In vitro MULTIPLICATION AND DNA FINGERPRINTING OF SELECTED HYBRIDS AND THEIR PARENTS IN *Anthurium andreanum* Linden.

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Thesis submitted in partial fulfilment of the requirement
for the degree of

Doctor of Philosophy in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2007

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DECLARATION

I hereby declare that this thesis entitled "***In vitro* multiplication and DNA fingerprinting of selected hybrids and their parents in *Anthurium andreanum* Linden**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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Certified that this thesis entitled "***In vitro* multiplication and DNA fingerprinting of selected hybrids and their parents in *Anthurium andreanum Linden***" is a record of research work done independently by Ms. Yasin Jeshima. K (2003-21-12) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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ICAR

TNAU

Guru, Raashi

My teachers

ACKNOWLEDGEMENT

With utmost reverence and deep sense of gratitude I express my indebtedness to

Allah for all the blessings showered on me at each and every moment in availing me this opportunity to do my Ph.D. programme and giving me the courage and strength to pursue this endeavour to completion.

Dr. Maya Devi. P, Associate Professor, Department of Plant Breeding and Genetics and Chairperson of the Advisory Committee for suggesting the problem, constant encouragement, valid opinions and kind treatment throughout the course of study and preparation of the thesis.

Dr. Manju. P, Professor and Head, Department of Plant Breeding and Genetics and member of the Advisory Committee for her sustained interest, inspiring encouragement and overwhelming support extended at all stages of work.

Dr. K. Rajmohan, Professor and Head, Department of Plant Biotechnology and member of the Advisory Committee for his timely advice and concern as a member of the Advisory Committee.

Dr. M. M. Viji, Associate Professor and Head and Dr. R. V. Manju, Assistant Professor, Department of Plant Physiology and member of the Advisory Committee for valuable guidance, constructive criticism, motivating inspiration and unstinted support throughout the research work, without which this piece of work would not have materialized.

Dr. V. G. Jayalekshmy, Associate Professor, Department of Plant Breeding and Genetics and member of the Advisory Committee for her unfailing support, generous help and critical scrutiny of the manuscript.

Dr. K.M. Abdul khadar, Dr. Roystephen, Dr. C.. Baskaran, Dr. K. B. Soni and Dr. Umamaheswaran for their scholarly suggestions and ungrudging help rendered at all stages of this research endeavour.

Dr.D.S.Radha Devi Professor, Department of Plant Breeding and Genetics for providing me the glasswares and chemicals intime to carry out this work.

Dr. Jayapragasam and Dr. Maheswaran, Professors at CPMB, TNAU for their valuable suggestions and guidance in planning my experiments.

All members of Teaching and Non-teaching Staff, Students and Research Associates of Kerala Agricultural University for their whole hearted co-operation and good will bestowed on me throughout this endeavour.

To my friends and family members for being my backbone and for their indispensable support throughout this research programme.

Indian Council of Agricultural Research for awarding ICAR- Senior Research Fellowship and Agricultural Scientists Recruitment Board for selecting me to avail the fellowship without which I would not be able to think about my Ph.D.

[Yasin Jeshima, K.]

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LIST OF ABBREVIATIONS

AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
2,4-D	2,4- di chloro phenoxy acetic acid
2ip	2- iso pentenyl adenine
ABA	Abscisic acid
AC	Activated charcoal
Ads	Adenine sulphate
BA	N^6 – benzyl adenine
CH	Casein hydrolysate
CI	Callus index
cm	Centi metre
CTAB	Cetyl trimethyl ammonium bromide
CW	Coconut water
DMSO	Di methyl sulphoxide
DNA	Deoxy ribonuclic acid
dNTPs	Deoxy neucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
g	Gram
GA_3	Gibberellic acid
h	Hour
HCl	Hydrochloric acid
IAA	Indole- 3- acetic acid
IBA	Indole-3-butyric acid
Kn	Kinetin
mg	Milli gram
mM	Milli molar
MS	Murashige and Skoog (1962)
NaCl	Sodium chloride

NaOH	Sodium hydroxide
NAA	ά- naphthalene acetic acid
ng	Nano gram
nm	Nano meter
°C	Degree Celsius
PVP	Poly vinyl pyrrollidone
RAPD	Random amplified polymorphic DNA
s	Second
TDZ	Thidiazuron
Tris	Tris (hydroxy methyl)amino methane
Tris-HCl	Tris (hydroxy methyl)amino methane hydrochloride
UV	Ultra violet
µl	Micro litre
µM	Micro molar

Introduction

1. INTRODUCTION

Anthurium is the largest genus in the family Araceae, encompassing more than 800 species. Anthurium is native to tropical American region ranging from Mexico, Costa Rica, Argentina and Cuba to Brazil. Anthurium with colourful inflorescences has been grown for cut flowers. The inflorescence which is known as spadix is having a candle composed of multitude of flowers. They are perfect having two-carpelled ovary and four anthers. Most of the anthurium species are having colourful and long-lasting inflorescences consisting of brightly coloured spathes with contrasting or complementary spadices.

Traditionally, anthuriums with colourful inflorescences have been grown for cut flowers. With the introduction of compact interspecific hybrids through breeding and the selection of somaclonal variants, the new commercially available types were developed.

Anthuriums can be propagated by seeds as well as by vegetative means. As it is a cross pollinated crop, the seedling progenies were found to be highly heterogenous showing wide variability and takes six to eight months from pollination to seed maturiuty. The seedlings require about three years to reach the first flowering. Mature plants will produce only one or two suckers per year. Suckers can also be induced by top cuttings. However, all these methods result insufficient multiplication rates for mass clonal propagation which paves the way for *in vitro* propagation to ensure mass clonal propagation.

The detection and exploitation of naturally occurring DNA sequence polymorphisms represent one of the most significant recent developments in molecular biology. Since its development, the Polymerase Chain Reaction (PCR) has revolutionized many standard molecular biological techniques, with modifications of the original procedure designed to suit a range of needs. One such variation generates a specific class of molecular marker termed RAPD [and

AP-PCR (Arbitrarily Primed PCR)]. Genetic improvement of any organism depends upon the existence, nature and extent of the genetic variability available for manipulation. Current and future plant breeding programmes will not only require access to this variability, but also will depend upon the conservation and management of biodiversity. RAPD provides a useful system to monitor levels of diversity detected between and within population. This procedure has the advantages of being technically simple, quick to perform, requires only small amounts of DNA and involves no radioactivity. RAPD is well suited for use in the large sample throughout systems required for plant breeding, population genetics and studies of biodiversity.

AP-PCR belongs to a recent generation of genotyping methods in which short oligonucleotides with randomly chosen sequences are used in a modification of classical PCR protocols. Such arbitrary primers generate a set of amplicons of varying numbers and size, providing the basis for typing varieties. This technique is methodologically easier, less time consuming and more cost effective than the older genomic typing methods particularly Pulsed Field Gel Electrophoresis.

In light of aforesaid views, an attempt was made to standardize *in vitro* multiplication and DNA fingerprinting of selected hybrids and their parents in *Anthurium andreanum* Linden through a systematic study with the objective of standardizing the protocol for *in vitro* multiplication and to differentiate the hybrids and their parents using a DNA fingerprinting tool RAPD using AP-PCR.

Review of Literature

2. REVIEW OF LITERATURE

This review highlights the research on the various techniques of *in vitro* propagation and molecular characterization of anthurium hybrids and their parents with special emphasis on somatic organogenesis, callus mediated organogenesis, somatic embryogenesis and RAPD analysis using AP-PCR amplification profiles.

2. 1 *In vitro* MULTIPLICATION

The first successful clonal multiplication through *in vitro* techniques was reported by Morel (1960) for Cymbidium orchid through meristem culture. Since then *in vitro* clonal propagation of ornamental plants gained momentum. The conventional methods of asexual propagation of tropical ornamental plants are inadequate to meet the increasing demand of quality planting materials. *In vitro* techniques have proved to be a better alternative to the conventional vegetative propagation methods. *In vitro* propagation is possible via enhanced release of axillary buds, somatic organogenesis and somatic embryogenesis (Murashige, 1977).

Several studies have been conducted in *in vitro* multiplication of anthurium. Pioneering studies on *in vitro* culture of anthurium were conducted by Pierik *et al.* (1974 a, b). There are reports on direct organogenesis, callus mediated organogenesis and embryo culture using explants like lamina, petiole, inflorescence stalk, spathe, embryo (Geier, 1982) and spadix. Methods of *in vitro* propagation of anthurium through different methods have been studied by many authors. Of the three different routes anthuriums are most successfully propagated via somatic organogenesis.

2. 1. 1 Surface Sterilization

Anthurium andreanum cv. tropical pink, premium red, white and tulip were surface sterilized by soaking in 32.5 per cent Benlate solution for 24h, further

sterilized by rinsing in 70 per cent alcohol for 45 seconds and soaking in 1.25% sodium hypochlorite for 15 minutes later rinsed 3 times in sterile distilled water for 15 minutes (Lightbourn and Prasad, 1990).

Dichloro iso cyanurate was found to be a very good disinfectant for micropropagation of plants. The microbial flora of the micropropagated plants showed a wide range of bacteria with predominantly Pseudomonas, Xanthomonas and Actinomyces. Parkinson *et al.* (1996) concluded from his work that the use of sodium dichloro iso cyanurate was highly stable both as tablets and as a solution maintained at the room temperature. Sterilization of shoots cultures which were heavily contaminated with bacteria was examined to identify the organism. It was also found that surface sterilization with dichloroisocyanurate was found to be effective as sterilization with a combination of mercuric chloride and calcium hypochlorite.

2. 1. 2 Explants and Medium

Leaf explants were found to produce callus in one and a half to two months on MS medium supplemented with 2 mg kinetin per litre. Regeneration was found to take a fairly long period of time and found to have a multiplication rate of 6 to 9 which varies with the genotype (Keller *et al.*, 1982). According to Malhotra *et al.* (1998) the leaf explants were found to be responsive for the *Anthurium andreanum* cv. Nitta, Osaki and Anouchka when cultured on modified MS medium having reduced concentration of ammonium sulphate at 200 mg per litre, supplemented with BA at 1 mg per litre and 2, 4-D at 0.1 mg per litre to get callus induction. Montes *et al.* (2000) concluded that the leaf explants from *Anthurium cubense*, as an alternative to *in vitro* culture of seeds, was used to produce white callus mass when subcultured on medium containing 4.7 μM Pectimorf to obtain a regeneration rate of up to 17 buds per explant.

Zens and Zimmer (1986) used shoot tip explants to produce callus cultures and adventitious shoots were found to increase significantly by changing the

ammoniacal and nitrate nitrogen levels from 1:1 to 1:5. The increase in productivity was inferred to differ between clones. Geier (1986 a, b) reported to have a nitrogen level of 200mg per litre to get regeneration in all the genotypes he tried. But Zimmer (1990) was of the opinion that seed explants when cultured *in vitro* can produce plantlets. These plants were used for clonal multiplication. The plantlets were maintained for a period of two to eleven years without variation.

Singh *et al.* (1991) investigated on spadix explants to get better capacity for regeneration than leaf segments on the modified Nitsch medium and plantlets derived from spadix segments were less variable. He also reported that the variation created was unable to be concluded to be of epigenetic or genetic variation which he found to occur at a very low rate.

Kuehnle and Sugii (1991) utilized petiole explants to dedifferentiate into callus on Pierik, modified Pierik and Fennie and Vanstaden medium. Cen *et al.* (1995) compared the effects of different levels of sucrose and glucose and found that three per cent glucose had the greatest inductive effect on callus formation. Petiole explants were found to be good in *Anthurium andreanum* cv. Mauritius orange, collected from the eight month old *in vitro* grown plantlets of selfed seed progenies over a culture duration of six to eight weeks in dark (Prakash *et al.*, 2001).

Zhang-Gui He *et al.* (2001) reported to have higher callus induction in *Anthurium andreanum* cultures on MS medium supplemented with 0.1 mg of BA per litre. About 1 cm size of the callus cultures were obtained in one month old cultures. In contrary to this Prakash *et al.* (2002) opined that *Anthurium andreanum* cv. Liver Red grown in Nitsch basal medium with coconut water facilitated faster germination of the embryo produced *in vitro*. But Yang-Yuan Hsin *et al.* (2002) confirmed the earlier report by culturing the lamina on a MS medium supplemented with 0.1 to 0.5 mg TDZ per litre that induced callus

formation in 98 to 100 percentages of the cultures. Whereas Lan-Qin Ying *et al.* (2003) concluded that promising results were recorded on N_6, Knudson C and ½ MS media for the leaf blade explant.

2. 1. 3 Callus Multiplication

Pierik *et al.* (1974 a, b) succeeded in callus induction and regeneration from embryo and tissues collected from seedlings. Later they were able to regenerate plantlets from non meristematic parts of mature plants. A modified MS medium supplemented with Cytokinin PBA [6- (benzyamino)-9-(2-tera hydro pyranyl)-9-purine] was used for callus induction. Optimum callus growth was obtained by incubation at 25°C in dark. They also reported that only 30 percentage of the adult genotypes studied were capable of forming callus. Pierik *et al.* (1975) studied thirty eight genotypes of *Anthurium andreanum*, and observed moderate to strong callus formation from leaf segments in thirty one types; very poor callus in four types and no response in three genotypes. According to Pierik (1975) there were striking differences in the growth rate among subcultured callus clones of *Anthurium andreanum.*

Callus multiplication was optimum in a liquid medium (Pierik, 1975; Pierik *et al.*, 1975). For this, leaf pieces with the callus were transferred to a liquid medium and placed on a shaker rotating at 120 rpm. Based on the detailed studies a scheme was proposed for the micropropagation of *Anthurium andreanum* and *Anthurium scherzerianum* by Pierik and Steegmans (1975), Pierik (1976) and Pierik *et al.* (1979 a). Leffering *et al.* (1976 a) and Leffering and Hoogstate (1977) found a close correlation between the ability of leaf explants to form callus and the further growth in subcultures. They also reported that in most genotypes callus growth was too slow and inconsistent to be exploited for large scale micropropagation. An alternative scheme was developed by Leffering and Soede (1978; 1979 a, b) made use of shoot proliferation as a means of multiplication. In this process multiple shoots were regenerated using 2-iP and BA from the callus developed by inoculation of leaf

lamina explants. They also found that the plantlets developed were more uniform. This was supported by Yu-kwang Jin *et al.* (1995) who opined that callus culture was more promising than shoot culture for micropropagation.

Geier (1986 a) inferred that *Anthurium scherzerianum* can be stimulated to produce callus on medium containing 0.1 mg per litre of 2,4-D and 1 mg per litre BA. These calli were found to gain regeneration on a medium with out 2, 4-D and with less BA at 0.2 to 0.5 mg per litre whereas Lightbourn and Prasad (1990) reported that Nitsch's medium with 0.05 to 1.0 mg of 2, 4–D per litre was good for better callus induction. The variety Tropical Red produced callus with in a range of 0.05 to 0.5 mg per litre of 2, 4–D.

Seven cultivars of *Anthurium andreanum* of Hawaiian types produced calli most successfully after two to three months on a modified Pierik medium containing 0.36 µM 2,4-D and 4.4 µM BA. Long term cultures of callus were maintained for twelve to thirteen months and were found to regenerate into plantlets. Adventitious plantlets were recovered from callus plated on a Kunisaki medium containing 22 µM BA (Kuehnle and Sugii, 1991).

Sreelatha *et al.* (1998) concluded that MS medium having quarter the strength of major nutrients supplemented with 2, 4-D and BA in MS medium was best for the callus induction and also suitable for callus multiplication.

This differed from the reports of Somaya *et al.* 1998 who investigated on micropropagation of *Anthurium andreanum* for rapid propagation. Seed explants were used for callus induction and found to be best for callus induction with 2.0 mg of 2, 4-D per litre while leaf, petiole, node and root explants gave the callus production with 0.1 mg 2, 4-D and 1.0 mg BA per litre.

Prakash *et al.* (2001) reported that the callus induction was good on MS medium with two per cent sucrose and 0.8 per cent agar supplemented with 2,4-

D, kinetin and BA. Callus was multiplied on the same medium by subculturing on every sixth week. Petiole explants were found to produce callus in six to eight weeks of culture duration in dark of 50 to 75 per cent of the cultures tried. Best combination found for callusing was MS with 1.0 or 0.5 mg 2, 4-D. Higher concentrations of 2, 4-D was observed to be toxic. Likewise an efficient method of plant regeneration from petiole explants was developed for *Anthurium andreanum* cv. Singapore Hybrid cultures initiated in MS medium containing IBA and kinetin with subculturing on every third day, 93.75 per cent callus induction was found in a treatment with 2 mg IBA and 6 mg kinetin per litre of MS medium (Dhananjaya and Sulladmath, 2003)

2. 1. 4 Regeneration

Novak and Nepustil (1980) obtained *Anthurium andreanum* callus clones with a high capacity for regeneration from leaf explants of flowering plants. Henny *et al.* (1988) reported that the *Anthurium andreanum* variety Southern Blush required ten to twelve months to reach marketable size in 150 mm pots. The initial cultures required full year for complete development of a rooted plantlet from callus and that was reduced in further multiplication cycle by generating multiple shoots within 5 months.

Shoot primordial initiation of *Anthurium andreanum* cv. Mauritius orange was noticed on ten month old callus cultures when transferred to MS basal medium (Prakash *et al.* 2001). Prakash *et al.*, (2002) worked on Liver Red and found that the first and the third leaves were recorded as the best explant for culturing on a medium supplemented with coconut water from 20 to 36 days after inoculation and 48 days to 88 days after inoculation respectively. In contrary Lan-Qin Ying *et al.* (2003) found that the period from explant to bud differentiation was 49 days which was 11 days to 31 days earlier than the period previously reported.

2. 1. 5 Multiple Shooting

In axillary shoot proliferation cytokinin was utilized to overcome the apical dominace of shoots and to enhance the branching of lateral buds from leaf axils. This enhanced release of axillary buds with cytokinin was discovered by Wickson and Thimman (1958).

Kunisaki (1980) obtained viable cultures of *Anthurium andreanum* from small vegetative buds as explants were grown into plantlets in a modified Murashige and Skoog (MS) medium supplemented with 15 per cent coconut water. He also observed that the best result was obtained when stem cuttings with two nodes from aseptically grown plantlets were cultured in medium containing BA 0.2 mg per litre. Higher BA concentrations produced more callus growth and stunted shoots. However, the major difficulty encountered with this route of propagation was the non availability of sterile cultures for initial explants. The high rate of contamination necessitated the use of minute explants of vegetative buds, which resulted in greater mortality of the cultures.

Seed explants after germination on Knudson C medium with 1 ppm of BAP at 28 °C found to produce 2000 plantlets in 15 months which was found to vary between genotypes (Zimmer, 1990). The seed explants inoculated were found to germinate and produced multiple shoots in about 12 weeks in 82 to 93 per cent of cultures in *Anthurium scherzerianum* in MS medium with NAA and BA. Seeds were found to produce caulogenic callus with new shoots where the productivity was mainly dependent on genotypes (Zens and Zimmer, 1986). Shoot proliferation was found to be greatest with 0.5 or 1.0 mg zeatin per litre (Soczek and Hempel, 1989).

Soczek and Hempel (1989) utilized single node fragments from the *in vitro* grown shoots placed on half strength MS medium containing BA, kinetin, zeatin or 2-iP at concentrations between 0.125 mg and 2.0 mg per litre. Callus

production increased with increasing levels of cytokinin concentration. According to Yu-kwang Jin *et al.* (1995) shoot multiplication and callus multiplication was found to be best on medium supplemented with 2iP, kinetin and BA. Callus culture was more promising than shoot culture for micropropagation. Shoot regeneration and growth were best in medium supplemented with 0.5 mg BA with 2.0 mg IAA per litre (Sreelatha *et al.*, 1998).

Geier (1987) found that the spadix and leaf segments can be used for micropropagation and some of the cultures were found to produce malformations and variations in the regenerated plants. In contrary to this Lightbourn and Prasad (1990) have taken leaf explants to inoculate for callus induction in Nitsh's basal medium and Sreelatha *et al.* (1998) developed multiple shoot in *Anthurium andreanum* on MS medium but did not found any variants.

Yang-Yuan Hsin *et al.* (2002) concluded that the shoot regeneration from the lamina of micropropagated anthurium plantlets were obtained while culturing on modified MS medium containing cytokinins. BA at 0.1 to 0.2 mg per litre or kinetin at 0.5 mg per litre induced 70 per cent of the explants to form adventitious shoots, while thidiazuron (TDZ) at 0.1 mg per litre induced only 63 per cent of the explants to form adventitious shoots. Direct plant regeneration from flowering plant derived lamina explants of *Anthurium andreanum* cv. Tinora Red and Senator was established on half strength MS medium with 1.11 µM BA, 1.14 µM IBA and 0.46 µM kinetin for shoot induction (Martin *et al.*, 2003).

Teng-Wheillan and Teng (1997) opined that by culturing homogenized inoculum in liquid or on the medium surface supported by a raft, adventitious shoots can be generated from *Anthurium andreanum*. The medium used was MS medium supplemented with 0.5 mg thiamine HCl, 0.4 mg pyridoxine HCl, 0.5 mg nicotinic acid, 20 mg Na-Fe EDTA, 15 per cent (v/v) coconut water, two per

cent sucrose, 2.2 to 4.4 μM BA and 0.9 μM 2, 4-D. The culture method as compared to solid culture did not affect the time required for inoculums to regenerate. The regeneration rate was affected mainly by inoculum size and it also influenced the regeneration frequency. All the regenerated shoots were normal, without any sign of hyperhydricity. Mu *et al.* (1999) micropropagated the anthurium plantlets after a lengthy surface sterilization procedure and kept over a medium containing BA 0.5 mg per litre.

Mass multiplication of *Anthurium andreanum* cv. Agnihotri was achieved by inoculating the axillary buds of *Anthurium andreanum* on a modified MS medium containing BA 0.8 mg per litre, vitamin B_5 0.5mg per litre, IAA 0.1 mg per litre, PVP 200 mg per litre and coconut water 150 ml per litre. The shoots were multiplied at a rate of 4.66 per explant (Mohanta and Paswan, 2001).

2. 1. 6 Rooting

Shoots isolated from callus cultured on a medium containing BA and 2, 4-D was found to produce roots when transferred to a basal medium with no growth regulators was confirmed by Geier (1986 b). The shoots regenerated with BA were found to thrive hard to develop roots and delayed in every cycle of multiplication. He also explained that the ammoniacal nitrogen at 720 mg per litre accelerated the root growth compared to 200 mg per litre. In contrary to this Lightbourn and Prasad (1990) reported that rooting was not affected by varying concentrations of ammonium nitrate but larger leaves and more prolific leaf production occurred with increased ammonium nitrate concentrations whereas for rooting of *Anthurium andreanum in vitro* developed shoots Yu-Kwang Jin *et al.* (1995) used IBA containing medium which was found to be most effective than IAA and NAA. According to Cen *et al.* (1995) half strength MS medium supplemented with 0.1 ppm NAA was best for inducing root formation.

Li-Jing and Li-J (1997) worked to find out the tissue culture technique which can be utilized for successful multiplication of the ornamental plant, *Anthurium*

andreanum. The plantlets were found to produce roots *in vitro* only when there was sufficient supply of exogenous auxins. Somaya *et al.* (1998) investigated on rooting of the cultured shoots and obtained the best combination on MS medium with 0.25 mg NAA per litre which increased the quality and number of roots produced.

Prakash *et al.* (2001) excised shoots raised from the shoot primordia of callus cultures to produce roots on MS basal medium with five per cent sucrose. Highest rooting of 80 per cent was observed by Mohanta and Paswan (2001) in MS basal medium supplemented with IAA 1.0 mg per litre. The survival rate was recorded as 60 per cent on Soilrite and Perlite 10: 1 mixture. Zhang-GuiHe *et al.*, (2001) inferred that the cultures tend to produce roots *in vitro* on subculturing into MS medium supplemented with 0.1 mg IBA per litre or 0.1 mg NAA per litre. The plantlets were formed easily with a survival rate of 85 per cent and more.

Dhananjaya and Sulladmath (2003) studied and reported that the MS medium supplemented with 1.5 mg IBA and 5 mg kinetin per litre was good for optimum root production *in vitro*. The root induction of the regenerated shoots were found optimum on half strength medium containing 0.54 µM NAA and 0.93 µM kinetin. More than 300 plantlets of each cultivar were harvested from a single source lamina within 200 days of culture out of which 95 per cent plantlets survived after acclimatization (Martin *et al.*, 2003).

2. 1. 7 Somatic Embryogenesis

Somatic embryogenesis is the development of embryos from somatic cells (Mascarenhas, 1989). Kuehnle *et al.* (1992) was the first to study the somatic embryogenesis in anthurium from callus. The production of secondary embryo was induced on the surface of the primary embrogenic calli without affecting the preformed ones. Half strength MS medium was found to produce somatic embryos with 1.0 to 4.0 mg per litre 2, 4-D and 0.33 to 1.0 mg per litre kinetin

which was having one per cent glucose and two per cent sucrose. Embryos were found to regenerate, in a medium containing 0.2 mg per litre BA and two per cent sucrose and placed in the light for conversion into plantlets.

Rajasekaran and Mohankumar (1994) could successfully induce somatic embryogenesis from leaf explants collected from the field using Nitsch and Nitsch (1965) as the basal medium.

Matsumato *et al.* (1996) has done histological analysis of somatic embryos derived from *in vitro* cultured laminas of *Anthurium andreanum* which showed bipolarity with the presence of shoot and root poles connected by pro-cambium. Vascular connections between the explants and somatic embryos were not observed. Storage of proteins, starch raphides as well as a suspensor like structure and an epidermis were observed in the somatic embryos. The origin of each somatic embryo was from a pro-embryonic cell complex or possibly from a single cell by direct embryogenesis. Both modes of somatic embryogenesis were found to raise from the mesophyll. When Hamidah *et al.* (1997a, b) supplemented the media with 18μM 2, 4-D and 6 per cent sucrose the leaf explants were found to produce micropropagated plantlets. Somatic embryos were found to regenerate on medium containing 0.46 μM kinetin.

2. 1. 7.1 Synthetic seed production

Synthetic seeds have been proposed as a new low cost, high efficiency propagation system which has opened new avenues in storage and delivery of new plant lines produced through biotechnological advances; encapsulation of cells, embryo, somatic tissue and somatic embryo have been attempted in several crops and has become increasingly popular as a simple way of handling cells, tissue and embryo and protecting them against external gradients and it is also an efficient storage and delivery system.

The concept of somatic embryo encapsulation to produce an analogue to true seeds was based on the similarity of somatic embryos with zygotic embryos in terms of morphology, physiology and biochemistry.

2. 1. 8 Factors affecting the *in vitro* growth

2. 1. 8.1 Macro Elements

The effect of macro element strength on the culture growth was studied by Yu-Kwang Jin *et al.* (1995) with varying concentrations of macroelement strength at 0.3,0.5,0.8,1.0,1.5 or 2 X of macroelements in MS medium on the organogenesis of shoot tip cultures of *Anthurium andreanum* and *Anthurium scherzerianum*. Shoot growth *in vitro* was enhanced on medium containing 0.3 to 0.8 X strength of macroelements. Symptoms of nutrient deficiencies were observed in plantlets as the culture duration was prolonged to a period of 6 months. MS basal medium was found to be the most effective medium for the multiplication and growth of shoots over a period of six months.

2. 1. 8. 2 Light Intensity

As per the reports of Lan-Qin Ying *et al.* (2003) different light treatments did not affect the callus induction in the petiole significantly, but the 24h and 10h per day light treatments were found to improve the bud differentiation compared to no light treatment.

2. 1. 8. 3 Systemic Infestation

Mu *et al.*, 1999 found that the *in vitro* cultivation can be used to eliminate the *Xanthomonas campestris pv. dieffenbachiae* which is a serious menace and found to be present in more than 12 per cent of the total field grown plants. This has also been reported by Kamemoto *et al.* (1990).

Tanabe *et al.* (1990) also inferred that the anthurium blight causing agent *Xanthomonas campestris pv. dieffenbachiae* survived in the tissue cultured medium for long time which also exhibited a cultivar based susceptibility to

pathogen on tissue cultured plants. Infections in *in vitro* were found not to express the blight symptom.

Norman and Alvarez (1994) opined that the latent infections of tissue cultured *Anthurium andreanum* caused by *Xanthomonas campestris pv.dieffenbachiae* were found to occur in the cultures for more than a year within the secondary shoots and was the primary source of infestation in the field.

2. 1. 9 *Ex vitro* Establishment

Dhananjaya and Sulladmath, (2003) have transferred the plantlets derived *in vitro* into a sterilized hardening medium containing coffee cherry husk, farm yard manure, soil and sand at 2:1:1:1 ratio. The survival percentage of the hardened plants was 80 per cent.

2. 2 MOLECULAR CHARACTERIZATION

With the advent of molecular markers, a new generation of markers has been introduced over the last two decades, which has revolutionized the entire scenario of biological sciences. DNA-based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, etc. They are no longer looked upon as simple DNA fingerprinting markers in variability studies or as mere forensic tools. Ever since their development, they are constantly being modified to enhance their utility and to bring about automation in the process of genome analysis. The discovery of PCR (polymerase chain reaction) was a landmark in this effort and proved to be an unique process that brought about a new class of DNA profiling markers. This facilitated the development of marker-based gene tags, map-based cloning of important genes, variability studies, phylogenetic analysis, synteny mapping, marker-assisted selection of desirable genotypes, etc. Thus giving new dimensions to concerted efforts of breeding and marker-aided selection that can reduce the time span of developing

new and better varieties will make the dream of super varieties come true. These DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analysed objectively (Joshi *et al.*, 1999).

Morphological and biochemical markers have been used successfully in the past to differentiate among the existing varieties. Markers like isozymes, which are protein based markers, have been used in the identification of polymorphisms (Gupta *et al.*, 2000). Different varieties cannot always be distinguished by the conventional methods because they are too similar, they have not grown to the stage in which the distinctive traits appear or their distinguishable traits are obscured by environmental factors (Buldewo and Jawfeerally, 2002). In such cases, molecular markers can be used.

2. 2.1 Molecular studies in anthurium

2. 2.1.1 DNA isolation

Hoopes and McClure (1981) investigated on the extraction of high quality DNA from spathe and leaf tissues. They found that the spathe tissues contain lower amounts of inhibitors and contaminants than the leaves and the spermine precipitation step was found to be required when using leaf tissues to remove contaminants and inhibitors. They could get amplifiable DNA only after spermine precipitation of DNA extracted using the CTAB protocol. They further modified Rowhani protocol for the extraction of clean amplifiable DNA from *Anthurium andreanum* without making use of hazardous compounds to obtain milligrams of high quality DNA from spathe tissue and found this to be ideal for extracting large amounts of anthurium DNA for further molecular use.

In *Anthurium andreanum* Buldewo and Jawfeerally (2002) studied different methods of DNA isolation. Those standard methods were found to produce DNA contaminated with polysaccharide like components and secondary metabolites. As per the reports of Rether *et al.* (1993) these contaminants co-

precipitate along with the DNA during ethanol precipitation and adversely affect the purity and suitability of the isolated DNA for further molecular use.

2. 2.1.2 RAPD in anthurium

Wang Jauyueh *et al.* (1999) in a study with RAPD markers for identification and genetic diversity analysis of anthurium cultivars derived twenty four DNA fragments from eight primers which were polymorphic and were used to distinguish fifteen anthurium cultivars. Cluster analysis from 160 RAPD fragments that were all amplified by eight primers divided the fifteen cultivars into three main groups. The results suggested that RAPD markers are useful in current breeding programmes of anthurium, allowing cultivar identification as well as the estimation of genetic similarity among genotypes which will be valuable in selecting the best parents to obtain new hybrid combinations.

In anthurium Ranamukhaarachchi *et al.* (2001) found that the RAPD markers were utilized to determine the genetic relationships of nine morphologically similar pot plant cultivars of anthurium. Of the twenty five arbitrary primers screened, nine generated DNA fingerprints that were used in computing the genetic distances and similarity co-efficient values. In this study using RAPD it was possible to distinguish the anthurium pot plant cultivars and their genetic relationships.

2. 2. 2 AP-PCR

By arbitrarily amplifying the variable regions of a genome using the methods proposed by Welsh and McClelland (1990) known as AP-PCR one can isolate or amplify specific DNA fingerprint in a reproducible manner. AP-PCR is cost effective procedure as well as having in-discriminatory ability for genotyping isolates Camarena *et al.* (1998).

Camarena *et al.* (1998) reported to have different stringencies in thermal cycling programme. They designed the programme with denaturation at 94°C for 5 min, followed by 44 cycles of 94°C for 1 min and ramp of 3 min 52 sec to 36°C for 1 min, a ramp of 2 min 24 sec to 72°C for 1min and a ramp of 1 min 18 s to 94°C followed by 10 min of extension at 72°C.

As per the reports of Kohler and Friedt (1999) for genetic characterization of sunflower interspecific hybrids using AP-PCR the annealing temperature of the primers was divided into groups of similar melting - point temperatures, while temperatures were set at 10 to 12°C lower than the average melting point temperature following the method proposed by Itakura *et al.* (1984). The thermal cycler was programmed for a first denaturation step of 1 min at 94°C followed by 45 cycles of 1min at 94°C, 1min at primer specific temperature and 2min at 72°C. Amplification products were resolved by gel electrophoresis in 2 per cent agarose in 0.5 per cent TBE along with 100bp ladder. Consistent AP-PCR can be achieved over a fairly broad range of temperatures. The primers ranging in size from 20 to 34 bases give a different pattern but have similar temperature dependent characteristics.

Materials and Methods

3. MATERIALS AND METHODS

The present study was undertaken in the Department of Plant Breeding and Genetics and the Biotechnology Laboratory attached to the department, College of Agriculture, Vellayani, Kerala during 2003 to 2006 to standardize the technique for micropropagation and molecular characterisation of selected hybrids and their parents with RAPD using AP-PCR markers in *Anthurium andreanum* Linden.

The materials and methods tried for the *in vitro* multiplication, Random Amplified Polymorphic DNA (RAPD) using AP-PCR are described in this chapter.

3.1 MATERIALS

The following twelve varieties and twelve hybrid genotypes of *Anthurium andreanum* showing variations in spathe colour, shape and size and other commercially valuable morphological characters generated through hybridization at the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani were utilized for the study.

List of selected varieties and hybrids used for the present investigations

3.1.1 Varieties

1. LR (Liver Red)
2. PR (Pompon Red)
3. W (White)
4. LJ (Lady Jane)
5. DT (Dragon's Tongue Red)
6. OG (Orange Glory)
7. KR (Kalympong Red)

Plate 1. Field view

8.	TR	(Tropical Red)
9.	OO	(Ordinary Orange)
10.	KO	(Kalympong Orange)
11.	MW	(Merengue White)
12.	FK	(Fla King)

3.1.1 Hybrids

13.	LJ X W	(Lady Jane X White)
14.	W X LJ	(White X Lady Jane)
15.	LJ X LR	(Lady Jane X Liver Red)
16.	PR X LR	(Pompon Red X Liver Red)
17.	PR X MW	(Pompon Red X Merengue White)
18.	PR X DT	(Pompon Red X Dragon's Tongue Red)
19.	PR X LJ	(Pompon Red X Lady Jane)
20.	FK X DT	(Flaking Red X Dragon's Tongue Red)
21.	FK X LR	(Flaking Red X Liver Red)
22.	OG X DT	(Orange Glory X Dragon's Tongue Red)
23.	OO X KR	(Ordinary Orange X Kalympong Orange)
24.	TR X MW	(Tropical Red X Merengue White)

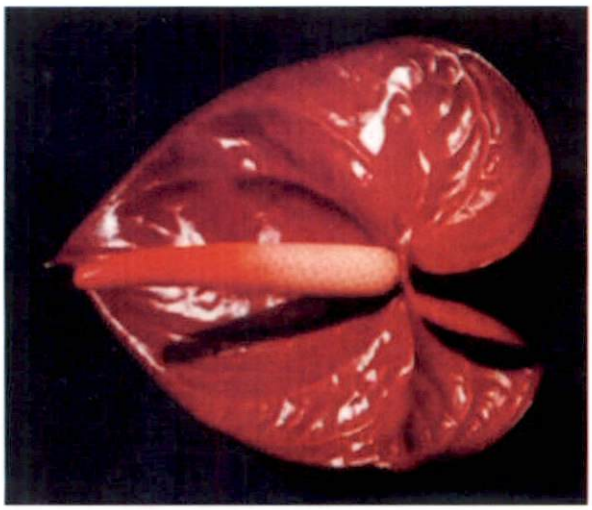
3.2 *IN VITRO* MULTIPLICATION

3.2.1 Explants

Explants of anthurium were taken from actively growing young plants. Segments of fully opened copper coloured tender leaves, petiole, candle, seed, shoot tip and immature unopened spadix were taken as explants for culture.

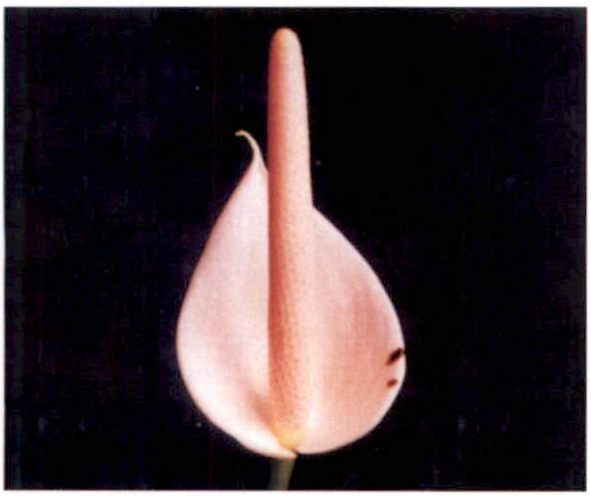
3.2.2 Collection and Preparation of Explants

Tender leaves with light copper brown colour of two to three days after unfurling, the distal portion of the petiole and immature unopened spadix with spathe were collected by excision with their petioles and stalks in an ice box. The cut ends were kept immersed in water inside the ice box. Culturing was

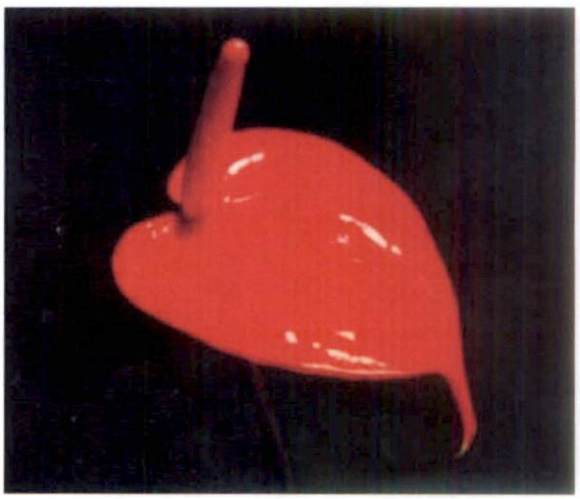


A. Liver Red

B. Pompon Red



C. White

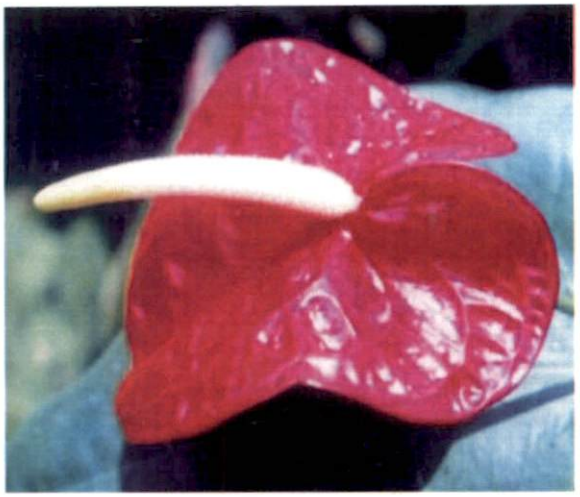


D. Lady Jane

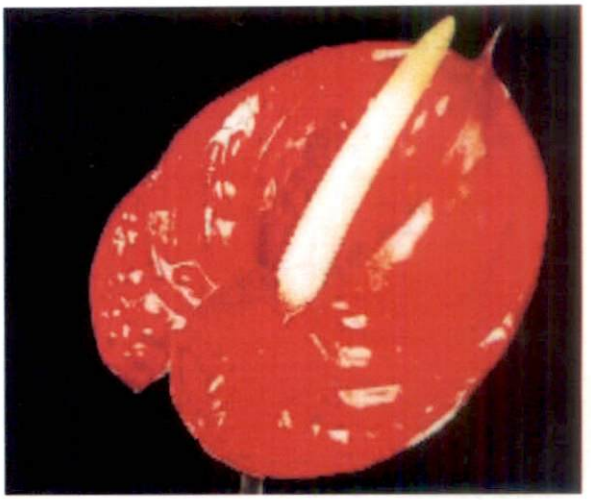
E. Dragon's Tongue

F. Orange Glory

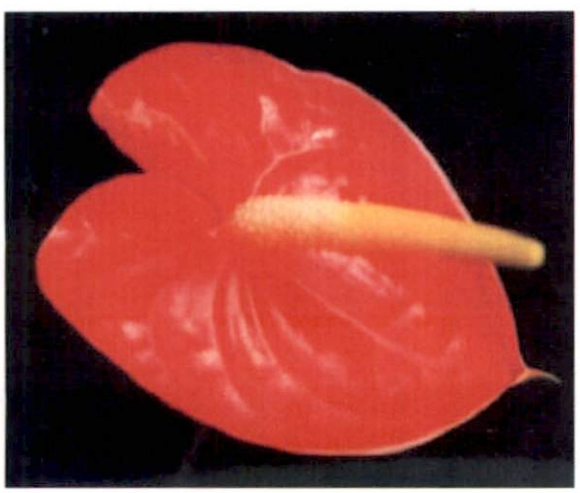
Plate 2. List of parents I



A. Kalympong Red

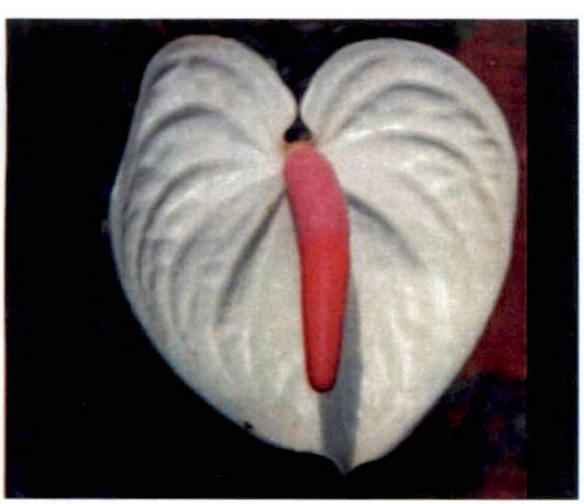


B. Tropical Red

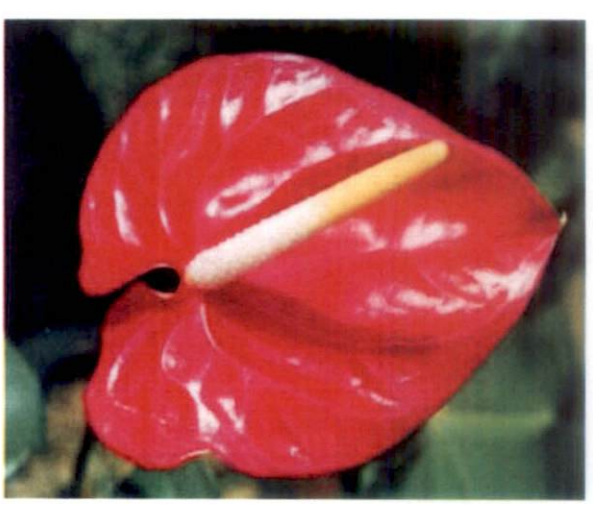


C. Ordinary Orange

D. Kalympong Orange

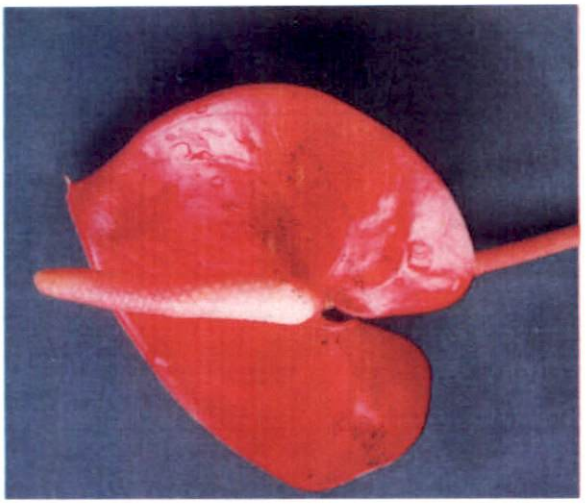


E. Merengue White

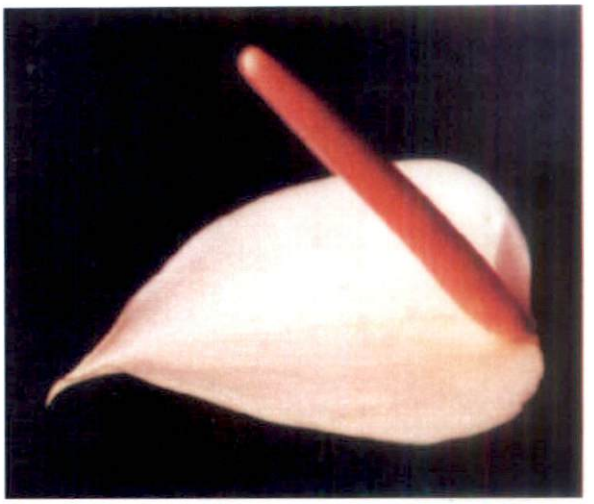


F. Fla King

Plate 3. List of parents II

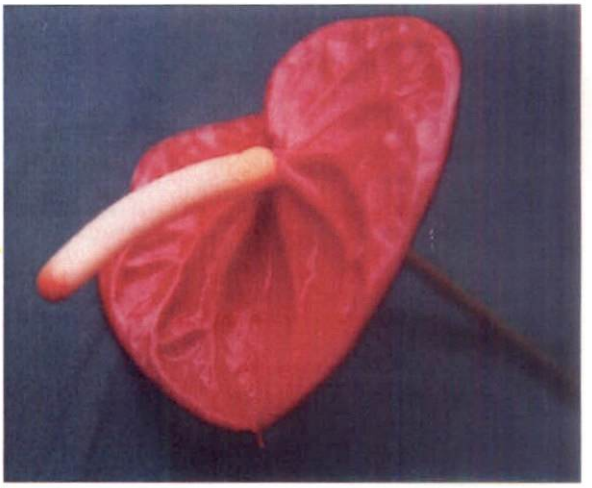


A. LJ X W



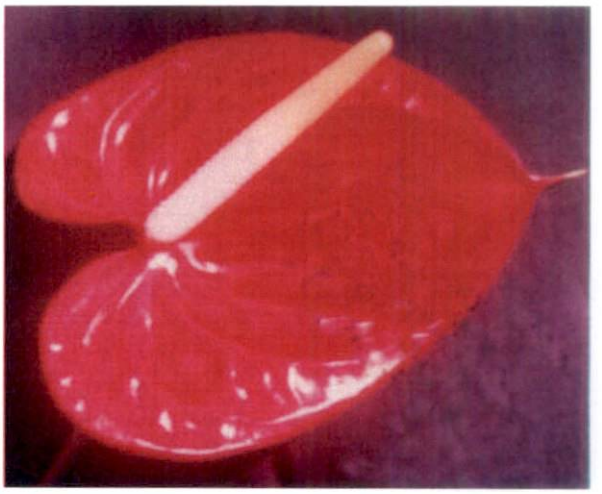
B. W X LJ

C. LJ X LR



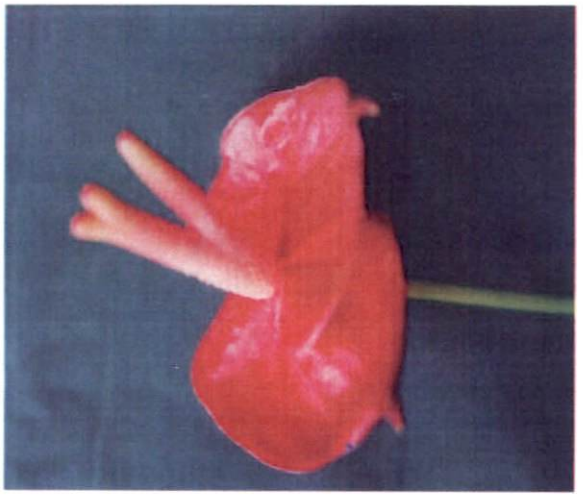
D. PR X LR

E. PR X MW



F. PR X DT

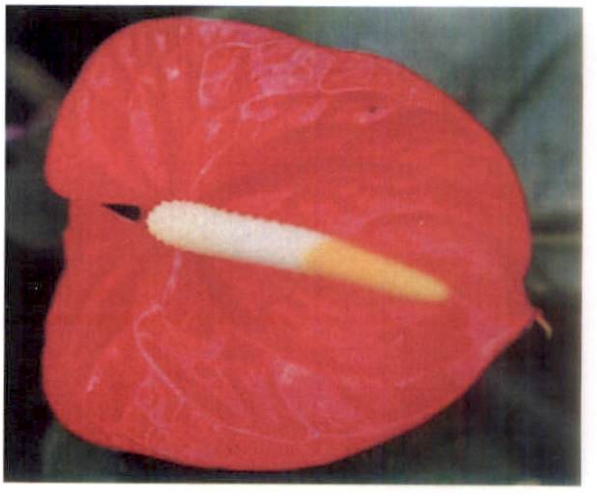
Plate 4. List of hybrids I



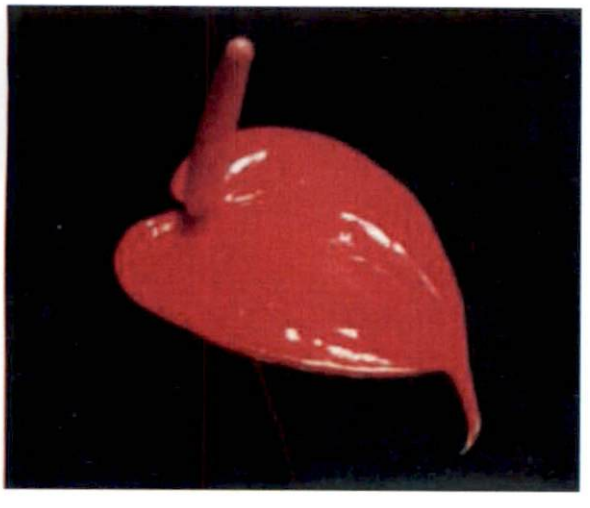
A. FK X DT

B. FK X LR

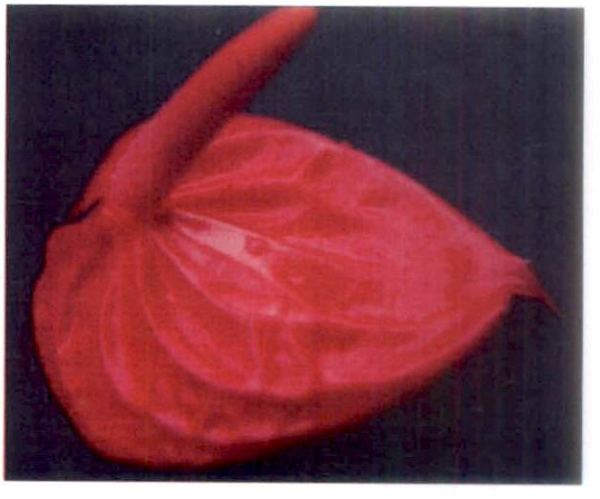
C. OG X DT



D. OO X KR



E. LJ X PR



F. TR X MW)

Plate 5. List of hybrids II

done within two to six hours after excision. The leaves were thoroughly washed in running tap water. The petioles and leaf lamina were separated and washed in distilled water containing few drops of a wetting agent viz. 'labolene'. They were further washed with distilled water to remove the traces of 'labolene'.

If the explants are collected during monsoon season and immediately after rainfall there will be high rate of microbial contamination. To prevent this, washed explants were treated with a systemic fungicide Bavistin (0.1%) for 20 minutes and antibiotic Streptomycin at 50 mg per litre for 10 minutes prior to surface sterilization.

3.2.3 Surface Sterilization

Surface sterilization of explants was carried out inside a laminar air flow chamber. The explants were dipped in 95 % Ethyl alcohol for 30 seconds and rinsed thoroughly in sterile double distilled water. The explants were cut into larger pieces for easy handling during sterilization. After cutting explants were treated using the surface sterilization agents and different treatment duration were employed as per Table 1. All the treatments were replicated twelve times. The explants after surface sterilization were rinsed thrice with sterile double distilled water.

Two varieties (Liver Red and Dragon's Tongue) and two hybrids (Ordinary Orange X Kalympong Red and Orange Glory X Dragon's Tongue) were selected as representative samples to standardize initial culture conditions. These lines and hybrids were subjected to different treatments. All the treatments were replicated twelve times. To have observations on survival percentage, callus multiplication, callus index and regeneration, twenty culture tubes free from contamination were considered to calculate the percentage response. Observations were recorded on the number of surviving cultures, callus initiation, callus multiplication, regeneration and rooting.

3.2.4 Media

The basal media used for the study were MS medium (Murashige and Skoog, 1962) and NN (Nitsch and Nitsch medium) at half and full strength as per Table

Table 1. Treatments tried to assess the effect of surface sterilization

S.No.	Treatment No.	Treatments	Duration
1.	TS_1	1.00 % Sodium hypochlorite	12 minutes
2.	TS_2	0.08 % Mercuric chloride	10 minutes
3.	TS_3	0.50 % Calcium hypochlorite	15 minutes
4.	TS_4	70.00 % Ethyl alcohol	20 minutes
5.	TS_5	95.00 % Ethyl alcohol 0.08 % Mercuric chloride 70.00 % Ethyl alcohol	1 minute 10 minutes 3 minutes

8 and Table 10. The chemicals used for the preparation of the culture media were of Analar Grade (AR) obtained from SRL, Merck and CDH. Standard procedures were followed for the preparation of media (Thorpe, 1980). Stock solutions of major and minor nutrients were prepared by dissolving the required quantity of chemicals in exact volume of double glass-distilled water. Plant growth regulators were first dissolved in 1N, NaOH or 95 per cent Ethanol and the volume made up with double glass-distilled water. The stock solutions were stored under refrigerated condition at 4 ° C.

The culture vessels used were 'Borosil' test tubes (25 X 150mm), Erlenmeyer flasks (150ml) and disposable Petri plates. They were kept for overnight soaking in diluted KOH solution and then cleaned thoroughly using 'Labolene', tap water and finally rinsed with double glass-distilled water. The washed glass wares were kept in hot air oven at 60 ° C for 2h to make them dry. They were autoclaved at 121 ° C and 1.06 Kg / cm^2 pressure for 45 minutes.

To prepare the media specific quantities of stock solutions were pipetted out into a 1000ml beaker. Sucrose and Myo-inositol were freshly added and dissolved. For specific treatments other additives were directly added while preparing the media.

Coconut water was collected fresh from freshly harvested eight month old tender coconuts. It was filtered using Whatman No.1 filter paper and added to the media. After adding all the media composition, except Agar and charcoal, the liquid mixture was subjected to pH checking. The pH was adjusted to 5.7 using 0.1 N NaOH and 0.1 N HCl using an electronic pH meter.

Agar was added to the medium while kept for boiling in a microwave oven with intermittent stirring using a glass rod till the Agar melts. Activated charcoal was added at this stage and mixed thoroughly to have uniform distribution. The medium was then poured into the pre-sterilized culture vessels using a glass

Table 2. Treatments tried to assess the effect of media on survival of explant

S. No.	Treatment No.	Treatments
1.	TM_1	½ MS
2.	TM_2	½ MS + AC
3.	TM_3	½ MS + AC + CW
4.	TM_4	M- MS
5.	TM_5	M- MS + AC
6.	TM_6	M- MS + AC + CW
7.	TM_7	½ NN
8.	TM_8	½ NN + AC
9.	TM_9	½ NN + AC + CW
10.	TM_{10}	M-NN
11.	TM_{11}	M-NN + AC
12.	TM_{12}	M-NN + AC + CW

MS – Murashige and Skoog medium

NN – Nitsch and Nitsch medium

AC – Activated Charcoal

CW – Coconut Water

funnel. The mouth of the culture vessels were plugged tightly with sterilized cotton, covered with aluminium foil, labelled and autoclaved at 121 °C and 1.06 Kg / cm^2 pressure for 20 minutes. After sterilization, the culture vessels were transferred to the culture room.

3.2.4.1 Inoculation

The tools and glass wares (blades, forceps, petri plates and beakers) required for inoculation were washed thoroughly, rinsed with double distilled water, covered with aluminium foil and kept inside an autoclavable poly propylene cover to autoclave at 121 °C and 1.06 Kg / cm^2 pressure for 40 minutes.

The cut ends of the sterile explants were excised off and then made into small pieces in a sterile petri plate with a sterile scalpel. The water droplets were finally removed on a sterilized blotting paper placed over a sterile petri dish.

To inoculate the explants on the culture medium, the cotton plugs were removed in front of the spirit lamp flame and the explants were placed on the medium.

3.2.4.2 Incubation

The inoculated test tubes were incubated at 25 °C in dark for callus induction and in light for shoot regeneration. Subculturing was done every three to four weeks. The explants were kept inoculated in different media composition to find out a suitable medium for further multiplication (Table 2, 9 and 11).

3.2.4.3 Callus Induction

The explants after standardizing for the survival on suitable media were further cultured on selected media with different hormone concentrations to get maximum callus induction (Table 3). For callus induction the culture flasks were kept in dark at 25 °C and subcultured every third week.

Table 3. Treatments tried to assess the effect of media and hormones on callus induction

S. No.	Treatment No.	Treatments
1.	TC_1	TM_3 + 10.74 µM NAA + 3.62 µM 2,4-D + 9.12 µM zeatin
2.	TC_2	TM_3 + 5.37 µM NAA + 2.26 µM 2,4-D + 9.12 µM zeatin
3.	TC_3	TM_3 + 3.62 µM 2,4-D + 9.12 µM zeatin
4.	TC_4	TM_3 + 9.12 µM zeatin
5.	TC_5	TM_6 + 10.74 µM NAA + 3.62 µM 2,4-D + 9.12 µM zeatin
6.	TC_6	TM_6 + 5.37 µM NAA + 2.26 µM 2,4-D + 9.12 µM zeatin
7.	TC_7	TM_6 + 3.62 µM 2,4-D + 9.12 µM zeatin
8.	TC_8	TM_6 + 9.12 µM zeatin
9.	TC_9	TM_9 + 10.74 µM NAA + 3.62 µM 2,4-D + 9.12 µM zeatin
10.	TC_{10}	TM_9 + 5.37 µM NAA + 2.26 µM 2,4-D + 9.12 µM zeatin
11.	TC_{11}	TM_9 + 3.62 µM 2,4-D + 9.12 µM zeatin
12.	TC_{12}	TM_9 + 9.12 µM zeatin
13.	TC_{13}	TM_{12} + 10.74 µM NAA + 3.62 µM 2,4-D +9.12 µM zeatin
14.	TC_{14}	TM_{12} + 5.37 µM NAA + 2.26 µM 2,4-D + 9.12 µM zeatin
15.	TC_{15}	TM_{12} + 3.62 µM 2,4-D + 9.12 µM zeatin
16.	TC_{16}	TM_{12} + 9.12 µM zeatin

3.2.4.4 Callus Multiplication

Callus induced were inoculated into different media compositions (Table 4) for faster multiplication. For this purpose a set of callus cultures were kept in dark and another in illumination.

3.2.4.5 Regeneration of Plantlets

To obtain plantlets the callus inoculated on different media compositions for regeneration (Table 5) were transferred to illumination at photon flux intensity of 30 to 50 μmol m^{-2} s^{-2} for 12 h per day at 25 ° C.

3.2.4.6 Somatic Embryogenesis

Callus cultures were transferred to media having different auxin and vitamin-H (D-biotin) concentrations (Table 6) for somatic embryo formation. The development of embryos *in vitro* was observed in binocular microscope with 40x and 100x objectives.

3.2.4.7 Synthetic Seed Production

Calli were transferred to a culture medium with different levels and combinations of hormones. The treatments were replicated thrice consisting twenty tubes per treatment. The per cent cultures inducing somatic embryos in each treatment were recorded.

3.2.4.7.1 Maturation and Germination

Somatic embryos were inoculated on to germination medium half strength NN supplemented with 1μM GA3. The sprouts were then transferred to shooting medium.

3.2.4.7. 2 Synthetic Seeds from Somatic Embryos

Somatic embryos were preconditioned on half strength NN medium with 0.5 M sucrose and 0.65 per cent agar. This was incubated at 25°C under 12 h photo period at a photon flux intensity of 30 to 50 μmol m^{-2} s^{-2} for one week.

Table 4. Treatments tried to assess the effect of media and hormones on callus multiplication

S. No.	Treatment No.	Treatments
1.	TN_1	TM_3 + 4.52 µM 2,4-D + 18.59 µM kinetin
2.	TN_2	TM_3 + 4.52 µM 2,4-D + 9.12 µM zeatin
3.	TN_3	TM_3 + 9.12 µM zeatin
4.	TN_4	TM_6 + 4.52 µM 2,4-D + 18.59 µM kinetin
5.	TN_5	TM_6 + 4.52 µM 2,4-D + 9.12 µM zeatin
6.	TN_6	TM_6 + 9.12 µM zeatin
7.	TN_7	TM_9 + 4.52 µM 2,4-D + 18.59 µM kinetin
8.	TN_8	TM_9 + 4.52 µM 2,4-D + 9.12 µM zeatin
9.	TN_9	TM_9 + 9.12 µM zeatin
10.	TN_{10}	TM_{12} + 4.52 µM 2,4-D + 18.59 µM kinetin
11.	TN_{11}	TM_{12} + 4.52 µM 2,4-D + 9.12 µM zeatin
12.	TN_{12}	TM_{12} + 9.12 µM zeatin

Table 5. Treatments framed to find out the influence of media and hormones on regeneration of plantlets

S. No.	Treatment No.	Treatments
1.	TR_1	TM_3 + 17.76 µM BA + 11.42 µM IAA + 2.05 µM biotin
2.	TR_2	TM_3 + 22.2 µM BA + 11.42 µM IAA + 4.09 µM biotin
3.	TR_3	TM_3 + 17.76 µM BA + 5.71µM IAA
4.	TR_4	TM_3 + 5.71µM IAA + 4.09 µM biotin
5.	TR_5	TM_6 + 17.76 µM BA + 11.42 µM IAA + 2.05 µM biotin
6.	TR_6	TM_6 + 22.2µM BA + 11.42 µM IAA + 4.09µM biotin
7.	TR_7	TM_6 + 17.76 µM BA + 5.71µM IAA
8.	TR_8	TM_6 + 5.71µM IAA + 4.09 µM biotin
9.	TR_9	TM_9 + 17.76 µM BA + 11.42 µM IAA + 2.05 µM biotin
10.	TR_{10}	TM_9 + 22.2µM BA + 11.42 µM IAA + 4.09µM biotin
11.	TR_{11}	TM_9 + 17.76 µM BA + 5.71µM IAA
12.	TR_{12}	TM_9 + 5.71µM IAA + 4.09 µM biotin
13.	TR_{13}	TM_{12} + 17.76 µM BA + 11.42 µM IAA + 2.05 µM biotin
14.	TR_{14}	TM_{12} + 22.2µM BA + 11.42 µM IAA + 4.09µM biotin
15.	TR_{15}	TM_{12} + 17.76 µM BA + 5.71µM IAA

Table 6. Treatments fixed for induction of somatic embryogenesis

S. No.	Treatment No.	Treatments
1.	TSE_1	TM_3 + 11.42 µM IAA + 4.09 µM biotin
2.	TSE_2	TM_3 + 5.71µM IAA
3.	TSE_3	TM_3 + 5.71µM IAA + 4.09 µM biotin
4.	TSE_4	TM_6 + 11.42 µM IAA + 4.09µM biotin
5.	TSE_5	TM_6 + 5.71µM IAA
6.	TSE_6	TM_6 + 5.71µM IAA + 4.09 µM biotin
7.	TSE_7	TM_9 + 11.42 µM IAA + 4.09µM biotin
8.	TSE_8	TM_9 + 5.71µM IAA
9.	TSE_9	TM_9 + 5.71µM IAA + 4.09 µM biotin
10.	TSE_{10}	TM_{12} + 11.42 µM IAA + 4.09µM biotin
11.	TSE_{11}	TM_{12} + 5.71µM IAA
12.	TSE_{12}	TM_{12} + 5.71µM IAA + 4.09 µM biotin

3.2.4.7. 3 Encapsulation

Preconditioned embryos were suspended in calcium free half strength NN medium (devoid of glycine) supplemented with 1.5 per cent sodium alginate and 0.5 M sucrose. This mixture was dispensed with a micropipette into 0.1 M calcium chloride.

3.2.4.7. 4 Preculture

Twenty minutes after encapsulation beads were pre cultured on modified half strength NN liquid medium supplemented with 0.75 M sucrose and three per cent DMSO in to 100 ml Erlenmeyer flasks for one day with out agitation. Beads were then transferred to fresh medium of same composition and incubated in darkness at 4°C for three days.

3.2.4.7. 5 Dehydration and Cryopreservation

Beads were desiccated in a sterile laminar air flow chamber. Dehydrated beads were transferred to 4 ml cryo vials and stored at – 80°C. On rewarming over a water bath at 25°C, the beads were transferred to culture medium for further evaluation of their germinability.

3.2.4.8 In vitro Rooting of Plantlets

Plantlets were transferred to media containing different concentrations of auxins for rooting (Table 7). Incubation was done in dark for a week and then transferred to light for further establishment.

3.2.4.9 Ex vitro Rooting of Plantlets

Elongated shoots with three to four leaves and 4cm length were subjected to *exvitro* rooting on sterile acid washed sand medium with ½ MS, Activated Charcoal and 5 µM IBA. Plantlets were kept under a photon flux intensity of 30 to 50 µmol m^{-2} s^{-2} 16h photoperiod.

Table 7. Treatments tried to assess the effect of media and hormones on rooting

S. No.	Treatment No.	Treatments
1.	R_1	TM_3 + 2.46 µM IBA + 5.37 µM NAA
2.	R_2	TM_3 + 4.93 µM IBA + 5.37 µM NAA
3.	R_3	TM_3 + 8 µM NAA
4.	R_4	TM_6 + 2.46 µM IBA + 5.37 µM NAA
5.	R_5	TM_6 + 4.93 µM IBA + 5.37 µM NAA
6.	R_6	TM_6 + 8 µM NAA
7.	R_7	TM_9 + 2.46 µM IBA + 5.37 µM NAA
8.	R_8	TM_9 + 4.93 µM IBA + 5.37 µM NAA
9.	R_9	TM_9 +8 µM NAA
10.	R_{10}	TM_{12} + 2.46 µM IBA + 5.37 µM NAA
11.	R_{11}	TM_{12} + 4.93 µM IBA + 5.37 µM NAA
12.	R_{12}	TM_{12} + 8 µM NAA

Table 8. Media composition for Murashige and skoog (1962) medium

Chemical composition	Quantity in mg per litre
KNO_3	1900.00
$NH_4 NO_3$	1650.00
$MgSO_4$	370.00
$CaCl_2.2H_2O$	170.00
KH_2PO_4	440.00
$FeSO_4.2H_2O$	27.80
Na_2 EDTA.$2H_2O$	37.30
$MnSO_4.4H_2O$	223.00
$ZnSO_4.7H_2O$	86.00
H_3BO_3	62.00
KI	8.30
$Na_2MoO_4.2H_2O$	2.50
$CuSO_4.5H_2O$	0.25
$COCl_2.6H_2O$	0.02
Inositol	100.00
Nicotinic acid	5.00
Pyridoxine HCl	5.00
Thiamine HCl	1.00
Glycine	20.00
Sucrose	20000.00

Table 9. Media composition for Modified Murashige and skoog medium

Chemical composition	Quantity in mg per litre
KNO_3	1900.00
$NH_4 NO_3$	412.50
$MgSO_4$	370.00
$CaCl_2.2H_2O$	170.00
KH_2PO_4	440.00
$FeSO_4.2H_2O$	27.80
Na_2 EDTA.$2H_2O$	37.30
$MnSO_4.4H_2O$	223.00
$ZnSO_4.7H_2O$	86.00
H_3BO_3	62.00
KI	8.30
$Na_2MoO_4.2H_2O$	2.50
$CuSO_4.5H_2O$	0.25
$COCl_2.6H_2O$	0.05
Inositol	100.00
Nicotinic acid	5.00
Pyridoxine HCl	5.00
Thiamine HCl	1.00
Folic acid	0.50
Biotin	0.05
Glycine	20.00
Cystine	1.00
Proline	2.00
Casein hydrolysate	10.00
Sucrose	20000.00

Table 10. Media composition for Nitsch and Nitsch (1969) medium

Chemical composition	Quantity in mg per litre
KNO_3	950.00
$NH_4 NO_3$	720.00
$MgSO_4$	182.00
$CaCl_2$	166.00
KH_2PO_4	68.00
$FeSO_4$	27.85
Na_2 EDTA	37.25
$MnSO_4$	25.00
$ZnSO_4$	10.00
H_3BO_3	10.00
Na_2MoO_4	0.25
$CuSO_4$	0.025
Inositol	100.00
Nicotinic acid	5.00
Pyridoxine HCl	0.50
Thiamine HCl	0.50
Folic acid	0.50
Biotin	0.05
Glycine	2.00
Sucrose	20000.00
IAA	0.10

Table 11. Media composition for Modified Nitsch and Nitsch medium

Chemical composition	Quantity in mg per litre
KNO_3	950.00
$NH_4 NO_3$	90.00
$MgSO_4$	182.00
$CaCl_2$	166.00
KH_2PO_4	68.00
$FeSO_4$	27.85
Na_2 EDTA	37.25
$MnSO_4$	25.00
$ZnSO_4$	10.00
H_3BO_3	10.00
Na_2MoO_4	0.25
$CuSO_4$	0.025
$COCl_2.6H_2O$	0.05
Inositol	100.00
Nicotinic acid	5.00
Pyridoxine HCl	0.50
Thiamine HCl	0.50
Folic acid	0.50
Biotin	0.05
Glycine	2.00
Cystine	1.00
Proline	2.00
Casein hydrolysate	10.00
Sucrose	20000.00
IAA	0.10

3.2.4.10 Hardening and Acclimatization

Plantlets of two to three centimetre long were removed from the culture flasks, treated with 0.2% Bavistin (Bavistin 50 WP) solution for 20 minutes and then drained to remove excess moisture. These rooted plantlets were planted in a tray or plastic disposable cups on sterilized fine river sand and kept in mist chamber with sufficient nutrient supply in the form of fertilizer mixture N:P:K-17: 17: 17 as placement application and as foliar spray of 1 % solution. To supplement carbon source, charcoal powder was added to the river sand during earlier stages of plantlets. Observations on survival percentage were recorded 30 days after planting.

3.3 STATISTICAL ANALYSIS

Completely Randomized Design (CRD) was followed for statistical analysis wherever necessary as per Panse and Sukhathme (1985).

3.4 MOLECULAR CHARACTERIZATION

Molecular characterization of twelve hybrids and their parents were carried out with RAPD using AP-PCR. Young leaf samples from each genotype were collected from the field for DNA isolation.

3.4.1 Genomic DNA Isolation

The extraction protocol was standardized by modifying the protocol proposed by Murray and Thompson (1980). Young copper coloured leaf tissues were used immediately after collection for DNA extraction. Leaf samples were collected and washed in running tap water and then with sterile water after chopping the leaves coarsely. After wiping off the water using tissue paper, the chopped leaves were pre-chilled at -80°C for half an hour with pestle and mortar and then pulverized in liquid nitrogen by rapid grinding to a fine powder.

The frozen powder was transferred to CTAB extraction buffer (2 per cent w/v CTAB, 100mM Tris HCl (pH 8.0), 20mM EDTA, 1.4mM NaCl and 0.2 per cent ß-mercapto ethanol) and incubated at 65°C for 45min with occasional shaking. After the treatment the contents were filtered using a sterile cloth filter and the filterate was collected in an eppendorf tube. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added after treating with protinase K and RNAase A treatments. The mix was inverted and centrifuged at 10,000 rpm for 10 min. The aqueous phase was reextracted with an equal volume of chloroform: isoamyl alcohol (24:1 v/v) and centrifuged at 10,000 rpm for 5min. The aqueous phase was collected and precipitated with ice cold isopropanal in the presence of 3 M sodium acetate at -80°C for 4h. The precipitate was collected after centrifuging the contents at 15,000 rpm for 12min and carefully pipetting out the aqueous phase. The precipitate was washed with 70 per cent ethanol and dried in laminar air flow chamber. The dried pellet was dissolved in low salt TE (100mM Tris buffer and 1mM EDTA) buffer. The purity of the DNA was analysed by running in 0.8 per cent agarose gel with 1 X TAE (Tris buffer, Glacial acetic acid and EDTA pH 8.0) buffer.

3.4.2 Quantification of DNA

The quantification of DNA is necessary before it is subjected to amplification. The quantification of DNA was carried out with the help of UV spectrophotometer (spectronic Genys 5).

The buffer in which the DNA was dissolved was taken in a cuvette to calibrate the spectrophotometer at 260 and 280nm. The optical density (OD) of the DNA samples dissolved in the buffer was recorded at both 260 and 280nm.

The quantity of DNA in the sample was estimated by employing the following formula

Amount of the DNA (ng / µl) = A_{260} x 50 X dilution factor / 1000

where A260 – absorbance at 260nm

The quality of DNA could be judged from the ratio of the OD values recorded at 260nm and 280nm. A_{260} / A_{280} ratio between 1.8 and 2.0 indicates good quality of DNA, where A_{280} is the absorbance at 280nm.

3.4.3 Standardization of AP-PCR Protocol

To determine the optimal conditions for the reliability and reproducibility of AP-PCR, the effects of different DNA template concentrations (5, 10, 25, 75 and 100 ng), DNA polymerase (at concentrations of 0.5, 1, 3, 5, 7 and 10u) arbitrary primers (a total of 60 primers included in the kit from Operon Technologies (at concentrations 10 and 20 pM), $MgCl_2$ concentrations (0.5, 1, 1.5, 2 and 2.5mM), annealing temperatures (25 to 45°C) and multiplication factors for degree centigrade in two different thermocycling conditions varying in ramp time were tested in triplicate for *Anthurium andreanum* varieties and hybrids (LR, KR, OO X KR and OG X DT).

The optimized PCR mixture consisted of 5u Taq polymerase; 15mM Tris-HCl (pH 8.8 at 25 °C); 2.5mM $MgCl_2$; 50 mM KCl; 50 µM each dATP, dGTP, dCTP and TTP; 10 pM primer and 50ng of template DNA for a final volume of 20µl. Cycling was performed (Antonio *et al.*, 1998) in a PCR machine (PTC 100) and consisted of the following steps

Intial denaturation at 90 °C

3 cycles of

94 °C for 25 seconds

35 °C for 25 seconds

72 °C for 2 minutes

followed by 40 cycles of

94 °C for 25 seconds

37 °C for 25 seconds

72 °C for 2 minutes

final extension of 72 °C for 7min and ∞ hold at 4 °C (Nowbuth *et al.*, 2005).

The amplified products were run in 1.6 per cent agarose gel with 1X TAE (Tris buffer, Glacial acetic acid and EDTA pH 8.0) buffer.

3.4.4 Data analysis

The reproducible bands were scored for their presence (1) or absence (0) for all the hybrids and parents. A genetic similarity matrix was constructed using Jaccards's similarity co-efficient methods (Jaccard, 1908).

$$S_j = a / (a+b+c)$$

Where

a – no of bands present in both the varieties

b – number of bands present in the first variety but not in the second one

c – number of bands in the second variety but not in the first

Based on the similarity coefficient, the distance between the genotypes was computed with the help of the software package NTSYS (version 2.02) using these values of distances, between genotypes, a dendrogram was constructed by following the UPGMA (Unweighted Pair Group Method for Arithmetic average) and Nei and Li's (1979) GD_{NL} methods. Association between the various genotypes was found out from the dendrogram.

Results

4. RESULTS

The results of the present investigations carried out on *in vitro* multiplication and DNA fingerprinting of selected hybrids and their parents in *Anthurium andreanum* Linden are presented under the following headings.

4.1 Morphological Characterization

4.2 *In vitro* Multiplication

4.3 Molecular Characterization (RAPD using AP-PCR)

4.1 MORPHOLOGICAL CHARACTERIZATION

The morphological description of the hybrids and their parents are presented in this chapter (Table 12).

4.2 *IN VITRO* MULTIPLICATION

The effects of various treatments designed to carryout are presented in this chapter.

4.2.1 Surface Sterilization

The observed data on surface sterilization treatments (Tables 13a, 13b, 13c, 13d and Fig. 1a, 1b, 1c, 1d) were found to be significantly different. One hundred percentage contamination free cultures were not obtained. TS_5 (double sterilization with 95 per cent ethyl alcohol for 1 min + 0.08 per cent mercuric chloride for 10 minutes + 70 per cent ethyl alcohol for 3 minutes) was found to be the best among the surface sterilization treatments, which was capable of producing more than 95 per cent contamination free culture. Next to it, TS_4 (70 per cent ethyl alcohol for 20 minutes) was found to be the best to produce more than 70 per cent contamination free cultures. Treatments TS_1 (1 per cent sodium hypochloride for 12 minutes), TS_2 (0.08 per cent mercuric chloride for 10 minutes) and TS_3 (0.5 per cent calcium chloride for 15 minutes) are also found to be significantly different but less effective in producing contamination free cultures. There are significant varietal differences for surface sterilization treatment in producing contamination free cultures.

Table 12. Morphological characters of hybrids and their parents

S. No.	Lines	Spathe colour	Shape	Texture
1.	LR	Dark red	B	DB
2.	PR	Chilli red	B	DB
3.	W	White	N	S
4.	LJ	Dark pink	N	S
5.	DT	Red	B	DB
6.	OG	Orange	B	DB
7.	KR	Purple	B	DB
8.	TR	Chilli red	B	DB
9.	OO	Dark orange	B	DB
10.	KO	Dull orange	B	DB
11.	MW	Milk white	B	DB
12.	FK	Red	B	DB
13.	LJ X W	Red	B	S
14.	W X LJ	White	N	S
15.	LJ X LR	Red	B	B
16.	PR X LR	Red	B	DB
17.	PR X MW	Red	B	DB
18.	PR X DT	Maroon	B	B
19.	PR X LJ	Red	B	B
20.	FK X DT	Dark red	B	DB
21.	FK X LR	Dark red	B	DB
22.	OG X DT	Purplish red	B	B
23.	OO X KR	Bright orange	B	DB
24.	TR X MW	Bright red	B	DB

B - Broad
N - Narrow
DB – Deeply blistered
B - Blistered
S - Smooth

Table 13a. Effect of surface sterilization treatments to produce contamination free explants in LR of *Anthurium andreanum* Linden (in % of contamination free explants)

S.No.	Treatments	Leaf	Petiole	Spadix	Candle	Seed	Shoot tip
1	TS_1	42.16	35.16	19.83	33.83	21.33	43.00
2	TS_2	73.50*	47.66*	26.16	45.33*	31.83*	44.00
3	TS_3	56.16	55.66*	47.50*	55.00*	44.50*	52.66*
4	TS_4	64.00*	55.16	52.83	57.33	58.83*	66.00*
5	TS_5	95.83*	87.66*	95.33*	96.00*	95.66*	95.33*

CD @ 0.05 - 6.23

Table 13b. Effect of surface sterilization treatments to produce contamination free explants in DT of *Anthurium andreanum* Linden (in% of contamination free explants)

S.No.	Treatments	Leaf	Petiole	Spadix	Candle	Seed	Shoot tip
1	TS_1	44.16	33.16	25.16	37.83	31.66	44.16
2	TS_2	74.50*	43.16*	33.66*	43.83	42.83*	74.50*
3	TS_3	62.16	45.83	53.50*	56.16*	47.83	62.16
4	TS_4	57.66	51.83	63.16*	62.33	66.83*	57.66
5	TS_5	95.83*	96.00*	98.00*	98.16*	97.16*	95.83*

CD @ 0.05 - 6.03

Table 13c. Effect of surface sterilization treatments to produce contamination free explants in OO X KR of *Anthurium andreanum* Linden (in % of contamination free explants)

S.No.	Treatments	Leaf	Petiole	Spadix	Candle	Seed	Shoot tip
1	TS_1	47.16	42.83	33.66	43.50	37.66	52.83
2	TS_2	83.00*	46.00	33.16	55.66*	47.00*	44.33
3	TS_3	72.16	54.66*	57.16*	64.83*	54.50*	54.33*
4	TS_4	65.16	59.00	64.50*	67.16	64.66*	75.33*
5	TS_5	96.16*	98.16*	97.33*	97.50*	96.83*	74.91

CD @ 0.05 – 4.70

Table 13d. Effect of surface sterilization treatments to produce contamination free explants in OG X DT of *Anthurium andreanum* Linden (in % of contamination free explants)

S.No.	Treatments	Leaf	Petiole	Spadix	Candle	Seed	Shoot tip
1	TS_1	45.16	43.83	36.16	44.16	43.00	52.50
2	TS_2	75.33*	47.00	34.00	53.16*	35.33	47.70
3	TS_3	76.50	56.50*	59.16*	62.83*	56.16*	56.16*
4	TS_4	64.50	62.33*	64.50*	64.33	62.83*	75.66*
5	TS_5	96.00*	96.16*	97.66*	97.50*	96.66*	93.16*

CD @ 0.05 – 4.19

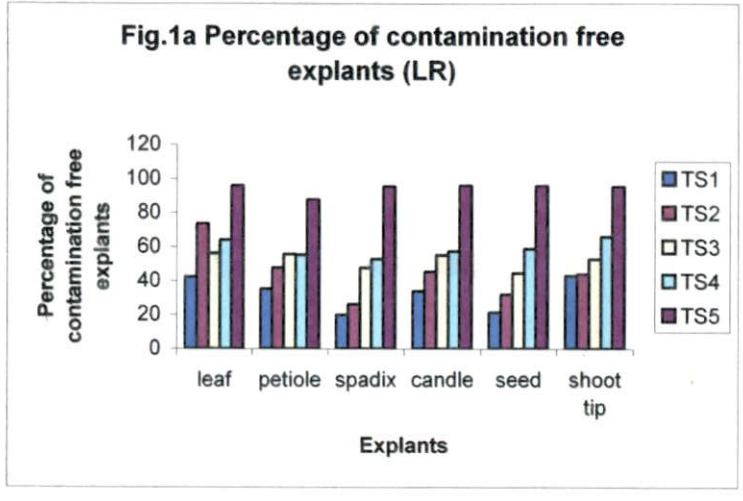


Fig.1a Percentage of contamination free explants (LR)
Percentage of contamination free explants
120
100
80
60
40
20
0
leaf
petiole
spadix
candle
seed
shoot tip
Explants
TS1
TS2
TS3
TS4
TS5

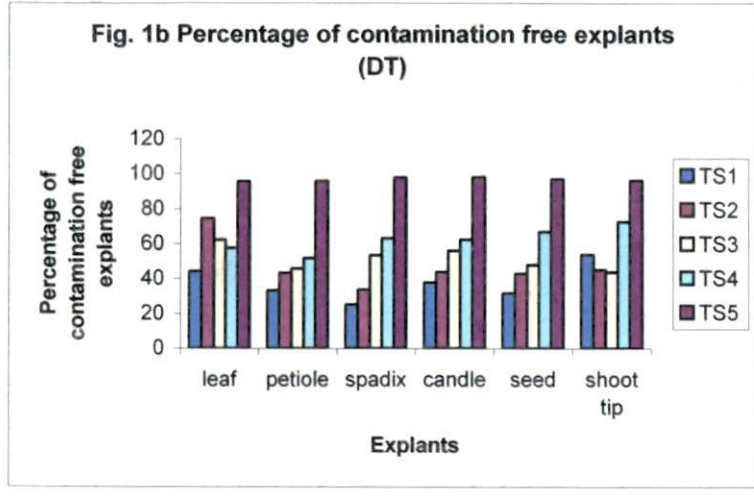


Fig. 1b Percentage of contamination free explants (DT)
Percentage of contamination free explants
120
100
80
60
40
20
0
leaf
petiole
spadix
candle
seed
shoot tip
Explants
TS1
TS2
TS3
TS4
TS5

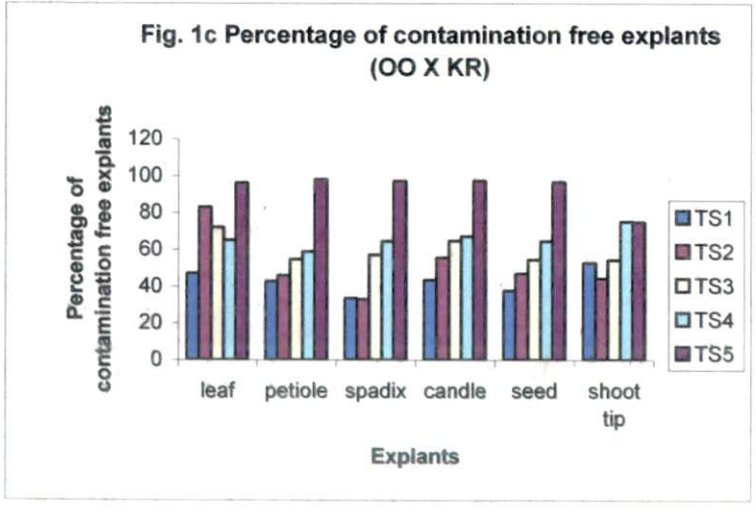


Fig. 1c Percentage of contamination free explants (OO X KR)
Percentage of contamination free explants
120
100
80
60
40
20
0
leaf
petiole
spadix
candle
seed
shoot tip
Explants
TS1
TS2
TS3
TS4
TS5

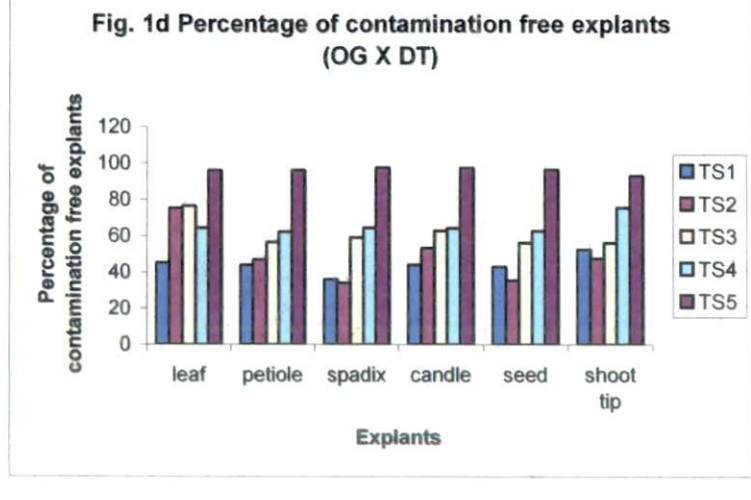


Fig. 1d Percentage of contamination free explants (OG X DT)
Percentage of contamination free explants
120
100
80
60
40
20
0
leaf
petiole
spadix
candle
seed
shoot tip
Explants
TS1
TS2
TS3
TS4
TS5

4.2.2 Media and hormones

4.2.2.1 Survival of explants

Six different explants viz. leaf lamina, petiole, spadix, candle, seed and shoot tip were used in the experiment. Among these explants, seed was the hardest and the candle was found to be the most sensitive. Candle explants were found to dry up faster than any other explant. Treatment TM_{12} (M-NN + AC + CW) recorded the highest survival rate for all the explants irrespective of variety and it was followed by TM_9 (½ NN + AC + CW), TM_6 (M-MS + AC + CW) and TM_3 (½MS + AC + CW). For spadix TM_6 and TM_9 are better even though on par. The lowest value was recorded in TM_1 (½ MS). TM_5 (M- MS + AC), TM_8 (½ NN + AC) and TM_{10} (M-NN) were found to be on par with each other. Analysed data shows significant variation among treatments (Table 14 and Fig. 2).

4.2.2.2 Callus induction

The differential response for the treatments fixed to get callus induction in twelve hybrids and their parents are represented as '+' for positive and as '-' for negative response (Table15a). The positively responding treatments were forwarded with variation for next set of treatments.

The observed data of callus induction for the 16 tried treatments were converted in to percentage response (Table 15 and Fig. 3). Among the 16 treatments, only eight were found to be responsive. All the eight treatments exhibited values ranging from 25 to 73.5 percentages. No treatment showed above 75 per cent response. Highest and lowest values were noticed in TC_{14} (TM_{12} + 5.37 µM NAA + 2.26 µM 2, 4-D + 9.12 µM zeatin) and TC_1 (TM_3 + 10.74 µM NAA + 3.62 µM 2, 4-D + 9.12 µM zeatin) respectively. Treatments were found to be significantly different from one another.

4.2.2.3 Days to Callus Induction

On perusal of data collected (Table 16 and in Fig. 4), hybrids were found to take more number of days to callus induction than varieties. A range of variation for days required for callus induction was from 72days for variety OG with treatment TC14 (TM_{12}

Table 14. Effect of media and hormones on survival of explants in *Anthurium andreanum* Linden varieties and hybrids (% of surviving explants)

S. No.	Treatment No.	Leaf	Petiole	Spadix	Candle	Seed	Shoot tip
1	TM_1	45.33	58.16	28.33	22.16	80.33	54.83
2	TM_2	52.16*	53.66	42.33*	40.33*	86.83*	63.16*
3	TM_3	87.50*	92.33*	85.16*	83.33*	92.33*	92.33*
4	TM_4	59.00	64.00	52.16	59.50	76.50	65.16
5	TM_5	72.50*	72.33*	63.83*	66.00*	86.33*	75.33*
6	TM_6	94.33*	94.66*	96.83*	90.50*	96.00*	93.00*
7	TM_7	63.33	66.66	54.50	54.83	63.50	63.33
8	TM_8	72.50*	72.33*	62.50*	78.83*	70.00*	74.33*
9	TM_9	95.83*	95.83*	95.50*	94.16*	94.50*	93.50*
10	TM_{10}	72.66	72.16	70.83	73.33	74.16	74.33
11	TM_{11}	91.33*	82.33*	87.50*	91.33*	84.33*	93.16*
12	TM_{12}	97.66*	98.50*	94.83*	95.83*	97.16*	96.50*

CD @ 0.05 – 5.35

Fig. 2 Effect of media and hormones on survival of explants

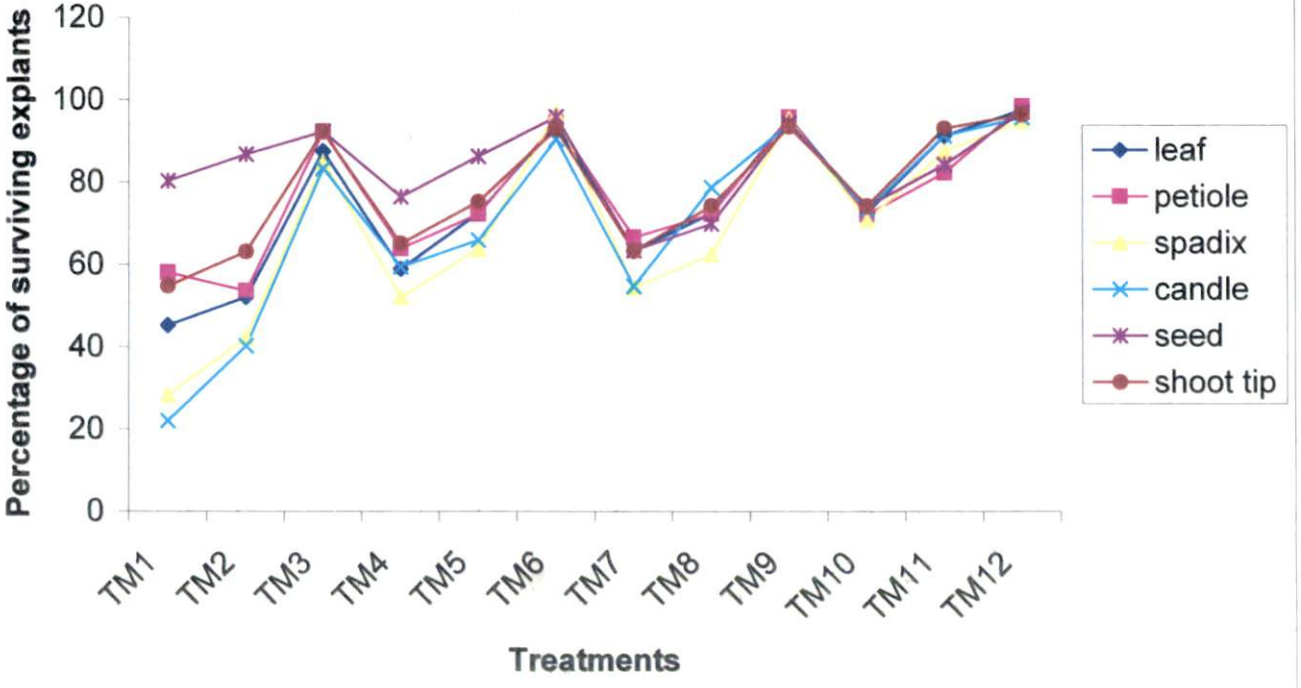


Table15. Effect of media and hormones on callus induction of *Anthurium andreanum* Linden varieties and hybrids (in % of cultures showing callus induction)

S. No.	Treatment No.	LR	LJ	OG	OO X KR	OG X DT	PR X DT
1	TC_1	25.00*	25.50*	26.00*	25.00*	25.50*	26.50*
2	TC_2	32.00*	30.00*	32.00*	31.50*	30.50*	32.50*
3	TC_3	0.00	0.00	0.00	0.00	0.00	0.00
4	TC_4	0.00	0.00	0.00	0.00	0.00	0.00
5	TC_5	40.00*	38.50*	38.00*	39.00*	39.50*	40.50*
6	TC_6	45.00*	46.50*	47.00*	46.00*	45.50*	47.50*
7	TC_7	0.00	0.00	0.00	0.00	0.00	0.00
8	TC_8	0.00	0.00	0.00	0.00	0.00	0.00
9	TC_9	54.00*	54.50*	55.50*	55.00*	58.00*	54.50*
10	TC_{10}	58.00*	59.50*	60.00*	59.00*	60.50*	58.50*
11	TC_{11}	0.00	0.00	0.00	0.00	0.00	0.00
12	TC_{12}	0.00	0.00	0.00	0.00	0.00	0.00
12	TC_{12}	0.00	0.00	0.00	0.00	0.00	0.00
13	TC_{13}	64.00*	63.50*	65.00*	65.50*	64.00*	63.00*
14	TC_{14}	71.00*	72.50*	73.00*	71.50*	72.00*	73.50*
15	TC_{15}	0.00	0.00	0.00	0.00	0.00	0.00
16	TC_{16}	0.00	0.00	0.00	0.00	0.00	0.00

CD @ 0.05 – 4.9

Table 15a. Effect of media and hormones on callus induction response

S. No.	Treatment No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	TC_1	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
2	TC_2	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
3	TC_3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	TC_4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	TC_5	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
6	TC_6	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
7	TC_7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	TC_8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	TC_9	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
10	TC_{10}	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
11	TC_{11}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	TC_{12}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	TC_{13}	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
14	TC_{14}	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
15	TC_{15}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	TC_{16}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 15b. Callus index (CI = Callusing percentage X Growth score)

S. No.	Treatment No.	LR	LJ	OG	OO X KR	OG X DT	PR X DT
1	TM_1	1.50	1.20	1.40	1.40	0.70	0.90
2	TM_2	3.00	5.80	3.00	3.50	3.00	4.00
3	TM_3	87.20	87.20	83.00	79.20	90.40	79.20
4	TM_4	5.80	5.60	5.80	5.80	5.40	5.80
5	TM_5	9.66	9.96	8.96	9.66	9.96	10.40
6	TM_6	109.40	109.40	181.20	110.80	100.40	114.20
7	TM_7	19.60	20.50	19.30	21.90	23.00	23.00
8	TM_8	29.70	74.00	31.00	34.60	74.60	88.00
9	TM_9	244.00	244.00	245.00	334.00	344.00	220.00
10	TM_{10}	74.00	88.00	93.00	93.80	93.80	102.40
11	TM_{11}	107.00	193.50	203.00	204.00	222.00	205.00
12	TM_{12}	375.00	337.00	366.00	388.00	388.00	396.00

Table 16. Effect of media and hormones on days to callus induction of *Anthurium andreanum* Linden varieties and hybrids

S. No.	Treatment No.	LR	LJ	OG	OO X KR	OG X DT	PR X DT
1	TC_1	92	98*	91	108*	110	94
2	TC_2	93	99	93	110	111	96
3	TC_5	97*	102*	96*	112*	113	99*
4	TC_6	96	100	94	110	112	97
5	TC_9	82	88*	81	98*	100	84
6	TC_{10}	80	87	80	96	98	83
7	TC_{13}	77	83	77	93	95	80
8	TC_{14}	75	78	72	88	91	75

CD @ 0.05 – 3

Fig. 3 Effect of media and hormones on callus induction

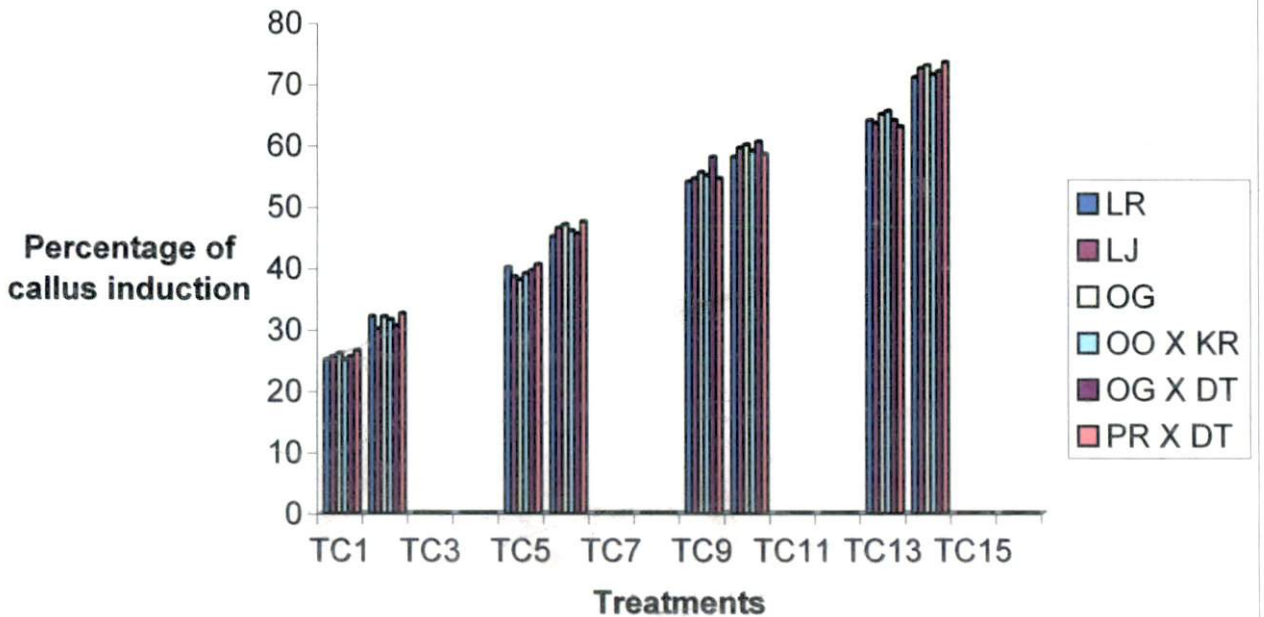
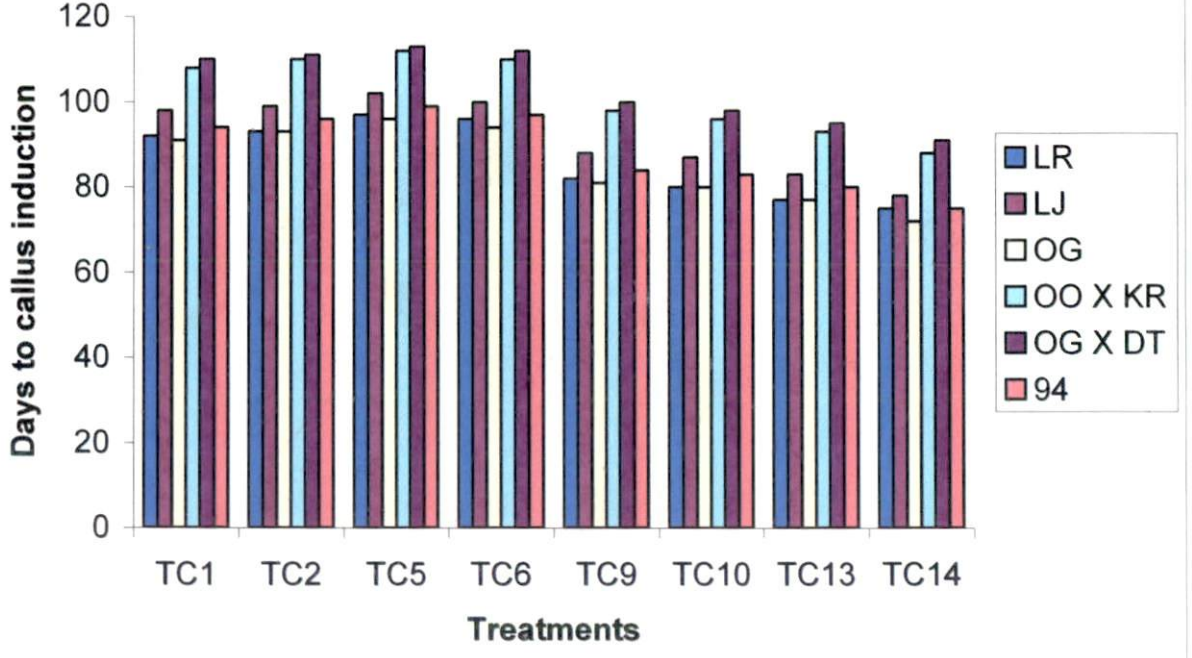
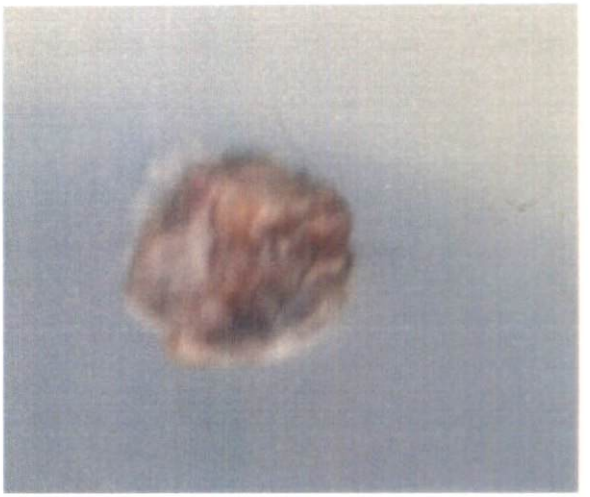


Fig. 4 Effect of media and hormones on days to callus induction

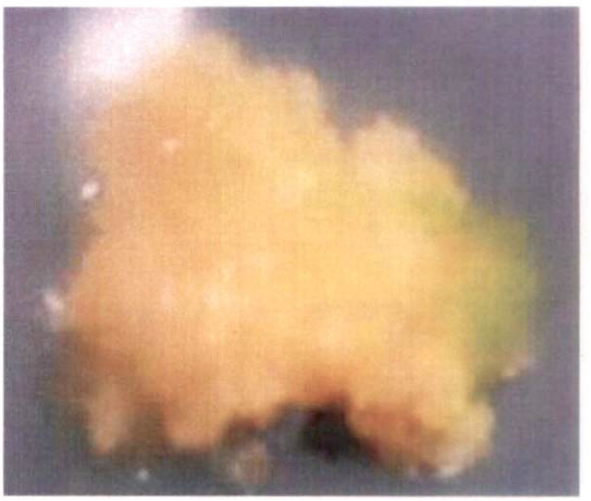


A. Callus induction



B. Callus initiation

C. Growing callus

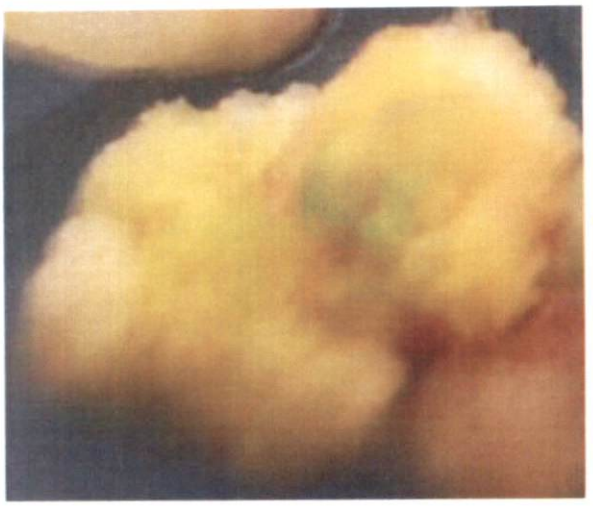


D. Callus multiplication

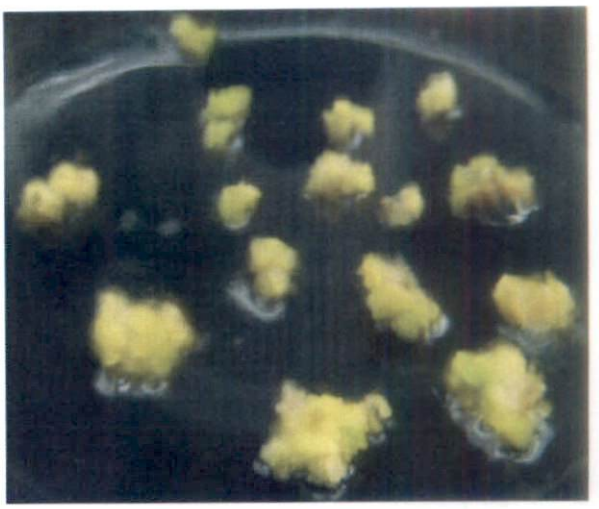
E. Regeneration

F. Young plant

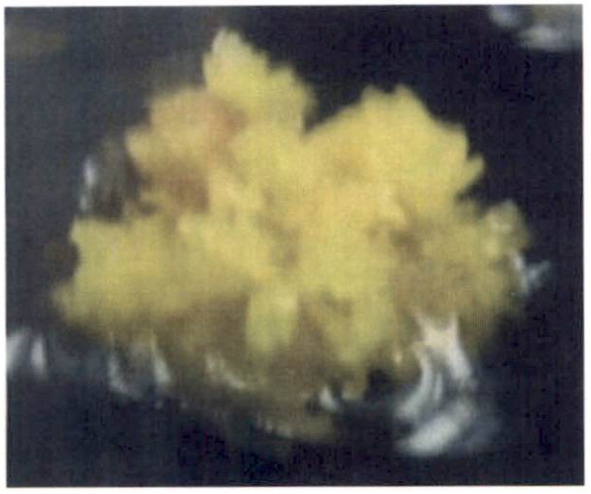
Plate 6. *In vitro* multiplication from lamina



A. Callus from petiole



B. Callus multiplication



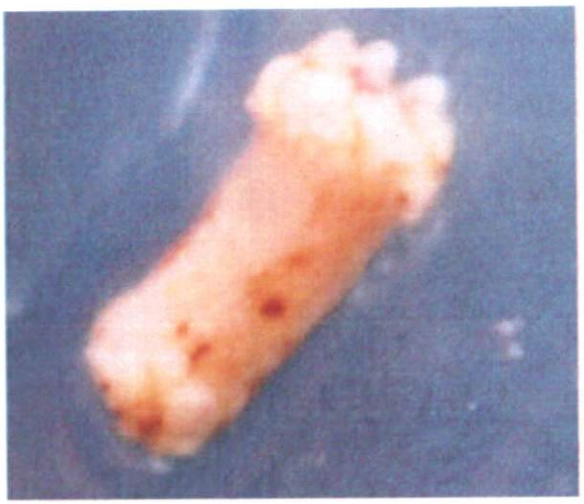
C. Embryogenic callus

D. Initial regeneration

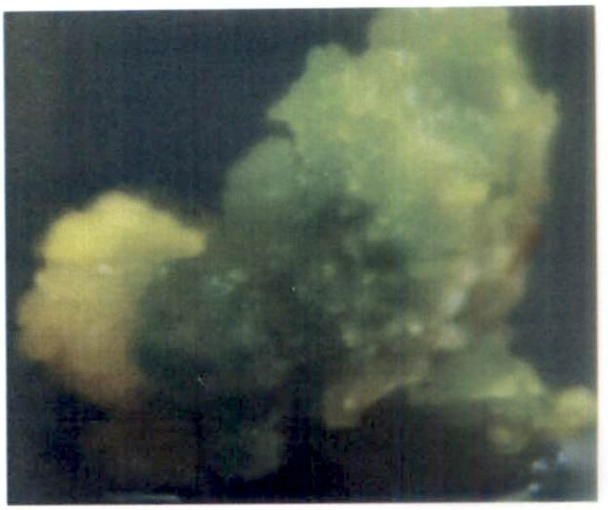
E. Regenerating plants

F. Young plants

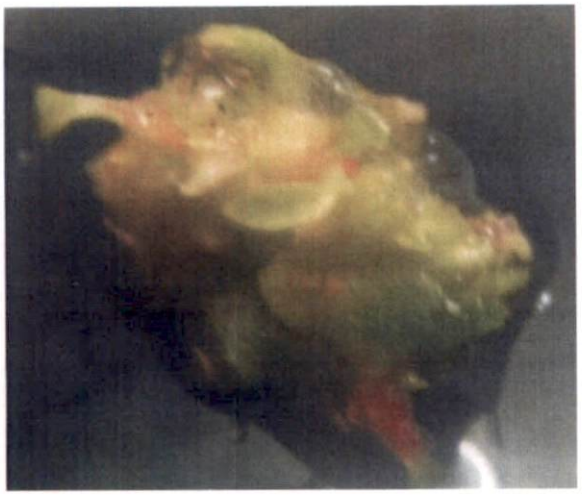
Plate 7. *In vitro* multiplication from petiole



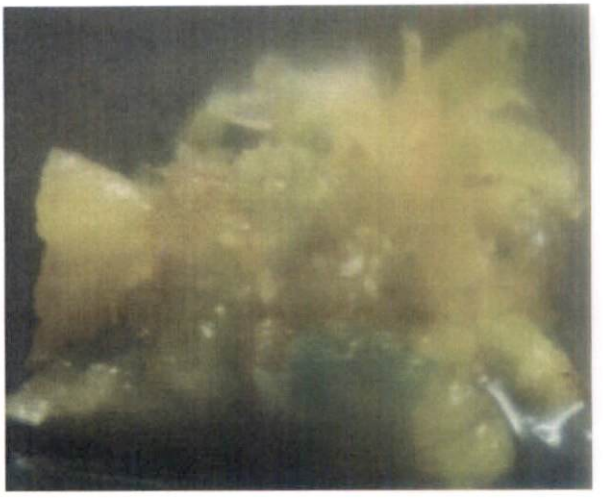
A. Callus initiation from candle



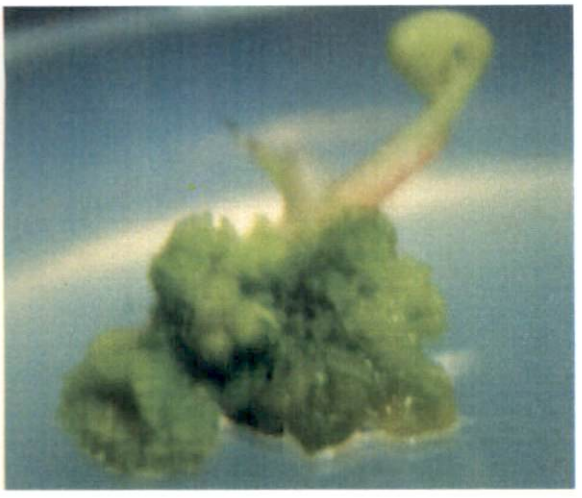
B. Embryogenic callus



C. Initiation of regeneration



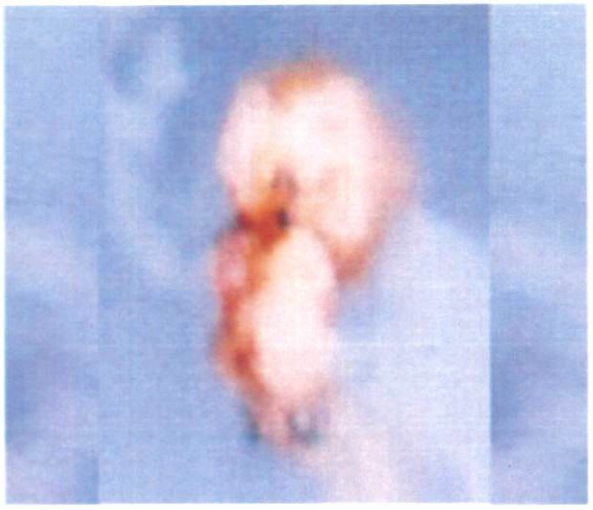
D. Miniature plants



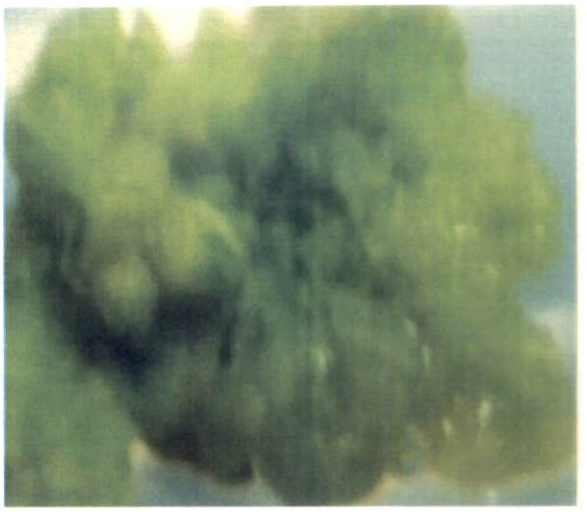
E. Single plant from callus

F. Regenerating plants from callus

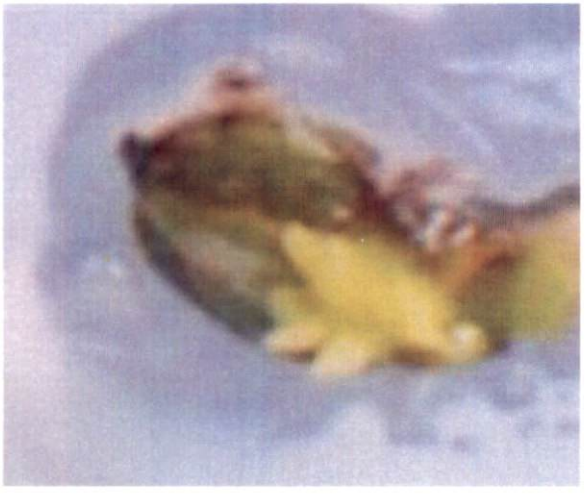
Plate 8. *In vitro* multiplication from candle



A. Callus from seed explant



B. Callus multiplication



C. Multiple shoot buds from seed

D. Single isolated shoot

E. Multiple shooting

F. Shoots kept for rooting

Plate 9. *In vitro* multiplication from seed explant

7 μM NAA + 2.26 μM 2, 4-D + 9.12 μM zeatin) to 113 days for OG X DT with (TM_6 + 10.74 μM NAA + 3.62 μM 2, 4-D + 9.12 μM zeatin). Among the eight nding treatments, TC_{14} (TM_{12} + 5.37 μM NAA + 2.26 μM 2, 4-D + 9.12 μM zeatin) ound to be the best in producing faster callus induction. TC_9 (TM_9 + 10.74 μM NAA 2 μM 2, 4-D + 9.12 μM zeatin) and TC_{10} (TM_9 + 5.37 μM NAA + 2.26 μM 2, 4-D + μM zeatin) were on par. When comparing other varieties and hybrids OO X KR was to take more time for callus induction.

4 Callus Multiplication

The analysed observations were tabulated in Table 17 and graphically represented g. 5. Callus weight ranged from 0.0662g to 3.9875g for TN_1 and TN_{12} respectively ety LR). The perusal of data on growth score and callus index indicated very good nse in treatment TN $_{11}$(TM_{12} + 4.52 μM 2, 4-D + 9.12 μM zeatin) followed by nent TN_{10} (TM_{12} + 4.52 μM 2, 4-D + 18.59 μM kinetin) and TN_9 (TN_9 + 9.12 μM n). TN_9 (TM_9 + 9.12 μM zeatin) and TN_8 (TM9 + 4.52 μM 2, 4-D + 9.12 μM zeatin) found to be on par. Like wise TM_{12} and TM_9 were found to be on par on the basis of th score (Table 18). When compared with TN_1 (TM_3 + 4.52 μM 2, 4-D + 18.59 μM in), TN_2 (TM_3 + 4.52 μM 2, 4-D + 9.12 μM zeatin) and TN_3 (TM_3 + 9.12 μM zeatin) least effective in callus multiplication.

.5 Regeneration

The entire callus cultures subjected to regeneration was found to regenerate pective of the source of explant and variety. Though they differ in their nutrient rements, they are found to be responsive (Plates 6 to 9).

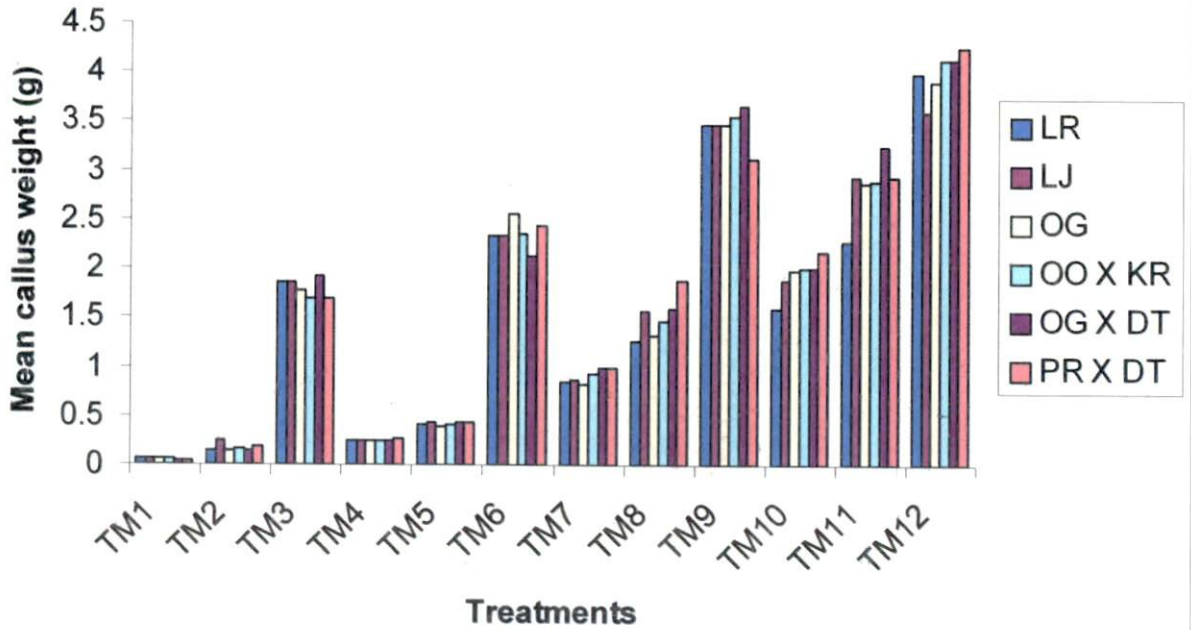
Number of days required for regeneration for three varieties and hybrids were rsed (Table 19 and Fig.7). The number of days required for regeneration ranges from days (TR_{14} (TM_{12} + 22.2μM BA + 11.42 μM IAA + 4.09μM biotin)) to 82.0 days in (TM_3 + 17.76 μM BA + 5.71μM IAA) for the hybrid OO x KR. The values were d to be significant for treatments and the variation between varieties and hybrids found to be insignificant. The treatments TR_1(TM_3 + 17.76 μM BA + 11.42 μM

ble 17. Effect of media and hormones on callus multiplication of *Anthurium dreanum* Linden varieties and hybrids (mean callus weight in grams)

S. No.	Treatments	LR	LJ	OG	OO X KR	OG X DT	PR X DT
1	TM_1	0.0662	0.0523	0.0614	0.0652	0.0342	0.0456
2	TM_2	0.1426	0.2513	0.1476	0.1582	0.1452	0.1789
3	TM_3	1.8561	1.8564	1.7658	1.6857	1.9256	1.6892
4	TM_4	0.2512	0.2414	0.2522	0.2532	0.2387	0.2587
5	TM_5	0.4125	0.4278	0.3892	0.4132	0.4256	0.4423
6	TM_6	2.3250	2.3254	2.5684	2.3547	2.1354	2.4257
7	TM_7	0.8365	0.8729	0.8275	0.9375	0.9872	0.9982
8	TM_8	1.2689	1.5783	1.3243	1.4759	1.5873	1.8776
9	TM_9	3.4581	3.4587	3.4685	3.5478	3.6587	3.1254
10	TM_{10}	1.5793	1.8778	1.9783	1.9987	1.9982	2.1775
11	TM_{11}	2.2779	2.9359	2.8759	2.8992	3.245	2.9359
12	TM_{12}	3.9875	3.5867	3.8979	4.1256	4.1325	4.2458

D @ 0.05 – 1.64

Fig. 5 Effect of media and hormones on callus multiplication



IAA + 2.05 µM biotin) and TR_{11}(TM_9 + 17.76 µM BA + 5.71µM IAA)are found to be on par while TR_{15} (TM_{12} + 17.76 µM BA + 5.71µM IAA)and TR_5(TM_6 + 17.76 µM BA + 11.42 µM IAA + 2.05 µM biotin) were also on par.

4.2.2.6 Somatic Embryogenesis

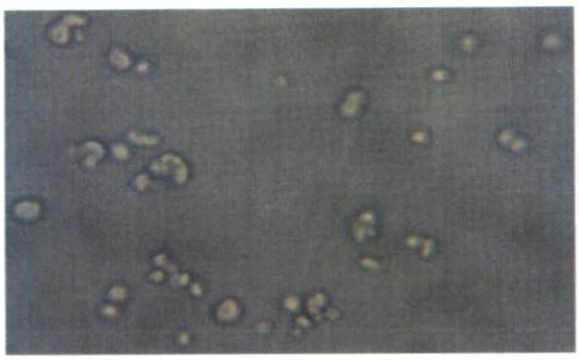
Wide range of variations from 4.3 to 72.5 percentages were recorded in TSE_5 (TM_6 + 5.71µM IAA) and TSE_{10} (TM_{12} + 11.42 µM IAA + 4.09µM biotin) respectively for somatic embryogenesis in the variety LJ. Each genotype was varying with the response to change in media composition in producing somatic embryogenesis (Table 18 Fig. 6). The treatments with MS and modified MS media were found to be insignificant when compared with NN and modified NN media. Among these modified NN was found to be the best one.

The microscopic study of *In vitro* embryo development (Plate 10) revealed that individual cells from the callus were found to enlarge and aggregate to form globular embryo which further develops into heart shaped and torpedo stage embryo. Further development revealed the similarity of development stages with a normal monocot embryo.

It was possible to store synthetic seeds for more than a week without loss of viability at –20 ° C for both hydrated and dried seeds. Seeds were capable of germinating and growing into viable plants (Plate 14).

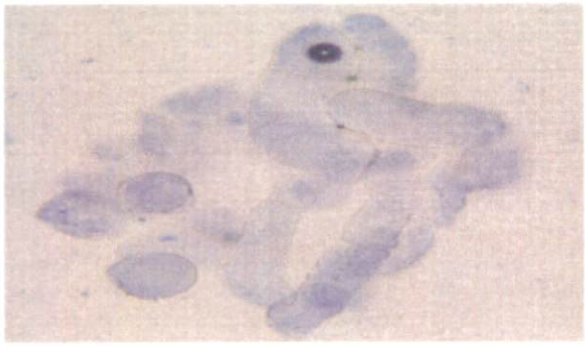
4.2.2.7 Emergence of First Leaf

Number of days required for emergence of leaf was calculated from the callus stage to regeneration and developing a first distinguishable leaf on a shoot (Table 20 and Fig. 8). The days required for emergence of the first leaf was directly correlated with the days required for regeneration. It ranges from 75.5 days (TR_{14} (TM_{12} + 22.2µM BA + 11.42 µM IAA + 4.09µM biotin)) in variety LR to 102 days (TR_3 (TM_3 + 17.76 µM BA + 5.71µM IAA)) for the hybrid OO x KR. The data did not exhibit any significant difference in leaf emergence but all treatments were found to be significant.TR_1 (TM_3 +



A. Individual cells from callus

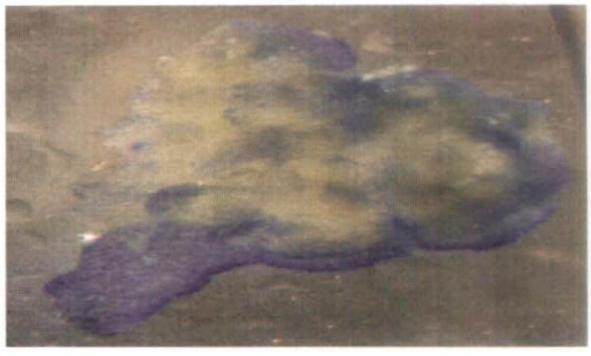
B. Basal and apical Cells



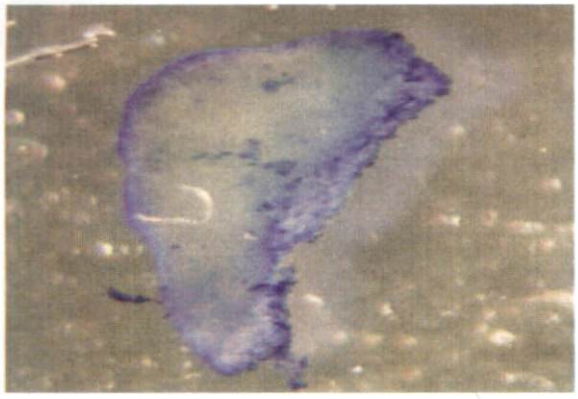
C. Proembryo formation by clustering

D. Spherical proembryo

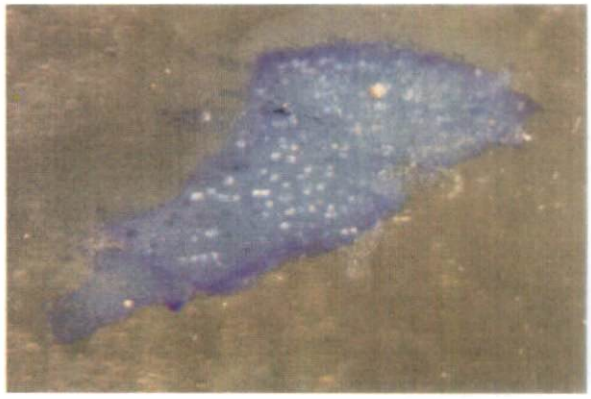
E. Initiation of heart shaped embryo



F. Heart shaped embryo



G. Torpedo stage embryo



H. Radicle and plumule elongation

Plate 10. Stages of *In vitro* Somatic Embryogenesis

A. Liver Red

B. Lady Jane

C. Orange Glory

E. Orange Glory X Dragons Tongue

F. Oirginal Orange X Kalympong Red

G. Pampon Red X Dragon's Tongue

Plate 11. *In vitro* developed plants

Table 18. Effect of treatments on somatic embryogenesis of *Anthurium andreanum* Linden varieties and hybrids (% of cultures exhibiting somatic embryogenesis)

S. No.	Treatment No.	LR	LJ	OG	OO X KR	OG X DT	PR X DT
1	TSE_1	12.50	11.00	14.00	13.00	9.00	15.00
2	TSE_2	7.50	8.60	5.00	4.50	12.00	14.00
3	TSE_3	8.50	4.60	9.80	12.00	14.00	11.00
4	TSE_4	29.00	24.00	23.50	28.00	27.00	21.00
5	TSE_5	5.00	4.30	8.50	7.50	11.00	12.00
6	TSE_6	17.00	15.00	14.00	27.00	21.00	19.00
7	TSE_7	51.50	49.00	58.00	57.00	59.00	52.00
8	TSE_8	33.00	31.00	30.00	39.00	35.00	31.00
9	TSE_9	47.00	49.00	42.00	51.00	47.00	42.00
10	TSE_{10}	68.50	72.50	64.50	69.00	71.00	65.00
11	TSE_{11}	48.00	45.00	43.00	49.50	47.00	42.50
12	TSE_{12}	59.00	57.50	62.00	63.50	59.00	57.00

CD @ 0.05 – 4.19

Fig. 6 Effect of treatmets on somatic embryogenesis

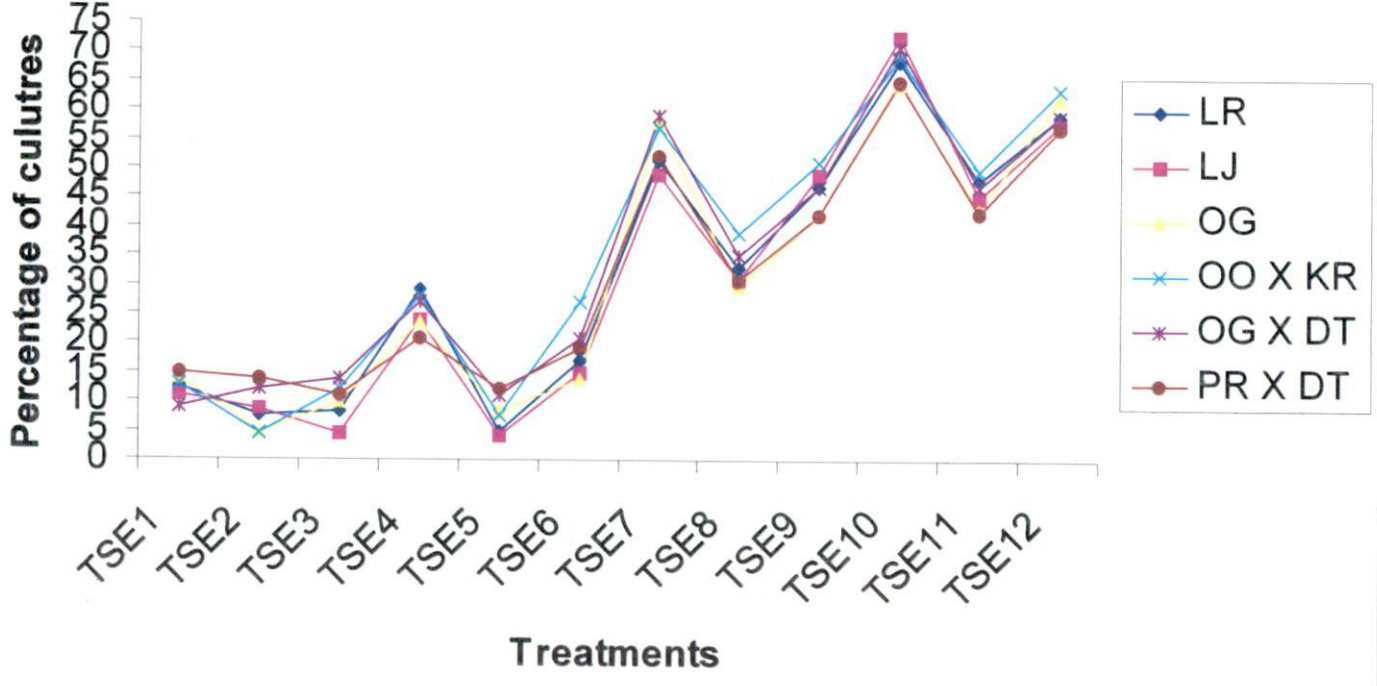


Table19. Effect of media and hormones on regeneration of *Anthurium andreanum* Linden varieties and hybrids (Days to regeneration)

S. No.	Treatment No.	LR	LJ	OG	OO X KR	OG X DT	PR X DT
1	TR_1	70.50	74.00	75.50	76.00	73.00	72.00
2	TR_2	67.50	67.00	67.50	72.00	70.00	69.00
3	TR_3	77.50	81.00	81.50	82.00	80.00	79.00
4	TR_4	74.50	78.00	78.50	79.00	77.00	76.00
5	TR_5	65.50	69.00	69.50	70.00	68.00	67.00
6	TR_6	62.50	66.00	66.50	67.00	65.00	74.00
7	TR_7	72.50	76.00	76.50	77.00	75.00	74.00
8	TR_8	79.50	73.00	73.50	74.00	72.00	71.00
9	TR_9	63.50	67.00	67.50	68.00	66.00	65.00
10	TR_{10}	60.50	64.00	64.50	65.00	63.00	62.00
11	TR_{11}	70.50	74.00	74.50	75.00	73.00	72.00
12	TR_{12}	67.50	71.00	71.50	72.00	70.00	69.00
13	TR_{13}	58.50	62.00	62.50	63.00	61.00	60.00
14	TR_{14}	55.50	59.00	59.50	60.00	58.00	67.00
15	TR_{15}	65.50	69.00	69.50	70.00	68.00	67.00
16	TR_{16}	62.50	66.00	66.50	67.00	65.00	64.00

Fig. 7 Effect of media and hormones on regeneration

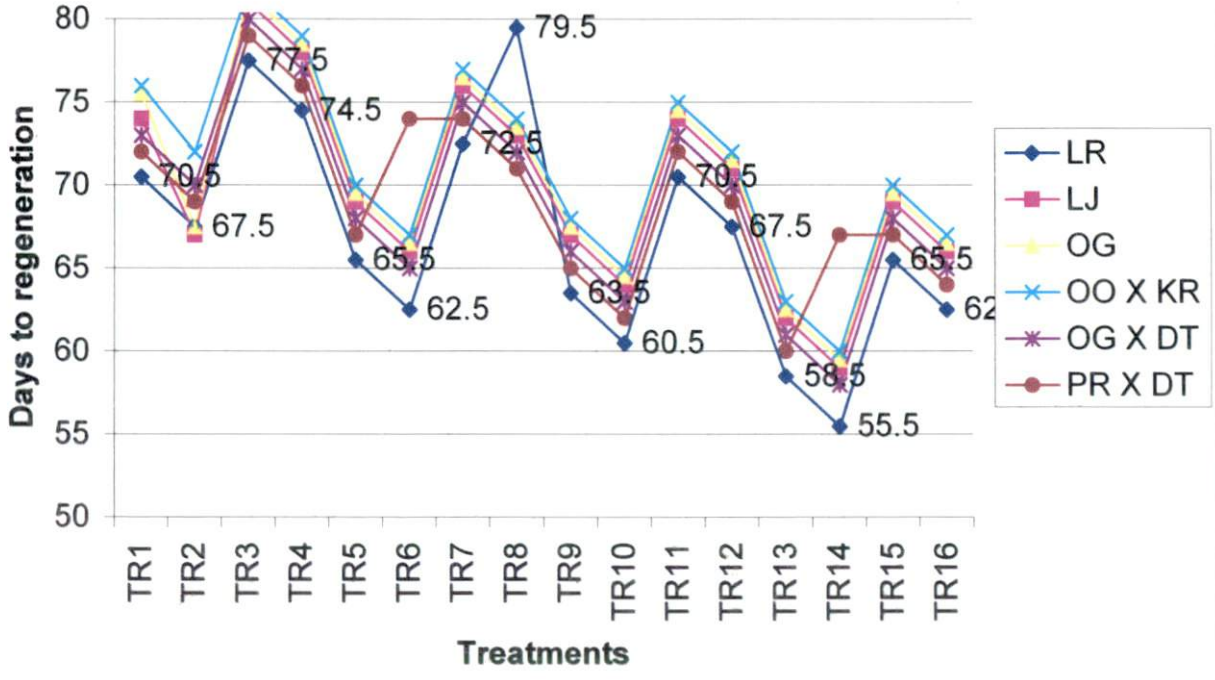
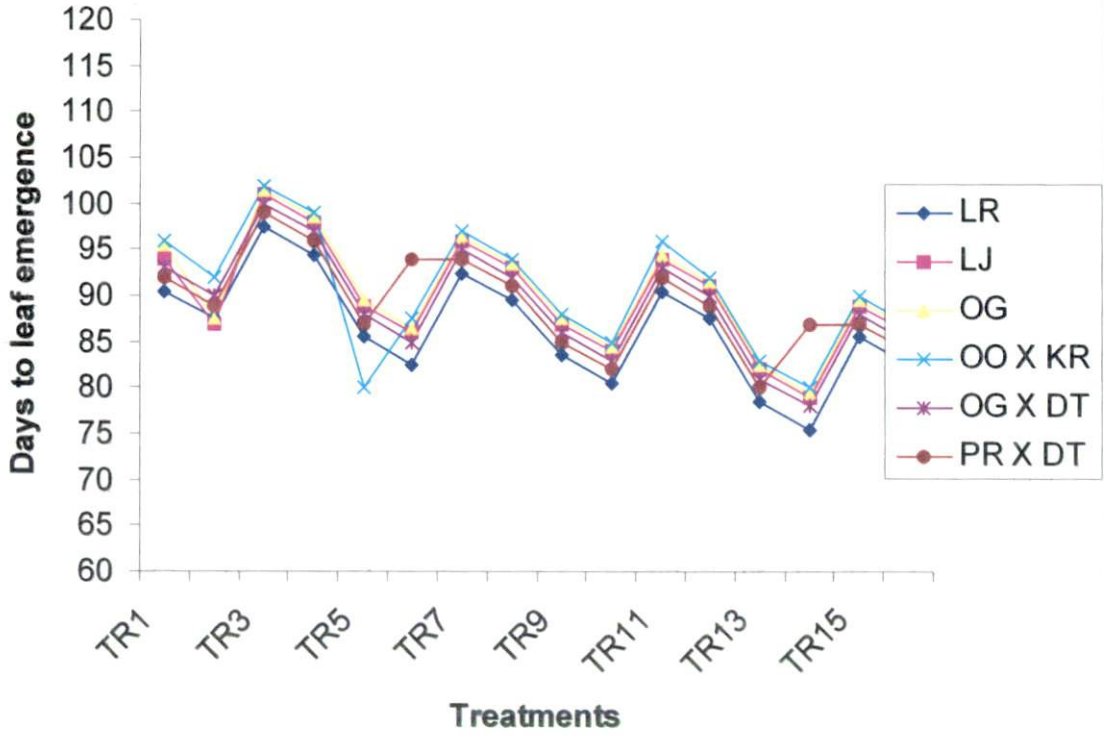


Table 20. Effect of media and hormones on days to emergence of first leaf in *Anthurium andreanum* Linden varieties and hybrids

S. No.	Treatment No.	LR	LJ	OG	OO X KR	OG X DT	PR X DT
1	TR_1	90.50	94.00	95.50	96.00	93.00	92.00
2	TR_2	87.50	87.00	87.50	92.00	90.00	89.00
3	TR_3	97.50	101.00	101.50	102.00	100.00	99.00
4	TR_4	94.50	98.00	98.50	99.00	97.00	96.00
5	TR_5	85.50	89.00	89.50	80.00	88.00	87.00
6	TR_6	82.50	86.00	86.50	87.50	85.00	94.00
7	TR_7	92.50	96.00	96.50	97.00	95.00	94.00
8	TR_8	89.50	93.00	93.50	94.00	92.00	91.00
9	TR_9	83.50	87.00	87.50	88.00	86.00	85.00
10	TR_{10}	80.50	84.00	84.50	85.00	83.00	82.00
11	TR_{11}	90.50	94.00	94.50	96.00	93.00	92.00
12	TR_{12}	87.50	91.00	91.50	92.00	90.00	89.00
13	TR_{13}	78.50	82.00	82.50	83.00	81.00	80.00
14	TR_{14}	75.50	79.00	79.50	80.00	78.00	87.00
15	TR_{15}	85.50	89.00	89.50	90.00	88.00	87.00
16	TR_{16}	82.50	86.00	86.50	87.00	85.00	84.00

Fig. 8 Effect of media and hormones on days to emergence of first leaf



17.76 µM BA + 11.42 µM IAA + 2.05 µM biotin) and TR_{11} (TM_9 + 17.76 µM BA + 5.71µM IAA) were on par. Similarly TR5 and TR15 were also on par.

4.2.2.8 In vitro Rooting

All the treatments tried were capable of inducing roots *in vitro*. Treatment R_3 (TM_3 + 4.93 µM IBA + 5.37 µM NAA) was found to be the best one followed by R_9 (TM_6 + 2.46 µM IBA + 5.37 µM NAA) and R_{12} (TM_{12} + 4.93 µM IBA + 5.37 µM NAA). The results exhibited that MS and modified MS media produced the best results for *in vitro* rooting when compared to NN and M- NN media (Table 21, Plate 12 and Fig. 9).

4.2.2.9 Hardening and Acclimatization

Plants when developed *in vitro* were hardened on a medium containing 1:1:1:1 sand: soil: composted coir pith: half MS salt solution. Plants required a minimum of 15 days hardening before transferring into potting area (Plate 12 and 13).

4.2.2.10 Problems in in vitro culturing

4.2.2.10.1 Direct organogenesis

Multiple shooting and direct regeneration or direct organogenesis was found to be non-responding to slow responding for hybrids and in case of parents it was slow responding. In total direct organogenesis failed to respond in the treatments tried.

4.2.2.10.2 Systemic infection

Recovery of systemically infected cultures which express out burst of *Xanthomonas compestris pv dieffenbachiae* in later stages of cultures were recovered by culturing on antibiotics Streptomycin and Rifambicin (50mg per litre) containing medium (Plate 15).

4.3 MOLECULAR CHARACTERIZATION

In the present study an attempt was made to determine the extent of genetic diversity in twelve selected hybrids and their parents for RAPD marker analysis using AP-PCR, making use of arbitrary primers to amplify DNA sequence in the genome.

A. Multiple shoots

B. Root initiation

C. Growing root

D. Positive geotropism

E. Multiple root formation

F. Hardening of rooted plants

Plate 12. Rooting of *in vitro* developed plants

A Hardening inside glass house

B. Acclimatized plant

C. Planting out

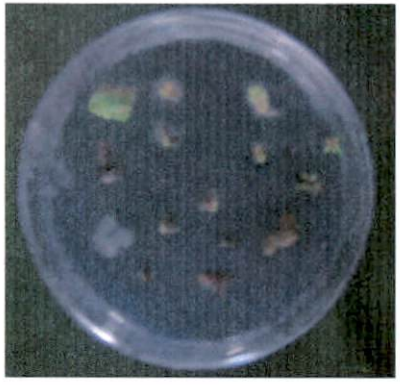
D. Transplanted OG X DT

E. Transplanted OO X KR

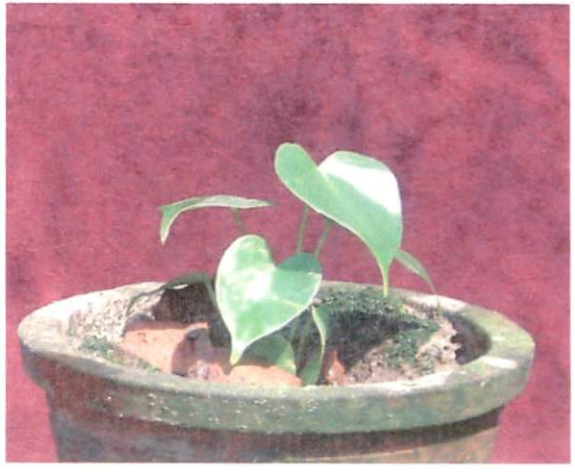
F. Transplanted PR X DT

Plate 13. Acclimatization of *in vitro* developed plants

Incomplete direct organogenesis



Infected culture recovered using kanamycin 50 mg per litre



In vitro developed plants in field condition

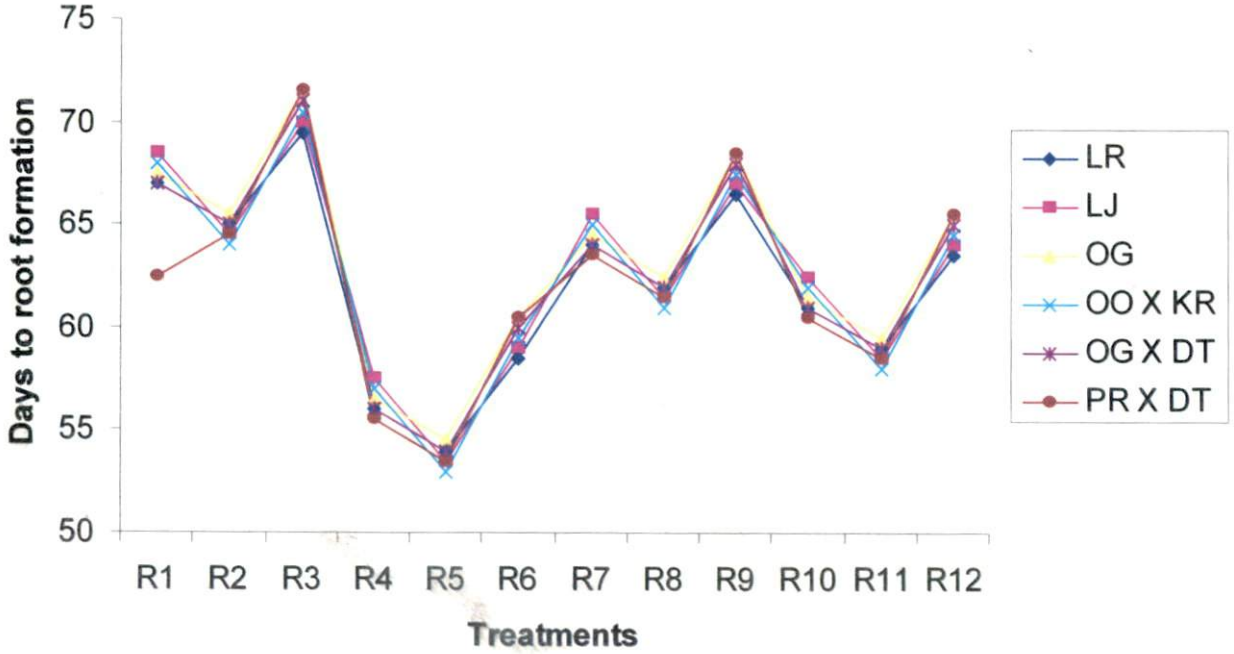
Plate 15. Problems in in vitro culturing

Table 21. Effect of media and hormones on rooting of *Anthurium andreanum* Linden varieties and hybrids (Days to root formation)

S. No.	Treatment No.	LR	LJ	OG	OO X KR	OG X DT	PR X DT
1	R_1	67.00	68.50	67.50	68.00	67.00	62.50
2	R_2	65.00	64.50	65.50	64.00	65.00	64.50
3	R_3	69.50*	70.00*	71.50*	70.50*	71.00*	71.50*
4	R_4	56.00	57.50	56.50	57.00	56.00	55.50
5	R_5	54.00	53.50	54.50	53.00	54.00	53.50
6	R_6	58.50	59.00	60.50	59.50	60.00	60.50
7	R_7	64.00*	65.50*	64.50*	65.00*	64.00*	63.50
8	R_8	62.00	61.50	62.50	61.00	62.00	61.50
9	R_9	66.50*	67.00*	68.50*	67.50*	68.00*	68.50*
10	R_{10}	61.00	62.50	61.50	62.00	61.00	60.50
11	R_{11}	59.00	58.50	59.50	58.00	59.00	58.50
12	R_{12}	63.50*	64.00*	65.50*	64.50*	65.00*	65.50*

CD @ 0.05 – 4

Fig. 9 Effect of media and hormones on rooting



Isolation of genomic DNA in *Anthurium andreanum* Linden was done using modified Murray and Thompson (1980) method. Tissues from young tender leaves were found to yield good quality of DNA.

The DNA yield for 24 accessions of *Anthurium andreanum* Linden ranged from 90to 420ng. The purity ratio of DNA ranged from 1.5 to 2.2 (Table 22).

To identify the promising primers for RAPD using AP-PCR analysis primer kits of A, B, K and J were screened using the DNA of five samples viz., LR, LJ X W, OG X DT, PR X MW and LJ X LR. The AP-PCR thermal cycling programme was standardized to have two sets of cycles with different stringency which was tried for amplification. Twenty five primers, out of the sixty decamer primers yielded amplification products indicating presence of sequence complementary to these primers in the DNA of selected five accessions of *Anthurium andreanum* Linden samples(Table 23).

From these DNA amplification profiles, seven promising primers were identified for RAPD analysis using AP-PCR based on performance in DNA amplification, production of highest number of polymorphic bands as well as intense bands and reproducibility. They were OPA 10, OPB15, OPA13, OPB20, OPB6, OPB8 and OPB18.

4.3.1 RAPD using AP-PCR

A total of 114 AP-PCR bands were generated by the 25 primers, of which 74.56 per cent were polymorphic (88 bands) and 26 were monomorphic. Ten primers showed high level of polymorphism out of which seven were selected. From the amplification profiles produced by the selected seven primers a total of 50 scorable bands (average of 7.143 bands per primer) were obtained of which only 8 were monomorphic and the rest were polymorphic. The number of bands ranged from 4 to 11 with an average of 7.143 per primer. The size of the amplicons in total ranged from 300bp to 1500bp. The extents of polymorphism of each primer tested were explained below.

Table 22. DNA yield and purity obtained in *Anthurium andreanum* Linden accessions

S. No.	Lines	OD @ 260 nm	Concentration in ng / 5µl	Purity
1	Liver Red	0.014	205	1.9
2	Pompon Red	0.007	100	2.2
3	White	0.005	70	1.6
4	Lady Jane	0.013	190	1.7
5	Dragon's Tongue red	0.004	55	1.5
6	Orange Glory	0.003	40	1.6
7	Kalympong Red	0.005	70	2.1
8	Tropical Red	0.018	265	2.0
9	Ordinary Orange	0.01	145	1.8
10	Kalympong Orange	0.017	250	1.8
11	Merengue White	0.010	145	1.5
12	Flaking Red	0.004	55	1.7
13	LJ XW	0.011	160	1.6
14	W X LJ	0.005	70	1.9
15	LJ X LR	0.005	70	2.0
16	PR X LR	0.008	115	1.8
17	PR X MW	0.005	70	1.5
18	PR X DT	0.004	55	1.6
19	FK X DT	0.003	40	1.6
20	FK X LR	0.004	55	1.8
21	OF X DT	0.014	205	1.8
22	OO X KR	0.008	115	1.7
23	LJ X PR	0.012	175	1.6
24	TR X MW	0.010	145	1.8

Table 23. Primers used in amplification of *Anthurium andreanum* Linden accessions

S.No.	primer	Primer sequence	Number of monomorphic bands	Number of polymorphic bands
1.	OPA1	CAGGCCCTTC	1	1
2.	OPA 2	TGCCGAGCTG	1	1
3.	OPA 3	AGTCAGCCAC	2	5
4.	OPA 4	AATCGGGCTG	3	1
5.	OPA 5	AGGGGTCTTG	1	2
6.	OPA 6	GGTCCCTGAC	-	-
7.	OPA 7	GAAACGGGTG	-	-
8.	OPA 8	GTGACGTAGG	-	-
9.	OPA 9	GGGTAACGCC	-	-
10.	OPA 10	GTGATCGCAG	1	7
11.	OPA 11	CAATCGCCGT	-	-
12.	OPA 12	TCGGCGATAG	-	-
13.	OPA 13	CAGCACCCAC	1	6
14.	OPA 14	TCTGTGCTGG	-	-
15.	OPA 15	TTCCGAACCC	2	2
16.	OPA 16	AGCCAGCGAA	-	-
17.	OPA 17	GACCGCTTGT	2	2
18.	OPA 18	AGGTGACCGT	-	-
19.	OPA 19	CAAACGTCGG	-	-
20.	OPA 20	GTTGCGATCC	-	-

Cont..

Table 23. Primers used in amplification of *Anthurium andreanum* Linden accessions

S.No.	primer	Primer sequence	Number of monomorphic bands	Number of polymorphic bands
21.	OPB1	GTTTCGCTCC	-	-
22.	OPB 2	TGATCCCTGG	2	2
23.	OPB 3	CATCCCCCTG	-	-
24.	OPB 4	GGACTGGAGT	1	2
25.	OPB 5	TGCGCCCTTC	-	-
26.	OPB 6	TGCTCTGCCC	-	4
27.	OPB 7	GGTGACGCAG	-	-
28.	OPB 8	GTCCACACGG	-	4
29.	OPB 9	TGGGGGACTC	-	-
30.	OPB 10	CTGCTGGGAC	2	3
31.	OPB 11	GTAGACCCGT	1	2
32.	OPB 12	CCTTGACGCA	2	2
33.	OPB 13	TTCCCCCGCT	-	-
34.	OPB 14	TCCGCTCTGG	-	-
35.	OPB 15	GGAGGGTGTT	1	6
36.	OPB 16	TTTGCCCGGA	-	-
37.	OPB 17	AGGGAACGAG	-	4
38.	OPB 18	CCACAGCAGT	-	11
39.	OPB 19	ACCCCCGAAG	-	-
40.	OPB 20	GGACCCTTAC	1	6

Cont..

Table 23. Primers used in amplification of *Anthurium andreanum* Linden accessions

S.No.	primer	Primer sequence	Number of monomorphic bands	Number of polymorphic bands
41.	OPJ1	CCCGGCATAA	-	-
42.	OPJ 2	CCCGTTGGGA	-	-
43.	OPJ 3	TCTCCGCTTG	-	-
44.	OPJ 4	CCGAACACGG	1	2
45.	OPJ 5	CTCCATGGGG	-	-
46.	OPJ 6	TCGTTCCGCA	1	2
47.	OPJ 7	CCTCTCGACA	-	-
48.	OPJ 8	CATACCGTGG	-	-
49.	OPJ 9	TGAGCCTCAC	-	-
50.	OPK 10	AAGCCCGAGG	-	-
51.	OPK 11	CATTCGAGCC	-	-
52.	OPK 12	GTCTCCGCAA	-	4
53.	OPK 13	CCAGCTTAGG	-	-
54.	OPK 14	CCGCCCAAAC	-	-
55.	OPK 15	TCTGTCGAGG	-	3
56.	OPK 16	CACCTTTCCC	-	-
57.	OPK 17	AGCGAGCAAG	-	-
58.	OPK 18	GAACACTGGG	-	4
59.	OPK 19	CCCTACCGAC	-	-
60.	OPK 20	GTGCAACGTG	-	-

Table 24. Similarity co-efficients between varieties

1.0000
0.4583 1.0000
0.4814 0.2812 1.0000
0.3461 0.3846 0.5769 1.0000
0.5185 0.3548 0.4687 0.4000 1.0000
0.5000 0.4375 0.5937 0.5333 0.7333 1.0000
0.3666 0.4000 0.4687 0.4482 0.4117 0.5294 1.0000
0.3870 0.4193 0.3243 0.4666 0.4705 0.5882 0.4285 1.0000
0.2857 0.4230 0.2727 0.3214 0.3870 0.4242 0.4827 0.4516 1.0000
0.4242 0.2972 0.4722 0.2972 0.5000 0.5263 0.4210 0.4736 0.4411 1.0000
0.4666 0.2857 0.5625 0.3235 0.5000 0.5277 0.4166 0.3947 0.4838 0.6285 1.0000
0.5652 0.4230 0.4000 0.3703 0.5925 0.5161 0.3437 0.5000 0.3571 0.4848 0.4838 1.0000

Table 25. Similarity co-efficients between hybrids

1.0000
0.6129 1.0000
0.4848 0.5312 1.0000
0.5312 0.4848 0.6000 1.0000
0.7037 0.5333 0.4516 0.5000 1.0000
0.4333 0.5357 0.4000 0.4482 0.3928 1.0000
0.4285 0.4705 0.6333 0.5312 0.3939 0.4827 1.0000
0.4722 0.5142 0.5294 0.7333 0.4848 0.3529 0.4324 1.0000
0.4687 0.4687 0.5862 0.7037 0.5357 0.4285 0.5666 0.6129 1.0000
0.4137 0.3666 0.2500 0.4285 0.5416 0.4166 0.2424 0.3750 0.4074 1.0000
0.4062 0.5000 0.3333 0.3750 0.3225 0.5200 0.3636 0.3714 0.3548 0.4400 1.0000
0.2580 0.3448 0.4074 0.4615 0.2962 0.2800 0.3928 0.3548 0.4400 0.3636 0.4166 1.0000

4.3.1.1 OPA 10

Nine amplicons (Table 26) were produced by OPA 10 (Plate 16) of which eight were found to be polymorphic giving 88.88 per cent polymorphism. The amplicons were numbered from A1 to A9. This primer produced five intense and four faint bands (Fig. 10). The size of the amplification products was found to exceed 1000kb.

The amplicon A1 was absent in accessions Liver Red, Lady Jane, Tropical Red, Lady Jane X White, Pompon Red X Dragon's Tongue Red and Orange Glory X Kalympong Orange. The amplicon A2 was absent in ten accessions viz. White, Lady Jane, Kalympong Red, Tropical Red, Lady Jane X White, White X Lady Jane, Pompon Red X Merengue White, Pompon Red X Dragon's Tongue Red, Orange Glory X Dragon's Tongue Red, Orange Glory X Kalympong Orange. Amplicon A3 was present in Kalympong Red, Kalympong Orange, Lady Jane X White, Pompon Red X Merengue White and Orange Glory X Dragon's Tongue Red. The amplicon A7 at 800bp size was found to be monomorphic. The last faint amplicon A9 was absent in six accessions viz. Pompon Red, Dragon's Tongue Red, Ordinary Orange, Lady Jane X Liver Red, Pompon Red X Lady Jane, Tropical Red X Merengue White. Amplicons were found to have a size range from 500bp and exceeds 1000kb.

4.3.1.2 OPB 20

Seven amplicons were produced by the primer OPB20 of which six were polymorphic giving a polymorphism of 85.71 per cent (Plate 19). The amplification products were numbered from D1 to D7 and having a size range between 300bp to 900bp. The monomorphic amplicon D4 was found with a size of 600bp. Accessions Liver Red, Pompon Red, White and Lady Jane were having only one monomorhic band like Orange Glory X Kalympong Orange and Tropical Red X Merengue White. All the bands were prominent, intense and bright. Amplicon D6 was absent in 13 accessions and was present in Ordinary Orange, Kalympong Orange, Merengue White, Lady Jane X White, White X Lady Jane, Lady Jane X Liver Red, Pompon Red X Liver Red, Pompon Red X Merengue White, Pompon Red X Lady Jane, Flaking Red X Dragon's Tongue Red, Flaking Red X Liver Red. Like wise D3 was present in Tropical Red, Ordinary

Orange, Kalympong Orange, Merengue White, Lady Jane X White, Lady Jane X Liver Red, Pompon Red X Liver Red, Pompon Red X Merengue White, Flaking Red X Dragon's Tongue Red, Flaking Red X Liver Red and Orange Glory X Dragon's Tongue Red (Table 27).

4.3.1.3 OPB 15

The primer OPB15 produced eight amplicons and the size ranged from 200bp to 800bp. They were numbered from B1 to B8 (Plate 17). Of the eight amplification products only one B4 was found to be monomorphic. Five intense and three faint bands were produced by this primer. The amplicon B1 was found to present in only two accessions viz. 10 and 19 which was a faint band near 900bp size. Accession Orange Glory X Kalympong Orange was found to have seven bands among which except B6 others were faint. The amplicon B2 was present in Kalympong Orange, White X Lady Jane, Pompon Red X Lady Jane, Orange Glory X Kalympong Orange. B3 was absent in Pompon Red, Lady Jane, Kalympong Red, Tropical Red, Ordinary Orange, Fla King, Lady Jane X Liver Red, Pompon Red X Liver Red, Pompon Red X Merengue White, Flaking Red X Dragon's Tongue Red, Flaking Red X Liver Red, Orange Glory X Dragon's Tongue Red, Tropical Red X Merengue White. Monomorphic B6 was found to present between 400bp and 500bp(Table 28).

4.3.1.4 OPA 13

The primer OPA 13 (Plate 18) produced seven markers. Amplicons C6, C7, C4 and C2 were intense bands and the other three were faint bands. Among these C1 was found to be unique and present in accession Pompon Red only which ranges near 800bp to 900bp. The amplicon C5 was monomorphic found to have a size between 400bp and 500bp. Among the seven amplicons C2 was found to be present in White, Kalympong Orange, Merengue White, Pompon Red X Liver Red, Flaking Red X Dragon's Tongue Red and Orange Glory X Dragon's Tongue Red. Amplicon C7 was found to present in all hybrids and in varieties Liver Red, White, Dragon's Tongue Red, Kalympong Orange, Merengue White and Fla King(Table 29).

4.3.1.5 OPB 6

In total the primer OPB6 produced only four bands (Plate 20). This primer produced amplicons with 100 per cent polymorphism. The amplicons were numbered from E1 to E4. Amplicon E4 was found to be present in Liver Red, Pompon Red, Tropical Red, Pompon Red X Dragon's Tongue Red, Pompon Red X Lady Jane, Flaking Red X Liver Red. Accession Fla King and White X Lady Jane produced no amplicons with the primer OPB6. The size of the amplified products ranged between 600bp and 1000bp. Amplicon E1 was absent in Liver Red, Pompon Red, Kalympong Red, Ordinary Orange, Fla King, Lady Jane X White, White X Lady Jane, Pompon Red X Merengue White, Orange Glory X Dragon's Tongue Red, Orange Glory X Kalympong Orange, Tropical Red X Merengue White. Accessions Liver Red and Pompon Red were having only one amplicon E4. Like wise accessions Orange Glory X Kalympong Orange and Tropical Red X Merengue White were having only E3(Table 30).

4.3.1.6 OPB 8

The primer OPB8 produced four amplification products (Plate 21). All these four markers F1 to F4 were polymorphic. Amplicon F3 was absent in accession Tropical Red X Merengue White and present in all other accessions. Amplicons ranged from 400bp to 1000bp. Amplicon F1 was faint one and present in accessions Dragon's Tongue Red, Orange Glory, Tropical Red, Fla King, Lady Jane X Liver Red and Pompon Red X Lady Jane. Amplicon F2 was present in Dragon's Tongue Red, Orange Glory, Tropical Red, Lady Jane X White, Pompon Red X Liver Red and in Flaking Red X Dragon's Tongue Red (Table 31).

4.3.1.7 OPB 18

Eleven amplicons were produced by the primer OPB18 (Plate 22). This primer produced 100 per cent polymorphic bands. The eleven amplicons produced were numbered from G1 to G11 (Plate 22). No amplicon was found to be monomorphic. The amplification products ranged from 200bp to more than 1000bp. There were six intense bands and five faint bands. The product G1 was present in accession Flaking Red X Dragon's Tongue Red only which was a faint band. Amplicon G2 was present in

Kalympong Red, Merengue White, Lady Jane X White, Pompon Red X Merengue White and Flaking Red X Dragon's Tongue Red. G3 was present in Dragon's Tongue Red, Tropical Red, Kalympong Orange, Fla King, White X Lady Jane and Flaking Red X Dragon's Tongue Red. The amplification product G11 was found in accession Orange Glory X Kalympong Orange only which was unique and faint. Amplicon G10 was present in Orange Glory X Kalympong Orange and Tropical Red X Merengue White. The amplicon G9 was present in Flaking Red X Liver Red, Orange Glory X Dragon's Tongue Red, Orange Glory X Kalympong Orange and Tropical Red X Merengue White. Near to 500bp size the amplification product G8 was present in White, Lady Jane and White X Lady Jane. This primer produced two unique amplicons(Table 32).

4.3.2 RAPD analysis

The amplification products (Table 26 to 32 Fig. 16 to 22) obtained were scored and illustrated. Pair wise genetic distances based on RAPD analysis using AP-PCR (Nei and Li Genetic Distance GD_{NL}) genetic distance similarity co-efficient values for twelve varieties and twelve hybrids ranged from 0.1875 to 0.7333 (Table 33) indicating the wider diversity.

From the cluster analysis, lines Tropical Red X Merengue White and Pompon Red were branched out from the dendrograms. Further more, lines Lady Jane X White and Pompon Red X Merengue White formed a monophyletic group in the tree. In addition three pairs of subclusters comprising Lady Jane X White and Pompon Red X Merengue White; Liver Red and Pompon Red X Dragon's Tongue Red and the third comprising Merengue White and Lady Jane X Liver Red were observed in cluster analysis.

The UPGMA analysis of the scored data and the Jaccard's similarity co-efficient values were used for the nested clustering to develop dendrogram (Fig. 23). This cluster forms two major clusters at 34 per cent similarity. The cluster I consisted of only one hybrid Tropical Red X Merengue White. The II cluster was divided into two in which cluster IIa was having only one member Pompon Red. Cluster IIb was further divided

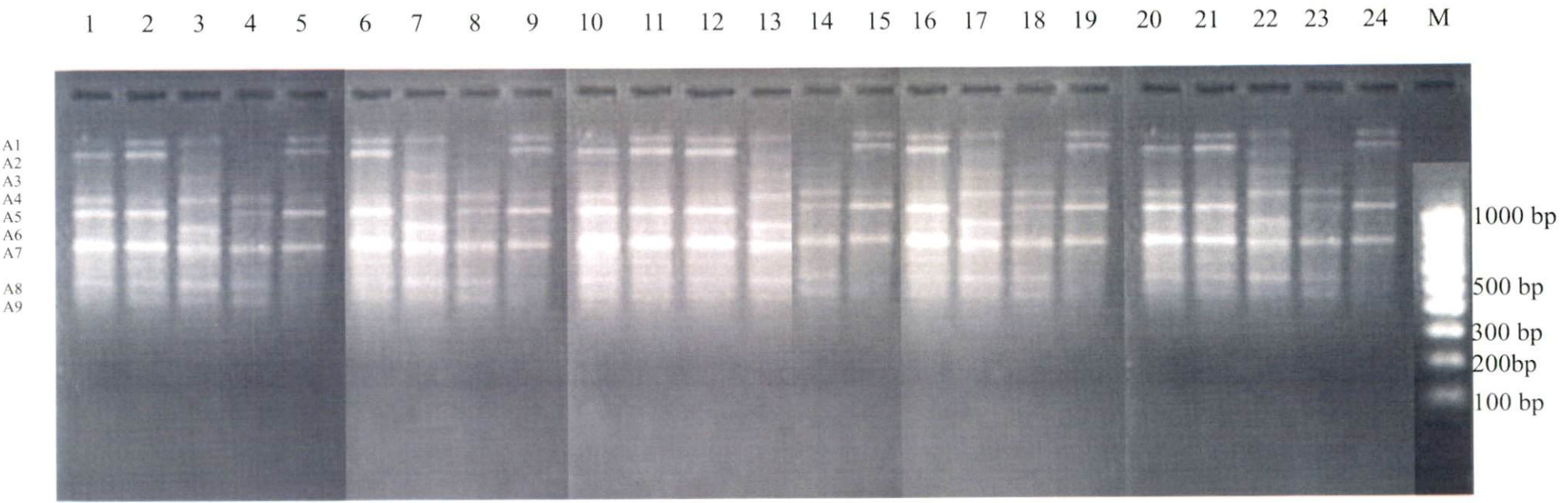


Plate 16. Amplification profile of the DNA of *Anthurium andreanum* Linden accessions using the primer OPA10

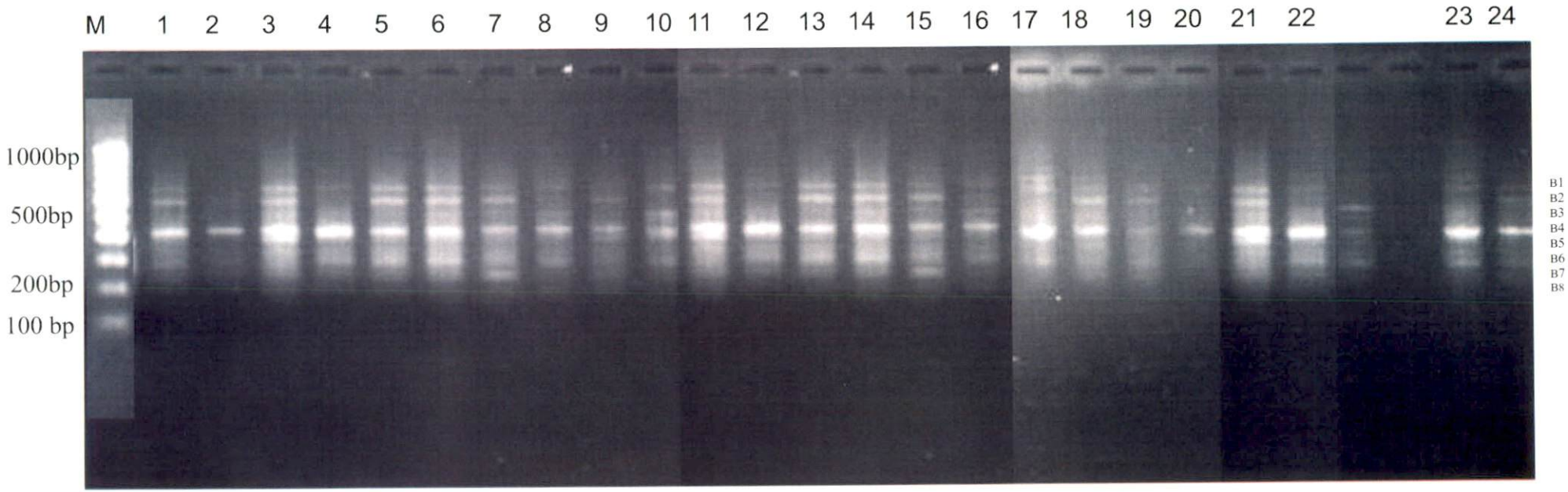


Plate 17. Amplification profile of the DNA of *Anthurium andreanum* Linden accessions using the primer OPB15

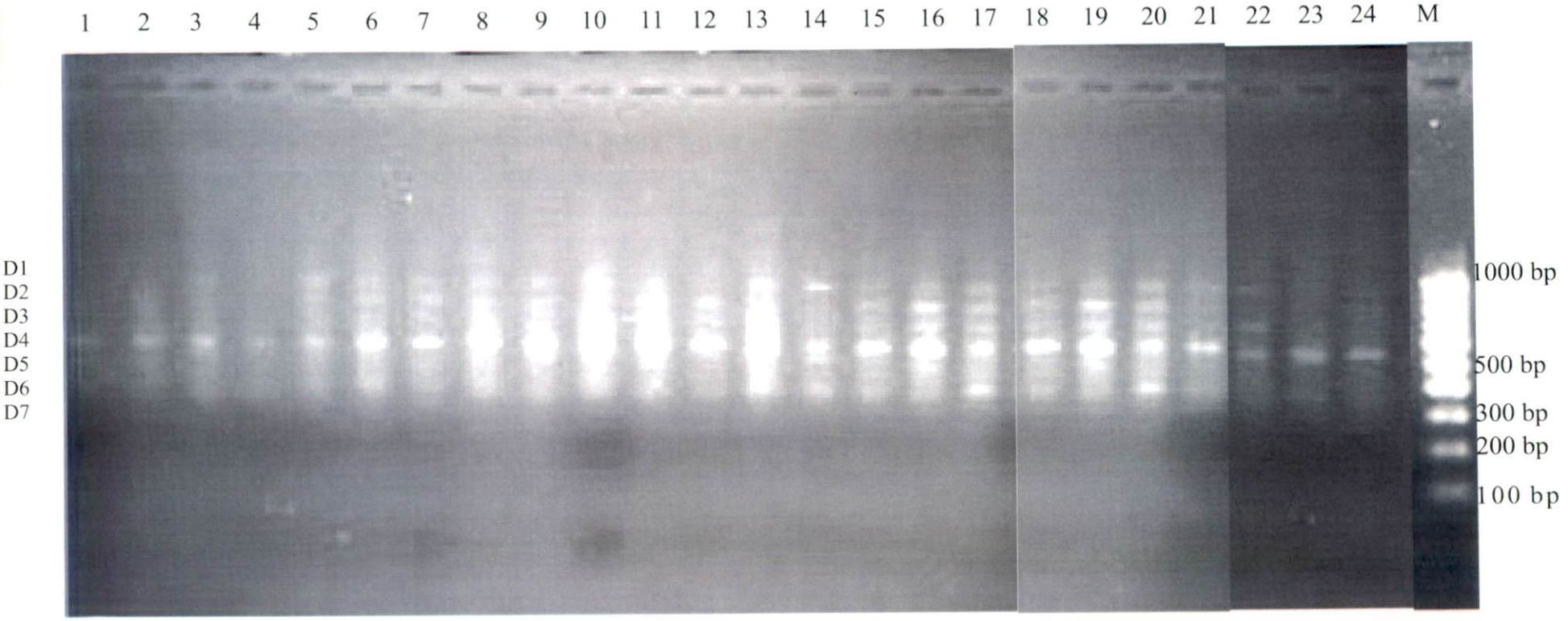


Plate 19. Amplification profile of the DNA of *Anthurium andreanum*Linden accessions using the primer OPB20

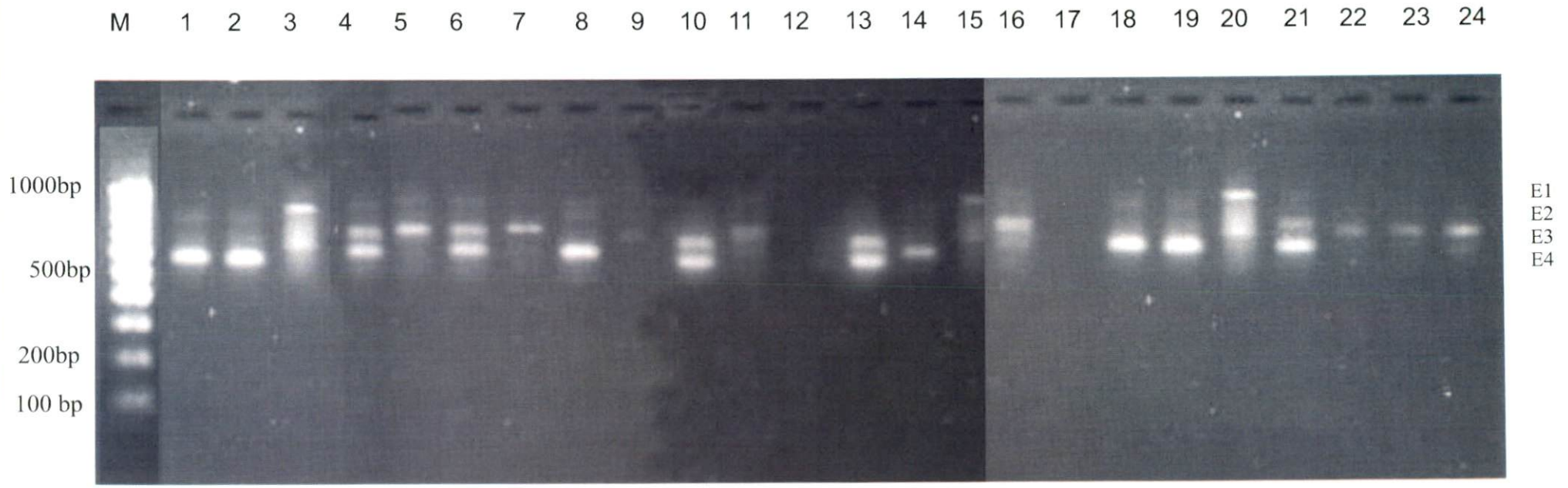


Plate 20. Amplification profile of the DNA of *Anthurium andreanum* Linden accessions using the primer OPB6

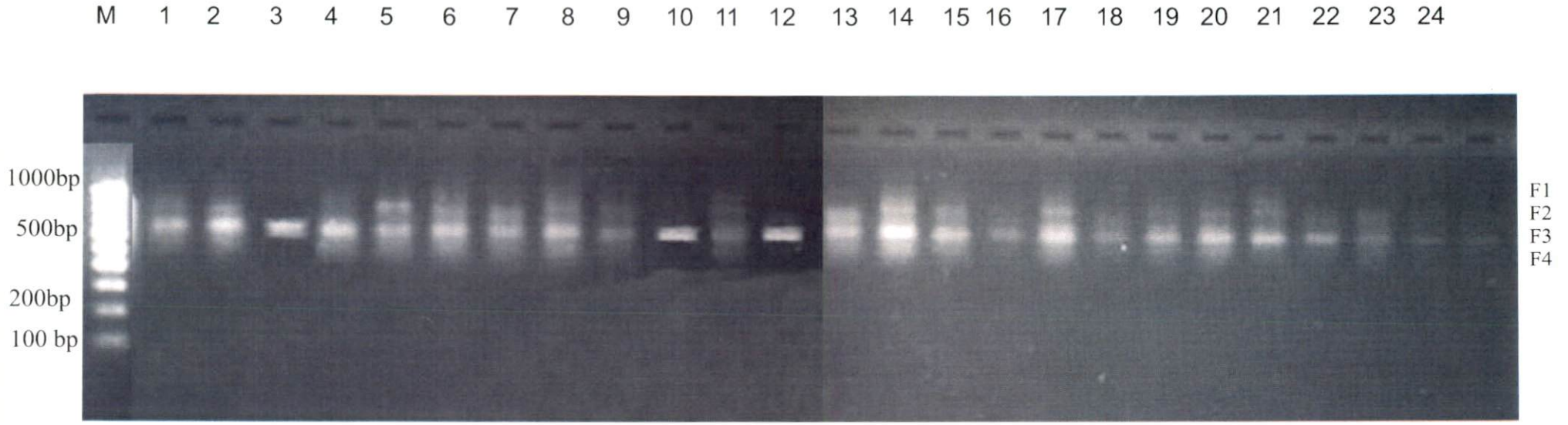


Plate 21. Amplification profile of the DNA of *Anthurium andreanum* Linden accessions using the primer OPB8

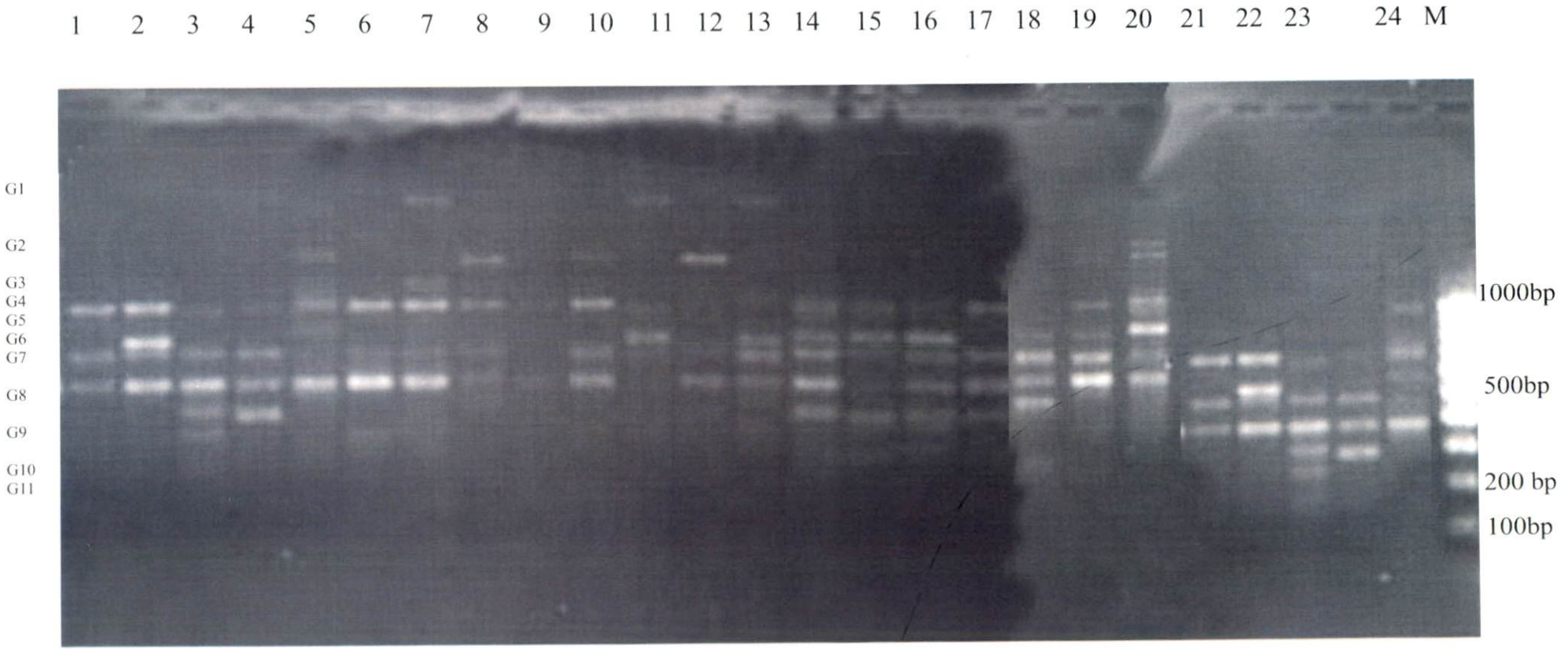


Plate 22. Amplification profile of the DNA of *Anthurium andreanum* Linden accessions using the primer OPB 18

Table26. Representation of amplification profile of DNA using primer OPA10

M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1	0	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1
A2	1	1	0	0	1	1	0	0	1	1	1	1	0	0	1	1	0	0	1	1	1	0	0	1
A3	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0
A4	1	0	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
A5	1	1	0	0	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1
A6	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
A7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A8	1	1	1	0	0	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0
A9	1	0	1	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0

Table 27. Representation of amplification profile of DNA using primer OPB 20

M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
D1	0	0	0	0	1	1	1	1	1	1	0	0	1	1	0	1	1	0	0	1	1	0	0	0
D2	0	0	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0	0	0
D3	0	0	0	0	0	0	0	1	1	1	1	0	1	0	1	1	1	0	1	1	1	0	0	0
D4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D5	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	1
D6	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	1	0	1	1	1	0	0	1
D7	0	0	0	0	0	0	1	1	1	1	1	0	1	1	1	0	1	0	1	0	1	0	0	0

Table28. Representation of amplification profile of DNA using primer OPB15

M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
B1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
B2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0
B3	1	0	1	0	1	1	0	0	0	1	1	0	1	1	0	0	0	1	1	0	0	0	1	0
B4	1	0	1	0	1	1	1	0	1	0	1	0	1	1	1	0	0	0	0	1	0	0	1	1
B5	0	0	1	0	0	1	0	0	0	1	1	0	0	1	0	0	0	1	1	1	0	0	1	0
B6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
B7	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0
B8	0	0	1	1	1	1	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	1	0

Table29. Representation of amplification profile of DNA using primer OPA13

M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
C1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	1	0	1	0	0
C3	0	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C4	0	1	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C6	0	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C7	1	0	1	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table30.Representation of amplification profile of DNA using primer OPB6

M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
E1	0	0	1	1	1	1	0	1	0	1	1	0	0	0	1	1	0	1	1	1	1	0	0	0
E2	0	0	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	0	0	1	1	0	0	0
E3	0	0	1	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	1	1	1
E4	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0

Table31.Representation of amplification profile of DNA using primer OPB8

M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
F1	0	0	0	0	1	1	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0
F2	0	0	0	0	1	1	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0
F3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
F4	0	1	0	1	1	1	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0

Table32.Representation of amplification profile of DNA using primer OPB18

M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
G1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
G2	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	0	0
G3	0	0	0	0	1	0	0	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0
G4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
G5	1	1	0	0	1	1	1	1	0	1	0	0	0	1	1	0	1	0	0	1	0	0	0	0
G6	0	1	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	1	1	0	0	0	0	0
G7	1	1	1	1	1	1	1	0	0	1	0	1	1	1	0	0	1	1	0	0	0	1	0	0
G8	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
G9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
G10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
G11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0

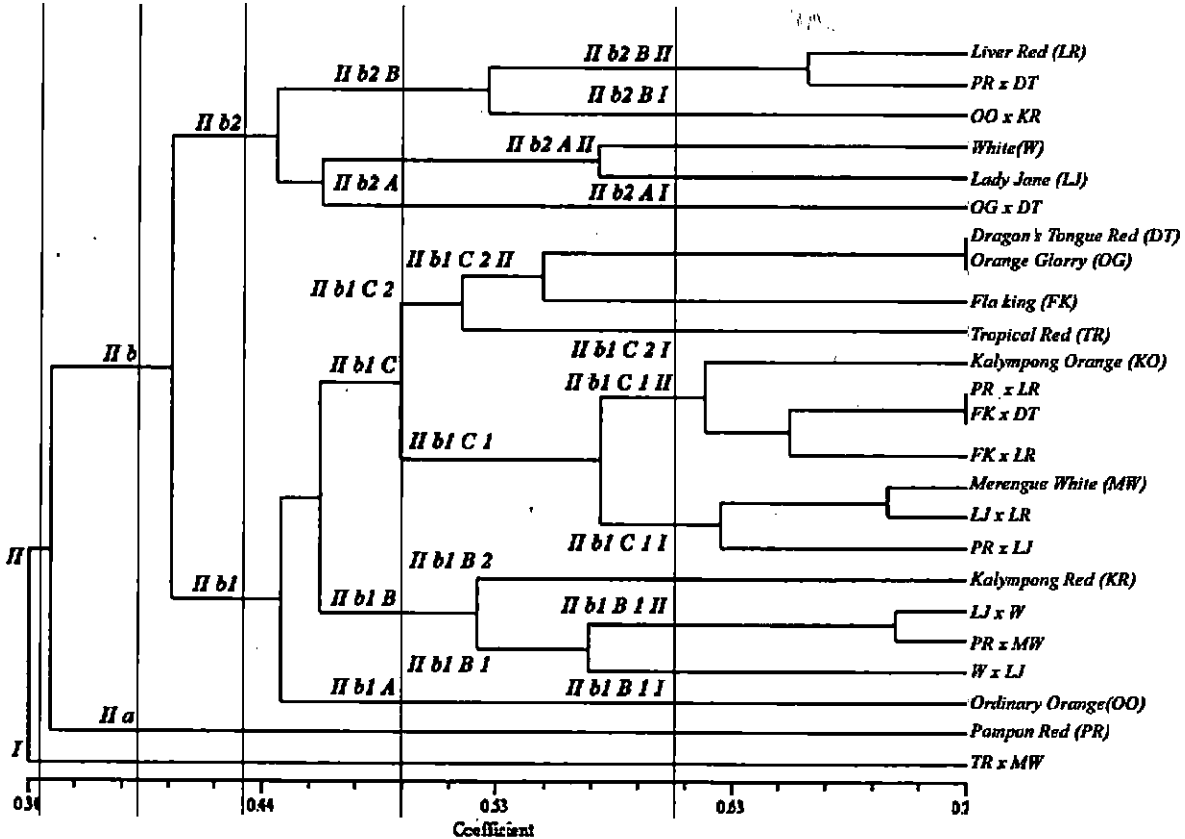


Plate 23./ Fig. 8 UPGMA - Dendrogram obtained from AP - PCR (RAPD) analysis of *Anthurium andreanum* Linden varieties and hybrids based on similarity coefficient values

Table 33. Similarity matrix of Anthurium hybrids and varieties based on Jaccard's similarity index

1.0000
0.4583 1.0000
0.4814 0.2812 1.0000
0.3461 0.3846 0.5769 1.0000
0.5185 0.3548 0.4687 0.4000 1.0000
0.5000 0.4375 0.5937 0.5333 0.7333 1.0000
0.3666 0.4000 0.4687 0.4482 0.4117 0.5294 1.0000
0.3870 0.4193 0.3243 0.4666 0.4705 0.5882 0.4285 1.0000
0.2857 0.4230 0.2727 0.3214 0.3870 0.4242 0.4827 0.4516 1.0000
0.4242 0.2972 0.4722 0.2972 0.5000 0.5263 0.4210 0.4736 0.4411 1.0000
0.4666 0.2857 0.5625 0.3235 0.5000 0.5277 0.4166 0.3947 0.4838 0.6285 1.0000
0.5652 0.4230 0.4000 0.3703 0.5925 0.5161 0.3437 0.5000 0.3571 0.4848 0.4838 1.0000
0.4482 0.2285 0.4545 0.3030 0.4411 0.4324 0.5312 0.4166 0.3750 0.4864 0.6250 0.4193 1.0000
0.5555 0.3437 0.5483 0.3437 0.5312 0.5142 0.4411 0.4166 0.3750 0.5714 0.6250 0.5172 0.6129 1.0000
0.4137 0.3548 0.3823 0.3548 0.6551 0.5294 0.3714 0.4705 0.5357 0.5000 0.7000 0.5357 0.4848 0.5312 1.0000
0.4137 0.3125 0.4687 0.3548 0.5000 0.5294 0.3333 0.5151 0.4827 0.6363 0.7000 0.5357 0.5312 0.4848 0.6000 1.0000
0.4615 0.3000 0.4193 0.3000 0.4062 0.4000 0.6071 0.4242 0.3793 0.5454 0.5000 0.4814 0.7037 0.5333 0.4516 0.5000 1.0000
0.6666 0.4400 0.5185 0.4400 0.4482 0.4838 0.2727 0.4193 0.1935 0.4117 0.5000 0.5416 0.4333 0.5357 0.4000 0.4482 0.3928 1.0000
0.4000 0.3030 0.3333 0.2285 0.4411 0.3947 0.2564 0.4166 0.4193 0.5714 0.6250 0.4666 0.4285 0.4705 0.6333 0.5312 0.3939 0.4827 1.0000
0.4062 0.2777 0.4571 0.2777 0.5294 0.5555 0.3684 0.5000 0.4687 0.6571 0.6666 0.4687 0.4722 0.5142 0.5294 0.7333 0.4848 0.3529 0.4324 1.0000
0.4444 0.3333 0.3636 0.2903 0.4375 0.4285 0.3529 0.5000 0.5769 0.5757 0.6333 0.5185 0.4687 0.4687 0.5862 0.7037 0.5357 0.4285 0.5666 0.6129 1.0000
0.4347 0.3076 0.5600 0.3600 0.2903 0.3333 0.3793 0.2727 0.2068 0.3939 0.3437 0.4583 0.4137 0.3666 0.2500 0.4285 0.5416 0.4166 0.2424 0.3750 0.4074 1.0000
0.5416 0.2258 0.5357 0.3571 0.3750 0.4545 0.2941 0.3142 0.2187 0.3888 0.4687 0.3928 0.4062 0.5000 0.3333 0.3750 0.3225 0.5200 0.3636 0.3714 0.3548 0.4400 1.0000
0.3478 0.2800 0.2758 0.2307 0.3103 0.2727 0.1875 0.2500 0.3750 0.3750 0.3666 0.4347 0.2580 0.3448 0.4074 0.4615 0.2962 0.2800 0.3928 0.3548 0.4400 0.3636 0.4166 1

into IIb1 and IIb2 at 40 per cent similarity level. IIb1 was having three sub clusters viz. IIb1A (Ordinary Orange), IIb1B with two more subsects viz. IIb1B2 (Kalympong Red) and IIb1B1. In IIb1B1, White X Lady Jane formed a separate cluster at 57 per cent similarity whereas Lady Jane X White formed a cluster with Pompon Red X Merengue White which was having more than 64 per cent similarity between them.

In subclusters of IIb1C, IIb1C1 is having Pompon Red X Lady Jane clustered with a pair of Merengue White and Lady Jane X Liver Red at 63 per cent co-efficient. The next subcluster IIb1C1II was having Pompon Red X Liver Red and Flaking Red X Dragon's Tongue Red in one cluster with 70 per cent similarity which were separated from Flaking Red X Liver Red at 65 per cent and Kalympong Orange at 61 per cent similarity co-efficient. In subcluster IIb1C2 Tropical Red was isolated; Fla King was separated from Dragon's Tongue Red and Orange Glory which were found to be related with 70 per cent similarity.

The second major subcluster IIb2 was divided into two more clusters IIb2A and IIb2B. IIb2B is having three members viz. Ordinary Orange X Kalympong Orange, Pompon Red X Dragon's Tongue Red and Liver Red. Among these three members Pompon Red X Dragon's Tongue Red and Liver Red were found to have 67 per cent similarities which were isolated from Ordinary Orange X Kalympong Orange. In the subcluster IIb2A Orange Glory X Dragon's Tongue Red forms a separate cluster with 46 per cent similarity. White and Lady Jane were formed a single cluster at 57 per cent.

Discussion

5. DISCUSSION

Anthurium is one of the most important commercial ornamental crops of the modern world which is now gaining importance. The cultivation practices are relatively simple. The colourful elegant cut flowers are highly prized for their long shelf life and they are fetching good price as they can be transported without damage.

The agroclimatic conditions of Kerala are highly conducive for the cultivation of anthuriums. Kerala is a product specific intensive floriculture zone for anthuriums. It is having great export potential for their beautiful flowers and handsome foliage in the current scenario. There is a great demand for new hybrids and varieties in the highly competitive international market as consumer preference is changing with regard to flower colour, shape and size within a short span.

Studies on *in vitro* propagation open new challenges for the creative ability of the scientist and desires of the consumers. Biotechnology, through cell culture and *in vitro* genetic manipulations are quite competent to meet the challenges of specific demands in order to tailor a crop to match the need of the people.

Anthuriums can be propagated by seeds as well as by vegetative means. As it is a cross pollinated crop, the seedling progenies were found to be highly heterogenous showing wide variability and takes six to eight months from pollination to seed maturiuty. The seedlings require about three years to reach the first flowering. Mature plants produced only one or two suckers per year. Suckers can also be induced by top cuttings. However, all these methods result insufficient multiplication rates for mass clonal propagation. *In vitro* propagation techniques become relevant in this context which [illegible] clonal propagation. Methods of *in vitro* propagation, mainly through [illegible]ganogenesis, have been standardized for few varieties of *Anthurium* [illegible]*n* (Pierik, 1976; Pierik *et al.*, 1974a, b; 1979 a) and *Anthurium scherzerianum* (Pierik and Steegmans, 1976; Geier, 1986 b). Not much work has been conducted in other species which shows wider variability in their requirements within species and

among varieties and hybrids. Clonal multiplication of *Anthurium andreanum* from stem sections of aseptically grown seedlings has been attempted by Kunisaki (1980). But in the experiment, the effect of only a single cytokinin (BA) has been tested. Somatic embryogenesis of callus developed from spadix explant of *Anthurium scherzerianum* has been reported by Geier and Reuther (1981) and Geier (1982). However, this mode of regeneration was found to be sporadic and the factors required for its consistent induction.

Though *Anthurium andreanum* is an important flower crop, methods of *in vitro* propagation via somatic organogenesis and somatic embryogenesis has not been standardized in *Anthurium andreanum* hybrids. Hence, there is a need for improving the rate of multiplication. Also, there is poor or nil development in *Anthurium andreanum* for standardized protocol. As per the views of Sreelatha (1992) the methods of somatic embyrogenesis, which ensures highly efficient rate of multiplication, has not been properly exploited for anthurium propagation. The present study was taken up with the objectives of improving the propagation efficiency of *Anthurium andreanum* Linden through all possible means of *in vitro* propagation. Standardization of media for the varieties and hybrids of *Anthurium andreanum* that are not having the protocols of *in vitro* propagation was attempted. Previously not much work has been conducted to overcome the recalcitrant nature exhibited by different explants in the species and hybrids of anthurium along with the existence of extreme variability. Hence, an attempt was made for the molecular characterization and contrasting of different species and hybrids of *Anthurium andreanum* with respect to their difference in *in vivo* phenotypic expression. Twelve varieties and twelve hybrids were selected for the study and the salient results are discussed in the following pages.

The *in vitro* response of plants is influenced by genotype in many cases. This influence is highly pronounced in the case of *Anthurium andreanum.* Protocols for *in vitro* propagation have been standardized for a few varieties of *Anthurium andreanum.* Hence the present investigation was taken up with the objective of standardizing the

media and culture condition for *Anthurium andreanum* hybrids and their parents and to characterize them at molecular level.

Physiological nature of the parent plant can account for the success of organogenesis in cell cultures. Recalcitrant nature in cultures may be attributed to the physiological status of the donor plant. The physiological expression and cell status is the direct response and expression of the genotype. The variation at genome level can be evaluated by molecular characterization using AP-PCR for RAPD which is an advanced technique with stringency in annealing temperature.

5. 1 *IN VITRO* MULTIPLICATION

5. 1. 1 Surface Sterilization

Microbial contamination has long been a major problem in the culture establishment of explants. Since plant parts are exposed in the field for a long time, they harbour various microorganisms and many of which penetrate into the plant tissue resulting in systemic infection. Cultures from such tissues are hence easily contaminated (Chan and Evans, 1990). In the present study also, high rate of fungal contamination was observed since the explants were collected from field grown plants. In order to minimize the rate of contamination, the explants were subjected to various surface sterilization treatments.

Lightbourn and Prasad (1990) used 32.5 per cent Benlate solution to soak in for 24h, sterilized by rinsing in 70 per cent alcohol for 45 seconds again soaking in 1.25 per cent sodium hypochlorite for 15 minutes then rinsed 3 times in sterile distilled water for 15 minutes. Parkinson *et al.* (1996) concluded from his work that the use of sodium dichloroisocyanurate was highly stable both as pre-prepared tablets and as solutions maintained at the room temperature. These two treatments were found to be tedious. Hence in the present investigation, simple and effective double sterilization techniques were tried.

Among the treatments tried to standardize surface sterilization, the double sterilization treatment with 95 per cent Ethyl alcohol dip for 1 minute, 0.08 per cent Mercuric chloride treatment for 10 minutes followed by 70 per cent Ethyl alcohol for 3 minutes was found to be very effective. This treatment was capable of producing more than 95 per cent contamination free cultures. There is significant varietal difference. Some genotypes are highly sensitive and explants like tender leaf lamina showed higher sensitivity for prolonged treatments. Death of explants was more in leaf lamina and spathe explants. Candle explants showed greater stability to treatments except for the mucilage exudation which was removed by excision and trimming after sterilization and wiping on a sterile blotting paper.

Surface disinfection treatments were standardized for the different explants. Irrespective of the explants and varieties, double sterilization was found to be effective. Among the explants, the highest number of sterile cultures was observed in double sterilization, followed by the treatment with 70 per cent Ethyl alcohol for 20 minutes. Majority of the contamination found in the cultures was due to the presence of systemic infection of *Xanthomonas compestris pv dieffenbachiae.* This directly influences the percentage of contamination occurred in the culturing condition and the size of explants which also play a major role in creating the bacterial contamination. Candle explants were found to exhibit more systemic infections than other explants and seed explants were found to be free from systemic infections. Leaf explants are highly vulnerable to exhibit systemic infections and are more sensitive; unable to recover even after treatments with antibiotics. The callus cultures exhibiting systemic infections can be recovered by kanamycin 50 mg per litre containing multiplication medium (Plate 15).

5. 1. 2 Explants and Medium

Although MS major nutrients are good to have a starting point for medium development, more dilute solutions may prove to be better in some circumstances. Adjustment of the ionic concentration becomes necessary when one or more ions at the normal level are inhibitory to a species. In *Anthurium andreanum* callus was observed only in a modified MS medium (with reduced major salt concentration). No callusing was

observed in a medium at normal strength. Half strength MS major nutrients with full strength micro nutrients have been found to be suitable for the *in vitro* culture of anthurium (Pierik *et al.*, 1974; Pierik and Steegmans, 1976). Kunisaki (1977) reported negatively for *in vitro* response of *Anthurium andreanum*. Kunisaki (1980) found that stem sections from aseptically grown plantlets gave best results in modified MS medium with 15 per cent coconut water and 0.2mg BA per litre.

Pierik *et al.* (1974 b) stated that for callus initiation twelve weeks were needed (84 days) and further eight weeks (56 days) were needed to transfer the culture from callus to shoot induction stage.The results of the present investigation indicated a wide range of requirements for different varieties and hybrids. It ranged from 72 days for variety OG (lesser than the earlier reports) to 113 days for the hybrid OG X DT (more than the earlier reports). Thus the days required for callus formation was influenced by the genotypes and treatments viz. medium and hormones.

Kunisaki (1980) used MS salts at full strength and recommended that MS major nutrients ¼ strength + micro nutrients at full strength can be recommended for multiple shoot induction in anthurium. Most of the workers in anthurium tissue culture have recommended reduced MS salts. This reduced salt requirement may be species specific. But the results of the present investigation also prove that all the varieties and hybrids tested in the experiment need only less quantity of major salts.

Sugars are indispensable in the basal medium as they are not only the source of carbon, but also involved in osmoregulation. Two per cent sucrose was employed by Kunisaki (1980) for multiple shoot induction. In contrast to the reports of Kunisaki (1980) the present study revealed that high sucrose content upto four per cent influenced significantly shoot formation, multiple shoot induction and regeneration. Though the anthuriums are epiphytic in nature the development of xylem and phloem were controlled by sucrose concentration and hence regeneration was successful at higher sucrose concentration but concentrations more than four per cent were found to be harmful. Callus induction, regeneration, multiple shoot formation and embryogenesis may differ

with carbon sources. In the present study, it was observed that glucose produced less number of shoots compared to sucrose. The advantage of sucrose over glucose may be derived from its more effective translocation to apical meristems (Butcher and Street, 1964). The number of shoots did not differ significantly in the treatments with different concentrations of agar.

Pierik *et al.* (1975) and Pierik (1976) found half strength MS as suitable one. The MS medium characterized by high concentration of mineral salts has been widely used for general plant tissue culture (Murashige, 1974).

Activated charcoal has the capacity to absorb the toxic substances and residual cytokinin from the medium (Fridborg *et al.*, 1978). In the present experiment the medium supplemented with activated charcoal showed better shoot growth and was found to absorb the phenolic exudations secreted from the inoculated explants. This was in accordance with the results of Anita (1996).

Pierik *et al.* (1979) obtained callus formation and regeneration in medium containing adenine -0.1mg, zeatin 1mg and 2, 4-D - 0.008 mg per litre. Similar results were observed in the present investigation also. Zhang-Gui He *et al.*, (2001) reported to have higher callus induction in *Anthurium andreanum* cultures on MS medium supplemented with 0.1 mg of BA per litre. In contrary to this Prakash *et al.* (2002) opined that *Anthurium andreanum* cv. Liver Red grown in Nitsch basal medium with coconut water facilitated faster germination of the embryo produced *in vitro*. But Yang-YuanHsin *et al.* (2003) confirmed the earlier report by culturing the lamina on a MS medium supplemented with 0.1 to 0.5 mg TDZ per litre induced callus formation in 98 to 100 percentages of the cultures whereas Lan-Qin Ying *et al.* (2003) concluded that promising results were recorded on N6, Knudson C and ½ MS media for the leaf blade explant. From the views of above authors, it was obvious that the requirement for each and every genotype varies considerably.

In most studies of *in vitro* culture of anthurium, MS medium has been used. In the present study, it was observed that Nitsch and Nitsch medium was better than MS medium for multiple shoot induction. Nitsch and Nitsch medium was especially suitable for morphogenesis, meristem culture and regeneration.

As the genotype showed different nutrient requirements for their survival and growth, the present investigation was planned to standardize the media by screening with modified MS, half strength MS, modified NN and half strength NN medium. Modified NN with activated charcoal and coconut water showed better response. Half strength NN with coconut water and activated charcoal, modified MS with activated charcoal and coconut water and half strength MS activated charcoal and coconut water were also found to support the explants without hindering the survival. In contrary to the reports of Sreelatha (1992) addition of inositol and glycine along with folic acid was found to be essential but the presence of small amount was inefficient. In the present investigation no callus initiation was observed when inositol was reduced to half of the reported quantity.

Leaf explants were found to produce callus by Keller *et al.* (1982). According to Malhotra *et al.* (1998) leaf explants were found to be responsive for the *Anthurium andreanum* cv. Nitta, Osaki and Anouchka when cultured on modified MS medium having reduced concentration of ammonium sulphate at 200 mg per litre, supplemented with BA at 1 mg per litre and 2, 4-D at 0.1 mg per litre to get callus induction. While Montes *et al.* (2000) concluded the leaf explants from *Anthurium cubense* as an alternative to *in vitro* culture of seeds. These were used to produce white callus mass and subcultured on medium containing 4.7 μM Pectimorf to obtain a regeneration rate of up to 17 buds per explant.

Zens and Zimmer (1986) used shoot tip explants to produce callus cultures and adventitious shoots were found to increase significantly but Zimmer (1990) was of the opinion that seed explants when cultured *in vitro* can produce plantlets. Singh (1994) investigated on spadix explants to get better capacity for regeneration than leaf segments on the modified Nitsch medium and plantlets derived from spadix segments were less

variable. Kuehnle and Sugii (1991) utilized petiole explants to dedifferentiate into callus on Pierik, modified Pierik and Fennie and Vanstaden medium. Petiole explants were found to be good in *Anthurium andreanum* cv. Mauritius orange by Prakash *et al.* (2001). These reports confirm the results of the present investigation that except spathe all other explants will produce regenerable calli which was contradictory to the reports of Anita (1996) and Sreelatha (1992).

5. 1. 3 Callus Induction and Multiplication

For any given species or variety, a particular explant may be ideal for successful plant regeneration. In several species explants consisting of shoot tips and isolated meristems which contain mitotically active cells are generally successful for callus initiation and subsequent plant regeneration (Murashige, 1974). In the present study, callus initiation was observed in the leaf, seed, spadix, shoot tip, candle and petiole explants. When explants from spathe and inflorescence stalk were used, callus formation was not obtained. Morphological and physiological status of the explants can account for the difference in the response. The less lignified tissues of leaf may facilitate easy de-differentiation process than the tissues of other plant parts.

Leffering *et al.* (1976 a, b) reported higher callusing capacity for apical portions of leaf in anthurium. In the present study, variation with respect to callus initiation was observed between basal and apical portions of leaf. In general for the basal portions of leaf, the number of days taken for callus initiation was 90 to 95 and for apical portions it ranges from 110 to 112 days. The difference in response between the basal and apical portions may be due to the difference in the physiological state as well as the number of cells undergoing dedifferentiation. Perhaps, more number of cells undergoes dedifferentiation in the basal portions of the leaf. Physiological state may account for the changes in the content of endogenous phytohormones, nutrients and metabolites. While comparing the days required for callus induction hybrids were found to take more number of days than varieties. In general, the hybrid OO X KR required more number of days for callus induction and multiplication when compared to other varieties or hybrids.

In anthurium, optimum callus formation and subsequent growth have been observed in continuous darkness by Pierik *et al.* (1975) and Pierik (1976). The present study also revealed that darkness was essential for callus initiation. The beneficial effect of darkness may be attributed to the etiolating effect. Reid (1972) reported that etiolated tissues may be less lignified, than the light grown tissues which facilitate easy de-differentiation. Herman and Hess (1963) proposed that the increased content of auxin co-factors in the etiolated tissues which increased the tissue growth was comparable to exogenously applied auxin. The explants became brown when exposed to light for 16h. Browning of the explants may be due to the oxidation of phenolic compounds under light. Inhibitory effect of light on callusing and further growth has been reported in monocots (Lowenberg, 1969; Pierik, 1974; Pierik and Steegmans, 1975).

In contrary to the present results which show callus formation and regeneration from all types of explants selected from the responding genotypes, Anita (1996) noticed response only from lamina. However, the present investigation confirms her results in the case of spathe explants which produced only swellings and no callus formation. According to the genetic control of morphogenesis, some genes exert their influence by regulating the effective levels of plant growth substances which may vary in different genotypes and thus account for the difference in spadix explants which produced only swellings.

Various treatments were tried for callus multiplication. The maximum fresh weight of callus was observed in PR X DT inoculated in NN medium with major nutrients at normal strength followed by OG X DT for the same composition in NN medium. From the economic point of view NN medium can be recommended for callus multiplication.

Pierik et al. (1974 a, b) succeeded in callus induction and regeneration from embryo and tissues collected from seedlings using a modified MS medium supplemented with Cytokinin PBA [6- (benzylamino)-9-(2-tera hydro pyranyl)-9-purine]. Optimum callus growth was obtained by incubation at 25°C in dark. Pierik et al. (1975) studied 38

genotypes of *Anthurium andreanum* and they observed moderate to strong callus formation from leaf segments in thirty one types; very poor callus in four types and no response in three genotypes. This indicates that the genotypes vary in their response to *in vitro* propagation. These results were supported by the striking differences in the growth rate among subcultured callus clones of *Anthurium andreanum* observed by Pierik (1975).The present investigation confirms the above reports out of twenty four only six genotypes were found to respond for the treatments planned for execution.

Sreelatha (1992) obtained callus induction only in leaf explants. Thus the view of Flick *et al.* (1983) stating that for any given species or variety, a particular explant may be necessary for successful plant regeneration, was over ruled by the results of the present investigation. This was already proved by Singh and Sangama (1990), Sreekumar *et al.* (1992) and Singh (1994) who reported callusing in petiole and spadix explant.

Liquid medium was used by Pierik, (1975) and Pierik et al. (1975) to culture leaf pieces with the callus. Based on the detailed studies a scheme was proposed for the micropropagation of *Anthurium andreanum* and *Anthurium scherzerianum* by Pierik and Steegamns (1975), Pierik (1976) and Pierik *et al.* (1979). Leffering *et al.* (1976 a) and Leffering and Hoogstrate (1977) found a close correlation between the ability of leaf explants to form callus and the further growth in subcultures. They also reported that in most genotypes callus growth was too slow and inconsistent to be exploited for large scale micropropagation. Multiple shoots regeneration method developed by Leffering and Soede (1978; 1979 a, b) using 2-iP and BA from the callus developed by inoculation of leaf lamina explants was a better method when compared to callus multiplication where there was least possibility to generate variations *in vitro*. This was opposed by Yu-kwang Jin *et al.* (1995) who opined that callus culture was more promising than shoot culture for micropropagation.

Geier (1986a) and Lightbourn and Prasad (1990) used MS medium and Nitsch's medium with 2, 4 –D for better callus induction, whereas Kuehnle and Sugii, (1991) used BA along with 2, 4-D. Sreelatha *et al.* (1998) concluded that MS medium having quarter

the strength of major nutrients supplemented with 2, 4-D and BA in MS medium was best for callus induction and also suitable for callus multiplication. This confirms the views of Somaya *et al.* (1998) who investigated on micropropagation of *Anthurium andreanum* using seed explants for callus induction and found MS medium with 2, 4-D as a suitable one. For leaf, petiole, node and root explants the author utilized BA along with 2, 4-D for callus induction.

According to Prakash *et al.* (2001) the callus induction was good on MS medium with two per cent sucrose and 0.8 per cent agar supplemented with 2,4-D, kinetin and BA. The best combination found for callusing was MS with 1.0 or 0.5 mg 2, 4-D. Higher concentrations of 2, 4-D was observed to be toxic. Cultures initiated in MS medium containing IBA and kinetin with subculturing on every third day produced callus induction in a treatment with 2 mg IBA and 6 mg kinetin per litre of MS medium (Dhananjaya and Sulladmath, 2003).

The results of the present experiment proved that callus induction required more specificity in the nutrient requirements. Out of sixteen media compositions tried, only eight were found to be responsive. These eight treatments were forwarded to next set of subculturing. The callus induction response ranged from 25 to 73.5 percentages. No treatment was effective more than 75 per cent. Highest response was recorded in modified NN activated charcoal and coconut water along with 5.37µM NAA, 2.26µM 2, 4-D, 9.12µM zeatin. The growth score and callus index indicated very good response in media containing modified NN with activated charcoal and coconut water along with 4.52µM 2, 4-D and 9.12 µM zeatin was found to provide maximum callus growth. Zeatin was found to be more effective when comparing with kinetin but ineffective in the absence of 2, 4-D.

Prakash *et al.* (2002) worked on Liver Red and found that the first and the third leaves were recorded to produce callus from 20 to 36 days after inoculation and 48 to 88 days after inoculation respectively.

Induction of callus growth was observed at the base of the explants in treatments with BA and 2iP whereas no callus growth was observed with kinetin. Leffering and Soede (1979) observed optimum branching of *Anthurium andreanum* shoots in a medium containing 13.7 μM kinetin. They also observed that BA and 2iP caused less branching and promoted callus growth. Callus growth at higher BA levels has been also reported by Kunisaki (1980).

Among the treatments, combination of 2, 4-D and zeatin was found to be the best. It stimulates callus formation and strongly antagonizes organized development. The low auxin requirement may be due to the high potency of the auxin which was used for callus initiation. The young developing leaf may be a rich source of endogenous auxins due to which lower exogenous application was required.

5. 1. 4 Regeneration

The initial cultures required full year for complete development of a rooted plantlet from callus and that was reduced in further multiplication cycles by generating multiple shoots within 5 months. Henny *et al.* (1988) reported that the *Anthurium andreanum* variety Southern Blush required ten to twelve months to reach marketable size in 150 mm pots.

Sreelatha (1992) reported enhanced release of axillary buds and the role of cytokinins in the culture establishment of anthurium shoot apices. Kinetin 2mg per litre and BA 1 mg per litre were found to be equally effective in inducing multiple shoots to a maximum of 4.5 shoots per culture. This was found to be a common phenomenon in the present investigation and all the successful treatments were able to produce multiple shoots. But somatic direct organogenesis was found to be a difficult task in the responding genotypes selected for the investigation. Direct organogenesis was reported by Leffering and Soede in 1979 a by the addition of 3mg 2iP and they reported callusing in medium containing 1 mg BA. Candle (spadix fragments) was found to have much higher capacity for regeneration than segments of leaf, petiole and inflorescence stalk.

Shoot primordial initiation of *Anthurium andreanum* cv. Mauritius orange was noticed on ten month old callus cultures when transferred to MS basal medium by Prakash *et al.* (2001). Lan-Qin Ying *et al.* (2003) was of the opinion that the period from explant to bud differentiation was 49 days which was 11 to 31 days earlier than the period previously reported.

Reduction in the concentration of BA for shoot elongation has been observed by Dantu and Bhojwani (1987) and Rajmohan (1985). Auxins in the medium promote cell elongation and may be useful to nullify the suppressive effect of cytokinin on shoot elongation (Lundergam and Janik, 1980).

Irrespective of the source of explant all the callus cultures were able to be converted into plantlets by redifferentiation. The number of days taken for regeneration ranges from 55.5 to 82. This variation was due to the varietal difference and difference in hormonal effect. Modified NN with activated charcoal and coconut water along with 22.2 µM BA, 11.42 µM IAA and 4.09 µM biotin was found to produce regenerants.

Photomorphogenesis was facilitated by pigments in the tissues which absorb radiation of particular wave lengths. Light was required for photomorphogenesis. Exposure to light had profound influence on multiple shoot formation. Induction of callus was observed at the basal portion of the explants under conditions of darkness. Several adventitious shoots were seen formed from the callus by non-inhibition of the growth of axillary shoots. Hence, exposure to light was found essential for enhanced release of axillary buds and for the maximum *in vitro* growth of culture by Hu and Wang (1983). Somatic organogenesis has proved to be the most successful route of *in vitro* propagation in *Anthurium andreanum* (Pierik, 1976; Pierik *et al.*, 1979; Sreelatha 1992; Nirmala and Singh, 1993; Satheeshkumar and Seeni, 1994).

5. 1. 5 Somatic Embryogenesis

There are reports on *in vitro* propagation of anthurium via somatic embryogenesis. Somatic organogenesis and embryogenesis were tried from explants

namely, leaf, petiole, spike, spathe and inflorescence stalk. In anthurium a low content of auxin with a high content of cytokinin has been reported to be suitable for embryogenic callus formation (Pierik *et al.*, 1975; Pierik *et al.*, 1979; Finnie and Van Staden, 1986; Geier, 1986b). Similar response was also apparent in the present instance.

Kuehnle *et al.* (1992) was the first to study the somatic embryogenesis in anthurium from callus. The production of secondary embryo was induced on the surface of the primary embrogenic calli without affecting the preformed ones. Half strength MS medium was found to produce somatic embryos with 1.0 to 4.0 mg per litre 2, 4-D and 0.33 to 1.0 mg per litre kinetin which was having one per cent glucose and two per cent sucrose. Embryos were found to regenerate in a medium containing 0.2 mg per litre BA and two per cent sucrose and placed in the light for conversion into plantlets.

Matsumoto *et al.* (1996) has done histological analysis of somatic embryos derived from *in vitro* cultured laminas of *Anthurium andreanum* which showed bipolarity with the presence of shoot and root poles connected by procambium. Vascular connections between the explants and somatic embryos were not observed. Storage of proteins, starch, and raphides as well as a suspensor like structure and an epidermis were observed in the somatic embryos. The origin of each somatic embryo was from a proembryonic cell complex or possibly from a single cell by direct embryogenesis. Both modes of somatic embryogenesis were found to rise from the mesophyll. When Hamidah *et al.* (1997a, b) supplemented the media with 18μM 2, 4-D and 6 per cent sucrose the leaf explants were found to produce plantlets. Somatic embryos were found to regenerate in a medium containing 0.46 μM kinetin.

The main difference between the mature embryos of monocotyledons *in vitro* and *in vivo* is the absence or presence of suspensor. The presence of single cotyledon is the terminal structure and the shoot initials present at the sides or hidden creating a heart shape. When the cotyledon starts growing the embryo will have a single cotyledon at the terminal end which is some what cylindrical in shape.

Callus on embryo induction medium was yellowish and friable, comprised of many discrete groups of small embryonic cells surrounded by large vacuolated cells. In contrast to the surrounding cells, the embryonic cells had a dense cytoplasm, conspicuous nucleus, and thin cell wall characteristic of early somatic embryogenesis in other monocot species (Ho and Vasil, 1983). After four weeks on embryo induction medium, callus contained embryos in the early globular stages.

Somatic embryos in the early globular stage consisted of a spherical region of eight to sixty four small cytoplasmically dense cells attached to an elongated region of six to fourteen large cells. This formation resembles the pattern known for early stage zygotic embryos of various monocot species where the globular embryo is attached to suspensor cells (Raghavan, 1976).

Within two weeks on embryo development medium, the globular embryos developed a bipolar shape. Embryos at this stage were comprised of cells larger than those at the globular stage. Bipolar embryos had an extended upper region that formed the cotyledon and the epicotyl, and a lower region that formed the radicle.

Mature embryos were opaque and attained a 4mm to 7 mm length within four to six weeks on embryo development medium. They possessed a distinct cotyledon, shoot apex and an elongated radicle. In addition, vascular tissue was apparent interconnecting the root and shoot meristems. Before plantlet formation, the shoot apex of mature embryos was fully differentiated and enclosed with a large coleoptile. This coleoptile resembled the coleoptile that covers the shoot apex in zygotic embryos of anthurium.

Under *In vitro* condition the embryo starts its first division to form a large enlarged basal cell and a small apical cell. In *in vitro,* condition single cells accumulate to form cells cluster and early proembryo. The embryo further grows into a globular embryo, heart shaped embryo and then into a growing torpedo with full differentiation of radicle and plumule.

Consistent somatic embryogenesis has not been reported in anthurium. Many treatments were tried for the induction of somatic embryos and irrespective of the source explant, the calli were able to be converted into somatic embryos. Somatic embryos were subjected to synthetic seed formation and seed storage treatments. Synthetic seeds were able to germinate to form viable plantlets (Plate 14).

5. 1. 6 Rooting and *Ex vitro* Establishment

In anthurium tissue culture, no special rooting treatments were needed and the shoots developed *in vitro* were found to develop roots spontaneously even in the absence of additional growth hormones in the supporting medium. The spontaneous root formation was not due to the carry over effect of the hormones supplied in the previous cultures for shoot formation. Irrespective of the supporting medium the shoots were able to form roots even in sterile sand supplied with sterilized compost materials.

Shoots isolated from callus cultured on a medium containing BA and 2, 4-D were found to produce roots when transferred to a basal medium with no growth regulators by Geier (1986a). The shoots regenerated with BA were found to thrive hard to develop roots and delayed in every cycle of multiplication. He also explained that the ammoniacal nitrogen at 720 mg per litre accelerated the root growth compared to 200 mg per litre. In contrary to this Lightbourn and Prasad (1990) reported that rooting was not affected by varying concentrations of ammonium nitrate but larger leaves and more prolific leaf production occurred with increased ammonium nitrate concentrations. Whereas for rooting of *Anthurium andreanum in vitro* developed shoots Yu-KwangJin *et al.* (1995) used IBA containing medium. This was found to be more effective than IAA and NAA.

Li-Jing and Li-J (1997) found that the plantlets produced roots *in vitro* only when there was sufficient supply of exogenous auxins. Somaya *et al.* (1998) reported rooting of the cultured shoots and obtained one of the best combinations on MS medium with 0.25 mg NAA per litre which increased the quality and number of roots produced.

Prakash *et al.* (2001) excised shoots arisen from the shoot primordia of callus cultures to produce roots on MS basal medium with five per cent sucrose. The highest rooting percentage of 80 per cent was observed by Mohanta and Paswan, (2001) in MS basal medium supplemented with IAA 1.0 mg per litre. The survival rate was recorded as 60 per cent on soilrite and perlite 10: 1 mixture. Zhang-GuiHe *et al.* (2001) inferred that the cultures tend to produce roots *in vitro* on subculturing into MS medium supplemented with 0.1 mg IBA per litre or 0.1 mg NAA per litre. The plantlets formed easily with a survival rate of more than 85 per cent.

Dhananjaya and Sulladmath, (2003) opined that the MS medium supplemented with 1.5 mg IBA and 5 mg kinetin per litre was good for optimum root production *in vitro*. The root induction of the regenerated shoots were found optimum on half strength medium containing 0.54 µM NAA and 0.93 µM kinetin. This was supported by Martin *et al.* (2003).

Ex vitro establishment of the *in vitro* generated plantlets is critical for successful clonal multiplication. The plantlets have to get acclimatized to the *ex vitro* conditions. Plants developed *in vitro* were hardened on a medium containing 1:1:1:1 sand: soil: composted coir pith: half MS salt solution. Plants required a minimum of 15 days hardening before transferring into main field. Excessive water loss and reduced uptake of water and nutrients cause problems in acclimatization.

In the present study, it was observed that plantlets (with at least two roots and three leaves) survived better than the micro shoots. Growth of the survived micro shoots was very slow compared to that of the plantlets; plantlets can absorb water and nutrients much easier than micro shoots. Water content of micro shoots will be less and there will be problems with desiccation. It was also observed that plantlets did require less hardening treatments. The rate of water loss through anthurium leaves of *in vitro* grown plantlets and field grown plants was comparable. This was contradictory to the results obtained for *ex vitro* establishment of other plants in which case more than double the rate of water loss was observed. The requirement of less hardening treatments in the

present study may be due to that the plantlets had sufficient water content (due to proper stomatal functioning) and food reserves. Murashige (1977) reported that the prolonged period of exposure to light built up sufficient food reserves to be utilized during the transformation period from partially heterotrophic to autotrophic growth of the plantlets, after transformation.

Texture and structure of the potting medium are important factors for the successful *ex vitro* establishment of plantlets. It was observed that sand was the best medium. Sand as the potting medium ensures proper drainage and sufficient aeration.

Addition of inorganic nutrients to the potting medium is essential for the normal growth of the potted plantlets (Amerson *et al.*, 1985). However, a negative influence of the nutrient solutions was apparent in anthurium. It is likely that plantlets had adequate nutrient reserves for supporting their survival and growth under *ex vitro* conditions in the absence of added nutrients.

5. 2 MOLECULAR CHARACTERIZATION

Molecular markers have been proved to be a fundamental and reliable tool for fingerprinting varieties, establishing the fidelity of progenies etc. The advent of automated PCR technology made a new set of markers available to scientists interested in comparing organisms at molecular level. Williams *et al.* (1990) first used RAPD markers which was performed on genomic DNA with random primers produced as short arbitrary oligonucleotides resulting in the amplification of several discreate DNA products.

The RAPD amplification generated can be classified into two types viz. constant (monomorphic) and variable (polymorphic). These differences can be sued to examine and establish systematic relationship (Rether *et al.*, 1993).

The genomic sequence of *Anthurium andreanum* has not yet been defined. As such, molecular techniques requiring no specific sequence information as in the case with RAPD can be used for its DNA analysis. The rationale behind this method is that the generation of RAPD markers is based on the probability that a DNA sequence, homologous to that of a short, oligonucleotide primer (tenmers for RAPDs) will occur at different sizes on opposite strands of a DNA template that is amplifiable by PCR (Waugh and Powell, 1992).

In the present investigation the specificity proposed by Welsh and MacClelland (1990) in effectively controlling the stringency of thermocycling reactions was used in RAPD (AP-PCR) analysis during PCR amplification of DNA fragments to make them highly reproducible and reliable.

Twelve hybrids and their parents developed in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani were used for the investigation to find out the extent of variability present in parents and hybrids. The results of AP-PCR marker was used for RAPD to cluster based on similarity co- efficient values. Distinct hybrids were identified with good quality parameters by molecular characterization and the results are discussed below.

The present investigation was carried out with seven primers selected out of sixty primers screened by amplifying the genomic DNA of five selected accessions. After screening the seven primers were used to amplify the twenty four accessions involved in the present investigation. The primers were selected based on their ability to produce maximum number of bands, reproducibility and the ability to produce polymorphism. Of the seven primers used, three were capable of producing cent per cent polymorphism (OPB 6, OPB 8 and OPB 18); one primer was reported to produce polymorphism by Buldewo and Jawfeerally (2002) and Puchooa and Sookun (2003).

A total of 114 AP-PCR bands were generated by the 25 primers, of which 74.56 per cent were polymorphic (88 bands) and 26 were monomorphic. Ten primers showed

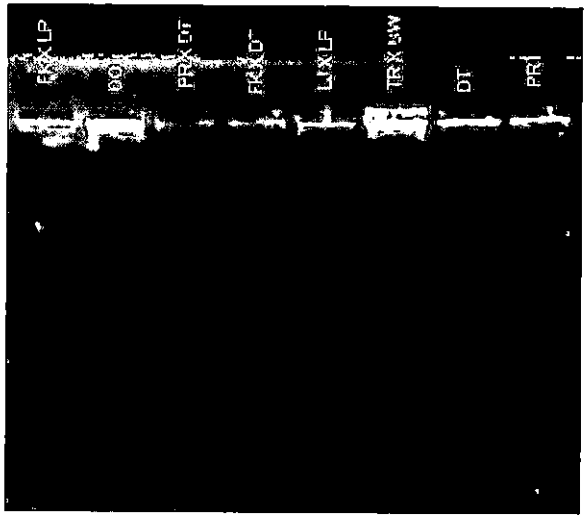
high level of polymorphism out of which seven were selected. This could be explained by the capability of individual primers to amplify the less conserved and highly repeated regions of the genomic DNA. There is high possibility for the amplified fragments to contain repeated sequences. The bands were reproducible. Bhat and Jarret (1995) suggested that the number of polymorphisms might be more important than the number of primers for the generation of stable phenogram and it would vary with plant material used for investigation and the sequences that are amplified.

The primer OPA-10 was unique as it could distinguish maximum number of the accessions tested. The highest number of scorable bands was given by OPB18. The AP-PCR profiles show the relatedness and diversity of the hybrids and varieties. The bands were found within 1.5kb from 100bp. Most of the bands were concentrated between 300bp and1200bp.

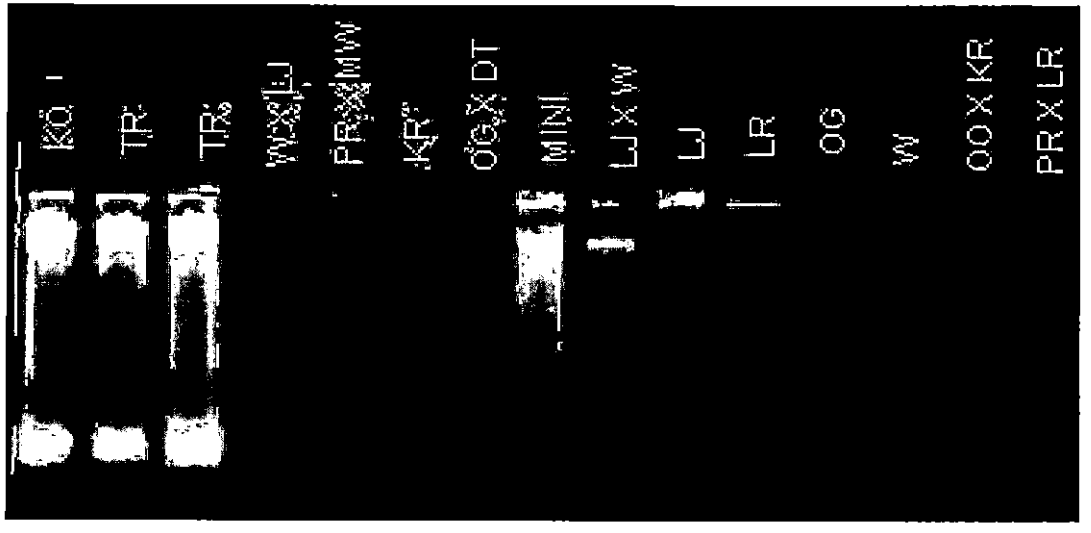
The previous reports of Ranamukhaarachchi *et al.* (2001), Buldewo and Jawfeerally(2002), Puchooa and Sookun (2003), Nowbuth *et al.*, (2005) showed less than 20 per cent variation among the accessions tested by the RAPD analysis. In contrary to the present investigation showed higher polymorphism and exhibited more than 70 per cent variation. The accessions chosen in the study were hybrids of anthurium and their parents. The divergence exhibited points out the efficiency of hybridization programme.

5. 2.1 DNA Isolation

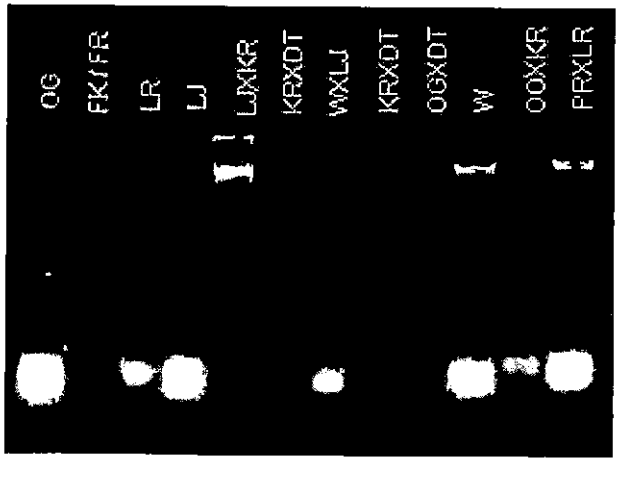
As the biochemical composition of plant tissues of species and hybrids varies considerably, it is difficult to use the same protocol for DNA isolation for different plant species. Even closely related species may require different DNA isolation procedures (Weising *et al.*, 1995). Among the different methods tried the method proposed by Murray and Thompson (1980) with modifications was found to yield DNA of good quality and quantity. Young emerging leaves and spathe were found to be good and suitable for DNA isolation (Plate 24). The quantity and quality of isolated DNA depends on the source of tissue as well as efficient disruption of plant cell wall (Babu, 2000). Mondal *et al.*(2000) suggested that tender leaves contain actively dividing cells with



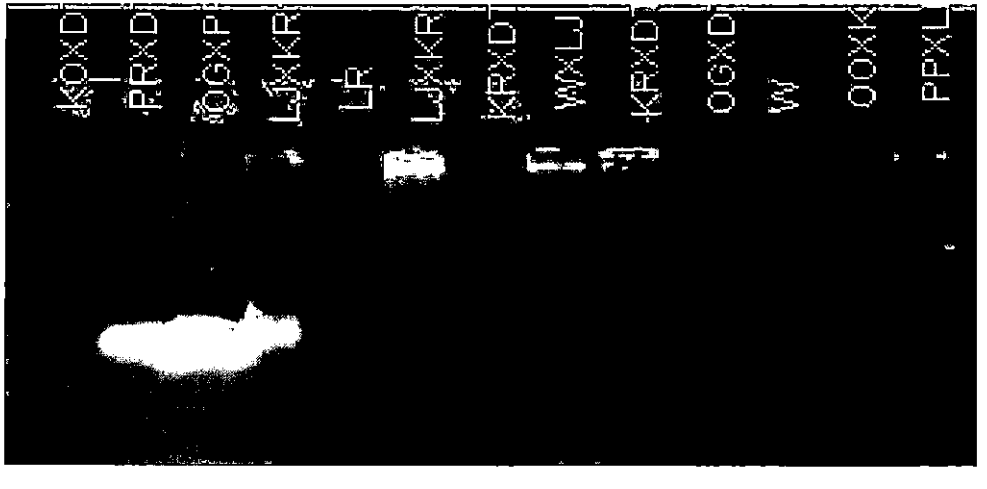
RNAase and Protinase treated



Without RNAase and Protinase treatment



Without RNAase treatment



Without PVP and chilling treatment

lesser intensity of extra nuclear material like proteins, carbohydrates and other metabolites that interfere with the isolation of nucleic acids which in turn improve the quality of DNA. Tender leaves also facilitate easy disruption for DNA extraction.

5. 2.2 RAPD (AP-PCR)

First step in designing an AP-PCR is choosing the primer and annealing temperatures which can be combined. One important requirement is to find an AP-PCR programme allowing optimal amplification of all loci when taken individually. This is achieved by adjusting the annealing and extension time and temperature with fixed ramp time.

As per the reports of Henegariu *et al.* (1997) in a 25 µl reaction volume, theoretically the four nucleotides should allow synthesis of about 6-6.5 µg of DNA. This amount should be sufficient for PCR reactions in which 1 or more primer or primer pairs are used at the same time. To work properly (besides the magnesium bound by the dNTP and the DNA), Taq polymerase requires free magnesium. This is probably the reason why small increases in the dNTP concentrations can rapidly inhibit the PCR reaction (Mg gets "trapped") whereas increases in magnesium concentration often have positive effects. The dNTP concentrations of about 200µM each are usually recommended for the Taq polymerase, at 1.5mM $MgCl_2$. The amount of DNA primer available during the PCR reaction influences the results. Primer concentration taken in a common PCR reaction (for example when amplifying a single locus) is about 100-500 nM. In an AP-PCR test using the primer concentrations it can be fixed between 15 to 500 nM each primer. It was fixed at 10pM in the present investigation. This test allowed the observation that too high and too low primer amounts need to be avoided. Too high primer concentrations may inhibit the PCR reaction whereas too low amount may not be sufficient.

The relationship between the concentration of magnesium and that of the dNTPs was investigated by Henegariu *et al.* (1997) to perform PCR with a random primer in reactions that contained 200, 400, 600 and 800 µM each dNTP, combined with 1.5, 2, 3, 4 or 5 mM $MgCl_2$. This test confirmed that any increase in dNTP concentration requires

an increase in the concentration of magnesium ions in order for the reaction to work. At 200 μM each dNTP, reaction worked at all magnesium concentrations, but for this primer it worked better at 3 mM (which is about double the recommended magnesium concentration for the amount of dNTP). At 800 μM each dNTP, reaction worked only above 3 mM magnesium. Different concentrations of a Taq polymerase were tested using primer OPB 15. The most efficient enzyme concentration seemed to be around 0.4μl or 2 Units / 25μl reaction volumes. Too much enzyme, possibly because of the high glycerol concentration in the stock solution, resulted in an imbalanced amplification of various loci and a slight increase in the background. Too little enzyme resulted in the lack of some of the amplification products.

PCR mixture with different DNA concentrations for PCR amplification using only one of the primers from the same mixture was tried. Varying DNA concentrations were used ranging from 5ng to 100ng. Again, the amount of PCR product decreases with the reduction in template DNA but less when only a primer is used. PCR program used the same annealing temperature fixed for the previous standardization.

Equimolar dNTP mixtures were used for AP-PCR amplification using two different PCR programmes, one at 65° C and the other at 72° C extension temperature. In general, there is a higher yield of PCR products at 72° C compared to the programme wherein 65°C was used. This shows that the 65° C extension temperature, negatively influenced amplification of some loci, while also making some unspecific products visible. It is likely that, for the short AP-PCR products used in these examples (below 500 bp), the higher annealing temperature is probably detrimental to the stability of the DNA helix, so less strands of DNA have the chance to become "copied" by the polymerase after annealing. Hence for the first three cycles lower annealing temperature was fixed at 35°C.

The amount of DNA primer available during the PCR reaction influences the results. Primer concentration taken in a common PCR reaction (for example when amplifying a single locus) is about 100-500 nM. In RAPD (AP-PCR) analysis of

anthurium accessions the amplicons produced had a molecular weight ranging from 100bp to 1500bp. But the previous reports in anthurium by Nowbuth *et al.* (2005) found a wider range of 1500 bp to 2600 bp as well as at 300bp also showing more bands at 500 to 1500 bp range. This may be due to the difference in the primers selected for the study and different thermal cycling conditions.

Numerous studies have supported the presence of a significantly high level of similarity among anthurium species and cultivars. Meiotic analysis (Sheffer and Croat,1983; Marutani *et al.,* 1993) indicated that most cut flower anthurium cultivars exhibited a high percentage of normal tetrads, cross compatibility studies further indicated their close relationship (Marutani *et al.,* 1988; Kamemoto and Kuchnle, 1996), Ranamukhaarachchi *et al.* (2001) showed the presence of low level genetic variability among flowering potted plant anthurium species using RAPD markers. The genetic variability accessible in a gene pool is normally considered as being the major resource available to breeders. The significantly low level of genetic variability detected among these cut flower anthurium cultivars highlighted the limited potential for crop improvement from the earlier reports. But the present study revealed the presence of variability among varieties selected and the hybrids produced from varieties.

Pair wise genetic distances based on AP-PCR (Nei and Li Genetic Distance-GD_{NL}) genetic distance co-efficient values for twelve varieties and twelve hybrids ranged from 0.1875 to 0.7333 indicating the wider diversity. The AP-PCR profiles show the relatedness and diversity of the hybrids and varieties. The bands were found within 1.5kb from 100bp. Most of the bands were concentrated between 300bp and 1200bp.

From the cluster analysis based on the dendrogram, TR X MW was found to be extremely different from the other accessions and its own parents showing the significance of hybridization. The hybrids like OO X KR, PR X DT, OG X DT, FK X LR and PR X MW are not closely related to either of their parents and hybrids were distinguished from others. Some hybrids like LJ X W and PR X MW; PR X LR and FK X DT shows 30 to 39 per cent similarity. This shows there is considerable variability

among the genotypes selected and can be further utilized for crop improvement. Confirming that, they were quite different from the other hybrids and varieties. Further more, Lady Jane X White and Pompon Red X Merengue White formed a monophyletic group in the tree indicating that they were distant from the other lines. In addition three pairs of sub clusters comprising Lady Jane X White and Pompon Red X Merengue White; Liver Red and Pompon Red X Dragon's Tongue Red and the third comprising Merengue White and Lady Jane X Liver Red were observed in cluster analysis.

In contrary to the earlier reports of Sheffer and Croat, 1983; Marutani *et al.*1988, 1993; Ranamukhaarachchi *et al.* 2001 considerable variability was found to exist as the material used here were derived by hybridization and AP-PCR analysis is highly specific than RAPD for fingerprinting to assess the extent of genetic variation among anthurium varieties and hybrids.

Summary

6. SUMMARY

The salient findings of the present investigation carried out on *in vitro* multiplication and DNA fingerprinting of selected hybrids and their parents in *Anthurium andreanum* Linden were enlisted in this chapter.

- Double sterilization with 95 per cent ethyl alcohol for 1 min; 0.08 per cent mercuric chloride for 10 minutes and 70 per cent ethyl alcohol for 3 minutes was found to be the best among the surface sterilization treatments, which is capable of producing more than 95 per cent contamination free culture. 100 per cent contamination free cultures were not obtained.

- Six different explants viz. leaf lamina, petiole, spadix, candle, seed and shoot tip were used in the experiment. Among these explants seed is the hardest and the candle is found to be most sensitive.

- Treatment M-NN + AC + CW recorded highest survival rate for all the explants irrespective of variety.

- Highest callus induction percentage was noticed in medium having M-NN + AC + CW + 5.37 µM NAA + 2.26 µM 2, 4-D + 9.12 µM zeatin. A range of variation for days required for callus induction was from 72 for variety OG with treatment M-NN + AC + CW + 5.37 µM NAA + 2.26 µM 2, 4-D + 9.12 µM zeatin to 113 for OG X DT with M- MS + AC + CW + 10.74 µM NAA + 3.62 µM 2, 4-D + 9.12 µM zeatin. When comparing other varieties and hybrids OO X KR was found to take more time for callus induction.

- Callus weight ranged from 0.0662g to 3.9875 for variety LR in ½ MS + CC + CW + 4.52 µM 2,4-D + 18.59 µM kinetin and M-NN + AC + CW + 9.12 µM zeatin respectively. The perusal of data on callus growth and callus

multiplication indicated very good response in treatment M-NN + AC + CW + 4.52 µM 2, 4-D + 9.12 µM zeatin.

- The entire callus cultures subjected to regeneration was found to regenerate irrespective of the source of explant and variety. Though they differ in their nutrient requirements they are found to be responsive.

- The number of days required for regeneration ranged from 55.5 days M-NN + AC + CW + 22.2µM BA + 11.42 µM IAA + 4.09µM biotin to 82 days ½ MS + AC + CW + 17.76 µM BA + 5.71µM IAA.

- Each genotype was varying with the response to change in media composition in producing somatic embryogenesis. It was possible to produce and store synthetic seeds for more than a week without loss of viability in hydrated as well as dried system.

- The treatments with MS and modified MS media were found to be insignificant when compared with NN and modified NN media. Among these modified NN was found to be the best one.

- The days required for emergence of first leaf is directly correlated with the days required for regeneration. This ranges from 75.5 days for M-NN + AC + CW + 22.2µM BA + 11.42 µM IAA + 4.09µM biotin to 102 days.

- All the treatments tried were capable of producing roots *in vitro*. Treatment M- MS + AC + CW + 4.93 µM IBA + 5.37 µM NAA was found to be a best.

- Plants developed *in vitro* were hardened on a medium containing 1:1:1:1 sand: soil: composted coir pith: half MS salt solution. Plants required a minimum of 15 days hardening before transferring into main field.

- Multiple shooting and direct regeneration or direct organogenesis was found to be a non-responding to slow responding for hybrids and in case of parents it is slow responding.

- Recovery of systemically infected cultures which express out burst of *Xanthomonas compestris* in later stages of cultures were recovered by culturing on antibiotics Streptomycin, Rifambicin and Kanamycin (50ppm) containing medium.

- The results confirm that anthurium can be successfully propagated using *in vitro* techniques. When comparing all the treatments varieties LR, LJ, OG and hybrids OO x KR, OG x DT, PR x DT are best suitable for mass multiplication using the candle explant and shoot tip explant.

- Isolation of genomic DNA in *Anthurium andreanum* Linden was done using modified Murray and Thompson (1980) method. Tissues from young tender leaves were found to yield good quality of DNA.

- The DNA yield for 24 accessions of *Anthurium andreanum* Linden ranged from 90to 420ng. The purity of DNA ranged from 1.5 to 2.33.

- A total of 114 AP-PCR bands were generated by the 25 primers in five selected genotypes, of which 74.56 per cent were polymorphic (88 bands) and 26 were monomorphic. Ten primers showed high level of polymorphism out of which seven were selected.

- Seven promising primers were identified for RAPD (AP-PCR) analysis based on performance in DNA amplification, production of highest number of polymorphic bands as well as intense bands and reproducibility viz. OPA 10, OPB15, OPA13, OPB20, OPB6, OPB8 and OPB18 primers were found to produce polymorphism in *Anthurium andreanum* Linden.

- A total of 50 scorable bands (average of 7.143 bands per primer) were generated by the selected seven primers of which only 8 were monomorphic and the rest were polymorphic. The number of bands ranged from 4 to 11 with an average of 7.143 per primer.

- Pair wise genetic distances based on RAPD (AP-PCR) [(Nei and Li Genetic Distance -GD_{NL})] genetic distance co-efficient values for twelve varieties and twelve hybrids ranged from 0.1875 to 0.7333 (table 33) indicating the wider diversity.

- The wide variability present between the hybrids and their parents and among the hybrids and varieties can be exploited for further crop improvement.

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In vitro MULTIPLICATION AND DNA FINGERPRINTING OF SELECTED HYBRIDS AND THEIR PARENTS IN *Anthurium andreanum* Linden

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Abstract of the
thesis submitted in partial fulfilment of the requirement
for the degree of

Doctor of Philosophy in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2007

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ABSTRACT

Anthurium is the largest genus in the family Araceae, encompassing more than 800 species. Native to tropical America of the region ranges from Mexico, Costa Rica, Cuba to Brazil and Argentina. Anthuriums with colourful inflorescences have been grown for cut flowers. In the inflorescence which is known as spadix is having a candle composed of multitude of flowers. They are perfect having two-carpelled ovary and four anthers. With the introduction of compact interspecific hybrids through breeding and the selection of somaclonal variants, the new commercially available types were developed.

Propagation is not easy for anthuriums and is considered a long-term crop which will take long time for the propagator to multiply. In the present investigation, an attempt was made to standardize *in vitro* multiplication and DNA fingerprinting of selected hybrids and their parents in *Anthurium andreanum* Linden.

The explants after standardizing for the surface sterilization methods and media were cultured on selected media with different hormone concentrations to get maximum callus induction. For callus induction, the culture flasks were kept in dark at 25 °C and subcultured every third week. Calli were transferred to regeneration medium and embryogenic calli induction medium. Regenerants were selected and placed in rooting medium; further hardened and transferred to the field condition.

Preconditioned embryos were suspended in calcium free half strength NN medium supplemented with 1.5 per cent sodium alginate and 0.5 M sucrose. This mixture was dispensed with a micropipette into 0.1 M calcium chloride. Twenty minutes after encapsulation, beads were pre cultured on modified half strength NN liquid medium supplemented with 0.75 M sucrose and three per cent DMSO into 100 ml Erlenmeyer flasks for one day without agitation. Beads

were then transferred to fresh medium of same composition and incubated in darkness at 4°C for three days. Beads were desiccated in a sterile laminar air flow chamber. Dehydrated beads were transferred to 4 ml cryo vials and stored at – 80°C. On rewarming over a water bath at 25°C, the beads were transferred to culture medium for germination.

Surface disinfection treatments were standardized for the different explants. Irrespective of the explants and varieties, double sterilization was found to be effective. Among the explants, the highest number of sterile cultures was observed in double sterilization, followed by the treatment with 70 per cent Ethyl alcohol for 20 minutes. Majority of the contamination found in the cultures was due to the presence of systemic infection of *Xanthomonas compestris pv dieffenbachiae.* This directly influences the percentage of contamination occurred in the culturing condition and the size of explants which also play a major role in creating the bacterial contamination. Candle explants were found to exhibit more systemic infections than other explants and seed explants were found to be free from systemic infections. Leaf explants are highly vulnerable to exhibit systemic infections and are more sensitive; unable to recover even after treatments with antibiotics. The callus cultures exhibiting systemic infections can be recovered by kanamycin 50 mg per litre containing multiplication medium.

In most studies of *in vitro* culture of anthurium, MS medium has been used. In the present study, it was observed that Nitsch and Nitsch medium was better than MS medium for multiple shoot induction. Nitsch and Nitsch medium is especially suitable for morphogenesis, meristem culture and regeneration.

As the genotypes showed different nutrient requirements for their survival and growth, the present investigation was planned to standardize the media by screening with modified MS, half strength MS, modified NN and half strength NN medium. Modified NN with activated charcoal and coconut water

showed better response. Half strength NN with coconut water and activated charcoal, modified MS with activated charcoal and coconut water and half strength MS with activated charcoal and coconut water were also found to support the explants without hindering the survival. Addition of inositol and glycine along with folic acid was found to be essential but the presence of small amount was inefficient. In the present investigation, no callus initiation was observed when inositol was reduced to half of the reported quantity.

Various treatments were tried for callus multiplication. The maximum fresh weight of callus 4.2458 g was observed in PR X DT inoculated in NN medium with major nutrients at normal strength followed by 4.1325 g in OG X DT for the same composition in NN medium. From the economic point of view, NN medium can be recommended for callus multiplication.

Among the treatments, combination of 2, 4-D and zeatin was found to be the best. It stimulates callus formation and strongly antagonizes organized development. The low auxin requirement may be due to the high potency of the auxin which was used for callus initiation. The young developing leaf may be a rich source of endogenous auxins due to which lower exogenous application is required.

Irrespective of the source of explants all the callus cultures were able to be converted into plantlets by redifferentiation. The number of days taken for regeneration ranged from 55.5 to 82. This variation is due to the varieties and difference in hormonal effect. Modified NN with activated charcoal and coconut water along with 22.2 μM BA, 11.42 μM IAA and 4.09 μM biotin was found to produce regenerants.

Each genotype was varying with the response to change in media composition in producing somatic embryogenesis. The treatments with MS and

modified MS media were found to be insignificant when compared with NN and modified NN media. Among these modified NN was found to be the best one.

Within two weeks on embryo development medium, the globular embryos developed a bipolar shape. Embryos at this stage were comprised of cells larger than those at the globular stage. Bipolar embryos had an extended upper region that formed the cotyledon and the epicotyl, and a lower region that formed the radicle. The main difference between the mature embryos of monocotyledons *in vitro* and *in vivo* is the absence or presence of suspensor. The presence of single cotyledon which is the terminal structure and the shoot initials present at the sides or hidden forming a heart shape. When the cotyledon starts growing the embryo will have a single cotyledon at the terminal end which was cylindrical in shape.

In anthurium tissue culture, no special rooting treatments were needed and the shoots developed *in vitro* were found to develop roots spontaneously even in the absence of additional growth hormones in the supporting medium. The spontaneous root formation was not due to the carry over effect of the hormones supplied in the previous cultures for shoot formation. Irrespective of the supporting medium the shoots were able to form roots even in sterile sand supplied with sterilized compost materials. For *in vitro* studies, the experiments were designed in Completely Randomized Block Design (CRD).

Molecular characterization of twelve hybrids and their parents were carried out with RAPD using AP-PCR. Young leaf samples from each genotype were collected for DNA isolation. Young copper coloured leaf tissues were used immediately after collection for DNA extraction. Leaf samples were pre-chilled at -80°C for half an hour and then pulverized in the presence of liquid nitrogen by rapid grinding to a fine powder. The frozen powder was used to extract the total genomic DNA using CTAB extraction buffer. The purity of the DNA was analysed by running in 0.8 per cent agarose gel with 1 X TAE buffer.

The optimized PCR mixture with 50ng of template DNA for a final volume of 20µl was used in thermal cycling in a PCR machine. The amplified products were run in 1.6 per cent agarose gel with 1X TAE (Tris buffer, Glacial acetic acid and EDTA pH 8.0) buffer.

A total of 114 AP-PCR bands were generated by the 25 primers, of which 74.56 per cent were polymorphic (88 bands) and 26 were monomorphic. Ten primers showed high level of polymorphism out of which seven were selected. Seven promising primers were identified for AP-PCR analysis based on performance in DNA amplification, production of highest number of polymorphic bands as well as intense bands and reproducibility. The primers OPA 10, OPB15, OPA13, OPB20, OPB6, OPB8 and OPB18 were found to produce polymorphism in *Anthurium andreanum* Linden. A total of 50 scorable bands (average of 7.143 bands per primer) were generated by the selected seven primers of which only 8 were monomorphic and the rest were polymorphic. The number of bands ranged from 4 to 11 with an average of 7.143 per primer. The reproducible bands were scored for their presence (1) or absence (0) for all the hybrids and parents. A genetic similarity matrix was constructed using Jaccards's similarity co-efficient methods.

From the cluster analysis based on the dendrogram, TR X MW was found to be extremely different from the other accessions and its own parents showing the significance of hybridization. The hybrids like OO X KR, PR X DT, OG X DT, FK X LR and PR X MW were not closely related to either of their parents. Some hybrids like LJ X W and PR X MW; PR X LR and FK X DT shows 30 to 39 per cent similarity. This shows there is considerable variability among the genotypes selected and can be further utilized for crop improvement.

Pair wise genetic distances based on RAPD [(AP-PCR) (Nei and Li Genetic Distance GD_{NL})] genetic distance co-efficient values for twelve varieties and twelve hybrids ranged from 0.1875 to 0.7333 indicating the wider diversity. The AP-PCR profiles showed the relatedness and diversity of the hybrids and varieties. The bands were found within 1.5kb from 100bp. Most of the bands were concentrated between 300bp and 1200bp.

The results confirmed that anthuriums can be successfully propagated using *in vitro* techniques and the hybrids and varieties shows the existence of considerable variability which can be further exploited for crop improvement.