Development and Characterisaton of an Inducible System of Betalain Synthesis in Cell Cultures of Beetroot (*Beta vulgaris*)

Thesis presented for the degree of

Doctor of Philosophy

by

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work

Signed: Vincent Mc Manus Date: 14.10.94

Candidate

Date:

ABBREVIATIONS

ABA	Abscisic Acid
Auf.	detection sensitivity(Absorbance units)
BAP	6-Benzylaminopurine
B5	Gamborgs B5 medium, Gamborg O.L. *et al.* 1968
2,4-D	2,4-Dichlorophenoxyacetic Acid
cm	centimetre(10^{-2} metre)
°C	degrees centigrade
DOPA	L-ß-3,4-Dihyroxyphenylalanine
gfr. wt.	gram fresh weight
g	relative centrifugal force
GA	Gibberellic acid
HPLC	High Performance Liquid Chromatography
i.d.	internal diameter
i.e.	that is
dm^3	litre
L & S	Linsmaier & Skoog medium, Linsmaier E.M. and Skoog F. 1965
mg	milligram
min	minute
cm^3	millilitre
mm	millimetre
mM	millimolar
M & S	Murashige & Skoog medium, Sigma Cat.N. M5524
mol/m/s	mol/metres/second
nm	nanometre(10^{-9})
nmol	nanomole
%	percentage
Pgo/PGo	Pgo medium, Degreef W.& Jacobs M. 1979
pH	$\log_{10}$ of the hydrogen ion concentration
T.L.C.	Thin Layer Chromatography
µ	micro(10^{-6})
v/v	volume per volume
W	Watt

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ABSTRACT

Development and Characterisation of an Inducible System of Betalain Synthesis in Cell Cultures of Beetroot (*Beta vulgaris*)

by

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The subject of this thesis was the development and characterisation of an inducible system for the production of betalains by red beet cultures. This was to be used as a model system to investigate the control of secondary metabolism in tissue culture. White lines of callus cultures of red beet, grown on solidified PGo salt cocktail medium containing 3% sucrose, 0.1mg/dm^3 2,4-D, 0.5mg/dm^3 kinetin were subjected to different light quantities and qualities, temperature and nutrient variations. In addition the effect of precursors was examined, feeding shikimic acid, tyrosine and DOPA in an attempt to see whether or not the control point could be detected from the biosynthetic pathway. It was also hoped to feed betalamic acid and cycloDOPA glucoside. However this necessitated the purification of standards in sufficient quantities so as to obtain these intermediates that did not exist commercially. This involved investigating different liquid chromatographic supports; Sephadex G-15, Dowex H^+ and cellulose(20μ). HPLC was also examined as a method to obtain standards.

The results demonstrate the inducing effect of light and temperature. However this induction was characterised by a heterogeneous appearance. This result was considered best for the study of a heterogeneous response but not for the study of control of secondary metabolism.

The feeding of precursors revealed that control was either further down the pathway at the intermediate betalamic acid or that each step of the biosynthetic pathway is turned on in concert in response to a signal.

CHAPTER 1. INTRODUCTION

The preoccupation of this research project was the study of the regulation of secondary metabolism in plant tissue culture. Plant tissue culture is the branch of science which investigates biochemical, physiological and developmental aspects of plant material under aseptic conditions. Secondary metabolites are those compounds produced by plants which are not essential to the maintenance and survival of individual cells but which may be necessary to the survival of the plant as a whole or which may perform some specialised function (Walton N.J. 1992). These secondary metabolites range from inactive dyes, cosmetics, fragrances, latex to biologically active drugs eg. morphine, vincristine (Hunter C.S. & Kilby N.J. 1990, Nippon-Kayahu 1990, Misawa M. 1980). Also included is the ability of the plant cell to biotransform a given product to another one by a series of complex chemical reactions too difficult to be done in the laboratory (Misawa M. 1980, Kurz W.G.W.& Constabel F. 1979). After production they are then secreted out into the environment or stored in the vacuole (Wink M. 1993). However, to a large extent, study to date has taken an empirical approach based on the end result being more important than an understanding of how the end result was obtained. The empirical approach has yielded some success (Van der Heiyden R. *et al.* 1988) but the overall outcome is that the scientific promises have not materialised for the industrial entrepreneur (Banthorpe D.V. 1994).

This was manifested by several phenomenon, either a high producing cell line could not be maintained (Deus-Neumann B. and Zenk M.H. 1984), a cell culture producing low amounts of secondary metabolite could not be enhanced, the fermentation technology used for yeast and bacteria was not appropriate or the producing cell culture was growing too slowly. However it is moreover the first two observations that have been the major stumbling blocks (Crawford D.L. *et al.* 1990).

It was initially thought that secondary metabolism was an integral part of morphological differentiation. This was based on the observation that some secondary metabolites only occurred in certain tissues, for example the occurrence of cardiac glycoside in the leaves of *Digitalis*, quinine and quinidine in the bark of *Cinchona*. The implication of this would have been that plant tissue culture would have been a dead end as a means of producing scarce and valuable secondary metabolites. That this may not be so is indicated by experiments on celery cultures producing the phlthalide flavouring. These were examined at different levels of differentiation from undifferentiated cells to torpedo-like embryos by Al-Abta and Collin (1978/1979). These results showed that the production of phlthalide was linked to chlorophyll development and not to morphological differentiation of the embryo (Whitaker R.J. and Evans D.A. 1985).

Another example of independence of morphological differentiation and secondary product synthesis this is where essential oils of *Ocismum basilicum* accumulate in the glycoside form and in the case of *Matricaria chamomilla* α-bisabolol is produced if a lipophilic layer is provided (Pétriard V. 1987). Previous to this it was believed that these metabolites could only be produced in the presence of lacticifers. Once this was realised attempts to turn on the biosynthetic pathway ensued.

A number of approaches were examined. As mentioned before high-producing cell lines were sought but these could not be maintained. In other cases it was found that using other plant sources in culture for the production of certain secondary metabolites was more effective than using the most obvious source. As an example, it was found that *Fedia cormicopiae* was better for the production of valepotriates than *Valeriana* species (Kurz W.G.W. and Constabel F. 1985). Other problems remain. Once groups have managed to get stable high producing cell lines, the qualitative nature of the production of secondary metabolites has to be assessed. For example quinine is the main alkaloid in *Cinchona ledgeriana* whereas in tissue culture two other alkaloids are produced; quinidine and cinchonine. In other cases the main secondary metabolite in *Cinchona* is anthraquinones and not alkaloids. To compound matters it has been found that a slight change in protocol manipulations between two laboratories can result in the change of cell characteristics from being a non-producing to a producing cell line (Whitaker R.J. and Evans D.A. 1985, Kurz W.G.W. and Constabel F. 1985). In other cases the secondary metabolite is present but only at very low levels. This results in a need for sensitive assays. These assays have revealed that the desired secondary metabolites are present in the expected model systems and that it is a question of increasing production (Rhodes M.J.C. 1994). It was eventually realised that the underlying cellular mechanisms were not being examined. These results highlight the problems associated with the empirical approach. Consequently no commercial progress could, on looking back, be made.

However techniques in plant cell culture have improved and a clearer vision of what is required has been obtained. This consists of selecting a stable, high-producing cell line growing optimally. To pursue this method is based on the fact that who is to say that the normal environmental and developmental state of the plant is optimum for secondary metabolism production (Pétiard V. 1987). This slightly more methodical approach resulted in the isolating of stable high nicotine-producing cell lines of *Nicotiana tabacum* and anthocyanin-producing cell lines of *Euphorbia millii* (Whitaker R.J. and Evans D.A. 1985). Despite this Hüsemann W., Amino S., Fischer K., Herzbeck

H. and Callis R. 1990 p373) have found that physiological and biochemical studies has furnished valuable information for the better understanding of basic questions about photoautrophy. The implication for secondary metabolism is a change in emphasis to an elucidation of what is happening at the cellular level. This change in direction has turned out to be a need for a fundamental study on differentiation of function of a plant cell (Dixon R.A. and Lamb C.J. 1990, Schmauder H.-P. and Doebel P.1990).

An initial analysis has revealed that secondary metabolism in linked to the general metabolism of the cell in a more complex way that was at first envisaged (Nessler C.L. 1994) especially if it is known that 12 different secondary pathways are known to operate within a cell (Rhodes M.J.C. 1994). An example of this is the terpenoid indole alkaloid biosynthetic pathway. Strictosidine, the universal precursor of all the known 3000 terpenoid indole alkaloids, is produced by the enzyme driven amalgamation of tryptamine and secologanin which are both indirectly derived from primary metabolism via highly regulated enzymes (Verpoort R. *et al.* 1993). Consequently when considering inducing factors such as light, temperature, the presence of phytopathogens, wounding and nutrient supply one must also be aware of other not so obvious criteria. These include, in addition to other enzyme pathways, stage of culture; it has been found that production of secondary metabolites may occur during the exponential phase (Hirano H. and Komamine A. 1994, Hirose M. *et al.* 1990) as opposed to during the stationary phase. Other factors include genetic stability (Kurz W.G.W. and Constabel F. 1979) which can influence the production levels during maintenance of cultures, internal cell compartmentation (Stevens L.H. *et al.* 1993) and aggregate size within a culture (Hanagata N. *et al.* 1993). Otherwise low producing cultures such as *Papaver bracteatum* producing the morphine alkaloid thebaine at 0.013% of dry weight will not improve.

This does not mean that there have been no successes to date. The most famous example of a commercial success is that of shikonin, a red naphthaquinone dye produced by Mitsui Petrochemical Ltd. in Japan using *Lithospermum erythrorhizon* (Ilker R. 1987). Kobayashi Y. *et al.* 1993 appear to have achieved a 500l pilot-scale jar fermenter production of anthocyanin by *Aralia cordata* without the use of light. Verpoorte R. *et al.* 1992 reviewed the current range of secondary metabolites that are either on the brink of commercial viability or are intensively studied

The plant cell's ability to be totipotent does not however permit it to do all things at the one time. Research to date has shown that for many activities the cell must grow first and attain a certain state before carrying out another activity (Curtin M.E. 1983).

This has meant that when using plant tissue culture systems for the production of secondary metabolites one has to produce the plant biomass first then induce secondary metabolism production by changing the culture conditions whether it be physical or chemical. Chattopadhyay S. *et al.* 1994 found that a higher accumulation of L-DOPA in *Mucuna pruriens f. pruriens* could be obtained using a two stage as opposed to a one stage culture cycle. It has been found that success in using plant tissue culture for producing compounds of commercial interest can best be obtained by a fundamental study on the immediate biosynthetic pathway of the secondary metabolite and its relationship with other synthetic pathways (Bokern M. *et al.* 1991). This is turn will permit tracing back to a genetic level which ultimately will allow us control over the regulation of the required synthetic pathway (Murray J.R. *et al.* 1994).

Plant tissue culture at present can be seen to be developing on two fronts 1. improving production technology for those cell cultures that are known to produce a desired secondary metabolite within cost efficient limits determined by the market place. 2. obtaining/improving secondary metabolite producing cell lines.

An assessment of commercial production can be preceded by 2 questions; (a.) can plant cells be grown in large amounts, (b.) is the means of production competitive in the existing market place. The first question can be answered positively (Verpoorte R. 1993). The second question can be answered by saying that the market price for the product must be high. This is due to the high cost of production resulting from large depreciation incurred due to the long production time.

The other developing front is the improving/obtaining of high producing cell lines. The bottom line for this facet will be that the cell line produce the same or superior levels of the secondary metabolite than the plant source. As said before this can be obtained by selecting for high producing cell lines. The other method, which will be more at the commercial level, is optimising the growth and production medium. This should be done at a fermenter level as one must bear in mind that the gas phases in a shake flask is completely different to that of a bioreactor (Verpoorte R. *et al.* 1993).

The approach of selecting for stable high producing cell lines has to date not yielded much success despite the fact that in the whole plant large amounts of the desired metabolite can be produced. This gap, as said before, has boiled down to a need to understand the regulation of product accumulation.

When examining regulation one must be aware of three facets A. *de novo*/enhancement biosynthesis of the product B. compartmentation of the product if it is not being excreted C. breakdown of the product. For the moment research appears to be

concentrating to a large extent on the first facet.

De novo synthesis/enhancement of the product will entail enzymes which need to be elucidated first if unknown. The elucidation and purification of enzymes may be problematic as in some cases the enzyme may belong to a series of isoenzymes which give rise to a series of products. In other cases the enzymes are specific for certain stereochemical positions. Once the enzyme has been found via electrophoresis, a range of chromatographic columns etc. and purification achieved, the genes to these enzymes can be cloned out. This will include determining the amino acid sequence which allow the construction of DNA/RNA probes so as to clone out the gene. It must be remembered that the last step is not as straight forward as it seems since initial DNA coding sequences can undergo major changes during passage from the nucleus outside to the cytoplasm and at the translation level into mRNA and finally protein synthesis. Once the gene(s) have been cloned out the genetic engineers can start work on promoting certain aspects of the cell metabolism. This will save greatly the time that has been taken up by conventional breeding. However one must make certain that increasing secondary metabolism will not interfere with the normal primary metabolism and associated growth of the cell.

There are a number of techniques of increasing secondary metabolism that can be envisaged once the gene(s) coding the enzymes of the pathway have been found. It is conceivable to insert a promoter-gene of enzyme construct into other model systems which may have attributes conducive to increased production. This is particularly the case of yeast and bacterial systems which have mechanism of excretion in the medium. The insertion of these promoter-gene constructs can be envisaged readily by the already common occurrence of promoter-reporter genes which have viral and bacterial origins (Nesseler C.L. 1994).

The method envisaged, at the present time for either inducing or at least increasing secondary metabolism by using different elements from foreign model systems, will depend on the problem involved. In some cases an enzyme is either missing or incapable of continuing a reaction in a certain direction. An example of this in where it is possible to transfer stilbene synthase gene from peanut to tobacco thus allowing the producing of the phytoalexin resveratral (Nessler C.L. 1994). However the problem with this approach is that one is changing the environmental conditions of the foreign gene. As a result it may develop into a phytotoxic situation and in the case of phytoalexins which are considered as a defense mechanism, the secondary metabolite may lose its desired activity. Another method for increasing secondary metabolite accumulation is

to target an enzyme to a specific subcellular compartment. However this is subject to the appropriate substrate being available. A third method that is being investigated is to redirect the normal flow of metabolite to or from the synthesis of the desired secondary metabolite. This will depend on how closely the secondary metabolism is linked to primary metabolism and how the overall pathway is regulated. In addition one will need to decide whether it is better to push the pathway by overexpressing an enzyme at the beginning of the pathway or pull by increasing the expression of an enzyme at the end of the pathway (Nessler C.L. 1994). The only possible obstacle to this approach is the extent to which the cell can accumulate elevated levels of a metabolite if it is not excreting it.

Increasing secondary metabolism accumulation can occur indirectly in the case where there is a drain on precursors or intermediates of the biosynthetic pathway. This can be achieved by using antisense RNA which would be complementary to the mRNA encoding for the enzyme causing the drain on the wanted biosynthetic pathway. This inhibition of certain enzymes can also occur via co-suppression. This method was accidentally discovered and is where an enzyme is overexpressed by the insertion of a second copy of the gene. The net result is the unexpected suppression of both the endogenous and the inserted second copy of the gene. This technique is still in a very early stage of development as the actual mechanics of action is not understood (Nessler C.L. 1994).

A more direct approach would be to control transcription via the expression of transcriptional activators. This, as said before, will entail a thorough understanding and elucidation of the enzymes of the pathway and the associated co-factors if there are any. This information may permit the production of secondary metabolites in cells that do not normally produce. This will be particularly useful in model systems where there is a heterogeneous response to induction. One is making the assumption that genetically engineering an increase/overexpression of such transcriptional factors will be passed on during cell division in culture. Thus it can be hoped that controlling the amount of transcriptions factors will permit the *ad libitum* command of where, when and how much of the secondary metabolite is to be produced

The most effective method for the study of such pathways is where the biosynthetic pathway is inducible (Whitaker R.J. and Evans D.A. 1985, Whitehead I.M. and Threlfall D.R. 1992). This is desirable as one wants to be able to detect changes in precursor and intermediate levels, enzyme activities, DNA and RNA developments (Murray J.R. *et al.* 1994). Relationship can thus be detected with greater ease and precision. The other

possibility is that of using constitutive pigment producing and non-producing cell lines (Zrijd J.-P. *et al.* 1982). However in such systems it is difficult to separate the relationship between cause (induction) and effect(secondary metabolite production) from other variables not related to pigment production. Examples of inducible systems include carotenoids, phycobilins (Jackman R.L. and Smith J.L.S. 1992) and phytoalexins (Whitehead I.M. and Threlfall D.R. 1992).

In conjunction with the need for an inducible system a number of additional criteria will dictate the choice of model system with respect to ease of experimentation. The more important of these aspects include that the secondary metabolite be pigmented, have a short and known enzyme pathway and produce a small number of easily quantified stable products. It was found that betalain production in red beet cultures fitted the required constraints.

Since a model system is required, that is the secondary metabolite does not have to be important in itself, it was considered best that the metabolite be a pigment/biochrome as it would facilitate measurement during experiments (Ellis D.E. 1988). These are chemical compounds absorbing specific wavelength of visible light. They should not be confused with structural colours (Hari R.K. *et al.* 1994), the predominant ones being tyndall blue colours(resulting from light scattering by very small particles eg: human eye, birds feathers) and iridescent colours(produced from interference with light by thin films or laminations eg: insects, fishes. Neither of the latter colours are extractable. There are a number of pigments that could possibly be studied: chlorophyll, carotenoids, flavonoids, phycobilins, betalains, melanins, anthraquinones, naphthaquinones, xanthophylls, anthocyanins (Hari R.K. *et al.* 1994). To help narrow down the choice other considerations had to be taken on board.

The pigment must be stable so as to measure the synthesis accurately. Pigment stability can be created by the plant cell or functional groups of the pigmented molecules called auxochromes (Hari R.K. *et al.* 1994) which preserve the excited state. This is achieved by the resulting increase in molecular size, the presence of aromatic ring system which permits maximal delocalisation of π - electrons thus leading to high-resonance energy and thermodynamic stability. One cannot afford to have a quick-sand situation where accumulation = synthesis minus degradation (Verpoorte R. *et al.* 1993). Degradation can occur during the culture stage as well as during extraction. A good indication of what pigments are stable outside the cell can be obtained from an industrial producers catalogue of pigment products (Overseal Ltd.). As for an idea of pigment stability in tissue culture no real work appeared to have been carried out.

The enzyme pathway must be elucidated. Studies on the control of secondary metabolites that have been successful, are characterised by the pathway being elucidated eg. anthocyanins. This will allow precursor and intermediate feeding studies to be backed up by enzyme activity and genetic studies so as to find the rate limiting step (Toguri T. *et al.* 1993). This will allow subsequent exploitation (Mol J. 1991, Gantet P. and Dron M. 1993).

It would be helpful if the pathway was short. This will limit the number of sites of regulation along the biosynthetic pathway. In addition other biosynthetic pathways could be a drain on the pathway under study by using precursors or intermediates of the former pathway if the latter pathway was long(isoprenoid biosynthetic pathway p146 Jackman R.L. and Smith J.L. 1992). This would complicate the interpretation of results. If one looks at figure 1.1 there are two main precursors before major changes take place. The only potential interfering biosynthetic pathway recorded in betalain-producing model systems is catecholamine biosynthesis in *Portulaca* callus (Endress R. *et al.* 1984). This would involve tyrosine and DOPA being diverted to form tyramine and dopamine.

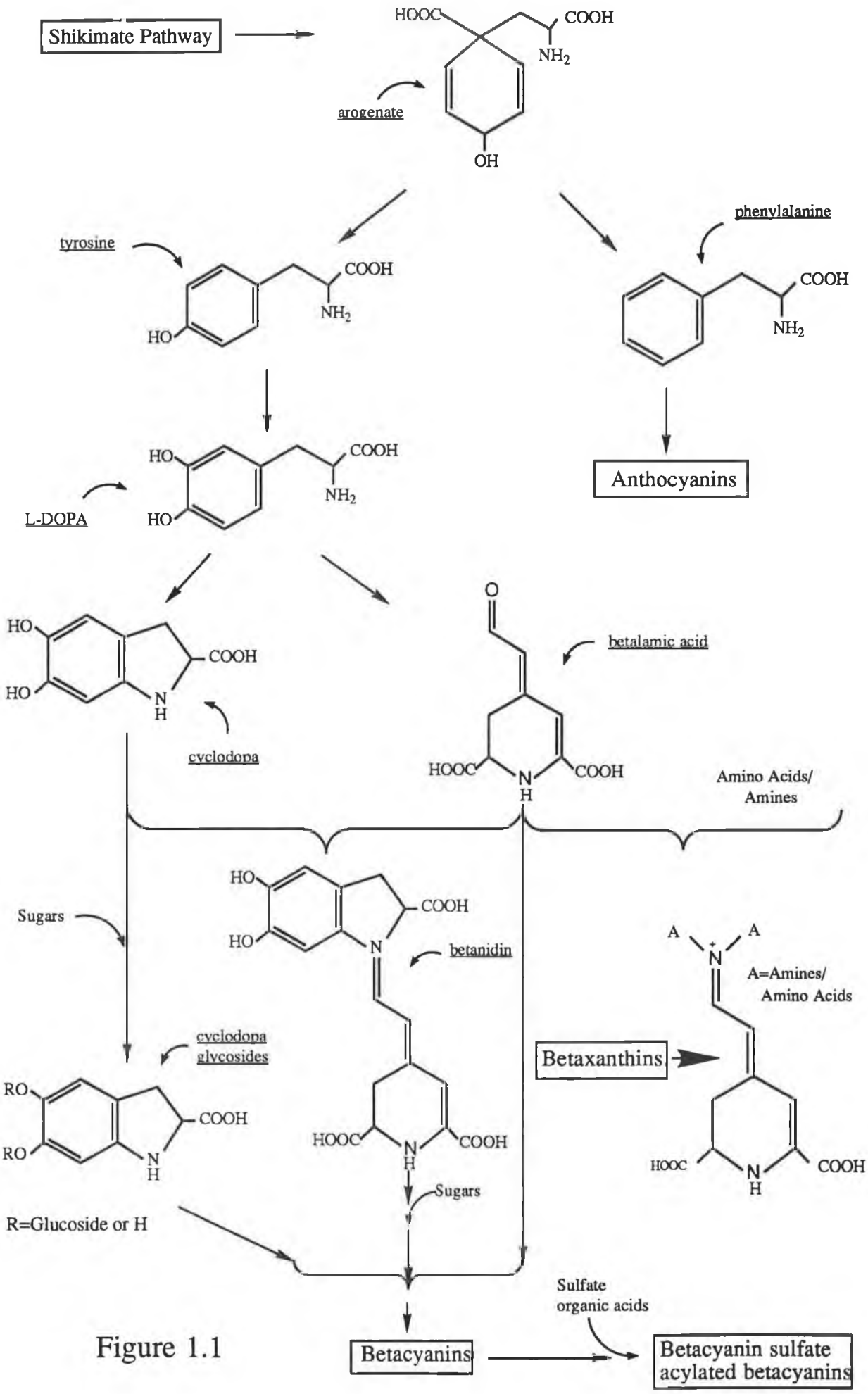

Shikimate Pathway
HOOC
COOH
NH2
arogenate
OH
tyrosine
COOH
NH2
HO
phenylalanine
COOH
NH2
Anthocyanins
HO
COOH
NH2
L-DOPA
HO
HO
COOH
N
H
cyclodopa
O
betalamic acid
HOOC
N
H
COOH
Amino Acids/
Amines
Sugars
HO
COOH
HO
N
betanidin
A
A
N
A=Amines/
Amino Acids
cyclodopa
glycosides
Betaxanthins
RO
COOH
RO
N
H
HOOC
N
H
COOH
HOOC
N
H
COOH
Sugars
R=Glucoside or H
Sulfate
organic acids
Betacyanins
Betacyanin sulfate
acylated betacyanins

Figure 1.1

In the case of betalain biosynthesis a number of steps are still a cause for investigation. The enzyme that catalyses DOPA to cyclodopa remains undetermined. The condensation of betalamic acid with an amino acid or amine is uncertain. One does not know if it is spontaneous or driven enzymatically. The time of glycosylation of betacyanin is still unsure, that is, it is not known whether glycosylation occurs at the betanidin level or at the cyclodopa level. For the moment it is thought that both processes can occur, which one depending on the species being studied (Steglich W. and Strack D. 1990).

It would be useful if the number of possible products be limited. This is to limit the amount of time in measuring the effect of induction experiments by having to analyze each individual product. It is probable that the more the number of products the likelihood of the need for increasingly sophisticated methods of analyze which would take up time. For example in one plant of *Catharanthus roseus* over 100 indole alkaloids can be isolated or flavonol quercitin forming as many as 80 different glycosides in plants (Rhodes M.J.C. 1994). When comparing the different pigment groups it was found that betalain synthesis in *Beta vulgaris* was best.

In the case of beetroot there are two pigments; the violet betanin(figure 1.2)

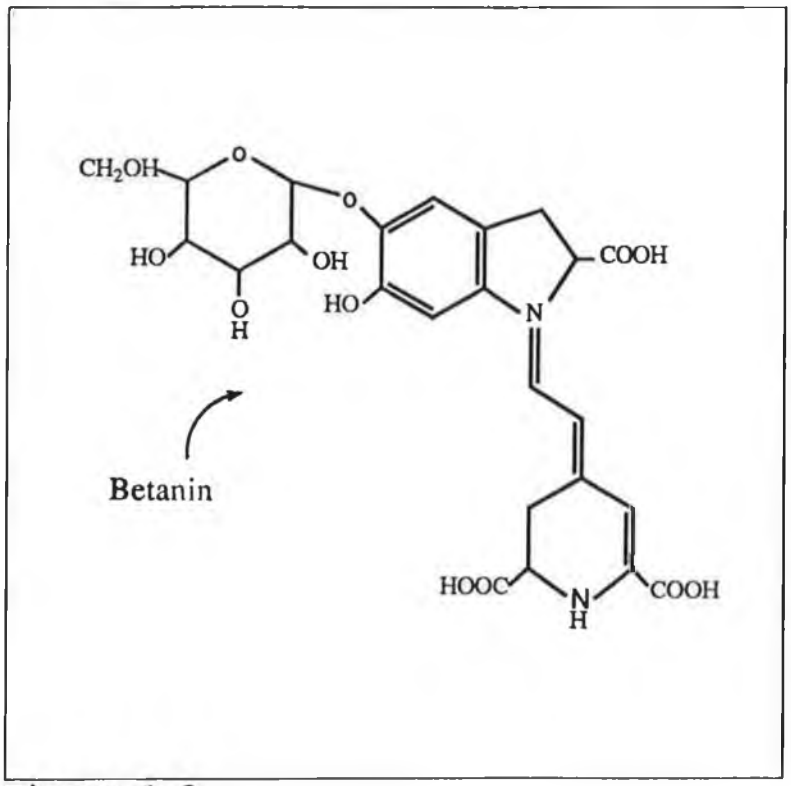


Figure 1.2

which accounts for 95% of total pigment and the yellow vulgaxanthin I(glutamine) and vulgaxanthin II(glutamic acid) (Figure 1.3).

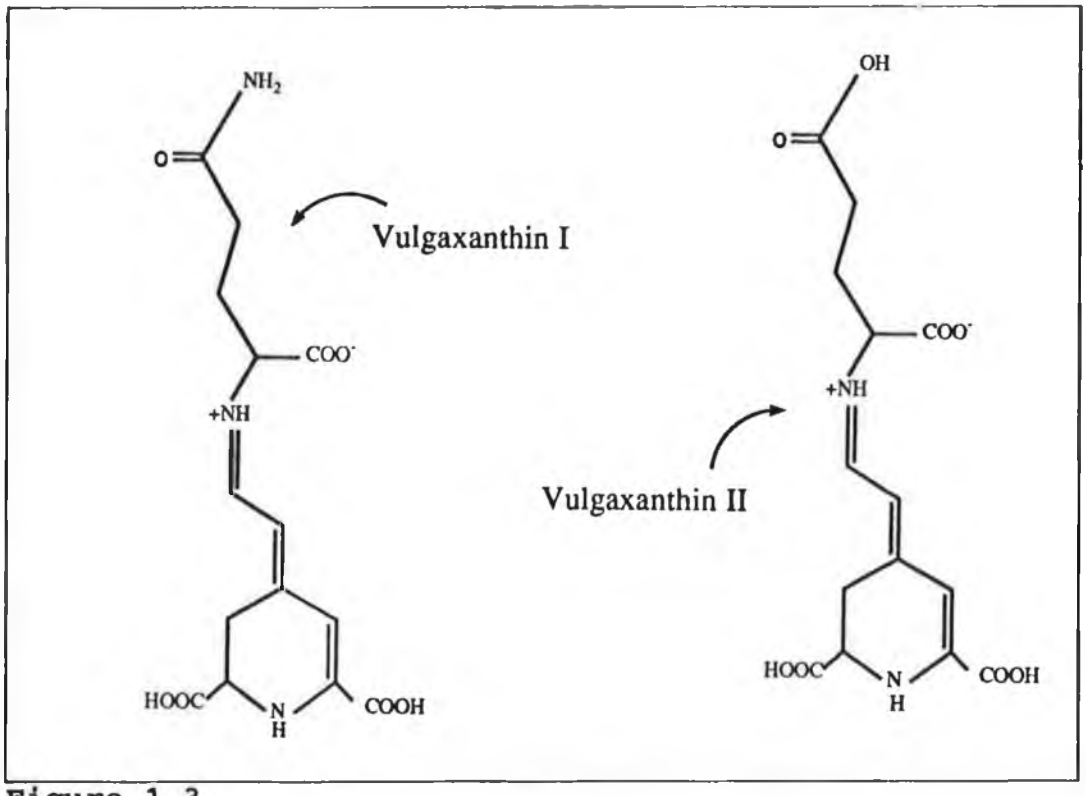


Figure 1.3

Additional research has shown that small amounts of other pigments can be produced by a given culture system. Strack D. *et al.* 1987 found that humilixanthin could be detected in yellow-coloured root of *Beta vulgaris*. This means that in cell culture other pigments, not normally associated with the studied system, could be synthesized. Bokern M. *et al.* 1991 detected the presence of lampranthin II(figure 1.4) in cell cultures of *Beta vulgaris*.

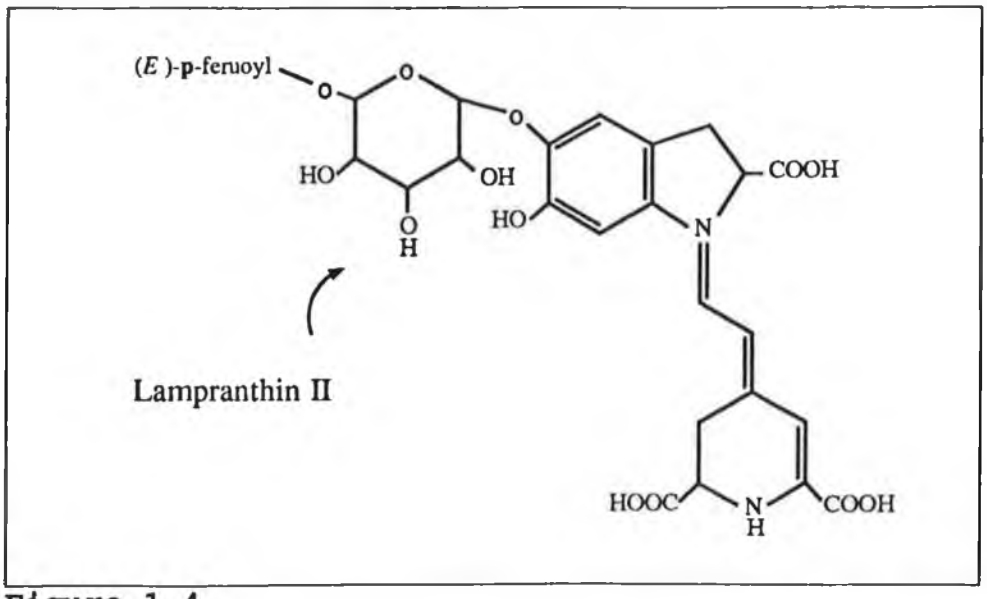


Figure 1.4

A spectrophometric method exists (Nillson T. 1970) for the measurement of betalains. This, in conjunction with a mathematical formula (Saguy I. *et al.* 1978 a,b), enables the calculation of both pigment absorbances in the one solution in addition to that of betalamic acid, a key intermediate. HPLC methods exist for more accurate determination (Forni E. *et al.* 1992, Attoe E.L. and vonElbe J.H. 1981, Schwartz S.J. and von Elbe J.H. 1980, Drdák M. and Vallová M. 1990, Strack D. and Reznik H. 1979, Strack D. *et al.* 1981, Adachi T. and Nakatsukasa M. 1983, Biano-Colomas J. and Hugues M. 1990, Huang A.S. and vonElbe J.H. 1985, Schwartz S.J. and vonElbe J.H. 1983, Watts A.R. *et al.* 1993).

As in a lot of research work where a substance is being examined a standard must be available for the purposes of a standard curve. If the substance is not produced commercially it must be obtained quite readily and in sufficient amounts to give purified standards. The advantage of betalains is that it can be obtained relatively easily from beetroot. Literature indicates that the pigments can be obtained via a mixture of Sephadex G-15, G-25 and cellulose columns, ion exchange, extractive fractionation and fermentation (Bilyk A. 1979, 1981, Colomas J. 1977, Adam J.P. *et al.* 1976, Adams J.P. and vonElbe 1977, Pourrat H. *et al.* 1983).

For the project to be able to concentrate on its objective it is best that protocols for callus and suspension cultures be worked out. Considerable time is needed to define the medium for a culture as salts, growth substances and sugar contribute to influence the morphology of the culture. The required culture morphology would be of friable form. The successful conditions however will vary depending on what species and/or variety is being studied. Literature surveys seemed to indicate that the appropriate material could easily be obtained (Bokern M. *et al.* 1991, Hunter C.S. and Kilby M.J. 1988, 1990, Janes D.A. 1988a, 1988b, Weller T.A. and Lasure L.L. 1981, Girod P.-A. and Zryd J.-P. 1987, 1991, Ripa PV. and Adler J.H. 1987)

Finally a commercial impetus will add weight to pursuing research in the area. It could even be a source of funding especially in times of financial constraints. In the case of betalains they are used as food colorants. As early as the end of the 19th century juice of pokeberry was added to wine to import a more desirable red colour (vonElbe J.H. 1977). Now it is used to a limited extent as they are heat and light labile (Pigment product brochure of Overseal Food Ltd. 1991). They are thus used in dairy products, ice creams, dry mixes, sugar confectionary, salami-style sausages, poultry, soft drinks (Altamirano R.C. *et al.* 1992, Jackman R.L. and Smith J.L. 1992, Havlíková L. *et al.* 1985, Pasch J.H. *et al.* 1975, Pasch J.H. *et al.* 1973).

At the moment betalain pigments can be obtained relatively cheaply by extracting from field crops. However bulk extraction in still somewhat inefficient spurring continued research in this process (Wiley R.C. and Lee Y.-N. 1978, Wiley R.C. *et al.* 1979, Abeysekere M. *et al.* 1990). But who is to say that fields devoted to producing dyes can be tolerated with increasing population and pollution of the worlds food producing zones!

Beetroot pigments appears to have attracted interest as early as 1876 when Bischoff emphasized the peculiarities of these anthocyanin-like plant pigments that contain nitrogen but are otherwise similar (Reznik H. 1981, vonElbe J.H. 1977). Further work on extraction occurred in 1937 with Pucker G.W., Curtis L.C. and Vickery H.B. A history of the development of research with respect to the characterisation and elucidation of structure can be seen in Piattelli M. 1976, Steglick W. and Strack D. 1990.

The literature can be divided into three sections:

1. the use of betalain production as a bioassay looking at the effect of light, growth substances,etc.(seedlings often used.)
2. using callus and suspension culture to produce betalains
3. studies on the viability of betalains as a food colorants

Jackman R.L. and Smith J.L. 1992 reviews studies carried out on betalains so as to determine their use as food colorants. Results showed that betalains were sensitive to various sources of radiation whether it be U.V., visible or gamma radiation (Sapers G.M. and Hornstein J.S. 1979, 1981, Attoe E.L. and vonElbe J.H. 1981, Aurstad K. and Dahle H.K. 1973), can be influenced by oxygen (Attoe E.L. and vonElbe J.H. 1982), organic acids, metal cations, sequestrents (Pasch J.H. and vonElbe J.H. 1979), is affected by water (Drdák M. *et al.* 1990, Pasch J.H. and vonElbe J.H. 1975, Altamirano R.C. *et al.* 1992) and can undergo severe degradation in heat (Saguy I. *et al.* 1978, 1979, Aronoff E.M. and Aronoff S. 1948, Aronoff S. and Aronoff E.M. 1948). Decolorization can also result from polyamines (Smith T.A. and Croker S.J.1985) and traces of heavy metals (Czapki J. 1990). However regeneration can spontaneously occur with time (von Elbe J.H. *et al.* 1981, Huang A.S. and vonElbe J.H. 1985) and can be promoted using isoascorbic acid (Bilyk A. *et al.* 1981, Bilyk A. and Howard M. 1982). This situation persists if decolorizing enzymes are not present. These degrading enzymes are usually cell bound with optimum activity at pH 3.5 at 40°C (Lashley D. and Wiley R.C. 1979, Zakharova N.S. *et al.* 1989, Wasserman B.P. and Guilfoy M.P. 1983, Shih C.C. and

Wiley R.C. 1981). Other characteristics of these pigments are that they are insoluble in organic solvents (vonElbe J.H. and Maing I.Y. 1973), they migrate as anions (Nilsson T. 1970, Piattelli M. 1976) and have different absorption maxima at different pH (vonElbe J.H. 1977, Savolainen K. and Kuusi T. 1978). The sugar moiety causes the glycons betacyanins to be hydrophilic resulting in the need to store under dry conditions or use dextrin (von Elbe J.H. 1977, Hendry B.S. 1992).

To date there have been several approaches taken to decipher the regulation of betalain production. On one hand the use of seedling and/or plant parts from *Beta vulgaris, Amaranthin* species and *Celosia* species and on the other hand the use of tissue culture systems in particular callus and suspension using *Chenopodium rubrum, Beta vulgaris, Phytolacca americana* and *Portulaca grandiflora.* In both areas of research various environmental factors have been employed to induce/inhibit pigment production with varying results depending on the species tested. However one notices that enzymology was hardly ever worked on. It is only in the last 10 years that a drive has been made to isolate and characterise the individual enzymes and understand the biosynthetic pathway. As a result little research has been carried out on the genetic front. Information to date can be summed up by saying pigment production is controlled by a triallelic system at the R locus having a complex structure with incomplete dominance (Wolyn D.J. and Gabelman W.H. 1989) on chromosome II (Butterfast 1968 see Piattelli M. 1981). This is somewhat confirmed by studies on grain amaranthus (Kulakow P.A. 1987). In this case embryo colour is controlled by two complementary epistatic genes with one locus determining the presence or absence of pigment and the other locus regulating the expression of pigment. These genetic studies seem to indicate that the proposed biosynthetic pathway (Fig. 1.1) is valid. The only other species of plant on which genetic analysis of betalain synthesis has been carried out is *Portulaca grandiflora* by Adachi during breeding experiments in Japan (Piattelli M. 1981). It is this type of work in conjunction with enzyme turnover studies including post-translational control that will result in a thorough understanding of the regulation of the overall process (p40 Mothes K. *et al.* 1985).

A good summary of studies on plant parts under varying environmental conditions can be found in Piattelli M. 1981. He divides these experiments into light and chemical induction. Since light is the easiest and neatest inducer to obtain and manipulate, a lot of work has been carried out. There are a number of general results:

1. that light is essential for those plants that do not produce pigment in the dark and will enhance production in those that can. Colomas J. 1975 demonstrates this by

removing the seed coats from the emerging cotyledons of *Amaranthus tricolor*.

2. that there may be two photoreceptors one in the red and another in the blue (Kochhar V.K. *et al.* 1981b, Göring H. and Dörfler M. 1981) and that cytokinin levels are increased. Obrenovic S. 1985, 1986, 1988, 1990 has looked at how light effect is mediated (Cu^{2+}/Mg^{2+} chelators, riboflavin, cyanide, SKF S25-A, rotenone, Cu(II) d-penicillamine). It was found that riboflavin acting on phytochrome and cyanide on flavins indicated that blue light-induced betacyanin formation does not occur via a separate reaction chain other than the phytochrome system contrary to previous assumptions. This is reiterated when the effect of Cu(II) d-penicillamine was examined. It was also found that Ca^{2+} ions do not play a role in the reaction chain conducting the light stimulus. Obrenovic had previously looked at the effect of purines (1983) and found that feeding purine could not selectively distinguish blue from light induction.
3. that photosynthesis may be the most important factor governing pigment synthesis (Giudici de Nicola M. *et al* 1972). However, any explanation for the regulation of pigment synthesis must account for pigment production in the dark.

Studies on the effect of precursors in the presence of light indicate that both precursors tyrosine and DOPA caused an increase in pigment production in hypocotyls of *Acetabularia mediterranea* (Guruprasad K.N. and Laloraya M.M. 1976). Zryd J.-P. *et al.* 1982 suggests that there could be compartmentation with respect to tyrosine and DOPA and that, for betanin production in cultures of red beet, tyrosine is better incorporated into the upper part of the betacyanin molecule and DOPA the betalamic part.

Bianco-Colomas J. 1980 indicates that control could be involved at the point of DOPA supply. This observation was again noted by Rink E. and Böhm H. 1985 who also shows that in leaves of *Portulaca grandiflora* the majority of fed DOPA is converted to betalamic acid (backing Zryd J.-P. *et al.* 1982). Control will eventually be revealed when a systematic comparative study of all precursors and intermediates is carried out. This can only occur when the enzymatic pathway has been elucidated (Bokern M. *et al.* 1991a, Knorr D. *et al.* 1993). To date studies have been based on tyrosine to DOPA.

Piattelli M. 1981 also examines the effect of chemical induction on particular plant growth substances. One of the major obstacles with growth substances is that they have such a wide variety of effects, some of which overlap. The general result is that kinetin can replace the effect of light where a process has an absolute light requirement and can

further stimulate dark existing processes (Wohlpart A. and Black S.M. 1973). Another hypothesis suggests that kinetin acts at the gene level and that in the case of dark synthesis of pigment, regulation is due to modulation of the activity of a gene which is already being expressed rather than gene activation. The other suspected role of kinetin is the control of availability of energy-rich compounds as demonstrated by the use of salicylaldoxine and 7,4-dinitrophenol which inhibit oxidative phosphorylation (Giudici de Nicola M. *et al.* 1972a, b). Guruprasad K.N. and Laloraya M.M. 1980 make the observation that GA inhibits betalain synthesis by diverting the precursor to other metabolic process. This situation is reversed by adding precursors. In the case of ABA initial speculation held that it acted at a membrane level. Guruprasad believes it blocks the genetic site of phytochrome action. Bianco-Colomas J. and Bulard C. 1981 shows that there is an interaction between cytokinin, fusicoccin (a substance which increases potassium uptake into cells), K+ and temperature acting on membrane permeability. The temperature effect on cytokinin action is examined again by Elliott D.C 1982 where it is stipulated that cytokinin action, in inducing the biosynthetic pathway involved in betacyanin synthesis in *Amaranthus tricolor* seedlings, is dependant on both membrane synthesis and function. This membrane synthesis is stimulated during a heat pretreatment at 40°C. Cytokinin action can also be influenced by Ca^{2+} depending on cytokinin type (Vallon U. *et al.* 1989) i.e. kinetinriboside is affected whereas with kinetin there is no effect. Cytokinin gene activating capacity may be the activation of replicon origins as suggested by Houssa C. *et al.* 1990 working on *Sinapis*. This idea is also suggested for 2,4-D in anthocyanin synthesis in *Daucus carota* where Ozeki Y. *et al.* 1990 shows that this plant growth substance affects transcriptional level in this system.

Studies on the interaction of light and cytokinin using the amaranthin bioassay has shown initially that light increased the level of cytokinin (Köhler K.H. *et al.* 1980) however a more rigorous analysis carried out by Kochhar V.K. *et al.* 1981a showed that there is no interaction between light and kinetin i.e. that phytochrome has a different path of action than kinetin. Despite this, pretreatment with kinetin can result in a interaction with phytochrome following illumination. This apparent interaction is observed by Bianco-Colomas J. *et al.* 1988 studying the effect of 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole(DRB) and its ability to distinguish light stimulation from cytokinin stimulation of amaranthin synthesis in *Amaranthus tricolor*. This hypothesis resulted from the observation that 7-(pentylamino-3-methylpyrazolo(4-3-d)pyrimidine (PAMPP), a well known competitive anticytokinin, inhibited the stimulating effect of

white light on amaranthin synthesis. Féray A. *et al.* 1992 come to the conclusion, when looking at the effect of polyamines in *Amaranthus caudatus* L. cv pendula, that certain polyamines, for example putrescine and diaminopropane, increases betacyanin content whereas others such as spermidine and sperimine causes an inhibition to a level below that of the control in the dark. It is noted that 10^3 times more of the polyamine is needed to achieve a similar level of betacyanin induction as that achieved by benzyladenine.

The other mode of research into betalains and betalain control is that of tissue culture. This involves the growth of plant material under aseptic conditions either on solid agar or in liquid suspension cultures. The development of molecular genetics has permitted experimenting with hairy root cultures since they have been found to be genetically more stable (Hamill J.D. *et al.* 1986, 1987, Taya M. *et al.* 1992, Porter J.R. 1991, Kino-Oka M. *et al.* 1992, Taya M. *et al.* 1994).

Despite this the study of regulation is best carried out using liquid suspension as it provides a more homogeneous environment around the cell. However this is subject to obtaining a relatively fine suspension culture. If obtaining a cell suspension proves difficult one can proceed with experiments using callus culture provided they are relatively friable.

Böhm H. and Rink E. 1988 reviews the use of tissue culture systems as a means of studying betalain production. They initially review the betalain producing tissue culture systems in relation to the types of media used. Irrespective of plant part source, betalain producing callus cultures have been selected and maintained for short to long periods of time. In general the callus inducing media was the same as the maintenance media except for variation in the growth substance combination and concentration. The production of betalains, depending on the species of plant, can either occur spontaneously in the dark or have to undergo induction by either a chemical or physical means. Subsequent production and accumulation generally reach maximum concentration in the stationary phase of cell development except for suspension cell cultures of *Phytolacca americana* where highest betacyanin accumulation rates occurred during the logarithmic growth phase. To date excretion has not been observed. Since the purple betacyanin predominates, work on the yellow betaxanthin-producing cultures is limited (Böhm H. *et al.* 1991, Piattelli M. *et al.* 1965, Trezzini G.F. and Zryd J.P. 1991). However it has been noted that there is a simultaneous occurrence of betalamic acid and betaxanthin (Reznick H. 1978).

The actual factors that induce betalain production will vary depending on the species.

The review by Böhm H. and Rink E. 1988 indicate that it is relatively difficult to get pigment production especially as it appears to be linked to differentiation. This observation applies for some varieties of *Beta vulgaris* but not for *Phytolacca americana*. Sakuta M. *et al.* 1987a, b looked at the effect of sucrose and nitrogen source on betacyanin accumulation and growth. They found that the water potential had to be taken into account when changing the sucrose concentration and that betacyanin accumulation was associated with nitrogen uptake. An in depth study on the effect of 2,4-D and 6-BAP on *Beta vulagaris* by Girod P.-A. and Zryd J.-P. 1991 showed that different combinations of auxin and cytokinin could influence the pigmentation of the final callus. This however was influenced by the history of the callus i.e. its initial colour and growth substance quantity and combination. Inhibitor studies using 5-azacytidine showed that demethylation, which is thought to permit gene activation (Kaeppler S.M. and Phillips R.L. 1993) was found to be only part of the answer and that the gene could be under negative regulation. Bokern M. *et al.* 1991 compared a red line suspension culture and a purple/red violet line and found that the purple could not persist in the dark whereas the less pigmented red line did. In the case of the red line dark production of pigment amounted to 26% of light production indicating the role of light as an enhancer.

Bokern M. *et al.* 1991 also points out that different species will react differently to the same treatment, in particular the administration of DOPA. He quotes Berlin J. *et al.* 1986 where tyrosine increased the pigment level in *Chenopodium rubrum* to back their result. The results of French C.J. *et al.* 1974, Elliott D.C. 1983, Rink E. and Böhm H. 1985 seem to go against Bokern M. *et al.* 1991 findings. In Böhm H. *et al.* 1991 the inability to get betaxanthin producing cultures from yellow petal could indicate that the supply of amino acids is lacking so much so that it is being channelled into growth processes. This could be directly related to the nitrogen metabolism of the cell which gives rise to glutamine (vulgaxanthin I) and glutamic acid (vulgaxanthin II).

The observation that pigment production is preceded by a dramatic tyrosine accumulation (Kishima Y. *et al.* 1991a) comes as a surprise suggesting that control is before tyrosine and not after it. This facet would have to be examined in other models being studied i.e. *Beta vulgaris, Chenopodium rubrum*, and *Phytolacca americana* so as to find out whether this aspect of control is model specific or a generalisation. It would be important also to look at enzyme level and biosynthetic intermediate fluctuations. In Kishima's case, which was the use of a bud, betalain peaked before the bud opened i.e. no light was needed. However a few months later Kishima (Kishima Y. *et al.*

1991b) was able to produce a callus cell line that could go white after 14 days in the dark and, following 7 days illumination, go violet again. Kishima also points out that obtaining a high producing cell line could be the result of either a higher metabolic activity of individual cells or an increase in the number of cells capable of producing betalain (Hall R.D. and Yeoman M.M. 1986, 1987). Sakuta M. *et al.* 1991 found a similar finding when looking at the effect of 2,4-D in betacyanin accumulation in suspension cultures of *Phytolacca americana*. Tyrosine level increase to 156% on the addition of 2,4-D with a resulting increase of betalain. The addition of tyrosine in the absence of 2,4-D appears to mimic the addition of 2,4-D. Sakuta points out that there could be different pools of tyrosine in the cytoplasm and vacuole. Rudat A. and Ehwald R. 1994, when looking at interspecies relationships, found that yellow callus cultures of *Chenopodium album* synthesised betacyanins in the presence of light, and duckweed *Wolffia arrhiza* used as a nurse culture. They also found that not all cells were induced to synthesize pigment as found by Girod P.A. and Zryd J.-P. 1987.

Within the last three years research appears to have stepped up on betalain synthesis with 3 groups on the european continent and two more in Japan. To justify this project, in light of the fact that one group in Japan have a readily inducible system based on *Phytolacca americana* producing betacyanin only and two other groups in Europe who are working on the enzymes, it is that; 1. we are working with beetroot in a different manner to that carried out in Switzerland and 2. despite the Japanese group studying in the same manner as us they are working on a different model system. As for the commercial spin off there is no doubt that extraction from field crop source is a lot cheaper. However as pointed out by Böhm H. and Rink E. 1988 cell culture eliminates the extracting procedures, the possible presence of betalain decolorizing enzymes and the characteristic smell geosmin (Pourrat H. *et al.* 1983, Havlíková K. *et al.* 1985) of beetroot. Experiments based on electrofusion of vacuole and protoplast membrane (Lackney V.K. *et al.* 1991) could overcome the last remaining obstacle with using plant cell cultures that is the storing of the required/desired metabolite in the vacuole.

CHAPTER 2. MATERIALS and METHODS

2.1 Initiation of Model System

2.1.1 Source of Beetroot Seeds

Seeds of seven varieties of red table beetroot (*Beta vulgaris* var Albium vereduna, *B.v.* var Globe, *B.v.* var Boltardy, *B.v.* var Burpees Golden, *B.v.* var Forino, *B.v.* var Monodet, *B.v.* var Detroit Little Ball) were obtained from Suttons Seeds Ltd., Torquay, England.

2.1.2 Sterilisation Procedure

1. Unless otherwise stated all materials and chemicals were sterilised by autoclaving (Portable autoclave, Express Equipment) for 20 mins. at 15psi prior to use.
2. All materials for autoclaving were sealed by two layers of aluminium foil.

2.1.3 Sterilisation of Seeds

This was carried out using a modified version of Kishima Y. 1991, Bohm H. *et al.* 1991

1. A 'sterilisation' tube was made by welding a piece of muslin/cheese cloth to one end of a plastic tube(diameter 27mm, height 95mm)
2. Seeds of one variety were placed in the sterilisation tube. The tube was immersed for 10 seconds in ethanol to lower the surface tension of the seed coat. This helps to reduce the amount of air bubbles which cling to the seed. It was ensured that the seeds were in direct contact with the ethanol by swirling the seeds on immersion.
3. The 'sterilisation' tube was subsequently transferred to a 10% v/v solution of commercial hypochlorite containing 5% - 15% chlorine based bleaching agent; sodium hypochlorite (about 1% available chlorine final concentration) for 10 min. The sterilisation tube was swirled every 2-3 minutes so as to ensure that the seeds were in direct contact with the chlorinated solution.
4. Finally the seeds were washed with sterile distilled water so as to remove the chlorinated solution. This consisted of three sterile beakers containing sterile distilled water. The tube was left in each beaker for 5 minutes during which the seeds were swirled so as to, again, ensure that the seeds were in direct contact with

the water.

The seeds were removed from the 'sterilisation' tube and transferred to an empty sterile Petri dish prior to placing on to a germinating medium. Transfer of seeds occurred within the succeeding 30 minutes.

2.1.4 Aseptic Germination of Seeds

Sterilised seeds were aseptically placed onto $20cm^3$ of solidified modified M&S medium(Table 1), pH at 5.6 using 1M NaOH, in a 90mm diameter petri-dish. Five seeds were used per dish.

TABLE 1

Modified Murashige and Skoog medium for nodal Cuttings

Ingredient	Concentration (mg/liter)
Murashige-Skoog; Basal Salt Mixture without Phytohormones Sucrose and Agar, Sigma, Cat.No. M5524	4610
Glycine (made as a 100 times stock)	0.2
Nicotinic acid (made as a 100 times stock)	0.5
Thiamine (made as a 100 times stock)	0.05
Pyridoxine HCl (made as a 100 times stock)	0.05
Inositol	100
Sucrose	30000
Agar Technical No.3 Oxoid Cat. No. L13	12000

Seedlings of 4 or more leaves were allowed to develop. Once the leaves attained about 2cm by 4cm in area they could be used for callus induction.

2.1.5 Callus Induction

Subsequently these leaves were aseptically cut, with a sterilised scalpel, into strips about 0.5 cm wide. 4-5 strips, adaxial side uppermost, were placed on a callus induction

medium(Table 2). When placing the leaf strips on the medium every attempt was made to make sure that the cut edge of the leaf was in direct contact with the medium.

2.2 Media Description and Growth Environment

2.2.1 Growth Conditions

Unless otherwise stated the following growth conditions applied; 16 hours light 8 hours dark with a temperature varying between 21°C - 25°C. Light was provided by a bank of 6 Philips fluorescent 125W warm white bulbs(figure 2.2.1)

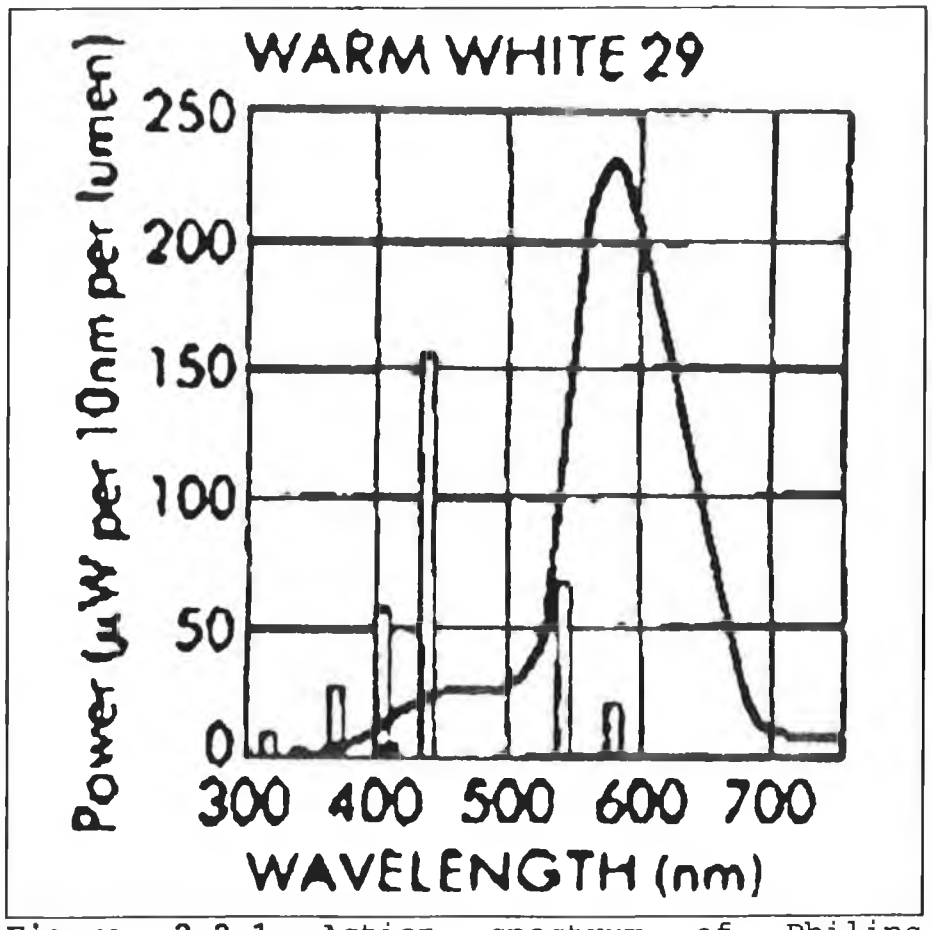


Figure 2.2.1 Action spectrum of Philips fluorescent 125W warm white bulbs

The range of light level was measured using a light meter(Skye Instruments Ltd., Unit 5, Ddole Industrial Estate, Llandrindod Wells, Powys LD1 6DF, UK.) and was found to vary between 6 - 14 $\mu molm^{-2}s^{-1}$ depending on the location in the growth room. Experimental light levels ranged between 8 - 14$\mu molm^{-2}s^{-1}$.

TABLE 2A (Macronutrients)

Made as a 10 times stock solution Ingredients	Concentration (mg/litre)		
	PGo medium	Freytag medium	Gamborg B5 medium
NaH_2PO_4	250	-	-
KCl	600	-	-
$(NH_4)_2SO_4$	400	-	134
$MgSO_4.7H_2O$	500	370	250
KNO_3	2000	1900	3000
$CaCl_2.2H_2O$	300	440	150
$NaH_2PO_4.2H_2O$	-	-	169.6

TABLE 2B (Micronutrients)

Made as a 1000 times stock solution Ingredients	Concentration (mg/litre)		
	PGo medium	Freytag medium	Gamborg B5 medium
H_3BO_3	10.62	6.2	3.0
$MnSO_4.H_2O$	1.68	-	-
$MnSO_4.4H_2O$	-	22.3	13.20
$ZnSO_4.7H_2O$	1.06	8.6	2.0
KI	1.583	0.83	0.75
$Na_2MoO_4.2H_2O$	0.0025	0.25	0.25
$CuSO_4.5H_2O$	0.0025	0.025	0.025
$CoCl_2.6H_2O$	0.0025	0.025	0.25

TABLE 2C (Stable Iron Solution)

made as a 100 times stock solution Ingredients	Concentration (mg/litre)		
	PGo medium	Freytag medium	Gamborg B5 medium
Na_2EDTA	37.3	37.3	-
$FeSO_4.7H_2O$	27.8	27.8	-
FeNaEDTA	-	-	40.0

TABLE 2D (Vitamins)

Made as a 1000 times stock solution Ingredients	Concentration (mg/litre)		
	PGo medium	Freytag medium	Gamborg B5 medium
p-Aminobenzoic Acid	-	0.2	-
Acorbic acid	-	0.4	-
D-Biotin	-	0.00025	-
Choline Chloride	-	0.2	-
Folic Acid	-	0.015	-
Niacin/Nicotinic Acid	1.0	0.5	1.0
D-Pantothenic Acid	-	0.4	-
Pyridoxine HCl	1.0	0.5	1.0
Riboflavin	-	0.015	-
Thiamine HCl	10.0	0.5	10.0
Inositol	100.0	-	100.0

TABLE 2E (Amino Acids)

Made as a 100 times stock solution Ingredients	Concentration (mg/litre)		
	PGo medium	Freytag medium	Gamborg B5 medium
L-Arginine(free base)	-	40.0	-
L-Asparagine(anhydrous)	-	40.0	-
L-Glycine	-	20.0	-
L-Phenylalanine	-	20.0	-
L-Tryptophan	-	40.0	-
L-Glutamine	-	60.0	-

TABLE 2F (Additional Ingredients)

Ingredients	Concentration (mg/litre)		
	PGo medium	Freytag medium	Gamborg B5 medium
Sucrose	30000	30000	30000
Kinetin	0.5	0.5	0.5
2,4- D	0.1	0.1	0.1

As shown in Table 2 three inorganic salt combinations were tested based on literature survey(Freytag A.H. *et al.* 1988, De Greef W. & Jacobs M. 1979, Hunter C.S.& Kilby N.J. 1990).

2.2.2 Media Preparation

As indicated in tables 1 and 2 stock solutions were used in making media preparations. When making up the individual stock solutions it was found that each constituent component had to be completely dissolved first before adding the next one. The iron solution may take a long time to dissolve even using a magnetic stirrer. Once all the components had been added, the pH of the aqueous medium was adjusted to 5.7 using 1.0 mM NaOH. In the case where precursors were added either 1.0mM NaOH or 1.0 mM NH_4OH was used to adjust the pH.

2.2.3 Plant Growth Regulators

Kinetin and 2,4-D were the two plant growth regulators used in this project. Kinetin was made as a stock solution and kept in the fridge. 10 mg of kinetin was weighed out in a polypropylene weighing boat (L.I.P. (Equipment & Services) Ltd., 111 Dockfield Road, Shipley, West Yorkshire, England BD17 7AS, Cat No. 30205) and drops of 1.0M NaOH added so as to 'wet' the kinetin crystals. Using a spatula the kinetin was forcibly dissolved into the 1.0M NaOH by crushing the kinetin crystals. Drops of the NaOH were added and the crushing motion continued until such time as the minimal amount of solution became clear. It was gradually made up to $100cm^3$ with distilled water. The same procedure was used for dissolving the 2,4-D. A $1mg/cm^3$ stock solution was made up in the latter case. The plant growth regulators were sterilised with the other media components.

2.3 Culture Manipulations

2.3.1 Subculture of Callus

Calli that developed after 1 month from the cut edges of the leaf strips, were transferred to fresh medium. This initial callus was hard and lumpy. The callus was subcultured every 3 weeks.

2.3.2 Callus Selection

This consisted of subculturing the callus every three weeks. Although the initial callus was hard and lumpy one could, after 3 subcultures, select for good growing white friable callus. Callus was kept in the dark.

2.3.3 Suspension Cultures

Attempts were made to set up violet, orange and white suspension cultures of B.v. var Globe. An empirical method was adopted using previous literature combinations and concentrations of kinetin and 2,4-D as a starting point. A batch of ten flasks of PGo medium containing 0.5mg/dm^3 kinetin, 0.1mg/dm^3 2,4-D were prepared for initial initiation and 2 subsequent subcultures followed by sieving using a 1mm square sieve. Initial initiation consisted of aspetically transferring callus from a three week old agar culture to a 250cm^3 flask containing 50cm^3 of sterilised Pgo salt cocktail liquid medium. Usually 1g of callus was tranferred. In the case of white suspensions white callus, that was at least three weeks in the dark, was used. In the case of violet suspensions calli, that were violet, were used. If a desirable suspension cultures, as characterised by a morphological homogeneous appearance and a doubling time of 4 days was obtained, the suspension line was continued.

2.3.4 Selection of Callus for Experimentation

The callus used was at least three weeks in the dark and only white pieces were used for experimental purposes.

2.3.5 Experimental Set-Up of Experiments Involving Callus Culture

Callus was weighed out on to 20cm^3 of solidified Pgo nutrient medium agar in 90 mm diameter Petri dishes. This consisted of;

1. A sterile medium-filled Petri dish was placed on a balance just outside the laminar flow and the balance zeroed. The Petri dish lid was kept on the petri dish to maintain sterility.
2. The Petri dish was brought into the laminar flow making every attempt to keep the Petri dish closed.

3. An estimated guess of callus was placed onto the medium.
4. Sterility was maintained by replacing the lid of the Petri dish back on and the closed Petri dish on the balance outside the laminar flow.
5. If the desired callus weight was not attained step 1 to 4 were repeated.
6. Resterilisation of heat stable material during the course of an experimental set up was achieved by flaming in a bunsen burner till red hot.

Five pieces of callus per plate and two plates per treatment was used.

Literature indicated that light played an important part in bringing about the induction of pigment synthesis, whether it be by turning on the biosynthetic pathway or increasing an already constitutive level of production. Since experimental material was set up in the light the plates were kept in the dark for one week so as to permit degradation / dilution of any pigments synthesis during the setting up of the experiment. This also permitted achieving a certain amount of homogeneity for result purposes. This procedure was used for experiments lasting in total 3 weeks. Otherwise in the case of simultaneous light and dark experiments lasting 4 weeks the callus was left over night in the dark after which the cultures were either left in the dark or exposed to light.

2.4 Light Experiments

2.4.1 Quantitative Effects

Plates were illuminated by 6 Philips fluorescent 120 warm white bulb(fig. 2.2.1). To achieve 1, 10, 100 µmol $m^{-2}s^{-1}$ (Photosynthetic Active Radiation) of light, levels were quantified by a light meter (Skye Instruments Ltd., Unit 5, Ddole Industrial Estate, Llandrindod Wells, Powys LD1 6DF, UK.). To vary the light levels in different experiments cheese cloth/muslin, bought from the haberdashery section of a department store, was used between the light source and the callus. It was found that the Petri dish lids absorbed light. As a result, when determining light levels, the light sensor was put under the Petri dish lid.

2.4.2 Qualitative Effects

For light quality experiments photographic gel(Lee Filters Limited, Central Way, Walwaorth, Andover, Hampshire, Andover 66247, UK. and Strand Lighting Limited,

Grant Way,(off Syon Lane), Isleworth, Middlesex, TW7 7QD, UK.) were used. Deep Green (139), Dark Blue (119), Primary Red (106) were chosen since their transmission spectra intersect minimally near the base line(Figure 2.4.2).

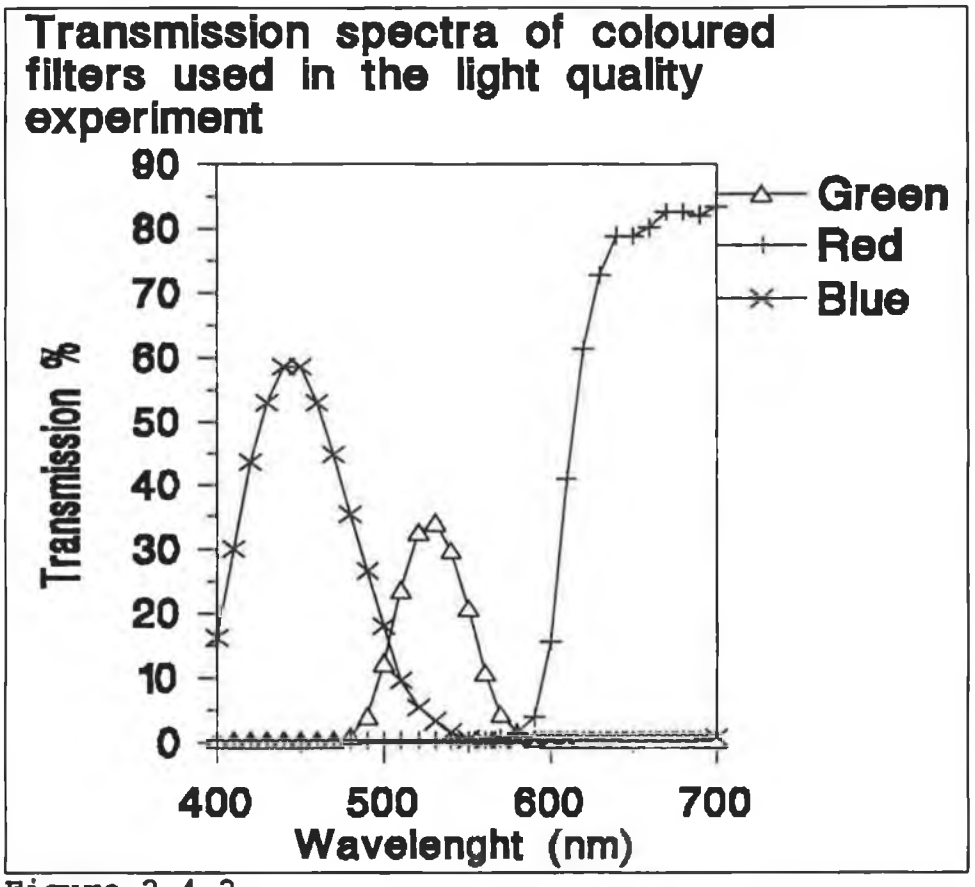


Figure 2.4.2

This feature is needed as one wants to look at the effect of one light quality at a given time.

Since the coloured filters, which were placed directly on the Petri dishes, caused a significant reduction in light levels, a shelving system fitted with three 30W fluorescent bulbs (Thorn 30W Warm White, G.B.) was used. The sides and bottom of the shelves were covered with aluminium foil (Irish Merchants, Dublin) so as to reflect the light thus maximising the light level. This set up resulted in a confined condition causing an accumulation of heat. Therefore a fan (ETRI, Modele 125 XR, 8 rue Boutard, 92200 Neuilly, France.) was used blowing from one end of the shelf to the other. The temperature stabilised between 25-27^{0}C. The use of certain coloured filters resulted in light levels during experiment not exceeding 7 µmol $m^{-2}s^{-1}$ for consistency purposes.

In the case of 2 hours heat treatment followed by 1 hour dark and 1 hour red light, heat was provided by an ordinary incubating oven (Memmert, West Germany.).

Temperature was checked by taking temperature readings of water in test tubes.

2.5 Precursor Experiments

These were carried out by adding appropriate concentrations of tyrosine (T-1020, Sigma), DOPA (D-9628, Sigma) and (-)shikimic acid (S-5375, Sigma).
In the case of tyrosine, a minimal amount of 1M NaOH was used to dissolve. It was then added to the aqueous salt cocktail (PGo).
In the case of DOPA it will oxidised in water over time and immediately in heat. To avoid the black oxidation product (probably due to a small chain polymerisation of the L-DOPA; this allows free circulation of electrons within the chain resulting in a black colour) an equimolar concentration, to that of DOPA, of ascorbic acid was added first to the aqueous medium then the appropriate DOPA concentrations. This caused a large acidity increase. When bringing the pH of the medium to pH 5.7 with 1mM NaOH one could only go as far as pH 5.4. This would take in excess of $5cm^3$ of the 1.0mM NaOH. Otherwise the ascorbic acid would be neutralised causing oxidation of the DOPA. Ascorbic acid controls were also carried out. It was realised later that the use of ammonia could remedy these problems. In the meantime an attempt was made to avoid the use of ascorbic acid by filter-sterilising (Sterilised filter, type HA, Cat. No. SFHA047LS, Milipore Corporation, Bedford, MA 01730) the DOPA-containing solutions. Unfortunately, despite the nutrient medium being at 50°C, the heat was sufficient to cause some spontaneous oxidation.
Shikimic acid was dissolved in the aqueous salt cocktail medium. Ammonia water was used to adjust the pH of the medium when studying the effect of shikimic acid.

2.6 Treatment of Experimental Samples

2.6.1 Extraction of Experimental Samples

Extraction was essentially according to J. Berlin *et al.* 1986 with certain modifications. On completion of the experiment, individual callus pieces were weighed then put into an Eppendorf for storage. In the case of bacterial or fungal contamination, calli nearest the contaminating organism were discarded. Degradation of betalains was occasionally evident after storage at -20°C thus the Eppendorfs were stored at -70°C until needed. It was found towards the end of the project that callus pieces in excess of 800mg could

not be used for trustworthy results. This is because when placing in an Eppendorf, samples, in excess of 800mg, had to be forced in. This caused lysis of cells and possibly the liberation of lysosomal enzymes probably causing cell and pigment degradation. The tissue could be seen to darken.

The callus samples were then freeze-dried. This was carried out by using a condenser (Hetosicc, Heto Lab Equipment, Denmark.) linked to a vacuum pump (Electromotors Ltd, AC motor, type BS220B). Freeze-drying occurred at ambient temperature (17-23°C) over at least 5 hours and at most overnight (16 hours).

To minimise rehydration of the freeze-dried samples, and the subsequent permanent loss of experimental material, the samples were promptly, or following a delay of 15 hours maximum at room temperature, ground up in situ using a closed forceps.

1 ml. of 50% aqueous methanol was added to each Eppendorf and mixed (40 Rotations/min) for 1 hour in a cold room(4°C +/- 1°C). Mixing consisted of a laboratory-made instrument. A plastic disc, 155mm in diameter, connected to an old LKB peristaltic pump head. Clips inserted in the disc held the Eppendorfs (Acknowledgement to Declan Doyle for much appreciated help). In later experiments it was realised that 1 hour mixing in a cold room could be omitted as the freeze-drying and grinding with a forceps in an Eppendorf broke up the cells sufficiently to release the pigment. Cell debris were spun down in a centrifuge (Heraeus christ, Biofuge A) at maximum speed (14926g) for 10 minutes at ambient temperature. The supernatant samples were diluted 1/10 and spectrophometric readings taken at 600nm, 537nm and 478nm. The samples were left for a period of not more than an hour at ambient temperature between the debris being spun down and the readings taken.

2.6.2 Quantisation of Pigments

Literature indicates that there are at least three pigments in crop beet root and cell suspension cultures of red table beetroot(*Beta vulgaris*)(Nillson T. 1970, Leathers R. & O'Riordan G. 1991, Bokern M. *et al.* 1991). One can thus presume that there are as many in callus cultures. As a result one cannot just simply take readings at the wavelength of maximum absorbance; 537nm for betanin, 478nm for vulgaxanthin I and II, 430nm for betalamic acid, 494nm for betanidin, 542nm for amaranthin. This is because of the intersection of absorption spectrum of the individual pigments(Figure 2.6.2)

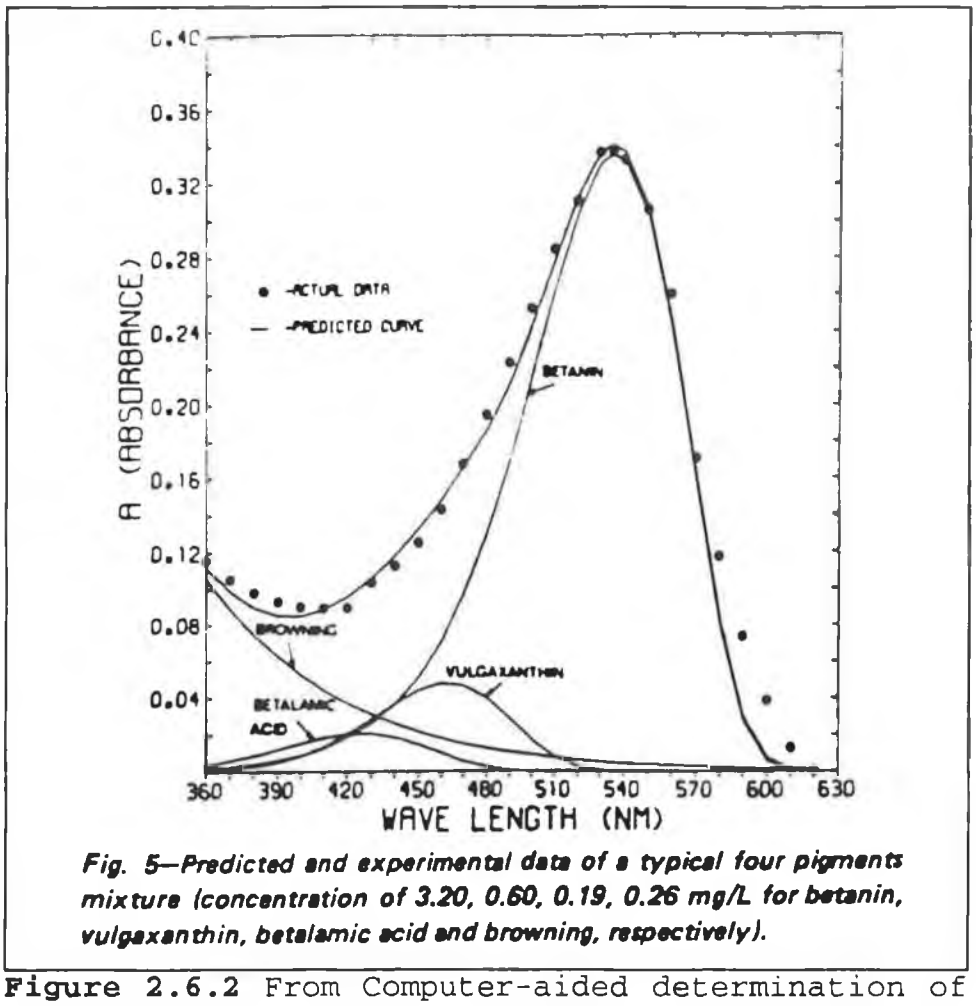


Fig. 5—Predicted and experimental data of a typical four pigments mixture (concentration of 3.20, 0.60, 0.19, 0.26 mg/L for betanin, vulgaxanthin, betalamic acid and browning, respectively).

Figure 2.6.2 From Computer-aided determination of beet pigments, Saguy I., Kopelman I.J. & Mizarahi S. 1978c

would interfere with the actual absorbance of a given compound. As a result an equation for the evaluation of actual absorbances of individual pigments in the same solution was used. This required the obtaining of absorbances at 600nm, 537nm and 478nm spectrophometrically once the samples were diluted 1 in 10. The equation used was that of Shin C.C & Wiley R.C. 1981 as follows;

$1.095*(A_{537} - A_{600})$ for the actual betanin absorbance

$A_{478} - 0.258A_{537} - 0.647A_{600}$ for the actual vulgaxanthin absorbance

This gave corrected absorbances which in turn were multiplied by the extinction coefficient (ε) 65000 for betanin and 48000 for the vulgaxanthins(23000 for betalamic acid, 49400 for betanidin). This method however does not distinguish between the two vulgaxanthins. Concentrations of both yellow vulgaxanthins and violet betanin were subsequently calculated using the Beer-Lambert law;

$$A = \varepsilon_\lambda * c * l$$

were A = absorbance or optical density

c = concentration moles/litres

l = 1 cm light path length

ε_λ = Molar extinction coefficient for the absorbing material at a wavelength lambda

2.7 Purification of Standards

2.7.1 Pigment Mixture

Fresh red table beetroot (*Beta vulgaris* var Boltardy or Primgold) bought on Dublin City's fruit & vegetable market was washed, peeled, diced into 2 cm^3 cubes and freeze-dried. A sophisticated freeze-drier (Supermodulyo Freeze Dryer & X3/SM Shelf Chamber Unit, Plant Model X3/SM) was used. This froze down the beetroot cubes, then, once the vacuum was turned on, increased the temperature to a fixed point (15°C was chosen so as to avoid heat degradation). Once the temperature of the probe in a beetroot cube reached the set-point temperature, the process of drying was considered finished. This could take up to 1.5 to 2 days.

The dried beetroot was ground into a powder using a mortar and pestle at room temperature. The powder was extracted twice with 100% methanol to obtain an extract enriched in betaxanthins. The remaining pulp was then extracted twice with 50% aqueous methanol to obtain an enriched betacyanins extract. These were allowed to stand overnight at ambient temperature to permit sugars to precipitate out. This is particularly the case of the 100% methanol extract. The methanol was removed by rotary vacuum evaporation at 30°C in a round bottom flask. Rotary evaporation of the 100% methanol extracts resulted in a thick syrup due to the presence of sugar. This was dissolved in a minimal amount of distilled water. The water was removed by freeze-drying in a round-bottom flask.

2.7.2 Separation of Pigments by Liquid Chromatography

The powder (or sticky material due to the presence of sugar) was dissolved in solutions depending on the column used. It was found that columns needed to be washed out with 5 to 7 times the volume of the column when changing solution; eg. washing out the storing solution (in the case of Sephadex G-15 0.2M NaOH) or changing from the

running buffer to the elution buffer. The mechanisms of column chromatography examined were (a.) molecular-sieving/adsorption using Sephadex G-15, adsorption / polarity using cellulose(20µm diameter), anion exchange using Dowex H^+ and ionic suppression technique/reverse phase HPLC using µBondapak C_{18} radial Pak.

2.7.2.1 Sephadex G-15

2.7.2.1.1 Citrate Buffer

A Sephadex G-15 matrix was washed through with a 0.1 M citrate buffer (citric acid and trisodium citrate) at pH 3.4. The enriched freeze-dried sample of betaxanthin was dissolved in the minimal amount of citrate buffer and applied to the column. The sample was allowed to enter the column and $1cm^3$ aliquots of citrate buffer added until such time as the sample had entered the column. A reservoir of citrate buffer was then attached to the column and sample allowed to go through at $1cm^3min^{-1}$ in a cold room. The volume in which the sample was dissolved did not exceed 5% of the column volume.

2.7.2.1.2 Acetic acid/HCl

It was found in the literature that Sephadex G-25 using 1% acetic acid could be used to separate beetroot pigments. This was based on a method by Drdak M. *et al.* 1990 and Bianco-Colomas J. 1977. Since Sephadex G-15 was readily available, it was decided to use it instead of the G-25. Column size used was 27mm diameter by 95mm height. Since literature suggested crystallising the pigments in 0.1% HCl it was decided to try using 0.1% HCl instead of 1% acetic acid. A similar effect was obtained in relation to the elution profile.

2.7.2.2 High Performance Liquid Chromatography

2.7.2.2.1 Preparatory

Preparative HPLC was carried out using a µBondapak C_{18}, particle size 10µm (Waters Radial-Pak, Cat. No. 85721) preceded by a Guard Pak µBondapak™ C_{18} HPLC precolumn insert No.88 held in a Guard Column (Waters Associates, Cat No. 88070).

Analytical apparatus consisted of two Model 510 pumps, solvent programmer Model 660 (Water Associates, Milford, MA), a U6K injector with a $1cm^3$ loop replaced later on by Rheodyne 7125 (Connetticut, California, U.S.A.) 20µm loop and a variable wavelength detector (Lambda-Max Model 481 LC Spectrophotometer) fitted with a flow cell, Model(97817). The chromatograms were monitored at 478nm since the calli were yellow in appearance.

The method according to Schwartz S.J. & vonElbe J.-H. 1980 for isocratic and gradient elution was used with some modifications. The column was developed with a gradient initially; using CH_3OH/0.05M KH_2PO_4 (18:82,v/v) adjusted to pH 3.5 with H_3PO_4 (solvent A) and the following solvent mixes: initial, 100% solvent A; final, 80% solvent A, 20% CH_3OH. Curve 8 on the programmer was followed for a duration of 9 mins. It was found that the isocratic elution gave broadly similar results as the gradient elution. $1cm^3$ aliquots were injected.

2.7.2.2.2 Quantitative

The same analytical apparatus and operating procedures were used. Experimental samples were dissolved in solvent A. It was found that when the sample were dissolved in either $1cm^3$ of 50% aqueous methanol or McIvaines buffer at pH 5.0 it gave additional interfering peaks, especially in the area of the desired peaks, and upset the base line.

2.7.2.3 Cellulose (20µm)

2.7.2.3.1 Plates

Based on continual literature survey it was found that good separation could be obtained using thin layer chromatography(TLC) on cellulose coated plates(Bilyk A. 1981a). This system consisted of developing first with a mix of 30% isopropanol, 35% ethanol, 30% distilled water and 5% acetic acid. Then drying the plate under a nitrogen atmosphere followed by 2 successive developments with a different solvent mix; 55% isopropanol, 20% ethanol, 20% distilled water and 5% acetic acid. Since sugars were still contaminating the separated pigments, attempts were made to improve the system. Based on theoretical advice from Dr. Padraig James, School of Chemical Sciences, D.C.U., a modified version of Bilyk was devised. The final solvent system was 50%

ethanol, 45% water and 5% acetic acid(Deirdre O'Sullivan, technician 1991/1992 whose help is acknowledged and appreciated).

2.7.2.3.2 Column

Once the solvents needed had been determined a 760mm by 50mm column was used to see whether cellulose could be used to separate the pink betacyanin from the yellow betaxanthins.

2.7.2.4 Dowex ion-exchange

It was found that this column support was used a number of times for separating pigments when carrying out studies on betalains (Piattelli M. & Minale L. 1964a, Pasch J.H. & von Elbe J.H. 1975 and von Elbe J.H. *et al.* 1972). This involved Dowex H^+ cation exchange (Dowex-50W, Sigma, Product No. 50X2-400) using 0.1% HCl for binding followed by elution with water.

Once sufficient quantities of violet betacyanin and yellow betaxanthins were obtained it had to be verified by high performance liquid chromatography(see above).

CHAPTER 3. RESULTS

3.1 Initiation of Model System

3.1.1 Effect of Different Salt Cocktails

The effects on cultures over time varied depending on the salt cocktail and the variety of beetroot. PGo was found to be best for growth, whiteness and friability. In the case of Freytag the calli remained lumpy and green. As for B5 growth was prohibited in some varieties with protrusions from calli appearing to develop in the 2 weeks following subculture. Subsequently attention was focused on PGo media so as to determine which variety would be best for experimental purposes. After about one year Forino was considered best for growth, whiteness and to a lesser extent friability. After 2 years Globe became even more desirable for all three characteristics. This became particularly apparent when maintenance plates were transferred from being kept in the light to being kept in the dark. Plates 3.1.1A-E show callus cultures of white, yellow and violet lines of *Beta vulgaris* var *Globe*

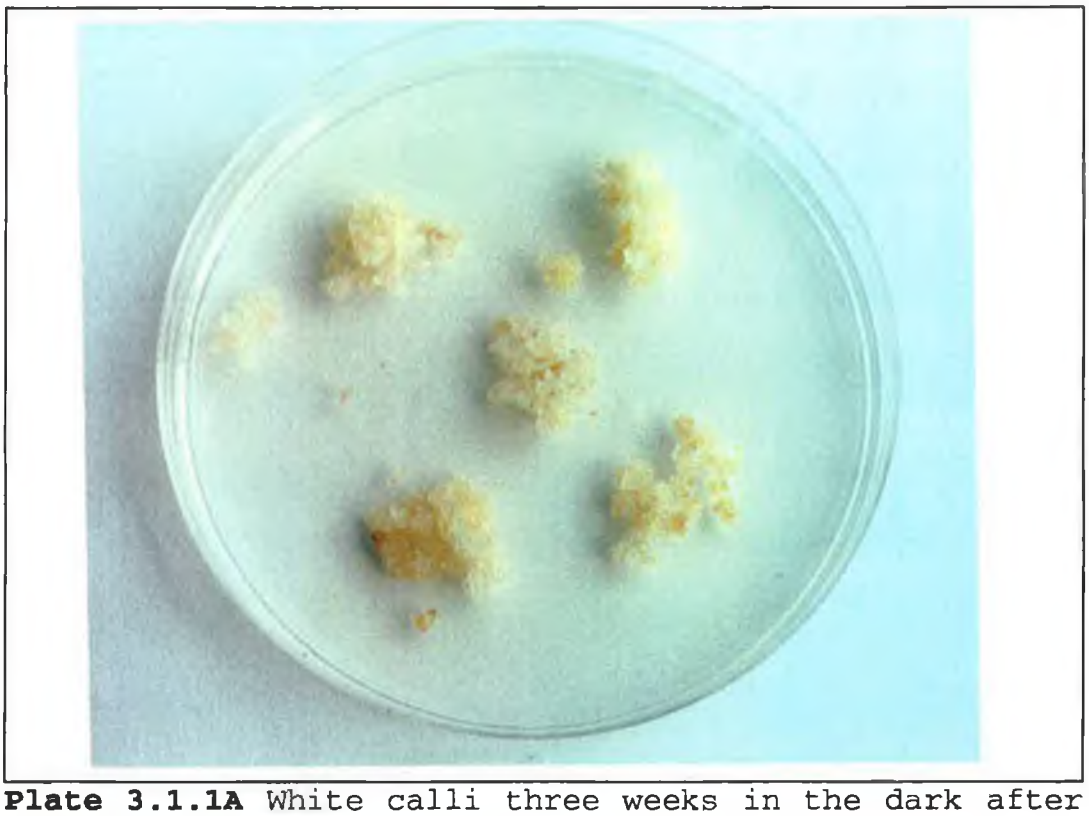


Plate 3.1.1A White calli three weeks in the dark after subculture

Plate 3.1.1B Yellow calli three weeks after subculture

Plate 3.3.1C Violet calli three weeks after subculture

Plate 3.1.1D White calli left for 1 week in the light after 3 weeks in the dark after subculture

Plate 3.3.1E Violet suspension culture, after sieving and further 3 subcultures

It was found that in addition to being able to initiate white callus cultures that yellow and violet cell lines could also be obtained. However the latter two tended to have a slower growth rate and, in the case of the violet cell line, necrose faster. The patchy/heterogeneous aspect of induction can be seen in plate 3.1.1D

Attempts at setting up suspension cultures indicated that homogenous suspension cultures would require further media manipulations. This is because even after sieving the cells through a 1mm mesh cell aggregates reappeared in the suspension cultures.

3.2 Methods Assessed for Efficiency in Separation of Pigments

For the purposes of the determination of the potential rate-limiting step it was necessary to obtain precursors and intermediates of the biosynthetic pathway. Tyrosine and DOPA were available commercially, however betalamic acid and cyclodopa-glucoside were not. It was thus envisaged to extract, separate and purify, from red table beet, vulgaxanthin and betanin. The purification of vulgaxanthin would provide standards necessary for HPLC analysis since calli were turning yellow on illumination. The purification of betanin would provide standards for HPLC and, on hydrolysis, provide a mixture of betalamic acid and cyclodopa glucoside (Schwartz S.J. & vonElbe J.H 1983). The following results concerns itself with the separation and purification of vulgaxanthin and betanin.

In an attempt to obtain an efficient and large scale production of pigment, a number of methods were examined. All column work used crude extracts obtained by two 100% methanol washes of freeze-dried red table beet followed by two 50% methanol washes. This results in an enriched vulgaxanthin and betanin extract respectively. Depending on the source material sucrose will be co-extracted. This sucrose can be partially removed by leaving the methanol extractions standing overnight. The methods investigated for pigment separation include the use of Sephadex G-15 using a number of different eluting solutions, Dowex H^+, cellulose(20μ) and reverse phase HPLC.

The properties of betalains dictated the method of separation. It has been found that on polyamide resins the retention of betacyanins decreases with increased glycosyl substitution, that betacyanins exhibit visible maxima between 534nm and 552nm (Piattelli M. & Minale L. 1964b) and between 474nm and 486nm for betaxanthins. The betacyanins are insoluble in organic solvents (vonElbe J.H. & Maing I.Y. 1973).

Initially it was considered essential to have a method which would permit a qualitative analysis of column work. Literature indicated that high performance liquid

chromatography would be the most suitable method.

3.2.1 High Performance Liquid Chromatography

3.2.1.1 Preparatory

In the continuing attempt to separate pigments so as to produce, initially standards, and subsequently enough material to derive intermediates of the biosynthetic pathway, in particular betalamic acid and cyclodopa glucoside, HPLC was investigated.

Figure 3.2.1 shows the results of preparative HPLC as a method of separating pigments for standards and further use. This method proved exceptionally good, giving neat separation of pigments especially in separating betanin from betanidin (Fig. 1.1). When comparing to the published chromatograms of Schwartz S.J.& vonElbe J.H. 1980(fig. 3.2.1A)

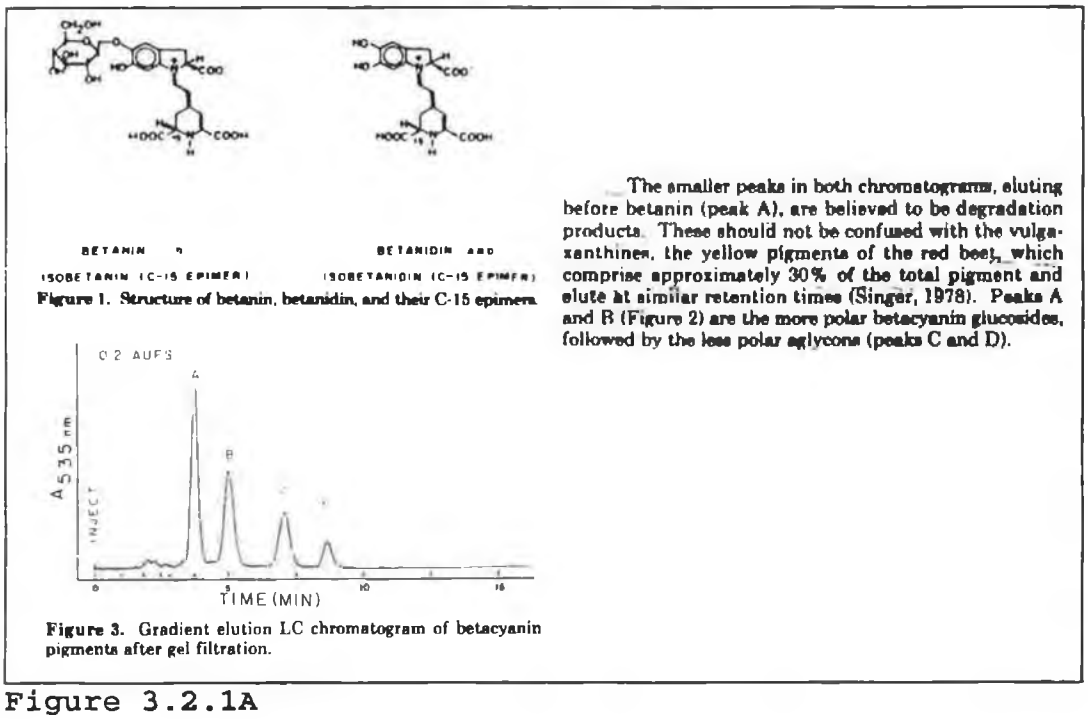


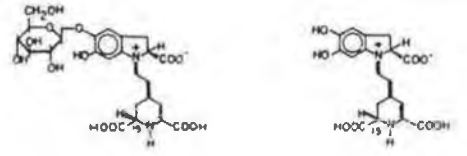
Figure 1. Structure of betanin, betanidin, and their C-15 epimers.

Figure 3. Gradient elution LC chromatogram of betacyanin pigments after gel filtration.

Figure 3.2.1A

it was found that the retention time for betanin(B) was bigger by 1-2 minutes. This also applies for isobetanin(C). In addition it appears that betanidin has merged into the isobetanin peak and that the last broad peak(D) in fig. 3.2.1B is isobetanidin. Irrespective of the result the method gave a low return of the end products in relation to the initial setting up of the HPLC system; 10 hours, 500cm^3 of solvent, 500mg of end product. This was evident given that these standards were to be used to produce intermediates in particular betalamic acid and cyclodopa glucoside.

Despite this it was decided to use the samples obtained in this manner as standards for HPLC analysis of experimental sample since HPLC necessitates only small quantities. It was also found that isocratic elution gave results just as effectively as gradient elution. It was felt that there was no net time gain if one took into account the time needed to reverse the gradient. Fig. 3.2.1B shows an isocratic elution from Schwartz S.J.& vonElbe J.H. 1980 and applies for chromatograms other than fig. 3.2.1C



BETANIN AND ISOBETANIN (C-15 EPIMER)

BETANIDIN AND ISOBETANIDIN (C-15 EPIMER)

Figure 1. Structure of betanin, betanidin, and their C-15 epimers.

The smaller peaks in both chromatograms, eluting before betanin (peak A), are believed to be degradation products. These should not be confused with the vulgaxanthines, the yellow pigments of the red beet, which comprise approximately 30% of the total pigment and elute at similar retention times (Singer, 1978). Peaks A and B (Figure 2) are the more polar betacyanin glucosides, followed by the less polar aglycons (peaks C and D).

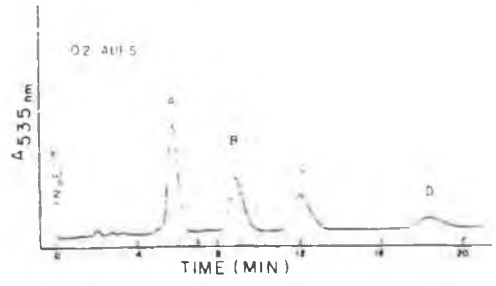


Figure 2. Isocratic LC chromatogram of betacyanin pigments after gel filtration.

Figure 3.2.1B

3.2.1.2 Qualitative

It was found that when experimental samples, that had been kept aside from normal spectrophometric analyses after harvesting, were extracted in either $1cm^3$ of 50% aqueous methanol or McIvaines buffer at pH 5.0, it gave additional interfering peaks, especially in the area of the desired peaks, and upset the base line. This disturbance in the base line consisted of a sharp sigmoidal 'blip' and is probably due to a refractive index alteration at the junction of the running buffer and the foreign extracting buffer. As a result callus samples used for HPLC analyses were extracted in the running buffer i.e. CH_3OH / 0.05M KH_2PO_4 (18:82v/v).

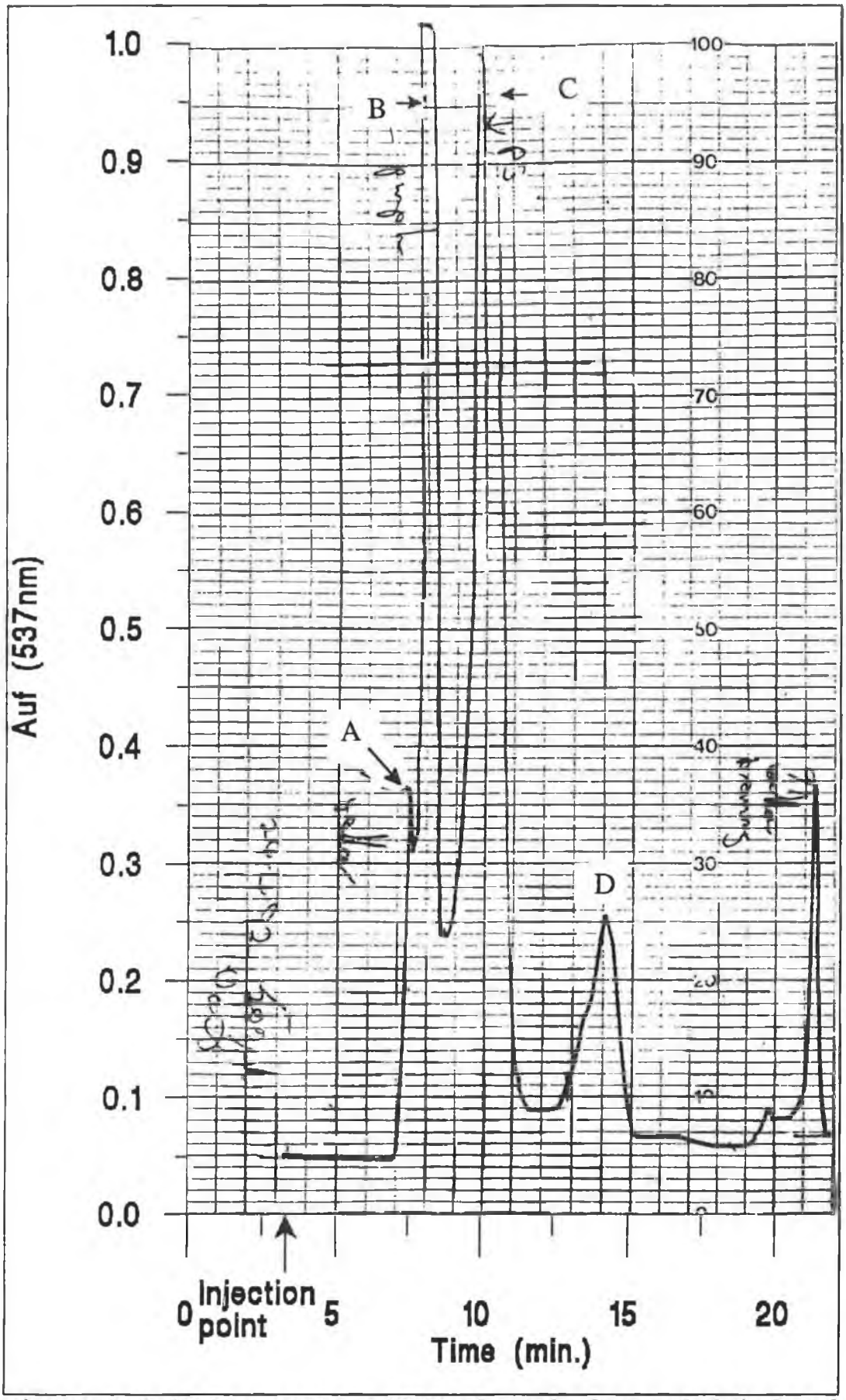


Figure 3.2.1C Preparatory HPLC of crude freezed-dried red beet dissolved in running buffer CH_3OH/KH_2PO_4 (18:82). Yellow betaxanthins(A), then betanin(B), isobetanin(C) and isobetanidin(D).

3.2.2 Pigment Separation using Liquid Chromatography

3.2.2.1 Sephadex G-15

3.2.2.1.1 Citrate pH 3.4

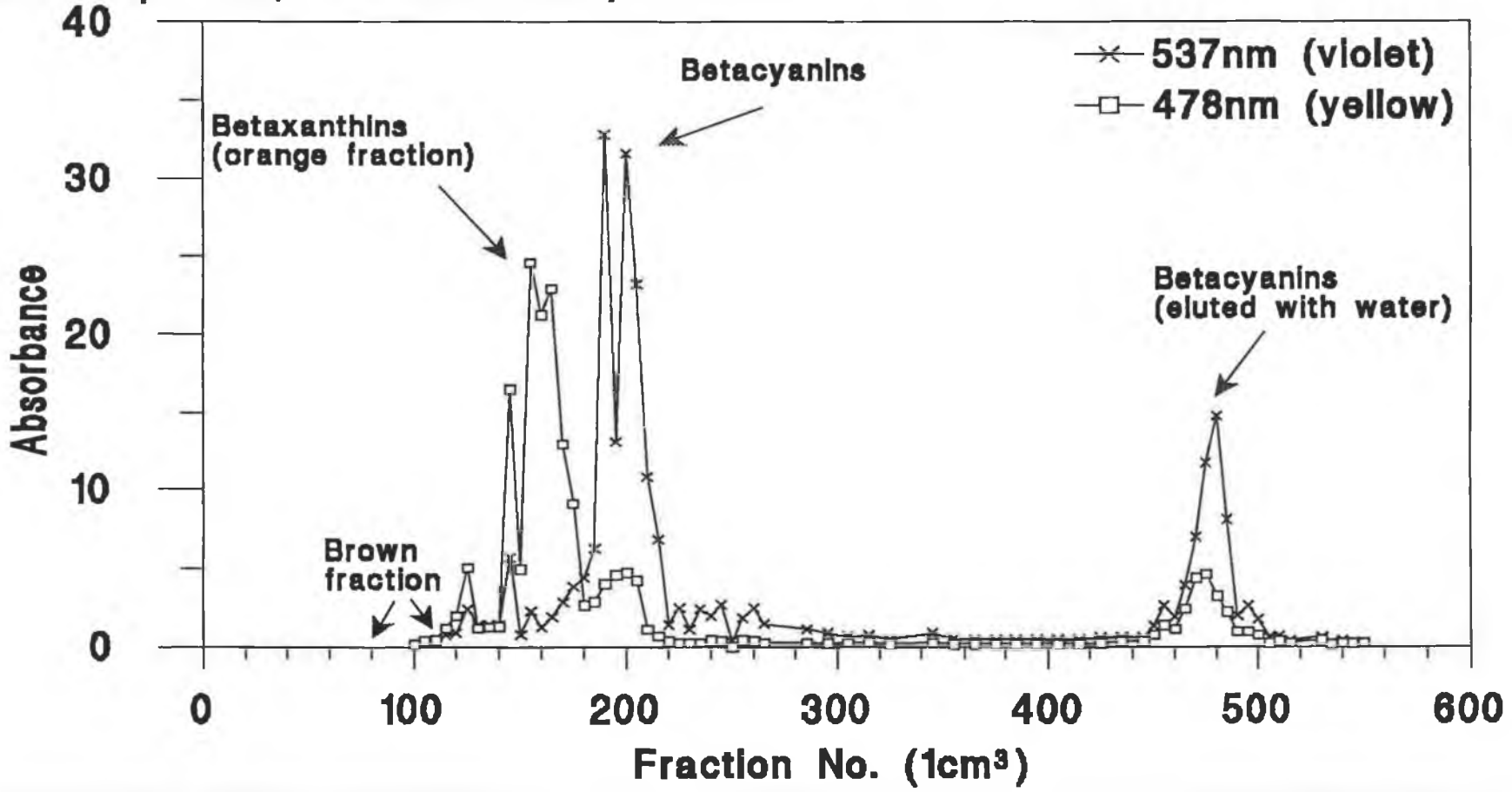
Adams J.P. & vonElbe J.H. 1977 found that Sephadex G-25 could be used to separate betalains via a certain amount of adsorption of the violet betacyanins while the yellow betaxanthins would elute off first. This exploits the charged nature of the gel in addition to its molecular sieving properties. This is contrary to the mode of action for which Sephadex was designed for that is molecular sieving. As Sephadex G-15 was readily available it was decided to use it instead of the G-25 as it has broadly similar properties with regard to molecular sieving and adsorption.

After trials with a 10mm by 100mm column, a 25mm by 650mm column(LKB, Pharmacia) was used. The results of elution of a betaxanthin enriched beet extract are shown in figure 3.2.2A. The elution profile was initially a light brown band, circa 35cm^3 (degradation products). This was followed by 41cm^3 of a brown orange band leading in to a 70cm^3 orange band. If the latter band was diluted it turned out to be the yellow betaxanthins. The next major fraction was a 150cm^3 red/violet band which is probably betanin. The violet pigments would eventually come off and, based on Adams J.P.& vonElbe J.H. 1977, is betanidin. This process could be speeded up by eluting with distilled water once all the yellow pigments had come off (fig. 3.2.2A). The concentrations of the betalains in the fractions was tentatively identified and quantified by their adsorption spectrum. The apparent presence of yellow betalains(max 478nm) in the last peak(375cm^3 fraction) results from the intersection of the absorption peaks of betacyanins and betaxanthins.

A clear separation between violet and yellow pigments was not possible for the bulk samples applied and quantities of end product wanted. In addition there remained the buffer salts to remove. This is particularly difficult given the molecular weights of betanin(549.5), betanidin(387.3), vulgaxanthin I(199.3) and vulgaxanthin II(226.3), and citrate buffer(critic acid 199.13, tri-sodium citrate 294.1). As a result the method was discontinued. Fig. 3.2.2B shows a HPLC analysis of the red/violet fraction. It appears that isobetanin(A) was isolated and that the other peak(B) is betanidin

Figure 3.2.2A

1g of the first 100% methanol extraction of crude freeze-dried red beet run through a 650mm by 25mm Sephadex G-15 column using, as running buffer, citrate pH 3.4, flow rate 1cm³/2.5mins.



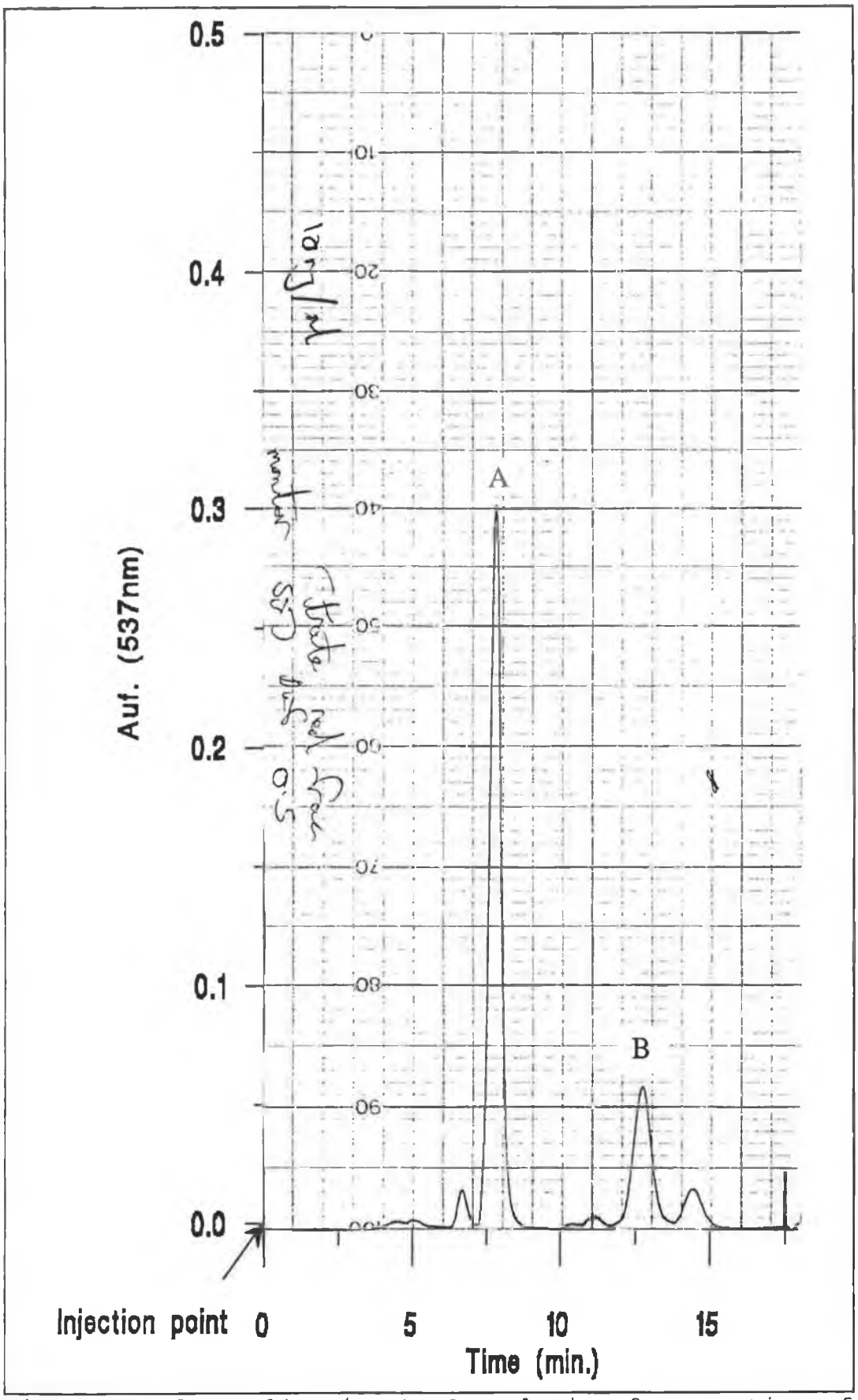


Figure 3.2.2B Qualitative HPLC analysis of separation of 100% methanol extraction of red beet run on Sephadex G-15 column eluted with citrate buffer pH = 3.5, red fraction, 20µl sample using a 20µl injection loop, betanin(A), betanidin(B).

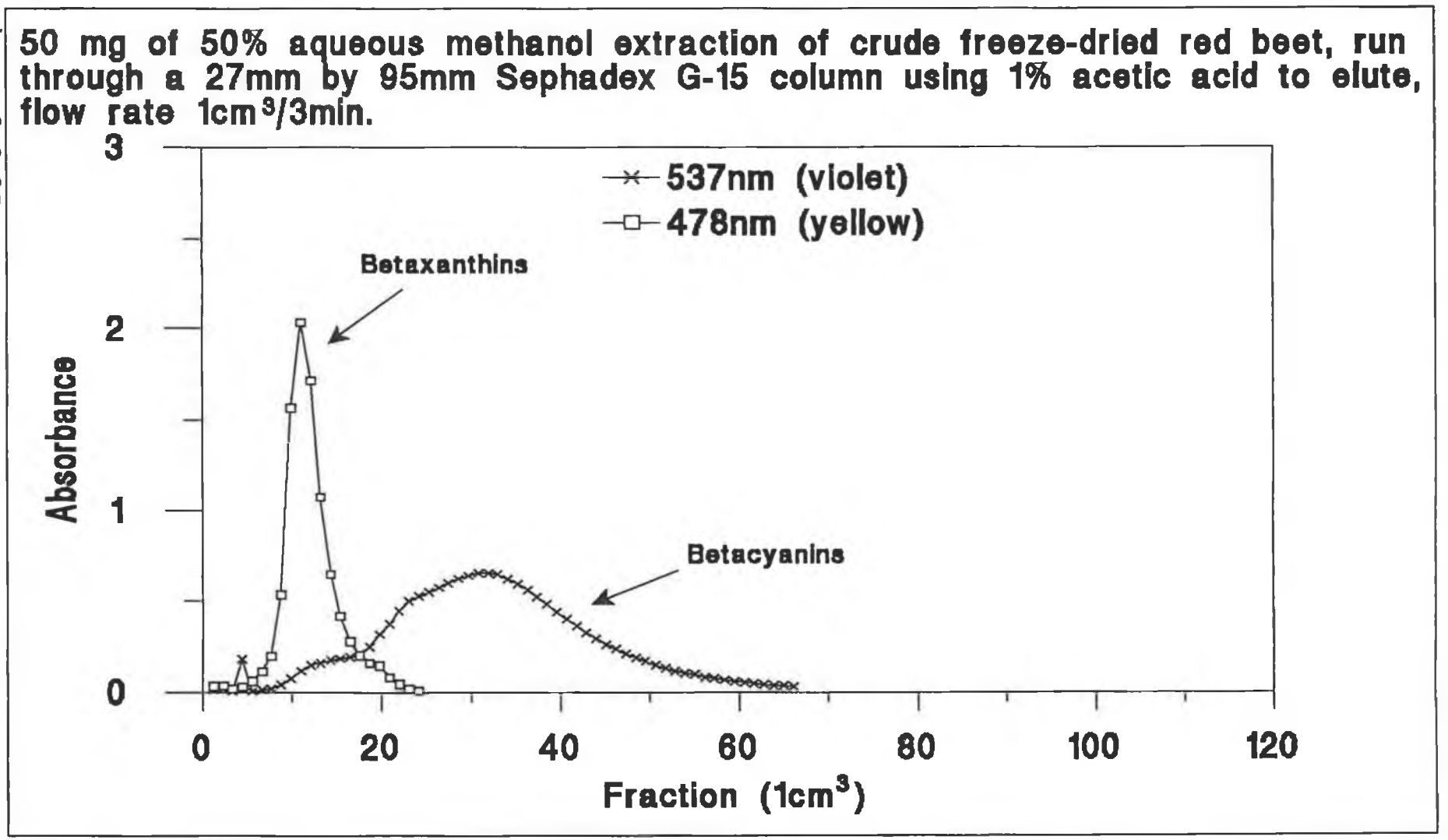
3.2.2.1.2 1% Acetic Acid

Literature (Altamirano R.C. *et al.* 1993) shows that Sephadex G-25 using 1.0 % acetic acid can be used to separate the yellow betalains from the violet. This method has the considerable advantage that the elution solvent is volatile and can be removed by rotary vacuum evaporation. Since Sephadex G-15 was readily available and, as mentioned above, has broadly similar properties with Sephadex G-25, it was decided to use this gel instead. The principle of separation is the same as in the previous column with a stronger initial adsorption of the violet pigments over the yellow resulting in the yellow betaxanthins eluting first followed by later elution of the violet betacyanins. However elution could be speeded up by applying water.

Figure 3.2.2C shows the results of elution of a 50% aqueous methanol extraction of freeze-dried red table beet using 1% acetic acid. The separation of betaxanthins and betacyanins was superior to that found with elution of citric acid (fig. 3.2.2A). It should be noted that some overlap of the peaks still occurs with appreciable encroachment of the red pigment peak into the yellow. Thus, while the method may be suitable for purification of betacyanins in high yields, contamination of the betaxanthin fraction by betacyanins makes the technique unsuitable for purification of betaxanthins. The technique, while showing improvements in separation over citric acid, needed further modification to improve separation.

Figure 3.2.2D shows a HPLC analysis of the violet fraction resulting from this method. The results do not tally with Schwartz S.J. & vonElbe J.H. 1980(fig.3.2.1A) in the sense that both peaks(A; betanin, B; betanidin) is a half to a minute late. However it was considered that given the available materials and apparatus and making the assumption that betanin is the major pigment in the fresh beetroot material, that this was going to be the result of the standard that was being purified.

Figure 3.2.2C


50 mg of 50% aqueous methanol extraction of crude freeze-dried red beet, run through a 27mm by 95mm Sephadex G-15 column using 1% acetic acid to elute, flow rate 1cm³/3min.
537nm (violet)
478nm (yellow)
Betaxanthins
Betacyanins
Absorbance
0
1
2
3
0
20
40
60
80
100
120
Fraction (1cm³)

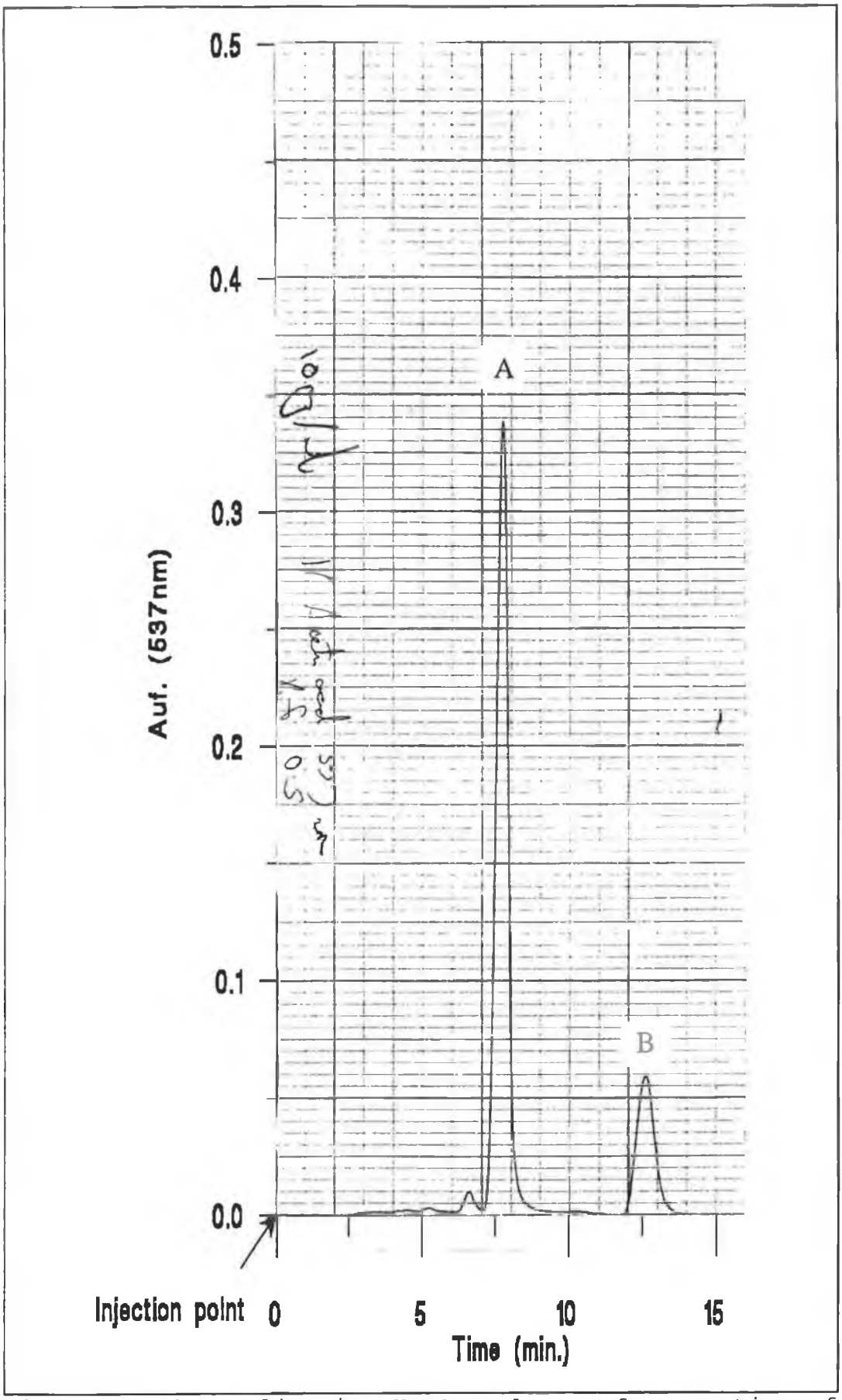


Figure 3.2.2D Qualitative HPLC analyses of separation of 50% aqueous methanol extraction of freeze-dried red beet on Sephadex G-15 eluted with 1% acetic acid, 20µl sample using a 20µl injection loop, betanin(A), betanidin(B).

3.2.2.1.3 0.1% HCl

The literature shows that 0.1% HCl can be used to crystalise pure betanin (Schwartz S.J. & von Elbe J.H. 1980) and that it in conjunction with Sephadex (Pasch J.H. & vonElbe J.H. 1979) could be used for pigment separation with possibly greater resolution. It was thus decided on using 0.1% HCl instead of 1% acetic acid as an elution buffer.

A similar effect was obtained in relation to the elution profile(Figure 3.2.2E). Separation was greatly superior to that of 1.0% acetic acid and clear separation of the major groups of pigments was achieved.

The technique however has one drawback. The adsorption effect could not be reproduced immediately after washing with 0.2M NaOH, then distilled water and finally equilibrating with either 1.0% acetic acid or 0.1% HCl. It was found that the Sephadex gel had to be left in 0.2M NaOH for at least two weeks before using again.

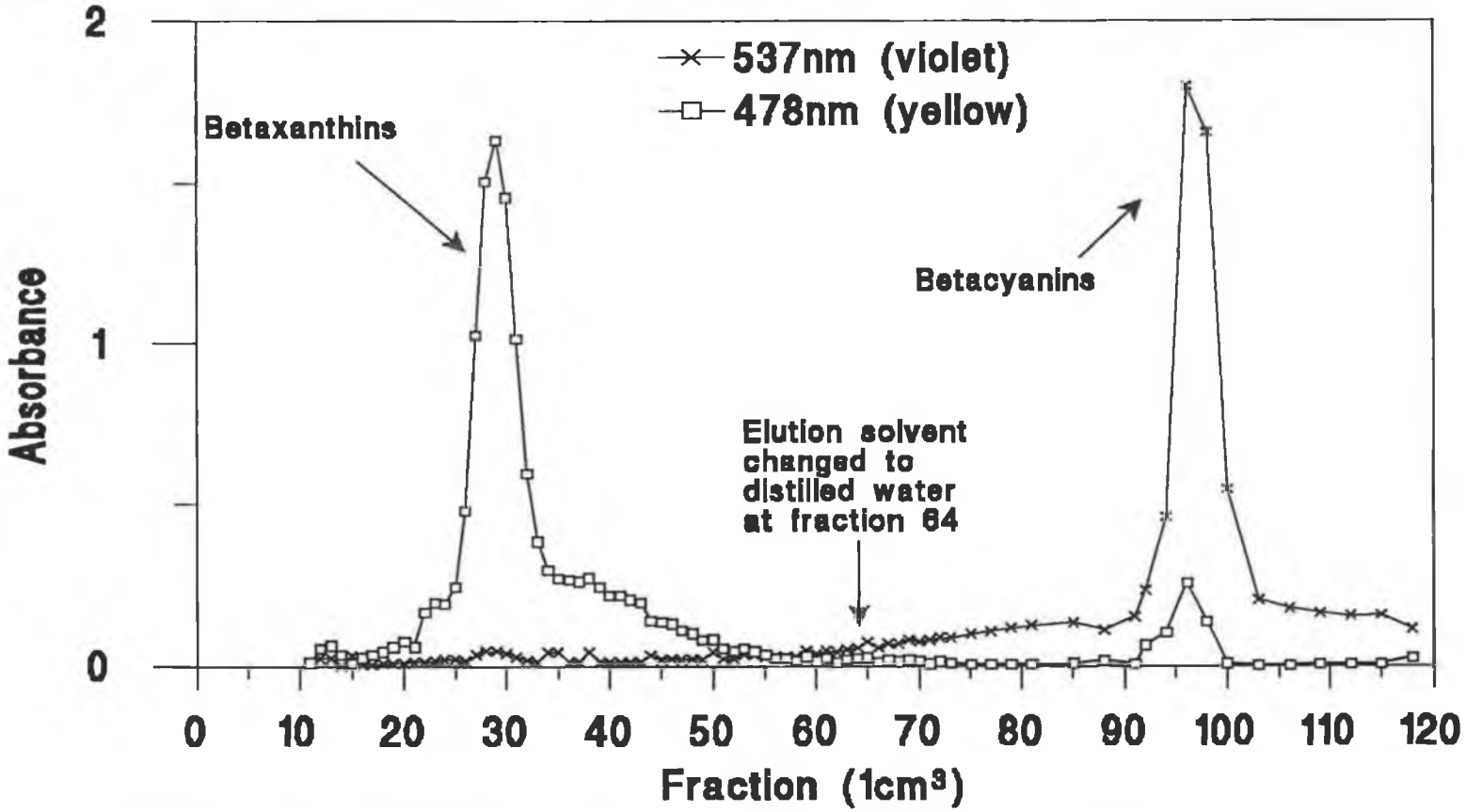
Figure 3.2.2F shows an HPLC analyses of the violet fraction resulting from 0.1% HCl elution on Sephadex G-15. Here the same results and reasons apply as in fig. 3.2.2D namely peak A represents betanin and peak B represents betanidin.

Note

Figures 3.2.2A-I All samples were dissolved in the equilibrating solution that is in citrate buffer, 0.1% HCl, 1.0% acetic acid or CH_3OH/0.05M KH_2PO_4 (18:82 v/v) depending on the column. In all cases the dissolved samples were spun down for 3 minutes at 14926g so as to precipitate any brown degradation products of the pigments and other unidentified suspended solid. This would reduced the amount of clogging of columns and deposition.

Figure 3.2.2B

50 mg of 50% aqueous methanol extraction of crude freeze-dried red beet, run through a 27mm by 95mm Sephadex G-15 column, eluting with 0.1% HCl then water once the yellow pigments had come off, flow rate 1cm^3/3min.



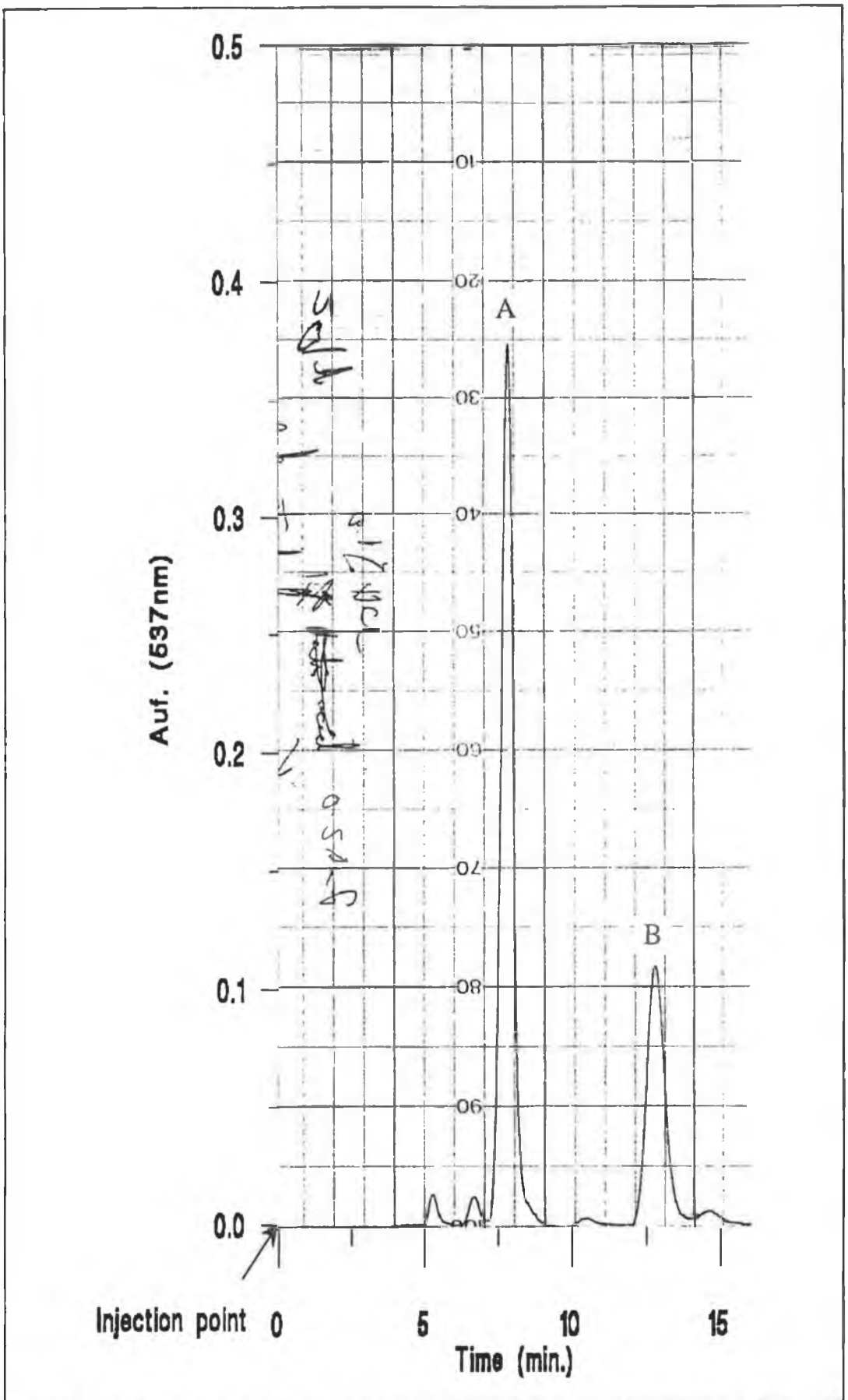


Figure 3.2.2F Qualitative HPLC analyses of separation of 50% aqueous methanol extraction of freeze-dried red beet on Sephadex G-15 eluted with 0.1% HCl, 20µl sample using a 20µl injection loop, betanin(A), betanidin(B).

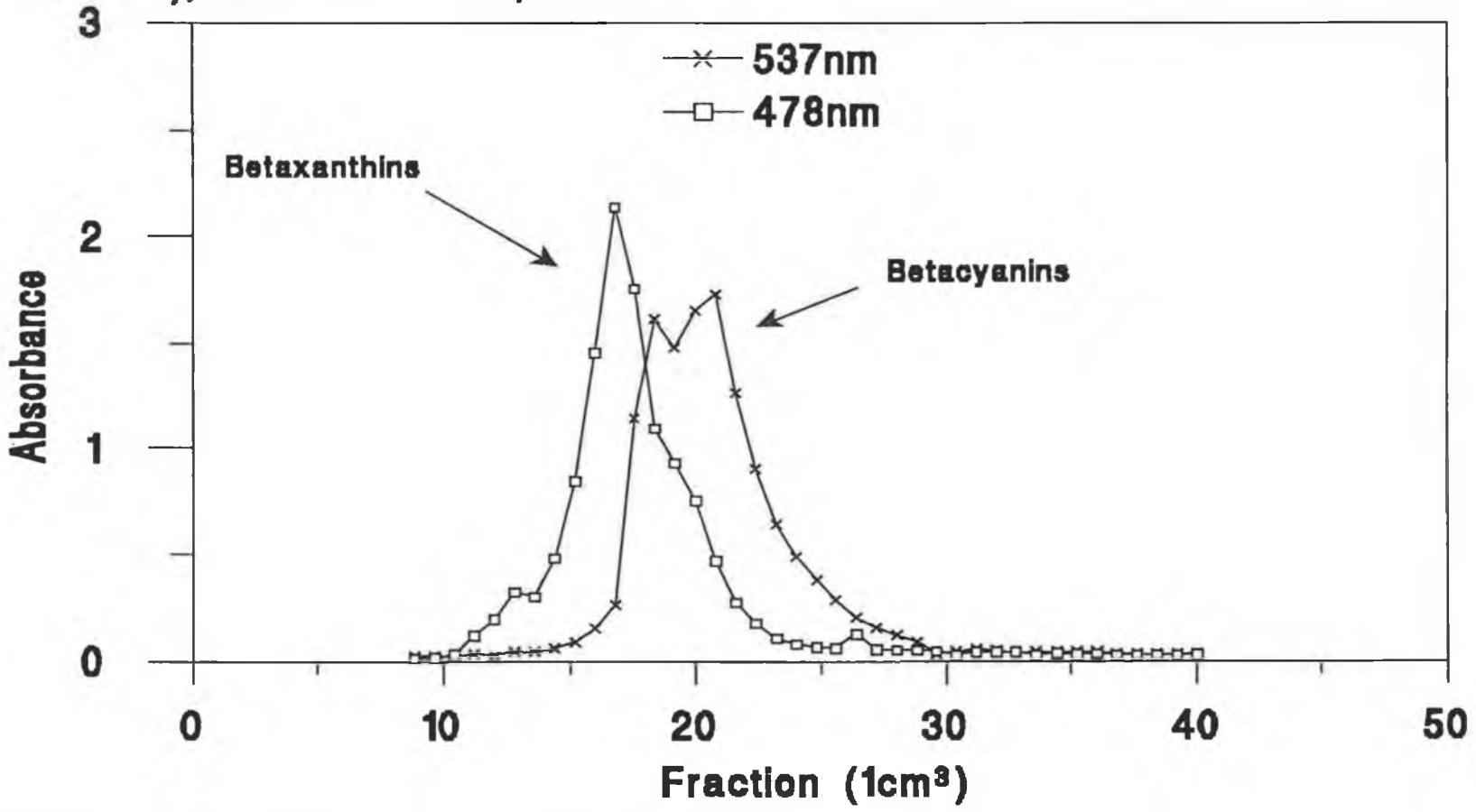
3.2.2.2 Cellulose 20μm

Initially during freeze-drying of semi-purified pigments it was found that total drying could not be obtained. It was thought that this might be due to large quantities of sugar still remaining in the freeze-dried aqueous methanol extractions of crude red beet. Literature survey indicated that separation of both sugars and pigments could be obtained using cellulose T.L.C. plates (Bilyk A. 1981). In this technique sucrose binds weakly to cellulose and always elutes ahead of the pigment. It was realised that for the purposes of practicality it would be desirable to carry out this separation using a column. This would avoid extracting the pigments from the cellulose on the plates thus reducing steps in the extraction procedure. Determination of the required solvents was carried out on T.L.C. plates. The final solvent system was 50% ethanol, 45% water and 5% acetic acid (Acknowledgements for much appreciated help to Deirdre O'Sullivan, technician 1991/1992).

Figure 3.3.2G shows the results of trials using cellulose in the form of a column. This system succeeded to a limited extent with a small column(22mm by 30mm). One could see a neat separation of the yellow pigments from the violet which tended to adsorb longer to the cellulose bed. The distance between the separated pigments was found to be narrow thus good separation could only be obtained if the column was poured perfectly. Despite this system working for the small column it did not work, as hoped, on a larger scale(70mm by 90mm) when bulking up was needed. This is because, given the nature of the cellulose used(20μm), the bigger the column, the slower the process. Secondly if pressure was used hard packing became a problem. Thirdly it was observed that the sugars tended still to come off with the yellow vulgaxanthins. This was observed on removal of the solvents via rotary vacuum evaporation and freeze-drying. One noticed a white appearance and a sugar texture. Figure 3.2.2H shows a HPLC analysis of the yellow fraction eluted from a cellulose column. It is thought that peak represents the vulgaxanthins based on a comparison of the chromatographs published by Vincent K.R. & Scholz R.G. 1978. The minor peak that follows may be the result of violet which did not separate from the betaxanthins.

Figure 3.2.2G

50 mg crude freeze-dried red beet dissolved in eluting solvent mix, run through a 27mm by 32mm cellulose column, eluting with solvent mix(ethanol:water:acetic acid 5:4:1), flow rate 1cm³/20min.



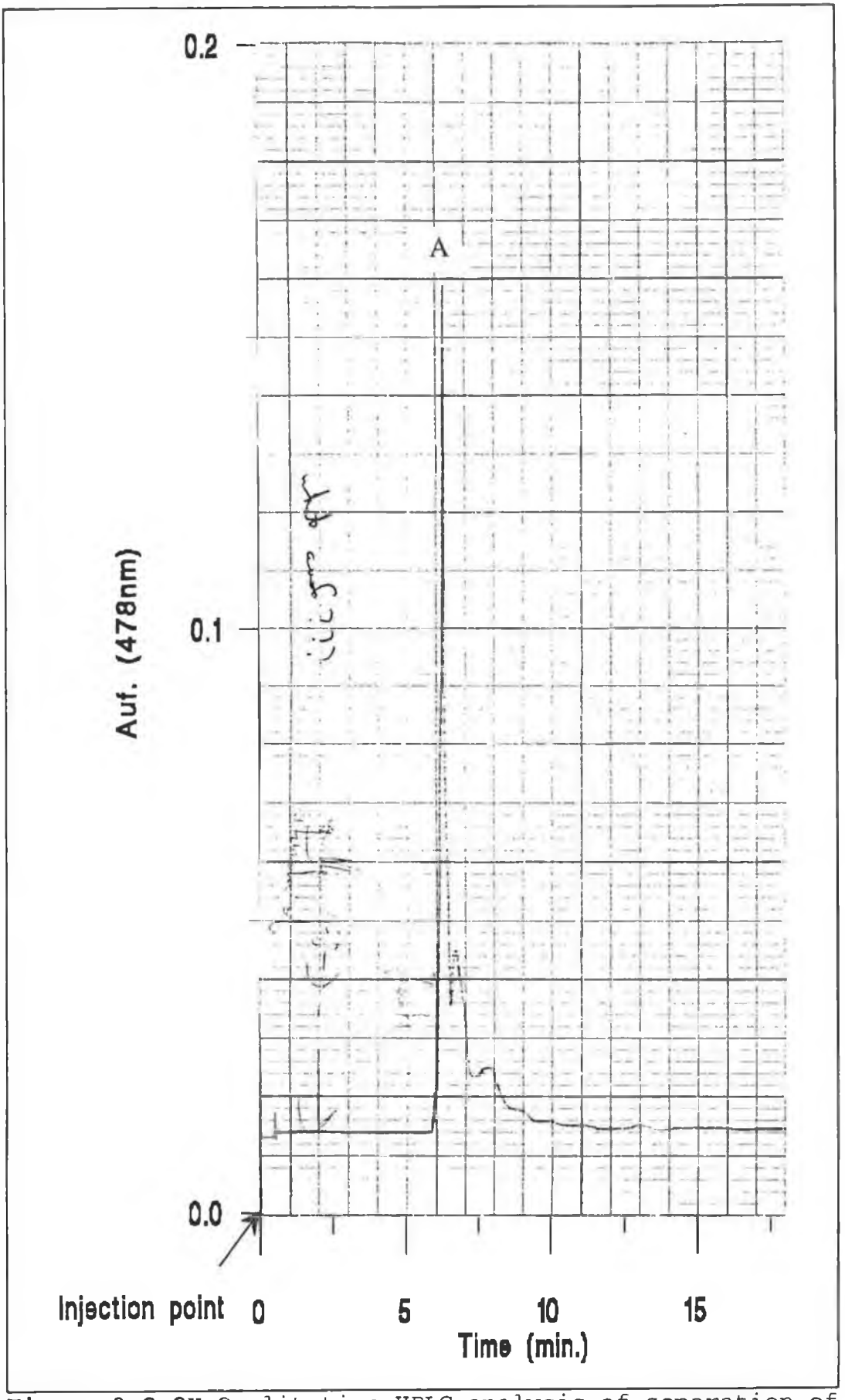


Figure 3.2.2H Qualitative HPLC analysis of separation of 50% aqueous methanol extraction of freeze-dried red beet on cellulose(20µm) column, yellow fraction, 100µ sample using a 1cm^3 injection loop, Vulgaxanthin ?(A)

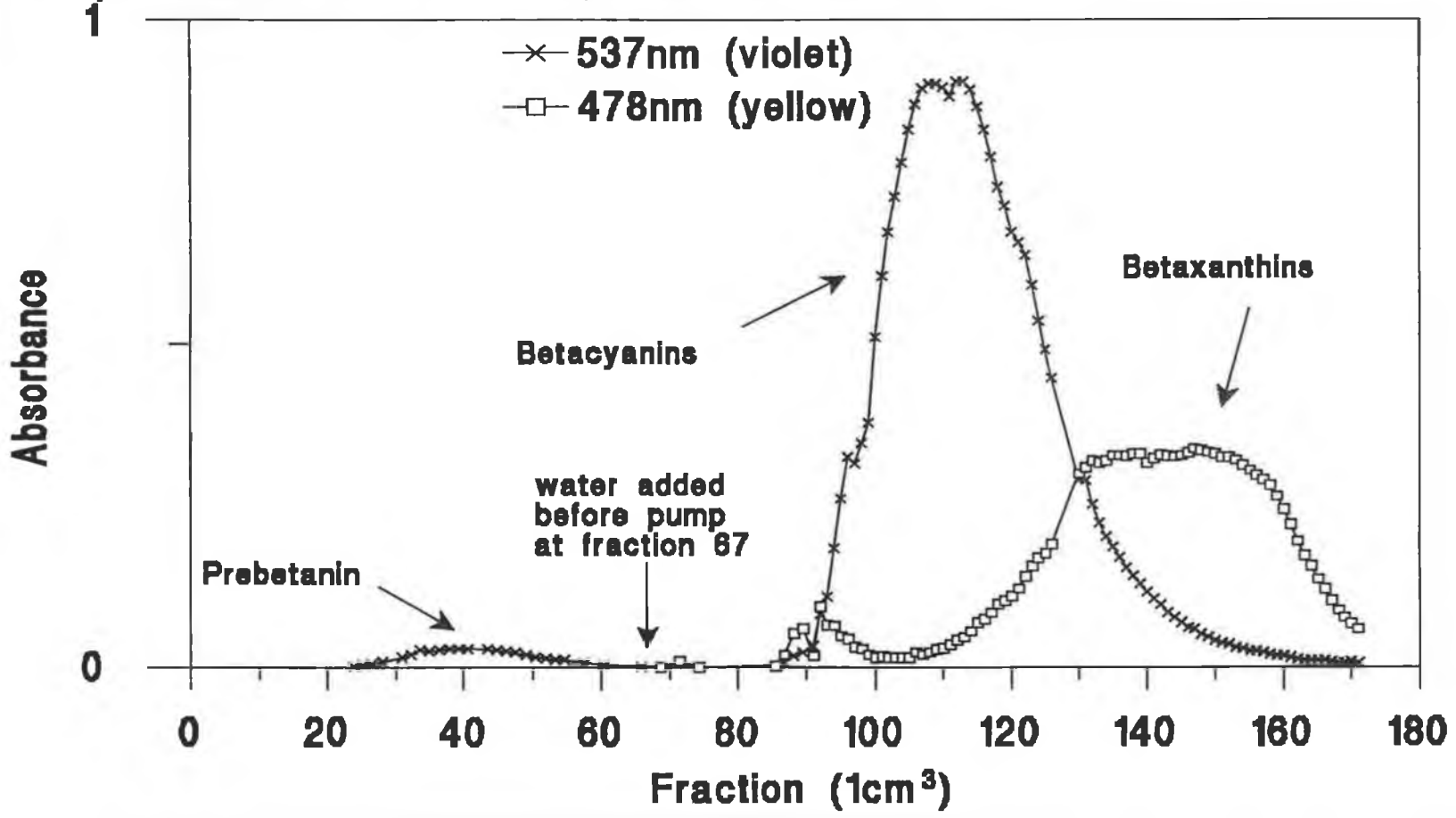
3.2.2.3 Dowex H^+

It was noticed in the literature survey that Dowex was used for the separation of pigments. Since this gel was available it was decided to investigate its potential for pigment separation. The principle of separation is somewhat different to what has been used up until now. Dowex is a strong ion-exchange matrix. It will bind molecules that have on overall negative charge. Once all other compounds of different charge have been eluted, the pH of the column can be changed by altering the pH of the eluting buffer. This results in a general change in the pH environment causing the desired molecules to disassociate from the Dowex matrix.

Figure 3.2.2I shows the results of 50% aqueous methanol extraction run through Dowex H^+. In this system the small amounts of prebetanin and isoprebetanin came off first(40cm^3 fraction) as predicted by Piattelli M. 1964. Then after changing the eluting solution from 0.1% HCl to distilled water the rest of the pigments came off. There was no clear separation from the yellow betaxanthins which came off on either side of the violet betacyanins. Despite this it was decided to use this system as a purification step in a two-stage protocol to separate prebetanin and isoprebetanin from betanin and vulgaxanthin.

Figure 3.2.2I

50 mg of 50% aqueous methanol extraction of crude freeze-dried red beet, run through a 25mm by 45mm Dowex-50W column eluting with 0.1% HCl then water once prebetanin had come off, flow rate 2cm^3/min.



3.3 Development of an Inducible Model System

The remaining sections of results are concerned with the development of an inducible model system and the elucidation of a possible rate-limiting step in the model system's biosynthetic pathway. The model system chosen was the production of betalain pigments in red beetroot in callus culture. Nutrient medium, light quantity and quality were considered as inducing agents. The elucidation of a possible rate-limiting step consisted of feeding different precursors at different concentrations. This will be looked at in the last section.

3.3.1 Change of Nutrients

3.3.1.1 Pgo to B5

A number of preliminary experiments were carried out to assess callus growth and pigment production of different salt cocktails. It was noticed during a transfer from Pgo to B5, when initiating examination of B5 as a salt cocktail, that there was inhanced pigment production. This suggested that an alteration in nutrient constituents could enhance pigment production. If one compares the contents of these two salt cocktails (section 2.2.1) one notices that there are differences in the levels of Cl^-, K^+, and NO_3. Stobart A.K *et al.* 1980 report the enhancment of pigment accumulation by K^+ ions in seedlings of *Amaranthus caudatus* L. and Sakuta M. *et al.* 1987 observe that betacyanin accumulation is related to nitrate uptake in *Phytolacca americana.* It was decided to quantify this observed pigment induction. Initially this would entail repeating a Pgo to B5 medium transfer followed by examining individual medium constituents. Figures 3.3.1.1A-J show the results of this experiment. Growth and pigment accumulation were quantified in callus grown on Pgo medium and after transfer to B5.

Figures 3.3.1.1A-J 30mg of callus were plated on to solidified agar containing either the Pgo or Gamborg's B5 cocktail of mineral salts. All values are a mean of 10 pieces of calli and include the standard error of the mean. 5 calli pieces per Petri dish in replicate was used per treatment. All experiments were set up as described in section 2.3.5. The plates were kept either in the light at 10-14 $\mu mol/m^2/s$ or the dark. Calli were harvested at weekly intervals and pigment extracted.

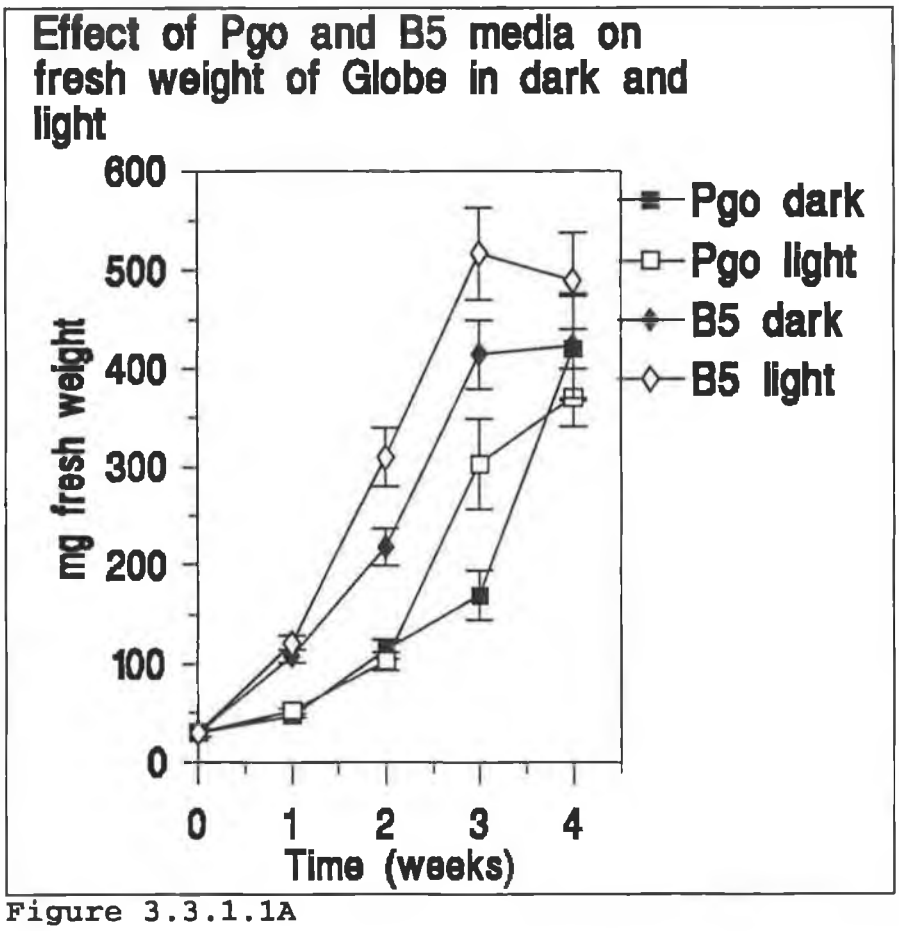


Figure 3.3.1.1A

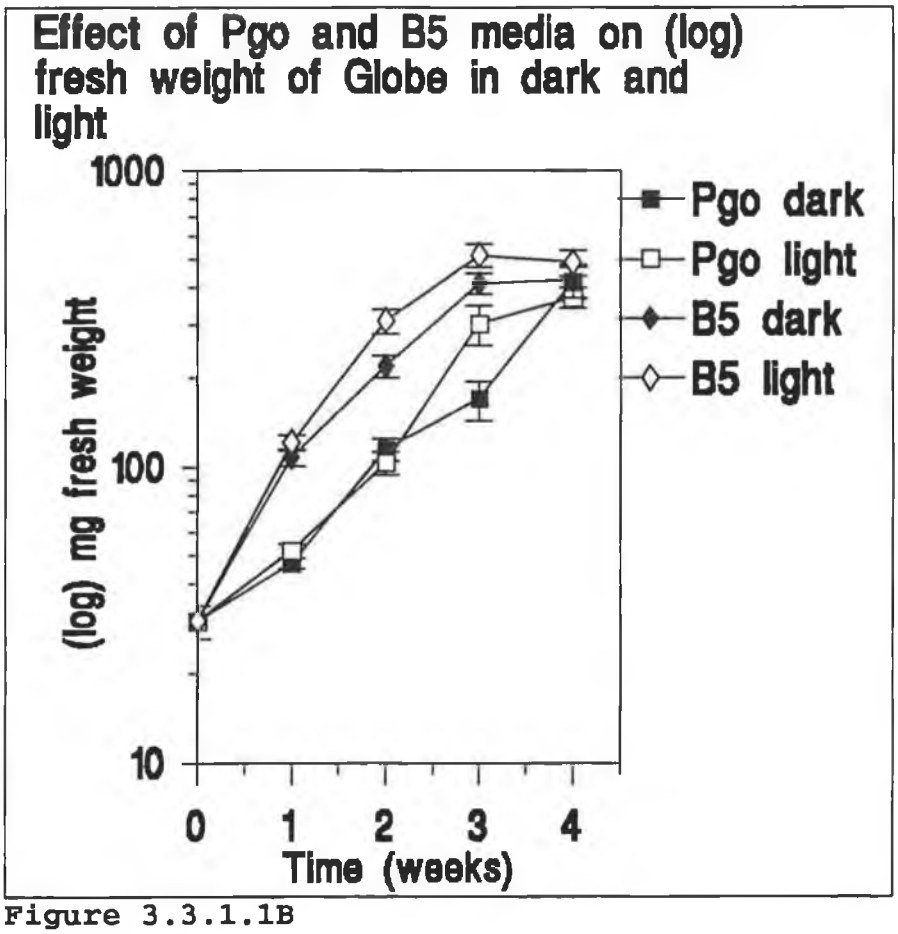


Figure 3.3.1.1B

Initial observations are that

A. Fig. 3.3.1.1A shows the results of assessments of the callus fresh weight. It was found that callus on B5 medium grew more quickly than that grown on Pgo and that growth was slightly more rapid in light than in dark.

   By plotting this data on a log scale (fig. 3.3.1.1B), the different growth phases can be identified, in particular a period spoken of as the logarithmic phase of multiplication (phase of exponential increase) whose identification is made easier to assess by the appearance of a straight line.

   Fig. 3.3.1.1B shows exponential growth over the first 2 weeks of culture for all cultures. The growth rate then declined, particularly for B5. This decline is presumably due to a depletion of nutrients in the medium which is only sufficient to support growth of callus up to approximately 450mg fresh weight per callus after which a decline in accumulation sets in.

B. In Figs. 3.3.1.1C & E one sees that total pigment per callus values, both for betanin and vulgaxanthin, were either constant or decreased in the dark while it increased in the light up to three weeks of culture. This corresponds to the period of active exponential growth in both Pgo and B5 (Fig. 3.3.1.1B).

   Again the results of Figs. 3.3.1.1C & E have been plotted on a log scale to facilitate an examination of production rates in Figs. 3.3.1.1D & F. Exponential increase in total pigment accumulation per callus can be detected only in Pgo in the light over the first 3 weeks.

C. Figs. 3.3.1.1G & H, showing the specific betanin and vulgaxanthin accumulations, reinforce the observations noted from the total pigment patterns. This is because total pigment in the dark is more or less constant but since the callus is still growing the existing pigment is being diluted out. As a result the specific pigment, that is pigment per unit callus, decreases. In the case of light, pigment production not only keeps up with growth but outstrips it up to 70-80 nmol/mg fresh weight. After this point specific pigment levels fall, co-incident with a decline in growth rate (fig. 3.3.1.1B). The difference in week 1 values, that is dark higher that light, reflects the influence of light. It suggests that light caused the increase in specific fresh weight accumulation to outstrip specific pigment during the first week of illumination. In other words growth was favoured to pigment accumulation. If one looks at Figs. 3.3.1.1I-J, which shows specific weekly accumulation of betanin, vulgaxanthin and fresh weight, one notices specific pigment accumulation decreases at the same time as specific fresh weight accumulation. The fact that specific

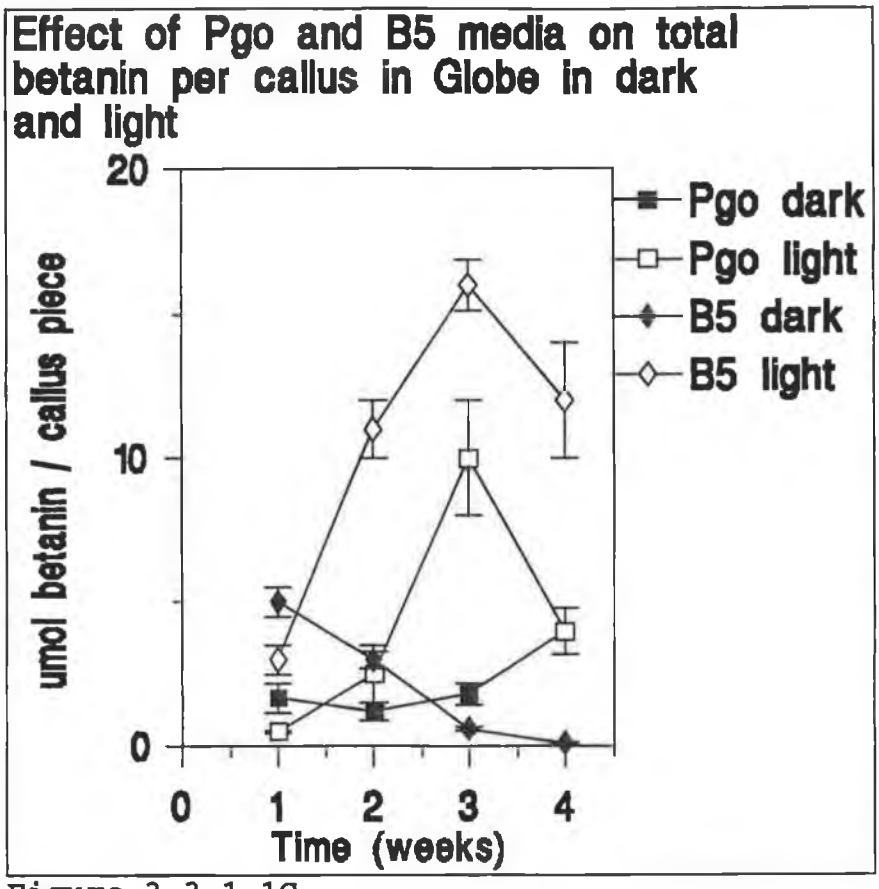


Figure 3.3.1.1C

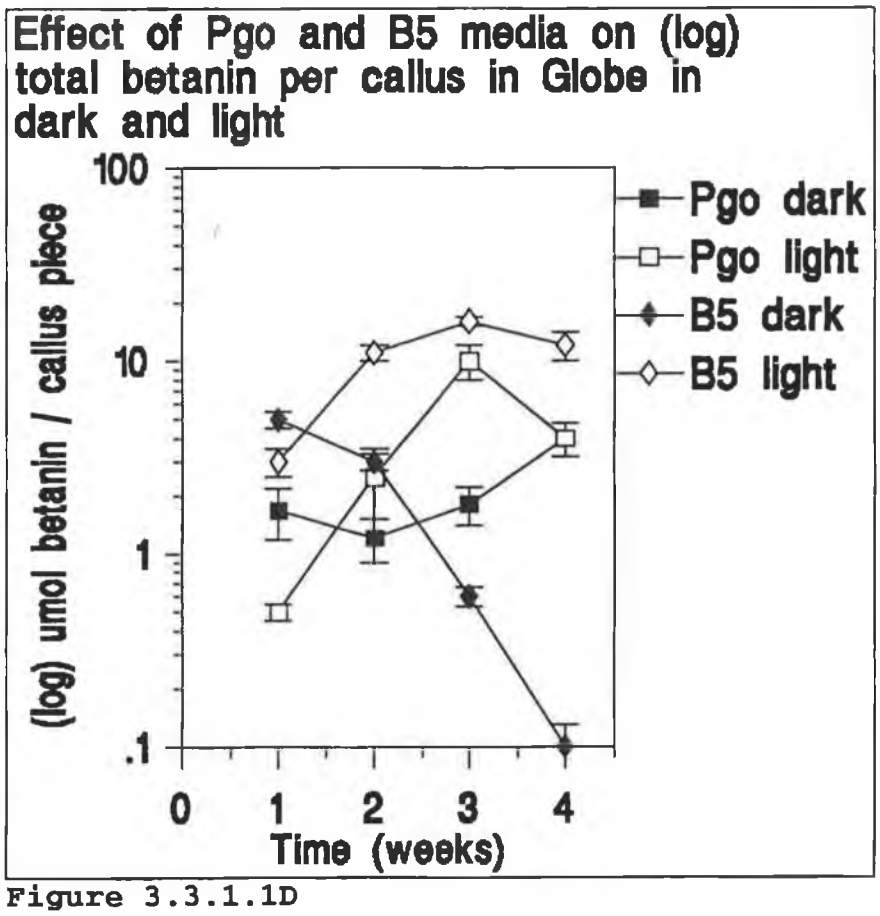


Figure 3.3.1.1D

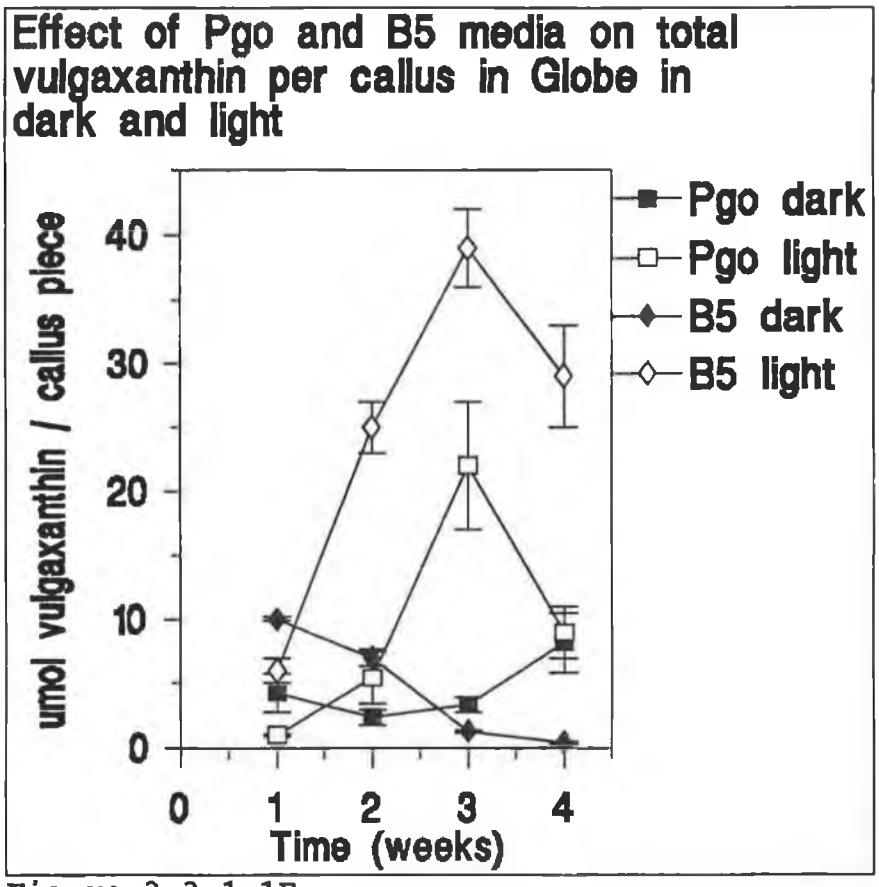


Figure 3.3.1.1E

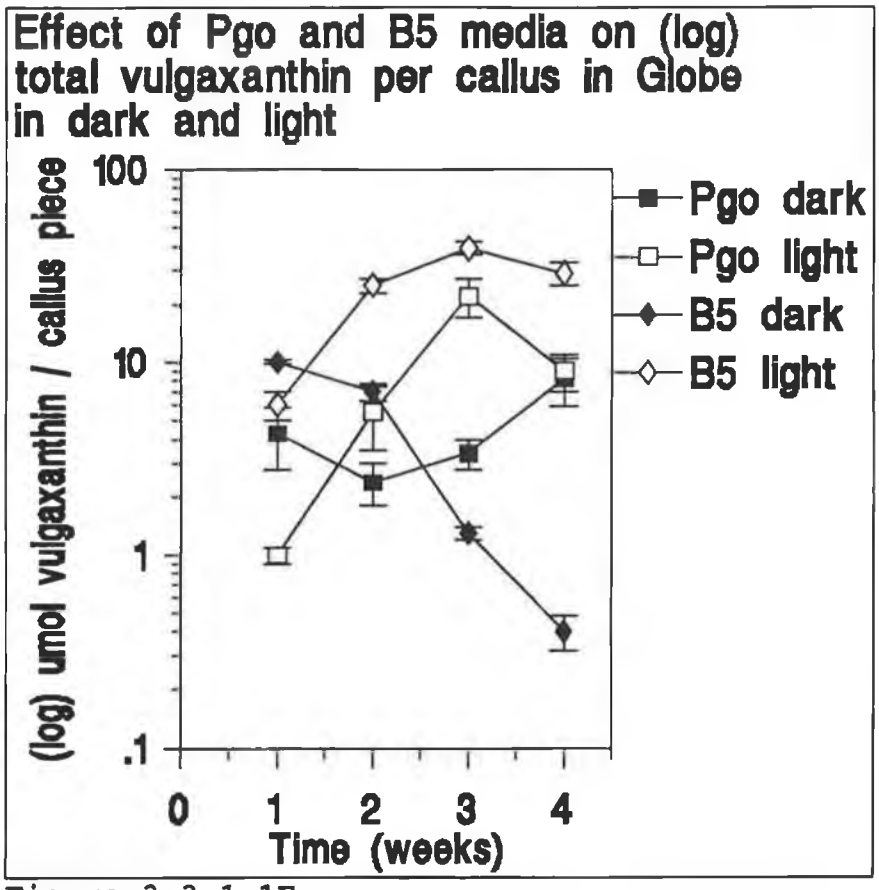


Figure 3.3.1.1F

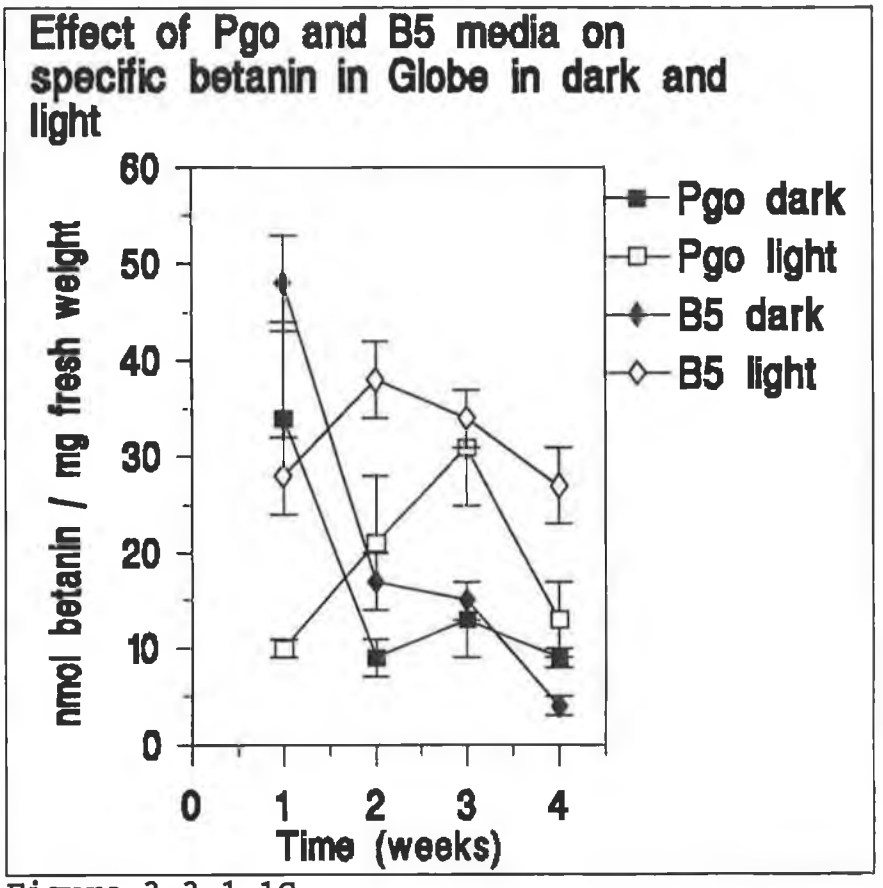


Figure 3.3.1.1G

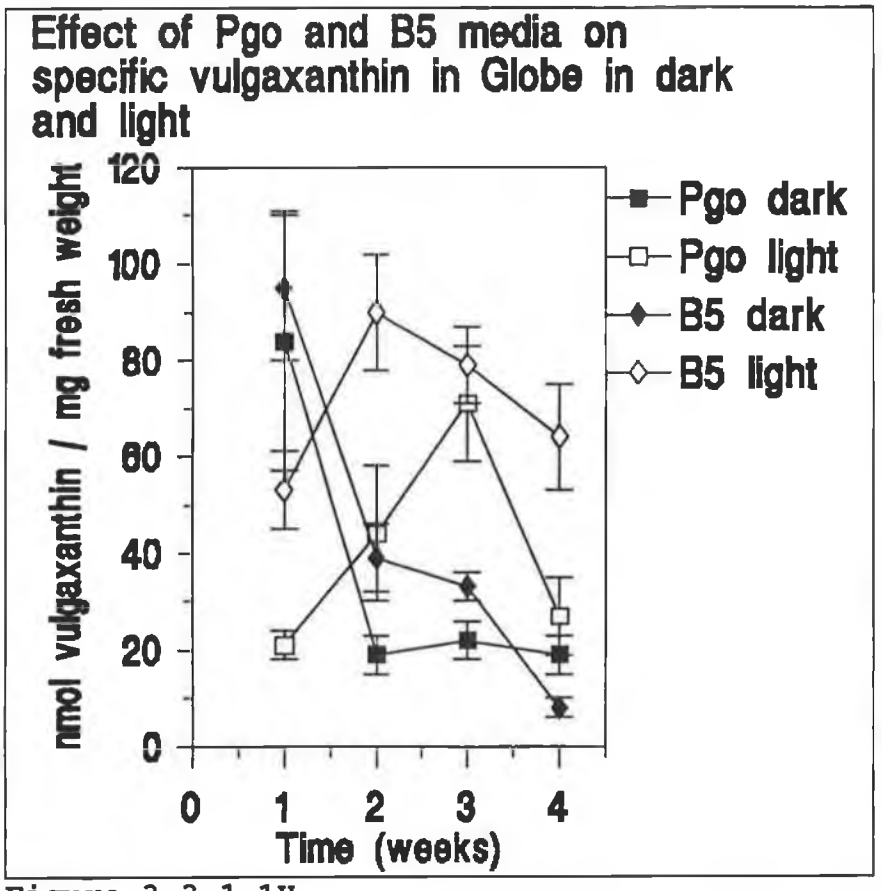


Figure 3.3.1.1H

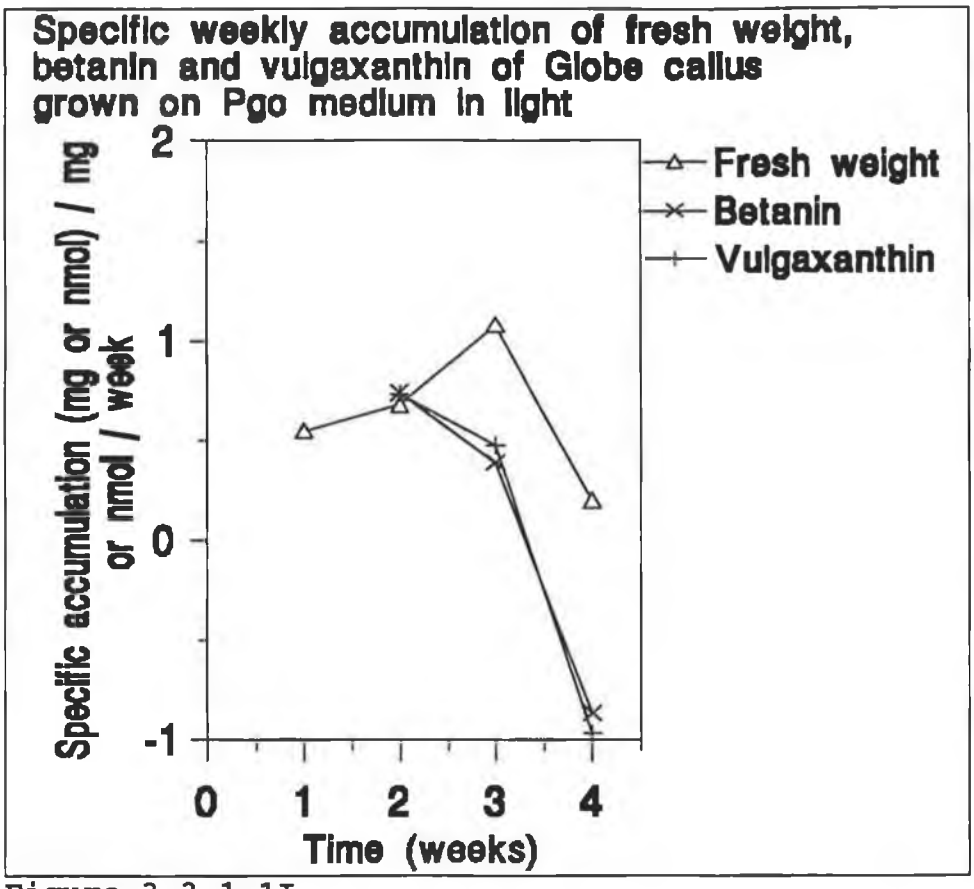


Figure 3.3.1.1I

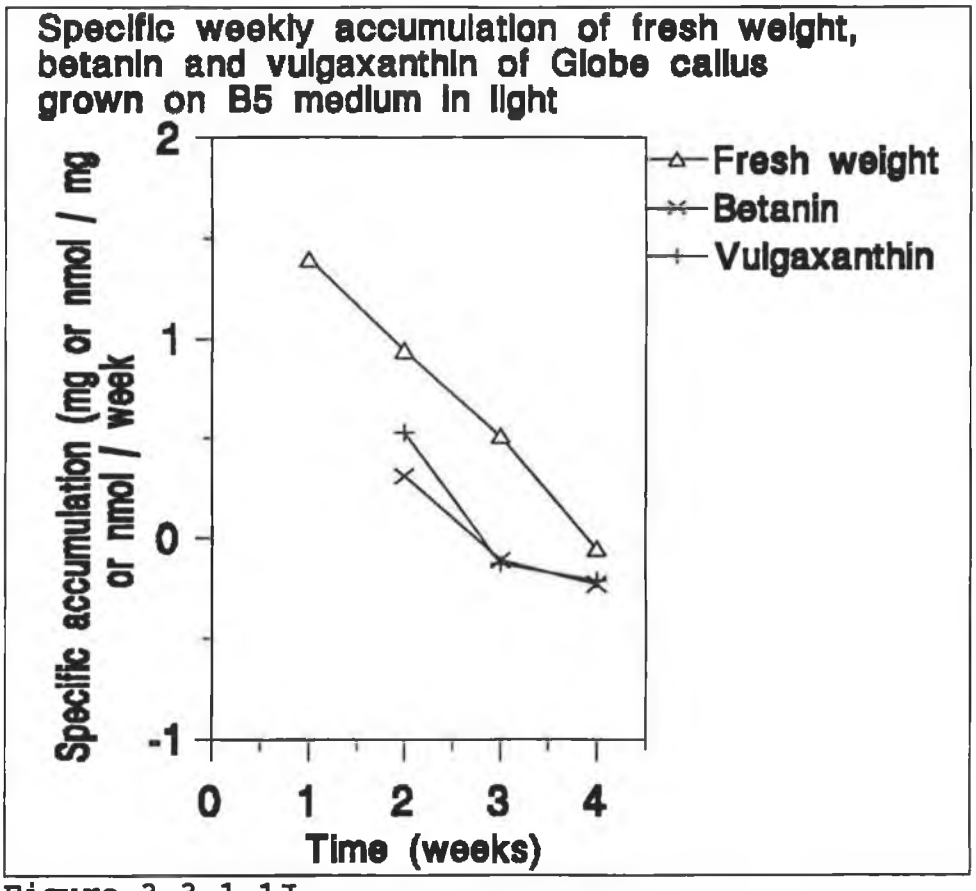


Figure 3.3.1.1J

pigment accumulation does not rise, except for B5, indicates that pigment accumulation is related to growth.

Despite callus growth and total pigment per callus production fairing better on B5, the two media produced similar results with pigment production only in the light (figs. 3.3.1.1G-H) and related to growth. Thus, transfer to B5 medium as a method of induction of pigment accumulation is not a useful technique for specifically promoting this accumulation. However the comparison of dark and light curves shows that light could be considered as an inducer/enhancer of pigment production. The apparent decrease in both specific and total pigment per callus accumulation in the light after 3 weeks is probably due to pigment degradation in the callus pieces which are in linear/stationary phase at this time. This emphasizes the importance of examination of growth and pigment production in the actively growing phases of the culture cycle. The light induction effect can be seen again when callus kept in the dark for three weeks is exposed to light (figs. 3.3.1.2A-F).

3.3.1.2 The Effect of Prolonged Dark Incubation

Since the transfer of calli can result in momentary traumatism due to the disruption of an equilibrated environment it was thought appropriate to examine whether or not preincubation in the dark would result in a higher pigment accumulation. This was based on the concept that a certain amount of biomass needs to be attained or a set stage of developement reached before accumulation of pigment can occur (Constabel F. & Nassif-Makki H. 1971).

Figures 3.3.1.2 A-F The experimental setup was as described for the previous experiments namely 5 pieces of calli per plate two plates harvested per week. In the case of the dark to light treatment two plates were left in the dark for 3 weeks then were transferred to light for a week. Calli were harvested at weekly intervals and pigment extracted.

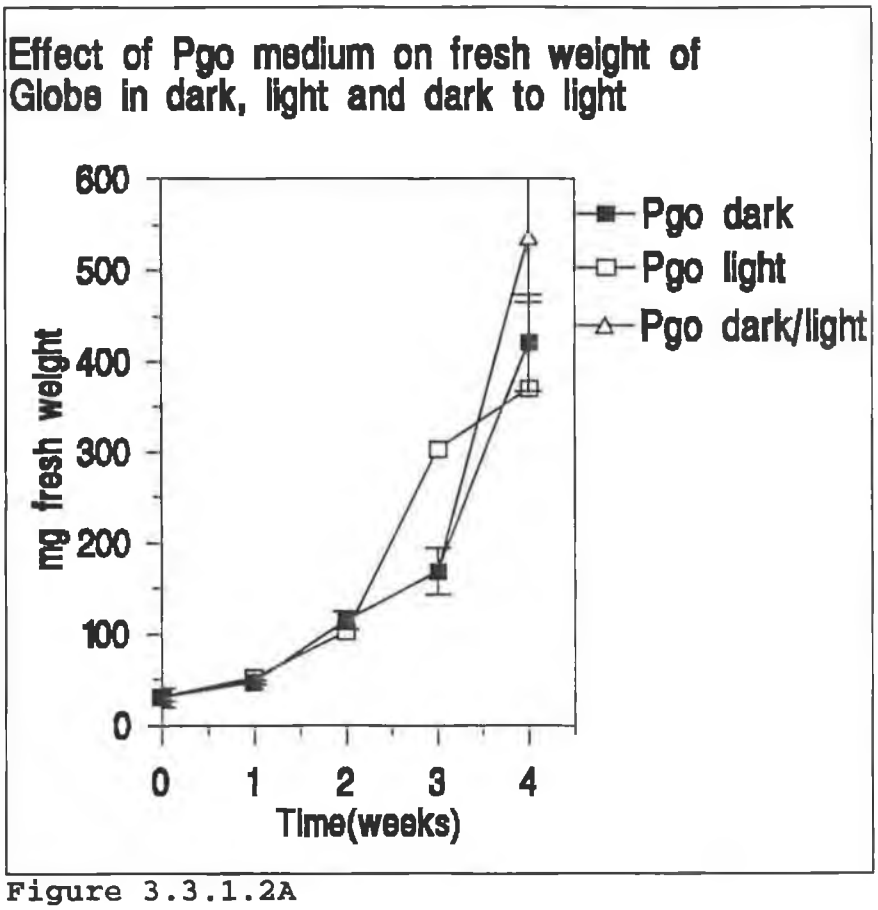


Figure 3.3.1.2A

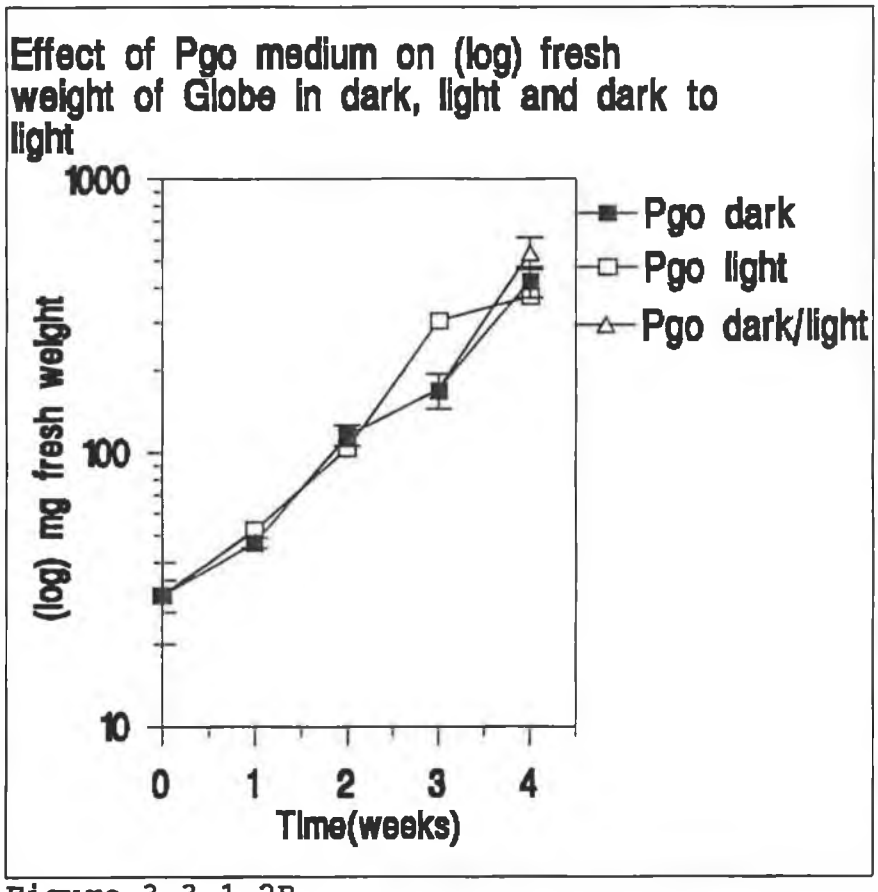


Figure 3.3.1.2B

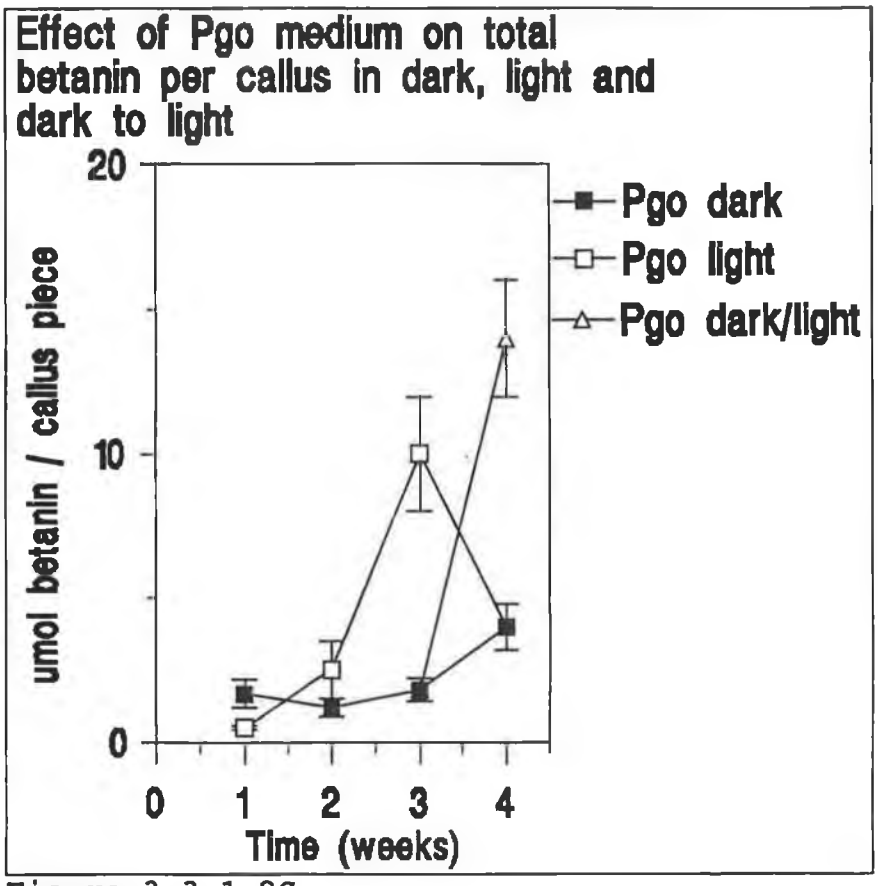


Figure 3.3.1.2C

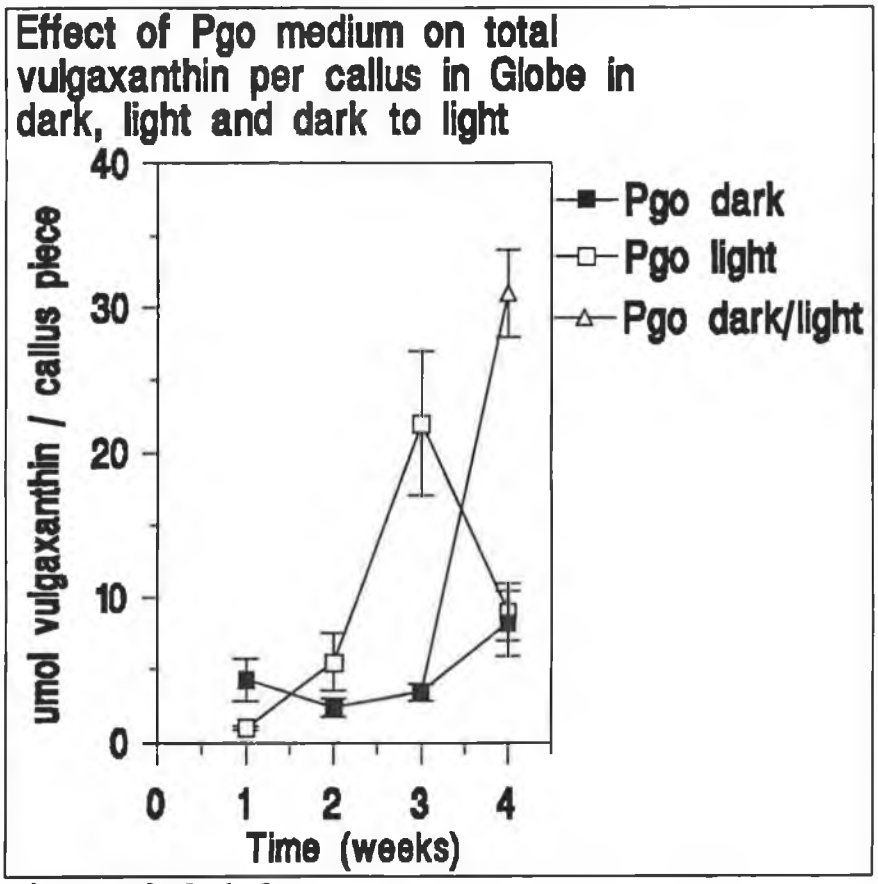


Figure 3.3.1.2D

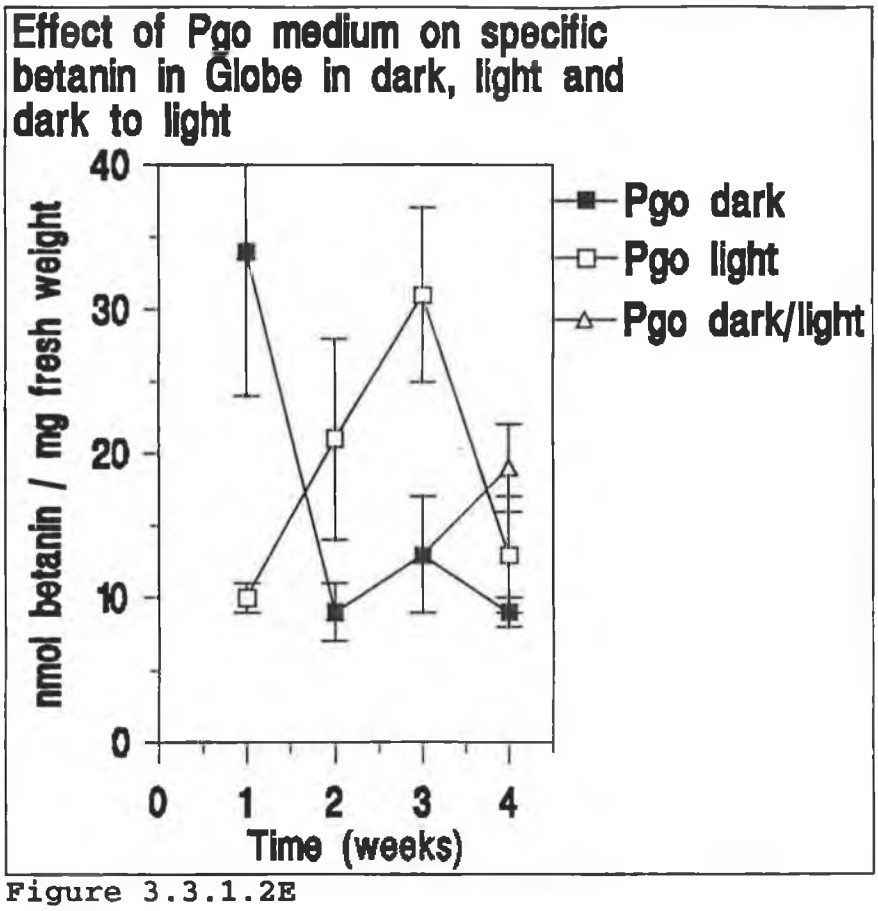


Figure 3.3.1.2E

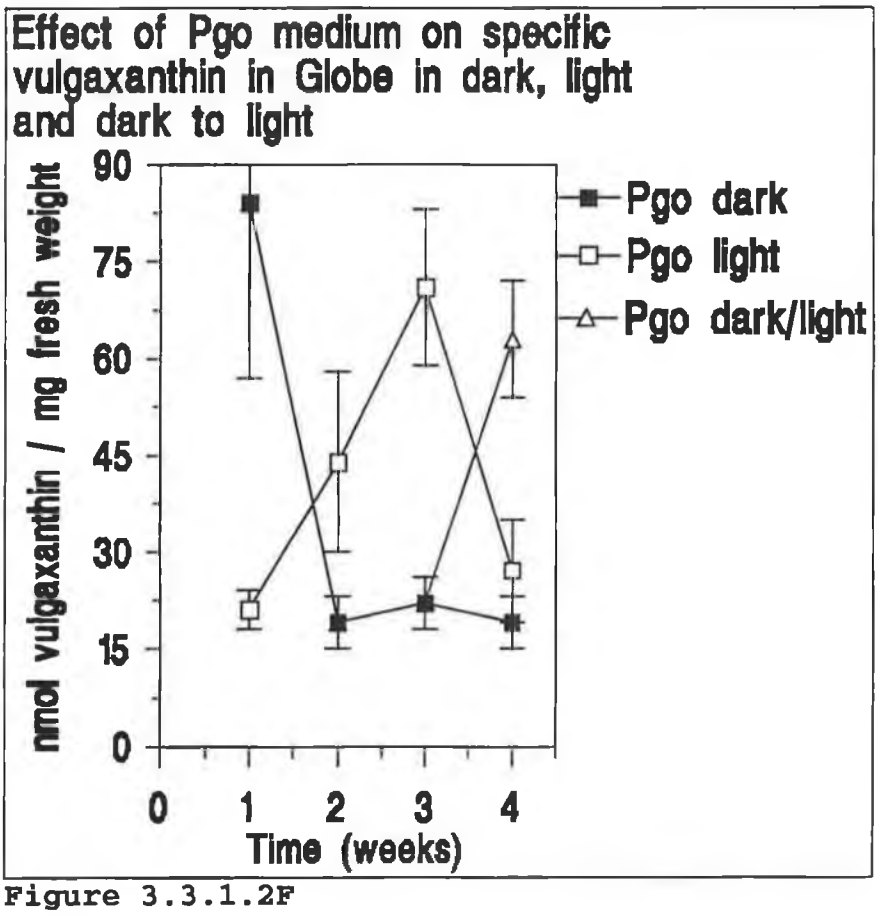


Figure 3.3.1.2F

One notices that the response of one week light after 3 weeks dark gives a higher fresh weight accumulation (fig. 3.3.1.2A) as well as a higher total pigment per callus accumulation, whether it be betanin (fig. 3.3.1.2C) or vulgaxanthin (fig. 3.3.1.2D).

In the case of specific accumulation, levels of vulgaxanthin after one week light preceded by 3 weeks dark is practically the same as 3 weeks light (fig. 3.3.1.2F).

In conclusion, these experiments demonstrate the importance of examining pigment production in actively growing cultures and the possibility of light induction of pigment.

3.3.1.3 Phosphate Omission

It has been found that light can enhance pigment production. This can be considered a good inducer of pigment accumulation. However during the Pgo to B5 experiments it was noticed that maximum pigment accumulation occurred late in the growth cycle (figs. 3.3.1.1A, 3.3.1.1D). This timing coincides with decelerating growth presumably due to nutrient exhaustion. This decrease in availability of a particular nutrient resulting in increase secondary metabolite production occurs in grape (*Vitis vinifera* L.) when there is an enhancement of intracellular accumulation of anthcyanins with low nitrate concentration (Do C.B. & Cormier F. 1991) and in carrot cultures producing increased anthocyanin pigment with decreasing PO_4^- (Fowler M.W. 1986). Constabel F. & Nassif-Makki H. observed a similar result in calli of red beetroot (Böhm H. & Rink L. 1988). They found that omitting phosphorous was a good inducer of pigment production in red table beetroot cultures. Figures 3.3.1.3A-F show the results of omitting phosphate from the nutrient salt cocktail.

Figures 3.3.1.3A-F The same culture conditions apply as in figs. 3.3.1.1A-H. 5 pieces of callus per Petri dish, 2 Petri dishes per treatment. Media was either Pgo salts with or without phosphorous. All values include standard error of the mean. All experimental Petri dishes were set up as described in section 2.3.5. The plates were kept either in the light at 10-14 $\mu mol/m^2/s$ or in the dark. From now on Pgo will be the medium for culture maintenance and experimental controls.

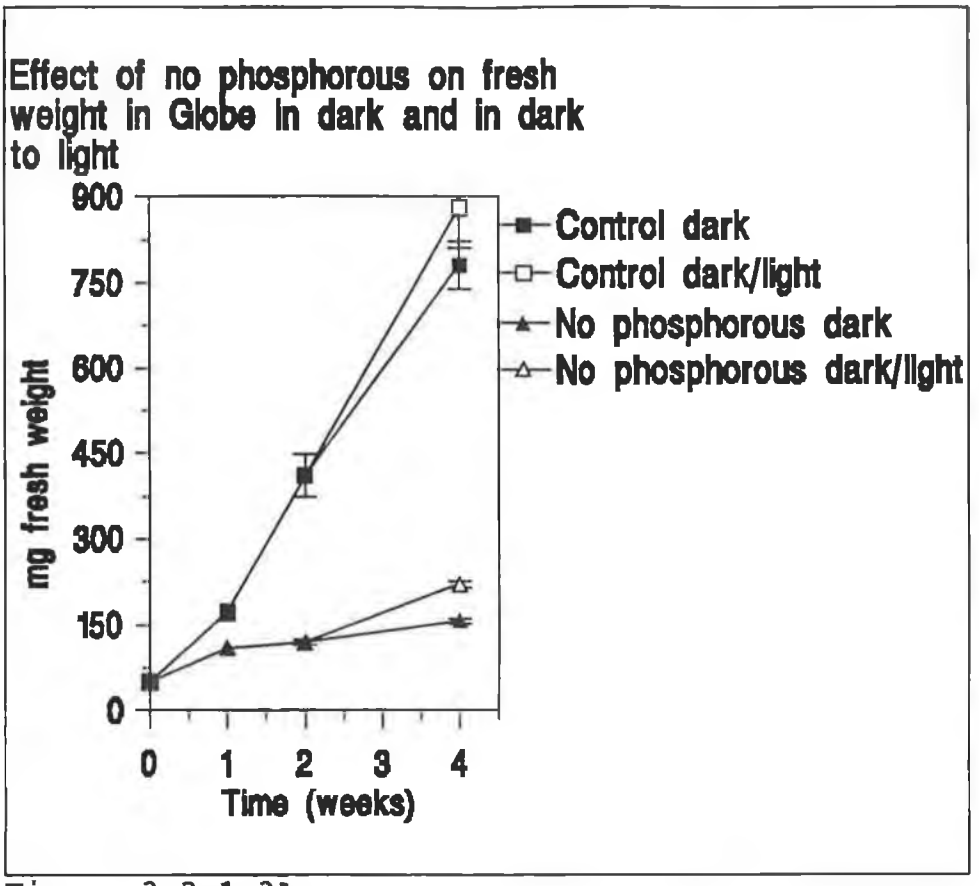


Figure 3.3.1.3A

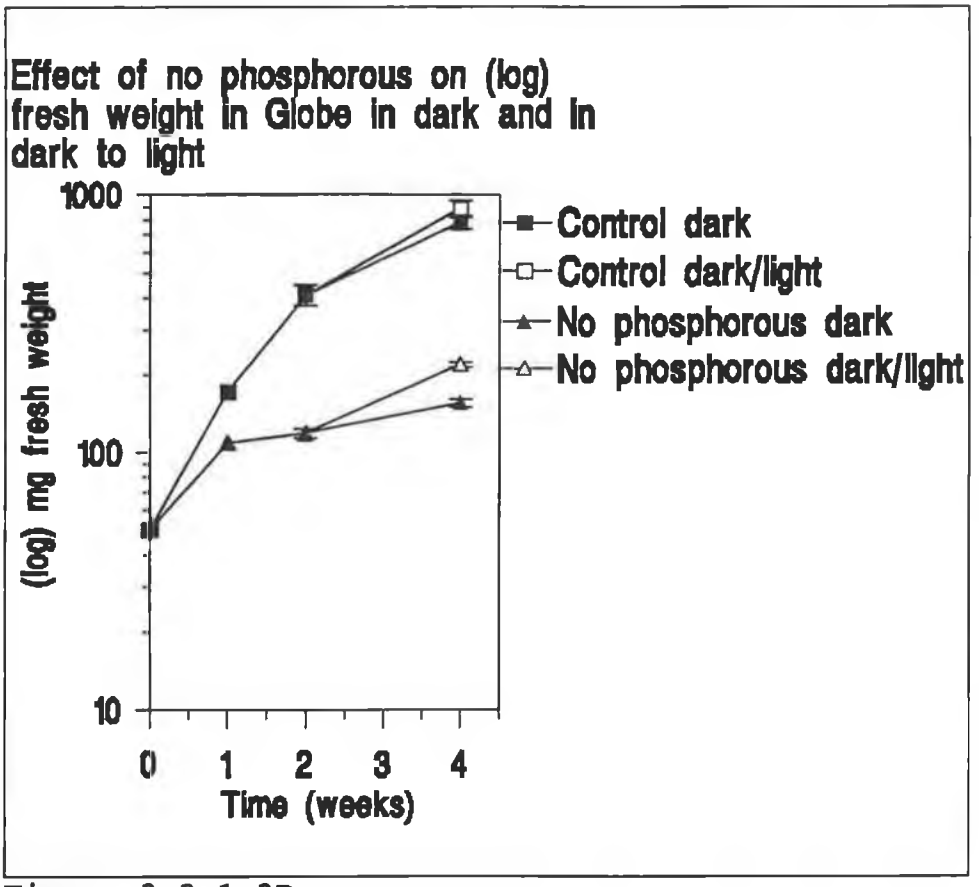


Figure 3.3.1.3B

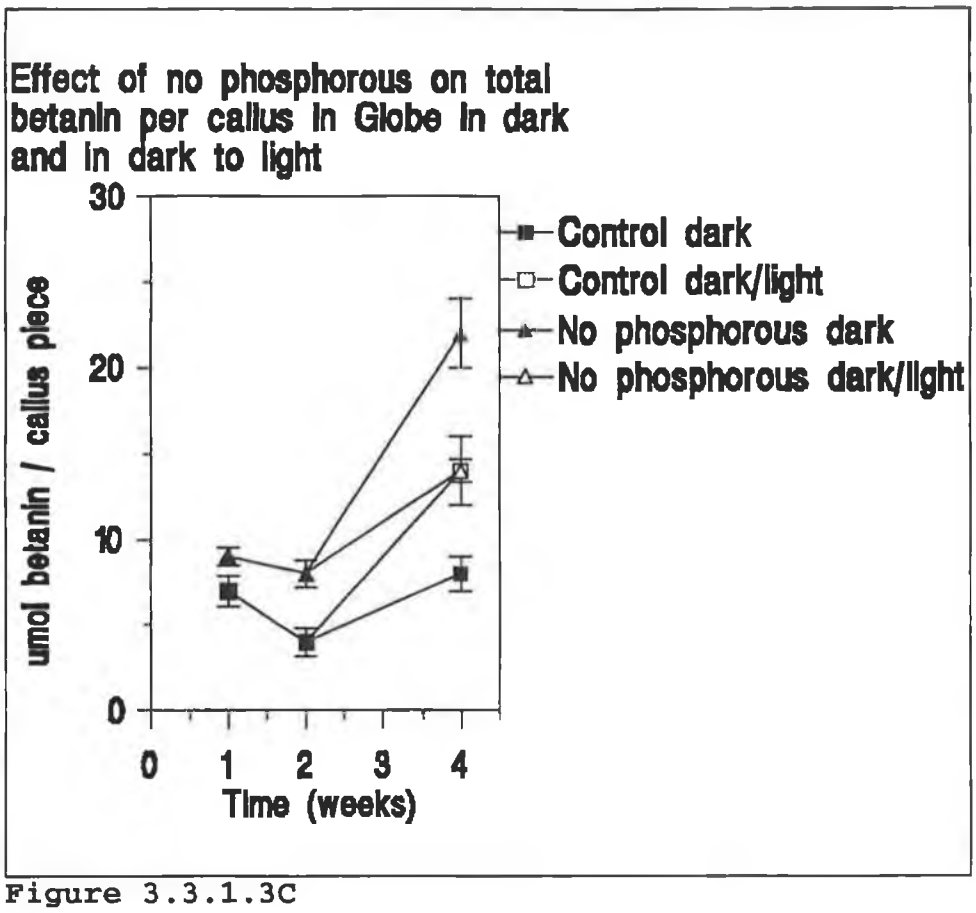


Figure 3.3.1.3C

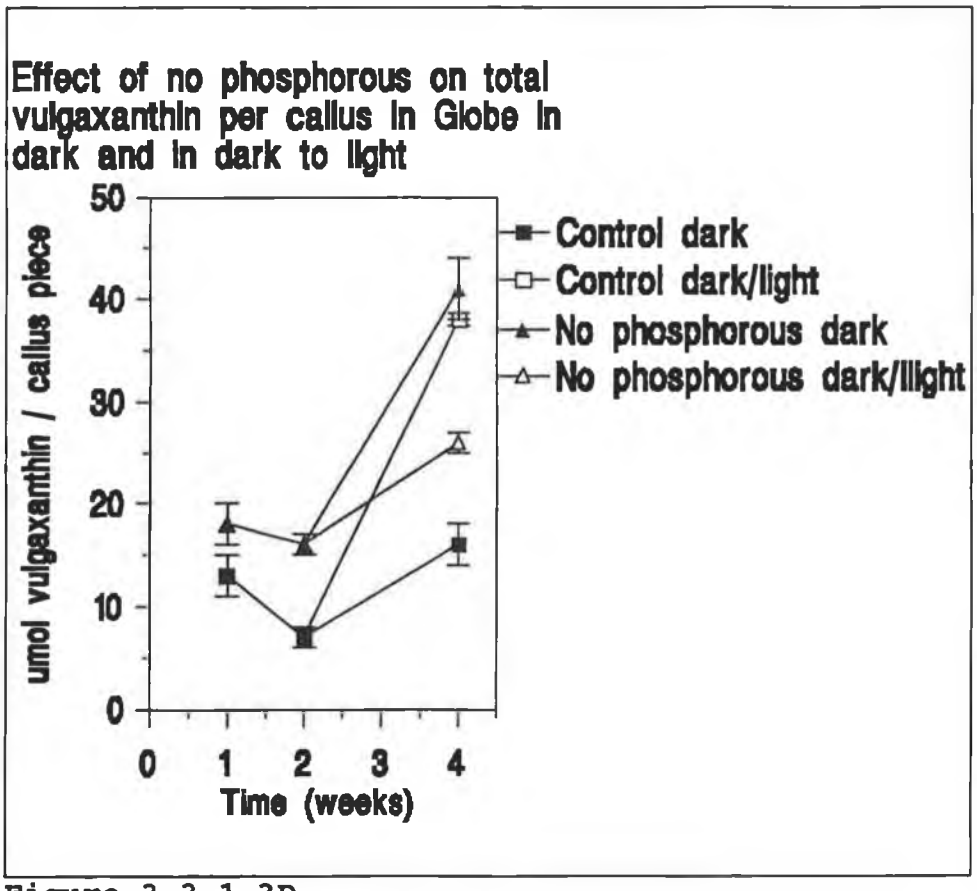


Figure 3.3.1.3D

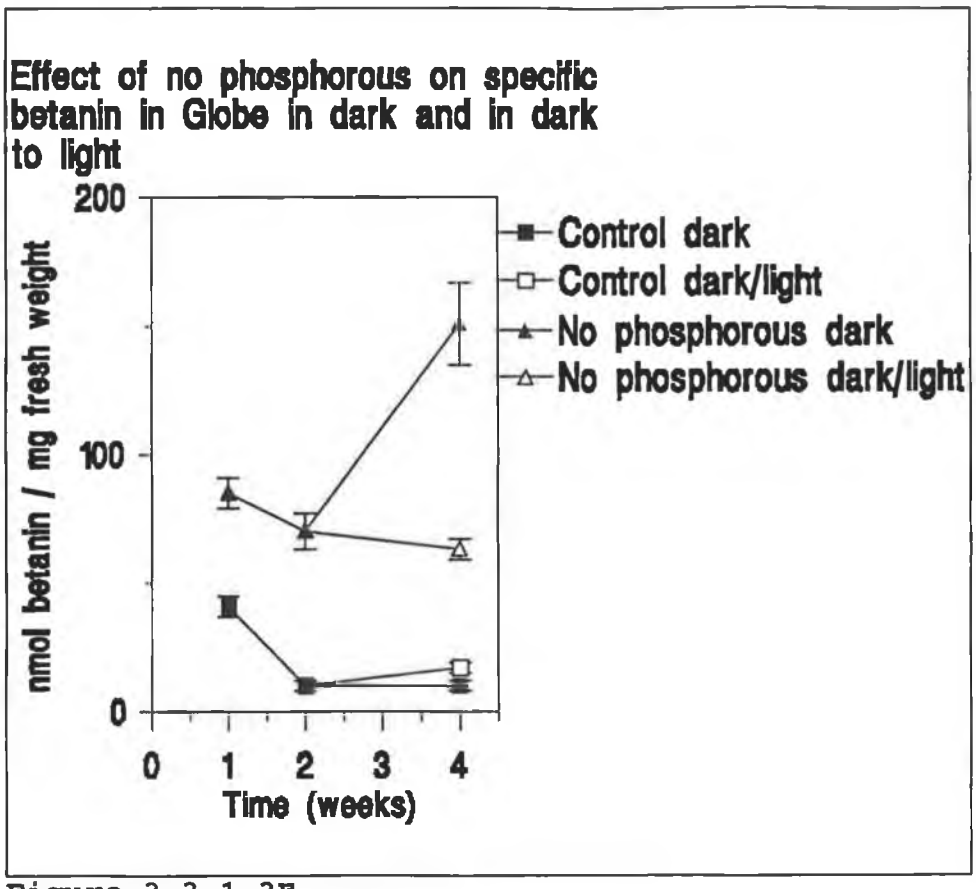


Figure 3.3.1.3E

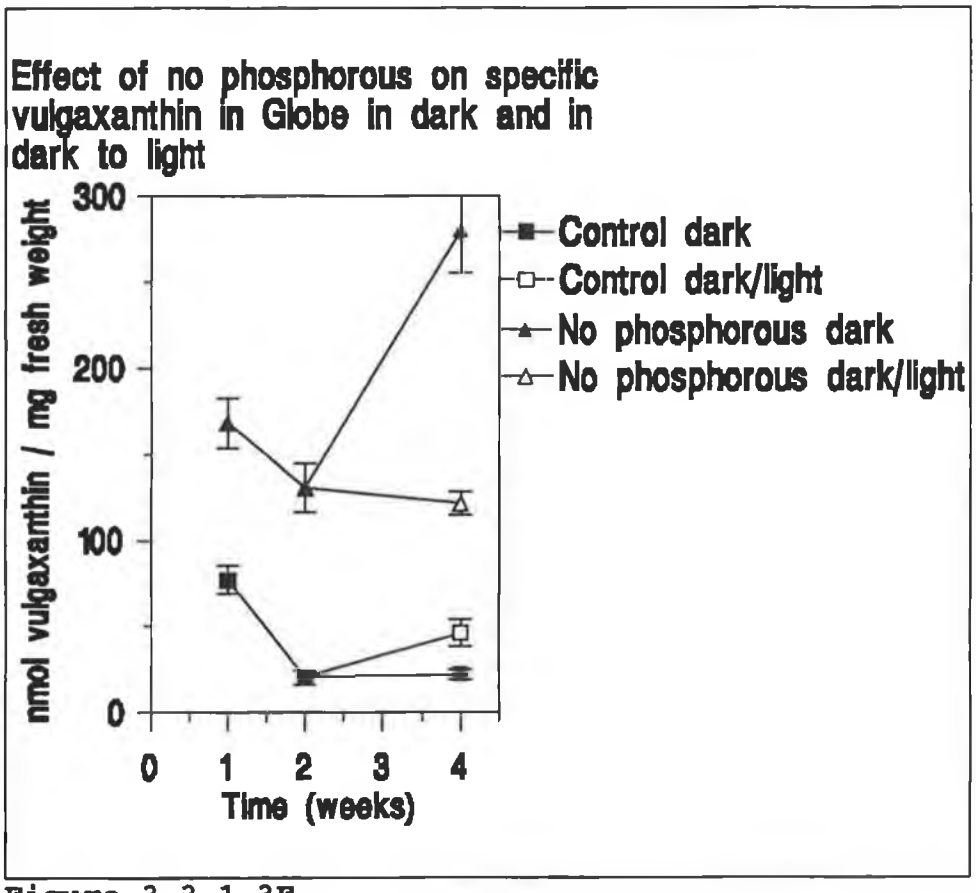


Figure 3.3.1.3F

Figures 3.3.1.3A-F show that;

A. callus deprived of phosphorous grew very slowly(fig. 3.3.1.3A). This was visually evident after one and half weeks. In addition the callus went black. Fig. 3.3.1.3B shows the limited logarithmic phase of multiplication.

B. Again as in the last experiment one sees the inducing effect of light in figs. 3.3.1.3C-D which show total pigment accumulation per callus. One notices that callus grown on medium without phosphorous had appeared to accumulate more pigment than that grown on the control (fig. 3.3.1.3C-D). One also notices that a higher value is to be found when callus, grown on medium without phosphorous, is kept in the dark and not in light. It should be mentioned at this point that Globe callus goes yellow when it becomes pigmented.

C. Figs. 3.3.1.3E-F show specific values and seem to indicate that callus grown on medium without phosphorous gave significantly higher values. However if one compares this to the fresh weight for callus starved of phosphorous one realises that this necrotic tissue is giving misleading results. This arises from the fact that high specific values of pigment accumulation would indicate yellow calli whether it be surface associated or not. It has been already mentioned in result (A.) that the visual appearance of the calli grown on medium without phosphorous was black. It can thus be concluded that the observed high values of pigment accumulation refers to the black pigments/substances due to necrotic and not yellow betalains. As a result one must leave the zero phosphorous aside and look solely at the control values.

These results reinforce the initial result that light is an inducer/enhancer of pigment accumulation. This can especially be seen in the rise in total vulgaxanthin per callus in callus grown in the light (fig. 3.3.1.3D). However they also indicate a possible source of 'false positives' in the form of dead material. This can be minimised by including dead material in the fresh weight measurements but omitting this during the actual extraction. Secondly it was found that the effect of nutrients or lack of nutrients can usually be seen after 2 weeks of culture. The rise in specific pigment in the control accumulation being a lot smaller than total per callus is probably due to the fact that pigment production appeared to occur only on the surface of the callus pieces. As a result despite total pigment per callus going up, the total amount of callus growth outstripped pigment production. Previous results showed that pigment production and growth decrease at high fresh weight values, presumably due to nutrient depletion. This is reinforced in this experiment. Despite fresh weight and total pigment per callus

accumulation being higher, specific pigment accumulation is lower in week 4 for the control values

3.3.2 Effect of Initial Inoculum

An observation, not evident from the graphs of the previous experiment, is that small pieces of calli, not more than 3mm in diameter, were deep yellow in colour. These small pieces of calli, which did not belong to the main 5 experimental pieces of calli, resulted from experimental tissue being accidently spread over the surface of the agar. Instead of the plate being discarded it was used for experimental purposes after most of the tissue had been removed. Specks of tissue, too small to be removed by forceps remained and thus developed. It was noticed, at the end of the experimental run, that the specks of tissue had grown slightly and had gone deep yellow in the presence of light. This raised the possibility that pigment production could be related to inoculum size.

In addition to the suspected inoculum effect it has been demonstrated in previous experiments that both growth rate and pigment production declined at high fresh weight values, probably due to nutrient depletion. An experiment to look at the effect of initial inoculum size would have the two possible benefits (1.) examine the effect of inoculum size on pigment production, (2.) determine a suitable inoculum size for optimal reproducibility and pigment production. This would also help to prolong the exponential phase of growth as depicted by a straight line on a log-linear plot of the growth curve. For the purposes of clarity growth curves resulting from cultures grown initially in the dark for 2 weeks then transferred into light, have been repeated when comparing to cultures grown in the dark for 4 weeks(figs. 3.3.2C-E). This is to show the effect of light.

Figures 3.3.2A to 3.3.2Q show the results of the effect of initial inoculum on fresh weight and pigment accumulation when either kept in the dark for 4 weeks or two weeks in the dark followed by exposure to light for two weeks.

Figures 3.2.2A-Q Experimental set up as described previously. Calli were either left in the dark for 2 weeks then transferred to light or left for 4 weeks in the dark. 5 pieces of callus per plate, two plates per treatment, harvested at weekly intervals and pigment extracted.

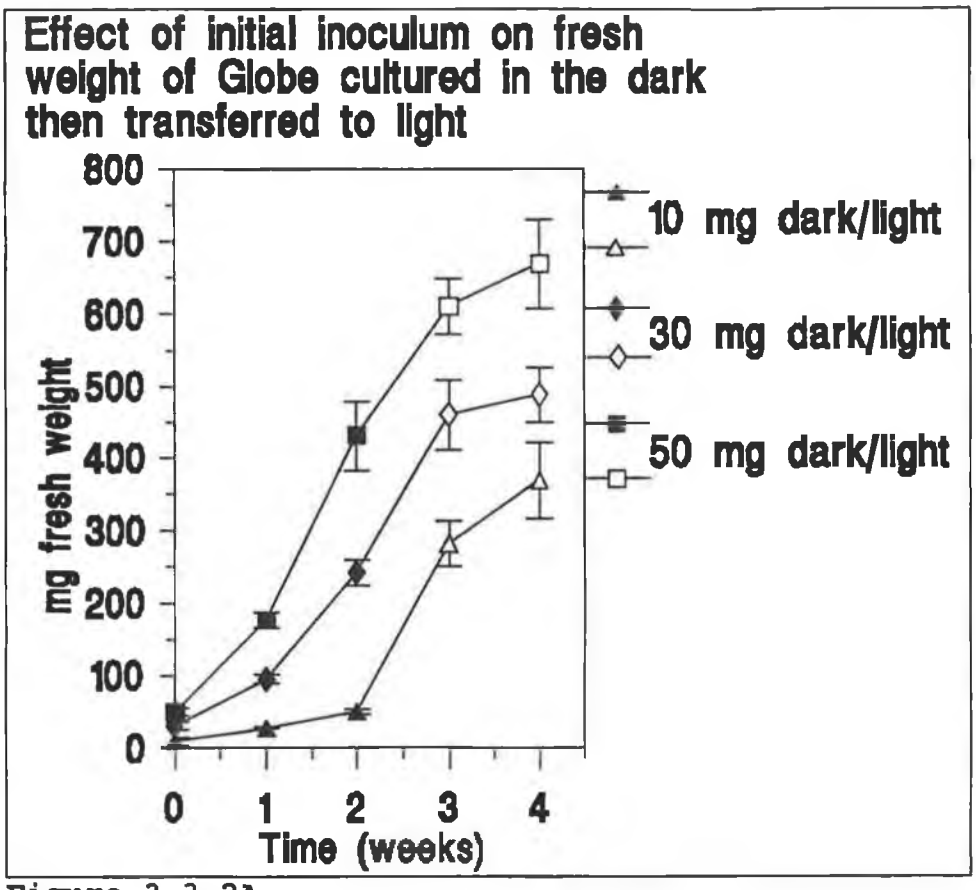
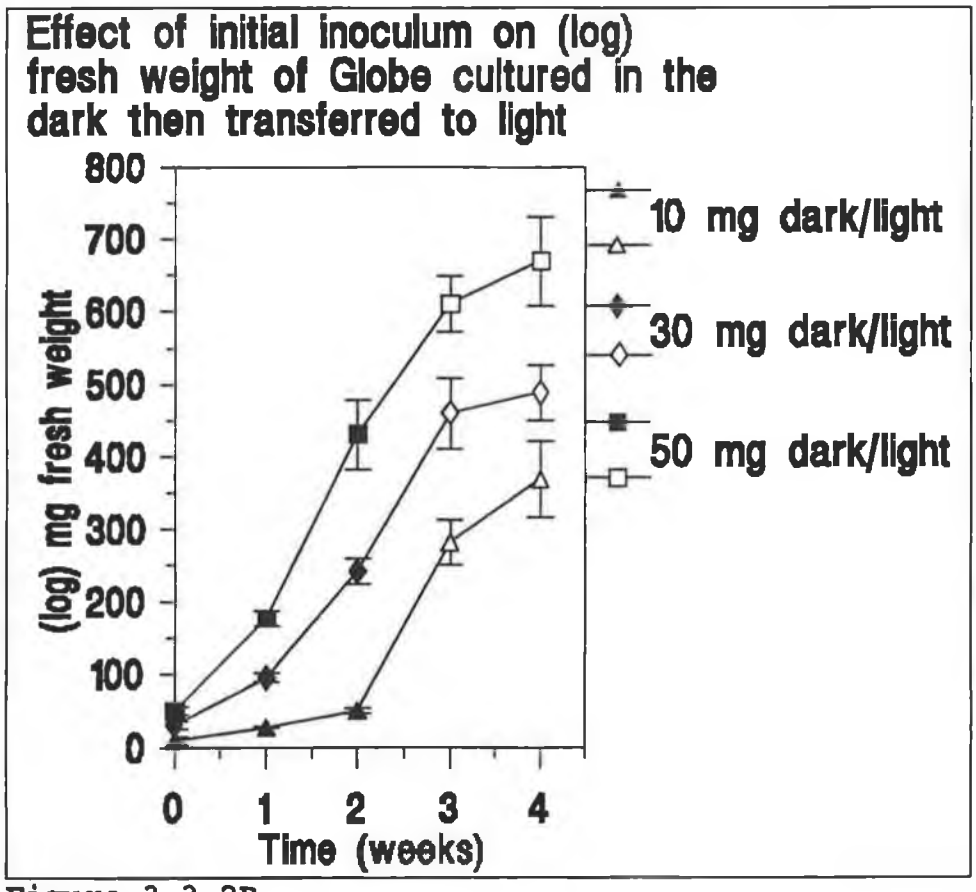


Figure 3.3.2A



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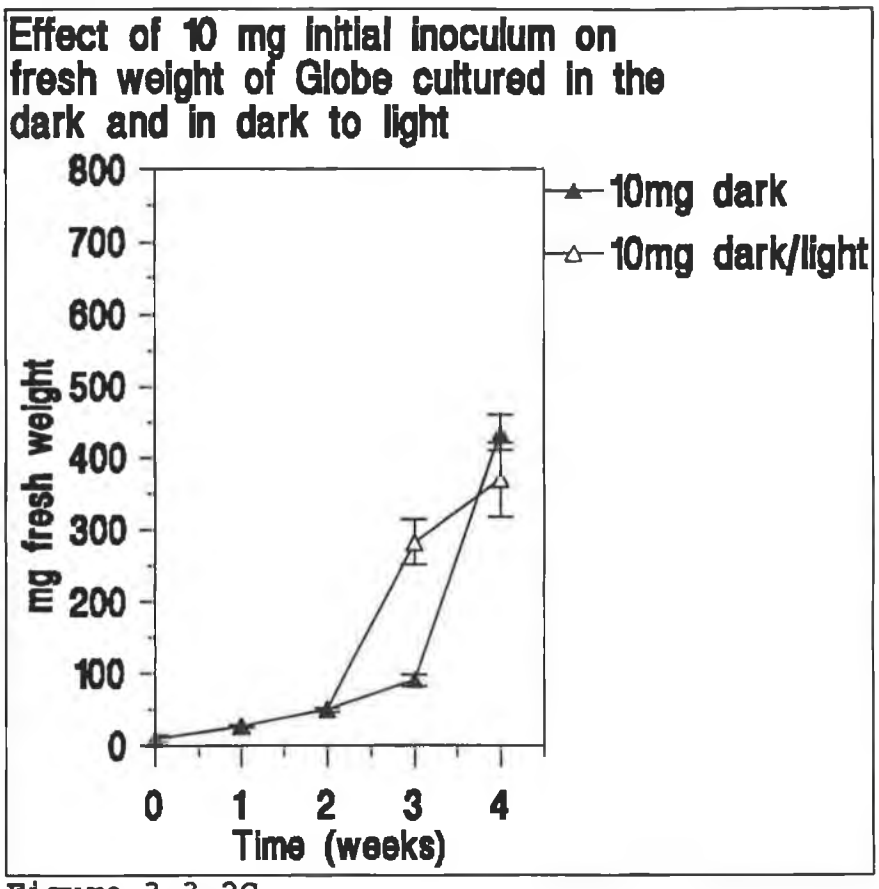

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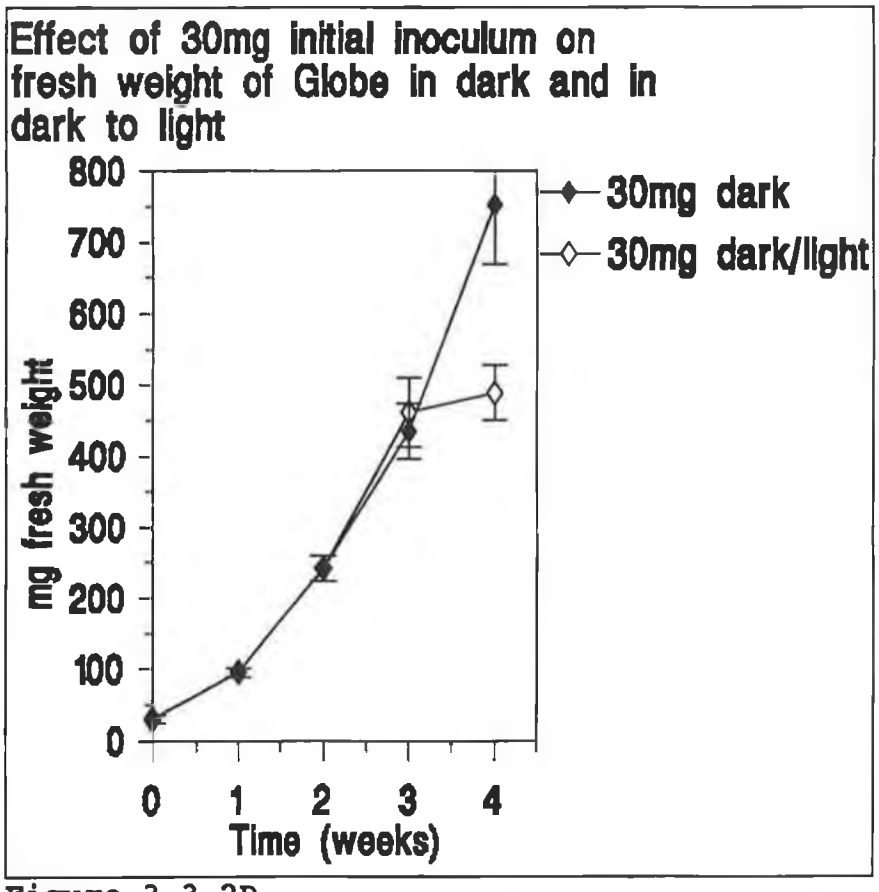


Figure 3.3.2D

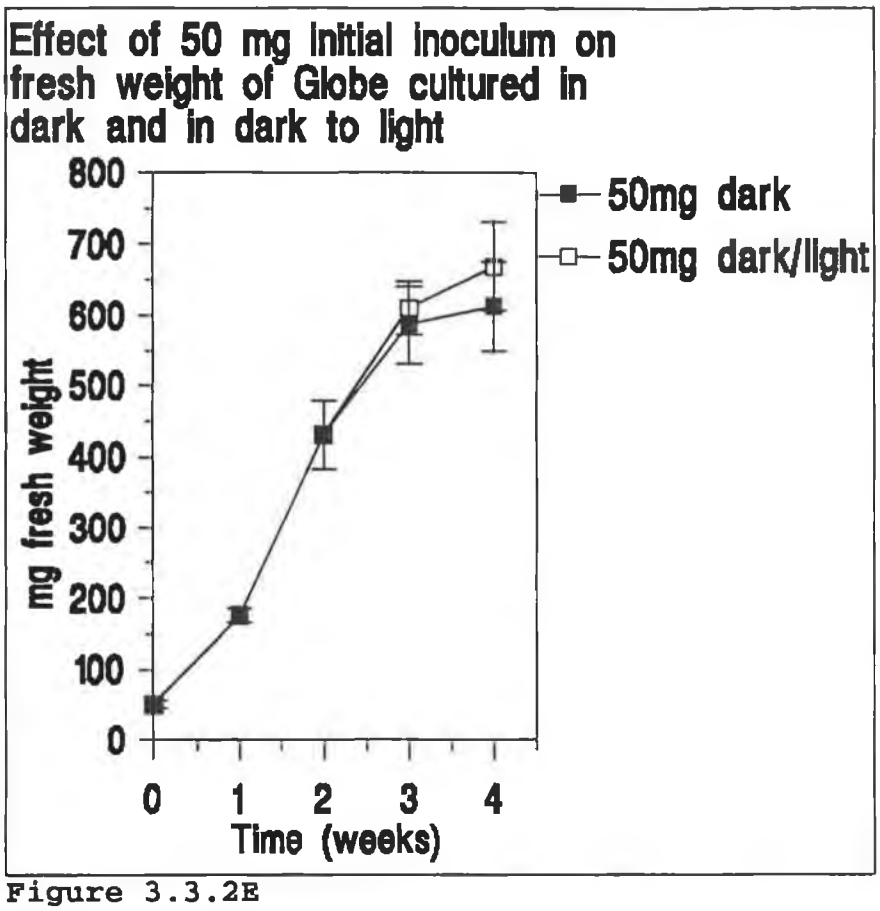


Figure 3.3.2E

A. Fig. 3.3.2A shows fresh weight accumulation. One finds that when initial inoculum is 30 mg there is a continual rise in fresh weight. In the case of 50 mg there is a slowing down of fresh weight accumulation after 2.5 weeks. As for a 10 mg initial inoculum growth is slow for the first 3 weeks then rises dramatically in week 4. This is confirmed by a log linear plot of these fresh weights over time(fig. 3.3.2B). If one analyses fig. 3.3.2B further one notices that exponential growth, as evidenced by points being in a straight line, appears to be the same for all initial inoculums up until around 450 mg fresh weight. This fresh weight is attained by 50 mg inoculum in 2 weeks, in 3 weeks by a 30 mg inoculum and 4 weeks by a 10mg inoculum. The suggestion therefore is that nutrients will become rate limiting after fresh weight accumulation has reached 450 mg and will thus interfere with final results. A different picture arises when calli are kept in the dark for 4 weeks. Figs. 3.3.2C-E give the impression that light retards growth. This is particularly seen when initial inoculums are 10 and 30 mg.

B. Figs. 3.3.2F-K illustrate what happens in the case of total pigment accumulation per callus. Total pigment per callus, given initial inoculum of 50 mg, whether it be betanin (fig. 3.3.2H) or vulgaxanthin (3.3.2K), appears to decrease and increase at

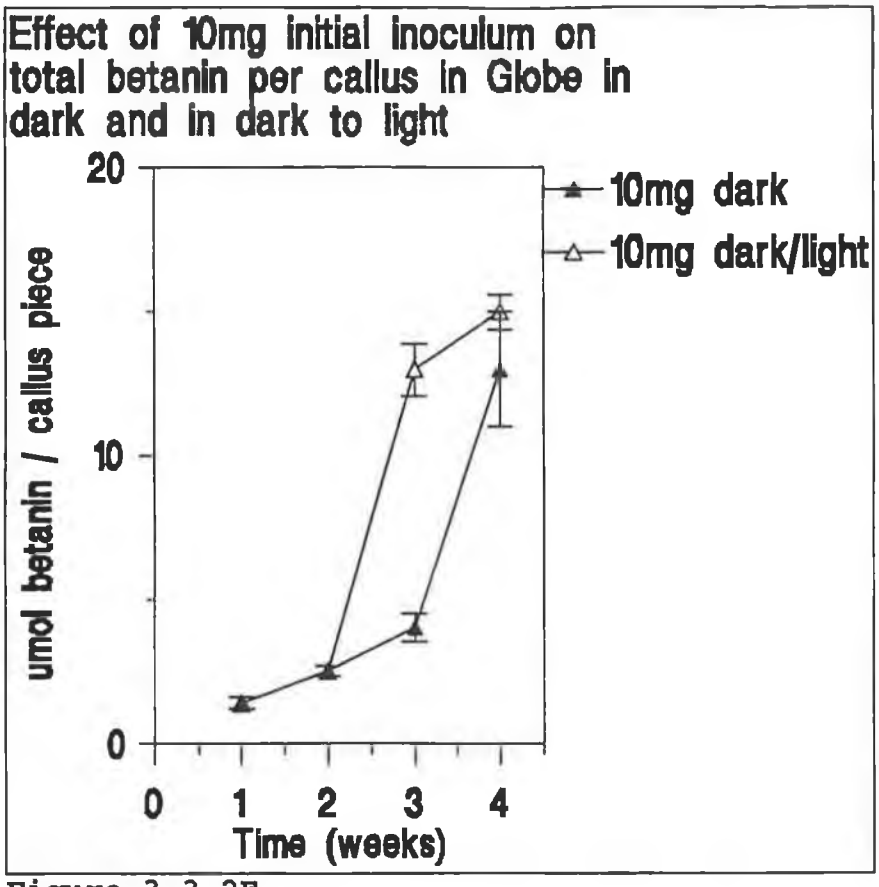


Figure 3.3.2F

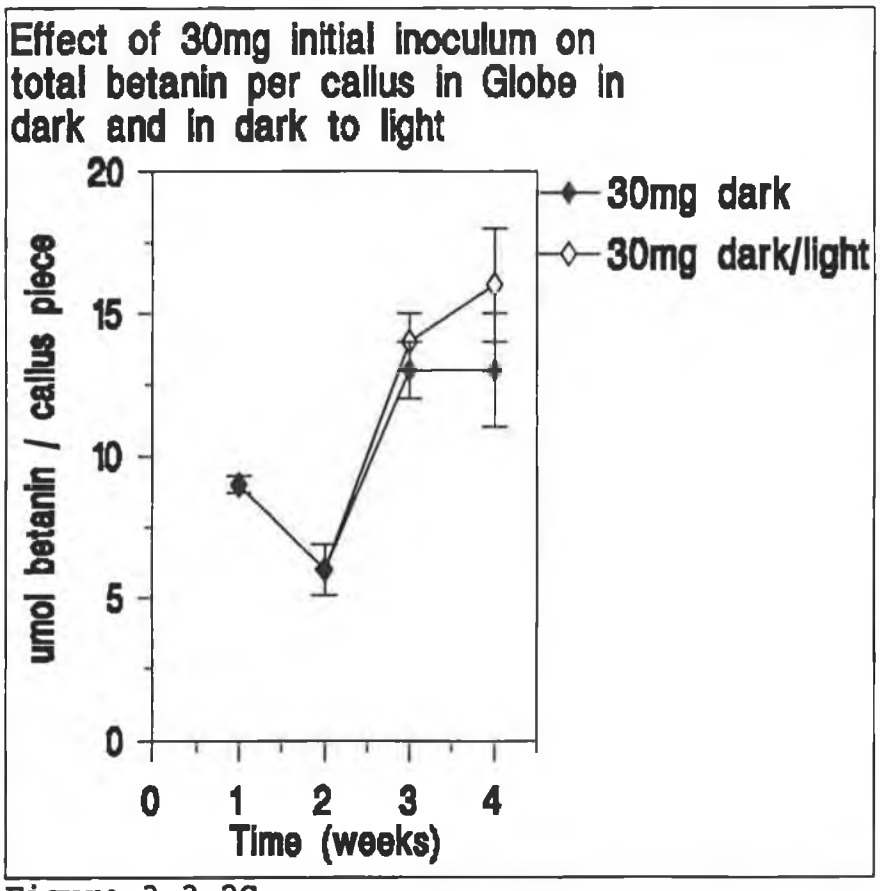


Figure 3.3.2G

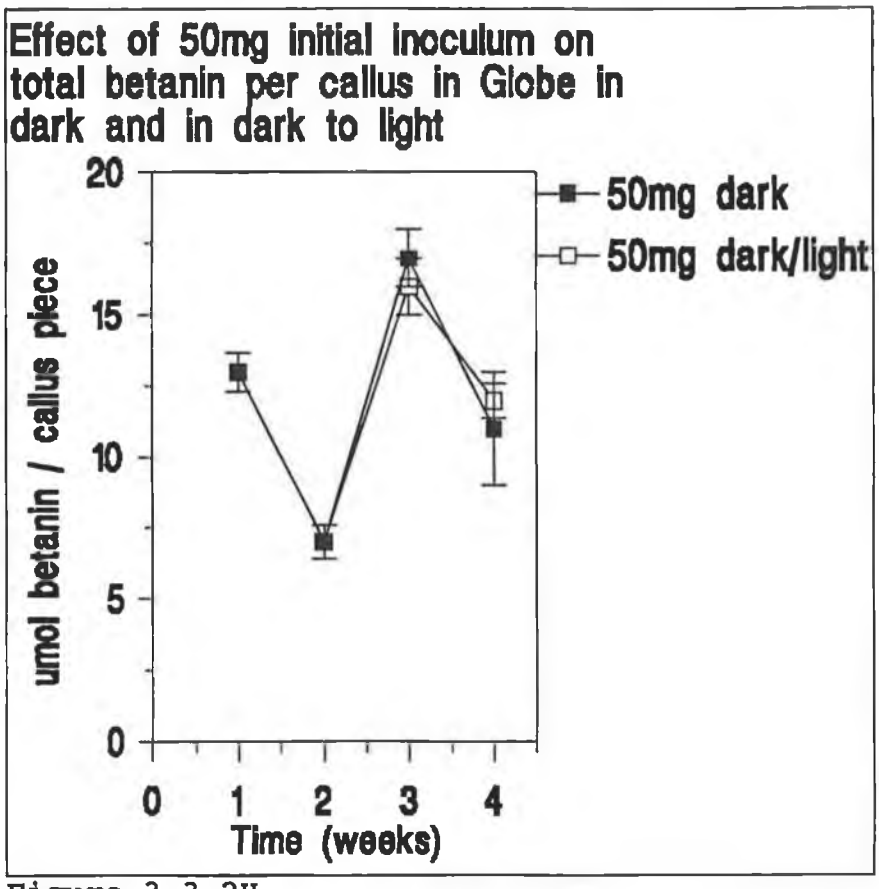


Figure 3.3.2H

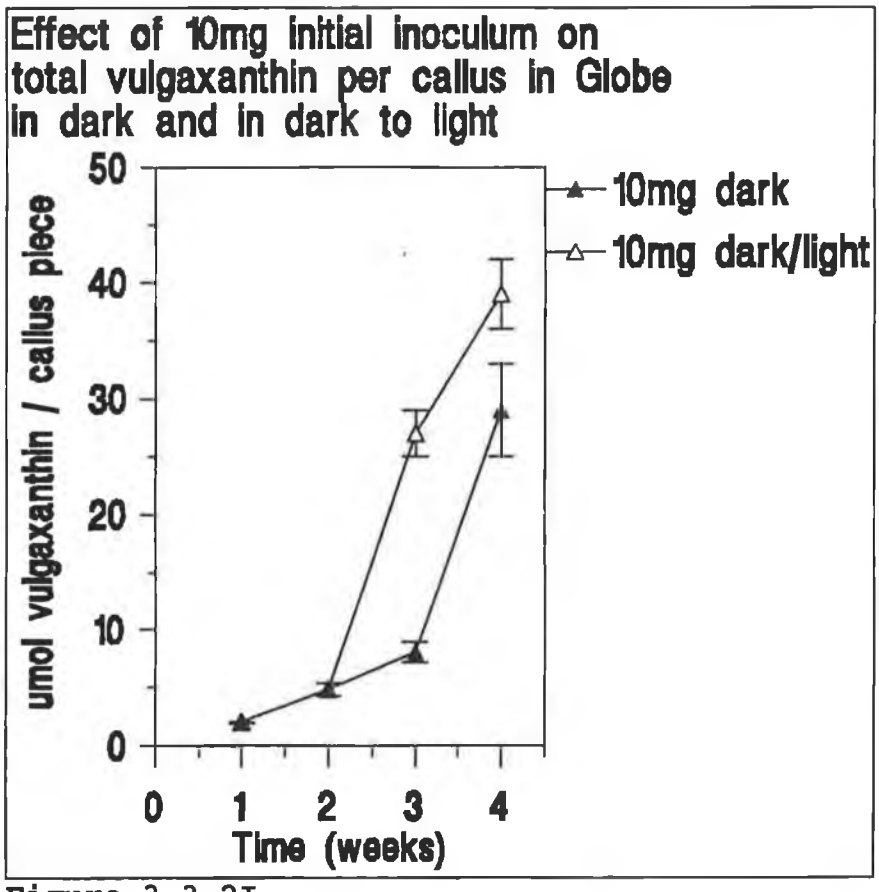
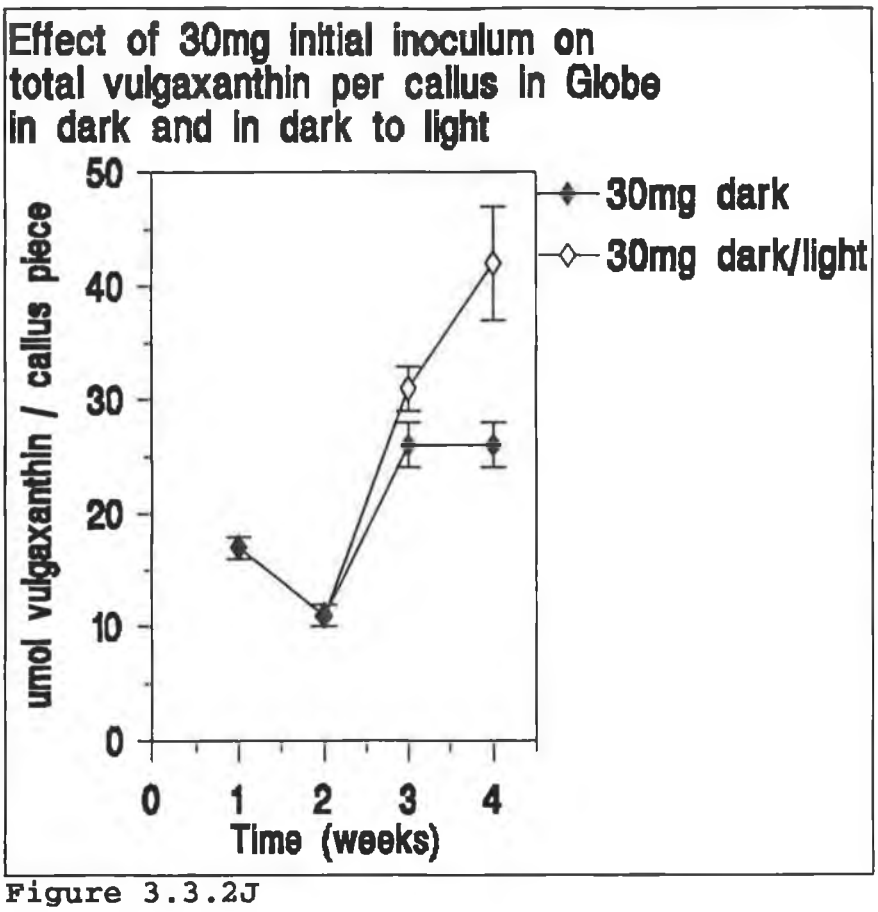


Figure 3.3.2I



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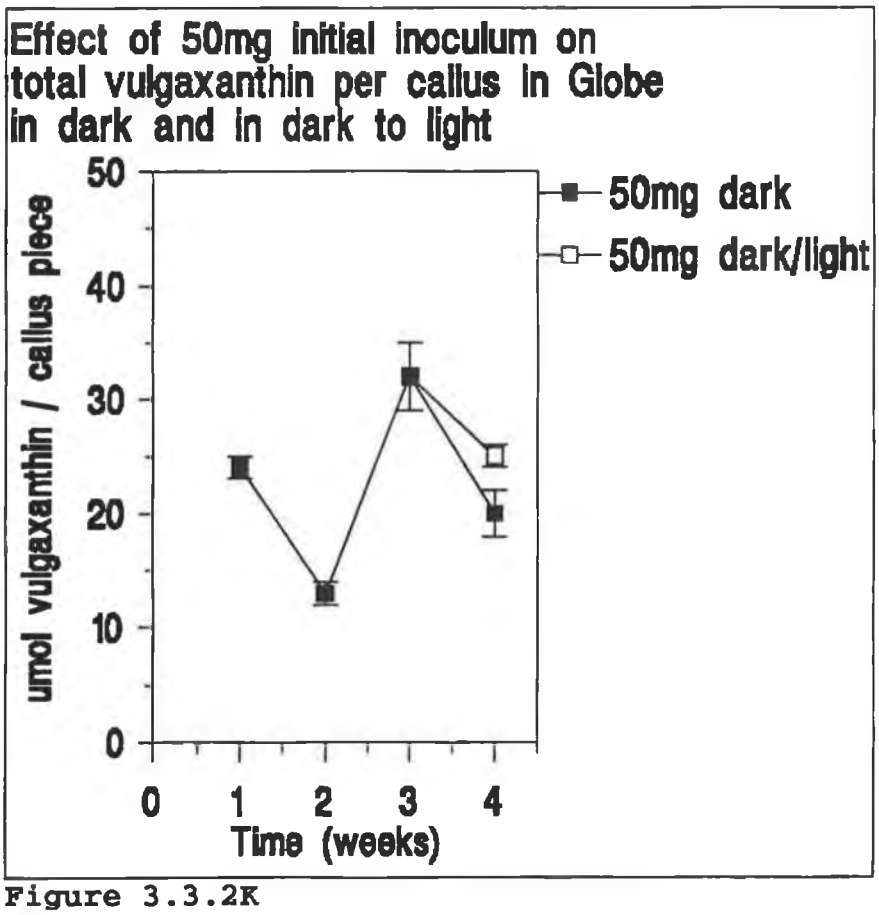


Figure 3.3.2K

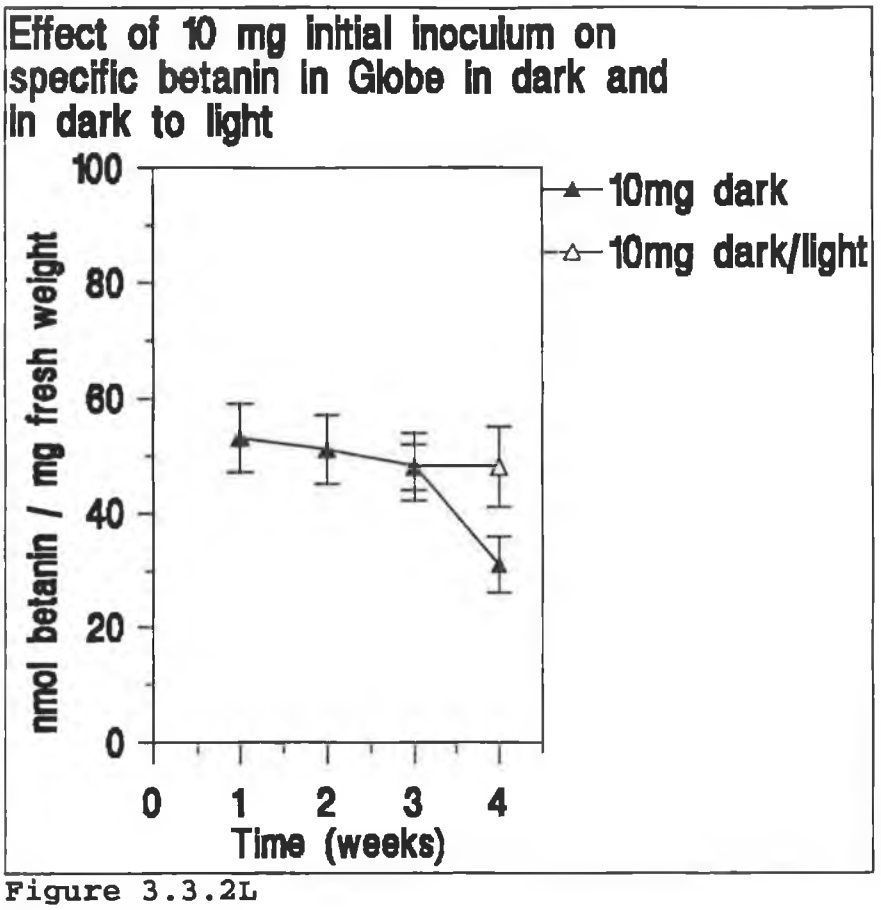
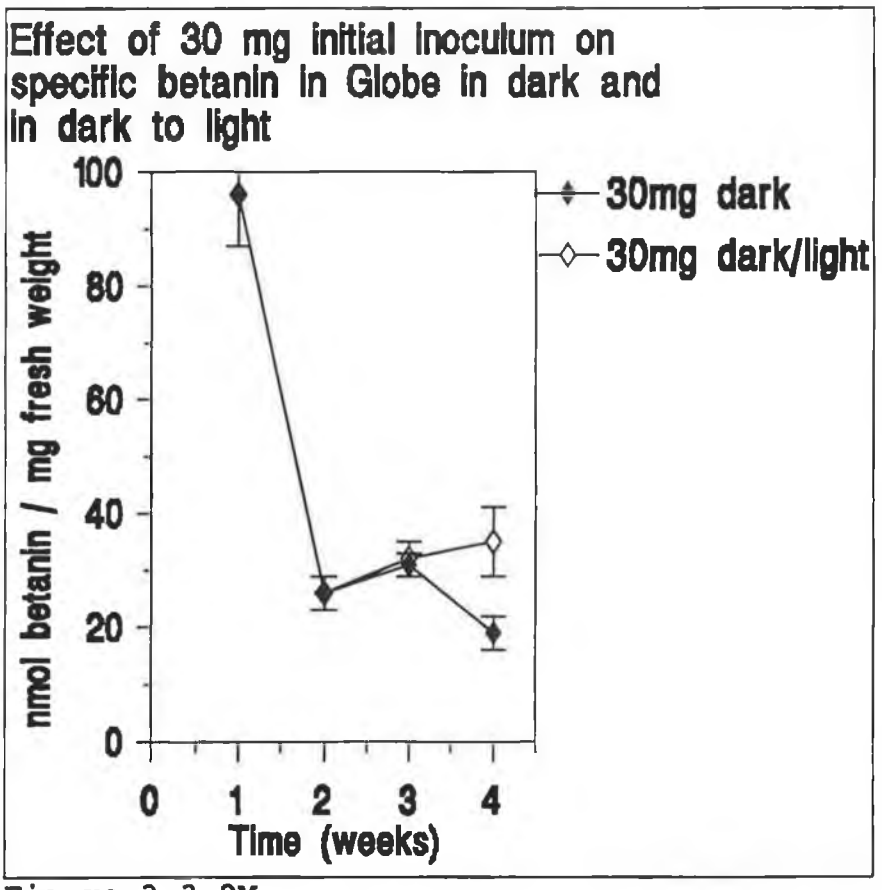


Figure 3.3.2L



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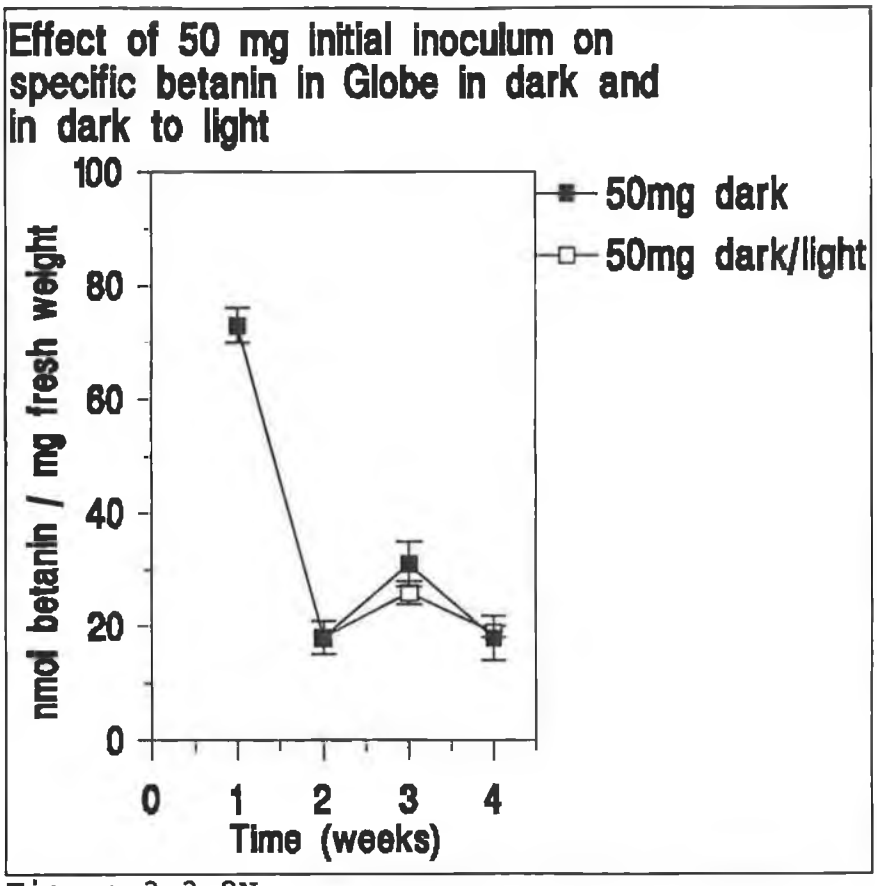


Figure 3.3.2N

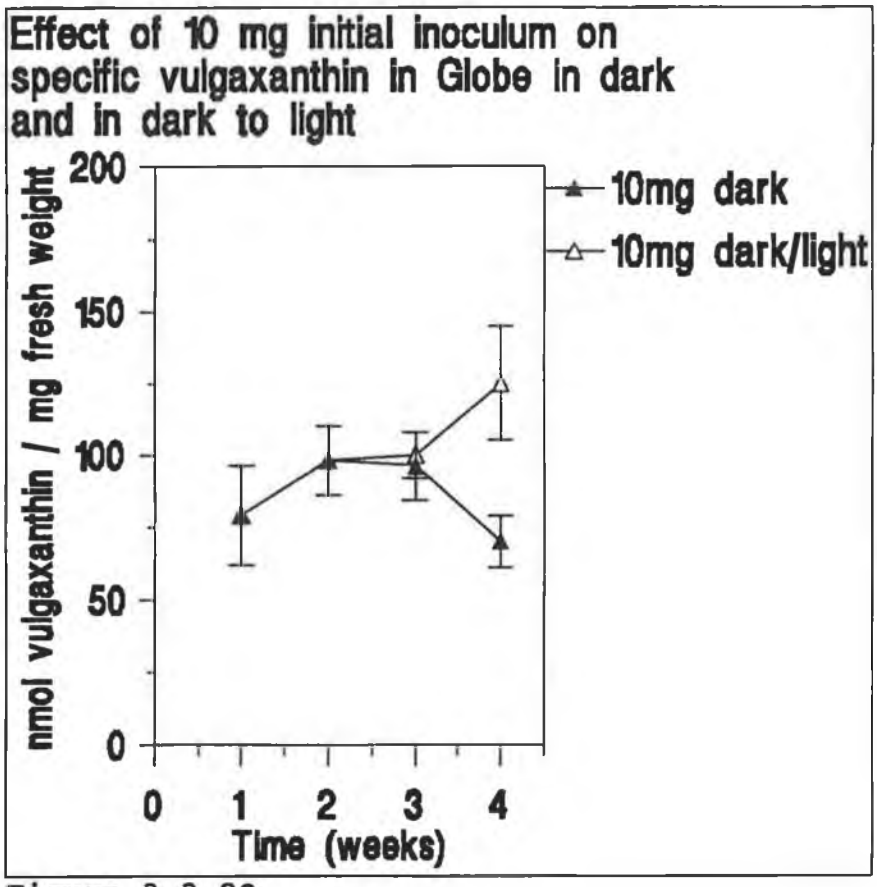


Figure 3.3.2O

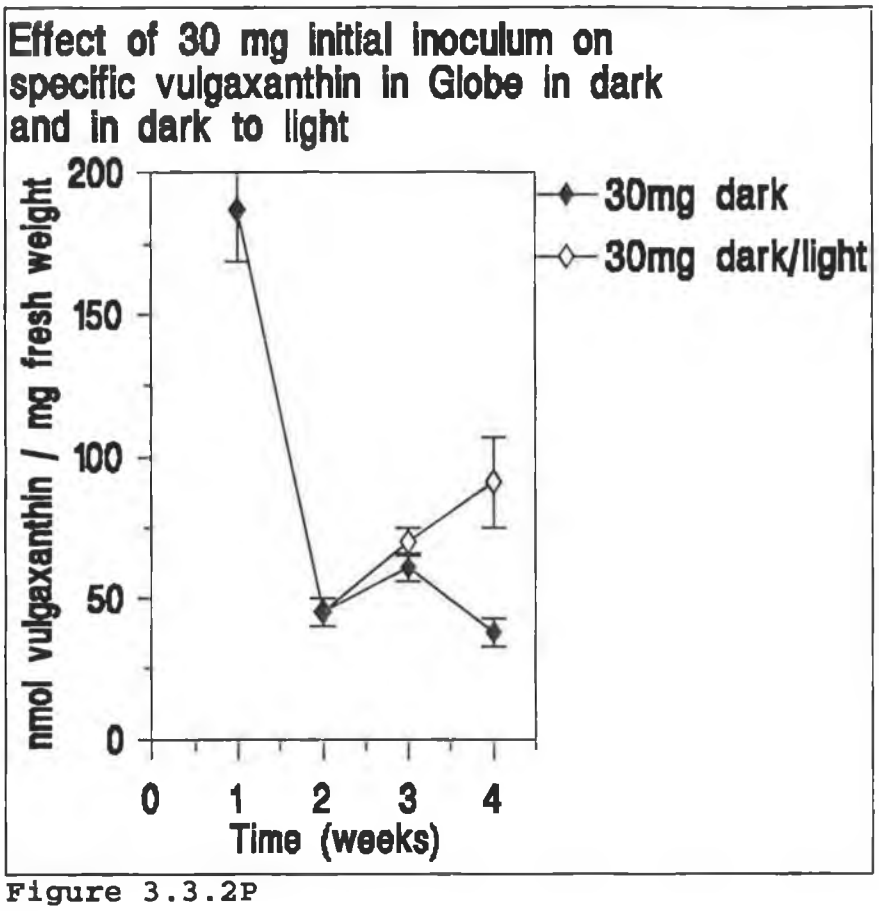


Figure 3.3.2P

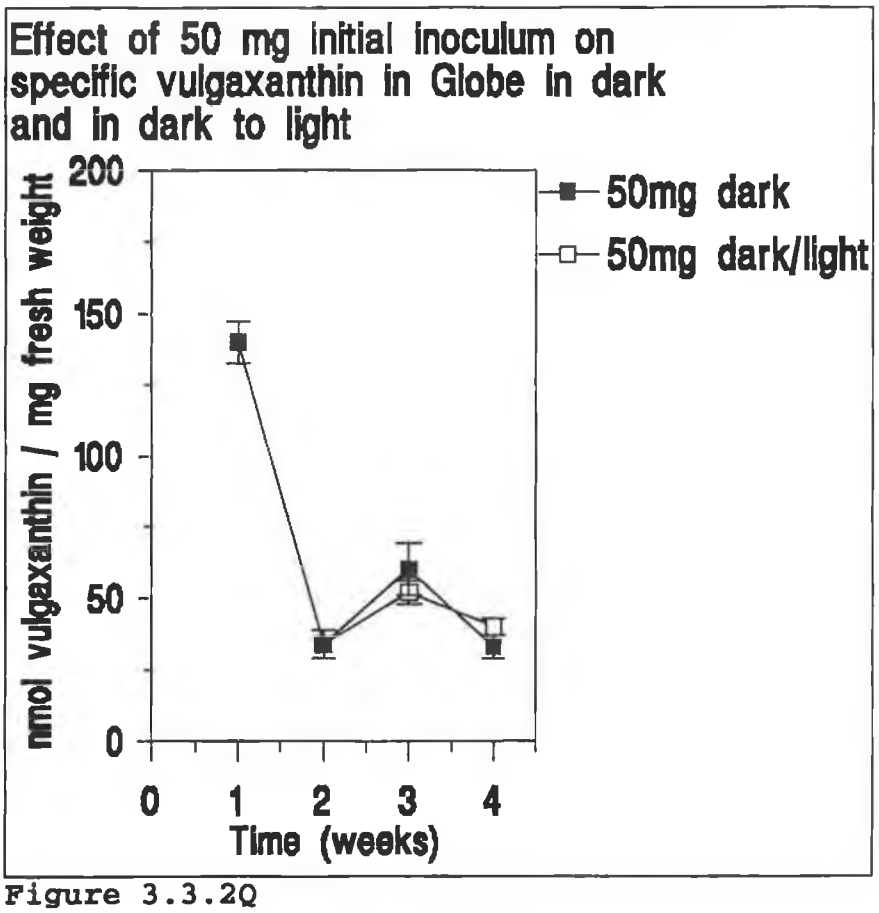


Figure 3.3.2Q

weekly intervals when kept in the dark. Despite considerable heterogeneity in pigment levels, there is a consistent light induction of pigment accumulation, but only in the second week of illumination(week 4). The dark values for week 3, which even exceed light values (figs. 3.3.2H), are the hardest to explain. It is probably a reflection of the heterogeneity of the callus. The total pigment values for callus, whose initial inoculums were 10 mg and 30mg (figs. 3.3.2F-G, 3.3.2I-J), are more in line with what is being expected, that is a continuous rise in the light. The steady rise in the dark for all inoculums may be the result of constitutive production of pigment. If one compares week 4 total pigment per callus values one gets the impression that 30 mg results in the highest pigment accumulation whether it be betanin (fig. 3.3.2F) or vulgaxanthin(fig. 3.3.2J). If this is tallied with fresh weight (fig. 3.3.2D) one notices the possible inverse relationship between pigment production and fresh weight accumulation.

C. Figs. 3.3.2L-Q show the specific pigment accumulations for betanin and vulgaxanthin. The general result is the expected decrease in the dark over time especially in the first two weeks of culture. One does see a slight rise, on illumination, in specific betanin values when initial inoculums are 10 and 30 mg (figs. 3.3.2L-M) but particularly in the case of vulgaxanthin(figs. 3.3.2O-P). One might suggest at this point a possible inverse relationship with growth(figs. 3.3.2C-D). The other point to note is the slow decrease in specific pigment accumulation when initial inoculum is 10 mg in the dark. This is because growth is initially sluggish as seen in figure 3.3.2A. If one compares week 4 specific pigment accumulations with those of fresh weight it can be seen that an initial inoculum of 10 mg results in the highest specific pigment values whether it be betanin(fig. 3.3.2L) or vulgaxanthin(fig. 3.3.2O) when cultures are maintained first in the dark for 2 weeks then transferred to light.

The overall result is that inoculum size did not cause a significant visible induction of pigment production whether it be yellow betaxanthins or pink betacyanins. However it did show that there is a light inducing/enhancing effect. Secondly that 30 mg instead of 50 mg could be used for setting up experiments since at 50mg suspected nutrient limitation tallies with decreased pigment accumulation. This nutrient limitation is less evident at 30mg and absent at 10mg. This will result in experiments being set up faster, less material being used and the reduced effect of possible nutrient deficiency occurring in week 4.

3.3.3 Effect of Light Quantity

Previous experiments showed a consistent and reproducible light induction of pigment accumulation. However this moderate light induction was not uniform over the entire callus piece but tended to occur as small clusters over the callus surface. It is thought that light was the limiting factor whether it be quantitative or qualitative. This arises from the fact that some papers indicate light levels of 50 umol/m^2/s when looking at anthocyanin production in *Brassica oleracca* and *Lycopersicon esculentum* Mill. (Mancinelli A.L. 1990), 95 umol/m^2/s (Agbariah K.T. & Roth-Bejerano N. 1990) when they were looking at the effect of light on the energy levels in *Commelina communis.* The low light level was examimed based on the fact that some light receptors are saturated within half a millisecond in midday summer sunlight (Smith H. & Whitelam G.C. 1990). In an attempt to examine this question different light levels were investigated; 1, 10 and 100 µmol/m^2/s. The results of this experiment can be seen in figures 3.3.3A-E.

Figures 3.3.3A-E The experimental Petri dishes were set up in the same way as in previous experiments. The different light levels were achieved using cheese cloth as described in Section 2.4.1. Light levels were measured using a light meter (Section 2.4.1). The experimental plates, once set up, were left in the dark for a week. Callus samples were harvested at 1, 3, 10, 30, 100 and 168(1 week) hour intervals after the week(168 hour) dark treatment. There is considerable overlay of error bars. An indication, therefore, of the largest error of the mean in figs. 3.3.3A-E is shown so as to permit comparison of data points.

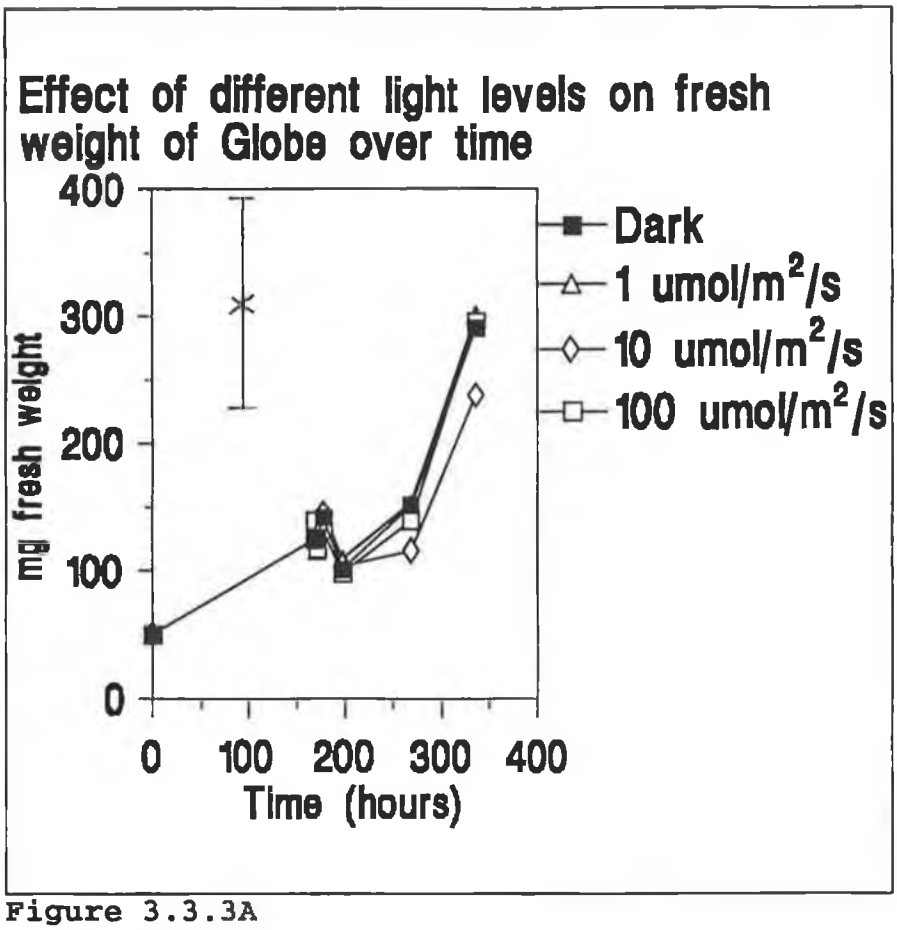


Figure 3.3.3A

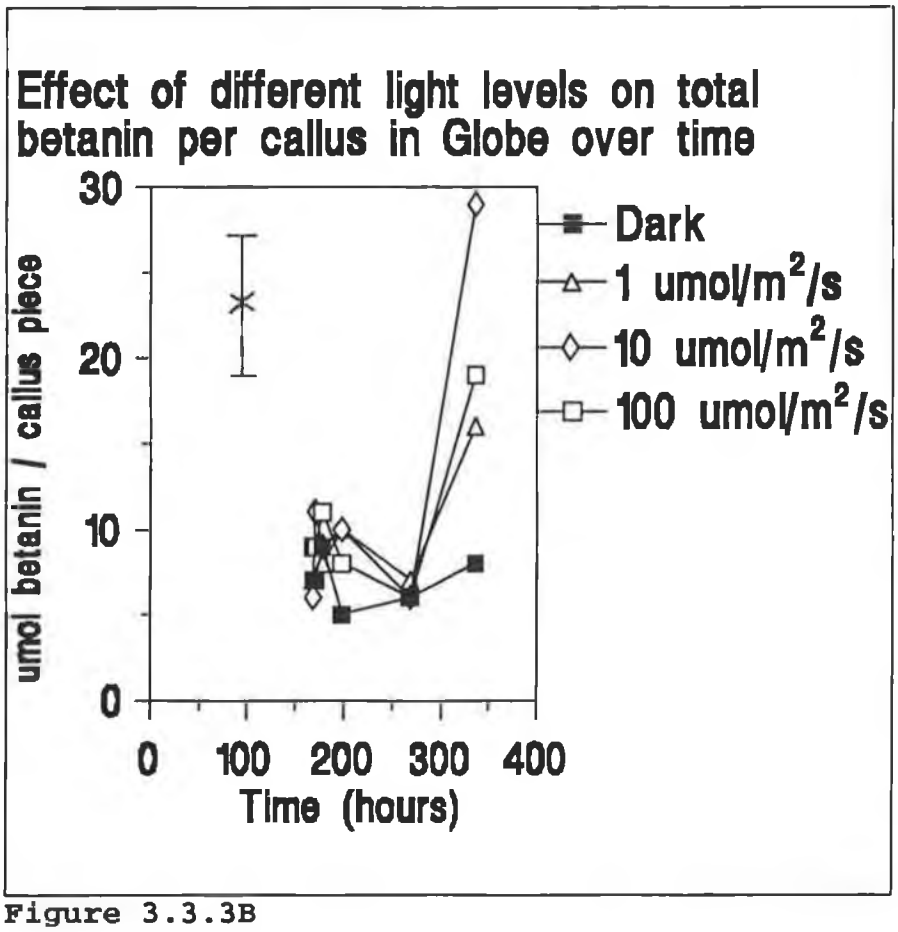


Figure 3.3.3B

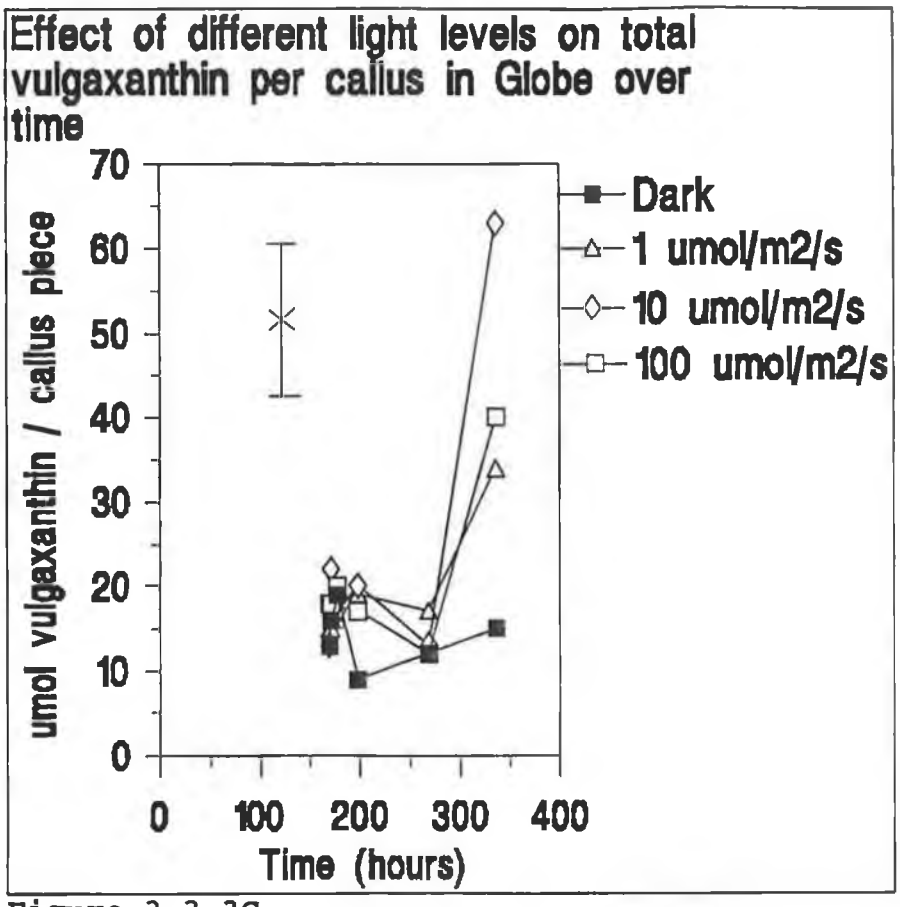


Figure 3.3.3C

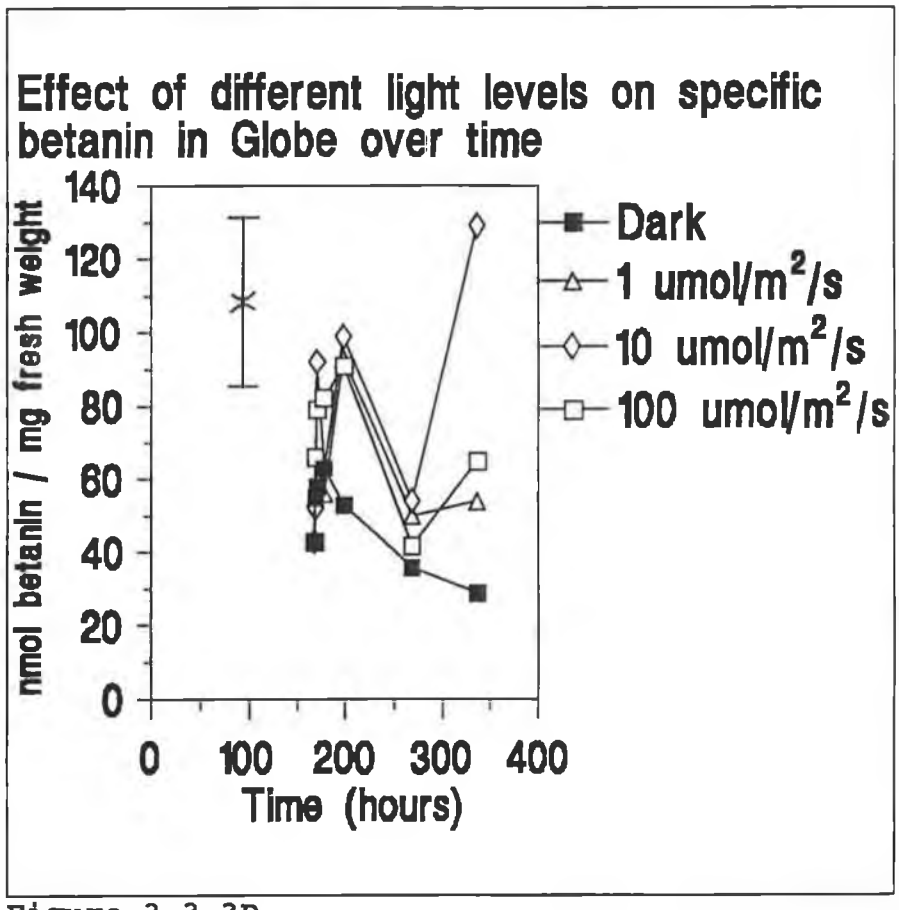


Figure 3.3.3D

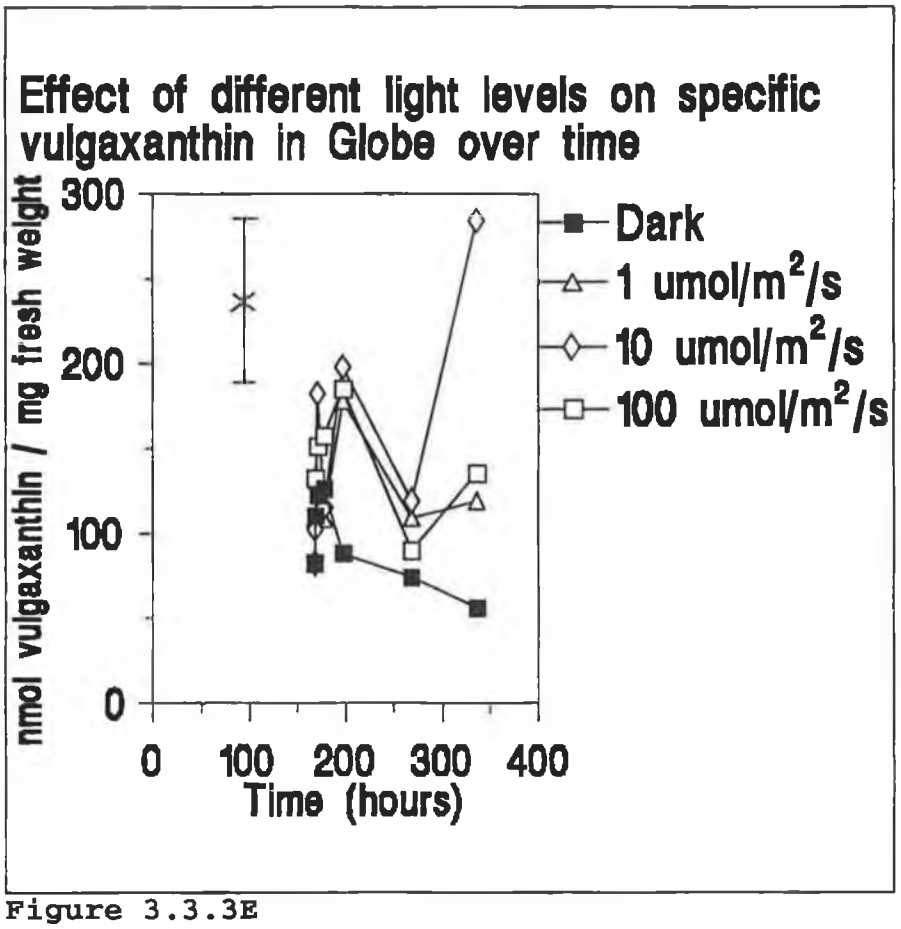


Figure 3.3.3E

A. Fig. 3.3.3A shows the results of increase in fresh weight with time which indicate that the different light levels did not significantly affect the corresponding growth curves.

B. that clear results can only be obtained after one week of illumination. A time period less than a week could give misleading results.

C. that total accumulation per callus (figs. 3.3.3B-C) appeared highest when the callus was illuminated at 10 µmol/m^2/s. Next best was 100 µmol/m^2/s followed by 1 µmol/m^2/s. One notices that the dark controls rise only slightly indicating the enhancing effect of light and the possible presence of constitutive production of pigment.

D. that specific pigment values(figs. 3.3.3D-E) reflect the trends found in the total pigment accumulation results, namely that 10 µmol/m^2/s gave the highest specific pigment accumulation. One also notices the decrease in specific pigment values in the dark control which is as expected.

E. When harvesting the calli it was observed, in the case of the 100 µmol/m^2/s treatment, that calli did not have the same appearance as calli grown under 1 and 10 µmol/m^2/s. They look shrunken and, to a lesser extent, dried out. There also were

excessive amounts of condensation indicating overheating of cultures. This is due to the fact that to achieve the light level required the plates were at 26cm away from a battery of six 125W fluorescent bulbs. It is thought that the excessive temperature, generated by the fluorescent bulbs, interfered with the results.

These results indicate that 10 μmol/m^2/s is the best light level to use to stimulate induction of pigment production and could be used in future experiments. This light level could be obtained on shelves that were adjacent to but not directly under the battery of lights used in this experiment.

3.3.4 Reproducibility of Light Effect at Different Temperatures

Previous experiments showed that growth was poor at the highest light level. Temperature measurements in the location under the battery of lights where the cultures were placed was found to be elevated in comparison to normal growth room temperatures. There was thus a need to find a suitable location in the growth room were the light level was at 10 μmol/m^2/s but with a lower ambient temperature than that under the light bulbs. The alternative location chosen was some shelving adjacent to the fluorescent lighting.

Figs. 3.3.4A-E show the results of the experiment to see whether or not one could use the shelves to the side of the battery of fluorescent tubes for the light induction experiments.

Figures 3.3.4A-E Experimental plates were set up as before. The plates were left one week in the dark. The calli were then left for 30 hours under the battery of lights as in the last experiment with muslin to reduce the light level to 10 μmol/m^2/s (light 26°C) or on the shelves (light 22°C) to the side of the lights. A dark control was also set up.

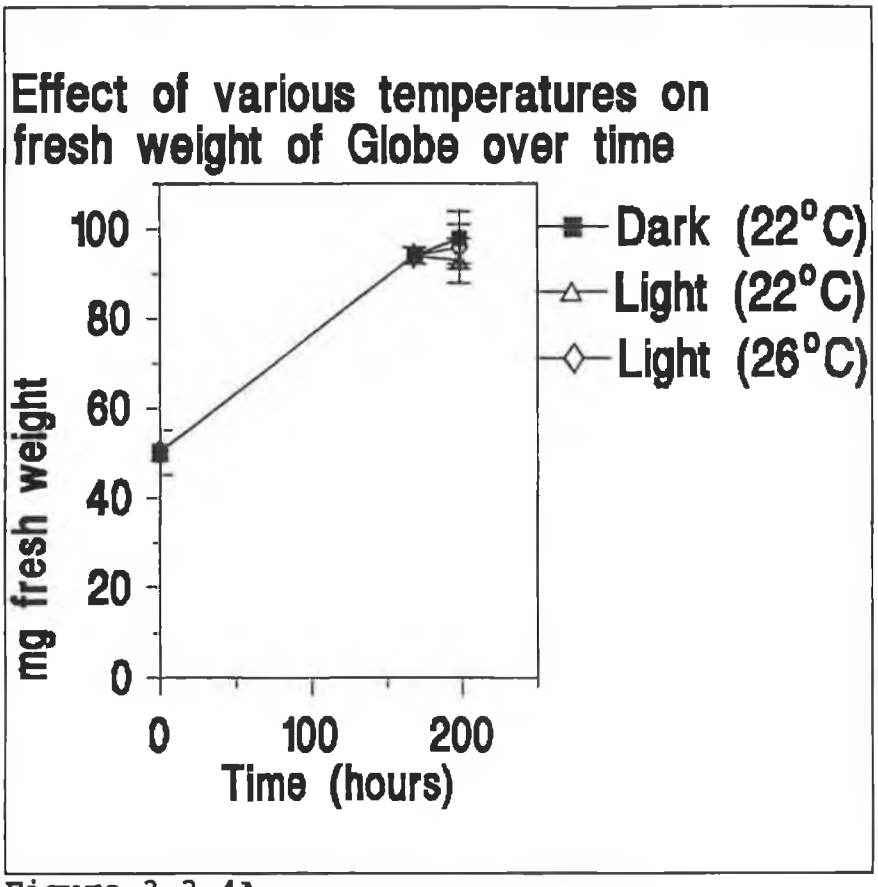


Figure 3.3.4A

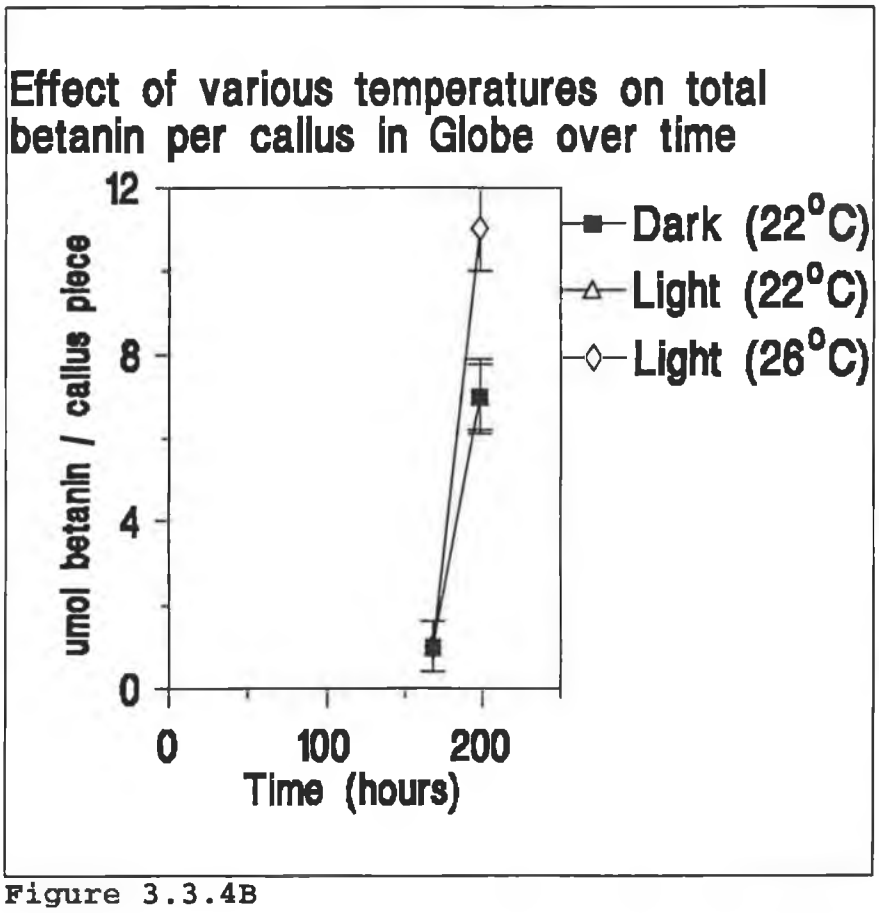


Figure 3.3.4B

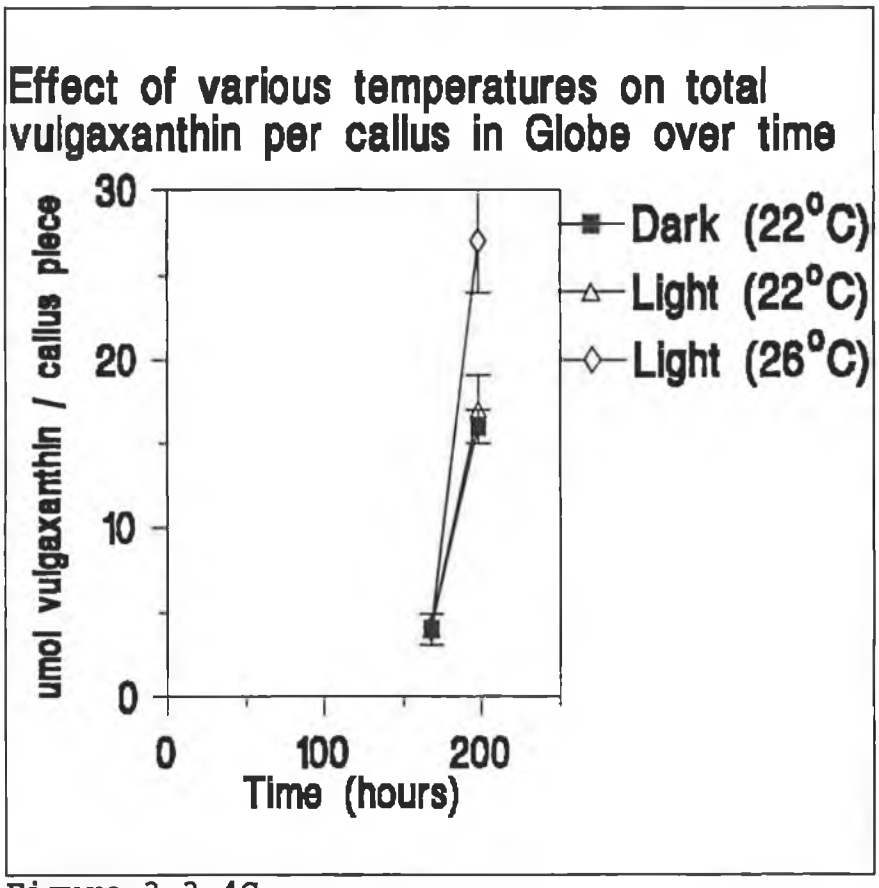


Figure 3.3.4C

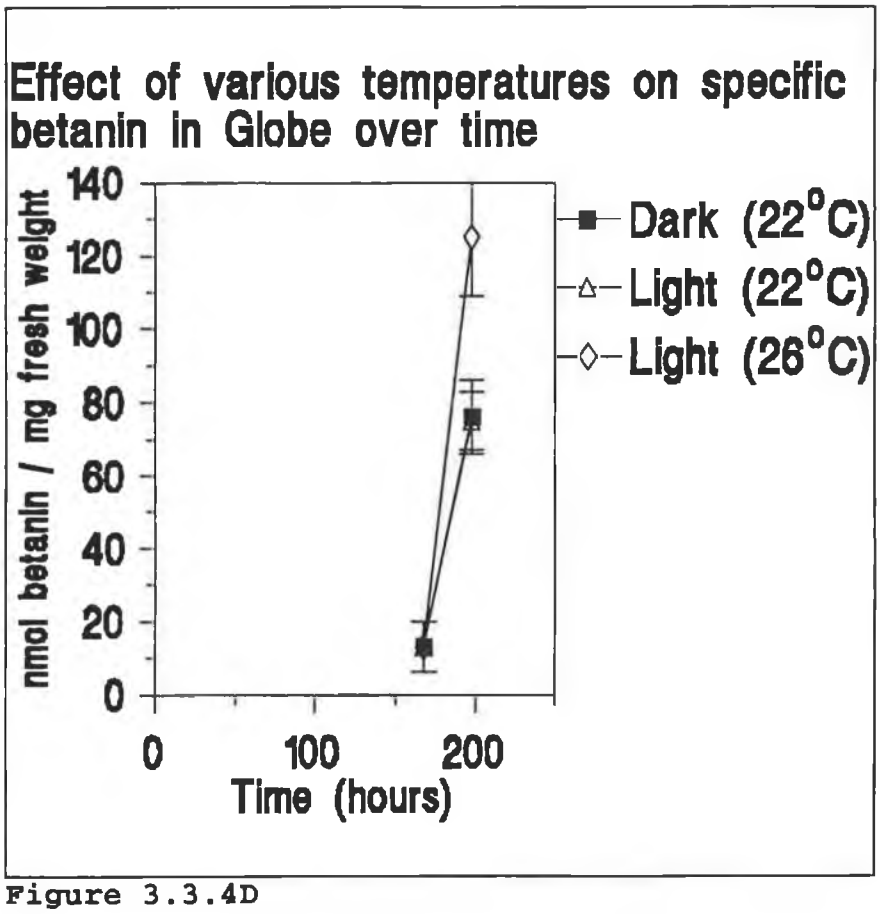


Figure 3.3.4D

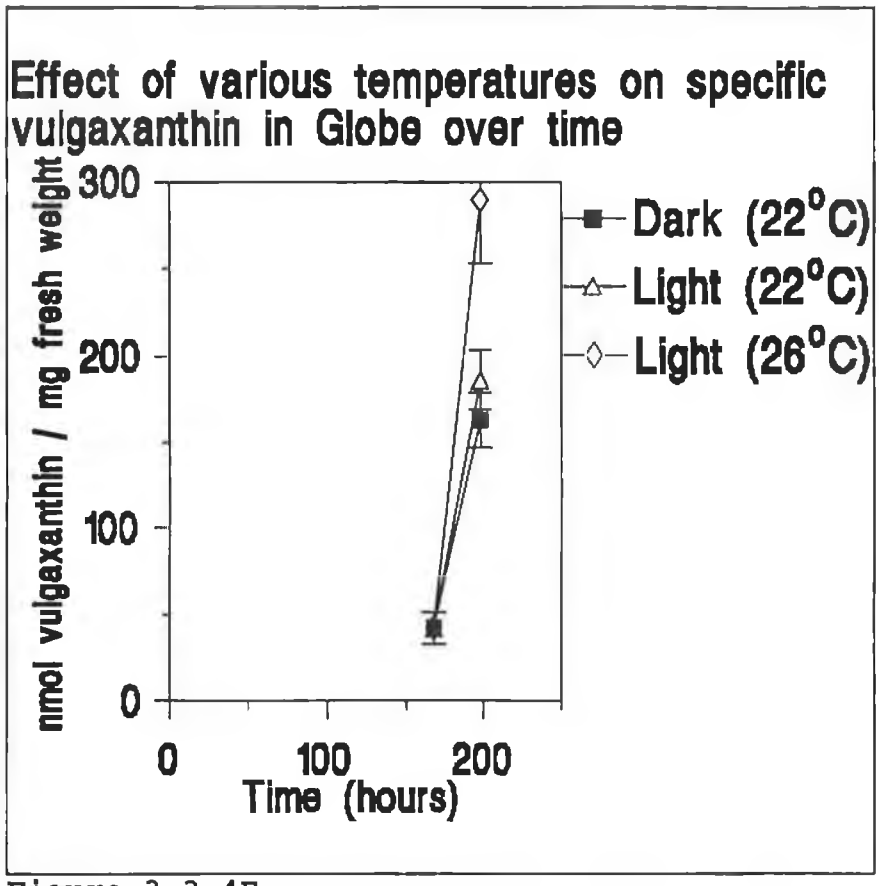


Figure 3.3.4E

A. Growth(fig. 3.3.4A) does not seem to differ significantly under the given treatments. 30 hours were chosen based on initial analysis of previous results. However 168 hour(1 week) exposure would have been more appropriate for the model system.

B. Figs. 3.3.4B-C show total pigment accumulation per callus. If one compares the 'light 26°C' values (10 µmol/m^2/s) with the 30 hour 10 µmol/m^2/s values from the last experiment one sees that there is a similarity; 11µmol(this experiment)/ 10µmol(last experiment) for betanin, 26µmol(this experiment)/19µmol(last experiment) for vulgaxanthin. The result seems to indicate that calli faired better when directly under the battery of lights.

C. Figs. 3.3.4D-E show specific pigment accumulation. The graphs reinforce the total pigment accumulation per callus trends in that the greatest accumulation of betanin and vulgaxanthin occurred when the cultures were incubated under the battery of lights where the temperature was 26°C.

One notices that both total and specific pigment accumulation increased 3-5 fold in the dark within 30 hours. This could be due to the way the experiment was carried out. All experimental plates, once set up, were kept in the same box for the initial one week

dark treatment. As a result the time needed to remove plates from the box for the purpose of the different light treatments may have been sufficient to enhance the constitutive pigment accumulation significantly enough to cause such a large pigment accumulation over a short period of time.

The results seem to indicate that the calli faired better when directly under the light. The experiment, however, was set up in such a way that the light levels were the same in both locations in the growth room. It thus seems that another factor can influence pigment production in the given model system. A slight temperature increase appeared to be the most likely possibility.

3.3.5 Effect of Temperature and Red Light

In an effort to optimise the light induction effect, it was decided to investigate the effect of heat shock pretreatment in calli since it was suspected that temperature was influencing pigment production. A method by Elliott D.C.(1979a,b, 1982) served as a starting point for this experiment. The model system in question was *Amaranthus tricolor* seedlings which was used to look at the effect of temperature on cytokinin induction. Elliott D.C. 1979a found that, despite betacyanin accumulation being inhibited at high temperatures, growth was unimpaired and there was still adequate uptake of cytokinin. On the otherhand a preincubation at an elevated temperature does not prevent subsequent induction at a lower temperature as there appears to be no irreversible change occurring during the preincubation treatment. It is thought that, in this particular case, elevated temperatures alter membrane properties in such a way so as to enhance cytokinin action.

Elliott D.C. 1979b subsequently looks at the interaction of temperature and red light. This is based on the fact that both red light acting on the phytochrome system and cytokinin in the dark are regulated by K^+ and Na^+ ions. Elliott D.C. found that the maximum response is achieved after 2 hours shift to 40°C followed by an hour at 25°C. The red light induction consisted of 30 mins plus 23.5 hours of further dark incubation at 25°C.

Elliott D.C. 1982 method of 1.5 hours at 40°C and 1.5 hours at 22°C was used as it was believed to be more appropriate for the experimental system i.e. calli in parafilm-sealed agar-containing Petri dishes. The continued incubation in the dark after the red light treatment was considered futile based on the observation that light promoted pigment synthesis. In her paper D.C. Elliott indicated 40°C as the temperature needed

for the 1.5 hour pretreatment/aging step. However from experience this temperature results in condensation building up. Consequently it was decided to test 29°C as well as 40°C, using 22°C as a control. Details of experimental procedure are given in legend for figures 3.3.5A-E.

Figures 3.3.5A-E. Experimental plates were set up as previously described. They were then left in the dark for a week. 2 plates(10 pieces of calli) were exposed to each temperature; 22°C (constant temperature room 22°C +/- 2°C where all experiments were carried out unless otherwise stated), 29°C (constant warm room) and 40°C (incubator; temperature was verified by taking the temperature of water in test tubes) and incubated for 1.5 hours. All plates were then return to 22°C for 1.5 hours in the dark. Finally the plates were exposed to 30 minutes red light(10 µmol/m^2/s) and subsequently harvested. The control consisted of 2 plates, with 5 pieces of calli per plate, kept in the dark at 22°C for the duration of the experiment.

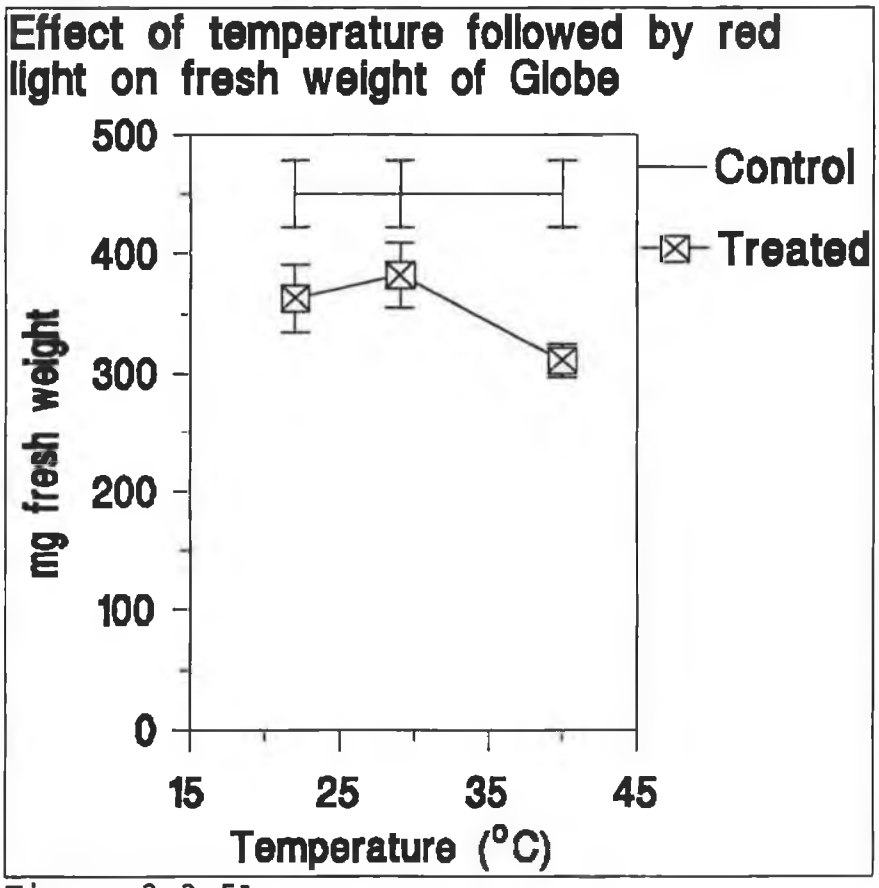


Figure 3.3.5A

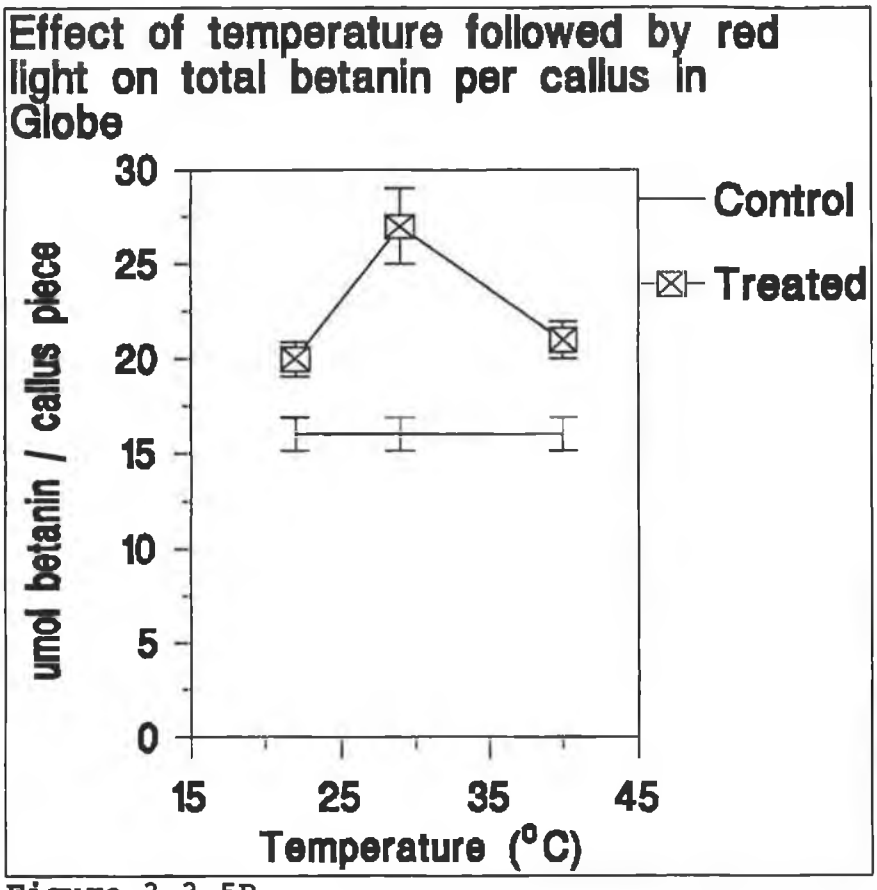


Figure 3.3.5B

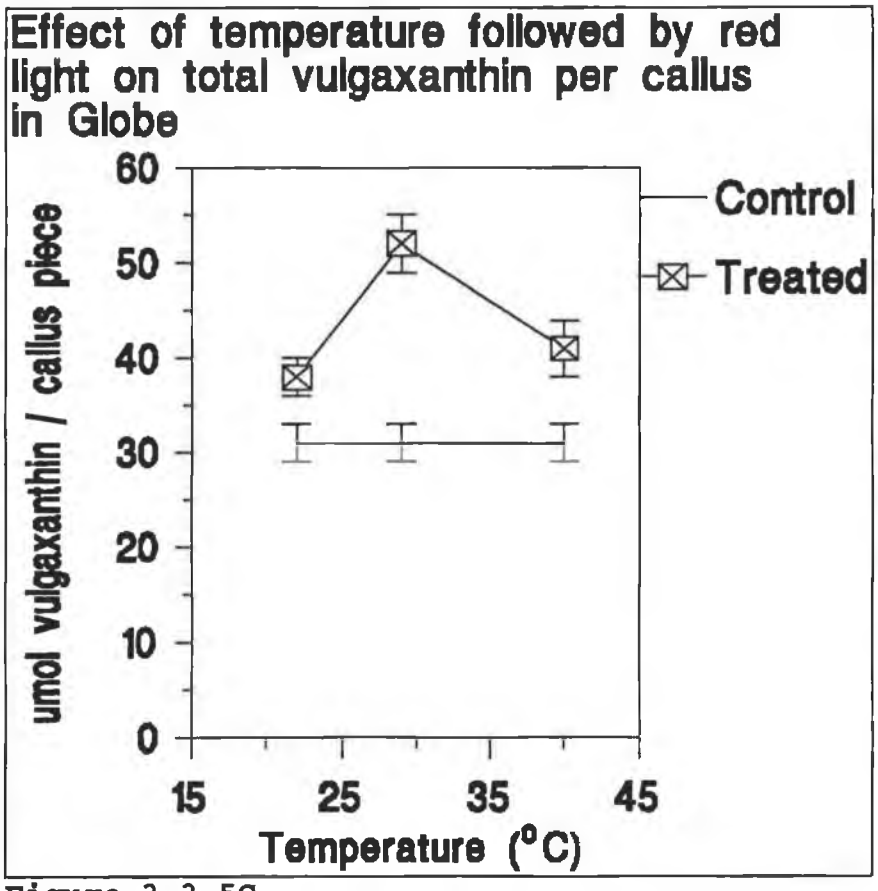


Figure 3.3.5C

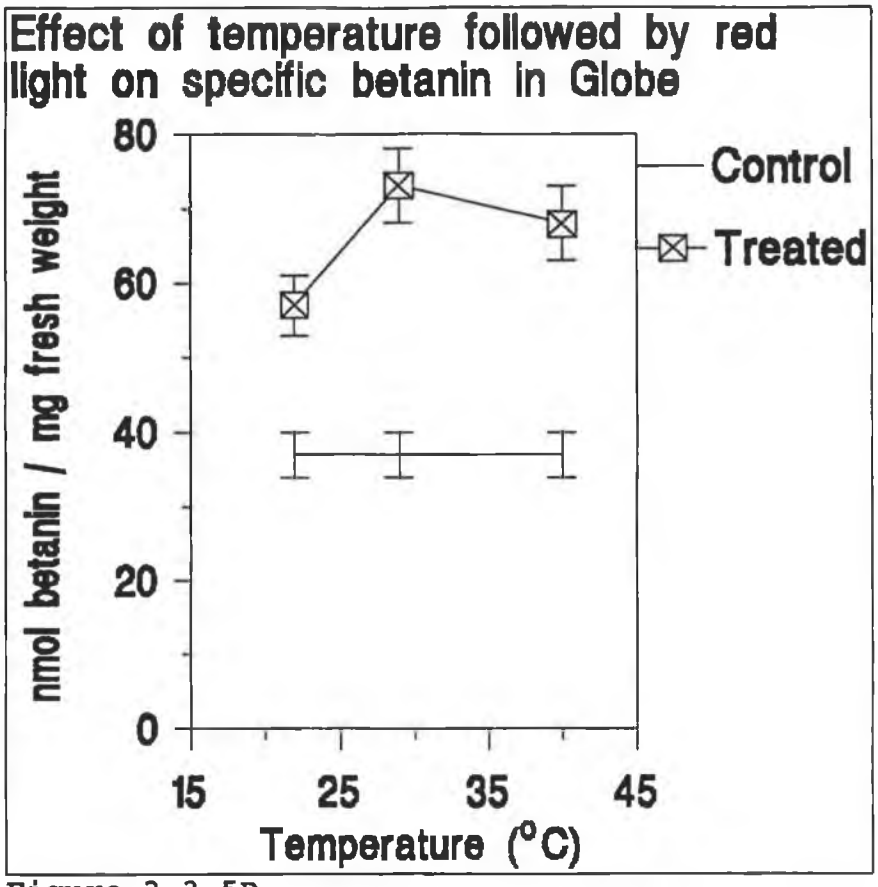


Figure 3.3.5D

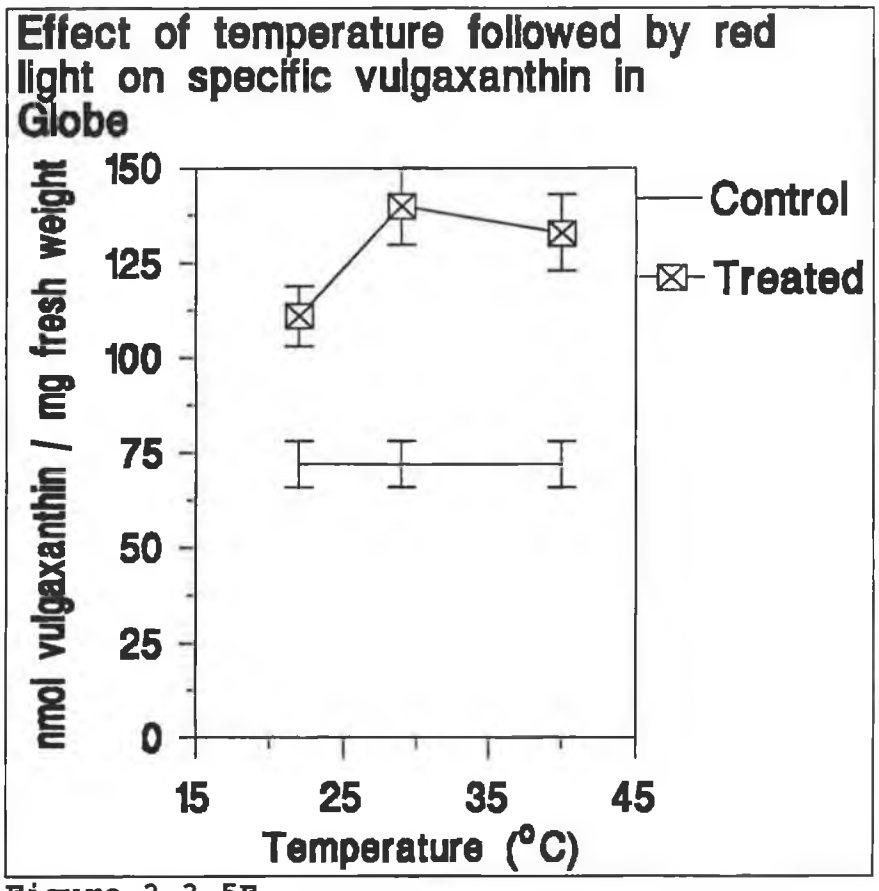


Figure 3.3.5E

Fig. 3.3.5A, showing the effect of temperature on fresh weight, demonstrates how 40°C can restrict growth. The differences in fresh weight may be the result of experimental error in the case of 22°C and 29°C and water loss at 40°C. This has implications on total pigment per callus and as can be seen it is nearly the same as those calli kept at 22°C.

The results seem to suggest that a heat pretreatment of 29°C promotes the effect of a subsequent treatment, in this case 30 minutes red light. However it was considered an apparent induction as no visible yellowing of calli was occurring that was typical of previous experiments lasting 4 weeks. The lack of yellowing is most probably due to the short time of exposure and low levels of pigment accumulated. The following experiment has a longer time exposure to red light at the higher temperature of 27°C than that of the control at 22°C.

3.3.6 Effect of Light Quality

The general conditions needed for a visible yellowing of the callus appeared to be 1(minimum) to 2(maximum) weeks in the dark followed by 1(minimum) week illumination at 10 μmol/m^2/s with an ambient temperature of between 25°C to 29°C. However these conditions did not give the wanted total yellowing of calli which would be optimal for a reproducible model system. To be able to use the experimental system it was desirable to produce as clear-cut an induction as possible. This can be achieved by both maximising induction in the light and minimising induction in the dark after subculture. The literature indicated that light quality played a role in causing pigment accumulation (Obrenovic S. 1986). This could be seen in cotyledons used as a model system for studies on the role of phytochrome(red/far red) and cryptochrome(blue) action (Mohr H. & Drumm-Herel H. 1983, Oelmüller R. & Mohr H. 1985). It was also found that safe green light, used during plant manipulation, caused a slight pigment induction (Obrenovic S. 1990, Nick P. *et al.* 1993). This was of interest as 1 week pigment accumulation values, registered in experiments, seemed to be high. It was thought that illumination during experimental set up was causing this unwanted pigment accumulation. Consequently it was decided to use blue, red and green coloured filters to illuminate calli. White light was used as a control. Figs. 3.3.6A-F show the results of this experiment.

Figures 3.3.6A-F Plates were set up as with previous experiments. Plates were kept one week in the dark then illuminated by three 30W fluorescent warm white tubes. Circular discs of colour filters producing red, green and blue spectra intersecting minimally (Lee Filters, Section 2.4.2) were placed between the light source and the calli. Muslin, placed between the colour filters and the light source, was used to reduced the light level, especially in the case of the red and white light, to 10 µmol/m^2/s. Ambient temperature was between 25°C - 28°C.

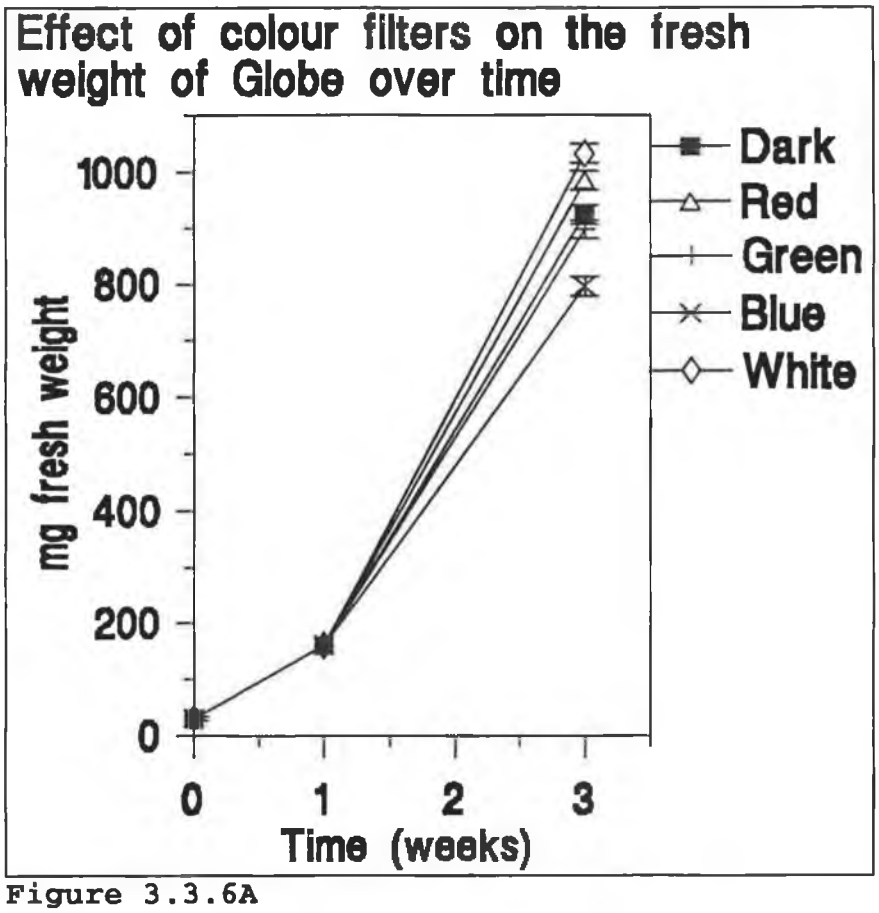


Figure 3.3.6A

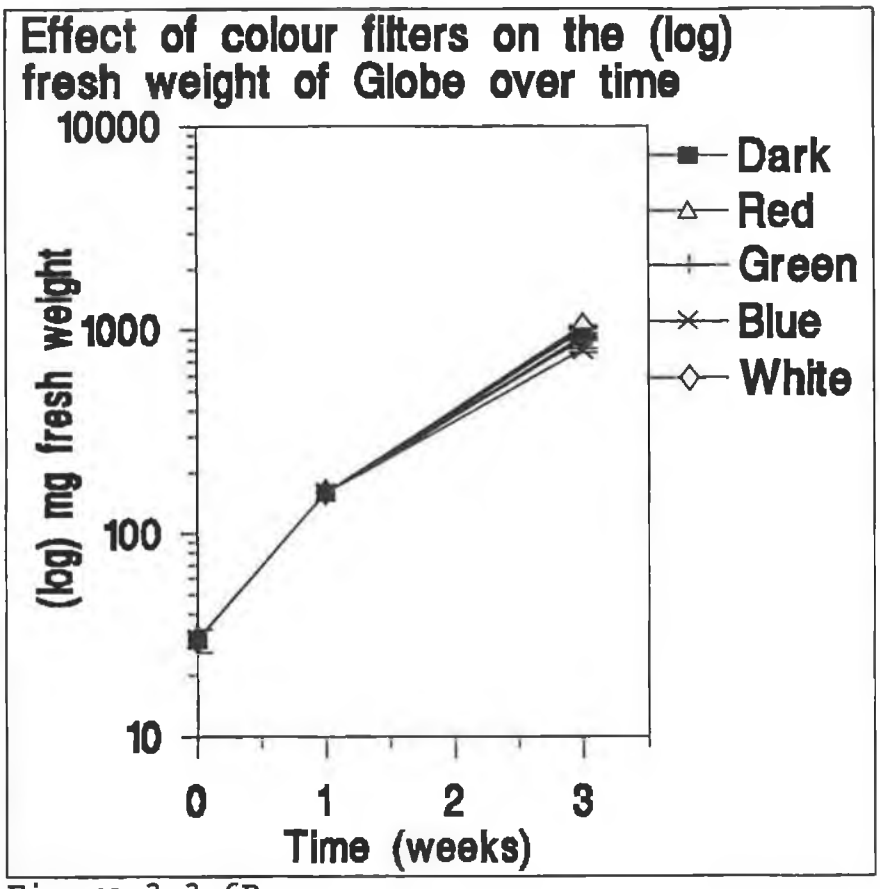


Figure 3.3.6B

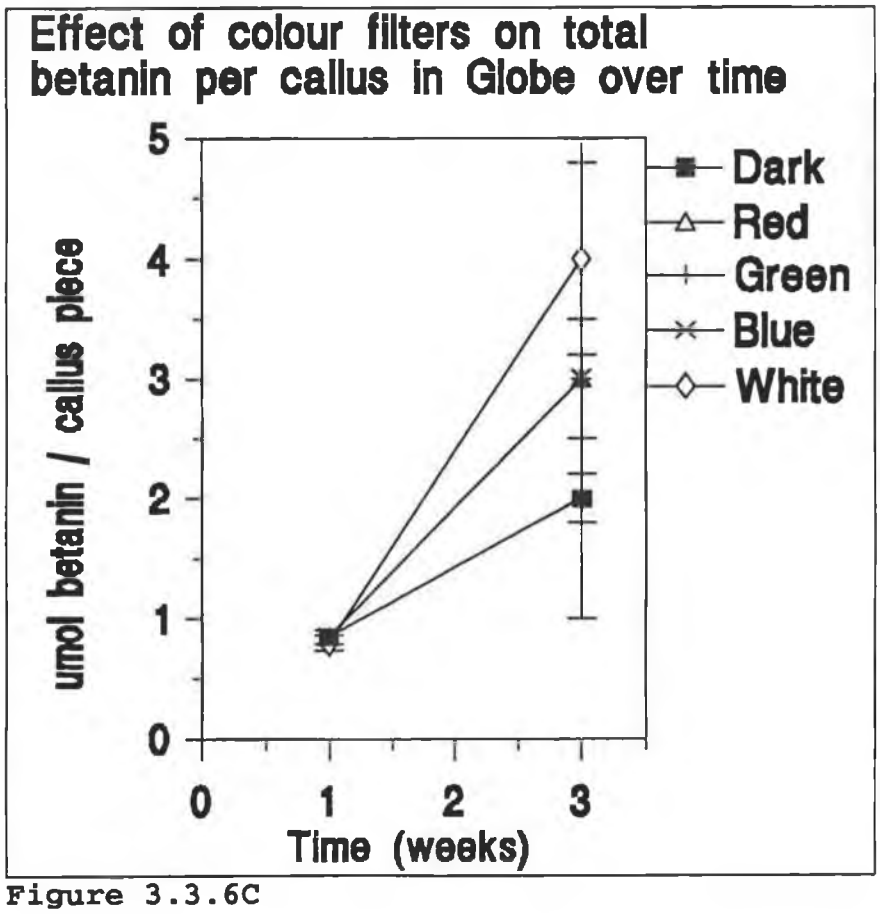


Figure 3.3.6C

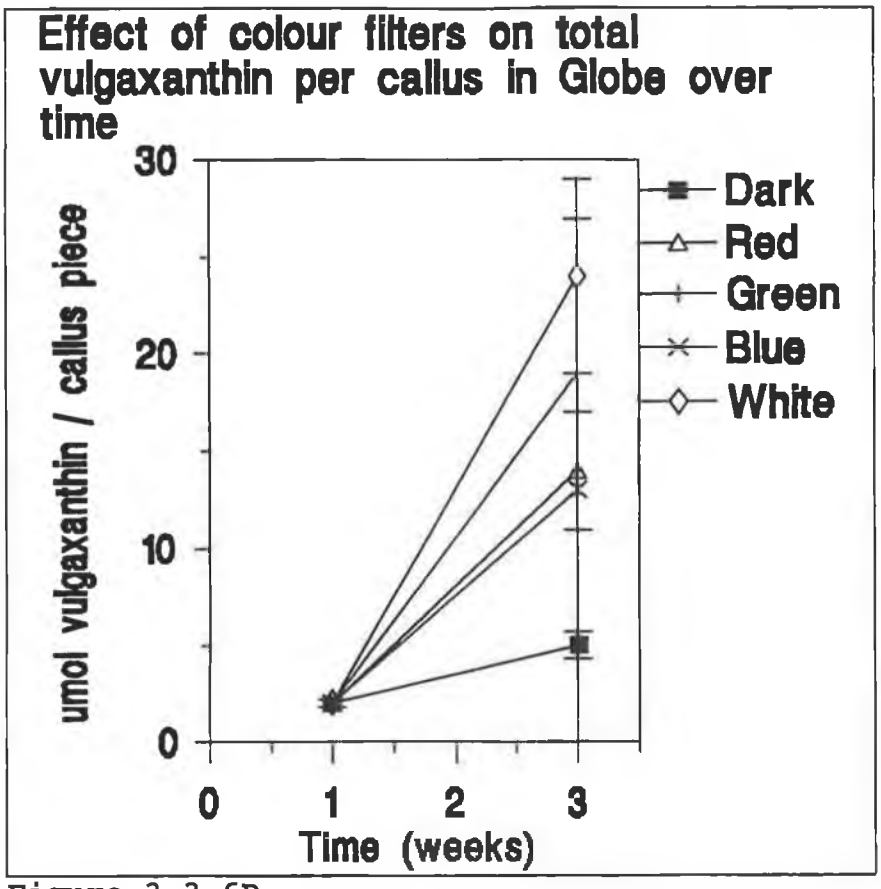


Figure 3.3.6D

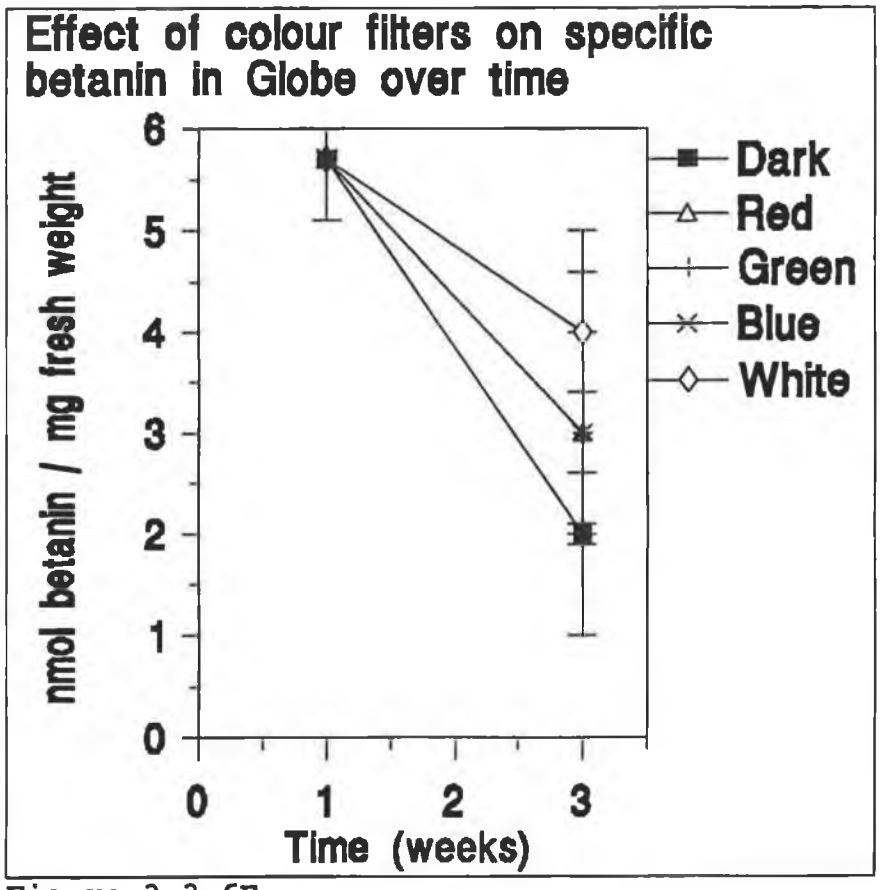


Figure 3.3.6E

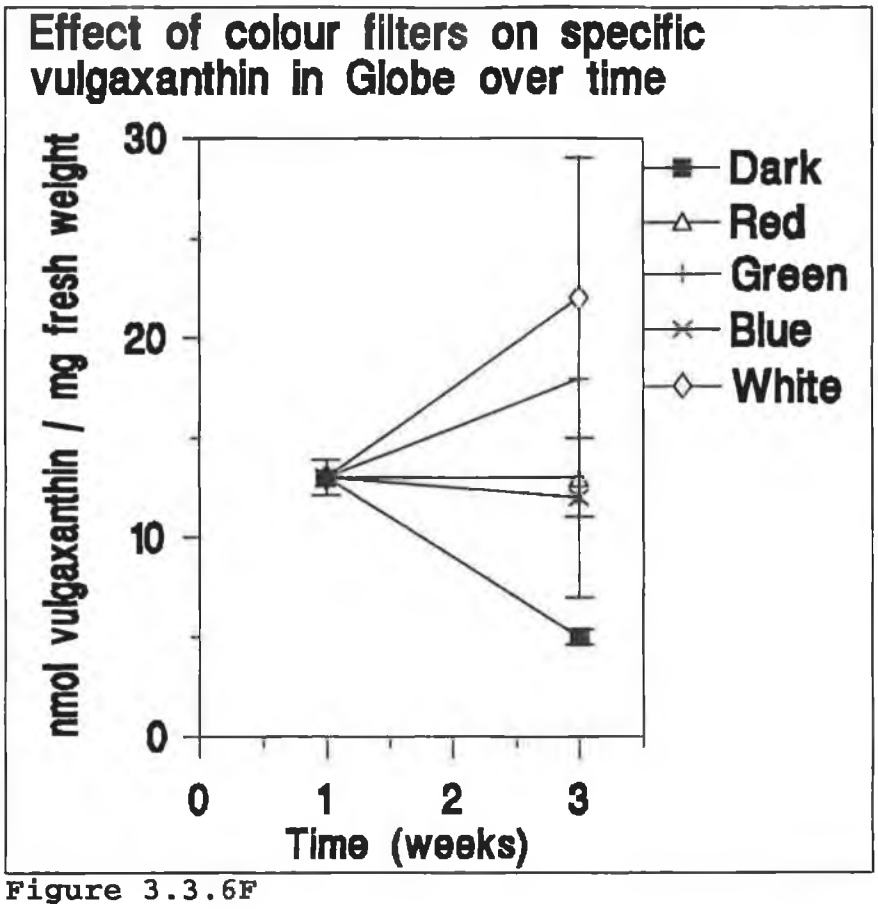


Figure 3.3.6F

It was found that;

A. Growth(fig. 3.3.6A) did not appear to be affected by any particular coloured filter. The lower value for calli grown under blue light is probably due to the heterogeneity of the calli. Despite the fact that there is no value for week 2 one can suspect a straight line for the first two weeks indicating that growth was in a logarithmic phase of multiplication(Fig. 3.3.6B).

B. Figs. 3.3.6C-D show total pigment accumulation per callus. Here the results are contrary to expectation. It appears that green filters give the same result as red and blue. This can partly be explained by observations made by Obrenovic S. 1986, 1990 and Nick P. *et al.* 1993. These authors indirectly imply that low levels of green light can induce pigment production via some parts of the phytochrome system which can become physiologically active given such a treatment.

C. Values for specific pigment accumulation(fig. 3.3.6E) are somewhat different to those for total pigment per callus accumulation. The steady decrease in specific betanin accumulation may be due to a combination of a number of factors. This will be outlined later on. The specific values for vulgaxanthin accumulation (fig. 3.3.6F) reinforce somewhat the trends of total vulgaxanthin per callus accumulation with dark

values decreasing and those of light increasing. Calli growth under red and blue light must have outstripped pigment production.

The results appear to suggest that green is just as effective in inducing/enhancing pigment production. The other point of interest is the decrease in specific betanin accumulation even when illuminated with white light. It is thought that the following reasons may explain this anomaly.

- It was noticed, before harvesting, that one of the plates of the pair, for each treatment, was less pigmented. It was also noticed that all the less pigmented calli were on one side(plates were arranged in 2 rows). It turned out that one of the fluorescent bulbs was expiring during the experiment.
- The other factor was that, as said before, Globe goes yellow due to the production of betaxanthins. As a result any betanin produced is in competition with betaxanthin production. This was particularly so given the experiment had an ambient temperature of 27°C which, based on the last experiment, is the optimal temperature for pigment induction.

Given these two observations plus the associated visible increase in pigmented tissue and a higher degree of pigmentation during the experiment, the net result was that specific betanin decreased, even when cultures were illuminated by white light.

3.4 Precursor Experiments

The approach to the project consisted of obtaining initially a non-pigment-producing white line then causing pigment production by applying an inducer whether it be physical or chemical. This would result in the white line going violet/yellow due to the production of pigment. This inducible system would then be used to examine a possible rate-limiting step and secondly to examine the mechanism by which the inducer activated the cell to produce pigment.

It is the following set of experiments that attempt to elucidate the rate-limiting step. This entailed adding precursors to the medium on which calli were grown. The precursor feeding experiments were based on the argument that the feeding of precursors at given points along the pathway may stimulate an increase in pigment accumulation. The precursor or intermediate that caused a significantly higher increase in pigment accumulation would indicate the location of the potential rate-limiting step.

Initially it was thought that precursor feeding in the dark should stimulate the biosynthetic pathway sufficiently and, if not, give a result more attributable to the effect of feeding precursors as opposed to the added interaction of light, as will be seen later on.

3.4.1 Dark Treatment of Precursor Feeding

The literature up until now has used two precursors, tyrosine and DOPA. Since the literature had indicated that tyrosine and DOPA stimulated pigment production in other model systems, it was decided to make the assumption that tyrosine and DOPA would do likewise in white callus cultures of red beet. The need to look at the effect of shikimic acid is based on the fact that, if induction of pigment accumulation is occurring in other model systems at the tyrosine and DOPA level, that the rate-limiting step may be up stream at the shikimic acid, chorismic acid or arogenate level. It could also just as well occur downstream at the conversion from L-DOPA to betalamic acid(fig. 1.1).

It is proposed to initially look at the results of feeding either shikimic acid, tyrosine or DOPA to callus in the dark then to make a comparison of the three precursors in an attempt to elucidate a possible rate-limiting step. The same will be done for callus fed with each of the precursors when grown in the light. An overall comparison will then be made.

The shikimic acid results are based on the feeding of shikimic acid to calli grown in the dark. Figs. 3.4.1A-F show the results of shikimic acid feeding.

Note: To facilitate comparisons between the 3 precursors, the same scaling of the y-axis has been used.

Figures 3.4.1A-F Experimental plates were set up as previously described. 5 pieces of calli per plate, two plates per treatment. Experimental plates were kept in a box at ambient temperature 22°C +/- 3°C and removed at weekly intervals. There was no one week dark pretreatment.

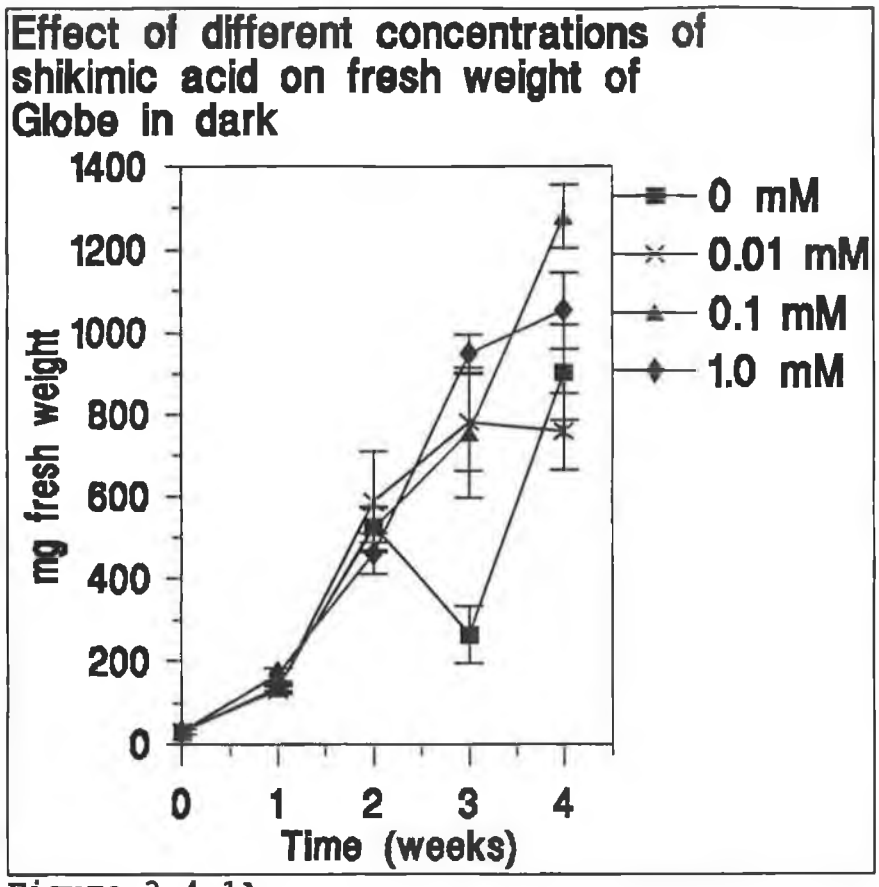


Figure 3.4.1A

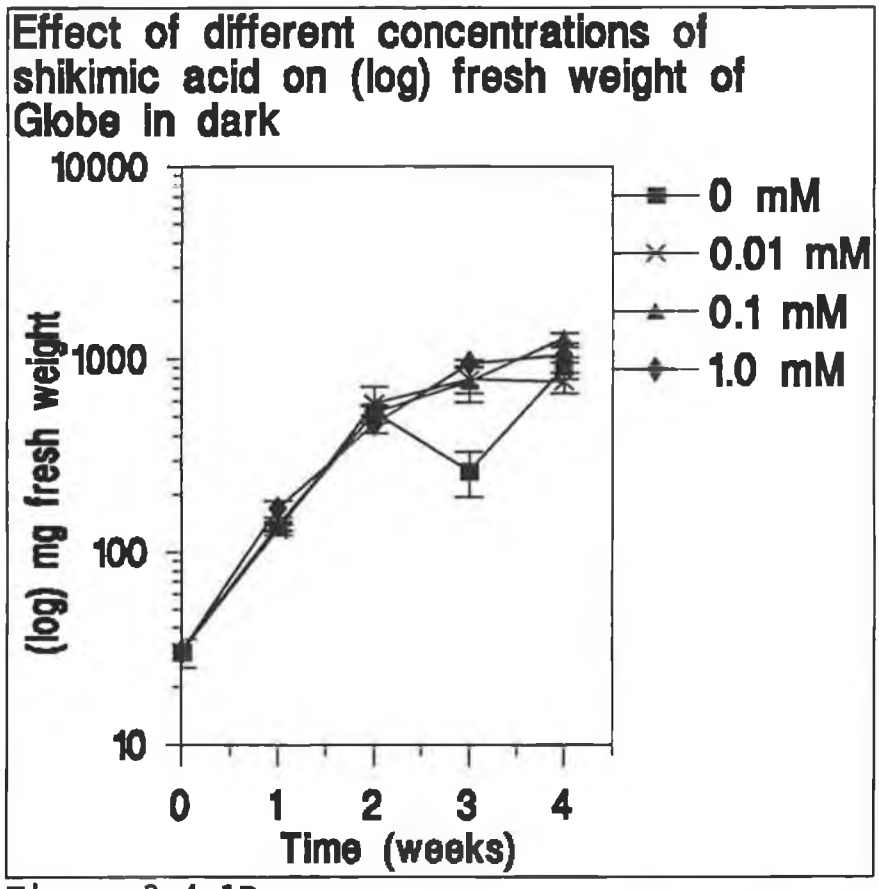


Figure 3.4.1B

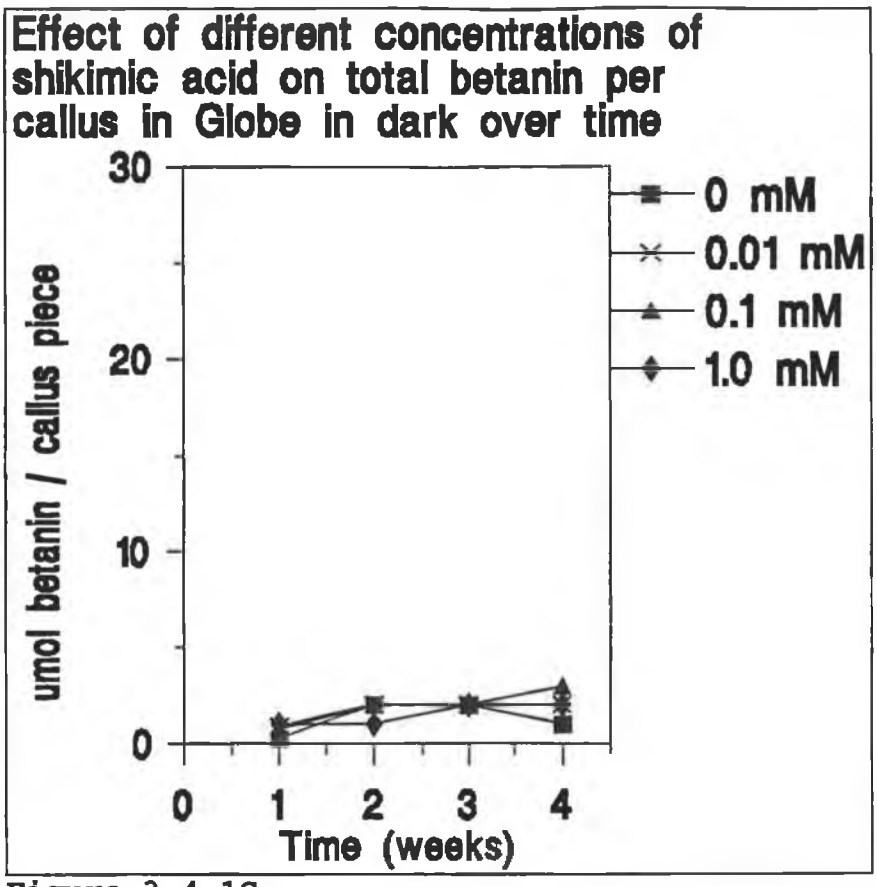


Figure 3.4.1C

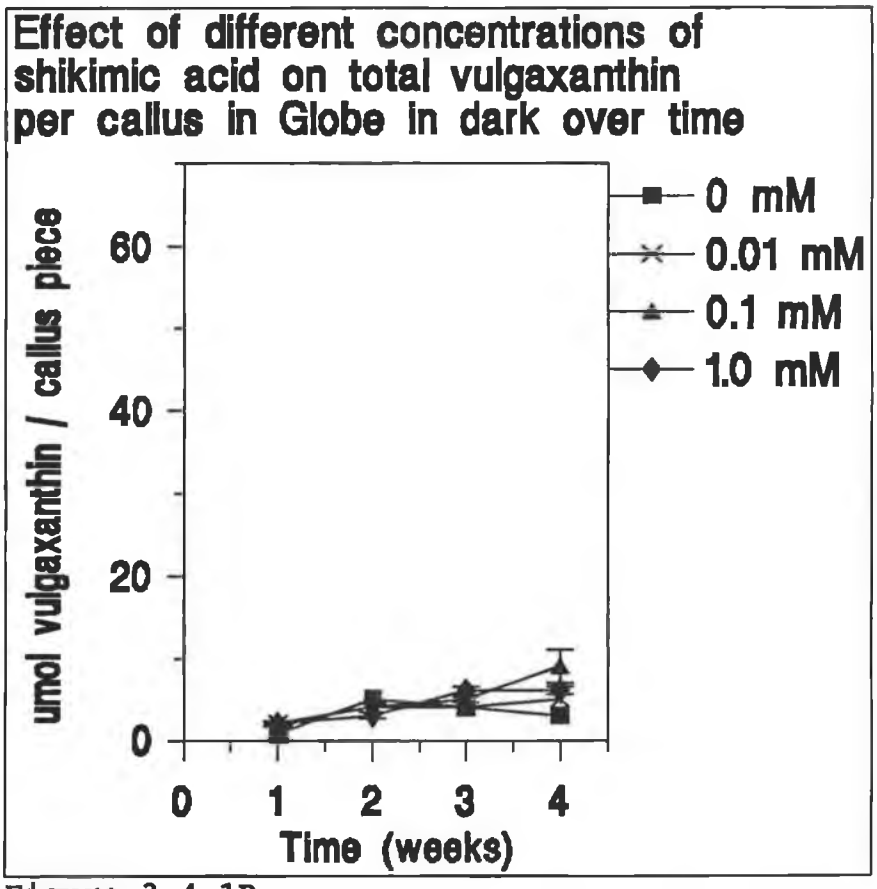


Figure 3.4.1D

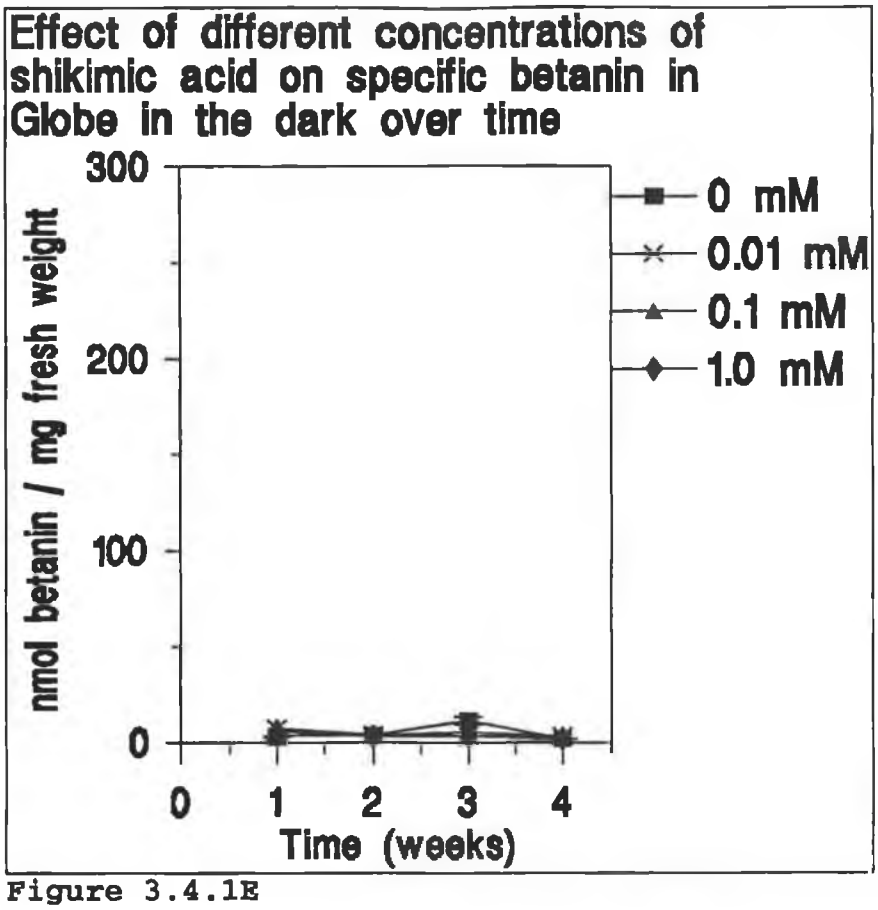


Figure 3.4.1E

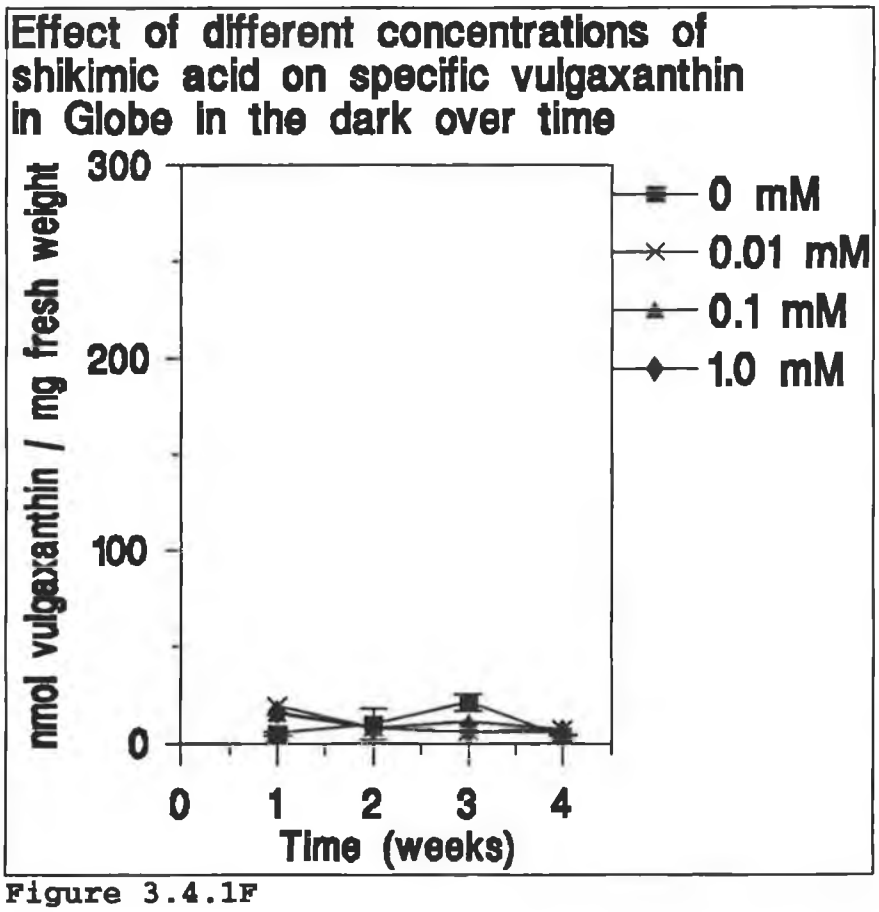


Figure 3.4.1F

A. Fig. 3.4.1A, showing fresh weight accumulation of callus when fed with different concentrations of shikimic acid, indicate that the different concentrations of the precursor has no real effect for the first two week after which differences emerge. The significant dip in week 3 for 0 mM shikimic acid is probably due to the heterogeneity of the calli. It is surprising to find that 0.1 mM fairs better that 1.0 mM and 0.01 mM in week 4. The logarithmic phase of multiplication lasts only for the first two weeks(fig. 3.4.1B) after which the growth curves level out promptly.

B. Total pigment accumulation per callus, whether it be betanin (fig. 3.4.1C) or vulgaxanthin (fig. 3.4.1D), rises during the first two weeks, after which it stabilises. It appears that 0.1 mM promotes the highest total pigment accumulation per callus after 4 weeks.

C. Figs. 3.4.1E-F reveals an unexpected rise in specific pigment accumulation in week 3 when no shikimic acid is given to calli. If one ignores for a moment the values for 0 mM shikimic acid one sees that specific values decrease with time in the dark. This does not come as a surprise based on previous results. The extent of the error bars seem to suggest that there is no effect of concentration on specific pigment content over time.

A general conclusion for these results is that shikimic acid hardly promotes pigment accumulation over time except for with 0.1 mM. Here one perceives a slight stimulating effect. The implication of these results can only be seen in relation to the effect of other precursors. Since tyrosine and DOPA were readily available it was decided to examine their effect on pigment accumulation. Figs. 3.4.1G-L show the results of feeding different concentrations of tyrosine to calli grown in the dark.

Figures 3.4.1G-L Experimental plates were set up as described previously. Since tyrosine dissolves with difficulty in water the calculated mg quantities needed were initially dissolved in a minimal amount of 1.0 M NaOH then added to the rest of the medium before the pH was adjusted to 5.7. 2 plates with 5 pieces of callus each were used per treatment. Plates were left in the dark at 22°C. The concentrations 0.1, 1.0 and 2.5 mM were chosen based on (1) the literature survey, (2) to be able to plot on a log scale if the results turned out in such a way that pigment production was closely linked to growth and (3) the solubility limit of tyrosine in water at 25°C (Merck Index). There was no preincubation of 1 week in the dark.

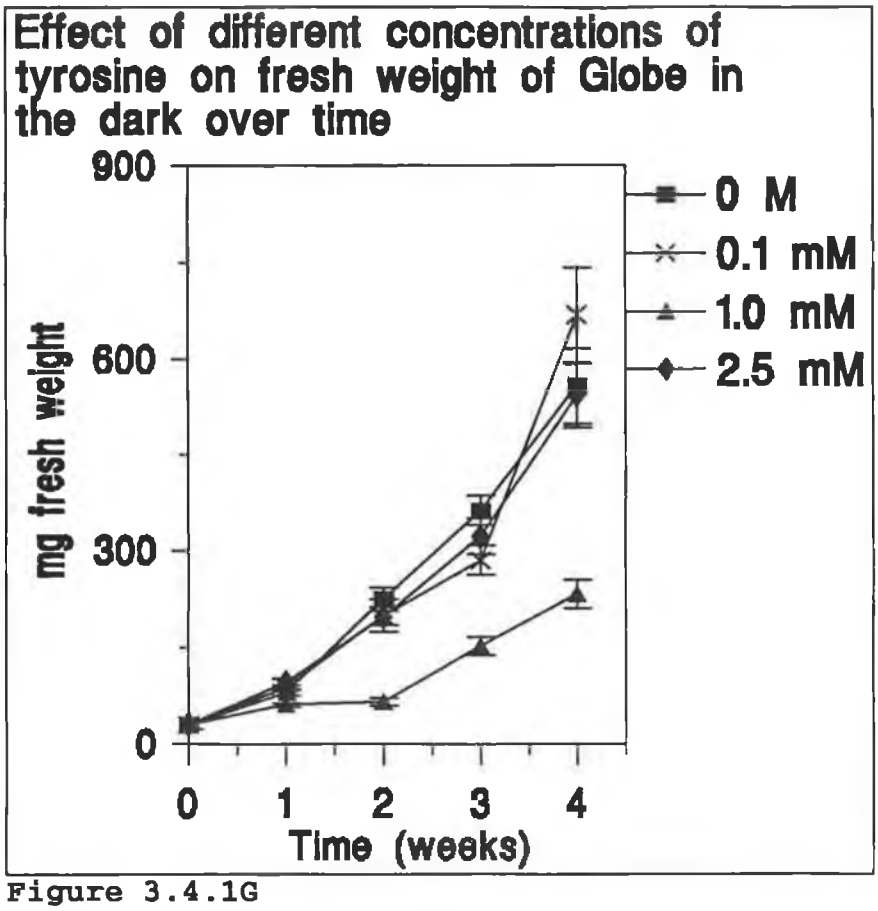


Figure 3.4.1G

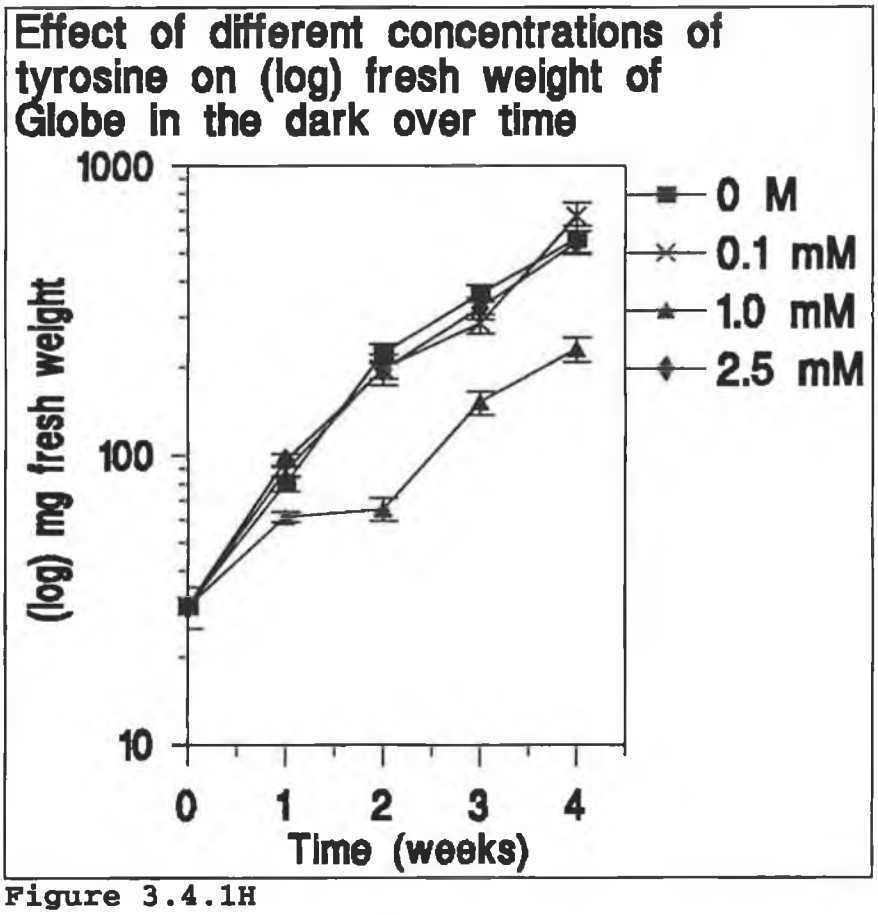


Figure 3.4.1H

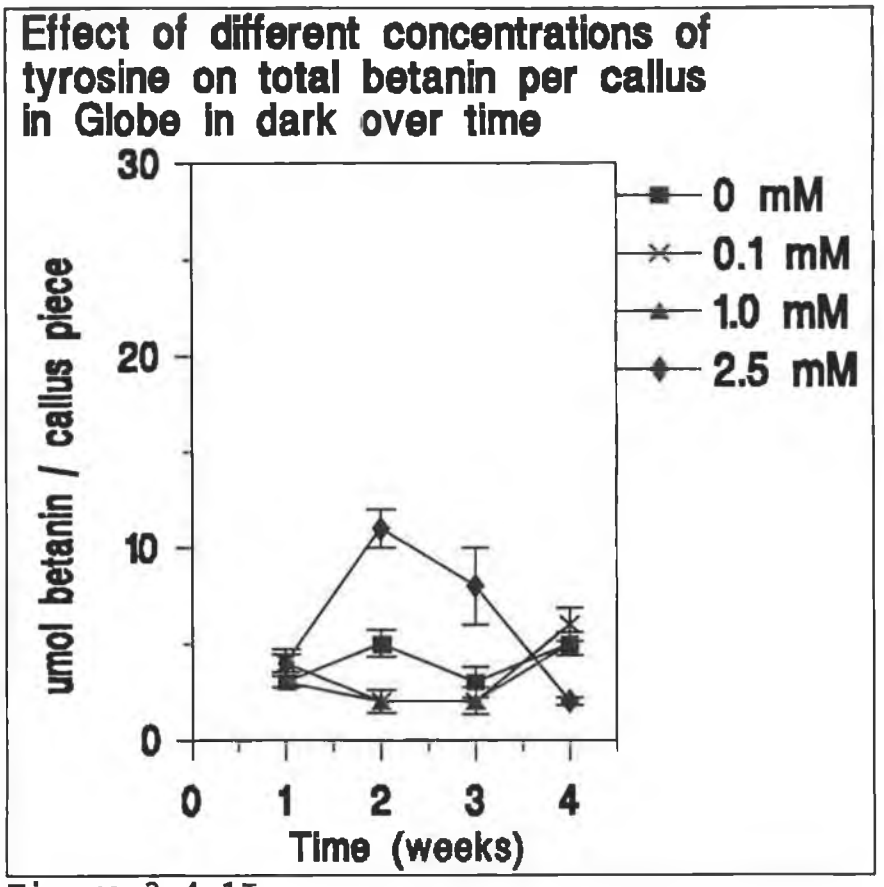


Figure 3.4.1I

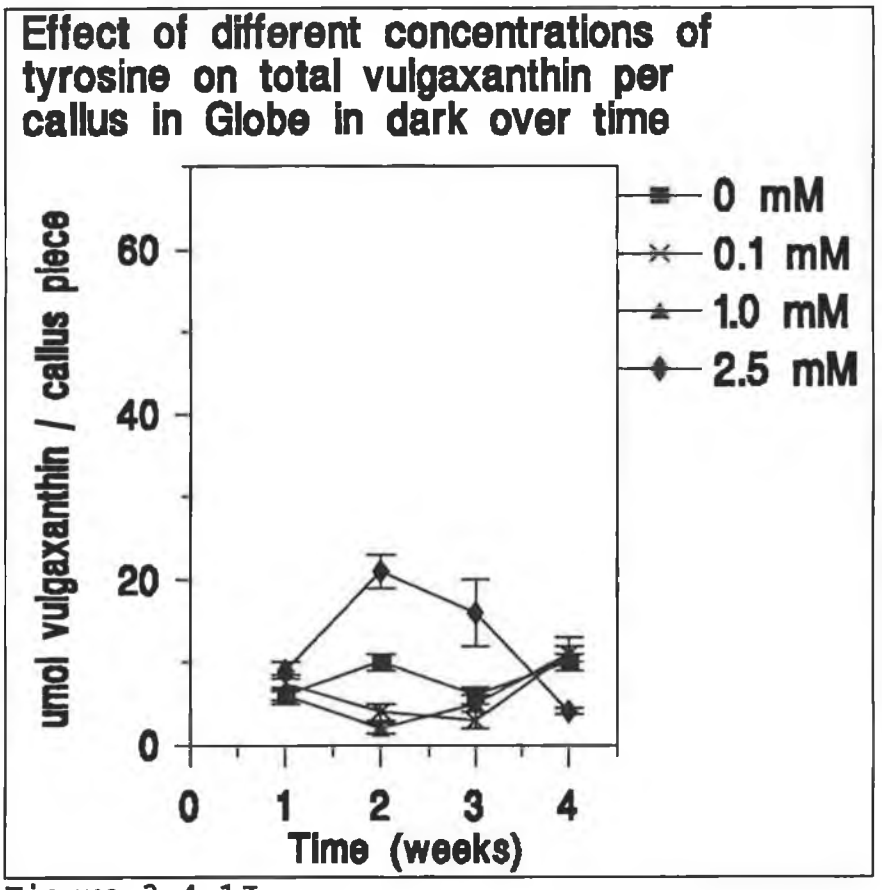


Figure 3.4.1J

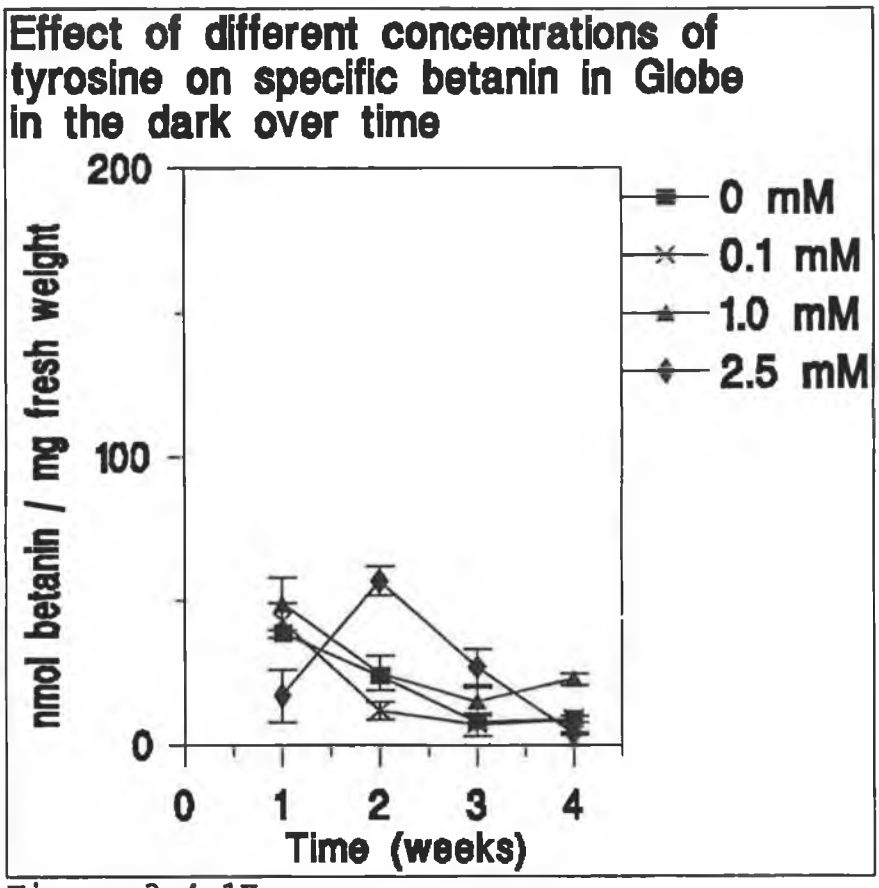


Figure 3.4.1K

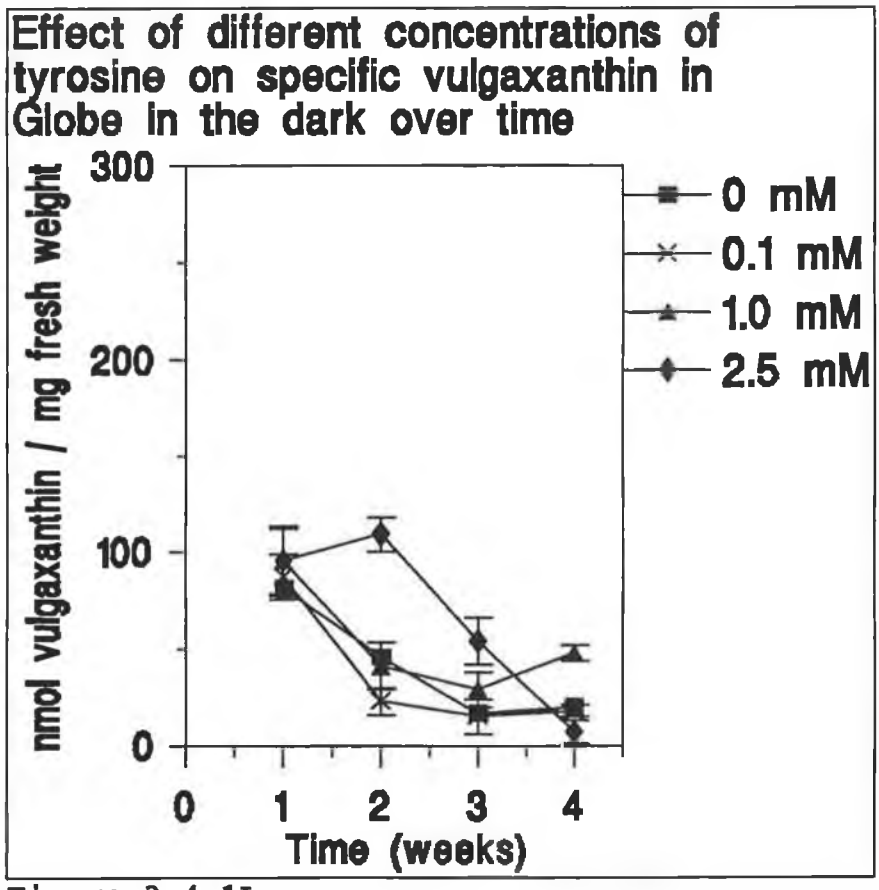


Figure 3.4.1L

The observations of figures 3.4.1G-L are as follows:

A. The effect of different concentrations of tyrosine on fresh weight(Fig. 3.4.1G) does not appear to produce any identifiable trends or variability. One notices that 1.0 mM tyrosine gives the lowest fresh weight accumulation after 4 week whereas the 0.1mM results in the highest. This is probably due to the heterogeneity of the callus. Fig. 3.4.1H indicates log linear growth in the first 2 weeks.

B. Figs. 3.4.1I-J show total pigment per callus accumulation when calli are grown on media containing different concentrations of tyrosine. The only clear result is that 2.5 mM tyrosine appears to induce pigment accumulation maximally after two weeks. Pigment accumulation in the dark over the 4 weeks is somewhat erratic for the other concentrations. However the final result is that pigment accumulation increases only slightly over time.

C. Specific values for pigment accumulation(figs. 3.4.1K-L) appear to reinforce observed trends. The delayed decrease in values for calli grown on 2.5 mM tyrosine tally with the total pigment accumulation trend. The slight rise in specific value in the 4^{th} week, in the case of calli grown on 1.0 mM tyrosine, is probably due to the lower fresh weight accumulation(fig. 3.4.1G) and a higher pigment accumulation rate.

In conclusion the control results show that cells grow in the dark without synthesizing large amounts of betanin or vulgaxanthin, and that pigment is therefore diluted out. The addition of tyrosine has only a small effect on pigment production, and none on growth.

When one compares the results of shikimic acid and tyrosine one sees that;

A. fresh weight accumulation was stimulated by shikimic acid (fig. 3.4.1A) with 1300mg accumulated after 4 weeks whereas with tyrosine(fig. 3.4.1G) total maximum accumulation was 700mg. In addition the phase of logarithmic multiplication only lasted 2 weeks in the case of shikimic acid(fig. 3.4.1B) whereas with tyrosine it lasted well into week 3(fig. 3.4.1H).

B. in the case of total pigment accumulation the situation is reversed. Tyrosine causes a tripling of total betanin(fig. 3.4.1I) compared to shikimic acid(fig. 3.4.1C) after 2 weeks. A four fold increase in total vulgaxanthin per callus is observed when tyrosine is fed (figs. 3.4.1J, 3.4.1D).

C. a comparison of results for specific pigment accumulation when callus is fed with shikimic acid(figs. 3.4.1E-F) or tyrosine(figs. 3.4.1K-L) gives the same type of picture. Tyrosine results in a higher specific pigment accumulation during the first week and half. Interestingly specific pigment accumulation for shikimic acid remains

constant throughout the 4 weeks. This may be due to growth being favoured.

The relevance of the results of shikimic acid and tyrosine can only be put into context on comparison with what happens when calli are fed with DOPA in the dark.

DOPA is a particularly difficult precursor to work with as it oxidises in water over time and spontaneously with heat. Up until now sterilisation has been carried out by autoclaving. Since autoclaving involves heat the oxidising of DOPA, during this procedure, can be avoided by adding equimolar concentrations of ascorbic acid to the nutrient medium before adding DOPA (Rink E.& Bohm H. 1985). The other method is by filter sterilizing instead of autoclaving the entire medium. This involves autoclaving a filter-sterilizing apparatus containing an appropriate filter.

When using ascorbic acid to prevent oxidising of DOPA, it was found that the results were being inflated. This is possibly due to the antioxidising effect of ascorbic acid which may have prevented pigment breakdown. This was only revealed when carrying out ascorbic acid controls.(results not shown).

The experiment was repeated, this time filter-sterilising the DOPA. The findings of calli being fed with DOPA and grown in the dark(Figs. 3.4.1M-R) will be examined initially in isolation then shall be compared with the previous observations on shikimic acid and tyrosine fed to calli grown in the dark.

Figures 3.4.1M-R Plates were set up as described before. Depending on the concentration filter-sterilisation occurred as follows:

In the case of 0.1 mM a minimal solution of the required quantity of DOPA was filter sterilised then added to the cooling autoclaved liquid nutrient agar medium under aseptic conditions.

In the case of 1.0 mM an aliquot of the total water needed was used to dissolve and filter-sterilise the DOPA. The rest of the total water needed was used for dissolving the nutrients and agar. The filter-sterilised DOPA was subsequently added aseptically to the cooling autoclaved nutrient agar.

In the case of 8.2 mM DOPA this concentration was not obtained because (1) a certain amount of water was needed to dissolve the agar and (2) since the maximum amount of DOPA, that could be dissolved in water at 25°C, was being used, the amount of water required for dissolving the agar, if taken from that water needed to dissolve the DOPA, would have forced the concentration of

DOPA, which was been dissolved with rest of the nutrient cocktail, to go over its solubility limit thus preventing filter-sterilisation which necessitates total solubility of the substance being filter-sterilised.

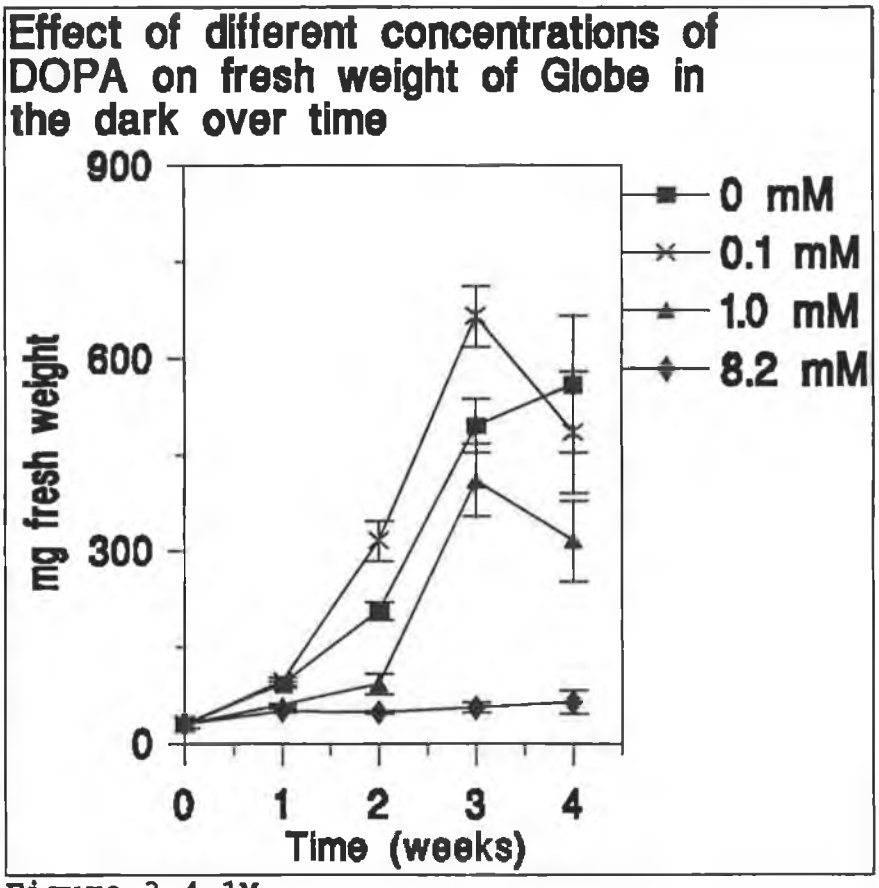


Figure 3.4.1M

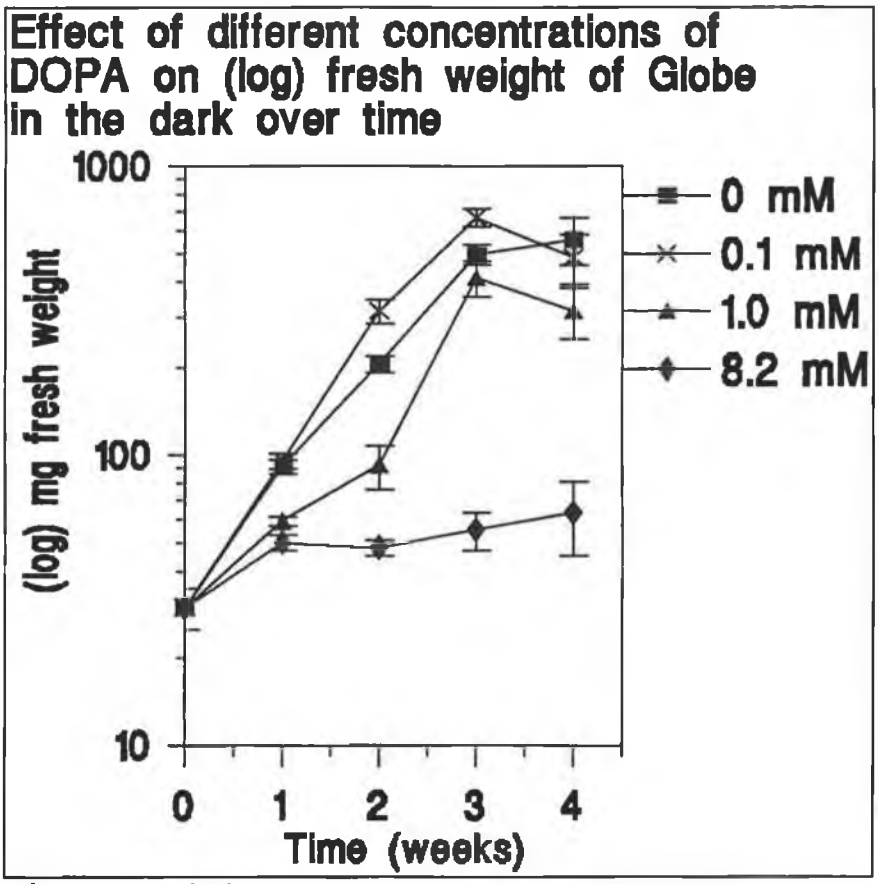


Figure 3.4.1N

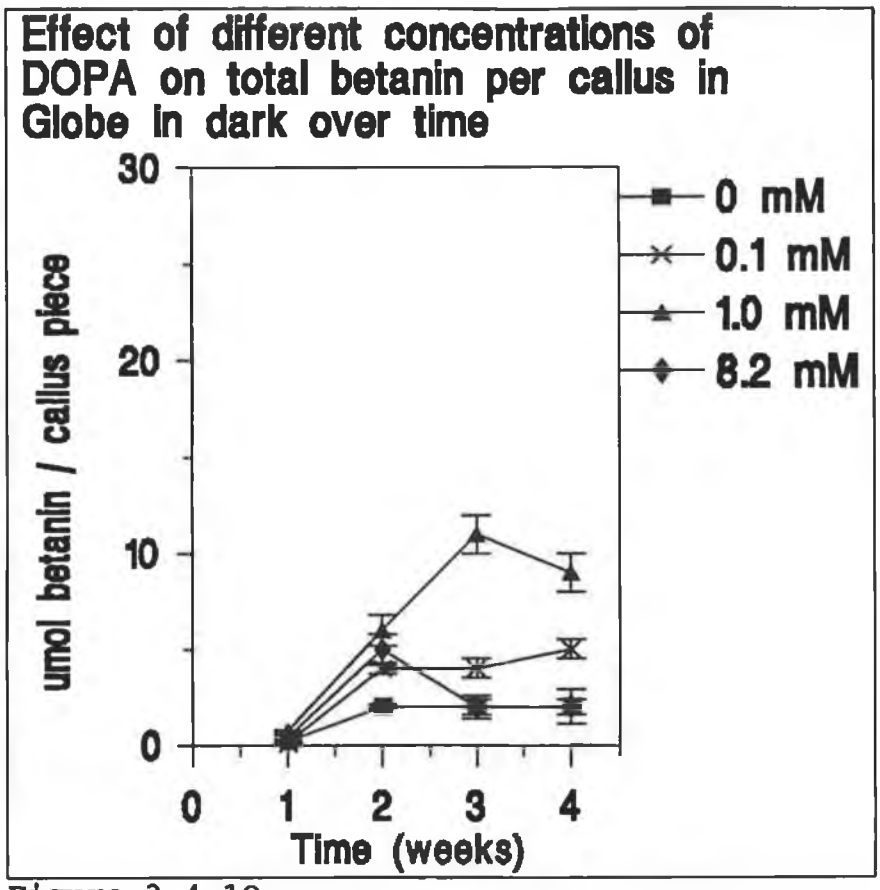


Figure 3.4.1O

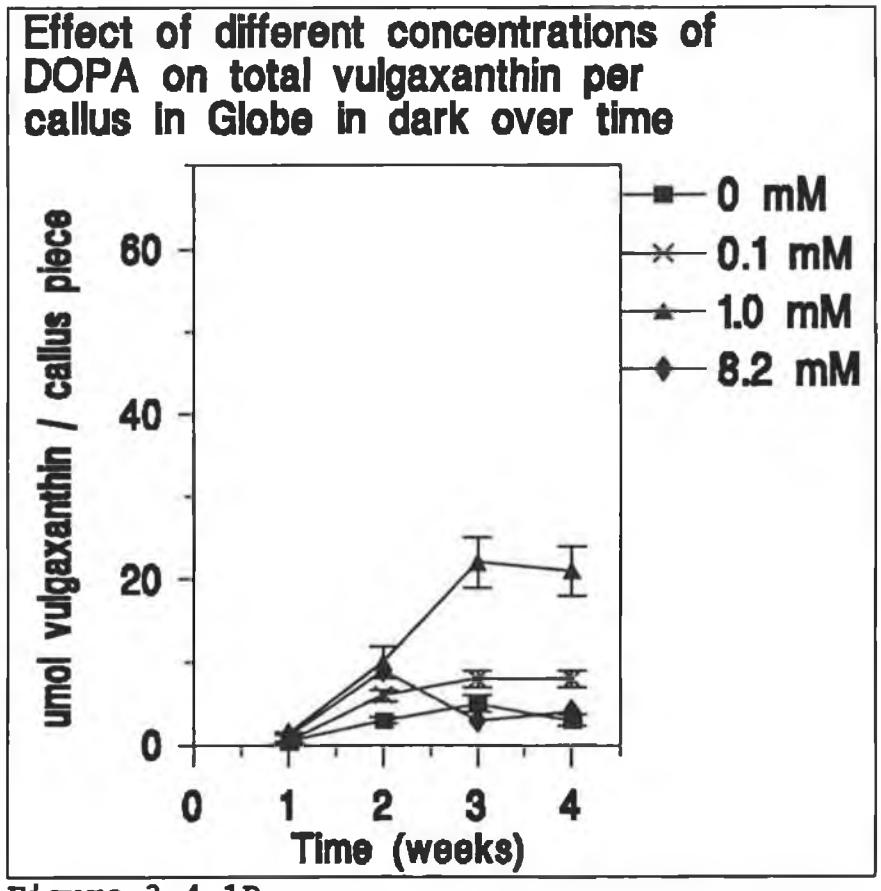


Figure 3.4.1P

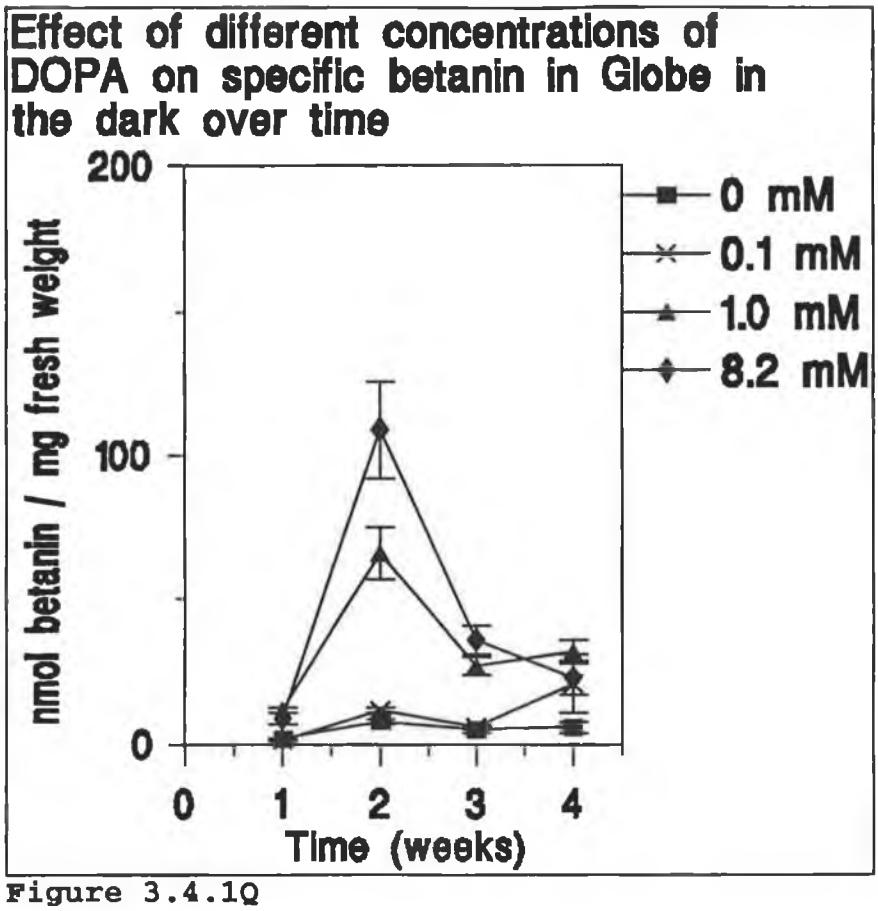


Figure 3.4.1Q

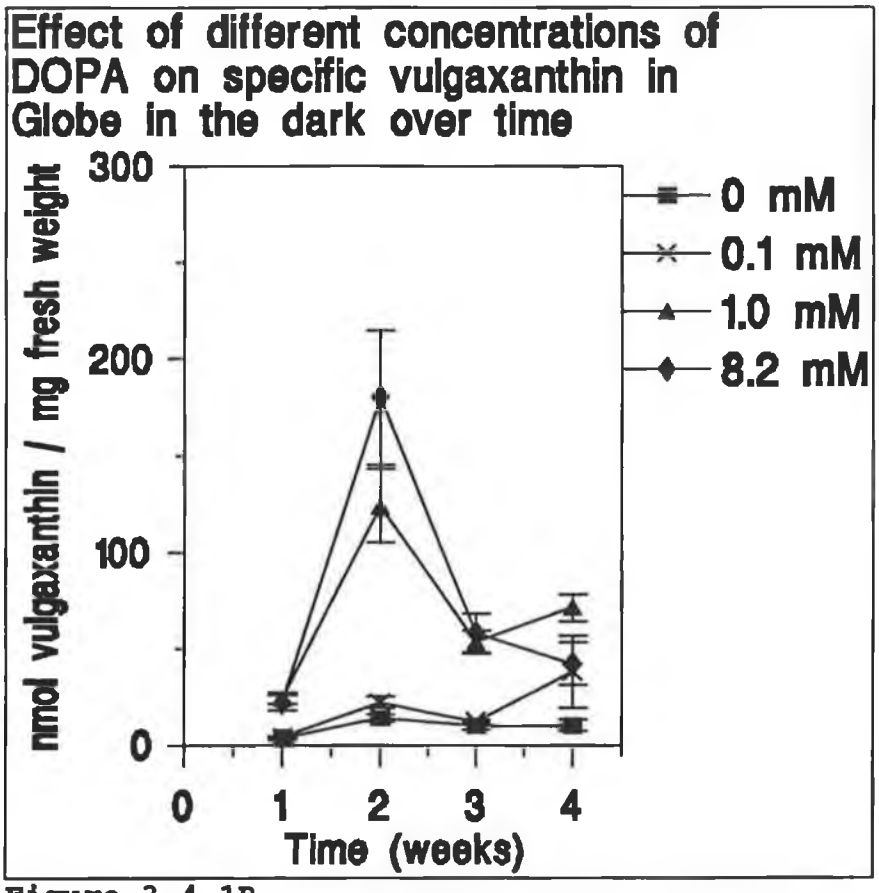


Figure 3.4.1R

A. Fig. 3.4.1M shows how, depending on the concentration, DOPA has a marked effect on fresh weight accumulation. This is particularly so with 8.2 mM DOPA where there was hardly any fresh weight accumulation over the 4 weeks. What is not evident from the graph is that calli pieces growing on the highest DOPA concentration went black after 1 week and necrosed. These growth trends are reflected in Fig. 3.4.1N which depicts the logarithmic phase of multiplication as a straight line. One sees that for the control and for DOPA concentrations up to 1.0mM there was exponential growth up to week 3 of culture after which growth ceases.

B. The fact that calli hardly grew, after the first week of culture, on medium containing 8.2 mM DOPA complicates the analysis of the corresponding pigment accumulation per callus whether it be total(figs. 3.4.1O-P) or specific(figs. 3.4.1Q-R). It appears that 1.0 mM DOPA results in the highest total betanin (fig. 3.4.1O) and vulgaxanthin (fig. 3.4.1P) accumulation per callus. The decrease in the fourth week may be due to the combined effects of decreased fresh weight accumulation and pigment accumulation. Alternatively it could be the result of the fact that pigment accumulation increases until growth stops at which stage pigment begins to breakdown due to initiation of cellular necrosis.

C. Figs. 3.4.1Q-R show that specific pigment values reflect those of total. The expected decrease can only be seen with the 1.0 mM after week 2 as opposed to week 1. This could be due to the lull in fresh weight accumulation during week 2(fig. 3.4.1N). The steady values for the control and the 0.1 mM DOPA could mean that pigment accumulation was at a par with fresh weight increase. It could also be postulated that the peak in week 2 is related to DOPA concentration. This peak in specific accumulation is probably related to a higher DOPA availability. However as the DOPA is being used by the callus pieces the DOPA concentration in the medium is going down. As a result there is less DOPA to fuel pigment production. In conjunction with this growth continues with the net result that in weeks 3 and 4 specific pigment decreases. In addition if one accepts that pigment production is related to growth and based on the result of the inoculum experiment which suggests that growth slows down significantly once 450mg fresh weight accumulation has been attained, it comes as no surprise that pigment should decrease towards the end of the experimental run. Despite being told to ignore the 8.2 mM curves because of necrotic tissue which is not apparent in the result, high values obtained for 8.2 mM indicate that necrotic tissue can give rise to 'false positives' due to interfering

substances.

When one compares the results of tyrosine and DOPA feeding in the dark, one gets a clearer picture of how the calli are reacting from the point of view of fresh weight and pigment accumulation.

A. Figs. 3.4.1G and 3.4.1M show that DOPA feeding has more of an effect on fresh weight accumulation than tyrosine, that fresh weight peaks at different times depending on precursor and concentration, that 0.1 mM concentration of either tyrosine or DOPA results in the highest fresh weight accumulation, that calli growth has a shorter exponential phase(figs. 3.4.1H & 3.4.1N) when fed with tyrosine.
B. If one compares treatments with total betanin per callus in mind (figs. 3.4.1I & 3.4.1O) one sees that accumulation peaks at different times, week 2 & 3, and with different concentrations, 2.5 mM tyrosine 1.0 mM DOPA, after which both decline. Peaks in accumulation are about the same for both precursors, 90 to 100 µmol of pigment per callus. The same story applies for total vulgaxanthin accumulation (figs. 3.4.1J & 3.4.1P). Accumulation curves for DOPA feeding are less erratic than for tyrosine especially for 0 and 0.1 mM concentrations.
C. Comparison of specific pigment values for betanin(figs. 3.4.1K & 3.4.1Q) appear to suggest that the highest concentration will occur in week 2, that neither precursor promotes more of an increase than the other and that specific pigment decreases in the dark. A similar trend is portrayed when comparing figs. 3.4.1L & 3.4.1R which show specific vulgaxanthin accumulation. Again as a remainder one must ignore values for 8.2 mM DOPA and in addition concentrate more so on vulgaxanthin values as the calli tended to go yellow when becoming pigmented.

A comparison can now be made of all the precursors with particular reference to the location of a rate-limiting step. Best fresh weight accumulation was observed with the feeding of shikimic acid(fig. 3.4.1A) however the higher concentrations of tyrosine(fig. 3.4.1G) and DOPA(fig. 3.4.1M) reduced if not inhibited fresh weight accumulation. The logarithmic phase of multiplication was shortest with shikimic acid(fig. 3.4.1B) and longest with DOPA(fig. 3.4.1N).

There appears to be a difference in total pigment accumulation in calli when grown on shikimic acid, tyrosine and DOPA. There was hardly any increase in total pigment accumulation when calli were fed with shikimic acid(figs. 3.4.1C-D) however at least a doubling with tyrosine(figs. 3.4.1I-J) and DOPA(figs. 3.4.1O-P) can be seen. On the

other hand maximum accumulation for both tyrosine and DOPA was achieved by different concentrations(2.5 mM tyrosine, 1.0 mM DOPA) at different times(2 weeks for tyrosine fig. 3.4.1J, 3 weeks for DOPA fig. 3.4.1P).

Specific pigment accumulation results tally well with those of total pigment. There is hardly any specific pigment accumulation when shikimic acid(figs. 3.4.1E-F) is fed to calli. The feeding of tyrosine(figs. 3.4.1K-L) results in a similar increase in specific pigment accumulation to DOPA(figs. 3.4.1Q-R), when one takes into account that the 8.2 mM result has to be considered as a 'false positive'.

Two main conclusions arise from these results. First that both tyrosine and DOPA caused pigment production of similar magnitude and shikimic hardly any. This implies, from a rate-limiting step point of view, that the potential block or control point is upstream from tyrosine. One however could also argue that an intermediate down stream along the biosynthetic pathway could also give a positive result but that it would be 10 times higher.

The other conclusion was that dead/necrotic tissue could give apparent high positive results. This is particularly seen with total pigment accumulation in calli fed with 8.2 mM DOPA. As said before one can avoid this by omitting the dead material during extraction but including it during fresh weight measurement.

3.4.2 Light Treatment of Precursor Feeding

The dark feeding of precursors indicates that the rate-limiting step is before tyrosine. If this is the case it should hold true for induction by environmental and/or chemical factors. Since a parallel set of experiments looked at the effect of light as an inducing factor, it was decided to repeat the feeding of precursors to calli but this time exposing them to light for the duration of the experiment. Figures 3.4.2A-Q show the results of these experiments.

It is proposed to look initially at the results concerning shikimic acid given to calli grown in the light, then tyrosine followed by that of DOPA. Subsequently compare the individual precursors with each other and finally all the results will be looked at, that is a comparison of dark and light results under the headings fresh weight, total and specific pigment accumulation.

Figures 3.4.2A-F Experimental plates were set up as described previously, namely 5 calli pieces per plate, 2 plates per treatment. The calli used for inoculating the experimental plates was at least 3 weeks in the dark. The light level used was 10 $\mu mol/m^2/s$ and the ambient temperature was between 20°C to 23°C. There was no 1 week dark incubation.

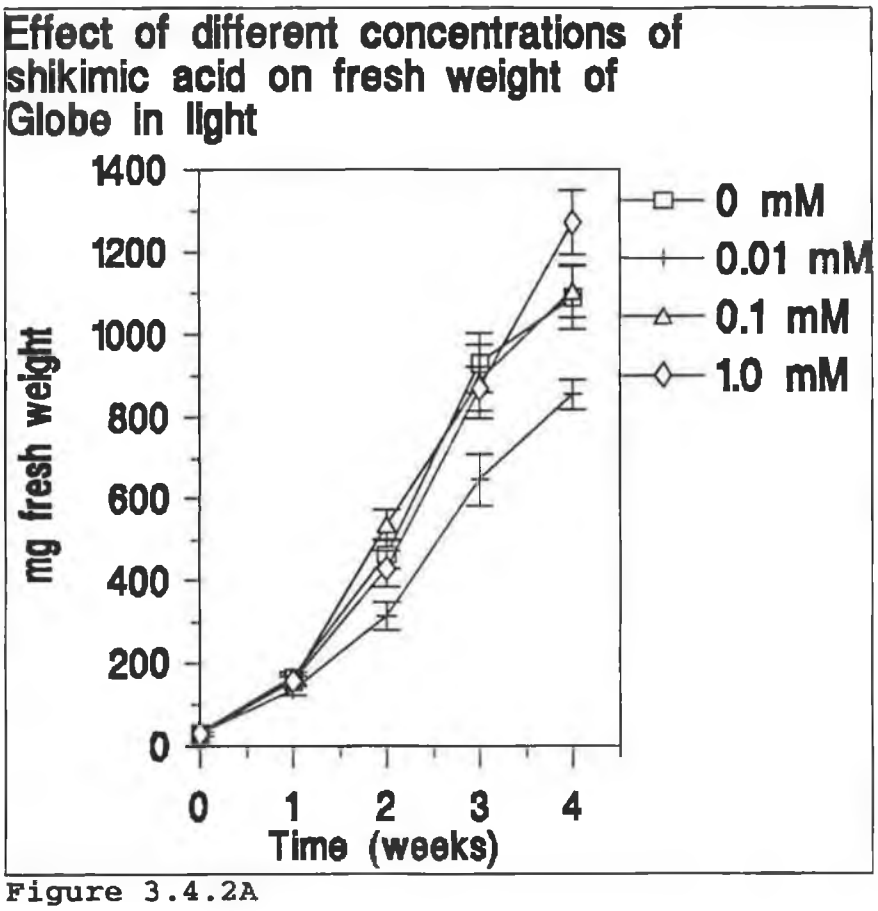


Figure 3.4.2A

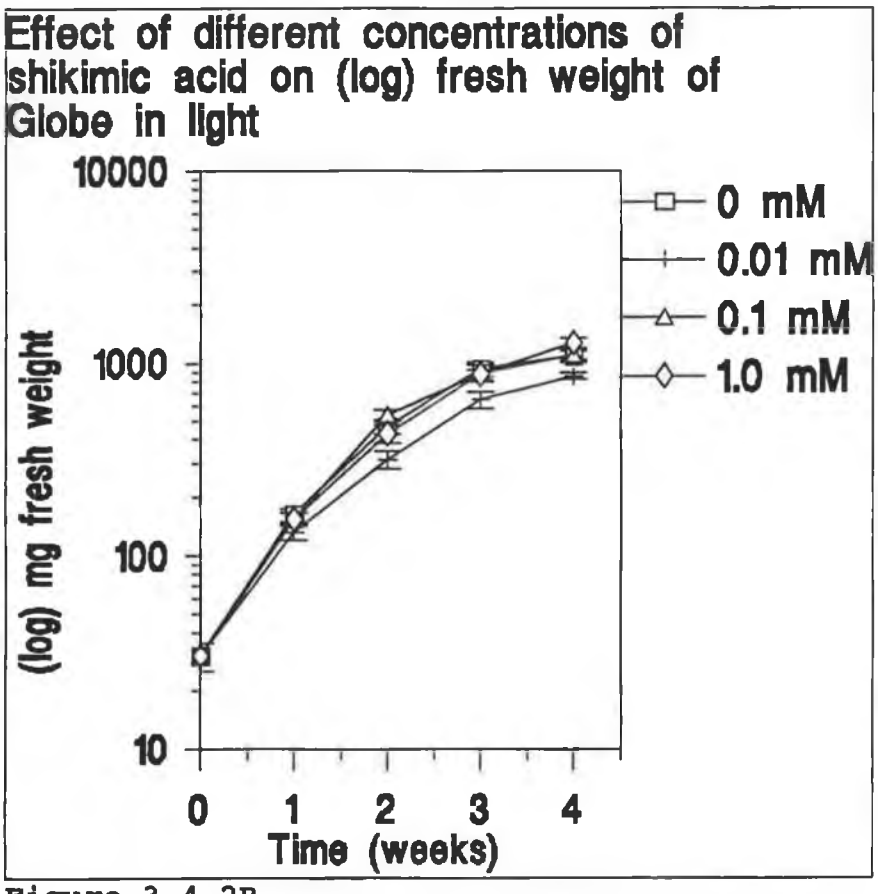
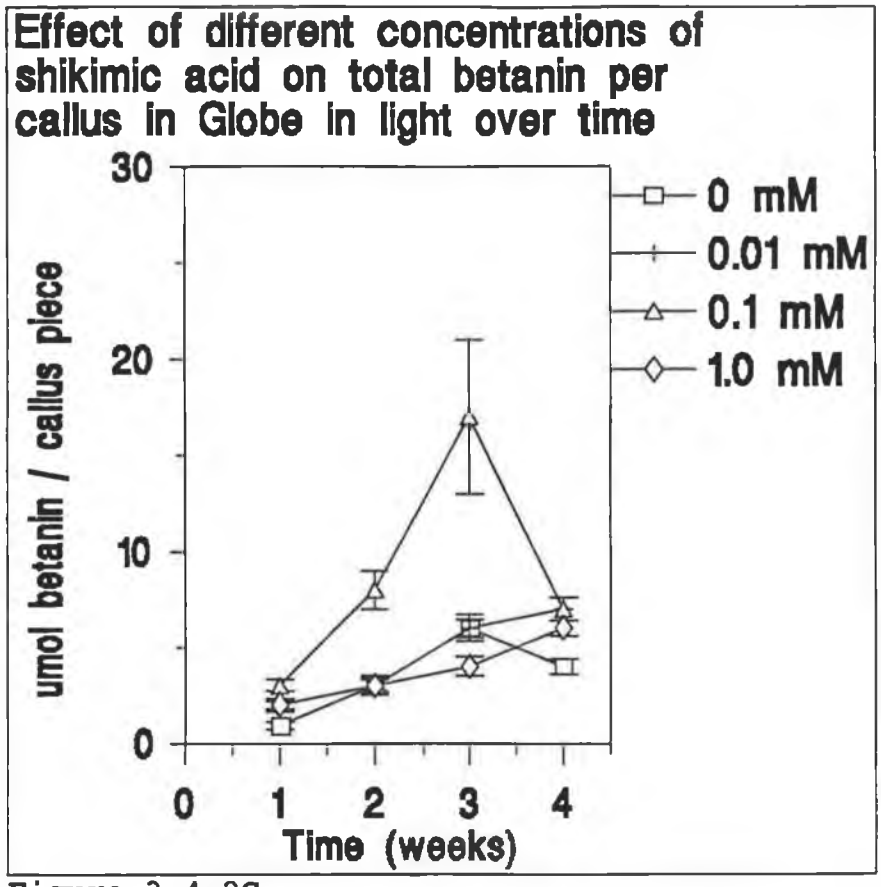


Figure 3.4.2B



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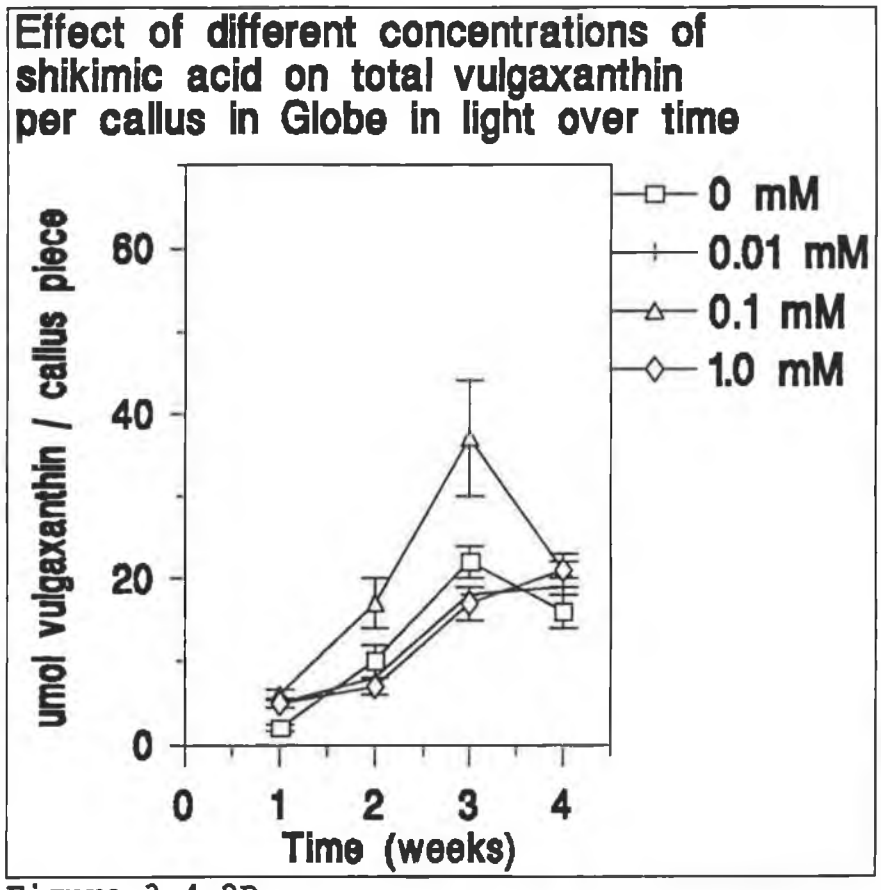

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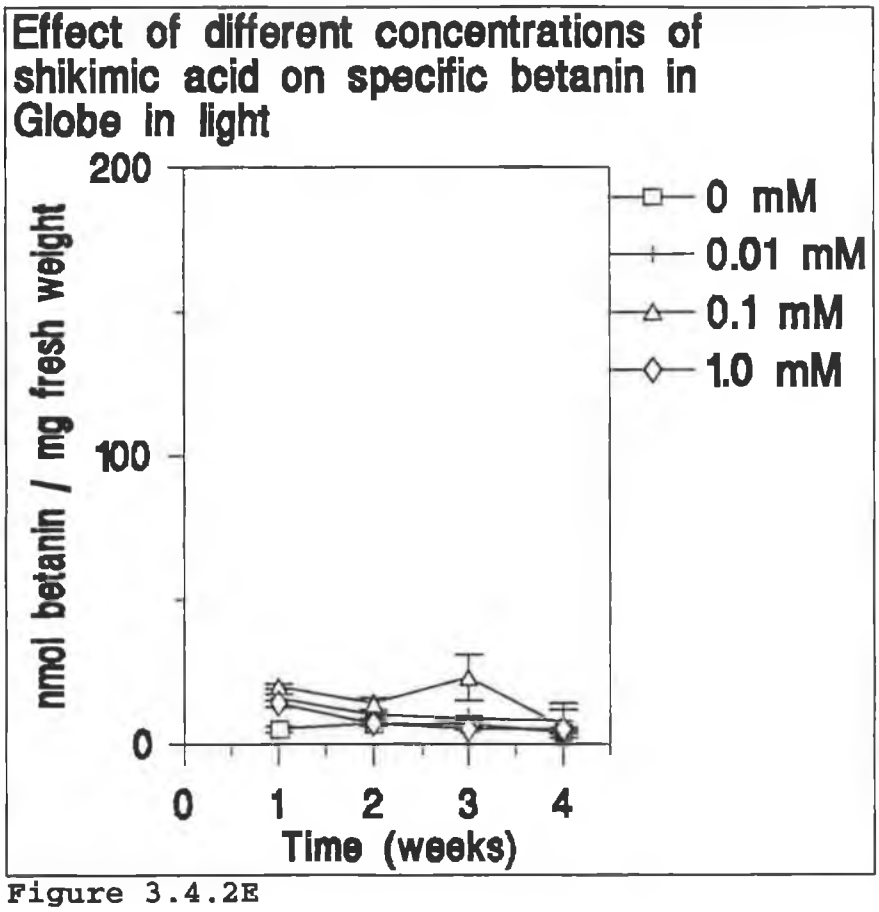


Figure 3.4.2E

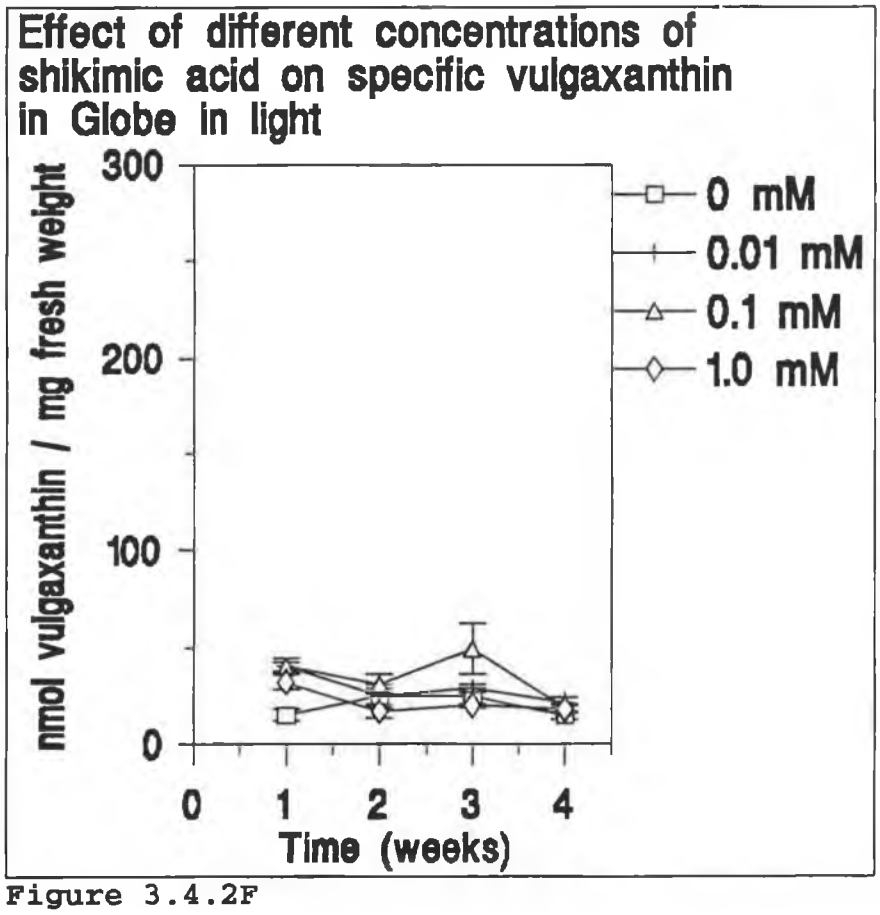


Figure 3.4.2F

A. Fig. 3.4.2A shows the increase in fresh weight over time of calli grown in the light on media containing different concentrations of shikimic acid. There is no identifiable trend with respect to concentration over time. However one does notice that 1.0 mM shikimic acid results in the highest fresh weight accumulation increase, 0.1 mM the lowest. Fig. 3.4.2B shows that there is no real logarithmic phase of multiplication over time.

B. Again as with fresh weight there is no apparent identifiable trend with total pigment accumulation per callus (figs. 3.4.2C-D). Peaking of accumulation occurs in week 3 with 0.1 mM shikimic acid. One notices that total vulgaxanthin per callus (fig. 3.4.2D) increases moreso over time than total betanin per callus (fig. 3.4.2C). This is as expected as calli tend to go yellow if they are going to become pigmented.

C. Figs. 3.4.2E-F show specific pigment values which tally somewhat with total pigment accumulation. This can be seen with a peak in week 3 for 0.1 mM which would imply that pigment production outstripped fresh weight accumulation. Despite similarities in values for week 1 and 4, one sees the expected decrease in specific values over time with a slight delay during week 3 for 0.01mM, 0.1mM and 1.0mM shikimic acid.

In conclusion despite total pigment increasing, specific pigment accumulation stays more or less the same in the light. The relevance of these results can only be judged after an examination of the feeding of tyrosine and DOPA.

Figures 3.4.2G-L Experimental plates were set up as in the previous experiments. For further details see legend for figures 3.4.1G-L. 10 $\mu mol/m^2/s$ was the light level used in this experiment.

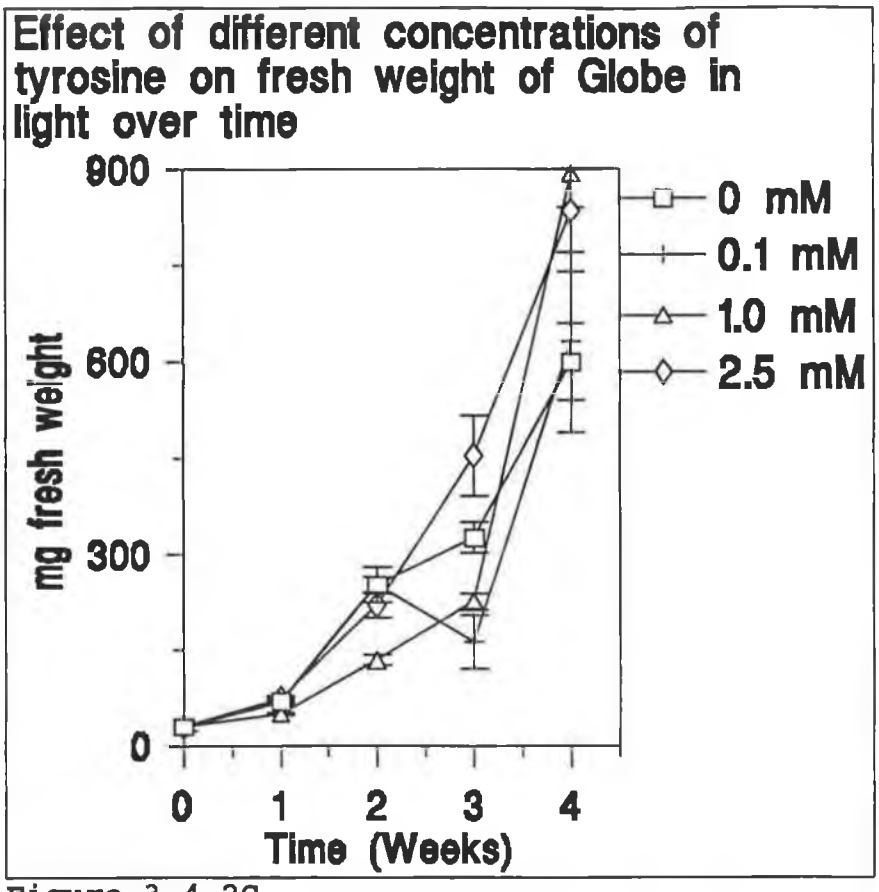
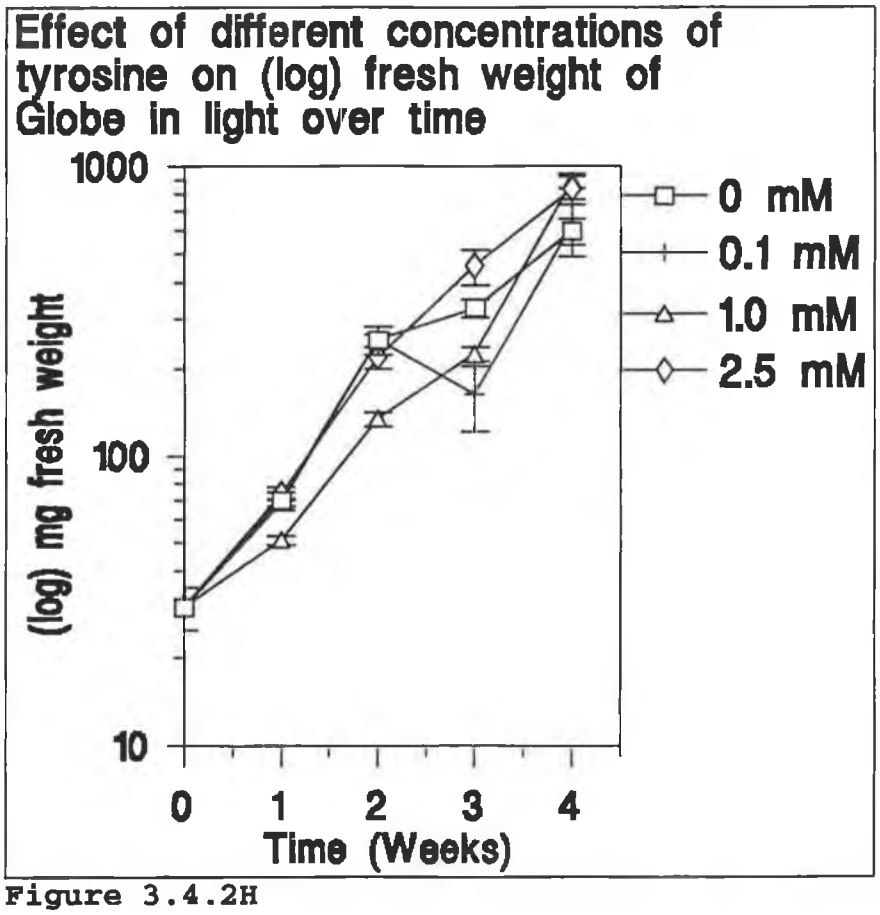


Figure 3.4.2G



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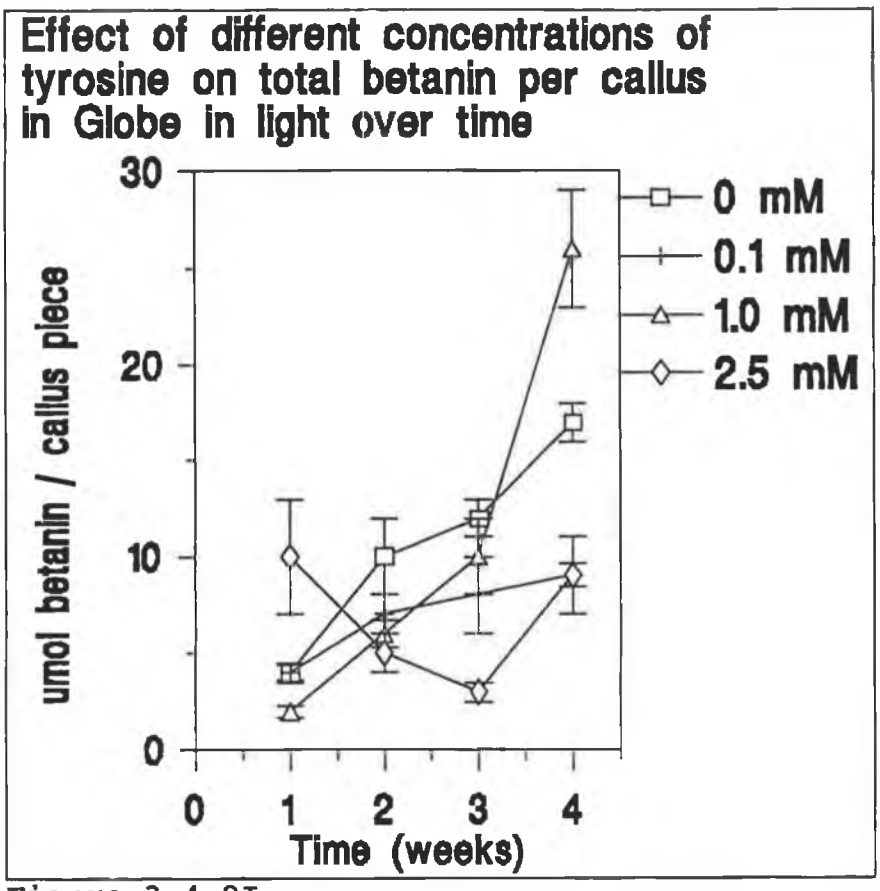

Figure 3.4.2I

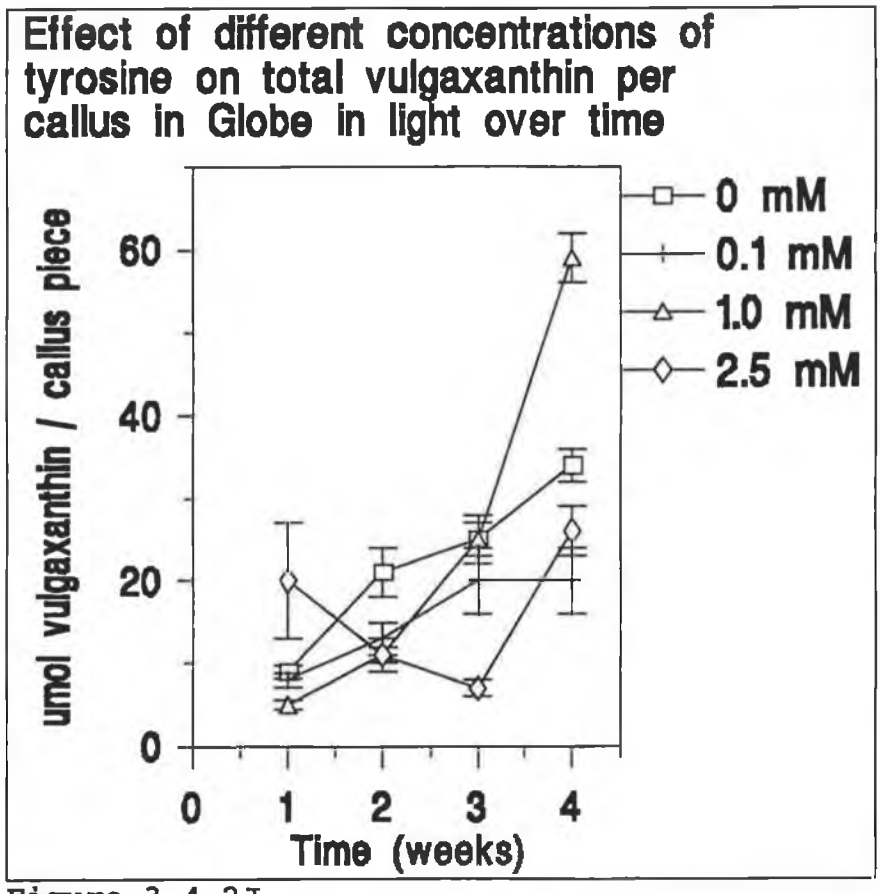

Figure 3.4.2J

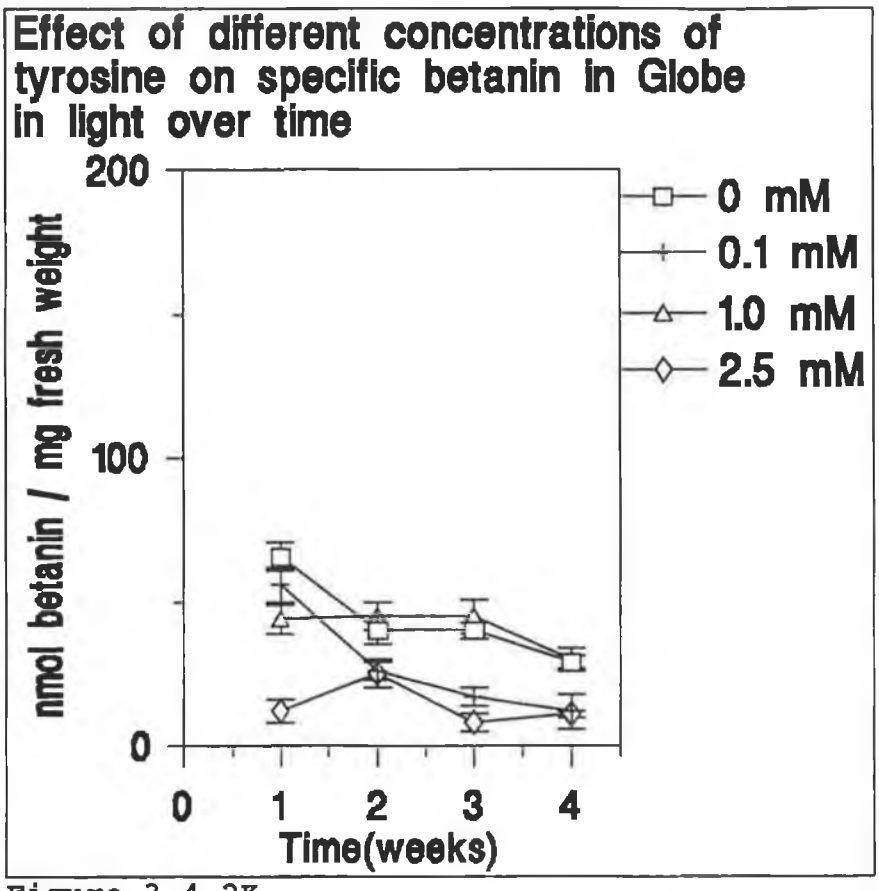

Figure 3.4.2K

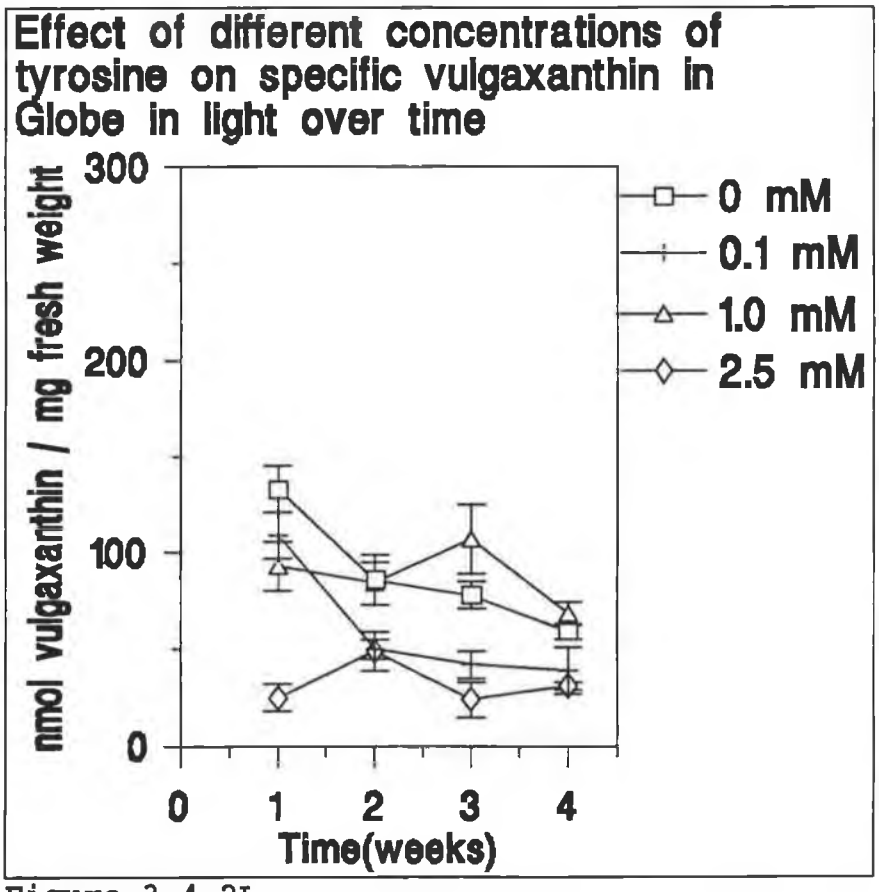

Figure 3.4.2L

The effect of tyrosine on calli grown in the light is as follows:

A. Fig. 3.4.2G shows the response of fresh weight of calli grown on medium containing tyrosine in light. One notices that tyrosine does not affect growth in any particular way except for 0.1 mM which is somewhat erratic. There is no apparent relationship between growth and tyrosine concentration. Exponential growth(fig. 3.4.2H) can only be seen in the first two weeks.

B. The total pigment per callus accumulation curves for betanin (fig. 3.4.2I) and vulgaxanthin (fig. 3.4.2J) look slightly chaotic. Again there is no consistent relationship between tyrosine concentration and pigment accumulation per callus over time. Only 0 mM and 1.0mM tyrosine, and to a lesser extent 0.1mM, give a steady rise in total pigment per callus accumulations for both pigments.

C. Figs. 3.4.2K-L shows specific pigment values which appear to decrease due to growth outstripping pigment accumulation. However depending on the concentration the decline is delayed. This tallies somewhat with total pigment per callus trends. The fact that 2.5 mM tyrosine is giving a result that is more or less stable might mean that pigment production is at a par with growth.

Despite error bars being present in certain graphs reference has not been made to them. However one does notice that there is considerable overlap or at least close proximity of error bars. This is a reflection of the poor reproducibility of the model system with respect to callus pieces or the fact that the system is not appropriate for the approach desired, in the sense that there was not a specific/clearcut response.

Before leaving the effect of tyrosine on calli grown in light to the side, the results of which will be more meaningful after those of DOPA have been examined, a comparison with shikimic acid can be made.

A. Figs. 3.4.2A & 3.4.2G shows how shikimic acid results in a higher fresh weight accumulation after 4 weeks. However the logarithmic phase of multiplication is longer when calli are fed with tyrosine(fig. 3.4.2H) than with shikimic acid(fig. 3.4.2B). Both precursors show no relationship between concentration and response of fresh weight accumulation. The irregular growth curve of 0.1 mM tyrosine(fig. 3.4.2G) may be due to calli heterogeneity.

B. Both shikimic acid and tyrosine promote similar total pigment per callus accumulations whether it be total betanin (figs. 3.4.2C, 3.4.2I) or total vulgaxanthin (figs. 3.4.2D, 3.4.2J). However maximum accumulation occurs with different concentrations (0.1 mM shikimic acid, 1.0 mM tyrosine) during different weeks

(week 3 for shikimic acid, week 4 for tyrosine). In both cases there is no regular relationship between response and precursor concentration.

C. Differences can be detected, however, between the different precursors and specific pigment accumulation. Tyrosine results in a generally higher specific accumulation at 70 nmol betanin(fig. 3.4.2K), 140 nmol vulgaxanthin(fig. 3.4.2L) compared to 25 nmol betanin(fig. 3.4.3E) and 50 nmol vulgaxanthin(fig. 3.4.2F) when shikimic acid is fed to calli. One point of similarity is the rise in specific vulgaxanthin accumulation in week 3 when fed with either 0.1 mM shikimic acid or 1.0 mM tyrosine.

Figures 3.4.2M-R show the results of DOPA fed to calli grown in the light for the period of the experiment.

Figures 3.4.2M-R Details of experimental set up can be found in legend for figures 3.4.1M-R. 10 $\mu mol/m^2/s$ was the light level used in this experiment.

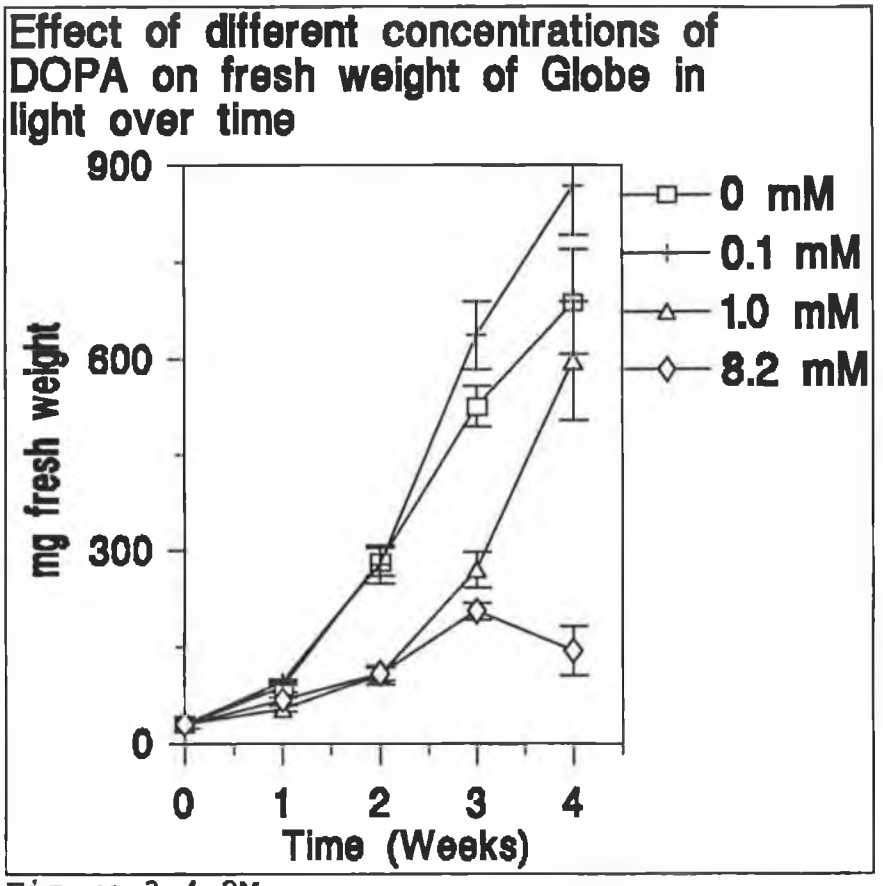

Figure 3.4.2M

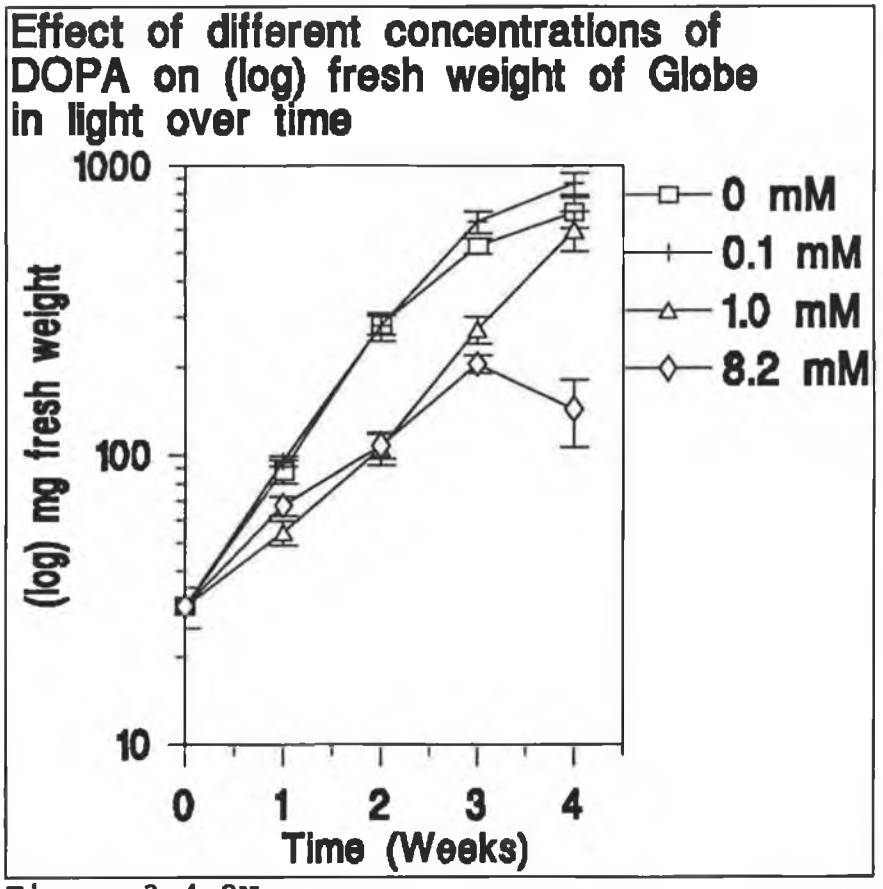

Figure 3.4.2N

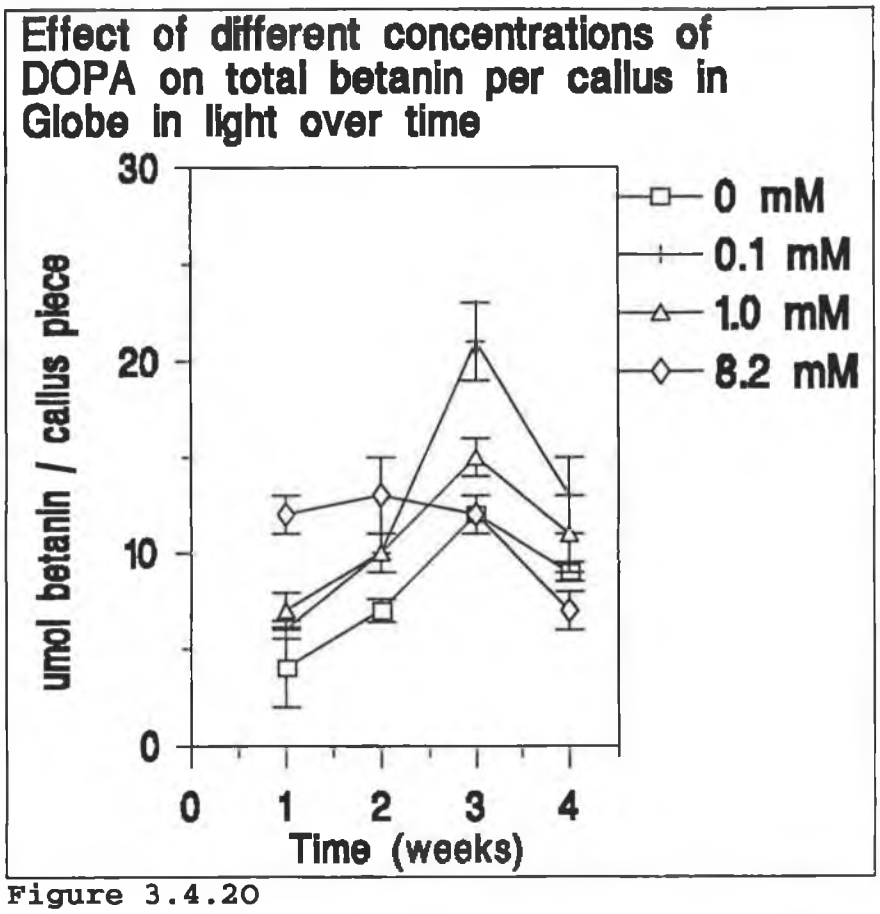

Figure 3.4.2O

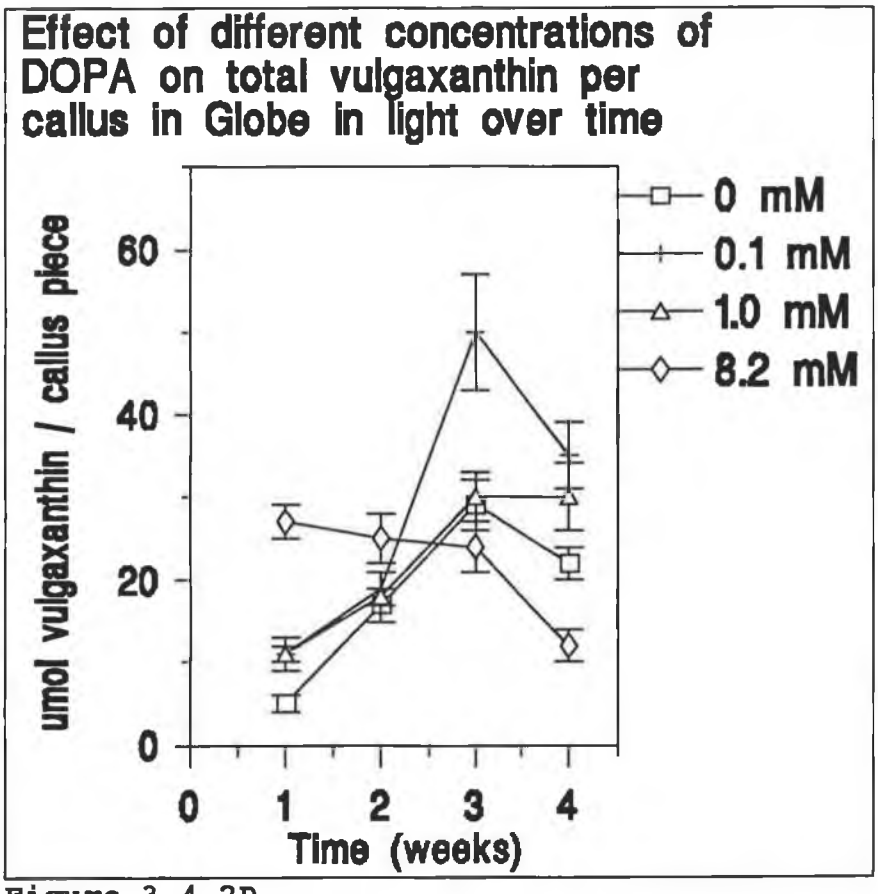

Figure 3.4.2P

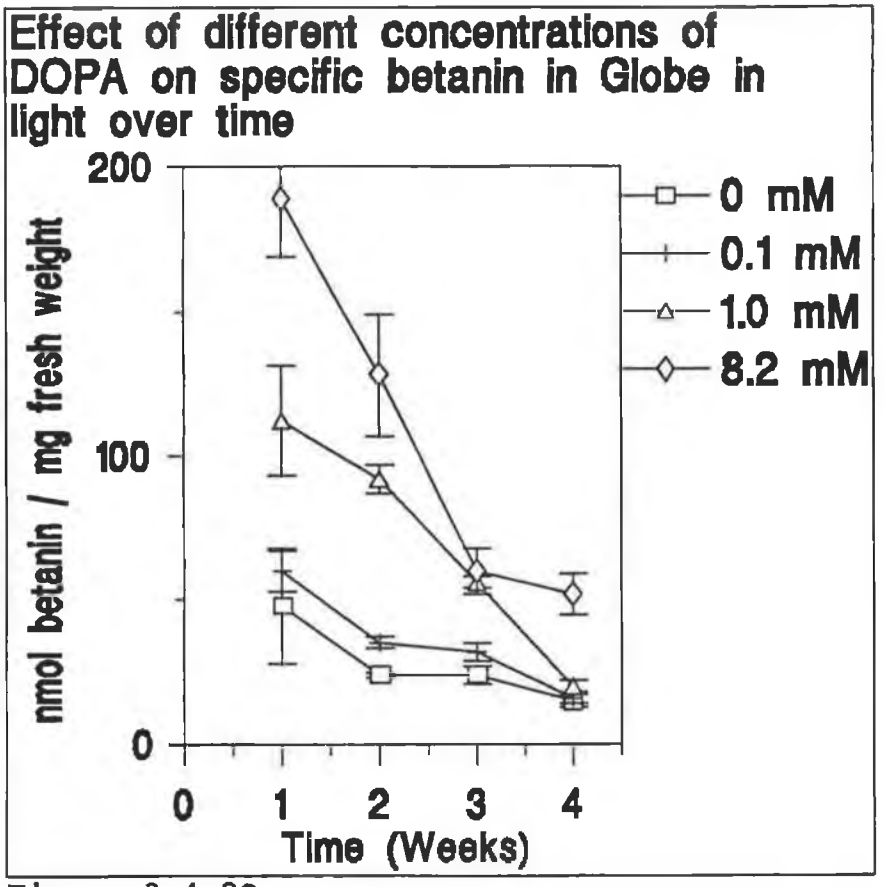

Figure 3.4.2Q

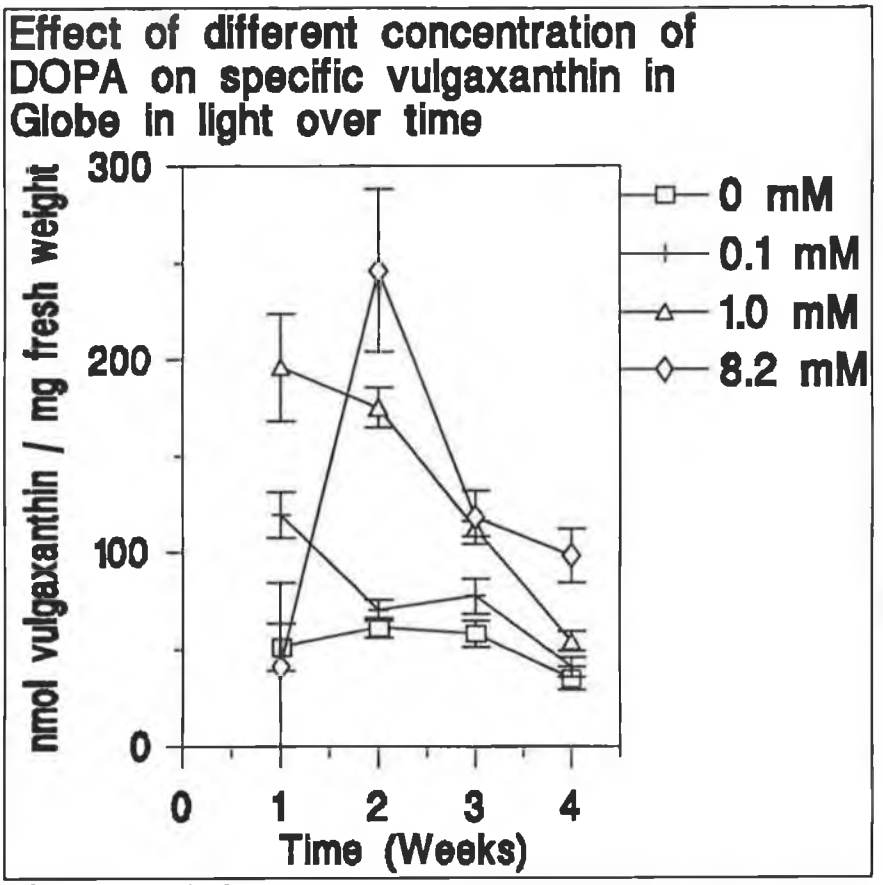

Figure 3.4.2R

A. Fresh weight(fig. 3.4.2M) accumulation seems to be affected by DOPA concentration, 0.1 mM causing the highest accumulation. The logarithmic phase of multiplication (fig. 3.4.2N) can be seen in some cases lasting up until week three; 1.0 mM DOPA.
B. In the case of total pigment per callus accumulation, whether it be betanin (fig. 3.4.2O) or vulgaxanthin(fig. 3.4.2P), it appears to peak during week 3 when calli are fed with 0.1 mM DOPA. 8.2 mM is the lowest as expected from fresh weight (fig. 3.4.2M).
C. The curves for specific pigment(figs. 3.4.2Q-R) are more clear cut. Again one notices that growth outstrips pigment production. As with the dark results, one must ignore 8.2 mM DOPA values as they are 'false positives' given that the calli were dead except for week 1. It appeared, from visual observation, that calli grown on 8.2 mM DOPA faired better up until week 1 in the light than in the dark. One also remarks, that relative to the control, there appears to be pigment production in the first week. Despite the specific values being influenced by fresh weight accumulation one can infer from the 1 week fresh weight accumulation and total pigment values that the higher the DOPA concentration the higher the total pigment accumulation.

Having looked at the DOPA results in isolation one can now compare them with those of tyrosine in the light. This comparison reveals the following:

A. Comparing fresh weight accumulation for Globe treated with tyrosine in the light(fig. 3.4.2G) and DOPA(fig. 3.4.2M) one notices that a similar magnitude of fresh weight accumulation was attained but only when fed with different concentrations i.e. 1.0 mM tyrosine, 0.1 mM DOPA. The logarithmic phase of multiplication, as evidenced by a straight line on a log-linear plot, is shorter when Globe in grown on medium with tyrosine(fig. 3.4.2H) than with DOPA(fig. 3.4.2N). There appears to be more variability in the response to DOPA concentrations than to tyrosine.
B. The main difference when comparing total pigment per callus accumulation, whether it be betanin or vulgaxanthin in Globe grown on tyrosine or DOPA (figs. 3.4.2I, O, J, P) in the light, is that peaking of pigment accumulation occurs at different times, week 3 or 4, depending on the concentration of the precursor. It appears that DOPA results in a higher pigment accumulation; 0.1 mM DOPA giving 50µmol vulgaxanthin (fig. 3.4.2P) as oppose to 1.0 mM tyrosine giving 60µmol vulgaxanthin (fig. 3.4.2J).

C. Graphs showing specific betanin and vulgaxanthin accumulation, when calli are grown on media containing tyrosine and DOPA(figs. 3.4.2K, Q, L, R), indicate that during the first week DOPA is more effective in promoting pigment production. In this particular case one can consider the 1 week values for 8.2 mM DOPA as being valid since the calli were not significantly necrosed. For the purposes of consistency and for determining the location of the possible rate-limiting step the values for 8.2 mM DOPA will be ignored. In spite of this DOPA still does appear to promote higher pigment accumulation than tyrosine.

Having looked at the effect of DOPA on calli grown in the light one can now see what indication light treatment of calli fed with the three different precursors gives as to the rate-limiting step. In passing, one notices that best fresh weight accumulation occurs with shikimic acid(fig. 3.4.2A) and that tyrosine(fig. 3.4.2G) and DOPA(fig. 3.4.2M) gives similar fresh weight results.

There appears to be no definite indication of a possible location of a rate-limiting step. The higher total betanin (fig. 3.4.2I) and vulgaxanthin (fig. 3.4.2J) accumulations per callus with tyrosine feeding, when compared to shikimic acid (figs. 3.4.2C-D) and DOPA (figs. 3.4.2O-P), could indicate a block at this point. When one takes into account the error bars of week 4 1.0mM tyrosine and week 3 0.1mM DOPA these values have to be considered as similar.

In addition the fact that specific values both for betanin (figs. 3.4.2E, K & Q) and for vulgaxanthin (figs. 3.4.2F, L & R) increase with each consecutive precursor may suggest that there is no particular point at which the enzyme pathway is blocked or that each enzyme has different rates of activity which increase dramatically given a signal.

One final comparison needs to be made; results of dark and light and how light affects the response of calli being grown on media containing one of the given precursors; shikimic acid, tyrosine or DOPA. It is proposed to look at each in turn starting with shikimic acid.

A. Figs. 3.4.1A & 3.4.2A, showing fresh weight accumulation for calli grown in the dark and light when fed with shikimic acid, indicate that light appears to have a different effect on fresh weight accumulation depending on the concentration of shikimic acid. The only similarity is that the same range of final fresh weight accumulations is attained and that 0.01 mM is the lowest in both cases. Figs. 3.4.1B & 3.4.2B reveals the logarithmic phase of multiplication for fresh weight over time.

If one looks closely only 1.0 mM shikimic acid in the dark has exponential growth for the first two weeks of culture.

B. Figs. 3.4.1C, 3.4.2C, 3.4.1D & 3.4.2D show total pigment per callus accumulation in calli fed with shikimic acid for both light and dark. The initial observation is that light promotes total pigment accumulation. This can be particularly seen for 0.1mM shikimic acid whether it be betanin (figs. 3.4.1C & 3.4.2C) or vulgaxanthin (figs. 3.4.1D & 3.4.2D). There is no identifiable trend for the effect of concentration over the 4 weeks whether it be dark (figs. 3.4.1C-D) or light (figs. 3.4.2C-D).

C. Contrary to the total pigment results there is no sizeable increase in specific pigment accumulation when calli are grown in light relative to being cultured in the dark. If one compares figs. 3.4.1E & 3.4.2E and figs. 3.4.1F & 3.4.2F one sees that there is barely a doubling and by different concentrations. Unexpected peaking occurs in both cases during week 3; 0 mM shikimic acid in the dark(figs. 3.4.1E-F), 0.1 mM shikimic acid in the light(figs. 3.4.2E-F). The latter tallies well with the total pigment results.

Comparing the results of dark and light treatment when calli are fed with tyrosine or DOPA reveals the following;

A. Comparing figs. 3.4.1G & 3.4.2G it appears that light promotes fresh weight accumulation somewhat when tyrosine is fed. However different concentrations predominate. Light seems to reduce the logarithmic phase of multiplication (figs. 3.4.1H & 3.4.2H). A similar situation is found when calli are grown on DOPA (figs. 3.4.1M & 3.4.1M). Light does not appear to affect the trend of response of concentrations up until week 3. In the case where DOPA is given to calli the length of the exponential growth phase is maintained both in dark and light (figs. 3.4.1N & 3.4.2N).

B. Figures 3.4.1I, 3.4.2I, 3.4.1J, 3.4.2J, show how dark and light promotes peaks in total accumulation per callus at different times and by different concentrations, 2.5mM dark (fig. 3.4.1I) 1.0 mM light(fig. 3.4.2I), when tyrosine is given to calli. There are no identifiable trends. However the general effect is of elevating the total pigment accumulation from 10 µmol betanin (fig. 3.4.1I), 20 µmol vulgaxanthin (fig. 3.4.1J) to 25 µmol betanin (fig. 3.4.2I), 60 µmol vulgaxanthin (fig. 3.4.2J). Likewise with total pigment per callus accumulation in calli given DOPA (figs. 3.4.1O, 3.4.2O, 3.4.1P, 3.4.2P). Light has the effect of elevating the level of pigment. However calli react differently under light and dark depending on the concentration of DOPA.

C. Specific pigment accumulation when calli are grown on tyrosine, whether in the dark or light, do not vary much in actual quantity accumulated(Figs. 3.4.1K-L, 3.4.2K-L). However dark seems to promote accumulation when calli are given 2.5 mM tyrosine (figs. 3.4.1K-L) and light(figs. 3.4.2K-L) appears to delay the decline in specific pigment i.e. promote pigment accumulation. As one looks at specific pigment accumulation, when DOPA is being fed to calli, one must ignore values for 8.2 mM DOPA in the dark(figs. 3.4.1Q-R) and for all weeks after week 1 in the light(figs. 3.4.2Q-R). The immediate noticeable effects are that (a.) peaking of specific pigment accumulation in the dark occurs in week 2(figs. 3.4.1Q-R), (b.) that increasing DOPA concentrations result in increasing response(figs. 3.4.2Q-R) in week 1, (c.) that light only increases the highest specific pigment accumulation slightly.

The overall conclusion from these experiments is that pigment accumulation does occur when tyrosine and DOPA are given to calli and that light has only a slight effect on increasing accumulation.

If one takes all graphs into account, namely figures 3.4.1A-R & 3.4.2A-R the following can be found;

A. that one of the more noticeable differences is that shikimic acid promotes fresh weight accumulation. The lowest is around 800 mg in week 4 in the case of shikimic acid(figs. 3.4.1A & 3.4.2A) and the highest in the case of tyrosine(figs. 3.4.1G & 3.4.2G) and DOPA(figs. 3.4.1M & 3.4.2M) is 800 to 900 mg in week 4. Again there are no identifiable trends, different concentrations peaking at different times. Light does not tend to have any particular effect on the logarithmic phase of multiplication(figs. 3.4.2B, 3.4.2H, 3.4.2N) except that it reduces the variation of response between the different concentrations that occur in the dark(figs. 3.4.1B, 3.4.1H, 3.4.1N).

B. When one looks at the effect of different precursors on total pigment per callus accumulation, one notices that in the dark (figs. 3.4.1C, I, O and 3.4.1D, J, P), shikimic acid results in the lowest total pigment per callus accumulation. The highest value is around 3 µmol for betanin (fig. 3.4.1C) and 10 µmol for vulgaxanthin (fig. 3.4.1D). In the case of tyrosine (figs. 3.4.1I-J) and DOPA (figs. 3.4.1O-P) the highest values are about the same but were achieved by different concentrations; 2.5 mM tyrosine and 1.0 mM DOPA. Light results in similar magnitudes (figs. 3.4.2C-D) of pigment accumulation to tyrosine (figs. 3.4.2I-J), to occur but at different times and different concentrations. The idea that the rate-limiting step could be after

shikimic acid is indicated by the higher values of total pigment per callus accumulation when calli are fed with DOPA; 20 µmols of a difference (figs. 3.4.2O-P).

C. The last point is borne out again by the specific pigment accumulation. Initial values for shikimic acid(figs. 3.4.1E-F) are 3 times lower than that of tyrosine(figs. 3.4.1K-L) and DOPA(figs. 3.4.1Q-R) in the dark. The only point of similarity is the unexpected peaking or delayed decline in specific pigment values. This occurs with different concentrations; this is barely seen with 0 mM shikimic acid(figs. 3.4.1E-F) as opposed to the highest values used with tyrosine(figs. 3.4.1K-L) and DOPA(figs. 3.4.1Q-R). The same observation applies when light is used. Again initial specific values of shikimic(figs. 3.4.2E-F) are lower than for tyrosine(figs. 3.4.2K-L) and DOPA(figs. 3.4.2Q-R).

As a general summary the graphs are saying that calli became more pigmented when given tyrosine and DOPA in the dark and that the rate-limiting step could be before tyrosine. However visual observations do not confirm this result especially in the case of the dark treatments. At this stage one could argue from another view point by saying that the rate-limiting step could be down stream at the level of betalamic acid and/or cyclodopa glycoside. It could be said that the all or nothing effect in not a valid approach to adopt given the nature of biological systems. It could be argued that as one moves down the enzyme pathway, that pigment accumulation could be increasing steadily at each precursor stage and that towards the end there is a dramatic increase, up to 100 times relative to increase occurring in prior steps. One would have had to grow calli on media containing either betalamic acid or cyclodopa glycoside. If these studies showed a similar level of pigment accumulation to DOPA it would only be at this stage that enzyme activity studies could be pursued.

Irrespective of the possible speculations that could be made or further experiments envisaged, the required level of pigmentation was not being obtained in any of the experiments. This is based (a.) the visual observation that not all calli became pigmented and when calli did it was only in the light and on the outside surface of the calli, (b.) error bars are large especially for the specific pigment values (c.) the fact that there were no continuous identifiable trends between concentrations means that the conclusions could be based on haphazard results and not attributable to the effect of individual precursors. These remarks also apply to the light induction experiments.

CHAPTER 4. DISCUSSION

It is proposed to discuss the results, in relation to previous literature, in four sections: 1. Initiation of model system, in particular callus cultures, 2. Extraction, separation and purification of pigments, 3. Development of the model system so that it could be induced to produce pigments, with particular reference to light as an inducer/enchancer and 4. an attempt to pinpoint the rate-limiting step by the feeding of precursors and intermediates of the biosynthetic pathway.

4.1 Initiation of Model System

Since a model system had to be initiated, three different salt cocktails were examined; Pgo, M & S and Gambourg B5. In addition 30g/dm^3 sucrose was used. The initial growth regulator combination and concentration used was 1.0mg/dm^3 2,4-D and 0.1mg /dm^3 kinetin. A subsequent literature survey found that a white cell line of red beet could be readily achieved by supplementing the medium agar with 0.1mg/dm^3 2,4-D and 0.5mg/dm^3 kinetin (Ripa P.V. & Adler J.H. 1987). At the beginning of the project all cell lines for each of the different salt cocktails were kept in the light. It was eventually realised that white cell lines could be achieved more efficiently in the dark.

When going through the literature one notices that not alot of work has been done on red beet callus or suspension cultures. It is only after a thorough search that one finds some reference to red *Beta vulgaris* cultures. As mentioned in the results section on model system initiation it was found that Pgo was the best salt cocktail for callus induction and maintenance of white friable calli in the dark. M & S was initially considered to give best growth based on previous literature on sugarbeet *Beta vulgaris*. However when using Freytags medium based on the M & S salt cocktail, calli remained green and lumpy and growth sluggish. No previous case of the use of M & S as media for red beet callus cultures could be traced except for the use of half strength M & S microelements by Girod P.A. & Zryd J.P. 1991. However they concentrated on the use of different sucrose, mannitol, 2,4-D and 6-BAP concentrations.

As said before most of the literature concerns itself with sugar *Beta vulgaris* which tends to be cultured on M & S (Doley W.P. & Saunders J.W. 1989, Joersbo M. *et al.* 1989). In these cases no amino acids were used and the vitamin complement was reduced from 10 to 3 vitamins and in other cases none at all. Doley W.P. & Saunders J.W. 1989 found that moist, white friable callus could be obtained on both growth regulator free and 1mg/dm^3 N^6-benzyladenine-containing medium. Kubaláková M. 1990 finds that transferring callus from L & S to M & S resulted in light yellow

spontaneously friable embryogenic calli.

It was found that B5 medium resulted in an improved friability of calli. However calli still persisted to be green and growth was still extremely sluggish for all of the varieties except Forino. In addition apparent morphogenesis, in the form of roots, was occurring in some cell lines, in particular Burpees and Detroit. This is also noted by Girod P. A. & Zryd J.-P. 1991 in old orange and violet lines. They conclude that the orange and violet phenotypes are part of a morphogenetic program. The only reference to the use of Gambourg B5 for the maintenance of white cells of red beet can be seen in Zryd J.-P. *et al.* 1982. However they do not give any details of callus characteristics other than that the cell line was habituated. B5 on the other hand has been used extensively for red cell lines. Constabel F. & Nassif-Makki H. 1971 grew 5 varieties of red beet on solid or liquid Gambourg *et al.* 1968 with 1ppm ($1mg/dm^3$)2,4-D. However they did not give any details of growth characteristics. The next reference is by Zryd J.-P. *et al.* 1982. They also use B5 but no growth regulators were added as the cultures were habituated. Again no growth characteristics were given. B5 salt cocktail can be seen in research by Bokern M. *et al.* 1991 and Hunter C.S. & Kilby N.J. 1990. Bokern M. *et al.* 1991 found that their pigmented cultures could be best initiated on Gambourg B5 supplemented with 2,4-D and kinetin $10^{-6}M$ then selected so that they could be maintained on growth regulator free agar medium. One of these cell lines could be subsequently transferred to M&S medium for selection on liquid medium. The authors used *Beta vulgaris* L. subsp. *vulgaris* var. *conditiva.* Hunter C.S. & Kilby N.J. 1990 used Gambourg's B5 medium (Flow Labs) 3.87g, 20g sucrose (not 30g as in this thesis), 0.1mg kinetin(6- furfurylaminopurine) , 1.0mg 2,4-D and $6g/dm^3$ agar(not 12 as in this thesis) for growing *Beta vulgaris* cv Boltardy.

Of the three salt cocktails Pgo was found to be the best for growth, whiteness and friability. This however only applied to Forino and Globe varieties. These characteristics remained valid for 3 to 4 weeks after subculture. This is contrast to Ripa P.V. & Adler J.H. 1987 using Pgo medium by DeGreef W. & Jacobs M. 1979 for growing Red Ball (catalog 6193-7, W. Atlee Burpee Co). This medium was in actual fact initially designed for sugarbeet. They used $1.0mg/dm^3$ indole acetic acid and $0.05mg/dm^3$ kinetin for callus induction from sterilised greenhouse plants. Cultures were kept in the dark. They found that white friable callus of red table beet could be obtained using $0.1mg/dm^3$ 2,4-D and $0.5mg/dm^3$ kinetin. They found that cultures had to be subcultured every 3 to 4 weeks otherwise by the 5^{th} week the calli would stop growing, would become pigmented red and/or orange, by the 6^{th} week calli would

become entirely pigmented and by the 8th week become brown. The implication here, from a pigment production angle, is that betalain accumulation is inversely related to growth.

Other than this reference no other trace of the use of Pgo for red beet cultures could be found. As mentioned before Pgo was designed or at least used for sugar beet varieties. This can be seen in most references dealing with sugar beet (Szabidas L. & Gaggero C. 1985, vanGeyt J.P.C. & Jacobs M. 1985, Lindsey K. & Jones M.G.K. 1989, LeDily F. *et al.* 1990). Despite each of these research groups using sugar beet (*Beta vulgaris*) and Pgo, all say they obtained friable calli with slight modifications of media supplements for example 1mg/dm^3 2,4-D and 0.5mg/dm^3 kinetin or 0.3mg/dm^3 benzylaminopurine with different combinations of naphthalene acetic acid or 2,4-D. What appears to be happening is that *Beta vulgaris* cultures can be initiated on any medium but specific morphology can occur via close selection and/or the use of growth regulators.

Finally other salt cocktails have been used. Girod P.A. & Zryd J.P. 1987 used a modified form of LS (Linsmarer E.M. & Skoog F. 1965) containing half strength salts, NH_4NO_3 being replaced by KNO_3, 10g/dm^3 sucrose(not 30g as in this thesis), 5g/dm^3 mannitol and 1.5mg/dm^3 thiamine-HCl adjusted to pH 5.8 prior to autoclaving. As the cultures were green habituated ones there was no need for plant growth regulators and a reduced amount of sucrose. Girod P.A. & Zryd J.-P. 1991 went a step further and elaborated their own medium cocktail for *Beta vulgaris* var bikores monogerm. This medium appears to have resulted in such a friable calli that calli scattered spontaneously when dropped in a 7% mannitol solution.

4.2 Pigment Extraction and Purification

All effective extractions of a substance can only be based on the awareness of the properties of the substance being extracted. The stability of betalains is greatest at pH 4.5 and over pH 7.0 they tend to degrade more rapidly. The betalain molecule is of amphoteric character. A typical betacyanin exists as a cation below pH 2, as an immobile Zwitterion at about pH 2, as an mono-anion between pH 2 and 3.5 and is a bis-anion between pH 3.5 and 7. In the latter case the three carboxyl groups of the betanidin chromophore are dissociated (Reznick H. 1981 p381). It is this aspect that is exploited the most and permits the use of gel filtration via adsorption and ion-exchange supports. The other main characteristics is the fact that betalains are water soluble and

that the betaxanthin are partially soluble in organic solvents such as methanol and ethanol (von Elbe J.H. & Maing I.-Y. 1973).

There were two levels of extraction and separation in the project; 1. extraction and separation of betalain pigments from fresh red table beet for the purposes of standards and intermediates of the biosynthetic pathway, 2. analysis of experimental samples. It is proposed to look at each in turn.

4.2.1 Extraction and Purification of Pigment for Standards

There were two methods of extraction of betalains from fresh table red beet available 1. water extraction 2. initial extraction of the freeze-dried beet with 100% methanol to get an enriched betaxanthin fraction followed by 50% methanol extraction of the remaining pulp. This method was based on extraction of experimental samples by Berlin J. *et al.* 1986. These initial crude extractions were subsequently freeze-dried or rotary-evaporated at 50°C depending whether the extraction used solvents or water. A similar sort of extraction is used by Alard D. *et al.* 1985. However in this case there was no attempt to separate the betaxanthins from the betacyanins. Otherwise extractions in the literature has been based on water followed by further separation and purification of the betacyanins and betaxanthins. Pourrat H. *et al.* 1983 stored these aqueous extractions in a cold room for 24 hours to allow proteins to precipitate.

Once extraction was completed separation of the betacyanins from betaxanthins could commence. High performance liquid chromatography was initially examined as a technique for preparatory separation of betalains. It was found that the amounts of product obtained with this method did not match the required amounts given the time and resources needed to set up this technique. This aspect is seen indirectly in the literature in the sense that there are very few references on its use. The first recorded use of HPLC for quantitative preparation of betalain pigments was made by Vincent K.R. & Scholz R.C. 1978. They were able to separate yellow from violet while monitoring at the appropriate wavelength using an isocratic elution. They used a reverse-phase μBondapak C_{18} column, PIC reagents, methanol and water. Schwartz S.J. & vonElbe J.H. 1980 used four 7.8mm i.d. by 61cm Bondapak C_{18}/Porasil B columns, particle size 32-75μ, connected in series. They used this system to separate out betanidin from betanin. However they indicate the use of Sephadex G-25, using 1% acetic, for initial separation of betacyanin and betaxanthins. Forni E. *et al.* 1992 were the next to use HPLC for preparatory purposes. They used an RP-18 50cm by 5cm i.d.

column using a two step elution; 0.1M phosphate pH 5/methanol (85:15) and 0.1M phosphate buffer pH 5/methanol (70:30).

As indicated previously Sephadex G-25 with 1% acetic acid was used to separate betacyanins from betaxanthins. It was decided initially to try out a modification of the protocol of Adams J.P. & vonElbe J.H. 1977 using Sephadex G-15. Citrate buffer was used, as an initial separation of yellow and pink pigments was wanted, not acetate buffer which was indicated as giving best resolution of betanin and betanidin. Despite the large scale separation giving yellow and violet fractions, it was realised that buffer salts would be present in the end product. A further literature search revealed that Sephadex could be used with 1% acetic acid (Adams J.P. & vonElbe J.H. 1977, Attoe E.L. & vonElbe J.H. 1981, von Elbe J.H. & Attoe E.L. 1986, Drdák M. *et al.* 1990a, Drdák M. & Vallová M. 1990b, Simon P. *et al.* 1993, Altamirano R.C. *et al.* 1993). The mode of action would be a mixture of adsorption of betacyanin, allowing the betaxanthins to pass through, and molecular sieving. The results show that there is a good separation of betacyanins from betaxanthins. The fact that 0.1% HCl was used to crystallise betanin and betanidin (Schwartz S.J. & vonElbe J.H. 1980) elution with 0.1% HCl was examined. This was to minimise any effect of acetic acid on pigment degradation. As the results show this caused the betacyanins to adhere to the column matrix while the yellow betxanathins eluted through. Elution of the betacyanins could be speeded up by changing the elution buffer to water.

Dowex was also found to have been used considerably over the years; Piattelli M. & Minale L. 1964a, vonElbe J.H. *et al.* 1972, Pasch J.H. & vonElbe J.H. 1975, Ulyanova N.S. *et al.* 1975, Colomas J. 1977. When using this method it was found that it was good for removing prebetanin and other minor betacyanins and that it agreed with the literature. However it did not separate the yellow vulgaxanthins well enough from the violet betacyanins. It was realised that for this method to work other columns, as indicated in the same literature, would have had to be used for example 7 Polyclar-AT (von Elbe J.H. *et al.* 1972) or that the extract be already free of vulgaxanthins.

A third method examined in this thesis was the use of cellulose. Despite this method separating extremely well the yellow from the violet pigments, it was dependent on the column being poured with high precision. In addition when bulking up to a bigger column it was found that the flow rate was drastically reduced to such an extent as to make the protocol impractical. This does not appear to have been encountered with Piattelli M. & Minale L. 1964 nor Bilyk A. 1981. In the case of the latter TLC was used.

On going through the literature one finds that one step is not sufficient to separate and purify an individual pigment. Numerous protocols have been developed over the years depending on the available resources to separate and purify pigments. VonElbe J.H. *et al.* 1974a review the initial techniques used for purifying pigments. The method of Piattelli M. & Minale L. 1964a was considered the most successful using cellulose initially to separate betacyanins from betaxanthins followed by Dowex 50W*2 to clean the betacyanin betanin from prebetanin and its hydrolysis by chromatography on polyamide to obtain betanidin. Despite Colomas J. 1977 comparing the use of Dowex 50*18 (200-400 mesh) with Sephadex G-15 for the purposes of pigment separation of experimental samples one can still consider this method for large scale red beet pigment separation. Colomas J. 1977 uses citric acid, NaCl and NaOH with a Dowex column and acetate buffer pH 4.5 with a Sephadex G-15 column. Unfortunately however these protocols leave the problem of separating the salts from the pigment. Adams J.P. & vonElbe J.H. 1977 overcomes this by rechromatographing but using 1% acetic acid in water to elute. Alternatively one can use solvents as did Bilyk A. 1979. This involved extractive fractionation using ethanol and various percentages of HCl. However it was found that sugar tended to accumulate. These sugars could be partially removed by initial fermentation of an homogenised red beet (Adams J.P. *et al.* 1976, Pourrat H. *et al.* 1983).

In summary an attempt to purify pigments in one step prove futile. It would have been probably been best to use Sephadex G-15 with 1% acetic acid or 0.1% HCl, Dowex and 0.1% HCl to clean up the resulting violet fractions, Sephadex G-15 and acetate buffer to separate betanin from betanidin. The buffer salts would be subsequently removed by again using Sephadex G-15 and 1% acetic acid. Some of the yellow and violet fractions obtained from the above column work would be put through HPLC so as to verify purity of fractions and acquire small quantities of purified standard for the purposes of HPLC analysis of experimental samples.

4.2.2 Analysis of Experimental Samples

For the purposes of analytical measurement of experimental samples the method of Berlin J. *et al.* 1986, working on cell suspensions of *Chenopodium rubrum*, was initially used. This method consisted of extracting experimental samples twice first with 100% methanol which would take out the yellow betaxanthins then with 50% to take out the betacyanins. The extractions were subsequently measured for betalain content by HPLC.

For the purposes of the project the second part of the procedure was not feasible. In addition it was found that the 100% methanol extraction was not all that effective in extracting just the betaxanthins (50% efficiency).

Consequently one 50% methanol extraction was carried out on the experimental samples. Measurements of pigment content were performed using a method by Nilsson T. 1970. This entailed a mathematical formula which gave actual absorbances of betaxanthins and betacyanins as opposed to the apparent absorbances due to background and, in the case of vulgaxanthins, interference from the betanin absorbance spectrum. This method was considered sufficient since a visible increase in callus pigmentation was being sought thus a quantitative method was all that was required. This mode of measurement was used by Wiley R.C. & Lee Y.-N. 1978, Shih C.C. & Wiley R.C. 1981 and Dr. Janes(Personal communication).

The use of HPLC however was considered more appropriate to verify the occurence of betalains in pigmented calli. The method used was that of Schwartz S.J. & vonElbe J.H. 1980 as all listed materials were available. It was found that the gradient elution did not improve the speed of separation over the isocratic elution. In addition, since the calli became yellow on pigmentation there was no need for the gradient which was mainly for the separation of betanin from the aglycon betanidin. The effectiveness of HPLC for analysis of betalains can be seen in the literature.

Strack D. & Reznick H. 1979 used an acetic acid, methanol and water mix with a gradient elution when looking for betaxanthin in members of the *Centrospermae*. They find that betalamic acid can be 50% degraded by 4% acetic acid within 5 minutes when monitoring at 436nm. Strack D. *et al.* 1981 used a LiChrosorb RP-18 column as they found it superior to the RP-8. It is surprising that the authors dissolved their samples in 40% methanol and not in their eluting buffer, the latter initially being 1.5% ortho-phosphoric acid in water changing linearly to ortho-phosphoric acid-gl.acetic acid-acetonitrile -water 1:5:20:35. They make reference to Schwartz S.J. & vonElbe J.H. 1980 as the alternative method. Adachi T. & Nakatsukasa M. 1983 used a 40mm by 250mm Zorbaz PC_8-10/s (Dupont) column with a gradient elution using just pH 2.5 phosphate buffer to 100% methanol. They found, using *Portulaca grandiflora* as a model system, that betaxanthin had to be monitored at 430nm, and not 478nm, since the latter wavelength tended to pick up betacyanins also. Pourrat A. *et al.* 1988 develop a HPLC system aimed for drug/pharmaceutical industrial monitoring of red beet colorants. They use a 250mm by 4mm 10µ Bondapak C_{18} (Walirc) column, methanol, KH_2PO_4 and H_3PO_4 for pH adjustment in a multistep gradient elution. They found that in doing so

they achieved an equivalent resolution using the same solvents and equipment as Schwartz S.J. & vonElbe J.H. 1980 but over a shorter time. Pigments of other model systems have been detected using HPLC. Bianco-Colomas J. & Hugues M. 1990 working on *Amaranthus tricolor*, Trezzini G.F. & Zryd J.-P. 1991a working on *Portulaca grandiflora* using a different method to that of Adachi T. & Nakatsukasa M. 1983 and Forni E. *et al.* 1992 working on *Opuntia ficus indica* (blood-red prickly pear).

It appears that HPLC was the best analytical procedure after Nilsson T. 1970 and the electrophorectical methods; Eppendorf photometer after preparative thin layer electrophoresis(Strack D. & Reznick H. 1979), preparative cellulose electrophoresis (Adachi T. & Nakatsukasa M. 1983). But now it appears that Smith M.A.L. *et al.* 1992 have developed an automated vision technique for immediate analysis and control of betalain-producing cell cultures.

4.3 Model System Induction

The third part of the discussion will look at the number of different ways that were examined to induce pigment production. These were (a.) nutrients whether it be whole media (Pgo to B5) or individual nutrients eg: phosphorous (b.) initial inoculum when setting up experiments (c.) light both quality and quantity, (d.) heat shock.

4.3.1 Nutrient Variation

In this thesis trials with different salt cocktails indicated that Pgo was the best for obtaining white friable calli. A transfer to B5 did not give any significant change in pigment accumulation. The experiment Pgo to B5 medium entailed a significant reduction in Cl^- ions(83%). The results of this thesis suggest that this reduction did not significantly affect the growth and pigment accumulation of calli. Published work, which looks at the effect of nutrients on pigment production in betalain producing systems, is limited. Constabel F. & Nassif-Makki H. 1971 found that the omission of phosphate from the nutrient medium resulted in a more intensive pigmentation of *Beta vulgaris* callus culture. Figs. 3.1.2A-F in this thesis would indicate this at first glance. However on a closer examination one sees that these results could be due to false positives caused by necrotic tissue. This necrosis was probably due to the accompanying sodium reduction not being counterbalanced(47mg). Somar 1989 found that Whites medium and that of M&S resulted in darkening of red beet calli whereas B5

was best for both fresh weight accumulation and pigment production. It was found in suspension cultures of *Chenopodium rubrum*(Berlin J. *et al.* 1986) and Phytolacca americana(Sakuta M. *et al.* 1986) that pigment levels increased with increasing phosphate. One gets the impression from the results and the literature that changes in phosphate levels result in different responses depending on the model system being studied.

4.3.2 Initial Inoculum

One of the findings of this thesis was that despite the initial inoculum size, callus growth appeared to slow down, if not cease, once 450mg total fresh weight accumulation was attained. This is probably the result of nutrient dificiency occurring. Pigment production, on the other hand, appeared to be associated with growth(figs. 3.3.2C, I). Callus inoculum experiments do not seem to have attracted much attention in the literature on betalain-producing model systems. The only reference to the amount of red beet callus used for inoculation onto agar plates was made by Girod P.A. & Zryd J.-P. 1987. They indicated the use of an initial inoculum of around 15mg. Berlin J. *et al.* 1986, when working on *Chenopodium rubrum* found that inoculum size was important as it affected the ability and the way in which cell cultures reacted to treatments. They found that with increasing inoculum size both the growth cycle and time of highest accumulation were shorten and specific accumulation enhanced. In contrast Moreno R.H.P. *et al.* 1993 found that, in suspension cultures of *Catharanthus roseus* the lower initial-inoculum-culture accumulated six times more ajamalicine than the higher density inoculum. A comparison between betalain and anthocyanin accumulation by Hirose M. *et al.* 1990 show that one of the major differences between the two pigments is that anthocyanins accumulate towards the end of the growth phase/the beginning of the stationary phase of the growth cycle. Results in this thesis indirectly indicate that betalain accumulation in linked to growth.

4.3.3 Light Quantity and Quality

The literature, however, is quite extensive on the study of light induction of betalain synthesis. Numerous model systems have been used from plantlets to cotyledons, callus and cell suspensions. Piattelli M. 1981, Böhm H. & Rink E. 1988 and Leathers R.R. *et al.* 1992 cover these facets in detail. When carrying out experiments in this thesis the

aim was to cause a significant reproducible induction of pigment production to the extent that the whole callus became visibly pigmented to the naked eye. This was judged necessary so as to facilitate further experiments which would probe the exact nature of the regulatory mechanism.

The project's finding is that this model system could not be used in the manner anticipated. This is probably due to an inherent property of the system. A similar finding is seen in Girod P.A. & Zryd J.P. 1987. Girod P.A. & Zryd J.P. went a step further and looked more closely at how green habituated cell cultures of red beet reacted to light. They found that on illumination calli developed a patch-like induction of pigment accumulation and that not all cells became pigmented. This was systematically found throughout this project. The authors also found that a minimum doubling time of 1.8 was needed before pigment production stabilized at a certain level after which pigment accumulation was independent of growth. It was also found that smaller calli grew faster and that light tended to increase the generation time, that is, slow down growth. This result is reinforced in a different manner in the initial inoculum experiment in this thesis, (Figs. 3.3.2A-B), in so far as growth slows down after a certain threshold of fresh weight accumulation has been attained. Figs. 3.3.2C-D show how light appears to slow down growth. Clonal variability was also found to be prelevant; this is characterised by a clone, when put back into dim light to be subcultured back again into intense light, does not display the original characteristics. This was seen throughout the experiments in the sense that the extent of light induction was never constant; calli would be variablely covered with pigmented patches and patches pigmented to varying degrees.

Bokern M. *et al.* 1991, when looking at accumulation of phenolic acid conjugates, betacyanins and sulphated, and acylated betacyanins in *Chenopodium rubrum* demonstrates, in passing, the effect of light. They also show that if they wanted to adopt the same approach as envisaged by this project, namely inducing a non- producing white line to a producing cell line, that only a certain cell type could be used. It was found that their high betacyanin producing cell line could not survive in the dark but that a cell line producing less pigment could, inspite of losing its colour. They find that light causes a 3.8 times increase in amaranthin accumulation. The reason for this is probably due to the finding of Hinz U. *et al.* 1992 namely that betalain accumulation is developmentally regulated. The extent of development in question has not being determined. Despite this, experiments in this thesis show the inductive effect of light and the presence of constitutive pigment production in the dark.

Previous literature which concerns itself with the effect of light on betalain synthesis, is mainly based on research using differentiated tissue. Wohlpart A. & Mabry T.J. 1968, when looking at dark synthesis of betalains in a variety of betalain-producing plants, found that light was not an absolute requirement for red beet. However only a subjective comparison is made between stem and leaves in the dark over 1 and 2 weeks. When a comparison was made with a light treatment of *Beta vulgaris* it was found that dark grown plants produced 15% of that grown in the light after 1 week and 25 - 30% after 2 weeks. The authors attributed this to the lack of carbohydrates. Rast D. *et al.* 1972 reiterates this point giving amounts of seedlings used and using $O.D._{536nm}$ for quantitative measurement. After 9 days of culture it was found that dark grown seedlings produced 33% the amount of pigment found in seedlings grown in the light. Giudici de Nicola M. *et al.* 1973, when looking at betaxanthin synthesis in *Celosia plumosa* seedlings, demonstrated the need for light for pigment synthesis. They show this by incubating a portion of the experimental seedlings initially in the light followed by dark treatment and comparing the response to that portion of seedlings which were continuously treated with light throughout the experiment. Berlin J. *et al.* 1986, working on pigment-producing cell suspensions of *Chenopodium rubrum,* found similar results as in this thesis for dark followed by light and continuous light and dark treatments. They record a 7 times increase on light illumination. They also noticed that light was not an absolute requirement for pigment accumulation. In contrast Somar 1989 patent on the use of red beet cultures for the production of betacyanin pigment using a reducing agent in the medium found that pigment cultures incubated in the light accumulated less pigment.

Light Quantity

Experiments on light intensity demonstrate that 10 $\mu mol/m^2/s$ was sufficient and that one week was needed at least before assessment of the effect of light could be judged. No work on light intensity has been carried out on *Beta vulgaris*.

However experiments looking at the duration of light treatment can be seen in other model systems in particular seedlings of *Amaranthus* and *Celosia.* Malaviya B. & Laloraya M.M. 1966 carried out a number of experiments with light and/or dark after light treatments. It was found that the longer the light induction the higher the pigment accumulation. This is seen somewhat in this thesis when it was found that at least one week was needed before measurements could be made. The authors however found that

dark incubation after a light pretreatment resulted in a decrease in pigment content. This reflects the role of light in maintaining pigment production. Piattelli M. *et al.* 1969 working on *Amaranthus tricolor* and Koehler K.-H. 1972 working on *A. caudatus* get a different response. They find that there is a decrease in pigment accumulation in light after 25 hours illumination. This is seen somewhat in the light intensity experiment in this thesis(figs. 3.3.3B-E). Kishima Y. *et al.* 1991 find that 30 hours is sufficient for a response. Kumon K. *et al.* 1990b, when looking at the epidermal tissue of *Phytolacca americana*, demonstrate light induction accumulation despite the light treatment alternating with 12 hours darkness every 12 hours. The trends tally well with the experiments of this thesis in so far as 4 weeks was required for a reasonable assessment of the effect of an inducer and secondly that a high variability was to be found in the data. They also found slight accumulation in tissue incubated in the dark. This adds to the list of Rast D. *et al.* 1972 of those model systems where light is not a absolute requirement for pigment accumulation. Kishima Y. *et al.* 1991b found that it was possible, with a high pigment producing callus line of *Portulacca grandiflora*, to cause visible light induction. In addition these calli could be transferred back into the dark, lose their pigment and still survive. This reiterates Girod P.A. & Zryd J.P. finding that the pigments are in a dynamic motion, that there is active pigment degradation. This can be somewhat supported by the presence of decolorising enzymes(Lashley D. & Wiley R.C. 1979, Shih C.C. & Wiley R.C. 1981, Elliott D.C. *et al.* 1983b, Wasserman B.P. & Guilfoy M.P. 1983, Zakharova N.S. *et al.* 1989, Kumon K. *et al.* 1990a, Zakharova N.N. *et al.* 1992). Böhm H. *et al.* 1991 observe a decrease in pigment accumulation when yellow *Portulaca grandiflora* cells were transferred to the dark. They also found that light affected growth causing nearly a 20% increase over dark grown cultures. In addition it was noticed that a peak in pigment accumulation occurred towards the end of the growth cycle during the stationary phase. This is contrary to what has been the case up to now in the literature and in the experiments in this thesis. This can be particularly seen in the mg experiment(Figs. 3.3.2A-E) if one takes the upper 450mg threshold into account. Also one can see this in the Pgo to B5 experiment (figs. 3.1.1K-P).

Light Quality

It is known, from the literature, that red light-activated phytochrome is involved in many plant processes. Since experiments in the project were not achieving a

systematically reproducible total callus pigmentation it was decided to see if light quality could improve the induction of pigment.

Experiments in this thesis show that white light results in the highest promotion of pigment production. Surprisingly green is the next highest. It was expected that green would give a similar response to dark grown calli. Red and blue irradiated calli give a similar response to each other. However this response is lower than that of green light.

No work appears to have been published on the effect of light quality on pigment production in *Beta vulgaris*. Most of the results in the literature concerning light quality, address the question of the role of phytochrome. This is achieved using red light followed by far red light.

Piattelli M. *et al.* 1969 demonstrate the role of phytochrome in seedlings of *Amaranthus tricolor*. However this does not tell us much about the rest of the visible spectrum since the red light alone did not result in the same amount of pigment accumulation as if the seedling was illuminated by white light. Rast D. *et al.* 1972 carry out similar experiments. They, on the other hand, find that red light alone could get higher results than white light. Giudici de Nicola M. *et al.* 1973b working on betaxanthin synthesis in *Amaranthus tricolor & Celosia plumosa* seedlings suggests the involvement of photosynthesis. Woodhead S. & Swain T. 1974 show that red light is not the only wavelength within white light that promotes pigment production. They found that red light accounts for only 15% of total pigment production. This is probably because 1W/cm of light was used as opposed to 20W/cm of white light. Colomas J & Bulard C. 1975 tackle this point by exposing *Amaranthus tricolor* seedlings to an absorption spectrum at 10nm intervals between 420nm-780nm. Unfortunately they did not do a constant energy/same exposure time for each 10nm interval. However despite this, whether one uses the result of the constant energy or constant exposure time, there is a peak in the blue and red region. The authors consider, as suspect, the direct action of phytochrome given the narrowness of the active zone and the maximum wavelength of 660nm. Kocchar V.K. *et al.* 1981b does an indepth study on red, farred, blue light and their interaction. They showed that phytochrome was involved, that there appears to be the existence of a specific blue/UV light photoreceptor whose effect can be partially reversed, that light quality and light quantity are important and that the photosynthetic pigments play no role in pigment accumulation. However one notices in this paper that there are no error bars associated with the result. The fact that the results of the thesis do not tally with previous literature may be due to the model system

chosen i.e. callus as oppose to seedlings.

4.3.4 Effect of Heat

The shelf/light experiment, the temperature followed by red light experiment and the colour filter experiment in this project all demonstrate the need for a cultivation temperature of around 29°C for optimal effect. Heat shock experiments are mainly to be found in research articles on molecular genetics and in particular gene regulation. However Elliot D.C. 1979, working on *Amaranthus tricolor* seedlings, applied this heat shock idea to seedlings which were used in a cytokinin bioassay. Her theory was that heat affected membrane properties in such a way that increased chances of substrates reaching the sites of enzyme activity and energy molecules becoming more available. This idea appears not to have been considered by other research groups. However if one looks closely at Wagner E. & Forsch S. 1971 one sees indirect evidence of the role of temperature. They found that a circadium rhythm in betacyanin accumulation in *Chenopodium rubrum* seedlings was initiated/synchronised by the cyclic temperature and light conditions. Rink E. & Böhm H. 1991 found that warm weather periods increase pigment synthesis in *Portulaca grandiflora.* Honda H. *et al.* 1993 working on *Phytolacca americana* indicate a cultivation temperature of around 28°C. A similar finding with respect to temperature is seen in Zhong J.-J. & Toshiomi Y. 1993 when looking at anthocyanin producing cell suspension cultures of *Perilla frutescens.* However Toivonen L. *et al.* 1992, Shichiyo Z. *et al.* 1993, Camm E.L. *et al.* 1993 get different results with a low-temperature 5-20°C causing an increase in anthocyanin accumulation. Hypothesis indicated include amplification of signal and alteration of membrane properties. It is probable that a number of physiological processes are occurring in the presence of heat in excess of biological norms. One must be aware of enzymes optimums, whether they be inhibitory or not, membrane permeability, co-factors, internal cytoplasm movements, disassociation kinetics, etc.

4.4 Rate-limiting Step Elucidation

The fourth part of the project was to try and determine the rate-limiting step in the biosynthetic pathway of betalains. The results of precursor feeding experiments in this project, as they stand, indicate a possible rate-limiting step before tyrosine or that each step results in a increase in pigment accumulation and that there is no one rate-limiting

step(Martin C. & Gerats T. 1993). The results appear to indicate that tyrosine has the same stimulating effect as DOPA during the dark but that light, despite increasing the overall pigment content relative to the dark, does not appear to expose the presence of precursors. Guruprasad K.N. & Laloraya M.M. 1976 fed 10^{-3} M tyrosine and DOPA to isolated hypocotyls of bei *Acetabularia mediterranea* in the light. There appeared to be no appreciable difference between the precursor despite a 200% increase in pigment accumulation over control. A similar result is found in this thesis. DOPA however has a higher stimulating effect in conjunction with light resulting in a doubling of the highest dark total pigment accumulation value.

A lot of precursor work over the last 20 years have confined itself to tyrosine and DOPA feeding. The determination of the rate-limiting step has been hampered by the lack of elucidation of the final steps of the pathway. A limited amount of work has been done using *Beta vulgaris*.

Constabel F. & Nassif-Makki H. 1971 showed that the use of tyrosine and DOPA did not promote betalain synthesis. They also notice a pronounced oxidation of DOPA. Zryd J.-P. *et al.* 1982, when comparing white and red callus lines of red beet, concluded after amino acid analysis and labelled tyrosine and DOPA feeding that regulation of DOPA metabolism appeared to influence pigment accumulation and that there appeared to be different entry points for tyrosine and DOPA into the biosynthetic pathway (Verpoorte R. *et al.* 1993). Leathers R. & O'Riordain G. 1991 found in an orange producing cell suspension culture of *Beta vulgaris* that the betaxanthin amino acid dopamine, which on combining with betalamic acid will give miraxanthin, accumulates to a far greater extent than miraxanthin. This would point to the enzyme that causes the condensation of amino acid to betalamic acid, as being the rate-limiting step in the biosynthetic pathway. However it could also be at the level of DOPA to betalamic acid conversion or the availability of DOPA. They also found growth-associated and non-growth-associated linked production of pigment in their cell suspension.

Work on other model systems does not appear to be able to give a definite answer as to were the rate-limiting step is, if one does exist. French C.J. *et al.* 1973 & 1974 looked specifically at precursor feeding and the effect of light. They show that dark 5mM DOPA results in 5 times more pigment accumulation than tyrosine after 48 hours. This was not the case in the experiments in this thesis. Colomas J. & Bulard C. 1975 found that the results of precursors feeding was independent of light induction. Giudici de Nicola M. *et al.* 1975 looked at the effect of precursor feeding indirectly when examining light control of pigment synthesis. The results of this thesis tally with those

of Giudici de Nicola M. 1975 in neither confirming nor denying a control point between tyrosine to DOPA. However they conclude that the rate-limiting step might be at the DOPA to the dihydropyridine moiety. This is based on the fact that cyclodopa failed to enhance amaranthin production in the dark. They also concluded from analysing the yellow pigment formation that *in vivo* spontaneous condensation of DOPA with betalamic acid or a chemical equivalent does not take place. Challice J.S. 1977, when looking at the effect of phenolic compounds showed that DOPA showed partial or no inhibition up to 0.1mg/cm^3 (0.5mM) with greater inhibition at 1mg/cm^3.

The experimental results of this project put forward the suggestion that there could be multiple control points along the biosynthetic pathway. This arises from the fact that DOPA feeding produces only a slightly greater increase in pigment accumulation than tyrosine and not a complete change from no pigment accumulation to 1000 times accumulation which the project was looking for. The former idea tallies somewhat with Stobart A.K. & Kinsman L.T. 1977 who showed that the addition of 5.5mM tyrosine resulted in 134% increase over water control and 5.5mM DOPA 141% increase in seedlings of *Amaranthus caudatus*. They concluded, based on other experiments using growth retardants and gibberelic acid, that regulation can occur at multiple steps along the enzymatic pathway, that is, before tyrosine, between tyrosine and DOPA and after DOPA. Bianco-Colomas J. 1980 looking at *Amaranthus caudatus* L. var *pendula* seedlings, which produce pigment in the dark, found that the feeding of DOPA in both dark and light produced 3 to 4 times as much amaranthin as the control. They found that the DOPA solution after 120 minutes at 23°C became progressively darker and finally black. Elliott D.C. 1983 working on *Amaranthus tricolor* seedlings found that there is no difference between 0.5mM and 5.0mM tyrosine except a 30% increase in the dark whereas 5.0mM DOPA resulted in a 100% increase over the 0.5mM treatment and 423% over the control. In a similar experiment in this thesis it can be seen that there is no appreciable difference between 0.1mM and 1.0mM tyrosine(fig. 3.4.1J) whereas in the case of DOPA 1.0mM results in a doubling of that of 0.1mM(fig. 3.4.1P) in pigment accumulation. Elliott concludes that there are three levels of control in her betalain producing model system 1. substrate supply/enzyme activity, 2. increase enzyme synthesis and/or activity, 3. tyrosine pool separate from cyclodopa, cyclodopa glucoside and amino acids.

Berlin J. *et al.* 1986, using 8 year old pigment producing cell suspension cultures of *Chenopodium rubrum*, found that the feeding of precursors to suspension cultures was dependent on initial inoculum of suspension cultures and time of feeding in the growth

cycle. As a result more experiments would have needed to be carried out on how best to use their model system before using it to elucidate a possible rate-limiting step. This would be required since Berlin found that feeding precursors reduced growth in some cases thus reducing pigment accumulation. This may have been an interfering aspect when using callus cultures in this project, that is time of feeding could not be controlled.

Sakuta M. *et al.* 1991 indicate that when studying the effect of tyrosine one cannot afford to have 2,4-D present as it causes degradation of the tyrosine. This may have affected dark treatments of tyrosine treatments in the project. They believe that the supply of precursor and enzyme control ultimately determined pigment accumulation. This is not borne out in the thesis and may be model system dependent. Wichers H.J. *et al.* 1993 find that high concentrations of 2,4-D causes suppression of L-DOPA accumulation and redirects tyrosine to protein metabolism. Bokern M. *et al.* 1991 working on *Chenopodium rubrum* suspension cultures reinforce this idea that the availability of tyrosine, in stationary phase, in the dark grown lighter violet cell line is 17% of the light-grown lighter violet suspension line. However the deep violet cell line, which cannot survive in the dark, there is 30% tyrosine and 198% DOPA of the light grown lighter pink cell line. Bokern M. believes that control could be at an amino acid level. They indicate how precursor supply can be affected by other biosynthetic pathways and the importance of precursor supply whatever the level of the pathway. This could point to either separate site of precursor formation and pigment synthesis, as suggested by Leathers R. & O'Riordain G. 1991 or the lack of appropriate enzymes.

Rink E. & Böhm H. 1985, 1991 found that after feeding DOPA to 16 species of betalain-producing plants, irrespective of whether individual species could synthesize both betacyanin and betaxanthin or not, there was occurrence of betaxanthins in all cell lines. The implication is that DOPA can act as an elicitor. This was not the case in the project. Kishima Y. *et al.* 1991a when working on *Portulaca grandiflora* buds, found that (a.) betalain pigmentation in petals was initiated after a dramatic tyrosine accumulation but that (b.) DOPA levels remained constant during pigment formation. (c.) On the other hand white petals show a large DOPA accumulation indicating block after DOPA. Hirano H. *et al.* 1992, when looking at the effect of cytokinin on tyrosine levels in pigment producing cell suspensions of *Phytolacca americana*, did, in passing, a comparison of the effect of tyrosine and no tyrosine. They showed a 25% increase in betacyanin absorbance/gfr. wt. They also showed that betalain production was not linked to general metabolism in their model system but that is was moreso influenced by precursor supply and the biosynthetic activity after tyrosine.

An explanation for the varied tyrosine and DOPA results may reside in the hypothesis by Riley P.A.(1993) in relation to melanogenesis. The initial two precursors are tyrosine and DOPA as for the betalain biosynthesis(Palumbo A. *et al.* 1990). Riley then stipulates that tyrosine goes to DOPA via dopaquinone and cyclodopa(fig. 4.4.1)

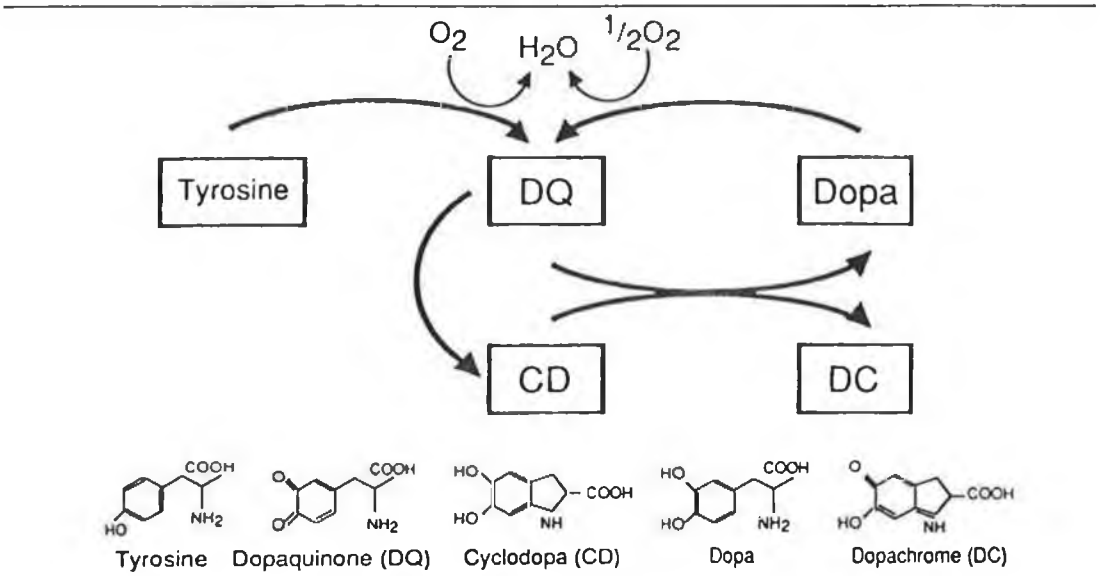

Fig. 1. Phase 1 melanogenesis. Schematic representation of phase 1 melanogenesis consisting of a series of steps commencing with the oxidation of tyrosine to dopaquinone (DQ) by the enzyme tyrosinase followed by the dismutation of dopaquinone, through the intermediacy of cyclodopa (CD), to give rise to 3,4-dihydroxyphenylalanine (Dopa) and dopachrome (DC). Dopa is reoxidised by tyrosinase to give dopaquinone. The structural formulae of the compounds referred to are shown in the lower portion of the figure.

Figure 4.1.1 P.A. Riley's hypothesis of the DOPA/dopaquinone redox cycle

via a tyrosinase-catalyzed oxidation to give dopaquinone which spontaneously undergoes endocyclic ring formation to give rise to cyclodopa. Cyclodopa is rapidly oxidised by redox exchange with dopaquinone generating respectively dopachrome and DOPA and setting up a DOPA/dopaquinone redox cycle. This may explain Zryd J.P. *et al.* 1982 observations that fed tyrosine is mostly found in the cyclodopa part of the betacyanin molecule and DOPA mostly found in the betalamic acid part. One could thus envisage a control point at the cyclodopa level as well as at the formation of betalamic acid

In conclusion for a more definite estimation of the nature of the rate-limiting step the feeding of betalamic acid would have been necessary as well as a detection of variations levels of intermediates such as cyclodopa and cyclodopa glucosides if they exist. This would then permit a search for the remaining enzymes of the pathway and subsequently a pin-pointing of the rate-limiting step/steps.

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