STUDIES ON TISSUE CULTURE SYSTEM FOR THE PRODUCTION OF FOOD COLOURS FROM *BETA VULGARIS* L.

Thesis Submitted to the University of Mysore

for the award of the degree of

Doctor of Philosophy

in

BIOTECHNOLOGY

by

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Dedicated to Beloved Parents,
Brothers and Sisters

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DECLARATION

I hereby declare that this thesis entitled **"STUDIES ON TISSUE CULTURE SYSTEM FOR THE PRODUCTION OF FOOD COLOURS FROM *BETA VULGARIS* L."** submitted to the University of Mysore for the award of the degree of ***Doctor of Philosophy*** in **Biotechnology,** is the result of work carried out by me in Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, under the guidance of **Dr. G.A.Ravishankar** during the period September 1999- September 2006.

I further declare that the results of this work are not previously submitted for any degree or fellowship.

Date: **Mohamed Yahya Khan**

Place: Mysore

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CERTIFICATE

I hereby certify that the thesis entitled **"STUDIES ON TISSUE CULTURE SYSTEM FOR THE PRODUCTION OF FOOD COLOURS FROM *BETA VULGARIS* L."** submitted by Mr. Mohamed Yahya Khan to the University of Mysore for the award of the degree of ***Doctor of Philosophy*** in **Biotechnology,** is the result of work carried out by him in Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, under my guidance during the period September 1999- September 2006.

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Place: Mysore

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Research supervisor

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Mohamed Yahya Khan

LIST OF ABBREVIATIONS

2,4-D	2, 4-Dichlorophenoxy acetic acid
2-iP	2-isopentinyl adenine
ABA	Abscisic acid
BAP	Benzyl amino purine
BCIP	5-Bromo-4-chloro-3-indoyl phosphate
bp	base pair
DNA	Deoxy ribonucleic acid
DW	Dry weight
EDTA	Ethylene diamine tetra acetic acid
Fig	Figure
FW	Fresh weight
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kb	Kilo base
Kg	Kilo gram
Kn	Kinetin
l	Litre
LB	Luria Bertani
LS	Linsmaier & Skoog
M	Molar
ml	Milli litre
mM	Milli moles
MS	Murashige and Skoog
NAA	α- Naphthalene acetic acid
OD	Optical density
PCR	Polymerase chain reaction
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TE	Tris-EDTA
Tris	Tris (hydroxy methyl) amino methane
U	Unit
µg	Micro gram
µM	Micro moles
µm	Micro meter
%	Percentage
°C	Degree centigrade
NBT	Nitro blue tetrazolium
AOS	Active oxygen species

SOD	Superoxide Dismutase
CAT	Catalase
APX	Ascorbate peroxidase
GR	Glutathione reductase
POD	Peroxidase
GOT	Glutamic oxaloacetic transminase
GPT	Glutamic pyruvic transminase
LDH	Lactic dehydrogenase
ALP	Alkaline phosphatase
WBC	White blood cell
RBC	Red blood cell
GA_3	Gibberellic acid
H_2O_2	Hydrogen peroxide
YEM	Yeast Extract Manitol
CTAB	Cetyl Trimethyl Ammonium Bromide
FW	Fresh weight
BLN	Betalain
rpm	Revelutions per minute
g	Grams
PVP	polyvinylpyrollidone
Hb	Haemoglobin
NADH	Nicotinamide adenine dinucleotide
STDV	Standard deviation

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CHAPTER -I

GENERAL INTRODUCTION &

REVIEW OF LITERATURE

1. INTRODUCTION

Colour in one form or another, has been added in foods for centuries. The art of making coloured candy is shown in paintings in Egyptian tombs as far back as 1500 BC. Spices and condiments were coloured at least 500 years ago. The first reports regarding the use of derived colour of minerals dated from the nineteenth century. Lead chromate and copper sulfate were used to pigment candies and sauerkraut, but in the pigmentation process arsenic and other venomous impurities were added frequently. Also, in that epoch began the use of tar colourants and other petroleum derivatives in the processing of foods, medicines, and cosmetics (FDA, 1993). Until the middle of the nineteenth century, the colourants used in cosmetics, drugs and foods were of natural origin from animals, plants and minerals. That changed with the discovery of the first synthetic dyestuff, mauve, by Sir William Henry Perkin in 1856. The German dyestuff industry rapidly developed a large number of 'coal tar' colourants and they rapidly found applications in the food and cosmetic industries. At the turn of the century, over 700 synthetic colourants were available for use in foods in the US (Francis, 1995).

Traditionally colours have also been added for decorative purposes and to disguise low quality foods. Everyone is sensitive to the colour of foods and appetite is stimulated or dampened in direct relation to the observer's reaction to colour. Factors that evaluate food quality are colour, flavor, and texture, but colour is considered the most important of them, because if it is not appealing consumers will not enjoy the flavor and texture of any given food (Newsome, 1986).

1.1. Importance of natural colourants

Since the early civilizations, natural products were used to give an attractive presentation to man-made products. Saffron and other species were used frequently to provide yellow colour in a variety of foods and evidence exists that butter was pigmented with these products. Synthetic colourants have been used for many years, in 1938 it was recognized the use of approximately 200, in 1970 and nowadays only seven can be used in food pigmentation (FDA/IFIC 1993, FNB, 1971) To emphasize this situation, it should be pointed out that up to 1986, 356 patents on natural colourants have been registered and for the synthetics only 71 (Francis, 1986). Nowadays, the number of advantages of natural over synthetic colourants have been increased because of the pharmacological properties of the

natural pigments. In 1994, the market of natural colourants had an estimated value of $ 250 million U.S. Dollars, and 65% of this corresponded to colour additives for food, with an annual growth rate of 5 to 10% in relation to 3 to 5% for the synthetic pigments. On the other hand, some products have a good market value only if they are coloured with natural products: in the manufacture of Cheddar cheese, only the annatto pigment is used. In the pigmentation of poultry products synthetic colourants are not adequate (Marusich and Bauernfeind, 1981).

1.2 Health aspects of natural colours

There are several reports on health benefits of natural colours. Moreover considerable research is under way to find their health promoting effects. Anthocyanins and related flavonoids have been very much in news lately for a wide variety of health claims, which include anticarcinogenic, anti inflammatory, antihepatoxic, antibacterial, antiviral, antiallergenic, antithrombotic and antioxidant effects. Several studies have shown that betalains are a good source of natural antioxidant, (Kahkonen *et al*., 1999**,** Kanner *et al*., 2001, Escribano *et al*.,1998). Betalains have also been shown to be antimutagenic in the Ames tester strains (Edenharder *et al*., 1994), and antitumor promoting in mouse skin and lung bioassays (Kapadia *et al*., 1996). Curcumin obtained from turmeric is known since 1950s that it has strong antioxidant effects. It inhibits lipid peroxidation and has a positive antioxidant effect for hemolysis and lipid peroxidation in mouse erythrocytes (Francis, 1995).

Table 1. Approved Colours for Food Industry in the European Union and in The Food and Drug Administration (FDA) of USA

Colour	CEE	FDA
Certifiable		
Red allure AC	No	Yes (red # 40)
Brilliant blue	No	Yes (blue #1)
Carmosine	Yes (E 122)	No
Erithrosine	Yes (E 127)	Yes (red # 3)
Fast green FCF	No	Yes (green # 3)
Indigotine	Yes (E 132)	Yes (blue # 2)
Ponceau 4R	Yes (E 124)	No
Sunset yellow FCF	Yes (E 110)	Yes (Yellow # 6)
Tartrazine	Yes (E 110)	Yes (Yellow # 5)
Citric red # 2	No	No
Exempt of certification		
Annatto extract	Yes	Yes
Dehydrated red beet	Yes	Yes
Ultramarine blue		Yes
Canthaxanthin	No	Yes
Caramel	Yes	Yes
β-apo-8'-carotenal	No	Yes
β-carotene	Yes	Yes
Dactylopuis coccus extract	Yes	Yes
Meal of cotton seeds	Yes	Yes
Iron glutamate	No	Yes
Skin grape extract	Yes	Yes
Iron oxide	No	Yes
Fruit juices	Yes	Yes
Vegetable juices	Yes	Yes
Algae meals	No	No
Tagetes and extracts	Yes	No
Carrot oil	Yes	Yes
Oil of corn endosperm	Yes	Yes
Paprika	Yes	Yes
Paprika oleoresin	Yes	Yes
Riboflavin	Yes	Yes
Saffron	Yes	Yes
Titanium dioxide	No	Yes
Turmeric	Yes	Yes
Chlorophyll	Yes	No
Xanthophylls, flavoxanthins, rubiaxanthins	Not all	Not all

1.3 Market trends

There are no reliable published statistics on the size of the colour market, however on a global scale a reasonable estimate would be $940m (Downham and Collins, 2000)

In terms of individual sector size, it is estimated that the split is:

- Synthetic colours - $400 m.
- Natural colours - $ 250 m (of which $100m is in the USA)
- Nature Identical Colours – $189 m.
- Caramel colours – $100 m.

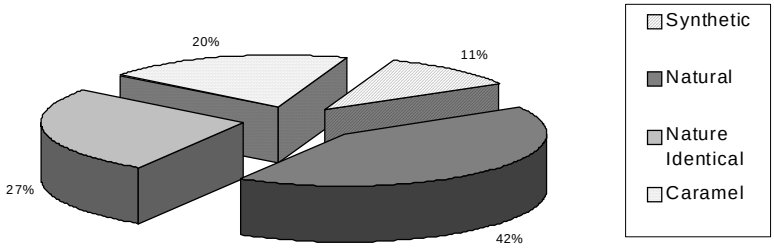

Figure 1. Percentage market share of food colours (the global food colour market) Adapted from Downham and Collins, 2000

1.4 Trends in natural colours

The natural colours market is reported to be growing twice as fast as that of synthetic colours. Overall there is a general increase in demand for natural ingredients. Many consumers associate natural products with superior quality and a good, natural-looking colour in a food or beverage will signal high quality whilst a washed out or artificially bright

product can give the opposite impression. Also, in relation to colours the fact that they are derived from well-known sources such as beetroot, grapes, cabbage and paprika, makes the consumers feel safer and thus recognition and acceptance are easier. Some of the natural colour pigments have only recently been recognized for their possible health effects. Those pigments, that are presently acknowledged for their nutritional properties are a number of carotenoids and anthocyanins. Natural carotenoids include carotenes, lutein and lycopene and have been recognized as antioxidants that are linked to the prevention of degenerative diseases (Giovannuci *et al*., 1995). It is a fact that epidemiological evidence of the nutritional benefits of fruit and vegetables points to a range of carotenoids rather than a single carotenoid providing these benefits. Another consequence of the health trend is the rising interest in and demand for reassurance concerning product quality, food safety and production methods. The demands on the natural colours industry will not only be for organic colours but also for a generally higher level of information with regard to production methods. In general, competition is increasing within the food and beverage industries and greater pressure is put on new product development. In their search to differentiate, product developers are looking at new ingredient options and natural colours are one of many possibilities. Simultaneously the natural colours industry is required continuously to bring forward new colouring opportunities to match the increasing demands of their customers. Future developments are expected to concentrate on improvements of well-known technologies within the formulation and processing of existing colour pigments. A further development of microencapsulation will particularly be in focus due to the many superior advantages of this technology. Besides the new technological solutions, natural colour manufacturers are also looking at a number of new pigment sources. One of the limitations in developing totally new colour formulations is the lengthy and costly safety testing and regulatory approval process. Therefore, 'untapped' sources of raw materials that conform to current regulations give valuable options to develop new colour products. Recent examples of these are blade carrot, red cabbage and algae beta-carotene.

Since 1960s people have shown a clear preference for natural products, including pigments, because more nutritious and healthy characteristics have been associated with them. Remarkably, food scientists consider that this consumer trend will be maintained in

the future (Downnham and Collins, 2000). In this sense, food technologists will continue affronting the problems of availability and stability of natural pigments in order to replace the synthetic ones. On the other hand, it has been clearly established that technology development has introduced new methodologies or processes to avoid the intrinsic instability and solubility problems of natural pigments, and today most of these problems can be solved through technological processes. In addition, the pigment research area is very wide, and natural pigments with improved characteristics (new colours and improved stability) have been discovered. Thus, a more adequate and updated legislation will provide a broad range of natural colours, and importantly with better stability characteristics (Delgado-Vargas *et al.* 2000).

However better production systems for natural pigments will be required for higher productivities in smaller area. Then research interests will evolve, and two areas will be future of natural pigments production. The generation of crops with improved characteristics, and pigment production at the industrial level and under controlled conditions. In order to produce such crops, the understanding of the involved metabolic pathways must be in place to have a complete vision of the biosynthesis and regulation of pigment production. Remarkably, the most impressive advances in these aspects have been reached using molecular biology techniques, and this will continue to be so in the near future.

Interestingly, model systems for pigment production under controlled conditions are now available, but production at the industrial level has not been feasible yet. Thus, carotenoids production by yeasts, bacteria and fungi, anthocyanin and betalain production by plant tissue cultures require the development of improved biotechnological approaches for feasibility and economics of production.

1.5 Betalains

1.5.1 Definition

The term "betalains" was introduced by Mabry and Dreiding (1968) this was supported by structural and biogenetic considerations. In a strict sense, betalains do not belong to alkaloids because they are acidic in nature due to the presence of several carboxyl groups. Originally, betalains were called "caryophyllinenroth" and successively renamed "rubenroth" and "chromoalkaloids" (Piatelli, 1976, Reznik, 1981).

Chemically, betalain definition embraces all compounds with structures based on the general formula shown in Figure 2 therefore, they are immonium derivatives of betalamic acid (Piatelli 1981, Strack *et al*., 1993). The chromophore of betalains can be described as a protonated 1, 2, 4, 7, 7 -pentasubstituted 1,7 – diazaheptamethin systems (Piatelli, 1976).

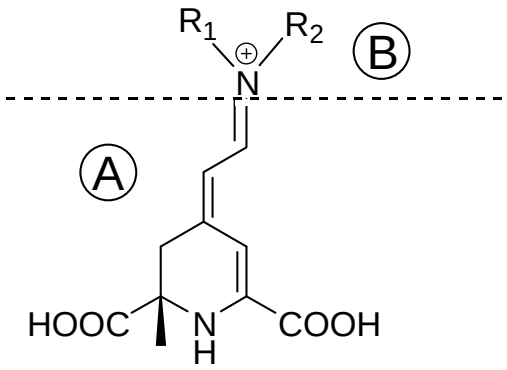

Figure 2. Betalain general formula. (A) Betalamic acid moiety is present in all betalain molecules. (B) The structure will represent a betacyanin or a betaxanthin, depending on the identity of R1 and R2 residues. (Adapted from Böhm and Rink, 1988).

Betacyanin structures (Figure 3A) show some variations in the acyl groups and sugar moieties, while betaxanthin (Figure 3B) exhibits the same dihydropyridine moiety but show conjugation with several amines and amino acids.

Betanidin is the basic structural unit of most of the betacyanins, followed by its C_{15} epimer, the isobetanidin (Piatelli and Minale, 1964). A considerable number of different

betacyanins can be obtained with glycosidation of one of the hydroxyl groups located at positions 5 and 6 (Figure 3A).

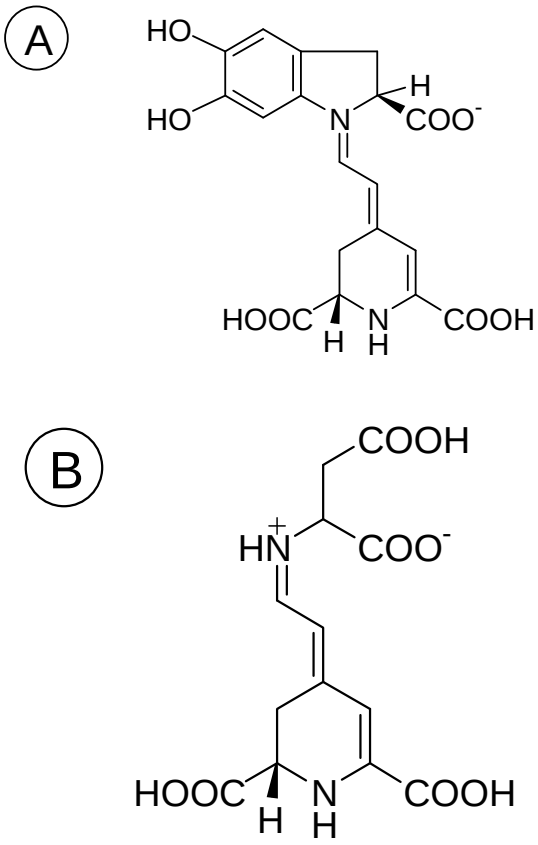

Figure 3. Betanidin is an example of betacyanins (A), while miraxanthin II is of betaxanthins (B). (Adapted from Piatelli, 1981and Strack *et al*., 1993).

Betaxanthins are constituted of different proteinogenic and non-proteinogenic amino acids, as well as biogenic amine – conjugated moieties of betalamic acids. More than 200 amino acids found in plants may potentially give rise to betaxanthin structure (Strack *et al*., 1993). The archetypal compound representing betaxanthins is the indicaxanthin, isolated from prickly pear (cactus fruits of *Opuntia ficus – indica*) (Piatelli, 1976).

1.5.2 Classification

Betalines can be divided into two structural groups, the yellow betaxanthins (from Latin *beta*, red beet and Greek *xanthos*, yellow) and red – purple betacyanins (c*yanos*, blue

colour), depending on R_1-*N*-R_2 moieties. More than 50 betalains are well known, and all of them have the same basic structure, in which R_1 and R_2 may be hydrogen or an aromatic substituent. Their colour is attributable to the resonating double bonds. conjugation of a substituted aromatic nucleus to the 1, 7 – diazaheptamethinium from 480 nm in yellow betaxanthins to 540 nm in red purple betacyanins (Strack *et al*., 1993).

In some earlier papers, the terms "betalains, "betacyanins", and "betaxanthins" were used; the terminal letter "e" was added by Fisher and Dreiding (1972) so the names would conform to the I.U.P.A.C. nomenclature; at present, these terms can be used without the terminal "e".

Betacyanins and betaxanthins can be classified using their chemical structures. Betacyanin structures show variations in their sugar (e.g., 5-O-$_D$-Glucose) and acyl groups (e.g. feruloyl), whereas betaxanthins show conjugation with a wide range of amines (e.g., glutamine) and amino acids (e.g. tyrosine) in their structures.

1.5.3. Distribution

Among higher plants the occurrence of betalains is restricted to the Caryophyllales (Mabry *et al*., 1963) and those found in certain higher fungi such as *Amanita, Hygrocybe* and *Hydrosporus* (Strack *et al*., 1993). Betalains of higher plants are in different organs (Rosendal *et al*., 1989). They produce red, yellow, pink, and orange colours in *Aizoaceae* and Portulacaceae flowers (Trezzini and Zryd, 1991b), and Portulacaceae flower, and purple pigmentation in Cactaceae fruits and in red beet root (Chenopodiaceae) (Piatelli and Imperato, 1969). *Bougainvillea* (Nyctagynaceae) possesses a wide range of colours (Piatelli and Imperato, 1970), they also in seeds of Amaranthus (Bianco-Colomas and Hugues 1990), in leaves of *Teloxis* and in stems (Arenas et al., 1993).

Common names and classification of different betacyanins and betaxanthins are standardized, and they are usually assigned in agreement with their botanical genus (Piatelli, 1976). In the betacyanin group, amaranthin – I was obtained from *Amaranthus tricolor*, betanin from *Beta vulgaris* and gomphrenin-I from *Gomphrena globosa*. While in the betaxanthin group, miraxanthin occurs in flowers of *Mirabilis jalapa*, vulgaxanthin I and II

have been found in root of *Beta vulgaris*, and portulaxanthin has been isolated from the petals of *Portulaca grandiflora* (Piatelli 1981).

Up to date more that 50 structures of naturally occurring betalains have been identified. A considerable number of different betacyanins may be derived from two basic compounds, betanidin (2S, 15S) and isobetanidin (2S, 15R) by glycosidation of one of the hydroxyl groups located at position 5,(Piatelli and Minale, 1964) for example, betanin, which occurs as the 5-*O*-glucoside, and the less-occurring position 6, for example. Gomphrenin-II, which is a 6-*O*-glucoside. No betacyanin is known to have both positions substituted with sugar residues (Piatelli, 1981). A few biosides are known amaranthin the betanidin 5-*O*-[2-*O*-(β- D -glucopyranosyluronic acid) β-D-glucopyranoside], and its epimer isoamaranthin is in the leaves of *Amaranthus caudatus* (Bianco-Colomas, 1980). Moreover, two epimeric betacyanins, bougainvillein-r-I and isobougainvillein-r-I, have been isolated from *Bougainvillea glabra* (Piatelli and Imperato, 1970).

There are about 15 natural occurring betaxanthins; the indicaxanthin from *Opuntia ficus – indica* was the first crystallized (Piatelli, 1981). In total eight of the naturally occurring betaxanthins contain non protein amino acids (Trezzini and Zyrd, 1991a).

1.6 Biosynthesis biochemistry and Molecular biology

1.6.1 Biochemistry

The determination of the chemical structure of betalains and of their biosynthetic intermediates contributed to the establishment of the corresponding biosynthetic pathway in addition, feeding experiments with isotopically labeled precursors and *in vitro* cell cultures were important tools in such discovering (Böhm and Rink 1988, Piatelli, 1981).However, very few enzymes involved in betalain synthesis have been purified and characterized (Leathers *et al*., 1992, Schwitzguebel *et al*., 1991, Strack *et al*., 1993). Figure 4 summarizes the propounded biosynthetic pathway. In detail, betalains are considered secondary metabolites; they derive from shikimic acid and from the tyrosine aminoacid (Piatelli, 1976). In their basic structure, the phenyl group is bonded to a lateral *n-propyl* chain giving place to a $C_6 – C_3$ unit (Berlin *et al*., 1986). Biogenesis of betalains from tyrosine has not been

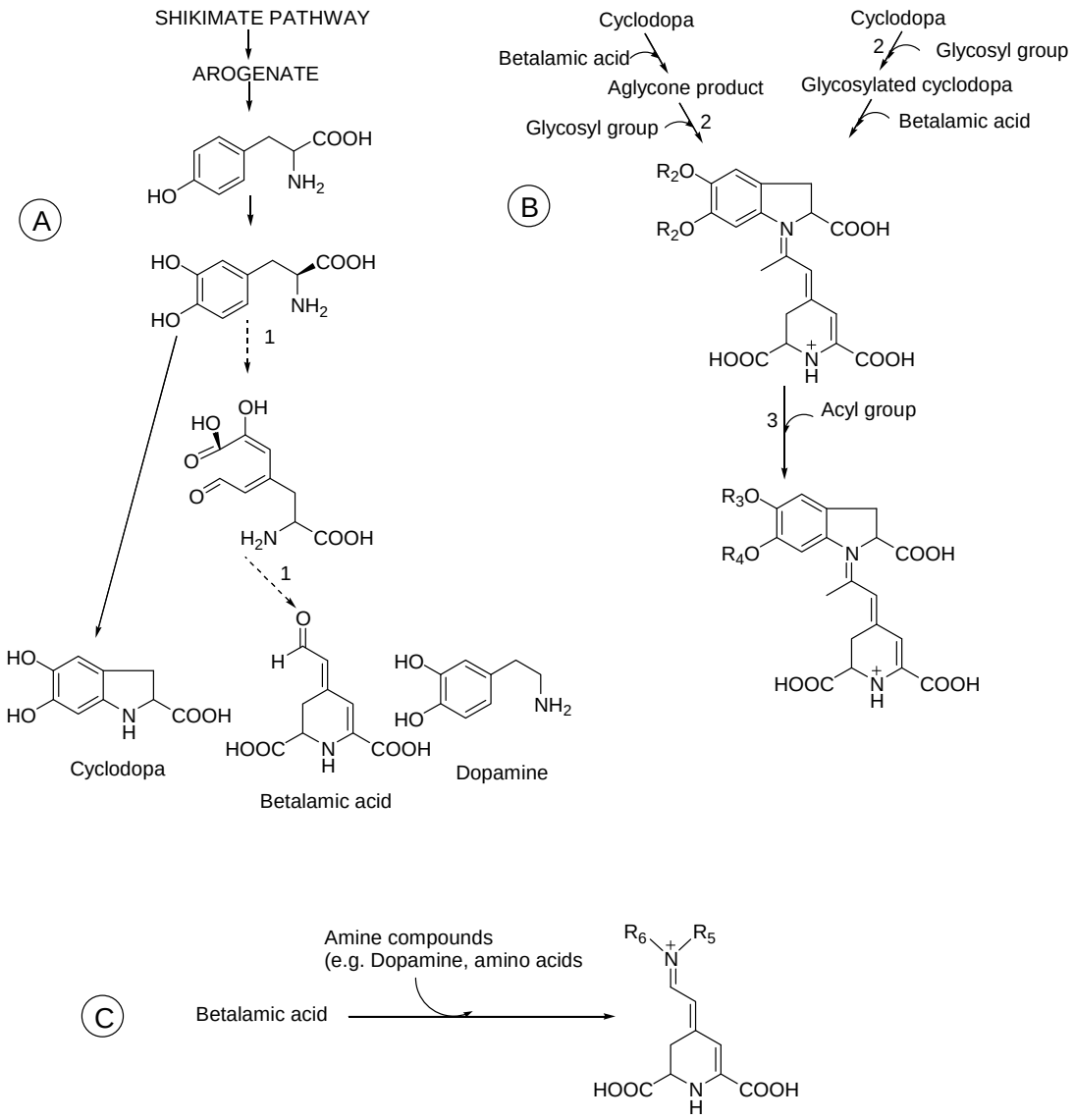

Figure 4 . Pathway proposed for betalain biosynthesis. (A) Initial stage, (B) betacyanin biosynthesis. Betacyanins may be glycosylated at position R_1 and R_6 and or acyglycosylated at position R_3 and R_4. (B) Betaxanthin biosynthesis. R_5 and R_6 represent lateral chains of amine compounds. Some of the enzymatic activities involved in betalain biosynthesis are (1) DOPA 4,5-dioxygenase, (2) glycosyltransferase, and (3) acyltransferase. (Adapted from Böhm and Rink 1988, Strack *et al*., 1993)

thoroughly understood, and only a few enzymes involved in the biosynthetic route have been identified (Bokern *et al*., 1992, Leathers *et al*., 1992). Two molecules of tyrosine are required in the biosynthesis of one molecule of betacyanin or betaxanthin. Initially, two molecules of 3-hydroxy-L-phenylalanine (L-DOPA) are formed (Mueller *et al*., 1996, Piatelli, 1981). The hydroxylation of tyrosine to L-DOPA is recognized as the first step in the biogenesis of betalains because experiments with radioactive precursors have shown good incorporation of labeled [C^{14}] - tyrosine into amaranthin and betanin, the major betacyanins in *Amaranthus tricolor* seedlings and red-beet (*Beta vulgaris*), respectively (Elliott, 1983). Mueller *et al.,* (1996) characterized a tyrosinase from pileus of *Amanita muscaria*. This enzyme was located only in the coloured parts of the fungi, and it was demonstrated that tyrosinase catalyzes the reaction of tyrosine hydroxylation to L-DOPA, confirming its involvement in betalain biosynthesis, tyrosinase also shows diphenolase activity, and it seems to be an heterodimer of two subunits with molecular weights of 27 and 30 kDa, something unusual for tyrosinases.

On the other hand, one L-DOPA molecule is transformed into DOPA-quinone, which is spontaneously converted to cyclo-DOPA, while betalamic acid is formed from the second molecule of L-DOPA by means of a reaction catalyzed by DOPA-dioxygenase (Mueller *et al*., 1997). In the proposed metabolic pathway betalamic acid arises from L-DOPA, which is cleaved at the C4-C5 bond of the aromatic ring to form an intermediate (4,5-seco-DOPA) that is cyclized, forming an heterocyclic system. It has been suggested that 4,5-seco-DOPA is produced by an "extradiolcleavage" of L-DOPA.

The oxidative disruption of the L-DOPA ring appears to be analogous to the aromatic rings fission of catechols, and the last reactions are catalyzed by specific dioxygenase. These enzymes contains Fe ion (both ferric and ferrous states) as an essential cofactor. DOPA 4,5-Dioxygenase was isolated from *A. muscaria*. This enzyme as other extradiolcleaving dioxygenases is an oligomer that catalyzes the 4,5-extradiol disruption of L-DOPA leading, via 4,5-seco DOPA to betalamic acid. It was shown by affinity chromatography that DOPA 4,5-dioxygenase is composed of a varying number of identical 22-kDa subunits. Another DOPA dioxygenase (DOPA 3,4 – dioxygenase) was also

extracted from *A. muscaria*; this enzyme catalyzes the 2,3-ring-opening reaction that yields the muscaflavin pigment, a compound that has never been found in plants. The betalamic acid may condense with the imino-group of cyclo-DOPA to produce the red-purple betacyanin (Figure 4B) or with the imino or amino group of amino acids to give the yellow betaxanthins (Figure 4C) (Strack *et al*., 1993).

Betacyanin biosynthesis (Figure 4B). Betacyanins are formed through the reaction of cyclo-DOPA with betalamic acid followed by glycosylation, or by condensation of the cyclo-DOPA glycosides with betalamic acid (Heuer *et al*., 1996). In general, it could be mentioned that condensation of betalamic acid with cyclo-DOPA to betanin formation and the subsequent glucosylation reaction to betanidin5-O-β-glucoside, main compound of red-beet, remain unknown. Complete glycosylation takes place with cyclo-DOPA followed by condensation with betalamic acid. On the other hand, free betanidin can be stored as the main component in betalain – producing cells, and it has been shown that betanidin can be the main receptor of UDP-glucose from glycosyl-transferase during the betanin biosynthesis (Heuer and Strack, 1992, Heuer *et al*., 1996). It may be possible that the sequence of reactions depends on the plant genus.

Betanin glycosylation catalyzed by uridine 5'-diphospho-glucose: betanidin 5-O-β-glucosyltransferase (5-GT) was demonstrated by Heuer and Strack (1992) who described the occurrence of one of the two proposed pathway of betacyanin formation, the transfer of glucose to 5-hydroxygroup of betanidin in the formation of betanin.

On the other hand, Heuer *et al*.,(1996) described a new glucosyltransferase, the 6-O-glucosytransferase (6-GT) that catalyzes the regiospecific transfer of glucose to the 6-hydroxygroup of betanidin in the formation of gomphrenin I, analogs to 5-GT. Both GT were extracted from cell cultures of *Dorontheanthus bellidiformis*. The 5-GT showed three isoforms and the 6-GT only one; both enzymes are monomers with a molecular weight near 55 kDa. Nowadays, it has been shown that both enzymes catalyze the indiscriminate glucose transfer from UDP-glucose to hydroxyl groups of betanidin, flavonols,

anthocyanins, and flavones. Interestingly, it was observed that GT catalyzes the formation of 7-O-glucosides, but in a minor extent (Vogt *et al*., 1997). Based on these results, it could possibly be that 5-GT and 6-GT have a phylogenetic relation with flavonoids glucosyltransferases. Subsequently, acylation of glycosylated betanidins is through acyl group donation from 1-O-acylglucosides. It is important to point out that this reaction catalyzed by an acyltransferase seems to be an exclusive biosynthetic mechanism of betalain-producing plants, contrasted with the analogous reaction in the biosynthesis of flavonoids where acylated flavonoids are produced by the hydroxycinnamoyl-coenzyme-A pathway (Bokern *et al*., 1992).

Another enzyme involved in betacyanin formation is an acyltransferase (HCA), which catalyzes the transfer of hydroxycinnamic acids from 1-O-hydroxycinnamoyl-β-glucose to the C_2 hydroxy group of glucuronic acid of betanidin 5-O-glucuronosylglucose (amaranthin) in *Chenopodium rubrum* (Bokern *et al*., 1992) the products formed are celosianin II (feruloylamaranthin). This enzyme exhibits a molecular weight near 70 kDa.

HCA could also catalyze the formation of 4-coumaroyl and feruloyl-derivatives in *Beta vulgaris* (lampranthin II), *Comphrena globosa* (gomphrenin III), *Lamppranthus sociorum* (celosianin I and II), and *Iresine lindenii* (lampranthin II) (Bokern *et al*., 1992). Betaxanthin biosynthesis (Figure 4C). However, it has been suggested the interchange of basic compounds as one of the main routes (Strack *et al*., 1993). A spontaneous condensation between betalamic acid and an amine group inside the vacuole has been based on genetic and biochemical studies on clones of *P. grandiflora*. In a recent work, (Hempel and Böhm, 1997) found two new betaxanthins in hairy root cultures of *Beta vulgaris var*. Lutea, vulgaxanthin III, and IV, when the culture media was supplemented with the corresponding L-amino acids. This feeding experiment provides arguments for a spontaneous condensation of betalamic acid with amino acids or amines in the course of betaxanthin biosynthesis.

In other experiments, the administration of L-DOPA to violet petals of flowers in *Portulaca grandiflora* led to the biosynthesis of betaxanthins not present in natural plants. These results show that L-DOPA administration elicits the formation of betaxanthins, which are absent in untreated flowers. In addition, nine L-and D-isomers of amino acid were supplied to seedlings and individual hairy roots strains of *Beta vulgaris*, var. Lutea. The increment of betaxanthin concentration and also the appearance of new betaxanthins was observed (Hempel and Böhm, 1997). Therefore, the theory of a spontaneous condensation of betalamic acid with amino acids or amines in the course of betaxanthin biosynthesis is supported.

On the other had, the activity of a betacyanin and betaxanthin-decolouring enzymes has been postulated in *Beta vulgaris*, (Lashley and Wile, 1979, Shih and Wiley, 1981) *Amaranthus tricolor* (Elliott, 1983), and *Phytolacca americana* (Kumon *et al*., 1990) the results suggest there is an enzyme complex with two acidic and two basic enzymes that contain a metal ion in the active site. Earlier works described this complex like a "peroxidase" enzyme (Im *et al*., 1990). Parra and Munoz (1997) confirmed that horseradish peroxidase catalyzes the oxidation of betanin. HPLC analysis showed a red intermediate and several yellow products (presumably of a polymeric nature) being betalamic acid, one of the final products. Zaharova *et al.,* (1995), described the occurrence of a betalain oxidase in three species of *Amaranthus*. This enzyme was found mainly in cell walls of *Amaranthus caudatus*, and it was suggested regulating the activity of this enzyme during plant tissue extraction could increase that pigment yield.

Watson and Goldman (1997) reported dominant alleles at two tightly linked loci (R and Y) of red beet. These alleles are involved in the production of betalain pigment. In addition, it has been shown that several alleles in these loci influence the production and distribution of betalains. The authors also suggested the existence of genes that play an important role in betalain synthesis.

1.6.2 Molecular Biology of betalain Biosynthesis

Very few of the enzymes have been purified and characterized that are involved in the biosynthesis of betalains (Hinz *et al*., 1997). The first enzyme involved in betalain biosynthesis was isolated from the mushroom *Amanita muscaria*. The only enzyme activities that have been described from higher plants are enzyme preparations that catalyze the glycosylation of betanidin and enzymes involved in betalain degradation, but work regarding genes is scarce. Two clones encoding polyphenol oxidase were isolated from a cDNA library constructed from a log-phase suspension culture of *Phytolacca americana,* producing betalains. Spatial and temporal expressions were investigated by northern blot analysis of total RNA from various organs of *Phytolacca* plants. Transcripts of the two clones were found to be 2.1 and 2.3 kb. Both transcripts were present only at substantial levels in the ripening of betalain containing fruits (Joy *et al*., 1995). Hinz *et al*., (1997) described the cloning and regulation of the gene *dod*A from basidiomycete *A.muscaria.* This constitutes the first effort to clone genes of the betalain biosynthetic pathway. *dod*A codes for a DOPA dioxygenase. The DNA library was constructed from the cap tissue of young specimens where betalain synthesis had not been yet begun. Southern blot analysis showed that a single copy gene in *A. muscaria*, encodes DOPA dioxygenase. (Hinz *et al*., 1997) Mueller *et al*., (1997), transformed white petals of *Portulaca grandiflora* with the *dod*A gene of *A.muscaria*, and the formation of yellow and violet spots that contained betalain and muscaflavin pigments was observed, indicating that the fungal enzyme DOPA dixoygenase was expressed in an active form in plants.

Tyrosinase activity was highlighted and partially purified in callus cultures of *Portulaca grandiflora* and red beet (reviewed by Strack *et al* 2003) which is a bifunctional enzyme which manages both the hydroxylation of tyrosinase into DOPA and the oxidation of DOPA into dopaquinone. Recently a tyrosinase-hydroxylase activity from betacyanin producing callus cultures from *P. grandiflora* was separated from a polyphenol-oxidase activity (Yamamoto *et al*., 2001) which is contradictory to the existence of a bifunctional tyrosinase or PPO. A bifunctional PPO was isolated from snapdragon flowers (*Antirrhinum majus*),

catalyzing the hydroxylation and oxidation of chalcones leading to aurones. cDNAs have been obtained for both betanidin 5-*O*-glucosyltransferase and betanidin 6-*O*-glucosyltransferase from *Dorotheanthus bellidiformis* species (reviewed by Strack *et al.,* 2003). Sequence comparision of these two enzymes show that only 19% of the amino acids are conserved, suggesting a paraphyletic origin of these two glucosyltransferases (reviewed by Strack *et al.,* 2003). A novel plant 4, 5-extradiol dioxygenase involved in betalain biosynthesis has been identified. This gene catalyses the last enzymatic reaction of the betalamic acid biosynthesis, opens the way to study the complete architecture of betaxanthins and betacyanins pathways.

1.7 Methodological Aspects

1.7.1. Extraction

Betalines-containing materials (raw plant or cell culture) are generally macerated or ground. Pigments can be extracted with pure water, cold, or at room temperature, although in most cases the use of methanol or ethanol solutions (20 to 50% v/v) is necessary to achieve complete extraction (Piatelli, 1981). Sometimes, the necessity of an aerobic juice fermentation (e.g., *Saccharomyces cerevisiae, Aspergills niger*) in order to reduce free sugars and then to increase the betacyanin content has been reported (Pourrat *et al*., 1988). In both procedures, the inactivation of degradative enzymes by a short heat treatment of the extract (70°C, 2 min) could the desirable, although this may destroy some of the pigments. Betacyanins can be precipitated by a slight acidification with hydrochloric acid or with acidified ethanol (0.1 to 1% HCl); subsequently, by the addition of 95% aqueous ethanol yields betaxanthins (Bilyk, 1974, Piatelli and Minale, 1964).

Degradation of betanin may occur very fast and destruction of complex pigments should also be avoided, because acid-acylated betacyanins are rapidly deacylated and such pigments can be over looked (Strack *et al*., 1993). In such a situation, extraction should be carried out with cold water for long-term and darkness conditions.

1.7.2 Separation

1.7.2.1 Ion exchange and column chromatography

Ionic – exchangers are the most widely used adsorbents in separation; then gel filtration is used (Piatelli and Minale, 1964). In a simple and rapid procedure, plant extract must be stirred with the ion exchanger resin (e.g., Dowex 50W-X2, Merck I, DEAE-Sephadex A25, etc.), which adsorbs the betalains (nonionic interaction). Subsequently, resin is washed with aqueous HCl (0.1% v/v) and pigments are eluted with water followed by final separation on a chromatographic column (e.g. Polyamide, Polyclarc-AT, or polyvinylpyrrolidone, Sephadex G-15 and G-25). The chromatographic and electrophoretic properties from unknown plant materials can be compared with those reported in the literature for known pigments e.g., Piatelli and Minale (1964), for betacyanins and acylated betalains, Piatelli and Imperato (1969) Von Elbe *et al*., (1972) Piatelli (1976) and Steglich and Strack (1991) for betalains in general.

1.7.2.2. Electrophoresis and thin layer chromatography (TLC)

Paper electrophoresis using pyridine and formic or acetic acid as solvents or in cellulose are common and reliable methods for betacyanin detection (Powrie and Fennema, 1963), because they migrate first as immobile zwitterions (pH 2), followed as monoanions (pH 2 to 3.5), and finally as *bis-anions* (pH 3.5, 7.0). In the case of betaxanthins, the mobility may be related to indicaxanthin, and betacyanins are related with the mobility of betanin (Piatelli and Minale, 1964). Electrophoresis can be carried out using pyridine-citric acid solvent, voltage gradient of 5.6 volts/cm, and a temperature of 4°C (Von Elbe *et al*., 1972). Capillary zone electrophoresis has been used for the analysis of betalains, particularly from *Beta vulgaris.* (Stuppner and Egger, 1996) This technique was carried out with a fused-silica capillary at 15°C and at a constant voltage of -22 kV, and it has permitted the separation of betanin, isobetanin and their corresponding aglycones. Bilyk (1981) developed a preparative TLC system in a 0.5 – mm cellulose-coated plate using two different mobile phase: isopropanol ethanol–water–acetic acid in a ratio of 6 : 7 : 6 : 1 (v/v) in the second one. When acid is incorporated in the developing solvent, betalain mobility on the TLC

plate is facilitated due to protonation of the betacyanin carboxyl group. The acid anion provides an electrically neutral system by its interaction with the quaternary nitrogen. The same effect occurs with betaxanthins (Bilyk, 1981). Good betaxanthin separations was also obtained on diethylaminoethyl cellulose plates using isopropanol-water-acetic acid (13:4:1 v/v) (Strack *et al*., 1993).

1.7.2.3. High performance liquid chromatography (HPLC)

The HPLC technique has become the method of choice for chromatographic separation, rapid quantification, and tentative identification of betalains. The first application was done by Vicent and Scholz (1978) using a C_{18} column with a gradient run using tetrabutylammonium in paired ion system as the mobile phase. The most useful column supports are C_8 and C_{18} column with a gradient run using tetrabutylammonium in paired ion system as the mobile phase. The most useful column supports are C_8 and C_{18} reversed phase with particle size between 3 to 10 μm, while the most used solvents are water -methanol or water-acetonitrile mixtures, acidified with acetic, formic, or phosphoric acid (Strack *et al*., 1993). HPLC elution order of pure crystalline pigments was as follows: betanin, betanidin, isobetanin and isobetanidin (Schwartz and Von Elbe, 1980). This evidence was based on an acid hydrolysis of the glycosides to yield aglycones and isomerization of betanin to isobetanin occurring (Vicent and Scholz, 1978).

Pourrat *et al*., (1988) analysed a fermented beet root extract using a reversed phase C_{18} column and ion pairing and methanol-water as mobile phase, and the elution order was betanin, isobetanin, betanidin, isobetanidin, and prebetanin for the betacyanins and vulgaxanthin I followed by vulgaxanthin II for the betaxanthins. Another good example of betaxanthin characterization was carried out by Trezzini and Zryd (1991a.).

1.7.3 Spectroscopy

Betalain analysis as that of other coloured compounds has been based basically on UV-visible spectroscopy. As a matter of fact, red violet betacyanins absorbs around λmax = 540 nm, while yellow betaxanthins at λmax = 480 nm, and the starting studies of betalain identification were supported in this Methodology (Piatelli, 1981). In addition, structural

modifications of betalains have been followed by UV-visible spectroscopy (Mabry, 1980, Mabry and Dreiding 1968, Piatelli, 1981). However, in the 1980s spectroscopy showed enormous progress and nowadays chemical characterization must be carried out considering at least HPLC separation and UV-visible, MS, and NMR spectroscopies rigorous characterization of betanin, lampranthins, cellosianins, neobetanin, among others, was established (Steglich and Strack, 1991, Strack *et al*., 1993, Stuppner and Egger, 1996).

1.7.4 Quantification and pigmenting efficiency

Nilsson (1970) developed a method to measure both red and yellow betalains of *Beta vulgaris* without prior separation of betacyanins and betaxanthins. Their quantitative determination mainly involved spectrophotometry, where the absorbance at the maximum wavelength (λ) is translated into concentration by means of the appropriate absorptivities. Another method is based on electrophoretic separation of individual pigments followed by the measurement of the colour intensity of the separated bands in a densitometer. The result was expressed as peak area in cm^2, which was determined with the aid of an integrator after correcting the baseline; thus, the results are translated to concentration comparing them with a betanin standard curve (Von Elbe *et al*., 1972).

A computer aided determination, based on previously reported absorptivity values, has been performed by Saguy *et al*., (1978). This method uses a nonlinear curve fitting of the spectrum with a predicted function of the individual pigments (e.g., betanin, betalamic acid, vulgaxanthin-I). The proposed procedure is rapid and accurate, avoiding the laborious and time-consuming separation steps. Schwartz and Von Elbe (1980) developed a method to quantify individual betalains by HPLC using the molar absorptivity of each pigment instead of absorptivity values. This method provides a more accurate determination of the total betalain content. Sapers and Hornstein (1979) reported the Hunter colour values for standardized dilution of juice from 48 red beet cultivars. “a” values varied over a relatively narrow range being slightly lower in samples with higher “b” values. The analyses of the 20 most highly pigmented cultivars produced values in the range 8.5 to 17.2. Interestingly, it was reported that “b” values could be used in the estimation of the betaxanthin-betacyanin.

1.8 Importance as Food colours – stability, processing and production

1.8.1 Stability in Model System

When betalains are used as food colourants, colour stability is a major concern. There are several factors that have been recognized to affect the stability of these pigments

1.8.1.1 pH

The hue of betalains is unaffected at the pH between 3.5 to 7, the values of most foods are in this range. Betalain solution in this pH range showed a similar absorption pattern for betacyanins and betaxanthins. Betacyanin wavelength (λ) maximum is in the range 537 to 538 nm, while betaxanthin maximum is between 475 to 477. Below pH 3.5, λ shifts toward a lower wavelength, and above 7 the change is toward a longer wavelength, out of the pH range 3.5 to 7 the intensity of the visible spectra decreases (Huang and Von Elbe, 1987).

Stability of betanin solution is pH dependent. Huang and von Elbe (1985, 1987), have shown that optimal pH for maximum betanin stability in the presence of oxygen is between 5.5 to 5.8. Red beet solutions showed their maximum stability at pH 5.5, the normal pH for beets. In addition, vulgaxanthin was most stable between pH 5.0 to 6.0, and it was more stable in juice than in purified extracts, while optimal pigment stability in reconstituted powders was noted at pH 5.7 (Singer and Von Elbe, 1980). Fermented and concentrated preparation of betalain from garambollo (*Myrtillocactus geometrizans*) at pH values tested (4, 5, and 6), was found to be very stable at pH 5, pure pigments of garambollo were 15% more stable than red beet at pH 5.5, however in crude form beet extract was stable than garambullo extract. Garambullo pigments demonstrated higher pH stability than beet root pigments showing 1% loss of betalains compared to 35% in beet root on first day. Betalains from several *Amarathus* species tested for pH stability, *Amaranthus cruentus* showed greatest stability between pH 5-7 at 25^{o}C, especially at 5.6 (Cai *et al*., 1998a). In the pH range 5-7 the spectral curves were very close, their λ_{max} values were all 535nm, and the colour of the pigment solution was constant (Cai *et al*., 1998 a, b). Betacyanins obtained from different species of *Opuntia* were showed to be thermally stable at pH 5 (Castellar *et al*., 2003).

1.8.1.2. Temperature

Temperature also shows a clear effect of betalain stability (Drdak and Vallova, 1990). Thermal kinetic degradation of betanin has been evaluated by several authors (Altamirano *et al*., 1993, Drdak and Vallova, 1990, Huang and Von Elbe, 1987, Huang and Von Elbe, 1985, Saguy *et al*., 1978, Von Elbe *et al*., 1974). It has been reported that thermostability of betanin solutions is pH dependent and partially reversible. Heating of betanin solutions produces a gradual reduction of red colour, and eventually the appearance of a light brown colour. Von Elbe *et al*., (1974) observed a first order reaction kinetics for betanin degradation by heating. Saguy *et al*., (1978) developed a thermal kinetic degradation model using a nonlinear least-square technique; this model enables one to predict the retention of betalains under variable conditions of temperature and time. Thermal degradation of betalains produced activation energies E_A) in the range 17 to 21 Kcal-mol^{-1} for the forward reaction, whereas the reverse reaction showed values between 0.6 to 3.5, (Huang and Von Elbe 1987, Saguy *et al*., 1978) It was also reported that E_A values showed a pH dependence. Betalamic acid and cyclo DOPA-5-*O*-glycoside have been reported as the probable intermediates for betanin degradation (Huang and von Elbe 1985, Saguy *et al*., 1978). Interestingly, the regeneration (reverse reaction) involves a Schiff's base condensation of the amine group of cyclo DOPA-5-*O*-glycoside with the aldehyde group of betalamic acid, betanin is rapidly formed when both compounds are mixed in solution (Huang and Von Elbe, 1985. Altamirano *et al*., (1993) reported the lowest stability of betanin and the lowest E_A in a water ethanol model system, supporting the idea the first step of the thermal betanin degradation is the nucleophilic attack on the >N + = CH- structure of betanin. Ethanol has a high electron density on the oxygen atom; therefore, it is a strong nucleophilic agent that diminishes the betanin stability. A linear behavior was shown for the depletion of the garambullo pigment after it was exposed to different temperatures, and it followed a first order reaction. The colour of the pigment decreased when the temperature increased, with a *k* of 1.18 x 10^{-5} min^{-1} and a $T_{1/2}$ of 58650 ± 1980 min at 0^{o}C and a k of 1.54 x 10^{-1} min^{-1} and a $T_{1/2}$ of 4.49 ± 0.28 min at 100^{o}C, but the garambullo pigments were more stable in comparison to beet pigmenst (Reynoso *et.al.*1997**)**.

1.8.1.3 Light

Von Elbe *et al*. (1974) found that rate of betanin degradation increased 15.6% after pigment day light exposure at 15°C. Degradation of light-exposed betalains followed a first-order kinetic. In addition, it was observed that degradation was higher at pH 3.0 (k=0.35 $days^{-1}$) than at pH 5.0 (k = 0.11 $days^{-1}$), when betacyanins were exposed to fluorescent light. On the other hand, at darkness conditions betacyanins were most stable (k = 0.07 $days^{-1}$) (Sapers and Hornstein, 1979). Attoe and Von Elbe (1981) showed an inverse relationship between betalain stability and light intensity in the range 2200 to 4400 lux. It is explained that visible light absorption excites π electrons of the pigment chromophore to a more energetic state (π∗). This would cause a higher reactivity or lowered activation energy for the molecule (E_A = 25 $Kcal.mol^{-1}$ in darkness and 19.2 in illumination). Aurstad and Dahle (1973) reported the effect of UV and gamma irradiation on betanin stability, total pigment destruction was reported by the treatments with 120 h of UV radiation or with 100 krad of gamma radiation. Nevertheless, these results, the photodegradation mechanisms for betalins remain to be determined. Reynoso *et.al.*(1997) have shown a slight increase in stability under light conditions of red beet and garambullo pigments in the presence of 0.1% ascorbic acid. When temperature was increased, the degradation of *Amaranths* betalain pigments accelerated with a *k* of 2.25 x 10^{-4} min^{-1} and a $t_{1/2}$ of 275 min at 40°C and a *k* of 3.71 x 10^{-2} min^{-1} and $t_{1/2}$ of 19 min at 100°C in presence of light (Cai *et al*., 1998a).

1.8.1.4 Water activity

Recognizing the importance of water in many degradation reactions, it is not surprising that water activity (a_w) is included among the primary factors affecting the betalain stability and / or colour of a food product containing these pigments (Von Elbe, 1977) because the degradation reaction does involve water, the greatest stability of betalains has been reported in foods or model systems of low moisture and a_w. (Cohen and Saguy, 1983) Pigment degradation follows first order kinetics, and stability increases with decreasing a_w. (Saguy *et al*., 1984). It has been established that a_w has a pronounced exponential effect on pigment stability. Pigment stability decreases in one order of magnitude when a_w was increased from 0.32 to 0.75 (Cohen and Saguy, 1983).

On the other hand, Simon *et al*., (1993) studied the influence of aw on the stability of betanin in various water alcohol model system. In all cases, it was observed a rate constant dependence with a_w (Von Elbe, 1987). The increase in stability of betanin with decreasing a_w may be attributable to reduced mobility of reactants or limited oxygen solubility. Consequently, high moisture content produces a high degradation rate. Furthermore, specification of a_w alone without the moisture content is not enough to predict pigment stability. A 10 month storage study of aqueous and dry betalains from *Amaranthus* at 4°C and 25°C showed that dried samples had very high pigment retention of 93.4% at 4°C and 78.2% at 25°C, where as the aqueous extract had 62.3% at 4°C and only 18.3% at 25°C after only 2 months of storage (Cai *et al*., 1998a).

1.8.1.5. Oxygen

Oxygen causes a product darkening and loss of colour. Von Elbe *et al*., (1974) stored buffered betanin solutions at pH7 under atmosphere of air and nitrogen for 6 days at 15°C; it was observed that colour degradation increases up to 15% due to air conditions. Betanin reacts with molecular oxygen, producing pigment degradation in air saturated solutions (Attoe and Von Elbe, 1985). Degradation kinetic under air atmosphere follows a first order model, but deviates from first-order in the absence of oxygen. As mentioned above betanin degradation is a partially reversible reaction, (Attoe and Von Elbe, 1985) and it has been reported that in order to increase the recovering of pigment it is necessary to have the sample under low levels of oxygen. Thus heated betanin solutions (pH 4.75, 130 min, 15°C) under low oxygen levels showed an increased betanin retention from 54 to 92% (Huang and Von Elbe, 1987). Reaction reversibility was responsible for the deviation from the first order degradation kinetics of betanin in the absence of oxygen. Several methods have been reported to prevent the destruction or to improve the stability of pigments, including degassing, addition of antioxidants and stabilizers, control of pH, minimal heat treatment, among others, (Altamirano *et al*., 1992, Attoe and Von Elbe, 1985, Bilyk *et al*., 1981, Han *et al*., 1998, Pasch and Von Elbe, 1979) and these efforts have been directed to their application in food products.

1.9 Processing and Stability in Foods

The sensitivity of betalains to different factors suggests that their application as food colourants is limited. Based on these properties, betalains can be used in foods with a short shelf-life, produced by a minimum heat treatment, and packaged and marketed in a dry state under reduced levels of light, oxygen and humidity (Rayner, 1993, Von Elbe, 1977).

Betanins have several application in foods, such as gelatins desserts, confectioneries, dry mixes, poultry, dairy and meat products (Counsell *et al*., 1979, Von Elbe, 1977). Table 2 summarizes some applications of betalain pigments in food products. The amount of pure pigments required in these foods groups to obtain the desired hue is relatively small and for most applications does not exceed 50 ppm of betalains, calculated as betanin. Problems associated with betalain degradation and pigment recovery during the processing operations are of economic importance and must be solved to betalains to displace the application of synthetic dyes in some food products. The effectiveness of commercial betalains depends largely on a continuous availability of highly pigmented sources, the use of cold and modified storage atmospheres prior to processing, efficient enzymatic control, handling practices, extraction procedures, purification, concentration, and finishing operations e.g. freeze, spray, and vacuum drying).

Nowadays, beet roots represent the main commercial source of betalains (concentrates or powders) (Pszczola, 1998). Many factors during the pre and post harvest period and during processing influence the recovery of these natural beet colourants. In addition, recent efforts are centered around the betalain content in red beets through selective breeding. Initially, high pigment content is very important. The average pigment content of beets is approximately 130 mg/100 g fresh weight, (Sapers and Hornstein, 1979, Von Elbe *et al* ,. 1972, Von Elbe 1977) but new red beet varieties produce around 450 to 500 mg / 100 g fresh weight. Furthermore, this value is increasing, as advanced selection is developed (Pszczola, 1998).

Table 2. Application of Beet Root Powder as Natural Colour in Food Products.

Food products	Shade	Level
Dairy products		
Strawberry yogurt	Rose-pink	0.09%
Ice creams	Pink	0.25%
	Rose-pink	0.20%
Meat products:		
Sausages	Pink	600 mg/100g
Cooked ham	Pink-brown	0.17%
Dry powder beverages	Strawberry	1.2%
	Raspberry	1.5%
	Blackcurrant	1.0%
Water ices	Strawberry-red	0.5 to 1.0%
	Raspberry	0.5 to 1.0%
Marzipan	Pastel-red	0.4%
	Bluish-red	2 mg/cm^2
Baked goods	Pink-brown	2.5%
Biscuit creams	Pink	0.28%
	Brown	1.6%
Hard candies	Pink	0.1%
Jellies	Raspberry-red	0.2%
Fruit cocktails	Raspberry-red	2.0%

Commercial preparations of beet pigment for use as food colourants are available as either juice concentrates (produced by concentrating juice under vacuum to 60 to 65% total solids) or powders (proceed by freeze or spray drying). These preparations contain from 0.3 to 1% of pigments (Blok *et al*., 1981, Cerezal and Nunez, 1996, Cerezal *et al*., 1994, Von Elbe, 1977). They show a variety of colours, depending on their content of yellow pigments, and may have a beet-like odor and flavor. The remainder of the solids is mainly sugars (75 to 80%), ash (8 to 10%), and protein (10%). On a laboratory scale, betalains can be obtained by employing reverse osmosis, (Lee *et al*., 1982) ultrafiltration, (Bayindiril *et al*., 1988, Real *et al*., 1993) solid liquid extraction, (Lee and Wiley, 1981, Wiley *et al*., 1979) and diffusion (Wiley and Lee 1978) . These processes have been shown to be efficient on the recovery of betalains from raw beet tissue when compared with conventional hydraulic techniques. As approximately 80% of beet juice solids consist of fermentable carbohydrates and nitrogenous compounds, a fermentation process to remove these materials has been widely employed (Drdak *et al*., 1992). The yeast *Candida utilis* and *Saccharomyces cerevisiae* have been used in the fermentative process. The powder obtained from fermented

juice contained five to seven times as much as the betacyanin obtained in the powder from raw juice (on a dry weight basis).

1.10 Production of Betalains by Plant Tissue Culture

Plant cell and tissue culture has been a very useful tool in the study of various aspects of biochemistry, enzymology, genetics, and biosynthesis of betalains, (Leathers *et al*., 1992) and, interestingly, betalain production by plant cell culture will represent an excellent option in the future; it has a number of advantages over conventional procedures. Mainly with this methodology, it is possible to control quality and availability of pigments independently of environmental changes (Dornenburg and Knorr, 1997). Nevertheless, the productivity of the bioreactor systems must be increased over 0.168 mg/g dry weight/day (Jimenez *et al*., 1996 Stafford, 1991) and the cost reduced below$0.15 U.S. dollars/l in order to be considered economically feasible (Jimenez and Gutierrez, 1999). Thus, a successful betalain production will depend on process optimization to maximize yields, and consequently, suitable downstream recovery techniques must be available (Pszczola, 1998).

Betalain production has been detected in cell cultures of plant species belonging to five families of Caryophyllales (Böhm and Rink, 1988). Betalain accumulation of betalains in beet callus culture was reported and also has been demonstrated in cell culture of *P. grandiflora*, (Endres, 1976) *A. tricolor*, (Bianco-Colomas and Hugues, 1990) *O. microdasys*, (Jimenez *et al*., 1992) *C. rubrum* (Berlin *et al*., 1986). Plant cell cultures are generally deep-red or purple coloured; it means that betacyanins are dominant over betaxanthins. Some models do not produce betaxanthins. It has been also established that main betacyanin in most of the studies models is betanin. Schwitzguebel *et al*., (1991) observed that individual *Beta vulgaris* callus cultures contained cells exhibiting a variety of colours either non-pigmented, yellow, orange, red or purple. The range of observed pigmentation was due to the presence of the deep red-purple betacyanins and to the yellow betaxanthins contained within the cell vacuole.

Recently, plant hairy roots have become of interest as an alternative for cell culture because their infinite and active proliferation in a phytohormone-free medium and their

ability to synthesize and accumulate valuable betalains at comparable levels to those found in plants (Taya *et al*., 1992). Extracellular production of betalains accompanied by pigment release has been obtained in hairy root cultures under oxygen starvation (Kino-oka and Tone, 1996). However, both plant cell and hairy root cultures are influenced by a variety of physical and chemical factors implicated in the production of betalain pigments, and some of them, such as growth regulators, light, nitrogen, carbon, and microelements, are widely discussed by Böhm and Rink (1988) and Leathers *et al*., (1992).

In addition, it is important to consider that betalain production by cell or hairy root cultures are mainly empirical, and it is not supported by a strong knowledge of the underlying mechanisms of biosynthesis and regulation. Nevertheless, in some instances, such as in the production of *B.vulgaris* betalains, it is possible to obtain cultures producing specific pigments in comparable or even larger quantities than in the tissues of the original plant (Stafford, 1991).

Hempel and Böhm (1997) administrated nine L-amino acids to hairy root cultures of *Beta vulgaris* var. Lutea. Two betaxanthins, portulaxanthin II and vulgaxanthin I, were produced predominantly, while minor quantities of muscaauri-VII, dopaxanthin, and indicaxanthin were synthesized *de novo*. These results are very important because the possibility of betaxanthin production at commercial level is opened, and interestingly, red beet is one of the best known betalain production models, being easiest the methodology standardization. Nowadays, the selection of a bioreactor and cultivation techniques for optimal culture growth and betalain production is one of the most important issues to be solved (Jimenez and Gutierrez, 1999). Much data are now available about the growth and production kinetics in suspension cultures (Leathers *et al*., 1992) however, most of them are not suitable for the design of large scale processes, and the growth and production kinetics must be studied under well defined conditions and at different steady states using the type of bioreactor selected for the large-scale process (Jimenez and Gutierrez, 1998).

1.11 *Beta vulgaris* Linn. (Chenopodiaceae)

A genus of glabrous succulent herbs with swollen roots containing sugars, commonly called beets, found in Europe and the Mediterranean region and cultivated in various parts of the world. The cultivated beets include sugar beet, garden beet, leaf beet, chard or swiss chard and mangels.

1.11.1 Beet root (garden beet or table beet)

The garden beet is a succulent, tuberous herb with mostly basal, ovate to oblong-ovate leaves and greenish flowers in panicles. It is cultivated as a vegetable almost throughout India producing a large swollen fleshy root and a rosette of leaves in the first year and in the second year, flowers and seeds. It has been known for a long time and its use as a root crop. Beet root is known to have originated in the Mediterranean region and the adjacent regions of central and West Asia. It is considered to have arisen by selection from within a wide range of diversity of the leafy beets; intermediate forms between leafy beets and beet root are found in Asia Minor and Central Asia.

Beet varieties are grouped on the basis of the shape and time of maturing of roots. The two varieties which are most commonly grown in India are 'Crimson Globe' and 'Detroit Dark Red', both having globular-oval roots. For the table, types with the deepest blood colour and predominance of succulent tissue are preferred. The important types of beet root cultivated in India with their morphological characters are given in Table 3

1.11.2 Cultivation of red beet

Beet root is essentially a cold season crop and is hardy to frost. The optimum temperature for its growth is 16^{o}C. The plants require plenty of sunshine. The crop can be grown on a variety of friable soils. It is sensitive to soil acidity and yields better in pH range of 5.8 to 7.0. Two types of beets are met with, the round rooted and the long rooted. The former matures earlier and yields better on shallow soils (The Wealth of India, 1985).

The soil is adequately manured with farmyard manure (25 to 37 tonnes/ha); fermenting manure is avoided as it produces mis-shapen roots. The crop removes about 2 kg of nitrogen, 4 kg of phosphoric acid and 4 kg of potash per ton of root yield. For an average

soil, the recommended manurial doses are 60-70 kg nitrogen, 100-120 kg phosphorus, and 60-70 kg of potash per hectare. Nitrate sources of nitrogen are preferred. The crop has a relatively high boron requirement, its deficiency leading to black spot or dry rot disease (The Wealth of India, 1985).

Sowing is done from mid-August to December in the plains and during March-July in the hills. Several successive sowings at intervals of 2-4 weeks ensure a continuous harvest of marketable roots. In some parts of South India, the crop is sown throughout the year.
The seeds are mixed with coarse sand before sowing. The field is laid out into ridges 15-22 cm high and 45 cm apart. The seeds (5-6 kg/ha) are dibbled on both sides of ridges and covered lightly with earth. The field is then irrigated if the soil is not moist at sowing time. The seeds usually germinate in 3-4 days. In some parts seedlings are raised in nursery and transplanted in the field but this practice tends to produce ill-developed roots. The plants are later thinned to a distance of 15 cm (The Wealth of India, 1985).

Frequent weeding is done during the early stages of growth; later the plants are not disturbed. Spraying of common salt (200 g/I) eradicates most of the weeds. The crop requires frequent irrigation. Fortnightly watering with liquid manure encourages rapid growth and tender roots. Slow growth leads to woody roots. High temperature causes zoning in the roots instead of the desirable solid colour. Under cool weather the roots develop high sugar content (The Wealth of India, 1985).

1.11.3 Cultivation of seed crop

The plants produce seed only above elevations of 1,200m in India in the Kulu valley in Himachal pradesh and in the Nilgiris in South India. They are cross-pollinated, generally by wind. As such for growing seed crop of commercial varieties about 1.6 km of isolation is required between two varieties. For production of nucleus and foundation seeds 3.2 km of isolation is required (The Wealth of India).

The mature roots serve as a seed material for the production of seeds. Hence the fields from which the roots are to be collected are subjected to close scrutiny and on the basis of foliage characters, all off-type plants are pulled out and rejected. Roots from such rogued field are examined on the basis of their external appearance and then selected for growing a seed crop. Roots having uniform and rich flesh-colour with indistinct rings are selected as planting material. For the commercial seed crop it is preferable to plant the

whole root. For production of nucleus seed crop, the roots are cut off one-third or one-fourth of their length (The Wealth of India, 1985).

The mature roots of the crop grown for vegetable are left *in situ* and they develop suckers. At this stage, irrigation is withheld for about fifteen-days coinciding with the dry weather. During Nov-Dec in Kulu valley and Janurary in the Nilgiris, the roots are lifted out of the soil. The shoots are trimmed without injuring the crown. Such roots are ready for the planting in the field in pits of 0.3 m^3 at a spacing of 60 X 60 cm or 60 X 45 cm, keeping the terminal bud just above the ground level. Watering is done carefully and judiciously for about 15 days so that the root establishes itself properly (The Wealth of India, 1985).

TABLE 3. Characteristics of some varieties of garden beet grown in India

Variety	Roots	Leaves	Remarks
Detroit Dark Red	Globe-shaped, symmetrical with small tap roots, deep blood red inside. Retains excellent quality until roots become large and fully grown	Small, glossy, dark green tinged with maroon	Comparatively late, widely cultivated
Crimson Globe	Globular to flattened Globe, medium red, small tap roots, flesh crimson with indistinct Zones	Bright green with maroon shades	Heavy yielder, sweet, edible even when raw
Egyptain Turnip Rooted	Roots as larges as turnip. Dark crimson colour. zones absent	------	High yielding, good for salads and preserves
Early Wonder	Slightly flattened, alternate zones of purplish red and purplish pink flesh in warm weather. Flesh dark coloured	--------	
'Sutton's Blood Red'	Long beet, flesh blood red	-------	Good strain

* The Wealth of India, 1985.

1.11.4 Diseases and Pests

The crop is generally free from serious diseases and pests and losses if any, are minor. The seed crop in kulu valley is especially free of insect pests and diseases. Among fungal diseases caused in the field, leaf spot due to *Cercospora beticola* is quiet common. It causes small circular brown spots, which later coalesce and cause blight. The affected leaves die prematurely. Treating the seeds with Ceresan (8gm/kg of seeds) or subjecting them to hot water treatment before sowing, and spraying the crop with Bordeaux mixture (1%) controls the disease. *Puccinia betae-bengalensis* commonly infects the leaves of beet root in the plains of North India causing pustules on the leaf blade. Beet root is also susceptible to a root rot due to *Rhizoctonia solani* (The Wealth of India, 1985)

Pests- Beet root is susceptible to a number of pests. *Trichodorus* sp., the stubby root worm, is an ectoparastie, which feeds on the root tips. The roots become brown and small, the young seedlings are badly affected (The Wealth of India, 1985). Wireworms feed on the underground portions of the beet plants and cause tunnels or scars. Other pests are the flea beetles, webworm and the leaf miners. Malathion can control these. Root knot due to the nematode *Meloidogyne javanica* is severe in the plains. It produces knotting of the lateral roots, reduced tap root size, and stunted and chlorotic plants.

Boron deficiency- Beets have a relatively high boron requirement; its deficiency causes heart rot or internal black spot. The chief symptoms are necrosis of the petiole, browning, and premature unfolding of leaves, and a dry root rot. Application of 22-85 kg/ha of borax along with other balanced fertilizers helps in avoiding the disease (The Wealth of India, 1985).

1.11.5 Yield

The yield of beet roots varies between 20 and 25 tonnes/ha and that of seed betweeen 1 and 2 tonnes/ha (The Wealth of India, 1985).

1.11.6 Storage

Both the tops and roots of beet wilt very quickly under normal conditions. At freezing temperature and a relative humidity of 95-98% the roots can be stored up to two months. Larger beets have a better keeping quality. For long storage the tops are cut off close to the roots and all diseased of decaying matter removed. Radiation helps in preventing sprouting which otherwise sets in after three months. The seeds are sun-dried thoroughly for storage. They are stored in sealed containers and have al life of four years.

1.12 Micropropagation

Micropropagation using axillary bud proliferation has been the most genetically conservative method of *in vitro* mass multiplication (D' Amato 1977). Nearly 50,000 plant varieties have been propagated so far by this method (Vasil 1994). Ornamentals and horticultural species constitute majority of the plants propagated by this method (Debargh and Zimmerman 1991). Micropropagation also finds its application in plant breeding for maintenance and multiplication of special genotypes of hybrids or new cultivars including those derived from genetic engineering (Wenzel 1992). In general micropropagation is extremely useful, when there is shortage of healthy seeds or planting material and, for raising disease-resistant clones (Conger 1981). Micropropagation offers other advantages such as a) minimum requirement of plant material for initiation of culture b) higher output per unit area c) availability of plantlets irrespective of seasons, d) higher health status of aseptically maintained plants, e) desirable characteristics of the clones, f) induced regeneration of recalcitrant woody species, and g) easy in vitro root induction in recalcitrant shoots (Pierik 1993).

Micropropagation also provides plant material ideal for international exchange, and also for germplasm conservation. The great advantage of micropropagation is its capacity to generate thousands of propagules within a short duration of time at any period of the year. Moreover, micropropagation industry is environment-friendly with high employment potential.

Though micropropagation system has many advantages, the limited numbers of plantlets that are produced, involving high costs, have led to stagnation of industry. Thus micropropagation has been found to be economically viable only in high per-unit value

crops like ornamentals, fruits and nut trees. Vegetable and other horticulture crops have to be made cheaper by reducing the cost of production, while at the same time increasing productivity.

Micropropagation consists of the following steps as described by Murashige and Skoog (1962): stage I- establishment of aseptic cultures from primary explant; stage II - multiplication of shoot cultures and stage III-*in vitro* rooting, hardening and transplanting to soil. In micropropagation procedure, the labour involved is in preparation of the nutrient media and its dispensing into culture vessels, manual transferring of explants into the media, cutting and transferring of the shoot cultures into growth media and subsequently into rooting media before hardening.

1.12.1 Automation in micropropagation

In order to offset the high cost of production automation has been attempted to bring down the labour use and enhance production**.** This would make the process viable for large volume, low per-unit value crops, such as vegetables like tomato, potato, celery etc. or plantation crops such as rubber, coffee, tea etc.

Completely integrated mechanical/biological automated micropropagation process has been developed in recent years. The process involves growing compact mass of shoot meristem in bioreactors, which were then processed, separated, and distributed automatically into a rooting medium. Rooted plantlets were automatically planted into soil, using a planting machine. It was claimed that using this process, labour cost was reduced by about 85%. Viable propagules were selected using machine vision system and planted into nutrient media automatically. However, the robot assisted process is expensive, requires highly skilled and trained labour and is unaffordable to any industry. Moreover, the robot-assisted process has been reported to be used only in stage I and does not apply to other stages of micropropagation, which are equally, if not more, labour intensive.

1.12.2 Micropropagation using Bioreactors

Scale-up of shoot cultures using bioreactors has also been proposed as a means of cutting down the cost of production. Akita and Shigeoka (1994) used 500 L bioreactor for mass propagation of multiple shoots of S*tevia rebaudiana*, resulting in nearly 140-fold

increase in shoot weight, over a period of 4 weeks. *In vitro* production of potato tubers in 10 L jar fermentor was demonstrated by Akita and Takayama (1994). A semi-continuous, medium surface level control method resulted in production of tubers, which were identical to those developed *in vivo* on dry weight basis. These tubers were sprouted in room conditions and directly planted into soil without hardening.

1.12.3 Commercialization of micropropagation

Over the years, micropropagation has been evolved from a basic research tool to a commercial enterprise, which play supplementary role to conventional breeding techniques in enhancing agricultural production. Since the establishment of the first commercial tissue culture laboratory in 1970, more than 600 industrial units have become fully operational all over the world, producing millions of plant.

Though there has been exponential increase in the number of commercial micropropgation units worldwide, the demand for tissue culture products have been ever increasing, a far exceeding production. Majority of production is in ornamentals, orchids, other cut flowers and pot plants, followed by horticultural crops.

1.13 Transformed Hairy Root Cultures

Hairy root and the crown gall tumor are two plant diseases caused by two gram-negative soil bacteria, *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens*, respectively. Depending on the strains of *Agrobacterium* involved, one or both of the two pieces (TL and TR) of the Ti-(tumor inducing) or Ri- (root inducing) plasmid are transferred from the bacteria into the nuclear genome of the host plant. The transferred T-NDA derived from the Ti-plasmid causes the plant cells to proliferate, to form crown gall tumors, and in the case of the Ri-plasmid, the extensive formation of adventitious roots at or near the site of infection. The transformed plant tissues are also directed by T-DNA genes to produce unusual metabolites called opines, that serve as specific nutrients for the bacteria (Chilton *et al*., 1982, Chilton, 2001).

The molecular mechanism of T-DNA transfer to the plant is most likely the same for both *Agrobacterium* species, but the physiological basis is totally different (Gelvin, 1990).

The physiological basis of the hairy root disease is not totally understood. Alteration of auxin metabolism in transformed cells has been supposed to play an important role in expression of the hairy root phenotype (Gelvin, 1990, Zambriski *et al*., 1989). TL-DNA plays the major role in hairy root induction, and the genes encoding auxin synthesis have a somewhat accessory role (Carderilli *et al*., 1987, Palazon *et al*., 1997). Auxin is necessary for hairy root induction, but it seems likely that auxin does not play a role in T-DNA expression in transformed plant cells (Carderilli *et al*., 1987). Physiological studies have indeed shown that the transformed cells are more sensitive to extracellularly supplied auxins than the normal roots (Shen *et al*., 1988). Spano *et. al*. (1987) have suggested that the genes responsible for increased sensitivity of hairy root cells to auxin are located on the TL-DNA. On the other hand, hairy roots of *Hyoscyamus muticus* L. have been demonstrated to tolerate high auxin levels. The sensitivity is most probably restricted to certain plant species (Vanhala *et al*., 1998).

1.13.1 Characterization of *Agrobacterium* plasmids

Several classes of both Ri- and Ti- plasmids have been characterized. The plasmids are large (200 to greater than 800 kb) and contain one or two regions of T-DNA and a *vir* (virulence) region, all of which are necessary for tumorgenesis (Gelvin, 1990, Zhu *et al*., 2000). The classification of plasmids depends to a large extent on the type of opines that the plasmids direct the infected plants to synthesise. The Ri-plasmids are grouped into two main classes according to the opines synthesized by hairy roots. Agropine-type strains (e.g., A4, 15834, LBA9402, 1855) induce roots to synthesize agropine, mannopine and the related acids, and mannopine-type strains (e.g., 8196, TR7, TR101) induce roots to produce mannopine and the corresponding acids (Rhodes *et al.,* 1990). Other types of opines (e.g., cucumopine, mikimopine) have also been described (Davioud *et al.,* 1988, Isogai *et al.,* 1988).

The Agropine-type Ri-plasmids are very similar as a group, and a quite distinct group from the mannopine-type plasmids (White *et al.,* 1985). Perhaps the most studied Ri-plasmids are agropine-type strains, which are considered to be the most virulent and therefore more often used in the establishment of hairy root cultures (Rhodes *et al.,* 1987).

The use of *Agrobacterium* as a vector is based on its unique capacity to transfer a piece of its own T-DNA into the nuclear genome of plant cells. Any DNA placed between the borders will be transferred to a plant cell. This property has been extremely useful for the introduction of new genes into plants, either for research or for practical applications (Tinland, 1996).

1.13.2 The genes responsible for hairy root formation

The T-DNA of the agropine-type Ri-plasmid consists of two T-DNA regions designed the TL-DNA and TR-DNA (White *et al.,* 1985). Each of the T-DNA fragments spans a 15-20 kb region, and they are separated from each other by at least 15kb of non-intergrated plasmid DNA. These two fragments can be transferred independently during the infection process (Vilaine and Casse-Delbart, 1987). White *et.al.* (1985) made a comparison between the T-DNA region of the agropine and mannopine-type Ri-plasmids and the octopine and nopaline-type Ti-plasmids. The genes encoding auxin synthesis (*tms* 1 and *tms* 2) and agropine synthesis (*ags*) have been localized on the TR-DNA of the agropine type Ri-plasmid (White *et al.,* 1985, Cardarelli *et al.,* 1987). The mannopine type Ri-plasmids contains only one T-DNA that shares considerable DNA sequence homology with TL of the agropine-type plasmids (Gelvin, 1990).

Mutation analysis of the TL-DNA has led to identification of four genetic loci, designed locus *rolA*, *rolB*, *rolC* and *rolD* loci. It was also shown that *rolA*, *rolB*, and *rolC* play the most important role in hairy root induction. In particular, *rolB* seems to be the most crucial in the differentiation process of transformed cells, while *rolA* and *rolC* provide with accessory functions (Palazon *et al.,* 1997).

Although the TR-DNA is not essential for hairy root formation it has been shown that the *auxl* gene harboured in this segment provided to the transformed cells with an additional source of auxin. It is known that *aux* genes play a significant role in the morphology and alkaloid production of transformed roots of *Datura metel* and *Duboisia* hybrid.

1.13.3 Mechanism of *Agrobacterium*-plant cell interaction

One of the earliest stages in the interaction between *Agrobacterium* and a plant is the attachment of the bacterium to the surface of the plant cell. A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds, such as acetosyringone, that active the *vir*-region of the bacterial plasmid. It has been shown that the *Agrobacterium* plasmid carries three genetic components that are required for plant cell transformation (Zambryski *et al.,* 1989). The first component, the T-DNA that is integrated into the plant cell is a mobile DNA element. The second one is the virulence area (*vir*), which contains several *vir* genes. These genes do not enter the plant cell but together with the chromosomal DNA (two loci), cause the transfer of T-DNA. The third component, the so-called border sequences (25 bp), resides in the *Agrobacterium* chromosome. The mobility of T-DNA is largely determined by these sequences, and they are the only *cis* elements necessary for direct T-DNA processing.

Zupan and Zambryski (1995) have described in detail the mechanism for the transfer of T-DNA into the plant cell. The early steps of the transfer are relatively well studied but the mechanics of integration are not completely understood. According to Zambryski (1988), it is a multistep process involving recombination, replication and repair activities, most likely mediated by host cell enzymes. The overall process of integration is probably very similar to any illegitimate recombination of foreign DNA into eukaryotic genomes (Tinland, 1996). Comprehensive reviews on *Agrobacterium* transformation are there by Zhu *et.al.* (2000) and Zupan (2000).

1.13.4 Establishment of hairy root cultures

The transformation is induced on aseptic, wounded plants or plant parts by inoculating them with a thick, viable *A. rhizogenes* suspension. After 1-4 weeks, when roots emerge at the site of inoculation, they are individually cut off and transferred into a hormone-free growth medium e.g., Murashige and Skoogs (1962) or Gamborg's *et.al.* B5 (1968), containing antibiotics to kill the bacteria. The protocol of the establishment of hairy

root cultures has been described in detail by (Sevon, 1998, Deno *et al*., 1987, Vanhala et.al.,1995).

The susceptibility of plant species to *Agrobacterium* strains varies greatly. However, plant species, which were shown to be insusceptible to *A. rhizogenes*, e.g., strain A4, have been successful transformed with other strains (Jung and Tepfer 1987, Hamil *et al.,* 1989). Significant differences were observed between the transformation ability of different strains of *Agrobacterium* (Vanhala *et al.*, 1995, Banerjee *et al.,* 1994). The age and differentiation status of plant tissue can also affect the chances of successful transformation. The level of tissue differentiation also determines the ability to give rise to transformed roots after *A. rhizogenes* inoculation (Trypsteen *et al.,* 1991). Successful infection of some species can be achieved by the addition of acetosyringone (Godwin *et al.,* 1991).

The genetic transformation can be confirmed by assaying the opines. Opine production can, however, be unstable in hairy roots and may disappear after a few passages (Flores *et al.,* 1987). For this reason, detection of T-DNA by Southern blot hybridization is often necessary to confirm the genetic transformation (Tepfer, 1984). The polymerase chain reaction (PCR) simplifies the detection of transformation (Hamill *et al.,* 1991, Sevon *et al.,* 1997).

1.13.5 Characteristics of the Hairy Roots Cultures

Hairy roots are fast growing and laterally highly branched, and are able to grow in hormone-free medium. Moreover, these organs are not susceptible to geotropism anymore. They are genetically stable and produce high contents of secondary metabolites characteristic to the host plant. The secondary metabolite production of hairy roots is stable compared to other types of plant cell culture. The alkaloid production of hairy roots cultures has been reported to remain stable for years (Sevon *et al.,* 1998). The secondary metabolite production of hairy roots is highly linked to cell differentiation. Alkaloid production decreased clearly when roots were induced to form callus, and reappeared when the roots were allowed to redifferentiate (Sevon *et al.,* 1998, Flores *et al.,* 1987). An interesting characteristic of some hairy roots is their ability to occasionally excrete the secondary

metabolites into the growth medium (Sevon *et al.,* 1992). However, the extent of secondary product release in hairy root cultures varies between species (Hamill *et al.,* 1986, Parr and Hamill, 1987, Boitel-Conti *et al.,* 1995).

The average growth rate of hairy roots varies from 0.1 to 2.0 g dry weight/litre/day. This growth rate exceeds that of virtually all-conventional roots and is comparable with that of suspension cultures. However, the greatest advantage of hairy roots compared to conventional roots is their ability to form several new growing points and, consequently, lateral branches (Oksman-Caldentey *et al.,* 1996). The growth rate of hairy roots may vary greatly between species, but differences area also observed between different root clones of the same species (Mano *et al.,* 1986, Oksman-Cladentery *et al.,* 1994). The pattern of growth and secondary metabolite production of hairy root cultures can also vary. Secondary production of the hairy roots of *Nicotiana rustica* L. was strictly related to the growth, whereas hairy roots of *Beta vulgaris* L. exhibited non-growth-related product accumulation (Hamil et al., 1986). In the case of the hairy roots of *Scopolia japonica* and *H. muticus*, the secondary products only started to accumulate after growth had ceased (Vanhala *et al.,* 1995, Sevon *et al.,* 1992, Nabeshima *et al.,* 1986).

1.13.6 Improvement of the Production in Transformed Root Cultures

Hundreds of plant species have been successfully transformed to hairy roots Tepfer (1990) and Giri and Narasu (2000). For the past ten years hairy roots have also been investigated as a potential source of pharmaceuticals (Oksman-Cladentey and Hiltunen 1996, Giri and Narsu, 2000). The comparison of hairy roots is not always possible, since the product yield is calculated in many different ways (e.g., mg/g f.w. or d. w., mg/flask, % of d.w., mg/l), and all the parameters are not always given. Additionally, high contents of the product could be associated with poor growth, and thus the real productivity (mg/l) remains low. The secondary metabolites of hairy roots are strictly limited to those that are normally produced in the roots. On the other hand, if the biosynthesis of secondary metabolites normally takes place in the green parts of plants, it is necessary to utilize modified hairy roots, e.g., “green hairy roots” or, alternatively, transformed shoot teratomas (Flores *et al.,* 1987, Saito *et al.,* 1992).

1.13.7 Optimization of the medium and growth conditions for hairy roots

Several physical and chemical factors have been found that could influence the growth and productivity of hairy root cultures. However, hairy roots are not so easily modified by changing the culture conditions as cell suspension cultures (Toivonen, 1993). Several studies have been made on the effect of medium composition on growth and the production of secondary metabolites. Most of the investigations have been carried out with hairy roots of *Catharanthus roseus* L. (Toivonen *et al.,* 1991) and Solanaceous species (Oksman-Caldenty *et al.,* 1994, Hilton and Wilson, 1995).

Factors such as the carbon source and its concentration, ionic concentration of the medium (Christen *et al.,* 1992a), pH of the medium (Morgan *et al.,* 2000), light (Christen *et al.,* 1992b), phytohormones (Vanhala *et al.,* 1998, Rhodes *et al.,* 1994, Arro *et al.,* 1995), temperature (Toivonen *et al.,* 1991) and inoculum (Bais 2000, Mano *et al.,* 1986, Bhadra and Shanks, 1995), are known to influence the growth and metabolite production of hairy roots.

Gamborg's B5 medium is the most widely used medium for the hairy roots of many species (Hilton and Wilson, 1995). Supplementation of heavy metal ions, such as Cu^{2+}, has been shown to stimulate metabolite production (Sevon *et al.,* 1992, Christen *et al.,*1992a).

Toivonen *et.al.* (1991) studied the effect of varying concentrations of sucrose, phosphate, nitrate and ammonium on growth and indole alkaloid alkaloid production in hairy root cultures of *C. roseus*. They found that low nutrient levels enhanced alkaloid production, but biomass yields were maximal in media containing high concentrations of sucrose and ammonia.

Hairy root cultures of *H. muticus* also produce the highest hoscyamine content at a sucrose concentration of 30 g/l, but higher than this stimulated the growth of the hairy roots. The root clones of *H. muticus* could not utilise ammonium as the sole nitrogen source (Oksman-Caldenty *et al.,* 1994). Ammonium had a strong influence on the growth of hairy roots of *A. belladonna* while nitrate had clear effect on the alkaloid production (Bensaddek *et al.,* 2001).

The hairy roots of different species behave differently in the same culture conditions. Hilton and Wilson (1995) investigated the growth and uptake of sucrose and minerals ions by six tropane alkaloid-producing transformed root cultures and found that their requirements for certain minerals ions varied when grown in batch cultures on Gamborg's B5 medium. Individual hairy root clones can also have different optimum concentrations of sucrose or mineral ions (Oksman-Cladenty *et al.,* 1994). The different requirements make optimization work difficult, because the culture conditions have to be optimized separately for each species and for individual clones.

1.13.8. Elicitation of secondary metabolites in hairy root cultures

Elicitation is one of the methods that have been used to enhance secondary metabolities of cell cultures (Eilert, 1987). Those compounds, which defend the plants against micro-organisms, namely, phytoalexins, are often easily formed in response to the elicitors, but the accumulation of the alkaloids of interest has not usually been induced. Although the use of elicitors does not directly increase the metabolite content of hairy roots, cell permeability increases and this often has positive effect on the formation of secondary metabolites (Brodelius *et al.,* 1989).The fungal elicitors and agents that increase the excretion of desired compounds have on occasions been combined successfully in the treatment of hairy roots of *C. roseus* (Buitelaar *et al.,* 1993).

Enhancement of cell permeability may increase the formation of secondary products, because feedback inhibition and intracellular degradation of the products decrease. The economical benefit of the production process also depends on the capacity of the producing cells to secrete the desired metabolite into the surrounding medium. Permeability of plant membranes for the release of secondary metabolites has often been connected with the loss of viability of the plant cells treated with permeabilizing agents and methods. Some attempts have been made to increase the permeability of the hairy roots. Biotic elicitors including solvents and detergents have been reported to release the products from hairy roots into the medium without any loss of viability and production capacity of the hairy roots (Buitelaar *et al.,* 1993, Pitta-Alvarez *et al.,* 2000). Cusido and co-workers (1999) reported that tween 20

treatments encouraged both growth and alkaloid productivity of hairy roots of *Datura metel* L.

Chitosan has been used as an effective elicitor, but it also enhances the permeability of the cells (Sevon *et al.,*1992, Dornenburg and Knorr, 1995), Permeabilization studies with chitosan have mainly been performed with cell suspension cultures which, however, are not directly connected with hairy roots. This polycationic polysaccharide induces pore formation in the plasmalemma of the cell cultures of *Chenopodium rubrum*. It has been suggested that pore formation is related to the degree of the deacetylation (positive charges) of the chitosan. Consequently, highly charged chitosan polymers induce a higher degree of pore formation and cause faster secondary product release than the less charges ones. This means that there is a critical charge density, which leads to loss of cell viability.

1.13.9 Large Scale Cultivation of Transformed Roots

Much work has been carried out with bioreactors and process development during the last decades. Design of the mixing system for bioreactors has been the most problematic. Mechanical agitation is seldom suitable for roots because they are susceptible to shear stress that causes disorganization and callus formation, with consequently lowered productivity.

Conventional stirred-tank reactors have been successfully applied to hairy roots even though the mixing system of such bioreactors has been reported to cause shear damage (Davioud *et al.,* 1989). However, the best growth characteristics were obtained with bioreactors without mechanical stirring. The use of airlift reactors makes it possible to avoid shear stress completely, and have been used for the growth of hairy roots. Wilson, 1997, 1990) have described droplet reactors in which the medium is sprayed over the roots and periodically sucked out, the roots being in contact with the air for most of the time. The most promising bioreactors for the cultivation of hairy roots seem to be so-called wave reactors. This reactor system has three components: a rocker unit, the disposable bioreactor chamber, and the measuring and control units. The wave reactor is a mechanically driven reactor system. The energy input is caused by movement. This reactor has been demonstrated to increase the growth of hairy root cultures producing tropane alkaloids and ginsenosides

significantly more than optimized stirred reactors, rotating drum reactors and droplet phase reactors (Eibl *et al.,* 1999) Pilot-scale studies with wave reactors are currently running up to 100 litre working volume. More comprehensive reviews on scale-up for large-scale cultivation of hairy roots are presented by Eibl *et al.* (2002), Guillon *et al*., (2006 a, b).

1.13.10 Oxidative stress and antioxidant enzymes in hairy root cultures

Much of the injury caused to plants by environmental stress is associated with damage inflicted by active oxygen species (AOS) at the cellular level (Slesak *et al.*, 2003) It is now known that a variety of abiotic stresses including drought, salinity, extreme temperatures, high irradiance, UV light, nutrient deficiency, air pollution etc. cause molecular damage to plants either directly or indirectly through the formation of AOS (Kavita *et al.* 2001, Lin and Kao 2000). These AOS, also unbalance the cellular redox system in favor of oxidized forms, inactivate enzymes, cause lipid peroxidation, and potentially damage DNA (Kavita *et al.* 2001). To scavenge these toxic radicals plant cells have developed a complex system of antioxidant enzymes that includes superoxide dismutases (SOD), Catalases (CAT), Ascorbate peroxidases (APX), Glutathione reductases (GR), which have specific subcellular locations (Foyer *et al.,* 1997). The enzymatic process basically involves dismutation of O_2 by SOD that generates another partially reduced oxgen species H_2O_2. Normally the enzymes catalase, peroxidaes and ascorbate peroxidase take care of the cellular H_2O_2 (Tewari *et.al.* 2002, Alscher *et al.* 1997). The enzymatic mechanism of detoxification also involves dehydroascorbate reductases and glutathione reductases. These enzymes play a regulatory role in heavy metal induced oxidative stress. Understanding the biochemical detoxification strategies that plants adopt against oxidative stress induced by accumulated metal ions is a key to manipulate heavy metal tolerance in plants. Redox metals like iron and copper generates AOS via Fenton- type reaction while non-redox-active metals like Cadmium, and Zinc generates oxidative stress by alternative pathways (Hendry and Brocklebank, 1985).

The overproduction of antioxidant enzymes provides a way to study the role of enzymes in the antioxidant system and the constitution of oxidative stress tolerance to biotic as well as abiotic type of stresses. Abiotic and biotic elicitors have an important role in the production of high value metabolites in plant cell cultures (Threlfall and Whitehead, 1988).

It has been reported that elicitation of cultured pant cells stimulates a burst of oxidative activity, which can lead to the dismutation of a variety of susceptible compounds (Apostol *et al* 1990). Metal ions are used to elicit the formation of secondary products in tissue culture system (Threlfall and Whitehead, 1988). Manipulation of the concentration of microelements in the culture media represents a strategy to increase the production of secondary metabolites in plant cell cultures (Jimenez and Guteirrez, 1998, Akita *et aI.*, 2001)

Superoxide dismutase (SOD: EC 1.15.1.1) is a metallo enzyme which catalyses the dismutation of superoxide radicals (O_2-) to molecular oxygen and hydrogen peroxide. These reactive radicals, which are toxic byproducts of oxygen metabolism, can oxidize membrane fatty acids and proteins and damage DNA. SOD has been widely used as an antioxidant (Larson 1988). SOD is found in many microorganisms, higher plants and animals. SOD has been detected in pak-bung and horseradish hairy roots (Kino-oka *et al*. 1991), and carrot hairy root cultures (Kim *et. al.* 1994). Peroxidases (PODs: EC 1.11.1.7) represent a class of ubiquitous enzymes widely distributed through out the plant kingdom. As multifunctional enzyme PODs have been associated with several physiological processes like regulation of growth and cell expansion (Goldberg *et al*. 1986), They also play a key role in several stress related processes such as browning and wound healing (Espilie, 1986). These enzymes have been extensively used as an important reagent for clinical diagnostics and various enzymatic assays. POD has been detected in hairy roots of *Armoracia lapathifolia* (horseradish) Flooco *et al*. (1998), carrot hairy roots (Kim & Yoo, 1996).

1.14 Pharmacological and antioxidant effects of betalains

Although structurally related to alkaloids, betalains have no toxic effects in the human body, as can be deduced from the fact that they are present in considerably high amounts in certain food stuffs, such as red-beet, prickly pear fruits, and *Amaranthus* seeds (Böhm and Rink, 1988). Therefore, betalains represent a safe natural alternative to some synthetic colour additives that are currently in use. On the other hand, in betanin tested for mutagenic and carcinogenic activity an absence of mutagenicity in five *Salmonella typhimurium* strains was observed, and it did not initiate or promote hepatocarcinogenesis in levels of 50 mg/kg of weight of pure betanin or diets containing 2000 mg/kg of betacyanin

(Schwartz *et al*., 1983). Notwithstanding, after ingestion of these products (particularly red beet), betanin occasionally appears in the urine, an effect known as beeturia or betaninuria. The etiology and mechanisms of this disorder are still controversial (Piatelli, 1976). They have however, received attention because betanin has shown antiviral and antimicrobial activities (e.g., *Pythium debaryum*, a pathogenic fungi in red-beet). Medicinally beet root is employed as a popular flock remedy to stimulate the immune system and for the treatment of liver and kidney diseases, and also as a special diet in the treatment of cancer (Chevallier, 1996). The extracts of beet root showed promising *in vitro* chemopreventive effect in TPA (1,2-O-tetradecanoyl pharbol-1,3-acetate) induced Epstene-Barr early antigen activation activity assay and in addition an *in vivo* anti tumor evaluation in the mouse skin and lung bioassay clearly showed betanin to be a potent cancer chemopreventive agent (Kapadia *et al*., 1996). However, in both cases the action mechanisms are still unknown. The importance of some natural pigments as nutraceutical ingredients was reviewed (Pszczola, 1998). For example, yellow betaxanthins, in addition to their potential role as natural food colourant, may be used as a means of introducing essential dietary amino acids into foodstuffs, giving rise to an "essential dietary colourant"(Leathers *et al*., 1992).

There is currently considerable interest in dietary antioxidants as bioactive components of food (Pszczola, 1998). There is also evidence suggesting that some natural colourants may be nutritionally important antioxidants and that their presence in the diet may reduce the risk of cardiovascular diseases, cancer, and other diseases associated with ageing (Kritchevsky, 1999; Rao and Agrwal, 1999; Mazza, 2000). Most research has been focused on natural colourants such as carotenoids, anthocyanins (flavonoids), and curcuminoids, which exhibit antioxidant, anti-inflammatory, and antitumor promoting effects (Wang *et al*., 1999; Espin *et al*., 2000; Shi and Lemaguer, 2000). Studies on extracts of beet root tissues have shown a broad range of antioxidant activities (Cao *et al*., 1996; Vinson *et al*., 1998). Recently several studies on the antiradical and antioxidant activity of betalains from beet roots have been published (Escribano *et al*., 1998; Zaharova and Petrova, 1998; Pedreno and Escribano, 2000; Kanner *et al*., 2001; Pavlov *et al*., 2002; Cai *et al*., 2003). It was found that extracts of lyophilized beet root tissue to possess significant phase II enzyme inducing and antioxidant activity (Wettasinghe *et al*., 2002).

Objectives of thesis

- To establish clones of hairy roots using different strains of *Agrobacterium rhizogenes*, different explants from beet root plants and different varieties of *Beta vulgaris* L for selection of superior clone looking at growth, morphological characteristics and pigment production.
- To study effect of medium components i.e. major and minor nutrients to enhance the growth and production of betalains in *Beta vulgaris* L. hairy roots.
- Influence of growth regulators on growth and production of betalains in *Beta vulgaris* L. hairy roots.
- To study the stability of betalains obtained from the hairy root cultures of *Beta vulgaris* L. and strategies employed to improve the stability.
- Metal ion stress studies to know the antioxidant enzyme status in hairy root cultures of *Beta vulgaris* L.
- Short term subacute and acute safety evaluation of betalain extracts of beet roots and beet hairy roots in experimental animals.
- Protocol development for micropropagation of *Beta vulgaris* L. using shoot tips from seedling and mature beet root plants.
- Field evaluation of tissue culture derived plants from mature shootips and seedling grown plants to study the growth and production of betalains.

CHAPTER -II

MATERIALS AND METHODS

2.0 Materials and Methods

2.1 Plant material

Beta vulgaris L. Detroit dark red, Sutton blood red, Crimson globe and Ashoka varieties of seeds were obtained from local market, Mysore, India.

2.2 Glassware

The glassware used in the experiments like conical flasks, culture tubes, pipettes, petridishes, and measuring cylinders were of Borosil and Vensil brand. The culture bottles and polypropylene bottle caps were procured from M/s. Varsha storage racks Bangalore, India.

2.3 Chemicals

All the chemicals used for plant culture in this study were from Sigma Co. Ltd. St. Louis USA and Hi-Media India Ltd. Bombay, India. The chemicals and solvents used were of analytical grade.

2.4 Surface sterilization and seed germination aseptically

The seeds were rinsed in 70% ethanol for 30 seconds and thoroughly washed in running tap water for 1 hr followed by washing in bavestine fungicide for 10 min. The cleaned seeds were surface sterilized in aqueous solution of 0.15% (w/v) HgCl2 for 5-7 min. in sterile deionized water, followed by five rinses with sterile distilled water to remove the residual mercuric chloride. MS basal media (Murashige and Skoog, 1962) supplemented with 3% sucrose (Hi media, India), the pH of the media was adjusted to 5.8 prior to gelling with 0.8% agar agar (Hi media India). The gelled media was autoclaved at 1.06 kg cm^{-2} pressure and 121^{o} C for 15 min. The seeds were inoculated on to the MS basal media aseptically in a laminar air flow chamber (Kirloskar India), which was swabbed with 70% ethanol and irradiated with ultraviolet light for 45 min, and incubated at 25 ± 2^{o} C under cool light (4.41 Jm^{-2} s^{-1} 16 hr day^{-1}).

2.5 Establishment of axenic hairy root cultures

2.5.1 Induction of hairy roots

Agrobacterium rhizogenes strains LMG-150, A 20/83, A2/83 and A4 were cultured for 3 days on solid YEM medium (Verveliet *et al*., 1975). Cotyledons and stem from axenically grown 12-15 days old seedlings of *Beta vulgaris* L. were immersed in 24 hour old bacterial suspension for 10 min and the explants were removed and placed on sterile filter paper (Whatman #3) to remove excess of bacterial suspension, and they were inoculated onto MS basal solidified medium containing 8 g/l Agar agar and 30 g/l sucrose and incubated at 25 ± 2°C in dark.

After 8-10 days, roots emerged from the infection site, the roots about 2-3 cm in length were excised and transferred to MS basal media containing 500 mg L-1 carbenicillin, and subcultured 2-3 times with 7 days interval on to fresh MS media containing carbenicillin to achieve complete decontamination. The axenic roots were inoculated to MS basal liquid media in 150 ml Erlenmeyer flasks containing 40 ml of media and incubated in dark on a rotary shaker at a speed of 90-100 rpm at 25 ± 2oC. Roots were harvested at interval of 25 days.

2.5.2 Confirmation of hairy roots

About 10 g each fresh weight of normal and hairy roots were homogenized with a mortar and pestle using acid washed and neutral sand in 1 ml of extraction buffer comprising of 0.1 M Tris HCl, 0.5 M sucrose, 0.1% ascorbic acid, 0.1 % cystein HCl at pH 8 (Otten and Schilperoort, 1978). The slurry was centrifuged at 5000 g for 10 min. the supernatant concentrated to 2 ml was used for opine assay. 5 µl of the concentrate, each from the transformed and non-transformed roots, were spotted separately on Whatman #3 chromatographic paper along with the standard mannopine (Sigma, USA) on anodal side (Otten and Schilperoort, 1978). The spotted samples were subjected to high voltage paper electrophoresis (1.5 KV) at 15 Vcm-1 for 30 min. in a buffer of formic acid, acetic acid, and water (3:6:91v/v/v) at pH 1.9. After drying overnight, the electrophoretogram was stained with silver nitrate (Trevelyan *et al*., 1950) and later fixed with sodium thiosulphate solution (10% v/v in water) and washed in tap water.

2.5.3 PCR analysis

The hairy roots were washed in NaOH (200 ml) and SDS (1%) for 5 minutes and then rinsed in sterile deionized water to remove the bacteria. The roots were dried on sterile filter paper and frozen quickly in liquid nitrogen, and genomic DNA was extracted according to CTAB (Dellaporta *et al*., 1983). In the polymerase chain reaction specific primers for *rol A* gene were used to amplify the gene fragment. The expected PCR product was 360 bp band for the amplified *rol A* gene. The amplification protocol for *rol A* was 4 minutes denaturation at 94^{o}C followed by 35 cycles of 1 minute denaturation at 94^{o}C, 1 minute annealing at 55^{o}C and one min extension at 72^{o}C with a final extension of 10 min at 72^{o}C. The oligonucleotide primers adopted at 72^{o}C. The oligonucleotide primers adopted to amplify the *rol A* gene on the T-DNA of *A. rhizogenes* were designed according to (Hamil *et al.*, 1991) and procured from Bangalore Genie India Pvt. Ltd. The oligonucleotides used were 5'-AGA GAA TTA GCC GGA CTA-3' and the reverse primer 5'-GTA TTA ATC CCG TGA GTT GTT –3'

Southern blot analysis was carried out by hybridizing the PCR amplified products with a *rol* A gene probe obtained from *A. rhizogenes* LMG-50 strain. The probe was labeled with digoxinin (DIG) DNA labeling kit from Boerhhinger Mannheim as described by the manufacturer.

2.5.4 Growth measurement of hairy roots

Growth of hairy roots of *Beta vulgaris* L. was determined by recording fresh and dry weights after 25 days from the mean of 5 replicates. The fresh weight was measured after removing culture medium by filtration through a pre weighed Whatman No.1 filter paper under vacuum. The dry weight was estimated after the same sample was dried at 60^{o}C for 48 hrs.

2.5.5 Extraction and estimation of betalains

Hairy roots (1 g) fresh weight was extracted in 10 ml (0.01%) acidified methanol using mortar and pestle, the homogenate was centrifuged and supernatant collected. The residue was extracted twice in 5 ml methanol for complete extraction of betalains. The supernatants were pooled and betalains were determined using Shimadzu UV- Visible

spectrophotometer at 540 nm for betacyanins and 480 nm for betaxanthins, by the method described by Nilsson (1970).

2.6 Factors influencing growth and production of betalains in hairy root cultures of *Beta vulgaris* L.

2.6.1 Influence of different media constituents

2.6.1.1 Effect of nitrates and phosphates

Effect of half and double the strength of nitrates and phosphates were studied on the growth and production of betalains. Nitrates are usually added to the medium as ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) at 20.6 mM and 18.8 mM and phosphate as potassium dihydrogen phosphate (KH_2PO_4) at 1.25 mM.

2.6.2 Effect of different pH on growth and production of betalains

As pH of the medium is known to play an important role on the growth and production of metabolites, pH levels ranging from 3.5-7.0 was adjusted before sterilizing the medium (MS) and their affects on growth and production of betalains were studied.

2.6.3 Effect of different sources of carbon on growth and production of betalains

Growth and betalain production in MS medium were investigated using sucrose (1-10% W/W) in addition maltose (3%), fructose (3%), glucose (3%) and fructose + glucose (1.5:1.5 %).

2.6.4 Effect of micronutrients on growth and production of betalains

The effects on the growth and production of betalains, of the following microelements manganese (Mn^{2+}; 100 μM/l), iron (Fe^{2+} 100 μM/l), molybdenum (Mo; 1.0 μM/l), Zinc (Zn^{2+}; 30 μM/l), cobalt (Co^{2+}; 0.1μM/l) and copper sulphate (Cu^{2+}; 1.0 μM/l), were evaluated at five times higher than the normal concentration of MS medium. The effect of cobalt at 5, 10 and 20 times the concentration in MS (control) was studied.

2.6.5 Effect of growth regulators on growth and production of betalains

The effect of different auxins and cytokinins on hairy root cultures of *Beta vulgaris* L. was studied. Growth regulators like Abscissic acid (ABA), 2,4-Dichlorophenoxyaceticacid (2,4-D), Indole-3-Acetic Acid (IAA), Indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), cytokinins Kinetin (Kn), 6-Benzylaminopurine (BAP) at different concentrations ranging from 0.1-10 mg/l, individually and in combinations and ethaphon at 5, 10, and 15 mg/l were investigated.

2.6.6 Effect of Gibberellin (GA_3)

The effects of exogenous feeding of GA_3 on transformed hairy root cultures of *Beta vulgaris* were studied. Hairy root cultures were fed with 0.1, 0.5, 5.0 and 10 mg/L GA_3 and growth and biosynthesis of betalains were monitored for a period of 25 days.

2.7 Stability studies of betalains obtained from hairy root cultures of *Beta vulgaris* L.

2.7.1 Preparation of betalain concentrate

Betalains were extracted from and beet hairy roots by crushing in a blender with 0.01% acidified methanol. The extracts obtained were centrifuged at 8000 g, the supernatant collected and the residue was extracted again for complete extraction of betalains. The supernatants obtained were pooled and concentrated by vacuum evaporation using flash evaporator. The concentrates obtained were 68.degree. Brix and contained 0.7% w/v betalains.

2.7.2 Betalain sample preparation for stability studies

The aqueous extracts for stability were prepared by adding 0.1 ml concentrates to 50 mM citrate phosphate buffers of required pH, and made up to 25 ml using volumetric flasks. These were used for stability studies at various temperatures, pH, and effects of metal ions, additives.

2.7.3 Effect of temperature and pH on betalains

Effect of temperature was studied at 30°C, 40°C, 50°C, 60°C, 80°C, in pH 3, 4, 5, 6,

and 7. Fifteen ml of betalain solution was taken in 100 ml glass test tubes and sealed with rubber cork to protect evaporation. The tubes were kept in water bath and temperature required was set. Initial betalain content was noted and at an interval of one hour betalain content was recorded up to 12 hours.

2.7.4 Effect of metal ions on thermal stability of betalains

The effect of the following metals Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} in form of their respective salts *i.e*., copper sulphate ($CuSO_4$), ferric chloride ($FeCl_3$) and ferrous sulphate ($FeSO_4$), Magnesium sulphate ($MgSO_4$), Manganese sulphate ($MnSO_4$) and Zinc sulphate ($ZnSO_4$) at 0.3 mM level were added to pigment extract separately and mixed thoroughly and 15 ml aqueous extracts were taken in 100ml test tubes and studied at 50^{o}C at pH 5 for 6 hours.

2.7.5 Effect of sugars and sugar alcohols on thermal stability of betalains

The effects of fructose, maltodextrin (mdx), starch and sugar alcohols glycerol and polyethylene glycol at 5 mM each were added in aqueous extracts of betalains from beet hairy root cultures and pH was adjusted to 5.0. Extracts 15 ml each were taken in 100 ml test tubes and sealed with rubber cork to avoid evaporation and heated in water bath at 50^{o}C. Initial absorbance was recorded then after one hour and 6 hours.

2.7.6 Effect of water activity (a_w) on thermal stability of betalains

Model systems with a water activity from 1.00 to 0.37 were prepared using water and glycerol Pasch and Von Elbe (1975). Betalain extracts were prepared at each water activity in test tubes and incubated at 50^{o}C. Absorbance readings were taken initially and for up to 3 hours thereafter.

2.7.7 Effect of temperature and pH on betalain concentrate

Effect of temperature was studied at 50^{o}C, 80^{o}C, and 95^{o}C in pH 3, 4, 5, 6, and 7. Fifteen ml of betalain concentrate (68.degree.brix) was taken in 100 ml glass test tubes and sealed with rubber cork to protect evaporation. The tubes were kept in water bath and temperature required was set. Initial betalain content was noted and at an interval of 5,10,15,30, 45 and 60 min betalain content was recorded.

2.7.8 Influence of ascorbic acid, isoascorbic acid, sodium ascorbate and sodium isoascorbate on stability of betalain concentrate obtained from hairy root cultures of *Beta vulgaris* L.

To 100 ml each of hairy root juice concentrates (68.degree. Brix, 0.7% betanin), 0.1 gm of ascorbic acid, sodium ascorbate and sodium isoascorbate was added separately. For heat stability study, 15 ml each of the colour concentrates were placed in test tubes and heated in a water bath at 95^{o} C for 20 minutes and after heating the tubes were immediately cooled keeping in ice water. The colour absorbance of the solutions was measured at 535 nm using a spectrophotometer.

2.7.9 Effect of citric acid on stability of betalain concentrate obtained from hairy root cultures of *Beta vulgaris* L.

To 100 ml each of of hairy root juice concentrates (68.degree. Brix, 0.7% betanin), 0.1 gm of citric acid was added separately. For heat stability study, 15 ml each of the colour concentrates were placed in test tubes and heated in a water bath at 95^{o} C for 20 minutes and after heating the tubes were immediately cooled keeping in ice water. The colour absorbance was measured at 535 nm using a spectrophotometer.

2.7.10 Effect of sodiumhexametaphosphate (SHMP), tetrasodiumpyrophosphate (TSPP) and sodiumacidpyrophosphate (SAPP) on stability of betalain concentrate from beet hairy root cultures

Chelating agents i.e., sodiumhexametaphosphate (SHMP), tetrasodiumpyrophosphate (TSPP) and sodiumacidpyrophosphate (SAPP), were added separately to a beet hairy root juice concentrate (68.degree. Brix, 0.7% betanin). These solutions in test tubes (15 ml each) were heated in a water bath at 95^{o}C for 5, 10, 15 and 20 minutes. After heating, the tubes were cooled immediately in ice water. The absorbance of heated solutions was measured at 535 nm using spectrophotometer.

2.8 Metal ion stress Studies

Effect of metal ions iron and copper on growth, production of betalains and antioxidant enzyme activities like superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in hairy root cultures of *Beta vulgaris* L.

2.8.1 Stress treatment

Metal ions used for the study were iron, copper. 0.1-0.12 g of hairy roots were inoculated in to 40 ml of hormone free MS liquid medium in 150 ml Erlenmeyer flasks, containing 100 µM to 1600µM iron as ferrous sulphate and 0.1 µM -1.6 µM copper as copper sulphate. MS basal liquid medium, containing iron at a level of 100 µM and copper at a level of 0.1µM, served as control. The cultures were incubated at 25±2°C under dark condition on a gyratory shaker at 90 rpm.

2.8.2 Extraction of super oxide dismutase (SOD) (E.C.1.15.1.1)

About 500 mg fresh hairy root samples were homogenized using mortar and pestle in 5 ml extraction buffer consisting of 50 mM phosphate, pH 7.8, in cold. The homogenate was centrifuged (Remi Centrifuge) at 7000 rpm for 10 min at 0^{o}C. To the supernatant, 4.3 g of ammonium sulphate was added and kept over night at 4^{o}C. The unwanted protein precipitate formed was dialysed against distill water to remove the salts. Partially purified SOD was used for the assay.

2.8.2.1 Superoxide dismutase assay (SOD)

Superoxide dismutase activity was assayed by using the photochemical NBT method. The assays were performed in the terms of Sod's ability to inhibit reduction of nitroblue tertazolium (NBT) to form formazan by superoxide (Bayer and Fidrovich, 1987). The assay system contained 9.9 mM L-methionine, 57 µM NBT, 0.025% (w/v) Triton X-100, and 0.044% (w/v) riboflavin in a total volume of 3 ml. The photo-reduction of NBT (formation of purple formazan) was measured at 560 nm and an inhibition curve was made against different volumes of extract. One unit of SOD was defined as those being present in the volume of extract that caused inhibition of the photo-reduction of NBT by 50%.

2.8.3 Extraction of catalase (CAT) (E.C.1.11.1.6)

About 500 mg hairy root samples were homogenized in 5ml mM Tris-Sodium hydroxide buffer (pH 8.0) containing 0.5 mM EDTA, 2% (w/v) PVP 0.5% (v/v) Triton-X 100 using a chilled mortar and pestle. The homogenate was centrifuged at 7000 rpm for 10 min at 4^{o}C and supernatant was used for enzyme assay.

2.8.3.1 Catalase assay (CAT)

Catalase activity was assayed according to the method of Beers and Sizer (1952) Assay mixture in a total volume of 1.5 ml contained 0.1 ml of 100 mM KH_2PO_4 buffer (pH 7.0), 400 µl of 200 mM H_2O_2 and 100 µl enzyme. Decrease in H_2O_2 was monitored at 240 nm. Enzyme specific activity is expressed as µmol of H_2O_2 oxidised min^{-1} mg^{-1} protein.

2.8.4 Extraction of peroxidase (POD) (E.C.1.11.1.1)

About 500 mg hairy root samples were homogenized in 5 ml of 20 mM borate buffer (pH 8.8) in cold using mortar and pestle. Homogenates were centrifuged at 8000 rpm for 10 min at 4^{o}C. Supernatant obtained was diluted with equal volume of ice cold 0.1 M acetate buffer (pH 5.4). This extract was used for POD assay.

2.8.4.1 Peroxidase assay (POD)

Peroxidase activity was assayed at 460 nm as described by Flanagan and Owens, (1985). The assay mixture contained 1 ml of sodium acetate buffer (pH 5.4), 1 ml of 50 mM guiacol (freshly prepared) and 0.5 ml of peroxidase extract. The reaction was initiated by the addition of H_2O_2 to a final concentration of 200 µM and was followed by the increase in OD at 460 nm. The activity was expressed in units per mg of protein. One unit was defined as change in 0.1 OD per minute per mg of protein.

2.8.5 Protein estimation

In all the enzymatic preparations protein was determined by Bradford method (1976) using bovine serum albumin (BSA) as standard.

2.8.6 Statistical analysis

Statistical significance was calculated for each sample by the students 't' test (Banat and Franklin, 1967).

2.9 Safety assessment of betalains obtained from hairy root cultures of *Beta vulgaris* L. in experimental rats.

2.9.1 Preparation of Betalain extract

Betalains were extracted once in a week from freshly harvested hairy roots and beet root, by crushing the biomass using a blender for 3 min and the homogenate obtained was filtered through double layer cheese cloth, the residue was extracted twice in 0.001% acidified water till complete betalains were released. The extract was freeze dried and the powder was used in feeding experiments.

2.9.2 Animals and maintenance

Rats- Adult (CFT-Swiss strain, 8-10 weeks old) of both sexes were used for the acute study, where as weanling male rats (28 days old) were employed for the subchronic toxicity study. Animals in each group were housed in individual cages, acclimatized for one week on control diet prior to the start of the study.

2.9.3 Diets and their preparation

Rats used for the acute studies were fed with the commercial pellet diet (M/s Gold Mohur, Lipton India Ltd.). For the subchronic study, basal diet containing (wheat flour 26%, ragi flour 26%, Bengal gram flour 27.2%, casein 5.6%, skimmed milk powder 4.52%, peanut oil 7.5%, calcium carbonate 0.68% and salt 2% W/W in g). Freeze dried powder of betalain extract from beet root and beet hairy roots was incorporated at two levels 1% and 5%. Fresh 10 kg batches were prepared once a week.

2.9.4 Acute toxicity in rats

Betalain extracts were administered intragastrically as a single dose (1,2 and 4 g/ kg body weight). The exact amount of betalain concentrate was calculated for each animal and administered by oral gavage by suspending freeze dried powder of the betalain extract in water. Prior to dosing, rats were fasted overnight. The rats were observed thoroughly for the onset of any immediate toxic signs and also during the observation period to record any delayed acute effects. All animals were sacrificed humanely after 14 days and selected vital organs were excised, blotted, weighed and processed for routine microscopic examination.

2.9.5 Subchronic toxicity in rats

Weanling male rats were randomly assigned to control and treatment groups(n=10). The rats were fed diets containing 0, 1 and 5% beet root and beet hairy root betalain extract powder. Diets and water were given *ad lib* for a continuous period of 90 days. daily food intake and weekly body weights were recorded.The animals were also observed thoroughly for the onset of any signs of toxicity. Terminally, all the rats were sacrificed humanely (over a 5 days period) under light ether anaesthesia, blood was collected in a vial containing 10% EDTA (anticoagulant) for haematological analysis and another part was collected without anti- coagulant and serum was obtained by keeping the tube in a slanting position for some time.Collected serum was subjected for biochemical analysis for various parameters such as protein, cholesterol, glutamic oxaloacetic transminase (GOT), glutamic pyruvic transminase (GPT), lactic dehydrogenase (LDH) and alkaline phosphatase (ALP). Care was taken that serum samples were completely free from hemolysis as the RBCs are rich in the enzymes studied. Haemolysis leads to the release of large amount of the enzymes resulting in erroneous results. Blood collected with anticoagulant was used for the measurement of % haemoglobin (%Hb), white blood cell count (WBC),red blood cell count (RBC) and packed cell volume.

2.9.6 Organ weights and histopathological studies

The following vital organs of each rat of both acute and sub-chronic studies were excised, blotted and weighed and the organ/body weight ratios calculated: adrenals, brain, heart, kidneys, liver, lungs, spleen and testes. Samples of these organs were fixed in Bouins

fixative embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin. Detailed microscopic examination was carried out on all organs of both control and treatment groups.

2.9.7 Measurement of haemoglobin content

Haemoglobin content of subchronic toxicity group was measured by cyanmethaemoglobin method using Drabkini's reagent. In alkaline condition haemoglobin and it's derivatives were oxidized in the presence of potassium ferricyanide and converted into methhaempglobin which react with potassium cyanide to form purple coloured cyanmethaemoglobin, which were monitored colorimetrically at 546 nm. Content of the samples were calculated comparing with the optical density of standard and expressed as % Hb content.

2.9.8 Measurement of white blood cells (WBC) count

WBC count was done by diluting 20 µl of blood with diluting fluid and the counting was done using hemocytometer and the results were expressed as number of cells/ml of blood.

2.9.9 Measurement of serum and liver protein

Serum and liver proteins were estimated by using the method of Lowry *et al*., (1951). Protein content was calculated using standard curve prepared using Bovine serum albumin.

2.9.10 Measurement of glutamate oxalo-acetate transaminase (GOT)

This was estimated by monitoring oxalo-acetate formed when α-ketoglutarate react with L-aspartate mediated by GOT. Oxaloacetate was made to react with 2,4-Dinitrophenyl hydrazine to form hydrozone producing brown colour in alkaline medium,which was monitored calorimetrically at 505 nm.The GOT content was calculated using standard diagnostic kit (SPAN Diagnostis, Gujarat, INDIA).

2.9.11 Measurement of glutamate pyruvate transaminase (GPT)

This was estimated by monitoring pyruvate formed when α-ketoglutarate reacts with L-alanine mediated by GPT. Pyruvate was made to react with 2,4-Dinitrophenyl hydrazine

to form hydrozone producing brown colour in alkaline medium,which was monitored calorimetrically at 505 nm. The GPT content was calculated using standard diagnostic kit (SPAN Diagnostis, Gujarat, India).

2.9.12 Measurement of alkaline phosphate (ALP)

ALP converts phenyl phosphate to inorganic phosphate and phenol. The phenol formed was made to react with 4-amino antipyrine in the presence of oxidixing agent potassium ferricyanide to form a orange–red complex and this was measured spectrophotometrically at 505 nm. The ALP content was calculated using standard graph with standard provided in diagnostic kit (SPAN Diagnostis, Gujarat, India).

2.9.13 Measurement of lactic dehydrogenase (LDH)

This was measured by monitoring pyruvate and NADH formed when lactate reacts with NAD, mediated by LDH. Pyruvate formed reacts with 2,4-Dinitrophenyl hydrazine to give hydrozone which gives brown colour at 440 nm. The LDH content was measured using the standard graph with pyruvate as standard using diagnostic kit (SPAN Diagnostis, Gujarat, India).

2.9.14 Measurement of cholesterol

This was estimated by monitoring the complex formed when cholesterol react with hot solution of ferric perchlorate, ethyl acetate and sulphuric acid resulting in the formation of lavender coloured complex which was measured at 560 nm. OD of the sample was compared with that of standard to know the cholesterol content.

2.9.15 Statistical analysis

The data were subjected to analysis of variance appropriate to completely randomized design and the means were separated using Duncan's new multiple range test (Steel and Torrie, 1980).

2.10 Protocol for micropropagation of *Beta vulgaris* L.

2.10.1 Plant Material

The seeds of *Beta vulgaris* L. var. Detroit dark red were obtained from the market and germinated in clay pots. 12-15 days old selected healthy seedlings were taken as the donor seedlings as the source of explants.

Shoot tips from mature beet root plants of uniform size and high betalain content were also selected and used for *in vitro* multiplication.

2.10.2 Preparation and sterilization of culture media

Glass distilled water was used for the preparation of stock solutions of growth regulators, reagents and culture media. 3% (w/v) sucrose and 100 mg/l mesoinositol was added to the media. Growth regulating substances like auxins and cytokinins were supplemented to the basal media. The auxins used were 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or naphthaleneacetic acid (NAA). The cytokinins used were benzyladenine (BA), Kinetin (Kn), or 2-isopentenyl adenine (2-ip). The growth regulators were added in the required concentrations (mg/l), to the basal medium. pH of all media used was adjusted to 5.8, and solidified with 1% agar. Media was sterilized at 121^{o}C for 20 min.

2.10.3 Explants surface sterilization and inoculation

Shoot tips excised from the 12-15 old seedlings and mature plants were washed in running tap water for 15 min to remove the dirt and soil particles adhering to them. The explants were then treated with liquid detergent (Tween-20) for 5min. All traces of detergent were removed by repeated washing in distilled water. Further sterilization procedures were carried out under aseptic conditions in a laminar airflow cabinet (Kirloskar, India), which was cleaned, with ethyl alcohol (70% v/v) and irradiated with ultraviolet light for 45 min. The cleaned explants were surface sterilized with 0.1% (w/v) mercuric chloride for 5-7 min followed by three rinses with sterile distilled water, to remove residual mercuric chloride. The explants were then inoculated in to the respective medium and incubated at 25 ± 2^{o} C under cool light of 4.41 Jm^{-2} s^{-1} 16 hr day^{-1} provided by white fluorescent tubes (40W, Philips, India).

2.10.4 Multiple shoot initiation

The surface sterilized shoot tips were inoculated on to MS medium containing BA, Kn, 2-ip at different levels. The cultures in screw cap bottles were maintained under cool light of 4.41 Jm^{-2} s^{-1} 16 hr day^{-1} provided by white fluorescent tubes (40W, Philips, India) at 25±2°C. The multiple shoot initiation was observed and the numbers of shoots formed and shoot length obtained were recorded after three weeks of culture.

2.10.5 Multiple shoot proliferation

Multiple shoots initiated on MS, supplemented with BA, Kn, 2-ip were used for further multiplication. 2-3 shoots were transferred onto 50 ml of media (semi solid) supplemented with cytokinins in combination with auxins. The cultures were maintained under cool light of 4.41 Jm^{-2} s^{-1} 16 hr day^{-1} provided by white fluorescent tubes (40W, Philips, India) at 25±2°C. Number of shoots developed and shoot length were recorded after three weeks.

2.10.6 In Vitro rooting

The shoots were excised from the cluster of shoots developed in proliferation medium and used for rooting *in vitro*. For induction of rooting, auxins such as IAA, IBA and NAA were supplemented to MS medium at different levels. Rooting was recorded after two weeks of culture.

2.10.7 Hardening and transplanting

The *in vitro* rooted plants were removed from the culture bottles, and washed in running tap water to remove the adhering agar and cleaned plantlets were planted in pots containing soil, sand and compost mixture (1:1:2). The potted plantlets were kept in green house under polyethylene hood at 90% relative humidity (RH). After one week, polyethylene hood was removed and the plantlets were hardened further in 60% RH for another one week. Hardened plants were transplanted in field. Survival rate and field performance of the *in vitro* derived plants were observed till the period of harvest.

2.11 Field evaluation

2.11.1 Experimental site

The experiment was conducted in winter season (November-February) of the year 2005 in Ramakrishna ashram garden plot Mysore.

2.11.2 Preparation of land

The experimental field before transplanting was ploughed, harrowed and smoothened. On this land the layout was prepared.

2.11.3 Fertilization

Farm yard manure at the rate of 10 tones/acre was applied in the soil. The fertilizers were applied as per recommended dose of 100-100-100 kg N (Urea), P_2O_5 (SSP) K_2O (Murate of potash)/ acre respectively 20 kg of nitrogen and entire dose of P_2O_5 and K_2O were applied before sowing in previously opened furrows by manual labour in each plot keeping spacing of 90 cm between two rows. Nitrogen (30 kg) was applied as top dressing after 10 days of transplanting. Further, nitrogen (30 kg) was applied at 30 days after transplanting and 20 kg of nitrogen was applied at 55 days of transplantation.

2.11.4 Seed bed preparation

Seed beds for raising the seedlings from the seeds of *Beta vulgaris* L. (Detroit dark red) were prepared. Seed rate of 50 gm/acre was considered and accordingly seed beds of size 7 meter long, 1 meter wide and 15 cm in height were prepared. Per bed 20 kg farm yard manure, 300 gm Di-ammonium phosphate and 100 gm of potash was applied.

2.11.5 Transplanting

Transplanting of *Beta vulgaris* L. seedlings was carried out after 25 days of sowing the seeds. The seedlings were transplanted at a distance of 60 cm by manual labour. The tissue cultured hardened plants were also transplanted to the field.

2.11.6 Irrigation

Two light irrigations were applied one at the time of transplanting and another 3 days after transplanting to each plot for ensuring proper establishment of the crop. Thereafter irrigation was applied to each plot at an interval of 6 days.

2.11.7 Weeding

To keep the crop free from weeds hand weeding was carried out after 10 days of transplanting and 30 days after transplantation.

2.11.8 Harvesting

Harvesting was carried out manually after three months from period of transplantation and 4 pickings were done at an interval of 5 days in both the types of plants.

2.12 Analysis of growth and betalain production

Plant samples were taken from borderlines of each plot. The first sample was taken 15 days after transplanting and thereafter at 30 days, 45 days, 60 days, and 90 days i.e. final harvest. Ten plants were randomly selected and were carefully uprooted. They were brought to the laboratory and adhering soil and sand particles were carefully washed out in running stream of water to clean the roots. The plant parts *viz.* Leaves, and roots were separated gently and following parameters mentioned below were recorded.

2.12.1 Fresh and dry weights of leaves per plant

The washed leaves were blotted on filter paper and fresh weight was recorded, then the leaves were kept in a hot air oven and dried at 70^{o}C till constant dry weight was obtained.

2.12.2 Fresh and dry weights of whole plant

The leaves weight and root weights were together considered as whole plant weight.

2.12.3 Betalain content in beet root

Betalain content was estimated by the method mentioned earlier

2.12.4 Statistical analysis

The data were subjected to analysis of variance appropriate to completely randomized design and the means were separated using Duncan's new multiple range test (Steel and Torrie, 1980).

CHAPTER -III

RESULTS

3.1. Induction and confirmation of transformed nature of hairy roots of *Beta vulgaris* L.

Objective of this study was to induce roots by using *Agrobacterium rhizogenes* and to confirm that the roots obtained from the region of infection are hairy roots. Initially transformation in the hairy roots obtained was checked by opine detection test (Fig.5), further it was confirmed by PCR analysis using *rolA* specific primer. PCR analysis of *rolA* gene provided the molecular evidence supporting the transformed nature of the hairy root with *rolA* primers, the expected size of 360bp were obtained in lanes containing DNA from transformed roots (Fig.6). Southern hybridization analysis further confirmed the transformed nature of the hairy roots and integration of the T-DNA into the hairy root chromosomal genome.

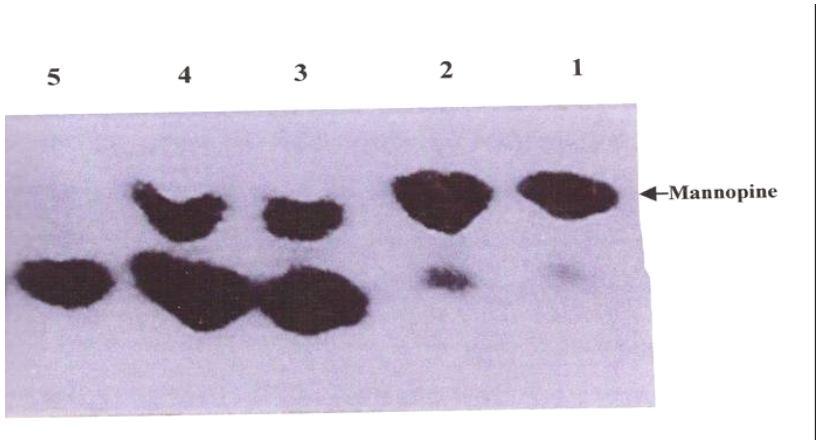

Figure 5. Opine analysis of roots derived from LMG-150, A2/83 and A20/83 strains of *A. rhizogenes*. Lane 1: Standard mannopine, Lane 2: Transformed roots of *Beta vulgaris* derived from LMG-150, Lane 3: Transformed roots of *Beta vulgaris* derived from A2/83, Lane 4: Transformed roots of *Beta vulgaris* derived from A20/83, Lane 5: Untransformed roots of *Beta vulgaris* L.

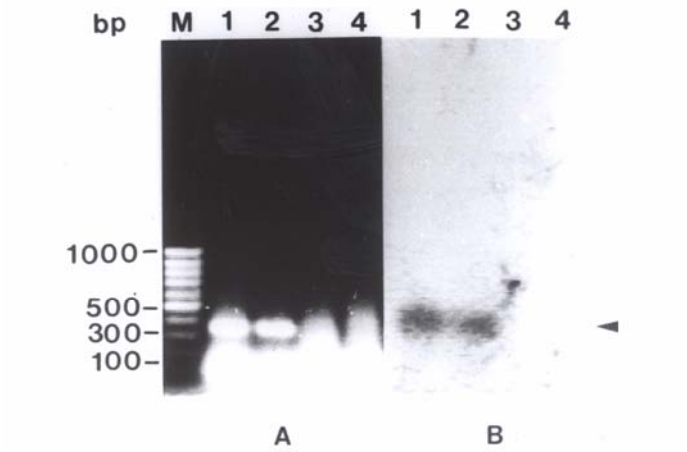

Figure 6. A. PCR analysis of *rol A* gene. Lane M-100bp marker, Lane 1&2. PCR amplification of *rol A* from transformed roots, Lane 3&4. PCR amplification of *rol A* from normal roots. B. Lane

1&2. DNA amplified from transformed roots, lane 3&4. DNA amplified from normal roots. Arrow mark indicates the amplification of *rol A* 360bp

3.2. Influence of different strains of *Agrobacterium rhizogenes* on induction frequency, growth and accumulation of betalain in hairy roots of *Beta vulgaris* L.

Objective of this study was to investigate the influence of different *A. rhizogenes* strains on frequency of hairy root induction, growth and morphology of the hairy roots obtained and production of pigments for the selection of best clone in all aspects for further studies. A systematic study using four different strains of *A. rhizogenes* for the evaluation of transformation frequency, growth and betalain production was carried out. Three strains, out of the four produced hairy root at the site of infection cultured on MS basal medium. *A rhizogenes* A4 did not produce hairy roots. The best root formation response as transformation frequency was in the following order LMG 150-95%, A 20/83 – 87% and A2/83 – 72% respectively (Table 4). Roots also showed difference in their morphology (Fig. 7). It was found that hairy roots obtained from LMG-150 were better in both growth and production of betalain. However there was a distinct difference in betalain production of different clones. Betalain production increased slowly during first seven days in all the hairy roots clones and then all the three lines behaved differently. In hairy roots obtained from LMG 150 strain, there was a rapid rise after 7 days and on 20th day 32.9 mg betalain /culture was recorded (Fig. 9). The time course of growth and

betalain production was studied on MS basal medium. There was an increase in biomass from 0.1 g FW at day one to 12.28 g FW/ culture at day 20 (Fig. 8)

Table 4. Influence of different strains of *Agrobacterium rhizogenes* an induction of hairy roots of *Beta vulgaris* L.

Bacterial Strain	Days for induction of hairy roots	Frequency of transformation %	Number of hairy roots after 3 weeks of cultures
LMG -			
150	5-7	95	39
A 20 / 83	6-9	87	32
A2/83	8-12	72	24
A4	-	0	0

Maximum of 10 explants were used for the transformation experiment.

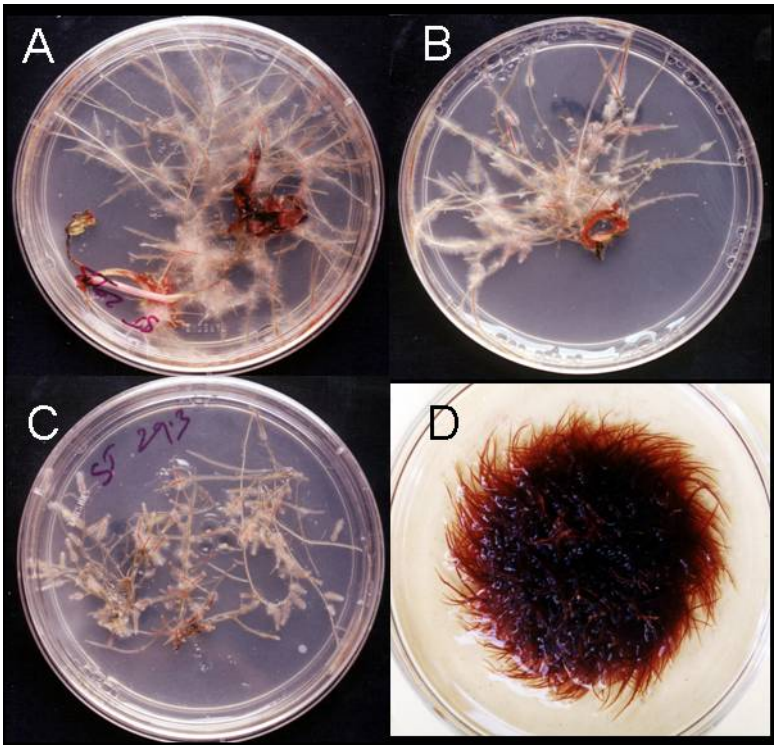

Figure 7. Hairy root of *Beta vulgaris* L. induced by (A) LMG-150, (B) A2/83, (C) A20/83 strains of *A. rhizogenes*. (D) Fully grown submerged cultivated hairy roots.

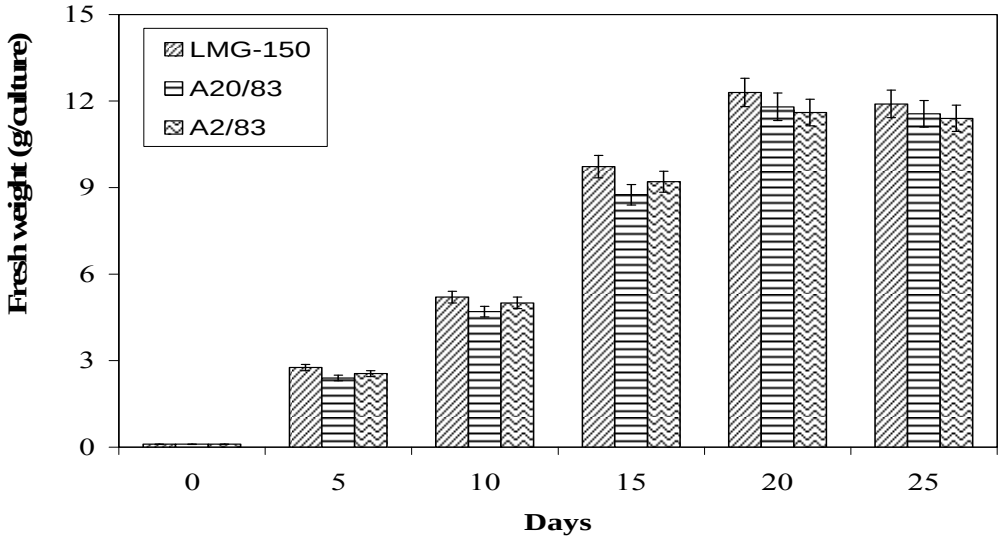

Figure 8. Influence of *A. rhizogenes* strains on growth of *Beta vulgaris* L hairy roots.

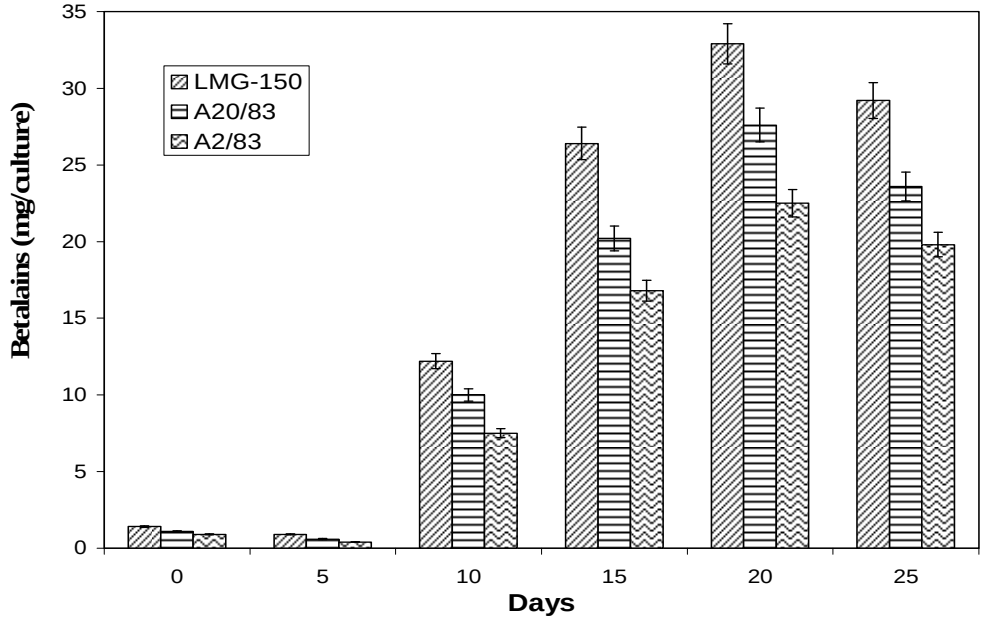

Figure 9. Influence of *A. rhizogenes* strains on accumulation of betalain in *Beta vulgaris* L

3.3 Induction of hairy roots from different explants excised from *in vitro* germinating seedling of *Beta vulgaris* L. using *A. rhizogenes* strain LMG – 150.

This study was carried out to understand the type and age of explants susceptibility to *A. rhizogenes* and their frequency of transformation and induction of hairy roots. Explants such as root, stem, leaf, hypocotyls and cotyledon were excised from 15 days old

in vitro germinating seedlings and co-cultivated with *A. rhizogenes* LMG – 150. Each explant showed a varying extent of root induction (Table 5). Maximum root induction frequency of 96% of cultured explants was observed using cotyledon explants, while other explants *i.e.* root 0.5%, stem 72%, leaf 88%, hypocotyls 64% transformation frequency respectively. Further the root induction response of cotyledon explants of different age was compared. Cotyledons isolated from 11-14 days old seedling showed maximum hairy root induction (Table 6). The time period required for root induction also varied with the age of cotyledon explants.

Table 5. Influence of different explants excised from *in vitro* germinating seedling on hairy root induction with *A. rhizogenes* LMG – 150.

Explant	Number of cultures infected with *A.rhizogenes*	Number of cultures showing hairy root induction	Hairy root induction%
Root	25	02	0.5
Stem	25	18	72
Leaf	25	22	88
Hypocotyls	25	16	88
Cotyledons	25	24	96

Table 6. Induction of hairy root from cotyledon explants of different age using *A. rhizogenes* LMG – 150.

Age of cotyledon (days after *in vitro* culture)	Number of explants used for infection	Number of explants showing hairy root induction	Time required for induction	Hairy root induction (%)
7	25	15	9-12	60%
11-14	25	24	5-7	96%
15-17	25	22	5-7	88%
17-20	25	20	10-14	80%

3.4. Induction of hairy roots on growth and betalains production pattern in four varieties of *Beta vulgaris* L

Induction of hairy roots in four varieties was carried out to study the difference in accumulation of betalains in hairy roots obtained from different varieties, a factor which is found in the field grown beet root varieties. Leaf cotyledon explants obtained from seedlings of four different commercial varieties cultivated widely all over India of *Beta vulgaris* ie. Detroit dark red, Sutton blood red, Crimson globe and Ashoka were infected with *A. rhizogenes* LMG-150 and hairy roots obtained. It was found that all four varieties produced hairy roots at the point of infection. Sutton blood red produced 100% hairy root induction followed by Detroit dark red 96%, Crimson globe 67% and Ashoka 53% (Table 7). Morphological differences were also found in hairy roots obtained from four varieties of hairy roots (Table 8).

Growth and production of betalains was investigated in hairy roots obtained from four varieties. There was no significant difference in the growth pattern of the hairy roots of four varieties and, a maximum biomass of 14.6 g FW /culture was recorded on 20th day. Significant differences were observed in accumulation of betalains and ratio of betacyanins to betaxanthins between the four varieties. Detroit dark red produced maximum betalains 32.7 mg/culture (Fig.12) in the ratio of 41% betacyanins to 59% (Fig. 10 and 11)

Table 7. Hairy root induction pattern in four varieties of *Beta vulgaris* seedlings infected with *Agrobacterium rhizogenes* LMG-150

Beta vulgaris variety	Number of cultures infected	Number of cultures showing hairy root induction	Number of hairy roots per culture after 3 weeks
Detroit Dark Red	25	24	31±1.2
Sutton Blood Red	25	25	32±1.7
Crimson globe	25	22	19±0.7
Ashoka	25	19	11±0.2

Table 8. Morphological characters of hairy roots obtained from four varieties of *Beta vulgaris* L.

Beet variety	Root Length (cm)	No. of lateral roots	Length of lateral roots (cm)	Colour	Hairyness	Thickness
Detroit Dark Red	8.5±0.31	12.2±0.3	1.92±0.05	Red	Dense	Thick
Sutton Blood Red	11.3±0.7	8.5±0.7	2.31±0.08	Red	Sparse	Thin
Crimson Globe	7.1±0.21	9.2±0.5	0.96±0.02	Yellowish	Dense	Thick
Ashoka	3.9±0.51	6.3±0.2	1.21±0.03	Yellowish	Sparse	Slender

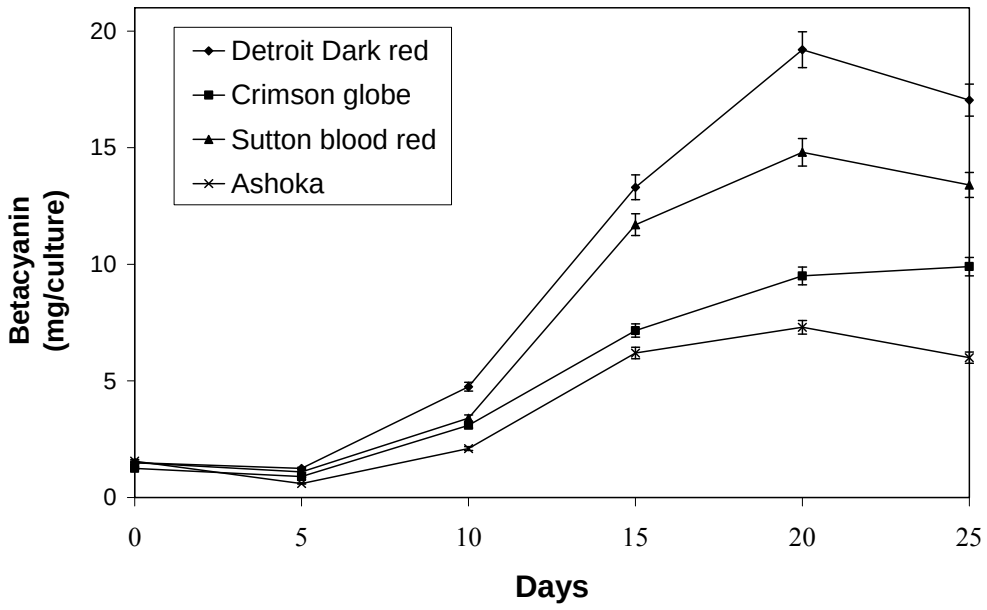

Figure10. Betacyanin content in four varieties of *Beta vulgaris* L. hairy roots.

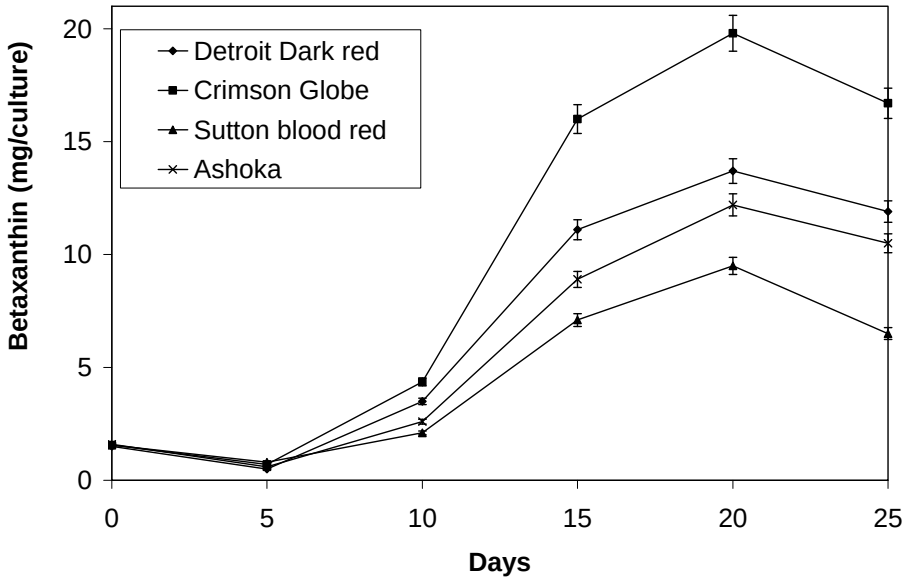

Figure 11. Betaxanthin content in four varieties of *Beta vulgaris* L. hairy roots.

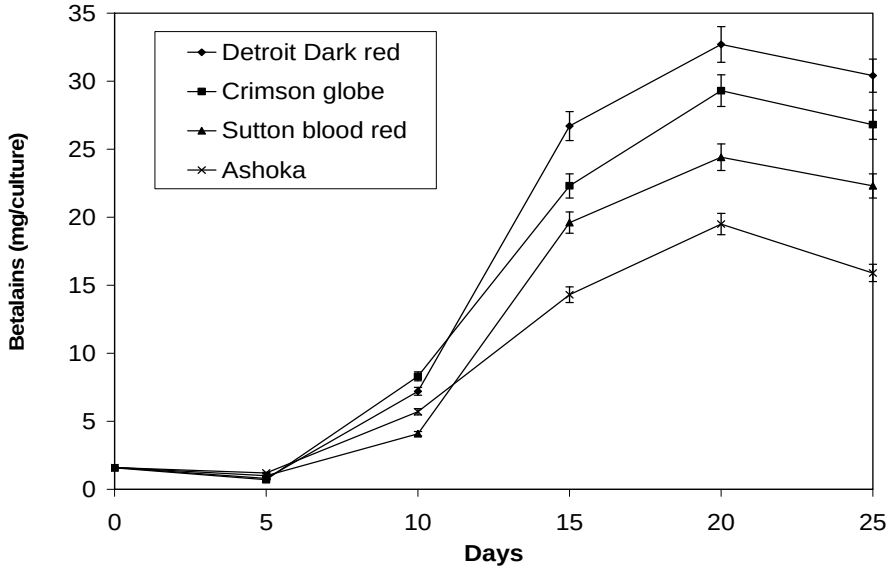

Figure12. Betalain content in four varieties of *Beta vulgaris* L. hairy roots.

betaxanthins, Sutton blood red variety hairy roots produced 24.3 mg/culture betalains with similar betacyanin to betaxantin ratio. Crimson globe variety hairy roots produced 29.3 mg/culture betalains with a ratio of 32.5% betacyanins to 67.5% betaxanthins, and Ashoka variety hairy roots produced 19.5 mg/culture betalains containing 37% betacyanins and 63% betaxanthins. As Detroit dark red variety hairy root cultures were found to be superior in all aspects, they were selected and used for all further experiments.

3.5. Influence of different medium constituents on growth and production of betalains in hairy root culture of *Beta vulgaris* L.

3.5.1. Effect of nitrates

The objective of this study was to investigate the effect of nitrates and phosphates and to standardize a particular level of both the major nutrients for better growth and production of betalains. The effects of various nitrate concentrations (⅛, ¼, ½, full, double and nitrate free) in MS were investigated for hairy root growth and betalain production. It was observed that nitrates at all the lower levels and nitrate free MS medium inhibited the growth of hairy roots. A maximum biomass accumulation (15.1 ± 0.5 g / culture) was found in double nitrates MS on 20th day and in nitrates free medium (4.5 ± 0.9g/culture) which is more than 50% lesser than in normal MS (Fig. 13). Nitrates at lower levels did not effect the betalain production significantly, but it was noticed that nitrates free and double nitrate medium inhibited the biosynthesis of betalains (22.8 and 20.07 mg / culture) respectively. A maximum amount of betalains (32.7 mg / culture) was produced on 20th day of culture in normal MS (Fig. 14).

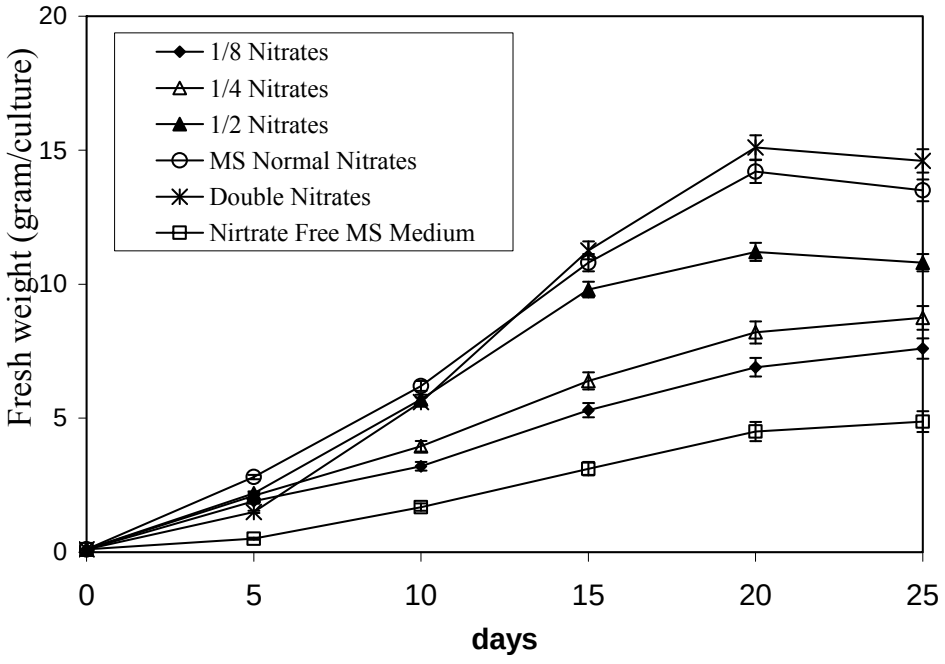

Figure 13. Effect of Nitrates on growth of *Beta vulgaris* L. hairy root cultures.

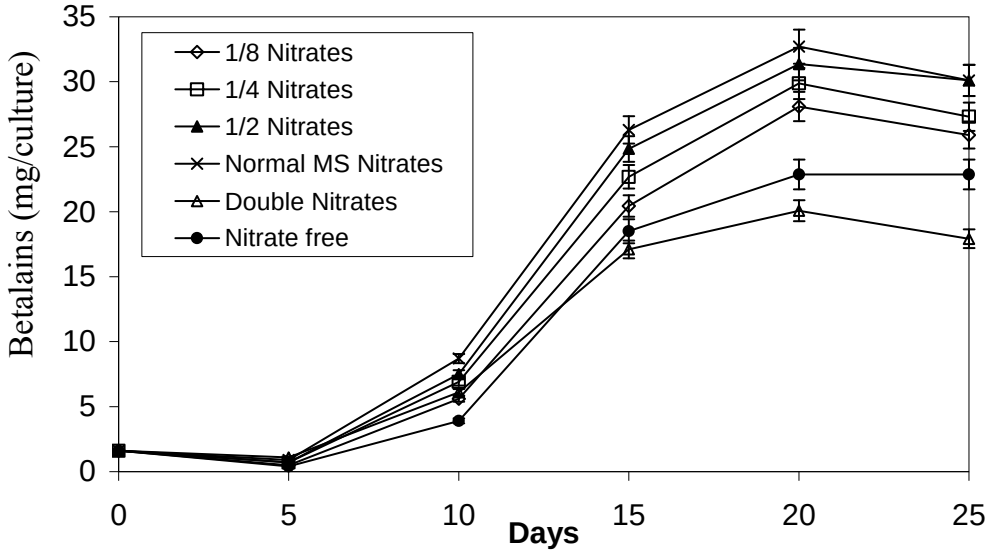

Figure14. Effect of Nitrates on production of betalains in *Beta vulgaris* L. hairy root cultures.

3.5.2. Effect of nitrate and ammonium at different levels

The effect of nitrogen concentration supplied in the MS medium as nitrates and ammonium was evaluated. The MS medium contains 39.4 mM nitrate (NO_3^-) and 20.6mM Ammonium (NH_4) respectively. Different ratios of nitrate and ammonium (39.4 : 20.6, 39.4 : 0.0, 0.0 : 20.6, 39.4 : 10.3) were tested. It was noticed that a low ammonium: nitrate ratio favored growth and accumulation of betalains (Fig. 15, Fig. 16). The ratio of 0 : 20.6 ie., ammonium alone inhibited the growth and production of betalains was reduced drastically, which was (6.1 ± 0.3 g / culture) and (11.2 ± 0.9 mg / culture) respectively.

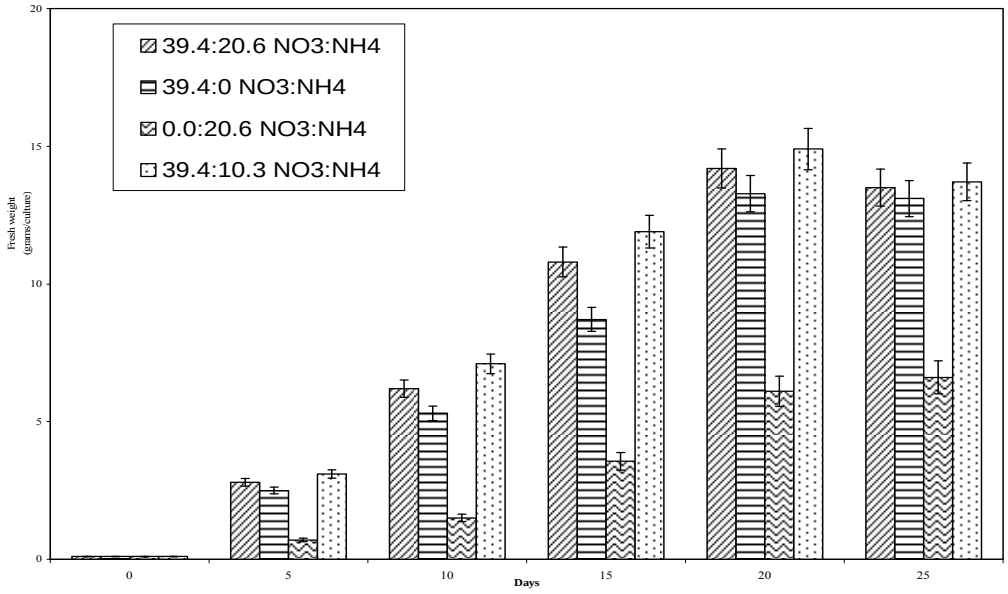

Figure15. Effect of Different ratio of nitrates and ammonium on growth of *Beta vulgaris* L. hairy roots.

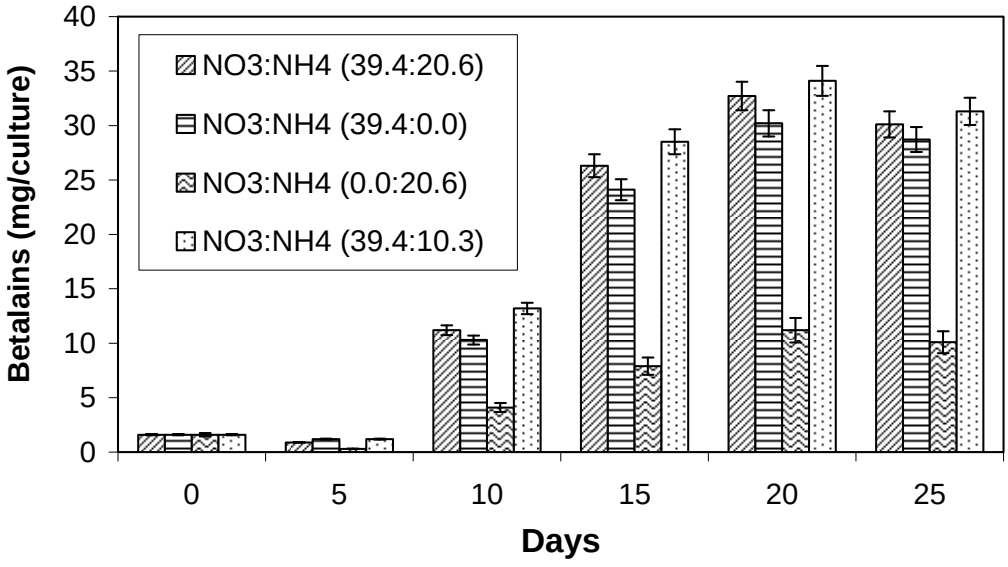

Figure 16. Effect of different ratio of nitrates and ammonium on production of betalains in hairy root cultures of *Beta vulgaris* L.

3.5.3. Effect of Phosphates

Influence of phosphates at different levels (⅛, ¼, ½, full, double and phosphate free) were investigated. It was found that lower levels of phosphate and phosphate free MS medium inhibited the growth of hairy roots (Fig. 17) but had great influence on

enhancement of betalain production. A maximum amount (45.2 mg/culture) betalains were recorded on 20th day in phosphate free medium. All the lower level of phosphates also showed significant increase in betalain production (Fig. 18). Double phosphate did not effect the growth of the hairy root but inhibited the biosynthesis of betalain significantly (25.1mg/culture).

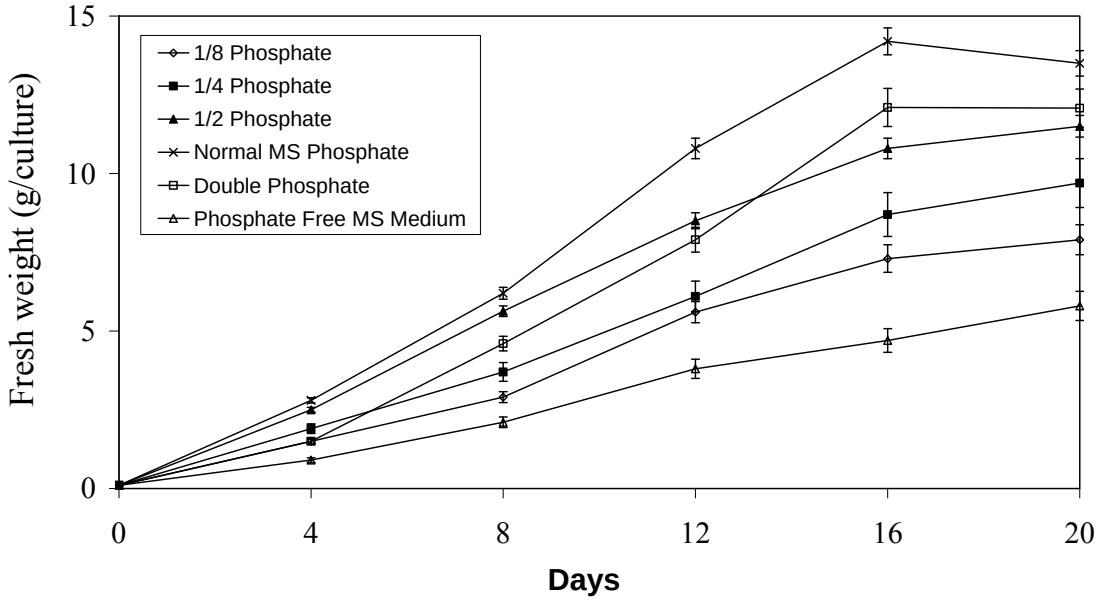

Figure-17 Effect of Phosphates on growth of *Beta vulgaris* L. hairy roots.

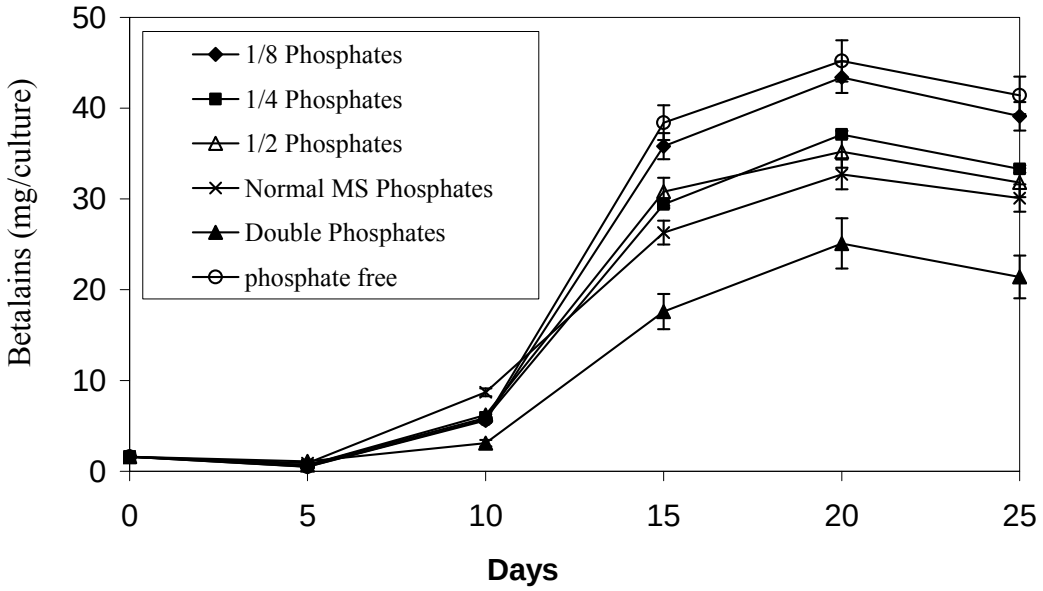

Figure 18. Effect of Phosphates on production of betalains in Beet hairy root cultures

In this above mentioned study on influence of nitrates and phosphates at various levels, specific concentration of nitrates, ratio of nitrate and ammonium and phosphate levels necessary for better growth and production of betalains have been standardized.

3.5.4. Effect of micronutrients on growth and production of betalains in hairy root cultures of *Beta vulgaris* L.

This study was done to know the effects of each micronutrient on growth and accumulation of betalains so that particular levels of micronutrients necessary can be standardized for enhanced growth and production of betalains. The effects on the production of betalains and hairy root growth of *Beta vulgaris* L. at increased concentration (×5) of the microelements tested are given in (Table 9). The microelements Co^{2+}, Mo^{2+} and Fe^{2+} had a positive effect on the growth and production of betalains. Zn^{2+} and Cu^{2+} inhibited the production of betalains but had no effect on growth. The highest accumulation of (45.7mg / culture) betalains was achieved with Co^{2+}, which was higher than the MS normal medium. The increment achieved after the increase of divalent ions, Mo^{2+}, Fe^{2+} was 20% lower than the one obtained in the increased concentration of Co^{2+}. Mn^{2+} did not show significant influence on the production of betalains. The microelements tested also had effect on betacyanin to betaxanthin ratio, compared to all other micronutrients and MS normal medium, Co^{2+} showed a ratio of 51:49 % betacyanin to betaxanthin whereas in others the betaxanthin content was high and betacyanin content was low.

Table 9. Effect of different microelements on the growth and production of betalains in hairy root cultures of *Beta vulgaris* L.

Divalent ion	[a] Conc. (μM)	Fresh weight (g/culture)	Dry weight (g/culture)	Betalains (mg/culture)	BC/BX ratio in %
MS (Normal)		14.51±0.53	2.04±0.06	32.7±1.23	46:54
Mn^{2+}	500	12.32±0.81	1.72±0.15	31.5±0.98	51:49
Fe^{2+}	500	12.73±0.46	1.52±0.23	34.3±0.83	55:45
Mo^{2+}	5	11.59±0.23	1.61±0.51	37.1±0.88	42:58
Zn^{2+}	150	13.95±0.37	1.79±0.91	19.3±0.63	37:63
Co^{2+}	0.50	13.82±0.60	1.81±0.83	45.7±1.57	51:49
Cu^{2+}	0.50	11.26±0.13	1.56±0.31	23.1±0.77	41:59

[a] value correspond to five fold MS concentration of divalent ions. Values are average of five replicates and ±SD. BC- Betacyanin, BX- Betaxanthin

Higher Co^{2+} concentration (10 and 20 µM) did not have much effect on growth and production of betalains (Fig.19). Beside the removal of Co^{2+} did not effect the growth but inhibited the synthesis of betalains. The maximum production of betalain achieved in MS normal (MSN) media and after the addition of 5µm Co^{2+} was 32.7 and 45.7 mg / culture respectively. The betacyanin to betaxanthin ratio did not change when Co^{2+} was increased.

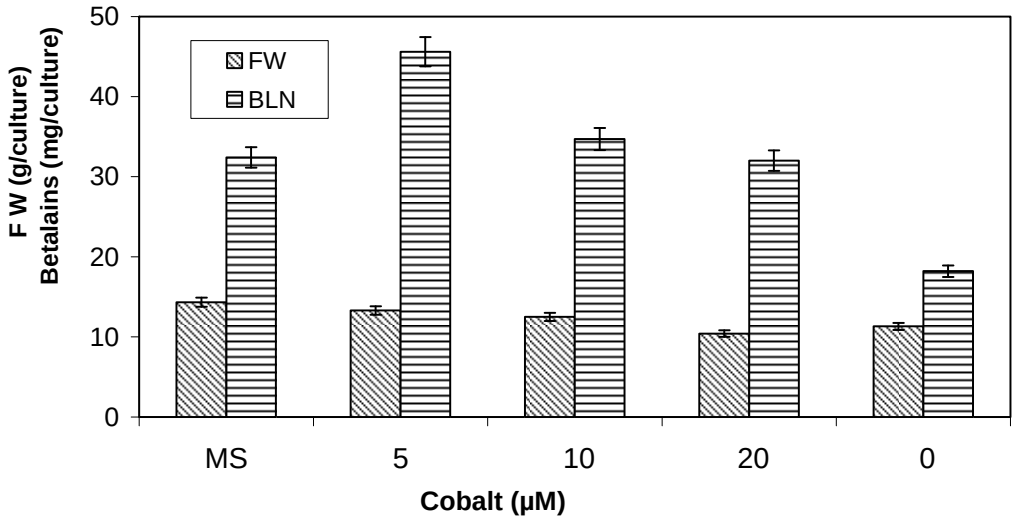

Figure 19. Effect of cobalt at different concentrations on growth and production of betalains in *Beta vulgaris* L. hairy root cultures.

Effect of zinc at different concentrations is shown in Fig. 20. The betalain content decreased with increasing zinc concentration in the medium, whereas removal of zinc effected the growth and accumulation of betalains. Enhanced accumulation of betalains 44.5 mg / culture was recorded, but the growth of hairy roots was slow and inhibited. In the absence of Zn, the betalain content increased to its maximum after 20 days (44.5 mg/ culture), whereas the betalain content in the MSN medium reached its maximum (32.4 mg/culture) after 15 days.

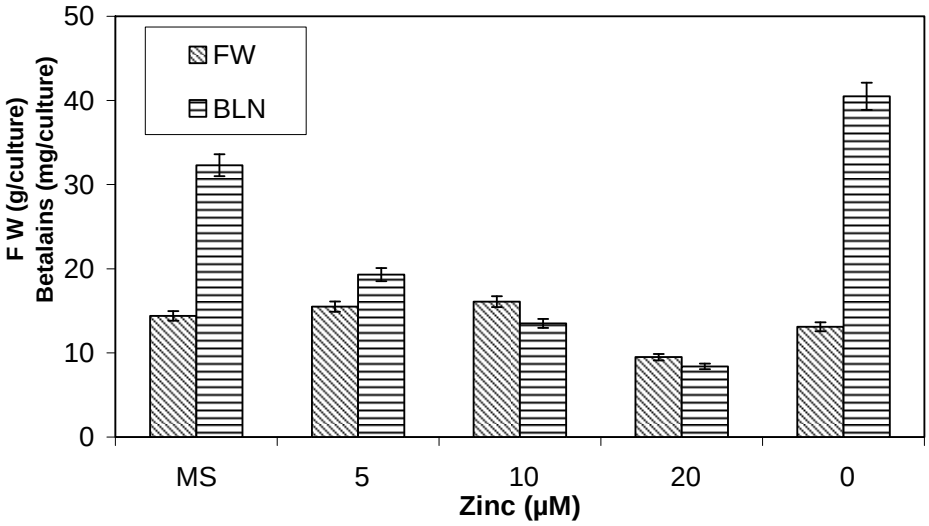

Figure 20. Effect of zinc at different concentrations on growth and production of betalains in *Beta vulgaris* L. hairy root cultures.

3.6. Effect of various basal media on growth and production of betalains in *Beta vulgaris* L. hairy roots

Influences of different basal medium were investigated on growth and production of betalains in beet hairy roots. Murashige and Skoog's (MS), Gamborg's (B5), Nitsch (N6) and White's media were tested. Root growth largely depended on the medium formulation. The best hairy root growth (14.2 g FW/culture) was achieved after 20 day culture in MS medium followed by B5 medium (13.7g FW/ culture) (Fig. 21). In other two media the maximum growth was achieved after 20 days (Nitsch – 10.8 g FW/culture, Whites – 8.46 g FW /culture). Different basal medium formulation affected betalain accumulation also. Highest betalain yield was recorded in MS (32.7 mg / culture) on 20th day of culture (Fig. 22). The production of betalains in B5 medium was similar to MS. In Whites (22.1 mg / culture) and Nitsch (16.6 mg / culture) betalains were obtained.

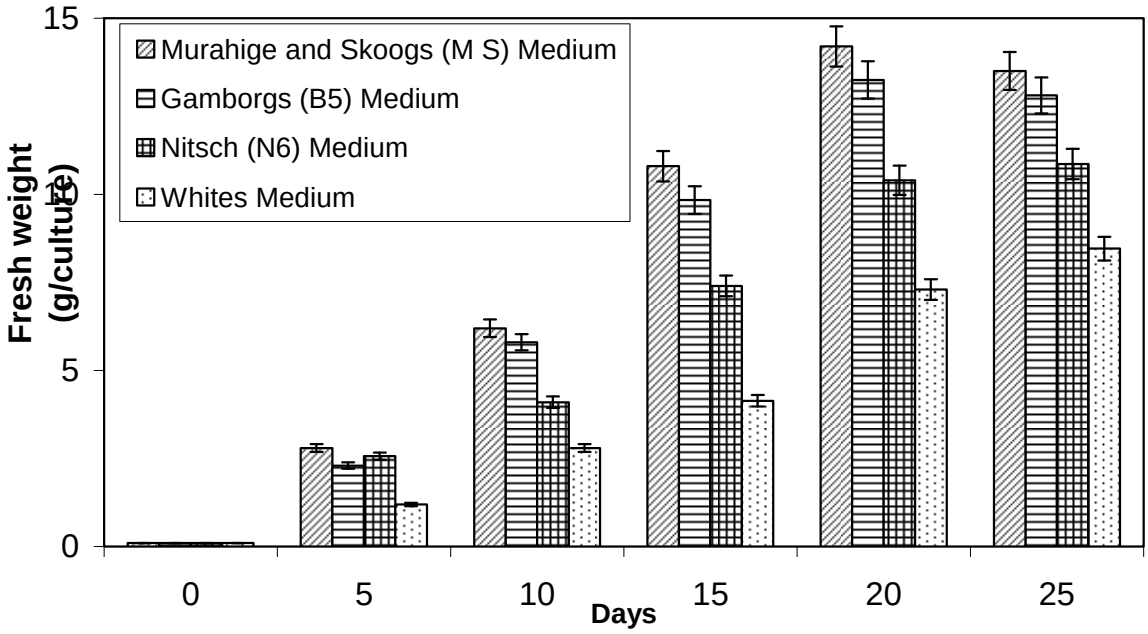

Figure 21. Effect of different basal medium on growth of *Beta vulgaris* L. hairy root cultures.

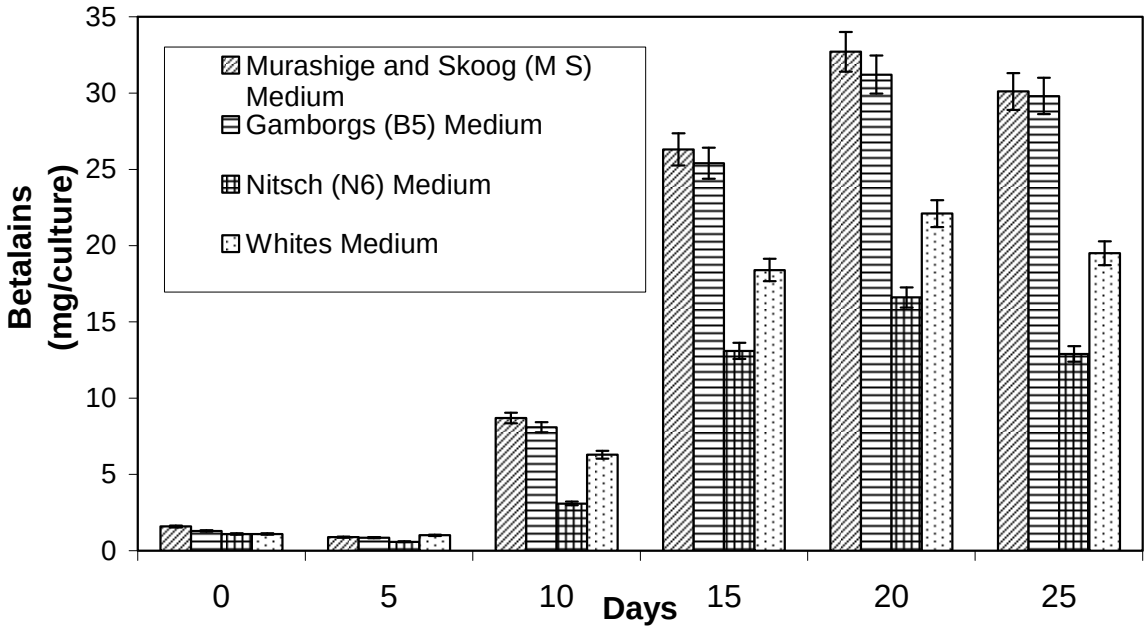

Figure 22. Effect of different basal medium on production of betalains in *Beta vulgaris* L. hairy root cultures.

3.7. Effect of pH on growth and production of betalains in *Beta vulgaris* hairy roots

Effect of pH ranging from (3.5-7.0) was investigated on growth and production of betalains in MS medium. The pH of the medium was adjusted to 3.5, 4, 4.5, 5.0 5.5, 5.8, 6.0, 6.5, 7.0, using 0.1 N NaOH. As shown in (Fig. 23) the pH levels ranging from 4.5-6.0, did not effect the hairy root growth at large and pH 5.8 was found to be optimal for maximum growth of hairy roots (14.2 g FW/ culture) on 20th day (Fig. 23). The pH levels below 4.5 inhibited the growth drastically and hairy root disintegration was noticed. Basic pHs 6.5 and 7.0 resulted in slow growth initially and death of cultures at later stages. Accumulation of betalain also found to be pH dependent, and pH levels ranging from (5-6) were supportive for biosynthesis of betalain, and maximum production (32.7mg / culture) was recorded on 20th day of culture in pH 5.8 (Fig. 24). In pH below 4.5, leaching of pigments into the medium was observed.

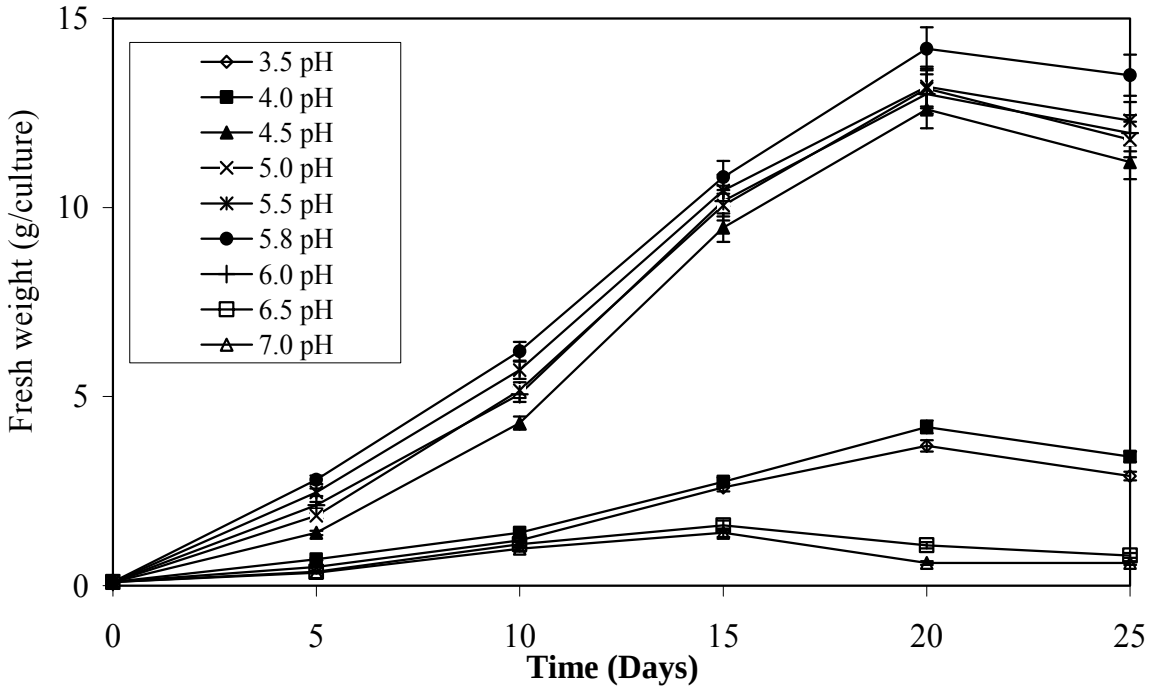

Figure 23. Effect of pH on growth of *Beta vulgaris* L. hairy root cultures.

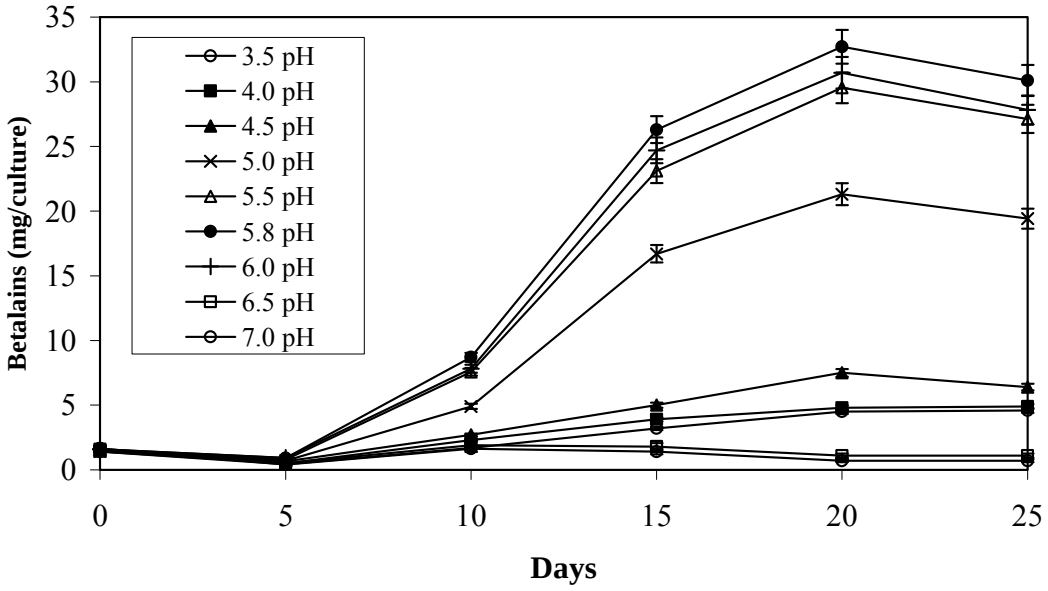

Figure 24. Effect of pH on production of betalains in *Beta vulgaris* L. hairy root cultures.

3.8. Effect of sucrose and other carbon sources on growth and production of betalain in transformed root cultures of *Beta vulgaris* L.

Objective of this study was to optimize the level of carbon source and the type of carbon source effective in influencing the growth of hairy roots and production of betalains. Sucrose, maltose, fructose, glucose and glucose + fructose were tested as carbon source. Results showed that the biomass accumulation increased with increasing sucrose concentration till 50g/l, and a maximum accumulation of 16.54 g FW/culture (Fig. 25) was recorded. On the other hand maximum betalain, 37.5 mg/culture was obtained at 20g/l (Fig.26). It was also noticed that between the different carbon sources individually and in combination, hairy root growth and production of betalains was higher in sucrose followed by maltose fructose and glucose (Fig.27 and 28). Thus sucrose was the suitable carbon source for *Beta vulgaris* hairy root growth and production of betalains. Sucrose at 3-5% was favorable for growth of hairy roots but 1-3 % influenced the accumulation of betalains.

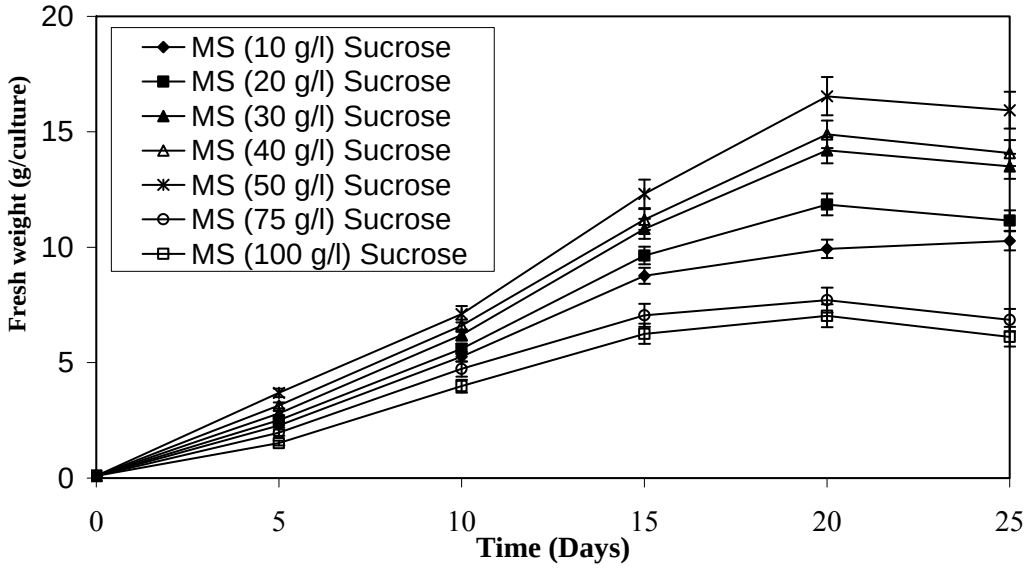

Figure 25. Effect of sucrose at different concentration on growth of *Beta vulgaris* L. hairy root cultures.

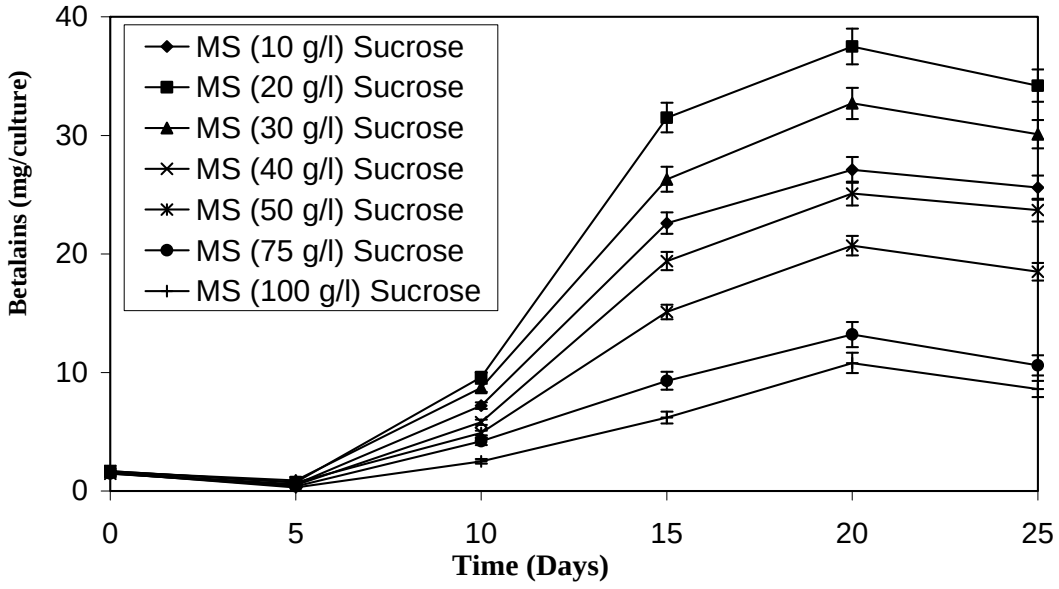

Figure 26. Effect of sucrose at different concentration on production of betalains in *Beta vulgaris* L. hairy root cultures.

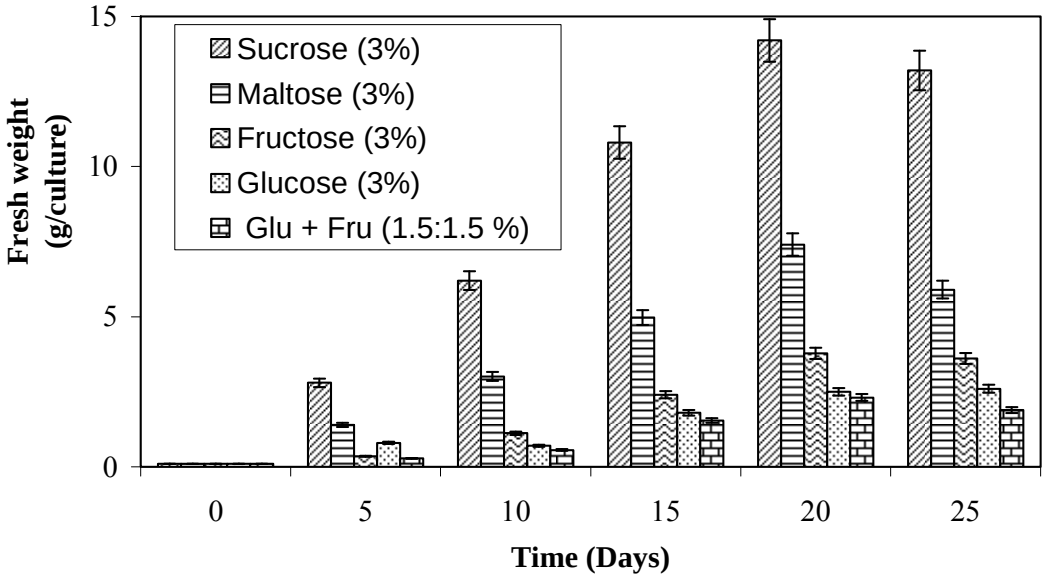

Figure 27. Effect of different sugars on growth of *Beta vulgaris* L. hairy root cultures.

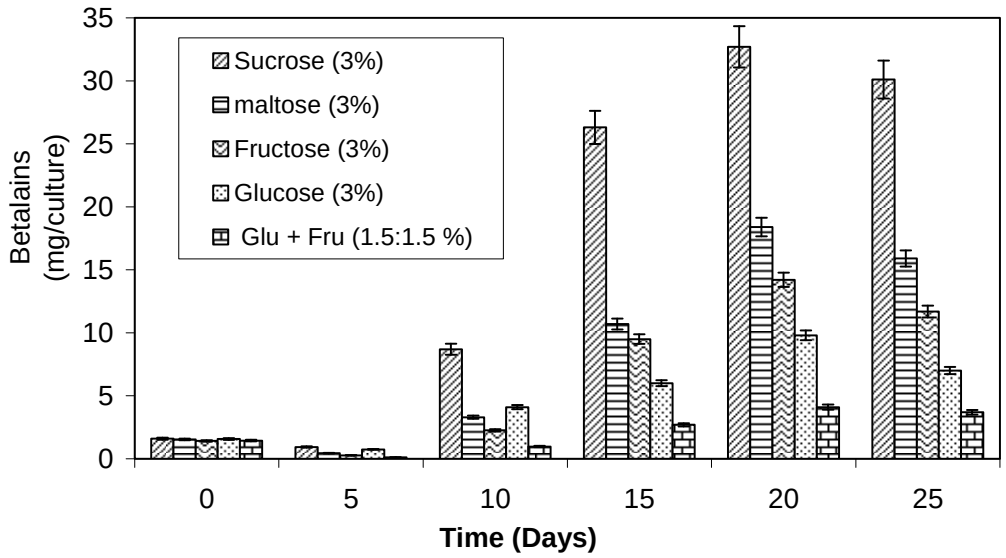

Figure 28. Effect of different sugars on production of betalains in *Beta vulgaris* L. hairy root cultures.

3.9. Influence of growth regulators on growth and production of betalains in hairy root cultures of *Beta vulgaris* L.

The objective of the study was to understand the individual effects of exogenously applied growth regulators on growth and betalain production in hairy root cultures of *Beta vulgaris* L. Effect of various treatments of auxins and cytokinins individually and in combination on hairy root growth and betalain accumulation was studied. It was observed that the growth of hairy root decreased with increase concentration of auxins to cytokinin ratio (Fig. 29, 30 and 31). Among the concentrations and combination of growth regulators tested maximum biomass was obtained in IAA 0.1 mg/l + Kn 0.01 mg/l (9.43 g FW/ culture) (Fig. 30), and betalain production in IBA 0.1 mg/l + Kn 0.01 mg/l (17.3 mg / culture) (Fig. 34). At high concentration of auxin to cytokinin ratio root disorganization was noticed and in NAA and 2-4 D, dedifferentiation and callus formation was seen.

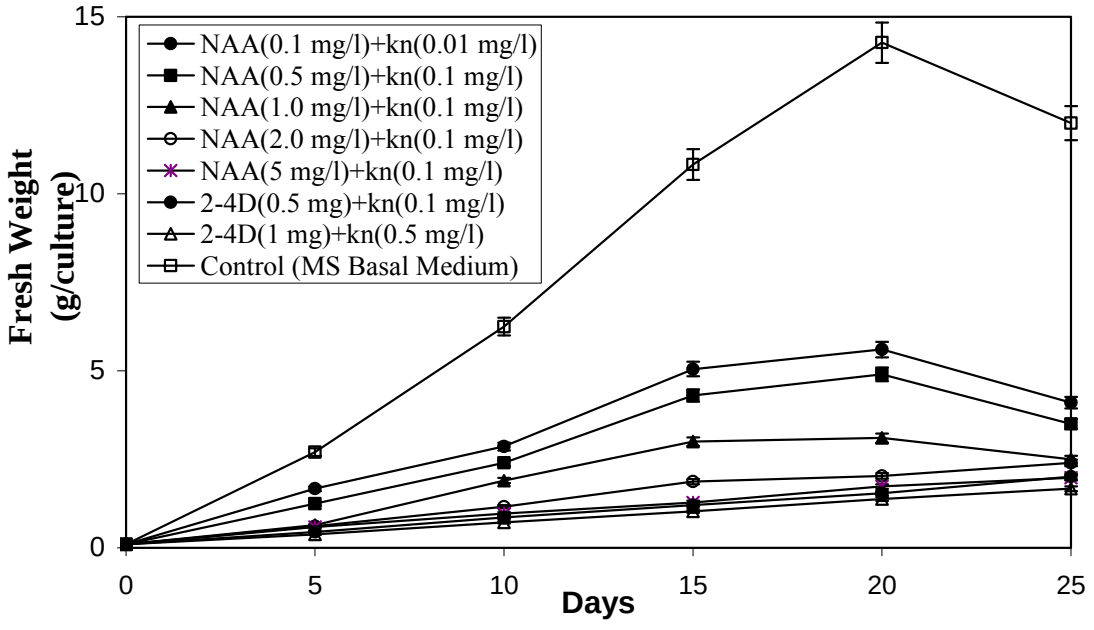

Figure 29. Effect of NAA + Kn and 2,4-D + Kn on growth of hairy root cultures of *Beta vulgaris* L.

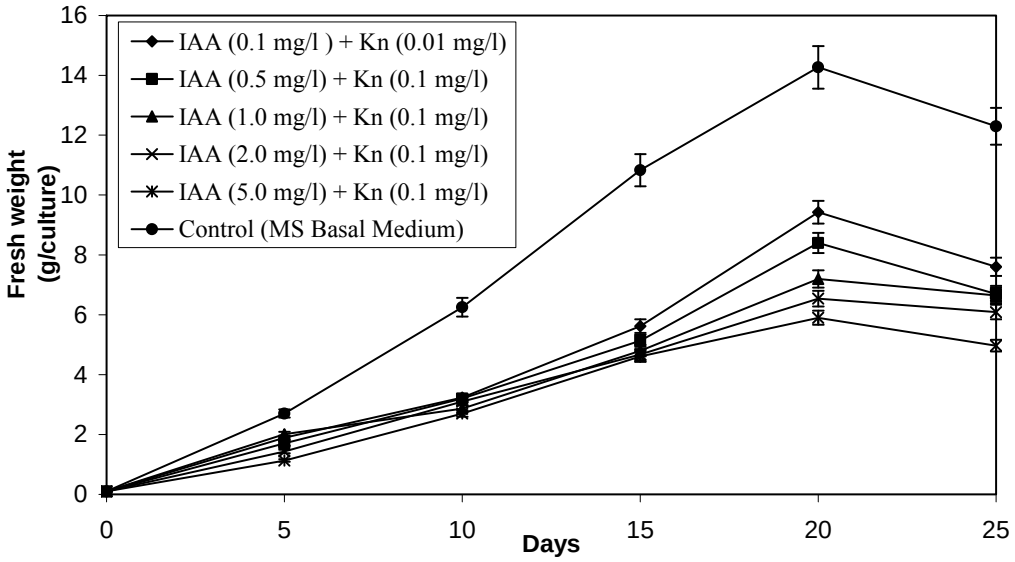

Figure 30. Effect of IAA + Kn on growth of hairy root cultures of *Beta vulgaris* L.

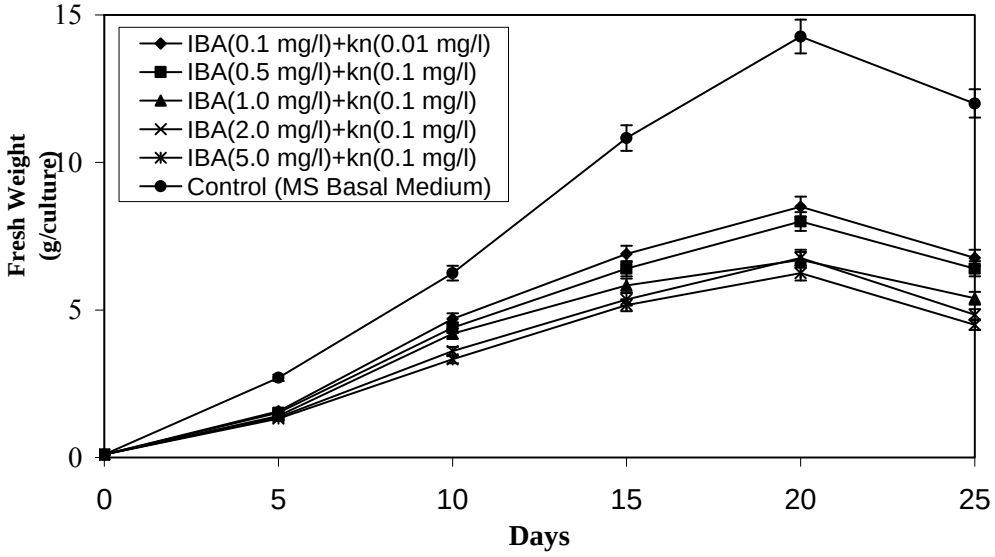

Figure 31. Effect of IBA + Kn on growth of hairy root cultures of *Beta vulgaris* L.

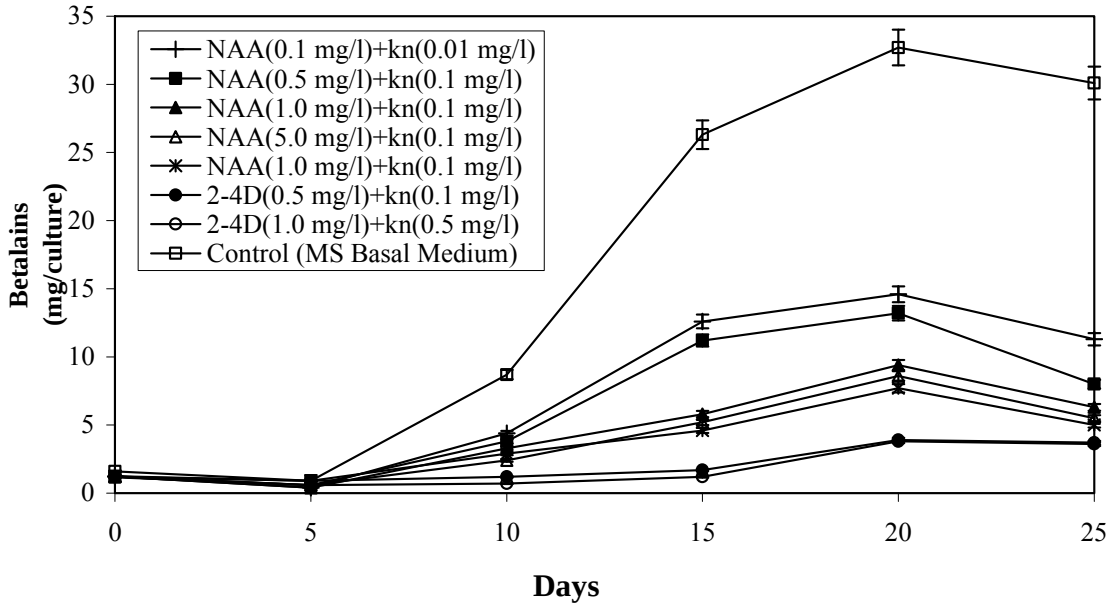

Figure 32. Effect of NAA + Kn on production of betalains in *Beta vulgaris* L. hairy root cultures.

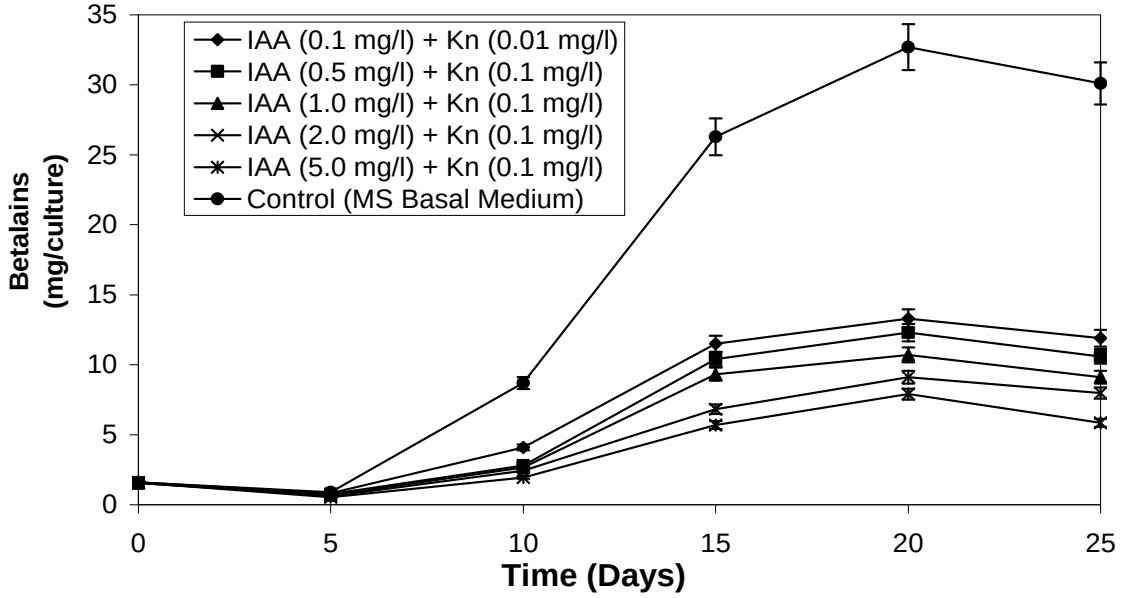

Figure 33. Effect of IAA + Kn on production of betalains in *Beta vulgaris* L. Hairy root cultures.

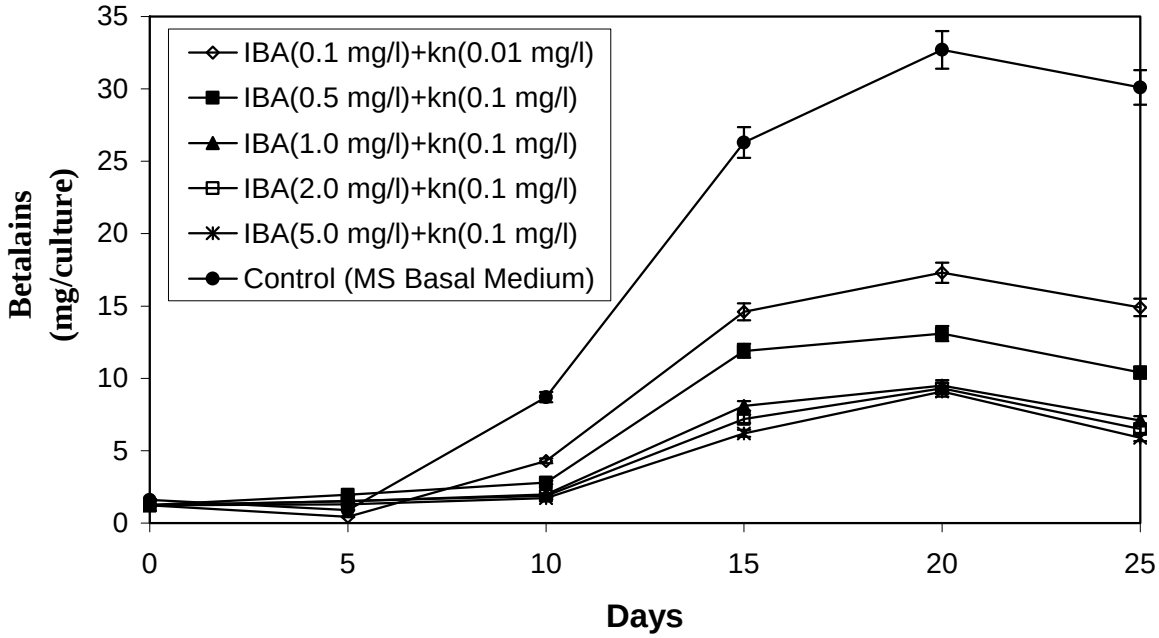

Figure 34. Effect of IBA + Kn on production of betalains in hairy root cultures of *Beta vulgaris* L.

In the individually tested growth regulators, generally growth of the hairy roots was inversely proportional to the concentration (Fig.35). NAA reduced the growth compared to IAA. Cultures provided with either NAA or IAA appeared to yield comparable levels of root biomass. Abscisic acid (ABA) provided cultures at all the levels especially 1 mg/l concentrations, produced the highest biomass (17.4 g FW/ culture), more than the control (MSB) (Fig. 35). Cultures incubated with ethaphon (Etphn) produced the least biomass yield (1.36 g FW/ culture) and the root cultures turned brown at later stages. The roots grown in BA, and Kn grew poorly. The increase and decrease in growth noticed in different growth regulators is due to the difference in profusion and number of lateral roots.

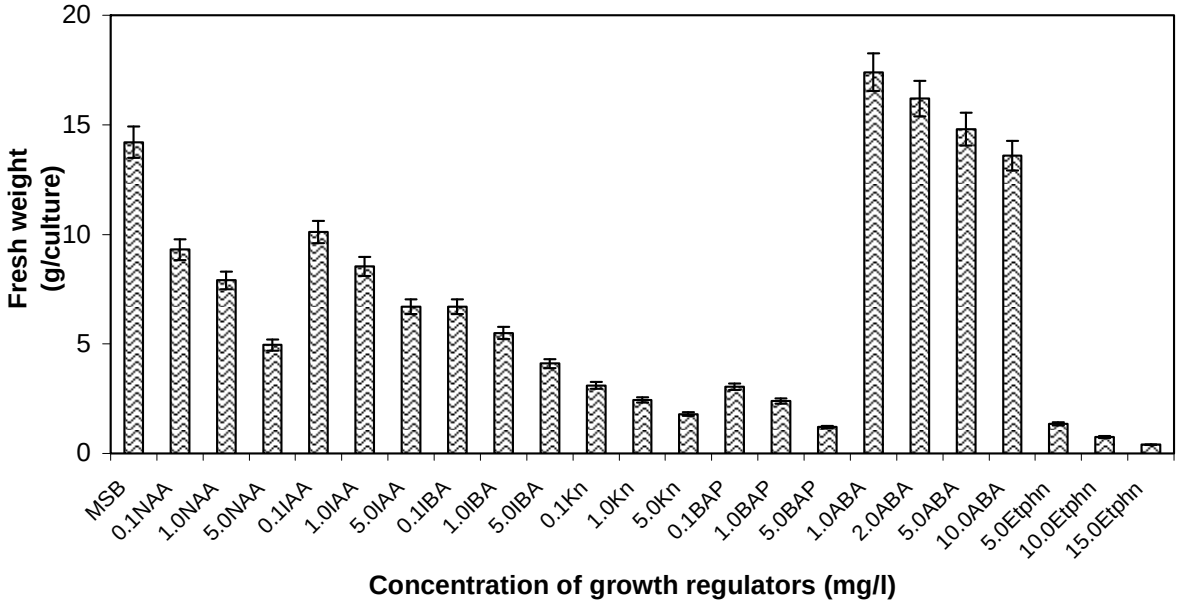

Figure 35. Effects of individual growth regulators on growth of *Beta vulgaris* L. hairy root cultures.

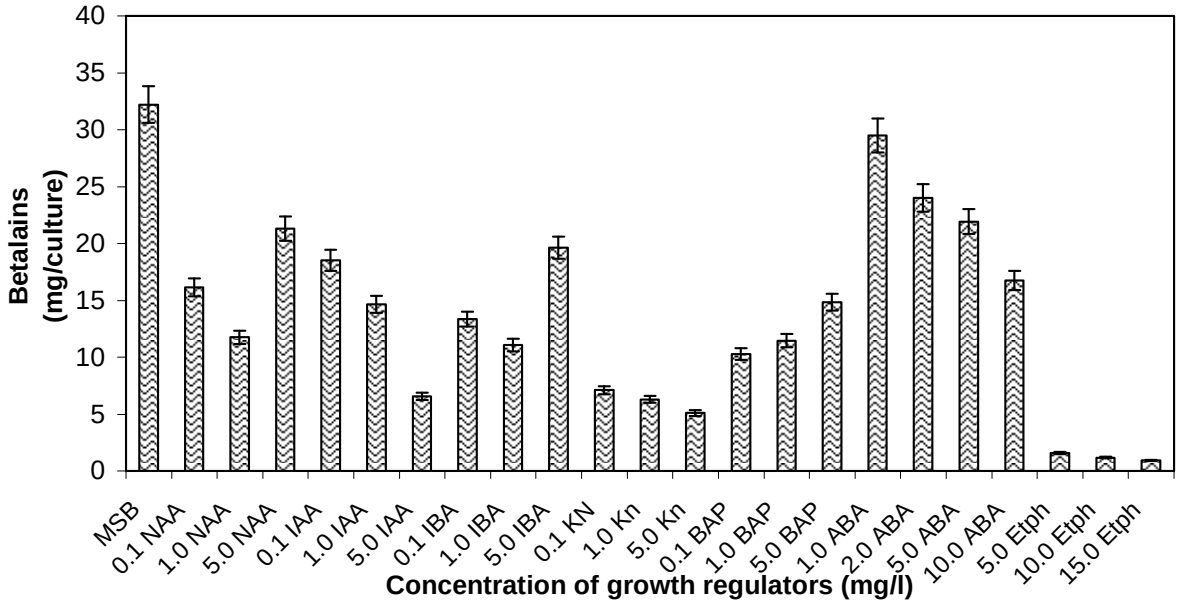

Figure 36. Effects of individual growth regulators on betalain production in *Beta vulgaris* L. hairy root cultures.

Of the seven categories of individual growth regulators tested shown in none of them produced betalains greater than control MS basal medium, but roots grown in medium with ABA produced the highest yield of betalains than all other growth regulators. It was observed that NAA and IBA at higher concentration produced more betalain than IAA. However between the cytokinins examined it was noticed that roots from all the concentrations of BAP produced more betalains. The cultures grow in ethaphon produced the least betalain production over all. This study was useful to investigate the individual effect these growth regulators have on growth and biosynthesis of betalain.

3.10. Effect of Gibberellin (GA_3) on hairy root cultures of *Beta vulgaris* L.

The effect of exogenous administration of GA_3 on beet hairy roots was studied. It was observed that treatment of GA_3, when fed at 1.0 mg to the media containing hairy root cultures of *Beta vulgaris* showed maximum biomass accumulation (19.31 g FW/ culture) on day 20, which is significantly more than control (14.27 g FW/culture). The treatment wherein GA_3 was administered at 5mg/l also showed an increase in biomass accumulation as compared to the control on day 20 (Fig.37), but the treatment of GA_3 at 10 mg/l showed an inhibitory effect on growth (Fig. 37). The betalain content increased with the increase is growth in 0.1-1.0 mg/l GA_3, treatment over the control on 20^{th} day and a maximum amount of (39.3 mg /culture) betalains recorded in 0.5 mg/l GA_3 treatment (Fig. 38). Exogenous administration of GA_3 influenced the branching pattern in hairy root cultures of *Beta vulgaris*.

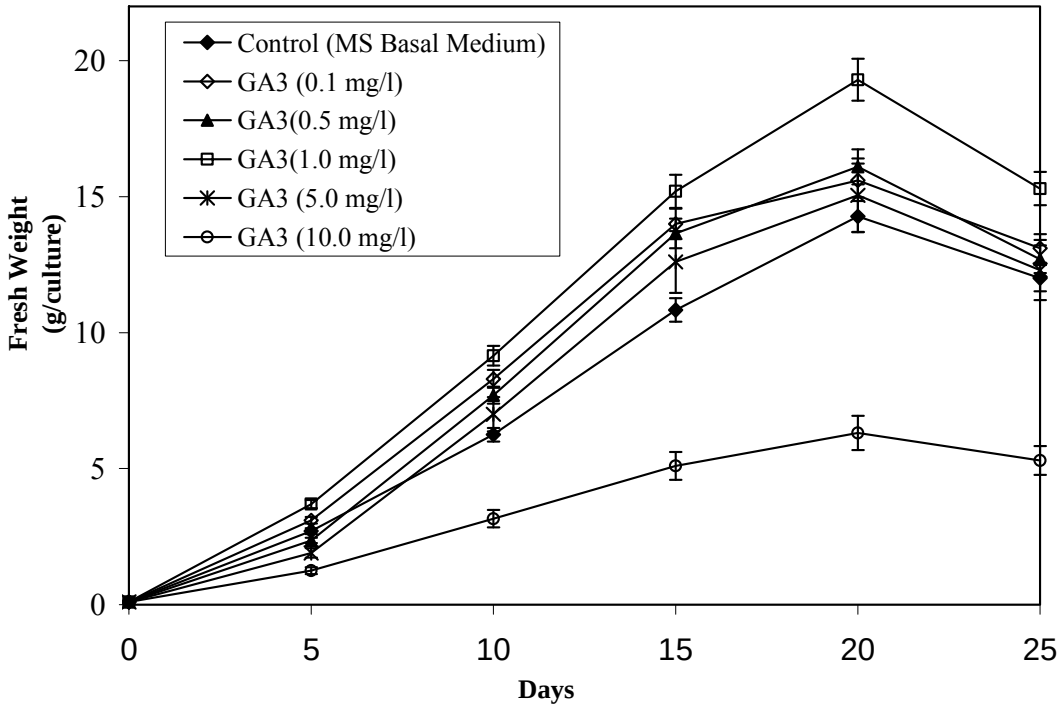

Figure 37. Effect of Gibberellic acid (GA_3) on growth of *Beta vulgaris* L. hairy root cultures.

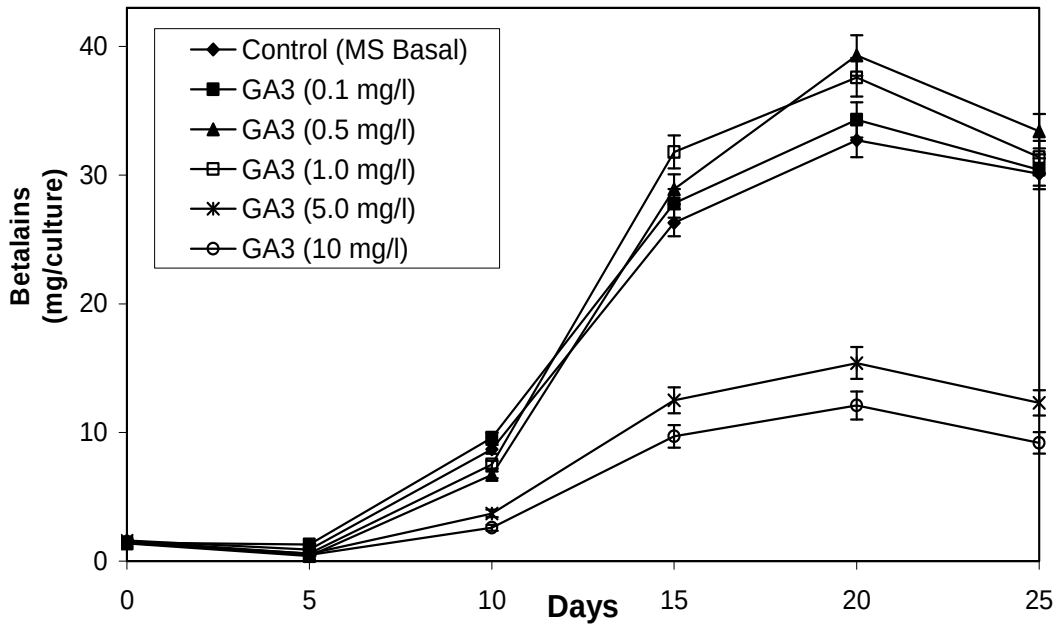

Figure 38. Effect of Gibberellic acid (GA_3) on betalain production in *Beta vulgaris* L. hairy root cultures.

3.11. Stability studies of betalains obtained from hairy root cultures of *Beta vulgaris* L.

Objective of the present study is to investigate the stability properties of betalains, as stability is a major constraint for natural colours to be used widely by food industries. Hence studies have been focused on the stability of beet hairy root pigments considering various parameters like different temperatures, pH levels, adding metal ions, and oxidants, water activity (a_w), which are responsible for lower stability of the betalains.

The influence of sugars and sugar alcohols, antioxidants ascorbic acid, isoascorbic acid, sodium ascorbate and sodium isoascorbate, citric acid and chelating agents such as sodium hexameta phosphate (SHMP), tetrasodium pyrophosphate (TSPP), and sodium acidpyro phosphate (SAPP) on stability of betalains were investigated.

3.11.1 Effect of temperature and pH on thermal stability of betalain concentrate

The thermal stability at different pH values (3, 4, 5, 6, and 7) was studied at 50^{o}C, 80^{o}C and 95^{o}C. It was observed that concentrate at 50^{o}C was stable compared to the other two temperatures. At pH 4, 5 and 6 less pigment degradation was found than pH 3 and 7. Highest stability was observed at pH 5, (40.6%) colour was remaining after one hour thermal treatment. At the same temperature in pH 3 and 7 about 19.8% and14.1% colour retained (Table 10). At 80^{o}C there was 80.5% colour loss at pH 4, 5 and 6 and at pH 3&7 about 90.9% and 94.8% colour was lost (Table11). Heating at 95^{o}C the degradation of pigment was higher and after one hour 7.8% colour retained at pH 5 (Table 12). The pH 4 and 5 influenced the stability of the betalain concentrate at all temperatures.

Table 10 . Effect of temperature (50°C) at different pH on stability of betalain concentrate (68. degree. Brix) from hairy root cultures of *Beta vulgaris* L.

pH	3.0	4.0	5.0	6.0	7.0
Time (min) 0	100%	100%	100%	100%	100%
5	83.3%	87.8%	91.5%	89.9%	82.3%
10	72.4%	75.1%	83.4%	75.4%	70.4%
15	52.1%	67.5%	71.2%	62.6%	50.1%
30	40.5%	48.7%	66.5%	55.1%	35.3%
45	31.3%	37.4%	51.1%	46.5%	23.7%
60	19.8%	31.2%	40.6%	39.3%	14.1%

The values represented are (%) remaining betalains, and the experiment was carried out in triplicate.

Table 11. Effect of temperature (80°C) at different pH on stability of betalain concentrate (68. degree. Brix) from hairy root cultures of *Beta vulgaris* L.

pH Time(min)	**3.0**	**4.0**	**5.0**	**6.0**	**7.0**
0	100%	100%	100%	100%	100%
5	71.3%	80.5%	86.2%	84.1%	70.4%
10	55.1%	67.1%	73.5%	64.9%	51.1%
15	42.6%	55.9%	59.8%	51.5%	36.7%
30	31.1%	36.1%	45.9%	40.2%	22.5%
45	24.7%	29.3%	32.1%	31.6%	10.4%
60	9.1%	13.2%	19.5%	18.5%	5.2%

The values represented are (%) remaining betalains, and the experiment was carried out in triplicate.

Table 12. Effect of temperature (95°C) at different pH on stability of betalain concentrate (68. degree. Brix) from hairy root cultures of *Beta vulgaris* L.

pH	3.0	4.0	5.0	6.0	7.0
Time (min) 0	100%	100%	100%	100%	100%
5	32.1%	68.2%	71.8%	65.5%	29.3%
10	21.7%	44.5%	53.5%	46.2%	15.4%
15	16.3%	27.9%	44.1%	32.5%	9.5%
30	7.1%	13.7%	31.9%	21.2%	2.1%
45	-	5.7%	20.5%	11.5%	-
60	-	-	7.8%	3.2%	-

The values represented are (%) remaining betalains, and the experiment was carried out in triplicate.

3.11.2 Effect of temperature and pH on betalains in aqueous solutions

Effect of temperature was studied at 30°C, 40°C, 50°C, 60°C, and 80°C, and pH 3, 4, 5,6, 7. It was noticed that at room temperature and pH 4, 5, and 6 the betalain remained quite stable (Fig. 40, 41 and 42), but at pH 3 and 7 degradation of colour was noticed after 6 hrs. At 40°C and pH 4, 5 and 6 the colour was stable up to 4 hrs and later a gradual degradation was noticed and at the end of 12 hrs 60% betalains were lost, at pH 3, and 7 degradation of colour was observed from the first hour and at the end 85% colour loss was recorded (Fig. 39 and 43). At 60°C gradual degradation of colour was observed from the first hour in all the pH and after 12 hrs 88% betalains were degraded. The degradation of betalains were drastic at 80°C from the first hour and it was complete with in 4 hrs at all the pH (Fig. 39, 40, 41, 42 and 43). It was found that pH 4 and 5 had a greater influence on the stability of the betalains at all temperatures.

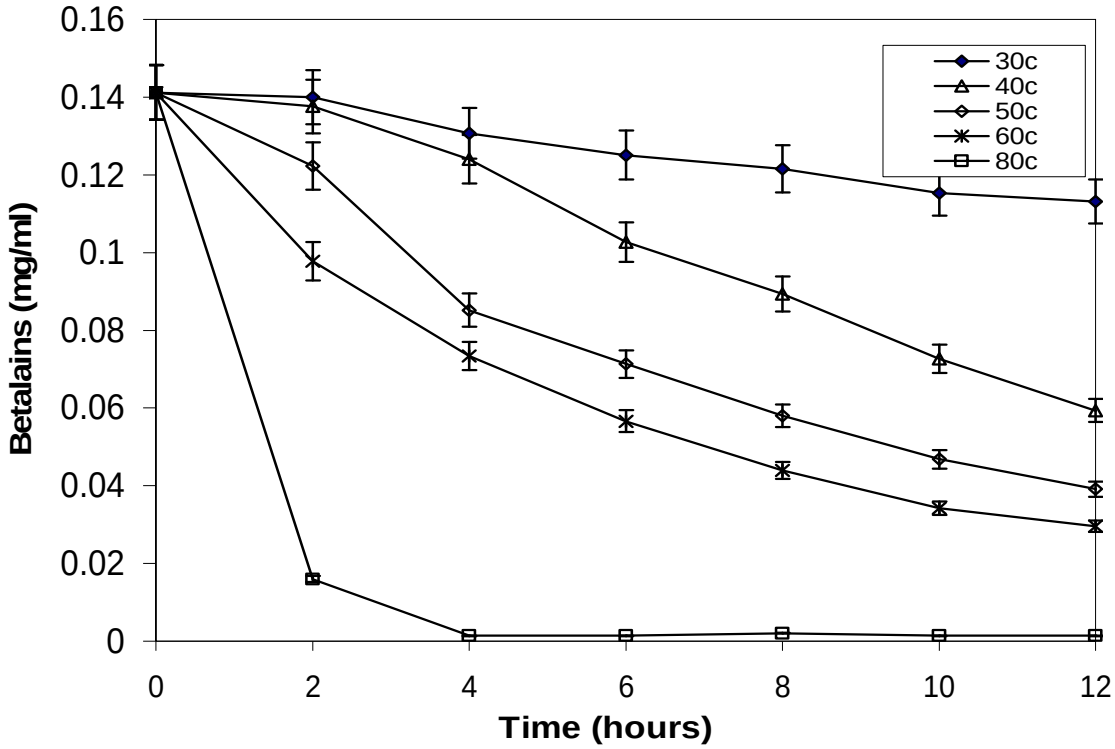

Figure 39. Thermal stability of Betalains at pH 3.0

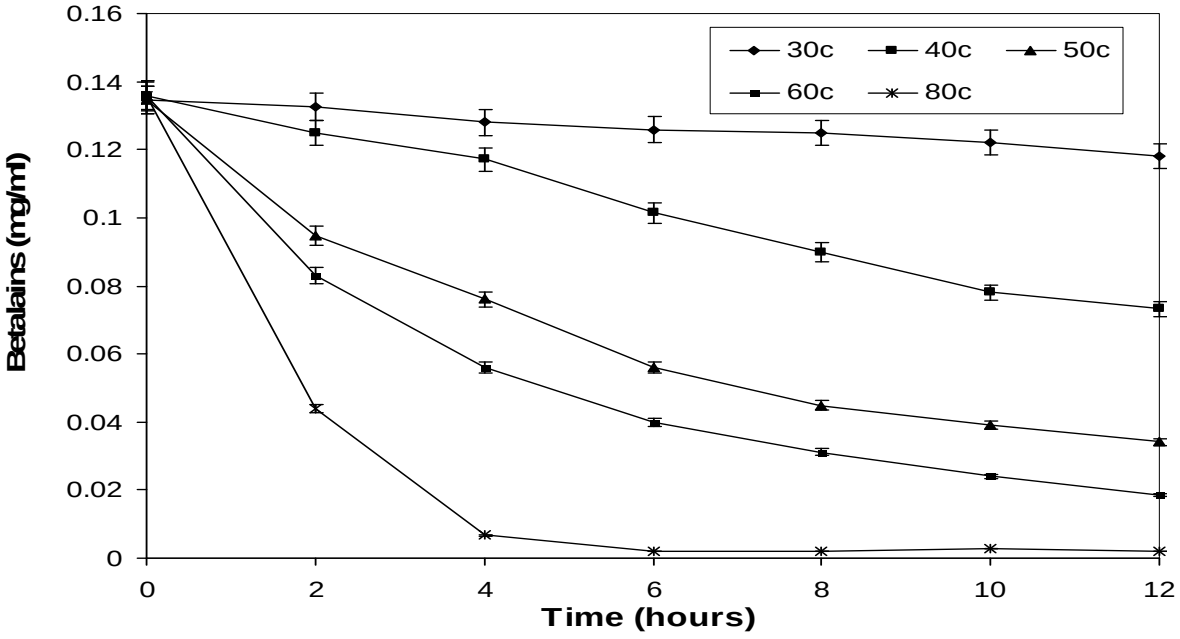

Figure 40. Thermal stability of Betalains at pH 4.0

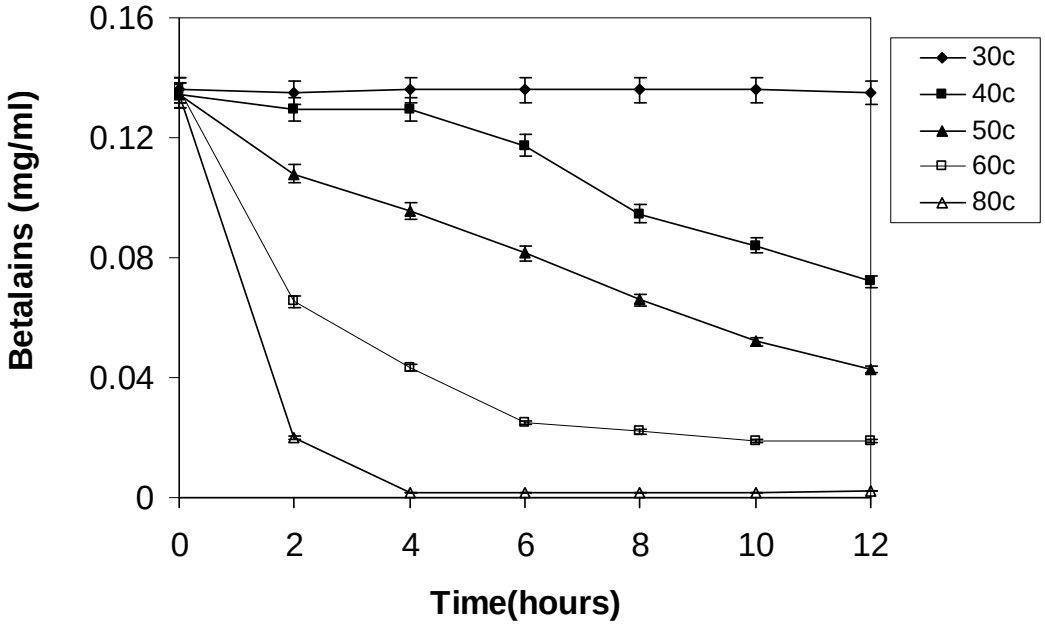

Figure 41. Thermal stability of Betalains at pH 5.0

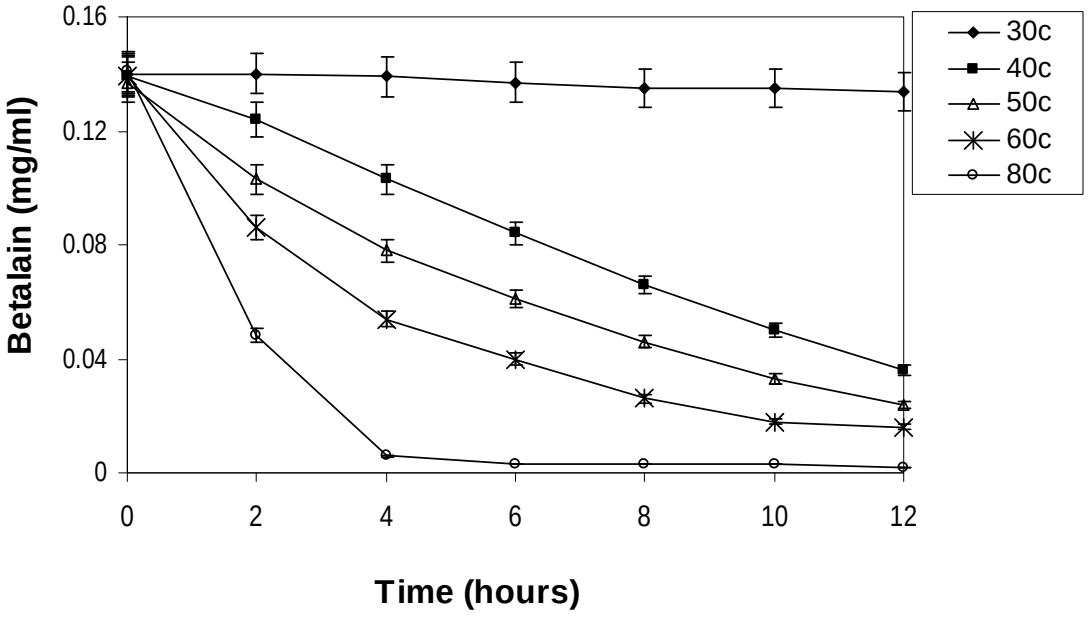

Figure 42. Thermal stability of Betalains at pH 6.0

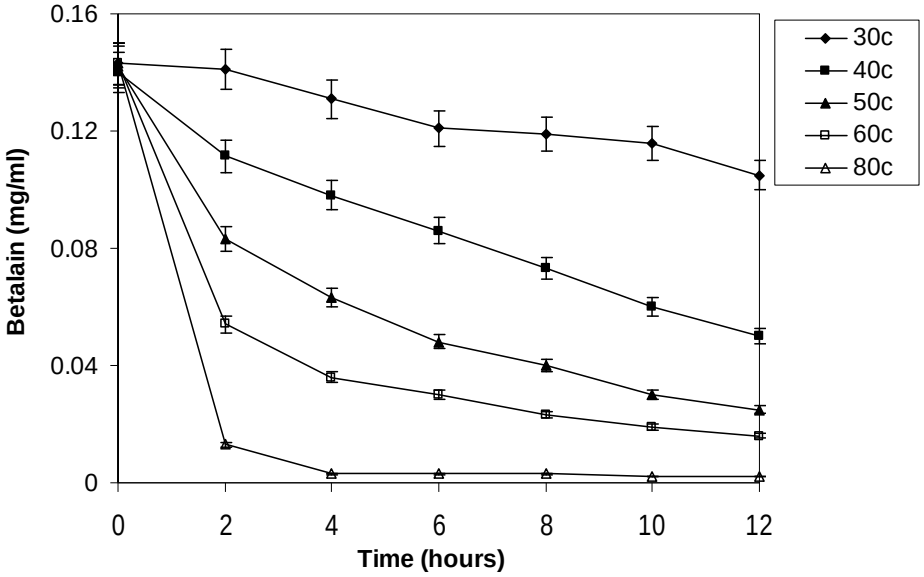

Figure 43. Thermal stability of Betalains at pH 7.0

3.11.3 Effect of metal ions on thermal stability of betalains

The effect of the following metals Cu^{2+}, Fe^{2+}, Fe^{3+} Mn^{2+}, Mg^{2+}, and Zn^{2+} at 0.3 mM level were studied at 50^{o}C at pH 5 for 6 hrs. It was observed that all the metals influenced the colour degradation to a large extent. In particular Mn^{2+} showed the strongest colour bleaching activity (94%). Cu^{2+}, Fe^{2+} and Fe^{3+} also facilitate the colour loss prominently (83, 82 and 80 % respectively), Zn^{2+} and Mg^{2+} were found to be comparatively less influential in degradation of betalains (48, 37 %) (Fig. 44).

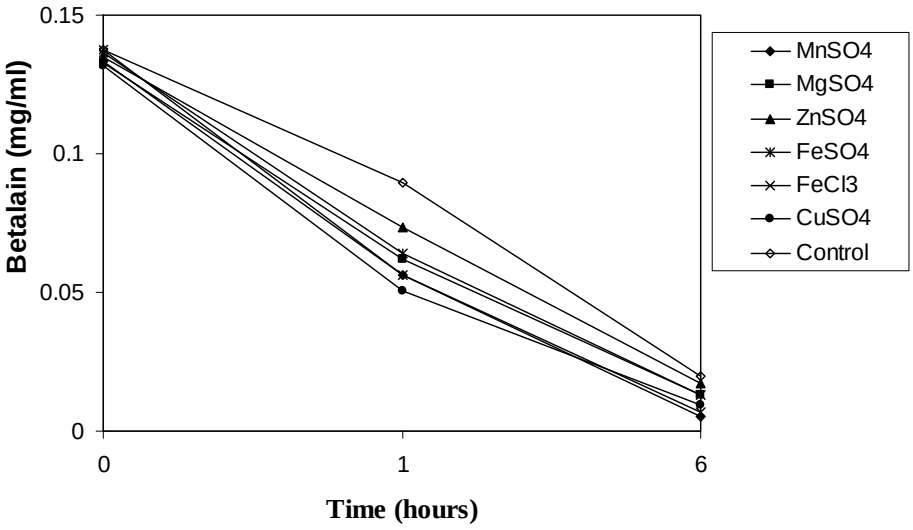

Figure 44. Effect of metal ions on stability of betalains at pH 5.0 at 50°C. (control: without metal ions)

3.11.6 Effect of water activity (a_w) on thermal stability of betalains from hairy root cultures

Water activity is very important in formulations with natural colours. Model systems with a water activity from 0.37 to 1.0 were prepared using water and glycerol and used for stability studies at 50°C for 3 hrs. It was observed that with the increase in water activity there was a gradual decrease in the colour. At 0.37 a_w from an initial absorbance of 1.72 after 3 hours absorbance of 1.32 was recorded, where as at a_w 1.0 the absorbance was 0.9. Hence a lower water activity helps the stability of betalains (Table.13).

Table. 13. Effect of water activity (a_w) on betalain stability (absorbance) at 50°C obtained from *Beta vulgaris* L. hairy root cultures.

Water activity (a_w)	0 hour	1.0 hour	2.0 hour	3.0 hour
1.00	1.64	1.35	1.09	0.99
0.95	1.56	1.33	1.09	0.98
0.87	1.60	1.36	1.13	1.05
0.74	1.58	1.37	1.14	1.07
0.63	1.61	1.38	1.17	1.10
0.47	1.64	1.42	1.25	1.20
0.37	1.72	1.51	1.35	1.32

The values represented are absorbance recorded at different hours.

3.11.5 Effect of polysaccharides and sugar alcohols as additives on thermal stability of betalains

The effects of fructose, starch, maltodextrin (mdx) and sugar alcohols, glycerol, polyethylene glycol (PEG) at 5mM each at 50°C at pH 5 were studied for 6 hrs. It was found that addition of both sugars and sugar alcohols acted as protectors against colour loss, the percentage of colour loss compared to control (without additives) was less and maltodextrin was found to be the best which show a retention of 32% of betalains and starch 23% followed by fructose 19% (Fig 45) compared to control where just 14% pigment was remaining. Polyethylene glycol protected the betalains at a higher rate by retaining 36%, which was the highest among sugars, and sugar alcohols, in glycerol 16%, (Fig. 46).

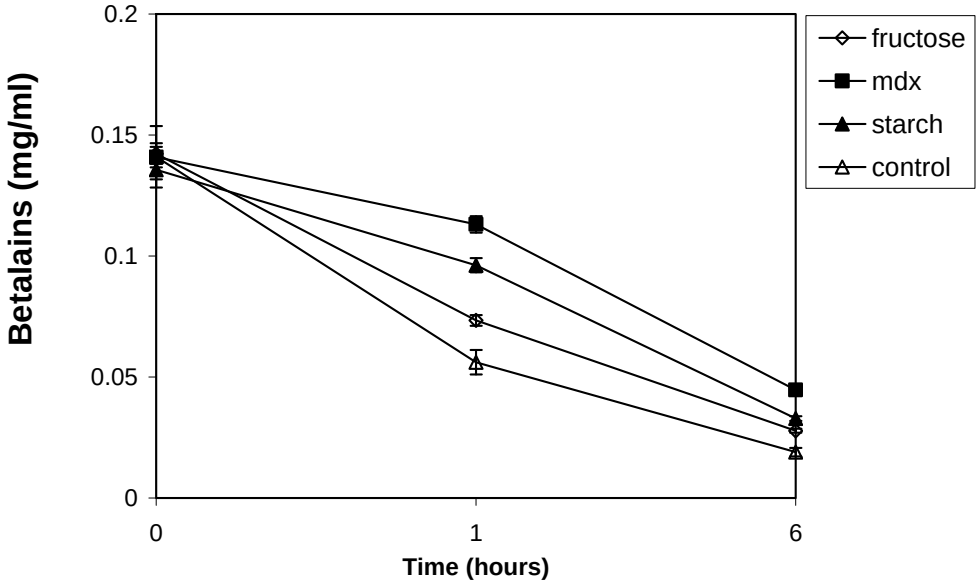

Figure 45. Effect of polysaccharides on thermal stability of betalains at pH 5.0 at 50°C.

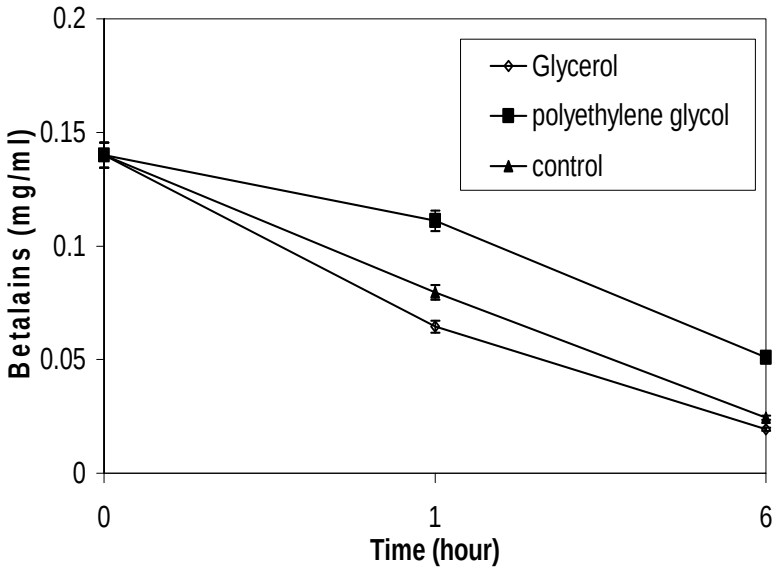

Figure 46. Effect of sugar alcohols on thermal stability of betalains at pH 5.0 at 50°C

3.11.6 Influence of ascorbic acid, isoascorbic acid, sodium ascorbate and sodium isoascorbate on stability of betalains obtained from hairy root cultures of *Beta vulgaris* L.

Influence of ascorbates at 0.1g w/v, was studied on stability of betalain concentrate at 93°C in pH 5.0. It was found that all the four ascorbates were effective in stabilizing the betalain concentrate. Sodium isoascorbate showed the highest stability retaining 57.5%

colour followed by sodium ascorbate 55.5%, isoascorbic acid and ascorbic acid 54.7% after 20 minutes heating where as 4.66% betalains remained in control (without additives). Hence ascorbic acid was found to be a good stabilizing agent for betalains (Table 14).

Table 14. **Effect of Ascorbates (0.1 g/100 ml) on thermal stability of betalain concentrate (pH 5.0) at 95°C obtained from beet hairy root cultures.**

Heating Time (min)	Control	Ascorbic acid (0.1 g)	Isoascorbic acid (0.1 g)	Sodium ascorbate (0.1 g)	Sodium isoascorbate (0.1 g)
0	100%	100%	100%	100%	100%
5	45.4%	91.5%	91.5%	92.5%	91.3%
10	23.2%	73.9%	73.9%	75.8%	74.5%
15	12.8%	65.1%	65.1%	64.1%	65.2%
20	4.66%	54.7%	54.7%	55.5%	57.5%

The values represented are (%) remaining betalains, and the experiment was carried out in triplicate.

3.11.7 Effect of citric acid on stability of betalains obtained from hairy root cultures of *Beta vulgaris* L.

Effect of citric acid at 0.1% w/v, on stability of betalain concentrate at 95°C in pH 5.0 was evaluated. After 20 minutes of thermal treatment it was observed that 35.7% retention of betalain which was higher than control without citric acid (4.66%). Citric acid was also found to influence the stability of betalains (Table 15).

Table 15. **Effect of Citric acid on thermal stability of betalain concentrate (pH 5.0) at 99°C obtained from hairy root cultures of *Beta vulgaris* L.**

Heating Time (min)	Control	0.1 g citric acid
0	100%	100%
5	45.4%	71.5%
10	23.2%	55.9%
15	12.8%	43.1%
20	4.66%	35.7%

The values represented are (%) remaining betalains, and the experiment was carried out in triplicate.

3.12 Effect of chelating agents sodiumhexametaphosphate (SHMP), tetrasodium pyrophosphate (TSPP) and sodiumacidpyrophosphate (SAPP) on stability of betalains from beet hairy root cultures

Influence of chelating agents were evaluated at 0.1% w/v at 95^{o}C, and it was found that SHMP was more influential in stabilizing betalains among the three by retaining 27.9% colour after 20 minutes of heating followed by TSPP 23.7% and SAPP 17.2% (Table 16).

Table 16. **Effect of sodiumhexametaphosphate (SHMP), tetrasodiumpyrophosphate (TSPP) and sodiumacidpyrophosphate (SAPP) on stability of betalain concentrate obtained from beet hairy root cultures at 95^{o}C and (pH 5.0)**

Heating time (min)	Control	SHMP	TSPP	SAPP
0	100%	100%	100%	100%
5	45.4%	78.3%	71.8%	65.1%
10	23.2%	56.7%	47.5%	51.4%
15	12.8%	39.1%	33.2%	24.3%
20	4.66%	27.9%	23.7%	17.2%

The values represented are (%) remaining betalains, and the experiment was carried out in triplicate.

3.12. Influence of iron and copper on antioxidant status in hairy root cultures of *Beta vulgaris* L.

The objective of this study was to obtain hairy root cultures of red beet rich in betalain and antioxidant properties for possible use as colourant and with functional attribute.

3.12.1. Superoxide Dismutase (SOD)

The effect of exposure to iron and copper on the antioxidant enzymes, superoxide dismutase, catalase and peroxidase was monitored over a period of 20 days to assess the changes in the antioxidant activities in relation to stress factor such as metal ions. The superoxide dismutase activity significantly increased on exposure of the hairy roots to both the metal ions at the first day and increased up to day five. The highest activity was found at 800 µM of iron (33.8 units /mg/ protein Fig.47) and 1.6 µM copper (40.2 units/mg protein Fig.48). The SOD activity was found to reduce gradually after five days and continued till 20th day.

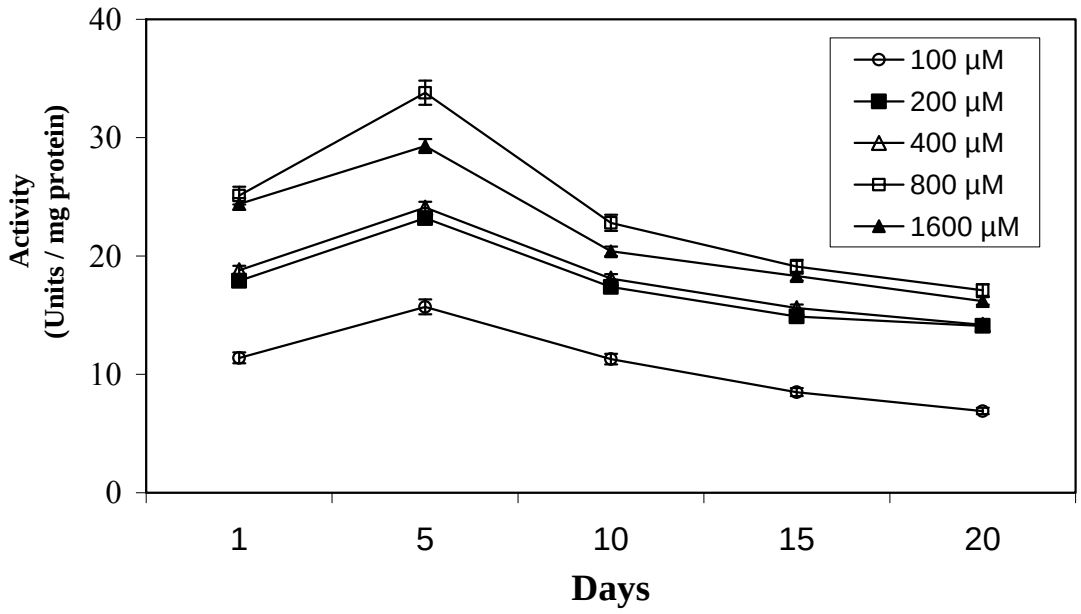

Figure 47. Effect of iron on superoxide dismutase activity in hairy roots of *Beta vulgaris* L.

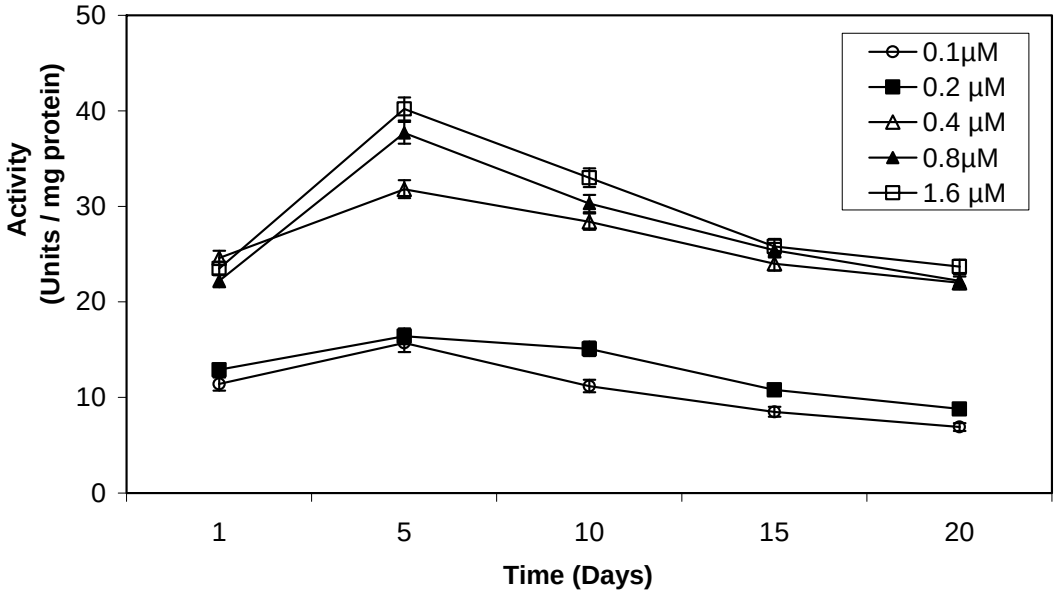

Figure 48. Effect of copper on superoxide dismutase activity in hairy roots of *Beta vulgaris* L.

3.12.2. Peroxidase

As shown in Fig.49 and 50. The peroxidase activity increased with increasing concentration of Fe and Cu. A gradual increase in the activity was noticed which continued till 20th day. Maximum activities were found at 400 µM iron (19.69 units/mg protein) and 0.4 µM copper (18.23 units/mg protein). The peroxidase activities were higher in both the metal ion treatments by 1.2 and 1.1 folds above the control respectively (Fig 49 and 50).

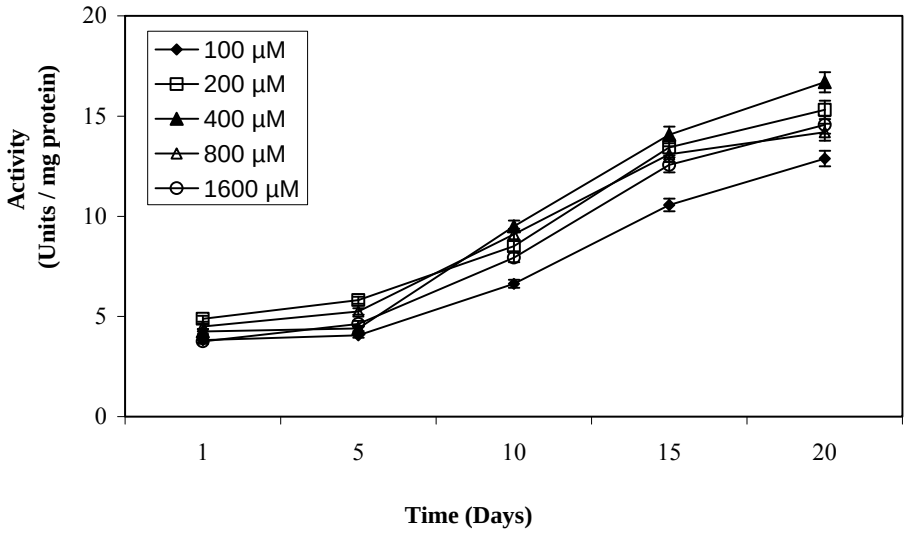

Figure 49. Effect of iron on peroxidase activity in hairy roots of *Beta vulgaris* L.

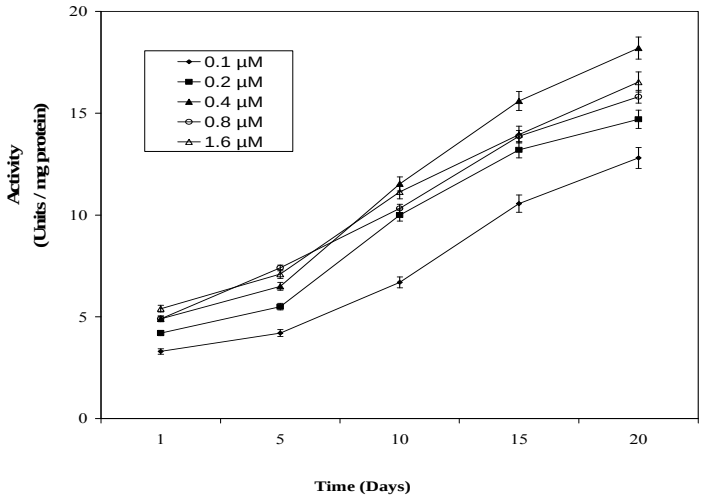

Figure 50. Effect of copper on peroxidase activity in hairy roots of *Beta vulgaris* L.

3.12.3. Catalase

Catalase activity did not show much enhancement, over the days but the maximum activity was found on the 5th day of culture gown in 800µM Fe (17.23 units/mg protein) and 0.4 µM Cu (21.75 units/mg protein). Catalase activity was higher by 1.5 and 1.2 folds over the control respectively (Fig 51 and 52). Decrease in catalase activity was observed in hairy root cultures after 5th day, but high enzyme activity was observed on all the days in hairy root cultures grown in 800 µM Fe and 0.4 µM Cu supplemented medium.

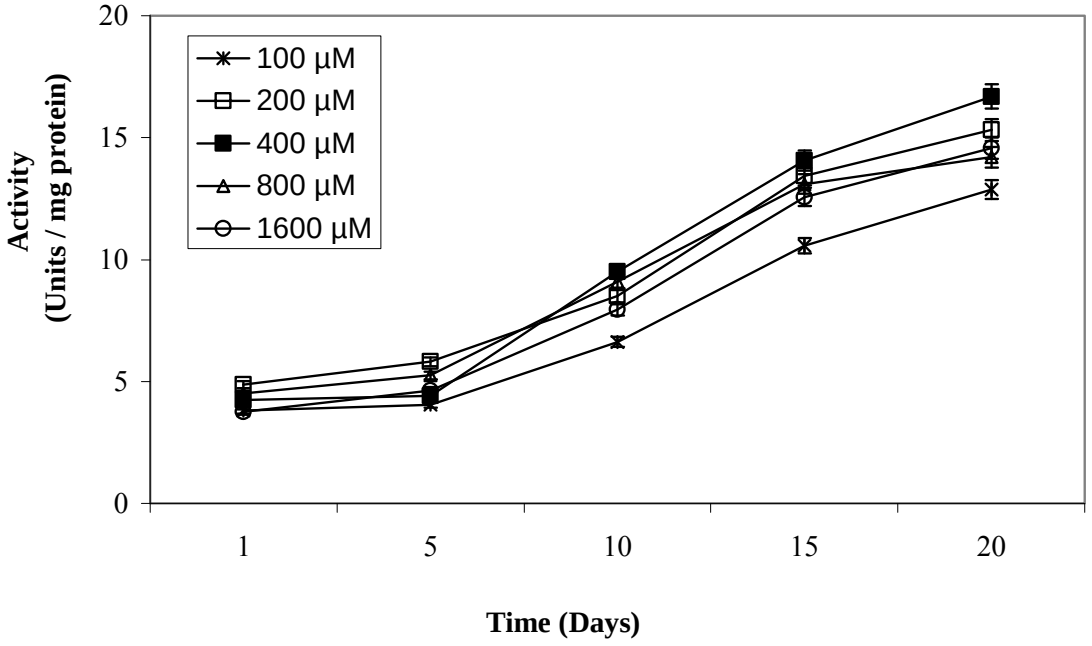

Figure 51. Effect of iron on catalase activity in hairy roots of *Beta vulgaris* L.

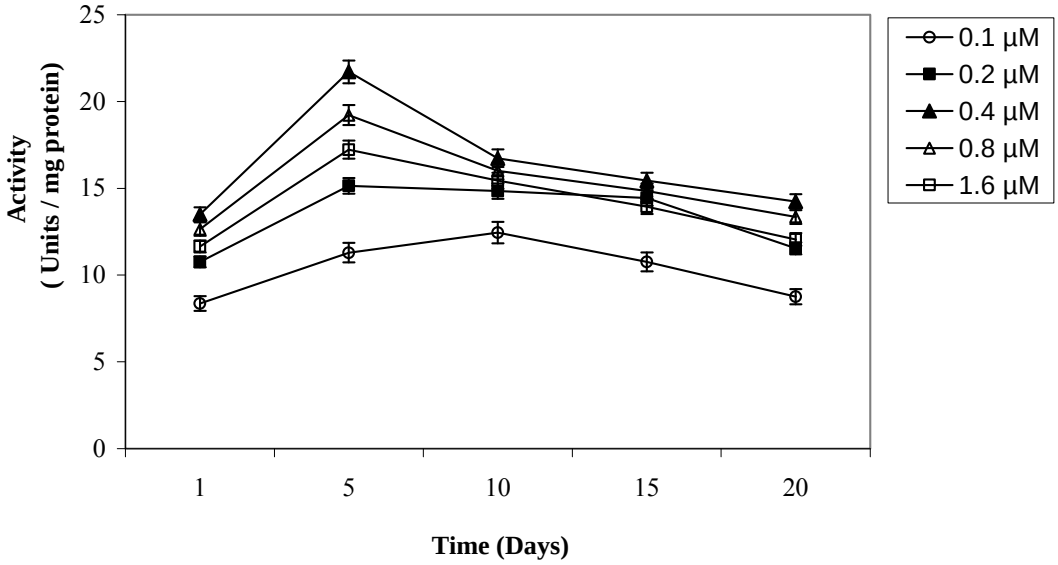

Figure 52. Effect of copper on catalase activity in hairy roots of *Beta vulgaris* L.

3.13 Safety assessment of betalains obtained from hairy root cultures of *Beta vulgaris* L. in experimental rat.

3.13.1 Acute toxicity studies in rats

3.13.1.1 Clinical signs and mortality

Rats administered betalain extract from Beet root and beet hairy roots did not develop any clinical signs of toxicity either immediately or during the post-treatment period even at the highest dose of 4 g/kg body weight. No alteration in food and water consumption was observed in any group. No mortality occurred in both sexes either immediate or during the 14 day observation period.

3.13.1.2 Food intake, body weight gain, organ weights and pathology

Oral administration of betalains did not cause any appreciable alterations in the feed intake during the 2 week period in both male (Table 17) and female rats (Table 18). Further, body weight gain during the observation period and the relative organ weights among the treated animals were comparable to controls. Animals of the BRE & HRE treatment group were active and similar to that of control group. Overall growth as measured by gain in body weight shows that in general male rats gained higher body weight when compared to females in all groups. Gain in weight ranged from 50-60 gram in male rats where as in females it was to the tune of 40-50 gram in all the groups. No significant difference was observed in the relative organ weight (g/100 body weight) of all groups in both sexes. Various tissues such as liver, lungs, kidneys and spleen of betalain

from beet root and beet hairy roots dosed animals showed no pathological alterations under light microscope.

Table 17. Acute dosing of Beet root extract (BRE) powder and Beet hairy root extract (HRE) powder to male rats and its effect on body weight and organ weights.

	Body weight (g)		Relative organ weight (g/100 g body weight)			
Dose (g/kg body weight)	Initial	Final	Liver	Lungs	Kidney	Spleen
Control	190.0±1.63	248.0±2.15	4.11±0.21	0.45±0.06	0.52±0.06	0.37±0.03
1.0 BRE	189.3±1.77	248.0±2.02	3.91±0.12	0.44±0.03	0.58±0.07	0.29±0.02
2.0 BRE	190.3±1.56	252.3±2.41	3.89±0.21	0.47±0.05	0.51±0.04	0.28±0.03
4.0 BRE	190.8±1.96	253.7±2.57	3.84±.20	0.46±0.03	0.53±0.06	0.39±0.04
1.0 HRE	188.0±2.41	258.0±2.74	3.93±0.09	0.45±0.03	0.53±0.04	0.33±0.03
2.0 HRE	190.5±1.89	258.2±1.96	4.09±0.15	0.46±0.03	0.49±0.04	0.28±0.06
4.0 HRE	188.8±2.06	247.8±3.97	3.80±0.14	0.46±0.03	0.52±0.05	0.31±0.05

BRE – Beet root extract, HRE – Hairy root extract. Each value is mean of 4 rats ±SD

Table 18. Acute dosing of Beet root extract (BRE) powder and Beet hairy root extract (HRE) powder to female rats and its effect on body weight and organ weights

	Body Weight (g)		Relative organ weight (g/100g body weight)			
Dose (g/kg body weight)	Initial	Final	Liver	Lungs	Kidney	Spleen
Control	157.3±1.29	186.3±2.28	4.03±0.14	0.57±0.03	0.62±0.03	0.27±0.03
1.0 BRE	152.2±1.89	187.7±2.98	4.26±0.22	0.62±0.03	0.68±0.07	0.23±0.02
2.0 BRE	152.0±2.36	194.3±3.01	4.17±0.33	0.56±0.06	0.66±0.04	0.26±0.06
4.0 BRE	149.4±1.86	189.0±2.76	4.21±0.22	0.56±0.06	0.60±0.05	0.26±0.06
1.0 HRE	152.2±1.73	189.8±3.22	4.20±0.33	0.59±0.02	0.58±0.06	0.24±0.04
2.0 HRE	149.0±1.91	197.5±3.20	4.25±0.12	0.61±0.03	0.61±0.03	0.26±0.03
4.0 HRE	154.2±1.88	194.7±3.43	3.86±0.21	0.57±0.04	0.64±0.04	0.25±0.04

BRE – Beet root extract, HRE – Hairy root extract. Each value is mean of 4 rats ±SD

3.13.2 Subchronic toxicity in rats

3.13.2.1 **Body weights and growth**

Betalain extract powder from beet root and beet hairy roots fed at 1 and 5 % did not cause any significant change in body weight gain in male rats. No deaths were observed in any of the groups during the 13 weeks of the experiment.

3.13.2.2 Food intake, and organ weights

In general incorporation of betalain extract powder even at 5 % level did not significantly affect the food intake in treatment groups. No meaningful difference in the actual food consumption was observed in the treatment groups throughout the 13 weeks experimental period (Table 19). As a result, growth of betalain fed rats was consistently similar to those of respective controls (Table 20). There were no marked differences in the group mean absolute or relative weights of various vital organs in the betalains treated rats compared to controls (Table 21).

Table 19: Weekly food intake pattern among male rats fed dietary Beet root extract (BRE) powder and Beet hairy root extract (HRE) powder for 90 days

Food intake (g/rat/wk)													
Group	1	2	3	4	5	6	7	8	9	10	11	12	13
Control	89.6 ±9.21	104.6 ±10.08	126.0 ±8.26	109.2 ±9.66	103.1 ±9.46	106.8 ±7.56	110.6 ±8.57	108.6 ±10.13	112.8 ±9.96	114.7 ±10.10	111.8 ±7.89	117.7 ±7.81	109.9 ±8.86
1% BRE	85.4 ±9.54	99.1 ±9.32	112.1 ±8.76	105.9 ±11.12	100.2 ±8.79	107.8 ±8.29	111.4 ±7.38	103.5 ±9.52	107.8 ±9.01	109.6 ±11.73	106.6 ±13.16	119.9 ±7.76	109.6 ±9.18
5% BRE	86.3 ±6.77	104.7 ±10.14	109.5 ±10.35	109.1 ±7.83	106.6 ±9.10	108.0 ±7.78	112.9 ±6.69	105.7 ±9.88	107.0 ±11.32	114.8 ±10.32	117.1 ±9.94	121.5 ±13.25	117.3 ±12.14
1% HRE	93.0 ±5.61	107.1 ±6.89	121.2 ±9,66	109.1 ±7.33	102.9 ±5.43	103.8 ±7.96	112.0 ±9.62	103.3 ±10.66	108.4 ±8.87	110.8 ±9.91	109.1 ±13.16	115.5 ±8.97	107.7 ±10.67
5% HRE	91.3 ±10.77	107.9 ±7.78	116.6 ±7.42	107.5 ±8.29	105.8 ±5.65	109.7 ±9.63	113.4 ±9.63	105.8 ±8.28	109.5 ±10.06	112.3 ±12.12	110.6 ±11.45	111.9 ±12.09	107.1 ±10.15

BRE – Beet root extract, HRE – Hairy root extract.Each value is mean of 10 rats ±SD. No significant difference (P≥ 0.05)

Table 20: Absolute weights of male rats fed dietary Beet root extract (BRE) powder and Beet hairy root extract (HRE) powder for 90 days

	WEEKS													
Group	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Control	102.4 ±2.11	134.4 ±9.39	157.2 ±11.57	205.2 ±7.78	224.6 ±8.43	243.7 ±8.63	267.1 ±9.14	281.5 ±6.98	299.7 ±7.76	302.1 ±8.93	313.3 ±8.45	335.8 ±11.39	345.2 ±11.00	354.8 ±10.17
1%BRE	101.5 ±4.45	130.8 ±7.69	167.1 ±12.74	194.4 ±9.95	216.1 ±7.72	233.5 ±7.05	255.7 ±8.49	270.5 ±7.74	285.5 ±9.19	298.5 ±9.36	305.3 ±9.16	315.4 ±10.42	327.7 ±10.18	343.5 ±11.26
5%BRE	101.5 ±1.90	131.0 ±6.44	165.6 ±10.67	191.3 ±10.04	212.0 ±6.98	235.1 ±8.33	249.4 ±8.06	266.5 ±8.77	282.7 ±8.19	299.4 ±9.12	312.3 ±9.38	325.6 ±9.63	333.7 ±9.91	342.4 ±10.53
1%HRE	102.6 ±4.00	135.2 ±6.05	175.8 ±9.46	204.3 ±10.12	229.8 ±8.79	250.7 ±7.96	267.1 ±9.15	278.8 ±8.67	293.6 ±6.92	308.0 ±9.16	320.4 ±8.86	333.9 ±10.16	341.6 ±11.04	350.3 ±10.38
5% RE	101.7 ±3,86	131.6 ±6.76	169.4 ±7.73	196.0 ±9.34	226.7 ±8.24	240.4 ±9.14	256.9 ±9.48	271.7 ±7.59	277.8 ±6.84	299.2 ±9.18	310.8 ±11.32	322.3 ±10.44	330.5 ±10.97	339.7 ±11.61

BRE – Beet root extract, HRE – Hairy root extract. Each value is mean of 10 rats ± SD. No significant difference (P≥ 0.05)

Table 21. Relative organ weights of male rats fed with dietary Beet root extract (BRE) powder and Beet hairy root extract (HRE) powder for 90 days

	Body weight	Relative weight (g/100 g weight)						
Group	Final	Liver	Kidney	Heart	Spleen	Adrenal	Brain	Testis
Control	354.8 ±10.17	3.46 ±0.21	0.58 ±0.02	0.28 ±0.02	0.19 ±0.02	0.019 ±0.005	0.49 ±0.02	0.80 ±0.06
1% BRE	343.5 ±11.26	3.45 ±0.36	0.57 ±0.07	0.29 ±0.03	0.18 ±0.03	0.020 ±0.003	0.50 ±0.05	0.85 ±0.04
5% BRE	342.4 ±10.53	3.39 ±0.33	0.58 ±0.05	0.29 ±0.02	0.19 ±0.05	0.019 ±0.002	0.50 ±0.03	0.87 ±0.03
1% HRE	350.3 ±10.38	3.32 ±0.35	0.55 ±0.04	0.28 ±0.05	0.20 ±0.02	0.021 ±0.006	0.50 ±0.02	0.85 ±0.07
5% HRE	339.7 ±11.61	3.40 ±0.29	0.56 ±0.05	0.27 ±0.03	0.18 ±0.04	0.019 ±0.004	0.50 ±0.07	0 .87 ±0.07

BRE – Beet root extract, HRE – Hairy root extract. Each value is mean of 10 rats ±SD No significant difference (P≥ 0.05)

3.13.3 Haematological profile

Feeding betalains even at 5% in the diet caused no significant alterations in Hb, RBC, WBC and differential count. The results of haematological examinations are summarized in Table 22. The haematological investigations revealed no changes.

Table 22. Haematological profile of male rats fed dietary Beet root extract (BRE) powder and Beet hairy root extract (HRE) powder for 90 days

					DC%					
Group	Hb (g/dl)	RBC (10^6µl)	WBC(µl)	PCV (%)	L	P	M	E	B	Blood picture
Control	14.15 ±0.83	10.14 ± 0.59	9325 ±1681	41.8 ±1.94	71	26	2	1	-	Normal
1% BRE	13.52 ±0.56	9.20 ±0.48	8916 ±1774	46.1 ±2.24	74	24	2	-	-	Normal
5% BRE	13.54 ±0.96	9.74 ±0.64	7708 ±1249	42.1 ±1.92	73	20	2	-	-	Normal
1% HRE	13.40 ±0.79	9.76 ±0.59	7508 ±1768	42.0 ±1.91	73	24	2	1	-	Normal
5% HRE	13.10 ±0.86	8.60 ±0.62	7625 ±1706	41.6 ±1.94	71	26	3	-	-	Normal

BRE – Beet root extract, HRE – Hairy root extract, Hb – hemoglobin, RBC – red blood cells, WBC – white blood cells, PCV – packed cell volume, DC – Differential counts, L – lymphocytes, P –polymorphs, M – monocytes, E – eosinophils,B basophils . Each value is mean of 6 rats ±SD. No significant difference (P≥ 0.05)

3.13.4 Histopathology

Gross examination did not reveal any abnormalities that could be ascribed to betalain feeding in rats. Further, on microscopic examination, no treatment related pathological lesions were evident in any of the vital organs irrespective of the betalain levels both from beet root and beet hairy roots.

3.13.5 Serum biochemical parameters

There were no marked alterations in any of the specific activities of enzymes, GPT, GOT, and ALP in betalain fed rats. A similar trend of result was observed with regard to the levels of serum constituents, proteins, cholesterol, and urea. Slight higher values in the serum protein values in the control and HRE groups did not differ when compared with other groups (Table 23).

Table 23. Serum enzymes and biochemical constituents of male rats fed dietary Beet root extract (BRE) powder and Beet hairy root extract (HRE) powder for 90 days

	IU/µl						
Group	GPT^{NS}	GOT^{NS}	ALP^{NS}	LDH^{NS}	Protein^{NS} (mg/ml)	Cholesterol^{NS} (mg/dl)	Urea ^{NS} (mg/ml)
Control	47.50 ±2.88	187.5 ±9.57	64.41 ±3.69	740 ±56.32	73.25 ±3.12	71.39 ±3.38	28.44 ±1.76
1% BRE	48.33 ±4.57	192.5 ±13.27	63.01 ±4.13	733 ±60.94	70.50 ±4.06	72.75 ±3.72	24.63 ±2.62
5% BRE	46.25 ±2.54	190.0 ±12.69	64.41 ±5.62	750 ±59.78	69.00 ±3.93	70.41 ±2.97	28.27 ±1.85
1% HRE	47.50 ±2.95	190.0 ±15.02	62.49 ±5.80	760 ±74.19	67.00 ±3.88	70.43 ±5.06	25.34 ±2.54
5% HRE	48.75 ±2.62	197.5 ±14.58	63.39 ±7.18	765 ±69.33	72.00 ±3.04	71.15 ±4.24	26.34 ±3.67

BRE – Beet root extract, HRE – Hairy root extract. Each value is mean of 6 rats ± SD

No significant difference (P≥ 0.05)

3.13.6 Hepatic enzymes and constituents

The specific activities of hepatic enzymes, GPT, GOT and ALP in the betalain fed rats were highly comparable to those of control (Table 24). Hepatic concentrations of proteins and cholesterol were in normal range in rats fed with betalain extract from beet root and beet hairy roots.

Table 24. Biochemical analysis in liver of rats fed with dietary Beet root extract (BRE) powder and Beet hairy root extract (HRE) powder for 90 days

Group	GPT^{NS}	GOT^{NS}	ALP^{NS}	Protein(mg/g)^{NS}	Cholesterol (mg/g^{NS}
Control	123.44 ±12.13	292.93 ±18.04	0.629 ±0.03	167.5 ±8.65	2.921 ±0.26
1% BRE	120.98 ±13.65	281.52 ±17.89	0.653 ±0.06	175.0 ±7.95	2.873 ±0.35
5% BRE	127.43 ±14.04	284.28 ±20.26	0.640 ±0.06	162.5 ±9.48	2.791 ±0.42
1% HRE	122.87 ±14.84	278.98 ±19.14	0.644 ±0.07	185.0 ±7.99	2.862 ±0.36
5% HRE	123.64 ±10.92	280.95 ±20.64	0.601 ±0.06	172.5 ±8.79	2.791 ±0.48

BRE – Beet root extract, HRE – Hairy root extract. Each value is mean of 6 rats ± SD No significant difference (P≥ 0.05).

3.14 Protocol standardization for micropropagation of red beet (*Beta vulgaris* L.) from seedling shoot tips.

The objective of this study was to develop a protocol for *in vitro* multiplication of *Beta vulgaris* plants, to obtain multiple shoots, rooting of the obtained multiple shoots and hardening of the rooted plants and successful field transplantation of the micropropagated plants.

3.14.1 Multiple shoots initiation

3.14.1.1 Effect of growth regulators on shoot multiplication

Multiple shoots were initiated from shoot tips of 15 days old seedlings after 2-3 weeks of culture. Of the different growth regulator combinations used for shoot multiplication treatment, of BA (2.0 mg/l) in combination with IBA (0.5 mg/l) resulted is an average of 9.4 ± 0.9 shoots per explant after three weeks of culture (Table 25;Fig. 53). Combination of BA (1.0 mg/l) with NAA (0.5 mg/l) also resulted in multiple shoots with an average of 7.3 ± 0.4 shoots per explant (Table 25). Treatment of BA with IAA, Kinetin (Kn) in combination with IAA or NAA also resulted in multiple shoots, but with lesser number of shoots than those of BA with IBA and NAA treatments. Treatment of BA with IBA resulted in better shoot length, followed by Kn and NAA compared to other treatments. Different levels of BA, Kn or 2-ip in combination with 2, 4-D (0.5 mg/l) resulted in nodular callusing without any shoot bud initiation.

Figure 53. Multiple shoots developed from shoot tip explants of *Beta vulgaris* L. MS medium supplemented with BA (2.0 mg/l) and IBA (0.5 mg/l).

Table 25. Multiple shoot initiation response of *Beta vulgaris* L. using various growth regulators.

Treatment (mg/l)	Multiple shoots	Shoot length (cms)
MSB	-	3.6±03
BAP (0.25) + IBA (0.1)	3.4 ± 0.5	3.7±0.9
BAP (0.5) + IBA (0.25)	4.1 ± 0.3	3.1±0.2
BAP (1.0) + IBA (0.5)	5.2 ± 1.3	2.0±0.5
BAP (2.0) + IBA (0.5)	9.4 ± 1.9	4.2±0.3
BAP (0.25) + NAA (0.1)	3.3 ± 0.5	3.6±0.4
BAP (0.5) + NAA (0.25)	4.7 ± 0.2	2.1±0.3
BAP (1.0) + NAA (0.5)	7.3 ± 0.4	2.7±0.5
BAP (2.0) + IAA (0.5)	5.2 ± 0.7	3.2±0.5
Kn (1.0) + IAA (0.5)	3.8 ± 0.5	2.8±0.3
Kn (2.0) + IAA (0.5)	4.3 ± 0.6	3.6±0.3
2-ip(1.0+IBA(0.2)	2.2 ± 0.2	3.1±0.5
2-ip(2.0)+NAA(0.5)	3.7 ± 0.6	2.5±0.2

mean ± SD (n = 10)

3.14.1.2 Effect of auxins on rooting of *in vitro* multiplied red beet shoots

Of the different auxins used for rooting of shoots *in vitro*, maximum number of roots were obtained in IBA (1.0 mg/l) treatment with an average of 17.2±1.1 roots per shoot (Table 26) as compared to 4.7±0.8 roots produced in MS based medium. NAA (0.5 mg/l) treatment also induced good rooting with an average of (13.6±1.2) roots per shoot (Fig 54, 55). Maximum root length was obtained in IAA with as average of (8.3±1.4 cm). NAA and IBA also induced rooting (Table 26). The roots formed in MS basal medium developed well with lateral branches and without callusing. In higher concentration auxin treatment roots were short and branching was less and callusing was noticed.

Table 26. Rooting response of *Beta vulgaris* L. to various auxins

Treatment	Number of roots	Root length (cms)
MSB	4.7±0.8	3.7±0.2
NAA(0.5)	13.6±1.2	5.3±0.7
IBA(0.5)	9.7±0.8	5.2±1.1
IBA(1.0)	17.2±2.1	6.3±0.5
IBA (2.0)	12.3±1.5	4.1±0.7
IAA(0.5	5.7±0.4	7.7±0.5
IAA(1.0)	8.6±0.7	7.3±0.3
IAA(2.0)	7.1±0.3	8.3±1.4

mean ± SD (n = 10)

Figure 54. *In vitro* rooting of shoot in *Beta vulgaris* L. Root induction on MS medium supplemented with IBA (1.0 mg/l).

Figure 55. *In vitro* rooted shoots of *Beta vulgaris* L. from seedling shoot tips

3. 14.2 Hardening, transplanting and field performance

The plantlets rooted *in vitro* were hardened in green house. The plantlets were kept for one week under polythene hoods at high relative humidity (RH 90%), later they were brought to a lower RH (60%) for further hardening for one week days. 85% plantlets survival was recorded during hardening. The hardened plants were transplanted in to field. The plants thrived well in the field at 81% survival rate.

3.15 In vitro multiplication of Beta vulgaris L. from shoot tips obtained from field grown high betalain yielding selected mature plants.

In field cultivated beet root a lot of variation in root size and pigment production was noticed. The objective of the present study was to obtain *in vitro* multiple shoots by selecting shoot tips of high betalain yielding mature plants to obtain a clone of high and uniform betalain producing plants and field evaluate the plants, to over come the problem of variability found in seed grown beet root.

Shoot tips excised from mature beet root ready to harvest plants yielding betalains were cultured on MS medium with several hormonal combinations. BAP (1.0 mg/l) + IBA (0.5 mg/l) produced highest number of multiple shoots (7.5 ± 1.3) shoots per shoot tip explant (Table 27; Fig. 56). Kn and NAA also resulted in multiple shoots formation (Kn 1.0 mg/l + NAA 0.5mg/l) and 5.8 ± 0.3 shoots. 2-ip and NAA also produced multiple shoots but at a lower rate 3.3 ± 0.1 shoots (Table 27). Cytokinins in combination with 2,4-D showed callusing without multiple shoot formation.

Table 27. Multiple shoot initiation response of *Beta vulgaris* L. from mature shoot tips in various growth regulators at different levels.

Treatment (mg/l)	Multiple Shoots	Shoot length (cms)
BA (0.5) + NAA (0.25)	3.3±0.8	2.1±0.3
BA (1.0)+NAA (0.5)	4.8±1.2	3.2±0.4
BA (2.0)+NAA (0.5)	5.2±0.5	2.1±0.2
BA (0.5)+IBA (0.25)	2.1±0.1	4.3±0.7
BA (1.0) + IBA (0.5)	7.5 ± 0.9	3.6±0.9
Kn (0.5) +NAA (0.25)	3.8 ±0.7	3.25±0.9
Kn (1.0) + NAA (0.5)	5.8±1.1	3.9±0.5
Kn (0.5) +IAA (0.25)	4.6±0.3	2.1±0.1
Kn (1.0)+IAA (0.5)	3.9±0.7	2.7±0.4
2-ip (0.5) +NAA (0.25)	2.7 ±0.3	1.8±0.3
2-ip (1.0) +NAA (0.5)	3.3±0.1	2.3±0.5

mean ± S.D (n = 10)

Figure 56. Multiple shoots developed from shoot tip explants of mature *Beta vulgaris* L. MS medium supplemented with BA (1.0 mg/l) and IBA (0.5 mg/l).

3.15.1 In vitro rooting of multiple shoots obtained from mature plants shoot tips

Shoots of length 3-5 cms that developed in multiplication medium were transferred to MS basal medium and MS with various auxins at different levels. Of the auxins used for rooting of shoots, maximum number of roots was obtained in NAA (1 mg/l) treatment with an average of 11.5 roots per shoot (Table 28; Fig 57). IAA (2.0 mg/l) treatment also induced good rooting with an average of 8.7 roots per shoot (Table 28). MS basal medium and IBA also induced rooting providing 3.2 and 5.4 roots per shoot. Maximum root length was obtained in IBA with an average of 5.9 cms. NAA at 0.5 and 1 mg/l level induced rooting with an average root length of 3.7 cm (Table 28). The roots were short and unbranched in higher auxin levels, and also callusing was observed at the shoot bases.

3.15.2 Hardening, transplantation and field performance

The plantlets rooted *in vitro* were hardened in green house. The plantlets were potted in plastic pots having mixture of soil and sand 1:1 and kept for a week under polyethylene hoods at high relative humidity (RH 90%). After a week, they were brought to a lower RH (RH 60%), for further hardening for another week. The hardened plants were transplanted to the field, plant, thrived well in the field at a survival rate of 85%.

Protocol for *in vitro* multiplication of *Beta vulgaris* plants obtained from matured explant and field cultivation is represented as a flow chart in Fig. 58. subsequently the comparison of seedling derived plants and tissue culture multiplied mature shoot explants (of selected high betalain yielding attribute) have been made.

Table 28. *In vitro* rooting response of *Beta vulgaris* L. in various auxins

Treatment (mg/l)	**No. of roots**	**Root length (cm)**
NAA (0.25)	7.8 ±0.85	2.5±0.31
NAA (0.5)	5.3±0.29	3.7 ±0.52
NAA (1.0)	4.5±0.35	3.9±0.41
IAA (0.2)	3.2±0.18	2.1±0.26
IAA (0.5)	5.1±0.79	4.5±0.71
IAA (1.0)	6.5 ±0.23	3.7±0.38
IAA (2.0)	8.7±0.13	1.9 ± 1.76
IBA (0.25)	2.9±0.38	2.5 ±0.59
IBA (0.5)	3.7 ±0.15	2.9±0.48
IBA (1.0)	5.4 ± 0.27	5.9 ±0.13
IBA (2.0)	3.5 ± 0.16	3.6 ±0.91
IBA (2.0)	3.2±0.19	4.5 ±0.37
MS (basal medium)	3..2±0.12	2.7±0.36

mean ± S.D (n = 10)

Figure 57. *In vitro* rooted shoots of *Beta vulgaris* L. obtained from mature shoot tips. Root induction on MS medium supplemented with IAA (2.0 mg/l).

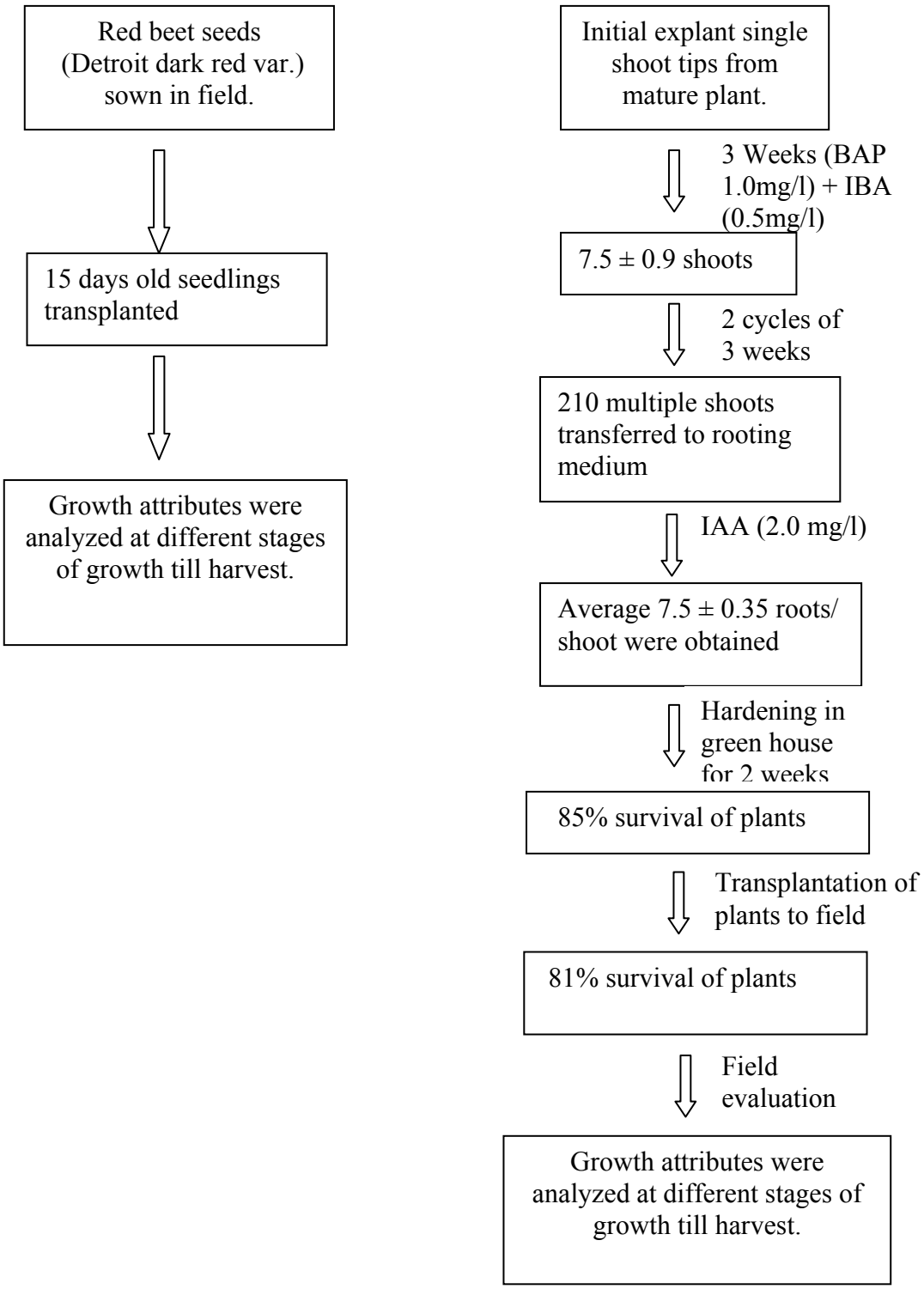

Figure 58. *In vitro* multiplication of *Beta vulgaris* L. shoot tips obtained from mature plants, and field evaluation of seedling and tissue culture obtained plants.

3. 15.3 Field evaluation and comparison of growth attributes of tissue cultured plants and seedling raised field plants of Beta vulgaris L.

a. Length of plant

The data on the length of plant for both tissue cultured plants obtained from shoot tips of mature plants and seedling raised plants recorded at different growth stages i.e. at transplantation, 15, 30, 45, 60 and 90 days after transplanting are presented in table 29. The length of the plant increased till 30 days after transplantation and remained constant till the harvest time. However tissue culture obtained plants showed uniformity and superiority over seedling raised plants (Table 27). The length of the plant increased till 45 days after transplantation and later it was constant till the harvest time of 90 days.

Table 29. Length of the plant (cm) of tissue culture and seedling raised *Beta vulgaris* L. plants at different stages of growth.

Type of Plants	Transplantation (0 day)	15 days	30 days	45 days	60days	90 days (Harvest)
T.C	8.75 ± 0.7	23.26 ± 1.3	61.36 ± 4.7	65.32 ± 5.4	65.51 ± 7.1	65.23 ± 5.3
S.P	5.35 ± 0.3	19.75 ± 0.9	45.25 ± 4.9	59.75 ± 4.9	59.6 ± 6.5	59.77 ± 6.2

T.C-Tissue cultured plants, S.P-Seed grown plants, mean ± SD (n = 10)

b. Length of the root

The data on length of root for tissue culture plants from shoot tips of mature plants and conventionally propagated plants recorded at various growth stages are presented in (Table 30). The length of root increased from transplantation stage to the final harvest stage. At transplanting stage the conventionally propagated plants showed more root length than tissue culture plants but later, 15 days after transplantation, the root length of tissue culture obtained plants was higher over seedling raised plants. Later from 45 days till harvest time no significant difference was found in the root length of tissue culture obtained plants and seedling raised plants.

Table 30. Length of root (cm) of tissue culture and seedling raised *Beta vulgaris* L. plants at various stages of growth.

Plant type	Transplantation (0 day)	15 days	30 days	45 days	60 days	90 days (final Harvest)
T.C	3.21 ± 0.2	5.31 ± 0.7	13.82 ± 1.4	15.23 ± 0.9	17.2 ± 1.5	18.96 ± 2.3
S.P	4.56 ± 0.2	7.74 ± 0.8	10.55 ± 1.1	13.82 ± 1.2	14.8 ± 1.7	17.51 ± 2.1

T.C-Tissue culture plants, S.P-Seed grown plants, mean ± S D (n = 10).

c. Fresh and dry weights of leaves

The data of fresh and dry weight of both tissue culture plants from shoot tips of mature plants and seedling raised plants at different stages of growth and are presented in (Table 31). The fresh weight of the leaves of tissue culture plants increased significantly as compared to seedling raised plants till harvest time after transplantation. The tissue culture obtained and seed grown plants in field are shown in Fig. 59 and 60.

Figure 59. Seed grown plants of *Beta valgaris* L. in field.

Figure 60. Tissue culture obtained plants *of Beta vulgaris* L. in field

Table 31. Fresh and dry weight of leaves (g) of tissue culture and seedling raised *Beta valgaris* L. plants at various stages of growth

Weight (g/plant)	Transplantation (0 day)	15 days	30 days	45 days	60 days	90 days (final Harvest)
FW (T.C)	3.30 ± 0.5	6.75 ± 0.7	139.3 ± 12.3	168.5 ± 10.5	185.5 ± 11.3	171.2 ± 12.4
DW (T.C)	0.28 ± 0.0	0.71 ± 0.09	12.85 ± 2.3	15.21 ± 3.6	17.31 ± 2.5	16.93 ± 2.7
FW (S.P)	2.10 ± 0.4	5.03 ± 0.6	125.1 ± 8.3	157.4 ± 7.2	174.1 ± 11.7	154.47 ± 11.9
DW (S.P)	0.25 ± 0.1	0.43 ± 0.05	11.47 ± 0.9	14.82 ± 1.5	16.45 ± 3.5	14.31 ± 1.7

FW- Fresh weight, DW- Dry weight. T.C- tissue culture plants, S.P-Seed grown plants, mean ± S D (n = 10)

d. Fresh and dry weight of whole plant

The fresh weight of whole *Beta vulgaris* L. plant increased consistently from transplantation stage up to the final harvest stage. Tissue cultured plants showed significant superiority over seedling raised plants (Table 32).

Table 32. Fresh and dry weight of whole plant (g) in tissue culture and seedling raised *Beta vulgaris* L. plants at various stages of growth

Weight (g/plant)	Transplantation (0 day)	15 days	30 days	45 days	60days	90 days (Final Harvest)
FW (T.C)	3.85 ± 0.2	7.93 ± 0.8	205.6 ± 10.2	415.5 ± 15.3	482.7 ± 17.5	526.9 ± 19.6
DW (T.C)	0.33 ± 0.01	0.69 ± 0.05	14.8 ± 1.3	37.2 ± 2.7	51.4 ± 3.9	55.8 ± 5.2
FW (S.P)	2.35 ± 0.3	6.55 ± 0.9	183.2 ± 9.7	389.7 ± 11.5	450.3 ± 13.8	494.0 ± 14.7
DW (S.P)	0.26 ± 0.04	0.66 ± 0.02	14.1 ± 2.5	34.1 ± 3.6	48.3 ± 3.7	41.0 ± 4.1

FW-fresh weight, DW -dry weight. T.C-Tissue culture plants, S.P-Seed grown plants, mean ± S D (n = 10)

e. Fresh weight and dry weight of root and betalain content.

The data of fresh and dry weight of roots of both tissue culture and seedling raised plants were recorded at different stages of growth and are presented in table 33 . Fresh weight of the root increased consistently after 15 day of transplantation till the harvest day. Difference in the root size ranging from 67.5 g to 406.5 g was observed in randomly selected 20 roots from individual seedling grown plants (Table 33; Fig. 61). In tissue culture obtained plants of mature shoot tips the root size was found to be uniform and the difference in weights was marginal (Table 33; Fig. 62).

Betalain content was evaluated in randomly selected 20 beet roots from both tissue culture and seed grown *Beta vulgaris* L plants. As reported earlier a lot of variation ranging from 9.5 mg/100g FW to 135.2 mg/100g FW was recorded (Table 33). In the plants obtained from *in vitro* multiplication of shoot tips of high betalain yielding mature plants the betalain content was fairly higher and uniform pigment content was noticed in the roots, it is between 122.5 mg/100 g FW to 138.2 mg/100g FW (Table 33).

Table 33. Fresh and dry weight of root biomass and betalain content in randomly selected beet root from seedling raised and micropropagated plants of shoot tips of mature *Beta vulgaris* plants.

Plant Sl. No.	Root biomass obtained in plants from seedlings (g)		Betalain content in roots from seedling raised plants (mg/100 g FW)	Root biomass obtained in plants multiplied *in vitro* through selected mature plants		Betalain content in roots from plants multiplied *in vitro* through selected mature plants (mg/100 g FW)
	Fresh weight	Dry Weight		Fresh weight	Dry weight	
1	175.3 cd	19.2 cd	9.5 d	345.3 ab	33.1 bc	135.3 bc
2	162.2 cd	17.2 cd	10.3 d	312.2 cd	29.7 de	129.5 cd
3	217.5 c	20.3 cd	17.5 d	320.8 c	33.5 bc	131.2 c
4	238.3 c	22.3 c	23.2 cd	350.7 ab	36.1 a	137.6 b
5	249.1 bc	23.5 c	36.5 cd	315.9 cd	30.2 d	138.2 b
6	275.6 b	28.6 bc	53.2 c	325.6 c	34.1 b	122.5 e
7	296.2 b	30.1 bc	61.5 bc	315.9 cd	30.7 d	125.9 de
8	197.6 cd	18.5 cd	76.3 bc	327.5 c	33.4 bc	131.6 cd
9	302.5 b	29.6 bc	103.7 b	357.1 a	33.9 b	135.7 bc
10	373.4 ab	37.2 ab	117.2 b	322.6 c	31.3 cd	130.2 cd
11	319.7 b	30.6 bc	135.2 ab	335.8 bc	30.5 d	133.5 c
12	238.3 c	23.1 c	96.3 b	312.6 cd	31.8 c	129.5 cd
13	214.7 c	20.6 cd	39.5 cd	306.7 d	29.5 de	126.2 d
14	364.8 ab	34.5 b	129.1 ab	333.5 c	32.9 bc	132.5 c
15	324.1 b	30.7 bc	17.5 d	351.8 a	36.2 a	130.7 cd
16	98.7 de	10.5 de	62.3 c	323.4 c	30.7 cd	142.1 a
17	406.5 a	41.2 a	106.9 ab	309.1 d	30.5 d	138.3 b
18	213.9 c	20.3 cd	83.5 bc	315.4 cd	29.9 d	135.7 bc
19	67.5 de	7.1 e	97.4 b	357.5 a	34.6 ab	122.5 e
20	312.6 b	32.2 b	82.5 bc	339.4 b	31.4 cd	128.3 d
mean	253.43	24.87	67.96	328.94	32.20	31.85
STDV	88.41	8.65	40.78	16.48	2.09	5.31

Means are separated by Dunkan's multiple range test.

Figure 61. Seed grown beet root plants showing variation in root size

Figure 62. Beet root plants obtained from tissue culture showing uniform root size.

CHAPTER -IV

DISCUSSION

4.1 Establishment of beet hairy root cultures and influence of different strains of *A. rhizogenes*

All three *Agrobacterium rhizogenes* strains exhibited significant differences in the ability of transformation, it was observed that LMG-150 gave the maximum % frequency infection as compared to other strains (Table 4). Several authors in various plant systems have also reported similar differences in induction of hairy roots using various strains of *A. rhizogenes* (Bais, 2000; Giri *et al.,* 2001; Lin *et al*., 2003; Thimmaraju, 2005). Further differences were also observed in hairy root morphology, growth and secondary metabolite production (Bias *et al.,* 2001; Lin *et al.,* 2003; Thimmaraju, 2005). Strains of *A. rhizogenes* are classified on the basis of their opine production (Rhodes *et al*., 1994). These mannopine strains are effective in root initiation and formation as they bear auxin-synthesizing genes (Filetici *et al*., 1987). The differences in virulence and morphology can be explained by the different plasmids harbored by the strains (Nguen *et al.,* 1992). They attribute the variation observed in secondary metabolite accumulation to the random T-DNA integration and the changes in the root genome (Abhyankar *et al.,* 2005). In some cases, it is reported that hairy roots obtained from single strain of *A. rhizogenes* induce changes in morphology and productivity (Mallol *et al.,* 2001). Hence the hairy roots derived from LMG-150, were selected over the roots derived from other two strains A20/83 and AZ/83 due to their faster growth, branching pattern and pigment production (Fig. 7 and 8). Hence LMG-150 strain was only used for infection in further studies on *Beta vulgaris* L. The systematic study using different strain of *A. rhizogenes* resulted in selection of better *A. rhizogenes* strain for induction and selection of better hairy root clone with respect to growth and morphological characteristics and betalain production.

4.2 Influence of different seedling explants, leaf cotyledon explants of different age and different varieties of *Beta vulgaris* L. on hairy root induction, growth and production of betalains

Among the different explants used, seedling cotyledon explants showed highest response in terms of transformation frequency and initiation of hairy roots at 96%, while other explants exhibited lower frequency of transformation (Table 5), and further age of the cotyledon explants also played an important role in induction of hairy roots. It was found that the cotyledon explants obtained from 11-14 days old seedling gave the highest transformation frequency *i.e.* 96% (Table 6). Several authors have observed differences in transformation frequency using various explants and explants at different age in *Beta vulgaris* and other plant species also (Dhakulkar *et al*., 2005; Thimmaraju, 2005). Zhao *et al*. (2004) has reported difference in transformation frequency between leaf blade and leaf petiole of *Saussurea medusa*. The difference in transformation frequency may be attributed to the susceptibility of the explants, which plays an important role in determining the frequency of hairy root induction.

Among the different varieties of *Beta vulgaris* L. it was observed that Detroit dark red variety was superior in growth, morphological characteristics and production of betalains. The roots were profusely branched, with many lateral branches. The growing tips were colourless and in active growing roots the pigmentation was mainly orange (this zone is from 0.5 cm to 1.0 cm long). The remaining parts of the roots were red, similar growth picture was observed by Hamil *et al*. (1986). The hairy roots grew intensively and maximum accumulated biomass on 20^{th} day was 14.6 g FW/culture in Detroit dark red and there was no significant difference in growth pattern between the varieties. At the end of the cultivation period the hairy root cultures had increased their mass over 20 times.

The productions of betalains in different varieties of *Beta vulgaris* are given in (Fig.12) The time course of betalain biosynthesis follows the growth. A maximum amount of betalains in all four varieties is accumulated in hairy roots on 20^{th} day of cultivation. Detroit dark red accumulated highest amount of betalains (32.7 mg/culture (Fig.12), which is several times higher than the other varieties. Similar studies have been carried out in *Beta*

vulgaris L. (Pavlov *et al*., 2002) in four different varieties. From this study the hairy root cultures obtained from Detroit dark red variety was selected as a prospective clone, which has stable morphological, growing and pigment biosynthesizing characteristics and, hence was used for further studies.

4.3 Influence of different medium constituents on growth and production of betalains in hairy root cultures of *Beta vulgaris* L.

In the present study the influence of nitrates phosphates, different basal medium and different pH on growth of hairy roots and betalain production was evaluated. It is well known that medium constituents exert notable effects on growth and metabolite formation by cultured plant cells (Fujita *et al.,* 1981a; Kino – Oka *et al.,* 1996). The concentration and type of inorganic nitrogen an important nutrient for plant cells, greatly affect the cell growth and secondary metabolism (Crawford and Glass, 1998). Nitrates at all the lower concentrations effected the growth, at ⅛th and ¼th level of nitrates growth inhibition was high (Fig 13). The production of betalains was not largely effected. Several authors have reported the effect of nitrates in various systems differently. Jacob and Malphatak (2005) have reported that increased levels of nitrates enhanced the growth but inhibited the production of solasodine in hairy root cultures of *Solanum khasianum*. Taya *et al.* (1994) has reported that decreasing the major nutrients in MS medium suppressed the growth of beet hairy roots but enhanced the betalain production. Doubling the nitrates slightly increased the growth of hairy roots (Fig. 13) while it had positive negative effect on accumulation of betalain (Fig.14).

Nitrogen in all its assimilable form is an essential element for the synthesis of several macromolecules, including DNA, RNA and proteins. Nitrates in addition of being an important macronutrient it also involved in signaling, plant growth and modulation of gene expression (Crawford and Glass, 1998; Stitt, 1992). Of the several nitrates and ammonium ratio tested, low ammonium: nitrate ratio favored the growth of hairy roots and production of betalains (Fig. 15 and 16). Wang and Tan (2002) have reported that a ratio of 50:10 nitrates to ammonium gave highest artemisinin productivity from *Artimisia annua* hairy roots. Ammonium alone inhibited the growth and betalain accumulation. In *Solanum*

khasianum (Jacob and Malphatak, 2005) reported that high nitrate: ammonium concentration increases the growth, while decrease solasodine production. Bensadek *et al*. (2001) have reported results similar to ours that reduced level of ammonium enhances the growth of *Atropa bellandona* hairy roots and production of alkaloids. Povlov *et al.* (2005) has reported that in beet hairy root cultures ammonium is completely consumed up to 3 days of culture at normal MS medium and the growth and betalain biosynthesis were correlated with ammonium ion limitation. Hence the reduction in growth at higher ammonium: nitrate ratio can be attributed to the toxic effect of ammonium ions remaining in the medium, which leads to acidification of the medium. Ammonium salts strongly inhibited the growth of *Buplerum falcatum* L. roots (Yamamoto and Kamura, 1997). Ammonium is very diffusive and easily accumulates in the tissues and becomes toxic if not metabolized, when ammonium concentration is low in the medium, most of the accumulated ammonium is metabolized by the cells, while it is high the remaining unmetabolized ammonium has inhibitory effect on the cell metabolism. Huang and Chan, (2006) have reported that an adequate quantity of NH^{4+} is necessary to enhance the growth and production of secondary metabolite in bioreactor studies of *Stizolobium hassjoo* hairy root cultures.

Phosphates at all the lower levels enhanced the production of betalains. At $\frac{1}{8}^{th}$ concentration (42.3 mg/ culture) betalains were recorded on 16^{th} day of culture, while the growth of hairy roots was not largely affected. Taya *et al.* (1994) similarly has reported an increase in betalain accumulation in beet hairy root at lower levels of phosphates and also in phosphate free MS medium. Jacob and Malpathak (2005) have reported that a low concentration of phosphates increase solasodine production in the stationary phase of *Solanum khasianum* culture.

Povlov and Bley (2006) have found that phosphate were consumed intensively and reached to 10-15mg in normal MS basal medium grown cultures and the concentration of phosphate remained constant till the end of cultivation. The relationship between phosphate uptake and growth and biosynthesis of betalain varies with different hairy root cultures.

4.4 Effect of micronutrients on growth and production of betalains in *Beat vulgaris* hairy roots

The effects on the production of betalains and hairy root growth of *Beta vulgaris* at the increased concentration of the microelements tested are given in (Table. 9) All the micronutrients except Cu^{2+} and Zn^{2+} had a positive effect on growth and betalains production. Co^{2+} was found to be the best among the all micronutrients in which a maximum amount 45.7 mg/culture betalains production was recorded (Table 9). The manipulation of the concentration of microelements in the culture media represents a strategy to increase the production of secondary metabolites in plant cell cultures (Jimenez Aparicio and Gutierrez – Lopez, 1998). Trace elements have been considered as abiotic elicitors or inducing factors (Survanalatha *et al.,* 1994), which trigger the formation of secondary metabolites (Verpoorte *et al.,* 1999). There are reports showing the effect of divalent ions on the production of secondary metabolites. Cu^{2+} and Cd^{2+} have a positive effect on alkaloid production *Catharanthus roseus*. Fe^{2+} (Mizukami *et al.,* 1977) and Cu^{2+} (Fujita *et al.,* 1981a) on the synthesis of shikonin and Mn^{2+} on the production of secondary metabolites (Fujita *et al.,* 1981b, Sri Andrijany *et al.,* 1999). Nevertheless their mechanism of action is not yet well understood. There are few reports showing the effect of addition of microelements on the production of betalains. Endres (1976) reported a negative effect on increase of Cu^{2+} on the accumulation of betacyanins in callus of *Protulaca grandiflora* and *Amaranthus* seedlings respectively, which is similar to our finding in beet hairy root cultures.

Savitha *et al.*, (2006) have reported inhibitory affect of Co^{2+} in *Beta vulgaris* L. at 10, 20 and 30 fold higher concentrations than MS medium, but in our study we found that a five fold increase in Co^{2+} concentration the growth and production of betalains enhanced enormously and at 10 and 20 folds it had no effect (Fig .19), this may be due to the difference in the hairy root clones. However, our findings agree with the effects reported for suspension culture of *Daucus carota* (Suvarnalata *et al.,* 1994). In that study an increase of 63.41% in the production of anthocyanins was achieved by the addition of Co^{2+}. Also a 6-fold increase of sapogenin steroid of *Agave americansis* was observed after the addition of 2 μM Co^{2+} (Sri Andrijany *et al.,* 1999). The use of microelements as stress factors or elicitors has induced the excretion of intracellular metabolites such as alkaloids, since they possibly

modified the plasma membrane permeability (Huang *et al.,* 1995). However, in our study it was not observed that increasing trace metal concentration induced the excretion of betalains of *B. vulgaris* hairy root cultures. Huang *et al.,* (1995) reported that 2.5 μm Co^{2+} stimulated 25 times the synthesis of L-DOPA by *Stizolobium harsjoo*. It was suggested that this increment in L-DOPA was due to a positive effect of ion on the activity of the enzyme phenol oxidase, since this divalent ion acts on prosthetic group. This enzyme is also related to the biosynthesis of betalains (Hinz *et al.,* 1997). Since the rate betacyanins to betaxanthins did not change, a possible effect of Co^{2+} should be exerted at the first steps of the metabolic pathway of betalains, where phenol oxidase participates. It remains unclear if the beneficial effects observed are due to the activation of certain enzymes or the hairy root response to the stress condition.

The growth and betalain content of hairy root cultures grown in the medium at higher concentrations of Zn^{2+} and without Zn^{2+} are shown in (Fig 20). In the absence of Zn, the betalain content increased to maximum of 40.5 mg /culture. The removal of zinc from the medium promoted betalain production. The increase in growth of hairy roots in the medium without zinc was slower than that in the medium with zinc. Akita *et al*., (2001) in beet root cell cultures, have reported results similar to our observation. Results show that zinc inhibited the betalain production although it was required for the growth of beet hairy roots. In conclusion, zinc deficiency (Fig. 20) enhanced the betalain production in hairy root cultures.

4.5 Influence of different basal medium on growth and production of betalains

MS and B5 basal media are the most widely used nutrient medium for hairy root cultures however choice of the culture medium is dependent on type of the hairy root cultures (Kim *et al*., 2002). Of the four media tested, MS was found to be the best for both growth and production of betalains producing 14.2 g FW/culture hairy roots and 32.7 mg/culture betalains. The growth of hairy roots was two fold higher than Nitsch (N6) medium (Nitsch and Nitsch 1969) (Fig. 21). Jin *et al.,* (2005) has similarly reported that MS medium was the best for growth of sesame hairy roots and production of recombinant fungal playtase. In *Saussurea medusa* hairy roots Nitsch (N6) medium was found to be influential

for growth and production of janceoidin (Zhao *et al.,* 2004). Dhakulkar *et al.,* (2005) has reported that B5 medium is effective for growth and production of verbascoside in hairy not culture of *Gmelina arboea* Roxb. Jacob and Malphatak (2005) have reported that in *Solanum khasianum* increased growth of hairy roots in B5 medium while solasodine production was high in MS Media. Hence the growth and production of secondary metabolite in various media depended on the plant species and in this case may be due to the specific nature of hairy root clone.

4.6 Influence of pH on growth and production of betalains in beet hairy root cultures

In our study we found that beet hairy root cultures were sensitive to pH.The growth and production of betalains correlated with the pH of the medium. A maximum production of 14.2 g FW/culture hairy root biomass and 32.7 mg/culture betalains were recorded (Fig.23). Both growth and production of betalains were supportive in the pH range 5-6. Bais (2000) has also reported the influence of pH on growth and production of phytochemicals in *Cichorium intybus* similar to our results. In *Polygonum tinctorium,* Young *et al.* (2000) has reported that hairy roots growth and indigo production was not effected at a range of 5 to 7, while it was inhibited at pH 4 and below which is similar to our finding in beet hairy root cultures where at lower pH there was disintegration, leaching of pigments leading to death of cultures. Merkil *et al.* (1997) has reported that pH range (5-5.9) did not effect the growth but had a pronounced effect on diosgenin production in hairy not cultures of *Trigonella foenum-graecum* L. It was noticed that when the pH is too low, levels of aluminum and hydrogen in the medium are toxic to the root systems of ginseng and if the pH is too high, micronutrients might be bound in forms unavailable for hairy root uptake (Sivakumar *et al.,* 2005). From this study it is evident that medium pH plays an important role in the growth and production of secondary metabolites, and was found that pH 5.8 is the best for beet hairy root cultures in all aspects (Fig. 23 and 24).

4.7 Influence of carbon source on hairy root growth and production of betalains

The sucrose concentration clearly affected both the growth and production of betalains in hairy root cultures of *Beta vulgaris* L. (Fig. 25 and 26) Optimal growth was obtained between 30 and 70g/l. It has been established that sucrose is energetically the most

suitable carbon source for the accumulation of *in vitro* plant cultures, particularly for the biosynthesis of secondary metabolites, but plant cells do differ however in the sequence in which they consume the inversion products of sucrose (Su, 1995; Ileva and Pavlov, 1997). The importance of sugars in controlling root processes is provided by reports that sugars help in regulating the expression of a significant number of plant root genes (Koch, 1996). Sucrose above 5% was found to be inhibitory for growth and production of betalains. A similar inhibitory affect of high sucrose concentration were reported on growth and pigment production in red beet hairy root cultures (Bhagyalakshmi *et al*., 2004) and cell suspensions of *Beta vulgaris* (Rodniguez *et al*., 1994), *Phytolacca americana* (Sakuta *et al*., 1987**)** and *Chenopodium rubrum* (Berlin *et al*., 1986). In this study it was also clear from reduced growth and betalain production in glucose + fructose (Fig.27 and 28), in comparison to sucrose fed cultures. Similar observation has been reported by Bhagyalakshmi *et al*., (2004) in *Beta vulgaris* hairy root cultures and seedlings. Rapid decline in sucrose in the medium was observed while no change in glucose and fructose levels in the medium was found one possible interpretation might be that external sucrose is presumably taken to the cell surface before it is hydrolyzed by extra cellular invertase (Shin *et al*., 2003). Sugar is probably degraded in the cell wall by released invertase, which has highest activity, and highest affinity for sucrose and the products of degradation are finally transported to the cytoplasm as hexoses (Ashira *et al.,* 1988**,** Masuda *et al.,* 1988**,** Van den Ende and Van Lacre 1995).

4.8 Influence of growth regulators on growth and production of betalains

The levels of growth regulators had influence on growth of hairy roots and increasing concentrations was inhibitory (Fig. 29, 30 and 31). Our result is consistent with the earlier reports of Weathers *et al.,* (2005) in *Artemisia annua*, Bais *et al.,* (2001) in *Cichorium intybus.* Robins *et al.,* (1996) and Saverweim *et al.,* (1992) have reported that the growth of the hairy roots is inversely proportional to the concentration of the hormone. Whereas Lin *et al.,* (2003) demonstrated that auxin had little influence on hairy root cultures of *Linum flavum*. Considering the results the transformation process with *Agrobacterium rhizogenes* results in clones that are sensitive to auxin or auxin over producers (Aroo *et al.,* 1995; Giri and Narasu, 2000). Although IAA and NAA yielded similar amounts of biomass growth and morphological expression of the growth was different. More and longer roots

formed in IAA than in NAA. These results are similar to those observed by Weathers *et al.,* (2005) in *Artemisia annua* and (Vanhala *et al.,* 1998) in *Hyoscymus muticus* hairy root cultures. Those cultures grown in ABA produced the highest amount of root biomass (Fig.35). In the single root growth studies, IAA appeared to be more stimulatory. Considering these results in the single root study and bulk root growth in flask, ABA increased the root biomass. Robins *et al.,* (1996) also reported that ABA stimulated an increase in root biomass in *Lotus corniculates,* while Vanhala *et al.,* (1998) did not observe any increase in *Hyoscymus muticus* which can be attributed that the ABA effect is species specific. Single and bulk roots grown in exogenously applied cytokinins did not yield much growth lateral root inhibition and root elongation, which is consistent with the work of Bais *et al.,* (2001) in *Chycorium intybus* and Weathers *et al.,* (2005). Hairy root growth and betalain accumulation decreased with increasing growth regulator concentration, in culture provided with IAA, NAA, ABA, BAP and Kinetin (Fig. 35 and 36). However cultures grown in ethaphon, 2-ip and 2,4-D yielded decreased amount of betalain (Fig.36). Except ABA all other growth regulators in beet hairy root cultures inhibited betalain production compared to roots grown in MS basal media (Fig.36).This result is similar to that of Weathers *et al.,* (2005) and Bais *et al.,* (2001) where increasing the auxin and cytokinin ratio resulted in decreased of secondary metabolite production.

4.9 Effect of Gibberellin (GA_3) on hairy root cultures of *Beta vulgaris* L.

GA_3 (0.1- 5.0 mg/l) influenced the growth and production of pigments in beet hairy root cultures, producing a maximum growth of 19.3 g FW/culture at 0.1 mg/l GA_3 and accumulation of betalains 39.3 mg/culture in 5.0 mg/l GA_3 treatments. Similar examples where added gibberellins have influenced growth of transformed root cultures and secondary metabolite production are reported by Liu *et al*., (1997) in *Artimisia annua*, Ohkewa *et al*., (1989) in *Datura innoxia*, Bais *et al*., (2001) in *Cichorium intybus* and Weathers *et al*., (2005) in *Artimisia annua* hairy root cultures. GA_3 administration showed increased branching patterns of transformed beet hairy roots and is similar to the report of Bais (2000) observed in *Cichorium intybus*. Hairy roots are generally considered to be independent of exogenous growth substances (Spane *et al.,* 1981) but there are reports where in exogenous administration of auxins and cytokinin has promoted growth in transformed root cultures.

The treatment with GA_3 leads to increase in growth and betalain production in hairy root cultures of *Beta vulgaris* L. that was associated with enhanced branching in the transformed roots.

4.10 Stability studies of betalains obtained from hairy root cultures of *Beta vulgaris* L.

Extended knowledge on betalain degradation and strategies for their stabilization is bound to increase demand on this underestimated pigment class. Betalains are known to be very sensitive to several factors including low pH, elevated temperatures or high water activity (Pasch and Von Elbe 1979; Huang and Von Elbe, 1987; Czapski, 1990; Altamirano *et al*., 1993**),** especially betalain thermal instability results in their restricted use. Therefore the stability studies have been carried out to understand the stability properties of betalains obtained from an unconventional source *i.e. in vitro* grown hairy root cultures of *Beta vulgaris* L. We have also applied some well known strategies to enhance the stability of the pigments.

The effect of temperature and pH on betalain concentrates was studied in the pH range 3-7. A maximum retention of 40.6% pigment in pH 5 was recorded at 50^{o}C, where as at 80^{o}C and 95^{o}C the loss of pigment was 80.5% and 92.2% respectively (Table. 10, 11 and 12). Similar results have been reported in beet root juice concentrates and betalains from cactaceae fruits (Reynoso *et al*., 1997; Altamirano *et al*., 1992). Application of betalains in concentrated preparations is advantageous for pigment stability since matrix effect of the vegetable juice together with water removal during processing result in low a_w values of the concentrate (Pasch and Von Elbe, 1975). In aqueous solutions at 30^{o}C the pigment was stable, and the pigment loss increased with the elevation of temperatures (Fig.39, 40, 41, 42 and 43), the thermal degradation of betains followed a first-order reaction kinetic and was dependent on pH. A first order degradation kinetic for betalains under aerobic conditions has been previously reported (Huang and Von Elbe, 1985; Huang and Von Elbe, 1987). During degradation of the betalains by temperature, the primary step involve a nucleophilic attack by water at C -11 positions on the betanin molecule, yielding cyclodopa-5-*O*-glycoside and betalamic acid (Huang and Von Elbe 1987). It is possible that betalamic acid cyclodopa-5-*O*- glycoside may undergo Schiff base condensation to regenerate betalain especially at

lower temperatures. However, betalamic acid is heat sensitive it may undergo aldol condensation or participate in Millard reaction making the pigment unavailable for the regeneration reaction. Similarly, the glycoside of cyclodopa-5-*O*-glycoside may be cleaved at high temperatures. It is also very susceptible to oxidation reaction, initiating polymerization to melanin type compounds. Thus as temperature increases, particularly in the presence of oxygen, irreversible betanin degradation is promoted. The quantity of betanin degradation and regeneration after thermal treatment depends not only on temperature and pH but also on the initial betanin concentration (Von Elbe *et al.,* 1974). As the initial betanin concentration increases so does the colour stability. Betanin may also yield isobetanin or decarboxylated betanin their formation is favored upon heating at pH 3-4 (Haung and Von Elbe, 1985). Colour loss can be minimized during processing and storage of betalain sources by choosing the respective temperature and pH regimes, as well as minimizing oxygen and light access (Delgado-Vargas *et al.,* 2000). Metals particularly those that contain two or more valance states, decreases the induction time and increases the oxidation rate of compounds. Metals can be prooxidant by transferring electrons there by releasing and forming free radicals (Reynoso *et al*., 1997). All the metal ions studied for the stability, were found to facilitate the degradation of betalains. In the result manganese had a greater effect (94%) on the degradation of betalain pigment than other metals of storage. Iron attacks the electrophilic center of betalains causing a loss of colour by destruction of the chromophore group.

Water activity is very important in biological systems and it has been reported that betalain stability increases with decrease in water activity (Pasch and Von Elbe 1979). Our results for beet hairy root pigments agree with these findings and a similar trend was found in this present study (Table 13).

Betalains are known to be sensitive to oxidation, which has an impact on their colour stability. Therefore, compounds such as ascorbic and citric acids have been used to counteract this phenomena due to the fact that ascorbic acid is a good stabilizer for its scavenger oxygen capacity in a closed system and citric acid can chelate metal ions such as iron which promote oxidation. In our study, it was found that addition of ascorbic acid to the pigment concentrate at 0.1g w/v protected their colour stability. In the concentrate of beet

hairy root pigment after 20 min heating at 95°C, 54.7% colour retention was recorded (Table 14). Ascorbic acid helps in stabilizing betalains possibly by partially neutralizing the electrophilic center of betanin through association with the positively charged amino nitrogen (Pasch and Von Elbe 1979). In our study citric acid also was found to improve the stability though being less effective than ascorbic acid, which is concurrent with the earlier reports (Han *et al*., 1998).

Chelating agents sodiumhexametaphosphate (SHMP), tetrasodiumpyrophosphate (TSPP) and sodiumacidpyrophosphate (SAPP) were also found to promote stability and SHMP proved to be the best chelating agent by retaining 27.9% pigment followed by TSPP 23.7% and 17.2% (Table 16), but they were not as effective as ascorbates.

From the above studies it was found that betalains obtained from hairy root cultures are sensitive to temperature, pH, water activity and metal ions. Hence during processing as a colourant in foods retention during and after heat treatment can be considerably increased by exclusion or removal of unfavorable conditions such as high temperatures, high water activity, metal ions, prolonged exposure to light and oxygen or by systemic utilization of common food additives such as ascorbic acid (antioxidant), citric acid and other chelating agents.

4.11 Influence of iron and copper on antioxidant status in hairy root cultures of *Beta vulgaris* L.

The SOD activity significantly increased on exposure of the hairy roots to both the metal ions after 24 hours. The highest activity was found at 800µM of iron 33.8 units/mg protein (Fig 47) and at 1.6 µM of copper 40.2 units/mg protein was found (Fig 48), that is 1.4 and 3.4 times higher than control, which is similar to as reported in carrot hairy root cultures by (Kim *et al*. 2002***)*** observed 12 times increased SOD activity in hairy roots of carrot at 0.3mM copper.

4.11.1 Influence of iron and copper on Peroxidase levels

Peroxidase activity was high on the first day. This may be due to the fact that culture was initiated by using the roots from a late exponential phase of growth as initial inoculum. The increase in peroxidase activity on the first day may also be due to mechanical injury involved during tissue transferring and the adaptation of roots on the fresh medium. The peroxidase activity decreased till the 5^{th} day and gradually increased till the 20^{th} day of culture. The highest activity was found at 200µM of iron 16.9 units/mg protein (Fig 49) and 04µM concentration of copper 19.4 units/mg protein (Fig 50). Kim *et al.* (2002) has reported peroxidase activity 9-19.2 units/ g FW in carrot hairy roots grown in various inducing chemicals. Increase of peroxidase activity at the end of cultivation can be explained by tissue aging when toxic peroxide accumulates in the cells resulting in increase in activity. The increase in peroxidase activity could also be attributed to the defense responses after metal ion stress.

From these results, it appears that by regulating the levels of iron it is possible to maximize the superoxide dismutase level in the hairy root cultures. In this study we have established the fact that beet hairy roots can produce large quantities of superoxide dismutase, peroxidase as well as betalains by inducing stress with iron and copper.

From these results, it is evident that process optimization is possible by growing hairy roots in regular MS medium during the phase of betalain production they may be exposed to iron resulting in higher yield of betalain and antioxidant factors. This has application for the production of colours and nutraceuticals.

4.12. Safety assessment of betalains obtained from hairy root cultures of Beta vulgaris L. in experimental rats

Betalains found in beet root are safe and used as a colourant since decades. In the present study pigments have been obtained from hairy root cultures which are obtained by the infection of soil bacterium *A. rhizogenes*. Safety evaluation of betalains from beet root have been carried out way back in 1970's (W.H.O technical report, 1974). This is the first report of safety evaluation on betalains obtained from hairy root cultures. Rats fed with betalain concentrate from beet root and beet hairy roots did not develop any clinical signs of toxicity either immediately or during the post treatment period even at the highest dose administered. No mortality occurred during the experimental period in acute toxicity studies.

Betalains fed at 1 and 5 % level did not cause any significant change in body weight gain in male rats. No meaningful difference in actual food consumption was observed in the treatment groups through out the 13 week experimental period. As result, growth of betaline fed rats was consistently similar to those of respective controls. Feeding betalains even at 5% in the diet caused no significant alteration in hemoglobin, RBC, WBC and differential count. No marked alterations were found in any of the specific activities of enzymes, GPT, GOT and ALP in betalin fed rats. The specific activities of hepatic enzymes in betalin fed rats were comparable with those of controls. Hence we conclude from this short term toxicity studies that betalines obtained from hairy root cultures and conventional beet root were not toxic. Similar studies have been carried out by (Naidu *et al*., 1999) in phycocyanin obtained from blue green algae *Spirulina platensis.*

4.13 Protocol for micropropagation of Beta vulgaris L. from seedling shoot tips and mature plant shoot tip explants and field evaluation of seed grown and mature shoot tip micropropagated plants

Since a lot of variation is found in root size and betalain content in beet root propagated conventionally from seeds (Gastztonyi *et al*., 2001). We attempted to produce consistently high level of betalain adopting micropropagation method. Seedling shoot tips were used to initially standardize *in vitro* multiplication whereas mature plants shoot tips to obtain plants having uniform root size and high pigment yield. Multiple shoots from shoot tip cultures were raised using different cytokinins at various levels and about 9.4±1.9 shoots from seedling shoot tip explant (Table 25) and 7.5±0.9 shoots from mature shoot tip explant (Table 27) were obtained. Similar results are reported by Harms *et al.*, (1983) in red beet. The shoots obtained were rooted *in vitro* using various auxins at different levels. The rooted plants obtained from mature plants shoot tip multiplication were hardened in green house and transferred to the field for field evaluation studies. During field evaluation several growth attributes were studied at different stages of growth. In shoots multiplied from mature shoot tips it was noticed that the growth attributes were superior and also the betalain production was fairly uniform. Hence these studies can be exploited for large scale propagation of red beet for the production of uniform roots and betalains.

CHAPTER -V

SUMMARY AND CONCLUSION

5.0 Summary

Plants are a vast resource of a number of chemical substances, which may be primary or secondary metabolites. Primary metabolites are those, which are directly synthesized and encoded by their respective genes or by basic photosynthetic process. Secondary metabolites are produced by plants, which play a major role in the defense mechanisms and adaptation of plants to their environment. These molecules largely contribute to plant fitness by interacting with the ecosystem. The plant secondary metabolites have been classified according to their biosynthetic pathways. Four large families are generally considered i.e. phenolics, terpenes, sterols and alkaloids. Plant secondary metabolites have been used from centuries in traditional medicine due to their large biological activities. They are also useful as food additives, flavours, colourants and pharmaceuticals. It has been established that despite rapid progress in applied chemistry 25% of the molecules used in pharmaceutical industry are still of plant origin.

Conventionally plant secondary metabolites are obtained from field grown plants. However recently plant cell cultures have been exploited for the production of various secondary metabolites of food and pharmaceutical importance. Roots are the most important part of the plant system and are a source of many valuable products such as pharmaceuticals, insecticides, colours, flavours, enzymes and others. They are the major sites of metabolite synthesis and storage, which exhibit indefinite growth because of the meristamatic activity of root tip meristem. Many compounds that are scarcely synthesized such as coumarins, caffeic acid esters and cathecins in undifferentiated cells are produced at higher levels in root cultures.

Among the *in vitro* culture systems, the cultured cell suspensions are not gained momentum because of their instability and non-uniformity of the product formation. Hence the differentiated organ cultures such as hairy root cultures are widely studied. Hairy root cultures are used as alternative production systems for secondary metabolites due to their tremendous potential to higher growth rate and uniform product formation. Being organized, they are amenable for scaling-up in bioreactors which is an added advantage. The metabolite pattern found in hairy roots is similar, if not always identical to that of plant roots. A major characteristic of hairy roots is the concomitant production

of secondary metabolites with growth. Hence it is possible to get a continuous source of secondary metabolites from actively growing hairy roots.

Colour in one form or the other has been added in foods for centuries. Natural colourants from plant sources are receiving growing interest from both food manufacturers and consumers in the continuing replacement of synthetic dyes. However substitution of synthetics with their natural alternatives presents a challenge due to higher stability of the former with respect to light, oxygen, temperature, and pH.

Betalains are red pigments found in abundance in *Beta vulgaris* L. roots. They have been used as natural additives for food, drugs and cosmetic products in form of beet juice concentrates or beet products. Recently several investigations on hairy root cultures of red beet are reported. Hairy root cultures of *Beta vulgaris* L are an alternative source for the continuous production of betalains.

In view of this background a series of experiments were designed and carried out to achieve the following objectives. The data generated from these studies form the core matter of this thesis. The objectives set for the present study were

- To establish clones of hairy roots using different strains of *Agrobacterium rhizogenes*, different explants from beet root plants and different varieties of *Beta vulgaris* L for selection of superior clone looking at growth, morphological characteristics and pigment production.
- To study effect of medium components i.e. major and minor nutrients to enhance the growth and production of betalains.
- Influence of growth regulators on growth and production of betalains in *Beta vulgaris* L. hairy roots.
- To study the stability of betalains obtained from the hairy root cultures of *Beta vulgaris* l. and strategies employed to improve the stability.
- Metal ion stress studies to know enhance the antioxidant enzyme status in hairy root cultures of *Beta vulgaris* L.
- Short term sub acute and acute safety evaluation of betalain extracts in experimental animals.
- Protocol development for micropropagation of *Beta vulgaris* L. using shoots tips from seedling and mature beet root plants.

- Field evaluation of tissue culture obtained plants and seedling grown plants to study the growth and production of betalains.

5.1 Induction and establishment of hairy root cultures

Hairy roots were induced by three different strains of *A. rhizogenes*, the best root formation response as transformation frequency was found in LMG-150. The hairy roots obtained from this *Agrobacterium* stain produced highest biomass (12.28 g FW/culture) and betalain pigment (32.9 mg/culture), significant morphological differences were also noticed between the roots obtained from different strains and roots obtained by LMG-150 stain was superior in differentiation, branching pattern and all other characteristics. Hence this *A. rhizogenes* strain was selected for induction of hairy roots of *Beta vulgaris* L. Different explants like root, stem, leaf and hypocotyls and cotyledons were examined and was found that leaf cotyledon explants were the best giving 96% hairy root induction frequency and the age of the cotyledon also had an impact on the induction frequency. Cotyledons obtained from 11-14 days old seedlings gave the best root induction frequency 96%. All the four varieties of beet root produced hairy roots and between the different varieties of *Beta vulgaris* L. Detroit dark red was found superior in all aspects producing 14.6 g FW/culture hairy root biomass and 32.7 mg /culture betalains.

5.2I Influence of different medium constituents on growth and production of betalains

The superior clone obtained was used for optimization of medium. Nitrates at different levels were studied and found that nitrates free MS medium and lower levels inhibited the growth, at lower levels pigment accumulation was not significantly effected. Nitrates present in MS normal concentration was found to be optimum for growth and production of betalains producing 14.6 g FW/ culture biomass and 32.7 mg/ culture betalains. Different ratios of nitrate: ammonium were tested and found that low ammonium: nitrate ratio favored superior growth and accumulation of betalains.

Phosphate free medium and at lower phosphate levels the growth of hairy roots was inhibited, but the pigment accumulation enhanced considerably. MS medium with regular phosphate level was optimum for growth 14.2 g FW/culture, but phosphate free medium was found to be good for betalain production 45.2 mg/culture.

Micronutrients are essential elements for the growth and production of secondary metabolites. Effect of these micronutrients at five times higher concentration than MS normal medium was studied. It was found that all the micronutrients had positive effect except Cu^{2+} and Zn^{2+}. Co^{2+} was found to be the best micronutrient in which 47.5 mg betalains were recorded on 20th day. Removal of zinc from MS medium had a positive effect on the production of pigments. From the above studies on medium constituents, we could optimize the amount of both macronutrients and micronutrients for better growth and production of betalains.

Influence of four different mediums was evaluated on the growth and pigment production of *Beta vulgaris* L. It was noticed that MS medium was superior over the other three mediums i.e. Gamborg's, Nitsch, and Whites medium.

Medium pH plays a important role in the growth and production of secondary metabolites. Hence we studied the influence of pH ranging from 3.5-7. It was recorded that pH between 4.5 - 6 supported the growth and production of betalains. pH below 4.5 and above 6 resulted in inhibiting the growth and production. Hence pH 5.8 was optimum for superior growth and production of pigments, which is generally used in most of the tissue culture studies.

Sucrose acts as the carbon source in, *in vitro* plant cell and tissue culture experiments. Studies were done to optimize the amount of sucrose and to know the effect of other carbon sources on growth and production of betalains. Sucrose at 3% was found to be optimal and also superior over all other sugars investigated.

Influence of growth regulators individually and in combination was studied. It was found that growth of hairy roots decreased as the concentration of each type of growth regulators was enhanced. Among the auxins and cytokinins studied maximum biomass accumulation was found in IAA + Kn combination and betalain production in IBA + Kn combination but was lower than MS basal medium. In the individually tested growth regulator, the hairy root growth and betalain accumulation decreased with increasing concentration of growth regulator. ABA produced the highest amount of betalain, but none of the growth regulators could produce betalains higher than MS basal medium.

Gibberellin influenced the growth and production of pigments in beet hairy root cultures. At 5 mg/l concentration maximum biomass accumulation 19.3 g FW/ culture and 39.3 mg/culture betalains were recorded. GA_3 at 10 mg/l inhibited both growth and betalain production. Hence an optimum concentration of GA_3 promotes the growth and production of pigments in *Beta vulgaris* L. hairy root cultures.

5. 3 Stability studies of betalains obtained from hairy root cultures of *Beta vulgaris* L.

Stability studies in betalain concentrate and aqueous extracts were carried out. In concentrate the pigments showed a higher stability to temperature and pH. Of the various pH levels tested, pH 5 was found to be the best in protecting the colour. All the metal ions influenced colour degradation to a large extent and Mn^{2+} exhibited the strongest bleaching activity. Higher water activity also inhibited the stability of betalains.

Addition of sugars, sugar alcohols, ascorbic acid in different forms, citric acid and chelating agents namely sodium hexametaphosphate, tetrasodiumpyrophosphate and sodiumacidpyrophosphate were found to protect the colour. Sodiumisoascorbate showed highest stability retaining 57.5% betalains even after heating for 20 min at 99°C. Hence the addition of this sugars, sugar alcohols, antioxidants and chelating agents prove to be protective for betalains at elevated temperatures and higher pH range.

5.4 Influence of iron and copper on antioxidant enzymes

The effect of exposure to iron and copper on antioxidant enzymes like SOD, CAT and POD was studied. Both iron and copper enhance the level of SOD at 800 µmol and 0.4 µmol concentrations. From these results, it is appears that, by regulating the levels of iron and copper it is possible to maximize the SOD level in the hairy root cultures. We have found that beet hairy roots can produce large quantities of SOD and peroxidase. From these results, it is evident that process optimization is possible by growing hairy roots in regular MS medium during the phase of betalain production they may be exposed to iron resulting in higher yield of betalain and antioxidant factors. This has application for the production of colours and nutraceuticals.

5.5 Safety assessment of betalains obtained from hairy root cultures of *Beta vulgaris* L. in experimental rats

Rats fed with betalain concentrate from beet root and beet hairy roots did not develop any clinical signs of toxicity either immediately or during the post treatment

period even at the highest dose administered. No mortality occurred during the experimental period in acute toxicity studies. Betalains fed at 1 and 5 % level did not cause any significant change in body weight gain in male rats. No meaningful difference in actual food consumption was observed in the treatment groups through out the 13 week experimental period. As a result, growth of betalain fed rats was consistently similar to those of respective controls. Feeding betalains even at 5% in the diet caused no significant alteration in hemoglobin, RBC, WBC and differential count. No marked alterations were found in any of the specific activities of enzymes, GPT, GOT and ALP in betalain fed rats. The specific activities of hepatic enzymes in betaline fed rats were comparable with those of controls. Hence we conclude from this short term toxicity studies that betalains obtained from hairy root cultures and conventional beet root were found to be safe.

5.6 Protocol for micropropagation of *Beta vulgaris* L.

Since a lot of variation is found in the betalain content of beet root obtained from seedling derived plants, this study was conducted to develop a protocol for *in vitro* multiplied red beet plants from seedlings shoot tips initially for standardization of protocol. Shoot tips of mature plants were in vitro multiplied to obtain clonal plants yielding uniform roots and high betalain content. From the field evaluation studies of both seedling and micropropagated plants, it was found that micropropagated plants were superior in growth, uniform roots and fairly high betalain production. From this study we could develop a protocol for uniform betalain yielding plants.

6.0 Conclusions

The results of the present study throw light on aspects of growth and pigment production in hairy root cultures of *Beta vulgaris* L. Efforts have been made to enhance the growth and betalain production by optimizing the medium constituents. Influence of growth regulators on the growth, morphology and pigment synthesis was studied. Effects of gibberellins were studied and it was found that it influences the growth, branching pattern and production of betalains. Metal ion stress enhanced the level of antioxidant enzymes and betalains in beet hairy root cultures.

Betalains obtained from hairy root cultures were studied for their stability at different temperatures, pH, and in presence of metals. Addition of antioxidants and chelating agents enhanced the pigment stability.

Short-term safety evaluation studies were carried out in experimental rats and found that the betalains obtained from hairy root cultures and conventional beet roots did not show any toxicity signs in the experimental animals. Hence hairy roots, may be considered for scale up and utilization as a source of pigments.

Micropropagation studies of red beet from seedling shoot tips and mature plant shoot tips were carried out. The multiple shoots obtained were rooted *in vitro*, hardened and grown successfully in field.

Field evaluation studies of tissue culture and seedling grown plants was carried out and growth aspects were analysed periodically. Tissue culture derived plants were found to be superior to the seedling plants in growth characteristics. Moreover micropropagated field grown plants produced fairly uniform levels of betalains. These studies will be helpful for the mass propagation of red beet plants to obtain beet root with consistent growth and pigment production.

Abhyankar, C., Reddy, V.D., Giri, C.C., Rao, K.V., Lakshmi, V.V., Prabhakar, S., Veeramani, M., Tippeswamy, B.S. and Bhattachrya, P.S. (2005) Amplified fragment length polymorphism and metabolomic profile of hairy root of *Psoralea corylifolia* L. *Phytochemistry*. **26 (20):** 2441-2457.

Akita, M., and Takayama S. (1994) Induction and development of potato tubers in a jar fermentor. *Plant Cell Tiss. Org. Cult.* **36**: 177–182.

Akita, M., Shigeoka T. (**1994**). Mass propagation of shoots of *Stevia rebaudiana* using a large scale bioreactor. *Plant Cell Rep.* **13**: 180-183.

Akita, T., Hina, Y. and Nishi, T. (2001) Effect of zinc deficiency on betacyanin production in cell suspension culture of table beet (*Beta vulgaris* L.). *Biosci. Biotechnol. Biochem* **65(4)**: 962-965.

Alscher, R.G., Donahue, D.L., and Cramer, C.L. (1997) Reactive oxygen species and antioxidant: relationship in green cells. *Physiol. Plant.* **100:** 224-233.

Altamirano, R.C., Drdak, M., Simon, P., Rajniakova, A., Karovicova, J., and Preclik, L. (1993) Thermal degradation of betanin in various water alcohol model systems. *Food Chem.* **46**: 73-75.

Altamirano, R.C., Drdak, M., Simon, P., Smelik, A. and Simko, P. (1992) Stability of red beet pigment concentrate in maize starch. *J. Sci. Food Agric.* **58**: 595-596.

Apostol, I., Heinstein, P.F., and Low, P.S. (1990) Rapid stimulation of an antioxidative burst during elicitation of cultured plant cells. *Plant Physiol*. **90:** 109-116.

Arenas, O.M., Sanchez, R.M., Del villar, M.A., Jimenez, A.A. and Davila, O.G. (1993) Obtencion de pigmentos de interes alimentario mediante el cultivade tejidos vegetales a partir de eqazote (*Teloxis ambriosoides* L.) *Biotechnologia.* **3** (34):AV1-AV9.

Arro, R.R.J., Develi, A., Meijers, H., Van de Westerlo, E., Kemp, A.K., Croes, A.F. (1995) Effect of exogeneous auxin on root morphology and secondary metabolism in *Tagetes patula* hairy root cultures. *Physiol. Plant.* **93**:233-240.

Ashira, H., Harikost, T., Li, X.N., Segishima, K. and Yamashita, Y. (1988) Profiles of enzymes involved in glycolysis in *Catharanthus roseus* cells in batch suspension culture. *J. Plant Physiol.* **133**: 38-45.

Attoe, E.L. and Von Elbe, J.H. (1981) Photochemical degradation of betanin and selected anthocyanins. *J. Food Sci.,* **46:** 1934-1937.

Attoe, E.L. and Von Elbe, J.H. (1985) Oxygen involvement in betanin degradation - Effect of antioxidants. *J. Food Sci*., **50**: 106-110

Aurstad, K., and Dahle, H.K. (**1973)** The effect of heat treatment, UV and gamma radiation on some beet root pigments, *Z Lebensm. Unters-Forsch.,* **151**:171-174.

Bais, H.P. (2000) Studies on the growth of hairy root culture of *Cichorium intybus* L. and production of phytochemicals. Ph.D. Thesis University of Mysore, India.

Bais, H.P., Sudha, G., George, J. and Ravishankar, G.A. (2001) Influence of exogenous hormones on growth and secondary metabolite production in hairy root cultures of *Cichorium intybus* L. CV. Local. *In Vitro Cell. Dev. Biol. Plant* **37**: 293-279.

Banat, C.A., and Franklin, N.L. Statistical analysis in chemistry and chemical industry. John Wiley and Son's Inc. New York, USA.

Banerjee, S., Naqvi, A.A., Mandal, S. and Ahuja, P.S. (1994) Transformation of *Withania somnifera* L. by *Agrobacterium rhizogenes* - infectivity and phytochemical studies. *Phytothe.r Res.,* **8**: 452-455.

Bayindirli, A., Yildiz, F. and Ozilgen, M. (**1988)** Modeling of sequential batch ultrafiltration of red beet extract. *J. Food Sci.,* **53(5)**: 1418-1422.

Beers, R.F., and Sizer, I.W. (1952) Colorimetric method for estimation of catalase. *J. Biol. Chem.* **195:** 133-139.

Bensaddek, L., Gillet, F., Sancedo, J.E.N. and Finia, M. (2001) The effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *Atropa belladonna* hairy roots. *J. Biotechnol*., **85:** 35-40.

Berlin, J., Sieg, S., Strack, D., Bokern, M. and Harms, H. (1986) Production of betalains by suspension cultures of *Chenopodium rubrum* L. *Plant Cell, Tiss. Org Cult,,* **5(2)**: 163-174.

Beyer, W.F., and Fridovich, Y. (1987) Assaying for superoxide dismutase activity: Some large consequences of minor changes in conditions. *Analytical Biochem.* **161:** 559-566.

Bhadra, R. and Shanks, J.V. (1995) Statistical design of the effect of inoculum conditions on growth of hairy root cultures of *Catharanthus roseus. Biotechnol Tech.,* **9:** 681-686.

Bhagyalakshmi, N., Thimmaraju, R. and Narayan, M.S. (2004) Various hexoses and di-hexoses differently influence growth, morphology and pigment synthesis in transformed root cultures of red beet (*Beta vulgaris*). *Plant Cell Tiss. Org. Cult*. **78**: 183-195.

Bianco-Colomas, J. (1980) Qualitative and quantitative aspects of betalains biosynthesis in *Amaranthus caudatus* L, var. pendula seedlings, *Planta,* **149**:176-180.

Bianco-Colomas, J. and Hugues, M. (1990) Establishment and characterization of a betacyanin producing cell line of *Amaranthus tricolor* inductive effects of light and cytokinin. *J. Plant physiol.,* **136:** 734-739.

Bilyk, A. (**1974)** A Extractive fractionation of betalains. *J. Food Sci,,* **44**: 1249-1251.

Bilyk, A. (1981) Thin-layer chromatographic separation of beet pigments, *J, Food Sci*.**, 46**: 298-299

Bilyk, A., Kolodij, M.A., and Sapers, G.M. (1981) Stabilization of red beet pigments with isoascorbic acid. *J. Food Sci.* **46:** 1616-1617

Biotel-Conti, M., Gontier, E., Laberche, J.C., Ducrocq, C. and Sangwan-Norreel B.S. (1995) Permeabilization of *Datura innoxia* hairy roots for release of stored tropane alkaloids. *Planta Med.,* **61:** 287-290.

Block, J.E., Amudson, C.H. and Von Elbe, J.H. (**1981)** Energy requirement of beet colourant production. *J Food Proc Eng*., **5(1)**: 67-75.

Böhm, H. and Rink, L. (**1988)** In: Cell culture and somatic cell genetics of plants, Academic Press, New York, 449-463.

Bokern, M., Heuer, S. and Stracj, D. (**1992)** Hydroxycinnamic acid transferases in the biosynthesis of acylated betacyanins: purification and characterization from cell cultures of *Chenopodium rubrum* and occurrence in some other members of the Caryophyllales. *Bot, Acta,*, **105**: 146-151.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein–dye binding. *Anal. Biochem.* **72**:248-254.

Brodelius, P., Funk, C., Häner, A. and Villegas, M.A. (1989) Procedure for the determination of optimal chitosan concentration for elicitation of cultured plant cells. *Phytochemistry*, **28:** 2651-2654.

Buitelaar, R.M., Leenen, E.J.T.M., Geurtsen, G., Aede Groot and Tramper, J. (1993) Effects of the addition of XAD-7 and of elicitor treatment on growth,

thiophene production, and excretion by hairy roots of *Tagetes patula*. *Enzyme Microb Technol.,* **15:**670-676.

Cai, Y., Mei-Sun and Corke, H. (2003) Antioxidant activity of betalains from plants of the Amaranthaceae. *J.Agri. Food Chem.* **51:** 2288-2294.

Cai, Y.Z., Sun, M. and Corke, H. (1998a) Colourant properties and stability of *Amaranthus* betacyanin pigments. *J. of Agri and Food Chem* 46: 4491-4495.

Cai, Y.Z., Sun, M., Wu, H.X., Huang, R.H., and Corke, H. (1998b) Characterization and quantification of betacyanin pigments from diverse *Amaranthus* species. *J. of Agri and Food Chem* 46: 2063-2070

Cao, G., Sofic, E., and Prior, R.L. (1996) Antioxidant capacity of tea and common vegetables. *J. Agri. Food. Chem.* **44:** 3426-3431.

Cardarelli, M., Spano, L., De Paolis, A., D Mauro, M.L., Vitali, G. and Costantino, P. (1987) Identification of the genetic locus responsible for non-polar root induction by *Agrobacterium rhizogenes*. *Enzyme Microb Technol.,* **5:** 385-391.

Castellar, R., Oban, J.J., Alacid, M., and Fernandez-Lopez J.A. (2003) Colour properties and stability of betacyanins from Opuntia fruits. *J. Agri. Food. Chem.* **51:** 2772-2776.

Cerezal, M.P. and Nunez, L.D. (**1996)** Caracterizacion del colorante de remolacha roja (*Beta vulgaris* L.). *Alimenraria, enerofebrero*: 91-94.

Cerezal, M.P., Pino, A.J. and Salabarria, Y. (1994) Red beet (*Beta vulgaris* L.) colourant stability in the form of a concentrated liquid. *Tecnologia Alimentaria,,* **293(4):** 7-16.

Chevaltier, A. (1996) The encyclopedia of medicinal plants. New York: DK publishing Inc. p.176.

Chilton, M.D. (2001) *Agrobacterium* A Memoir. *Plant Physiol*.**, 125**: 9-14

Chilton, M.D., Tepfer, D.A. Petit, A., David, C., Casse-Delbart, F. and Tempe, J. (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. *Nature,* **295**:432-434

Christen, P., Aoki. T. and Shimomura, K. (**1992a**) Characteristics of growth and tropane alkaloid production in *Hyoscyamus albus* hairy roots transformed with *Agrobacterium rhizogenes* A-4. *Plant Cell Rep.,* **11:** 597-600.

Christen, P., Aoki. T. and Shimomura, K. (**1992b**) Characteristics of growth and tropane alkaloid production in *Hyoscyamus muticus* and *Atropa belladonna*. *Planta Med.* **61**: 345-350.

Cogner, B.N. (1981) Cloning agricultural plants via *in vitro* techniques CRC press, Boca Raton, Florida. p. 165-168.

Cohen, E. and Saguy, I. (1983) Effect of water activity and moisture content on the stability of beet powder pigments. *J. Food Sci.,* **48**:703-707.

Counsell, J.N., Jeffries, G.S. and Knewstrub, C.J. (1979) Some other natural colours and their applications. In: Counsell J.N. and Dunastable, J.A., Eds., Natural Colours for Foods and Other Uses. Applied Science, London, pp 122-151.

Crawford, N.M. and Glass, A.D.M. (1998) Molecular and Physiological aspects of nitrate uptake in plants. *Plant Sci*; 3,389 – 95.

Cuisdo, R.M., Palazón, J., Piñol, M.T., Bonfill, M. and Morales, C. (1999) *Datura metel*: *in vitro* production of tropane alkaloids. *Planta Med.*, **65:** 144-148.

Czapski, J. (1990) Heat stability of betacyanin in red beet juice and in betanin solutions. *Z. Lebensur. Unters. Forsch*. **191**: 275-278.

D' Amato. (1977) Cytogenetics of differentiation in tissue and cell cultures. In: Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture. Reinert, J., and Bajaj, Y.P.S Eds. Springer-Verlag, Berlin, p. 343-357.

Davioud, E., Kan, C., Hamon, J., Tempé, J. and Husson, H.P. (1989) Production of indole alkaloids by *in vitro* root cultures from *Catharanthus trichophyllus*. *Phytochemistry.,* **28:** 2675-80.

Davioud, E., Petit, A., Tate, M.E., Ryder, M.H. and Tempe, J. (1988) Cucumopine -A new T-DNA-encoded opine in hairy root and crown gall. *Phytochemistry*, **27**: 2429-33

Deberg, P.C., and Zimmerman, R.H. (1991) Micropropagation: Technology and application. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Delgado-Vargas, H., Jimenez, A.R. and Lopez, P.O. (2000) Natural pigments carotenoids, Anthocyanins and betalains. Characteristics, biosynthesis, processing and stability. *Crit. Rev. Food Sci. and Nutri*. **40**: 173-289.

Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) A plant DNA minipreparation. Version II. *Plant Mol. Biol. Rep.*, **1:** 19-21.

Deno, H., Yamagata, H., Emoto, T., Yoshioka, T., Yamada Y. and Fujita Y. (1987) scopolamine production by root cultures of *Duboisia myoporoides* –II. Establishment of hairy root culture by infection with *Agrobacterium rhizogenes*. *J. Plant Physiol*. **131**:315-323.

Dhakulkar, S., Ganapathi, T.R., Sujata, B. and Bapat, V.A. (2005) Induction of hairy roots in *Gmelina arborea* Roxb. and production of verbascoside in hairy roots. *Plant Sci* **169:** 812-818.

Dornenbrug, H. and Knorr, D. (**1997)** Challenges and opportunities of metabolite production from plant cell and tissue culture. *Food Technol*., **51(11):** 47-54.

Dörnenburg, H. and Knorr, D. (1995) Strategies for the improvement of secondary metabolite production in plant cell cultures. *Enzy. Microb. Technol*., **17:** 674-684.

Downham, A. and Collins, P. (2000) Colouring our foods in the last and next millennium. *Int. J. Food Sci. Tech*. **35**: 5-22

Drdak, M. and Vallova, M. (**1990)** Kinetics of the thermal degradation of betanin. *Die Nahrung*. **34**: 307-310.

Drdak, M., Altamirano, R.C., Rahniakova, A., Simko, P., Karovicova, J. and Benkovska, D. (1992) Red beet pigment composition-Effects of fermentation by different strains of *Saccharomyces cervisiae. J. Food Sci.*, **57(4)**: 935-936.

Edenharder, R., Kurz, P., John, K., Burgard, S., Seeger, K. (1994) *In vitro* effect of vegetable and fruit juices on the mutagenicity of 2-amino-3-methylimidazole [4,5-f]quinoline, 2-amino-3,4-dimethylimidazole [4,5-*f*]quinline, 2-amino-3,4-dimethylimidazole-[4,5-*f*]quinoxaline. *Food. Chem. Toxicol., 32: 443-459.*

Eibl, R., Eibl, D. In: Oksman-Caldentey K-M, Barz W, editors, (**2002)** Eds.; Bioreactors for plant cell and tissue cultures. Plant Biotechnology and Transgenic Plants. New York Marcel & Dekker.

Eibl, R., Lettenbauer, C., Eibl, D. and Röll, M. (1999) Experiences in the application of the wave bioreactors. *Bioforum.*, **3:** 110-112.

Eilert, U. (1987) Vol 4: Cell culture and somatic genetics of plants In: Constabel F, Vasil IK, editors, Eds. Elicitation methodology and aspects of application.

San Diego, New York, Berkley, Boston, London, Sydney, Tokyo, Toronto Academic press Inc**,** p. 153-88.

Elliott CD, (1983) The pathway of betalain biosythesis: Effect of cytokinin on enzymic oxidation and hydroxylation of tyrosine in *Amaranthus tricolour* seedlings. *Physiol, Plant.,* **59:** 428-437.

Endres, R. (1976) Betacyanin akkununlateas in Kallus Van Portulacca grandiflora Var. JR unterdemcinfluss van phytohormomem and Cu^{2+} ion and interschiedlichen grundmedian. *Biochem. Physiol. Flanz*. **169:** 87-98.

Escribano, J., Pedrenp, M.A., Garcia-Carmona, F., Munoz, R. (1998) characterization of the antiradical activity of various betalains from *Beta vulgaris* L. roots. *Phytochem. Anal., 9: 93-98.*

Espelie, K.E., Franceschi, V.R. and Kollattukudy, P.E. (1986) Immunocytochemical localization and time course of appearance of an ionic peroxidase associated with suberization in wound-healing potato tuber tissues. *Plant Physiol*. **81:** 487-492.

Espin, C.J., Soler-Rivas, C., Wichers, H.J., and Garcia-Vignera J. (2000) Anthocyanin based natural colourants: A new source of antiradical activity for food stuffs. *J. Agri. Food. Chem.* **48:** 1588-1592.

FDA/IFIC (**1993).** Regulation of colour additives, Food and Drug Administration, FDA/IFIC Brochure.

Filetici, P., Spano, L. and Constantino, R. (1987) Conserved region of T-DNA in different *Agrobacterium rhizogenes* root inducing plasmids. *Plant Mol. Biol*., **9:** 19-26.

Fisher, N. and Dreiding, A.S. (1972) Biosynthesis of betalains. On the cleavage of the aromatic ring during enzymatic transformation of DOPA into betalamic acid. *Hel, Chim. Acta.,* **55**:649-652.

Flanagan, A.M.J., and Owens, J.N. (1985) Peroxidase activity in relation to suberization and respiration in white spruce (*Picea glauca* voss.) seedling roots. *Plant Physiol*. **79:** 103-107.

Flooco, C.G., Alvarez, M.A. and Giulietti (1998) Peroxidase production *in vitro* by *Armoracia lapathifolia* (horse radish) transformed root cultures: effect of elicitation on level and profile of isoenzyme. *Biotechnol. Appl. Biochem.* **28**:333-338.

Flores, H.E., Hoy, M.W. and Pickard, J.J. (1987) Secondary metabolites from root cultures. *Trends Biotechnol.*, **5:**64-68.

FNB (**1971)** Food Colours, Food and Nutrition board, National Academy of Sciences, Washington, D.C.**.**

Foyer, C.H., Lopez-Delgado, H., Dat, J.F., and Scoot, I.M. (1997) Hydrogen peroxide and glutathione-associated mechanisms of acclamatory stress tolerance and signaling. *Physiol. Plant.* **100:** 241-254.

Francis, F.J. (1986) Handbook of Food Colourant Patents Ist, edn., Food and Nutrition Press. Westport, CT.

Francis, J. (**1995)** Carotenoids as colourants. The World of Ingredients. Sept. Oct: 34-38.

Fujita, Y., Hara, Y., Agino, T. and Suga, C. (1981a) Production of shikonin derivatives by cell suspension cultures of *Lithospermum* e*rythrorhizon* I. Effects of nitrogen sources on the production of shikonin derivatives. *Plant Cell Rep*., **1:** 59-60.

Fujita, Y., Hara, Y., Suga, C. and Marimoto, T. (1981b) Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon* II. A new medium for the production of Shikonin derivatives, *Plant Cell. Rep*. **1:** 61-63.

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res.*, **50:** 151-58.

Gasztonyi, M.N., Daood, A., Hajos, M.T., and Biacs, P. (2001) Comparision of red beet (*Beta vulgaris* var. Conditiva) varieties on the basis of their pigment components. *J. Sci Food Agri*. **81:** 932-933.

Gelvin, S.B. (1990) Crown gall disease and hairy root disease. *Plant Physiol.*, 92**:** 281-285.

Giovannuci, E. Ascherio, A, Rimm, E.B. (1995) Intake of carotenoids and retinol in relation to risk of prostate cancer. *J. Natl. Cancer. Inst*. **87(23)**: 1767-76

Giri, A. and Narasu, M.L. (2000) Transgenic hairy roots: recent trends and applications. *Biotechnol. Advances* **18**: 1-22.

Giri, A., Sarish, T.R., Dingra,V. and Narasu, M.L. (2001) Influence of different strains of Agrobacterium rhizogenes on induction of hairy roots and artimisinin in *Artemisia annua*. *Curr. Sci*. **81**: 378-382.

Godwin, I., Todd, G., Ford Lloyd, B. and Newbury, H.J. (1991) The effects of acetosyringone and pH on *Agrobacterium* mediated transformation vary according to plant species. *Plant Cell Rep.*, **9:** 671-675.

Goldberg, R., Imberty, A., Liberman, M. and Prat R. (1986) In: Grepin H. Penel C, Gaspar T. Eds. *Molecular and Physiological Aspects of Plant Peroxidases.* University of Geneva Press, Geneva, pp. 208-220.

Guillon, S., Tremouillaux-Guiller, J., Pati, P.K., Rideau, M., and Gantet, P. (2006 a) Harnessing the potential of hairy roots: dawn of a new era. *Trends in Biotechnol,*. **24 (9):** 403-409

Guillon, S., Tremouillaux-Guiller, J., Pati, P.K., Rideau, M., and Gantet, P. (2006 b) Hairy root research: recent scenario and exciting prospects commentary. *Current Opinion in Plant Biol.* **9(3):** 341-346.

Hamil, J.D., Ransley, S., Spencer, A., Todd, G. and Rhodes, M.J.C. (1991) The polymerase chain reaction in plant transformation studies. *Plant Cell Rep.* **21:** 221-224.

Hamil, J.U., Parr, A.J., Robins, R.J. and Rhodes, M.J.C. (1986) Secondary product formation by cultures of *Beta vulgaris* and *Nicotiana rustica* transformed with *Agrobacterium rhizogenes*. *Plant Cell Rep.*, **5:** 111-114.

Hamill, J.D., Robins, R.J. and Rhodes, M.J.C. (1989) Alkaloid production by transformed root cultures of *Cinchona ledgeriana*. *Planta Med.,* **55:** 345-347.

Han, D., Kim, S.J. and Kim, D.M. (1998) Repeated regeneration of degraded red beet juice pigments in the presence of antioxidants. *J. Food Sci,*. **63(1):** 69-72.

Harms, C., Baktir I., and Oertli, J.J. (1983) Clonal propagation *in vitro* of red beet (*Beta vulgaris* ssp.) by multiple adventitious shoot formation. *Plant Cell. Tiss. Org Cult.* **2:** 93-102.

Hempel, J. and Bohm, H. (**1997)** Betaxanthin pattern of hairy roots from *Beta vulgaris* var. Lutea and its alteration by feeding of amino acids, *Phytochemistry*, **44(5):** 847-852.

Hendry, G.A.F., and Brocklebank, J. (1985) Iron induced oxygen radical metabolism in water logged plants. *New Phytol.* **101:** 199-206.

Heuer, S., Vogth, T., Bohm, H. and Strack, D. (1996) Partial prurification and characterization of UDP-glucose: betanidin 5-*O*- and 6-*O*-

glucosyltransferases form cell suspension cultures of *Doretheanthus bellidiformis* (Burm, F.), *Planta*. **199:** 244-250.

Heuer, S. and Strack, D. (1992) Synthesis of betanin from betanidin and UDP-glucose by protein preparation from cell suspension cultures of *Dorotheanthus bellidiformis* (Burm, F.). *Planta*, **186**: 626-228.

Hilton, M.G. and Wilson, P.D.G. (1995) Growth and uptake of sucrose and mineral ions by transformed root cultures of *Datura stramonium, Datura candida aurea, Datura wrightii, Hyoscyamus muticus and Atropa belladonna*. *Planta Med.*, **61:**345-350.

Hinz, U.G., Fivaz, J., Girod, P.A. and Zyrd, J.P. (1997) The gene coding for the DOPA dioxygenase involved in betalain biosynthesis in *Amanita muscaria* and its regulation. *Mol. Gen. Genet*., **256:** 1-6.

Huang, A.S. and Von Elbe, J.H. (**1985)** Kinetics of the degradation and regeneration of betanin. *J. Food Sci.* **50**: 1115-1120.

Huang, A.S. and Von Elbe, J.H. (**1987)** Effect of pH on the degradation and regeneration of betanin. *J. Food Sci.* **52**: 1689-1693.

Huang, S., Cheng, S. and Taung, W. (1995) Strategy for inducing pertinent cell line and optimization of the medium for *Stizolobium harsjoo* producing L-DOPA. *J. Ferment. Bioeng*., **79(4)** 342-347.

Huang, S.Y. and Chan, S.N. (2006) Elucidation of the effects of nitrogen source on proliferation of transformed hairy roots and secondary metabolite productivity in a mist trickling reactor by redox potential measurement. *Enzyme Microb. Technol*., **38:** 803-813.

Ilieva, M. and Parda, A. (1997) Rosmarinic acid production by *Lavundula vera* cell suspension culture. *Appl. Micorbiol. Biotechnol.*, **47:** 683-688.

Im, J.S., Parkin, K.L. and Von Elbe, J.H. (1990). Endogenous polyphenoloxidase activity associated with the "Black ring" defect in canned beet (*Beta vulgaris* L.) root slices. *J. Food Sci.* **55(4):** 1042-1059.

Isogai, A., Fukuchi, N., Hayashi, M., Kannada, H., Harada, H., Suzuki, A. (1988) Structure of a new opine mikimopine, in hairy root induced by *Agrobacterium rhizogenes*. *Agric Bio Chem* **52:** 3235-3237.

Jacob, A. and Malpathak, N. (2005) Manipulation of MS and B5 components for enhancement of growth and solasodine production in hairy root culture of *Solanum khasianum* chalke. *Plant Cell Tiss. Org. Cult*. **80:** 247-257.

Jimenez, A.A. and Gutierrez, L.G. (**1998)** Production of food related colourants by culture of plant cells. The case of betalains. In: Shahidi, F, Kolodziejczyk P, Whitaker J, Lopez-Munguia A, and Feller G, Eds. Chemicals via Higher Plant bioengineering. Plenum press. pp-195-210.

Jimenez, A.A., Davila, O.G., Villegas, G.T. and Del Villar, M.A. (**1992)** Obtencion de colourants de interes alimentario por cultivo de celulas de *Opuntia microdasys* Lehm) Pfeiff., *Rev. Latinoamer. Quim*. **23(1):** 5-8.

Jimenez, A.A., Gutierrez, L.G. and Rodriguez, M.M. (**1996)** Caracterizacion relogica e hidrodinamica del cultio de celulas de betabel (*B.vulgaris L*) productoras de betalainas en un bioreactor tipo tanque agitado. In: Hubinger, M., Murr, F.X., Aguilera, J.M., Eds., Propiedades fisicas de los alimentor Vol. I. Valencia: CYTED-UPV. 105-112

Jin, V.H., Chun, J.A., Han, M.O., Lee, J.W., Yi, Y.B., Lee, S.W. and Chung, L.H. (2005) Sesame hairy root cultures for extra cellular production of recombinant fungal phytase. *Proc*. *Biochem*. **40:** 3574-3762.

Joy, R.W., Sugiyama, M., Fukuda, H. and Komamine, A. (**1995)** Cloning and characterization of Polyphenol oxidase c-DNAs of *Phytolacca americana, Plant Physiol*. **107(4):** 1083-1089.

Jung, G., and Tepfer, D. (1987) Use of genetic transformation by the Ri T-DNA of *Agrobacterium rhizogenes* to stimulate biomass and tropane alkaloid production in *Atropa belladonna* and *Calystegia sepium* roots grown *in vitro*. *Plant Sci.,* **50:** 145-51.

Kahkonen, M.P., Hopia, A.I., Heikki, J.V., Ranha, J.P., Pihleja, K., Kujala, T.S., Heionen., M. (1999) Antioxidant activity of plant extracts containing phenolic compounds. *J. Agri. Food. Chem.,* **47:** 3954-3962.

Kanner, J., Heral, S., and Granit, R. (2001) Betalains-Anew class of dietary cationized antioxidants. *J. Food Chem.,* **49:** 5178-5185.

Kapadia, G.J., Tokuda, H., Konoshima, T., and Nishino, T. (1996) Chemoprevention of lung and skin cancer by *Beta vulgaris* (beet) root extract. *Cancer Lett.,* **100**: 211-214.

Kavita, S., Ritambhara, G.K., Shalini, V., and Dubey, R.S. (2001) Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedlings. *Plant Sci.* **161 (6):** 1135-1144.

Kim, J.H., Lee Y.W., and Yoo Y.J. (1994) Biosynthesis of superoxide dismutase by carrot hairy roots. *Biotechnol. Lett.* **16**: 581-538.

Kim, Y.H., Yoo, Y.J. (1996) Peroxidase production for hairy root cell culture. *Enz. Microb. Technol*. **18**:531-538.

Kim, Y.J., Wyslouzil, B.E. and Weatness, P.J. (2002) Secondary metabolism of hairy root cultures in bioreactors. *In Vitro Cell Dev Biol-Plant*; **38:** 1-10.

Kino-Oka, M. and Tone, S. (**1996)** Extracellular Production of pigment from red beet hairy roots accompanied by oxygen preservation. *J. Chem. Eng. Jpn.* **29(3):** 488-493.

Kino-oka, M., Taya, M. and Tone, S. (1991) Production of superoxide dismutase from plant hairy root by the effect of nitrogen source in their cultures. *Kagaku kogaku Ronbu* **17**: 1012-1018.

Koch, K.C. (1996) Carbohydrate modulated gene expression in plants. *Ann. Rev. Plant Physilol. Plant. Mol. Biol*. **47**: 590-540.

Kritchevsky, S.B. (1999) ß-Carotene, carotenoids and the prevention of coronary heart disease. *J. Nutr*. **129:** 5-8.

Kumon, K., Sasaki, J., Sejima, M., Takeuchi, Y. and Hayashi, Y. (1990) Betacyanin-decolorizing enzymes from *Phytolacca americana*, *Plant Cell Physiol.* **31(2);** 233-240.

Larson R.A. (1988) The antioxidants of higher plants. *Phytochemistry* **27**:969-978.

Lashley, D. and Wile, R.C. (1979) A betacyanin decolorizing enzyme found in red beet tissue, *J. Food. Sci.* **44:** 1568-1569.

Leathers, R. R., Darvin, C. and Zyrd, J. P. (1992) Betalain producing cell cultures of *Beta vulgaris* L. var. bikores (red beet). *In vitro Cell Dev. Biol.* **28:** 39-45.

Lee, Y.N. and Wiley, R.C. (**1981)** Betalain yield from a continuous solid liquid extraction system as influenced by raw product, post harvest and processing variable. *J. Food Sci.* **46:** 421-424.

Lee, Y.N., Wiley, R.C., Sheu, M.J. and Schlimme, D.V. (1982) Purification and concentration of betalains by ultrafiltration and concentration of betalains by ultra filtration and reverse osmosis, *J. Food Sci.* **47(2):** 465-471.

Lin, C.C., and Kao, C.H. (2000) Effect of NaCl stress on H_2O_2 metabolism in rice leaves. *Plant Growth Regu*. **30:** 151-155.

Lin, H., Kwok, K.H. and Doran, P.M. (2003) Development of *Linum flavum* hairy root cultures for production of coniferin. *Biotech Lett* **25:** 521-525.

Liu, C.Z., Wang, Y.C., Onyang, F., Ye, H. C. and I G.F. (1997) Production of artimisinin by hairy root culture of *Artemisia annua* L. *Biotech Lett* **19:** 927-929.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randell, R.J. (1951) Protein measurements with the Folin phenol reagent. *J. Biol Chem*. **193:** 265-275.

Mabry, T.J. (**1980)** Betalains. In: Bell, E.A. and Charlwood, B.V. Eds, Encyclopedia of Plant Physiology, Vol. 8, Secondary Plant Products, Springer-Verlag, Berlin, 513-533.

Mabry, T.J. and Dreiding, A.S. (1968) The betalains, In: Mabry R, Alston, E. and Runeckles, V.C. Eds, Recent Advances in Phytochemistry, Vol. l., Appleton Century Crofts, New York, 145-160.

Mabry, T.J., Taylor, A. and Turner, B.L. (**1963)** The betacyanins and their distribution, *Photochemistry*, **2**: 61-64.

Mallol A, Cusido RM, Palazon J, Banfill M, Merales C, Pinol M. T. (2001) Ginsenoside production in different phenotypes of *Panax ginseng* transformed roots. *Phytochemistry*, **57(3):** 365 – 371.

Mano, Y., Nabeshima, S., Matsui, C. and Ohkawa, H. (1986) Production of tropane alkaloids by hairy root cultures of *Scopolia japonica*. *Agric Biol Chem.*, **50:** 2715-22

Marusich, W.I., and Bauerfeind. (1981) Oxicarotenoids in poultry feeds. In: Bauerufeind J.C, Ed. Carotenoids as colourants and vitamin A precursors. Academic Press, New York. P. 319-426.

Masuda, H., Toshimala, T. and Sugawale, S. (1988) Acid and alkaline invertases in suspension cultures of sugar beet cells. *Plant. Physiol*. **86:** 312-317.

Mazza, G.(2000) Health aspects of natural colours. In natural Food Colourants Science and Technology: Lauro, G.J., Francis, F.J., Eds: Marcel Dekker: Newyork pp: 289-314.

Merkil, A., Christen, P. and Kapetanidis, I. (1997) Production of diosgenin in hairy root cultures of *Trigonella faenum gracecum* L. *Plant Cell. Rep.* **16**: 632-636.

Mizukami, H., Karoshima, M. and Tabata, M. (1977) Effect of nutritional factor on Shikonin deravative formation in *Lithospermum* callus cultures. *Phytochemistry*, **16:** 1183-1186.

Morgan, J.A., Barney, C.S., Penn, A.H. and Shanks, J.V. (2000) Effects of buffered media upon growth and alkaloid production of *Catharanthus roseus* hairy roots. *Appl Microbiol Biotechnol.,* **53**: 262-65.

Mueller, L.A., Hinz, U., Uze, M., Sautter, C. and Zyrd, J.P. (1997) Biochemical complementation of the betalain biosynthetic pathway in *Portulaca grandiflora* by a fungal 3,4-dihydroxyphenylalanine dioxygenase, *Planta*, **203(2):** 260-263.

Mueller, L.A., Hinz, U., Uze, M., Sautter, C. and Zyrd, J.P. (**1996)** Complementation of betalain biosynthesis in *P. grandiflora* by a fungal DOPA-dioxygenase, *Experientia*. **522**: A20.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassys with tobacco tissue cultures. *Physiol Plant***., 15 :**473-479.

Nabeshima, S., Mano, Y. and Okhawa, H. (**1986)** Production of tropane alkaloids by hairy root cultures of *Scopolia japonica*. *Symbiosis* **2:** 11-8.

Naidu, A.K., Sarada, R., Manoj G., Khan, M.Y., Mahadevaswamy, M., Narashima Murthy, K., Ravishankar G.A., and Leela Srinivas. (1999) Toxicity assessment of phycocyanin-A blue colourant from blue green algae *Spirulina platensis*. *Food. Biotechnol.* **13:** 51-66.

Newsome, R.L. (**1986)** Food colours, *Food Technol*. **40(7):** 49-56.

Nguyen, B.T., Forlot, P. and Gucker, A. (1992) Establishment of hairy root cultures of *Psoralea* species, *Plant Cell Rep*. **11:** 424-427.

Nilsson, T. (**1970)** Studies into pigments of beet root, *Lantbruk-sheogskplans Annal*. **36**:179-219.

Nitsch, J.P., and Nitsch. C. (1969). Haploid plants from pollen grains. *Science* **163**: 85-87

Ohkewa, H., Kamada, H., Sudo, H., and Harade, H. (1989). Effects of gibberellic acid on hairy root growth in the *Datura innoxia J. Plant Physiol* **134:** 633-636.

Oksman-Caldentey, K.M. and Hiltunen, R. (**1996)** Transgenic crops for improved pharmaceutical products. *Field Crops Res***; 45;** 57-69.

Oksman-Caldentey, K.M., Sevon, N., Vanhala, L. and Hiltunen, R. (1994) Effect of nitrogen and sucrose on the primary and secondary metabolism of transformed root cultures of *Hyoscyamus muticus*. *Plant Cell Tiss. Org. Cult***.,** **38:** 263-72.

Otten, L.A.B.M. and Schiperoot, R.A. (1978) A rapid microscale method for the detection of lysopine and nopaline dehydrogenase activities. *Biochem. Biophys. Acta.,*. **527:** 497-500.

Paderno, M.A., and Escribano, J. (2000) Studying the oxidation and the antiradical activity of betalains from beet root. *J. Biol. Educ*. **35:** 49-51.

Palazon, J., Cusido, R.M., Roig, C. and Pifiol, M.T. (1997) Effect of rol genes from *Agrobacterium rhizogenes* TL-DNA on nicotine production in tobacco root cultures. *Plant Physiol Biochem.,* **35:** 155-62.

Parr, A.J. and Hamill, J.D. (1987) Relationship between *Agrobacterium rhizogenes* transformed hairy roots and intact, uninfected *Nicotiana* plants. *Phytochemistry*, **26**:3241-5.

Parra, J.M. and Munoz, R. (**1997)** An approach to the characterization of betanin oxidation catalyzed by horseradish peroxidase, *J. Agric. Food Chem*., **458**: 2984-298.

Pasch, J.H. and Von Elbe, J.H. (1975). Betanin degradation as influenced by water activity. *J. Food Sci*. **40**:1145-1146**.**

Pasch, J.H. and Von Elbe, J.H. (**1979)** Betanin stability in buffered solutions containing organic acids, metal cations, antioxidants or sequestrants, *J. Food Sci*. **44**:72-741.

Pavlov A. and Bley T (2006) Betalains biosynthesis by *Beta vulgaris* L. hairy root culture in a temporary immersion cultivation system *Proc. Biochem.,* **41 (4):** *848-852*

Pavlov, A., Georgier, V. and Ilieva, M. (2005) Betalain biosynthesis by red beet (*Beta vulgaris* L.) hairy roots culture, *J. Agric. Food Chem*., 40**:** 1531-1533.

Pavlov, A., Kovatcheva, P., Georgiev, V., Koleva, I., and Liieva, M. (2002) Biosyntheis and radical scavenging activity of betalains during the cultivation of red beet (*Beta vulgaris*) hairy root cultures. *Zeistsch. Naturforschrung C. A J. of Biosci* **57:** 640-644.

Piatelli, M. (1976) Betalains. In: Goodwin TW, Ed., Chemistry and Biochemistry of plant pigments Academic press New York**,** pp 560-596.

Piatelli, M. (**1981)** The betalains structure, biosynthesis and chemical taxonomy. In: Conn, E.E. Ed., The Biochemistry of plants: A comprehensive Treatise. Vol. 17. Secondary Plant Products, Academic Press, New York, 557-575.

Piatelli, M. and Imperato, F. (1969) Betacyanins of the family Cactaceae, *Phytochemistry*. **8:**1503-1507.

Piatelli, M. and Imperato, F. (1970) Pigments of *Bogainvillea glabra*, *Phytochemistry*. **9:**2557-2560.

Piatelli, M. and Minale, L. (**1964)** Pigments of Centrospermae. III. Distribution of betacyanins, *Phytochemistry***. 3:**547-557.

Piatelli, M., and Imperato, F. (1969) Betacyanin of the family cactaceae. *Phytochemistry* **18:** 1503-1507.

Pierik, R.L.M. (1993) Micropropagation: Technology and opportunities. In: Plant Biotechnology- Commwecial prospects and problems. Prakash, J., and Pierik, R.L.M Eds. Oxford and IBH Publishing, New Delhi., p. 9-22.

Pitta-Alvarez, S.I., Spollansky, T.C. and Giulietti, A.M. (2000) Scopolamine and hyoscyamine production by hairy root cultures of *Brugmansia candida*: Influence of calcium chloride, hemicellulase and theophylline. *Biotechnol Lett.,* **22:**1653-6.

Pourrat, A., Lejeunem, B., Grand, A. and Pourrat, H. (1988) Betalains assay of fermented red beet root extract by high performance liquid chromatography, *J. Food Sci.* **53(1):** 294-295.

Powrie, W.D. and Fennema, O. (**1963)** Electrophoretic separation of beet pigments, *J. Food Sci.* **28:** 214-216.

Pszczola, D.E. (**1998)** Natural colours: pigments of imagination, *Food Technol.* **52(6):**70-76.

Rao, A.V., and Agarwal, S. (1999) Role of lycopene as an antioxidant carotenoid in the prevention of chronic diseases: a review: *Nat. Res.* **19:** 305-323.

Rayner, P.B. (1993) Food and drink colours from natural sources, *Food Marketing Technol.* **7(1):** 9-10.

Real, S.E., Cerezal, M.P, and Ruiz, H.A. (1993) Empleode la ultrafiltraction en la purification del jugp de remolacha para colorante, *Alimentos,* **18(4).** 5-10.

Reynoso, R., Garcia, F.A., Morales, D. and Gonzalez, de Mejna (1997) Stability of Betalain pigments from a cactaceae fruit. *J Agri. Food. Chem*. **45:** 2884-2889.

Reznik, H. (**1981).** Betalains. In: Pigments in plants, Czygan, F.E.Ed., *Akademie Verlag.* 370-392.

Rhodes, M.J.C., Parr, A.J. and Giulietti, A. and Aird, E.L.H. (1994) Influence of exogenous hormones on the growth and secondary metabolite formation in transformed root cultures. *Plant Cell Tiss. Org Cult.,* **38:**143-51.

Rhodes, M.J.C., Robins, R.J., Hamill, J.D., Parr, A.J. and Walton, N.J. (1987) Secondary product formation using *Agrobacterium rhizogenes*- transformed "hairy root" cultures. *TCA Newletters* **53:**2-15.

Rhodes, M.J.C., Robins, R.J., Hamill, J.D., Parr, A.J., Hilton, M.G. and Walton, N.J. (1990) Secondary products from plant tissue cultures In: Charlwood BV, Rhodes MJC, editors, Eds, Properties of transformed root cultures. Oxford Clarendon Press, pp.201-25

Robins, M.P., Evans, J.E., and Morris, P. (1996) The effect of plant growth regulators on growth, morphology and condensed tannin accumulation in transformed root cultures of *Lotus carniculatus*. *Plant Cell. Tissue. Org. Cult* **44:** 219-227.

Rodniguez, M.M., Jimenez, A.A., Davilla, O.G. and Sepulvede, J.G. (1994) Effect of carbon source on cell growth and betalain production in cell suspension culture of *Beta vulgaris. Biotech. Lett* **16:** 853-858.

Rosendal-Jensen, S., Juhl-Nielsen, B. and Dahlgren, R. (1989) Use of chemistry in plant classification. *Rev. Latinoamer. Quim.*; Suppl **1:** 66-89.

Saguy, I., Goldman, M., Bord, A. and Cohen, E. (1984) Effect of oxygen retained on beet powder on the stability of betanin and vulgaxanthine-I, *J, Food Sci.*, **49**: 99-101.

Saguy, I., Kopelman, I.J. and Mizrahi, S. (1978) Computer aided determination of beet pigments, *J. Food Sci.* **43**: 124-127.

Saito, K., Yamazaki, M. and Murakoshi, I. (1992) Transgenic medicinal plants. *Agrobacterium* mediated foreign gene transfer and production of secondary metabolites. *J Nat Prod.,* **55**: 149-62.

Sakuta, M., Takagi, T. and Komamine, A. (1987) Effects of sucrose on betacyanin accumulation and growth in suspension cultures of *Phytolacca americana*. *Physiol. Plant.* **71:** 455-458.

Sapers, G.M. and Hornstein, J.S. (**1979)** Varietal differences in colourant properties and stability of red beet pigments, *J, Food Sci*. **44:** 1245-1248.

Saverweim, M., Wink, M. and Shimonure, K. (1992) Influence of light and phytohormones on alkaloid production in transformed root cultures of *Hyoscyamus albus J. Plant. Physiol.* **140:** 147-152.

Savitha, B.C., Thimmaraju, R., Bhagyalakshmi, N. and Ravishankar, G.A. (2006) Different biotic and abiotic elicitors influence betalain production in hairy root cultures of *Beta vulgaris* in shake flask and bioreactor. *Proc. Biochem.* **41:** 50-60.

Schwartz, S.J. (**1983)** Chemical alterations of betacyanin and chlorophyll pigments during processing and a toxicological investigation of betalain pigments, *Diss. Abst. Intern.* **43(11):** 3524-3525.

Schwartz, S.J. and Von Elbe, J. H. (**1980)** Quantitative determination of individual betacyanin pigments by high performance liquid chromatography, *J. Agric. Food Chem.,* **28:** 540-543.

Schwartz, S.J., Von Elbe, J.H., Pariza, M.W., Goldsworthy, T., and Pitot H.C. (1983) Inability of red beet betalain pigments to initiate or promote hepatocarcinogenesis. *Food. Chem. Toxicol*. **21 (5):** 531-535.

Schwitzguebel, J.P., Zryd, J.P. and Leathers, R. (1991) From plant cells to biotechnology. *Swiss Biotechnol,*. **9(1):** 17-24.

Sevon, N. (1997) Tropane alkaloids in hairy roots and regenerated plants of *Hyoscyamus muticus.* Helsinki, Finland PhD Thesis, University of Helsinki.

Sevon, N., Drager, B., Hiltunen, R. and Oksman-Caldentey, K.M. (1997) Characterization of transgenic plants derived from hairy roots of *Hyoscyamus muticus. Plant Cell Rep***., 14**: 738-742.

Sevon, N., Hiltunen, R. and Oksman-Caldentey, K.M. (1992) Chitosan increases hyoscyamine content in hairy root cultures of *Hyoscyamus muticus. Pharm Pharmacol Lett.,* **2:** 96-99.

Sevon, N., Hiltunen, R. and Oksman-Caldentey, K.M. (1998) Somaclonal variation in *Agrobacterium* transformed roots and protoplast-derived hairy root clones of *Hyoscyamus muticus*. *Planta Med*., **64:** 37-41.

Shen, W.H., Petit, A., Guern, J. and Tempe, J. (1988) Hairy roots are more sensitive to auxin than normal roots. *Proc Natl Acid Sci.,* **85:** 3417-3421.

Shi, J., and Le-Maguer, M. (2000) Lycopene in tomatoes. Chemical and physical properties affected by food processing. *Crit. Rev. Food Sci. Nutr.* **40:** 1-42.

Shih, C.C, and Wiley, R.C. (**1981)** Betacyanin and betaxanthin decolorizing enzymes in the beet (*Beta vulgaris* L.,) root, *J. Food Sci.* **47:** 164-166.

Shin,K.S., Chakrabarty, D., Ko, J.Y., Han, S.S., and Peak, K. (2003) Sucrose utilization and mineral nutrient uptake during hairy root growth of red beet (*Beta vulgaris* L.) in liquid culture. *Plant Growth Regu.* **39**: 187-193.

Simon, P., Drdak, M. and Altamirano, R. (1993) Influence of water activity on the stability of betanin is various water/alcohol model systems, *Food Chem.,* **46**: 155-158.

Singer, J.W. and Von Elbe, H.H. (1980) Degradation rates of vulgaxanthin-I. *J. Food Sci.* **45:** 489-491.

Sivakumar, G., Yu, K. W., Hahn, E.J. and Paek, K.Y. (2005) Optimization of organic nutrients for ginseng hairy root production in large scale bioreactors. *Current Sci.* **89**: 641-649

Slesak, I., Libik, M and Miszalski, Z. (2003) Superoxide dismutase activity in callus from the C_3-CAM intermediet plant *Mesembryanthemum crystallinum*. *Plant. Cell Tiss. Org. Cult*. **75:** 49-55.

Spane, A., Wullems, G.J., Schilperoont, R.A. and Constantino (1981) Hairy root: *In vitro* growth properties of tissues induced by *Agrobacterium rhizogenes* in tobacco plant. *Sci. Lett*. **23:** 299-305.

Spano, L., Mariotti, D., Pezzotti, M., Damiani, F. and Arcioni, S. (1987) Hairy root transformation in alfalfa (*Medicago sativa* L.) *Theor Appl Genet.,* **73:** 523-530.

Sri Andrijany, V., Indrayante, G. and Adi Soehano, (1999) Simultaneous effect of calcium, magnesium, copper and cobalt ions on sapogenin steroids content in Callus cultures of *Agave amaniensis*. *Plant Cell. Tiss. Org. Cult*., **55:** 103-108.

Stafford, A. (**1991)** The manufacture of food ingredients using plant cell and tissue cultures, *Trends Food Sci. Technol*., **5(2):** 116-122.

Steel R.D., and Torrie J.H. (1980) Principles and procedures of statistics, biometrical approach, 2nd ed. Mc Graw-Hill Book, Co. New York.

Steglich, W. and Strack, D. (1991) Betalains, In:Brosswi, A., Ed., The Alkaloids. Vol.39, Academic Press, Orlando. 1-62.

Stitt, M. (1992) Nitrate regulation of metabolism and growth. *Curr Opinion Plant Biol*, **2:** 178-186.

Strack, D., Steglich, W. and Wray, V. (**1993)** Betalains. In: Methods in Plant Biochemistry Vol. 8, Academic Press, Orlando. 421-450.

Strack, D., Vogt, T., Schliemann, W. (2003) Recent advances in betalain research *Phytochemistry* **62**: 247-269.

Stuppner, H. and Egger, R. (**1996)** Application of capillary zone electrophoresis to the analysis of betalains from *Beta vulgaris*, *J. Chromatogr. A.* **735:** 409-413.

Su, W.W. (1995) Bioprocessing technology for plant cell suspension cultures. *Appl. Biochem. Biotechnol,* **50:** 189-229.

Suvarnalatha, G., Rajendran, L., Ravishankar, G.A. and Venkatramana, L.V. (1994) Elicitation of anthocyanin production in cell cultures of Carrot (*Daucus carota* L) by using elicitors and abiotic stress. *Biotech. Lett.,* **16**: 1275-1280.

Taya, M., Mine, K., Kino-Oka, M., Tone, S. and Ichi, T. (1992) Production and release of pigments by culture of transformed hairy root of red beet, *J. Ferm. Bioeng.*,**73(1):** 31-36.

Taya, M., Yalkura, K., Kine Oka, M. and Jane, S. (1994) Influence of medium constituents on enhancement of pigment production by batch culture of red beet hairy roots. *J. Ferment and Biol.*, **2:** 215-217.

Tepfer D. (1990) Genetic transformation using *agrobacterium rhizogenes*. *Physiol. Plant*. **79**:140-146.

Tepfer, D. (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes:* sexual transmission of the transformed genotype and phenotype. *Cell,* **37:** 959-967.

Tewari, R.K., Kumar, P., Sharma, P.N., and Bisht, S.S. (2002) Modulation of oxidative stress responsive enzymes by excess cobalt. *Plant Sci.* **162 (3):** 381-388.

The Wealth of India (1985) A Dictionary of Indian Raw Materials & Industrial Products. Raw Materials, Volume I: A (Revised). 513, 54

Thimmaraju, R. (2005) Genetically transformed root cultures of red beet (*Beta vulgaris* L.) for the production of food colour and peroxidase Ph.D. Thesis, University of Mysore, India.

Threlfall, D.R., and Whitehead, I.M. (1988) The use of biotic and abiotic elicitors to induce the formation of secondary plant products in cell suspension cultures of solanaceae plants. *Biochem. Soc. Trans.* **16:** 71-75.

Tinland, B. (1996) The integration of T-DNA into plant genomes. *Trends plant Sci.*, **6:** 178-84.

Toivonen, L. (1993) Utilization of hairy root cultures for production of secondary metabolites. *Biotechnol Prog* **., 9:** 12-20.

Toivonen, L., Ojala, M. and Kauppinen, V. (1991) Studies on the optimization of growth and indole alkaloid production by hairy root culture of *Catharanthus roseus*. *Biotechnol Bioeng* **., 37:** 673-680.

Trevelyan W,F. Proctor, D.P., and Harrison, H.P., (1950) Detection of sugars on paper chromatography. *Nature.* **166:** 444-445.

Trezzini, G.F. and Zryd, J.P., (1991a) Characterization of some natural and semi-synthetic betaxanthins, *Phytochemistry*, **30(6):** 1901-1903.

Trezzini, G.F., and Zryd, J.P. (1991b) Two betalains from *Portulaca grandiflora*. *Phytochemistry* **30:** 1897-1899.

Trypsteen, M., Van Lijsebettens, M., Van Severen, R. and Van Montagu, M. (1991) *Agrobacterium rhizogenes*-mediated transformation of *Echinacea purpurea*. *Plant Cell Rep.*, **10:** 85-89.

Van den Ende, W. and Lacre, V. (1995) Purification and properties of a neutral invertase from the roots of *cichorium intybus*. *Physiol. Plant*. **93:** 241-248.

Vanhala, L., Eeva, M., Lapinjoki, S., Hiltunen, R. and Oksman-Caldentey, K.M. (1998) Effect of growth regulators on transformed root cultures of *Hyoscyamus muticus. J Plant Physiol.*, **153**: 75-81.

Vanhala, L., Hiltunen, R. and Oksman-Caldentey, K.M. (1995) Virulence of different *Agrobacterium* strains on hairy root formation of *Hyoscyamus muticus*. *Plant Cell Rep.,* **14:** 246-240.

Vasil, I.K. (1994) Automation of plant propagation. *Plant Cell Tiss and Org Cult.* **39:** 105-108.

Verpoorte, R., Vander Heijden, R., Ten Hoopen, H.J.G. and Memlink, J. (1999) Metabolic engineering of plant secondary metabolites pathways for the production of fine chemicals. *Biotech Lett.,* **21:** 767-479.

Verveliet, G., Holsters, M., Tenchy. H., Van Mantagu, M. and Schell, J. (1975) Characteristics of different plaque forming and defective temperate phages in *Agrobacterium* strains. *J Gen Verol.,* **26:** 33-48.

Vicent, K.R. and Scholz, R.G. (1978) Separation and quantification of red beet betacyanins and betaxanthins by high-performance liquid chromatography, *J. Agric. Food Chem.* **264:** 812-816.

Vilaine, F. and Casse-Delbart, F. (1987) Independent induction of transformed roots by the TL and TR regions of the Ri plasmid of agropine type *Agrobacterium rhizogenes. Mol Gen Genet* **., 206**: 17-23.

Vinson J.A., Hao, Y., Yu-Su,X., and Zubik, L. (1998) Phenol antioxidant quantity and quality in foods: Vegetables. *J. Agri. Food Chem*. **46:** 3630-3634.

Vogt, T., Zimmermann, E., Grimm, R., Meyer, M. and Strack, K. (1997) Are the characteristics of betanidin glycosyltransferases from cell- suspension cultures of *Doratheanthus bellidiformis* indicative of their phylogenetic relationship with flavonoid glucosyltransferases, *Planta*. **203(3)**: 349-361.

Von Elbe, J.H. (1977) The betalains. In: Furia, E. T., Ed., Current Aspects of Food Colourants, CRS Press Inc.Boca Raton. Pp. 29-39.

Von Elbe, J.H., Maing, I. and Amudson, C.H. (1974) Colour stability of betanin, *J. Food Sci.* **39**: 334-337.

Von Elbe, **J.H., Sy, S.H., Maing, I.Y. and Gabelman, W.H.** (**1972)** Quantitative analysis of betacyanins in red table beets (*Beta vulgaris*) *J. Food Sci.* **37**: 932-934.

Von Elbe. (1987) Influence of water activity on pigment stability in food products. In: Rockland, L.B., and Belichat, L.R. Eds. Water activity: Theory and Applications to Food, IFT Basic sym. Ser. Marcel Dekker, New York, p.55-83.

Wang, H., Nair, M.G., Strasburg,G.M., Chang, Y.C., Booren, A.M., Gray, J.I., and Dewin, D.L. (1999) Antioxidant and anti-inflammatory activities of anthocyanins and their aglycon cynidin from tart cherries. *J. Nat. Prod*. **62:** 294-296.

Wang,W. J. and Tan, R.X. (2002) Artemisinin production in *Artemisia annua* hairy root cultures with improved growth by altering the nitrogen source in the medium. *Biotechnol.Lett*. **24(14):**1153-1156.

Watson, J.F. and Goldman, I.L. (1997) Inheritance of a gene conditioning blotchy root colour in table beet (*Beta vulgaris* L.) *J. Heredity*, **886**: 540-543.

Weathers, P.J., Bunk, G., and McCoy, M.C. (2005) The effect of phytohormones on growth and artemisinin production in *Artemisia annua* hairy roots. *In Vitro Cell. Dev. Biol. Plant* **41**: 47–53.

Wenszel, G. (1992) Application of unconventional techniques in classical plant production. In: Plant Biotechnology-Comprehensive Biotechnology. II Supplement Fowler M.W., and Warren, G.S. Eds. Pergmon Press, England., p. 259-281.

Wettasinghe, M., Bollings, B., Plhak, L., Xiao, H., and Parkin, K. (2002) Phase II Enzyme-inducing and antioxidant activities of beet root (*Beta vulgaris* L.) Extracts from phenotypes of different pigmentation. *J. Agri. Food Chem.* **50:** 6704-6709.

White, F.F. Taylor, B.H., Huffman, G.A., Gordon, M.P. and Nester, E.W. (1985) Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. *J Bacteriol* **., 164:**33-44.

Wiley, R.C. and Lee, Y.N. (**1978)** Recovery of betalains from red beets by a diffusion-extraction procedure, *J. Food Sci.* **43**:1056-1058.

Wiley, R.C., Lee, Y.N., Saladini, J.J., Wyss, R.C. and Topalian, H.H. (1979) Efficiency studies of a continuous diffusion apparatus for the recovery of betalains from red table beet, *J. Food Sci.* **44(1):** 208-211.

Wilson, P.D.G. (1990) In: **Nijkamp HJJ, Van Der Plas LHW, Van Aartrijk J,** editors, Eds.; The cultivation of transformed roots from laboratory to pilot plant. Progress in plant cellular and molecular biology. Dordrech,-Boston, London Kluwer Academic Publishers, pp. 700-705.

Wilson, P.D.G. (1997) In: Doran PM, editor, Eds.; The Pilot-Scale Cultivation of Transformed Roots. Hairy Roots and Culture Application. Amsterdam Harwood Academic Publishers, pp. 179-190.

World Health Organization Tech. Rep. Ser., (1974) Eighteenth report of the joint FAO/WHO expert committee on food additives, *No:557 and No: 54*

Yamamoto, K., Kobayashi, N., Yoshitama, K., Teramoto, S., and Komamine, A. (2001) Isolation and purification of tyrosinase hydroxylase from callus cultures of *Portulaca grandiflora*. *Plant and Cell Physiol*. **42:** 969-975.

Yamamoto, O and Kamura K. (1997) Production of saikosaponin in cultured roots of *Bupleurum falcatum* L. *Plant tiss. Cult. Biotechnol 3,138-147.*

Young, A. C., Yu, H., Song, J., Chun, H. and Pack, S. (2000) Indigo production in hairy root cultures of *Polygonum tinctorium* Laur. *Biotech. Lett*.**, 22:** 1527-1530.

Zaharova, N.S. and Petrova, T.A. (1998) Relationship between the structure and antioxidant activity of various betalains. *Prikl. Biochem. Microbiol*., **237**:314-321.

Z**aharova, N.S., Petrova, T.A., Shcherbukhin, V.D. and Gins, V.K. (1995)** Betacyanin and betalain oxidase in different *Amaranthus sp*., *Appl. Biochem. Microbiol.,* **31(2):** 202-205.

Zambryski, P. (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Annu Rev Genet*.**, 22:** 1-30.

Zambryski, P., Tempe, J. and Schell, J. (1989) Transfer and function of T-DNA genes from *Agrobacterium* Ti-and Ri plasmids in plants. *Cell,* **56:** 193-201.

Zhao, D., Fu, C. and Chen, Y. (2004) Transformation of *Saussurea medusa* for hairy roots and janceosidin production. *Plant cells Rep*.**, 23:** 468-474.

Zhu, J., Oger, P.M., Schrammeijer, B., Hooykaas, P.J.J., Farrand, S.K. and Winnas, S.C. (2000) The bases of crown gall tumorigenesis. *J Bacteriol.,* pp.3885-3895.

Zupan, J. (2000) The transfer of DNA from Agrobacterium tumefaciens into plant: a feast of fundamental insight. *Plant* **23**: 11-28

Zupan, J.R. and Zambryski, P. (1995) Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiol.,* **107:**1041-1047.

CHAPTER -VI

BIBLIOGRAPHY