D.R., Dixon, M.A. (1995b) Mechanisms of drought response in *Thuja occidentalis* L. II. Post-conditioning water stress and stress relief. *Tree Physiology* 15:129-133.
- Eickmeier, W.G., Lebkuecher, J.G., Osmond, C.B. (1992) Photosynthetic water oxidation and water stress in plants. In *Water and Life*, Somero, G.N., Osmond, C.B., Bolis, C.L., editors, pp 223-239, Berlin, Heidelberg: Springer-Verlag.
- Enami, I., Kaneko, M., Kitamura, N., Koike, H., Sonoike, K., Inoue, Y., Katoh, S. (1991) Total immobilization of the extrinsic 33 kDa protein in spinach photosystem II membrane preparations. Protein stoichiometry and stabilization of oxygen evolution. *Biochimica et Biophysica Acta* 1060:224-232.
- Enami, I., Kitamura, M., Tomo, T., Isokawa, Y., Ohta, H., Katoh, S. (1994) Is the primary cause of thermal inactivation of oxygen evolution in spinach PSII membranes release of the extrinsic 33kDa protein or of Mn? *Biochimica et Biophysica Acta* 1186:52-58.

- Enami, I., Mochizuki, Y., Takahashi, S., Kakuno, T., Horio, T., Satoh, K., Katoh, S. (1990) Evidence from crosslinking for nearest-neighbor relationships among the three extrinsic proteins of spinach photosystem II complexes that are associated with oxygen evolution. *Plant and Cell Physiology* 31:725-729.
- Enami, I., Ohta, S., Mitsuhashi, S., Takahashi, S., Ikeuchi, M., Katoh, S. (1992) Evidence from crosslinking for a close association of the extrinsic 33 kDa protein with the 9.4 kDa subunit of cytochrome *b₅₅₉* and the 4.8 kDa product of the *psb I* gene in oxygen-evolving photosystem II complexes from spinach. *Plant and Cell Physiology* 33:291-297.
- Engels, D.H., Lott, A., Schmid, G.H., Pistorius, E.K. (1994) Inactivation of the water-oxidizing enzyme in manganese stabilizing protein-free mutant cells of the cyanobacteria *Synechococcus* PCC7942 and *Synechocystis* PCC6803 during dark incubation and conditions leading to photoactivation. *Photosynthesis Research* 42:227-244.
- Epron, D., Dreyer, E. (1993). Photosynthesis of oak leaves under water stress: maintenance of high photochemical efficiency of photosystem II and occurrence of non-uniform CO₂ assimilation. *Tree Physiology* 13:107-117.
- Epron, D., Dreyer, E. (1992). Effects of severe dehydration on leaf photosynthesis in *Quercus petraea* (Matt.) Liebl.: photosystem II efficiency, photochemical and nonphotochemical fluorescence quenching and electrolyte leakage. *Tree Physiology* 10:273-284.
- Epstein, E. (1972) *Mineral Nutrition of Plants - Principles and Perspectives*, p. 39, New York: John Wiley.
- Ettinger, W.F., Theg, S.M. (1991) Physiologically active chloroplasts contain pools of unassembled extrinsic proteins of the photosynthetic oxygen-evolving enzyme complex in the thylakoid lumen. *Journal of Cell Biology* 115:321-328.
- Evans, E., Brown, R.G., Wellburn, A.R. (1992) Chlorophyll fluorescence decay profiles of O₃-exposed spruce needles as measured by time-correlated single photon counting. *New Phytologist* 122:501-506.
- Flammersfeld, U., Wild, A. (1992) Changes in the constitution of thylakoid membranes in spruce needles during an open-top chamber experiment. *Botanica Acta* 105:348-354.
- Fling, S.P., Gregerson, D.S. (1986) Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. *Analytical Biochemistry* 155:83-88.
- Ford, C., Rosenberg, M.F., Shepherd, F.H., McPhie, P., Holzenburg, A. (1995) Photosystem II 3-D structure and the role of the extrinsic subunits in photosynthetic oxygen evolution. *Micron* 26:133-140.
- Foyer, C., Lelandais, M., Kunert, K.J. (1994) Photooxidative stress in plants. *Physiologia Plantarum* 92:696-717.

- Foyer, C.H., Lelandais, M., Harbinson, J. (1992) Control of the quantum efficiencies of photosystem I and II, electron flow, and enzyme activation following dark-to-light transitions in pea leaves. *Plant Physiology* 99:979-986.
- Frankel, L.K., Bricker, T.M. (1992) Interaction of CPa-1 with the manganese-stabilizing protein of photosystem II: identification of domains on CPa-1 which are shielded from *N*-hydroxysuccinimide biotinylation by the manganese-stabilizing protein. *Biochemistry* 32:11059-11064.
- Frankel, L.K., Bricker, T.M. (1995) Interaction of the 33-kDa extrinsic protein with photosystem II: identification of domains on the 33-kDa protein that are shielded from NHS-biotinylation in photosystem II. *Biochemistry* 34:7492-7497.
- Franzen, L.-G., Andreasson, L.-E. (1984) Studies on manganese binding by selective solubilization of photosystem-II polypeptides. *Biochimica Biophysica Acta* 765:166-170.
- Friso, G., Spetea, C., Giacometti, G.M., Vass, I., Barbato, R. (1994) Degradation of photosystem II reaction center D1-protein induced by UVB radiation in isolated thylakoids. Identification and characterization of C- and N-terminal breakdown products. *Biochimica et Biophysica Acta* 1184:78-84.
- Garstka, M., Kaniuga, Z. (1988) Linolenic acid-induced release of Mn, polypeptides and inactivation of oxygen evolution in photosystem II particles. *FEBS Letters* 232:372-376.
- Genty, B., Briantais, J.-M., Baker, N.R. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 990:87-92.
- Gillies, S.L., Vidaver, W. (1990) Resistance to photodamage in evergreen conifers. *Physiologia Plantarum* 80:148-153.
- Gilmore, A. M., Bjorkman, O. (1994a) Adenine nucleotides and the xanthophyll cycle in leaves. I. Effects of CO₂ - and temperature-limited photosynthesis on adenylate energy charge and violoxanthin de-epoxidation. *Planta* 192:526-536.
- Gilmore, A. M., Bjorkman, O. (1994b) Adenine nucleotides and the xanthophyll cycle in leaves. II. Comparison of the effects of CO₂ - and temperature-limited photosynthesis on photosystem II fluorescence quenching, the adenylate energy charge and violoxanthin de-epoxidation in cotton. *Planta* 192:537-544.
- Godde, D. (1992) Photosynthesis in relation to modern forest decline; content and turnover of the D-1 reaction centre polypeptide of photosystem 2 in spruce trees (*Picea abies*). *Photosynthetica* 27:217-230.

- Godde, D., Buchhold J. (1992) Effect of long term fumigation with ozone on the turnover of the D-1 reaction center polypeptide of photosystem II in spruce (*Picea abies*). *Physiologia Plantarum* 86:568-574.
- Godde, D., Dannehl, H. (1994) Stress-induced chlorosis and increase in D1-protein turnover precede photoinhibition in spinach suffering under magnesium/sulphur deficiency. *Planta* 195:291-300.
- Godde, D., Hefer, M. (1994) Photoinhibition and light-dependent turnover of the D1 reaction-centre polypeptide of photosystem II are enhance by mineral-stress conditions. *Planta* 193:290-299.
- Godde, D., Schmitz, H., Weidner, M. (1991) Turnover of the D-1 reaction center polypeptide from photosystem II in intact spruce needles and spinach leaves. *Zeitschrift fur Naturforschung* 46c:245-251.
- Gong, H. (1994) Light-dependent degradation of the Photosystem II D1 protein is retarded by inhibitors of chloroplast transcription and translation: possible involvement of a chloroplast-encoded proteinase. *Biochimica et Biophysica Acta* 1188:422-426.
- Graan, T., J.S. Boyer. 1990. Very high CO₂ partially restores photosynthesis in sunflower at low water potentials. *Planta* 181:378-384.
- Gray, J.C. (1982) Use of proteolytic inhibitors during isolation of plastid proteins. In *Methods in Chloroplast Molecular Biology*, Edelman, M., Halick, R.B., Chua, N.-H., editors, pp. 1093-1102, Amsterdam: Elsevier Biomedical Press.
- Grieu, P., Robin, C., Guckert, A. (1995) Effect of drought on photosynthesis in *Trifolium repens*: maintenance of photosystem II efficiency and of measured photosynthesis. *Plant Physiology and Biochemistry* 33:19-24.
- Grill, D., Pfeifhofer, H., Tschulik, A., Hellig, K., Holzer, K. (1988) Thiol content of spruce needles at forest limits. *Oecologia* 76:294-297.
- Grossnickle, S.C. (1989) Shoot phenology and water relations of *Picea glauca*. *Canadian Journal of Forest Research* 19:1287-1290.
- Grossnickle, S.C., J.E. Major. (1994). Interior spruce seedlings compared to emblings produced from somatic embryogenesis II) Stock quality assessment prior to field planting. *Canadian Journal of Forest Research* 24:1385-1396.
- Grossnickle, S.C., Major, J.E., Folk, R.S. (1994) Interior spruce seedlings compared to emblings produced from somatic embryogenesis I) Nursery development, fall acclimation and over-winter storage. *Canadian Journal of Forest Research* 24:1397-1407.

- Haag, E., Irrgang, K.-D., Boekema, E.J., Renger, G. (1990) Functional and structural analysis of photosystem II core complexes from spinach with high oxygen evolution capacity. *European Journal of Biochemistry* 189:47-53.
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, , pp 119, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- Hashimoto, A., Akasaka, T., Yamamoto Y. (1993) Characteristics of the assembly of the 33 kDa oxygen-evolving complex protein in the etioplasts and the developing chloroplasts of barley seedlings. *Biochimica et Biophysica Acta* 1183:397-407
- Havaux, M. (1992). Stress tolerance of photosystem II *in vivo* - Antagonistic effects of water, heat, and photoinhibition stresses. *Plant Physiology* 100:424-432.
- Havaux, M., Canaani, O., Malkin, S. (1986) Photosynthetic responses of leaves to water stress, expressed by photoacoustics and related methods. II. The effect of rapid drought on the electron transport and the relative activities of the two photosystems. *Plant Physiology* 82:834-839.
- He, J.X., Wang, J., Liang, H.G. (1995) Effects of water stress on photochemical function and protein metabolism of photosystem II in wheat leaves. *Physiologia Plantarum* 93:771-777.
- Hertwig, B., Streb, P., Feierabend, J. (1992) Light dependence of catalase synthesis and degradation in leaves and the influence of interfering stress conditions. *Plant Physiology* 100:1547-1553.
- Hideg, E., Spetea, C., Vass, I. (1994) Singlet oxygen production in thylakoid membranes during photoinhibition as detected by EPR spectroscopy. *Photosynthesis Research* 39:191-199.
- Hiramatsu, H., Mizobuchi, A., Mori, H., Yamamoto, Y. (1991) Relationship between the stability of antenna chl *a*-binding proteins CP43/47 and the accumulation of the extrinsic 30-kDa protein in PS II in *Euglena*. *Plant Cell Physiology* 32:881-889.
- Hofer, M.U., Santore, U.J., Westhoff, P. (1992) Differential accumulation of the 10-, 16- and 23-kDa peripheral components of the water-splitting complex of photosystem II in mesophyll and bundle-sheath chloroplasts of the dicotyledonous C₄ plant *Flaveria trinervia* (Spreng.) C. Mohr. *Planta* 186:304-312.
- Hoffman, N.E., Franklin, A.E. (1994) Evidence for a stromal GTP requirement for the integration of a chlorophyll *a/b*-binding polypeptide into thylakoid membranes. *Plant Physiology* 105:295-304.
- Holzenburg, A., Shepherd, F.H., Ford, C. (1994) Localization of the oxygen-evolving complex of photosystem II by Fourier difference analysis. *Micron* 25:447-451.

- Homann, P.H. (1987) The relations between the chloride, calcium, and polypeptide requirements of photosynthetic water oxidation. *Journal of Bioenergetics and Biomembranes* 19:105-123.
- Homann, P.H. (1988a) Structural effects of Cl^- and other anions on the water oxidizing complex of chloroplast photosystem II. *Plant Physiology* 88:194-199.
- Homann, P.H. (1988b) The chloride and calcium requirement of photosynthetic water oxidation: effects of pH. *Biochimica et Biophysica Acta* 934:1-13.
- Homann, P.H., Madabusi, L.V. (1993) Modification of the thermoluminescence properties of Ca^{2+} depleted Photosystem II membranes by the 23 kDa extrinsic polypeptide and by oligocarboxylic acids. *Photosynthesis Research* 35:29-39.
- Horton, P., Ruban, A.V., Walters, R.G. (1994) Regulation of light harvesting in green plants - Indication by nonphotochemical quenching of chlorophyll fluorescence. *Plant Physiology* 106:415-420.
- Hulbert, C., Funkhouser, E.A., Soltes, E.J., Newton, R.J. (1988) Inhibition of protein synthesis in loblolly pine hypocotyls by mannitol-induced water stress. *Tree Physiology* 4:19-26.
- Hulford, A., Hazell, L., Mould, R.M., Robinson, C. (1994) Two distinct mechanisms for the translocation of proteins across the thylakoid membrane, one requiring the presence of a stromal protein factor and nucleotide triphosphates. *The Journal of Biological Chemistry* 269:3251-3256.
- Hundal, T., Virgin, I., Strying, S., Andersson, B. (1990) Changes in the organization of photosystem II following light-induced D1-protein degradation. *Biochimica et Biophysica Acta* 1017:235-241.
- Irrgang, K.-D., Geiken, B., Lange, B., Renger, G. (1992) Disulfide bridge modifiers and sulfhydryl group blockers are inactivating the oxygen evolving enzyme of PSII from spinach, In *Research in Photosynthesis* Vol. II, Murata, N., editor, pp. 417-420, Netherlands: Kluwer Academic Publishers.
- Irrgang, K.-D., Shi, L.-X., Funk, C., Schroder, W.P. (1995) A nuclear-encoded subunit of the photosystem II reaction center. *The Journal of Biological Chemistry* 270: 17588-17593.
- Ivanov, A.G., Busheva, M.C., Velitchkova, M.Y. (1990) Surface density changes in isolated photosystem II membranes induced by depletion of the extrinsic polypeptides of the oxygen evolving system. *Zeitschrift fur Naturforschung* 45c:627-632.
- Jansson, S., Selstam, E., Gustafsson, P. (1990) The rapidly phosphorylated 25 kDa polypeptide of the light-harvesting complex of Photosystem II is encoded by the Type 2 *cab-II* genes. *Biochimica et Biophysica Acta* 1019:110-114.

- Jefferies, R.A. (1994) Drought and chlorophyll fluorescence in field-grown potato (*Solanum tuberosum*). *Physiologia Plantarum* 90:93-97.
- Jegerschold, C., Virgin, I., Stryring, S. (1990) Light-dependent degradation of the D1 protein in photosystem II is accelerated after inhibition of the water splitting reaction. *Biochemistry* 29:6179-6186.
- Jiang, Y., Macdonald, S.E., Zwiazek, J.J. (1995) Effects of cold storage and water stress on water relations and gas exchange of white spruce (*Picea glauca*) seedlings. *Tree Physiology* 15:267-273.
- Johnson, G.N., Rutherford, A.W., Krieger, A. (1995) A change in the midpoint potential of the quinone Q_A in photosystem II associated with photoactivation of oxygen evolution. *Biochimica et Biophysica Acta* 1229:202-207.
- Jursinic, P., Miller, N., Carpentier, R. (1990) Ruthenium red inhibition of oxygen evolution and specific release of the extrinsic 16 kDa polypeptide in a photosystem II preparation. *Photochemistry and Photobiology* 51:705-712.
- Kainulainen, P., Oksanen, J., Palomaki, V., Holopainen, T. (1992) Effect of drought and waterlogging on needle monoterpenes of *Picea abies*. *Canadian Journal of Botany* 70:1613-1616.
- Kaiser, W.M. (1987) Effects of water deficit on photosynthetic capacity. *Physiologia Plantarum* 71:142-149.
- Kaiser, W.M., Kaiser, G., Prachuab, P.K., Wildman, S.G., Heber, U. (1981) Photosynthesis under osmotic stress - inhibition of photosynthesis of intact chloroplasts, protoplasts, and leaf slices at high osmotic potentials. *Planta* 153:416-422.
- Kamachi, H., Tamura, N., Yoshihira, T., Oku, T. (1994) Photoactivation of the latent water-oxidizing complex in photosystem II membranes isolated from dark-grown spruce seedlings. *Physiologia Plantarum* 91:747-753.
- Kanervo, E., Maenpaa, P., Aro, E.-M. (1993) D1 protein degradation and *psbA* transcript levels in *Synechocystis* PCC 6803 during photoinhibition *in vivo*. *Journal of Plant Physiology* 142:669-675.
- Kaufmann, M.R. (1976) Stomatal response of Engelmann spruce to humidity, light, and water stress. *Plant Physiology* 57:898-901.
- Kim, J., Eichacker, L.A., Rudiger, W., Mullet, J.E. (1994a) Chlorophyll regulates accumulation of the plastid-encoded chlorophyll proteins P700 and D1 by increasing apoprotein stability. *Plant Physiology* 104:907-916.
- Kim, J., Klein, P.G., Mullet, J.E. (1994b) Vir-115 gene product is required to stabilize D1 translation intermediates in chloroplasts. *Plant Molecular Biology* 25:459-467.

- Kim, J.H., Nemson, J.A., Melis, A. (1993) Photosystem II reaction center damage and repair in *Dunaliella salina* (green alga)- Analysis under physiological and irradiance-stress conditions. *Plant Physiology* 103:181-189.
- Ko, K., Granell, A., Bennett, J., Cashmore, A.R. (1990) Isolation and characterization of cDNAs from *Lycopersicon esculentum* and *Arabidopsis thaliana* encoding the 33 kDa protein of the photosystem II-associated oxygen evolving complex. *Plant Molecular Biology* 14:217-227.
- Kojima, K., Yamamoto, N., Sasaki, S. (1992) Structure of the pine (*Pinus thunbergii*) chlorophyll *a/b*-binding protein gene expressed in the absence of light. *Plant Molecular Biology* 19:405-410.
- Komenda, J., Barber, J. (1995) Comparison of *psbO* and *psbH* deletion mutants of *Synechocystis* PCC 6803 indicates that degradation of D1 protein is regulated by the QB site and dependent on protein synthesis. *Biochemistry* 34:9625-9631.
- Koppelaar, R.S., Tschaplinski, T.J., Colombo, S.J. (1991) Carbohydrate accumulation and turgor maintenance in seedling shoots and roots of two boreal conifers subjected to water stress. *Canadian Journal of Botany* 69:2522-2528.
- Krieger, A., Weis, E. (1992) Energy-dependent quenching of chlorophyll-*a*-fluorescence: the involvement of proton-calcium exchange at photosystem 2. *Photosynthetica* 27:89-98.
- Krieger, A., Weis, E., Demeter, S. (1993) Low-pH-induced Ca^{2+} ion release in the water-splitting system is accompanied by a shift in the midpoint redox potential of the primary quinone acceptor Q_A . *Biochimica et Biophysica Acta* 1144:411-418.
- Kuhn, M., Fromme, P., Krabben, L. (1994) A 'membrane attached' α -helix: a conserved structural motif in bacterial reaction centres, photosystem I and chloroplast NADH-plastoquinone oxidoreductase. *Trends in Biochemical Sciences* 19: 401-402.
- Kukidome, H., Kobayashi, Y., Oku, T. (1986) Properties of photosystem II particles prepared from chloroplasts of spruce seedlings. *Journal of the Faculty of Agriculture Kyushu University* 30:267-274.
- Kulkarni, R.D., Golden, S.S. (1994) Adaption to high light intensity in *Synechococcus* sp. strain PCC 7942: Regulation of three *psbA* genes and two forms of the D1 protein. *Journal of Bacteriology* 176:959-965.
- Kusnetnov, V.V., Oelmüller, R., Sarwat, M.I., Porfirova, S.A., Cherepneva, G.N., Herrmann, R.G., Kulaeva, O.N. (1994) Cytokinins, abscisic acid and light affect accumulation of chloroplast proteins in *Lupinus luteus* cotyledons without notable effect on steady-state mRNA levels. *Planta* 194:318-327.

- Kuwabara, T., Reddy, K.J., Sherman, L.A. (1987) Nucleotide sequence of the gene from the cyanobacterium *Anacystis nidulans* R2 encoding the Mn-stabilizing protein involved in photosystem II water oxidation. *Proceedings of the National Academy of Science USA* 84:8230-8234.
- Kuwabara, T., Suzuki, K. (1995) Reversible changes in conformation of the 23-kDa protein of photosystem II and their relationship to the susceptibility of the protein to a proteinase from photosystem II membranes. *Plant and Cell Physiology* 36:495-504.
- Laemmli, U.K. (1970) Changes of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 227:680-685.
- Lamhamedi, M.S., Bernier, P.Y. (1994) Ecophysiology and field performance of black spruce (*Picea mariana*): a review. *Annales des Sciences Forestieres* 51:529-551.
- Lange, O.L., Fuhrer, G., Gebel, J. (1986) Rapid field determination of photosynthetic capacity of cut spruce twigs (*Picea abies*) at saturating ambient CO₂. *Trees* 1:70-77.
- Le Thiec, D., Dixon, M., Garrec, J.P. (1994) The effects of slightly elevated ozone concentrations and mild drought stress on the physiology and growth of Norway spruce, *Picea abies* (L.) Karst. and beech, *Fagus sylvatica* L., in open-top chambers. *New Phytologist* 128:671-678.
- Legocki, R. and Verma, D.P.S. (1981) Multiple immunoreplica techniques: screening for specific proteins with a series of different antibodies using one polyacrylamide gel. *Analytical Biochemistry* 111:385-392.
- Leone, A., Costa, A., Tucci, M., Grillo, S. (1994) Comparative analysis of short- and long-term changes in gene expression caused by low water potential in potato (*Solanum tuberosum*) cell-suspension cultures. *Plant Physiology* 106:703-712.
- Leverenz, J.W., Oquist, G. (1987) Quantum yields of photosynthesis at temperatures between -2°C and 35°C in a cold-tolerant C₃ plant (*Pinus sylvestris*) during the course of one year. *Plant, Cell and Environment* 10:287-295.
- Li, X., Henry, R., Yuan, J., Cline, K., Hoffman, N.E. (1995) A chloroplast homologue of the signal recognition particle subunit SRP54 is involved in the posttranslational integration of a protein into thylakoid membranes. *Proceedings of the National Academy of Science USA* 92:3789-3793.
- Lichtenthaler, H.K., Wellburn, A.R. (1983) Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochemical Society Transactions* 11:591-592.
- Lidholm, J., Gustafsson, P. (1992) A functional promoter shift of a chloroplast gene: a transcriptional fusion between a novel *psbA* gene copy and the *trnk* (UUU) gene in *Pinus contorta*. *The Plant Journal* 2:875-886.

- Lidholm, J., Szmidt, A.E., Hallgren, J.-E., Gustafsson, P. (1988) The chloroplast genome of conifers lacks one of the rRNA-encoding inverted repeats. *Molecular and General Genetics* 212:6-10.
- Liebl, U., Mockensturm-Wilson, M., Trost, J.T., Brune, D.C., Blankenship, R.E., Vermaas, W. (1993) Single core polypeptide in the reaction center of the photosynthetic bacterium *Helioobacillus mobilis*: structural implications and relations to other photosystems. *Proceedings of the National Academy of Science USA* 90:7124-7128.
- Lu, P., Biron, P., Breda, N., Granier, A. (1995) Water relations of adult Norway spruce (*Picea abies* (L) Karst) under soil drought in the Vosges mountains: water potential, stomatal conductance and transpiration. *Annale des Sciences Forestieres* 52:117-129.
- Lundmark, T., Hallgren, J.-E., Heden, J. (1988) Recovery from winter depression of photosynthesis in pine and spruce. *Trees* 2:110-114.
- Lutz, C., Steiger, A., Godde, D. (1992) Influence of air pollutants and nutrient deficiency on D-1 protein content and photosynthesis in young spruce trees. *Physiologia Plantarum* 85:611-617.
- Maier, C.A., Teskey, R.O. (1992) Internal and external control of net photosynthesis and stomatal conductance of mature eastern white pine (*Pinus strobus*). *Canadian Journal of Forest Research* 22:1387-1394.
- Manderscheid, R., Jager, H.-J. (1993) Seasonal changes in nitrogen metabolism of spruce needles (*Picea abies* (L.) Karst.) as affected by water stress and ambient air pollutants. *Journal of Plant Physiology* 141:494-501.
- Margolis, H.A. and Brand, D.G. (1990) An ecophysiological basis for understanding plantation establishment. *Canadian Journal of Forest Research* 20:375-390.
- Mariani, P., De Carli, M.E., Rascio, N., Baldan, B., Casadoro, G., Gennari, G., Bodner, M., Larcher, W. (1990) Synthesis of chlorophyll and photosynthetic competence in etiolated and greening seedlings of *Larix decidua* as compared with *Picea abies*. *Journal of Plant Physiology* 137:5-14.
- Masojidek, J., Hall, D.O. (1992) Salinity and drought stresses are amplified by high irradiance in sorghum. *Photosynthetica* 27:159-171.
- Masojidek, J., Trivedi, S., Halshaw, L., Alexiou, A., Hall, D.O. (1991) The synergistic effect of drought and light stresses in sorghum and pearl millet. *Plant Physiology* 96:198-207.
- Massacci, A., Jones, H.G. (1990) Use of simultaneous analyses of gas-exchange and chlorophyll fluorescence quenching for analysing the effects of water stress on photosynthesis in apple leaves. *Trees* 4:1-8.

- Mattoo, A.K., Edelman, M. (1987) Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32-kd herbicide-binding protein. *Proceedings of the National Academy of Science USA* 84:1497-1501.
- Mayfield, S.P. (1991) Over-expression of the oxygen-evolving enhancer 1 protein and its consequences on photosystem II accumulation. *Planta* 185:105-110.
- Mayfield, S.P., Bennoun, P., Rochaix, J.-D. (1987a) Expression of the nuclear encoded OEE1 protein is required for oxygen evolution and stability of photosystem II particles in *Chlamydomonas reinhardtii*. *The EMBO Journal* 6:313-318.
- Mayfield, S.P., Rahire, M., Frank, G., Zuber, H., Rochaix, J.-D. (1987b) Expression of the nuclear gene encoding oxygen-evolving enhancer protein 2 is required for high levels of photosynthetic oxygen evolution in *Chlamydomonas reinhardtii*. *Proceeding of the National Academy of Science USA* 84:749-753.
- Mazari, A., Camm, E.L. (1993) Effect of cytokinins on plastid development and photosynthetic polypeptides during organogenesis of *Pinus ponderosa* Dougl. cotyledons cultured *in vitro*. *Plant Cell, Tissue, and Organ Culture* 33:81-89.
- Mei, R., Green, J.P., Sayre, R.T., Frasch, W.D. (1989) Manganese-binding proteins of the oxygen-evolving complex. *Biochemistry* 28:5560-5567.
- Meierhoff, K., Westhoff, P. (1993) Differential biogenesis of photosystem II in mesophyll and bundle-sheath cells of monocotyledonous NADP-malic enzyme-type C₄ plants: the non-stoichiometric abundance of the subunits of photosystem II in the bundle-sheath chloroplasts and the translational activity of the plastome-encoded genes. *Planta* 191:23-33.
- Meyer, S., Hung, S.P.N., Tremolieres, A., de Kouchkovsky, Y. (1992) Energy coupling, membrane lipids and structure of thylakoids of Lupin plants submitted to water stress. *Photosynthesis Research* 32:95-107.
- Michel, H., Dissenhofer, J. (1988) Relevance of the photosynthetic reaction center from purple bacteria to the structure of Photosystem II. *Biochemistry* 27:1-7.
- Mishra, N., Francke, C., van Gorkom, H.J., Ghanotakis, D.F. (1994) Destructive role of singlet oxygen during aerobic illumination of the Photosystem II core complex. *Biochimica et Biophysica* 1186:81-90.
- Miyao, M., Murata, N. (1983) Partial disintegration and reconstitution of the photosynthetic oxygen evolving system: binding of 24 kilodalton and 18 kilodalton polypeptides. *Biochimica et Biophysica* 725:87-93.
- Miyao, M., Murata, N. (1989) The mode of binding of three extrinsic proteins of 33 kDa, 23 kDa and 18 kDa in the photosystem II complex of spinach. *Biochimica et Biophysica* 977:315-321.

- Miyao, M., Murata, N., Laval, J., Maison-Peteri, B., Boussac, A., Etienne, A.-L. (1987) Effect of the 33-kDa protein on the S-state transitions in photosynthetic oxygen evolution. *Biochimica et Biophysica Acta* 890:151-159.
- Mizobuchi, A. and Yamamoto, Y. (1989) Assembly of photosystem II polypeptides and expression of oxygen evolution activity in the chloroplasts of *Euglena gracilis* Z during the dark-light transition. *Biochimica et Biophysica Acta* 977:26-32.
- Moran, J.F., Becana, M., Iturbe-Ormaetxe, I., Frechill, S., Klucas, R.V., Aparicio-Tejo, P. (1994) Drought induces oxidative stress in pea plants. *Planta* 194:346-352.
- Munson, A.D., Margolis, H.A., Brand, D.G. (1995) Seasonal nutrient dynamics in white pine and white spruce in response to environmental manipulation. *Tree Physiology* 15:141-149.
- Murota, K.-I., Ohshita, Y., Watanabe, A., Aso, S., Sato, F., Yamada, Y. (1994) Changes related to salt tolerance in thylakoid membranes of photoautotrophically cultured green tobacco cells. *Plant and Cell Physiology* 35:107-113.
- Muthuchelian, K., Nedunchezian, N., Kulandaivelu, G. (1994) Acid rain: acidic mist-induced response in growth and photosynthetic activities on crop plants. *Archives Environmental Contamination and Toxicology* 26:521-526.
- Nakai, M., Goto, A., Nohara, T., Sugita, D., Endo, T. (1994) Identification of the Sec A protein homolog in pea chloroplasts and its possible involvement in thylakoidal protein transport. *The Journal of Biological Chemistry* 269:31338-31341.
- Nedunchezian, N., Kulandaivelu, G. (1993) Increased stability of thylakoid components in *Vigna sinensis* L. seedlings grown under ultraviolet-B enhanced radiation. *Photosynthetica* 29:369-375.
- Ni, B.-R., Pallardy, S.G. (1992) Stomatal and nonstomatal limitations to net photosynthesis in seedlings of woody angiosperms. *Plant Physiology* 99:1502-1508.
- Nievelstein, V., Vanderkerckhove, J., Tadros, M.H., Lintig, J.V., Nitschke, W., Beyer, P. (1995) Carotene desaturation is linked to a respiratory redox pathway in *Narcissus pseudonarcissus* chromoplast membranes. Involvement of a 23-kDa oxygen-evolving-complex-like protein. *European Journal of Biochemistry* 233:864-872.
- Nixon, P.J., Dyer, T.A., Barber, J., Hunter, C.N. (1987) The production of monospecific antibodies to D1 and D2 polypeptides of photosystem II. In *Progress in Photosynthesis Research*, Vol III, Biggens J., editor, pp. 779-782, Dordrecht, Netherlands: Martinus Nijhoff Publishers.
- Nobel, P.S. (1983) *Biophysical Plant Physiology and Ecology*, p. 75, San Francisco: W.H. Freeman and Co..

- Nohara, T., Nakai, M., Goto, A., Endo, T. (1995) Isolation and characterization of the cDNA for pea chloroplast SecA - evolutionary conservation of the bacterial-type SecA-dependent protein transport within chloroplasts. *FEBS Letters* 364:305-308.
- Odom, W.R., Bricker, T.M. (1992) Interaction of CPa-1 with the manganese-stabilizing protein of photosystem II: identification of domains cross-linked by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. *Biochemistry* 31:5616-5620.
- Ogren, E., Rosenqvist, E. (1992) On the significance of photoinhibition of photosynthesis in the field and its generality among species. *Photosynthesis Research* 33:63-71.
- Ono, T.-a., Izawa, S., Inoue, Y. (1992) Structural and functional modulation of the manganese cluster in Ca^{2+} -depleted photosystem II induced by binding of the 24-kilodalton extrinsic protein. *Biochemistry* 31:7648-7655.
- Ono, T.-a., Noguchi, T., Nakajima, Y. (1995) Characteristic changes of function and structure of photosystem II during strong-light photoinhibition under aerobic conditions. *Biochimica et Biophysica Acta* 1229:239-248.
- Oquist, G. (1982) Seasonally induced changes in acyl lipids and fatty acids of chloroplast thylakoids of *Pinus silvestris*. *Plant Physiology* 69:869-875.
- Oquist, G., Anderson, J.M., McCaffery, S., Chow, N.S. (1992a) Mechanistic differences in photoinhibition of sun and shade plants. *Planta* 188:422-431.
- Oquist, G., Chow, W.S., Anderson, J.M. (1992b) Photoinhibition of photosynthesis represents a mechanism for the long-term regulation of photosystem II. *Planta* 186:450-460.
- Oquist, G., Chow, W.S. (1992) On the relationship between the quantum yield of photosystem II electron transport, as determined by chlorophyll fluorescence and the quantum yield of CO_2 -dependent O_2 evolution. *Photosynthesis Research* 33:51-62.
- Oquist, G., Huner, N.P.A. (1991) Effects of cold acclimation on the susceptibility of photosynthesis to photoinhibition in Scots pine and in winter and spring cereals: a fluorescence analysis. *Functional Ecology* 5:91-100.
- Oquist, G., Strand, M. (1986) Effects of frost hardening on photosynthetic quantum yield, chlorophyll organization, and energy distribution between the two photosystems in Scots pine. *Canadian Journal of Botany* 64:748-753.
- Orlander, G. (1993) Shading reduces both visible and invisible frost damage to Norway spruce seedlings in the field. *Forestry* 66:27-36.
- Oswald, A., Streubel, M., Ljungberg, U., Hermans, J., Eskins, K., Westhoff, P. (1990) Differential biogenesis of photosystem-II in mesophyll and bundle-sheath cells of 'malic' enzyme NADP^+ -type C_4 plants. *European Journal of Biochemistry* 190:185-194.

- Ottander, C., Oquist, G. (1991) Recovery of photosynthesis in winter-stressed Scots pine. *Plant, Cell and Environment* 14:345-349.
- Palomares, R., Herrmann, R.G., Oelmüller, R. (1993a) Post-transcriptional and post-translational regulatory steps are crucial in controlling the appearance and stability of thylakoid polypeptides during the transition of etiolated tobacco seedlings to white light. *European Journal of Biochemistry* 217:345-352.
- Palomares, R., Herrmann, R.G., Oelmüller, R. (1993b) Anti-sense RNA for components associated with the oxygen-evolving complex and the Rieske iron/sulfur protein of the tobacco thylakoid membrane suppresses accumulation of mRNA, but not of protein. *Planta* 190:305-312.
- Patterson, M.T., Rundel, P.W. (1995) Stand characteristics of ozone-stressed populations of *Pinus jeffreyi* (Pinaceae): extent, development, and physiological consequences of visible injury. *American Journal of Botany* 82:150-158.
- Philbrick, J.B., Diner, B.A., Zilinskas, B.A. (1995) Construction and characterization of cyanobacterial mutants lacking the manganese-stabilizing polypeptide of photosystem II. *The Journal of Biological Chemistry* 266:13370-13376.
- Pierre, M., Savoure, A. (1990) Effects of water-stress and SO₂ pollution on spruce endoproteases. *Plant Physiology and Biochemistry* 28:95-104.
- Polle, A., Rennenberg, H. (1992) Field studies on Norway spruce trees at high altitudes: II. Defense systems against oxidative stress in needles. *New Phytologist* 121:635-642.
- Powles, S.B. (1984) Photoinhibition of photosynthesis induced by visible light. *Annual Review of Plant Physiology* 35:15-44.
- Putnam-Evans, C., Bricker, T.M. (1992) Site-directed mutagenesis of the CPa-1 protein of photosystem II: alteration of the basic residue pair ^{384,385}R to ^{384,385}G leads to a defect associated with the oxygen-evolving complex. *Biochemistry* 31:11482-11488.
- Rashid, A., Camm, E.L. (1994) Activities and stability of native and Triton-X-100-fractionated thylakoid membranes from white spruce and spinach. *Photosynthetica* 31:389-398.
- Raval, M.K., Ramaswamy, N.K., Nair, P.M. (1994) Mechanism of Mn^{II} oxidation by the extrinsic 33 kDa protein of photosystem II. *Plant Science* 98:141-150.
- Renou, J.-L., Gerbaud, A., Just, D., Andre, M. (1990) Differing substomatal and chloroplastic CO₂ concentration in water-stressed wheat. *Planta* 182:415-419.
- Rintamäki, E., Salo, R., Aro, E.-M. (1994) Rapid turnover of the D1 reaction-center protein of photosystem II as a protection against photoinhibition in a moss, *Ceratodon purpureus* (Hedw.) Brid. *Planta* 193:520-529.

- Ritchie, G.A., Hinckley, T.M. (1975) The pressure chamber as a tool for ecological research. *Advances in Ecology Research* 19:165-254.
- Roberts, D.R., Kristie, D.N., Thompson, J.E., Dumbroff, E.B., Gepstein, S. (1991) In vitro evidence for the involvement of activated oxygen in light-induced aggregation of thylakoid proteins. *Physiologia Plantarum* 82:389-396.
- Roberts, D.R., Thompson, J.E., Dumbroff, E.B., Gepstein, S., Mattoo, A.K. (1987) Differential changes in the synthesis and steady-state levels of thylakoid proteins during bean leaf senescence. *Plant Molecular Biology* 9:343-353.
- Robertson, E.J., Baker, N.R., Leech, R.M. (1993) Chloroplast thylakoid protein changes induced by low growth temperature in maize revealed by immunocytology. *Plant, Cell and Environment* 16:809-818.
- Robertson, E.J., Leech, R.M. (1995) Significant changes in cell and chloroplast development in young wheat leaves (*Triticum aestivum* cv Hereward) grown in elevated CO₂. *Plant Physiology* 107:63-71.
- Robinson, C., Cai, D., Hulford, A., Brock, I.W., Michl, D., Hazell, L., Schmidt, I., Herrmann, R.G., Klosgen, R.B. (1994) The presequence of a chimeric construct dictates which of two mechanisms are utilized for translocation across the thylakoid membrane: evidence for the existence of two distinct translocation systems. *The EMBO Journal* 13:279-285.
- Robinson, C., Klosgen, R.B. (1994) Targeting of proteins into and across the thylakoid membrane - a multitude of mechanisms. *Plant Molecular Biology* 26:15-24.
- Rova, M., Franzen, L.-G., Fredriksson, P.-O., Strying, S. (1994) Photosystem II in a mutant of *Chlamydomonas reinhardtii* lacking the 23 kDa psbP protein shows increased sensitivity to photoinhibition in the absence of chloride. *Photosynthesis Research* 39:75-83.
- Russell, A.W., Critchley, C., Robinson, S.A., Franklin, L.A., Seaton, G.G.R., Chow, W.S., Anderson, J.A., Osmond, C.B. (1995) Photosystem II regulation and dynamics of the chloroplast D1 protein in *Arabidopsis* leaves during photosynthesis and photoinhibition. *Plant Physiology* 107:943-952.
- Ruth, B., Weisel, B. (1993) Investigation on the photosynthetic system of spruce affected by forest decline and ozone fumigation in closed chambers. *Environmental Pollution* 79:31-35.
- Ryrie, I.J., Young, S., Andersson, B. (1984) Development of the 33-, 23-, and 16-kDa polypeptides of the photosynthetic oxygen-evolving system during greening. *FEBS Letters* 177:269-273.
- Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) *Molecular Cloning: a laboratory manual*. 2nd Edition, pp. 1.80-1.82, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

- Saradadevi, K., Raghavendra, A.S. (1994) Inhibition of photosynthesis by osmotic stress in pea (*Pisum sativum*) mesophyll protoplasts is intensified by chilling or photoinhibitory light: intriguing responses of respiration. *Plant, Cell and Environment* 17:739-746.
- Schmitz, H., Löffel, U., Weidner, M. (1993) The rate of protein synthesis in needles of Norway spruce (*Picea abies*): light stimulation, regulation through photophosphorylation, stress enhancement. *Physiologia Plantarum* 87:237-247.
- Schnettger, B., Critchley, C., Santore, U.J., Graf, M., Krause, G.H. (1994) Relationship between photoinhibition of photosynthesis, D1 protein turnover and chloroplast structure: effects of protein synthesis inhibitors. *Plant, Cell and Environment* 17:55-64.
- Schreiber, U., U. Schliwa, W. Bilger. (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynthesis Research* 10:51-62.
- Schulte P.G., Hinckley, T.M. (1985) A comparison of pressure-volume curve data analysis techniques. *Journal of Experimental Botany* 36:1590-1602.
- Schuster, G., Timberg, R., Ohad, I. (1988) Turnover of thylakoid photosystem II proteins during photoinhibition of *Chlamydomonas reinhardtii*. *European Journal of Biochemistry* 177:403-410.
- Seaton, G.G.R., Walker, D.A. (1990) Chlorophyll fluorescence as a measure of photosynthetic carbon assimilation. *Proceedings of the Royal Society of London B* 242:29-35.
- Seiler, J.R., Cazell, B.H. (1990) Influence of water stress on the physiology of growth of red spruce seedlings. *Tree Physiology* 6:69-77.
- Sharkey, T.D. (1990) Water stress effects on photosynthesis. *Photosynthetica* 24:651.
- Sharkey, T.D., Seemann, J.R. (1989) Mild water stress effects on carbon-reduction-cycle intermediates, ribulose biphosphate carboxylase activity, and spatial homogeneity of photosynthesis in intact leaves. *Plant Physiology* 89:1060-1065.
- Sheen, J.-Y., Sayre, R., Bogorad, L. (1987) Differential expression of oxygen-evolving polypeptide genes in maize leaf cell types. *Plant Molecular Biology* 9:217-226.
- Shen, J.-R., Inoue, Y. (1991) Low pH-induced dissociation of three extrinsic proteins from O₂-evolving photosystem II. *Plant and Cell Physiology* 32:453-457.
- Shen, J.-R., Inoue, Y. (1993) Binding and functional properties of two extrinsic components, cytochrome *c*-550 and a 12 kDa protein, in cyanobacterial photosystem II. *Biochemistry* 32:1825-1832.
- Shen, J.-R., Terashima, I., Katoh, S. (1990) Cause for dark, chilling-induced inactivation of photosynthetic oxygen-evolving system in cucumber leaves. *Plant Physiology* 93:1354-1357.

- Shen, J.-R., Vermaas, W., Inoue, Y. (1995) The role of cytochrome *c*-550 as studied through reverse genetics and mutant characterization in *Synechocystis* sp. PCC 6803. *The Journal of Biological Chemistry* 270:6901-6907.
- Shinohara, K., Murakami, A., Fujita, Y. (1992a) Biochemical characteristics of thylakoid membranes in chloroplasts of dark-grown pine cotyledons. *Plant Physiology* 98:39-43.
- Shinohara, K., Ono, T., Inoue, Y. (1992b) Photoactivation of oxygen-evolving enzyme in dark-grown pine cotyledons: relationship between assembly of photosystem II proteins and integration of manganese and calcium. *Plant and Cell Physiology* 33:281-289.
- Smith, B.M., Morrissey, P.J., Guenther, J.E., Nemson, J.A., Harrison, M.A., Allen, J.F., Melis, A. (1990) Response of the photosynthetic apparatus in *Dunaliella salina* (green algae) to irradiance stress. *Plant Physiology* 93:1433-1440.
- Soitamo, A.J., Zhou, G., Clarke, A.K., Oquist, G., Aro, E.-M., Gustafsson, P. (1994) Overproduction of the D1 protein of photosystem II reaction centre in the cyanobacterium *Synechococcus* sp. PCC 7942. *Plant Molecular Biology* 26:709-721.
- Staehelin, L.A. (1976) Reversible particle movements associated with unstacking and restacking of chloroplast membranes in vitro. *Journal of Cell Biology* 71:136-156.
- Stewart, J.D., Zine El Abidine, A., Bernier, P.Y. (1995) Stomatal and mesophyll limitations of photosynthesis in black spruce seedlings during multiple cycles of drought. *Tree Physiology* 15:57-64.
- Strand, M., Lundmark, T. (1987) Effects of low night temperature and light on chlorophyll fluorescence of field-grown seedlings of Scots pine (*Pinus sylvestris* L.). *Tree Physiology* 3:211-224.
- Strand, M., Lundmark, T. (1995) Recovery of photosynthesis in 1-year-old needles of unfertilized and fertilized Norway spruce (*Picea abies* (L.) Karst.) during spring. *Tree Physiology* 15:151-158.
- Strand, M., Oquist, G. (1988) Effects of frost hardening, dehardening and freezing stress on *in vivo* chlorophyll fluorescence of seedling of Scots pine (*Pinus sylvestris* L.). *Plant, Cell and Environment* 11:211-218.
- Streb, P., Michael-Knauf, A., Feirabend, J. (1993) Preferential photoinactivation of catalase and photoinhibition of photosystem II are common early symptoms under various osmotic and chemical stress conditions. *Physiologia Plantarum* 88:590-598.
- Stuhlfauth, T., Scheuermann, R., Fock, H.P. (1990) Light energy dissipation under water stress conditions - Contribution of reassimilation and evidence for additional processes. *Plant Physiology* 92:1053-1061.

- Stuhlfauth, T., Sultemeyer, D.F., Weinz, S., Fock, H.P. (1988) Fluorescence quenching and gas exchange in a water stressed C₃ plant, *Digitalis lanata*. *Plant Physiology* 86:246-250.
- Sundari, D.S., Raghavendra, A.S. (1990) Sensitivity of photosynthesis by spinach chloroplast membranes to osmotic stress *in vitro*: rapid inhibition of O₂ evolution in presence of magnesium. *Photosynthesis Research* 23:325-330.
- Sundby, C., McCaffery, S., Anderson, J.M. (1993) Turnover of the photosystem II D1 protein in higher plants under photoinhibitory and nonphotoinhibitory irradiance. *The Journal of Biological Chemistry* 268:25476-25482.
- Takahashi, H., Ehara, Y. (1992) Changes in the activity and the polypeptide composition of the oxygen-evolving complex in photosystem II of tobacco leaves infected with cucumber mosaic virus strain Y. *Molecular Plant-Microbe Interactions* 5:269-272.
- Takeuchi, T.S., Thornber, J.P. (1994) Heat-induced alterations in thylakoid membrane protein composition in barley. *Australian Journal of Plant Physiology* 21:759-770.
- Tamura, N., Cheniae, G.M. (1988) Photoactivation of the water oxidizing complex: the mechanism and general consequences to photosystem 2. In *Light-Energy Transduction in Photosynthesis: Higher Plant and Bacterial Models*, Stevens, Jr., S.E., Bryant, D.A., editors, pp. 227-242, Rockville, Maryland: The American Society of Plant Physiologists.
- Tan, W., Blake, T.J., Boyle, T.J.B. (1992) Drought tolerance in faster- and slower-growing black spruce (*Picea mariana*) progenies: II. Osmotic adjustment and changes of soluble carbohydrates and amino acids under osmotic stress. *Physiologia Plantarum* 85:645-651.
- Tanaka, S., Wada, K. (1988) The status of cysteine residues in the extrinsic 33 kDa protein of spinach photosystem II complexes. *Photosynthesis Research* 17:255-266.
- Tandy, N.E., De Giulio, R.T., Richardson, C.J. (1989) Assay and electrophoresis of superoxide dismutase from red spruce (*Picea rubens* Sarg.), loblolly pine (*Pinus taeda* L.) and scotch pine (*Pinus sylvestris* L.) - a method from biomonitoring. *Plant Physiology* 90:742-748.
- Tanimura, S., Kobayashi, M., Terashita, N., Takahashi, M. (1994) Sedimentation analysis of the state of aggregation of the oxygen-evolving photosystem II reaction center core complex and the extrinsic proteins. *Bioscience, Biotechnology and Biochemistry* 58:2172-2177.
- Temple, P.J., Riechers, G.H. (1995) Nitrogen allocation in ponderosa pine seedlings exposed to interacting ozone and drought stresses. *New Phytologist* 130:97-104.
- Teskey, R.O., Fites, J.A., Samuelson, L.J., Bongarten, B.C. (1986) Stomatal and nonstomatal limitations to photosynthesis in *Pinus taeda* L. under different environmental conditions. *Tree Physiology* 2:131-142.
- Theg, S.M., Filar, L.J., Dilley, R.A. (1986) Photoinactivation of chloroplasts already inhibited on the oxidizing side of photosystem II. *Biochimica et Biophysica Acta* 849:104-111.

- Thompson, L.K., Miller, A.-F., Buser, C.A., de Paula, J.C., Brudvig, G.W. (1989) Characterization of the multiple forms of cytochrome *b₅₅₉* in photosystem II. *Biochemistry* 28:8048-8056.
- Tiller, K., Link, G. (1993) Phosphorylation and dephosphorylation affect functional characteristics of chloroplast and etioplast transcription systems from mustard (*Sinapis alba* L.). *The EMBO Journal* 12:1745-1753.
- Toivonen, P., Vidaver, W. (1988) Variable chlorophyll fluorescence and carbon dioxide uptake in water-stressed white spruce seedlings. *Plant Physiology* 86:744-748.
- Towbin, H., Staehelin, T., Gordo, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proceedings of the National Academy of Science USA* 76:4350-4354.
- Tsel'niker, Y., Chetverikov, A.G. (1988) Dynamics of chlorophyll content and amounts of reaction centres of photosystems 1 and 2 in *Pinus silvestris* L. and *Picea abies* Karst. needles during a year. *Photosynthetica* 22:483-490.
- Valentini, R., Epron, D., De Angelis, P., Matteucci, G., Dreyer, E. (1995) *In situ* estimation of net CO₂ assimilation, photosynthetic electron flow and photorespiration in Turkey oak (*Q. cerris* L.) leaves: diurnal cycles under different levels of water supply. *Plant, Cell and Environment* 18:631-640.
- Valluri, J.V., Castillon, J., Newton, R.J., Soltes, E.J. (1989) Water stress-induced changes in protein synthesis of slash pine hypocotyls. *Journal of Plant Physiology* 135:355-360.
- Valluri, J.V., Treat, W.J., Newton, R.J., Cobb, B.G., Soltes, E.J. (1988) Protein synthesis in slash pine callus cultures exposed to water stress. *Tree Physiology* 4:181-186.
- van Leeuwen, P.J., Nieveen, M.C., van de Meent, E.J., Dekker, J.P., van Gorkom, H.J. (1991) Rapid and simple isolation of pure photosystem II core and reaction center particles from spinach. *Photosynthesis Research* 28:149-153.
- van Kooten, O., Snel, J.F.H. (1990) the use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynthesis Research* 25:147-150.
- van Wijk, K.J., van Hasselt, P.R. (1993) Photoinhibition of photosystem II *in vivo* is preceded by down-regulation through light-induced acidification of the lumen: consequences for the mechanism of photoinhibition *in vivo*. *Planta* 189:359-368.
- Vance, N.C., Zaerr, J.B. (1991) Influence of drought stress and low irradiance on plant water relations and structural constituents in needles of *Pinus ponderosa* seedlings. *Tree Physiology* 8:175-184.

- Vasilikiotis, C., Melis, A. (1994) Photosystem II reaction center damage and repair cycle: chloroplast acclimation strategy to irradiance stress. *Proceedings of the National Academy Science USA* 91:7222-7226.
- Vasilikiotis, C., Melis, A. (1995) The role of chloroplast-encoded protein biosynthesis on the rate of D1 protein degradation in *Dunaliella salina*. *Photosynthesis Research* 45:147-155.
- Virgin, I., Hundal, T., Strying, S., Andersson, B. (1990) Consequences of light induced D1-protein degradation on thylakoid membrane organization. In *Current Research in Photosynthesis* Vol. II, Baltscheffsky, M., editor, pp 423-426, the Netherlands: Kluwer Academic Publishers.
- Virgin, I., Strying, S., Andersson, B. (1988) Photosystem II disorganization and manganese release after photoinhibition of isolated spinach thylakoid membranes. *FEBS Letters* 233:408-412.
- Walker, D. (1987) *The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis*, pp 10-11, University of Sheffield, Sheffield, United Kingdom: Oxygraphics Ltd.
- Wang, W.-Q., Chapman, D.J., Barber, J. (1992a) Effect of cold treatments on the binding stability of photosystem II extrinsic proteins and an associated increase in susceptibility to photoinhibition. *Plant Physiology* 99:21-25.
- Wang, W.-Q., Chapman, D.J., Barber, J. (1992b) Inhibition of water splitting increases the susceptibility of photosystem II to photoinhibition. *Plant Physiology* 99:16-20.
- Watts, W.R., R.E. Neilson. 1978. Photosynthesis in Sitka spruce (*Picea sitchensis* (Bong.) Carr.) VIII. Measurements of stomatal conductance and $^{14}\text{CO}_2$ uptake in controlled environments. *Journal of Applied Ecology* 15:245-255.
- Webb, D.T., Webster, F., Flinn, B.C., Roberts, D.R., Ellis, D.D. (1989) Factors influencing the induction of embryogenic and caulogenic callus from embryos of *Picea glauca* and *P. engelmannii*. *Canadian Journal of Forest Research* 19:1303-1308.
- Wedler, M., Weikert, R.M., Lippert, M. (1995) Photosynthetic performance, chloroplast pigments and mineral content of Norway spruce (*Picea abies* (L.) Karst.) exposed to SO_2 and O_3 in an open-air fumigation experiment. *Plant, Cell and Environment* 18:263-276.
- Westhoff, P., Jansson, C., Klein-Hitpass, L., Berzborn, R., Larsson, C., Bartlett, S.G. (1985) Intracellular coding site of polypeptides associated with photosynthetic oxygen evolution of photosystem II. *Plant Molecular Biology* 4:137-146.
- White, E.E. (1986) A method for extraction of chloroplast DNA from conifers. *Plant Molecular Biology Reporter* 4:98-101.

- Wild, A. Flammersfeld, U., Moors, I., Dietz, B., Ruhle, W. (1988) Investigation on the photosynthetic membranes of spruce needles in relation to the occurrence of novel forest decline II. The content of Q_B-protein, cytochrome *f*, and P-700. *Zeitschrift fur Naturforschung* 43c:589-595.
- Wilkinson, L. (1990) *Sygraph: The System for Graphics*. Evanston, Illinois: SYSTAT, Inc.
- Xu, Q., Bricker, T.M. (1992) Structural organization of proteins on the oxidizing side of photosystem II. *The Journal of Biological Chemistry* 267:25816-25821.
- Xu, Q., Nelson, J., Bricker, T.M. (1994) Secondary structure of the 33 kDa, extrinsic protein of photosystem II: a far-UV circular dichroism study. *Biochimica et Biophysica Acta* 1188:427-431.
- Yachandra, V.K., DeRose, V.J., Latimer, M.J., Mukerji, I., Sauer, K., Klein, M.P. (1993) Where plants make oxygen: a structural model for the photosynthetic oxygen-evolving manganese cluster. *Science* 260:675-679.
- Yamamoto, N., Mukai, M., Kano-Murakami, Y., Ohashi, Y., Tanaka, Y., Ozeki, Y., Odani, K. (1991) Light-independent expression of *cab* and *rbcS* genes in dark-grown pine seedlings. *Plant Physiology* 95:379-383.
- Yamamoto, Y., Akasaka, T. (1995) Degradation of the antenna chlorophyll-binding protein CP43 during photoinhibition of photosystem II. *Biochemistry* 34:9038-9045.
- Yamamoto, Y., Kubota, F. (1987) Specific release of the extrinsic 18 kDa protein from spinach photosystem-II particles by the treatment with NaCl and methanol and its application for large-scale purification of the three extrinsic proteins of photosystem II without chromatography. *Biochimica et Biophysica Acta* 893:579-583.
- Zhang, L., Wang, J., Liang, H. (1995) Investigation of Ca²⁺-binding sites on the 33 kDa protein in photosystem 2 using Tb³⁺ as a fluorescence probe. *Photosynthetica* 31:203-208.
- Zhou, C., Yang, Y., Jong, A.Y. (1990) Mini-prep in ten minutes. *BioTechniques* 8:172-173.
- Zwiazek, J.J. (1991) Cell wall changes in white spruce (*Picea glauca*) needles subjected to repeated drought stress. *Physiologia Plantarum* 82:513-518.
- Zwiazek, J.J., Blake, T.J. (1989) Effects of preconditioning on subsequent water relations, stomatal sensitivity, and photosynthesis in osmotically stressed black spruce. *Canadian Journal of Botany* 67:2240-2244.