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Studies on the in vitro photosynthetic response and the culture environment in Pisum

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Studies on the *in vitro* photosynthetic response and the culture environment in *Pisum*.

Submitted by Vivian Hope for the degree of Ph.D.

of the University of Bath

1991

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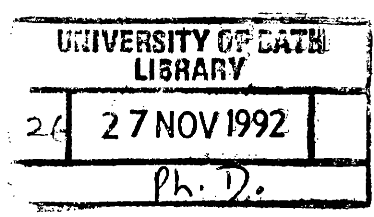
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For

Mishka (Andy)

and Mary

"It's a curious feeling to see the edge receding,
it seems the more I know, the more I don't know,
does this ever end?"

From "A Curious Feeling"
by Tony Banks.

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Abstract.

A series of experiments was performed to assess the *in vitro* photosynthetic physiology of pea (*Pisum sativum*) plantlets.

The time in culture and the type of explant used to initiate the cultures were both found to influence the photosynthetic ability of the resultant plantlets. Three week old cultures derived from shoot tips (apical explants) had photosynthetic abilities comparable to those of *in vivo* seedlings grown under similar conditions. The effects of varying concentrations of 6-benzyl aminopurine (BAP) and sugar in the culture media, and the growth cabinet light intensity were also investigated. BAP above 20 nM was found to inhibit photosynthesis and to reduce pigment content, while a media sucrose concentration of 30 g/l and a growth cabinet light intensity of 150 µE $m^{-2}s^{-1}$ produced cultures with optimal levels. The photosynthetic ability on a per chlorophyll basis (or the photosynthetic efficiency) was greatest at the higher light intensity (300 µE $m^{-2}s^{-1}$).

Measurements were made of the levels of some compounds associated with photoprotection and radical scavenging. At the highest light intensity cultures were found to have a greater ascorbate content and a lower chlorophyll to carotenoid ratio. This was indicative of a higher level of photoprotection. Ultrastructural studies showed that at high light intensities chloroplasts had a reduced lamellal membrane system and exhibited signs of structural damage.

Further experiments involved a modified culture vessel system that was designed to allow sampling of the internal atmosphere. Measurements of the carbon dioxide concentration in these culture vessels showed that this typically fell below the ambient atmospheric level during the light period. This might therefore limit the photosynthetic activity of the cultures.

The media sugar concentration, growth cabinet light intensity and the atmospheric carbon dioxide concentration are discussed in relation to the photosynthetic physiology and growth of *in vitro* grown plants.

Abbreviations.

A	. .	absorbance
ALA	. .	δ-aminolevulinic acid
ATP	. .	adenosine triphosphate
BAP	. .	6-benzyl-aminopurine
C3	. .	CO_2 fixation initially resulting in 3 carbon compounds
C4	. .	CO_2 fixation initially resulting in 4 carbon compounds
CAM	. .	crassulacean acid metabolism
C_{at}	. .	atmospheric CO_2 concentration
C_{in}	. .	CO_2 concentration inside culture vessel
C_{out}	. .	CO_2 concentration outside culture vessel
CPa	. .	chlorophyll protein complex of PSI
CPI	. .	chlorophyll protein complexes of PSII
DTNB	. .	5,5'-dithiobis-(2-nitrobenzoic acid)
DNA	. .	deoxyribonucleic acid
DW	. .	dry weight
EDTA	. .	ethylene diaminetetraacetic acid
Fig.	. .	figure
FW	. .	fresh weight
GSSH	. .	oxidised glutathione
H^+	. .	proton
IRGA	. .	infra red gas analyser
LHCP	. .	light harvesting chlorophyll protein complex(es)
MS	. .	Murashige & Skoog (1962) media salts
NaH_2PO_4	.	sodium phosphate
NAA	. .	naphthalene acetic acid
$NADPH/^+$	.	nicotinamide adenine dinucleotide phosphate oxidised/reduced
NDF(s)	.	neutral density filter(s)
$O_2{}^{\cdot -}$	. .	superoxide radical
PEP	. .	phosphoenol pyruvate
P.G.R.(s)	.	plant growth regulator(s)
ppm	. .	parts per million
psi	. .	pounds per square inch
PSI/II	.	photosystem one/two
RH	. .	relative humidity
RNA	. .	ribonucleic acid
RUBISCO	.	ribulose bisphosphate carboxylase
w/v	. .	weight per volume

Chapter 1

Introduction.

Plant material has been cultured *in vitro* for a variety of purposes: micropropagation, genetic manipulation, physiological studies, germplasm storage, pathogen elimination and for biotechnological uses. Plant culture involves many stages; the exact procedures used depend on the reason for culture, the plant species and to some extent on the particular laboratory.

The successful establishment and maintenance of cultures requires the selection of a suitable culture environment. Environmental factors will have optimum levels, which will depend upon the plant species and the culture process involved (George & Sherrington, 1984). These factors include:

i/ Temperature. This will affect the growth and physiological activity of the cultures. Temperatures in the range 20-25° Centigrade are typically used.

ii/ Humidity. If too low and the cultures will dry out, if too high and they may be prone to vitrification or necrosis.

iii/ Light. This will affect photomorphogenesis and photosynthesis. The wavelength, illumination period and intensity are all important.

iv/ Container. Its size in relation to the size of the culture(s) and its gas permeability will affect the

culture vessel's internal humidity and gas concentrations.

v/ Media composition. The levels of the various micro- and macro-nutrients, the pH, the presence of plant growth regulators (P.G.R.s), and the levels and types of sugar are important.

The success of the culture protocol will also be dependent on explant factors, such as the origin, polarity and age of the explants (George & Sherrington, 1984; Cohen, 1986).

Some of the above culture applications (micropropagation, genetic manipulation, germplasm storage and pathogen elimination) not only require the establishment and maintenance of cultures, but also the transfer of the products back to the *in vivo* environment. During this latter stage, weaning, many problems may arise. Cultures are not only susceptible to pathogens, but also to water loss and nutritional problems. This is because they are adapted to a sterile *in vitro* environment, with high humidity and the provision of a carbon and energy source (sugar).

The culture vessel environment has a high relative humidity (RH), approaching 100%. This affects both the morphology and anatomy of the *in vitro* grown plant material. It has been shown that the leaves of

cultured plantlets when compared to *in vivo* material have smaller amounts of epicuticular wax, fewer and abnormal stomata. As a result plantlets have a potentially high rate of water loss (Grout, 1975; Grout & Aston, 1978a; Sutter, 1982; Conner & Conner, 1984; Donnelly & Vidaver, 1984a; Short *et al*, 1984). Cultured leaves also have less spongy mesophyll and hence reduced dry matter content compared to greenhouse grown plants (Brainerd *et al*, 1981; Wetzstein & Summer, 1982 & 1983).

The transfer of plantlets from culture to *in vivo* is accompanied by a change in environmental conditions. The *in vivo* RH will be much lower than that in culture, thus if plantlets are transplanted directly from the culture vessel to the greenhouse or the field the majority will die due to their inability to control water loss (Grout, 1975). This can be overcome by weaning plantlets off high RH (Brainerd & Fuchigami, 1981). This has been achieved in three ways.

i/ Plantlets are initially transferred to a high humidity environment and the RH is then gradually reduced with time (For example: Grout, 1975; Smith *et al*, 1986). During this process there are no significant changes in the leaves persisting from culture, but new leaves developing become more like and finally the same as leaves of *in vivo* grown plants (Donnelly & Vidaver, 1984a; Donnelly *et al*, 1985).

ii/ The use of foliar antitranspirants to protect plantlets on transfer has been tried; these have however been reported to be ineffective (Sutter & Hutzell, 1984; Wardle *et al*, 1979).

iii/ Plantlets have also been adapted to lower RH in culture, by reducing the RH inside the culture vessel prior to the transfer to soil (Sutter & Langham, 1982; Wardle *et al*, 1983; Ziv *et al*, 1983; Short *et al*, 1984 & 1985).

Cultures can be, and have been, successfully adapted to lower humidities, either prior to or during the transfer to the *in vivo* environment.

The inclusion of sugar in the culture media provides cultures with a readily available carbon and energy source. This has been assumed to result in the development of cultures that are not photosynthetically competent, as the presence of sugar supersedes the need to synthesise sugars from CO_2. water and light energy. The cultures are therefore assumed to be either heterotrophic or mixotrophic, not photoautotrophic like *in vivo* grown plants. Moreover, in the absence of sugar cultures have been typically found to show poor growth (Langford, 1987).

The *in vivo* establishment of *in vitro* grown plantlets poses problems, as they are being

transferred to a sugar-free environment and thus need to be autotrophic. There are however instances in which plant cultures have been grown successfully on media lacking sugar, that is photoautotropically (Pospisilova *et al*, 1987). The development of photoautotrophic cultures would have several advantages over conventional micropropagation systems, for example, easier weaning as the plantlets would already be photosynthetically competent, and reductions in the culturing and weaning costs. The photosynthetic activity of plant cultures will be considered further.

1.1. Photosynthetic physiology: an overview.

This biochemical process is defined as the assimilation of carbon dioxide in the presence of light (an energy source) into carbohydrate with the production of oxygen (for further information see; Foyer, 1984; Halliwell, 1984; Lawlor, 1987).

This process occurs in a specialized plastid, the chloroplast. This organelle is bound by a double membrane. The outer membrane is freely permeable to many substrates, while the inner membrane regulates the passage of solutes by special transport systems. These membranes enclose the stroma, and an organized lamellar membrane system, the grana. The latter has stacked regions of lamellae that are interconnected by unstacked intergranal membranes. There may also be starch grains and a varying number of lipid bodies called plastoglobuli. The stroma also contains protein granules, RNA and DNA.

Chloroplasts develop from undifferentiated plastids, etioplasts which contain a distinctive membrane structure called the prolamellar body. The development of chloroplasts from these is induced by light. The ultrastructure of the chloroplasts is affected by the conditions under which they develop, as well as the plant species. Chloroplasts formed at low light levels ("shade" chloroplasts) have no starch grains, a larger number of broader granal stacks and fewer plastoglobuli than those

formed under high light ("sun" chloroplasts). The division between these "types" is not clear cut, and chloroplasts may adapt their internal structure when the environmental light conditions change (Lichtenthaler & Meier, 1984).

The lamellar membranes contain a high proportion of unsaturated lipids, the photosynthetic pigments (chlorophylls and carotenoids) and numerous proteins. The organization of these components of the photochemical apparatus is related to their function. This is summarized below (Fig. 1a & b).

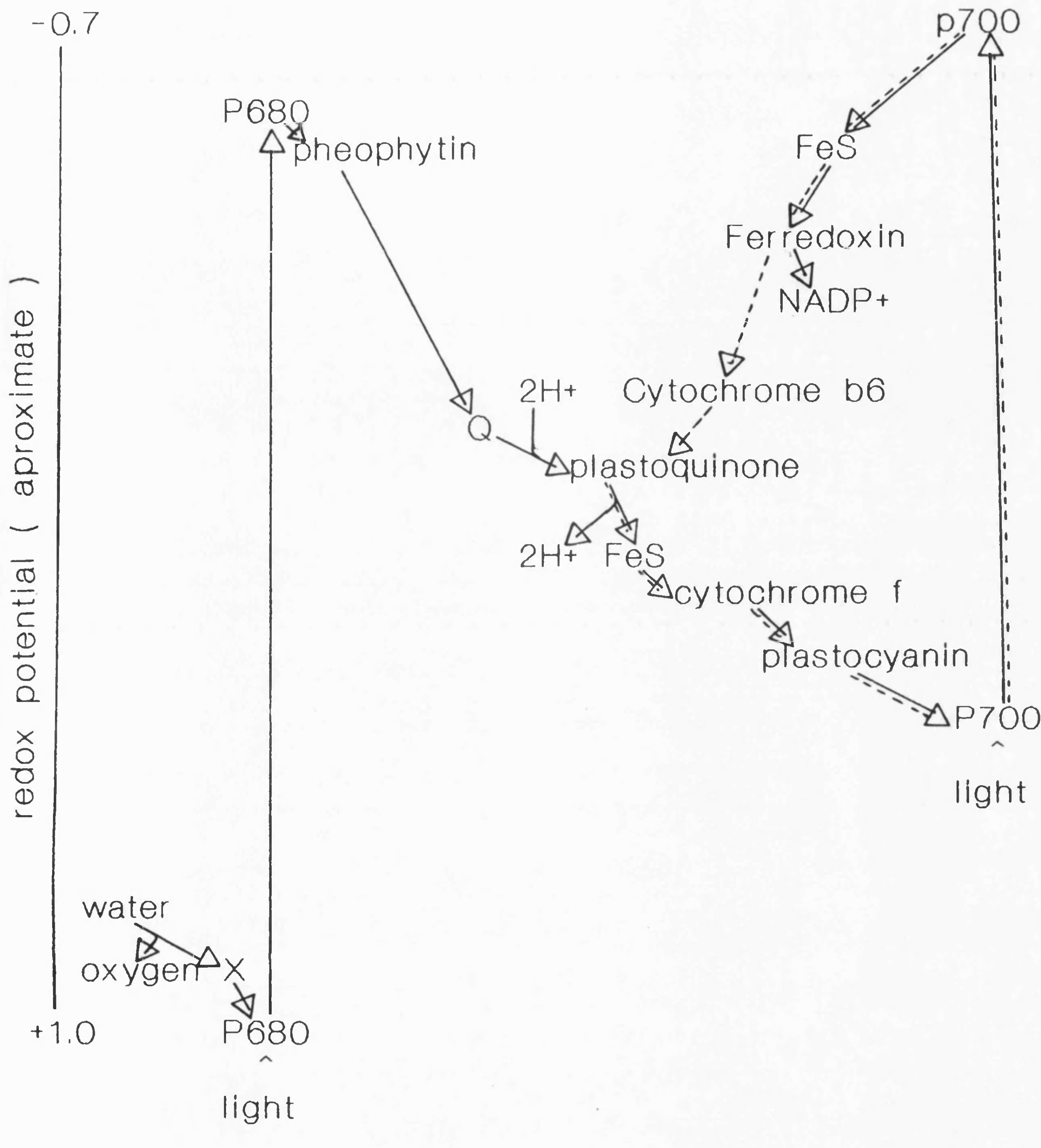
The pigments absorb light energy and channel it to the reaction centres of the two photosystems (Glazer & Melis, 1987). Photosystem I (PSI) consists of a chlorophyll protein complex (CP), CPI which contains the reaction centre p700, chlorophyll a, cartenoids and associated proteins. Photosystem II (PSII) is formed from CP_a, this contains various pigments mostly chlorophyll a, proteins and the reaction centre p680. CP_a is thought to have two types, $PSII_\alpha$ and $PSII_\beta$, which have differences in their pigment contents, and $PSII_\alpha$ may unlike $PSII_\beta$ associate in groups. There are also the light harvesting complexes (LHCP) which are pigment protein complexes containing carotenoids, chlorophyll a and all the chlorophyll b; these are usually associated with PSII.

The light energy, harvested by the

stroma
PSII
CPa
red
Fd
CF1
CF2
PQ
LHCP
X
cytb6
plc
PSI
thylakoid membrane
lumen

LHCP	light harvesting complex
PSI	photosystem I
CPa	chlorophyll-protein complex a
PQ	plastoquinone
X	water splitting complex
cytb6	cytochrome b6
plc	plastocyanin
PSII	photosystem II
red	NADP+ reductase
CF1/2	coupling factors, ATPase
Fd	ferredoxin

Fig. 1a
A diagrammatic representation of a chloroplast thylakiod showing the various components of the electron transport chain.



FeS = Iron sulpher proteins.
—— non-cyclic, ---- cyclic, electron flow.

Fig. 1b
A diagrammatic representation of the "Z" scheme for electron transport in chloroplast thylakoids.

pigments, is used to promote the splitting of water to yield oxygen (O_2), protons and electrons. The electrons are driven along the electron transport chain (Fig. 1a & b) to reduced NADP+. These reactions also lead to a proton gradient across the lamellar membrane, protons being accumulated in the lumen of the grana. This gradient is believed to be used to produce the energy-rich compound ATP. The ATP is formed as the protons return to the stroma through trans membrane ATPases.

The products of the light dependent reactions ATP and NADPH are used to fix carbon dioxide (CO_2) (Fig. 2). Ribulose-1,5-bisphosphate reacts with CO_2 to produce two molecules of 3-phosphoglycerate. This reaction is catalysed by the enzyme ribulose-1,5-bisphosphate carboxylase (RUBISCO). The 3-phosphoglycerate enters into a complex reaction cycle that regenerates the ribulose-1,5-bisphosphate and produces useful carbon-containing compounds. This cycle uses the ATP and reduced NADP+ produced by the electron transport system; these are required at certain stoichiometric levels. This cyclic reaction, commonly called the Calvin cycle, occurs in the stroma of the chloroplast. Some plant species, however, also fix CO_2 using phosphoenol pyruvic acid (PEP). There are two fixation pathways involving the use of PEP: C4 photosynthesis, which occurs in some monocotyledons particularly tropical grasses such as *Zea mays*, and the Crassulacean acid metabolism (CAM) photosynthesis which

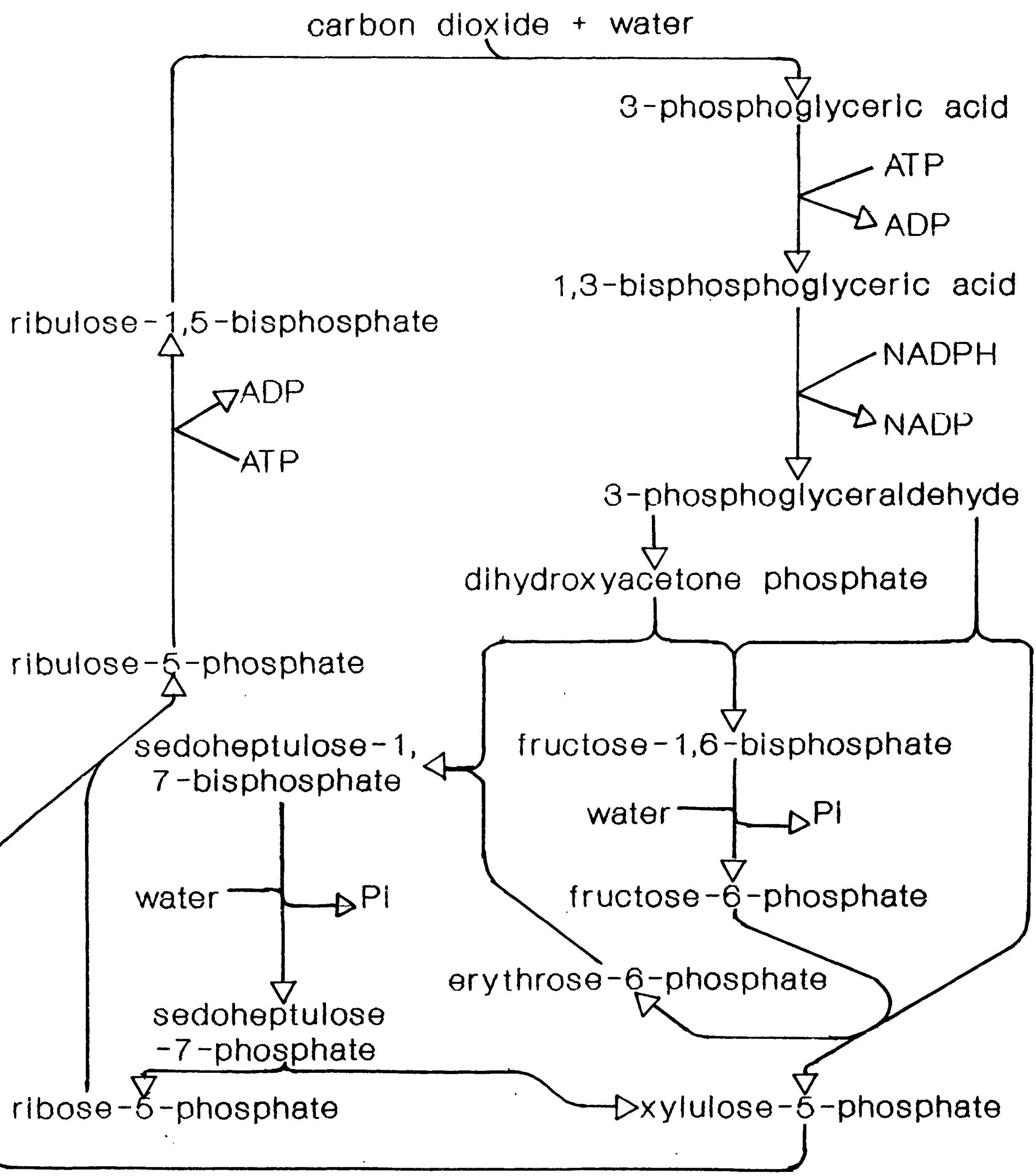


Fig. 2
A diagrammatic representation of the Calvin cycle.

is found in many succulents. The products of this CO_2 fixation are used to feed the Calvin cycle. Plants that directly fix CO_2 using ribulose-1,5-bisphosphate are called C3 plants.

The primary products of the Calvin cycle include triose phosphates, which are used as the basis for starch or sucrose synthesis. The synthesis of starch takes place in the chloroplast stroma, and that of sucrose in the cytosol of the cell. Triose phosphates are selectively counter exported from the chloroplast in exchange for orthophosphate. The relative rates of synthesis can be affected by the physiological need for sugar. A low requirement for sucrose, or "sink" demand, can result in reduced sucrose synthesis and a concomitant increase in the formation of starch. This may also lead to the inhibition of photosynthesis, or "source" production of sugar (Herold, 1980).

Although oxygen is essential for aerobic life forms it can be toxic (Asada & Takahashi, 1987). O_2 can form a number of highly reactive species that can be damaging. Oxygen itself can inhibit photosynthesis as it competes with CO_2 for the active sites of RUBISCO, in a process termed photorespiration (Ogren, 1984). Under certain circumstances the photosynthetic systems absorb more light than is needed; this excess energy would be destructive if not dissipated. During the de-excitation of excited triplet chlorophyll, via the carotenoid pigments, some of the electron flow may be directed to

reduce O_2 producing superoxide ($O_2{\cdot}^-$). This also supports the production of ATP without the formation of reduced NADP (Allen, 1976), thus this O_2 reduction may be important in maintaining the stoichiometric balance of NADPH and ATP required for efficient CO_2 assimilation. The reactive and potentially damaging O_2 species that are produced by these dissipation mechanisms may be scavenged by a number of reactions (Asada & Takahashi, 1987). These scavenging reactions are summarized below:

$$O_2{\cdot}^- + O_2{\cdot}^- + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2$$

$$\text{ascorbate} + 2H^+ + O_2{\cdot}^- \longrightarrow \text{Dehydroascorbate} + H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

$$\text{ascorbate} + 2H^+ + H_2O_2 \xrightarrow{\text{ascorbate peroxidase}} \text{Dehydroascorbate} + 2H_2O$$

The dehydroascorbate generated in the above reactions is reduced back to ascorbate by glutathione (Foyer *et al*, 1983). The oxidised glutathione (GSSH) is reduced by an energy requiring reaction that utilises reduced NADPH.

dehydroascorbate + 2xGlutathione ---> Ascorbate + GSSH

GSSH + NADPH + H^+ ---> 2xGlutathione + $NADP^+$

In addition to superoxide several other reactive radicals of O_2 can be formed. Singlet oxygen can be scavenged by quenchers such as the carotenoid pigments and α-tocopherol (Asada & Taskahashi, 1987). The highly reactive hydroxyl radical is probably scavenged by a mechanism of direct quenching by ascorbate, glutathione and α-tocopherol.

If these toxic species are not scavenged then they may cause damage by lipid peroxidation, inhibition of electron flow and loss of Calvin cycle activity by enzyme inhibition. For example, if H_2O_2 that is formed from $O_2\cdot^-$ accumulates, it will lead to the inactivation of certain of the Calvin cycle enzymes, in particular fructose-1,6-bisphosphotase and sedoheptulose bisphosphatase (Heldt *et al*, 1977; Kasier, 1979). Singlet oxygen can destroy chlorophyll molecules and causes the peroxidation of lipids (Asada & Takahashi, 1987).

1.2. Photosynthesis in culture.

Photosynthetic studies of plant tissue cultures have primarily concentrated on cultures of unorganized cells, such as cell suspension and callus cultures (see review Neuman & Bender, 1987). The study of photosynthesis in differentiated cultures has been limited to a few studies mostly relating to improving protocols for plantlet weaning.

1.2.1. Photosynthesis in unorganized cultures.

1.2.1.1. Cell suspension cultures.

Plant cell cultures in liquid media are often green, or can be greened by using suitable media supplements and/or changes in the culture environment. These cells have been shown to be mixotrophic with up to one third of their energy coming from photosynthesis (Horn & Dalton, 1984). Their photosynthetic activity was affected by the media sugar content and light intensity. From such mixotrophic cultures it has been possible to select photoautotrophic culture lines. These photoautotrophic cultures usually require elevated CO_2 concentrations in the culture vessel atmosphere (up to 2% CO_2) and sometimes higher light intensities. For example, LaRosa *et al* (1984) grew photoautotrophic liquid cell suspension cultures of potato on sugar free media using an atmosphere containing 2% CO_2 at a light intensity of 90-110 $\mu E\ m^{-2} s^{-1}$. These were obtained from

photomixotrophic cultures by gradually reducing the media sugar content from 25 g/l to 2.5 g/l. On transferring the cells to sugar free media, the CO_2 content of the culture vessel atmosphere was enriched. It has, however, been possible in some cases to obtain cultures that do not rely on elevated CO_2 concentrations, for example *Arachis hypogea* & *Daucus carota* (Neumann & Bender, 1987).

A common factor of these photomixotrophic and autotrophic cell cultures is a high level of phosphoenolpyruvate carboxylase (PEPcase) activity (Husemann, 1981; Husemann *et al*, 1979 & 1984; Nato *et al*, 1981 & 1985). Recent studies have shown that this enzyme normally associated with CO_2 fixation in C4 and CAM plants, occurs in many plant species (Aoyagi & Bassham, 1986). Its exact role in the fixing of CO_2 by cultures is not clear but evidence suggests that the products are used to provide carbon skeletons, as well as possibly directly feeding the Calvin cycle (Yamada 1982; Husemann *et al*, 1984; Neumann & Bender, 1987).

Production of carbon skeletons is associated with cell division. Photosynthetic mesophyll cells do not divide, hence the low PEPcase levels found in these cells from C3 leaves. High levels of PEPcase activity, however, are found in regions of plants were cell division occurs (Aoyagi & Bassham, 1986).

The productivity of photoautotrophic

cultures is usually lower than that of mixotrophic cultures of the same species grown on a carbon source (typically sucrose) containing media (Neumann & Bender, 1987). Much of the work on photoautotrophic cell cultures has been associated with general physiological studies, or for producing model systems for testing herbicides, or because of the potential biotechnological applications of such cultures (Neumann & Bender, 1987; Horn & Dalton, 1984).

1.2.1.2. Callus cultures.

Callus cultures are cell aggregates which have arisen by the unorganized growth of a plant tissue. The cells of calli are similar to those of cell suspension cultures and can be greened by careful modification of the culture environment, such as adding plant growth regulators to the media. For example, Ohria *et al* (1975) found that *Ruta* callus grown in the light was green, while that of soyabean became green when BAP was added to the media.

Green callus is capable of photosynthesis and has been shown to have high levels of PEPcase activity. The photosynthetic competence of callus cultures is affected by the same factors that affect cell suspension cultures: light intensity, CO_2 concentration and the carbon source content of the media (Neumann & Raffat, 1973; Berlyn *et al*, 1978; Bender, 1986).

1.2.2. Photosynthesis in plantlets.

Plantlets produced by plant tissue culture are typically derived from apices, axillary buds or as culture regenerants from callus or embryos. They are basically miniature plants growing in an enclosed sterile environment. Measurements of their photosynthetic activity have shown this to be typically lower than that of the *in vivo* grown plant material used for comparisons. Donnelly & Vidaver (1984b) found that at saturating light intensities Red Raspberry (*Rubus idaeus*) plantlets in culture had CO_2 uptake rates two to four times smaller than mature plant leaves. When cultures and seedlings were compared similar differences were found. For example, Grout & Aston (1978b) and Grout & Donkin (1985) with cauliflower (*Brassica oleracea*) found that glasshouse grown seedlings had a photosynthetic ability much higher (approximately 40x) than *in vitro* grown plantlets; they also had higher levels of RUBISCO and electron transport activity. Comparable results have been obtained for other plant species, for example Smith *et al* (1986) for Asian White Birch (*Betula platyphylla*) and Langford (1987) with *Rosa*. The validity of such comparisons between *in vivo* and *in vitro* grown plant material are difficult to judge, since plantlets and *in vivo* grown material have varying growth rates and ontogenies.

Cultures are grown in enclosed vessels that

are designed to reduce the chances of atmospheric microbiological contamination, and thus they have only a limited ability to exchange gases with the atmosphere. The gaseous environment of a culture vessel containing a plantlet that respires and possibly photosynthises will therefore be different from the surrounding atmosphere. Moreover, the culture environment is also different from *in vivo* in that cultures are typically grown on media containing a sugar carbon and energy source, and under an artificial light regime.

The photosynthetic activity measurements in the studies above used plant material that had been removed from the culture vessel, and placed into an assimilation chamber. During the measurement of CO_2 exchange the plant material was typically provided with a supply of atmospheric air and exposed to a high light level, that is, to conditions favourable to photosynthesis. These measurements were therefore not of the actual photosynthetic activity of the cultures (when growing in the culture vessel), but of their photosynthetic potential or ability under the conditions prevalent in an assimilation chamber.

The growth of plantlets under modified culture conditions has been shown in some instances to produce cultures which have photosynthetic abilities that are similar to and in some cases higher than those of comparable *in vivo* grown material. Three culture

environment factors could effect the photosynthetic activity: the sugar concentration of the media, as this provides an alternative carbon and energy source to photosynthesis; the light intensity, the photosynthetic energy source; and the CO_2 content, the level of the photosynthetic "substrate". All three have been shown to have affects on culture photosynthetic abilities.

a/ Media carbon source.

Decreasing the carbon source (sugar) content of the culture media has been shown to result in an increased photosynthetic ability of plantlet cultures. Total removal of the sugar, however, usually results in their death. Evers (1982) using cultures of shoot initials from Douglas Fir found that decreasing the sucrose concentration of the media increased the photosynthetic rate, but that the optimal sucrose concentration depended on the exact source of the explant. Langford & Wainwright (1986 & 1987) also found that lowering sucrose levels of the media increased the photosynthetic ability of cultures of *Rosa* cultivars. The optimum was dependent on the cultivar. They also found that when the sugar content of the media was reduced over successive subcultures, the photosynthetic ability of the plantlets increased, though 10g/l sucrose was the limiting level and below that photosynthetic ability decreased. However, Pospisilova *et al* (1987) grew culture regenerants of *Nicotiana tabacum* on sucrose-free media. These cultures had a photosynthetic ability at saturating light intensity

(1130 μE $m^{-2}s^{-1}$) similar to *in vivo* seedlings grown in sand containing a nutrient solution.

b/ Light intensity.

It has been shown that increasing the growth cabinet light intensity can increase the photosynthetic ability of cultures of some plant species; however excessively high light intensities may reduce it. This is usually associated with a drop in chlorophyll content as the light intensity is increased. Evers (1982) found that Douglas fir cultures grown at 43 Wm^{-2} had lower photosynthetic rates than those grown at 22 Wm^{-2}. Donnelly & Vidaver (1984) found that Red Raspberry (*Rubus idaeus*) cultures grown over the range 2 to 6 Klux had little difference in their photosynthetic rates, though the rate was always lower than that for field grown material. The pigment levels were higher for cultures grown at lower light intensities and comparable to *in vivo* levels. Lee *et al* (1985) with cultures of Sweetgum (*Liquidamber styraciflua*) found that medium light intensities (155 μE $m^{-2}s^{-1}$) produced cultures with higher photosynthetic ability than low or high light intensities (50 & 315 μE $m^{-2}s^{-1}$). They also found that Sweetgum seedlings, grown on perlite watered with nutrient solution, had their maximum CO_2 fixation rate at the highest cabinet light intensity. At all light intensities they had lower photosynthetic abilities and pigment content per unit leaf area than the cultures. Moreover, Langford (1987) found that cultures of rose cultivar

Peace had their maximum photosynthetic ability at an intermediate light intensity, and the pigment content decreased as the light intensity was increased (light intensities used were 3, 10 & 20 Wm^{-2}). Medium and high light intensities appear to enhance the photosynthetic ability of plantlets of some species.

c/ CO_2 concentration of the culture vessel atmosphere.
The CO_2 concentration inside the culture vessel has been shown to potentially limit photosynthesis. Fujiwara *et al* (1987) found in a study with cultures of eight genera of ornamental plant that CO_2 accumulated in the vessel during the dark period, and reached a level between 3000 and 9000ppm by the end of the dark period. However, the CO_2 concentration decreased rapidly at the onset of the light period dropping to 90ppm or less; this decrease in the CO_2 concentration was probably due to photosynthesis. The resultant low CO_2 concentration would have in turn limited the photosynthetic activity of the cultures, which was estimated to be 100x less than their calculated photosynthetic ability. The estimated CO_2 uptake by the cultures was negative, therefore their main carbon source was probably the media sugar. This inhibition of photosynthesis could possibly be overcome by raising the CO_2 content of the culture vessel atmosphere. Lasko *et al* (1986) found that when grape shoots were transferred from culture to nutrient solution-soaked vermiculite, an elevated CO_2 content increased growth, photosynthesis and rooting. Moreover, Kozai *et al* (1987) found that the

growth of *Limonium* hybrid cultures was increased at elevated CO_2 concentrations. This was thought to be due to the increase in the CO_2 concentration promoting photomixotrophic growth.

Combinations of the above three factors may produce even greater differences in the photosynthetic abilities of the cultures. Modification of the above factors may have advantageous effects on the photosynthetic competence of cultures, but may also have other effects on plantlet growth. For example, high and low media sugar concentrations reduced the growth of rose cultures (Langford, 1987), while the DW of *Ribes nigrum* cultures increased concomitantly with light intensity (Flegmann & Wainwright, 1984). These other effects may be undesirable.

Micropropagation produces plantlets *in vitro* that have photosynthetic rates that are typically lower than *in vivo* grown plants (see above). When these plants are transferred to soil they need to develop fully functional photosynthetic systems as they are no longer provided with a carbon source. Studies of plantlets during weaning have shown, at least for some species, that the leaf material from culture degenerates as new leaves are produced. The new leaves had a greater photosynthetic competence than the leaves produced in culture. The overall photosynthetic ability of plants improved with time (age) after weaning (Grout & Aston, 1978b & Grout & Donkin, 1985 with Cauliflower (*Brassica*

oleracea); Grout & Millam, 1985 with Strawberry (*Fragaria ananassa*); Donnelly & Vidaver, 1984 & Donnelly *et al*, 1984 with Red Raspberry (*Rubus ideaus*)). Tagging experiments have also shown that the leaves produced immediately after transplanting are transitional in their photosynthetic ability between those from culture and those of *in vivo* established plants (these have a similar photosynthetic rate to *in vivo* grown plants). Leaves formed successively during weaning have increasing photosynthetic competence (Donnelly & Vidaver, 1984b; Donnelly *et al*, 1984; Short *et al* , 1984; Grout & Millam, 1985).

Lasko *et al* (1986) found that elevated CO_2 concentrations during the weaning process increased the photosynthetic CO_2 assimilation (photosynthetic activity) of Grape Vine (*Vitus*) plantlets, and thus the plantlets showed a greater weight increase than plantlets from a non-elevated CO_2 concentration regime. Donnelly *et al* (1984) found that light intensity affected photosynthetic ability of the transplanted red raspberry plantlets.

It has been found that plantlets typically have a photosynthetic apparatus that functions ineffectively, and on transplanting they are thus not capable of photoautotrophic growth. Photoautotrophic growth can be developed during weaning and this can be assisted by careful control of the weaning environment. However the production of photoautotrophic plantlets

could remove or greatly reduce this need for "photosynthetic weaning", and thus speed the formation of actively growing *in vivo* plants from *in vitro* grown plantlets.

1.2.3. Measuring the photosynthetic activity of *in vitro* grown plantlets.

Photosynthesis can be measured in many ways. Methods which have been used for the measurement of the photosynthetic activities of cultures are briefly described below.

1.2.3.1. Photosynthetic ability.

The photosynthetic ability or potential of cultures (see section 1.2.2.) is their photosynthetic activity under conditions favourable to photosynthesis, that is, adequate or high environmental light intensities and CO_2 concentrations. These conditions are different from those found in the culture vessel and are comparable to those found in favourable *in vivo* environments.

a/ Infra red gas analysis under standard conditions.
The plant material, either leaves or whole plantlets, is removed from culture and placed in the sample chamber of an Infra Red Gas Analyser (IRGA). The IRGA is set up as for standard laboratory use, that is, a high light intensity and a stream of dried atmospheric air (for examples of use see: Grout & Aston, 1978b; Evers, 1982; Donnelly & Vidaver, 1984b; Lee *et al*, 1985; Langford &

Waiwright, 1987). One of the problems with this relatively quick method is that it relies on the placing of the samples in an air stream. This forced ventilation provides a very different environment to that in the culture vessel, where there is only limited air movement.

b/ $^{14}CO_2$ fixation.

Vials containing ^{14}C sodium bicarbonate are placed into a vessel containing the plantlet. This vessel is sealed and left for a set period of time, after which the plant material is removed. The ^{14}C incorporation is ascertained by scintillation counting of the digested plant material (Grout & Aston, 1978b; Short *et al*, 1984). This method is slow and results in the destruction of the plant material.

c/ Oxygen exchange.

Samples of leaf material are placed in an oxygen monitor or electrode, where they are submerged in a reaction solution (Smith *et al*, 1986). This method only uses small samples of the plant material and a liquid environment.

1.2.3.2. Actual photosynthetic activity.

This is an indication of the actual photosynthetic activity of the cultures when growing in the culture vessel, that is, the photosynthetic rate under the conditions prevalent in the culture vessel. This can be measured or estimated as follows.

a/ Measuring photosynthesis under culture conditions.

The CO_2 content and RH in the culture vessel are measured during the light period. The conditions in the analysis chamber are then adjusted to match these levels. The photosynthetic activity can then be measured as in section 1.2.3.1. a or b. However this depends on first measuring, and then successfully reproducing, the culture environment, which may vary from one culture vessel to another within a treatment. This procedure is therefore both slow and complex to perform.

b/ Calculation from measured changes in CO_2 levels.
The CO_2 content inside the culture vessel is measured throughout the diurnal cycle, and the rate of exchange of gasses between the vessel and the atmosphere is also measured. These and the atmospheric CO_2 concentration can then be used to calculate estimates of the photosynthetic activity and ability (Fujiwara *et al*, 1987).

The measurement of photosynthetic activity by infra red gas analysis has been the most common procedure. This method has been used in this study.

1.4. Objectives of this study.

1.4.1. A model system.

Physiological studies of plants have mainly concentrated on certain species such as members of the genera *Nicotiana* and *Phaseolus*, *Zea mays* and *Pisum sativum*. These particular plant species have been studied due to their ready availability in large quantities. They also have the advantage of having levels of secondary metabolites and other metabolic by-products that do not interfere with physiological studies. Physiologically they have been extensively elucidated and furthermore, standard techniques for their physiological analysis have been established. They have been used, for example, in studies on the effects of environmental and chemical treatments. Use of such a species would therefore be advantageous in studies on the effects of environmental factors on photosynthesis in plant tissue cultures.

1.4.2. Tissue culture of *Pisum sativum*.

Studies on tissue cultures of *P. sativum*, the garden pea, have primarily been concerned with the elimination of viruses and clonal propagation, especially in relation to germplasm storage. Certain viral diseases of the pea are known to be seed born; by culturing apical meristems it may be possible to eliminate such viruses from pea stocks (Kartha *et al*, 1974). The culture of meristems may also be useful in germplasm storage.

Meristems are frozen and cold stored, they can then be thawed and plants regenerated by *in vitro* culture when required. Pea meristems have been frozen for 26 weeks, with 61 % being capable of regeneration (Kartha *et al*, 1978). Work on the clonal propagation of peas has also been concerned with the development of more effective propagation systems through the modification of the culture conditions (Kartha *et al*, 1974; Griga *et al*, 1984 & 1986). For example, the growth and proliferation of the cultures depends on the origin of the explant and the concentration of benzyl aminopurine (BAP), a cytokinin in the media (Gould *et al*, 1987). Others workers have regenerated peas from callus cultures, but the genetic stability of the regenerants was found to deteriorate with time in culture (Malemburg, 1979; Hussey & Gunn, 1984; Rablo *et al*, 1984). This genetic instability may be useful in future *P. sativum* breeding programmes. Although micropropagation is not a major method of propagation for peas, tissue culture techniques may have uses in controlling pathogens, germplasm storage and breeding programmes.

The fact that the physiology of *P. sativum* has been extensively elucidated, and that tissue culture techniques have been developed, make *Pisum sativum* a suitable species for the study of plant culture physiology.

1.4.3. The aims of this project.

The photosynthetic ability of plant culture systems have been studied to varying degrees. Plant cell and callus cultures have been the most extensively studied. There have, however, only been a limited number of studies on plantlets, and many of these have been concerned with improving weaning protocols.

The aim of this study was to expand the available information on the photosynthetic ability and physiology of plant tissue cultures by using a "model system", plantlets of *Pisum sativum*. The effects of culture factors such as the media sugar content, the ambient light levels and the culture vessel CO_2 concentrations on photosynthesis have been investigated, with a view to producing cultures that are more photosynthetically competent prior to weaning.

Chapter 2.

General Methods and Materials.

The methods described in this section are those that have been used in more than one experiment. Details of the experiments performed and protocols specific to individual experiments are given in Chapter 3.

a/ Materials.

Chemicals were obtained from either BDH Laboratory Supplies, Poole, U.K. or Sigma Chemical Company Ltd., Poole, U.K., and were of Analar grade where possible. Substances obtained elsewhere have the source indicated.

b/ Experimental Design.

The cultures and plants, used in this study, were grown under controlled conditions in growth cabinets (Section 2.1.). The design of the experiments did not need, therefore, to allow for consideration, in the analysis of the results, of any significant changes due to variation in the environmental conditions (other than the experimental treatments). A block experimental design was therefore unnecessary. The conditions in the growth cabinets may, however, have varied slightly with time and, furthermore, there may also have been edge affects (small variations in conditions between the centre and the sides of the cabinet chamber). To reduce any affects from such factors the experiments were performed twice, and samples for analysis (usually leaves) were derived, where possible, from a number of randomly selected plants or plantlets. The number of replicate results obtained

for each treatment depended upon the amount of plant material available, and therefore, the experimental culture environment as this affected the growth of the cultures. An excess number of cultures was usually initiated to allow for any losses through contamination or damage during manipulations. The replicate results from each run of the experiment were pooled (n = total number of replicates for each treatment from both runs of an experiment).

c/ Procedural reliability.

The experimental techniques employed in an investigation have to be reliable if results are to be regarded as valid. The procedures adopted, therefore, need to give consistent (precise) as well as accurate results for samples with identical levels of the parameter being measured. The consistency of the experimental techniques employed can be checked by applying them to identical (or comparable) samples of the material to be analysed. This could be achieved by either using identical samples (sub-lots of a sample or its extract) for the assays, or ontogenetically comparable samples of plant material (grown under standard conditions) for the IGRA and pigment measurements. Such procedural checks were applied to the experimental techniques used in this study; they were initially employed during the establishment of the laboratory protocols for the techniques, and further

checks were also made at the same-time as the experimental measurements. The results of these checks showed only small variations (for an example see Appendix 1, Fig. A1), indicating, therefore, that the procedures were consistent. The techniques employed in this investigation were calibrated, where possible, by using standards containing known levels of the parameter under investigation (Appendix 1).

d/ Statistics.

The results obtained for each experiment were analysed to reveal any significant differences, across both the whole range of the treatments used, and also between pairs of results. The statistical significance of the results was, therefore, ascertained either by calculating the standard errors of the means (SEM), or by performing the analysis of variance (Anova) test. The SEM were typically used to compare data from experiments involving a variety of growth periods, and the results of assays and weight determinations. The Anova test was used on the results from the gas exchange measurements and pigment determinations of the experiments involving a range of treatment levels. The significance of the data analysed using SEM was ascertained using a (Student's) t-table (where the degrees of freedom were equal to n-1) to ascertain confidence intervals. Before doing an Anova test the data were analysed for normal distribution and for homogenous treatment variances. Following the Anova

tests the results were further analysed using Fishers Multiple Comparison Procedure. This test made pair-wise comparisons of the treatment means that were based on the proceeding Anova test (for further details see Appendix 2, part 2.). The statistical analysis was carried out using Minitab (7.1) on a Gould mainframe computer. The results of the statistical analyses undertaken have been included in this thesis. For the data that has been presented in tabular form, the results of the statistical analyses are given with the mean values in Chapter 3. Where the results have been presented graphically, any significant changes have been noted on the figures, and details of the analyses undertaken given, with the numerical results (mean values), in Appendix 2.

2.1. The establishment of cultures.

Seeds of *Pisum sativum* var. Meteor (Booker Seeds Ltd., Sleaford, U.K..) were sterilized by exposure to 70% alcohol solution for 1 min and 10 to 14% sodium hypochlorite solution containing a few drops of "Tween" (a wetting agent) for 30 min. They were then washed three times in sterile distilled water and subsequently soaked in this for 2 hours (Kartha *et al*, 1978; Hussey & Gunn, 1984; Griga *et al*, 1986). The seeds were germinated aseptically in plastic jars on moist germination paper.

After one week the seedlings were dissected under sterile conditions to yield two types of explant; nodal, obtained by cutting out the cotyledonary node and apical, obtained by cutting off the shoot tip (Griga *et al*, 1986) (Fig. 3). These explants were placed onto Murashige & Skoog (1968) (MS) media (Flow Laboratories, Andover, U.K..), 30 ml in sterile 175 ml glass jars normally containing 30 g l^{-1} sucrose and solidified using 6 g l^{-1} Tissue Culture agar (MC 29, labm/Amersham, Bury, U.K..). The pH of this basal media was adjusted to 5.7 prior to sterilization. The proliferation medium for the nodal explants in addition contained plant growth regulators (P.G.R.s) (1 µM benzyl aminopurine (BAP) and 20 µM naphthalene acetic acid (NAA)) (Kartha *et al*, 1974; Griga *et al*, 1984; Griga *et al*, 1986).

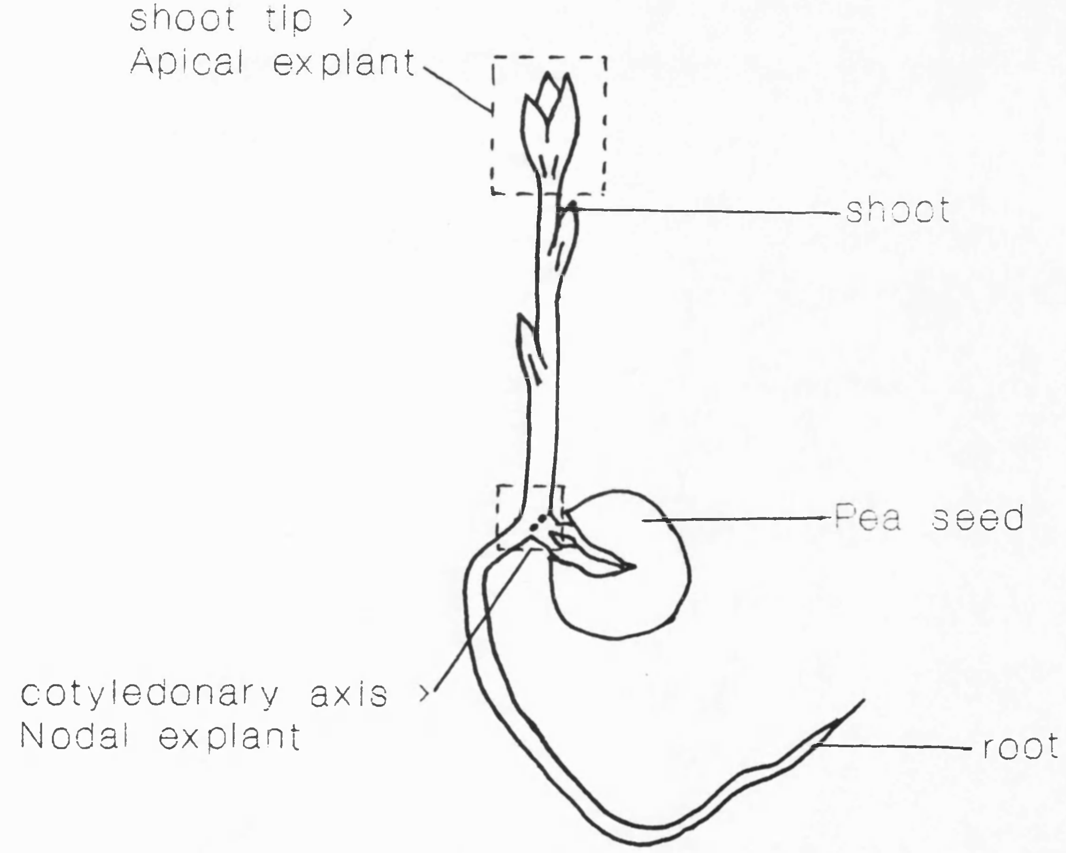


Fig. 3
A diagrammatic representation of a pea seedling showing the regions used as explants.

The jars were covered with the smaller half of a sterile petri dish (45 mm diameter, Sterilin Ltd., Hounslow, U.K..) and placed in a growth cabinet at 25 °C with a 16-hour day at a light intensity of 150 µE $m^{-2}s^{-1}$ (warm white flourescent tubes, five feet (approximately 1.5 m), 65 W, Thorn EMI, U.K..).

The media, distilled water, germination paper and culture vessels were sterilized by autoclaving at 121 °C, 15 psi., for 15 min, using either an automatic autoclave or a pressure cooker. All aseptic procedures were carried out in a laminar airflow cabinet, the working surface of which was sterilized with 70% ethanol. The instruments used were sterilized by immersing in 70% ethanol and flaming.

2.2. The measurement of photosynthetic activity, respiratory rates and the pigment levels.

Photosynthetic ability as CO_2 uptake and the dark respiration rate as CO_2 production were measured using an Infra Red Gas Analyser (IRGA) (Analytical Development Co. Ltd., Hoddesdon, U.K., Series 225.) in differential mode. After the growth period, samples of leaf tissue were removed from the cultures (or plants) and approximately 0.1 g samples placed in the IRGA analysis chamber.

The light source for the photosynthetic measurements was provided by four 100 W tungstan lamps (Decorspot 95, Thorn EMI, U.K..). Heating of the analysis chamber by these lamps was prevented by a fan and a running water heat filter (Fig. 4). The light reaching the analysis chamber (380 µE $m^{-2}s^{-1}$) was measured using a "Licor" light meter (model Li 185) and could be reduced by using neutral density filters (NDFs) mounted on glass plates.

The dark respiration rate (CO_2 production) and the photosynthetic rate (CO_2 uptake in the light) were calculated from the chart recorder trace (Fig. 4). The following equation was used (Long, 1982):

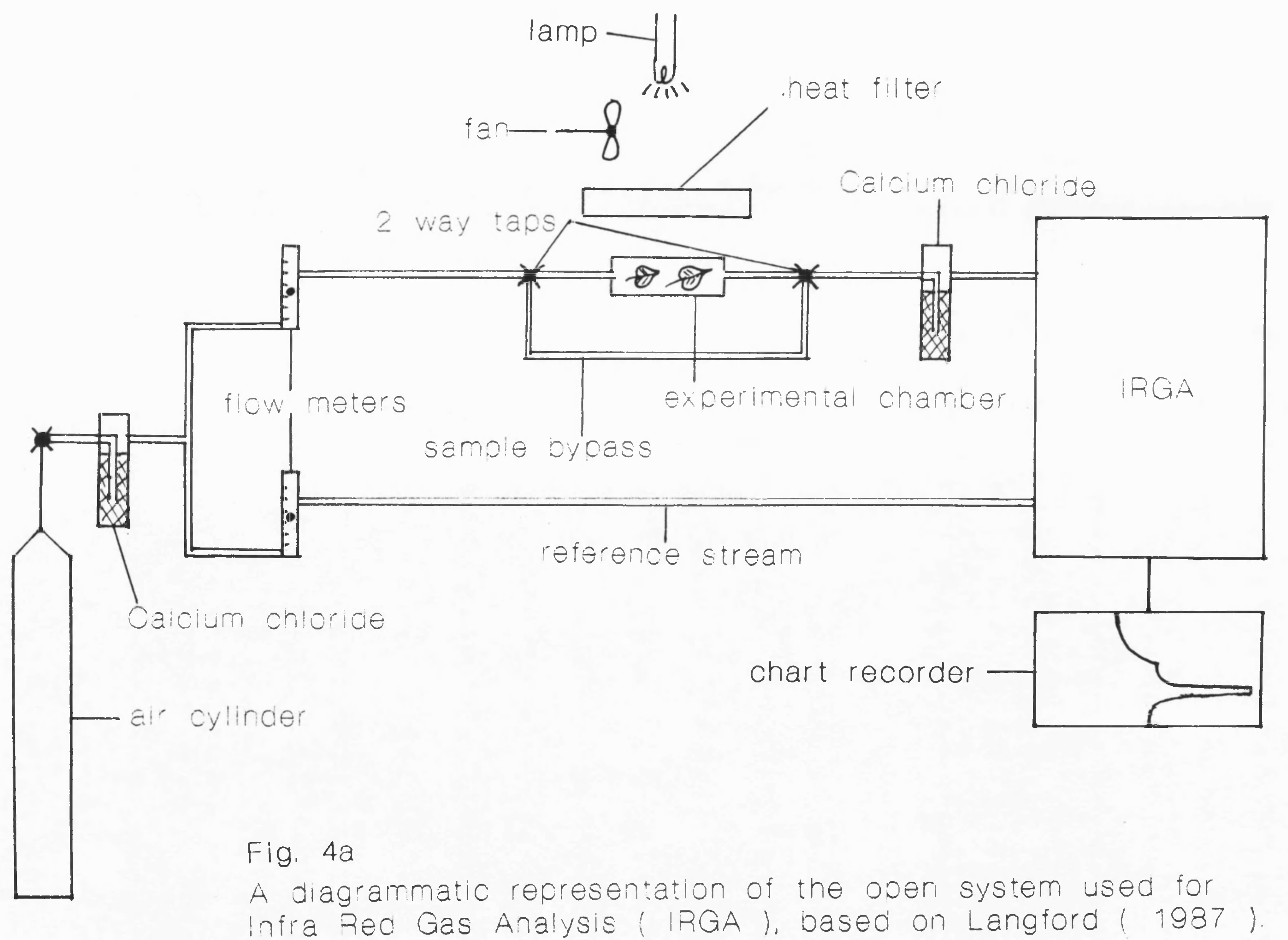


Fig. 4a
A diagrammatic representation of the open system used for Infra Red Gas Analysis (IRGA), based on Langford (1987).

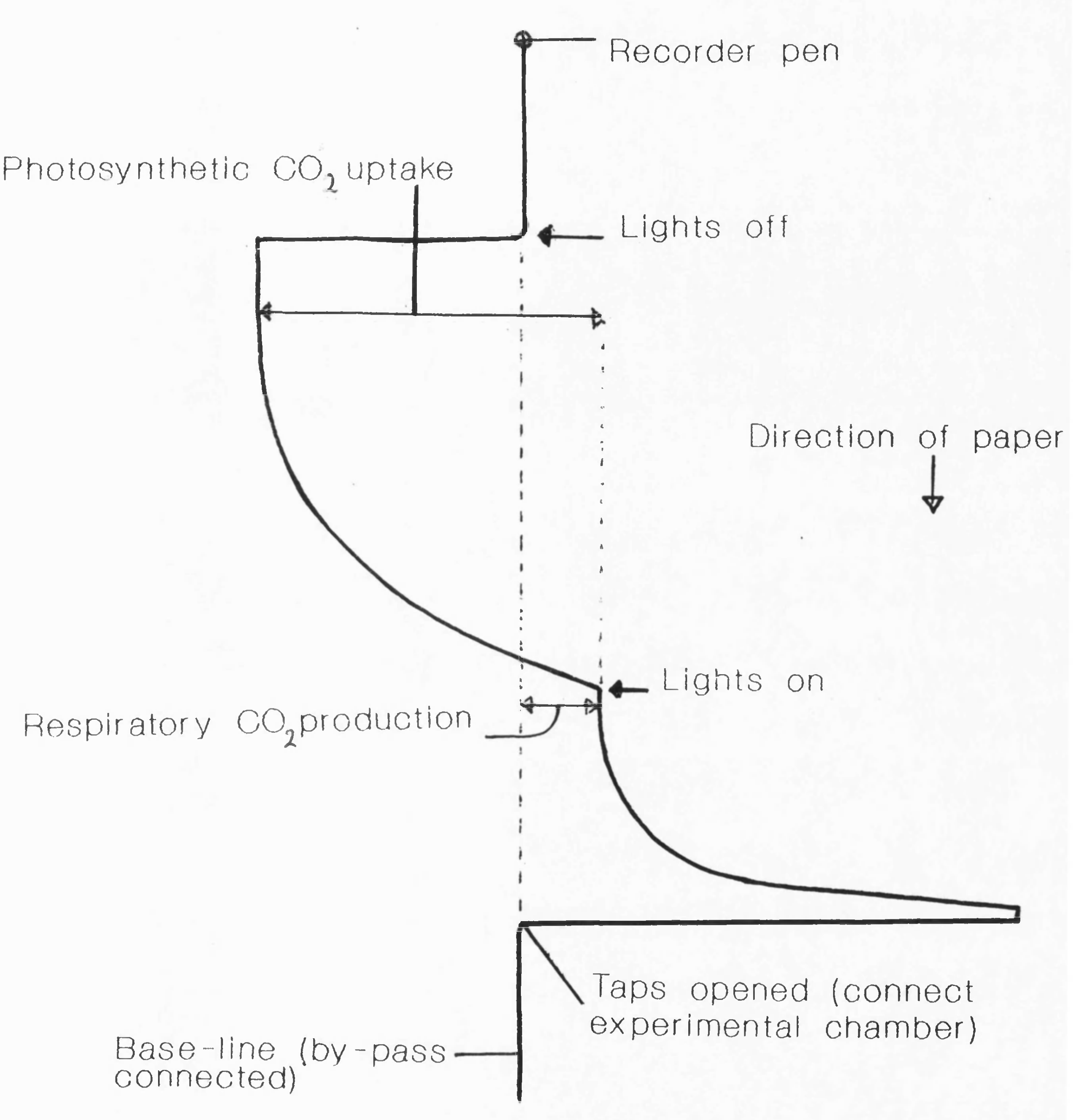


Fig. 4b
A diagrammatic representation of an IRGA chart recorder trace.

$$\text{Photosynthetic or respiratory rate } (\mu mol\ CO_2\ hr^{-1} gFW^{-1}) = \frac{f\ \Delta Co_2}{FW}$$

f = IRGA flow rate, m^3/hr.

ΔCo_2 = Change in carbon dioxide concentration in analysis chamber of IRGA, $\mu mol/m^3$.

FW = Fresh weight, g.

The pigment content of leaf material used for photosynthetic and respiratory measurements was ascertained by extracting the pigments in a known volume of 96% ethanol for 40 hours, at 4 °C and in darkness (Lichtenthaler & Wellburn, 1983). The absorbance (A) was then measured at 470, 649 and 665 nm using a recording UV/visible spectrophotometer (Shimadzu UV-200, Kyoto, Japan.) with a 10 mm fixed cell length.

$$\text{Chlorophyll a } (C_a) \text{ mg/ml} = 13.95\ A_{665} - 6.88\ A_{649}$$

$$\text{Chlorophyll b } (C_b) \text{ mg/ml} = 24.96\ A_{649} - 7.32\ A_{665}$$

$$\text{Carotenoids } (Cr) \text{ mg/ml} = \frac{1000\ A_{470} - 2.05C_a - 114.8C_b}{245}$$

Lichtenthaler & Wellburn (1983).

2.3. The sugar concentration of the culture media.

The sugar concentration of the media was measured by using the anthrone assay (Trevelyan & Harrison, 1952). Samples of media to be analysed were molten and diluted 1:250 with distilled water. One ml samples of the diluted media were layered above 5 ml of 0.2% anthrone (dissolved in 70% sulphuric acid) in test tubes, which were cooled and mixed. The tubes were heated in a boiling water bath for 10 min and, after rapid cooling, the absorbances were read at 620nm using a spectrophotometer (PU 8650 Visible, Pye Unicam, Cambridge, U.K..).

A standard curve was prepared using 1:250 diluted media with known sucrose concentrations of 10, 30 and 50 g l^{-1} (see Appendix 1, Fig. A1).

Media samples that could not be analysed immediately were stored frozen at -20 $^{\circ}$C.

2.4. Fresh and dry weight determinations.

The cultures were removed from the culture vessels and transferred to pre-weighed petri dishes. These were then re-weighed and the fresh weights (FW) determined. The petri dishes containing the cultures were then placed in a 40 ^{0}C oven for 4 days, after which they were transferred to a desiccator containing dried silica gel. A day later the dishes were weighed again and the dry weights (DW) calculated.

Chapter 3.

Experiments and Results.

3.1. Effect of time in culture, plant growth regulators and types of explant on the photosynthetic potential.

3.1.1. Introduction.

The explant, the culture environment and media composition can all have effects on the growth, development and physiology of plant tissue cultures (George & Sherrington, 1984). More specifically, the source of the explant (Evers, 1982), the time in culture (Langford & Wainwright, 1987) and the culture conditions have been shown to affect the photosynthetic physiology of cultures. Knowledge of the effects of these factors on the growth, development and physiology of the cultures is therefore needed, if the effects of other parameters are to be investigated.

Cultures derived from the two explant types, apical and nodal, were subcultured at three-weekly intervals on both basal medium and proliferation medium. The growth and photosynthetic physiology of these cultures and of *in vivo* pea seedlings, grown under similar conditions, were investigated.

3.1.2. Materials & Methods.

a/

Twenty explants of each type (per run of the experiment) were cultured on both basal (MS) medium and proliferation medium (basal (MS) medium plus P.G.R.s) and then

subcultured four times at three weekly intervals. The apical cultures were subcultured by removing the shoot tips to fresh medium. The nodal explants had any shoots removed and were then subcultured to fresh medium (Fig. 5). The leaf material left after each subculture was used to measure photosynthetic ability, respiration rate and pigment content. The FW and DW were also determined for the three-week old cultures.

b/

Apical explants (approximately 200 for each run of the experiment) were cultured on basal (MS) media. After three weeks, 50 of the resultant cultures were subcultured to fresh basal (MS) medium. The photosynthetic ability, respiration rate and pigment contents were ascertained for leaf material taken from two-, three-, four-, five- and six-week old non-subcultured cultures and form five- and six-week old subcultured cultures.

c/

Pea seeds were soaked in water for 3 hours and then sown in half seed trays containing universal compost (Fisons F2 Compost, Ipswich, U.K..). The seed trays were placed in the growth cabinet used for growing the cultures and watered regularly. The photosynthetic abilities, respiration rates and pigment contents were determined for leaf material removed from two-, three-, four-, five- and six-week old intact seedlings.

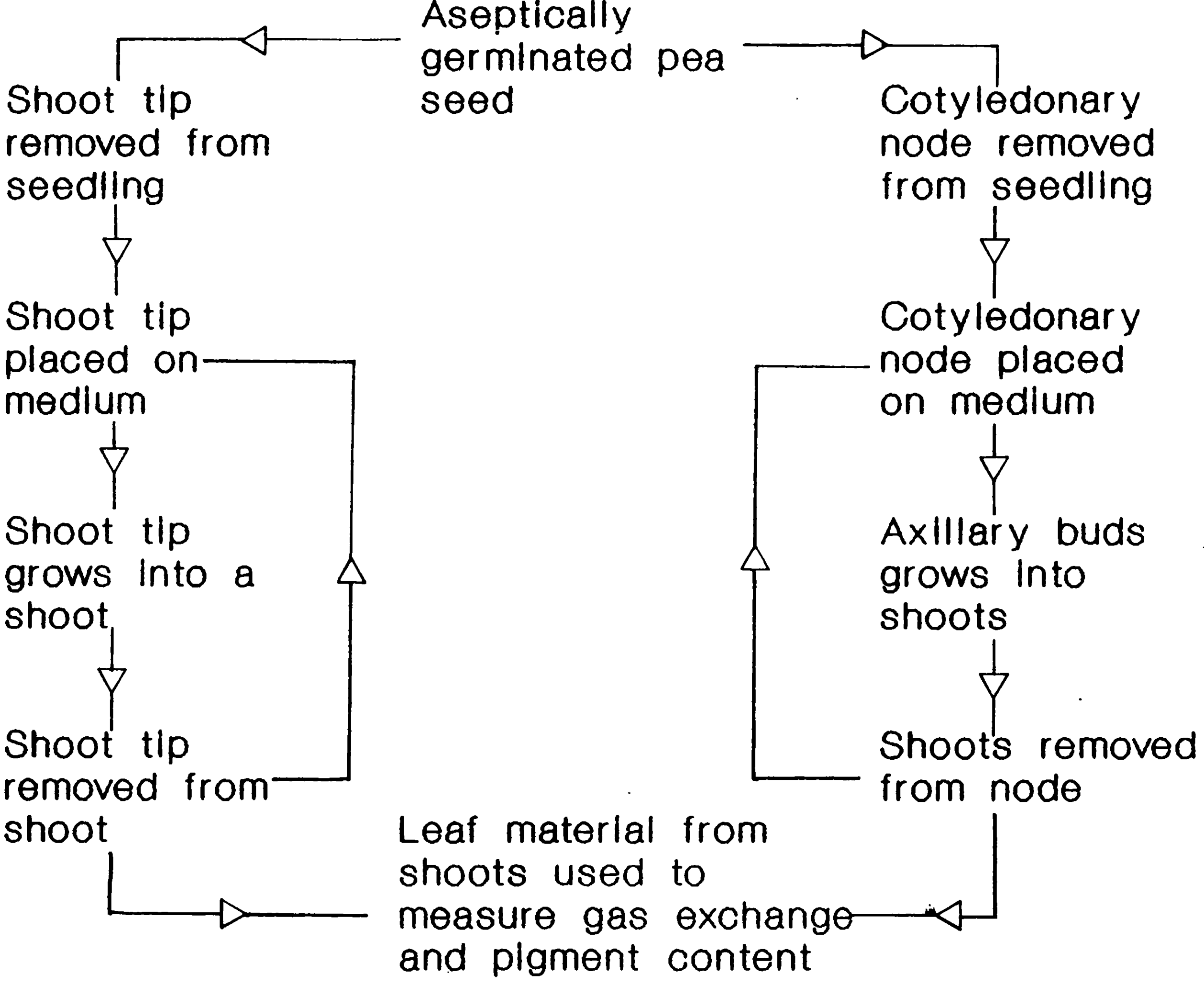


FIG. 5
A summary of the subculturing protocols used for both apical and nodal cultures.

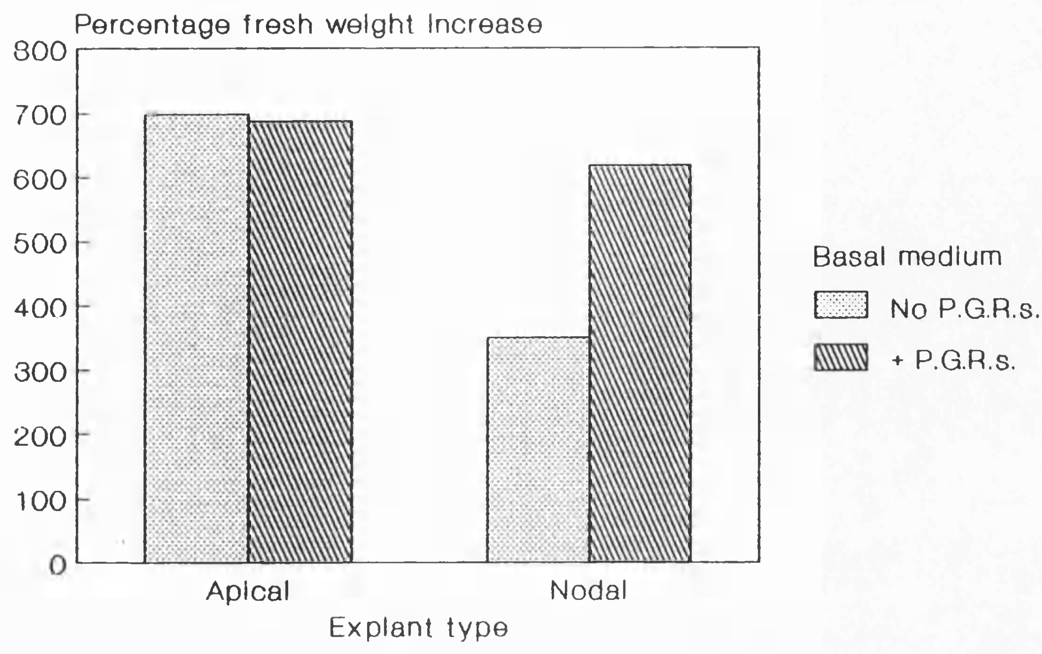
3.1.3. Results.

a/

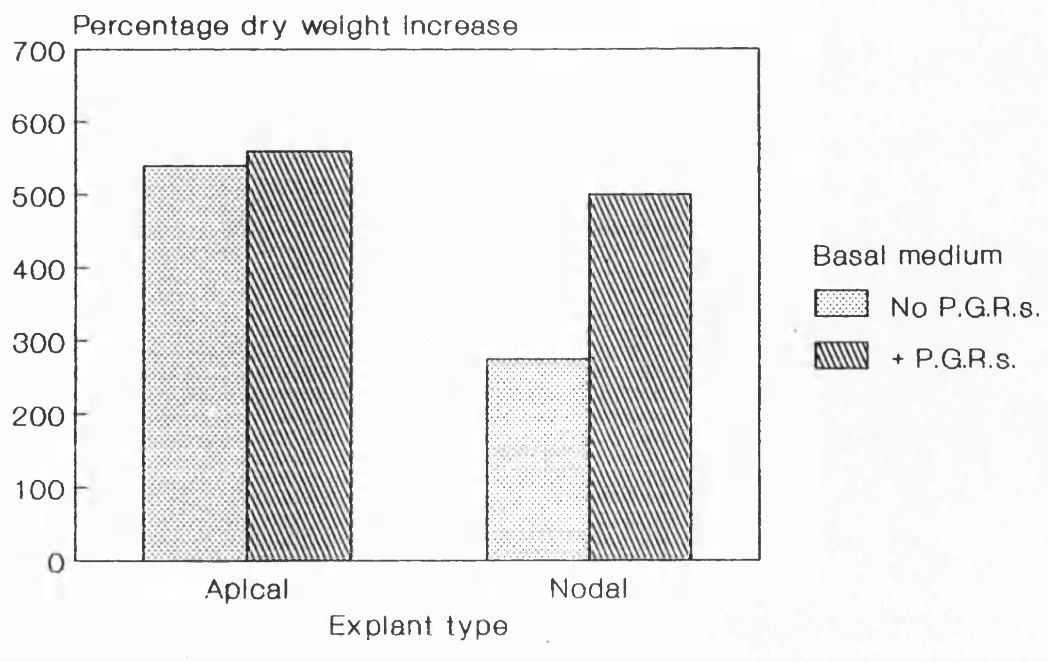
The presence of P.G.R.s (20 µM NAA and 2 µM BAP) in the medium resulted in a greater FW and DW for the 3 week old nodal cultures (Fig. 6). The FW and DW for the apical cultures were similar on both media types. For all four culture/medium combinations the FW and DW showed an increase over those for the explants, showing that the two culture types grew on both media. This growth was due to the development of shoots, roots and/or callus formation by the explants. For the nodal cultures on basal medium, callus formation was probably the main cause of the weight gain, as only a little shoot material was formed.

The shoots harvested from the nodal cultures at the end of each three week subculture period were of the same age, because they had arisen from axillary buds since the explants were last subcultured. The apical cultures were maintained by subculturing the same shoot tips every 3 weeks; therefore the shoots were three weeks older at every subculture and at the fourth subculture they were flowering and beginning to senesce, the shoots having been in culture for 12 weeks. The morphology of the cultures was dependent on both the explant source and the presence of P.G.R.s in the medium (Fig. 7). The presence of P.G.R.s caused shoots to arise from the nodal cultures

Fig. 6 Fresh and dry weight increases of apical and nodal cultures after three weeks.



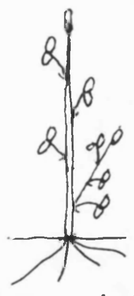
n = 16
Fresh weights for nodal cultures significantly different (at 5% level).



n = 16
Dry weights for nodal cultures significantly different (at 5% level).

P.G.R.s = 20 μM NAA + 2 μM BAP

Fig. 7 Diagrammatic representation of apical and nodal cultures grown on the different media.

	Explant Type	
Media	Apical	Nodal
basal, MS only	One shoot with leaves looks like a minature *in vivo* plant.	Very few shoots or leaves arising from buds, shoots look like miniature *in vivo* plants.
basal (MS) + P.G.R.	 Main shoot with side shoots and small leaves.	Many shoots with small leaves arising from buds.

P.G.R. = 20 µM NAA + 2 µM BAP

and the apical cultures to form side shoots. Both culture types also had smaller leaves in the presence of P.G.R.s.

The photosynthetic ability of the leaves of the apical cultures on basal medium without P.G.R.s was, at the time of the first subculture (3 weeks), significantly higher than that of apical cultures on media containing P.G.R.s and nodal cultures on both types of medium (Fig. 8). At later subcultures all cultures had similar and relatively constant photosynthetic abilities. The dark respiration rates showed no significant trends (Fig. 8).

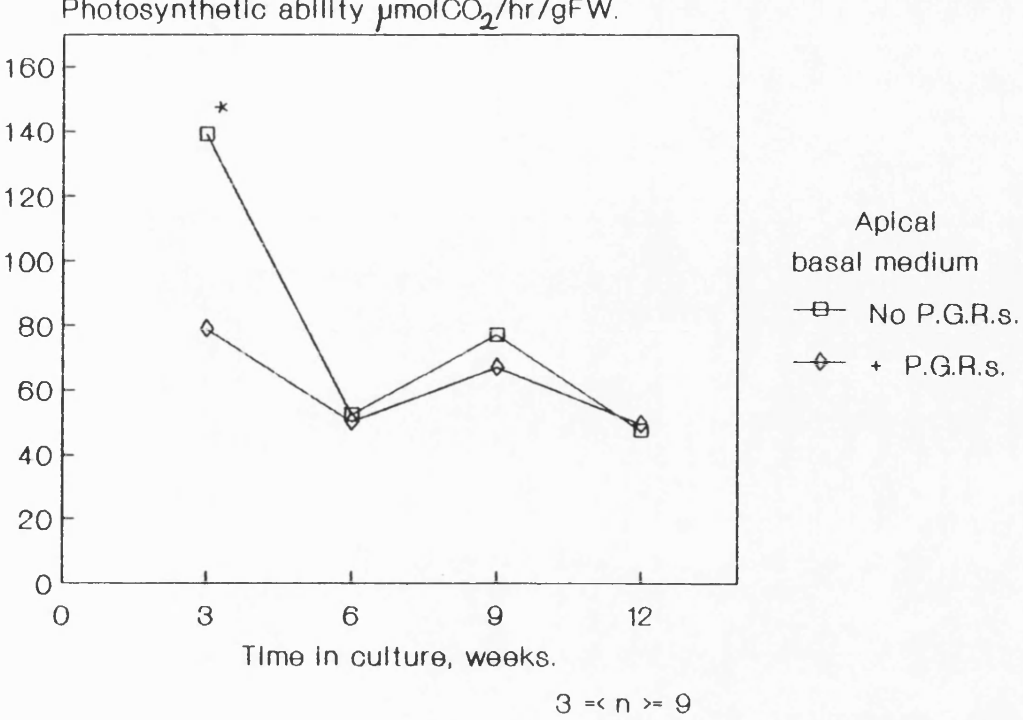
The pigment content of the leaves (Fig. 9, 10 & 11) produced by the cultures showed no obvious trends during the period of the experiment. However, they tended to decline with time, and the chlorophyll levels were lower for cultures grown on media containing P.G.R.s, but only significantly lower for apical cultures at the first and second subcultures.

b/

The two to four week old non-subcultured apical cultures had leaves with similar photosynthetic abilities (Fig. 12), while those five and six weeks old had lower photosynthetic abilities. The leaves of the subcultured cultures had a lower photosynthetic ability than those of the non-subcultured cultures when five weeks old.

The respiration rates (Fig. 12) and pigment contents (Table 1) of the leaves were constant

Fig. 8 a/ Variation of photosynthetic ability with time in culture for apical and nodal cultures.



3 =< n >= 9
* this value is significantly different (at 5% level).

Photosynthetic ability µmolCO2/hr/gFW.

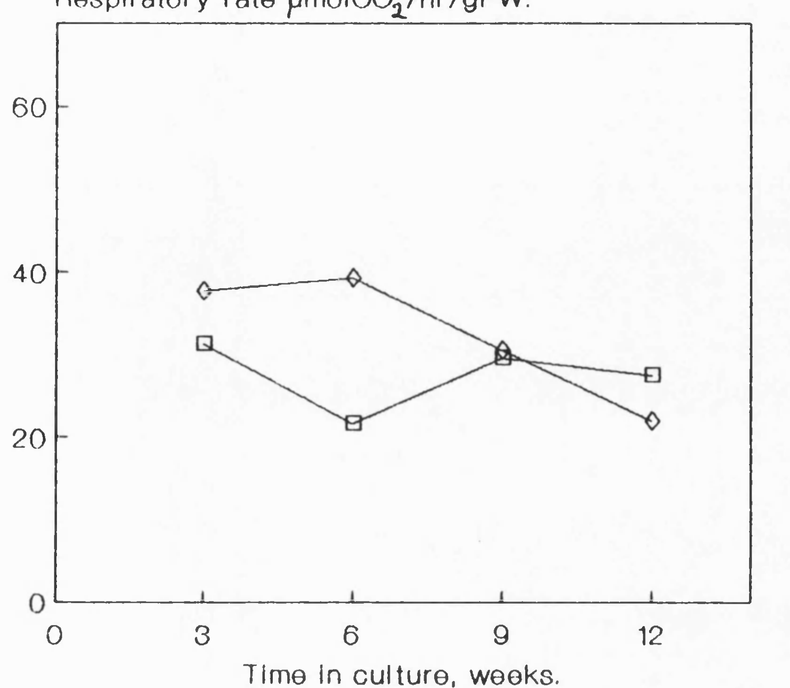
160
140
120
100
80
60
40
20
0
0
3
6
9
12
Time in culture, weeks.

Nodal
Basal medium
No P.G.R.s.
+ P.G.R.s.

2 =< n >= 7
No significant differences.

P.G.R.s = 20 µM NAA & 2µM BAP.

Fig. 8 b/ Variation of the respiratory rate with time in culture for apical and nodal cultures.



Apical

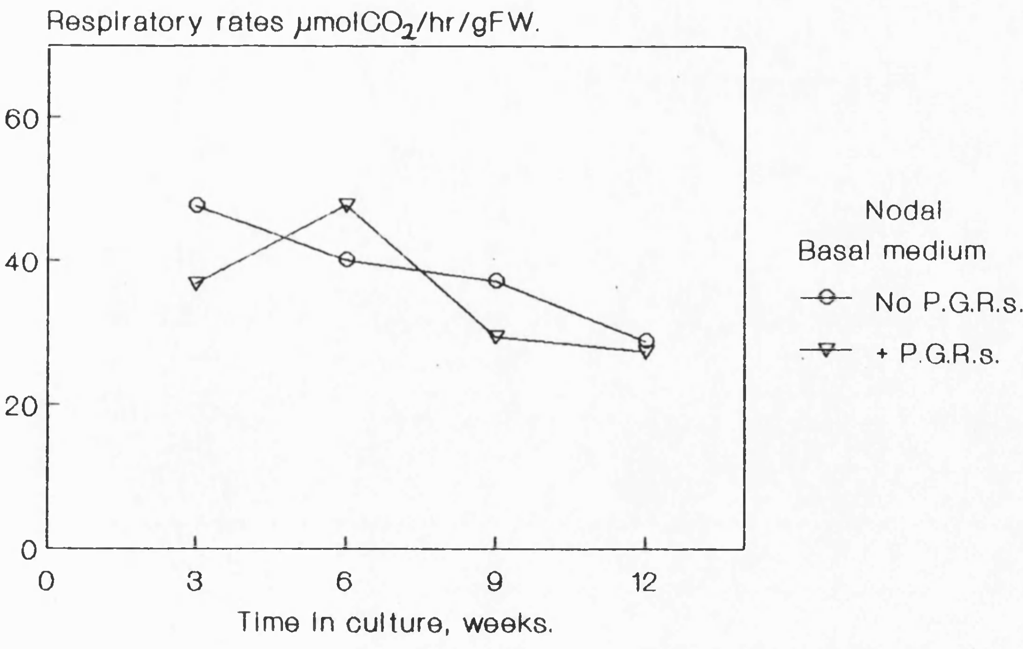
basal medium

No P.G.R.s.

+ P.G.R.s.

3 =< n >= 9

No significant differences

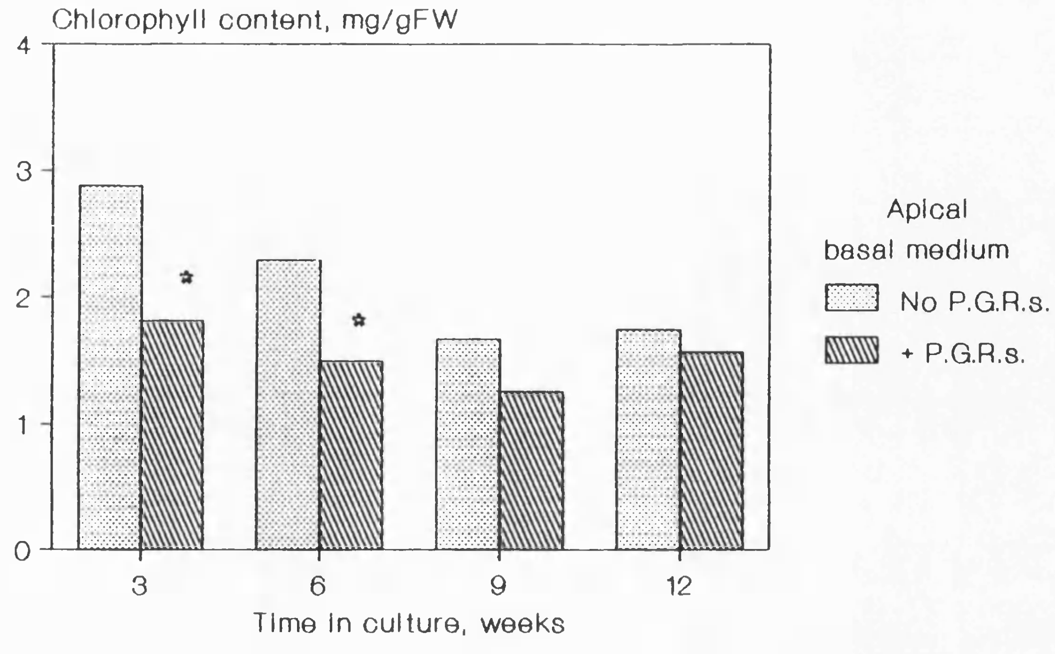


2 =< n >= 7

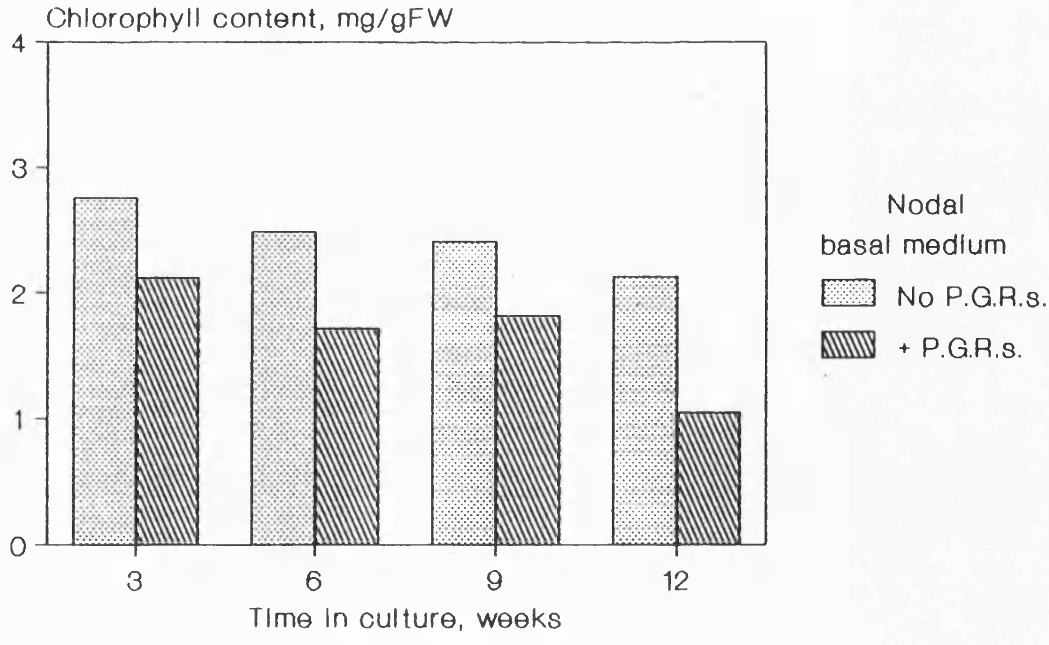
No significant differences.

P.G.R.s = 20 µM NAA & 2µM BAP.

Fig. 9 Variation of chlorophyll content with time in culture.



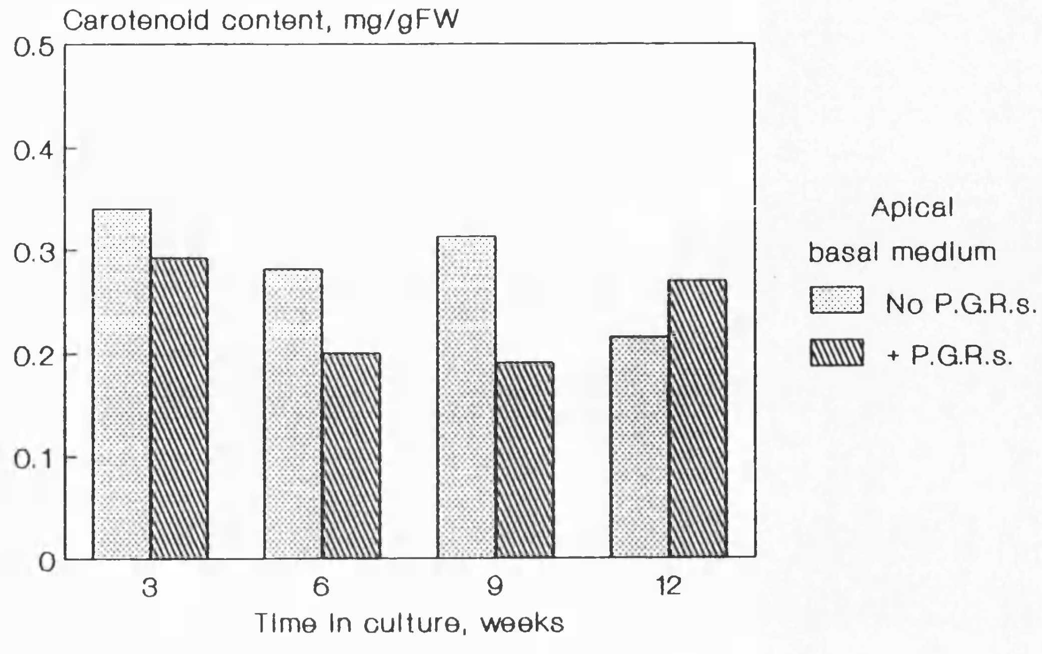
3 =< n >= 9 * significantly lower.
For basal medium no P.G.R.s decrease in chlorophyll significant (at 5% level).



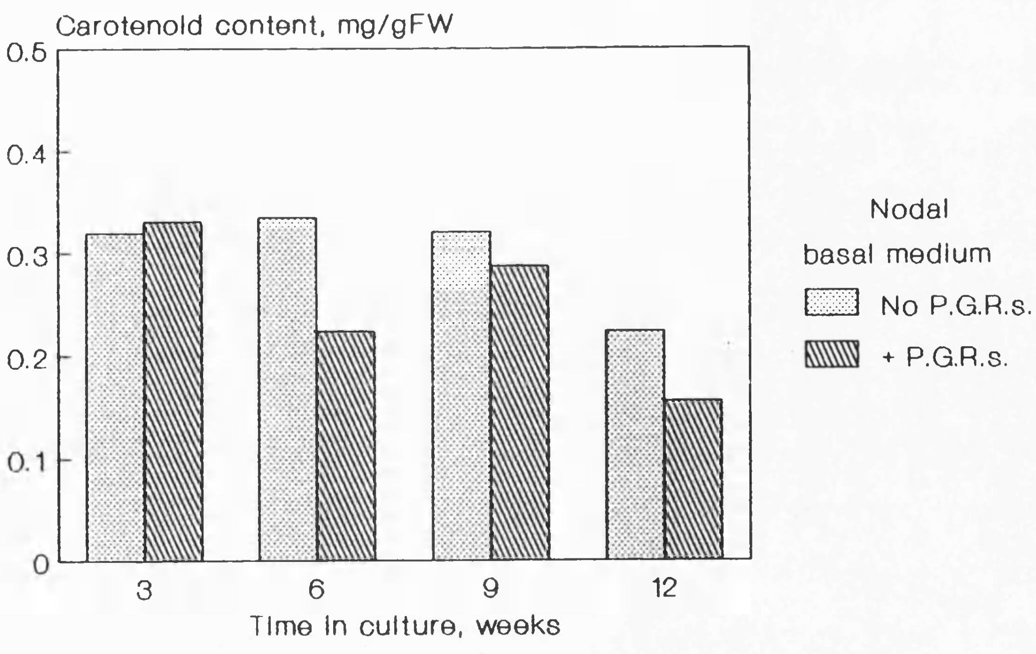
2 =< n >= 7
No significant differences.

P.G.R.s = 20 μM NAA + 2 μM BAP.

Fig. 10 Variation in the carotenoid content with time in culture.



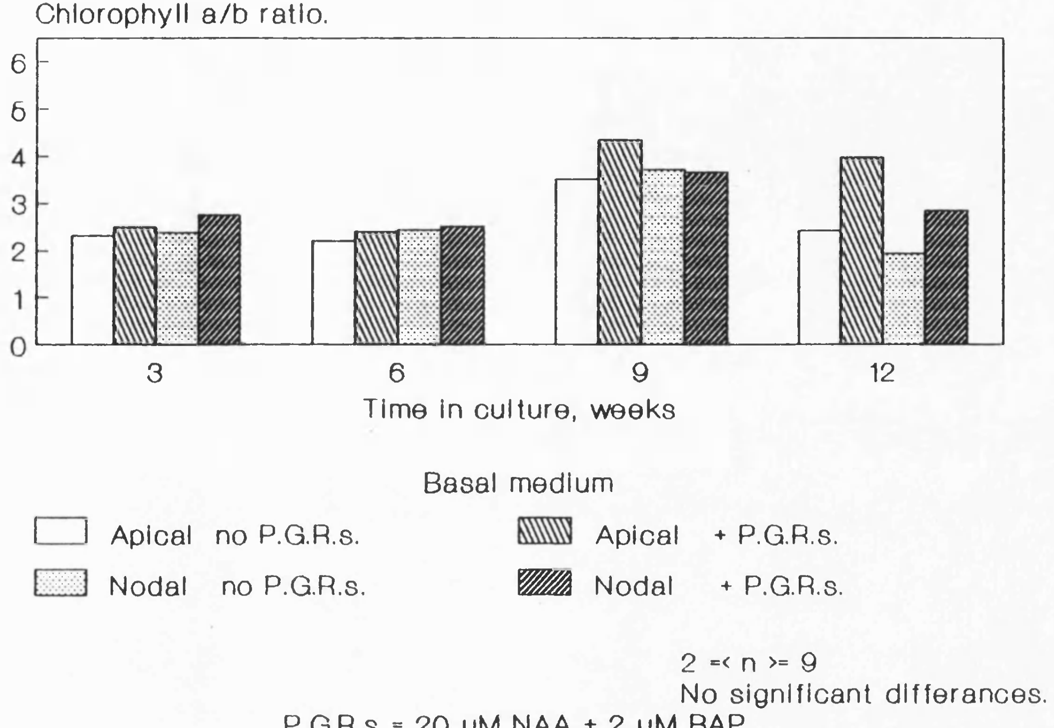
3 =< n >= 9
For basal medium no P.G.R.s, decrease in carotenoids significant (at 5% level).



2 =< n >= 7
No significant differences.

P.G.R.s = 20 μM NAA + 2 μM BAP

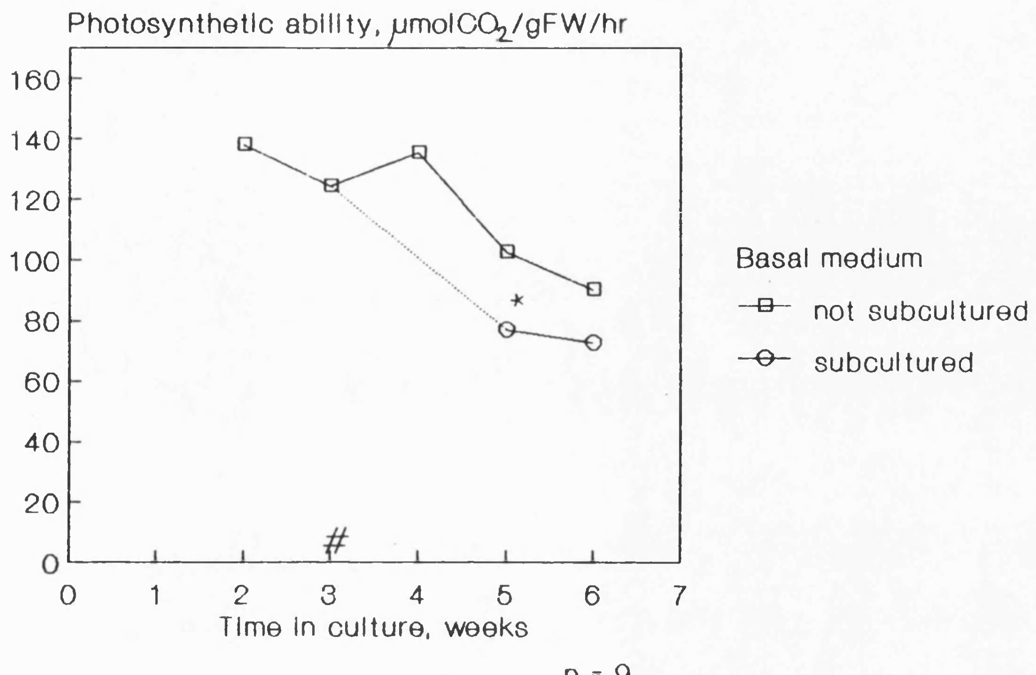
Fig. 11 Variation of the chlorophyll a/b ratio with time in culture.



2 =< n >= 9
No significant differances.

P.G.R.s = 20 μM NAA + 2 μM BAP.

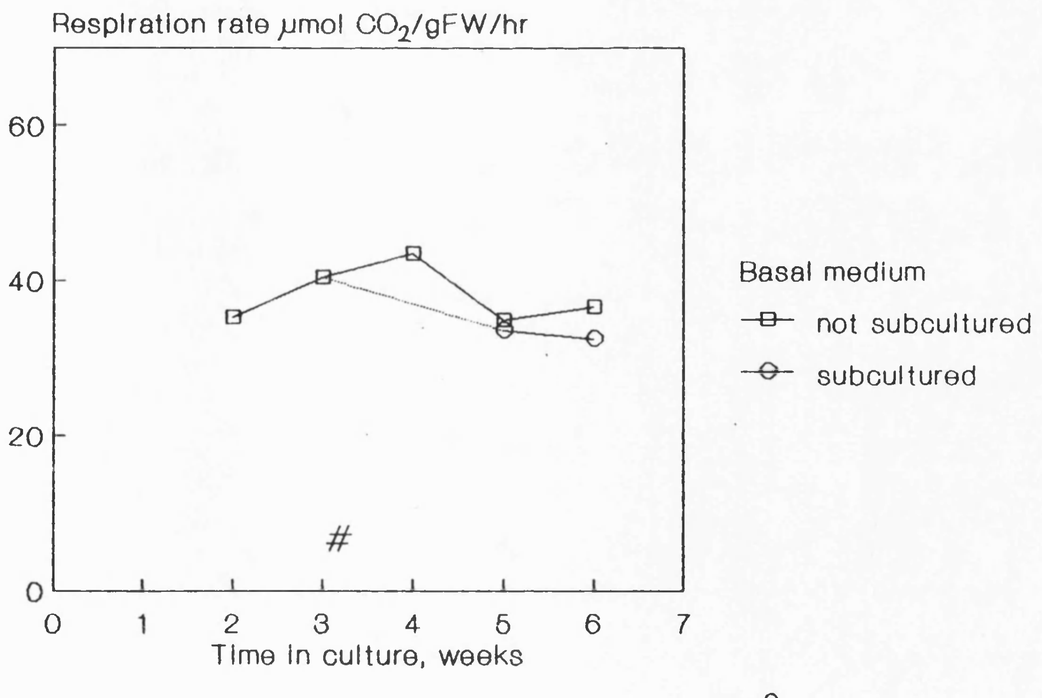
Fig. 12 a/ Variation in photosynthetic ability of apical pea cultures with time.



n = 9
* value significantly different (at 5% level).

time of subculturing.

Fig. 12 b/ Variation in the respiration rate of apical pea cultures with time.



n = 9
No significant differences

time of subculturing.

Table 1 Effect of time in culture on the pigment content of the leaves of apical pea cultures on MS medium, measured at weekly intervals.

Time in culture	Total Chlorophyll		Chlorophyll a/b ratio		Cartenoids	
weeks	mg/gFW	SEM		SEM	mg/gFW	SEM
not sub-cultured						
2	2.082	0.132	2.71	0.04	0.286	0.026
3	2.637	0.130	2.40	0.07	0.329	0.016
4	2.619	0.138	2.54	0.04	0.340	0.012
5	2.686	0.235	2.20	0.19	0.254	0.037
6	2.251	0.290	3.52	0.50	0.343	0.055
* sub-cultured						
5	2.599	0.181	2.09	0.18	0.238	0.037
6	2.245	0.338	4.37	0.84	0.303	0.054

basal media, no P.G.R.s.

* Subcultured after 3 weeks in culture, culture apex transferred to fresh media.

n = 9 No significant differences.

for both the non-subcultured and subcultured apical cultures over the six-week period.

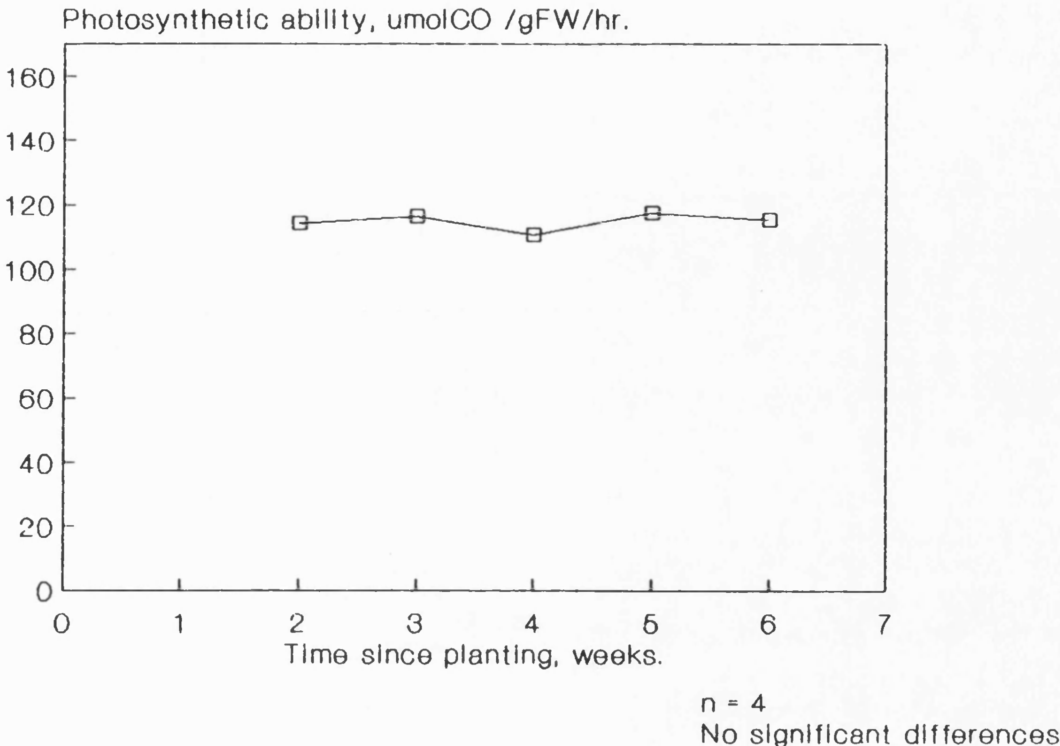
c/

The pigment contents (Table 2), photosynthetic abilities and respiration rates (Fig. 13) were constant for the growth cabinet grown two- to six-week old pea seedlings.

3.1.4. Discussion.

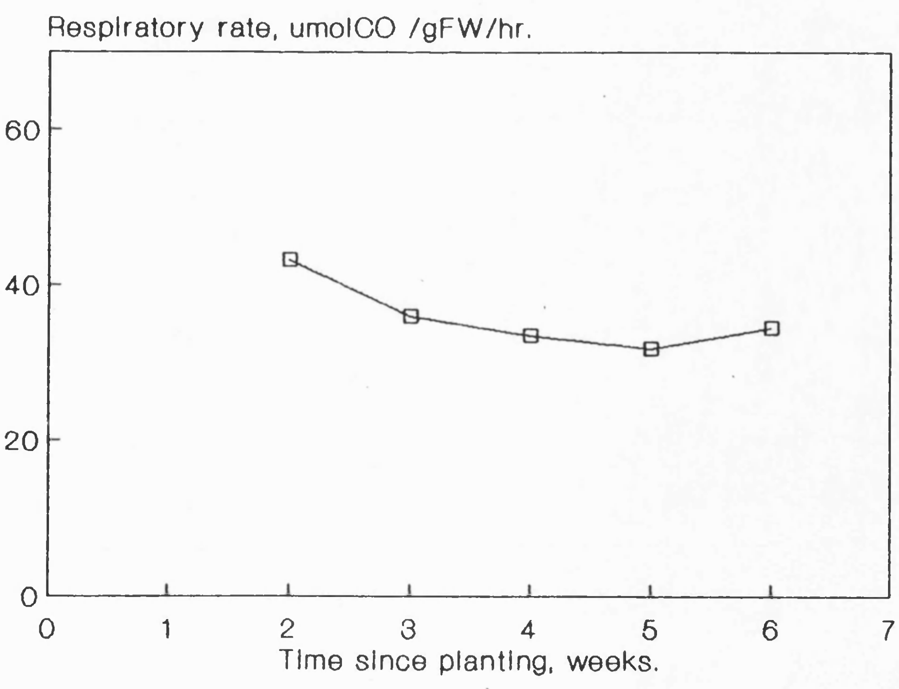
The FW and DW gains for the apical cultures on both media types were similar, but their photosynthetic abilities were different (Fig. 6 & 8). Moreover, the nodal cultures had similar photosynthetic abilities on both media types, but different FW and DW. This suggests that the growth of the cultures was not related to their photosynthetic abilities. However, the photosynthetic measurements were not taken under the conditions prevalent in the culture vessel, but rather at those in the IRGA analysis chamber: a higher light intensity; a constant atmospheric CO_2 concentration; and a lower humidity. The photosynthetic ability therefore, reflects the cultures potential for photosynthesis and not the actual photosynthetic activity. Photosynthetic activity of the cultures in the *in vitro* environment will probably be different from the measured photosynthetic ability of their leaves. This may explain why the FW and DW of the cultures were unrelated to the measured photosynthetic abilities. The media sugar will also have provided the cultures with a potential carbon and energy

Fig. 13 a/ Variation of photosynthetic ability with age for pea seedlings.



n = 4
No significant differences

Fig. 13 b/ Variation of the respiratory rate with age for pea seedlings.



n = 4
No significant differences

Table 2 The change in the pigment content with age of leaves from pea seedlings.

Age*	Total Chlorophyll		Chlorophyll a/b ratio		Cartenoids	
weeks	mg/gFW	SEM		SEM	mg/gFW	SEM
2	2.537	0.103	2.50	0.09	0.354	0.015
3	2.396	0.215	2.49	0.10	0.301	0.012
4	2.435	0.099	2.32	0.08	0.314	0.026
5	2.789	0.089	2.27	0.05	0.332	0.006
6	2.601	0.110	2.26	0.10	0.323	0.015

* Seeds sowed at week 0, into universal compost in half seed trays. These were placed in a growth cabinet, 25^{0} Centigrade, 16 hr day length (150 μE $m^{-2}s^{-1}$) and watered regularly.

n = 4 No significant differences.

source. The growth of the cultures may have been dependent on both the products of photosynthesis and the media sugar. The results reported here give no indication of the relative contributions that the two carbon and energy sources make to the growth of the cultures.

The development of the cultures was shown to be affected by the presence of P.G.R.s in the media, nodal cultures growing significantly better in the presence of the P.G.R.s provided. The two explant types; apical meristem (apical cultures) and axillary buds (nodal cultures) produced morphologically different cultures, which showed a similar response to the presence of P.G.R.s (Fig. 7). The particular combination of P.G.R.s used here caused, as intended, shoot proliferation, but resulted in reduced leaf size.

The photosynthetic abilities of the pea seedlings grown in compost in the growth cabinet were higher than those of the *in vitro* cultures, other than the three week old (first subculture) apical cultures on basal media (Fig. 8, 12 & 13). Lee *et al* (1985), with sweetgum cultures and seedlings, found that the cultures attained a higher net level of photosynthesis than the seedlings. However Grout & Aston (1978b) with cauliflower, Donnelly & Vidaver (1984b) with raspberry, Smith *et al* (1986) with Asian White Birch and Langford (1987) with *Rosa* found that cultures had a lower photosynthetic activity than *in vivo* produced plant material. While Pospisilova *et al* (1987), with *Nicotiana*

tobacum found that seedlings and plantlets had similar photosynthetic activities. *In vitro* and *in vivo* produced plant materials may have differences in their patterns and rates of growth, and have different origins. Considering these differences comparisons should be made with caution. However, the results of the some of the studies mentioned above do show that the photosynthetic abilities of cultures can, at least in certain instances, reach levels comparable to those attained *in vivo* .

The higher photosynthetic ability of the apical cultures on basal media (Fig. 8) also suggests that the photosynthetic ability of the pea cultures was possibly dependent upon the source of the explant. Evers (1982), with Douglas Fir found that the source of the explants (buds) affected photosynthetic ability of the resultant cultures.

The photosynthetic measurements show that apical cultures on basal media had a constant initial photosynthetic ability, but after four weeks in culture the photosynthetic ability decreased (Fig. 12). This fall was greater when the cultures were subcultured. When subculturing, only the culture apex was transferred to the fresh media. This removal of the greater part of the plantlet may have been the cause of the larger decline in the photosynthetic ability. Studies of *Rosa* cultivars (Langford & Wainwright, 1987) found that photosynthetic ability of the cultures derived from "nodal" type explants gradually increased with time in

culture (over a 24 week period). The pigment levels, although variable, did not show any comparable changes. In contrast, Smith *et al* (1986), with cultures of Asian White Birch, found that the photosynthetic activity of the cultures declined with time after subculturing. Therefore, it appears the photosynthetic ability of plant cultures can change with the time. This effect of time is probably dependent on the species, the origin of the cultures and their age. Studies of a variety of other plant species and culture systems are needed to confirm this.

The pigment contents of the leaves, were either similar to or lower than the *in vivo* levels, and tended to declined with time in culture (Fig. 9, 10 & 11, Tables 1 & 2), although the only significant decrease was for the apical cultures on basal media. This decline could be either a cause of or a result of the decreased photosynthetic abilities of the cultures. However the changes in the photosynthetic abilities of the cultures may be at least partly due to physiological factors not related to the pigment levels. Grout & Donkin (1985) and Langford (1987) suggested that the photosynthetic ability of cultures is not only limited by the pigment levels, which they found to be lower *in vitro* than *in vivo*, but also by biochemical factors, such as the activity of the photosynthetic enzymes, for example RUBISCO. Moreover, Grout & Donkin (1985) found lower levels of RUBISCO and electron transport activity, as well as decreased

chlorophyll levels, in cauliflower cultures.

The presence of P.G.R.s in the media reduced the pigment contents (Fig. 9, 10 & 11), and adversely affected the initial photosynthetic ability (Fig. 8) of the apical cultures. These effects could be due to the P.G.R.s either preventing the cultures attaining higher levels of photosynthetic activity, or advancing the decline in culture photosynthetic activity that occurs with time. These results however give no indication as to which, if either, of these is the cause of this fall. Investigations have suggested that cytokinins such as BAP are involved in promoting plastid development and pigment formation (Teyssendier de la Serve *et al*, 1985; Chernyad've *et al*, 1986; Novak *et al*, 1986; Schneider & Szweykowka, 1987). These effects however are dependent on concentration, and may well be modified by the presence of other P.G.R.s. Therefore, concentrations and combinations of P.G.R.s other than that used here, may have had different effects on the photosynthetic physiology of the cultures.

The measured respiratory rates (Fig. 8, 12 & 13) showed no trends, and those for the *in vitro* and *in vivo* grown plant material were similar. Therefore, the culturing of peas, under the conditions used here, had no apparent affect on their respiratory physiology. Donnelly & Vidaver (1984b) found that under certain culture conditions plantlets of red raspberry had comparable

respiration rates to *in vivo* grown material. Moreover, Smith *et al* (1986) found that plantlets and seedlings of Asian White birch, grown under similar environmental conditions, had comparable respiratory rates.

The FW and DW of the nodal cultures (Fig. 6), and therefore their growth rates, were different on the two media types. As respiration provides the energy for growth this difference in growth should, therefore, have been accompanied by differences in the respiratory physiology of the cultures. The respiration rates measured here, however, were obtained using leaves removed from the cultures, not whole plantlets. Leaves were used for the gas exchange measurements as these are the major photosynthetic organ, their photosynthetic rate was, therefore, comparable to that for whole plantlet. Mature photosynthetic leaves, unlike say shoot tips, are however not major areas of growth, and therefore, their respiratory physiology was probably not representative of the whole plantlets. Furthermore, the dark respiration rates of the leaves were not measured under the conditions prevalent in the culture vessel, but those of the IRGA analysis chamber, a low humidity and a constant atmospheric O_2 level. The respiration rates measured were, therefore, not representative of the respiratory physiology of the whole plantlet in the culture vessel. Further studies are therefore needed to ascertain the respiratory rates of whole plantlets, thus allowing the effects of culture parameters, such as, the time in

culture and the presence of P.G.R.s in the culture medium, on the respiratory physiology to be elucidated.

These experiments suggest that the explant source, time in culture and the presence of P.G.R.s in the media can affect the photosynthetic physiology of cultures. The effects of these factors are variable, and they may interact and be dependent on other parameters, such as the plant species and cultivar. Cultures have been shown to be capable of attaining levels of photosynthetic activity comparable to those found *in vivo*. This high level of culture photosynthetic activity may be related to the culture environment. By changing environmental parameters such as the media sugar concentration (an alternative energy and carbon source to photosynthesis), the cabinet light intensity (the energy source of photosynthesis) or the ambient carbon dioxide concentration in the culture vessel (photosynthetic carbon source), it may be possible to attain greater photosynthetic activity *in vitro*, or sustain it for a longer period. The photosynthetic activity of cultures has previously been shown to be affected by such parameters, for example, growth cabinet light intensity and media sugar on Rose cultures (Langford, 1987). The effects of light intensity and media sugar content on apical cultures need to be investigated, and further study is also needed into the effects of the P.G.R.s.

3.2. Benzyl aminopurine (benzyladenine) concentration and photosynthetic potential.

3.2.1. Introduction.

Cytokinins have been shown to affect chloroplast development and physiology, including the levels of chlorophyll and the activity of photosynthetic enzymes such as RUBISCO (Parthier, 1979). More specifically, cytokinins are thought to be involved in promoting plastogenesis. They act at the molecular level, modulating gene expression (Teyssendier de la Serve, 1985) and affecting protein formation. They have also been shown to stimulate the formation of chloroplast grana (Chernyad'ev *et al*, 1986) and to increase the photosynthetic rate of *Phaseolus* primary leaves (Camri, 1986).

The effects of cytokinins *in vitro* have been found to be varible. They typically cause greening of callus and cell cultures, as well as promoting growth and cell division (Laetch & Boassman, 1972; George & Sherrington, 1984). The effects often depend on the type of cytokinin applied (Nowak *et al*, 1975) and its concentration. The different P.G.R.s tend to interact (as they do *in vivo*), for example, changing the relative concentration of applied auxins and cytokinins can promote different forms of culture growth (George & Sherrington, 1984).

In the previous section (3.1.) two P.G.R.s

were used, NAA and BAP. The effect the cytokinin BAP on photosynthetic physiology was investigated further. An experiment was performed to assess the effect of a range of BAP concentrations on the photosynthetic activity of apical pea cultures.

3.2.2. Materials & Methods.

Apical explants were grown on basal (MS) media containing BAP at the following concentrations: 0; 0.002; 0.02; 0.2; 2; 20 & 200 µM. Twenty five explants were initiated per concentration. After a three week culture period, leaf material was harvested and used for the measurement of photosynthetic and respiratory gas exchange, and pigment content.

3.2.3. Results.

Concentrations of BAP 0.02 µM and below had no effect on the photosynthetic ability and pigment content of the apical cultures (Fig. 14, 15, 16 & 17). At higher concentrations BAP had an inhibitory effect, consistently reducing the photosynthetic ability (Fig. 14) to about half that for cultures grown on BAP-free media. The range of BAP concentrations used had no effect on the dark respiration rates of the cultures (Fig. 14).

The chlorophyll and carotenoid contents (Fig. 15 & 16) were also reduced by BAP concentrations of

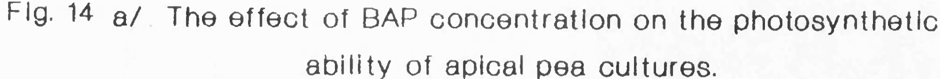
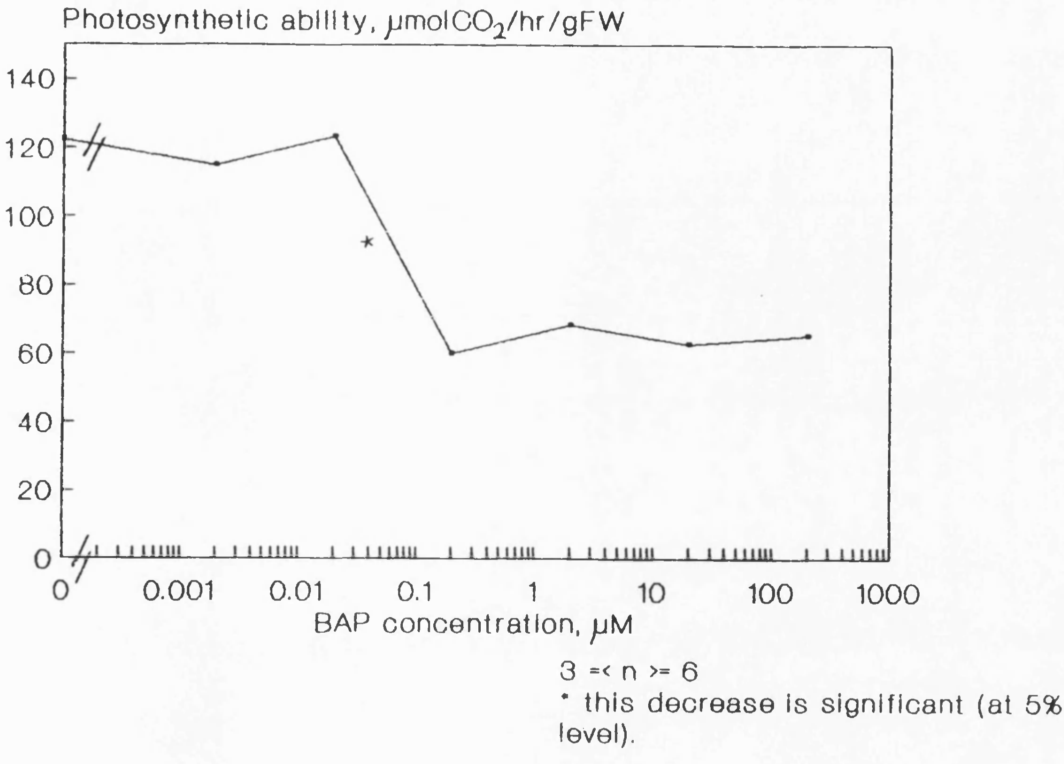
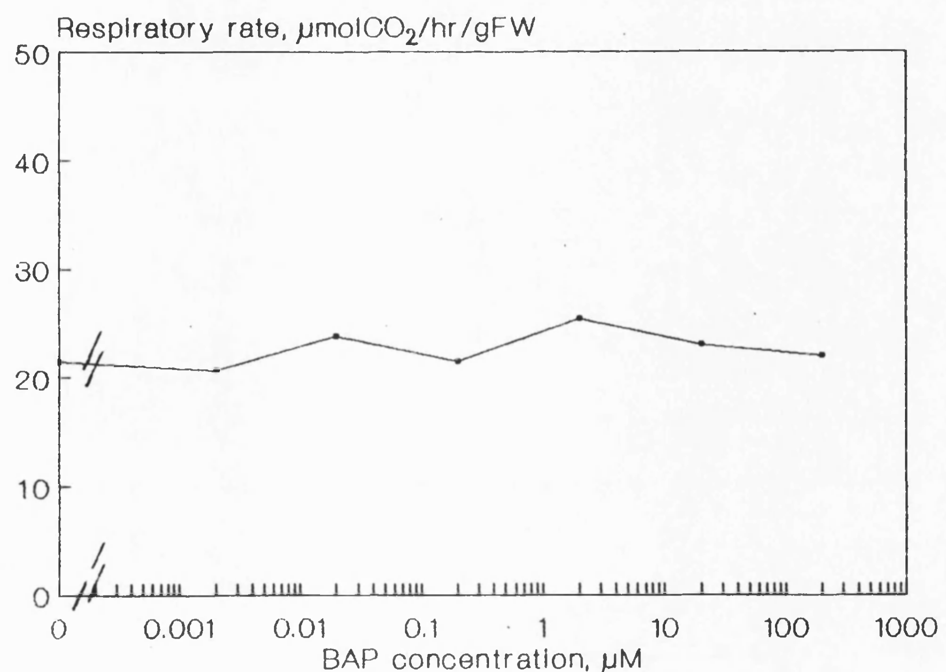


Fig. 14 a/ The effect of BAP concentration on the photosynthetic ability of apical pea cultures.



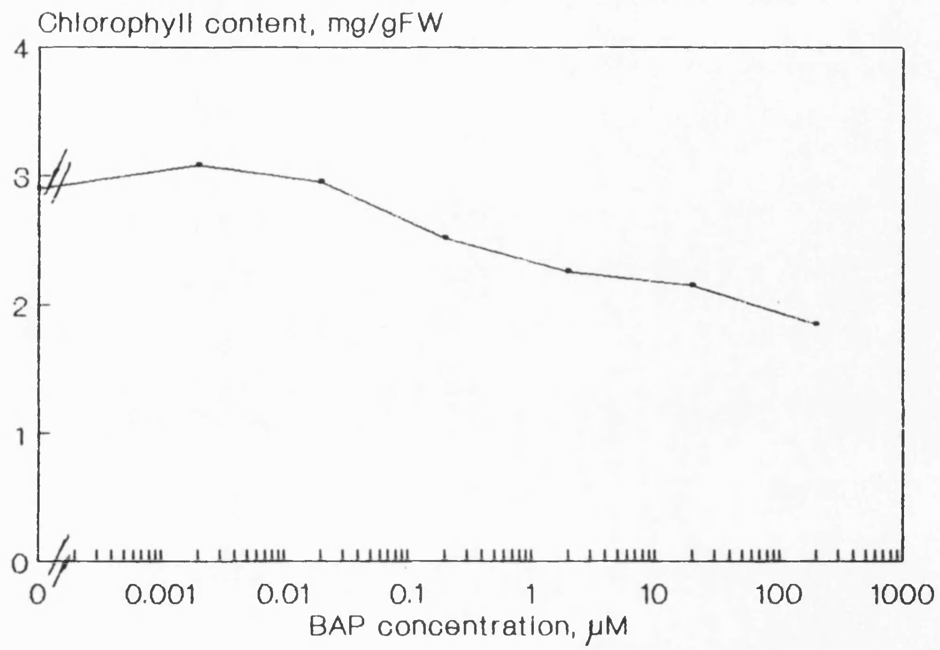
3 =< n >= 6
* this decrease is significant (at 5% level).

Fig. 14 b/ The effect of BAP concentration on the respiratory rate of apical pea cultures.



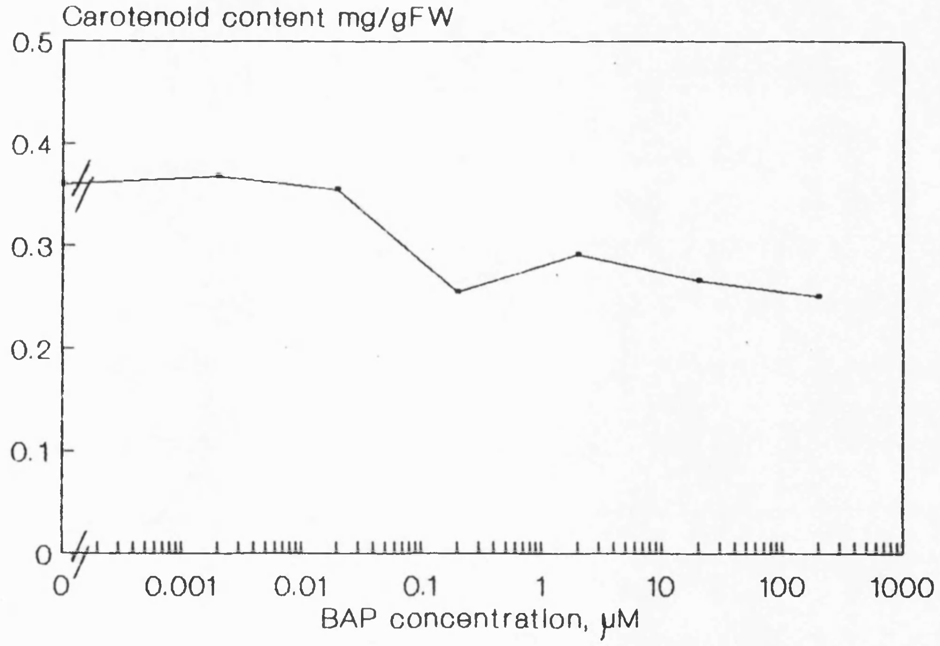
3 =< n >= 6
No significant differences.

Fig. 15 The effect of BAP concentration on the chlorophyll content of apical pea cultures.



3 =< n >= 6
The overall decrease in chlorophyll content is significant (at 5% level).

Fig. 16 The effect of BAP concentration on the carotenoid content of apical pea cultures.



3 =< n >= 6
The overall decrease in the carotenoid levels is significant (at 5% level).

0.2 µM and above, the chlorophyll level decreased gradually by one third. The chlorophyll a/b ratio (Fig. 17) remained constant throughout the range of BAP concentrations tested.

The photosynthetic ability per unit chlorophyll (Fig. 18) was significantly lower at 0.2 µM BAP than at all the other BAP concentrations. At 2 and 20 µM BAP the photosynthetic ability per unit chlorophyll was significantly lower than at 0-0.02 µM and 200 µM BAP.

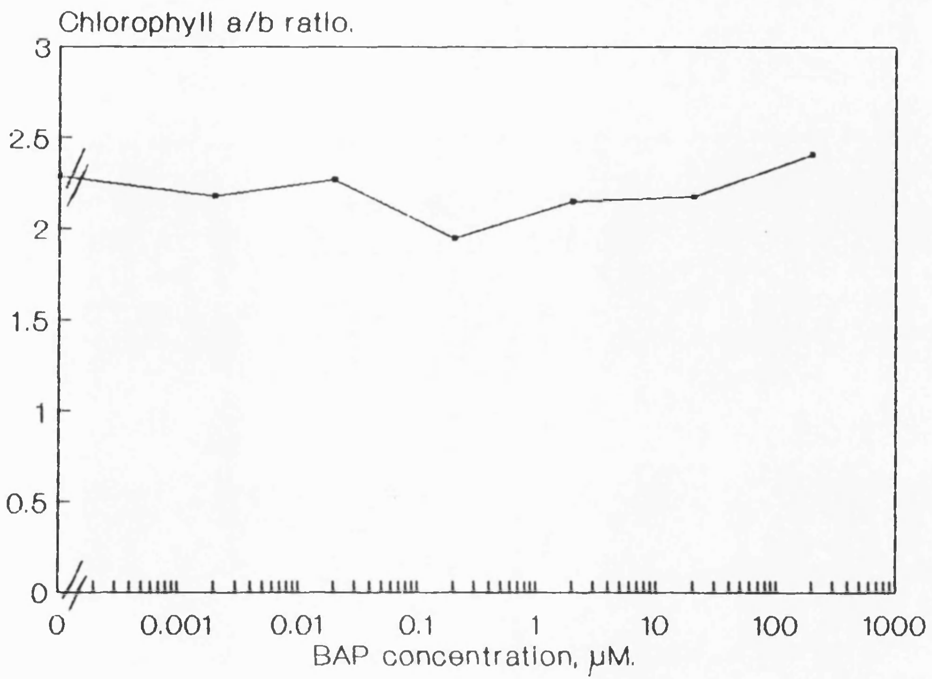
The presence of BAP in the media produced cultures that were morphologically different to those grown on P.G.R.-free media. The culture leaf size appeared to decrease as the BAP concentration was increased. This change was not quantified.

3.2.4. Discussion.

Concentrations of BAP 0.2 µM and above reduced the photosynthetic ability and pigment content of apical cultures, whereas lower concentrations had no effect (Fig. 14, 15, 16 & 17), the respiratory rates of the cultures were, however, unaffected. These results show that the effects of BAP, and possibly other cytokinins, on photosynthetic systems of *in vitro* grown pea material are dependent on concentration.

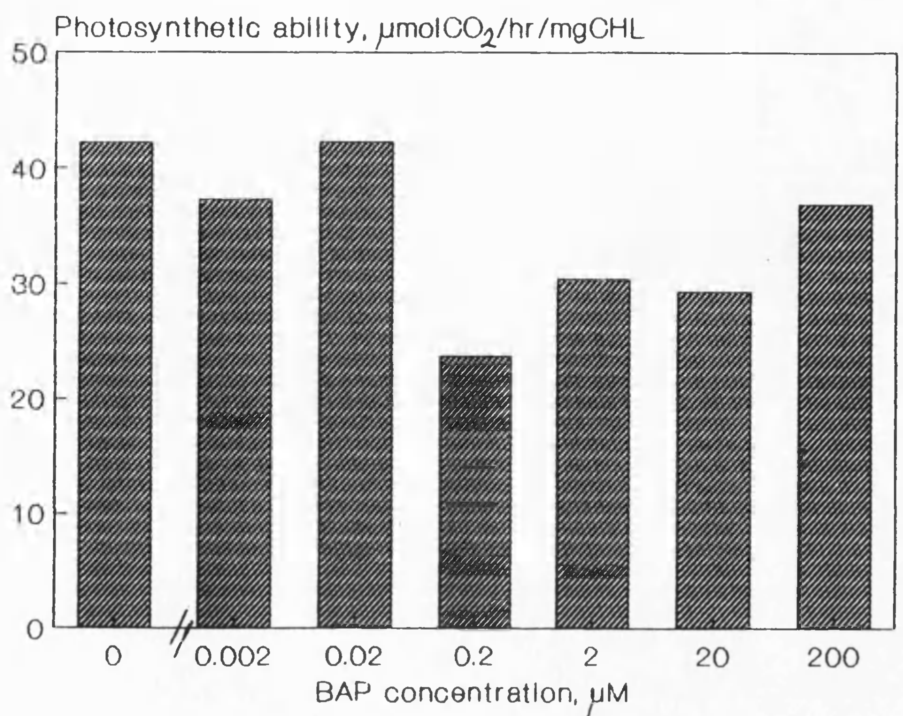
Studies on callus and cell suspension cultures have shown that cytokinins, including BAP, promote plastid development and pigment production

Fig. 17 The effect of BAP concentration on the chlorophyll a/b ratio of apical pea cultures.



3 =< n >= 6
No significant changes.

Fig. 18 The effect of BAP concentration on the photosynthetic ability (per unit chlorophyll) of apical pea cultures.



3 =< n >= 6

CHL = chlorophyll

Photosynthetic ability for the cultures on 0.2 µM BAP media is significantly lower than those for cultures on all other BAP concentrations.
Photosynthetic abilities for cultures on 2 & 20 µM BAP are significantly lower than values for cultures on 0 - 0.02 & 200 µM BAP.
Photosynthetic abilities for cultures on 0 - 0.02 & 200 µM BAP show no significant differences.

All significances tested at the 5% level.

(Neumann & Raafat, 1973; Sayer *et al* 1975; Parthier, 1979). Nowak *et al* (1986) showed that green callus of *Dianthus caryophyllus* gradually lost the ability to synthesize chlorophyll over successive subcultures, but could be maintained green by adding cytokinins to the culture medium. Kinetin reduced the loss slightly, while in the presence of BAP the chloroplasts maintained a high chlorophyll content. The added cytokinins probably caused the production of proteins associated with the light harvesting complex (Schneider & Szweykowka, 1987). Moreover, Teyssendier de la Serve *et al* (1986), have shown that cytokinins are involved in modulating the expression of genes encoding protein components of the light harvesting chlorophyll a/b complex in tobacco cell cultures.

The effects of cytokinins on plants and plantlets are less clear. The different cell types and tissues present may respond differently, and the effects may be modified by the presence of endogenous P.G.R.s, as well as being dependent on concentration. However, cytokinins have been shown to retard senescence (Roberts & Hooley, 1988) and are thought to promote chloroplast development and function (Parthier, 1979). For dark-grown cucumber cotyledons, chlorophyll formation in the light could be promoted by adding cytokinins. The increase in the chlorophyll level was proportional to the cytokinin concentration. The level of δ-aminolevulinic acid (ALA), a chlorophyll precursor, was also increased in the

treated cotyledons, probably as a result of the cytokinin inducing the production of ALA-synthase (Fletcher & McCullagh, 1971; Fletcher *et al*, 1973). This response of cotyledons to changes in the concentration of applied cytokinins has been used as a bioassay for this group of P.G.R.s. The photosynthetic rate of *Phaseolus vulgaris* L. plants was increased by applying BAP to the primary leaves, and there was also a greater increase in the DW of the aerial parts of the treated plants, but reduced increase in the DW of the roots (Camri, 1986). It was concluded that the application of BAP, not only stimulates photosynthetic activity, but also affected the allocation of photosynthetic assimilates. Chernyad'ev *et al* (1984) found that when applied to the leaves BAP increased photosynthetic CO_2 uptake or RUBISCO activity in a variety of plant species. Moreover, Chernyad'ev *et al* (1984 & 1986) with *Betula vulgaris* found that the photosynthetic rates were either increased or not affected by the application of BAP to the leaves. The response depended on the concentration of BAP applied and the time after application when the photosynthetic rate was measured. Concentrations of BAP between 1-5 mg/l gave the highest rates. The photosynthetic activity increased initially then declined to the level of the controls. However, Wild *et al* (1981) found that kinetin did not effect the rate of CO_2 uptake (photosynthetic activity) by the primary leaves of *Sinapis alba*. Thus it appears that the effects of cytokinins on the photosynthetic physiology of differentiated plant material are variable;

in some instances they have been shown to increase chlorophyll levels and photosynthetic rates.

The reduction of the photosynthetic ability and pigment contents of apical pea cultures by BAP (Fig. 14, 15 & 16), does not preclude other cytokinins from enhancing photosynthesis, Nowak *et al* (1986) found that the effect may be dependent upon the particular cytokinin used (see earlier). Kaul & Sabharwal (1971) found that kinetin induced greening of non-green callus, but had an inhibitory effect on chlorophyll formation in green callus. Their results also suggested that the relative concentrations of media sucrose and kinetin were also important in controlling culture growth, chlorophyll levels and protein synthesis. The effect of cytokinins may depend not only on their concentration, but also on the tissue and possibly other factors such as the sugar content of the media.

The sudden fall and then gradual rise in the photosynthetic abilities per unit chlorophyll as the BAP concentration was increased (Fig. 18) suggest that the BAP may have a two-fold effect on the photosynthetic physiology of apical pea cultures. The initial sharp fall was possibly due to the BAP inhibiting the photosynthetic biochemistry at concentrations of 0.2 µM and above. The gradual increase in the photosynthetic ability per unit chlorophyll as the BAP concentration was increased further may have been due to a corresponding decrease in

the chlorophyll content.

The presence of the BAP also caused morphological changes to the cultures; these may have been related to the decreased photosynthetic abilities. Conversely, the decline in the photosynthetic ability and pigment contents of the leaves may have been due to the changes in the culture growth patterns, and not due to the direct effects of BAP on the photosynthetic systems. There may, for example, have also been changes in the culture DW and leaf areas. If the photosynthetic abilities and pigment contents had been expressed on either a DW or a leaf area basis, different trends might have been observed. The respiration rates (Fig. 14) of the cultures leaves were, however, not affected by the range BAP concentrations used here. This suggests that the BAP may not have had an indirect affect on the physiology of the cultures leaves; as such an indirect effect may well have affected the respiratory physiology of the cultures leaves, and not just their photosynthetic physiology.

This experiment has shown that the presence of BAP in the culture media can reduce the photosynthetic ability and pigment content of the leaves of apical pea cultures when expressed on a FW basis. These changes might be due to the BAP affecting other aspects of the cultures physiology, producing a secondary decrease in the photosynthetic parameters. The cause of

this "inhibition" is unknown and merits further investigation. The changes in the culture morphology and the effects of other cytokinins, such as kinetin, also need to be investigated.

3.3. Effect of sugar concentration and light intensity on the photosynthetic potential, sugar uptake and growth.

3.3.1. Introduction.

The photosynthetic abilities of plant tissue cultures have been shown to be affected by the media sugar concentration and the growth cabinet light intensity.

Plant tissue cultures are usually only grown on a media containing a known concentration of a single sugar, typically sucrose, in the range 2-4% w/v. The effects of sugars on culture photosynthesis and, particularly, chlorophyll contents, are well documented. Most of the studies have been on cell and callus cultures. High media sucrose concentration inhibited chlorophyll synthesis in spinach cell cultures (Dalton & Street, 1977) and low media sucrose content produced tabacco callus that was darker green, but showed reduced growth (Barg & Uniel, 1976). High media sugar has been shown to reduce photosynthesis (Herold, 1980). Evers (1982) found that the photosynthetic ability of Douglas Fir cultures was related to the sucrose content of the media. High sugar may also change the number and morphology of chloroplasts, and affect ALA synthesis (Pamplin & Chapman, 1975). Other readily utilisable sugars, such as fructose, produce similar effects (Dalton & Street, 1977). The concentration of sugar in the culture media therefore, affects culture growth, high

sugar levels inhibiting photosynthesis and chlorophyll synthesis and low levels reducing culture growth.

Light is essential for the normal growth and development of plant tissue cultures (George & Sherrington, 1984). Both the quantity (day length and intensity) and quality (wavelength) are important. Light quality can affect growth; more specifically, blue light is needed for normal plastid development, chlorophyll formation and culture growth, while red light has an inhibitory effect (Berger & Bergman, 1967; Kamiya *et al*, 1981; Ni *et al*, 1985 a & b; Norton & Norton, 1986). The amount of light the cultures receive can affect their growth (Flegmann & Wainwright, 1984). Lee *et al* (1985) with sweetgum, Langford (1987) with Rose and Desjardins *et al* (1988) with Strawberry, all found that the photosynthetic activity of cultures was effected by light intensity, with medium light intensities typically giving the highest rates.

Variations in the light intensity may lead to variations in temperature within the growth cabinet. Langford (1987) found that in air-conditioned growth cabinets the temperature inside the culture vessels varied, increasing with the light intensity, the variation was however small, 1-2 ^{0}C. It was concluded that such a small variation would not have any significant effect on the growth of the cultures.

A series of experiments was conducted to

investigate the effects of both light intensity and media sucrose concentration, alone and in combination, on the photosynthetic ability and pigment contents of apical pea cultures.

3.3.2. Materials & Methods.

The photosynthetic ability, dark respiration rates and pigment contents were ascertained for leaf material taken from three-week old apical cultures grown as follows:

a/ On media (MS) containing 0, 10, 20, 30, 40 and 50 g/l sucrose at a growth cabinet light intensity of 150 $\mu E\ m^{-2} s^{-1}$.

b/ On basal media (MS containing 30 g/l sucrose) at growth cabinet light intensities of 50, 100, 150, 240 and 330 $\mu E\ m^{-2} s^{-1}$.

c/ On media (MS) containing 10, 30 and 50 g/l sucrose at each of the following growth cabinet light intensities 50, 150 and 300 $\mu E\ m^{-2} s^{-1}$. The FW and DW were also ascertained for these cultures, and the sugar content of the media at the end of the three week culture period was measured using the Anthrone assay.

In the experiments where the cultures were grown under a range of growth cabinet light intensities, photosynthesis was measured in the IRGA at four different light intensities, obtained by placing neutral density

filters (NDFs) between the IRGA analysis chamber and the light source (Table 3).

3.3.3. Results.

a/ Low and high media sucrose concentrations caused a small, but not statistically significant, decrease in the photosynthetic ability of the leaves of the apical pea cultures on P.G.R.-free media (Fig. 19). The dark respiration rate (Fig. 19) for cultures grown on media containing 10 g/l sucrose was significantly lower than those for cultures grown on media containing 20, 30 & 50 g/l sucrose.

The pigment content (Fig. 20, 21 & 22) increased with the media sugar concentration, and cultures grown on media containing 40 and 50 g/l sucrose had significantly higher levels of chlorophyll than cultures grown on media containing 10 g/l sucrose. Cultures grown on media containing low sucrose also had reduced carotenoid levels (Fig. 21). The chlorophyll a/b ratios (Fig. 22) remained relatively constant.

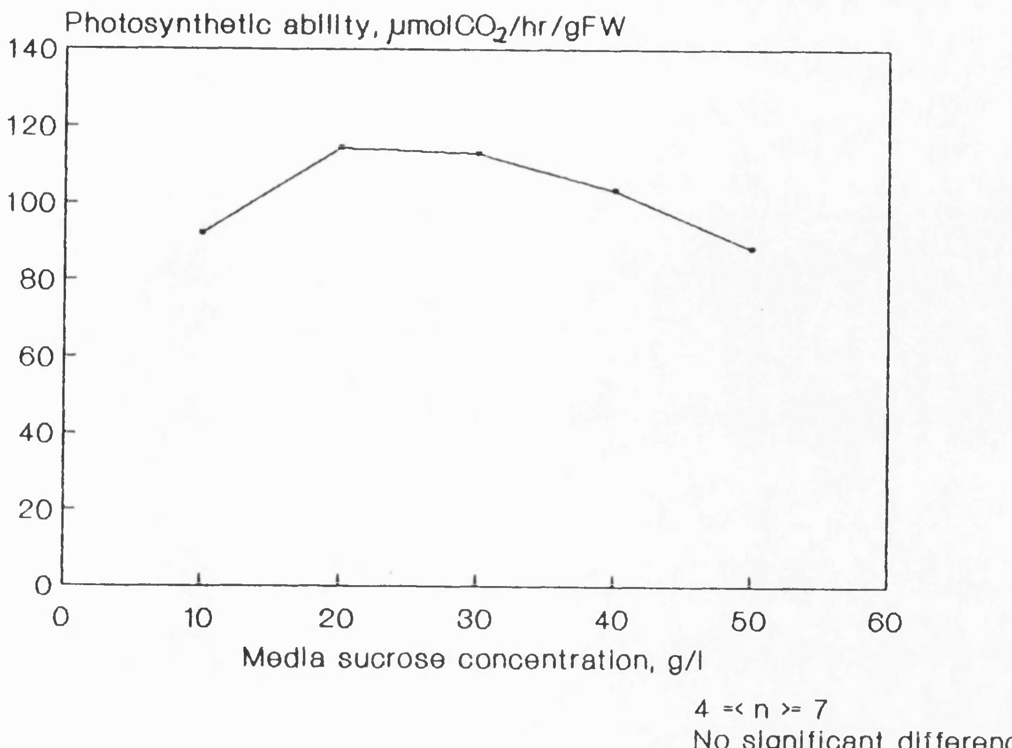
The results for sucrose free media were single readings as follows: photosynthetic ability, 92.8 $\mu molCO_2\ hr^{-1}gFW^{-1}$; total chlorophyll, 2.3 mg/gFW; carotenoids, 0.25 mg/gFW; chlorophyll a:b, 2.03. The cultures did not readily grow on this media and produced only a minimal amount of leaf material.

Table 3 Effect of Neutral Density Filters (NDFs) on light intensity in the IRGA analysis chamber.

No. NDFs	Light intensity	
	$\mu E\ m^{-2} s^{-1}$	%
0	380	100
1	140	37
2	73	19
3	29	8

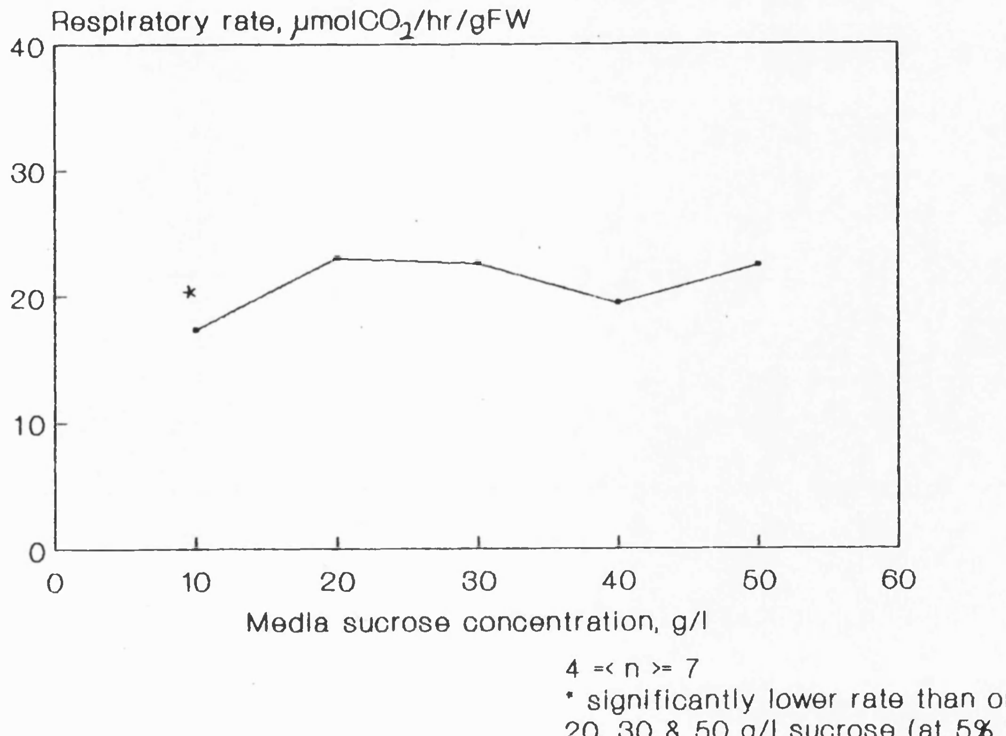
NDFs were placed between the light source and the IRGA analysis chamber.

Fig. 19 a/ The effect of various media sugar concentrations on the photosynthetic ability of apical pea cultures.



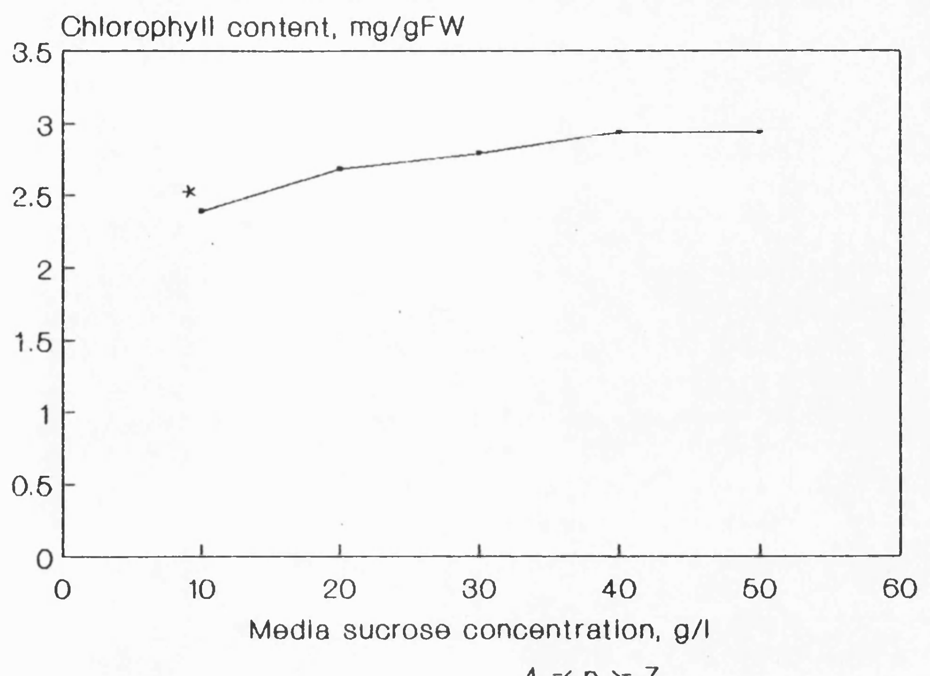
4 =< n >= 7
No significant differences.

Fig. 19 a/ The effect of various media sugar concentrations on the respiration rate of apical pea cultures.



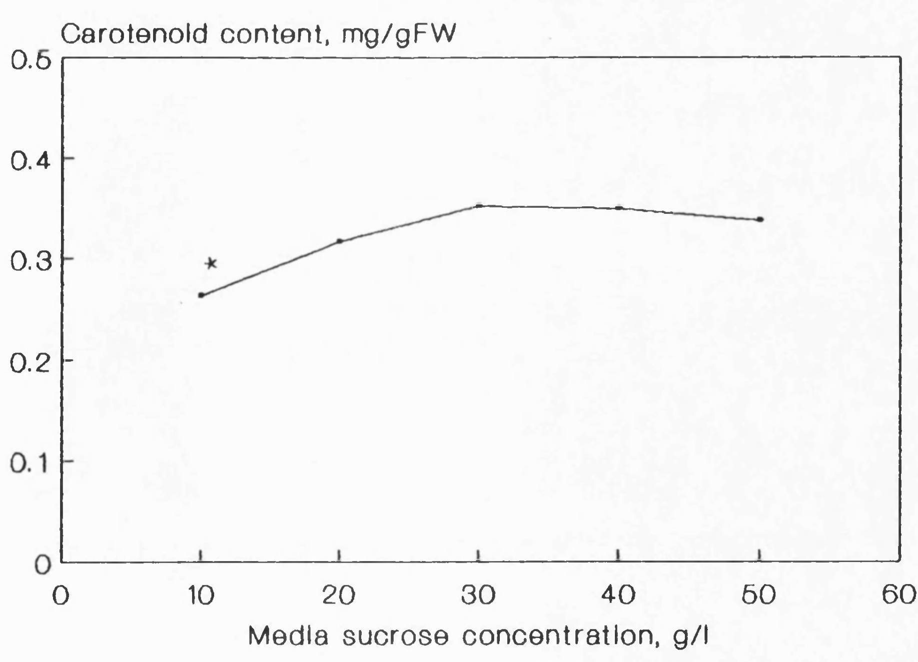
4 =< n >= 7
* significantly lower rate than on 20, 30 & 50 g/l sucrose (at 5% level)

Fig. 20 The effect of various media sugar concentrations on the chlorophyll content of apical pea cultures.



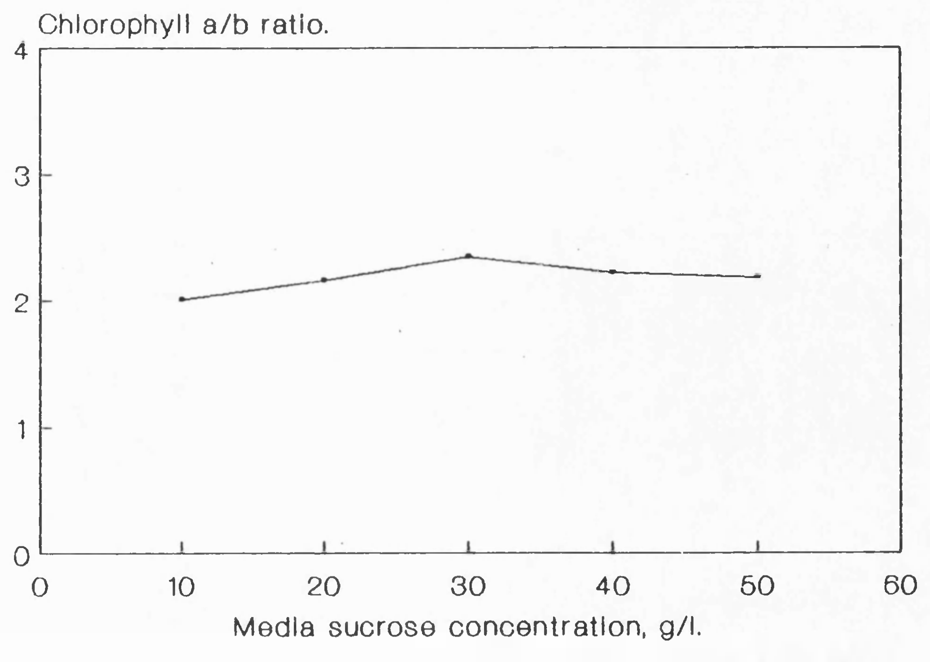
4 =< n >= 7
*Chlorophyll content significantly lower than at 20-50 g/l sucrose (5% level).

Fig. 21 The effect of various media sugar concentrations on the cartenoid content of apical pea cultures.



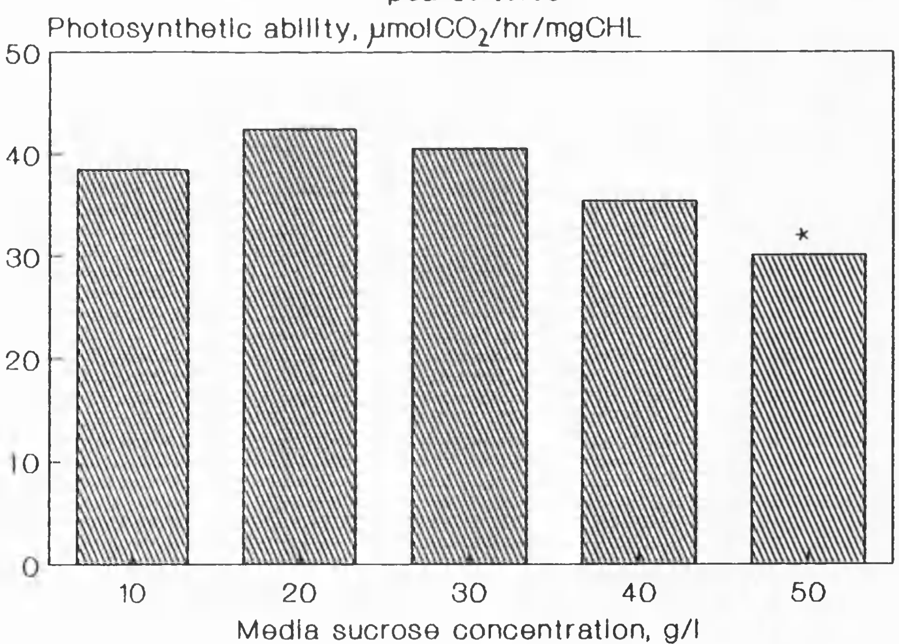
4 =< n >= 7
* Significantly lower carotenoid content than at 30-50 g/l sucrose (5% level).

Fig. 22 The effect of various media sucrose concentrations on the chlorophyll a/b ratio of apical pea cultures.



4 =< n >= 7
No significant differences

Fig. 23 The effect of various media sucrose concentrations on the photosynthetic abilities (per unit chlorophyll) of apical pea cultures.



4 =< n >= 7
*significantly lower than photosynthetic ability at 10-30 g/lsucrose (5% level).

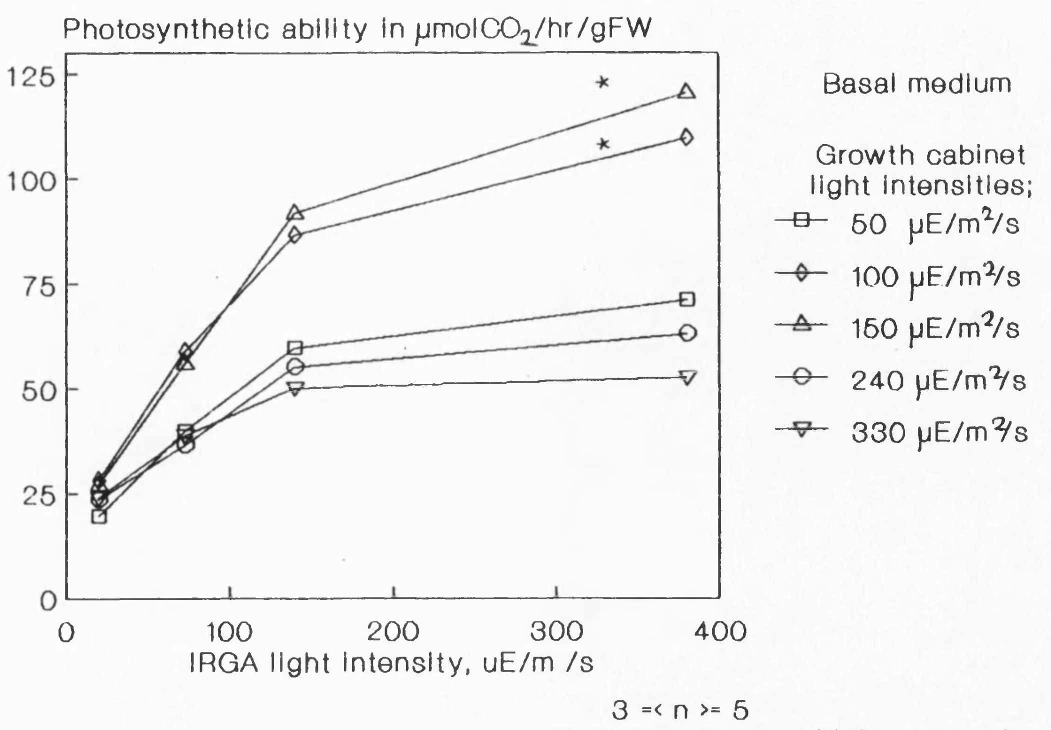
The photosynthetic rates per unit chhlorophyll were significantly lower for cultures grown on media containing 50 g/l sucrose (Fig. 23), but constant for the other sucrose contents.

b/ Reducing the light intensity in the IRGA analysis chamber decreased the photosynthetic rate attained by leaf samples from the apical cultures (Fig. 24). As the light intensity in the IRGA analysis chamber was reduced, the decline in the photosynthetic rate was intially small, but the rate of decline increased as the light intensity fell below about 140 $\mu E\ m^{-2} s^{-1}$ (1 NDF).

Cultures grown at light intensities above and below 150 $\mu E\ m^{-2} s^{-1}$ showed a similar responses curves to the cultures grown at 150 $\mu E\ m^{-2} s^{-1}$, but those grown at 50, 240 and 330 $\mu E\ m^{-2} s^{-1}$ had decreased photosynthetic rates and pigment contents (Fig. 25 & 26). Cultures grown at 100 $\mu E\ m^{-2} s^{-1}$ had a similar photosynthetic rate to cultures grown at 150 $\mu E\ m^{-2} s^{-1}$ but they contained less pigment. Those cultures grown at the higher light intensities had leaves with the lowest pigment contents.

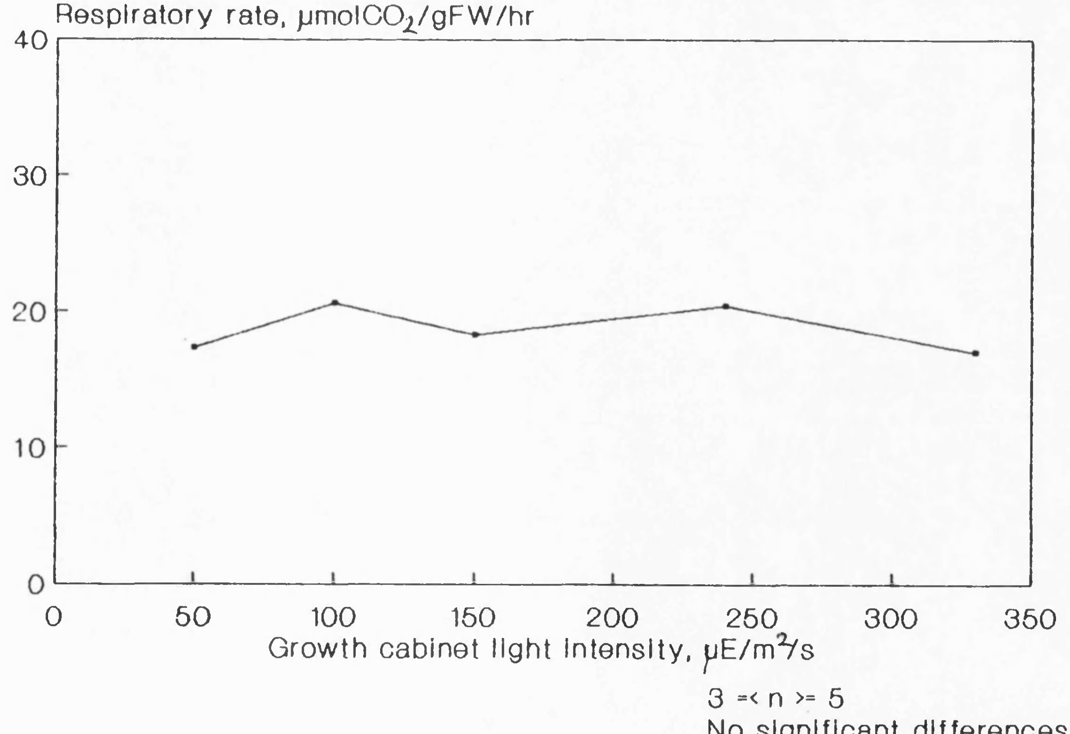
The growth cabinet light intensity had no significant effect on the dark respiration rates of the cultures (Fig. 24). The cultures grown at the highest light intensity had the lowest chlorophyll a:b ratio (Fig. 27) and the highest photosynthetic rates per unit chlorophyll (Fig. 28).

Fig. 24 a/ The effect of a range of growth cabinet light intensities the photosynthetic ability of apical pea cultures



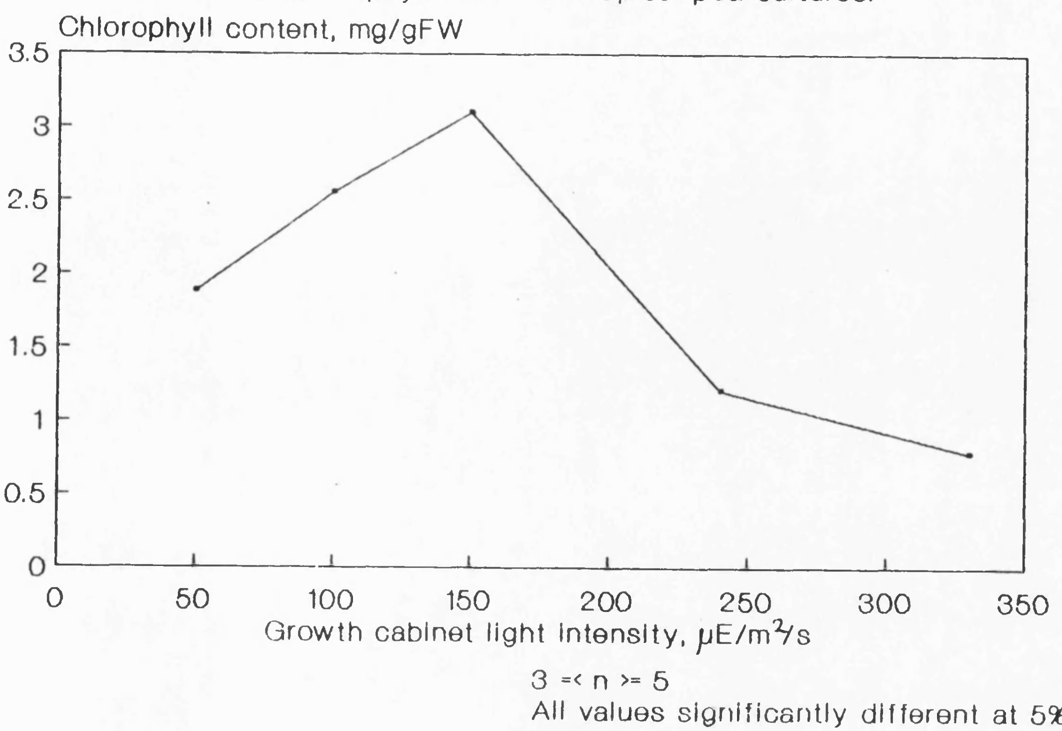
3 =< n >= 5
* significantly higher at 5% level.

Fig. 24 b/ The effect of a range of growth cabinet light intensities on the respiratory rate of apical pea cultures.



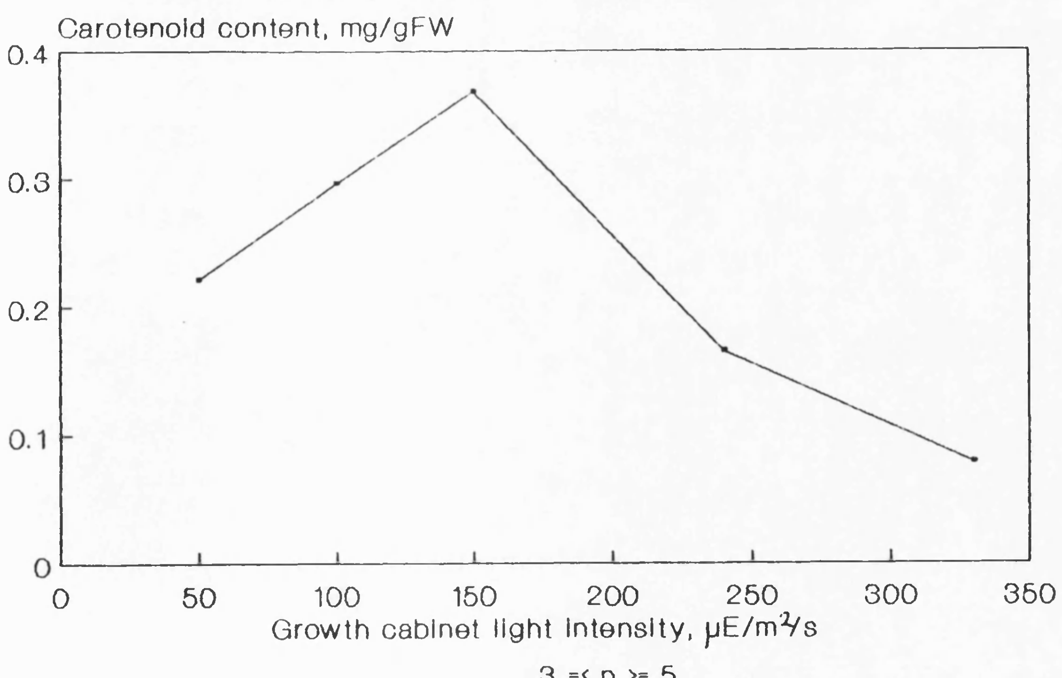
3 =< n >= 5
No significant differences.

Fig. 25 The effect of a range of growth cabinet light intensities on the chlorophyll content of apical pea cultures.



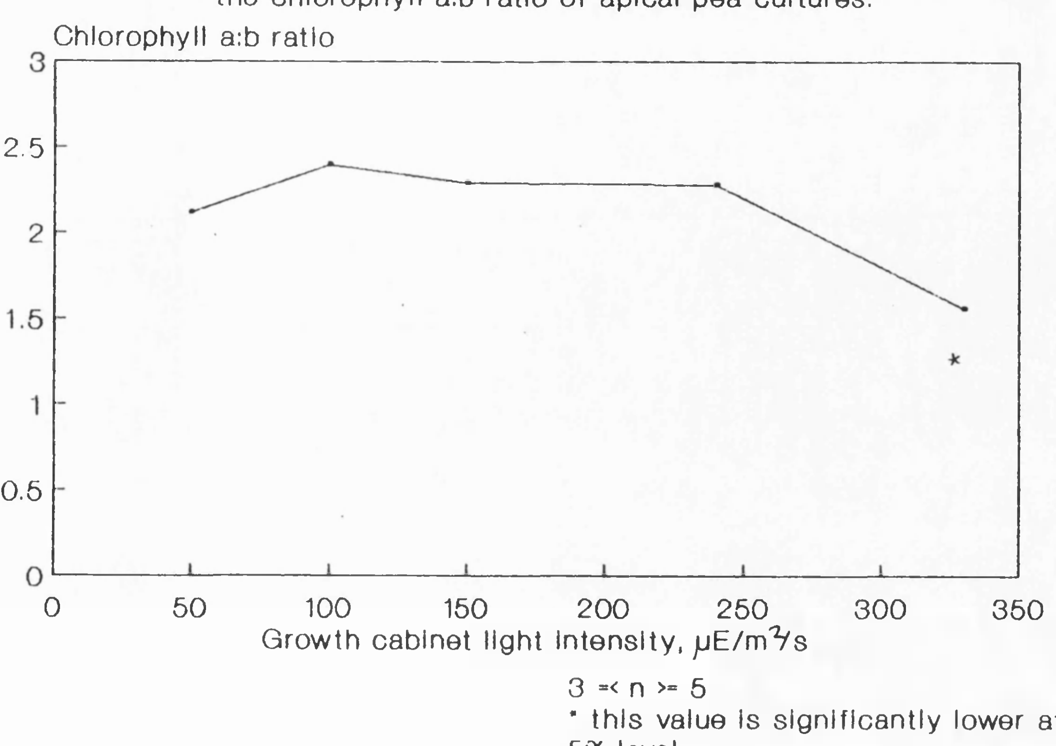
3 =< n >= 5
All values significantly different at 5% level.

Fig. 26 The effect of various growth cabinet light intensities on the carotenoid content of apical pea cultures.



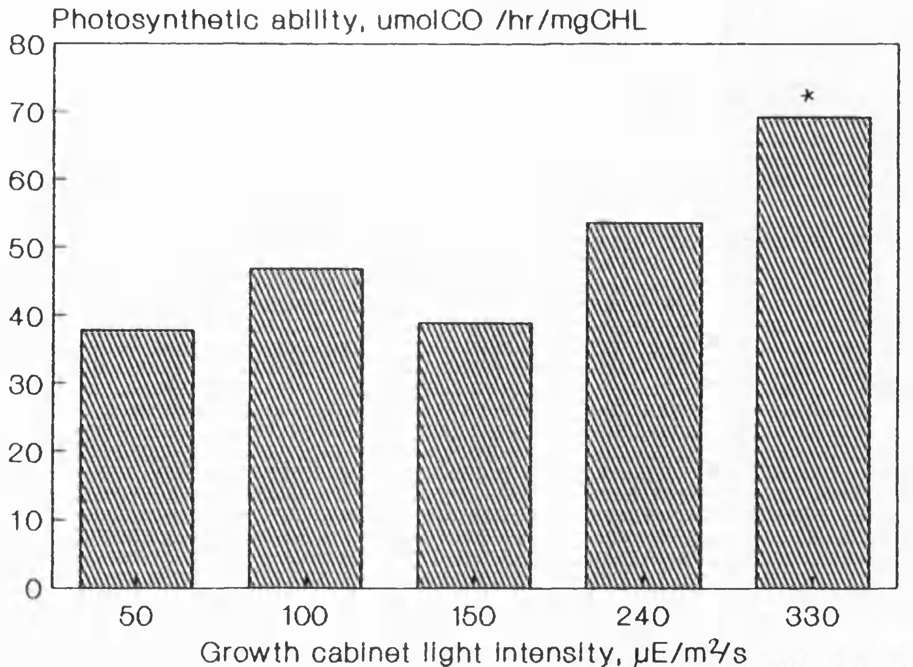
3 =< n >= 5
All values significantly different at 5% level.

Fig. 27 The effect of a range of growth cabinet light intensities on the chlorophyll a:b ratio of apical pea cultures.



3 =< n >= 5
* this value is significantly lower at 5% level.

Fig. 28 The effect of a range of growth cabinet light intensities on photosynthetic ability (per unit chlorophyll) of apical pea cultures.

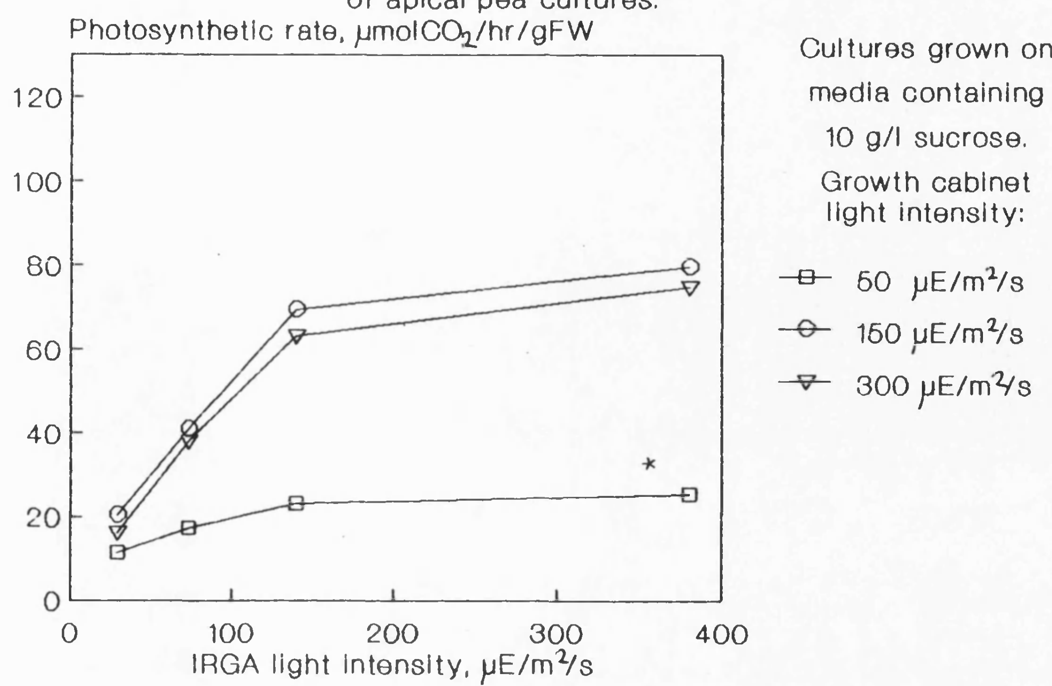


3 =< n >= 5
* significantly higher at 5% level.

c/ Apical cultures grown on media containing 10 g/l sucrose at 50 µE $m^{-2}s^{-1}$ had leaves with very low photosynthetic rates and pigment contents (Fig. 29, 30 & 31). Cultures grown on this media at the higher light intensities had higher and similar photosynthetic rates. Those grown at 150 µE $m^{-2}s^{-1}$ had higher pigment levels. The cultures grown at the higher media sucrose concentrations also had their highest photosynthetic rates and pigment contents at 150 µE $m^{-2}s^{-1}$. Cultures grown at either the intermediate or high media sugar concentrations had similar photosynthetic abilities at either the low and high light intensities.

For the cultures grown at a light intensity of 150 µE $m^{-2}s^{-1}$, the highest photosynthetic rates were found in those cultures grown on medium containing 30 g/l sucrose, and the photosynthetic rate of a culture grown on 10 g/l medium was comparable to that of a culture grown on 50 g/l medium. At this light intensity the pigment content was lower for cultures on media containing 10 g/l sucrose (Fig. 30 & 31). The cultures grown at 50 µE $m^{-2}s^{-1}$ had their highest photosynthetic rates and pigment contents at media sucrose concentrations of 30 and 50 g/l. The highest photosynthetic rate for those cultures grown at a light intensities of 300 µE $m^{-2}s^{-1}$ was on media containing 10 and 30 g/l sucrose, the pigment contents of the leaves were constant for these cultures. The chlorophyll a:b ratios showed no trends (Fig. 32).

Fig. 29 a/ The effect of various growth cabinet light intensities and media sucrose concentrations on the photosynthetic ability of apical pea cultures.



n = 3 or 4
* significantly different

Photosynthetic rate, μmolCO2/hr/gFW

120
100
80
60
40
20
0

0
100
200
300
400

IRGA light intensity, μE/m2/s

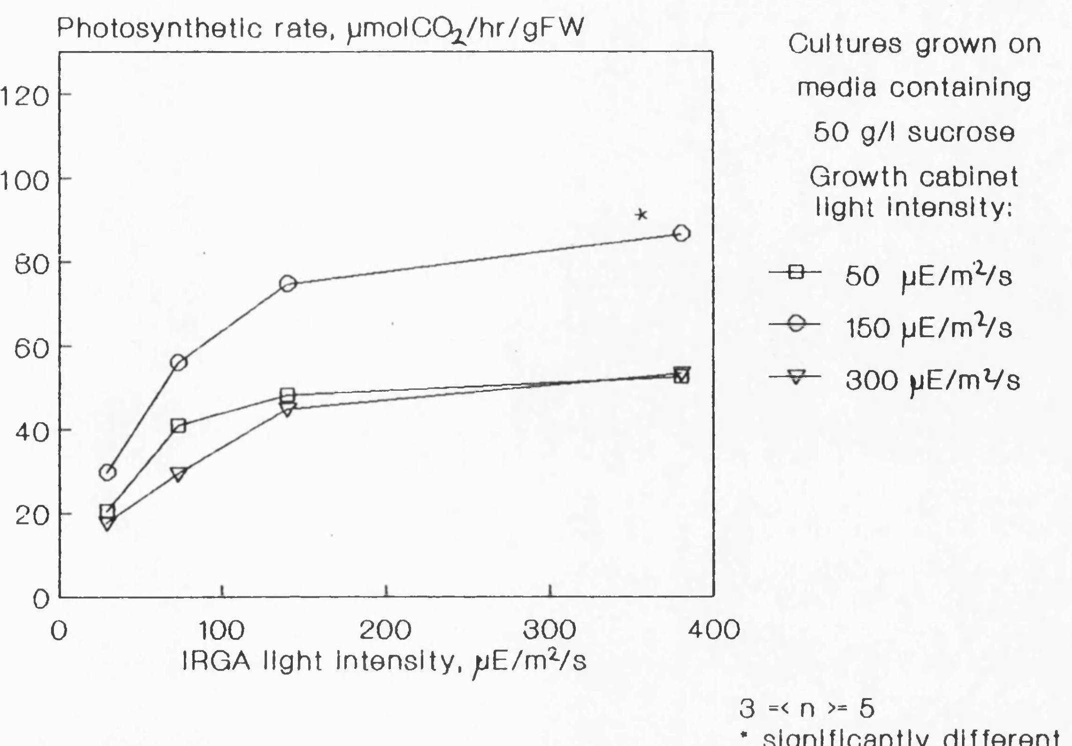
*

Cultures grown on
media containing
30 g/l sucrose
Growth cabinet
light intensity:

50 μE/m2/s
150 μE/m2/s
300 μE/m2/s

3 =< n >= 5
* significantly different

Fig. 29 continued.



3 =< n >= 5
* significantly different

For cultures grown at:

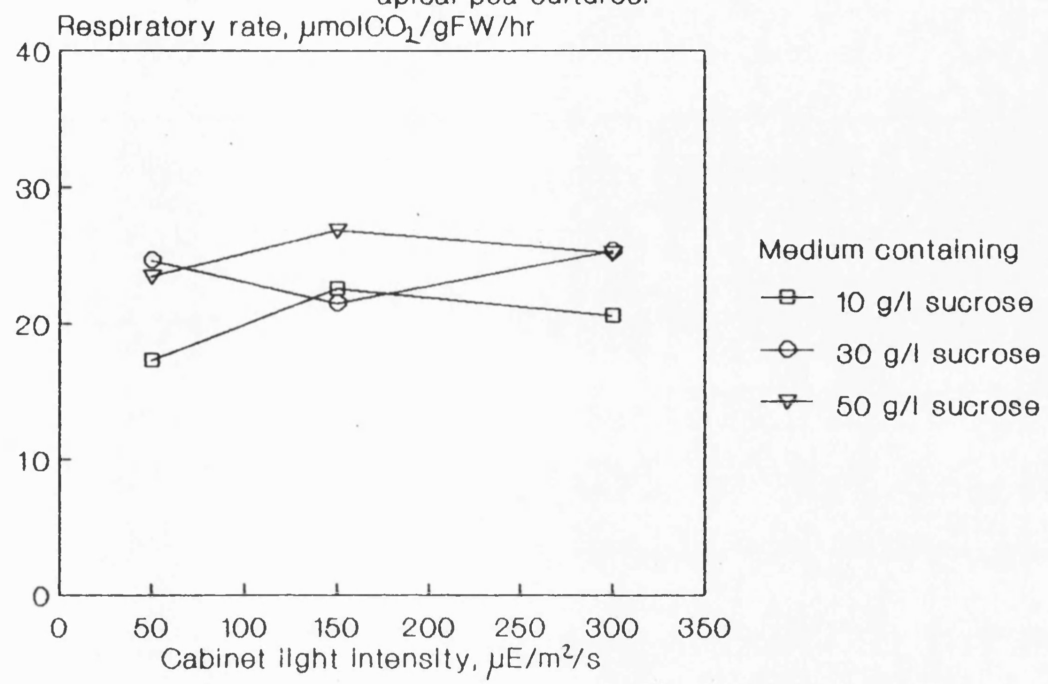
the highest light intensity, those on media containing 50 g/l sucrose had a significantly lower photosynthetic ability.

the intermediate light intensity, those on media containing 30 g/l sucrose had a significantly higher photosynthetic ability.

the lowest light intensity, those on media containing 10 g/l sucrose had a significantly lower photosynthetic ability.

All the significances were tested at the 5% level.

Fig. 29 b/ The effect of various growth cabinet light intensities and media sugar concentrations on the respiratory rates of apical pea cultures.



3 =< n >= 5
No significant differences.

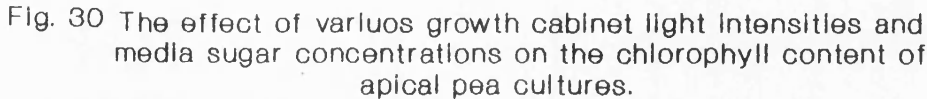
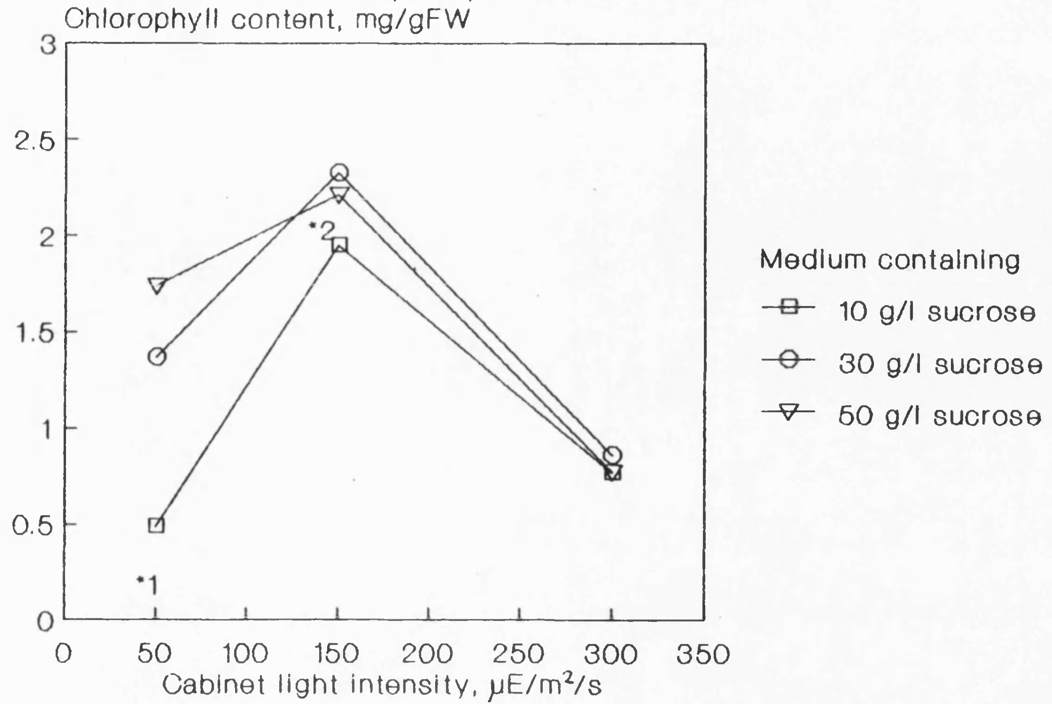


Fig. 30 The effect of variuos growth cabinet light intensities and media sugar concentrations on the chlorophyll content of apical pea cultures.



3 =< n >= 5

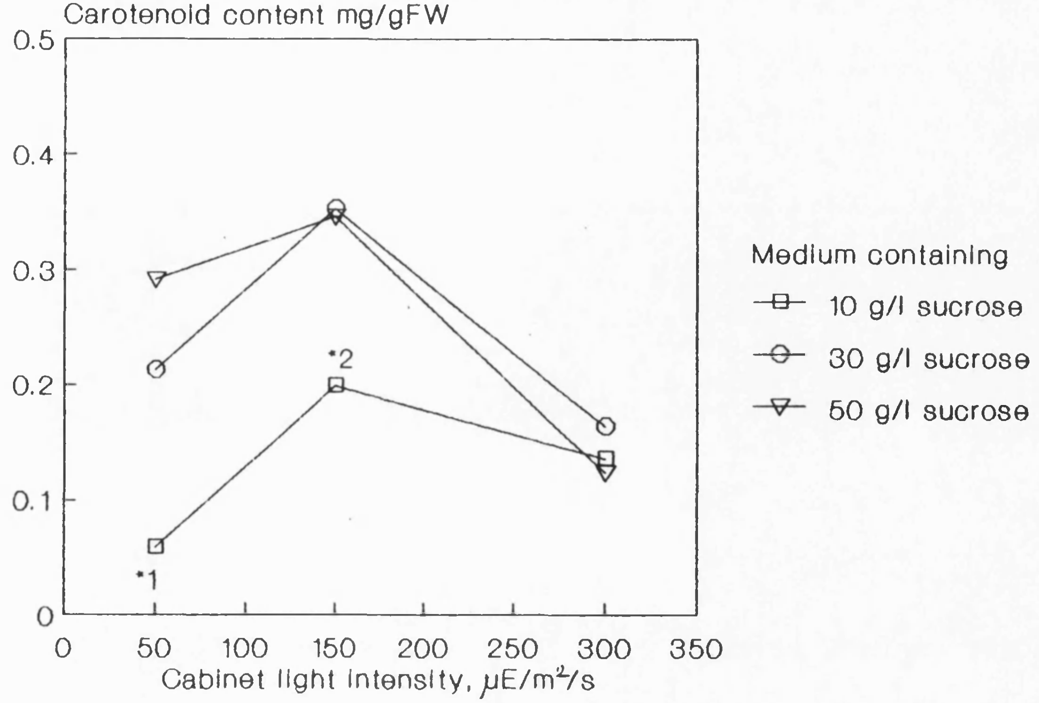
Notes;

*1 All chlorophyll contents significantly different at this light intensity.

*2 Cultures on media containing 10 g/l sucrose have significantly lower chlorophyll contents than those on media containing 30 g/l at this light intensity.

For cultures grown on media containing either 30 or 50 g/l sucrose the chlorophyll contents are significantly different at all light intensities. For cultures grown on a medium containing 10 g/l sucrose the chlorophyll contents are the same at the lowest and highest light intensities.

Fig. 31 The effect of various growth cabinet light intensities and media sugar concentrations on the carotenoid content of apical pea cultures.



3 =< n >= 5

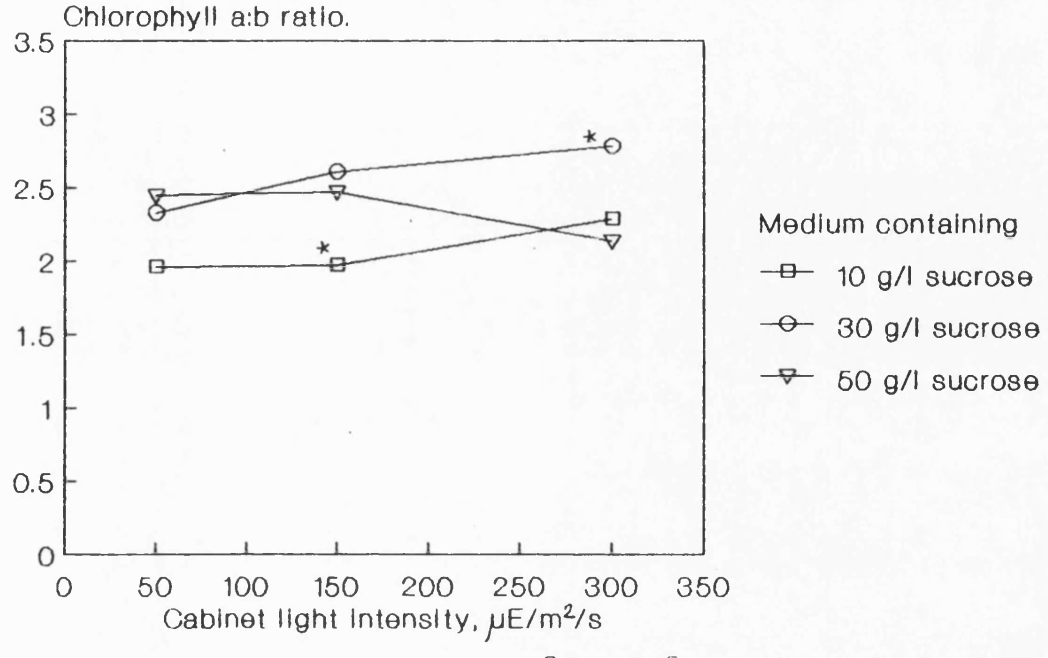
Notes;

*1 All carotenoid contents significantly different at this light intensity.

*2 This value is significantly lower than the others at this light intensity.

At 10 g/l sucrose, carotenoid content lower at lowest light intensity than at medium and high light intensities.
At 30 g/l sucrose, caritenoid content higher at medium light intensity than at low and high light intensities.
At 50 g/l sucrose, carotenoid content lower at highest light intensity than at low and medium light intensities.

Fig. 32 The effect of various growth cabinet light intensities and media sugar concentrations on the chlorophyll a:b ratio of apical pea cultures.



3 =< n >= 5

* value significantly different from the others at same light intensity.

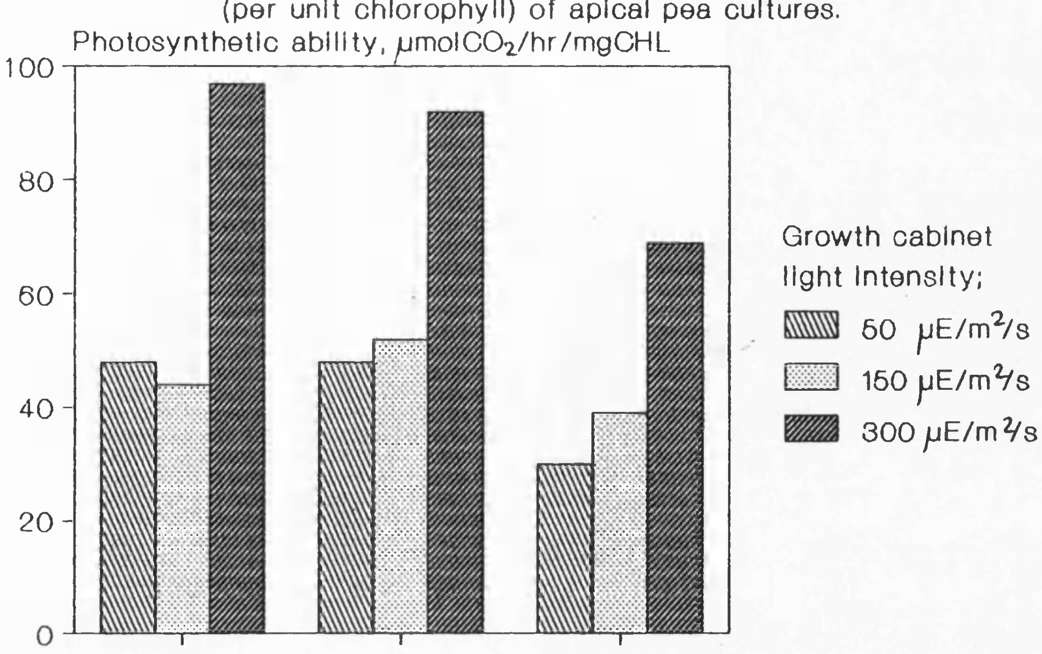
The dark respiration rates of the cultures (Fig. 29) showed no significant trends as both the media sucrose concentration and the growth cabinet light intensity were varied. The lowest value was obtained for cultures grown at the lowest light intensity on media containing the lowest sucrose concentration.

The leaves of the cultures grown on all media sugar concentrations at light intensities of 50 µE $m^{-2}s^{-1}$ and 150 µE $m^{-2}s^{-1}$ had a similar photosynthetic rate per unit chlorophyll (Fig. 33). For cultures grown at the highest light intensity, 300 µE $m^{-2}s^{-1}$, the rate was higher, but was reduced at the highest sugar concentration, 50 g/l.

The FW and DW of the apical cultures increased as both cabinet light intensity and media sugar concentration were increased (Fig. 34). Cultures grown at 50 µE $m^{-2}s^{-1}$ on media initially containing 10 g/l sucrose had the lowest FW and DW, while those grown at 300 µE $m^{-2}s^{-1}$ on media initially containing 30 and 50 g/l sucrose, and at 150 µE $m^{-2}s^{-1}$ on 50 g/l sucrose media, had the highest. The other combinations gave intermediate values.

The media sugar content always decreased over three week culture periods (Fig. 35). As the initial media sugar concentration was increased the amount of sugar lost from the media increased. For cultures grown on media containing 10 and 30 g/l sucrose, the cabinet

Fig. 33 The effect of various growth cabinet light intensities and media sucrose concentrations on the photosynthetic ability (per unit chlorophyll) of apical pea cultures.



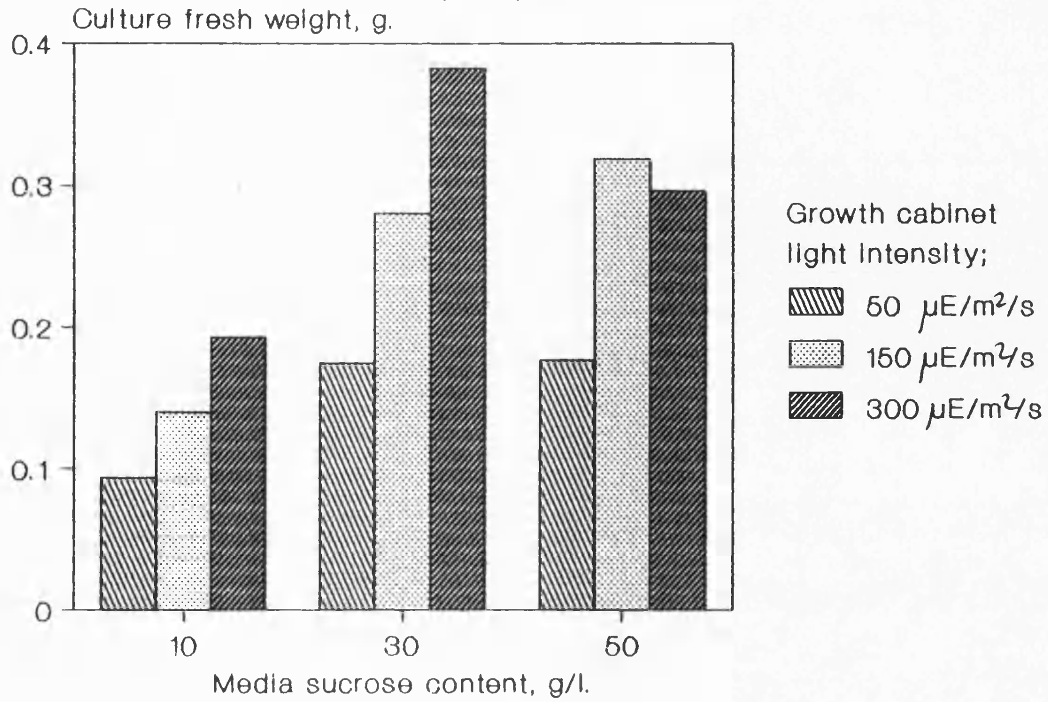
3 =< n >= 5

Notes;

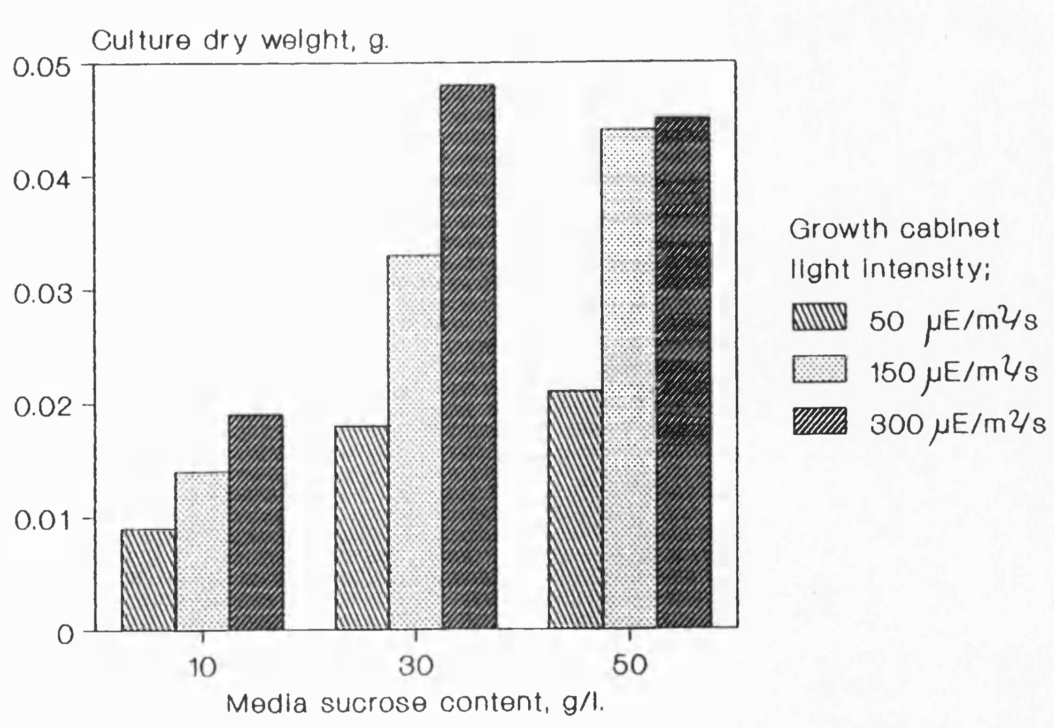
At each media sucrose concentration, the photosynthetic ability per unit chlorophyll is significantly higher for cultures grown at the highest light intensity.

For cultures grown at the highest light intensity, the photosynthetic ability per unit chlorophyll is significantly lower on media containing 50 g/l sucrose.

Fig. 34 The effect of various growth cabinet light intensities and media sucrose concentrations on the fresh and dry weights of apical pea cultures.

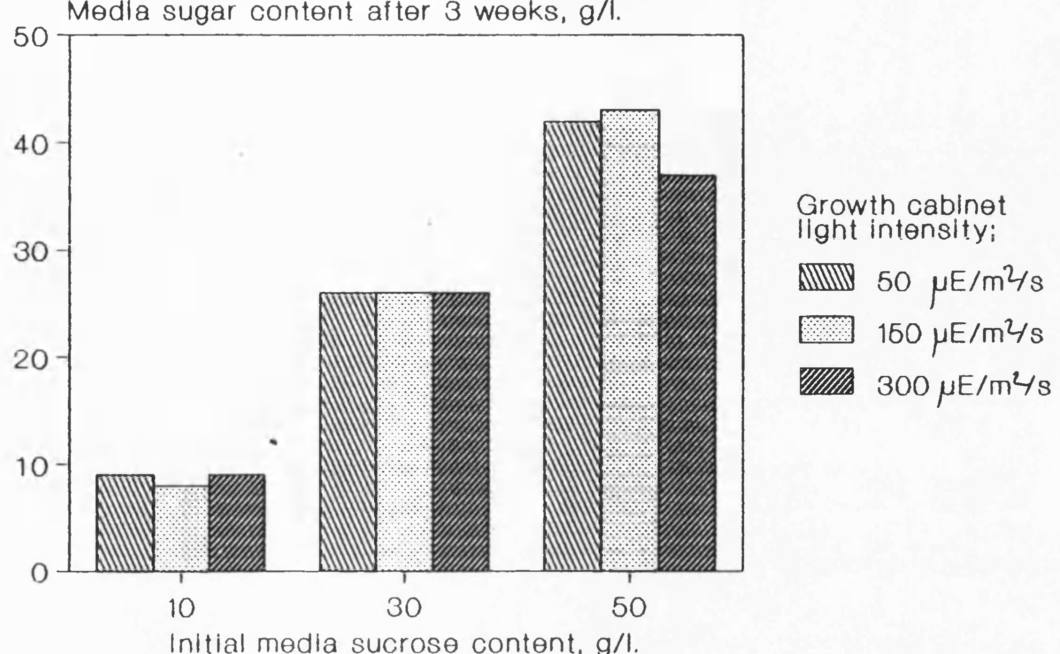


n = 26



n = 26

Fig. 35 The effect of various growth cabinet light intensities on the media sugar content after a three week culture period, for apical pea cultures.



n = 9
No significant differences.

light intensity did not affect the loss of sugar over the three week culture period. For cultures grown on media initially containing 50 g/l sucrose, the amount of sugar remaining at the end of the culture period decreased as the light intensity increased, but not significantly.

3.3.4. Discussion.

The non significant variation in photosynthetic ability and the change in the pigment content of the leaves when the initial media sugar concentration was varied (Fig. 19, 20, 21 & 22) suggests that the sugar concentration of the media may have affected pigment content. This could have been due to effects on pigment production or breakdown. These changes in pigment content in turn might have affected photosynthesis, though this process might have been affected directly by the media sucrose concentration.

The sugar concentration of the culture medium has though been found to have a significant effect on the photosynthetic ability of cultures of other plant species. Evers (1982), using bud cultures of Douglas Fir, found that there was a correlation between lower medium sucrose content and higher photosynthetic carbohydrate production. Cultures of *Rosa* cultivars (Langford & Wainwright, 1987; Cappellades *et al*, 1991) were found to have higher photosynthetic activity and lower pigment contents at low medium sucrose concentrations. Moreover, the photosynthetic abilities and pigment contents were

also found to increase as the medium sucrose content was reduced over a number of subcultures (Langford & Wainwright, 1987), although 10 g/l sucrose was found to be limiting to growth. Cappellades *et al* (1991), found that the amount of starch stored in chloroplasts of rose cultures increased as the medium sugar content was increased, and suggested that this starch accumulation might be responsible for the reduced photosynthesis, due to the high chloroplast carbohydrate levels blocking the production of more carbohydrate. Rose (Langford, 1987) plantlets were found not to grow on sucrose-free media, and a similar result was obtained in thus study. Kozai *et al* (1988a), however, using potato shoot tip cultures, were able to grow cultures on a medium lacking sucrose. Similar results have also been obtained with tobacco (Pospisilova *et al*, 1987), carnation (Kozai *et al*, 1988b), strawberry (Kozai *et al*, 1991a) and *Brassica compestris* (Kozai *et al*, 1991b) cultures (Sections 4.1 & 4.3).

Langford (1987) with rose cultures found that the photosynthetic ability per unit chlorophyll was lower at higher medium sucrose concentrations, and a similar response was obtained with apical pea cultures (Fig. 23). The photosynthetic rates per unit chlorophyll give an indication of the efficiency of the photosynthetic apparatus, as the amount of carbon dioxide fixed per unit of the light energy harvesting pigments. High medium sugar concentrations decrease this

photosynthetic efficiency. This supports the hypothesis that at high medium sugar concentrations carbohydrate accumulation reduces the photosynthetic activity, by inhibiting the formation of more carbohydrate.

High concentrations of medium sugar have been shown to reduce chlorophyll levels in a variety of plant cell and callus cultures (Pamplin & Chapman, 1975; Dalton & Street, 1976; Barg & Umiel, 1977). This may be the result of sucrose inhibiting the production of chlorophyll at the ALA synthesis stage (Pamplin & Chapman, 1975). However, Rose plantlets (Langford, 1987) had lower chlorophyll levels at low sugar concentrations, and a similar result was obtained in this study.

The lower dark respiration rate for cultures on media containing 10 g/l sucrose (Fig. 19), suggests that low concentrations of media sugar may have restricted the respiratory physiology of the cultures. As sugars, such as sucrose, provide the energy source for respiration, the media sugar concentration might, therefore, be expected to have an affect on the rate of this metabolic process. Capellades *et al* (1991) found that rose cultures had their lowest dark respiration rates on media containing an intermediate sucrose concentration (range used was 1-5%). However, carnation (Kozai *et al*, 1988b) and potato (Kozai *et al*, 1988a) cultures grown on sucrose-free media were found to have lower respiratory rates than those grown on media

containing sucrose (20 g/l). The effects of the media sugar concentration on the respiratory physiology of cultures appear to be variable, and are possibly dependent on the plant species.

The medium sugar concentration has also been shown to affect cultures growth, with both high and low concentrations being inhibitory; for example, Constantaine (1983) with *Prunus domestica* and Langford (1987) with *Rosa*. Pua & Chong (1985), with cultures of apple, found that high and low medium sugar concentrations inhibited culture growth, and that the growth was also dependent on the type of sugar used. These findings suggest that cultures have a sugar optimum, high or low sugar concentrations reducing the photosynthetic activity, chlorophyll content and growth; however the responses depend upon the cultivar or species.

Increasing or decreasing the cabinet light intensity caused significant decreases in the photosynthetic physiology of the apical pea cultures (Fig. 24, 25, 26 & 27), but did not effect their respiration rates (Fig. 24). Lee *et al* (1985) with sweetgum, Langford (1987) with Rose cultivars and Desjardins *et al* (1988) with strawberry have obtained similar changes in the photosynthetic physiology of cultures. However, Kozai *et al* (1990), with *Cymbidium* plantlets, found the photosynthetic ability increased as the growth cabinet light intensity was raised from 35 to

226 µE $m^{-2}s^{-1}$. Infante *et al* (1989), using cultures of *Actinidia deliciosa* grown under a range of cabinet light intensities (30 to 250 µE $m^{-2}s^{-1}$), found that the photosynthetic activity increased as the cabinet light intensity was raised to 70 µE $m^{-2}s^{-1}$ and thereafter remained constant. The range of light intensities used in those two instances was smaller than that used in this study, and higher light levels may have caused reductions in the photosynthetic activities of those cultures. Donnelly & Vidaver (1984b), with cultures of red raspberry grown under a range of light intensities from 2-6 Klux, found no change in photosynthetic activity, but cultures grown at the highest light intensity had a higher dark respiration rate. The light intensity has, therefore, been shown to affect the photosynthetic activity of plantlets; however the response depends on both the light intensity and the plant species.

The reduced photosynthetic rates and pigment content of the cultures grown at the higher light intensities could have been due to damage to the pigment production systems, or destruction of the pigment by high light levels. The high light levels, may also have affected some other aspect of the photosynthetic system, such as the chloroplast structure. The decrease at low light intensities could be due to limited development of the photosynthetic apparatus, or to poorly developed photo-protection mechanisms. Such changes could be likened to the differences found *in vivo* between sun and

shade leaves. High light levels may cause the breakdown of chlorophyll, and the development chloroplasts containing a few small thylakoids with high photosynthetic activity. At low light levels the leaves have, chloroplasts containing extensive thylakoids with much lower photosynthetic activity (Lichtenthaler *et al*, 1981; Grumbach & Lichtenthaler, 1982; Longstreth *et al*, 1985).

The photosynthetic rate per unit chlorophyll (Fig. 28) was significantly higher at the highest light intensity. Langford (1987) and Lee *et al* (1985) also obtained higher photosynthetic rates per unit chlorophyll at higher light intensities. Photosynthetic efficiency was greater at higher cabinet light intensities, that is more CO_2 was being fixed per unit chlorophyll than at the lower light levels. At high light intensities, the plantlets may possibly be developing chloroplasts like those of sun leaves.

Light intensity has also been previously shown to affect culture growth. Cultures of *Ribes nigrum* have an optimal light intensity for proliferation, and their DW increases with light intensity (Flegmann & Wainwright, 1984). However Langford (1987), using cultures of *Rosa*, found that light intensity did not affect multiplication or FW, although Bresson *et al* (1982) did find that the multiplication rate of rose cultures had an optimal light level. Moreover, both of

these investigations noted a decrease in shoot vigour as light levels were increased.

Growth cabinet light intensity has been shown to affect the culture's photosynthetic ability, cultures typically having an optimum light level. These differences are possibly due to changes in culture growth and chloroplast development, as well as being associated with changes in the pigment content.

When both the initial medium sugar concentration and the growth cabinet light intensity were varied, the combined effects on the photosynthetic physiology of the apical pea cultures were typically larger than when only one was varied (Fig. 29, 30, 31 & 32). It appears that both the initial medium sucrose concentration and the growth cabinet light intensity have effects on photosynthetic physiology of apical pea cultures, the interaction of these two factors possibly being synergistic. For example, at low light and low sugar the photosynthetic ability and pigment content were lower than for cultures grown at the intermediate sugar concentration and light intensity. The low light may lead to shade plant-like leaves, that is to chloroplasts with extensive thylakoids but low photosynthetic activity. The photosynthetic physiology though could be further limited by the low medium sucrose probably restricting the growth and development of the cultures.

The photosynthetic rates per unit

chlorophyll (Fig. 33) show that the photosynthetic efficiency was increased by high light and decreased by high sugar. The cultures developing under high light would probably make efficient use of their photosynthetic pigments and have well developed photo-protection mechanisms. High medium sugar levels probably provide an easily available alternative carbon and energy source to photosynthesis and so possibly restrict the development or operation of the photosynthetic apparatus.

The highest FW and DW gains (Fig. 34) were for the cultures grown on high sucrose concentration media at both the high and intermediate light intensities and on intermediate sucrose concentration media at the highest light intensity. These combinations gave potential access to the largest amounts of energy, that is to the greatest light intensities and sugar concentrations. Chong & Pua (1985) and Pua & Chong (1984 & 1985), using apple cultures on a range of carbon sources at different concentrations, found that the culture weight gains were dependent on the sugar, its concentration and the apple cultivar. The FW & DW of carnation plantlets were found to increase as the sucrose content of the media was increased from 1 to 3 % (Kozai & Iwanami, 1988), and a similar result was also obtained with potato cultures (Kozai *et al*, 1988a). Wainwright & Flegmann (1984) showed that the DW of blackcurrant cultures on rooting media increased with light intensity.

The loss of sugar from the media (Fig. 35)

during the culture period was most probably due to sugar uptake by the cultures, with the sugar providing energy and raw materials for growth. The amount of sugar lost from the media increased as the initial media sucrose concentration was raised. Champion (1982), using shoot cultures of *Cinchona ledgeriana* found that the sugar content of the medium decreased with time in culture and that this sugar uptake was related to the growth of the cultures. Kozai *et al* (1991a), with strawberry plantlets cultured on media initially containing 20 g/l sucrose, found that the sucrose content of the culture medium decreased with time, declining to approximately 10 g/l after 21 days. However these workers also found that the glucose and fructose content of the media increased with time (approximately 5 g/l of each of these two sugars was present after 21 days). Therefore, they concluded that cultures net sugar uptake was only a small proportion of the total media carbohydrate. *Dendrobium* cultures (Hew & Mah, 1989) have been shown to take up sugar from media, the rate of carbohydrate uptake increasing as the medium sucrose concentration was raised from 1 to 2 %. The cultures secreted both acid and alkali invertase, with the activity of these increasing with time, and the sucrose was shown to be hydrolysed before uptake. Therefore, cultures absorb sugars from the culture media; however only a small fraction of the total medium sugar is taken up, and the amount taken up may be related to the initial sugar content of the media.

The dark respiration rates of the cultures were, in certain instances, lower for those grown on media containing the lowest sucrose concentration (Fig. 19 & 29). As sugars provide the energy source for respiration such variation might be expected. The sugar concentration has been found to affect the respiratory rates of cultures of other plant species (Capellades, *et al* 1991; Kozai *et al* ,1988 a & b). The light intensity was not found to affect the cultures respiratory rates (Fig. 24 & 29). Donnelly & Vidaver (1984b) found, however, that the highest light intensities (6 Klux) increased the dark respiration rate of raspberry cultures.

The FW and DW of the cultures (Fig. 34), unlike the measured dark respiration rates, changed when both the sugar concentration of the media and the growth cabinet light intensity were varied. The growth of the cultures was, therefore, also dependent on these environmental factors. Moreover, as growth requires respiratory energy, similar changes in the cultures respiratory physiology might also have been expected. The dark respiration rates were, however, measured under the conditions prevalent in the IRGA analysis chamber not the culture vessel, and using leaves removed from the cultures not whole plantlets. The measured respiratory rates may, therefore, have not been indicative of those of the whole plantlets in the culture vessel (Section 3.1.4.).

The reduction in the respiration rates of the culture leaves (Fig. 19) by the lowest media sucrose concentration, suggest that the media sugar content may have had a substantial effect on the respiratory physiology of the cultures. This might have been expected as sugars are the respiratory "substrate". Light intensity might have had an indirect affect respiratory physiology of the cultures, via the photosynthetic production of sugar or by stimulating growth. These results are, however, inconclusive as to the effects of media sugar and growth cabinet light intensity on the respiratory physiology of the cultures. Further more detailed investigation are, therefore, needed using whole plantlets instead of leaf material.

Cultures grown on medium with the intermediate sugar concentration (30 g/l) and at the intermediate light intensity (150 $\mu E\ m^{-2}s^{-1}$) had the highest photosynthetic ability and pigment content. These cultures did not, however, attain the greatest FW and DW or have the highest photosynthetic rate per unit chlorophyll. There was also no clear relationship between the uptake of sugar from the medium and photosynthetic ability. These results suggest that the cultures were dependent on both photosynthesis and medium sugar as sources of energy for growth, as the higher initial medium sucrose concentrations and growth cabinet light intensities gave the greatest culture growth. The

relative contributions these two energy sources made to the growth of the cultures probably depended on their relative levels in the culture environment.

The growth of cultures under a range of light intensities has been shown to affect their photosynthetic abilities. The changes may possibly be related to changes in the cultures ultrastructure and their ability to protect against photo-oxidative damage, therefore the effect of light intensity on culture ultrastructure and photoprotection mechanisms need investigation (Section 3.4.). Furthermore, these experiments give no indication as to the gas composition inside the culture vessel. The concentration of CO_2, the photosynthetic "substrate", in the culture vessel will affect the culture's level of photosynthesis *in situ*. This too needs investigation.

3.4. Chloroplast ultrastructure and photoprotection mechanisms.

3.4.1. Introduction.

In section 3.3. it was shown that the cabinet light intensity affects the photosynthetic ability, pigment content and growth of apical pea cultures.

Light intensity has been shown to affect the ultrastructure of sun and shade leaves, and high- and low-light plants. Shade type chloroplasts have a greater number of thylakoids per granum, a higher degree of stacking of the thylakoids into grana, and broader grana than are found in sun type chloroplasts. Sun-type chloroplasts also have large starch grains (Lichenthaler *et al*, 1981). Lee *et al* (1985) showed that light intensity affected the ultrastructure of chloroplasts in both plantlets and seedlings of *Liquidambar styraciflua* L. (Sweetgum).

Highly reactive oxygen derived radicals, such as superoxide, can be produced as by-products of photosynthesis, particularly at high light levels. These radicals can inhibit photosynthesis and other processes by, for example, damaging membranes (Pryor, 1978) or inactivating enzymes (Kaiser, 1979; Charles & Halliwell, 1980). Glutathione and ascorbate are active in protecting

chloroplasts against this photo-oxidative damage (Asada & Takahashi, 1987) by scavenging these radicals (see Section 1.1.). The levels of these scavengers have been shown to vary with environmental conditions and plant age. For example, levels increase in greening leaves (Gillham & Dodge, 1985), and decrease with the onset of senescence (Thomas & Stoddart, 1980).

In this section the effect of various growth cabinet light intensities on the ultrastructure of the chloroplasts of apical pea cultures has been studied. The levels of glutathione and ascorbate in leaves of these cultures were also ascertained.

3.4.2. Methods and Materials.

Apical pea cultures were grown at growth cabinet light intensities of 50, 150 & 300 µE $m^{-2}s^{-1}$ on basal medium (MS containing 30 g/l sucrose). After three weeks leaf material was harvested from the cultures and used for ultrastructural studies or to assay levels of glutathione and ascorbate. The ultrastructure of *in vivo* pea seedlings grown at 150 µE $m^{-2}s^{-1}$ was also studied.

3.4.2.1. Ultrastructure: The preparation of material for transmission electron microscope.

Pieces of leaf material (1 mm^2) were fixed overnight in 2.5% glutaraldehyde in 50 mM cacodylate buffer at 4°C in the dark. The material was then washed

in this buffer (2 x 10 min) and post fixed with osmium tetroxide in distilled water for one hour. After washing in distilled water, the leaf material was dehydrated through an acetone gradient (2 x 15 min in 30%, 50%, 70% & 90%, and 4 x 15 min in 100%).

The samples were then placed in a 50:50 resin:acetone mixture and left overnight at room temperature on a rotor. The following day the resin acetone mixture was replaced by pure resin; after 24 hours this was replaced by fresh resin. The material was then transferred to fresh resin in block moulds, which were placed in a 70 °C oven to harden. The resin blocks were cooled and sectioned using an ultramicrotome, the sections were floated onto copper grids and stained using solutions of uranyl acetate and lead citrate (7 min in each) and washed four times with distilled water. The sections were examined in a Joel (JEM 1200EX II) transmission electron microscope.

3.4.2.2. The measurement of glutathione and ascorbate levels.

a. Glutathione

Leaf material (0.25 gFW) was ground in 10 ml of 0.5% sulphosalicylic acid, and 2.3 ml of this was then spun at 4000 rpm in a MSE chillspin centrifuge (Fisons, Crawley, U.K.) for 10 min.

The following reaction mixture was then made up in a 3 ml cuvette: 875 µl 125 mM of buffer (Na H_2PO_4/6.3 mM EDTA); 375 µl of supernatant; 875 µl of 0.6 mM NADPH (in buffer); 250 µl of 6 mM DTNB (in buffer); and 125 µl of glutathione reductase (10 units/ml, in buffer). The reaction was followed at 412 nm using a recording spectrophotometer (Shmadzu UV-200, Kyoto, Japan.). A standard curve was prepared using known amounts of glutathione (Law *et al*, 1983; Polge, 1989).

b. Ascorbate

Leaf material (100 mgFW) was ground in 5 ml of Tricine-NaOH buffer (pH 7.8), left to stand for 10 min. and then spun for 10 min at 3000*g* in an MSE Centaur 1 (Fisions, Crawley, U.K.). The supernatant was mixed with 0.3 g acid washed charcoal and filtered using Whatman no.1 filter paper.

Two ml of the extract was then mixed with 1 ml of 2,4-dinitrophenol-hydrazine (2% in 90% sulphuric acid) and one drop of 10% thiourea (in 70% ethanol). The mixture was heated in a boiling waterbath for 15 min, and then cooled to 0°C, whereupon 2.5 ml of 80% sulphuric acid was added. The absorbance was read at 530 nm using a spectrophotometer (PU 8650 visible, Pye Unicam, Cambridge, U.K.). A standard curve was established using known amounts of ascorbate (Mukherjee & Choudhuri, 1983).

3.4.3. Results.

The glutathione content of the leaves of the apical pea cultures was not affected by growth cabinet light intensities under the conditions tested. The ascorbate content increased, however, as the cabinet light intensity was raised (Table 4).

The chlorophyll to carotenoid ratios (Table 5) were calculated using the pigment content data obtained in section 3.3. (Fig. 30 & 31). For cultures grown at the highest growth cabinet light intensity this ratio was significantly lower.

Light intensity affected the chloroplast structure (Plates 1, 2 & 3). Leaves from cultures grown at 50 $\mu E\ m^{-2}s^{-1}$ had chloroplasts with well formed lamella and clearly defined grana, but with few starch grains. At 150 $\mu E\ m^{-2}s^{-1}$ the membrane structure was similar, but there were prominent starch grains. The chloroplasts of leaves from cultures grown at 300 $\mu E\ m^{-2}s^{-1}$ had a poorly formed membrane system, starch grains and numerous lipid globules. The chloroplasts of the material grown at 150 $\mu E\ m^{-2}s^{-1}$ appeared similar to those of the *in vivo* controls (Plate 4).

Table 4 Glutathione and ascorbate concentrations of the leaves of three-week old apical pea cultures grown under a range of light intensities.

Cabinet light intensity $\mu E\ m^{-2} s^{-1}$	Glutathione mg/g FW	Ascorbate mg/g FW
50	1.28	2.5
150	1.07	3.0
300	1.17	3.7*
	n = 8	n = 10

* this value is significantly higher (at the 5% level) than the value at the lowest light intensity.

No significant changes in the glutathione concentration.

Medium contained 30 g/l sucrose and no P.G.R.s.

Table 5 The chlorophyll to cartenoid ratio of the leaves of three week old apical pea cultures grown under a range of light intensities.

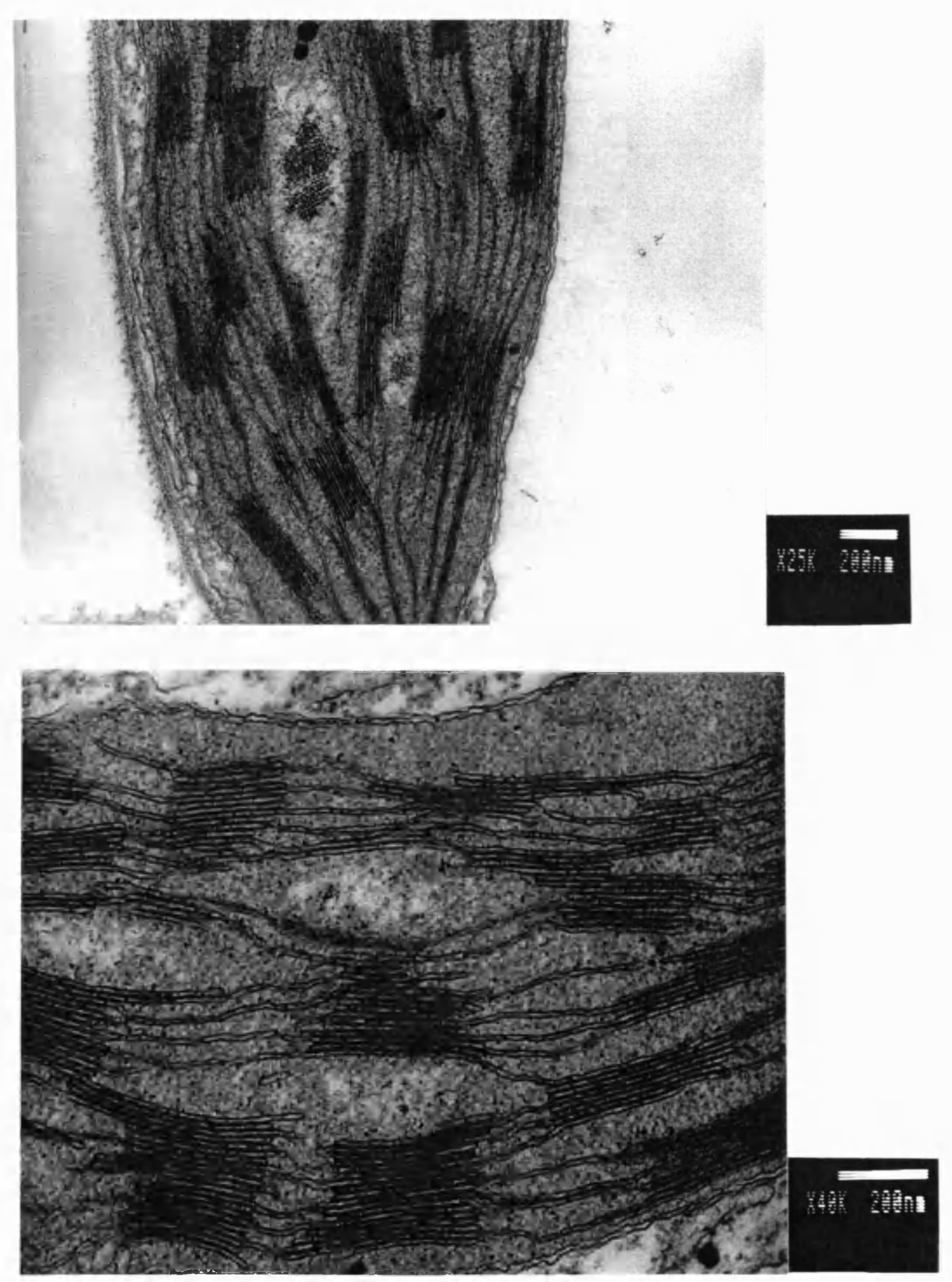
Cabinet light intensity $\mu E\ m^{-2} s^{-1}$	Chlorophyll: carotenoid ratio
50	6.44
150	6.79
300	5.34*

3 =< n >= 6

* this value is significantly lower (at the 5% level) than the values at the other light intensities.

Medium contained 30 g/l sucrose and no P.G.R.s.

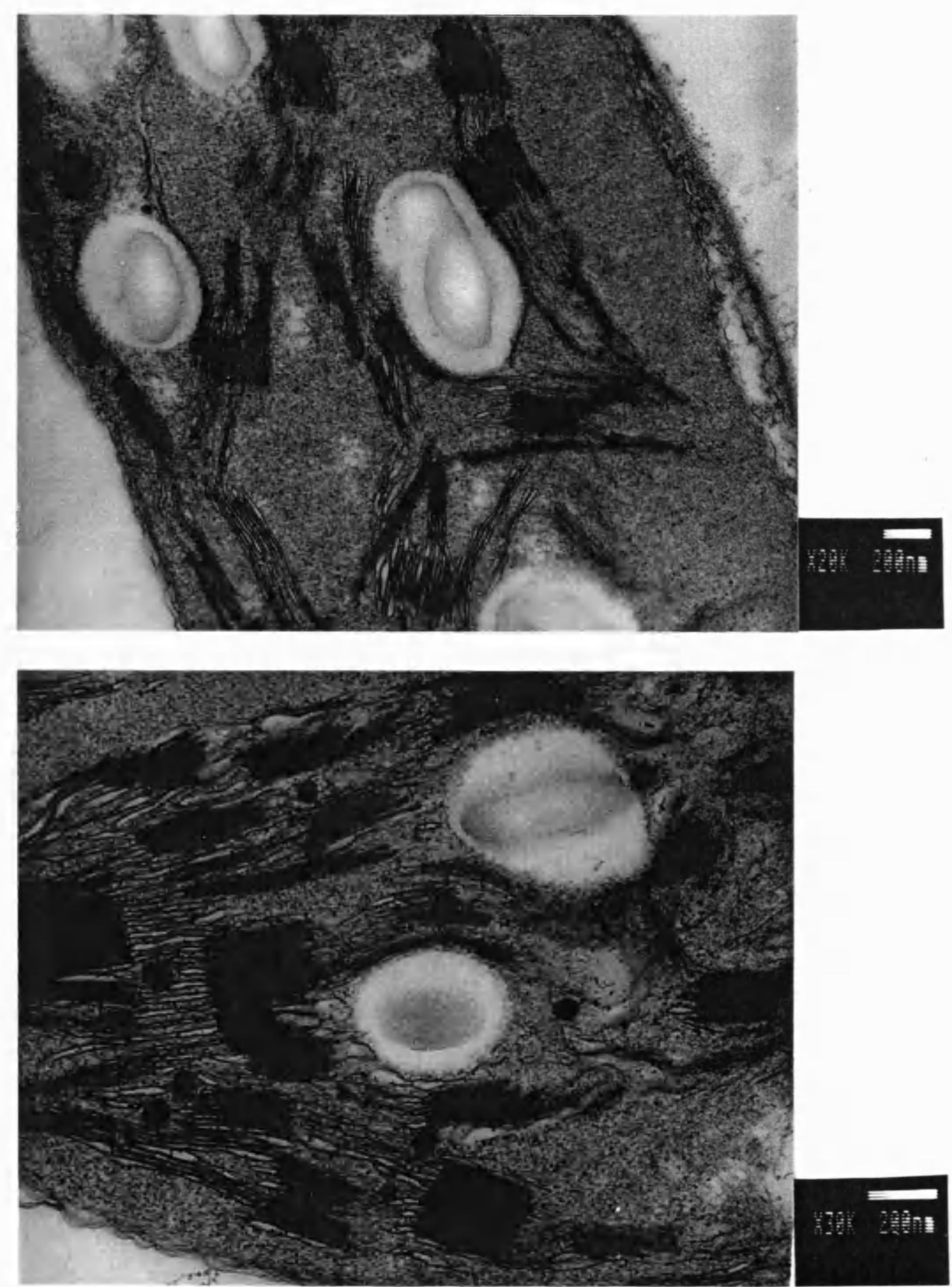
Plate 1. Micrographs of chloroplasts of apical pea cultures grown at a cabinet light intensity of 50 $\mu E\ m^{-2} s^{-1}$.



Note:
the clearly defined grana, and the abscence of strach grains and lipid globules.

Cultures grown on basal medium(MS, no P.G.R.s & 30 g/l sucrose).

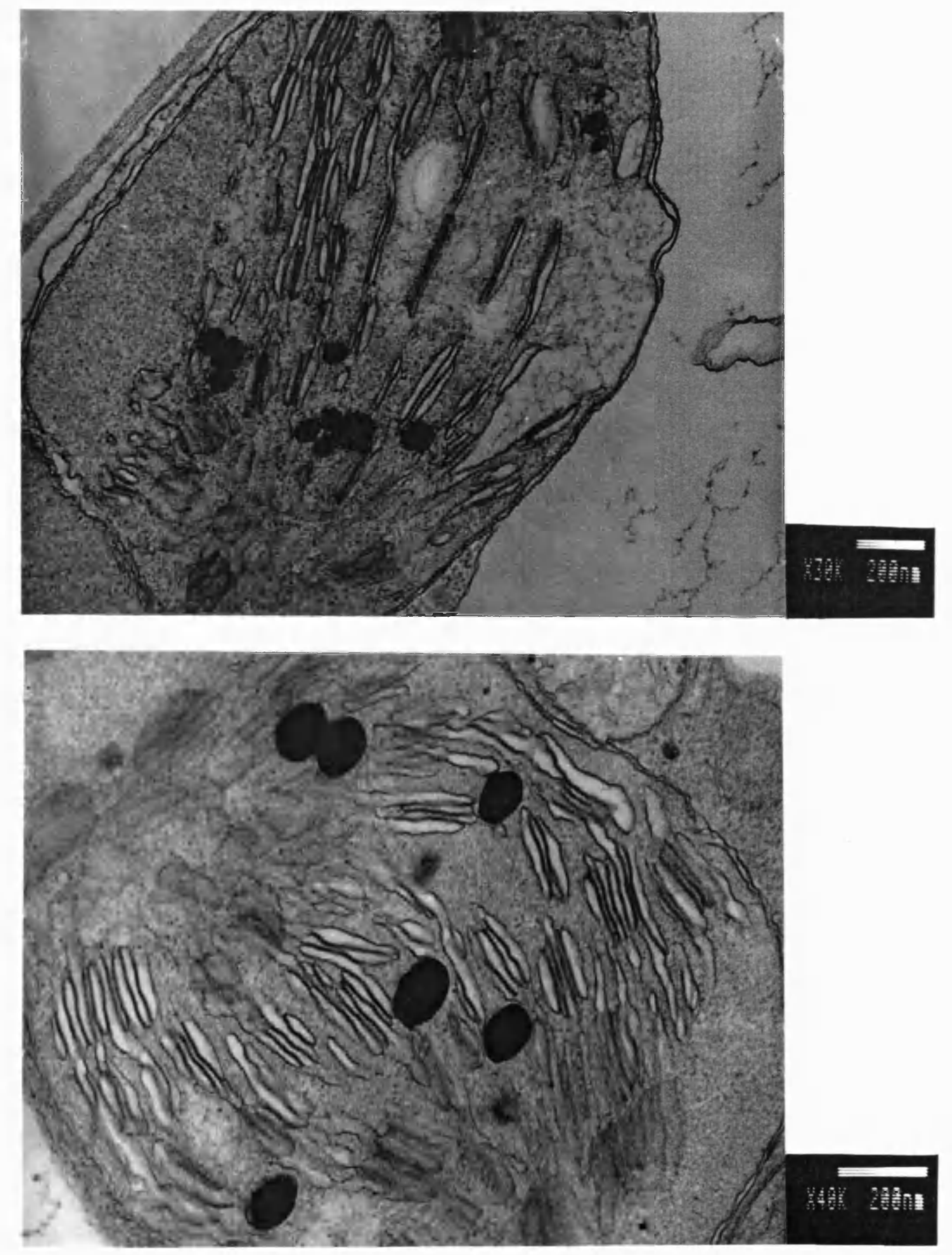
Plate 2. Micrographs of chloroplasts of apical pea cultures grown at a cabinet light intensity of 150 $\mu E\ m^{-2} s^{-1}$



Note:
extensive membrane system, presence of starch grains and the small number of lipid globules.

Cultures grown on basal medium (MS, no P.G.R.s & 30 g/l sucrose).

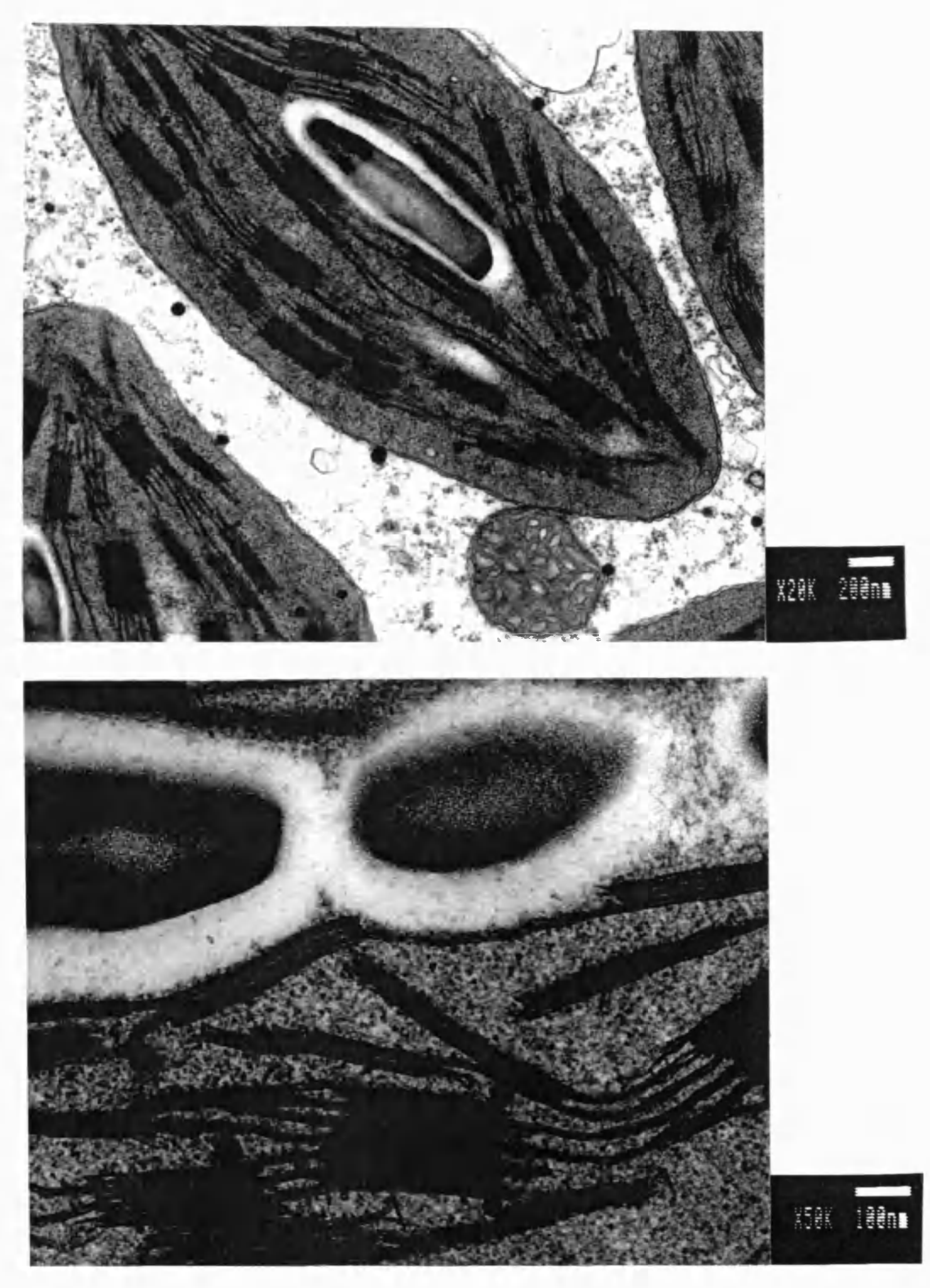
Plate 3. Micrographs of chloroplasts of apical pea cultures grown at a cabinet light intensity of 300 $\mu E\ m^{-2} s^{-1}$



Note:
the disrupted membrane system and numerous lipid globules.

Cultures grown on basal medium (MS, no P.G.R.s & 30 g/l sucrose).

Plate 4. Micrographs of chloroplasts of *in vivo* pea seedlings grown at 150 $\mu E\ m^{-2}.s^{-1}$



Seedlings grown in universal compost.

3.4.4. Discussion

Both glutathione and ascorbate are involved in the scavenging of toxic oxygen derived radicals, which are produced as by-products of photosynthetic activity (Halliwell, 1982). O_2 is reduced at photosystem I, producing superoxide, which can in turn can form products such as hydrogen peroxide and the hydroxyl radical (Asada & Takahashi, 1987). These radicals may adversely affect photosynthesis and other processes, by damaging membranes (Pryor, 1978) or inactivating enzymes (Kaiser, 1979; Charles & Halliwell, 1980). The O_2 reduction that produces these radicals is thought to allow the synthesis of ATP without the production of NADP+, thus providing a mechanism for varying the ratio of these compounds (Allen, 1976). The light driven assimilation of CO_2 requires the correct stoichiometeric balance of ATP and NADP+. Moreover, the reduction of O_2 maintains the operation of the electron transport system by preventing its over-reduction when the CO_2 levels are low, and furthermore dissipates excess light energy that would otherwise damage the photosynthetic apparatus (Furbank, 1984). As light levels are raised photosynthetic activity will increase, so there could be a concomitant rise in the production of these toxic radicals. An increase in the levels of glutathione and ascorbate might be expected to remove these radicals and thus prevent or reduce photo-oxidative damage to the photosynthetic apparatus.

Ruffle (1989) found that for leaves of pea seedlings, grown at two growth cabinet light intensities, the level of ascorbate was approximately two times higher for those that had been grown at the higher light level (300 µE $m^{-2}s^{-1}$). The glutathione content was initially found to be the same at both light levels; however the level decreased slightly with time for the seedlings that had been grown at the lower light level (150 µE $m^{-2}s^{-1}$). This concurs with the results reported here for apical pea cultures (Table 4), where the glutathione level was relatively constant, while the ascorbate level increased, as the light intensity in the growth cabinet was raised. The increase in the ascorbate level *in vitro* was smaller however than that for *in vivo* pea leaves (Ruffle, 1989). Gillham (1986) found that the ascorbate content of pea plants varied seasonally and that this variation was related to seasonal variation in light intensity. Thus, both pea cultures and whole plants have been shown to possess increased levels of ascorbate and hence greater photo-protection, when the environmental light intensity was elevated.

Protection against photo-oxidative damage is also provided by the carotenoid pigments. These are only 30-40 % efficient at transferring harvested light energy to the photosynthetic reaction centres. The main function of these pigments is to dissipate singlet oxygen

and to quench excited chlorophyll (Lawlor, 1987; Asada & Takahashi, 1987). The chlorophyll-to-carotenoid ratio of pea seedlings was shown to increase during the transition from etiolated to green plants (Gillham & Dodge, 1986), and this was related to changing requirements for photoprotection during this period. In Section 3.3. measurements were made of the chlorophyll and carotenoid contents of apical pea cultures grown under a range of light intensities (Fig. 30 & 31). The levels of these two pigments showed similar changes as the light intensity was increased, however the chlorophyll to carotenoid ratio (Table 5) was lower at the highest light intensity. Therefore, there were parallel changes in the levels of the carotenoids and chlorophylls at the low and medium light intensities, but at the higher light intensity there was proportionally more carotenoid. The level of photo-protection provided by the carotenoids to the chlorophyll pigments was therefore greater at higher light intensities.

The changes in the ultrastructure of the plantlet chloroplasts suggest that they were "adapting" to the various light levels. The internal chloroplast membranes, the thylakoids, contain the components of the photosynthetic apparatus (Lawlor, 1987; see Section 1.2.), including the light harvesting chlorophyll-protein complexes (LHCP), the photosynthetic reaction centres

(photosystems, PSI & II) and the associated electron transport system. $PSII_{\alpha}$ (numerous associated LHCP) is found in grana membranes, while $PSII_{\beta}$ (few associated LHCP) is found in the stroma exposed membranes, as is PSI. The degree of organisation of the membranes into grana and the extent of the thylakoids is therefore related to the photosynthetic ability of the chloroplasts, and thus of the leaf. Sun leaves, for example, have smaller grana than shade leaves, and so less LHCP (associated with PSII). They need to harvest proportionally less of the available light energy (Lichtenthaler & Meier, 1985), though they have higher levels of other pigment components. The presence of starch grains would suggest that the carbohydrate level in the chloroplast, due to either the photosynthetic production of sugar or its import, is such that there is a storable surplus. More, and larger, starch grains are for example, found in sun leaves (Lichtenthaler *et al* , 1981).

Cultures grown at 50 $\mu E\ m^{-2} s^{-1}$ had chloroplasts with numerous well formed grana, but few starch grains (Plate 1). They therefore possessed an extensive photosynthetic apparatus, able to harvest as much as possible of the limited avaliable light energy. The chloroplasts were similar to those of *in vivo* shade leaves. At 150 $\mu E\ m^{-2} s^{-1}$, the thylakoid system was similar, but there were more starch grains (Plate 2). The

chloroplasts had an extensive photosynthetic apparatus, which was operating more efficiently and producing surplus carbohydrate which was stored. The chloroplasts from leaves grown at 300 $\mu E\ m^{-2} s^{-1}$ had a limited membrane system and showed signs of structural breakdown (Plate 3) such as swollen membranes, poorly defined grana, and numerous lipid globules (possibly the products of membrane breakdown). This suggests that the chloroplasts were being damaged by the high light levels. The light energy absorbed by the pigments exceeded that needed for photosynthesis, and it appears that this surplus energy was not being safely dissipated. As excess light was available, a smaller chloroplast membrane system may have developed, than at low light intensities. This smaller membrane system could possibly have been similar to those of *in vivo* sun leaves. The cultures, however, would still have been able to attain a viable photosynthetic rate.

Lee *et al* (1985) grew seedlings and cultures of sweetgum under a similar range of light intensity to that used here with apical pea cultures. The chloroplasts of the plantlets had well organised grana at high light intensities and disorganised grana at low light intensities, and furthermore the chloroplasts were always devoid of starch. The observations for the sweetgum seedlings, however, were different to those of the Sweetgum plantlets and similar to those for the

apical pea cultures.

Capellades *et al* (1991) found for rose cultures that the number and size of the starch grains in the chloroplasts, and the starch content of the leaves, increased as the sucrose concentration of the media was raised from 1 to 5%. Therefore, medium sugar concentration can affect the chloroplast carbohydrate level, and might inhibit the formation of more sugar by photosynthesis. The photosynthetic rates of the rose cultures were lower at the higher sugar concentrations (Cappellades, 1991). The presence of starch grains may also have reduced the area of the photosynthetic membranes. The effects of sugars on pea cultures are probably similar, but need investigation. These findings suggest that in culture the formation and structure of the chloroplasts is dependent on the culture conditions, such as light intensity and media sugar concentration. The response may also be dependent on the plant species.

For apical pea cultures grown at the high light intensity, the physiological studies have shown an increased level of compounds associated with photo-protection, ascorbate (Table 4) and carotenoids (Table 5), while the ultrastructural studies show a reduced thylakoid membrane system (Plates 1 to 4). The photosynthetic ability and pigment contents of apical pea cultures have been shown to have an optimal level at the

intermediate light intensity (Section 3.3, Fig. 24, 25, 26, 29, 30 & 31), while the highest photosynthetic rates per unit chlorophyll and FW and DW gains (Section 3.3, Fig. 28, 33 & 34) were for cultures grown at the highest light intensity. At high light levels the photosynthetic activity of the cultures may be limited by photo-oxidative damage to the reduced internal membrane system (like that of sun leaf chloroplasts), even though there was an increased level of ascorbate and a decrease in the chlorophyll to carotenoid ratio. However, the photosynthetic systems present still operated efficiently, and thus the cultures achieve the greatest growth (Fig. 34). Cultures at the low light level had an extensive membrane system adapted to the low light environment, similar to those of the chloroplasts of shade leaves, and had a photosynthetic apparatus that was less efficient. They thus had lower FW & DW gains and growth. Hence, when the enviromental light intensity was changed, apical pea cultures showed changes in photosynthetic physiology and ultrastructure, and these changes were comparable to the differences between sun and shade leaves.

3.5. The measurement of the carbon dioxide levels inside a modified culture vessel and the *in situ* photosynthetic rate.

3.5.1. Introduction.

The previous sections have shown that the growth and physiology of apical pea cultures can be affected by their culture environment. Photosynthetic abilities, pigment content, photoprotection mechanisms, ultrastructure and FW and DW were affected by the medium sucrose content and the growth cabinet light intensity.

The photosynthetic activity of the plant material will also be affected by the availability of the photosynthetic "substrate", CO_2. Studies on other culture vessel systems have shown that the methods of closing these, to prevent contamination, provide a barrier to the free diffusion of gases. Different types of vessel closure have varying resistances (DeProft *et al*, 1985; Kozai *et al*, 1986). The culture jars used in the experiments in the previous sections were closed by using the basal halves of 45mm petri dishes. This method of closure will probably have restricted diffusion and thus the exchange of gases between the atmosphere and the enclosed culture vessel. The composition of the gas mixture inside the culture vessel may as a result have been significantly different from that of the atmosphere, due to the production and/or consumption of gases by the photosynthetic, respiratory and ethyne metabolisms. Changes in the gas content of the culture

vessel may have in turn affected the photosynthetic activity of the cultures. Studies of other culture systems (DeProft *et al*, 1985; Kozai *et al*, 1987), have shown that the concentration of CO_2 in the culture vessels was different from that of the atmosphere. This may have affect on the photosynthetic activity and growth of the cultures (Kozai *et al*, 1987; Solarova, 1989).

A series of experiments was conducted on a modified culture system, which was designed to permit sampling of the gases inside the culture vessel. This allowed the *"in situ"* measurement of the photosynthetic ability of the cultures.

3.5.2. Methods and Materials.

Apical cultures were grown, in conical flasks, under the following light intensities: 50; 150; and 300 $\mu E\ m^{-2} s^{-1}$, on MS medium containing 10, 30 and 50 g/l sucrose. After three weeks, the following were ascertained: the CO_2 content of the air inside the culture vessel; the *"in situ"* and leaf photosynthetic abilities and respiration rates; pigment content; FW; and medium sugar content. The CO_2 content of the culture vessel air was measured at intervals, starting half an hour before the end of the dark period and finishing nine hours into the light period.

3.5.2.1. The establishment of apical pea cultures in conical flasks.

Apical explants were established in 100 ml conical flasks containing 30 ml of medium (MS without P.G.R.s). The flasks were plugged loosely, to prevent the vessel from being absolutely gas tight, using a sterile Size 36 rubber bung. Two lengths of glass tubing passed through each bung, to which sterile 10 ml syringes were attached (Fig. 36) using silicon rubber tubing. This experimental culture vessel was based on that devised by Champion (1982). The cultures were grown for three weeks at 25°C with a 16 hour day length as in section 2.1..

3.5.2.2. Measurement of the carbon dioxide content inside the culture vessel.

The method used was based on that of Clegg *et al* (1978). The IRGA was set in differential mode, and dried nitrogen was passed through at a rate of 400 ml/min. Gas samples were introduced into the nitrogen stream leading to the analysis chamber of the IRGA by injection (Fig. 37). The IRGA was calibrated by the injection of gas samples of known volume and CO_2 concentration, and the calibration was checked during each set of measurements.

The gas content of the flasks was mixed by twice flushing the flasks using the two 10 ml syringes. Gas samples (1 ml) were then taken from the culture vessels, using a 1 ml hypodermic syringe with needle. As each sample was taken the internal volume

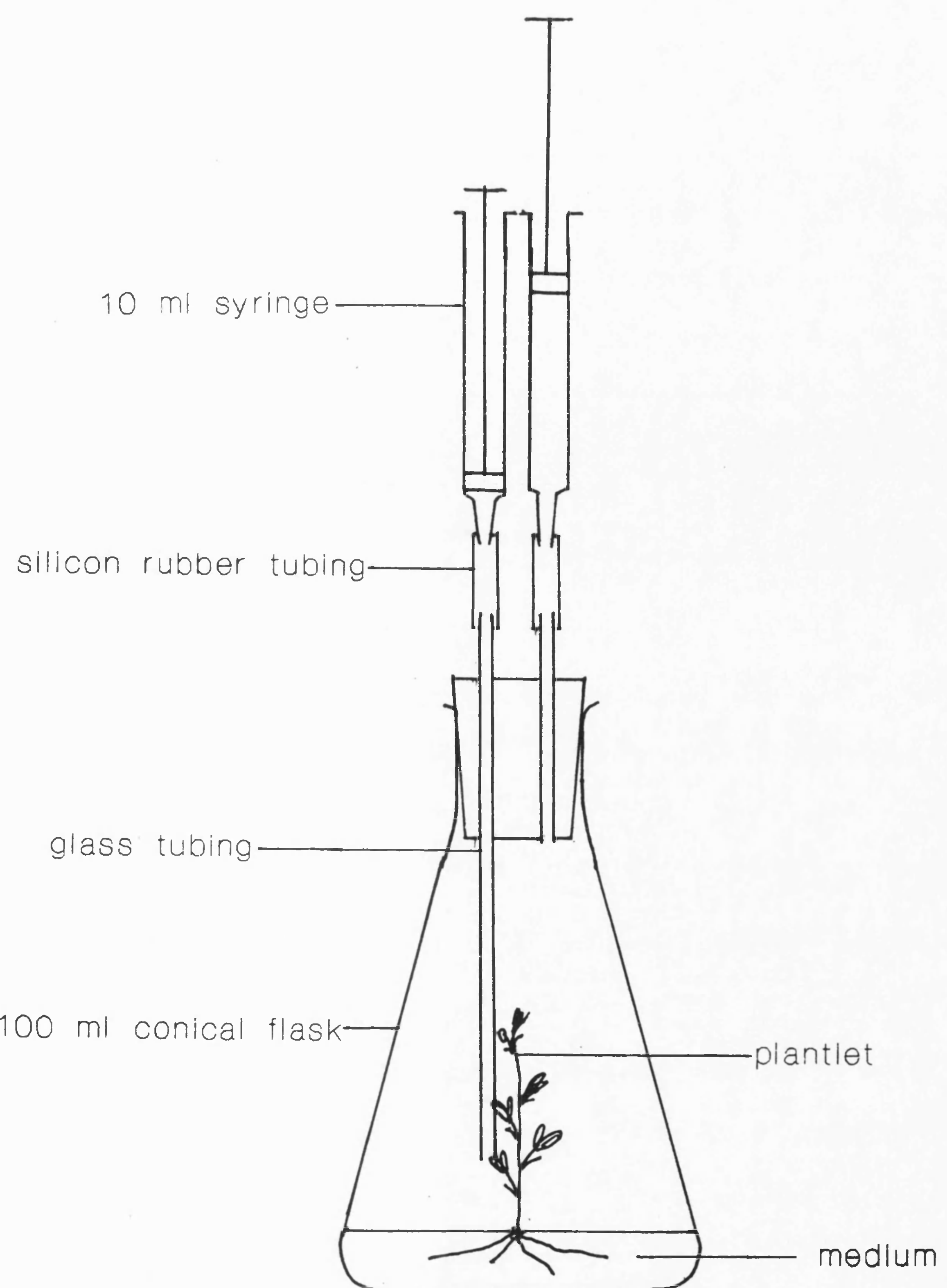


Fig. 36
A diagrammatic representation of the culture vessel used to allow sampling of the enclosed air.

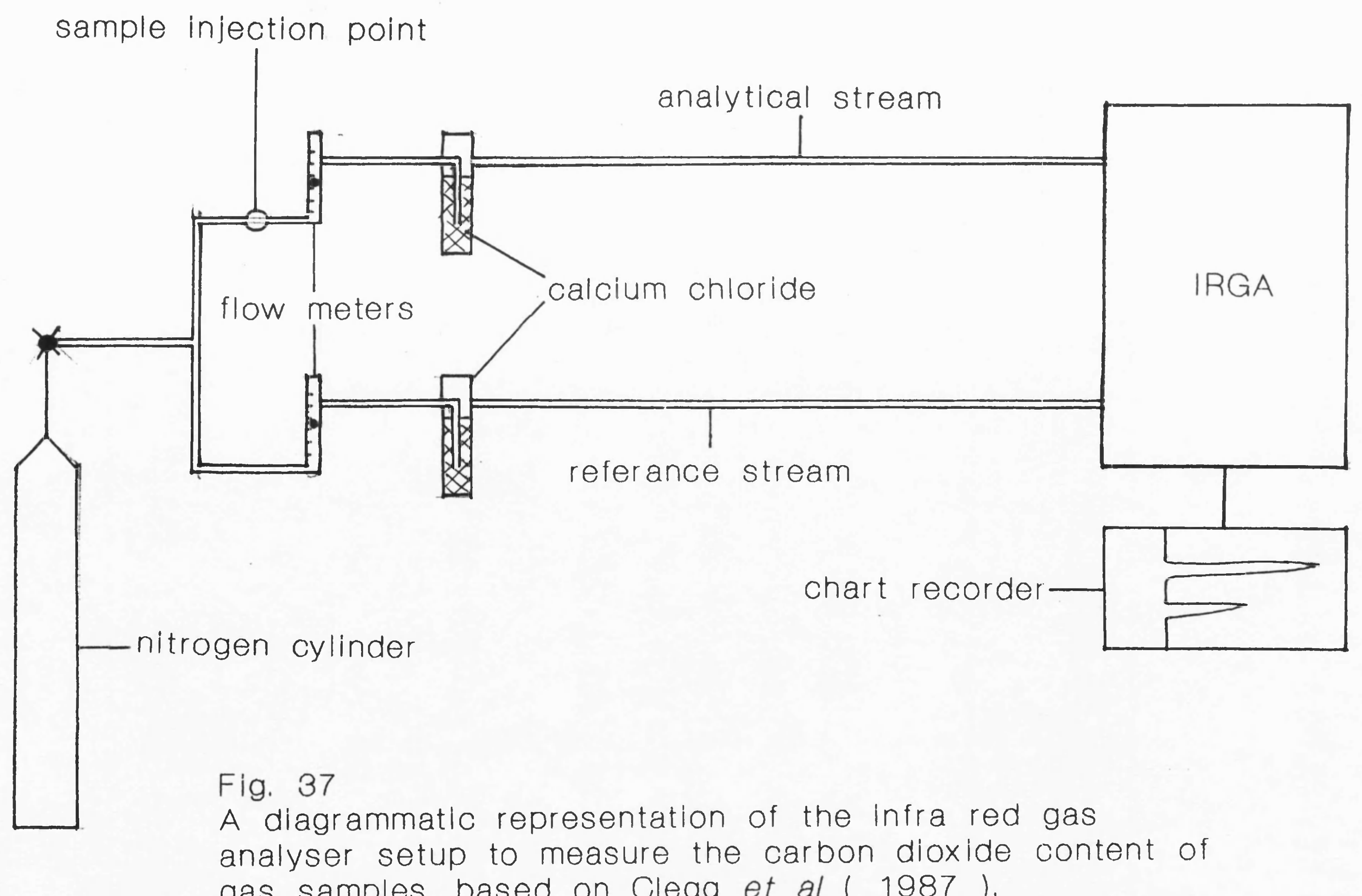


Fig. 37
A diagrammatic representation of the infra red gas analyser setup to measure the carbon dioxide content of gas samples, based on Clegg *et al* (1987).

of the culture vessel was reduced by 1 ml, using one of the 10 ml syringes.

3.5.2.3. Measurement of photosynthesis and respiration *"in situ"*.

The enclosing bung was removed from the flasks. They were then flushed with air (from a cylinder), and connected to the IRGA in place of the usual sample chamber (Fig. 38). The IRGA was used as described in Section 2.2..

3.5.3. Results.

The culture vessels used here were not ideal and need further development. There were a number of related problems.

1/ The loose placement of the rubber bung, was necessary to prevent the culture vessels from being gastight. This led to easy bung dislodgement and thus the free entry of atmospheric air and contaminants.

2/ The closure of the flasks with bungs probably led to the flasks being more gastight than jars closed with half petri dishes.

3/ The bungs shaded the cultures from the overhead illumination.

The dislodgement of the bungs introduced errors into the results, due to contamination (loss of replicates) and changes in gas permeability of the vessel (varying the

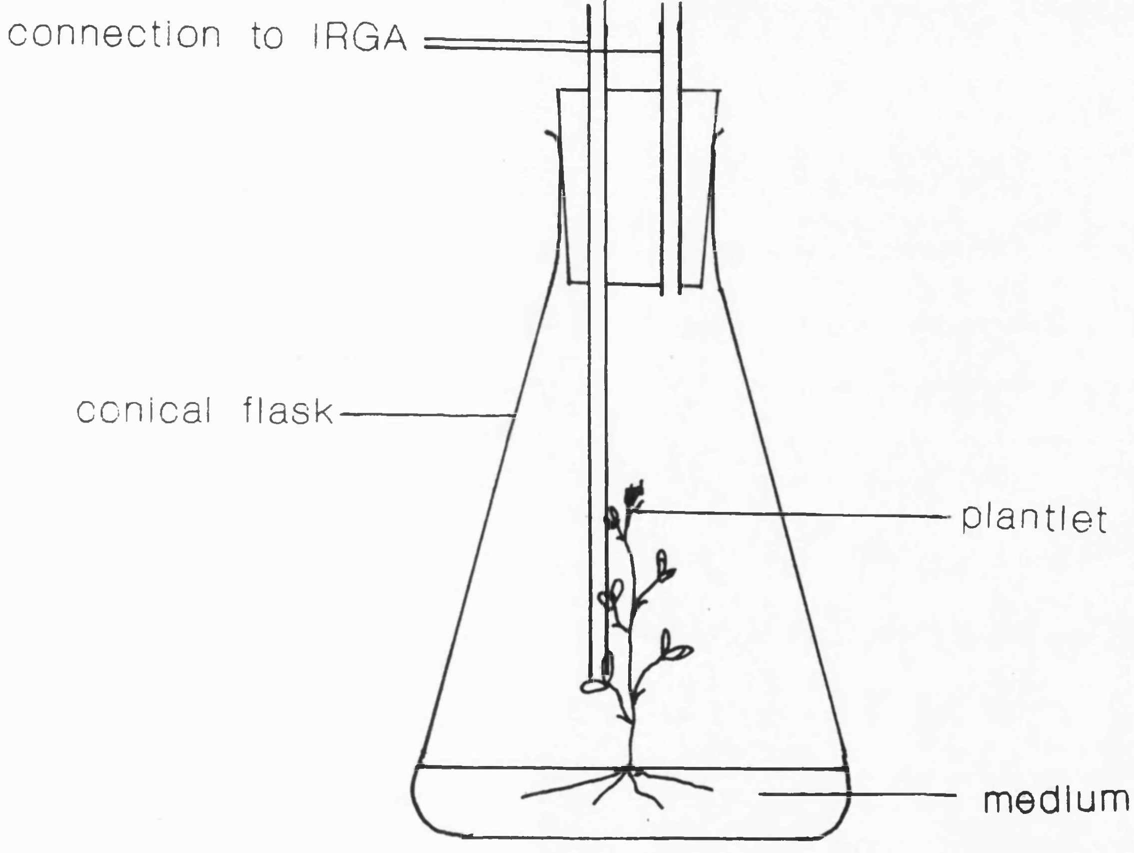


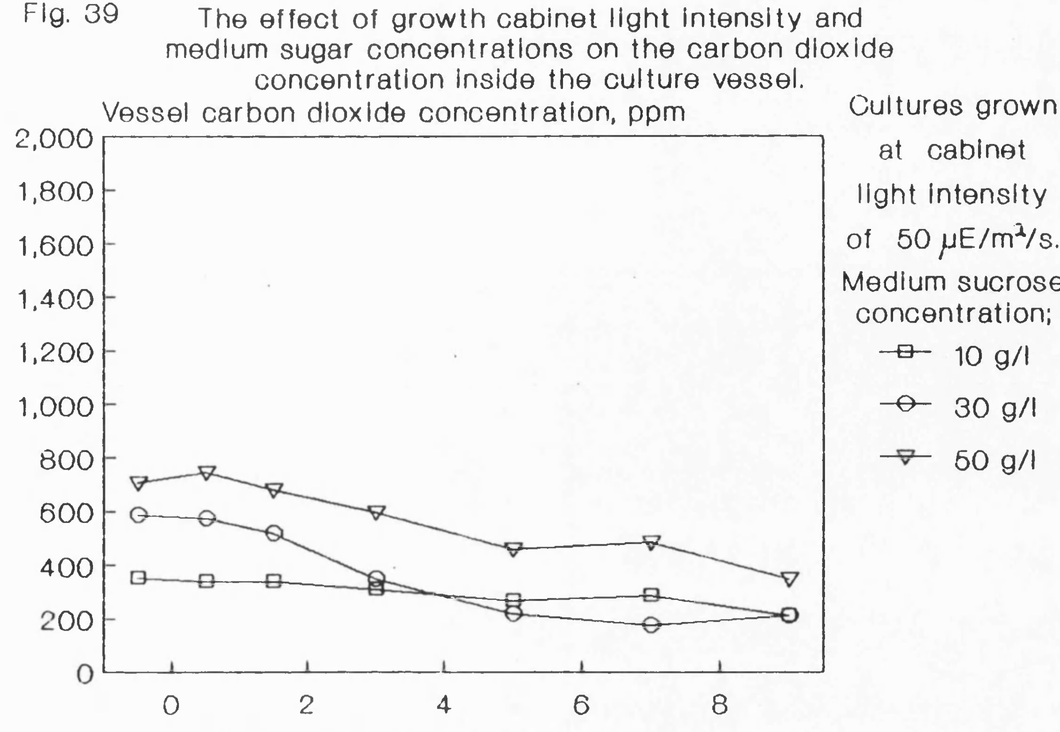
Fig. 38
A diagrammatic representation of the system used for the measurement of culture photosynthesis *in situ*.

culture environment). The cultures were typically elongated in appearance, with less leaf material than the jar cultures. The poor growth was probably due to restricted gas exchange with the atmosphere, which would lead to disadvantageous levels of CO_2, O_2 and water vapour inside the culture vessel. Direct comparison between the culture vessels was therefore not possible.

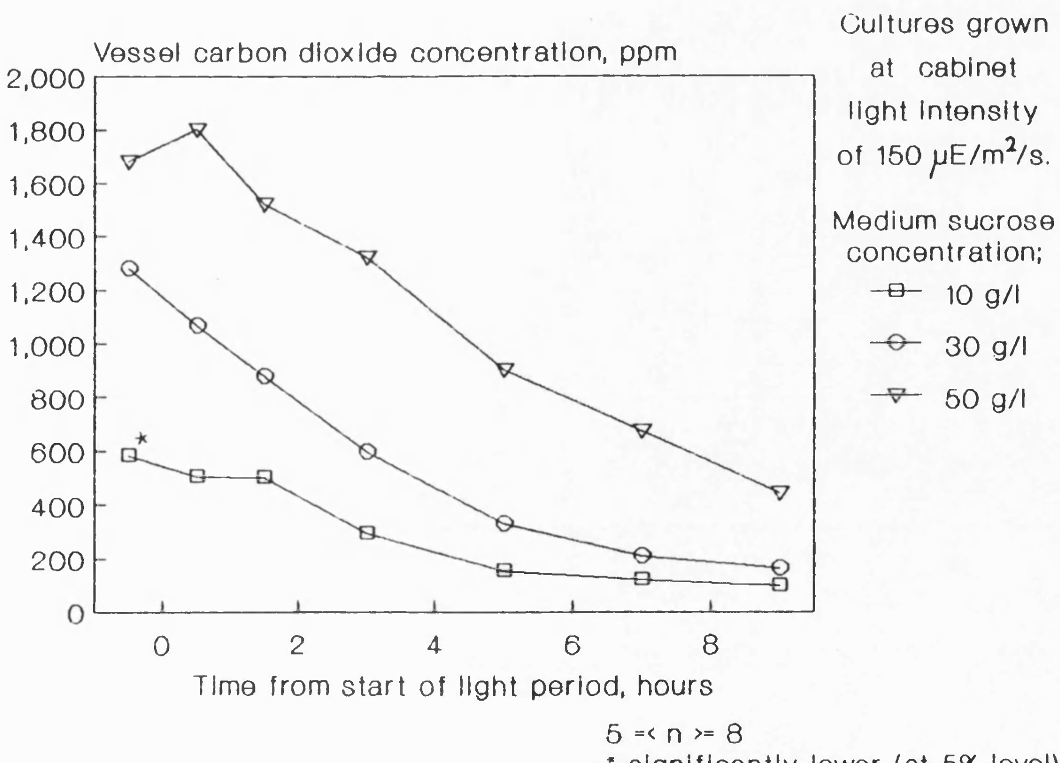
It would have been preferable to use modified jars with tubes attached to their sides. This was not possible since the jars were made of soda glass, and the manufacture and design of modified jars, from other types of glass, such as Pyrex, was prohibited by lack of time.

The CO_2 content of the culture vessels fell for all conditions during the $9^1/_2$ hours sampling period (Fig. 39). There was an initial rapid fall shortly after the commencement of the light period; the rate of fall then decreased gradually. The CO_2 level at the end of the sampling period had in some cases dropped below atmospheric, and was stable. The CO_2 content at the end of the dark period/beginning of the light period, was related to the culture conditions. At all light intensities the CO_2 concentration was highest for cultures grown on medium initially containing 50 g/l sucrose and lowest for cultures grown on medium containing 10g/l. This variation was not significant for the cultures grown at 50 $\mu E\ m^{-2} s^{-1}$. For cultures grown on medium containing 10 g/l sucrose the light level had no

Fig. 39 The effect of growth cabinet light intensity and medium sugar concentrations on the carbon dioxide concentration inside the culture vessel.

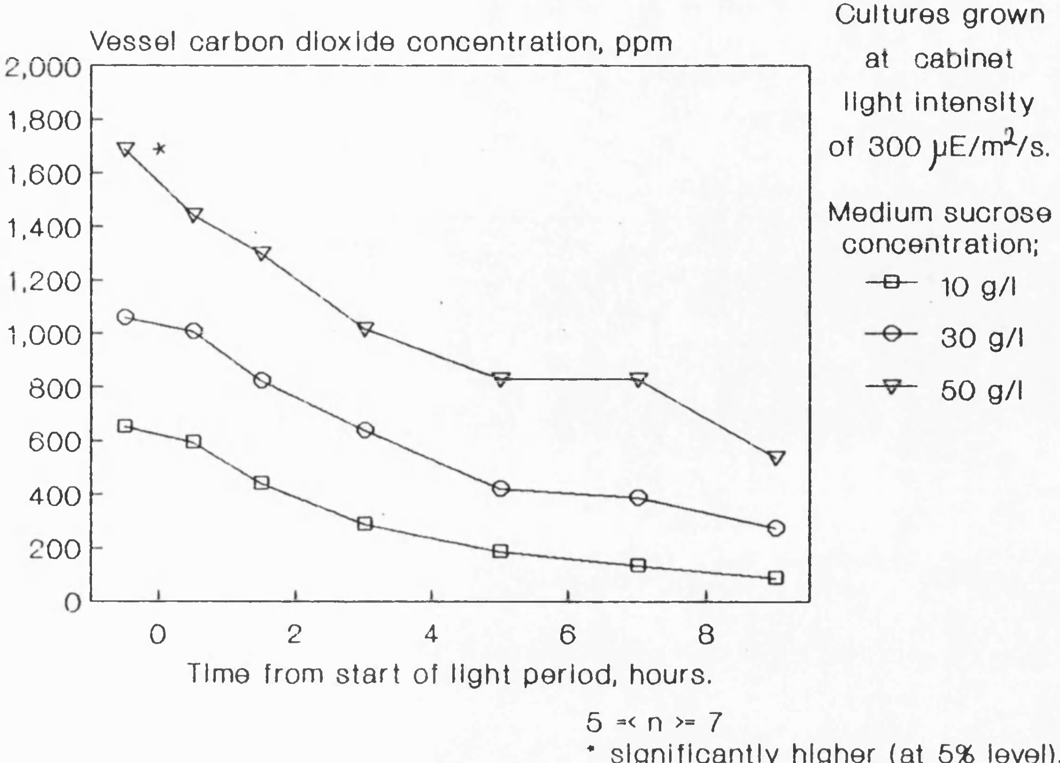


4 =< n >= 7
No significant differences.



5 =< n >= 8
* significantly lower (at 5% level).

Fig. 39 continued,



For cultures grown on media containing 30 & 50 g/l sucrose, the culture vessel carbon dioxide concentrations were significantly lower at the lowest cabinet light intensity. For cultures grown on medium containing 10 g/l sucrose there were no significant differences between culture vessel carbon dioxide concentrations at the different growth cabinet light intensities.

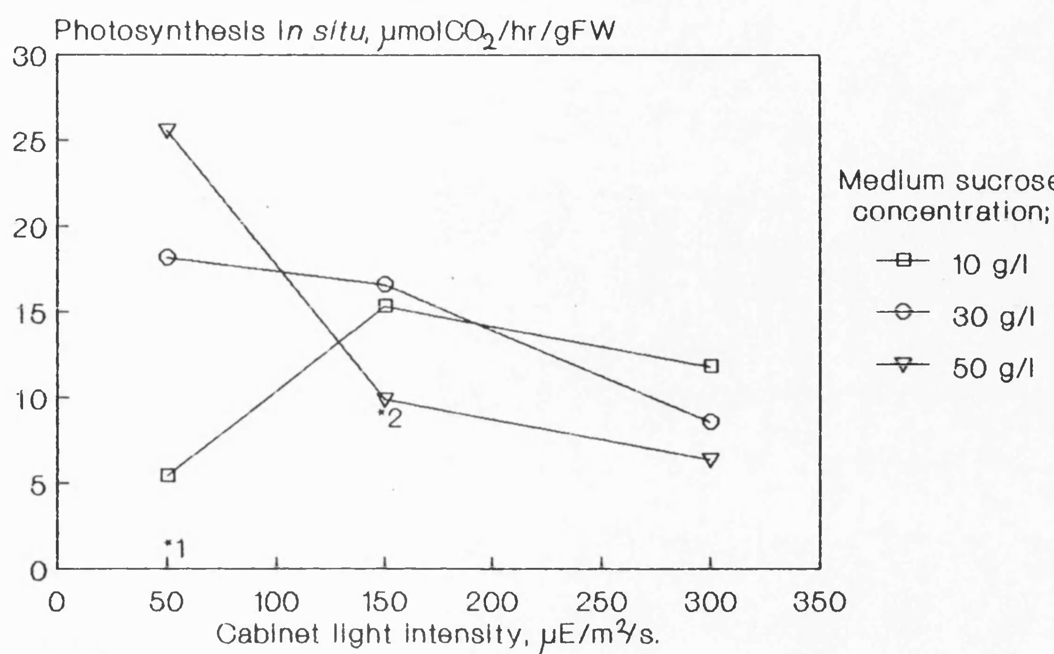
All significances tested at the 5% level.

significant effect on the vessel CO_2 content. For cultures with initial medium sucrose content of 30 and 50 g/l, the levels were higher at light intensities of 150 and 300 µE $m^{-2}s^{-1}$.

The *"in situ"* photosynthetic ability is the photosynthetic ability per FW of plantlet, measured while the plantlet was still in the culture vessel. This varied with the initial medium sucrose content and growth cabinet light intensity (Fig. 40). The highest rate was for cultures grown at the lowest light intensity on media containing 50 g/l sucrose. For those cultures grown at a light intensity of 50 µE $m^{-2}s^{-1}$ the photosynthetic ability decreased as the initial medium sugar content was reduced, however for those cultures grown at 300 µE $m^{-2}s^{-1}$ the rate increased. Cultures grown at a light intensity of 150 µE $m^{-2}s^{-1}$ had the same *"in situ"* photosynthetic ability on media initially containing 10 and 30 g/l sucrose, and a lower rate on medium initially containing 50 g/l. Increasing the light intensity for cultures grown on media containing any of the initial medium sucrose concentrations affected the *"in situ"* photosynthetic ability. The rate increased for cultures grown on medium initially containing 10 g/l and was reduced on media containing 30 and 50 g/l.

The photosynthetic abilities (that is photosynthetic ability per leaf FW) (Fig. 41) and the pigment contents (Fig. 42, 43 & 44) of the cultures leaves had only limited replication due to the small

Fig. 40 a/ The effect of growth cabinet light intensity and medium sucrose concentration on the *in situ* photosynthetic ability of apical pea cultures.



4 =< n >= 8

*1 All values significantly different at this light intensity.

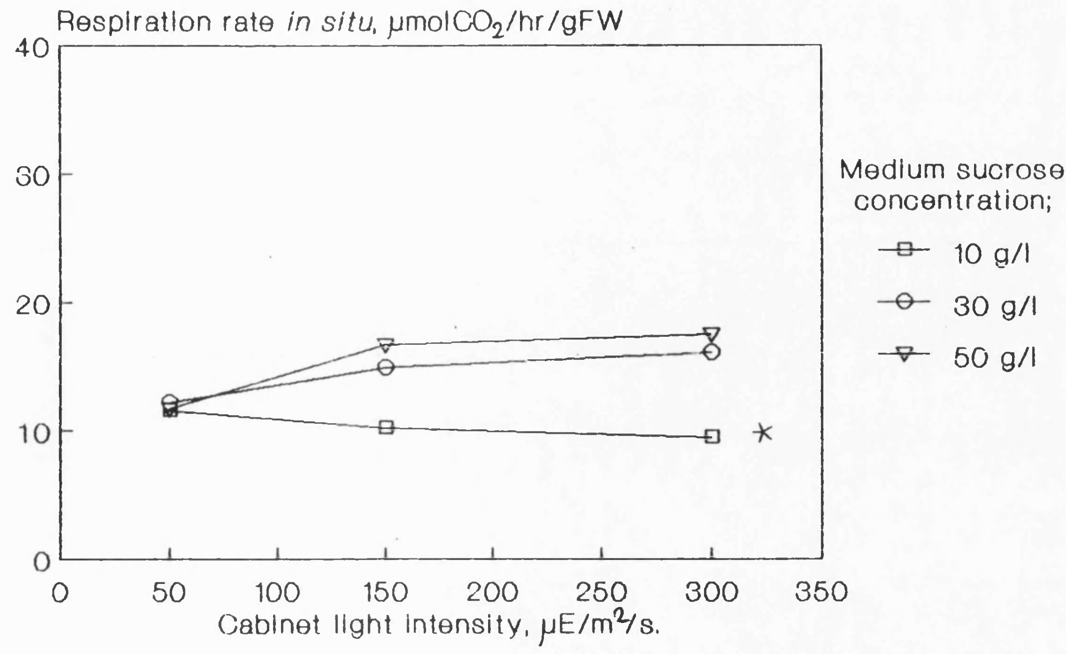
*2 This value significantly lower than that for cultures grown on medium containing 30 g/l sucrose at the same light intensity.

For cultures on medium containing:

10 g/l sucrose, the photosynthetic ability is significantly lower at the lowest light intensity.
30 g/l sucrose, the photosynthetic ability is significantly lower at the highest light intensity.
50 g/l sucrose, the photosynthetic ability is significantly higher at the lowest light intensity.

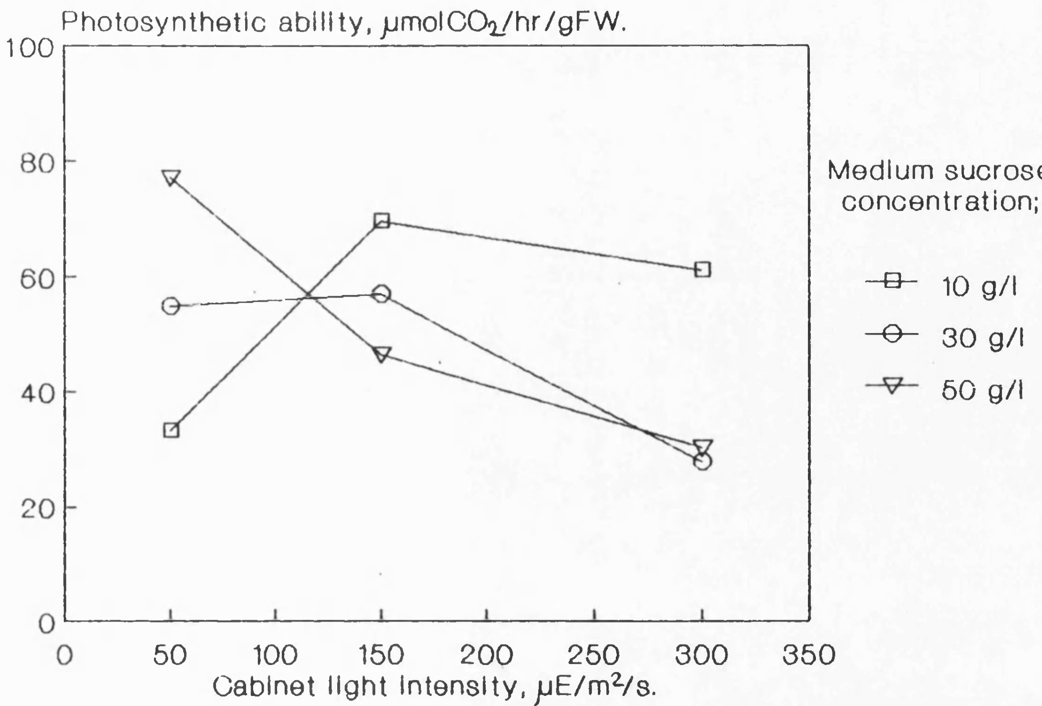
All significances tested at the 5% level.

Fig. 40 b/ The effect of growth cabinet light intensity and medium sucrose concentration on the *in situ* respiratory rate of apical pea cultures.



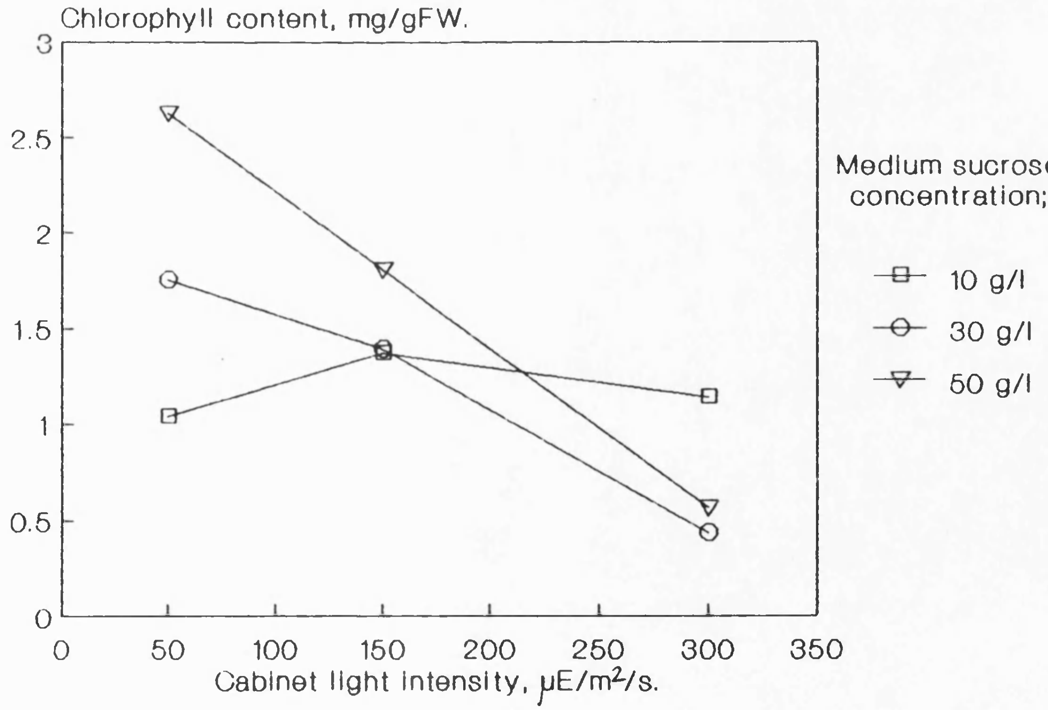
4 =< n >= 8
* value significantly lower than for 50 g/l sucrose (at 5% level).

Fig. 41 The effect ofgrowth cabinet light intensity and medium sucrose concentration on the photosynthetic ability of apical pea cultures.



n = 2

Fig. 42 The effect of growth cabinet light intensity and medium sucrose concentration on the chlorophyll content of apical pea cultures.



n = 2

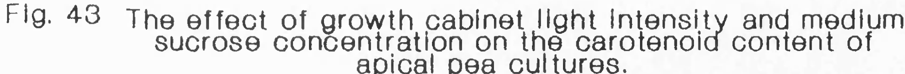
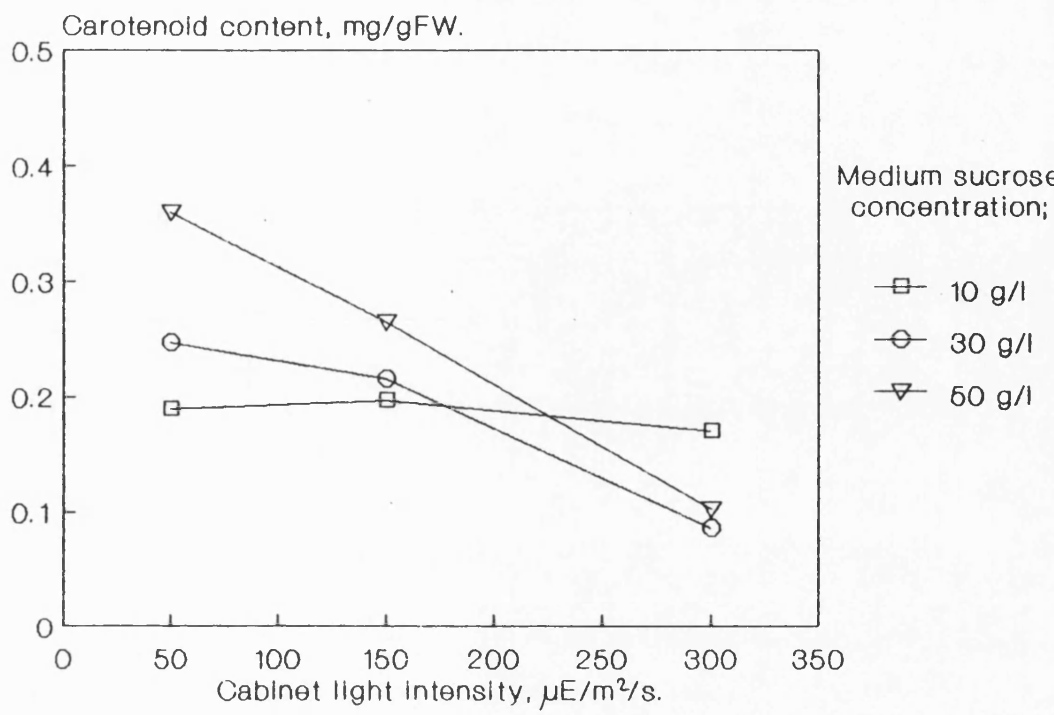
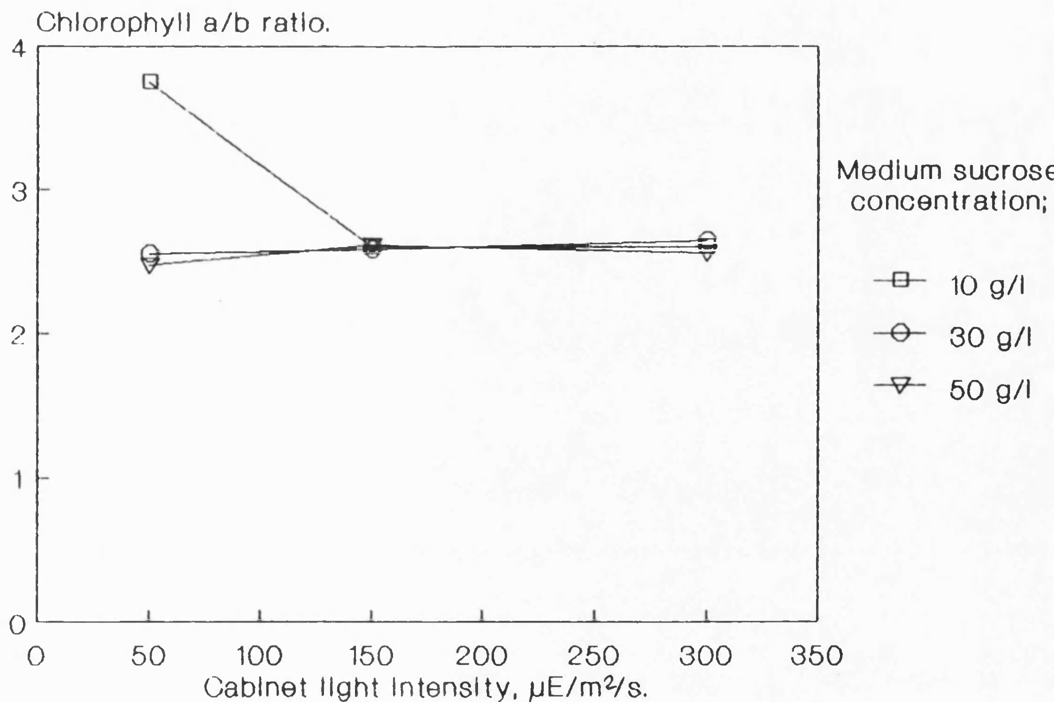


Fig. 43 The effect of growth cabinet light intensity and medium sucrose concentration on the carotenoid content of apical pea cultures.



n = 2

Fig. 44 The effect of growth cabinet light intensity and medium sucrose concentration on the chlorophyll a/b ratio of apical pea cultures.



n = 2

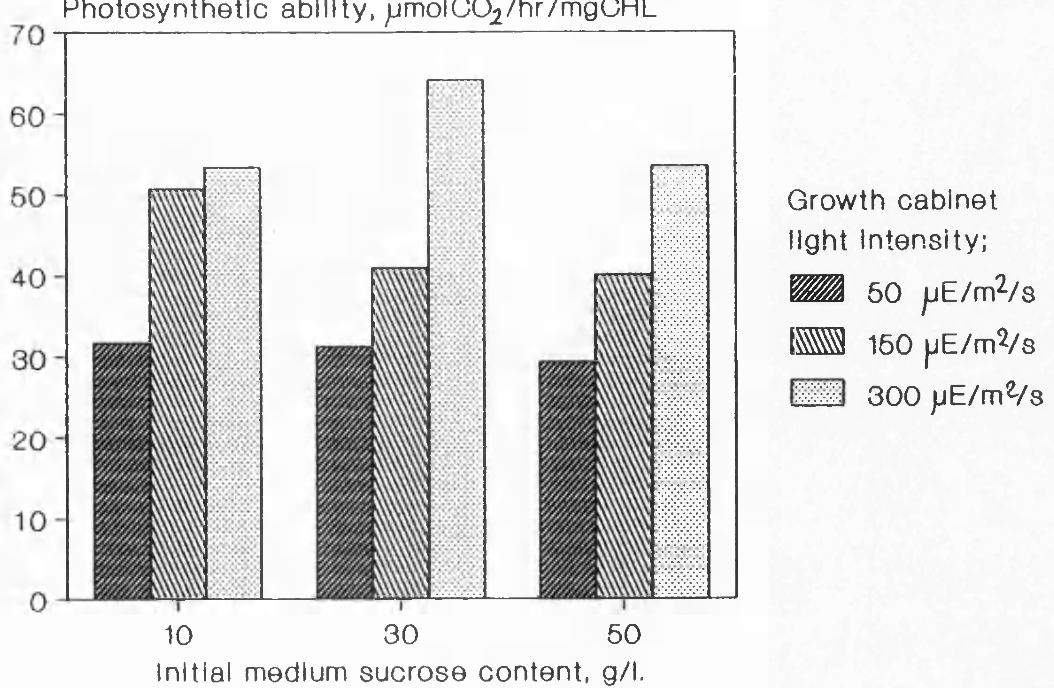
amount of plant material available. The photosynthetic measurement (Fig. 41) and pigment contents (Fig. 42 & 43) showed trends similar to those for the *"in situ"* photosynthetic abilities (Fig. 40). Pigment levels were highest for cultures grown on media that initially containing 30 and 50 g/l sucrose at the lowest light level; pigment content then fell as the light intensity was raised. The chlorophyll a:b ratio was relatively constant (Fig. 44). The photosynthetic abilities, showed similar trends to the *"in situ"* photosynthetic abilities, but were lower. This was due to these measurements being based on different FW.

The *"in situ"* respiratory rates (Fig. 40) of the cultures did not show any significant differences as the growth cabinet light intensity and media sugar concentration were varied. The leaf respiration rates had only limited repilcation (n=2) and the readings were highly variable, the mean values have therefore not been presented.

The photosynthetic rates per unit chlorophyll of the culture leaves were not affected by the initial medium sucrose content, but increased as the growth cabinet light intensity was raised (Fig. 45).

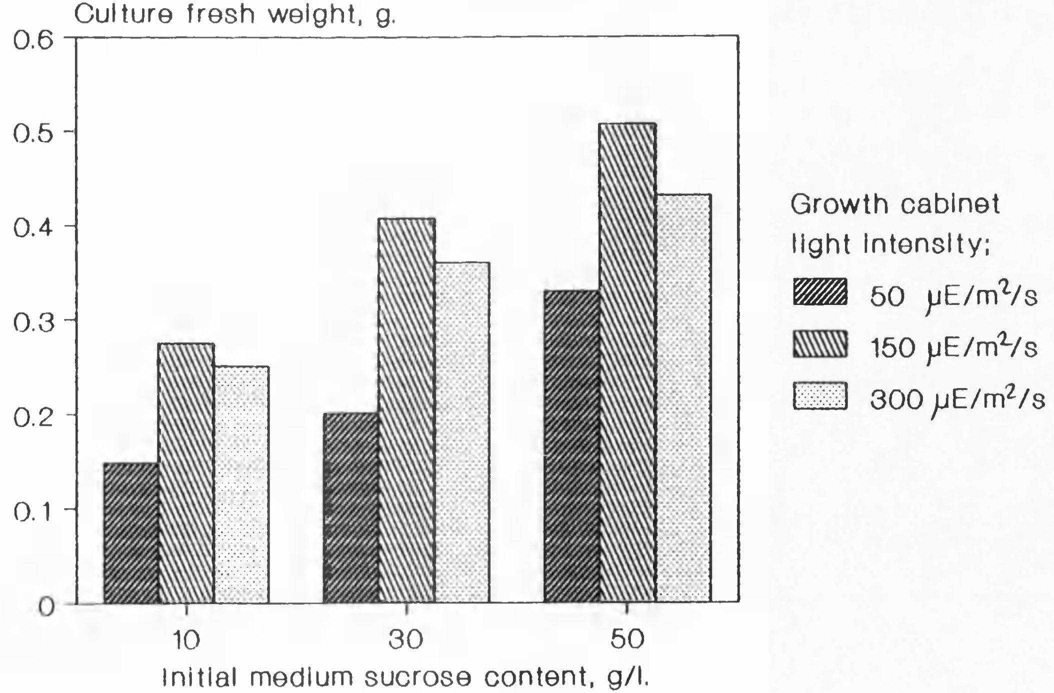
At all light intensities, the FW of the cultures increased (Fig. 46) as the initial sucrose concentration of the medium was raised. The FW were lowest at the lowest light intensities, for all of the

Fig. 45 The effect of growth cabinet light intensity and medium sucrose concentration on the photostynthetic ability (per unit chlorophyll) of apical pea cultures (flask).



n = 2

Fig. 46 The effect of growth cabinet light intensity and medium sucrose concentration on the fresh weight of apical pea cultures (flask).



4 =< n >= 8

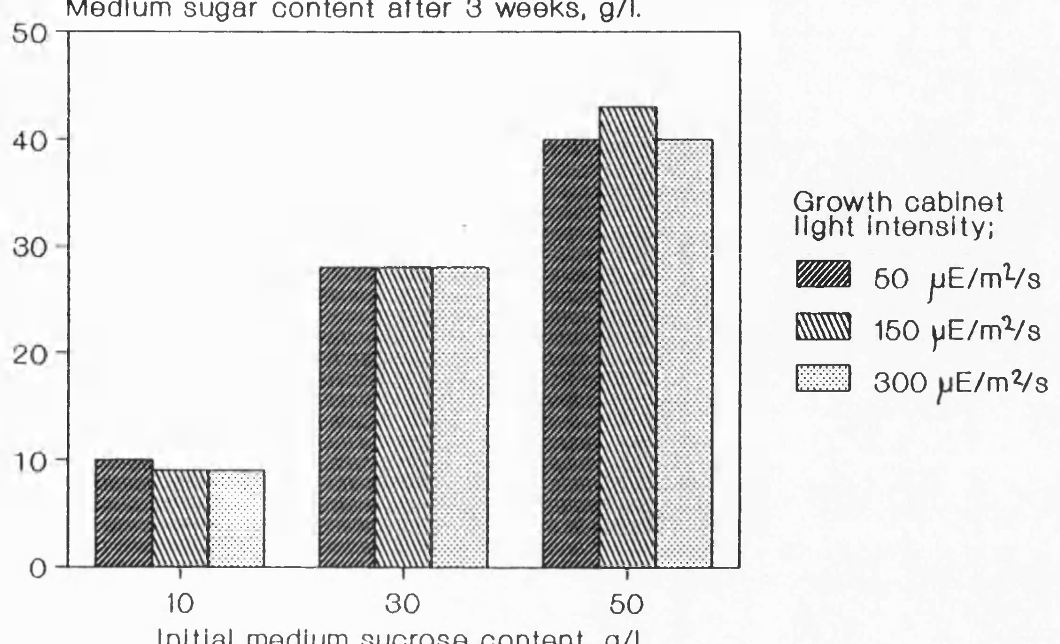
medium sucrose concentrations. Due to the limited amount of plant material available the culture DW were not measured.

The sugar remaining in the medium at the end of the three week culture period (Fig. 47), was similar to or less than that initially present. The decrease, was always proportionally larger for cultures grown on medium initially containing 50 g/l sucrose. The growth cabinet light intensity had no effect on the concentration of sugar remaining in the medium.

3.5.4. Discussion

The CO_2 concentration of the air inside the cultures vessels was related to the initial medium sucrose content and growth cabinet light intensity. As the initial sucrose content of the media was raised, the level of CO_2 inside the culture vessels at the end of the dark period increased (Fig. 39). Solarova (1989), using cultures of *Nicotiana tabacum* L. c.v. White Burley on media containing 0, 1, 2 & 4 % sucrose (at 100 µE $m^{-2}s^{-1}$), measured the culture vessel CO_2 content using a flow through potentiometric method and obtained similar results. The more sucrose that was present in the medium, the more substrate there was available to be metabolised. Cultures that were grown at the intermediate and high light intensities had similar levels of CO_2 present at the end of the dark period, at a given medium sucrose content, while those at the lowest light intensity had

Fig. 47 The effect of groth cabinet light intensity on the medium sugar concentration after a three week culture period, for apical pea cultures (flask).



n = 12
No significant differences.

lower levels. Desjardins *et al* (1988), with raspberry cultures, found that the CO_2 concentration in the culture vessel at the end of the light period increased with the light intensity. These results suggests that at the low light intensity the cultures were less physiologically active, possibly showing lower respiratory production of CO_2; however, the "*in situ*" dark respiration rates showed no significant changes under the range of growth cabinet light intensities used (see later). A certain level of light might, therefore, have been required by the cultures for full growth and development.

Fujiwara *et al* (1987), Desjardins *et al* (1988), Infante *et al* (1989) and Solarova (1989) all found that the CO_2 content in the culture vessel fell at the onset of the light period, and was below the ambient atmospheric level during most of the subsequent illumination. For example, the CO_2 content of culture vessels containing raspberry cultures fell to between 100-200 ppm two hours into the light period substantially less than the atmospheric CO_2 level of 330 ppm (Desjardins *et al*, 1988). Similarly, for apical pea cultures the CO_2 content in the culture vessel was for part of the light period below the ambient atmospheric level (approximately 330 ppm). This would have restricted the photosynthetic activity of the cultures. In a number of studies (Desjardins *et al*, 1988 & 1989; Kozai & Iwanami, 1988; Infante *et al*, 1989; Kozai *et al*, 1991 a & b) the CO_2 content of culture vessels has been

maintained around or above the ambient atmospheric level by elevating the CO_2 content of the atmosphere surrounding gas permeable culture vessels. This elevation of the vessel CO_2 content resulted in increased photosynthetic activity and growth, especially at higher light levels (see final discussion, Section 4.3.).

The photosynthetic ability of the cultures changed with the initial medium sucrose content and growth cabinet light intensity (Fig. 40 & 41). For cultures grown on the medium containing the highest initial sucrose level (50 g/l), the photosynthetic ability decreased as the light intensity was raised. The cultures may have required a certain level of photosynthetic activity for successful growth. They may therefore have developed a more extensive photosynthetic apparatus at the lower light level, in order to utilise the limited light energy available. This extensive photosynthetic apparatus could have given the cultures a potentially high photosynthetic activity, as measured at the high light intensity in the IRGA. Secondly, as the growth cabinet light intensity was raised the operational efficiency of the photosynthetic apparatus would be increased, thus producing more sugar, whereupon, a smaller photosynthetic apparatus would probably be adequate. Moreover, the amount of sugar available to these cultures, already high as the medium contained 50 g/l sucrose, would therefore be increased further as the light intensity was raised. This could lead to an excess

of available carbohydrate, which in turn might be stored, probably as starch. The storage of any excess carbohydrate within the chloroplast may have limited the development of the photosynthetic apparatus or shaded the lamellae, and thus reduced photosynthetic ability (Thorne & Keller, 1974). The high sugar concentration may have also have inhibited photosynthesis, by limiting the production of more sucrose or its photosynthetic precursors (Herold, 1980; Still & Quick, 1989). Such an inhibition of photosynthesis by sugar concentration could be compared to the interaction between "sink" and "source" of whole plants.

For cultures grown at the lower initial medium sucrose level (10 g/l), the photosynthetic ability was lowest at the lowest light intensity. The low light and low sucrose would result in impaired growth and development of the cultures, as insufficient energy was available. At the higher light intensities more light energy was available, allowing the photosynthetic apparatus to operate more efficiently, making further growth and development, including that of the photosynthetic systems, possible. The responses of the cultures on the medium with intermediate initial sucrose (30 g/l) were intermediate to those on the high and low sucrose media.

Solarova (1989), using tobacco plantlets, had found that as the media sugar content was raised from 1 to 4 % the photosynthetic rate decreased (light

intensity 100 μE $m^{-2}s^{-1}$). Evers (1982), with Douglas Fir cultures, and Langford & Wainwright (1987), with rose obtained similar responses. Kozai *et al* (1990), with *Cymbidium* plantlets, found that increasing the cabinet light intensity (from 35 to 226 μE $m^{-2}s^{-1}$, at 25 °C on a medium containing 20 g/l sucrose) increased the net photosynthetic activity; however Langford (1987), with rose and Lee *et al* (1988), using sweetgum, found that the cultures had there maximum photosynthetic ability at an intermediate light intensity. These results and those for apical pea cultures support the supposition that the photosynthetic ability of cultures is dependent on the medium sugar content and the cabinet light intensity. Furthermore, the effects of these factors and others, such as the culture vessel CO_2 concentration and the plant species, are probably interdependent.

The changes in pigment content (Fig. 42, 43 & 44) reflect those of the photosynthetic abilities. The pigment content decreased as the growth cabinet light intensity was raised. Comparable changes have been observed in studies with *in vivo* grown material. For example, studies using radish seedlings have shown that a high environmental light intensity reduces the pigment content of the plants (Grumbach & Lichtenthaler, 1982). The pigments were also shown to have a higher rate of turnover at the high light intensity. These variations were accompanied by differences in the association of the pigments with the photosystems and in the chloroplast

ultrastructure. Plants may have different "types" of leaves/chloroplasts depending on the light environment under which they develop. Those formed under high light are termed sun leaves/chloroplasts, and those under low light conditions shade leaves/chloroplasts (Lichtenthaler *et al*, 1981; Lichtenthaler & Meier, 1984). The dividing line between these types is not clear, and there may be gradual variation between them.

The light energy available to the plantlets grown at the higher light intensities may have been excessive, and thus may have caused photo-oxidative damage to the photosynthetic apparatus, therefore reducing the cultures photosynthetic abilities and pigment contents. Furthermore, conditions that limit photosynthesis, such as low CO_2 concentrations and high sugar levels, may reduce the photosynthetic requirement for light energy. Under such conditions there would be a greater potential for photo-oxidative damage, particularly at the higher light levels, unless there was also an increase in the levels of the photoprotection mechanisms. The cultures low pigment levels and photosynthetic abilities at the higher light intensities may, therefore, have been due to the culture environment limiting photosynthetic activity, and thus leading to an increase in the incidence of photo-oxidative damage.

In Sections 3.3. it was shown that

photosynthesis in apical pea cultures (grown in culture jars) was dependent on the culture conditions. Intermediate light intensities and sucrose concentrations gave the highest photosynthetic abilities. The highest pigment concentrations were obtained under the same conditions, and also at higher sugar concentrations. The results for the apical pea cultures in flasks, grown under a similar range of conditions, concur with the general conclusion that the photosynthetic physiology is dependent on the culture conditions, although the actual responses for the two culture systems were different.

The photosynthetic rates per unit chlorophyll (Fig. 45) show that the efficiency of pigment use declined with light intensity, and was not affected by initial medium sucrose content. Increasing the cabinet light intensity increased the photosynthetic efficiency, but the photosynthetic ability and pigment content fell. This could be due to the fact that plentiful source of energy at high light intensities reduced the need for a large photosynthetic apparatus. It appears that in this case the smaller photosynthetic apparatus present intercepted the required energy. For the cultures on medium initially containing 50 g/l sucrose, the storage of carbohydrates by the chloroplasts or the high sugar concentration may also have reduced the photosynthetic ability. The effects of the initial medium sucrose concentration on photosynthesis were related to the cabinet light intensity. High light high sucrose and low

light low sucrose gave low rates, whereas low light and high sucrose gave high rates. The other combinations were intermediate.

The actual photosynthetic activities of the cultures under the conditions prevalent in the culture vessel have not been measured. These could have been ascertained by a number of methods, see Section 4.4.ii. for details.

The changes in the photosynthetic abilities of the cultures may have been, at least in part, due to changes in the activities of Calvin cycle enzymes, such as Ribulose-bisphosphate carboxylase (RUBISCO). Measurements of the activity of this enzyme were initiated, but due to temporal constraints and problems with obtaining reliable results, they were not followed to completion (no results are available). The activity of RUBISCO was to have been measured spectrophotometerically by following the 3-phosphoglycerate-dependent oxidation of NADH using a coupled enzyme assay (Racker, 1962; Lilly & Walker, 1974). The protocol to be used was that adopted by Knox & Dodge (1985). Measurements of the activity of this enzyme would have given an indication of the cultures ability to fix CO_2 using the Calvin Cycle, that is their biochemical capacity for photosynthesis. Moreover, such measurements would indicate to what extent the photosynthetic ability of the cultures was affected by biochemical factors, such as, enzyme levels and activities, as opposed to

other physiological factors.

Cauliflower cultures (Grout & Donkin, 1985) were found to have reduced levels of RUBISCO activity, as well as lower CO_2 fixation rates, compared to seedlings and the *de novo* formed foliage of cultures transplanted to *in vivo* conditions. Moreover, work by Grout & Price (1987) indicated that the reduced photosynthetic CO_2 uptake and RUBISCO activity of strawberry plants, were possibly due to the presence of sucrose in the culture media. Furthermore, their results also suggested that the presence of sugar in the media may have irreversibly reduced the RUBISCO activity of the cultures leaves.

Measurements of, the levels and activities, of photosynthetic enzymes, such as RUBISCO, are needed, if a greater understanding of the photosynthetic physiology of plant tissue cultures is to be reached. The results of these biochemical analyses, with the data from CO_2 exchange analysis, ultrastructural studies and pigment measurements, would allow discussion of the effects of the culture environment on the various aspects of the plantlets photosynthetic physiology. The photosynthetic physiology of cultures could have been influenced by the environmental conditions as a result of these affecting, one or more, of a number of physiological factors, such as: directly affecting enzyme activities and levels; interacting with substrate or end-product regulation; and affecting the structure and

integrity of the chloroplasts. Furthermore, studies of other enzymes associated with CO_2 fixation and carbon metabolism, such as PEPcase (Section 1.2.1.1.), and the following of the uptake and incorporation of ^{14}C labelled CO_2 and sucrose, would be useful in ascertaining the roles of CO_2 fixation and media sugar uptake in culture growth. For example, Desjardins *et al* (1989) found that in cultures of strawberry the activities of RUBISCO and PEPcase were both effected by the culture environment. Furthermore, they also found that the changes in the activity of PEPcase were more closely correlated to the uptake of CO_2 than the changes in the activity of RUBISCO (Section 4.3). The biochemistry of plantlets carbon metabolism needs further more extensive investigation.

The dark respiration rates (Fig. 40) of the flask grown pea cultures were not affected by the range of growth cabinet light intensities and media sucrose concentrations used in this study. However, potato (Kozai *et al*, 1988a) and tobacco (Kozai *et al*, 1990) cultures grown on media containing no added sugar were found to have lower respiratory rates than cultures grown on media containing sugar. Moreover, the results for the jar cultures (Fig. 19 & 24), suggested that low media sugar concentrations may have reduce respiratory activity. The dark respiration rates measured here were, unlike those for the jar cultures, for whole plantlets. The readings were, however, made under the conditions

prevalent in the IRGA analysis chamber (atmospheric O_2 levels and a low humidity), not those of the cultures vessel (which would probably have had O_2 concentrations and the relative humidities different from the atmospheric levels, due to the vessels restricted gas permeability). The measured respiratory rates may, therefore, have not been representative of the respiratory physiology of the cultures when growing in the culture vessel.

The measured photosynthetic abilities and respiration rates of the cultures (Fig. 40) suggest that the level of CO_2 uptake (photosynthesis) is comparable to the level of CO_2 production (respiration). The cultures would, therefore, probably have had a negative net carbon balance, as there would be no photosynthetic CO_2 fixation during the dark period. The cultures would probably have overcome this carbon in-balance by taking-up sugars from the media. The measurements of both of these metabolic processes were, however, made under the conditions prevalent in the IRGA analysis chamber, not the culture vessel. Therefore, they are probably not indicative of the actual levels of CO_2 exchange by the plantlets.

The FW (Fig. 46), and therefore the growth, of the flask grown cultures varied as the culture condition were changed. As respiration provides the energy for growth, the rate of this process would be expected to show similar variations. The lack of any significant changes in the dark respiration rates of the

cultures as the conditions were varied, may have been due to any affects being "masked". This "masking" could have been the consequence of, firstly, the measurements having been made under conditions that were different from those prevalent in the culture vessel, and secondly, due to the results for each treatment showing a high degree of variability. The respiratory physiology of cultures, therefore, needs further more extensive investigation. Moreover, the actual respiratory rates of the cultures under the conditions prevalent in the culture vessel need to be reliably measured.

As the initial media sucrose concentration was raised, the culture FW increased (Fig. 46). Solarova (1989) obtained an increase in FW as the medium sugar concentration was raised with tobacco cultures. Increasing the media sugar concentration would result in a concomitant increase in the levels of energy and carbon available, and thus greater plant growth. The increase in the uptake of sugar from the medium, with increasing initial medium sucrose concentration (Fig. 47) confirms this. The effect of the light intensity was less clear. This did not effect the uptake of sugar from the medium, though the low cabinet light intensity resulted in lower FW. This could have been due to the photosynthetic systems functioning inefficiently at the lowest light intensities. This process was considered to be an important factor in the growth of the cultures, even though sucrose was also available as an carbon and energy

source.

These results have shown that both the medium sucrose concentration and photosynthetic ability contribute to the growth of the cultures. The relative importance of these to the growth of the cultures depends on the culture conditions, the medium sucrose level, the cabinet light intensity and the CO_2 concentration in the culture vessel. The flask and jar cultures (Section 3.3.) showed different responses to the range of sugar concentrations and growth cabinet light intensities, maximum photosynthetic abilities and pigment contents being attained under different conditions. This was probably due to differences in the environmental conditions inside the two experimental culture vessels, due to their different methods of closure. Without full knowledge of these differences direct comparisons between the cultures from the two vessels are not possible.

Chapter 4

Concluding Discussion.

Plantlets *in vitro* were considered to have only limited photosynthetic ability, and thus to require sugar as a source of carbon and energy for growth. The growth of these cultures was thought to be either heterotrophic (using only the medium sugar) or mixotrophic (using a combination of the medium sugar and photosynthesis). Recent work (Kozai, 1991; Section 3.1.) however, has shown that plantlets can have high photosynthetic abilities and are therefore potentially capable of photoautotrophic growth (Kozai, 1988 & 1991) when provided with a suitable culture environment.

A number of studies have shown (for examples see Chapter 3, Fig. 19, 23, 24, 28, 29, 33, 40, 41 & 45; also Evers, 1982; Lee *et al*, 1985; Langford, 1987; Kozai, 1991) that not only the growth and development, but also the photosynthetic ability (potential) of the cultures is influenced by the culture environment. In this study the photosynthetic physiology was affected by the medium sugar concentration, the growth cabinet light intensity and the availability of CO_2 in the culture vessel. The effects of these will be discussed further in the following sections.

The time in culture was, at least in some instances, shown to influence the photosynthetic ability (see Section 3.1). These changes may have been due to cultures adapting their photosynthetic potential to the culture environment. For example, apical pea cultures

probably had their photosynthetic activity limited by the culture environment, hence the decrease in their photosynthetic abilities with time (Fig. 8 & 12).

The presence of BAP in the culture medium has at certain concentrations been shown to have a negative effect on photosynthesis in apical pea cultures (Section 3.2.). BAP at certain concentrations appears to inhibit photosynthetic physiology and to reduce levels of the photosynthetic pigments (Fig. 14, 15, 16, 17 & 18). However, studies of cultures of other species and of *in vivo* grown plants have shown cytokinins to more typically have positive effects on photosynthesis. The apparent reduction in the photosynthetic ability and pigment content for the leaves of apical pea cultures, may however, have been an artifact. The BAP also affected other aspects of the cultures growth, such as leaf area, which may have affected the measured photosynthetic parameters.

The time in culture and the BAP concentration have both been shown to have effects on photosynthesis. Moreover, these effects were also related to the explant source and plant species (Sections 3.1. & 3.2.). The effects of BAP and other cytokinins require further more detailed investigation. The effect of time in culture needs to be studied in greater range of plant species.

The measured dark respiration rates of the

cultures were, unlike their photosynthetic abilities, not significantly affected by the range of growth cabinet light intensity used in this study (Fig. 24, 29 & 40). The results are inconclusive as to the effects of the sugar concentration of the media on the respiratory physiology of the cultures (Fig. 19, 29 & 40); in some experiments low sugar levels were found to significantly reduced the respiratory rates. The leaf and *in situ* respiratory rates measured in this study may, however, have not been indicative of the respiratory physiology of the plantlets (Sections 3.3.4. & 3.5.4.). The respiratory physiology of the cultures needs further more extensive investigation, with the respiration rates being measured using whole plantlets and under the conditions prevalent in culture vessels.

4.1. Sugar and photosynthesis.

The growth, development and physiology of plant tissue cultures, such as those of three-week old apical pea cultures, was shown to be affected by the media sugar concentration (Sections 3.3.1., 3.3.4., 3.5.1. & 3.5.4.). The effects of sugar on the photosynthetic physiology of *in vitro*-produced plantlets has been likened to the interaction of the sinks and sources of *in vivo*-grown plants.

Carbohydrates are the primary products of photosynthesis and their synthesis in higher plants takes place mainly in the adult leaves. Carbohydrates are subsequently exported to other organs and young leaves (metabolic "sinks"), but a substantial fraction remains in the leaves (the "source" organ), being either temporarily stored or metabolised to satisfy the synthetic and energetic needs of the "source" (Azcon-Bieto, 1986). However, more strictly the "source" can be defined as the photosynthetic organelle, the chloroplast. The chloroplast membrane can therefore be defined as the boundary between the "sink" and the "source", "sinks" being all the non-photosynthetic parts of the plant, including the roots, the storage organs and the cytoplasm of the photosynthetic cell (Herold, 1980). The later definition has been adopted here.

Manipulation of sinks to increase or decrease the plant's demand for sugar has been shown to

change the rate of photosynthesis (Neales & Incoll, 1968; Herold, 1980). For example, Sweet & Wareing (1966) found that when the shoot apices (sink) of pine seedlings were removed the photosynthetic rate of the seedlings was reduced after 8 days. In other words, when the sink demand was decreased the production of sugar by the sources was reduced. Thorn & Koller (1976) found that when potted soyabean plants, excepting one leaf, were completely shaded the rate of photosynthesis increased in the unshaded leaf. The photosynthetic rate of a similar leaf in a totally unshaded plant showed no increase. Thus an increase in sink demand, caused by the shading, increased the production of sugar by the source, the unshaded leaf. These manipulations of sources and sinks may have caused changes to other factors unrelated to the sugar concentration, such as the levels of P.G.R.s, which may have indirectly caused the changes in photosynthetic activity (Herold, 1980). Source responses have however been induced by sink manipulation in a number of species and some of the manipulations have been less severe.

The path by which the photosynthetic (source) response is linked to the change in demand for sugar (sink) is not clear. Three possible mechanisms have been postulated: a/ P.G.R.s; b/ carbohydrate levels; c/ orthophosphate levels (Herold, 1980).

Studies have suggested that high sugar levels can inhibit sucrose synthesis in the cytosol of

the photosynthetic cell (Stitt & Quick, 1989). Two of the enzymes in the triose phosphate to sucrose pathway have been shown to regulate the synthesis of sucrose, sucrose phosphate synthase and fructose-1,6-bis-phosphotase. These enzymes are regulated by multiple mechanisms that allow the rate of sucrose synthesis to be increased in response to a rising supply of photosynthate, and to decrease the rate in response to falling demand for sucrose (Stitt & Quick, 1989). High sugar levels lead to the accumulation of triose phosphates and hence the depletion of the free phosphate in the cytosol. These low phosphate levels will reduce the export of the triose phosphates from the chloroplast in counter-exchange for cytosol phosphate. This will in turn stimulate the production of starch in the chloroplast as the triose phosphates accumulate and may lead to the reduction in photosynthetic activity. This feedback inhibition is thought to act as a short term regulator of photosynthetic production (Stitt & Quick, 1989).

Longer term adaptation may take place if low sink demand and thus high cytosol sugar levels are maintained. Firstly, the formation of starch grains in the chloroplast as triose phosphate levels rise may distort or shade the photosynthetic membranes (Azcon-Bieto, 1986). Secondly, the accumulation of photosynthetic products, such as triose phosphates, may inhibit photosynthesis. Stitt *et al* (1991) found with transgenic mutant tobacco plants, that sink regulation

involved changes in the concentrations of certain enzymes. Levels of some of the Calvin cycle enzymes were decreased, while levels of certain the glycolytic enzymes increased. It was suggested that medium to long term adjustments to high leaf carbohydrate levels may lead to reduced levels of Calvin cycle activity (photosynthesis) and increased levels of respiration.

The medium sugar concentration has been shown to effect the photosynthetic physiology and growth of shoot cultures of a variety of plant species (Evers, 1982 with Douglas Fir; Langford, 1987 and Cappalledes *et al*, 1991 with rose; this study, Fig. 19, 29, 34, 40, 41 & 46 for apical pea cultures). These effects may be compared to the source/sink interactions of *in vivo* grown plants. Cultures have been shown to take up sugar from the culture medium (Fig.'s 35 & 47), the amount increasing as the medium sugar concentration was raised.

The level of sugar available to the cultures could be high, due to photosynthesis and/or absorption from the medium. It can be postulated that the cultures sinks might be "inundated" with carbohydrate. If this occurs, it could in turn lead to the accumulation of sugars in the cytosol of the photosynthetic cells, and hence may cause the formation of starch (probably in starch grains) in the chloroplasts (sources) and possibly lead to reduced photosynthetic activity, by feedback inhibition. This possible response may be mediated by decreased levels of free phosphate in the cytosol of the

photosynthetic cells, blocking counter-exchange of triose phosphates out of the chloroplasts. Longer-term adjustments may also be hypothesized, particularly for cultures on media with high sugar concentrations, where the greatest amount of sugar was taken up. The high sugar levels may result in changes in the levels of the photosynthetic enzymes, thus reducing the photosynthetic ability (potential) of the chloroplasts.

The responses of the cultures to changes in the medium sugar concentrations support these hypotheses. The decrease in the photosynthetic rate per unit chlorophyll (Fig. 23, 33 & 45) when the medium sucrose level was raised suggests that the high sugar concentration was inhibiting photosynthetic biochemistry. The high sugar levels may therefore have reduced the levels of the photosynthetic enzymes. Cappellades (1991) with cultures of rose, found greater amounts of starch in the leaves, more starch grains in the chloroplasts and reduced photosynthetic activity in culture grown on media containing high concentrations of sucrose. This suggests that an excess of sugar was available to these cultures and was reducing their photosynthetic activity.

These findings suggest that high media sugar concentrations probably have an effect on plant tissue cultures that is comparable to the effect of low sink demand on source activity in *in vivo*-grown plants.

Low or no medium sugar levels resulted in

reduced growth, photosynthetic activity and pigment contents (Langford & Wainwright, 1987, for rose cultures; Section 3.3., for apical pea cultures). This may have been due to low levels of sugar restricting the growth and development of the cultures, including the photosynthetic systems.

In certain instances cultures have been successfully grown on sugar-free media. In most cases the cultures were grown in gas permeable vessels, typically in an atmosphere with an elevated CO_2 concentration. These cultures were also often grown at high light intensities. The cultures involved were usually obtained by regeneration, and had comparable photosynthetic abilities to cultures grown on media containing sugar or to seedlings grown under similar environmental conditions. For example, Kozai *et al* (1988b) with carnation, and Kozai *et al* (1988a) with potato, grew cultures on a sugar-free medium in gas permeable culture vessels in an atmosphere with an elevated CO_2 concentration and high light intensity. Moreover, Pospisilova *et al* (1987) and Solarova (1989) with tobacco, and Kozai *et al* (1991) with *Brassica campestris* grew cultures on sugar-free media in gas permeable culture vessels at atmospheric CO_2 concentrations. The *Brassica campestris* cultures were additionally grown at a high light intensity. The presence of higher CO_2 concentrations (photosynthetic substrate) in the culture vessel and high light intensities (photosynthetic energy

source) would lead to increased photosynthetic activity by the cultures, adequate to maintain growth in the absence of sugars (Section 4.3).

The sugar concentration of the culture medium has not only been shown to affect the photosynthetic ability of the cultures, but their growth and multiplication (Sections 3.3.1. & 3.3.4.). The medium sugar concentration has also been shown to be involved in vitrification (Ziv *et al*, 1983; Langford, 1987), due to the effect of sugar concentration on the medium's osmotic potential. These effects should be considered when selecting a media sugar concentration for a culture protocol.

4.2. Light and photosynthesis.

Light intensity has been shown to affect the growth and development of plantlets including apical pea cultures (Sections 3.3.1., 3.3.4., 3.5.1. & 3.5.4.). The effect of light on the photosynthetic physiology of these cultures was compared to the differences found *in vivo* between "sun" and "shade" leaves, and between plants grown at high and low light intensities (high- and low-light plants).

Plants have been shown to possess the capacity for an ontogenetic adaptation of their photosynthetic apparatus to the incident light intensity. Leaves and chloroplasts developed under high light intensities (sun leaves and high-light plants) have been found to be structurally and physiologically different from those formed at lower light intensities (shade leaves and low-light plants) (Lichtenthaler & Meier, 1984). These "types" of light adaptation have been found in a great variety of plant species.

Typical differences between plant material grown at different light intensities are summarized below. These changes, however, are not universal, and will depend on the plant species and environmental conditions.

High light compared to low light (Lichtenthaler *et al*, 1981 & 1982; Lichtenthaler & Meier, 1984; Longstreth *et*

al, 1985):

Leaves from high-light/sun plants typically have a smaller surface area and are thicker, having more layers of mesophyll cells than those from low-light/shade environments. They also have a higher photosynthetic rate, DW and stomatal density.

Chloroplasts from high-light/sun plants have a lower thylakoid frequency, narrow and low granal stacks, and a higher proportion of non-appressed membranes than those from low-light/shade plants. They also have a higher starch level and differences in pigment content, with more pigment associated with PSI and less LHCP.

The ultrastructural studies of the apical pea cultures (Section 3.4., Plates 1, 2 & 3) showed a reduced thalykoid system at high growth cabinet light intensities, but not an increased number of starch grains. Lee *et al* (1985) found with cultures of sweetgum that the number of thylakoid membranes increased as the growth cabinet light intensity was raised and that the chloroplasts were always devoid of starch. In both studies the highest photosynthetic abilities (on a FW basis for apical pea cultures, and a leaf area basis for sweetgum) were found at the intermediate growth cabinet light intensities. For cultures of the rose cultivar Peace (Langford, 1987) the maximum photosynthetic ability was similarly found at an intermediate light intensity. The photosynthetic abilities on a unit chlorophyll basis

for sweetgum (Lee *et al*, 1985), rose (Langford, 1987) and apical pea (Fig. 33) cultures were larger at the highest cabinet light intensities. The largest FW and DW were also for the apical pea cultures grown at the highest light level (Fig. 34). Apical cultures grown in flasks had their highest photosynthetic abilities per unit chlorophyll (Fig. 45) and FW (Fig. 46) at the highest light intensities.

The higher photosynthetic ability per unit chlorophyll, higher FW and DW and the reduced chloroplast thylakoid system for apical pea cultures at higher growth cabinet light intensities suggest that the chloroplasts were undergoing a sun-shade type adaptation, although there was no increase in the number of starch grains (Plates 1, 2 & 3) with light intensity, which suggests that there was no change in the chloroplast starch contents. This may have been be due to photosynthetic activity, but not the photosynthetic ability (potential), being limited in the culture vessel by the low concentration of CO_2 throughout most of the light period (Section 4.3.). The difference in the ultrastructural changes between the apical pea cultures and those of sweetgum (Lee *et al* , 1985) as the light intensity was raised may have been the result of interspecific differences. This variation needs further investigation, and studies should be conducted with a greater range of plant species.

The measured levels of the photoprotection

compounds, ascorbate and glutathione (Table 4), show that as with *in vivo* grown seedlings the level of ascorbate increased as the light level was raised. The increase in the ascorbate level of the apical pea cultures was however smaller than that for the pea seedlings (Gillham, 1986; Ruffle, 1989). Moreover, the chlorophyll-to-carotenoid ratio was shown to decrease as the light intensity was raised (Table 5), the carotenoids providing the chlorophylls with protection against photo-oxidative damage. The ultrastructural studies of the chloroplasts of apical pea cultures showed that those grown at high light intensities had the smallest membrane system, this might have been due to adaptation to the high light, (see above). However, there were prominent plastoglobuli (lipid bodies) which are often associated with membrane breakdown. Considering the smaller *in vitro* increase in the ascorbate levels, the limited presence of starch grains and the evidence of membrane breakdown, it might be concluded that there was possibly some photo-oxidative damage to the "high light" chloroplasts. This may have been enhanced by the low culture vessel CO_2 concentration during most of the light period, which would have limited the photosynthetic utilization of the harvested light energy. The excess light energy not being safely dissipated, might cause photo-oxidative damage.

The effect of light intensity on the photosynthetic systems of apical pea cultures showed similarities to the effect of light on *in vivo* grown

plants, that is, high- and low-light plant or sun and shade plant adaptations. These changes are accompanied by changes in the photoprotection mechanisms. The presence of sugar in the culture media and the limited availability of CO_2 in the culture vessel may result in a reduction in the culture's photosynthetic activity, and there may therefore be an increased potential for photo-oxidative damage, since less of the harvested light energy will be required. There may also be effects on the adaptations of the chloroplast and leaves to the different light intensities.

Varying the growth cabinet light intensity has been shown not only to affect the photosynthetic ability and chloroplast ultrastructure of cultures, but also to affect culture growth and development (Sections 3.3.1. and 3.3.4.). When selecting a growth cabinet light intensity for a culture protocol, in addition to the effects of the light intensity on the photosynthetic physiology, the effect of light on culture growth and development should also be considered.

4.3. Carbon dioxide concentration and photosynthesis.

The CO_2 concentration inside the culture vessel (C_{in}) has been shown to vary during the diurnal light dark cycle (Sections 3.5.3. & 3.5.4.). This variation in the composition of the air enclosed in the culture vessel may have affected the respiratory and photosynthetic activity of the cultures.

Fujiwara *et al* (1987), using cultures of eight genera of ornamental plant in "relatively air-tight" culture vessels, found that the C_{in} droped to 70-80 ppm within two hours of the commencement of the light period (light intensity 65 uE $m^{-2}s^{-1}$). The atmospheric CO_2 content (C_{at}) was approximately 340 ppm. The C_{in} at the end of the dark period was in the range 3000 to 9000 ppm. When more gas-permeable culture vessels were used the C_{in} fell to around 100-200 ppm during the photoperiod (Kozai *et al*, 1987, 1988a & b, & 1990a; Desjardins *et al*, 1988; Kozai & Iwanami, 1988; Kozai & Sekimoto, 1988). These results are comparable to those obtained with flask-grown apical pea cultures (Fig. 39). The high C_{in} at the end of the dark period was the result of the respiratory production of CO_2 from carbohydrate. The decrease in the C_{in} at the start of the light period was probably due to the consumption of CO_2 during photosynthesis. The resultant decrease in the level of this gas to below atmospheric levels would have limited the photosynthetic activity of the cultures.

The calculated photosynthetic activities were found to be 1000x lower than the estimated maximum rates or photosynthetic potential (ability) for cultures of 15 ornamental plant species (Fujiwara *et al*, 1987). This difference was probably due to the low CO_2 concentration in the culture vessel (C_{in} 50-70 ppm) during the light period limiting the photosynthetic activity in the vessel. A smaller difference between the photosynthetic ability and the photosynthetic activity (which was one third lower) in the culture vessel was obtained with cultures of *Cymbidium* grown in gas permeable culture vessels, at a C_{in} of 250 ppm (Kozai *et al*, 1990a).

Pospisilova *et al* (1987) found that ontogenically comparable cultures and seedlings of tobacco grown under similar conditions (C_{at} 340 ppm, light intensity 110 µE $m^{-2}s^{-1}$) had similar photosynthetic abilities at saturating light levels (1130 µE $m^{-2}s^{-1}$, C_{at} 340ppm). When seedlings and plantlets were grown *in vitro* (in test tubes on MS medium) under indentical conditions (sugar and light) they showed similar growth responses and comparable photosynthetic activity trends when the culture conditions were changed (Kozai *et al*, 1990b & 1991b). The photosynthetic abilities of apical pea cultures (in jars) were under certain culture conditions comparable to those of *in vivo* grown seedlings (Fig. 8, 12 & 13). The actual photosynthetic activity in the vessels however was probably much lower, being limited by

a low CO_2 level.

These studies appear to support the hypothoesis that plantlets have a potential photosynthetic competence that is comparable to that of seedlings grown under similar conditions. The photosynthetic activity of the cultures, however, was limited by the "air" enclosed in the culture vessel which had a CO_2 concentration below C_{at}, during most of the light period.

This limitation of *in vitro* photosynthetic activity by the CO_2 concentration could be overcome by raising C_{in}. Three ways for achieving this have been suggested (Kozai, 1991):

1/ increased vessel permeability to gases, higher C_{in} at C_{at}.

2/ CO_2 enrichment of culture room atmosphere (C_{ou}), higher C_{in} by raising C_{ou} above C_{at}.

3/ supplying CO_2 directly to the culture vessel (for vessels of large volume), elevating C_{in} directly.

In a number of studies the first two of these mechanisms have been used either alone or in combination. The effects of the medium sugar concentation and growth cabinet light intensity on photosynthetic activity have also been investigated for cultures grown at elevated C_{in}.

Kozai & Iwananimi (1988), found that elevated CO_2 concentration (C_{ou} 1000-1500 ppm) and low medium sugar (0 or 10 g/l) under a high light level (150 µE $m^{-2}s^{-1}$) promoted the growth of carnation node cultures grown in test tubes closed with molded plastic caps. The photosynthetic abilities of these cultures increased significantly with time in the CO_2-enriched environment, while remaining unchanged for the non-enriched treatments. The media sugar concentration had no effect. Comparable results have been obtained for plantlets of *Cymbidium* (Kozai *et al*, 1990a) and strawberry (Kozai *et al*, 1991a). Desjardins *et al* (1988), grew strawberry and raspberry plantlets under a range of light intensities (80, 150 & 250 µE $m^{-2}s^{-1}$) and CO_2 concentrations (C_{ou} 600, 1800 & 3000 ppm), they found that higher light intensities and CO_2 concentrations increased the photosynthetic activity of strawberry cultures and the stomatal density of raspberry cultures. *In vitro* grown plantlets and seedlings have been found to have comparable growth and photosynthetic responses to changes in C_{in} (Kozai *et al*, 1990b & 1991b); high C_{in} promoted photosynthesis and growth. Desjardins *et al* (1989) measured the PEPcase and RUBISCO activities of strawberry plantlets grown under a range of light intensities and CO_2 concentrations. Those plantlets that were grown at both the highest light intensity and CO_2 concentration had the greatest CO_2 assimilation rate and PEPcase activity. The change in the PEPcase activity was more

closely correlated to the change in the CO_2 assimilation rate than RUBISCO activity. High levels of PEPcase activity have also been found in photosynthetically competent cell cultures (Section 1.2.1.1.). The exact role of CO_2 fixation by PEP in cultures is however uncertain. In cell cultures it has been suggested that the products may provide carbon skeletons for growth, as well as feeding the Calvin cycle (Neumann & Bender, 1987). The role of PEP in the fixation of CO_2 by cultures needs further investigation.

The investigations described above have shown that elevated of C_{in}, high light intensities and low medium sugar concentration promote the development of photosynthetically competent cultures. However, Kozai *et al* (1990c) found that the culture media used, as well as the sugar concentration and gas permeability of the culture vessel (gas concentration), affected the growth of carnation cultures. Moreover, Kozai *et al* (1991a) found that photoautotrophically grown strawberry plantlets (on MS medium, no sugar and a high C_{ou}) depleted the culture medium of phosphates faster than photomixotrophic cultures (growing on MS medium plus sucrose). This low medium phosphate level (approximately 5 % of that initially present after 21 days) may have limited the growth of the cultures. These workers suggested that the growth and development of photoautotrophic cultures might be enhanced by using a modified culture media.

For apical pea cultures grown in flasks the CO_2 content of the culture vessel atmosphere (C_{in}) fell (Fig. 39), however the decrease was "slower" than those obtained in other studies (Fujiwara *et al*, 1987, see above). The accumulation of CO_2 in the vessels seemed to be dependent on the medium sugar concentration and light intensity. High medium sugar content, and high and intermediate cabinet light intensities, gave at the end of the dark period, higher C_{in}. These changes in the C_{in} were probably due to variations in the actual respiratory and photosynthetic activities of the cultures (but not necessarily the measured respiratory rates and photosynthetic abilities, as the conditions under which these measurements were made differed from those in the culture vessel). High medium sugar increases the carbohydrate available for respiration, and higher light intensities the energy for photosynthesis, thus, on high sugar media there may be a higher respiratory production of CO_2, but the photosynthetic activity may be reduced by the presence of the alternative source of sugar. At higher light levels the higher CO_2 levels may be the result of the light stimulating culture growth and development, and thus respiration. High light will also stimulate the photosynthetic fixation of carbon, and thus the utilization of the available CO_2. This photosynthetic activity would be dependent on the photosynthetic ability (potential) of the cultures, which in turn would be affected by the culture conditions. The photosynthetic

ability was probably directly related to C_{in}, however the effects of light intensity and media sugar concentration on photosynthetic ability are interdependent (Sections 4.1. & 4.2.), so there may be a similar interdependence between these environmental factors and C_{in}.

The photosynthetic ability of the cultures was shown to be affected by the culture conditions (Sections 4.1. & 4.2.). The actual photosynthetic activity of the culture during the normal course of the culture process is probably much lower, as has been found for cultures of other species (Fujiwara *et al*, 1987, see above). Photosynthetic activity was not measured here for the apical pea cultures under the conditions prevalent in the culture vessel. Such measurements should be possible with a modified culture vessel and IRGA gas stream, which approximately reproduces the conditions in the culture vessel. Furthermore, the effect of raising C_{in} has not been investigated for apical pea cultures.

4.4. Concluding Remarks

i/ Summary of conclusions.

The medium sugar concentration, growth cabinet light intensity and the CO_2 content of the culture vessel atmosphere (C_{in}) have all been shown to influence the photosynthetic ability, and probably the photosynthetic activity, of apical pea cultures. These responses were comparable to those that have been obtained with a variety of other species and culture systems. The effects of these environmental factors on cultures were shown to be interrelated.

The effects of the medium sugar concentration on photosynthesis had similarities with the interaction of the "sinks" and "sources" of *in vivo* grown plants. Further, the effects of light intensity on the cultures were comparable with the differences found between sun/high-light and shade/low-light plants *in vivo*. High light also resulted in an increase in the levels of the photoprotection compound ascorbate and a decrease in the chlorophyll-to-carotenoid ratio.

The low CO_2 concentration inside the culture vessel during much of the light period could have been responsible for the limited the photosynthetic activity of the cultures. This was probably the primary cause of the cultures growth not being photoautotrophic, and thus of their reliance on the media sugar. The prevention of this CO_2 shortfall by elevating C_{in} has

been shown to lead to cultures growing photoautotrophically, particularly at elevated light levels and on low-sugar or sugar-free media (Kozai, 1991).

The length of time that the pea apices spent in culture was shown to decrease their photosynthetic ability and pigment contents. The presence of the cytokinin BAP was also shown to have a negative effect on photosynthesis, however this latter change may have been an artifact and thus needs more detailed investigation.

The measured dark respiration rates suggested that the respiratory physiology of the cultures may have been restricted by low media sugar concentrations, but uneffected by the range growth cabinet light intensity used. Further more detailed investigations are, however, needed to confirm these findings.

ii/ Further work.

Further work is needed to expand the range of plant species studied. The effects of sugar concentration, light intensity and CO_2 concentration, alone and in combination, on the photosynthetic activity and ability of plantlets should be examined. The effects of these environmental manipulations on the culture growth, physiology and ultrastructure, also need more detailed

investigation. Particulary attention should be given to the effects of sugar and light, either alone or in combination, on chloroplast ultrastructure, and the effect of light levels on the photoprotection mechanisms.

The flask culture vessel system requires refinement to overcome the problems discussed in Section 3.5.3. and to allow elevation of C_{in}. Further, a systems needs to be developed to allow the measurement of photosynthesis under the conditions prevalent in this culture vessel, that is, the actual photosynthetic activity of the cultures. This could be done in one of three ways:

1/ use of a system by which the photosynthetic activity is calculated from the measured changes in the CO_2 content of the culture vessel, similar to that used by Fujiwara *et al* (1987);

2/ modification of the IRGA system so that the gas composition (CO_2 concentration and water vapour) and light intensity are the same as those found in the culture vessel.

3/ study of the incorporation of radioactively labelled CO_2.

Measurements of the levels and activities of photosynthetic enzymes, such as Rubisco, and the following of the incorporation of radioactively labled CO_2 and sugars, would be useful in attaining a greater

understanding of the role of photosynthesis in culture growth. Furthermore, such studies would also allow a greater understanding of the effects of the culture environment on photosynthetic physiology.

iii/ Future perspectives.

The further study of the effects of sugar, light and CO_2 on culture growth and physiology should allow the development of improved culture protocols. This may lead to further optimization of micropropagation systems and so reduced costs or improved yields. It has been shown that cultures have a considerable photosynthetic potential (ability) and thus under certain conditions photoautotrophic culture growth should be possible. Such growth has already been achieved for a few species (Kozai, 1991). The production of photoautotrophically growing cultures should be possible with a wide range of plant species, including pea, however, the development of the necessary culture systems requires further research and development.

The development of photoautotrophic plantlet cultures and thus micropropagation systems will probably have advantages over the conventional systems of micropropagation. High light and CO_2 would not only promote photosynthesis but may also stimulate culture growth. Gas permeable vessels may have a lower internal RH, therefore weaning the plants to *in vivo* conditions should be simpler. The use of sugar free media would

reduce the risks of microbiological contamination, particularly if an inorganic supporting agent is used instead of agar. Losses due to contamination could be reduced, handling the cultures would be easier, and the use of larger vessels would be more practicable. Such photoautotrophic micropropagation systems still need development.

Further studies of the effects of sugar and light on the growth and physiology of cultures will show how they compare with the effects of these factors on *in vivo* grown plants, that is, "sink/source" interactions and sun/high-light - shade/low-light differences. This could lead to a greater understanding of photosynthesis and the possible development *in vitro* models to aid future investigations.

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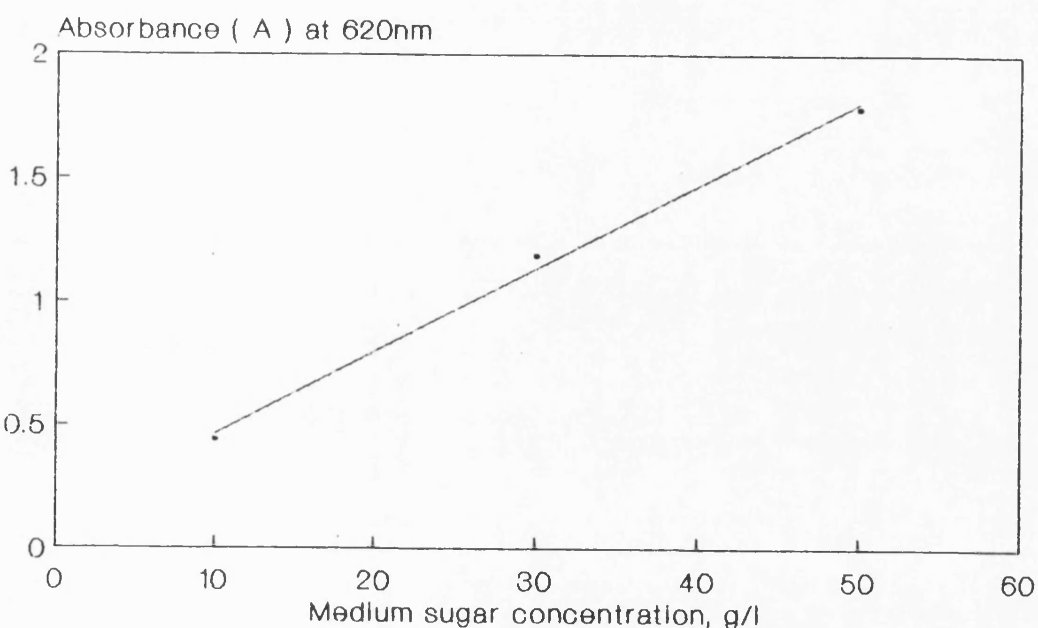
Appendices

Appendix 1

Standards for the Biochemical Assays.

Fig. A1
Anthrone assay for media sugar.
Calibration line.



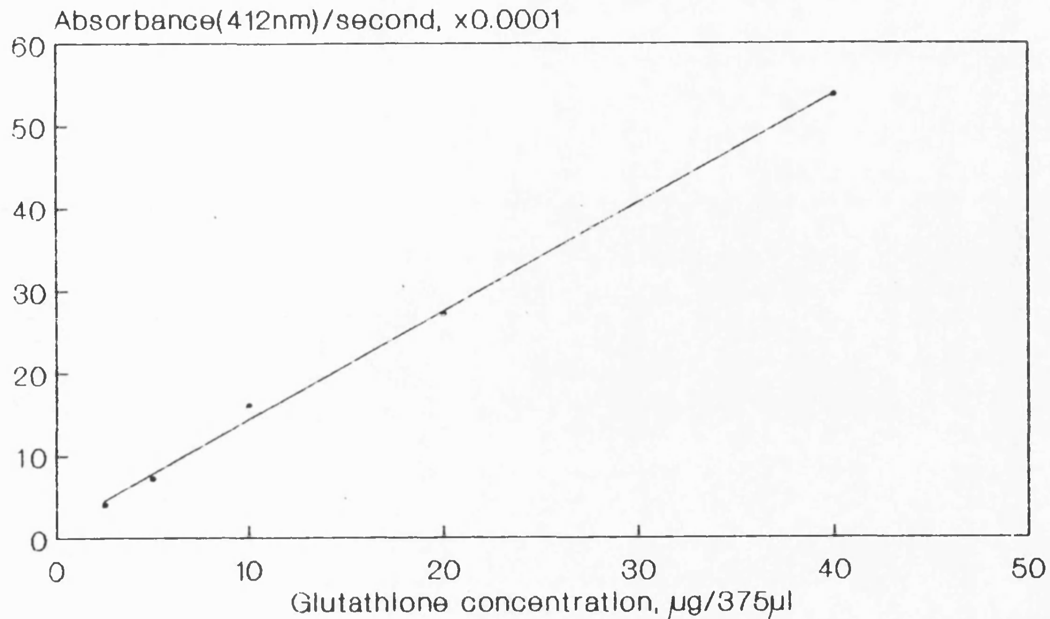
The line was ploted using points
calculated by regression.
n = 35

The procedural reliability of the Anthrone sugar assay was checked. For example; absorbance readings for a set of standards (jars containing media but no plantlets) are given below. Two samples of molten media were taken from each culture jar and their sugar contents assayed;

Initial media sugar concentration g/l	Absorbance Jar 1		Jar 2		Jar 3	
10	0.371	0.378	0.403	0.396	0.452	0.461
30	1.131	1.026	0.963	0.972	1.144	1.123
50	1.700	1.744	1.765	1.756	1.745	1.709

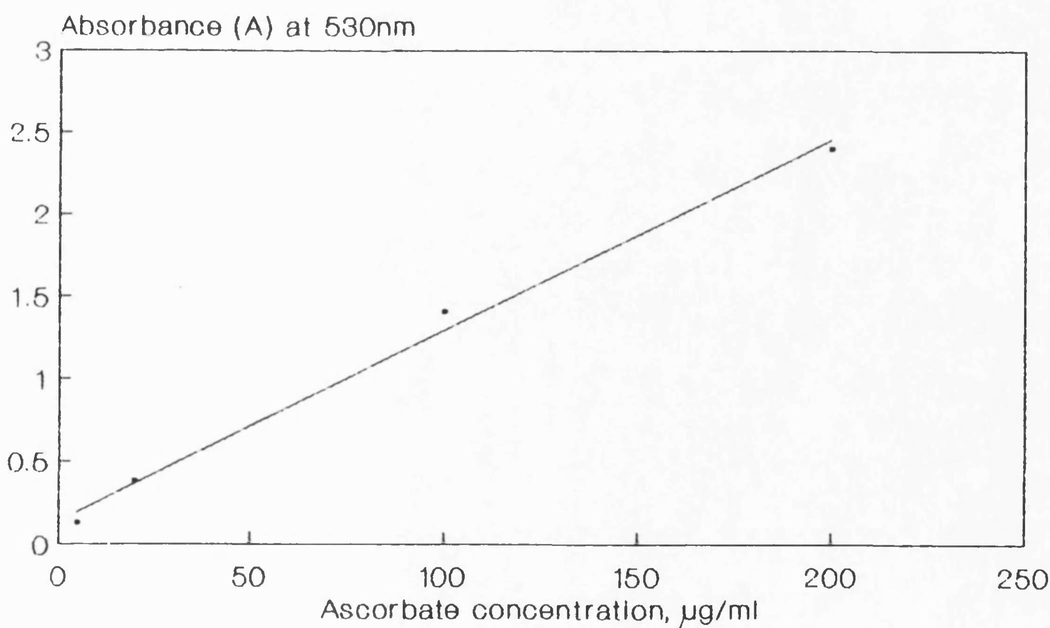
The differences between the samples from the same jar are small, indicating that the assay procedure is reliable. The variation between the jars is larger, this is probably due to differences in the jars sugar content (from the added sucrose and agar, after autoclaving and three weeks in a growth cabinet). This variation is still, however, small in relation to the total sugar content.

Fig. A2
Glutathione assay.
Calibration line.



The line was ploted using points
calculated by regression.
n = 8

Fig. A3
Ascorbate assay.
Calibration line.



The line was ploted using points
calculated by regression.
n = 10

Appendix 2

Means and Statistical Analysis.

Part 1:

Mean values and their standard errors (SEM).

In this appendix the means values and their SEM's are given for the appropriate fig. and tables, by section.

Abbreviations.

P.G.R.(s)	. .	plant growth regulators, 20µM naphthaleneacetic acid & 2µM 6-benzyl-aminopurine
MS	. .	Murashige & Skoog (1962) media salts
basal media.	.	MS salts media containing 30 g/l sucrose

Section 3.1. Subculturing.

Fig. 6; fresh (FW) and dry (DW) weigths of the two explant types and of three week old cultures.

Material	Fresh Weight		Dry Weight	
	g	SEM	g	SEM
Apical explant	0.046	0.004	0.005	0.0005
Nodal explant	0.044	0.004	0.004	0.0005
Apical cultures				
basal media	0.321	0.047	0.027	0.003
basal media + P.G.R.s	0.316	0.021	0.028	0.002
Nodal cultures				
basal media	0.154	0.015	0.011	0.002
basal media + P.R.G.s	0.272	0.026	0.020	0.002

n = 16

Fig. 8 a/; photosynthetic ability of pea cultures.

Media	Time in culture	Photosynthetic ability					
		n	Apical		n	Nodal	
	weeks		$\mu molCO_2$ /hr/gFW	SEM		$\mu molCO_2$ /hr/gFW	SEM
basal media							
	3	9	139.0	4.4	7	76.5	3.9
	6	9	52.3	4.8	5	84.4	5.6
	9	4	77.1	10.9	4	86.2	7.9
	12	6	47.6	3.9	2	74.9	
basal media + P.G.R.s							
	3	7	78.9	4.9	6	82.8	7.2
	6	5	50.2	5.8	6	69.9	3.6
	9	3	67.0	8.6	5	88.6	5.6
	12	5	49.3	9.2	5	58.2	5.4

Fig. 8 b/; respiratory rate of pea cultures.

Media	Time in culture	Respiratory rate					
		n	Apical		n	Nodal	
	weeks		μmolCO_2 /hr/gFW	SEM		μmolCO_2 /hr/gFW	SEM
basal media							
	3	9	31.2	3.2	7	47.6	4.5
	6	9	21.6	4.1	5	40.1	5.7
	9	4	29.5	2.2	4	37.2	3.4
	12	6	27.4	1.7	2	28.9	
basal media + P.G.R.s							
	3	7	37.9	2.1	6	36.9	1.5
	6	5	39.3	8.5	6	47.8	7.1
	9	3	30.5	3.3	5	29.4	4.3
	12	5	21.9	3.0	5	27.4	2.6

Fig.s 9, 10 & 11; pigment content of the leaves of pea cultures.

Time in culture	n	Total Chlorophyll		Chlorophyll a/b ratio		Cartenoids	
weeks		mg/gFW	SEM		SEM	mg/gFW	SEM
Apical							
basal media							
3	9	2.882	0.166	2.31	0.15	0.343	0.013
6	9	2.293	0.064	2.19	0.10	0.282	0.015
9	4	1.668	0.189	3.51	0.08	0.313	0.044
12	6	1.749	0.071	2.42	0.24	0.215	0.026
basal media + P.G.R.s							
3	7	1.814	0.099	2.49	0.28	0.293	0.033
6	5	1.500	0.132	2.40	0.13	0.200	0.018
9	3	1.256	0.202	4.36	2.59	0.191	0.038
12	5	1.566	0.059	3.99	0.79	0.270	0.037
Nodal							
basal media							
3	7	2.755	0.218	2.39	0.24	0.320	0.021
6	5	2.489	0.179	2.43	0.02	0.335	0.027
9	4	2.410	0.526	3.73	1.63	0.321	0.022
12	2	2.135		1.93		0.224	
basal media + P.G.R.s							
3	6	2.199	0.138	2.76	0.14	0.331	0.014
6	6	1.717	0.202	2.52	0.18	0.224	0.020
9	5	1.821	0.281	3.67	0.94	0.288	0.013
12	5	1.045	0.211	2.85	0.34	0.156	0.036

Fig. 12;
a/ photosynthetic ability of apical pea cultures.

Media	Time in culture	Photosynthetic ability			
		Apical		Apical Subcultured	
	weeks	µmolCO_2 /hr/gFW	SEM	µmolCO_2 /hr/gFW	SEM
basal media					
	2	138.0	6.2		
	3	124.4	6.2		
	4	135.4	6.1		
	5	102.7	4.2	77.0	2.4
	6	90.2	4.9	72.5	7.6

b/ respiratory rate of apical pea cultures.

Media	Time in culture	Respiratory rate			
		Apical		Apical Subcultured	
	weeks	µmolCO_2 /hr/gFW	SEM	µmolCO_2 /hr/gFW	SEM
basal media					
	2	35.2	1.6		
	3	40.4	2.1		
	4	43.4	2.8		
	5	34.4	3.1	33.6	2.5
	6	36.6	2.5	32.4	1.4

n = 9

Fig. 13;
a/ photosynthetic ability of pea seedlings.

Age	Photosynthetic ability	
weeks	$\mu molCO_2$ /hr/gFW	SEM
2	114.2	2.0
3	116.3	7.6
4	110.6	8.3
5	117.5	10.4
6	112.2	8.1

b/ respiratory rate of pea seedlings.

Age	Respiratory rate	
weeks	$\mu molCO_2$ /hr/gFW	SEM
2	43.2	2.5
3	35.9	3.6
4	33.4	4.3
5	31.7	5.2
6	34.4	2.9

seedlings grown in universal compost at 150 $\mu E\ m^{-2}s^{-1}$.

n = 4

Section 3.3. Sugar & Light.

Fig. 34; fresh and dry weights of apical cultures.

The Fresh Weights of 3 week old cultures, g

Conditions		growth cabinet light intensity $\mu E\ m^{-2} s^{-1}$			
		50	150	300	
Initial media sugar content g/l	10	0.095	0.140	0.188	
		0.007	0.011	0.017	SEM
	30	0.175	0.281	0.383	
		0.010	0.018	0.017	SEM
	50	0.177	0.319	0.297	
		0.008	0.021	0.032	SEM

The Dry Weights of 3 week old cultures, g

Conditions		growth cabinet light intensity $\mu E\ m^{-2} s^{-1}$			
		50	150	300	
Initial media sugar content g/l	10	0.009	0.014	0.019	
		0.001	0.001	0.001	SEM
	30	0.018	0.033	0.048	
		0.001	0.002	0.003	SEM
	50	0.021	0.044	0.045	
		0.001	0.002	0.004	SEM

media MS salts containing sucrose concentrations given in the table.

n = 26

Fig. 35; sugar content of media after a three week culture period.

Sugar content of media after 3 week culture period, g/l					
Conditions		growth cabinet light intensity µE $m^{-2} s^{-1}$			
		50	150	300	
Initial media sugar content g/l	10	9	8	9	
		0.9	1.1	1.0	SEM
	30	26	26	26	
		1.6	2.2	2.6	SEM
	50	42	40	37	
		6.3	6.4	5.8	SEM

media, MS salts containing the sucrose concentration given in the table.

n = 9

Section 3.4. Photoprotection.

Table 4; glutathione and ascorbate concentrations of the leaves of three week old apical pea cultures grown under a range of light intensities.

Material cabinet light intensity	Glutathione		Ascorbate	
$\mu E\ m^{-2} s^{-1}$	mg/g FW	SEM	mg/g FW	SEM
50	1.28	0.13	2.5	0.17
150	1.07	0.06	3.0	0.20
300	1.17	0.07	3.7	0.20
	n = 8		n = 10	

MS media.

Section 3.5. Flask Cultures.

Fig. 47; sugar concentration of media after the three week culture period.

Sugar content of media after 3 week culture period, g/l

Conditions		growth cabinet light intensity $\mu E\ m^{-2} s^{-1}$			
		50	150	300	
Initial media sugar content g/l	10	10	9	9	
		0.8	2.2	0.5	SEM
	30	28	28	29	
		0.9	1.1	1.4	SEM
	50	40	44	40	
		1.8	1.3	1.8	SEM

media, MS salts containing sucrose concentrations given in the table.

n = 12

Part 2:

Mean values, plus the results of their statistical analysis by the analysis variance test (Anova) and Fishers multiple comparison procedure are given in this appendix. All cultures were grown on media containing Murashige & Skoog (1962) media salts. The media contained 30 g/l sucrose unless otherwise stated. For all data the table produced by the Anova test is given first. This is then followed by the results of Fishers multiple comparison test and the mean values.

Fishers Multiple comparison test is summarised bellow:

Fishers LSD = $t_{(f)} \sqrt{s_p^2 (1/n_1 + 1/n_2)}$

Where: $t_{(f)}$ = (Students) t-value for f degrees of freedom.

f = is the degrees of freedom for the error analysis of the Anova test.

s_p^2 = the mean square (MS) for the error analysis from the Anova test.

$n_1/_2$ = number of replicates for each of the two treatments being compared

The LSD is then used to calculate a confidence interval. The difference between the two treatment means is calculated and the LSD is then added and subtracted from this difference. If the resultant two numbers are either, both positive or both negative numbers, the treatments are significantly different from each other.

Abbreviations.
general:
- BAP . . 6-benzyl-aminopurine, µmol
- L . . growth cabinet light intensity, $\mu E\ m^{-2} s^{-1}$
- FW . . fresh wieght, g
- S . . media sugar concentration, g/l

Anova table columns:
- DF . . degrees of freedom
- SS . . sums of squares
- MS . . mean squares
- P . . probability

above the mean values:
- CART . . carotenoid content, mg/gFW
- CHL . . chlorophyll content, mg/gFW
- RESP . . Respiration rate, $\mu mol\ gFW^{-1} hr^{-1}$
- PHS . . photosynthetic ability, $\mu mol\ gFW^{-1} hr^{-1}$ or $\mu mol\ mgCHL^{-1} hr^{-1}$

Fishers multuple comparison tables:
- * . . difference significant (at 5% level)
- \- . . difference not significant

Section 3.2. BAP concentration.

Fig. 14:
a/ photosynthetic ability

source	DF	SS	MS	F	P
factor	6	21955	3659	35.68	0
error	22	2256	103		
total	28	24211			

BAP	PHS	0	0.002	0.02	0.2	2	20
0	122.2						
0.002	114.8	-					
0.02	123.4	-	-				
0.2	60.1	-	-	-			
2	68.3	*	*	*	-		
20	62.3	*	*	*	-	-	
200	65.4	*	*	*	-	-	-

a/ respiratory rate

source	DF	SS	MS	F	P
factor	6	63.6	10.6	0.83	0.557
error	22	279.6	12.7		
total	28	343.2			

BAP	PHS	0	0.002	0.02	0.2	2	20
0	21.5						
0.002	20.7	-					
0.02	23.8	-	-				
0.2	21.5	-	-	-			
2	25.4	-	-	-	-		
20	22.9	-	-	-	-	-	
200	22.0	-	-	-	-	-	-

Fig. 15: chlorophyll content

source	DF	SS	MS	F	P
factor	6	5.051	0.842	6.82	0
error	22	2.716	0.123		
total	28	7.768			

BAP	CHL	0	0.002	0.02	0.2	2	20
0	2.90						
0.002	3.08	-					
0.02	2.95	-	-				
0.2	2.52	-	-	-			
2	2.26	*	*	*	-		
20	2.15	*	*	*	-	-	
200	1.85	*	*	*	*	-	-

Fig. 16: carotenoid content

source	DF	SS	MS	F	P
factor	6	0.06783	0.01131	3.74	0.01
error	22	0.06652	0.00302		
total	28	0.13435			

BAP	CART	0	0.002	0.02	0.2	2	20
0	0.360						
0.002	0.368	-					
0.02	0.354	-	-				
0.2	0.255	*	*	*			
2	0.292	-	-	-	-		
20	0.266	*	*	*	-	-	
200	0.251	*	*	*	-	-	-

Fig. 17: chlorophyll a:b ratio

source	DF	SS	MS	F	P
factor	6	0.6051	0.1009	2.14	0.089
error	22	1.0365	0.0471		
total	28	1.6416			

BAP		0	0.002	0.02	0.2	2	20
0	2.29						
0.002	2.18	-					
0.02	2.27	-	-				
0.2	1.95	*	-	*			
2	2.15	-	-	-	-		
20	2.18	-	-	-	-	-	
200	2.41	-	-	-	*	-	-

Fig. 18: photosynthesis per unit chlorophyll

source	DF	SS	MS	F	P
factor	6	1323.3	220.5	14.18	0
error	22	342.2	15.6		
total	28				

BAP	PHS	0	0.002	0.02	0.2	2	20
0	42.2						
0.002	37.3	-					
0.02	42.3	-	-				
0.2	23.7	*	*	*			
2	30.4	-	-	-	*		
20	29.3	*	*	*	*	-	
200	36.9	-	-	-	*	*	*

Section 3.3. Sugar & light.

Media sugar concentration.

Fig. 19:
a/ photosynthetic ability

source	DF	SS	MS	F	P
factor	4	2912	728	2.05	0.125
error	21	7471	356		
total	25	10383			

S	PHS	10	20	30	40
10	92.0				
20	114.3	-			
30	113.0	-	-		
40	103.3	-	-	-	
50	88.3	-	-	-	-

a/ respiration rate

source	DF	SS	MS	F	P
factor	4	114.9	28.7	2.27	0.095
error	21	265.4	12.6		
total	25	380.0			

S	PHS	10	20	30	40
10	17.3				
20	23.0	*			
30	22.6	*	-		
40	19.4	-	-	-	
50	22.4	*	-	-	-

Fig. 20: chlorophyll content

source	DF	SS	MS	F	P
factor	4	0.9788	0.2447	4.54	0.008
error	21	1.1328	0.0539		
total	25	2.1117			

S	CHL	10	20	30	40
10	2.39				
20	2.69	-			
30	2.79	*	-		
40	2.94	*	-	-	
50	2.94	*	-	-	-

Fig. 21: carotenoid content

source	DF	SS	MS	F	P
factor	4	0.26325	0.005918	7.59	0.001
error	21	0.016374	0.000780		
total	25	0.040048			

S	CART	10	20	30	40
10	0.263				
20	0.317	*			
30	0.352	*	-		
40	0.349	*	-	-	
50	0.337	*	-	-	-

Fig. 22: chlorophyll a:b ratio

source	DF	SS	MS	F	P
factor	4	0.2811	0.0703	2.53	0.071
error	21	0.5827	0.0277		
total	25	0.8638			

S		10	20	30	40
10	2.01				
20	2.16	-			
30	2.35	-	-		
40	2.22	-	-	-	
50	2.18	-	-	-	-

Fig. 23: photosynthesis per unit chlorophyll

source	DF	SS	MS	F	P
factor	4	532.4	133.1	3.44	0.026
error	21	811.9	38.7		
total	25	1344.3			

S	PHS	10	20	30	40
10	38.5				
20	42.5	-			
30	40.5	-	-		
40	35.4	-	-	-	
50	30.1	*	*	*	-

Growth cabinert light intensity.

Fig. 24:
a/ photosynthetic ability

source	DF	SS	MS	F	P
factor	4	17148	4287	36.77	0
error	17	1982	117		
total	21	19131			

L	PHS mean	50	100	150	240
50	71.1				
100	109.8	*			
150	120.5	*	-		
240	63.0	-	*	*	
330	52.5	*	*	*	-

a/ respiration rate

source	DF	SS	MS	F	P
factor	4	51.0	12.8	1.14	0.373
error	17	190.9	11.2		
total	21	241.9			

L	PHS mean	50	100	150	240
50	17.3				
100	20.6	-			
150	18.3	-	-		
240	20.4	-	-	-	
330	17.0	-	-	-	-

Fig. 25: chlorophyll content

source	DF	SS	MS	F	P
factor	4	16.3697	4.0924	103.39	0
error	17	0.6729	0.0396		
total	21	17.0426			

L	CHL	50	100	150	240
50	1.88				
100	2.35	*			
150	3.10	*	*		
240	1.21	*	*	*	
330	0.79	*	*	*	*

Fig. 26: carotenoid content

source	DF	SS	MS	F	P
factor	4	0.249575	0.062394	166.65	0
error	17	0.006365	0.000375		
total	21	0.255940			

L	CART	50	100	150	240
50	0.221				
100	0.296	*			
150	0.368	*	*		
240	0.165	*	*	*	
330	0.078	*	*	*	*

Fig. 27: chlorophyll a:b ratio

source	DF	SS	MS	F	P
factor	4	2.1621	0.5480	16.94	0
error	17	0.5501	0.0324		
total	21	2.7422			

L		50	100	150	240
50	2.12				
100	2.40				
150	2.29	-	-		
240	2.28	-	-	-	
330	1.56	*	*	*	*

Fig. 28: photosynthesis per unit chlorophyll

source	DF	SS	MS	F	P
factor	4	2988.9	747.2	8.37	0.001
error	17	1517.5	89.3		
total	21	4506.4			

L	PHS	50	100	150	240
50	37.8				
100	46.8	-			
150	38.9	-	-		
240	53.6	*	-	*	
330	69.1	*	*	*	*

Media sugar concentration & growth cabinet light intensity.

Fig. 29:
a/ photosynthetic ability

at the highest light intensity in the sample chamber of the infra red gas analyser.

source	DF	SS	MS	F	P
factor	8	22780	2848	14.64	0
error	27	5251	194		
total	35	28031			

			50			30			10	
S	L	PHS	300	150	50	300	150	50	300	150
50	300	53.3								
	150	86.4	*							
	50	52.5	-	*						
30	300	79.3	*							
	150	120.8		*		*				
	50	65.3			-	-	*			
10	300	74.8	*			-				
	150	79.6		-			*		-	
	50	25.5			*			*	*	*

similar results were obtained at the other sample chamber light intensities.

a/ respiration rate

source	DF	SS	MS	F	P
factor	8	263.1	32.9	1.47	0.214
error	27	603.4	22.3		
total	35	866.4			

			50			30			10	
S	L	PHS	300	150	50	300	150	50	300	150
50	300	25.2								
	150	26.9	-							
	50	23.5	-	-						
30	300	25.5	-							
	150	21.5		-		-				
	50	24.6			-	-	-			
10	300	20.7	-			-				
	150	22.6		-			-		-	
	50	17.3			-			-	-	-

Fig. 30: chlorophyll content

source	DF	SS	MS	F	P
factor	8	16.3437	2.043	33.22	0
error	27	1.6602	0.0615		
total	35	18.0039			

			50			30			10	
S	L	CHL	300	150	50	300	150	50	300	150
50	300	0.775								
	150	2.213	*							
	50	1.742	*	*						
30	300	0.863	-							
	150	2.328		-		*				
	50	1.368			-	*	*			
10	300	0.773	-			-				
	150	1.948		-			*		*	
	50	0.487			*			*	-	*

Fig. 31: carotenoid content

source	DF	SS	MS	F	P
factor	8	0.35274	0.05882	20.24	0
error	27	0.05274	0.00218		
total	35	0.41155			

			50			30			10	
S	L	CART	300	150	50	300	150	50	300	150
50	300	0.124								
	150	0.346	*							
	50	0.292	*	-						
30	300	0.163	-							
	150	0.353		-		*				
	50	0.212			*	-	*			
10	300	0.135	-			-				
	150	0.199		*			*		-	
	50	0.059			*			*	*	*

Fig. 32: chlorophyll a:b ratio

source	DF	SS	MS	F	P
factor	8	2.531	0.316	3.01	0.015
error	27	2.836	0.105		
total	35	5.367			

			50			30			10	
S	L		300	150	50	300	150	50	300	150
50	300	2.14								
	150	2.47	-							
	50	2.45	-	-						
30	300	2.78	*							
	150	2.61		-		-				
	50	2.33			-	-	-			
10	300	2.29	-			*				
	150	1.97		*			*		-	
	50	1.94			-			-	-	-

Fig. 33: photosynthesis per unit chlorophyll

source	DF	SS	MS	F	P
factor	8	18269	2248	14.76	0
error	27	4177	155		
total	35	22445			

			50			30			10	
S	L	PHS	300	150	50	300	150	50	300	150
50	300	69.6								
	150	39.5	*							
	50	30.1	*	-						
30	300	93.7	*							
	150	52.8		-		*				
	50	48.0			-	*	-			
10	300	97.2	*			-				
	150	41.7		-			-		*	
	50	53.3			*			-	*	-

Section 3.5. flask cultures.

Fig. 39: carbon dioxide concentration.

at the end of the dark period.

source	DF	SS	MS	F	P
factor	8	10724819	1340602	10.17	0
error	44	5802834	131883		
total	52	16527653			

			50			30			10	
S	L	CO_2	300	150	50	300	150	50	300	150
50	300	1688								
	150	1682	-							
	50	707	*	*						
30	300	1058	*							
	150	1280		-		-				
	50	587			-	*	*			
10	300	652	*			-				
	150	582		*			*		-	
	50	352		-				-	-	-

similar results were obtained at other sampling times.

Fig. 40:
a/ photosynthesis *in situ*

source	DF	SS	MS	F	P
factor	8	1625.4	203.2	8.51	0
error	44	1049.9	23.9		
total	52	2675.4			

			50			30			10	
S	L	PHS	300	150	50	300	150	50	300	150
50	300	6.3								
	150	9.9	-							
	50	25.6	*	*						
30	300	8.6	-							
	150	16.6		*		*				
	50	18.2			*	*	-			
10	300	11.8	-			-				
	150	15.3		-			-		-	
	50	5.4			*			*	*	*

b/ respiration *in situ*

source	DF	SS	MS	F	P
factor	8	422.3	52.8	1.24	0.3
error	44	1873.9	42.6		
total	52	2296.2			

			50			30			10	
S	L	PHS	300	150	50	300	150	50	300	150
50	300	17.5								
	150	16.7	-							
	50	11.7	-	-						
30	300	16.1	-							
	150	14.9		-		-				
	50	12.2			-	-	-			
10	300	9.5	*			-				
	150	10.2		-			-		-	
	50	11.6			-			-	-	-

Fig. 46: fresh weight

source	DF	SS	MS	F	P
factor	8	0.5996	0.0749	4.37	0
error	44	0.7547	0.0172		
total	52	1.3543			

			50			30			10	
S	L	FW	300	150	50	300	150	50	300	150
50	300	0.431								
	150	0.507	-							
	50	0.373	-	-						
30	300	0.251	-							
	150	0.407		-		-				
	50	0.201			*	*	*			
10	300	0.251	*			-				
	150	0.275		*			-		-	
	50	0.148			*			-	-	-