

**STUDIES ON THE BIOSYNTHESIS OF BETALAINS IN CELL CULTURES
OF *BETA VULGARIS* L.**

by

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DECLARATION

I hereby declare that this thesis was composed by myself, and the work described herein to be my own, except where indicated otherwise.

MOHD SHAIB JAAFAR
EDINBURGH, 1991

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ABBREVIATIONS

B5	Gamborg's B5
butyl-PBD	2-(4'-tetra-butylphenyl)-5-(4"-biphenyl) -1,3,4,-oxadiazole
°C	degree Centigrade
ca	circa, about
cm	centimetre
cpm	counts per minute
cv	cultivar
d	day
2,4-D	2,4-dichloro phenoxy acetic acid
dpm	disintegration per minute
EDTA	ethylenediamine-tetraacetic acid
et al	et alia
eg.	example
FIG.	figure
FW	fresh weight
g	gramme
xg	times the force of gravity
h	hour
i.e.	that is
K	kinetin
l	litre
M	Molar
mg.l ⁻¹	milligramme per litre
min	minute
MS	Murashige and Skoog
ml	millilitre
μl	microlitre
mm	millimetre
μmol	micromole
N	nitrogen
nm	nanometre
No.	number
OD	optical density
ppm	parts per million
PCV	packed cell volume
PGR	plant growth regulator

RGR	relative growth rate
R _f	distance moved by a compound relative to the solvent front
RITA	Radioactivity Intelligent Thin-Layer Analyser
rpm	revolution per minute
s.e.	standard error of the mean
s	second
SH	Schenk and Hildebrandt
TLC	thin-layer chromatography
uv	ultra-violet
v/v	volume per unit volume
W	White's
w/v	weight per unit volume

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ABSTRACT

Callus was obtained from three cultivars of *Beta vulgaris*, Boltardy, Burpee's Golden and Albina veredunda, and their pigment contents examined. Cv. Boltardy was chosen for subsequent studies because it contains relatively high amounts of the red betacyanins and the yellow betaxanthins. Suspension cultures obtained from friable callus were grown in MS, SH, B5 and White's media, and the best growth was obtained on SH medium, while the most intense pigmentation occurred in White's medium. B5 medium supports growth as well as betalain accumulation, and was used subsequently for the remainder of the investigation. 2,4-D, within the range tested, enhanced growth with increasing concentration, and tends to repress betaxanthin accumulation, but does not affect betacyanin. The effects of sucrose concentration within the range 2-16% were examined, and concentrations above 8% were found to be deleterious to both growth and pigment accumulation. The optimum concentration was found to be 4%, where biomass yield and betalain yield were highest and the growth period was also extended. Within the range 5.4-6.0, pH did not have any significant effect on betalain content, nor did inoculum size, although the cultures mature more quickly at a high initial cell density.

A close relationship was shown to exist in illuminated cultures between growth and betalain accumulation. In an

experiment involving feeding of radioactive L-tyrosine, maximal betacyanin synthesis occurred during the active growth phase, whilst maximum betaxanthin formation occurred toward the end of the growth cycle. It was also shown that betacyanin degradation occurred toward the end of the growth cycle, during which betacyanin synthesis decreased and betaxanthin synthesis increased concomitantly. These results suggested that there was a differential regulation of betacyanin and betaxanthin synthesis and accumulation.

Light was shown to stimulate betalain synthesis and accumulation, and it did not appear to be an essential prerequisite for betalain biosynthesis, but accumulation of the betalains increased with the level of irradiance. However, pigment persisted in dark-grown cultures after many subcultures, with certain cells showing a high capability for pigment production. This suggests that there is an alternative non-light dependent route to the betalains in these cells. A high variability within the cultures was also observed.

CHAPTER 1
INTRODUCTION

1.1 THE BETALAINS

Betalains (Fig. 1.1) are naturally occurring water-soluble nitrogenous pigments, and are the products of secondary metabolism. They are often classified as alkaloids although they are not alkaloids in a strict sense because of the presence of several carboxyl groups which render the molecules acidic in nature. There are two groups of betalains, the red-violet betacyanins and the yellow-orange betaxanthins.

1.1.1 Occurrence in Nature

Betalains occur in the vacuoles of cells in various parts of the plant and are characteristic of most families in the order *Centrospermae*. Interestingly, although betalains and anthocyanins are the main pigments responsible for the orange to violet hues in plants, their occurrence is mutually exclusive (Kimler *et al.*, 1970). Betalains do not occur in animals or micro-organisms, but are present in at least one mushroom, the Fly Agaric (*Amanita muscaria*) (see Döpp *et al.*, 1982; Mabry, 1980). In plants, betalains can be found in almost every structure, including the subterranean organs. Betaxanthins are usually found together with the betacyanins (Nilsson, 1970), although at a lower concentration.

Fig. 1.1

The general structure of betalain. (a) betalamic acid moiety (b) R^1-N-R^2 , residue of cyclo-DOPA in betacyanins (c) R^1-N-R^2 , residue of amino acid or amine in betaxanthins.

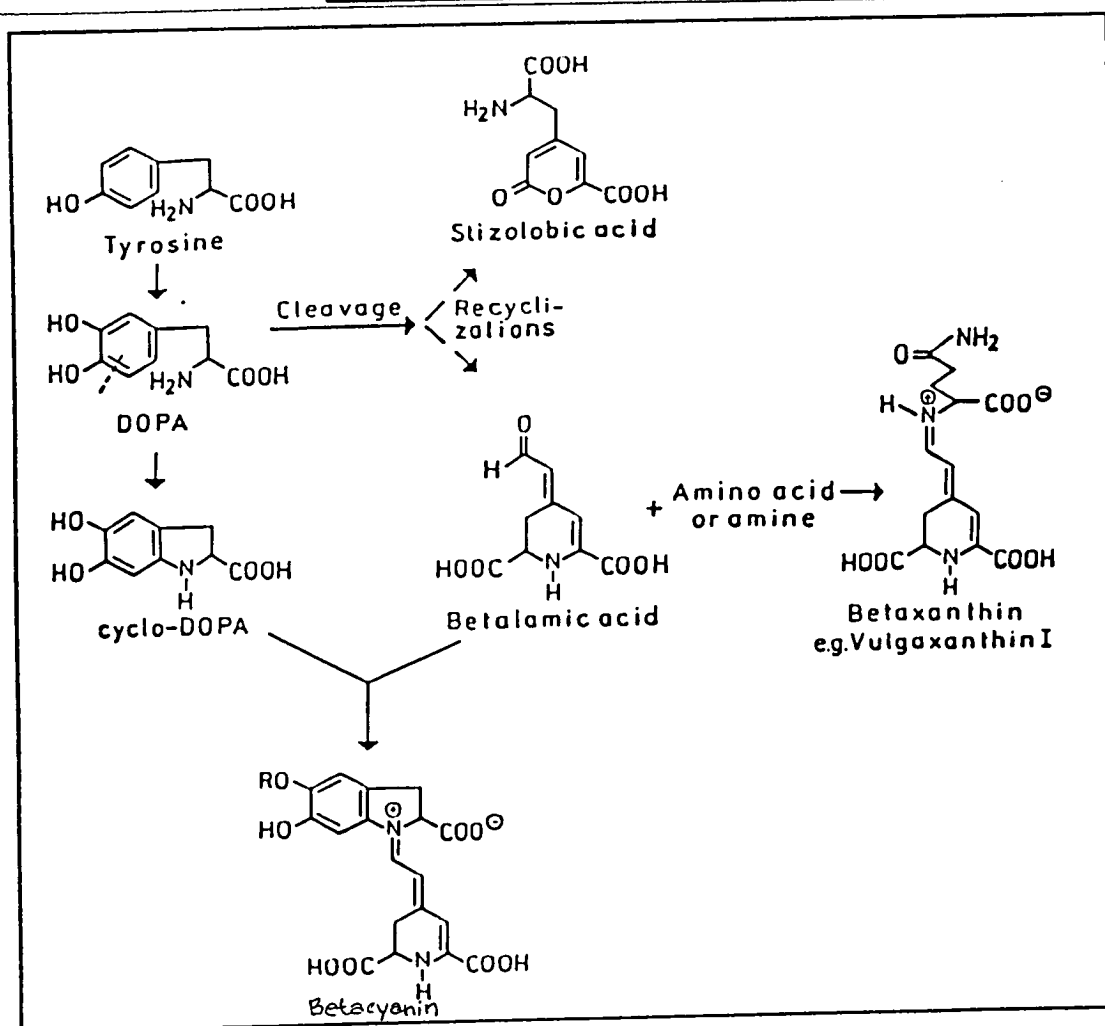
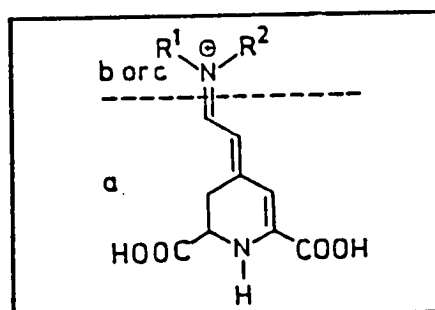


Fig. 1.2.

The proposed biosynthetic pathway for betalains (after Schütte and Liebisch, 1985)

1.1.2 Functions of the Betalains

In general, the presence of a brightly coloured pigment may function as a signal or warning to other organisms (Luckner, 1990). However, betalains in the perianths of flowers or in fruits, most probably act as an attractant to animals to help in pollination and seed dispersal. It has been shown that injury to a tissue induces betalain formation, probably a self-defence mechanism, since betalains have been shown to inhibit viral reproduction (Sosnova, 1970). Betanin-containing beet seedlings were also found to have a higher resistance to 'damping off'. When the fungus responsible for this disease was grown in liquid culture containing 50 p.p.m. of betanin, growth was reduced by 50% (Kimler, in Mabry, 1980). Betalains have also been shown to inhibit IAA oxidase and can counteract the inhibitory effects of IAA on the elongation of wheat roots (Stenlid, 1976), but there was no indication that it plays a role as a regulator of IAA activity *in vivo*.

1.1.3 The Biosynthesis of Betalains.

A scheme for the biosynthetic pathway leading to the betalains (Fig. 1.2) has been suggested by Schütte and Liebisch (1985), based on results from tracer experiments with intact plants and plant parts. Judging by the structure of betacyanin and betaxanthin, it would seem

probable that both betalains share the same pathway. Furthermore, incorporation of radioactive tyrosine and L-DOPA (dihydroxyphenylalanine) into both type of betalains has been reported (Impellizzeri and Piattelli, 1972; Zryd *et al.*, 1982; Endress *et al.* 1984).

In addition to the betalains, radioactive tyrosine has also been incorporated into L-DOPA and cyclodopa (Zryd *et al.* 1982). The absence of tyrosine incorporation into L-DOPA in colourless cells (Constabel and Haala, 1968) provides some evidence, albeit negative for the existence of L-DOPA as an intermediate in betalain synthesis. Betalamic acid also occurs in a number of species of the Centrospermae, and is possibly an intermediate to all of the betalains (Kimler *et al.*, 1971). Chang *et al.* (1974) have provided further support to this suggestion by demonstrating the incorporation of labelled L-DOPA into betalamic acid. In a recent study with extracts from the mushroom *Amanita muscaria*, Terradas and Wyler (1991) have demonstrated the existence of a putative intermediate between L-DOPA and betalamic acid, i.e. 4,5 secodopa. However, in a similar study (Girod and Zryd, 1991), another intermediate was found to be α -hydroxy- γ -alanylmuconic ϵ -semialdehyde.

From the evidence available in the literature, it would seem that the reactions between L-tyrosine and L-DOPA and between DOPA and betalamic acid are the most important in

the biosynthesis of betalains (see reviews by Mabry, 1980; Piattelli, 1981; Böhm and Rink, 1988).

1.1.3.1 Formation of L-DOPA.

The entry point to the biosynthetic pathway leading to the betalains is through L-tyrosine, which is hydroxylated to L-DOPA probably by tyrosinase, (see Endress , 1977, 1979).

1.1.3.2 Formation of the dihydropyridine moiety.

According to the biosynthetic scheme shown in Fig. 1.2, one molecule of L-DOPA is converted into cyclodopa, a reaction possibly catalysed by a phenol oxidase. Constabel and Haala (1968) have shown that this enzyme group containing the phenoloxidases was more active in pigmented cells of *B. vulgaris* compared to colourless cells (Constabel and Haala, 1968). In parallel, another molecule of L-DOPA undergoes an extradiol ring cleavage between the 4 and 5 positions (Fischer and Dreiding, 1972; Impellizzeri and Piattelli, 1972; Chang *et al.*, 1974; Girod and Zryd, 1991; Terradas and Wyler, 1991), and is recycled to form the dihydropyridine moiety present in all betalains, via betalamic acid.

The reaction sequence between L-DOPA and betalamic acid has been studied in the mushroom *A. muscaria*. Terradas and Wyler (1991) found that 4,5 secodopa was an intermediate in the above step, and suggested that an enzyme similar to the metapyrochatechase found in bacteria and animals was responsible for this reaction. Meanwhile, Girod and Zryd (1991) have isolated the enzyme 3,4 DOPA 4,5 di^xoxygenase and shown that it catalyses the conversion of L-DOPA to α -hydroxy- γ -alanylmuconic ϵ -semialdehyde. These quite different results show the present state of uncertainty of how the betalains are synthesised.

1.2 BETALAINS AS A FOOD COLOURING AGENT.

1.2.1 The Use of Colour Additives in Food

Colour permeates various aspects of our lives, including the enhancement of the visual appeal of food. In fruits and vegetables, for instance, it signals freshness and readiness for consumption. In the UK, about 75% of all food supplies are processed before they reach the consumer (Timberlake and Henry, 1986), and the use of colouring agents is common. According to Timberlake and Henry (1986), manufacturers add colourants to foods for the following reasons:

- a. to make food attractive and hence stimulate the appetite and enhance enjoyment;
- b. to replace colour lost during processing;
- c. to provide colour to otherwise colourless or dull looking foods;
- d. to cover up faults and non-uniformity of appearance.

In general, the addition of colouring agents does not change the nutritional quality of processed foods, however, it does influence customers' preference and often leads to lasting first impressions.

Of all the food additives, none give rise to greater controversy than food colours (Timberlake and Henry, 1986). Concern about food additives has been voiced for some time and recently this concern has been heightened (eg. Busch, 1991; Harlander, 1991; Timberlake and Henry, 1986), bringing a response from the authorities. At the beginning of the century, 80 colourants were recommended in the USA, but only seven were approved. By 1987, only nine colourants were approved (Kassner, 1987). Many of the colourants previously used present a health hazard, and have been banned in certain countries, for example the FD & C Red No. 2 and No. 4 in the USA (Pasch and von Elbe, 1978). Among the currently permitted colourants in the UK, amaranth (E123), sunset yellow (E110) and tartrazine (E102) have also been associated with certain allergies and diseases and their use in foods has been discouraged or even banned in certain countries (Timberlake and Henry, 1986).

1.2.2 Food Colouring From Plants.

The fear aroused by the adverse effects of synthetic dyes has shifted attention to natural colours. In the period 1969-1984, 356 patents were issued on natural colourants and only 71 on synthetic dyes (Francis, 1987).

Plant pigments are a natural choice since they are isolated directly from plants and may be similar in cultures derived from that plant. Indeed, these naturally occurring pigments have been in use since early times. In spite of this, however, the toxicology of these compounds must be evaluated before approval can be given for use in foods. This is because some secondary metabolites might be toxic even at low concentrations, and therefore the risk involved must be weighed against the benefits of using them in food (Busch, 1991).

The major plant pigments used commercially today are carotenoids, anthocyanins, betalains, and chlorophylls. Some of the best known orange food colourants include annatto, saffron and gardenia. With a market value for food colourants estimated at 150-200 million US\$ in 1985 (Ilker, 1987), natural pigments from plant sources have a comfortable niche among the other food colourants.

1.2.3 Beetroot Red.

Beetroot red (E162) is a food colouring agent obtained from the storage root of *B. vulgaris*. The commercial preparation includes an extraction under acidic conditions and evaporating the extract to dryness. The resulting powder contains betacyanins and a lesser amount of betaxanthins (Timberlake and Henry, 1986). Beetroot red

is water-soluble and stable in the pH range 3 - 7. However, it tends to be degraded by heat, light and oxygen, and therefore is used mostly in short-life dairy products such as cured meat, ice cream, soft drinks and various kinds of dessert preparations (see Pasch and von Elbe, 1978; Weller and Lasure, 1981 and references therein; Timberlake and Henry, 1986).

1.3 PLANTS AS A SOURCE OF SECONDARY COMPOUNDS.

1.3.1 Secondary Metabolism and Secondary Products.

In all living organisms, primary metabolism is the common vehicle for the maintenance of life. The reactions are similar, and involve low molecular weight compounds of relatively simple structure. In contrast, secondary metabolism does not seem to be required by the organism for cell maintenance and growth, but may have an important function in the well-being of the plant and survival of the species. For example, plants are known to produce phytochemicals (eg. allelochemicals, phytoalexins) that determine the behaviour of other organisms interacting with them (see reviews by Haslam 1986; Harborne, 1990; Barz *et al.*, 1990).

The scope of secondary metabolism is vast and involves generally complex chemical structures. Secondary metabolism depends on primary metabolism for its input of intermediates, and it is not easy to distinguish with certainty between primary and secondary metabolites. However, there are certain features (Luckner, 1990), in addition to those described above, which are more characteristic of secondary metabolites, which includes the following :

- a. compounds are taxonomically restricted in distribution;
- b. enzymes, precursors, intermediates and products tend to be compartmentalised;
- c. the phase-dependent expression of secondary metabolism is an aspect of cell specialisation brought about by the integration in the programs of differentiation and development of the producer organism.

1.3.2. Secondary Products Useful to Man.

Since early times man has exploited the genetic diversity of the plant kingdom for his food, fibre, building material, and medicine supply. The domestication of plants which signals early civilisation has made the relationship between man and plant more intimate, and hence developed related practises such as selection, breeding and processing. Amongst these products from plants, is an array of secondary products which includes fine chemicals like pharmaceuticals, gums, dyes, food flavours, and fragrances. The production of these compounds, once solely by direct extraction from plants presents problems due to the difficulty and instability of supplies, has steadily been replaced by chemical synthesis (Fowler, 1987). However, most of the fine chemicals are complex structures and may be chiral molecules which are difficult or even impossible to synthesise chemically (Wink, 1990). Hence, about a quarter of prescriptions in

the USA and probably in Western Europe (Curtin, 1983; Phillipson, 1990; Fowler ~~et al~~, 1990) are derived from higher plants.

1.4 PLANT CELL CULTURES AS A SOURCE OF SECONDARY COMPOUNDS

1.4.1 The Potential of Plant Cell Cultures.

Biotechnology provides a new set of tools for improving the variety, productivity and quality of plant products. It provides a system that is free of seasonal, climatological and political intervention (Ilker, 1987). Plant cell culture, in particular, offers a more reliable and predictable source of secondary compounds than conventional means of production. The extraction of products from cultured cells is probably simpler than from non-uniform plant organs and, at least in theory, production is more easily controlled. In the general areas of natural products synthesis from plants (Fowler, 1987), plant cell cultures can contribute in three principal ways:

- a. as an alternative source of established products;
- b. as a source of novel active principles ('lead compounds') and enzymes;
- c. as a source of biotransformation systems for upgrading precursor molecules.

Despite the major advances made in the field of plant tissue and cell culture, the production of secondary metabolites from plant cell cultures has only been

successfully commercialised in one system, the production of shikonin from *Lithospermum erythrorhizon* cell cultures (see Fujita, 1988). Since then (1983), no other commercial system has been publicised although the production at an industrial scale of berberine from cell cultures of *Coptis japonica* has been studied and commercial production is anticipated. This inability to harness cultures for the commercial production of secondary compounds is mainly due to the high costs of production, exacerbated by low yield and a long period of production (Fowler, 1987; Fujita, 1988). Fowler (1987) has discussed the economics of secondary metabolite production, and suggested that it is possible to reduce production cost if cell growth rate, biomass yield and product yield can be increased, in addition to shortening the total process period (Reinhard^{et al.}, 1989; Lambie, 1990).

1.4.2 The Relationship between Growth and Development, and Product Accumulation.

The relationship between growth and secondary metabolism is an important aspect in studies involving plant cell culture (see Yeoman *et al.*, 1980; Lindsey and Yeoman, 1985; Yeoman, 1987^b; Komamine *et al.*, 1989). Growth rate and biomass yield, besides product yield, are important determinants for system productivity (Fowler, 1987). In most of the cases reported so far, growth is found to be

inversely related to secondary product formation and active secondary metabolite production occurs towards the end of the culture cycle, at the downturn of growth (see for example Phillips and Henshaw, 1977; Yeoman *et al.*, 1980, 1982, 1990). In several Solanaceous species, alkaloid accumulation occurs towards the end of the growth phase (Lindsey and Yeoman, 1983). In *Vitis* and *Portulaca* cell cultures, anthocyanin and betaxanthin accumulation respectively, were maximal at the stationary phase (Hirose *et al.*, 1990; Böhm *et al.*, 1991). This inverse relationship between growth and product accumulation has been explained in terms of a metabolic switch (Phillips and Henshaw, 1977; Yeoman *et al.*, 1980). In an environment where primary and secondary metabolism compete for common precursors, the slowing down of growth results in the channelling of the precursors into secondary metabolic pathways (Phillips and Henshaw, 1977; Lindsey and Yeoman, 1984, Lindsey, 1985, 1986; Hall and Yeoman, 1991).

However, there are also some cases in which growth and secondary metabolism are closely related. For example, podophyllotoxin, betacyanin, and carotenoid accumulation were maximal during the period of active growth in *Podophyllum*, *Phytolacca* and *Bixa* cell cultures, respectively (Kadkade, 1982; Sakuta *et al.*, 1986; Boyd, 1991). Reinhard *et al.* (1989) have also shown that the conversion of β -methyldigitoxin to β -methyldigoxin was

highest at the logarithmic growth phase and lower at the lag and stationary phases.

In a number of cell culture systems, secondary metabolite formation occurred during the lag phase, for example in the production of germichrysone in *Cassia* (Noguchi and Sankawa, 1982), phenolics in tobacco (Cvikrova *et al.*, 1988), ferruginol in *Salvia* (Miyasaka *et al.*, 1985) and carotenoids in *Bixa* (Boyd, 1991) cell cultures. Since there was no growth detected during this period, it is possible that this was an extension of the accumulation associated with the downturn of growth.

1.4.3 Differentiation and Secondary Metabolite Production.

Often, the accumulation of secondary metabolites in cell cultures is associated with biochemical and structural differentiation (Lindsey and Yeoman, 1985; Collin, 1987; Yeoman, 1987^b). In cell cultures of *Atropa* (Thomas and Street, 1970), *Papaver* (Kamimura and Nishikawa, 1976), carrot (Ozeki and Komamine, 1981), and *Digitalis* (Hagimori *et al.*, 1980), organogenesis seems to stimulate or induce secondary metabolite production. Indeed cardenolide synthesis in *Digitalis* cell cultures was enhanced during somatic embryogenesis (Kuberski *et al.*, 1984). Furthermore, Tabata *et al.* (1972) have shown that the

metabolic activity pertaining to the biosynthesis of tropic acid, a precursor of alkaloids was lower in dedifferentiated cells. All these observations support the view that secondary metabolism may be triggered by cell differentiation, specialisation or organisation. A similar situation might also prevail in immobilised cell cultures, in which it was suggested (Yeoman *et al.*, 1982) that conditions conducive to secondary metabolite formation in whole plant were mimicked.

However, in some cultures, secondary metabolites are produced at a higher level in poorly differentiated and disorganised cells. Steroid and sapogenin accumulation, for example, was higher in undifferentiated cells than in cultures differentiating roots (Kaul and Staba, 1968) or bulbils (Sharma and Khanna, 1980). Tissue specialisation in these cultures does not appear to be a prerequisite for secondary product accumulation. Furthermore, in *Atropa* and *Hyoscyamus* cell cultures (Collinge, 1987) no correlation was detected between cell differentiation and tropane alkaloid production.

1.5 INCREASING THE YIELD OF SECONDARY PRODUCTS IN PLANT CELL CULTURES.

The overall yield of a production system as discussed by Fowler (1987), depends on the growth rate, biomass yield and product yield. In fact, the success of shikonin production (Fujita, 1988) by *Lithospermum erythrorhizon* cell cultures was partly due to the ability to maximise all of these criteria.

1.5.1 Selection of High-Yielding Cell Lines.

The choice of plant material is likely to be important in determining the yield capacity of cell cultures. Cell lines derived from high-yielding plants are known to produce higher levels of secondary metabolites than low-yielding plants (Zenke ~~et al~~ 1977; Kinnersly and Dougall, 1980b). Although this is not a generally established phenomenon, it is certainly worth further consideration.

The fact that cell cultures are derived from tissues that may contain cells of a different genetic make up, and give rise to cultures displaying genotypic variation (Yeoman and Forche, 1980); provides an opportunity for selecting cells with particular characteristics such as the over-production^t of a particular secondary product. Selection of cell lines from callus, cell suspensions and

protoplasts have been successfully exploited for the improvement of secondary product yield (see Tabata *et al.*, 1978; Fujita, 1988; Collin and Dix, 1990). Yamamoto and Mizuguchi (1982) succeeded in obtaining a high producing cell line of *Euphorbia millii* by recurrent selection of highly pigmented cell aggregates. A similar technique was employed with cell cultures of *Lithospermum* (Fujita and Tabata, 1987), *Coptis* (Fujita, 1988) and *Ipomoea* (Nozue *et al.*, 1987) cell cultures. These selection procedures were possible because the compounds of interest were coloured.

An important aspect of the cell line selection procedure, is to maintain a stable level of metabolite production in the clones. In heterogeneous cell culture systems, productivity tends to diminish with time, probably due to a selection pressure which favours growth, and which gradually leads to the elimination of the originally dominant producing cells. This phenomenon has a parallel in crop breeding, in which traits like disease resistance might be lost when emphasis is given to increasing yield. However, selection of high-producing cell lines from cell culture systems has been shown to be successful in restoring the original productivity of the parent line (for example Zenk *et al.*, 1977; Morris *et al.*, 1989).

It may now be possible to promote high yields of secondary metabolites by the use of genetically engineered plant material. Genetic engineering and recombinant DNA

technology is a powerful tool, and although its use in the field of secondary metabolite production has not been widely studied, the potential seems to be real. The introduction and expression of foreign genetic material has been shown to affect the biosynthesis of phenylpropanoids (Liang *et al.*, 1989) and protoalkaloids (Songstad *et al.*, 1990, 1991) in tobacco plantlets. The use of antisense procedures (Mol *et al.*, 1990) also may make it possible to block the formation of product-degrading enzymes, or to suppress the activity of enzymes competing for common precursors, thus eliminating side chain reactions. Another suggested approach is to modify the promoter sequence of developmentally regulated genes so that they would always be active and the product would be synthesised continuously (Yeoman *et al.*, 1990).

1.5.2 Manipulation of the Culture Conditions.

In order to harness the full synthetic potential of a cell line, it is necessary to provide an optimum environment. Here, the most important criterion is to enable the expression and efficient operation of the specific biosynthetic pathway leading to the compound of interest.

Foremost on this agenda is the choice of basic medium. Over the years, many media formulations have been developed, most of them aimed at maximising growth. Therefore these media might not be the best for secondary metabolite production, and therefore some adjustments might be required to the level of individual components.

The effects of various medium components on cell growth and secondary metabolite production have been reviewed by Dougall (1980) and Mantell and Smith (1983). A few studies on the evaluation of individual components have also been reported (Kato *et al.*, 1977; Fujita *et al.*, 1981; De-Eknamkul and Ellis, 1985). By combining results from experiments on the optimisation of individual media components, an 'optimum' medium can be obtained, for example in *Lithospermum* cell cultures (Fujita *et al.*, 1981). However, this approach has not always been successful, for example in *Anchusa* cultures (De-Eknamkul and Ellis, 1985) where changes in the concentration of individual components at the same time, results in a lower

yield. It is likely therefore, that it is the balance and interactions between the media components which are important and these should be worked out for each individual system.

In some of the cell cultures of betalain-producing plants it has been reported that betalain was not produced on certain media. For example, callus of *Portulaca grandiflora* remained colourless on modified ^{MS} (Nagata and Takebe, 1971) and SH media, but when cultures were grown on White's and MS media, betacyanin formation was detected.

The most important nutrients, and those that have received most attention are carbohydrate (sugar), nitrogen (nitrate and ammonium), and phosphate. In general, the effects on secondary metabolism brought about by these nutrients are associated with growth and cell division. However, more specific roles of the medium components have been reported, for example nitrogen sources can influence the pH of the medium (Wetherell and Dougall, 1976), and the effects of cations such as Mg^{2+} on enzymatic reactions (Hagimori *et al.*, 1983).

Sucrose apart from its role as a carbon source also influences the osmotic potential of the medium (Rudge and Morris, 1986; Do and Cormier, 1991), and this can result in an increase in secondary product accumulation.

In *Chenopodium rubrum* cell cultures (Berlin *et al.*, 1986), the effect of a range of sucrose concentrations (2-10%) was tested, and maximal betacyanin occurred at a concentration of 2%. Meanwhile, Sakuta *et al.* (1987) have reported that betacyanin accumulation increased with the concentration of sucrose up to 88 mM, and no further increase occurred at higher concentrations. This result is in agreement with the suggestion made by Wohlpert and Black (1973) that a certain level of sucrose is needed for betacyanin formation and that above this level sucrose has little effect on betacyanin accumulation.

The source of nitrogen is also critical in some cases, for example shikonin production was inhibited by ammonium, and therefore only nitrate was used with *Lithospermum* cell cultures (Fujita *et al.*, 1981). In addition, Ikeda *et al.* (1976) have shown that good growth and ubiquinone formation were observed when nitrate was used as the sole nitrogen source. In *Portulaca grandiflora* cell cultures, replacement of ammonium with nitrate increased betacyanin accumulation substantially (see Böhm and Rink, 1988). On the other hand, the ratio of ammonium to nitrate has been shown to be important in determining growth and secondary metabolite accumulation. Ikeda *et al.* (1976) have found that a higher ammonium to nitrate ratio increased accumulation and reduced growth. On the contrary, Fujita *et al.* (1981) showed that decreasing the ammonium concentration increased yield of shikonin-derivatives but

reduced growth in cultures of *Lithospermum*. In *Vitis* cell cultures (Yamakawa *et al.*, 1983), anthocyanin production was maximal when the molar ratio of $\text{NH}_4\text{-NO}_3$ was 1:1. These workers have further shown that the C:N ratio was also an important factor in anthocyanin production. In *Phytolacca americana* cell cultures, increasing total nitrogen within the range 0-40mM (ratio of $\text{NH}_4\text{-NO}_3$ is 1:2) resulted in increases in betacyanin accumulation (Sakuta *et al.*, 1987^a). Increasing ammonium level enhanced growth but reduced betacyanin level, while increasing nitrate level increased betacyanin, without a significant effect on growth. In earlier studies, ammonium and nitrate levels higher than those of the original B5 medium changed the growth rate (Berlin *et al.*, 1986) but not the betacyanin concentration in cell cultures of *B. vulgaris* (Constabel and Nassif-Makki, 1971) and *Chenopodium rubrum* (Berlin *et al.*, 1986).

The effects of phosphate have been studied in a range of species, for example, by Knobloch and Berlin (1981), Nettleship and Slaytor (1974) and Lindsey (1985) and it has been shown that phosphate limitation induced secondary metabolite accumulation. In cells of *B. vulgaris* (Constabel and Nassif-Makki, 1971), the elimination of phosphate resulted in increased pigment formation. However, the same condition applied to cell cultures of *Chenopodium rubrum* (Berlin *et al.*, 1986) and *Phytolacca americana* (Sakuta *et al.*, 1986) significantly reduced

betacyanin content. Subsequent addition of phosphate to the medium increased the betacyanin level.

Among the micro-nutrients, Cu^{2+} has been shown to inhibit betacyanin accumulation in callus of *Portulaca grandiflora* but acted differently in MS and White's media. This component is considered by Berlin *et al.* (1986) as a co-factor rather than as a nutrient.

Plant growth regulators (PGRs), particularly the auxins and cytokinins are important components of the culture medium. Although there are a number of cultures which are independent of PGRs (for example Berlin *et al.* 1986), the type and concentration, and the balance of auxin and cytokinin is often critical in determining secondary metabolite production (see reviews by Dougall, 1980; Mantell and Smith, 1983; Fowler, 1990). Constabel and Nassif-Makki (1971) observed no betacyanin formation in callus of *Beta vulgaris*, *Amaranthus caudatus*, and *Celosia argentea* when grown on medium containing 1 mg.l^{-1} 2,4-D, but the reduction and elimination of 2,4-D led to pigment formation. Further, they have shown that substituting 2,4-D with NAA induced pigment formation in all the species investigated, and the increase in pigmentation was paralleled by a decrease in growth rate. In addition, the addition of GA_3 led to root formation and enhanced betacyanin accumulation. McCormick (1972) has also reported that root and betacyanin formation occurred

simultaneously when cultures of *B. vulgaris* were transferred from an auxin-containing medium to an auxin-free medium. In contrast, he has also shown that in *Phytolacca americana*, differentiation of roots did not result in betacyanin formation. Meanwhile, the important effect of cytokinin (kinetin) on betalain production was shown in dark-grown seedlings of *A. retroflexus* (Bamberger and Mayer, 1960) and *P. americana* cell cultures (Endress, 1976), where betacyanin accumulation was observed. Recently, Girod and Zryd (1991^b), have shown that by changing the ratio of auxin to cytokinin, the phenotypic character (colour) of *B. vulgaris* cells could be altered. They have suggested that PGRs act as indicator signals that induce changes in the secondary metabolic pathways of cultured plant cells.

In addition to the usual nutrients, precursor feeding (Brodelius and Nilsson, 1980; Lindsey and Yeoman, 1984) and the use of elicitors (Eilert et al., 1987) have also been shown to induce and direct the biosynthetic pathways leading to the formation of desired compounds. Precursors have been used in studies involving the effects of light on betalain synthesis (for example French et al., 1973; Giudici de Nicola, 1974), and in studies to determine the biosynthetic pathway leading from tyrosine to DOPA, and finally to the betalains (for example Zryd et al., 1982), but specific studies on the use of precursors for increasing betalain production are not known.

Amongst the physical conditions that can be manipulated for increasing yield are, light, pH, temperature and aeration. Light is known to affect the production of secondary metabolites (see review by Mantell and Smith, 1983; Towers and Yamamoto, 1985). Contrasting effects of light on the production of secondary metabolites have been reported, for example the inhibition of nicotine production in tobacco cultures (Ohta and Yatazawa, 1978), and the stimulation of anthocyanin production by *Haplopappus* cultures (Stickland and Sunderland, 1972). Perhaps surprisingly, light is not an essential prerequisite for the production of betalains by most plants and cell cultures, however, pigment production is generally higher in seedlings (Wohlpert and Mabry, 1968; Rast et al., 1972) and in cell cultures (Berlin et al., 1986) exposed to light.

However, in some culture systems, light does seem to be necessary, for example pigmented cultures cells of *Portulaca grandiflora* (Liebisch and Böhm, 1981) and *Beta vulgaris* (Girod and Zryd, 1985) became colourless when transferred to the dark, and pigment reappeared upon subsequent transfer back to the light. In seedling leaf tissue, Wohlpert and Black (1973) observed an enhanced betacyanin content in tissue pretreated with a high light intensity than with a low light intensity. They suggested that photostimulation is realised by the activation of genes coding for the enzymes involved in pigment

biosynthesis. In *A. caudatus* seedlings light stimulates betacyanin accumulation and the activity of PAL, the enzyme responsible for the conversion of L-phenylalanine to cinnamic acid, both co-substrates of betalain synthesis (Woodhead and Swain, 1974).

Surprisingly, callus of *P. grandiflora* was reported to incorporate ^{14}C tyrosine into betacyanin more in the dark than in the light (Endress et al., 1984). Further evidence of light independent betalain synthesis is provided from work with for example, *Amaranthus retroflexus* (Bamberger and Mayer, 1960) and *Chenopodium rubrum* (Berlin et al., 1986). However, in the latter case betacyanin accumulation was stimulated by blue light. Based on observations on the variegation intensities of callus of *Beta vulgaris* Girod and Zryd (1987) have suggested that light acts only as a revealing factor in betalain formation, and that the determining factor is an intrinsic property of the cells.

pH of the medium can influence uptake of nutrients and permeability of membranes (Butenko, 1968). The effects of pH on the production of secondary metabolites is exemplified by *Ipomoea* cultures accumulating tryptophol (Veliky, 1977).

The effects of temperature on product accumulation have not received much attention, except in a few studies, for example in *Catharanthus* cell cultures (Fowler, 1988), a dramatic effect on growth and product accumulation was observed in cultures grown under different temperature regimes.

Last but not least among the components of the physical environment, perhaps, is the aeration and mixing of cells. This is vital, especially in large-scale production systems in fermentors or bioreactors (see reviews by Mantell and Smith, 1983; Fowler, 1988), but does not present problems in the shake flasks used in this study.

The nature of cell aggregation/dispersion also determines the metabolic performance in certain systems. Whilst some cultures are able to produce secondary compounds in fine suspensions, there are reports on the beneficial effects of cell aggregation (for example Kuboi^{and Yamada}, 1978a,b). In relation to this, cell immobilisation (Lindsey and Yeoman, 1986) can contribute substantially to product yield. Under these conditions, there is a high cell to cell communication, and favourable conditions for secondary metabolite production mimicking the situation found in the plant.

1.6 AIMS AND OBJECTIVES.

The aim of this project was to study the production of betalains in cell cultures of *Beta vulgaris*, in order to begin to understand the regulation of betalain accumulation. In order to achieve this the following objectives must be fulfilled:

1. To establish cultures from three cultivars of *B. vulgaris*.
2. To determine the pigment composition and select a suitable cultivar for detailed studies.
3. To determine the effects of cultural conditions on betalain accumulation.
4. To determine the relationship between growth and betalain synthesis and accumulation.
5. To determine the effects of light on synthesis and accumulation of the betalains.

CHAPTER TWO
MATERIALS AND METHODS

2.1 PLANT MATERIAL.

The varieties used in this investigation were Boltardy, Burpee's Golden and Albina veredunda, the red, yellow and white varieties, respectively, of the table beet, *Beta vulgaris* L. Seeds were obtained from Thompson and Morgan (Ipswich) Ltd., England. Callus and suspension cultures of variety Boltardy were a kind gift from Dr. C.S. Hunter of the Plant Sciences Group, Bristol Polytechnic.

2.2 TISSUE AND CELL CULTURE.

2.2.1 Preparation of Culture Media.

Four basal media were used in this investigation. They were Gamborg's B5 (B5) medium (Gamborg *et al.*, 1968), Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) and White's (W) medium (White, 1963). The constituents of these media are shown in Table 2.2.1.

The media were prepared from powdered mixtures obtained from Imperial Laboratories Ltd., Twyford, England, and stored at 5°C. An appropriate amount of the mixture (Table 2.2.2) was dissolved in distilled water. Sucrose

COMPONENTS	AMOUNT (mg.l ⁻¹)			
	B5	MS	SH	W
Macro-nutrients:				
CaCl ₂ .2H ₂ O	150.00	440.00	200.00	-
Ca(NO ₃) ₂	-	-	-	208.50
KCl	-	-	-	65.00
KH ₂ PO ₄	-	170.00	-	-
KNO ₃	3000.00	1900.00	2500.00	80.00
MgSO ₄ .7H ₂ O	250.00	370.00	400.00	720.00
Na ₂ EDTA	-	-	20.00	-
NaH ₂ PO ₄ .2H ₂ O	169.60	-	-	18.70
NH ₄ NO ₃	-	1650.00	-	-
(NH ₄) ₂ SO ₄	134.00	-	-	-
NH ₄ H ₂ PO ₄	-	-	300.00	-
Micro-nutrients:				
FeNa EDTA	40.00	36.70	-	4.59
FeSO ₄ .7H ₂ O	-	-	15.00	-
CoCl ₂ .6H ₂ O	0.025	0.025	0.10	-
CuSO ₄ .5H ₂ O	0.025	0.025	0.20	0.001
H ₃ BO ₃	3.00	6.20	5.00	1.50
KI	0.75	0.83	1.00	0.75
MnSO ₄ .4H ₂ O	13.20	22.30	13.20	7.00
MoO ₃	-	-	-	0.001
Na ₂ MoO ₄ .2H ₂ O	0.25	0.25	0.10	-
ZnSO ₄ .7H ₂ O	2.00	8.60	1.00	3.00
Organic constituents:				
Glycine	-	2.00	-	3.00
i-inositol	100.00	100.00	1000.00	-
Nicotinic acid	1.00	0.50	5.00	0.50
Pyridoxine HCl	1.00	0.50	0.50	0.10
Thiamine HCl	10.00	0.10	5.00	0.10

MEDIUM				
	B5	MS	SH	W
AMOUNT (g.l ⁻¹)	3.874	4.71	4.463	1.31

(FSA Laboratory Supplies, Loughborough, England) was added to the medium at 20 g.l^{-1} , as a carbon source. Plant growth regulators, Kinetin (0.1 mg.l^{-1}) and 2,4-D (0.02 mg.l^{-1}) (both supplied by Sigma Chemicals Ltd., U.K) were then added in the form of stock solutions. The pH of the media was adjusted to 5.8 with M KOH and M HCl (both from BDH Ltd., Poole, Dorset, England). The media were then dispensed into 250 ml and 100 ml Erlenmeyer flasks (Pyrex, England) at 50 ml and 25 ml, respectively, and the flasks covered loosely with a double layer of aluminium foil before autoclaving.

For solid medium, 0.6% (w/v) Bacto agar (Difco Laboratories, Detroit, U.S.A.) was added to the medium after the pH was adjusted. After autoclaving the medium was cooled to about 60°C , and then poured into 50 mm disposable Petri dishes (Bibby Sterilin Ltd., Staffs, U.K.) and left to solidify in the air-flow cabinet before use.

2.2.1.1 Preparation of kinetin and 2,4-D stock solutions.

Stock solutions of 100 mg.l^{-1} kinetin and 2,4-D were prepared by dissolving 0.01 g of kinetin and 2,4-D separately into 5 ml of 0.1 M KOH and the solution made up to 100 ml with distilled water. The stock solutions were

kept at 5°C in a refrigerator, and replaced every 3 months.

2.2.2 Sterilisation Techniques.

2.2.2.1 Sterilisation by moist heat.

All media, distilled water, glassware and nylon sieves were sterilised by moist heat (steam) in an autoclave at 121°C and 103.4×10^3 Pa. The process time was 20 min, except for liquid volumes greater than 250 ml, when the process time was as shown in Table 2.2.3. All items were covered or wrapped up in aluminium foil prior to autoclaving.

2.2.2.2 Sterilisation with ethanol.

All work surfaces in the air-flow cabinets were swabbed down with absolute ethanol before being used, together with external surfaces of culture vessels and other glassware.

TABLE 2.2.3.

Autoclaving time for liquid (Biondi and Thorpe,1981).
Liquid in larger volumes takes a little longer for the innermost regions to reach the sterilising temperature of 121°C. However, once this temperature is attained, it is maintained for a longer time due to the slower rate of cooling down.

Vol of liquid per container (ml)	Minimum time at 121°C .
75	20
250-500	25
1000	30
1500	35
2000	40

2.2.2.3 Sterilisation with ethanol and flaming.

Instruments such as forceps, scalpels and spatula spoons were immersed in absolute ethanol. They were flamed briefly and cooled down for a few seconds before making contact with sterile items.

2.2.2.4 Sterilisation with sodium hypochlorite.

To obtain sterile seedlings, seeds were sterilised according to the method of Reinert and Yeoman (1982) by immersing in a solution of 20% (v/v) sodium hypochlorite (10-14% available chlorine - A & J Beveridge Ltd., Edinburgh) containing 0.05% (v/v) Tween-80 (Sigma Ltd., Poole, Dorset, England). The seeds were first placed in a glass tube (25 x 250 mm) covered at the lower end with muslin cloth and trapped by a piece of nylon mesh placed towards the middle of the tube to prevent them from floating. The seeds were agitated periodically over a 10 min period to facilitate contact with the sterilant, and then rinsed five times with sterile distilled water.

2.2.3 Sterile Technique.

Sterilised plant materials, cultures, nutrient media and instruments were handled using aseptic techniques and all aseptic manipulations were carried out in an air-flow cabinet. Hands were cleansed and rubbed with a disinfectant, Hibisol hand-rub (ICI Pharmaceuticals, U.K) before starting aseptic work.

2.2.4 Decontamination and Cleaning of Glassware and Equipment.

Contaminated cultures were autoclaved at 121°C and 103.4×10^3 Pa for 30 minutes to kill all micro-organisms, before being cleaned. All glassware and equipment was first washed under running tap water, and then soaked overnight in a 2% (v/v) concentrate of R.B.S 25 (Chemical Concentrates [Ltd], Kent England) solution. They were then brushed clean and rinsed three times under running tap water, twice with distilled water, before being left to dry in the hot-air oven or at room temperature.

Glassware and equipment used for handling radioisotopes were first soaked in a 2% (v/v) of R.B.S 25 solution for 48 h, washed thoroughly under warm, running tap water and then further soaked and cleaned as described above.

2.2.5 Initiation and Maintenance of Cultures.

2.2.5.1 Culture conditions.

Cultures were kept in an air-conditioned growth room maintained at $25 \pm 1^{\circ}\text{C}$ and a R.H. of 80%. Lighting, either at $10 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ or $20 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ was provided continuously by Thorn natural light fluorescent tubes (Thorn EMI, U.K.). Liquid cultures were agitated on an orbital shaker at a speed of 98 r.p.m. with an amplitude of 8 mm. Dark-grown cultures were kept in black plastic boxes placed alongside the light-grown cultures on the shaker.

2.2.5.2 Initiation of callus cultures.

Seeds were sterilised according to the procedure described in Section 2.2.2.4. Prior to this, the seeds were scarified by rubbing between two pieces of sand paper to remove the dead tissues which harboured a multitude of micro-organisms. After sterilisation, the seeds were placed on solid B5 medium containing 0.1 mg.l^{-1} kinetin and 0.02 mg.l^{-1} 2,4-D. The Petri dishes were sealed with a double layer of parafilm (American National Can, Greenwich, U.S.A) to exclude micro-organisms and to prevent desiccation. Seedlings emerged after 4-21 d and were ready to be used as explants after a further 6-8 w.

Segments of the hypocotyl, petiole and leaf blade, 2 mm in length, were excised from the seedlings using sterile forceps and a scalpel, and inoculated onto solid B5 medium. The Petri dishes were sealed with a double layer of parafilm. Callus developed in 2-4 w and was large enough to be transferred to fresh medium after a further 6-8 w.

2.2.5.3 Initiation of suspension cultures.

Suspension cultures were initiated from friable callus. About 3 g fresh weight of callus was added to 50 ml of liquid medium contained in a 250 ml flask. The callus was broken into small pieces using a perforated spatula spoon, and the flasks covered with a double layer of aluminium foil. The cultures were then agitated continuously on a shaker as described in section 2.2.5.1. A high density of inoculum is helpful in the early establishment of the cultures.

2.2.5.4 Maintenance of callus and cell suspension cultures.

Callus cultures were maintained on solid B5 medium supplemented with 0.1 mg.l^{-1} kinetin and 0.02 mg.l^{-1} 2,4-D. They were sub-cultured by transferring pieces ca. 1 cm in diameter using sterile forceps every 3 to 4 w.

Suspension cultures were maintained in liquid B5 medium as above and were sub-cultured every 14 d by transferring ca. 2 g of cells per 250 ml flask using a sterile perforated spatula spoon. Cells in suspension became very aggregated during culture. Hence, it was necessary to pass the cell aggregates through a 2 mm nylon mesh every 2-3 sub-cultures to maintain uniform cultures. All aseptic manipulations were carried out in an air-flow cabinet under aseptic conditions. Manipulations involving dark-grown cultures were performed under a safelight (Lee HT 139 primary green filter, LEE Colortran International, London). These cultures were maintained in black plastic boxes or wrapped in two layers of aluminium foil.

2.3 RADIOLABELLING OF SUSPENSION CULTURES.

Suspension cultures were injected with 20 μ l (1 μ Ci) of ^{14}C -(U)-L-Tyrosine (Sigma Chemical Co. Ltd., U.K.) under aseptic conditions and the cultures returned to the shaker. After a 24 h labelling period the cultures were harvested for analysis.

2.4 MEASUREMENT OF CULTURE GROWTH.

2.4.1 Determination of Culture Fresh Weight.

Culture fresh weight is a more accurate measurement of growth than PCV. It was used to calculate the total metabolite accumulation in this study. Cell suspensions were vacuum-filtered through a Whatman No. 1 filter paper using a Buchner funnel. The weight of the culture was taken immediately.

2.4.2 Determination of the Cell Population Density.

The cell population density, or simply the cell number, is useful in showing the kinetics of cell multiplication in a culture. The cell number was determined according to the method of Reinert and Yeoman (1982) using a Hawksley

crystallite haemocytometer (grid volume 1.8 μ l). 100 mg of freshly harvested cells were suspended in 2 ml of a solution containing 12% (w/v) chromium trioxide and 10% (v/v) HCl (both chemicals supplied by BDH Ltd., Poole, England) in 10 ml plastic centrifuge tubes. The cells were disrupted gently with a glass rod to break up large aggregates and left at room temperature for 4 h. They were then incubated in a water bath at 60°C for 20 min. Finally, the suspensions were agitated vigorously on a vortex mixer for 15 s before counting under a microscope. Six grids were counted for each sample and the cell number calculated according to the following formula:

$$\text{Cell No} = \frac{\text{Vol of macerate} \times \text{Cell count}}{\text{Vol above grid}}$$

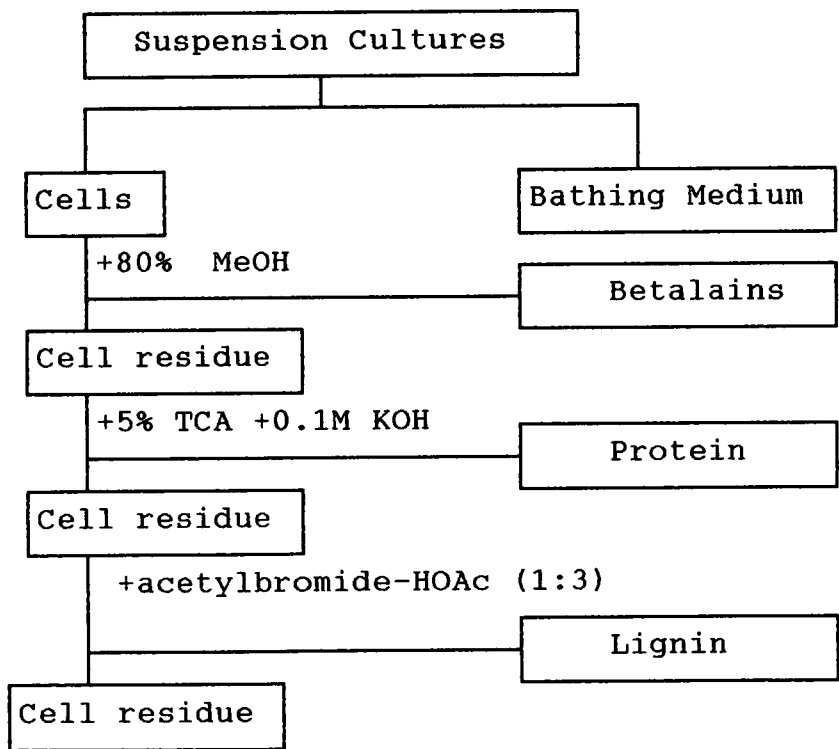
Samples were diluted with distilled water when the count per grid was greater than 200, to ensure good counting efficiency.

2.5 ANALYTICAL TECHNIQUES

2.5.1 Extraction of Betalains and Tyrosine-derived Compounds.

The extraction of betalains and other components of the culture, except lignin, was conducted at room temperature under normal light condition. However, samples were put on ice and kept away from light between operations to prevent compound degradation. A scheme for the extraction procedure is shown in Fig. 2.5.1.

FIG. 2.5.1.
A Scheme for the Extraction of Compounds of Interest.



2.5.1.1 Extraction of betalains from cells.

Freshly harvested cells were homogenised in ca. 2 ml of 80% methanol (MeOH) with a pestle and mortar. The homogenate was then poured into a 50 ml centrifuge tube together with the washings from the pestle and mortar. The volume was made up to ca. 10 ml, and the tubes centrifuged at 2000 xg for 10 min. The pellet was reextracted twice more with ca. 10 ml of 80% MeOH and centrifuged as above. The combined extracts were collected in a 250 ml round-bottom flask. The extract was then dried down *in vacuo* at 35°C using a rotary evaporator and the residue dissolved in 1 ml distilled water and centrifuged in an Eppendorf tube at ca. 12,000 xg for 5 min to remove solid impurities. The supernatant was then placed in a 2 ml amber serum bottle sealed with an aluminium crimp top (both from Anachem, Scotland), and saturated with nitrogen prior to storage at -40°C.

2.5.1.2 Extraction of protein from the cells.

Cell residue after methanol extraction (section 2.5.1.1) was left to stand overnight in 10 ml of 0.1 M KOH at room temperature to dissolve the protein. The cell wall material was then spun down at 3,000 xg for 15 min, and kept for further analysis. The supernatant containing the protein was treated with 10 ml of 5% (w/v) TCA, and left

standing on ice to allow the protein to precipitate completely. The protein was then spun down at 3,000 xg for 15 min as above, and the supernatant discarded. This protein pellet was subsequently dissolved in 10 ml of 0.1 M KOH and kept in the fridge at 5°C before subjecting the solution to scintillation counting.

2.5.1.3 Extraction of lignin from the cells.

The cell residue remaining after protein extraction (section 2.5.1.2) was washed with 80% MeOH and then dried in an oven at 60°C for ca. 24 h in a 15 ml screw-capped test tube. The extraction procedure was carried out in a fume cupboard according to the method of Johnson *et al.* (1961). Five ml of acetylbromide-acetic acid (1:3) solution was added to each sample in the screw-capped test tube and the lid put on loosely. The samples were then incubated in a water bath at 70°C for 30 min with frequent agitation. They were then left to cool down to room temperature. Forty five ml of 2 M NaOH was then added to neutralise the excess acetylbromide. Finally, 0.5 ml of 7.5 N hydroxylammonium chloride was added to each sample to remove the bromine and polyamide. The solution was kept in the fridge at 5°C before being subjected to scintillation counting.

2.5.2 Analysis of Betalains by Spectrophotometry.

The absorption spectra of the cell extracts and the optical densities (OD) of betacyanin and betaxanthin were determined using a Pye-Unicam SP8-100 and a Beckman DU-64 spectrophotometer. Betacyanin was measured at a wavelength of 535 nm and betaxanthin at 477 nm (see Fig. 2.5.2). Betacyanins and betaxanthins are the two major groups of pigments routinely obtained from these cell cultures.

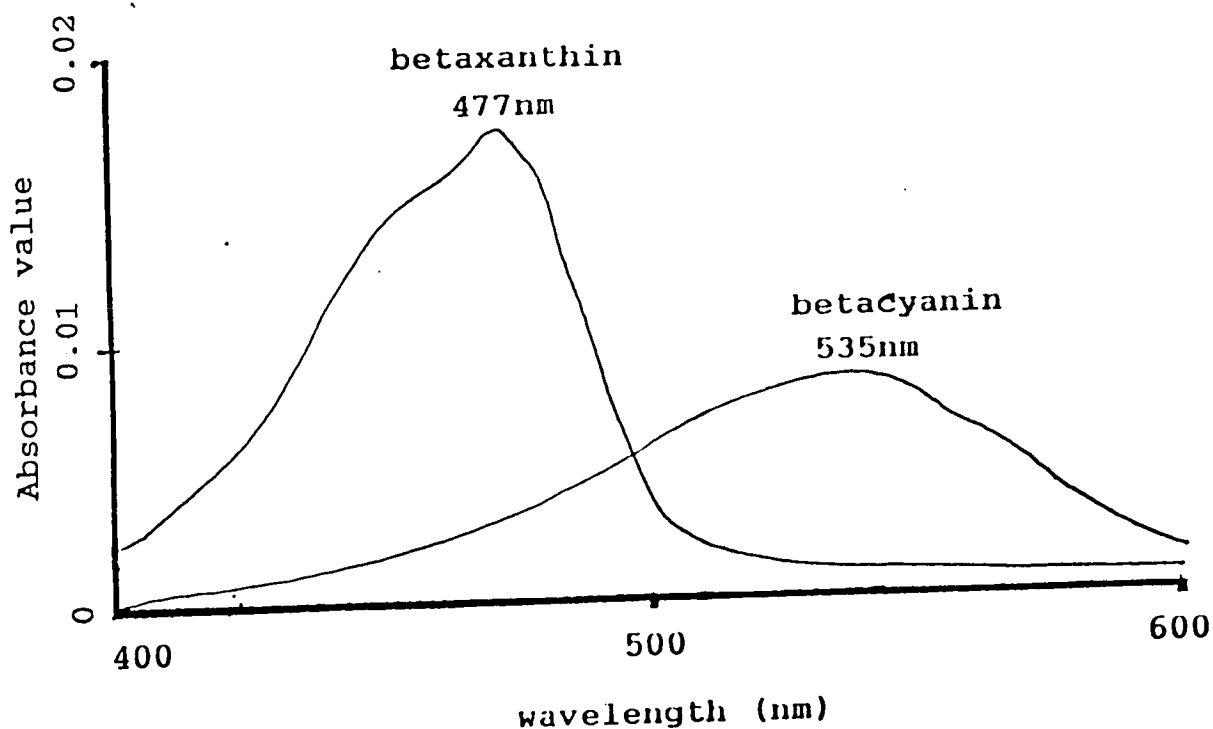
2.5.3 Analysis of Betalains and Amino Acids by Thin-Layer Chromatography (TLC).

2.5.3.1 One-dimensional thin-layer chromatography.

Betalains and amino acids were separated according to the simplified method of Bilyk (1981). The methanol extracts were spotted onto a cellulose-coated aluminium plate (Merck, Darmstadt, Germany) and run up to 15 cm in a solvent mixture consisting of isopropanol-ethanol-water-acetic acid (6:7:6:1). The chromatograms were then dried under a stream of nitrogen and stored in the dark at 5°C. Coloured spots/bands of the betalains were readily visible under white light (Fig. 2.5.3).

FIG. 2.5.2.

An OD scan showing the absorption maxima for betacyanin and betaxanthin. The pigments were separated on TLC plates (2.5.3.1), and the spots scraped out, and eluted by dissolving them in distilled water, centrifuging at 10, 000 x g for 5 min, and finally, subjecting the supernatant to spectrophotometric scanning.



2.5.3.2 Detection of p-coumaric acid and L-tyrosine on TLC plates using uv light.

p-coumaric acid was readily visible under uv illumination as a dark purplish spot running close to the solvent front, whilst L-tyrosine appeared as a dark spot with an R_f of 0.4.

2.5.3.3 Detection of L-tyrosine on TLC plates using ninhydrin

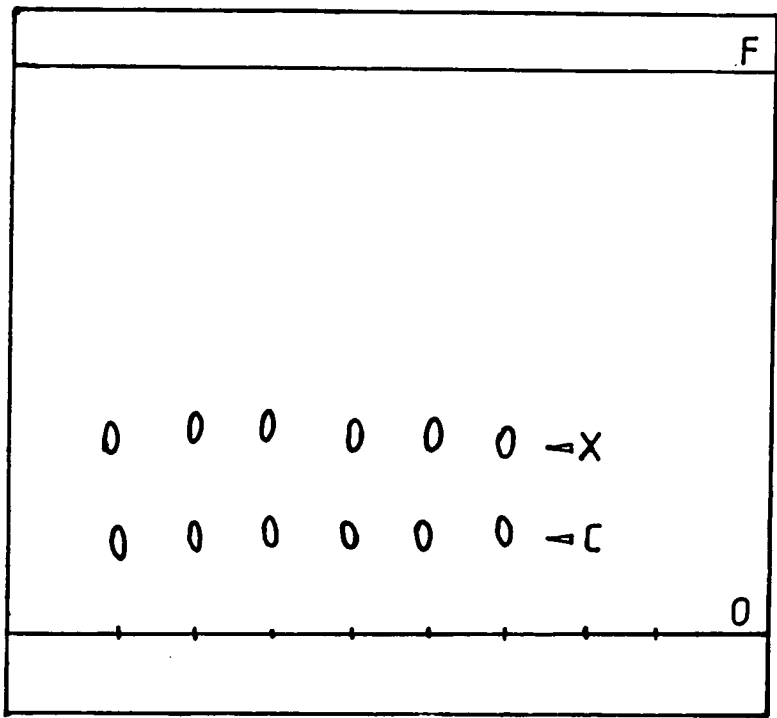
Ninhydrin spray reagent containing 0.2% (w/v) Ninhydrin in EtOH was also used to detect the betalains and other amino compounds. Ninhydrin reacts with the amine groups producing purple spots which appear after the plates have been dried at 110°C for ca 10 min.



FIG. 2.5.3.

Separation of betacyanin and betaxanthin by TLC (see 2.5.3) on cellulose.

X: betaxanthin F: solvent front
C: betacyanin O: origin



2.5.3.4 Autoradiography.

Chromatograms from TLC (section 2.5.3.1) were dried under a stream of nitrogen and autoradiographed in order to locate the radioactive compounds and to determine the distribution of radioactivity after feeding the cells with ^{14}C -(U)-L-tyrosine. Autoradiography was carried out by placing an 18 x 24 cm sheet of Hyperfilm-MP (Amersham, U.K.) on the chromatograms previously positioned in a light-proof cassette. This operation was carried out under safelight (Ilford 914). The cassette was sealed and labelled, then kept at -70°C . After 2 w of exposure to the radioisotope the cassette was taken out to allow the film to reach room temperature before being developed using a Gevamatric 60 automatic developer (Agfa-Gevaert, Germany).

2.5.3.5 RITA.

RITA (Radioactivity Intelligent Thin Layer Analyser) was used for rapid measurements of radioactivity in one-dimensional TLC. RITA scans were carried out on an Isomess IM-3000 Radio-TLC-Analyser (Nuclear Interface).

2.5.4 Determination of Radioactivity in Compounds by Liquid Scintillation Counting

Quantitative analysis of radioactivity in the biosynthetic products was carried out by liquid scintillation counting using an Intertechnique SC3000 scintillation counter.

2.5.4.1 The scintillation fluid.

The scintillation fluid or cocktail was made up of 0.61% (w/v) butyl-PBD in toluene-Triton X (2:1) (both chemicals from BDH, Poole, Dorset, U.K.). This was kept in a dark bottle and prepared fresh before use.

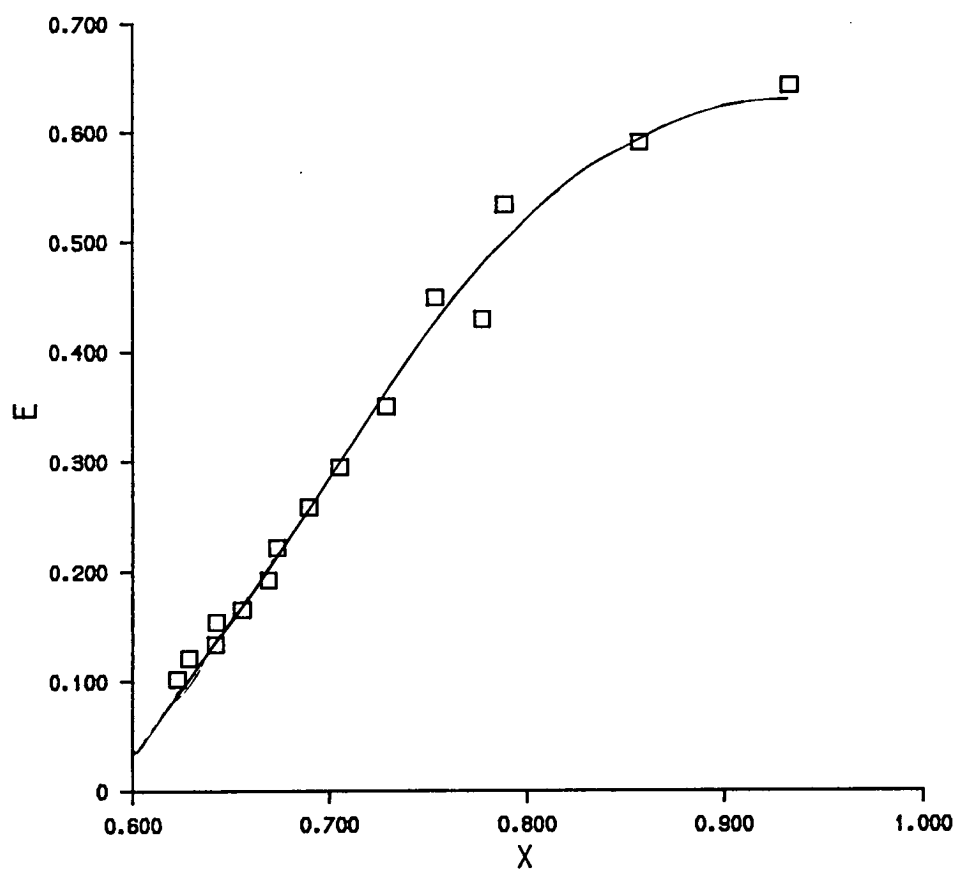
2.5.4.2 Liquid scintillation counting.

Radioactive spots from TLC plates were scraped off using a scalpel blade and eluted by dissolving them in 0.5 ml distilled water, centrifuging at 10,000 x g for 5 min, and finally, placing the supernatant in 5 ml polythene insert vials. The scintillation fluid was then added at the ratio of at least 1:10 (sample-scintillant). The solution was mixed well and then counted for 10 min at an error of 0.5%. Results expressed in counts per minute (cpm) were converted into disintegrations per minute (dpm) using the external standards ratio method. This requires

a quench correction curve (see Fig. 2.5.4) obtained by counting a known activity of ^{14}C -(U)-L-tyrosine quenched at different levels by the addition of 0-290 μl of acetone. The x-values (machine efficiency) were plotted against the counting efficiency (%) which is derived by dividing cpm by actual (calculated) counts.

FIGU 2.5.4.

The quench correction curve. Actual cpm was calculated by multiplying cpm obtained from the counter, with counting efficiency determined from this curve.



2.6 MATHEMATICAL AND STATISTICAL ANALYSIS OF RESULTS.

Mathematical and statistical analyses were carried out using a statistical package programme, Minitab Release 7.2 (Ryan *et al.*, 1985) available from the Edinburgh University Computing Services.

2.6.1 Calculation of the Standard Error of the Mean.

Unless otherwise stated, experiments in this study consisted of three replicates per treatment. The standard error (s.e.) of the mean was calculated using the following formula:

$$\text{s.e.} = \text{s.d.} / \sqrt{n} ,$$

where,
$$\text{s.d.} = \sqrt{[\Sigma (X - \bar{X})^2 / n-1]}$$

2.6.2. Calculation of Correlation Coefficient.

The associaton between two variables was measured by determining the Pearson product moment correlation, r , where:

$$r = \frac{[\sum (X - \bar{X})(Y - \bar{Y})]}{\sqrt{[\sum (X - \bar{X})^2 \sum (Y - \bar{Y})^2]}}$$

2.6.3. Statistical Analysis Using the paired t-test.

The means of two different treatments were compared using the paired t-test. In order to determine that two treatments (means) were different the calculated t-values must be greater than the tabulated values for a certain degree of freedom (df) and level of confidence.

2.6.4. Statistical Analysis Using One-Way Anova.

The means of more than two different treatments were compared using the one-way analysis of variance. The null hypothesis that the population mean, $\mu = 0$, was rejected if the F-value was greater than the P-value.

CHAPTER THREE

RESULTS

This chapter is divided into three main Sections (3.1, 3.2, and 3.3). Section 3.1 deals with experiments on the establishment of callus and suspension cultures of *Beta vulgaris* L, and optimisation of the growth medium constituents. In Sub-section 3.1.1, a series of experiments are described on the effects of explant source (3.1.1.1), level of Kinetin and 2,4-D (3.1.1.2), and size of inoculum (3.1.1.3) on the establishment and growth of callus cultures from cvs Boltardy, Burpee's Golden and Albina veredunda. The morphological and cellular characteristics of the three cvs are examined in 3.1.1.4. Sub-section 3.1.2 includes experiments that show the effects of basal salts composition (3.1.2.1), 2,4-D (3.1.2.2), sucrose concentration (3.1.2.3), inoculum size (3.1.2.4) and pH (3.1.2.5) on the growth of suspension cultures of cv Boltardy. In Section 3.2, a series of experiments are presented in which the effects of light on growth and pigment production were investigated. Particular attention was paid to the level of light irradiance, timing of light application, and finally an experiment to show the importance of light for the biosynthesis of betalains.

Lastly (Section 3.3), the effects of light on the flux of metabolites down the biosynthetic pathway leading to the betalains were investigated, which involved feeding the cultures with ^{14}C -(U)-L-tyrosine and following movement of the label down the pathway into betacyanin and

betaxanthin.

3.1 ESTABLISHMENT OF CALLUS AND SUSPENSION CULTURES OF *Beta vulgaris* L.

A necessary prerequisite to this project was to set up protocols for the establishment and maintenance of cultures. Sub-section 3.1.1 includes experiments performed on the establishment and growth of callus cultures from three cultivars of *B. vulgaris* L. (i.e. Boltardy, Burpee's Golden and Albina veredunda), paying particular attention to the effects of explant source (3.1.1.1), levels of cytokinin (kinetin) and auxin (2,4-D) (3.1.1.2), and size of the inoculum (3.1.1.3).

In 3.1.1.4 the morphological and cellular characteristics of cultures of the three cultivars of *B. vulgaris* viz. Boltardy, Burpee's Golden and Albina veredunda are examined.

Sub-section 3.1.2 deals with experiments on the establishment and maintenance of suspension cultures of *Beta vulgaris* L. cv. Boltardy, and the optimisation of growth conditions including the effects of different basal salts formulations, i.e. Murashige and Skoog (MS), Gamborg's B5 (B5), Schenk and Hildebrandt (SH), and White's media (WH) (3.1.2.1), concentration of auxin

(3.1.2.2), concentration of sucrose (3.1.2.3), size of inoculum (3.1.3.4) and pH (3.1.3.5).

3.1.1 The Establishment and Maintenance of Callus Cultures of *Beta vulgaris* L.

The experiments included in this sub-section describe the methods for the establishment and maintenance of callus cultures, and the characteristics of cultures of three cultivars, i.e. Boltardy, Burpee's Golden and Albina veredunda.

3.1.1.1 Determination of the best source of explant for callus initiation.

Various parts of seedlings and plantlets of *B. vulgaris* L. have been used for the initiation of callus, eg. cotyledon, hypocotyl, and leaf petiole (Girod and Zryd, 1987), root tip (Weller and Lasure, 1981) and apical meristem (Ripa and Adler, 1987). The limited evidence available (see Holden, 1989) show that explant origin does not usually affect culture initiation and growth, but may, at least in the initial stages of culture, affect secondary metabolite accumulation (eg. Nagel and Reinhard, 1975; Dhoot and Henshaw, 1977). The aim of this preliminary experiment was to determine the most

responsive part of the seedling to callus inducing conditions.

Sterile seedlings of *Beta vulgaris* L. cv. Egyptian Turnip were used as the source of explants. Segments of the hypocotyl, leaf petiole, cotyledon (leaf blade), and roots measuring 2 mm in length were placed onto the surface of 25 ml of Gamborg's B5 medium, solidified with 0.6% agar in a 9 cm disposable Petri dish and supplemented with 0.1 mg.l^{-1} Kinetin and 1 mg.l^{-1} 2,4-D. Cultures were left on growth racks at $25 \pm 1 \text{ }^{\circ}\text{C}$ under $10 \pm 2 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.). Each treatment consisted of 10 plates each with 5 explants. Callus formation was recorded after 6 w.

The results presented in Table 3.1.1 indicate that the hypocotyl explants showed the best response, followed by the root, cotyledon and petiole. Subsequently hypocotyl segments were used to establish callus cultures.

TABLE 3.1.1

Callus formation from different parts of the seedlings of *B. vulgaris* L. cv. Egyptian Turnip, recorded 6 w after inoculation.

EXPLANT ORIGIN	NO. OF EXPLANTS INOCULATED	% CALLUSING EXPLANT
hypocotyl	50	70
cotyledon	50	44
petiole	50	28
root	50	56

N.B. Callusing explants are those with a cell mass growing at the cut edge.

3.1.1.2 The effects of 2,4-D and kinetin on
callus initiation and growth of
B. vulgaris L.

The importance of the effects of auxin and cytokinin concentration on callus growth are well documented (eg Krikorian *et al.*, 1987; Linsmaier and Skoog, 1965; Mantell and Smith, 1983). However, cultures vary in their requirement for plant growth regulators. This experiment was carried out to optimise the level of 2,4-D and kinetin in the medium for callus induction and subsequent growth of the tissue culture.

Hypocotyl segments, 2 mm in length, obtained from 8 w old sterile seedlings of cvs. Boltardy, Burpee's Golden and Albina veredunda were inoculated onto the surface of 25 ml of Gamborg's B5 medium solidified with 0.6% agar, in 9 cm disposable Petri plates, supplemented with the following combinations of kinetin (K) and 2,4-D:

T1 - 0.04 mg.l^{-1} K + 0.02 mg.l^{-1} 2,4-D
T2 - 0.04 mg.l^{-1} K + 1.0 mg.l^{-1} 2,4-D
T3 - 0.1 mg.l^{-1} K + 0.02 mg.l^{-1} 2,4-D
T4 - 0.1 mg.l^{-1} K + 1.0 mg.l^{-1} 2,4-D

A treatment consisted of 3 Petri plates, each with 5 explants. Plates were left on a growth rack at $25 \pm 1 \text{ }^{\circ}\text{C}$ under $10 \pm 2 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn

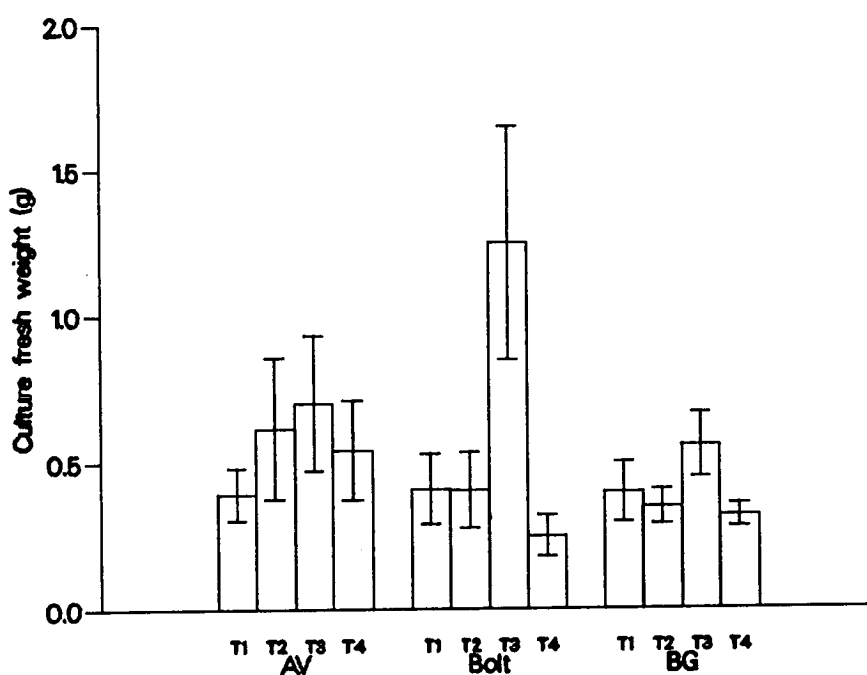
natural light fluorescent tubes (Thorn EMI, U.K.). Fresh weight of the cultures was taken after 12 wk.

Statistical analysis (Appendix 6.1) shows that treatment effects were significant in cvs Boltardy (Bolt) and Burpee's Golden (BG), but not in cv Albina veredunda. Fig. 3.1.1 shows that Treatment T3 was the most conducive to growth in cultures of cv Bolt and equal with T1 in cv BG. In BG, 2,4-D was more critical within the range of concentrations tested.

From the above results it was concluded that the medium supplemented with 0.1 mg.l^{-1} kinetin and 0.02 mg.l^{-1} 2,4-D (T3) was generally the best. In subsequent work callus cultures were initiated and maintained with this PGR combination.

FIG. 3.1.1

Comparison of the growth of callus after 12 w on medium supplemented with different levels of kinetin and 2,4-D. Each value is the mean of 3 replicates $\pm$ s.e. AV-Albina veredunda; Bolt-Boltardy; BG-Burpee's Golden.



Treatments:

T1: 0.04 mg.l^{-1} K + 0.02 mg.l^{-1} 2,4D

T2: 0.04 mg.l^{-1} K + 1.0 mg.l^{-1} 2,4-D

T3: 0.1 mg.l^{-1} K + 0.02 mg.l^{-1} 2,4-D

T4: 0.1 mg.l^{-1} K + 1.0 mg.l^{-1} 2,4-D

3.1.1.3 The effects of inoculum size on the growth of callus cultures of *B. vulgaris* L.

Observations on callus obtained from the previous experiments showed that small pieces of callus were more likely to die upon sub-culture. Growth too, was adversely affected if cultures were shaken or disturbed. This experiment was carried out to determine the optimum size of callus pieces for sub-culture and to examine the effects of breaking down the callus on sub-culture into small pieces.

Callus was obtained from 14 d old healthy growing calli of cv Bolt. Pieces measuring 3, 5, 10 and 15 mm in diameter were inoculated onto the surface of 25 ml of Gamborg's B5 medium solidified with 0.6% agar, in 9 cm disposable Petri plates, and supplemented with 0.1 mg.l^{-1} kinetin and 0.02 mg.l^{-1} 2,4-D. In addition to these, three 3 mm callus pieces were grouped together, touching, and treated as if they were one piece. Cultures were grown at $25 \pm 1 \text{ }^{\circ}\text{C}$ under $10 \pm 2 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.). Each treatment consisted of 10 plates with one piece of callus. After 3 w, survival rate of the cultures was recorded.

The
Results (Table 3.1.2) show that pieces measuring 10 - 15 mm in diameter were established successfully upon sub-

culture. However, smaller pieces did not survive consistently; 5 mm callus pieces had a survival rate of 50%, and 3mm callus pieces 20%. Grouping of the 3 mm callus pieces did not improve the survival rate suggesting that these calli were very sensitive to disturbances in their micro-environment. Subsequently, callus cultures were maintained routinely by the transfer of 10-15 mm fragments onto fresh media.

TABLE 3.1.2

A comparison of the survival rate of *B. vulgaris* cv Bolt. callus pieces of different sizes, upon transfer to fresh media. Observations were made 3 w after sub-culture.

Inoculum Size (mm)	% survival
15	100
10	100
5	50
3 (individually)	20
3 (in groups of 3)	20

N.B. Surviving calluses are those that remained healthy and managed good growth as soon as transferred to fresh media, while others showed necrosis of a part or the whole of the callus.

3.1.1.4 Characterisation of callus cultures derived from cv Boltardy, Burpee's Golden and Albina veredunda.

The aim of this experiment was to select a cultivar for subsequent studies based on pigment composition. Using the methods already described, three callus lines were established from the cvs Boltardy, Burpee's Golden and Albina veredunda. These cultures were examined microscopically in order to determine the proportions of pigmented (red, violet, yellow, orange, green) cells and the absorption spectra of culture extracts were also measured. To determine the proportions of pigmented cells in each callus, the culture was separated into small aggregates by gently applying pressure from a spatula spoon. The small aggregates were then suspended in liquid Gamborg's B5 medium and sucked in and out of a Pasteur pipette *ca.* 20 times before examination under an Olympus IM inverted microscope (Olympus Optical Co., Tokyo, Japan). About 1,000 cells were counted with each sample to determine the proportions of pigmented cells. Absorbance spectra of extracts were made according to the methods described in Section 2 (2.5.2).

FIG. 3.1.2

Callus cultures of three cultivars of *B. vulgaris* L. (a) Boltardy (b) Burpee's Golden (c) Albina veredunda, at d 14.

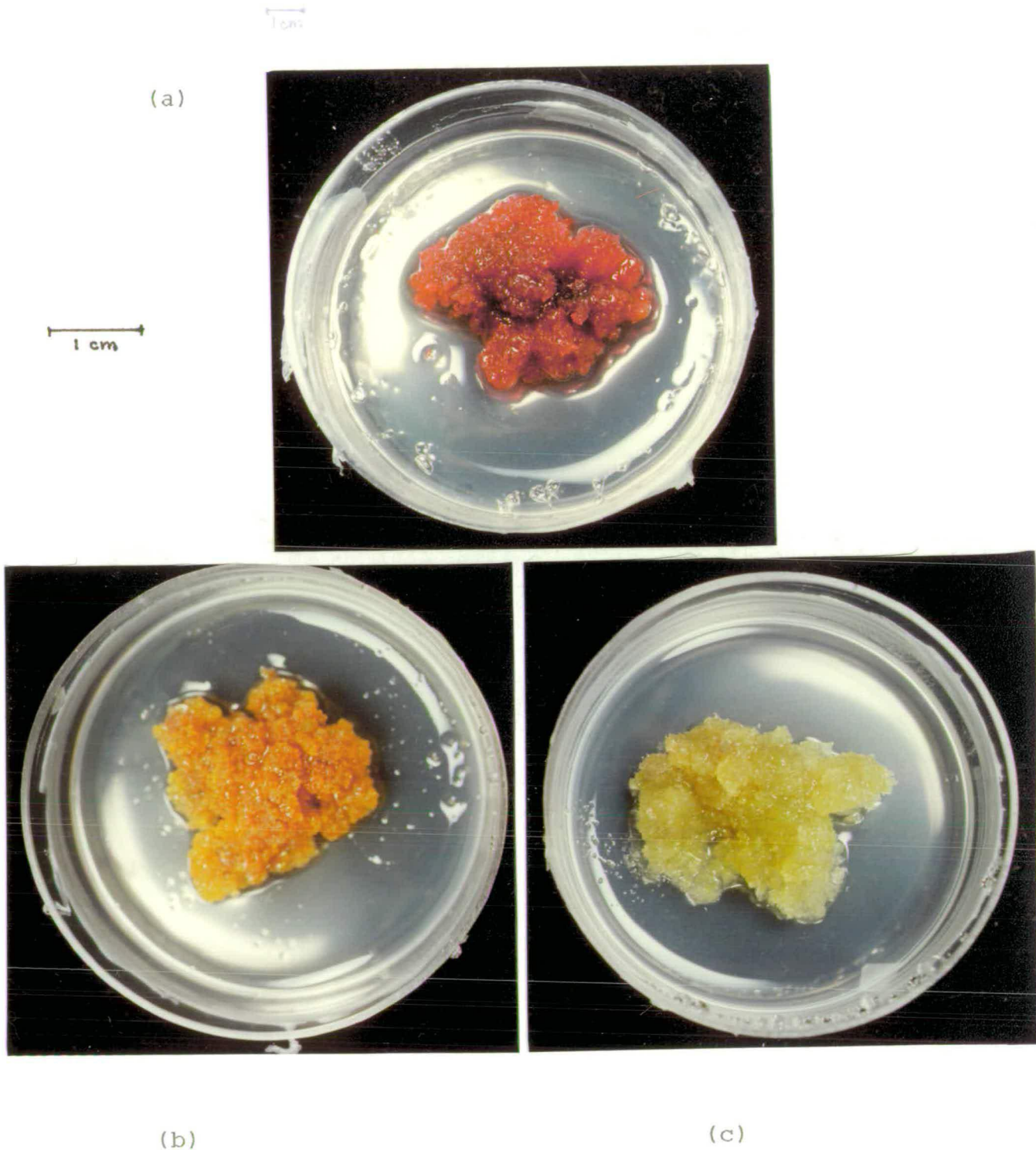
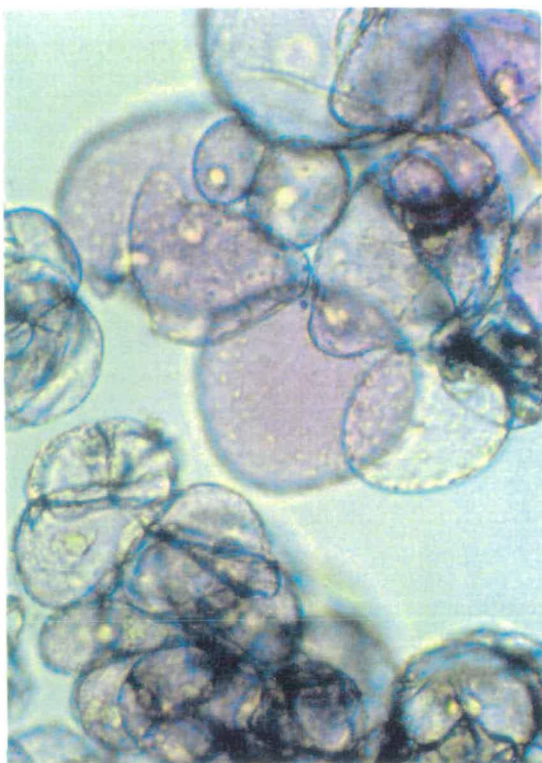


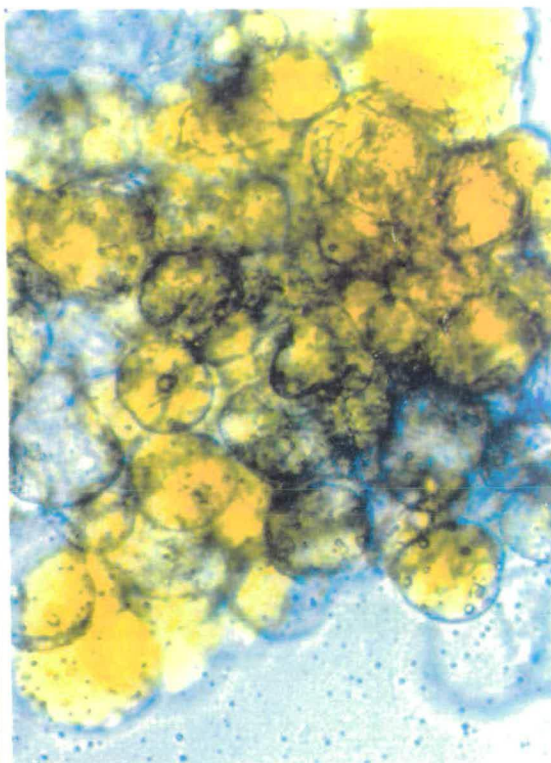
FIG. 3.1.3

Photomicrographs of cells from ^{d14} callus cultures of *B. vulgaris* L. cvs Boltardy (a), Burpee's Golden (b) and Albina veredunda (c).

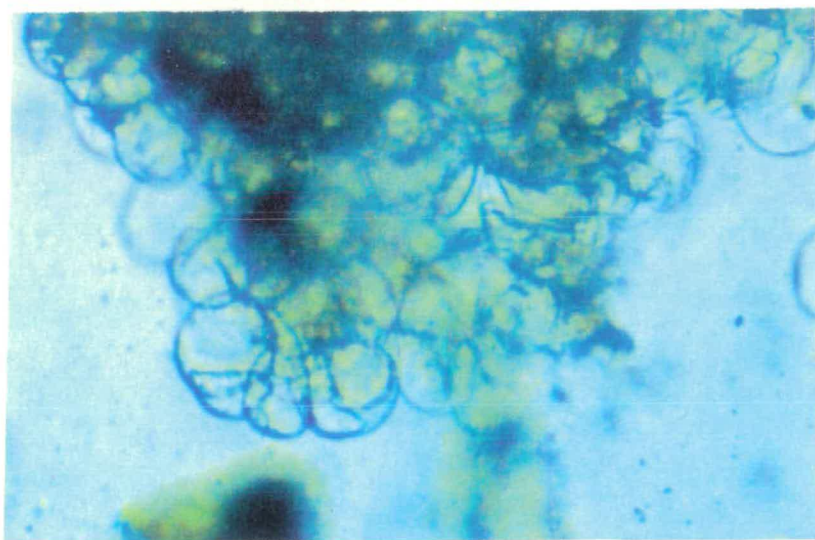
a.



b.



c.

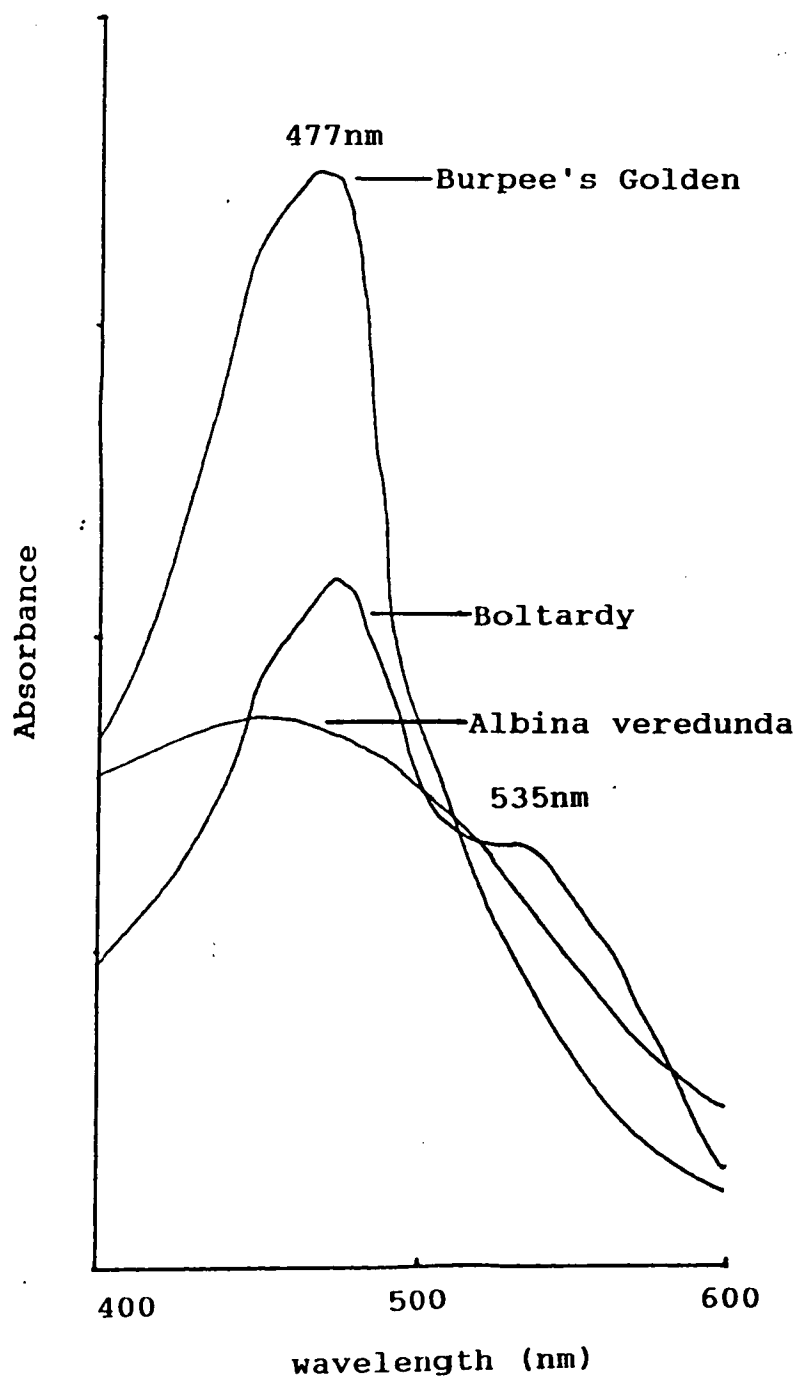


Figs 3.1.2 (a-c) show the appearance of the cultures. Cv Boltardy was red, cv Burpee's Golden yellow and cv Albina veredunda greenish white. However, a closer examination of the cultures under the microscope revealed that each culture was made up of cells of different colours (Figs. 3.1.3 (a-c). Cv Boltardy showed the most varied composition: colourless (28%), yellow (3%), orange (25%), red (3%) and violet (40%) cells. Cultures of the other two cvs consisted of a relatively uniform population of cells with respect to pigmentation. Cv B. Golden was made up of colourless (30%) and yellow (70%) cells, with occasional red-violet cells; whilst cv Alb. veredunda consisted almost totally of pale greenish cells which were presumably photosynthetic, although red-violet cells were found occasionally.

Extracts of the cultures were examined spectrophotometrically. The absorption spectra in Fig. 3.1.4 show that two major pigments were present in cv Boltardy with absorbance maxima at 477 nm (betaxanthin) and 535 nm (betacyanin). Extracts of cv B. Golden showed a single peak at around 477 nm, whilst those of cv Alb. veredunda showed no distinct peak. Pigments were separated by Thin-layer Chromatography and eluted from the cellulose before determination of the spectra (Fig. 2.5.2)

FIGURE 3.1.4

The absorption spectra of aqueous extracts from *B. vulgaris* L. cvs Boltardy, B. golden and Albina veredunda. Extracts were prepared using the method described in Sub-section 2.5.3.



3.1.2 The Establishment of Suspension Cultures of *Beta vulgaris* L. cv Boltardy

Suspension cultures were established from friable callus obtained by the method described in Sub-section 3.1.1. Attempts to establish suspensions from compact calli often resulted in failure, and always resulted in the regeneration of roots. In the following series of experiments attempts were made to optimise the growth in suspension cultures. The basal salts mixture (MS, B5, SH, and WH), auxin concentration, inoculum size, sucrose concentration, and pH were all examined. In addition the betalain content of the cultures was determined.

3.1.2.1 The effects of basal salts formulation on growth and pigment accumulation in suspension cultures.

Cell cultures of *Beta vulgaris* L. have been maintained in various basal salts formulations, eg. Murashige and Skoog, MS (Constabel and Nassif-Makki, 1971), Gamborg's B5 (Hunter, 1988), Linsmaier and Skoog, LS (Girod and Zryd, 1987), and PG (see Ripa and Adler, 1987). The aim of this experiment was to determine the optimum salts formulation for culture growth as well as pigment accumulation in suspension cultures of the beetroot cv Boltardy. Four

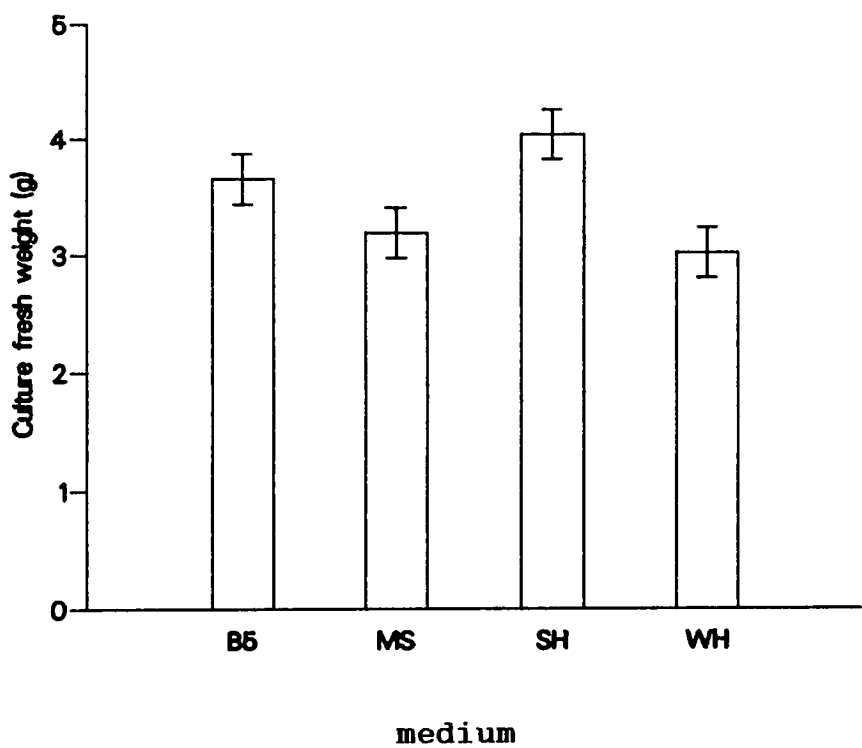
kinds of basal salts were used, i.e. MS, SH, B5 and WH.

Fourteen day old cells grown previously in B5 medium were suspended in the four different media. A 10 ml aliquot of each suspension containing ca. 1 g FW of cells was inoculated into each 100 ml Erlenmeyer flask containing 15 ml of the respective medium. All media were supplemented with 0.1 mg.l^{-1} kinetin and 0.02 mg.l^{-1} 2,4-D and their pH adjusted to 5.8. Each treatment consisted of 3 replicates. Cultures were placed on a shaker rotating at 98 rpm (8mm throw) at $25 \pm 1^\circ\text{C}$ and under $10 \pm 2 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn, EMI, U.K.). After 14 d, the cultures were harvested and their fresh weight and pigment content determined according to the methods described in Sub-sections 2.4.2 and 2.5.2, respectively.

Statistical analysis of results (Appendix 6.2) showed that growth was significantly influenced by the composition of the basic medium. From the results presented in Fig. 3.1.5, it would appear that SH is marginally superior to the others in terms of its ability to support growth, and is followed closely by Gamborg's B5 medium. MS and WH media support growth to the same extent but less than SH and B5.

FIG. 3.1.5

The effects of different basal salts formulations (medium): B5, MS, SH and WH, on the growth of suspension cultures of *B. vulgaris* cv Boltardy after 14 d. Each value is the mean of 3 replicates $\pm$ s.e.



Visual observation of the cultures showed that those in WH medium were the most intensely coloured (red), followed by B5, SH and MS media respectively.

Gamberg's B5 was now chosen as the standard medium for future experimentation because it supports growth as well as pigment accumulation.

3.1.2.2 The effects of 2,4-D on culture growth and pigment accumulation in suspension cultures of *Beta vulgaris* L. cv Boltardy

Auxin (2,4-D) has been reported to affect the production of betacyanin in cell cultures of *Phytolacca americana* (Sakuta *et al.*, 1986; Komamine *et al.*, 1989; Hirose *et al.*, 1990). In *B. vulgaris*, Constabel and Nassif-Makki (1971) have shown that at 1 mg.l^{-1} of 2,4-D the formation of betacyanin was inhibited, but when the concentration of 2,4-D was reduced, pigment reappeared. In this experiment the effects of low levels of 2,4-D (0.02 and 0.2 mg.l^{-1}) on culture growth and pigment accumulation in suspension cultures of *B. vulgaris* L. cv Boltardy were investigated. An additional treatment without 2,4-D was included.

Cells were obtained from 14 d old cultures maintained in Gamborg's B5 medium supplemented with 0.1 mg.l^{-1} kinetin and 0.02 mg.l^{-1} of 2,4-D, and washed twice with 200 ml of Gamborg's B5 medium containing no PGRs so as to remove any trace of 2,4-D which might be carried over. 2 g FW of cells was inoculated into 250 ml Erlenmeyer flasks containing 50 ml of Gamborg's B5 medium supplemented with 0, 0.02 and 0.2 mg.l^{-1} of 2,4-D. The flasks were then covered with a double layer of aluminium foil and placed on a shaker rotating at $98 \pm 2 \text{ rpm}$, at $25 \pm 1 \text{ }^{\circ}\text{C}$ under continuous light of $10 \pm 2 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$ supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.). Each

treatment was replicated twice. After 14 d, cultures were harvested and fresh weight and betalain content determined by the methods described in Sub-sections 2.4.2 and 2.5.2, respectively.

Analysis of variance (Appendix 6.3) indicates a significant influence of 2,4-D on culture growth as well as on pigment accumulation. Culture growth decreases with decreasing concentration of 2,4-D (Fig. 3.1.6a). On the contrary, pigment accumulation generally showed an inverse correlation (Fig. 3.1.6b). Lowering the 2,4-D concentration in the medium, or omitting it, resulted in a much higher level of betaxanthin but little effect on betacyanin.

FIG. 3.1.6.

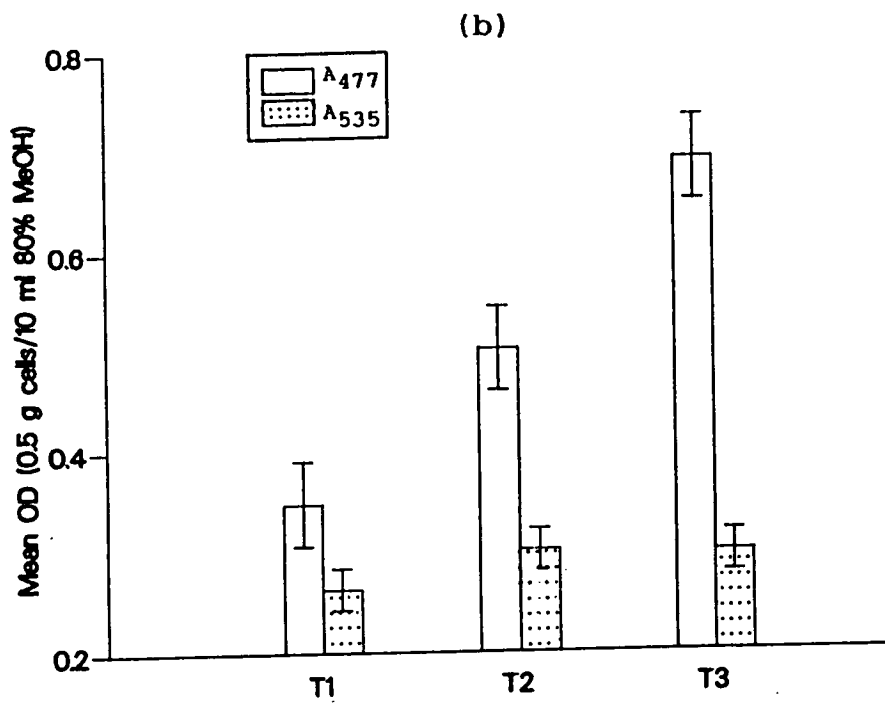
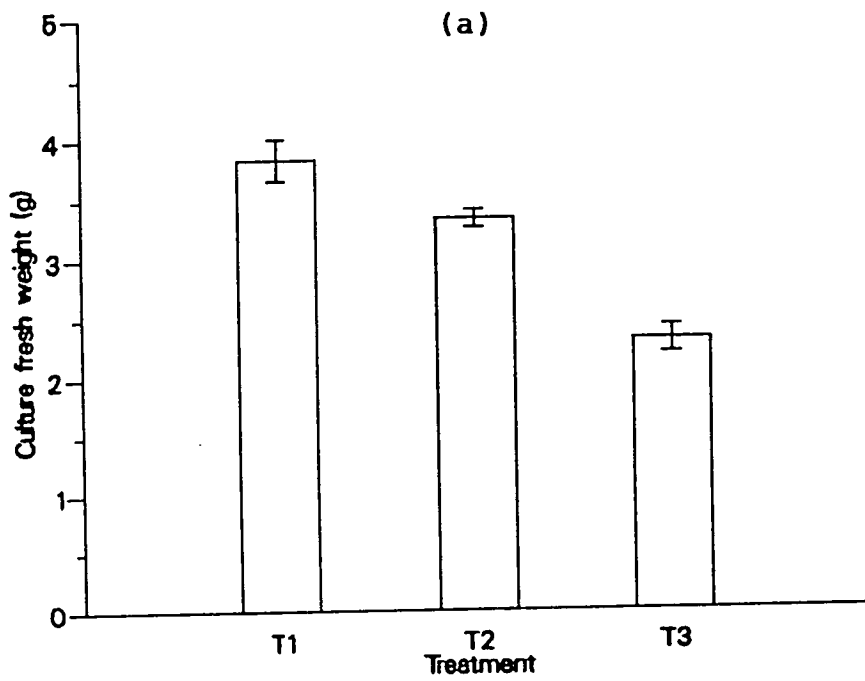
The effects of 2,4-D concentration on (a) culture growth and (b) the accumulation of betacyanin (A_{535}) and betaxanthin (A_{477}) in suspension cultures of *B. vulgaris* cv Boltardy. Each value is the mean of two replicates $\pm$ s.e.

Treatment:

T1: 0.2 mg.l⁻¹ 2,4-D

T2: 0.02 mg.l⁻¹ 2,4-D

T3: 0 mg.l⁻¹ 2,4-D



3.1.2.3 The effects of sucrose concentration on cell growth and betalain accumulation in suspension cultures of *Beta Vulgaris* cv. Boltardy.

The previous experiment examined the effects of 2,4-D on growth and betalain accumulation. In this experiment, the effect of sucrose, another important media component, was studied. Sucrose is the main source of carbon in tissue culture medium, besides its ability to support growth, sucrose has also been shown to influence the production of secondary metabolites (see review by Dougall, 1980). The aim of this experiment was to investigate the effects of various sucrose concentrations, i.e. 2%, 4%, 8%, and 16%, on growth and betalain accumulation over a period of 35 d, and to determine the optimum level required in suspension cultures of *B. vulgaris* cv Boltardy.

Cells were obtained from suspensions maintained in Gamborg's B5 medium containing 2% sucrose and grown at 25 ± 1 °C under $20 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.). Sixty cultures were prepared at d 0 by inoculating ca. 1 g FW of cells into 100 ml Erlenmeyer flasks containing 25 ml of Gamborg's B5 medium supplemented with 0.1 mg.l^{-1} kinetin and 0.02 mg.l^{-1} 2,4-D, and with the following concentrations (w/v) of sucrose: 2%, 4%, 8%, and 16%. Cultures were grown at 25 ± 1 °C on a shaker rotating at

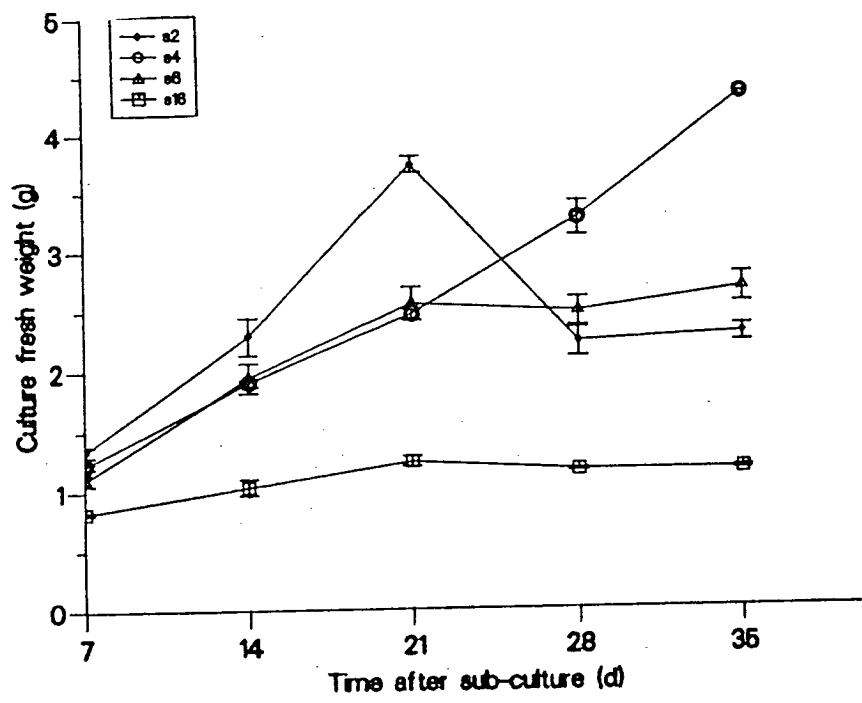
98 ± 2 rpm under 10 ± 2 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.). Three flasks were harvested every 7 d up to and including d 35 and the fresh weight and betalain content determined by the methods described in Sub-section 2.4.2 and 2.5.2, respectively.

i. The effects of initial sucrose concentration on growth.

The effects of initial sucrose concentration on growth are shown in Fig. 3.1.7. At a sucrose concentration of 2%, culture FW increased rapidly until day 21, then fell sharply when the cells autolysed and started to release pigments into the medium which turned from red to black within 2-3 d. At 8% and 16% growth followed a similar trend with an increase until d 21, but then the FW remained constant until the end of the experiment. At 4% sucrose, cells initially grew at the same rate as those grown in 8% sucrose over the first 21 d but then continued to increase in fresh weight until the end of the experiment (35d). Cultures at this stage remained healthy without showing any signs of nutrient stress.

FIG. 3.1.7

The effects of initial sucrose concentration on growth in suspension cultures of *B. vulgaris* cv Boltardy. Each point is the mean of 3 replicates $\pm$ s.e. s2, 2% sucrose ; s4, 4% sucrose; s8, 8% sucrose; and s16, 16% sucrose (w/v).

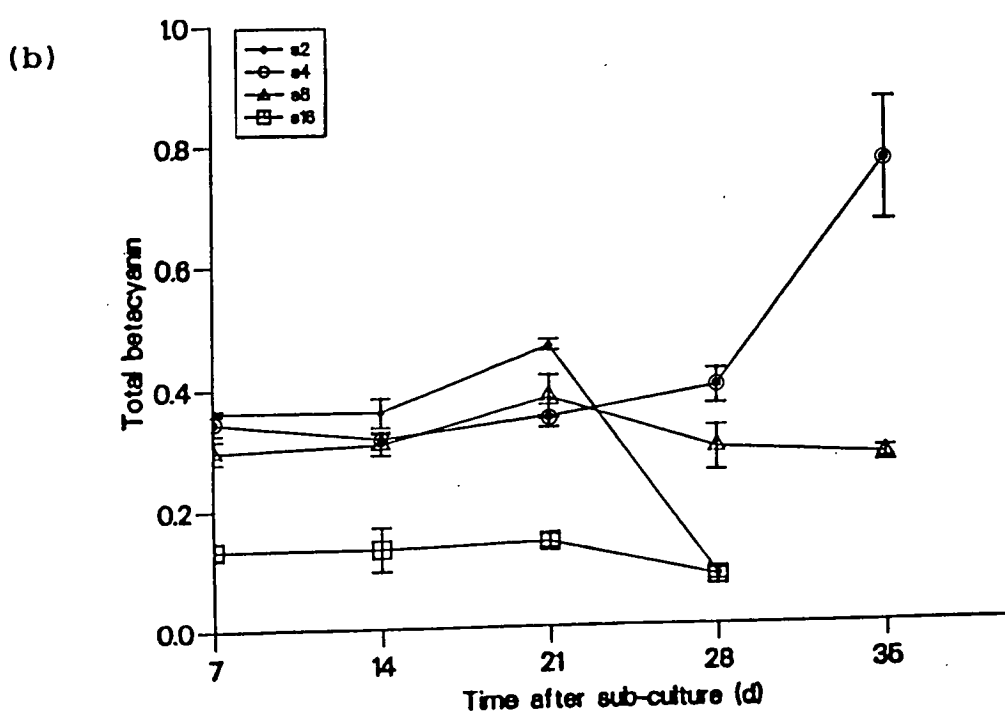
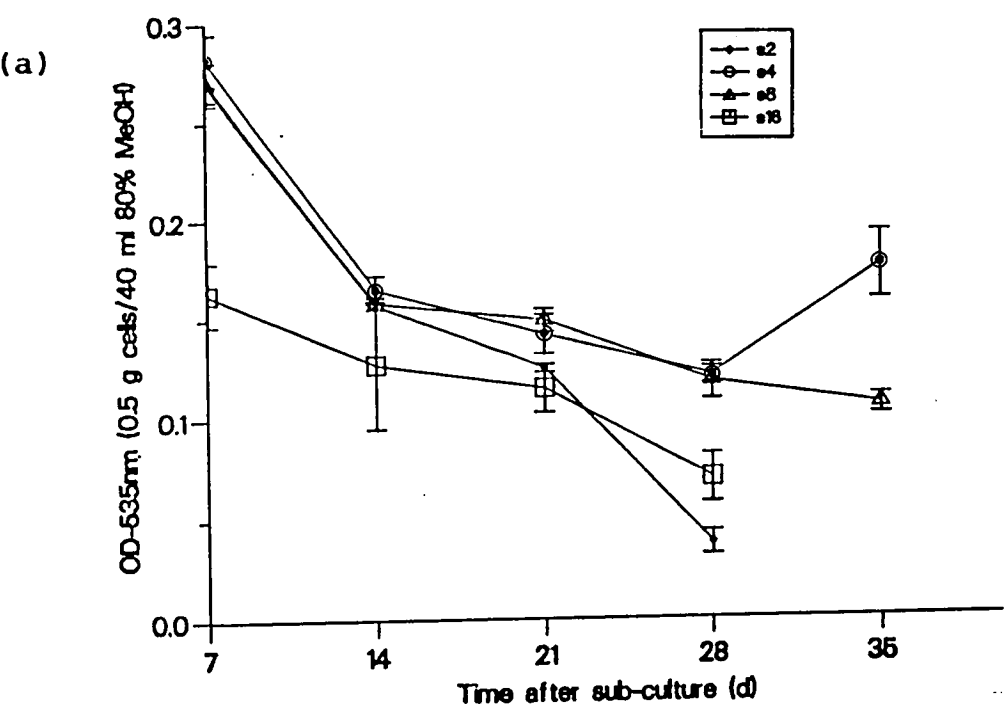


ii. The effects of initial sucrose concentration on betacyanin accumulation.

Figs. 3.1.8 (a) and (b) show the pattern of betacyanin accumulation on a per unit FW, and per culture basis, respectively. In all cultures except for the 16% treatment in which pigment level had been reduced considerably in the previous 7 d of culture, there was a sharp drop in betacyanin content per unit FW during the first 14 d, then the decrease became more gradual towards the end of the experiment, except for cultures in 4% sucrose which showed an increase in betacyanin content at d 35. Meanwhile, the total pigment accumulated as shown in Fig. 3.1.8b was lowest throughout the experiment in cultures with 16% sucrose. Pigment accumulation in 2% sucrose was the highest until about d 21 when the cells began to autolyse. Production in cultures with 4% and 8% sucrose were slightly lower than those in 2% sucrose until d 35 when cultures with 4% sucrose began to accumulate the highest amount of pigment recorded between the treatments. The general downward trend in pigment concentration was probably due to the lower light irradiance used, i.e. $10 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$, compared to $20 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ for the stock cultures used to initiate this experiment.

FIG. 3.1.8

The effect of initial sucrose concentration on betacyanin accumulation in suspension cultures of *B. vulgaris* cv Boltardy (a) OD per unit FW (b) OD per culture. Each value is the mean of 3 replicates $\pm$ s.e. s2, 2% sucrose; s4, 4% sucrose; s8, 8% sucrose; s16, 16% sucrose (w/v).



*iii. The effects of initial sucrose concentration on
betaxanthin accumulation*

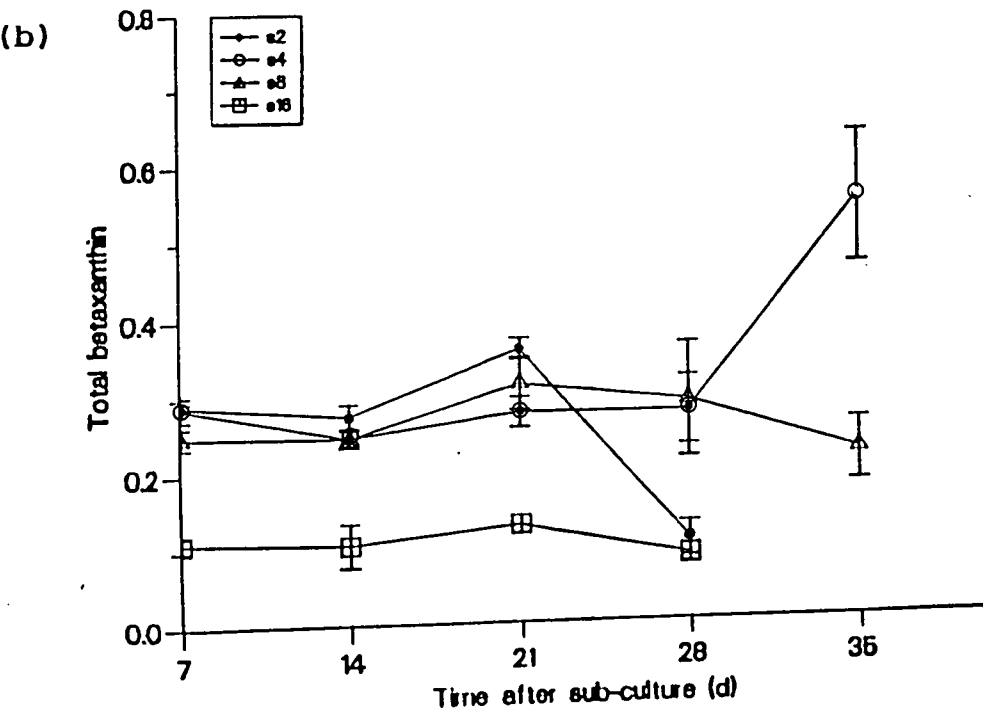
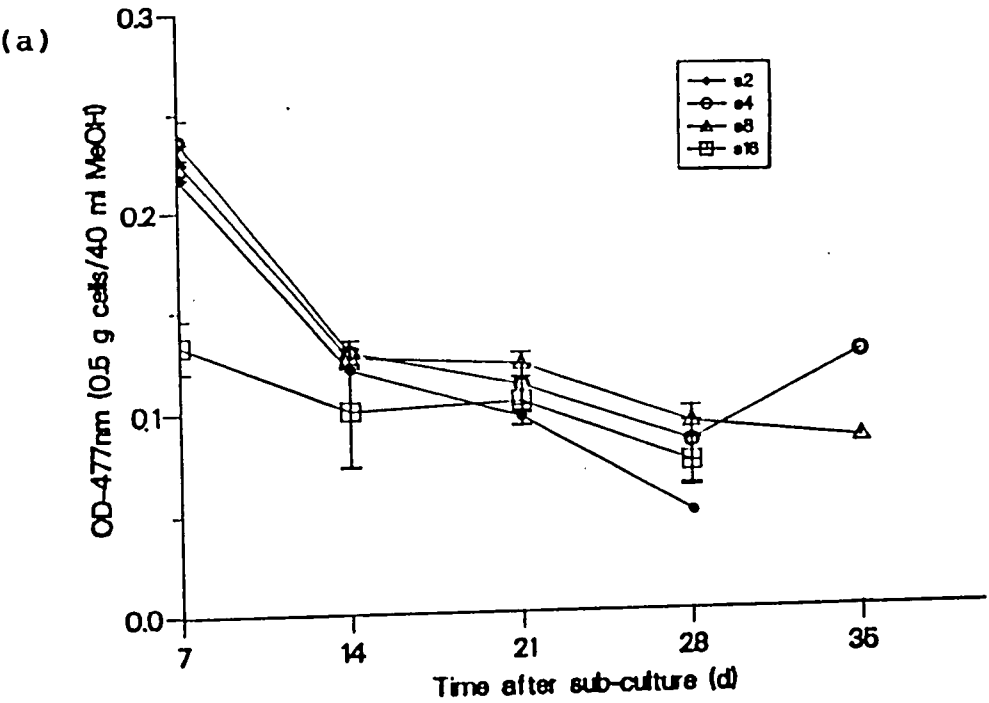
The pattern of betaxanthin accumulation (Fig. 3.1.9 a-b) is similar to that of betacyanin.

Summary

The above results showed that at sucrose concentrations exceeding 8%, growth and pigment accumulation were adversely affected. A sucrose concentration of 4% produced the highest amount of the betalains, although this was achieved after 35 d of incubation. A concentration of 2% was shown to be the best for experimental purposes since it is able to provide rapid growth as well as a high production of the betalains in a shorter time. This concentration was used in subsequent experiments.

FIG. 3.1.9

The effects of initial sucrose concentration on betaxanthin accumulation in suspension cultures of *B. vulgaris* cv Boltardy (a) OD per unit FW (b) OD per culture. Each point is the mean of 3 replicates $\pm$ s.e. s2, 2% sucrose; s4, 4% sucrose; s8, 8% sucrose; s16, 16% sucrose (w/v).



3.1.2.4 The effects of inoculum size on growth and pigment accumulation in suspension cultures of *Beta vulgaris* L cv Boltardy.

Growth and secondary product accumulation may change with volume of medium and the ratio of cells to volume (Staba, 1969). In this study (3.1.1.3) the survival and growth of *B. vulgaris* cv Boltardy callus cultures upon transfer to fresh media has been shown to be dependent on the amount of inoculum. In this experiment an attempt was made to examine the effects of inoculum size on the growth and pigment content in suspension cultures, and to determine the optimum inoculum size for a successful transfer to fresh medium.

Cells were inoculated using a perforated spatula spoon. Ca. 2, 4, and 6 g fw of cells were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of Gamborg's B5 medium supplemented with 0.1 mg.l^{-1} kinetin, 0.02 mg.l^{-1} 2,4-D, and 2% (w/v) sucrose. The flasks were then covered with a double layer of aluminium foil, and placed on a shaker rotating at $98 \pm 2 \text{ rpm}$ at $25 \pm 1 \text{ }^{\circ}\text{C}$ with continuous light of $10 \pm 2 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$ supplied by Thorn natural light (Thorn EMI, U.K.) fluorescent tubes. After 14 d, cultures were harvested and their fresh weights and betalain contents determined according to the methods described in Sub-section 2.4.2 and 2.5.2, respectively.

The results of the statistical analysis (Appendix 6.4) and those in Fig. 3.1.10 show that there was a significant difference amongst the treatments though these were very small. Fig. 3.1.10 shows that there was little difference in the relative growth rates (RGR) between T1 and T3 and none between other treatments. There was no difference in either betacyanin or betaxanthin content.

Since there was little difference between the treatments, T2 (ca. 2g FW of cells) was used as the standard inoculum size in culture maintenance. It was also observed that cultures in T3 were beginning to show signs of nutrient depletion at the end of the experiment, i.e. the cells were turning brown.

FIG. 3.1.10

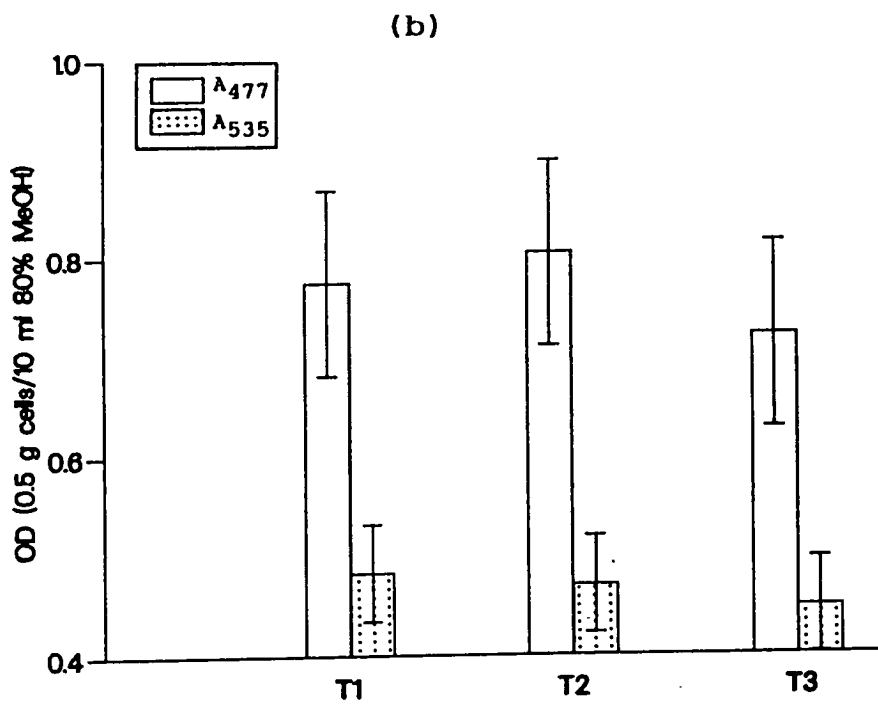
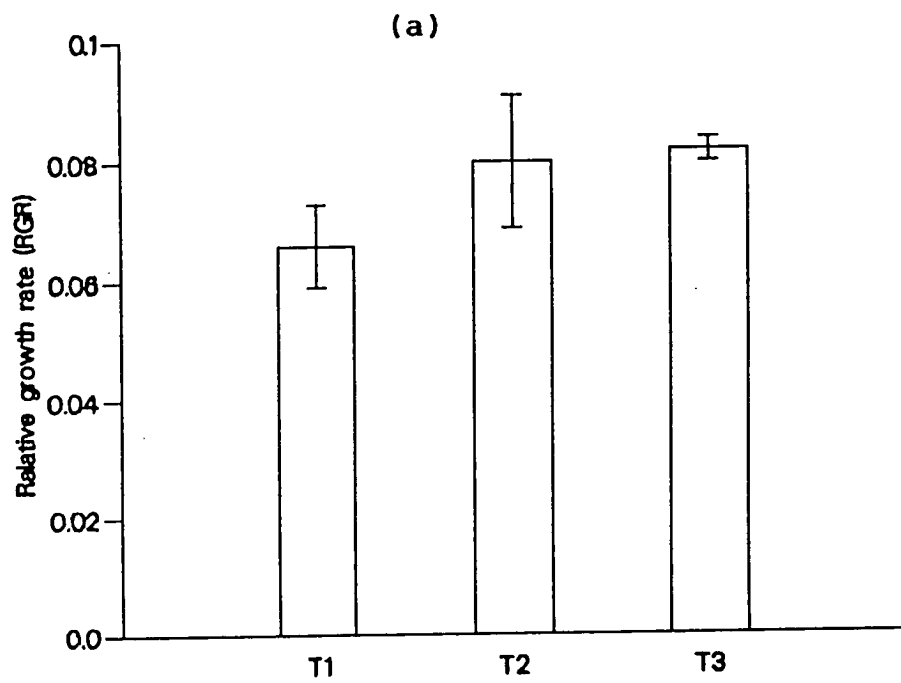
The effects of inoculum size on culture growth (a) after 14 d , and levels of betacyanin and betaxanthin accumulated (b) in suspension cultures of *B. vulgaris* L. cv Boltardy . Each value is the mean of 3 replicates $\pm$ s.e.

Treatment:

T1 = ca. 2 g FW of cells

T2 = ca. 4 g FW of cells

T3 = ca. 6 g FW of cells



**3.1.2.5 The effects of pH on betalain
accumulation in suspension cultures of
B. vulgaris cv Boltardy.**

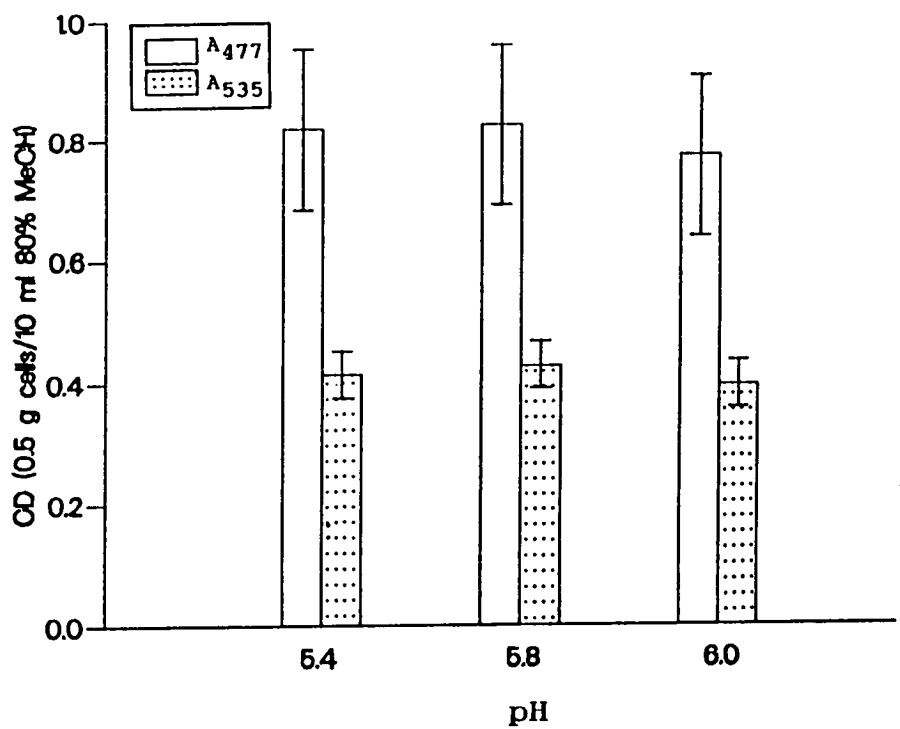
It is known that the pH of the culture medium can influence the growth and secondary metabolism of cell cultures (eg. Veliky, 1977). The aim of this experiment was to examine if initial pH of the growth medium affected pigment accumulation in suspension cultures of *B. vulgaris* cv Boltardy.

Approximately 2 g FW of cells were inoculated into six 250 ml Erlenmeyer flasks containing 50 ml of Gamborg's B5 medium supplemented with 0.1 mg.l^{-1} of kinetin, 0.02 mg.l^{-1} of 2,4-D, and 2% sucrose, with the pH at 5.4, 5.8 or 6.0. Cultures were grown at $25 \pm 1^\circ\text{C}$ on a shaker rotating at $98 \pm 2 \text{ rpm}$ under $10 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.). Cultures were harvested at d 14 and analysed for their betalain content according to the methods described in Sub-section 2.5.2.

The results presented in Fig. 3.1.11 and the analysis of variance in Appendix 6.5 show no significant difference in betalain content between any of the treatments so that within the pH range 5.4 - 6.0 there was no detectable effect on pigment accumulation.

FIG. 3.1.11

A comparison of the effect of pH on the amount of betacyanin (A_{535}) and betaxanthin (A_{477}) accumulated in 14 d old suspension cultures of *B. vulgaris* L. cv Boltardy. Each value is the mean of three replicates $\pm$ s.e.



Summary

The initiation of callus cultures was best achieved using hypocotyl explants. Once initiated the best growth of callus was observed on a medium with 0.02 mg.l^{-1} 2,4-D and 0.1 mg.l^{-1} kinetin. The optimum size of inoculum was found to be 10-15 mm in diameter (ca. 2 g FW). Cultures initiated with smaller pieces of callus showed a poor rate of survival.

Determination of the pigment content of callus cultures showed that cv Boltardy produced significant amounts of betacyanin as well as betaxanthin, while cv Burpee's Golden produced mainly betaxanthin. Cv Albina veredunda did not produce appreciable amount of either of the pigments.

In suspension cultures derived from friable callus, Gamborg's B5 was shown to be the best compromise for growth and pigment production. SH medium was shown to be the best for growth, and cultures in WH medium produced most red pigment. There was a positive correlation between 2,4-D concentration in the medium and growth over the range 0 - 0.2 mg.l^{-1} . Growth increased with increasing levels of 2,4-D, However, the effect on betaxanthin accumulation was the reverse, whereas betacyanin was not affected. Of the two betalains, betaxanthin seems to be more sensitive to changes in 2,4-D

concentration. A concentration of 0.02 mg.l^{-1} was shown to be the optimum level and gave a good response in both growth and pigment accumulation. Sucrose concentration was shown to influence growth and pigment accumulation. At concentrations higher than 8%, growth and betalain accumulation were adversely affected. The highest production of pigments was obtained in medium with 4% sucrose after 35 d of culture. A concentration of 2% was shown to be the optimum as cultures grew faster and accumulated more pigments in a shorter time. No effect of pH over the range 5.4 - 6.0 was detected on the level of pigment accumulated.

3.2. THE EFFECT OF LIGHT ON THE SYNTHESIS AND ACCUMULATION OF BETALAINS IN SUSPENSION CULTURES OF *B. VULGARIS* L. CV BOLTARDY.

The importance of light in the biosynthesis of the betalains has been demonstrated in seedlings (Wohlpart and Black, 1973) and in cell cultures of the beetroot (Girod and Zryd, 1987), as well as in a few other species of the order *Centrospermae*. However, little is understood of the mechanism of light control in these systems. It has also been suggested that betalain accumulation may be determined by the stage of development of the cells (Constabel and Nassif-Makki, 1971). This section includes experiments carried out to determine (1) if there is any correlation between culture growth and betalain accumulation, and (2) the effect of light on the synthesis and accumulation of these pigments.

3.2.1 Preliminary Experiment on the Effects of Light and Dark Treatments on Betalain Accumulation.

The aim of this experiment was to examine whether light was necessary for the accumulation of betalains in suspension cultures of *B. vulgaris* cv Boltardy.

Cells were obtained from 14 d old stock suspension cultures grown under $10 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.). Two g FW of cells were inoculated into four 250 ml Erlenmeyer flasks containing 50 ml of Gamborg's B5 medium supplemented with 0.1 mg.l^{-1} of kinetin, 0.02 mg.l^{-1} of 2,4-D, and 2% sucrose. Dark treated cultures were wrapped up in a double layer of Aluminium foil. All cultures were placed on a shaker and grown under standard growth conditions (Section 2.2.5.1) ^{with $10 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light}. Each treatment was replicated twice. After 14 d, cultures were harvested and their betacyanin and betaxanthin content determined by the methods described in Section 2.5.2.

The results presented in Table 3.2.1 and Appendix 6.6 show that both betacyanin and betaxanthin were accumulated to a significantly higher level in cultures grown in the light. In cultures deprived of light there was a 32% reduction in betacyanin, and a 60% reduction in betaxanthin.

From this it would appear that light is important in determining the level of betalains accumulated by the cultures. However, these results are only from cultures at one particular growth stage, i.e. those at d 14. In the next experiment the pattern of pigment accumulation was examined in cells at different stages of growth to determine any correlation between the stage of development and the response of the cells to light.

Table 3.2.1

The level of betacyanin (A₅₃₅) and betaxanthin (A₄₇₇) accumulated in ^{14 d old} light and dark-grown cultures of *B. vulgaris* cv Boltardy. Each value is the mean of 2 replicates ± s.e.

TREATMENT	A ₄₇₇	A ₅₃₅
Light	0.7213 ± 0.0515	0.2800 ± 0.0150
Dark	0.2925 ± 0.0075	0.1913 ± 0.0035

3.2.2 A Time-Course Study of the Growth and Betalain Accumulation in Light and Dark-Grown Cultures.

In the previous experiment, light was shown to influence the levels of betacyanin and betaxanthin accumulated in the cells. The suggestion that the level of betacyanin accumulated may be determined by the growth stage of the cells prompted this experiment. Accordingly, the aim of this experiment was to examine the relationship between betalain accumulation and culture growth within one culture cycle in the light and in the dark.

Cells were pooled from 14 d old stock cultures grown under $10 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.), and suspended in fresh Gamborg's B5 medium supplemented with 0.1 mg.l^{-1} of kinetin, 0.02 mg.l^{-1} of 2,4-D, and 2% sucrose (Section 2.2.5.1.). Forty five flasks were inoculated on d 0 using a sterile wide-bore pipette with the tip removed. A 10 ml aliquot containing ca. 0.79 g FW of cells was added to 15 ml of the same medium contained in each 100 ml Erlenmeyer flask. Cultures were incubated at $25 \pm 1^\circ\text{C}$ on an orbital shaker rotating at $98 \pm 2 \text{ rpm}$ (8 mm throw). Light-grown cultures were supplied with $1 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light irradiance from Thorn natural light fluorescent tubes (Thorn EMI, U.K.), while dark-grown cultures were placed in black plastic boxes and incubated alongside the

other flasks on the shaker. Three flasks were harvested from each treatment every 4 d up to and including d 28. Measurements were made of culture fresh weight, cell number, and betalain content according to the methods described in Section 2.4.2 and 2.5.2, respectively.

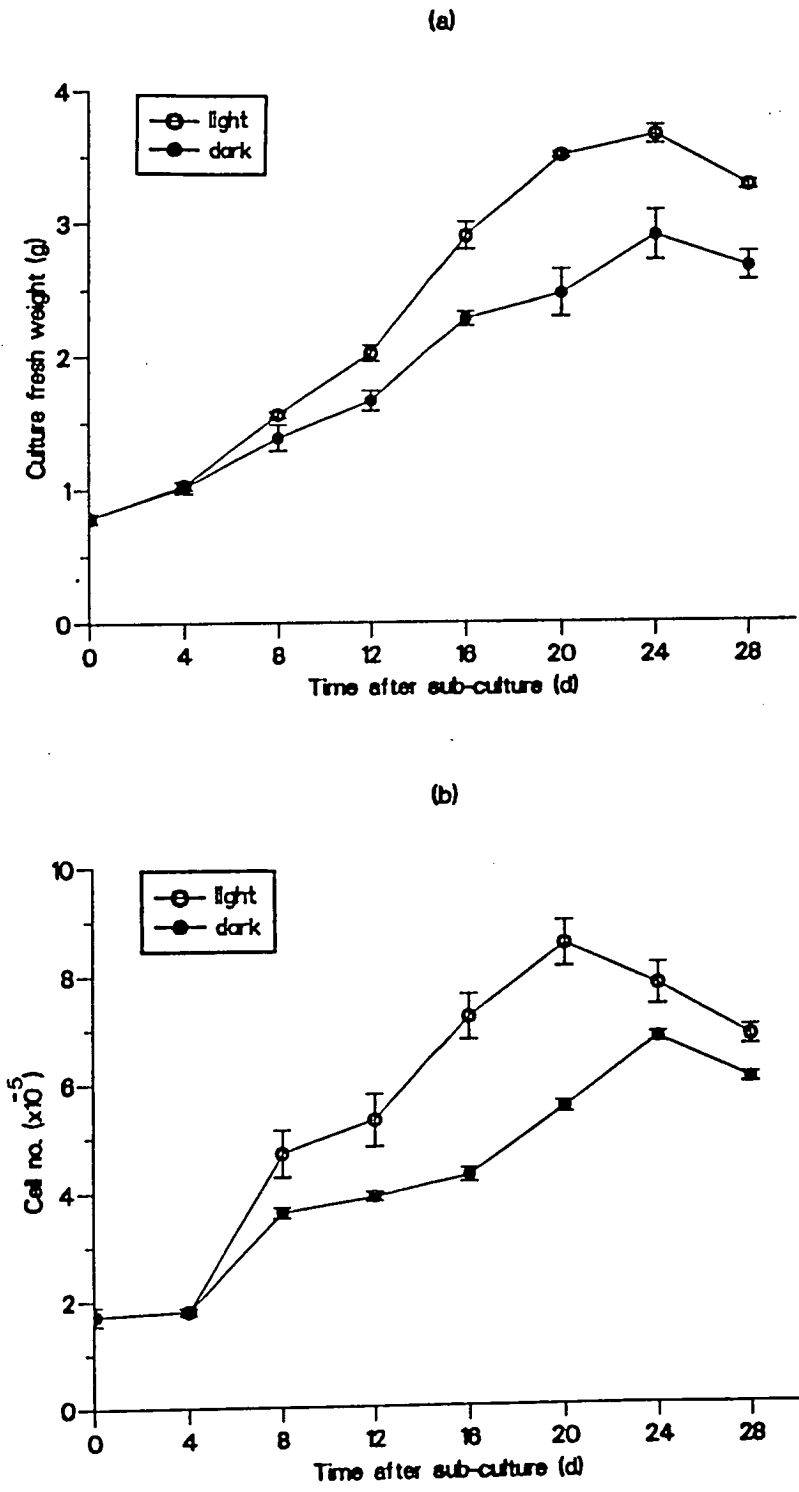
i. Kinetics of culture growth (FW and cell number).

Figs. 3.2.1 (a) and (b) show the pattern of culture growth on the basis of FW and cell number, respectively. Throughout this experiment culture growth was generally lower in the dark. There was a lag phase of 4 d in both treatments. In light-grown cultures the cell number began to increase rapidly between d 4 and d 16, then slowed down until d 20 after reaching a maximum, remained constant until d 24, and then began to fall until the end of the experiment. The change in culture FW was similar to that in cell number.

In dark-grown cultures, the pattern of growth (cell number) is quite similar to that observed in light-grown cultures, except that the maximum value was achieved later, i.e. at d 24. Thereafter, the cell number dropped until the end of the experiment. Growth in terms of FW showed a similar pattern.

FIG. 3.2.1

Culture growth in light and dark-grown suspension cultures of *B. vulgaris* cv Boltardy. (a) culture fresh weight (b) cell number. Each point represents the mean $\pm$ s.e. (n=3).



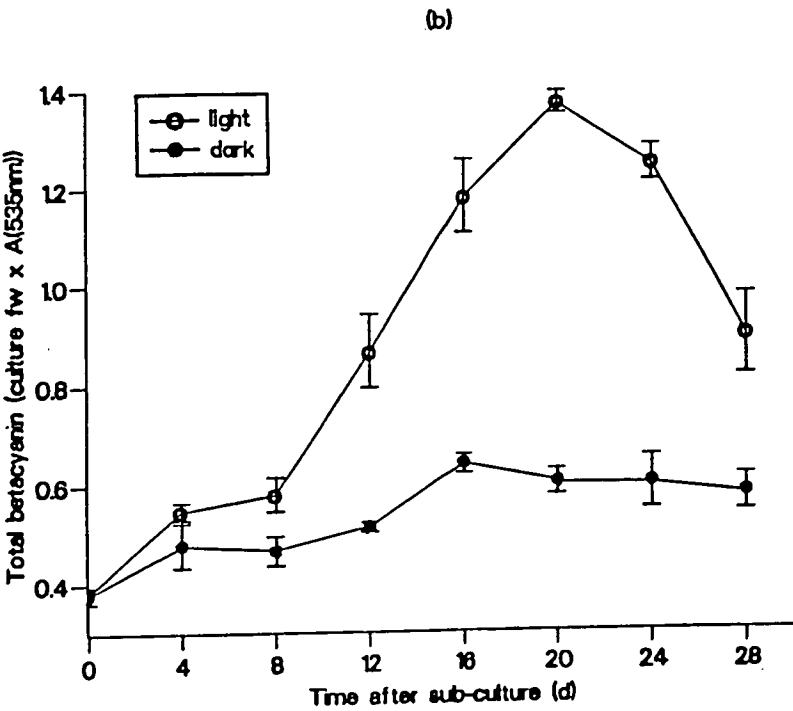
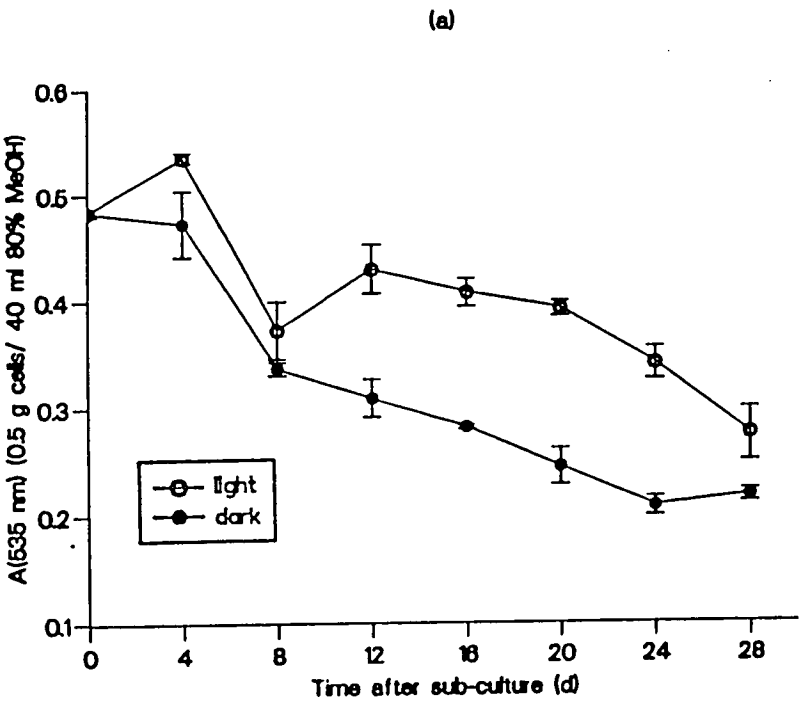
ii. Kinetics of Betacyanin Accumulation.

The results are presented on an OD per unit FW (Fig. 3.2.2a) and on an OD per culture (Fig. 3.2.2b) basis, respectively. Pigment accumulation was in general lower in dark-grown cultures. In light-grown cultures, the betacyanin content per unit FW increased a little during the lag phase. After d 4, it fell sharply until d 8, following the rapid increase in cell number, before increasing again until d 12. It then remained constant until d 20. By this time, the increase in cell number had ceased. In contrast, the OD per culture increased during the lag phase, remained steady until d 8, and increased again until it reached a maximum at d 20. Thereafter, it fell until the end of the experiment.

In dark-grown cultures, the betacyanin content per unit FW remained steady during the lag phase. It then fell sharply until d 8, remained constant until d 12, and continued to fall until the end of the experiment. OD per culture increased a little during the lag phase, remained steady until d 12, and increased again until d 16 when it reached a maximum. It then remained constant until the end of the experiment.

FIG. 3.2.2

Betacyanin accumulation in light and dark-grown suspension cultures of *B. vulgaris* cv Boltardy. (a) OD per unit FW (b) OD per culture. Each point represents the mean $\pm$ s.e. (n=3).



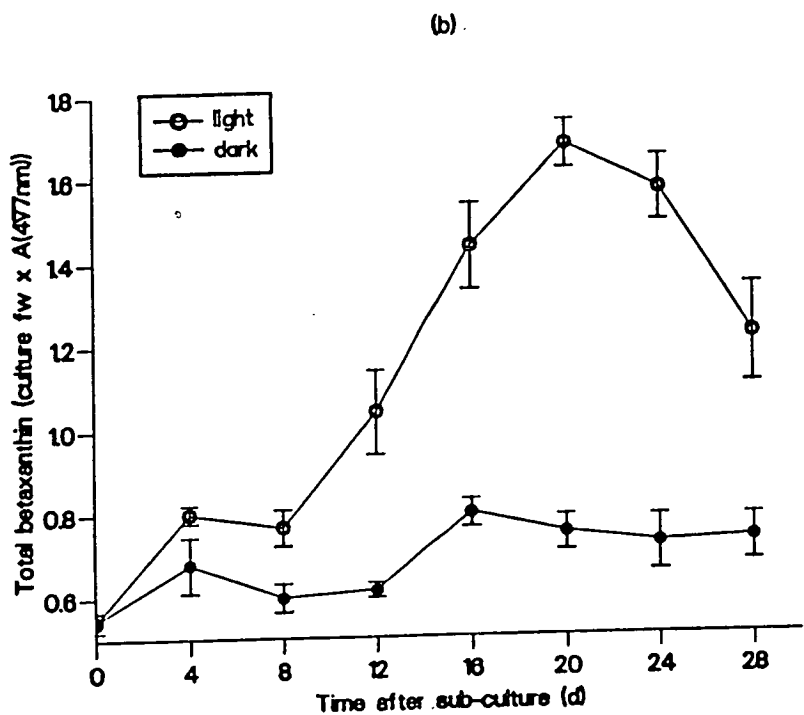
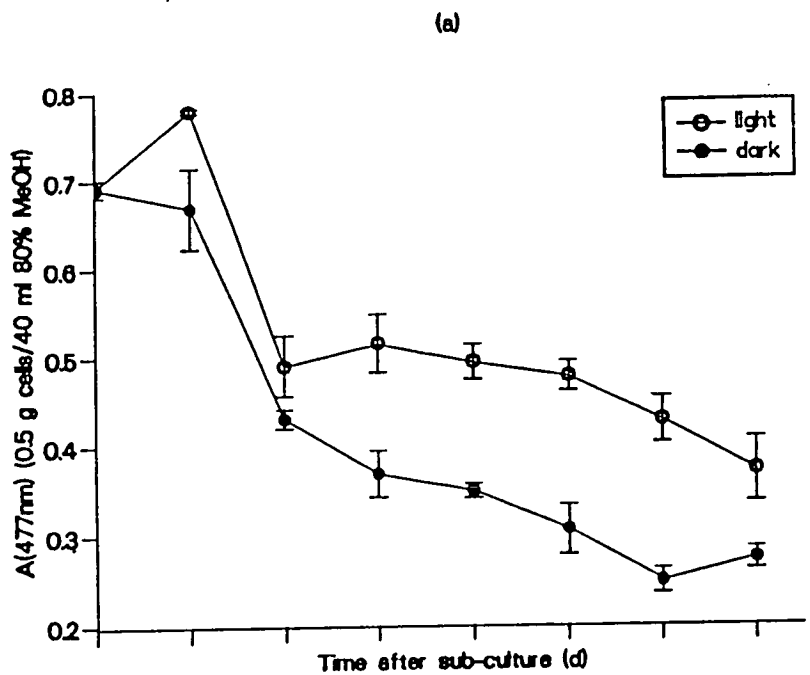
iii. Kinetics of Betaxanthin Accumulation.

The kinetics of betaxanthin accumulation are shown in Fig. 3.2.3 (a) and (b). As for betacyanin, betaxanthin accumulation was generally lower in dark-grown cultures. In light-grown cultures, betaxanthin content per unit FW increased initially during the lag phase, and then fell sharply until d 8. It then remained steady until d 20, before falling again until the end of the experiment. OD per culture increased during the lag phase and remained steady until d 8. It then increased until d 20 when it reached a maximum, and remained steady until d 24, before falling towards the end of the experiment.

In the dark, betaxanthin accumulation per unit FW and per culture showed a similar pattern to betacyanin accumulation.

FIG. 3.2.3

Betaxanthin accumulation in light and dark-grown suspension cultures of *B. vulgaris* cv Boltardy. (a) OD per unit fw (b) OD per culture. Each point represents the mean $\pm$ s.e. (n=3).



Summary

The growth pattern in light and dark-grown cultures was similar, except that cultures in the dark displayed a longer stationary phase and growth was generally less than cultures in the light. In the light, there was a strong correlation between culture growth and total pigment accumulated. However, cellular pigment content seemed to decrease when cell number increased rapidly during the early growth phase, increased a little (betaxanthin) or remained constant (betacyanin) during the late growth phase and dropped again when cells ceased dividing.

However, content per unit FW was significantly lower at the end of the culture cycle than at the beginning of the experiment. Here the change in the amount of light irradiance given to the cultures was the likely cause of this anomaly. Clearly the level of irradiance was sub-optimal for the synthesis and accumulation of the betalains. In order to determine whether this was the case an experiment was designed to examine the effects of varying the level of light given to the cultures and this is described next.

3.2.3 The Effects of Light Irradiance Level on Pigment Accumulation.

The previous experiment showed that cultures grown under a condition of low light irradiance did not achieve the level of pigment accumulated in the original cultures. The aim of this experiment was, therefore, to ascertain the extent to which pigment accumulation is influenced by the level of light irradiance.

Cultures were set up as described in Sub-section 3.2.1. Four levels of irradiance (1, 5, 10, and 20 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) were achieved simply by placing cultures at varying distances from the light source. Light irradiance was measured using a LI-COR Steady State Photometer, Model LI-1600. Each treatment consisted of 3 replicates. Cultures were harvested at d 14, and the pigment content was determined by the methods described in Sub-section 2.5.2.

The results presented in Table 3.2.2 together with the analysis of variance (Appendix 6.7) show that there was a significant influence of light irradiance on both the accumulation of betacyanin and betaxanthin. The results presented in Table 3.2.1 also show that cultures subjected to 20 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light irradiance accumulated the highest levels of pigments, whilst cultures exposed to 1 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light irradiance accumulated the least. There was no difference in the levels of pigment

TABLE 3.2.2.

The effects of various levels of light irradiance on the accumulation of betacyanin (A535) and betaxanthin (A477) in 14 d old suspension cultures of *B. vulgaris* cv Boltardy. Each value is the means of 3 replicates $\pm$ s.e.

IRRADIANCE LEVEL ($\mu\text{mol.m}^{-2}.\text{s}^{-1}$)	A ₄₇₇	A ₅₃₅
1	0.375 $\pm$ 0.015	0.210 $\pm$ 0.003
5	0.543 $\pm$ 0.025	0.276 $\pm$.011
10	0.515 $\pm$ 0.009	0.277 $\pm$ 0.008
20	0.702 $\pm$ 0.008	0.346 $\pm$ 0.003

accumulated in cultures grown either at $5 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ or at $10 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ (controls).

Summary

Exposure of cultures to different levels of light irradiance influences betalain accumulation. Therefore, the use of a high light irradiance would seem to be a good strategy to improve pigment yield. Whether this increase in the ability of the cultures to accumulate enhanced amounts of the betalains at high light intensity was permanent, was examined in the next experiment.

3.2.4 Changes in the capacity of cultures to accumulate betalains after transfer from a low to a high level of irradiance.

It was shown in the preceding experiment that changes in the amount of irradiance can influence the level of betalain accumulated. This provides a ready and controllable means of increasing product yield in *Beta* cultures. However, such a stimulation achieved by transfer from a low to a high level of irradiance may be transitory and this must be ascertained before proceeding with this study. This, was the aim of this experiment.

The experiment was set up as described in sub-section 3.2.1. Suspensions were grown initially under $10 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of irradiance in the first passage, and then exposed to $20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ for the next two consecutive passages. Two flasks were harvested at the end of each passage (14 d) and their betacyanin and betaxanthin contents determined according to the methods described in Section 2.5.2.

Betacyanin and betaxanthin accumulation increased dramatically upon the first transfer of cultures to the higher level of irradiance (Table 3.2.3). However, when cultures were subsequently maintained at this enhanced level of irradiance, the amount of pigment accumulated was reduced to that amount accumulated under a low level of

irradiance.

The results of this experiment show that the enhancement of betalain content by exposure to a high level of irradiance is transitory and in order to stabilize the effects of changing the light environment (irradiance level) cultures should be grown for an extended period at the higher level of irradiance. In the following experiment an attempt will be made to examine the kinetics of culture growth and pigment accumulation in a stabilised light environment, as well as in the dark.

TABLE 3.2.3

The effects of transferring cultures from a low to a higher level of irradiance on betalain accumulation in 14 d old suspension cultures of *B. vulgaris* cv Boltardy. Each value is the mean of two replicates, $\pm$ s.e.

PASSAGE/IRRADIANCE	A ₄₇₇	A ₅₃₅
1ST (10 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$)	0.460 $\pm$ 0.022	0.257 $\pm$ 0.017
2ND (20 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$)	0.702 $\pm$ 0.008	0.346 $\pm$ 0.003
3RD (20 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$)	0.467 $\pm$ 0.007	0.246 $\pm$ 0.003

3.2.5 The effects of light on the growth and betalain accumulation in suspension cultures.

The aim of this experiment was to characterise the kinetics of culture growth and pigment accumulation within one culture cycle under stabilised light and dark conditions..

Cells were pooled from 18 d old stock cultures grown for four passages under $20 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light (2.2.5.1), and suspended in Gamborg's B5 medium supplemented with 0.1 mg.l^{-1} of kinetin, 0.02 mg.l^{-1} of 2,4-D, and 2% sucrose. Forty five flasks were inoculated on d 0 using a sterile wide-bore pipette with the tip removed. A 10 ml aliquot containing ca. 0.8 g (fw) of cells was placed in 15 ml of the same medium contained in a 100 ml Erlenmeyer flask. Cultures were incubated at $25 \pm 1^\circ\text{C}$ on an orbital shaker rotating at $98 \pm 2 \text{ rpm}$ (8 mm throw). Light-grown cultures were supplied with $20 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light irradiance from Thorn natural light fluorescent tubes (Thorn EMI, U.K.), while dark-grown cultures were placed in black plastic boxes and incubated alongside the other flasks on the shaker. Three flasks were harvested from each treatment every 4 d up to and including d 28. Measurements were made of culture fresh weight, cell number, and betalain content according to the methods described in Section 2.4.2 and 2.5.2.

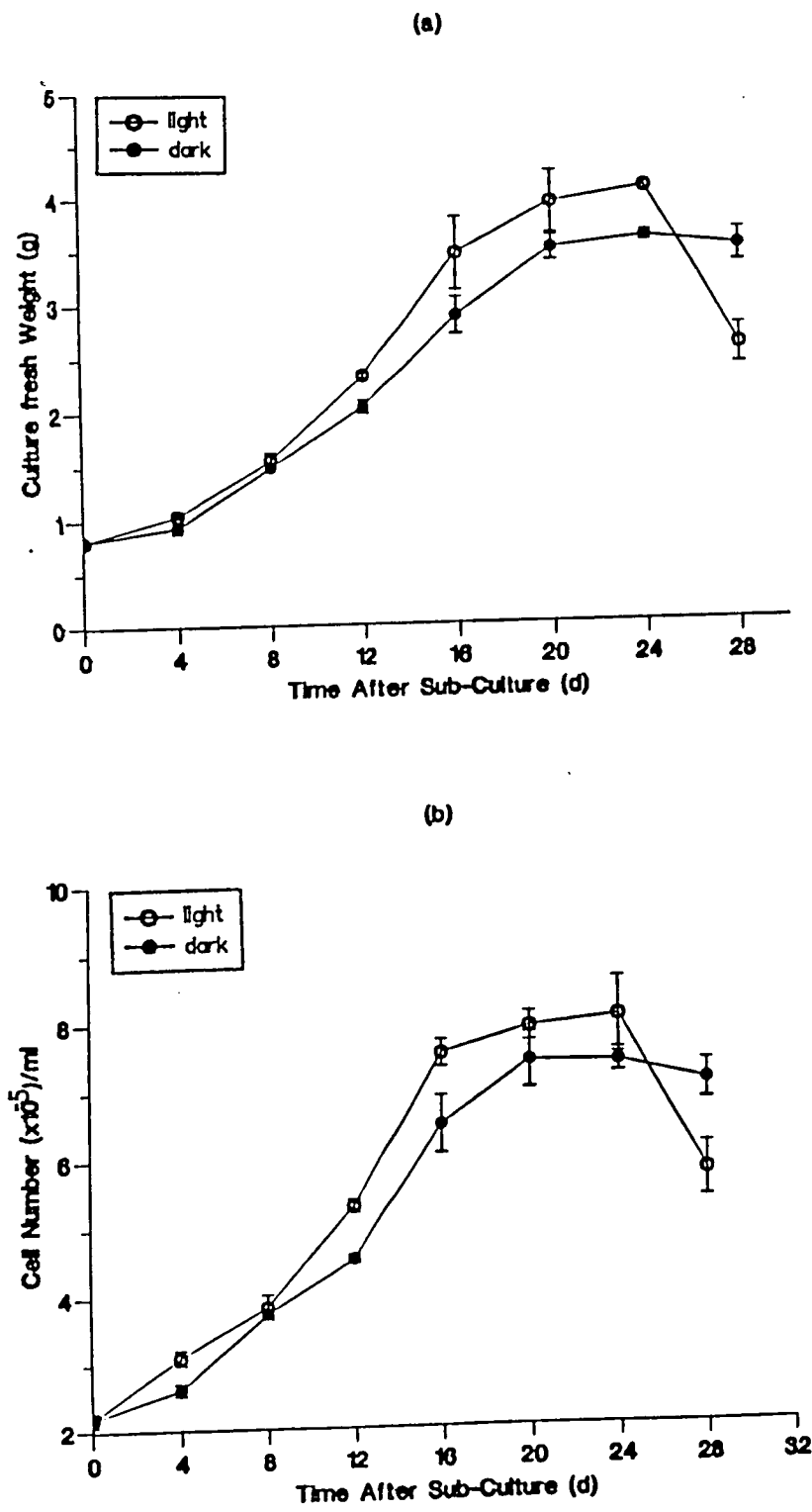
i. The Kinetics of Cell Growth in Light and Dark-Grown Cultures.

The growth pattern of light and dark-grown cultures is shown in Fig. 3.2.4 (a) and (b). In light-grown cultures, there was an increase in cell number by d 4. The length of the lag phase was not clearly defined as the first sample was taken at d 4. Subsequently, the number of cells and fresh weight of the culture increased rapidly until d 16 and then remained constant until d 20. After this period of increase the cells began to autolyse, releasing the red pigments into the medium, then the whole culture turned black within 2-3 d.

The growth pattern of dark-grown cultures was similar to that of light-grown cultures. Growth, in general, was slightly less than in light-grown cultures.

FIG. 3.2.4

Culture growth in light and dark-grown suspension cultures of *Beta vulgaris* cv Boltardy. (a) culture fresh weight (b) cell number. Each point represents the mean $\pm$ s.e. (n=3)



ii. Betacyanin Accumulation in Light and Dark-Grown Cultures.

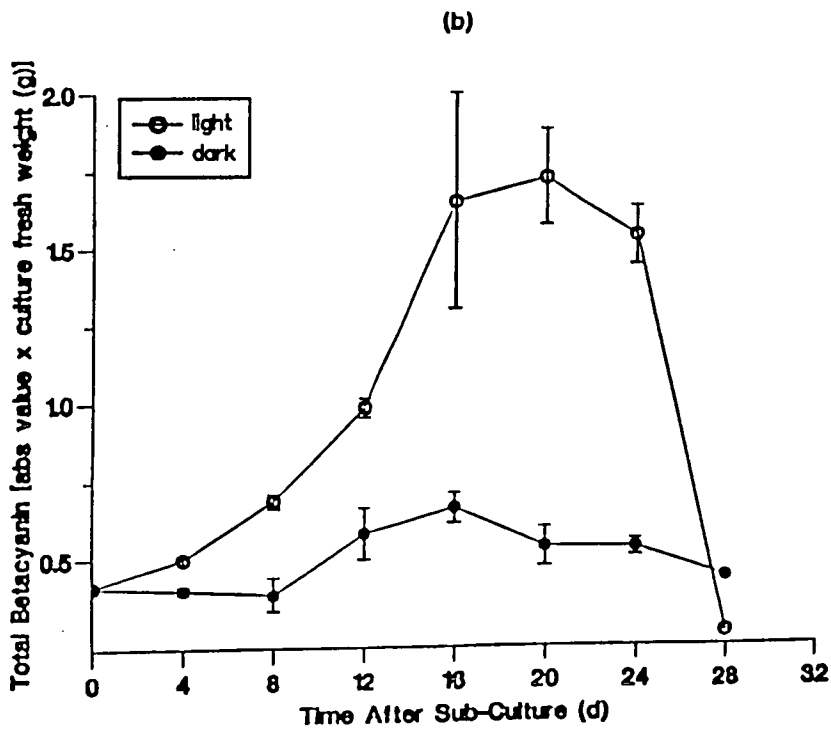
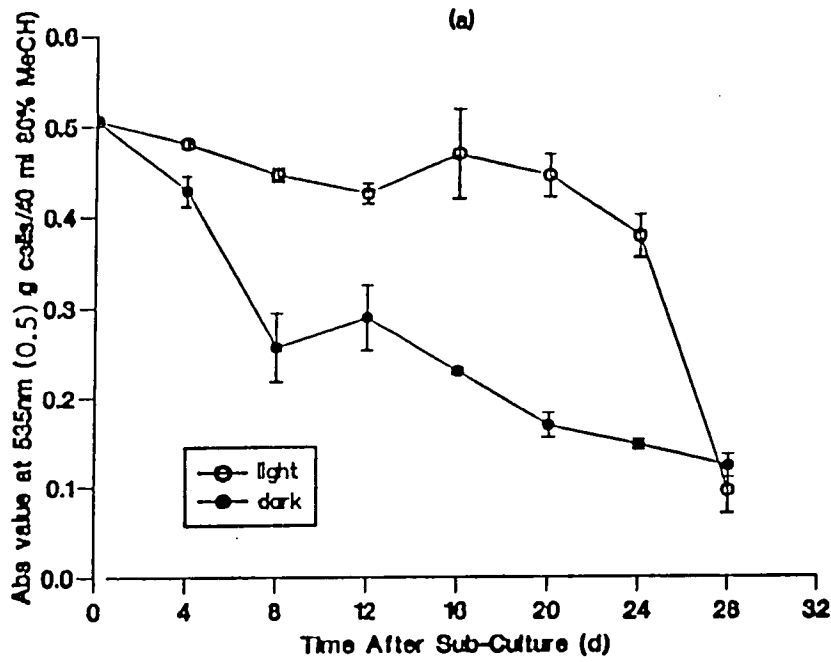
The results presented in Figs. 3.2.5 (a) and (b) show the pattern of betacyanin accumulation on an OD per unit fresh weight and on an OD per culture basis, respectively. The OD per culture was obtained by multiplying the total culture fresh weight by the OD per unit weight, and represents the total production of betacyanin in the culture.

In light-grown cultures the betacyanin content per unit fresh weight fell initially until d 12, and then remained steady until d 20 when it fell again reaching a very low value by the end of the experiment (d 28). In contrast, the total pigment per culture showed a pattern similar to the growth curve and increased until d 16 remaining constant until d 24 then falling steeply reaching a very low value by d 28. The drop in betacyanin at the end of the culture cycle coincides with the visible deterioration of the cultures.

In dark-grown cultures, the pigment per unit weight decreased until day 8, then remained constant until d 12, finally falling until the end of the experiment. The total betacyanin per culture did not increase until d 12, then more or less remained at a steady level for the rest of the experiment.

FIG. 3.2.5

Accumulation of betacyanin in light and dark-grown suspension cultures of *Beta vulgaris* cv Boltardy (a) OD per unit fresh weight (b) OD per culture. Each point represents the mean $\pm$ s.e. (n=3).



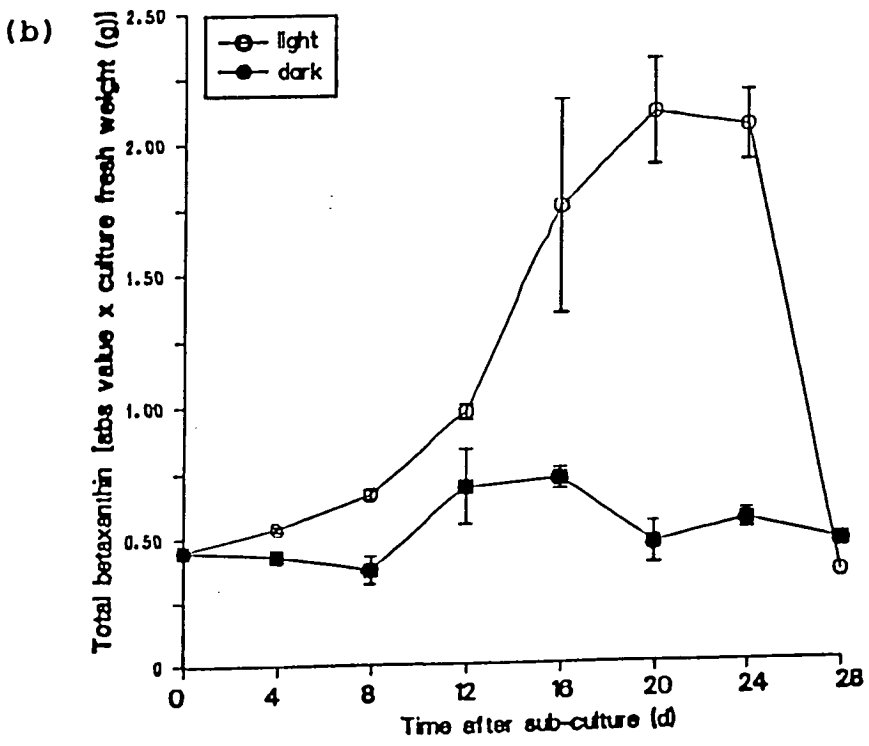
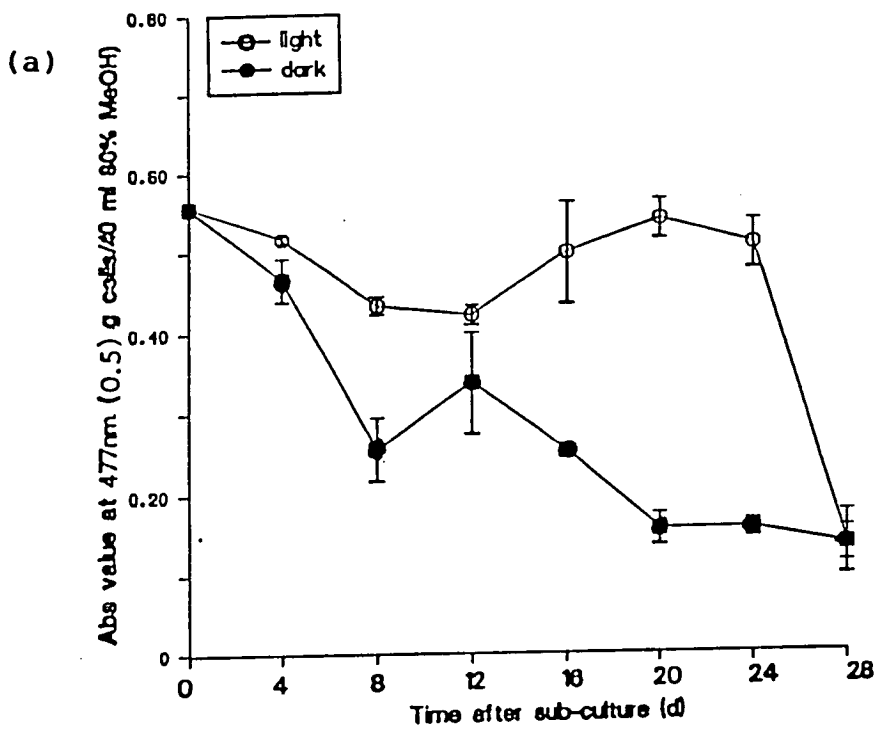
iii. Betaxanthin Accumulation in Light and Dark-Grown Cultures.

The results presented in Figs. 3.2.6 (a) and (b) show the pattern of betaxanthin accumulation on an OD per unit fresh weight and on an OD per culture basis, respectively. In light-grown cultures, the betaxanthin per unit fresh weight fell initially until day 12, and then increased until day 20, before dropping again. The OD per culture shows a trend similar to the growth pattern.

In dark-grown cultures, the OD per unit weight dropped initially until day 8, remained constant until d 12 and then decreased steadily reaching a minimum value on d 24. The betaxanthin per culture started to increase after day 8 and reached a maximum by day 12, remained constant until d 16 and then reaching a minimum value by d 20.

FIG. 3.2.6

Accumulation of betaxanthin in light and dark-grown suspension cultures of *Beta vulgaris* cv Boltardy (a) OD per unit fresh weight (b) OD per culture. Each point represents the mean $\pm$ s.e. (n=3).



iv. TLC Analysis of the Betacyanins and Betaxanthins.

Fig. 3.2.7 shows a TLC separation of the pigments in the aqueous extract obtained from samples taken at regular intervals during the growth cycle. There seems to be no qualitative differences between the light and dark-grown cultures as far as major pigments are concerned, although the intensities of the coloured bands vary.

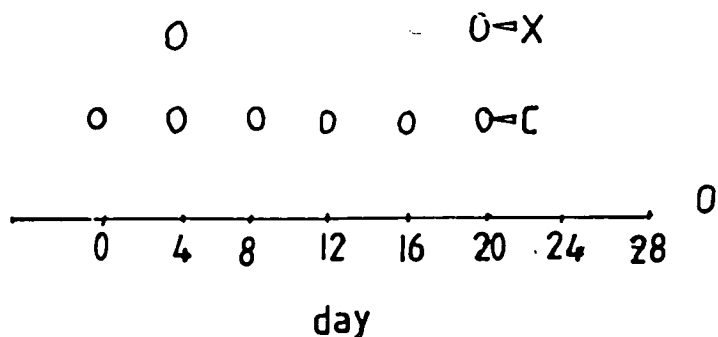
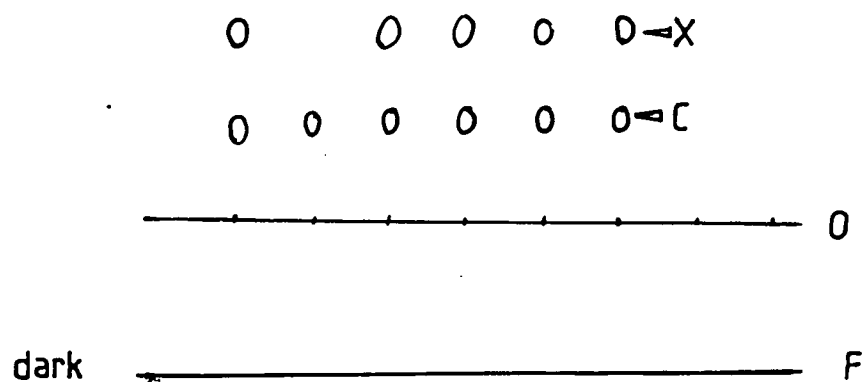
TLC separation of the betacyanin and betaxanthin pigments on cellulose. Extracts were obtained from light and dark-grown cultures. Plates were run in isopropanol-ethanol-acetic acid-water (6:7:6:1) up to 15 cm.

0: origin

X: betaxanthin

light

F



Summary.

The growth pattern of the cultures was similar in light and dark, although cultures in the light grew slightly better. However, pigment accumulation as measured by the OD per unit weight and OD per culture was strongly influenced by the availability of light. In the light there was a marked correlation between growth (cell number and fresh weight) and pigment accumulation (betacyanin and betaxanthin). Cellular content of betacyanin seems to be less affected by the stage of culture growth. This, was more pronounced with betaxanthin, where pigment accumulation was highest during the late growth phase. It is interesting to note that both pigments persisted in dark-grown cultures. Whether light is absolutely necessary for the synthesis of the betalains is still unclear and was examined later in this section. However, the next experiment examines the possibility of targetting light at the most productive period of the culture cycle.

3.2.6 The responsiveness of cultures to light at different growth stages in the accumulation of the betalains.

In the previous experiment, it was shown that the betaxanthin content in cells was at a maximum during the late phases of growth, although this relationship was not so clear with betacyanin. In this experiment, an attempt was made to evaluate the ability of cells at different stages of growth to respond to the light stimulation of betalain synthesis.

Cells were obtained from stock cultures grown under $20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of irradiance. About 2 g FW of cells were inoculated into 50 ml of Gamborg's medium supplemented with 0.1 mg.l^{-1} of kinetin, 0.02 mg.l^{-1} of 2,4-D, and 2% sucrose. Cultures were grown at $25 \pm 1^\circ\text{C}$ on a shaker rotating at $98 \pm 2 \text{ rpm}$, under $20 \pm 2 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.). Cultures were divided into three groups, each receiving a light stimulus during the periods: d 0 - d 4; d 5 - d 16; and d 17 - d 21. In between these periods of light exposure cultures were wrapped in a double layer of Aluminium foil. Each treatment consisted of three flasks. At the end of the experiment (d 21), cultures were harvested and their betacyanin and betaxanthin contents determined according to the methods described in Section 2.5.2

The data presented in Table 3.2.4 show that cultures receiving light in the lag phase accumulated the least of both pigments but all the treatments showed a positive response. Cultures given light during the second and third stages of the growth cycle accumulated a similar amount of betacyanin and betaxanthin which was higher than cells treated in the lag phase. From these results, it is clear that cells in the later stages of the growth cycle are more responsive to the light stimulation of betalain synthesis.

In all the experiments described so far in this section, betalains persisted when cultures were transferred to conditions without light. It may then be possible that biosynthesis of betalains can occur without light. This possibility is tested in the next experiment.

TABLE 3.2.4

A comparison on the ability of cells at different growth stages to accumulate betacyanin (A₅₃₅) and betaxanthin (A₄₇₇) after 21 d of culture. Each value is the mean OD (0.5 g cells/20 ml 80% MeOH) of three replicate $\pm$ s.e.

PERIOD OF LIGHT STIMULATION	A ₄₇₇	A ₅₃₅
d 0 - d 4	0.756 $\pm$ 0.009	0.564 $\pm$ 0.042
d 5 - d 16	0.891 $\pm$ 0.047	0.712 $\pm$ 0.062
d 17 - d 21	0.937 $\pm$ 0.023	0.719 $\pm$ 0.028

3.2.7 Pigment Accumulation in Cultures Grown Continuously in the Dark.

In previous experiments, cultures grown in the dark accumulated both betacyanin and betaxanthin, although the levels diminish towards the end of the culture cycle. In this experiment, cultures were grown continuously in the dark for a prolonged period and the pigment levels measured at the end of each culture cycle. From this it should be possible to determine if light is an absolute necessity for the biosynthesis of betalains in these cultures.

Cultures originated from suspension stock cultures grown under $10 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light irradiance. Two g FW of cells were inoculated under green safelight (Lee filters HT 139 primary green, LEE Colortran International, London) into 250 ml flasks containing 50 ml of Gamborg's B5 medium supplemented with 0.1 mg.l^{-1} kinetin and 0.02 mg.l^{-1} 2,4-D, and maintained in black plastic boxes as described in 2.2.5.1. At each sub-culture time (d 14) betacyanin and betaxanthin contents of the cultures were determined according to the methods described in Section 2.5.2. A microscopic examination of the cells was also carried out, as described in 3.1.1.4, to determine the pattern of pigmentation in the cultures.

Results are presented in Table 3.2.5. The levels of betaxanthin and betacyanin accumulated at the end of the first passage decreased by about 60% and 55%, respectively. This drop in the pigment levels continued in the second passage, and by the end of the fourth passage of culture, the levels of betaxanthin and betacyanin accumulated were only 12% and 10%, respectively, that of the original cultures. Level of pigment accumulation remained more or less constant for the rest of the experiment up to the fifth passage. Observations on these cultures (Fig. 3.2.9) showed that the cells remained coloured in the dark. At the end of the experiment, pigmented cells were estimated to be 55% of the cell population, whilst the rest of the cells were colourless. From this population of pigmented cells, less than 20% were highly pigmented, whilst the others showed a weak pigmentation. This contrasts with the original cultures which comprised 80% pigmented, and 20% unpigmented cells. Within the pigmented cell population, 75% of the cells were highly pigmented.

From these results it would appear that cultures are capable of synthesising betalains in the dark. However, the amount of pigment accumulated was far less than in light-grown cultures due to the lower proportion of cells accumulating the pigments, as well as a lower average amount accumulated in the pigmented cells.

TABLE 3.2.5

Betacyanin (A₅₃₅) and betaxanthin (A₄₇₇) accumulation in suspension cultures grown continuously in the dark. The length of a passage was 14 d. Each OD value was determined from a pooled sample taken from two flasks .

PASSAGE NO.	A ₄₇₇	A ₅₃₅
0	0.556	0.506
1	0.328	0.285
2	0.068	0.068
3	NA*	NA*
4	0.065	0.054
5	0.068	0.049

* OD not available

Summary

1) Light does not appear to be an absolute prerequisite for the accumulation of betalains. However, light does considerably enhance the level of pigment accumulated.

2) The lower amount of pigment accumulated in cultures grown in the dark is due to a lower proportion of pigmented cells, as well as a lower average cellular pigment content.

3) The level of light irradiance has an effect on pigment accumulation. The transfer of cultures from a low to a high level of irradiance resulted in a higher accumulation of the betalains. However, this change in the capacity to accumulate a higher amount of pigments was not sustained.

4) There is a strong correlation between growth and total pigment accumulated in light-grown cultures. Betacyanin is accumulated throughout the growth cycle, however, betaxanthin seems to accumulate more during the later stages of growth.

5) Cells in the later stages of growth were more responsive to the light stimulation of betalain accumulation.

6) No qualitative differences in betalains were detected

between light and dark-grown cultures.

3.3 THE EFFECTS OF LIGHT ON THE INCORPORATION OF ^{14}C -TYROSINE INTO BETACYANIN AND BETAXANTHIN.

In the previous section, light was shown to be an important factor determining the level of betacyanin and betaxanthin in suspension cultures. In this section, radioactively-labelled tyrosine, supposedly a primary precursor of the betalains (see Böhm and Rink, 1988) was used to study the pattern of distribution of label, and hence the biochemical changes that lead to the formation of betacyanin and betaxanthin in cells at different stages of growth, in light and dark.

Eighteen cultures were prepared by inoculating ca. 1 g FW of cells obtained from 14 d old stock cultures into 25 ml of Gamborg's B5 medium in 100 ml Erlenmeyer flasks containing 0.1 mg.l^{-1} Kinetin and 0.02 mg.l^{-1} 2,4-D. Suspensions were cultured at $25 \pm 1 \text{ }^{\circ}\text{C}$ on a shaker rotating at $98 \pm 2 \text{ rpm}$ (8 mm amplitude). Half of the cultures were placed under $20 \pm 2 \text{ } \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of light supplied by Thorn natural light fluorescent tubes (Thorn EMI, U.K.), and the other half in the dark, in black plastic boxes. At d 3, d 14, and d 21, 3 flasks for each treatment were injected under sterile condition with 1 μCi of ^{14}C -(U)-L-tyrosine. After a 24 h labelling period, the cultures were harvested and their FW determined according to the methods described in 2.4.2. Incorporated

radioactivity was determined in the spent medium, the 80% MeOH fraction, protein, lignin, and cell residue, as shown in Fig. 2.5.1. Extraction of the betalains and amino acids, protein and lignin were carried out according to the methods described in 2.5.1.1, 2.5.1.2 and 2.5.1.3, respectively. Separation of compounds in the 80% MeOH extract was carried out by TLC on cellulose (2.5.3.1), and to facilitate identification, L-DOPA, ^{14}C -L-tyrosine, and ^{14}C -p-coumaric acid were separated on the same plate. Distribution of label in the relevant compound was analysed by autoradiography (2.5.3.5), liquid scintillation counting (sub-section 2.5.4) and RITA (2.5.3.5).

i. Distribution of label

Analysis of culture components by liquid scintillation counting (Table 3.3.1) shows that ^{14}C -(U)-L-tyrosine was metabolised and converted into a range of compounds by both primary and secondary metabolic pathways. Between 4.5 - 17.3% of the original total activity was present in the 80% Methanol extract. Incorporation into protein at all sampling time was rather high, between 11.5 - 24.3%, which shows that a substantial proportion of the precursor was channelled into primary metabolism. Incorporation into lignin, an important sink in tyrosine metabolism

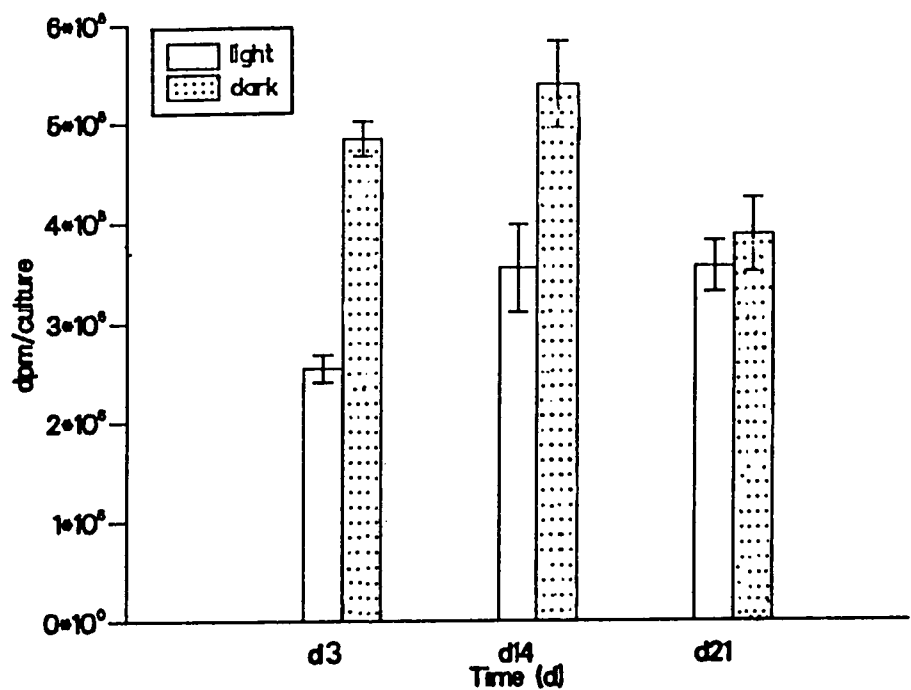
(Young *et al.*, 1966), was between 3.6 - 38.3%. In the final residue, which was black and probably included melanin, another end product of tyrosine metabolism (Young *et al.*, 1966) the radioactivity detected was between 11.5 - 24.3%. Finally, the activity in the spent medium, which contained unused ^{14}C -L-tyrosine together with labelled metabolites released from the cells, was between 1.7 - 7.6%. The levels of incorporation at any particular growth stage were higher in the light in the 80% MeOH fraction and lignin, but were lower for protein.

ii. Incorporation into protein.

Fig. 3.3.1 shows that the incorporation of label was much higher in dark-grown cultures at d 3 and d 14 prior to the onset of the stationary phase (d 21). Light-grown cultures at d 3 incorporated ca. 12% of the total activity applied into protein. The amount incorporated in the light was higher in d 14 cultures, but similar in d 21 cultures. In dark-grown cultures, incorporation into protein at d 3 was ca. 22% of total activity applied, and ca. 18% in d 14 cultures, and less in d 21 cultures.

FIG. 3.3.1

Incorporation of radioactivity into protein by suspension cultures of *B. vulgaris* cv. Boltardy grown in the light and in the dark. Cultures were labelled with ^{14}C -(U)-L-tyrosine for 24 h at d 3, d 14, and d 21. Each value is the mean of 3 replicates $\pm$ s.e.



iii. Incorporation into betacyanin.

Fig. 3.3.2 shows that the levels of incorporation into betacyanin were always higher in the light than in the dark and the highest incorporation was achieved by cultures at d 14. Incorporation into cultures at d 3 and d 21 was less than at d 14.

iv. Incorporation into betaxanthin.

As with betacyanin, incorporation was always higher in light-grown cultures. However, the effect of culture age contrasted with that of betacyanin. The level of incorporation (Fig. 3.3.3) increased as the cultures grew older and was maximal at d 21.

v. Incorporation into lignin.

The pattern of incorporation into lignin shown in Fig. 3.3.4, has also been reported for many other systems accumulating a secondary metabolite (eg Phillip and Henshaw, 1977; Lindsey and Yeoman, 1986) and is similar to that of betaxanthin. The amount of incorporation increased through the culture period and was maximal at d 21.

FIG. 3.3.2

Incorporation of radioactivity into betacyanin (A_{535}) by suspension cultures of *B. vulgaris* cv. Boltardy grown in the light and in the dark. Cultures were labelled with ^{14}C -(U)-L-tyrosine for 24 h at d 3, d 14, and d 21. Each value is the mean of 3 replicates $\pm$ s.e.

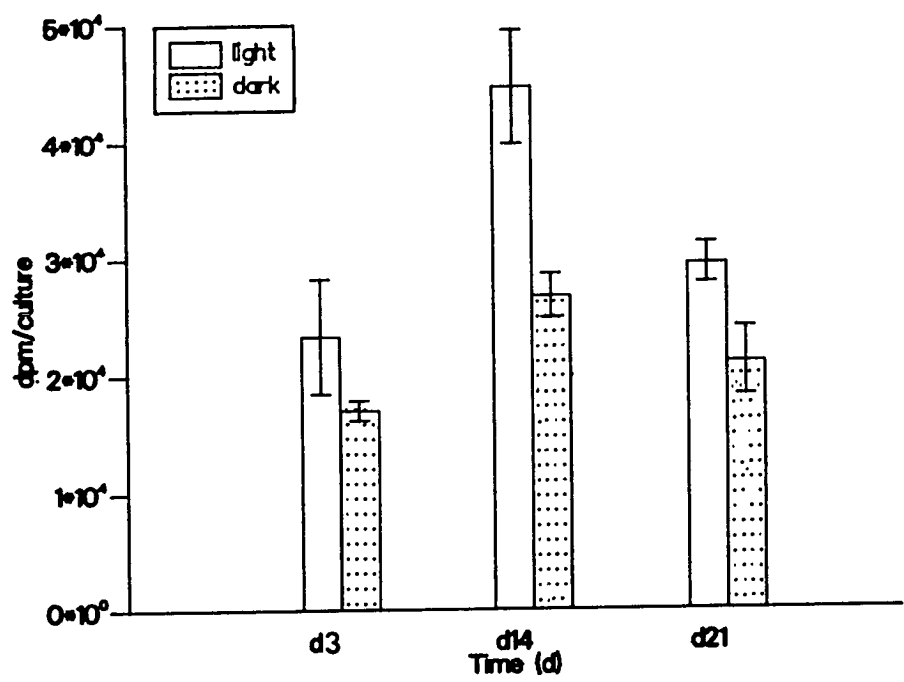


FIG. 3.3.3

Incorporation of radioactivity into betaxanthin (A_{477}) by suspension cultures of *B. vulgaris* cv. Boltardy grown in the light and in the dark. Cultures were labelled with ^{14}C -(U)-L-tyrosine for 24 h at d 3, d 14, and d 21. Each value is the mean of 3 replicates $\pm$ s.e.

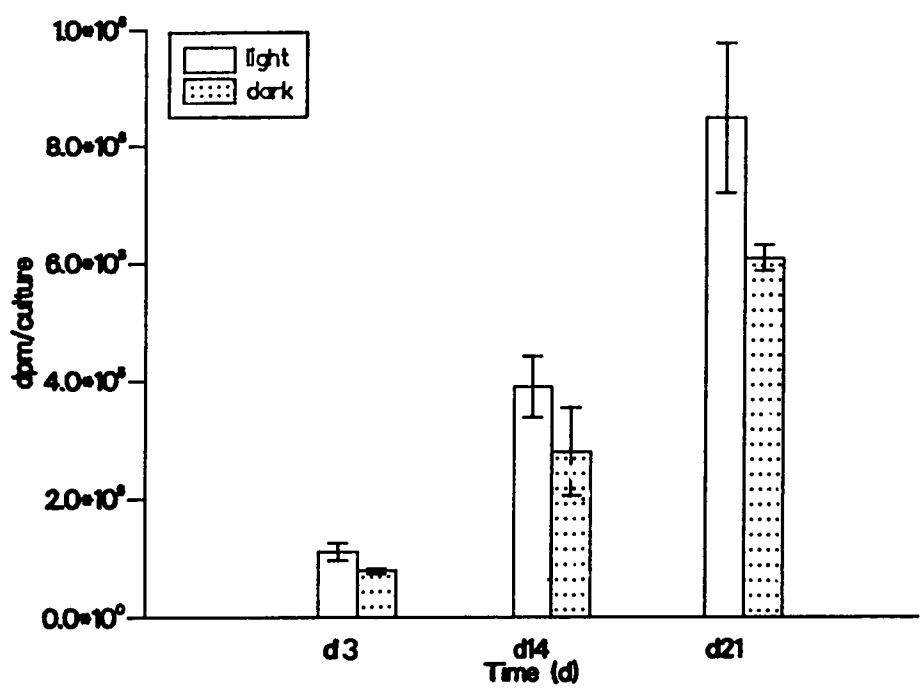
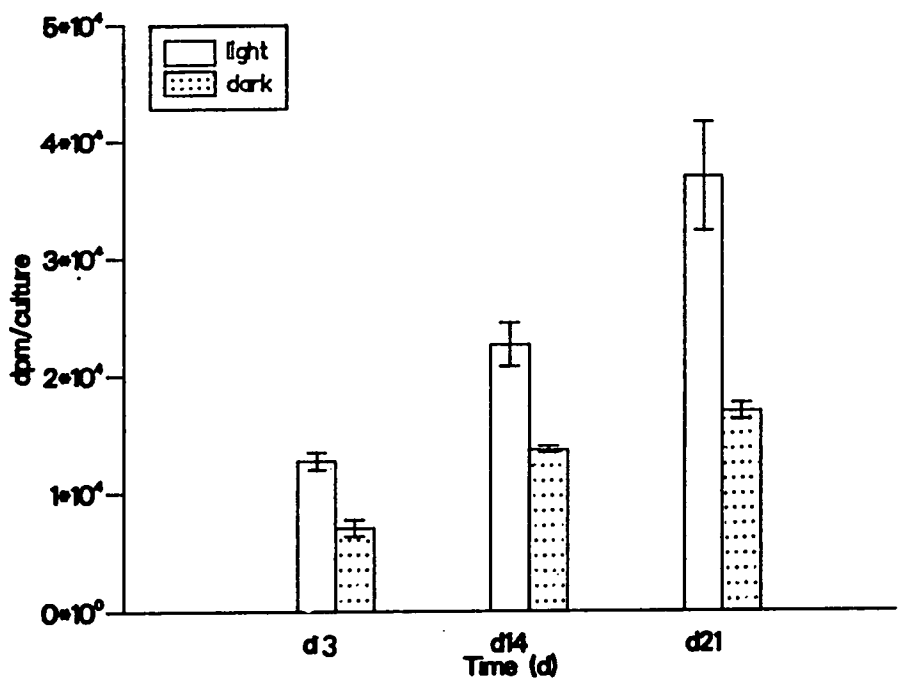


FIG. 3.3.4

Incorporation of radioactivity into lignin by suspension cultures of *B. vulgaris* cv. Boltardy grown in the light and in the dark. Cultures were labelled with ^{14}C -(U)-L-tyrosine for 24 h at d 3, d 14, and d 21. Each value is the mean of 3 replicates $\pm$ s.e.

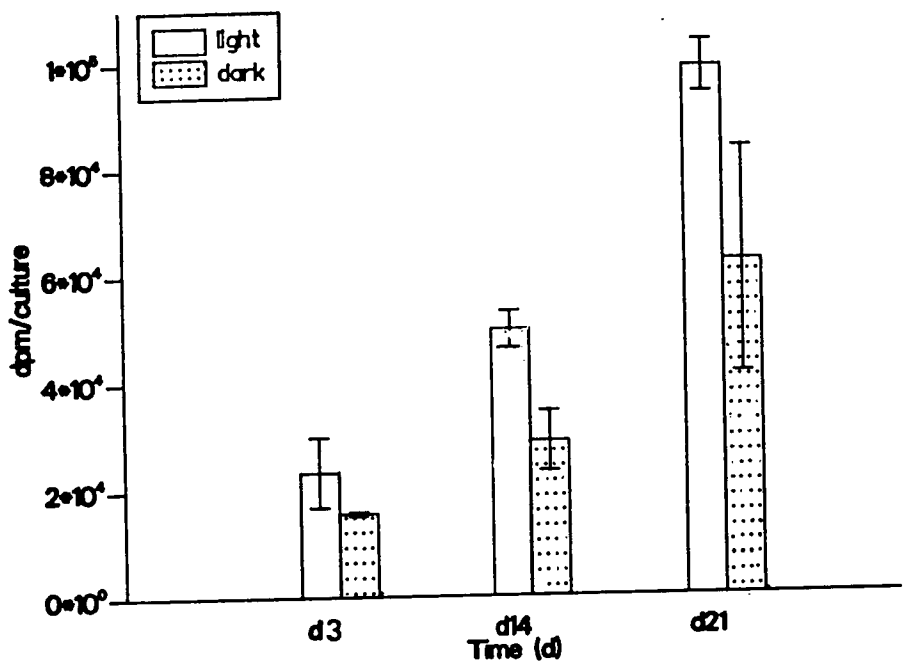


vi. Residual Radioactivity in tyrosine.

The amount of ^{radioactivity in}unincorporated tyrosine (Fig. 3.3.5) in the cell extract increased at each sampling time through the culture cycle. Small differences between the amount in the dark and the light were evident at all sampling times with the highest amount always in the light treatment.

FIG. 3.3.5

Amount of radioactivity in unincorporated tyrosine in suspension cultures of *B. vulgaris* cv. Boltardy grown in the light and in the dark. Cultures were labelled with ^{14}C -(U)-L-tyrosine for 24 h at d 3, d 14, and d 21. Each value is the mean of 3 replicates $\pm$ s.e.

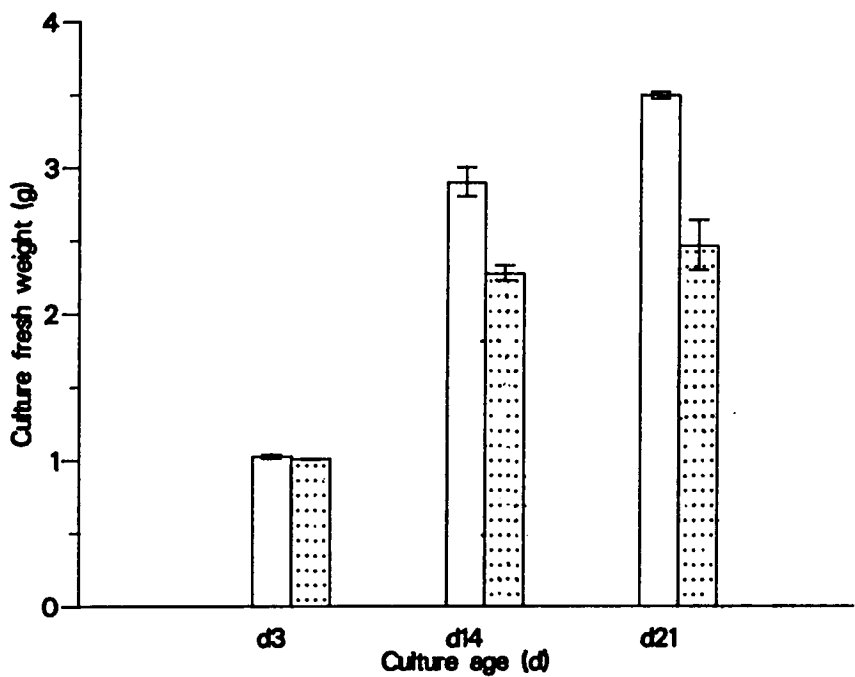


vii. Growth of Cultures.

Fig. 3.3.6 shows the growth of suspension cultures in terms of culture FW. At d3 there was hardly any increase in culture FW (inoculum size was ca. 1 g FW) in the light and in the dark. However, cultures in the light showed a large increase in their FW by d 14, and a small increase between d 14 and d 21. Cultures in the dark showed a large increase by d 14, but the culture FW remained constant at d 21. At d 14 and d 21 cultures in the light showed a better growth in terms of culture FW.

FIG. 3.3.6.

Growth of suspension cultures of *B. vulgaris* cv Boltardy. Each value is the mean of 3 replicates $\pm$ s.e., taken at d3, d14, and d21.



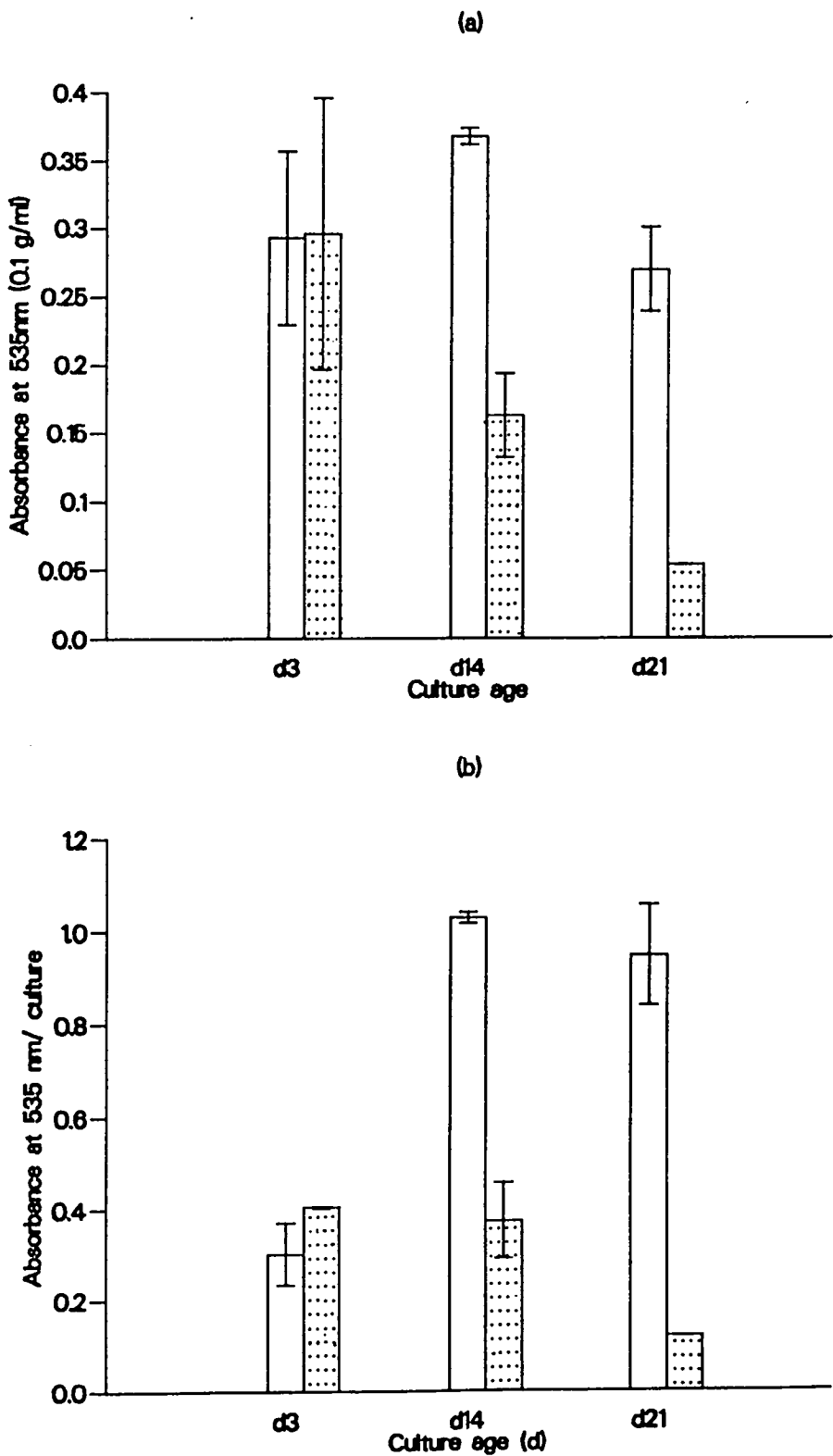
viii. Betacyanin Accumulation.

Fig. 3.3.7 (a) shows the accumulation of betacyanin on a per g FW basis. In the light, betacyanin accumulation per unit FW was similar at d 3, d 14 and d 21. There was no difference in the level of accumulation in the latter cultures. Meanwhile, cultures in the dark showed a reduced level of accumulation per unit FW as the culture progressed.

On a per culture basis (Fig. 3.3.7b) betacyanin accumulation in the light increased sharply at d 14 and was slightly higher at d 21. In the dark, the level of accumulation in d 3 and d 14 cultures were the same, but were less in d 21 cultures.

FIG. 3.3.7.

Betacyanin accumulation in suspension cultures of *B. vulgaris* cv Boltardy at d 3, d 14 and d 21 on (a) per g FW, and (b) per culture basis. Each value is the mean of 3 replicates $\pm$ s.e.



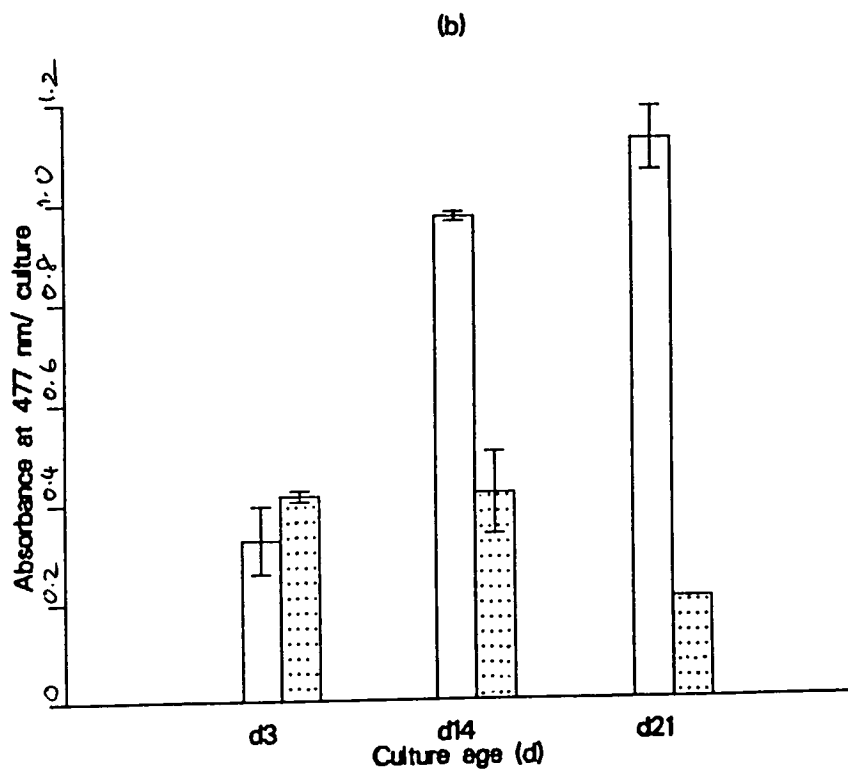
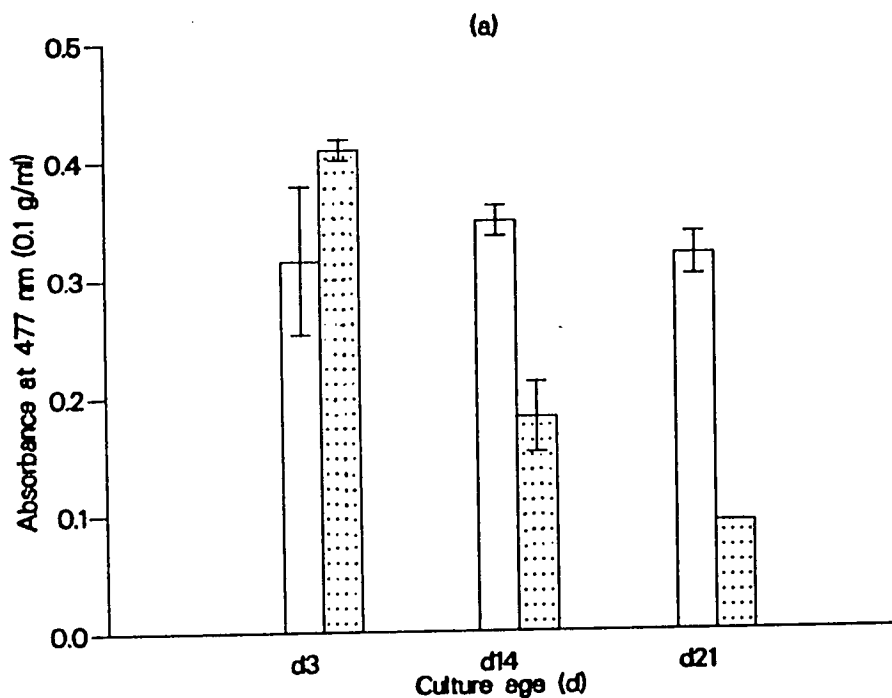
ix.. Betaxanthin Accumulation.

Fig. 3.3.8 (a) shows the level of betaxanthin accumulation on a per g FW basis. In the light, there was no difference in the level of accumulation between cultures of different ages, however, those in the dark showed a reduced level of accumulation as the culture progressed.

On a per culture basis (Fig. 3.3.8-b), d 14 cultures in the light showed a large increase in the level of betaxanthin accumulated. Cultures at d 21 showed no further increase. In the dark, the level of accumulation was the same in d 3 and d 14 cultures, but dropped in d 21 cultures.

FIG. 3.3.8.

Betaxanthin accumulation in suspension cultures of *B. vulgaris* cv Boltardy at d 3, d 14 and d 21 on (a) per g FW, and (b) per culture basis. Each value is the mean of 3 replicates $\pm$ s.e.



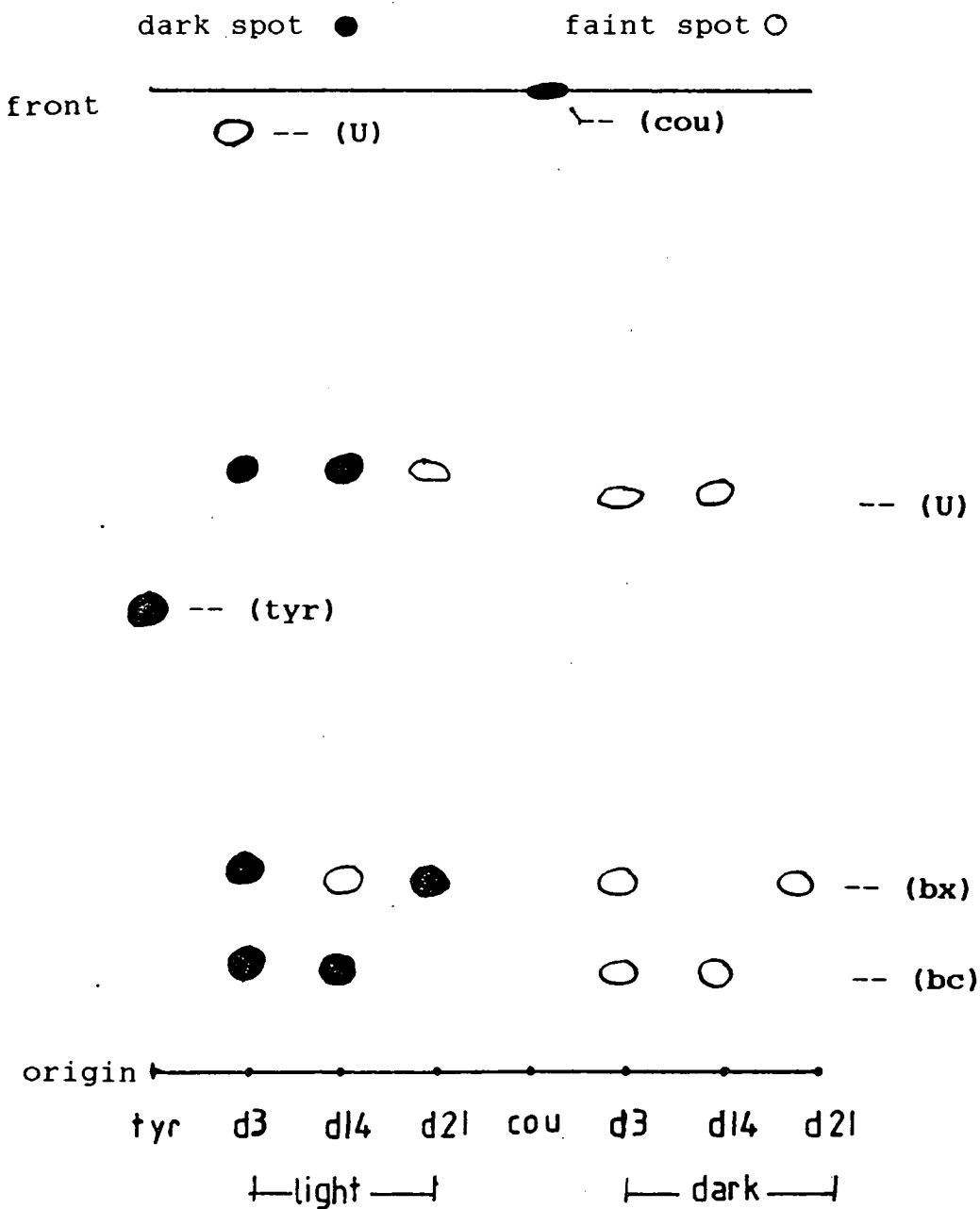
x. RITA and Autoradiographic analysis of label distribution.

Compounds in the 80% MeOH extracts were separated by TLC (sub-section 2.5.3) and the chromatogram subjected to RITA analysis. Results of the RITA scan showed that the amount of radioactivity present was too low to give any clear result.

Fig. 3.3.9 is a diagram of an autoradiograph prepared by exposing a one dimensional TLC separation of the components in the 80% MeOH fraction. The activity in all the compounds as indicated by the intensity of the spots shows that the incorporation rate at any specific growth stage was higher in the light. Activity was detected in betacyanin in d 3 and d 14 cultures in both light and dark. For betaxanthin, activity was detected in cultures of all ages in the light. However, cultures at d 14 showed a fainter spot. In the dark, d 3 and d 21 cultures showed faint but visible spots. No spots were detected that corresponded with L-tyrosine or p-coumaric acid. However, unknown spots with R_f 's around 0.75 appeared in all light-grown cultures and d3 and d 14 cultures grown in the dark. Another distinct spot was observed in d 3 light-grown cultures, which was close to the front, but did not coincide with p-coumaric acid.

FIG. 3.3.9.

A diagram of an autoradiograph of a TLC plate showing the distribution of radioactivity, in compounds in the 80% MeOH extracts, obtained from light and dark-grown cultures labelled with ^{14}C -tyrosine. (bc) betacyanin, (bx) betaxanthin, (tyr) tyrosine, (cou) p-coumaric acid, (U) unknown.



Summary.

The results of this section show that both betacyanin and betaxanthin were synthesised at all three stages of the culture cycle in the light and in the dark. However, the rate of synthesis of both betalains as measured by the incorporation of labelled tyrosine was higher in the light. It was also shown that the patterns of synthesis of betacyanin and betaxanthin were different in the light, with maximum incorporation into betacyanin occurring at d 14 during active growth, while incorporation into betaxanthin was highest at the downturn of growth. Incorporation of ^{14}C -(U)-L-tyrosine into lignin was also shown to be higher in the light and tended to increase with time. Meanwhile, incorporation of ^{14}C -(U)-L-tyrosine into protein was generally higher in the dark. In the light, higher incorporation occurred towards the end of the culture growth, whilst the opposite was observed for cultures grown in the dark.

CHAPTER FOUR
DISCUSSION

Many species of the *Centrospermae* are capable of accumulating betalains, either betacyanins (red-violet) or betaxanthins (yellow-orange), or both (Piattelli, 1981). *Beta vulgaris* L. , the species chosen for this study, accumulates both betacyanins and betaxanthins simultaneously. This property of *Beta* plants to accumulate both betacyanins and betaxanthins can also be observed in cultures of the species (Girod and Zryd, 1987) and enables studies to be made of the biosynthesis of these pigments. In culture, the accumulation of the betalains in a variety of species from the *Centrospermae* can either occur simultaneously with growth (Sakuta *et al.*, 1981; Berlin *et al.*, 1986) or when growth is slowing down (eg. Constabel and Nassif-Makki, 1971; Hirose *et al.*, 1990). Also, in all of the studies carried out so far light has been shown to considerably enhance betalain yield in cultures, although it is still uncertain whether light is an essential pre-requisite for betalain synthesis (see review by Böhm and Rink, 1988). In this discussion the production of betalains is considered in relation to growth and light.

The discussion is divided into three sections. In the first (4.1) the factors affecting the growth and accumulation of betalains in cultured cells are examined. The second section (4.2) considers the relationship between growth and the accumulation of betalains in suspension cultures. Finally (4.3) the discussion

concentrates on the effects of light on the ability of cultures to synthesise and accumulate betacyanins and betaxanthins.

4.1 FACTORS AFFECTING GROWTH AND ACCUMULATION OF BETALAINS IN CULTURED CELLS OF *Beta vulgaris*

i. Source of Explant

In higher plants, especially the dicotyledons (Allan, 1991), almost any part of the plant can be used to initiate callus (Butenko, 1968; Street, 1969; and Thomas and Davey, 1975). However, young tissues are more responsive than mature tissues (Wernicke and Brettell, 1980; Lindsey and Yeoman, 1985). In this study, callus was successfully initiated from hypocotyl, root, petiole and cotyledon explants of *B. vulgaris* L. cv Egyptian Turnip, however, callus was induced most readily from the hypocotyl followed by root, cotyledon and petiole (3.1.1.1). Similar observations were made by Welander (1974) when comparing hypocotyl and root explants of *B. vulgaris*, while Haddon and Northcote (1976) found that in bean (*Phaseolus vulgaris*) the most rapid rate of callus initiation was obtained from hypocotyl, epicotyl and shoot, with a less rapid response from pith and cotyledon explants. Differences in the ability of different tissues to respond in culture is widely acknowledged (eg.

Krikorian *et al.*, 1987) and could be explained by the fact that in certain tissues, such as the hypocotyl, there are more actively dividing cells compared, for example to the cotyledon (Haddon and Northcote, 1976). Another reason could be related to the endogenous level of hormones and sensitivity of the tissue (Welander, 1974). The hypocotyl, for instance, due to its activity in metabolite transport, might contain the necessary growth factors, the amount of which in some other tissues may be sub-optimal. In mature plants, stem explants have been shown to be an important source of callus (see review by Butenko, 1968). However, King (1980) has suggested that this differential response is due to random, critical events that happen during the early phases of explant exposure to the culture medium.

In this study, irrespective of explant origin, callus obtained was similar in appearance. Initially the calluses were cream in colour, with speckles of red and yellow cells which later dominated the callus, thus indicating their ability to accumulate betalains. This is not surprising, perhaps, since betalains are found all over the plant (Appendix 6.8) and seedlings. The absence of pigments initially in the proliferating callus could be due to a disturbance in the expression of primary and secondary metabolism (Aitchison *et al.*, 1977), as conditions became stabilised and the biochemical totipotency of the cells was realised (Zenk, 1978; Lindsey

and Yeoman, 1985). While there is little evidence to suggest that explant source significantly affects culture growth (Haddon and Northcote, 1976; Holden, 1989), reports on the influence of culture origin on the production of secondary metabolites are conflicting. On the one hand, it was shown that secondary product formation is influenced by the plant part used to initiate the culture (eg. Nagel and Reinhard, 1975; Kadkade 1982) and on the other, differences were not observed (Speake *et al.*, 1964; Dhoot and Henshaw, 1977), although the levels of accumulation in intact organs were different.

So far this discussion has centered around the influence of various explant sources, supposedly of the same genotype. At a different level, the ability of cultures to produce secondary metabolites is broadly determined by their genetic composition (Lindsey and Yeoman 1985). In this study (3.1.1.4) it has been shown that although cv Boltardy and Burpee's Golden are capable of accumulating both betacyanin and betaxanthin, and despite presumably sharing the same metabolic pathway the ratio between these pigments is quite different, i.e. 1:1.8 and 1: 3.9 respectively. This result suggests that the ability of cultures to synthesise and accumulate particular metabolites depends on the biosynthetic capability of the plant. Zenk *et al.* (1977) have shown that cultures derived from high-yielding *Catharanthus roseus* plants accumulated greater amount of serpentine than cultures

from low-yielding plants. A similar observation was reported for the nicotine content of tobacco cultures (Kinnersley and Dougall, 1980). However, Constabel (1967) has interestingly shown that colourless explants of *B. vulgaris* were able to produce betalains suggesting that the secondary metabolism of a cell culture may be independent of the biochemical status of the explant (Böhm and Rink, 1988). Ibrahim *et al.* (1971) have further shown that callus derived from tuber, cotyledon or storage roots of carrot and Jerusalem artichoke were capable of *de novo* synthesis of anthocyanin.

ii. Effects of the basal medium

Plant cell culture media can influence growth and many other plant processes, including the secondary metabolism of cell cultures (eg. Zenk *et al.*, 1977; Fujita and Tabata, 1987). It has been shown (see review by Böhm and Rink, 1988) that certain basal media support the formation of betacyanin in *Portulaca grandiflora* callus, while others did not. In this study, cultures were grown on MS, SH, B5 and WH basal salts formulations, supplemented with the same amount of PGR and sucrose, and the effects on growth and pigment formation were examined.

SH was shown (3.1.2.1) to be superior to the other media in supporting growth, followed closely by B5. MS and WH media support growth to the same extent but less than SH and B5. However, cultures in WH medium were the most intensely pigmented (red-violet) followed by those grown in B5, SH and MS media, respectively. The contrasting effects of WH and SH media on growth and pigment accumulation were obvious. SH medium, is apparently highly capable of supporting growth but does not seem to be suitable for pigment accumulation, whilst the opposite seems to be true with WH medium. In this case, the relationship between growth and secondary metabolite accumulation influenced by media composition was seen to be antagonistic, as generally believed in plant secondary metabolism (Phillips and Henshaw, 1977; Yeoman *et al.*, 1980; Lindsey and Yeoman, 1983). A similar observation was made with *Lithospermum* cell cultures (Fujita and Tabata, 1987) in which cultures in WH medium yielded shikonin, but none was present in cultures grown in LS medium. These effects of the culture medium on growth and product accumulation could be explained by the salt composition and concentration, especially that of phosphate and nitrate in WH and SH medium, respectively. Furthermore Böhm and Rink (1988) have pointed out that SH, and a modified MS medium with four times the phosphate and half the nitrate concentration (Nagata and Takabe, 1971) did not support the production of betacyanin in callus cultures of *Portulaca grandiflora*, but pigment appeared

when cultures were grown on WH and MS media, in which the overall salts concentration was lower.

However, cultures grown in B5 and MS media in this study appeared to show similar levels of growth and pigment accumulation, from which it would seem that these two attributes of the culture system are closely related. This direct relationship between growth and secondary product formation has also been observed in some other studies, for instance in *Catharanthus* cultures (Stafford *et al.*, 1985).

It is important to point out that the above results, with different media (3.1.2.1), obtained in this study are from only one sampling time, and might not reflect the true capability of the media tested. The effects and utilisation pattern of individual nutrients varies and might be more important in determining the level of product formed than the total salts composition alone (see reviews by Dougall, 1980; Mantell and Smith, 1983).

iii. The effects of 2,4-D and Kinetin.

The results presented in 3.1.1.2 show a differential response in growth of callus derived from 3 different cvs of *B. vulgaris*. Cv Albina veredunda responded equally to all the combinations of PGRs. Since these cultures were

greenish in colour and presumably partially photosynthetic (Bergmann, 1977), they were less affected by an exogenous supply of nutrients and growth factors. Cv Boltardy clearly grew well in a medium with 0.1 mg.l^{-1} kinetin and 0.02 mg.l^{-1} 2,4-D. Cv Burpee's Golden showed the same response to this PGR combination, but also grew equally well in medium with 0.04 mg.l^{-1} kinetin and 0.02 mg.l^{-1} 2,4-D. From the result with cv Burpee's Golden, it would seem that the presence and concentration of 2,4-D is more critical than kinetin in influencing growth.

However, in many studies, 2,4-D has been shown to affect secondary metabolite production (see review by Sakuta and Komamine, 1987). In this study (3.1.2.2), the elimination of 2,4-D or the reduction of 2,4-D concentration in the culture medium resulted in reduced growth (culture FW) of cell suspensions of cv Boltardy, but an increase in the level of betaxanthin accumulated; whereas betacyanin accumulation was not significantly affected. A similar effect to that described for betaxanthin has been observed for betacyanin in other laboratories. In a study with *Beta* callus cultures Constabel and Nassif-Makki (1971) reported that the removal of 2,4-D from the culture medium induced betacyanin formation, which was not produced by cells in a medium with 1 mg.l^{-1} 2,4-D. In contrast, in cell cultures of *Phytolacca americana*, 2,4-D actually promoted betacyanin accumulation (Sakuta *et al.*, 1986); Komamine *et al.*, 1989; Hirose *et al.*, 1990). Sakuta *et al.* (1991)

have shown that 2,4-D elimination decreased intracellular tyrosine, a putative precursor of betacyanin (see Böhm and Rink, 1988), and the incorporation of radioactivity from labelled tyrosine into betacyanin. The indifferent response of betacyanin accumulation to the level of 2,4-D in the medium, reported in this study, does not rule out the possibility that some of the 2,4-D might be carried over from the stock suspensions. The importance of 2,4-D has been confirmed by Sakuta *et al.* (1991) who have suggested that besides the availability of the common precursor tyrosine, there is an important regulatory step controlling the formation of betacyanin from tyrosine which is affected by this PGR.

However, in this study, an increase in the concentration of 2,4-D led to a reduction in the amount of betaxanthin but did not affect the amount of betacyanin accumulated, which would seem to suggest that the effect of 2,4-D is not the result of a general effect on betalain accumulation, but rather a specific effect on the accumulation, or possibly, the formation of different betalains. The differential effects of PGR on various kinds of betalains accumulated by *Beta* suspension cultures have also been reported by Girod and Zryd (1991). They have suggested that beetroot cells when given the appropriate stimuli, such as specific PGRs and medium combinations, are capable of switching on or off a series of reactions leading to the formation of the various

betalains. A similar observation was reported from studies with *Cinchona ledgeriana* suspension cultures (Harkes *et al.*, 1985) accumulating various secondary products; anthraquinones, indole alkaloids and quinoline alkaloids. It is therefore, possible that the differential effects of 2,4-D observed in the present study and those observed by Girod and Zryd (1991) and Harkes *et al.* (1985) support the possibility that individual betalains may be regulated independently.

iv. Effects of initial sucrose concentration.

Sucrose is the most common source of carbon used in plant tissue culture media (Maretzki, 1974). It not only supports growth and metabolism but can also influence other plant processes, including the production of secondary metabolites (see review by Dougall, 1980; Mantell and Smith, 1983). In general, increasing the sucrose concentration in the culture medium above that normally used for cell growth and maintenance results in product enhancement (eg. Davies, 1972; Zenk *et al.*, 1977; Mizukami *et al.*, 1977). However, product inhibition by sucrose has also been observed (Ikeda *et al.*, 1976). Product formation may be coupled with growth and cell proliferation (Sakuta *et al.*, 1987c), or otherwise (Matsumoto *et al.*, 1976). In the present study the effects of various initial sucrose concentrations, ranging

from 2 to 16% (w/v) were examined.

Results (3.1.2.3) show that at 8% and above, growth and total betalain yield were both adversely affected, while maximal growth was achieved at 4% sucrose. Growth inhibition by sucrose concentrations higher than 'normal' has also been reported for carrot suspension cultures (Ozeki and Komamine, 1985). Do and Cormier (1990) have suggested that the same response in *Vitis* cells is due to osmotic stress. This has also been suggested by Ibrahim (1987).

The effect on betalain accumulation in the present study could not be attributed to sucrose concentration alone since cultures were affected by the lower irradiation level prevailing in this experiment. However, there were indications that specific pigment accumulation (cellular content) was stimulated at a sucrose concentration of 4 and 8%. Betalain content was, by the end of the experiment, highest in media with 4% sucrose, and still rising. A similar stimulatory effect of high sucrose concentration (up to a limit) was also observed in anthocyanin production by *Vitis* cell suspensions (Yamakawa *et al.*, 1983; Do and Cormier, 1990, 1991), betacyanin production by *Phytolacca americana* cultures (Sakuta *et al.*, 1987b), and carotenoid production by *Bixa orellana* cultures (Boyd, 1991). Yamakawa *et al.* (1983) have also shown that the C/N (carbon to nitrogen) ratio is also an

important determining factor. This shows that interactions with other factors could also influence product accumulation.

Results presented herein also showed that total betalain yield is very much dependent on growth. Cultures in 2% and 4% sucrose, in which growth was good, accumulated the highest levels of betacyanin and betaxanthin. Similar observations were made by Mizukami *et al.* (1972) in *Lithospermum* callus and by Sakuta *et al.* (1987b) in *Phytolacca* suspensions producing betacyanin. Results in this thesis also showed that cultures in 4% sucrose had a prolonged growth phase when compared to other sucrose treatments, and as pigment accumulation is growth-related as suggested by Sakuta *et al.* (1987c), a sucrose concentration which supports growth would be expected to provide conditions for high betalain productivity.

v. Effects of initial pH.

pH of the culture medium may affect the availability of nutrients (Butenko, 1968; Minocha, 1987), plant growth regulators (Kaiser and Hartung, 1981), cell division (Basu *et al.*, 1988) and other plant processes. Studies on the effects of pH on secondary metabolite production are few. Nonetheless, pH of the medium has been shown to influence the intracellular pH (Hildebrandt *et al.*, 1945) and hence

the accumulation of secondary metabolites (Kurkdjian, 1982).

In this study (3.1.2.5), it was shown that there was no significant difference in the levels of betacyanin and betaxanthin accumulated within a pH range of 5.4 to 6.0. This is in agreement with the suggestion that, in most cases, plant cell cultures are capable of normalising the pH of the medium (Hagimori *et al.*, 1983; Butenko *et al.*, 1984; Ozias-Akins and Vasil, 1985; Collinge, 1987) and this is achieved by regulation of the efflux and influx of ions and protons (Scragg, 1991) across the cellular membranes. In contrast to this, it has been shown (Kurkdjian, 1982; Veliky, 1977) that production of secondary metabolites may be affected if the pH of the medium is maintained constant throughout the culture period. Hence, manipulation of the medium pH may be considered as a form of elicitation (Wink, 1985). The results in this study have also shown that the pH optimum for betacyanin and betaxanthin production in this study is rather wide compared to, for instance, the 0.2 pH unit reported for the growth of rose cell cultures (Nesius and Fletcher, 1973).

vi. Effects of inoculum density.

Relatively little attention has been given to the effects of inoculum density on secondary metabolism of cultured plant cells. The effects on cell growth have been noted in large-scale suspension cultures (eg. Fowler, 1977). Induction of PAL (phenylalanine ammonia-lyase) was shown to be influenced by inoculum density (Hahlbrock and Wellman, 1973), which means that subsequent effects on related secondary metabolism are possible.

Results presented in this thesis (3.1.2.4) showed that cultures initiated with a high cell density (6 g FW; ca. 3×10^5 cells/ml) reached a slightly higher FW after 14 d, compared to those initiated with a low (2 g FW; ca. 1×10^5 cells/ml) inoculum density, and there was no difference between cultures initiated with 4 g FW (3×10^5 cells/ml) and either 2 or 6 g FW. However, this may not indicate the true capability of the cultures for growth, since, if cultures were allowed to grow to the stationary phase, the final FW could be comparable (Thomas and Davey, 1975). Furthermore, cultures with low inoculum densities are known to have a longer lag phase and also a longer period of exponential growth (Stuart and Street, 1969).

The effect on the levels of betacyanin and betaxanthin accumulated was negligible within the range of inoculum

densities tested. In contrast, Ozeki and Komamine (1985) have shown that in carrot suspension cultures, the maximum anthocyanin yield was maximal at an inoculum density of 4.5×10^5 cells/ml. In carrot suspensions, however, growth is significantly increased at higher inoculum densities, and the high accumulation observed by these workers is probably a manifestation of the contrasting relationship between growth and anthocyanin accumulation (eg. Forrest, 1969, Stickland and Sunderland, 1972). However, this may not be the case with betacyanin accumulation (Sakuta *et al.*, 1986), and since growth is not much affected by the level of inoculum density, as found in this study, this should explain the observed levels of accumulation of betacyanin and betaxanthin.

4.2 GROWTH AND THE PRODUCTION OF BETALAINS.

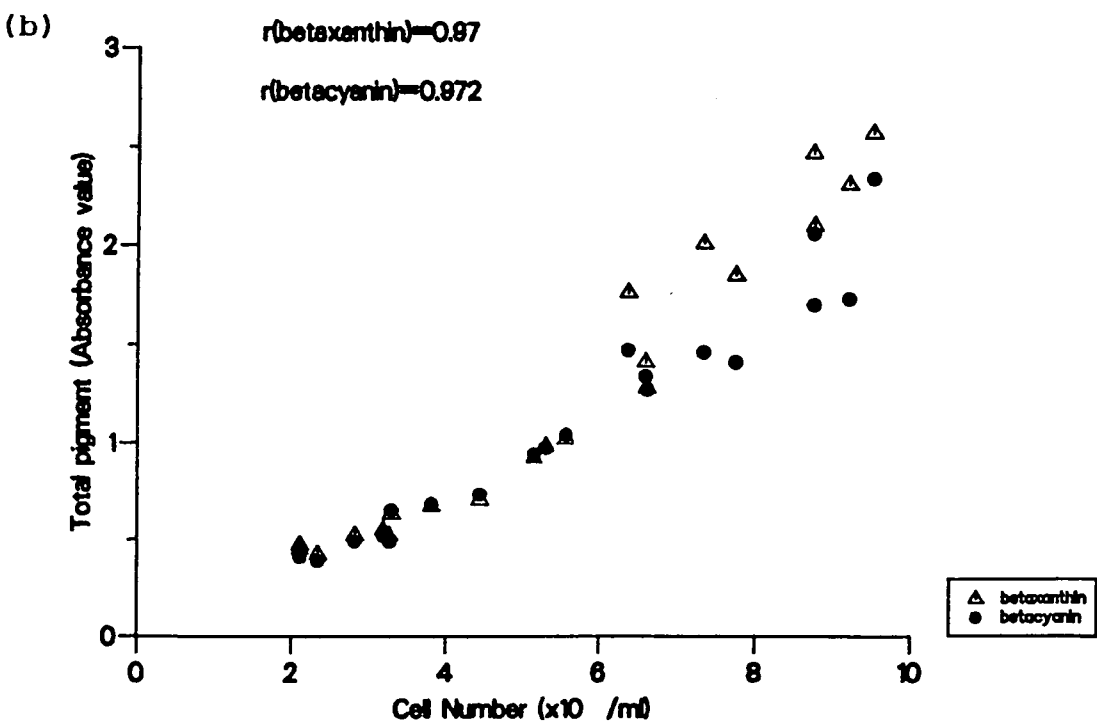
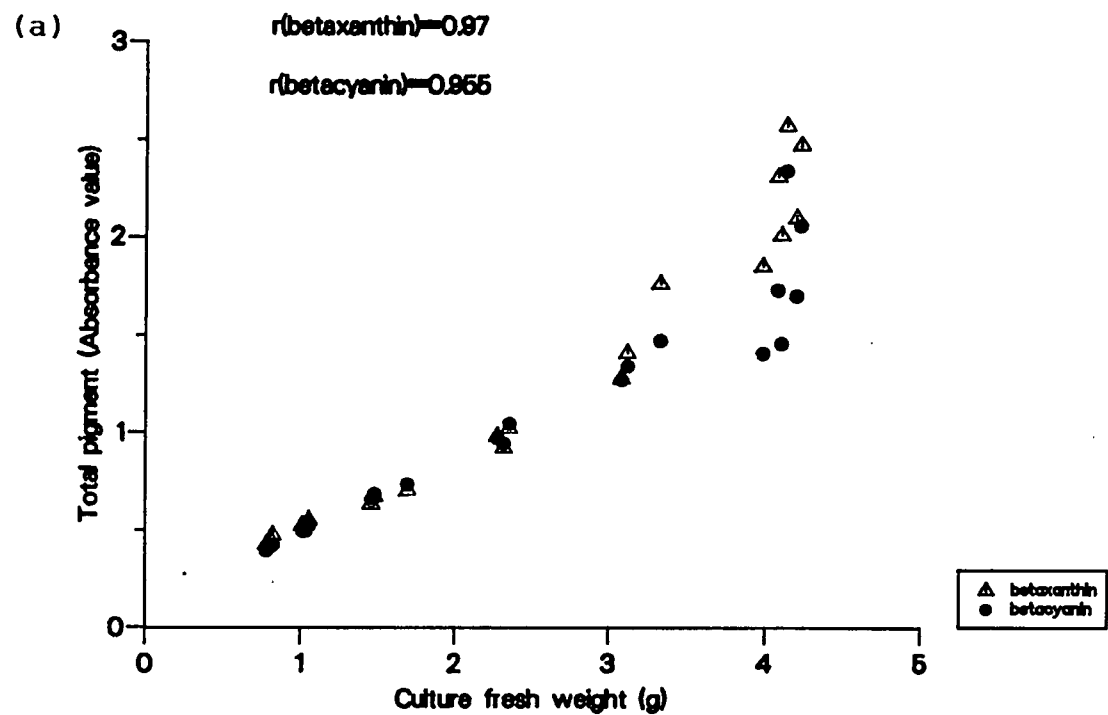
i. Growth-related Accumulation of Betalains.

The relationship between growth and secondary metabolite production is an important aspect of research with plant cell cultures. It is generally acknowledged that in most cases an inverse relationship exists between these two attributes of the culture system (for example Phillips and Henshaw, 1977; Yeoman *et al.*, 1980). Results of many studies support this hypothesis (for example Kaul and Staba 1969; Davies, 1972; Lindsey and Yeoman, 1983; Yeoman *et al.*, 1983; and Hall and Yeoman, 1986). However, this inverse relationship is not universal and there are a number of tissue culture systems in which there is a close link between growth and secondary metabolite production (for example Constabel *et al.*, 1971; Kadkade, 1982; Sakuta *et al.*, 1986; and Hirose *et al.*, 1990).

In the present study (3.2.5) there is a close correlation between growth and the total betalain (betacyanin and betaxanthin) accumulated in light-grown cultures of *Beta*. This close relationship between product and culture fresh weight is shown by the high correlation coefficient, r , which is 0.970 for betaxanthin and 0.955 for betacyanin as shown in Fig. 4.1. The relationship between product and cell number is equally high, the correlation coefficient for betaxanthin and betacyanin being 0.970 and 0.972

Fig. 4.1.

A diagram showing the relationship between growth and the amount of betalain accumulated in light-grown cultures of *Beta vulgaris* cv Boltardy. A 95% confidence interval was used.



respectively. These results are similar to those from other studies on betacyanin production reported by Sakuta *et al.* (1986) for *Phytolacca americana* and Hirose *et al.* (1990) for *Chenopodium rubrum*, where accumulation was maximal during the logarithmic phase of the culture cycle. Similar observations have been reported for other culture systems accumulating metabolites other than betalains, eg. anthocyanin production by *Haplopappus gracilis* cells in continuous culture (Constabel *et al.*, 1971), volatile oil production by *Ruta graveolens* cultures (Corduan and Reinhard, 1972) podophyllotoxin production by *Podophyllum peltatum* callus (Kadkade, 1982), and sapogenin production by cell cultures of *Agave wightii* (Sharma and Khanna, 1980).

It has been shown that, where product accumulation is maximal during the stationary phase, culture productivity can be improved by maintaining cultures in this phase (Mantell and Smith, 1983). Conversely, in cultures where product accumulation is growth-dependent, maintaining cells in a state of proliferation is a sensible way of achieving high product yield. Results from this study with *Beta* (3.1.2.3) have shown that cultures grown in a medium with 4% sucrose have a longer growth phase than cultures on 2% sucrose and show a higher level of betalain accumulation. Therefore, it would seem that the optimisation of sucrose concentration could provide a way for maximising profit in large-scale production systems by

reducing the number of process runs per year, as the production period is extended (Reinhard et al., 1989; Fowler, 1988)

ii. Differential Regulation of Betacyanin and Betaxanthin Accumulation.

Constabel and Nassif-Makki (1971) have suggested that betalain accumulation in cell cultures is influenced by the developmental stage of the cells. In this study, it was shown (3.3) that cultures at three stages of the culture cycle were capable of synthesising and accumulating both betacyanin and betaxanthin. However, there appears to be a differential regulation of the synthesis and accumulation of these two groups of betalains. Betacyanin synthesis was shown to occur maximally mid-way through the growth cycle (14d) when cell proliferation was highest, and declined at the end of the growth cycle (21d). During this period of maximal synthesis, the ratio of betacyanin to betaxanthin per unit fresh weight was 1.06 and fell to 0.84 at the end of the growth cycle. The total amount of betacyanin accumulated was also maximal at the mid-growth stage, and remained constant until the end of the growth cycle, despite the cultures showing a reduced level of synthesis. This suggests that the betacyanin accumulated was either not being degraded or that synthesis and degradation were

equal. This would support the results reported by Sakuta *et al.* (1986), Komamine *et al.* (1989), and Hirose *et al.* (1990). Furthermore, Hirose *et al.* (1990) have shown that inhibition of growth by the application of aphidicolin (APC), an inhibitor of DNA synthesis, or the removal of phosphate led to an inhibition of betacyanin synthesis. Restoration of phosphate and removal of APC returned growth and betacyanin accumulation back to normal. All these results point to a close positive correlation between growth (cell division) and betalain accumulation.

Interestingly, it was also shown in this study (3.3) that betaxanthin synthesis and accumulation increased with time and was maximal at the end of the growth cycle (d21), when growth had ceased. On the other hand, betaxanthin per unit fresh weight was more or less constant throughout the growth cycle. This late period of active synthesis and accumulation of betaxanthin in cultures towards the end of the growth cycle is similar to the majority of cases of secondary metabolite production in cultured cells (for example Phillips and Henshaw, 1977; Yeoman *et al.*, 1980), except that, in addition betaxanthin synthesis also occurred earlier during active growth and was unrelated to physiological age. It is also interesting to note that although the incorporation of ^{14}C -tyrosine into lignin increased with culture age as the culture differentiated, the rate of incorporation into protein was still high at the downturn of growth. Yet again, this shows that growth

and betalain accumulation are in general, closely related. Here, the results with betalains do not provide evidence of a switch from primary to secondary metabolism. In an analogy to the model presented by Yeoman *et al.* (1982), the switch in the case of betalain synthesis is always open, although to different extents. To understand the regulation of the switching mechanism, a detailed study of the enzymes involved in the biosynthesis of betalains is necessary. A few studies have been carried out on this aspect (Kumon *et al.*, 1990; Girod and Zryd, 1991a) but the results are very preliminary.

The differential influence of culture age on the synthesis of betacyanin and betaxanthin reported in this study (3.3) points to a regulatory point after tyrosine in the metabolic pathway. Results (Table 3.1) show that at mid-growth stage the combined incorporation of ^{14}C into betacyanin and betaxanthin was similar to that at the end of the culture cycle. However, at the mid-growth stage, 67% of the radioactivity went into betacyanin, and by the end of the growth cycle 57% of the radioactivity was found in betaxanthin. This shows that during the period between these two stages of the growth cycle, there was a decrease in the incorporation into betacyanin, and a corresponding increase in the incorporation into betaxanthin. This suggests that a common precursor is preferentially channelled into betacyanin synthesis at the mid-growth stage, and into betaxanthin synthesis at the end of the

growth cycle. Indeed, it has been suggested that the production of secondary metabolites is a phase-dependent expression of genes controlling the availability of substrates and enzymes, and is a part of cell specialisation (Luckner, 1990). This situation in which two closely linked metabolites appear to differ in their synthetic behaviour is an interesting one and merits further investigation.

The differential regulation of betacyanin and betaxanthin synthesis and accumulation was also observed in an experiment on the effects of 2,4-D (3.1.2.2), where it was shown that reduction in the concentration or omission of auxin from the medium resulted in decreased growth and increased betaxanthin accumulation, whereas the amount of betacyanin remained unchanged. This result is difficult to explain in terms of growth and is inconsistent with a positive relationship between growth and betalain accumulation. In a recent communication, Sakuta *et al.* (1991) have made a suggestion, based on their results with *Phytolacca americana* cell cultures, that besides the supply of the supposed precursor, tyrosine, there may be another regulatory step in the biosynthetic pathway leading from tyrosine to betacyanin that is influenced by 2,4-D. A similar regulatory mechanism might exist in the *Beta* cultures used in this study. Whether this step/s will affect the formation of betacyanin and betaxanthin to different extents, however, is another question.

4.3 LIGHT AND BETALAIN ACCUMULATION

Light can influence growth and development, and the production of secondary metabolites in plant cell cultures. The stimulatory and inhibitory effects on secondary metabolite formation and accumulation have been reported for many species (see review by Seibert and Kadkade, 1980; and Mantell and Smith, 1983). In other cases (eg. Ikeda *et al.*, 1977) light seems to have no effect on the production of secondary products. In the production of the betalains, the effect of light is not very clear (see review by Böhm and Rink, 1988) and contrasting observations have been reported. In this study the effects of light on growth and the synthesis and accumulation of betacyanin and betaxanthin were investigated and will now be discussed.

i. Is light a necessity in betalain accumulation?

Results presented in this study (3.2.7) show that light does not appear to be an absolute prerequisite for the accumulation of the betalains. Cultures maintained in the dark for six passages still displayed a low level of the pigments. Further observations (results not shown) showed that these cultures when maintained for nine months in darkness were still pigmented. In spite of this, there is

still a possibility of light leakage during aseptic transfer of the cultures under safelight condition (see Appendix 6.9). This effect of low fluence irradiation under safelight condition has been observed in seedling tissue of *Amaranthus* (Obrenovic, 1990). However, further evidence for the suggestion that betalain formation does not have an absolute requirement for light was obtained from the experiment with labelled tyrosine (3.3). Here, ^{14}C was detected in betacyanin and betaxanthin isolated from light as well as dark-grown cultures labelled with ^{14}C -(U)-L-tyrosine.

Upon transfer from light to dark the pigment content fell sharply (Table 3.2.5) and beyond the second passage pigment levels remained at a constant low level. Wohlpart and Mabry (1968) have shown in their study with seedlings of fourteen species of the *Centrospermae*, that ten of the species were able to produce betacyanin and betaxanthin in the dark. Accumulation of betalains in the dark has also been reported by Kumon *et al.* (1990) in epidermal tissue of *Phytolacca americana*, Berlin *et al.* (1986) in *Chenopodium rubrum* suspension cultures, and Endress *et al.* (1984) in callus of *Portulaca grandiflora*. In a radioactive feeding study, Endress *et al.* (1984) have further shown that the incorporation of radioactive tyrosine into betacyanin was higher in cultures grown in the dark. However, in contrast to these results, it has also been reported (see review by Böhm and Rink, 1988,

Kishima et al., 1991) that highly pigmented cell cultures of *Portulaca grandiflora* and *Beta vulgaris* grown in the light became colourless when transferred to the dark, and the pigmentation of these cultures was restored on transfer back to the light. However, the results presented in this thesis and most of those reported elsewhere show that light does not seem to be an absolute requirement for the accumulation of the betalains. Moreover, studies with seedlings of *Amaranthus* (Bamberger and Mayer, 1960; Köhler, 1972; Giudici de Nicola^{et al.}, 1972), and epidermal tissue of *Phytolacca americana* (Kumon et al, 1990a) have demonstrated that kinetin can replace light, suggesting that there is an alternative, non light-dependent mechanism in the pathway leading to the formation of the betalains. The appearance of the betalains in subterranean structures in the intact plant may also be an indication of the non-dependence of betalain accumulation on light, although the site of biosynthesis of betalains has not been confirmed, and the pigments or their precursors may be transported from the aerial parts of the plant to the underground structures.

ii. Light enhancement of betalain accumulation.

Although light is not generally required for the accumulation of the betalains, its presence (see results in 3.2) clearly enhanced the level of accumulation. These results support those reported for seedlings (Wohlpert and

Mabry, 1968; Rast et al, 1972) and cell cultures (Girod and Zryd, 1987) accumulating betalains. Other results presented in this thesis (3.3) also support this suggestion. For example, in 3.3, the levels of ^{14}C -tyrosine incorporated into betacyanin and betaxanthin were higher in the light than in the dark at each sampling time, although the ratio of incorporation between the two treatments varies with culture age.

In a preliminary experiment (3.2.1), cultures transferred from light to dark accumulated 32% less betacyanin and 60% less betaxanthin than the light-grown cultures. From this result, it could be argued that the difference in the amounts of betacyanin and betaxanthin is due to different rates of synthesis or degradation of the two groups of betalains in the dark. The latter seems more likely, as the ratio of ^{14}C -tyrosine incorporation into betacyanin and betaxanthin (i.e. synthesis) at d 14 was the same in the light as in the dark, i.e. 1.7 (see 3.3). It could be that transfer of cultures from light to dark promotes conditions that lead to the differential degradation of betaxanthin. It is therefore possible that part of the effect of light in promoting betalain accumulation is due to a repression of degradative enzymes. According to Haslam (1986), the control of secondary metabolism in the majority of cases, is effected either by repression of the synthesis of enzymes or by direct inhibition of enzyme activity. Betacyanin degrading enzymes have been

reported in tissues that accumulate betacyanins (eg. Elliot et al, 1983; Kumon et al, 1990a,b). Elliot et al (1983) have shown that the root was the richest source of these enzymes, and that the low activity observed in the shoot could be due to the presence of an unknown inhibitor which was shown to be soluble in acetone. Kumon et al (1990a) demonstrated the presence of certain substances in the cortex tissue that inhibit light and kinetin-induced betacyanin accumulation, and further reported (Kumon et al, 1990b) the presence of betacyanin degrading enzymes which, however, occurred simultaneously with the pigment. Whether a similar group of enzymes is present in light-grown cultures in this study, is unknown.

iii. Effects of level of Irradiance.

The quality and quantity of light can influence the production of secondary metabolites (see review by Seibert and Kadkade, 1980, Mantell and Smith, 1983), but in this study only the latter aspect of light was studied. The light source used (2.5.2) provided a wide spectrum of light, since red (Piattelli, 1981) and blue (Berlin et al, 1986) light are known to be involved in betalain synthesis. Light irradiance is known to influence the level of accumulation of anthocyanin, for example in *Vitis* (Yamakawa et al, 1983) and in *Catharanthus* (Hall and Yeoman, 1986) suspension cultures. In the present study,

Yeoman, 1986) suspension cultures. In the present study, the results (3.2.3) show that generally, betalain accumulation increased with the increasing level of irradiance. However, in one experiment (3.2.4) it was shown that the increase in betalain accumulation upon the transfer of cultures from a low level of irradiance to a high irradiance level was only transitory. In the subsequent passage under high irradiance, the amounts of betalains accumulated reverted back to those obtained at the lower level of irradiance. Therefore, it would seem that the initial effect brought about by increased light was due to a sudden sharp response to the stress provided by a 'high' light intensity. However, these results were obtained at only one sampling time, i.e. at mid-growth stage and therefore must be considered with caution as they might not present firm evidence of a positive correlation between level of irradiance and the amount of betalain accumulated. Nevertheless, results (3.2.2) from a time-course study show that cultures grown under low irradiance did accumulate less betalains than the cultures from which they were derived. Under this ($1 \mu\text{mol.m}^{-2}\text{s}^{-1}$), as well as under normal light condition of $20 \mu\text{mol.m}^{-2}\text{s}^{-1}$ (see 3.2.5), the levels of betalain accumulation were more or less the same during the period of mid-growth to the end of the growth cycle. Therefore the earlier observations that light irradiance positively influence betalain accumulation seem to be valid. This is further supported by the results of Girod and Zryd (1987), with

green habituated callus of *B. vulgaris* which could be induced to form betalains only at high light intensities, and when the cultures were transferred back to low a lower light intensity, the proportion of pigmented cells was reduced.

iv. Mechanism of Light Regulation.

It is not possible to determine the exact mechanism of light action from the results presented in this thesis. Nevertheless, the possibility of a negative regulation of the level of inhibitors and betalain degrading enzymes cannot be ruled out. This might seem to be relevant in cultures transferred from the light to the dark, as shown in the results in 3.3, in which betacyanin degradation was implicated. As discussed earlier, a degradation mechanism could be invoked in a non-photosynthetic environment (dark) to enable cells to utilise secondary products such as betalains for maintenance and growth. The suggestion that secondary products are an 'overflow' from primary metabolism, the biosynthesis of which, maintains the cells during conditions of nutrient imbalance brought about by nutrient depletion (Foster, 1949; Haslam, 1986), is relevant to this theory. The fact that these cells were heterotrophic does not rule out this possibility.

On the other hand, a positive regulatory role of light has

been suggested by Giudici de Nicola *et al.* (1974) whereby under continuous light, the photosynthetic system may be responsible for most of the light action leading to the formation of the betalains. The higher level of betalain synthesis in the light than in the dark (3.3), implies that the activities of certain enzymes were raised. Since both betacyanin and betaxanthin are similarly affected by light, the possibility that light influences the earlier sequence of reactions seems likely. Woodhead and Swain (1984) have reported that the activity of ^{phenylalanine ammonia lyase} (PAL), and the formation of hydroxycinnamic acid and betacyanin were similarly induced by light. Since phenylalanine and its derivatives are common precursors for the betalains (Mabry and Dreiding, 1968), an increase in their activity could lead to an increase in the formation of the betalains. However, precursor feeding studies suggested that light influences the reactions leading to the formation of the dihydropyridine moiety of betalains (French *et al.*, 1973; Giudici de Nicola *et al.*, 1974). French *et al.* (1973) further suggested that light affects the reactions between tyrosine and DOPA, as well as between DOPA and betacyanin. Since it is impossible to ascertain whether enzymes or substrate are the limiting factor, further detailed investigations are required that involve radioactive feeding and enzyme studies.

It was also observed that cells grown in the dark were generally less aggregated and had thinner cell walls.

materials, lignin included. Kuboi and Yamada (1978a,b) showed a positive correlation between cell aggregation on the one hand, and lignification and activities of secondary metabolic enzymes (shikimate dehydrogenase, cinnamic acid 4-hydroxylase, caffeic acid- and 5-hydroxyferrulic acid O-methyltransferases) on the other, in tobacco suspension cultures. Results reported in 3.3 in this thesis, in which the incorporation of ^{14}C -tyrosine into lignin was higher in the light, also support this correlation. Since lignin formation is related to cell differentiation (see review by Wiermann, 1981), the effect of light reported in this study could be through the control of cytodifferentiation.

The other effect of light seems to be on the competence of cells to produce betalains. In the dark, it was observed that certain cells, often clusters of cells, were capable of producing high amounts of pigments judged by their colour intensity. In 3.2.7 these amounted to about 10% of the pigmented cells. Girod and Zryd (1987) suggested that competence is a factor inherent to the cells and that light is required for the induction of betalain formation. On the contrary, the presence of highly pigmented cells in the dark point to a kind of autonomy to light, which could be a characteristic of genetically modified cells. It would be interesting to see if this relates to kinetin-induced betalain formation, or even habituation (Berlin et al, 1984; Böhm et al, 1991).

In all the studies involving cultures at different growth stages reported in this thesis, physiological age has an over-riding influence on product accumulation. It is apparent that here, as expected, there is a regulation by genes controlling secondary metabolism which are phase-dependent as proposed by Luckner (1990).

FUTURE WORK

Results of the studies presented in this thesis and discussed in the preceding sections have brought to attention some areas that need further investigation.

Firstly, it has been shown that cultural factors can influence growth and product accumulation. A detailed study on the effects of nutrients, auxin and cytokinin, and the light requirement might be carried out to optimise betalain production.

Beta cultures are shown to be highly heterogeneous and sensitive to the micro-environment. Girod and Zryd (1991b) have shown that cells are capable of phenotypic changes brought about by the manipulation of medium and PGR composition. This means that a stable and homogeneous population could be obtained by selection procedures and by controlling the cultural conditions. By studying the pattern and nature of culture variability, culture productivity could be improved.

The most important point perhaps is the study of the biosynthesis of the betalains and its regulation. Results discussed in this thesis show that product synthesis and accumulation are closely related to growth and the physiological age of the cultures. It is therefore, important to investigate the flux of metabolites down the

pathway leading to the formation of the betalains, and this could be achieved through radioactive feeding of precursors, and studies on the enzyme activities over a growth cycle. This will bring forward a clearer scenario on the biosynthetic pathway from which competing reactions, and rate-limiting factors and steps can be determined.

Light has been shown to stimulate betalain synthesis and accumulation, but the presence of highly-productive cells in the dark and the fact that kinetin has been reported to substitute for light merits further investigation. This could be important in the industrial production of the betalains.

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APPENDICES.

Appendix. 6.1.

One-way ANOVA on the effects of 2,4-D and kinetin concentrations on the growth of callus of *B. vulgaris* cvs Boltardy, Burpee's Golden and Albina veredunda. (P=0.05)

a. Albina veredunda:

SOURCE	DF	SS	MS	F	P-value
FACTOR	3	0.155	0.052	0.46	0.717
ERROR	8	0.897	0.112		
TOTAL	11	1.052			

b. Burpee's Golden:

SOURCE	DF	SS	MS	F	P-value
FACTOR	3	0.0998	0.0333	1.54	0.278
ERROR	8	0.1729	0.0216		
TOTAL	11	0.2727			

c. Boltardy:

SOURCE	DF	SS	MS	F	P-value
FACTOR	3	1.844	0.615	4.14	0.048
ERROR	8	1.188	0.149		
TOTAL	11	3.032			

Appendix 6.2.

One-way ANOVA on the effect of basal salt formulations on growth (FW) in suspension cultures of *B. vulgaris* cv Boltarday. (P=0.05)

SOURCE	DF	SS	MS	F	P-value
FACTOR	3	1.8927	0.6309	13.37	0.002
ERROR	8	0.3775	0.0472		
TOTAL	11	2.2703			

Appendix 6.3.

One-way ANOVA on the effect of 2,4-D on growth and betalain content in suspension cultures of *B. vulgaris* cv Boltardy. (P=0.05)

a. Growth (FW):

SOURCE	DF	SS	MS	F	P-value
FACTOR	2	2.4012	1.2016	35.80	0.008
ERROR	3	0.1006	0.0335		
TOTAL	5	2.5018			

b. Betaxanthin:

SOURCE	DF	SS	MS	F	P-value
FACTOR	2	0.12051	0.06025	33.54	0.009
ERROR	3	0.00539	0.00180		
TOTAL	5	0.12590			

c. Betacyanin:

SOURCE	DF	SS	MS	F	P-value
FACTOR	2	0.001879	0.000939	2.19	0.259
ERROR	3	0.001287	0.000429		
TOTAL	5	0.003166			

Appendix 6.4.

One-way ANOVA on the effects of size of the inoculum on growth and betalain accumulation in suspension cultures of *B. vulgaris* cv Boltardy. (P=0.05)

a. Growth (RGR):

SOURCE	DF	SS	MS	F	P-value
FACTOR	2	0.000309	0.000154	1.42	0.368
ERROR	3	0.000325	0.000108		
TOTAL	5	0.000634			

b. Betaxanthin:

SOURCE	DF	SS	MS	F	P-value
FACTOR	2	0.00707	0.00353	0.40	0.699
ERROR	3	0.02624	0.00875		
TOTAL	5	0.03330			

c. Betacyanin:

SOURCE	DF	SS	MS	F	P-value
FACTOR	2	0.00112	0.00056	0.24	0.802
ERROR	3	0.00703	0.00234		
TOTAL	5	0.00815			

Appendix 6.5.

One-way ANOVA on the effect of initial pH on pigment content in suspension cultures of *B. vulgaris* cv Boltardy. (P=0.05).

a. Betaxanthin:

SOURCE	DF	SS	MS	F	P
FACTOR	2	0.0047	0.0023	0.13	0.882
ERROR	6	0.1090	0.0182		
TOTAL	8	0.1136			

b. Betacyanin:

SOURCE	DF	SS	MS	F	P-value
FACTOR	2	0.00155	0.00077	0.51	0.623
ERROR	6	0.00905	0.00151		
TOTAL	8	0.01060			

Appendix 6.6.

Statistical analysis (paired t-test) on the effect of light and dark on the accumulation of betalain in suspension cultures of *B. vulgaris* cv Boltardy. (P=0.05).

a. Betaxanthin:

N	MEAN	SD	SE	t	p-value
2	0.4290	0.0834	0.0590	7.27	0.087

b. Betacyanin:

N	MEAN	SD	SE	t	p-value
2	0.0885	0.0262	0.0185	4.78	0.13

Appendix 6.7.

One-way ANOVA on the effect of level of irradiance on betalain accumulation in suspension cultures of *B. vulgaris* cv Boltardy. ($P=0.05$).

a. Betaxanthin:

SOURCE	DF	SS	MS	F	P-value
FACTOR	2	0.049020	0.024510	25.72	0.001
ERROR	6	0.005718	0.000953		
TOTAL	8	0.054738			

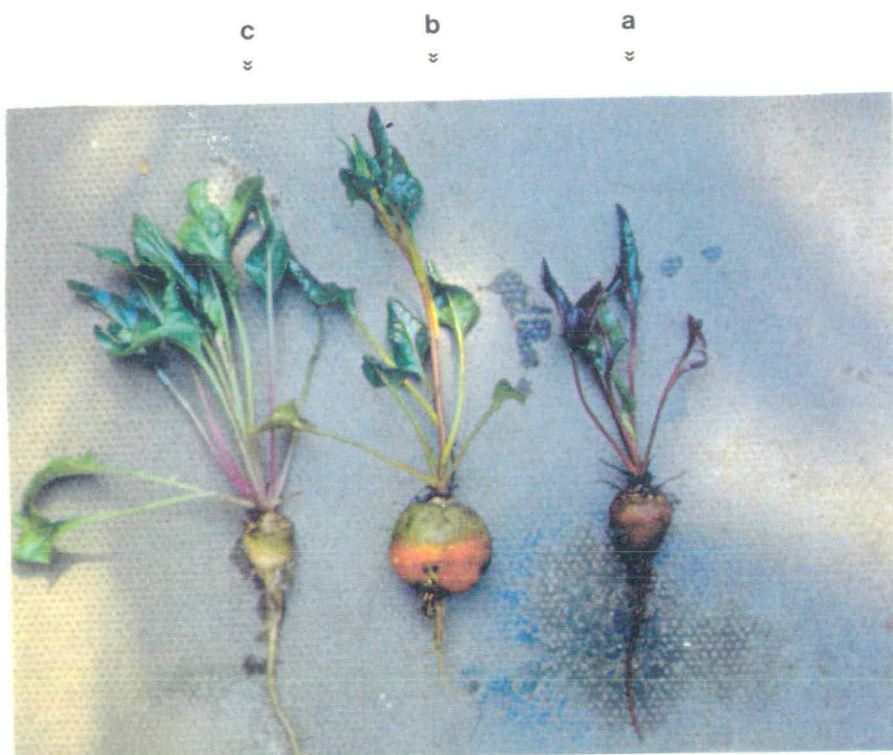
b. Betacyanin:

SOURCE	DF	SS	MS	F	P-value
FACTOR	2	0.006613	0.003306	17.54	0.003
ERROR	6	0.001131	0.000189		
TOTAL	8	0.007744			

Appendix 6.8.

B. vulgaris plants showing pigmentation of the structures.

a. Boltardy, b. Burpee's Golden, c. Albina veredunda.



Appendix 6.9.

Lighting condition during aseptic transfer of dark-grown cultures of *B. vulgaris* cv Boltardy.

Aseptic transfer of cultures was carried out under safelight condition obtained by covering fluorescent lights with a layer of primary green plastic filter (HT 139, LEE Colortran International, London), in a dark transfer room. The light quality is as shown in the graph below. No light irradiance (in $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) was detected at 60 cm below the light source, at the level of work surface. Each culture was exposed for about 3 min.

