THE DEVELOPMENT OF CHLOROPHYLL A FLUORESCENCE TECHNIQUES AND THEIR APPLICATION TO PROBLEMS IN CONIFER SEEDLING PRODUCTION

by

Kenneth P. Murphy

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APPROVAL

Name: KENNETH MURPHY

Degree: Master of Science

Title of Thesis:

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Examinining Committee:

Chairman: Dr. R.C. Brooke, Associate Professor

Dr. V.G.R. Lister, Assistant Professor, Senior Supervisor, Dept. of Biological Sciences, SFU,

Dr. W.E. Vidaver, Professor, Dept. of Biological Sciences, SFU

Dr. C.D. Hawkins, Research Physiologist, MacMillan Bloedel Ltd., Nanaimo, B.C., Public Examiner

Date Approved Aug. 10th 1990.
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Title of Thesis/Project/Extended Essay

The Development of Chlorophyll A Fluorescence Techniques and Their Application to Woodlean Fir Conifer Seedling Production

Author:

(Kenneth P. Murphy)

(date)

9 August 1990

(signature)
Abstract

A fluorometer for measuring chlorophyll a fluorescence in conifer seedlings was constructed and tested. It was subsequently used as an adjunct to established methods of seedling testing in two contrasting applied problems of nursery conifer seedlings.

The development phase included hardware, and associated software for controlling the fluorometer and acquiring the fluorescence data. Secondary software was also created for, management, analysis and presentation of the data.

Following the testing of the apparatus to ensure the viability of the data and to establish satisfactory protocols, $F_O$ (initial fluorescence) was investigated to test its suitability as a basis for the normalization of corrected variable fluorescence ($F_{var}$) data which, after normalization, is termed $F_v$. The results showed that $F_O$ has the necessary characteristics to act as a basis for representing $F_v$ data, and also showed that $F_O$ has some additional advantageous attributes. These may make $F_O$ a better basis than dry weight or surface area, for the representation of apparent photosynthetic rates and perhaps other physiological values as well.
The fluorometer was then used to test the effects of atmospheric pollution and water stress in nursery grown conifers in two separate trials. Ambient levels of ozone pollution were found to have no significant effect on the seedlings. Water stress was detected using the fluorometer system but fluorescence was not found to be significantly better than less sophisticated methods, at detecting water stress in white spruce seedlings. Advantages of $F_0$ are detailed for use in practical applications and especially as a rapid non-destructive estimate of plant size.
Dedication

To Miss Tippet
Acknowledgements

My thanks to Dr. Geoff Lister who has helped me throughout the course of this work, with both biological and technical aspects of chlorophyll a fluorescence. I would also like to extend my appreciation for his more than generous support during the writing of this manuscript.

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<tr>
<td>A/D board</td>
<td>Analogue to digital converter board.</td>
</tr>
<tr>
<td>a.c.</td>
<td>Alternating Current.</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate.</td>
</tr>
<tr>
<td>APS</td>
<td>Apparent photosynthesis.</td>
</tr>
<tr>
<td>ASCII</td>
<td>American Standard Code for Information Interchange.</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate.</td>
</tr>
<tr>
<td>B.C.</td>
<td>British Columbia.</td>
</tr>
<tr>
<td>B.C.M.O.F.</td>
<td>British Columbia Ministry Of Forests.</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide.</td>
</tr>
<tr>
<td>d.c.</td>
<td>Direct Current.</td>
</tr>
<tr>
<td>F_D</td>
<td>Intermediary dip in fluorescence transient $F_0$ to $F_P$.</td>
</tr>
<tr>
<td>F_M</td>
<td>Secondary peak in variable fluorescence.</td>
</tr>
<tr>
<td>F_I</td>
<td>Intermediary point of fluorescence transient $F_0$ to $F_P$.</td>
</tr>
<tr>
<td>F_O</td>
<td>Initial fluorescence.</td>
</tr>
<tr>
<td>F_P</td>
<td>Initial peak of variable fluorescence.</td>
</tr>
<tr>
<td>F_S</td>
<td>Quasi steady state inflection minimum occurring after $F_P$.</td>
</tr>
<tr>
<td>F_T</td>
<td>Steady state fluorescence.</td>
</tr>
<tr>
<td>F_v</td>
<td>Corrected normalized variable fluorescence.</td>
</tr>
<tr>
<td>F_var</td>
<td>Corrected variable fluorescence.</td>
</tr>
<tr>
<td>IRGA</td>
<td>Infra Red Gas Analyzer.</td>
</tr>
<tr>
<td>LHC</td>
<td>Light-harvesting Complex.</td>
</tr>
<tr>
<td>LSD</td>
<td>Least Significant Difference.</td>
</tr>
<tr>
<td>N</td>
<td>Replicate number.</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate.</td>
</tr>
<tr>
<td>NSR</td>
<td>Not Satisfactorily Restocked.</td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically Active Radiation (400nm – 700nm wave-band).</td>
</tr>
<tr>
<td>P680</td>
<td>Photosystem II reaction centre.</td>
</tr>
<tr>
<td>P700</td>
<td>Photosystem I reaction centre.</td>
</tr>
<tr>
<td>PS I</td>
<td>Photosystem one.</td>
</tr>
<tr>
<td>PS II</td>
<td>Photosystem two.</td>
</tr>
<tr>
<td>QA</td>
<td>Primary quinone electron acceptor of PS II.</td>
</tr>
<tr>
<td>Q_B</td>
<td>Secondary quinone electron acceptor of PS II.</td>
</tr>
<tr>
<td>Q_e</td>
<td>Energy dependant fluorescence quenching.</td>
</tr>
<tr>
<td>Q_g</td>
<td>Photochemical fluorescence quenching.</td>
</tr>
<tr>
<td>R fd</td>
<td>Fluorescence decrease ratio.</td>
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<tr>
<td>Rd</td>
<td>Dark respiration.</td>
</tr>
<tr>
<td>R.G.C.</td>
<td>Root Growth Capacity.</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis System.</td>
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<td>S.F.U.</td>
<td>Simon Fraser University.</td>
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Chapter One

Introduction

Can chlorophyll a fluorescence measurements be used as a physiological assay of conifer seedlings to enable improvement in the success of their production and subsequent outplanting? This question, which grew out of research done previously at Simon Fraser University (S.F.U.) (Wiltens 1975, Fink 1978, Hawkins 1981, Toivonen 1986), formed the basis of my thesis, and the work described here is divided into three parts:

1) Development of a fluorometer system.
2) Devising a protocol for using the fluorometer system.
3) Application of the system and techniques developed in 1) and 2) to practical problems of conifer seedling physiology and operational nursery production.

This chapter will detail the two basic themes of the thesis: chlorophyll a fluorescence, and the production of quality conifer seedlings in British Columbia.
Chlorophyll a fluorescence

Chlorophyll a fluorescence is the name given to light which is promptly emitted from photosynthetically active tissue during illumination with light of certain wavelengths. This activating light is termed photosynthetically active radiation (PAR, waveband 400nm to 700nm) and is approximately equivalent to the visible light range (Larcher 1980). Light which is incident on plants has one of three possible fates: to be absorbed, to be reflected, or to be transmitted (Larcher 1980). The physical configuration of the tissue and the wavelength of the photon determines which event will take place. Only light absorbed by photosynthetic pigments is important as a source of chlorophyll a fluorescence (Papageorgiou 1975). Light emitted by plants, again has various possible fates, for example: to be reabsorbed and degraded to heat in radiationless de-excitation, to be reabsorbed and utilized in photochemistry, or to be re-emitted as even longer wavelength photons (Nobel 1983). Chlorophyll a fluorescence only dissipates a relatively small portion (≈ 4%) of the energy coming from light absorbed by the photosynthetic pigments.

Perhaps the first person to see chlorophyll a fluorescence was N.J.C. Muller who in the latter part of the Nineteenth century observed the phenomenon by eye using tinted glasses.
(Schreiber 1983). It is Kautsky however, who is now best remembered as the pioneer of chlorophyll \textit{a} fluorescence with his work beginning in the early 1930's which led to the description of fluorescence induction. Fluorescence induction is the characteristic rise in fluorescence intensity when a plant is reilluminated after a period in darkness, and the graph of this change in fluorescence intensity plotted against time is known as a Kautsky curve (Fig 1.1a). The intensity of fluorescence emission varies during fluorescence induction and therefore this phenomenon was called variable chlorophyll \textit{a} fluorescence.

In conifers there are many photosynthetic pigments; chlorophylls, pheophytins, and carotenoids, which absorb light in the PAR waveband, and these make up the light-harvesting complexes (LHC) (Danks \textit{et al.} 1983). The efficiency with which light is absorbed depends on the particular pigment and the wavelength of the light; however the physical process by which light energy is captured is the same for all pigments. When a photon of PAR interacts with a pigment, an electron in a chromophore (a group of atoms in a pigment molecule which absorb light energy), is moved into an excited state (Lawlor 1987). The greater the energy of the photon the higher the excited state an electron will achieve when the photon is absorbed. Decay from the higher excited states to the first excited singlet state occurs only by the radiationless loss of energy to
heat. Some of the excitation energy from the first excited singlet state is transferred, either by rapid excitation transfer or inductive resonance, between pigment molecules until the energy reaches a special chlorophyll $a$ molecule at the reaction centre. There are two types of reaction centres "P680" [linked to photosystem II (PS II)] and "P700" [linked to photosystem I (PS I)] which have peak absorbances of 680nm and 700nm respectively. Up to 20% of the energy absorbed by the pigment molecule array attached to a reaction centre is channelled into the photosystems, independent of which type of pigment originally captured the energy (Kok et al. 1967).

Energy reaching a reaction centre is utilized to transfer an electron from the reaction centre chlorophyll to either pheophytin and then the quinone primary electron acceptor ($Q_A$) in PS II, or ferredoxin in PS I (Govindjee and Coleman 1990); this is the process of charge separation. Once the reaction centre has expelled an electron to the primary acceptor using energy from the antennae pigments, it has to be rereduced before it can accept more energy and emit another electron (Nobel 1983). Charge separation using energy from light is a very important process in the biosphere (Danks et al. 1983). The photosystems harness the energy of charge separation to produce carbohydrates and the two photosystems are linked together to capture the energy required (Lawlor 1987). The fate of the energy after being
transferred from a reaction centre, in the form of an excited state electron, depends on which photosystem is being considered, and the discussion here will be mainly limited to PS II which is the major source of chlorophyll a fluorescence (Bonventura and Myers 1969, and Rabinowitch and Govindjee 1969).

The primary electron acceptor, pheophytin, passes the energy with the electron to the quinone $Q_A$ and subsequently to the secondary quinone electron acceptor ($Q_B$). Once $Q_B$ has accepted two electrons it can then diffuse into the plastoquinone pool (Nobel 1983). The electrons pass down the electron transport chain to $P_700$ and are re-excited with more energy from light collected by the photosynthetic pigments linked to PS I. Eventually they are sequentially transferred to NADP (nicotinamide adenine dinucleotide phosphate) and used in the carbon reduction cycle (Calvin cycle) to reduce carbon dioxide ($CO_2$) (Rabinowitch and Govindjee 1969).

This process of absorbing light energy and using it to move electrons from water is optimal for green plants. However, if the amount of light energy absorbed is more than the photosystems can cope with damage may occur. This can be prevented or reduced by several processes which include non radiative dissipation of energy as heat, carbon assimilation, passing electrons to oxygen in PS I, and
chlorophyll a fluorescence (Krause 1988 and Nobel 1983). The advantage of there being more than one method for dissipating excess energy is that one method alone would be more easily overwhelmed.

Fluorescence occurs: (1) when the primary acceptor is already reduced and the P680 reaction centre is energized from the antennae pigments. No electrons can be transferred to QA until it has been reoxidized, and energy arriving at the reaction centre must be dissipated in some other way. This situation is not uncommon because the reoxidization of QA takes longer than the transfer of excited electrons to it (Nobel 1983). Therefore the processes which underlie variable fluorescence begin after light energy has been absorbed and transferred to P680, and the factor determining the variable fluorescence intensity is the redox state of QA (Renger and Schulze 1985). (2) at the antennae pigments, where chromophores can release a photon instead of undergoing radiationless decay, or passing energy to other pigment molecules. In both cases the fluorescence acts (on a small scale) as a control mechanism for light energy throughput, and is in the form of long wavelength photons (usually greater than approximately 675nm) because of Stokes' or red shift.

Fluorescence emission is a dynamic process, common to all photosynthetic organisms, and factors which affect the
photosynthetic systems may cause changes (manifested almost immediately) in fluorescence emission. This, combined with the non-destructive nature of ambient temperature chlorophyll a fluorescence, makes fluorescence induction a valuable tool in plant physiology (Lichtenthaler et al. 1986). Fluorescence induction is however only one of several types of chlorophyll a fluorescence measurements. Other measurements include chlorophyll a fluorescence spectrum analysis, which is used by some researchers as a means to indicate plant stress. An index can be derived from the ratio of fluorescence intensity at two different wavelengths 690nm and 735nm which indicates changes in chlorophyll content of tissue. This ratio is used for detecting changing chlorophyll content which can result from stresses such as drought, mechanical injury, or insufficient mineral nutrient uptake (Lichtenthaler and Rinderle 1988).

Measurements of energy-dependent fluorescence quenching (Qe), and photochemical fluorescence quenching (Qq) are also used extensively (Schreiber et al. 1986). Qe represents the lowering of fluorescence intensity due to proton accumulation within the lumen, and the value Qq is a measure of the relative extent of QA reduction. These values for photochemical and non-photochemical fluorescence quenching give an indication of the efficiency of the electron transfer system whilst it is functioning. Low temperature and nanosecond time-scale measurements are also used, however this thesis is only concerned with induction
kinetics, which is one of the more common measurements of fluorescence.

Examples of Kautsky curves are presented in figures 1.1a and 1.1b, which show the same data plotted on linear and logarithmic time scales. Logarithmic time-scale representation of fluorescence induction data was used extensively during the experiments described in chapters 3, 4, and 5, and has the advantage of allowing comparison of the rapid initial phase with the slower changes which occur subsequently.

To facilitate description of these types of curve a nomenclature has come into common usage which consists of letter names given to the common features (Papageorgiou 1975). Figures 1.1a and 1.1b, show actual data and are presented to accompany the following guide to the features of Kautsky curves; note that all of the features mentioned will not necessarily be present in every Kautsky curve.

$F_0$ is the first feature of the Kautsky curve and occurs directly after illumination of dark pre-treated tissue (Govindjee et al. 1990). In the fluorometer used during this work $F_0$ occurs immediately after the shutter becomes fully open (see chapter 2). $F_0$ is traditionally taken to represent "the fluorescence level at a physiological state in which all the intersystem
Figure 1.1a

An example of a Kautsky curve, fluorescence intensity during induction plotted against time for a white spruce seedling. The nomenclature of the features is modified from Papageorgiou (1975), see text for details.
Figure 1.1b

Fluorescence induction data (as presented in figure 1.1a) plotted against the logarithm of time. This system of representation has some advantages over the Fig. 1.1a linear scale plot. Using this new technique the F₀ plateau is clearly visible as are other features of the curve such as F₁ and Fₚ. Thus the early features of chlorophyll a fluorescence induction can be seen on the same plot as the later features. Note that the shutter opening and rebound features are also visible. As with figure 1.1a, the nomenclature is modified from Papageorgiou (1975), see text for details.
intermediates are oxidized" and was thought to be a "constant yield component" (Papageorgiou 1975) i.e. not affected by physiochemical processes taking place in the thylakoid membrane. Recent advances in measuring systems have shown however that $F_0$ is not a constant value in the way that it was previously considered (Fraser et al. 1987, and Krause 1988), and it is now referred to variously as initial, ground, dead, or instantaneous fluorescence (Lichtenthaler and Rinderle 1988, and Bjorkman and Powles 1984). $F_0$ has been used as an estimate of the quantity of active photosynthetic pigment (Toivonen 1986). All the induction curve features following $F_0$ are commonly referred to, en masse, as variable fluorescence ($Fv$).

$F_I$ and $F_D$ These two adjacent features occur soon after $F_0$ (within 500ms of illumination), $F_D$ being a 'dip' or change in the rate of the fast fluorescence rise between $F_0$ and $F_p$. $F_I$ is controlled by the rate of charge separation in PS II, and $F_D$ is dependent on the equilibrium between the two electron acceptors $Q_A$ and $Q_B$, in the inter-system electron transport chain. These two features ($F_I$ and $F_D$) were not quantitatively analyzed during the course of this work and therefore will not be discussed in detail.

$F_p$ and $F_S$ $F_p$ is the first maximum (or peak), and is caused by the reduction of the primary electron acceptor $Q_A$. $F_S$ is a minimum which is reached after a decline in variable
fluorescence from \( F_p \), and is due to the reoxidation of \( Q_A \) from the PS I side of PS II.

\( F_M \) is a tertiary maximum which succeeds \( F_p \) in some induction measurements especially if low actinic light is used or if the tissue has not become fully dark pre-treated. There may be more than one maximum subsequent to \( F_p \) and these are often termed \( F_{M1} \), \( F_{M2} \) etc. They are caused by a reduction in the trans-thylakoid proton gradient because of increased availability of ADP (Adenosine diphosphate) which leads to a decline in \( Q_e \) (non-photochemical) quenching. Increased availability of NADP, and accelerated electron transport leads to increases in both \( Q_q \) and \( Q_e \) quenching causing \( F_M \) to decline again (Walker 1988).

\( F_T \) is the steady state fluorescence, reached after a period of minutes, when dynamic equilibrium has become established in the photosystems. This steady state will continue while conditions remain constant.

In many chlorophyll fluorometers a specific small area of the leaf is illuminated with a known quantity of light and the resulting fluorescence induction curves from different samples are then readily comparable because the results are based on a per unit tissue area basis (Bolhar-Nordenkampf et al. 1989). Because of their narrow leaf shapes and three dimensional cross sections, many conifer species do not lend
themselves to this type of system. To circumvent this problem an integrating sphere is used (initially developed by Toivonen (1986)) see chapter 2, which provides relatively uniform light distribution to all tissue surfaces. Fluorescence is also reflected by the interior surface of the sphere into a detector. This system however does not necessarily assay a consistent area of tissue and thus the normalizing basis is lost. \( F_0 \) is therefore used as a substitute because tissue area is difficult to measure non-destructively in needle leafed conifers. Underlying factors connected with this approach are discussed in some of the later chapters.

This introduction does not attempt to present great detail of the processes which underlie chlorophyll a fluorescence, reference should be made to one or more of the following for a more detailed account if required (Papageorgiou 1975, Schreiber and Bilger 1987, Lichtenthaler and Rinderle 1988, Bolhar-Nordenkampf et al. 1989, and Barber et al. 1989).

Seedling Production

Concern about the rate of successful regeneration of forest stands in British Columbia compared to the rate of logging
has led to increasingly large numbers of seedlings being planted each year in an attempt to reduce the problem of the N.S.R. (not satisfactorily restocked) backlog. Clear cutting is the most common type of logging system used in British Columbia (B.C.), and one of the most successful ways to restock areas which have been clear cut is direct replanting with a successionaly dominant species, such as was harvested. This is not a perfect solution because late successional species are often not as vigorous in open conditions compared to other less desirable species. However such replanting is effective in reducing the time of regeneration and so there is a large demand for seedlings and particularly for species such as white spruce (Picea glauca [Moench] Voss) and interior Douglas-fir (Pseudotsuga menziesii [Mirb.] Franco). To keep up with the demand for seedlings [which is approximately 240 million per year (Willingdon pers. comm. 1990)], many seedlings are grown as plugs in styroblocks which facilitate mechanization and handling (a typical type 313 block holds 160 seedlings). Despite the sophistication in some nursery practices, methods to assay for seedling quality are quite limited both in the nursery and post storage. Thus little is known about the overall vigor of the seedlings which makes nursery practices difficult to assess. In turn, predictions about outplant success rates are also more difficult, and there is little detailed information available to help foresters with planting strategy, or long term management decisions.
The most commonly used stock quality tests (excluding morphological measurements) subject seedlings to predetermined environmental conditions (which may or may not be stressful), and use various means to assess the response of the seedlings after a predetermined interval. Two such tests are root growth capacity (R.G.C.) (or root growth potential, R.G.P.), and -18°C cold hardiness (storability) test. There are also other less common tests such as, foliar composition analysis and hormone analysis. However even R.G.C. which has been studied by many researchers and is one of the most common stock quality tests, is considered to be insufficient on its own as an indicator of stock quality (Binder et al. 1988). This highlights the problem of individual tests having limited scope, which is in some ways akin to a physician deciding, on the results of a blood pressure test, whether a patient is healthy or not. In other words, despite the rationale underlying any single test, it is probably not wide ranging enough to comprehensively estimate overall seedling quality. The link between chlorophyll a fluorescence and physiological status gives this technique great potential for supplementing current quality assessment methods. The major potential benefit is the ability of chlorophyll a fluorescence to indicate problems in the photosynthetic systems or in other physiological processes which indirectly effect photosynthesis, to do this non-destructively, and before
they are noticeable in other parameters. However the research done in applying chlorophyll a fluorescence technology to conifer seedlings is sparse, and there are both practical problems and physiological questions remaining to be answered before full benefit can be reaped from chlorophyll a fluorescence technology.

The Aims Of The Thesis

The aim of this work was to produce a chlorophyll a fluorescence assay system with sufficient flexibility to allow testing of various operating protocols, and then to use the system to establish acceptable protocols. From this foundation the aim then was to investigate the properties of various features of fluorescence induction and relate these to conifer seedling physiology. Finally, to test the ability of the fluorometer system in a practical situation, the effects of atmospheric ozone pollution on conifer seedlings in a lower mainland nursery were investigated.
Chapter Two

The Machinery And Techniques Of Chlorophyll A fluorescence

Introduction

This chapter details the development of a fluorometer system, and the techniques associated with its use. The initial section deals with the fluorometer hardware, and is followed by a description of the related software which was developed in parallel. Secondary software for data reduction and presentation, and the details of the actual protocol used to obtain the measurements presented in chapters 3, 4, and 5, are included in the latter part of this chapter.

The Fluorometer System Hardware

The fluorometer is composed of a bright incandescent light source which supplies the actinic light. This light passes through two coloured filters and an electronic shutter, into a hollow sphere. Green plant tissue to be assayed is placed inside the sphere and illuminated to induce fluorescence emission from chlorophyll a. The fluorescence (long wavelength red light) intensity is then measured by a light detector positioned over a port in the sphere's surface.
The fluorescence detector is screened from the actinic light by a second pair of filters which intercept its shorter wavelengths. Some of the mechanics of this system are based on principles modified from earlier work done at S.F.U. especially by P. Toivonen and this debt to earlier researchers is acknowledged. Figures 2.1 and 2.2 complement the following detailed description of the fluorometer hardware. The capital letters in the text refer to individual components of Figure 2.1.

The light source:
A 24 volt, 200 watt, tungsten halogen projector bulb (type EJL), powered by direct current (d.c.) from a rectifier, is used to provide the actinic light (K). The reason for the use of d.c. to power the bulb is to achieve a steady light intensity. Alternating current (a.c) power creates a flicker in the intensity at 60Hz which is registered by the fluorescence detection part of the system. The 24 volt bulb was powered at 18 volts as this provides sufficient light intensity to drive fluorescence induction. This low operating voltage extensively prolonged the life of the bulb. Light output intensity is dependent on the voltage supplied to it. This could be used to set a consistent light intensity for the fluorescence, but is not recommended because the emission spectrum of the bulb changes with voltage which may affect the plant tissue. To circumvent this problem all the measurements were taken using the same
voltage and when changes in light intensity were required an iris diaphragm was used in the light train. Even at 18 volts however the bulb generates a considerable quantity of infra-red radiation which must be removed from the beam before it enters the sphere.

The cooling system:
To prevent heat from the bulb being transferred to the tissue in the sphere a pair of heat filters (H and I) are used in the light path. The first filter (H) is heat reflecting glass, which reflects much of the near infra-red energy back towards the bulb. The second is a heat absorbing filter (I) made of specially tempered glass and connected to an aluminum plate, which conducts heat from the filter into the metal casing of the fluorometer. Both filters are transparent to PAR light.

To deal with the heat generated by the bulb and that reflected back by the first filter a chimney (J) is located immediately above the bulb mounting. A 12 volt d.c. fan (P), (Archer 273-243) positioned directly below the bulb pushes air upwards across the bulb and heat filters, and out of the chimney. Heat transmitted to the casing of the fluorometer is prevented from being conducted to the sphere by a conical light collector (C) made of heat resistant glass and fitted with a rubber gasket. This effectively
insulates the metal sphere from the lamp housing part of the fluorometer.

The lamp housing:
The bulb is situated in an aluminum case, painted black on the outside to promote heat radiation. The lamp housing also contains the coloured filters which modify the actinic light. The first coloured filter (F) is yellow glass (Corning CS 3-71) which reduces background signal by blocking wavelengths shorter than 460 nm (see fluorescence detector, and background signal sections below). The second filter (E) is blue glass (Corning CS 4-96) and these two combine to give a green actinic light (460 - 625 nm). This waveband is used to minimize interference with the fluorescence detector. The access of the light into the sphere is then controlled by an electronic shutter.

The electronic shutter:
This unit (G) (Uniblitz 225L) is situated between the coloured filters and the heat filters. In this position it is protected from much of the bulb's heat, and in turn it protects the blue filter from continuous high light levels. This is important because the blue filter can be easily damaged by excessive absorbed energy. Shutter opening and closing is controlled by a voltage signal from the computer, which activates a peripheral shutter controller. The controller uses a 115v supply to energize the
electromagnetic coils and pull the shutter blades open against restraining springs. A voltage signal from the computer causes the shutter controller to switch power to the coils on and off. The opening time of the shutter is relatively consistent and takes approximately 3ms. The force of the shutter opening mechanism which pulls the two blades apart causes them to bounce back slightly after they reach the fully open position, this causes a slight but noticeable dip in the fluorescence signal at this early part of the induction curve (Fig. 2.3, curve A). This is not a problem for the measurement of either $F_0$ or $F_v$ curve values. After the actinic light has passed the shutter blades the beam is collated by a segment of parabolic reflector which sits against a thin rubber gasket to connect the lamp housing to the sphere.

The sphere:
The 22.5 cm diameter sphere is made of aluminum, in two halves, one fixed against the lamp housing part of the system as described, the other detachable. At the base of the rim of both hemispheres is a small semicircular notch which fits around the stem of the seedling while the shoot is in the sphere. This allows the seedling to remain in its original container during the fluorescence assay. When the two hemispheres are clamped together a seal between them is
Figure 2.1
Side on schematic representation of the fluorometer. An iris diaphragm (not shown) used occasionally to control the actinic light intensity, was inserted in the light train between the sphere and the glass light collector (C). Key to the symbols:
A - Actinic light detector port.
B - Fluorescence light detector.
C - Glass light collector at the sphere light input port.
D - Carrying handle.
E - Blue glass actinic light filter.
F - Yellow glass actinic light filter.
G - Electronic shutter.
H - Heat absorbing filter, mounted on a heat conducting plate.
I - Heat reflecting filter.
J - Heat vent chimney.
K - Tungsten halogen bulb.
L - Shutter plug connector.
M - Bulb power connector plug.
N - Fan power connector plug.
P - Fan, mounted horizontally.
Q - Light dispersing cone.
R - Air exhaust port.
S - Removable hemisphere.
formed by a thin neoprene gasket. The inside surface is coated with highly reflective, wavelength neutral, white paint (Kodak White Reflectance Coating). The paint has a matte finish which helps to diffuse the actinic light such that an even illumination of the tissue in the sphere is achieved (Toivonen 1986). Positioned immediately in front of the light input port is a solid cone (Q). This disperses the incoming light onto the walls of the sphere and has its apex at the centre of the light input port with its base parallel to the port (Morissette 1988). The dimensions of the cone (6.7 cm in height x 5.7 cm in diameter at the base) ensure that there is no direct light pathway from the 2.5 cm diameter input port to tissue located in the sphere (Baker et al. 1989). Indirect illumination in such a sphere is approximately the same everywhere no matter how unequal the direct illumination. For these reasons a sphere is used in preference to a spot probe type system (Schreiber et al. 1986, Öquist and Wass 1988, and Bolhar-Nordenkampf et al. 1989). Spot probe type fluorometers assay a specific area of tissue during each fluorescence determination to permit comparison between individual measurements, but this is not easy to accomplish with conifers because of the needle type leaves. The sphere integrates the fluorescence emitted by the tissue which can then be measured by a detector at any point on the surface of the sphere. Therefore the integrating sphere allows fluorescence measurements independent of the shape of the plant and is thus well
suited to the measurement of the three dimensional needle like leaf form of white spruce and Douglas-fir which were used in this work.

Other environmental factors inside the sphere are also of considerable consequence to the fluorescence measurements, because plants begin to acclimate to the environment inside the sphere during the fluorescence assay, and this may have significant effects on the results.

**The sphere environment:**
The small volume of the sphere makes it especially susceptible to environmental changes. As has already been mentioned the sphere is isolated as much as possible from temperature changes in the lamp housing. Another environmental factor which is prone to change is the gaseous composition, which is liable to be modified by the photosynthetic and respiratory actions of the tissue being measured. To keep the atmosphere inside the sphere consistent during the assay, air from outside the laboratory is continuously pumped, at a low rate, into the sphere and escapes through an exhaust vent (R). This keeps the concentration of CO₂ in the sphere near the ambient level independent of the room atmosphere which may have an artificially high CO₂ concentration due to the presence of the operator. With a stable environment inside the sphere, the fluorescence yield of the plants perceived by the
detector is of more value as an indicator of current physiological status.

The fluorescence detector:
The fluorescence emitted by the tissue in the sphere, in response to the actinic light, is measured by a battery-powered silicone photodiode (Devar 529-2-5). This is located at a small port flush with the inside surface of the sphere which allows it a large angle of detection. To prevent the actinic light itself interfering, the detector is shielded by two coloured filters. These filters are blue and red (Corning CS 7-59, and CS 2-64 respectively), and in conjunction with the actinic light filters, allow only wavelengths greater than 670 nm to pass to the detector (Toivonen and Vidaver 1984). The detector is sensitive to very low light intensities and has a linear response to light intensity of greater than six orders of magnitude. During operation a small voltage is continuously present in the circuit even in complete darkness; this can be measured by the computer and is called the trickle voltage, or the dark offset voltage. The detector responds to light intensity by controlling the additional amount of voltage in the circuit which is then amplified before it reaches the computer.
The external amplifier:
The maximum voltage signal produced by the fluorescence detector in response to light of fluorescence wavelengths is small (approximately 0.1 volts) and needs to be amplified before it can be readily detected by the computer. The optimum range for the computer is 0 - 1 volt because this gives greatest definition of the incoming signal. To achieve the required amplification, and attain optimum utilization of the 0-1 volt range, a simple battery powered amplifier is used in line between the detector and the computer. The amount of amplification is manually adjustable in increments between factors of 2 and 400. Amplification required depends on the quantity and physiological status of the photosynthetic tissue being measured and on the intensity of the actinic light (see chapter 3).

The actinic light meter:
Along with the fluorescence light detector there is also an actinic light meter (Li-Cor LI-185A) which monitors the PAR light intensity (µmol quanta m\(^{-2}\) s\(^{-1}\)) inside the sphere. The quantum flux probe for this second detector is located in a special port in the surface of the sphere, and the cosine correction factor of the probe means that the meter registers light from all parts of the sphere. An actinic light intensity in the sphere of 95 µmol quanta m\(^{-2}\) s\(^{-1}\) is used for all the routine fluorescence assays. This
corresponds to an input light intensity of $1.2 \times 10^4 \, \text{mmol quanta m}^{-2} \text{s}^{-1}$ as measured at the light input port. The actinic light values are recorded on a chart recorder (Metrohm model E478) linked to the light meter. This meter is used for monitoring the actinic light intensity which excites fluorescence in the plant tissue, and also for measuring the absorbency of the plant tissue. Absorbency is the quantity of the actinic light which is absorbed by the plant tissue, expressed as a percentage of full light when the sphere is empty, and is therefore a measure of sample size.

The analogue to digital converter board:
The voltage signal which reaches the computer after being amplified is in an analogue form. This is not directly readable by the computer which understands only digital data. To deal with this problem a special computer circuit board, called an analogue to digital converter board (A/D board) is used, into which the analogue voltage signal is fed. The board (Metrabyte DASH-16) can be manually set to read d.c. voltages of different ranges, up to 0-5 volts, but the larger the range selected the lower the accuracy of the voltage determination. This is because the A/D board has a 12 bit processor allowing definition of the chosen voltage range into $2^{12}$ or 4096 divisions. For this reason the smallest range (0 - 1 volt) was used for this work, giving a 0.00024 volt definition interval. The A/D board uses
sophisticated electronics to translate the analogue signal into equivalent digital information at a user-specified rate up to a maximum of 50kHz. In this form the signal from the detector is then used by the fluorometer system software in the computer to produce Kautsky curves.

The Fluorometer System Software

The purpose of the fluorometer system software, sometimes referred to as data acquisition software (although it does more than acquire data), is to allow the user to control the collection of data from the fluorescence detector and review the accrued data. The software developed was written in Turbo Pascal and allows certain important parameters to be preset by the user before the fluorescence assay procedure is started. These are:-

1) the duration of the assay.
2) the frequency of sampling of the fluorescence signal.
3) changes in sampling frequency during the assay.

The duration of the fluorescence assay will depend on which fluorescence parameters the user wants to obtain. For example a full fluorescence induction curve requires a
Figure 2.2

Diagrammatic representation of the fluorometer system components and connections. ALM, actinic light meter; ALD, actinic light detector; FD, fluorescence detector; Amp, external amplifier; A/D board, analogue to digital data conversion board.
minimum of 300 seconds, whereas an $F_0$ value can be determined with a one second assay. The frequency of sampling the signal determines the accuracy of the results. At high sampling rates the accuracy is increased but only at the expense of collecting more data points. For this reason the higher rates of digitization available using the Dash-16 A/D board are not normally employed during routine assays. Changes in sampling frequency during an assay, as used by Kocsanyi (1988), may be used to achieve high accuracy in the kinetics of the initial part of the induction curve, where the magnitude of the fluorescence signal is changing rapidly. This is followed by a slower sampling frequency for the rest of the induction to reduce memory requirements.

In the work presented in this thesis three standard combinations of settings were used for all the data collection. These combinations were:

1) a very short duration collection at a high sampling rate for the measurement of the background signal, which is the amount of fluorescence present in the sphere when there is no tissue present, and includes the trickle voltage. The use of this data will be explained later in the chapter.

Duration 0.2s, sampling frequency 5000Hz.
2) a short duration collection to capture values of $F_0$ and $F_p$.
Duration 5s, sampling frequency 1000Hz.

3) a long duration collection to measure the whole induction curve including $F_T$. The first five seconds are sampled at a high rate, and then to reduce memory requirements the rate is reduced for the remaining five minutes of the assay.
Part i) duration 5s, sampling frequency 1000Hz.
Part ii) duration 300s, sampling frequency 2Hz.

Following collection of the data from the fluorescence detector it is helpful for the user to have a visual record, both to assess the assay and to be reassured that there was no interference in the signal. To do this the data is presented graphically on the computer monitor, with the voltage from the detector on the ordinate axis plotted against the time on the abscissa. The user may also want to institute recording of the data into permanent memory, either on an internal hard disk or onto floppy diskettes. Data can be stored in either of three formats: Lotus, ASCII (American Standard Code for Information Interchange), or binary. Lotus format (which is similar to ASCII) was employed for the majority of the work as Lotus 123 software was used for later data analysis. Binary format takes up considerably less memory space but is less compatible with commercial software programs.
The last important feature of the fluorometer software is to control triggering of the fluorescence run, including shutter activation. Shutter operation and data collection are integrated, and are instituted by pressing a specific key on the computer keyboard. Shutter opening and closing is induced by an analogue signal generated by the A/D board, and the data acquisition is synchronized with this so that the fluorescence intensity during the shutter opening is monitored. Collecting the data is however only half of the procedure, as data in its 'raw' form must be processed to be useful as a tool in plant physiology.

The Data Processing Software

After the raw data has been collected and stored, the voltage values must then be translated into a form which allows the individual assays to be compared with each other. This data processing is done by software written to run inside the commercially available Lotus 123 spreadsheet environment. The software processes each assay individually.

Background Signal:
Even when the sphere is empty of plant tissue a positive fluorescence reading will be obtained from the system
because of stray light and the trickle voltage of the fluorescence detector. This is called the background signal (Fig. 2.3, curve B), and to determine its amplitude a background light signal measurement (see above) is taken each time the fluorometer is used. The plant tissue assay results must be corrected for this background signal before further processing.

Data correction:
A linear regression analysis is performed on the background signal data (Fig. 2.3B), using only the section of data collected after the shutter is fully open. The value determined by this analysis is then divided by the amplification factor used when the background signal was recorded. This is called the correction value. The correction value is subtracted from the values recorded from each individual plant assay once they have been divided by the amplification factor used during the assay. Effectively this removes from the fluorescence measurements, variations due to the stray light and the trickle voltage in the empty system. The resulting data is called 'corrected' after it has been processed to this point, and the next step is normalization.

Data normalization:
The area of tissue assayed with the integrating sphere system will vary with each individual plant. Therefore to
make the measurements comparable a common basis must be used to represent the data. Surface area is not used because it is exceptionally difficult to measure accurately in conifers. Dry weight could be used but this would negate the benefit of the non-destructive nature of the fluorescence assay, so \( F_0 \) is used for this purpose and the variable fluorescence data is presented on a per unit \( F_0 \) basis. The assumptions that \( F_0 \) is both a valid and good basis for the presentation of fluorescence data are examined in some of the proceeding experiments reported in chapters 3, 4 and 5.

The \( F_0 \) value is selected from the data when the shutter first becomes fully open (Fig. 2.3, curve A). At this point there is typically a short-lived plateau lasting about a millisecond and the amplitude of this point is recorded as \( F_0 \). This value is then used for normalization by the following formula:

\[
\frac{F_{\text{var}} - F_0}{F_0} = F_v
\]

Where:

\( F_{\text{var}} \), is the variable fluorescence corrected for signal amplification and dark offset components.
\( F_0 \), is the initial value of the Kautsky curve when the shutter has fully opened, and which has also been corrected for dark offset and amplification.

Thus the variable fluorescence data is then in units of \( F_0^{-1} \), which appears to solve the problem of using different amounts of photosynthetic material (see chapter 5).

**Variable fluorescence features:**
The variable fluorescence data may contain various peaks, dips, and plateaus, and the software searches for certain of the more common features. The \( F_p \) value is discerned by taking the value of the highest peak which occurs within the initial five seconds of the assay. The \( F_T \) value is recorded from the steady state which occurs at the end of the long assay period. The time taken to achieve \( F_p \) from the onset of illumination is also determined by cross checking the \( F_p \) value with the time co-ordinate. Once these values have been read from the data they are also used to calculate compound fluorescence values such as Rfd (fluorescence decrease ratio) (see Equation 4.2) (Lichtenthaler and Rinderle 1988).

**Kautsky curves:**
Finally the corrected and normalized data are graphed against time to give Kautsky curves (Fig. 1.1a) (Papageorgiou 1975). The data can also be plotted against the logarithm
Figure 2.3

A: the initial 50ms of the fluorescence transient collected from a white spruce seedling. The plateau which occurs after the initial rapid rise in the fluorescence intensity, i.e. when the shutter has opened fully, represents $F_0$. The noticeable dip in the fluorescence intensity after the $F_0$ plateau is caused by shutter bounce.

B: the complementary background signal to curve A measured in the empty sphere on the same day.

C: the trickle voltage at the time of the other two measurements.
of time to highlight the early phases of fluorescence induction (Fig. 1.1b). The values of $F_O$, $F_p$ and $F_T$ etc. from the data analysis are printed out in tabular form and the complete set of corrected and normalized data values from each assay are saved in permanent memory for possible future reference. To illustrate how all the above mentioned factors are used in practice a sample procedure is given below.

Sample Procedure

This section describes the typical fluorescence assay procedure used in the experiments presented in chapters 3, 4, and 5. It assumes that the seedlings are ready for the fluorescence assay, meaning that any lower needles, up to approximately 4cm above the root collar, are trimmed off to allow the seedlings to be placed into the sphere more easily.

The first action is to switch on the actinic light, which requires approximately 20 minutes to attain a constant illumination intensity. The cooling fan starts automatically when the light is switched on. Next the shutter controller, the computer (AST Premium 286, IBM PC-AT compatible), the actinic light meter, and its chart recorder are switched on (Fig. 2.2). The data acquisition program is booted and used to open the shutter. Light then enters the
sphere and causes the actinic light meter to respond; the intensity can then be monitored as it reaches equilibrium. The first plant is placed into the dark box and the timing clock started to count-down the 20 minute dark pre-treatment period (see chapter 3).

Next, the pump which feeds air into the sphere is started, and the external amplifier and the fluorescence detector are switched on. A test run of five seconds duration is then conducted using the largest of the remaining plants as the sample. To do this the shutter is closed and the plant is placed into the sphere. After a few minutes in the darkened sphere the assay is conducted. The results are then displayed on the computer monitor screen and used to estimate the amount of amplification required for the batch of plants. This is then set manually on the external amplifier. Any problems with the general working of the system will also be noticeable in the results at this point.

Once the actinic light intensity has stabilized as monitored by the actinic light meter, the background signal is measured for 0.2 seconds with the sphere empty and the room in darkness. The data acquisition program is then set up as required by the user for the sample assays (see detail above). After the exact 20 minute dark pre-treatment period has elapsed, the plant is transferred, in total darkness,
into the sphere and the fluorescence assay conducted with the room in darkness.

After the fluorescence assay, the raw data are displayed graphically plotted against time on the computer monitor and then saved in permanent memory for later correction and normalization.

Once all the plants to be tested have been dark pre-treated and assayed, the background signal is again determined using a short duration run, and the fluorometer system is shut down by cutting the power to all the electrical components.

The raw data collected during the fluorescence assays is later fed through the data processing software. This uses the two background signal runs and the amplification factors to correct the raw data. The software then determines the $F_0$ value of each individual seedling and uses it to normalize the data which is subsequently saved to the hard disk. The fluorescence induction curves of the data can be plotted at this point and tables of values derived from the curves printed out.

Finally, values such as $F_0$ and $F_P$ can be analysed using statistical software packages. In the experiments presented in chapters 3, 4, and 5, SAS (Statistical Analysis System)
version five was used on a mainframe computer to analyse the data, but:

"It is as well to understand what you can do before you learn to measure how well you seem to have done it." (Tukey 1977).

and the next chapter details work done to this end.
Chapter Three

The Establishment Of Working Protocols.

An Investigation Of Dark Pre-treatment and Actinic Light Intensity.

Introduction

Before many of the physiological or applied aspects of chlorophyll a fluorescence can be investigated knowledge must be gained about what is actually being measured and whether the measurements which are taken allow more complex questions to be addressed. Thus there is a need to make sure that the current techniques are sound and valid, to give confidence that the conclusions from their use are worthwhile. This is especially important as the general application of this technique to disciplines such as agriculture and forestry proceeds. This chapter, following on from the development of the physical fluorometer system and the data acquisition and processing systems, deals with the generation of two basic protocols involved in the practical use of the fluorometer: i) the dark pre-treatment of the samples before they are assayed, and ii) the light intensity with which the samples are illuminated during the fluorescence induction measurements (actinic or excitation light intensity). These two factors were investigated a)
because there were no standards to act as a guide to what durations and intensities were acceptable for measurements, and b) because these factors are very often at the discretion of the user of a fluorometer, though occasionally actinic light intensity is preset in some commercial systems.

Since its discovery chlorophyll \(a\) fluorescence has been used by many researchers (for example Toivonen and Vidaver 1984, Schreiber and Bilger 1987, Schneckenburger and Bader 1988, and Öquist and Wass 1988). However, despite its numerous applications (Lichtenthaler et al. 1986) little has been discovered about the relationship of dark pre-treatment (commonly and perhaps erroneously called dark adaptation), to the measurements of \(F_0\) and \(F_v\) in conifers, achieved by using it.

Dark pre-treatment is necessary for fluorescence induction in the fluorometer system used in this work, but do \(F_0\) and \(F_v\) respond in the same manner to its duration? Other physical components of the measuring system may also vary (actinic light intensity, for example) and responses to these need to be known too. If the two components \(F_v\) and \(F_0\) are not similar in their response to possible variables, then the comparability of measurements of fluorescence kinetics in integrating spheres is limited to those made under identical conditions. For this reason it is necessary
to determine acceptable standards for these potentially variable factors when taking fluorescence measurements. Perhaps the most important conditions, as already mentioned, are dark pre-treatment and actinic light intensity, though other factors such as temperature may also have a perceptible effect (Neubauer and Schreiber 1987). Once the effect of possible variables is known then viable protocols can be developed to permit valid comparison of fluorescence measurements. To begin addressing these questions, two series of experiments were conducted, firstly with dark pre-treatment, and secondly with actinic light intensity.

**Dark Pre-treatment**

It is known that the kinetics of fluorescence induction depend on the time of dark pre-treatment, and that during periods of darkness PS II acceptor pools (i.e. the quinone primary acceptor QA) become oxidized (Buschmann and Buchanan-Bollig 1983). Oxidation of the primary acceptor pool takes a finite length of time, but how long is sufficient? Various researchers working on different plant species have reached disparate conclusions about the need for, and the optimum duration of dark pre-treatment (Sivak and Walker 1983, Sivak et al. 1985, Andreeva and Tikhonov 1983, and Karavaev et al. 1985). To answer these questions for white spruce a set of seedlings was assayed for both F₅₀,
and Fv over a range (from 1 minute to 1 hour) of dark-pretreatments and also with an almost zero dark-pretreatment time of < 10ms.

Materials and methods

Measurements of chlorophyll a fluorescence (refer to chapter 2) were taken using white spruce seedlings on five consecutive days during the last week of April. The styroblock grown seedlings were 2.0 stock of seedlot 4208 (Quesnel area, elevation 1250m, latitude 53'19" N, longitude 121'51" W), which ranged in height from 13.5cm to 24.5cm. and in root collar diameter from 2.9mm to 4.5mm. All the seedlings used in the experiment were originally grown under standard nursery conditions by Pelton Reforestation Ltd. of Maple Ridge, and had been transferred to an ambient temperature greenhouse at S.F.U. where they had remained over winter (minimum temperature 1.5°C). A set of five seedlings was selected at random prior to the experiment. On each of the five days of the experiment the same set of five seedlings was measured for fluorescence using eight different dark pre-treatment times each day. The (approximately) zero dark pre-treatment time was achieved by closing and re-opening the shutter whilst the seedling was being illuminated in the sphere. This gave a very short dark-pretreatment of less than 10ms. The order of the eight
treatments was random each day and the order in which the five trees were measured was random within each treatment. The measurements were taken only between 10:00 hrs. and 16:00 hrs. to minimize potential diurnal effects which were noticed in earlier unpublished work, though some researchers have reported no diurnal patterns in \( F_0 \) and \( F_v \) (Buschmann and Buchanan-Bollig 1983). After the first three days of measurements the new foliage produced by the trees some weeks prior to the experiment when the seedlings began to flush, was stripped off using scissors leaving only the one year old foliage produced in the previous year to be measured during the last two days of the experiment. This was done to allow the \( F_v \) and \( F_0 \) response of the first year and the new second year needles to be differentiated.

**Results**

\( F_0 \) was high when the seedlings were not given any perceptible dark pre-treatment time (less than 10ms), compared to measurements taken after some dark pre-treatment (between 1 and 60 minutes) where \( F_0 \) was significantly lower. \( F_0 \) remained relatively constant over the whole range of dark pre-treatment times between one minute and one hour (Fig. 3.1). A marked decrease is also obvious in the absolute value of the \( F_0 \) of the trees after the new foliage was
Figure 3.1
Response of $F_0$ to dark pre-treatment time. The two curves show the same set of trees measured before and after the removal of foliage produced in the current season. Tukey's LSD is indicated.

--- Original and new foliage, number of replicates $N = 10$.
○○○ Original foliage only, $N = 10$. 

0.00 0.02 0.04 0.06
Fo (relative units)

0 10 20 30 40 50 60 70
Dark pre-treatment time (minutes)
Figure 3.2
Response of $F_p$ to dark pre-treatment time. The two curves show $F_p$ values from the same set of trees measured before and after the removal of foliage produced in the current season. Tukey's LSD is indicated.

--- --- Original and new foliage $N = 10$.

.. . Original foliage only $N = 10$. 
stripped off. However, the shape of the response to different lengths of dark pre-treatment is not appreciably affected by the removal of the new foliage.

The $F_p$ level increases substantially as soon as dark pre-treatment of one minute or greater is given, and it continues to rise, although not uniformly, with increasingly long periods of dark pre-treatment (Fig. 3.2). The response of the $F_p$ value to dark pre-treatment was not significantly affected by the removal of the new foliage.

Discussion

The large decrease in $F_0$ when the seedlings were measured after dark pre-treatments of one minute or more, is misleading. When no appreciable dark pre-treatment is used and a measure of the $F_0$ is taken, what is probably being recorded is the $F_T$ value of the seedling under those particular conditions, making comparison invalid. This shows that very short dark pre-treatment times in the order of a few milliseconds are not sufficient to allow a meaningful $F_0$ to be measured. This is probably because the steady state fluorescence $F_T$ decays to zero slower than the pigment beds become de-energized (photon absorption to charge separation occurs in a few microseconds) (Youvan and Marrs 1987), and the high $F_0$ value produced after a 10ms
dark period is composed of the $F_O$ of the tissue supplemented by the residual steady state fluorescence $F_T$, which has not decayed to zero before the re-illumination.

In a seedling which has had a period of dark pre-treatment of one minute or longer, the $F_O$ is relatively constant whatever the duration of the dark pre-treatment period. This suggests that the source of $F_O$ fluorescence, which is commonly considered to be the photosynthetic pigments of the light-harvesting complex (LHC) (Papageorgiou 1975), reaches a steady state very soon after being placed in the dark. This may be because they merely act as a 'funnel' for collecting and then transferring excitation energy from absorbed PAR light. Light capture and transfer are very rapid processes (Witt 1975). Thus once the light input ceases there is only a short period of time before the pigments have transferred all the absorbed light energy to the reaction centres or it has been lost as heat or fluorescence (Govindjee et al. 1990). After these processes are complete, any re-illumination of the pigments will give rise to an $F_O$ which is independent of the dark pre-treatment time. Such an $F_O$ would not be independent of dark pre-treatment time however if the configuration of the pigments changed with increasingly long periods of time spent in darkness. This may be the case for tissue left in darkness over a period of days such that etiolation begins to occur. In some tissues of certain species exposure to light causes
ultrastructural changes in the photosynthetic apparatus (Lichtenthaler et al. 1982). Whether any similar effect is caused by periods of darkness is unknown, however the results obtained in this experiment suggest that little or no effect is caused by the period of darkness per se. In fact there is no significant difference between the average $F_0$ of the set of seedlings after being dark pre-treated for 1 minute or after being dark pre-treated for 1 hour. This leads to the conclusion that the $F_0$ measurement of a tissue is almost independent of the period of dark pre-treatment, however the time taken for steady state fluorescence $F_T$ to decay to zero does limit the minimum dark pre-treatment time which can be used.

After the seedlings were stripped of their new foliage there was an obvious decline in the $F_0$ values which is consistent with the hypothesis that the $F_0$ value is an estimate of the size of the active photosynthetic pigment (Krause and Weis 1984). The response of the stripped seedlings to dark pre-treatment is the same as the control seedlings thus $F_0$ which is apparently independent of dark pre-treatment time is also independent of tissue age in trees at least at this stage of development. This again suggests that $F_0$ may almost be considered to be a morphological measurement.

In contrast to the $F_0$ values of the seedlings, the $F_p$ values increase with increasingly long dark pre-treatment periods.
As with \( F_0 \), \( F_p \) measured without appreciable dark pre-treatment is probably \( F_T \), which in general is considerably lower than \( F_p \). This would agree with the findings of other researchers that when no dark pre-treatment is used \( F_v \) is lower and declines more rapidly to steady state \((F_T)\) due to faster energy distribution to PS I than when the seedlings are given dark pre-treatment prior to fluorescence assay (Buschmann and Buchanan-Bollig 1983). When the seedlings were given dark pre-treatment there was a rise in the \( F_p \) value which does not appear to follow a simple relationship with the duration of the dark pre-treatment. As the tissue is moved from light to darkness, no more energy arrives at the P680 reaction centre, the site of a large percentage of \( F_v \) (Barber et al. 1989), and the energy already in the photosystems will progress along the usual pathways to PS I and beyond. As it seems unlikely that the rate of this quenching varies due to the onset of darkness, a more plausible explanation for the changing response of \( F_p \) to dark pre-treatment period might lie in the changing levels of the proton gradient which is built up during photosynthesis and which drives the production of ATP (adenosine triphosphate) from ADP (adenosine diphosphate) and inorganic phosphate, (Schreiber and Bilger 1987). When a plant moves from light to darkness the 'emptying' of energy from the photosystems is rapid; by contrast the proton gradient may take much longer to dissipate. Also the rate of dissipation may depend on the concentration
difference, such that the higher the gradient the more rapid the decay. This may account for the asymptotic shape of the response of \( F_p \) to dark pre-treatment period. With a large proton gradient remaining when the seedling is re-illuminated the backlog of excitons in PS I will be small and therefore \( F_p \) will also be small (Sivak and Walker 1983). After the light has been on for a period of time the proton gradient is increased again, due to trans-membrane transfer by the inter-photosystem electron carriers and \( F_v \) falls. Weis et al. (1987) suggested that \( \Delta pH \) exerts a strong control on the electron transport rate between PS II and PS I. and slow relaxation of non-photochemical quenching is consistent with slow decay of thylakoid \( \Delta pH \) after a reduction in light intensity.

For \( F_p \), similarly to \( F_0 \), the removal of new foliage had no significant effect on the response to dark pre-treatment duration. This indicates again that an age difference of one year between tissue does not affect the response of the tissue to dark pre-treatment. Therefore when \( F_p \) is measured and the raw data normalized to \( F_0 \), as set out in chapter 2, the values obtained may be representative of the tissue physiology independent of tissue age as well as size. This does not however accord with results of Brooke et al. (pers. comm.) which indicated an elevated \( F_p \) in new needles compared to the original tissue in white spruce seedlings.
Conclusions

Neither the response of the $F_0$ nor the $F_p$ component to dark pre-treatment were affected by the age of the tissue measured. This linked with the fact that size of tissue used is not a determining factor of normalized $F_p$ opens the possibility of using small portable fluorometer systems to assess large mature plants.

The curvilinear response of $F_p$ to dark pre-treatment time indicates that for the measurement of $F_v$ in white spruce seedlings at this developmental stage, it is important that the dark pre-treatment time be consistent at a predetermined value so that separate samples are comparable. A value in excess of 30 minutes is probably wise if this is practical because the response is least variable after this amount of dark pre-treatment. In practice, a standard value of perhaps 20 minutes may be more convenient, and will be satisfactory as long as it is applied accurately, and adhered to consistently.

The measurement of $F_0$, however, does not appear to require an accurate, standardized dark pre-treatment time, and in cases where $F_0$ is the only value required (as it might be if $F_0$ is being used as an indicator of photosynthetic tissue size) the results suggest that any dark pre-treatment time
can be used, with consistent results providing it is long enough for Fv to decay to zero. This may allow significant time-saving if dark pre-treatment times as short as 60 seconds are used.

Finally, changes in $F_p$ do not parallel those of $F_o$ in regard to dark pre-treatment. This presents the problem that measurements of Fv taken using one dark pre-treatment period cannot be readily compared to measurements of Fv characteristics taken using another dark pre-treatment period, unless a curve as in Fig. 3.2 is used to correct for the effect on Fv values caused by the difference in dark pre-treatment period. In practice, considering the time required to construct such a curve, the application of this type of induction kinetics is limited to the use of consistent dark pre-treatment periods so that Fv values obtained are comparable, at least for a given species, seedlot and age.

Actinic Light Intensity

Materials and methods

Five one year old white spruce seedlings of seedlot 4208, grown under the same conditions as described in the dark pre-treatment experiment, were used in this experiment. The
seedlings were measured for fluorescence (see chapter 2) on four different days, at a series of different actinic light intensities (no other types of measurements were made). The actinic light intensity was adjusted with the iris diaphragm (see Fig 2.1).

A day prior to the experiment the seedlings were brought from an unheated greenhouse and placed by a window in the laboratory, in supplemented ambient light (100-250 μmol quanta m\(^{-2}\) s\(^{-1}\)). For one half of the experiment the seedlings were first measured for fluorescence (for 5s) using a low intensity actinic light, with each subsequent measurement using a successively higher light intensity. Between measurements the seedlings were again placed by the window for at least 20 minutes before being dark pre-treated for the next measurement. When all five seedlings had been measured the light intensity was changed by manipulation of the diaphragm and the seedlings were again dark pre-treated and measured. The second half of the experiment was conducted in the same way as the first, except that the seedlings were tested using the highest actinic light intensity first followed by successively lower intensities. All measurements were done between 10:00 hrs. and 16:00 hrs. to reduce any potential effect from diurnal rhythms. The temperature during the measurements ranged from 21-24°C and the light intensities used were between 0.25 μmol quanta m\(^{-2}\) s\(^{-1}\), and 105 μmol quanta m\(^{-2}\) s\(^{-1}\).
Results

$F_0$ has a linear relationship with actinic light intensity (Fig. 3.3). This was true for each of the days and for both the directions in which the light intensity was adjusted i.e. from low intensity to high, or from high intensity to low. Even at very low intensities the linear relationship is notable. The relationship of $F_P$ to the light intensity (Fig. 3.4) shows much greater variation than that with $F_0$. Overall, the $F_P$ increases as the actinic light intensity increases, but the gradient of the relationship appears to depend on the direction in which the light intensity was changed. When the light intensity was decreasing the gradient is less than when the light intensity was increasing. Both seem to have a shoulder or curve in their response to light intensity and neither are as linear as the $F_0$:light intensity relationship.

Discussion

The linear relationship of light intensity and $F_0$ seems to indicate that $F_0$ is a physical phenomenon, as has been proposed previously by some researchers (Schreiber and Bilger 1987). As more light is supplied to the tissue more energy is directly re-emitted from the light harvesting
Figure 3.3

The response of $F_0$ in white spruce seedlings to different actinic light intensities. Results from both parts of the experiment (refer to text) are combined because there is no significant difference between them. Tukey's LSD is indicated. $N = 5$. 
Figure 3.4

The response of Fp to different actinic light intensities. The two curves show values obtained when the light intensity was being successively increased ( o—o ), and when the light intensity was being successively decreased ( +.....+). Tukey's LSD is indicated. N = 15.
pigments as fluorescence. The linear response also indicates that $F_0$ has good characteristics as a measure of the size or amount of the photosynthetic tissue. This is because there is no obvious confounding influence due to the physiology of the tissue in the $F_0$-light intensity relationship. A different response in $F_0$ at one light intensity than at another would have indicated that the $F_0$ value was dependent on the reaction of the tissue to the light and not simply on the quantity of the photosynthetic pigment. However the results clearly show that this was not the case.

As with $F_0$, $F_p$ rises with increasing light intensity, but the response is not as linear as that of $F_0$. The relationship between $F_p$ and light intensity when the light intensity was rising is relatively linear although the gradient decreases somewhat as the light intensity increases. Low light intensities mean less impediment and more efficiency of throughput of excitation energy than high light intensities. In other words, photochemical efficiency in PS II declines in high light (Karavaev et al. 1985, and Horton et al. 1988). When the light intensity used is successively being reduced the gradient of the response is rather less. One reason for this might be that the photosynthetic system of the seedlings acclimates to the light used in the previous measurement, even though the
assays were only 5 seconds long, and the seedlings were placed in light between measurements. When a sample of photosynthetic tissue is illuminated with strong light the photophysiological processes which take place are of greater magnitude than those induced by lower light intensity. Thus when a seedling is remeasured at a lower light intensity the photosystems may be somewhat more responsive than in a plant kept under lower light i.e. the proton gradient will be slightly greater. The result is that the fluorescence kinetic response is of a lower magnitude because the seedling is able to utilize a greater percentage of the energy. There is also a delay until electron transport system adjusts to a lower photon flux density and fewer electrons, and this may be partially responsible for the lag in the $F_p$ values when the light intensity was decreasing rather than increasing (Stitt et al. 1989).

One effect which was predicted, but which did not appear in the results of this experiment, was a levelling off of the $F_p$ response to light intensity at the higher light intensities. As more light is supplied to a seedling, more excitons will be transferred to the PS II reaction centres and thus more energy will be removed to the primary electron acceptors (Nobel 1983). At some light intensity, the transfer of energy from P680 to the primary electron acceptor will be saturated and $F_p$ will reach a maximum. However there is no indication that a maximum was reached in
this experiment, though $F_p$ is known to saturate at high actinic light intensities (Neubauer and Schreiber 1987). This fluorometer may not provide a high enough actinic light intensity to approach the saturation level of the photosystems.

Conclusions

Both the $F_0$ and $F_v$ components of fluorescence kinetics are affected by the light intensity used to measure them. The responses of these two components were not identical, which suggests that it would be unwise to attempt to compare values of $F_v$ measured using different light intensities.

The difference in the response of $F_p$ to light intensity due to the light intensity being increased compared to it being decreased, implies that standardizing the light regime imposed on the tissue prior to the dark pre-treatment period may be important for obtaining reliable results.

The direct linear response of $F_0$ to the light intensity indicates that $F_0$ is largely independent of the physiology of the tissue being measured and thus is ideal in this respect for use as a measure of the size of the active photosynthetic pigment complement, or more generally as an estimate of tissue size.
Chapter Four

An Investigation Of Drought Stress In White Spruce Seedlings
Using Chlorophyll A fluorescence

Introduction

Growing conifer seedlings is a large scale business in B.C. because of the large number of seedlings needed to restock stands which have been logged. This dictates that bulk growing and handling methods be used to produce the seedlings for this restocking. The main basis of the seedling bulk production system in modern tree nurseries is automation stemming from the use of standard size styrofoam blocks (styroblocks). One problem with the use of styroblocks however compared to bare root techniques is that of watering. The problem arises from an accumulation of factors the first of which is that each cavity has only a small diameter (generally 3 cm, for white spruce seedlings) and is separate from the adjacent cavities. This means that the catchment area for water is very small, and transfer of excess water from one cavity to another is not possible. Other factors compound this problem, firstly the mixture of peat and vermiculite used as a growing medium, which is difficult to re-wet once dry, has a tendency to become compressed in the cavity. Secondly what little depth is
left at the top of the mixture is filled with coarse grit in an attempt to reduce the incidence of fungal and liverwort growth, which can also hinder water penetration. Another inherent problem is the small volume of the cavities, which leaves the seedlings with restricted reserves of water. These factors predispose cavity grown seedlings to drought and makes them susceptible to injury (Van Eerden 1974).

The media packing problem reduces the rate of drainage and insinuation of water into the lower part of the cavity; and the lack of an indentation in the top of the media means that any water not immediately absorbed during watering, runs off. To counteract these problems one obvious solution would be to simply increase the amount of watering, however if more water is applied more is wasted since the amount of run-off is large. High watering rates although promoting growth can also cause delayed budset, or second flushing which can be detrimental because new tissue is susceptible to frost damage (Duryea 1984). Under-watering can reduce the growth rate of the seedlings, reduce resistance to other stresses and cause seedling death (Timmis and Tanaka 1976, and Vidaver et al. 1988a). Reduced growth rates may also be a problem because the B.C.M.O.F. (British Columbia Ministry of Forests) seedling stock quality standards (for outplanting) are based principally on shoot morphological attributes.
Some nurseries use a system of weighing a block of seedlings and when the weight drops to a particular value, due to drainage, evaporation and transpiration, the surrounding batch of seedlings is watered. Routine watering is also used, for example: 3 times per week, 10,000 liters per million cavities (Van Eerden 1974). Whether these are acceptable systems is hard to judge because they give no measure of how well the seedlings are doing. An alternative approach to this problem is to directly monitor seedling water stress.

Finding an alternative system is however, made harder because conifers do not show obvious morphological symptoms of drought stress until the stress is very severe. This allied with varying degrees of xerophytic properties depending on species and provenance, makes judgements regarding watering especially difficult. Chlorophyll $a$ fluorescence may be an ideal tool in this situation because both electron transport between PS II and PS I and also water splitting in algae are known to be sensitive to drought stress (Wiltens et al. 1978). Previous experiments (Brooke et al. 1988, Toivonen and Vidaver 1987, and Vidaver et al. 1988b) have shown that variable fluorescence is affected by drought stress. To test the value of chlorophyll $a$ fluorescence for monitoring water stress, an experiment was designed to examine the effects of drought stress on chlorophyll $a$ fluorescence in white spruce seedlings. A
broad set of morphological and physiological seedling parameters were measured in addition to chlorophyll a fluorescence to reveal potential correlations and to demonstrate those parameters which are superior indicators of drought stress.

Materials and Methods

Six styroblocs of 1.0 white spruce seedlings (Picea glauca) of seedlot 8534 (elevation 1060m, latitude 54'50"N, longitude 124'W, near Prince George) were brought to S.F.U. from Pelton Reforestation Ltd. in mid July. The six blocks were randomly allocated into three adjacent groups (two blocks per group). Two groups were positioned underneath a canopy, a mansard roof type structure designed to keep rain off the seedlings. The canopy was made of transparent polyvinylchloride (80% PAR transmission) stretched over a wooden frame, open at the base on all four sides to allow air circulation. The group of seedlings outside the canopy, the "ambient" treatment, were watered regularly to field capacity (with maximum intervals of three days), and were also open to rainfall. One of the two groups under the canopy was also watered at the same time as the "ambient" seedlings, this treatment was called "watered". The third set of seedlings, also under the canopy, received no water
after the beginning of the experiment, this treatment was called "dry".

At two to three day intervals, a sample of seven seedlings was chosen randomly from each treatment and used for a series of physiological and morphological measurements. Seedlings from randomly selected styroblock coordinates were taken from each treatment, the same coordinates were used for each treatment on any one sample date. To reduce edge effect trees from the outer two rows of the styroblocks were not selected.

The 21 seedlings selected on each date were measured for fluorescence (for 305s) in a random order, using the method described in chapter 2. Following fluorescence assay, three trees from each of the three treatments ("ambient", "watered" and "dry") were used for determinations of apparent photosynthesis (APS) and dark respiration (Rd) of the shoots by measuring the carbon dioxide exchange rate in a closed system infra-red gas analyzer (IRGA) apparatus. The seedlings selected for APS measurement were first placed in light of 375 μmol quanta m\(^{-2}\) s\(^{-1}\) intensity for 15 minutes and then for 30 minutes in light of 650 μmol quanta m\(^{-2}\) s\(^{-1}\) intensity. The measurements of gas exchange rates were taken using a Beckman model 865 IRGA with a high pressure sodium light (400 watt Poot Elektra, type PC 1078/N lamp, with a General Electric Lucalox LU400/40 bulb) at a light
intensity of 650 μmol quanta m⁻² s⁻¹ (85% – 90% light saturation). The rate of change of carbon dioxide concentration was also measured in darkness to give the dark respiration rate. The APS and Rd rates were calculated using equation 4.1.

The IRGA was calibrated using a standard CO₂ mixture, and 100% nitrogen was used to set zero. Water vapor in the air stream from transpiration mimics carbon dioxide by absorbing infra-red radiation, this can cause errors and distort the results. Therefore transpired water vapor was removed from the circulating air by an ice jacket water vapor condenser, thus maintaining a constant relative humidity in the system. For consistency the seedlings were tested for CO₂ exchange rate over a predetermined range of CO₂ concentrations (305ppm to 374ppm) which brackets mean ambient levels.

Following fluorescence and gas exchange rate determinations a representative group of five needles was taken from each seedling and used to determine needle water potential. This was done with a leaf press (Decagon Devices, Inc. J-14 Leaf Press). Hydraulic pressure is applied to the needles until moisture is forced from the needle cells. The amount of pressure required to achieve this is considered by Rajendrudu et al. (1983) as an estimate of daytime needle water potential, although it may be argued that the value obtained is only a measure of leaf osmotic potential.
Equation 4.1

Calculation of APS and Rd rates from rates of CO₂ exchange in a closed system IRGA.

\[ \Delta \text{ ppm min}^{-1} \times \frac{\text{volume}}{1000} \times \frac{1}{1000} \times \frac{P}{101.3} \times \frac{273}{T} \times 1.964 \times 60 = R \]

Where:
- \( \Delta \text{ ppm min}^{-1} \) = change, in parts per million per minute.
- 273 = standard temperature in °K.
- \( T \) = temperature of system in °K.
- \( P \) = pressure in kPa.
- 101.3 = standard pressure in kPa.
- \( \frac{\text{volume}}{1000} \) = volume of the system in liters.
- \( \frac{1}{1000} \) = ppm to ml conversion.
- 1.964 = mgCO₂ l⁻¹ at standard temperature and pressure.
- 60 = minutes to hours conversion.
- \( R \) = APS or Rd in mgCO₂ hr⁻¹ seedling⁻¹.

Seedling height (root collar to base of the apical bud) was also recorded before shoot detachment for measurement of shoot xylem water potential using a Scholander pressure-bomb (PMS Instrument Co.). Lastly the shoots were separated into stem and needles for dry weight determination and oven dried at 89°C for 48 hours before being cooled and weighed.

On selected days while seedling assays were conducted, a separate set of five seedlings from the "dry" treatment were selected at random from the styroblocks and re-watered. These seedlings were then assayed for gas exchange and
fluorescence three days later to assess physiological recovery.

The data collected during the experiment were analyzed using the data processing software described in chapter 2. SAS was used to calculate analyses of variance, and apply mean separation tests to the data, and also to calculate correlation coefficients.

Results

Morphological Values:
Over the course of the experiment there was no significant change in height or dry weight (stem or needle). These values were however all slightly larger (approximately 3% for height) in the "ambient" treatment than in either of the other treatments, where the seedlings were under the canopy. As expected, shoot and needle water potentials of the "dry" treatment seedlings decreased during the experiment and were significantly lower than those of the watered seedlings after 8 days of drought for shoot xylem water potential, and after 14 days for needle water potential. Neither F₀ nor absorbency were significantly different due to treatment but both became significantly lower over the course of the experiment.
Physiological values (APS and Rd):

There was a very consistent pattern in the gas exchange measurements for both the APS and Rd rates. In the seedlings subjected to the "dry" treatment, rates declined as the water stress increased, whilst those of the other two treatments which were watered (i.e. "ambient" and "watered"), remained high (Fig. 4.1 and Fig. 4.2). This was the pattern whether the rates are expressed as per gram needle dry weight, per unit $F_O$, or per seedling. On a 'per $F_O$' or 'per seedling' basis Rd was significantly greater in the "watered" treatment than in the "dry" treatment and in turn greater in the "ambient" treatment than the "watered" treatment.

Physiological values (fluorescence):

The common features of the variable fluorescence curve all exhibited a very similar behavior in response to the treatments. For the drought treated seedlings, following an initial consistent small rise, all the variable fluorescence features declined as the severity of the drought increased (Fig. 4.3). The values of $F_p$, $F_s$, and $F_T$, as well as the overall maximum ($F_{max}$) all have a maximum amplitude in the "watered" treatment, followed by slightly lower values in the "ambient" treatment with their lowest values in the "dry" treatment (Fig. 4.4).
The derived value, Rfd, was calculated after Lichtenthaler et al. (1986) but modified to take into account the different amounts of tissue being measured in each fluorescence induction (see Equation 4.2). The amplitude of Rfd is reported to indicate the potential photosynthetic activity of tissue such as leaves (Lichtenthaler et al. 1986, and Lichtenthaler and Rinderle 1988). Rfd was significantly higher in the "dry" treatment than in the two watered treatments but only after 12 days without watering (Fig. 4.5). Another derived value $\frac{F_p}{F_T}$ ($F_p$ divided by $F_T$), which was calculated as a possible alternative to Rfd, was higher in the droughted seedlings when the drought was severe, but was very variable in all three treatments (Fig. 4.6). Both Rfd and $\frac{F_p}{F_T}$ exhibited a dramatic decline in the final measurement. The value of $F_p$ minus $F_T$ ($F_p-F_T$), an approximate measure of the degree of photosynthetic quantum conversion (Lichtenthaler and Rinderle 1988), was somewhat lower in the droughted seedlings than in the other two treatments, though not significantly so (data not presented).

The time for the induction kinetics to reach certain major features shows differences due to the treatment the seedlings received. The time taken to attain $F_p$ or $F_{\text{max}}$ was longer in "ambient" or "watered" seedlings than in the "dry" seedlings. The time to achieve the minimum $F_S$ however shows a reversal of this pattern with the "dry" treatment.
seedlings taking more time to reach the minimum than the seedlings of either the "ambient" or the "watered" treatments (Fig. 4.7).

**Equation 4.2**

Calculation of the Rfd value modified from Lichtenthaler and Rinderle (1988). The original equation was for probe type fluorometers and the modified equation compensates for the use of \(F_0\) as the basis of the normalization of variable fluorescence measurements.

\[
Rfd = \frac{F_p - F_T}{F_T + F_0}
\]

The seedlings which were chosen at random from the "dry" treatment during the experiment and re-watered, showed some recovery (three days after re-watering) in APS even after enduring shoot water potentials down to -2.5 MPa (Fig. 4.8), and showed some recovery from potentials lower than -3 MPa. The variable fluorescence features, such as \(F_p\), of the seedlings which were re-watered, were in accordance with the drought stress they had endured. Seedlings which had endured less drought had greater values for \(F_p\) three days after being re-watered.

**Discussion**

Despite the precautions taken to keep shading to a minimum,
the PAR light intensity under the canopy was approximately 20% lower than ambient, and the canopy may also have reduced wind velocity around the seedlings. These factors may be the cause of the slightly lower dry weights and heights at the conclusion of the experiment in the seedlings which were under the canopy.

Shoot and needle water potential remained steady in the "ambient" and "watered" treatments throughout the experiment, but decreased sharply in the "dry" treatment as the number of days since last watering increased. The decreased water potentials were due to a decline in water available to replace that used in cell physiological processes and lost by transpiration.

$F_0$ is reported to be unaffected by drought in species as diverse as *Pinus radiata* (Conroy *et al.* 1986) and grape *Vitis vinifera* (Downton and Millhouse 1984), and here too there was no significant effect on $F_0$ due to either the watering regime or the presence of the canopy. The decline in both $F_0$ and absorbency in all three treatments during the experiment is consistent with seasonal changes in the pigment complement of the seedlings (see chapter 5). In early autumn, there is a seasonal decrease in the chlorophyll content of Douglas-fir seedlings (Hawkins and Lister 1985). As $F_0$ declined the needle dry weight also declined, giving a weak linear relationship (see Fig. 4.9).
This indicates that \( F_0 \) has some correlation to needle dry weight, which would be the case if \( F_0 \) is, as thought, a measure of the quantity of photosynthetic pigment active during the fluorescence assay.

\( F_0 \) was tested experimentally as a basis for APS rate normalization because of its relationship to needle dry weight. Data presented in this way was compared to APS rates presented on a per unit dry weight, and produced analogous results suggesting that either method is acceptable. \( F_0 \) may have some advantage over dry weight because it is linked to the actual rate of photosynthesis, and also it is non-destructive (for further discussion see chapter 5).

The depression effect on APS and Rd of increasing drought stress was clearly demonstrated (Fig. 4.1 and 4.2). In contrast the rates were more or less constant for the seedlings kept in the two watered regimes. Kaiser (1987) noted that photosynthetic rates were diminished under drought stress conditions. A noticeable decline in the APS rate in the "dry" treatment was apparent at shoot water potentials of only -1.1 MPa, which occurred after four days of drought. A slightly longer time than this was needed for the Rd rates of the droughted seedlings to fall significantly below those of the controls.
The $F_p$, $F_s$ and $F_T$ features of variable fluorescence, all showed a marked decline in the seedlings under the "dry" treatment regime (Fig. 4.3). This agrees with the findings of Bjorkmann and Powles (1984) who reported that under water stress,

"...the variable ($F_v$) fluorescence yield at 692 nm was strongly quenched but there was little effect on the instantaneous ($F_0$) fluorescence. These results indicate an inactivation of the primary photochemistry associated with photosystem II."

$F_p$, $F_s$ and $F_T$ all appear to react proportionately to the severity of the water stress the seedlings are experiencing, and there is a relatively linear relationship between these values and seedling shoot water potential. For example, Fig. 4.4 shows $F_p$ versus shoot water potential including a regression of the "dry" treatment results. This data suggests that each of the three variable fluorescence values act as measures of seedling drought stress. The decline in $F_v$ with increasing drought stress may be due to photoinhibition, a reversible reduction of photosynthetic carbon assimilation capacity caused by PAR intensities in excess of the photosynthetic requirement of the plant (Krause 1988). A decline in the rate of electron transport resulting from photoinhibition may result in a corresponding falling of $F_v$ yield. This would therefore suggest a relatively linear effect of stress on electron transport rates. The actual mechanisms of photoinhibition have not been definitively elucidated as yet, but one possibility is
that P680+ is prone to damage from excitons and the probability of this occurring is therefore dependent on light intensity and the rate of electron donation to P680+ from the water oxidation complex (Cleland and Melis 1987). If this mechanism is in operation, the results from white spruce suggest that water oxidation is adversely affected by drought stress. Another possible method of photoinhibition is light induced structural changes in the Q_B electron acceptor (possibly at the 32 kDa binding protein), when resynthesis is slower than the rate of degradation (Krause 1988). This would lead to reduced electron transport, which is thought to induce reduced efficiency of the P680 reaction centre (Krause 1988). A mechanism of this type would also explain the observed decline in Fv.

The seedlings which were not subject to significant water stress ("ambient" and "watered" treatments) showed relatively large variability in F_p, F_s and F_T (Fig. 4.4), which could confound the direct use of fluorescence values as indicators of drought stress. This variability may be due in part, to the seasonal decline in photosynthesis, reflected in APS and Fv data, known to occur in late summer in conifers (Lister et al. 1967, Senser and Beck 1978).

Fluorescence quenching after F_p, increased with the severity of the drought stress until the seedlings were so severely stressed that they did not recover (Fig. 4.6). This agrees
with the results of Conroy et al. (1986) and Wiltens et al. (1978).

Fv was not significantly lower in the droughted seedlings compared to the control seedlings until the drought stress had become very severe (Fig. 4.3 for example). This may indicate that drought stress is manifested in two linked components, a stomatal component and a non-stomatal component, sometimes referred to as being chloroplast level stress (Lawlor 1983, Bjorkman and Powles 1984). The effects of the two components are difficult to separate (Woolhouse 1983) but they may be revealed by the difference between the effects of drought measured by APS and those measured by fluorescence assay. Drought stress causes the APS rates to be noticeably lower in the droughted seedlings at water potentials below approximately -1.2 MPa (compare with the control seedlings at -0.6 to -0.8 MPa), whereas there is no marked difference in the Fv values until the water potential has reached almost -5 MPa (Fig. 4.4). The initial effect of drought stress is probably the restriction of CO₂ as the stomates close, this will affect the APS rates once the reduced supply of CO₂ becomes the limiting factor on photosynthesis. At this point in the drought, there may be no direct effect on the electron transport systems within the photosystems. The APS rates continue to decline and Fv also begins to decline as the drought becomes severe.
Figure 4.1

The response of apparent photosynthesis rate per unit \(F_o\) to increasing drought stress ("dry" treatment). Control treatments ("ambient" and "watered") are also shown. +, "watered" treatment; o, "dry" treatment; *, "ambient" treatment. Tukey's LSD is indicated. \(N = 7\) (each point represents mean value of 7 seedlings).
Figure 4.2

Response of Rd to drought stress with control treatments included. +, "watered" treatment; o, "dry" treatment; *, "ambient" treatment. Tukey's LSD is indicated, $N = 7$. 
Figure 4.3

The response of various features of the variable chlorophyll a fluorescence induction curve to drought stress in the "dry" treatment seedling. *, \( F_p \); o, \( F_T \); +, \( F_s \); Tukey's LSD is indicated, \( N = 7 \).
Figure 4.4

The response of Fp to drought stress in all three treatments. The regression line of the "dry" treatment is shown. Tukey's LSD is indicated, N = 7. +, "watered" treatment; o, "dry" treatment; *, "ambient" treatment.
Figure 4.5

The response of Rfd to increasing drought stress in white spruce seedlings. The control treatments are also shown for comparison. Tukey's LSD is indicated. N = 7; +, "ambient" treatment; *, "watered" treatment; o, "dry" treatment.
This may be the so-called non-stomatal component in which the actual photosystems and/or associated mechanisms begin to be affected. Because of the reduced efficiency of CO₂ assimilation, there is more energy from absorbed light to be dissipated by other routes (Kaiser 1987). This may cause the initial rise in Fv noticed at moderate water stress. Many researchers have reported that drought stress caused Fv to decrease (Wiltens et al. 1978, Bjorkman and Powles 1984, and Conroy et al. 1986) and the results with white spruce showed a large decline in Fv as drought stress increased (Fig. 4.3). However, prior to the large decline, all Fv features exhibited an initial small increase with the onset of drought stress. A similar small rise in Fv for various plant species under drought stress, before the commonly observed large decline has been reported by Wu and Todd (1988). As CO₂ assimilation decreases even more, damage to the photosystems may result from electrons passing to oxygen, producing superoxide, OH⁻ radicals, and H₂O₂ (Lawlor 1983). This may also explain why drought stress has been reported to be much more severe in plants exposed to high light during drought, and why drought stress symptoms resemble photoinhibition (Lawlor 1983, Bjorkman and Powles 1984, Downton and Millhouse 1984, Kaiser 1987).

Seedlings were re-watered after reaching various drought stress severities. The purpose of this was to attempt to determine the point at which permanent damage occurred.
Seedlings which reached water potentials less than approximately -4 MPa showed no recovery of photosynthesis (Fig. 4.8), and little variable fluorescence. Cell turgor is thought to be important in drought stress, and it may explain this lack of physiological recovery observed after exposure to severe stress (Kaiser 1987). Plants that can control turgor and prevent plasmolysis are more resistant to drought and in most species there is a high mortality rate once turgor is lost and this may be the deciding factor which dictates whether the seedlings recovered after re-watering (Downton and Millhouse 1984). In many seedlings which had been exposed to relatively severe drought conditions a partial recovery was noticed when they were re-watered. This may be due to increasing photoinhibition which although leading to permanent photodamage, protects the tissue from complete destruction and allows at least partial recovery when the drought stress is ameliorated (Krause 1988).

The compound or derived measures of fluorescence, which are used as indices of seedling physiological health, also show potential as indicators of drought stress but they too exhibited high variability in the seedlings not subjected to drought. In this category are $F_p/F_T$ and $Rfd$ (Lichtenthaler 1987). For $F_p/F_T$ a ratio greater than approximately 2.5 was only seen in the seedlings which were drought stressed; for the non-drought stressed seedlings ratios less than 2 were
the norm (Fig. 4.6). For the last set of measurements done on the droughted seedlings the $F_p/F_T$ ratio was negative, but this may be a mathematical quirk because at this point the $F_v$ curve was effectively flat and the seedlings were probably dead (there was no recovery upon re-watering). These results may be evidence that $F_p/F_T$ ratio does provide a gauge of water stress although like the direct factors $F_p$ and $F_T$, $F_p/F_T$ did not become markedly different from the controls until the drought was well advanced. The Rfd value showed a similar pattern to that of the $F_p/F_T$ ratio. As the water stress increased the Rfd value increased correspondingly except when the droughted seedlings were so severely stressed as to be nearly dead (Fig. 4.5). Again as with the other fluorescence factors which were measured, the Rfd values of the controls were variable enough to nullify its potential as a practical gauge of drought stress. This problem was common to all the values based on variable fluorescence and may have been just an artifact of autumnal inactivation of photosynthesis (Hawkins and Lister 1985). In which case $F_p$, $F_T$, Rfd, and $F_p/F_T$ may all be found to be useful in detecting water stress in plants in some situations as has been the case with other investigations (Conroy et al. 1986, Havaux et al. 1988).

The time taken for fluorescence induction to achieve one of the three recorded features $F_p$, $F_S$ or $F_T$ was also measured
The ratio of $F_P:F_T$ in relationship to shoot water potential for all three treatments. Tukey's LSD is indicated, $N = 7$. +, "watered" treatment; o, "dry" treatment; *, "ambient" treatment.
Figure 4.7

The time taken for the seedling induction curves to reach points $F_p$, and $F_s$ following shutter opening in "dry" treatment seedlings. To enable both curves to be presented on one axis the $F_s$ time values were all divided by 30. Note that the highest shoot water potential values on the curve are comparable to those of watered seedlings. Tukey's LSD is indicated for each curve, $N = 7$. +, $F_s$; and o, $F_p$. 

87
Figure 4.8

The recovery of previously drought stressed seedlings, presented as APS rates (per unit $F_o$) measured 3 days after re-watering versus the shoot water potential of the seedlings immediately prior to re-watering. The overall mean APS rate of the "watered" treatment seedlings is shown by the horizontal line. Tukey's LSD is indicated $N = 5$.  

"dry" treatment.
Figure 4.9

The relationship between $F_0$ and seedling needle dry weight. All three treatments are included as there was no significant treatment effect for these two factors. $N = 7$, i.e. each point represents the mean value from 7 seedlings. Correlation coefficient $R = 0.89$, significant at 0.0001.
and the results give evidence that as drought increased the Fp point was reached sooner after exposure to light, and the Fs minimum point was reached later (Fig. 4.7). This suggests that backup of electrons in the electron transport system is more rapid when the plant is drought stressed which leads to an earlier Fp. This may be because as stress increases, the electron transport quenching pools become depleted so that they are exhausted sooner than in seedlings which are not under stress. Slower electron transport from PS I due to depletion of carbon assimilation intermediates may cause Fs to be increasingly delayed as the drought stress intensifies.

**Conclusions**

F₀, and other morphological factors of the seedlings were unaffected by 27 days of drought stress. F₀ was found to have a significant linear relationship with needle dry weight.

APS and Rd rates were both reduced by the drought stress, but the basis on which APS was presented (i.e. per unit F₀, or per unit needle dry weight) was found not to effect the results.

Variable fluorescence factors (Fp, Fs, and Ft) all declined
with increasing drought stress, in a linear relationship with shoot xylem water potential, and with needle water potential (data not shown). The two component theory of drought stress (Bjorkman and Powles 1984) was supported by the results as APS showed a much faster response to drought than Fv. Compound measures of fluorescence such as $F_p/F_T$ and Rfd were similar to basic variable fluorescence characteristics as indicators of drought. $F_p$ was reached sooner in the drought stressed seedlings compared to the controls, while $F_S$ was reached later due to drought stress. APS rates are probably a better method for detecting minor water stress in white spruce than chlorophyll $a$ fluorescence techniques; but physical measurements such as shoot or needle water potentials are cheaper and easier than either APS or Fv measurements, and are therefore probably most suitable for practical use. However there is a proviso to this, water potential measurements provide no information about damage caused by drought stress and this limitation may need to be compensated for by the use of a physiological assay. Variable chlorophyll $a$ fluorescence values such as $F_p$ and $F_T$ may be of most use, as tools, for the investigation of the effects of severe drought stress, as opposed to use in routine water status monitoring.

Finally there may be a possibility of using chlorophyll $a$ fluorescence not as a tool for monitoring water stress but for monitoring the response of plants to water stress. In
this way fluorescence could be used in breeding programs to select for drought tolerance on the basis of photosynthetic resilience to drought.
Chapter Five

Chlorophyll A Fluorescence As A Tool For The Detection Of Damage Caused By Atmospheric Ozone Pollution.

Introduction

Airborne ozone pollution in the lower mainland of B.C. is known to have a marked effect on agricultural production. Wilson (1984) estimated the loss to be about $9 million in economic terms in 1984 due to reduced growth. This raises concerns about the effect of such pollution on the forest nursery industry in the lower mainland. Lower mainland nurseries in B.C. produce large quantities of conifer seedlings for reforestation purposes (about 80 million per year, or approximately one third of the total number produced in B.C.) (Willingdon, pers. comm.). Any adverse effect on the seedlings may have serious implications on the future pace of reforestation in the province.

Ozone is an allotropic trimeric form of oxygen produced by the photochemical action of ultraviolet radiation in the upper atmosphere. Ozone produced in this way tends to remain in the upper atmosphere where it acts as a screen for ultraviolet radiation (between approximately 210nm to 290nm), and thus benefits the biosphere. Ozone however, can
also have a destructive effect on organic tissue because of its capacity as a potent oxidizer. Ozone is produced in the lower atmosphere by nitrogen oxide-hydrocarbon reactions, and reacts with and oxidizes organic matter on contact, causing injury. Thus, even in low concentrations, ozone can be destructive to plant and animal tissue. Many researchers have reported that ozone has adverse effects on tree species both in morphology and in physiology (Jensen 1982, Hogsett et al. 1985, Reich and Amundson 1985, and Shafer et al. 1987). Reduced height, and root and shoot dry weights were observed in ten species of forest tree by Kress and Skally (1982). Reduced photosynthetic rates were described by Sasek and Richardson (1989), and Koziol (1984) reported altered carbohydrate metabolism.

In the lower mainland region of B.C., ozone has been constantly monitored (over the last decade) at different locations both in the Fraser valley and on the north shore mountains. The concentrations generally range from <10 μg m\(^{-3}\) to 85 μg m\(^{-3}\) with highs up to 240 μg m\(^{-3}\). The lowest values occur during the night with the daily high typically in mid-afternoon (approximately 15:00 hours). The seasonal peak usually occurs in late spring (May) (Coligado, pers. comm.). To address the concern about the effects of atmospheric pollution on conifer seedling production and because of the lack of information concerning this problem, an experiment was devised. This examined the effects of the
ambient levels of airborne pollution (specifically ozone) in conifers at one of the large forest nurseries (Ministry of Forests Nursery, Surrey B.C.). Both physiological and morphological indicators were measured to give a clear overall view of the effects, if any, that the current atmospheric pollution has on conifer seedlings. Coastal Douglas-fir was selected as the trial species because it is widely grown in the lower mainland.

Chlorophyll a fluorescence was seen as a potentially useful tool to aid in this type of investigation because of the reported effects of ozone on the photosynthetic rates of trees (Reich and Amundson 1985). This type of exploratory experiment was also seen as a good chance to test the utility of the chlorophyll a fluorescence system in a practical application. Results from atmospheric pollution experiments and even nutrient deficiency experiments have indicated that chlorophyll a fluorescence is able to detect damage to plants significantly earlier than other physiological or morphological assays are able to (Schreiber et al. 1978, and Baillon et al. 1988)

The aim of the experiment was to determine if the health and vigor of nursery seedlings is affected by current air pollution levels. This was done by testing both physiological and morphological parameters of conifer seedlings during their initial growth phase, from
germination to the end of the first season when the seedlings would be lifted for cold dark storage.

**Materials and Methods**

Coastal Douglas-fir seedlings of seedlot 4390 (Duncan, Vancouver Island, altitude 700m, latitude 48°17' N, and longitude 123°42' W) were seeded in mid April 1989, into PSB 313 styroblocks. On May 2 the blocks were randomly allocated to six plots at Surrey Nursery, such that each plot had 16 styroblocks (with 160 seedlings per block) in total. Four of the plots were located in open top chambers made of transparent plastic with a metal frame and standing 3m tall with a diameter of approximately 2.4m (see Fig. 5.1a). The remaining two being open plots in line with the chambers, and identical to the chambered plots except for the enveloping chambers. The styroblocks were placed in their allotted plots at the beginning of May, and thinned as per typical commercial nursery practice in mid May. Rails were used under the edges of the blocks to raise them off the ground about 4cm, enough to allow air pruning. The six plots accommodated three ozone treatments which were: ambient, filtered, and unfiltered. These three treatments allowed the determination and separation of effects on the seedlings due to the chamber and due to the ambient atmospheric pollution. The seedlings in the two open plots
received the ambient ozone treatment (because they were naturally open to the atmosphere). All four of the chambers had air pumped into them from 09:00 to 17:00 each day by large fans connected to each chamber, these fans forced air into the double skin of the chambers from where it was dispersed into the interior through an array of perforations in the lower half of the inner membrane. Two of the chambers were fitted with charcoal filters which removed hydrocarbons and ozone from the incoming air-stream; this was the filtered treatment. The other two chambers were not fitted with filters and therefore the seedlings received normal atmospheric air; this was the unfiltered treatment. All the plots were periodically weeded, supplied with nutrients (as necessary), and at weekly intervals the styroblocks were re-randomised within each treatment (see Fig. 5.1b).

Each month between May and October 1989 at intervals of approximately four weeks, random samples of three seedlings from each plot were taken to S.F.U. for morphological and physiological status assays. Three seedlings from each of the six plots, one seedling from each of three styroblocks, were randomly selected (the outer row of each styroblock was not used to reduce edge effects). The seedlings were collected the day previous to the assays and transported to the laboratory in styrofoam cool boxes with their roots
Figure 5.1

a) The allocation of seedlings to the plots on May 2, 1989, with a view of two of the chambered plots (left and centre) and one open plot (right) prior to the seedlings being stationed on it. The open plot shows the rails which the stryroblocks were set on to allow air pruning of the roots.

b) View of three of the four chambered plots during the weekly re-randomization, the fan unit is just visible to the right of the nearest chamber.
c) The layout of the styroblocks within one of the chambered plots; the same layout was used in the open plots as well. The inlet port for the ozone monitor can be seen hanging above the seedlings and, in the top left hand corner, the perforations in the inner membrane are visible.
wrapped with wet paper towels inside plastic bags. At S.F.U. they were set into separated styroblock plugs, watered, and placed outdoors. The next day they were removed from their wrappings and again watered. Each of the seedlings was measured for fluorescence and absorbency according to the methods described in Chapter 2, and following this they were measured for gas exchange using the protocol described in chapter 4. Seedling height, root collar diameter and the temperature and barometric pressure were also recorded. Finally the seedlings were oven dried for 48 hours at 85°C to obtain dry weight measurements of the stems and needles.

Concomitant measurements were also done during the course of the experiment on the ozone concentrations at Surrey Nursery and around the lower mainland. The ozone concentrations at 6 locations in the nursery plots were measured. Four directly above the seedlings in each of the chambered plots (see Fig. 5.1c), one at foliage height over one of the open plots, and the sixth at 3m above the open plots. These measurements were done by means of a Scaniavalve system which sampled each of the six pick-ups for four minutes every thirty minutes, feeding air samples to a (5513340 Bendix Chemiluminescent) ozone monitor. Weather data, specifically radiation, temperature and humidity were also measured on a continuous basis directly beside the plots.
Figure 5.2

Respiration rates on a needle dry weight basis, showing the differences which occurred during the course of the growing season and the noticeable increase in October. The significantly higher respiration rate in the open plot ambient treatment compared to the two chambered treatments during certain months is discussed in the text. Tukey's LSD is indicated, N = 6.
Watering was done according to nursery practice by using overhead sprinklers with six nozzles above each plot. PAR light measurements were also recorded within and around the chambers using a light meter (Li-Cor, LI-185).

Results

The only parameter which showed a significant effect due to the atmosphere filtering treatments was shoot respiration on a per unit of needle dry weight basis (see Fig. 5.2). None of the other parameters showed any significant treatment effects. However almost all the measured parameters did show significant effects over the time course of the experiment (see Table 5.1) except APS presented on a per unit $F_0$ basis.

Morphological measurements:— both shoot and needle dry weight increased markedly each month up until late September. The seedlings in the unfiltered tank then declined in dry weight the following month. Though the seedlings in the filtered tank and in the open ambient plots continued to increase in dry weight into October. Seedling height increased in all three treatments approximately equally throughout the duration of the experiment. Root collar diameter and absorbency also increased throughout the course of the experiment regardless of the ozone treatment.
Gas exchange measurements: - APS when presented on a per seedling basis showed a general increase over the season with a small reduction after the September measurement in the unfiltered treatment (Fig. 5.3). When presented on a per gram dry weight basis the seedlings in the open plots showed a decline in APS between May and October (data not shown). With \( F_0 \) as a basis for the APS values there was no significant differences between the APS rates during the experiment. Respiration per gram dry weight declined from May to September then increased in October (see Fig. 5.2). Also the respiration per gram dry weight was significantly greater in the seedlings in the ambient plots than in those in either of the two chamber treatments.

Fluorescence measurements: - \( F_0 \) did show a marked trend similar to dry weight, height, and absorbency (see Table 5.2). \( F_0 \) increased until September and then declined in October. \( F_P \) was variable with the highest values occurring in September but with no overall pattern. \( F_T, F_S \) and \( F_M \) all showed rises from May to September but in October \( F_M \) and \( F_T \) dropped below their September values and only \( F_S \) rose after September.

Environmental measurements: - Ozone concentrations, measured above the open plots, rarely exceeded 60 ppb, and the highest daily maximum was 79 ppb (June 11). In general
the daily maxima were in the order of 40 ppb. Temperature was found to be approximately 1°C higher inside the chambers than in the open plots. PAR light in the chambers was reduced by a small amount (approximately 10%) due to the polyvinylchloride walls though ultra violet light was reduced more severely. The relative humidity inside the chambers was on average 3% lower than in the open plots.

Discussion

From the results it seems that the seedlings were not adversely affected by the atmospheric concentrations of ozone which occurred, unless the effects were apparent only in other mechanisms or in magnitudes less than were detectable. Traits such as height, root collar diameter, and foliage dry weight have been found to be affected by ozone at levels only slightly above ambient (generally between 40ppb and 60ppb) in Loblolly pine seedlings (Pinus taeda); though genetic variation caused varying sensitivity such that ambient ozone concentrations showed no effect on some seedlings whilst being very significant in others (Shafer and Heagle, 1989). Thus adverse effects in Douglas-fir seedlings may have been offset to some extent by reduced sensitivity due to genetic variation. Pitch pine seedlings (Pinus rigida [Mill.]) are known to exhibit intra-specie variation in sensitivity to ozone (Scherzer and McClanahan
Other research has shown that Douglas-fir is relatively insensitive to ozone pollution compared to other conifer species. A study done over the course of a single growing season using simulated ambient ozone concentrations found that Ponderosa pine and Lodgepole pine seedlings exhibited significant signs of visible injury whereas Douglas-fir seedlings showed no response under identical ozone exposure (NCASI 1989). Another factor which may have lead to the lack of significant effects of ambient compared with filtered air treatments is that ozone damage can be attenuated by plants during periods of low ozone concentration, especially in darkness (Schreiber et al. 1978). There were also considerable periods of time during the course of the experiment when the ambient ozone was at low concentrations. The low daily maxima of around 40 ppb were also not conducive to severe stress when compared to the concentration of 120 ppb designated in the United States as being "air pollution" (Singh et al. 1980).

Variable chlorophyll a fluorescence measures such as $F_T$ and $F_P$ did not vary significantly; this may be because there was no damage to the shoot physiology in the seedlings due to airborne pollutants. Ozone has been shown to increase $F_T$ and decrease $F_P$ and $F_M$ parts of the induction curve and only after prolonged exposure to cause a decline in $F_O$ (Schreiber et al. 1978). The decline in $F_O$ seen after August in this
experiment was not due to ozone damage however as it was evident in all treatments.

The dry weight, height, root collar diameter and absorbency of the seedlings all increased over the course of the experiment as the seedlings grew. All the morphological factors correlate extremely well with each other and this includes $F_0$ (see table 5.2). These results support the possibility of using absorbency or $F_0$ as measures of seedling size. Both of these measurements have distinct advantages over dry weight because they are instantaneous and non-destructive. $F_0$ is probably a better measure for physiological purposes because it estimates the size of the photosynthetic pigment complement of the seedlings whereas absorbency is dependent on total tissue area and colour. $F_0$ and absorbency showed a nearly linear relationship until early autumn after which absorbency was more closely correlated to dry weight. The relationship between $F_0$ and needle dry weight is approximately linear for the initial few months but then deviates from this after August (see Fig. 5.4), the same time at which the $F_0$ values started to decline. After August (see Fig. 5.3), the APS rate declines and so does the $F_0$ but the needle dry weight continues to increase. This indicates that the amount of photosynthetic tissue in the seedlings was increasing as the seedlings grew, but began to decline in September and October from a
Table 5.1

Analysis of variance summary for treatment effects of the morphological and physiological values recorded over the period of six months on Douglas-fir seedlings at Surrey Nursery. Fisher ratio (F) values and degrees of freedom (df) are presented. Regime refers to the ozone treatments (ambient no tank, ambient tank, and filtered tank). APS, apparent photosynthesis; Resp., respiration; ***, significant at 0.001 level; **, significant at 0.01 level; ns, not significant.

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<th>APS /tree</th>
<th>APS /g dry weight</th>
<th>APS /FO</th>
<th>Resp. /tree</th>
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maximum in August at which time the dry weight of both the stem and needles was still increasing. This suggests that whilst the seedlings were still accumulating dry weight in October the size of their photosynthetic pigment complement was decreasing. This may be a physiological preparation for winter, where greater sturdiness may be of more benefit to the seedlings than rapid photosynthesis. $F_o$ is known to decline in autumn and winter as a portent of changes in the pigment system (Hawkins and Lister 1985). A reduction in the photosynthetic tissue in autumn may confer an advantage on the seedlings by reducing the amount of light captured by the photosynthetic pigments. With less light captured by the pigments there is less energy transferred to the photosystems which consequently lessens the chance of overload when the temperature is very low and energy disbursement from the photosystems is slowed (Öquist and Ogren 1985). This may be one reason why a reduction in the photosynthetic pigment during autumn is advantageous to seedlings during the winter. The $F_o$ parameter closely followed the APS rates in the seedlings in a directly linear relationship (see Fig. 5.5). This, combined with the strong evidence for $F_o$ as a good measure of morphological size suggests that using $F_o$ as a basis for the normalization of various other measurements would be an improvement over other currently used values such as dry weight. Measurements of APS and Rd rates are
Table 5.2

Correlation coefficients (R) of selected pairs of morphological and physiological parameters, n = 107.

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<th>Parameter</th>
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<tr>
<td>F₀ : APS per seedling</td>
<td>0.95</td>
<td>significant at 0.0001</td>
</tr>
</tbody>
</table>
Figure 5.3

The relative progression of apparent photosynthesis, Fo, and needle dry weight of Douglas-fir seedlings during the first growing season (mean of all three treatments). This shows how Fo follows the physiological factor (APS) much more closely than does the needle dry weight which at present is perhaps the most commonly used basis for representing APS in conifers (i.e. \( \mu g CO_2 h^{-1} \) gram needle dry weight\(^{-1} \)). Tukey's LSD is shown for each of the three parameters, \( N = 18 \).

Note: the values recorded in May were set to unity and those recorded in latter months are relative to the May readings.
The combined results from all three treatments show how needle dry weight and $F_0$ had an almost direct relationship during the summer, which was then lost in early autumn as the $F_0$ began to decline even though the needle dry weight was still increasing. Tukey's least significant difference (LSD) is indicated, $N = 18$. 

Figure 5.4
Figure 5.5

Apparent photosynthesis per seedling shows a very high correlation with the initial fluorescence $F_0$. The relationship remained even when the seedlings began to be less photosynthetically active in the early autumn. Tukey's LSD is indicated, $N = 18$. 
examples of where $F_0$ could be used as the normalizing factor to improve these physiological estimates. At present many values which are used as estimates of different physiological processes are presented in units relative to dry weight, or surface area of the plant. Gas exchange measurements are often presented as $\mu$gCO$_2$ h$^{-1}$ gram dry weight$^{-1}$ or $\mu$gCO$_2$ h$^{-1}$ cm$^{-2}$ and as Sestak, Jarvis and Catsky (1971) pointed out succinctly:

"Herein lies an unresolved problem. What is the most appropriate plant characteristic on which to base the quantitative expression of photosynthetic rate?"

They went on to say:

"The rate of a process yields maximum information about the process itself if it is expressed on the basis of a plant characteristic which limits or at least strongly influences the process. Leaf dry weight does not seem to be a very logical basis on which to express photosynthetic rate since it has little or no direct relationship with radiation absorption... It is generally used purely for convenience for leaves the area of which is difficult to estimate."

In 1990 this is still an unresolved problem and it is clear that there is a place for an appropriate new basis for both photosynthetic and respiratory rate data, as well as other measures, and the results suggest that $F_0$ has the desirable characteristics required. The quantity of active photosynthetic pigment which $F_0$ represents may limit, and does at least influence, the rate of the process of photosynthesis.
This linked with the fact that \( F_0 \) is not adversely affected by complex tissue shape, and may be non-destructively determined, make it an ideal candidate to replace needle dry weight.

APS rates given on the basis of \( \mu gCO_2 \ h^{-1} \ F_0^{-1} \) are directly comparable with other measurements presented per unit \( F_0 \) as long as a standardized measurement routine was used (see Chapter 3). In the case of this experiment, young plants measured in May can be compared with more mature ones measured in October. This may not be so for APS rates expressed in terms of \( mgCO_2 \ h^{-1} \ g \) needle dry weight\(^{-1} \). Whereas \( F_0 \) is a measure of the quantity of active photosynthetic pigment and as such is strongly correlated with the photosynthetic process (see table 5.2), needle dry weight is not reflecting a characteristic which strongly influences the process of photosynthesis. In May when the seedlings had recently germinated their dry weight was low compared to the size of the photosynthetic tissue but by October when the seedlings had increased in bulk the dry weight was considerably higher and, in part, this was not due to an increase in photosynthetic tissue but to other matter which had been accumulated. In contrast, \( F_0 \) remains a measure of photosynthetic tissue throughout the life of the plant and is therefore a more satisfactory basis on which to base APS rates which are to be compared between plants of significantly different ages.
Conclusions

Ambient levels of ozone and other airborne hydrocarbon pollutants had no detectable effect on Douglas-fir seedling growth and development for either the morphological or the physiological parameters measured during this experiment in the initial growing season. This does not however, rule out the possibility of delayed adverse effects, though it would seem likely that any significant effects on the seedlings would have been detectable with the methods employed in this experiment. These results go somewhat against the findings of other researchers previously mentioned (Schreiber et al. 1978, Shafer and Heagle 1989, Sasek and Richardson 1989). One explanation for this might be that the experiment was conducted to evaluate the effects of the ambient ozone pollution which the seedlings are naturally subjected to, and during the summer of 1989 the levels of ozone were quite low compared to those of previous years, where the ozone concentration has been above 60ppb on a regular basis (Coligado pers. comm.). Also, other research has often used enhanced ozone levels by controlled application of ozone rather than testing the effect of naturally occurring amounts. Adding ozone in this manner may result in a greater and more easily detectable effect on the tissue than was the case with this study.
appeared to act as a morphological measurement as predicted by the data in chapters 3 and 4, and this may allow improvements in the techniques of representing certain types of measurements, especially photosynthetic rate data for plants with difficult to measure surface areas.
Chapter Six

Summary Discussion

Development of the computer controlled fluorometer assay system, highlighted the associated problems and benefits of this type of system, and some of these are listed below.

Benefits:

1) Many functions were integrated so that the operator was relieved of some of the repetitive work. This is probably one of the largest benefits because shutter release, sampling frequency, sample duration, data analysis and data presentation, could all be pre-selected or pre-set and invoked by single keystrokes. There was probably also a sizeable subsidiary benefit from the resulting reduction of human errors.

2) The fluorescence data was already in a format which allowed computerized analysis.

3) Minimal labour was required to obtain normalized fluorescence data, thus data can be acquired easily and with high accuracy.

4) Ongoing costs were small.
Problems:

1) Data compression was difficult and time consuming, which reduced the effective rate at which usable data could be obtained.

2) The system was very dependant on the computer and was therefore susceptible to breakdowns.

3) Two general problems with computerized systems are that they can be difficult to implement, and the data produced can be difficult to test for validity, although it tends to be believed.

Automated curve reading to obtain values such as $F_O$, $F_P$, $F_S$, and $F_T$ was more successful than expected and facilitated data analysis. Overall, computerization was found to enhance the performance of the fluorometer assay system.

The non linear responses of $F_V$ to both dark pre-treatment and actinic light intensity shows a need for standardized procedures for variable fluorescence measurements. A long (greater than one hour) dark pre-treatment is required for relaxation of the proton gradient, however short dark pre-treatment times were found to be sufficient for the assessment of $F_O$, with the minimum time limited only by the decay of $F_V$. 
was shown to be effective as a measure of tissue size and found to have certain similarities to, and advantages over, other estimates of size such as surface area or dry weight. The close relationship of $F_0$ to the photosynthetic system (one of the primary physiological mechanisms) gives it great relevance to physiological assessments whilst still being independent of physiology. Because of this, and because it is non-destructively determined, $F_0$ is a good candidate in some circumstances to replace values such as chlorophyll content, leaf area and foliar dry weight. The many possible uses may include representation of gas exchange rate (as already demonstrated in chapters 4 and 5), and leaf area index, traditionally leaf area per unit ground area, but which could be replaced by $F_0$ unit ground area$^{-1}$ (Hunt 1978). Such methods of data presentation may yield new insight into plant growth, as was found with APS $F_0^{-1}$ values in Douglas-fir, which remained almost constant during the first growing season though the seedlings underwent a ten fold increase in height.

APS, and Rd rate changes, detected drought stress in white spruce seedlings before it was noticeable in changing $F_v$ values. However, $F_v$ did react to severe drought which caused all the features of the variable fluorescence induction curve to decline. Drought stress also caused the kinetics of the induction process in the seedlings to change, with $F_p$ occurring earlier and $F_s$ being delayed. The
indeterminate response of Fv to mild drought probably makes fluorescence assay ineffective as an ongoing water stress monitor, but it may be useful for selecting for drought tolerance in the photosynthetic system of seedlings. Values (such as Rfd), derived from basic features of fluorescence induction curves were not found to be better at determining drought stress than the basic features themselves.

Ambient hydrocarbon and ozone pollution levels in the lower mainland region of B.C. were not found to be immediately deleterious to Douglas-fir seedlings. This result, although negative, does indicate that the effects of episodic ozone pollution (as found in the lower mainland of B.C.) at the relatively low levels occurring in 1989 were not debilitating to Douglas fir nursery stock tested over the course of a whole growing season.


Personal Communications

Brooke, R.C., G.R. Lister, C. O'Reilly, and W. Vidaver, Simon Fraser University, Burnaby B.C. 1990.
