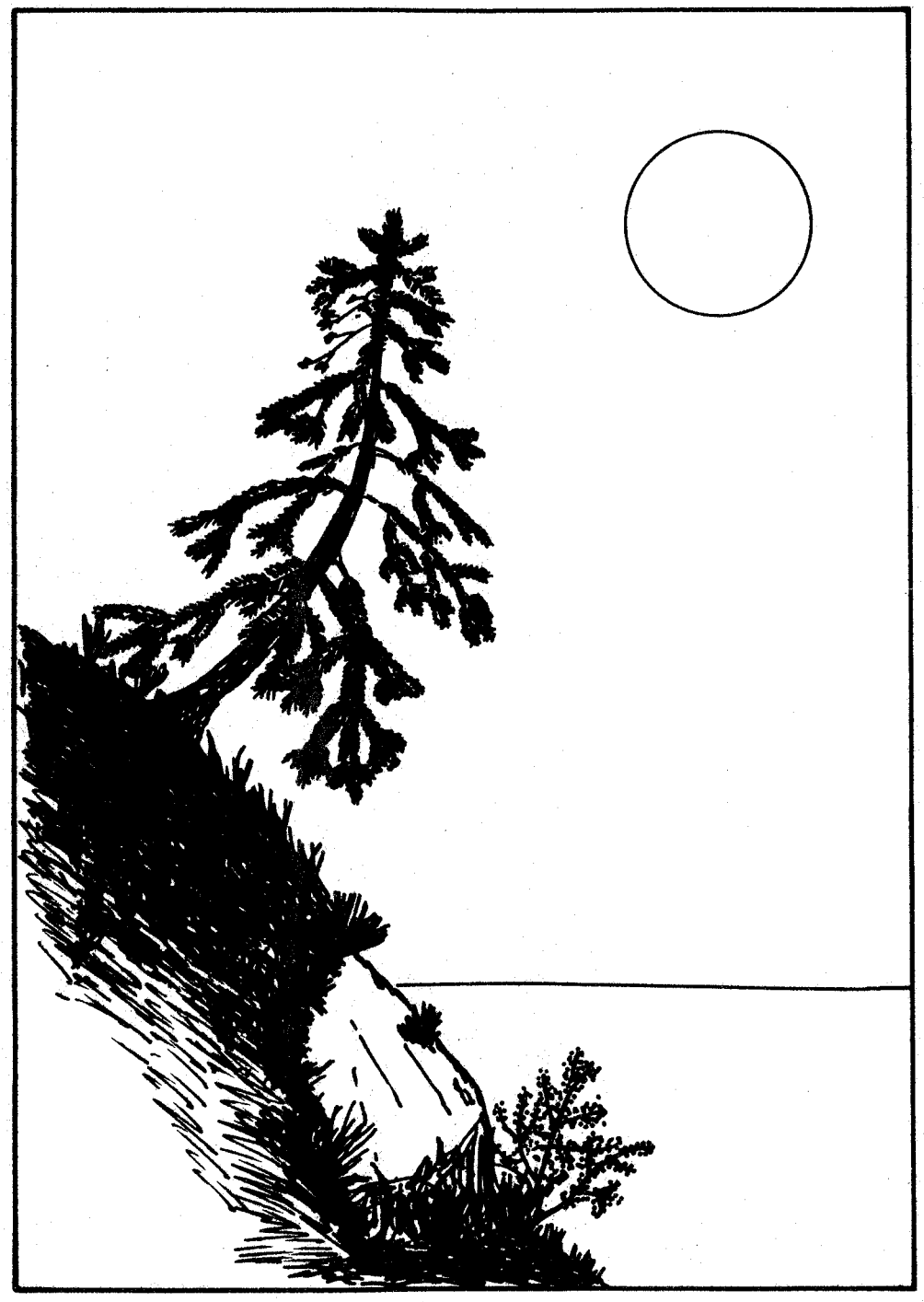

WINTER INACTIVATION OF PHOTOSYNTHESIS IN *ABIES AMABILIS (DOUGL.) FORBES*

by

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B.Sc., Simon Fraser University, 1973

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in the Department
of
Biological Sciences

SIMON FRASER UNIVERSITY
July 1978

APPROVAL

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Degree: Master of Science

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Winter Inactivation of Photosynthesis in Abies amabilis

Author:

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ABSTRACT

Winter Inactivation of Photosynthesis in
Abies amabilis (Dougl.) Forbes

The variable fluorescence of chlorophyll *a in vivo* has been used in this study to examine the photosynthetic activity of the subalpine conifer *Abies amabilis (Dougl.) Forbes* during winter. The normal fluorescence response (Kautsky effect) was greatly altered by prolonged cold weather, presumably indicating photosynthetic inactivation. Incubation of detached inactive needles at 20°C for 10 - 24 hours resulted in a recovery of a response similar to that of active needles. This recovery required the presence of water, but not light.

Various environmental factors such as freezing, desiccation, rehydration, and prolonged darkness, were examined for their possible roles in the winter inactivation phenomenon. The photosynthetic apparatus was unaffected by most freezing treatments although partly desiccated needles suffered some permanent damage. Desiccation at 20°C produced a response closely resembling the field inactive response, but recovery from this condition after rehydration did not occur. Less severe desiccation allowed full photosynthetic recovery following rehydration. Though the fluorescence response induced by long dark periods was markedly different from that of untreated needles, it did not resemble the field inactive response.

Further examination of the field response showed that in some inactive needles, an active type response could be induced by increasing the excitation light intensity, suggesting that different levels of inactiva-

tion are possible.

The exact sequence of events leading to inactivation of photosynthesis in *A. amabilis* cannot be described fully at this time. Freezing is known to be a prime requirement, and the water status of the needles at the time of freezing appears to be important. Daylength seems not to be directly related to inactivation, although the ability to become inactive under a given set of environmental conditions could be influenced by photoperiod. Photosynthetic inactivation in *A. amabilis* cannot be considered a true dormancy in the classical sense for two reasons. First, the occurrence of inactivation is highly dependent on the position of the needles on the tree and, second, the only requirements for full recovery of photosynthesis are the presence of water and the onset of warmer conditions.

The inactive response appears to be related to a decrease in the turnover rate at the PS II reaction center. Some possible causes for this decrease, and its adaptive significance in relation to winter photosynthesis, are discussed in the text. Winter inactivation seems to be a direct effect of environment on the photosynthetic apparatus and the mechanism of photosynthetic deactivation (decreased CO_2 uptake) reported for other subalpine conifers during winter may be similar to that described here.

ACKNOWLEDGEMENTS

I would like to thank Professor William Vidaver, my senior supervisor, for his enthusiastic support during the course of the study, and, in particular, for his untiring assistance in the preparation of this manuscript. I would also like to extend my appreciation to the members of the supervisory committee, Drs. G.R. Lister, R.C. Brooke, and L.M. Srivastava for their continued support throughout the study.

I wish to extend my sincere gratitude to Dr. U. Schreiber, whose unfailing support during many hours of discussion and criticism, and invaluable experience in the field of chlorophyll fluorescence analysis, helped to make this investigation possible.

Special thanks are due to my wife, Patricia Duggan, for her understanding and encouragement throughout the entire period of this project.

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LIST OF ABBREVIATIONS

1)	Chl*a*	:	Chlorophyll *a*
2)	Chl*b*	:	Chlorophyll *b*
3)	DCMU	:	3- (3,4-dichlorophenyl)-1,1-dimenthylurea
4)	I	:	When followed by a number (eg. 2.0×10^3 ergcm^{-2} sec^{-1}) I represents the excitation light intensity produced by the fluorometer light emitting diode
5)	O,I,D,P,S,M,T	:	Features of the Kautsky Fluorescence Transient described in the text (Page 11 and 12a)
6)	PAR	:	Photosynthetic active radiation (400 - 700 nm)
7)	PS I	:	Photosystem I
8)	PS II	:	Photosystem II
9)	P_{678}	:	Fluorescing chl*a* within PS II
10)	P_{680}	:	PS II reaction center chl*a*
11)	P_{700}	:	PS I reaction center chl*a*
12)	Q	:	PS II primary electron acceptor
13)	X	:	PS I primary electron acceptor
14)	Z	:	Watersplitting complex
15)	µE	:	micro einstein

CHAPTER I

INTRODUCTION

Section I: Winter Inactivation of Photosynthesis

There is a marked drop in the photosynthetic output of conifers from continental and subalpine climatic zones coinciding with the onset of cold weather. Photosynthesis in subalpine conifers drops to zero with the occurrence of deep frosts, and subsequently a negative CO_2 balance is maintained for months (Bourdeau, 1959; Pisek and Winkler, 1958). Where frosts are less severe the period of negative CO_2 balance is shorter and trees of these localities are able to take advantage of midwinter warm periods. Tranquillini (1964) demonstrated inactivation in *Pinus cembra* where photosynthesis was severely inhibited by needle temperatures below $-4^{o}C$. Bamberg *et al.* (1967), also with *Pinus cembra*, showed that the decrease in photosynthetic capacity normally accompanying cold weather also occurs to some extent in trees maintained under constant light and temperature, concluding that either the trend towards decreased photosynthesis had set in prior to the experiment or there was an endogenous factor regulating photosynthesis. They also demonstrated that inactive plant material brought into a warm greenhouse recovered much of its summer photosynthetic rate after several hours to a day or more. Schwarz (1971) found that the speed with which winter dormant plants regained positive CO_2 uptake rates after being warmed in the laboratory was dependent both on the species tested and on the extent of dormancy. Similar cases of low temperature inactivation have been reported for Douglas fir by Pharis (1970) and for bristlecone pine by Schulze, *et al.* (1967). On the other hand,

Fry and Phillips (1977) demonstrated that the suppression of photosynthetic rates in conifers was not found in the relatively mild winter conditions of southwest England, and concluded that cold inactivation of conifer photosynthesis was more characteristic of continental and subalpine environments than it was of temperate climates. They did report changes in chloroplast organization and starch content, but were unable to correlate these features to any drop in photosynthetic rates. A winter drop in photosynthetic capacity was reported by van den Driessche (personal communication) for Douglas fir growing under the mild climatic conditions of Vancouver Island.

It appears that some interaction between daylength and freezing temperatures is involved with the drop in photosynthetic rates found in high latitude (Vowinkel, *et al.*, 1975) and high elevation conifers during winter (Hiede, 1974). However, Bamberg, *et al.* (1967) demonstrated the possibility that an endogenous rhythm is involved. There is general agreement that prolonged freezing is the ultimate cause of the inactivation of photosynthesis in conifers (Bamberg, *et al.*, 1967; Pharis, 1970; Schulze, 1967; Schwarz, 1971; and Tranquillini, 1964) although the actual mechanisms involved remain unknown.

It was found that in *Abies amabilis (Dougl.) Forbes**, a subalpine conifer of the Coast Ranges of British Columbia, the variable chlorophyll fluorescence response typical of healthy active plant material is lost

* Authorities for species names are according to Hitchcock & Cronquist (1973)

under certain winter conditions, and is regained only after 10 - 24 hours incubation in the laboratory. Figure 1-1 compares an active response of this species typical of warm periods, with the inactive response found under cold conditions. The inactive response indicates major changes in the operation of the photosynthetic partial reactions at the level of either pigments and/or electron transport, and the purpose of this study is to examine the changes that take place, and how these relate to the observed inactivation of photosynthesis.

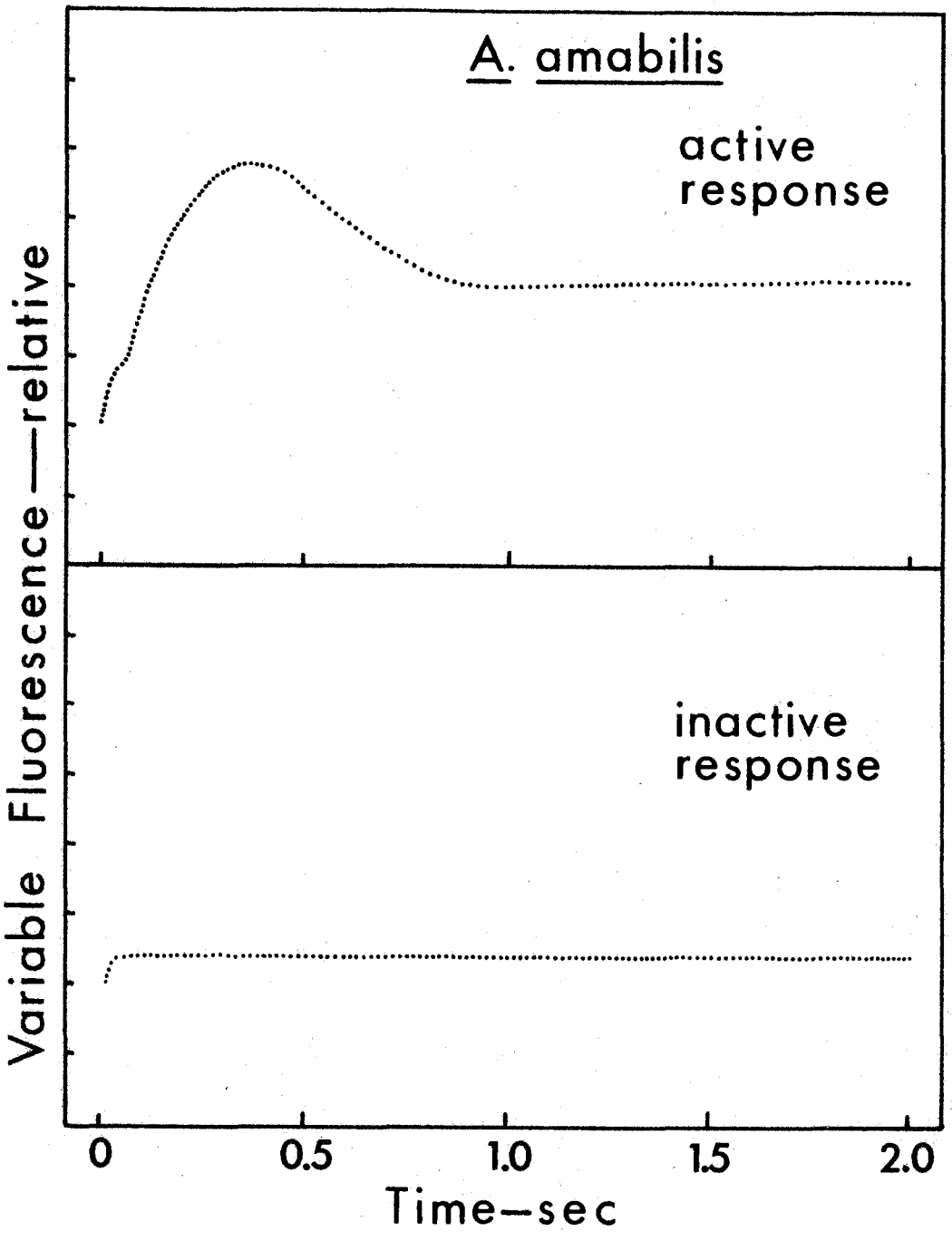
The technique of fluorescence induction analysis* has been used to investigate the influence of environmental variables on several plant species. Schreiber, *et al.* (1978) used it to examine effects of ozone on photosynthesis in developing bean leaves. Similarly, desiccation effects on photosynthesis in marine algae were studied by Wiltens, *et al.* (1978). Temperature effects on fluorescence induction in several plant species were analysed by Schreiber and Berry (1977), Schreiber, *et al.* (1976), and Armond, *et al.* (1978). These studies clearly show that the fluorescence induction response is influenced by environmental variables, and that

* Only a very superficial introduction into the field of fluorescence measurement is within the intended scope of this discussion. For a more complete understanding of this complex subject the works of Govindjee (Govindjee and Papageorgiou, 1971; Munday and Govindjee, 1969) and Butler (1972) may be helpful. In addition the papers by Davis, et al. (1976), Krause (1974), Strasser and Sironval (1974), Bonaventura and Meyers (1969), and Murata and Sugahara (1969), provide specific information about the relationship between fluorescence and partial processes like oxygen evolution, and energy distribution changes. Important work relating fluorescence to the stability of membranes in desert plants has been carried out for several years at the Carnegie Institute in Palo Alto, California (Schreiber and Armond, 1978, Berry et al., 1975). Samuelsson and Oquist (1977) have described a method for assessing the primary productivity of aquatic systems utilizing fluorescence. An excellant review of information relating to fluorescence is that of Papageorgiou (1975).

Figure 1-1 Active and inactive fluorescence responses in *A. amabilis* needles collected in late October 1975.

$I = 2.0 \times 10^3$ erg $cm^{-2}sec^{-1}$

(I, in this format, represents the excitation light intensity of the fluorometer light emitting diode. Where it is not followed by a similar number-letter notation it refers to a feature of the variable fluorescence response.)


A. amabilis
active response
inactive response
Variable Fluorescence—relative
0
0.5
1.0
1.5
2.0
Time—sec

information about the extent to which photosynthetic capacity is affected by environmental factors can be obtained from analysis of the induction transients.

Using this chlorophyll *a* (Chl*a*) fluorescence induction analysis the phenomenon of winter inactivation of photosynthesis in *A. amabilis* was examined. The study has three major emphases: First, the environmental conditions which were thought to induce inactivation were systematically investigated. Second, the mechanisms by which winter photosynthetic activity is regulated were considered. Finally, the whole problem of inactivation and recovery was analysed in terms of present understanding of the contributions of photosynthetic partial reactions to the overall process of photosynthesis.

Section 2: Chl*a* Fluorescence Induction

When a photon is absorbed by a molecule an electron is raised from a low energy level to a higher level. These energy levels are the ground state and the first (or higher) excited singlet states respectively. The wavelengths of light which can be absorbed by any particular molecule are determined by the available energy states in the electron orbitals. As light comes in discrete packets or quanta of energy, it can only be absorbed in quantum steps where stable electron configurations are available. The orbitals of electrons from simple elements are few and well defined so that their absorption spectra are characterized by very narrow bands. In more complex molecules like chlorophyll, the interaction between different

parts of the molecule as well as between the molecule and its immediate environment produces a multiplication of energy states leading to much wider absorption bands. Chl*a* absorbs strongly in the blue and red regions of the visible spectrum and only weakly in the green because of fewer available energy states in the latter region.

Once light energy has been absorbed by chlorophyll it can be dissipated in several ways. Some are nonproductive forms of energy release like heat loss, fluorescence, and phosphorescence; others lead towards the conservation of light energy and its conversion into chemical energy. In the same way that absorption of energy is regulated by available energy states, so energy dissipation is likewise determined. After the absorption of a blue quantum the high energy state relaxes, releasing heat in a series of small steps, over the large number of available energy states. When the electron reaches a level in which fewer electron states exist, it must make larger quantum steps in order to regain the ground state. It is these large jumps which give rise to fluorescence. Thus blue light, in addition to giving off a quantity of heat, also may result in fluorescence in the red part of the spectrum.

Fluorescence is by no means an inevitable consequence of light absorption. An excited chlorophyll molecule can return to the ground state by transferring its energy to a second chlorophyll. In the closely packed array of pigments in thylakoid membranes, this resonance transfer of energy fromone molecule to the next is much more likely than the emission of fluorescence. In a living green plant only about 3% of the absorbed light energy

is given off as fluorescence (Govindjee and Govindjee, 1975). Resonance transfer is the means by which energy moves from the pigment molecule where it was initially absorbed to the reaction center where it is changed to chemical energy.

Although the exact nature of the reaction center is not precisely understood, its operation in the conversion of light to chemical energy has been studied extensively (for a recent review, see Sauer, 1975). Closely associated with the reaction center pigment molecule (P_{680}) is an electron donor (Z) and an electron acceptor (Q). The reaction center complex of $ZP_{680}Q$ is thought to respond to the absorption of a quantum of light in the following way:

$$ZP_{680}Q \xrightarrow{h\nu} ZP_{680^*}Q \longrightarrow ZP_{680}{}^{+}Q^{-} \longrightarrow Z^{+}P_{680}Q^{-}$$

In this model, the excited chlorophyll (P_{680^*}) loses its electron to Q and then extracts a replacement from Z and is, therefore, temporarily in the oxidized state (Amesz and Duysens, 1977; Butler, 1972).

Research in the 1950's and early 1960's on photophosphorylation (Arnon, *et al.*, 1954), chromatic transients in red algae (Blinks, 1957), the red drop phenomenon (Emerson and Rabinowitch, 1960), the function of the chloroplast cytochromes (Hill and Bendall, 1960) and the discovery of P_{700} (Kok, 1960) has led to the formulation of a photosynthetic scheme requiring two sequential light reactions coupled by an electron transport system. The refined model, known as the Z-scheme, remains as a working

hypothesis for the pathway of photosynthetic energy conversion. Figure 1-2 outlines a model of the scheme.

Coupled to the sequential transfer of electrons from water to NADP+ is the transfer of protons from the stroma into the thylakoid interior. The resulting pH differential is thought to be the driving force behind photophosphorylation (Avron, 1977). There is also recent evidence suggesting a second type of phosphorylation which depends on the electrical field resulting from the charge separation across the thylakoid membrane at the Photosystem II (PS II) reaction center (Avron, 1977; Graber, *et al.*, 1977). Cyclic phosphorylation occurs when electrons from Photosystem I (PS I), instead of reducing ferredoxin and eventually NADP+, are recycled into the electron transport chain between PS I and PS II, resulting in the transfer of protons, before returning to the PS I reaction center (for a recent review of phosphorylation see Boyer, *et al.*, 1977). The products required for dark CO_2 fixation, NADPH and ATP, are generated near the external surface of the thylakoid membrane and then become available to the Calvin cycle.

After a period of darkness, all plants show characteristic changes in Chl*a* fluorescence yield when exposed to light (Kautsky effect: Kautsky and Hirsch, 1931). The complex interaction between PS I and II involving electron transport, ion fluxes, the direct but variable coupling between the respective pigment systems, in addition to the relationship between the light reactions and the Calvin cycle, contributes towards this variable fluorescence response (for a recent review of fluorescence induction, see

Figure 1-2 Photosynthetic electron transport adapted from Govindjee and Govindjee (1975)

Z : watersplitting enzyme complex

PS II : photosystem II reaction center

Q : PS II primary electron acceptor

PQ : plastoquinone

cyt : several electron transport cytochromes

PC : plastocyanin

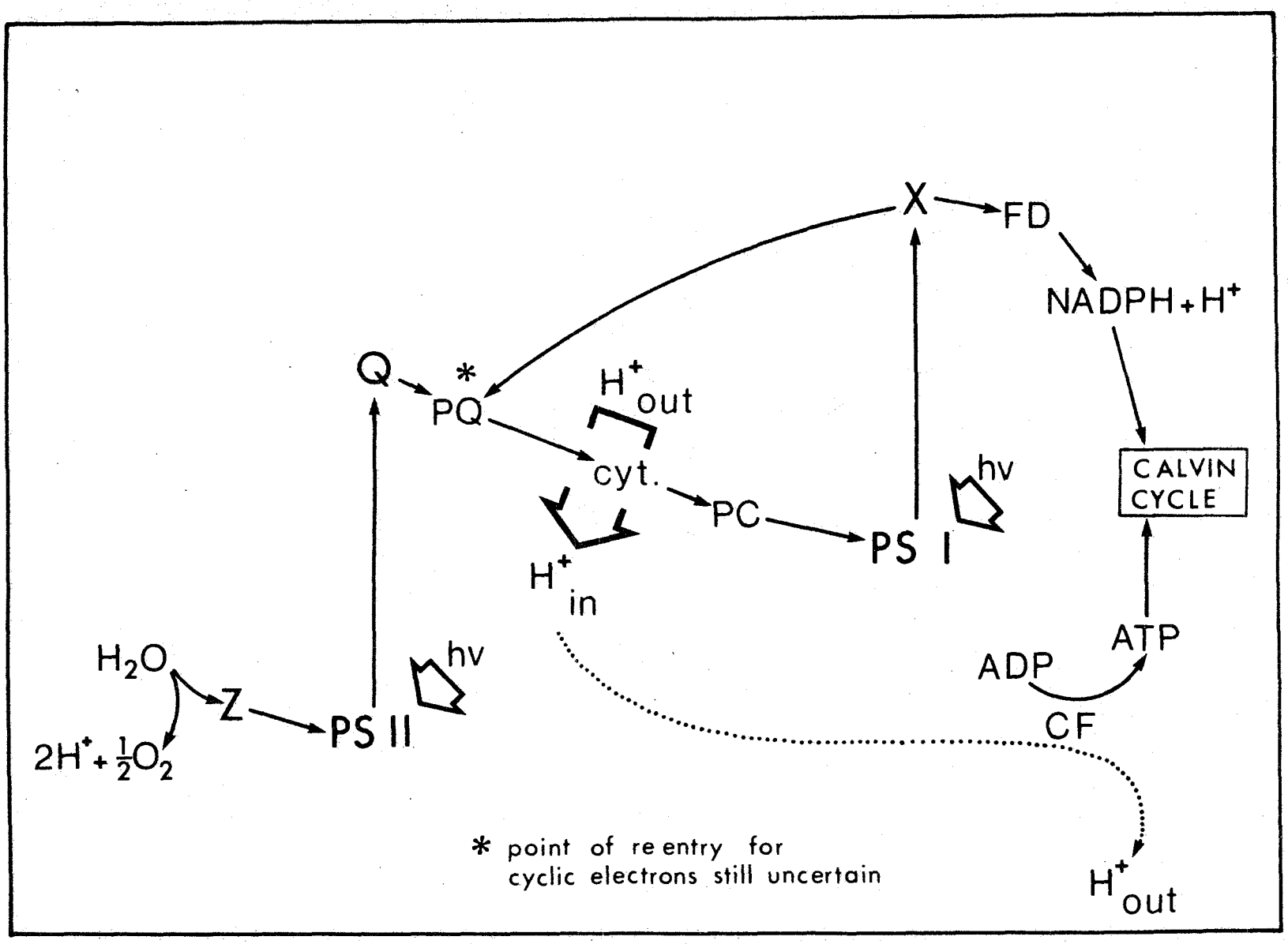
PSI : photo system I reaction center

X : PSI primary acceptor

FD : ferridoxin

CF : phosphorylation coupling factor

NOTE: Water splitting takes place after an accumulation at 3-4 positive charges at Z. The water splitting reaction donates 4 electrons to Z to replace those extracted by the light reaction at PS II. Exact stoichiometry of electron flow itself is not attempted in this figure.


X
FD
NADPH+H+
Q
*
PQ
H+ out
cyt.
PC
PS I
hv
CALVIN CYCLE
H+ in
H2O
Z
PS II
hv
ATP
ADP
CF
2H+ + ½O2
* point of re entry for cyclic electrons still uncertain
H+ out

Papageorgiou, 1975).

At room temperatures, most fluorescence originates from PS II (more particularly from a small percentage of the chlorophyll close to the reaction center, Chl*a* 678, and its intensity is thought to reflect the momentary supply of singlet Chl*a* excitation of PS II (Papageorgiou, 1975). The ability of PS II to utilize this excitation is controlled primarily by the availability of oxidized Q, the PS II primary electron acceptor. Since Q is a one electron carrier (Erixon and Butler, 1971) the reaction center is closed when the condition $Z^{+}P_{680}Q^{-}$ exists, and remains closed until Q is able to give up its electron to the secondary acceptor pools. This explains why the addition of DCMU, which blocks electron transport away from Q, results in a high fluorescence yield since all the PS II reaction centers become closed.

As a first approximation, fluorescence increases are a result of PS II activity while fluorescence quenching is caused by a number of factors including the following:

1. the reoxidation of Q by PS I acting through the secondary electron acceptors, which facilitates energy conversion at the PS II reaction center,

2. spillover of energy from PS II pigments to PS I pigments decreasing fluorescence intensity by reducing the migration of excitation energy towards the PS II reaction center,

(Murata and Sugahara, 1969),

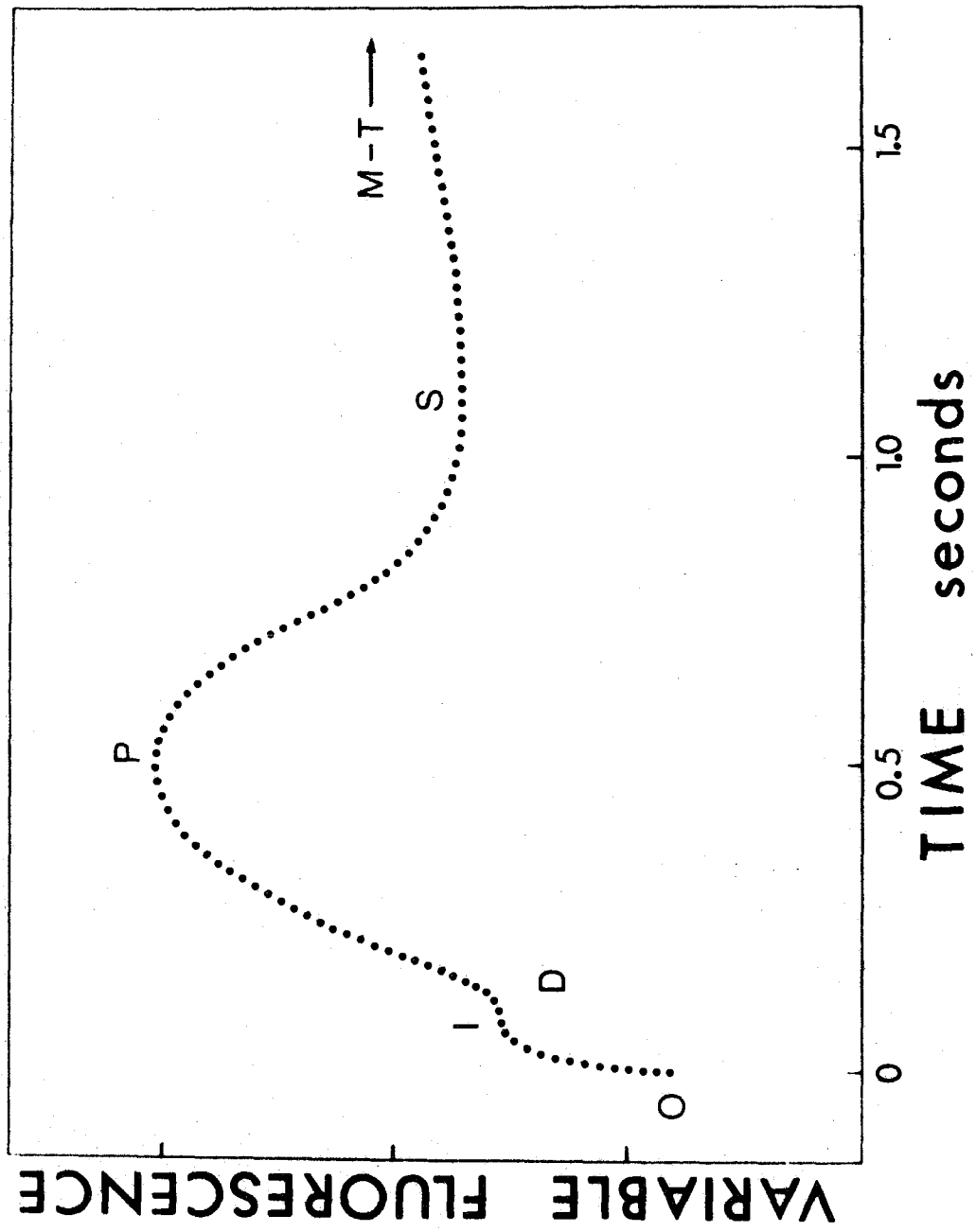
3) quenching by molecular oxygen (Schreiber and Vidaver, 1974).

Since the purpose of this study is to relate inactivation of photosynthesis in *A. amabilis* to environmental influences associated with winter conditions, and the Chl*a* fluorescence yield reflects the photosynthetic potential of a plant, changes in fluorescence induction have been used in the laboratory to determine the roles of low temperature, light intensity and desiccation in relation to the problem of winter inactivation of photosynthesis. In this study inactivation is taken to mean the loss of activity of the light reactions of photosynthesis as determined by Chl*a* fluorescence analysis. Reactivation means the recovery of the light reactions to an active state.

Figure 1-3 illustrates a typical fluorescence induction time course and represents the response of a photosynthetically active *A. amabilis* needle to a dark/light transition. The features of the time course (O, I, D, P, S, M, and T) are interpreted primarily according to the review by Papageorgiou (1975). A state of quasi-equilibrium (T) is attained when all partial processes reach relatively constant levels of activity. It is at this time that photosynthesis reaches a steady production rate under any given set of conditions (H_2O, light, temperature, and CO_2), and no further changes occur in fluorescence yield.

Figure 1-3 Hypothetical conifer fluorescence response. Interpretation is based primarily on that of Papageorgiou (1975).

Feature	Explanation
O-I rise	Initial reduction of Q by PS II; conversion of the water splitting system to the S_3 state.
I-D dip	Oxidation of Q by PS I via intersystem electron transport.
D-P rise	PS I acceptors temporarily exhausted, resulting in rapid reduction of Q.
P-S decay	Reoxidation of Q as PS I acceptors begin to clear. Quenching of fluorescence by molecular oxygen in pseudocyclic phosphorylation (Schreiber and Vidaver, 1974). Initiation of Calvin cycle.
S-M-T	Poorly understood part of the fluorescence response. Related to photophosphorylation and membrane conformational processes.


VARIABLE FLUORESCENCE
TIME seconds
0
0.5
1.0
1.5
O
I
D
P
S
M-T

Any environmental change may affect any one or all of the components of the variable fluorescence response. A change in T-level reflects a change in the overall rate of photosynthesis, while a change in any of the other components indicates specific effects on photosynthetic partial processes. It is these changes in time course components which are used in this study as indicators of the influence of environmental variables on photosynthetic activity.

Grigor'ev, *et al.*, (1973) compared fluorescence induction in C_3 and C_4 plants and found that carbon pathway was a factor in determining some aspects of the response. Since leaf metabolism has an influence on fluorescence induction, there is also a possibility that the fluorescence responses are different in the major plant groups. It seemed important to be able to distinguish between genetically determined variability and the influence of environmental factors on fluorescence induction responses. Consequently, a preliminary study comparing plants from several taxonomic divisions under different environmental conditions was carried out.

Figures 1-4 and 1-5 illustrate fluorescence transients for a number of plants representing major plant groups. Each of the responses clearly shows typical features of the Kautsky effect, as did samples of *Ginkgo, Psilotum, and Equisetum* (data not shown). Close examination of the time courses presented in Figure 1-4 and 5 reveal that all of the responses except for the dicot (Figure 1-5 C) are fundamentally alike kinetically. This distinction between the dicot sample and the representatives of all the other green plant divisions has persisted in all observations of

Figure 1-4 Fluorescence response of a green alga, moss, and a fern. Samples were given a 2 second light treatment (dashed line), dark adapted for 5 minutes, then given a 60 second light treatment (dotted line).

All samples were dark adapted for 30 minutes at $20^{o}C$ prior to the initial 2 second light treatment. A low excitation light intensity ($I = 0.7 \times 10^{3}$ erg $cm^{-2}sec^{-1}$) was used to allow maximum separation of the transient features in time.

A) unidentified green alga (fresh water : not keyed out).

B) unidentified moss species (not keyed out).

C) *Blechnum spicant* (L) *Roth.* - deer fern.

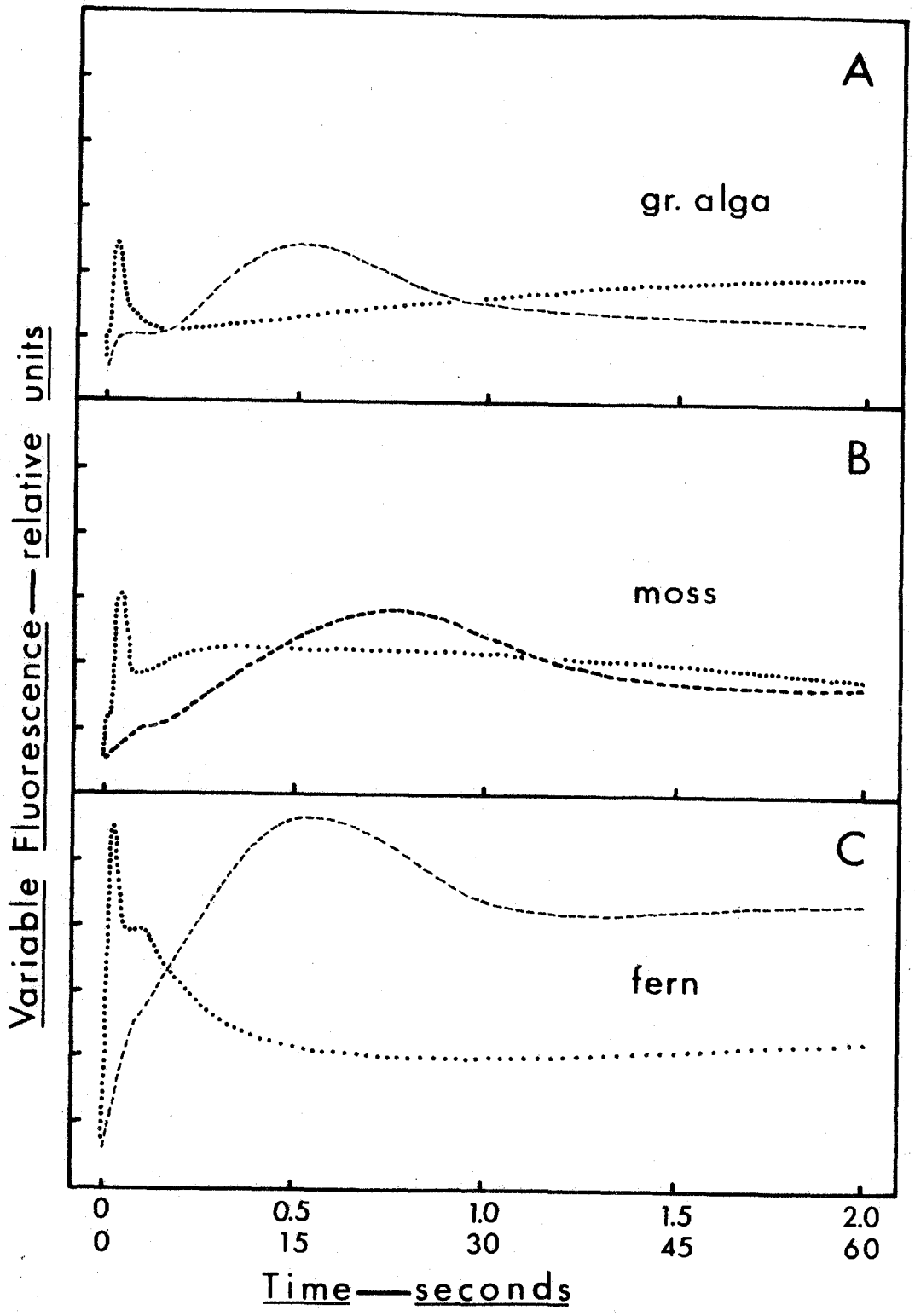

A
gr. alga
B
moss
C
fern
Variable Fluorescence — relative units
0
0
0.5
15
1.0
30
1.5
45
2.0
60
Time — seconds

Figure 1-5 Fluorescence response of a conifer, cycad, and a dicot angiosperm. Conditions as in Figure 1-4.

A) *Tsuga heterophylla* - Western hemlock

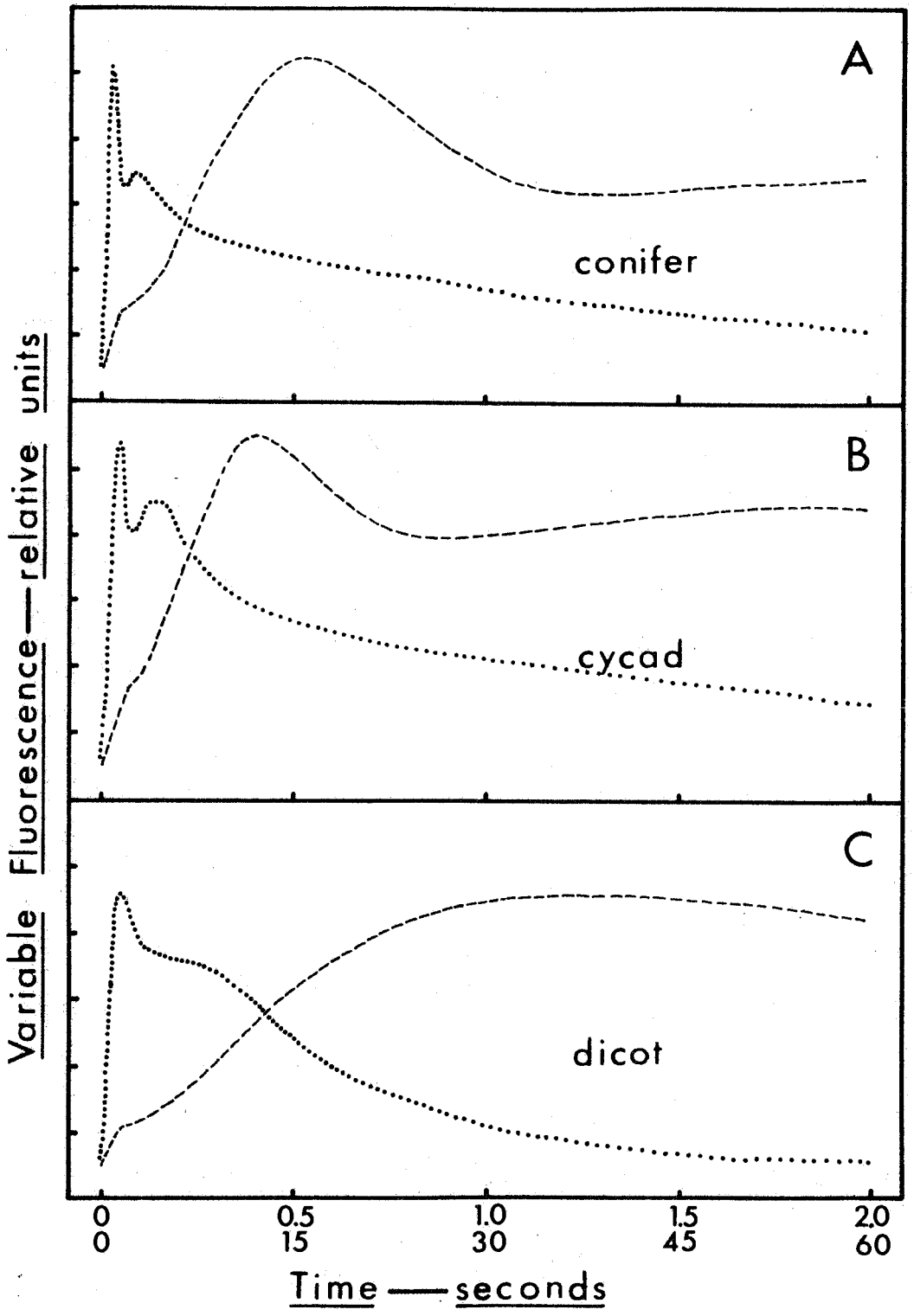
B) *Cycad species*

C) *Vaccinium parvifolium* Smith - red huckleberry

- - - - Fluorescence response during 2 second light treatment

..... Fluorescence response during 60 second light treatment

NOTE: The slow P-S decay in red huckleberry is typical of all angiosperms tested to date, including both monocots and dicots.


A
conifer
B
cycad
C
dicot
Variable Fluorescence — relative units
0
0
0.5
15
1.0
30
1.5
45
2.0
60
Time — seconds

angiosperms (including monocots) made to date (see Schreiber, *et al.*, 1977, for further examples). The components of the rise to P are generally slower and the decay to S is invariably slower in angiosperms than in all other green plants. While the significance of these differences cannot now be explained in terms of chloroplast structure, its persistence suggests that in the angiosperms, the way in which components of the photosynthetic apparatus interact with each other is not precisely the same as in the lower plants. In this respect the gymnosperms, of which *A. amabilis* is a representative, resemble lower plants. Perhaps this feature is worth investigating as one of the properties contributing to the success of the angiosperms as a group.

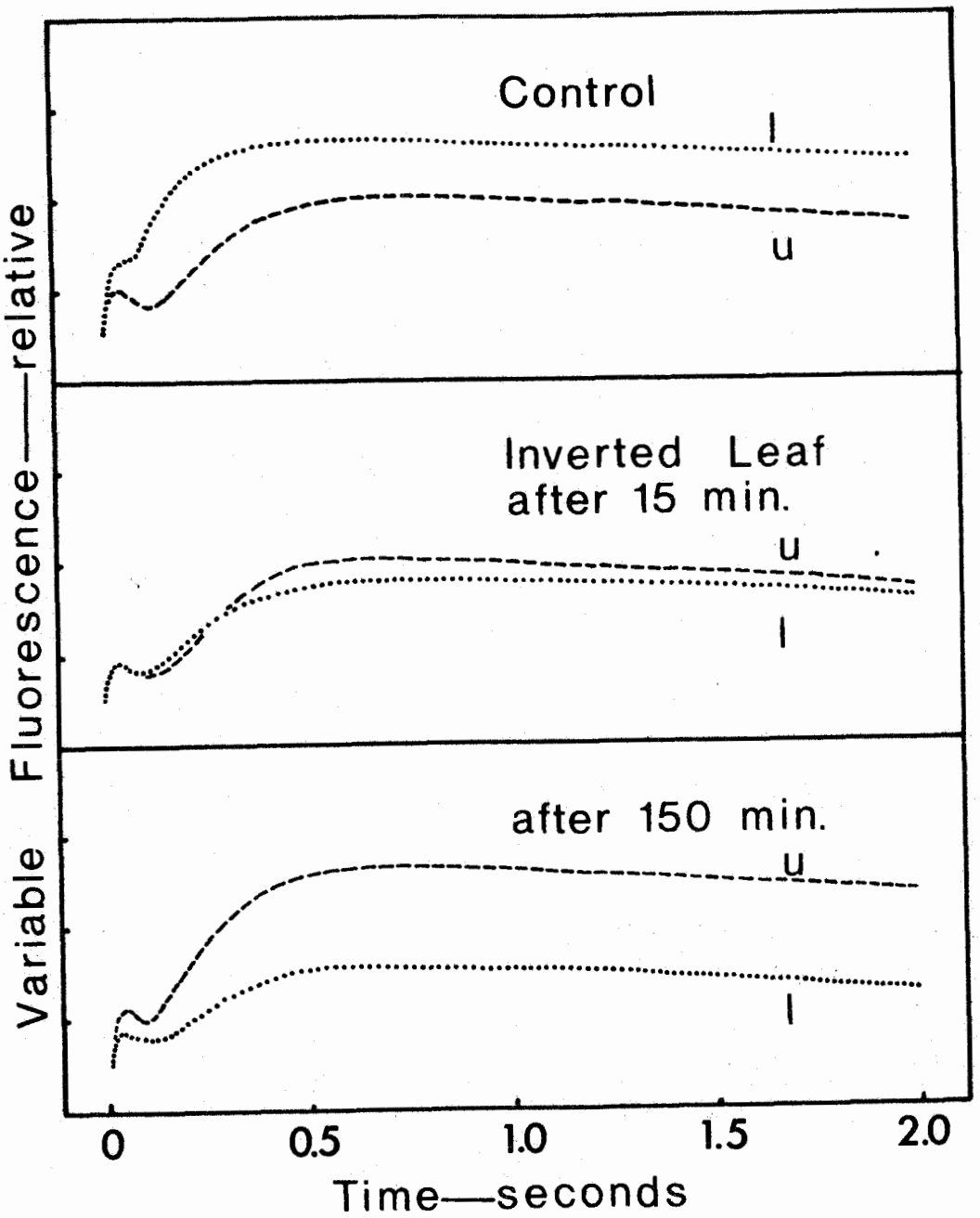
It was observed during the course of this study that the fluorescence responses of the upper and lower needle surfaces in *A. amabilis* are distinctly different. This difference is characteristic of all leaf surfaces examined to date (Schreiber, *et al*, 1977). It appears that at least some of the observed difference can be attributed to the fact that the development of the upper side of the leaf takes place at considerably higher light intensities than the lower side. The control response in Figure 1-6 exemplifies the difference. Similar differences are observed when the upper surface of leaves growing under sun and shade conditions are compared (see Figure 3 of Schreiber, *et al.*, 1977). To some extent, the lower leaf surfaces can be induced to respond like the upper surfaces, and vice versa, simply by inverting the leaves in the light for a period of time (Figure 1-6).

Figure 1-6 Effect of leaf inversion on fluorescence in *Hedera helix* L. Outdoor leaves were inverted at mid day and samples taken every 30 minutes. Outside light intensity was 1900 - 2100 uE m^{-2} sec^{-1}. Leaves were dark adapted for 60 minutes prior to fluorescence measurements.

------ upper leaf surface response (u)

..... lower leaf surface response (l)

$I = 1.6 \times 10^{3}$ erg $cm^{-2}sec^{-1}$


Control
l
u
Inverted Leaf
after 15 min.
u
l
after 150 min.
u
l
Variable Fluorescence——relative
0
0.5
1.0
1.5
2.0
Time——seconds

A mature needle of *A. amabilis* lives several years and undergoes many cycles of winter inactivation and spring reactivation. In any leaf, during its lifetime, the fluorescence response is subject to a number of controlling influences. Among these are:

1. the genetically determined components of the photosynthetic apparatus,

2. modifications of the photosynthetic apparatus related to conditions under which development occurs,

3. leaf morphology,

4. the response of the photosynthetic apparatus in mature leaves in adjusting to a change in environmental conditions.

In this study, the primary concern is with environmentally induced changes in mature leaves, and attempts were made to minimize the other determinants now that their significance was appreciated. This was done by restricting all determinations to the upper leaf surface of mature first year needles, and by attempting to account for the modifications related to variations in conditions under which development occurred.

EXPERIMENTAL DETERMINATIONS OF FLUORESCENCE INDUCTION IN *ABIES AMABILIS (DOUGL.) FORBES*

Materials and Methods

Abies amabilis needles were collected from subalpine locations on the south-facing slope of Mt. Seymour (1300 meters), in the Coast Range Mountains of south-western British Columbia.

The Mt. Seymour habitat of *A. amabilis* is characterized in winter by extensive cold periods below the freezing temperature of the needles (-4°C or lower). Snow cover can be 2 or more meters and last up to eight months. Soil water content remains high during winter in most habitats and the soil is seldom frozen (Brooke, *et al.*, 1970). The region is subject to temperature fluctuations over an interval of a few hours between several degrees below 0°C and +10 - 15°C even in late spring and early fall.

Detached branches of *A. amabilis* were stored in loosely packed snow in the dark for the trip back to the laboratory. Unless otherwise stated, all experiments were done in the dark at 20°C ($\pm$2°C).

Fluorescence measurements were made with the portable fluorometer developed by Schreiber, *et al.*, (1975). Excitation light was produced by a light emitting diode (Monsanto MV 5020; Monsanto Commercial Products Co., Cupertino, California, U.S.A.), peak wavelength approximately 660 - 670 nm at a forward current of 50 mA. Fluorescence was separated from excitation light with a 3 mm thick cutoff filter (Corning CS 7-69; Corning Glass Works,

New York), in place over the photodetector, and therefore, only long wavelength fluorescence (>710 nm) was measured. Fluorescence emission was measured with a combination phototransistor-operational amplifier (Bell and Howell 529-2-5; Bell and Howell, Conn.) as the photodetector.

The fluorometer output was displayed on either one of two dual beam storage oscilloscopes. For field measurements and the desiccation studies a portable Tektronix (Beaverton, Oregon) model 214 was used, and for the rest of the experiments a Tektronix (Beaverton, Oregon) model 5103N Oscilloscope was used.

Excitation light emitted by the LED was measured with a YSI-Kettering Radiometer (model 65; Yellow Springs Instrument Co., Yellow Springs, Ohio), and ranged from 0.3 - 2.4 x 10^3 erg $cm^{-2}sec^{-1}$. Outdoor light measurements were made with a Lambda Quantum/Radiometer/Photometer (model LI 185, Lambda Instrument Corp., Lincoln, Nebraska) and are presented as $\mu E\ m^{-2}sec^{-1}$ (PAR).

A number of factors combine to influence the shape of fluorescence responses of plants. Among these are:

1) light intensity at the time of collection,

2) time allowed for dark adaptation (in part determined by 1),

3) the physiological state of the plant, especially in

relation to dessication (see Chapter II section 2) and temperature.

During the actual measurement, the following influences became important:

1) light intensity of excitation,

2) wavelength of excitation light,

3) availability of oxygen to the sample, particularly where repetitive measurements are made,

4) movement of the sample during measurement can result in misinterpretation of results.

The techniques used in this study to prepare samples for fluorometric analysis were standardized in the following way:

1) all plants were dark adapted for at least 30 minutes prior to measurement. Longer periods were not required to give reproducible responses and the responses were stable during dark periods up to several days (see Chapter II Section 2, Dark Storage),

2) during dark adaptation, all plant material used was kept

in black plexiglas boxes which were periodically opened to prevent anaerobiosis,

3) leaf material was kept over wet filter paper, while branches and individual needles were kept with their stems or petiole ends in water,

4) sample movement was reduced by placing a weight on the sample to hold it on the probe so that vibrations were at a minimum.

The light intensity of excitation varied from 0.7×10^3 erg $cm^{-2}sec^{-1}$ to 2.4×10^3 erg $cm^{-2}sec^{-1}$. A low light intensity slows down the various features of the fluorescence response and reduces their amplitude. This is compensated for by:

1) increasing the sensitivity of the oscilloscope and,

2) reducing the sweep rate (number of divisions per second) of the oscilloscope.

Increasing the excitation light intensity speeds up the early parts of the fluorescence response and makes it difficult to detect the I-D transient, but slows down the P-S decay since the processes quenching fluorescence do not increase to the same degree as processes giving rise to fluorescence.

The range of light intensities used here provides good separation of features in active needles of *A. amabilis*. Below 0.7×10^3 erg $cm^{-2}sec^{-1}$, the fluorescence response is reduced to an O-I rise followed by a horizontal line (very similar to the winter inactive response in *A. amabilis*). At this excitation light intensity, the rate of fluorescence quenching is high enough to prevent an appreciable reduction of the pool of Q.

The fluorescence yield under these conditions is very low and scope sensitivity must be increased. This contrasts with the winter inactive response in that the fluorescence yield of the active and inactive response (though different) are of the same order of magnitude.

Different light intensities have been used in different experiments throughout this study and are indicated in the figure legends. During any particular experiment, light intensity was kept constant, and any variations in samples due to differences in chlorophyll density or differences in the geometry of needle, LED, and photodetector were eliminated by equalizing "0" levels through oscilloscope sensitivity.

An exception to this rule of equal light intensity during an experiment is found in Chapter II section 2 (Levels of Reactivation). In this particular case of reactivating needles, increasing excitation light intensity resulted in the appearance of the Kautsky features in needles thought to be inactive. This does not occur in completely inactive needles within the range of intensities available in the apparatus.

Again, it should be pointed out that all determinations of fluorescence were made on the upper needle surface of first year needles.

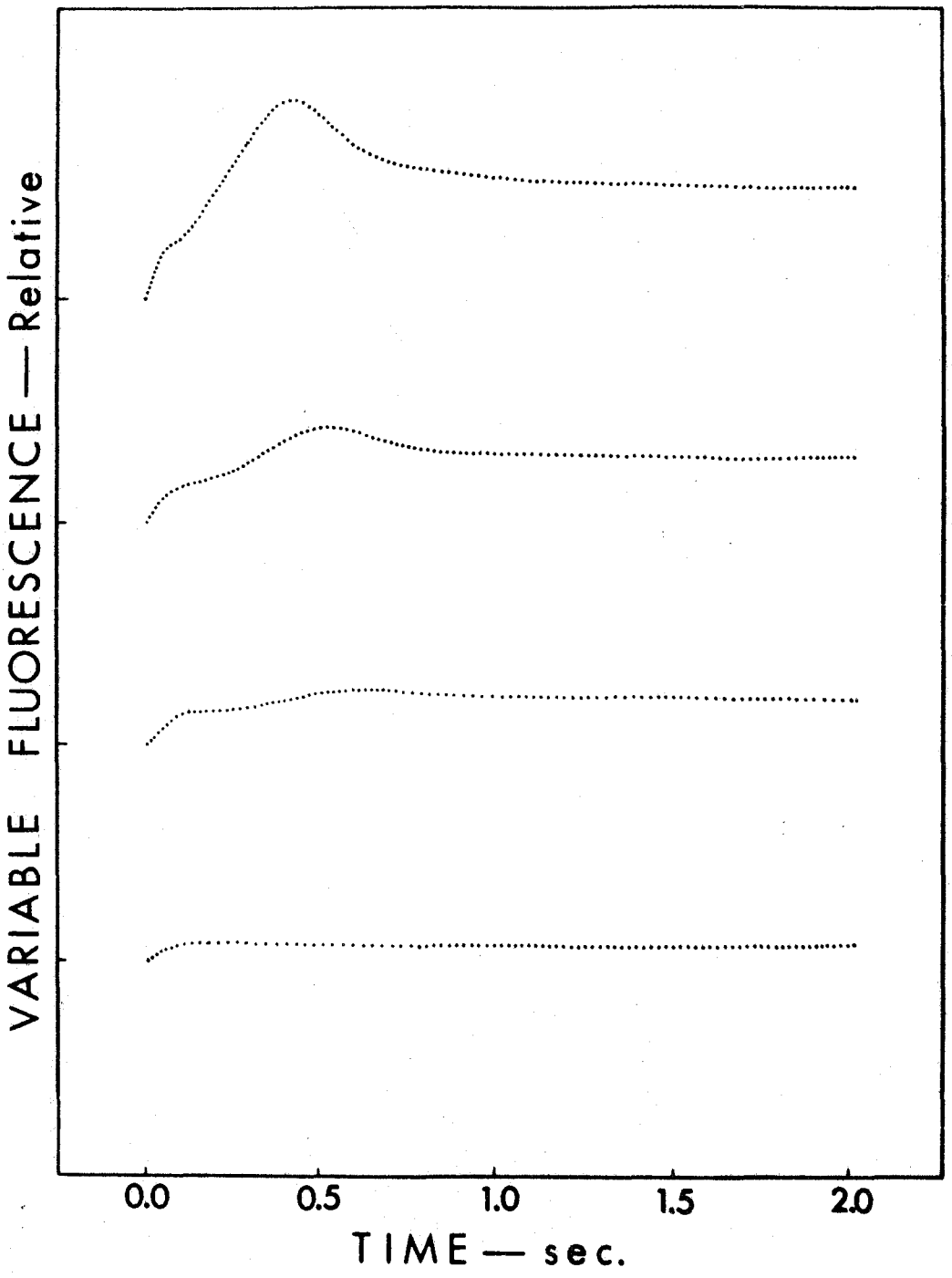
Section 1: Field Investigations

During extended periods of freezing temperatures it was found that the active fluorescence response of *A. amabilis* needles is no longer present.

Brief warming does not restore the active response. Recovery differed in individual needles and it took from 10 to as much as 24 hours at 20°C to fully restore the variable fluorescence response. Light was not necessary but the samples had to be stood with their petiole ends in water or recovery was much slower or did not occur at all. The inactive response (Figure 1-1) is characterized by a short O-I rise followed by a flat line. Recovery is accompanied by a gradual reappearance of the active response (Figure 2-1). The O-I rise of the inactive response represents only a small part of the initial reduction of Q by PS II and little else in the way of photosynthetic activity. The early recovery features indicate the beginning of electron transport and water splitting activity (i.e. I-D dip, D-P rise, P-S decay).

Many collections of needles from Mt. Seymour (1300 meters elevation) were made in the fall (September - November), and winter (December - February) months of 1975 - 77. It was anticipated that observations of fluorescence activity of the needles during the period just prior to and

Figure 2-1 Recovery from winter inactivation in *A. amabilis* needles collected in November (1976) during a cold period. Needles were kept in darkness with their petiole ends in water, and fluorescence was measured periodically. Figure responses are averaged. The time required to go from the field inactive response (bottom curve) to the active response (top curve) was 10 - 24 hours depending on the needle. Some needles showed photochemical activity (curve second from the bottom) in as little as 2.5 hours.


VARIABLE FLUORESCENCE — Relative
0.0
0.5
1.0
1.5
2.0
TIME — sec.

just after the onset of winter conditions would lead to a description of the environmental events resulting in the inactivation of the light reactions, and also to a description of those photochemical processes initially affected. Field observations made as little as three days apart, however, failed to show well defined transitional responses between the active and the inactive state, and the sequence of environmental conditions preceeding inactivation was not determined precisely. The state of the photosynthetic apparatus during inactivation was therefore deduced from the characteristics of the fluorescence response during recovery to the active state.

The level of photochemical activity in *A. amabilis* needles appeared to be closely associated with climatic conditions prevailing at the time of collection. During the winter months of 1976 - 77, when snowfall was very light in the coastal mountains and freezing temperatures seldom were encountered for more than two or three days at a time, field determinations showed the needles to be continually active right through until spring.

In mid-spring (May, 1976), when sub-freezing conditions were still a common occurrence, responses reflecting varying levels of activity were observed in needles from different heights on the same tree (Figure 2-2). The type of response was dependent on the location of the needles in relation to the snow cover. On warming to 20^{o}C, samples buried under 20 - 40 cm of snow and samples fully exposed had typically active responses; needles from around the snow line showed the flat response common to needles measured in midwinter after long freezing periods; in all cases

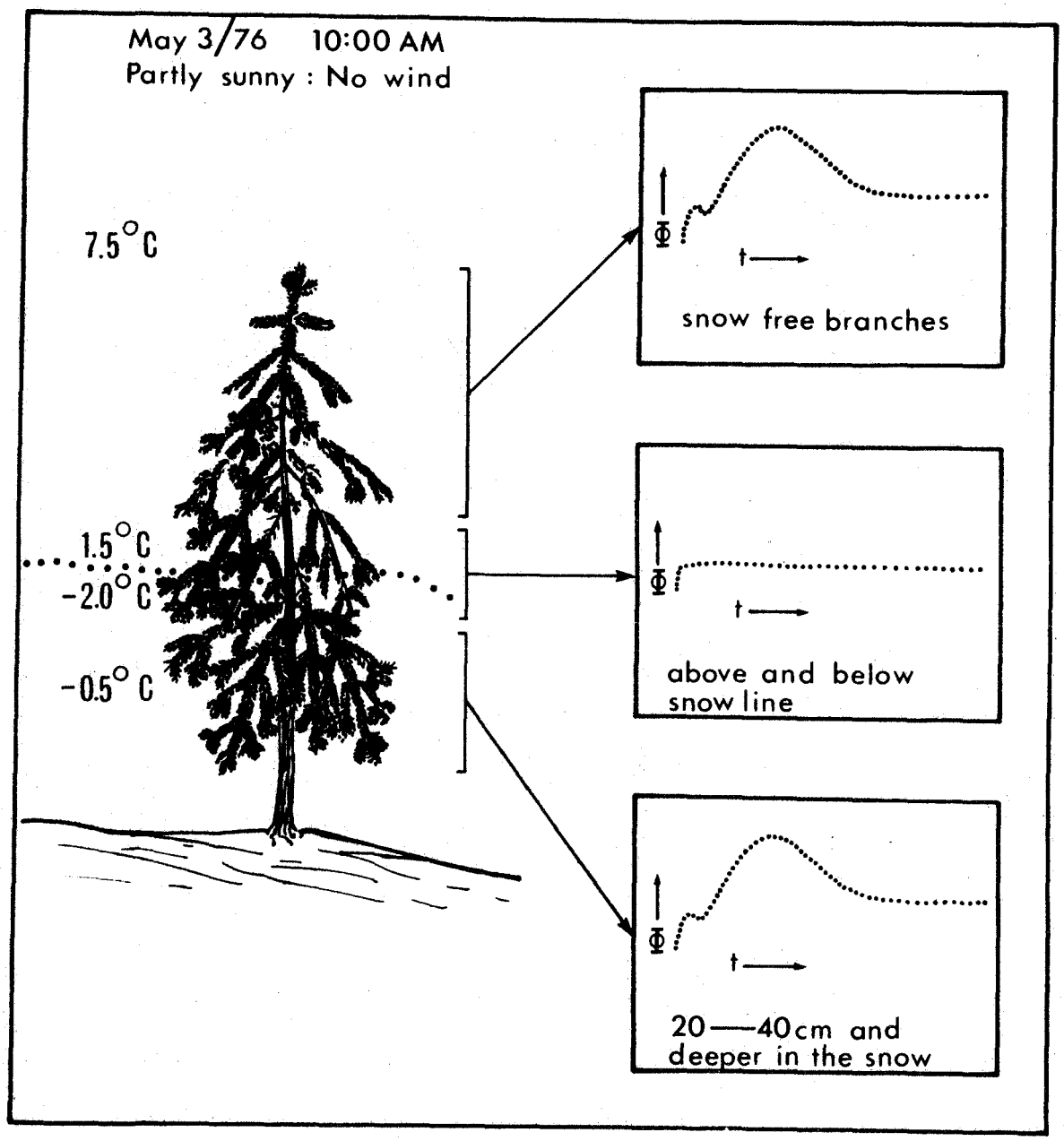
Figure 2-2 Effect of microclimate on fluorescence activity in *A. amabilis* during spring recovery. Measurements were recorded in the field after 30 minutes dark time at approximately 7.5°C. Several trees were sampled (10 observations of each type). Figure responses are averaged. Snowline samples were collected at a level 5 cm above and 5 cm below the snow surface.

$\underline{\Phi}$ = fluorescence intensity

t = time

..... snow surface

I = 2.0×10^3 erg $cm^{-2} sec^{-1}$

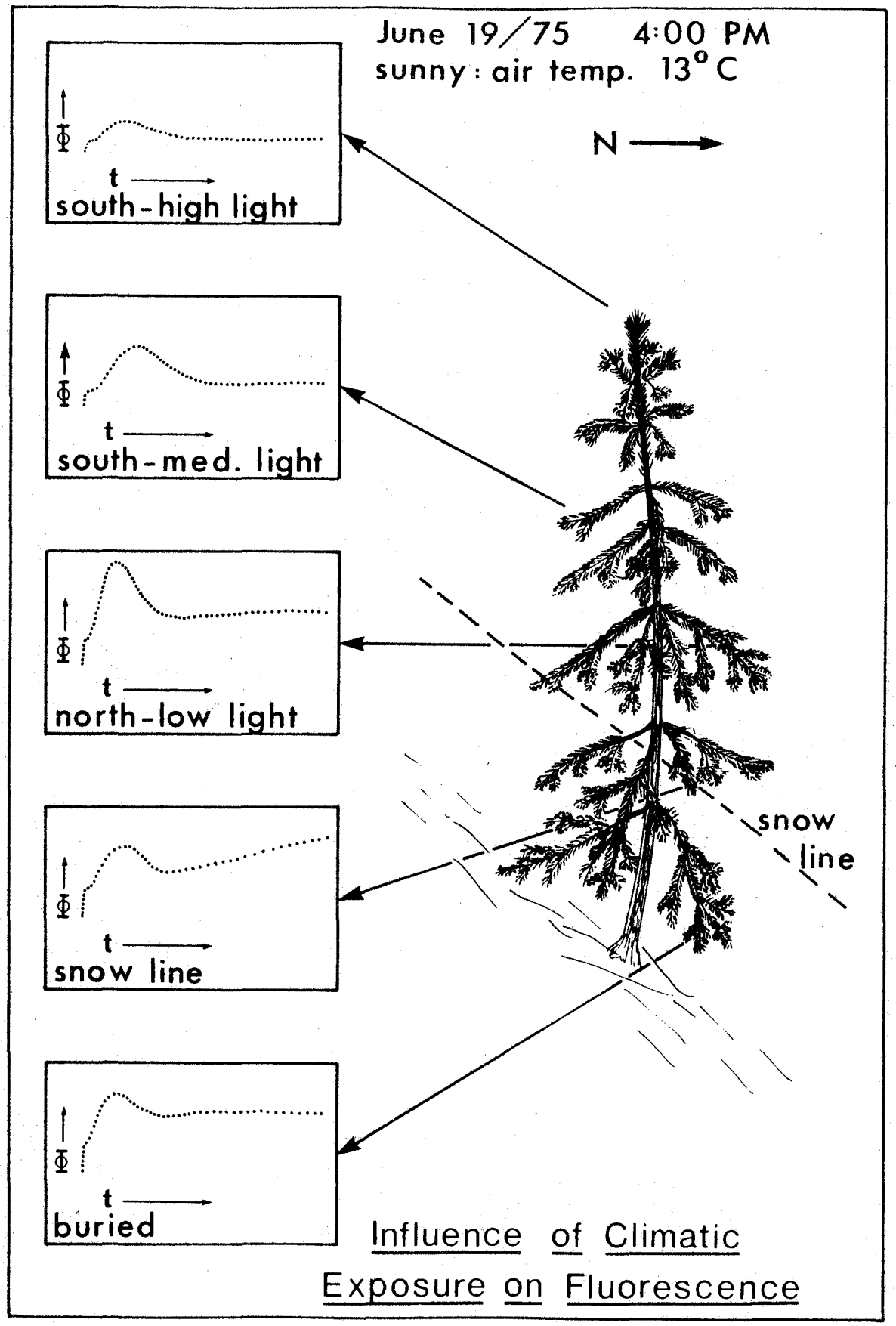

May 3/76 10:00 AM
Partly sunny : No wind
7.5°C
1.5°C
-2.0°C
-0.5° C
Φ
t
snow free branches
above and below snow line
20—40cm and deeper in the snow

observed during several periods of investigation, it was found that buried needles retained an active response as long as the subsurface snow temperature remained above -4°C; below this temperature they were inactive. Tranquillini (1964) showed that photosynthesis in *Pinus cembra* continued down to -4°C at which time the needles froze. It was found in this study that detached needles of *A. amabilis* froze between -5°C and -12°C. Temperatures this low were seldom encountered at snow depths exceeding 20 cm. Exposure to temperatures below -4°C appears to be required to inactivate needles from any part of the tree. Similar exposures to low temperatures also appeared to inactivate the needles of other conifers present in the same area (*Tsuga mertensiana (Bong.) Carr. and Chamaecyparis nootkatensis (D.Don) Spach.*

During transitional periods with the approach of warmer weather, it was apparent that the sun side of trees regained activity faster than the shaded side. It remains uncertain as to whether the sunlight plays a direct role in reactivation in the field or if this effect is simply due to increased temperature, since light was found not to be essential for recovery.

While the role of light in reactivation following winter inactivation is uncertain, the amount of light available to needles during the onset of summer conditions clearly influences the fluorescence response. Figure 2-3 illustrates responses of needles from different exposures of a tree in mid-June when daytime air temperatures were quite high (13°C). None of the needles showed the inactive response, even those at the snow line. All

Figure 2-3 Range of fluorescence responses in needles of *A. amabilis* during early summer. Measurements were recorded in the field after 30 minutes dark time at 13°C. Single tree sampled; four needles of each type. Figure shows single typical responses. At the time of collection the south high-light needles were fully exposed to direct sunlight, the south medium light needles were partly shaded, and the north low light needles were fully shaded. No direct light measurements were made. Subsurface snow temperature was uniformly at 0°C. No inactive needles were found under these mild conditions. See Figure 2-5 for a response similar to the snowline response.



Influence of Climatic Exposure on Fluorescence

of them displayed responses that reflect their position on the tree, especially in relation to the amount of light they presumably had received. The center curve (north low-light) is what would be expected for a shade plant (Schreiber, *et al.*, 1977). Proceeding upwards, the curves progressively resemble those expected for sun plants, in that the O-I and D-P rises are shortened, and the time to reach P is somewhat longer. The lower two curves are more complex since they reflect both the effects of exposure to low light intensities and relatively recent low temperatures. In the snow line needles, the O-I rise is similar to the north low-light response but the D-P rise is shorter and the S-M rise is very pronounced so that M is now the fluorescence maximum. Buried needles have a D-P rise which is intermediate between the snowline and the north low-light samples but do not have a pronounced S-M rise. In the snowline and buried samples P is not delayed as it is in the high light samples.

In summary, the following can be said about winter inactivation and recovery:

1. Sufficiently low temperatures will inactivate all the needles on the tree.

2. Incubation at above freezing temperatures is all that is required to reactivate the needles. Proximity to the air - snow interface appears to delay reactivation under generally mild conditions.

3. The types of responses observed with reactivation suggests that there are interactions between light and temperature, even though light is not necessary for the initiation of recovery.

Mature needles of *A. amabilis* are clearly resistant to damage from the low temperature extremes encountered in their subalpine environment. This contrasts with most angiosperm leaves and with developing conifer needles which are killed or damaged by freezing (Levitt, 1972). Obviously, any subalpine or arctic plant which retains its leaves in winter must have mechanisms by which freezing or thawing damage in avoided. The nature of these mechanisms is poorly understood; however, the technique of Chl*a* fluorescence induction analysis is also ideal for laboratory investigations into the mechanisms of winter inactivation. Consequently a series of experiments was carried out using this technique and are reported on below.

Section 2: Laboratory Investigations

Winter inactivation of conifer needles occurs under conditions where temperatures reach sub-freezing levels, available light is minimal both in intensity and duration of the photoperiod, and needle water content tends to be low. Any and all of the factors have been associated with the onset of dormancy or other cessation of metabolic activity in plants (Levitt, 1972). The role of any or perhaps all of these environmental variables could be important in the inactivation of *A. amabilis* needles. The investigations reported on here examine the influence of these variables, indep-

endently from each other in some cases, and also effects of their interactions in others. As shown from the following experiments, inactivation is most probably a consequence of desiccation while the needles are exposed to sub-freezing temperatures.

Responses to Freezing

Many experiments involving freezing of *A. amabilis* needles were carried out. For the most part these used presumably fully hydrated samples attached to branches. Little influence of single freezing events on variable fluorescence was observed when the needles were thawed. However, under the conditions used in the following experiments, several changes in the variable fluorescence transients were noted. These experiments suggest that winter inactivation of needles in the field is not simply a consequence of exposure to low temperature.

Figures 2-4 and 2-5 summarize aspects of the freezing responses in *A. amabilis* . Figure 2-4 shows the response of detached needles cooled in darkness to -13°C at 2°C per minute, and after 24 hours, rapidly thawed just prior to fluorescence measurement. Some of the needles were frozen dry (Figures 2-4, A-B) and the others frozen with their petiole ends in water (Figures 2-4, C-D). On thawing, the ends of both wet and dry frozen needles were kept in water. Upon thawing, the 10 dry frozen needles showed active responses, and 7 out of 10 had slightly slower P-S decays than the others. Those needles frozen standing in water had markedly different responses. A few were like the dry frozen samples in having a P-S decay.

Figure 2-4 Response of *A. amabilis* to freezing. Twenty needles of a single tree were frozen at $2^{o}C$ per minute, half of them with their petiole ends in air (A - B), and the other half in water (C - D). After 24 hours at $-13^{o}C$, the needles were quickly thawed by removing them to $20^{o}C$ air temperature, and fluorescence was recorded. After 3 days with their ends in water the needles were again measured to determine recovery (B-D).

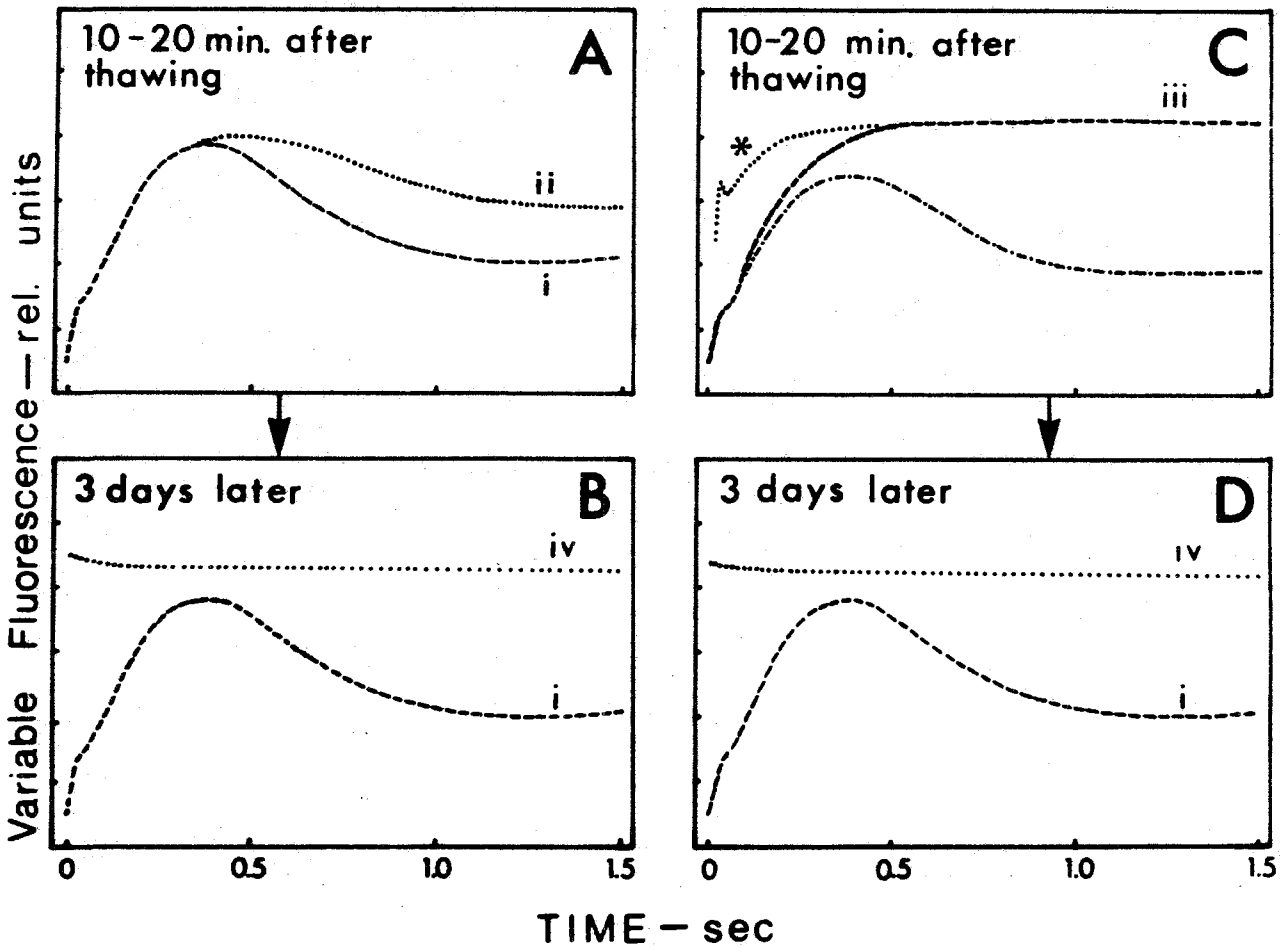
i) response typical of active needles; 3 of 10 needles in A, and 2 of 10 needles in C gave this response

ii) response indicating reduction in PS I activity; 7 of 10 needles in A gave this response

iii) response indicating weak PS I photoreduction; 8 of 10 needles in C gave this response. Persistence of an I-D dip during repeated light treatments (curve marked *) indicates that the PS I reaction center and electron transport are still active.

iv) this response indicates a lack of fluorescence quenching, and is usually associated with dead or dying needles; 3 of 10 needles in B showed this response, but only 1 of 10 in D (wet frozen) showed it, and this needle had previously shown a type i response immediately upon thawing.

$I = 2.4 \times 10^{3}$ erg $cm^{-2} sec^{-1}$


10 - 20 min. after thawing
A
ii
i
10-20 min. after thawing
C
iii
*
3 days later
B
iv
i
3 days later
D
iv
i
Variable Fluorescence — rel. units
0
0.5
1.0
1.5
TIME — sec

Eight of 10 showed the typical O-P transients, but appeared to have no P-S decay. Actually there was a much delayed decay from P which took over 5 minutes or more to reach a level approximately 50% of the maximum. These samples with a slow P-S decay following the initial 1.5 second illumination displayed a recurring I-D dip (marked with an asterisk, Figure 3-4, C) reminiscent of a partly light adapted response in a fully photosynthetically active sample (Schreiber and Vidaver, 1976), but the very slow P-S decay suggests that these samples have only weak photoreduction by PS I.

After three days incubation at 20°C in the dark, the situation had changed considerably. Some of the dry frozen samples had deteriorated to a flat response and were either dead or dying. However, all of the wet frozen samples which had shown the slow P-S decay appeared to have fully recovered.

These results suggest that:

1. This single freezing treatment alone does not give rise to the inactive response observed in the field, though other combinations of freezing and thawing events may do so,

2. The capacity for active photosynthesis at the time of thawing does not necessarily indicate the subsequent viability of the needles; some of the dry frozen needles which appeared active immediately on thawing were dead or dying a few days later (Figure 2-4, B), whereas the

wet frozen samples, which appeared to have weak photo-reductive capacity upon thawing, remained viable and recovered full activity after the same 3 day period,

3. The delayed P-S decay can be interpreted as a weak PS I photo-reduction because: a) PS II and watersplitting are functional as indicated by the O-I and D-P rises, b) PS I reaction centers and electron transport are functioning as shown by the recurring I-D dip, and, c) reduced PS I electron acceptors are not being reoxidixed, as indicated by the high fluorescence yield.

More rapid cooling ($5^{o}C$/minute to $-13^{o}C$) of fully hydrated needles produced the following results shown in Figure 2-5. All determinations were made as soon as possible after thawing. The results of this experiment are best visualized by lengthening the period of the response from a few seconds to a minute or more so that the later transients (S-M-T) are apparent. It should be noted that 30 seconds into the response, the fluorescence yield in the unfrozen needles (control) is lower than the O-level and it continues to decrease for the remainder of the response. In the frozen samples, the O-I rise is unchanged, the D-P rise is greatly reduced in height and occurs somewhat earlier than in the controls, the S-M-T part of the response is broadened, and in particular, M-T is not as steep. The fluorescence maximum is now M as in the snowline response of Figure 2-3. The T level is now higher than in the controls.

Figure 2-5 Response to rapid freezing in *A. amabilis*. 10 needles were frozen in darkness at 5°C per minute, to -13°C, for 1 hour, then rapidly thawed by moving them immediately to 20°C air.

A) control

B) 7.5 minutes dark adaptation after thawing

C) 180 minutes dark adaptation after thawing

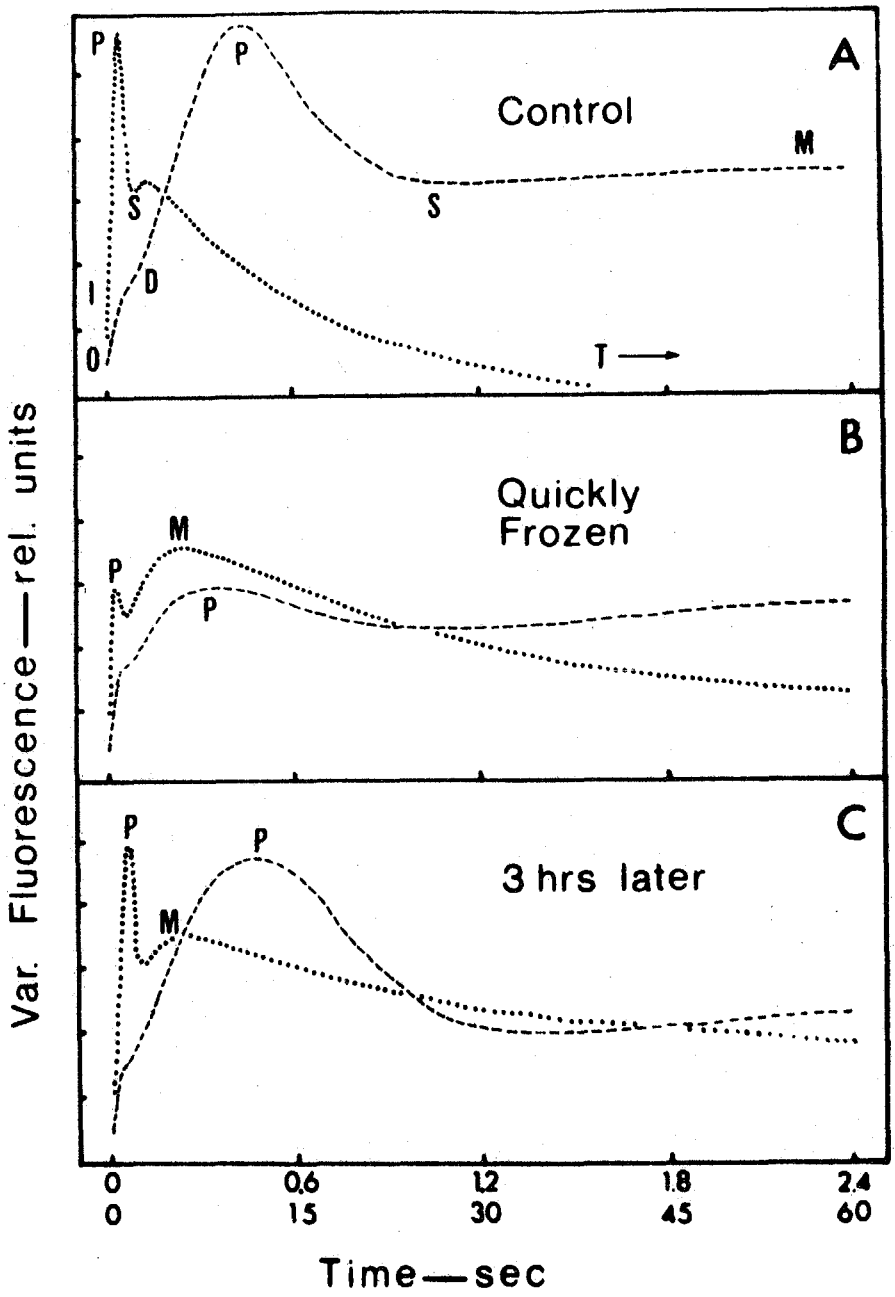
----- response during 2.4 second light treatment

..... response during 60 second light treatment

Note particularly the decreases in the height of P in the quickly frozen needles relative to the control.

It should be noted that the horizontal axis does not represent zero fluorescence yield.

* Temperature of a single needle was monitored during cooling by direct attachment of a thermocouple. Rates of thawing were not measurable using this technique.


A
Control
P
P
M
S
S
I
D
O
T
B
Quickly
Frozen
M
P
P
C
3 hrs later
P
P
M
Var. Fluorescence — rel. units
0
0
0.6
15
1.2
30
1.8
45
2.4
60
Time — sec

After three hours in darkness at 20°C (Figure 2-5, C) P has recovered significantly. The S-M-T phase of the response has not recovered during this period and the T level is even higher than immediately after thawing. In this experiment there seem to be at least two apparently independent effects on the photosynthetic system, one on watersplitting (resulting in the drop in P) which tends to recover during the three hour period, and the other affecting processes after PS I, which do not recover during this time.

The two experiments presented here demonstrate that freezing treatment alone did not reproduce the winter inactive response. They also indicate that the hydration state of the needles, in conjunction with freezing, does modify the functioning of the photosynthetic apparatus, as seen in the changes in the fluorescence response, and probably plays some role in inactivation.

Response to Desiccation and Rehydration

The water status of the needles during freezing appears to be related to winter inactivation. Water must be supplied for the full recovery of inactive needles brought from the field to the laboratory. The influence of needle water content on the fluorescence response was therefore examined.

Active needles collected in November (1975) were first fully hydrated by standing them in vials with their petiole ends in water. After 24 hours the needles were weighed, their fluorescence responses recorded, and then

they were placed dry in open vials in the dark. After a period of desiccation the needles were reweighed and then rehydrated by adding water to the bottom of the vials.

In this study, water content (WC) represents the amount of water remaining in the needles relative to the initial amount following 24 hours with their petiole ends in water. This is expressed as a percentage so that 50% WC means the needle contained half of the water it had at saturation.

Needle water content ranged from 35 - 65% with 24 hours desiccation and 3 - 45% after 48 - 72 hours. Figure 2-6 illustrates changes in fluorescence response with decreasing water contents. The responses are arbitrarily divided into 8 classes based on their overall characteristics and proceed from a typical active fluorescence response to a flat line response with no variable fluorescence. After 24 hours, most needles showed class 3 or 4 response types, while after 48 - 72 hours most were in response class 5 - 8.

The characteristics of the response classes are as follows:

1. <u>100 - 75% water content</u>

   This initial stage of desiccation produced little or no effect on the early transients from O through S.

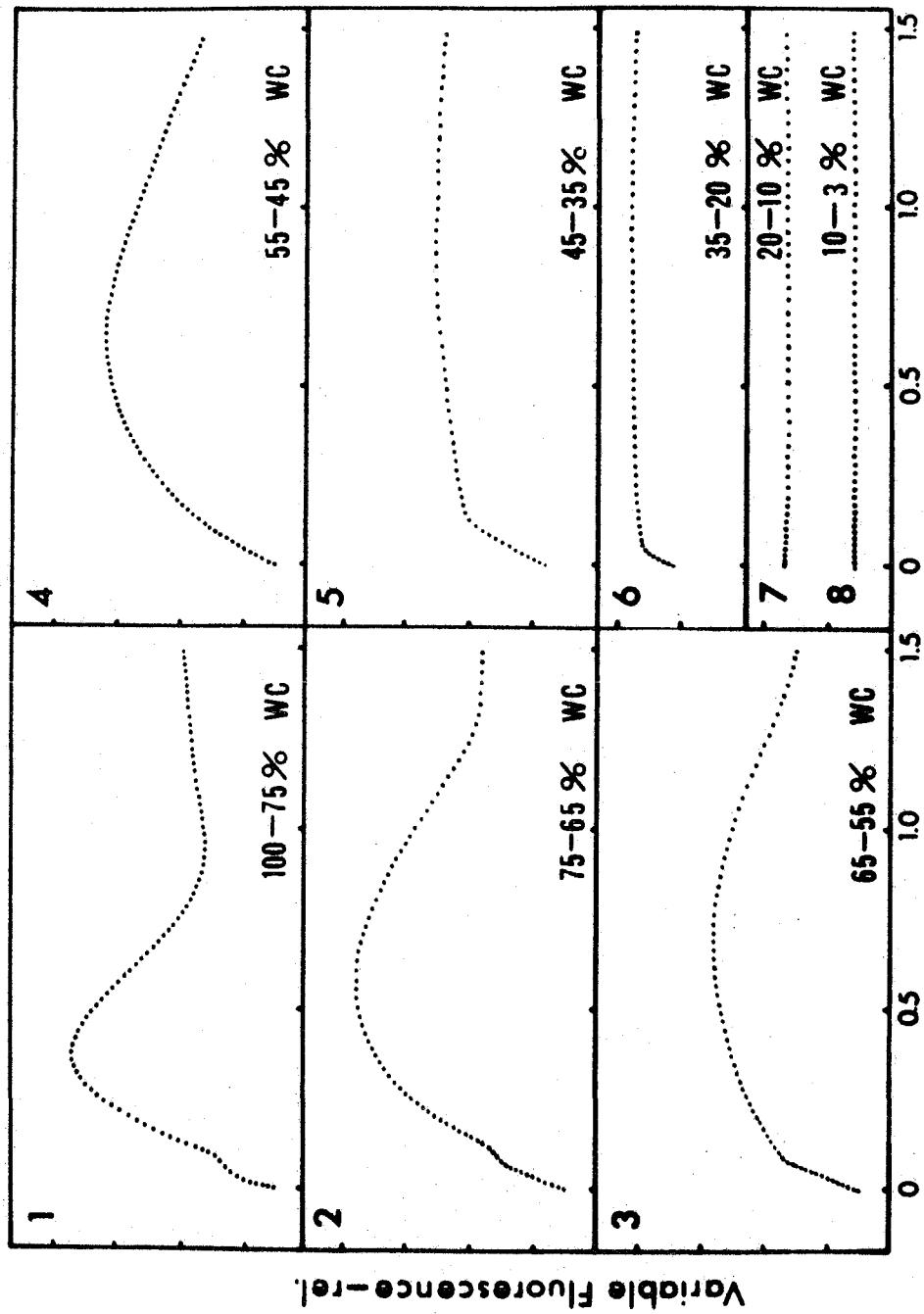
Figure 2-6

1. Effect of desiccation in *A. amabilis*. Thirty active needles from several trees, collected in November (1975) from Mt. Seymour (1300 meters) were water saturated during 24 hours dark storage with their petiole ends in water, then weighed and placed in dry, open vials for periods up to 72 hours. Needles were weighed at 24 hour intervals and their fluorescence responses recorded. Because needles were dried at different rates, the responses shown reflect the desiccation state of the needles, not the time after desiccation started. There are transitions between each of the response classes shown.

   Note : The O-level remains roughly the same up to Class 6 where it begins to rise. This trend continues in Class 7 but is reversed in Class 8.

2. %WC = percent by weight of water remaining in the needles relative to their saturated water content.

3. $I = 2.0 \times 10^3$ erg $cm^{-2} sec^{-1}$


1
100—75% WC
2
75—65% WC
3
65—55% WC
4
55—45% WC
5
45—35% WC
6
35—20% WC
7
20—10% WC
8
10—3% WC
0
0.5
1.0
1.5
0
0.5
1.0
1.5
Variable Fluorescence—rel.
Time——seconds

2. 75 - 65% water content

Fluorescence yield remained high in this stage of drying. However, P is delayed and the P-S decay is not as rapid as in the initial class.

3. 65 - 55% water content

A number of overlapping effects occur in this stage of desiccation. The D-P rise cannot be distinguished from the I-D dip. The fluorescence maximum has dropped considerably, and is further delayed, as is the position of S. The O-I rise appears unchanged indicating that PS II is still able to reduce Q.

4. 55 - 45% water content

The O-I rise is less steep and no shoulder is evident in this phase of dessication in comparison with the previous response class. The latter may result from weakened PS I activity. If so, this could also explain the anomolously higher fluorescence yield compared to the previous response class. Within the 1.5 second light exposure S is no longer reached.

5. 45 - 35% water content

This response class is notable for at least two things. The overall fluorescence yield is much lower than in any of the previous response classes. Although somewhat slower, the O-I rise shows little alteration whereas the other transients appear to be almost eliminated, suggesting that PS I activity and electron transport are no longer working.

6. 35 - 20% water content

In this stage of desiccation the fluorescence yield remains about the same as in the previous class. However, the O-level is slightly higher and the O-I rise correspondingly reduced in height, which is the first indication that PS II reaction centers are being affected. Later transients are entirely missing at this stage. The needles in this class show a response very similar to that of winter inactivated needles (Figure 2-1), however, needles of this class and also classes 7 & 8 do not recover the active response with rehydration, even after 4 - 5 days.

7. 20 - 10% water content

The overall fluorescence yield in this class varies but it is often markedly higher than in classes 5 and 6. At this stage of desiccation all the induction features associated with variable fluorescence are absent.

All photosynthetic activity appears to have ceased. Differences in overall fluorescence yield suggest variations in the extent to which desiccation has altered the physical state of the antennae pigment molecules (Wiltens, 1975).

8. 10 - 3% water content

The overall fluorescence yield is barely detectable in this response class. Desiccation has proceeded to the extent that the chlorophyll has lost contact with an aqueous environment and no longer fluoresces (Wiltens, 1975).

The first detectable effect of desiccation on *A. amabilis* needles appears to be on partial reactions which influence the P-S decay although initially electron transport appears relatively unaffected. The intermediate stages of desiccation (Response classes 3, 4 and 5) appear to be associated with decreases in both watersplitting activity and electron transport. In the later stages of desiccation (Response classes 6 - 8) the PS II reaction centers progressively lose their ability to reduce the

primary acceptor Q.

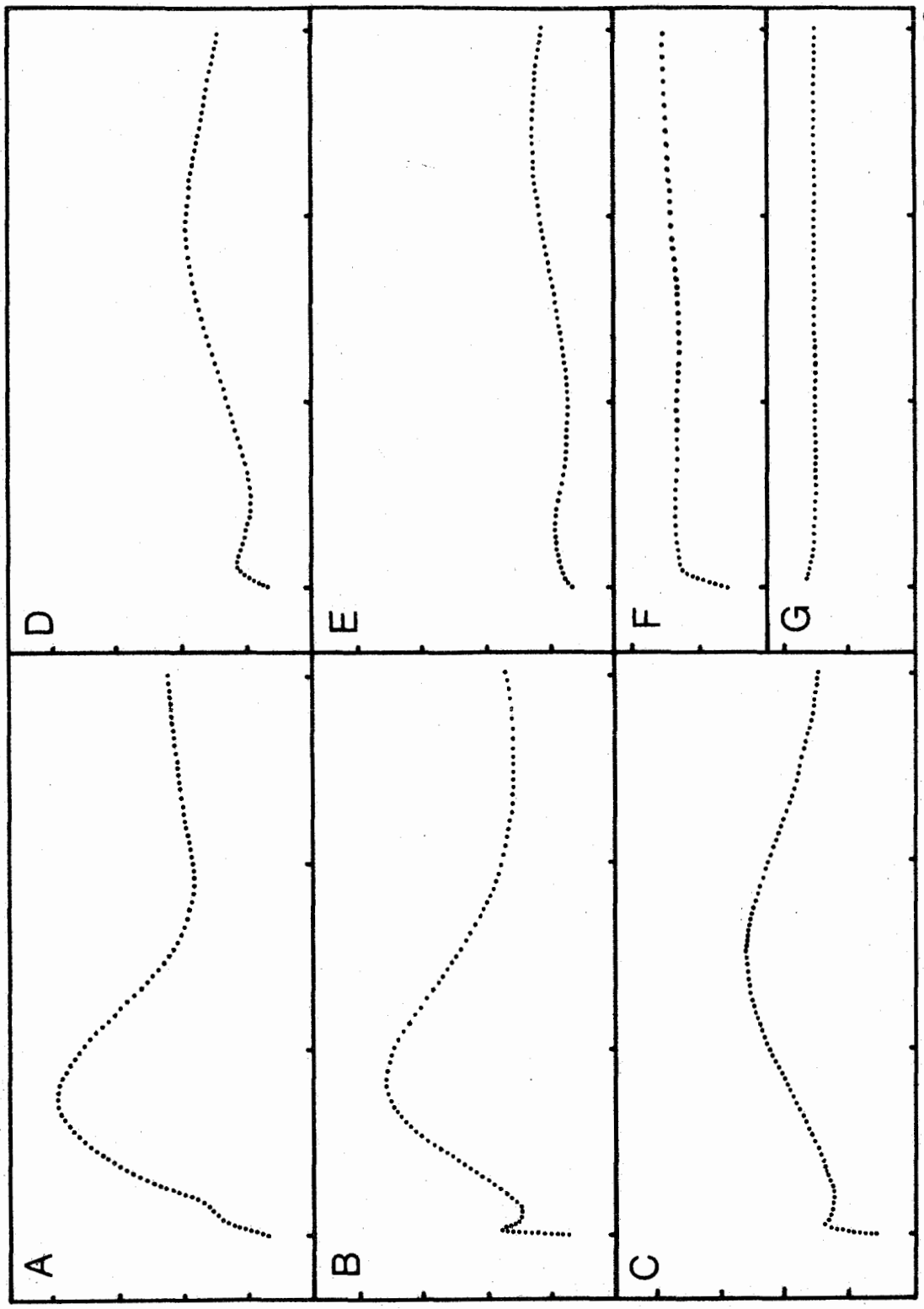
Thus desiccation proceeds with a gradual inactivation of photosynthetic partial processes until in response classes 7 and 8, no further activity can be detected. While O-level fluorescence yield may change, variable fluorescence has been eliminated.

Needles partially inactivated by desiccation can recover their photosynthetic activity on rehydration. The rehydration responses of previously desiccated needles were examined. Recovery was found to be dependent on the extent to which desiccation affected the photosynthetic partial processes. The samples were rehydrated by submerging their petiole ends in water for 24 - 48 hours in darkness before fluorescence induction was recorded. Recovery occurred only in those needles not more severely desiccated than those of response class 5 (Figure 2-6). Following rehydration, about 50% of the class 5 needles regain a typical active response.

In Figure 2-7, curves A through E represent the various stages of recovery. After 24 hours rehydration, the needles which will eventually recover full activity show induction responses similar to those in Figure 2-7, D-E. At this time water content determinations indicate that the needles are fully hydrated. With more time, these needles pass through activity stages represented by C-A. The time for any given needle to reach any particular stage is somewhat variable but by 72 hours all the needles will have reached the activity stage of A, presumably reflecting complete recovery of the photosynthetic apparatus from the effects of

Figure 2-7 Recovery of activity with rehydration in *A. amabilis*. Thirty needles ranging from 3-45% water contents (Fig. 2-6, classes 5 - 8) were rehydrated with their petiole ends standing in water. After 24 hours in darkness, the fluorescence responses of the needles were measured. Those needles previously desiccated below 35% water content do not recover and exhibit rehydration responses F or G. Half of the needles desiccated to between 35 - 45% water contents also did not recover and show response F after 24 hours (8 of 15 needles). The remainder of these needles show response E after 24 hours and by 72 hours have progressed through stages D - A. Different needles advance at different rates.

$I = 2.0 \times 10^3$ erg $cm^{-2} sec^{-1}$


A
B
C
D
E
F
G
0
0.5
1.0
1.5
0
0.5
1.0
1.5
Time—seconds
Variable Fluorescence—rel.

desiccation. Those needles from response Class 5 (Figure 2-6) which do not recover, and all those from Classes 6 - 8, responded similarly to F - G (Figure 2-7), even after 72 hours. The Class F response is characterized by some residual PS II reaction center activity, while in G this is no longer detected.

From these results it appears that recovery from desiccation is possible only if inactivation does not proceed to the stage where PS I activity can no longer be detected in the fluorescence response (Class 6, Figure 2-6). The PS II reaction centers remain functional at desiccation stages from which recovery does not take place after rehydration.

Recovery of activity in desiccated needles does not appear to be simply the obverse of inactivation. This suggests that the order in which photosynthetic partial processes become inactivated is different from that in which they are reactivated. Effects on electron transport appear with relatively little desiccation as evidenced by the disappearance of the I - D dip (Figure 2-6, response Classes 3 - 4) and PS II reaction center activity persists to very low water contents (response Class 6). On recovery, the initially predominant activity is quenching by the electron transport system (Figure 2-7, B - E). Watersplitting appears to be the last process to fully recover. Perhaps the inactivation-reactivation progression should be expected to differ since in these experiments inactivation is a direct consequence of decreased needle water content while reactivation takes place in needles which have already been fully rehydrated. Recovery clearly requires the reordering of the photosynthetic apparatus following rehy-

dration.

Dark Storage

Many aspects of winter dormancy in plants are regulated by photoperiod, including leaf senescence in deciduous trees. There is a possibility that winter inactivation in *A. amabilis* needles is, to a greater or lesser extent, under photocontrol. *A. amabilis* branches were kept fully hydrated in a 8/16 light-dark cycle at $+1^{o}/-3^{o}C$ for up to 5 weeks. Needles examined throughout this period never showed an inactive response. Since this treatment did not induce inactivation, the effects of extended periods of total darkness, both at $20^{o}C$ and $-4^{o}C$ were examined.

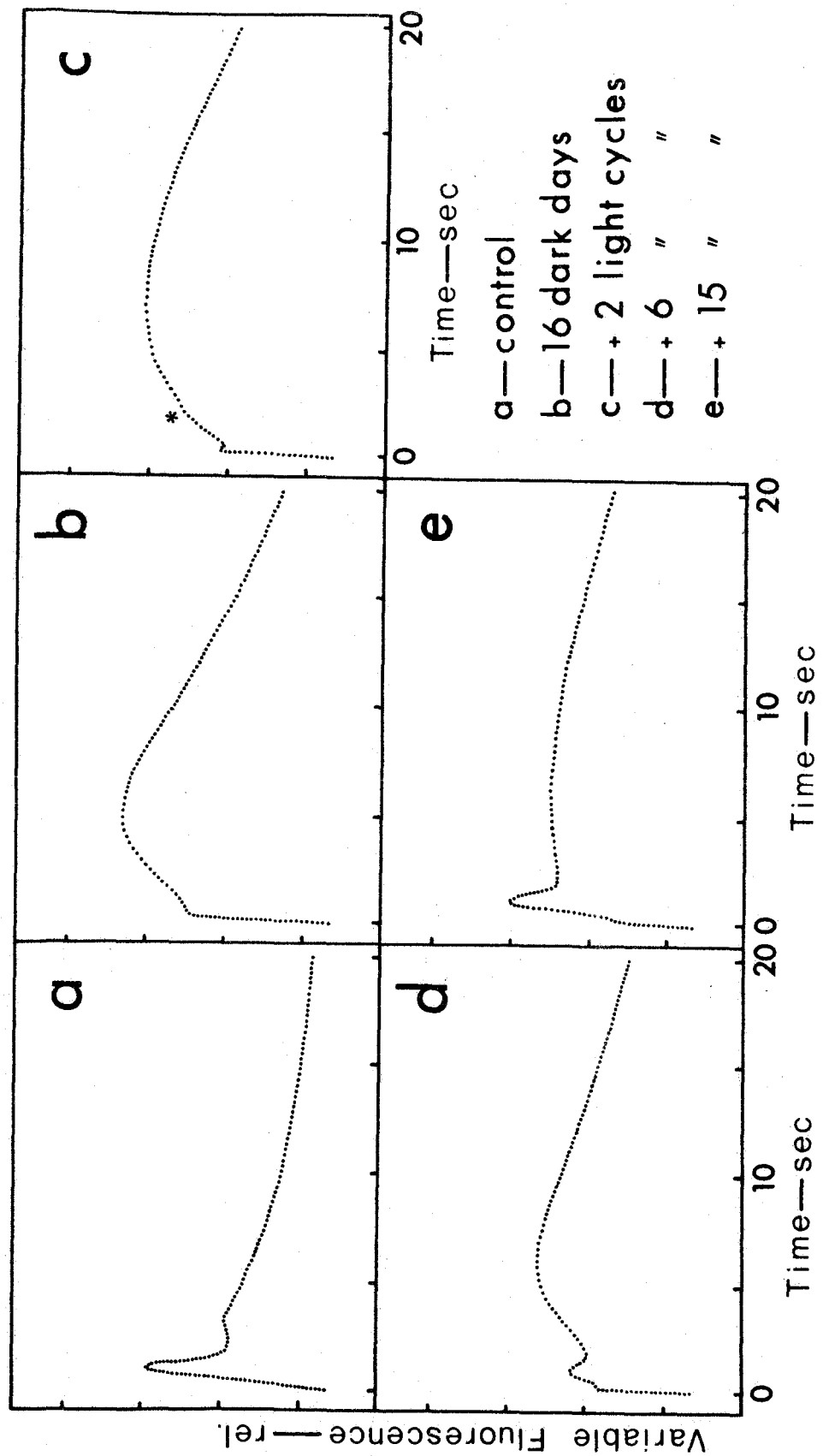
The results of the extended dark storage treatment at $20^{o}C$ (Figure 2-8, a, b) show several effects of darkness on fluorescence induction, but tend to show that extended darkness alone does not induce inactivation. Compared to the control (a), sixteen days of darkness (b) resulted in a high O-I rise and P appears diminished. M has increased markedly but the M - T decay seems little affected. These results suggest that watersplitting activity is lost to some extent but the increase in O-I is difficult of interpret. PS I activity seems to remain reasonably effective in quenching fluorescence. It was found that fluorescence returned to an approximation of the control response after a number of 5/19 hour light-dark cycles (Figure 2-8, c - e). Under these conditions the D-P transient gradually regenerates, but by now there seems to be some loss of ability of PS I to quench fluorescence in the later parts of the response, perhaps

Figure 2-8 Effect of dark storage in *A. amabilis* needles collected from Mt. Seymour (1300 meters elevation) during mild weather in mid January (1976). Several branches were kept with their stems in water, in black boxes for 16 days (2-8 b). Recovery after exposure to 5/19 hour light/dark cycles is indicated by responses c through e.

The control response a was recorded 4 hours after the needles were collected. The I-level of a is at approximately 1.5 units. The I-levels of responses b - e are all apparent in the figures.

* The asterisk in c signifies a persistent though barely detectable peak occurring before 3 seconds. Later stages of recovery suggest that this early peak is probably P which is partly reduced in amplitude during dark storage, and partly masked by other parts of the dark storage response. See also Figure 2-9.

$I = 2.4 \times 10^{3}$ erg $cm^{-2} sec^{-1}$


a
b
c
d
e
Variable Fluorescence — rel.
Time—sec
0
10
20
a—control
b—16 dark days
c—+ 2 light cycles
d—+ 6 "
e—+ 15 "

due to a decrease in CO_2 fixation rather than to a loss of PS I reaction center activity (note the I-D dip and strong P-S decay of response e, Figure 2-8).

Thus the light reactions seem to recover from dark storage, but by this time (31 days from the beginning of the experiment) the needles may be low on substrates or perhaps mineral nutrients.

Similar effects of dark storage were found in four other conifers (Figure 2-9), suggesting that the effects found in *A. amabilis* may be typical for conifers in general.

Moist needles from branches stored for 10 weeks at $-4^{o}C$ in the dark, showed responses quite different from those stored at $20^{o}C$ (Figure 2-10). The initial response to light (A) demonstrates a completely active photosynthetic system. After a 7.5 minute dark interval (B), during which time a healthy, untreated needle would have become almost completely dark adapted, the dark-stored needle appears to have lost most of its PS II activity. Recovery of PS II activity has hardly begun even after a further 30 minutes dark interval (C). Although the significance of these results is not understood at this time, the initial response (A) does indicate that low temperature can preserve an almost normal active photosynthetic response during extended periods of darkness.

In the field, *A. amabilis* fir branches may spend up to 5 months under conditions of low light intensity, buried in snow. On the other hand, all

Figure 2-9 Effect of dark storage on four conifer genera, *Abies*, *Pinus*, *Cedrus*, and *Tsuga*. Branches of four conifers collected from gardens on Burnaby Mountain during February (1976) were kept in darkness on wet filter paper for 9 days at $20^{o}C$. Corresponding control responses for the dark storage treatments are shown by genera on the left side of the figure.

----- fast responses (0 - 1.8 seconds)

..... slow responses (0 - 45 seconds)

$I = 2.4 \times 10^{3}$ erg $cm^{-2}sec^{-1}$

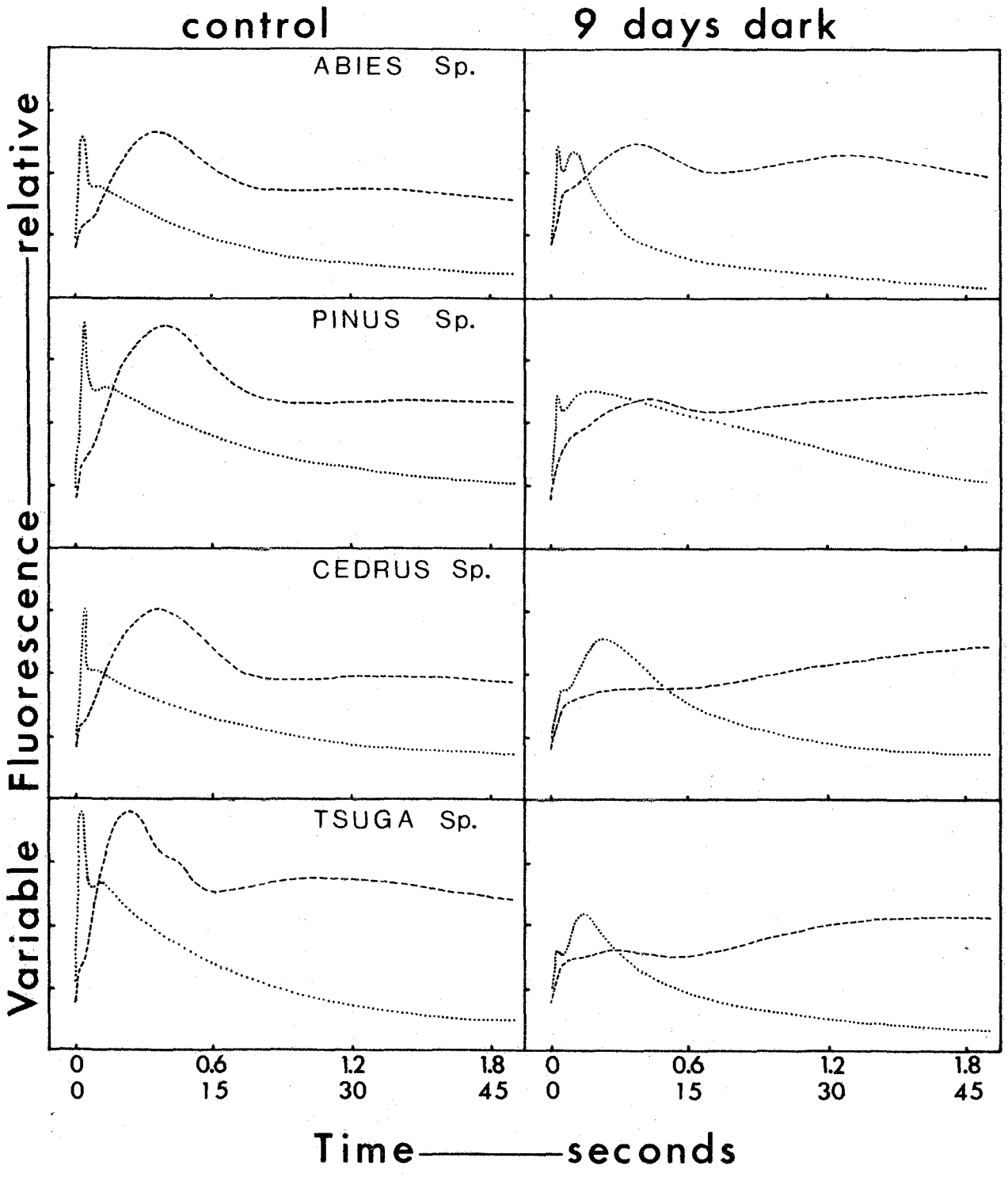

control
9 days dark
ABIES Sp.
PINUS Sp.
CEDRUS Sp.
TSUGA Sp.
Variable Fluorescence——relative
0
0
0.6
15
1.2
30
1.8
45
0
0
0.6
15
1.2
30
1.8
45
Time——seconds

Figure 2-10 Temperature dark storage interaction in *A. amabilis*. Branches were kept in darkness at $-4^{o}C$ for 10 weeks, then incubated in darkness for 20 hours at $20^{o}C$ prior to fluorescence measurement. Figure shows single typical response.

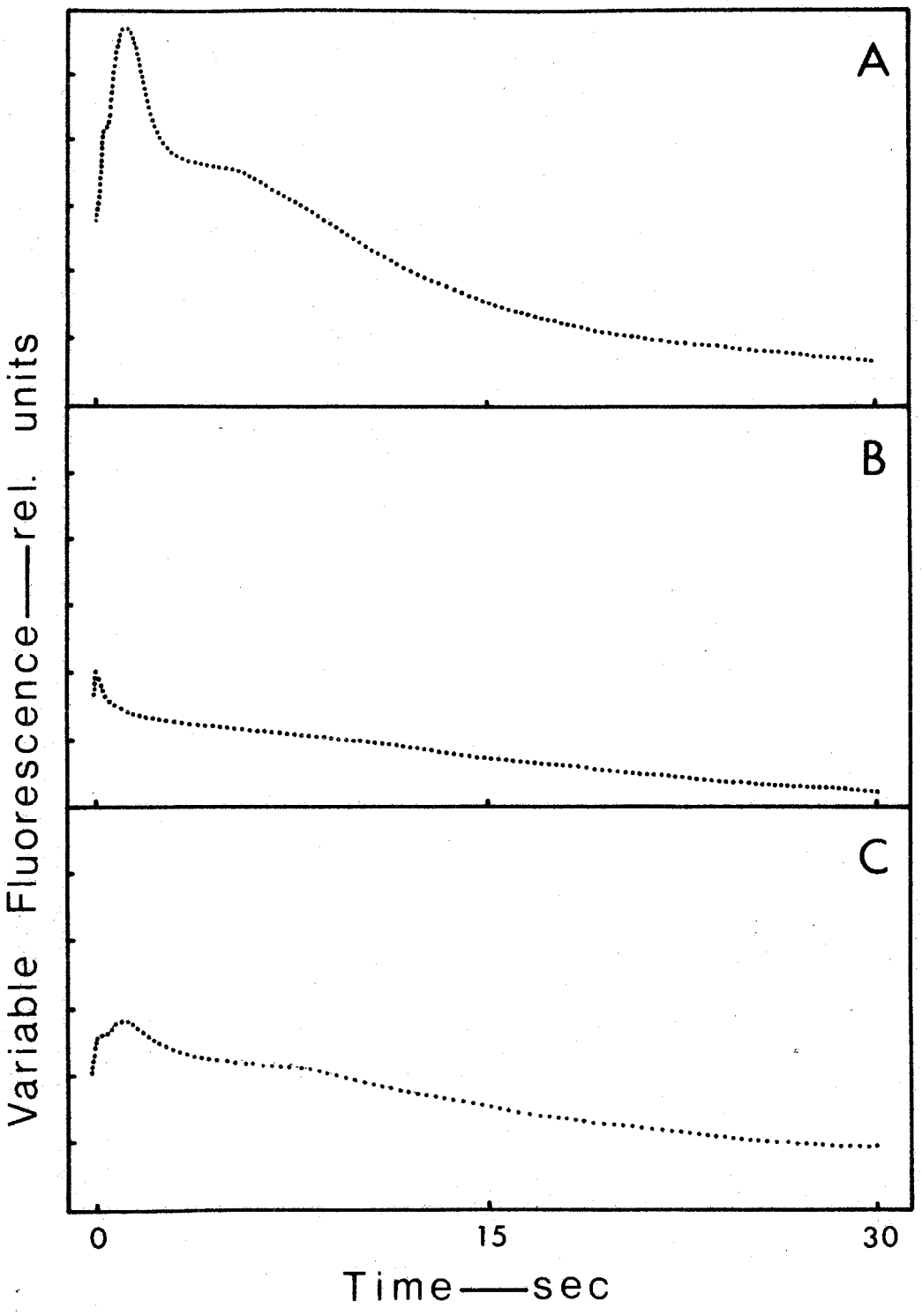
A) needle's first exposure to light

B) same needle after 7.5 minutes dark time

C) same needle after 30 minutes additional dark time

$I = 2.4 \times 10^{3}$ erg $cm^{-2}sec^{-1}$

NOTE: C response O-level is intermediate between A and B.


A
B
C
Variable Fluorescence——rel. units
0
15
30
Time——sec

the needles on a tree sometime or other experience winter inactivation, suggesting that continuous near-darkness is not a requirement for inactivation.

Levels of Reactivation

Reactivation appears not to be a simple switching on of photosynthesis. Whether or not photochemical activity is detectable in needles presumed to be inactive may depend on the excitation light intensity presumably reflecting different levels of activity in individual needles (Figure 2-11).

The needles tested were from branches collected from the field under cold conditions (November, 1977); in having only an O-I rise, all initially appeared inactive with moderate light intensity (I = 4, Figure 2-11 A). When light intensity was increased to I = 5 (see figure legend, Figure 2-11), 40 - 50% of the needles showed what might be termed an incipient active response in that the components of the induction time course were clearly discernable even though they were of very low magnitude. In about 30% of the needles, increasing to I = 5 did not result in the appearance of any additional fluorescence transients, but on a further increase to I = 6, these needles now showed the incipient response (Figure 2-11 B). The remainder of the needles did not show the incipient response at I = 6. Recovery in all of these needles was similar to that shown in Figure 2-1. In addition, it should be noted that needles collected after extensive periods of cold weather also do not show the incipient response at I = 6.

Figure 2-11 Levels of reactivation in *A. amabilis*. Water saturated needles collected in November 1977, were dark adapted for 2 hours, and their fluorescence responses measured at different light intensities.

A) needles in this class (40 - 50% of the total) showed an incipient active response at I = 5

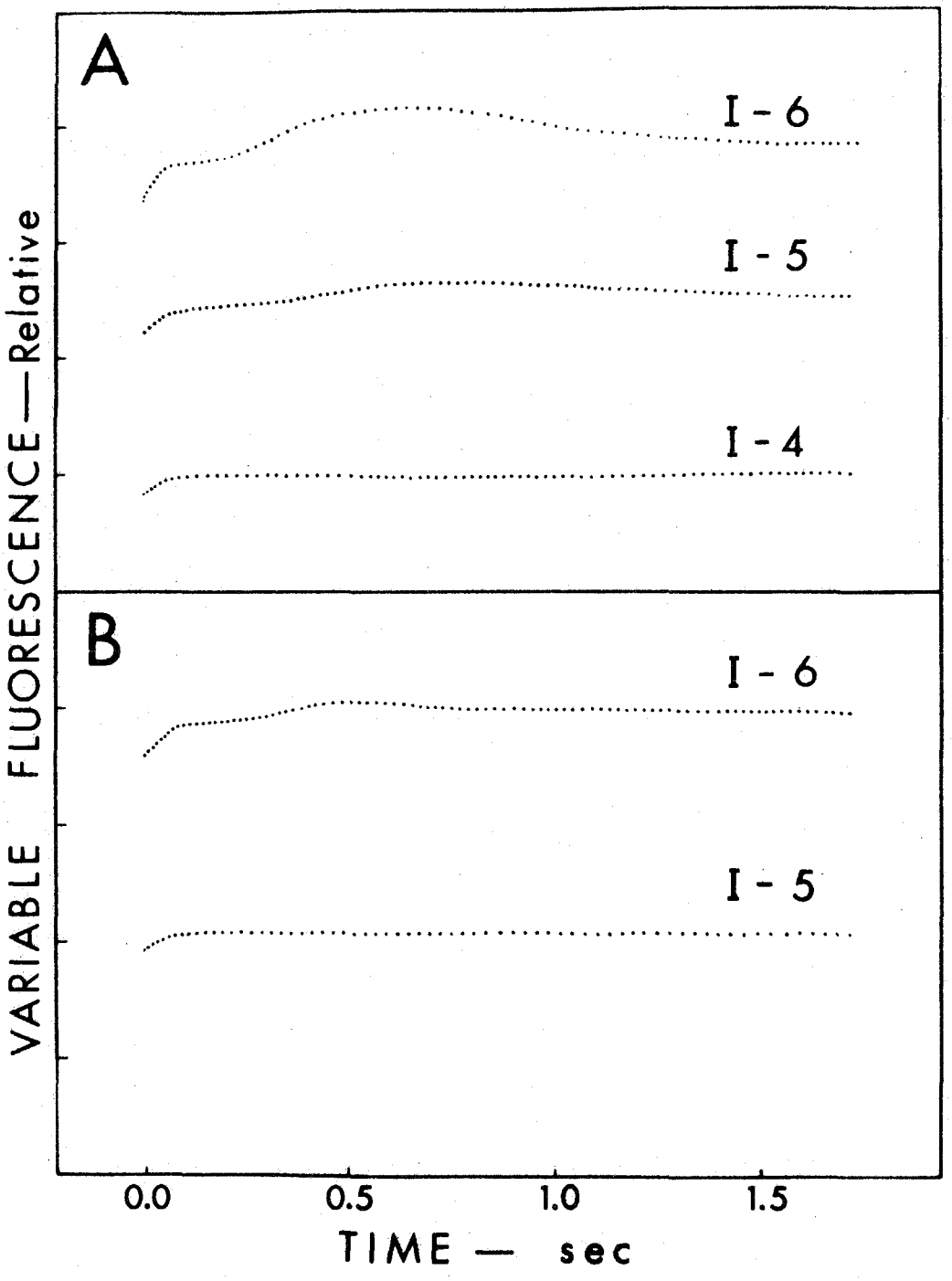
B) needles in this class (approximately 30% of the total) showed an incipient response at I = 6

The remaining needles did not exhibit active fluorescence responses at either I = 5 or I = 6. Needles collected during previous winter cold periods did not exhibit the incipient response with increased light intensity.

I = 4 ..... 1.6 erg $cm^{-2}sec^{-1}$

I = 5 ..... 2.0 " " "

I = 6 ..... 2.4 " " "


A
I - 6
I - 5
I - 4
B
I - 6
I - 5
VARIABLE FLUORESCENCE — Relative
0.0
0.5
1.0
1.5
TIME — sec

Active needles are like the photosynthetic organs of other vascular plants in that they produce a strong Kautsky transient at I = 2* (see Figures 1-4 and 1-5), but not at I = 1*, and it was assumed that this is a characteristic of green plant chloroplasts and represents what might be considered a light compensation level with respect to variable fluorescence. This experiment shows that the light intensity required to generate a variable fluorescence response is not constant, and further that it may reflect different activity levels in the samples.

* I = 1 = 0.3×10^3 erg $cm^{-2} sec^{-1}$
I = 2 = $0.7 \times$ " " " "

CHAPTER III

DISCUSSION AND CONCLUSIONS

Section 1: Review of Experimental Results

A variety of strategies are used by plants of temperate and more rigorous climatic regions to endure winter with its unfavourable temperatures, photoperiods, and extremes of humidity. Many plants become dormant or otherwise inactive yet rapidly resume metabolic activity with the onset of spring. In the cases of annual and deciduous plants these strategies are at least superficially obvious. On the other hand, in many conifers and other plants which maintain their leaf canopies throughout the winter, the means by which structural and functional integrity of leaves is maintained over winter are poorly understood.

It has been shown for some conifer species that needle metabolic activity is reduced in winter to very low levels or ceases altogether. In *A. amabilis*, photosynthetic activity is absent and little if any carbon exchange presumably takes place. Whether or not this metabolic shut down contributes to overwinter survival of the needles has not been shown, and the way the phenomenon is regulated has not been demonstrated previously.

Winter inactivation in *A. amabilis* is nevertheless probably an adaptive response serving to protect the needles from damage arising from the climatic extremes encountered during winter. Inactivation of the photosynthetic apparatus appears to be a common phenomenon and presumably accounts for the winter drop in conifer photosynthesis observed by other

workers. Recovery of photosynthetic activity with the onset of warmer conditions has been extensively studied but the mechanisms are not known. The present investigation may provide some insight into these mechanisms.

The interpretation of field and laboratory measurements made during the course of this study relating to inactivation and reactivation of photosynthesis in *A. amabilis* are summarized as follows:

1. During the coldest period of winter in which measurements were made all needles observed were inactive, as indicated by the absence of variable fluorescence.

2. Unless subsurface snow temperatures dropped below about $-4^{o}C$ buried needles remained active.

3. With the onset of warmer conditions there is a differential reactivation of needles on a tree dependent on their proximity to the snow line. This may come about because temperatures at the snowline are often considerably lower than either subsurface or ambient air temperatures.

4. After 10 - 24 hours at $20^{o}C$ in the laboratory, inactive needles from the field were reactivated. This recovery took place in the dark when the petiole ends of the needles were submerged in water; no recovery was observed in needles kept in water-saturated air.

5. The inactive response was not induced in needles frozen in the laboratory at $2^{o}C$ per minute to $-13^{o}C$ and thawed after 24 hours. Water-stressed needles under this treatment did suffer more injury than those kept fully hydrated.

6. Freezing at $5^{o}C$ per minute to $-13^{o}C$ with immediate thawing decreased the fluorescence yield of detached needles to some extent, but recovery was substantially complete after 3 hours at $20^{o}C$.

7. Desiccation results in a progressive inactivation of the photosynthetic partial processes until no further activity can be detected.

8. Recovery from the desiccated state is characterized by a gradual return of the active response and takes place only in needles not desiccated to the extent that PS II activity begins to disappear.

9. Desiccation of needles to 20 - 35% water content produces a response closely resembling the winter inactive response, but at $20^{o}C$ these needles do not recover after rehydration.

10. Neither a short photoperiod coupled with low, non-freezing temperatures, nor extended periods of darkness induced the inactive response.

11. Needles which show the inactive response may not all be in the same physiological state, since in individual needles an incipient active response appears over a range of excitation light intensities.

12. None of the laboratory treatments resulted in an inactive response which exactly matched the winter inactive response. However, desiccation and freezing did produce responses which paralleled field observations.

Section 2: Interpretation of Inactivation Phenomena by Fluorescence Induction Analysis

Certain features of the fluorescence induction time course can be related to the participation of discrete photosynthetic partial reactions. Therefore, some of the influence of environmental factors, or experimental conditions on the time course can, with some degree of certainty, be attributed to effects on one or more of these partial reactions. This approach has been used previously in consideration of freezing effects on detached needles (see Chapter II section 2) and other aspects of this study. The same approach may identify those parts of the photosynthetic apparatus most affected during winter inactivation.

Needles in midwinter show a flat fluorescence response, consisting of an O-I rise followed by a horizontal line (Figure 1-1). After incubation under warm conditions in the dark, fluorescence returns to normal. The time needed is dependent on temperature and the availability of water.

Increasing the excitation light intensity resulted in a variable response in some needles which show flat responses at lower intensities. In addition, it was found that needles which gave a weak variable response (one in which the features were delayed and reduced in amplitude) at relatively high intensity (I = 5, see Chapter II, section 2) showed a flat response when the intensity was reduced slightly. The fact that more light is required suggests the possibility that the contribution of PS II in inactive needles is much lower than in active needles, which could account for the loss of variable fluorescence found in material collected from the field in midwinter. All other plant material tested, including active needles, gave strong variable fluorescence responses at light intensities which produced flat responses in inactive or only partially active needles.

There are a number of ways by which a reduction in PS II activity could occur.

1. Some PS II reaction centers could be inactivated, but direct evidence for this is unavailable.

2. The amount of energy reaching the PS II reaction centers could be decreased.

3. Watersplitting may be inhibited so that fewer electrons are available to PS II. This has been shown to account for changes in the variable fluorescence in other

systems. (Papageorgiou, 1975).

A progressive deactivation of watersplitting could account for both the decrease in photosynthetic rates and also for the complete cessation of photosynthesis in conifer needles.

Another way in which the energy reaching the PS II reaction centers could be reduced is from an overall decrease in the transfer efficiency of one chlorophyll molecule to the next, within the light gathering apparatus. It could also be accomplished if the light-harvesting complex (LHC) became detached from the PS II core particle (Armond, *et al.*, 1977).

Schreiber and Armond (1978) were able to demonstrate that one of the principle effects of heat damage in isolated chloroplasts was a reduction in the transfer efficiency of Chl*b* to *a* which they attributed to an uncoupling of the LHC from the reaction center complex of PS II. Their method was to determine the fluorescence intensity of DCMU and NH_2OH poisoned chloroplasts under light exciting primarily Chl*a* (430 nm) and light exciting Chl*b* (480 nm). It was found that under 480 nm light, fluorescence intensity dropped off faster as the temperature was raised than it did under 430 nm light, indicating that Chl*b* was losing its ability to transfer energy to Chl*a*.

If the same drop in Chl*b*-excited Chl*a* were found in inactive needles relative to active needles, it would be strong argument in favour of a decoupling process occuring during inactivation.

Figure 3-1 outlines how this hypothesis might apply during a freeze - thaw cycle. When freezing occurs, ice formation in the intercellular spaces causes removal of water from the cells. An equilibrium is reached between the amount of water which goes to form ice and that which remains in the cytosol. It is known that the light harvesting complex is particularly sensitive to water stress (Alberte and Thornber, 1977) and the dehydration induced by ice formation in this hypothesis is thought to cause a decoupling between the LHC and the reaction center complex, so that energy transfer is greatly reduced. At warmer temperatures the ice melts and water returns to the cells. Reattachment of the LHC to the PS II core particle is thought to occur during this phase, and the degree of reattachment would coincide with the increase in apparent PS II strength in the fluorescence response (it is also possible that the LHC's, to a greater or lesser extent, are resynthesized rather than reattached). Metabolic activity may be required for this recovery since the process takes considerable time (up to 24 hours or more) and is temperature dependent.

This investigation has not led to a definitive description of the inactivation phenomenon in *A. amabilis* needles. Laboratory studies do suggest however, that inactivation and subsequent reactivation progress as follows:

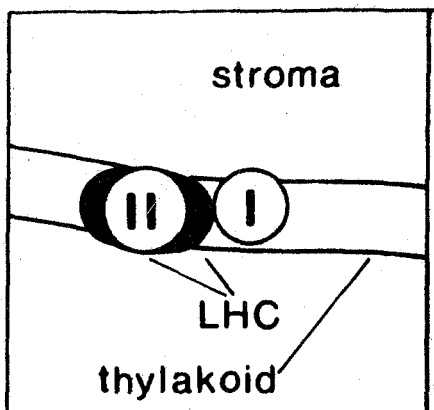
1. During freezing, events occur affecting PS II so that, after thawing, its turnover rate is decreased.

Figure 3-1 Thylakoid organization during ice formation: an hypothesis of winter inactivation. Hypothesis is based on the assumption that an uncoupling effect takes place between the light-harvesting complex, and the PS II reaction center particle. Model of thylakoid membrane adapted from Armond, *et al.* (1977).

II = PS II core particle

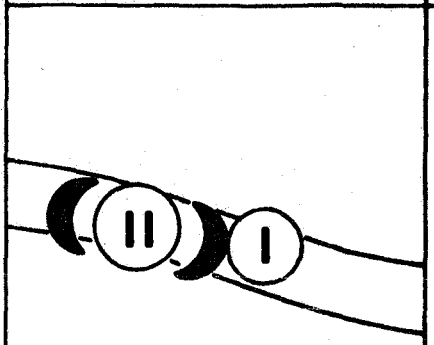
I = PS I core particle

LHC = Light harvesting complex (chlorophyll a/b protein)



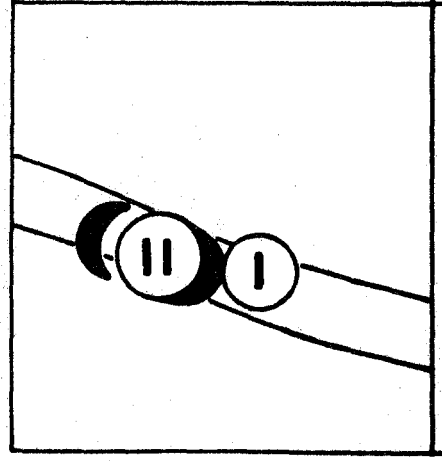
DURING FREEZING

Formation of extra-cellular ice removes water from cells



FROZEN EQUILIBRIUM

Water stress uncouples light harvesting complex from the PS II core particle



AFTER THAWING

Temperature after thawing regulates the rate of coupling of LHC to PS II

2. Electron transport and PS I appear less affected during inactivation because: a) the fluorescence yield of inactive and partially active needles is very low compared to that of active needles, and, b) inactivation of either electron transport or PS I would be expected to cause fluorescence to rise rather than decrease, since any activity of PS II would not now be quenched.

3. During reactivation, the increase in fluorescence yield is a gradual process which seems to reflect primarily a return of observed PS II activity. The presence of both an I-D dip and a P-S decay in the incipient active response tends to confirm the hypothesis that the major influence of inactivation is on those partial reactions directly linked to PS II.

4. Any effect of inactivation on Calvin cycle, photophosphorylation, or NADP+ reduction cannot be inferred from the inactive fluorescence response; however, laboratory experiments suggest that one or more of these may be affected also (Figures 2-4, 2-5). That several effects may combine to produce winter inactivation seems possible. It is certainly true in heat-damaged chloroplasts where damage occured close to PS II just prior to an uncoupling effect of the light harvesting pigments from the PS II reaction center particle (Schreiber and Armond, 1978).

The level of photochemical activity during inactivation and recovery has been described in some detail. In the inactive state the PS II reaction centers are operating at a slower rate than in the active state, so that the rate of transport of electrons away from Q is greater than the supply. This keeps the fluorescence yield low and prevents the occurence of the normal Kautsky transients. A major question that remains unanswered is what happens during the inactive state to the energy that formerly was converted at the PS II reaction center when the needle was fully active? At present no definitive answer can be provided because the precise reason for the slowdown in the PS II turnover rate is not known. There are several alternative methods of quenching excitation:

1) The excess could be converted to heat,

2) it could be quenched by oxygen via a mechanism involving the carotenoids (Papageorgiou, 1975),

3) excess energy could be diverted to PS I by an increase in energy spillover (Murata and Sugabara, 1969),

4) the rate of the trade reaction from Q to PS II could be increased (Butler, 1972),

5) pigments might be photo-oxidized by excess energy.

Perhaps the most likely sink for an excess of light energy is in its conversion into heat. At the low temperatures encountered during winter this is not likely to prove dangerous. Some pigment degradation might result during inactivation but the recovery process indicates no lasting effect of inactivation on the photosynthetic apparatus takes place.

Increased spillover to PS I is also likely since, as in the case of conversion into heat, the mechanism of energy spillover is firmly established and, in addition, it has been shown in this study that PS I is largely unaffected during inactivation and is, therefore, available for energy dissapation (perhaps by cyclic phosphorylation or some other process).

It should also be remembered that some energy conversion may still take place at PS II in the inactive state. All that can be stated is that the rate of turnover is much lower.

The Chl*a* fluorescence induction analysis has proved to be invaluable in this investigation of the phenomenon of winter inactivation, and is a sensitive indicator of the effect of environmental influence on the photosynthetic apparatus. The data are easy to obtain and interpret in field and laboratory situations. Provided the samples are dark-adapted the level of photosynthetic activity can be determined in a few seconds. The instrumentation is inexpensive and highly portable, and since the method is non-destructive, individual samples can be assayed many times while undergoing experimental treatments. Compared with the techniques

of CO_2 exchange and C^{14} exchange, the results obtained from fluorescence measurements are relatively unambiguous. The technique should have wide applications in studies of this nature, since it can identify specific sites of influence of environmental variables that can only be alluded to by other methods.

Section 3: Inactivation and Overwintering

Inactivation is clearly associated with the conifer habit of maintaining a needle canopy throughout the winter. This loss of photochemical activity may be related to the survival of the needles during the stress conditons of winter.

One obvious function of inactivation could be to protect the reaction centers from damage resulting from an excess of light energy during times when the normal channels of energy dissipation are inoperative. Any of the previously proposed mechanisms for regulating the turnover rate at PS II could accomplish this end (see Chapter III section 2). Such damage is known to occur in other photosynthetic systems where the normal channels are blocked (Ridley, 1977; van Hasselt and Strikwerda, 1976).

It has been shown here that water is essential for reactivation even in fully hydrated needles. Also, active needles when frozen under water stress appear to suffer more damage than fully hydrated needles. It may be that inactivation serves to prevent photosynthetic activity during times when the needles are in danger of being exposed to low temperatures

and water stress. Neither low temperature nor moderate water stress by themselves, appeared to injure the needles. Desiccation of needles has been related to the upper tree line in several conifer species (Baig and Tranquillini, 1976; Wardle, 1968). Perhaps the limit of high latitude forests is determined by similar constraints. It is possible that the distribution of conifers is limited to regions where needle inactivation takes place prior to exposure to the desiccation associated with sub-freezing temperatures. If this is indeed the case, then winter inactivation of photosynthesis in *A. amabilis* is clearly an adaptive response.

A mechanism such as winter inactivation may be adaptive, but not necessarily in relation to the habitat where individuals of a species are found. For example, resistence to desiccation injury may be found in localities where the chances of desiccation are very slight. Needles of *A. amabilis* were always found to be close to their water saturation levels regardless of the conditions under which they were collected, yet they proved remarkably resistant to short term effects of desiccation down to 45% water content.

If winter inactivation is related to water availability, and in other habitats this becomes more important where frozen soil may prevent the uptake of water by roots and where water is removed from the needles by direct sublimation, then its adaptive significance may be in preventing photosynthesis from starting up when liquid water is not freely available. A number of factors may combine to limit the availability of water during periods when photosynthesis is otherwise feasible :

a) frozen soil may prevent uptake,

b) frozen trunks or branches could block water uptake,

c) shallow soils in exposed areas could conceivably dry out.

Several questions may be asked relating to the problem of photosynthetic inactivation:

a) What is the cost to the plant of reactivation?

b) If energy is required to reactivate, is the number of times a needle can reactivate limited?

c) Does reactivation depend on the occurrence of a favourable set of environmental conditions or is it regulated, in part by some endogenous control mechanism such as phytochrome or hormones?

d) Are short warming trends in otherwise extremely cold periods ignored by the plant?

A possibility that cannot be ignored is that the delay in reactivation is caused by the necessity to reorganize part of the photosynthetic system which is injured in the sequence of water removal during ice form-

ation. Inactivation would then be thought of as an inevitable price for existing in a habitat where repeated freezing is routine, and reactivation would represent the successful restoration of a functional photosynthetic apparatus. In this situation, photosynthesis would normally take place during short warm periods except for the need to re-order a damaged photosynthetic apparatus. If this were the case then inactivation could be thought of as a consequence of environmental conditions (rather than an adaptive mechanism) while reactivation would be an adaptive response necessary to restore photosynthetic activity to the needles. At the present time distinction between these possibilities is not feasible.

Throughout this study the term dormancy has been deliberately avoided in reference to winter inactivation of conifer needles. This was done for the following reasons. In the classical sense, a dormant plant is one in which metabolic activity resulting in growth and development is proceeding at very low levels or may be difficult to detect. Activity is usually resumed in response to particular stimuli operating through endogenous regulatory systems. Provision of conditions of temperature, light, and humidity normally conducive to growth and development are not sufficient to restore activity unless some dormancy breaking event perceived by the endogenous regulatory system takes place. For many plants the dormancy breaking 'trigger' is lengthening of days to more than a certain number of hours. The success of dormancy as an overwintering strategy depends on suppressing physiological reactivation until such time as winter injury is unlikely.

In constrast, the reactivation of conifer needles does not appear to be 'triggered' by any special event. All that is required is that they be exposed to sufficient moisture and high enough temperatures. Light is not required, the time of year is not important, and no endogenous regulation appears to be involved.

The location of the needle on a tree is much more important in determining probability for reactivation than daylength. In true dormancy the whole plant would be expected to become and remain dormant until such time as the triggering event took place. While such dormancy may be involved in needle flushing by conifer trees, it does not appear to be operative in individual mature needles during winter.

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