THE DEVELOPMENT OF A PHOTOPHYSIOLOGICAL ASSESSMENT SYSTEM FOR WHITE SPRUCE [PICEA GLAUCA (MOENCH.)VOSS.] SEEDLINGS AND MICROPROPAGATED PLANTLETS

by

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M.Sc.,University of Guelph, 1980

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

November, 1985

APPROVAL

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DEGREE: Doctor of Philosophy

TITLE OF THESIS: The Development of a Photophysiological Assessment System for White Spruce (Picea glauca (Moench.) Voss.) Seedlings and Micropropagated Plantlets.

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The Development of a Photophysiological Assessment System for White Spruce (*Picea glauca* (Moench.) Voss.) Seedlings and Micropropagated Plantlets.

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December 5, 1985
(date)

ABSTRACT

This thesis is concerned with the development of an assessment system for both young spruce seedlings and plantlets produced via improved micropropagation techniques. The application of the instrumentation and techniques is demonstrated in the present study.

The assessment protocol involved improved techniques to measure O_2 exchange as well as the evaluation of stomatal control of water loss. Optical instrumentation, which was designed to measure whole plant chlorophyll fluorescence, provided nondestructive indicators of photosynthetic activity and shoot size. Correlations were found between seedling fluorescence and gas exchange under conditions of soil and atmospheric water stress. These correlations allowed the estimation of levels of water stress and suggested the probable photosynthetic site(s), that were affected, as well.

Rooted plantlets used in studies were obtained via improved micropropagation techniques developed from reports of previous investigators. Studies comparing the physiology of seedlings to plantlets were done. These studies led to three conclusions concerning probable causes of the impaired physiology of micropropagated white spruce plantlets. First, the type of support medium used *in vitro* can influence on post-transfer CO_2 uptake. Second, effects of the *in vitro* environment, exclusive of the treatments imposed during micropropagation of plantlets,

also have significant influence on subsequent photophysiology. Third, other unknown factors in the *in vitro* environment, to which only plantlets are subject, are responsible for their deterioration once they are transferred to soil. These conclusions provide a basis for understanding what components of the *in vitro* environment must be modified in order to produce vigorous spruce plantlets via micropropagation techniques.

This thesis provides a test protocol useful in assessing the physiological status of micropropagated plantlets as well as determining probable causes for any detrimental effects due to the *in vitro* environment which are identified. The instrumentation and techniques presented here can easily be applied to other plant production situations, such as in conventional tissue culture and nursery propagation, forest nurseries, and field and greenhouse horticultural crop systems.

DEDICATION

To Alison and Sam with love.

ACKNOWLEDGEMENTS

I wish to express my appreciation to Dr. W. Vidaver for his guidance and the freedom which he allowed. Thanks to Dr. G. Lister for many valuable discussions and ideas and his review of my thesis, and to Dr. L. Albright for his input and review of my thesis. I would like also to extend my gratitude to Dr. W.D. Binder for providing me with seed as well as his suggestions and careful review of the thesis. Special thanks to E.B. Dumbroff, for acting as the external reviewer. Most of all, thanks to Alison and Sam for putting up with me for the duration.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Many problems can limit micropropagation of plantlets[1]. Major limitations include successful surface sterilization of the original explant, beneficial pretreatments prior to culture, and development of favorable protocols for shoot or embryoid induction (18,30,48). Once shoots are established, success of the propagation protocol is dependent on consistent rooting and then finally on acclimatization for survival and vigorous growth in vivo. For many plants the protocols for plantlet production are reasonably successful (18), but large losses are usually incurred at transfer to soil (2,6,75,106).

A review of pertinent literature indicates a variation of survival rates of outplanted progeny (2,6,18,28,48). With some species this survival rate is quite acceptable while in others it may be a major limitation to the success of the micropropagation program (2,18). There have been a few reports of the characterization of the physiology of plantlets of horticultural material at the transfer stage (12,19,25,28,115). The results of these studies have been interpreted as indicating poor development of the photosynthetic apparatus and an inherent lack of stomatal function.

Stomatal movements are commonly thought to be controlled by changes in the environmental conditions. However, there are

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[1] young plants produced via vegetative means

several reports demonstrating the modification of stomatal behaviour with the application of exogenous hormones (1,9,99,102). Stomatal closure under severe water stress conditions can be impaired by cytokinin application (1,102). These reports have implications for the stomatal behaviour of plantlet material that has been exposed to cytokinins during development in vitro. The concentrations of cytokinins to which angiosperms in culture are continuously exposed may be responsible for the lack of stomatal function (19,48). In contrast, conifers are exposed to hormone pulses only during bud or root induction treatments (2,6,84,108,109). Conifer plantlets would not be subject to the accumulation of cytokinins that would occur under a continuous exposure regime. If there is a demonstrated presence of normal function in stomata of conifer plantlets, it would be an indication that the lack of stomatal function found in some angiosperms is due to continuous hormone exposure and not some other component of the in vitro environment.

Physiology specific to micropropagated white spruce plantlets would require investigation of other relationships. The choice of the assay system requires some thought.

1.1 Choice of Physiological Assessment

The development of an assessment system for the determination of the physiological status and functioning of plantlets at the time of transfer to soil is necessary. The assay should be nondestructive and relatively simple to perform. These features would be particularly useful in tissue-culture tests as they would allow sequential, repeated assessments of each sample. Therefore, responses to environmental stimuli could be studied with fewer samples. The lower requirement in numbers would allow the elucidation of problems in plantlets before the proliferation rates of the protocol were large. This is essential in conifers such as spruce, where consistent production of shoots is not common and subsequent rooting of these shoots is also inconsistent (2,6).

Photosynthesis is a major determinant in growth and survival of plants, and it is also very sensitive to environmental stress (5,69,76,78,90,92,122). Therefore, it is a vital indicator of the plant's physiological state of being. Techniques for photosynthetic assessment can also be applied in nondestructive situations. Two of the most useful techniques are gas exchange and chlorophyll a fluorescence assays. The ideal gas exchange system consists of probes for both CO_2 and H_2O determinations. There is extensive literature on gas exchange system configurations (59), therefore the set-up of such an assay system is straightforward. Techniques for chlorophyll

fluorescence measurement have been developed previously, but the probe (87) has not been flexible enough to accomodate the measurement of spruce seedlings or micropropagated plantlets. The design of a new probe is required for the measurement of such material.

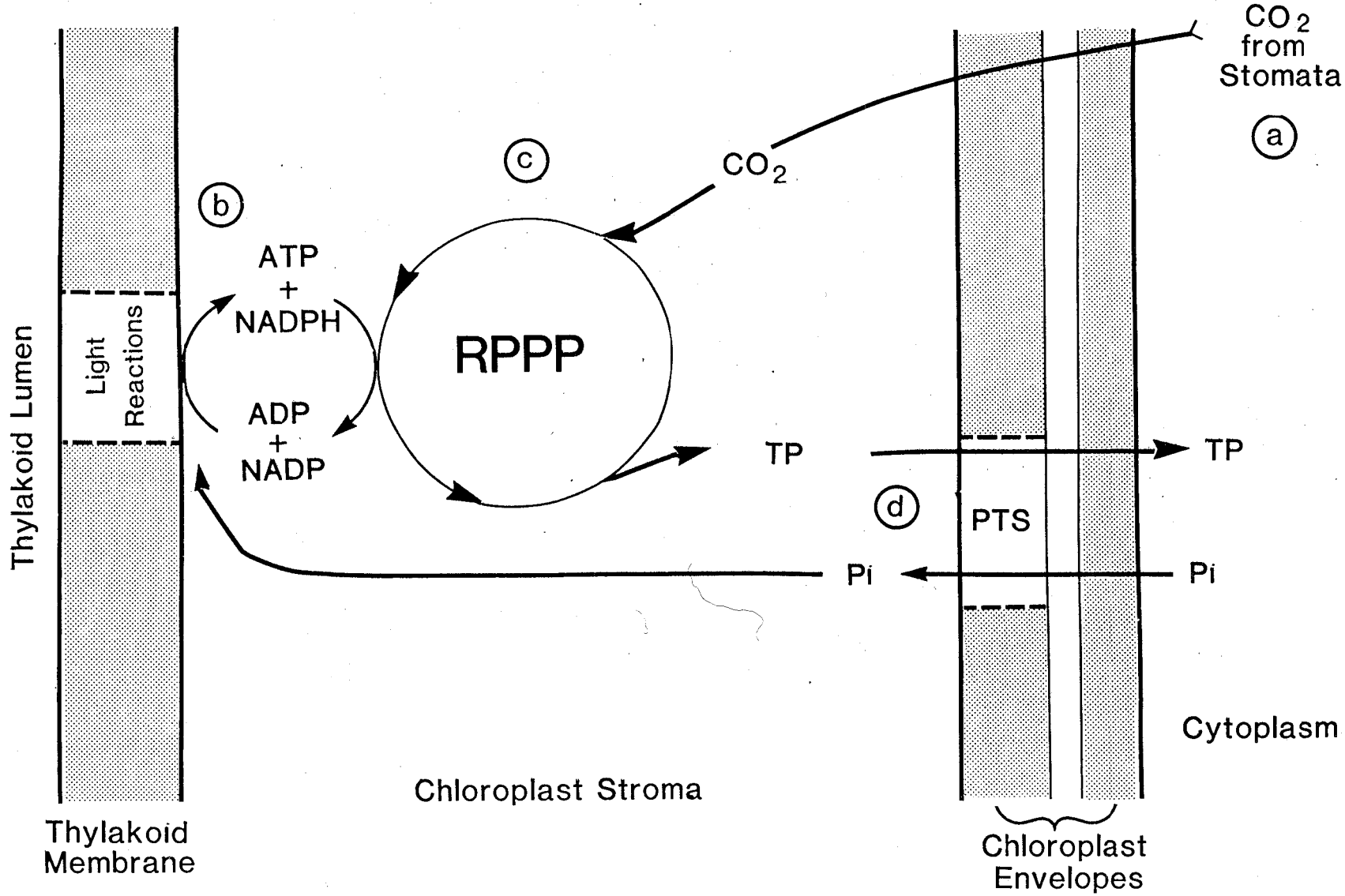
1.2 Limitations on CO_2 Uptake

There are several possible factors that may limit CO_2 uptake and they can be grouped into either the input side or the output side of the Calvin cycle. Figure 1.1 schematically represents the major components involved in photosynthesis. On the input side limitations may be imposed by the stomata (a), the light reactions (b), and the dark reactions (c). Limitations on the output side may be due to effects on triose phosphate utilization (d). These possible limitations and their detection will be discussed next.

The first limitation to be considered is the control of the availability of CO_2 by the stomata. This is regulated through stomatal closure and can be estimated by calculating stomatal conductance and then internal CO_2 partial pressures (36,91,93,111). The calculations are as follows:

$$\text{Conductance} = \frac{P \times E}{e_i - e_a}$$

Figure 1.1 - Schematic representation of CO_2 assimilation processes. Letters a - d represent potential sites for limitation of CO_2 uptake. (modified from Edwards and Walker (34)) Abbreviations: RPPP = Reductive Pentose Phosphate Pathway (Calvin cycle), TP = Triose Phosphate, PTS = Phosphate Translocatory Site, and Pi = Inorganic Phosphate.


CO_2 from Stomata
a
CO_2
c
b
ATP
+
NADPH
RPPP
ADP
+
NADP
TP
TP
d
PTS
Pi
Pi
Thylakoid Lumen
Light Reactions
Cytoplasm
Chloroplast Stroma
Thylakoid Membrane
Chloroplast Envelopes

where, P is the atmospheric pressure in kPa, E is the rate of transpiration in μmoles m^{-2} s^{-1}, e_i is the internal vapor pressure (assumed to be saturation vapor pressure) in kPa, and e_a is the ambient vapor pressure in kPa, then,

$$\text{Internal } CO_2 \text{ Partial Pressure} = \frac{(g - E/2)C_a - AP}{g + E/2}$$

where, g is the conductance to CO_2 (g is .0625 x the conductance to H_2O) in μmoles m^{-2} s^{-1}, C_a is the ambient CO_2 partial pressure in kPa, and A is the net assimilation rate in μmoles m^{-2} s^{-1}.

Two other sites of possible limitation are located in the mesophyll parenchyma, they are; the photoelectron transport system and the dark reaction components in the chloroplast stroma (22,23,91,111). Effects on photoelectron transport can be seen nondestructively, using techniques such as chlorophyll a fluorescence induction determinations (54,64,83,88,97). Effects on the dark reaction components, however, are not readily measurable using nondestructive, whole plant approaches. Therefore, their effects must be inferred by a process of elimination.

Control of CO_2 uptake may also be exerted on the output side of photosynthesis by the rate of utilization of triose phosphates. If there is a low demand for photosynthate by the

plant, then there will be poor utilization of the triose phosphates produced by photosynthesis (41,42,105,114,121). The uptake of inorganic phosphate is dependent on a phosphate translocatory site (PTS, Fig. 1.1) and this is linked to triose phosphate export. Therefore, if triose phosphates are not utilized a phosphate deficiency develops within the chloroplast stroma. This deficiency reduces the inorganic phosphate (P_i) available for photophosphorylation and thus slows or shuts down ATP generation and subsequently the regeneration of ribulose bisphosphate (47,50,91,114). This essentially shuts, or at least slows, down the reductive pentose phosphate pathway (the dark reactions) and little or no carbon is fixed by the chloroplast.

The rate of triose phosphate export is dependent on the photosynthate requirements of the plant. Parts of the plant that require a net photosynthate import are called "sinks", whereas photosynthetically active organs act as "sources" for the nutrients required by the "sinks". As a sink requires photosynthate, the triose phosphates are utilized for sugar production in the cytoplasm. The rate of triose phosphate export governs the rate of P_i influx into the stroma. The metabolic activity and size the "sinks" will thus govern P_i influx and therefore regulate CO_2 uptake rates. Comparison of the CO_2 uptake rates of plantlets and seedlings must therefore be based on the understanding of whether the "sink" size and activities in the two materials are comparable.

Previous workers have looked at the selection of uniform material with respect to developmental age (17,35,55,56,70,73) , for the purpose of reducing variability caused by differences in plant size and sink development. Plastochron indexing (35) has proven to be very useful for culling out possible anomolous results that would otherwise have not been recognized. This principle of Plastochron indexing can be extended to deal with the comparisons of material from differing developmental origins such as micropropagated plantlets and conventially propagated plants. To deal with tissue-cultured material and normal material comparisons, there has to be some kind of examination as to what constitutes a valid comparison. One objective of this thesis is to make some statements concerning the selection of a valid comparison on a physiological basis.

1.3 Principles of Assessment

Tissue-cultured material is very sensitive to small vapor pressure deficits once transferred to *in vivo* conditions. To alleviate this problem, it is standard practice to place the plantlets under misting tents or in high humidity chambers for at least the first month after transfer to soil (6,19,28,48). Assessments that measure response to vapor pressure deficits might therefore help identify the degree of sensitivity and, perhaps, possible causes for it.

An assessment system for investigating the photosynthetic function of plantlets as well as responses to vapor pressure deficits can yield basic information on the seedlings. There is at least one report showing the diagnostic utility of vapor pressure deficit tests in plants (13). The purpose of this thesis is to show some of the potential of such an assay system for the evaluation of the physiological state of both plantlets and seedlings.

1.4 Thesis Objectives

This thesis deals with the development of an assay protocol consisting of both gas exhange measurements and fluorescence determinations. The development of a satisfactory nondestructive technique for measurement of sample size is a part of the assay development. Next the understanding of responses to water stress conditions is undertaken. This allows meaningful interpretation of subsequent results when plantlets and seedlings are compared. The micropropagation of white spruce plantlets makes up the next portion of the thesis. This section discusses the steps required to produce plantlets via seedling explants. The final portion of the thesis reports on the results of an investigation into comparisons involving plantlets and seedlings.

CHAPTER II

THE ASSESSMENT SYSTEM

2.1 Introduction

The routine assessment of both young spruce seedlings and plantlets requires the set up of instrumentation appropriate to the plantlet and seedling size. This involves a need for an appropriate gas exchange measurement system for both CO_2 and H_2O, a chlorophyll a fluorescence probe system, and a method to nondestructively estimate plant or shoot size. This last requirement was essential in order to carry out a sequential, nondestructive assay. This chapter will deal with the development and set up of the instrumentation, and it will establish the significance of output data in relation to water stress response of spruce seedlings.

Gas Exchange Measurement

There are many designs of gas exchange systems to measure photosynthesis (59). These systems can be grouped as either open or closed. Closed, as the name implies describes a system in which a plant is placed in a closed loop and the depletion of the CO_2 from the total loop volume is measured over time. This system is not readily amenable to the measurement of response to humidity or steady-state transpiration rates, because of the

constant accumulation of atmospheric moisture due to sample transpiration. In order to use a closed system for transpirational measurement and humidity response tests, it must be equipped with a sophisticated humidity feedback control system (59)

The open system configuration readily accomodates atmospheric component modification (i.e. humidity and CO_2 and O_2 partial pressures). Since air passes through the system only once and there is no accumulation of atmospheric moisture, transpiration rates can easily be measured by psychrometry or hygrometry (100). However an open system requires a continuous supply of fresh air. Air can be supplied from an outdoor source, bottled air, or from a common laboratory air system. Common laboratory air was selected and required removal of undesirable components such as oil particles, and volatiles from the compressors (e.g. ethylene) that are released due to the action of heat on the compressor lubricant.

The open gas exhange system was developed to accomodate both the material of interest (seedlings and plantlets) and to be: 1. capable of measuring CO_2 uptake of small samples, 2. capable of measuring transpiration rates and 3. flexible enough to allow modification of test humidity very quickly.

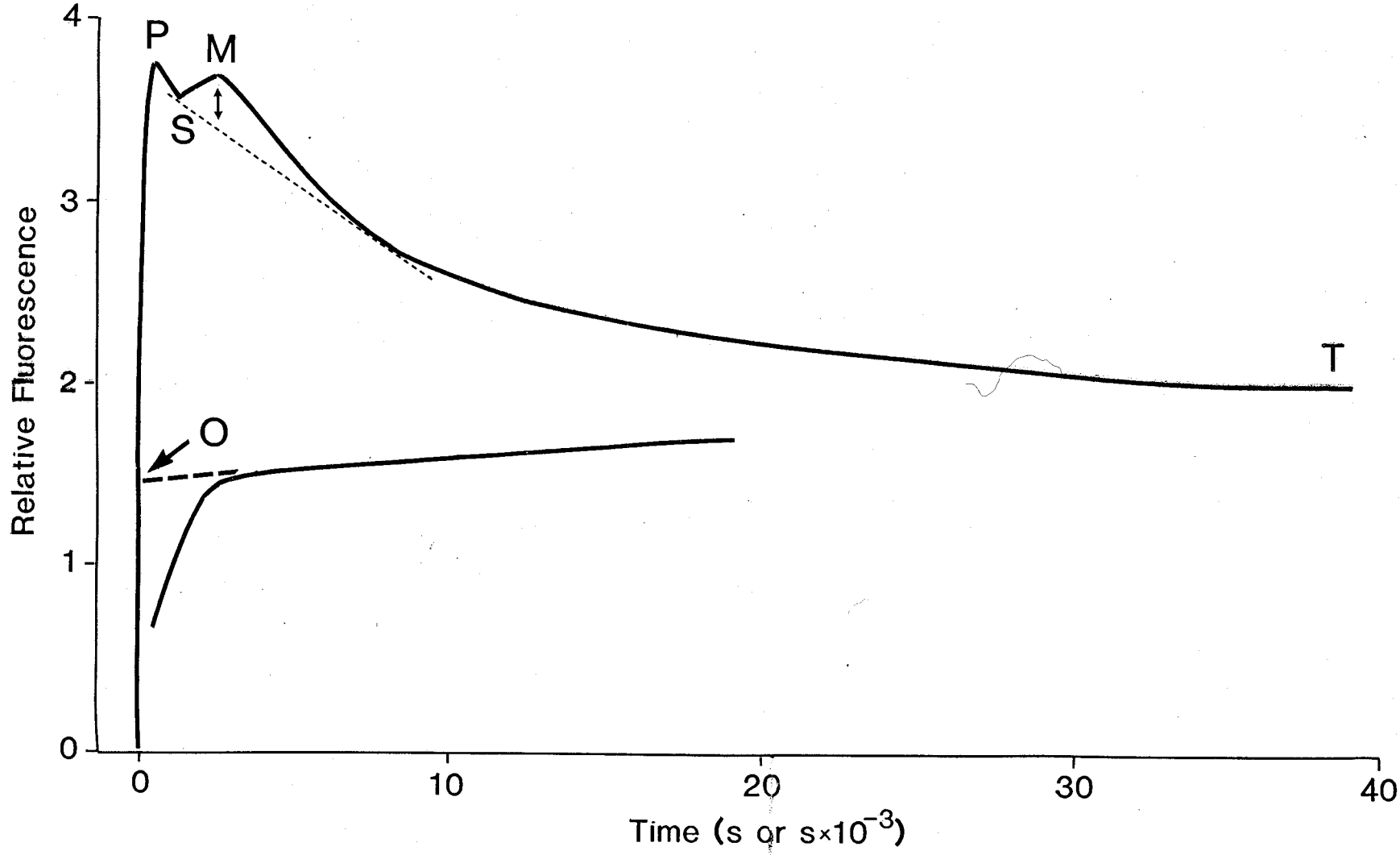
Fluorescence Measurement

The pre-existing probe initially used for *in vivo* chlorophyll *a* fluorescence (87) was found to be unsatisfactory for use with spruce seedling material. Shortcomings of the previous probe system are that it can only readily assess a two dimensional surface and the amount of continuous surface required for consistent sampling is much greater than found on a spruce needle. Therefore, in order to work with spruce seedlings and plantlets, the development of a more flexible probe system was required.

Estimation of Sample Size

The task of estimating sample size nondestructively is difficult achieve, particularly in conifers. In conifers, most workers use shoot volume or dry weight determinations as the basis for expression of sample size. In work with conifers that have relatively flat needles, such as Sitka spruce, an estimation of needle area has been done by measurement of area of needle silhouette (68). However, none of these can be considered nondestructive measurements. A recent report indicated the possibility of using an integrating sphere with light absorption measurement capabilities for the reliable estimation of whole plant chlorophyll content (71). O-level fluorescence (Fig. 2.1) is also a reflection of total chlorophyll content (64,72).

Figure 2.1 - Fluorescence induction time courses for a one year old white spruce seedling. A fast time course of 20 ms allows the calculation of O-level fluorescence and a slow time course of 40 s allows the recording of the induction curve. This is a typical induction curve after a 5 minute dark adaptation. O-level is calculated by extrapolating the fast time-course slope to the y-axis at time zero. P-level is calculated by the height of the P peak from the x-axis. M-level is calculated from the height of the M peak from a line which intersects both a tangent of the P-S decay and a tangent of the M-T decay simultaneously. Relative P- and M-levels are calculated by normalizing the previously calculated values against the O-level value.


P
M
S
O
T
Relative Fluorescence
0
1
2
3
4
0
10
20
30
40
Time (s or s×10^{-3})

13b

Therefore a study was done to determine whether O[1] correlated with total seedling chlorophyll. Correlations between chlorophyll content and other parameters were also investigated to show if O could be an indirect estimator of, for example, needle volume.

Correlations of Gas Exchange and Fluorescence for Seedlings

The significance of response measured by gas exchange and chlorophyll a fluorescence was important in order to correlate assessment results to the physiology of the final test material (i.e. plantlets). There have been numerous reports of the use of fluorescence determinations to give indications of stress response to various environmental factors (34,58,65,89). It is known that fluorescence induction curves can indicate levels of water stress (8,27,44). However, there has not been a study with respect to the significance of the amplitude of fluorescence response in relation to CO_2 uptake responses.

Numerous reports have discussed the antiparallel symmetry of fluorescence induction kinetics with the induction kinetics of photosynthetic O_2-evolution and CO_2 uptake (16,58,81,82,96,98,113). These works indicated that the typical S-M-T feature shown in figure 2.1 is coincident with CO_2 uptake induction. Re-examination of data from Ireland et al. (58) shows that the amplitude of M[2] is definitely correlated to CO_2 uptake

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[1] O = O-level fluorescence

[2] M = relative M-level fluorescence

under a test where plant material was exposed to a large range of ambient temperatures. These reports suggest that fluorescence is linked with CO_2 uptake and that M may be a good indicator of effects on photoelectron transport.

It was with these reports in mind that a series of studies was carried out to examine CO_2 uptake, stomatal behaviour, and fluorescence induction of spruce seedlings that were exposed to either soil or atmospheric water stress. A small number of the tests were performed at U.B.C. in Dr. P. Jolliffe's laboratory, using a gas exchange system described by Ehret and Jolliffe (32). This was an expedient measure to allow work until an infrared gas analyzer was available to our laboratory.

Fluorescence response in relation to induction illumination levels was examined to corroborate the interpretation of the results from the water stress studies. If fluorescence level changes are related to effects on the photoelectron transport system, then changes to varying light exposure would be expected to be parallel to changes found for other photosynthetic processes such as the CO_2 uptake response to light (17,30,90).

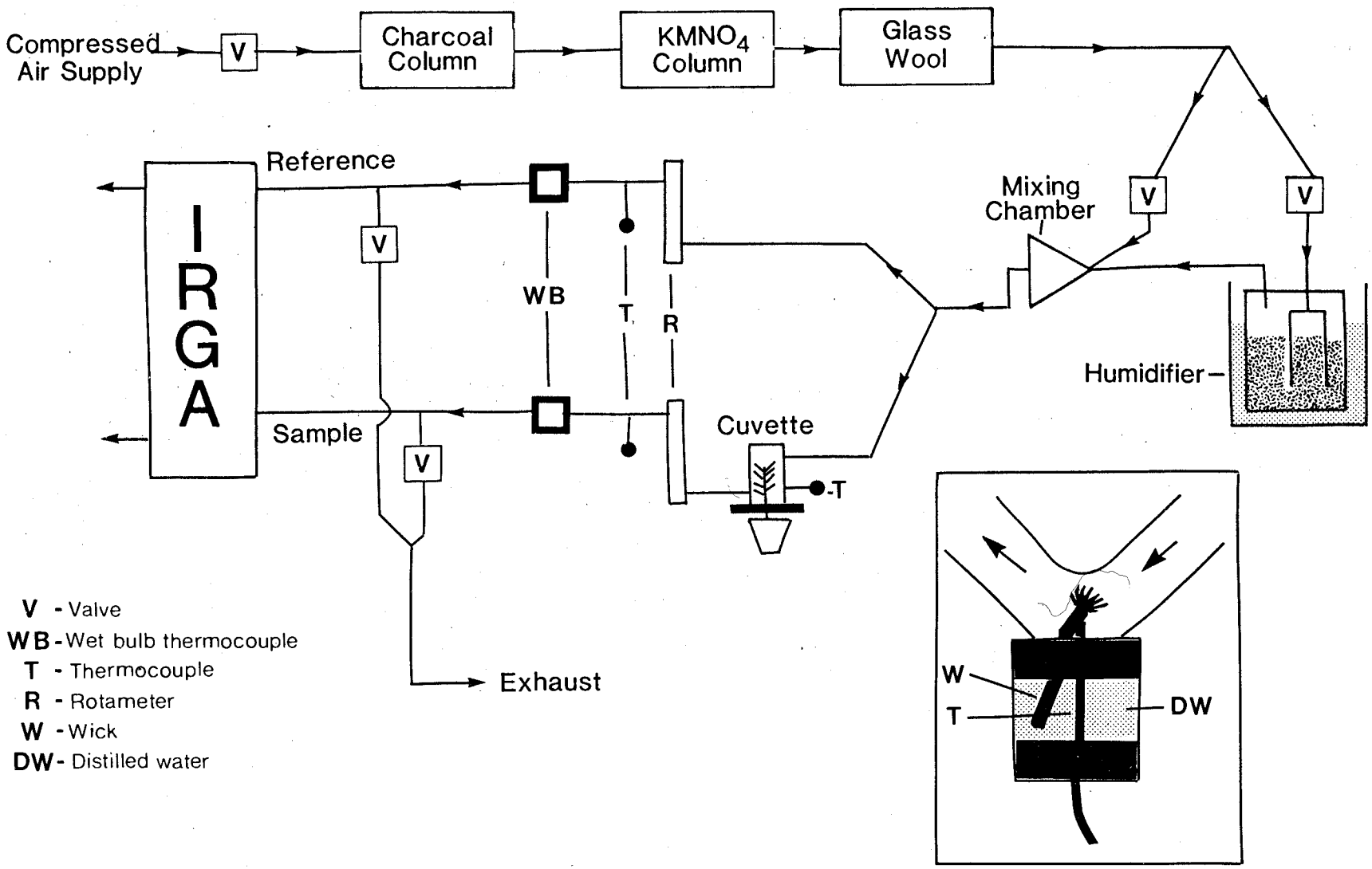
2.2 Materials and Methods

Gas Exchange

The compressed airstream was cleaned by two columns of absorbant material placed in series at the gas outlet. The first column was packed with granular activated charcoal (20-40 mesh), which is known to absorb many organic volatiles and inorganic pollutants such as O_3 and SO_2 (101). The second column was packed with $KMnO_4$ which had been adsorbed onto screened "Perlite" (W.R. Grace & Co., Ltd., Cambridge, Mass.). This second absorbant is very effective in removing ethylene and other light organic volatiles from air streams (86).

Figure 2.2 shows a schematic representation of the gas exchange system. Compressed air input into the system is controlled by a needle valve. The air passes through the two absorbant columns and then through the glass wool filter. The airstream is then split, the flow through either branch is controlled by a needle valve. One branch contains a humidifier which consists of a cheesecloth wick through which incoming air must pass. This is contained within a sealed glass vessel. The humidifying vessel is submerged in a water bath which is set at 2 to 4C above ambient temperature. The water bath is required to counteract the cooling that occurs when water in the humidifying vessel evaporates into the airstream.

Figure 2.2 - Schematic diagram of the gas exchange system. The inset shows the details of the wet bulb thermocouple assembly of the psychrometers.


Compressed Air Supply
V
Charcoal Column
KMNO4 Column
Glass Wool
Mixing Chamber
V
V
Humidifier
IRGA
Reference
V
WB
T
R
Sample
V
Cuvette
T
Exhaust
V - Valve
WB - Wet bulb thermocouple
T - Thermocouple
R - Rotameter
W - Wick
DW - Distilled water
W
T
DW

Once the air leaves the humidifier, it rejoins the parallel, non-humidified branch of the split stream and the two streams are allowed to mix in an Erlenmeyer flask. The resultant air mixture is split again into two streams. One stream passes through a rotameter (#604, Matheson Co., Inc., E. Rutherford, N.J.) and across a dry thermocouple and then through a wet bulb thermocouple chamber (100) which is detailed in the inset of figure 2.2. This air then passes through a cold thimble (cooled with dry ice and acetone) and through the reference cell of an ADC-225-MK3 infrared gas analyser (The Analytical Development Co., Ltd., Hertfordshire, Eng.), set up to detect CO_2. The second stream passes through a gas exchange cuvette designed to contain spruce seedlings or plantlets and then through a route similar to the first stream. This air passes through the sample cell of the infrared gas analyser.

The cuvette is illuminated with a CGE projector lamp (Quartz iodide, 650 watts, 125 volts) with a water bath imposed between the cuvette and the light source, acting as a heat filter. Light levels for gas exchange measurements were maintained at 440 μmoles quanta m^{-2} s^{-1} (PAR). The light levels were determined with a LiCor model LI-185A light meter fitted with a quantum flux detector head (LiCor Inc., Lincoln, Nebraska).

Humidity of the air during measurements was adjusted by controlling air flow through the humidifier branch of the air stream. The humidity was allowed to stabilize before plant material was placed into the gas exchange system.

The thermocouple psychrometers were calibrated against a EG&G thermoelectric dewpoint hygrometer (Cambridge Instruments Inc., Massachusetts). Subsequent recalibration was done once a month to ensure the stability of the pyschrometers.

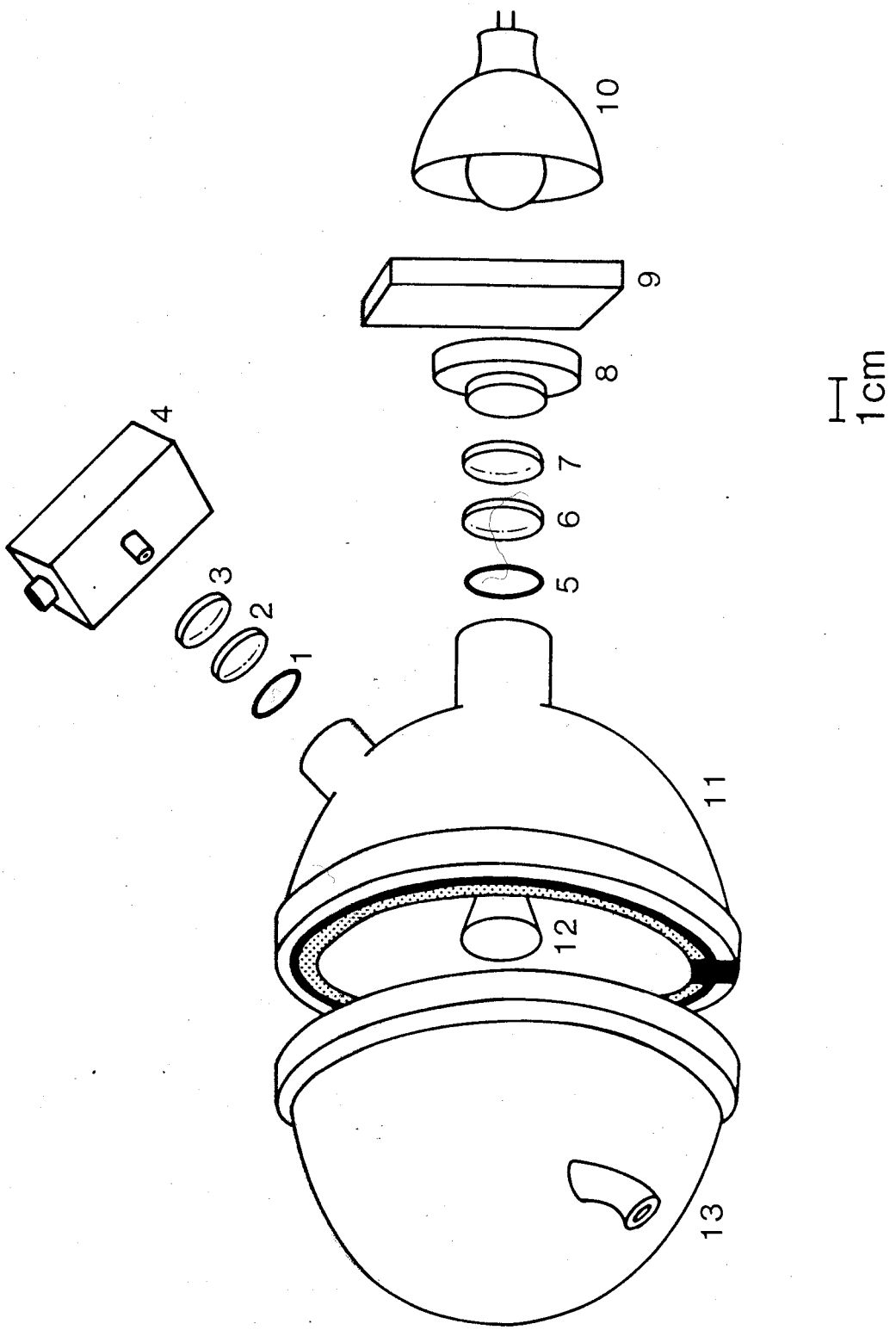
When CO_2 response curves were developed, a CO_2 absorbant column was added at the air input. The soda lime column was installed parallel to a blank branch, so that air flow between the branches could be adjusted, with needle valves, to produce the desired CO_2 levels for the ingoing test air.

Fluorescence Measurement

The integrating fluorescence probe system consists of two separable hemispheres, an assembly of light source components, and a fluorescence emmission detection assembly, all of which fit together as one unit. A data acquisition system (an oscilloscope and a strip chart recorder) and a stabilized dc power supply are the only accessories required to operate the probe system.

An exploded view of the probe system is seen in figure 2.3. The internal diameter of the sphere is 10 cm. The hemispheres are constructed of fiberglass. The interior is first coated with aluminum paint, which then followed with several layers of Kodak White Reflectance Coating (Eastman Kodak Co., Rochester, N.Y.). The excitation light port is 1 cm in diameter and a diffusion cone (measuring 2 cm at base and 2 cm in height) is suspended in

Figure 2.3 - An exploded schematic diagram of the integrating fluorometer probe. The indicated parts are; 1. an o-ring, 2. a CS 7-59 light filter, 3. a CS 2-64 filter, 4. light detector assembly, 5. an o-ring, 6. a CS 4-96 filter, 7. a CS 3-71 filter, 8. a photographic shutter, 9. a heat filter, 10. a prefocused projector lamp, 11. the stationary hemisphere, 12. a dispersion cone, and 13. the movable hemisphere with gas ports. (from Toivonen and Vidaver (107))


10
9
8
7
6
5
4
3
2
1
11
12
13
1cm

front of this port to ensure that the incoming excitation light is diffuse. A detector port, situated approximately 45° from the light port, is 0.5 cm in diameter.

A sponge rubber gasket is bonded to the stationary hemisphere, ensuring a light- and air-tight seal when the movable hemisphere is attached to the assembly. A rounded notch cut into the the flanges of both hemispheres allows insertion of the stem of a spruce seedling or plantlet. This notch is also lined with sponge rubber gasket to seal around but not injure the stem. The sphere is closed using four screw fasteners. The atmosphere within the sphere can be modified via the gas ports built into the movable hemisphere.

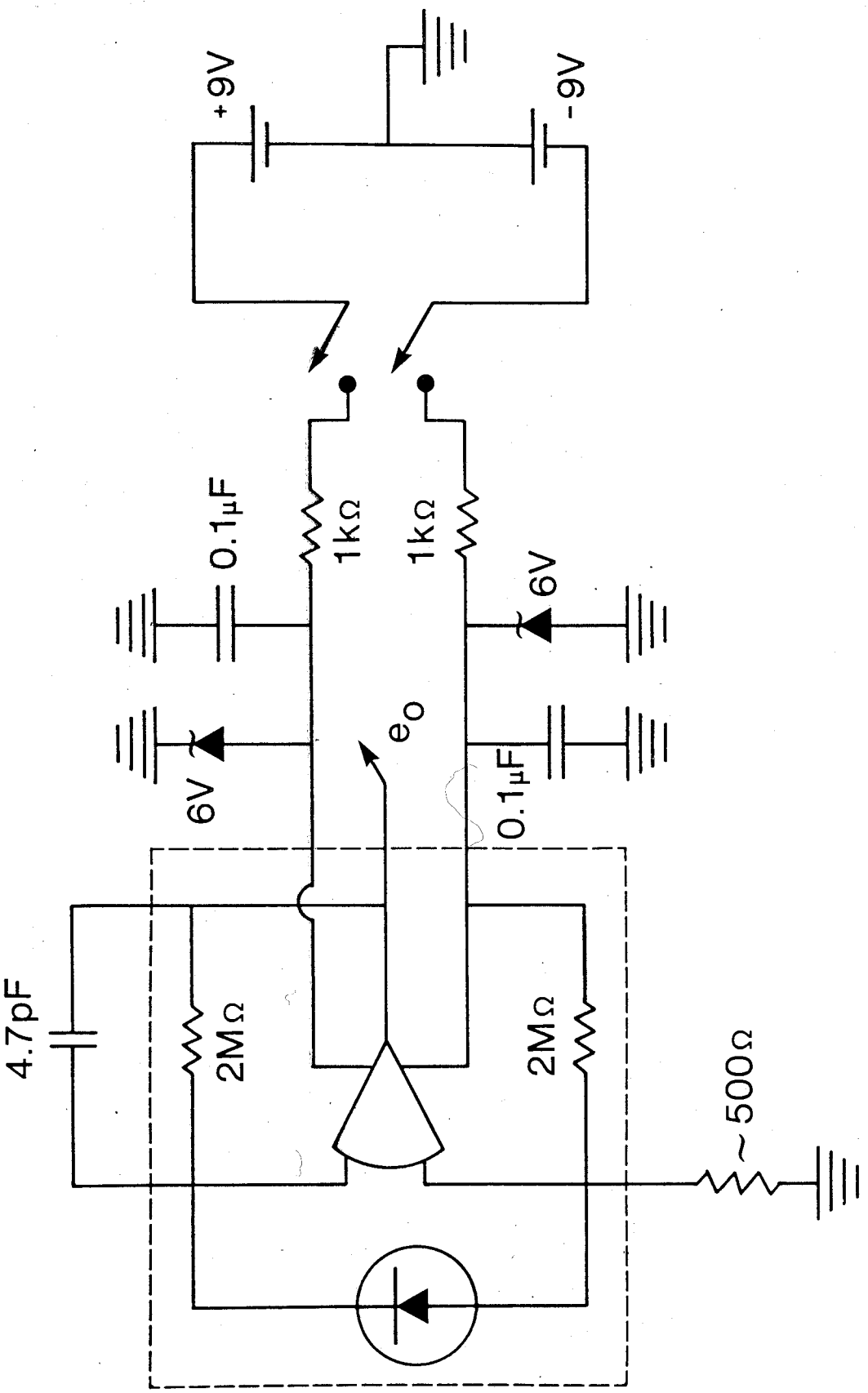
Excitation light is produced by a prefocused Sylvania EFP projector lamp (12 V, 100 W). The lamp is powered by a Topaz 12 V dc regulator. The light, from the lamp, passes through a heat filter, then Corning CS 3-71 and CS 4-96 filters (Fig. 2.4). The duration of the excitation light exposure is controlled by a photographic shutter (Fig. 2.3). Light levels entering the sphere can be controlled by adjusting the shutter diaphragm aperture. The excitation light assembly is air-cooled.

Fluorescence emmissions are isolated from the excitation light by Corning CS 7-59 and CS 2-64 filters (Fig. 2.4) placed in front of the optical detector. The detection system (Fig. 2.5) is built around a Devar 529-2-5 integrated optical detector package (Devar Inc., Bridgeport, CT). The remainder of the

Figure 2.4 - Transmission spectra for the filters used in the integrating fluorometer probe. The CS 4-96 and CS 3-71 filters are used at the excitation light port. The CS 7-59 and 2-64 filters are used in front of the light detector. Note the chlorophyll a fluorescence emission spectrum.(from Toivonen and Vidaver (107))

RELATIVE TRANSMISSION OR
FLUORESCENCE EMISSION
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
400
500
600
700
800
900
WAVELENGTH (nm)
CS 4-96
CS 3-71
CS 7-59(a)
CS 2-64
CS 7-59(b)
CHLOROPHYLL 'a'
FLUORESCENCE
SPECTRUM

Figure 2.5 - Schematic wiring diagram for the light detector assembly. The portions within the dashed box represent the detector package supplied by Devar.(from Toivonen and Vidaver (107))


+9V
-9V
0.1μF
1kΩ
1kΩ
6V
6V
e_o
0.1μF
4.7pF
2MΩ
2MΩ
~500Ω

system consists of a bias voltage produced by two 9-V transistor batteries and regulated to 6-V with Zener diodes. A 4.7-pF capacitor is used to damp signal overshoot and a variable resistor ($\simeq$ 500 Ω) to ground adjusts dark offset.

Estimation of Sample Size

O-level fluorescence determinations were made on samples of white spruce seedlings. The shoot volumes were then determined volumetrically, using 5 ml pipets. Needle volume was determined by removal of the needles and remeasurement of the of the bare shoot volume. Chlorophyll from needles was extracted using cold 80% acetone and the total chlorophyll content of the needles was determined spectrophotometrically using a Beckman model 35 spectrophotometer, and the equations of Arnon (3). The residues and extracted components of each sample were conserved and fully dried at 100°C. The total dry matter of each sample was then weighed on a microbalance.

Sampling Procedure

Plant material to be tested was placed into a clear Plexiglas chamber in which the atmospheric humidity was maintained at saturation. It was found that plant material required 20 minutes in the preconditioning chamber for complete adaptation to the light regime used for the testing. The adaptation was inferred from the time required by the plant material to reach steady-state CO_2 uptake rates once inserted

into the gas exchange cuvette. Once preadapted, three to five minutes were required to reach the steady-state uptake rate of a sample. Fresh room air was continually pumped through this preconditioning chamber, with an aquarium pump, to ensure that there was no significant CO_2 depletion. The plants while in the chamber were illuminated with a light source identical to the source used for the actual gas exchange measurements.

Each sample was measured in light until 10 continuous minutes of steady-state uptake was noted. The cuvette was then blacked out for 5 minutes to allow measurement of dark respiration rates. Following this, the sample was removed from the cuvette and placed into the integrating fluorometer probe.

The sample was allowed to dark adapt for 5 minutes in the closed integrating fluorometer before an O-level determination was made. The O determination was made with a 20 ms light exposure. One minute after the O determination, the plant material was illuminated and simultaneous fluorescence recorded for 2 to 3 minutes on a chart recorder. Figure 2.1 shows a typical fluorescence time course. The reliability of the results using the 5 minute dark adaptation was tested in a study with a series of dark times ranging from 15 s to 15 min. The results are shown in figures 2.6 and 2.7. The 5 minute dark adaptation time appears to be beyond a time threshold where no further

Figure 2.6 - Changes in M-level fluorescence with time of dark adaptation previous to induction measurement. N = 3, ± standard error. This figure shows that M is at a maximum level with 5 minutes of dark adaptation.

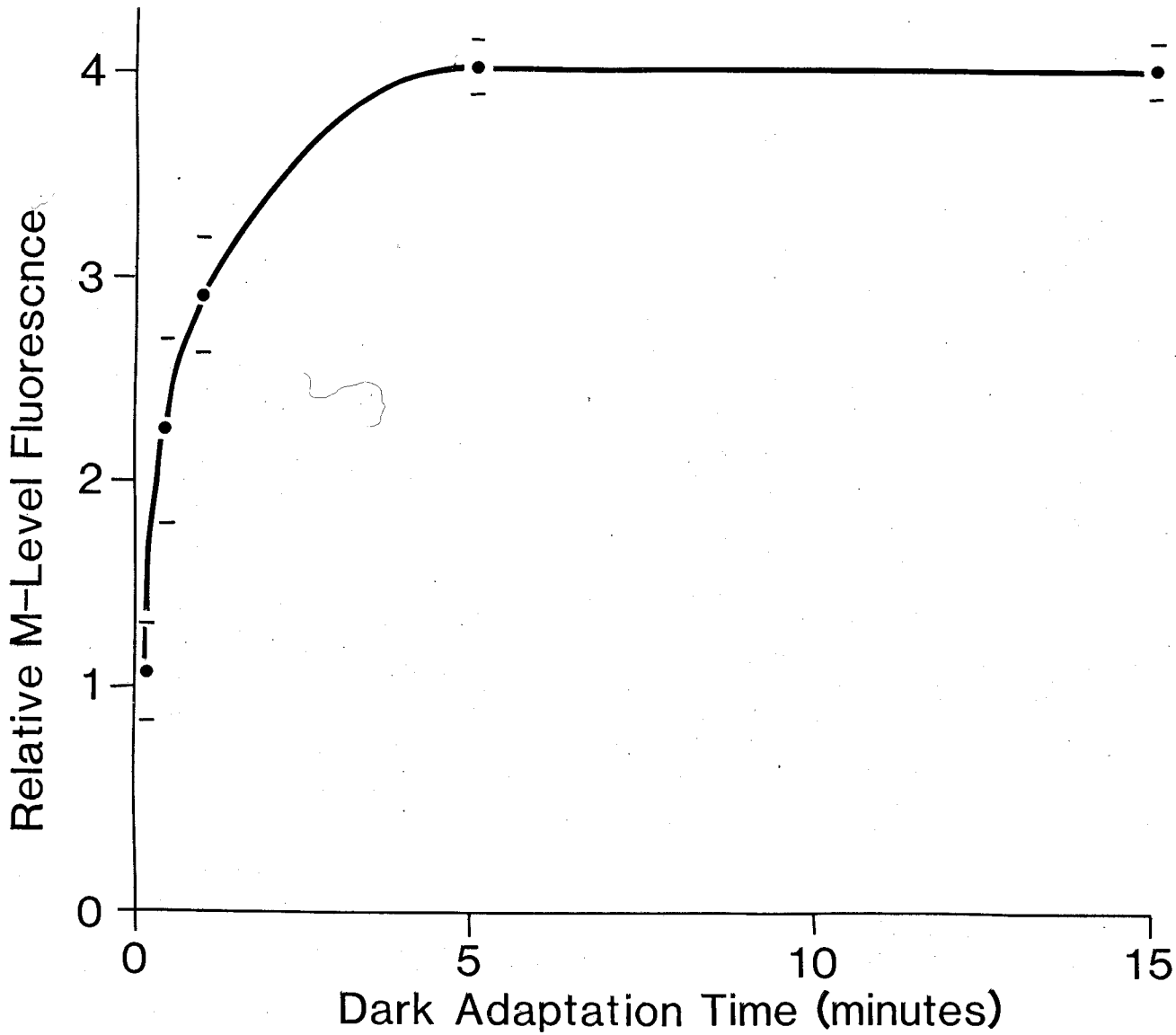
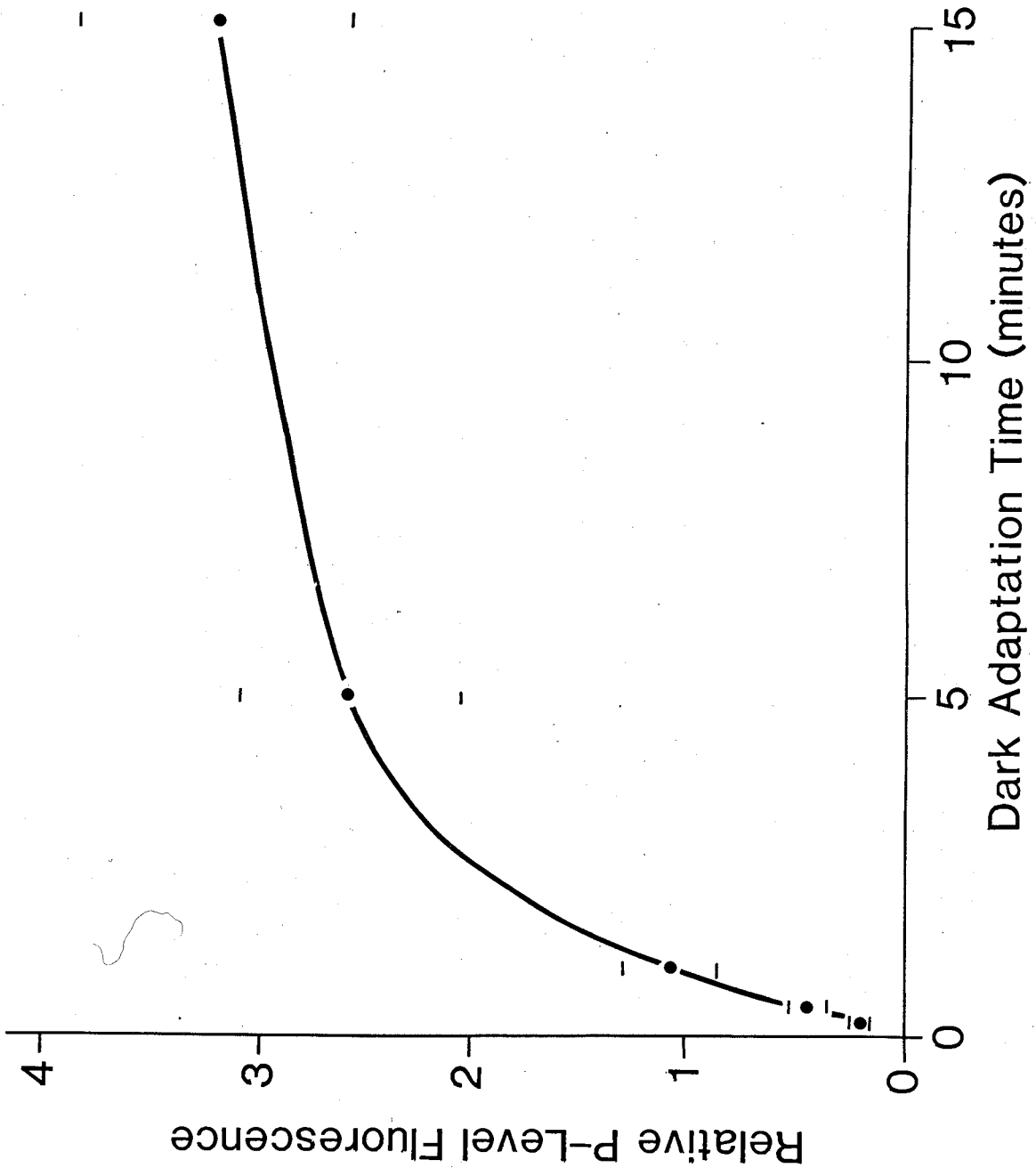

Relative M-Level Fluorescnce
0
1
2
3
4
0
5
10
15
Dark Adaptation Time (minutes)

Figure 2.7 - Changes in P-level fluorescence with time of dark adaptation previous to induction measurement. N = 3, ± standard error. This figure shows that P is approaching, but has not reached, a maximum value with 5 minutes of dark adaptation.


Relative P-Level Fluorescence
0
1
2
3
4
0
5
10
15
Dark Adaptation Time (minutes)

change in M amplitude occurs. P[3] at 5 minutes is relatively stable.

Responses to Vapor Pressure Deficits

The first series of tests involved investigation of responses of two-month old white spruce seedlings to vapor pressure deficits. The seedlings were grown in culture tubes in agar-solidified medium (described as root elongation medium in chapter 3). The seedlings were transferred to soil and were well-watered prior to testing. The assessment for steady-state CO_2 uptake, stomatal conductance, and fluorescence was carried out as described in the sampling procedure. The first test vapor pressure deficit was .66 kPa (i.e. 75% RH at 22C) and subsequent tests on the same material was made at 1.59 kPa vapor pressure deficit (i.e. 40% RH at 22C). Air flow was maintained at 1 l min^{-1} for the test. The seedling was placed in the gas exchange system at 75% RH for 15 min to measure the steady-state uptake and was then transferred to the fluorometer for an induction determination. Air flow in the probe was maintained at 2 l min^{-1}. Immediately following the induction determination, the seedling was placed back into the gas exchange system where the humidity had been lowered to 40% RH. Fifteen minutes was again required to obtain a steady-state uptake rate. The seedling was again transferred to the fluorometer for a second induction determination. The procedure was repeated at 40% RH twice more.

---

[3]P = relative P-level fluorescence

To calculate conductance, an estimate of total needle surface area was required. An estimation was based on examination of micrographs of needles of both plantlets and seedlings. A conversion of volume to area was developed from the measurements of cross-sectional area. For convenience, the needles were assumed to be uniform in cross-sectional area along their length. The equation developed for conversion was, area = k x volume, where k = 27.78 cm^{-1}.

Response to Soil Water Stress

In the study involving soil water stress, one year old greenhouse grown seedlings were separated into two groups and one group was subjected to normal watering while the other had water withheld for a period of up to 10 days. Both groups were tested at a relative humidity of 45 ± 3% and according to the general sampling procedure as outlined previously. Part of these tests were carried out at Dr. Jolliffe's laboratory. After steady-state CO_2 uptake was obtained, the seedlings were transferred to the fluorometer for induction determination.

Regression analysis was performed according to procedures outlined by Steel and Torrie (103).

Further Correlations

A series of tests were carried out to show that M displays the same characteristic response to light as do other processes of photosynthesis. This would support the hypothesis that there is a direct link between fluorescence response and processes involved with CO_2 uptake. Seedlings were placed in the fluorometer for a 5 minute dark adaptation and fluorescence induction was carried out under varying light intensities. The light intensity was controlled by the shutter diaphragm and incoming light intensity was measured with a LiCor light meter fitted with a quantum flux head. The minimum light intensity was determined by the minimum aperture of the diaphragm. Both P and M were calculated.

2.3 Results and Discussion

Estimation of Sample Size

Table 2.1 shows that there is a good correlation of total chlorophyll content of the seedlings and the O. The relationship between total chlorophyll and needle volume or dry weight provides an indirect correlation of O and these two measures of sample size. It is apparent from this study that for the population of seedlings used there is a good argument for using O as a nondestructive estimator of plant size.

Table 2.1 - Correlations for O-level, total chlorophyll, volume and dry weight of one year old spruce seedlings.

Correlation	Equation	R-value
O-level vs Total Chlorophyll	y = 36.66x + 0.48	0.81*
Volume vs Total Chlorophyll	y = 0.78x - 0.01	0.81*
Dry Weight vs Total Chlorophyll	y = 0.20x + 0.01	0.83*

* - n=14, R-values are significant at the 99% level.
Note: O-levels are expressed as millivolts, total chlorophyll as milligrams, volume as milliliters, and dry weight as grams.

Responses to Vapor Pressure Deficits

Figure 2.8 shows the response of a seedling to the treatment imposed on it. The responses seen are typical of the seedlings tested. The progress of responses shown for this seedling does indicate sequential changes, with exposure time to the large vapor pressure deficit induced by the atmospheric humidity of 40% RH.

The CO_2 uptake drops when the humidity was lowered to 40% from 75% (Fig. 2.8 a&b). It then stayed at that level for a second 15 minute exposure to 40% RH, but in the third 15 minute exposure to 40% RH the CO_2 uptake dropped to a much lower level. Relative P-level fluorescence increased when the humidity was first lowered and fell significantly with each subsequent exposure to the low humidity (Fig. 2.8,c). M-level fluorescence, in contrast, remained at a constant level until the third time interval at 40% RH, when the second CO_2 uptake drop was seen (Fig. 2.8,d). The pattern of needle conductance follows that of the CO_2 uptake (Fig. 2.8,e). However when the internal CO_2 levels are calculated (Fig. 2.8,f), it becomes apparent that the stomatal response is parallel to CO_2 rather than causal of the uptake control.

The last statement becomes clear if one examines an internal CO_2 concentration response curve for such a seedling (Fig. 2.9). The ambient CO_2 concentration is indicated by C_a on the x-axis.

Figure 2.8 - Photophysiological responses of a two month old white spruce seedling to lowering of test humidity. Graphs represent; a. test humidity vs time, b. CO_2 uptake vs time, c. P-level fluorescence vs time, d. M-level fluorescence vs time, e. calculated conductance vs time, and f. estimated depression of internal CO_2 partial pressure relative to ambient.

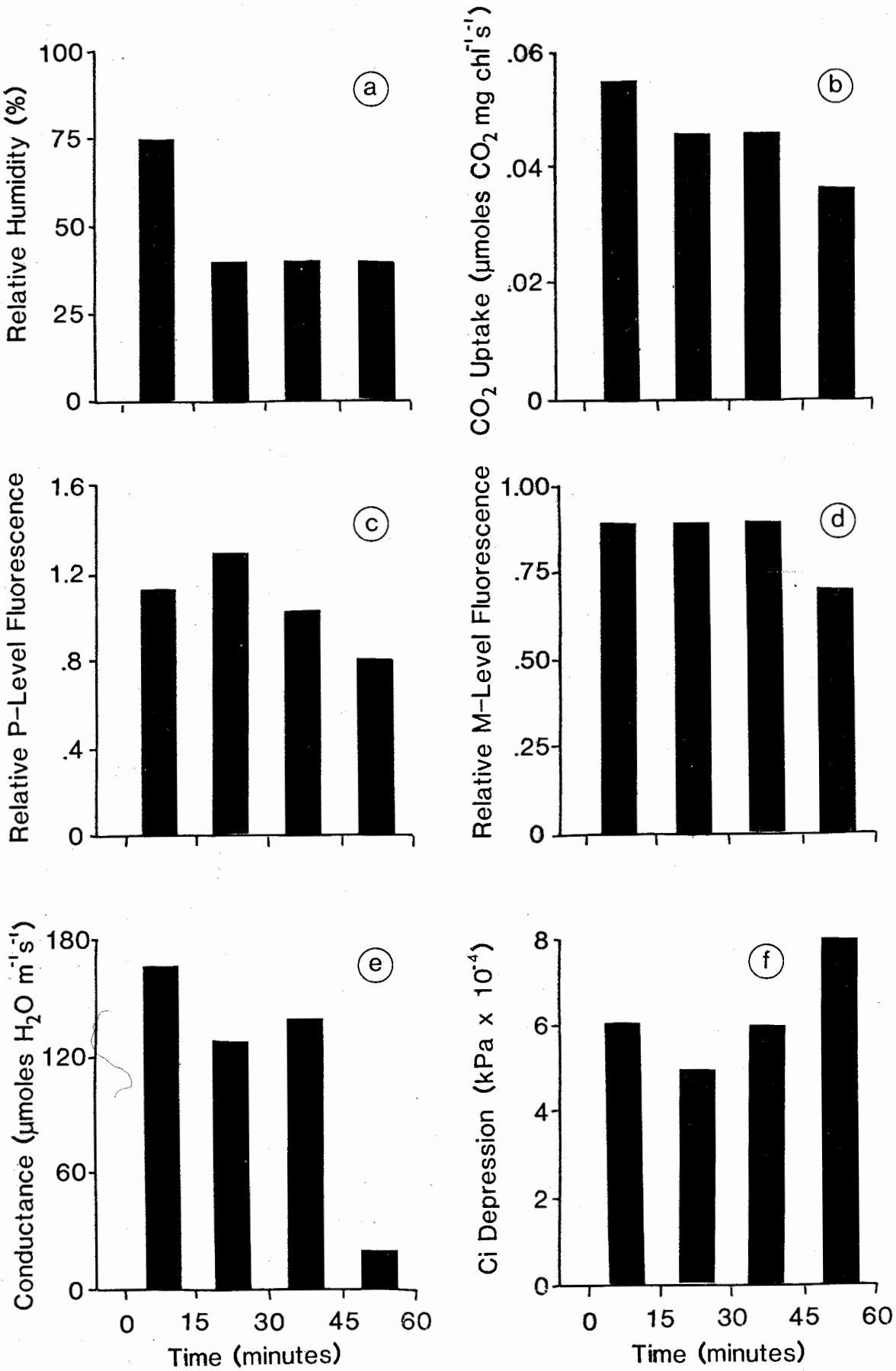
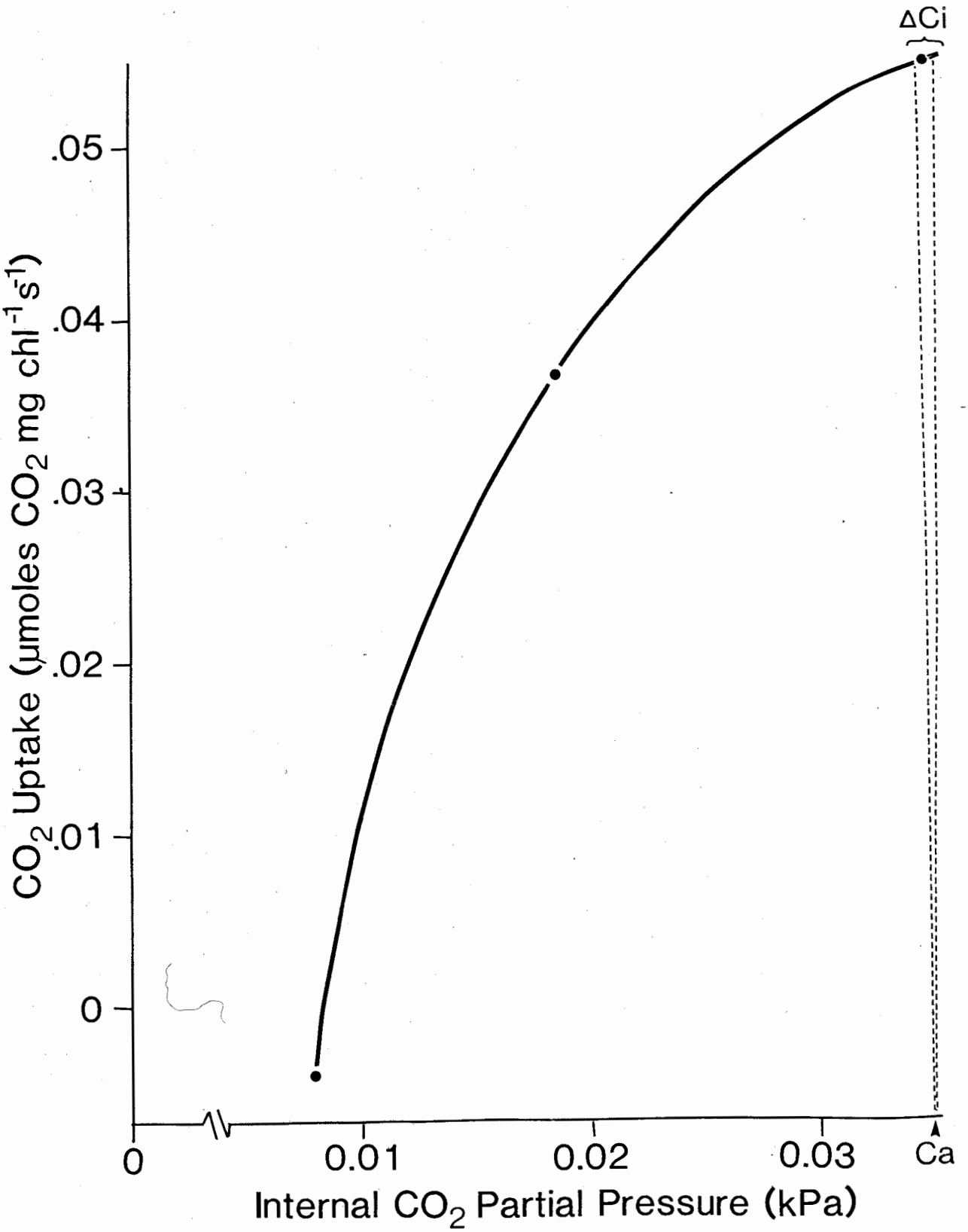

a
Relative Humidity (%)
100
75
50
25
0
b
CO_2 Uptake (μmoles CO_2 mg chl^{-1} s^{-1})
.06
.04
.02
0
c
Relative P-Level Fluorescence
1.6
1.2
.8
.4
0
d
Relative M-Level Fluorescence
1.00
.75
.50
.25
0
e
Conductance (μmoles H_2O m^{-1} s^{-1})
180
120
60
0
f
Ci Depression (kPa x 10^{-4})
8
6
4
2
0
0 15 30 45 60
Time (minutes)
0 15 30 45 60
Time (minutes)

Figure 2.9 - CO_2 uptake response of a two month old white spruce seedling to variation in internal CO_2 partial pressure. Abbreviations: ΔCi - depression of internal CO_2 partial pressure relative to ambient, Ca - ambient CO_2 partial pressure.


ΔCi
.05
.04
.03
.02
.01
0
CO2 Uptake (μmoles CO2 mg chl-1 s-1)
0
0.01
0.02
0.03
Ca
Internal CO2 Partial Pressure (kPa)

Ideally, if there were no stomatal resistance to CO_2 diffusion into the needles, then the maximum rate of uptake, shown by the intersect of the vertical line at the external concentration and the response curve, occurs. Looking at the depression of internal CO_2 relative to the ambient levels, it becomes apparent that the stomatal closure cannot be attributed to any significant limitation of the uptake rate.

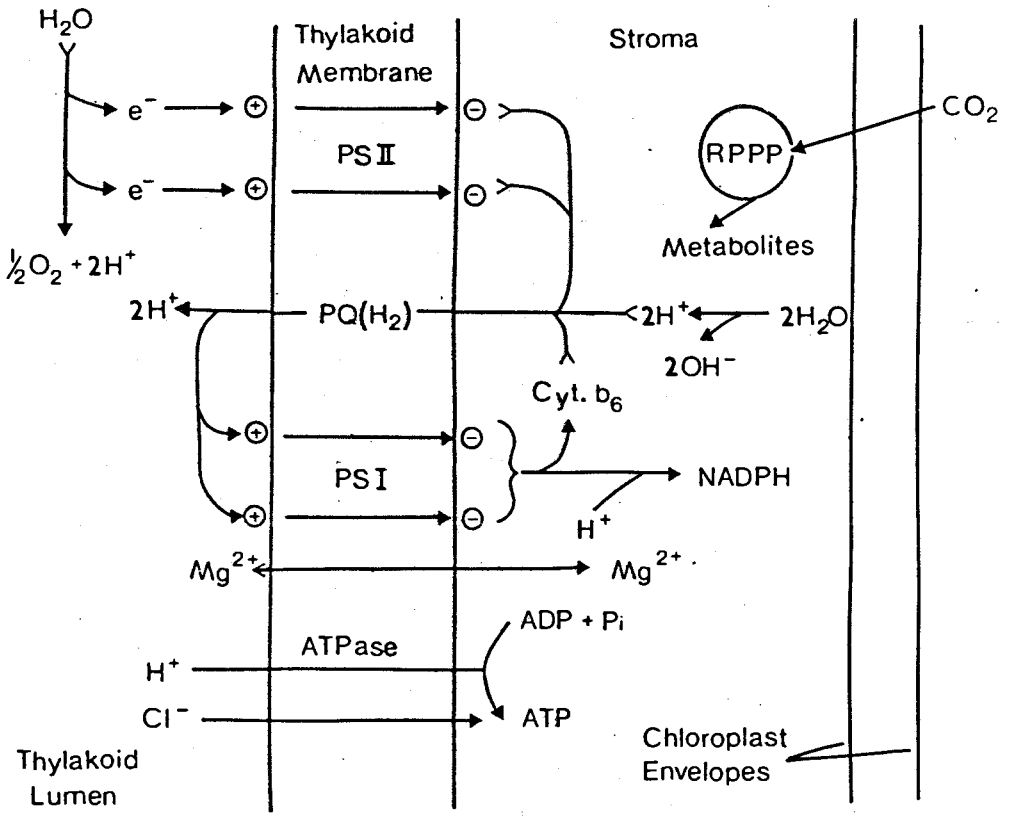
Fluorescence induction responses as seen on figure 2.8 (c and d) provide information which allows interpretation of the course of events. The changes that occur in P are not necessarily indicative of a direct cause for the uptake response. Two processes can affect P, they are; 1. water-splitting, and 2. the quenching potential of the photoelectron transport system beyond PS I (63,82). If water-splitting is decreased by water stress, then the P would drop due to a shortage of electrons available for fluorescence emission. It is known that water-splitting does not increase under water stress conditions (31,60), therefore the response seen on the initial humidity drop cannot be due to water-splitting effects. Mild water stress impairs the activity of the dark reaction enzymes (7,31,69), and therefore the utilization of the reducing power generated by the photoelectron transport system. This would create an electron back pressure, if the water-splitting was not reduced at the same time. The result of such a course of events would be an increase in P.

The decrease in P in the second and third time intervals at 40% RH indicates a net reduction in water-splitting. The height of P, as discussed above, is determined by effects on both water-splitting and electron quenching on the far side of PS I. If further decrease in PS I quenching occurs simultaneously to impairment in water-splitting then the P would be the net result of the opposing effects on electron quenching and water-splitting. There is no effect on CO_2 uptake for the first drop in P, meaning that whatever is happening is of no measurable consequence to the photophysiology.

At the last exposure interval both M, P,and CO_2 uptake dropped. This indicates that when both M and P drop there is a measurable effect on photosynthesis. The M response indicates that there may be a second phase of response of CO_2 uptake to the water vapor pressure deficit. The first phase was related to effects on processes beyond the photoelectron transport system, whereas this second response may well be related to an effect on the coupling of electron transport to membrane potential establishment and ATP production. The mechanism of this second response phase can be explained with a hypothesis which incorporates several separate reports with respect to fluorescence and the operation of the photelectron transport system.

The hypothesis for the mechanism of the M-level fluorescence response can best be discussed with reference to figure 2.10. It is accepted that P-level fluorescence represents the point in

Figure 2.10 - Consequences of illumination on the photosystem. On illumination, electrons are donated from water splitting to excited chlorophyll in PS II. Transfer of electrons to PS I via plastoquinone (PQ) requires protons which are taken up from the chloroplast stroma. Stromal pH rises, Mg^{2+} moves as a counter ion. In order to establish the electrochemical gradient across the thylakoid membrane, electrons are cycled from PS I to cytochrome b_6 which then feeds them through PQ again. ATP concentrations rise as H^+ is shuttled via the ATPase in photophosphorylation. Both ATP and NADPH are available for the reductive pentose phosphate pathway (RPPP).(modified from Edwards and Walker (34))


H_2O
Thylakoid Membrane
Stroma
e^-
e^-
PS II
CO_2
RPPP
Metabolites
$\frac{1}{2}O_2 + 2H^+$
$2H^+$
$PQ(H_2)$
$2H^+$
$2H_2O$
$2OH^-$
Cyt. b_6
PS I
NADPH
H^+
Mg^{2+}
Mg^{2+}
ADP + Pi
ATPase
H^+
Cl^-
ATP
Thylakoid Lumen
Chloroplast Envelopes

time, during photosynthetic induction, when PS I pools are most highly reduced (10,64). Decay from P-level involves the quenching of electrons from PS I by reduction of NADP, disulfide groups, and ferredoxin (31). However this linear flow cannot generate a significant thylakoid transmembrane electrochemical gradient (21,38). The establishment of this gradient is required to ; 1. establish ATP synthesis, and 2. activate dark reaction enzymes. ATP synthesis involves the protonation of the thylakoid lumen such that the export from the lumen will drive the ATPase (31,45,74,77). The activation of the dark reaction system requires alkalinization of the chloroplast stroma, accumulation of Mg^{2+} and Cl^- into the stroma, and the initiation of ATP production (16,40,45,79). The development of the transmembrane electrochemical gradient by the mechanisms at plastoquinone (PQ) of photoelectron transport accomplishes this (21,31,45,62,77,91).

In order to develop the gradient without developing a surplus of reducing power, cyclic electron flow is established around PS I (21,45,49,51,77). Electrons on the oxidized side of PS I can be fed back to PQ via cytochrome b_6. This process will reduce the electron demand from PS II. Oxidation of Q by the electron transport chain will then occur at a slower rate, and Q will remain highly reduced. The result of this would be an increase in fluorescence to yield the rise to M as shown in figure 2.1. Once an electrochemical gradient is established Mg^{2+} migrates as a counter ion to the stroma to re-establish an ionic

balance, which was disturbed by H^+ transport into the thylakoid. ATP synthesis would instantaneously begin, the dark reactions would be initiated, and reducing power (i.e. NADPH) and ATP would be utilized. Cyclic electron flow reduces the availability of reducing power required by the oxidizing side of PS I for photosynthesis. One way in which the PS II electrons might be transferred is suggested by several investigators (4,8,11,49). They suggest that proteins of the thylakoid membrane become phosphorylated, causing a conformational change in the membrane. This allows spillover of electrons directly from PS II to PS I , allowing partial circumvention of the electron transport chain through PQ. This would remove electron pressure from Q and result in the M decay.

The hypothesis presented here is supported by other work that has involved studies on the processes and phenomena under discussion. Previously it was considered that PQ linked H^+ transport was directly responsible for ATP synthesis (51). Recently it has been found that the relationship between electron transport and ATP synthesis is poor (21,38), indicating an indirect link between the processes. It is accepted that ATP synthesis is dependent primarily on proton gradients and photophosphylation is driven by the export of H^+ from the thylakoid to the the stroma (62,74,77). This would be consistent with the lack of direct association of between electron transport and photophosphorylation.

There is also evidence indicating that the functioning of the thylakoid membrane affects all the above processes and the phenomena of M-level fluorescence (96). All effects on fluorescence and photosynthesis can be seen to occur simultaneously, however the key process is that of the establishment of the electrochemical gradient. Any effect (due to water or any other stress) seen on the process of the initiation of the gradient would be expected to be, and is, seen in the M-level fluorescence phenomena (121). Concurrent with M changes would be parallel effects on ATP synthesis and on CO_2 uptake which has been shown in the results preceeding this discussion. Water stress might affect the initiation process by reducing water-splitting to the point where electron availability is too low to establish the normal magnitude of a potential gradient.

Responses to Soil Water Stress

A quantification of the correlation between M and CO_2 uptake could be accomplished in a test where water status was varied. The best system with which to work was that of soil water stress. Effects seen with vapor pressure induced water stress are identical to those seen in soil water stress (90), indicating that there should be no problem relating results to the vapor pressure deficit studies.

The relationship between M and CO_2 uptake are shown in figure 2.11. To ensure that water stress was in fact responsible

for the decrease of CO_2 uptake, stomatal conductance calculations were done (Fig. 2.12). The relationship is consistent with work that has shown a similar correlation between stomatal conductance (or stomatal resistance) and CO_2 uptake under varying levels of water stress (17,92,117). M varies dramatically with CO_2 uptake under conditions of poor water status (Fig. 2.12) which is represented by the lower portion of the curve in figure 2.11. As water status improves and CO_2 uptake rates increase, the relationship between M and CO_2 uptake declines (i.e. the slope of the curve decreases). This is consistent with previous work (7,23) which showed that photoelectron transport is limiting at low leaf water potentials and becomes nonlimiting as leaf water potentials increase.

Figure 2.13 shows that there is no consistent response of P and CO_2 uptake. This is due to the dual nature of P-level control that was discussed previously. Although water-splitting is affected under these stress conditions, there are also possible effects on the electron accepting side of PS I. These effects would counteract water-splitting effects on P fluorescence under the conditions imposed. In other words, P may fluctuate with varying levels of water stress. The net P would be dependent on the relative effects of the water stress on the water-splitting and the oxidising side of PS I.

Figure 2.11 - M-level fluorescence versus CO_2 uptake of one year old white spruce seedlings subjected to varying levels of soil water stress. The equation of the fitted curve is: $y = 0.944x - 1.180x^2 - 0.029$. The multiple correlation coefficient (R) is 0.94, which is significant at the 99% level.

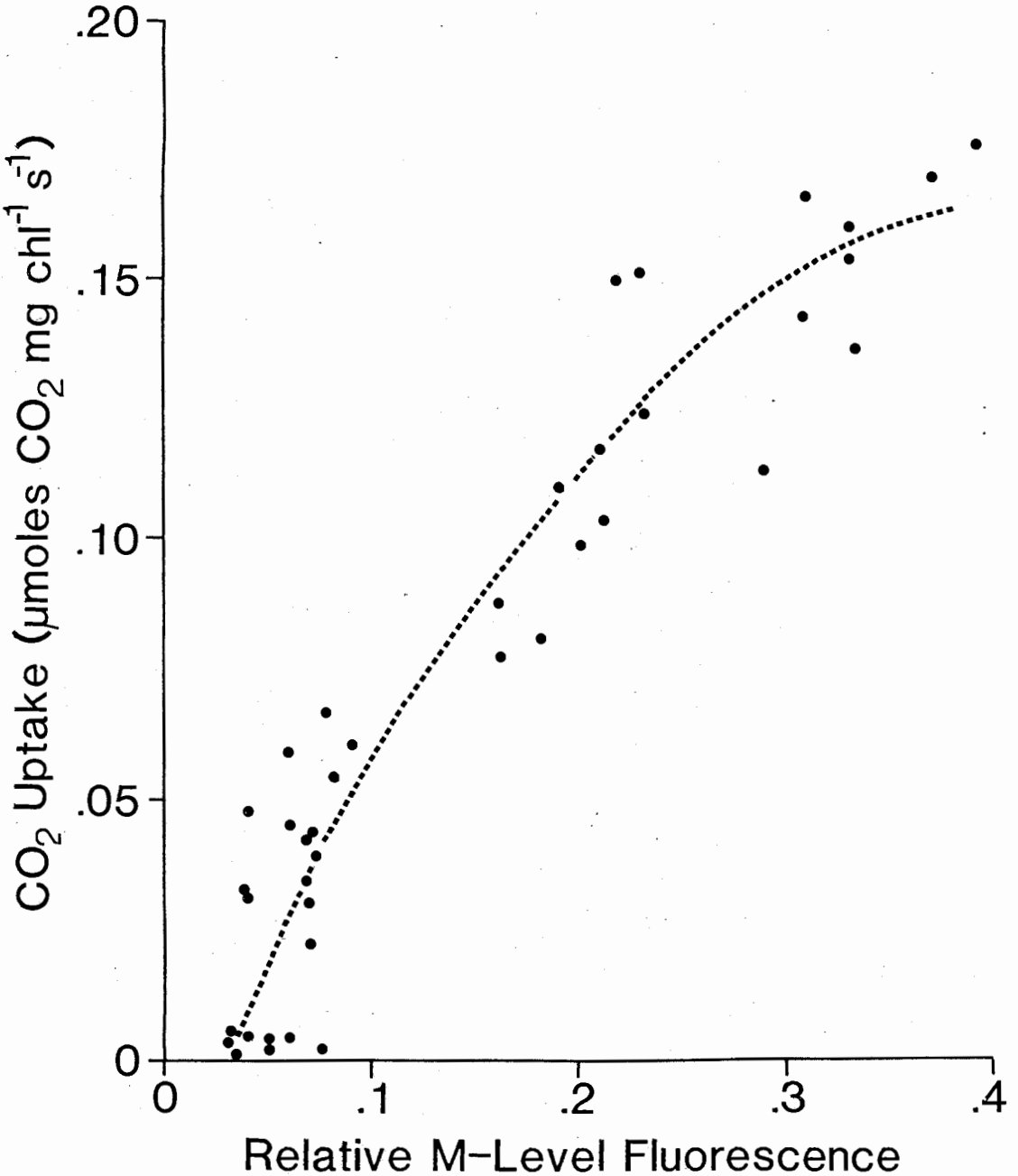

.20
.15
.10
.05
0
0
.1
.2
.3
.4
CO_2 Uptake (μmoles CO_2 mg chl^{-1} s^{-1})
Relative M-Level Fluorescence

Figure 2.12 - Conductance versus CO_2 uptake of one year old white spruce seedlings subjected to varying levels of soil water stress. The equation of the fitted curve is: $y = 0.022 + 0.0018x + 0.0000052x^2$. The multiple correlation coefficient (R) is 0.94, which is significant at the 99% level.

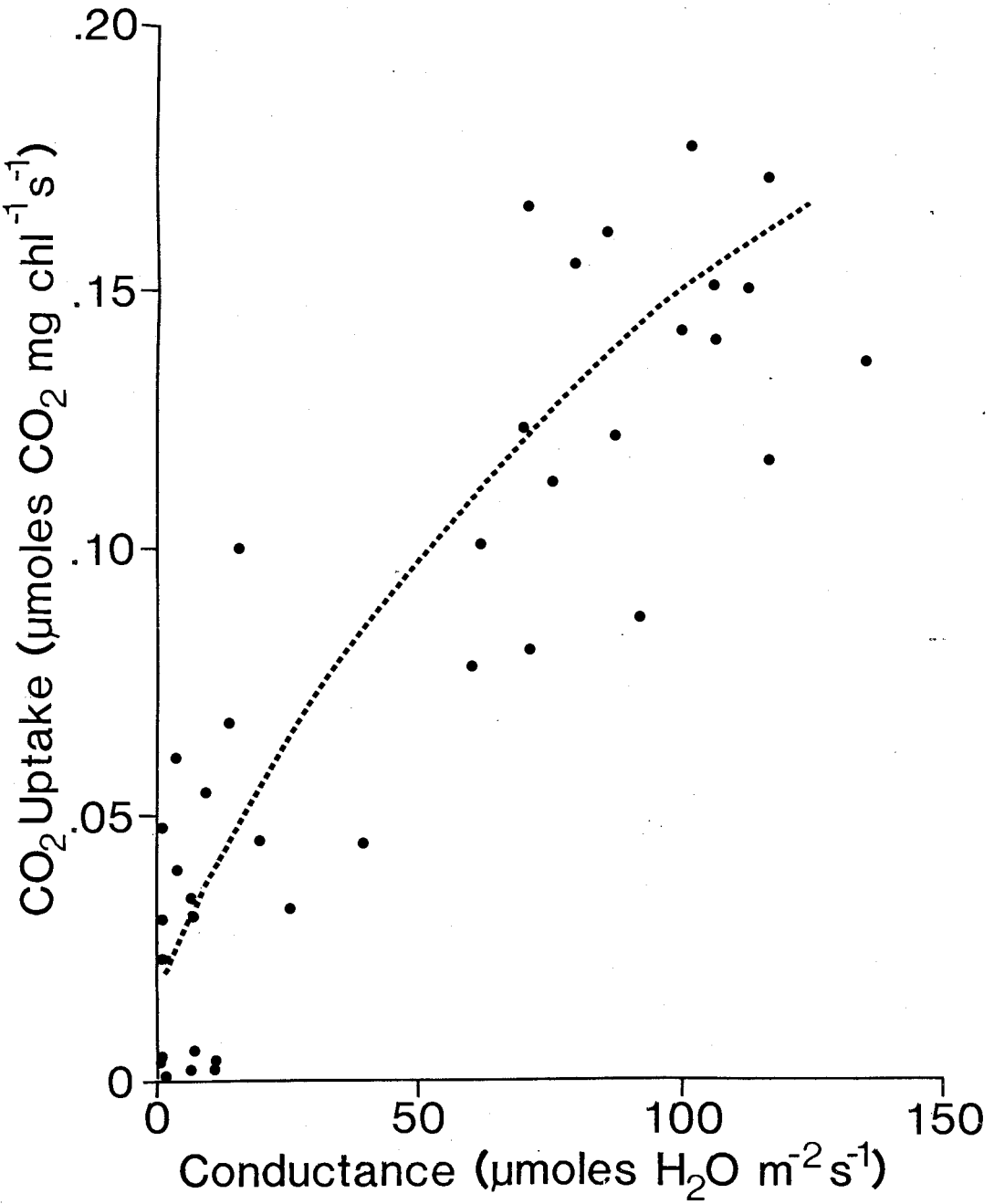
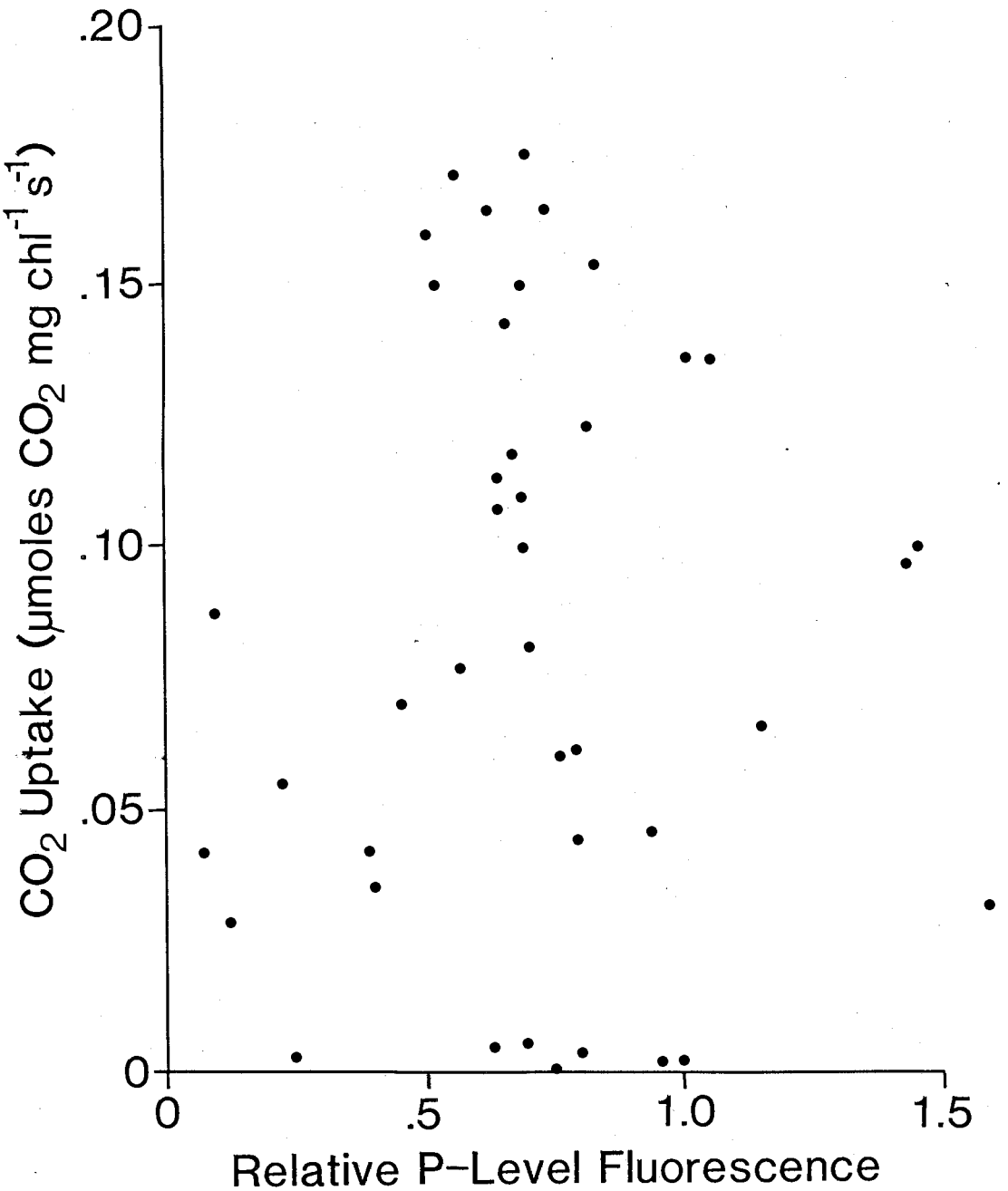

.20
.15
.10
.05
0
0
50
100
150
CO_2 Uptake (μmoles CO_2 mg chl^{-1} s^{-1})
Conductance (μmoles H_2O m^{-2} s^{-1})

Figure 2.13 - P-level fluorescence versus CO_2 uptake of one year old white spruce seedlings subjected to varying levels of soil water stress. No significant correlation was found.


.20
.15
.10
.05
0
0
.5
1.0
1.5
CO_2 Uptake (μmoles CO_2 mg chl^{-1} s^{-1})
Relative P-Level Fluorescence

The possibility that stomatal resistance (or conductance) change was responsible for the drop in CO_2 uptake must be considered. If the internal partial pressure of CO_2 is calculated, it becomes apparent that little if any reduction in the uptake rate is due to stomatal control (Fig. 2.14). Therefore the drop in CO_2 uptake and stomatal conductance must be parallel phenomena. The stomatal response, however, limits further water loss (i.e. it increases water use efficiency). The CO_2 uptake response is due to the mesophyll water stress imposed by the transpirational water loss (7,69,73,76,112). This mesophyll stress can occur because the stomatal closure response is due to epidermal water potential, not to mesophyll water status (94).

Further Correlations of Fluorescence

Figures 2.15 and 2.16 show the response of the P and M to increased light. The response of M is comparable to that seen for CO_2 uptake, ATP generation, and the establishment of the transthylakoid electrochemical gradient (17,49,79,85).

It is also important to note that P is dependent on light intensity; however, the response pattern is different. At the higher light intensities the decrease in amplitude was linear and only when the light intensity was reduced appreciably was there a large decrease in P. Water-splitting is known to decline with decreasing light intensity (23,31,64). This information

Figure 2.14 - CO_2 uptake response of 2 - one year old white spruce seedlings to variation in internal CO_2 partial pressure. One seedling is well-watered and the other is suffering from soil water stress. The dotted lines estimate the points on the curves to which the stomates are limiting uptake in the the test series. Abbreviations: ΔCi - depression of internal CO_2 partial pressure relative to ambient, Ca - ambient CO_2 partial pressure.

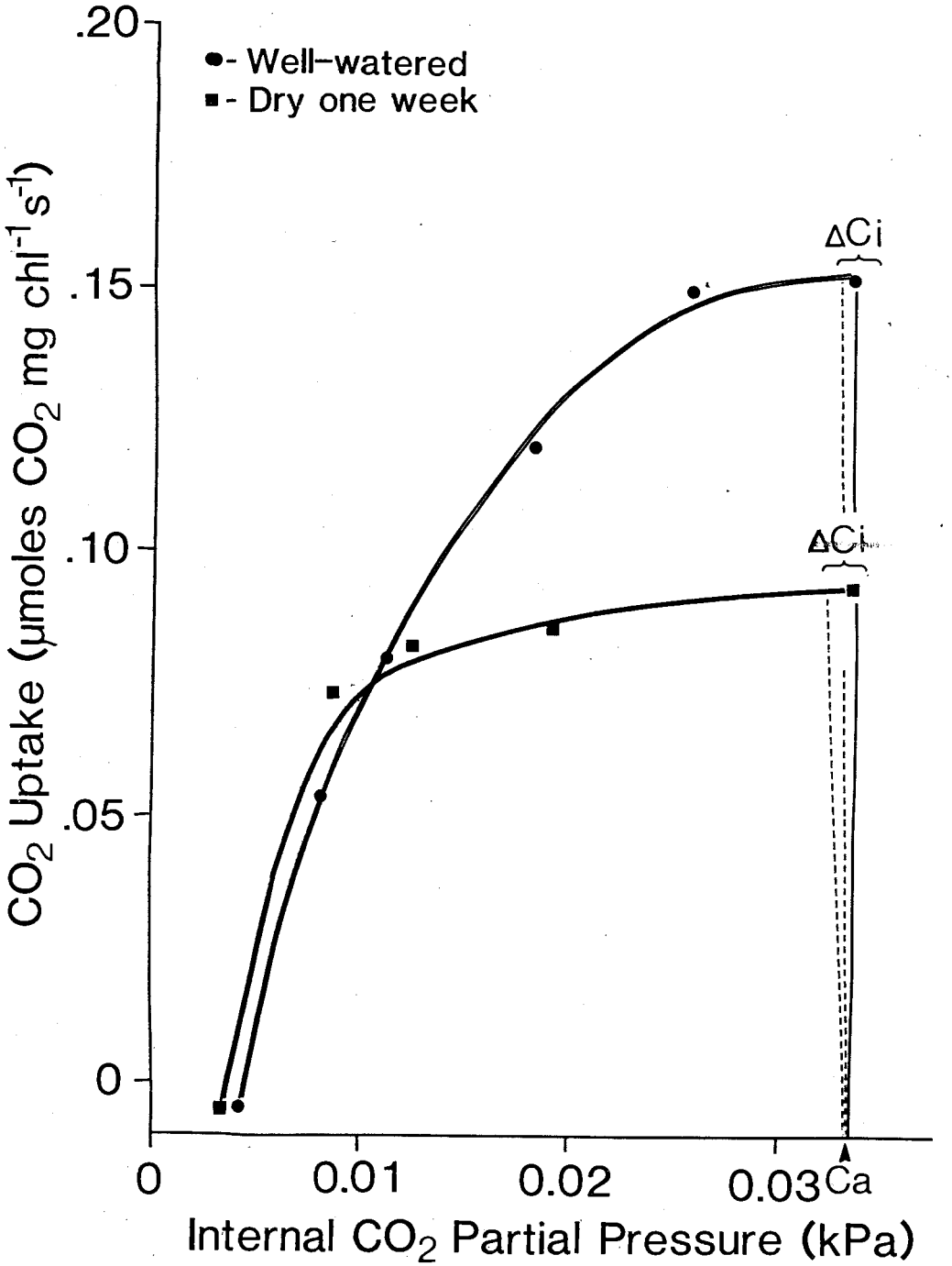

.20
.15
.10
.05
0
●- Well-watered
■- Dry one week
ΔCi
ΔCi
CO2 Uptake (μmoles CO2 mg chl-1 s-1)
0
0.01
0.02
0.03
Ca
Internal CO2 Partial Pressure (kPa)

Figure 2.15 - The change in P-level fluorescence response with excitation illumination intensity for one year old white spruce seedlings. N = 3, ± standard error.

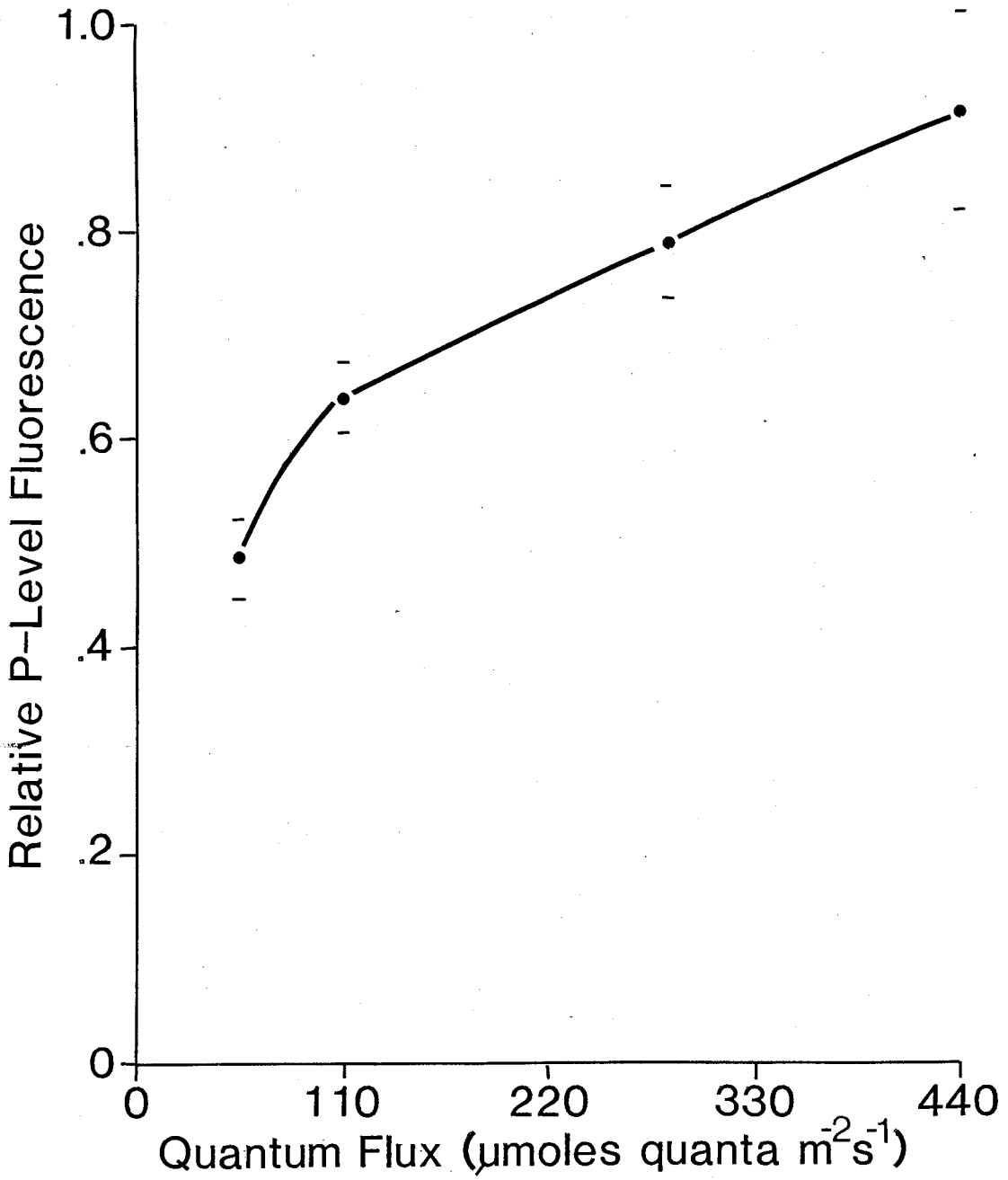
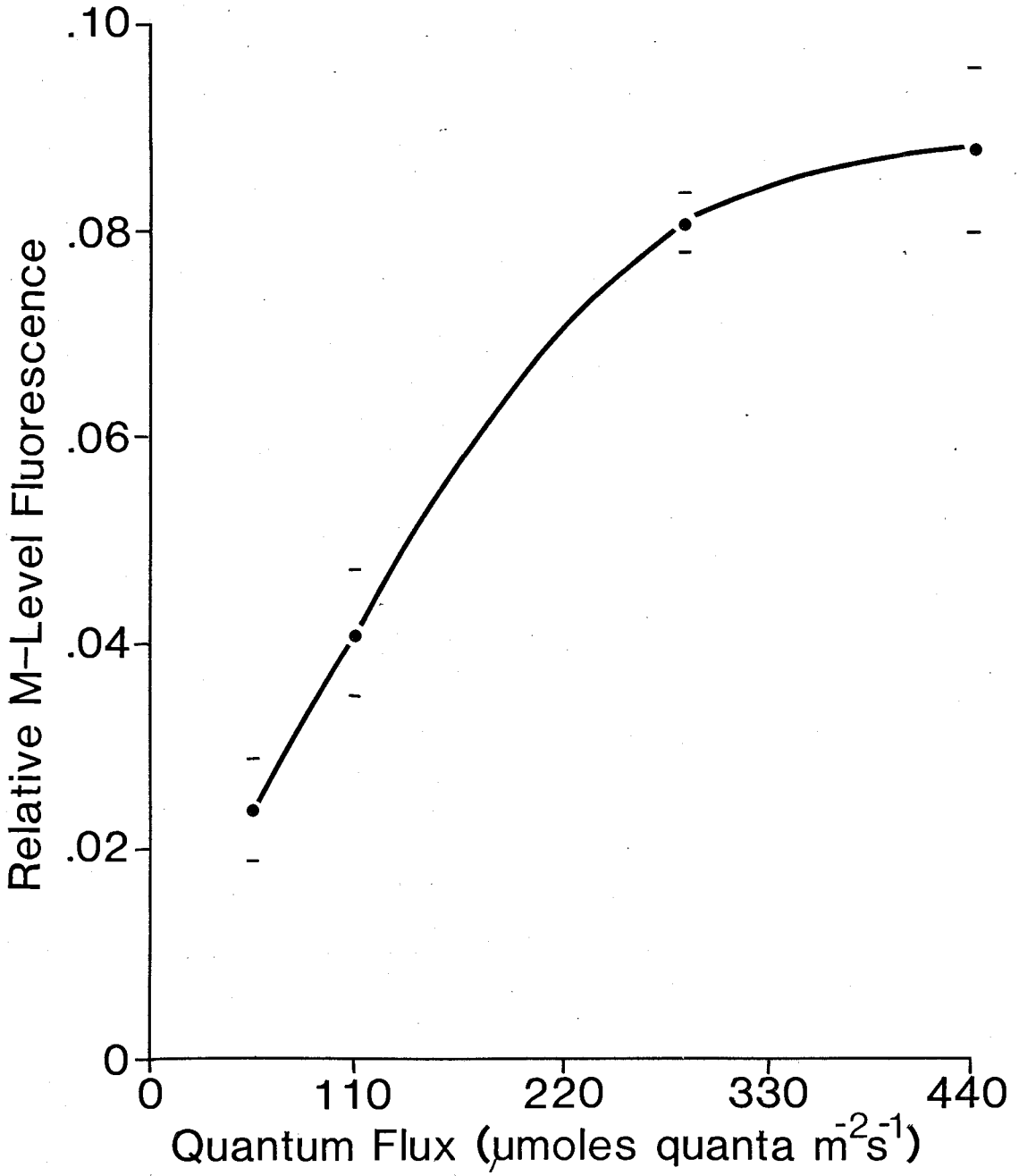

1.0
.8
.6
.4
.2
0
Relative P-Level Fluorescence
0
110
220
330
440
Quantum Flux (μmoles quanta m-2s-1)

Figure 2.16 - The change in M-level fluorescence response with excitation illumination intensity for one year old white spruce seedlings. N = 3, ± standard error.


.10
.08
.06
.04
.02
0
Relative M-Level Fluorescence
0
110
220
330
440
Quantum Flux (μmoles quanta m-2s-1)

allows an explanation for the course of events seen in M and P. First, when light levels are slightly reduced, water-splitting is directly affected. This is shown by the decrease in P. As light levels are further reduced, water-splitting declines to the point where it cannot supply sufficient electron flow for the development of a maximal transthylakoid electrochemical potential gradient. This effect is shown by a significant decrease in M. It would be expected that this M decline is associated with a reduction in ATP synthesis and CO_2 uptake. These observations are consistent with Keck and Boyer (60), in that water-splitting is affected before significant effects on a dependent process such as photophosphorylation can be seen. The changes seen in this experiment are due to only one component of P-level control, that is the electron transport from PS II (i.e. from water-splitting). Therefore, the connection between P-level and M-level is obvious, unlike the situation with the water stress studies, where P-level was governed both by water-splitting rates and the quenching potential at PS I.

2.4 Conclusions

The assessment system discussed in this chapter can provide useful information concerning the degree of water stress in seedlings. It also suggests the sites of the water stress response. In two month old seedlings, which are extremely sensitive to vapor pressure deficits, the test protocol showed a

definite and rapid water stress response. The characterization of the degree of this kind of response is useful for differentiating between the physiological status of plantlets and seedlings.

The integrating fluorometer can be used in conjunction with gas exchange measurements (for physiological studies) as well as by itself in the determination of plant size. Calibrations must be developed for plants of interest prior to the use of O-level fluorescence as reliable estimate of total chlorophyll content of a plant. Parameters such as total shoot volume can also be estimated if they are required.

The results of the water stress studies show that the M response consistently correlates to CO_2 uptake when spruce seedlings are subjected to either vapor pressure deficits or to soil water stress. The M decrease is not an initial response to water stress but occurs when the stress level has increased to a point where a serious reduction of PS II water-splitting is expected. The progression of responses seen over time with the increase of vapor pressure deficits in sensitive seedlings are as follows:

The first response includes a rise in P , a reduction in needle conductance, and a drop in CO_2 uptake. The next phase of response shows a decrease in the elevated P , stabilization of needle conductance and CO_2 uptake. A further exposure produces a another drop in P and an initial drop in M, and a second drop in conductance and CO_2 uptake.

The initial increase in P is probably due to a reduction in demand for electrons from the photoelectron transport system. This may indicate reduced activity of important enzymes associated with CO_2 uptake.

The second response reflects a stabilization of stomatal control and CO_2 uptake. P decreases at this stage, indicating an impairment of water-splitting, however, there is no measureable correlation of a decrease in P to photosynthetic CO_2 uptake.

The third response stage indicates that a second phase in water stress has been reached. In this phase actual effects might be due to changes in the photoelectron transport system and may be caused by a critical reduction in water-splitting.

It is this second phase of stress that shows a correlation to CO_2 uptake when plants under a broad range of water stress levels are assessed. The results indicate that impairment of photoelectron transport can be important in limiting CO_2 uptake under the study conditions, whereas stomatal limitation is negligible. Effects of other factors in the mesophyll are important even at low levels of water stress. Keck and Boyer (60) have shown that electron transport is first affected and later photophosphorylation is limited as water potentials are lowered. These two phases of fluorescence response, seen in this study, are consistent with their observations.

CHAPTER III

MICROPROPAGATION OF WHITE SPRUCE

3.1 Introduction

There are three sources for propagule derivation of micropropagated plantlets, these are; 1. axillary budding, 2. adventitious budding or embryogenesis directly from tissue explants or single cells, and 3. adventitious budding or embryogenesis from callus (57). Adventitious budding has been most widely used in conifer tissue culture (2,6). Material produced via adventitious budding is known to have a moderate risk of genetic mutation and can potentially produce hundreds of progeny per explant (30,57).

In many instances tissue culture micropropagation is used as the method by which to obtain initial multiplication of disinfested material for breeding purposes (18). In the ornamental plant industry, it is the best route by which to get novel or improved mutants into production in a very short time (48). Where material is readily available via other means or volume demand is not high, as in fruit trees, the costs of micropropagation make the process uneconomical (48). In forestry, there is a need for multiplication of difficult to propagate species such as white spruce (2,30) and for the propagation of resistant individuals of a population suffering

losses from disease (eg. the problem of blister rust in white pine) (30). Minimal improvements in desired attributes (wood volume, disease and stress resistance) can make micropropagation techniques cost effective (30). There is a report of initial large scale outplantings of a few species (2). These programs utilized plantlets produced adventitiously from juvenile explant material. Apparently these plantlets do not thrive in the in vivo environment. The reason for this is probably due to the in vitro environment which precludes plant development that is suitable for survival in the in vivo environment (2,12,19,24,25,28). The study in the next chapter will address this problem.

The first report of white spruce micropropagation was by Campbell and Durzan (15). They worked with the hypocotyl material of apparently sterile seedlings. In 1984, Rumary and Thorpe described a protocol for the micropropagation of white spruce from epicotyls of seedlings (84) which is similar to those developed for other conifers (6,18). The first stage involves the surface sterilization of seed with sodium hypochlorite, followed by germination in vitro, and then the epicotyl is excised from the hypocotyl of the seedling. The epicotyl is then placed on a Shenck-Hildebrandt (S-H) medium containing benzyl adenine (BA) and isopentyl adenine (2-*i*P) to induce bud development. The explants are subsequently transferred to the S-H medium minus hormones. After several transfers, the explants show substantial shoot development.

Shoots over 1 cm in length were excised and rooted on charcoal and vermiculite support medium, with indolebutyric acid (IBA). This protocol was selected as a model for micropropagation of the white spruce that is used in the study reported in chapter 4.

3.2 Materials and Methods

The protocol of Rumary and Thorpe (84) was used here in a modified form, as it appeared to show promise of reasonable numbers of shoots per explant and success in rooting. Several modifications, however, were found necessary to achieve success in our laboratory. White spruce [*Picea glauca*(Moench.)Voss.] seed, seedlot 4119, was provided by Dr. W. Binder (B.C. Ministry of of Forests, 4300 North Road, Victoria). The seed source location was Prince George, B.C. and the seeds had a reported germination of 82%.

The first modification was that of the seed surface sterilization procedure. Thirty percent H_2O_2 was more satisfactory than sodium hypochlorite. The seeds were imbibed for 24 hours in running cold water and then sterilized in a solution of 30% H_2O_2 for 20 minutes. Three drops of 100x diluted Tween 20 (Fisher Sci. Co., Fairlawn, N.J.) were added to every 50 ml of sterilizing solution. At the end of the treatment, the seeds were rinsed 3-4 times with sterile distilled water. The

Table 3.1 - A modified Shenck-Hildebrandt Medium (after Rumary and Thorpe (84))

Nutrient	Media Concentration
	(mM)
$NH_4H_2PO_4$	2.6
KNO_3	25.0
$CaCl_2.2H_2O$	1.4
$MgSO_4.7H_2O$	1.6
$FeSO_4.7H_2O$	55.0
$Na_2.EDTA$	55.0
	(μM)
H_3BO_3	80.0
KI	6.0
$Na_2MoO_4.2H_2O$	0.4
$CoCl_2.6H_2O$	0.4
$MnSO_4.4H_2O$	60.0
$ZnSO_4.7H_2O$	35.0
$CuSO_4.5H_2O$	0.8
	(mg l^{-1})
Nicotinic Acid	5.0
Pyridoxine.HCl	0.5
Thiamine.HCl	5.0
Glycine	7.0
Inositol	10.0
L-Asparagine	10.0
pH = 5.7 ± 0.1	

Note: These concentrations represent a full-strength medium.

seeds were then ready for germination.

The sterilized seeds were placed in petri plates with a support medium of 5% activated, acid-washed charcoal (#C4386, Sigma Chem. Co., St. Louis, Mo.) in vermiculite. The support medium was saturated with a half-strength S-H medium as (Table 3.1), and 1% sucrose (w/v). The plates were sealed with parafilm, wrapped in foil and placed in a refigerator at 5C for 7 days.

On removal from the cold, the plates were warmed to room temperature and the foil wrap removed. The plates were transferred to an incubator with a 16-hour photoperiod (50 μmoles quanta m^{-2} s^{-1}) at 25°C. By three weeks most seeds had germinated and cotyledons had expanded. The first true needles were beginning to emerge. At this point epicotyledonary explants (84) were excised and transferred to petri plates containing an initiation medium consisting of full-strength S-H medium (Table 3.1) with 3% sucrose (w/v), BAP (10 mg l^{-1}), 2-*i*P (10 mg l^{-1}), and 0.8% Difco Bacto agar (6,75,84). The epicotyls were positioned so that the cut end was imbedded about 1 mm into the medium. The cotyledons were appressed to the medium as well. These cultures were placed into the same incubator conditions as for germination.

Swelling or shoot clump formation could be seen at the shoot axis, three to four weeks later. The explants were then transferred to plates containing a hormone-free S-H medium with

2% sucrose (w/v) and Difco Bacto agar. These cultures were placed into an incubator with a 16-hour photoperiod and a day/night temperature of 22/18C. The light intensity at plate height was 50 μmoles quanta m^{-2} s^{-1}. Explants were transferred to fresh hormone-free medium every three weeks until the shoot clumps had reached the diameter of about 1 cm. This occurred from 6 to 9 weeks after hormonal induction.

Once the shoot clump had reached 1 cm in diameter, it was divided into four pieces and transferred to a hormone-free S-H medium (Table 3.1) containing 2% sucrose (w/v) and 0.5% charcoal (w/v). The cut sides of the subcultured explants were imbedded 1-2 mm into the medium. The shoot clumps were allowed to remain on this medium for 3 weeks, at which time they were transferred to a full-strength hormone-free medium, without charcoal. Once individual shoots had developed to a length of 1 cm or more, they were excised from the clump and placed into root initiation medium.

Root initiation medium consisted of half-strength S-H salts, 1% sucrose (w/v), 0.9% Difco Bacto agar (w/v), and 10 mg l^{-1} of indolebutyric acid (IBA) (6,75). The shoots were placed under a dark cloth for the first 7 days of the root initiation treatment. Then the material was placed into an incubator with a 16-hour photoperiod and a day/night temperature regime of 22/18C with light at about 30 μmoles quanta m^{-2} s^{-1}. After 4 weeks the shoots were transferred from the root initiation medium to root elongation medium. This medium was identical to the root

initiation medium except for the absence of IBA. Root formation took 6 to 8 weeks.

3.2 Results and Discussion

Fluorescent illumination proved not to be satisfactory for explant growth or bud induction, so incandescent bulbs were also installed in addition to cool-white fluorescent tubes. Bud proliferation and growth under this lighting was much improved. This observation is consistent with work of other plant physiologists on the importance of light quality in growth and development of many plant species (52,53,116), including bud dormancy of conifers (26).

Some shoots from an explant would be ready for rooting 4 months after the initiation of the explant with the cytokinin medium. The number of shoots available for rooting from one explant at one time would be 4 to 8. The total number of shoots initiated on one explant varied from about 8 to 50. Factors most contributory to shoot losses were incubator malfunction and contamination of explants subsequent to surface sterilization and transfer into culture.

Rooting success was low, about 20%. There may be ways to improve this, including the modification of photoperiod. It is known that rooting of many conifers is dependent on both light quality and photoperiod (26,116). The material may require

further lighting modifications of light quality and duration. Many incubators would be required for this kind of study; therefore this study is not presently feasible in our laboratory.

Charcoal was useful in promoting elongation of shoots and in improving shoot appearance. The charcoal medium reduced the incidence of water soaked shoots. Previous investigators have shown that charcoal is beneficial in culture (6,80,108,118,119), and there is evidence indicating that it adsorbs toxic accumulations of metabolites. Gould and Murashige (43) have also demonstrated the accumulation of a growth inhibitor (berberine) in Nandina cultures. These reports suggest that without charcoal, tissues in culture have to deal with accumulations of growth inhibitors. These inhibitors affect explant growth, differentiation, and survival.

3.4 Conclusions

The protocol used here was modified from that of Rumary and Thorpe (84). The protocol requires more work in order to make it routinely successful. Points that may improve the protocol involve greater emphasis on selection of seed that would give greater consistency in growth and bud induction response (i.e. "plus" seed) and examination of factors that might possibly improve the rooting response. Light quality and photoperiod

should definitely be examined in relation to rooting.

The protocol seems to produce a reasonably good number of shoots from the original epicotyledonary explants. A similar system has already been shown to be adaptable to larger scale programs where outplanting is being attempted (2).

CHAPTER IV

ASSESSMENT OF SEEDLINGS AND PLANTLETS

4.1 Introduction

The progression of response to water stress by seedlings is shown in chapter 2, and it is this response sequence that may be important in separating the plantlets and seedlings. However, environmental components must be investigated in order to elucidate the causes of the responses seen. One component may be the support medium used *in vitro*. There have been many reports concerning the importance of root development on photophysiology (14,37,95,102) and water relations of plants (39,67,120). Rooting medium is known to be a major contributor to the determination of root morphology ,as well as young seedling growth and development (20,33,80,99). Other components may be the medium and atmospheric water potential in the culture vessels (12,115) and the hormone additions used during culture (29).

The studies in this chapter include an investigation of the effect of rooting media support components on the sensitivity of seedlings and plantlets to transient vapor pressure deficits. There is also an examination of the selection criteria for seedlings that are used as comparisons for plantlets when making statements as to relative photosynthetic competence.

4.2 Materials and Methods

Seedling Comparison Selection

Seedlings and plantlets were grown from the same seed source. CO_2 uptake as it varies with age was examined in a series of age groups, from 2 months to 18 months. All seedlings were grown in a greenhouse, watered as required, and fertilized once every three weeks with a half strength S-H mineral salts solution (Table 3.1). Plant material was transferred to the laboratory one hour before CO_2 uptake measurements were made.

Each sample was enclosed within the gas exchange cuvette and connected to the gas exchange system. The test relative humidity was adjusted to 75% and the cuvette internal temperature was measured as 22 ± 3C. Light intensity was 440 μmoles quanta m^{-2} s^{-1} (PAR). Material was maintained in the cuvette until 10 consecutive minutes of steady state CO_2 uptake were noted. Following this, O-level fluoresence was determined for each sample, and subsequently an estimate of total chlorophyll content was made using the O-level relationship from chapter 2.

A separate group of seedlings grown under greenhouse conditions was sampled for dry weight of shoots and roots. This was done in order to quantify the dry matter mass of potential sinks (i.e. the root and stem). Four seedlings per age group were sampled, and the roots were severed from the shoot portions. All samples were dryed in an oven for 4 days at 100C.

Then, the weights of root and shoot portions were determined on a microbalance.

Effects on CO_2 uptake, due to the seedling transplant procedure were investigated. Spruce seedlings grown for two months in soil in the incubator were selected for this test. Half of the seedlings were left in situ while the other seedlings were transplanted to new pots. CO_2 uptake measurements were made the next day. The test humidity was 75%, the chamber temperature was 22 ± 3C, and light intensity was 440 μmoles quanta m^{-2} s^{-1} (PAR).

Seedling and Plantlet Assessment

Plantlets were produced according to the protocol outlined in chapter 3. Seedlings were grown axenically in culture tubes on 4 different rooting media. These media were, 1. an agar-solidified media identical to the root elongation medium used in the micropropagation protocol, 2. a paper bridge support, wetted with a liquid medium of the nutrient composition used in the root elongation medium , 3. a vermiculite support medium saturated with the liquid medium, and 4. a soil-mix support medium saturated with the liquid medium. Two-month-old seedlings and rooted plantlets were transferred to soil-mix one day before initial assessment was made. The transplanted material was kept under high humidity in the incubator by means of a polypropylene tent.

The seedlings and plantlets were placed in the light pre-conditioning chamber one hour before gas exchange measurement. Material was first assessed at 75% RH for 15 minutes and subsequently fluorescence measurements were made. Then, the humidity of the gas exchange supply air was reduced to 40% and the samples were reassessed for gas exchange and fluorescence induction response. The humidity of the integrating fluorometer was maintained at the test humidity. One replication of all treatments took one day.

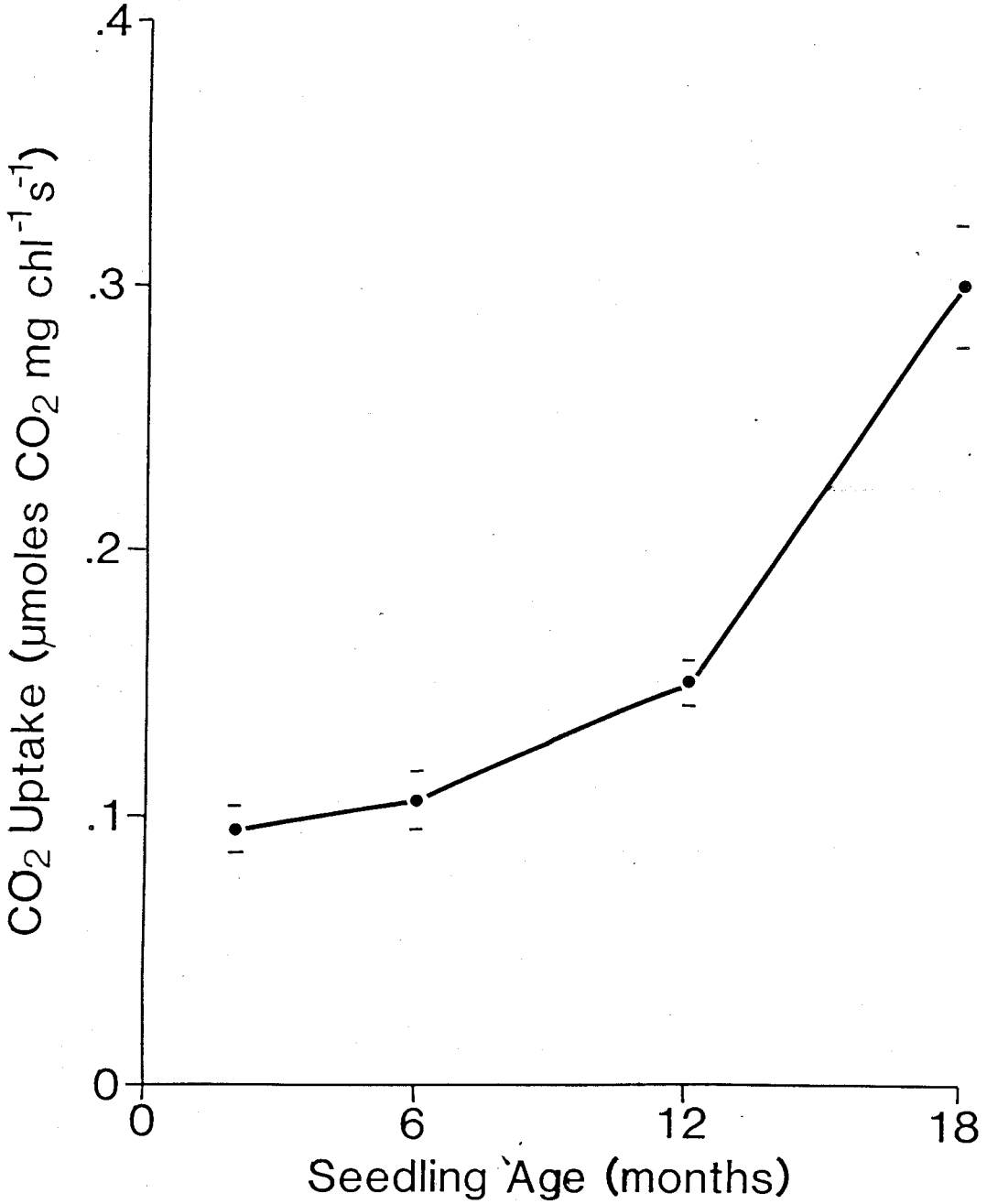
Data for comparisons were analyzed using analysis of variance and Duncan's Multiple Range Means test. Where simple comparisons were made, data were presented with standard error calculations. The principles and procedures used in the statistical analyses are outlined by Steel and Torrey (103).

4.3 Results and Discussion

Seedling Comparison Selection

Figure 4.1 shows the progressive change of apparent CO_2 uptake with increase in seedling age. The increase is quite dramatic, especially at the 12 to 18 month age interval. This indicates that the comparison of plantlets to 18 month old seedlings would give a totally different interpretation than the

Figure 4.1 - Changes in CO_2 uptake with age of white spruce seedlings. This figure shows the increase in CO_2 uptake for spruce seedlings as they grow from two months to 18 months. N = 4, ± standard error.


.4
.3
.2
.1
0
CO_2 Uptake (μmoles CO_2 mg chl^{-1} s^{-1})
0
6
12
18
Seedling Age (months)

comparison of uptake rates of plantlets and 2 month old seedlings. Therefore the correct equivalent in seedling age must be chosen before comparisons are made. It happens that the visually determined equivalent is approximately a 2 month old seedling.

The reason for this progressive increase is probably due to an increase in sink sizes of seedlings with age. This is supported by the data in figure 4.2. The root dry weight increased with age in a pattern similar to the CO_2 uptake rate increase. The shoot weight increased with age and as age increased the proportion going to shoot dry weight accumulation increased. Other work has shown that root growth in young plant material influences photosynthesis (55,56,66). Sink activity and size are determinants of the magnitude of CO_2 uptake (41,50,105,121).

It is clear from this discussion that two-month-old seedlings are a good comparison with respect to absolute CO_2 uptake rates of plantlets produced via micropropagation. The development of plantlets and two-month-old seedlings do appear to be visually comparable.

The transplant of seedlings reduces CO_2 uptake (Table 4.1). This "transplant shock" which reduces CO_2 uptake may be related to the disruption of the root system. The comparison of plantlets to <u>in vivo</u> seedlings must be with seedlings that have been transplanted in order to be similar to the plantlets which

Figure 4.2 - Dry weight accumulation with age of white spruce seedlings. This figure shows the increase in root dry matter of seedlings to one year and the slower increase in shoot dry matter. N = 4, ± standard error.

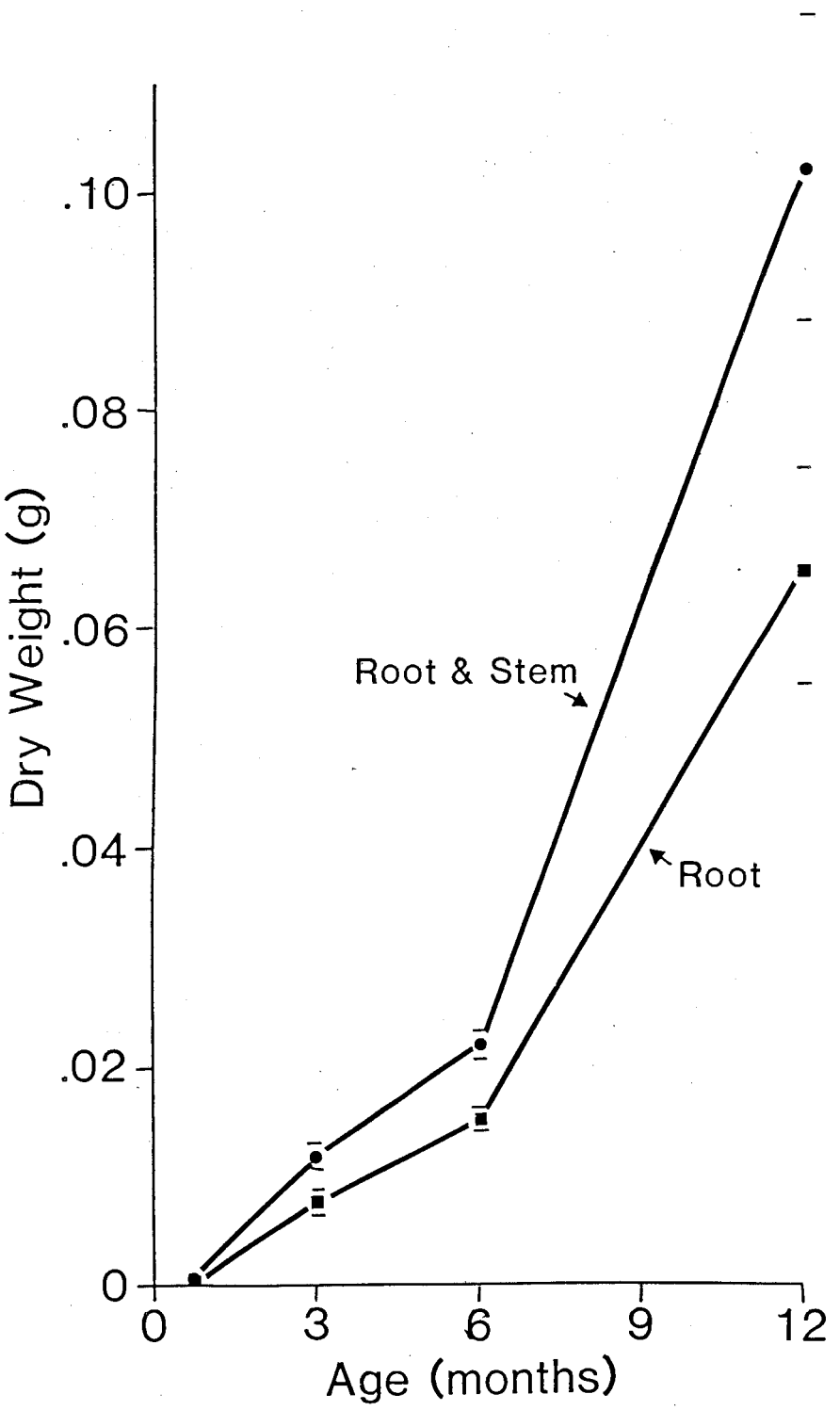

.10
.08
.06
.04
.02
0
Dry Weight (g)
Root & Stem
Root
0
3
6
9
12
Age (months)

Table 4.1 - CO_2 uptake and respiration rates of two month old white spruce seedlings that were either left *in situ* or transplanted one day before assessment.

Treatment	CO_2 Uptake (μmoles CO_2/mg chl/s)	Respiration (μmoles CO_2/mg chl/s)
No Transplant	0.114 ± 0.006	0.018 ± 0.003
Transplant	0.065 ± 0.014	0.017 ± 0.006

Note: ± standard error, n = 3.

have undergone a disruption during transfer to soil from the test tube.

Seedling and Plantlet Comparison

The CO_2 uptake rates for the plantlets are not appreciably different from the uptake rates of the *in vivo* grown 2 month old seedlings (Table 4.2). There was no difference in uptake rates of the plantlets and the seedlings that had been grown axenically in an agar supported medium. Seedlings grown on either the paper bridge or the soil-mix support media had uptake rates significantly higher than the plantlets or seedlings grown on agar. These results suggest that the support medium might influence the CO_2 uptake rates of the material grown in culture. This may indicate that the sink size and/or sink activity in the seedlings grown on agar is poor. Rancillac et al. (83) have reported that agar medium does produce a plantlet with less vigor.

The uptake rates are probably governed by the requirements of photosynthate sinks. Development of the seedling *in vivo* is modified by the incubator environment which had a dry atmosphere (40 - 50% RH) relative to the test tube environment (approaching 100% RH). In the incubator, plant growth is below its potential, whereas seedlings grown in culture tubes (except in agar)

Table 4.2 - Comparison of the net CO_2 uptake rates of transplanted white spruce plantlets and seedlings grown either *in vivo* or *in vitro*.

Treatment	CO_2 Uptake (μmoles CO_2/mg chl/s) 1 Day After Transplant	30 Days After Transplant	Percent Change After 30 Days
Plantlet from Agar	0.056 a (±.003)	0.021 a (±.005)	-62+
Seedling from Agar	0.058 a (±.005)	0.032 ab (±.001)	-45+
Seedling from Soil *in vivo*	0.065 ab (±.014)	0.093 d (±.006)	+43+
Seedling from Vermiculite and Charcoal	0.078 ab (±.002)	0.064 cd (±.004)	-18+
Seedling from Liquid	0.097 b (±.006)	0.040 abc (±.008)	-59+
Seedling from Soil *in vitro*	0.098 b (±.005)	0.059 bc (±.001)	-40+

Note: Those values followed by the same letter in a column are not significantly different according to a Duncan's Multiple Range Test at the 95% level.
N = 3, ± standard error.
+ - Percentage change is significant at the 95% level.

are free of most of the environmental restrictions to which the in vivo seedlings are exposed. The agar medium might be imposing a restriction on root growth and development, despite the fact that the tube environment is otherwise favorable for growth.

The absolute CO_2 uptake rates decline for most treatments after thirty days in a humidity tent in the incubator (Table 4.2). The decline in the uptake for the seedlings grown in vermiculite and charcoal is much less than for the other seedlings grown in vitro. The decline for the plantlets is similar to the drop for most of the seedling treatments. The in vivo grown seedlings were the only treatment to show a net increase in uptake rates over the month.

These results indicate that there is an effect attributable to the support medium. Agar, soil-mix, and liquid media are poor with respect to production of a seedling that maintains vigor over time in vivo . Vermiculite-charcoal support medium produces the most vigorous plant, in vitro. However, the in vitro environment consistently produces a plant that does not perform well over time after transfer to the in vivo environment. The normal seedlings seemed to be more vigorous after the 30 day hold in the high humidity.

The above stated points can be further considered when O-level fluoresence measurements are examined (Table 4.3). O-level fluorescence is considered an estimator of plant

Table 4.3 - Comparison of O-level fluorescence values of white spruce plantlets and seedlings grown either *in vivo* or *in vitro*.

Treatment	O-level Fluorescence (millivolts) 1 day after Transplant	30 days After Transplant	Percent Change After 30 Days
Plantlet from Agar	2.37 b (±.23)	2.4 ab (±.82)	+1.26
Seedling from Agar	2.17 ab (±.14)	2.17 ab (±.03)	0
Seedling from Soil *in vivo*	1.57 a (±.27)	1.50 a (±.21)	-4.46
Seedling from Vermiculite and Charcoal	2.73 b (±.38)	2.93 ab (±.38)	+7.33
Seedling from Liquid	2.73 b (±.35)	3.17 b (±.23)	+16.12
Seedling from Soil *in vitro*	2.73 b (±.34)	3.30 b (±.24)	+20.88

Note: Those values followed by the same letter in a column are not significantly different according to a Duncan's Multiple Range Test at the 95% level.
N = 3, ± standard error.

size. It is apparent from the O-level determinations that the shoot material of both the soil-mix and liquid media grew significantly over one month. However, the CO_2 uptake per mg chlorophyll decreased. The demand for photosynthate did not increase along with the foliar increase. This may indicate poor root growth and activity, since the root system is a major photosynthate sink in young seedlings (35,56,66). Therefore, there is more photosynthetic area at 30 days providing a similar or smaller net demand for photosynthate which means that the CO_2 uptake per chlorophyll would decrease.

The seedlings in agar and the plantlets showed essentially no increase in foliar material as expressed by O-level (Table 4.3). The CO_2 uptake in both materials drops significantly as well (Table 4.2). This indicates that the physiology of this material is deteriorating over this time period. Foliar growth is stagnant and photosynthate demand is decreasing. These observations may be related to root growth and activity. Photosynthetic capacity and chlorophyll synthesis depends highly on root activity, primarily the vigor of the root tips, and if the root growth is stopped or slowed, the shoot growth and photosynthetic activity can be curtailed significantly (14,37,99). The roots of the seedlings grown on agar are visually quite different from roots of material grown in other support media. The agar roots are stubby and thick, brittle and lack branching. These roots are probably nonfunctional.

The seedlings grown in vermiculite-charcoal showed a minor increase in foliar growth. Along with this increase there was a decrease in unit CO_2 uptake for the vermiculite-charcoal seedlings. Despite an insignificant decrease in foliage, there was a large increase in uptake for the *in vivo* grown seedlings. Seedlings grown on vermiculite and charcoal show a similar response as the seedlings grown on liquid or in soil-mix supports. However the magnitude of decline in uptake is smaller for the charcoal-vermiculite grown seedlings. The *in vivo* grown seedlings show an increase in photosynthate demand over the 30 days. This can be attributed to root growth (35,56,66). The *in vivo* grown seedlings are physiologically well established by 30 days.

The medium may be a major determinant of growth and vigor of the material on transfer to soil. Agar support medium yields seedlings with the poorest long term vigor, followed by liquid-paper bridge and soil-mix, then vermiculite-charcoal support media. The seedlings grown *in vivo* show the best situation, that is, an increase in photosynthetic CO_2 uptake on a per unit chlorophyll basis. The seedlings grown in vermiculite charcoal most closely approach this situation. The plantlets were very similar, with respect to foliar growth and change in CO_2 uptake, to the seedlings grown in agar suggesting that the medium affects the plantlet physiology.

Table 4.4 shows the respiration measurements of the material over the 30 days. On the first day after transplant there was no

Table 4.4 - Comparison of the respiration rates of transplanted white spruce plantlets and seedlings grown either *in vivo* or *in vitro*.

Treatment	Respiration (μmoles CO_2/mg chl/s) 1 day After Transplant	Respiration (μmoles CO_2/mg chl/s) 30 days After Transplant	Percent Change After 30 days
Plantlet from Agar	0.022 a (±.002)	0.015 b (±.002)	-32+
Seedling from Agar	0.011 a (±.010)	0.007 a (±.001)	-36
Seedling from Soil *in vivo*	0.017 a (±.006)	0.008 a (±.001)	-53+
Seedling from Vermiculite and Charcoal	0.018 a (±.003)	0.005 a (±.001)	-72+
Seedling from Liquid	0.020 a (±.003)	0.006 a (±.001)	-70+
Seedling from Soil *in vitro*	0.017 a (±.002)	0.006 a (±.001)	-64+

Note: Those values followed by the same letter in a column are not significantly different according to a Duncan's Multiple Range Test at the 95% level.
N = 3, ± standard error.
+ - Percentage change is significant at the 95% level.

significant difference in respiration of treatments. On day 30 however, the plantlets had high rates of respiration compared to all other material. Both the seedlings grown on agar and the plantlets showed the same magnitude of change. The absolute magnitude of the respiration of the plantlets at 30 days may reflect a serious physiological breakdown that is not occuring in the seedlings, since the CO_2 uptake is also poor for the plantlets.

The last statement may be emphasized by examination of the changes occuring in the absolute needle conductance over the 30 day period (Table 4.5). The magnitude of change of the conductance of the plantlets was very small compared to all other material. This indicates that the there may be a physiological dysfunction developing. Both the seedlings grown on agar and the plantlets have higher conductances relative to the other seedlings grown *in vitro*. The seedlings grown *in vivo* had the highest conductance.

The test of response to increasing vapor pressure deficits from .66 kPa to 1.59 kPa also showed some interesting relationships. Table 4.6 shows the response on day one after transfer. Both the plantlets and the *in vivo* grown seedlings showed the greatest reduction in CO_2 uptake when the test vapor pressure deficit was increased. The seedlings grown on agar showed the smallest decrease in uptake. The other three *in vitro*

Table 4.5 - Comparison of the needle conductances of transplanted white spruce plantlets and seedlings grown *in vivo* or *in vitro*.

Treatment	Conductance (μmoles $H_2O/m^2/s$) 1 Day After Transplant	30 Days After Transplant	Percent Change After 30 Days
Plantlet from Agar	487 a (±78)	482 ab (±89)	-1
Seedling from Agar	604 a (±47)	337 ab (±24)	-44+
Seedling from Soil *in vivo*	922 b (±64)	641 b (±25)	-30+
Seedling from Vermiculite and Charcoal	430 a (±29)	239 a (±13)	-44+
Seedling from Liquid	421 a (±38)	276 a (±32)	-34+
Seedling from Soil *in vitro*	458 a (±17)	210 a (±19)	-54+

Note: Those values followed by the same letter in a column are not significantly different according to a Duncan's Multiple Range Test at the 95% level.
N = 3, ± standard error.
+ - Percentage change is significant at the 95% level.

Table 4.6 - Changes in CO_2 uptake, conductance, internal CO_2 concentration (C_i), and fluorescence induction parameters when the test humidity is lowered from 75% to 40% RH. Tests with white spruce plantlets and seedlings grown either *in vivo* or *in vitro*, one day after transplant.

Treatment	Change in CO_2 uptake (%)	Change in Conductance (%)	Change in C_i (kPa x 10^{-4})	Change in Fluorescence P (%)	Change in Fluorescence M (%)
Plantlet from Agar	-32±17	-54±9	-6.17±.32	30±16	0
Seedling from Agar	-9±3	-58±7	-5.37±0.65	12±4	0
Seedling from Soil *in vivo*	-38±22	-58±1	-5.97±0.75	10±8	0
Seedling from Vermiculite and Charcoal	-21±8	-62±3	-6.27±0.46	7±8	0
Seedling from Liquid	-26±6	-40±6	-4.50±2.22	10±3	0
Seedling from Soil *in vitro*	-22±8	-61±9	-6.40±0.56	2±7	0

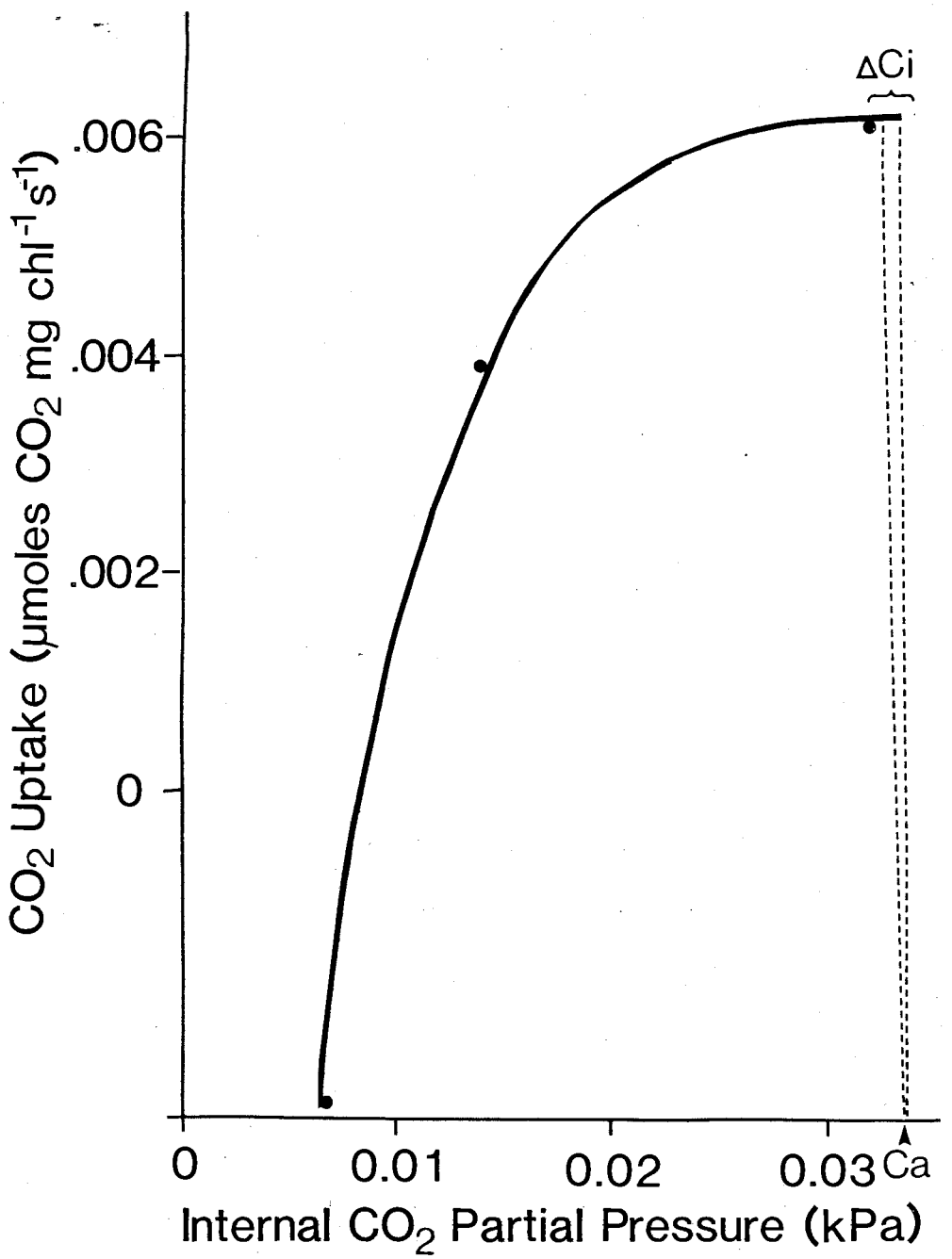
Note: N = 3, ± standard error.

grown seedlings showed an intermediate response. Changes in foliar conductance indicate that the stomata of all material showed a relatively similar magnitude of response. In none of the material did the internal CO_2 appear to become limiting (Table 4.6 and Figure 4.3). The relative P-level fluorescence responded in a similar magnitude as did CO_2 uptake. Lack of M-level fluorescence change indicates that there was no major physiological stress produced by the treatment.

The magnitudes of P-level fluorescence responses and CO_2 responses for all *in vitro* grown material were roughly equivalent. The disimilarity of the *in vivo* grown seedlings P-level fluorescence and CO_2 uptake responses is probably due to the decline of P, once a maximum is reached. This phenomenon was demonstrated in chapter 2. The fluorescence responses show that none of the plantlets or seedlings were water stressed to the point where the photoelectron transport system became limiting on CO_2 uptake. This is important since the assessment is designed to be nondestructive. The seedlings and plantlets were only subjected to a stress which caused the first phase of water stress response (chapter 2). Therefore, none of the material was exposed to serious level of stress.

These interpretations suggest that the seedlings grown *in vivo* are more sensitive to transient water stress than seedlings grown in test tubes. The reason for this sensitivity can be explained by a review of table 4.6, showing the absolute conductance values for the material. The conductance of the *in*

Figure 4.3 - CO_2 uptake response of a white spruce plantlet to variation in internal CO_2 partial pressure. Abbreviations: ΔCi - depression of internal CO_2 partial pressure relative to ambient, Ca - ambient CO_2 partial pressure.


ΔCi
.006
.004
.002
0
CO_2 Uptake (μmoles CO_2 mg chl^{-1} s^{-1})
0
0.01
0.02
0.03
Ca
Internal CO_2 Partial Pressure (kPa)

vivo seedlings is greater than the conductance of the other material. The susceptibility to initial transient vapor pressure deficit increases would be greater, because the flux of water vapor out of the foliage would be greater in material with higher initial conductance. Stomatal responses, though, were equal to those of other material on a relative scale.

The reason for the difference in sensitivity between the seedlings grown on agar and plantlets is possibly due to a reduced mesophyll resistance to internal water stress that is related to abnormal internal anatomy. The conductance of the agar grown seedlings was significantly lower than the conductance of the in vivo seedlings. Therefore, the susceptibility to water stress for the agar grown seedlings under test conditions would be significantly lower than for the in vivo seedlings. The susceptibility of the plantlet compared to the in vitro grown seedlings indicates a major difference in water stress control, even though needle conductance was similar. The reason may be related to either internal needle anatomy and/or to problems of water conductance through the shoot. There have been reports that the internal anatomy of plantlet foliage is disorganized (12,24,61,110) and this type of anatomy could be responsible for greater susceptibility to water loss when the water potential gradient across the stomata was increased by the lowering of humidity. A similar kind of response could be expected if the water conducting system beyond the needles were not fully functional, for example, if the

root-shoot interface was poorly developed.

Both in vivo grown seedlings and plantlets grown on agar are the most sensitive to water stress that is induced by increasing the environmental vapor pressure deficit. The seedlings have generally a greater conductance to begin with and so the susceptibility can be related to the lower inherent control of water loss in transient stress conditions. However, that does not mean that the in vivo grown seedling remains as susceptible over longer periods of exposure to the water stress. This is known to be the case because transplanted seedlings will easily survive outside the humidity tent whereas plantlets will not. Plantlets are very susceptible to vapor pressure deficits despite the fact that they have stomatal function comparable to in vivo grown seedlings.

Responses on day 30 after transplant show that the material did not change in ranking of response to water stress to any great extent. The water stress responses are indicated by the CO_2 uptake and fluorescence responses to the change in test humidity. It must be noted that the in vivo grown seedlings were less susceptible to the test water stress than on day 1. This may be due to a decrease in conductance from the initial value over the 30 day period (Table 4.7).

The plantlets appear to be even more susceptible to water stress on day 30, even though the conductance was similar to that on day 1. Along with this was a noted deterioration of the

Table 4.7 - Changes in CO_2 uptake, conductance, internal CO_2 partial pressure (Ci), and fluorescence parameters when the test humidity is lowered from 75% to 40% RH. Tests with white spruce plantlets and seedlings grown *in vivo* or *in vitro*, 30 days after transplant.

Treatment	Change in CO_2 uptake (%)	Change in Conductance (%)	Change in Ci (kPa x 10^{-4})	Change in Fluorescence P (%)	Change in Fluorescence M (%)
Plantlet from Agar	-41±14	-49±4	-6.30±0.51	2±13	0
Seedling from Agar	-14±12	-31±11	-5.40±0.03	11±4	0
Seedling from Soil *in vivo*	-28±4	-25±10	-5.20±0.66	24±5	0
Seedling from Vermiculite and Charcoal	-18±6	-37±4	-5.60±0.46	3±10	0
Seedling from Liquid	- 14±1	-30±11	-5.83±0.19	11±4	0
Seedling from Soil *in vitro*	-22±9	-25±4	-6.17±0.46	12±5	0

Note: N = 3, ± standard error.

visual appearance of the roots. This would tend to indicate that inadequate root function is a significant contributor to the water stress sensitivity of the plantlets. This does not preclude the possibility that the internal needle anatomy may also be contributory to stress susceptibility.

4.4 Conclusions

There are several points brought out by these studies. First, the CO_2 uptake competence of micropropagated spruce plantlets is similar to that of a comparable spruce seedling. Secondly, there is physiological deterioration of the plantlets over a 30 day period after transfer to soil. Lastly, there are inherent problems within the plantlet, indicated by susceptibility to water stress induced by transient increase of vapor pressure deficits from .66 kPa to 1.59 kPa.

Stomatal function in white spruce plantlets is similar to normal seedlings. This is in contrast to reports on horticultural species (19,24,104). The white spruce plantlets were exposed to exogenous cytokinins only at the outset of culture, whereas the standard protocol for the reported horticultural species involved continual exposure of the explants to some level of exogenous cytokinin. Such treatment can render stomata insensitive to environmental stimuli that would normally cause closure.

Three conclusions concerning the probable causes for impaired physiology in micropropagated white spruce plantlets are apparent. First, the type of support medium used *in vitro* can have influence on post-transfer CO_2 uptake. The best support medium was found to be made up of 5% charcoal in vermiculite. Second, effects of the *in vitro* environment, exclusive of the treatments imposed during micropropagation of plantlets, also have significant influence on subsequent photophysiology. This is demonstrated by the differences found between seedlings grown *in vivo* and *in vitro*. Third, other unknown factors in the *in vitro* environment, to which only plantlets are subject, are responsible for their deterioration once they are transferred to soil. This progressive deterioration of the plantlets was not observed in the *in vitro* grown seedlings. The cause may well be associated with development induced by the hormonal treatments during the micropropagation procedure.

The use of gas exchange and whole plant fluorescence techniques allow the estimation of stress response of both plantlets and seedlings to vapor pressure transients. The system developed in this thesis permits seedling and plantlet assessments to be made over time after transplant to soil. This adds a second dimension to the physiological characterization of both seedlings and plantlets. The probable sub-cellular sites of stress effects could be determined by analysis of the gas exchange and fluorescence data. The nondestructive nature of the vapor pressure deficit regime was confirmed as well.

The present results indicate the potential utility of the assessment procedures that were developed during these studies. The assessments show short term as well as long term physiological differences between plantlets and seedlings as well as between seedlings grown under various conditions. The tests of the various support media emphasized the importance of the rooting medium for success of plantlet culture. These procedures can be helpful when plantlet numbers do not allow their use in large tests of environmental factors that may be significant with respect to postculture physiology. They may be even more useful in large scale assessments of micropropagated material, as they would allow a clear characterization of the photophysiological status. Elimination or modification of environmental factors that are not favorable to photophysiological development could then be effected without waiting to see a long term survival and vigor response of outplanted material.

CHAPTER V

GENERAL DISCUSSION

This work set out to compare the photophysiological characteristics of micropropagated white spruce plantlets and whole seedlings from the same seedlot. Plantlets were produced in order to allow assessments to be made. Development of the assessment involved reworking of existing methods and instrumentation to suit the material under study. This involved setting up a gas exchange system and designing fluorescence instrumentation. Development of correlations allowed both the nondestructive estimation of plant size and interpretation of fluorescence responses with respect to CO_2 uptake and water status. Transient vapor pressure deficits were utilized in comparing the physiological responses of plantlets and seedlings in short term tests. Physiological changes, of all material, over a 30 day period were used for long term comparison.

Chapter 2 discusses the successful development of the assessment protocol as well as utilization of O-level fluorescence as an estimate of plant size. Relationships between fluorescence and CO_2 uptake, with respect to both soil and atmospheric water stress, were found. P-level fluorescence appeared to be a sensitive indicator of water stress during transient stress tests. An initial P-level increase is inferred to be due to an effect on the dark reactions of photosynthesis. As water stress increases, impairment of water splitting is

suggested by a P-level decrease. Effects on light reactions could be attributed to CO_2 uptake limitation when M-level fluorescence changes were noted.

Under water stress conditions the P-level is affected by two opposing control processes. Reduction in electron supply from PS II due to impairment of water-splitting would decrease P-level. On the other hand, quenching PS I by dark reaction components could also be impaired and this would cause a P-level increase. Examination of P-level and M-level relationships could be better seen under conditions where only electron supply would affect the system, i.e. in a test where light levels were varied. It was apparent that P-level dropped in a linear manner at higher ranges of illumination. M-level fluorescence fall was not apparent until P-level dropped to a considerable degree. These results demonstrate the progression in time of the response to increasing water stress. Effects on photosynthesis due to the photoelectron transport system are only seen after severe impairment of water-splitting. Before that time, reductions could primarily be attributed to direct impairment of dark reactions. Differences in the of degree of water stress can be distinguished.

Micropropagation procedures similar to Rumary and Thorpe (84) proved to be moderately successful. However, further work is required to make the protocol more consistent. The main limitation is the consistent production of functional roots on propagated shoots.

Studies comparing whole seedlings and micropropagated plantlets gave an indication of the power of the assay protocol. The gas exchange and fluorescence instrumentation was used to indicate relative levels of water stress induced by transient increases in vapor pressure deficits. The treatment resulted in the differentiation of the physiological status of seedlings and plantlets. The nondestructive nature of the water stress was confirmed by the fluorescence responses.

While similar levels of water stress occurred in plantlets and *in vivo* grown seedlings, the reason for the stress lay at different sites. Stress in the seedlings was due to degree of stomatal aperture. In contrast, the stress response of the plantlets seemed to be independent of stomatal aperture. Root support medium, as well as the culture environment, had significant bearing on physiology of material. At least three levels of effects influence plantlet physiology, these are : 1. the propagation protocol, 2. the rooting medium, and 3. the *in vitro* environment.

The obvious indication from this work is that physiological problems created by plantlet propagation environment are not simple and solution may involve many approaches. Rooting of plantlets, *in vitro*, on medium that will produce greatest vigor and resistance to water stress is one approach. These results together with work of Rancillac et al. (83) indicate that, for rooting, the use of agar can produce plantlets of poor vigor. Rooting might be also improved with the introduction of

mychorrhizal symbionts. This approach apparently is proving successful with a Betula species (46).

Other factors in the culture environment that may affect the growth and development of plantlets or shoots should also be investigated. Modification of media water potential has been shown, in some cases, to have dramatic effects on foliage development and morphology in culture (12,61,110). Effects due to hormone additions throughout the culture procedure should be studied, as they can affect shoot physiology and morphology (29) and stomatal behaviour (1).

All of the above suggested studies could easily be monitored using the assessment system developed here. The assessment would allow relatively rapid and multiple measurement over time so that improvements in rooting or the culture environment could be identified. This is the strength and utility of this nondestructive photophysiological assay. The result of such improvements would be a potential reduction of plantlet loss at the transfer from culture tube to soil stage of micropropagation.

The information that this assessment system yields could be useful in other plant production systems. All types of plantlets produced via micropropagation or other vegetative means could be assessed for their physiological status in a similar fashion as described here. Plants grown in the field (i.e. forest seedlings, crop and horticultural plants) can be assessed in

situ to determine their physiological status or their response to environmental stresses. This system has the flexibility to allow measurements to be taken in many situations in order to get an *in situ* assessment.

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