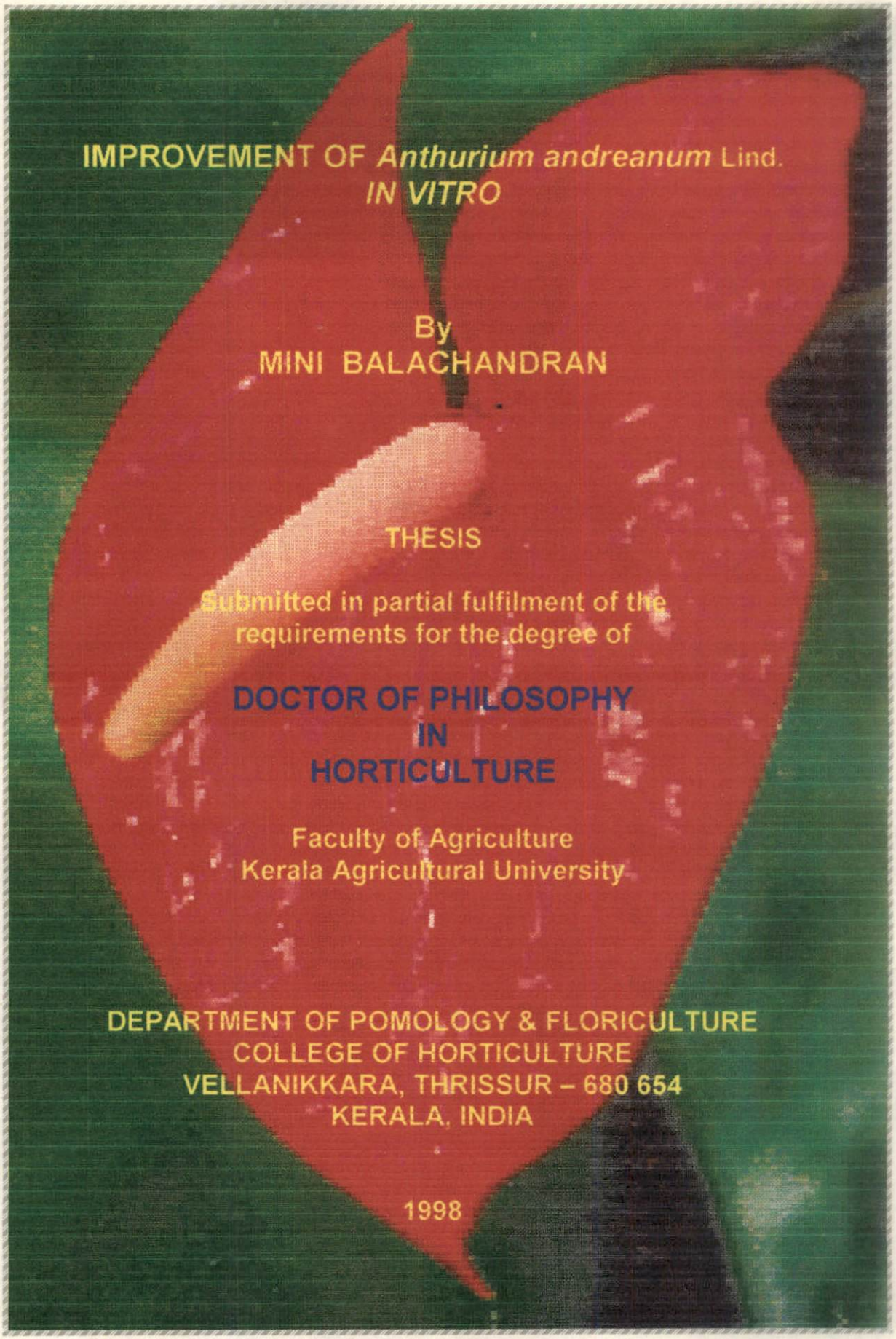

IMPROVEMENT OF *Anthurium andreanum* Lind. *IN VITRO*

By
MINI BALACHANDRAN

THESIS

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
IN
HORTICULTURE

Faculty of Agriculture
Kerala Agricultural University

DEPARTMENT OF POMOLOGY & FLORICULTURE
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR – 680 654
KERALA, INDIA

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1998

DECLARATION

I hereby declare that the thesis entitled **"Improvement of *Anthurium andreanum* Lind. *in vitro*."** 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 to me of any degree, diploma, fellowship, associateship or other similar title, of any other University or society.

Vellanikkara,
12.11.1998.

MINI BALACHANDRAN

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CERTIFICATE

Certified that the thesis entitled **"Improvement of *Anthurium andreanum* Lind. *in vitro*"** is a record of research work done independently by **Smt.Mini Balachandran**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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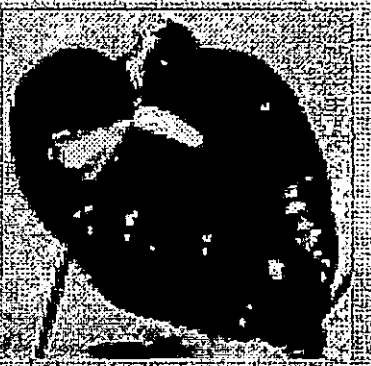
MINI BALACHANDRAN

Dedication



Dedicated to

my

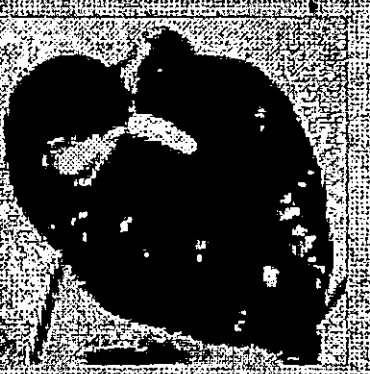
Loving Husband

&

Son

ARVIND MURALI

Contents



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Introduction

1. INTRODUCTION

Aesthetics integrated with science and given an economic dimension is Floriculture. Rarity and diversity derived from moving subtle variability in shape, colour, pattern and vase life make it an attractive economic proposition.

The baffling phenomenon of unusually slow growth and development of plants coupled with long life of cut flowers of anthurium and orchids have made them the focal point of attraction everywhere in the world always. The poor share of tropical flowers in the world market at present has been attributed mainly to shortage of healthy planting material with improved flower characters and disease vulnerability, which stems from lack of scientific comprehension and technology for producing rarity, variability and large-scale multiplication of these tropical flowers.

Consequent to the energy crisis, which occurred in early seventies, demand for tropical cut flowers and ornamental plants popularly known as 'Tropical exotics' has increased tremendously. Large scale production of elite clones through micropropagation or somatic embryogenesis, production of disease-free propagule and meaningful development of plant varieties utilising cellular, molecular and whole plant breeding technique hold the promise for a flourishing Indian Floriculture industry.

Successful and scientific floriculture demands a complete and comprehensive package on all aspects of production and management. The

thrust of this project has been to fill this lacuna in respect of availability of novel types and their planting materials.

Plant biotechnologies can contribute both to rapid multiplication of crop species and crop improvement. *In vitro* micropropagation techniques as well as other crop biotechnologies like somatic embryogenesis, *in vitro* radiation breeding, somaclonal variation, haploid plants, somatic hybridisation and genetic engineering are increasingly contributing to the growth of the world market of cut flowers and potted plants. The most popular and widely commercialised global application of biotechnology has been in the sphere of plant tissue culture, particularly in micropropagation. The emergence of the tissue culture industry in India in the past decade has been stupendous. Its usefulness in the current global scenario can hardly be overemphasised vis-à-vis traditional propagation methods which are inadequate to meet the ever-burgeoning demand for timely and clean planting material. India contributed over 22 million of the 680 million *in vitro* plants sold globally (Chadha, 1996). Micropropagation is of special significance to anthurium (*Anthurium andreanum* Lind..), being multiplied asexually.

There are two ways by which plant regeneration can be achieved. One is through callus from various explants and other is without intervening callus from embryo and axillary bud explants. Of late, production of somatic embryos is being utilized for rapid multiplication of plants.

Artificial seeds or synseeds consisting of tissue culture derived somatic embryos encased in a protective coating have been suggested as powerful tool for mass production of elite plant species. Advantages of artificial seeds over somatic embryos for propagation include ease of handling and potential long-term storage. In view of the high volume, low cost of production and subsequent propagation, this technique is considered as an alternative method of propagation (Redenbaugh *et al.*, 1987). Micropropagation through synthetic seeds or synseeds holds tremendous potential in the anthurium production industry, because of the feasibility of rapid, large scale propagation of desired genotype at a reduced cost of production (Marsolais *et al.*, 1991).

In recent years anthuriums, which are exclusively grown for cut flower production, are being brought under tissue culture for purpose of mass propagation. There is a lot of demand for anthurium cut flower, but the production is far behind the demand. Anthurium flowers are imported from Mauritius during seasons of peak demand in Thiruvananthapuram in Kerala, a major anthurium growing state in the country. Major producers of anthuriums are Hawaii, Mauritius, Martinique, Guadeloupe, Sri Lanka and West European countries, mainly the Netherlands . In Hawaii *A. andreanum* remains the most important cut flower crop valued at $ 9.9 million in the year 1989 (Criley, 1989). However, there are only eleven laboratories involved in investigation and commercial micropropagation in 16 EEC countries. This shows that there is a necessity to enhance the production of both cut flower and number of micropropagated plants in order to meet the demand.

The commercial potential of plant tissue culture, in anthurium micropropagation industry has not yet been fully realised. Though success has been reported in propagation of anthurium through tissue culture, no reports are available regarding the development of synseeds or artificial seeds. Crop improvement through radiation breeding *in vitro* and generation and selection of somaclonal variants have also not been attempted. This was due, in part, to the slow growth rate of plants in culture coupled with the time required for weaning, which both push up the cost of production. Adding to these problems, differences in morphogenetic potential in vitro of genotypes of anthurium have been observed (Pierik, 1975; Eapen and Rao, 1985) and protocols have been evolved only for a few genotypes of *A. andreanum*. In popular cut flower varieties like 'Dragon's Tongue' micropropagation techniques was attempted (Thomas, 1996) but could not be perfected due to problems confronted *in vitro,* such as improper development of foliage and prolonged period of callus induction and multiplication. Improvement of cultural conditions and refining protocols that would lead to faster and cheaper production systems is of considerable significance.

Besides red, orange, white, pink and coral coloured varieties, cultivars of obake (bicoloured spathe), novelties (tulip types), brown spathe and double flowering anthuriums are receiving considerable importance. Although a lot of breeding work is going on in different parts of the world, creation of yellow and blue anthuriums, improved shape, stem length, new colours, productivity,

suckering ability, long vase life and disease resistance are characters worth improving through *in vitro* techniques.

In general, trend in cut flower crops and ornamental plants viz., "different is better" certainly applies to anthurium as well. A slight difference in colour of spathe, angle between spadix and spathe or any other desirable character may open new avenues for growers and traders .

The traditional view of somatic mutation in higher eukaryotes is that they are rare, roughly 10^{-6} in frequency and of little consequence in population dynamics. Evidences from a large and growing numbers of reports have shown that mutations are present at much higher than expected frequencies when higher plant cells or tissues are induced to proliferate *in vitro* for even a brief period of time and these variations generated termed as 'Somaclonal variation'.

The potential of somaclonal variation for crop improvement has been suggested frequently. In many vegetatively propagated crops significant and notable attempts have been made to generate and utilize somaclonal variation for crop improvement. Generation and selection of somaclonal variants have assumed significance among biotechnological approaches of crop improvement since techniques like genetic engineering is much more sophisticated and cost involved.

Plant cell culture has provided a rapid and exciting option for obtaining increased genetic variability (Choudhary, 1998). *In vitro* techniques of breeding

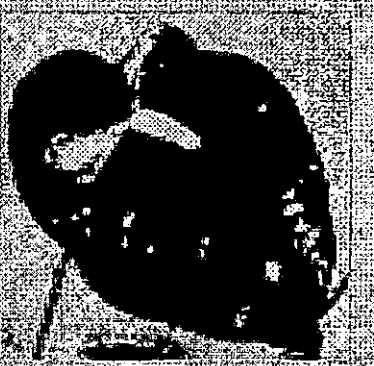
are useful to obtain desirable mutants and to restrict chimera formation (Broertjes *et al.*, 1976).

Traits like flower pigmentation, shape and size are often controlled by single genes and offer scope for genetic upgradation through somaclonal variant selection using calli proliferated upon seed, leaf blade, petiole, spadix and root explants. Somaclonal variation may be supplemented with application of radiation breeding as well (Seeni, 1994).

Inclusion of anthurium in the existing cropping pattern and the need for production of quality flowers for export require quality planting materials and varieties suitable for our polycropped conditions is evolved urgently. This study formed part of a programme designed to achieve this broad objective. Considering the above aspects the present experiments were conducted with the following objectives:

1. To refine the protocol and establish micropropagation system in *A.andreanum.*
2. To attempt somatic embryogenesis to produce synseeds through encapsulation of somatic embryos.
3. To attempt crop improvement *in vitro* through irradiation of callus and shoot tips.
4. To generate somaclonal variants by repeated subculturing and using high concentration of cytokinins.

Review of Literature



2. REVIEW OF LITERATURE

Anthuriums have gained a lot of importance in recent years as cut flower crop and as a foliage plant. Growing interest on this crop has motivated many researchers to work on various aspects. Developments in the field of tissue culture and biotechnology have made provision for better crop improvement and faster multiplication of selected clones and varieties. A perusal of literature so far published reveals that work done on anthurium is meagre. However, experimental evidences accumulated over the years on *in vitro* propagation and crop improvement through biotechnologies in anthuriums and other ornamental plants are reviewed in the following pages.

2.1. In *vitro* propagation through tissue culture

Plant tissue culture has revolutionised the anthurium industry world wide (Choudhary, 1998). Spectrums of explants are successfully used for micropropagation.

2.1.1. Explants

For *in vitro* propagation of anthurium, the segments of leaf petiole, spathe, spadix, pedicel, vegetative buds, shoot tips and roots are used as the source of explants.

Leaf segments have been widely used as explants by many research workers (Pierik, 1976; Novak and Neptusil, 1980; Geier 1982; Eapen and Rao, 1985; Geier, 1986a; Keller *et al.*, 1986; Geier, 1987; Kuehnle and Sugii, 1991 Singh and Sangama, 1991; Kuehnle *et al.*, 1992: Nirmala and Singh, 1993 and Matsumoto *et al.*, 1996) in different species of anthurium. Callus mediated plantlets were obtained from leaf explant by Novak and Neptusil (1980), Geier (1982), Sreelatha(1992) and Thomas(1996).

The presence of midrib in the leaf segments has been reported to have a greater influence of callusing in *A. andreanum* in both liquid and solid media (Pierik, 1976). The intensity and frequency of callus was found to be highest in leaf sections cultured with midrib veins in *A. patulum* (Eapen and Rao 1985). Geier (1986a) found that presence or absence of midrib had no effect on callusing in leaf explatns A. *scherzerianum.* On the contrary, according to Nirmala and Singh (1993) leaf section with midrib responded better to BA and 2,4-D application while producing callus. Position of the leaf from where the explant is excised and surface (abaxial) touching the media is also important for better callus production (Geier, 1986 a). Leaf explant of *A. andreanum* formed callus 1.5 -2.0 months on MS medium supplemented vvith 2 mgl^{-1} Kinetin (Keller *et al.*, 1986). Regenerative callus was obtained from leaf segments on modified Nitsch medium found to be poorly regenerative (Singh and Sangma, 1991). Kuehnle *et al.*, (1 992) obtained callus from *in vitro* grown plants, with in a month of culture under dark conditions in four cultivars of *A. andreanum* such as UH

780, UH 965, UH1060 and UH 1003. Nirmala and Singh (1993) also obtained regenerative callus on Nitsch medium supplemented with BA and 2,4-D from leaf sections. Matsumoto *et al.*, (1996) derived somatic embryos from *in vitro* cultured leaf lamina of *A. andreanum cv.* Anuenue.

Petiole segments of *A. scherzerianum* showed low regeneration compared to that of spadix segments (Geier, 1982). However, Eapen and Rao (1985) obtained good regeneration in *A.patulum* and Kuehnle and Sugii (1991) in *A. andreanum* using the same explant.

Geier (1982) reported that spadix fragments of *A. scherzerianum* cultivated *in vitro* showed high capacity for regeneration than segments of leaf petiole and spathe under darkness on modified Nitsch medium. Singh and Sangma (1991) and Nirmala and Singh (1993) obtained similar results in *A. andreanum.*

Zen and Zimmer (1986) reported that formation of callus and adventitious shoots by shoot tip explants could be improved significantly by increasing the NH_4-N concentration. Regenerative callus was obtained by Soczeck and Hampel (1989) from single node fragments of *in vitro* grown shoots of *A. andreanum* when cultured on half strength MS medium containing various concentrations of different cytokinins. Cen *et al.*, (1993) reported the superiority of light over darkness in induction and growth of callus as well as the further

growth of the plantlets derived from adventitious buds. Nirmala and Singh (1993) also reported similar results on liquid Vacin and Went medium.

Nirmala and Singh (1998) stated that leaves were the best explant in terms of per cent and intensity of callusing with better differentiation ability. Leaf sections with midrib showed better response than those without midrib. Multiple shoot production was more in the case of shoot tips, than single nodes of *in vitro* origin. Callusing was better with leaf explants which showed the highest degree of callusing (5.00) with maximum per cent response (98.60), followed by leaf with petiole (96.80), petiole segments (86.8) and leaf section without petiole (74.40).

When shoot tips from *in vitro* grown seedlings were used as explants, 100 per cent survival was observed in all the cytokinin treatments (Nirmala, 1989). The maximum number of shoots was observed with kinetin 2.0 mgl^{-1} as well as BA 1.0 mgl^{-1}. Treatment with kinetin showed no callus growth and in treatment with BA and 2-ip callus growth was observed at the base of the explant. Treatment with MS inorganic salts as well as sucrose did not influence multiple shoot formation. One-fourth strength of MS major nutrients with full strength of micronutrients was ideal for multiple shoot induction. Glucose produced less number of shoots than sucrose. The longest shoot was observed at 0.4 per cent agar. Light was necessary for the enhancement of axillary branching from which many adventitious shoots were produced (Nirmala and Singh, 1993).

Segments of leaf, petiole, spathe, spike and inflorescence stalk were used as explants for callus initiation. Modified MS medium with reduced salt concentration was ideal for callus initiation in all the species. Combination of 2,4-D and BA were efficient in initiating callus. In *A. andreanum* 2,4-D 0.08 mgl^{-1} and BA 1.0 mgl^{-1} was ideal for callus initiation. Combination of 2,4-D 0.2 mgl^{-1} and BA 1.0mgl^{-1} was the best for callus initiation in *A. veitchii.* In *A. grande* callus initiated best with 2,4-D 0.5 mgl^{-1} and BA 1.0 mgl^{-1}. Inositol when reduced to half the normal concentration influenced callus initiation. Continuous darkness was necessary for callus initiation and growth. Basal portions of the leaf responded better than the apical portions(Nirmala, 1989).

Thomas (1996) conducted a study to optimise the *in vitro* propagation technique via somatic organogenesis in five anthurium varieties. Segments of tender leaves, petiole and immature spadix were the explants used for culture. Among the three explants tried the leaf explants with the smallest vascular bundles recorded the maximum per cent of sterile cultures compared to the petiole and spadix explants. The variety 'Dragon's Tongue' produced the best caulogenic callus capable of regeneration. The maximum number of cultures initiating callus was recorded in modified MS medium with a reduced ammonium nitrate level of 200 mgl^{-1} supplemented with BA 0.5 mgl^{-1} + 2,4-D 0.5 mgl^{-1}. Sucrose 30 gl^{-1} was found to be better carbon source and callus formation took place only when the cultures were kept under darkness. Regeneration was

obtained in the MS medium supplemented with BA 0.5 l^{-1}, IAA 2.0 mgl^{-1}, sucrose 30 gl^{-1} and agar 6 gl^{-1} under light. Addition of casein hydrolysate 150 mg l^{-1} favoured better shoot proliferation. Kinetin 1.5 mgl^{-1} + IAA 3.0 mgl^{-1} was found to be the most promising growth regulators for shoot proliferation. Improvement in the growth of shoot was observed when cultured on MS medium supplemented with activated charcoal I.0 gl^{-1} (Thomas, 1996).

2.1.2 Sterilization of explants

Several reports are available on the sterilization requirements of explants. Lightbourn and Prasad (1990) recommended 24 hours soaking of leaf explants of *A. andreanum* in benomyl (32.5%), followed by 45 second dip in alcohol (70%), soaking for 15 minutes in sodium hypochlorite (1.25%), and by rinsing in sterile water for 15 minutes. Kunisaki (1977) reported that leaf should be sterilized using 0.26 per cent sodium hypochlorite + two drops of Tween 20 for 45 minutes and rinsing in sterile water thrice. Geier (1986a) for *A. scherzerianum* leaves suggested a dip for a few seconds in 70 per cent ethanol, followed by sodium hypochlorite (15gl^{-1}active chlorine) + 0.5 ml Tween 20 for 10 to 15 minutes, then three rinses for 10, 30 and 60 minutes.

Kunisaki (1977) suggested soaking nodes of *A. andreanum* for 20 minutes in 0.52 per cent volume of sodium hypochlorite (10% chlorax) with two drops of Tween 20 for sterilization.

Pierik (1975) recommended that surface sterilization of seeds by soaking for 15 minutes in 3 per cent sodium hypochlorite, followed by three washes of sterile water for 30 minutes. Then the dissected seeds were treated for 20 minutes with one per cent sodium hypochlorite, followed by a wash in sterile distilled water for 30 minutes. He also reported that, for adult plant organs, dip in 96 per cent alcohol for a few seconds, followed by 30 minutes treatment with one per cent sodium hypochlorite in combination of two drops of Tween 20 and rinsing thrice With sterile water was required.

Eapen and Rao (1985) reported that in *A. patulum,* for leaves, petioles, pedicel and spathe of three-year-old plants, a dip in 70 per cent alcohol, 5 minutes in 0. 1 per cent mercuric chloride, with 0.25 mill Tween 20 and three rinses with sterile water was essential. Thomas (1996) found that surface sterilization of leaf explants with mercuric chloride(0.1 %) for 8 minutes to be optimum.

2.1.3. Nutrient medium

The success of plant tissue culture as a means of plant propagation is predominantly influenced by the nature of the culture medium used. Plant tissue culture media provide major and minor nutrient elements and carbohydrates. Improved results were obtained by providing trace amount of organic compounds, notably vitamins, amino acids and plant growth regulators (George and Sherrington, 1984). Most of the reports on anthurium are based on MS and

Nitsch media. Pierik *et al.*, (1974) cultured embryo on modified MS medium containing half strength macro elements, micro elements, sucrose (3%) and organic constituents(except adenine, IAA and kinetin) and Difco agar (0.078%). Further, he modified the macroelements of MS medium for callus induction, callus subculture, sprout regeneration and rooting of sprouts. But, macro-elements and other organic constituents were maintained without change. It was also found that, lower concentrations of NH_4NO_3 were an essential factor in the induction of adventitious shoots in callus tissue of almost all genotypes. Fersing and Lutz(1977) reported that medium supplemented with yeast extract stimulated shoot growth in *A.scherzerianum* but restricted in *A. andreanum*. Where as according to the report of Eapen and Rao (1985) half strength MS except Fe-EDTA along with vitamins and 0.6 per cent agar was found to be most suitable for the induction of callus mediated plant regeneration from leaf sections with midrib and petiole segments in *A. patulum.*

For the culture of spadix segments of *A. scherzerianum, Geier(*1982) employed modified Nitsch medium (Nitsch, 1969) which contained low level of ammonium nitrate (100 mgl^{-1}). Further, he also made standard modifications to suit the culturing of leaf segments. It was also reported that NH_4NO_3 was most suited for callus and shoot induction from shoot tip explants in liquid medium. Zen and Zimmer (1986) reported that MS solid medium supplemented with BA and NAA was found to be good for the production of callus mediated multiple shoots from seeds *of A.scherzerianum.* Root formation was not affected by

varying concentration of ammonium nitrate in MS medium (Lightbourn and Prasad, 1990). Good and regenerative calli were obtained from leaf explants of *A. andreanum* on modified Pierik medium containing 2,4-D and BA after 2-3 months. Petiole explant callused best on Pierik, Modified Pierik and Dinnie and Van Staden media. While multiple plantlets were derived from callus subcultured onto Kunisaki medium (Kuehnle and Sugii. 1991).

Modified Nitsch medium was found to be good for regeneration of plantlets from spadix segments (Singh and Sangma, 1991). Leaf explants when cultured onto media containing the combination of 2 per cent sucrose and I per cent gelrite produced more somatic embryos than on half strength MS medium with 0.7 per cent Bacto agar (Kuehnle *et al.*, 1992). MS medium supplemented with 3 per cent glucose had the greatest inductive effect on callus formation as compared to that of sucrose (Cen *et al.*, 1993). Leaf sections formed good callus on Nitsch medium containing BA and 2,4-D, spadix segments on MS with BA and 2,4-D, whereas vegetative buds callused in Vacin and Went medium(Nirmala and Singh, 1993). Reduced concentrations of MS major nutrient elements and sucrose didnot significantly influence the production of multiple shoots (Sreelatha *et al.*, 1994).

2.1.4 Growth regulators

Growth and morphogenesis in *in vitro* cultures are regulated by interaction and balance between the growth regulators supplied with the medium

and growth substances produced endogenously by cultured cells. Besides, many synthetic growth regulators may in fact modify the level of endogenous growth substances, some times in a fashion, which is heritable over many cell generations (George and Sherrington, 1984). The break through made in tissue culture is the discovery that root and shoot initiation is basically regulated by interaction between two hormonal substances namely auxin and cytokinin (Skoog and Miller, 1957).

Among the four concentrations of BAP (0,1, 2 and 4 $mg1^{-1}$), 1 mgl^{-1} BAP was found optimal for the production of maximum number of shoots (18.4/explant) in the case of A. *andreanum* cv.Crinkle Red. The number of shoots produced ranged from 1 to 48 per explant. The explant initially formed protocorm like structures, which acted as the base for production of multiple branches. Although the other higher concentrations were effective in induction of multiple shoots most of them were lanky and vitrified. The process of induction of multiple branches and the growth were rapid when two-noded cuttings were used than single nodes (Narayanaswamy, 1998-personal communication).

2.1.5. Callus induction and subculture

Generally a high concentration of auxin and a low concentration of cytokinin in the basal medium promotes cell proliferation and callus formation. Pierik *et al.*, (1974) reported that in *A. andreanum* different organs of adult plant

were capable of forming callus at 1.5 mgl^{-1} PBA added to modified MS medium. Callus produced from leaf, spathe, petiole and pedicel could be maintained on basal medium supplied with PBA 1 mgl^{-1} and NAA 0. 1 mgl^{-1}. Modified MS liquid medium was found to be good for callus growth. Presence of NAA in the solid medium caused root formation (Pierik, 1975). Further, Pierik (1976) reported that callus induction can be achieved with PBA (1 mgl^{-1}) + 2,4-D (0.08 $mg1^{-1}$), which can be subcultured with PBA (I mgl^{-1}), in the medium. With BA (I mgl^{-1}) and 2,4-D (0.I mgl^{-1}) Nitsch medium could induce callus from spadix segments (Geier, 1982) and leaf segments (Geier, 1986a) of *A. scherzerianum.* Leaf, pedicel, spathe and petiole segments produced pink coloured callus in *A. patulum* (Eapen and Rao, 1985).

The leaf explant of *A. andreanum* formed callus in 1.5 - 2.0 months on MS medium supplemented with 2 mgl^{-1} 2,4-D (Keller *et al.*, 1986). Variation in ploidy level was observed in the case of callus derived from shoot (Geier, 1988). Seeds of *A. scherzerianum* produced caulogenic callus or callus with new shoots and productivity depended on genotype and it decreased with increased NH_4NO_3 ratio in the medium (Zen and Zimmer, 1988). Callus production increased as cytokinin concentration was increased from 0.125 to 2.O mgl^{-1} in *A. andreanum* (Soczeck and Hempel,1989). Best callusing was seen on medium supplemented with 0.5 mgl^{-1} 2,4-D in the case of *A. andreanum* cv. 'Tulip'. Whereas in the case of 'Tropical Pink' 0.05-0.5 mgl^{-1} 2,4- D was found to be effective (Lightbourn and Prasad, 1990). Embryogenic callus was obtained from

basal ends of cut leaf blade explants within one month of culture. MS medium supplemented with glucose was found to be good for callus induction compared to that of sucrose (Cen *et al.*, 1993). MS medium containing 1 mgl^{-1} each of 2-ip and BA induced callus (Sreelatha *et al.*, 1994). Regenerative callus was obtained on the media supplemented with 2-ip in *A. andreanum* cvs. 'Hazarija' and 'Ingrid' and *A. scherzerianum* cv.'Belinda'(Yu and Paek,1995).

2.1.6. Shoot bud differentiation

Low auxin and high cytokinin levels in the medium is a general requirement for shoot bud differentiation though, Pierik *et al.*, (1974) reported that sprout formation occurs in callus spontaneously on transferring the culture to light. Pierik (1976) obtained good plant regeneration from callus on solid MS medium as compared to that of liquid medlia. Culture media supplemented with yeast extract stimulated shoot growth in *A. scherzerianum* but restricted in *A. andreanum* (Fersing and Lutz 1977). Similarly, medium containing kinetin (3 mgl^{-1}) was found to be more effective in enhancing shoot differentiation as compared to that of BA and 2-ip(Leffring and Soede, 1979a). Subculturing of callus (3-6 times) on hormone free, modified Nitsch medium resulted in the production of true to type multiple shoots at 2000 lux light for 14 hours/day (Geier, 1982).

Eapen and Rao (1985) reported that the best response for shoot differentiation was obtained with BA (1.0 mgl^{-1}) and 2,4-D (0.1 mgl^{-1}). Kinetin and

2-ip stimulated shoot differentiation to lesser degree, while zeatin was ineffective in the case of *A. patulum.*

Jaruwan and Boonyen(1987) reported that callus grown on modified Pierik callus induction media(PCI media) With 2-ip could induce shoot formation faster than those in PCI media with BA. Callus grown on MS media with NAA (0.0, 0. 1, 0.5, 1.0 or 2.0 mgl^{-1}) could not induce any shoot formation.

Maximum shoot multiplication was observed on MS medium supplemented with NAA and BA from seeds of *A. scherzerianum (Zen* and Zimmer, 1988). Lightbourn and Prasad (1990) obtained same results in *A. andreanum* cvs. 'Tulip' and 'Tropical Pink' at 0.2-0.8 mgl^{-1} BA. Sreelatha *et al.,* (1994) reported that kinetin (2mgl^{-1}) and BA (1 mgl^{-1}) were effective in the production of shoots. Treatments with kinetin did not produce callus, whereas BA and 2-ip induced callus. Yu and Paek (1995) reported similar results in *A. andreanum* cvs.'Hazarija' and 'Ingrid' *and A.scherzerianum* cv.'Belinda'.

2.1.7. Rooting

Geier (1982) reported highest rooting of shootlets on hormone free Nitsch medium maintained at 200-lux light duration of 14 hours per day. Rooting occurred after four weeks on cytokinin medium in the case of *A.andreanum* (Kraft *et al,* 1983), Hormone-free medium and 720 mgl^{-1} NH_4NO_3 accelerated

root formation (Geier, 1986 a). Varying concentrations of ammonium nitrate did not influence root formation.

Shoots rooted spontaneously and the plantlets survived better than microshoots, *ex vitro.* The plantlets required less hardening treatments. Sand was the best potting medium for planting out. A treatment with VAM (*Glomus constrictum* and *G. etunicatum*) was beneficial for the survival as well as growth of the plantlets *ex vitro* (Sreelatha *et al.*, 1994).

Ajithkumar (1993) reported that a combination of BA 0.5 mgl^{-1} and IAA 2.0 mgl^{-1} was found to be the best for *in vitro* rooting plantlets. *In vitro* plantlets 2.5-3.0 cm long with 3-4 leaves and two or more roots were ideal for transplanting, supporting highest survival percentage (90-100). Agar at 0.7 per cent recorded shortest time (10.54 days) for root initiation and the number of roots per shoot decreased by increasing its concentration in the medium. While the length of root increased with increase in agar concentration, Sucrose level maintained at normal level (3.0 %) was found to be the best, for *in vitro* rooting. Among the media soilrite was the best for *ex vitro* establishment of anthurium plantlets but containers showed no uniform response with various growth factors. Plastic pots grown plants produced maximum number of leaves at fortnightly intervals. Regarding leaf area, at second and fourth fortnight, plastic pot and at third fortnight polythene cover was found to be the best container.

The plants grown in polythene cover with soilrite media recorded the maximum number of roots and maximum length of roots at two months after transplanting.

Thomas (1996) reported that rooting of shoots occurred simultaneously in proliferation media containing kinetin 1.5 mgl^{-1} and IAA 3.0 mgl^{-1}.

2.1.8. Response of genotypes

Pierik (1975) showed that growth rate was strongly depended on the genotype in *A. andreanum.* Most of the genotypes showed good response to the reduced level of NH_4NO_3, for sprout regeneration (Pierik, 1976). In *A. scherzerianum* also, genotype strongly determined the regeneration ability. When leaf segments of 18 genotypes were incubated on low NH_4NO_3 (200 mgl^{-1}) containing medium, three genotypes didnot show any regeneration, five produced only callus, where as 10 produced caulogenic callus in the last group. The average number of shoots per explant was less than one in five genotypes, 1-10 in three genotypes and more than 10 in two genotypes (Geier, 1986a; 1986b). In a study involving five varieties of *A. andreanum* viz., Dragon's Tongue, Pompon Red, Flaking, Honeymoon Red and Nitta, cv.Dragon's Tongue produced only caulogenic callus, whereas others produced callus which regenerated into multiple shoots(Thomas, 1996).

2.2 Somatic embryogenesis and production of synseeds

The production of "seeds" by coating a matrix around cells, somatic tissues, somatic embryos and obtaining plants from these encapsulated embryos is termed as synthetic seed or 'artificial seeds' (Redenbaugh *et al.*, 1986). To make the production of somatic embryos practically applicable, a delivery system must be available by which the embryos survive, develop further (when cultured on media) and give rise to plants (Pierik, 1987). Encapsulation of cells, embryos, somatic tissues and somatic embryos have been attempted in several plants (Kitto and Janick, 1985; Redenbaugh *et al.*, 1986) and has became increasingly popular as a simple way of handling cells, tissues and embryos and protoplasts protecting them against strain and strong external gradients (Bapat and, Rao, 1988; Singh, 1992) and as efficient delivery system (Datta and Potrykus, 1989). They provide a unique opportunity for the conservation of endangered and threatened germplasm (Mathur *et al.*, 1989) and plant tissue cultures are the ideal material for the same.

Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. Reinert (1959) gave the first report of somatic embryogenesis in carrot cultures. General pattern of *in vitro* embryogenesis includes direct initiation from differentiated tissue and indirect initiation via callus intermediary. Direct embryogenesis proceeds from

embryogenically determined cells (Kato and Kateuchi, 1963), Indirect embryogenesis requires differentiation of the cells, callus proliferation and differentiation of embryogenic cells (Sharp *et al.*, 1980). The positive results are limited to a few species, but are a more rapid mode of plant regeneration (Evans *et al.*, 1981). It requires auxin medium for the induction of embryos and a medium devoid of growth regulators for its maturation (Ammirato, 1983). Hussey (1986) also reported that the level of growth regulators in the culture medium, particularly when the auxin level was lowered, there was the chance for the embryo formation.

Successful somatic embryogenesis had been reported in various crops (Murashige, 1978, Sharp *et al.*, 1979; Vasil and Vasil, 1980). Cheng and Raghavan (1985) could obtain somatic embryos in hyocyamus where pattern of development was similar to that of zygotic embryos. The somatic embryos were formed from friable calli produced from petiole and ovary explants in medium containing 2,4-D. Embryogenesis was initiated when, the friable calli were cultured in a medium lacking auxin. In *Nardostachys jatamansi,* it was found that embryogenesis could be initiated from callus upon subculture to a medium containing lesser auxin (NAA) and more cytokinin (kinetin) while the concentrations of NAA was to be more and kinetin less for callus initiation as reported by Mathur (1993). Induction of somatic embryogenesis by different growth regulators was observed in leaf disc cultures of *Nicotiana tabaccum* L. by Gill and Saxena (1993). Successful somatic embryogenesis has also been

reported in crops like Coffea (Sondahl *et al., 1979), Carica papaya* (Litz and, Connover, 1982), *Malus pumila* (James *et al., 1984), Oryza sativa* (Ram and Nabors, 1984), *Citrus limon (Carini et al.,* 1994) and *Elaeis guinensis (Teixeira et al.,* 1994).

Somatic embryogenesis and successful plant regeneration was also reported recently in many ornamental plants like begonia (Zhang *et al.*, 1988), rose (Rout *et al.*, 1989), carnations (Frey *et al.*, 1992), camellia (Pedroso and Pais 1993), chrysanthemum (Pavingerova *et al.,* 1994) and cyclamen (Kreuger, *et al.,* 1995).

2.2.1 Somatic embryogenesis in *Anthurium*

In vitro propagation of anthurium hybrids is widely used commercially, but has shortcomings in being either relatively slow or unreliable with occasional somaclonal variation. Somatic embryogenesis is an alternative micropropagation method. Recently, Kuehnle and Sugii (1991) described an embryogenic like callus of *A. andreanum* cultured on medium containing 2,4-D and BAP. Leaf explants produced callus most successfully after 2-3 months on a modified Pierik medium and petiole explant callused best on Pierik, modified Pierik and Finnie and Van Staden media. Plants were readily obtained from callus but regeneration appeared to be organogenic than embryogenic and it varied among the genotypes tested.

Geier (1982) observed some embryoid formation from spadix derived callus of *A. scherzerianum*, but plants were not recovered, Kuehnle *et al.*, (1992) described a method for the production of somatic embryos and subsequent plant regeneration for *A. andreanum* hybrids. Whole leaf blade explants derived from plantlets grown *in vitro* formed translucent embryogenic calli at their basal ends within one month of culture in the dark. Embryogenesis was induced on modified half strength Murashige and Skoog (MS) medium supplemented with 1.0 to 4.0 $mg1^{-1}$ 2, 4-D and 0.33 to 1.0 mgl^{-1} kinetin.

Leaf discs of *A. andreanum* produced somatic embryos in modified Nitsch and Nitsch medium containing BAP, kinetin and 2,4-D. The embryos were germinated into plantlets on a modified MS medium containing BAP (Rajasekharan and Kumar, 1994).

Histological analysis of somatic embryos derived from *in vitro* cultured laminas of *A. andreanum* showed bipolarity with the presence of shoot and root poles connected by procambium (Matsumoto *et al.*, 1996). The origin of somatic embryos was from a proembryonic cell, complex or possibly from a single cell by direct embryogenesis.

2.2.2 Somatic embryogenesis in other ornamental monocots

Radojevic *et al.*, (1987) obtained somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of *Iris pumila*.

Embryogenic callus was successfully induced on Murashige and Skoo Medium containing 2,4-D 1.0 $mg1^{-1}$. Somatic embryos were differentiated in the same medium. Further development of embryos was achieved in liquid-medium. Havel and Novak (1988) report somatic embryogenesis in *Allium carinatun* from callus cultures derived from roots on BDS medium without 2,4-D.

In *Freesia refracta* epidermal cells of young inflorescence segments cultured on modified N6 medium containing [AA 2 $mg1^{-1}$ and BAP 3 mgl^{-1} produced embryoids directly which could develop into new plants (Wang *et al.*, 1994). Somatic embryogenesis was also achieved by transferring pale yellow translucent callus induced on surface of similar explants cultured on MS medium containing IAA 2 $mg1^{-1}$, BAP 0.5 mgl^{-1} and or NAA 0.5 mgl^{-1} on to a modified N_6 medium containing IAA 2 mgl^{-1} and BAP 3 mgl^{-1} (Wang *et al.*, 1994).

Laublin *et al.*, (1991) reported somatic embryos in *Iris pseudocorus, I. versicolor and I. setosa*. Callus was induced on root cultures on induction media containing 2, 4-D (45 or 22.5 µM), NAA (5.4 1µM) and kinetin (0.5 µM). Somatic embryos were developed after transfer of callus on to regeneration media containing 9 or 22 µM BAP and 21uM TIBA or 9 µM BAP and 4 µM TI BA.

Lilien-Kipnis *et al.*, (1992) obtained somatic embryogenesis and plant regeneration from inflorescence explants of *Nerine manselli* in liquid culture. Compact but friable meristematic callus was obtained in liquid medium containing 0.25 µM NAA, 10 µM BAP and paclobutrazol 2.5 mg l^{-1}. Exclusion of

paclobutrazol from proliferation medium resulted in the by 2-ip development of embryonic masses. Differentiation of somatic embryos was enhanced and these germinated in growth regulator free medium.

Radojevic and Subotic (1992) reported plant regeneration of *Iris setosa* through somatic embryogenesis. Embryogenic calli were induced from mature zygotic embryos on Murashige and Skoog (MS) medium supplemented with 2,4-D 5 mg 1^{-1}, kinetin 1 mg 1^{-1}, x-proline 250 mgl^{-1} and casein hydrolysate250 mgl^{-1} . Somatic embryos were regenerated in to plantlets in MS medium with 1 per cent sucrose and 1 mg IAA + 3 mg kinetin + 1 mg GA_3 per litre:

Embryogenic callus was initiated from inner tissues of protocorm like bodies of cymbidium cultured on Murashige and Skoog (MS) medium containing 2 $mg1^{-1}$ NAA + 0.5 $mg1^{-1}$ 2,4-D. These embryogenic calli produced proembryoid like structures after 10 days of culture, which become globular embryos differentiated into protocorms and plantlet after being transferred to the hormone free MS medium (Begum *et al.*,1994).

Stefaniak (1994) produced friable embryogenic callus and somatic embryos of four gladiolus cultivars on Murashige and Skoog (MS) medium with various concentrations of 2,4-D or NAA from corm slices, young leaf bases and whole, intact plantlets. Somatic embryos transferred on to MS hormone-free medium regenerated into plantlets. Jehan *et a]*., (1994) developed a method of rapid multiplication of *Iris pallida* and *I. germanica* by somatic embryogenesis.

The optimum medium for embryogenic callus on leaf bases, ovaries, petals rhizome apices was Murashige and Skoog (MS) medium supplemented with proline 2.9 gl^{-1}. Following transfer of callus to embryogenic expression medium, somatic embryos appeared on the callus surface , which developed, to plantlets on the same medium.

Somatic embryogenesis and plantlet regeneration were achieved from an ornamental bamboo, *Otatea acuminata azetocorum by* culturing zygotic embryo explants on both Murashige and Skoog (MS) medium and Gamborg medium supplemented with 2,4-D, benzyl amino purine and 2 per cent sucrose. Over 95 per cent of these embryoids were germinated into plantlets (Woods *et al.*, 1994).

Rout and Das (1994) reported somatic embryogenesis in 3 species of ornamental bamboo. Embryogenic callus was obtained following the culture of nodal explants from *in vitro* grown seedlings and excised mature zygotic embryos of *Bambusa vulgaris, Dendrocalamus giganteus and D. strictus* on Murashige and Skoog (MS) medium supplemented with 0.5 $mg1^{-1}$ kinetin, 2.0 $mg1^{-1}$ 2,4-D, 10 $mg1^{-1}$ adenine sulfate and 3 per cent sucrose in the light or dark. Somatic embryos germinated into normal plants and were transferred to soil with 95 per cent success.

Dias *et al.*, (1994) observed somatic embryogenesis and plant regeneration in the tissue cultures of *Geonoma gamiova*. Immature zygotic

embryos from young fruits were inoculated on semi-solid medium containing Murashige and Skoog (MS) salts, Morel vitamins, 1.5 g l^{-1} activated charcoal, 100 $mg1^{-1}$ 2,4-D and 2-ip . One month after inoculation embryogenic masses formed which later regenerated embryoids on medium with 20 mgl^{-1} 2,4-D. These embryoids germinated on growth regulator free medium.

Somatic embryogenesis was obtained from suspension callus of gladiolus (Tomotsune *et al.*, 1994). The suspension callus was induced from sprouted cormel in MS and Skoog (MS) medium containing various concentrations of NAA. Somatic embryogenesis was obtained when callus was placed on growth regulator free medium or medium containing 0.1 mg 1^{-1} BAP.

Atta-Alla and Van (1996) observed indirect somatic embryogenesis from leaves of *Yucca aloifolia.* The embryogenesis occurred on half strength Murashige and Skoog (MS) medium containing 2,4-D 2 $mg1^{-1}$ and BAP 1.0mgl^{-1}. Direct embryogenesis also occurred to a lesser extent. Plantlets were regenerated on media without growth regulators.

2.2.3. Encapsulation of somatic embryos

Murashige (1977) first coined the term artificial seed and in simple terms it means a somatic embryo entrapped in a biodegradable synthetic polymer coating that acts as an artificial seed coat. Kamada (1985) broadened the scope of artificial seed technology by defining this somatic propagule as a

capsule prepared by coating a cultured matter such as a piece of tissue or an organ which can grow into a complete plant body along with nutrients with an artificial covering.

Redenbaugh *et al.*, (1986) encapsulated somatic embryos of alfalfa, celery and cauliflower as single beads to produce individual somatic artificial seeds. The somatic embryos were mixed in 3.2 per cent sodium alginate, dropped individually into a 50 mM solution of calcium chloride and allowed to undergo complexation for 30 minutes to form an alginate bead.

Kitto and Janick (1985) produced synthetic seeds by encapsulating somatic embryos of carrot. Synthetic seed coats were applied to asexual embryos of carrot by mixing equal volumes of embryo suspension and a 5 per cent solution of polyethylene oxide (Polyox WSR -N 750) and dispensing 0.2 mi drops of this mixture into teflon sheets. The drops got dried to form detachable wafers consisting of embryo suspension embedded in polyox.

Synthetic seeds were developed also for mulberry (Bapat *et al.*, 1987), sandal wood (Bapat and Rao, 1988) and *Valeriana wallichii* (Mathur *et al.*, 1989). In pineapple, a 2.5 per cent solution of sodium alginate upon complexation with 75 mM of $CaCl_2$. $2H_20$ solution gave optimal, firm and round beads within an ion exchange duration of 30 minutes. The encapsulated beads stored at room temperature (30^oC) retained their regeneration capacity for 30 days when put in cotton wool moistened with MS basal salts (Prabha, 1993).

Keshavchandran *et al.*, (1993) reported that differentiating calli obtained from immature inflorescence of *Vetiveria zizanioides* (L) Nash, were successfully encapsulated in to beads using sodium alginate and calcium chloride at suitable concentrations to produce firm, round beads. The beads started differentiating into shoots when put on regeneration medium in 4 days. The encapsulated explants could be successfully stored at 40°C for 45 days without loosing their capacity to regenerate. The encapsulated explants differentiated and regenerated shoots earlier than non-encapsulated explants. Root initiation was also slightly precocious. Encapsulating the differentiating calli resulted in economizing on the medium, space and time.

Somatic embryos of *Asparagus cooperi* were encapsulated as single embryos approximately 4-6 mm in diameter to produce individual synthetic seeds. The highest plant regeneration (32.2%) was obtained with 3.5 per cent sodium alginate and 50 mM calcium chloride. After storage for 90 days at 2°C, the conversion frequency of artificial seed was 8.3 per cent (Ghosh and Sen, 1994). Dias *et al.*, (1994) produced synthetic seed of *Geonoma gamiova* by encapsulating somatic embryos in sodium alginate.

Encapsulating somatic embryos using 3 per cent sodium alginate and 50 mM calcium chloride developed artificial seeds of *Pelargonium hortorum*. The resultant synthetic seeds germinated normally and produced flowering plants within 12-14 weeks (Gill *et al.*, 1994).

2.2. 4. Storage and regeneration studies

Regeneration or plantlet conversion is defined as the per cent of somatic embryos that produce complete plant with normal phenotype. Conversion has been accepted as more appropriate term in relation to artificial seeds.

Plant tissue culture is the ideal material for the conservation of germplasm. This is possible through advanced technologies such as cryopreservation, low temperature storage, and artificial seeds etc. They help in long term storage so that plant could be obtained whenever the need arises and also help in easy handling because of small like zygotic seeds and reduce the cost of transportation with easy handling.

In *Spathoglottis plicata.,* a perusal of earlier literature reveals that protocorm without any hydrogel around them would loose the capacity to regenerate when kept for long storage as such (Datta and Potrykus, 1989). Protocorms stored 20°C and 0°C were crystallized and then thawed, didnot retains any water and became messy and failed to grow. At 25°C, the protocorms were dead and at 15°C protocorms had fungus growth on them. However, protocorms stored. This indicates that protocorm, as such cannot be stored and should be immediately cultured.

2.3. Somaclonal variation in ornamental crops

Somaclonal variation is the variation displayed amongst plants regenerated from cell cultures (Larkin and Scowcroft, 1981). As a method to create genetic variability, somaclonal variation is unique. It has been reported in more than 22 species that include monocots and dicots, sexually and asexually reproducing species. In general, frequency of variation could be as high as 30-40 per cent for the number of plants showing some type of variation and from 0.2 to almost 3 per cent for a particular trait. The frequencies of variation are high enough that desirable variants can be identified and isolated. There are two types of variation - epigenetic or developmental variation and heritable variation. Heritable variation results due to changes in genetic constitution. Somaclonal variation can be used to improve yield and quality, to get plants resistant to abiotic and biotic stresses and also to create variability in crops which do not set seeds. Growth regulators like 2,4-D and BA are considered to effect greatest number of variability. Rates of variation are reported to increase with an increase in overall concentrations of growth regulators (Nehra *et al.*, 1992).

Proliferation rate *in vitro* is regarded as the causative factor for inducing somaclonal variation. Cultures proliferated at excessive rate show more variation than those grown at moderate rates did. Repeated subculturing thereby could result in induction of somaclonal variation. Factors like cultivars (Rietveld *et al.*, 1993), explant source, mode of regeneration, culture conditions etc., could induce somaclonal variation. Induction of somaclonal variation in *A.*

andreanum has not been reported and literature available in other anthurium species and other ornamental crops are reviewed.

2.3.1. Somaclonal variation in anthuriums

High level of organogenesis in cultures resulted in ploidy variation in *A. scherzerianum* (Geier, 1988). Chromosome counts of plants regenerated from shoot forming callus showed little variation in ploidy. However, repeated subculturing revealed increasing cytogenetic instability, loss of shoot forming ability and habituation particularly when portion of callus lacking shoot initials were isolated and subcultured separately (Geier, 1988).

2.3.2. Somaclonal variation in other ornamental crops

First commercial cultivar through somaclonal variation was released in *Pelargonium sp.* Symmetrical flowers, large and fertile stamens and seed set were the traits seen in the variant (Skirvin and Janick, 1976). Selection of petunia cells for tolerance to herbicide N (Phosphonomethyl glycine) has been reported (Shah *et al.*, 1986).

Somaclonal variants of commeciral value were obtained in *Dendranthema morifolium* by an intermediate callus stage using foliar and petal explants (Khalid *et al.*, 1989; Malaure *et al.*, 1991). In kalanchoe, phenotypic variation depended on the cultivars and method of propagation (Schwaiger and Horn, 1988).

Griesbach (1989) developed a procedure for inducing somaclonal variation in hemerocallis tissue culture. The frequency of genetic variation, however, was reported to be very high and a commercial variety 'Yellow Tinkerbell ' was released.

Plants of *Lavandula officinalis from* callus cultures derived from various explants cultured on LS basal medium supplemented with BA 10 mgl^{-1} showed some alterations in inflorescence and leaf morphology (Panizza *et al.*, 1990).

Laneri (1990) reported somaclonal variants among plantlets from *in vitro* cultured immature flower buds of cymbidium. The somaclones produced racemes of flowers with a slightly longer labellum and more pointed, yellow - brown rather than cinnamon tepals.

Growth regulators induced epigenetic variation was reported in *Kalanchoe blossfeldiana* (Huitema *et al.*, 1990). Plantlets regenerated from leaf discs cultured on MS medium supplemented with high concentration of NAA and 2-ip showed epigenetic instability, as shown by fasciation and aberrant phyllotaxis.

In *Cyclamen persicum,* the frequency of altered plants ranged from 4.5 to 36.3 per cent. The variations observed were in growth habit, flower

shape and colour, and chlorophyll pattern and shape of leaves (Schwenkel and Grunewaldt, 1990).

Begonia X elatoir plantlets, regenerated from leaf discs, callus showed differences in flower morphology, flower size, plant height, plant morphology, and number of flowers per plant (Jain, 1993). Similar somaclonal variations were reported in *Saintpaulia ionantha* (Jain, 1993; El-Mardi *et al.*, 1993) and *Zinnia marylandica* (Stieve *et al.*, 1992).

Arene *et al.*, (1993) reported that plants regenerated from callus in *Rosa hybrida* CV. Meirutral exhibited variation in number, colour and shape of petals, growth habit and height.

Growth hormonal influence on somaclonal variation in ornamental plants has been reported. In *Dendranthema morifolium,* altered characteristics like exposed tubular florets and decreased flower diameter, petal number, peduncle length and leaf area were seen in regenerated plants from young inflorescence cultures (Fei and Zhou, 1994).

In Rudbeckia, plants regenerated through callus cultures exhibited variation in terms of flower shape, number of ray florets/flower, flower colour, polyploidy and aneuploidy (Khilbas, 1995).

Hadi and Bridgen (1996) developed somaclonal variants of *Torero fourneieri* cv. Compacta Blue resistant to spider mite *(Tetranychus urticae)* and green house whitlefly *(Trialeurodes vaporariorum)* through callus cultures.

2.4. Induced mutagenesis in ornamental plants under *in vitro* culture

Bajaj et *al.*, (1971) based on a study of direct and indirect effect of gamma irradiation on the seeds, seedlings, callus, excised roots, ovules and embryos has observed that, callus tissue culture are more radioresistant than intact seedlings.

In two cultivars of chrysanthemum, Mabuchi and Kawada (1975) reported that, gamma irradiation of shoot tip culture resulted in the production of plants healthy enough to transplant. The higher the radiation dose the lesser was the survival rate. A few the plants that withered were those irradiated with a dose less than 2000 Gy.

An effective chemical mutagenesis procedure for *Petunia hybrida* cell suspension culture was reported by Coljin *et al.*, (1979). Among the various chemical mutagens tested Nitrosoguanidine was the most effective one.

Roest *et al.*, (1980) irradiated the detached leaves of two genotypes of *Begonia heimalis* with different doses of X-rays and when the leaf disc explants were cultured *in vitro* about 30 per cent of plantlets produced after two

cycles were mutants with respect to colour, size and form of leaves and flowers. Majority of the mutants (98.5 %) was found to be non-chimeric.

Sunnio *et al.*, (1984, 1986) proposed a procedure for *in vitro* mutation breeding of potato. Two hundred and thirty five plants obtained from buds of cultivar ' Desiree ' cultured on modified MS medium were irradiated with 3 kR of gamma rays and single node pieces subcultured twice. After about 40 days VM_4 plants were cut into single node pieces and transferred to fresh medium. Among the 1094 plants established, 158 mutations were detected. Variations were observed with respect to leaf size and shape (36), leaf colour (dark green or pale green)(39) and flower colour (white or dark Purple)(24), 1 of flower shape (exerted style), 7 of anthocyanin deficient stems, 5 of dwarf type and 46 of tuber skin colour (yellow, dark purple or spotted). Of 102 mutants, 78 were apparently homogenous while 25 appeared to be chimeric.

Duron and Decourtye (1986) reported that when Weigela cv.'Bristol Ruby cultured *in vitro* was gamma irradiated with doses 20-60Gy. Bud survival, rhizogenesis and cutting growth was found to be affected at doses of 30 Gy and few buds survived at 60 Gy dose. Mutants produced from irradiated buds appeared to be homogeneous at first but after 2-3 years 40 per cent proved to be periclinal chimeras.

Axillary shoot from *in vitro* derived microshoots of two lines of gerbera (A 26 and 82/19/16) were irradiated with X- ray doses between 10 and

25 Gy by Walther and Sauer (1986a). During 16 weeks of post-irradiation culture, the radiosensitivity was estimated based on the explants, survival rate, number of developed shoots on the first cut off date (27 days after irradiation) and the cumulative number of axillary shoots on four subsequent dates. They observed that higher X-ray doses resulted in greater inhibition of shoot generation and radiation induced damage was higher in A 26 than in 82/19/16.

Kleffel *et al.*, (1986) obtained homogenous ' whwh ' mutants in poinsettia by X-ray irradiation (10-60 Gy) of immobilized embryonic cells, heterozygous cells for anthocyanin synthesis (whwh). Mutation rate increased vvith increasing X-ray doses reaching 8.9 per cent at 60 Gy, but the survival rates decreased with increasing doses.

Walther and Sauer (1986b) observed that tetraploid rose cultivar responded in a different manner to X-ray irradiation of *in vitro* derived microshoots. Based on their studies with six tetraploid rose cultivars, it was suggested that radiosensitivity of any cultivar can be estimated by determining the survival rate of explants, the productivity of axillary shoots and inhibition of shoot development on the first day of cut off. A broad spectrum of variability was induced by applying X-ray doses between 25 and 60 Gy to basal segment on *in vitro* derived microshoots of rose cultivar ' Ilseta ' followed by repeated cutting of axillary shoots from treated mother plants. The mutation comprised of 73 per cent flower mutants with variation in size, colour and number of petals, 14 per

cent with altered growth and 13 per cent with modified leaves. They also observed that a period of about 9 months was required to, select the mutants ready for grafting in to rootstock.

Tissue cultures derived from flowering buds of aractostaphylos were grown in the dark on MS medium supplemented with 10 mgl^{-1} kinetin. The cultures were gamma irradiated in their 10th to 18th subculture five times at an interval of four weeks with doses of 2.5 to 5 Gy or with doses of between 2.5 to 160 Gy. Compared with the control the growth of the irradiated culture decreased with increasing radiation doses. The highest dose (160 Gy) was lethal to calli. None of the radiation treatments induced embryogenesis. The number of very large cells in the calli increased with increasing radiation doses (Duskova *et al.*, 1988).

Wilson (1993) reported that gamma irradiation of axillary buds delayed bud break, reduced percentage of bud break, multiple shoot production and rooting efficiency and also induced morphological variation in leaf and growth pattern. The estimated value for LD_{50} was 38 Gy under *in vitro* culture. Exposure of multiple shoots to gamma rays induced several morphological abnormalities and reduced the shoot production and rooting.

In *Lilium lavidii* var. Wilimottiae plantlet regeneration was decreased when irradiated bulb scales were cultured on MS medium containing BA, NAA

and colchicine, with or without irradiation. Variations in leaf thickness, leaf colour, bulb size and other attributes were found (Wang *et al.*, 1989).

Ahloowalia (1992) reported that gamma irradiation of explants cultured *in vitro of Dendranthema morifolium* produced mutants differing from parent plant in flower shape, petal number, and leaf size and shape.

Gamma irradiation of *in vitro* petal cultures at a dose of 40 Gy on the fourth day of culture produced variants of horticultural interest in carnation (Simard, 1992).

Peng *et al.*, (1995) reported nine chrysanthemum varieties developed by radiation breeding and micropropagation by exposing to tissue culture. Similar induction of mutants, by irradiating pedicel from immature flower buds with gamma ray dose of 8 Gy was reported by Latado *et al.*, (1996).

2.5. Screening of *in vitro* mutants and somaclonal variants

In vitro regenerated mutants and somaclonal variants could be screened based on morphological, biochemical and chromosomal analysis. In many cases, researchers have not used an early method of selection of variant plants. The selection is therefore realised at the stage of whole plants grown in the green house or field (Auge *et al.*, 1995). However, it is clear that the utility of this technique is considerably augmented if one uses a method of choosing (or 'screening') cells buds, or plants as early as possible, which will help the

manipulator avoid handling a very large number of non-variants and less useful plants.

The use of gel electrophoresis of different isozyme systems was investigated as a possible tool to identify cultivars of *A. andreanum* (Kobayashi *et al.,* 1987). They were able to characterize the different varieties using combined data for peroxidase (PX), malate dehydrogenase (MDH) and phosphogluco isomerase (PGI).

Klerk *et al.*, (1990) concluded that in *Begonia X hiemalis the* difference in the value of standard deviation may be used to assess difference in the extent of variation.

Differences in banding pattern observed for leaf peroxidase was used in identification of rose hybrids (Yoneda *et al.*, 1993). In *Cereus peruviana* isocitrate dehydrogenase (IDH), acid phosphatase (ACP), peroxidase (PX) and esterase (EST) enzyme systems were considered as good markers for investigating possible genetic variation in plant population regenerated from calli cultures (Mangolin *et al.*, 1994).

Cytological analysis of somaclonal variants regenerated from young inflorescence cultures of *D. morifolium* genotype 'Jin Chao' indicated the increased frequency of aneuploidy (Fei and Zhou, 1994). Lagging

chromosomes and micronuclei were observed at meiosis, frequency of which was higher among regenerated plants from single node culture.

A phenotypic, quantitative assay for measurement of somaclonal variation in begonia based on coefficient of variation was described and compared to randomly amplified polymorphic DNA (RAPD) analysis on the same plants. RAPD analysis sometimes failed to identify clonal variation when plants showed severe mutations (Bauman, 1995).

Jeong *et al.,* (1996) suggested that variation, in morphological characteristics of *Lilium hansonii* can be studied using isozymes, esterase and peroxidase.

In cymbidium, genetic variation among cultivars was detected using isoenzymes. The identification potential increased with the number of isoenzymes used. *In vitro* derived protocorm like bodies could be used successfully for the isoenzyme analysis (Obara-Okeyo, 1997).

Materials and Methods

3. MATERIALS AND METHODS

The investigations were carried out at the Plant Tissue Culture Laboratory, Kerala Horticultural Development Programme (KHDP - R & D), College of Horticulture, Kerala Agricultural University, and Vellanikkara during 1995 to 1998. The materials utilized and the methodology followed are described in this chapter. The investigations presented here comprised of five major experiments:

3.1. Refining the protocols and establishing micropropagation system

3.2. Somatic embryogenesis and encapsulation of somatic embryos for production of synthetic seeds

3.3. Induction of somaclonal variation

3.4. *In vitro* mutagenesis

3.5. Screening somaclonal variants and mutants

3.1. Refining the protocol and establishing micropropagation system

Details of the experiments taken up to improve the micropropagation technique developed for *A. andreanum* cv. Dragon's Tongue are presented below:

3.1.1 Primary explants

Explants were taken from actively growing adult plants receiving uniform cultural practices (KAU, 1996) and maintained adjacent to the Plant

Tissue Culture Laboratory. Tender leaves (most recently opened fully unfurled), petiole, spadix and seeds were used as the sources of explants.

3.1.1.1 Collection and preparation of explants

Selected leaves were excised along with their petioles. The leaves were first washed gently in running tap water. The petioles were then removed and the leaves washed in distilled water containing a few drops of the wetting agent 'Tween 20'. They were further washed two to three times with distilled water to remove the traces of the wetting agent.

The distal portion of the petiole and spadix were prepared as in the case of tender leaves. The seeds after removing the mucilagenous coat were also prepared as described before. Spadices of different age such , as spadix with tightly coiled spathe, partially opened spathe and spadix nearing seed set was used for the study.

Explants from *in vitro* regenerated plants such as., leaves, single nodes, petiole and roots were also used for culturing, As they were derived from aseptic cultures the explants were not subjected to surface sterilization procedures.

3.1.1.2 Surface sterilization of explants

Surface sterilization of explant materials was carried out inside the laminar airflow chamber. The leaves were wiped with cotton wool dipped in 70 per cent ethyl alcohol. They were then cut into smaller pieces of 5 cm^2 and treated with one per cent sodium hypochlorite for 15 minutes. The treated leaf

Table 1 Surface sterilization treatments tested for various explants from *A. andreanum* cv. Dragon's Tongue

Surface sterilants	Concentration	Duration (Min)
Mercuric chloride	0.1	5
"	0.1	8
"	0.1	10
Sodium hypochlorite	0.5	10
"	0.5	20
"	1.0	10
"	1.0	20
Ethyl alcohol (70%) wipe + mercuric chloride	0.1	5
"	0.1	8
"	0.1	10
Ethyl alcohol (70%) wipe + sodium hypochlorite	0.5	10
"	0.5	20
"	1.0	10
"	1.0	20
Emisan (0.1%) dip 3 for 3 minutes + mercuric chloride	0.1	5
"	0.1	8
"	0.1	10
Emisan (0.1%) dip 3 minutes + sodium hypochlorite	0.5	10
"	0.5	20
"	1.0	10
"	1.0	20
Ethyl alcohol (70%) wipe + emisan (0.1%) dip for 3 minutes + mercuric chloride	0.1	5
"	0.1	8
"	0.1	10
Sodium hypochlorite (5%) dip for 20 minutes + mercuric chloride	0.1	8
"	0.1	8
"	0.1	10

pieces were then rinsed four or five times with sterile distilled water to remove the traces of surface sterilant on the explant. Petiole, seeds and spadix explants were also sterilized in the same manner. Spadix with tightly coiled spathe was sterilized in a two step fashion - initially along with spathe and finally after removing the spathe.

For fine tuning the surface sterilization protocols, different surface sterilants at various concentrations were tested. The details of such surface sterilization treatments are given in Table I.

There were three replications for each treatment with ten culture tubes per replication. Observations on per cent survival of cultures and the extent of contamination were recorded after three weeks of culturing the explants.

3.1.1.3. Inoculation and incubation

All the inoculations were carried out under strict aseptic conditions in a laminar air flow cabinet. Sterilized forceps, petridishes, surgical blades and blotting paper were used. The leaf explants after surface sterilization were given a fresh cut along the margins to remove the portion that had become brown due to surface sterilization. Such surface sterilized leaf segments were further cut into square pieces of 1 cm^2. Pieces with and without midrib were used for inoculation. Similarly, the two ends of the petiole and spadix were also given a fresh cut and sliced into pieces of 1cm and 8 mm respectively. The explants were finally dried on a sterile blotting paper prior to inoculation, The

seeds after surface sterilization were also blotted and dried on a blotting paper and then transferred on to the media.

3.1.2. Secondary explants

The seeds (primary explant) were induced to germinate *in vitro* and produce plantlets. Such plantlets were used as the source of secondary explants. Leaf, petiole, single nodes, and roots were used for the induction of callus and for somatic embryogenesis studies.

3.1.3. Culture media

The basal media used for the study were half MS (Murashige and Skoog, 1962) with half the concentration of inorganic salts and full concentration organic constituents), Modified MS (MMS - Pierik, 1976), Nitsch (Nitsch and Nitsch, 1969), NW (inorganic salts of Nitsch + Whites vitamins), B_5 (Gamborg *et al.*, 1968) and BM (inorganic salts of B_5 + MS vitamins). The compositions of different basal media tried are given in Appendix I, II and III.

3.1.4. Medium preparation

The various chemicals used for preparation of the medium were of analytical grade from SISCO Research Lab (SRL), British Drug House (BDH), Merck and Sigma.

Standard procedures were followed for the preparation of media (Thorpe, 1980). Stock solutions of salts of major- and minor-elements, vitamins and amino acids were prepared by dissolving the required quantity of chemicals in

specific volumes of distilled water. Whereas, plant growth substance were initially dissolved in dilute acid /alcohol and further volume made up with distilled water. Stock solutions of salts of major and minor nutrients were prepared first and were stored under refrigerated condition in amber coloured bottles. The growth regulators and vitamin stocks were prepared separately and fresh stocks were prepared at six-week interval.

All the glassware used for the preparation of the media were washed with water containing a few drops of Tween 20 and rinsed with distilled water. Specific quantities of stock solutions were pipetted into beakers or steel containers of required size. Sucrose and myo-inositol were added fresh and dissolved for specific treatment requirements. Glucose, casein hydrolysate (CH) and glutamine were also added fresh. Coconut water (CW) when used (after deproteinization) was collected from freshly harvested tender coconuts. The volume was made up using double glass-distilled water. The pH value of the solution was adjusted between 5.7 and 5.8 using 0.1N NaOH or 0.1N HCl. Agar was added to the medium and final volume was made up to required quantity. The medium was then heated by placing the beaker or the container used on a heating mantle and stirred thoroughly for uniform mixing till the agar got dissolved. The medium was poured hot to oven sterilized culture tubes (Borosilicate test tubes of size of 10.0 x 2.5 cm and 15.0 x 2.5 cm) which were previously rinsed thoroughly twice with distilled water. The containers with the medium were then tightly plugged with non-absorbent cotton wool plugs. They

were then autoclaved at 121 ^{0}c and 1.06 kgcm^{-2} pressure for 20 minutes. After cooling, the media were stored in an air-conditioned culture room for further use.

3.1.5. Callus induction

Attempts were made to induce callus from different *in vivo* and *in vitro* derived explants as explained earlier. All the explants were subjected to various treatment combinations as shown in Table 2, for callus induction. Growth regulator treatments tried for callus induction *in vitro* derived explants are presented in Table 3. Each treatment was replicated three times with each replication consisting of ten culture tubes. All the cultures were incubated under 24 hours dark conditions.

3.1.5.1. Observations recorded:

1. Number of cultures initiating callus
2. Days taken for callus induction
3. Degree of callusing

3.1.6. Regeneration of multiple shoots from callus obtained from different explants

The cultures after callus multiplication were transferred to the regeneration medium developed by Sreelatha (1992). MS medium containing BA 0.5mg/l, IAA 2.0 mg/l, sucrose 30 g/l and agar 6.0 g/l was used. The cultures were incubated in a culture room with a light intensity of 3000 lux and a photoperiod of 16 hd^{-1}. For further refinement of protocols with the objectives

Table 2. Treatment combinations tested for callus induction from different *in vivo* explants

Sl. No.	Growth regulator combinations (mg l^{-1})
1	BAP 0.3 + 2,4-D 0.08
2	BAP 0.5 + 2,4-D 0.08
3	BAP 0.5 + 2,4-D 0.3
4	BAP 0.5 + 2,4-D 0.5
5	BAP 0.5 + 2,4-D1.0
6	Kinetin 0.3 + 2,4-D 0.08
7	Kinetin 0.5 + 2,4-D 0.3
8	Kinetin 0.5 + 2,4-D 0.5
9	Kinetin 0.75 + 2,4-D 0.5
10	Kinetin 0.5 + 2,4-D 1.5
11	Kinetin 0.75 + 2,4-D 2.0
12	Kinetin 1.0+ 2,4-D 1.0
13	Kinetin 1.0+ 2,4-D 2.0
14	Kinetin 1.0+ 2,4-D 3.0

Media –MMS, Nitsch , Nitsch-White, B_5, $^1/_2$ MS
No. of Replications = Three (One replication = 10 Culture tubes)

Table 3 Treatment combinations tried to induce callus from *in vitro* derived explants

Sl. No	Treatment (mg l^{-1})	Media
1	BAP 1.0 + 2,4-D 0.10	MMS & Nitsch
2	BAP 1.0 + 2,4-D 0.25	"
3	BAP 1.0 + 2,4-D 0.50	"
4	BAP 1.0 + 2,4-D 1.0	"
5	BAP 1.0 + 2,4-D 2.0	"
6	BA P1.0 + 2,4-D 4.0	"
7	Kinetin 1.0 + 2,4-D 0.1	MMS & Nitsch
8	Kinetin 1.0 + 2,4-D 0.25	"
9	Kinetin 1.0 + 2,4-D 0.50	"
10	Kinetin 1.0 + 2,4-D 1.0	"
11	Kinetin 1.0 + 2,4-D 2.0	"
12	Kinetin 1.0 + 2,4-D 4.0	"
13	IBA 0.5 + BAP 2.0	"
14	2,4-D 4.0 + kinetin 0.5	"

No. of replications = Three
One replication = 10 culture tubes

Table 4 a. Treatments tested for early shoot regeneration and proliferation

Treatment (mg l^{-1})	Media
BAP 0.1	MS, MS, Nitsch and combination of Nitsch and White's media MMS
BAP 0.2	"
BAP 0.5	"
BAP 1.0	"
BAP 1.5	
Kinetin 0.1	"
Kinetin 0.2	"
Kinetin 0.5	"
Kinetin 1.0	"
Kinetin 1.5	"
BA P2.0 + GA_3 0.5	"
BAP 2.0 + GA_3 1.0	"
BAP 2.0 + GA_3 2.0	"
Kinetin 2.0 + GA_3 0.5	"
Kinetin 2.0 + GA_3 1.0	"
Kinetin 2.0 + GA_3 2.0	"
Kinetin 1.0 + IAA 2.0	
Kinetin 1.5 + IAA 2.0	"
BA 1.0 + IAA 2.0	"
BA 1.5 + IAA 2.0	"
BAP 1.0 + Kinetin 1.0 + IAA 1.0	"
BAP 1.0 + Kinetin 1.0 + IAA 2.0	"
2,4-D 1.0 + BAP 0.75 + kinetin 0.75	"
2,4-D 1.0 + BAP 1.0 + kinetin 1.0	"
2,4-D 2.0 + BAP 0.75 + kinetin 0.75	"
2,4-D 2.0 + BAP 1.0 + kinetin 1.0	"

Table 4b. Media tested for shoot regeneration from callus induced from spadix explants

Growth regulator- mgl^{-1}	Culture media tested
BAP 0.1	MMS
BAP 0.2	½ MS
BAP 0.5	Nitsch
BAP 1.0	NW
BAP 1.5	B_5
BAP 1.0	
BAP 1.0 + kinetin 1.0	
BAP 2.0 + kinetin 2.0	
BAP 4.0 + kinetin 4.0	

of early callus regeneration, increased shoot proliferation, elongation of shoots and proper leaf development, the callus were subjected to various treatment combinations as in Tables 4 and 5. There were three replications having 10 culture tubes each.

3.1.6.1. Observations recorded:

1. Per cent cultures with multiple shoots
2. Numbers of days taken for regeneration of multiple shoots
3. Number of shoots/culture
4. Number of leaves/shoot
5. Width of leaves

3.1.7. Improvement of shoot proliferation and leaf enlargement

Regenerated shoots were subjected to different treatments for further refinement for increased shoot proliferation and leaf enlargement (Table 6, 7 and 7). Treatments were replicated three times with 10 culture tubes per replication.

3.1.7.1.Observations recorded

1. Number of shoots /cultures
2. Length of shoot
3. Number of leaves/shoot
4. Width of the leaf

Table 5. Different carbon sources and their concentrations tried for leaf enlargement *in vitro*

Sl. No.	Carbon source	Concentration (g l^{-1})
1	Sucrose	20
2	"	30
3	"	40
4	Glucose	20
5	"	30
6	"	40
7	Sucrose + glucose	30
8	Sucrose + glucose	30

Culture media = Nitsch + BAP 0.2 mg l^{-1}

Table 6. Media supplements incorporated into the culture media for leaf enlargement and shoot proliferation

Sl. No.	Media supplements	Concentration (mg l^{-1} / ml l^{-1})
1	Glutamine	100
		200
		400
2	Casein hydrolysate	100
		150
		200
3	Coconut water (ml l^{-1})	100
		150
		300

Culture media = Nitsch + BAP 0.2 mg l^{-1}

Table 7. Different culture vessels used for culturing shoots

Sl. No.	Culture vessel	Size/volume
1	Culture(Jam) bottle	300 ml
2	Conical flask	100 ml
3	Conical flask	200 ml
4	Test tubes	25 x 150 mm without rim

3.1.8. Planting out and hardening

Rooted plantlets were carefully removed from the culture vessels and washed thoroughly to remove the adhering agar. Preliminary observations were recorded just before planting out. Different treatments were tried to refine the hardening procedures adopted for stage IV establishment of tissue culture plantlets of anthurium.

Containers like Minipot (mud), Protray and Sachet were used for planting. Different potting mixes like sand, mixture of sand and cocopeat (1:1) were tried forprimary establishment of plantlets. Effect of cluster- and individual- planting during stage IV on establishment of plantlets was investigated. Beneficial effects of incorporating vesicular-arbuscular mycorrhizae (VAM)- *Glomus sp.* to the potting mix were also studied.

3.1.8.1.Observations recorded

1. Per cent establishment of plantlets
2. Growth parameters
    a) Length of the shoot
    b) Number of leaves/shoot
    c) Width of the leaves

3.2. Somatic embryogenesis and encapsulation of somatic embryos

3.2.1. Induction of somatic embryoids

3.2.1.1. Explants

Tender leaves and seeds from mature plants grown in green house, and the explants derived from *in vitro* grown plants (leaves, petioles, single nodes and roots) were used for the study. After surface sterilization (same as in section 3.1.1.2) they were subjected to various treatments for induction of somatic embryoids (Table 9).

3.2.1.2. Media

The basal media evaluated were half MS, Nitsch, NW, B_5, BM. Thirty treatment combinations were evaluated for their ability to induce somatic embryoids (Table 9). The treatments involved combination of plant growth substances (2,4-D, IBA, BAP, kinetin and GA_3). The treatments were replicated three times.

3.2.1.3 Observation recorded

Number of cultures initiating callus and embryo mass.

3.2.1.4 Effect of Culture conditions

Studies were conducted to find out the effect of culture conditions on the initial establishment of the embryogenic cultures. Placing cultures on culture-racks covered by black cloth provided darkness and cool white fluorescent

Table 8. Treatments tested for induction of somatic embryogenesis

Notations	Media combinations
T_1	□ MS + 2,4-D 0.5 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 200 mg l^{-1} + CW 10% + glucose 10 g l^{-1} + sucrose 20 g l^{-1}
T_2	□ MS + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 200 mg l^{-1} + CW 15% + glucose 10 g l^{-1} + sucrose 20 g l^{-1}
T_3	$^1/_2$ MS + 2,4-D 2.0 mg l^{-1} + kinetin 0.3 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g l^{-1} + glucose 10 g l^{-1}
T_4	□ MS + 2,4-D 4.0 mg l^{-1} + kinetin 0.3 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
T_5	□ MS + 2,4-D 2.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
T_6	□ MS + kinetin 1.5 mg l^{-1} + IAA 3.0 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 30 g
T_7	□ MS + 2,4-D 4.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + + sucrose 30 g + glutamine 200 mg l^{-1}
T_8	□ MS + 2,4-D 4.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 30 g
T_9	□ MS + 2,4-D 4.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 40 g
T_{10}	□ MS + 2,4-D 4.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 50 g
T_{11}	Nitsch + 2,4-D 0.5 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 200 mgl^{-1} + sucrose 20 g + glucose 10 g.
T_{12}	Nitsch + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 200 mgl^{-1} + sucrose 20 g + glucose 10 g.
T_{13}	Nitsch + 2,4-D 2.0 mg l^{-1} + kinetin 0.3 mg l^{-1} + glutamine 200 mgl^{-1} + sucrose 20 g + glucose 10 g.
T_{14}	Nitsch + 2,4-D 4.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + glutamine 200 mgl^{-1} + sucrose 20 g + glucose 10 g.
T_{15}	Nitsch + 2,4-D 4.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + glutamine 200 mgl^{-1} + sucrose 30 g
T_{16}	Nitsch white + 2,4-D 1.0 mg l^{-1} + kinetin 0.25 mg l^{-1} + glutamine 200 mgl^{-1} + sucrose 20 g+ glucose 10 g.

Contd.....

Table 8 contd.....

T_{17}	NW + 2,4-D 1.5 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 200 mgl^{-1} + sucrose 20 g + glucose 10 g.
T_{18}	NW + 2,4-D 2.0 mg l^{-1} + kinetin 0.75 mg l^{-1} + glutamine 200 mgl^{-1} + sucrose 20 g + glucose 10 g l^{-1}
T_{19}	B5 + GA 5 mg l^{-1} + glutamine 200 mg l^{-1} + casein hydrolysate 100 mg l^{-1}
T_{20}	B5 + 2,4-D 1.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + glutamine 200 mg l^{-1} + casein hydrolysate 100 mg l^{-1}
T_{21}	B5 + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 200 mg l^{-1} + casein hydrolysate 100 mg l^{-1}
T_{22}	BM + IBA 0.5 mg l^{-1} + BAP 0.5 mg l^{-1} + glutamine 200 mg l^{-1} + casein hydrolysate 100 mg l^{-1}
T_{23}	BM + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 400 mg l^{-1} + casein hydrolysate 100 mg l^{-1}
T_{24}	BM + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 200 mg l^{-1} + casein hydrolysate 100 mg l^{-1}
T_{25}	BM + 2,4-D 2.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + glutamine 200 mg l^{-1} + casein hydrolysate 100 mg l^{-1}
T_{26}	Whites + 2,4-D 1.0 mg l^{-1} + kinetin 0.25 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
T_{27}	Whites + 2,4-D 2.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
T_{28}	Whites + 2,4-D 4.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
T29	Whites + 2,4-D 2.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + glutamine 400 mg l^{-1} + casein hydrolysate 100 mg l^{-1}
T_{30}	Whites + 2,4-D 2.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + glutamine 200 mg l^{-1} + casein hydrolysate 100 mg l^{-1}

tubes, giving a light intensity of 3000 lux provided the light (Photoperiod of 16 hd^{-1}).

3.2.1.5 Observation recorded

Number of cultures initiating embryo mass.

3.2.1.6 Effect of Media supplements

Different concentrations of various media supplements (coconut water, glutamine, sucrose and casein hydrolysate) were evaluated to identify their ability to induce somatic embryoids (Table 10). The explants were transferred to fresh media at two weeks interval about 5-6 times for the induction of somatic embryoids.

3.2.1.7 Observation recorded

Number of cultures initiating somatic embryoids.

3.2.2 Maturation of somatic embryoids

The initiated somatic embryoids were transferred to the maturation medium. For maturation of somatic embryoids half MS, Nitsch and NW medium were used. The treatments involved combination of different plant growth substances (2,4-D, kinetin and ABA), glutamine, casein hydrolysate and sucrose (Table 11). The treatments were replicated 10 times.

3.2.2.1. Observations recorded

Four weeks after transfer to maturation media the following observations recorded.

1. Per cent of live cultures
2. Number of embryoids per culture
3. Colour of embryos

3.2.2. 2 Effect of light on maturation of somatic embryos

Effect of light on maturation of somatic embryoids was also studied.

3.2.2.2.1 Observations recorded

1. Per cent live cultures
2. Number of embryoids/culture
3. Size of embryoids

3.2.2.3 Effect of sucrose concentration on maturation

Effect of different concentrations of sucrose (20, 30 or 40 gl^{-1}) on maturation of somatic embryos was studied.

3.2.2.3.1 Observations recorded

Same as in section 3.2.2.2.1

Table 9. Treatments tested to evaluate the effect of media supplements on induction of somatic embryoids

Notation	Media combinations -mg l^{-1})
Mg_1	NW + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 200 mg l^{-1}
Mg_2	NW + 2,4-D 1.0 mg l^{-1}+ kinetin 0.mg l^{-1}+ glutamine 400 mg l^{-1}
Mg_3	NW + 2,4-D 1.0 mg l^{-1} + kinetin 0.15mg l^{-1} + glutamine 600 mg l^{-1}
Mc_1	NW + 2,4-D 1.0 mg l^{-1}+ kinetin 0.15 mg l^{-1} + glutamine 100 mg l^{-1} + coconut water 10%
Mc_2	NW + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 100 mg l^{-1} + coconut water 5%
Mc_3	NW + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 100 mg l^{-1} + coconut water 20%
Mn_1	NW + 2,4-D 1.0mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 100 mg l^{-1} + Casein hydrolysate 100 mg l^{-1}
Mn_2	NW + 2,4-D 1.0 mg l^{-1}+ kinetin 0.15 mg l^{-1} + glutamine 100 mg l^{-1} + Casein hydrolysate 200 mg l^{-1}
Mn_3	NW + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 100 mg l^{-1} + Casein hydrolysate 300 mg l^{-1}

3.2.3. Germination of somatic embryoids

The somatic embryoids from the maturation media were transferred to the germination media.

Twelve treatment combinations were tried for germination of somatic embryoids (Table 12). All the treatments were replicated 10 times.

3.2.2.2.1 Observations recorded

1. Germination of somatic embryoids
2. Size of embryoids
3. Colour of embryoids

3.2.4 Encapsulation of somatic embryoids and production of synthetic seeds/synseeds

Somatic embryos at early cotyledonary stage and shoot tips were isolated individually with a pair of fine forceps and cultured for 24 hours on basal Nitsch medium solidified with 0.6 per cent agar. The somatic embryos were then dipped for one minute in sodium alginate or agar (2,3, 4 or 5 %) prepared in liquid Nitsch media and then dropped individually using a bend-tip forceps in conical flask (250 ml) containing 50 ml of calcium chloride solution (50 or 25 mM). The suspension was agitated at 180 rpm for 40 minutes on a horizontal shaker (Certomat, Braun, Germany). The beads formed in the solution were recovered by decanting excess calcium chloride solution and rinsing the beads four times with sterile distilled water. Encapsulated somatic embryos and shoot

Table 10. Treatments tested for maturation of somatic embryoids

Notation	Media combinations
M_1	1/2MS + 2,4-D 0.5mg l^{-1} + kinetin 0.15mg l^{-1} + ABA 0.1 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g
M_2	□MS + 2,4-D 1.0mg l^{-1} + kinetin 0.15 mg l^{-1} + ABA 0.1 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g
M_3	□MS + 2,4-D 1.0 mg l^{-1}+ kinetin 0.15 mg l^{-1}+ ABA 0.2 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g
M_4	□MS + 2,4-D 1.0 mg l^{-1}+ kinetin 0.15 mg l^{-1} + ABA 0.2 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g
M_5	Nitsch + 2,4-D 0.5mg l^{-1} + kinetin 0.15mg l^{-1} + ABA 0.1 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g
M_6	Nitsch + 2,4-D 0.5 mg l^{-1}+ kinetin 0.15 mg l^{-1} + ABA 0.2 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g
M_7	Nitsch + 2,4-D 1.0 mg l^{-1}+ kinetin 0.15 mg l^{-1}+ ABA 0.1 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g
M_8	NW + 2,4-D 0.5 + kinetin 0.15 mg l^{-1}+ ABA 0.1 mg^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g
M_9	NW + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + ABA 0.1 + glutamine 400 mg l^{-1} + sucrose 30 g

tips or synseeds were cultured on basal Nitsch media immediately after encapsulation or stored at low temperature (4°C). At different intervals (10, 20 or 30 days) the synseeds were taken out for recording observations.

3.2.4.1. Observations recorded

1. Number of days taken for germination
2. Embryo conversion

3.3. Induction of somaclonal variation

Induction of variation *in vitro* was attempted by methods like repeated subculturing and employing high concentrations of cytokinins.

3.3.1 Repeated subculturing

Fresh callus was used for the experiment. The calli were subcultured at three weeks intervals for ten cycles. Plantlets were regenerated from calli from different cycles of subculturing. The best media identified for shoot elongation and multiplication from the present study were employed as the culture media for shoot induction from callus of different subcultures. There were three replications with ten culture tubes per replication. The plantlets were evaluated morphologically for somaclonal variants.

3.3.1.1 Observations recorded

Plantlets from each subculture were evaluated for following growth parameters.

Table 11. Treatments tested for inducing germination of somatic embryoids

Notation	Media combinations
G_1	½MS + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_2	½MS + BAP 0.1 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_3	½MS + BAP 0.2mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_4	½MS + BAP 0.5mg l^{-1}+ glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_5	Nitsch + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_6	Nitsch + BAP 0.1 mg l^{-1}+ glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_7	Nitsch + BAP 0.2mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_8	Nitsch + BAP 0.5mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_9	NW + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g glucose 10 g
G_{10}	NW + BAP 0.1mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_{11}	NW + BAP 0.2 mg l^{-1}+ glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g
G_{12}	NW + BAP 0.5mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g + glucose 10 g

1. Number of propagules produced at each subculture
2. Number of leaves
3. Width of leaves

3.3.2 Effect of high concentrations of cytokinins

Induction of somaclonal variation *in vitro* was attempted by employing high concentrations of cytokinins like benzyl adenine (BA) and kinetin in the culture media. Calli for induction of shoots were cultured on to media containing high concentrations of these cytokinins. Initially very high concentrations like 10, 15 or 20 mgl^{-1} of the cytokinins were employed. But, it was observed that at concentrations above 15 mgl^{-1}, only swelling of the shoot was effected. So, further trials were carried out with concentrations of 10, 12.5 or 15 mgl^{-1}.

3.3.2.1. Observations recorded

1. Shoot doubling time
2. Number of shoots/culture
3. Length of longest shoot
4. Number of leaves
5. Number of roots

Plants obtained through repeated subculturing and by the use of high cytokinin levels were screened morphologically for somaclonal variants at *in vitro* and *ex vitro* development stages, also using biochemical and cytological techniques.

3.4 *In vitro* Mutagenesis

Fresh callus and excised shoot tips cultured on basal MS (1962) media were exposed to gamma irradiation in the range of 100-400 Gy. From preliminary trial it was found that doses above 100 Gy is lethal for all the explants. So, further trials were carried out using gamma ray doses of 25, 50, 75, 100 or 150 Gy. After irradiation, calli were subcultured on to shoot regeneration media and shoot tips were cultured on to shoot multiplication media standardized from the present study.

3.4.1 Observations recorded

1. Days taken for shoot regeneration from callus
2. Shoot doubling time
3. Per cent culture showing shoot regeneration
4. Number of shoots/culture
5. Length of the largest shoot
5. Number of leaves/shoot

3.4.2 Observations recorded after planting out

Growth parameters were recorded after stage IV transplanting, at periodical intervals.

1. Height of the plant
2. Girth of the shoot
3. Number of leaves
4. Length of leaves

5. Width of leaves

Plants obtained through *in vitro* mutagenesis were also subjected to screening as in the case of plants from repeated subculturing and use of high concentrations of cytokinins.

3.5 Screening somaclonal variants and mutants

3.5.1 Isozyme studies

3.5.1.2 Methods

For the separation of multiple forms of enzymes, polyacrylamide gel electrophoresis was carried out using vertical slab gel electrophoresis unit of Centre for Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara.

Acrylamide monomers ($CH=CHCONH_2$) were polymerised with bis-acrylamide (CH_2 (NH CONH $=CH_2$) bis) to obtain the gel. Freshly prepared ammonium persulphate was used as catalyst and N, N, N', N' tetra methylene diamine (TEMED) as chain initiator.

Polyacrylamide gel was preferred because of its chemical inertness, high resolution, and ease in handling and easiness in preparation.

3.5.1.3 Enzymes assayed

Electrophoresis and isoenzyme variation determination were done for the following enzymes:

1. Peroxidase

2. Esterase

3. Glutamate oxaloacetate transaminase

The above enzymes were selected, as they are commonly occuring plant enzymes.

3.5.1.4. Preparation of samples

In vitro regenerated plantlets from Ten serial subcultures and four (4) irradiated cultures (25, 50, 75 or 100 Gy) were selected for the study. Leaf samples were thoroughly washed in cold water and blotted dry. Samples were then extracted by maceration of tissue with a pre-chilled mortar and pestle along with extraction buffer (Kobayashi *et al.*, 1987). Different treatments were tried to standardise the extraction buffer (Table 13). Tris buffer 0.05 M (pH 7.3) containing 0.2M sucrose, 0.002M diethyl dithiocarbamic acid, B- mercaptoethnol 10 µl, Triton -10 µl and PVPP one mg. B- mercaptoethnol and PVPP was added after adjusting the pH to 7.3. Samples extracted were centrifuged at 30, 00 rpm for 15 minutes in a Remi refrigerated centrifuge below 4°C. From the different proportions of sample and buffer tried, it was found that a sample buffer ratio of 1:3 was ideal for getting sufficient volume of extract in required concentration. After centrifuging, the supernatant was transferred to labelled vials and stored below subzero temperature. Fresh samples were collected each day as it was found that in the case of anthurium the stability of enzymes was very poor even under subzero temperature.

3.5.1.5. Preparation of gel

Analysis of the enzymes was carried out in an anionic system. Ten per cent polyacrylamide gel was used to evaluate the enzyme system. The following stocks were prepared:

1. 30% monomer- 60.0 g acrylamide + 1.6 g bis + H_2O to 200 ml (Stored at 4^0C)
2. Resolving gel buffer - 36.3 g Tris base (pH 8.8) H_2O to 200ml

Composition of the resolving gel is presented in below.

Composition of the resolving gel

1.	Monomer	3.33 ml
2.	Resolving buffer	2.5 ml
3.	Water	4.1 ml
4.	Ammonium persulphate	50 µl
5.	TEMED	10 µl

3.5.1.6.1. Electrode buffer

The stock solution was prepared by dissolving tris and glycine making up the volume to one litre with distilled water, keeping the pH at 8.3. The stock buffer was diluted to different ratios, 1:9, 1:2, and 1:1 before use and the response in each ratio studied.

The slab gel unit of the Centre of Molecular Biology and Biotechnology was used for the study. The size of slab gel was 10cm X 8cm. After preparing the working solution, it was gently poured in between the glass plates kept in polymerisation stand. Polymerisation was achieved within 45 minutes to one hour. Staking gel did not give any added advantage and hence was not used.

After polymerisation, the gel along with glass plate was removed to electrophoresis apparatus. The upper and lower trays of the unit were filled with electrode buffer. Upper trough was connected to cathode and the lower one to anode.

Electrophoresis was carried out at 4°C. A constant current of 25 mA per slab was maintained throughout the run. Bromophenol blue (0.002%) in the imidazole buffer (pH 7.0) was used as the tracer dye.

3.5.1.6 Enzyme assays

3.5.1.6.1 Peroxidase

A gel concentration of 10 per cent acrylamide was found best for peroxidase enzyme separation in *A. andreanum*.

Table 12. Details of extraction buffers for leaf samples

Extraction buffer	
(1)	Tris buffer 0.2 M pH 7.3 + PVPP
(2)	Tris buffer 0.2 M (pH 7.3) + sucrose 0.20 M + Diethyl dithio carbomic acid 0.002 M + □ mercaptoethanol 10 Fl + PVPP
(3)	Tris buffer 0.2 M (pH 7.3) + sucrose 0.20 M + 0.002 M Diethyl dithio carbamic acid + + 10 µl Triton-X + 10 µl PMSF + 10 µl β-mercapto ethanol + PVPP

Table 13. Composition of resolving gel

Resolving gel (10%)		
Monomer	:	3.33 ml
Resolving gel buffer	:	2.5 ml
Water	:	4.1 ml
TEMED	:	10 Fl
Ammonium persulphate	:	50 Fl (0.1 g APS H_2) to 1.0 ml)

Gel buffer - Tris hydrochloride pH 8.9

Electrode buffer - Tris-glycine pH 8.3

3.5.1.6.1.1 Staining solution (modified from Shaw and Kohen, 1968)

100ml solution contained:

0.2 M acetate buffer pH 5.6 - 100ml

Benzidine - 0.1g

Hydrogen peroxide - 0.4ml

Fresh stain was prepared each time. Acetate buffer and benzidine were mixed, heated, cooled and filtered and then hydrogen peroxide was added to the mixture. The gels were immersed in staining solution for about half an hour. As the bands faded on standing for long time photographs were taken on the same day of staining.

3.5.1.6.2 Esterase

The gel concentration, gel buffer and electrode buffer, were the same as that were used for peroxidase.

3.5.1.6.2.1 Staining solution (modified from Shaw and Kohen, 1968)

100ml of staining solution contained:

Phos A- Na_2 H PO_4 (0.2M), pH 8.8 -10ml

Phos B- Na_2H PO_4 (0.2M), pH 4.16 - 50ml

Fast blue RR -100mg

Naphthyl acetate in 50 % acetone - 2ml

Distilled water - 40ml

After the run was over, the gel was taken out and incubated in staining solution at 37°C for 45-60 minutes till brown bands appeared. The gels were destained in seven per cent acetic acid. The bands remained stable for many days.

3.5.1.6.3 Glutamate oxaloacetate transaminase

The gel concentration and gel and electrode buffer were the same as those were used for peroxidase,

3.5.1.6.3.1 **Staining solution** (Shaw and Kohen, 1968)

100ml of staining solution contained:

L-aspartic acid	- 532 mg
α-keto glutaric cid	- 72mg
pyridoxal 5'phosphate	- 50mg
St violet B-salt	- 400mg
0.1 M phosphate buffer pH 7.0	- 100ml

Fast blue B-salt was added before use. Gel was incubated in staining solution for 15 to 20 minutes till reddish orange bands developed.

3.5.1.7 Nomenclature of Isozymes adopted in the present study

The following abbreviations designated the enzymes:

1. Peroxidase - PRX
2. Esterase - EST
3. Glutamate oxaloacetate transaminase - GOT

3.5.2 Cytological studies

For screening somaclonal variant or mutant if any, among regenerated plants from the present study, cytological studies were carried out. Young actively growing root tips for the study were collected between 11.00 am and 12.30pm, which was found to be the peak time for mitotic division. The collected root tips were washed in water and then subjected to a pre-treatment with 0.002M 8-hydroxy quinoline for five hours at 10°C. The root tips were then washed in water and fixed for 24 hours in Carnoy's fluid (Alcohol: acetic acid: chloroform - 3:1:1, to which a few drops of ferric chloride was added as mordant) at room temperature. The fixative was changed once or twice to clear the cytoplasm and the roots were then washed and stored in 70% ethanol. For squash preparation the root tips were then hydrolysed in 1N HCl and kept for five minutes. The terminal one millimetre of deeply stained portion of the root tips alone was taken for squash preparation.

The slides were observed under a magnification of 1000x on a Nikon microscope. Various division stages were observed and the chromosome behaviour was recorded.

For karyomorphological studies, only those cells showing well-dispersed chromosomes with straight or almost straight arms were selected. Such selected stages were used for taking photomicrographs.

3.5.2.1 Photomicrography

The photomicrographs were taken with the help of Pentax photomicrograph equipment.

3.6. Statistical analysis

The data collected were analysed as per completely randomised design (Sunderraj *et al.*, 1972) wherever required.

Results

4.RESULTS

The salient results of the investigation carried out to refine the protocol for micropropagation and crop improvement *in vitro* of *A. andreanum* cv. Dragon's Tongue are presented in this chapter.

4.1 Refining the protocol and establishing micropropagation system for *A. andreanum* cv. Dragon's Tongue

4.1.1 Surface sterilisation

4.1.1.1 *In vivo* explants

4.1.1.1.1 Leaf explants

Significant differences among different surface sterilisation treatments were observed for the control of rate of contamination and survival of cultured explants. Surface sterilisation with mercuric chloride (0.1%) for eight minutes along with ethyl alcohol (70%) wipe was found to be very effective for controlling the rate of contamination of the leaf explants as it showed maximum survival (99.14%) of the cultures (Table 14). Mercuric chloride (0.1%) for 10 minutes along with ethyl alcohol (70%) wipe was equally effective and recorded a survival rate of 98.76 per cent. Without ethyl alcohol wipe surface sterilisation, mercuric chloride 0.1 per cent treatment for eight minutes recorded 87.08 per cent culture survival and the same concentration for 10 minutes recorded a survival rate of 88.82 per cent. Sodium hypochlorite as surface sterilant at a concentration of 1.0 per cent for 20 minutes also was effective in reducing the rate of contamination and recorded a survival rate of 80.70%. But, the treatment caused browning of tissues and further response of explants to callusing was

prevented. The browning of tissues was accelerated when the treatment was combined with ethyl alcohol (70%) wipe. The survival of cultures was minimum (15.58%) with sodium hypochlorite 1.5 per cent treatment for a longer duration of 20 minutes along with alcohol wipe.

4.1.1.1.2 Petiole explants

The combined surface sterilisation treatments involving ethyl alcohol (70%) wipe, emisan (0.1 %) dip for three minutes and mercuric chloride (0.1 %) treatment for 8 minutes recorded maximum survival of cultures (87.28%) (Table 15). A combination of sodium hypochlorite (5%) for 10 minutes and mercuric chloride (0.1%) for eight minutes also recorded good per cent survival of explants (81.50). But, the major limitation of treatment was tissue browning caused consequent to surface sterilisation. Treatment using mercuric chloride alone (0.1% for five minutes) recorded poor response with respect to cutting down the rate of contamination and increasing survival of explants (20.26%).

4.1.1.1.3 Spadix explants

A sequential treatments involving ethyl alcohol (70%) wipe, emisan (0.1%) dip for three minutes and mercuric chloride (0.1%) for 10 minutes recorded the maximum survival (87.59%) of spadix explants cultured *in vitro* (Table 16). This was closely followed (86.56%) by the treatment combinations as above with a change in duration of mercuric chloride (0.1%) treatment to eight minutes. Surface sterilisation of explants from spadices with mercuric

Table 14. Effect of different surface sterilants on percentage culture survival of leaf **explants** of *Anthurium andreanum* cv. Dragon's Tongue

Surface sterilants	Treatments		Cultures free from microbial contamination (%)
	Concentration (%)	Time (minutes)	
Sodium hypochlorite	0.5	10	61.16
	"	20	54.04
	1.0	10	54.85
	"	20	80.70
	1.5	10	77.39
	"	20	50.32
Ethyl alcohol (70%) wipe + Sodium hypochlorite	0.5	10	56.68
	"	20	66.31
	1.0	10	71.28
	"	20	37.85
	1.5	10	22.47
	"	20	15.58
Mercuric chloride	0.1	5	71.73
		8	87.08
	"	10	88.82
Ethyl alcohol (70%) wipe + Mercuric chloride	0.1	5	71.42
		8	99.14
		10	98.76
CD (0.05)			8.15

Observations taken three weeks after culture.
Number of replications = Three (3)
(One replication = 10 culture tubes)

chloride alone failed to cut down the contamination resulting in poor survival of explants.

4.1.1.1.4 Seed explants

Surface sterilisation treatment involving emisan (0.1%) dip for three minutes, and mercuric chloride (0.1%) for eight minutes was found to be very effective for seed explants as it showed 87.85 per cent culture survival. In the combined treatment as above reducing the duration of mercuric chloride (0.1%) treatment to five minutes resulted in reduction of rate of survival of explants (84.83%). Though a combination of treatments involving sodium hypochlorite (5%) and mercuric chloride (0.1%) each for a duration of 10 minutes recorded good rate of survival (84.48%), seed explants were sensitive to sodium hypochlorite and the tissues turned brown consequent to surface sterilisation which prevented further response *in vitro*.

4.1.2 Somatic organogenesis

Investigations were carried out using different explants viz., leaves, petiole, spadix and seed from field grown plants and explants from *in vitro* raised seedlings. The influence of 70 (for *ex vitro* explants) and 24 (for *in vitro* derived explants) media combination treatments on the induction of callus was studied. Callusing was observed in all the explants tried.

Table 15. Effect of different surface sterilants on per cent culture survival of **petiole explants** of *Anthurium andreanum* cv. Dragon's Tongue

Surface sterilants	Treatments		Cultures free from microbial contamination (%)
	Concentration (%)	Time (minutes)	
Mercuric chloride	0.1	5	20.26
		8	29.19
		10	52.43
Ethyl alcohol (70%) wipe + Mercuric chloride	0.1	50	34.49
		80	46.78
		10	30.05
Ethyl alcohol (70%) wipe + Emisan (0.1% dip 3 mts + Mercuric chloride	0.1	5	72.21
		8	87.28
		10	74.04
Sodium hypochlorite (5%) 10 mts + Mercuric chloride	0.1	5	78.32
		8	81.50
		10	62.45
CD (0.05)			9.14

Observations taken three weeks after culture.
Number of replications = Three (3)
(One replication = 10 culture tubes)

Table 16. Effect of different surface sterilants on percentage culture survival of **spadix explants** of *Anthurium andreanum* cv. Dragon's Tongue

Surface sterilants	Treatments		*Cultures free from microbial contamination (%)
	Concentration (%)	Time (minutes)	
Mercuric chloride	0.1	8	41.79
		10	54.02
		15	34.59
Emissan (0.1%) dip 3 mts + Mercuric chloride	0.1	8	74.31
		10	76.83
		15	77.90
Ethyl alcohol (70%) wipe + emisan (0.1%) dip 3 mts + Mercuric chloride	0.1	8	86.56
		10	87.59
		15	76.39
CD (0.05)			8.97

*Observations taken three weeks after culture.
Number of replications = Three (3)
(One replication = 10 culture tubes)

4.1.2.1 *In vivo* explants

4.1.2.1.1 Leaf explants

Out of 70 treatment combinations tried, only fourteen (14) treatments showed the positive response to induction of callus (Table 18). The maximum number of cultures initiating callus (86.50%) was recorded by the treatment combination involving NW + kinetin 0.5 mg l^{-1} + 2,4-D 0.3 mg l^{-1} (Plate 2). This was *on par* with the treatment combination involving NW + kinetin 0.3 mg l^{-1} + 2,4-D 0.08 mg l^{-1} which recorded 86% culture showing callus induction. The rest of the treatments tried showed a response ranging from 39.0% to 77.50%.

Cultures initiating callus were more when the concentration of auxin (2,4-D) was lower than the concentration of cytokinin (kinetin) in the treatment. The treatment combination 2, 4-D 0.3mgl^{-1} + kinetin 0.5 mg l^{-1} recorded the maximum per cent cultures initiating callus (Table 18) in MMS, Nitsch and NW media (76.00%, 77.50 % and 86.50 % respectively). But, an increase in concentration of the auxin (2,4-D 1.0 mgl^{-1} + kinetin 0.5 mgl^{-1} treatment combination) reduced the per cent cultures initiating callus in half MS (39%) and Nitsch media (52%) respectively. In MMS and NW media, the same treatment combination did not produce any callus. The treatment combinations with B_5 media did not show any effect on leaf explants.

The number of days taken for callus induction was minimum (51 days) in the treatment combination with NW + kinetin 0.5 mg l^{-1} + 2,4-D 0.3 mg l^{-1} glutamine 200 mg l^{-1} (Table 18). The treatments, NW + kinetin 0.3 mg l^{-1} + 2,4-

Plate 1. *Anthurium andreanum* cv. Dragon's Tongue

Plate 2. Callus induction in leaf explant of *A. andreanum* cv. Dragon's Tongue

Table 17. Effect of different surface sterilants on percentage culture survival of **seed explants** of *Anthurium andreanum* cv. Dragon's Tongue

Surface sterilants	Treatments		*Cultures free from microbial contamination (%)
	Concentration (%)	Time (minutes)	
Mercuric chloride	0.1	5	56.98
		8	37.80
		10	22.56
Sodium hypochlorite	5.0	10	57.42
		15	56.79
		20	63.95
Emisan (0.1%) dip 3 mts + Mercuric chloride	0.1	5	84.83
		8	87.85
		10	76.47
Emisan (0.1%) dip 3 mts + Sodium hypochlorite	5.0	10	49.42
		15	55.40
		20	46.36
Sodium hypochlorite (5%) 10 mts + Mercuric chloride	0.1	5	76.93
		8	83.34
		10	84.48
CD (0.05)			8.06

*Observations taken three weeks after culture.
Number of replications = Three (3)
(One replication = 10 culture tubes)

D 0.08 mg l^{-1} and NW 2,4-D 0.5 mg l^{-1} + kinetin 0.5 mg l^{-1} recorded 51.50 and 56.00 days respectively for callus induction. Other treatments recorded a range between 60 to 97 days for callus initiation (Plate 2).

4.1.2.1.2 Spadix explants

Out of the 65 treatment combinations tried only seven (7) treatments recorded positive response of callus induction in spadix explants (Table 19; Plate 3)). The maximum number of cultures initiating callus (59.85%) was recorded by the treatment half MS + 2,4-D 0.3 mg l^{-1} + kinetin 0.5 mg l^{-1}. It was *on par* with the treatment combination half MS + 2, 4-D 0.08 mg l^{-1} + kinetin 0.3 mg l^{-1} which recorded 58.18% cultures initiating callus. Per cent cultures initiating callus was lowest (35.02) in the treatment combination Nitsch + 2,4-D 0.03 mgl^{-1} + Kinetin 0.5 mgl^{-1}. It was also noted that callus induction per cent was strongly depended on the growth regulator composition of the culture media. Concentration of auxin must be lower than that of cytokinin (2,4-D 0.3mgl^{-1} + Kinetin 0.5mgl^{-1}) for callus induction in all the basal media tried. MMS and B_5 media was not effective for callus induction in explants from spadices anyway.

The treatment combination Nitsch + 2,4-D 0.3 mgl^{-1} + kinetin 0.5 mgl^{-1} took the maximum number of days (81.66) for callus induction which was also highly dependent on the culture media (Table 19). The number of days taken for callus induction was the minimum (52.00) in the treatment combination half MS + 2,4-D 0.08 mg l^{-1} + kinetin 0.3 mg l^{-1}. When the growth regulator concentration in the treatment combination was increased (2,4-D 0.03 mg l^{-1} +

Plate 3. Callus multiplication from spadix explant.

Plate 4. Callus induction from *in vitro* derived leaf explant

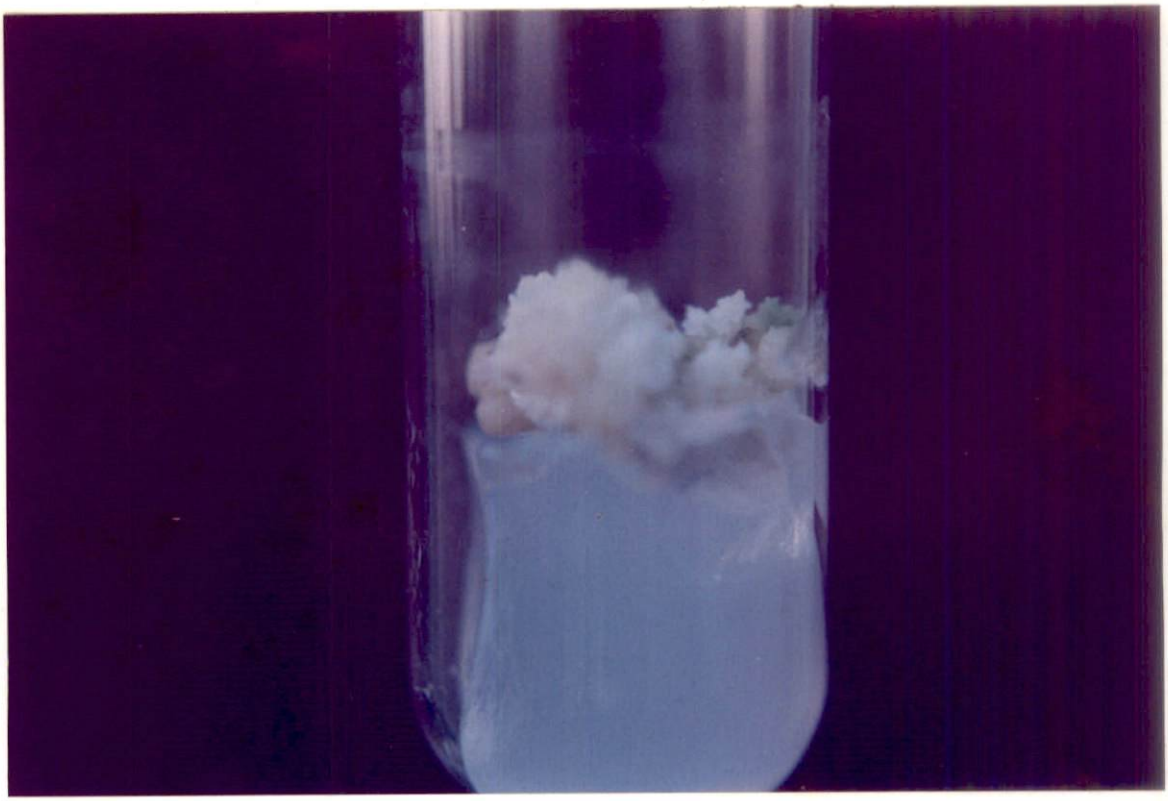


Table 18. Effect of different treatments on callus induction from **leaf explants** of *A. andreanum* cv. Dragon's tongue

Treatment combinations* (Media + growth regulator mg l^{-1})	Cultures initiating callus (%)	Days for callus induction
MMS + BAP 0.3 + 2,4-D 0.08	54.33	90.33
MMS + BAP 0.5 + 2,4-D 0.5	74.66	97.25
MMS + kinetin 0.5 + 2,4-D 0.3	76.00	93.33
□MS + kinetin 0.3 + 2,4-D 0.08	45.00	97.00
□MS + kinetin 0.5 + 2,4-D 0.3	43.16	97.35
□MS + kinetin 0.5 + 2,4-D 0.5	60.00	90.00
□MS + kinetin 0.5 + 2,4-D 1.0	39.00	95.00
Nitsch + kinetin 0.3 + 2,4-D 0.08	77.50	59.50
Nitsch + kinetin 0.5 + 2,4-D 0.3	68.50	62.50
Nitsch + kinetin 0.5 + 2,4-D 0.5	76.66	60.00
Nitsch + kinetin 0.5 + 2,4-D 1	52.00	57.00
NW + kinetin 0.3 + 2,4-D 0.8	86.00	51.50
NW + kinetin 0.5 + 2,4-D 0.3	86.50	51.00
NW + kinetin 0.5 + 2,4-D 0.5	66.50	56.00
CD (0.05)	8.59	9.80

*The treatments which induced callus only mentioned.
Number of replications = Three (3) (one replication = 10 culture tubes)
MMS - Modified Murashige and Skoog (1962) medium
NW - Inorganic salts of Nitsch and Whites' vitamins

kinetin 0.3 mg l^{-1}) the explants took more number of days (57.33) for callus initiation. Other treatment combinations tried recorded number of days for callus induction, ranging from 60.00 to 81.66 days.

4.1.2.1.2.1 Behaviour of different development stages of spadix to callus induction

Data presented in Table 20 shows that callus induction was depended on the stage of harvest of the spadix. Intense callusing was observed only in the case of explants derived from spadix with coiled spathe, which showed callusing in all the basal media tried along with growth regulators. But, the callus produced showed regeneration potential only in half MS media. The next best stage of harvest of spadices for explants was spadix with partially opened spathe, which recorded moderate callusing in half MS and low callusing in Nitsch and Nitsch-White media. But, only the callus produced in half MS media showed regeneration potential. Spadices collected from other development stages failed to show callus induction.

4.1.2.1.3 Petiole explants

Among the 70 treatment combinations tried, callus induction with petiole explants was noticed only in 6 treatments. However, response was noticed only in 5 treatment combinations for seed explants (Table 21). As in the previous cases, in petiole- and seed-explants also lower concentration of auxin over cytokinin favoured callus induction. In the case of petiole explants, maximum per cent of cultures inducing callus (61.00) was recorded by the treatment combination NW + 2,4-D 0.5 mgl^{-1} + kinetin 0.75 mgl^{-1}. The lowest per cent of

Table 19. Effect of various treatments on callus induction from **spadix explants** of *A. andreanum* cv. Dragon's tongue

Treatment combination* (Media + growth regulator mg l^{-1})	Cultures initiating callus (%)	Days for callus induction
MS + 2,4-D 0.08 + KN 0.3	58.18	52.00
MS + 2,4-D 0.3 + KN 0.5	59.85	57.33
MS + 2,4-D 0.5 + KN 0.5	51.01	64.66
Nitsch + 2,4-D 0.08 + KN 0.5	35.02	81.66
NW + 2,4-D 0.1 + KN 0.3	42.09	67.00
NW + 2,4-D 0.3 + KN 0.5	45.83	67.00
NW + 2,4-D 0.5 + KN 0. 5	43.70	60.00
CD (0.05)	5.82	6.06

Number of replications =Three (one replication = 10 culture tubes)
NW - Inorganic salts of Nitsch and White's vitamins

cultures inducing callus (35.41) was recorded by the treatment combination MMS + 2,4-D 0.3 mgl^{-1} + kinetin 0.5 mgl^{-1}. Among the different culture media tried, NW media showed the maximum response (61.00%) and MMS recorded the minimum response (35.41%). Half MS and B_5 media failed to induce callus in petiole explants. Days taken for callus induction was minimum (61.00) in the treatment combination NW + 2,4-D 0.5 mgl^{-1} + kinetin 0.75 mgl^{-1} compared to 85.00 days in case of explants cultured onto N + 2,4-D 0.5 mgl^{-1} + kinetin 0.3 mgl^{-1}.

4.1.2.1.4 Seed explants

Seed explants did not show callusing when cultured onto MMS media. Explant cultured onto NW media showed more cultures showing callus induction than those cultured on to Nitsch media. Maximum per cent of callus induction (35.33) was recorded by NW + 2,4-D 0.5 mg l^{-1} + kinetin 0.75 mg l^{-1} (Table 21). Other growth regulator combinations on Nitsch or NW media recorded a callus induction per centnin the range of 31-33 per cent.

Days taken for callus induction were almost the same (61.33) as in the case of petiole explants. The treatment which was most effective in early callus induction (61.33 days) was NW + 2,4-D 0.5 mg l^{-1} + kinetin 0.75 mg l^{-1}. This treatment combination was significantly superior to other treatments for both per cent callus induction as well as days taken for callus induction from petiole and seed explants.

Table 20 Behaviour of floral explants as influenced by the stage of harvest Of spadicess

Stage of harvest	½MS		Nitsch		Nitsch + White	
	Degree of callusing *	Regene-ration**	Degree of callusing *	Regene-ration**	Degree of callusing *	Regene-ration**
Tightly coiled spathe	-	-	-	-	-	-
Coiled spathe	+++	R	+++	NR	+++	NR
Partially opened	++	R	+	NR	+	NR
Cupped spathe	-	-	-	-	-	-
Fully opened spathe	-	-	-	-	-	-
Near to seed set	-	-	-	-	-	-

* 2,4-D 0.30 mg l^{-1} + kinetin 0.5 mg l^{-1}
** Kinetin 0.5 mg l^{-1} + BA 0.5 mg l^{-1}
+ Low callusing ++ Medium callusing +++ Intense callusing
(-) No callusing (R) Regeneration (NR) No regeneration

4.1.2.2 In *vitro* derived explants

Out of 43 treatment combinations tried, only 10 recorded positive results in leaf and petiole explants. Here also lower level of auxin over cytokinin recorded the better results for per cent callus induction as well as number of days taken for callus induction (Table 22 a and b).

4.1.2.2.1 Leaf explants

Callus induction per cent was maximum (75.33) in the case of leaf explants cultured onto Nitsch + BAP 1.0 mg l^{-1} + 2,4-D 0.1 mg l^{-1} (Table 22a; Plates 4, 5, 6). Minimum number of days taken (51.66) for callus induction was recorded by the treatment combination MMS + BAP 1.0 mg l^{-1} + 2,4-D 0.1 mg l^{-1}. Among the basal media tried, per cent callus induction was maximum (average of 75.33) in Nitsch media but the days taken for callus induction was less in the case of explants cultured onto MMS media (51.77)(Plate 4).

4.1.2.2.2 Petiole explants

Per cent callus induction (82.00) was maximum in the treatment combination NItsch + BAP 1.0 mgl^{-1} + 2,4-D 0.1 mgl^{-1} (Table 22a). Minimum per cent callus induction (62.66) was recorded by the treatment combination MMS + BAP 1.0 mgl^{-1} +2,4-D 0.5 mgl^{-1}. Days taken for callus induction was minimum (47.00) in the treatment combination Nitsch + kinetin 1.0 mgl^{-1} + 2,4-D 0.5 mgl^{-1}.

Table 21. Effect of different treatments on callus induction from **petiole** and **seed explants** of *A. andreanum* cv. Dragon's Tongue

Treatment (Media + growth regulator mg l^{-1})	Petiole		Seed	
	Callus induction (%)	Days taken for callus induction	Days taken for callus induction	Callus induction (%)
MMs + 2,4-D 0.5 + KN 0.5	41.15	74.00	-	-
MMs + 2,4-D 1.0 + KN 0.5	35.41	82.00	-	-
N + 2,4-D 0.5 + KN 0.3	45.20	85.00	67.66	31.60
NW + 2,4-D 0.5 + KN 0.5	47.67	75.67	72.00	31.66
NW + 2,4-D 0.3 + KN 0.5	58.51	70.00	76.66	32.94
NW + 2,4-D 0.5 + KN 0.75	61.00	61.00	61.33	35.33
CD (0.05)	15.16	20.86	11.74	24.77

Replication = Three (3) (One replication = 10 culture tubes)
NW - Inorganic salts of Nitsch and Whites' vitamins

Plate 5. Profuse growth of callus on subculturing

Plate 6. Formation of shoot initials in callus culture towards the end of culture cycle

And the maximum days taken (64.33) for callus induction was recorded by MMS + BAP 1.0 mgl^{-1} + 2,4-D 0.25 mgl^{-1}.

4.1.2.2.3 Nodal explants

Out of the 43 treatment combinations tried, only 10 combinations recorded good callusing. Here also lower auxin level over cytokinin favoured callus induction (Table 22b). Per cent callus induction was maximum (78.66) in the treatment combination Nitsch + kinetin 1. 0 mgl^{-1} + 2,4-D 0.5 mgl^{-1}. Minimum per cent of callus induction (60.33) was recorded by the treatment combination MMS + BAP 1.0 mgl^{-1}+ 2,4-D 0.1 mgl^{-1}. The maximum number of days (72.33) for callusing was recorded by Nitsch + BAP 1.0 mgl^{-1} +2,4-D 0.5 mgl^{-1}.

4.1.2.2.4 Root explants

Root explants from aseptic cultures showed maximum callus induction (81.00%) when cultured onto Nitsch + BAP 1.0 mg l^{-1} + 2,4-D 0.1 mg l^{-1}, which took 68.00 days for callus induction (Table 22b; Fig 1; Plate 7). However, the minimum number of days (59.33) for callus induction from aseptic root explants was recorded by the treatment combination MMS + BAP 1.0 mg l^{-1} + 2,4-D 0.1 mg l^{-1}. Per cent callus induction was minimum (71.00) in the treatment combination Nitsch + kinetin 1.0 mgl^{-1} + 2,4-D 0.5 mgl^{-1}

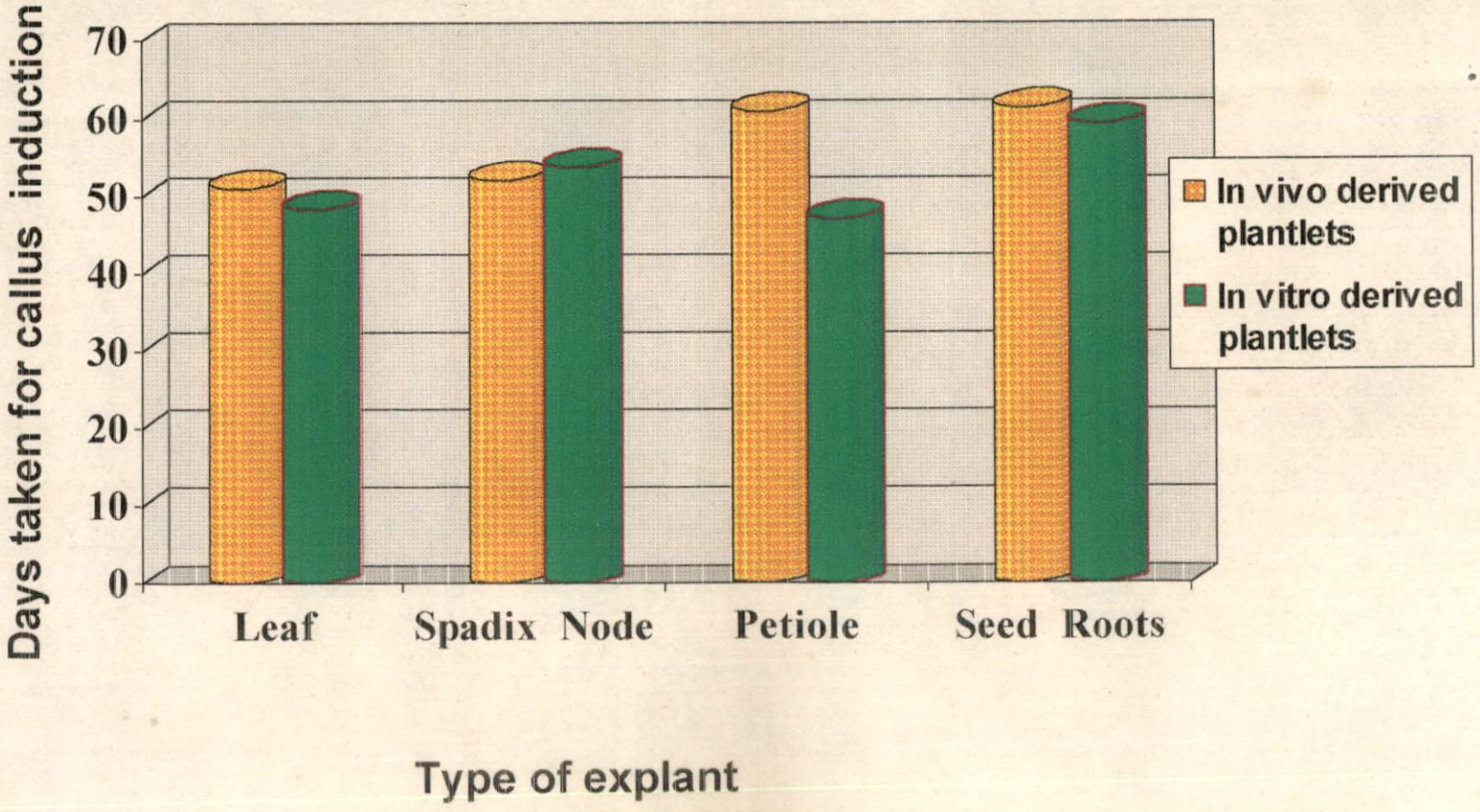
Table 22a Effect of different plant growth regulators on callus induction from ***in vitro derived leaf and petiole*** **explants** of *A. andreanum* cv. Dragon's Tongue

Treatment (Media + growth regulator mg l^{-1})	Leaf		Petiole	
	Callus induction (%)	Days taken for callus induction	Callus induction (%)	Days taken for callus induction
MMS + BAP 1.0 + 2,4-D 0.1	65.67	51.66	72.00	48.67
MMS + BAP 1.0 + 2,4-D 0.25	40.66	52.33	68.00	63.00
MMS + BAP 1.0 + 2,4-D 0.5	39.00	61.34	62.66	64.33
MMS + KN 1.0 + 2,4-D 0.1	67.33	55.67	74.33	58.66
MMS + KN 1.0 + 2,4-D 0.5	72.00	54.33	80.33	51.33
Nitsch + BAP 1.0 + 2,4-D 0.1	75.33	68.66	82.00	51.00
Nitsch + BAP 1.0 + 2,4-D 0.25	58.00	72.00	78.33	62.00
Nitsch + BAP 1.0 + 2,4-D 0.5	60.67	73.33	71.00	61.66
Nitsch + KN 1.0 + 2,4-D 0.1	67.33	57.30	74.67	50.00
Nitsch + KN 1.0 + 2,4-D 0.5 + glutamine 100 mg l^{-1}	67.33	48.36	80.33	47.00
CD (0.05)	6.46	4.75	3.30	3.63

Number of replication = Three (3) (One replication = 10 culture tubes)
MMS - Modified Murashige and Skoog (1962) medium

Figure 1

Influence of explants on the number of days taken for callus induction



4.1.2.3 Effect of various treatments on the degree of callusing from the different explants

4.1.2.3.1 *In vivo* explants

In the case of leaf explants, intense callusing was observed (Table 23) in the treatment combination NW + 2,4-D 0.3 mgl^{-1} + kinetin 0.5 mgl^{-1} and NW +2,4-D 0.5 mgl^{-1} + kinetin 0.75 mgl^{-1}. Callus produced was pale green in color and globular in shape. The callus produced showed a good regeneration potential.

Medium callusing (Table 23) was observed with petiole explants in the treatment combination NW + 2,4-D 0.5 mgl^{-1} + kinetin 0.75 mgl^{-1}). The callus produced was pale yellow and friable. But, the callus didnot show the potential for regeneration.

4.1.2.3.2 In *vitro* explants

Among the explants tried root explants showed (Table 24) maximum intense callusing in the treatment combinations Nitsch + kinetin 1.0 mgl^{-1} + 2,4-D 0.1 mgl^{-1} and Nitsch + kinetin 1.0 mgl^{-1} +2,4-D 0.25 mgl^{-1}. Intense callusing was also noted in leaf explants from (Nitsch + kinetin 1.0 mg l^{-1} + 2,4-D 0.25 mgl^{-1}) and petiole (Nitsch + kinetin 1.0 mgl^{-1} + 2,4-D 0.1mgl^{-1}). The callus produced in all the treatments above showed good shoot regeneration potential. When concentration of auxin was increased over the concentration of cytokinin (kinetin 1.0mgl^{-1}) all the explants produced low callusing and they showed rhizogenesis.

Table 22b. Effect of different plant growth regulators on callus induction from ***in vitro* derived root and single node explants** of *A. andreanum* cv. Dragon's Tongue

Treatments (Media + growth regulator mg l^{-1})	Single node		Root	
	Callus induction (%)	Days taken for callus induction	Callus induction (%)	Days taken for callus induction
MMS + BAP 1.0 + 2,4-D 0.1	72.00	53.66	77.66	59.33
MMS + BAP 1.0 + 2,4-D 0.25	60.33	61.00	79.66	72.33
MMS + BAP 1.0 + 2,4-D 0.5	60.66	66.00	75.66	69.66
MMS + KN 1.0 + 2,4-D 0.1	68.00	60.66	75.00	76.33
MMS + KN 1.0 + 2,4-D 0.5	71.00	63.66	75.66	60.00
Nitsch + BAP 1.0 + 2,4-D 0.1	72.00	69.00	81.00	68.00
Nitsch + BAP 1.0 + 2,4-D 0.25	72.00	69.66	79.00	66.00
Nitsch + BAP 1.0 + 2,4-D 0.5	62.00	72.33	72.33	64.66
Nitsch + KN 1.0 + 2,4-D 0.1	78.00	55.33	76.00	66.66
Nitsch + KN 1.0 + 2,4-D 0.5	78.66	54.66	71.00	60.66
CD (0.05)	3.57	3.54	3.03	3.03

Number of replication = Three (3) (One replication = 10 culture tubes)
MMS - Modified Murashige and Skoog (1962) medium

Plate 7. Callus induction from *in vitro* root explant

Plate 8. Shoot regeneration from callus derived from leaf

Table 23 Behaviour of floral explants as influenced by the stage of harvest

Stage of harvest	2MS		Nitsch		Nitsch -White	
	Degree of callusing *	Regeneration**	Degree of callusing *	Regeneration**	Degree of callusing *	Regeneration**
Tightly coiled spathe	-	-	-	-	-	-
Coiled spathe	+++	R	+++	NR	+++	NR
Partially opened	++	R	+	NR	+	NR
Cupped spathe	-	-	-	-	-	-
Fully opened spathe	-	-	-	-	-	-
Near to seed set	-	-	-	-	-	-

* 2,4-D 1.0 mg l^{-1} + kinetin 0.3 mg l^{-1}
** Kinetin 0.5 mg l^{-1} + BA 0.5 mg l^{-1}
+ Low callusing ++ Medium callusing +++ Intense callusing
(-) No callusing (R) Regeneration (NR) No regeneration

Table 24. Effect of different concentrations of 2,4-D in combination with kinetin 1.0 mg l^{-1} on the degree of callusing from different *in vitro* derived explants

Media - Nitsch

1.0 mg l^{-1} kinetin + 2,4-D mg l^{-1}	Petiole	Node	Leaf	Root
0.10	+++(R)	++ (R)	++ (R)	+++ (R)
0.25	++(R)	+ (R)	+++ (R)	+++(R)
0.50	+	+	+	++ (R)
1.00	+	+	+	++
2.00	++ (E)	++	++ (E)	+
4.00	+(Rr)	+(Rr)	+(Rr)	+ (Rr)

(+) Low callusing (++) Moderate callusing (+++) Good callusing
(R) Regeneration of shoot (E) Embryogenic (Rr) Regeneration or root

Except nodal explants, all the others from aseptic cultures (leaf, petiole and root) showed good callusing. Leaf and petiole explants produced embryogenic callus in the treatment combination, Nitsch + 2,4-D 2.0 mg l^{-1} + kinetin 0.5 mg l^{-1}. Among the *in vitro* explants, maximum callus multiplication was exhibited by the root explants (Table 24).

4.1.2.4 Effect of media on callus proliferation

Among the *in vivo* explants tried only leaf and spadix explants performed better in terms of proliferation of callus (Table 25). In the case of leaf explants, cultures initiating callus was maximum (86.5%) with NW media. Growth score (3.91) and callus index (338.21) was also maximum in NW media. The lowest growth score (1.41) and callus index (60.85) was recorded by half MS media. In the case of spadix explants, cultures initiating callus was maximum (58.15 %) in half MS media, and the lowest (35.02%) in Nitsch media. The growth score also, showed a similar trend with maximum (3.6) in half MS and minimum in Nitsch media.

Callus induction was maximum at 0.1 mg l^{-1} of 2,4-D in all the *in vitro* explants tried - leaf (90.18%), petiole (95.91%), node (83.50%) and roots (90.92%) (Table 26). It was seen that, addition of glutamine 200 mg l^{-1} had a positive effect on callus induction. The treatment combination Nitsch + 2,4-D 0.1 mg l^{-1} + kinetin 1.0 mg l^{-1} recorded the maximum per cent callus induction in leaf, petiole, node and root explants (67.33, 74.67, 78.00 and 76.00 respectively). The addition of glutamine increased further the per cent callus induction to

Table 25. Effect of culture media on callus initiation and proliferation

Growth regulators - 2,4-D 0.3 mg l^{-1} + kinetin 0.5 mg l^{-}

Culture media	Leaf			Spadix		
	Cultures initiating callus (%)	Growth score*	Callus index (%)	Culture initiating callus (%)	Growth score	Callus index (%)
½ MS	43.16	1.41	60.85	58.15	3.60	209.44
MMS	74.66	2.16	161.26	-	-	-
Nitsch	82.00	3.56	291.92	35.02	1.00	35.02
NW	86.50	3.91	338.21	53.70	2.31	124.05
Mean	71.58	2.76	213.06	48.96	2.30	122.80

MMS - Modified Murashige and Skoog (1962) medium
NW - Inorganic salts of Nitsch and Whites' vitamins
Number of replications =Three (one replication = 10 culture tubes) growth score

Table 26. Effect of culture media on callus initiation and proliferation from *in vitro* derived explants

Growth regulators - BAP 1.0 mg l^{-1} + 2,4-D 0.1 mg l^{-1}

Explants	MMS			Nitsch		
	Cultures initiating callus (%)	Growth score*	Callus index (%)	Cultures initiating callus (%)	Growth score	Callus index (%)
Leaf	65.67	2.45	160.85	75.33	3.00	226.00
Petiole	72.00	2.80	201.60	82.00	3.24	265.68
Node	72.00	1.91	137.52	72.00	2.00	144.00
Root	77.66	3.10	240.74	81.00	3.50	283.35
Mean	71.83	2.57	185.17	77.58	2.94	229.79

MMS - Modified Murashige and Skoog (1962) medium
Number of replications=Three (One replication = 10 culture tubes)

Table 27. Callus induction as influenced by different concentrations of 2,4-D in combination with kinetin (1 mg l^{-1}) in *in vitro* derived explants

	Media -Nitsch + 200 mg l^{-1} glutamine + Kinetin 1.0 mg l^{-1} Callus induction (%)			
Treatment 2,4-D mg l^{-1}	Leaf	Petiole	Node	Root
0.1(without glutamine)	67.33	74.67	78.00	76.00
0.1	90.18	95.91	83.50	90.92
0.25	86.47	77.83	75.26	83.75
0.50	74.84	74.96	76.08	80.83
1.00	72.48	75.45	76.20	77.08
2.00	40.92	31.61	53.13	49.62
CD (0.05)	14.74	17.89	8.69	11.94

Number of replications = Three (one replication = 10 culture tubes)

Table 28 Shoot regeneration from leaf explant derived calli as influenced by different auxin and cytokinins and their combinations

			Media - Nitsch		
Growth regulators (mg l^{-1})	Shoot regeneration (%)	Days for shoot induction	Mean number of rootable shoots/culture	Length of longest shoot (cm)	Width of largest leaf (cm)
BAP 0.1	55.60	40.10	7.67	4.07	0.48
0.2	71.00	42.00	8.67	4.13	0.62
0.5	92.00	46.00	12.30	4.00	0.62
1.0	88.00	36.60	12.33	3.27	0.62
1.5	77.00	39.67	11.66	3.77	0.60
Kinetin 0.1	48.33	42.66	7.33	4.27	0.50
0.2	56.66	38.00	7.33	4.43	0.45
0.5	83.00	45.00	9.33	3.86	0.48
1.0	75.67	35.33	8.67	3.66	0.40
1.5	72.33	41.00	8.33	3.13	0.41
BAP 1.0 + IAA 2.0	54.00	45.33	13.00	4.53	0.34
BAP 1.5 + IAA 2.0	66.67	50.00	12.67	4.70	0.36
Kinetin 1.0 + IAA 2.0	55.33	55.33	7.33	3.66	0.41
Kinetin 1.5 + IAA 2.0	60.00	60.65	9.33	3.56	0.35
Kinetin 1.0 + BAP 1.0 + IAA 2.0	46.66	60.65	6.00	3.03	0.28
CD (0.05)	6.15	3.41	1.03	0.23	0.001

Number of replications = Three (3)
(One replication = 10 culture tubes)
Observation taken after 12 weeks of subculturing of callus

90.18, 95.91, 83.50 and 90.92 for leaf, petiole, node and root explants respectively. In all the explants tried, the lowest per cent of callus induction was recorded at 2,4-D 2.0 mg l^{-1}. It is evident from the data (Table 26) that increasing the concentration of auxin over cytokinin had a negative effect on callus induction.

Overall performance of *in vitro* explants(Table 27) in Nitsch media was better (mean CI =229.79) than in MMS media (mean CI = 229.79). Maximum callus index was recorded by root explants (283.35) in Nitsch media followed by petiole explants (265.68).

4.1.2.5 Shoot regeneration from callus

4.1.2.5.1 Effect of growth regulators

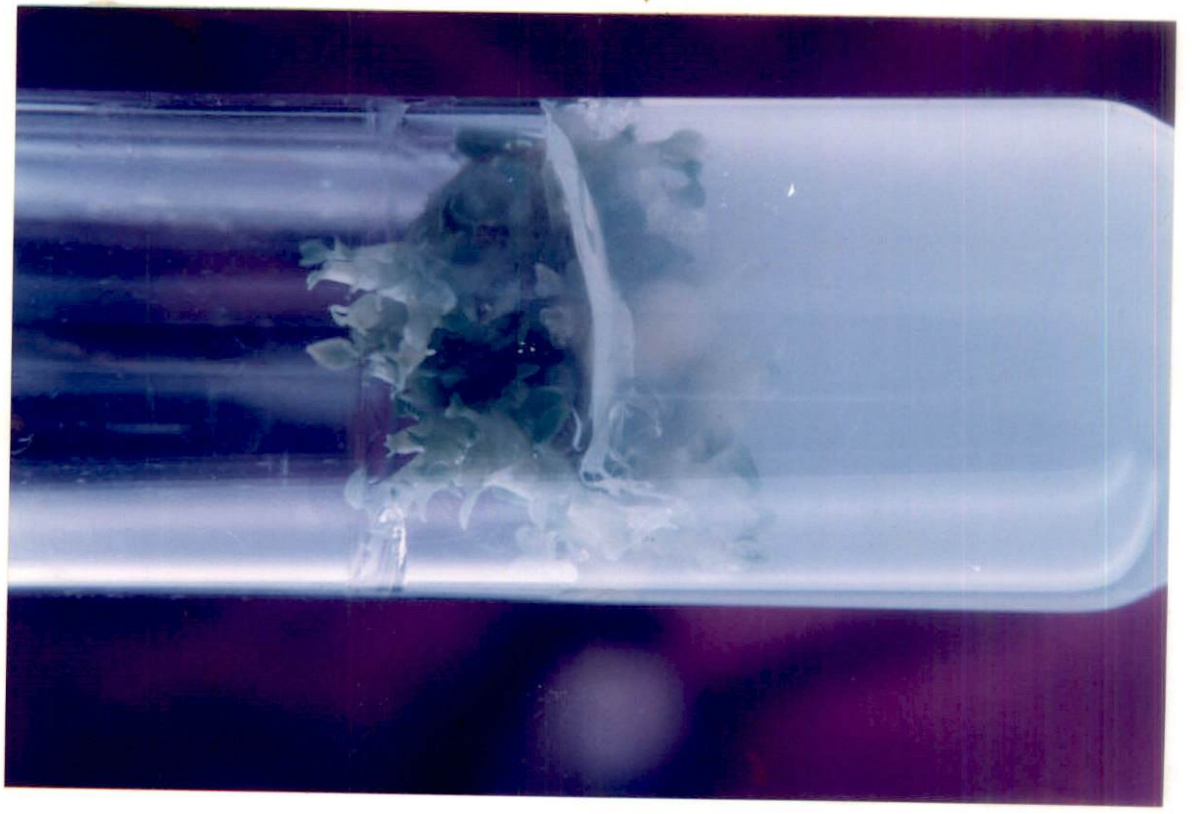
4.1.2.5.1.1 *In vivo* explants

4.1.2.5.1.1.1 Leaf explants

Out of 79 treatment combinations tried for shoot regeneration, success was obtained only in 15 treatment combinations (Table 28; Plate 8). Maximum shoot regeneration (92%) was observed in the treatment BAP 0.5 mg l^{-1} and minimum (66%) in the treatment combination of cytokinins with auxin (BAP 1.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + IAA 1.0 mg l^{-1}). Shoot regeneration was better when cytokinins used alone than in combination with auxin, as evident from the data that the treatment combination BAP 1.0 mg l^{-1} + IAA 2.0 l^{-1} and kinetin 1.0 mg l^{-1} + IAA 2.0 mg l^{-1} recorded minimum shoot regeneration (54.00% and 55.33% respectively). Cytokinins used alone (BAP 1.0 mg l^{-1} and kinetin 1.0 mg l^{-1})

Plate 9. Shoot regeneration from callus induced in spadix explants

Plate 10. *In vitro* regenerated anthurium plant with well developed leaves and roots



recorded the maximum shoot regeneration (92.8% and 83.00% respectively) among the different treatments tried(Table 28).

The least number of days for shoot regeneration (35.33) was recorded in the treatment kinetin 1.0 mg l^{-1} whereas kinetin 1.0 mg l^{-1} + BAP 1.0 mg l^{-1} + IAA 1.0 mg l^{-1} took the maximum number of days (60.65) for shoot regeneration (Table 28). Days taken for shoot regeneration was less when cytokinin was used alone than in combination with auxin, which is evident from the data that BAP 1.0 mg l^{-1} + IAA 2.0 mg l^{-1} took 45.33 days for shoot regeneration as against 36.60 days taken by BAP 1.0 mg l^{-1}. Number of rootable shoots/culture was maximum (13.00) in the treatment combination BAP 1.0 mg l^{-1} + IAA 2.0 mg l^{-1} and was minimum (6.00) in the case of callus cultured on to kinetin 1.0 mg l^{-1} + BAP 1.0 mg l^{-1}. Number of shoots produced also was better in BAP (12.33) and BAP (13.00) combinations than in kinetin (8.67) and its combinations (7.33).

The length of shoots was maximum (4.70 cm) in the treatment combination BAP 1.5 mg l^{-1} + IAA 2.0 mg l^{-1} and minimum (3.03 cm) in BAP 1.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + IAA 1.0 mg l^{-1}. Different concentrations of BAP recorded the maximum width of leaf (0.62 cm) and the treatment combination BAP 1.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + IAA 1.0 mg l^{-1} recorded the lowest width of leaf (0.62 cm).

All the treatment combinations with GA_3 resulted in yellowing of the green callus and no regeneration occurred.

4.1.2.5.2 Effect of media

Culture media significantly influenced the shoot regeneration from callus (Table 29). Maximum shoot regeneration was observed in Nitsch media, which recorded the least number of days (45) for shoot regeneration as well as maximum number of shoots (12.30). The next best media for shoot regeneration was found to be Nitsch + White (83.00%) followed by half (80.10%). Shoot regeneration was minimum (64.90%) in half MS media, which took 62.67 days for shoot regeneration and the only 6.97 shoots.

4.1.2.5.3. Effect of different treatments on shoot regeneration from spadix derived callus

Out of the 40 treatment combinations tried, only 11 treatment combinations were recorded successful in shoot regeneration. In the case of spadix explants a combination of cytokinins recorded better effect on shoot regeneration than they are used individually. Shoot regeneration per cent was maximum (71.42) in the treatment combination BAP 2.0 mg l^{-1} + kinetin 2.0 mg l^{-1} and minimum (24.55) in the treatment Nitsch + BAP 0.5 mg l^{-1}. The number of days taken for shoot regeneration was maximum (67.41) in the treatment BAP 1.5 mg l^{-1} but lowering the concentration of BAP (0.5 mg l^{-1}) and the strength of the media(half MS) reduced the time taken for (40.33 days) for regeneration of shoots. Maximum number of shoots (14.50) was recorded in the treatment combination BAP 2.0 + kinetin 2.0 mg l^{-1} . The shoots with minimum length (2.90 cm) was observed in Nitsch media supplemented with BAP 0.2 mg l^{-1}. Width of the longest leaf (0.55 cm) was maximum in half MS media with BAP 1.0 mg l^{-1}

Table 29. Shoot regeneration from leaf explants derived callus as influenced by different basal media

Growth regulator - BAP 0.5 mg l^{-1}

Media	Shoot regeneration (%)	Days taken for shoot regeneration	Mean number of rootable sprouts/culture
1/2MS	64.90	62.67	6.97
MMS	80.10	50.33	10.33
Nitsch	92.00	45.00	12.30
Nitsch + White	83.00	49.98	10.69
B5 + M	65.07	55.00	7.66
CD (0.05)	9.20	5.10	1.83

Number of replications = Three (3)
(One replication = 10 culture tubes)
B5 + M = Inorganic salts of B5 + MS vitamin

Table 30. Effect of different treatments on shoot regeneration from callus obtained from spadix explants

Treatment (mg l^{-1})	Shoot regeneration (%)	Days for shoot regeneration	Mean No.of rootable Shoots/ culture	Length of longest shoot (cm)	Width of largest leaf (cm)
MMS + BAP 0.5	55.46	61.76	7.62	3.50	0.45
MMS + BAP 1.0	36.00	65.42	8.00	3.20	0.48
MMS + BAP 1.5	41.52	67.41	8.42	3.20	0.45
□MS + BAP 0.5	59.91	49.50	8.50	4.00	0.47
□MS + BAP 1.0	64.35	47.21	9.00	3.50	0.47
□MS + BAP 1.5	52.46	40.33	8.30	3.67	0.45
MMS + BAP2.0 + Kinetin 2.0	67.21	42.56	10.21	4.20	0.55
□MS + BAP 2.0 + kinetin 2.0	71.42	45.00	14.50	4.50	0.50
Nitsch + BAP 0.1	25.60	60.00	7.34	3.70	0.39
Nitsch + BAP 0.2	26.00	59.91	8.25	2.90	0.42
Nitsch + BAP 0.5	24.55	62.00	8.50	3.67	0.42
CD (0.05)	12.31	0.26	1.86	0.43	0.04

Number of replications = Three (3)
(One replication = 10 culture tubes)
Observation taken after 12 weeks of subculturing callus

Table 31. Leaf enlargement in *in vitro* derived shoots as influenced by different basal media

Growth regulator - BAP 0.5 mg l^{-1}

Basal Media	Number . of shoots/culture	Length of largest shoot (cm)	Width of largest leaf (cm)	Number of leaves
MS	6.16	2.76	0.36	3.33
□MS	8.96	3.23	0.46	3.66
Nitsch	15.90	4.63	0.63	4.33
NW	11.00	3.96	0.56	4.33
CD (0.05)	3.68	0.72	0.09	0.62

Number of replications = Three (3)
(One replication = 10 culture tubes)
NW - Inorganic salts of Nitsch + White's vitamins

Plate 11. Effect of culture vessel (conical flask) on leaf enlargement

Plate 12. *Ex vitro* developmental stages of micropropagated anthurium plantlets

+ kinetin 1.0 mg l^{-1} and minimum (0.39 cm) in Nitsch media fortified with BAP 0.1 mg l^{-1}.

Data presented in Table 30 reveals that shoot regeneration is influenced by the culture media used. Among the different basal media used, MS media showed better response than MMS and Nitsch media. A comparison of the number of days taken for callus induction using BAP 0.5 mg l^{-1} revealed that it was minimum in half MS media (49.50) compared to MMS(55.46) and Nitsch(62.00). Number of shoots produced was also maximum (14.50) in half MS compared to MMS media (10.21). Response to shoot regeneration treatments using NW media and B_5 media was found to be negative.

4.1.2.6 Leaf enlargement

4.1.2.6.1 Effect of media

Number of shoots per culture was maximum (15.90) in Nitsch media (Table 31) which recorded maximum length of shoot (4.63 cm), width of leaf (0.63 cm) and the number of leaves (4.33). It was found that there was a significant difference between the culture media on their effect on leaf enlargement in *in vitro* regenerated plantlets. Effect of Nitsch media was more pronounced than MS media which recorded less number of rootable shoots/culture (6.16) length of shoot (2.76 cm), width of leaf (0.36 cm) and number of leaves (3.33).

4.1.2.6.2 Effect of carbon source

Higher concentration of sucrose (20 gl^{-1}) and lower concentration of glucose (10 gl^{-1}) (Table 32) in combination recorded the maximum length of shoot (4.36 cm) and width (0.68 cm) of leaf. Number of leaves produced per shoot was maximum (4.66) in sucrose 30 gl^{-1}. But, when concentration of glucose in glucose-sucrose combination was (20 gl^{-1}) increased over sucrose (10 gl^{-1}) length of leaf (3.03 cm), number of leaves (3.66) and width of leaves (0.56 cm) reduced. When sucrose and glucose were used independently, effect of sucrose was better than glucose and 30 gl^{-1}sucrose recorded a shoot length of 4.03 cm, 4.66 leaves and a leaf width of 0.46 cm. Where as, glucose recorded only a shoot length of 2.26 cm, 2.66 leaves and a leaf width of 0.36 cm. Among the treatments tried, a combination of sucrose (20 g gl^{-1}) glucose (10 gl^{-1}) gave the best response.

4.1.2.6.3 Effect of media supplements

Treatment involving glutamine 200 mg l^{-1} (Table 33) recorded the maximum length of shoot (5.03 cm), number of leaves (4.33) and largest width of the leaf (0.63 cm). Casein hydrolysate (100-200 mg l^{-1}) recorded a length of shoot ranging from 3.21 to 4.20 cm and width of the largest leaf ranged from 0.33 cm to 0.41 cm. Fortifying the culture media with coconut water resulted in a shoot length ranging from 2.36 to 3.43 cm and the width of the leaf ranging from 0.43 to 0.50 cm. Length of the shoot was minimum (2.36) in the treatment with coconut water 15%. Width of the leaf was lowest (0.33 cm) in the treatment with casein hydrolysate 200 mg l^{-1}.

Table 32. Leaf enlargement in micropropagated shoots as affected by carbon sources

Media - Nitsch + BAP 0.5 mg l^{-1}

Carbon source	Concentration (g l^{-1})	Length of longest shoot (cm)	Number of leaves	Width of largest leaves (cm)
Sucrose	20	3.16	3.33	0.31
	30	4.03	4.66	0.46
	40	2.60	2.33	0.36
Glucose	20	2.26	2.66	0.33
	30	2.06	2.66	0.36
	40	1.96	2.33	0.31
Sucrose + glucose	20 + 10	4.36	4.33	0.68
Sucrose + glucose	10 + 20	3.03	3.66	0.56
CD (0.05)		0.54	0.59	0.08

Number of replications = Three (3)
(One replication = 10 culture tubes)

Table 33. Influence of media supplements on leaf enlargement in micropropagated shoots

Growth regulator = BAP 0.5 mgl^{-1}

Basal media : Nitsch

Media supplements	Concentration ($mg\ l^{-1}$)	Length of largest shoot (cm)	Number of leaves	Width of largest leaf (cm)
Glutamine	100	4.40	3.33	0.53
	200	5.03	4.33	0.63
	400	4.53	4.33	0.53
Casein hydrolysate	100	4.20	3.33	0.35
	150	3.21	3.33	0.41
	200	4.16	3.66	0.33
Coconut water (%)	10	3.43	3.33	0.45
	15	2.36	3.66	0.43
	20	3.03	3.66	0.50
CD (0.05)		0.48	0.35	0.05

Number of replications = Three (3)
(One replication = 10 culture tubes)

4.1.2.6.4 Effect of culture vessels

Among the culture vessels tested maximum width of the leaf (0.80 cm) was in shoots cultured in conical flask 200 ml(Table 34). Shoots cultured in culture bottles also recorded better leaf width (0.74 cm), but culture survival was very poor (28.44%). Number of shoots produced/culture was also maximum (22.00) in 200 ml conical flask followed by 100 ml conical flask (17.33)(Fig 2 & 3). However, culture survival was maximum (88.67%) in test tubes.

Spontaneous rooting of shoots occurred when shoots were allowed to remain in culture for 2-3 weeks. so separate rooting treatments were not required.

4.1.2.7 Hardening

4.1.2.7.1 Effect of containers and planting method on establishment of plantlets

Minipots (mud) recorded the maximum establishment (Table 35) after two weeks of planting out (80.66% in individual planting and 85.33% in cluster planting). *Ex vitro* establishment of plantlets was the least(7.33%) when cluster planting was done in sachet. None of the plantlets survived cluster planting in protray. Height of the plantlet after two weeks was maximum in the case of those planted in mud pots(5.57 cm in single planting and 5.17 cm in cluster planting). After 6 weeks also, the same pattern of results was observed. Width of the leaf was also maximum in the case of plantlets in mini pots under both single- and cluster– planting.

Table 34. Effect of different culture vessels on foliar development in micropropagated shoots

Culture media - Nitsch + BAP 0.2 mg l^{-1}

Culture vessels	Per cent survival of cultures	Number of shoots/culture	width of the largest leaf (cm)
Test tubes	88.67	11.66	0.68
Conical flask			
100 ml	84.16	17.33	0.73
200 ml	79.66	22.00	0.80
Culture bottles	28.44	11.30	0.74
CD (0.05)	24.00	4.56	0.06

Number of replications = Three (3)
(One replication = 10 culture tubes)

Figure 2

Effect of culture vessels on the production of multiple shoots *in vitro*

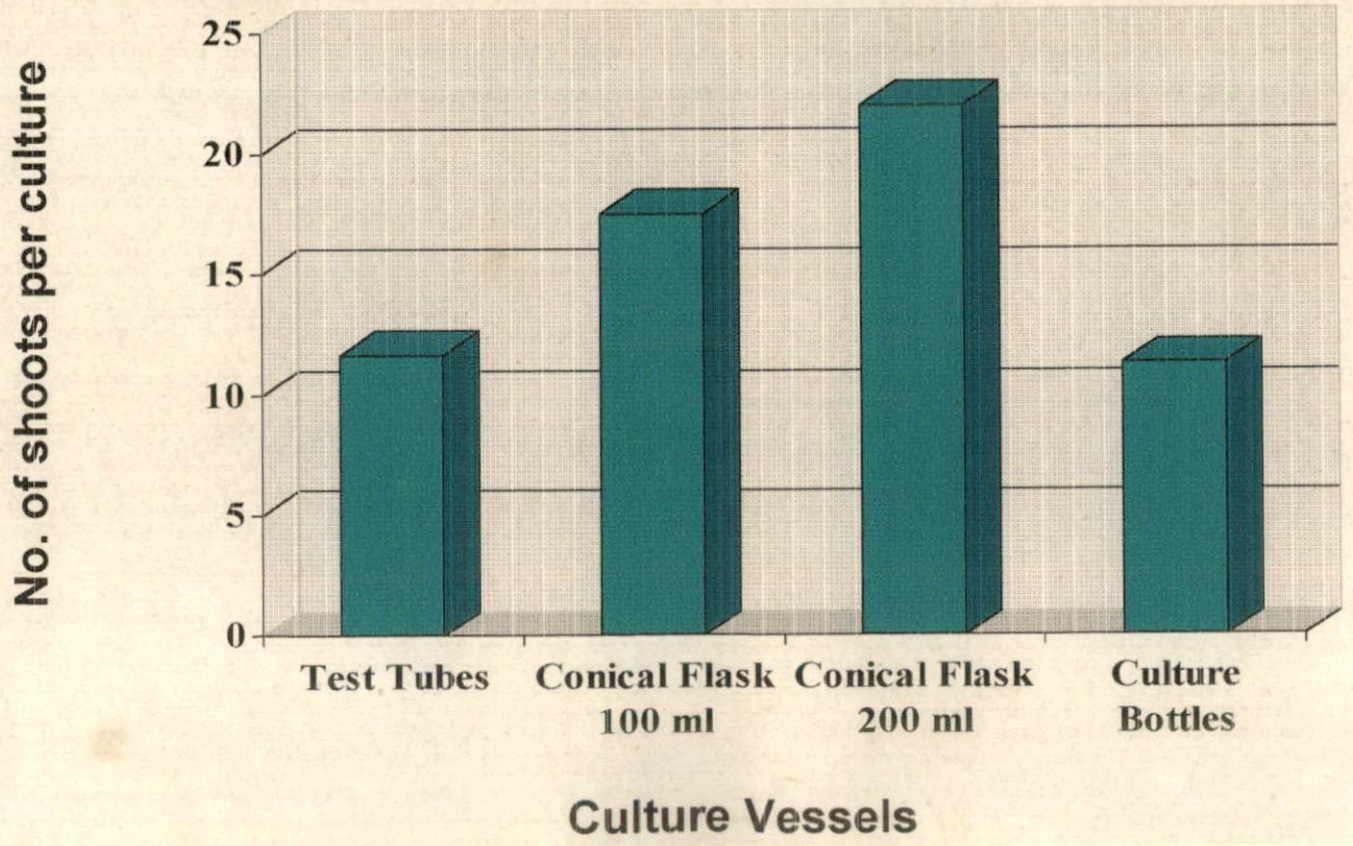


Figure 3

Width of leaf of *in vitro* regenerated plantlets as influenced by the culture vessels

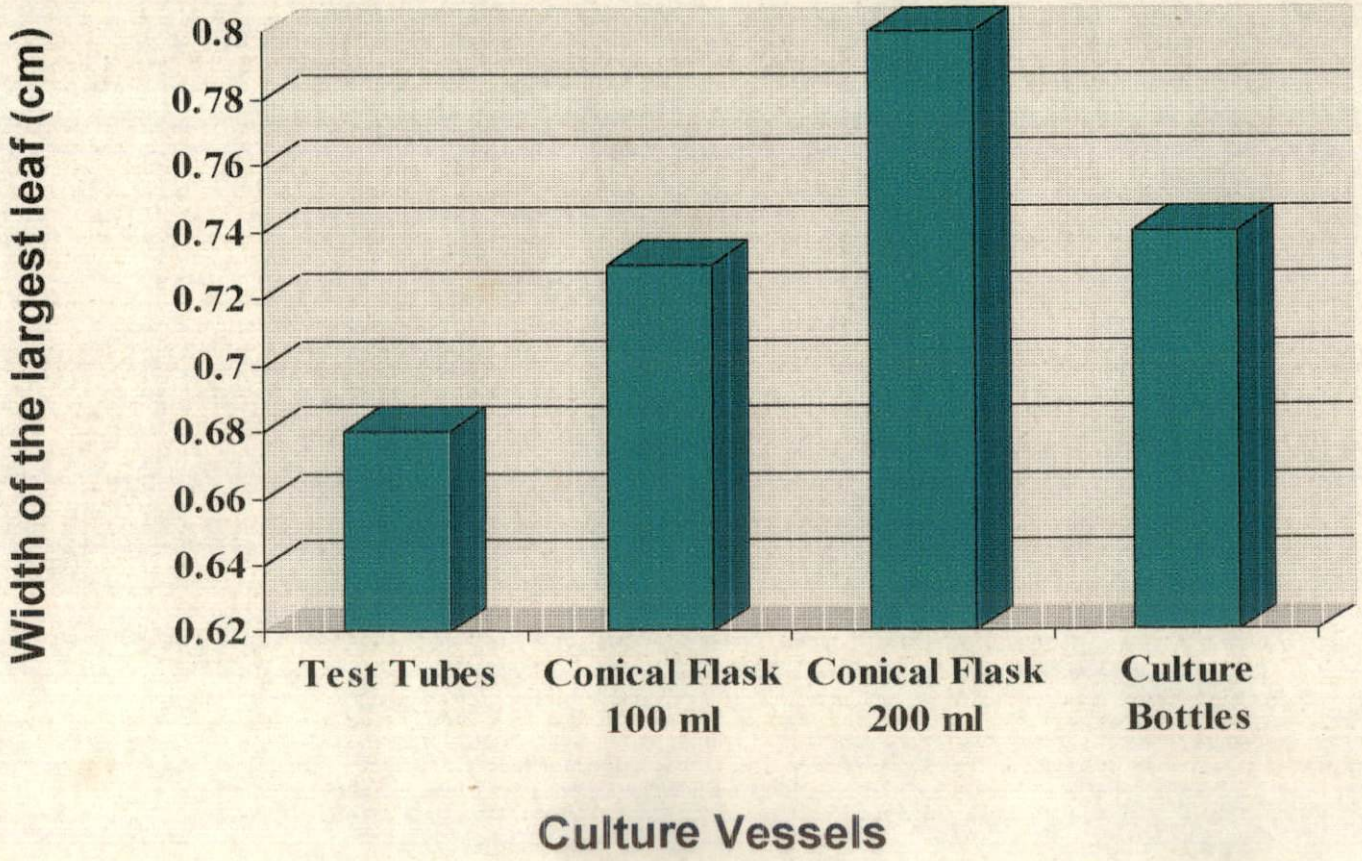


Plate 13. Well established anthurium plants after hardening

Plate 14. Effect of incorporation of VAM (*Glomus sp.*) into potting mixes on establishment of plantlets *ex vitro*
Potting mix without VAM (Left) *and* with VAM (Right)

Table 35 Growth and development of *in vitro* regenerated plantlets under different methods of hardening

Potting media = Coarse sand

Type of planting	Containers	No. of plants planted out	Establishment after two weeks (%)	At the time of planting			Two weeks after planting			Six weeks after planting		
				Height (cm)	No.of leaves	Width of leaves (cm)	Height (cm)	No.of leaves	Width of leaves (cm)	Height (cm)	No. of leaves	Width of leaves (cm)
Single plantlets	Minipot (mud)	25	80.66	4	5.33	0.78	5.57	5.67	0.87	5.77	6.33	0.96
	Protray	25	45.34	4.00	5.00	0.75	4.83	5.66	0.80	5.13	5.67	0.90
	Sachet	25	20.67	4.33	5.33	0.77	4.73	5.34	0.87	4.90	6.00	0.88
Cluster planting	Mini pot	10	85.33	4.33	5.33	0.77	5.17	6.00	0.96	5.37	5.66	1.00
	Sachet	10	7.33	4.33	5.33	0.77	4.33	5.33	0.78	4.33	5.33	0.80
CD (0.05)			20.60	0.34	0.38	0.03	0.44	0.40	0.06	0.44	0.44	0.06

All the plantlets were maintained under uniform cultural practices

Planting out stage - 9 months

It was found that (Table 35) for better initial establishment of plantlets cluster planting (85.33%) was better than single-planting However, further growth was better in the case of plantlets in single-planting. Accordingly, plant height was increased to 5.57 cm and 5.77 cm after two- and six –weeks respectively. At the same time, plantlets planted in individual-planting recorded a plant height of only 5.17 cm after two weeks and 5.37 cm after six weeks. Width of the largest leaf was maximum (1.00 cm) in cluster planting after six months as compared to 0.96 cm in individual planting.

4.1.2.7.2 Effect of potting media

Among the different pottting mixes tried for primary establishment of the *in vitro* raised plantlets *extra vitrum*, maximum establishment (93.33%) was observed (Table 36) in the mixture of coarse sand and cocopeat (1:1), which was closely followed by coarse sand inoculated with VAM (*Glomus sp.*) (91%). The growth rate of plantlets was also more in a mixture of coarse sand and cocopeat. The height of the platlets was increased to 5.5 cm after six weeks from 4.5 cm at the time of planting. But, plantlets planted out in in coarse sand showed a growth difference of only 0.58 cm between at the time of planting and 6 weeks after planting. There was no significant influence of potting mixes on the number of leaves produced per plant. However, the maximum width of leaf after six weeks of planting (1.00 cm) was recorded by coarse sand + cocopeat (1:1).

After primary establishment of plantlets extra vitrum, they were transplanted into bigger pots with potting mix recommended for growing of

Table 36 Growth and development of *in vitro* regenerated plantlets as influenced by different potting mixes at stage IV

Potting media	Percent establishment of plantlets after two weeks	At the time of planting			Six weeks after planting		
		Height (cm)	No of leaves	Width of leaves (cm)	Height (cm)	No of leaves	Width of leaves (cm)
Coarse sand	86.67	4.50	5.66	0.78	5.08	6.00	0.86
Coarse sand + Coco peat (1:1)	93.33	4.50	5.33	0.80	5.50	5.67	1.00
Coarse sand inoculated with VAM (*Glomus sp*)	91.00	4.33	5.33	0.77	5.30	5.66	0.90
CD (0.05)	7.02	0.45	0.62	0.03	0.51	0.52	0.17

Plate 15. Effect of method of planting on establishment of plantlets *ex vitro* (single planting Vs cluster planting)

Plate 16. Well established cluster planted plantlets after hardening

bigger plants of anthurium. Evaluation of growth parameters (Table 37) showed that all the plants survived on transplanting to bigger pots. Average height of plants after two- and six-months was 5.97 cm and 20.50 cm respectively. Canopy spread was 16.83 cm after six months of growth. Length of the leaf was more (10.57 cm) than width (5.73 cm) (Table 37). Early suckering was observed in all the plants and produced on an average 2-3 suckers per plant.

4.2 Somatic embryogenesis

4.2.1 Induction of somatic embryoids

4.2.1.1 Explants

The response of the different explants with respect to the induction of somatic embryoids was studied. Three types of explants such as, tender leaves, immature seeds from *ex vitro* growing plants and *in vitro* derived explants (leaves, petiole, single nodes and roots) taken from *in vitro* grown seedlings wre employed (Table 38). All the explants responded to callus induction. But, embryogenic calli were observed only in the case of *in vitro* derived leaves, petiole and the immature seeds. So, further investigations were carried out only with explants from *in vitro* leaves, petiole and immature seeds. Maximum embryogenic ability (53.60%) was recorded by *in vitro* leaf explants(Table 38).

Further the experiments were directed to determine the embryogenic ability of the selected explants (Table 38). *In vitro* leaves produced maximum (53.00%) (Plate 12) embryogenic frequency followed by petiole (18.90%) (Plate 13) and seeds (8.33%)(Plate 14). Number of somatic embryoids produced per

Table 37. Performance of *in vitro* regenerated plantlets at different period after hardening

Potting mix = Coarse sand + Charcoal pieces + Brick pieces (1:1:1))

Period/Stage of observation	Height (cm)	Canopy spread (cm)	Width of largest leaf (cm)	Length of leaf (cm)
2 months after planting	5.97	9.76	2.00	4.03
4 months after planting	13.30	16.83	4.37	7.07
6 months after planting	20.50	16.83	5.73	10.57
CD (0.05)	7.30	4.15	1.94	3.35

Number of plants = Ten

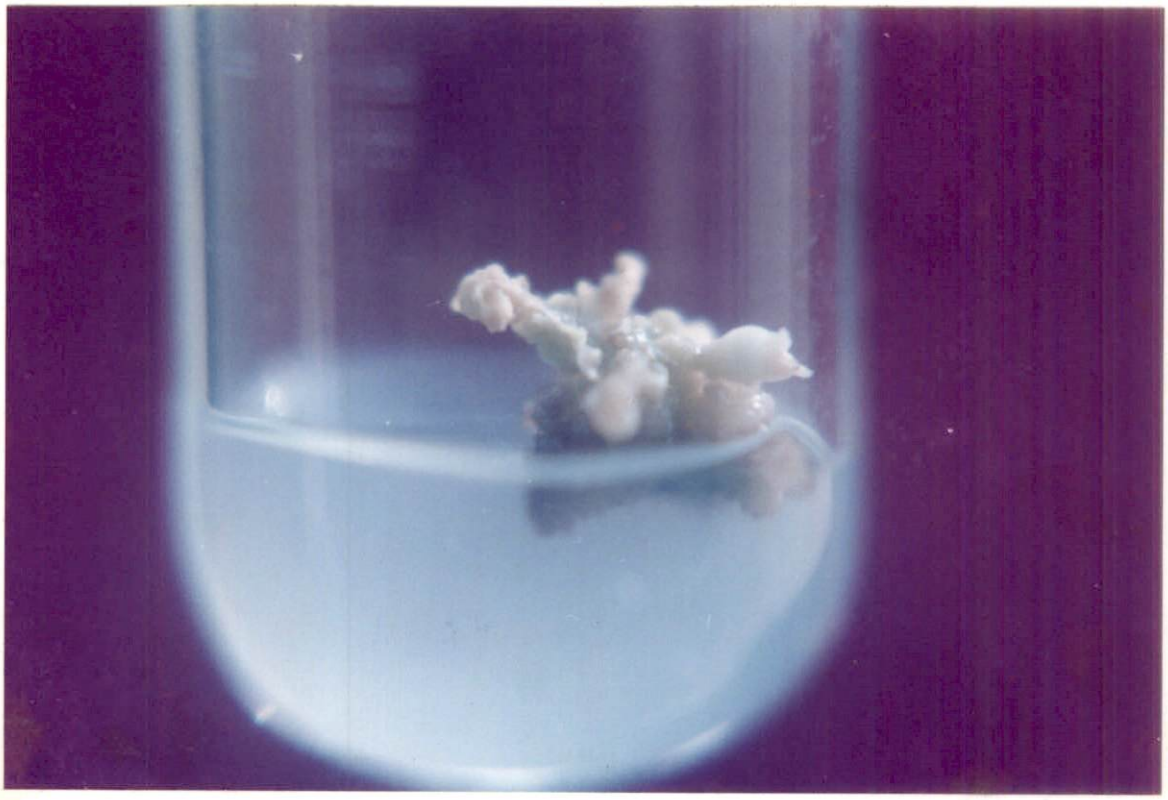
Table 38. Induction of somatic embryos as influenced by the type of explants

Type of Explants		Cultures initiating callus (%)	Cultures initiating embryogenic callus/ embryoids (%)
In vivo explants			
Tender leaves	T_1	66.50	-
	T_2	86.00	-
Immature Seeds	T_1	72.00	8.33
	T_2	76.66	5.60
***In vitro* explants**			
Leaves	T_1	67.30	53.00
Petiole	,,	80.33	18.90
Nodal explants	,,	72.00	-
Root	,,	81.00	-
Mean		65.22	21.45

T_1 = NW + 2,4-D 1.0 mg l^{-1} + kinetin 0.25 mg l^{-1} + glutamine 200 mg l^{-1}
T2 = N + 2,4-D 1.0 mg l^{-1} + kinetin 0.25 mg l^{-1} + glutamine 200 mg l^{-1}

Plate 17. Somatic embryogenesis from *in vitro* derived leaf callus

Plate 18. Somatic embryogenesis from *in vitro* derived petiole explants



+-explant was 5-6 in leaf explants and 3-4 in petiole and seed explants. Somatic embryos from leaf callus showed maximum germination (4-7%). So *in vitro* leaves were selected as the explant source for the further studies.

4.2.1.2 Effect of growth regulators

Out of 30 treatment combinations tried only 18 treatment combinations recorded the positive results (Table 39), among which treatments, per cent culture initiating embryogenic callus was maximum (67.30) in the treatment combinations half MS + 2,4-D 4.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + sucrose 30 g l^{-1} + glutamine 200 mg l^{-1} (T_7) and Nitsch + 2,4-D 4.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + sucrose 20 g l^{-1} + glucose 10 g l^{-1} + glutamine 200 mg l^{-1} (T_{14}). But, among the embryogenic calli induced cultures, maximum (53.58%) induction of somatic embryoids was noticed in the treatment combination NW + 2,4-D 2.0 mg l^{-1} + kinetin 0.75 mg l^{-1} + glutamine 200 mg l^{-1} +sucrose 20 g l^{-1} + glucose 10 g l^{-1} (T_{18}). Cultures producing embryogenic callus was minimum (34%) in the treatment combination NW + 2,4-D 1.5 mg l^{-1} + kinetin 0.15 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g l^{-1} + glucose 10 g l^{-1}. But, the per cent cultures producing somatic embryoids was better in (45.37%) the above treatment. Least per cent cultures initiating somatic embryoids (3.68%) was recorded by the treatment combination half MS + 2,4-D 4.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 50 gl^{-1}. In the above treatment, when sucrose concentration was reduced to 30 gl^{-1}cultures initiating embryoids were slightly increased (5.70%). The treatment combination half MS + 2,4-D 2 mg l^{-1} + kinetin 0.3 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g l^{-1} + glucose 10 g l^{-1}(T_3) and half

Table 39. Response of *in vitro* leaf explants to somatic embryoid induction treatments

Treatments*	Cultures initiating embryogenic callus (%)	Cultures initiating embryoids (%)
T1	64.67	33.58
T2	65.66	35.69
T3	39.33	-
T4	41.00	20.48
T5	48.33	9.50
T6	39.33	-
T7	67.30	6.67
T8	49.67	5.70
T9	49.67	5.66
T10	50.00	3.68
T11	61.67	31.55
T12	50.66	22.00
T13	66.67	10.30
T14	67.30	14.36
T15	66.67	10.00
T16	49.80	45.33
T17	34.00	45.37
T18	61.29	53.58
CD (0.06)	4.29	6.37

*Observations taken 3 months after culture

MS + IAA 3.0 mg l^{-1} + kinetin 1.5 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 30 g l^{-1} (T_6) eventhough produced embryogenic calli, none of them were able to induce somatic embryoids.

4.2.1.3 Basal media

Half strength MS basal media, Nitsch media, combination of Nitsch inorganic salts and vitamin component of White media (NW), B_5 media and combination of B_5 and half MS media were used for the production of somatic embryoids (Table 40). Among the media tried, NW media recorded the maximum cultures initiating somatic embryos (55.58%). In the same media when concentration of growth regulators was reduced, the response was slightly reduced (45.33%). Minimum per cent of somatic embryo induction was (5.66) recorded in half MS media. B_5 and BM media didnot have any response to embryoid induction treatments

4.2.1.4 Culture conditions

A dark period of 24 hours was found to be very essential for the embryoid induction from all the explants with all the treatments employed.

4.2.1.5 Media supplements

Different concentrations of glutamine, coconut water and casein hydrolysate were supplemented to the media to know the effect of media supplements (Table 41) on somatic embryoid induction. Among the media supplements added, glutamine 200 mg l^{-1} (Mg_1) recorded the maximum per cent

Table 40. Comparison of growth regulator supplements to basal media used in induction of somatic embyos

Basal media	Growth regulator supplements (mg l^{-1})	Response (%)
1/2MS	2,4-D 0.5 + KN 0.15 + sucrose 20 + glucose 10 g + glutamine + Agar 0.2%	33.65
	2,4-D 1.0 + KN 0.15 + sucrose 20 + glucose 10 + glutamine 200 + Agar 0.2%	35.69
	2,4-D 2.0 + KN 0.5 + sucrose 20 + glucose 10 + glutamine 200[1] + Agar 0.6%	9.67
	2,4-D 4.0 + KN 0.3 + sucrose 20 + glucose 10 + glutamine 200 + Agar 0.6%	20.33
	2,4-D 4.0 + KN 0.5 + sucrose 20 + glucose 10 +glutamine 200 + Agar 0.6%	6.67
	2,4-D 4.0 + KN 1.0 + sucrose 20 g + glucose 10 + glutamine 200 + Agar 0.6%	5.66
Nitsch	2,4-D 0.5 + KN 0.15 + glucose 10 g sucrose 20 + glutamine 200 Agar 0.6%	31.00
	2,4-D 1.0 + KN 0.15 + sucrose 20 + glucose 10 g + glutamine 200 + Agar 0.6%	22.00
	2,4-D 2.0 + KN 0.3 + sucrose 20 + glucose 10 + glutamine 200 + Agar 0.6%	10.35
	2,4-D 4.0 + KN 0.5 + sucrose 20 + glucose 10 + glutamine 200 + Agar 0.6%	14.36
Nitsch white (NW)	2,4-D 1.0 +KN 0.25 + sucrose 20 + glucose 10 + glutamine 200 + Agar 0.6%	45.33
	2,4-D 1.5 + KN 0.5 + sucrose 20 + glucose 10 + glutamine 200 + Agar 0.6%	55.58
CD(0.05)		7.53

*Observations taken 3 months after culture

of cultures initiating embryoids (53.66) and the same treatment also recorded the maximum number of embryoids pert culture (6.00). Minimum response of cultures initiating embryoids (25.00%) was recorded by casein hydrolysate 100 mg l^{-1} (Mn_1) and the number of embryoids produced per culture was also less (2.36) in the treatment. Minimum number of embryoids per culture was recorded (2.00) in casein hydrolysate 200 mg l^{-1} (Mn_2). Number of embryoids per culture was better (3.67) in media fortified with coconut water 15% than in the treatments with casein hydrolysate.

Per cent cultures initiating embryoids was better in leaf explants than in petiole explants(Table 41). Among the media supplements, glutamine 200 mg l^{-1} (Mg_1) recorded maximum cultures (20.70%) initiating embryoids. Number of embryoids produced per culture (3.60) was also maximum in the same treatment. The response of cultures initiating embryoids was minimum (9.70%) in the treatment casein hydrolysate 300 mg l^{-1} (Mn_3). The number of embryoids per culture was minimum(1.32) in the treatments with 10% coconut water (Mc_1), casein hydrolysate 200 mg l^{-1} (1.00)(Mn_2) and 300 mg l^{-1} (Mn_3)(1.00). From the results presented in Table 41 it can be seen that glutamine 200 mg $l^{-1}$and 400 mg l^{-1} was superior to all the other media treatments in terms of per ent embryoid induction and number of embryoids produced per culture.

4.2.2 Maturation of somatic embryoids

Total nine treatments involving various combinations of abscisic acid (ABA), sucrose and basal media were tried for the maturation of somatic embryos (Table 42). All the cultures did not survive the transfer to the

Table 41. Effect of media supplements on induction of somatic embryoids in different *in vitro derived* explants

Treatments	Leaf		Petiole	
	Cultures initiating embryoids (%)	Number of embryoids/ culture	Cultures initiating embryoids (%)	Number of embryoids/ culture
Mg_1	53.66	6.00	20.70	3.60
Mg_2	51.00	3.67	17.00	2.35
Mg_3	43.30	2.33	20.30	2.35
Mc_1	33.67	2.00	11.67	1.00
Mc_2	32.00	3.67	12.33	1.67
Mc_3	32.33	2.33	13.00	1.32
Mn_1	25.00	2.36	12.33	1.32
Mn_2	31.33	2.00	13.33	1.00
Mn_3	30.38	2.69	9.70	1.00
CD (0.05)	5.65	2.02	2.49	0.27

*Observations taken 3 months after culture

Table 42 Effect of culture media on maturation of somatic embryoids

Treatments	Live cultures (%)	Size of embryoids (cm)	Colour of embryoids
M_1	79.10	0.18	Cream to pale green
M_2	75.00	0.18	Pale green of
M_3	68.00	0.18	Pale green of
M_4	59.33	0.2	Pale green of
M_5	64.24	0.18	Cream
M_6	64.45	0.2	Cream
M_7	78.67	0.2	Cream
M_8	74.00	0.2	Cream
M_9	64.00	0.2	Cream
CD (0.05)	4.22	NS	

Observation taken 5 weeks after culture
Number of replications = 10

6+6+maturation media. Maximum live cultures (79.10%) were recorded in the treatment half MS + 2,4-D 0.5 mg l^{-1} + kinetin 0.15 mg l^{-1} + ABA 0.1 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g l^{-1} (M_1). The treatment was *on par* with the treatment combination Nitsch + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + ABA 0.1 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g (M_7), as this treatment recorded 78.67% live cultures. Live cultures was least (59.33%) in the treatment combination half MS + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + ABA 0.2 mg l^{-1} + glutamine 400 mg l^{-1} + sucrose 30 g (M_4). There was no significant variation observed between treatments for the size of embryoids that ranged between 0.18 cm - 0.2 cm. The colour of the embryoids varied from cream to pale green.

Data presented in Table 42 reveals that half MS media (79.10%) was the most effective media for somatic embryoid maturation. It was closely followed by Nitsch media which recorded 78.67 per ent live cultures. But, the size of the embryoid was 0.18 cm or less in all the treatments employing half MS media. However, the size of the embryoid was almost the same (0.2 cm) in treatments employing NW media. Hence, in terms of size of embryoids, NW media proved to be a better culture media compared to half MS and Nitsch.

4.2.2.1 Effect of sucrose on maturation

Three treatments involving different sucrose levels(20, 30 or 40 g l^{-1}) were tried for the maturation of somatic embryoids (Table 43). The size of the embryoid was reduced (0.10cm) when the sucrose concentration was increased to 40 g. Reducing the sucrose concentration to 20 g/l also had negative effect (0.16 cm size). The optimum concentration for improved size of the embryoid

(0.20 cm) was found to be 30 g/l. Culture survival also more in light condition (64.56%) than in dark (50.33%).

4.2.2.2 Culture conditions

Size of the embryoid and the number of embryoids were influenced by light (Table 44). Improvement in the size of the embryoid (0.22 cm) was noticed when embryogenic cultures were exposed to light conditions. In the cultures maintained in dark conditions, the size of the embryoid was only 0.16 cm. However, the average number of embryoids per culture was higher (3.60) when the cultures were kept in darkness

4.2.3 Germination of somatic embryoids

All the tested treatments did not induce germination of embryoids. Among the treatments that induced germination, maximum per cent of cultures showing germination (48.32%) was noticed in the treatment combination half MS + BAP 0.1 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g l^{-1} + glucose 10 g l^{-1}. The minimum (24.56%) germination of cultures was observed in the NW + BAP 0.1 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g l^{-1} + glucose 10 g l^{-1}. The size of the embryoid increased to 0.5 cm in the treatment combination half MS + glutamine 200 mg l^{-1} + sucrose 20 g l^{-1} + glucose 10 g l^{-1} and in NW + BAP 0.1 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g l^{-1} + glucose 10 g l^{-1} and in NW + BAP 0.1 mg l^{-1} + glutamine 200 mg l^{-1} + sucrose 20 g l^{-1} + glucose 10 g l^{-1}. In all the other treatments the size of the embryoid was 0.4 cm. In the germination media the colour of the embryoid changed to pale green to dark green(Plate 20).

Table 43. Effect of sucrose on maturation of somatic embryoids

Media - NW + 2,4-D 1.0 mg l^{-1} + Kinetin 0.15 mg l^{-1} + ABA 0.1 mg l^{-1} + CH 100 mg l^{-1} + glutamine 400 mg l^{-1}

Sucrose concentration (g)	Live cultures (%)	Size of embryoids (cm)
20	59.33	0.16
30	64.00	0.20
40	60.45	0.10
Mean	61.26	0.15

Number of replications = 10

Table 44. Light vs dark on maturation of somatic embryoids

Media - NW + 2,4-D 1.0 mg l^{-1} + kinetin 0.15 mg l^{-1} + ABA 0.1 mg l^{-1} + CH 100 mg l^{-1} + glutamine 400 mg l^{-1} + Sucrose 30 g

Physical condition of incubation of cultures	Live cultures (%)	Size of embryoids (cm)
Light	64.56	0.22
Dark	50.33	0.16
Mean	57.46	0.19

Number of replications = 10

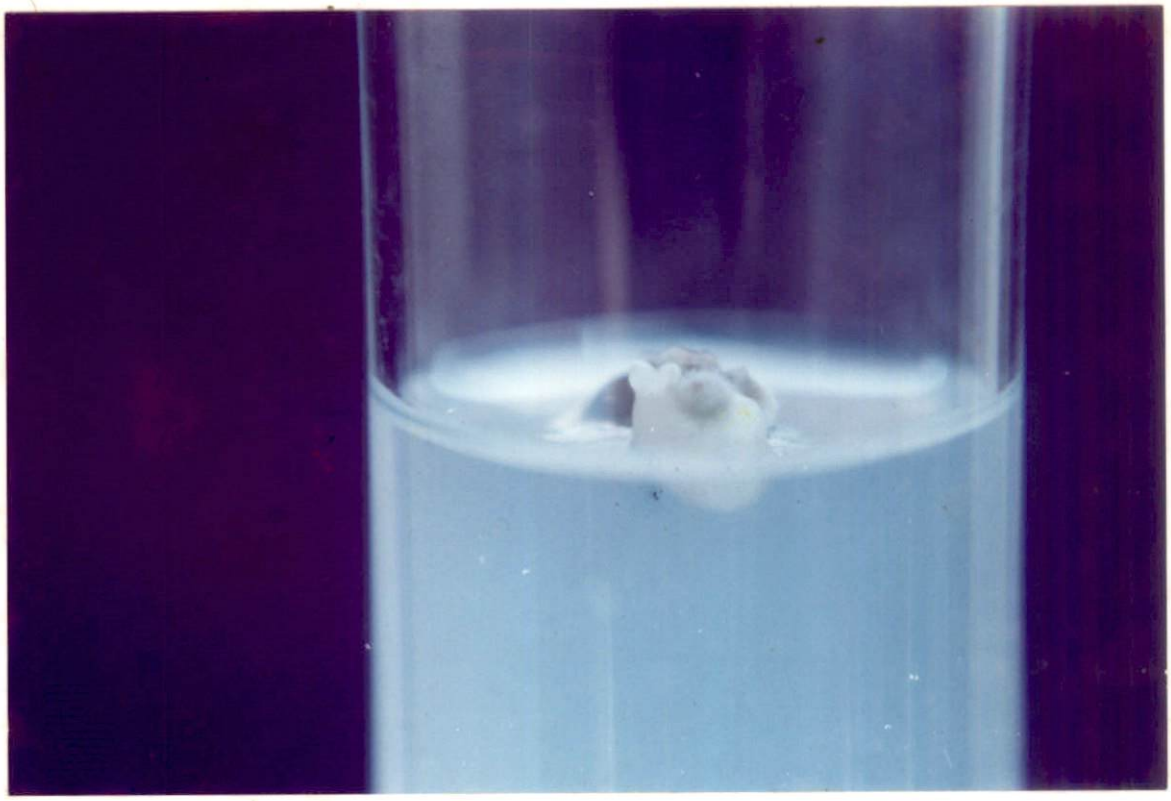
Table 45. Effect of culture media on germination of somatic embryoids

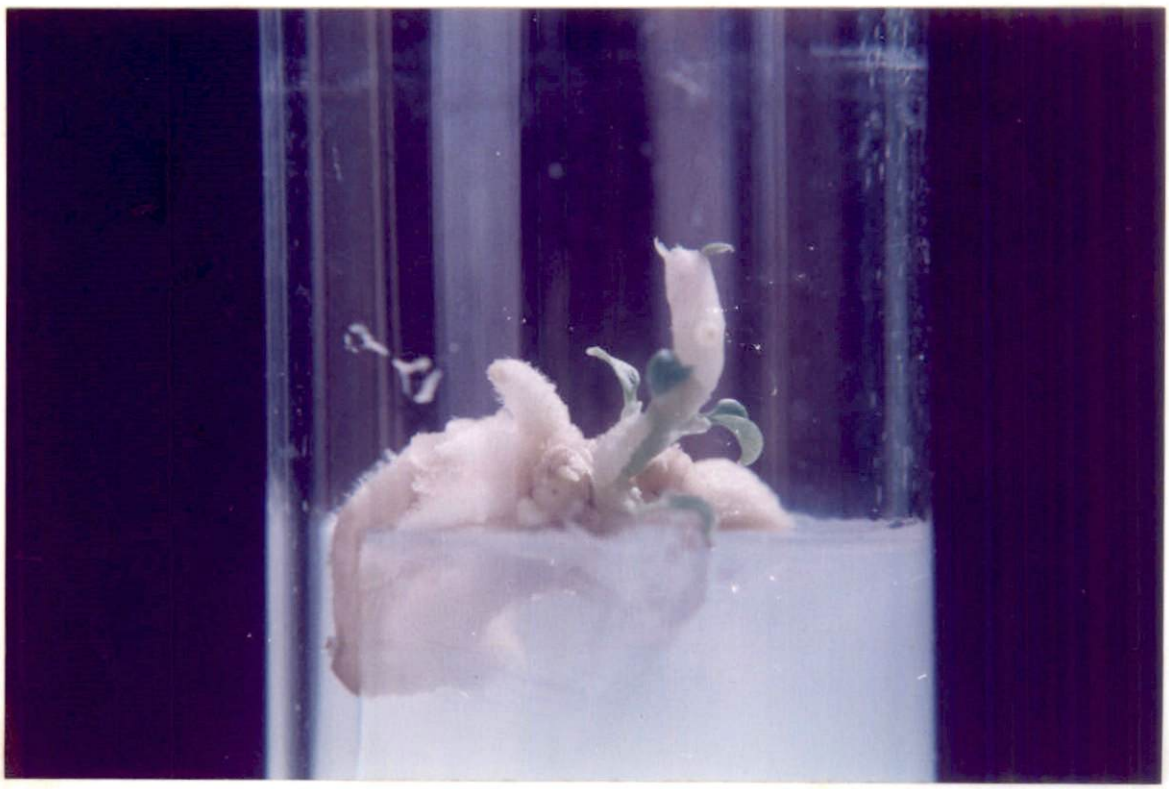
Treatments	Live cultures (%)	Colour of embryoid	Size of embryoids (cm)
G_1	45.57	Pale green	0.5
G_2	48.32	Dark green	0.4
G_3	39.50	Dark green	0.4
G_4	-	-	-
G_5	32.00	Pale green	0.4
G_6	29.25	Pale green	0.4
G_7	-	-	-
G_8	-	-	-
G_9	-	-	-
G_{10}	24.56	Yellow	0.5
G_{11}	-	-	-
G_{12}	-	-	-

Observations taken 6-7 weeks after culture

Plate 19. Somatic embryogenesis from immature seeds

Plate 20. Germination of somatic embryoids





Increasing the growth regulator concentration in the culture media resulted in increased browning of embryoids. It was also noted that, for germination and development transferring the culture to liquid media and changing the culture media at two weeks interval is very essential. Germination occurred in presence of dark. Rooting occurred frequently and spontaneously. After two months of culturing on embryo conversion medium plantlets were selected for planting out.

Embryogenic ability of different explants of anthurium cv. Dragon's Tongue differed significantly (Table 46). *In vitro* derived leaves recorded the maximum response, which showed 53 per cent of cultures with frequency of embryogenic callus. The number of somatic embryos per explant (5.88) and their germination was also maximum(47.00%) when *in vitro* derived leaves were used as the explant . Petioles of *in vitro* leaves produced a mean number of 3.67 somatic embryos per culture but, the embryogenic frequency was less.

4.2.4 Encapsulation and conversion of somatic embryos

Sodium alginate and calcium chloride were used as gelling and complexing agents respectively, for encapsulation (calcium-alginate hydrogel) of somatic embryos.

The firmness and texture of the gel was changed considerably with variation in the concentration of sodium alginate (Table 47). For instance, the capsules produced with higher concentrations (5% sodium alginate) were very hard and suppressed the germination of somatic embryos and subsequent

Table 46. Embryogenic ability of *A. andreanum* cv. Dragon's Tongue

Type of explant	Frequency of embryogenic callus (%)	Somatic embryo/explant	Germination of somatic embryos (%)
Leaves (*in vitro*)	53.00(a)	5.88 (a)	47.00(b)
Petiole (*in vitro*)	18.90(a)	3.67(a)	11.25(b)
Immature Seeds	8.33(c)	1.67(c)	12.45(d)
CD	24.39	1.11	20.38

Number of replications =Three (3)
(One replication=10 Culture tubes)

(a) NW + 2,4-D 1.5 mgl^{-1} + kinetin 0.15 mgl^{-1} + sucrose 20 g l^{-1}+ glucose 10 g + glutamine 200 mgl^{-1}
(b) 1/2MS + BAP 0.2 mgl^{-1} + sucrose 20 gl^{-1}
(c) Nitsch + 2,4-D 2.0 + kinetin 0.3 + sucrose 20 gl^{-1}+ glucose 10 gl^{-1} +glutamine 200 mg l^{-1}
(d) Nitsch + Sucrose 20 gl^{-1}

Table 47. Suitability of varying concentrations of sodium alginate for encapsulation of somatic embryos developed from leaf embryogenic callus

Media: 1/2MS + BAP 0.1 mg l^{-1}

Concentration of sodium alginate (%)	Firmness	Conversion frequency (%)
2	Very soft	7.30
3	Medium (proper consistency)	32.67
4	Hard	3.33
5	Very hard	0.0
	CD (0.05)	12.71

Conversion frequency = Number of encapsulated embryos germinated

Table 48 Conversion frequency of somatic embryos/encapsulated with sodium alginate in different concentrations of $Cacl_2.2H_2O$

Concentration of Sodium alginate (%)	Concentration of $CaCl_2.2H_2O$ (mM)	Conversion (%)
3	25	24.67
	50	32.60
	CD (0.05)	7.30

growth. Employing three per cent sodium alginate obtained beads of proper consistency (Plate 21). The firmness of gel capsule developed with 2 per cent sodium alginate was unsatisfactory for synthetic seed handling and storage.

Calcium chloride at 50 mM (Table 48) and sodium alginate at 3 per cent level were more effective in producing a proper gel cover consistency around the somatic embryos. The highest conversion frequency and the consequent maximum plantlet regeneration (32.67%) was obtained with three per cent sodium alginate and 50 mM calcium chloride (Table 48).

4.2.4.1 Effect of media on conversion frequency

Different basal media (Table 49) were evaluated to increase the conversion rate of encapsulated embryos. The conversion frequency of encapsulated embryos and non-encapsulated embryos, in different basal media differed were significantly different (Table 49). Non-encapsulated embryos showed a higher frequency (48.32%) of conversion when cultured on half strength MS medium. The lowest conversion rate for non-encapsulated embryos (24.53%) was noticed in NW medium. In comparison to non-encapsulated embryos, the conversion frequency for encapsulated embryos without storage was low. The conversion frequency of somatic embryos was maximum (32.67%) on half strength MS medium and minimum (9.70 %) with those cultured on Nitsch media. Time required for germination of encapsulated and non-encapsulated somatic embryos varied (Table 50). Non-encapsulated embryos took only 6-7 weeks for germination, whereas, encapsulated embryos took 8-9 weeks.

Table 49. Effect of different culture media on conversion of somatic embryos

Gelling agent for encapsulation - 3% Sodium alginate
Complexing agent - 50 mM Ca $Cl_2 .2H_2O$

Culture media	Conversion frequency (%)	
	Encapsulated embryos	Non-encapsulated embryoids
MS	32.67	48.32
Nitsch	9.70	29.50
NW	12.00	24.53
CD (0.05)	12.94	16.56

4.2.4.2 Regeneration from encapsulated embryos/synseeds

Minimum number of days taken for germination was recorded by those cultured onto half MS medium (29.55 days), which recorded a germination per cent of 30 when cultured fresh without storage at low temperature. But, the germination per cent increased (32.50) when half strength media was supplemented with BAP 0.1 mg l^{-1} (Plate 22). Germination of encapsulated embryos (10.00%) was poor when they were cultured afresh on to NW + BAP 0.2 mg l^{-1}. (Table 50).

4.2.4.3 Viability of encapsulated embryos/synseeds

Germination per cent of encapsulated embryos increased after low temperature storage at 4°C (Table 50). Ten days after low temperature storage germination per cent was increased to 35.56 in half strength MS medium when half strength media was supplied with BAP 0.1 mg l^{-1}. Twenty (20) days after low temperature storage germination per cent was further increased to 38.56. Increasing the concentration of BAP in half strength MS media to 0.2 mg l^{-1} reduced the germination (28.25%). It was observed that germination on transfer to half MS media decreased to 20 per cent after 30 days and to 3.50 per cent after 60 days of low temperature storage. In all the media combinations there was a decrease in the germination of the synthetic seeds after 30 days of low temperature (4°C) storage.

A comparison was made between encapsulated and non-encapsulated embryoids to determine their germination and viability after the storage at low

Table 50. Viability of encapsulated somatic embryos and shoot tips (Synseeds)

Treatments	Days taken for germination	Germination (%)				
		Fresh synseeds without storage*	10 days after storage**	20 days after storage**	30 days after storage**	60 days after storage**
1/2MS	29.55	30.00	35.56	38.56	20.00	3.50
□MS + BAP0.1 (2% sucrose)	30.00	32.50	36.00	38.00	21.20	3.50
½ MS + BAP 0.2 (2% sucrose)	38.50	25.00	27.50	28.25	18.20	3.00
□MS MS + BAP 0.5 (2% sucrose)	-	-	-	-	-	-
Nitsch (N)	32.25	16.00	17.50	24.50	22.00	6.40
Nitsch + BAP 0.1 (2% sucrose)	39.00	19.50	10.00	18.80	15.33	3.00
N + BAP 0.2 (2% sucrose)	-	-	-	-	-	-
N + BAP 0.5 (2% sucrose)	-	-	-	-	-	-
NW	-	-	-	-	-	-
NW + BAP 0.1	35.53	12.50	14.00	14.33	13.50	2.50
NW + BAP 0.2	36.30	10.00	10.00	10.67	9.60	3.00
NW + BAP 0.5	-	-	-	-	-	-

Data not subjected to statistical analysis
* Cultured fresh without storage
**Lowtemperaturestorage

temperature (Table 51). Prior to storage germination per cent was maximum (48.32) in non-encapsulated embryoids. After ten days storage (ambient temperature) germination per cent of non-encapsulated embryoids was considerably reduced (6.00%). Prior to storage encapsulated embryoids recorded germination per cent of 30.00 and after storage of ten days it was 30.40 per cent. Whereas, non-encapsulated embryoids after storage for prolonged periods at ambient temperature failed to germinate. However, in the case of encapsulated embryos germination per cent considerably reduced (15.50%) after storage at ambient temperature. The non-encapsulated embryoids did not survive low temperature storage. Whereas in the case of encapsulated embryoids the per cent germination increased after low temperature storage. After ten days of low temperature storage, the germination per cent was 35.56, which was further increased to 38.56 after 20 days. However, after 30 days the per cent germination was reduced significantly. Hence, it is obvious that the viability of somatic embryoids it can be concluded that viability of the somatic embryoid can be improved by encapsulation, and subsequent low temperature storage for 20 days. Storing the encapsulated embryoids upto 20 days and then putting them to germinate can improve the germination per cent of synseeds (Table 51). The time taken for germination was also significantly reduced consequent to storage at low temperature storage (Fig. 4). However, low temperature storage of encapsulated somatic embryos for longer days took more days for their germination, though less than that of those stored at ambient temperature (Table 51).

Table 51 Germination of encapsulated and non-encapsulated somatic embryos after storage

Media – 1/2MS

Duration of Storage (Days)	Per cent germination of encapsulated somatic embryos		Non-encapsulated somatic embryos	
	Germination (%)	Days ken for germination	Germination (%)	Days ken for germination
Control (Ambient temperature)	30.00	39.7	48.32	30
10	30.40	40.00	6.00	32
15	30.25	40.50	-	-
20	25.50	40.50	-	-
Low temperature				
10	35.56	32.00	-	-
20	38.56	35.00	-	-
30	20.00	35.00	-	-

Crystallisation of non -capsulated somatic embryos took place after low temperature storage.
Data was not subject to statistical analysis

Figure 4

Influence of duration of low temperature storage on germination of synseeds

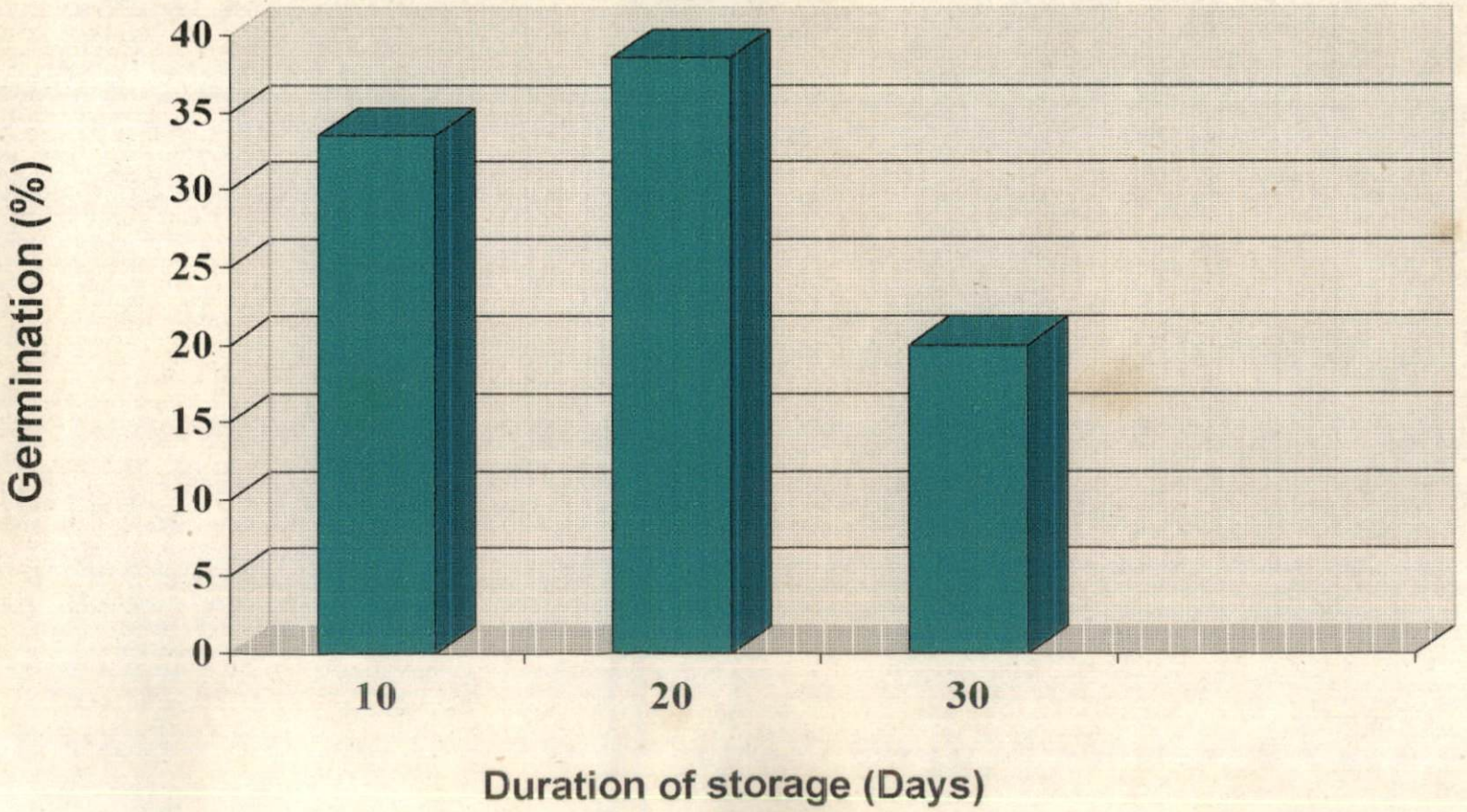


Plate 21. Encapsulated somatic embryos

Plate 22. Germination of encapsulated seeds

4.3 Induction of somaclonal variation

4.3.1 Effect of repeated subculturing

Repeated subculturing was carried out at three weeks interval to assess the variation induced to cultured plants. During the culture period, there was an increase or decrease in multiplication rate of axillary shoots as recorded in Table 52.

4.3.1.1 Time taken for Shoot regeneration

There was a significant difference among the subcultures tried with respect to the time taken for regeneration of shoots and the number of shoots produced per culture (Table 52). Minimum number of days for regeneration was recorded (19.33 days) by the seventh subculture followed by sixth subculture (19.67 days). The callus from tenth subculture took the maximum number of days (30.06 days) followed by those from nineth subculture (27.60 days).

4.3.1.2 Number of rootable shoots

Maximum number of rootable shoots (7.00) were produced by the callus from eighth subculture, followed by those from seventh and sixth subcultures (6.00 and 6.33 respectively). Increase in the number of shoots started from the fourth subculture onwards (5.30) up to eighth subculture (7.00) and thereafter the number of rootable shoots were reduced (Table 52).

Table 52 Response of *A. andreanum* cv. Dragon's tongue culture to repeated subculturing

Number of subculture	Shoot doubling time (days)	Number of rootable shoots after 3 weeks	Other features
1	26.66	3.00	Green healthy plants with small leaves
2	24.67	3.33	Healthy plants with medium sized leaves
3	23.00	3.33	Healthy plants with medium sized leaves
4	21.80	5.30	Healthy plants with medium sized leaves
5	20.10	5.70	Healthy plants with well developed leaves
6	19.67	6.33	Healthy plants with well developed leaves
7	19.33	6.00	Healthy plants with well developed leaves
8	23.33	7.00	Leaf size slightly reduced
9	27.60	5.20	Slightly pale plants with smaller leaves
10	30.06	3.50	Slightly pale plants with smaller leaves
CD (0.05)	2.00	0.89	

Total culture period 30 weeks
Replications =Three (One Replication = 10 culture tubes)

4.3.1.3 Effect of repeated subculturing on growth and development upon transplanting

4.3.1.3.1 Height of plant

Plants regenerated from the sixth subculture recorded the maximum height of plants after two months of growth (8.20 cm) but after four months of planting out plants of third subculture recorded the maximum height (16.67 cm). After six months of growth, maximum height was recorded by plants regenerated from the fifth (23.66 cm) and sixth (23.60 cm) subculture. Plant height after two months of growth was the lowest (5.97 cm) in the case of plants regenerated from the first subculture. However, after fourth and sixth month of growth, plants from eighth and ninth subculture recorded the lowest height (Table 53).

4.3.1.3.2 Canopy spread

No significant difference in canopy spread was noticed among plants regenerated from different subcultures after two months of growth (Table 53). However, after two months of establishment of plants maximum canopy spread (10.23 cm) was recorded by plants from fifth subculture followed by those from first (9.97 cm), seventh (9.97 cm) and fourth (9.86 cm). But, after four months of growth, plants from fifth subculture recorded a significant improvement in canopy spread (18.96 cm) over plants regenerated from other subcultures. Canopy spread was minimum (12.83 cm) in the case of plants from sixth subculture. Values recorded for canopy spread after 6 months of growth differed

Table 53 Effect of repeated subculturing on growth and development of plantlets after hardening

Number of subculture	Two months				Four months				Six months			
	Height of the plant (cm)	Canopy spread of the plant (cm)	Leaf width (cm)	Leaf length (cm)	Height of the plant (cm)	Canopy spread of the plant (cm)	Leaf width (cm)	Leaf length (cm)	Height of the plant (cm)	Canopy spread of the plant (cm)	Leaf width (cm)	Leaf length (cm)
1	5.97	9.76	2.00	4.03	13.30	14.83	4.36	6.56	20.50	16.85	5.73	10.56
2	6.33	9.74	1.66	2.03	13.00	16.10	2.27	2.43	20.33	17.20	4.00	6.00
3	6.43	9.76	1.60	1.97	12.33	14.66	2.10	2.83	20.33	16.83	3.80	7.16
4	7.50	9.90	1.83	2.17	14.33	14.16	2.33	3.50	21.67	16.00	4.17	7.60
5	8.20	10.23	1.96	3.30	16.07	18.96	3.00	7.13	23.66	19.00	6.16	11.23
6	8.26	9.77	1.40	2.27	15.70	12.83	2.67	4.76	23.60	15.30	4.50	7.83
7	7.43	9.97	1.90	2.56	14.17	14.00	2.20	3.56	21.00	16.10	5.16	8.03
8	7.46	9.97	1.37	1.40	14.66	14.50	2.10	2.00	22.00	14.83	4.33	6.50
9	6.90	9.86	1.50	2.00	16.50	14.13	2.17	2.93	23.16	14.00	3.73	5.17
10	7.00	9.83	1.66	2.17	16.67	16.80	2.13	3.17	23.00	13.67	3.70	4.97
CD(0.05)	0.47	NS	0.18	0.41	0.85	1.00	0.38	0.91	0.74	0.76	0.47	1.09

significantly plants regenerated from fifth subculture recorded the maximum canopy spread (19.00 cm).

4.3.1.3.3 Average length of the leaf

Leaf length after two months of growth was maximum (4.03 cm) in plants regenerated from first subculture. After four and six months of growth, length of leaf was maximum 7.13 cm and 11.23 cm respectively) in plants from 5th subculture (Table 53). Length of the leaf two months (1.4 cm) and four months (2.00 cm) was the least in case of plants from eighth subculture. But, after six months, plants obtained from 10th subculture recorded the least leaf length (4.97 cm).

4.3.1.3.4 Average width of leaf

Upon planting out, the width of leaf (Table 53) was maximum in plants regenerated from the first subculture after two months (2.00 cm), four months (4.36 cm) and six months (5.73 cm). The width of leaves after six months was the lowest (3.73 cm) in plants regenerated from 10th subculture. A trend in response of reduction in the width of leaf with advancing subculture cycles was noticed at all stages of growth (Table 53).

4.3.1.3.5 Effect of repeated subculturing on individual leaf area

Maximum leaf area (16.57 cm^2) after fourth month and sixth month (46.32 cm^2) of growth was recorded by plants obtained from the first subculture (Table 54; Fig. 4; Plate 24). Even after six months of growth leaf area was only 9.11

Table 54 Effect of repeated subculturing on leaf area of Anthurium plants after transplanting

Treatment (Subculture)	Leaf area (cm^2)	
	4 months	6 months
1	16.572	46.32
2	0.639	19.35
3	0.019	15.77
4	1.623	18.80
5	11.391	40.48
6	5.020	21.42
7	1.410	25.98
8	0.371	16.20
9	-	9.81
10	-	9.11
CD (0.05)	3.12	6.72

Figure 5

Effect of repeated subculturing on leaf area of anthurium plants six months after transplanting

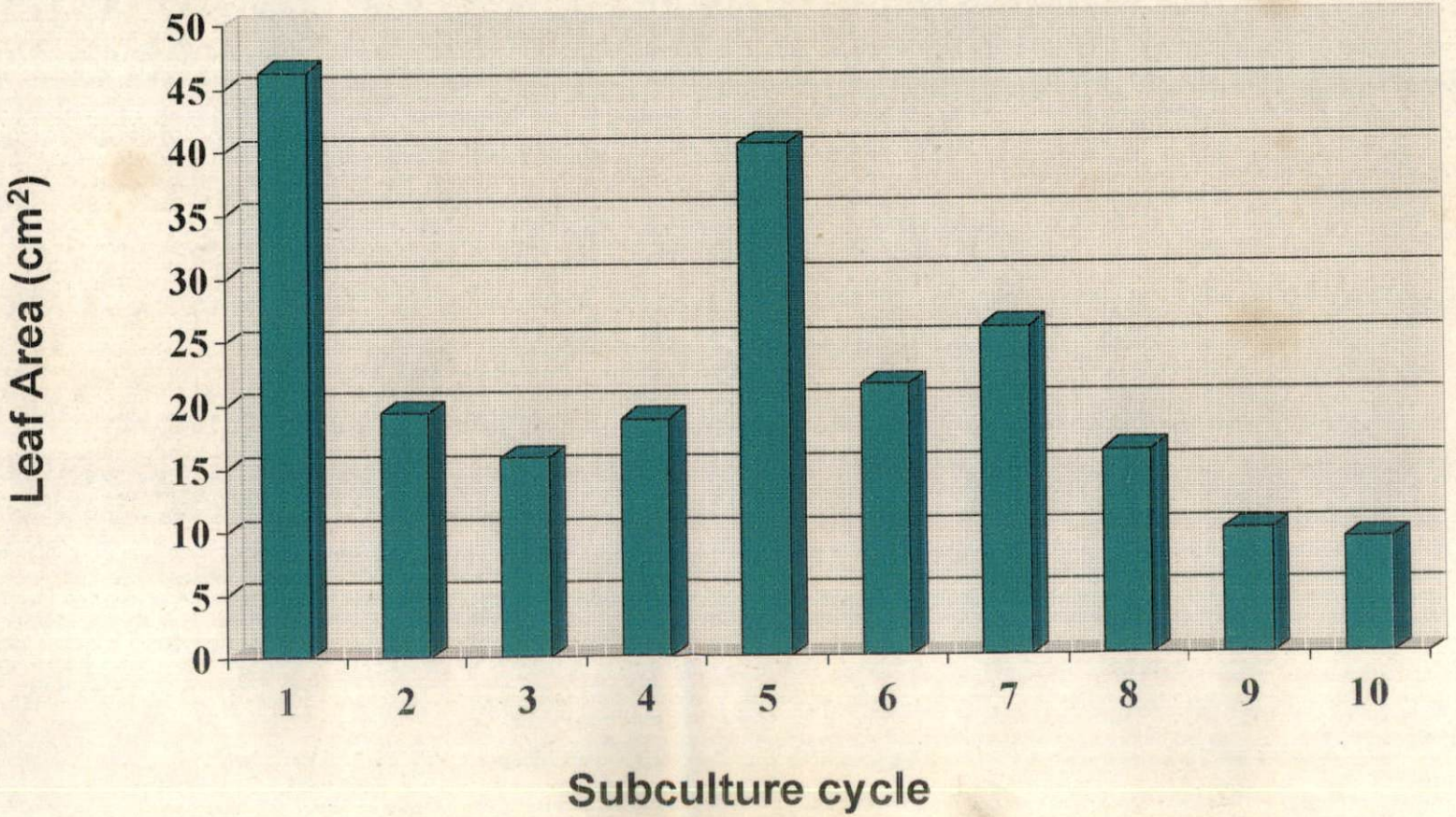
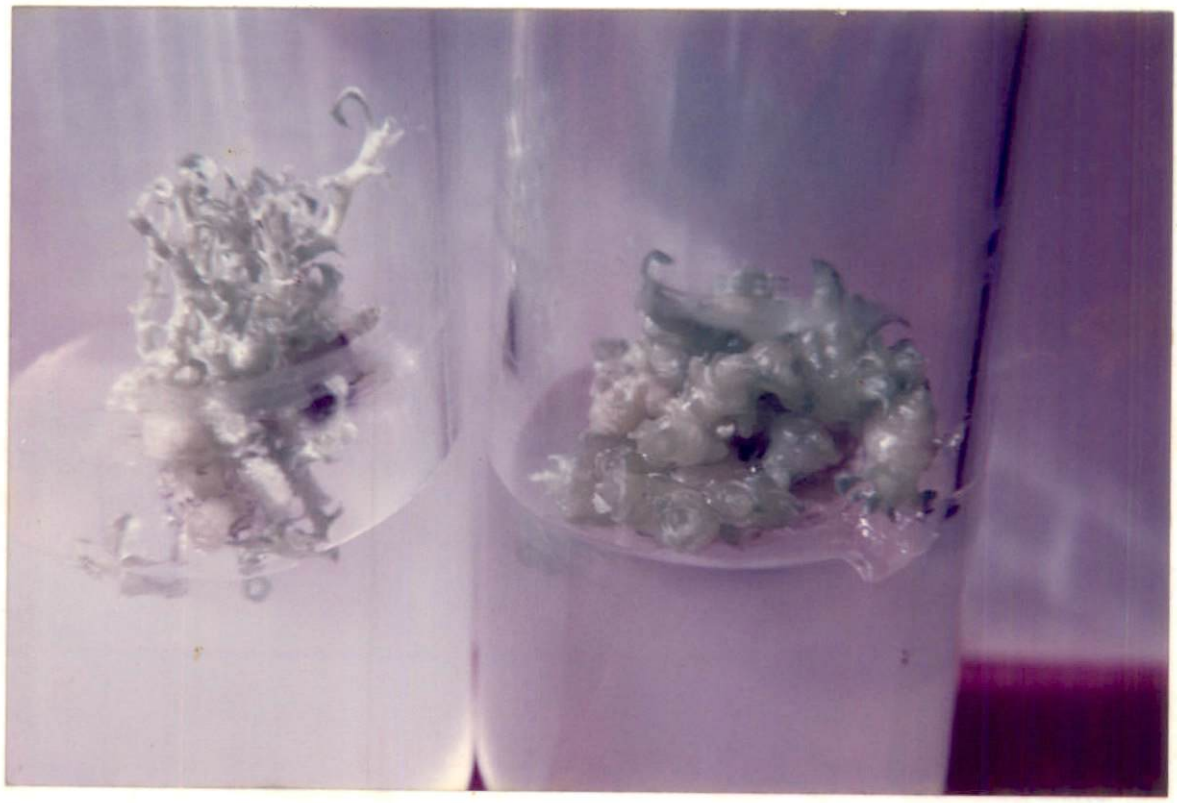


Plate 23. Response of *in vitro* cultured shoots to high concentration of cytokinins

Plate 24. Variation in leaf area due to repeated culturing



cm^2 in plants from 10th subculture and 9.81 cm^2 in plants obtained from ninth subculture (Plate 24). Though uniform trend in response was absent, it was seen that the individual leaf area was reduced with plant regenerated from advanced subculture cycles.

4.3.2 Effect of high concentrations of cytokinins on induction of somaclonal of variation

Total nine treatments using different media and high concentrations of cytokinins were tried to induce variation (Table 55). Plantlets regenerated from callus cultured onto media containing concentrations above 20mg l^{-1} showed no upward growth and only swelling of the explants and irregular shootlets were seen. Hence, further trials were carried out with concentrations of cytokinins limiting to 15 mgl^{-1} (Plate23).

4.3.2.1 Days taken for shoot regeneration

Number of days taken for shoot regeneration (47.67 days) was the lowest in the case of callus cultured onto the culture media Nitsch + BAP 10.0 mg l^{-1} followed by Nitsch + BAP 12.5 mg l^{-1} (49.00). Maximum number of days (62.33) for regeneration was taken by callus cultured onto half MS media fortified with BAP 15.0 mg l^{-1}. From the results presented in Table 55, it can be seen that the response of growth regulators to shoot regeneration varied with the basal media employed. With the same concentrations of the growth regulator BAP, Nitsch media was effective in early induction of shoots than ½ MS media . Between the different cytokinins, BAP was effective in early induction of shoots than Kinetin.

Table 55 Effect of high concentrations of cytokinins on growth response of *A. andreanum* cv. Dragon's Tongue

Treatment (mg l^{-1})	Days taken for shoot regeneration (days)	Number of shoots per culture	Length of longest shoot (cm)	Number of leaves in the longest shoot	Number of roots	Other features
Nitsch + BAP 10.0	47.67	8.58	1.46	4.00	3.33	All the cultures showed simultaneous rooting and the leaves produced were rudimentary.
Nitsch + BAP 12.5	49.00	10.00	1.46	4.00	4.33	
Nitsch + BAP 15.0	49.67	11.00	1.53	3.33	4.00	
Nitsch + Kinetin 10.0	57.00	4.43	0.93	2.33	3.33	Only swelling of the plants. No vertical growth.
Nitsch + Kinetin 12.5	58.00	4.67	0.83	3.00	3.67	
Nitsch + Kinetin 15.0	60.33	5.33	0.73	2.00	2.00	
MS + BAP 10.0	62.33	2.00	0.80	2.00	2.30	Only swelling of the shoots.
MS + BAP 12.5	62.60	2.30	0.70	2.00	2.30	
MS + BAP 15.0	62.33	3.00	0.65	2.00	2.00	
CD (0.05)	5.58	1.56	0.21	0.51	0.56	

Replications = Three (One replication - 10 culture tubes)

4.3.2.2 Number of shoots per culture

Maximum number of shoots per culture were observed in the treatment Nitsch + BAP 15 mg l^{-1} (11.00) followed by Nitsch + BAP 12.5 mg l^{-1} (10.00). Minimum number of shoots per culture was recorded by the treatment combination half strength MS + BAP 10.0 mg l^{-1}(Table 55) . Nitsch media was significantly better in induction of more number of shoots per culture than ½ Ms media. Similarly, BAP induced more number of shoots in comparison to kinetin.

4.3.2.3 Length of longest shoot

Shoots of maximum of the shoot (1.53 cm) was observed in the media combination Nitsch + BAP 15.0 mg l^{-1} and of minimum length (0.65 cm) in half strength MS + BAP 15.0 mg l^{-1}. Nitsch media than ½ MS media supported better development of shoots for its length. Among the cytokinins, BAP favoured better shoot length and its effect was more pronounced when supplemented in Nitsch media (Table 55)

4.3.2.4 Number of leaves and roots

The number of leaves as well as shoots was maximum with cultures maintained in Nitsch media (Table 55). Maximum number of leaves (4.00) was recorded by Nitsch + BAP 10.00 mgl $^{-1}$ was maximum with shoots produced in Nitsch + BAP 12.5 mgl^{-1}. As with the previous growth parameters, number of leaves and roots were promoted better by Nitsch- than ½ MS -media.

4.4 *In vitro* mutagenesis

The callus induced from leaves from the callus regeneration phase and shoot tips were the explants selected for radiation breeding induced *in vitro* using γ- ray. Initially they were subjected to irradiation doses ranging from 100-400 Gy (Table 56). From the preliminary studies, it was found that doses above 200 Gy was lethal for callus as well as shoot tip explants. Further, though multiplication from irradiated callus at a low rate was observed with 200 Gy, they failed to regenerate into shoots. In case of shoot tips, occasionally meristem dropped off. Consequent to the above observations, the dose was fixed again at 25, 50, 75 and 100 Gy. Effect of irradiation on callus showed that as the irradiation dose increased callus multiplication rate was reduced (Table 57). Callus irradiated at lower doses (25 Gy) regenerated well than those at higher doses. Callus irradiated at higher doses (150 Gy) showed lowest survival (80%).

Per cent callus regeneration (Table 58) of all the irradiated callus was less than the unirradiated callus (88%). Among the doses used for irradiation, callus regeneration was maximum (83.43%) at the lower dose (25 Gy). At higher doses (150 Gy) per cent callus regeneration was reduced drastically (36.25) and took maximum number of days (62.67) for regeneration from callus.

Table 56. Response of various explants of *A. andreanum* cv. Dragon's Tongue to different doses of irradiation with g rays

Treatments (Gy)	Survival (%)			
	Callus (a)	Colour	Shoot tip (b)	Condition of meristem
10	85.00 (R)	Pale green	80.67 (M)	Normal
20	65.50 (NC)	Pale yellow	42.00 (NC)	Occasionally meristem dropped off
30	Dried	Dark brown	Dried	Yellowish brown
40	Dried	Dark brown	Dried	Yellowish brown

(R) = Callus regeneration observed
(M) = Shoot multiplication
(NC) = No further change
(a) = Nitsch white + BAP 1.5 mg l^{-1} (media for regeneration from callus)
(b) = Nitsch + BAP 0.5 mg l^{-1} (media for regeneration from shoot tips)
Number of replications = Three (One replication = 10 culture tubes)

Table 57. Effect of γ irradiation on regeneration/multiplication from callus

Treatment (Gy)	Callus survival (%)	Colour of callus	Degree of multiplication after one month
0	100.00	Pale yellow, friable	+++ (HR)
0.25	100.00	Pale yellow, friable	+++ (HR)
0.50	99.00	Pale green, globular	++ (R)
0.75	100.00	Pale green, globular	++ (R)
1.00	95.00	Yellow, compact	+ (R)
1.50	80.00	Yellow, compact	+ (R)

HR = Highly regenerative
R = Regeneration
\+ = Low callusing
++ = Medium callusing
+++ = Intense callusing

Number of replications=Ten(10)

Table 58. Effect of different doses of γ irradiation on callus regeneration of *A. andreanum* cv. Dragon's Tongue

Media: Nitsch + BAP 1.0 mg l^{-1}

Irradiation dose (Gy)	Callus Regeneration (%)	Days taken for callus regeneration
Control	88.30	45.66
0.25	83.43	49.00
0.50	75.12	53.33
0.75	64.73	55.33
1.00	61.75	58.00
1.50	36.25	62.67
CD (0.05)	0.10	4.30

Number of replications = 10

4.4.1 Effect of γ-ray on multiple shoot production from callus

4.4.1.1 Number of shoots

Number of shoots produced was less in the case of irradiated callus Table 59) compared to control (9.00 shoots per culture). Number of rootable shoots produced was maximum (5.33) from callus irradiated with 25 Gy. Number of shoots produced per culture decreased steadily with an increase in the dose of irradiation and the lowest number of shoots (1.66) were produced by those irradiated with a dose of 150 Gy.

4.4.1.2 Length of longest shoots

Length of the longest shoots also reduced steadily consequent to irradiation (Table 59). When shoots from unirradiated callus recorded 2.46 cm length after two months, regenerated shoots from irradiated callus recorded a range between 1.06-1.27 cm. Among the different doses, 25 Gy recorded maximum length (1.27 cm) and at 150 Gy recorded the minimum length (1.06 cm).

4.4.1.3 Number of leaves

Control recorded an average number of 4.6 leaves per plant, but irradiated cultures produced leaves in the range of 2.00-3.33 leaves per plant. The number of leaves produced decreased with an increase in dose of irradiation employed (Table 59).

Table 59 Effect of different doses of γ irradiation on shoot multiplication from irradiated callus of *A. andreanum* cv. Dragon's Tongue

Media - Nitisch + BAP 0.5 mg l^{-1}

Irradiation dose (Gy)	Number of shoots/culture	Length of longest shoot (cm)	Number of leaves
0.00	9.00	2.46	4.60
0.25	5.33	1.27	3.33
0.50	4.66	1.20	3.00
0.75	3.33	1.13	2.66
1.00	2.33	1.10	2.30
1.50	1.66	1.06	2.00
CD (0.05)	1.85	0.37	0.71

* Number of replications =Ten(10)
* Observations taken after 8 weeks

4.4.1.4 Time taken for shoot tip regeneration

Non -irradiated shoot tips took only 48 days for regeneration (Table 60). But, irradiated shoot tips depending on the dose took 58-85 days for regeneration. Lower doses (25 Gy) took lesser days (58.50) and higher doses (100 Gy) took 85 days for shoot tip regeneration. Gamma irradiation dose of 150 Gy was lethal for shoot tips.

4.4.1.5 Regeneration from shoot tips

Though 92 per cent of the non- irradiated culture showed regeneration, there was a drastic reduction in regeneration from irradiated shoot tips. With higher doses (100 Gy) the per cent regeneration was only 22.50 per cent compared to 25 Gy which recorded 70 per cent of cultures showing regeneration(Table 60).

4.4.1.6 Number of shoots

Non-irradiated shoot tips produced the maximum number of shoots (6-8 shoots/culture). But, the irradiated cultures produced lesser number shoots ranging from 2.0 to 4.5. Among the different doses of irradiation, 25 Gy recorded maximum number of shoots and the lowest number of shoots (2.0) was minimum (2.0) with100 Gy (Table 60).

4.4.1.7 Length of shoot and number of leaves

Length of shoot (2.2 cm) and number of leaves (3-4) recorded by plantlets regenerated from cultures irradiated with 25 Gy were comparable to those in plantlets from non-irradiated cultures (Table 60).

4.4.1.8 Response after transplanting

The data on different morphological characters are given in Table 61. Height and diameter of the plant, number of leaves, width and length of leaves (Plate 25) were reduced remarkably due to irradiation. This retarding effect was minimum in lower doses compared to higher doses.

4.4.1.8.1 Height of the plant

Height of the plant regenerated from non-irradiated calli was recorded at four months and six months after transplanting. After four and six months of growth, when control plants recorded a height of 13.30 cm and 20.50 cm respectively, plants from callus irradiated with the lowest dose of y-ray (25Gy) recorded only 6.03cm (after 4 months) and 13.60 cm (after 6 months). It was found that at the higher dose of irradiation, the height of the plant was reduced significantly. Height of the plant was 4.60cm (after 4 months) and 9.40 (after 6 months). Hence, it can be concluded from the data presented in Table 61 that dwarfing of regenerated plantlets was caused due to irradiation of the callus.

Table 60. Response of shoot tips to different doses of γ irradiation

Media - Nitsch + BAP 0.05 mg l^{-1}

Treatment (Gy)	Time taken for shoot tip regeneration	Regeneration (%)	Number of shoots/culture	Length of the longest shoot	Number of leaves
0	48.00	92.00	6.8	2.0	3-4
25	58.50	70.00	4.5	2.2	3-4
50	70.00	68.50	4.0	1.9	3-4
75	78.67	45.45	4.0	1.8	3-4
100	85.00	22.50	2.0	1.5	2-3
150	NC	NC	-	-	-
CD(0.05)	6.77	15.7	0.92	0.37	-

Number of replications =Four(4)

4.4.1.8.2 Canopy spread

Four months after transplanting maximum canopy spread (16.75cm) was recorded by plants regenerated from non-irradiated cultures those from 150 Gy treatment recorded the minimum (10.16cm). Canopy spread decreased with an increase in dose of irradiation and 150 Gy treatment recorded the minimum canopy spread (7.77cm). But, the pattern of development changed after four months of growth and the plants from irradiated cultures showed an active horizontal growth and consequently 50 Gy treatment recorded the maximum canopy spread (17.10 cm) which was more than the untreated plants.(16.85cm). The canopy spread of plants regenerated from callus irradiated with the lowest dose (25Gy) was also considerably improved (16.00cm) after 6 months. Though good canopy spread was recorded by all the different irradiation treatments after 6 months of growth, the rate of increase in canopy spread in the case of plants from highest dose of irradiation (150Gy) was less (Table 61).

4.4.1.8.3.1 Number of leaves

There was not much variation observed for the number of leaves produced in plants from irradiated callus (Table 61). But, at higher doses of irradiation (100 Gy and 150 Gy) there was a reduction in the number of leaves produced (5-6leaves/plant). This trend in response continued even after six months of growth.

Table 61 Effect of doses of -ray on growth parameters of plants regenerated from irradiated callus

Treatment (Gy)	Plant height (cm)		Canopy spread		Number of leaves	
	4month	6 months	4 months	6 months	4 months	6 months
Control(5)	13.30	20.50	16.75	16.85	6.66	7.66
25 (6)	6.03	13.60	9.83	16.60	7.60	8.60
50 (4)	6.16	13.76	10.16	17.10	7.60	8.66
75 (5)	6.00	13.60	9.80	16.73	7.33	7.66
100 (7)	5.40	13.26	9.50	16.00	6.00	6.33
150 (6)	4.60	9.40	7.77	10.67	5.30	6.00
CD (0.05)	0.40	0.44	0.59	1.69	0.74	0.83

4.4.1.8.4. Width, length and color of the leaves

Effect of irradiation with γ-rays on length, width and color of leaves was also recorded (Table 62) after four months and six months of growth. Maximum width (5.05 cm and 6.03 cm after four and six months respectively) was recorded by plants from callus irradiated with 50Gy which was more than those recorded by untreated plants. Higher dose of irradiation recorded a reduction in width of the leaf. And at 150 Gy the width of leaf was very much reduced (2.00 cm and 3.03 cm after four months and six months respectively.

The length of leaves was maximum in non-irradiated plants at four - (6.54 cm) as well as six -months (10.64 cm) stage. Among regenerated plants, those from callus irradiated with 50 Gy recorded better length (5.60 cm and 7.56 cm after four and six months respectively). The length of the leaves was the lowest in plants from treatment width highest dose (150 Gy irradiation)

No difference in leaf colour was noticed among plants regenerated from callus irradiated width different dose of irradiation.

4.4.1.8.5 Leaf area

Effect of irradiation on leaf area indicated that, maximum leaf area was recorded by plants from non-irradiated callus, 16.72 cm^2 and 40.11 cm^2 after four months and six months of growth(Table 62; Fig. 6; Plate 25). The lowest leaf area (0.37 cm^2) after four months and six months (2.58 cm^2) was recorded in the case of plants regenerated from callus irradiated with 150 Gy. A decreasing trend in the leaf area was observed from 75 Gy onwards. Among the

Table 62. Effect of doses of g-ray on length, width and colour of leaves of plants regenerated from irradiated callus upon transplanting

Treatments (Gy)	Width of leaf (cm)		Length of leaf (cm)		Colour of leaves
	4 months	6 months	4 months	6 months	
0.00 (5)*	4.86	5.73	6.54	10.64	Dark green
0.25 (8)2+	3.56	5.43	4.73	7.53	"
0.50 (8)2	5.05	6.03	5.60	7.56	"
0.75 (7)2	3.66	4.57	4.17	7.43	"
1.00 (7)3	2.03	4.20	3.53	5.63	"
1.50 (8)2	2.00	3.03	3.13	3.10	"
CD (0.05)	0.80	0.80	0.59	1.20	

* Number of plants evaluated
\+ Number of leaf variants

Table 63. Variation in leaf area of plants of *A. andreanum* cv. Dragon's Tongue after transplanting induced by irradiation

Treatment (Gy)	Leaf area (cm^2)		Frequency of variation
	4 months	6 months	
0.00 (5)*	16.72	40.11	Nil
0.25 (8)2+	8.03	25.55	25.00
0.50 (8)2	16.34	31.21	25.00
0.75 (7)2	6.86	20.47	37.50
1.00 (8)3	3.02	13.11	37.50
1.50 (5)2	0.37	2.58	40.00
CD (0.05)	4.00	6.84	-

* Number of plants evaluated
\+ Number of leaf variants

Figure 6

Leaf area of *in vitro* regenerated plants after 6 months of growth as influenced by the dose of gamma irradiation

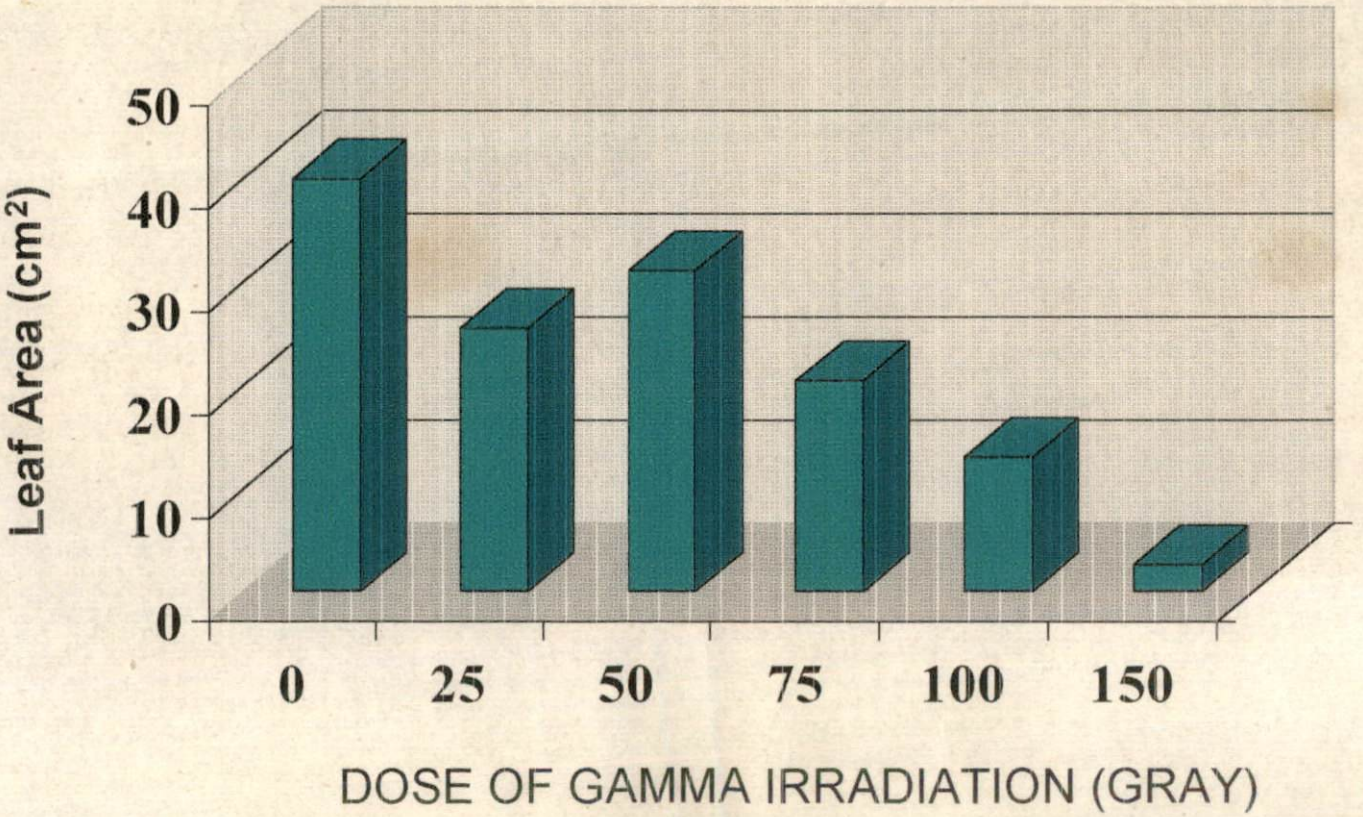


Plate 25. Variation in the leaf area in micropropagated plants due to irradiation with γ– rays (L to R : 150, 100, 50, 25 Gy and Control)

Plate 26. A comparison of plants regenerated irradiated (L) and unirradiated (R) callus

different irradiation doses treatment with 50 Gy recorded the maximum leaf area at four - (16.34 cm^{-2}) as well as six-months (31.21 cm^{-2}) stage, though less than the untreated plants.

The frequency of variation was found to increase with an increase in the dose for irradiation and accordingly the maximum variation (40%) was noticed at 150 Gy treatment (Table 63). Early suckering was observed in plants from untreated cultures after six months. However, no suckering was observed in any of the plants regenerated from irradiated callus (Plate 26).

4.5. Screening somaclonal variants

4.5.1. Isoenzyme electrophoresis

4.5.1.1. Evaluation of different procedures for enzyme extraction

Leaves were selected as source of enzyme extraction. Mature leaves gave better result for peroxidase and young leaves performed better for esterase enzymes. No result was observed with GOT enzymes. Initially sample buffer ratio was standardised (Table 64). Among four treatment tested sample buffer ratio 1:3 gave better quality of supernatant and bands developed were sharp brown for peroxidase and pale bands were observed with esterase enzymes. Ratio 1:4 gave pale bands in peroxidase but no staining observed in esterase enzyme. Leaf extract obtained by T_3 method (Table 65) displayed no tissue browning. After centrifugation a clear pale green supernatant was collected. Zymogram obtained from these samples exhibited least amount of streaking and the sharpest bands. Pale brown macerates were observed in the first extraction

Table 64 Evaluation of sample buffer ratio on the intensity/colour of bands

Sample (leaf): Buffer	Response	
	Esterase	Peroxidase
1 : 1	–	No supernatant recovered after centrifugation
1 : 2	–	Only 0.1 ml of supernatant recovered, bands developed were pale
1 : 3	Pale brown bands	Sharp brown bands observed
1 : 4	No staining	Pale bands resolved

Table 65. Effect of different methods of enzyme extraction from the leaf tissues of *A. andreanum* cv. Dragon's Tongue

Extraction buffer	Result
Tissue (leaf) + Tris buffer 0.2 M + (pH 7.3 PVPP	Slight brouning of the extract and no bands or spots developed on the gel
Tissue (leaf) + Tris buffer 0.2 M (pH 7.3) + sucrose 0.20 M + 0.002 M diethyl dithio carbamic acid + 10 Fl b-mercaptoethanol + a pinch of PVPP	No bands/spots developed on the gel
Tissue (leaf) + Tris buffer 0.2 M (pH 7.3) + sucrose 0.20 M + 0.002 M diethyl dithio carbonic acid + 10 Fl Triton - x + 10 Fl PMSF + 10Fl b mercaptoethanol + a pinch of PVPP	Produced sharp bands on the gel

method. The zymogram obtained from the 2nd method produced only pale brown streaks.

4.5.1.2. Effect of sample buffer ratio on the recovery of supernatant and intensity of bands

When the sample buffer ratio was 1:1 no supernatant recovered after centrifugation. 1:2 ratio produced supernatant, but the bands resolved were pale and 1:3 ratio produced the sharpest bands. Increasing the buffer ratio again decreased the colour of the bands.

4.5.1.3 Tank buffer ratio

Among the treatments tried 1 part tank buffer (Tris-glycine) 1 part water produced the maximum bands (4) among the treatments tried. 1:9 ratio did not recovered any bands. In 1:2 ratio 3 bands produced, out of that 2 were pale and one was thick and dark.

4.5.1.4 Effect of gel buffer concentration:

Out of the three types of gel prepared (Table 66), 10% polyacrylamide gave better resolution of bands for peroxidase and esterase enzymes. Twelve per cent polyacrylamide produced pale brown streaks in esterase.

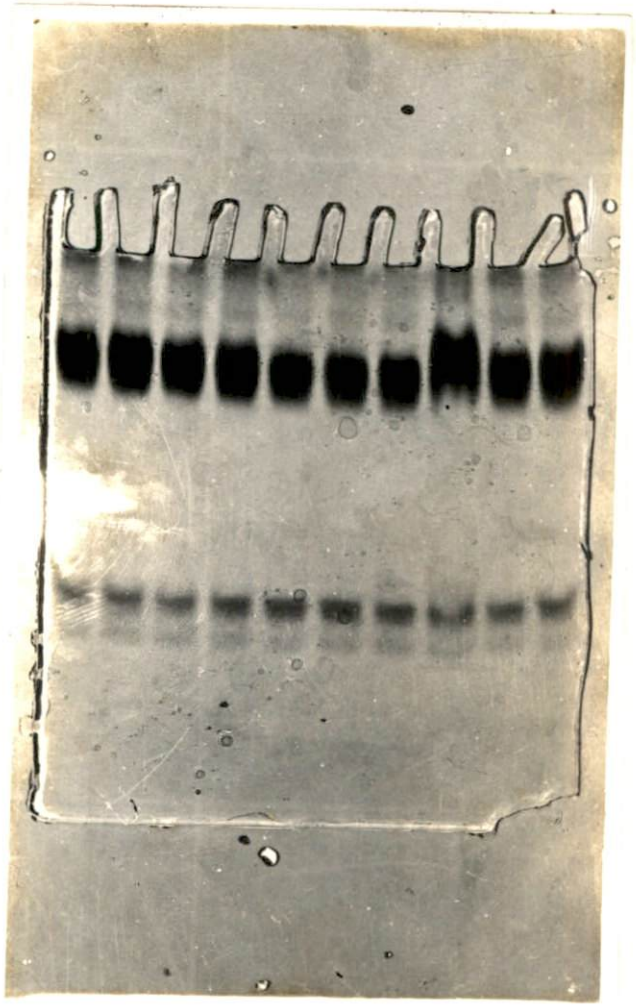
Table 66 Response of different ratios of tank buffer tried on the resolution of banding pattern

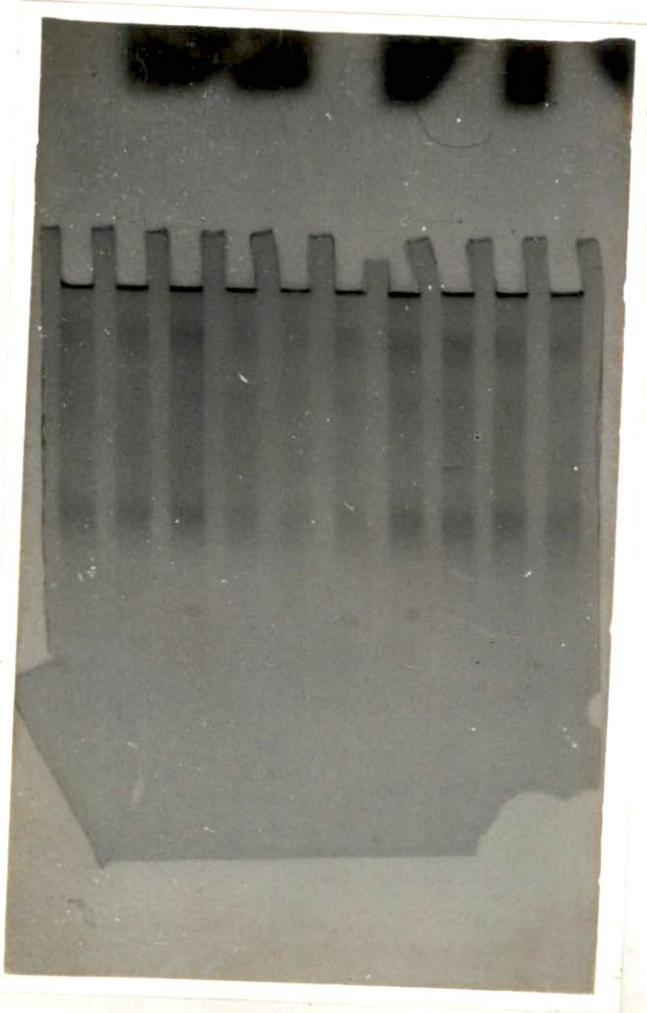
Enzyme : Peroxidase

Tank buffer : water (Tris-glyine)	Response
1 : 0	No resolution of bands
1 : 1	4 sharp brown bands recovered
1 : 2	2 medium & 2 one heavy band recovered

Plate 27. Zymogram of plants of *A. andreanum* regenerated from different subcultures for peroxidase enzyme
(L eft to Right: First to Tenth subculture)

Plate 28. Zymogram for esterase enzyme showing the effect of repeated subculturing
(L eft to Right: First to Tenth subculture)





4.5.1.5. Effect of different tank buffer dilutions on resolution of banding pattern

Among the 5 treatments tried T_5 gave better results. Totally five and two bands were resolved in T_5 (1:5) for peroxidase and esterase respectively. The intensity of each zymogram was of the order, one heavy, one medium and three light bands for peroxidase and two light bands in case of esterase in the same treatment.

Three isoenzymes assayed for screening variation among 10 serial subcultures revealed that there was no variation for peroxidase and esterase isoenzyme among the plants regenerated from callus which was subjected to repeated subculturing at three weeks interval, for induction of somaclonal variants. All the subcultures showed same type of banding pattern. Zymogram of peroxidase isoenzyme had a banding pattern with 5 bands with similar mobility and the zymogram of esterase isoenzyme exhibited a banding pattern with 2 bands with similar mobility. No result was obtained with GOT isoenzyme, as there was no zymogram development in the samples studied.

Three isoenzymes assayed for detecting the variants present in the irradiated plants revealed that there was no variation in banding pattern between plants regenerated from callus irradiated with the different doses of irradiation. All the irradiation doses tried exhibited medium bands with poor mobility. But, in the zymogram developed by the plants regenerated from non-irradiated callus showed five bands, which exhibited good mobility.

Table 67. Effect of gel buffer concentration on resolution of bands

Concentration(%)	Peroxidase	Esterase	GOT
6	No bands /staining	No bands /staining	No bands/ staining
10	Better resolution of bands	Better resolution of bands	No change
12	No bands/staining	Pale brown streak	No change

Composition of gel buffer is given in Chapter 3

Table 68. Response of different dilution of Tank buffer (Tris-glycine) on resolution of banding pattern

	Response (Intensity)		
Tank buffer dilution (Tris Glycine:Water)	Peroxidase	Esterase	GOT
1:0	No change	No change	No change
1:1	1-2 heavy and 1-2 medium bands but poor mobility	"	"
1:2	"	"	"
1:3	"	1-2 medium bands resolved, mobility was very poor	"
1:5	One very heavy one medium and 3 pale bands resolved. Good mobility (till 2/3 distance of the gel)	2 pale bands resolved; movement was good and covered 2/3rd distance of the gel	"

Table 69. Variation in isozyme banding pattern among tissues from plantlets regenerated from different Subcultures

Enzymes	Subculture cycles/ Number of bands 1 2 3 4 5 6 7 8 9 10	Pattern of bands
Peroxidase	5 5 5 5 5 5 5 5 5 5	one very,heavy, one medium, 3 light bands
Esterase	2 2 2 2 2 2 2 2 2 2	Light bands
GOT	No response	–

Table 70. Isozyme banding pattern in tissues of plants regenerated from irradiated callus

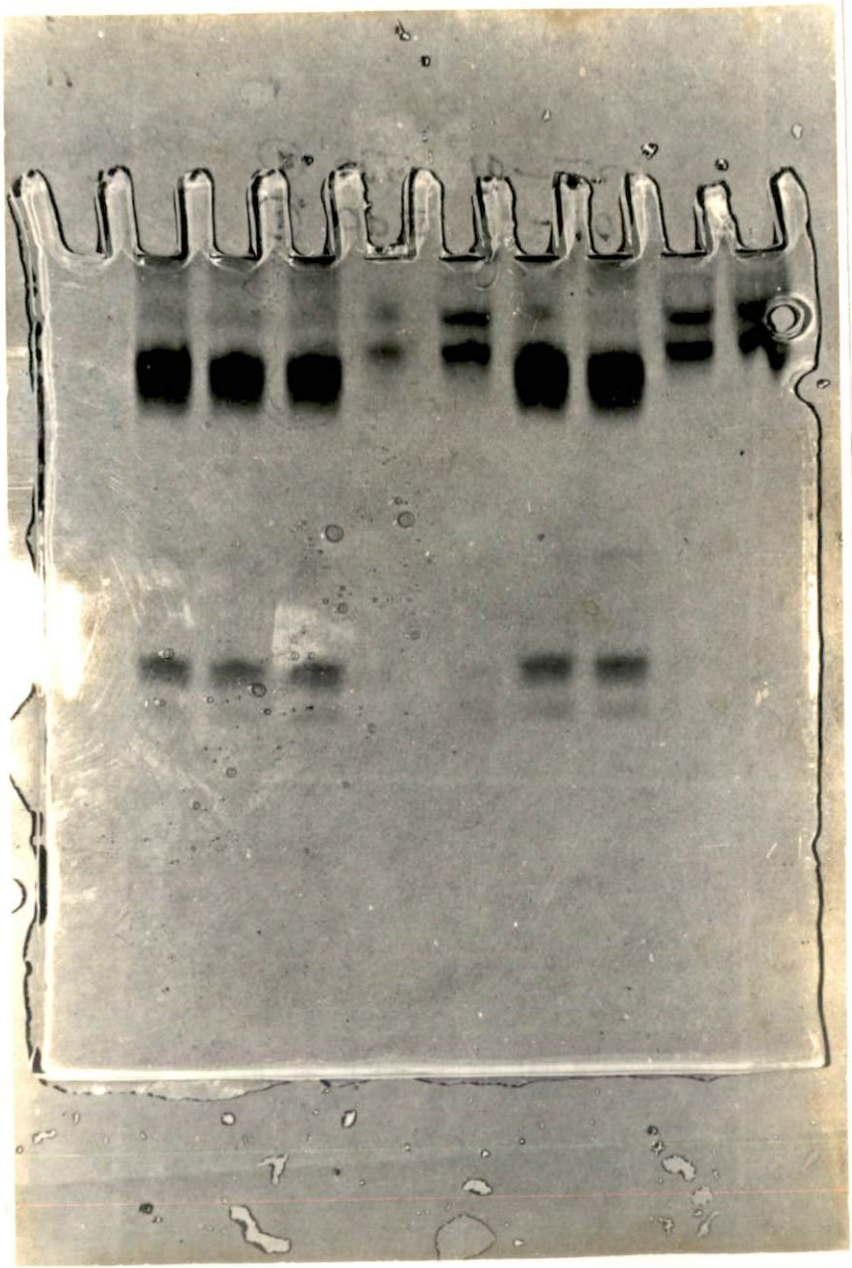
Isoenzyme system		Pattern of bands
	Number of bands	
	25 50 75 100 150	
Peroxidase enzyme	2 2 2 2 2	Light bands
Esterase	Bands not developed	–
GOT	Bands not developed	–

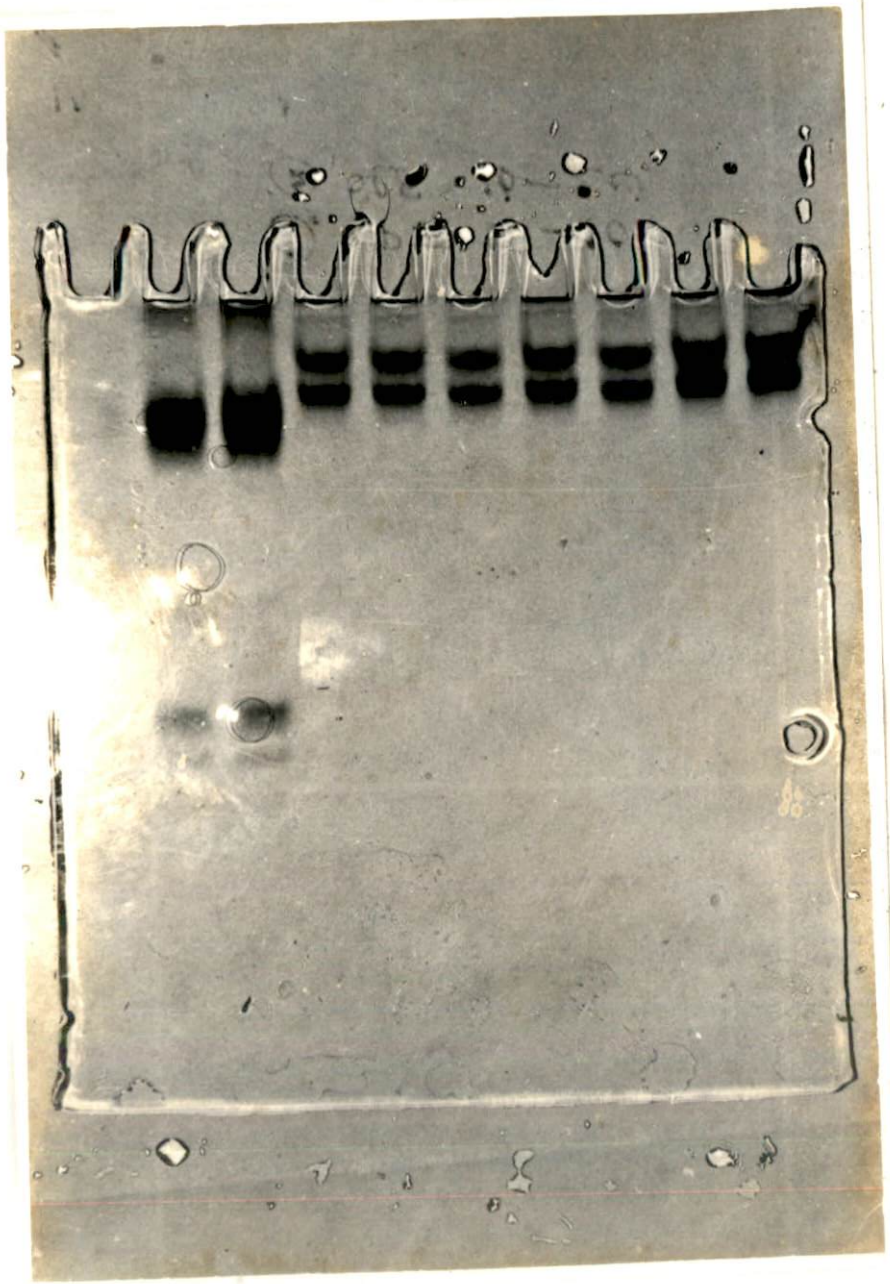
Table 71. Evaluation of peroxidase enzyme activity in three different cultivars of anthurium

Characters	Dragon's Tongue	Liver Red	White
Staining intensity	Medium-Heavy	Medium-Heavy	Light
Movement	Till 2/3 of the gel	Till 2/3 of the gel	Till ¼ of the gel
Number of bands	5	5	2

Plate 29. Zymogram for peroxidase enzyme showing the effect of irradiation
(Left to Right : Control, 150, 100, 75, 50 and 25 Gy)

Plate 30. Zymogram for peroxidase enzyme of three cultivars of anthurium





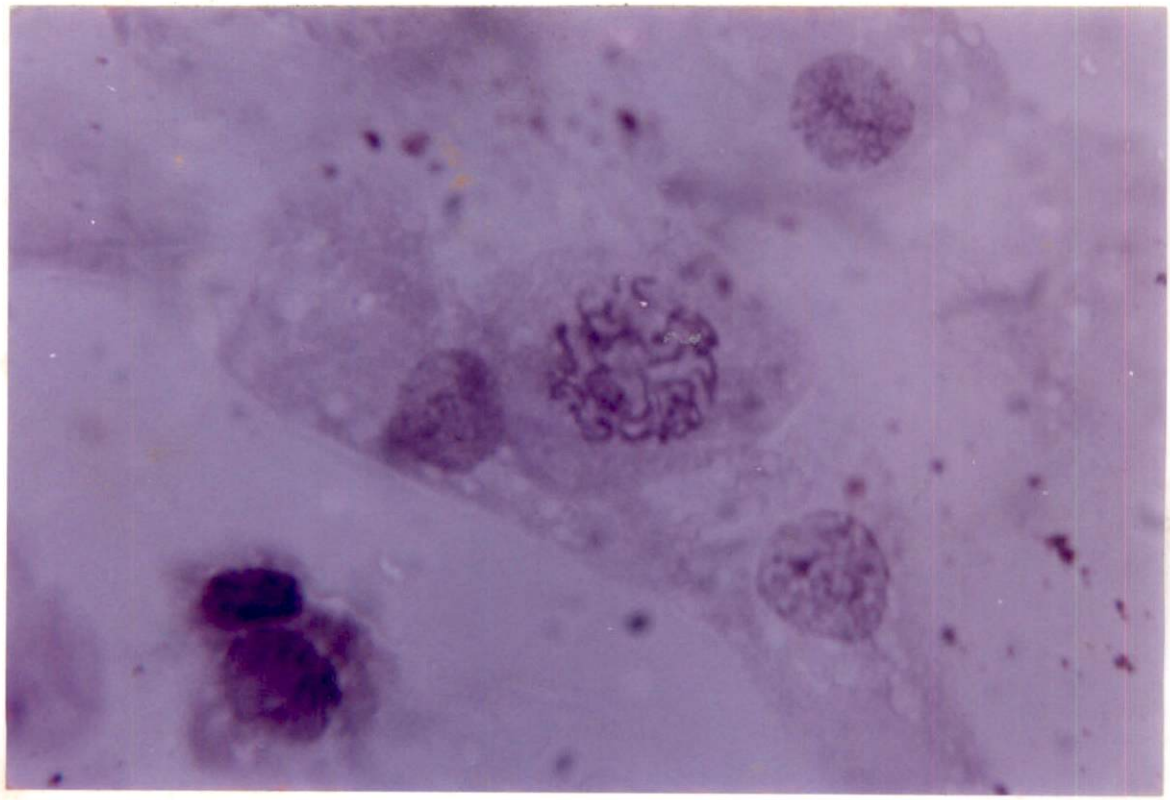
Eventhough no isoenzyme variation was observed among plants from different subcultures tried(Plate 27 and 28) and different doses of irradiation (Plate 29) of the cultivar 'Dragon's Tongue, isoenzyme variation was observed between two cultivars of different colours-white and red. Red cultivars such as Dragon's Tongue and Liver Red exhibited similar banding patterns with 5 bands each exhibiting similar mobility. But, in the White cultivar, banding pattern was entirely different with only 2 bands resolved exhibiting poor mobility (Plate 30).

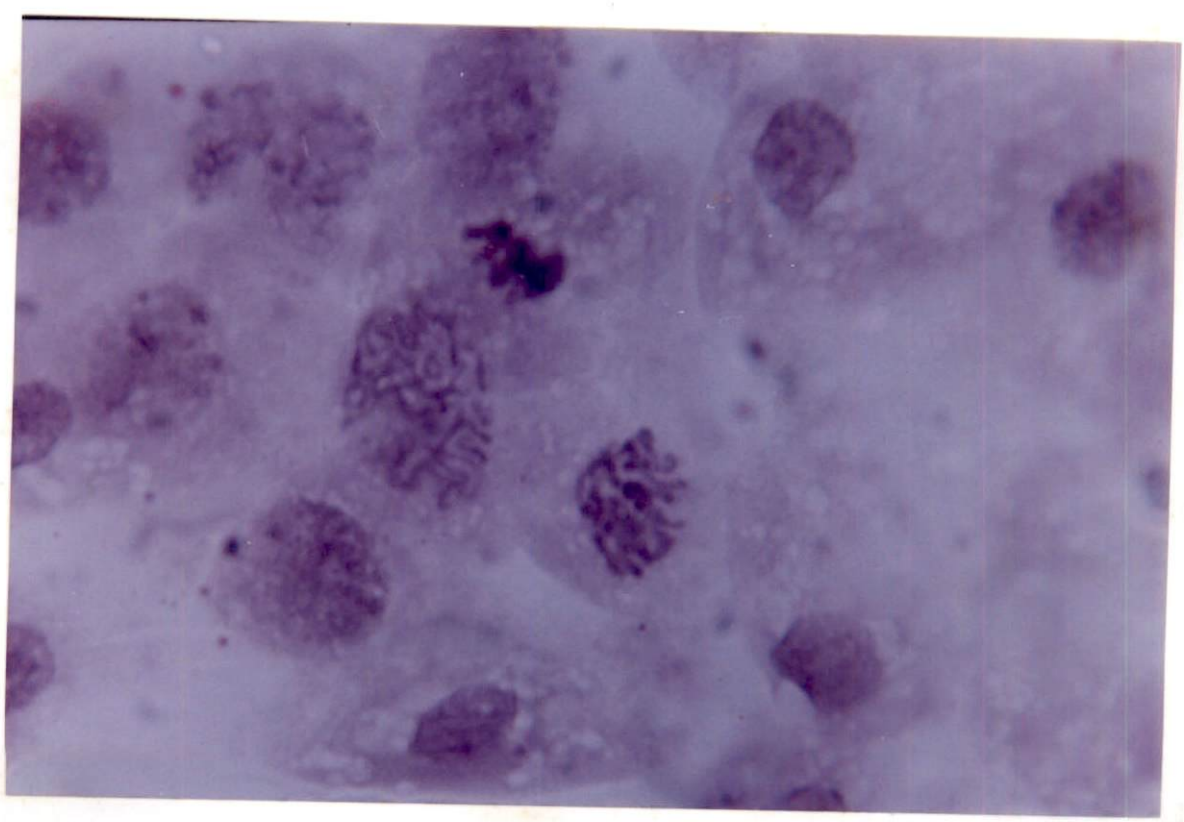
4.5.2. Cytological studies

Mitotic behaviour of the chromosome was studied and the results are presented. Mitosis of the root tip cells was studied in all the plants regenerated from 10 subcultures obtained by repeated subculturing and those from cultures irradiated with four levels of gamma irradiation. For all the plants regenerated from different repeated subcultures and irradiated cultures, somatic chromosome number of 30 + 2B was recorded (Plate 31). Mitosis was regular and all the dividing cells appeared normal. The B chromosomes of all the cultures in the present study were seen to be deeply stained, smaller in size than the smallest A chromosome of the genome, Since, plants different cultures had the same chromosome number (2n=30+2B) the karyograph for the plants from 5 th subculture (Plate 31) and those irradiated with 50 Gy (Plate 32) have been shown as a representative figure.

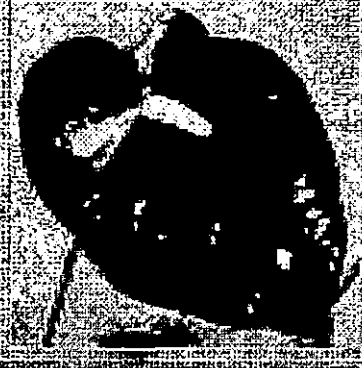
Plate 31. Mitotic behaviour of chromosomes of plants from the 5th subculture (2n=30+2B)

Plate 32. Mitotic behaviour of the chromosomes in plants regenerated from cultures irradiated with 50 Gy of γ rays





Discussion



5. DISCUSSION

Establishing and sustaining anthurium cut flower industry requires quality, disease free planting material and new varieties with improved flower characters and disease resistance. Less than expected performance of imported varieties and the 'Jamaican experience' of risk of introducing dreaded diseases which had even wiped out an already established anthurium flower industry, involved in importing exotic varieties from overseas warrants developing varieties indigenously. Mass propagation of elite clones through micropropagation or artificial- or synthetic- seeds /synseed-technology and meaningful development of varieties utilising *in vitro* techniques of crop improvement hold the promise for establishment and sustenance of anthurium industry, an infant one, in India. Refining protocols of establishing micropropagation system and *in vitro* crop improvement were the foci of the study.

A popular cut flower variety grown in the state 'Dragon's Tongue' was chosen as the experimental genotype. It is a shy suckering variety and produces inflorescence with maroon red spathe and cream coloured spadix positioned at an angle of 40^0 (Plate I) (Mercy and Dale, 1992).

Slow growth of plantlets *in vitro* and the time required for weaning are the major constraints in micropropagation of anthurium. In lieu of the difference in morphogenetic potential *in vitro* (Pierik 1975: Eapen and Rao, 1985) micropropagation protocols have been developed only for few varieties. *In vitro*

propagation protocol developed for popular variety like 'Dragon's Tongue' (Thomas, 1996) is far from the point of adoption for large scale multiplication due to its inherent problems of development of caulogenic call and improper foliage development, prolonged duration for callus induction and multiplication.

The salient results of the experiments covering protocol development for micropropagation, artificial seed technology, inducing somaclonal variation through repeated subculturing and media manipulation, creation of mutants through radiation breeding *in vitro* and screening of variants and mutants are discussed in this chapter.

5.1. Refining the protocol

5.1.1. Micropropagation

Murashige (1978) has recognised three major steps in tissue culture of any plant:

Step I : Establishment of aseptic cultures

Step II : Multiplication of propagule and

Step III : Preparation for re-establishment of plants in soil.

The requirements at each stage are different from one another.

5.1.1.1 Explants

Of various explants tried for callus induction and multiplication such as tender leaf sections, petiole segments, spadix segments, seeds and explants from aseptic cultures (leaf, petiole, single nodes and roots) the results have

shown that leaf sections and explants from the aseptic cultures are the best explants in terms of per cent callusing and intensity of callusing. A perusal of earlier literature shows that leaf sections have been widely used as explants by various workers. (Pierik *et al.*, 1974: Pierik 1975; Pierik 1976; Pierik *et al.*, 1979; Eapen and Rao, 1985; Geier, 1986a; Geier, 1986b; Lightbourn and Prasad, 1990; Kuehnle and Sugii 1991; Nirmala and Singh, 1998). The reason for this may be the better response and easy availability of the explant material. Explants obtained from aseptically grown plantlets slowed very good response because they are well acclimatized to the culture conditions and actively growing with most of the cells in meristematic condition. Root sections obtained from the aseptically grown plants recorded maximum response in terms of per cent callusing and intensity of callusing among the *in vitro* explants tried. Higher response of root explants may be due to the anatomical position of the explant on the mother plant, where polarity plays an important role.

The spadix explants recorded the minimum number of days for callus induction but number of cultures callusing was less (59.85 %) compared to other explants due to bacterial contamination. Geier (1986) reported that losses of spadix explants due to contamination were very high in *A. scherzerianum*. Another important observation noticed in the case of spadix explants was its high multiplication rate. They produced globular pale green calli, which have good potential to become plantlets. Early callus induction noticed in the spadix explants may be due to the fact that they are actively growing with most of the cells in meristematic condition. Callus induction and intensity of callusing were

low with seeds and petiole explants. It is reported that the plantlets derived from spadix are less variable (Singh *et al* ., 1991).

5.1.1.2. Surface sterilization

To remove the microbes present on the explants different surface sterilization treatments were given to various explants used. Explants from different parts may vary in their sensitivity to bleach solutions (George and Sherrington, 1984). As with most of the plant species, the higher percentage of explant survival reported in the present study depended on the duration of treatment, kind of explants and surface sterilants. Results of the investigation have indicated that, a combination of surface sterilants is more effective than using any single surface sterilant for all the type of explants. Microbial contamination is a major problem in the establishment of cultures in *in vivo* explants. Since plant parts are exposed, they harbour various microorganisms and many of which penetrate into the plant tissue resulting in systemic infection. cultures from such tissue are hence easily contaminated (Chen and Evans, 1990).

Among the explants, the highest per cent of sterile cultures (99.14) was observed in leaf sections using ethyl alcohol (70%) wipe + mercuric chloride 0.1% for eight minutes (Table 14). The per cent of sterile cultures was lower in case of explants from petiole (87.28), spadix (87.59) and seeds (87.85) even after the addition of emisan 0.1% dip for 3 minutes. A longer duration of surface sterilization with ethyl alcohol wipe + emisan 01% dip for 3 minutes + mercuric chloride 0.1% for 10 minutes was given for the spadix explants because the

spadix surface is not smooth like leaves or petioles and may harbour more microorganisms because of its surface composition.

Studies showed that vascular bundle of the leaves were smaller compared to that of other explants. So there will be less translocation of microorganisms along smaller conducting vessels (Sreelatha 1992). Thus the reduced microbial contamination of leaf explants (0.86%) may be due to smaller vascular bundles in their tissues.

5.1.1.3. Culture initiation

5.1.1.3.1. Media

NW medium [macronurients of Nitsch (Nitsch & Nitsch 1965) and vitamin components of Whites (Whites, 1943)] was found to be good for the initiation of cultures in leaf explants (86.50%), petiole (61.00%), seeds (35.33%) and for the explants derived from aseptically grown seedlings-leaf (75.33%), single nodes (78.66%) and roots (81.00%). Rajasekharan and Kumar (1994) observed the positive effect of NW media for vegetative mass propagation of *A. andreanum* and *A. scherzerianum* from somatic tissue- and organ-cultures. In the present study, basal Nitsch media showed better response than MMS media for callus initiation. But, in the case of spadix explants, half strength MS media recorded the maximum response (59.85 %). Nirmala (1989) observed that MMS medium supported callus growth and plantlet differentiation in spadix explants. Anu (1998) also observed the positive effect of half strength MS media and callus induction in explants from spadix. The basal medium requirement is highly tissue dependent and varies from one variety to another.

5.1.1.3.2. Influence of growth regulators

The importance of growth regulators in plant cell culture came in 1955 with the discovery of cytokinins as regulators of cell division (Murashige and Skoog, 1962). Among auxins, 2,4-D is the most potent and where it stimulates callus cultures, it strongly antagonize organised development (Murashige, 1974). Cytokinins are used in combination with auxin for initiation as well as maintenance of callus cultures.

For callus induction, various combinations and concentrations of growth regulators were added to the medium. Among the various combinations and concentrations of auxins and cytokinins tried in the present study, callusing was highest (86.50%) with kinetin 0.5mg/l + 2,4-D 0.3mg/l in leaf explants. Spadix segments showed better callusing (59.85%) at 2,4-D 3mg/l + kinetin 0.5 mg/l and 2,4-D 0.5mg/l + kinetin 0.75 mg/l recorded 61.00% and 35.33% callusing for petiole sections and seed explants respectively. In the case of *in vitro* explants kinetin 1.0mgl^{-1} + 2,4-D 1.0 mgl^{-1} + glutamine 200 mgl^{-1} recorded the maximum per cent (90.18) callusing. In all the treatment combinations, concentrations of 2,4-D were found to be more critical for the explants of *in vivo* origin. Among the growth regulator combinations, low concentration of 2,4-D over cytokinin was found to be favourable for callus induction. Addition of glutamine improved the per cent callus induction in *in vitro* derived explants but no effect on *in vivo* explants was noticed. For *in vitro* derived explants equal concentrations of auxin and cytokinin recorded favourable response. This difference in response may

probably be due to the difference in the endogenous hormonal levels in the explants.

In terms of callus multiplication, it was found that the quantum of callus was more in *in vivo* explants than *in vitro* explants. The calli produced from *in vitro* explants were more or less embryogenic and tendency was more or less towards development of plantlets than caulogenesis and then to rhizogenesis. The reason may be due to the difference in the endogenous hormonal levels between the *in vivo*- and *in vitro*-derived explants.

5.1.1.3.3 Culture Media

Among the culture media tried for callus regeneration, Nitsch media recorded the maximum sprout regeneration. The number of days taken for regeneration was also reduced to 45. But for spadix explants, sprout regeneration was maximum in half strength MS (1962) medium. Nirmala and Singh (1998) reported that leaf segments and petiole explants showed response when cultured onto MMS medium and for explants from spadix, Nitsch medium was more suitable. So, it is clear that the response vary with the kind of explant used. It may be due to the specific requirement of each explant and also the fact that ionic composition is more important than mere quantity of the nutrients used in the medium.

5.1.1.3.4. Culture conditions

Unlike the callus induction, callus multiplication and organogenesis was found to be promoted by light. The dark grown callus cultures contain plastids lacking chlorophyll. When they are transferred to light the plastids develop the green pigment chlorophyll and become chloroplasts (George and Sherrington, 1984). Chlorophyll is required for photosynthesis, which in turn promotes shoot regeneration.

5.1.1.3.5 Effect of growth regulators

The interaction and balance between the plant growth substances supplied in the medium regulate growth and morphogenesis in vitro. In the present study, BAP0.5mg/l recorded the maximum sprout regeneration per cent (92.00%) in the case of leaf explants. The treatment also showed better shoot production (12.30). Days taken for regeneration from callus was minimum (35.33 days) with 1.0 mg/l concentration of kinetin. Maximum number of rootable shoots (13.00) were produced when cytokinin (BAP 1.0 mgl^{-1}) was combined with auxin (IAA 2.0mgl^{-1}). IAA with BAP in most of the cases induced callus and shoot formation and response was also found to be good (Nirmala, 1995). The length of the shoots was higher (4.7cm) in the BAP + IAA combination (1.5mgl^{-1} + 2.0mgl^{-1}). Auxin in the regeneration medium promotes cell elongation and may be used to nullify the suppressive effect of cytokinins on shoot regeneration (Lundergan and Janick, 1980). In the case of organogenesis from callus induced from spadix explants 1/2 Ms medium showed higher sprout regeneration (71.42%).

5.1.1.4. Callus multiplication and regeneration

5.1.1.4.1. Explants

Among the various *in vivo* explants used, leaf and spadix explants showed maximum callus multiplication (CI 338.21 and 209.44 respectively). Explants from petiole and seed showed comparatively poor multiplication and regeneration. Regeneration of shoots from callus also showed a similar trend in response with leaf (92.00%) and spadix (71.42%) explants. According to Sreelatha (1992) morphological and physiological stages of the explant can account for the difference in response. The less lignified tissue of the leaf may facilitate easy de-differentiation process than the tissue of other plant parts. In the present study, comparison of in *vivo* and in vitro explants which took 45 days in presence of BAP 2.0 mgl^{-1} + kinetin 2.0 mgl^{-1}. Plants regenerated from spadix explants are less variable(Singh *et al.*, 1991).

5.1.1.4.2. Leaf enlargement

The present study clearly showed that leaf development in culture is a process determined by the cumulative effect of a number of factors such as, type of media, carbon source, media supplements and the culture vessel. Use of Nitsch media fortified with a combination of sucrose 20 gl^{-1} + glucose 10 gl^{-1} along with 200 mgl^{-1} glutamine and culturing shoots in conical flask (200ml) recorded the maximum width of the leaf (0.74cm).

5.1.1.4.3. Rooting, transplanting and hardening

Rooting of the plantlets was spontaneous and no special treatment was required. The high level of endogenous auxin and the prolonged exposure to light (for shoot proliferation) might have enhanced spontaneous rooting of shoots. Transplanting was taken up when the plantlets had developed 4-5 leaves and 3-4 roots.

5.1.1.4.4. Effect of planting method and containers during hardening

For the quicker initial establishment and maximum survival of plantlets at stage IV, cluster planting was found suitable as it recorded 85. 33% establishment after two weeks. More the leaf area, the more could be the photointerception and stored energy. So, the method of planting and the containers which can produce larger leaf area could be taken as the ideal one for stage IV hardening and planting out of *in vitro* regenerated plantlets and the consequent establishment of plantlets. Cluster planting in mini pots (mud pots) recorded maximum individual leaf size measured as width (0.96cm) and survival and establishment (85.33%) after two weeks of planting out. Among the media tried, coarse sand + Cocopeat (1:1) recorded maximum establishment of plantlets *ex vitro* and leaf width (1.00cm) after six weeks. After six weeks of hardening they were transplanted to mud pots, in a mixture of sand + brick pieces + coconut husk pieces + charcoal (1:1:1:1).

5.2. Somatic embryogenesis

In vitro propagation of anthurium hybrids is widely used commercially, but has shortcoming in being either relatively slow (bud culture) or unreliable with occasional somaclonal variation (callus culture). But the plants developed from somatic embryos are true to type. As transgenic plants have been recovered from somatic embryos treated with *Agrobacterium tumafaciens* (Mc Granhan *et al.*,., 1988; 1990) and particle bombardment (Fitch *et al.*,., 1990), development of an embryogenic system would be important in facilitating micropropagation and genetic engineering of anthuriums.

5.2.1.Explants

Among the *in vivo* explants used in the present study, only seed explants showed response(8.33 %) to embryogenesis induction treatments. Among *in vitro* explants used, eventhough callusing was observed from all the explants, only leaves (53.00%) and petiole (18.90%) produced embryogenic callus. For indirect somatic embryogenesis certain tissue explants gave a better yield of embryogenic calli and embryoids (William and Maheswaran, 1986) and according to Pedroso and Pais (1995) explants region specific to embryogenic competence occurred in the leaf, stem segments and cotyledons of *Camellia sp.* Excised zygotic embryos, particularly immature ones have permitted development of embryogenic cultures in many recalcitrant plants (Ammirato 1983).

Red varieties of anthurium are observed to be showing high degree of recalcitrance *in vitro*. Anu (1998) reported that none of the treatments tried was able to induce somatic embryogenesis in a red variety of anthurium.

In the present study, the possible use of *in vitro* leaves for the production of somatic embryoids in anthurium was seen. Increased recalcitrance observed with *in vivo* explants of anthurium in general and red varieties in particular, with respect to somatic embryogenesis induction treatments could be overcome by the use of *in vitro* derived leaf explants as observed in the present study.

5.2.2.Surface sterilization

Survival of the explants from seeds were maximum (87.85%) when they were subjected to the treatment, emisan 0.1per cent dip for 3minutes + mercuric chloride 0.1 per cent for 8 minutes. Explants from *in vitro* culture didnot require any surface sterilization treatment. Effective use of mercuric chloride as a surface sterilant at 0.1per cent level has been reported in dendrobium (Devi 1992) and many other ornamental plants.

5.2.3. Induction of somatic embryoids

Within two months of culture in dark with subculturing at two weeks interval onto fresh media, translucent embryogenic calli were observed along the cut surface of both whole leaf blade and petiole explants derived from *in vitro* cultures. In the case of seeds, embryogenic calli produced was from hypocotyl.

Leaf and petiole explants produced abundant embryogenic calli when cultured on NW medium, but for seeds ideal media for embryogenic callus induction was Nitsch media. The concentration of inorganic salts in the basal medium influenced the initiation of somatic embryoids. Response of petiole explants to somatic embryogenesis was less (18.90%) compared to that of explants from leaves (53.00%). Similar effect was noted by Kuhnle (1992) when petiole explants of the genotype tested were inferior in somatic embryo production compared to whole leaf blades.

For the initiation of somatic embryoids from embryo mass produced from leaves, 2,4-D 2.0 mgl^{-1} + kinetin 0.75 mgl^{-1} recorded the maximum response (53.00%). Increasing kinetin from 0.3mgl^{-1} to 0.5 mgl^{-1} while holding the 2., 4-D concentration at 2.0 mgl^{-1} improved the response from 9% to 20%. The results obtained in the present study is in agreement to the findings of an earlier study in anthurium (Kuehnle, 1992).

A supply of reduced nitrogen was required in the form of NH_4^+ ion and /or as an amino acid such as glutamine or alanine is beneficial at the embryo initiation stages (Wetherell and Dougall 1976). In the present study also, supplementing the media with glutamine 200mgl^{-1} recorded the maximum response in terms of embryogenic culture initiation (ranging from 43 to 53 %). An absolute necessity for glutamine in the culture media was seen in the present study, for the initiation and maturation of somatic embryoids.

5.2.4. Maturation of somatic embryoids

It became evident that a major limiting factor in the development of synthetic seeds was the quality the somatic embryos in terms of their ability to germinate readily and form vigorous quality seedling plants. The criteria for the maturation of somatic embryoids were the size of the embryoids. The growth inhibitor abscissic acid (ABA) influenced the quality of embryoids in the present study. Various concentrations of ABA (0.1 to 0.5mgl^{-1}) were tried along with 2,4-D, kinetin and glutamine for the maturation of somatic embryos. Number of embryoids/culture was maximum in the treatment combination involving NW media + 2,4-D 10mgl^{-1} + kinetin 0.15 mgl^{-1} + ABA 0.1mgl^{-1} + glutamine 400mgl^{-1} + sucrose 30 gl^{-1}. Elevated osmolarity can effect both the induction of embryogenic growth as well as embryo maturation (Ammirato, 1985). But, in the present study increased sucrose levels did not show any added effect on embryoid maturation. Sucrose level 30gl^{-1} recorded the maximum number (3.70/culture) and size (0.2cm) of embryoids. Sucrose supplied in the culture media at a concentration of 30 gl^{-1} was found to be a better carbon source for callus initiation in anthurium (Thomas, 1996).

The effect of light on maturation of somatic embryoids was studied. Average number of embryoids per culture (3.60) and the size of embryoids (0.22cm) were favoured by light. Dewald *et al.*, (1989) found that incubating the maturing embryogenic cultures in darkness was beneficial.

5.2.5. Germination of somatic embryoids

Poor germination is typical in many embryogenic culture systems. In the present study, germination of somatic embryoids was attempted using twelve different treatment combinations. In certain treatments abnormal development was observed. Growth regulatory factors and osmolarity were recognised as important factor for controlling developments in somatic embryogenesis (Dewald *et al.*,, 1989). Of the different media tested to germinate somatic embryos, half strength MS medium with pH 5.6 + glutamine 200mgl^{-1} + sucrose 20gl^{-1} + glucose 10 gl^{-1} recorded the maximum germination (46%). After germination and development of plantlets, the cultures were moved to continuous light regime. The plantlets became green, elongated and ready for transfer to *extra vitrum* conditions.

The whole regeneration cycle from somatic embryos into plantlets beginning with the induction of embryos, passing through the phase of induction, maturation and germination took about five months.

5.2.6. Encapsulation and conversion of somatic embryos for production of synthetic seeds

The production of synseeds by coating a matrix around cells, somatic tissues, somatic embryos and obtaining plants from these encapsulated embryos is termed as synthetic seed /synseeds or artificial seeds (Redenbaugh *et al.*, 1986). Hydrogel is the material used for encapsulation. It forms a thin coat covering the inside material, there by protecting it from outside stress and strain.

In the present study, sodium alginate was used as the gelling agent. Sodium alginate at 3% concentration gave beads that gave enough protection to the plant material, easy handling and good regeneration. It is the best hydrogel that could be used for encapsulation because of easy complexation with calcium chloride (50μM) through ion exchange, biologically non-damaging, biodegradable, universal availability and low price (Mathur, 1993).

5.2.7. Storage and regeneration studies

Regeneration or plantlets conversion is defined as per cent of the somatic embryos that produce complete plant with a normal phenotype. Conversion has been accepted as more appropriate term in relation to artificial seeds.

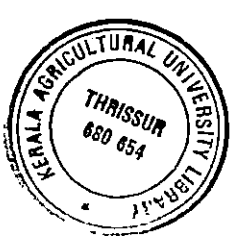
Plant tissue is the ideal material for the conservation of germplasm. This is possible through cryopreservation and low temperature storage of artificial seeds.

In this connection, the present study was directed to identify the storage behaviour of encapsulated and non-encapsulated somatic embryos. It was observed that those non-encapsulated somatic embryos when kept for long time lost their viability. This is further evident from the work of Datta and Portykus (1989). This indicates that somatic embryos as such cannot be stored and should be immediately cultured or encapsulated.

5.2.8. Regeneration of encapsulated somatic embryos

Sodium alginate encapsulation was tried by many workers (Bapat and Rao, 1983) and reported to be the best hydrogel for maximum significant variation observed between the regeneration of encapsulated and non-encapsulated embryos in all the media combinations tried. This may be due to the fact that non-encapsulated embryos are directly in contact with the conversion medium as opposed to encapsulated somatic embryos. Conversion was maximum in half strength MS (1962) medium by non-encapsulated embryos (48.32 %) followed by 32.67 % in the case of encapsulated embryos. Addition of low concentration of cytokinin (BAP 0.1mg/l) to the media increased the germination per cent in encapsulated (32.00%) and non-encapsulated (48.00%) embryos.

Conversion frequencies of encapsulated and non-encapsulated embryos stored at 40C were varied. Conversion frequency of encapsulated embryos improved after low temperature storage for a period of 10-20 days whereas non-encapsulated embryos did not germinate after the different storage periods. Similar response of somatic embryos was evidenced also in the works of Bapat and Rao, 1990. Hence, from the present study it can be concluded that for the storage of somatic embryos encapsulation is essential. Conversion frequency of the encapsulated somatic embryos can be improved by low temperature storage. Encapsulated somatic embryos can be stored without loss of viability up to 20 days.



The present study demonstrated that somatic embryogenesis from *A. andreanum* cv. 'Dragon's Tongue' is influenced by the explant, conditions of incubation of cultures (light), culture media, sucrose concentration, presence of glutamine and genotype. The protocol developed herein may not be competitive with current large scale propagation methods, but do have the potential for commercial exploitation, if the rate of somatic embryogenesis and conversion of embryos to plantlets can be improved. The protocol developed from the present investigation may be useful for recovering unique genotype generated by mutation or genetic engineering techniques.

5.3. Induction of somaclonal variation

An attempt to induce somaclonal variation in anthurium was attempted by subjecting callus to repeated subculturing and culturing onto media containing higher concentrations of cytokinins. The details of somaclonal variants induced by the above methods are discussed

5.3.1. Repeated subculturing

It was reported that the cultures maintained *in vitro* for a long period are excellent source of variation (Barbier and Dalieu, 1980). The degree of variation increased slightly with subculture time in *Dendranthema sp.* (Fei and Zho, 1994). In the present study, variation was observed among plantlets for growth and development *in vitro*. Number of shoots produced per culture increased from fourth subculture onwards. Early multiple shoot production occurred in sixth and seventh subcultures. Tenth subculture took maximum time for shoot doubling.

Plants from ninth and tenth subculture had small leaves and were of pale green colour. There was an increase in the number of shoots from 4th to 8th subculture which may be due to freshly cut shoot base being exposed to renewed levels of cytokinin in the medium at each subculture. It was reported that continuous subculturing modifies the physiological state of the plant in such a way that it favours the revitalisation of innate dormant vegetative buds (David 1982).

After transplanting, variation was noted in growth habit of plants from 9th and 10th subcultures. The leaf size was reduced compared to plants from other subcultures. But, the frequency of such variation was only 2-3%. Early suckering was noted in all the plants regenerated from different subcultures up to the 8th subculture. Quantitative aspects of somaclonal variation was reported by Schwenkel and Grunwaldt (1990) in cyclamen . They observed variation in growth habit, flower shape and shape and size of the leaves in *in vitro* regenerated plants. Israeli *et al.*, (1992) observed that dwarfism was the most commonly occurring variation in morphological characters among the somaclonal variants in banana.

5.3.2. High concentration of cytokinins

Concentrations of cytokinins above 15 mg/l resulted only in swelling of the explants. So the concentrations for treatment combinations were again refixed as 10,12.5 and 15 mgl^{-1}. Though, shoot multiplication occurred in all the cases vertical growth was absent and leaves produced were rudimentary. Nirmala and Singh (1998) noted similar effects of high concentrations of cytokinins earlier in anthurium. High concentrations of cytokinins induced an

inhibitory effect on shoot formation. Fasciation and aberrant phyllotaxis of regenerated shoots were promoted by increasing concentrations of growth regulators, especially of cytokinins in Kalanchoe tissue culture(Huitema *et al.*, 1990).

5.3.2. *In vitro* mutagenesis

Radiobiological studies on plant tissue cultures could provide basic information on cell growth behaviour, their radio-sensitivity and induction of mutations. Shoot tips from aseptic cultures and callus were subjected to varying does of γ- irradiation. Initially they were subjected to γ- irradiation treatments at 100-400 Gy. Maximum survival consequent to irradiation of the shoot tips (85%) and callus (80%) was observed at 100 Gy. At higher doses above 200 Gy, explants turned yellowish to brown colour and no further response was seen. Bajaj *et al.*, (1970) also noted yellowing of callus at higher does. Hence, in the present study the dose was refined in the range of 25 Gy to 150 Gy. Here also lower doses of γ-irradiation recorded good multiplication from callus and shoot tips. Increasing doses of gamma-irradiation had negative effect. In the case of ionising radiation, several kinds of events may occur such as disruption to water molecule, DNA, enzymes, growth substances etc., leading to disturbances in the mechanisms responsible for homeostasis in the cell. As a result of above events different responses are observed consequent to irradiation treatment with gamma rays, commonly employed in radiation breeding (Walther and Kaudro, 1981). In the present trials it was also noted that there is

striking difference in the radiosensitivity of shoot tips and calli. Shoot tips were more sensitive to radiation and callus tissue being more tolerant.

In the case of callus regeneration also, higher doses of irradiation reduced the per cent of callus regeneration and increased the days taken for regeneration.

Stimulation of growth of callus at a lower dose of 25 Gy was observed in the present investigation. Similar reports were recorded by Bajaj *et al.,* (1970).

In the case of shoot tips, gamma irradiation delayed the bud break and also reduced the per cent regeneration. Number of shoots produced per culture was also reduced as a result of irradiation. Plants obtained from tissues irradiated with lower doses of γ-rays showed slight increase in the length of leaves, but number of leaves produced was not affected.

5.4. Screening somaclonal variants

Regenerates from tissue cultures often show much genetic variation. Such variation induced by culture conditions is called somaclonal variation (Larkin and Scowcroft, 1981). It provides new genetic variability to the advantages of the plant breeder. These somaclonal variants may be genetic or epigenetic. The ornamentals in which somaclonal variation has been induced and exploited commercially are fuchsias, begonia and chrysanthemums. The variants include alterations in plant pigmentation, plant vigour and size, leaf and flower morphology, diseases tolerance or resistance etc. (Chadha, 1993). There

are different methods to assess the somaclonal variation like morphological markers, isozyme markers and cytological techniques.

5.4.1 Morphological variation

5.4.1.1 Repeated subculturing

The results showed a shifting of growth rate among the plants from different subcultures at monthly intervals. But leaf length and width showed a significant variation among the different subcultures in repeated subculturing. Plants from early subcultures recorded better leaf area compared to the last subcultures showing variation in the leaf area of the regenerated plants from repeated subculturing process. Plants from ninth and tenth subcultures recorded lowest leaf area and showed poor growth and development of plants.

5.4.1.2. *In vitro* mutagenesis

Height, canopy spread and leaf area of the non-irradiated plants were much better than the irradiated plants. Among the plants regenerated from irradiated callus mutants from lower doses (25 and 50 Gy) recorded better growth though poorer than control, compared to those obtained from higher doses (100 and 150 Gy). Height, canopy spread and leaf area were considerably reduced at 100 and 150 Gy. Several workers in a number of crops (Walther and Saucer, 1986b) reported retarding effect of higher doses of γ-rays used for irradiation. Most significant variation observed was the reduction in leaf size among plants obtained from irradiating with doses of 100 and 150 Gy.

5.4.2 Isoenzyme studies

Poor zymogram quality seen in the present study, resulting from dark streaking seems to be related to tissue browning noted during the grinding process. Ku and Fretz (1978) reported that sample preparation for electrophoresis could be complicated by interaction occurring between proteins and other cell matter such as carbohydrates, phenolic compounds, hydrolytic and oxidative enzymes. It was also reported that a buffer containing reducing agents such as ascorbic acid, mercaptoethanol, metabisulphite and a phenolic oxidase inhibitor diethyl dithiocarbamic acid was effective in enzyme extraction from tannin rich *Camellia japonica*. Phenol removal can be accomplished by addition of PVPP (Kobayashi *et al.*, 1987). In the present study also, procedures were developed to extract active enzymes from anthurium leaves. Clear zymogram were obtained from only one of the three extraction methods examined, which consisted of grinding leaf tissue in extraction buffer containing phenol oxidase inhibitor, reducing agent and detergent (to break peroxisome) and PVPP. Sample buffer ratio for the best quality zymograms was found to be 1:3. Polyacrylamide(10%) was selected to evaluate the enzyme systems. Tank buffer was diluted to 1:4 for the better resolution of bands. Earlier studies on electrophoresis in anthurium revealed that out of the seven enzymes studied peroxidase was the most useful isoenzyme system for distinguishing cultivars (Kobayashi *et al.*, 1987). In the present study also peroxidase was found to be the most stable, and was expressed in different subcultures as well as in irradiated cultures. Data on peroxidase suggested that, there was no difference

among the banding patterns of plants from different subcultures and irradiated cultures. But variation was observed between the control and irradiated cultures. Five bands resolved in control with good mobility, but only two bands were resolved in irradiated cultures. Variation in banding pattern was also observed between the cultivars, red cultivar (Liver Red) showing five bands and white cutivar (White) showed two bands of medium intensity.

For esterase enzyme also, banding patterns with two bands with good mobility in different subcultures was seen. But, there was no response shown by plant extracts from irradiated cultures. However, the potential of isozymes as a tool in genetic studies of anthurium and cultivar identification seems to be indicated by the data from the study.

5.4.2 Cytological studies

Mitosis of the root tip cells studied in all the subcultures and irradiated cultures of *Anthurium andreanum* cv. Dragon's Tongue recorded a somatic chromosome number of 2n =30+2B. This is in conformity with the earlier observation of Bindhu (1992) in different varieties of *A. andreanum.* Sheffer and Croat (1983) reported that the most common somatic number of the genus anthurium is 30 and B chromosomes are basic features of the genus Anthurium. Studying the chromosome behavior of in vitro regenerate *A. scherzerianum*, Geier (1988) reported that, as long as high level of organogenesis is maintained in cultures, the risk of change in ploidy is negligible. In the present study, no correlation were found between zymotype and morphotype or ploidy levels. Studying isoenzyme variation is *Colocasia esculenta*, Lebot and Aradhya (1992)

reported a similar observation of lack of isoenzyme studies, morphological- and cytological evaluation.

Summary

6. SUMMARY

For refining and establishing micropropagation systom for *A. andreanum* cv. Dragon's Tongue, studies were carried out, to solve many of the problems faced in the micropropagation of the variety such as, improper foliage development, prolonged period for callus induction and multiplication. Investigations were conducted also to explore alternative explants for micropropagation; induction of somatic embryogenesis and development of artificial seed or synthetic seed/synseed- technology; crop improvement through induction of somaclonal variation and radiation breeding *in vitro.* The salient findings of the study are summarised in this chapter.

1. Surface sterilization with 70% ethyl alcohol wipe followed by 0.1 % mercuric chloride for eight minutes recorded maximum survival of cultures (99.74%) in the case of leaf explants.

2. A combination of surface sterilization treatments involving ethyl alcohol (70%) wipe + emisan (0.1%) dip for three minutes followed by mercuric chloride (0.!%) for eight minutes recorded maximum survival (87.28 %) of the petiole explants.

3. For spadix explants, ethyl alcohol (70%) wipe + emisan (0.1%) dip for three minutes followed by mercuric chloride (0.1%) for 10 minutes recorded maximum survival (87.56%) of the cultures.

4. For immature seeds emisan (0.1%) dip for three minute followed by mercuric chloride (0.!%) for eight minutes recorded maximum culture survival(87.85%).

5. Callus induction was maximum (86.50 %) in the leaf explants, when cultured onto the media NW + Kinetin 0.5 mg/l + 2,4-D 0.3mg/l + glutamine 200 mg/l.

6. Number of days taken for callus induction varied with the explants. Days for callus induction from leaf explants was reduced to 51 days compared to 90 days reported earlier.

7. *In vitro* explants and explants from spadix showed good response to callus induction treatments.

8. Callus index (CI) was maximum in the case of *in vivo* leaf explants (338).

9. Among the media tried for callus treatments, NW media recorded maximum response in all the explants tried with the exception of spadix, in which half MS gave the maximum response.

10. The treatment combination NW + kinetin 0.5 mgl^{-1} + 2,4-D 0.3 mgl^{-1} recorded the maximum per cent cultures initiating callus (86.50) from leaf explants.

11. In the case of spadix explants, the treatment combination ½ MS + 2,4-D 0.1-mgl^{-1} + kinetin 0.5 mgl^{-1} was most effective and recorded the maximum per cent cultures (59. 85) initiating callus.

12. In the case of petiole and seed explants, callus induction percent was maximum (61% and 35 % respectively) in the treatment combination NW + 2,4-D 0.5mgl^{-1} + kinetin 0.75mgl^{-1}.

13. Shoot regeneration was maximum (92%) in the treatment combination Nitsch + BAP 0.5 gl^{-1} in the case of leaf explants. In the case of spadix explants $^1/_2$ MS + BAP 2.0 mgl^{-1} + kinetin 2.0 mgl^{-1} recorded the maximum response (71.42%).

14. Leaf development was a cumulative effect of media (Nitsch), carbon source (sucrose 20gl^{-1} + glucose 10 gl^{-1}), media supplements (glutamine) and the culture vessels (conical flask – 200 ml capacity).

15. For quicker establishment of the plantlets *ex vitro*, cluster planting gave the best results (85.33%).

16. Among the potting media tried for stage IV establishment of *in vitro* regenerated plantlets, a mixture of cocopeat + coarse sand (1:1) gave the best results (93.33%). Incorporation of vesicular-arbuscular mycorrhizae (VAM), *Glomus sp.* into the potting mix improved the growth of plantlets *ex vitro*.

17. Among the explants tried for induction of somatic embryogenesis, *in vitro* derived leaves gave the maximum response (53%).

18. Maximum response (55.58 %) to somatic embryogenesis was recorded by the treatment combination Nitsch-White + 2,4-D 1.5 mgl^{-1} + kinetin 0.5 mgl^{-1} + sucrose 20g/l + glucose 10g + glutamine 200 mgl^{-1} + agar 0.6%.

19. Light favoured embryo maturation. Lowering sucrose concentration does not have any added effect on embryoid maturation . Also lower level (0.1mgl^{-1}) of ABA favoured embryo maturation.

20. Germination of somatic embryoids was the highest in the treatment combination, half MS + BAP 0.1mgl^{-1} + glutamine 200 mgl^{-1} maintained in dark.

21. Total time required for induction of somatic embryoids to germination was 7-8 months.

22. Calcium chloride at 50 µM and sodium alginate at 3 per cent level were more effective in the encapsulation of somatic embryos.

23. Conversion frequency for encapsulated somatic embryos can be improved from 30.5 to 39.00 per cent after low temperature storage for 20 days.

24. For long term storage of encapsulated embryos, low temperature storage is very essential, as viability of non-encapsulated somatic embryos was very poor.

25. Conversion frequency of somatic embryos prior to storage was more in the case of non-encapsulated embryoids(48.32 %) than in encapsulated ones (32.67%).

26. Frequency of conversion when cultured onto ½ MS media was maximum in non-encapsulated embryoids (48.32%) compared to encapsulated embryoids (32.67%).

27. The non-encapsulated somatic embryos did not survive the low temperature storage.

28. The low temperature storage of encapsulated somatic embryoids increased the per cent germination and reduced the days taken for germination.

29. Rudimentary leaves were observed for plantlets regenerated from nineth and tenth subculture. The leaf area of such plants was very poor, even after transplanting.

30. Suckering was observed in all plants regenerated up to 10th subculture after 6 months of transplanting.

31. Plants developed in media supplemented with high concentration of cytokinins showed abnormal development with rudimentary leaves and poor vertical growth of the shoots.32. Irradiation dose above 150 Gy was lethal to callus as well as for shoot tips.

33. Maximum response from callus (regeneration and multiplication) and shoot tips (multiplication) were observed at lowest dose of irradiation (25 Gy).

34. Number of shoots per culture was maximum (5.33) at 25 Gy than at 150 Gy, which recorded only 1.66 shoots per culture

35. No suckering was seen in any of the plants from irradiated cultures even after six months.

36. Height, plant spread and leaf area of the plants from irradiated callus was smaller than those from non-irradiated explants.

37. Most significant variation observed was reduction in leaf area of plants/mutants regenerated from explants irradiated with 100 and 150 Gy of γ–ray.

38. Among the different isozymes studied, peroxidase was found to be most stable. Peroxidase was expressed in plant samples from different subcultures as well as irradiated cultures.

39. Variation in banding patterns for peroxidase enzyme was observed between the control and the irradiated cultures.

40. Cytological studies revealed that plants from the different subcultures and irradiated cultures of anthurium, cv. Dragon's Tongue recorded a somatic chromosome number of 2n =30+2B.

In conclusion, the present study provides a method of micropropagation for anthurium cv. Dragon's Tongue using immature spadix as the explants. The existing protocol for cv. Dragon's Tongue has been refined by overcoming many of the problems faced with, while adopting it. Also, it has been made more efficient and effective for mass propagation by way of significantly reducing the number of days for callus induction. The number of days was reduced to 51 days as against 90 days reported previously. Proper foliar development from caulogenic calli reported previously could be achieved by media manipulation. Improvement in the leaf size of *in vitro* regenerated plantlets and the consequent better survival could be achieved. Better hardening practices for the successful establishment of the plantlets were standardised. The method of somatic embryogenesis in anthurium cv. Dragon's Tongue and production of synthetic seeds has been reported for the first time. Also identified the potential of isozymes as a tool in genetical studies of anthuriums and in cultivar

identification. The detailed procedure for isozyme studies in anthuriums was standardized.

Formulating a nutritive enclosure for somatic embryos for encapsulation and production of synseeds and standardising storage conditions for synseeds; follow up studies (including field evaluation) on induction of somaclonal variants by repeated subculturing and media manipulation by the use of high concentrations of cytokinins and mutants through radiation breeding are future lines of work indicated.

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Abstract

IMPROVEMENT OF *Anthurium andreanum* Lind. *IN VITRO*

By

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ABSTRACT OF A THESIS

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ABSTRACT

For refining and establishing micropropagation system for *Anthurium andreanum* cv. Dragon's Tongue, studies were carried out, to solve many of the problems faced in the micropropagation of the variety, such as, improper foliage development, prolonged period for callus induction and multiplication. Investigations were conducted also to explore alternative explants for micropropagation, induction of somatic embryogenesis and development of artificial seeds, crop improvement through induction of somaclonal variation and radiation breeding *in vitro.* The study was carried out during 1995-98 at the Plant Tissue Culture Laboratory, Kerala Horticulture Development Programme (R & D), Kerala Agricultural University, Vellanikkara.

Surface disinfestation with 70 per cent ethyl alcohol wipe followed by treatment with 0.1 per cent $HgCl_2$ for eight minutes recorded maximum survival (99.74%) of cultures in the case of leaf explants. For spadix explants, ethyl alcohol wipe (70%) + emisan (0.1%) dip for three minutes followed by $HgCl_2$ (0.1%) for 10 minutes recorded maximum survival (87.56%) of the cultures. Among the different explants tried (*in vivo* and *in vitro* derived explants) callus induction was maximum in the case of *in vivo* leaf explants. Callus was initiated (86.50%) within 51 days compared to 90 days reported earlier, when cultured in darkness on to the culture media, Nitsch-White (NW) + kinetin 0.5mgl^{-1} + 2,4-D 0.3 mgl^{-1} + sucrose 20 gl^{-1}+ glucose 10 gl^{-1} + agar 6.0 gl^{-1}. Spadix explants and *in vitro* derived explants (leaves, nodes, petiole and roots) showed

good response to callus induction treatments. Among the *in vitro* derived explants, root explants recorded the maximum callus multiplication.

Callus induction from spadix explants was better (59.85%) in half strength MS basal medium supplemented with 2,4-D 0.3 mgl^{-1} + kinetin 0.5 mgl^{-1} +Sucrose 30gl^{-1} + agar 6gl^{-1}. Maximum shoot regeneration (92%) was observed after 46 days in Nitsch media supplemented with BAP 0.5 mgl^{-1} in the case of leaf callus. In the case of callus derived from spadix explants, half strength MS media supplemented with BAP 2.0 mgl^{-1} + kinetin 2.0 mgl^{-1} recorded maximum response (71.42). Multiplication rate (22 per culture vessel) of the shoots and growth and development of the leaves and shoots were better in conical flasks (200ml size). For quicker establishment of the plantlets, mud pots gave the best results (85.33%). Incorporation of vesicular -arbuscular mycorrhizae (VAM) *Glomus sp.,* into the potting mix improved the growth of the plantlets *ex vitro.*

Among the explants tried for somatic embryogenesis, *in vitro* derived leaves (53%) and petiole (18.90%) and immature seeds (8.33%) showed positive response. Induction of somatic embryoids was observed in the media, Nitsch-White (NW) supplemented with 2,4-D 1.5 mgl^{-1} + kinetin 0.15 mgl^{-1}+ sucrose 20gl^{-1}+ glucose 10gl^{-1}+ glutamine 200mgl^{-1} + agar 6 gl^{-1} in explants derived from in *vitro* leaves and petiole. For immature seeds, response was observed in Nitsch media supplemented with 2,4-D 2.0 mgl^{-1} + kinetin 0.3 mgl^{-1} + sucrose 20gl^{-1} + glucose10 gl^{-1}+ glutamine 200 mgl^{-1}. Germination of the somatic embryoids was highest in half strength MS media supplemented with BAP 0.1mgl^{-1} + glutamine 200 mgl^{-1}. Viability of the somatic embryoids was

observed to be very low (5-10 days). Encapsulation of somatic embryoids was achieved with calcium chloride at 50 µM and sodium alginate at 3 per cent level. After encapsulation, somatic embryoids can be stored up to 20 days without much loss in capacity for germination (15.50%). Germination per cent of encapsulated somatic embryos was improved (39%) after a low temperature storage (4 ^{0}C) for 20 days.

Rudimentary leaves were observed in plantlets regenerated from ninth and tenth subculture. The colour of the leaves in such plantlets was observed to be pale green. After transplanting also, the plants exhibited poor leaf growth. In such plants the leaf area remained smaller than other plants. But, chlorophyll development was normal.

For radiation breeding using γ-rays, the irradiation doses above 150 Gy were found to be lethal to callus as well as for shoot tips. Maximum response in terms of plant height, plant spread and leaf area was recorded at lower dose of 50 Gy. Most significant variation observed in *in vitro* regenerated plantlets compared to mother plants was the reduction in leaf area of mutants regenerated from explants irradiated with 150 Gy. Height of the plant was also less at higher doses of γ–irradiation.

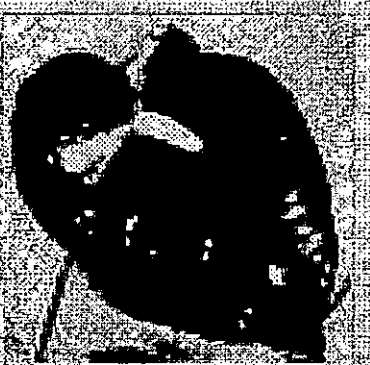
The plants obtained from the trial on induction of somaclonal variation and radiation breeding were screened for possible mutants and somaclonal variants using morphological characters, biochemical markers and cytological technique. Morphological characters were recorded for observing possible

variability, at periodical intervals after transplanting. Dwarf mutants were observed at higher doses of γ–irradiation.

The biochemical studies using isozymes revealed no difference among the plants regenerated from different subcultures and different doses of irradiation. But, difference was observed between the non-irradiated and the irradiated plants for the number of bands produced. Peroxidase isoenzyme was found to be the most stable and was expressed in plants regenerated from different subcultures as well as those from irradiated cultures. Five bands were resolved in the case of plants from the repeated subculturing and two bands were resolved in the case of those regenerated from irradiated cultures.

Cytological study showed no alteration in the somatic chromosome number, which remained uniform at 2n=30+2B, in all the plants regenerated from the different subcultures and the irradiated cultures.

Appendices



APPENDIX I

Composition of Murashige and Skoog media (MS) (Murashige and Skoog 1962)1000 ml

Element	Quantity(mg)
Inorganic	
$NH_4 NO_3$	1650.00
KNO_3	1900.00
$CaCl_2$	440.00
$MgSO_4$	370.00
$KH_2 PO_4$	170.00
NaEDTA	37.30
$FeSO_4$	27.80
$H_3 BO_3$	6.20
$MnSO_4$	22.30
KI	0.83
Na molybdate	0.25
Ca SO_4	0.025
$COCl_2$	0.025
Organic	
Myoinositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.10
Glycine	2.00
Sucrose	30000.00
Agar	8000.00

APPENDIX II

Composition of Nitsch (1969) 1000ml

Element	Quantity(mg)
Inorganic	
NH_4NO_3	720.00
KNO_3	950.00
$CaCl_2$	166.00
$MgSO_4$	185.00
KH_2PO4	68.00
H_3BO_4	10.00
$Mn\ SO_4$	25.00
$ZnSO_4$	10.00
Na molybdate	0.25
$CuSO_4$	0.025
FeSO4	27.80
Na EDTA	37.30
Organic	
Inositol	100.00
Nicotinic acid	5.00
Pyridoxine HCl	0.5
Thiamine HCl	0.5
Glycine	2.00
Folic acid	0.50
Biotin	0.05
Agar	9000.0
Sucrose	20000.00

APPENDIX III

Composition of Modified Murashige and Skoog (MMS) (Kuehnle and Sugii, 1991) 1000ml.

Elements	Quantity(mg)
Inorganic	
NH_4NO_3	1650.00
KNO_3	1900.00
$CaCl_2$	440.00
$MgSO_4$	370
KH_2PO_4	170.00
NaEDTA	37.30
$FeSO_4$	27.80
H_3BO_3	6.20
$MnSO_4$	22.30
$ZnSO_4$	8.60
KI	0.83
Na Molybdate	0.25
$CaSO_4$	0.025
$COCl_2$	0.025
Organic	
Myoinositol	100.00
Nicotinic acid	0.50
Pyridoxine	0.50
ThiamineHCl	0.10
Glycine	2.00
Sucrose	30000.00
Agar	8000.00