

**APPARATUSES AND METHODS FOR ENHANCEMENT AND
DETECTION OF PHOTOSYNTHETIC COMPETENCE IN TISSUE
CULTURED PLANTLETS.**

by

Sylvain Langis Dubé

B.Sc. (Agr.), Macdonald College of McGill University, 1981

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the department
of
BIOLOGICAL SCIENCES

© Sylvain L. Dubé 1990
SIMON FRASER UNIVERSITY
June, 1990

All rights reserved. This thesis may not be reproduced in whole or in part,
by photocopy or other means, without the permission of the author.

APPROVAL

Name: SYLVAIN DUBE

Degree: Doctor of Philosophy

Title of Thesis:

APPARATUS AND METHODS FOR ENHANCEMENT AND DETECTION OF PHOTOSYNTHETIC
COMPETENCE IN TISSUE CULTURED PLANTLETS

Examining Committee:

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

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

Dr. C.L. Kemp, Associate Professor, Dept. of
Biological Sciences. SFU

~~Dr. G.R. Lister, Assistant Professor, Dept. of
Biological Sciences, SFU~~

Dr. L.M. Srivastava, Professor, Dept. of Biological
Sciences SFU, Public Examiner

Dr. N.H. Haunerland, Assistant Professor, Dept. of
Biological Sciences, SFU, Public Examiner

Dr. J.F. Bornman, Research Fellow, Dept. of Plant
Physiology, University of Lund, Lund, Sweden,
External Examiner

Date Approved

July 11, 1990

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

APPARATUSES AND METHODS FOR
ENHANCEMENT AND DETECTION OF
PHOTOSYNTHETIC COMPETENCE IN
TISSUE CULTURED PLANTLETS

Author: _____

(signature)

SYLVAIN G. DUBE

(name)

July 13, 1990

(date)

ABSTRACT

In recent years, the potential of biotechnology to create new types of plants with desirable properties not found in nature, has enormously stimulated interest in plant cell and tissue culture. This new interest has revealed a need for establishing a firm scientific understanding of the biochemical, physiological and developmental activities of plants growth *in vitro*. Much of the difficulty, failure and expense of tissue culture micropropagation occurs at the acclimatization stage or when plantlets are transferred from *in vitro* to *ex vitro* conditions. This project concentrates on the development of apparatuses and methods directed toward understanding physiological characteristics of plantlets grown *in vitro*.

In an attempt to characterize the physiological requirements for photoautotrophic growth *in vitro*, an Aseptic Gas Exchange and Hydroponic System (AGEHS) has been designed and developed to accommodate tissue cultured plants. Interfaced to a PC type computer, the AGEHS controls and monitors several parameters relevant to plant growth. Young plantlets treated with flow of air or CO₂-enriched air and controlled relative humidity, with or without exogenously supplied sucrose were grown under low and high photon flux densities (PFD).

An important aspect of this work is the development of an Integrating Fluorometer Data Acquisition System (IFDAS). To characterize photosynthesis and electron transport activity of very small plants, an integrating sphere fluorometer with elevated excitation light quanta and automated controls was designed. The system, interfaced with software

for instrument control and data acquisition allows for automation and reliability. A method of data handling for normalization of variable fluorescence has also been incorporated. Parameters such as absorbed light quanta (I_{ABS}) and relative number of photosynthetic reaction centres can be determined and displayed with each digitized fluorescence induction curve. Correlation studies were performed on data acquired simultaneously from gas exchange measurements and fluorescence.

Using Generalized Linear Modeling (GLM) statistics, analysis of the photosynthetic characteristics of plantlets grown under AGEHS conditions helped define requirements for *in vitro* plant growth. Controlled acclimatization *in vitro* can potentially alleviate the needs for *ex vitro* acclimatization. The apparatuses and methods developed here can be applied to other studies in plant physiology.

RESUME

APPAREILS ET METHODES POUR L'AMELIORATION ET LA DETECTION DE LA CAPACITE PHOTOSYNTHETIQUE DES IMPLANTS CULTIVES *IN VITRO*

Depuis tout récemment, la capacité de la biotechnologie de créer de nouveaux types de plantes avec des propriétés encore non découvertes dans la nature a suscité un nouvel intérêt dans les techniques de cultures des plantes *in vitro*. Ce nouvel intérêt nous a indiqué un grand besoin de bâtir une connaissance de base scientifique, tout particulièrement dans les domaines de la biochimie, de la physiologie et de la morphogénèse des plantes cultivées *in vitro*. La plupart des difficultés, échecs et coûts des techniques de la micropropagation proviennent au moment de l'acclimatation ou du repiquage *ex vitro*. Ce projet met l'emphase sur le développement d'appareils et méthodes conçues spécialement pour l'étude des caractéristiques physiologiques des implants cultivés *in vitro*.

Afin de caractériser les besoins physiologiques du développement photo-autotrophique *in vitro*, un appareil contrôlant automatiquement les échanges gazeux et les pompages de solutions hydroponiques en milieu aseptique (AGEHS) fut développé et construit. Avec l'aide d'un ordinateur de type 'personnel', cet appareil contrôle et mesure plusieurs paramètres nécessaires à la croissance des plantes. De jeunes implants traités avec échange d'air ou d'air enrichi au CO₂ sous humidité contrôlée avec ou sans saccharose supplémentaire furent cultivés sous faible ou forte intensité lumineuse.

Une autre facette de ces travaux consiste au développement d'un fluorimètre à sphère intégratrice contrôlé par un logiciel d'acquisition des données. Afin de caractériser l'activité photosynthétique et le transport des électrons chez de petites plantes telles que celles cultivées *in vitro*, il était nécessaire de développer un fluorimètre à sphère intégratrice capable de produire une lumière d'excitation d'intensité lumineuse élevée pourvu d'un contrôle automatisé. Ce système, grâce à un logiciel de contrôle et d'acquisition de données, permet l'automatisation et une fiabilité accrue. Une méthode de traitement des données permettant de normaliser les signaux de la fluorescence variable fut également développée et incorporée au système. Des paramètres tels que quanta de lumière absorbée (I_{ABS}) et le nombre relatif des centres de réactions photosynthétiques peuvent être calculés et ajoutés aux données des courbes individuelles d'induction de la fluorescence numérisées. Des études de corrélation furent établies sur des données d'échanges gazeux et de fluorescence échantillonnées simultanément.

En employant des méthodes statistiques de Model Linéaires Généralisés (GLM), l'analyse des caractéristiques photosynthétiques des implants cultivés avec l'AGEHS permet de reconnaître les besoins des implants *in vitro*. Lorsque l'acclimatation est contrôlée *in vitro*, il apparaît possible d'éviter l'acclimatation *ex vitro*. Les appareils et méthodes décrits sont applicables à plusieurs autres domaines reliés à la physiologie végétale.

DEDICATIONS (DEDICACES)

A Jeannine, ma mère, qui m'a toujours dit:

L'instruction ça n'a pas de prix et ça vaudra toujours plus qu'un gros 'char'.
Surtout lorsqu'elle entendait le voisin faire crisser les pneus de son immense
véhicule automobile.

A mon père, Hilaire, qui m'a souvent dit:

*Mon ptit gars, je ferais n'importe quoi pour que tu n'aies pas à gagner ta
vie dans le bois comme un bûcheron.*

Notre amour pour la forêt sera toujours le même, il diffère seulement dans
la façon de la voir grandir...

...je pense que leurs promesses et la mienne ont de bonnes
chances d'être remplies.

A Anne, mon amie et mon amante qui s'est donnée pour que je
réussisse ce projet et au plus vite...et bel et bien jusqu'à la dernière
minute...

ACKNOWLEDGEMENTS

I would like to thank Bill Vidaver, my senior supervisor for his encouragement, his valuable criticisms, support, and the freedom he allowed. Many thanks to Dr. Lister, for the very valuable discussions and loan of equipment. I wish to thank Dr. Kemp for his encouragement and discussions. Thanks to all faculty and staff of the Department of Biological Sciences.

I am thankful to the granting agencies that were involved in various parts of this project in particular NRC-PILP (to Clay's Nurseries and Laboratories), NSERC (to Dr. Vidaver), FRDA and The B.C. Ministry of Forests (to Vidaver *et al.*) and Simon Fraser University (Vice-President Special Research Fund and others).

I wish to extend my gratitude to those who quickly understood that biologists really need help with electronics; the staff of the Electronic Shop. Without them things would have been much different... Many thanks also to the staff of the Machine Shop and Glass Shop.

Endless thanks to Jyoti Vithlani for taking so good care of my little plants and teaching me how to bake chapatis.

Special thanks to all my colleagues and friends, including André Lévesque for helping me tame the GLIM statistical package. I wish to thank a special friend, Dr. Alex Harper for his visions and priceless encouragements.

Thanks to Anne for putting up with me and my hair loss.

TABLE OF CONTENTS

APPROVAL	ii
ABSTRACT	iii
RESUME	v
DEDICATIONS (DEDICACES)	vii
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS	ix
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF EQUATIONS	xiii
CHAPTER 1	1
GENERAL INTRODUCTION	1
1.1 PLANT TISSUE CULTURE	1
1.1.1 The <i>in vitro</i> environment	6
- energy source	6
- gas exchange	6
- nutrition	7
1.2 PHOTOSYNTHESIS	8
1.3 STRATEGIES OF <i>IN VITRO</i> PLANTLET ACCLIMATIZATION	13
1.4 OBJECTIVES OF RESEARCH PROJECT	14
1.5 ASSESSMENT OF PHOTOSYNTHETIC COMPETENCE	14
CHAPTER 2	16
AN <i>IN VITRO</i> ENHANCED ACCLIMATIZATION SYSTEM FOR TISSUE CULTURED PLANTLETS	16
2.1 INTRODUCTION	16
2.2 MATERIAL AND METHODS	19
2.2.1 Description of system	19
- Relative humidity.	22
- Temperature measurement	22
- Control and measurement of inlet CO ₂	23
- Measurement of CO ₂ outlet.	24
- Control of gas flow rates	25
- Growth chamber units	25
- Computer control and data collection	26
2.2.2 Plant material and growth conditions	31
2.3 RESULTS AND DISCUSSION	32
2.4 CONCLUSION	35
CHAPTER 3	37
PHYSIOLOGICAL ASSESSMENTS AND PROCEDURES	37
3.1 INTRODUCTION	37
3.2 MATERIALS AND METHODS	38
3.2.1 Integrated Fluorescence Data Acquisition System (IFDAS)	38
- Hardware components of the system.	42
- Data acquisition software.	44
3.2.2 Gas exchange.	50
3.2.3 Growth and water status measurements	53
3.2.4 Statistical analysis	53
3.3 RESULTS	54
3.3.1 Determination of F_O and I_{ABS}	54
3.3.2 Examples of application of the IFDAS	58

3.4 DISCUSSION	60
3.5 CONCLUSION	60
CHAPTER 4	62
PHOTOSYNTHESIS AND GROWTH OF PLANTLETS GROWN WITH VARIOUS AMOUNTS OF EXOGENOUS SUCROSE	62
4.1 INTRODUCTION	62
4.2 MATERIALS AND METHODS	64
4.2.1 Experimental design	64
4.2.2 Physiological assessments	66
4.3 RESULTS	67
4.3.1 Effects of sucrose concentration	67
4.3.2 Effects of growth photon flux density (PFD)	75
4.3.3 Effects of gas exchange	75
4.3.4 Effects of CO ₂ -enrichment	76
4.4 DISCUSSION	77
4.5 CONCLUSION	81
CHAPTER 5	83
GROWTH AND PHOTOSYNTHESIS OF PLANTLETS GROWN WITH GAS-EXCHANGE AND HYDROPONIC MEDIUM FLOW	83
5.1 INTRODUCTION	83
5.2 MATERIALS AND METHODS	85
5.3 RESULTS	87
5.3.1 Effects on plantlet growth	87
5.3.2 Effects on photosynthesis	93
5.4 DISCUSSION	97
5.5 CONCLUSION	100
CHAPTER 6	103
INDUCTION AND DETECTION OF EFFECTS OF EXCESS LIGHT IN TISSUE CULTURED PLANTLETS	103
6.1 INTRODUCTION	103
6.2 MATERIALS AND METHODS	105
6.3.1 Effects on fluorescence characteristics	107
6.3.2 Effects on CO ₂ gas-exchange rates	107
6.4 DISCUSSION	112
6.5 CONCLUSION	113
CHAPTER 7	115
SUMMARY AND CONCLUSIONS	115
7.1 INTRODUCTION	115
7.2 THE PLANT TISSUE CULTURE ENVIRONMENT	116
7.3 PROSPECTS OF THE AGEHS	118
7.4 FLUORESCENCE AND GAS-EXCHANGE ASSESSMENT SYSTEMS	119
7.2 CONCLUSION	120
APPENDIX	121
ABBREVIATIONS	121
REFERENCES	123

LIST OF TABLES

Table 2.1. Parameters displayed and saved by the AGEHS	28
Table 4.1. List of treatments for the study of combined effects of sucrose and <i>in vitro</i> growth conditions	65
Table 5.1. AGEHS and culture tube treatments and their code symbols	86
Table 5.2. Parameters of fluorescence induction kinetics	94
Table 6.1. List of excess PFD treatments and their code symbols	106

LIST OF FIGURES

Figure 1.1. Organization of the thylakoid membrane	11
Figure 2.1. Schematic diagram of the Aseptic Gas Exchange and Hydroponic System (AGEHS)	21
Figure 2.2. AGEHS recordings of parameters for a 24 hour period	30
Figure 2.3. Recordings of temperature, RH and CO ₂ uptake	34
Figure 3.1. Diagram of the Integrating Fluorometer Data Acquisition System (IFDAS)	41
Figure 3.2. Diagram of the Gas Exchange Assessment System	52
Figure 3.3. Determination of F_O	55
Figure 3.4. Comparison of two methods for the estimation of F_O	56
Figure 3.5. Regression analysis and correlation coefficients for determining plant mass	57
Figure 3.6. Photoinactivation of photosynthetic activity of nursery grown conifer seedlings	59
Figure 4.1. Total dry weight of shootlets	69
Figure 4.2. CO ₂ uptake and dark respiration rates of shootlets grown under AGEHS conditions	70
Figure 4.3. CO ₂ uptake and dark respiration rates of shootlets grown in culture tubes	71
Figure 4.4. Relative water content of shootlets grown under AGEHS (less hydroponic system) and culture tube conditions	72
Figure 4.5. F_P fluorescence characteristics of shootlets grown under AGEHS (less hydroponic sys.) and culture tube conditions	73
Figure 4.6. Time to reach $F_T=0$	74
Figure 5.1. Dry weight (DW) of shootlets	88
Figure 5.2. Relative water content (RWC) of shootlets	89
Figure 5.3. Specific leaf weight (SLW)	90
Figure 5.4. CO ₂ uptake and dark respiration rates of shootlets	91
Figure 5.5. Parameters of fluorescence induction kinetics	92
Figure 5.6. Correlation between PSU size and photosynthetic gas exchange	96
Figure 6.1. Fluorescence induction kinetics of plantlets	109
Figure 6.2. Changes in F_P fluorescence and ratio F_O/I_{ABS}	110
Figure 6.3. Changes in photosynthetic rates and their correlation with F_O/I_{ABS} ratios	111

LIST OF EQUATIONS

<i>Equation 1.1.</i> $PA + hv = P^*A + P^*A -$	9
<i>Equation 2.1.</i> $F = (C_{in} - C_{out}) \times f \times \frac{44000}{22414} \times 10^{-6} \times \frac{273}{1+273} \times \frac{P}{101.3} \times 3600$	29
<i>Equation 3.1.</i> $k_{ST} = L_{ST} / I_0$	45
<i>Equation 3.2.</i> $L_{ST} = k_{ST} I_s$	45
<i>Equation 3.3.</i> $F(t) = F_{MEAS}(t) - L_{ST} D_s$	45
<i>Equation 3.4.</i> $I_{ABS} = I_0 - I_s$	46
<i>Equation 3.5.</i> $F_{VAR}(t) = \frac{F(t) - F_0}{F_0}$	47
<i>Equation 3.6.</i> $RC_{II} = \alpha_2 \phi_2 I_{ABS} t$	48
<i>Equation 3.7.</i> $\phi_2 = 1 - \frac{F_0}{F_{MAX}}$	48
<i>Equation 3.8.</i> $RWC = (IFW - IDW) / IFW$	53
<i>Equation 4.1.</i> $RWC = (tFW - tDW) / tFW$	66
<i>Equation 4.2.</i> $F_{VAR}(t) = \frac{F(t) - F_T}{F_T}$	66
<i>Equation 5.1.</i> $SLW = IDW / \text{leaf area}$	87

CHAPTER 1

GENERAL INTRODUCTION

1.1 PLANT TISSUE CULTURE

Plant tissue culture consists of a number of techniques for growing plant cells, tissues and organs; often referred to as *in vitro* micropropagation. One of the well recognized uses of tissue culture is as an efficient, safe and fast method of plant propagation. It has become firmly established as an economically important means of propagation especially for ornamental plants, woody angiosperms and many crop species. Numerous fields of plant sciences such as phytopathology, ecology, physiology, genetic and applied sciences such as biotechnology, genetic engineering and others are benefiting from plant tissue culture techniques. Meristem culture to eliminate virus from infected plants is widely practised. The technique is particularly useful when cultivars of pathogen-tested plants must be increased rapidly (Murashige and Huang, 1987). Plant tissue culture permeates plant biotechnology and cements together its various aspects; to a large extent the tissue culture revolution has occurred because of the needs of this new plant biotechnology (Cocking, 1986).

Haberlandt (1902)¹ is generally acknowledged as the first to attempt plant tissue culture (Gautheret, 1983; Murashige and Huang, 1987). His experiments, on the culture of isolated cells as well those of

¹ Krikorian, A.D., and D.L. Berquam. 1969. Translated the original German version of this paper of Haberlandt into English.

other early investigators were unsuccessful. Two decades later, demonstration of totipotency and, ultimately the division of isolated cells in culture coupled with whole plant regeneration were demonstrated by the successful root-tip culture of Kotte (1922) and Robbins (1922). However, plant tissue culture as a propagation technique became reality when the auxin, IAA (Kogl et al. 1934) and the cytokinin, kinetin (Miller et al. 1956), were used as additions to the culture medium. Morel (1960) not only succeeded in eliminating mosaic virus from cymbidium orchids but discovered that not only one plant could be derived from one explant but "millions". Morel's methods became a standard (Murashige and Huang, 1987).

Somatic embryogenesis is a process by which somatic cells develop into entire plants through a series of stages characteristic of zygotic embryo development. It has now been reported for many higher plants, angiosperms and gymnosperms and represents the most striking confirmation of totipotency (Wann, 1988). Although, few woody species have been regenerated by this process in comparison with herbaceous plants, applicability of these techniques to forest regeneration is now conceivable. Although plant tissue culture techniques can potentially become the most convenient way of propagating large numbers of clonal cultivars they are not without serious difficulties.

Current procedures can be laborious, costly and slow, while more efficient methods such as somatic cell embryogenesis, an adventitious method², result in high incidence of genetic variants (Murashige and Huang, 1987). Despite successes obtained with many plant cultivars

² In contrast to enhanced axillary branching which is less prone to genetic variations.

others, of equal importance, have resisted attempts to micropropagate them, either entirely or with such poor efficiency that commercial production is not feasible. For some plants, when propagation is accomplished, survival after transplanting from culture conditions to a less controlled environment is poor and makes the method cost ineffective. Those obstacles are attributable to inherent methods and more importantly, to a lack of systematic knowledge in the basic parameters and their influences on plant tissue culture development. The conventional tissue culture hardware is the first problem; culture vials were simply borrowed from the microbiology laboratory and adopted by tissue culturists because of their convenience. This usage appears to have been based primarily on the ability of the vials to prevent contaminants from entering and infecting the plant material and the organically based nutrient medium. While convenient, this use imposes a serious limitation to plant growth due to severely reduced gas exchange (Fujiwara *et al.*, 1987; Kozai *et al.*, 1986; Pospisilova *et al.*, 1987; Solarova, 1989). A second problem arises from the empirical approach to media formulation and decisions involving optimal environmental parameters to sustain *in vitro* plant growth. The current approaches are based on a limited number of criteria which include number of survivors, number of axillary shoots per culture cycle, "greenness", "vigor", "hardiness", "normalcy", and "trueness-to-type" (Donnelly, 1984). Proliferation or multiplication rate of plantlets combined with morphological characteristics such as shoot height and number of roots are amongst the most recorded parameters used to evaluate the performance of a micropropagation protocol. While the survival rate after *ex vitro* transplant appears to be determined by the success of the prior technical approach, acclimatization strategies may

incur elevated costs in both labor and facilities with accompanied lengthy delays in production (Driver and Suttle, 1987; Grout and Donkin, 1987). Acclimatization has been defined as a process, controlled by humans, to adapt an organism to an environmental change (Brainerd and Fuchigami, 1981; Donnelly and Vidaver, 1988). Survival rates of *ex vitro* transplanted plantlets vary widely (Aitken-Christie and Thorpe, 1984; Driver and Suttle, 1987; Dunstan and Turner, 1984; Murashige and Huang, 1987).

The unexpected and costly burden of re-establishing explants after *in vitro* propagation has revealed our need to understand *in vitro* plant systems. Fortunately, interest has been rising in the physiology of growth and development of plants grown under tissue culture conditions. It is reported that tissue and organ structures (Blanke and Belcher, 1989; Dhawan and Bhojwani, 1987; Donnelly and Vidaver, 1984a; Fabbri *et al.*, 1986; Sutter and Hutzell, 1984; Sutter and Langhans, 1982) as well as the physiological activities of plantlets at the stage of transfer *ex vitro* are very different from autotrophically grown plants (Donnelly and Vidaver, 1984b; Donnelly *et al.* 1984; Grout and Donkin, 1987; Grout and Millam, 1985; Kozai *et al.*, 1988; Pospisilova *et al.*, 1987; Pospisilova *et al.*, 1988; Shimada *et al.*, 1988; Toivonen, 1985).

There has been an increased interest in the transplant or *ex vitro* physiological performance of tissue-cultured plantlets (Desjardins *et al.* 1987; Donnelly and Vidaver, 1984b; Fujiwara *et al.* 1988; Grout and Donkin, 1987). It has previously been suggested that a major factor in the vulnerability of cauliflower transplants to stress was poor development of the photosynthetic system *in vitro*, resulting in wilting and desiccation due to poor epicuticular and cuticular wax formation (Fuchigami *et al.*, 1981;

Grout and Aston, 1977; Grout and Donkin, 1987; Short *et al.*, 1987; Wardle *et al.* 1983) or lack of stomatal function (Blanke, and Belcher, 1989; Brainerd and Fuchigami, 1981; Conner and Conner, 1984; Fuchigami *et al.*, 1981; Wardle *et al.* 1983). The physiological and developmental effects of the conditions under which the plantlets were differentiated appear to play a definite role in the *ex vitro* survivability of plantlets. New leaves of red raspberry transplants formed during the first month *ex vitro* tended to have intermediate morphology and physiological characteristics between *in vitro* expanded leaves and greenhouse controls (Donnelly, 1984). Once developed, the leaves have very limited capacity for adjustment to a new set of environmental conditions. Abnormalities arising from *in vitro* culture need to be corrected. It thus appears necessary to supply conditions which will allow *in vitro* developed leaves to persist after transfer.

Photosynthesis is a major determinant in growth and survival of plants and is very sensitive to the energy balance of the processes triggered by the physico-chemical environment. Its optimization should enhance morphological and physiological development necessary for a full photoautotrophic characterization. Optimization of photosynthesis in tissue-cultured plantlets *in vitro* should be achievable through careful manipulations of growth conditions.

There is need for developing assessment methods that allow continuous monitoring of changes in physiological performance with the changes in environmental conditions *in vitro*. Photosynthetic performance can be assessed with various techniques. The preferred techniques are nondestructive such as CO₂ gas exchange, which allows repetitive

measurement with little impact on the plants. The major difficulty with conventional tissue culture techniques is that the explants are confined to a static, semi-sealed or completely sealed environment. Sampling a single parameter, not only potentially breaks the necessary aseptic conditions but also disturbs all the other conditions which limit the interpretation of the measured effects of the parameter investigated. Once sugars and other organic addenda are removed from the medium formulation, physiological assessment is simplified (Fujiwara *et al.* 1988; Hayashi *et al.*, 1988). However, little information is available regarding the combined effects of sugars, growth regulators, mineral nutrients, the gas atmosphere, on growth and morphological development of the plants. Kozai *et al.*, (1988) have developed an automated mass-propagation system to enhance autotrophic development by increasing CO₂ levels of the atmosphere surrounding conventionally capped culture tubes. However, this system does rely on the passive diffusion of gases between the culture tube and its stopper and does not provide for replenishment of nutrition medium.

1.1.1 The *in vitro* environment

The *in vitro* environment can be described in terms of three main aspects which differ from that normally experienced by plants developed *ex vitro*.

- energy source

Photoautotrophic plants or plant organs rely on sun as the primary energy source. *In vitro* culture implies that the explants are placed into

culture tubes or other vessels on a medium containing nutrient minerals, organic addenda such as growth regulators, vitamins and an exogenous source of carbon and energy such as sucrose or glucose (Murashige and Skoog, 1962). Light levels are usually well below the compensation point. High levels of sugars which are present in most cell and tissue culture media have been implicated in the repression of chloroplast development and photosynthetic capacity (Kozai *et al.*, 1988; Neuman and Bender, 1987).

- gas exchange

When organic addenda are supplied, sterile conditions are essential. However, closed or partially sealed culture vessels used in tissue culture restrict the exchange of atmospheric gases. Plants consume O₂ and release CO₂ in the dark or when light levels are below the compensation point. Under elevated PFD, *in vitro* plantlets consume all available CO₂ until CO₂ compensation is reached (Solarova, 1989). The *in vitro* humidity being close to saturation may only induce poor transpirational control. Plantlets normally exposed to an atmosphere nearly saturated with water vapor appear to possess non-functional stomata and are essentially devoid of leaf cuticle. *In vitro*, they acquire little capacity to regulate water loss (Sutter and Langhans, 1982).

- nutrition

Tissue culture of plants requires complex media. Most often formulations were derived empirically, under conditions that limit photoautotrophic development, and have been selected on the basis of

how well the plantlets proliferated, developed or survived on them. All media contain sufficient mineral salts and enough of an organic energy source (sugar) to meet the needs of the *in vitro* culture during the entire developmental period. Other substances such as amino acids, organic acids or vitamins may also be present. Understanding of the physiological effects of added hormones and organics, other than sugar, is severely limited. There is a possibility that excess of certain mineral nutrients curtail the development of functional roots or even photosynthetic functions. Ammonium salts were found in several studies to be linked to a physiological disorder called vitrification or vitreousness in plant tissue cultures (Daguin and Letouze, 1986; Kevers *et al.* 1984; Meulemans *et al.*, 1987). It also appears that the ratio of $\text{NH}_4^+:\text{NO}_3^-$ plays an important role in morphogenesis (Selby and Harvey, 1990). Other difficulties arise with reduced availability of certain minerals caused by precipitation. Ferric phosphate and ferrous phosphate precipitate with autoclaving, which makes iron inaccessible to the plants (Vyskot and Bezdek, 1984) and possibly phosphate also. The metabolism of the plants generally shifts the initial pH of the weakly buffered MS medium thus inducing chemical instability of the medium. The low inorganic phosphate content of the Murashige-Skoog medium is known to be a rapidly limiting factor of photosynthetic efficiency in some culture systems (Dalton *et al.*, 1983).

1.2 PHOTOSYNTHESIS

When light is absorbed by chloroplasts some photons are utilized to promote a series of reactions with the ultimate result that H_2O and CO_2 are converted into carbohydrates. This series of reactions leads to the

reduction of NADP⁺ to generate NADPH and the phosphorylation of ADP to ATP, two primary temporary energy storage products. Aerobic organisms retransform this energy originating from photosynthesis by oxidative phosphorylation in the mitochondria. Photosynthesis and oxidative phosphorylation through respiration occur in chloroplasts and mitochondria respectively and eucaryotic photosynthetic organisms, such as plants, possess both organelles. Respiration is roughly the reverse of photosynthesis; it requires O₂ and releases CO₂ while the latter requires CO₂ and releases O₂.

Photosynthetic organisms, in order to produce organic compounds, need to channel light energy to provide electrons necessary for the enzymatic reactions of CO₂ assimilation. In higher plants light energy is initially captured by pigments such as chlorophyll **a** (Chl **a**), chlorophyll **b** (Chl **b**), and carotenoids, organized in protein-lipid bilayer membrane systems (Marder and Barber, 1989). Antennae are pigment-containing complexes concerned with the interception of light and the propagation of the exciton energy towards the reaction centres (RCs). RCs form the photochemical nuclei of the photosynthetic system; their function is to convert exciton energy to chemical energy. Electron transfer components transport electrons to and from the reaction centres by catalysing redox reactions such as:



The primary functions of the electron transport components are to generate reducing power by reducing **A**, and once **A**⁻ is reduced,

prevent the rereduction of the electron acceptor P^+ by reoxidation of the electron donor A^- . The reduction of the strong oxidant P^+ is permitted through a four step enzymatic reaction, water oxidation. The reduction of $NADP^+$ to generate NADPH is accomplished with the oxidation of A^- . The passage from ground state of P to an excited form P^* allows primary charge separations which are the photochemical force driving electrons to higher redox potentials. Electron transport takes place in two main stages in order to supply the energy requirement for the redox reactions. Those stages are associated with two types of protein complexes in higher plants, the P700-Chl a -protein and the P680-Chl a -protein constituting the reaction centres which are part of photosystem I (PS_I) and photosystem II (PS_{II}) respectively. The arrangement of PS_I , PS_{II} , the various electron carriers, donors and acceptors is shown in *figure 1.1* (Marder and Barber, 1989). The electro-chemical potential generated across the thylakoid membranes is the driving force for the formation of ATP (Nicholls, 1982). Energy coupling factors make use of the fact that the passage of electrons through the electron transport chain is coupled to the vectorial pumping of protons into the thylakoid lumen. The potential available to the chloroplast coupling factor (CF_0 - CF_1), proton-driven ATP synthase, is used to phosphorylate ADP. In higher plants, a major portion of photosynthetically-produced ATP and NADPH is consumed in CO_2 fixation and other enzymatic reactions. Of importance are the pathways for assimilation of nitrate and sulphate, which both require input of ATP and reducing power.

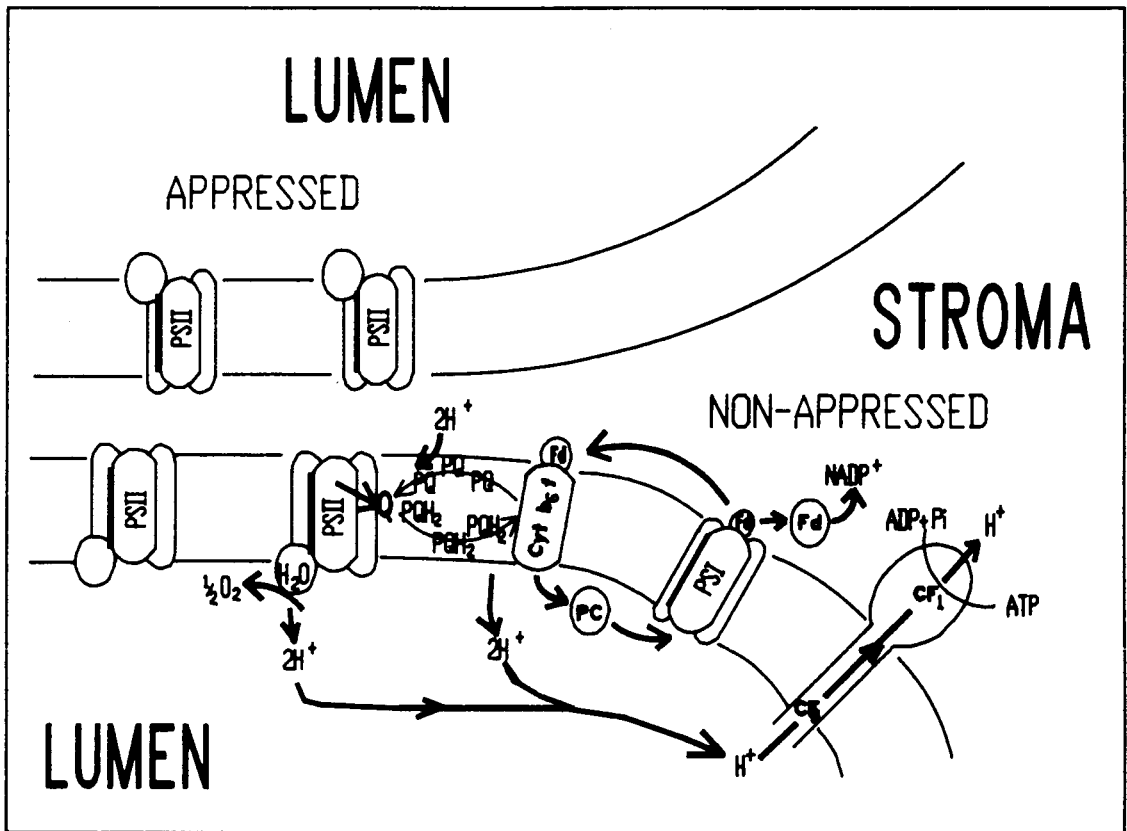


Figure 1.1. Organization of the thylakoid membrane. The figure depicts the four types of membrane-embedded complexes, PS_{II}, PS_I, Cyt b₆-f and CF₀-CF₁ distributed between appressed and non-appressed regions of the thylakoid. The first three complexes undergo electron transfer reactions with mobile components plastoquinone (PQ/PQH₂), plastocyanin (PC) and ferredoxin (Fd) to allow transfer of reducing equivalents from water to NADP⁺ coupled to pumping of protons into the lumen. This creates an electrochemical potential gradient which is then used by the CF₀-CF₁ complex to drive ATP synthesis. (Marder and Barber, 1989)

Not all light conventionally absorbed by leaves result in useful photosynthetic reactions. Dynamic changes in the composition and organization of the thylakoid membrane are essential for optimization and protection of the photosynthetic machinery in response to an everchanging environment. Short-term and long-term strategies for acclimation have been developed by plants. The short-term acclimation involves reorganization of existing membrane components while long-term requires changes in the composition of the photosynthetic membrane (Anderson and Andersson, 1988). When long-term strategies are unable to prevent damage due to a sudden stress and when short-term regulatory mechanisms fail to dissipate excess energy, damage occurs (Powles, 1984).

Several factors may limit the photosynthetic CO₂ assimilation process. Limitations to the rate of photosynthesis can be divided among three general classes: (1) the supply or utilization of CO₂, (2) the supply or the utilization of light, (3) the supply or utilization of phosphate (Sharkey, 1985). Mesophyll and stomatal resistance to CO₂ diffusion can limit the supply to the carboxylation reaction. Systems that consist of probes for CO₂ and H₂O exchanges are generally straightforward and provide information regarding mesophyll and stomatal limitations of photosynthesis and (Sharkey *et al.*, 1982; von Caemmerer and Farquhar, 1981) are applicable to tissue-cultured plantlets (Toivonen, 1985). *In vivo* chlorophyll *a* fluorescence techniques provide information on potential sites of electron transport limitations to CO₂ assimilation (Govindjee *et al.*, 1967; Krause and Weis, 1984; Lavorel and Etienne, 1977; Sivak and Walker, 1985).

Photosynthetic unit (PSU)³ sizes can also concurrently be estimated with fluorescence induction methods (Malkin *et al.*, 1981; Malkin and Fork, 1981). Differences in over-all photosynthetic rates can therefore be established on a PSU basis, which brings further understanding of the mechanisms of energy transfer and of the adaptive mechanisms in plants (Mauzerall and Greenbaum, 1989). Determinations of relative PSU sizes, using the equations of Malkin *et al.*, (1981), can be achieved with an integrating sphere configured to measure concurrent light quanta absorption (I_{ABS}) and fluorescence. The methods of Malkin and co-workers can be simplified by the use of the directly measured parameter I_{ABS} . Photosynthetic carbon assimilation reported on PSU size estimations allow determinations of quantum efficiency and can be estimated nondestructively in intact plants. These methods applied to tissue-cultured plants can bring insight on the effects of the *in vitro* environment on their photosynthetic development.

1.3 STRATEGIES OF IN VITRO PLANTLET ACCLIMATIZATION

To minimize the effects of applied and sustained stress factors implied in *ex vitro* acclimatization, PFD is usually reduced to minimal levels, humidity tents are used to minimize leaf transpiration and "damping off" pesticides are applied systematically for until about a month after transfer to soil. The strategy employed and described in this thesis is to immediately favor photoautotrophic potential while the plantlets are maintained under *in vitro* conditions. This requires the provision of control over the growth

³ APPENDIX I lists abbreviations and the page of their first occurrence in the text.

parameters necessary for normal plant growth: light, water and nutrients, moisture, CO₂ and O₂ in a temperature controlled environment. An automated assessment system for investigating the immediate effects of controlled micro-environmental changes was designed and used to enhance growth and photosynthetic ability of plantlets prior to transfer *ex vitro*.

1.4 OBJECTIVES OF RESEARCH PROJECT

The primary objective of this thesis is to proffer methods by which physiological competence could be assessed and monitored by quantitative performance criteria in order to characterize *in vitro* growth requirements. The secondary objective is to suggest that when important parameters of growth conditions are subject to feed-back control, the process can ease or even eliminate the need for acclimatization at transplant.

1.5 ASSESSMENT OF PHOTOSYNTHETIC COMPETENCE

An assay protocol consisting of simultaneous gas exchange measurements and fluorescence determinations was designed to diagnose plantlet photosynthetic competence reached prior to and after controlled *in vitro* acclimatization. To understand the prerequisites for photosynthetically enhanced growth, effects of exogenously supplied sucrose were studied under conditions that would potentially minimize sugar requirement. Another section of this thesis analyzes the effects of artificially induced photoinhibition of photosynthesis in conventionally produced plantlets. These photoinhibition responses are used to compare

photosynthetic characteristics in plantlets acclimatized *in vitro* with those in unacclimatized plantlets.

CHAPTER 2

AN *IN VITRO* ENHANCED ACCLIMATIZATION SYSTEM FOR TISSUE CULTURED PLANTLETS

2.1 INTRODUCTION

The most deterministic element of plant tissue culture methodology and technology is the presence of exogenously supplied sugars as an energy and carbon supplement. Although necessary in the early stages of the propagation protocol, they have been linked to several problems in plant cell and tissue culture. After uptake, hexose phosphates are phosphorylated and channelled into metabolism via glycolysis. Phosphorylation requires P_i . The major products of photosynthesis being exported from the chloroplasts to the cytoplasm by the phosphate translocator require inorganic phosphate. A low ratio of P_i /organic P would eventually induce feedback inhibition and reduce the activity of the Calvin cycle (Neuman and Bender, 1987). There is a fine balance between the activities of glycolysis and photosynthesis when sugars are supplied which appears to depend on the availability of P_i . Optimization of the activity of the Calvin cycle could only be achieved with increasing photon flux density (PFD) to levels that encourage net photosynthesis with concomitant adjustments in the availability of exogenously supplied sugars.

Plantlets grown under conventional tissue culture conditions cannot achieve full photosynthetic capacity because the CO_2 concentration inside the vessel is too low during most of the light period

(Fujiwara *et al.*, 1987). In stoppered vials, dark respiration generated increases in CO₂ concentration up to 9000 μl l⁻¹ and soon after the light is turned on, CO₂ concentration decreases to 90 μl l⁻¹. Similar results were obtained by others (Pospisilova *et al.* 1988; Solarova, 1989). Those observations lead to the conclusions that plantlets are photosynthetically active under those conditions and that CO₂ availability for photosynthesis can only depend on activities of hexose catabolism to regenerate reducing equivalents. It appears reasonable, at this stage, to speculate that under those circumstances, inavailability of reducing equivalents would cause plantlets to experience photoinhibition and possibly photodamage. Several approaches have been used to increase the supply of CO₂ in the gas atmosphere of tissue culture vials. Improving the gas permeability of the culture vial closure has been accomplished by using gas permeable polypropylene film (Mahlberg *et al.*, 1980), and by increasing CO₂ concentration in the air surrounding the culture vials capped with semi-sealed closures (Howard *et al.*, 1983; Kozai *et al.* 1987a; Kozai *et al.* 1988; Infante *et al.*, 1989; McHale, 1985; Mousseau, 1986). In all those studies mentioned, none depicted control of growth conditions which allowed direct gas exchange with the tissue cultured material nor control of the relative humidity inside the vials. Instead, plantlets remained in vials semi-sealed with types of stoppers which allowed only a passive and limited diffusion of gases to and from the outside of the vessels. The driving force for transpiration is the difference in water vapor partial pressure within the leaf and in the atmosphere beyond the leaf boundary layer. Inside the vials, water vapor constantly saturates the boundary layer, therefore, the leaves are not subjected to the driving force of transpiration and all the benefits it incurs to plant metabolism. A

reasonable approach would be to provide plantlets with air that would disturb the boundary layer with non-limiting levels of CO₂ and known high moisture content.

All media have been formulated empirically from experiments using conventional tissue culture practices. Most media formulations for minerals, growth regulators, sugars and organic supplements were originally derived for procedures to grow callus of given plants (Gamborg *et al.*, 1976). Very little attention has been devoted to understanding the physiological and biochemical aspects of plant tissue culture nutrition compared to aspects dealing with desirable morphogenetic characteristics. Generally speaking, nutrients are not routinely replenished, with the assumption that initial balance and content of nutrients would support growth optimally for the duration of the passage. Hyperhydric malformations, known as the vitrification disorder in tissue cultured plants, have been associated with the presence of NH₄⁺ ions in the culture medium (Daguin and Letouzé, 1986; Letouzé and Daguin, 1983). Kevers *et al.* (1984) hypothesised that vitrification results from a burst of ethylene controlled by the peroxidase-IAA-oxidase system initiated by stress caused by the presence NH₄⁺ ions in the cells. Ammonium toxicity, to some extent can be prevented by glutamate dehydrogenase since the specific activity of this enzyme is affected by increases in intracellular ammonia due to the presence of NH₄⁺ in the medium (Zink, 1989). The levels of ribulose-1,5-bisphosphate carboxylase (Rubisco) were affected by high NH₄⁺/NO₃⁻ ratios (Mehrer and Mohr, 1989). In higher plants, ammonia assimilation occurs via the glutamate synthase cycle which probably operates in the chloroplasts and is light-stimulated (Wallsgrave *et al.*,

1983). However, ammonium nutrition avoids energy expenditure in plants in contrast to nitrate reduction (15-16 ATP (mol NO₃⁻)⁻¹) (Salsac *et al.* 1987). The mechanisms of NH₄⁺-induced vitrification in tissue cultured plants are certainly not well understood. However improving photosynthetic activity, reducing the NH₄⁺/NO₃⁻ ratio in the medium formulation and allowing a better gas exchange within the culture vials are plausible approaches that can reduce or eliminate occurrence of vitreousness due to presence of NH₄⁺.

The purpose of designing an automated *in vitro* system is to provide controlled conditions that would take into account the limitations cited above. An *in vitro* system should provide plantlets with optimal conditions for CO₂ and H₂O vapor exchange under high PFD with sustained supply of water and nutrients, all under aseptic conditions. A computer interface is required since a fair amount of automation for data collection and device control is necessary.

2.2 MATERIAL AND METHODS

2.2.1 Description of system

The *in vitro* or Aseptic Gas exchange and Hydroponic System (AGEHS)** was designed to accommodate two chamber units (CU-1 and CU-2). The dimensions of the cylinder shaped glass chamber units are: 13 cm diam. x 35 cm long. The effective growth area in each chamber is 330 cm². With minor modifications of the various circuits, more chamber units

** Also named "GASSY-JACK" because of the sounds it constantly produced and mainly after a call at 3:00 AM from the security agents informing me that the water lines popped and flooded the laboratory, including the PC computer...

can be added. The AGEHS monitors several conditions relevant to plant growth such as temperature, relative humidity (RH), CO₂ concentration and labels collected data with the activities of the automated devices such as photoperiod and PFD controls, medium pumping and drainage cycles, time and duration of calibration cycles of the interfaced Infrared Gas Analysers (IRGAs). *Figure 2.1* is a schematic diagram of the AGEHS.

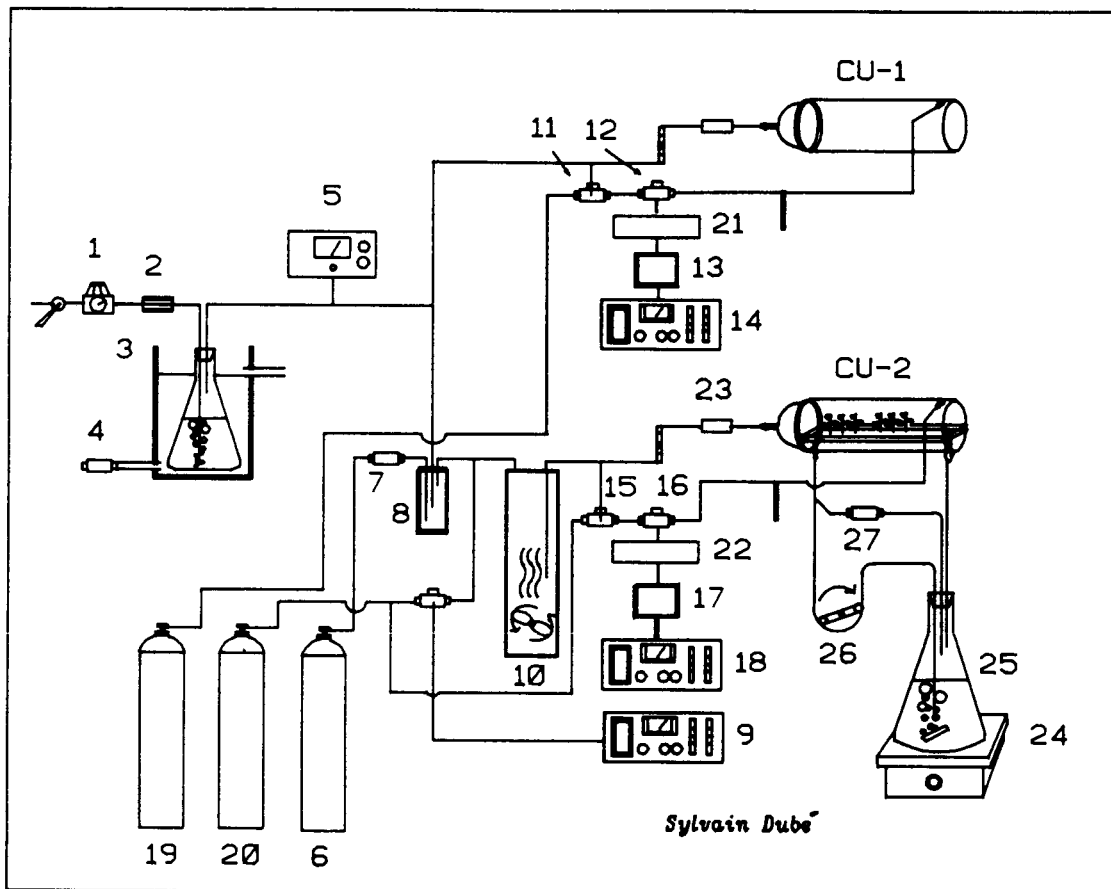


Figure 2.1. Schematic diagram of the Aseptic Gas Exchange and Hydroponic System (AGEHS). Air and liquid lines are shown; electrical lines are omitted for clarity. Only one chamber unit is shown with hydroponic medium line connections. Refer to text for description of the various components.

- *Relative humidity.*

Prior to water vapor generation, a pressure regulator (1) attenuated the pressure fluctuations of the air lines from the university compressed-air system. The air was filtered by an in-line 0.45 μm pore size filter (2) to remove dust particles and oil droplets. Water vapor was generated by bubbling filtered compressed air into a flask containing distilled water surrounded by a thermoregulated water bath. The temperature of the water bath (3) was controlled by the Central Processor Unit (CPU) to generate water vapor to preset level by alternating warm and cool cycles of the water bath. The solenoid valve (4) allowed admission of pulses of cool water into the water bath. Water vapor thus generated was continuously monitored with a dew point hygrometer (5) (EG&G, Cambridge Instruments Inc., Massachusetts) placed in air lines before distribution to each circuit. The relative humidity of the air inside the chamber units was determined by calculating the ratio of water vapor pressure at the dew point divided by the tabulated water vapor pressure at the temperature of the glass chamber as measured by a temperature transducer pressed against the wall of the chamber units.

- *Temperature measurement*

Temperature at various parts of the system was measured with an array of 6 solid state temperature transducers AD590 (Analog Devices™) calibrated in the physiological range of temperature giving a precision of 0.2 °C. Temperature transducers were pressed against the outside glass wall of each chamber unit or near the culture tube controls. A fourth

transducer was placed in the output stream line to monitor air temperature exiting a chamber unit. The fifth and sixth transducers monitored temperature of the medium solution and the temperature of the thermoregulated water bath respectively.

- Control and measurement of inlet CO₂

The humidified air stream was divided into two branches, one for each chamber unit. For the normal air (CU-VN) chamber unit (CU-1), the CO₂ concentration remained at ambient level ca. 360 µl l⁻¹ while for the enriched-air (CU-VE) chamber unit (CU-2) the ambient air was enriched with CO₂ to increase the concentration to a preset level, ca. 4500 µl l⁻¹. CO₂ was provided from a compressed gas cylinder (6) by opening a solenoid valve (7) for pulse injections in the air stream. The CO₂ enriched air was pre-mixed in a flask (8) and continuously sampled by an Infra Red Gas Analyzer (IRGA) (9) to determine amounts and duration of CO₂ pulses in the air in order to bring the concentration to the preset level. The effects of pulsing CO₂ was attenuated by mixing the air in a storage tank (10). It was possible to program changes of CO₂ concentrations provided the IRGAs were calibrated within the range of those changes. Sampling of the normal air stream for determining intake CO₂ concentration was achieved by operating solenoid valves (11 and 12) to divert the sampled flow to the desiccator (13) before measurement by IRGA (14), an ADC-225 MK3 (Analytical Development Co., Ltd., Eng.) calibrated in the absolute mode. Sampling of the CO₂ enriched air for determining intake CO₂ concentration was achieved by operating solenoid valves (15 and 16) to divert sampled air to the desiccator (17) and then to the IRGA (18)

(Beckman) calibrated also in the absolute mode. Base line zeroing of the IRGAs was done manually every 2 days with compressed pure nitrogen gas for the normal air IRGA and 1500 $\mu\text{l l}^{-1}$ CO_2 for the enriched air IRGA (standard gas cylinders and line circuits are not shown in the figure). Upper scale intermittent calibrations were achieved automatically every 4 hours by measuring the voltage deflections when standard gas streams from different compressed gas cylinders (19 and 20) were diverted to IRGA (14) and IRGAs (9 and 18) respectively. Compensations in $\mu\text{l l}^{-1}$ of CO_2 were calculated from the voltage deflections measured during calibration. The standard gases used for the normal air and CO_2 enriched air were 320 and 5000 $\mu\text{l l}^{-1}$ (CO_2 mixed in air) respectively. All gas exchange circuits were configured in an open mode with the IRGAs (Sestak *et al.*, 1971)

- Measurement of CO_2 outlet.

Gas outputs from individual chamber units were measured for CO_2 uptake or respiration rates. The gas outlet of the CU-VN chamber unit, was diverted to IRGA (14) by operating solenoid valve (12); while for the gas outlet of the CU-VE chamber unit was diverted to IRGA (18). For CU-VE gas outlet, air flow rates were measured with a variable area flow-meter (21), (GilmontTM), manually recorded at regular intervals. For CU-VN, gas outlets were automatically monitored with a mass flow meter (22), (Aalborg, AFM 2600-PRO). Due to their range of calibration, IRGAs dedicated to measurements of the enriched air, had their precision to ca. 125 $\mu\text{l l}^{-1}$.

- Control of gas flow rates

Flow rates of both CU-VN and CU-VE were adjusted to sweep across the plantlets inside the chamber units on the basis of the amounts of CO₂ required to offset photosynthetic demand and to provide leaf boundary layer disturbance for an adequate gas exchange with the plantlets. In the case of the CU-VN, the air flow rate was routinely adjusted to maintain a ca. 10-35 $\mu\text{l l}^{-1}$ depletion of CO₂ from the inlet gas stream. Whereas for the CU-VE, the air stream was maintained at a fairly low and constant flow rate such as to induce a measurable depletion of CO₂ by the plantlets. The pre-set flows were maintained during the dark period and any increments or adjustments were made manually.

- Growth chamber units

Each chamber unit was independently configured with gas and liquid medium ports. The inlet gas port consisted of an autoclavable 0.22 μm pore size in-line filter (Gelman Science, MiniCapsule™) (23) clamped to the head cover of the glass cylinders. The design and the dimensions of the glass cylinders were such that they offered adequate air movements, light capture, medium circulation, and capability to withstand autoclaving. Liquid media circulation was achieved by pumping in and draining out by gravity through a single port built at the bottom and excess media was expelled through an adjustable overflow port at the other end of the chamber unit. A magnetic stirrer (24) continuously stirred the bulk medium in a sealed 5 l capacity Erlenmeyer flask (25). Tubing connections to and from the chamber units were made with clamped Tygon™. Pressure adjustments, air movements and aeration

of the medium were made possible through the connection with the overflow port and the medium flask. A peristaltic pump (26) delivered medium automatically every four hours to flood the plantlet support (Rockwool, Grodan™) for a few seconds. The drainage of the medium was achieved by controlling a solenoid valve (27). The glass cylinder, microfilter, rockwool support, tubing and liquid medium contained in the flask were all autoclavable as one unit. The chamber units were only opened in a laminar flow cabinet for aseptic transfer of the plantlets.

- Computer control and data collection

Electrical connections to all peripheral devices for data acquisition and control were established through an Analog to Digital (A/D) card (Metrabyte DASH-16) installed in an expansion slot of an IBM PC type computer. Analog outputs from the IRGAs and the dew point hygrometer were delivered to acquisition instrumentation amplifiers AD552 (Analog Devices™) for amplification to meet the range of the A/D card (0-5V). Nonlinearity of the signals from most instruments was corrected by applying quadratic regressions determined from previous calibrations. The peristaltic pumps and solenoid valves were controlled by activating 120 V_{AC} solid-state relays from dedicated output ports of the A/D card. Programming has been done in BASIC language (Microsoft QuickBASIC™). Level of relative humidity, concentration of CO₂ in the enriched air stream, frequency of pumping and duration of contact of the medium with the rockwool support were parameters automatically controlled to preset values. The parameters displayed and saved to floppy disks are detailed in *table 2.1*. The data was saved in ASCII

(American Standard Code for Information Interchange) characters for easy importing into Database or Spreadsheet programs.

Table 2.1. Parameters displayed and saved by the AGEHS. Every 5 min on a 24 h basis over the entire duration of the experiments all parameters were saved in ASCII characters for analysis and plotting. Time is expressed in min from 0:00 hour of the current day. Rates of CO₂ exchange are the rates of the all plantlets present in each chamber unit (CU).

PARAMETER	UNIT
Date	
Time of the day	min from 0:00hr
Photoperiod	min
Current activity of the system	reserved codes
T of the air exiting the chamber units	°C
T of the medium flasks	°C
T inside growth cabinet	°C
T of the water bath	°C
Relative humidity	ratio of vapor pressures
Air flow	ml s ⁻¹
Barometric pressure	kPa
PFD	μmol photons m ⁻² s ⁻¹
CO ₂ concentration from each IRGA	μl l ⁻¹
Rates of CO ₂ exchange	mg CO ₂ h ⁻¹ CU ⁻¹

Rates of CO₂ exchange were calculated according to equation.

2.1 (After; Sestak *et al.*, 1971).

$$F = (C_{in} - C_{out}) \times f \times \frac{44000}{22414} \times 10^{-6} \times \frac{273}{T+273} \times \frac{P}{101.3} \times 3600 \quad (2.1)$$

where **F**=flux density of CO₂ [mg h⁻¹], (C_{in}-C_{out})=difference in CO₂ concentration of air streams before and after a chamber unit [μl l⁻¹], *f*=air flow [ml s⁻¹], *T*=temperature [°C], *P*=barometric pressure [kPa]¹. Rates of CO₂ exchange were calculated and recorded every 5 min on a 24 h basis over the entire duration of the experiments except during short periods for zeroing IRGAs to base line.

¹ Barometric pressure and PFD were entered manually every day.

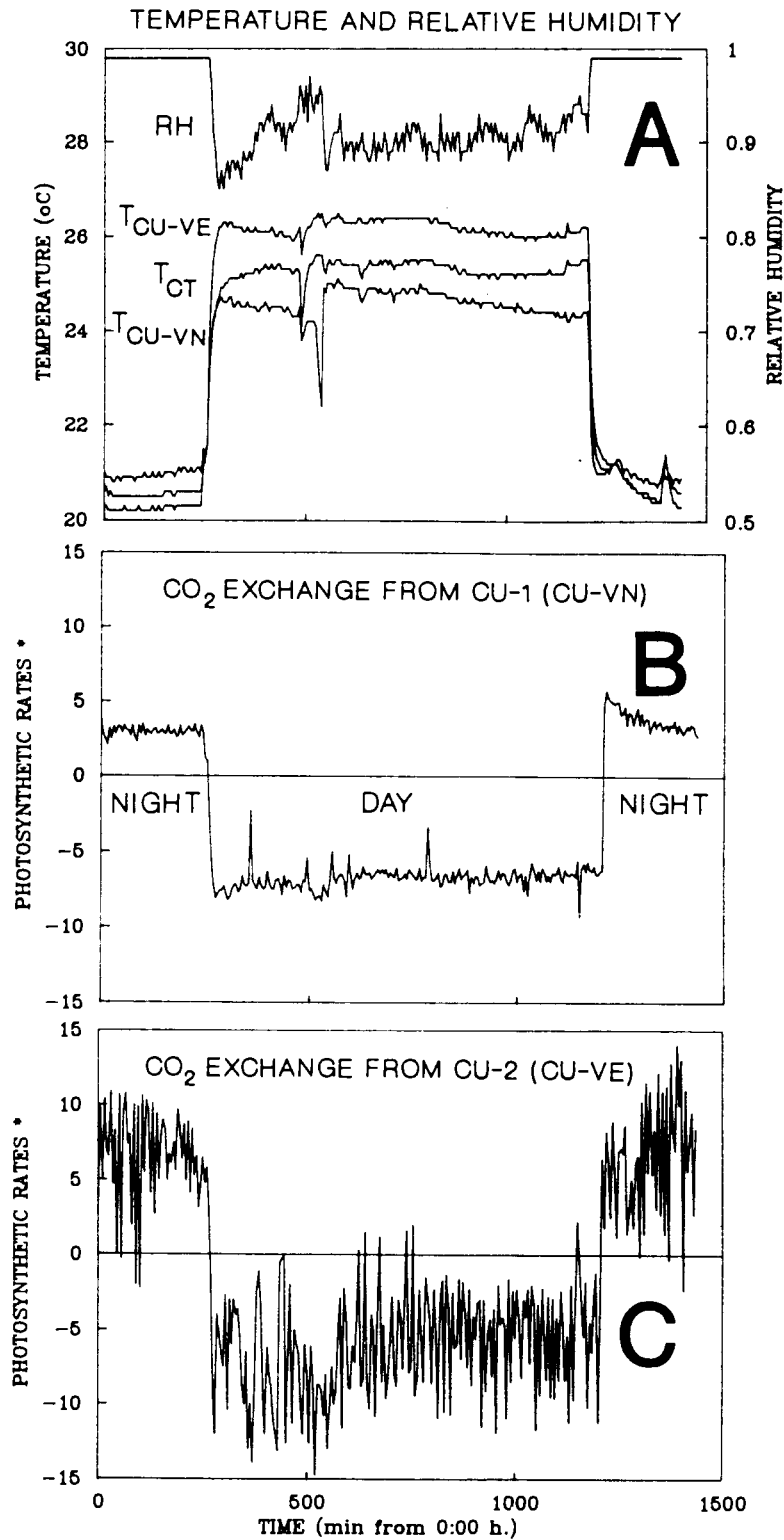


Figure 2.2. AGEHS recordings of parameters for a 24 hour period. Temperature and RH (Fig. 2.3A). Gas exchange rates from plantlets in CU-1 or CU-VN (Fig. 2.3B). Gas exchange rates from plantlets in CU-2 or CU-VE (Fig. 2.3C). *Gas exchange rates are in $\text{mg CO}_2 \text{ h}^{-1} \text{ CU}^{-1}$.

2.2.2 Plant material and growth conditions

Chrysanthemum (*Chrysanthemum X morifolium* Ramat. cv. Envy) plantlets derived from cloned shoot tips were routinely subcultured on a modified MS medium (Murashige and Skoog, 1962). Screening for non-endogenously contaminated plantlets was performed by growth young explants on Bacto Agar, glucose and nitrogen rich media. For the multiplication and acclimatization stage the basal medium had NH_4NO_3 salts reduced to 5.1 mM, and KNO_3 increased to 37 mM, (or : 2x of MS medium for NH_4^+ ions and NO_3^- respectively). The multiplication medium formulation contained BAP [1 mg l^{-1}], IBA [0.5 mg l^{-1}], GA_3 [1 mg l^{-1}] and sucrose [20 g l^{-1}], gelled with a mixture of Difco Agar and Gelrite. All media were adjusted to pH 5.6 before autoclaving. During multiplication, plantlets were grown in 25 mm diameter culture tubes capped with KimKap™ stoppers or *Majenta*™ jars 275 cm³ vol., (Majenta Corp., Chicago). Light was provided from cool white fluorescent tubes of photosynthetically active radiation (PAR) at a rate of $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. At transfer of shootlets to the chamber units, the liquid medium formulation contained IBA [3 mg l^{-1}], GA_3 [1 mg l^{-1}], no BAP and sucrose [5 g l^{-1}] unless stated otherwise.

At the beginning of all experiments, inoculated plantlets had 3-4 leaves and no roots (shootlets). The chamber units along with the control culture tube treatments were positioned inside a growth cabinet where light was provided from a mixture of cool white fluorescence tubes, incandescent light bulbs and a high pressure sodium lamp. Temperature and photoperiod were 25: 20 °C (day: night), 14: 10 h, (day:night)

respectively. RH inside the growth cabinet was ambient and not controlled. All experiments ended on the 15th day after the start.

2.3 RESULTS AND DISCUSSION

Figure 2.2 shows examples of data collected and calculated by the AGEHS. Recorded temperatures for each chamber unit ($T_{\text{CU-VN}}$, $T_{\text{CU-VE}}$) and a culture tube treatment (T_{CT}) during a 24 hour are shown in Fig 2.2A. The temperature traces indicate small differences between treatments. There is, during the day, a relatively constant ca. 2 °C difference between the CU-VE and the CU-VN cylinder. These discrepancies were partly due to uneven heat distribution inside the growth cabinet. For the CU-VN treatment, CU-1 was located closer to the ventilation system inside the growth cabinet and when the lamps are all turned on, location effects are more accentuated. The minor oscillations in the temperature traces are due to the heating vs cooling cycles of the incubator. Relative humidity is also reported. At night, RH reached saturation levels due to inadequacy of the cooling cycle of the thermoregulated water bath. However during the day, the range of RH was within acceptable limits of the preset value. There is a very direct dependence effect of temperature and relative humidity as shown by the major spikes of temperature recordings. Those are immediately echoed in the relative humidity trace. Photosynthetic rates of both CU-VN and CU-VE chamber units are displayed in Fig. 2.2B and 2.2C respectively. Values below the zero line indicate CO₂ uptake rates while values above the line indicate respiration rates. Instrumentation and scale of calibration differed between treatments. The traces of gas exchange rates for the CU-VN (CU-1), are very smooth compared to the CU-VE (CU-2). Precision differences

may come from at least three causes; first, scale of calibration of the instrument; second, the air flow rates being much slower in the CU-VE treatment, the gas lines may not get totally flushed between cycles; third, the choice of the instrument (the available instrument was more than 20 years old). In *Fig. 2.3*, results displayed are averages of data from light and dark cycles for each day for an entire 15 day experiment. RH is displayed on a 0-1.00 scale. The day and night cycle pattern of RH is very noticeable throughout the entire cycle. Recorded temperatures from only one temperature transducer are displayed using the same axis as the gas exchange data, although the units are different. Gas exchange data shown for CU-VE and CU-VN chamber units are equal for the first 5 days. Differences in both photosynthesis and respiration start to arise on the 5th day when PFD is increased. As the final PFD setting is made, on the 9th day, those differences become more accentuated.

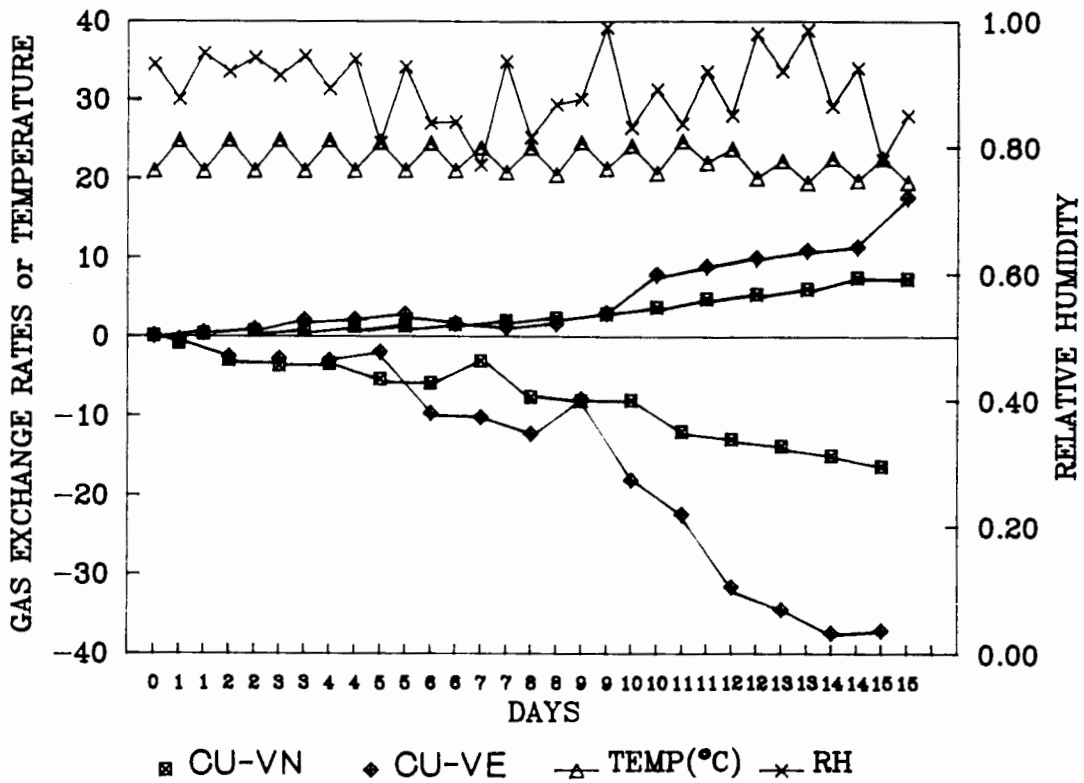


Figure 2.3. Recordings of temperature, RH and CO₂ uptake for 15 days. Results shown are averages of all 5 min recordings for each light and dark cycles for a 15 day period. Gas exchange rates expressed per chamber unit are shown for both, CU-VE and CU-VN. *Gas exchange rates are in mg CO₂ h⁻¹ CU⁻¹.

2.4 CONCLUSION

Despite serious limitations imposed by several ineffective hardware components, the results show the potential of this system for *in vitro* gas-exchange studies. It is possible with the system configuration, as shown in Fig. 2.1, to easily follow changes in photosynthesis with increments of PFD and/ or modifications of other parameters. This allows monitoring of effects of accelerated changes in conditions which can potentially induce photoautotrophy.

As total biomass and photosynthetic capacity increase, more CO₂ is needed during the photoperiod. This requires large volumes of air flow through the CU-VN chamber to the point where, moisture control, filtration and other functions of the system become inadequate. In most cases, experiments had to be terminated due to hardware limitations. One of many advantages of using a CO₂ enrichment configuration is the possibility of supplying all CO₂ and moisture at much lower flow rates. CU-VE flow rates through the chamber unit need not to be very fast and as frequently adjusted as for the CU-VN. To avoid wide fluctuations in the CU-VE gas exchange rates, a compromise can be found between CO₂ concentration and flow rate. However, with slower flow rates, risks of rapid desiccation are minimized.

Rapid contamination was often revealed within hours upon transfer of shootlets from the multiplication stage (in culture tubes) to the AGEHS system; probably caused by bacteria and yeasts. This contamination, qualifiable as acute, does not necessarily have any serious effects on plantlet growth but invade the rooting support and

hydroponic solution and the CO₂ released overwhelms the monitored gas-exchange rates. It became necessary to subject explants to a methodical decontamination scheme for several weeks prior to transfers. Application of surface decontamination protocols alone were inadequate. Systemic contaminants are likely to be the cause (Constantine, 1986). High turbulence of moist air in addition to the presence of sucrose in the medium provide excellent conditions for such contaminants to reveal themselves and spread. The use of carefully selected antibiotics in the early stages of the micropropagation scheme may help alleviate this serious problem.

With additional increments of PFD, the potential benefits or unfavorable effects of CO₂ enrichment could have been further tested. With the growth cabinet available, PFD was limited to a maximum of ca. 325 $\mu\text{mol m}^{-2} \text{s}^{-1}$, still only one sixth PFD on a sunny day.

CHAPTER 3

PHYSIOLOGICAL ASSESSMENTS AND PROCEDURES

3.1 INTRODUCTION

Photosynthesis is a major determinant of survival of plants and is an excellent indicator of the plants' physiological state. At present, with the refinement of various techniques, photosynthesis can be characterized by determining its potential for energy utilization and dissipation. Applications of adequate methods of assessment for tissue-cultured plantlets may help elucidate their needs for survivability in both, *in vitro* and *ex vitro* environments. Among the important criteria for plantlet transplant success are capacities for autotrophy and the control of water relations reached at the onset of transplant. Capacity for autotrophy can be assessed using techniques such as CO₂ gas exchange and *in vivo* chl *a* fluorescence with appropriate assay protocols.

The way in which PFD during *in vitro* growth affects photosynthetic performance and pigment content of plantlets *ex vitro* was first studied by Donnelly, (1984). To acquire information on photoautotrophic competence, the criteria that qualify plantlets as being competent, need to be resolved. In this present study, the rationale is to measure photosynthesis of the plants before and after imposition of controlled stress conditions or before and after stress is alleviated. Photosynthetic competence is determined by looking at how the net light quanta absorbed (I_{ABS}) are utilized *via* useful photochemistry such as CO₂ assimilation and dissipated *via* fluorescence emission. When whole shoots

of plants are assessed, both the yield and kinetics of fluorescence emission are relevant.

When normalized to dry or fresh weights, photosynthetic gas exchange rates of plants generated under tissue culture conditions are likely to be underestimated. If leaf area is used, it cannot take into account the thickness of the leaves and thus misrepresents the volume of tissue which is responsible for the CO₂ exchange rates. As mixotrophic means of nutrition enormously influence the way tissue cultured plantlets accumulate dry weight, a more appropriate parameter needs to be used. Net light quanta absorbed (I_{ABS}) by the plant material under investigation is proposed and used throughout for a substitute of dry weight for normalization of photosynthetic parameters.

A pre-existing fluorescence probe was available for integrating fluorescence determinations (Toivonen, 1985). However, modification and improvement of this laboratory prototype were required to accomplish reliable data acquisition for all parameters studied.

3.2 MATERIALS AND METHODS

3.2.1 Integrated Fluorescence Data Acquisition System (IFDAS)

Optical integrating spheres have previously been used for various applications in plant physiology studies. These include determination of chlorophyll (Macdowall, 1982), quantum yield for photosynthesis (Idle and Proctor, 1983; Öquist *et al.*, 1978), quanta absorption spectra (Öquist *et al.*, 1978), fluorescence emission (Toivonen and Vidaver, 1984; Morissette *et*

al., 1988). Data from fluorescence transients acquired by an integrating fluorometer represent an average of the entire sample, not influenced by the leaf size or geometry of the sample and therefore allows a consistent interpretation of results. This ability to obtain data indicating the response of the intact sample permits its application to studies incompatible with the conventional type of fluorometer capable of measuring the signal from only a small area of a sample such as with those described by Bolhar-Nordenkamp *et al.*, (1989).

Automation of fluorometer data acquisition with the use of a microcomputer not only provides fast and accurate data analysis (Öquist *et al.*, 1988; Morisette *et al.*, 1988) but provides means for reproducible measurements through precise control of parameters such as excitation light (I_0) and temperature.

The first requirement in fluorescence data analysis is the determination of a precise estimate of the initial rise of fluorescence F_0 . This is also called dark level fluorescence and it represents the fluorescence level at a physiological state in which all the intersystem intermediates are oxidized (Lavorel and Etienne, 1977; Papageorgiou, 1975) and should not consist of any variable fluorescence component (see Bilger and Schreiber, 1986). This represents emission from the chl a molecules of the light-harvesting and antenna matrices associated with PS_{II} occurring prior to the excitation energy inducing photochemical electron transport via the PS_{II} reaction centers (P680) (Baker and Bradbury, 1981). Since the time course of relative variable fluorescence yield is of particular interest, it is usually obtained from the subsequent normalization ($F_{VAR}(t) = (F(t) - F_0) / F_0$) of fluorescence signals, which removes the

contribution of the so called dead or initial fluorescence. Slow opening times of magnetic shutters produce a variable fluorescence component to F_0 thus require a standard method of determining the F_0 parameter in order to perform reliable estimates of F_0 . Excitation PFD is also critical to F_0 determination. As described by Lavorel and Etienne (1977), fluorescence intensity is directly dependent on absorbed PFD. Since F_0 is directly proportional to excitation PFD, the precise control of the latter in an integrating sphere system provides a means for comparing F_0 values and net absorption of light quanta by the sample (I_{ABS}). A block diagram of the IFDAS is shown in *Fig. 3.1*.

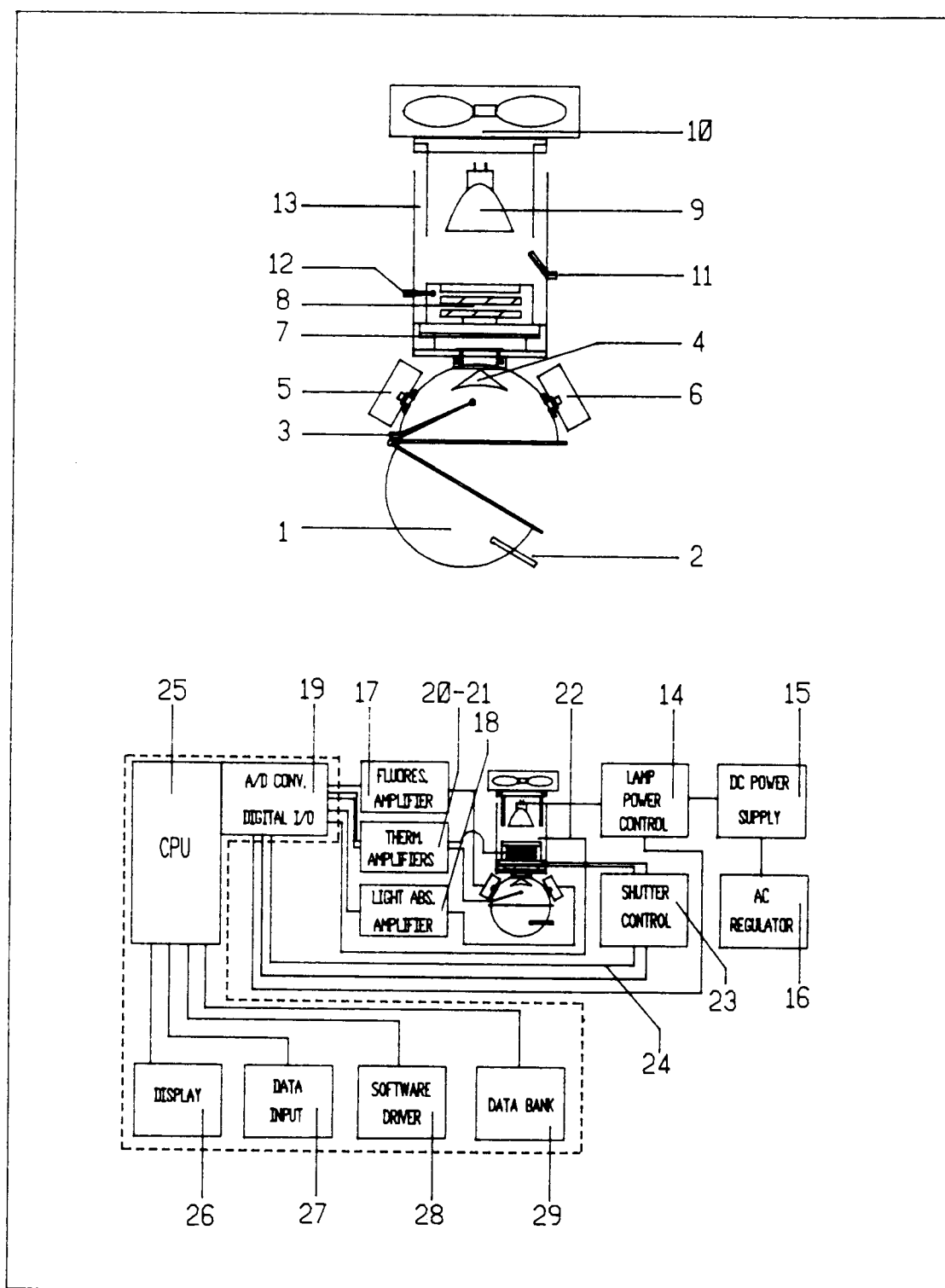


Figure 3.1. Diagram of the Integrating Fluorometer Data Acquisition System (IFDAS). Detailed description is given in the text.

- Hardware components of the system.

Sphere module. For the examples described below, an aluminium sphere of 10 cm inside diameter (1) composed of two separable hemispheres within which the sample is placed. Its diameter is selected on the basis of the size of the samples. Various sphere diameters can be easily mounted to the light source module. Ports on the surface of the sphere accommodate a gas inlet (2) and outlet to provide for atmospheric gas flow through the sphere. A telethermometer (3) monitors air temperature in the sphere to ensure experimental data is collected within proper temperature range. Three other ports accommodate the focussed excitation light beam dispersed by a diffusion cone (4), the fluorescence photodetector (5) and a light level photodetector (6).

Light source module. (13). Air cooled by a fan (10), the light source (9) of intensity monitored by a detector (11) is from a prefocussed Sylvania ELC projector lamp (24 V, 250 W) passing through Corning glass filters (CS 3-71 and CS 4-96) to isolate the excitation wave band between 460 and 620 nm. Both filters are enclosed in a circulating water jacket (8) for protection against heat. An electronic photographic shutter Uniblitz 225L (7), computer triggered, controls the excitation time and the duration of a measurement. A shutter control circuit (23) provides for opening and closing the photographic shutter at user selected intervals. The closed and open positions signal (24) of the shutter blades is assessed by the Central Processing Unit (CPU) (25) which allows

testing of shutter operations. The time required to fully open the shutter is about 3 ms. Light levels applied to the sphere can also be adjusted without opening the shutter by monitoring with the light detector (11). The shutter is controlled *via* the digital output ports (I/O) of the A/D board. The voltage at the lamp is either CPU-controlled or manually adjusted by a powerstat (14) in series with a regulated DC power supply (15) and a constant voltage transformer (16). When using a 10 cm inside diameter sphere, light levels can be maintained at $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ inside the sphere. Excess heat on the filters can be detected by monitoring the electrical output of the temperature probe (12).

Fluorescence and light detector modules. Fluorescence is detected with a photodetector (Devar S-529-02-1). Fluorescence wave bands are discriminated against excitation light using filters as described in Toivonen and Vidaver (1984). A second photodetector (Devar 529-01-1) unprotected by filters detects sphere quantum flux density. Outputs of both photodetector circuits are delivered to high accuracy data acquisition instrumentation amplifiers AD552, (Analog DevicesTM) (17) (18). Both photodetector signals are digitized simultaneously in D.M.A. (Direct Memory Access) mode from two different A/D channels of the A/D board (19). This actually splits the maximal sampling rate (50kHz) in half for each channel. Both telethermometer circuits requiring amplification (20-21) are connected to two separate A/D channels while the light source intensity detection circuit (22) is connected to a fourth A/D channel.

A/D converter. An analog to digital converter (19) MetraByte DASH-16 (MetraByte Corp. Taunton, Mass., U.S.A.) capable of 50 kHz data collection in Direct Memory Access (D.M.A.) mode installed internally in the expansion slot of a PC/AT type of computer (25), converts signals to digital form for computer processing of system control, data acquisition, analysis and storage.

- Data acquisition software.

The computer program designed to operate the instrument and acquire various signals has been developed in BASIC (Microsoft QuickBASIC 4.5). It carries out the following functions:

Control of I_0 . Using the signal from the sphere light level photo-detector (6), I_0 is measured and adjusted to ensure it is at a constant and sufficient level to stimulate usable fluorescence emission from the sample. Measurement of the light levels without the sample, pre-calibrated with a LICOR LI-185A fitted with a quantum flux detector (LiCor Inc, Lincoln, Nebraska), provides an accurate estimate of I_0 . When I_0 falls outside of a preset range, the CPU issues a signal to the operator or automatically adjusts the power supply (14). Units are in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Corrections for dark signal (D_S) and stray light (L_{ST}). D_S and L_{ST} are both measured from the fluorescence photodetector circuit. Data collection is set to start as the shutter is triggered to open. The electrical energization of the shutter coils takes about 1 ms. This allows time to collect enough data points to determine D_S which is

the contribution of the detection circuits in the absence of light in the sphere. An average of the first 15 data points is used to estimate the height above the abscissa. This value establishes D_S (Fig. 3.3) and corrects for offset drifts with changes of gain of the amplifier. With the plant absent from the sphere, the same mathematical method as for the determination of F_O (shown below) is applied to determine L_{ST} . This also corrects for any background light and background fluorescence. D_S is subtracted from L_{ST} ; the latter is directly proportional to I_O (Equ. 3.1, Equ. 3.2):

$$k_{ST} = L_{ST} / I_O \quad (3.1)$$

Therefore,

$$L_{ST} = k_{ST} I_S \quad (3.2)$$

in the presence of a sample in the sphere. The determination of the stray light constant (k_{ST}) is done periodically during a session. The net fluorescence signal can thus be expressed as the following:

$$F(t) = F_{MEAS}(t) - L_{ST} D_S \quad (3.3)$$

where $F_{MEAS}(t)$ is the time course of uncorrected fluorescence signal. $F(t)$ is the net fluorescence signal. Units of $F(t)$ are in mV.

Determination of F_O . F_O is the fluorescence emitted by the sample chlorophylls (minus reabsorption) before the onset of measurable photochemistry and is proportional to the total number of excited

chlorophyll molecules. Except for a small contribution from the early rise in variable fluorescence, the kinetics of the increase in PFD within the sphere during shutter opening and the PFD dependent rise in fluorescence are similar and do not influence the calculation of F_0 appreciably. The F_0 rise-time is about 1 ns. Since the shutter opening time is on the order of 1 ms, the value of F_0 can only be estimated and slightly corrected for the shutter opening time.

Determination of I_{ABS} . I_0 is the quantum flux density in the sphere in the absence of a plant. I_S is the quantum flux density in the sphere in the presence of a plant immediately after shutter opening. The total quantum flux density absorbed by the sample, I_{ABS} is determined by:

$$I_{ABS} = I_0 - I_S \quad (3.4)$$

Units are in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

F(*t*) data collection. To acquire and store the time course fluorescence emission data ($F(t)$) (27), two or more different sampling rates for each sample are required: one fast rate during the initial phase of emission to obtain a F_0 value at $> 10^4$ points s^{-1} and the remaining at much slower rates (1 to 10^2 points s^{-1}) selected by the user and determined by the nature of the information desired.

Normalization of the data points. Once completed, the fluorescence time courses are normalized. The purpose is to remove the contribution of the F_0 component, which is accomplished for every data point of the emission time course using the relationship:

$$F_{\text{VAR}}(t) = \frac{F(t) - F_0}{F_0} \quad (3.5)$$

where F_0 is the extracted value of the initial emission in mV. $F_{\text{VAR}}(t)$ is the relative variable fluorescence emission. Possible changes in the value of F_0 during the course of measurement are not accommodated in this formulation.

Signal averaging. Unlimited numbers of time courses of normalized fluorescence emission can be averaged. The mean and the corresponding S.E. of every point of the averaged curve are determined and displayed. Averaging is accomplished by adding the corrected values $F_{\text{VAR}}(t)$ at each sampling point (t) on the fluorescence emission time course and dividing by the number of sample curves. The S.E. is also calculated for every corresponding sampling point (t) which is used to draw the error band below and above the averaged curve. This allows comparison of regions of averaged curves with same regions of other curves. In this manner, statistical differences between groups of fluorescence curves can be established comparing delimited regions of the curves instead of using isolated parameters such as F_p or F_M (see Fig. 5.5 for nomenclature).

Estimation of the relative amount of Reaction Centers (RC_{II}). From normalized fluorescence data points and the rate of photon absorption by the sample, it is possible to estimate the concentration of RC_{II}. The principle of the method lies in the equivalence between the number of quanta which bring about the fluorescence change and the magnitude of the electron acceptor pool. The formula, first obtained by Malkin *et al.*, (1981) is as follows (Equ. 3.6):

$$RC_{II} = \alpha_2 \phi_2 I_{ABS} t \quad (3.6)$$

where RC_{II} is an estimate of the amount of reaction centers of PS_{II}, α_2 is the fraction of absorbed light directly absorbed by PS_{II}, ϕ_2 is the maximal efficiency of photochemistry in open reactions centers of PS_{II}, I_{ABS} is the rate of light absorption (in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) by the sample and t is the average time of the fluorescence rise or the time to reduce Q_A (F_{MAX}) or the induction time. When measured after a long dark adaption with saturating excitation, F_p fluorescence level could be substituted for F_{MAX} . According to Malkin *et al.*, (1981) and Malkin and Fork, (1981) it is possible to estimate ϕ_2 from the assumption that it is equal to the excitation trapping efficiency in PS_{II} (Equ. 3.7):

$$\phi_2 = 1 - \frac{F_0}{F_{MAX}} \quad (3.7)$$

$\alpha_2 = 0.5$, is a tentative factor of light distribution into PS_{II}. Since the

extinction coefficient of chlorophyll *in vivo* was not determined in the present study, the units of *equation 3.6* are relative. However, since the parameter I_{ABS} can be determined directly, the original equation can be considerably simplified. The reciprocal of RC_{II} will be referred to as the PSU size of PS_{II} .

User instructions. The program provides the user with guidelines for data acquisition, processing, and analysis steps and tutorials for overall operation of the system (28).

Reference to a data bank. Newly acquired data (27) can be compared with previously stored data (29) and evaluated in relation to established responses of particular species or varieties under conditions similar to the test conditions of the sample plant, for examples: development stage, stress levels, viability, photoinhibition and physiological inactivation of photosynthesis. This is done either visually or with a software program which compares new data with data stored in library-files containing $F_{VAR}(t)$ curves previously interpreted and known to be relevant to the actual test.

Display of $F_{VAR}(t)$ data. Any previously collected data can be plotted onto the screen monitor and/or either produced on a hard copy from a pen plotter or printer (26). The values of F_O , I_{ABS} and other parameters accompanying $F_{VAR}(t)$ data can be retrieved and printed.

Automation of data collection operations. The program has been designed to conduct all the operations listed above with a minimum of user intervention.

3.2.2 Gas exchange.

A schematic outline of the gas exchange assessment system is shown in *Fig. 3.2*. Filtered compressed air containing 1.5% O₂ (1) was bubbled in a thermoregulated bath (2) of distilled water (3) to increase its water vapor pressure as measured by a dew point hygrometer (4), (EG&G, Cambridge Instruments Inc., Massachusetts). RH of the air stream in the gas exchange cuvette was controlled to remain at 90-95%. The air flow through either branch is controlled by a needle valve (5) (6). The rates of air flow entering the cuvette were measured with a mass flow meter (Aalborg, AFM 2600-PRO) (7). The temperature at the leaf level was measured with a telethermometer (8) with a probe (YSI-42SC) (9) inserted in the gas exchange cuvette. For both the reference and analytical air streams, the moisture was removed before entering the IRGA by passing it through desiccators (10) (11), test tubes immersed in an acetone-dry ice bath (-60°C). CO₂ exchange rates in the light and in the dark were measured with an IRGA (12), (ADC-225 MK3, The Analytical Development Co., Ltd., England.) in an open system configuration. The gas exchange cuvette (13) was fitted inside the sphere of the fluorometer to carry out both assessments simultaneously. A plantlet with its basal end immersed in a 2 ml narrow neck glass vial (14), was inserted in the gas exchange cuvette. Incident PFD (350 μmol photons m⁻² s⁻¹) was measured inside the sphere with the gas exchange cuvette but in the absence of plantlets. Electrical signals from all instruments were collected by an analog to

digital converter (A/D) card (15) installed inside a PC/AT type computer (16). The temperature inside the cuvette remained unchanged during measurements. Respiration from the roots, when present, could not significantly affect the measured rates of CO₂ exchange of the upper parts due to the slow diffusion of gases from water. The rates of gas exchange are reported as in *Equ. 2.1* and normalized to I_{ABS} .

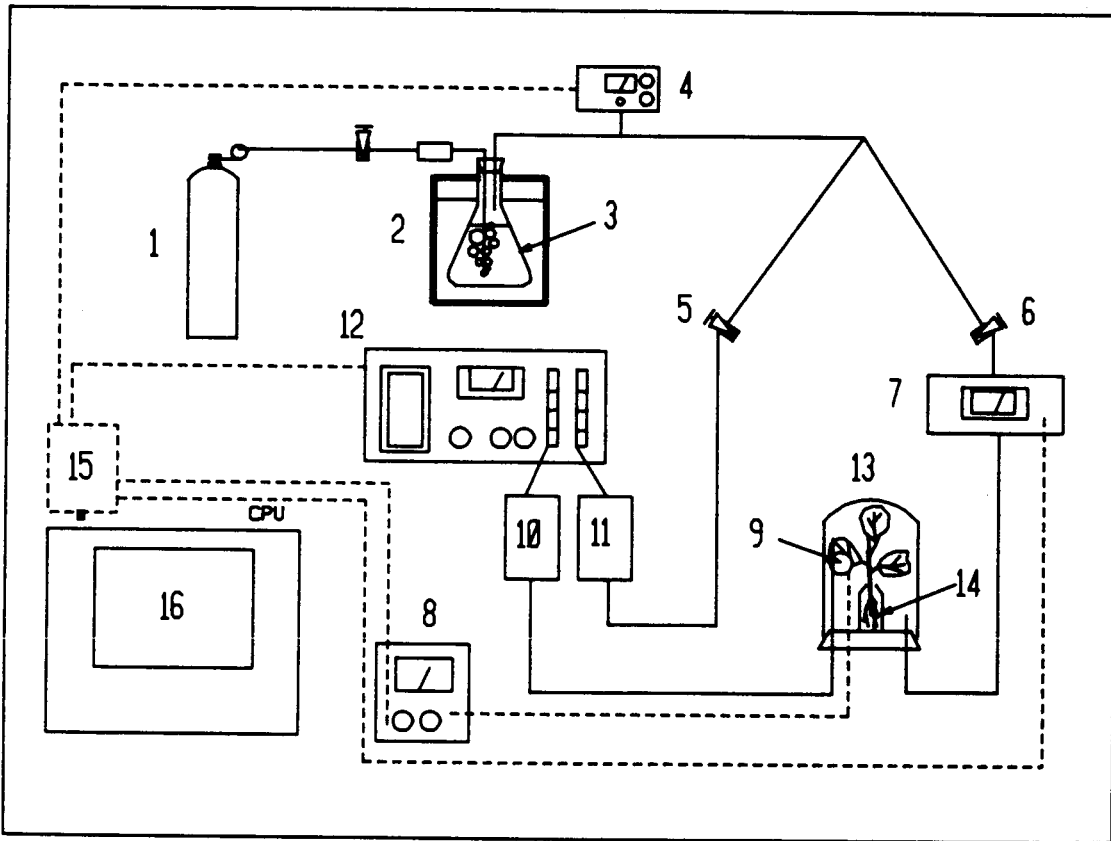


Figure 3.2. Diagram of the Gas Exchange Assessment System. Solid lines represent gas connections for CO₂ exchange measurements and dash lines represent electrical connections between instruments and A/D card. A detailed description is given in the text.

3.2.3 Growth and water status measurements

Immediately after fluorescence and gas exchange measurements, the fresh weight (FW) of the plantlets was measured with an analytical balance. Total leaf area dimensions were calculated from the weight of photocopied images of the fresh leaves. Dry weight (DW) measurements consisted of measuring the mass of the leaves and shoots 12 h after they were removed from an oven (85 °C for 36 h). Total dry weight (tDW) consisted of the addition of the leaves dry weight (lDW) and the shoot dry weight (sDW). Relative water content (RWC) of leaf tissue was calculated as:

$$\text{RWC} = (\text{IFW} - \text{lDW}) / \text{IFW} \quad (3.8)$$

according to Havaux and Lannoye, (1983). Results are reported on a natural log ($\log_{2.718}$) scale.

3.2.4 Statistical analysis

Results from most experiments of this project were analyzed using Generalized Linear Interactive Modelling (GLIM) developed by the Royal Statistical Society. The process of model fitting and statistical modelling depicted by GLIM is an iterative process. After an initial model is developed, usually fitted to a regression line (e.g. $Y_i = \mu + \beta_0 + \beta_1 X_i + \epsilon$), residuals are plotted, model assumptions checked and parameter estimates examined. Transformations of the original data *i.e.* from DW to $\log_{2.718}(\text{DW})$ can be made to minimize the error term " ϵ ". The coefficients

(β_0 , β_1 , etc.) or variables were included in a model if their significance determined by the deviance drop from a less precise to a more precise model was tested with calculation of the **F** statistics. When a straight-line regression model was not adequate, the data are fitted to a curved trend model (e.g. $y = \alpha + bx + cx^2$). This is known as a quadratic curve where the term " cx^2 " describes the possible deviation from linearity (Altkin *et al.*, 1989; Payne, 1987)

3.3 RESULTS

3.3.1 Determination of F_O and I_{ABS}

A double regression algorithm applied to the initial fluorescence signal rise provides a consistent estimation of F_O . This is achieved by solving two regression line equations and estimating the value of the point where both lines meet (*Fig. 3.3*). This intersection is an estimate of F_O . Once F_O is obtained its value is stored for subsequent data processing. The slope of the second regression line is stored for further analysis since it refers to the rate of reduction of the Q_A pools. Measurement units of F_O are in mV. In *figure 3.4*, two different methods for estimating F_O are shown. The method of line intersection indicates more consistent results when the plant material is subjected to conditions that alter the flow of electrons in the electron transport chain. A comparison between I_{ABS} and F_O as estimators of sample size is shown in *figure 3.5*. I_{ABS} has a higher correlation with shoot fresh weight than does F_O and therefore a better estimate of plant size.

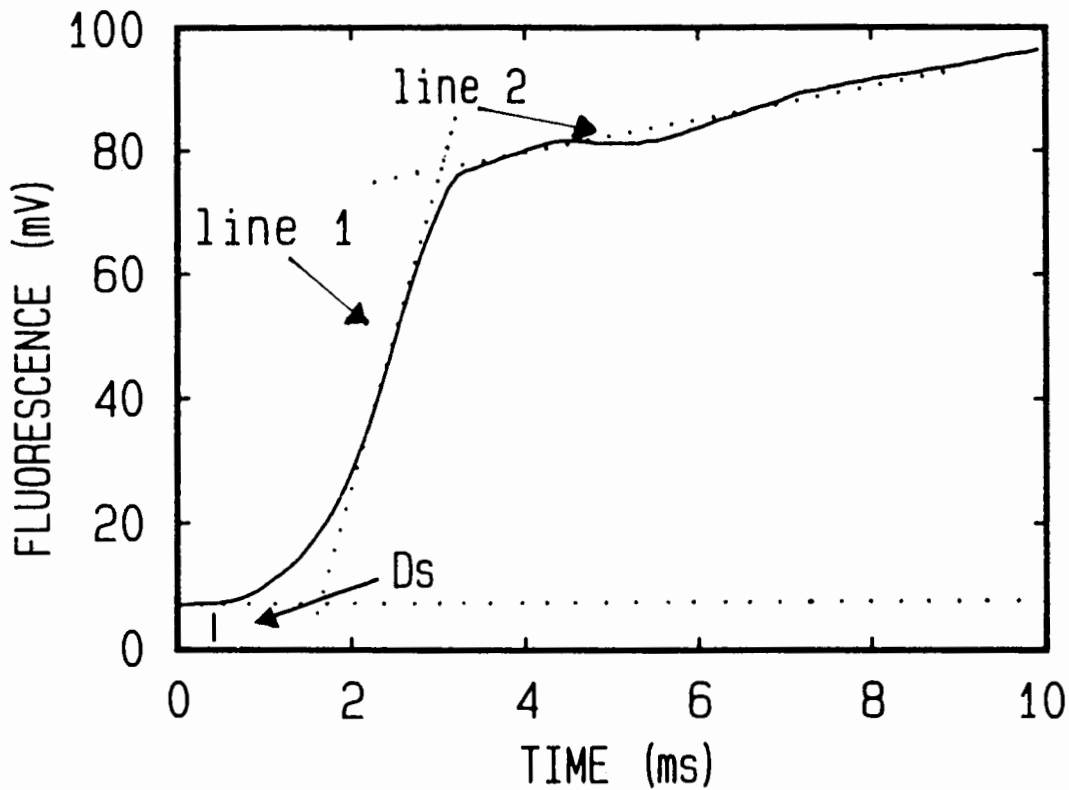


Figure 3.3. Determination of F_0 . Leaf discs were punched of trifoliolate leaves of kidney bean plants *Phaseolus vulgaris* L. cv. Top Crop and floated in HEPES buffer solution for one hour in darkness. The discs were held on the tip of a needle placed in the center of the sphere. Rate and duration of data collection for F_0 determination were set to collect at 25 kHz for 10 ms. Line 1 represents data points of the maximum rise of fluorescence due to shutter opening while line 2 reflects the rate of reduction of Q_A pools. The point where those two lines meet is used the estimate of $F_0 + D_s$. The contribution of L_{ST} to the fluorescence rise is not shown in the figure.

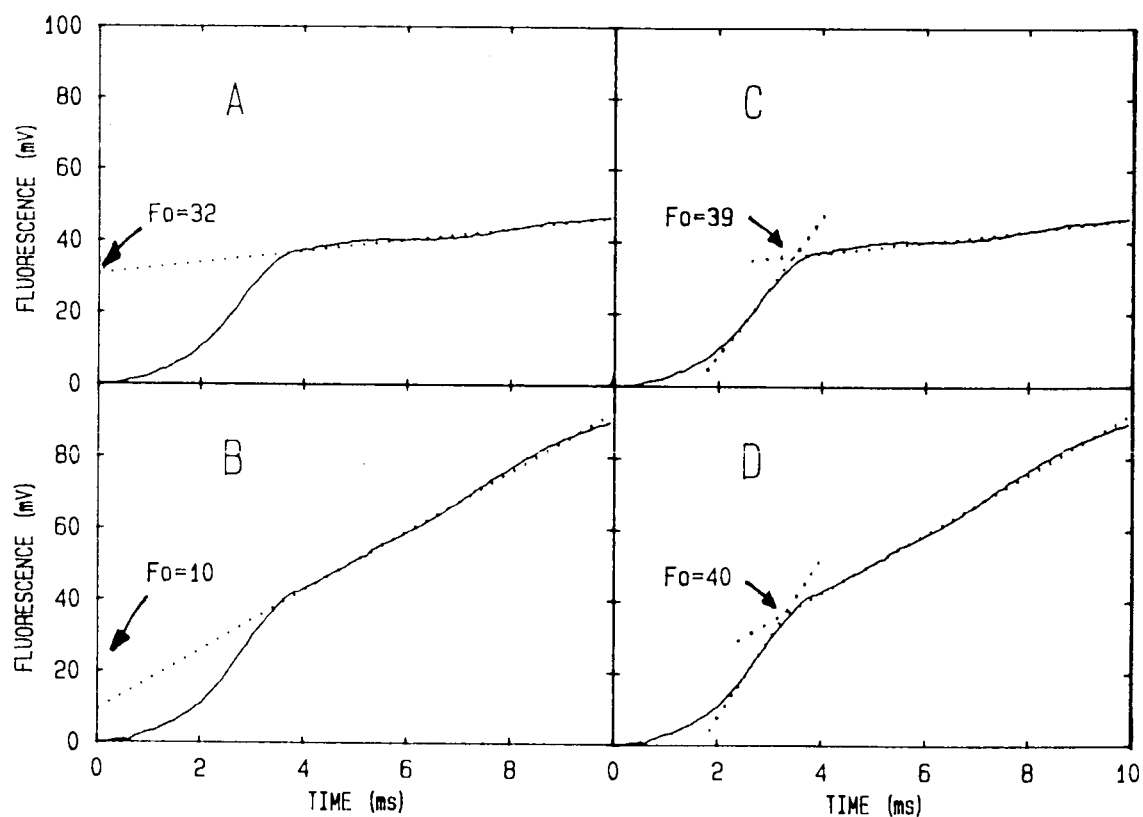


Figure 3.4. Comparison of two methods for the estimation of F_0 . Plant material was treated as in *figure 2*, except for discs in *figure 3B* and *3D*, where DCMU was added to the buffer solution prior to floating the discs. Estimates of the inflection point at the time the shutter is completely opened (C and D) are preferred over the extrapolation method (A and B) since with DCMU the increased rates of Q_A reduction would cause an underestimation of F_0 . The kinetics of the electron transport chain are immediately reflected as shown in the F_0 rise when DCMU is present.

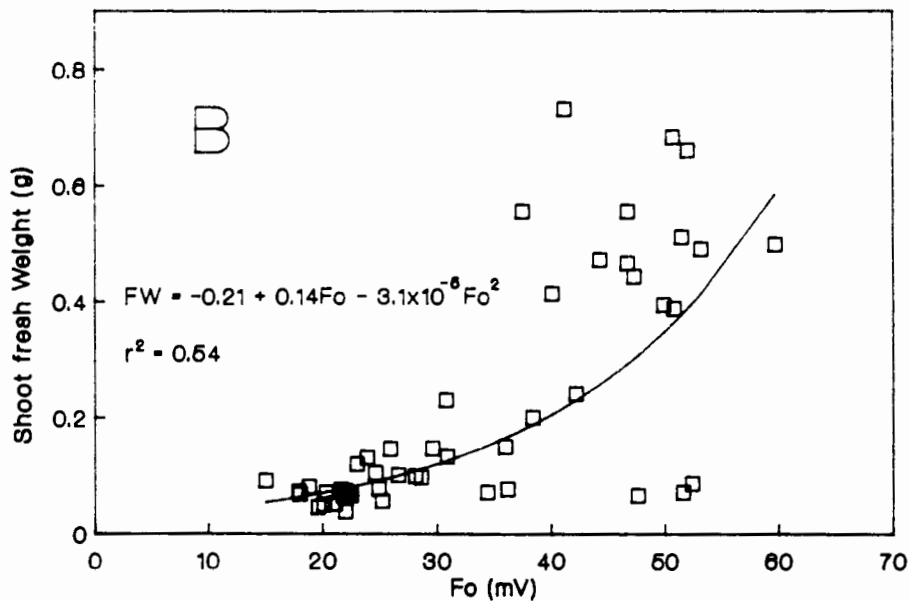
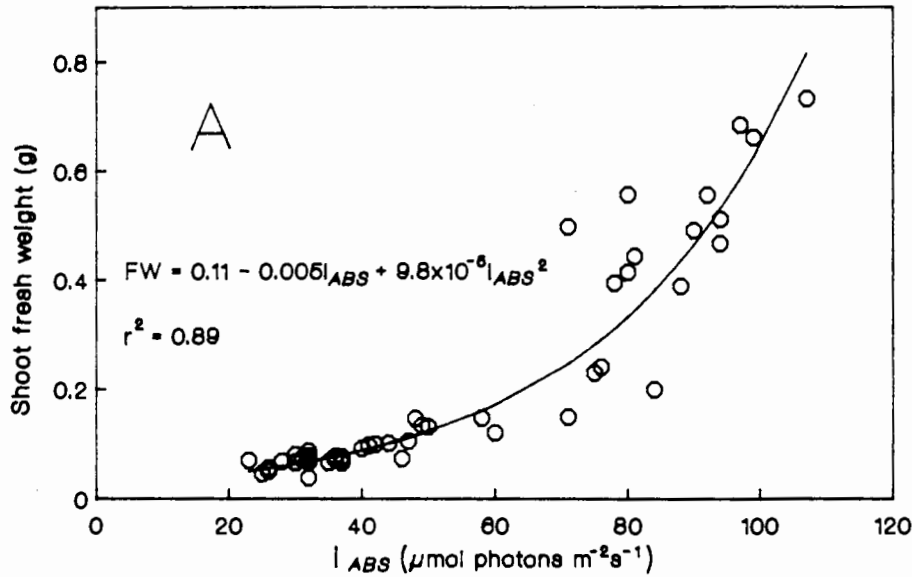


Figure 3.5. Regression analysis and correlation coefficients for determining plant mass. Comparison is made between estimates of I_{ABS} (A) with estimates of F_o (B) in order to determine plant fresh weight. Both parameters were measured simultaneously using tissue-cultured plantlets of *Chrysanthemum X morifolium* Ramat. (cv. Envy).

3.3.2 Examples of application of the IFDAS

The absorption of light in excess of what can be dissipated by energy conversion processes such as carbohydrate synthesis, heat and fluorescence, may result in a reduction in light-limited and light-saturated CO₂ uptake and by a concomitant decrease in the rate and photon yield of electron transport. Long term exposure to excess light can result in photodestruction of photosynthetic pigments (Powles, 1984; Krause, 1988).

Application of an integrating fluorometer which can accommodate whole shoots of conifer seedlings integrates the fluorescence signal from all needles. It minimizes the response extremes from older to younger parts of the shoot and provides averaged responses therefore giving a coherent indication of the status of the whole plant. Reversible inactivation of photochemical water splitting has been linked to the disappearance of $F_{VAR}(t)$ in response to stress or other environmental variables (Toivonen and Vidaver, 1988; Vidaver *et al.*, 1988). Figure 3.6 shows an example of $F_{VAR}(t)$ responses to combined cold temperatures and light of nursery grown conifer seedlings. Values of F_p level from seedlings treated with light (Fig. 3.6C) were much lower than values from seedlings treated in the absence of light (Fig. 3.6D). Values of the ratio F_O/I_{ABS} remained identical between the treatments (data not shown).

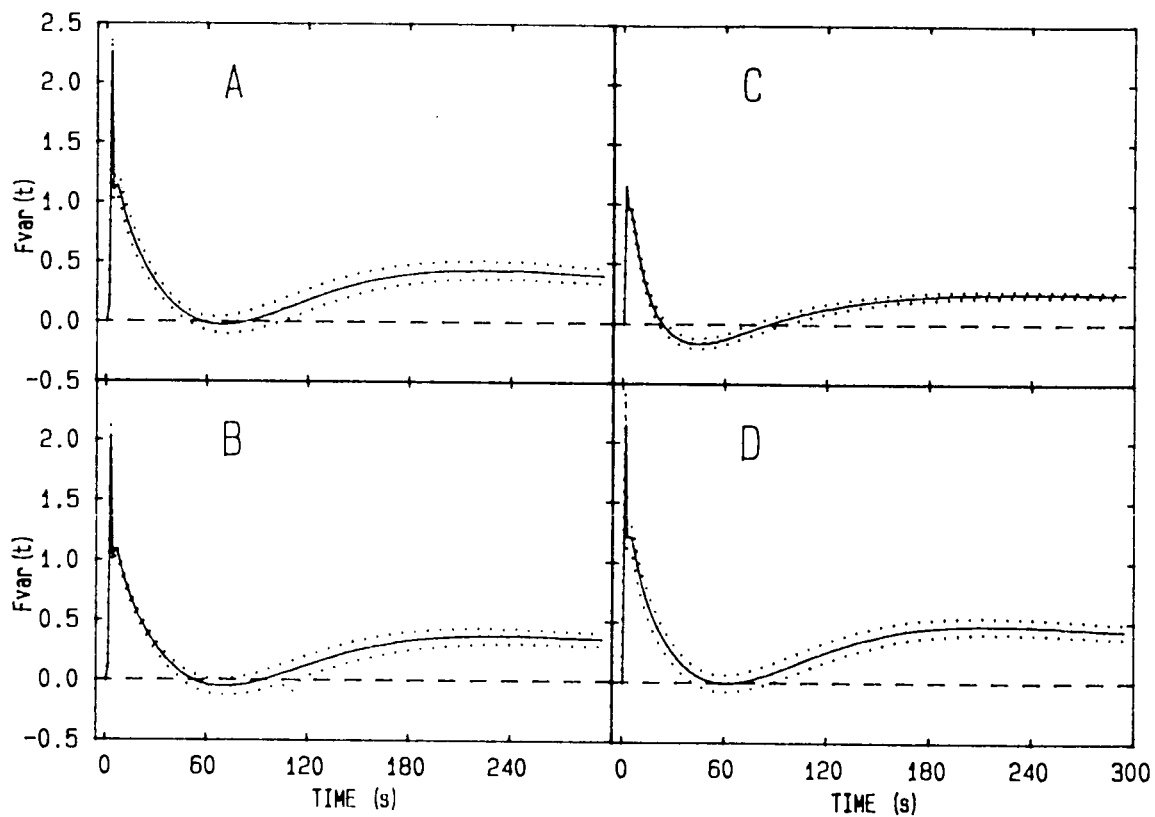


Figure 3.6. Photoinactivation of photosynthetic activity of nursery grown conifer seedlings. Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings were analyzed before (A) and after (C) a treatment of $450 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 2°C for 100 min. Another set of seedlings were measured before (B) and after (D) the same treatment without exposure to light. Every curve is an average of fluorescence signals from different samples ($n=5$). The dotted lines represent the upper and lower limits of the S.E. of the mean calculated for every point along the averaged fluorescence curve. Values of the ratio F_O/I_{ABS} were not different between the treatments (not shown).

3.4 DISCUSSION

The techniques of $F_{VAR}(t)$ assessment provide a reliable means for determining how photosynthetic activity is affected by experimental and environmental conditions. Integrating sphere fluorometers provide for repeated sampling of individual, intact plants which permits the study of effects of almost any variable on any population of samples over any period of time. The problem of using samples of different sizes (e.g. number of excited chlorophylls) is overcome by using a normalization procedure based on an adequate and reliable estimation of the parameter F_O . Determination of light quanta absorbed provides tools for monitoring changes in growth of the samples in situations where repeated measurements are made over time. The ratio F_O/I_{ABS} can also provide insights to the early events of photosynthetic reactions in intact plants, it thus allows normalization of F_O to light quanta absorbed. It is therefore easier to follow changes in F_O being affected by energy quenching phenomena. It can increase or decrease depending on treatments while I_{ABS} remains relatively constant over the course of fluorescence measurement.

3.5 CONCLUSION

The fluorometer should extend the usefulness of chlorophyll fluorescence assessment to laboratory and field studies in ecophysiology, agriculture, forestry and horticulture. The IFDAS is useful in assessing photosynthetic activity in individual plants or organs at intervals of from a few seconds to days, months or even years. Care should be taken when

using samples of various geometry or extreme sizes since folding of leaves or large leaves would modify light distribution patterns around the sample and induce an altered quantum yield of fluorescence. Very large samples would affect the kinetics of fluorescence in reducing the amounts of light in the sphere (I_s) which affect the rates of photochemistry. A similar description of the same fluorescence apparatus is being published by Dubé and Vidaver, (1990).

Measuring light quanta absorbed by the plants allows the study of the different energy dissipation processes. When interfaced with an IRGA, the fluorometer system can potentially provide information of quantum efficiency versus energy dissipation rates and processes. Automation of fluorescence data acquisition with the application of a microcomputer interfaced to adequate software greatly extends the usefulness of chlorophyll fluorescence assessment to laboratory and field studies in many applications of plant physiology.

CHAPTER 4

PHOTOSYNTHESIS AND GROWTH OF PLANTLETS GROWN WITH VARIOUS AMOUNTS OF EXOGENOUS SUCROSE

4.1 INTRODUCTION

It is not clear how and to what extent exogenously supplied sugars are beneficial for growth and development of *in vitro* plantlets. Most media formulations for micropropagation protocols require at least 2% sucrose. It is well known that the high levels of sugar, especially sucrose, in the medium repress chloroplast development and chlorophyll synthesis. Incubation in 2% sucrose, of *in vivo* excised cotyledons of *Sinapis sp.* caused a reduction in chlorophyll level and reduced CO₂ fixation (Moore *et al.*, 1974). In cell culture systems, deleterious effects of sucrose are also reported; however in most cases cell cultures grow faster in a sugar supplemented medium (Bender *et al.*, 1987; Neumann and Bender, 1987). In carrot tissue cultures chlorophyll synthesis is suppressed by 3% sucrose (Edelman and Hanson, 1972). Gradual decreases of sucrose in the medium failed to induce photoautotrophy in rose shoots (Langford and Wainwright, 1987). The inability to grow photosynthetically active cultured tissues on a sugar-free medium supports the argument that synthesis of sucrose by means of photosynthesis is limited in tissue cultures. Factors other than simple organic or mineral nutrients seem to be limiting. The gaseous atmosphere of the culture environment, because of the nature of the closure, is certainly altered by the metabolism of the plants either by exhaustion of CO₂ in the light (Fujiwara *et al.*, 1987; Infante, *et al.*, 1989; Kozai *et al.* 1986; Pospisilova *et al.*, 1988; Solarova, 1989), potentially

accompanied by O₂ build up in the light. This latter condition is detrimental to photosynthetic pigments as it leads to photodamage (McHale *et al.*, 1987). Effects of O₂ concentrations on photosynthesis during callus tissue growth (McHale *et al.*, 1987; Smolov *et al.* 1983) and in plantlets (Shimada *et al.* 1988) were also investigated. Increasing O₂ levels produces a decline in the rate of net photosynthesis, and this inhibitory effect diminishes as CO₂ levels are increased.

Efforts to induce photoautotrophic development in tissue cultures *in vitro* have been successful only recently. In most studies reported, photoautotrophic tissues were obtained by disturbing the atmosphere surrounding semi-sealed culture tubes with CO₂ enriched air and increasing photon flux density (PFD) to above 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Infante, 1989) and completely removing sucrose from the medium, (McHale, 1985; Kozai *et al.*, 1987). However, in all studies mentioned above, even when the culture tubes were placed in a CO₂ enriched atmosphere, gas exchange was still restricted by a semi-sealing stopper. Under high PFD, plantlets inside the vials were capable of depleting CO₂ to levels below 200 $\mu\text{l l}^{-1}$ during the photoperiod (Kozai *et al.*, 1986). Their results indicated that a sucrose supply, although not necessary, was still beneficial to plantlet growth.

To understand the effects of sugars in the medium on photosynthesis and plant growth, a more desirable approach is to supply plantlets with non-limiting amounts of CO₂. An experimental setup was designed whereby CO₂ concentration was continuously monitored and adjusted according to plantlets' demand while they were grown on various amounts of exogenously supplied sucrose and under high PFD.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

To determine the combined effects of PFD, sucrose and the gas atmosphere on their development *in vitro*, shootlets were individually cultured on 10 ml of gelled medium containing different concentrations of sucrose, 0, 5, 20, 40, 80 g l⁻¹, corresponding to 0%, 0.5%, 2%, 4%, 8% respectively. The shootlets were distributed amongst six treatments where PFD was gradually increased over the course of the experiment (Varied) or kept constant at ca. 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Low) as indicated (Tab. 4.1). Growth PFD, in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, was set at ca. 150 for the first 3 days; ca. 200 for the next 4 days; ca. 320 for the next 5 days; and finally lowered to ca. 280 for the last 3 days. RH in both chamber units was controlled as shown in *figure 2.1*.

Table 4.1. List of treatments for the study of combined effects of sucrose and *in vitro* growth conditions. For all treatments, shootlets were cultured on gelled media containing various concentrations of sucrose. They were grown under AGEHS (less the hydroponic system) conditions or under conventional tissue culture conditions.

GROWTH VIAL*	PFD	AIR EXCHANGE	SYMBOL
Chamber Unit	Varied	Normal	CU-VN
Chamber Unit	Varied	Enriched	CU-VE
Culture Tube	Varied	KimKap	CT-VK
Culture Tube	Low	KimKap	CT-LK
Culture Tube	Varied	Sealed	CT-VS
Culture Tube	Low	Sealed	CT-LS

* within each treatment sucrose was 0%, 0.5%, 2%, 4%, 8% and 3 replica per treatment-sucrose combination

4.2.2 Physiological assessments

On the 15th day, plantlets were removed from their growth environment and individually analyzed for their photosynthetic ability concurrently with *in vivo* chlorophyll *a* fluorescence and rates of CO₂ gas exchange. Plantlet leaf fresh weight (IFW) and leaf dry weight (IDW) were determined. Total dry weight (tDW) was obtained from the addition of IDW and stem dry weight (sDW). Relative water content (RWC) was calculated using the following equation:

$$RWC = (tFW - tDW) / tFW \quad (4.1)$$

To gain precision in the statistical analysis and modelling, the values RWC, tFW, tDW were all transformed and reported on a natural log scale ($\log_{2.718}$). The values of the abscissa were determined by calculating the natural log of the sucrose concentration, $\log_{2.718}(\text{SUC}\% + 1)$.

For the purpose of analyzing the effects of sucrose and other treatments on the time (*t*) it requires for $F_{\text{VAR}}(t)$ to reach the nul value, $F(t)$ was normalized to F_{T} using:

$$F_{\text{VAR}}(t) = \frac{F(t) - F_{\text{T}}}{F_{\text{T}}} \quad (4.2)$$

where F_{T} is the extracted value of $F_{\text{VAR}}(t=300)$ in mV (refer to *Equ.* 3.3). This time (*t*) feature expressed in seconds after the start of the fluorescence kinetics induction indicates the relative rate of overall fluorescence quenching.

4.3 RESULTS

4.3.1 Effects of sucrose concentration

In all test tube treatments (*Fig. 4.1B and 4.1C*), tDW increased with increasing sucrose concentration but sucrose became inhibitory when supplied above 2% in both the CU-VE and CU-VN exchange treatments. (*Fig. 4.1A*). Both photosynthetic rates and respiration rates are strongly affected by sucrose. Plantlets exposed to high light levels and some air exchange showed a drastic inhibitory response with increasing sucrose. With AGEHS conditions, photosynthetic rates are adversely affected with any concentration of sucrose and almost reach net respiration in the light in plantlets grown with 8% sucrose (*Fig. 4.2A*). When the culture tubes are semi-sealed or sealed, the trends in photosynthetic rates are reversed until sucrose concentrations reach 0.5% and 2% respectively; after the 2% threshold, photosynthesis declines rapidly (*Fig. 4.3A*). *Figure 4.2B* shows a reduction in respiration rates of plantlets grown under AGEHS with increases of sucrose. Almost opposite trends are found when plantlets are grown in culture tubes until sucrose concentration reaches 4%, after which respiration starts to be inhibited by sucrose (*Fig. 4.3B*). Relative water content (RWC) slightly decreases as sucrose concentration increases in semi-sealed or sealed culture tube treatments (*Fig.4.4B*). This effect of sucrose on RWC is not observed in enhanced air exchange treatments (*Fig.4.4A*). The relationship between F_p -level chl a fluorescence (F_p) and sucrose concentration is not exactly linear (*Fig.4.5A and 4.5B*). F_p declines from a control level ($F_p=3-3.5$), (refer to *Fig. 6.1*) as sucrose concentration is raised in AGEHS treatments but gradually increases to reach an optimal level at 2% sucrose and declines afterward when plantlets are grown in

culture tubes (Fig. 4.5B). The fluorescence quenching characteristics are strongly affected by sucrose (Fig 4.6A and 4.6B). Generally speaking, increasing sucrose in the medium increases the time required for $F_{VAR}(t)$ to be equal to zero. However, in that respect, little sucrose ca. 0.5%-2%, appears to be an optimal amount for plantlets grown in culture tubes (Fig. 4.6B). In practice, values of t could not be greater than 300 s, as it is the assay protocol time limit for the duration of fluorescence kinetics measurements.

TOTAL DRY WEIGHT OF SHOOTLETS

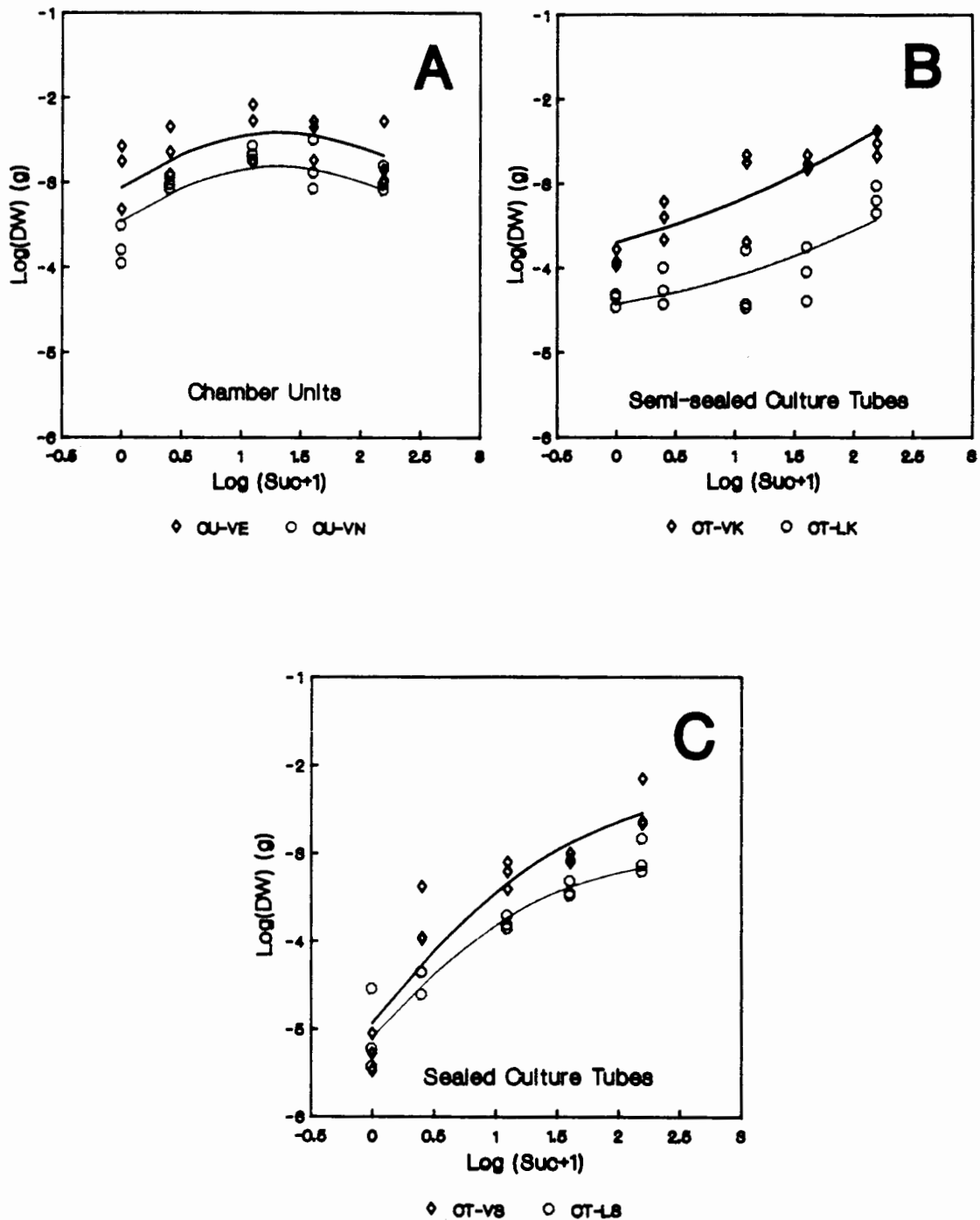


Figure 4.1. Total dry weight of shootlets. Both the tDW and sucrose concentration are expressed on a log scale. Comparison is made between shootlets grown under AGEHS conditions (less hydroponic system) with either CU-VN or CU-VE and conventional tissue culture conditions; semi-sealed (KimKap) or sealed culture tubes under varied PFD respectively (CT-VK, CT-VS) or low PFD (CT-LK, CT-LS).

PHOTOSYNTHETIC RATES

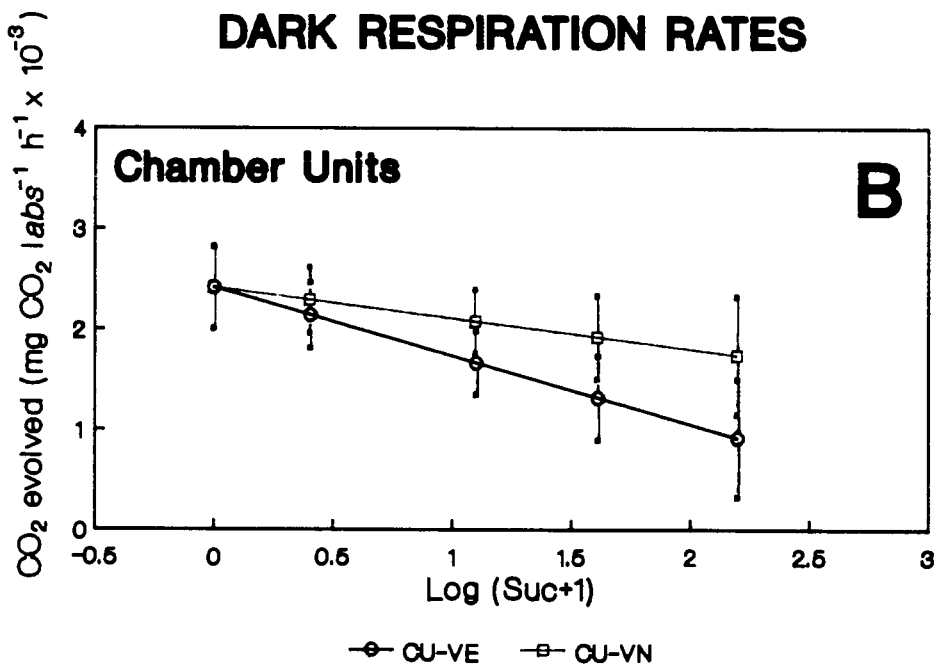
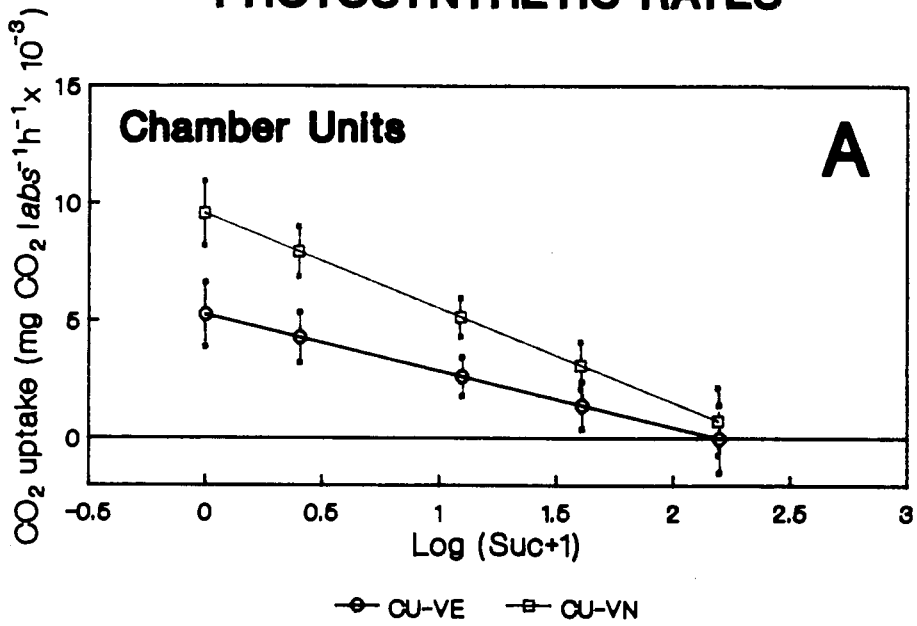


Figure 4.2. CO₂ uptake and dark respiration rates of shootlets grown under AGEHS conditions, CU-VE and CU-VN treatments. Methods of assessment are described in *chapter 3*.

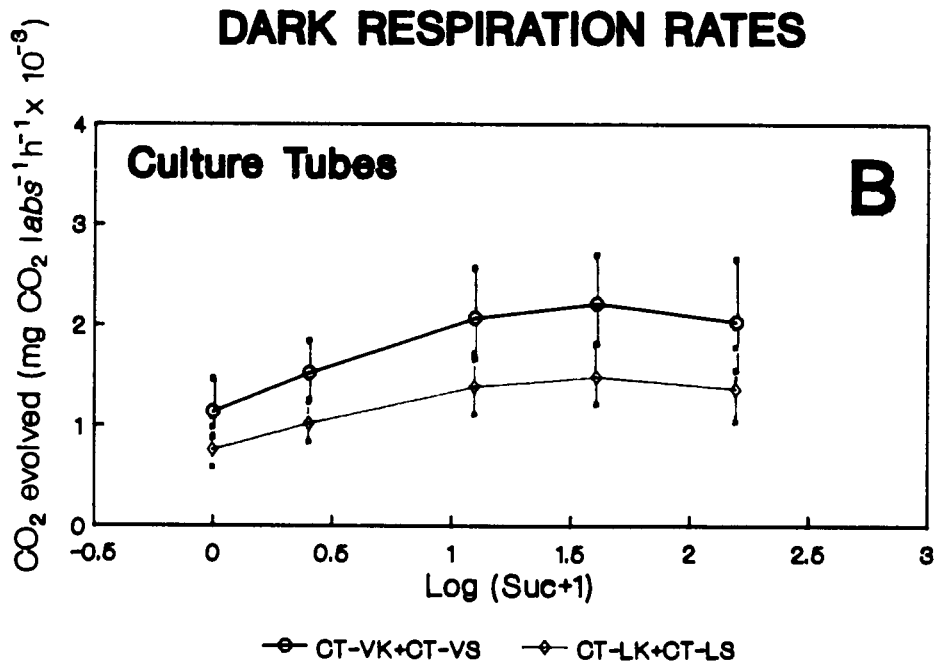
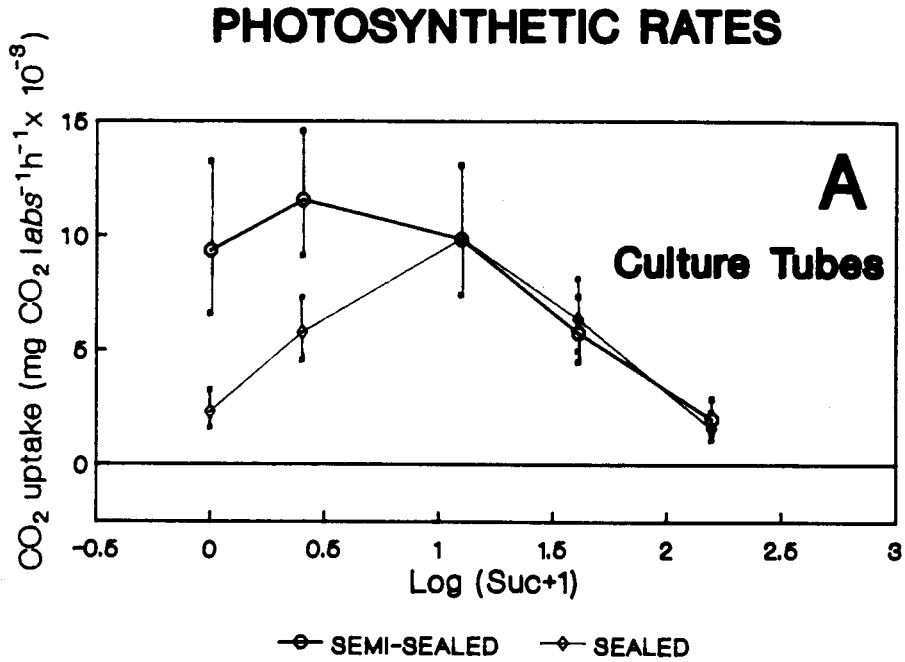
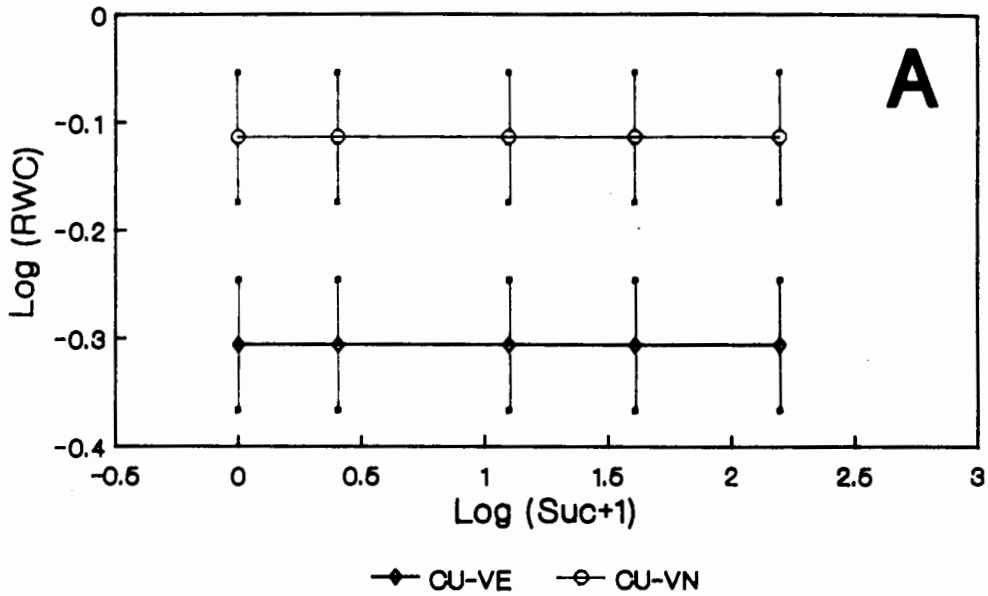


Figure 4.3. CO_2 uptake and dark respiration rates of shootlets grown in culture tubes. The culture tubes were semi-sealed (KimKap) or sealed and placed under varied PFD respectively (CT-VK, CT-VS) or low PFD (CT-LK, CT-LS). Methods of assessment are described in *chapter 3*

RELATIVE WATER CONTENT OF LEAVES Chamber Units



RELATIVE WATER CONTENT OF LEAVES Culture Tubes

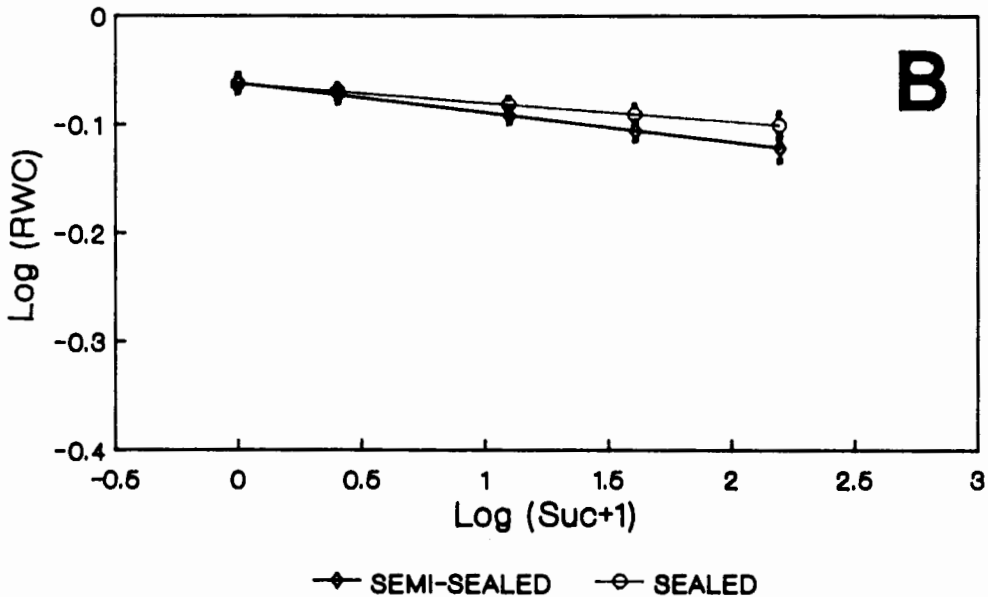
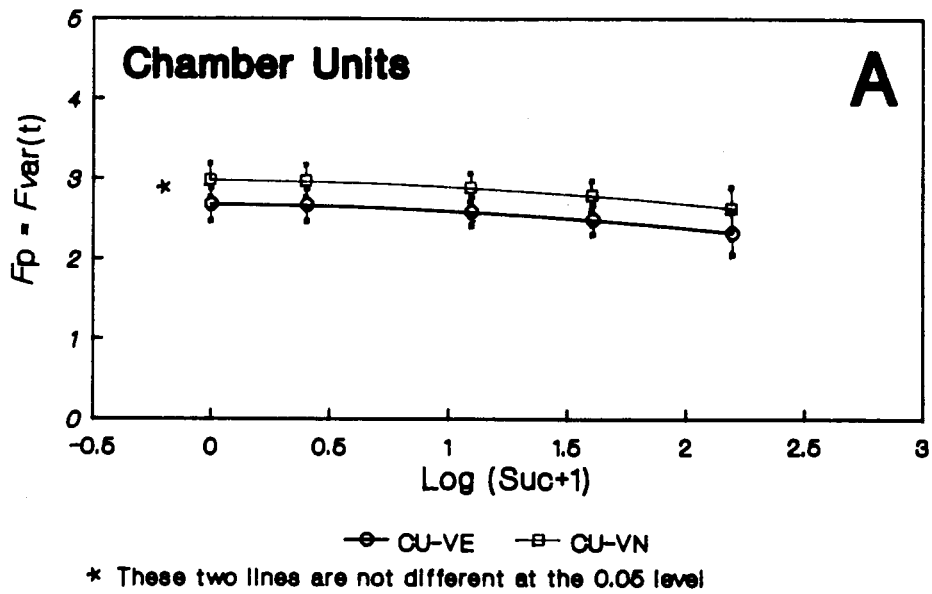


Figure 4.4. Relative water content of shootlets grown under AGEHS (less hydroponic system) and culture tube conditions. The culture tubes were semi-sealed (KimKap) or sealed and placed under elevated PFD respectively (CT-VK, CT-VS) or low PFD (CT-LK, CT-LS). RWC was determined according to equation 4.1.

F_p FLUORESCENCE



F_p FLUORESCENCE

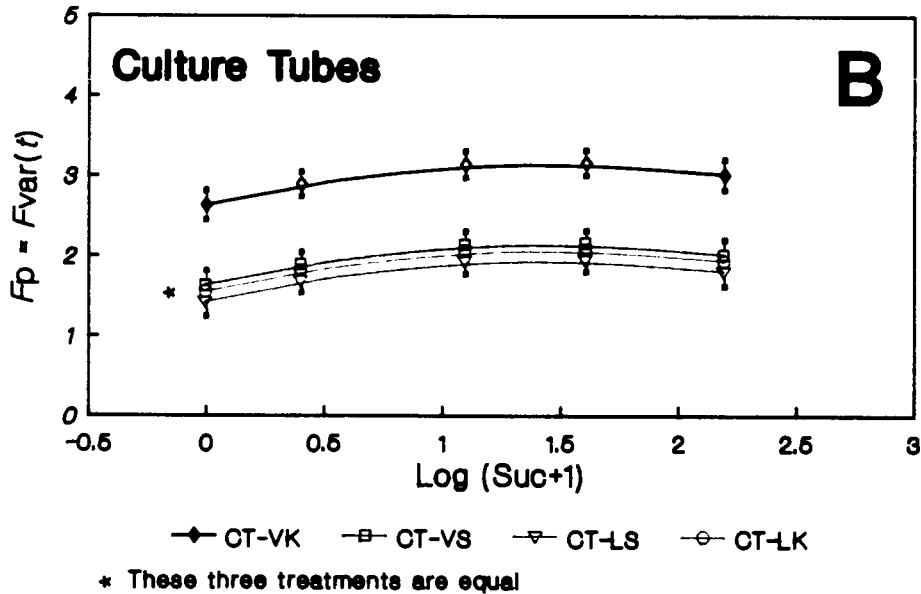
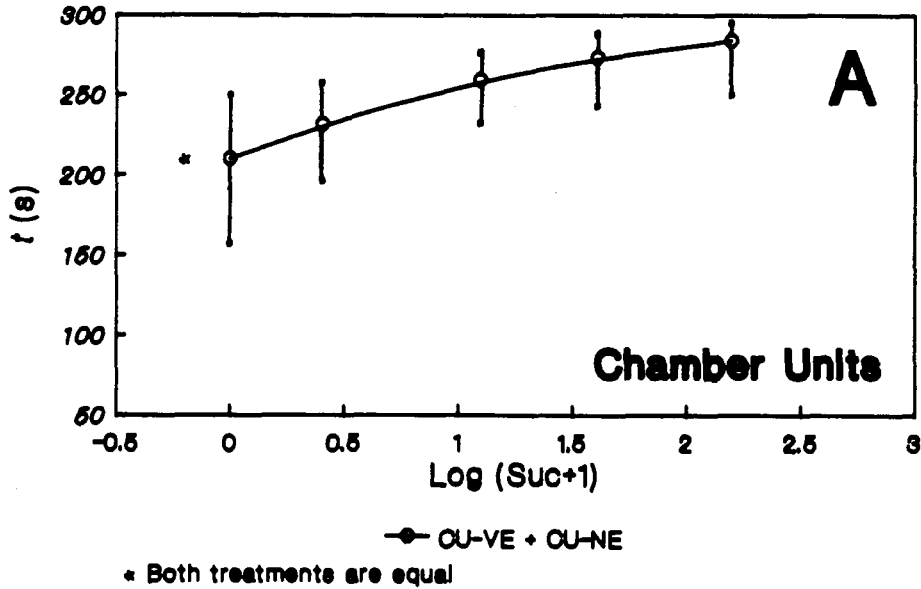


Figure 4.5. F_p fluorescence characteristics of shootlets grown under AGEHS (less hydroponic system) and culture tube conditions. The culture tubes were semi-sealed (KimKap) or sealed and placed under varied PFD respectively (CT-VK, CT-VS) or low PFD (CT-LK, CT-LS). Fluorescence induction kinetics measurements were made according to methods described in *chapter 3*.

TIME TO REACH $F_T = 0$



TIME TO REACH $F_T = 0$

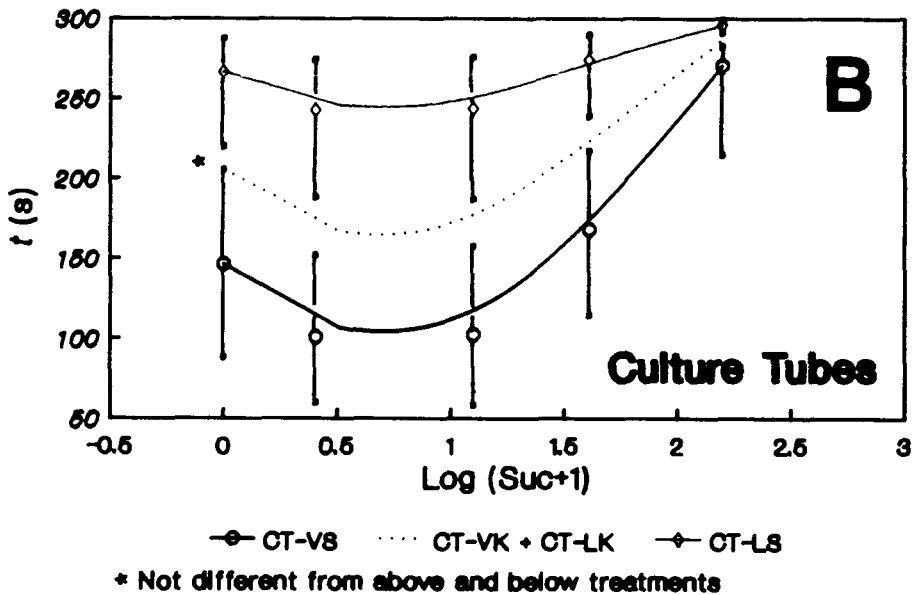


Figure 4.6. Time to reach $F_T=0$. Time t to reach the abscissa of the normalized fluorescence from shootlets grown under AGEHS (less hydroponic system) and culture tube conditions. The culture tubes were semi-sealed (KimKap) or sealed and placed under varied PFD respectively (CT-VK, CT-VS) or low PFD (CT-LK, CT-LS). Fluorescence induction kinetics measurements were made according to methods described in *chapter 3*.

4.3.2 Effects of growth photon flux density (PFD)

The effects of PFD could not be tested between both AGEHS treatments. However, elevated (varied) PFD increases growth of plantlets mainly when the culture tubes are not sealed (*Fig. 4.1B*). Sucrose does not alter this difference at almost any concentration except when the vials are sealed and the benefits of elevated PFD appear at higher sucrose concentrations. Photosynthetic rates are not affected by the PFD under which plantlets were grown. However respiration rates are higher when the plantlets are grown under higher PFD. This difference is not uniformly distributed amongst sucrose treatments as seen from the different curvatures of the regression lines. It is more significant when sucrose reaches 4% (*Fig 4.3B*). The effects of elevated PFD on F_p can only be observed in plants grown in semi-sealed culture tubes. F_p is back to control levels with the combined effects of elevated PFD and 2% sucrose. In sealed vials, at either PFD, F_p remained fairly low (*Fig. 4.5B*). The time t required to reach $F_T=0$ is considerably increased when plantlets are grown under low PFD compared to values of t from plantlets grown under high light (*Fig. 4.6B*). Differences in t due to light could not be observed when plantlets are grown in semi-sealed vials.

4.3.3 Effects of gas exchange

As seen by the absence of sucrose in the medium, the major differences in growth appear when gas exchange is favored in the vials. When the culture tubes are sealed (*Fig. 4.1C*), the plantlets do not grow without sucrose. Intermediate tDW values are found when the vials are

semi-sealed. However, growth is much improved when gas exchange is controlled and is only slightly affected by the presence of sucrose as seen by the gentle curvature of the regression line (*Fig. 4.1A*). Photosynthesis is strongly affected by the type of closure of the culture tubes. When no sucrose is supplied photosynthesis of plantlets is almost four times higher when grown in unsealed culture tubes. Respiration rates were not affected by the type of culture tube closure. The RWC of plantlets is affected by the type of closure of the culture tubes only when sucrose concentration reaches 4% and beyond (*Fig. 4.4B*). It is difficult to isolate the effects of gas exchange on F_p fluorescence in culture tube treatments as those effects are combined with those of growth PFD (*Fig. 4.5B*). It appears that an elevated growth PFD is inducing elevated F_p fluorescence levels. Differences in values of t , time to reach $F_T=0$, are not significant between semi-sealed (CT-VK+CT-LK) and sealed vial (CT-VS+CT-LS) treatments (*Fig. 4.6B*).

4.3.4 Effects of CO₂-enrichment

Growth of plantlets is enhanced when CO₂-enriched air (CU-VE) is used (*Fig. 4.1A*). The effects of sucrose concentration on the differences of growth between CU-VN and CU-VE could not be detected, since the model predicts parallel regression lines for all sucrose concentrations. When sucrose treatments were considered together, CU-VE was responsible for the largest plants. CO₂-enrichment caused reductions in the photosynthetic rates. However, as sucrose concentration rose, no difference could be observed as it reached 4% and 8% (*Fig. 4.2A*). Respiration rates were not different when exogenous sucrose was not

supplied. However, as sucrose concentration rose, CU-VE caused more drastic reductions in respiration than with CU-VN (Fig. 4.2B). Very strong effects of CO₂-enrichment were observed on the RWC of plantlets. The RWC of plantlets grown under CU-VE were 17% lower than plantlets grown in CU-VN (Fig. 4.4A). There is no significant difference of F_p fluorescence with CO₂-enrichment (Fig. 4.5A). No effect of CU-VE can be observed on the time to reach $F_T=0$ (Fig. 4.6A).

4.4 DISCUSSION

Sucrose has a very deterministic effect on growth and photosynthesis of plantlets. When the culture vials are semi-sealed or sealed, it becomes essential for plant growth and normal photosynthetic development, especially in sealed vials. When CO₂ is supplied optimally, photosynthesis becomes altered upon any addition of exogenous sucrose while dark respiration decreases to levels comparable to those found in culture tube grown plantlets at the 8% level (Fig. 4.2B and 4.3B). With the data presented in this study, it appears that even under adequate gas atmosphere conditions, some exogenously supplied sucrose is beneficial to plantlet growth (e.g. in the range of 0.5% to 1%). Those findings are in agreement with the statements of Kozai *et al.*, (1987a, 1987b) and could indicate that under the present experimental conditions, incident PFD was still not saturating photosynthesis. Under CO₂-enrichment plantlets have lower photosynthetic rates. The more rapid accumulation of endogenous sugars with the extra CO₂ may lower the rate of photosynthesis and even more so with exogenously supplied sucrose. There is an absence of significant effects of elevated PFD on the CO₂ uptake rates of plantlets grown in culture tubes in contrast to respiration, which is somewhat

increased in plantlets grown under higher PFD. It can be speculated that increased respiration in plantlets grown under elevated PFD may be attributable to a higher carbohydrate status. In plant cell cultures, elevated respiration rates have been associated with a high carbohydrate status (Azcon-Bieto *et al.*, 1983; Azcon-Bieto and Osmond, 1983; Hrubec *et al.*, 1985; Volenec and Nelson, 1984).

The observed slower fluorescence quenching characteristics from plantlets grown with elevated exogenous sucrose (*Fig. 4.6*), further support the evidence for carbohydrate feedback inhibition of photosynthesis. There is a fine balance between the rate of regeneration of ribulose biphosphate from triose phosphate and synthesis of sucrose from triose phosphate. Availability of orthophosphate (P_i) plays a definite role on photosynthetic carbon assimilation and on the nature of final products of photosynthesis (Walker and Sivak, 1985). Sequestration of P_i prevents sucrose synthesis, exportation of triose phosphate to the cytosol and reduces ribulose biphosphate regeneration. When pools of P_i are incorporated into phosphorylated intermediates, photosynthesis is inhibited and only recovers when sucrose synthesis is activated (Stitt and Grosse, 1988; Stitt and Schreiber, 1988). Such inhibition is rapidly reflected on the rate of electron transport activity. It is slow in spinach chloroplasts when phosphorylation is prevented by the absence of phosphate (Saha *et al.*, 1971). Evidence that fluorescence induction transients are influenced by carbohydrate feedback inhibition through sequestration of cytoplasmic P_i is also reported by several authors (Brooks, 1986; Conroy *et al.* 1986; Dietz *et al.*, 1984; Stitt and Grosse, 1988; Stitt and Schreiber, 1988; Walker, 1981; Walker and Sivak, 1985). ATP synthesis can also be limited by

reduced amounts of P_i (Giersch and Robinson, 1987). The shape of the fluorescence induction curve, especially the $F_S-F_M-F_T$ transients depends, in a complex manner, on the ATP/ADP- P_i ratio (Horton, 1983; Walker, 1981). During the present experiment, accumulations of anthocyanin pigments in petioles and stems of plantlets were observed. Coloration consistently increased with increasing sucrose concentration in the media. Phosphorus deficiency may cause accumulation of anthocyanin pigments. Langford *et al.*, (1987) also observed anthocyanin pigments in plantlets grown with 4% sucrose in the medium.

Fluorescence curves of plantlets grown in tissue culture rarely showed intricate $F_S-F_M-F_T$ transients although F_P levels are comparable to the levels observed in *in vivo* plants. The combined effects of P_i sequestering due to exogenous sucrose and limited amounts of P_i in the medium are probably responsible for the reduced photosynthetic activity *in vitro*. The inorganic phosphate content of the Murashige-Skoog medium is known to be a rapidly limiting factor of photosynthetic efficiency in some culture systems (Dalton *et al.*, 1983).

The osmotic potentials of the culture media were not compensated by non-metabolic osmotica since this was judged unnecessary. The range of water potential [-0.212 to -0.836 MPa] generated by the presence of sucrose could not significantly influence water splitting activity to detectable levels as depicted by values of F_P fluorescence. Available studies on effects of water potential on F_P fluorescence did not indicate effects above ca. -0.8 MPa (Govindjee *et al.*, 1981; Stuhlfauth *et al.*, 1988). From the present data, the observed patterns of F_P can not be attributed to effects of water potential, the

results from *figure. 4.5A* being somewhat inconsistent with those of *figure. 4.5B* regarding sucrose effects.

The rather low F_p values observed in plantlets grown in sealed culture tubes are likely to be attributable to photoinhibition (Critchley, 1988; Powles, 1984). Conditions in sealed culture tubes are such that even under low PFD, CO_2 concentrations inside the vial become very rapidly limiting which leads to consequences such as severe limitations of the carbon reduction cycle. The energy generated through the electron transport chain exceeding the demand and being not dissipated, results in the inactivation of the photochemical reaction centers. CO_2 molecules become more available with increased respiration with increasing sucrose in the media, photosynthesis is therefore less inhibited, a situation found in semi-sealed and especially sealed culture tubes (*Fig. 4.3A* and *4.3B*). This is supported by the results shown in *Fig. 4.5B* where F_p values increase with sucrose in the medium. Results in *Fig. 4.6B* also support such evidence where more rapid quenching is observed when some sucrose is present in the medium.

Photorespiration is one means of effectively dissipating excess photochemical energy (Powles and Osmond, 1979). Conditions which lead to increased photorespiration would be present in the CT-VK. The degree of photoinhibition being less in those plantlets, as depicted by measured F_p values (*Fig. 4.5B*), it can be speculated that some degree of protection was offered through increased photorespiration, the rest being through reduction of CO_2 being made available from respiration with little gas exchange.

4.5 CONCLUSION

There are major differences in the photosynthetic capacity between plantlets grown in AGEHS (less hydroponic flow) and culture tubes. Given the growth conditions provided during this experiment it appears beneficial to provide some exogenous sucrose to the plantlets for optimal growth. However, careful inference should be exercised when interpreting these results. Technical limitations did not allow further increases of PFD in the growth cabinet. The observed changes in performance upon increases in PFD suggest that the latter could have been boosted even further. At certain developmental stages, elevated PFD may totally alleviate the need for exogenous sugar in the medium. Water stress did not seem to be a limiting factor for photosynthesis, at least in plantlets grown with enhanced normal-air exchange, its long term effect would certainly be to reduce growth. Evapotranspiration being considerably elevated under AGEHS growth conditions causes rapid depletion of water from the medium. Desiccation of gelled media is a serious problem, even under atmospheres of near water vapor saturation. A controlled flow of aerated liquid medium to replace gelled media would minimize dangers of desiccation and uniformly distribute non-limiting amounts of nutrients and water to all plantlets. Other experiments should be designed whereby a hydroponic flow of medium would help to further test the requirements of exogenously supplied sugars and to what extent growth could be enhanced *in vitro* when water availability is not limiting.

Media formulations will need considerable modifications with the development of *in vitro* gas exchange systems. Amounts and kinds of certain addenda will need to be adjusted in order to support enhanced

photosynthetic rates and development. For example, P_i should not be limiting as it plays an important role in energy transfer and in the regulation of metabolism.

CHAPTER 5

GROWTH AND PHOTOSYNTHESIS OF PLANTLETS GROWN WITH GAS-EXCHANGE AND HYDROPONIC MEDIUM FLOW

5.1 INTRODUCTION

Conventional tissue culture practices are labor intensive. Normally, during any stage of the propagation scheme, plantlets are kept undisturbed on nutrient medium for a minimum of 4 to 8 weeks (Murashige and Skoog, 1962). The assumption being that the nutrients provided are sufficient to last and remain in balance for the entire course of a stage. Accumulation of plantlet metabolites in the medium may reach toxic levels and repress growth of the plants (Murashige and Huang, 1987). To replenish medium or change its composition, tissue cultured plants need to be physically handled.

Oxygen must be available for *in vivo* root production, although requirements vary with species (Hartman and Kester, 1975). Greenhouse and nursery industries devote much effort in providing soil mixtures for better rooting and growth, a primary requirement is for adequate aeration. Hypoxia affects the amounts of sucrose taken up by flood intolerant species (Jenkin and Rees, 1986). In solidified agar media, inhibition of root development due to hypoxic conditions is likely. This need for oxygen seems to be ignored by most tissue culture workers. The type of support medium used *in vitro* may affect oxygen availability to developing roots. Toivonen, (1985) found that vermiculite supplemented with activated charcoal, compared to agar media, was the best support

for post-transfer physiological development. This observed enhancement in the physiological performance may come from a better aeration in the support medium provided with vermiculite. The properties of this material such as water-holding capacity, good porosity and large cation exchange capacity are well recognized in the horticulture sector and therefore it is extensively used as a growth substrate (Tingey *et al.*, 1982). A few other studies report that vitrification symptoms have sometimes been linked to roots being in a hypoxic environment such as solidified agar (Debergh, 1983; Gebhardt, 1985).

Liquid nutrient replenishment systems for tissue cultured plants designed to reduce labor costs have been suggested (Aitken-Christie and Jones, 1987; Maene and Debergh, 1985; Tisserat and Vandercook, 1985). However, none of the systems described in those studies included concurrent implementations of other growth enhancing parameters such as high PFD and gas exchange. This chapter demonstrates the feasibility and benefits of supplying aerated liquid medium pulses to *in vitro* tissue cultured plantlets. A support medium, rockwool, which shares several properties with vermiculite is used in this system.

Any of several factors may account for the limited photosynthetic CO₂ assimilation in plantlets grown *in vitro*. The relative size of the photosynthetic unit (PSU) may potentially characterize the photosynthetic light acclimation of plantlets grown under low versus high PFD. The 'size' of the PSU is defined as the number of light-absorbing pigment molecules contributing excitation energy to a reaction center (Mauzerall and Greenbaum, 1989). Since there exist large differences in PSU size between sun and shade leaves (when PSU size is defined as the number of

chlorophyll molecules per electron transport chain (Björkman, 1981)), a measure of relative PSU size is relevant to determine the differences in response of photosynthesis in plantlets grown under the *in vitro* environments. Simultaneous use of CO₂ gas exchange and integrating sphere fluorometry, permits the study of some of these factors by comparing plantlets grown under conventional and enhanced (*i.e.*, AGEHS) *in vitro* conditions.

5.2 MATERIALS AND METHODS

For all four treatments, as depicted in *table* 5.1, the shootlets were cultured onto Grodan™ rockwool plugs. The shootlets in culture tube treatments received 10 ml of medium at the beginning of the experiment only. The chamber units and one culture tube treatment were placed under an increasing PFD regime while the second culture tube treatment remained under low PFD *ca.* 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. PFD growth in the cabinet was adjusted to *ca.* 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a mixture of fluorescent tubes and incandescent bulbs for the first 3 photoperiods and was increased to *ca.* 325 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a high pressure sodium light for the remaining 12 days of the experiment. Neutral density filters were used to control PFD for the low light culture tube treatment. RH in both chamber units was controlled to remain at 95% for the first 10 days and to gradually decrease to 75% towards the end of the experiment. The medium contained mineral salts plus 5 g l⁻¹ sucrose for the first 5 days of the growth cycle. In both chamber units, it was then changed to mineral salts only until the end of the experiment. The medium in the culture tubes remained unchanged.

Table 5.1. AGEHS and culture tube treatments and their code symbols. PFD either varied according to a known schedule or remained low throughout the experiment. Air exchange in the chamber units was either a controlled flow of normal air or CO₂-enriched air, while for the culture tubes air flow was restricted by a semi-sealing closure called KimKap. The root zone of the plantlets in both chamber units was periodically flooded with hydroponic medium while plantlets in culture tubes remained on a gelled medium.

GROWTH VIAL	PFD	AIR EXCHANGE	MEDIUM	SYMBOL
Chamber Unit	Varied	Normal	Hydroponic	CU-VNH
Chamber Unit	Varied	Enriched	Hydroponic	CU-VEH
Culture Tube	Varied	KimKap	Gelled	CT-VKG
Culture Tube	Low	KimKap	Gelled	CT-LKG

The growth parameter, specific leaf weight (SLW), *i.e.* amount of leaf dry weight per total leaf area, used with this particular set of data is calculated with the following equation:

$$\text{SLW} = \text{IDW} / \text{leaf area} \quad (5.1)$$

Values of SLW are expressed in mg cm⁻².

5.3 RESULTS

5.3.1 Effects on plantlet growth

Plantlets grown under AGEHS conditions accumulated 3 times as much DW as those from the best culture tube treatment. Plantlets from the CT-VKG treatment were only 1.6 times the DW of CT-LKG plantlets (*Fig. 5.1*). CU-VEH treated plantlets did not gain dry weight more than the CU-VNH treated plantlets. The relative water content of culture tube plants was slightly higher than AGEHS treatments which might indicate that moisture and/or water availability was close to being the same under both growth conditions (*Fig. 5.2*). Again, values of relative water content of CU-VEH and CU-VNH plants are similar. Each treatment has a significantly different effect on SLW (*Fig. 5.3*). CO₂-enrichment increased SLW the most and low light culture treatment (CT-LKG) the least, with a difference between CU-VEH and CU-VNH being marginal.

DRY WEIGHT OF SHOOTLETS

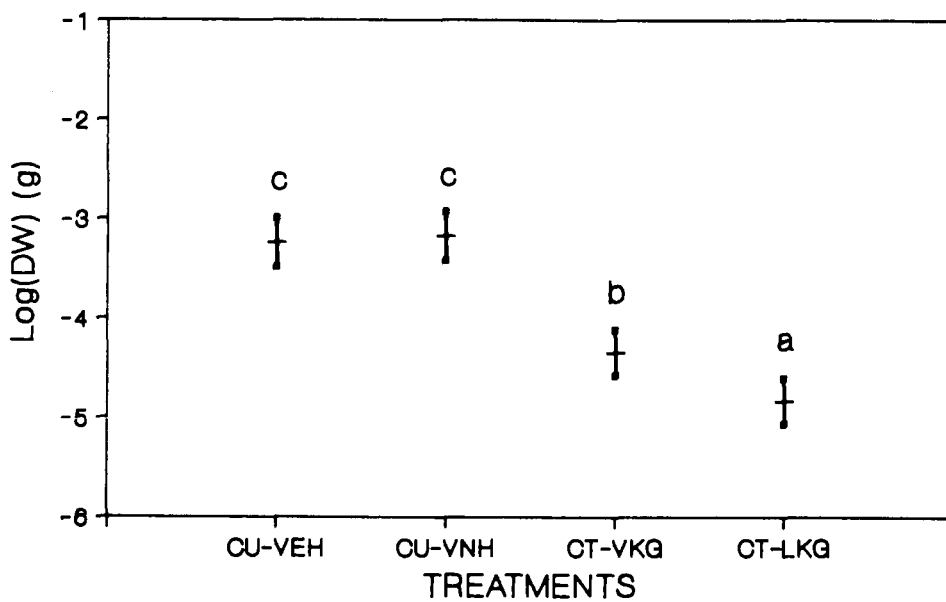


Figure 5.1. Dry weight (DW) of shootlets. Comparison is made between shootlets grown under AGEHS conditions with either normal air (CU-VNH) or CO₂-enriched air (CU-VEH) and conventional tissue culture conditions either under elevated (CT-VKG) or low PFD (CT-LKG). Treatments with the same letters are not significantly different at the 0.05 level.

RELATIVE WATER CONTENT

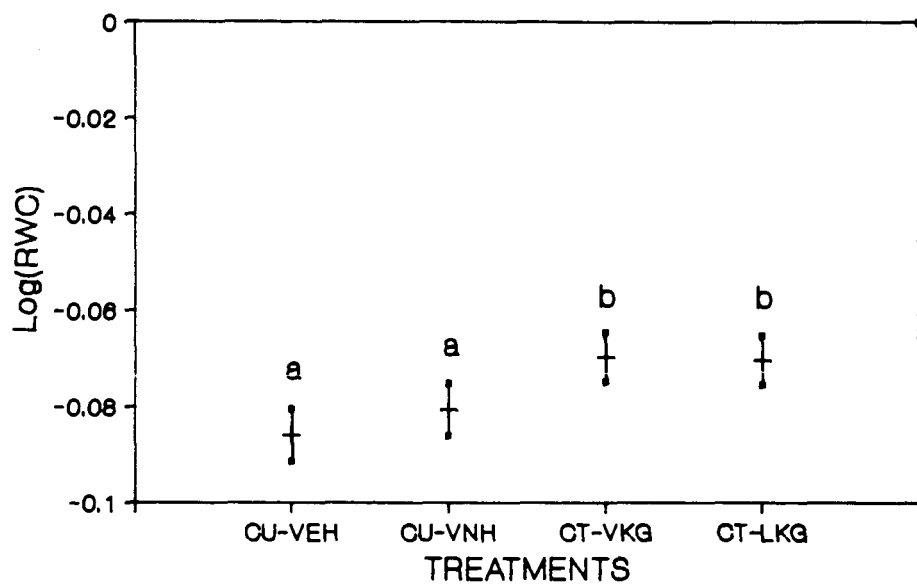


Figure 5.2. Relative water content (RWC) of shootlets. Comparison is made between shootlets grown under AGEHS conditions with either normal air (CU-VNH) or CO₂-enriched air (CU-VEH) and tissue culture conditions either under elevated (CT-VKG) or low PFD (CT-LKG). Treatments with the same letters are not significantly different at the 0.05 level.

SPECIFIC LEAF WEIGHT

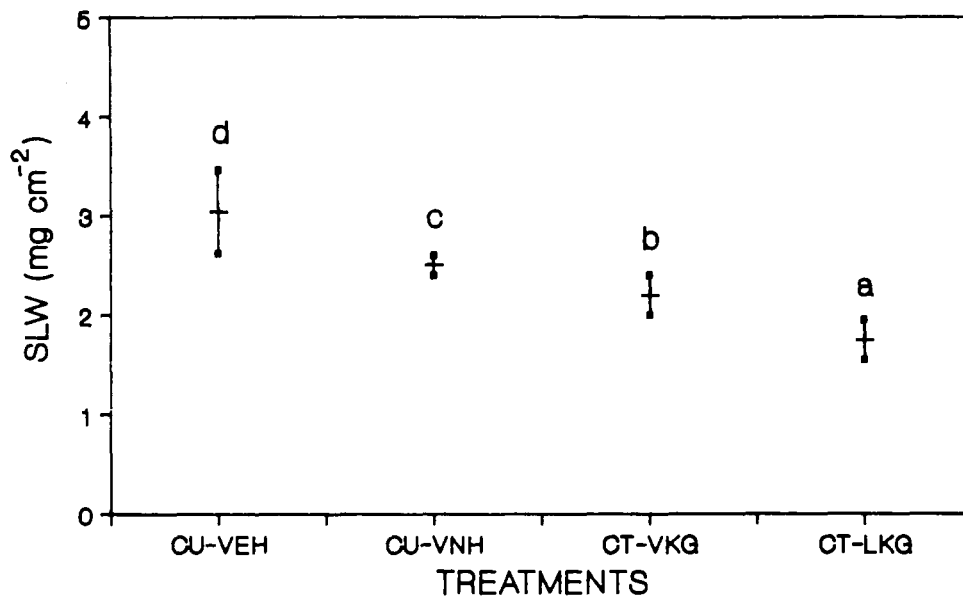
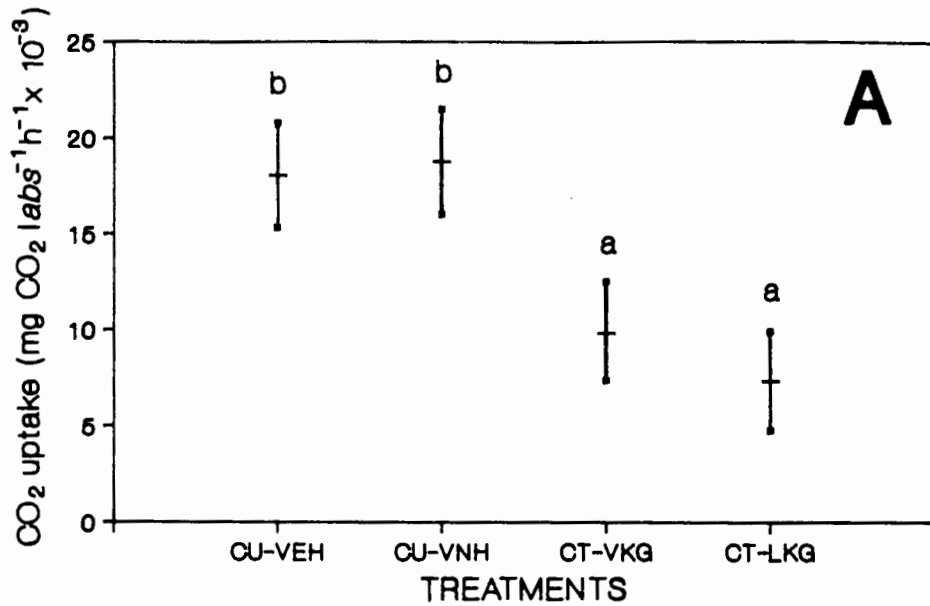


Figure 5.3. Specific leaf weight (SLW). SLW is the ratio of leaf DW per unit leaf area expressed in mg cm⁻². Comparison is made between shootlets grown under AGEHS conditions with either normal air (CU-VNH) or CO₂-enriched air (CU-VEH) and tissue culture conditions either under elevated (CT-VKG) or low PFD (CT-LKG) Treatments with the same letters are not significantly different at the 0.05 level.

PHOTOSYNTHETIC RATES



DARK RESPIRATION RATES

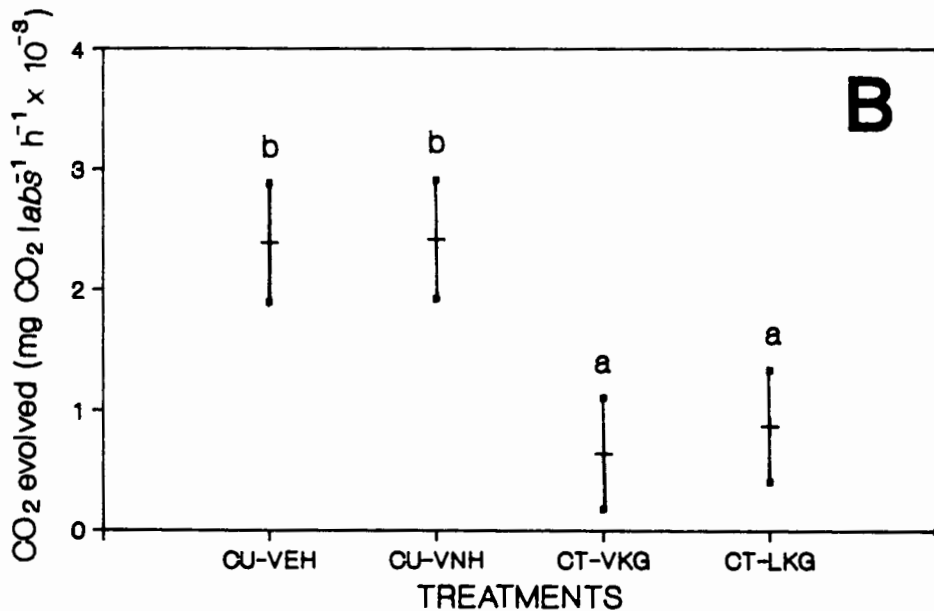


Figure 5.4. CO₂ uptake and dark respiration rates of shootlets. Rates are expressed in mg CO₂ l abs⁻¹ h⁻¹ x 10⁻³. Assessment methods are described in *chapter 3*. The plantlets were grown under AGEHS conditions with either normal air (CU-VNH) or CO₂-enriched air (CT-VEH) and tissue culture conditions either under elevated (CT-VKG) or low PFD (CT-LKG). Treatments with the same letters are not significantly different at the 0.05 level.

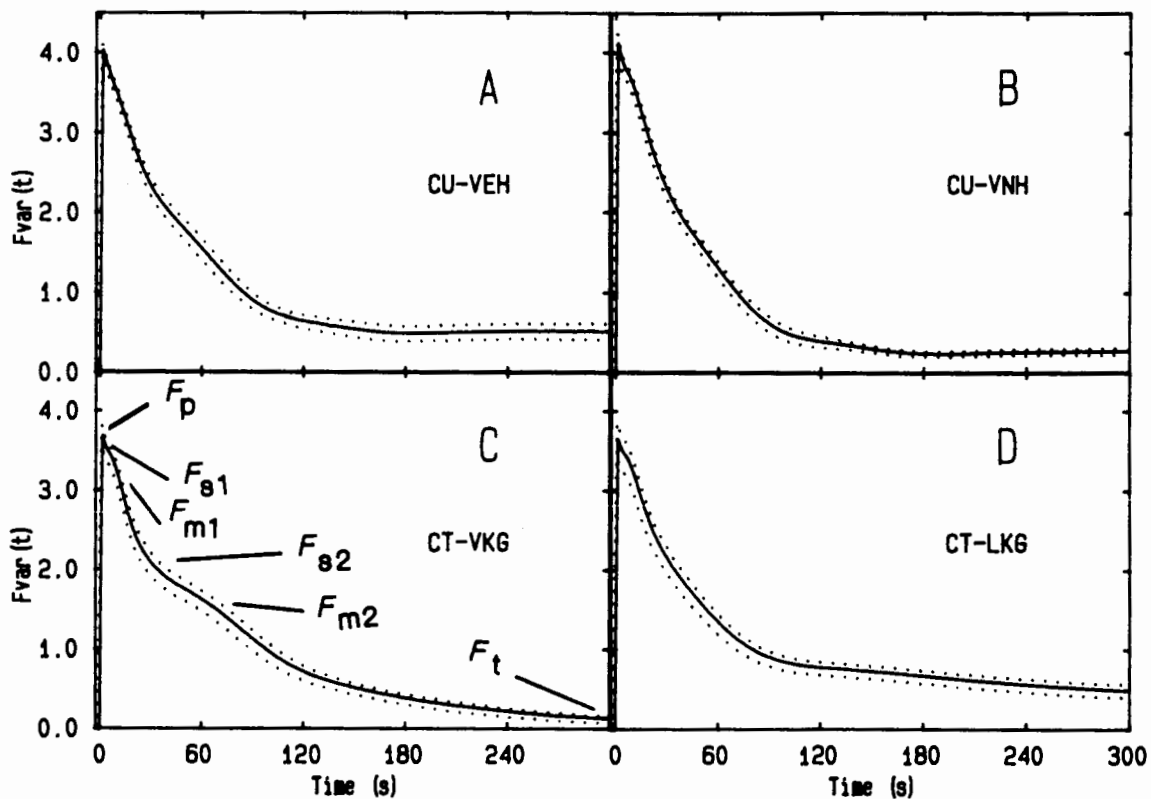


Figure 5.5. Parameters of fluorescence induction kinetics. Shootlets were dark adapted for 60 min prior to fluorescence induction. The plantlets were grown under AGEHS conditions with either normal air (CU-VNH) or CO₂-enriched air (CU-VEH) and tissue culture conditions either under elevated (CT-VKG) or low PFD (CT-LKG). The dotted lines enclose an area above and below the averaged fluorescence induction curve which represents the S.E. of the mean. The number of samples for averages of CU-VEH and CT-VNH is 7 and for CT-VKG and CT-LKG is 8. Characteristic inflection points of the induction curves are indicated in Fig. 5.5C. Assessment methods are described in *chapter 3*.

5.3.2 Effects on photosynthesis

The photosynthetic and dark respiration rates of plantlets grown under AGEHS conditions were much higher than those grown in culture tubes. However, rates between CU-VEH and CU-VNH plantlets are not different (Fig. 5.4). The kinetics of *in vivo* chl *a* fluorescence induction are very similar amongst all treatments. However, secondary features such as F_{M1} , F_{M2} , (Fig. 5.5C), (for nomenclature of fluorescence transients, see Andreeva, 1983; Papageorgiou, 1975) are more accentuated in plantlets from the high PFD culture treatment. F_T tends to be somewhat higher in the CT-LKG treatment; the lowest are found in high PFD culture tube (CT-VKG) treated plantlets. Under AGEHS conditions F_T -level values are intermediate to those found in culture tube plantlets (Tab. 5.2). F_p -level values are all statistically equal.

Table 5.2. Parameters of fluorescence induction kinetics. Comparison is made between shootlets grown under AGEHS conditions with either normal air (CU-VNH) or enriched air (CU-VEH) and conventional tissue culture conditions either under elevated (CT-VKG) or low PFD (CT-LKG). Fluorescence was measured according to methods and apparatus described in *chapter 3*. F_p is the height of the peak of the major fluorescence transient while F_T is the height of the fluorescence curve transient reached at 300 s of fluorescence induction. Values are shown with S.E. Treatments with identical letters are not significantly different at the 0.05 level.

TREATMENT	n	F_p (S.E.)	F_T (S.E.)
CU-VNH	7	4.13 0.07a	0.44 0.10 bc
CU-VEH	7	4.31 0.08a	0.22 0.04 abc
CT-VKG	8	3.88 0.13a	0.13 0.04 a
CT-LKG	8	3.90 0.22a	0.48 0.08 c

The correlation analysis between relative PSU size and photosynthesis reveals a good segregation of the data into three distinct groups (*Fig. 5.6*). These groups appear to segregate on the basis of growth PFD. The CU-VEH and CU-VNH treatments form a single group with the highest rates and smallest PSU sizes. CT-LKG plantlets exhibit a reduced photosynthetic ability per reaction center compared to plantlets also grown in culture tubes but at elevated PFD (CT-VKG).

RELATIVE PHOTOSYNTHETIC UNIT SIZE

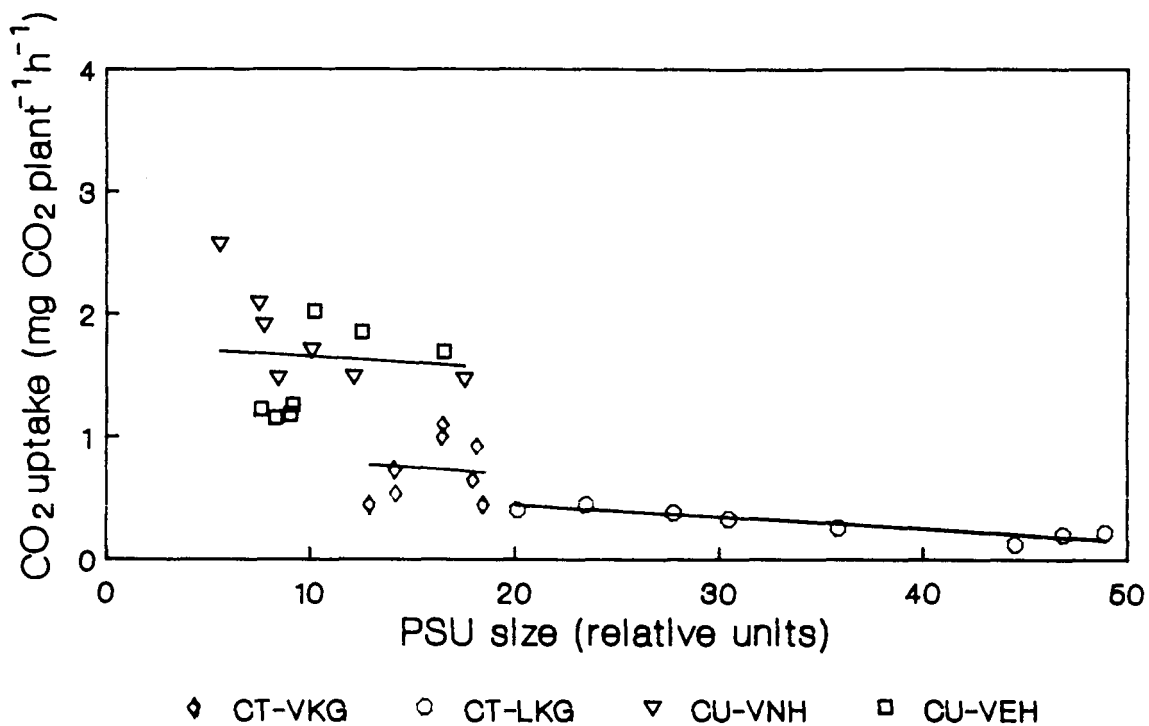


Figure 5.6. Correlation between relative PSU size and photosynthetic gas exchange. Comparison is made between shootlets grown under AGEHS conditions with either normal air (CU-VNH) or CO₂-enriched air (CU-VEH) and tissue culture conditions either under elevated (CT-VKG) or low PFD (CT-LKG). CO₂ gas exchange and fluorescence were measured simultaneously according to methods described in *chapter 3*.

5.4 DISCUSSION

Plantlet growth is significantly increased with the combination of enhanced-air exchange and hydroponic medium flow. It is even more interesting to note that these differences were gained in only 15 days. Increased DW in plantlets grown with CO₂ enrichment in tissue culture systems are also reported in other studies (Kozai *et al.*, 1987a; Kozai and Iwanami, 1988; Mousseau, 1986; Solarova *et al.* 1989). However, a difference in DW between CU-VEH and CU-VNH treatments is not seen here. The constant regulation of the flow of air sweeping across the *in vitro* plantlets which brings all necessary CO₂ may account for this enhancement of growth in CU-VNH which is not seen in the previous studies. Photosynthesizing plantlets do deplete CO₂ from the air enclosed in sealed vessels to levels near the CO₂ compensation point (Solarova, 1989). With regulation and air movement, the CO₂ content of the air surrounding the leaves of plantlets is non-limiting at all times. Under these conditions CO₂-enriched air flow may not confer any further advantage to plantlet photosynthesis over normal air flow. The decreased relative water content in AGEHS treated shootlets may come from the increased ability to accumulate photosynthates due to increased photosynthetic activity.

Photosynthetic rates are far higher in AGEHS treatments, which may account for the increased growth resulting from these treatments. Respiration being also much increased may indicate a higher carbohydrate status in the plantlets' foliage under AGEHS conditions. Other studies on effects of CO₂ enrichment have indicated that higher

respiration rates could be attributable to higher nonstructural carbohydrate status (Hrubec *et al.* 1985). As indicated by the height of F_p -level fluorescence, water splitting activity appeared normal in all treatments with no indication of photoinhibition. The F_T -levels shown in the CT-LKG treatment (Fig. 5.5D) are illustrative of an overall reduced quenching ability which may indicate a reduced Calvin cycle activity which plays an important role in balancing the redox state of the electron-transport chain. Under assessment PFD, rates of electron transport would be higher than normally encountered during growth. The activities of the enzymes of the dark reactions of photosynthesis would need further adjustments to cope with the unexpected increased electron transport activity which is not limiting, according to the observed height of F_p level. Under conditions of high electron transport activity and lowered utilization of photosynthate the electron transport intermediate Q_A becomes highly reduced (Dietz *et al.*, 1985). However, plantlets from the CT-VKG treatment had the lowest F_T -level which suggests that there is increased utilization of stored photosynthetic energy. There is increased energy consumption with photorespiration which is reflected in lower phosphorylation potential. Fluorescence kinetics are directly affected by changes in phosphorylation potential (Osmond, 1981). The more pronounced secondary fluorescence transients seen in CT-VKG may be an indication of altered phosphorylation potential linked to increased utilisation of energy. Since electron transport activity appears not to be limiting and CO_2 uptake rates are comparable to those of CT-LKG treated plantlets the electron transport may be devoted to other processes. Direct reduction of O_2 via Mehler processes could occur (Osmond, 1981). These results compare with those of the previous chapter. The correlation study

between the relative PSU size and photosynthesis indicates that under conventional tissue culture conditions, the efficiency of the reaction centers is lower. That is seen by extending the line of the CU-VEH and CU-VNH treatments to the 'y' ordinate of *figure 5.6*, which ordinate is significantly higher than the two others, that is with the same value of relative PSU size. The CT-VKG treated plantlets being grown under the same PFD as the plantlets in the AGEHS treatments would be expected to have the same ordinate. The results depict differences in the relative PSU sizes on a basis probably other than acclimation to growth. The relative decrease in photosynthetic CO₂ uptake for the same PSU size in the CT-VKG treated plantlets can be considered to express a decrease in the number of active chlorophyll molecules.

Specific leaf weight (SLW) has been commonly reported in other studies on the effects of CO₂ enrichment (Wulff and Strain, 1981). An increase in SLW is one of the most observed effects on growth under CO₂ enrichment (Hoddinot and Jolliffe, 1988; Hofstra and Hesketh, 1975; Hrubec *et al.* 1985; Hurd, 1968; Neales and Nichols, 1978; Vu *et al.*, 1989; Wulff and Strain, 1981). In plant tissue cultures, effects of CO₂ enrichment on SLW are also reported (Mousseau, 1986; Solarova *et al.*, 1989). High SLW indicates a denser leaf and is often attributed to increased leaf starch content (Hoddinot and Jolliffe, 1988). In this experiment, SLW increases as PFD is increased or as both increased PFD and CO₂ are provided. This statement is supported from the results of increased respiration rates. There is also a significant correlation between respiration rates, photosynthetic rates and SLW (data not shown).

5.5 CONCLUSION

Short term photosynthetically enhanced growth is possible when elevated PFD, enhanced air-exchange and hydroponic medium flow are provided together. This enhancement is achievable through careful increments of light quanta, balanced with increments of air flow and/or CO₂ content in air which seem to be necessary to avoid potential photoinhibition and premature water exhaustion from gelled media. In non-regulated systems such as in conventional culture tube environments, the depletion of CO₂ by the photosynthesizing plantlets is likely to cause excess electron flow. This would in turn, induce a photoinhibition response and possibly photooxidation resulting in loss in photosynthetic efficiency. Available water to satisfy the evapotranspiration demand is the next limitation, after PFD and air flow are provided adequately. A hydroponic flow implementation seems to offer a reasonably good solution to the problem of rapid desiccation of gelled media under rapid air flow conditions. In comparison with data from the previous chapter of this thesis, growth, water content and photosynthetic rates all improved under similar growth conditions but supplemented with ways to replenish water loss from the plantlet support. This particular set of data does not directly show the benefits of hydroponic flow nevertheless, the comparison can be made. Because the plants grew so much in 15 days, serious hardware limitations of the AGEHS became obvious; air flow rates through the CU-VNH chamber unit dictated from the calculated CO₂ requirements, could no longer be achieved and line pressure problems arose. The benefits of CO₂-enrichment being to provide adequate amounts of CO₂ without the draw backs of an elevated air flow, otherwise, from the results

of this short term experiment, CO₂ enrichment did not confer any other benefit to the plantlets. With a longer term experiment, this question could probably be answered.

Since photosynthetic electron flow is not limiting in *C. morifolium* at this stage of *in vitro* development other factors than inadequate development of electron transport carriers must be limiting. Photosynthetic activity of cauliflower plantlets *in vitro* and *ex vitro* has been studied in comparison with seedlings by Grout and Donkin, (1987). Their study indicated that *in vitro* plantlets have levels of electron transport activity similar to seedlings. However, they demonstrated that carbon fixation was limited by low RuBPase activity. Lack of reducing equivalents appear to be the cause since plantlets grown under conventional methods or with a semi-sealing closure may also need to recycle CO₂ through dark respiration and photorespiration; sucrose being necessary to replenish a pool of carbon building blocks. Osmond, (1981) and Powles and Osmond, (1979), report that CO₂ recycling through photorespiration is one means of effectively dissipating excess photochemical energy when CO₂ supply to illuminated leaves is limiting.

PFD levels have deterministic effects on the photophysiological development. Low light grown plants are more deeply colored *i.e.* have more chlorophyll. One of the determining factors for the photochemical activity of plants besides electron transfer reactions is the size of PSU or the photochemical activity per leaf dry matter, the number of chlorophyll molecules per reaction center. The minimum size of a PSU is that of the RC itself (Mauzerall and Greenbaum, 1989). From the results depicted in this present study, factors accompanying light effects appear to play

important roles in the final resolution of the photosynthetic efficiency. It can be speculated that under elevated PFD and restricted air-exchange conditions such as found in plantlets of the CT-VKG treatment, photoinhibition or even photodamage may have resulted in lower photosynthetic rates per PSU. This particular aspect needs to be further investigated.

In plant tissue culture studies, the determination of the absolute size of a PSU would allow a more detailed understanding of the adaptation of photosynthetic apparatus to changes in the environment, especially in order predict acclimatization potential.

CHAPTER 6

INDUCTION AND DETECTION OF EFFECTS OF EXCESS LIGHT IN TISSUE CULTURED PLANTLETS

6.1 INTRODUCTION

The absorption of light in excess of what can be dissipated by the energy conversion processes such as carbohydrate synthesis, heat and fluorescence, may result in a reduction in light-limited and light-saturated CO₂ uptake and by a concomitant decrease in the rate and photon yield of electron transport (for reviews, see Powles, 1984, Krause, 1988). Leaves, algal cells or isolated chloroplasts vary in their ability to cope with excess light energy depending on their origin and their previous physiological adaptations resulting from acclimation. Photoinhibition is thought to occur when PS_{II} cannot dissipate excess excitation energy via useful photochemistry (Osmond, 1981). As used here, photoinhibition refers to light-dependent decline in photon yield. Conditions which result in more photons being more absorbed than required to drive photosynthesis are inducible of photoinhibition. Photoinhibition does not imply photooxidation or photoregulation although it may be the result of either or both phenomena. Under high PFD and severe stress, pigments can be destroyed (photooxidation and/or peroxidation) and serious damage may result, often culminating in senescence or death of the organism. Conditions which reduce the activities of the photosynthetic carbon reduction cycle and/or the carbon oxidation cycle and dark respiration, increase damage effects including the photoinhibitory effects of high

irradiance (Powles, 1984). Plants exposed to temperature or osmotic stress usually become photoinhibited at lower PFD than if unstressed, presumably because of a decreased capacity for energy dissipation (Ögren and Öquist 1985; Strand and Öquist 1985a, 1985b; Strand and Öquist 1988).

The inhibition of electron transport capacity is also evident as a change in chlorophyll fluorescence yield. In intact leaves at room temperature, the kinetics of chl *a* fluorescence (F_{VAR}) was lowered by exposure to a higher PFD (Critchley and Smillie, 1981). Changes in F_O are also observed throughout photoinhibition inducing treatments (Bradbury and Baker, 1986; Krause, 1988).

Conditions prevailing *in vitro* are such that plantlets do not develop full photosynthetic capacity (Chap. 5; Donnelly and Vidaver, 1984b). Furthermore, close examination of the conditions of growth encountered in conventional tissue culture systems leads to the hypothesis that unless growth PFD is kept very low, these conditions are likely to induce photoinhibition or even photodamage. When transplanted *ex vitro*, the effects of the *in vitro* conditions, including growth PFD, will determine the susceptibility of the plantlets to photoinhibition or photodamage. The characterization of the effects of excess PFD on photosynthesis in tissue cultures is yet poorly documented. This chapter is aimed at providing some guidance for the assessment of photoinhibitory responses in plant tissue cultures.

6.2 MATERIALS AND METHODS

All treatments were done using Chrysanthemum (*Chrysanthemum X morifolium* Ramat. cv. Envy) plantlets derived from cloned shoot tips and routinely subcultured on multiplication formula (see *chapter 2*). Prior to dark adaption for fluorescence and gas-exchange measurements, plantlets were removed from their growth vials, and stems with 3-4 leaves were cut under water to prevent breaking the xylem water column and placed in distilled H₂O in a 2 ml flask. All dark adaption times were 60 10 min. Physiological assessments were according to methods described in *chapter 3*. They were performed before and after excess light or control treatments. All measurements were preceded by a dark adaption period. The treatments consisted of exposing shootlets to CO₂ depleted air flow saturated with water vapor for a duration of 100 min under elevated PFD. Two levels of PFD were chosen for treatment, ca. 500 and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. PFD was provided from a 650 W quartz halogen lamp, filtered through a 10 cm wide water bath. PFD, measured with a LICOR LI-185A fitted with a quantum flux detector (LiCor Inc, Lincoln, Nebraska) was controlled to desired levels by placing layers of cheesecloth between the water bath and the gas-exchange cuvette in which shootlets were enclosed for exposing their leaves to treatment gases. CO₂ depleted air was obtained by scrubbing CO₂ from compressed air with a 2 l column of soda lime. Treatments are as indicated in *table 6.1*. Shootlets from the ME-LIGHT treatment were returned to 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PFD and normal-air for 3 hours to allow recovery.

Table 6.1. List of excess PFD treatments and their code symbols. Treatments consisted of exposing tissue cultured plantlets to CO₂-depleted and humidified gas under elevated PFD. PFD is expressed in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Recovery and control treatments were done in air with ambient levels of CO₂. A recovery (REC-RY) treatment was done with plantlets exposed to 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (ME-LIGHT).

TREATMENT	GAS	PFD	TIME EXPOSED (min)	SYMBOL
Medium	no CO ₂	500	100	ME-LIGHT
Recovery	air	40	180	REC-RY
High	no CO ₂	1000	100	HI-LIGHT
Control	no CO ₂	10	100	CTRL

6.3 RESULTS

6.3.1 Effects on fluorescence characteristics

Fluorescence kinetics measurements, showed the effects of extended exposure of tissue cultured shootlets to excess light quanta under conditions that generally result in photoinhibition (*Fig. 6.1*). Typical F_p values for untreated plantlets were between 3.5 and 4.5 (*Fig. 6.1A*, before (B) and *Fig. 6.1C*, before (B)). After exposure to CO₂-free air for more than one hour under 500 or 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the fluorescence transients of treated plantlets indicated an altered electron transport activity (*Fig. 6.1A*, after (A) and *Fig. 6.1C*, after (A)). However, plantlets exposed to CO₂-free air under every low light conditions (CTRL) were not affected at any point of the fluorescence transients (*Fig. 6.1D*). Compared to prior-treatment values, F_p decreased by more than 50% in both ME-LIGHT and HI-LIGHT treatments, however, HI-LIGHT caused even further decreases to F_p values below 1.0 (*Fig. 6.2A*). Shootlets allowed to recover for three hours, significantly increased in F_p value but not to control levels. Changes in the ratio of F_o/I_{ABS} were observed in all treatments except controls (*Fig. 6.2B*). HI-LIGHT values, although comparable to CTRL were significantly lower than ME-LIGHT and REC-RY.

6.3.2 Effects on CO₂ gas-exchange rates

CO₂ uptake rates were similar prior to treatments (*Fig. 6.3A*). Photosynthetic rates of treated shootlets increased with the experimental procedure alone. Except for HI-LIGHT, photosynthetic rates increased with all treatments. The rate with HI-LIGHT was significantly lower than with the

other treatments and showed a significant decline from the initial rate (*Fig. 6.3A*). Correlation analysis of the change in CO₂ uptake rates with the changes in the ratio F_O/I_{ABS} reveals a linear relationship between these two parameters (*Fig. 6.3B*). HI-LIGHT shootlets segregate as a separate group, with values of both CO₂ uptake changes and the ratio F_O/I_{ABS} changes being negative as well as of lower origin than the other treatments which are positive and of higher origin respectively.

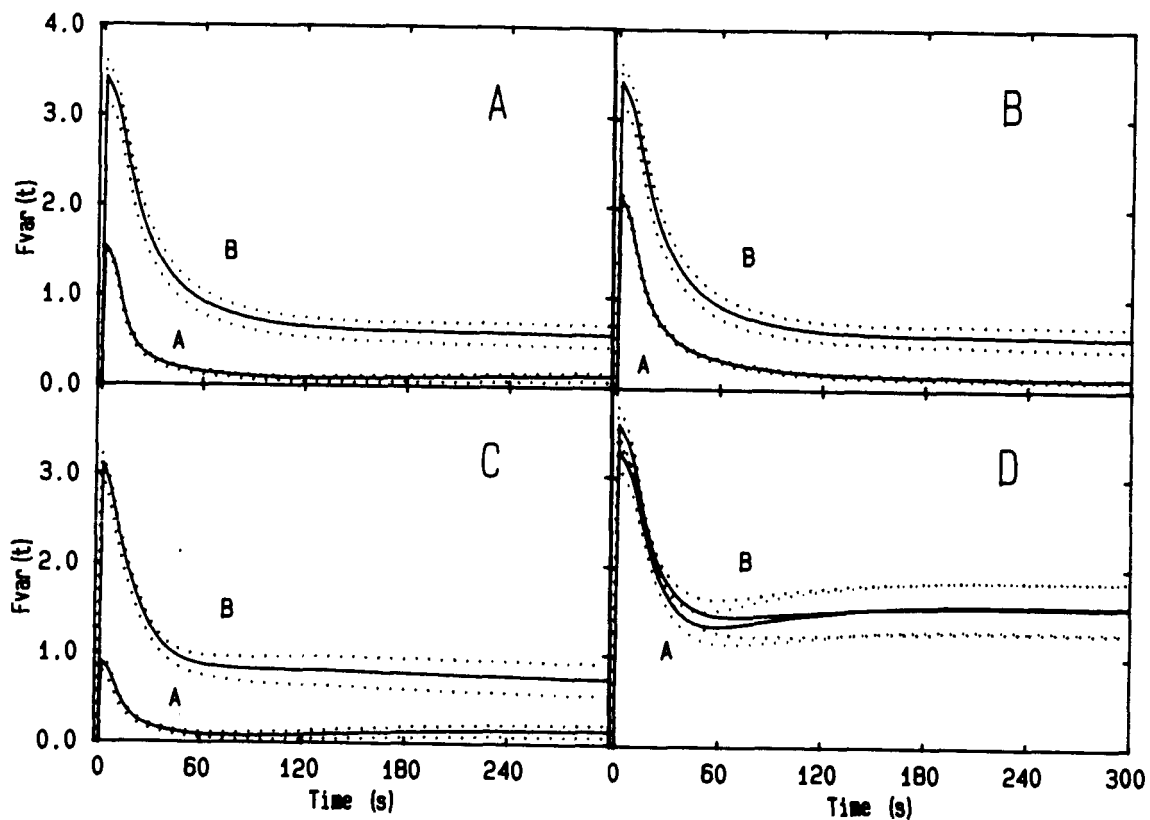
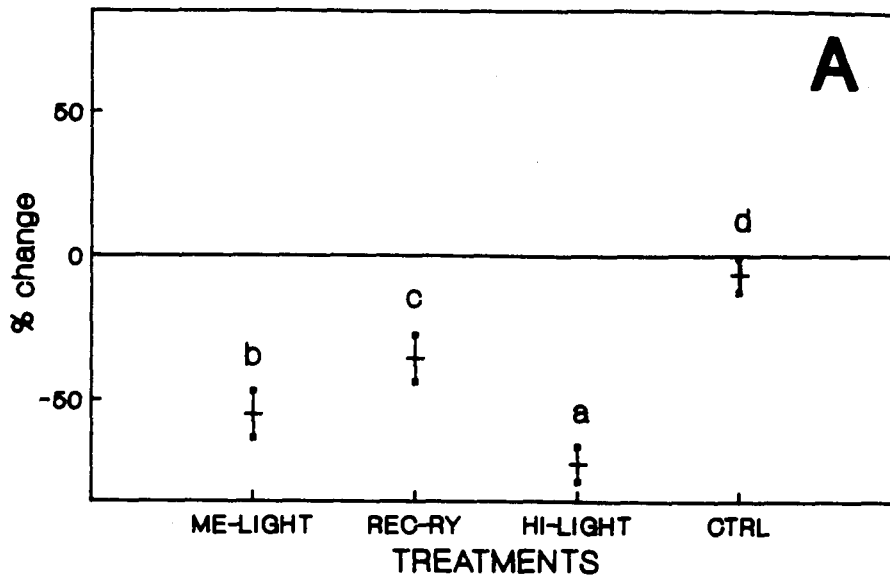


Figure 6.1. Fluorescence induction kinetics of plantlets. Plantlets were treated with excess PFD. They were previously grown at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ PFD. Prior to and after every treatment, plantlets were assayed for fluorescence. These curves are marked B (Before) or A (After) respectively. They were exposed to CO_2 depleted humidified gas under elevated PFD and allowed to recover under low PFD in ambient humidified air. Controls consisted of exposing plantlets to CO_2 depleted humidified air under very low PFD. Curves show an average of 5-9 samples. The dotted lines above and below the curves represent the S.E. of the mean. *Fig. 6.1A*, ME-LIGHT; *Fig. 6.1B*, REC-RY; *Fig. 6.1C*, HI-LIGHT; *Fig. 6.1A*, CTRL. Symbols are as in *Tab. 6.1*.

CHANGES IN F_p FLUORESCENCE



CHANGES IN THE RATIO F_0/I_{ABS}

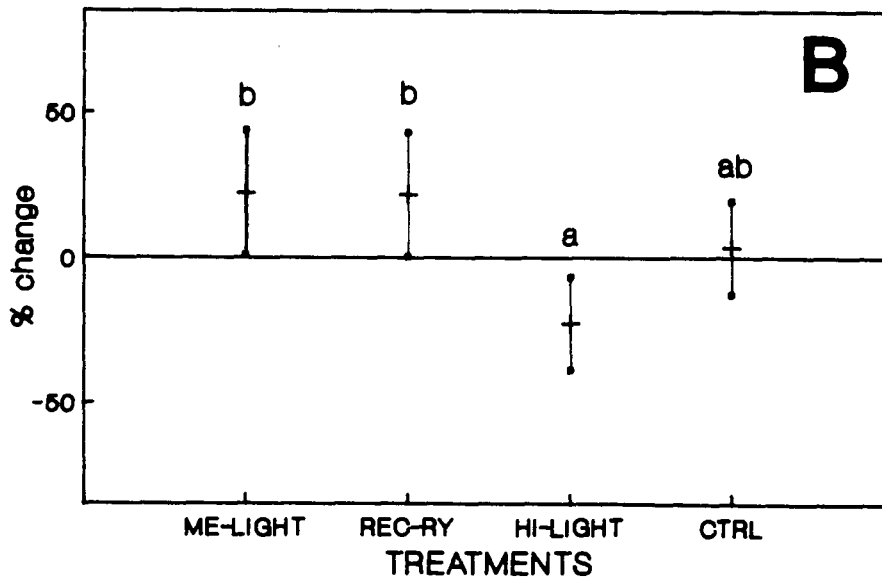
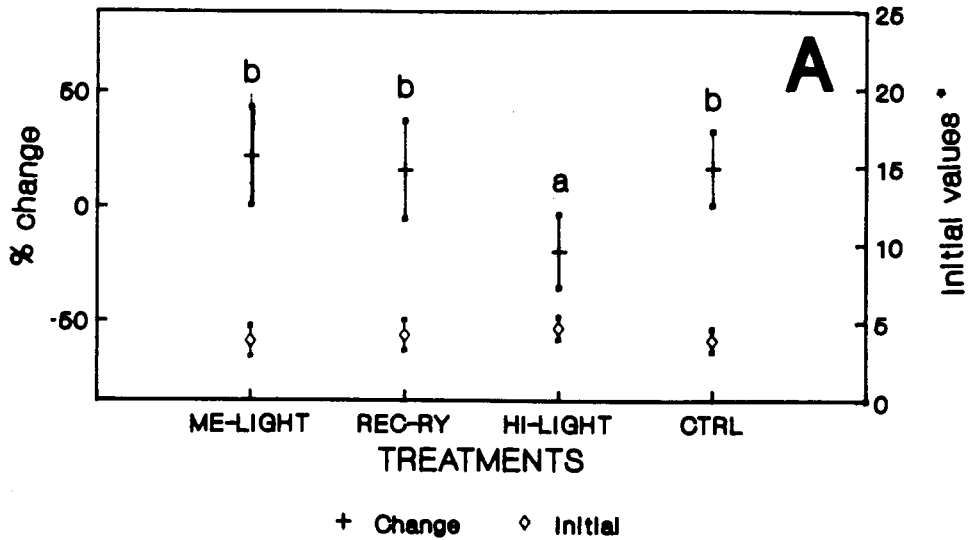


Figure 6.2. Changes in F_p fluorescence and ratio F_0/I_{ABS} . Plantlets were treated with excess PFD. They were previously grown at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ PFD. Prior to and after every treatment, plantlets were assayed for fluorescence. The results are the differences between prior to and after every treatment. Error bars represent a 95% confidence interval of the mean and treatments with the same letters are not different at the 0.05 level. Fluorescence induction is discussed in *chapter 3*.

PHOTOSYNTHETIC RATES



CHANGES IN F_o/I_{abs} AND CO_2 UPTAKE

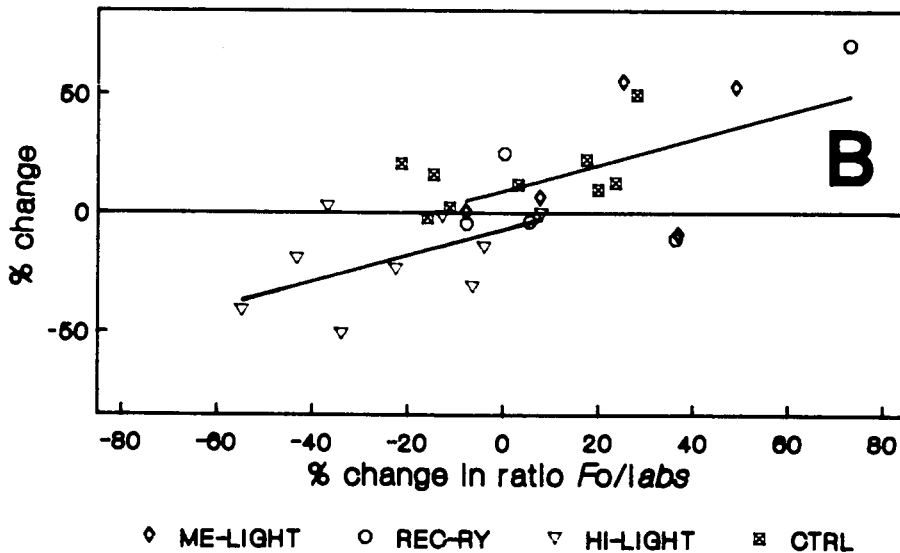


Figure 6.3. Changes in photosynthetic rates and their correlation with F_o/I_{ABS} ratios. The results are shown as differences prior to and after every treatment. In Fig. 6.3A, the initial values of CO_2 uptake rates are also shown and scaled on the second 'y' axis. In Fig. 6.3B, the changes in the ratio F_o/I_{ABS} are plotted against the changes in CO_2 uptake rates. Error bars represent a 95% confidence interval of the mean and treatments with the same letters are not different at the 0.05 level. The methods are described in chapter 3.

6.4 DISCUSSION

C. morifolium plantlets measured at the multiplication stage are capable of net photosynthesis. Two distinctly observable physiological responses arise from this experiment. First, an increase in photosynthetic rates seen in the CTRL treatment results from exposure of plantlets to the physiological assessment scheme. This increase could be attributed to changes in activity of the carbon reduction cycle e.g., RuBPCase activity, upon transition from growth PFD to the assessment PFD. This interpretation is suggested by the work of Dujardyn and Foyer, (1989). ME-LIGHT showed a similar increase in CO₂ uptake. However, the fluorescence kinetics determined from these plantlets showed that electron transport was affected. Upon return to the assessment PFD, RuBPCase activity appears to remain high and outweigh any reduction of electron transport activity. Exposure to 1000 μmol photons m⁻² s⁻¹ resulted in a marked decrease in photosynthetic activity also evidenced by reduced electron transport as well as reduced CO₂ uptake rates.

Changes in F_O level with high PFD treatments are reported elsewhere (Bradbury and Baker, 1986; Krause, 1988) also when determined with rapid induction systems such as the PAM chlorophyll measuring system (Schreiber and Bilger, 1987; Quick and Stitt, 1989). However, discriminations on the basis of responses of F_O do not always appear valid.

According to Bradbury and Baker, (1986), during the first 5 min of a photoinhibitory treatment, an observed rise in F_O was attributed to a decline in PS_{II} photochemistry, possibly due to a loss of functional reaction centres. The subsequent fall in F_O and the continuous decline in F_{MAX} was

suggested to result from increased radiationless deexcitation of the PS_{II} reaction centre and antennae pigments (Bradbury and Baker, 1986). These authors also suggested that two processes contribute to photoinhibition. In sun plants, a decrease of F_O would be indicative of a decline in primary photochemical efficiency due to a lower energy transfer from the LHC_{II} to PS_{II} reaction centers (Bilger and Schreiber, 1986). Weis and Berry (1987) found correlations between a decrease in the F_O level and the increase in energy quenching at high PFD. Although, the various quencher coefficients were not measured in the actual experiment, the lower origin of the HI-LIGHT regression line shown in Fig. 6.3B, may be an indication of still another quenching mechanism.

6.5 CONCLUSION

When light is provided in excess or when conditions lead to a potential rate of photochemistry exceeding the rates of carbon metabolism, quenching of F_O as a result of an altered state of PS_{II} centers together with a lower F_p level are indications of mechanisms of regulation of the rate of photochemistry. Plantlets grown under *in vitro* conditions may be capable of net photosynthetic carbon reduction. However, when the plantlets are exposed to light several times higher than their growth PFD, characteristic declines in photosynthetic activity rapidly develop (photoinhibition). Changes in the fluorescence kinetic patterns in response to high PFD treatments are good indicators of impairment of photosynthetic functions. The overall quenching of the fluorescence curves (F_{VAR}) may be an indication of altered photochemistry, in spite of this manifestation, little information is obtained about processes, sites of

damage or even degree of reversibility, if any. Resolution of these two photoinhibitory phenomena is needed in order to elicit requirements for successful acclimatization *in vitro* or *ex vitro*.

The ratio, F_0/I_{ABS} , is particularly useful in the non-destructive study of conditions that affect the yield of fluorescence in whole plants of different sizes. This ratio allows discrimination between the contributions of sample size and the changes in photochemistry to the F_0 parameter, a difficulty not easily solved with other fluorescence detection systems.

CHAPTER 7

SUMMARY AND CONCLUSIONS

7.1 INTRODUCTION

This project clearly demonstrates the feasibility of growth plantlets with improved photosynthetic capacity while under aseptic conditions. It also indicates that growth rates could be much improved as a result of enhanced photosynthesis. This work also opens up new approaches to plant tissue culture techniques. It shows that through careful and balanced manipulations of the culture conditions it is possible to impose favorable physiological changes in plantlets. Several scientific and practical uses may arise from the application of AGEHS technology. New areas of research opened by the use of this system are its applicability to techniques of somatic embryogenesis, conifer tissue cultures, automation of micropropagation schemes and many others.

Physiological characteristics of micropropagated plantlets could be deduced from the application of appropriate assessment methods. This involves the development of an innovative system whereby physico-chemical parameters of the environment are controlled and monitored and also requires refined physiological assessment instrumentation based on fluorescence assessment and CO₂ gas exchange techniques.

Current plant tissue culture techniques do have flaws. The most unequivocal is the lack of flexibility regarding control of the physico-chemical environment. Another obstacle is a lack of knowledge in how to

provide modifications of the physico-chemical environment and how any modification affects plant growth.

7.2 THE PLANT TISSUE CULTURE ENVIRONMENT

In *in vitro* propagation aseptic conditions are mandatory. Although necessary in the early stages, supplied sugars remain a very deterministic element to the tissue culture methods. *Chapter 2* describes a system in which some parameters are controlled with the intent of acclimatizing plantlets while they are shifted from mixotrophic to photoautotrophic nutrition. This feature alone constitutes an innovation.

The optimum PFD needed by cultures of various organs and tissues differs among stages of micropropagation, explant type and species (Economou and Read, 1987). More importantly, optimization of PFD in any stage will depend on the balance of all other relevant parameters. For example, it is widely believed that exogenously supplied sugar is required for plantlet growth and development as well as survivability under a given set of growth conditions. However, such belief can only be denied if the PFD factor is tested with all other parameters optimally supplied. This study indicates that in semi-sealed and especially sealed culture vials, sucrose helps to minimize photoinhibition due to increased availability of reducing equivalents through increased respiration. The exclusion of any beneficial effects of exogenous sucrose could not be made in this study since growth PFD probably did not replace mixotrophy in *in vitro* Chrysanthemum plantlets.

In sealed vials, respiration is the sole source of carbon for photosynthetic assimilation. Under such conditions, growth is dependent on and limited by respiration, not photosynthesis, as CO₂ can only be recycled in the dark. Photoinhibitory responses are likely to begin soon after the lights are turned on and as CO₂ is depleted by photosynthesis. It is well established from this study as well as from others that photosynthetic electron transfer is not limiting in plantlets at any post-initialization stage of propagation. Limitations are therefore imposed from other parts of the photosynthetic process *i.e.* more towards the sites of dark reactions.

In semi-sealed vials such as those closed with stoppers that allow only limited air-exchange, photosynthesis must stop when CO₂ is depleted. When elevated PFD is provided, passive diffusion of gases can not suffice the demand for photosynthesis. Under assessment PFD (350 μmol photons m⁻² s⁻¹), the average plantlet required at least 500-800 ml min⁻¹ of air for optimal availability of CO₂. Again, respiration being mostly fueled by exogenous sugars, when present, provides a limited protection against deleterious effects of excess light. This capacity to avoid extensive photoinhibition may be linked to the capacity to use respiratory pathways.

Under growth conditions with enhanced gas-exchange, light and availability of water and/or certain nutrients seem to become the next limiting factor. When plants are grown *in vivo* with suboptimal phosphorus they have lower photosynthetic rates and lack secondary fluorescence transients such as F_{S1} , F_{M1} , F_{S2} , F_{M2} (Brooks, 1986). Plantlets grown *in vitro* tend to lack secondary fluorescence transients. Since many media formulations for tissue culture are derived empirically, for callus or other

tissues grown under conditions suboptimal for photosynthesis, substantial modifications are necessary with the changeover to photoautotrophy. With organic addenda removed, such as sugars and vitamins, autoclaving media is unnecessary. This would prevent excessive precipitation of phosphate salts and would therefore be more available to the plantlets.

7.3 PROSPECTS OF THE AGEHS

The main features of the Aseptic Gas Exchange and Hydroponic System is the direct measurement of photosynthesis *in vitro*. Photosynthetic gas-exchange characteristics, when adequately measured and interpreted, as the plantlets are growth, allow feedback control upon the physico-chemical environment. The benefits of such a system are summarized as follows:

- it enhances growth of plantlets.
- it allows *in vitro* acclimatization since it monitors and enhances photosynthetic development.
- it helps prevent photoinhibition or even photodamage since it indicates immediate effects of increasing PFD and/or changing relative humidity.
- it allows changes of the medium and medium formulation without handling the plantlets.

7.4 FLUORESCENCE AND GAS-EXCHANGE ASSESSMENT SYSTEMS

The Integrated Fluorescence Data Acquisition System (IFDAS) has, since its construction, demonstrated its usefulness in several applications of plant physiology studies (Brooke *et al.* 1990; Melakeberhan *et al.* 1990; Vidaver *et al.* 1990; Vidaver *et al.* 1988). This present study extends its applicability to plant tissue culture physiology.

Features such as elevated incident or excitation PFD for integrated fluorescence induction studies, combined with the light quanta absorption (I_{ABS}) with the computer interface are innovations inherent to this system. A limitation of this system is due to a low shutter speed (3 ms), which inevitably induces variable chl *a* fluorescence that results in a slight over estimation of the F_0 value. The IFDAS does not provide saturating flashes for the study of the various quenchers during induction of photosynthesis, a minor limitation which prevents the comparison of data obtained with pulse fluorometers.

When combined with photosynthetic gas exchange measurement, the IFDAS provides the capability for direct monitoring of quantum yield of photosynthesis. The information acquired by both assessment systems can help to further examine the correlations between quantum yield to changes in fluorescence kinetics. During this study, the relationship between fluorescence and CO₂ uptake was frequently verified. In most cases, when plants were under conditions less favorable for photosynthesis (or stress), fluorescence characteristics were more closely correlated to the rates of CO₂ uptake. Similar observations were reported in other studies (Adams III *et al.* 1990; Toivonen, 1985).

7.5 CONCLUSION

It is clear from this work that the physico-chemical environment has profound effects on the physiological development of plantlets grown *in vitro*. Optimization of these parameters do confer potential for enhanced photosynthetic capacity in order to improve growth rates *in vitro*. The application of non-destructive assessment methods such as those elaborated in this study, should result in a better understanding of the requirements for optimal plant tissue culture growth.

APPENDIX

ABBREVIATIONS

α_2 (fraction of light directly absorbed by PS _{II})	48
A (the primary electron acceptor)	9
A/D (Analog to Digital converter card)	26
AGEHS (Aseptic Gas Exchange and Hydroponic System)	iii
ASCII (American Standard Code for Information Interchange)	26
BAP (the cytokinin)	31
CF₀-CF₁ (coupling factor of ATP synthesis)	10
Chl <i>a</i> (chlorophyll <i>a</i>)	9
Chl <i>b</i> (chlorophyll <i>b</i>)	9
CO₂ (Carbon dioxide)	iii
CPU (Central Processing Unit of a PC computer)	22
CT-LK (Culture tube treatment under low PFD and semi-sealed with KimKap)	65
CT-LKG (Culture tube treatment under low PFD, semi-sealed with KimKap and gelled medium)	86
CT-LS (Culture tube treatment under low PFD and sealed)	65
CU-VE (Chamber unit under varying PFD and configured for CO ₂ -enriched air flow)	33
CT-VK (Culture tube treatment under varying PFD and semi-sealed with KimKap)	71
CT-VS (Culture tube treatment under varying PFD and sealed)	65
CU-VN (Chamber unit under varying PFD and configured for normal air flow)	23
CU-VNH (Chamber unit under varying PFD, configured for normal air flow and hydroponic system)	86
CU-VEH (Chamber unit under varying PFD, configured for CO ₂ -enriched air flow and hydroponic system)	86
CT-VKG (Culture tube treatment under varying PFD, semi-sealed with KimKap and gelled medium)	86
CTRL (Control treatment)	106
CU-1 and CU-2 (Chamber units 1 and 2 of the AGEHS)	19
Cyt b₆-f (Cytochrome b ₆ -f complex of the electron transport chain)	11
DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)	56
D.M.A. (Direct PC computer Memory Access)	44
D_s (dark signal from electronic circuitry)	44
DW (Dry Weight of the sample)	53
F_d (ferredoxin)	11
F_{MAX} ($F_{VAR}(t)$ when Q_A is maximally reduced)	58
F_{MEAS}(t)} (gross fluorescence signal, in mV)	45
F_{M1} (First secondary peak of fluorescence)	93
F_{M2} (Secondary secondary peak of fluorescence)	93
F_O (dead or initial rise of fluorescence, in mV)	39
F_P (primary peak of the variable fluorescence rise)	48
F_{VAR}(t)} (normalized net variable fluorescence)	39
F_S (Secondary dip of fluorescence quenching)	79
F_T (steady state variable fluorescence level)	66

FW (Fresh Weight of the sample)	53
GA3 (Gibberellic Acid)	31
GLIM ((Generalized Linear Interactive Modeling)	53
GLM (Generalized Linear Modeling)	iv
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)	55
HI-LIGHT (High PFD treatment)	106
I_{ABS} (Light quanta absorbed by the sample)	iv
IFDAS (Integrated Fluorescence Data Acquisition System)	iii
I₀ (quantum flux density in the sphere)	39
I/O (digital Input and Output ports of the A/D card)	43
IRGA (Infra Red GAs Analyzer)	23
I_s (quantum flux density in the sphere with sample)	46
k_{ST} (stray constant)	45
IDW (Leaf DW)	53
L_{ST} (stray and background fluorescence, in mV)	44
ME-LIGHT (Medium treatment)	106
MS (Murashige and Skoog, 1962., medium formulation)	31
μ (mu, the twelfth letter of the Greek alphabet, signifies 10 ⁻⁶)	23
O₂ (molecular oxygen)	7
φ₂ (maximal efficiency of photochemistry of PS _{II})	48
NH₄⁺:NO₃⁻ (ratio of Ammonium to Nitrate salts)	8
NADP⁺ (nicotinamide adenine dinucleotide phosphate)	9
NADPH (the reduced form of NADP ⁺)	9
P⁺ (the oxidized primary electron donor)	10
PAR (Photosynthetically Active Radiation, 400-700nm)	31
PC (plastocyanin)	11
PC (Personal Computer)	iii
PFD (Photon Flux Density, mol quanta m ⁻² s ⁻¹ , 400 to 700 nm)	iii
Pi (H ₂ PO ₄ or inorganic phosphate)	16
PQ/PQH₂ (Plastoquinone-plastoquinol couple)	11
PS_I (photosystem I)	10
PS_{II} (photosystem II)	10
PSU (Photosynthetic Unit)	19
P680 (photochemical electron donor of photosystem II)	10
P700 (photochemical electron donor of photosystem I)	10
Q_A (primary quinone acceptor of PS _{II})	48
REC-RY (Recovery treatment from ME-LIGHT plants)	106
RC_{II} (Reaction Center of PS _{II})	48
RCs (Reaction Centers)	9
RH (Relative Humidity <i>i.e.</i> ratio of vapor pressures)	20
Rubisco (Ribulose bisphosphate carboxylase/oxygenase enzyme)	18
RWC (Relative Water Content)	53
sDW (Stem DW)	53
S.E. (Standard Error of the mean)	47
tDW (Leaf and stem DW)	53

REFERENCES

- Adams III, W.W., B. Demmig-Adams, K. Winter, and U. Schreiber.** 1990. The ratio of variable to maximum chlorophyll fluorescence from photosystem II, measured in leaves at ambient temperature and at 77K, as an indicator of the photon yield of photosynthesis. *Planta*, **180**: 166-174.
- Aitkin, M., D. Anderson, B. Francis, and J. Hinde.** 1989. Statistical Modeling in GLIM. Oxford Statistical Science Series 4. G.K. Eagleson, D.A. Pierce, and B.W. Silverman (Eds).
- Aitken-Christie, J., and C. Jones.** 1987. Towards automation: Radiata pine shoot hedges *in vitro*. *Plant Cell Tissue Organ Culture*, **8**: 185-196.
- Aitken-Christie, J. and T.A. Thorpe.** 1984. Clonal propagation of gymnosperms. *In: Cell Culture and Somatic Cell Genetics of Plants. Volume 1. Laboratory Procedures and their Applications.* I.K. Vasil (Ed.), Academic Press Inc., Orlando. Fla., pp. 82-95.
- Anderson, J.M., and B. Andersson.** 1988. The dynamic photosynthetic membrane and regulation of solar energy conversion. *Trends Biochem. Sci.* **13**: 351-355.
- Andreeva, A.S.** 1983. The influence of preirradiation on the chlorophyll fluorescence kinetics in leaves of *Vicia faba*. *Photosynthetica*, **17**: 515-522.
- Azcon-Bieto, J., H. Lambers, and D.A. Day.** 1983. Effect of photosynthesis and carbohydrate status on respiratory rates and involvement of the alternative pathway in leaf respiration. *Plant Physiol.*, **72**: 598-603.
- Azcon-Bieto, J., and C.B. Osmond.** 1983. Relationship between photosynthesis and respiration. *Plant Physiol.*, **71**: 574-581.
- Baker, N.R., and M. Bradbury.** 1981. Possible applications of chlorophyll fluorescence techniques for studying photosynthesis *in vivo*. *In: H. Smith, (Ed.), Plants and the Day spectrum*, Academic Press, pp. 355-373.
- Bender, L., B. Pauler, and K.-H. Neumann.** 1987. On carbohydrate metabolism of cultured carrot root explants. *Plant Cell Tissue Organ Culture*, **8**: 135-146.
- Bilger W. and U. Schreiber.** 1986. Energy dependent quenching of dark-level chlorophyll fluorescence in intact leaves. *Photosynth. Res.*, **10**, 303-308.

- Björkman, O.** 1981. Responses to different quantum flux densities. *In: O.L. Lange, P.S. Nobel, C.B. Osmond, and H. Ziegler (Eds.), Physiological Plant Ecology I, Encyclopedia of Plant Physiology, Vol. 12a, Springer-Verlag, Berlin, pp. 57-107.*
- Blanke, M.M., and A.R. Belcher.** 1989. Stomata of apple leaves cultured *in vitro*. *Plant Cell Tissue Organ Culture*, **19**: 85-89.
- Bolhar-Nordenkamp, H.R., S.P Long, N.R. Baker, G. Oquist, U. Schreiber, and E.G. Lechner.** 1989. Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Functional Ecology*, **3**: 497-514.
- Bradbury, M., and N.R. Baker.** 1986. The kinetics of photoinhibition of the photosynthetic apparatus in pea chloroplasts. *Plant Cell Environ.*, **9**: 289-297.
- Brainerd, K.E., and L.H. Fuchigami.** 1981. Acclimatization of aseptically cultured apple plants to low relative humidity. *J. Amer. Soc. Hort. Sci.*, **106**: 515-518.
- Brooke, R. C., Lister G. R. , P. Toivonen, S. Dubé, W. Binder, and W. Vidaver.** 1990. Variable chlorophyll fluorescence as an indicator of the effects of water deficits of White spruce [*Picea glauca* (Moench) Voss] and Douglas Fir [*Pseudotsuga menziesii* (Mirb.) Franco] seedlings. (in preparation).
- Brooks, A.** 1986. Effects of phosphorus nutrition on ribulose-1,5-bisphosphate carboxylase activation, photosynthetic quantum yield and amounts of some Calvin-cycle metabolites in Spinach leaves. *Aust. J. Plant Phys.*, **13**: 221-237.
- Cocking, E.C.** 1986. The tissue culture revolution. *In: Plant Tissue Culture and its Agricultural Applications*. L.A. Withers and P.G. Alderson (Eds.), Butterworths, London, England, pp. 3-21.
- Conner, L.N., and A.J. Conner.** 1984. Comparative water loss from leaves of *Solanum laciniatum* plants cultured *in vitro* and *in vivo*. *Plant Sci. Lett.* **36**: 241-246.
- Constantine, D.R.** 1986. Micropropagation in the commercial environment. *In: L.A. Withers and P.G. Alderson (Eds.), Plant Tissue Culture and its Agricultural Applications*. Butterworths, London, pp.175-186.
- Conroy, J.P., R.M. Smillie, M. Koppers, D. Bevege, and E.W. Barlow.** 1986. Chlorophyll *a* fluorescence and photosynthetic and growth responses of *Pinus radiata* to phosphorus deficiency, drought stress, and high CO₂. *Plant Physiol.*, **81**: 423-429.
- Critchley, C.** 1988. The molecular mechanism of photoinhibition - facts and fiction. *Aust. J. Plant Physiol.*, **15**: 27-41.

- Critchley, C., and R.M. Smillie.** 1981. Leaf chlorophyll fluorescence as an indicator of high light stress (photoinhibition) in *Cucumis sativus* L. *Aust. J. Plant Physiol.*, **8**: 133-141.
- Daguin, F., and R. Letouzé.** 1986. Ammonium-induced vitrification in cultured tissues. *Physiol. Plant.*, **66**: 94-98
- Dalton, C.C., K. Iqbal, and D.A. Turner.** 1983. Iron phosphate precipitation in Murashige and Skoog media. *Physiol. Plant.*, **57**: 472-476.
- Desjardins, Y., A. Gosselin, and S. Yelle.** 1987. Acclimatization of *ex vitro* strawberry plantlets in CO₂ enriched environments and supplementary irrigation. *J. Am. Soc. Hortic. Sci.*, **112**: 846-851.
- Dhawan, V. and S.S. Bhojwani.** 1987. Hardening *in vitro* and morpho-physiological changes in the leaves during acclimatization of micropropagated plants of *Leucaena leucocephala* (LAM.) deWit. *Plant Sci.*, **53**: 65-72.
- Dietz, K.-J., S. Neimanis, and U. Heber.** 1984. Rate-limiting factors in leaf photosynthesis II. Electron transport. *Biochim. Biophys. Acta*, **767**: 444-450.
- Dietz, K.-J., U. Schreiber, and U. Heber.** 1985. The relationship between the redox state of Q_A and photosynthesis in leaves at various carbon-dioxide, oxygen and nitrogen regimes. *Planta*, **166**: 219-226.
- Donnelly, D.J.** 1984. Epigenetic variation in the anatomy and physiology of tissue cultured Red raspberry plantlets and transplants. Doctoral Thesis, Simon Fraser University.
- Donnelly, D.J., and W.E. Vidaver.** 1984a. Leaf anatomy of Red raspberry transferred from culture to soil. *J. Am. Soc. Hortic. Sci.*, **109**: 172-176.
- Donnelly, D.J., and W.E. Vidaver.** 1984b. Pigment content and gas exchange of Red raspberry *in vitro* and *ex vitro*. *J. Amer. Soc. Hort. Sci.*, **109**: 177-181.
- Donnelly, D.J., and W.E. Vidaver.** 1988. Glossary of Plant Tissue Culture. Theodore R. Dudley, (Ed.), *Advances in Plant Sciences Series*, Vol. 3. Dioscorides Press, Portland, Or., 141pp.
- Donnelly, D.D., W.E. Vidaver, and K. Kolbow.** 1984. Fixation of ¹⁴CO₂ in tissue cultured Red raspberry prior to and after transfer to soil. *Plant Cell Tissue Organ Culture*, **3**: 313-317.
- Driver, J.A. and G.R.L. Suttle.** 1987. Nursery handling of propagules. *In: Cell and Tissue Culture in Forestry*, Vol. 2; specific principles and methods: Growth and developments. Bonga and Durzan (Eds.) Dordrecht, Martinusnyhoff. pp. 320-335.
- Dubé S., and W. Vidaver.** 1990. An integrating fluorometer data acquisition system. *Plant Physiol. Biochem.*, **28**: 539-546.

- Dujardyn, M., and C.H. Foyer.** 1989. Limitation of CO₂ assimilation and regulation of Benson-Calvin cycle activity in Barley leaves in response to changes in irradiance, photoinhibition, and recovery. *Plant Physiol.*, **91**: 1562-1568.
- Dunstan, D.I., and K.E. Turner.** 1984. The acclimatization of micropropagated plants. *In: Cell culture & somatic cell genetics of Plants.* I.K. Vasil (Ed.), Volume 1, Laboratory procedures and Their Applications. Academic Press Inc., Orlando, Fla. pp. 123-129.
- Economou, A.S., and P.E. Read.** 1987. Light treatments to improve efficiency of *in vitro* propagation systems. *HortSci.*, **22**: 751-754.
- Edelman, J., and A.D. Hanson.** 1972. Sucrose suppression of chlorophyll synthesis in carrot-tissue cultures. *J. Exp. Bot.* **23**: 469-478.
- Fabrizi, A., E. Sutter, and S.K. Dunston.** 1986. Anatomical changes in persistent leaves of tissue-cultured strawberry plants after removal from culture. *Sci. Hortic.*, **28**: 331-337.
- Fuchigami, L.H., T.Y. Cheng, and A. Soeldner.** (1981). Abaxial transpiration and water loss in aseptically cultured plum. *J. Amer. Soc. Hort. Sci.*, **106**: 519-522.
- Fujiwara K., T. Kozai, and I. Watanabe.** 1987. Fundamental studies on environments in plant tissue culture vessels. (3) Measurement of CO₂ gas concentration in closed vessels containing tissue cultured plantlets and estimates of net photosynthetic rates of the plantlets. *J. Agr. Met.*, **43**: 21-30.
- Fujiwara, K., T. Kozai, and I. Watanabe.** 1988. Development of a photoautotrophic tissue culture system for shoot and/or plantlets at rooting and acclimatization stages. *Acta Hortic.*, **230**: 153-158.
- Gautheret, R.J.** 1983. *Plant Tissue Culture: A History.* *Bot. Mag. Tokyo*, **96**: 393-410.
- Gebhardt, K.** 1985. Development of a sterile cultivation system for rooting of shoot tip cultures (Red raspberries) in duroplast foam. *Plant Sci.*, **39**: 141-148.
- Giersch, C., and S.P. Robinson.** 1987. Regulation of photosynthetic carbon metabolism during phosphate limitation of photosynthesis in isolated spinach chloroplasts. *Photosyn. Res.*, **14**: 211-227.
- Govindjee, W.J.S. Downton, D.C. Fork, and P.A. Armond.** 1981. Chlorophyll *a* fluorescence transient as an indicator of water potential of leaves. *Plant Sci. Lett.*, **20**: 191-194.
- Govindjee, G. Papageorgiou, and E. Rabinowitch.** 1967. Chlorophyll fluorescence and photosynthesis. *In: G.G. Guilbault (Ed.), Fluorescence Theory, Instrumentation and Practice, Chap. 12, pp. 511-564.*

- Gamborg, O.L., T. Murashige, T.A. Thorpe, and I.K. Vasil.** 1976. Plant tissue culture media. *In Vitro*, **12**: 473-478.
- Grout, B., and M.J. Aston.** 1977. Transplanting of cauliflower plants regenerated from meristem culture I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. *Hort. Res.*, **17**: 1-7.
- Grout, B.W.W. and M.E. Donkin.** 1987. Photosynthetic activity of cauliflower meristem cultures *in vitro* and at transplanting into soil. *Acta Hortic.*, **212**: 323-327.
- Grout, B.W.W. and S. Millam.** 1985. Photosynthetic development of micropropagated strawberry plantlets following transplanting. *Ann. Bot.*, **55**: 129-131.
- Hartman, H.T., and D.E. Kester.** 1975. *Plant Propagation. Principles and Practices.* Prentice-Hall, Inc., Englewood Cliffs, New Jersey. p. 257.
- Havaux, M., and R. Lannoye.** 1983. Chlorophyll fluorescence induction: A sensitive indicator of water stress. in *Maize plants.* *Irrig. Sci.*, **4**: 147-151.
- Hayashi, M., M. Nakayama, and T. Kozai.** 1988. An application of the acclimatization unit for growth of carnation explants, and for rooting and acclimatization of the plantlets. *Acta Hortic.*, **230**: 189-194.
- Hoddinott, J., and P. Jolliffe.** 1988. The influence of elevated carbon dioxide concentrations on the partitioning of carbon in source leaves of *Phaseolus vulgaris*. *Can. J. Bot.*, **66**: 2396-2401.
- Hofstra, G., and J.D. Hesketh.** 1975. The effects of temperature and CO₂ enrichment on photosynthesis in soybean. *In: Environmental and biological control of photosynthesis.* R. Marcelle. (Ed.), Dr. W. Junk bv Publishers, The Hague. pp. 71-80.
- Horton, P.** 1983. Effects of change in the capacity for photosynthetic electron transfer and photophosphorylation on the kinetics of fluorescence induction in isolated chloroplasts. *Biochim. Biophys. Acta*, **724**: 404-410.
- Howard, J.A., K.L. Mitchell, and K.E. Peterson.** 1983. Effects of exogenous CO₂ on shoot initiation in tobacco cultures. *Plant Sci. Lett.*, **31**: 261-268.
- Hrubec, T.C., J.M. Robinson, and R.P. Donaldson.** 1985. Effect of CO₂ enrichment and carbohydrate content on the dark respiration of soybeans. *Plant Physiol.*, **79**: 684-689.
- Hurd, R.G.** 1968. Effects of CO₂ enrichment on the growth of young tomato plants in low light. *Ann. Bot.*, **32**: 531-542.
- Idle D.B. and Proctor C.W.** 1983. An integrating sphere leaf chamber. *Plant Cell Environ.*, **6**, 437-439.

- Infante, R., E. Magnanini, and B. Righetti.** 1989. The role of light and CO₂ in optimizing the conditions for shoot proliferation of *Actinidia deliciosa* *in vitro*. *Physiol. Plant.*, **77**: 191-195.
- Jenkin, L.E., and T.A. Rees.** 1986. Effects of lack of oxygen on the metabolism of shoots of *Typha angustifolia*. *Phytochemistry*, **25**: 823-827.
- Kevers, C., M. Coumans, M.-F. Coumans-Gilles, and Th. Gaspar.** 1984. Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. *Physiol. Plant.*, **61**: 69-74.
- Knight, S.L., C.P. Akers, S.W. Akers, and C.A. Mitchell.** 1988. *Minitron II* system for precise control of the plant growth environment. *Photosynthetica*, **22**: 90-98.
- Kogl, F., Haagen-Smith, and H. Erxleben.** 1934. Über ein neues auxin ("hetero-auxin") aus harn. *Z. Physiol. Chem.* **228**: 90-103.
- Kotte, W.** 1922. Wurzel Meristem in Gewebekultur. *Ber. Dsch. Bot. Ges.*, **40**, 269-272.
- Kozai, T., K. Fujiwara, and I. Watanabe.** 1986. Fundamental studies on environments in plant tissue culture vessels. (2) Effects of stoppers and vessels on gas exchange rates between inside and outside of vessels closed with stoppers. *J. Agric. Met.*, **42**: 119-127.
- Kozai, T., and Y. Iwanami.** 1988. Effects of CO₂ enrichment and sucrose concentration under high photon fluxes on plantlet growth of carnation (*Dianthus caryophyllus* L.) in tissue culture during the preparation stage. *J. Japan Soc. Hortic. Sci.*, **57**: 279-288.
- Kozai, T., Y. Iwanami, and K. Fujiwara.** 1987a. Effects of CO₂ enrichment on the plantlet growth during the multiplication stage. *Plant Tissue Culture Lett.*, **4**: 22-26.
- Kozai, T., C. Kubota, and I. Watanabe.** 1988. Effects of basal medium composition on the growth of carnation plantlets in auto- and mixotrophic tissue culture. *Acta Hortic.*, **230**: 159-166.
- Kozai, T., H. Oki and K. Fujiwara.** 1987b. Effects of the CO₂ enrichment and sucrose concentration under high photosynthetic photon fluxes on growth of tissue-cultured *Cymbidium* plantlets during the preparation stage. *In*: G. Ducate, M. Jacob, and A. Simeon, (Eds.). *Symposium Florizel: Plant micropropagation in Horticulture Industries*, BPTC Group (Belgium Plant Tissue Culture Group), Arlon, Belgium. pp. 135-141.
- Krause G. H.** 1988. Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. *Physiol. Plant.*, **74**, 566-574.
- Krause, G.H., and E. Weis.** 1984. Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. *Photosyn. Res.*, **5**: 139-157.

- Krikorian, A.D., and D.L. Berquam.** 1969. Plant cell and tissue cultures: the role of Haberlandt. *Bot. Rev.* **35**: 5988.
- Lakso, A.N., B.I. Reisch, J. Mortensen, and M.H. Roberts. J.** 1986. Carbon dioxide enrichment for stimulation of growth of *in vitro* propagated grapevines after transfer from culture. *Amer. Soc. Hortic. Sci.*, **111**: 634-638.
- Langford, P.J., and H. Wainwright.** 1987. Effects of sucrose concentration on the photosynthetic ability on rose shoots *in vitro*. *Ann. Bot.*, **60**: 633-640.
- Lavorel, J., and A.-L. Etienne.** 1977. *In vivo* chlorophyll fluorescence. In: J. Barber (Ed.), *Primary Processes of Photosynthesis*, Elsevier/North-Holland Biomedical Press, pp. 203-268.
- Letouzé, R., and F. Daguin.** 1983. Manifestation spontanée et aléatoire d'une croissance anormale en culture *in vitro*. Recherche de marqueurs métaboliques. *Rev. Can. Biol. Exp.*, **42**: 23-28.
- Macdowall F.D.H.** 1983. An integrating sphere for the rapid nondestructive estimation of whole-plant chlorophyll content. *Can. J. Bot.*, **61**, 3072-3079.
- Maene, L. and P. Debergh.** 1985. Liquid medium additions to established tissue cultures to improve elongation and rooting *in vitro*. *Plant Cell Tissue Organ Culture*, **5**: 23-33.
- Mahlberg, P.G., P. Masi, and D.R. Paul.** 1982. Use of polypropylene film for capping tissue culture containers. *Phytomorphology*, **32**, 397-399.
- Malkin, S., P.A. Armond, H.A. Mooney, and D.C. Fork.** 1981. Photosystem II photosynthetic unit sizes from fluorescence induction in leaves. Correlation to photosynthetic capacity. *Plant Physiol.*, **67**: 570-579.
- Malkin, S., and D.C. Fork.** 1981. Photosynthetic units of sun and shade plants. *Plant Physiol.*, **67**: 580-583.
- Marder, J.B., and J. Barber.** 1989. The molecular anatomy and function of thylakoid proteins. *Plant Cell Environ.*, **12**: 595-614.
- Mauzerall, D., and N. L. Greenbaum.** 1989. The absolute size of a photosynthetic unit. *Biochim. Biophys. Acta*, **974**: 119-140.
- McHale, N.A.** 1985. Conditions for strict autotrophic culture of tobacco callus. *Plant Physiol.*, **77**: 240-242.
- McHale, N.A., I. Zelitch, and R.B. Peterson.** 1987. Effects of CO₂ and O₂ on photosynthesis and growth of autotrophic tobacco callus. *Plant Physiol.*, **84**: 1055-1058.
- Mehrer, I., and H. Mohr.** 1989. Ammonium toxicity: description of the syndrome in *Sinapis alba* and the search for its causation. *Physiol. Plant.*, **77**: 545-554.

- Melakeberhan, H., P.M.A. Toivonen, W.E. Vidaver, J.M. Webster, and S. Dubé.** 1990. The influence of *Bursaphelenchus xylophilus* on the water potential and water-splitting complex of photosystem II of *Pinus sylvestris*. (submitted).
- Meulemans, M., D. Dekegel, and G. Vanheule.** 1987. Ammonium toxicity in potato shoot culture. *Acta Hortic.*, **212**: 263-266.
- Miller, C.O., F. Skoog, F. Okumura, M.H. von Saltza, and F.N. Strong.** 1956. Isolation, structure and synthesis of kinetin: a substance promoting cell division. *J. Am. Chem. Soc.* **78**: 1375-1380.
- Moore, K.G., A. Illsley, and P.H. Lovell.** 1974. Effects of sucrose on petiolar carbohydrate accumulation and photosynthesis in excised *Staples* cotyledons. *J. Exp. Bot.*, **25**: 887-898.
- Morel, G.M.** 1960. Producing virus-free cymbidiums. *Amer. Orchid Soc. Bull.* **29**: 495-497.
- Morissette, J.C., P.C. Meunier, and R. Popovic.** 1988. Automatic integrating fluorometer using an Apple-II and Scope-85. *Rev. Sci. Instrum.*, **59**: 934-936.
- Mousseau, M.** 1986. CO₂ enrichment *in vitro*. Effect on autotrophic and heterotrophic cultures of *Nicotiana tabacum* (var. Samsun). *Photosyn. Res.*, **8**: 187-191.
- Murashige, T., and L.-C. Huang.** 1987. Cloning plants by tissue culture : early years, current status and future prospects. *Acta Hortic.*, **212**: 35-42.
- Murashige, T., and F. Skoog.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, **15**: 473-497.
- Neales, T.F., and A.O. Nichols.** 1978. Growth responses of young wheat plants to a range of ambient CO₂ levels. *Aust. J. Plant Physiol.*, **5**: 45-59.
- Neuman, K.-H., and L. Bender.** 1987. Photosynthesis in cell and tissue culture systems. *In*: Green C.E., D.A. Somers, W.P. Hackett, D.D. Biesboer (Eds.), *Plant Tissue and Cell culture, Proceedings of the Vth International congress on Plant Tissue and Cell Culture, held at the University of Minnesota August 3-8, 1986*. Alan R. Liss, Inc. New York, pp. 151-165.
- Nicholls, D.G.** 1982. *In*: *Bioenergetics, An Introduction to the Chemiosmotic Theory*. Academic Press, Inc., New York.
- Ögren, E. and G. Öquist.** 1985. Effects of drought on photosynthesis, chlorophyll fluorescence and photoinhibition susceptibility in intact willow leaves. *Planta*, **166**, 380-388.

- Öquist G., Hällgren J.-E. and L. Brunes.** 1978. An apparatus for measuring photosynthetic quantum yields and quanta absorption spectra of intact plants. *Plant Cell Environ.*, **1**, 21-27.
- Öquist G. and Wass R.** 1988. A portable microprocessor for measuring chlorophyll fluorescence kinetics in stress physiology. *Physiol. Plant.*, **73**, 211-217.
- Osmond, C.B.** 1981. Photorespiration and photoinhibition. Some implications for the energies of photosynthesis. *Biochim. Biophys. Acta*, **639**, 77-98.
- Papageorgiou G.** 1975. Chlorophyll fluorescence: An intrinsic probe of photosynthesis. *In: Bioenergetics of Photosynthesis*, Govindjee, (ed.), Academic Press, New York, pp. 319-371.
- Payne, C.D. (Ed.)** 1987. The GLIM System, Release 3.77. Generalised Linear Interactive Modelling, Part 1, 2, 3. Numerical Algorithms Group Ltd. Oxford. UK.
- Pospisilova, J., J. Catsky, J. Solarova, and I. Ticha.** 1987. Photosynthesis of plant regenerants. Specificity of *in vitro* conditions and plantlet response. *Biol. Plant.*, **29**: 415-421.
- Pospisilova, J., J. Solarova, J. Catsky, M. Ondrej, and Z. Opatmy.** 1988. The photosynthetic characteristics during the micropropagation of tobacco and potato plants. *Photosynthetica*, **22**: 205-213
- Powles, S.B.** 1984. Photoinhibition of photosynthesis induced by visible . *Annu. Rev. Plant Physiol.*, **35**: 15-44.
- Powles, S.B., and C.B. Osmond.** 1979. Photoinhibition of intact attached leaves of C₃ plants illuminated in the absence of both carbon dioxide and of photorespiration. *Plant Physiol.*, **64**: 982-988.
- Quick W.P. and M. Stitt.** 1989. An examination of factors contributing to non-photochemical quenching of chlorophyll fluorescence in barley leaves. *Biochim. Biophys. Acta*, **977**: 287-296.
- Robbins, W.J.** 1922. Cultivation of excised root tips and stem tips under sterile conditions. *Bot. Gaz.*, **73**: 375-390.
- Saha, S., R. Ouitrakul, S Izawa, and N.E. Good.** 1971. Electron transport and photophosphorylation in chloroplasts as a function of the electron acceptor. *J. Biol. Chem.*, **246**: 3204-3209.
- Salsac, L., S. Chaillou, J.-F. Morot-Gaudry, C. Lesaint, and E. Jolivet.** 1987. Nitrate and ammonium nutrition in plants. *Plant Physiol. Biochem.*, **25**: 805-812.
- Schreiber, U., and W. Bilger.** 1987. Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurements. *In: J.D. Tenhunen et al. (Ed.), Plant Response to Stress*, NATO ASI Series, Vol G15, Springer-Verlag, Berlin, Heidelberg.

- Selby, C., and B.M.R. Harvey.** 1990. The influence of composition of the basal medium on the growth and morphogenesis of cultured Sitka spruce (*Picea sitchensis*) tissues. *Ann. Bot.*, **65**: 395-407.
- Sestak, Z., J. Catsky, and P.G. Jarvis (Eds.).** 1971. Plant photosynthetic production. Manual of Methods. Dr. W. Junk N.V. Publishers, The Hague, pp. 161-166.
- Sharkey, T.D.** 1985. Photosynthesis in intact leaves of C₃ plants: physics, physiology and rate limitation. *Bot. Rev.*, **51**: 53-105.
- Sharkey, T.D., K. Imal, G.D. Farquhar, and I.R. Cowan.** 1982. A direct confirmation of the standard method of estimating intercellular partial pressure of CO₂. *Plant Physiol.*, **69**: 657-659.
- Shimada, N., F. Tanaka, and T. Kozai.** 1988. Effects of low O₂ concentration on net photosynthesis of C₃ plantlets *in vitro*. *Acta Hortic.*, **230**: 171-175.
- Short, K.C., J. Warburton, and A.V. Roberts.** 1987. *In vitro* hardening of cultured cauliflower and chrysanthemum plantlets to humidity. *Acta Hortic.*, **212**: 329-334.
- Sivak, M.N., and D.A. Walker.** 1985. Chlorophyll *a* fluorescence: can it shed light on fundamental questions in photosynthetic carbon dioxide fixation? *Plant Cell Environ.*, **8**: 439-448.
- Smolov, A.P., V.S. Polevaya, and T.N. Smolova.** 1983. Effect of the concentration of O₂ on O₂ and CO₂ exchange of heterotrophic and photoheterotrophic rye tissue culture. *Sov. Plant Physiol.*, **30**: 210-215.
- Solarova, J.** 1989. Photosynthesis of plants regenerants. Diurnal variation in CO₂ concentration in cultivation vessels resulting from plantlets photosynthetic activity. *Photosynthetica*, **23**: 100-107
- Stitt, M., and H. Grosse.** 1988. Interactions between sucrose synthesis and CO₂ fixation I. Secondary kinetics during photosynthetic induction are related to a delayed activation of sucrose synthesis. *J. Plant Physiol.*, **133**: 129-137.
- Stitt, M., and U. Schreiber.** 1988. Interactions between sucrose synthesis and CO₂ fixation. III. Response of biphasic induction kinetics and oscillations to manipulation of the relaxation between electron transport, Calvin cycle, and sucrose synthesis. *J. Plant Physiol.*, **133**: 263-271.
- Strand, M., and G. Öquist.** 1985a. Inhibition of photosynthesis by freezing temperatures and high light levels in cold-acclimated seedlings of Scots pine (*Pinus sylvestris*). - I. Effects on the -limited and -saturated rates of CO₂ assimilation. *Physiol. Plant.*, **64**: 425-430.

- Strand, M., and G. Öquist.** 1985b. Inhibition of photosynthesis by freezing temperatures and high light levels in cold-acclimated seedlings of Scots pine (*Pinus sylvestris*). - II. Effects on chlorophyll fluorescence at room temperature and 77K. *Physiol. Plant.*, **65**: 117-123.
- Stuhlfauth, T., D.F. Sultemeyer, S. Weinz, and H.P. Fock.** 1988. Fluorescence quenching and gas exchange in a water stressed C₃ plant, *Digitalis lanata*. *Plant Physiol.*, **86**: 256-250.
- Sutter, E.G., and M. Hutzell.** 1984. Use of humidity tents and antitranspirants in the acclimatization of tissue-cultured plants to the greenhouse. *Sci. Hortic.*, **23**: 303-312.
- Sutter, E., and R.W. Langhans.** 1982. Formation of epicuticular wax and its effect on water loss in cabbage plants regenerated from shoot-tip culture. *Can. J. Bot.*, **60**: 2896-2902.
- Tingey, D.T., S. Raba, K.D. Rodecap, and J.J. Wagner.** 1982. Vermiculite, a source of metals for *Arabidopsis thaliana*. *J. Am. Soc. Hortic. Sci.*, **107**: 465-468.
- Tisserat, B., and C.E. Vandercook.** 1985. Development of an automated plant culture system. *Plant Cell Tissue Organ Culture*, **5**: 107-117.
- Toivonen, P.M.A.** 1985. The development of a physiological assessment system for White spruce [*Picea glauca* (Moench.) Voss.] seedlings and micropropagated plantlets. Doctoral thesis. Simon Fraser University.
- Toivonen P. and W. Vidaver.** 1988. Chlorophyll *a* fluorescence and CO₂ uptake in water-stressed white spruce seedlings. *Plant Physiol.*, **86**, 744-748.
- Vidaver, W., G.R. Lister, W. Binder, P.M.A. Toivonen, S.L. Dubé, and R. Brooke.** (1990). Photochemical regulation: Protection from photodamage in conifer seedlings. *Tree Physiol.*, (submitted).
- Vidaver W., P. Toivonen, G. Lister, R. Brooke, and W. Binder.** 1988. Variable chlorophyll *a* fluorescence and its potential use in tree seedling production and forest regeneration. Proc. Combined Mtg. of Western Forest Nursery Associations USDA For. Serv. Gen. Tech. Report, RM-167, pp. 127-132.
- Volenec, J.J., and C.J. Nelson.** 1984. Carbohydrate metabolism in leaf meristems of tall fescue. *Plant Physiol.*, **74**: 590-594.
- von Caemmerer S., and G.D. Farquhar.** 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta*, **153**: 376-387.
- Vu, J.C.V., L.H. Allen, jr, and G. Bowes.** 1989. Leaf ultrastructure, carbohydrates and protein of soybeans grown under CO₂ enrichment. *Environ. Exp. Bot.*, **29**: 141-147.

- Vyskot B. and M. Bezdek.** 1984. Stabilization of the synthetic media for plant tissue and cell cultures. *Biol. Plant.*, **26**: 132-143.
- Wainwright H. and J. Scrace.** 1989. Influence of *in vitro* preconditioning with carbohydrates during the rooting of microcuttings on *in vivo* establishment. *Sci. Hortic.*, **38**: 261-267.
- Walker, D.A.** 1981. Secondary fluorescence kinetics of spinach leaves in relation to the onset of photosynthetic carbon assimilation. *Planta*, **153**: 273-278.
- Walker, D.A., and M.N. Sivak.** 1985. Can phosphate limit photosynthetic carbon assimilation *in vivo*? *Physiol. Vég.*, **23**: 829-841.
- Wallsgrave, R.M., A.J. Keys, P.J. Lea, and B.J. Mifflin.** 1983. Photosynthesis, photorespiration and nitrogen metabolism. *Plant Cell Environ.*, **6**: 301-309.
- Wann, S.R.** 1988. Somatic embryogenesis in woody species. *Hortic. Rev.*, **10**: 153-181.
- Wardle, K. E.B. Dobbs, and K.C. Short.** 1983. *In vitro* acclimatization of aseptically cultured plantlets to humidity. *J. Am. Soc. Hortic. Sci.*, **108**: 386-389.
- Weis E., and J.A. Berry.** 1987. Quantum efficiency of photosystem 2 in relation to 'energy' dependent quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta*, **894**, 198-208.
- Wulff, R.D., and B.R. Strain.** 1982. Effects of CO₂ enrichment on growth and photosynthesis in *Desmodium paniculatum*. *Can. J. Bot.*, **60**: 1084-1091.
- Zink, M.W.** 1989. Regulation of ammonia-assimilating enzymes by various nitrogen sources in cultured *Ipomea* spp. *Can. J. Bot.*, **67**: 3127-3133.