SYNOPSIS

Annexure- I

INTRODUCTION

Color is a physical property or phenomena possessed by certain class of chemical compounds that are capable of absorbing and reflecting the electromagnetic waves in the visible region (400-700nm) as perceived through vision (Masland, 1996) by those biological organisms possessing cone cells. Such chemical compounds are called as **pigments** and the part of the pigment that absorbs or reflects the light are called as **chromophores.** Of the different types of plant pigments, carotenoids possess a special concern and attention due to its diverse structural and functional properties that are well known. Till date, more than 650 types of carotenoids were identified and reported (Otles and Cagindi, 2008). Plant pigments with special reference to food colorants were four types *viz*., carotenoids (annatto), anthocyanins, betalains (beetroot pigment) and curcumin (Davies, 2004). These four pigments along with cochineal, account for over 90% of the market (quantitatively) for natural food colorants (Hendry, 1996).

Annatto pigment is produced on the aril portion of the seeds of *Bixa orellana* (Fam: Bixaceae) – a tree native to Brazil – that is responsible for the reddish orange color appearance. Bixin – a unique apocarotenoid (monomethyl ester) – is the major constituent of annatto pigment (Aparnathi and Sharma, 1991). Annatto pigment is well known for its usage in dairy industry for cheese and butter (50 percent), fish processing (20 percent), confectionaries (10 percent), cosmetics and other major food grade coloring industries (20 percent). In general, oil-soluble extracts and oil suspensions are commercialized at 0.2-0.3% and 4% pigment content respectively; whereas, water-soluble extracts and spray dried products are at 5% and 14% respectively (Francis, 1999). Maximum levels of annatto pigment that can be used in various food formulations were specified by the expert committee of FAO/WHO (JECFA, 2001). Annatto pigment was extracted mostly by using chloroform. There are various processes available for annatto pigment extraction including the one from CFTRI Resource Center, Hyderabad (730/DEL/2000)

On analyzing the requirements for advancement of science in annatto towards the better utility of the crop for better prospects and better understanding, the present study was

formulated and framed with six objectives viz., i) standardization of tissue culture protocol for efficient mass multiplication; ii) studies on the levels of annatto color during developmental stages of fruits and elicitor mediated enhancement of annatto color under *in vitro* and *in vivo*; iii) down stream processing of annatto pigment; iv) molecular characterization of germplasm by RAPD; v) regulation of the lycopene cleavage dioxygenase gene using anti-sense approach for production of lycopene; and vi) developing an efficient transformation protocol for *Bixa orellana* for *in vitro* production of annatto pigment.

WORK DONE

This study has been presented in the thesis with five chapters in a detailed manner. **Chapter 1** describes the introduction and importance of this study along with a mention of the objectives of this study. **Chapter 2** deals with the review of literature that covers the literature with respect to annatto and also the basic principles and mechanism involved in the working of the objectives of the study. **Chapter 3** includes materials and methods that are involved in this study in a detailed manner. **Chapter 4** deals with studies carried out and implication of results in the light of existing knowledge in this field. **Chapter 5** provides the highlights of results (in the form of summary) obtained from this study along with future perspectives that can be looked in scientifically in the near future. Commonly used buffers, protocols, maps of vectors used in this study and the sequence of *lco* gene cassette were provided in appendix as a separate section after which the bibliography involved in this study was given in reference as a separate section. A brief section involving outputs achieved from this thesis was also given along with the copies of reprints of the published papers of the study involved in this thesis was also provided. Brief contents of the chapters are given below.

ORGANISATION OF THE THESIS

CHAPTER I: INTRODUCTION: This chapter deals with a brief introduction of annatto with regard to its value based on food applications and its market share among various food colorants and its importance of study with framing of objectives.

CHAPTER II: REVIEW OF LITERATURE: This chapter describes review of literature under different sections that includes about the tissue culture in annatto, elicitation, molecular markers, downstream processing, cloning and transformation for gene expression studies.

CHAPTER III: MATERIALS AND METHODS: This chapter deals with the materials used for the present study like seeds that are collected from the backyard of our department and chemicals from different firms, and the methodologies that are used for this study in different sections as mentioned in review of literature. All chemicals were purchased from Hi-Media, Mumbai; all hormones were purchased from Sigma-Aldrich, USA; all antibiotics that are used for transformation studies were procured from Duchefa, Germany; all solvents were purchased from Rankem chemicals, Mumbai; and all glasswares were purchased from Borosil, Mumbai. Restriction enzymes that are involved in this study were procured from MBI fermentas, Germany; *Z-Taq* polymerase enzyme was procured from TaKaRa Bio Inc., Japan. RAPD primers were procured from Operon biotechnologies, USA. T-tailed vectors for cloning of PCR products were procured from Promega Corporation, USA. All plastic wares like PCR tubes, eppendorf tubes, etc., were procured from Axygen Inc., USA. Genomic DNA extraction kit was procured from Sigma-Aldrich, USA. Gel elution kit and plasmid kit were procured from Qiagen, GmbH, Germany.

For tissue culture, different types of explants *viz*., nodal shoot tip, single node, shoot tip, hypocotyl, cotyledonary leaves, leaves, etc., were obtained from in vitro raised seedlings. Water soluble polysaccharides were extracted from the twigs (5-7 mm thick) of three varieties classed based on fruit shapes for its further analyses.

CHAPTER IV: RESULTS AND DISCUSSION: This chapter presents the results obtained in this study in six different sections. A brief outline of results obtained from this study was described in different sections below.

1. Establishment of efficient mass multiplication protocol through tissue culture. Acid scarified and surface sterilized seed of *B orellana* were inoculated onto the MS medium (Murashige and Skoog, 1962) supplemented with GA_3 (2.89 µM) and triacontanol (0.011 µM) for germination. Different parts of germinated seedlings were used as explants for

standardization of tissue culture protocols under *in vitro* conditions. Four different protocols are standardized for organogenesis under *in vitro* conditions by using phenylacetic acid; triacontanol; thidiazuron; and polyamines.

Two-step protocol using phenylacetic acid had been standardized that produces simultaneous multiplication and elongation which can be further taken for *in vitro* rooting and establishment. Through this protocol, it is possible to reduce one-third of the time involved in production of regenerated plantlets. Nodal shoot tip explant responded by producing a maximum of 34.3 ± 3.2 shoots when cultured on to MS medium supplemented with BA (31.08 µM) in combination with PAA (14.08 µM). MS medium supplemented with BA (13.32 µM) in combination with PAA (7.34 µM) produced a maximum shoot length of 5.0 ± 0.9 cm and simultaneously producing 6.1 ± 1.4 new shoots in a month's time. Best rooting response was found when the elongated shoots were inoculated on to MS medium supplemented with IBA (4.90 µM) by producing a maximum root length of 4.8 ± 0.52 cms and at an average of 4.11 ± 0.75 number of roots in four to six weeks time.

Triacontanol is a non-traditionally used plant growth regulator that exhibited numerous shoot buds and with simultaneous shoot multiplication in short time. Nodal shoot tip explants produced a maximum of 213.6 ± 12.5 shoot buds and 18 ± 1.4 primary shoots when inoculated onto MS medium containing TRIA (11.2 µM), BA (8.87 µM), and IAA (0.05 µM) with 80% response. MS medium containing 6.66 µM BA and 4.9µM IBA had used for elongation; a maximum of 18 ± 1.5 shoots with length of 45 ± 2.2 mm were produced. Elongated shoots (~ 4.0 cm) when inoculated on to MS medium supplemented with 4.9 µM IBA for rooting as mentioned in the above protocol. Plantlets that are established in the field with this hormonal combination produced enhanced pigment content than the control ones.

Thidiazuron is an efficient cytokinin that produces shoot multiplication and elongation. While using rooted hypocotyls as explants onto the MS medium supplemented with 9.08 µM of thidiazuron and 0.25% v/v coconut water leads to the production of adventitious shoots from hypocotyl tips. A maximum of 20-22 shoots per explant with a shoot length of 10-12 mm were obtained. Hence obtained shoots were inoculated onto the MS medium supplemented with

6.66 µM BA with 4.9 µM IBA that produced shoots of length 4.71 cms in four weeks time. These shoots were used to inoculate onto the rooting medium as mentioned in the previous protocols.

Polyamines are the non-traditional plant growth regulators. The nodal shoot tip explants inoculated onto MS media containing BA (6.66 µM) and IBA (4.9 µM) supplemented with 1000 µM and 800 µM of putrescine produced shoot multiplication and shoot elongation respectively with a maximum response of 12.8 ± 0.52 shoots and the maximum shoot elongation of 7.3 ± 0.31 cms.

Rooted plantlets from any of the above mentioned protocols were transplanted into the pots containing sand: soil: and compost mixture (1:1:2 w/w) and maintained under green house conditions for a month time for hardening and followed by their field transfer which resulted in approximately 70-80% of the rooted plantlets survived.

Also in this present study, establishment of cell suspension cultures were done using MS medium supplemented with 1.07 µM NAA and 10.2 µM BA. The callus obtained in the solid medium was used to inoculate onto the liquid MS medium supplemented with the same hormones.

Direct somatic embryogenesis protocols had been standardized by using immature zygotic embryonic stalk as explants. Ten to twelve primary somatic embryos are obtained from single explant in sixteen to eighteen weeks time, when the immature zygotic embryonic stalk were inoculated onto the MS medium containing B5 vitamins (Gamborg *et al.*, 1968) supplemented with 0.44 µM BA; 0.054 µM NAA; 2.89 µM GA_3; 0.02 µM TIBA; and 0.011 µM TRIA. These primary somatic embryos when inoculated onto the same medium produced secondary somatic embryos and maintained by sub-culturing them once in six weeks to eight weeks time. Somatic embryos from the callus are obtained when the callus suspension cultures were inoculated onto the MS medium supplemented with 4.44 µM BA; 40 µM $AgNO_3$; and 0.011 µM TRIA. These were sub-cultured once in six to eight weeks time onto the same medium composition for maintenance. Direct or callus mediated somatic embryos were inoculated onto the MS medium supplemented with 0.44 µM BA; 0.11 µM NAA; 2.89 µM GA_3;

0.02 µM TIBA; and 0.011 µM TRIA to produce rooting for somatic embryos. This medium composition is similar to the medium composition that is used to produce primary somatic embryos except that it has two fold increases in its NAA concentration. The regenerated plantlets are maintained onto the same medium composition till they are kept for hardening and field establishment.

2. Elicitor mediated enhancement of annatto pigment during ontogeny of fruit. Different types of biotic and abiotic elicitors were used for enhancement of annatto pigment content on the standing crops. Annatto pigment content was estimated once in ten days during the ontogeny of fruit as reported by McKeown and Mark (1962).

Of the different biotic elicitors used, best responded was the aqueous extracts of *Rhizopus oligosporus* at 0.5% v/v exhibiting 5.3% w/w pigment yield when compared to 1.8% w/w with respect to control. Of the different abiotic elicitors, hormones and plant growth regulators used, best responded one was methyl jasmonate at 10 µM concentration yielding 6.6% w/w when compared to 1.6% w/w in control.

Of the germplasm from different places in India, total annatto pigment yield has been estimated and found that, germplasm collected from Wayanad (Kerala) exhibited a maximum of 2.5% w/w pigment content.

3. Downstream processing of annatto pigment. Present study on downstream processing was mainly focused towards identification of the best solvent system using different solvents and its combinations for extraction efficiency of annatto pigment from the seeds. This is mainly to increase the annatto pigment yield by extracting the pigment from the seeds to maximum by increasing the elution efficiency through the optimized solvent combination. Also, three methods of extraction *viz*., mechanical abrasion, magnetic stirrer and sonication were tried for the efficiency of extraction and found that irrespective of the solvent combination used, mechanical abrasion method of extraction proved to be the best.

Among different solvent combinations tried, solvent combination Chloroform and Dichloromethane at 1:1 ratio v/v gave maximum yield of 2.4% w/w than any other solvents in single or in combination. Coconut oil yielded a maximum of 2.9% w/w pigment content than

the other vegetable oils used for extraction. These extracts were confirmed by using different analytical procedures like HPLC, FT-IR, NMR and MS.

4. Molecular characterization of germplasm by RAPD & chemotaxonomic markers. Genomic DNA isolated from the seedlings of *B orellana* germplasm collected from different parts of India were subjected to polymorphism using 36 different decamer primers and the polymorphic and monomorphic bands were identified and were used to establish a correlation matrix along with the Jaccard's similarity index. In this present study, the decamer primers that are subjected to polymorphism produced a total of 424 RAPD markers. Total number of polymorphic markers obtained was 419 that accounts for 98.8% of polymorphism. A maximum polymorphic marker of 23 was produced by the primer OPI 1. Jaccard's similarity index shows that the germplasm collected from the two places *viz*., Wayanad (Kerala) and Coimbatore (Tamil Nadu) exhibited maximum similarity; and, Wyanad (Kerala) and Neyveli (Tamil Nadu) showed maximum diversity.

Identification and characterization between the three varieties based on fruiting types were done by using chemotaxonomic markers. It was found that polysaccharides from twigs of hemispherical fruiting varieties contain 38% rhamnose whereas it is absent in case of obovate ones and is 2% in conical ones. Similarly, glucose content incase of obovate variety is 34% whereas, incase of conical and hemispherical ones it is 17% each.

5. Regulation of *lco* gene using anti-sense approach for production of lycopene. *Lycopene cleavage dioxygenase* (*lco*) gene was isolated from genomic DNA using primers designed from sequence at NCBI database and cloned initially in a T-tailed vector (pGEM-T easy). The gene fragment was then removed from the T-tailed vector using *Not*I restriction enzyme (GC-GGCCGC) and cloned into the pRT100 vector that is linearized using *Bsp*120I restriction enzyme (G-GGCCC). This step in cloning is required to obtain a complete gene cassette with CaMV 35 S promoter and CaMV poly A signal for the *lco* gene for its cloning into a binary vector for its further transformation.

Ligated product was transformed into *E. coli* JM109 strain and plated onto ampicillin containing plates for selection of colonies containing the vector. Recombinant vector was

identified by plasmid isolation and are confirmed for its orientation by restriction analyses using *Xho*I (C-TCGAG) and *Sca*I (AGT-ACT) restriction enzymes. Of the randomly selected fifteen colonies, based on the restriction analyses, it was found that clone 1 was sense construct; clone 6 was double pRT100 ligated vector; clone 7 was tandem sense construct; and clone 12 was an anti-sense construct and the remaining were self ligated ones. Since the vector pRT100 lacks *Lac*Zα, blue-white screening for selection of recombinant clones was not possible.

Gene cassette of clone 12 containing the anti-sense construct of *lco* gene was isolated using restriction enzymes from the recombinant vector and cloned into the MCS of binary vector pCAMBIA 1305.2. Clone 12 was first linearized with *Sca*I (AGT-ACT) and further the eluted ones was digested with *Sda*I (CCTGCA-GG) to produce a compatible ends with the linearized pCAMBIA 1305.2 binary vector using *Pst*I (CTGCA-G) restriction enzyme. Being neoschizomers, *Sda*I and *Pst*I restriction enzymes produce a compatible 3' extension sequence of TGCA that had been used for ligation.

The ligated product was initially transformed into *E. coli* JM109 strain and plated onto the kanamycin containing plates for selection. Since the MCS of pCAMBIA 1305.2 vector contains LacZα gene, transformed cultures were plated onto medium with X-gal and IPTG and incubated overnight at 37 ^{O}C for colonies with blue-white selection. White colonies obtained were used for the confirmation of the insert of gene cassette in the MCS. Recombinant plasmid pCAMBIA 1305.2-LCOAS was transformed into *Agrobacterium tumefaciens* GV3101 which were further used for plant transformation studies.

6. Developing an efficient transformation protocol for *Bixa orellana.* *Agrobacterium tumefaciens* GV3101 harboring pCAMBIA 1305.2 binary vector was used for establishment of transformation protocols. In case of *in vitro* conditions, somatic embryos that are established were used as explants and for *in vivo*, floral dip methodology has been established. Initially hygromycin sensitivity test was performed using somatic embryos and found that 10 mg L^{-1} was the minimal concentration at which somatic embryos die off.

Somatic embryos were co-cultivated with overnight grown cultures of *A. tumefaciens* GV3101 that has an absorbance value of 1.0 at 600 nm. Along with the culture during co-

cultivation, 100 µM of acetosyringone was added to enhance the transformation efficiency and co-cultivation time was standardized to forty-five minutes. They were dry blotted and inoculated onto primary somatic embryogenesis medium containing 10 µM of acetosyringone. In two to five days, when the bacterial growth was seen, they were washed in sterile dH_2O containing 500 mg L^{-1} cefotaxime and inoculated onto the primary somatic embryogenesis medium containing 10 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime. Transgenic nature of the newly produced secondary somatic embryos was confirmed by molecular and histochemical means.

CHAPTER V: SUMMARY AND CONCLUSION: A birds-eye-view of the achievements and highlights obtained from this study are summarized and given below.

- Protocols for *in vitro* organogenesis and callus suspension cultures were established that can be used for commercial propagation.
- Protocol for somatic embryogenesis through direct and indirect means were obtained that can be used further for transformation protocol.
- Enhancement of annatto pigment for the standing crop using elicitor mediated approach with a maximum of 3.3 folds.
- Downstream processing of annatto pigment for efficient extraction of pigment was standardized.
- Identification and characterization of germplasm using RAPD and identification of three varieties based on fruiting types using chemotaxonomic markers were done that can be used for screening elite clones at seedling stage itself.
- Isolation of *lco* gene and its cloning in pRT100 vector in anti-sense orientation.
- Cloning of *lco* gene cassette in pCAMBIA 1305.2 binary vector was done.
- Protocol for *Agrobacterium* mediated *in vitro* transformation using somatic embryos as explants or floral dip method for *in vivo* transformation has been established that can be used further for genetic engineering studies.