
An Abstract

Submitted for the Degree of

DOCTOR OF PHILOSOPHY

In

MOLECULAR BIOLOGY AND BIOTECHNOLOGY

To

Faculty of Science

UNIVERSITY OF KALYANI

By

CHAYANIKA BHATTACHARYA, M.Sc.

Under the supervision of Dr. T.K. Bandyopadhyay

DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY

UNIVERSITY OF KALYANI

KALYANI 741235

INDIA

2014
Abstract

Floriculture is a very important part of the horticultural industry. Flowering plants are used within the floriculture either in the cut-flower industry, where the harvested flowers are the final product, or in the flowering pot plant industry, where the plants are sold for landscaping purposes. Besides their aesthetic value, they are important for their economic uses, such as for cut blooms and for extracting perfumes and other products. The commercial production of cut flower is rising worldwide. Its economic value has significantly augmented over the last two decades and there is a great probability for further growth in both domestic and international markets. At the global level, at present flora business is around US$ 176 billion, which is expanding day by day and with an annual average growth rate of 10.3 per cent and is expected to reach US$ 250 billion by 2025 (Global Horticulture Market Outlook 2015). Flowers and foliage accounted for around 52.45 per cent, and live plants, bulbs and cuttings accounted for 47.55 per cent of total floriculture products at global trade (APEDA 2014). In view of floriculture exports from India, an exponential growth of floriculture products was being observed in 2006-07 when the exports reached to 649.6 crores of Rupees. But since then, a down fall was noticed to 340.14 crores in 2007-2008, 368.81 crore in 2008-9, 294.46 crore in 2009-10 and 296.04 in 2010-11 which may be explained by the downfall on the world economy. However, an overall rise with the establishment of a large number of exports oriented cut flower units which has given recognition for Indian floral industry in the international market and the growing dry flower industry that contribute to 60-70% of the export values. With this, little rise in export value was found 365.32 crore in last two years in 2011-12, 423.23 crore in 2012-13 (APEDA 2014).

Anthurium is native to Central and South American countries such as Ecuador, Colombia, Peru, Brazil and Venezuela. Anthurium is gaining popularity in floriculture industry because of its beautiful, attractive bright eye catching spathe colour, attractive long lasting vibrant inflorescence, candle-like spadix, prolonged vase life and higher returns per unit area. Moreover, desirable horticultural attributes contributed to the
dominance of the *Anthurium* industry by Hawaiian growers for much of the second half of the 20\textsuperscript{th} century (Bliss et al. 2012). *Anthurium* species are grown worldwide with the highest production in the United States and The Netherlands. *Anthurium andraeanum* and other species comprise the majority of the cut-flower production in the Netherlands and other tropical and subtropical regions including Hawaii (Matsumoto and Kuehnle 1997). In 2008, 81.4 million stems of *Anthurium* were sold on the Dutch auctions for 39.0 million €. In Hawaii, *Anthurium* are the top cut-flower with 6.3 million stems sold for $3.5 million in 2008 (HDOA Agricultural Development Division and USDA National Agricultural Statistics Service 2011; Matsumoto et al. 2013).

The cut flower industry mainly depends on elite cultivars and the hybrids of such plants that in turn depend on the availability of true to type planting materials. However, in most of the cases, conventional propagation cannot be the suitable option for solving the high market demand. Therefore, micropropagation is the best way for mass multiplication of disease and pest free plantlets at faster rates than conventional procedures. Micropropagation is the practice of rapidly multiplying stock plant material to generate a large number of progeny plants, using modern plant tissue culture methods. Maximum productivity is achieved by determining optimal environmental conditions and nutrient medium composition for shoot multiplication and rooting. The vast range of plants including annual, biennials and perennials and woody plants are commercially micropropagated (Stimart, 1986). The term somaclonal variation is closely related with the *in vitro* propagation through tissue culture. Somaclonal variation is defined as quantitative and qualitative (phenotype and genetic) alterations occurs among the clonally-propagated somaclones (Larkin and Scowcroft, 1981). Somaclonal variation is an undesirable consequence in true to type plant propagation but desirable for the searching of genetic variation. Somaclonal variation can be detected by two ways: i) Morphological screening-Visual screening during acclimatization in the green house, ii) Molecular markers assisted screening procedure, The last method is more acceptable in the present scenario of epigenetic research. Recent developments in genetic engineering
together with the progress in gene identification and isolation have enabled specific alterations of single traits in already successful varieties. These techniques allow the transfer of foreign genes in to plants to extend the available gene pool. Such transfer of genes to plants is not restricted to genes from other plant species, rather genes from other kingdoms, such as bacterial or even viral genes, can also be inserted. The modification of flower colour, incorporation of floral fragrance, disease and insect resistance, modified plant architecture, reducing flowering time, abiotic stress resistance, and increased vase life of flowers are the important traits that can be targeted in any florist plant. *Agrobacterium tumefaciens* mediated genetic transformation is well accepted in the transgenic research. The unique protocol for individual variety must be standardized by using the reporter or marker genes, before taking any major transgenic research.

On the above mentioned background the following objectives were taken for experimentation and the key achievements are presented in three chapters.

1. Germlasm dependent *in vitro* multiplication (Somatic embryogenesis and Organogenesis) (Chapter I)

(A) Optimization of a reproducible and effective protocol for the establishment of *in vitro* culture of four cultivars (Cancan, Midori, Tinora and Fantasia) of *Anthurium andraeanum* from leaf explants.

(B) Optimization of proliferation of embryogenic callus in subsequent cultures.

Among all the tested genotypes, Tinora showed the highest frequency (90%) of callus induction on semi-solid MS medium supplemented with N6-Benzylaminopurine (BA) (0.26 μM) and 2, 4 dichlorophenoxyacetic acid (2,4-D) (0.36 μM) and the similar PGR combinations stimulated rapid callus initiation in other two genotypes (Cancan - 76.6% and Fantasia- 86.6%). However, the initiation of callus required little bit higher concentration of BA (0.35 μM) along with 2,4-D (0.36 μM) in Midori and induced 83.3% explants to form callus. The proliferation of calli in different cultivars was also standardized based on their fresh weight increase. The medium supplemented with 4.44
μM BA and 0.36μM 2,4-D significantly increased highest amount of callus fresh weight in Cancan (0.57±0.05g), Midori (0.64±0.04), Tinora (0.65±0.38g) and Fantasia (0.55±0.02g). The morphological differences among the different varieties were also well established in the present study. Cancan produced compact green callus with a mixture of friable and granular regions; Midori generated light green compact, grannular callus with distinct green patches; Fantasia developed compact hard callus with distinct granular structure on it and Tinora proliferated light yellow, compact, friable callus with occasional nodular green patches.

(C) Induction, maturation and conversion of somatic embryos from embryogenic callus. Attempts have also been made to study the influence of various factors like genotype, growth regulators and basal media composition on the somatic embryogenesis.

The culture media tried for callus induction and proliferation did not produce any embryo like structure after a long time of culture. The comparative analysis on the effect of ammonium nitrate concentration in the culture medium revealed its role on the induction of somatic embryos. From the statistical analysis it is clearly indicated that 15 mM ammonium nitrate significantly induced highest number of somatic embryos in the three cultivars namely Cancan, Midori, and Tinora. In Fantasia no significant variations was observed in 10 and 15 mM ammonium nitrate containing media. The modified MS medium (MMS4) supplemented solely with 4.44 μM BA induced significantly highest number of somatic embryos in Cancan (26.0±2.1), Midori (24.0±2.0), Tinora (22.6±2.4) and Fantasia (25.3±2.3). The morphological differences of somatic embryos were detected in different genotypes. For maturation and conversion of somatic embryos the MMS4 basal medium was used and BA was supplemented in different concentrations from 4.44 μM to 0.27 μM. Significantly highest numbers of matured embryos were observed in culture medium having 0.27 μM of BA. The different stages of somatic embryo maturation and their conversion in different varieties are explained by the microphotographs.
(D) Establishment of a novel protocol of direct somatic embryogenesis and subsequent regeneration from the leaf explants of cultivar Fantasia, without any intervening callus phase.

In this section, a novel protocol of direct somatic embryogenesis and subsequent regeneration from the leaf explants of cultivar Fantasia has been described. The MS (Murashige and Skoog, 1962) basal medium supplemented with 0.27 μM N6-Benzylaminopurine (BA) and 2.68 μM α-Naphthalene acetic acid (NAA) induced the highest percentage (83.3 %) of leaf explants for embryogenesis as well as the highest number of somatic embryos (15.33 ± 1.4) per explant. Subsequent culture in MS basal media supplemented with BA (0.27 μM) and sucrose (29.2-175.2 mM), somatic embryos matured into coleoptile and scutellum embryos before it further differentiated into distinct shoot meristem.

(E) Study of the potentiality of in vitro generated leaf petioles and nodes for indirect organogenesis through the meristemoid formation and shoot regeneration.

The modified MS basal media (half strength of macro nutrients) supplemented with BA (2.21 μM) combined with Kinetin (KN) (1.38 μM) and Indole acetic acid (IAA) (1.71 μM) produced 83.3 %, 93.3 %, 96.6 % and 90.0 % meristemoids in Cancan, Midori, Tinora and Fantasia respectively. In vitro generated nodal segments were found more efficient than leaf petiole explants for the production of meristemoids in all four cultivars. Micro-shoots were produced from meristemoids of all four varieties.

(F) Confirmation of occurrence and development of direct and indirect somatic embryogenesis and also organogenesis of Anthurium andraeanum by histological and SEM analysis.

Occurrence and development of direct and indirect somatic embryogenesis and also organogenesis of Anthurium andraeanum was evaluated by histological and SEM analysis. During initiation the SEM analysis revealed the smooth walled pro-embryos from the cut end of leaf explant and histologically 16-celled pro-embryos were found to be attached with a distinct suspensor like structure. The pro-embryo structures were
further divided and protruded as distinct globular appearance having distinct protoderm and suspensor. In some cases the globular structures formed in cluster and surface topography of them clearly indicated protoderm differentiation, the characteristic feature of somatic embryogenesis. The embryos were further differentiated into club shaped structure. The appearance of closed vascular tissue and the accumulation of lipid globules, tannin and calcium oxalate in the cells of embryo manifested their similarity with zygotic embryo.

In organogenesis report, the histological images of both explants revealed the dividing cells were located at the epidermal layer which were smaller in size than the surrounding cells and contain densely stained nuclei. After several subsequent divisions, the cells formed the hard nodular structure called meristemoid. SEM analysis clearly demonstrates the cell walls and hard, compact surface of meristemoid tissues. Shoot buds formed from meristemoid surface was demonstrated histologically. Shoot bud germination was clearly observed by SEM analysis.

(G) Development of a suitable hardening and field acclimatization procedure for transfer and maintenance of in vitro regenerated plants in field and flowering conditions of *Anthurium andraeanum*.

Development of a suitable hardening and field acclimatization procedure for transfer and maintenance of *in vitro* regenerated plants in field and flowering conditions of *Anthurium andraeanum* is equally important to the protocol of embryogenesis. However, the process of acclimatization of micropropagated plants to the soil environment has not fully been emphasized by different workers. Consequently, the transplantation stage continues to be a major bottleneck in the micropropagation of many plants. Plantlets or shoots that have grown *in vitro* have been continuously exposed to a unique microenvironment that has been selected to provide minimal stress and optimum conditions for plant growth. Plantlets were developed within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity.
These contribute a culture-induced phenotype that cannot survive the environmental conditions when directly placed in a greenhouse or field. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field. In this work we have realized that potting mixture is very important role in hardening. During the acclimatization of plants derived from direct somatic embryogenesis, the highest mean survival percentage of primary hardened plants (85 %) was observed in potting mixtures containing cocopeat, sand and vermiculite (1:1:1), after 30 days. About 90 % of the plants subjected to secondary hardening survived after 60 days with same potting mixture. Among the cultivars the survival frequency of primary hardened plants, derived from indirect somatic embryogenesis and organogenesis, varied from 68.6 – 95.5 %. During secondary hardening 66.6 – 93.3% of plants survived after 1-month in bigger pot in the same potting mixture.

2. Assessment of genetic polymorphism (Chapter II)

(A) Molecular marker (RAPD and ISSR) based study for the assessment of genetic fidelity of direct and indirect somatic embryo derived plantlets to delineate somaclonal variations.

In our present study, 15 RAPD and 10 ISSR primers were used to check the genetic status of randomly selected 9 regenerated clones of four cultivars of *Anthurium andraeanum* obtained via indirect somatic embryogenesis and Fantasia obtained via direct somatic embryogenesis. All these primers produced clear and consistent banding profile in 9 randomly selected regenerants. However, we found no polymorphism in the regenerants obtained by direct somatic embryogenesis and indirect somatic embryogenesis of three cultivars. However, considerable amount (17.7% for RAPD and 18.8% for ISSR) of DNA polymorphism was found in case of indirect somatic embryogenesis of Tinora regenerants and remaining three produced true-to-type plants. Our first finding supports the fact that the direct somatic embryogenesis system is more genetically stable than those regenerated via callus mediated somatic embryogenesis.
Similarity index based on Jaccard’s coefficients for the regenerated clones was also higher in RAPD markers (Mean similarity index 0.85) than that of ISSR markers (Mean similarity index 0.853). Cluster analysis was performed based on the Jaccard’s (Jaccard, 1908) similarity coefficient matrices, calculated from RAPD, ISSR and RAPD-ISSR pooled data. Dendrograms were generated on the basis of RAPD, ISSR and RAPD-ISSR combined similarity matrix data by unweighted pair group method with average (UPGMA) cluster analysis. The product moment correlation (r) and the Mantel test statistic (Z) were calculated to measure the degree of relationship between the similarity matrices obtained and combined (RAPD and ISSR) data and the correlation was significant (0.239).

(B) Determination of genetic variability among the different generations of in vitro propagated plantlets through molecular marker techniques (RAPD and ISSR).

We also evaluated the genetic variability among the different generations by RAPD and ISSR analysis of Cancan and Midori. 12 RAPD and 10 ISSR primers produced respectively 104 and 95 bands in cultivar Cancan, out of which, 18 (17.3%) and 20 (21 %) bands were polymorphic. Similarly, 15 RAPD and 9 ISSR primers produced 98 and 88 bands respectively in cultivar Midori. Among them, 16 RAPD bands (16.3%) and 17 ISSR bands (19.3%) were found polymorphic. The RAPD-ISSR combine analysis was performed and revealed that maximum similarity index was observed between sub-culture generations 4 and 5 for both Cancan and Midori.

3. Genetic transformation through Agrobacterium tumefaciens (Chapter III)

(A) The preliminary trials for incorporation of two transgenes namely Gus and nptII in nodal segments and leaf petioles of Anthurium andraeanum cv. Midori.

This is a preliminary report on genetic transformation of Anthurium andraeanum cv. Midori. In the present investigation, different parameters including pre-culture duration, infection time, co-cultivation period and effect of acetosyringone concentration
were evaluated and were optimized for Agrobacterium mediated transformation with in nodal segments and leaf petioles of Anthurium andraeanum cv. Midori. Our study revealed that infecting the explants after 7 days of preculture for at least 45 mins with Agrobacterium tumefaciens (LBA 4404) suspension in the presence of 100 μM acetosyringone followed by a cocultivation for 3-4 days in the dark gave rise to maximum transient and stable transformation frequency. The putative transgenic regenerated plantlets were selected on respective regeneration media supplemented with kanamycin. However, 8 plantlets regenerated from node derived meristemoids on kanamycin-containing media. A transformation frequency of 2.9% transformation efficiency was found when nodal segments were used as explants. However, the multiplication of transformed shoots in in vitro condition is under process.

(B) Confirmation of transient expression through Gus-histochemical assay

The transient expression of transformed tissue was evaluated by histo-chemical Gus assay. A transient transformation frequency [(number of GUS-positive plants/number of explants) × 100] of 3.3% transformation efficiency was found when nodal segments were used as initial explants. The presence of blue colour in all transgenic tissues stained with X-gluc reagent clearly demonstrated the presence of GUS gene in those transformed tissues.

(C) Confirmation of stable gene expression through molecular analysis.

Genomic PCR analysis was carried out on the 8 Kanamycin resistant (KanR) plants using the specific primers for the nptII gene. Amplification of a 616-bp fragment was observed from 8 of the KanR plants, demonstrating the presence of the nptII transgene in these plants. No fragment was amplified using genomic DNA extracted from non-transformed control (NC) plants as the template. We also tested the DNA isolated from these eight plants by PCR with primers for the uidA gene, further confirming the presence of the uidA transgene. A 1,203-bp fragment was amplified from all eight
putative transgenic plants, whereas this fragment was not detected in untransformed plants and the negative control. The GUS assay along with genomic PCR clearly demonstrated the efficacy of the *Agrobacterium* mediated transformation protocol of a monocot, *Anthurium andraeanum* cv. Midori optimized in the present thesis.

**References:**

APEDA 2014- website: http://agriexchange.apeda.gov.in


