IMPROVEMENT OF PROPAGATION EFFICIENCY OF *Anthurium* SPECIES *IN VITRO*

By

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THESIS

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requirement for the degree

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DECLARATION

I hereby declare that the thesis entitled "Improvement of propagation efficiency of *Anthurium* species *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, dipioma, associateship, fellowship or other similar title of any other University or Society.

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LIST OF ABBREVIATIONS

Auxins:

IAA	Indole acetic acid
IBA	Indole butyric acid
2,4-D	2,4 - dichlorophenoxyacetic acid
2,4,5-T	2,4,5 - trichlorophenoxyacetic acid
NAA	Naphthaleneacetic acid

Cytokinins:

BA	Benzyladenine
2ip	2 isopentenyl adenine

Other growth regulators:

ABA	Abscisic acid
GA	Gibberellic acid
CW	Coconut water

Explants:

L	Leaf
P	Petiole
S	Spathe
Sp	Spike
In	Inflorescence stalk

Media:

MS	Murashige and skoog (1962)
SH	Schenck and Hildebrandt (1972)
LS	Lin and Staba (1961)
B_5	Gamborg et al.(1968)

Others:

AC	Activated charcoal
CD	Critical difference

NS	Not significant
CI	Callus Index
VAM	Vesicular arbuscular mycorrhiza
OD	Optical density.

Introduction

INTRODUCTION

Economic aspects of ornamental horticulture are as important as aesthetic ones. The floriculture products of commercial importance mainly consists of cut flowers and ornamental foliage plants. Cut flowers constitute 45 per cent of the total world trade in floricultural products. The use of cut flowers in home decoration has become an integral part of living in human society. Production of ornamental foliage plants has gained much importance in the recent years in places like Bangalore, Calcutta, Delhi and Trivandrum. There has been a great awareness of the usefulness of house plants for home decoration. With the growing population, lack of open space and development of multistoreyed housing systems, people have to depend largely on indoor plants for decorating their surroundings.

There is tremendous potential for cultivation of flowers and ornamental foliage plants in India. The availability of favourable soil and climatic conditions in different regions of the country makes it possible for the production of important flower crops almost all through the year in some parts of the country. Besides their demand in the export markets, the requirement for cut flowers and other foliage plants in the domesitc trade has also developed. But the commercial production has not kept pace with the increasing demands.

Anthurium constitutes the largest genus of the family Araceae. They are valued for their colourful longlasting flowers and handsome foliage. They are gaining popularity as one of the

most important commercial ornamental crops of the modern world. Flowering type anthuriums are exclusively grown for cut flowers and foliage type anthuriums cater to the demands for indoor decorations. Anthuriums are slow growing and produce only six to eight new leaves per year. Most of the species are of tropical climate. Kerala, with its unique climate, is highly congenial for anthurium cultivation. However, production and marketing of the crop are still in infancy in the State. Recently, there has been an increasing demand and interest in commercialisation of the crop. Now a days in vitro propagated plantlets are being imported by nursery owners. There is tremendous potential for this new commercial crop, both at home and foreign market.

Anthuriums are commonly propagated by seeds. They can also be propagated by suckers or cuttings. Seed set is not commonly observed (except in Anthurium andreanum) under Kerala conditions. Propagation by cuttings as well as by suckers is very slow. In vitro propagation with its high rate of multiplication becomes relevant in this context. Methods of in vitro propagation, mainly through somatic organogenesis, have been standardized for Anthurium andreanum (Pierik, 1976; Pierik et al., 1974; Pierik et al., 1979) and Anthurium scherzerianum (Pierik and Steegmans, 1976; Geier, 1986). Although methods have been standardized for these species, there is possibility for improving the rate of multiplication. Also, there are species of Anthurium for which the standardized method is not effective. Clonal multiplication of A. andreanum, with stem-sections from aseptically grown plantlets has been attempted by Kunisaki (1980). But in the

experiment, effect of only a single cytokinin (BA) has been studied. In Anthurium scherzerianum, somatic embryogenesis from spadix callus has been reported by Geier and Reuther (1981) and Geier (1982). However, this mode of regeneration occured only sporadically, and the factors required for its consistent induction have yet to be defined. Differences in morphogenetic potential of explants/genotypes of anthuriums have been observed by Pierik (1975), Leffring et al. (1976) and Eapen and Rao (1985). In this respect, no biochemical studies have been attempted.

The relevance of the present study becomes evident in this context. The objectives of the study are:

(i) To improve the propagation efficiency of Anthurium species through enhanced release of axillary buds, direct/callus - mediated somatic embryogenesis and direct/callus-mediated organogenesis.

(ii) Biochemical characterization of recalcitrant nature, if any, exhibited by different species/genotypes.

(iii) To optimise the techniques for maximum ex vitro establishment of plantlets.

Review of Literature

REVIEW OF LITERATURE

Although plant tissue was first successfully cultured by White in 1934, Morel's success with rapid in vitro propagation of Cymbidium orchids (1960) was the major stimulus to the application of tissue culture techniques to the propagation of floriculture crops. Other orchids viz. cattleyas, dendrobiums, vandas and phalaenopsis have been clonally propagated through shoot tip culture. The extensibility of tissue culture to other ornamentals was confirmed by Murashige et al. (1974) in gerbera, Harper (1976) in ferns, Jones and Murashige (1974) in bromeliads, Hartman (1974) in caladium. The use of tissue culture for rapid clonal increase has developed very rapidly for herbaceous ornamental plants, and for some crops like orchid, it has already given the industry a great boost. As procedures have been developed to encompass a wider array of plants, tissue culture could attain an important role in commercial floriculture for the production of uniform disease free plants.

Murashige (1974) suggested three possible routes for in vitro propagule multiplication: (a) enhanced release of axillary buds, (b) production of adventitious shoots through organogenesis and (c) somatic embryogenesis.

Routes of in vitro propagation

A Enhanced release of axillary buds

Georges Morel (1960) was the pioneer in applying shoot tip culture as a clonal multiplication tool. He was successful in cloning the orchid, Cymbidium and since then, in vitro clonal

multiplication gained momentum. Many herbaceous horticultural species have been successfully multiplied using this technique. The success has been partially due to the weak apical dominance and strong root regenerating capacities of many herbaceous plants (Hu and Wang, 1983).

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

Kunisaki (1980) obtained high yields of viable cultures of Anthurium andreanum with the use of small explants of vegetative buds. Explants were grown into plantlets in modified Murashige and Skoog medium, supplemented with 15 percent coconut water. He also observed that the best clonal increase was obtained when stem sections (2-nodal) from aseptically grown plantlets were cultured in medium containing BA 0.2 mg/l. Higher BA concentrations produced more callus growth and stunted shoots.

In spathiphyllum, Fonnesbech and Fonnesbech (1977) obtained four shoots per stem explants, after 12 weeks of culture on a nutrient agar medium and grown with 18 h light and 6 h darkness at 24°C.

Knauss (1977) reported that lateral buds or shoot tip from Dieffenbachia picta, when cultured on MS medium, produced disease-free plantlets. In Dieffenbachia exotica, the presence of either 2ip or kinetin was prerequisite for in vitro shoot formation

(Voyatzi and Voyatziz, 1989). They also observed that 2ip at 16mg/l produced more shoots than Kinetin at 2 mg/l.

In carnation, for multiplication of shoots, Earle and Langhans (1975) cultured the shoots on MS liquid medium supplemented with 9.3µM kinetin and 0.11µMNAA. On the other hand, Jelaska and sutina (1977) used agar medium supplemented with the same growth regulators.

Clusters of adventitious shoots were induced on soft stem sections of *amaryllis* by treatment with NAA (0.64µM) in liquid culture for 12 hours and afterward transferring them to a medium containing 2.2 to 4.4µM BA (Hussey, 1977). Axillary branching was also developed on the same medium.

Harney (1982) used MS medium supplemented with 0.9µM BA and 1.0 µM NAA, for regeneration of plants from shoot tips of geranium.

Shoot tip explants of snapdragon when placed on MS medium supplemented with 18.6 µM kinetin and 11.42 µM 1AA, formed multiple shoots (Pfister and Widholm, 1984).

In chrysanthemum (cv. Pink Camino, Princess Anee and Super Yellow Spider) multiple shoot formation occured in apical meristem cultured on modified MS medium supplemented with 1 mg/l BA and 0.1 mg/l 1AA (Gertsson and Andersson, 1985). Ahmed (1986) achieved the best growth and propagation rates of meristem apices of chrysanthemum when cultured on media containing the macro elements on MS, vitamins, and BA and NAA each at 1 mg/l. Heat

treatment of the meristem tips accelerated the growth and they survived better than the untreated control (Ahmed and Andrea, 1987). Multiple shoot formation from shoot tip explants of Chrysanthemum morifolium was determined by the size of the explant and combination of growth regulators. Treatment with 0.2 mg/l NAA and 2 mg/l kinetin with or without 0.2 mg/l GA_3 gave good multiple shoot formation (Sangwan et al., 1987).

Curir et al. (1988) cultured buds (explants) of some rose cultivars for three days in presence of activated charcoal to obtain an enhanced growth of primary explants. These were, then transferred to a fresh basal medium with thiamine (2 mg/l) and myo-inositol (100 mg/l), for proliferation. Shoot explants of hybrid-tea, floribunda and miniature rose cultivars were best micropropagated in MS, Lee and de Fossard and Gamborg media, enriched with 0.5-1.0 mg/l BA (Alekhno and Vysotskii, 1986). Compared with the standard solid medium the propagation coefficient was improved when a double phase medium comprising of 7-8 mm solid layer with MS salts (0.5 mg/l thiamin, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 1.0 mg/l ascorbic acid, 100 mg/l meso-inositol, 0.2-1.0 mg/l BA, 30000 mg/l sucrose and 15-20000 mg/l agar) and a liquid medium overlay of the same composition was used for shoot proliferation (Alekhno and Vysotskii, 1987). In "Golden Times" roses, axillary bud growth and morphogenesis were the best, when buds from the middle nodal position of soft-wood stems were cultured in the presence of BA (Mederos and Rodriguez, 1987). Valles and Boxus (1987) reported an

enhanced proliferation of shoots of several Rosa hybrida cultivars with BA at 1 mg/l.

Anderson (1980) perfected tissue culture propagation procedure for rhododendron. He observed that the explants did not respond to their maximum potential when grown on MS medium. They were apparently affected by general salt toxicity as shown by foliage chlorosis and browning of the stems. Anderson found it necessary to modify the MS formula by reducing the ammonium nitrate and potassium nitrate to approximately 0.25 strength and by adding twice the strength of ferrous sulphate and Na_2EDTA. These changes dramatically improved the propagule multiplication and culture health. Five-cm shoot tips of rhododendron (P.J.M hybrids) showed better establishment than 2 cm shoot tips. Microshoot proliferation was the best with 2ip at 5 and 10 mg/l (Ettinger and Preece, 1985). Norton and Norton (1986) compared the activity of BA and 2ip in shoot proliferation and found that BA treatment resulted in necrosis in six cultivars. They also observed more shoot proliferation with 2ip.

Hussey (1977) could obtain precocious outgrowth of axillary shoots from axillary buds of gladiolus when cultured on nutrient medium containing BAP. In Gladiolus flanaganii, axillary bud growth from corm explants was stimulated when cultured on MS medium + kinetin at 0.5 mg/l (Dickens et al., 1986). Shoot cultures of cultivars `Friendship', `Her Majesty' and `American Beauty' were initiated from axillary buds excised from cold-stored corms. These shoots were multiplied on MS medium + BA (0.5 mg/l) and the shoots elongated only when BA was omitted from

the medium or when its level was reduced to 0.1 - 0.2 mg/l (Dantu and Bhojwani, 1987). Apical and lateral buds of corms were used as explants for shoot proliferation on MS medium supplemented with low levels of NAA and BA or kinetin at varying concentrations (Lilien-Kipnis and Kochba, 1987).

In Senecio cruentus (cv. Hansa), shoot tips derived seedlings germinated in vitro were used for shoot multiplication which was best accomplished on MS gerbera medium supplemented with 2.8 µM IAA (Cockrel et al., 1986).

Gerbera (cv. Arendsoog and Super Giant Yellow) shoots, cultured on medium containing 50% MS nutrients + 5 mg/l BA + 0.1 mg/l IAA + 1.0% agar, developed ten shoots per explant after four weeks (Huang and Chu, 1985). Cultivars Auriza-Karmezin and Sonia showed good proliferation in vitro with BA at 1 mg/l (Zakhrova, 1987).

Ault and Black (1987) propagated Ferocactus acanthodes by culturing apical explants from seedlings germinated in vitro. Axillary shoot proliferation was accomplished by culture on MS mineral salts supplemented with 9 g/l agar, 87.6 m M sucrose, 1.1 mM $Na_2H_2PO_4$. H_2O, 0.9 m M inositol, 0.2 m M adenine, 46.5 µM kinetin, 5.4 µM NAA, 8.1 µM nicotinic acid, 4.9 µM pyridoxine HCl and 3.0 µM thiamine HCl.

In a tree crop like gauva, Amin and Jaiswal (1987) reported that axillary buds were enhanced and it was observed that BA (4.5 µM) without any auxin and gibberellin gave the best shoot multiplication.

Hosier et al. (1985) achieved shoot proliferation of lingonberry from shoot tips cultured on a low salt medium supplemented with 2ip.

Multiple shoots of chinese chestnut, from axillary buds of juvenile shoots, were best produced with BA at 0.44 µM. Higher concentrations of BA (4.44 µM and 44.4 µM) inhibited buds from sprouting and promoted callus growth (Qu-guang et al., 1986).

Vuylsteke and DeLanghe (1985) studied the feasibility of in vitro propagation of bananas and plantains. Highly proliferative growth of adventitious buds was obtained by culturing preexisting meristems on a medium with high cytokinin. Wong (1986) tried in vitro multiplication of 22 banana cultivars, from shoot tip explants which could be induced to produce multiple shoots in the presence or absence of aplical dome. Wide variations among the cultivars in their multiplication rate in response to cytokinins were observed. BA was reported to be more effective than kinetin. Main apices of sucker buds and lateral buds of the banana clone "Poyo" were stimulated by a high concentration of BA in the absence of auxin (Mateille and Foncelle, 1988).

B Somatic organogenesis/embryogenesis

Generally, a high concentration of auxin and a low concentration of cytokinin in the medium promote abundant cell proliferation with the formation of callus (Skoog and Miller, 1957). On the ohterhand, low auxin and high cytokinin concentration in the medium result in the induction of shoot

morphogenesis. Auxin, alone or in combination with a very low concentration of cytokinin, is important in the induction of root primordia. Somatic organogenesis can be direct or callus mediated (Evans et al., 1981) and is useful in inducing genetic variability or to recover preexisting natural genetic variability.

Several studies have been conducted in the micropropagation of Anthurium andreanum and A scherzerianum. Plantlet regeneration has been obtained via callus from cultured embryos and explants of leaf lamina, petiole, inflorescence stalk, spathe and spadix, or without intervening callus from embryo and axillary bud explants. Pioneering studies were conducted by Pierik and collaborators (1974 a,b). They succeeded in the induction of regeneration, first from embryo and seedling tissues and later from non meristematic parts of mature plants. A modified MS medium supplemented with a cytokinin (2BA) was used. Optimum growth of the callus tissue was obtained at 25°C in darkness. Genotypical variation in response to in vitro culture was also observed.

Callus multiplication was observed best in a liquid medium (Pierik 1975, Pierik et al., 1975). For this leaf pieces with the callus was transferred to a liquid medium which was placed on a shaker rotating at 120 rpm. Based on the detailed studies a scheme was proposed for the micro propagation of A. andreanum and A. scherzerianum (Pierik, 1976, Pierik and Steegmans, 1975; Pierik et al., 1979).

Leffring et al. (1976) studied factors affecting callus formation on leaf tissues of Anthurium. Disinfection of the tissue for 20 minutes resulted in less damage than disinfection for 30 minutes. Tissue from along the central vein produced callus better than that from along the leaf margin and tissue from the apical half of the leaf produced callus better than that from the basal half. Leffring and Soede (1978; 1979 a,b) proposed a new method of tissue culture for anthuriums based on the production of side shoots from callus. Addition of 2ip at 3 mg/l to the medium resulted in wide spread shoot formation. Novak and Nepustil (1980) observed that callus clones with a high capacity for regeneration were derived from leaf explants of flowering plants. Anthurium andreanum hybrids Ellrina and Porzellan were propagated by tissue culture (Kraft et al., 1983).

Several shoots were regenerated from the callus which was subjected to series of subculture and these shoots were rooted in cytokinin free medium. Finnie and Van staden (1986) achieved plantlet regeneration using a modified MS medium at 25 ± 2°C with a 16 h light/18 h dark cycle. Keller et al. (1986) obtained callus from leaf explants on MS medium supplemented with 2 mg kinetin/l. Geier (1982 and 1986) successfully propagated A. scherzerianum by tissue culture. Callus formation was stimulated in darkness on a medium containing 0.1 mg/l 2,4-D and 1 mg/l BA. Shoots were developed on a medium without 2,4-D and with less BA (0.2-0.5 mg/l). Rooting of shoots required 4 weeks of light. It was observed that some genotypes were more easily propagated from flower spikes than from leaf cuttings. However, during initial

stages of culture they showed considerable stability and rarely formed callus or shoots. Germinated seeds when transferred to Knudson C medium with 1 ppm BA produced large number of plantlets (Zimmer and Bahneman, 1982). Genotypic variation of shoot proliferation of in vitro germinated seed was observed by Zens and Zimmer (1986 and 1988). Shoot production was decreased with increased NH_4: NO_3 ratio.

In Agave sp. callus have been established from leaves cultured in media containing 0.3 µM BA and 1.0 µM 2,4-D (Hunault, 1974) or from seed fragments cultured in media supplemented with 23 µM kinetin and 4.5 µM 2,4-D (Groenewald et al., 1977). Adventitious shoots were obtained by transferring callus into media containing 0.9 µM 2,4-D and 4.6 µM kinetin. In Agave fourcroydes, the NO_3^- : NH_4^+ balance in the medium was a key factor controlling callus growth and organogenesis in rhizome cultures (Robert et al., 1987).

Adventitious vegetative bud formation on leaf, petiole or inflorescence segments is the method for plantlet formation in begonia. Hilding and Welander (1976) reported an increased shoot production with increasing levels of BA from 0.01 to 1.0 mg/l but rooting was hindered unless high NAA (0.5 mg/l) was present. NAA alone at 10 mg/l resulted in formation of callus only. Various combination of N and K (5-25mM and 1-20mM respectively) on shoot production were also tried. A higher percentage of shoot production was observed as the K level increased while the N level remained low. With increasing NAA (0.01 to 1 mg/l) and decreasing BA (0.1 to .01 mg/l) concentration, the percentage of

petiole explants forming only shoots decreased where as those forming only roots increased (Welander, 1977). Leaf segments produced many buds during 70 days on MS medium containing 1 mg/l BA and 1 mg/l NAA (Iida et al., 1986). Somatic embryos from leaf segments of Begonia fimbristipula were induced when cultured on liquid SH medium supplemented with 0.125 mg/l 2,4-D, 0.25-0.5 mg/l BA and 10% (v/v) coconut milk (Lan-Ying et al., 1988).

Petru and Landa (1974) reported that shoot formation occured on the callus tissues of carnation derived from isolated hypocotyl segments on modified MS medium supplemented with 22 µM IAA and 11.8 µM kinetin. Takeda (1978) reported that shoots could be induced from the basal segments of immature as well as mature leaves by using MS medium supplemented with BA and NAA. Immature petals, on MS medium containing 5.4 µM IAA and 4.4 µM BA, produced multiple shoots (Kakehi, 1978).

In Dracaena, callus was induced on which adventitious shoots were regenerated (Mee, 1978; Chua, et al., 1981). Mostly 2,4-D (2.3 to 13.5 µM) was used for callus induction.

Organ formation and regeneration of entire plants of cyclamen were reported from cultured petiole, leaf blade, pedicel, anther and ovary explants. Morel (1975) reported regeneration from leaf tissue. Geier (1977) was well as Geier et al. (1979) achieved only callus and scarce root formation from petiole segments. In pedicel segments cultured in the presence of 5.7 µM IAA and 4.4 µM BA, they observed either few roots or some isolated leaves, depending on the age of explants. It was

also observed that only those medium containing an auxin and a cytokinin induced cell proliferation from anthers maintained in continuous darkenss; illumination inhibited callus formation and caused deep brown pigmentation of the explants. Hawkes and Wainwright (1987) attempted to subculture adventitious leaves on media containing BA as sole growth regulator. High levels of BA (4.4 and 11.1 µM) caused leaf distortion, whereas at low levels of BA (0.4 to 2.2 µM), proliferation was too slow.

Organogenesis was the only route observed for in vitro regeneration of geranium plants. Callus was induced to differentiate and to produce shoots (Chen and Galston, 1967; Skirvin and Janick, 1976). Growth regulators required for callus induction ranged from 2.3 to 46.4 µM kinetin and 1-5 µM NAA or from 0.6 to 5.7 µM IAA. Shcherbakova et al. (1977) reported the beneficial effect of 2,4-D at 5 mg/l on the growth of isolated Pelargonium roseum tissues. It was also observed that growth was effected by darkness. Organized structures indicative of somatic embryogeneis was observed by Abo El-Nil and Hildebrandt (1976).

In gladiolus, plantlets were successfully regenerated from callus, from meristem tips, buds or from the flower stalk. The best callus production was induced on MS medium in the presence of high levels of NAA or 2,4-D. Explants from the flower stalk and the shoot tip produced callus within 1-3 weeks on media with 26.9 or 53.8 µM NAA and 2.3 µM kinetin (Ziv et al., 1970; Bajaj et al., 1983).

Ruffoni and Sulis (1988) achieved regeneration from callus in Gerbera jamesonii hybrida on half strength MS nutrients, sucrose and agar supplemented with IAA and a high concentration of BA.

Apical meristems of Japanese morning glory grew up to plantles on BM + BA (4.4 µM) or BM + kinetin (4.6 µM). The treatment also promoted compact and nodular callus from which several shoot buds arose and developed into complete plants (Bapat and Rao, 1977). On BM + 2,4-D agar medium, yellowish, soft callus was produced and globular embryos developed in the callus (Rao and Harada, 1974).

In Lilium, embryos used for in vitro culture varied considerably in size. Myodo (1962) studied the effects of several medium factors on embryo growth. The MS medium was found to be better suited for embryo growth; a sucrose concentration of 58.4 - 116.8 m M and NAA concentration of 0.54-54 nm were optimal. Most experiments were carried out at 25°C in the dark (Stimart and Ascher, 1978).

Internodal sections of flower stalks of Phalaenopsis produced several protocorm like bodies around 100 days after explanting and these proliferated and grew into plantlets (Homma and Asahira, 1985). Leaf segments of Phalaenopsis amabilis produced protocorm like bodies when cultured on MS medium supplemented with 1 ppm NAA + 10 ppm adenine + 10 ppm BA. These protocorm like bodies developed into plantlets on Kundson C medium supplemented with 100 ppm inositol, 1 ppm thiamine HCl,

1ppm nicotinic acid and 2 g/l peptone (Tanaka and Sakanishi, 1985).

Rao et al. (1973) placed leaf discs and stem internode sections of Petunia hybrida and P. inflata plants on MS + 2,4-D (0.45 µM) medium containing 58 mM sucrose and 0.8% agar. These cultures, when incubated at 22-30°C under 5000 lux light, showed rapid development of soft friable callus from which globular embryos were initiated. Sangwan and Harada (1976) also demonstrated somatic embryogenesis with the same Petunia spp. on the same media compositions. The callus was subcultured to liquid MS + 2,4-D (0.45 µM) + kinetin (0.46 µM) and after 4 weeks somatic embryos were observed as free floating structures.

Khosh-Khui and Sink (1982) found that optimal conditions for the production of friable callus in rose include the establishment of callus lines in the dark. Lloyd et al. (1988) reported adventitious shoot formation from callus cultures of R. persica X Xanthima on MS media supplemented with 4.4 - 8.8 µM BA and 0.54 - 1.62 µM NAA.

In snapdragon, shoot and root explants from young seedlings were induced to form callus on MS modified medium containing 2,4-D (0.45 µM or NOA (1.25 µM) + 10% CW (Sangwan and Harada, 1975). These calli, when transferred to regenerating media - BM + IAA (5.37 µM) + kinetin (2.32 µM) or NOA (9.90 µM) + 10% CW, produced many shoots within 4-6 weeks (Pfister and Widholm, 1984). Embryos were formed in the callus in the presence of 2,4-D (4.50 µM) and NOA (1.25 µM) with or without coconut Water (Sangwan and Harada, 1975; Rao et al., 1976).

Plantlet regeneration through somatic embryogenesis from tissues of cotyledon, hypocotyl and immature/mature embryos were reported in camellia. Zhuang an Liang (1985) reported that embryogenesis occured on MS medium with the addition of BA or combination with NAA. Kato (1989) achieved embryogenesis on MS medium supplemented with GA_3 while Ana Maria and Barciela (1990) observed embryo regeneration on MS medium without growth regulators. It was also observed that embryonic axes collected in September produced more somatic embyos when compared with the embryonic axes collected in October.

Jarret et al. (1984) obtained asexual embryos from callus derived from axillary bud shoot tips of sweet potato when cultured on MS medium supplemented with 2,4-D. The embryogenic callus to auxin-free medium resulted in germination of embryos. Chee et al. (1990) observed embryo formation on basal medium containing 3 per cent sucrose and no growth regulators. The percentage of embryos forming shoots was increased by 4 µM BAP, but BAP reduced whole plant formation and promoted callusing at the root axis.

Hammet and Davey (1986) established a culture sequence for the induction of somatic embryogenesis from cultured zygotic embryos of soybean. Embryos were cultured on B_5 medium supplemented with 2% sucrose, 0.1 mg/l IBA and 10% (v/v) coconut milk. Somatic embryos were also initiated from immature cotyledons when explanted on to a medium containing moderately high levels of auxin. Parrot et al. (1988) observed that a

reduced exposure to auxin (10-14 days on 10 mg/l NAA) enhanced shoot meristem development. Interaction between sucrose and auxin was reported by Lazzeri et al. (1988). Embryogenesis was maximum on media supplemented with low to intermediate levels of sucrose (1 or 2%) and NAA (6.25 or 12.5 mg/l).

Somatic embryos were induced from the primary callus of celery arising from leaf blade explants placed on MS medium supplemented with 9 µM 2,4-D (Kim, 1989). Embryogenic suspension cultures were also established from petiole and maintained on a medium containing 2.3 µM 2,4-D and 0.88 µM BA. Addition of mannitol (3-4%) increased the number of single somatic embryos and improved their differentiation and development (Nadal et al., 1989).

Leaf explants of rye when cultured on MS medium with different concentrations of 2,4-D, produced embryogenic callus from which plantlets were obtained (Linacero and Vazqjez, 1986).

In Iris, embryogenic callus was initiated from explanted germinated seeds on MS medium containing 5% sucrose and 2,4-D at 1.0 mg/l (Radojevic et al., 1987).

Preece (1989) observed that in white ash, more embryogenic callus was formed on media with 1 µM 2,4-D than on media with 10 µM 2,4-D. BA at 5 µM was also found to be beneficial in the production of somatic embryos.

In cucumber embryogenesis was induced on MS medium supplemented with 2,4-D at 2.0 mg/l and kinetin at 0.5 mg/l

(Chee, 1990). The embryos were developed on MS medium with NAA (1.0 mg/l) and kinetin (0.5 mg/l).

Factors influencing success of in vitro propagation

Explant

Type

Novak and Nepustil (1980) obtained Anthurium andreanum callus clones with a high capacity for regeneration from leaf explants of flowering plants. Geier (1982) observed a much higher capacity for regeneration of spadix fragments in A. scherzerianum when compared to segments of leaf, petiole, inflorescence stalk or spathe. However, work by the same author in 1986 revealed that cutting from young leaves were highly regenerative than cuttings from older leaves.

In hyacinth, the basal part of the leaf shows higher regeneration (Hussey, 1975) and in the inflorescence stem attached to the pedicel, the distal part has the highest regeneration ability (Paek and Choi, 1982).

Grunewaldt (1977) reported in vitro propagation of some gesneriads viz. achimenes, saintpaulia, streptocarpus and gloxinia, from small pieces of lamina. Gloxinia, saintpaulia and streptocarpus also produced plants from petiole segment while stem pieces produced plants only with achimenes.

Leaf explants and explants formed from the apical part of the embryo were reported to be the most suitable material for in vitro propagation in date palm (Falcone and Marcheschi, 1988).

King and Morehart (1988) studied the influence of various explants viz. shoot-tip, nodal or internodal sections on mophogenesis in Osage-orange. It was observed that shoots proliferated from both shoot-tip and nodal sections, but not from internodes.

Rao et al. (1988) observed that fresh cotyledons followed by leaves were the best source of explants for induction and growth of callus in Azadiracta indica.

The stem explants were more responsive than the petiole explants with respect to callus induction, growth and differentiation in mature leguminous trees viz. Albizzia lebbeck, Cassia fistula, C. Siamea (Gharyal and Maheswari, 1990).

Surface disinfection

Explants are usually cut to a size larger than that of the final one, surface sterlized and trimmed to the final size before being transferred to the culture vessel (Hussey, 1979). The most commonly used surface sterilant is sodium hypochlorite. For softer tissues, a dilution to lower strength may be needed; but anything below 0.5% may prove ineffective (Sommer and Caldas, 1981). Concentrations ranging from 1% to 10% (Kuo and Tssay, 1977) have been used. Generally a drop of detergent is added to the surface sterilant. Mercuric chloride is another commonly used surface sterliant.

Culture medium

Basal medium

Wide variety of media have been reported. The choice depends on the plant species and intented use of the culture. The Murashige-Skoog (1962) medium, characterized by high concentrations of mineral salts has been widely used for general plant tissue culture and specifically for morphogenesis and plant regeneration (Murashige, 1974).

Growth substances

Auxins and cytokinins are inevitable components of plant tissue culture media. BA has been the most effective cytokinin for meristem, shoot tip and bud cultures, followed by kinetin (Murashige, 1974). Several Scientists have reported 2-isopentenyl adenine as the best cytokinin for multiple shoot induction and callus regeneration (Ettinger and Preece, 1985; Voyatzi and Voyatziz, 1989). Lo et al. (1980) reported that high cytokinin content was deleterious to the initiation and elongation of roots of both monocotyledonous and dicotyledonous plants. Young actively growing plant parts has been described as an active site for auxin biosynthesis. However, in anthurium a low auxin has been reported to be suitable for callus formation and further growth and regeneration (Pierik et al., 1975; Pierik, 1979; Finnie and Van Staden, 1986).

Culture conditions

Not much studies have been carried out to reveal the optima for the culture conditions such as temperature, light, humidity etc. which influence the growth and development of plant materials in culture.

Most workers in anthurium observed optimum callus formation and subsequent growth in continuous darkness at temperatures around 25°C while Finnie and Van Staden (1986) reported better callus production in a light environment. In the case of spadix derived cultures of A. scherzerianum, light favoured the persistence of floral differentiation patterns (Geier and Reuther, 1981).

In Begonia, the optimal temperature for vegetative bud formation was reported to be between 20 and 25°C (Takayama and Misawa, 1982).

Loewenberg (1969) reported that light inhibited callus growth in cyclamen. Beneficial effect of darkness on callusing and further growth have also been reported in freesia (Bajaj and Pierik, 1974; Pierik and Steegmans, 1975) and in fuchsia (Bouharmont and Dabin, 1986).

Optimum temperature for in vitro culture systems have not always been accurately determined, but temperatures in the range of 20-27°C are used most frequently.

Humidity is essential to successful in vitro culture; dehydration of the cultures may occur if the humidity external to the culture vessels is low unless the vessles are tightly sealed. Usually the relative humidity is maintained in the vicinity of 70 percent.

Preculture environmental influences

Stock plant nutrition

Healthy plant material is normally considered to be the superior choice for initiating in vitro cultures, and thus nutrition of the stock plant becomes of obvious importance. Shoot proliferation from tomato leaf segment explants was reduced when the explants were taken from stock plants that were excessively vigorous because of high nitrogen levels in their nutrient medium (Read et al., 1984).

Season

In Lillium speciosum, bulb scale explants obtained during the spring and fall seasons regenerated freely, whereas those taken during summer or winter months hardly ever produced bulblets (Robb, 1957). Stichel (1959) noticed seasonal variation in both the rate of contamination and the morphogenetic response of tuber segments in cyclamen. Explants treated during the months of May to July showed only 25 per cent visible contamination whereas during the remaining seasons, upto 90 per cent contamination was observed (Geier et al., 1979).

Rooting

Rooting of in vitro regenerated plants does not always have to be carried out in vitro (Mc Cown and Amos, 1979). Direct rooting of rootless shoots of anthurium is possible but is not recommedned because it requires very long periods of time, success is inconsistent, and losses are considerable (Geier, 1990). In Dracaena, Debergh and Maene (1981) achieved rooting of shoots when planted in rockwool saturated with a watery solution

of IBA at 9.8 µM. However in many situations *in vitro* rooting is found to be better than *ex vitro* rooting. Auxin is considered essential for root initiation. Among the auxins, NAA and IBA are widely used for root induction (Samartin *et al.*, 1986; Iida *et al.*, 1986; King and Morehart, 1988). In camellia, Samartin *et al.* (1986) observed better rooting with IBA than with NAA. Other auxin like IAA was also found to induce rooting *in vitro* (Curir *et al.*, 1988).

Though auxins are considered essential for rooting, several workers have reported better rooting in a medium free of plant growth substances (Nair *et al.*, 1984; Maria and Segura, 1989).

Sometimes root induction fails at high salt concentration regardless of the types of hormone present. Abundant rooting was observed when the salt concentration was reduced to one-half of the standard strength (Iida *et al.*, 1986; Omura *et al.*, 1987; King and Morehart, 1988; Maria and Segura, 1989).

Cytological changes

Numerical or structural changes in chromosomes are reported to be associated with *in vitro* regeneration of plants (Larkin and Scowcroft, 1981). In *Anthurium scherzerianum*, Geier (1987) observed tetraploid variants in plants regenerated from spadix and leaf segments. However, the extent of variability was less than that usually observed in seed-propagated cultivars. Numerical changes have been observed in callus cultures of tobacco (Sacristan and Melchers, 1969), ornithogalum (Hussey, 1976), geranium (Skirvin and Janick 1976), petunia (Santos and Handro, 1983), chrysanthemum (Khalid *et al.*, 1989).

With respect to apical meristem culture, such variations are rare (Ancora et al., 1981).

Ex vitro establishment

Physical, chemical and biological properties of the potting media and the atmospheric conditions during post-transfer growth are important in the establishment of in vitro regenerated plantlets, which have been planted out.

Potting media

Geier (1990) observed that plantlets of anthurium could be established without losses in a peat/sand media.

Damino (1979) reported that either pure peat or a mixture of 1:1 sand and peat was suitable as the potting medium for strawberry plantlets. Kyte and Briggs (1979) observed that porous potting mixture of peat: perlite: composted bark (1:1:1) was the best for rooting tissue-cultured rhododendrons. Sand as the potting medium was found to be the best for jack plantlets by Ramesh (1990).

Humidity

Chrysanthemum plantlets, cultured under low relative humidity, exhibited high rate of mortality (Wardle et al., 1983). Short et al. (1987) reported that cauliflower and chrysanthemum plantlets cultured at 80 per cent relative humidity had increased way deposition on their leaves. When they were transferred, water loss from the leaves was reduced and better ex vitro establishment resulted.

Vesicular arbuscular mycorrhize (VAM)

Survival and growth of *in vitro* cultured plantlets were increased when treated with VAM (Kiernan *et al.*, 1984; Ponton *et al.*, 1990; Ramesh, 1990).

The increased growth of mycorrhiza treated plants was due to enhanced mineral uptake (St. John, 1980). The increased plantlet establishment, consequent on mycorrhizal treatment of the medium was due to decreased plant injury (Menge *et al.*, 1978), increased water uptake and transport (Safir *et al.*, 1971) and low infection of the plantlets by soil-borne pathogens (Schenck, 1981).

Biochemical characterization

It has been shown that IAA is destroyed by the oxidative action of peroxidases *in vitro*. The endogenous auxin level is controlled by such destructive enzymes and thus, by changes in the internal hormonal milieu, organogenetic differentiation or dedifferentiation could be induced. On the other hand, multiple molecular forms of enzymes in development and differentiation have been investigated in many plant species (Scandalios, 1974) and in plant tissue culture (Scandalios and Sorenson, 1977). In this context, peroxidase isozymes and their auxin destructive activity were studied in various organs (Yoneda and Endo, 1970) and callus (Yoneda and Endo, 1969) of *Pharbitis nil*. Syono (1979) demonstrated the increase of inhibitors of IAA-destruction activity in the induction period of auxin non-requiring tobacco calli from auxin requiring ones. Kevers *et al.* (1981) showed

that an organogenetic-habituated sugar beet callus contained higher levels of auxin protectors, higher peroxidase activity and a large number of peroxidase isozymes than a non organogenetic one.

Materials and Methods

MATERIALS AND METHODS

The present investigations were carried out at the Plant Tissue Culture Laboratory of the Department of Horticulture, College of Agriculture, Vellayani during 1990 to 1992. Four species of Anthurium (Plates I to IV), namely, Anthurium andreanum Lind. (both pink and red colour spathes), A. crystallinum Lind. & Andre, A. veitchii Mast. and A. grande Hort. were selected for the study.

The striking features of the species are as follows:

Anthurium andreanum is an epiphyte with some what creeping habit of growth using aerial roots for anchorage. The species is native of South West Columbia. The plant is erect with long, heart-shaped green leaves. The showy cordate spathe is wavy and puckered with pendent spadix tipped yellow.

Anthurium crystallinum is native of Columbia and Peru. From the central crown with thick fleshy roots, arise wiry petioles, circular in cross section, carrying large decorative heart-shaped velvetty leaves of stiff leathery texture, glistening emerald green with contrasting net work of white veins, 25 to 45 cm long with basal lobes overlapping, acutely angled at the thickened juncture; long stalked inflorescence with slender yellowish green spadix and linear green spathe.

Anthurium veitchii is native of Columbia. Plant with pendent, showy leaves upto 1 m long, cordate at base, curved lateral veins sunken giving a quilted look.

Plate I Anthurium andreanum

Plate II Anthurium crystallinum

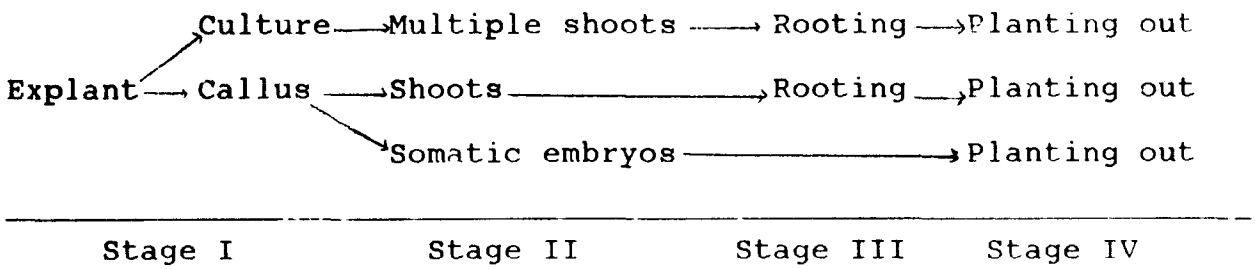
Plate III Anthurium veitchii

Plate IV Anthurium grande

Anthurium grande is native of Bolivia. Beautiful velvetty species from Yungas, large heart-shaped, pointed leaf with net work of pale veins.

The general *in vitro* multiplication procedures adopted for the study is presented as follows:

(Enhanced release of axillary buds)



General *in vitro* culture techniques

The chemicals used were of analytical grade from British Drug House (BDH), Sisco Research Laboratories (SRL), Merck or Sigma. Standard procedures (Biondi and Thorpe, 1980) were adopted for the preparation of the media. The pH of the media was adjusted between 5.8 and 6.0. Semisolid media containing 0.7 g/l agar (BDH) were used. Borosil brand test tubes of O.D and length (mm) of 25 x 100 and 25 x 200 and conical flasks (100 and 150 ml) were used. Sterilization of media and glasswares were done at 1.0 kg/cm^2 for 20 minutes. All aseptic manipulations were carried out in a laminar air flow chamber. Cultures were incubated at 26 ± 2°C, relative humidity ranging from 55 to 65 per cent with a 16 h photoperiod (40 μ E $m^{-2}s^{-1}$) except in cases where complete darkness was required. Cultures for callus initiation and callus multiplication were kept in darkness.

In vitro multiplication procedures

The methods adopted for the study are given in Table 1. Explants were washed thoroughly in distilled water followed by sterile water. They were then dipped in 0.1 per cent bavistin for 20 minutes and subjected to surface sterilization using chemicals as per Table 2. A few drops of the wetting agent 'laboline' were added to the washing solutions as well as sterilising agents.

The explants, after surface-sterilization, were rinsed (at least five times) with sterile water. The explants were given a fresh cut to remove the portions which became brown due to surface-sterilization and then they were transferred to the medium.

A. Enhanced release of axillary buds

Shoot tips from culture were used as explants for the study. Shoot tips were taken from seedlings grown *in vitro*. For this, ripe berries were removed from the spadix, seeds were squeezed out, washed in tap water and the pulp was removed. Seeds were then washed with sterile water, surface-sterilized with 4 per cent sodium hypochlorite for 10 minutes and then rinsed (at least five times) with sterile water. Seeds were germinated *in vitro* in MS (1962) medium. Two nodal stem segments from the germinated seeds were transferred to different treatments. The various treatments tried on enhanced release of axillary buds are given in Tables 3 and 4.

Table 1. *In vitro* multiplication procedures

Method of multiplication	Explant
(i) Enhanced release of axillary buds	Shoots tips (from culture)
(ii) Somatic organogenesis	Leaf Petiole Inflorescence stalk Spike Spathe
(iii) Somatic embryogenesis	Leaf (from field as well as from culture) Petiole Inflorescence stalk Spike Spathe

Table 2. Surface sterilization

Explant	Sterilant	Concentration	Time
Leaf	Sodium hypochlorite	1.0%	30 minutes
	or		
	Mercuric Chloride	0.1%	30 minutes
Petiole	Sodium hypochlorite	2.0%	30 minutes
Inflorescence Stalk	Sodium hypochlorite	2.0%	30 minutes
Spike	Sodium hypochlorite	3.0%	15 minutes
Spathe	Sodium hypochlorite	1.0%	30 minutes

Table 3. Cytokinins tested on enhanced release of axillary buds

Basal medium : MS

Treatment	Level
BA	0.1, 0.2, 0.5, 1.0 and 2.0 mg/l
Kinetin	0.1, 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l
2ip	0.1, 0.2, 0.5, 1.0 and 2.0 mg/l

Table 4. Carbon source, MS inorganic salts and agar concentration tested on enhanced released of axillary buds

Basal medium MS supplemented with kinetin 2.0 mg/l .

Treatment	Level
Sucrose	1.0, 2.0, 3.0 and 4.0%
Glucose	3.0%
Inorganic salts (Major and minor)	$\frac{1}{4}$, $\frac{1}{2}$, 1.0 times the normal strength
Agar	0.4, 0.5, 0.6, 0.7 and 0.8%

Effect of darkness on enhanced release of axillary buds was recorded. Multiple shoot formation on Nitsch medium supplemented with kinetin (2.0 mg/l) was also observed.

Observations

Observations were made, on number of cultures survived, number of growing cultures, number of shoots and length of the longest shoot, forty five days after inoculation.

B. Somatic organogenesis/embryogenesis

Explants of anthurium were taken from actively growing immature parts. Segments of leaves and petioles were taken three to four days after unfurling. Segments (2.0 cm diameter) of leaf and spathe were surface-sterilized, edges of the disc were given a fresh cut and then each disc was divided into half before transferring to medium. Segments of petioles, inflorescence stalk and spike were cut into pieces of 1.0 cm length.

Since both organogenesis and embryogenesis were callus mediated, treatments for callus initiation were tried in common.

The plants were selected from commercial nurseries and hence, the response of individual plants to in vitro culture was tested to screen out any non-responding types. Plants which responded to in vitro culture were selected for further studies.

Callus initiation

Basal medium used for callus initiation was MS medium modified by Pierik, 1976 (Appendix II). Studies were conducted on the effect of various plant growth substances (Table 5),

Table 5. Plant growth substances tested on callus initiation

Plant growth substances (mg/l)	Basal medium - Modified MS															
	A. andreanum					A. crystallinum				A. veitchii				A. grande		
	L	P	S	Sp	In	L	P	Sp	In	L	P	S	Sp	L	P	
2,4-D 0.02 + BA 1.00	L															
2,4-D 0.04 + BA 1.00	L															
2,4-D 0.06 + BA 1.00	L															
2,4-D 0.08 + BA 1.00	L	P	S	Sp	In	L	P	Sp	In	L	P	S	Sp	L	P	
2,4-D 0.10 + BA 1.00	L				In	L				L				L	P	
2,4-D 0.20 + BA 1.00	L				In	L	P			L	P	S		L	P	
2,4-D 0.50 + BA 1.00	L	P			In	L	P			L				L		
2,4-D 1.00 + BA 1.00	L					L				L				L		
2,4-D 0.10 + BA 2.00	L															
2,4-D 0.50 + BA 2.00	L															
2,4-D 1.00 + BA 2.00	L															
2,4-D 2.00 + BA 2.00	L															
2,4-D 2.00 + BA 1.00						L										
2,4-D 0.08 + 2ip 3.00	L	P	S			L				L		S		L	P	
2,4-D 1.00 + 2ip 1.00	L	P	S			L				L		S		L	P	
2,4-D 1.00 + 2ip 2.00	L															
2,4-D 0.08 + Kinetin 0.50	L															
2,4-D 0.08 + Kinetin 1.00	L															
2,4-D 0.08 + Kinetin 2.00	L															
2,4-D 0.20 + Kinetin 0.50	L															
2,4-D 0.20 + Kinetin 1.00	L															
2,4-D 0.20 + Kinetin 2.00	L															

Contd.....2

Plant growth substances (mg/l)	A. andreanum					A. crystallinum				A. veitchii				A. grande	
	L	P	S	Sp	In	L	P	Sp	In	L	P	S	Sp	L	P
2,4-D 0.50 + Kinetin 0.50	L														
2,4-D 0.50 + Kinetin 1.00	L														
2,4-D 0.50 + Kinetin 2.00	L														
2,4-D 1.00 + Kinetin 0.50	L														
2,4-D 1.00 + Kinetin 1.00	L														
2,4-D 1.00 + Kinetin 2.00	L														
2,4-D 0.10 + BA 1.00 + 2ip 3.00	L					L				L		S		L	
NAA 0.08 + BA 1.00	L														
NAA 0.10 + BA 1.00	L														
NAA 1.00 + BA 1.00	L														
NAA 0.08 + Kinetin 1.00	L														
NAA 0.10 + Kinetin 1.00	L														
NAA 1.00 + Kinetin 2.00	L														
IAA 1.00 + BA 1.00	L														
IAA 5.00 + Kinetin 1.00	L														
IAA 10.00 + Kinetin 1.00	L														
2,4-D 1.00 + NAA 1.00+ Kinetin 1.00	L														
2,4, 5-T 0.50 + BA 0.50	L														
2,4, 5-T 0.50 + BA 1.00	L														
2,4, 5-T 1.00 + BA 1.00	L														
2,4, 5-T 2.00 + BA 1.00						L				L					

L - Leaf P - Petiole S - Spathe Sp - Spike In - Inflorescence stalk

inorganic salts and inositol (Table 6) of the medium on callus initiation. Effect of other basal media on callus initiation was also compared (Table 7).

Observations

Observations on callus initiation were made at weekly intervals. Growth of the callus was assessed based on a visual rating (with score 1 to the smallest and score 4 to the largest). The mean score was expressed as the growth score G. Callus Index (CI) was computed by multiplying per cent explants initiating callus with the growth score.

Studies were also conducted on the effect of light (16 and zero hours), effect of source of explants (leaf, petiole, spathe, spike and inflorescence stalk), position of the leaf explant (basal or apical) and the effect of season of explant collection on callus initiation.

Observations were made on number of live explants under condtions of light and darkness. Various sources of explants were compared based on the number of sterile cultures and their in vitro response with respect to callus initiation. Callusing of different positions (basal or apical) of leaf explants was also observed.

Seasonal variation on callus initiation was studied. For this explants (leaf) were inoculated at monthly intervals. Observations were taken on callus initiation.

Table 6. MS inorganic salts and inositol on callus initiation

Basal medium : MS				
Treatments	*A. andreanum*	*A. crystallinum*	*A. veitchii*	*A. grande*
MS A full + 2,h-D 0.08 mg/l + B A 1.00 mg/l *	L		L	
MS A modified + 2,4-D 0.08 mg/l + B A 1.00 mg/l *	L		L	
MS A full + 2,4-D 0.50 mg/l + B A 1.00 mg/l *		L		L
MS A modified + 2,4-D 0.50 mg/l + B A 1.00 mg/l *		L		L
MS A modified _ 2,4-D 0.08 mg/l + B A 1.00 mg/l + inositol full *	L			
MS A modifed + 2,4-D 0.08 mg/l + B A 1.00 mg/l + inositol half *	L			

* Apppendi. 1 and

Table 7. Comparison of basal media on callus initiation

Treatment	Supplemented with	A. andreanum	A. crystallinum	A. veitchii	A. grande
Modified MS*	2,4-D 0.08mg/l+BA 1.00mg/l	L	L	L	L
Nitsch	2,4-D 0.08mg/l+BA 1.00mg/l	L	L	L	L
Nitsch	2,4-D 0.1 mg/l+BA 1.00mg/l	L			
Nitsch	2,4-D 0.08 mg/l+Kinetin 1.00mg/l	L			
Nitsch	2,4-D 0.08mg/l+Kinetin 1.00mg/l	L			
Nitsch	NAA 0.08mg/l+Kinetin 1.00mg/l	L			
Nitsch	NAA 0.1mg/l+Kinetin 1.00mg/l	L			
SH	2,4-D 0.08mg/l+BA 1.00mg/l	L	L		
LS	2,4-D 0.08mg/l+BA 1.00mg/l	L			
B_5	2,4-D 0.08mg/l+BA 1.00mg/l	L			

* Appendix II

Callus multiplication

For the study a known weight (approximately 0.2 g) of callus was transferred to media for various treatments (Table 8).

Observations were made on fresh weight of callus after 40 days of subculture. The callus was wrapped in sterilized aluminium foil and dried (till constant weight was recorded) in an oven at 50-60°C to find out the dry weight. Fresh weight multiplication rate was calculated as the ratio of final fresh weight to the initial fresh weight. Mitotic index of the various treatments was also observed.

The above observations were made on three callus pieces per treatment.

Sprout regenration and growth

The callus after proliferation was transferred to media (Table 9) for regeneration and further growth of the regenerated shoots.

Three cultures were observed per treatment. Number of cultures initiating sprouts, number of shoots per culture and length of the longest shoot were observed 30 days of culture.

Induction of somatic embryo

The callus was also transferred to media for the induction of somatic embryo. Treatments tested for embryoid formation are given in Table 10.

Observations were made for embryoid-like structures on three cultures per treatment after 30 days of culture.

Table 8. MS inorganic salts, other basal media tested on callus multiplication

Supplemented with BA 1.0 mg/l

Basal medium	Treatment	Level
MS	Inorganic salts (major elements)	$\frac{1}{4}$, $\frac{1}{2}$, 1.0 times the normal strength
SH		
LS		

Table 9. Treatments on sprout regeneration and growth of the shoots

	Basal medium - MS
Treatment	Level
Ammonium nitrate + BA 1.0 mg/l	1/8, 1/4, 1/2 and 1.0 times the normal strength
BA + NAA	0.5 + 1.0 mg/l
BA + NAA	1.0 + 1.0 mg/l
BA + IAA	0.5 + 0.5 mg/l
BA + IAA	0.5 + 2.0 mg/l
BA + IAA	1.0 + 2.0 mg/l
IAA	2.0 mg/l
BA	2.0 mg.l*
2ip	2.0 mg/l*
Kinetin	2.0 mg/l*
BA + CW	2.0 mg/l + 20%
2ip + CW	2.0 mg/l + 20%
BA + Phluoroglucinol	1.0 mg/l + 500 mg/l*
BA + AC	1.0 mg/l + 0.1%*

* Treatments tried only in A. grande

Table 10. Treatments tested on somatic embryo formation

Basal medium - MS

Treatment	Level
BA	0.5 mg/l
BA	1.0 mg/l
2, 4-D + BA + GA	0.1 + 0.5 + 1.0 mg/l
2, 4-D + BA + GA	0.1 + 1.0 + 1.0 mg/l
2, 4-D + BA + GA	0.1 + 2.0 + 1.0 mg/l
2, 4-D + BA + GA	0.2 + 0.5 + 1.0 mg/l
2, 4-D + BA + GA	0.2 + 1.0 + 1.0 mg/l
2, 4-D + BA + GA	0.2 + 2.0 + 1.0 mg/l
2, 4-D + BA	0.1 + 0.1 mg/l
2, 4-D + BA	0.5 + 0.5 mg/l
2, 4-D + BA	1.0 + 0.5 mg/l
2, 4-D + BA + CW	0.1 + 0.1 mg/l + 20.0%
2, 4-D + BA + CW	1.0 + 0.1 mg/l + 20.0%
2, 4-D + BA + Aspargine	0.1 + 0.1 + 600 mg/l
2, 4-D + Kinetin	0.1 + 0.5 mg/l
ABA	0.5 mg/l
ABA	1.0 mg/l
ABA	2.0 mg/l
Glucose + Mannitol	3.0 + 3.0%
Glucose + Mannitol	3.0 + 4.0%
Glucose + Mannitol	3.0 + 5.0%

Rooting

Shoots regenerated *via* somatic organogenesis as well as enhanced release of axillary buds, rooted spontaneously. However, the following treatments were tried for rooting.

(i) IBA 0.1 mg/l

(ii) IBA 0.5 mg/l

(iii) IBA 1.0 mg/l

(iv) BA (0.5 mg/l) + IAA (2.0 mg/l)

MS medium was used. In addition to the above treatments, MS medium (without plant growth substances) at half concentration was also tried.

Observations were made on days taken to rooting and number of roots.

Ex vitro establishment

1. Planting out

Plantlets with at least two roots as well as shoots without roots were carefully removed from the containers, washed thoroughly to remove the adhering agar and then transferred to sterilized sand medium (moisture per cent 19) in plastic pots. Observations were taken on number of plants survived after 30 days of planting out.

2. **Hardening**

The potted plantlets were kept in plastic basins and covered with a polythene sheet to maintain high humidity. For lowering the humidity during the later period of acclimatization, the polythene sheet was gradually uncovered.

Survival of plantlets without hardening was also observed.

3. Water loss through leaves

A comparitive study was made of the water loss per unit area at regular intervals from the leaves of in vitro grown plantlets, acclimatized plantlets (ne leaves and persistent leaves) and field grown plants. The excised leaves (with petioles) were kept immersed in distilled water for three hours at 25°C under diffused light (20 m E $m^{-2}S^{-1}$) provided by cool white fluorescent tubes. They were then taken out and the petioles were excised. After gently wiping the leaves with dry blotting paper, they were placed in dry petri dishes with abaxial surface facing up. Water loss from each leaf was estimated by recording the weight at regular intervals of 15 minutes for a total period of 105 minutes on an electronic digital balance (Sarotorius make with an accuracy of ± 0.1 mg). Throughout the experiment, a temperature of 29 ± 1°C and a relative humidity of 59 per cent prevailed. After this, the leaf area was estimated and loss of water per unit area per unit time was calculated.

4. Potting media

The following potting media were used for the study:

(i) Sand alone

(ii) Sand + Peat moss (1:1)

(iii) Sand + Perlite (1:1)

(iv) Sand + Charcoal (1:1)

(v) Sand + Cowdung (dried and powdered) (1:1)

(vi) Sand + Cowdung (dried and powdered) + Perlite (1:1:1)

(vii) Sand + Cowdung (dried and powdered) + Charcoal (1:1:1)

The potting media were autoclaved at 1.0 kg/cm^2 for 20 minutes and used.

Observations were recorded on the number of plantlets survived after 45 days of planting out.

5. Mineral salt solution for irrigation

The following treatments were used.

(i) 1/10 MS inorganic salt solution (pH 5.7)

(ii) ½ MS inorganic salt solution (pH 5.7)

(iii) 1:0:5:1 g/l Urea : Superphosphate : Muriate of Potash (pH 5.7)

(iv) Hoagland 2 solution

(v) Tap water (as control)

The plantlets were irrigated with 5ml of the solutions/tap water. Observations were recorded on survival of plantlets after 45 days.

6. Vesicular arbuscular mycorrhizae (VAM)

During the first part of the experiment, the following five strains of VAM were compared with control, on the survival of plantlets.

(i) Acaulospora morroweae

(ii) Glomus constrictum

(iii) Glomus fasciculatum

(iv) Glomus etunicatum

(v) Glomus mossae

VAM were incorporated to the potting medium (at the time of planting out) through infected root bits of guinea grass.

A detailed experiment was conducted with two strains of VAM, namely, Glomus constrictum and G. etunicatum against the control, to test the effect on growth of the plantlets. Observations were recorded on increase in height and fresh weight of plantlets (10 number/treatment) after 45 days of the treatment. Total leaf area, number of roots, length of the longest root and dry weight of shoots and roots were also recorded. The plantlets were analysed for the major and minor nutrients to assess the uptake.

Cytological studies

Roots (2 to 3 mm in length) were excised (between 11.30 a.m. and 12.30 p.m.), pretreated with 0.002 M 8- hydroxy quinoline for four hours (at 10°C) and fixed in carnoy's fluid (3:1:1 ethanol : acetic acid : chloroform) for 24 hours. The root tips were then hydrolysed for 15 minutes in IN HCl at 60°C, squashed in two per cent aceto carmine and observed for numerical changes in chromosomes.

Mitotic index for the treatments on callus multiplication was recorded. For this the callus piece was pretreated, fixed and hydrolysed as above; squashed in 2.0 per cent acetocarmine and observed. Mitotic index was calculated as the ratio of dividing cells to the total number of cells, multiplied by 100.

Histological analysis

Fresh, hand sections of callus were taken; stained in dilute safranin and observed for the status of embryoid-like structures. Cross sections of different explants were taken for comparison of the size of vascular bundles.

Biochemical characterization of species and explant sources

Different explant sources as well as the different species selected for the study were analysed for C/N ratio, contents of protein and total carbohydrate and activities of enzymes namely α - amylase, peroxidase and phenol oxidase. Polyacrylamide gel electrophoresis was done to study the isozymes of peroxidase.

C/N ratio

Total carbon and nitrogen content was estimated as per the standard procedures suggested by Jackson (1958) and the C/N ratio of the four Anthurium species as well as of the different explants were determined.

Protein

Different sources of explants (leaf, petiole, inflorescence stalk, spike and spathe) of Anthurium andreanum and leaves from the other three species (A. crystallinum, A. veitchii and A. grande) were analysed for protein content by the Folin-Lowry method (Plummer, 1988).

Total carbohydrate

Estimation of total carbohydrate in the different explant sources of anthurium as well as the four species of Anthurium was done by the anthrone method (Plummer, 1988).

α - amylase activity

The α - amylase activity was estimated by measuring (at 540 nm) the increase in reducing sugars using 3,5-dinitrosalicylate reagent when an alkaline solution of 3,5-dinitrosalicylic acid was reduced to 3-amino- 5-nitrosalicylic acid.

Buffered starch (2.5 ml), 1.0 ml phosphate buffer (0.1 M, pH 6.7) and 0.5 ml sodium chloride solution were pippetted out into tubes which were maintained at 37°C in a water bath. Added 0.5 ml of the enzyme extract and incubated at 37°C for 30 minutes. Added 0.5 ml of 2 M NaOH followed by 0.5 ml dinitrosalicylate reagent. The tubes were then heated for five minutes in a boiling water bath, cooled and read the extinction at 540 nm against a blank (Plummer, 1988).

Phenol oxidase activity

Phenol oxidase activity was measured by standard procedures suggested by Mahadevan and Sridhar (1982).

Peroxidase activity

Peroxidase activity was measured using pyrogallol method suggested by Mahadevan and Sridhar (1982).

Polyacrylamide gel electrophoresis

The tissue from each sample was homogenised in 100 mM phosphate buffer (pH 7.0) using a prechilled mortar and pestle in an ice bath. The homogenates were centrifuged at 3°C for 20 minutes and enzyme extracts equivalent to 200 µ g were loaded per well for acrylamide gel electrophoresis to detect the peroxidase isozyme activity (Anbalagan, 1985).

Isozymes were separated on 7.5 per cent polyacrylamide gels.

Stock solutions

A. Acrylamide stock

(i) 44:0:8

Acrylamide	44.0 g
Bis	0.8 g
Water to	100 ml

(ii) 30:8

Acrylamide	38.0 g
Bis	0.8 g
Water to	100 ml

B. Buffers

(i) separating gel buffer

Tris 1.5 M, 18.15 g

Dissolved in 50 ml water, adjusted the pH to 8.8 with concentrated HCl, made up the final volume to 100 ml.

(ii) Stacking gel buffer

Tris (0.5 M) 6.5 g

Dissolved in 50 ml water, adjusted the pH to 6.8 with concentrated HCl and final volume made upto 100 ml.

(iii)Lower tank buffer

Tris 1.21 g dissolved in water and pH adjusted to 6.5 with HCl (concentrated) and made upto 1 litre.

4. Upper tank buffer

Tris 0.6 g in 500 ml water

Glycine 0.35 in 500 ml water

Glycine added to Tris and pH adjusted to 8.8.

C. Procedure for gel preparation

(i) Separation gel

Equal volumes (6.81 ml) of acrylamide stock (i) and the separating gel buffer, 150 µl TEMED and 250µl of Ammonium persulphate were pippetted out into a beaker; volume of the solution was made upto 40 ml, mixed and poured into the gel mould and left to polymerise for 15 minutes.

(ii) Stacking gel

Equal volumes (7.88 ml) of acrylamide stock (ii) and the stacking gel buffer, 150 µl TEMED and 250µl Ammonium persulphate were pippetted out into a beaker; volume made up to 40 ml and the solution was mixed. The well forming comb was placed in position and the stacking gel solution was poured into the gel mould.

D. Loading the enzyme extract

Electrophoresis was carried out in Broviga-slab gel electrophoresis unit. The upper and lower tanks of the unit was filled to the level with the respective buffers. Enzyme extracts (200 µl) were loaded into the wells. Electrophoresis was carried out at constant current (50 m A) and at a voltage of 60 volts till the marker dye entered the separating gel. The voltage was then increased to 120 volts. At the end, the gels were removed and incubated in the appropriate staining solutions to detect the banding pattern.

E. Visualisation

The gel was incubated in a solution (for 20-30 minutes) containing benzidine (0.2 g), acetic acid (0.5 ml), hydrogen

peroxide (30.0%, 0.2 ml) and water (100 ml). After the incubation, gel was briefly washed in distilled water, stained for 10 minutes in 0.1 per cent ammonium sulphate solution and for 12 hours in 0.2 per cent ammonium sulphate solution.

Statistical analysis

Statistical analysis was done in completely randomised design, wherever necessary as per Panse and Sukhatme (1978).

Correlation coefficient among various weather parameters with callus index was computed from the following equation:

$$r = \frac{\Sigma xy - \frac{\Sigma x \, \Sigma y}{n}}{\sqrt{\left(\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right)\left(\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right)}}$$

Economics of production of anthurium plantlets

The cost of production of anthurium plantlets using leaf explants was worked out based on the facilities of the tissue culture laboratory of the Department of Horticulture, College of Agriculture, Vellayani, having a potential of maintaining 600 cultures for callus multiplication, 1200 cultures for sprout regeneration, 2400 cultures for shoot proliferation, growth and rooting and 2400 cultures of elongated shoots with roots (ready to be planted out). One Scientist (Rs. 2000/- p.m.) and one Assistant (Rs. 1200/- p.m.) were considered necessary for the work. The number of initial explants was 600. Expecting 50 per cent callus initiation, the number of callus initiating cultures was 300. The duration of callus initiation was 50 to 75 days; for callus multiplication 45 days; for sprout regenration 25 days;

for shoot proliferation, growth and rooting 45 days and for elongation of rooted shoots 60 days. The total number of shoots produced by this time (250 days) was 2400 x 25x. Expecting 10 plantlets (ready to be planted out) from each culture, the total number of plantlets, planted out at 25 days' interval, was calculated as 24000. Based on the survival ex vitro, the number of plantlets produced per year was worked out. The total cost of production involved per year was worked out; the cost of building, equipments, glasswares and miscellaneous items having been distributed over the years according to their potential durability. The cost of production of a single anthurium plantlet was worked out from the total cost of production and the number of plantlets produced per year.

Results

RESULTS

Attempts were made to improve the propagation efficiency of Anthurium species in vitro through enhanced release of axillary buds and somatic organogenesis/embryogenesis. Four species of Anthurium, namely, A. andreanum (pink and red). A. crystallinum, A. veitchii and A. grande were selected for the study. The results are presented in the following pages.

A. Enhanced release of axillary buds

Sterile seedlings to be used as the source of explants could be obtained only in Anthurium andreanum (pink). Hence, the results of the various treatments could be obtained only in this species.

Effect of cytokinins

Culture establishment and multiple shoot formation

The results of various cytokinin treatments are presented in Table 11. Cent per cent survival as well as growing cultures were observed in all the treatments tried. However, the number of multiple shoots differed among the treatments. Cent per cent cultures with multiple shoots were observed with BA (0.5 mg/l). 2ip (2.0 mg/l) as well as kinetin (1.0 and 2.0 mg/l). No multiple shoots were observed with 2ip 0.1 mg/l. Treatments with BA 0.2, 0.5, 1.0 and 2.0 mg/l and 2ip 1.0 and 2.0 mg/l induced callus growth at the basal portion of the shoot explants. Several adventitious shoots were produced from the callus, making it difficult to identify the axillary shoots. Increasing

Table 11. Effect of cytokinins on survival, growth and multiple shoot formation

Basal medium : MS

Treatment (mg/l)	Per cent survival	Per cent growing culture	Per cent growing culture with multiple shoots	Callus growth
BA 0.1	100.00	100.00	33.33	-
" 0.2	"	"	66.67	+
" 0.5	"	"	100.00	+
" 1.0	"	"	83.33	+
" 2.0	"	"	83.33	+
2ip 0.1	"	"	0.00	-
" 0.2	"	"	33.33	-
" 0.5	"	"	33.33	-
" 1.0	"	"	66.67	+
" 2.0	"	"	100.00	+
Kinetin 0.1	"	"	33.33	-
" 0.2	"	"	50.00	-
" 0.5	"	"	83.33	-
" 1.0	"	"	100.00	-
" 2.0	"	"	100.00	-

+ Callus growth observed

Plate V. BA inducing axillary and adventitious shoots

Plate VI. Kinetin inducing only axillary shoots

concentrations of BA and 2ip induced more callus growth whereas with kinetin, no callus growth was observed at all the levels tried.

Number of shoots

Significant variation was observed among the treaments for the number of shoots produced (Table 12). The number of shoots produced was 4.50 for BA (1.0 mg/l) and kinetin (2.0 mg/l) and 4.33 for BA (0.5 mg/l). Among these, treatments involving BA produced axillary as well as adventitious shoots (Plate V) whereas treatment with kinetin produced only axillary shoots (Plate VI). The number of shoots was reduced with kinetin 3.0 mg/l (3.33) and 4.0 mg/l (2.67).

Length of the longest shoot

Length of the longest shoot significantly differed among the treatments (Table 12). The length of shoots recorded with 2ip 1.0 mg/l (1.57 cm), BA 0.5 mg/l (1.52 cm) and 2ip 2.0 mg/l (0.88 cm) was on par. The longest shoot in the case of kinetin (2.0 mg/l) was 0.52 cm long.

Effect of MS inorganic salts

There was no significant difference among the treatments for the number of shoots as well as length of the longest shoot (Table 13).

Table 12. Effect of cytokinins on the number of shoots and the length of the longest shoot

Basal medium : MS

Treatment (mg/l)	Number of shoots	Length of the longest shoot (cm)	Callus growth
BA 0.1	0.67	0.03	-
" 0.2	3.00	0.56	+
" 0.5	4.33	1.52	+
" 1.0	4.50	0.63	+
" 2.0	2.17	0.35	+
2ip 0.1	-	-	-
" 0.2	0.33	0.23	-
" 0.5	0.33	0.18	-
" 1.0	2.00	1.57	+
" 2.0	3.50	0.88	+
Kinetin 0.1	0.33	0.27	-
" 0.2	0.50	0.28	-
" 0.5	1.83	0.38	-
" 1.0	2.83	0.27	-
" 2.0	4.50	0.52	-
CD	2.41**	0.90**	

\+ Callus growth observed

** Significant at 1% level

Effect of sucrose

None of the treatments recorded significant difference (Table 13).

Effect of glucose

Glucose (3.0%) produced less number of shoots (3.33) compared to sucrose (3.0%) where the number of shoots was 4.50.

Effect of agar

Data presented in Table 13 indicated no significant difference among the treatments for the number of shoots. Length of shoots was influenced by Agar concentration. Agar 0.4 per cent produced significantly longer shoots (Table 13). Increasing the concentration of agar suppressed the shoot growth.

Effect of Nitsch medium

Effect of Nitsch medium on multiple shoot formation was compared with that of MS medium. The mean number of shoots was less (2.67) in Nitsch medium compared to that in MS medium (4.50).

Effect of light

Effect of light for zero and 16 h was compared on multiple shoot formation. Early shoot induction was observed with 16 h of light. Callus induction at the base of the explant was observed in darkness.

Table 13. Effect of MS inorganic salts, sucrose and agar on the number of shoots and the length of the longest shoot.

Basal medium : MS + kinetin 2.0 mg/l

Treatment		Number of shoots	Length of the longest shoot (cm)
MS major nutrients	¼ Conc.	2.67	0.75
" " "	½ Conc.	3.00	0.80
MS minor nutrients	¼ Conc.	2.50	0.83
" " "	½ Conc.	3.33	1.38
" major & minor nutrients full Conc.		4.50	0.52
	CD	NS	NS
Sucrose	1.0%	2.66	0.45
"	2.0%	3.00	0.48
"	3.0%	4.50	0.52
"	4.0%	2.50	0.52
	CD	NS	NS
Agar	0.4%	3.00	0.95
"	0.5%	2.83	0.67
"	0.6%	3.17	0.72
"	0.7%	4.50	0.52
"	0.8%	4.15	0.28
	CD	NS	0.24

B. Somatic organogenesis/embryogenesis

Callus initiation

Effect of plant growth substances

The studies were initially made in A. andreanum (pink and red). Since response was obtained only for A. andreanum with pink spathe colour, the results unless otherwise specifically mentioned, relate to this type.

Among the 32 combinations (Table 14) of plant growth substances tried, 2,4-D (0.08 mg/l) + BA (1.0 mg/l) recorded the maximum number of cultures (50.35%) initiating callus, the highest growth score (1.86) and CI value (93.65). The number of cultures initiating callus was less (20.00%) at higher and lower concentrations of 2,4-D. Combinations of 2,4-D and BA were better than the other combinations of growth substances in inducing callus.

In A. crystallinum none of the treatments could produce positive response. In A. veitchii, the maximum number of cultures initiating callus (43.33%) was recorded for 2,4-D (0.2 mg/l) + BA (1.0 mg/l). The maximum growth score was 1.00 and the maximum CI value was 43.33. In the case of A. grande, the most effective treatment for A. andreanum could induce callus only in 20 per cent of the cultures. Higher concentrations of 2,4-D were found to be better in inducing callus in this species. A combination of 2,4-D (0.5 mg/l) + BA (1.0 mg/l) was the best, inducing callus in 61.67 per cent cultures. In this case, the maximum growth score was 1.50 and the maximum CI value was 92.51.

Table 14. Effect of plant growth substances on callus initiation and growth

Explant : Leaf
Modified MS Medium

Plant growth substances (mg/l)	Per cent cultures initiating callus				Growth Score				Callus Index			
	A	C	V	G	A	C	V	G	A	C	V	G
2,4-D 0.02 + BA 1.00	20.00				1.00				20.00			
" 0.04 + "	20.00				1.00				20.00			
" 0.06 + "	16.67				1.00				16.67			
" 0.08 + "	50.35	0	20.00	20.00	1.86	0	1.00	1.00	93.65	0	20.00	20.00
" 0.10 + "	33.33	0	20.00	20.00	1.00	0	1.00	1.00	33.33	0	20.00	20.00
" 0.20 + "	20.00	0	43.33	40.00	1.00	0	1.00	1.00	20.00	0	43.33	40.00
" 0.50 + "	0	0	0	61.67	0	0	0	1.50	0	0	0	92.51
" 1.00 + "	0	0	0	60.00	0	0	0	1.00	0	0	0	60.00
" 0.10 + BA 2.00	0			0					0			
" 0.50 + "	0			0					0			
" 1.00 + "	0			0					0			
" 2.00 + "	0			0					0			
2,4-D 2.00 + BA 1.00		0				0				0		
" 0.08 + 2ip 3.00	0	0	20.00	60.00	0	0	1.00	1.00	0	0	20.00	60.00
" 1.00 + 2ip 1.00	0				0				0			
" " + 2ip 2.00	0				0				0			
2,4-D 0.08 + Kinetin 1.00	0				0				0			
2,4-D 0.10 + BA 1.00 + 2ip 3.00	0	0	0	33.33	0	0	0	1.00	0	0	0	33.33

(Contd...)

Table 14 (Contd.)

Plant growth substances (mg/l)	Per cent cultures initiating callus				Growth Score				Callus Index			
	A	C	V	G	A	C	V	G	A	C	V	G
NAA 0.08 + BA 1.00	0					0			0			
" 0.10 + "	0					0			0			
" 1.00 + "	0					0			0			
" 0.08 + kinetin 1.00	0					0			0			
" 0.10 + kinetin 1.00	0					0			0			
" 1.00 + "	0					0			0			
" " + kinetin 2.00	0					0			0			
IAA 1.00 + BA 1.00	0					0			0			
IAA 5.00 + kinetin 1.00	0					0			0			
IAA 10.00 + kinetin 1.00	0					0			0			
2,4-D 1.00 + NAA 1.00 + kinetin 1.00	0					0			0			
2,4,5-T 0.50 + BA 0.50	0				0				0			
+ BA 1.00	0				0				0			
2,4,5-T 1.00 + BA 1.00	0	0			0	0			0	0		
2,4,5-T 2.00 + BA 1.00		0	0			0	0			0	0	

A : Anthurium andreanum
C : Anthurium crystallinum
V : Anthurium veitchii
G : Anthurium grande

Various combinations of 2,4-D and kinetin were also tried for initiating callus from in vitro grown leaves (Table 15). Among the treatments, 2,4-D (0.2 or 1.0 mg/l) + kinetin (0.5 mg/l) induced callus in 66.67 per cent cultures. The maximum growth score of 1.75 and CI value of 116.67 were observed in 2,4-D (0.2 mg/l) + kinetin (0.5 mg/l). Callusing was reduced with increasing concentrations of kinetin.

Effect of MS inorganic salts and inositol

Callusing was observed in modified MS medium with reduced MS major nutrients concentration whereas no callusing was observed in MS medium with normal concentration (Table 16). Similar result was obtained with A. veitchii. In A. grande, MS major inorganic salts at normal concentration produced callus in 50 per cent cultures whereas modified MS major inorganic salts produced callus in 61.67 per cent cultures.

No callusing was observed when inositol was reduced to half concentration.

Effect of different basal media

The maximum number of cultures initiating callus (50.35%), growth score (1.86) and CI (93.65) value were recorded in modified MS medium (Table 17). Callusing was less with the other basal media, namely, Nitsch, SH and LS whereas no callusing was observed in B_5 medium.

Table 15. Effect of plant growth substances on callus initiation and growth in *in vitro* grown leaves

Modified MS medium

Plant growth substances (mg/l)				Per cent culture initiatin callus	Growth score	Callus Index
2,4-D	0.08	+ Kinetin	0.50	0	0	0
"		+ "	1.00	0	0	0
"		+ "	2.00	0	0	0
2,4-D	0.20	+ "	0.50	66.67	1.75	116.67
"		+ "	1.00	33.33	1.00	33.33
"		+ "	2.00	0	0	0
2,4-D	0.50	+ "	0.50	33.33	1.00	33.33
"		+ "	1.00	0	0	0
"		+ "	2.00	0	0	0
2,4-D	1.00	+ "	0.50	66.67	1.00	66.67
"		+ "	1.00	0	0	0
"		+ "	2.00	0	0	0

Table 16. Effect of MS inorganic salts and inositol on callus initiation

Treatment	Per cent cultures initiating callus				Growth Score				Callus Index			
	A	C	V	G	A	C	V	G	A	C	V	G
MS* A full + 2,4-D 0.08mg/l + BA 1.00 mg/l	0		0		0		0		0		0	
MS** A modified + "	50.35		20.00		1.86		1.00		93.65		20.00	
MS* A full + 2,4-D 0.5 mg/l + BA 1.00 mg/l		0		50.00		0		1.00		0		50.00
MS** A modified + "		0		61.67		0		1.50		0		92.51
Modified MS** + 2,4-D 0.08 mg/l + BA 1.00mg/l + inositol full	50.35				1.86				93.65			
Modified MS** + 2,4-D 0.08 mg/l + BA 1.00mg/l + inositol half	0				0				0			

* Appendix I
** Appendix II

A : Anthurium andreanum
C : Anthurium scherzerianum
V : Anthurium veitchii
G : Anthurium grande

Table 17. Comparison of different basal media on callus initiation

Treatment	Per cent cultures initiang callus				Growth Score				Callus Index			
	A	C	V	G	A	C	V	G	A	C	V	G
Modified MS + 2,4-D 0.08mg/l + BA 1.00 mg/l	56.35	0	20.00	20 00	1.86	0	1.00	1.00	93.65	0	20.00	20.00
Nitsch + 2,4-D "	38.89	0	0	0	1.50	0	0	0	58.34	0	0	0
" + 2,4-D 0.10 mg/l+ BA 1.00 mg/l	0					0			0			
" + 2,4-D 0.08 mg/l+ kinetin 1.00 mg/l	0					0			0			
" + NAA 0.10 mg/l + kinetin 1.00 mg/l	0					0			0			
" + NAA 0.10 mg/l+ kinetin 1.00 mg/l	0					0			0			
SH + 2,4-D 0.08 mg/l + BA 1.00 mg/l	20.00	0			1.50	0			30.00	0		
LS + " "	16.67				1.00				16.67			
B5 + " "	0				0				0			

A : Anthurium andreanum
C : Anthurium crystallinum
V : Anthurium veitchii
G : Anthurium grande

Effect of light

An exposure to 16 h of light inhibited initiation of callus from leaf explants. The explants turned brown within 15 days.

Comparison of different explant sources

The data on contamination, number of sterile cultures, days taken to callusing and the number of cultures initiating callus are given in Table 18. The highest number of sterile cultures (59.09%) was obtained from leaf explants (Figure 1). Only leaf explants initiated callus whereas only swelling was observed when explants were taken from spike, petiole and inflorescence stalk. Spathe explants did not exhibit swelling or callusing. Average number of cultures initiating callus was 51.78 per cent for leaf explants from which callus was initiated in 52.33 days.

Comparison of basal and apical portion of leaf explants

Comparison of basal and tip portions of leaf explants on callus initiation is presented in Table 19. In A. andreanum (pink), basal portions of leaf produced callus in 55.20 days whereas apical portions produced callus in 75.20 days. Basal portions of leaf initiated callus in 60.00 days in A. veitchii whereas apical portions produced no callus. However, in A. grande, both basal and apical portions produced callus in 42.00 days. Basal protions of leaf in A. andreanum (pink) produced callus in 50.35 per cent cultures whereas apical portions produced callus only in 6.25 per cent cultures. The growth scores were 1.86 and 1.00 and the CI values were 93.65 and 6.25 for basal and apical portions, respectively. In A. veitchii, basal

Table 18. Comparison of different explants on callus initiation

Explant	Average percentage of contamination	Per cent sterile cultures	Number of days taken to callus initiation	Per cent cultures initiating callus
Leaf	40.91	59.09	52.33	51.78
Petiole	78.06	21.94	0	0
Spike	79.86	20.14	0	0
Spathe	54.00	46.00	0	0
Inflorescence Stalk	78.75	21.25	0	0

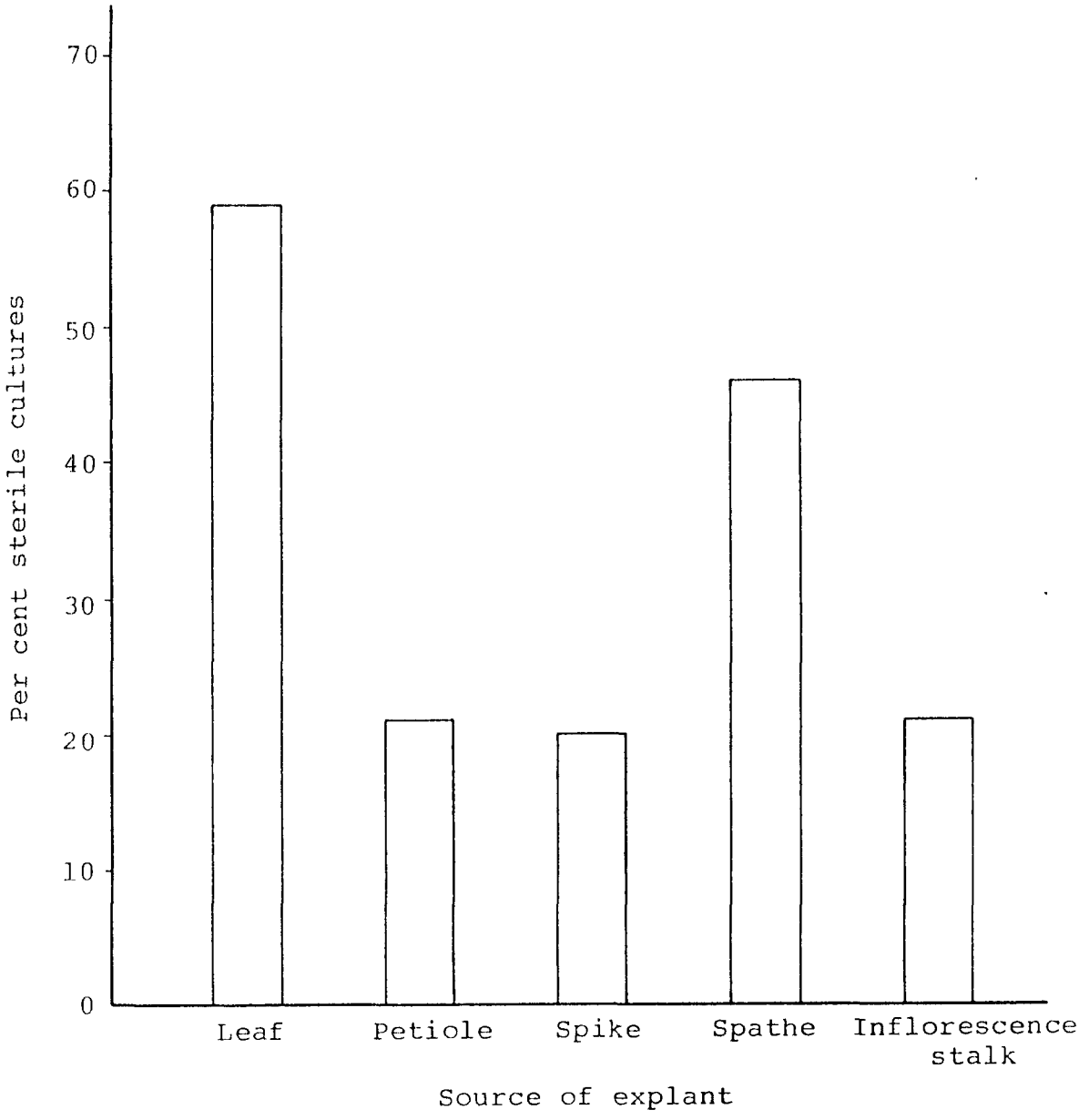


Fig.1 Comparison of different explant sources for callus initiation

Table 19. Comparison of differnt portions of leaf explants on callus initiation

Portion of leaf	Number of days taken to callus initiation			Per cent cultures initiating callus			Growth score			Callus index		
	A	V	G	A	V	G	A	V	G	A	V	G
Basal	55.20	60.00	42.00	50.35	43.33	61.67	1.86	1.00	1.50	93.65	43.33	92.51
Apical	75.20	-	42.00	6.25	-	61.67	1.00	-	1.50	6.25	-	92.51

A : Anthurium andreanum
V : Anthurium veitchii
G : Anthurium grande

portions of leaf initiated callus in 43.33 per cent cultures with a growth score 1.00 and CI value 43.33. Basal and apical portions of leaf in A. grande produced callus in 61.67 per cent cultures with growth score 1.50 and CI value 92.51.

Effect of season of explant collection on callus initiation

Weather parameters like maximum temperature, minimum temperature, relative humidity and rainfall during the months in which explants were collected and the mean CI values for the respective months for A. andreanum (pink) and A. veitchii are given in Tables 20 and 21 respectively. Coefficient of correlation of mean CI value with weather parameters is given in Table 22. A negative correlation (not significant) between mean CI values and maximum and minimum temperature was observed in both the species whereas a positive correlation was observed (not significant) between mean CI value and relative humidity and rainfall.

Callus multiplication

Effect of MS inorganic salts

MS major nutrients at full concentration recorded (Table 23) the maximum fresh weight (3.15 g) of callus followed by one-forth MS major nutrients concentration (3.11 g). Fresh weight multiplication rate was maximum (15.75) in MS medium at full concentration. The maximum dry weight (1.76 g) was also recorded in this treatment. Mitotic index was the maximum (1.65) for MS major nutrients at one-forth concentration.

Table 20. Weather parameters and mean Callus Index for Anthurium andreanum (pink)

Month	Maximum temperature (°C)	Minimum temperature (°C)	Relative humidity (%)	Rainfall (mm)	Mean CI
August 1990	29.45	23.65	78.00	27.90	112.87
September 1990	30.70	24.02	77.50	78.20	0
October 1990*	-	-	-	-	-
November 1990*	-	-	-	-	-
December 1990*	-	-	-	-	-
January 1991	30.80	22.30	77.80	28.60	150.00
February 1991	-	-	-	-	-
March 1991	32.30	23.98	76.10	40.00	3.33
April 1991	33.40	25.40	78.70	31.20	60.00
May 1991	33.20	25.75	78.66	87.40	0
June 1991	29.50	24.00	80.20	669.30	80.10
July 1991*	-	-	-	-	-
August 1991*	-	-	-	-	-
September 1991	30.70	24.10	76.90	22.40	38.33
October 1991	29.91	23.69	80.53	205.80	16.67
November 1991	30.22	23.16	81.60	247.10	52.41
December 1991	30.40	21.90	74.30	20.00	0
January 1992	30.29	20.40	73.32	35.00	36.00
February 1992	30.87	21.92	74.41	0	130.20
March 1992	32.06	22.22	72.47	0	14.29
April 1992	33.10	25.47	75.57	6.00	8.33
May 1992	31.72	24.55	78.06	80.90	4.17

* Explants not collected during these months

Table 21. Weather parameters and mean Callus Index for Anthurium veitchii

Month	Maximum temperature (°C)	Minimum temperature (°C)	Relative humidity (%)	Rainfall (mm)	Mean CI
April 1991	33.40	25.40	78.70	31.20	0
May 1991*	-	-	-	-	-
June 1991	29.50	24.00	88.20	669.30	20.00
July 1991*	-	-	-	-	-
August 1991*	-	-	-	-	-
September 1991	30.70	24.10	76.90	22.40	0
October 1991*	-	-	-	-	-
November 1991	30.22	23.16	81.60	247.10	0
December 1991	30.53	23.99	74.30	20.00	10.00
January 1992*	-	-	-	-	
February 1992*	-	-	-	-	-
March 1992*	-	-	-	-	-
April 1992*	-	-	-	-	-
May 1992	31.72	24.55	78.06	80.90	15.00

* Explants not collected during these months

Table 22. Correlation coefficient between Callus Index and weather parameters in A. andreanum (pink) and A. veitchii

Species	Coefficient of correlation			
	Maximum temperature	Minimum temperature	Relative humidity	Rainfall
A. andreanum	-0.3807^{NS}	-0.3056^{NS}	0.1911^{NS}	0.0972^{NS}
A. veitchii	-0.4037^{NS}	-0.1130^{NS}	0.4457^{NS}	0.6019^{NS}

Table 23. Effect of MS inorganic salts (major nutrients) on callus multiplication

Treatment	Initial weight of callus 0.2 g			
	Fresh weight(g)	Dry weight(g)	Fresh weight multiplication rate	Mitotic index %
MS major nutrients full	3.15	1.76	15.75	1.20
MS major nutrients half	2.67	1.34	13.35	0.65
MS major one-fourth	3.11	1.58	15.55	1.65
Modified MS	1.32	0.28	6.60	0.40

Effect of different basal media

MS (one-forth major nutrients concentration) medium recorded (Table 24) the maximum fresh weight (3.11 g) as well as dry weight (1.58 g) of callus. Fresh weight as well as dry weight of callus was less in the other basal media (SH and LS).

Induction of somatic embryos

Somatic embryo formation was not observed in the treatments tried. However, some embryoid-like structures were observed in treatment with 2,4-D + BA + CW (0.1 mg/l + 1 mg/l + 20.00%).

Shoot regeneration and growth

The effect of various treatments on shoot regeneration and growth of shoots in A. andreanum (pink) is presented in Table 25. Cent per cent regeneration was recorded in all the treatments. However, the maximum number of shoots per culture (6.50) as well as length of the longest shoot (1.20 cm) were recorded for BA (0.5 mg/l) + IAA (2.0 mg/l). Many shoots with suppressed growth was produced with BA 1.0 mg/l. In general, IAA treatments produced longer shoots. In A. veitchii BA (0.5 mg/l) + IAA (2.0 mg/l) recorded a shoot number of 5.60 (Plate VII) whereas in A. grande (Plate VIII) the number of shoots was less (2.33). Less number or no shoot was produced in the other treatments (Table 26) which were tried to improve the regeneration of shoots in A. grande.

Rooting

The effect of various treatments are given in Table 27. The minimum number of days (10.00) taken to initiate roots was

Table 24. Comparison of basal media for callus multiplication

Initial weight of callus 0.2 g

Treatment	Fresh weight(g)	Dry weight(g)	Fresh weight multiplication rate	Mitotic index %
MS (major nutrients $\frac{1}{4}$)	3.11	1.58	15.55	1.65
SH	2.00	0.87	10.00	0.60
LS	1.37	0.60	6.85	0.45

Table 25. Effect of treatments on shoot regeneration and growth in Anthurium andreanum (pink)

Basal medium : MS

Treatment	Per cent culture initiating shoot	Number of shoots per culture	Length of the longest shoot (cm)
Ammonium nitrate 1/8 times the normal strength + BA 1.0mg/l	100	5.9	0.56
Ammonium nitrate ¼ times the normal strength + BA 1.0mg/l	100	5.9	0.55
Ammonium nitrate ½ times the normal strength + BA 1.0mg/l	100	5.6	0.56
Ammonium nitrate 1.0 times the normal strength + BA 1.0 mg/l	100	5.8	0.52
BA 0.5 mg/l + IAA 0.5 mg/l	100	5.2	0.62
BA 0.5 mg/l + IAA 1.0 mg/l	100	5.8	0.68
BA 0.5 mg/l + IAA 2.0 mg/l	100	6.5	1.20
BA 1.0 mg/l + IAA 2.0 mg/l	100	5.7	0.89
IAA 2.0 mg/l	100	5.2	0.68

Table 26. Effect of treatments on shoot regeneration and growth in Anthurium grande

Basal medium : MS

Treatment	Per cent cultures initiating shoot	Number of shoots per culture	length of the longest shoot (cm)
Ammonium nitrate 1/8 times the normal strength + BA 1.0 mg/l	0	0	0
Ammonium nitrate 1/4 times the normal strength + BA 1.0 mg/l	0	0	0
Ammonium nitrate 1/2 times the normal strength + BA 1.0 mg/l	33.33	0.33	0.21
Ammonium nitrate 1.0 times the normal strength + BA 1.0 mg/l	66.67	0.67	0.33
BA 1.0 mg/l + NAA 1.0 mg/l	0	0	0
BA 2.0 mg/l	0	0	0
BA 2.0 mg/l + CW 20%	66.67	2.00	0.36
2ip 2.0 mg/l	0	0	0
2ip 2.0 mg/l + CW 20%	0	0	0
Kinetin 2.0 mg/l	33.33	0.67	0.54
BA 0.5 mg/l + IAA 2.0 mg/l	66.67	2.33	1.10
BA 1.0 mg/l + Phluroglucinol	0	0	0
BA 1.0 mg/l + AC 0.1%	33.33	0.33	0.58

Plate VII. Anthurium veitchii shoot regeneration

Plate VIII. Anthurium grande shoot regeneration

recorded for BA (0.5 mg/l) + IAA (2.0 mg/l). The average number of roots was 5.33 in this treatment. MS (half strength) without any plant growth substances initiated roots within 9.00 days, but had less number of roots (4.00).

Ex vitro establishment

1. Planting out

Plantlets with at least two roots survived better (83.33%) than shoots without roots (33.33%). Growth of the survived shoots (without roots) was very slow compared to rooted plantlets.

2. Hardening

Plantlets with hardening as well as without hardening treatment recorded the same survival (80.00%) and growth ex vitro.

3. Water loss through leaves

The results indicated that the rate of water loss through leaves of in vitro grown anthurium plantlets was 21.78 ng per unit area per unit time (Appendix III). Leaves (both newly produced and persistent) of acclimatized plantlets had reduced water loss. Water loss through leaves of field grown plants was 19.43 ng per unit area per unit time. There was not much difference in the rate of water loss through leaves of in vitro plantlets and field grown plants (Figure 2).

4. Potting media

The data on survival (in percentage) of plantlets in different potting media are presented in Table 28. The maximum survival (83.33%) was observed (Figure 3) in sand alone (as the potting medium).

Table 27. Effect of treatments on rooting

Basal medium : MS

Treatment	Days taken to rooting	Number of roots
IBA 0.1 mg/l	16.33	7.33
IBA 0.5 mg/l	10.33	6.67
IBA 1.0 mg/l	18.67	5.00
BA (0.5 mg/l) + IAA (2.0 mg/l)	10.00	5.33

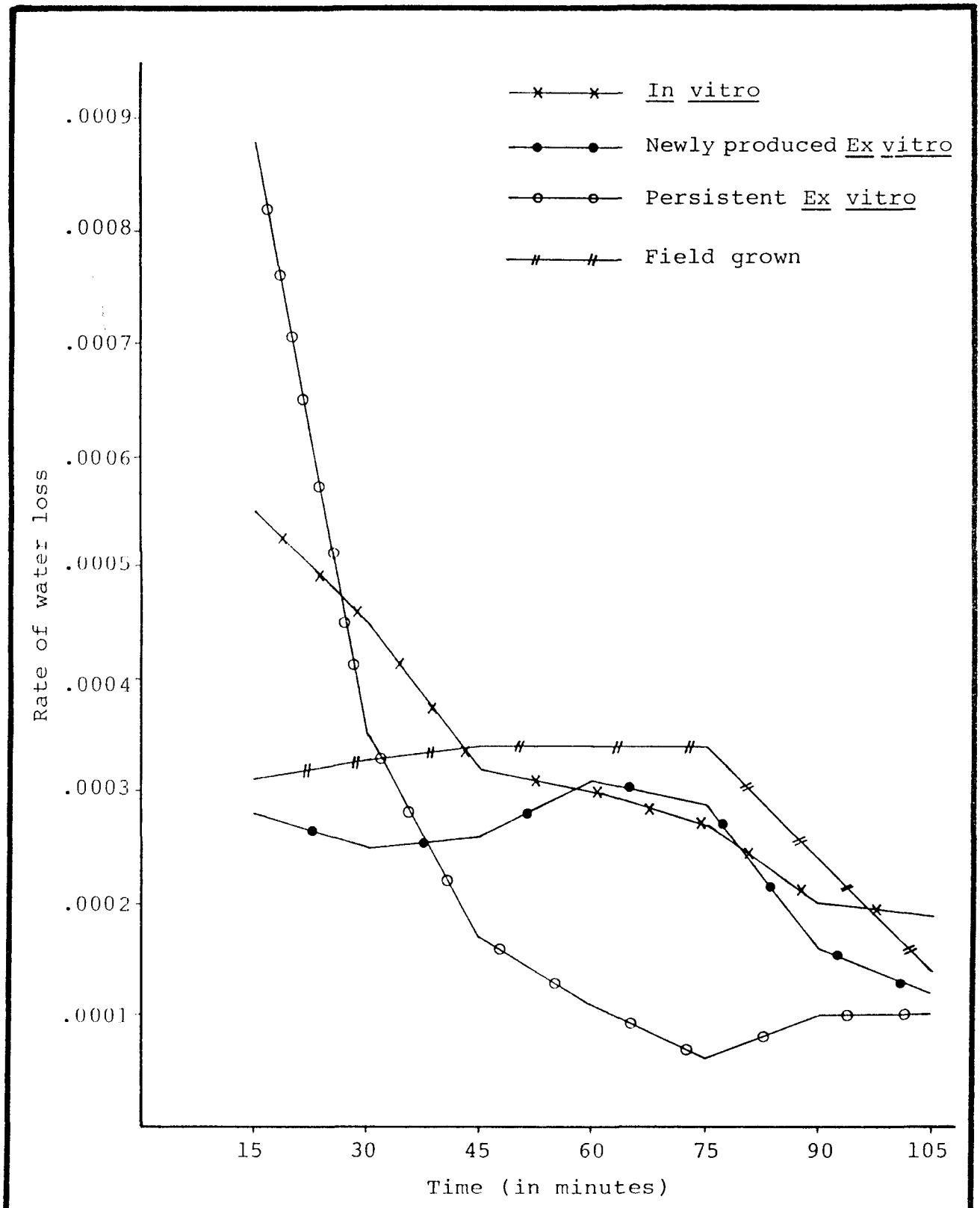


Fig.2 Rate of water loss through leaves

Table 28. Effect of potting media on ex vitro establishment of plantlets

Potting media	Survival (%)
Sand alone	83.34
Sand + Peat moss (1:1)	66.67
Sand + Cowdung (dried and powered) (1:1)	50.00
Sand + Perlite (1:1)	50.00
Sand + Charcoal (1:1)	66.67
Sand + Cowdung (dried and powdered) + Perlite (1:1:1)	66.67
Sand + Cowdung (dried and powdered) + Charcoal (1:1:1)	66.67

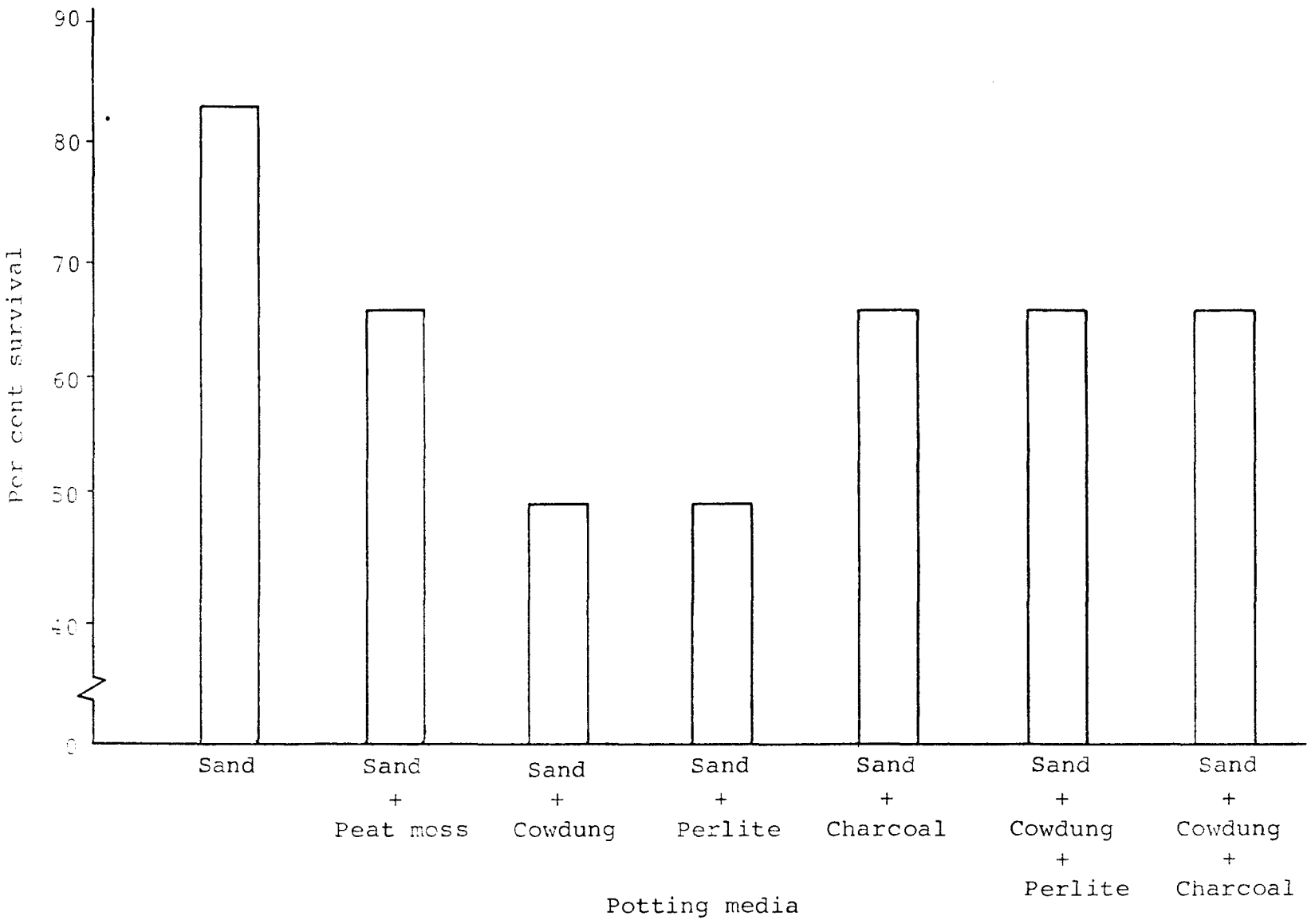


Fig.3 Effect of potting media on ex vitro establishment of plantlets

5. Mineral salt solution for irrigation

Tap water supported the maximum survival (80.00%) of the plantlets (Table 29). This was followed by 40.00 per cent survival in MS inorganic salt solution (1/10 concentration) and 20.00 per cent survival in Hoagland 2 solution.

6. Vesicular arbuscular mycorrhizae

Among the five strains compared with control, for survival of plantlets, Glomus constrictum treated plantlets recorded cent per cent survival (Table 30) followed by 60.00 per cent survival in plantlets treated with G. etunicatum, and Acaulospora morroweae, and in the control.

The data on the effect of VAM (G. constrictum and G. etunicatum) on growth of the plantlets are presented in Table 31. The increase in height was more (46.02% and 43.24%) in VAM-treated plantlets compared to the control (19.67%). Increase in the fresh weight was the maximum (40.61%) in the plantlets treated with G. etunicatum. The VAM-treated plantlets showed significantly increased leaf area (25.74 cm^2 and 22.54 cm^2) compared to the control (14.79 cm^2). The number of roots and length of the longest root were on par in all the treatments whereas fresh and dry weight of roots were significantly high in the VAM-treated plantlets. Data on nutrient analysis of plantlets are given in Table 32. An increased content of N, P, K, Ca, Mg and Zn was present in the VAM-treated plantlets.

Table 29. Effect of mineral salt solution used for irrigation on survival of plantlets

Treatment	Survival (%)
1/10 MS inorganic salt solution (pH 5.7)	40.00
¼ MS inorganic salt solution (pH 5.7)	0
1:0.5:1 g Urea:Super phosphate: Muriate of potash/l (pH 5.7)	0
Hoagland 2 solution	20.00
Control (tap water)	80.00

Table 30. Effect of vesicular arbuscular mycorrhizae on survival of plantlets

Treatment	Survival (%)
Acaulospora morrowae	60.00
Glomus constrictum	100.00
Glomus etunicatum	60.00
Glomus fasciculatum	40.00
Glomus mossae	40.00
Control	60.00

Table 31. Effect of vesicular arbuscular mycorrhizae on growth of plantlets

Treatment	Per cent increase in height	Per cent increase in fresh weight	Leaf area (cm^2)	Number of roots	Length of roots (cm)	Fresh weight of roots (g)	Dry weight of roots (g)
Glomus constrictum	46.02	20.96	22.54	5.40	2.26	0.4344	0.0375
Glomus etunicatum	43.24	40.61	25.74	6.20	6.90	0.5264	0.0387
Control	19.67	12.33	14.79	4.20	4.46	0.2577	0.0168
		CD	5.96**	NS	NS	4.88*	6.82**

Table 32. Nutrient analysis

Treatment	Nutrients (in percentage)					
	N	P	K	Ca	Mg	Zn
Glomus constrictum	0.24	0.44	3.28	1.95	0.87	0.15
Glomus etunicatum	0.13	0.44	3.25	1.91	1.03	0.16
Control	0.12	0.32	2.09	1.91	0.59	0.04

Cytological studies

Observations on mitotic chromosome number of 100 plantlets randomly selected from 500 regenerated plantlets, revealed (Plate IX) a diploid nature with the normal count of 2n = 30 + 2B chromosomes.

Histological analysis

No bipolar structure was observed in the fresh sections of callus. The embryoid-like structures showed an adventitious origin of roots.

There was difference in the size of the vascular bundles in the different explants. Cross sections of leaf and spathe showed smaller vascular bundles compared to that of petiole spike and inflorescence stalk (Plates X to XIV).

Biochemical characterization

C/N ratio

C/N ratio of different explants of A. andreanum (pink) as well as of the leaves of A. andreanum (red), A. crystallinum, A. veitchii and A. grande, are given in Table 33. Among the different explants of A. andreanum (pink), leaf had the highest value (165.46). The lowest C/N ratio was recorded for petiole (4.94). Leaf explant of A. andreanum (red) had a low C/N ratio (9.32) compared to pink type. Other species of Anthurium also recorded low C/N ratios.

Protein

Spathe had the highest protein content (1.71%) among the different explants of A. andreanum (pink). Leaves of A. andreanum

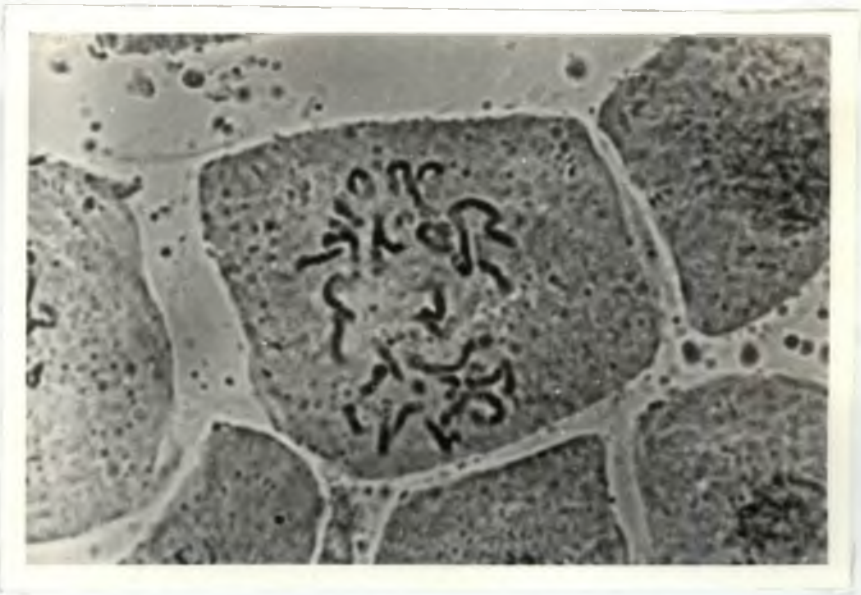


Plate IX. Mitotic chromosomes in in vitro regenerated Anthurium andreanum (pink)

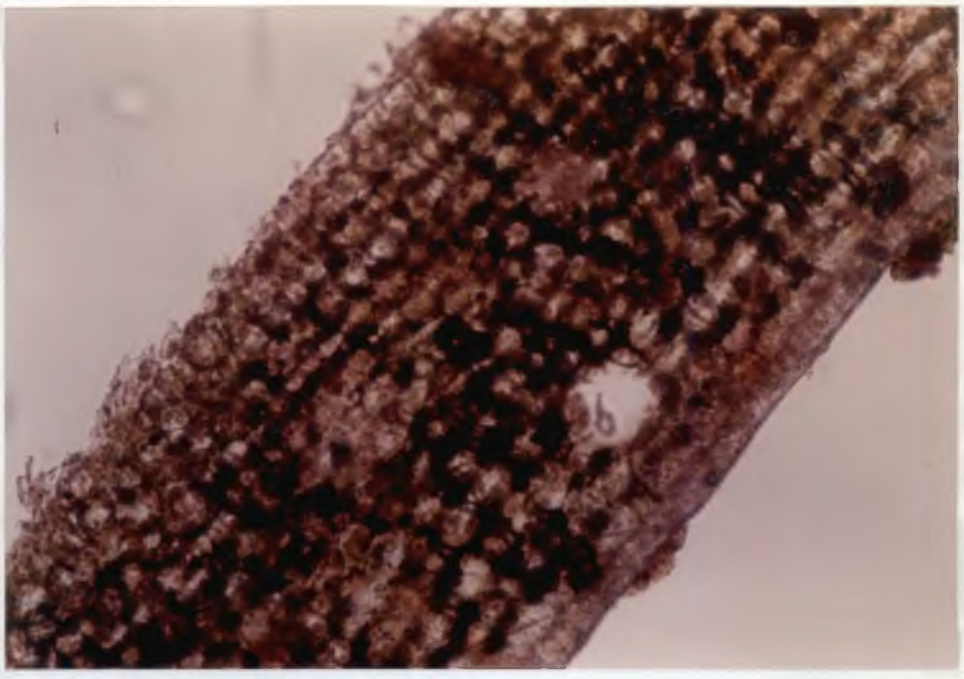


Plate X. Vascular bundles of leaf (C.S)
Magnification 100 x

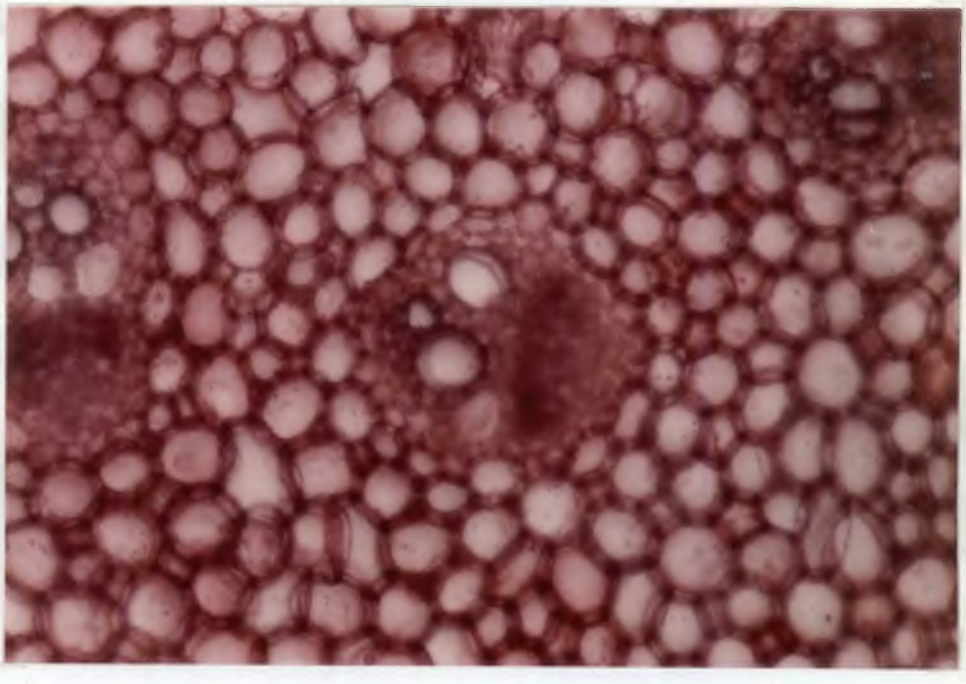


Plate XI. Vascular bundles of petiole (C.S)
Magnification 100 x

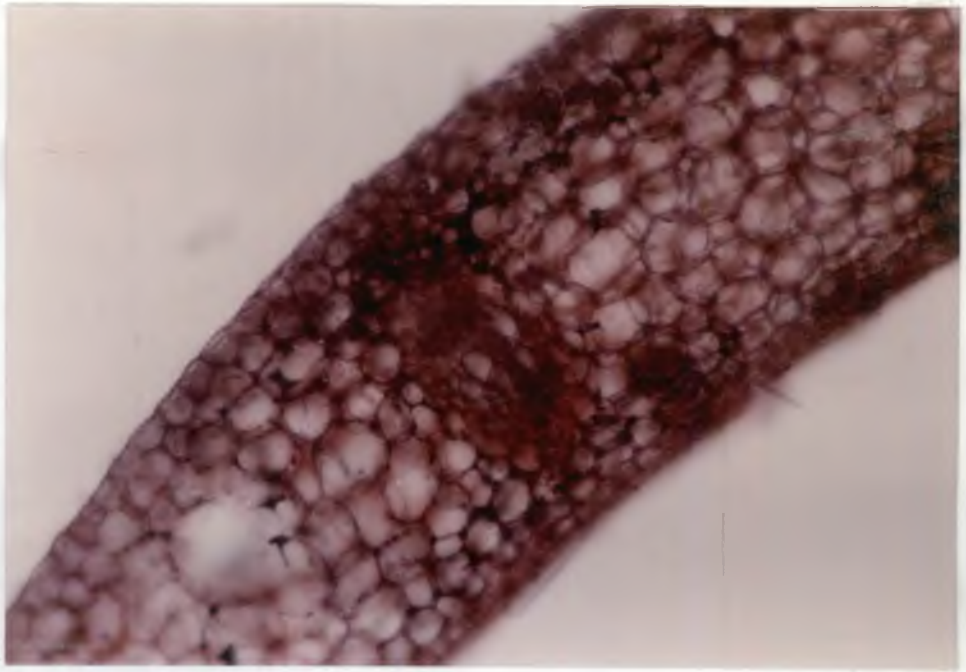


Plate XII. Vasuclar bundles of spathe (C.S)
Magnification 100 x

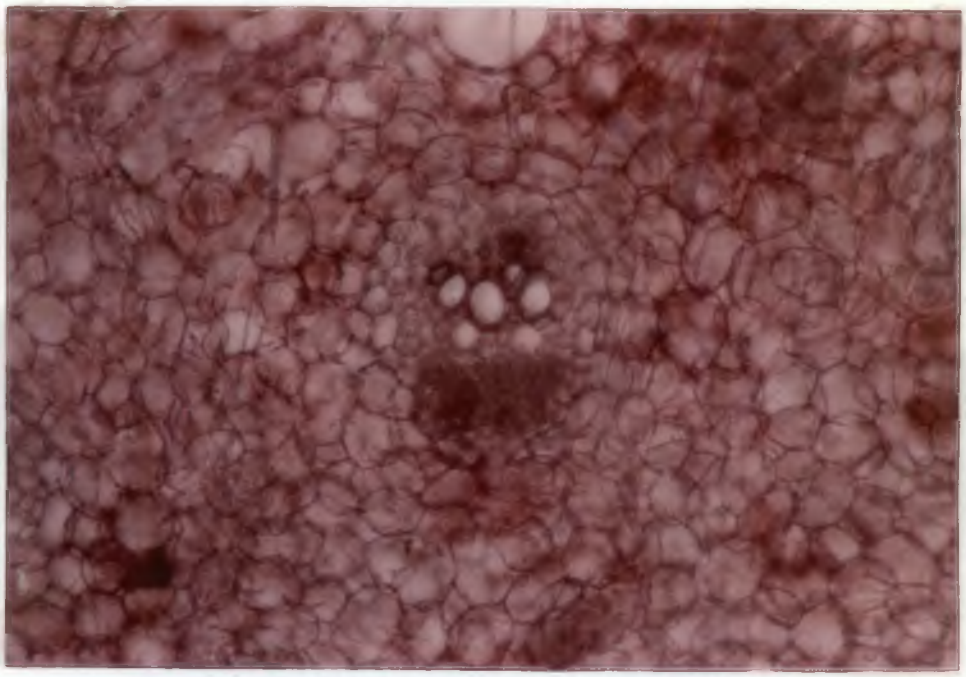


Plate XIII. Vascular bundles of spike (C.S)
Magnification 100 x

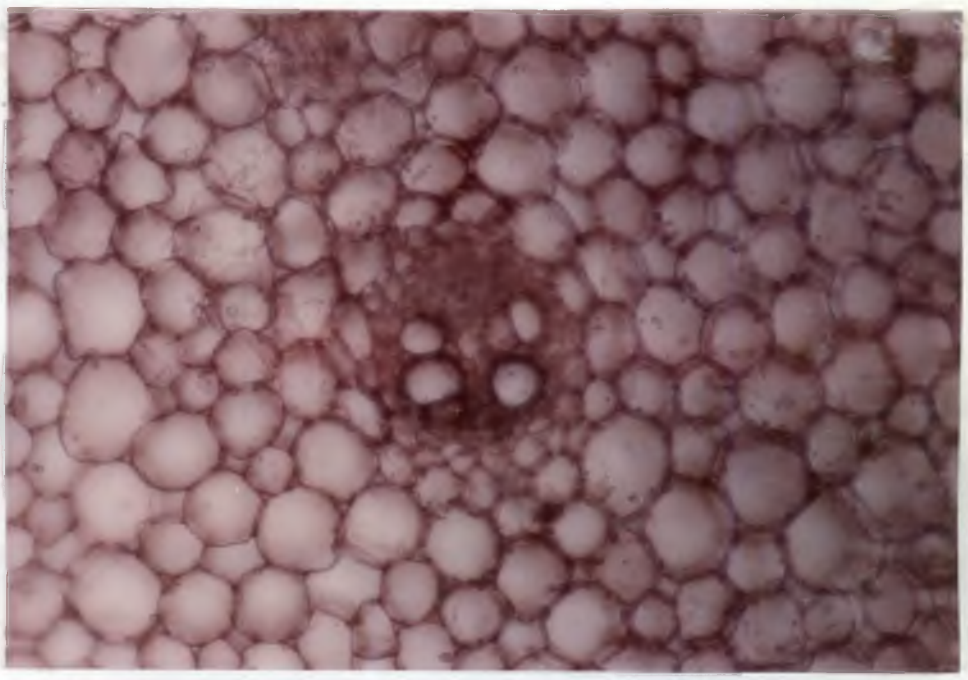


Plate XIV. Vascular bundles of inflorescence stalk (C.S)
Magnification 100 x

(red) recorded lesser content of protein (0.28%) compared to leaves of A. andreanum (pink). Among the other species, A. crystallinum had the highest leaf protein (1.19%).

Total carbohydrate

Among the different explants of A. andreanum (pink), the highest content of total carbohydrate (0.17%) was recorded in leaf (Table 33). Leaves of A. andreanum (red) recorded a higher content of total carbohydrate (0.19%). Among the other species, leaves of A. crystallinum recorded the highest content of total carbohydrate (0.48%).

α-amylase activity

Spike recorded (Table 34) the highest activity of α-amylase (0.0745 mg sugar formed/mg protein/min) among the different explants of A. andreanum (pink). The minimum activity (0.0086 mg sugar formed/mg protein/min) was observed in spathe. Leaves of A. andreanum (red) also had less activity of α-amylase (0.0025 mg sugar formed/mg protein/min). Among the other species tested, the maximum activity was observed in A. veitchii.

Phenol oxidase activity

The rate of change in optical density (at 495 nm) at 60-second interval (upto three minutes) and the average rate of change in the optical density per minute are given in Table 35. The average change in optical density was the maximum (0.064) in leaf explant compared to other explants in A. andreanum (pink). Leaves of A. andreanum (red), A. crystallinum, and A. grande had

Table 33. C/N ratio, content of protein and total carbohydrate and in vitro response in Anthurium spp.

Species		C/N ratio	Protein (%)	Total carbohydrate (%)	In vitro response	
					Dedifferentiation Mean CI	Redifferentiation No.of shoots
A. andreanum (pink)						
	Leaf	165.46	0.41	0.17	93.65	6.50
	Petiole	4.94	0.47	0.04	0	0
	Spike	16.20	1.09	0.14	0	0
	Spathe	17.08	1.71	0.10	0	0
Inflorescence stalk		20.23	0.59	0.06	0	0
A. andreanum (red)						
	Leaf	9.32	0.28	0.19	0	0
A. crystallinum	Leaf	65.31	1.19	0.48	0	0
A. veitchii	Leaf	75.85	0.52	0.10	43.33	5.60
A. grande	Leaf	57.81	0.70	0.47	61.67	...

Table 34. α-amylase activity and *in vitro* response in *Anthurium* spp.

Species		Activity of amylase mg sugar formed/ mg protein/min	*In vitro* response	
			Dedifferentiation Mean CI	Rediffer entiation No. of shoots
A. andreanum (pink)				
	Leaf	0.0182	93.65	6.50
	Petiole	0.0302	0	0
	Spike	0.0745	0	0
	Spathe	0.0086	0	0
Inflorescence	stalk	0.0661	0	0
A. andreanum (red)				
	Leaf	0.0025	0	0
A. crystallinum	Leaf	0.0839	0	0
A. veitchii	Leaf	0.0848	43.33	5.60
A. grande	Leaf	0.0724	61.67	2.33

a less change in optical density whereas it was high A. veitchii. Among the different explants of A. andreanum (pink), petiole, spike, inflorescence stalk and spathe showed an increasing trend in rate of change in the optical density while in the leaf a decreasing trend was observed.

Peroxidase activity

The rate of change in optical density (at 420 nm) and the average rate of change in optical density of the different explants of A. andreanum (pink), leaves of A. andreanum (red), A. crystallinum, A. veitchii and A. grande are given in Table 36. Petiole had the highest average rate of change in optical density (0.0027) among different explants of A. andreanum (pink). Leaves of A. andreanum (red) recorded a higher average rate of change in optical density (0.0033) compared to leaves of A. andreanum (pink). Among the other species, A. veitchii showed the highest rate of change in optical density (0.0033).

Peroxidase isozymes

Polyacrylamide gel electrophoresis was done to study the banding pattern of peroxidase isozymes in different explants of A. andreanum (pink), leaves of A. andreanum (red), A. crystallinum, A. veitchii and A. grande. No separate banding pattern was observed in all the cases (Plates XV and XVI). However, differences were observed in the activity of peroxidase enzyme.

Table 35. Phenol oxidase activity and in vitro response in Anthurium spp.

O.D : 495 nm

Species		Rate of change in O.D at 60 seconds interval			Average change in O.D/min	In vitro response	
		60	120	180		Dedifferentiation Mean CI	Redifferentiation No. of shoots
A. andreanum (pink)							
	Leaf	0.062	0.070	0.059	0.064	93.65	6.50
	Petiole	0.037	0.054	0.057	0.049	0	0
	Spike	0.015	0.019	0.023	0.019	0	0
	Spathe	0.003	0.006	0.009	0.006	0	0
Inflorescence	stalk	0.016	0.019	0.021	0.019	0	0
A. andreanum (red)							
	Leaf	0.012	0.036	0.037	0.028	0	0
A. crystallinum							
	Leaf	0.012	0.005	0.010	0.00[illegible]	0	0
A. veitchii	Leaf	0.041	0.082	0.079	0.067	43.33	5.60
A. grande	Leaf	0.005	0.008	0.008	0.007	61.67	2.33

Table 36 Peroxidase activity and in vitro response in Anthurium spp.

O.D : 420 nm

Species		Rate of change in O.D at 60 seconds interval			Average change in O.D/min	In vitro response	
		60	120	180		Dediffer-entiation Mean CI	Redifferentiat No. of shoots
A. andreanum (pink)							
	Leaf	0.001	0.000	0.001	0.0006	93.65	6.50
	Petiole	0.006	0.001	0.001	0.0027	0	0
	Spike	0.003	0.000	0.002	0.0017	0	0
	Spathe	0.001	0.000	0.001	0.0006	0	0
Inflorescence stalk		0.003	0.000	0.001	0.0013	0	0
A. andreanum (red)							
	Leaf	0.005	0.004	0.00	0.0033	0	0
A. crystallinum	Leaf	0.001	0.001	0.000	0.0006	0	0
A. veitchii	Leaf	0.007	0.003	0.002	0.0033	43.33	5.60
A. grande	Leaf	0.004	0.000	0.001	0.0017	61.67	2.33

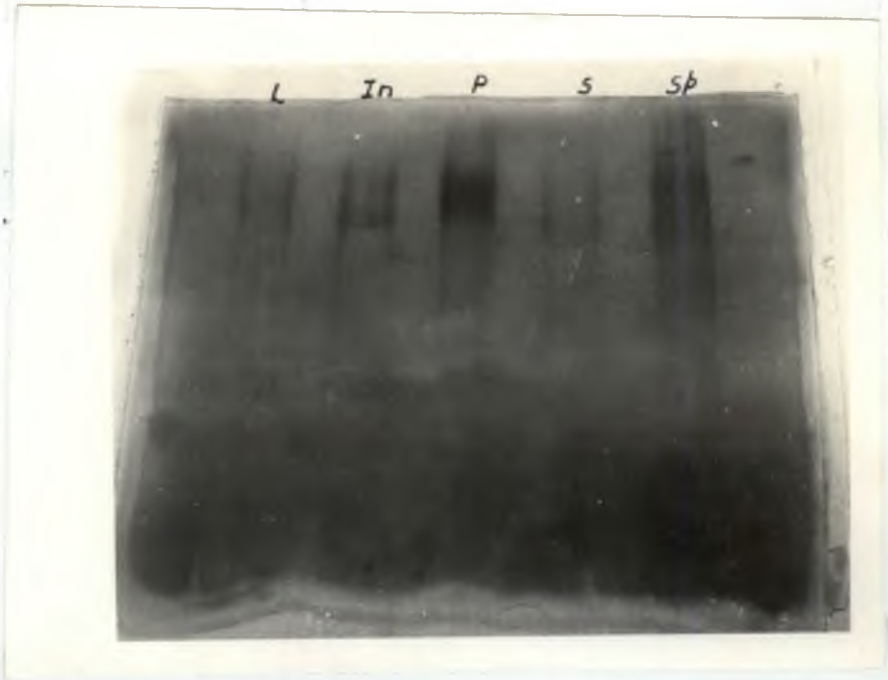


Plate XV. Isozyme (peroxidase) analysis in different explants of Anthurium andreanum (pink)

L : Leaf; P : Petiole; S : Spathe
Sp: Spike; In: Inflorescence stalk

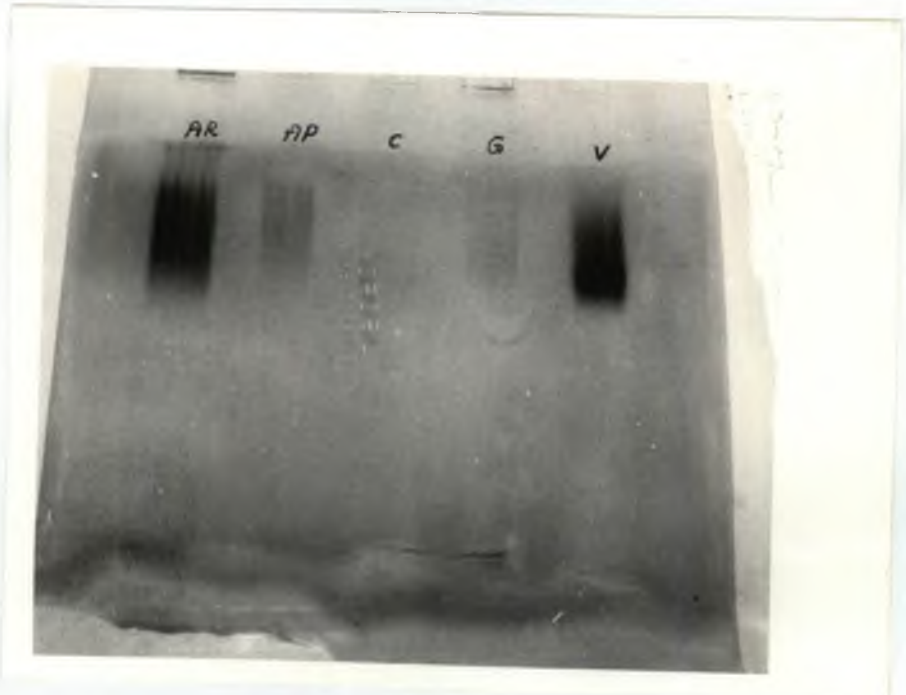


Plate XVI. Isozyme (peroxidase) analysis in different species of Anthurium

Ap : A. andreanum (pink); AR : A. andreanum (red)
C : A. crystallinum; V : A. veitchii
G : A. grande

Comparison of biochemical characterization and *in vitro* response of pink and red types of *Anthurium andreanum*

The results of the biochemical characterization of pink and red types of *A. andreanum* and their *in vitro* response are compared in Table 37. *A. andreanum* (pink) showed a higher C/N ratio, higher content of protein, increased activity of α-amylase and phenol oxidase. Peroxidase activity (average change in O.D/min) was less in the pink type compared to the red type.

Economics of production of anthurium plantlets

The details of calculation of economics of production of anthurium plantlets, based on the existing facilities of the tissue culture laboratory of the Department of Horticulture, College of Agriculture, Vellayani, are presented in Table 38. The total number of plantlets produced per year was estimated to be 96000. Expecting 80 per cent survival *ex vitro*, the number of established plantlets was 76800. The total cost involved per year was worked out to be Rs. 230578/-. The cost of production of a single plantlet was Rs.3.00/-.

Table 37. Biochemical characterization and *in vitro* response of pink and red types of *Anthurium andreanum*

Explant : Leaf

A. andreanum type	C/N ratio	Protein %	Total carbohydrate %	α-amylase activity mg sugar formed/mg protein/min	Phenolosidase activity average change in O.D/min	Peroxidase activity average change in O.D/Min	*In vitro* response Dedifferentiation Mean CI	Redifferentiation No. of shoots
Pink	165.46	0.41	0.17	0.0182	0.064	0.0006	93.65	8.50
Red	9.32	0.28	0.19	0.0025	0.028	0.0033	0	0

Table 38. Economics of production of anthurium plantlets

I. Cost of production

No.	Particulars	Total cost (Rs.)	Expenditure per year (Rs.)
1.	Glassware	60000/5 yrs.	12000.00
2.	Chemicals	20000/5 yrs.	4000.00
3.	Wooden racks (5 no.) with flourescent tubes and fittings	10000/15 yrs.	6666.00
4.	Equipment		
	4.1. Autoclave (1 no.)	8000/10 yrs.	8000.00
	4.2. Laminar air flow chamber (1 no.)	40000/10 yrs.	4000.00
	4.3. Air conditioner (2 nos.)	50000/10 yrs.	5000.00
	4.4. Balance (1 no.)	10000/20 yrs.	500.00
	4.5. pH meter (1 no.)	4000/10 yrs.	400.00
	4.6. Refrigerator (1 no.)	7500/10 yrs.	750.00
	4.7. Heating mantle (1 no.)	1000/5 yrs.	200.00
	4.8. Double glass distillation unit (1 no.)	10000/10 yrs.	1000.00
5.	Cotton, Inoculation aids, foils etc.	2000/yr	2000.00
6.	Pots, potting media and nursery expenditure	120000/yr	120000.00
7.	Electricity & maintenance charges	20000/yr	20000.00
8.	Buildings and furnishings	200000/50 yrs.	4000.00
9.	Salary		
	9.1. Salary of one Scientist (pay Rs.2000/p.m.)	24000/yr	24000.00
	9.2. Salary of one assistant (pay Rs.1200/p.m.)	14400/yr	14400.00
10.	Interest (@ 12%) on capital assets		3662.00
	TOTAL		230578.00

II. Number of plantlets produced per year

Number of days	Callus initiation	Callus multiplication	Sprout regeneration	Shoot proliferation, growth and rooting	Elongated shoots with roots	Number of plantlets planted out
75	300					
120		600 cultures				
145			1200 cultures			
190				2400 cultures		
250					2400 cultures with (2400 x 25 x no. of shoots)	
275						24000
300						24000
325						24000
350						24000
		TOTAL				96000

Expecting 80 per cent survival ex vitro;
Number of plantlets produced per year is 76800.

III. Cost of production of a single plantlet

Total cost involved per year Rs 230578

Number of plantlets produced per year 76800

Cost of production of one plantlet Rs. 3.00

Discussion

DISCUSSION

Anthuriums are usually propagated by seeds as well as by vegetative means. Under Kerala conditions, natural seed set is not commonly observed in all the species of Anthurium. Being a cross pollinated crop, the seedling progeny are highly heterogeneous. Also it takes six to twelve months from pollination to seed maturity. The seedlings require two to three years for flowering. Mature plants produce one or two suckers in an year. Suckers can also be induced by top cuttings. However, all these methods result in insufficient multiplication rates for mass clonal propagation. In vitro propagation techniques become relevant in this context. It ensures mass clonal propagation. Methods of in vitro propagation, mainly through somatic organogenesis, have been standardized for Anthurium andreanum (Pierik, 1976; Pierik et al., 1974; 1979) and Anthurium scherzerianum (Pierik and Steegmans, 1976; Geier, 1986). Not much work has been conducted in the other species. Clonal multiplication of A. andreanum from stem sections of aseptically grown seedlings, has been attempted by Kunisaki (1980). But in the experiment, the effect of only a single cytokinin (BA) has been tested. Somatic embryogenesis from spadix callus of A. scherzerianum has been reported by Geier and Reuther (1981) and Geier (1982). However, this mode of regeneration was sporadic and the factors required for its consistent induction have to be defined.

Anthuriums are important flower crops. Although methods of in vitro propagation via somatic organogenesis has been

standardized in A. andreanum, there is possibility for improving the rate of multiplication. Also, there are species of Anthurium for which the standardized method for A. andreanum is not effective. Method of somatic embryogenesis, which ensures highly efficient rate of multiplication, has not been properly exploited for anthurium propagation. The present study was taken up with the objectives of improving the propagation efficiency of Anthurium species through enhanced release of axillary buds and somatic organogenesis/embryogenesis. Standardization of media for the species of Anthurium not having the protocols of in vitro propagation, was also attempted. Not much work has been conducted to analyze and overcome the recalcitrant nature exhibited by different explants/types/species of Anthurium. Hence, an attempt was made for the biochemical characterization and contrasting of different explants/types/species of Anthurium with respect to their difference in in vitro response. Four species of Anthurium namely, A. andreanum, A. crystallinum, A. veitchii and A. grande were selected for the study. The salient results are discussed in the following pages.

For enhanced release of axillary buds, the role of cytokinins in the culture establishment of anthurium shoot apices was analysed. Cent per cent survival as well as growing cultures were observed in all the cytokinin treatments tested for the enhanced release of axillary buds. Kinetin 2.0 mg/l and BA 1.0 mg/l were equally effective in inducing multiple shoots and the maximum number of shoots in these treatments was 4.50. Induction

of callus growth was observed at the base of the explant in treatments with BA and 2ip whereas no callus growth was observed with kinetin. Leffring and Soede (1979) observed optimum branching of A. andreanum shoots in a medium containing 13.7 µM (3.0 mg/l) kinetin. They also observed that BA and 2ip caused at less branching and promoted callus growth. Callus growth at higher BA levels has been also reported by Kunisaki (1980) and Qu-guang et al. (1988). Length of the longest shoot was the maximum (1.57 cm) in 2ip 1.0 mg/l. In the present study it was observed that higher concentrations of cytokinins suppressed the shoot growth. This was contradictory to the result obtained by Leffring and Soede (1979) who reported optimum number of shoots (6.10) with kinetin 3.0 mg/l. However, they used a different mode of culture, liquid, for their studies. This might account for the difference in the response.

Kunisaki (1980) used MS salts at full strength for multiple shoot induction in anthurium. In the present study, the number of shoots and the length of the longest shoot did not differ significantly in the treatments with reduced MS salts. It was observed that MS major nutrients 1/4 + micro nutrients at full strength can be recommended for multiple shoot induction in anthurium. Most of the workers in anthurium tissue culture have recommended reduced MS salts. This reduced salt requirement may be species-specific.

Sugars are indispensable in the basal medium as they are not only the source of carbon, but also involved in osmoregulation. Two per cent sucrose was employed by Kunisaki (1980) for multiple

shoot induction in A. andreanum. The present study revealed that sucrose content can be reduced to one per cent as it did not significantly influenced multiple shoot formation. The reduced sucrose requirement may be attributed to the epiphytic nature of the species. Kunisaki et al. (1972) have observed that no supplemental sucrose is required for monopodial orchids. The reduced sucrose requirement can also be due to the fact that the explant, which is taken from newly germinated seedlings, may be rich in sugar. Induction of multiple shoots may differ with carbon sources. In the present study, it was observed that glucose produced less number of shoots compared to sucrose. The advantage of sucrose over glucose may be derived from its more effective translocation to apical meristems (Butcher and Street, 1964).

The number of shoots did not differ significantly in the treatments with different concentrations of agar. The maximum length (0.95 cm) was observed at the lowest level of agar (0.4%) tried. The optimum branching of anthurium shoot reported by Leffring and Soede (1979), was in liquid medium. However, there is a chance for the production of highly fragile vitrified shoots in liquid culture which will cause difficulties in handling of the cultures and successful transplantation of the shoots (Davis et al., 1977). Improved shoot growth at low agar level has been reported by Sagawa and Kunisaki (1990). However, low levels of agar cannot be recommended because the medium will not get solidified enough and as such, handling of the cultures may be

difficult.

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

Photomorphogenesis is facilitated by pigments in the tissues which absorb radiation of particular wave lengths. Light is required for photomorphogenesis. Exposure to light had profound influence on multiple shoot formation. Indunction of callus was observed at the basal portion of the explants under conditions of darkness. Several adventitious shoots were seen formed from the callus, inhibiting the growth of axillary shoots. Hence, exposure to light was found essential for enhanced release of axillary buds. Exposure to light has been found to be essential for the maximum *in vitro* growth of culture by Hu and Wang (1983).

Somatic organogenesis/embryogenesis was tried from explants namely, leaf, petiole, spike, spathe and inflorescence stalk. In anthurium, a low content of auxin + a high content of cytokinin has been reported to be suitable for callus formation (Pierik *et al*., 1975; Pierik, 1979; Finnie and Van Staden, 1986; Geier, 1986). Similar response was also apparent in the present instance. Among the treatments, combination of 2,4-D and BA was found to be the best. In *A. andreanum*, 2,4-D (0.08 mg/l) + BA (1.0 mg/l) was the best for callus initiation. In *A. veitchii*,

callusing was the best at slightly increased levels of 2,4-D (0.2 mg/l). A still higher concentration of 2,4-D (0.5 mg/l) was found to be the best in A. grande. Concentrations of 2,4-D higher than 0.5 mg/l did not improve the response. Among the auxins 2,4-D is highly potent. It stimulates callus formation and strongly antagonizes organized development. The low auxin requirement may be due to the high potency of the auxin 2,4-D, which was used for callus initiation. The young developing leaf may be a rich source of endogenous auxins due to which lower exogenous application is required. Lower levels of auxins like 2,4-D (0.1 mg/l) with higher levels of BA (1.0 mg/l) have been reported to induce in DayLily (Krikorian et al., 1987).

Although MS major nutrients are a good starting point for medium development, more dilute solutions may prove to be better in some circumstances. Adjustment to the ionic concentration becomes necessary when one or more ions at the normal level are inhibitory to a species. In A. andreanum and A. veitchii, callus was observed only in a modified MS medium (with reduced major salt concentration). No callusing was observed in a medium at normal strength. In A. grande, callusing was the best (CI 92.51) in the modified MS medium compared to that in MS medium at normal strength (CI 50.00). This response may be species - specific. Half strength MS major nutrients with full strength micro nutrients has been found to be suitable for the in vitro culture of anthurium (Pierik et al., 1974; Pierik and Steegmans, 1976).

Addition of inositol to the media is not essential; but the presence of small amounts is beneficial. In the present instance, no callus initiation was observed when inositol was reduced to half concentration.

Surface-disinfection treatments were standardized for the different explants. Among the explants, the highest number of sterile cultures (59.09%) was observed in leaf, followed by spathe (46.00%). A comparison of the size of vascular bundles (of the different explants) and incidence of microbial contamination was attempted. It was observed that vascular bundles of leaf was the smallest compared to that of other explants. There may be less translocation of pathogens along smaller conducting vessels. The reduced microbial contamination in the leaf explants may be due to the smaller vascular bundles.

For any given species or variety, a particular explant may be ideal for successful plant regeneration. Explants consisting of shoot tips or isolated meristems, which contain mitotically active cells, have been generally successful for callus initiation and subsequent plant regeneration (Murashige, 1974) in several species. In the present study, callus initiation was observed in the leaf explants whereas only swelling of the explants was observed when explants from petiole, spike and inflorescence stal were used. Morphological and physiological status of the explants can account for the difference in the response. The less lignified tissues of leaf may facilitate easy de-differentiation process than the tissues of other plant parts.

Leffring et al. (1976) reported higher callusing capacity for apical portions of leaf in anthurium. In the present study also, variation in the response, with respect to callus initiation, was observed between basal and apical portions of leaf. In general, basal portions responded better than the apical portions. In the basal portions of leaf, the number of days taken for callus initiation was 55.20 in A. andreanum and 60.00, in A. veitchii. Apical portions of leaf took 75.20 days for callus initiation in A. andreanum whereas in A. veitchii no callusing was observed. The difference in response between the basal and the apical portions may be due to the difference in the physiological state as well as the number of cells undergoing de-differentiation. Perhaps, more number of cells undergo de-differentiation in the basal portions of leaf. Physiological state may account for the changes in the content of endogenous phytohormones, nutrients and metabolites.

In anthurium, optimum callus formation and subsequent growth have been observed in continuous darkness by Pierik et al. (1975) and Pierik (1976). The present study also revealed that darkness was essential for callus initiation. The beneficial effect of darkness may be attributed to the etiolation effect. Reid (1972) reported that etiolated tissues may be less lignified, than the light grown tissues, which facilitate easy de-differentiation. Herman and Hess (1963) proposed an increased content of auxin cofactors in the etiolated tissues which increased the tissues

specificity to exogeneously applied auxin. The explants became brown when exposed to light for 16 h. Browning of the explants may be due to the oxidation of phenolic compounds under light. Inhibitory effect of light on callusing and further growth, has been reported in cyclamen (Lowenberg, 1969), in freesia (Bajaj and Pierik, 1974; Pierik and Steegmans, 1975) and in fuchsia (Bouharmont and Dabin, 1986).

Various treatments were tried for callus multiplication. The maximum fresh weight of callus (3.15 g) was observed in MS medium with major nutrients at normal strength followed by MS medium with major nutrients at 1/4 strength (3.11 g). MS major nutrients at 1/4 strength had the highest mitotic index (1.65). From the economic point of view, MS major nutrient at 1/4 strength can be recommended for callus multiplication.

Shoot regeneration and growth of the shoots were the best in MS medium when BA was reduced to 0.5 mg/l with the addition of IAA 2.0 mg/l. Higher levels of BA in the culture medium enhance more cell division which may result in callus growth. Growth of shoots may be reduced in such cultures. Reduction in the concentration of BA for shoot elongation has been observed by Dantu and Bhojwani (1987) and Rajmohan (1985). Auxins in the medium promote cell elongation and may be useful to nullify the suppressive effect of cytokinin on shoot elongation (Lundergan and Janick, 1980).

In anthurium tissue culture, no special rooting treatments

was found necessary. Rooting of shoots occured spontaneously in the shoot proliferation medium itself. Rooting was also observed in media without any plant growth substance. The high level of endogenous auxins and the prolonged exposure to light (for shoot proliferation) might have enhanced spontaneous rooting of shoots.

Ex vitro establishment of the in vitro generated plantlets is critical for successful clonal multiplication. The plantlets have to get acclimatized to the ex vitro conditions. Excessive water loss and reduced uptake of water and nutrients cause problems in acclimatization. In the present study, it was observed that plantlets (with at least two roots) survived better than the microshoots. Growth of the survived microshoots was very slow compared to that of the plantlets. Plantlets can absorb water and nutrients much easier than microshoots. Water content of microshoots will be less and there will be problems with desiccation. It was also observed that plantlets did require less hardening treatments. The rate of water loss through anthurium leaves of in vitro grown plantlets and field grown plants was comparable. This was contradictory to the results obtained in the ex vitro establishment of jack plantlets (Ramesh, 1990) in which case more than double the rate of water loss was observed in in vitro leaves. The requirement of less hardening treatments in the present study, may be due to that the plantlets had sufficient water content (due to proper stomatal functioning) and food reserves. Murashige (1978) reported that the prolonged period of exposure to light built up sufficient food reserves to

be utilized during the transformation period from partially heterotrophic to autotrophic growth of the plantlets, after transplnatation.

Texture and structure of the potting medium are important factors for the successful ex vitro establishment of plantlets. It was observed that sand was the best potting medium. Sand, as the potting medium ensures proper drainage and sufficient aeration. Ramesh (1990) has also reported sand as the best potting medium for ex vitro establishment jack plantlets.

Addition of inorganic nutrients to the potting medium is essential for the normal growth of the potted plantlets (Brown and Sommer, 1982; Amerson et al., 1985). However, a negative influence of the nutrient solutions was apparent in anthurium. It is likely that plantlets had adequate nutrient reserves for supporting their survival and growth under ex vitro conditions in the absence of added nutrients (Rahman, 1988). Negative influence of nutrient solutions on survival and growth of jack plantlets has been reported by Ramesh (1990).

Vesicular arbuscular mycorrhizae have been found beneficial in improving the growth of in vitro generated plantlets. In anthurium, the plantlets treated with VAM (Glomus constrictum) recorded cent per cent survival ex vitro. The VAM (G. constrictum and G. etunicatum) treated plantlets showed better growth compared to that of control plantlets. Nutrient analysis recorded an increased content of major and minor nutrients namely N, P, K,

Ca, Mg and Zn. The beneficial effects of VAM, like increased nutrients status, enhanced phytohormone activity (Allen et al., 1980) and photosynthetic efficiency (Sivaprasad and Rai, 1984) might have helped the plantlets in their early establishment and better survival. The mycorrhizal mycelia explore large volumes of soil (St. John, 1980) and help the plants with increased nutrient uptake. Their possible utilization might have contributed to the enhanced plant growth with respect to plant height, fresh weight of plants and total leaf area. The increased uptake of Zn which is required for the synthesis of IAA within the plant, might have caused increased phytohormone activity. The beneficial effect of VAM on in vitro grown plantlets has been reported by several workers (Blal et al., 1990; Fogher et al., 1986; Gonzalez-Chavez et al., 1987; Granger et al., 1983 and Ramesh, 1990).

Limitted cytological examinations of root tip squashes made on hundred randomly selected anthurium plantlets from five hundred plantlets recorded a normal diploid chromosome count of 2n = 30 + 2B chromosomes. The plantlets had resulted from enhanced release of axillary buds and callus-mediated somatic organogenesis. Cytological stability has been observed in both the cases. No genetic variability was noted among the plantlets upto six to eight months after transplanting. Genetic stability in the case of axillary shoots has been attributed to the properties of the meristematic line involved in their origin. The meristematic line, consisting of specific cells in more or less fixed position, exercises a strict control over the mitotic

events (Bonga, 1982; Vasil, 1985). According to Hussey (1979), mutated cells, if any, in a multicellular shoot apex will form only limited areas of tissue which eventually remain suppressed in the meristematic region. Shoot apex culture has been found to be genetically stable by Ancora et al. (1981) and Rajmohan (1985). Little variation in ploidy level has been reported by Geier (1988) in A. scherzerianum plantlets regenerated via callus-mediated somatic organogenesis. In anthurium the callus is highly organized and it can be termed as organoid colonies. It is likely that the control mechanisms ensuring genetic stability in shoot meristems are still active in such cultures (Geier, 1988).

Physiological status of the parent plant can account for the success of organogenesis in cell cultures. Recalcitrant nature in cultures may be attributed to the physiological status of the donor plant. Hence an attempt was made to correlate the in vitro response of different explants/types/species of Anthurium with their biochemical characterization and contrasting. C/N ratio, content of protein and total carbohydrate and activities of enzymes, namely, α-amylase, phenol oxidase and peroxidase were analysed. Wide variations were observed in all these parameters in the different explants, types and species of Anthurium. However, conclusions could not be drawn from the present study. Further work needs to be conducted in these aspects. Biochemical studies with respect to C/N ratio, contents of protein, total carbohydrate and endogenous hormone and activities of the enzymes may be conducted in different explants/types/species at different developing ages and seasons.

The season of the year can influence callus initiation from explants, especially when the donor plant is field grown. In the present study also, seasonal variation was observed with respect to callus initiation from leaf explants. However, much work is to be done for a consecutive period of atleast three years before concluding the seasonal effect. The variation may be due to changes in endogenous auxins. Seasonsal variations in the concentration of endogenous auxins have been reported by Wodzicki (1978).

Consistent somatic embryogenesis has not been reported in anthurium. Many treatments were tried for the induction of somatic embryos. Though somatic embryogenesis was not apparent, some embryoid-like structures were observed. A detailed investigation is found essential to follow up this lead.

In the present instance, the cost of production of a single anthruium plantlet was worked out to be Rs. 3.00/-. The cost was worked out, based on the existing facilities of the tissue culture laboratory of the Department of Horticultre. The cost can be reduced by augmenting the physical facilities to accomodate more number of cultures of anthurium.

It is worthwhile to suggest some future prospects from the results of the present investigations. Somatic embryogenesis, capable of ensuring highly efficient rate of multiplication,

needs detailed studies. The work may be taken up with more number of treatments for the induction of somatic embryos and thoroughly analysing the induction of embryoids, histologically. In the present instance, no callusing was observed in A. andreanum (red) and A. crystallinum. The absence of the response may be a genotype effect. Further work is necessary to develop a protocol for in vitro propagation in these types. Biochemical studies are needed in different explants of responding and non-responding types in anthurium at the different developing ages and seasons. These studies will be useful in the selection of better responding types as well as the best season for in vitro culture.

The present investigations aimed at the isolation and multiplication of phenotypic variants. However, phenotypic variations could not be studied because variations were not apparent in the early stages of growth. Moreover, the crop require atleast one year for flowering after planting out. Selection of desirable variants and their multiplication is also suggested as future studies.

Summary

SUMMARY

Attempts were made to improve the propagation efficienty of Anthurium species through enhanced release of axillary buds and callus-mediated somatic organogenesis/embryogenesis, in the plant tissue culture laboratory of Department of Horticulture, College of Agriculture, Vellayani during 1990-92. Four species of Anthurium, namely, A. andreanum Lind. (pink and red), A. crystallinum Lind. & Andre, A. veitchii Mast. and A. grande Hort. were selected for the study.

Shoot tips from in vitro germinated seedlings were used as explants for enhanced release of axillary buds. The explants were subjected to treatments for enhanced release of axillary buds. Since the explants could be obtained only in A. andreanum (pink), the results could be obtained only in this species.

Segments of leaf, petiole, spathe, spike and inflorescence stalk were used as explants for somatic organogenesis / embryogenesis. Callus initiation in the different explants was compared. Seasonal influence on callus initiation was stuided by collecting explants (leaf) at monthly intervals. Ex vitro treatments were attempted to maximise the establishment of the plantlets. Biochemical characterization of explants and species were conducted. The results of the stuides were examined for correlation with in vitro response.

The salient findings of the above study are:

Cent percent survival as well as growing cultures was observed in all the cytokinin treatments tested for enhanced

release of axillary buds. Kinetin 2.0 mg/l and BA 1.0 mg/l were equally effective in inducing multiple shoots. The maximum number of shoots in these treatments was 4.50.

No callus growth was observed in treatments with kinetin. In treatments with BA and 2ip, callus growth was observed at the base of explants.

The number of shoots and length of the longest shoot did not differ significantly in different concentrations of MS inorganic salts. One forth strength of MS major nutrients and full strength of minor nutrients was ideal for multiple shoot induction.

Multiple shoot formation was not influenced by one percent sucrose in MS medium.

Three percent glucose produced less number of shoots than three percent sucrose.

The number of shoots was not influenced by different concentrations of agar. However, it influenced the length of shoots. The maximum length (0.95 cm) was observed at 0.4 percent agar.

The number of shoots (2.67) was less in Nitsch medium than in MS medium (4.50).

Light induced early shoot induction. In darkness, callus induction was observed at the base of the explant.

In A. andreanum, 2.4-D 0.08mg/l and BA 1.0mg/l recorded the maximum number of cultures (50.35%) initiating callus, the highest growth score (1.85) and CI value (93.65). In A. crystallinum, none of the treatments could induce callus. In A. veitchii, 2.4-D 0.2mg/l and BA 1.0 mg/l induced callus in 43.33 per cent cultures with growth score 1.00 and CI value 43.33. A combination of 2.4-D 0.5 mg/l and BA 1.0 mg/l was ideal for callus initiatian in A. grande.

In A. andreanum and A. veitchii, callus initiation was observed only in modified MS medium with reduced salt concentration. In A. grande, callus was reduced in MS medium at normal salt concentration. No callusing was observed when inosital was reduced to half concentration of the normal (100 mg/l).

The leaf explant, with the smallest vascular bundles, had the highest number of sterile cultures (59.09%).

Basal portion of leaf was ideal for callus initiation. The number of cultures initiating callus, growth score and CI value were higher for basal protions than the apical portions.

Conclusions could not be drawn from studies on seasonal influence on callus initiation.

Continuous darkness was necessary for callus initiation and subsequent growth.

One-forth strength of MS major nutrients, supplemented with BA 1.0 mg/l, recorded callus fresh weight of 3.11g with the highest mitotic index (1.65%).

Attempts, made on callus-mediated somatic embryogenesis, were not successful.

Shoot regeneration and growth were the best in MS medium with BA 0.5 mg/l and IAA 2.0 mg/l.

Rooting of shoots occurred spontaneously. Hence no rooting treatments were necessary.

Plantlets survived better than microshoots, when transplanted. Less hardening treatments were found necessary for the plantlets.

Sand was the best medium for planting out. Survival of plantlets irrigated with nutrient solutions was poor.

The VAM (G. constrictum and G. etunicatum) treated plantlets had better survival and growth then the control plantlets.

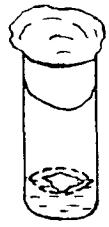
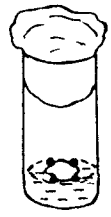
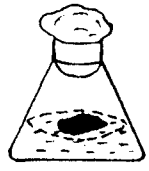
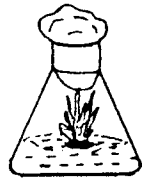
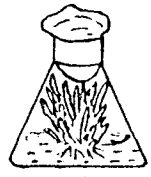
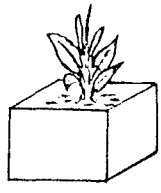
Cytological examinations of the root tip squashes made on random plantlets at planting out, recorded a normal diploid chromosome count of 2n = 30 + 2B chromosomes.

Results of the biochemical studies could not be correlated with in vitro response.

The cost of production of single plantlet was worked out to be Rs.3.00.

PROTOCOL

Species: Anthurium andreanum (pink)

Factors influencing		Stages & Duration
Explant-Leaf		Explant
1% Sod. hypochlorite		Surface-sterilization
Modified MS + 2,4-D 0.08 + BA 1.0 mg/l		Explanting
		50-75 days
Modified MS + 2,4-D 0.08 + BA 1.0 mg/l		Callus initiation
		45 days
MS (macro elements ¼) + BA 1.0 mg/l		Callus multiplication
		30 days
MS + BA 0.5 + IAA 2.0 mg/l		Sprout regeneration
		50-60 days
MS + BA 0.5 + IAA 2.0 mg/l		Shoot proliferation, growth & rooting
		20-30 days
Potting medium - Sand		Planting out
		30 days
		Garden pots

A. andreanum:Plant regeneration from leaf tissues cultured in vitro

a : Explant (leaf)
b : Callus initiation
c : Callus multiplication
d : Shoot regeneration
e : Elongated shoots with roots
f : Plantlets in plastic pots
g : Plantlets in garden pots

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* Originals not seen.

Appendices

Appendix I

Composition of Murashige and Shoog (1962) medium

Series made into	Particulars	Quantity per litre	Weight taken	Volume made up	Volume pipetted
	{NH_4NO_3	1650 mg	16.5 g}		
	{KNO_3	1900 mg	19.0 g}		
A	{$MgSO_4$ $7H_2O$	370 mg	3.7 g}	250 ml	25 ml
	{KH_2PO_4	170 mg	1.7 g}		
B	$CaCl_2$ $2H_2O$	440 mg	8.8 g	100 ml	5 ml
	{H_3BO_3	6.2 mg	620 mg}		
	{$MnSO_4$ H_2O	22.3 mg	2.23 g}		
C	{$ZnSO_4$ $7H_2O$	8.6 mg	860 mg}	100 ml	1 ml
	{KI	0.83 mg	83 mg }		
	{Na_2MoO_4 2 H_2O	250 mg	25 mg }		
D	{$FeSO_4$ $7H_2O$	27.8 mg	2.78 g}	500 ml	5 ml
	{NaEDTA	37.3 mg	3.73 g}		
E	{$CoCl_2$ $6H_2O$	25 µg	2.5 mg}	100 ml	1 ml
	{$CuSO_4$ $5H_2O$	25 µg	2.5 mg}		
	{Thiamine HCl	0.1 mg	10 mg }		
F	{Pyridoxine HCl	0.5 mg	50 mg }	100 ml	1 ml
	{Nicotinic acid	0.5 mg	50 mg }		
	Inositol	100 mg	100 mg		
	Glucose	30 g			

Appendix II

Composition of Murashige and Shoog (1962) modified by Pierik (1976)

Series made into	Particulars	Quantity per litre	Weight taken	Volume made up	Volume pipetted
A	NH_4NO_3	825 mg	8.25 g	250 ml	25 ml
	KNO_3	950 mg	9.50 g		
	$MgSO_4\ 7H_2O$	370 mg	3.7 g		
	KH_2PO_4	85 mg	850 g		
B	$CaCl_2\ 2H_2O$	440 mg	8.8 g	100 ml	5 ml
C	H_3BO_3	6.2 mg	620 mg	100 ml	1 ml
	$MnSO_4\ H_2O$	22.3 mg	2.23 g		
	$ZnSO_4\ 7H_2O$	8.6 mg	860 mg		
	KI	0.83 mg	83 mg		
	$Na_2MoO_4\ 2\ H_2O$	250 mg	25 mg		
D	$FeSO_4\ 7H_2O$	27.8 mg	2.78 g	500 ml	5 ml
	NaEDTA	37.3 mg	3.73 g		
E	$CoCl_2\ 6H_2O$	25 µg	2.5 mg	100 ml	1 ml
	$CuSO_4\ 5H_2O$	25 µg	2.5 mg		
F	Thiamine HCl	0.1 mg	10 mg	100 ml	1 ml
	Pyridoxine HCl	0.5 mg	50 mg		
	Nicotinic acid	0.5 mg	50 mg		
	Inositol	100 mg	100 mg		
	Glucose	30 g			

Appendix III

Water loss through unit area leaves

Time(in minutes)	Mean weight of leaves (g)			
	In vitro	Nearly produced Ex vitro	persistent Ex vitro	Field grown
0	0.02064	0.02180	0.02250	0.02716
15	0.02009	0.02152	0.02162	0.02685
30	0.01964	0.02127	0.02127	0.02652
45	0.01932	0.02101	0.02110	0.02618
60	0.01902	0.02070	0.02099	0.02584
75	0.01875	0.02041	0.02093	0.02550
90	0.01855	0.02025	0.02083	0.02526
105	0.01836	0.02013	0.02078	0.02512

Rate of water loss through leaves

Treatment*	Rate of water loss Time (Minutes)							Rate of water loss (ng) per unit area per unit time
	15	30	45	60	75	90	105	
In vitro	0.00055	0.00045	0.00032	0.00030	0.00027	0.00020	0.00019	21.78
Newly produced ex vitro	0.00028	0.00025	0.00026	0.00031	0.00029	0.00016	0.00012	15.90
Persistent ex vitro	0.00088	0.00035	0.00017	0.00011	0.00006	0.00010	0.00010	16.38
Field grown	0.00031	0.00033	0.00034	0.00034	0.00034	0.00024	0.00014	19.43

* Replication number - 2

IMPROVEMENT OF PROPAGATION EFFICIENCY OF *Anthurium* SPECIES *IN VITRO*

By

SREELATHA, U.

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the
requirement for the degree

Doctor of Philosophy in Horticulture

Faculty of Agriculture
Kerala Agricultural University

Department of Horticulture
COLLEGE OF AGRICULTURE
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ABSTRACT

Attempts were made, to improve the propagation efficiency of Anthurium species through enhanced release of axillary buds and callus-mediated somatic organogenesis/embryogenesis, in the plant tissue culture laboratory of the Department of Horticulture, College of Agriculture, Vellayani during 1990-92. Four species of Anthurium namely, A. andreanum, A. crystallinum, A. veitchii and A. grande were selected for the study.

Shoot tips from in vitro grown seedling were used as explants for the enhanced release of axillary buds. Cent percent survival as well as growing cultures was observed in all the cytokinin treatments. The maximum number of shoots (4.50) was observed with kinetin 2.0 mg/l as well as BA 1.0 mg/l. Treatments with kinetin was free of callus growth. In treatments with BA and 2ip, callus growth was observed at the base of the explant. Treatments with MS inorganic salts as well as sucrose did not influence multiple shoot formation. One forth strength of MS major nutrients with full strength of micro nutrients was ideal for multiple shoot induction. Glucose produced less number of shoots than sucrose. One percent sucrose did not influence multiple shoot induction. The longest shoot (0.95cm) was observed at 0.4 percent agar. Light was necessary for the enhancement of axillary buds. In darkness, callus growth was observed, from which many adventitious shoot were produced.

Segments of leaf, petiole, spathe, spike and inflorescence stalk were used a explants for callus initiation. Combinations

of 2,4-D and BA were efficient in initiating callus. In A. andreanum, 2,4-D 0.08 mg/l and BA 1.0 mg/l was ideal for callus initiation. Combination of 2,4-D 0.2 mg/l and BA 1.0 mg/l was the best for callus initiation in A. veitchii. In A. grande, the best callus initiation was observed with 2,4-D 0.5 mg/l and BA 1.0 mg/l.

Modified MS medium with reduced salt concentrations was ideal for callus initiation in all the species. Inositol when reduced to half concentration (of the normal) influenced callus initiation. The leaf explant (with the smallest vascular bundles) among the other explants, had the highest number of cultures free of microbial contamination. Basal portions of leaf responded, better than the apical portions, to in vitro culture. Continuous darkness was necessary for callus initiation and growth.

MS medium with 1/4 strength major nutrients was ideal for callus multiplication. Attempts, made on callus-mediated somatic embryogenesis, were not successful. Shoot regeneration and growth of the shoots were the best in MS medium with BA 0.5 mg/l and IAA 2.0 mg/l.

No rooting treatments were required as the shoots rooted spontaneously. Plantlets survived, better than microshoots, ex vitro. The plantlets required less hardening treatments. Sand was the best potting medium for planting out. Nutrient solutions when used for the irrigation the plantlets, had a negative influence on the survival of plantlets. Treatments with VAM

(Glomus constrictum and G. etunicatum) was beneficial for the survival as well as growth of the plantlets.

Cytological examinations of the root tip squashes made on random number of plantlets, at planting out, showed a normal diploid chromosome count.

Attempts, to correlate the biochemical properties with in vitro response, of different explants as well as species, were not successful.

Based on the existing facilities of the plant tissue culture laboratory of the Department of Horticulture, College of Agriculture, Vellayani, the cost of single anthurium plantlet was worked out to be Rs.3.00/=.