DEVELOPMENT OF MICROPROPAGATION SYSTEMS AND NEW *in vitro* STRATEGIES FOR SOME IMPORTANT ORNAMENTAL PLANTS

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DEVELOPMENT OF MICROPROPAGATION SYSTEMS AND NEW *in vitro* STRATEGIES FOR SOME IMPORTANT ORNAMENTAL PLANTS

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Dedicated to my

Beloved Parents

Smt. and Sri M. Bhaskara Rao

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BANGALORE

C E R T I F I C A T E

This is to certify that the thesis entitled **"DEVELOPMENT OF MICROPROPAGATION SYSTEMS AND NEW in vitro STRATEGIES FOR SOME IMPORTANT ORNAMENTAL PLANTS"** submitted by Mr. M.B. RAVINDRA in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in HORTICULTURE to the University of Agricultural Sciences, Bangalore is a record of bonafide research work carried out by him under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or other similar titles.

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INTRODUCTION

I. INTRODUCTION

The idea of plant tissue culture was conceived by German botanist Haberlandt in 1902 who believed that plant cells are 'Totipotent' i.e., with proper environment and nutrition they have the capacity to regenerate the whole plant. Plant tissue culture was viewed as a primary tool for botanical investigations to understand the phenomenon of morphogenesis. Application of plant tissue culture in commercial horticulture dawned with the successful in vitro orchid multiplication by Morel (1960). He observed that when excised shoot tips of cymbidiums were cultured, instead of developing into leafy shoots, they formed a spherical body with rhizoids at base (protocorms), ultimately giving rise to many plantlets. This technique not only revolutionized orchid industry, but also gave impetus for the application of tissue culture technique for rapid propagation of horticultural plants. Currrently, tissue culture is the most preferred method for the propagation of most of value added ornamentals like orchids, anthuriums, begonias and saintpaulias on commercial scale. Peperomias.

Plant cell and tissue culture has distinct advantages over the conventional methods in the propagation and improvement of horticultural crops, these include:

- Rapid multiplication of elite cultivars and desirable hybrids.
- Production of disease free plants which have high commercial value.
- Year round production of desired cultivars.
- Anther culture for production of haploids.
- Induction of mutation and variation at cellular level for crop improvement.
- Protoplast culture and somatic hybridization.
- Encapsulation of somatic embryos and protocorms.

Tissue culture is the sole method by which most of ornamentals and other commercial plants are being reproduced commercially in countries like Holland, USA, Germany, Japan, Thailand and Brazil, where mostof the sophisticated modern tissue culture laboratories have been established. These laboratories not only mericlone the desired types but also do the indexing of specific pathogen free plants (SPF) through the use of ELISA (enzyme linked immuno sorbent assay). In recent years, tissue culture has been supplemented with protoplast culture, fusion to produce somatic hybrids with desired characteristics and faster rate of propagation.

The increasing awareness of the potentialities of plant cell culture for rapid multiplication, improvement and conservation of germplasm has provided substantial

impetus for research. Hence, the present investigations were carried out with the following objectives.

i) To develop suitable protocol for micropropagation of Saintpaulia ionantha Wendl.

ii) To develop suitable strategies for rapid in vitro multiplication of Begonia 'Lucerna' and Cymbidium aloifolium Sw.

iii) To study the effect of growth regulators and culture conditions for callus initiation in Anthurium scherzerianum Schott.

iv) To assess the efficacy of encapsulation and regeneration of encapsulated plant material of Cymbidium aloifolium and Saintpaulia ionantha.

v) To develop suitable strategies for obtaining haploids in Saintpaulia ionantha by anther culture.

vi) To develop protocols suitable for isolation of protoplasts from leaves of Dendrobium 'Jaquelyn Thomas'. Daucus carota

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Technical innovations and resulting changes are the key to the process of economic development. Dramatic advances in biology augur a third agricultural revolution involving biotechnology, a catch-all term that include both cell and DNA manipulation. With regard to some of the applications of this technology, a mention can be made of micropropagation, induction of androgenic haploids, synthetic seeds and somatic hybridization as aids to crop improvement programmes.

As the basic structural unit of multicellular organisms, the cell is involved in a complex system of inter-relationships. It is both functionally autonomous and part of a graded morphological and physiological hierarchy of tissues, organs and organism. The concept of cell theory of Schleiden (1838) and Schwann (1839) is the basis of plant cell, tissue and organ culture. Haberlandt (1902) first attempted cultivation of isolated plant cells in vitro on a nutrient medium and this lead to many important discoveries of this century. Haberlandt initiated the technique of plant tissue culture, which has since been tremendously exploited both for basic and applied aspects of plant research, encompassing haploidy, mutation and mutagenesis, somatic embryogenesis, soma-clonal variation, protoplast fusion, which are helpful in genetic manipulation and molecular biology of plant genes

(Bajaj, 1977; Barz et al., 1977; Lindsey and Yeoman, 1983; Mantell et al., 1985) and even for commercial exploitation (Murashige, 1974; Reinert and Bajaj, 1977; Yeoman, 1986).

Major breakthroughs in plant tissue culture were achieved after the discovery of auxins and cytokinins. The identification and purification of indole-3-acetic acid (IAA), the first known growth regulator, by Kogl et al. (1934) and then by Thimann (1935) made it possible to control the growth of plant cells and tissues. It was not until 1957 that kinetin was discovered and the idea of synergistic effects of auxins and cytokinins in promoting cell division in tobacco triggered the imagination of physiologists (Skoog and Miller, 1957). The pioneering experiments of White (1934, 1939), Gautheret (1939), Morel and Martin (1952), Skoog and Miller (1957), Reiert (1958), Steward et al. (1958), Morel (1960), Cocking (1960, 1977), Murashige and Skoog (1962), Vasil and Hilderbrandt (1965), Guha and Maheshwari (1966), Carlson et al. (1972) and Murashige (1974, 1978) are often cited as the land marks in the developmental phases of plant tissue culture.

2.1 Tissue culture of ornamental plants

The multiplication of ornamental plants for the horticultural industry has provided the first and at present, by for the largest practical application for the

science of plant tissue culture (George and Sherrington, 1984).

An important technique, which later became a viable horticultural practice, was developed by Ball (1946), who successfully raised transplantable whole plants of nasturtium and lupine by culturing their shoot tips with a couple of leaf primordia. However, the demonstration of the practical usefulness of this important technique must be credited to Morel and Martin (1952) who for the first time recovered virus free Dahlia plants from infected individuals by excising and culturing their shoot tips in vitro. The basis of this approach is that even in a virus infected plant, the cells of the shoot tip are either free of virus or carry a negligible amount of pathogen. The technique has since then been widely used with a variety of plant species of commercial importance. However, a major stimulus for application of plant tissue culture techniques to the propagation of ornamental species may be attributed to the early work by Morel (1960) on the propagation of orchids in culture and to the development and wide spread use of a new medium with high concentrations of mineral salts by Murashige and Skoog (1962).

While applying the technique of shoot tip culture for raising virus free plants of the orchid Cymbidium, Morel (1960) also realized the potentiality of this method

for the rapid propagation of these plants. It was possible to produce 4 million genetically identical plants from a single bud in a period of one year. This revolutionised the orchid industry, and gave impetus to the application of shoot tip culture for rapid cloning of other plant species. Currently, in vitro propagation strategies have been developed for a number of economically important plant species. Excellent reviews on the tissue culture propagation of ornamentals (Holdgate, 1977; Hughes, 1981; Read and Hosier, 1986; Ammirato et al., 1990) bulbs (Hussey, 1977; Krikorian and Kann, 1986), flower crops (Stimart, 1986), foliage plants (Henny et al., 1981; Hartmann and Zetler, 1986) orchids (Rao, 1977; Sagawa, 1984; Griesbach, 1986; Singh, 1987) have been published.

Murashige (1974) listed three possible methods for in vitro micropropagation, viz., (i) enhanced release of axillary buds, (ii) production of adventitious shoots through direct or indirect organogenesis and (iii) somatic embryogenesis.

Release of axillary buds

Stimulating axillary branching by in vitro culture of nodal explants is the commonest technique of micropropagation (Lawrene, 1981). Wickson and Thimann (1958) discovered that the cytokinins could release the lateral buds from apical dominance in an intact stem tip.

The method of axillary bud multiplication was slower, and genetically aberrant plants were virtually absent (Murashige, 1974). These methods have been used on a wide range of species from foliage plants to woody ornamentals (Lane, 1982). Greater success in herbaceous plants has been particularly due to the weak apical dominance and strong root regenerating capacity (Hu and Wang, 1983). Applications of this technique in elimination of phytopathogens has gained vital importance. It has made a significant impact on the foliage industry as a means of controlling such trouble some diseases as dasheen mosaic virus in case of Dieffenbachia and caladium (Hartmann, 1974).

Adventitious shoots

Direct adventitious organogenesis has more potential than the induction of axillary buds for mass clonal propagation. A single leaf or petiole may produce thousands of shoot buds, each genetically identical to the explant (Hughes, 1981). The poptentiality of many ornamental plants like Begonia, Saintpaulia to develop adventitious shoots has been exploited in many micropropagation strategies (Hartmann and Kester, 1975; Jungnickel, 1976). Production of adventitious shoots through indirect organogenesis viz., callus mediated somatic organogenesis is not recommended for clonal

propagation, but may be ideal for recovery of useful variant lines. Such genetic variability may be agriculturally useful when integrated into existing breeding programmes (Evans et al., 1984). Sacristan and Melchers (1969) reported that long time cultures resulted in tissue culture induced variability in chromosome number in both the callus and in the plants regenerated from it.

Somatic embryogenesis

The greatest potential for clonal multiplication is through somatic embryogenesis, where, technically a single isolated cell can produce first an embryo, then a complete plant. Somatic embryoenesis has been demonstrated in several highly morphogenic species to date including carrot, Nigella, Antirrhinum and Petunia (Hughes, 1981). The idea of using somatic embryo as an artificial seed was initiated by Murashige (1977). Conversion efficiency of somatic embryos to plants, better handling techniques of somatic embryo, fluid drilling and encapsulation of embryos have all been suggested as necessary criteria for potential delivery systems of artificial seed (Murashige, 1978; Drew, 1979; Redenbaugh et al., 1984).

2.1.1 Saintpaulia ionantha Wendl.

The common house plant known as African violet is a member of family Gesneriaceae. A major hope for in

vitro techniques as applied to Saintpaulia ionantha was an improvement in mass propagation. African violets have traditionally been propagated by leaf cuttings and are responsive to in vitro cultures. The large number of commercial propagators currently using in vitro technique for Saintpaulia, would suggest that, despite apparent problems, the benefits of the system are significant when compared to conventional methods (Grout, 1990).

Initiation of culture

Explants: The first step in any plant cell or tissue culture system is to obtain a suitable explant. The type of explant, its size, age and the manner in which it is cultured can all determine whether the cultures can be successfully maintained and morphogenesis could be induced (Murashige, 1974).

In Saintpaulia, various explants such as petiole (Bilkey et al., 1978; Harney and Knap, 1979), leaf lamina (Jacob et al., 1980; Chang, 1985; Chen et al., 1987), floral parts (Vazquez and Short, 1978) and anthers (Smith et al., 1981; Norris et al., 1982) have been successfully used for organogenesis and for further multiplication.

The petiole is the organ of origin of adventitious shoots in conventional propagation of Saintpaulia ionantha, but an early report suggested that they had no

regenerative properties in vitro (Kukulczanka and Suszynska, 1972). Success with petiole explants infected with Agrobacterium tumefaciens was reported (Rao and Morel, 1973) and thereafter a number of techniques for establishing regenerating cultures appeared in the literature (Flores et al., 1976; Grunewaldt, 1976; Bilkey et al., 1978 and Xu, 1984). The data collected by Bilkey et al. (1978) indicated the value of this method by which they could produce 5000 commercially acceptable plants within 3-4 months from a single petiole.

Regeneration from leaf lamina explant is perhaps the most widely employed technique for the in vitro propagation of Saintpaulia ionantha (Grout, 1990). Characteristically, callus tissue begins growth at the cut surfaces of the explant and adventitious organ production is from the callus developed at cut surfaces of the explants (Kukulczanka and Suszynska, 1972; Flores et al., 1976; Grunewaldt, 1976; Start and Cumming, 1976; Zhang et al., 1982; Cassells and Plunkett, 1984). The most suitable explants are taken from the body of the leaf lamina, because peripheral portions have been shown to be less productive in vitro (Start and Cumming, 1976).

Saintpaulia explants are succulent and thus are sensitive to higher concentration of sterilizing agents. Le and Collett (1981) advocated tissue culture recycling based on stored axenic materials as the explant source

which avoid the cost of maintaining mother plants and also avoid the use of sterilization agents which might kill the tissues of the explants. To eliminate possible variation due to difference in the physiological status of the explants prior to culture, Cassells et al. (1986) used young axenic leaves and obtained different morphogenic response.

Molgaard et al. (1991) followed a new method of homogenization of shoot buds from Saintpaulia ionantha petals using a blender for rapid multiplication. The homogenates of shoot buds when cultured induced many clumps of shoot buds.

Composition of culture medium

Different media such as MS (Bilkey et al., 1978); Vazquez and Short, 1978; Harney and Knap, 1979; Jacob et al., 1980; Chang, 1985; Ioannou, 1987), Huang and Murashige medium (Smith and Norris, 1983), Margara medium (Margara and Piollart, 1985), LS medium (Lercari et al., 1986) and B_5 medium (Flores et al., 1976) were used for successful regeneration of plantlets in vitro. Bilkey and Cocking (1981) reported that the MS medium has resulted in shoot regeneration from only petiole explants containing an epidermis. Whereas, B_5 medium gave shoots both in the presence and absence of epidermis.

Plant growth regulators

Generally a high concentration of auxin and a low concentration of cytokinin in the basal medium promotes abundant cell proliferation with the formation of callus (Skoog and Miller, 1957). On the other hand, low auxin and high cytokinin concentration in the medium results in the induction of shoot morphogenesis. Auxin alone or in combination with a very low concentration of cytokinin is important for the induction of root primordia.

Callus initiation and shoot bud differentiation

Initiation and growth of callus was reported by Bilkey and Cocking (1981) in petiole cross sections when explanted on MS medium supplemented with NAA 0.1 mgl^{-1} and BA 0.5 mgl^{-1}. Schlegel (1983) obtained a callus ring around which many shoots were produced when leaf stalk was cultured on MS medium containing NAA 1 mgl^{-1} and BA 1 mgl^{-1} Chen et al. (1987) reported the initiation of callus and plantlets on leaf stalks when cultured on MS medium supplemented with NAA (0.1 to 0.5 mgl^{-1}) and kinetin (0.5 - 2.0 mgl^{-1}).

In a study, while comparing the effects of BA and kinetin as a source of cytokinins, Bilkey et al. (1978) revealed that BA produced greater callus growth and subsequently greater number of differentiated plantlets as compared to kinetin, they also noted that high cytokinin

concentrations marked the regenerative capacity of petioles. When 2,4-D at 1 mgl^{-1} was used in MS medium thin cell walled callus was produced on petiole sections (Bilkey and Cocking, 1982).

Start and Cumming (1976) reported the initiation of callus from leaf sections on a medium having higher concentration of NAA and BA. Vazquez et al. (1977) obtained callus from leaf discs within 35 days of culture on MS medium supplemented with NAA (2 mgl^{-1}) and BAP (0.2 mgl^{-1}) and they have also noticed shoot and root differentiation from the callus in the same medium.

Direct organogenesis

Some of the ornamental plants can be multiplied by direct shoot regeneration, where shoot buds can be freely regenerated directly on leaf explants without the formation of any interveining callus phase. Direct adventitious shoot formation has been reported on petiole (Bilkey et al., 1978; Harney and Knap, 1979) and leaf segments (Vazquez et al., 1977; Jacob et al., 1980) resulting in highly proliferative shoot mass and a very rapid rate of propagation. In Saintpaulia both adventitious shoots and axillary shoots arise on shoots formed initially from adventitious buds in an in vitro culture (George and Sherrington, 1984). Leaf blades cultured on MS medium supplemented with NAA and BA at 1

mgl^{-1} produced vegetative buds within 4 weeks (Jacob *et al.*, 1980).

Subculturing

Many kinds of tissue culture plantlets can be subcultured once they are established. Subculturing often becomes imperative to maintain the culture or to increase its volume (George and Sherrington, 1984).

In an attempt to improve the rate of propagation of *Saintpaulia*, Start and Cumming (1976) subcultured individual plants on to a modified MS medium containing a ratio of 1.0 NAA : 0.74BA. Theoretically, this method could produce a million fold increase of plants per annum. Grunewaldt (1976) grew three cultivars of *Saint paulia* for 6 years at five passage per year without any added growth regulators.

The ability of axenic leaves to produce vegetative buds was retained during successive subcultures when cultured on a basal medium of Margara and MS medium (Margara and Piollart, 1985). Reist and Le (1987) used shoot clusters obtained by *in vitro* cultures for further multiplication by series of subculturing for several months.

Rhizogenesis

A few plants produce roots during multiple shoot formation. But in most of the instances, the presence of cytokinin inhibits root formation and a separate medium for induction of roots has to be used. Since, auxin is essential for root initiation, majority of rooting media contain this growth regulator as a supplement. The root elongation phase has been found to be very sensitive to auxin concentrations. At the same time very high concentrations of auxins inhibited root elongation (Thimann, 1977).

In Saintpaulia, incubation of leaf discs and excised floral parts on MS medium supplemented with NAA (1 mgl^{-1}) and kinetin (0.2 mgl^{-1}) resulted in the development of roots within 35 days of culture (Vazquez et al., 1977). For considerable growth of shoot and good rooting, subculturing of plantlets into hormone free medium has been suggested (Cooke, 1977; Jacob et al., 1980; Grout, 1990). Whereas, Start and Cumming (1976) found a second medium high in auxins, essential for root formation before transfer of plantlets into soil. Chang (1985) reported the rooting of plantlets in a low auxin content (0-0.01 ppm NAA) rooting medium.

In another study, the rootless plantlets when transplantd to commercial soil mix, these plants

established well showing the production of roots in the soil medium (Harney and Knap, 1979).

Hardening and planting out

The commercial success of any microprapagation technique depends upon the ease and efficiency with which the plantlets can be established in soil. The transfer of plantlets from the culture to soil requires meticulous and careful stepwise procedure. It is essential to maintain the plants under a very high relative humidity (90-100%) for the first 10-15 days by keeping them under mist or covering them with polythene/polypropylene covers. Partial defoliation of plantlets at the time of transplantation may also be beneficial (Bhojwani, 1980).

To achieve optimum transpslanting success, Grout (1990) selected plantlets after a growth period of 4 weeks in hormone free medium, which had atleast three healthy leaves all greater than 1 cm across and a well developed root system. Jacob *et al*. (1980) obtained a 100 per cent success in survivability of plantlets when they were hardened at 20-25^{o}C and at high relative humidity

Physical, chemical and biological properties of the potting mixture and the atmospheric conditions during post-transfer growth are important in the establishment of *in vitro* regenerated plants. Higher rates of success was

achieved by Flores *et al*. (1976) in survivability of transplanted plantlets in 1:1 mixture of peat moss and soil, whereas, Ioannou (1987) transplanted plantlets to a 2:1 mixture of Sphagnum peat and soil.

In vitro variation in plants

The question of variation in phenotype that may arise following culture *in vitro* has been raised in *Saintpaulia* (Grunewaldt, 1980; Cassels and Plunkett, 1986).

The extent to which variation has been reported may be indicative of the frequency of generation of somaclonal variants in the leaf culture system, particularly when the amount of callus is significant (Larkin and Scowcroft, 1981). During the *in vitro* culture a fluted leaf variant was observed in *Saintpaulia* leaf cultures (Cassells and Plunkett, 1986). They have also noticed variation in flower colours, leaf shape, altered pigmentation which was varying from simple leaf flecking to an almost completely albino appearence. Whereas, Vazquez *et al*. (1977) observed uniformity *in vitro* propagated plants, which had no morphological or cytological changes.

2.1.2 *Begonia*

Begonias are the wonderfully diverse group of plants with various growth habits and there are over 2475

different species and cultivars which are being grown (Thompson and Thompson, 1981). Amongst the various groups, cane type has 215 different species and cultivars which are grown for their attractive foliage and long lasting pendulous inflorescence. As the available information on *in vitro* studies of cane type cultivar *Begonia* 'Lucerna' is very scanty, the relevant literature of *in vitro* cultures of other species and cultivars of *Begonia* has been reviewed here.

Many attempts have been made to grow *Bigonia* plants by using tissue culture, but most of them were on the effects of plant growth regulators or physiochemical conditions on differentiation and growth (Takayama, 1990).

Among the different *in vitro* propgation studies, explants such as leaf segments (Reuter, 1980; Takayama and Misawa, 1981), petiole (Bigot, 1981), stem segments (Bigot, 1981) in *Begonia hiemalis*; leaf segments (Arora *et al.*, 1970) in *Begonia rex* and leaf segments (Nakayama *et al.*, 1981) in *Begonia tuberhybrida* have given sucessful results in multiplication.

Ringe and Nitsch (1968) obtained both vegetative and floral buds from explants of petiole and floral stalks of *Begonia* 'Lucerna' cultured on Knop and MS medium supplemented with IAA ($5-7 \times 10^{-7}$M) and BA (5×10^{-7} and different concentrations of adenine ($0-3 \times 10^{-4}$M).

MS medium is the most widely used medium for Begonia tissue cultures (Nakayama et al., 1981; Roest et al., 1981; Simmonds, 1984; Peck and Cumming, 1984). However, other media such as LS (Reuter, 1980, Reuter and Bhandari, 1981) and Margara (Margara and Piollart, 1983) have also been tried successfully for culturing Begonia explants.

The liquid shake cultures by using modified MS medium for Begonia hiemalis resulted in rapid production of large number of plantlets indicating the importance of liquid culture in propagation (Takayama and Misawa, 1981; Simmonds and Werry, 1987).

Leaf tissues of Begonia feastii cultured on MS medium supplemented with 0.2 mgl^{-1} NAA and 2 mgl^{-1} BA initiated callus after 2 weeks and plantlets were produced after 2 months (Li, 1983). Guang et al. (1983) obtained callus and differentiation of plantlets on leaf explant of Begonia fimbristipula when cultured on MS medium supplemented with 0.1 ppm 2,4-D, 2.5 ppm NAA and 0.25 ppm kinetin.

The petiole explants of Begonia tuberhybrida cultured on medium having 2,4-D (0.15 mgl^{-1}), NAA (1 mgl^{-1}), BAP (0.9 mgl^{-1}) induced callus after 15 days. The complete cycle from induction of callus to flowering of the rgenerated potted plants took 6-7 months which was

less than the time taken for propagation by seeds or cuttings (Viseur and Lievens, 1987).

One of the methods of plantlet formation in tissue culture is based on adventitious bud formation on leaf petiole or inflorescence segments. Although vegetative buds are easily regenerated from young leaf and petiole segments of Begonia hiemalis, combination of auxins and cytokinins are fundamental pre-requisites for differentiation in vitro (Takayama, 1990).

Benzyladenine at 1.3 μM BA or kinetin at 4.6 μM in combination with 8.4 μM NAA was sufficient for the formation of numerous vegetative buds (Takayama and Misawa, 1981). Similar results were reported by Reuter (1980), Reuter and Bhandari (1981) and Bigot (1981). Whereas, lower cytokinin concentrations at 0.22 μM BA (Welander, 1977) or 0.08 to 0.88 μM BA (Appelgren, 1985) were successful in inducing vegetative buds in Begonia hiemalis. Simmonds and Werry (1987) obtained large number of adventitious buds on petiole explants of Begonia hiemalis on MS medium supplemented with NAA (5×10^{-7}M) and BAP (4×10^{-7}M).

In Begonia tuberhybrida, the leaf sections including a major vein differentiated into buds on MS medium supplemented with 1.0 mgl^{-1} NAA and 5 mgl^{-1} BA, and the rooting was induced in a medium having IBA 2 mgl^{-1}

(Peck and Cumming, 1984). Root induction and further growth was observed in MS medium supplemented with higher NAA concentrations in case of Begonia hiemalis (Takayama and Misawa, 1982).

2.1.3. Cymbidium

Cymbidiums which belong to sympodial group of orchids are propagated conventionally by splitting of plants at the sympodial joints so that more of the axillary buds at the bases of pseudobulbs can be stimulated to develop.

The orchids could be asexually multiplied by tissue culture technique (Morel, 1960, 1964a, b) which has led to an enormous increase in the number of plants mostly artificial hybrids in cultivation. Apart from getting virus free Cymbidium plants by culturing apical meristem, Morel (1960) made three discoveries of far-reaching importance: (1) the cultured orchid meristem developed into a mass of tissue morphologically identical to the protocorm, (2) this protocorms some times developed a shoot and root, but quite often it spontaneously produced a number of lateral protruberances or protocorm like bodies, (3) the number of protocorms obtained from a single meristem could be enhanced artificially by cutting the protocorms into pieces and culturing. Each piece

proliferated and more protocorms formed which could be regenerated into plantlets (Morel, 1964b).

Meanwhile, it had been discovered that, if the meristems were cultured in a liquid medium kept constantly in agitated motion, the number of protocorm like bodies could be greatly increased (Wimber, 1963, 1965).

Many more orchid genera have been successfully cloned by this tissue culture technique, since then Murashige (1974) enumerated 22 genera and Arditti (1977) listed 35, including some intergeneric hybrids.

The source of explants for tissue culture techniques among orchids was originally the apical meristem of a young shoot. Terminal and axillary buds from shoot tips containing meristems have also been used on many occassions (Sagawa, 1961; Morel, 1963; Sagawa and Shoji, 1967; Vajrabhaya and Vajrabhaya, 1970; Fonnesbech, 1972; Sagawa and Kunisaki, 1982; Choi et al., 1989).

Trials have also been carried out with a number of other organs in orchids. Reports have been made of callus formation and plantlet regeneration from juvenile material such as protocorms (Pierik and Steegmans, 1972) seedling leaf bases (Champagnat et al., 1970). Leaves have provided explant material in several instances (Churchill et al., 1973; Sagawa and Kunisaki, 1982).

The two most popular media used for orchid tissue culture are Vacin and Went (1949) and Knudson C medium (Knudson, 1946). These media are originally devised for orchid seed germination and they are much simpler than those devised for the tissue culture of other plant materials. Different species of Cymbidium were successfully cultured on modified Vacin and Went medium (Sagawa et al., 1966; Gu et al., 1987). Media defined by Morel (1965), Knudson C (1946) and MS (1962) have also given good results in cultured Cymbidium explants (Kano, 1965; Steward and Mapes, 1971; Thompson, 1971; Kim and Kako, 1984; Shimasaki and Uemoto, 1991).

Two types of media have been used for the propagation of orchids by tissue culture techniques, namely solid and agitated liquid media (Stewart, 1989). Wimber (1963) introduced the use of a liquid medium, in flasks on a rotary shaker. In case of Cymbidium, he compared liquid medium with that of Morel's (1960) method using a solid medium and found that the growth of the explants was 100 per cent greater in the agitated liquid medium. Singh and Prakash (1985) used suspension culture technique for immature embryos of Epidendrum radicans.

Subsequent to the development of different media for orchid tissue culture, several modifications were made to the media by changing the ingredients and their quality and quantity (Arditti, 1977). The most important

development in the medium was the incorporation of growth substances which include auxins, cytokinins, vitamins and other additives like coconut water and banana pulp.

Cymbidium protocorms cultured on Knudson C medium with Nitsch micro elements , produced plantlets within 2 months. When the medium was supplemented with 0.1 mgl^{-1} NAA, about half of the protocorms produced shoots (Ueda and Torikata, 1969). Matsuri et al. (1970) reported that in Cymbidium the protocorm was not affected by the addition of NAA at rates upto 1 ppm. But a higher concentrations of BA (10 ppm) with NAA increased the number of shoots. Root formation was affected little by 0.1 ppm NAA, however, BA inhibited it at higher concentrations. Shoot apices of Cymbidium sazanami , when cultured on MS medium supplemented with 0.1 ppm BA , enhanced leaf elongation, differentiation and the formation of protocorm like bodies. Whereas, NAA at 0.5 ppm inhibited these things (Kim and Kako, 1984). Shoot tips from lateral buds of Cymbidium pseudobulb induced multiple protocorm like bodies (PLB) on a modified liquid medium of Vacin and Went containing 1 mgl^{-1} BA. When these PLB's were transferred to Vacin and Went liquid medium having 0.4 mgl^{-1} the PLB formed shoots and eventually plantlets (Gu et al., 1987). Wang (1988) reported the formation of PLB within 4-6 months when shoot meristems and axillary buds of Cymbidium ensifolium and

Cymbidium goeringii were cultured on MS or White medium supplemented with 10 per cent coconut water and 5 mgl^{-1} NAA.

Rhizome development and subsequent plantlet regeneration was observed by Shimasaki and Uemoto (1991), when apical flower buds of Cymbidium goeringii were explanted on modified MS medium containing 0.1 mgl^{-1} BA and 10 mgl^{-1} NAA.

2.1.4 Anthurium

Anthurium belongs to the family Araceae and has more than 500 known species (Criley, 1986). Anthuriums are grown for their long lasting and attractive colours. Conventionally, Anthuriums are propagated by seeds. The time required from pollination to the maturity of seed is about 6-7 months for A. andreanum and 10-12 months for A. scherzerianum and it takes 2-3 years for evaluation of seedling raised plants. Moreover, seed propagated cultivars are poor in uniformity. Vegetative propagation by terminal cuttings and stem sections with dormant buds results in multiplication rates clearly insufficient for mass propagation. Hence, the recent development of efficient micropropagation techniques opened up entirely new and promising prospects for Anthurium breeding and improvement (Geier, 1990).

Tissue culture of Anthurium andreanum was first reported by Pierik et al. (1974), who reported callus from embryos and young parts of adult plants.

Plantlet regenerations has been obtained via callus from cultured spathe (Pierik, 1975; Finnic and Staden, 1986), leaf (Pierik, 1976; Fersing and Lutz, 1977; Singh and Sangama, 1991) and petiole explants (Finnic and Staden, 1986) in Anthurium andreanum and from spadix (Geier and Reuther, 1981), leaf segments (Geier, 1986) in Anthurium scherzerianum. Plantlets without the intervention of callus has also been obtained by axillary buds of Anthurium andreanum (Kunisaki, 1980).

In most studies of in vitro cultures of Anthurium, basic media derived from the MS formula (Murashige and Skoog, 1962) have been used (Geier, 1990). Modifications of Nitsch medium (Nitsch, 1969) also have been used for culturing explants of Anthurium scherzerianum (Geier and Reuther, 1981).

Investigatins by Geier (1986) revealed the beneficial effect of low (2.5 mM) NH_4NO_3 levels on regeneration from Anthurium scherzerianum leaf explants, and this low ammonium medium was found to be an absolute requirement for both callus and shoot induction in a number of genotypes. The ratio of ammonium to nitrate was also shown to be a critical in growth and shoot production

of subcultured Anthurium scherzerianum callus clones (Zens and Zimmer, 1986).

Though optimum callus formation was observed in continuous darkness (Pierik et al., 1974; Pierik, 1975; Singh and Sangama, 1991) in many cases, Finnic and Staden (1986) reported better callus production in light. Apparently, A. scherzerianum callus has no specific light requirement, since successful subculturing has been performed in continuous darkness (Geier and Reuther, 1981) as well as under 16/8 light/dark regime (Zens and Zimmer, 1986). In the case of spadix derived cultures of A scherzerianum, light favoured the persistence of floral differentiation patterns (Geier and Reuther, 1981).

Callus from leaf explants of A. scherzerianum was first obtained by Pierik and Steegmans (1975, 1976) under conditions almost identical to those employed in A. andreanum. Fersing and Lutz (1977), who compared regeneration from explants of both species, found some what lower potential for callus formation in Anthurium scherzerianum when tissues were cultured on modified MS medium supplemented with 4.4 µM BA and 0.45 µM 2,4-D, but addition of 2 gl^{-1} yeast extract while being inhibitory to A. andreanum, strongly promoted callus formation in A. scherzerianum.

In A. scherzerianum, BA at 1 mgl^{-1} and 2,4-D at 0.1 mgl^{-1} in modified Nitsch medium induced callus from spadix explants, whereas, for shoot formation and multiplication BA at 0.5 mgl^{-1} in Nitsch medium found to be optimum (Geier, 1987).

2.2 ENCAPSULATION

Recent advances in the biotechnological research in relation to tissue culture, comprises the techniques which enable the efficient plant production, storage, handling and delivery of regenerated propagules. To facilitate easier handling and delivery of some of these tissue culture induced plant material and cell lines, a technique known as encapsulation has been developed recently, which is still in its infancy.

In the encapsulation of plant materials such as, somatic embryos (Kitto and Janick, 1985; Redenbaugh et al., 1986;) axillary buds (Bapat et al., 1987; Bapat and Rao, 1990), protocorms (Singh, 1991) and cell suspensions (Bapat and Rao, 1988), the matrix which covers the plant materials forming a bead or capsule should be pliable enough to cushion and protect the plant tissue against desiccation, yet sufficiently rigid to allow for rough handling of the capsule during production, storage, transportations and culturing. The matrix usually comprises of alginate, delivers sufficient nutrients, a

growth-developmental agent and other chemical or biological compounds necessary for germination or conversion and survivability of plant material. Encapsulated material could be easily packed in bottles, thus saving the space and ensuring viability and survival rate. These materials form an efficient delivery system enabling easy transport (Bapat et al., 1987).

Though, encapsulation technique is being practiced in many micro-organisms, plantlets and enzymes for the production of specific compounds from the cell, the origin of the idea of encapsulation for plant propagation systems is difficult to determine. However, the encapsulation of somatic embryos to use them as somatic or synthetic or artificial seeds may have been considered as a biotechnological application after the discovery of somatic embryos by Steward et al (1958) and Reinert (1958).

The credit of using encapsulation technique for the production of synthetic seeds goes to Kitto and Janick (1980) who used soluble resins as artificial sead coat and Redenbaugh et al. (1984) who used an alginate gel for encapsulating somatic embryos.

There are many encapsulating agents such as agar, polyox, alginate, gelrite, agarose, carrageenan and polyacrylamide. Polyox, a water soluble ethylene oxide

polymer, as an artificial coat for somatic embryos has been reported in carrot (Kitto and Janick, 1985). Recently nitrocellulose and ethylcellulose have also been used for encapsulation (Wadhawa et al., 1989). However, sodium alginate which is water soluble and forms a completely permeable gel with calcium chloride at room temperature is found to be suitable in production of synthetic seeds (Redenbaugh et al., 1984, 1986, 1987; Bapat and Rao, 1988, 1990; Bapat et al., 1987; Datta and Potrykus, 1989; Singh, 1991).

Redenbaugh et al. (1986) encapsulated alfalfa, celary and cauliflower somatic embryos with alginate matrix forming a single embryo beads which were 4 mm in diameter. Encapsulated alfalfa and celeary somatic embryos developed into plants in sand trays or in transplant plugs at frequencies of 7 and 10 per cent respectively. Bapat and Rao (1988) successfully encapsulated somatic embryos and actively growing cell suspensions of sandal wood in 3 per cent sodium alginate matrix and obtained 10-16 per cent germination from somatic seeds when cultured on a suitable medium. They have also noted the reviewal of encapsulated somatic embryos and cell lines after storage for 45 days at $4^{o}C$, indicating the suitability of this method for preserving desirable elite genotypes.

The artificial seeds obtained by encapsulation of barley embryos maintained the germination capacity for atleast 6 months, whereas non-encapsulated embryos did not survive more than 2 weeks in 4°C storage (Datta and Potrykus, 1989).

Apart from somatic embryos, other plant parts like shoot buds are also used for the production of synthetic seed by encapsulation. Bapat et al. (1987) used axillary buds from in vitro grown mulberry plants for encapsulation, and successfully germinated this encapsulated axillary buds into plantlets in a suitable medium. They also could store these encapsulated axillary buds for 45 days in 4°C temperature without affecting the survivability. Use of different fungicides such as Carbendinzin, Benomyl and Bavistan in alginate matrix of encapsulated axillary buds were found to be beneficial in preventing contaminations and increased the survival of the buds (Bapat and Rao, 1990) showing the suitability of encapsulated buds in a system acceptable at farm level.

Singh (1991) reported the suitability of encapsulation in easy and novel delivery systems for orchid propagation by using 2.5 per cent sodium alginate as the gel matrix for encapsulating protocorms of Spathoglottis plicata. The germination of encapsulated protocorms upto 70 per cent after 180 days storage in 4°C

was also observed when encapsuslated protocorms were transferred to modified Vacin and Went (1949) medium.

2.3 ANTHER CULTURE

Long before the discovery of *in vitro* androgenesis in *Datura innoxia* by Guha and Maheshwari (1964), haploids had been known for their potential uses in plant breeding and basic research. In the majority of crop species, the frequency of naturally occuring haploids, even at its best is too low to be of any use (Prakash and Giles, 1987). The simplicity and ease with which haploids can be produced in large number by *in vitro* anther culture has stimulated a great deal of activity in the production and utilization of haploids for fundamental and applied genetics (Hu and Zeng, 1984).

Saintpaulia ionantha, which is a modern ornamental plant of considerable economic importance has potential as a model system for assessing the value of haploid plants in crop improvement programmes (Grout and Weatherhead, 1980). Further more, the availability of homozygous plants could lead to the production of new, commercial cultivars.

Hughes *et al*. (1975) reported the production of haploid plants by culturing immature anther from unopened flower buds of *Saintpaulia ionantha*. They obtained a frequency of 1-5 plantlets per anther in 20 per cent of

the anthers. Late uninucleate pollen was one of the prerequisite for regeneration in a study in which callus from a single anther was reported to be the source of upto 200 haploid plantlets in the 10 weeks period from culture initiation (Weatherhead et al., 1982). The frequency of anthers producing such callus within the culture population was as high as 80 per cent.

Smith et al. (1981) cultured anthers from immature unopened flower buds in Blaydes medium having 5 mgl^{-1} IAA and 0.5 mgl^{-1} kinetin and obtained plantlets directly without interveining callus. These plants were about two-thirds the size of the parent even after 2 years. In another study, haploid plant having phenotypic differences in leaf margin were obtained by Norris et al. (1982). They indicated the usefulness of haploid plants in breeding programmes to obtain miniatures for direct use as ornamental plants for mutation studies and also the diploidized plants could serve as parents for hybrid seed production.

2.4 PROTOPLAST ISOLATION

Plant protoplasts represent the finest single cell system and offer exciting possibilities in the fields of somatic cell genetics and crop improvement. In culture, isolated protoplasts often perform better then single, whole cells (Nagata and Takaba, 1971) and therefore serve

as an excellent starting material for cell cloning and development of mutant lines (Shepard et al., 1980). However, the feature of isolated protoplasts that has brought them into limelight is the ability of these naked cells to fuse with each other resulting in somatic hybrids.

Cocking (1960) for the first time demonstrated the large scale isolation of protoplasts by enzymatic method. The commercial preparations of the enzymes for protoplast isolation were first employed by Takabe et al. (1968). Power and Cocking (1968) demonstrated that the two enzymes viz., macerozyme and cellulase can be used together for isolating protoplasts at a faster rate.

Protoplasts can be isolated from a variety of plant tissues and cultured cells. However, it has often proved difficult to obtain consistently high yields of viable protoplasts from fresh tissues. The most important factor responsible for such variability appears to be the physiological condition of the source tissue or plant, which affects the susceptibility of the protoplasts (Uchimiya and Murashige, 1974; Watts et al., 1974; Zaitlin and Beachy, 1974; Shepard and Totten, 1975).

Though isolation of protoplasts have been reported successfully in many ornamental plants, the regeneration into plantlets from protoplasts is restricted to around 46

ornamental species (Griesbach, 1988). Approximately 60 per cent of the regenerated species are either from the Petunia or Nicotiana genera.

Protoplasts were readily isolated enzymatically from both petal and petiole sections of Saintpaulia ionantha (Grout, 1990), by an enzymatic treatment of 0.5 per cent (W/V) Onozuka R10 cellulase for 16 hours.

Eventhough protoplasts have been isolated from many orchids, regeneration of plantlets from protoplast is very limited. In 1982 the American Orchid Society offered a prize of $ 50,000 (US) to the first person who successfully fuse protoplasts from two widely divergent orchid species and plants obtained from this must be carried to flowering size (Sheehan, 1986).

Seeni and Abraham (1984) screened more than two dozens of wild and cultivated species and hybrids of orchids for isolation of protoplasts from mesophyll and root tissues. They used a 5 ml standard enzyme mixture at pH 5.5 containing macerozyme R10 (0.5%), cellulase Onozuka R10 (2.0%) MES (5 mM), $CaCl_2$ (1 mM) and sorbitol for 6 hours in isolating the protoplasts. They could get highest yield of protoplasts from tender leaf bases of Cymbidium aloifolium, whereas significant variations in yield were observed among different hybrids of Dendrobium. Price and Earle (1984) reported some success in obtaining

protoplasts from leaf and petal of Dendrobium 'Louis Bleriot' and also from leaf tissues of Vanilla planifolia.

The concentration of the enzyme solution and duration of incubation, are the main factors in obtaining viable protoplasts. The incubation period in the enzyme solution may be as short as 30 minutes (Nagata and Ishii, 1979) or as long as 20 hours (Vasil and Vasil, 1979). Evans and Bravo (1984) stated that the fundamental property of isolated protoplasts is their osmotic fragility and hence, the need for suitable osmotic stabilizer in the enzyme solution, the protoplast washing medium and the protoplast culture medium. Mannitol and sorbitol, separately or in combination, have been used most often as osmoticum for the isolation of protoplasts. Callus as a source for isolation for protoplast has been reported in Hibiscus syriacus by Yan-Xiu et al. (1991).

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The experiments were carried out at the Orchid Laboratory of the Indian Institute of Horticultural Research, Hessaraghatta, Bangalore. The plants were maintained in the temperature and humidity controlled green house attached to the laboratory. Standard cultural practices for growing the plants were followed.

3.1 REQUIREMENTS

3.1.1 Chemicals

Glycine, nicotinic acid, pyridoxine HCl, thiamine HCl, myo-inositol and other plant growth regulators used were of "cell culture tested" grade from Sigma Chemicals, St. Lousis, USA. Agar from BDH chemical for initial cultures and gelrite from Kalco Division of Merc and Co., USA., for further growth and establishment of cultures were used as gelling agent. Cellulase, pectinase and MES (2-[N-Morpholino] ethane sulfonic acid) from Sigma Chemicals, St. Louis, USA and Onozuka R-10 Macerozyme from Yakult Hosha Co. Ltd., Japan were used for protoplast isolation. All the remaining chemicals used in the preparation of tissue culture media were of the analytical grade obtained from local firms such as BDH, Glaxo, SD Fine Chemicals and Hi-media Chemicals.

3.1.2 Glasswares and its washing

The required glasswares were procured from corning/borosil. For the initiation of cultures, tubes having baculate caps were used, where as for further cultures 100 and 150 ml flasks were used. Glasswares were cleaned by soaking in 5 per cent chromic acid, solution for 6 hrs, followed by rinsing with tap water and cleaning with detergent solution. Then glasswares were throughly washed in tap water and rinsed twice in double distilled water. The glasswares were then dried in an hot air oven to remove the moisture and were stored in dustproof area till the use.

3.2 CULTURE MEDIA

3.2.1 Selection of culture media

Suitable media required for present studies were selected depending on species used. The composition of different media used in the present study are listed in Table 1-8.

3.2.2 Adjuvants to basal medium

For establishment of cultures, initiation of callus/morphogenesis of plantlets and rhizogenesis, the basal medium was supplemented with growth regulators such as auxins : NAA (2-naphthalene acetic acid), IBA-(indole-3-butyric acid), 2,4-D (2,4-dichlorophenoxy acetic acid)

Table 1. Composition of Murashige and Skoog medium (Murashige and Skoog, 1962)

Ingredient	Quantity/litre
Potassium nitrate	1900.00 mg
Ammonium nitrate	1650.00 mg
Calcium chloride	440.00 mg
Magnesium sulphate	370.00 mg
Potassium dihydrogen phosphate	170.00 mg
Sodium EDTA	37.30 mg
Ferrous sulphate	27.80 mg
Manganese sulphate	22.30 mg
Zinc sulphate	8.60 mg
Boric acid	6.20 mg
Potassium iodide	0.83 mg
Sodium molybdate	0.25 mg
Copper sulphate	0.025 mg
Cobalt chloride	0.025 mg
myo-inositol	100 mg
Nicotinic acid	0.5 mg
Pyridoxine HCl	0.5 mg
Thiamine	0.1 mg
Glycine	2.0 mg
Sucrose	30.00 g
Distilled water to make volume upto	1000.00 ml
Agar OR	8.00 g
Gelrite	2.50 g

Table 2. Composition of B_5 basal medium (Gamborg *et al.*, 1968)

Ingredient	Quantity/litre
Potassium nitrate	2500.00 mg
Calcium chloride	150.00 mg
Magnesium sulphate	250.00 mg
Sodium dihydrogen Phosphate	150.00 mg
Ammonium sulphate	134.00 mg
Manganese sulphate	10.00 mg
Zinc sulphate	2.00 mg
Boric acid	3.00 mg
Potassium iodide	0.75 mg
Copper sulphate	0.025 mg
Sodium molybdate	0.25 mg
Cobalt chloride	0.025 mg
Sodium Fe EDTA	43.00 mg
myo-inositol	100.00 mg
Thiamine Hcl	10.00 mg
Nicotinic acid	1.00 mg
Pyridoxine-Hcl	1.00 mg
Sucrose	20.00 g
Distilled water to make volume upto	1000.00 ml
Agar	8.00 g

Table 3. Compositions of Margara (1978) N_5K medium.

Element	Quantiy/litre
Potassium nitrate	75.75 mg
Ammonium nitrate	80.0 mg
Calcium nitrate	265.5 mg
Magnesium sulphate	246.0 mg
Potassium chloride	372.5 mg
Potassium dihydrogen phosphate	136.0 mg
Zinc sulphate	0.5 mg
Boric acid	0.5 mg
Potassium iodide	0.01 mg
Copper sulphate	0.1 mg
Sodium molybdate	59.0 ug
Ferrous sulphate	35.0 mg
Sodium EDTA	30.0 mg
Mangnese chloride	0.1 mg
Sucrose	30.0 g
Agar	8.0 g
Distilled water to make volume upto	1000.0 ml

Table 4. Composition of Margara (1978) $N_{45}K$ medium.

Ingredient	Quantiy/litre	
Potassium nitrate	1818.0	mg
Sodium nitrate	85.0	mg
Ammonium nitrate	720.0	mg
Calcium nitrate	944.0	mg
Megnesium sulphate	246.0	mg
Potassium chloride	377.5	mg
Potassium dihydrogen phosphate	136.0	mg
Zinc sulphate	0.5	mg
Boric acid	0.5	mg
Potassium iodide	0.01	mg
Copper sulphate	0.1	mg
Sodium molybdate	59.0	ug
Ferrous sulphate	35.0	mg
Sodium EDTA	30.0	mg
Manganese chloride	0.1	mg
Sucrose	30.0	g
Agar	8.0	g
Distilled water to make volume upto	1000.0	ml

Table 5. Composition of Vacin and Went (1949) medium.

Ingredient	Quantity/litre	
Tri-calcium phosphate	200.0	mg
Potassium nitrate	525.0	mg
Mono patossium acid phosphate	250.0	mg
Magnesium sulphate	250.0	mg
Ammonium sulphate	500.0	mg
Manganese sulphate	6.8	mg
Ferric tartarate	28.0	mg
Sucrose	20.0	g
Agar	8.0	g
Distilled water to make volume upto	1000.0	ml

Table 6. Composition of Knudson C medium (Knudson, 1951).

Ingredient	Quantiy/litre
Calcium nitrate	1.0 g
Ammonium sulphate	0.5 g
Potassium phosphate	250.0 mg
Magnesium sulphate	250.0 mg
Ferrous sulphate	250.0 mg
Manganese sulphate	7.5 mg
Sucrose	20.0 g
Distilled water to make volume upto	1000.0 ml
Agar	8.00 g

Table 7. Composition of Nitsch medium (Nitsch, 1969)

Ingredient	Quantity/litre
Potassium nitrate	950.00 mg
Ammonium nitrate	720.00 mg
Magnesium sulphate	185.00 mg
Calcium chloride	166.00 mg
Potassium dihydrogen phosphate	68.00 mg
Boric acid	10.00 mg
Manganese sulphate	25.00 mg
Zinc sulphate	10.00 mg
Sodium molybdate	0.25 mg
Ferrous sulphate	27.80 mg
Sodium EDTA	37.30 mg
myo-Inositol	100.00 mg
Nicotinic acid	5.00 mg
Pyridoxine HCl	0.50 mg
Thiamine HCl	0.50 mg
Folic acid	2.00 mg
Glycine	0.05 mg
Biotine	0.05 mg
Sucrose	20.00 g
Distilled water to make volume upto	1000.00 ml
Agar	8.00 g

Table 8. Composition of Blaydes medium (1966).

Ingredient	Quantity/litre
Potassium dihydrogen phosphate	300.0 mg
Potassium nitrate	1000.0 mg
Ammonium nitrate	1000.0 mg
Calcium nitrate	347.0 mg
Magensium sulphate	35.0 mg
Potassium chloride	65.0 mg
Potassium iodide	0.8 mg
Zinc sulphate	1.5 mg
Boric acid	1.6 mg
Manganese sulphate	4.4 mg
Glycine	2.0 mg
Thiamine HCl	0.1 mg
Nicotinic acid	0.5 mg
Pyridoxine HCl	0.1 mg
Sodium EDTA	32.0 mg
Sucrose	30.0 g
Gelrite	2.0 g
Distilled water to make volume upto	1000.0 ml

and cytokinins : BA (6-benzyladenine), Kn (kinetin). The growth regulators were added to the basal medium either alone or in various combinations and concentrations as required in the experiments.

3.2.3 Stock solution

Stock solutions of micronutrients and vitamins were prepared by dissolving known quantity of each chemical separately in known volume of double distilled water Growth regulators such as IBA, NAA and 2,4-D were first dissolved in a minimum quantity of ethyl alcohol and the volume was made up with double distilled water. Kinetin, BA and tri-calcium phosphate were first dissolved in 1 N HCl and then the volume was made up. They were stored in amber coloured bottles in refrigerator at $4^{o}C$.

3.2.4 Preparation of media

For preparing the nutrient media required quantity of solution of micronutrients, growth regulators and vitamins were taken in a boiling flask. Required quantity of macronutrients were weighed and added. The volume was made up by adding double distilled water. The pH was adjusted to 5.8 by using 1 N NaOH or 1 N HCl. For preparing the solid medium, required quantity of gelling agent such as agar (8 gl^{-1} or gelrite (2.5 gl^{-1}) was added to the solution and digested by careful warming.

The media thus prepared were dispensed into culture tubes or Erlenmyere's flask at a rate of 25 ml or 50 ml per container respectively. These containers were either capped with baculate caps for tubes or plugged with non adsorbant cotton plugs for flasks and kept for autoclaving.

3.2.5 Autoclaving

The containers having medium were autoclaved at a pressure of 1.06 kg/cm^2 (121^oC) for 15 minutes in a horizantal autoclave. After autoclaving they were stored in the culture room at 25 ± 2^oC.

3.3 TRANSFER AREA AND ASEPTIC MANIPULATION

All the aseptic manipulations like surface sterilization, preparation, inoculation of explants, subculturing and encapsulation were carried out in a laminar air flow chamber. Before using the laminar air flow bench, the working surface was surface sterilized with iso-propyl alcohol. During the course of transfer, all the surgical instruments were dipped in ethyl alcohol, flamed and cooled before use. After every sterile transfer operation, the laminar airflow hood was cleaned and swabed with iso-propyl alcohol and kept closed.

3.4 CULTURE CONDITIONS

The cultures were kept under light or dark conditions as required by the experiment. The prepared cultures were incubated in an airconditioned room at a temperature of 25 ± 2°C in white fluorescent light (2000 Lux) under photoperiodic regime of 16 hrs light and 8 hrs dark cycles. Cultures which required darkness were kept in BOD incubator calton 152 - 6 CF with light control. Liquid cultures which required shaking were put on Emenve rotary shaker at 80 rpm.

Source and preparation of explants, their culturing, subculturing of different species for micropropagation, anther culture, encapsulation and protoplast isolation are covered under seperate headings.

3.5 MICROPROPAGATION

3.5.1 Saintpaulia ionantha Wendl.

The plants were obtained from the Department of Horticulture, Lalbagh, Bangalore and were grown under green house conditions.

3.5.1.1 Initiation of culture

Healthy, pink coloured flowering Saintpaulia ionantha plants which were grown under green house were selected as a source of explants. Fully matured leaves, approximately 6 x 5 cm, with 4 - 5 cm long petiole were

Table 9. Micropropagation strategies adopted in some ornamental plants

Species/variety		Explants	Method of multiplication
Saintpaulia ionantha	(a)	Leaf	IM : Callus, Adventitious shoot buds.
	(b)	Petiole	DM : Adventitious shoot buds.
Cymbidium aloifolium	(a)	Protocorms	
	(b)	Nodal segments from in vitro plants	DM : Multiple shoots.
Begonia 'Lucerna'	(a)	Leaf	
	(b)	Petiole	IM : Callus, adventitious shoots.
	(c)	Stem segments	
Anthurium scherzerianum	(a)	Leaf	
	(b)	Petiole	IM : Callus.

DM : Direct morphogenesis; IM : Indirect morphogenesis (callus mediated).

separated and immersed in tap water for 15 minutes. These leaves with petiole were treated with either 5 per cent sodium hypochlorite solution with 2-3 drops of Tween 20 for 40 minutes or with freshly prepared saturated chlorine water diluted with sterile water at 1:1 concentrations for 5 minutes. After this, leaves were washed in sterile water for 3-4 times to remove the traces of sterilizing agents from the material. Leaf lamina and petioles were separated and dissected aseptically into following dimensions.

Leaf discs - 1 cm diameter

Petiole sections - 4-5 mm thick sections

These dissected explants were cultured to find out suitable medium and optimum concentrations of growth regulator combinations for induction of callus and differentiation.

3.5.1.2 Recurrent propagation using axenic leaves as explants

Since the leaf explants taken from mother stock were sensitive to sterilizing agents, use of axenic leaves as the explant source from *in vitro* plants were tried. In this method, axenic leaves approximately 1 x 1 cm were separated aseptically and cultured to standardise the suitable growth regulator concentration for direct and indirect organogenesis.

Fig. 1. *Saintpaulia ionantha* Wendl.

Fig. 2. *Begonia* 'Lucerna'

3.5.1.3 Callus culture

The green friable callus, obtained in the initial culture was cut into 0.5 cm cube aseptically and cultured on MS medium to find out the optimum concentration of growth regulators for prolifertion and differentiation.

3.5.1.4 Subculturing

The plantlets which were obtained from *in vitro* culture were sub-cultured to know the effect of different combinations of growth regulators on further multiplication and rooting. The axenic plantlets of 1.5 cm height were sub-cultured aseptically at regular intervals to find out the rate of multiplication.

3.5.1.5 Hardening and transfer of plantlets

Young plantlets were taken out from the flasks, thoroughly washed with water to remove all traces of media and transferred to pots to study the suitable potting mixture in community pots under different hardening treatments.

3.5.1.6 Recording of observations

All the cultures were examined every day and the data were recorded after the removal of contaminated cultures. Depending upon the rate and type of growth,

cultures were scored by using following observation and notations.

Abbrevations	Type of response
-	Nil
+	Low
++	Moderate
+++	High
++++	Intense
% R	Percentage of explant response
NR	Nature of response
C	Callus
CC	Colour of callus
NC	Nature of callus
SB	Shoot buds
PL	Plantlets
R	Roots

Where ever possible the actual number of shoot buds and plantlets were recorded. For responses like callus, shoot buds, plantlets and roots, observations were recorded treatmentwise.

3.5.2 Begonia 'Leucrna'

The plants were obtained from Division of Horticulture, University of Agricultural Sciences, GKVK,

Bangalore, and were grown under green house condition. Fully expanded mature leaves with petiole and internodal stem segment of 1 cm thick were selected and washed in running tap water for 15 minutes. These leaf materials were treated with freshly prepared chlorine water diluted with sterile water at 1:1 concentration along with 2-3 drops of Tween 20 for 5 minutes. However in case of petiole and stem segments the sterilizing treatment was increased to 8 minutes. These explants were then washed thoroughly 3-4 times with sterile water to remove traces of sterilizing agents and then dissected aseptically into following dimensions.

Leaf discs with vein	-	1 cm diameter
Petiole segment	-	6-8 mm thick sections
Stem segment	-	6-8 mm thick sections

These explants were inoculated on MS medium with various concentrations of growth regulators for obtaining morphogenesis.

3.5.2.2 Recording of observations

As given under 3.5.1.6

3.5.2 Cymbidium aloifolium Sw.

The plants were obtained from Western Ghats and maintained in Orchidarium at Indian Institute of Horticultural Research, Bangalore. Axenic plant materials

Fig. 3. Cymbidium aloifolium Sw.

were used as explant for culturing. The 3 months old protocorms obtained by seeds cultured on Knudson 'C' medium were used to initiate the culture.

The green protocorms, 2 mm in diameter were taken out aseptically from flasks and cultured on the modified liquid and solid Vacin and Went and MS medium at different growth regulator combinations. For further multiplication, multiple shoots obtained in vitro were dissected to get single nodes of 1.5 cm length each having an axillary bud and cultured on medium having various combinations of growth regulators.

Observations recorded

1. Proliferation of protocorms
2. Differentiation into plantlets and roots
3. Multiplication rate

3.5.4 Anthurium scherzerianum Schott.

The plants were obtained from Beena Nursery, Trivandrum and maintained in green house conditions. For leaf and petiole cultures, leaves 6-7 cm in length from a healthy plants were selected. After washing in tap water for 5 minutes they were dipped in alcohol for 10 seconds and treated with freshly prepared chlorine water diluted with sterile water at 1:1 concentration along with 2-3 drops of Tween 20 for 10 minutes. Then these explants

Fig. 4. Anthurium scherzerianum Schott.

were washed thoroughly 3-4 times with sterile water to remove the traces of chlorine and then dissected aseptically into following dimentions.

Leaf segment with vein - 1 cm^2

Petiole segment - 6 mm thick sections

The explants were cultured on to different medium having different concentration of growth regulators and incubated for initiation of callus.

3.6 ENCAPSULATION

3.6.1 Plant materials for encapsulation

3.6.1.1 Cymbidium aloifolium Sw.

Three months old green protocorms of size 2 mm in diameter which werr obtained from culturing seeds in Knudson 'C' medium were selected for encapsulation.

3.6.1.2 Saintpaulia ionantha Wendl.

Petiole and leaf sections of 3-4 mm in size, adventitious shoot buds and green friable callus obtained during initial *in vitro* cultures from MS medium were taken as material for encapsulation.

3.6.2 Encapsulation technique

The plant materials of Cymbidium and Saintpaulia were mixed separately with 100 ml of autoclaved 2-4 per cent sodium alginate (Sigma Cat A-7128) gel prepared in MS

basal liquid medium. These plant materials were mixed with the gel and were dropped individually with sterile bent tip forceps into an autoclaved 50 mM solution of $CaCl_2$, $2H_2O$ (1.36 g/150 ml). On complexation, calcium alginate beads of 6-7 mm were formed around the plant material and these beads were allowed to remain in the solution for 30 minutes on a rotary shaker (80 rpm) at 25°C for 30 minutes. The alginate surface embeded materials (beads) were rinsed in sterile water, surface dried with sterile filter paper and cultured dirctly on Vacin and Went liquid medium supplemented with 0.4 mg l^{-1} BA for protocorms beads or MS medium supplemented with IBA and BA at 1 mg l^{-1} for Saintpaulia petiole section, shoot bud and callus beads.

One set each of these beads were stored at 4 and 0°C in the dark or kept in 25°C 16 h/8 h light/dark condition in sterile dry petridishes sealed with parafilm. All these operations were carried out aseptically.

Stored beads were cultured on liquid Vacin and Went medium supplemented with 0.4 mgl^{-1} BA for Cymbidium protocorm beads and MS medium supplemented with IBA and BA $1mgl^{-1}$ for saint paulia at different intervals to assess the regeneration capacity. A set of non-encapsulated plant material was also kept at 25/4/0°C in sterile dry petridishes as control. The stored and cultured beads

were observed for their germination as in the case of Cymbidium and regeneration and callusing in different material of Saintpaulia.

3.7 ANTHER CULTURE

Immature unopened flower buds of 4-6 mm in length were excised from Saintpaulia ionantha plants grown in green house and washed in water for 15 minutes. These were surface sterilized by using saturated chlorine water containing 2-3 drops of tween 20, for 5 minutes. After repeated rinsing with sterile water the anthers were dissected and were transferred to culture tubes containing experimental media. Observations on extent of callus and regeneration were recorded.

3.8 ISOLATION OF PROTOPLASTS

For isolation of protoplasts, third tender leaves from the top of Dendrobium 'Jaquelyn Thomas' were taken. 600 mg of leaf material was preplasmolysed in 20 ml of 13 per cent mannitol solution for 2 hrs. The leaf material was cut into small pieces and incubated in 5 ml of enzyme solution (Table 10) in dark for differnt periods. After incubation, undigested tissue was removed by filteration by 100 μ nylon screen and the filtrate was mixed with equal volume of 1 M sucrose solution. This aliquot was centrifuged at 120 x G for 3 minutes to purify the protoplasts. Protoplasts were removed from floating

Fig. 5. Dendrobium 'Jaquelyn Thomas'

layers and resuspended in 2 ml of 0.3 M sorbitol and the viable protoplasts were isolated and observed under phrase contrast microscope. Observations of release of intact protoplasts were made at different incubation timings and various enzyme treatments.

Table 10. Composition of enzyme mixture for isolation of of protoplasts.

Ingredient	Concentrations
Cellulase	0-5% - 2.0%
Pectinase	0.5%
Macerozyme	0.2% - 0.4%
Sorbitol	5 mM
MES	5 mM
pH	5.5

3.9 STATISTICAL ANALYSIS

The data was analysed to rank the means from the highest to the lowest to identify the appropriate treatments as advocated by Mize and Chun (1988) for tissue culture experiments.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Results obtained from the present investigations conducted on 'Development of micropropagation systems and new in vitro strategies for some important ornamental plants' are presented under the following headings.

1. Micropropagation
2. Encapsulation
3. Anther culture
4. Protoplast isolation

4.1 MICROPROPAGATION

4.1.1 Saintpaulia ionantha Wendl.

4.1.1.1 Effect of different nutrient media during initiation of culture

The experiment was conducted to study the effect of different nutrient media on leaf and petiole explants of Saintpaulia ionantha. Five different media were used in the present study which include MS, 1/2 strength MS, Margara N_5K, Margara $N_{45}K$ and Gamborg's B_5 medium. These medium were supplemented with NAA 0.5 mgl^{-1} and BA 0.5 mgl^{-1} for various responses. The morphogenetic response of leaf discs and petiole sections were assessed at the end of 10 weeks period and are presented in Table 11.

a. Leaf disc explants

The per cent of leaf disc cultures responded to morphogenesis varied depending or the media used. 85 per

Table 11. Effect of different nutrient media on explants of Saintpaulia ionantha.

Treatment*	LEAF DISCS					PETIOLE SECTIONS				
			Frequency of organogenesis					Frequency of organogenesis		
	NR	%R	Callus	SB	Roots	NR	%R	Callus	SB	Roots
MS	C+SB+R	85	++++	+++	++	C+SB+R	70	+++	++	+
1/2 MS	C+SB+R	60	+	+	+	C+SB+R	55	+	+	+
Margara N_5K	C+SB+R	50	+	++	+	C+SB+R	20	+	+	+
Margara $N_{45}K$	C+SB+R	60	++	++	+	C+SB	45	+	+	-
B_5	C+SB	65	+	++	-	C+SB	55	+	+	-

* : Medium supplemented with $0.5mgl^{-1}$ NAA + $0.5mgl^{-1}$ BA.
NR : Nature of response; %R : Percentage of response; SB : Shoot buds; C : Callus; R : Roots.

- : Nil; + : Low; ++ : Moderate; +++ : High; ++++ : Intense.

cent of the cultures responded to the MS medium where as it was lower (50%) in case of Margara N_5K medium. Initiation of callus was observed on leaf discs tried on different media. The leaf discs which initiated callus later turned into callus mass which was green in colour and friable in nature. MS medium resulted in intense callusing of leaf discs whereas Margara $N_{45}K$ medium induced moderate callus. Slight callusing was recorded on leaf discs cultured on 1/2 strength MS, Margara N_5K and B_5 medium.

The differentiation of callus into adventitious shoot buds was recorded in all the media tried. However, MS medium resulted in higher intensity of differentiation into shoot buds which ultimately grew into plantlets. Margara N_5K, Margara $N_{45}K$ and B_5 media helped in moderate differentiation into shoot buds. Whereas a fewer shoot buds were differentiated from the callus of leaf discs cultured on 1/2 strength MS medium.

Root initiation from the clump of callus and shoot buds was observed in treatments MS, 1/2 MS, Margara N_5K and Margara $N_{45}K$ medium. B5 medium failed to initiate roots. MS medium induced moderate intensity of rooting. The shoot buds differentiated from callus on B_5 medium were very small and took longer time to grow into plantlets.

b. Petiole sections

Petiole explants responded to all the media tried with varieng percentage of response. The per cent response was higher in MS medium (70%) and lowest in Margara N_5K medium (20%).

Callus initiation and shoot bud differentiation were observed in all the treatments of different media. Callus proliferation was higher in MS medium and was very low in all other media. Similarly MS medium differentiated moderate number of shoot buds from callus of petiole sections, whereas all other treatments differentiated only few shoot buds. The rooting intensity was low in MS, 1/2 MS and Margara N_5K medium whereas Margara $N_{45}K$ and B_5 medium failed to induce rooting from callus and shoot bud clusters.

Leaf disc explants have given better results in callusing and its differentiation when compared to petiole sections.

It is evident from the Table 11 that Saintpaulia ionantha leaf discs and petiole sections rerspond well on MS medium. Hence, all the further studies of standardisation of growth regulators for callusing and differentiation were conducted on MS medium.

4.1.1.2 Effect of growth regulators on explants of Saintpaulia ionantha

The effect of different growth regulator concentrations and combinations were experimented to assess the morphogenic rersponses of petiole section and leaf disc explants and the results are presented in Table 12-14.

Effect of NAA and BA

a. Leaf disc explants

Leaf disc explants did not respond to either MS medium without any growth regulators or individual NAA and BA concentrations. Even higher levels of NAA (1 mgl^{-1}) with lower levels of BA (0.5 mgl^{-1}) and lower levels of NAA (0.5 mgl^{-1}) with higher levels of BA (1.0 mgl^{-1}) did not result any morphogenic response on leaf discs. However, equal concentrations of NAA : BA combinations (0.5:0.5, 1:1, 2:2) resulted in callus initiation, differentiation of shoot buds and root initiation.

The per cent explants responded was highest (80%) at NAA 0.5 mgl^{-1} + BA 0.5 mgl^{-1} combinations followed by NAA 1 mgl^{-1} + BA 1 mgl^{-1} combinations (50%).

Callusing and differentiation

Days taken for callus initiation varied from 23-34 days among the treatments. NAA 0.5 mgl^{-1} + BA 0.5 mgl^{-1}

Table 12. Response of Saintpaulia ionantha explants to NAA and BA concentrations and combinations during initiation of cultures.

Treatment (mgl^{-1})		LEAF DISCS									PETIOLE SECTIONS								
				Callus				Differerntiation					Callus				Differentiation		
		NR	%R	DI	EX	CC	NC	DD	SB	R	NR	%R	DI	EX	CC	NC	DD	SB	R
NAA	BA																		
0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	0.5	C+SB+R	80	28	++++	YG	F	62	32	++	C+SB+R	70	21	+++	G	F	68	26.5	+
0.5	1	-	-	-	-	-	-	-	-	-	C+SB	30	48	+	G	F	71.3	11	-
1	0.5	-	-	-	-	-	-	-	-	-	C+SB+R	30	42	+	YG	F	77	8	++
1	1	C+SB+R	60	34	++	B	F	60.5	13.5	+	C+SB+R	40	25	+	G	F	67	15	+
2	2	C+SB+R	40	23	+	B	F	57	15.5	+	C+SB+R	40	21.5	+	G	F	59	10	+

NR : Nature of response; %R : Percentage of response; DI : Days taken for initiation; EX : Extent of callusing;
CC : Colour of callus: YG : Yellowish green; G : Green; B : Brown; NC : Nature of callus; F : Friable;
DD : Days taken for differentiation; SB : Shoot buds; R : Roots;

- : Nil; + : Low; ++ : Moderate; +++ : High; ++++ : Intense.

Fig. 6. Saintpaulia ionantha : Leaf discs callusing on MS medium enriched with NAA (0.5 mgl^{-1}) + BA (0.5 mgl^{-1})

Fig. 7. Saintpaulia ionantha : Differentiation of shoot buds from leaf callus

Fig. 8. Saintpaulia ionantha : Differentiated shoot buds developing into plantlets

combination resulted in initiation of callus from 28 days (Fig.6) induced yellowish green friable callus. The proliferation of callus was intense. As the concentrations of NAA + BA increased there was an inverse relation in extent of callusing and the callus proliferated was brownish in colour and friable in nature.

The differentiation of callus was recorded after 62 days of culture in the case of NAA 0.5 mgl^{-1} + BA 0.5 mgl^{-1} (Fig.7). Whereas, in higher combinations of NAA + BA the time taken for differentiation was less. Initially there was a production of few hairy like rhizoids on callus mass and then leading to differentiation into adventitious shoot buds. NAA at 0.5 mgl^{-1} + BA at 0.5 mgl^{-1} resulted in highest (32) number of shoot buds differentiation, whereas at higher NAA + BA combinations there was a lesser degree of differentiation. From the base of the callus and shoot bud cluster the root initiation was observed which was moderate in the case of treatment combination of 0.5 mgl^{-1} NAA + 0.5 mgl^{-1} BA (Fig.8). Whereas, at higher concentrations of NAA + BA the root intensity was low.

b. Petiole sections

Petiole sections did not respond to MS basal medium having no growth regulators and also to individual NAA and BA concentrations. However, all the combinations

of NAA and BA has resulted in organogenetic response with a varied degree of success. The per cent response was highest (70%) in combination NAA 0.5 mgl^{-1} + BA 0.5 mgl^{-1}.

Callusing and differentiation

Callus initiation started after 21 days of culture in the case of petiole sections cultured on MS medium supplemented with NAA 0.5 mgl^{-1} + BA 0.5 mgl^{-1} (Fig.9). Higher concentration of NAA (1 mgl^{-1}) with lower concentration of BA (0.5 mgl^{-1}) and lower concentration of NAA (0.5 mgl^{-1}) with higher concentration of NAA (0.5 mgl^{-1}) with higher concentrations of BA (1.0 mgl^{-1}) took longer time for callus initiation. In all the treatments the colour of the callus was yellowish green to green which was friable in nature. There was a high degree of proliferation of callus at 0.5 mgl^{-1} NAA + 0.5 mgl^{-1} BA. Whereas, other NAA and BA combinations resulted in lower amount of callusing.

The friable mass of callus differentiated in to adventitious shoot buds after 68 days of culture in the case of NAA 0.5 mgl^{-1} + BA 0.5 mgl^{-1}. At higher combination of NAA and BA (2 mgl^{-1} each) the time taken for differentiation was low (59 days), whereas it was 77 days for treatment NAA 1 mgl^{-1} + BA 0.5 mgl^{-1}. NAA (0.5 mgl^{-1}) + BA (0.5 mgl^{-1}) combination resulted in differentiation into 26.5 adventitious shoot buds and

Fig. 9. *Saintpaulia ionantha* : Petiole section callusing on MS medium enriched with NAA ($0.5 mgl^{-1}$) + BA ($0.5 mgl^{-1}$)

Fig. 10. *Saintpaulia ionantha* : Nodular callus and roots from petiole explant cultured on MS medium supplemented with NAA ($1 mgl^{-1}$) + kinetin ($1 mgl^{-1}$)

lower intensity of rooting. At NAA 1 mgl^{-1} + BA 0.5 mg

combinations, though there was reduction in differ tiation of shoot buds (8) the root intensity was modera whereas at 0.5 mgl^{-1} NAA + 1.0 mgl^{-1} BA there was no r initiation.

When compared to leaf discs, the petiole sectic initiated callus at an earlier day, but differentiati took more time and the resulted shoot buds were lower number.

Effect of NAA and kinetin

Leaf disc and petiole section explants did n respond to the kinetin at 1 mgl^{-1} concentration. Where both the explants responded to NAA and kinetin different combinations (Table 13). NAA 0.5 mgl^{-1} + 0.5 mgl^{-1} initiated callus from explants at an earlier da when compared to other treatments. At this combinatic the callus initiated was yellowish green in colour a later turned to dark brown with nodular type of call (Fig.10). Though the intensity of callus was higher, failed to differentiate. These dark brown nodular call initiated only hairy rhizoid like organs and afte continuous culture there were formation of roots in som cultures and ultimately these cultures became senescent Similarly,all the NAA + kinetin combinations tried on bot

Table 13. Response of Saintpaulia ionantha explants to NAA and kinetin combinations during initiation of cultures.

Treatment (mgl^{-1})		LEAF DISCS									PETIOLE SECTIONS								
				Callus				Differentiation					Callus					Differentiation		
NAA	Kn	NR	%R	DI	EX	CC	NC	DD	SB	R	NR	%R	DI	EX	CC	NC	DD	SB	R
0	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	0.5	C+Rh	60	27	+++	DB	N	-	-	++[a]	C+Rh	60	18	++	DB	N	-	-	+[a]
0.5	2	C	50	38	++	DB	N	-	-	-	C	30	34	+	DB	N	-	-	-
1	1	C+Rh	70	31	++	DB	N	-	-	-	C+Rh	75	24	+++	DB	N	-	-	++
2	2	C+Rh	60	32	+	DB	N	-	-	-	C+Rh	40	25	++	DB	N	-	-	-

a : Many rhizoids were formed from the callus which led to the development of roots after continued cultures.
NR : Nature of response; %R : Percentage of response; DI : Days taken for initiation; EX : Extent of callusing;
CC : Colour of callus: DB : Dark brown; NC : Nature of callus;
DD : Days taken for differentiation; SB : Shoot buds; R : Roots; Rh : Rhizoids.

- : Nil; + : Low; ++ : Moderate; +++ : High; ++++ : Intense.

the explants resulted only in dark brown nodular callus which failed to differentiate into shoot buds.

Effect of IBA and BA

a. Leaf disc explants

Leaf disc explants responded to different IBA and BA combinations. 70 per cent of the cultures responded to IBA and BA combination at 0.5 mgl^{-1} (Table 14).

Callusing and differentiation

Treatment combination of IBA 1 mgl^{-1} + BA 1 mgl^{-1} resulted in moderate callusing. Treatment combinations of 0.5 mgl^{-1} IBA + 0.5 mgl^{-1} BA and 1 mgl^{-1} IBA + 1 mgl^{-1} BA resulted in slight callusing. The callus obtained was green in colour and friable in nature. The initiation of callus and diffeerentiations of callus was earlier (30 and 68 days respectively) in combinations of IBA and BA at 2 mgl^{-1}. There was not much variation in differentiation of callus into shoot buds among the treatments. The highest number of shoot buds (16) were resulted from IBA and BA at 1 mgl^{-1} combination. In all the treatments root initiation was observed with a lower intensity.

b. Petiole sections

Response of petiole explants was higher (60%) in treatment combination of IBA 0.5 mgl^{-1} + BA 0.5 mgl^{-1} and IBA 1 mgl^{-1} + BA 1 mgl^{-1}. Petiole sections induced

Table 14. Response of Saintpaulia ionantha explants to IBA and BA combinations during initiation of cultures.

Treatment (mgl^{-1})		LEAF DISCS									PETIOLE SECTIONS								
				Callus				Differentiation					Callus				Differentiation		
		NR	%R	DI	EX	CC	NC	DD	SB	R	NR	%R	DI	EX	CC	NC	DD	SB	R
IBA	BA																		
0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	0.5	C+SB+R	70	32	+	G	F	74	12	+	C+SB+R	60	30	+	G	F	76.5	8.5	+
1	1	C+SB+R	65	35	++	G	F	73.5	16	+	C+SB+R	60	31	+	G	F	74	14	+
2	2	C+SB+R	40	30	+	G	F	64	13.5	+	C+SB+R	30	32	+	G	F	66	6.5	+

NR : Nature of response; %R : Percentage of response; DI : Days taken for initiation; EX : Extent of callusing;
CC : Colour of callus; G : Green; NC : Nature of callus; F : Friable; DD : Days taken for differentiation;
SB : Shoot buds; R : Roots;

- : Nil; + : Low; ++ : Moderate; +++ : High; ++++ : Intense.

callus, but the intensity of callus was lower in all the treatment combinations.

The maximum number of shoot buds (14) was differentiated from the callus of petiole explants cultured on MS medium supplemented with IBA 1 mgl^{-1} + BA 1 mgl^{-1}. The root initiation was recorded in all the treatment combinations but the intensity was low.

4.1.1.3 Recurrent culture

The axenic leaves obtained from in vitro grown plants which need no sterilizing treatments, were used as explants during recurrent cultures. The results obtained during recurrent cultures of axenic leaf explants cultured on MS medium supplemented with various concentrations and combinations of growth regulator are summarised in Tables 15-17.

Response of axenic leaf explants to NAA and BA

Axenic leaf explants failed to show any response to MS basal medium without any growth regulators (Table 15). BA at concentrations from 0.5 mgl^{-1} to 3 mgl^{-1} responded resulting in direct morphogenesis without intervention of callus (Figs.11-12). However, at higher concentrations of BA (4 mgl^{-1}) it failed to respond. At 0.5 mgl^{-1} BA the shoot bud differentiation started on 25th day resulting in 24.5 shoot buds per culture which lead to

Table 15. Response of axenic leaf explants of Saintpaulia ionantha to NAA and BA concentrations and combinations during recurrent cultures.

Treatment (mgl^{-1})		Callus				Differentiation				
		NR	DI	EX	CC	NC	ND	DD	SB	PL	R
NAA	BA										
0	0	-	-	-	-	-	-	-	-	-	-
0	0.5	SB	-	-	-	-	DM	25	24.5	13	-
0	1	SB	-	-	-	-	DM	23	18	8	-
0	2	SB	-	-	-	-	DM	21	14	*	-
0	3	SB	-	-	-	-	DM	32	8	*	-
0	4	-	-	-	-	-	-	-	-	-	-
0.5	0	-	-	-	-	-	-	-	-	-	-
1	0	C+SB+R	33	+	B	F	IM,DM	41	7	2	++
3	0	-	-	-	-	-	-	-	-	-	-
0.5	0.5	C+SB+R	31	++	G	F	IM,DM	38	36	21	++
1	1	C+SB+R	23	+	G	F	IM,DM	28	20.5	11	+
1	2	-	-	-	-	-	-	-	-	-	-
1	4	-	-	-	-	-	-	-	-	-	-
2	1	C	30	+	B	N	No differentiation				
2	2	C+SB	31	++	B	F	IM	52	8	*	-
2	4	C	33	++	B	N	No differentiation				
4	1	-	-	-	-	-	-	-	-	-	-
4	4	-	-	-	-	-	-	-	-	-	-

* - No further growth of shoot buds after initiation of 2 weeks.
NR - Nature of response; DI-Days taken for initiation;
EX - Extent of calluaing; CC-Colour of callus; NC-Nature of callus;
ND - Nature of differentiation; DD-Days taken for differentiation;
SB - Shoot buds; PL-Plantlets; R-Roots; G-Green; B-Brown
- : Nil; +: Low; ++ : Moderate; +++: High; ++++: Intense.

the formation of 13 plantlets after 8 weeks of culture. At higher concentrations of BA (2 mgl^{-1} and 3 mgl^{-1}) there was a reduction in shoot bud differentiation and also these shoot buds failed to grow after 2 weeks of initiation. All the individual BA treatments which responded to differentiation of shoot buds failed to initiate roots.

Individual concentrtations of NAA at 0.5 mgl^{-1} and at 3 mgl^{-1} failed to respond. However, 1 mgl^{-1} NAA resulted in slight callusing after 33 days of culturing. There were both direct and indirect morphogenesis resulting in 7 shoot buds.

Treatment combination having NAA and BA at 0.5 mgl^{-1} initiated greenish, friable callus from axenic leaf explants. The intensity of callus was moderate. These explants on same concentration initiated shoot buds directly on 38th day and subsequently there was a differentiation of callus into shoot buds. The shoot buds (36) which were differentiated both from callus and directly from explants resulted in formation of 21 planlets with moderate rooting from the base of the plantlet clusters.

NAA and BA at 2:1 and 2:4 combinations resulted in brownish nodular callus which failed to differentiate

Fig. 11. Saintpaulia ionantha : Production of adventitious shoot buds directly from axenic leaf explants cultured on MS medium supplemented with BA (0.5 mgl^{-1})

Fig. 12. Saintpaulia ionantha : Development of plantlets directly from axenic leaf cultured on MS medium enriched with BA (1.0 mgl^{-1})

Fig. 13. Saintpaulia ionantha : Axenic leaf explant inducing callus and shoot buds on MS_1 medium enriched with IBA (1 mgl^{-1}) + BA (1 mgl^{-1})

either indirerctly or directly. NAA 2 mgl^{-1} + BA 2 mgl^{-1} combination resulted in moderate callusing on explants and there after differentiated into shoot buds via callus. However, these shoot buds failed to grow into plantlets.

Response of axenic leaf explants to IBA and BA

The axenic leaf explants cultured on medium supplemented with either IBA alone (1 mgl^{-1}) or with various combinations of IBA and BA responded in callusing and differentiation into shoot buds through direct or indirect morphogenesis (Table 16). However, combinations of IBA 4 mgl^{-1} + BA 1 mgl^{-1} failed to respond. The extent of callusing was low at lower concentrations of IBA (0.5 and 1 mgl^{-1}) combined with BA at 1-3 mgl^{-1}. As the NAA concentrations (2 mgl^{-1} and 3 mgl^{-1}) increased along with 1 mgl^{-1} BA there was moderate callusing. The initiation of callus started at one end of the explant and subsequently the shoot buds differentiated from explants directly followed by differentiation of callus. The maximum number of shoot buds (52) were rrecorded from the treatment of 1 mgl^{-1} IBA +1 mgl^{-1} BA (Fig.13) which ultimately resulted in formation of 27 plantlets. However, rooting was low in this treatment. IBA at 1 mgl^{-1} resulted in rooting from callus and plantlet clump at an higher intensity.

Table 16. Response of axenic leaf explants of Saintpaulia ionantha to IBA and BA combinations during recurrent culture.

Treatment (mgl^{-1})			Callus				Differentiation				
		NR	DI	EX	CC	NC	ND	DD	SB	PL	R
IBA	BA										
1	0	C+SB+R	32	+	G	F	IM,DM	36	15	4	+++
0.5	0.5	C+SB+R	30	+	G	F	IM,DM	32.5	12	5	+
1	1	C+SB+R	26	+	G	F	IM,DM	28	52	27	+
1	2	C+SB+R	34	+	G	F	IM,DM	36	32.5	18	+
1	3	C+SB	30.5	+	G	F	IM,DM	32	31*	-	-
2	1	C+SB+R	34.5	++	B	F	IM,DM	38.5	36.5	17	++
3	1	C+SB+R	35	++	B	F	IM,DM	38	15.5	7	++
4	1	-	-	-	-	-	-	-	-	-	-

* : No further growth of shoot buds after initiation of 2 weeks.
NR : Nature of response; DI: Days taken for initiation;
EX : Extent of callusing; CC: Colour of callus; NC: Nature of callus;
ND : Nature of differentiation; DD : Days taken for differentiation;
SB : Shoot buds; PL: Plantlets; R: Roots; G: Green; B: Brown;
F : Friable; IM: Indirect morphogenesis; DM: Direct morphogenesis

-: Nil; +: Low; ++ : Moderate; +++: High; ++++: Intense.

Table 17. Response of axenic leaf explants of Saintpaulia ionantha to 2,4-D and BA combinations during recurrent culture.

Treatment (mgl^{-1})		Callus					Differentiation				
		NR	DI	EX	CC	NC	ND	DD	SB	PL	R
2,4-D	BA										
0.5	0	C	32	+	B	N	No differentiation				
0.5	0.5	C	31	+	B	N	No differentiation				
1	0	-	-	-	-	-	-	-	-	-	-
1	1	-	-	-	-	-	-	-	-	-	-

NR : Nature of response; DI: Days taken for initiation;
EX : Extent of callusing; CC: Colour of callus; NC: Nature of callus;
ND : Nature of differentiation; DD : Days taken for differentiation;
SB : Shoot buds; PL: Plantlets; R: Roots; B: Brown; N: Nodular

-: Nil; +: Low; ++ : Moderate; +++: High; ++++: Intense.

Response of axenic leaf explants to 2,4-D and BA

Axenic leaf explants responded to 2,4-D alone at lower (0.5 mgl^{-1}) concentration and to 2,4-D and BA at 0.5 mgl^{-1} combination. These callus resulted in formation of brownish nodular type of callus which ultimately failed to differentiate (Table 17).

4.1.1.4 Callus culture

A series of experiments were conducted using a combination of different growth regulators to determine the effect on callus proliferation and differentiation from green friable callus during callus culture.

Response of callus to NAA and BA

Effect of NAA and BA and their combinations on callus was investigated and the results are furnished in Table 18.

MS basal medium without any growth regulators failed to proliferate the callus, but there was a differentiation of callus resulting in lower number of shoot buds which ultimately developed into plantlets (4) after 6 weeks. This treatment induced intense rooting of plantlets. NAA alone at 0.5 mgl^{-1} resulted in proliferation of yellowish green friable callus to a lower extent which subsequently differentiated into shoot buds. These shoot buds developed to form 16.5 plantlets per

Table 18. Response of NAA concentrations and NAA + BA combinations on callus of Saintpaulia ionantha.

Treatment (mgl^{-1})			Callus			Differentiation		
		NR	EX	CC	NC	SB	R	PL
NAA	BA							
0	0	SB+R	-	-	-	+	++++	4
0.5	0	C+SB+R	+	YG	F	++	++	16.5
2	0	C	++	LB	N	-	-	-
0.5	0.5	C+SB+R	+++	G	F	++	++	12
1	0.5	C	++	LB	N	-	-	-
0.5	1	C+SB+R	++	G	F	+	++	7.5
1	1	C+SB+R	++	G	F	++	++	10.5
4	0.5	-	-	-	-	-	-	-

NR : Nature of response; EX : Extent of callusing; CC : Colour of callus;
NC : Nature of callus; SB : Shoot buds; R : Roots;
PL : Plantlets; YG : Yellow green; G : Green; LB : Light brown;
F : Friable; N : Nodular.

-: Nil; +: Low; ++: Moderate; +++: High; ++++: Intense.

culture with a moderate rooting. However, NAA at higher concentration (2 mgl^{-1}) proliferated light brown nodular callus which failed to differentiate. Similarly, NAA at 1 mgl^{-1} + BA 0.5 mgl^{-1} proliferated light brown nodular callus which became recalcitrant.

NAA 0.5 mgl^{-1} + BA 0.5 mgl^{-1} combination proliferted green friable callus to a greater extent (Fig.14), but differentiation was moderate leading to development of 12 plantlets per culture after 6 weeks.

Response of callus to IBA and BA

Effect of IBA and BA on callus was investigated and results are summerised in Table 19. BA at 1 mgl^{-1} failed to proliferate the callus but differentiated the callus into shoot buds to an higher extent resulting in 23 plantlets. But this treatment failed to induce rooting. IBA at 1 mgl^{-1} concentration proliferated green friable callus to a moderate extent but differentiation of shoot buds was low with higher intensity of rooting. Treatment involving IBA 1 mgl^{-1} + BA 1 mgl^{-1} proliferated callus resulting in higher extent of shoot bud differentiation and formation of 29 plantlets after 6 weeks of culturing. However, the root initiation was lower in this case. IBA at 2 mgl^{-1} + BA at 0.2 mgl^{-1} proliferated brown nodular callus which failed to differentiate into shoot buds.

Table 19. Response of BA concentrations and IBA + BA combinations on callus of Saintpaulia ionantha.

Treatment (mgl^{-1})			Callus			Differentiation		
		NR	EX	CC	NC	SB	R	PL
IBA	BA							
0	0.5	C+SB+R	+	YG	F	+++	+	21.5
0	1	SB	-	-	-	+++	-	23
1	0	C+SB+R	++	G	F	+	+++	3
0.5	0.5	C+SB+R	++	G	F	+++	+	18.6
1	1	C+SB+R	++	G	F	+++	+	29
2	0.2	C+Rh	++	B	N	-	-	-

NR : Nature of response; EX : Extent of callus; CC : Colour of callus;
NC : Nature of callus; SB : Shoot buds; R : Roots; PL : Plantlets;
YG : Yellowish green; G : Green; B : Brown; F : Friable;
N : Nodular.

-: Nil; +: Low; ++: Moderate; +++: High; ++++: Intense.

Response of callus to NAA and kinetin

The results of treatments of NAA and kinetin combinations on callus are furnished in Table 20. Combinations of NAA 0.5 mgl^{-1} + kinetin 0.5 mgl^{-1}, NAA 1 mgl^{-1} + kinetin 2 mgl^{-1} and NAA 2 mgl^{-1} + kinetin 0.2 mgl^{-1} proliferated callus to a lower extent resulting in brownish nodular callus which failed to differentiate. There was a initiation of hairy rhizids to a lower extent which did not grow further. However, lower concentration of NAA (0.2 mgl^{-1}) with higher concentration of kinetin (2 mgl^{-1}) resulted in proliferation of brownish friable callus which differentiated into shoot buds giving 3 plantlets. This treatment did not initiate roots.

Response of IBA and kinetin

The results of the experiment on the effect of IBA and kinetin on callus are presented in Table 21.

Kinetin alone at 1 mgl^{-1} and higher concentration of kinetin (2 mgl^{-1}) with lower concentrations of IBA (1.0 mgl^{-1}) resulted in proliferation of greenish friable callus leading to differentiation. Kinetin at 1 mgl^{-1} resulted in formation of 11.5 plantlets per culture but failed to initiate roots. IBA and kinetin at 0.5 mgl^{-1} + 0.5 mgl^{-1} and 1.0 mgl^{-1} + 1.0 mgl^{-1} combinations resulted in proliferation of brownish nodular callus which ultimately failed to differentiate.

Table 20. Response of NAA and kinetin combinations on callus of Saintpaulia ionantha.

Treatment (mgl^{-1})			Callus			Differentiation		
		NR	EX	CC	NC	SB	R	PL
NAA	Kn							
0.5	0.5	C	+	B	N	-	-	-
1	1	C	+	B	N	-	-	-
2	0.2	C	+	B	N	-	-	-
0.2	2	C+SB	+	B	F	+	-	3

NR : Nature of response; EX : Extent of callus; CC : Colour of callus;
NC : Nature of callus; SB : Shoot buds; R : Roots;
PL : Plantlets; B : Brown; N: Nodular; F : Friable.

-: Nil; +: Low; ++: Moderate; +++: High; ++++:

Table 21. Response of kinetin and IBA + kinetin combinations on callus of Saintpaulia ionantha.

Treatment (mgl^{-1})			Callus			Differentiation		
		NR	EX	CC	NC	SB	R	PL
IBA	Kn							
0	1	C+SB	+	G	F	++	-	11.5
0.5	0.5	C	+	B	N	-	-	-
1	1	C	++	B	N	-	-	-
1	2	C+SB+R	+	G	F	+	+	8

NR : Nature of response; EX : Extent of callus; CC : Colour of callus;
NC : Nature of callus; SB : Shoot buds; R : Roots;
PL : Plantlets; G : Green; B : Brown; N: Nodular; F : Friable.

-: Nil; +: Low; ++: Moderate; +++: High; ++++:

ig. 14. Saintpaulia ionantha : Proliferation of callus and differentiation from callus mass cultured on MS medium enriched with NAA (0.5 mgl^{-1}) + BA (0.5 mgl^{-1})

ig. 15. Saintpaulia ionantha : Multiplication of plantlets from axenic plantlet during subculturing (MS + IBA 1 mgl^{-1} + BA 1 mgl^{-1})

In another experiment tried on callus, various 2,4-D and BA combinations failed to respond for further proliferation or differentiation. After 2 weeks of culturing, the callus turned dark and became senescent.

The brownish nodular callus which was obtained in the initial culture failed to either proliferate or differentiate when cultured on MS medium having different concentrations of growth regulators and their combnations.

4.1.1.5 Subculturing of plantlets for multiplication during recurrent cultures

The axenic plantlets which were produced in vitro were subcultured on MS medium supplemented with various growth regulator combinations for further multiplication.

Effect of NAA and BA on axenic plantlets during subculturing

The effect of NAA and BA concentrations and combinations was investigated to know the potential treatments which can support a rapid rate of micropropagation (Table 22).

The plantlet subcultured on MS basal medium without any growth regulators did not result in multiplication of plantlet. But there was a better growth of the plantlet leading to initiation of higher intensity of roots per plant.

Plantlets subcultured on medium having BA at 0.5 mgl^{-1} and 1 mgl^{-1} did not induce callusing at the base, but there was induction of shoot buds on leaf lamina and at axils of leaf resulting in production of many plantlets. BA at 1 mgl^{-1} concentration induced 45 plantlets at the end of 10 weeks without any root initiation.

Combinations of NAA and BA at various concentrations induced callusing at base of the plantlet and there was multiplication of adventitious shoot buds mainly from leaf lamina or from axils of leaf. The callus produced at the base also differentiated into shoot buds, but intensity of such differentiation was very low. NAA 1 mgl^{-1} + BA 1 mgl^{-1} combination resulted in proliferation of shoot buds at a very high leading to development of 48.5 plantlets per culture. The differentiation was through direct and indirect morphogenesis. However, the extent callus mediated differentiation was low. These plantlets initiated only few roots. NAA at higher concentration (2 mgl^{-1}) with BA at 0.5 mgl^{-1} and 2 mgl^{-1} resulted in proliferation of callus to a greater extent. The whole plantlet in this case converting into brownish nodular callus which failed to differentiate.

NAA at 4 mgl^{-1} + BA 0.5 mgl^{-1} resulted in lower extent of brownish nodular callus at base and the plantlet became malformed without any further differentiation.

Table 22. Effect of NAA and BA concentrations and combinations on *Saintpaulia ionantha* axenic plantlets for multiplication during subculturing.

Treatment (mgl^{-1})			Callus			Differentiation		
		NR	EX	CC	NC	ND	PL	R
NAA	BA							
0	0	R	-	-	-	-	1	+++
0	0.5	PL+R	-	-	-	DM	28	+
0	1	PL	-	-	-	DM	45	-
1	0	C+PL+R	+	G	F	IM,DM	5	++
0.5	0.5	C+PL+R	+	G	F	IM,DM	28	+
1	1	C+PL+R	+	G	F	IM,DM	48.5	++
2	0.5	C	+++	B	N	No differentiation		
2	2	C	+++	B	N	No differentiation		
4	0.5*	C	+	B	N	No differentiation		

* : Plantlet became malformed.

NR : Nature of response; EX : Extent of callus; CC : Colour of callus;
NC : Nature of callus; ND : Nature of differentiation; R : Roots;
PL : Plantlets; G : Green; B : Brown; N: Nodular; F : Friable;
DM : Direct morphogenesis; IM : Indirect morphogenesis.

-: Nil; +: Low; ++: Moderate; +++: High; ++++:

Effect of IBA and BA on axenic plantlets during subculturing

Plantlets subcultured on medium containing IBA 1 to 3 mgl^{-1} initiated greenish friable callus at the base of the plantlet and there was multiplications of plantlets both directly and indirectly (Table 23). These plantlets induced moderate rooting. However, these plantlets were smaller in size. Similarly, various combinations of IBA and BA intitiated callus at base and differentiated shoot buds both directly and indirectly via callus. However, the intensity of differentiation of callus was low when compared to direct differentiation from plantlet. The root initiation was observed in all these combinations but with a lower intensity. Combination of IBA 1 mgl^{-1} + BA 1 mgl^{-1} resulted in production of maximum number of plantlets (52.5) per culture with a lower intensity of rooting (Fig.15).

Effect of NAA and kinetin on axenic plantlets during subculturing

Kinetin at 0.5 and 1.0 mgl^{-1} did not initiate callus, but formation of few plantlets with a low intensity of rooting was observed. Kinetin at 1.0 mgl^{-1} induced 12.5 plantlets per culture after 10 weeks of subculturing (Table 24). NAA 0.5 mgl^{-1} + BA 0.5 mgl^{-1} and NAA 1.0 mgl^{-1} + BA 1.0 mgl^{-1} combinations resulted in initiation of brownish nodular callus at base of the

Table 23. Effect of IBA concentrations and IBA + BA combinations on *Saintpaulia ionantha* axenic plantlets for multiplication during subculturing.

Treatment (mgl^{-1})			Callus			Differentiation		
		NR	EX	CC	NC	ND	PL	R
IBA	BA							
1	0	C+PL+R	+	G	F	IM,DM	8	++
2	0	C+PL+R	+	G	F	IM,DM	7.5	++
4	0	C+PL+R	+	G	F	IM,DM	9.5*	+++
0.5	0.5	C+PL+R	++	G	F	IM,DM	28	+
0.5	1	C+PL+R	+	G	F	IM,DM	32	+
1	0.5	C+PL+R	+	G	F	IM,DM	28.5	+
1	1	C+PL+R	+	G	F	IM,DM	52.5	+
2	0.5	C+PL+R	+	G	F	IM,DM	31.5	+
2	1	C+PL+R	+	G	F	IM,DM	38.5	+
4	1	C+PL+R	++	G	F	IM,DM	18.5	++

* : Plantlets became malformed.

NR : Nature of response; EX : Extent of callus; CC : Colour of callus;
NC : Nature of callus; SB : Shoot buds; Rh : Rhizoids; R : Roots;
PL : Plantlets; G : Green; F : Friable; DM: Direct morphogenesis;
IM : Indirect morphogenesis.
-: Nil; +: Low; ++: Moderate; +++: High; ++++:

Table 24. Effect of kinetin concentrations and NAA + kinetin combinations on *Saintpaulia ionantha* axenic plantlets for multiplication during subculturing.

Treatment (mgl^{-1})			Callus			Differentiation		
		NR	EX	CC	NC	ND	PL	R
NAA	Kn							
0	0.5	PL+R	-	-	-	DM	6.5	+
0	1	PL+R	-	-	-	DM	12.5	+
0.5	0.5	C+PL	+	B	N	DM	6	+
1	1	C+PL+R	+	B	N	DM	11.5	+
2	2*	-	-	-	-	-	-	-
2	0.5*	-	-	-	-	-	-	-

* : Plantlets became malformed.

NR : Nature of response; EX : Extent of callus; CC : Colour of callus;
NC : Nature of callus; SB : Shoot buds; R : Roots;
PL : Plantlets; B : Brown; N : Nodular; DM : Direct morphogenesis.

-: Nil; +: Low; ++: Moderate; +++: High; ++++:

plantlet which failed to differentiate. But the shoot buds formed directly on plantlet leading to formation of multiple plantlets. Treatment combinations of 2 mgl^{-1} NAA + 2 mgl^{-1} kinetin and 2 mgl^{-1} NAA + 0.5 mgl^{-1} kinetin did not respond to further proliferation. The plantlet that was subcultured became malformed ultimately showing no growth of the plantlet.

Effect of IBA and kinetin on axenic plantlets during subculturing

All the IBA and kinetin combinations responded resulting in callusing at base and multiplication of plantlets. Combination of IBA 1 mgl^{-1} + kinetin 1 mgl^{-1} induced brownish nodular callus which failed to differentiate (Table 25). However, the plantlet directly differentiated adventitious shoot buds leading to formation of 18 plantlets with moderate rooting. Treatment combination of IBA 1 mgl^{-1} and kinetin 2 mgl^{-1} resulted in friable callus at base. The direct and indirect morphogenesis was observed resulting in 24.5 plantlets. These plantlets were malformed and few varigated types were observed.

The multiple plantlets obtained from various combinations of growth regulator were smaller in size with a low intensity of rooting. Hence, these plantlets were further subcultured in growth regulator free MS medium for rooting and growth of the plantlets.

Table 25. Effect of IBA and kinetin combinations on Saintpaulia ionantha axenic plantlets for multiplication during subculturing.

Treatment (mgl^{-1})			Callus			Differentiation		
		NR	EX	CC	NC	ND	PL	R
IBA	Kn							
0.5	0.5	C+PL	+	G	F	IM,DM	8.5	-
1	1	C+PL+R	++	B	N	DM	18	++
1	2	C+PL+R	+	B	F	IM,DM	24.5*	+
1	3	C+PL+R	+	G	F	IM,DM	6*	+

* : Plantlets became malformed and some variegated plants were observed

NR : Nature of response; EX : Extent of callus; CC : Colour of callus;
NC : Nature of callus; SB : Shoot buds; R : Roots;
PL : Plantlets; G : Green; B : Brown; F : Friable; N : Nodular.
IM : Indirect morphogenesis; DM : Direct morphogenesis.

-: Nil; +: Low; ++: Moderate; +++: High; ++++:

Multiplication rate of Saintpaulia ionantha plantlets at continuous subculturing

The results of the studies conducted to assess the rate of multiplication of the plantlets on continuous subculturing at 10 weeks interval upto 8 passages are presented in Table 26.

The multiplication medium used in this study contained MS with IBA 1 mgl^{-1} + BA 1 mgl^{-1}, which was found to be optimum for rapid multiple plantlet production from earlier studies were adopted here.

The third subculture recorded the maximum multiplication rate resulting in 61.5 plantlets per culture. Though there were little variations in multiplication rate at each serial subculture the axenic plantlets did not lose the capacity to regenerate till the last subculture tried (8th subculture). On an average the multiplication rate was 51.56 plantlets per culture.

Variegation and albino type of plantlets

Some of the plantlets on a continued cultures showed albino (Fig.16) or variegated leaves. The extent of this type of plantlets was very low. When these varigated leaves were cultured on MS medium supplemented with NAA and BA at 1 mgl^{-1} there was a proliferation of callus (Fig.17) and differentiation (Fig.18). However,

Fig. 16. Saintpaulia ionantha : Albino type of plantlets from continuous in vitro cultures

Fig. 17. Saintpaulia ionantha : Variegated leaves inducing callus

Fig. 18. Saintpaulia ionantha : Differentiation of shoot buds from variegated leaves

Table 26. Influence of continuous subcultering at 10 week interval on the multiplication rate of plantlets of Saintpaulia ionantha

Medium : MS + IBA $1mgl^{-1}$ + BA $1mgl^{-1}$

Serial subculture number	Number of plantlets/ culture
Control (MS*)	1
I	52.5
II	48.5
III	61.5
IV	53.0
V	45.5
VI	48.0
VII	54.5
VIII	49.0
Mean	51.56

MS* - Basal medium without any growth regulators

the regenerated plantlets did not show any variegation. The albino leaves when cultured failed to respond.

4.1.1.6 Response of growth regulators on *in vitro* rooting and plant growth

In an effort to induce good rooting the micro-propagated plantlets were subcultured on MS basal medium without any growth regulator (Fig.19) and MS with different IBA and NAA concentrations. The data were recorded after 6 weeks of subculture and are presented in Table 27.

The plantlets subcultured on growth regulator free MS basal medium, took longer time (26.5 days) to initiate rooting when compared to MS with IBA or NAA concentrations. However, MS basal medium induced higher intensity of rooting (Fig.20) and there was no further multiplication of plantlets. The plantlet which was subcultured had a good growth. MS with different concentrations of IBA and NAA resulted in moderate rooting with slight callus at the base and there was further multiplications of plantlets. The plantlets obtained from these treatments were small and roots per plantlets was low.

Table 27. Effect of growth regulators on *in vitro* rooting and plant growth of *Sapintpaulia ionantha* plantlets.

Treatments	Days taken for root initiation	Root intensity after 6 weeks of culture	Number of Plantlets	Growth of Plantlets
MS*	26.50	+ + +	1	+ + +
MS + IBA $1mgl^{-1}$	15.33	+ +	8.0	+
MS + IBA $2mgl^{-1}$	14.50	+ +	7.5	+
MS + NAA $1mgl^{-1}$	18.50	+ +	5.33	+
MS + NAA $2mgl^{-1}$	17.00	+ +	4.5	+

MS* - MS basal medium without any growth regulators

Fig. 19. Saintpaulia ionantha : Plantlet subcultured on MS basal medium for rooting

Fig. 20. Saintpaulia ionantha : Plantlet showing intense rooting on MS basal medium

Fig. 21. Saintpaulia ionantha : In vitro raised plant in pot containing mixture of soilrite + sand + compost (2:1:1 v/v)

4.1.1.7 Preparation and hardening of plantlets for establishment in pots

The data on influence of age of plantlets, maintenance of humidity and potting mixture on the survival of the plantlets were assessed and the results are presented in Table 28.

a. Age of plantlets

Age of the plantlet at the time of planting out was found to influence their survivality The plantlets without any roots viz., at root initiation stage did not survive in the pots even with adequate maintenance of humidity and right potting mixture. Delaying in planting out till three weeks after root initiation which had elongated roots has given better survival percentage (85%). However, the plantlets after four weeks of root initiation survived upto 80 per cent.

b. Maintenance of humidity

Maintenance of humidity during hardening of plantlets in pots viz., during the initial 3 weeks of planting out was found to influence the survivality. The plantlets after 3 weeks of root initiation were transferred to study the differnt humidity treatments. The plantlets which were transferred to pots kept open in green house with regular watering failed to survive. Covering the plantlets with a glass beaker with regular

Table 28. Influence of age of plantlets, maintenance of humidity and potting mixture on the survivality of the *in vitro* produced *Saintpaulia ionantha* plantlets.

Treatments	Survival %
A*a Age of the plantlets	
1. Root intiation (without any roots)	0
2. 2 Weeks after root initiation	65
3. 3 Weeks after root initiation	85
4. 4 Weeks after root initiation	80
B. Maintenance of humidity	
1. Keeping in the open (green house)	0
2. Covering with glass beaker	70
3. Covering with polythene cover	90
C* Potting mixture	
1. Sand	30
2. Sand + Soil (1:1 v/v)	55
3. Sand + Compost (1:1 v/v)	65
4. Soilrite + Sand + Compost (2:1:1 v/v)	85

* - Plantlets were covered with polythene cover for 3 weeks to maintain the humidity

a - Rooting was induced in MS basal medium.

watering to maintain the humidity resulted in 70 per cent survivability of plants. Polythene cover as a covering material to maintain humidity during hardening treatment was found to be most suitable with 90 per cent survival of plantlets. After three weeks, the humidity was reduced gradually to make the plants adapt to green house condition.

c. Potting mixture

The plantlets were transferred to community pots having different potting medium. The plantlets were covered with polythene covers to maintain the humidity. The most suitable medium was found to be mixture containing soilrite + sand + compost (2:1:1 v/v), which resulted in 85 per cent survival of the plantlets (Fig21). Sand + compost (1:1 v/v) medium recorded 65 per cent survivability of the plantlets. The least survivality (30%) was recorded in a potting medium of pure sand.

4.1.2 Begonia 'Lucerna'

The petiole, leaf and stem explants of Begonia 'Lucerna' were cultured on MS medium supplemented with different growth regulator concentrations and combinations for callusing and differentiation and the results are presented in Tables 29-32.

a. Petiole sections

Effect of NAA and BA

The petiole sections cultured on MS medium having NAA and BA concentrations and combinations initially swelled and were green for about 4 months. The swelling started at 3 weeks from the cut ends and only few treatments initiated morphogenesis (Table 29). MS basal medium without any growth regulators showed no response of callusing. NAA at lower concentrations (1 mgl^{-1}) resulted in slight callusing at one basal end of petiole sectors after 35 days of culture. Root initiation was recorded subsequentlly from the callus which elongated giving 3 roots and this culture failed to differentiate into shoot buds (Fig.22). This response was observed only in 10 per cent of the cultures. Higher concentrations of NAA (2 and 4 mgl^{-1}) did not influence petiole for callusing.

BA at 1, 2, 4 mgl^{-1} did not influence the petiole sections in organogenesis, but there was a slight swelling at one end of the petiole sections which ultimately turned dark and became senescent. BA at higher concentrations (5 mgl^{-1}) resulted in swelling and callusing of petiole after 32 days of culturing. The intensity of callus was low which was brownish in colour and compact in nature. This callus ultimately turned dark with globular head like callus mass which failed to differentiate.

Table 29. Response of Begonia 'Lucerna' petiole explants to NAA and BA concentrations and combinations.

Treatment (mgl^{-1})				Callus		Differentiation	
		NR	%R	EX	CC	PL	R
NAA	BA						
0	0	S	-	-	-	-	-
1	0	C+R	10	+	B	-	+
2	0	S	-	-	-	-	-
4	0	S	-	-	-	-	-
0	1	S	-	-	-	-	-
0	2	S	-	-	-	-	-
0	4	S	-	-	-	-	-
0	5	C	40	+	DB	-	-
1	1	S	-	-	-	-	-
1	2	C+PL+R	60	+++	G	18	+
1	5	C	40	+	DB	-	-
2	2	S	-	-	-	-	-
2	5	C+PL+R	40	+	G	6	+
4	1	S	-	-	-	-	-
4	4	S	-	-	-	-	-

NR : Nature of response; %R : Percentage of explants responded;
EX : Extent of callus; CC : Colour of callus; PL : Plantlets;
R : Roots; S : Swell; B: Brown; DB: Dark brown; G: Green

-: Nil; +: Low; ++: Moderate; +++: High; ++++ : Intense.

.g. 22. Begonia 'Lucerna' : Rooting from petiole explant cultured on MS medium enriched with NAA (1 mgl^{-1})

ig. 23. Begonia 'Lucerna' : Callus initiation from one end of petiole explant cultured on MS medium supplemented with NAA (1 mgl^{-1}) + BA (2 mgl^{-1})

ig. 24. Begonia 'Lucerna' : Proliferation of callus from petiole section cultured on MS medium + NAA (1 mgl^{-1}) + BA (2 mgl^{-1}), note light and dark colour callus

Among the differet combinations, NAA 1 mgl^{-1} + BA 2 mgl^{-1} and NAA 2 mgl^{-1} + BA 5 mgl^{-1} resulted in callusing and differentiation into shoot buds leading to formation of plantlets. However, combination of 1 mgl^{-1} NAA + 5 mgl^{-1} BA resulted only in dark brown compact callus which failed to differentiate. Combinations of NAA 1 mgl^{-1} + BA 2 mgl^{-1} responded to an extent of 60 per cent resulting in higher extent of callusing and the callus was green in colour. In this case, initially there was callusing at one end of petiole section (Fig.23) which was light green in colour later turning to dark green colour. The callusing was restricted to cut ends of petiole sections and differentiation was recorded only from dark green coloured callus (Fig.24). This treatment recorded maximum number of plantlets (18) with low intensity of rooting after 20 weeks of culture. These plantlets when subcultured on same medium there was a higher production of multiple plantlets(24) within a period of 10 weeks with moderate rooting (Fig.27).

Combinations of NAA 2 mgl^{-1} + BA 5 mgl^{-1} resulted in slight callusing which was green and friable which ultimately resulted in differentiation into 6 plantlets with low rooting.

Fig. 25. Begonia 'Lucerna' : Differentiation of shoot buds from callus of petiole explants cultured on MS medium + NAA (1 mgl^{-1}) + BA (2 mgl^{-1})

Fig. 26. Begonia 'Lucerna' : Differentiation of plantlets and roots from callus of petiole explants cultured on MS medium + NAA (1 mgl^{-1}) + BA (2 mgl^{-1})

Fig. 27. Begonia 'Lucerna' : Production of multiple plantlets from axenic plantlet when subcultured on MS medium + NAA (1 mgl^{-1}) + (2 mgl^{-1})

Table 30. Response of Begonia 'Lucerna' petiole explants to IBA and BA combinations.

Treatment (mgl^{-1})				Callus		Differentiation	
		NR	%R	EX	CC	PL	R
IBA	BA						
1	0	S	-	-	-	-	-
1	1	S	-	-	-	-	-
1	5	S	-	-	-	-	-
2	2	C+PL	40	+	G	7	-
2	5	S	-	-	-	-	-
3	3	S	-	-	-	-	-

NR : Nature of response; %R : Percentage of explants responded;
EX : Extent of callus; CC : Colour of callus; PL : Plantlets;
R : Roots; S : Swell; G : Green.

-: Nil; +: Low; ++: Moderate; +++: High; ++++ : Intense.

Table 31. Response of Begonia 'Lucerna' petiole explants to 2,4-D and BA concentrations and combinations.

Treatment (mgl^{-1})		NR	%R	Callus		Differentiation	
				EX	CC	PL	R
2,4-D	BA						
0	1	S	-	-	-	-	-
1	0	C	30	+	B	-	-
0.5	0.5	S	-	-	-	-	-
1	1	C	40	++	B	-	-
1	5	S	-	-	-	-	-
2	2	S	-	-	-	-	-
2	5	S	-	-	-	-	-

NR : Nature of response; %R : Percentage of explants responded;
EX : Extent of callus; CC : Colour of callus; PL : Plantlets;
R : Roots; S : Swell; B : Brown.

-: Nil; +: Low; ++: Moderate; +++: High; ++++ : Intense.

Effect of IBA and BA

Most of the IBA + BA combinations tried resulted in swelling of the petiole sections initially (Table 30) which later turned dark leading to senescence. However, combination of IBA 2 mgl^{-1} + BA 2 mgl^{-1} recorded 40 per cent response. In this treatment, petiole sections callused at one end of the petiole section which was green in colour. After 45 days of culturing this callus differrentiated into shoot buds forming 7 plantlets.

Effect of 2,4-D and BA

The petiole explants resulted in swelling at the cut ends after 3 weeks of culture in all the treatments (Table 31). However, 2,4-D alone at 1 mgl^{-1} and combination of 2,4-D 1 mgl^{-1} + BA 1 mgl^{-1} resulted in callusing of petiole sections at swollen end. 2,4-D at 1 mgl^{-1} resulted in 30 per cent response with a slight brownish, compact callus. At 2,4-D 1 mgl^{-1} + BA 1 mgl^{-1} combination there was a moderate callusing. Callus produced from both the treatments turned dark after 4 weeks of initiation without any differentiation.

b. Leaf sections

Effect of NAA and BA on leaf explants

The leaf sections with vein were cultured on MS medium supplemented with various concentrations and

combinations of NAA and BA for morphogenesis and results are furnished in Table 32.

All the individual concentrations of NAA and BA and MS basal medium failed to respond any results in leaf explants. However, combinations of 1 mgl^{-1} NAA + 1 mgl^{-1} BA, 1 mgl^{-1} NAA + 2 mgl^{-1} BA and 1 mgl^{-1} NAA + 5 mgl^{-1} BA resulted in callusing and differentiation. The callusing was restrictd to vein and rest of the lamina portion dried without any response. At 1 mgl^{-1} NAA + 1 mgl^{-1} BA combination, there was a slight callusing which was brownish in colour, resulting in differentiation of shoot buds which later developed into 2 plantlets. The root initiation was recorded in this culture with a lower intensity. Treatment combination of 1 mgl^{-1} NAA + 2 mgl^{-1} BA rsulted in slight callusing and differentiation leading to formation of 4 plantlets without any rooting. Combination of NAA 1 mgl^{-1} + BA 5 mgl^{-1} responded to an extent of 40 per cent resulting in moderate callusing and differentiation. This treatment resulted in formation of 11.5 plantlets per culture without any rooting.

The leaf sections tried on different concentrations and combinations of IBA + BA and 2,4-D BA did not respond for callusing and differentiation.

Table 32. Response of Begonia 'Lucerna' leaf explants to NAA and BA concentrations and combinations.

Treatment (mgl^{-1})		NR	%R	Callus		Differentiation	
				EX	CC	PL	R
NAA	BA						
0	0	-	-	-	-	-	-
1	0	-	-	-	-	-	-
2	0	-	-	-	-	-	-
0	1	-	-	-	-	-	-
0	2	-	-	-	-	-	-
1	1	C+PL+R	15	+	B	2	+
1	2	C+PL	30	+	B	4	-
1	5	C+PL	40	++	B	11.5	-
2	2	-	-	-	-	-	-
2	5	-	-	-	-	-	-

NR : Nature of response; %R : Percentage of explants responded;
EX : Extent of callus; CC : Colour of callus; PL : Plantlets;
R : Roots; S : Swell; B : Brown.

-: Nil; +: Low; ++: Moderate; +++: High; ++++ : Intense.

c. Stem segments

The stem segments which were cultured on MS medium supplemented with NAA and BA concentrations and combinations did not respond. However, there was a swelling at the basal cut end and hairy out growth was observed on the segments. These explants were green for 4-5 months and then turned dark without any callusing and differentiation.

4.1.3 Cymbidium aloifolium Sw.

Effect of liquid and solid medium of Vacin Went and MS on protocorms

The protocorms of 2 mm in diamter obtained from seed culture were used as initial material for culturing. These protocorms were cultured on liquid and solid medium of Vacin and Went and MS supplemented with different 2,4-D and BA concentrations to study the proliferation of protocorms like bodies and differentiation of shoots. The results obtained after 16 weeks of culture are presented in Table 33. The protocorms proliferated giving many protocorm like bodies (PLB) both from liquid as well as solid medium tried (Fig.28). The proliferation of PLB's and their differentiation into shoots were better in liquid medium when compared to solid medium.

Liquid Vacin and Went medium supplemented with 0.4 mgl^{-1} BA resulted in intense proliferation of PLB's

Table 33. Influence of medium and growth regulators on protocorms of *Cymbidium aloifolium*.

Treatment	Response	PLB	MSh	R
Liquid medium				
VW + 0.2mgl^{-1} 2,4-D	PLB+MSh	+	5	-
VW + 0.2mgl^{-1} 2,4-D+0.2mgl^{-1} BA	PLB+MSh	+++	26	-
VW + 0.4mgl^{-1} 2,4-D+0.4mgl^{-1} BA	PLB+MSh	++	16	-
VW + 0.4mgl^{-1} BA	PLB+MSh+R	++++	42.5	+
MS + 0.4mgl^{-1} BA	PLB+MSh	++	18	-
Solid medium				
VW + 0.2mgl^{-1} 2,4-D+0.2mgl^{-1} BA	PLB+MSh	+	3	-
VW + 0.4mgl^{-1} BA	PLB+MSh	++	10.5	-
MS + 0.2mgl^{-1} 2,4-D+0.2mgl^{-1} BA	PLB	++	-	-
MS + 0.4mgl^{-1} BA	PLB+MSh	+	4	-

VW : Vacin and Went medium; MS : Murashige and Skoog medium;
PLB : Protocorm like body; MSh : Multiple shoots; R : Roots.

-: Nil; + : Low; ++ : Moderate; +++ : High; ++++ : Intense.

Fig. 28. Cymbidium aloifolium : Proliferation of protocorm resulting in formation of PLB's

Fig. 29. Cymbidium aloifolium : Differentiation of shoots from PLB's

leading to differentiation of 42.5 multiple shoots per culture (Fig.30). However, the root intensity recorded was low. Liquid Vacin and Went medium supplemented with 0.2 mgl^{-1} 2,4-D + 0.2 mgl^{-1} BA resulted in higher proliferation of PLB's leading to differentiation of 26 multiple shoots without root formation. At higher concentration of 2,4-D and BA (Liquid vaccin and went + 0.4 mgl^{-1} 2,4-D + 0.4 mgl^{-1} BA) there was a decrease in PLB formation and their differentiation. Liquid Vacin and Went medium supplemented with only 2,4-D at 0.2 mgl^{-1} resulted in poor froliferation rate leading to differentiation of 5 multiple shoots.

Liquid MS supplemented with 0.4 mgl^{-1} BA resulted in moderate proliferation of PLB's leading to formation of only 18 multiple shoots.

Among the different solid media, Vaccin and Went supplemented with 0.4 mgl^{-1} BA resulted in moderate proliferation of protocorms giving 10.5 multiple shoots for culture. Whereas, MS + 0.4 mgl^{-1} BA resulted in 4 multiple shoots. Protocorm cultured on solid MS + 0.2 mgl^{-1} 2,4-D + 0.2 mgl^{-1} BA resulted in moderate multiplication of PLB's. These failed to differentiate even after 6 months of culture.

Effect of media and growth regulators on nodal segments obtained from axenic shoots

The nodes of about 1.5 cm having lateral buds obtained from shoots of in vitro cultures are cultured on liquid and solid medium to assess the multiplication of shoots and results are presented in Table 34.

Liquid Vacin and Went medium supplemented with 0.2 mgl^{-1} 2,4-D + 0.2 mgl^{-1} BA resulted in 20.5 multiple shoots at the end of 8 weeks of culture. Root initiation was recorded to a lower extent in this case. The maximum number of multiple shoots (32) was recorded from the cultures having liquid Vacin and Went medium + 0.4 mgl^{-1} BA.

In all the solid medium tried there was not much multiplication of shoots (Fig.31). MS basal medium and MS + IBA 1 mgl^{-1} did not induce any multiple shoots. However, the nodal segments grew and formed plantlets with good growth and moderate rooting.

Multiplication rates in nodal segments

The results of the studies conductd to assess the rate of multiplication of the shoots on continuous subculturing upto 6 passages at 8 weeks interval are presented in Table 35. The multiplication medium used in this study contained liquid Vacin and Went supplemented with 0.4 mgl^{-1} BA, which was found to be optimur for rapid

Table 34. Influence of medium and growth regulators on nodal segments of *Cymbidium aloifolium*.

Treatment	PLB	MSh	R
Liquid medium			
VW + 0.4mgl^{-1} BA	-	32	-
VW + 0.2mgl^{-1} 2,4-D + 0.2mgl^{-1} BA	-	20.5	+
Solid medium			
VW + 0.4 mgl^{-1} BA	-	8.5	-
MS basal	-	1	++
MS + 1mgl^{-1} NAA + 1mgl^{-1} BA	-	4	+
MS + 1mgl^{-1} IBA + 1mgl^{-1} BA	-	2	+
MS + 1mgl^{-1} IBA	-	1	++

VW : Vacin and Went medium; MS : Murashige and Skoog medium;
PLB : Protocorm like body; MSh : Multiple shoots; R : Roots.

-: Nil; + : Low; ++ : Moderate; +++ : High; ++++ : Intense.

Table 35. Influence of continuous subculturing at 8 weeks interval on the multiplication rate of *Cymbidium aloifolium* shoots.

Serial subculture number	Number of shoots per culture
I	32.0
II	28.5
III	36.5
IV	28.5
V	34.0
VI	30.5
Mean	30.66

Medium : Vacin and Went + 0.4 mgl^{-1} BA.

g. 30. Cymbidium aloifolium : Production of multiple shoots from protocorm cultured on liquid Vacin and Went medium with 0.4 mgl^{-1} BA

g. 31. Cymbidium aloifolium : Production of shoots from nodal segment cultured on solid medium

multiple shoot initiation from earlier studies were adopted here.

The number of multiple shoots obtained per culture after each subculture followed more or less same trend with a mean of 30.66 multiple shoots per each passage.

The multiple shoots obtained were subcultured on solid MS basal medium for shoot enlargement and rooting before transferring into pots. The plants transferred to potting mixture of soilrite + sand and + compost (2:1:1 v/v) with a hardening treatment of covering plants with glass beakers for 2 weeks resulted in good survival (90%) and establishment in the pots.

4.1.4 *Anthurium scherzerianum* Schott.

Effect of media and growth regulators on petiole and leaf explants

The petiole and leaf explants were cultured on Vacin and Went and modified Nitsch medium supplemented with various growth regulator combinations. These cultures were incubated in dark or light/dark (16 hrs/8 hrs) condition for obtaining callus. The results of response of leaf and petiole explants are presented in Table 36.

Leaf section with midrib cultured on solid Vacin and Went medium supplememnted with 2,4-D 1 mgl^{-1} + BA 1 mgl^{-1} and incubated in light/dark condition initiated

Table 36. Influence of media and growth regulators on leaf and petiole explants of Anthurium scherzerianum incubated in light/dark and dark conditions.

Treatments	Leaf sections				Petiole sections	
	NR	%R	EX	D	EX	D
*Light/dark incubation						
VW +2,4-D 1mgl^{-1} +BA 1mgl^{-1}	C	30	+++	-	-	-
VW +2,4-D 0.2mgl^{-1} +BA 0.2mgl^{-1}	-	-	-	-	-	-
VW +2,4-D 1mgl^{-1}	-	-	-	-	-	-
VW +BA 1mgl^{-1}	-	-	-	-	-	-
N_1 +2,4-D 0.1mgl^{-1} +BA 1mgl^{-1}	-	-	-	-	-	-
N_2 +2,4-D 0.1mgl^{-1} +BA 1mgl^{-1}	-	-	-	-	-	-
N_3 +2,4-D 0.1mgl^{-1} +BA 1mgl^{-1}	-	-	-	-	-	-
Dark incubation						
VW +2,4-D 0.2mgl^{-1} +BA 0.2mgl^{-1}	C	20	+	-	-	-
VW +2,4-D 1mgl^{-1} +BA 1mgl^{-1}	-	-	-	-	-	-
N_1 +2,4-D 0.1mgl^{-1} +BA 1mgl^{-1}	-	-	-	-	-	-
N_2 +2,4-D 0.1mgl^{-1} +BA 1mgl^{-1}	C	10	+	-	-	-
N_3 +2,4-D 0.1mgl^{-1} +BA 1mgl^{-1}	-	-	-	-	-	-

* : Cultures incubated in 16 hour light and 8 hour dark conditions.

VW : Vacin and Went medium.
N_1 : Nitsch medium with reduced ammonium nitrate (200mgl^{-1})
N_2 : Nitsch medium with reduced ammonium nitrate (500mgl^{-1})
N_3 : Nitsch medium with normal ammonium nitrate (720mgl^{-1})
NR : Nature of response; %R : Percentage of response;
EX : Extent of callusing; D : Differentiation.

. 32. Anthurium scherzerianum : Callusing of leaf section cultured on Vacin and Went medium with 2,4-D (1 mgl^{-1}) + BA (1 mgl^{-1})

callus after 72 days of culture. From midrib portion of the lamina higher intensity of callusing was observed which was geenish and globular. This callus continued to proliferate slowly without any differentiation (Fig.32). Leaf segment cultured on modified Nitsch medium and incubated in light/dark condition did not show any response.

Leaf explants cultured on Vacin and Went + 0.2 mg 2,4-D + 0.2 mg BA and modified Nitsch medium having lower ammonium nitrate (500 mg) in the medium + 1 mgl^{-1} BA and 0.1 mgl^{-1} 2,4-D) initiated callus under dark condition. The mid rib of the leaf lamina initiated dark callus at one end after 3 months of culture. After 2 months of callusing there was no response of differentiation leading to death of callus and explant.

The petiole sections cultured on different medium and incubation conditions did not result in callusing. However, there was little swell in some cases, ultimately leading to death of the explants after 3-4 months.

4.2 ENCAPSULATION

Encapsulation of protocorms of Cymbidium aloifolium and leaf sections, petiole sections, callus and shoot buds of Saintpaulia ionantha weretried using sodium alginate as a gelling agent to form beads.

Effect of various concentrations of sodium alginate

Sodium alginate (Sigma A 7128) at 2, 2.5, 3, and 4 per cent was used to encapsulate the plant material (Table 37). The best suited concentration was 2.5 per cent of sodium alginate prepared in MS basal medium. The beads formed at this concentration had a proper consistency and was found suitable for further studies. At 2 per cent sodium alginate, the beads formed were soft and were found unsuitable. Whereas, at higher concentrations (3 and 4%), beads formed around plant material were hard and found unsuitable for encapsulation.

Suitability of plant material for encapsultation

Cymbidium protocorms were found suitable (Fig.33) in forming beads and hence was chosen for the study (Table 38). In the case of Saintpaulia ionantha, petiole sections (Fig.35), callus (Fig.36) and axenic shoot buds (Fig.37) formed beads and were suitable for encapsulation. The leaf sections did not form proper beads because of flat nature of leaf sections and this material was not used for further studies.

Regeneration capacity

The encapsulated beads were tested for their regeneration capacity soon after encapsulation and also after different storage time at various temperatures.

Table 37. Effect of various concentrations of sodium alginate on the encapsulation of Cymbidium and Saintpaulia plant material.

Source	Concentration (% W/V)	Remarks
Sigma (A.7128)	2.0	Very soft beads, unsuitable for encapsulation.
	2.5	Proper consistency, suitable for encapsulation.
	3.0	Hard and beaked beads, unsuitable for encapsulation.
	4.0	Hard beads, unsuitable for encapsulation.

Table 38. Suitability of *Cymbidium* protocorms and different *Saintpaulia* plant material for encapsulation.

Species	Plant material	Suitability
Cymbidium aloifolium	Protocorms	Forms beads, suitable
Saintpaulia ionantha	Leaf segments	Does not form beads, unsuitable.
	Petiole sections	Forms beads, suitable
	Callus pieces	Forms beads, suitable
	Shoot buds	Forms beads, suitable

Fig. 33. Encapsulated beads of Cymbidium aloifolium protocorms

Fig. 34. Regeneration of encapsulated protocorms of Cymbidium aloifolium after storage of 10 weeks at 4°C

Regeneration capacity of encapsulatd and non-encapsulated protocorms of Cymbidium aloifolium

The non-encapsulated protocorms which were cultured before encapsulation on liquid Vacin and Went medium supplemented with 0.4 mgl^{-1} BA resulted in regeneration to an extnt of 100 per cent leading to formation of multiple shoots (Table 39). The non-encapsulated protocorms stored in different temperatures for different time did not show any regeneration capacity.

The encapsulated protocosm beads which were cultured on liquid Vacin and Went medium supplemented with 0.4 mgl^{-1} BA, soon after encapsulation resulted in 100 per cen regeneration. The protocorms which were encapsulated and stored in petridishes at 25°C temperature started emerging through the matrix of bead indicating 60 per cent regeneration capacity. However, after 35 days at 25°C the beads dried and the protocorms dessicated.

The encapsulated protocorms stored at 4°C temperature germinated well when cultured on medium (Fig.34). However, the regeneration capacity decreased to 20 per cent after 10 weeks of storage. There was a moderate regeneration (50%) at 8 weeks of storage. Encapsulated protocorms stored in 0°C temperature did not regenerate when cultured on appropriate medium. The

Table 39. Regeneration capacity of encapsulated and non encapsulated protocorms of Cymbidium aloifolium.

Treatment	Storage temperature (°C)	Storage time (weeks)						
		0	2	4	6	8	10	12
Encapsulated protocorms	NS	100						
	25		*					
	4		80	75	60	50	20	-
	0		-	-	-	-	-	-
Non encapsulated protocorms	NS	100						
	25/4/0**		-	-	-	-	-	-

NS : No storage; Protocorms were cultured before and after encapsulation without any storage.

* : Encapsulated protocorms stored at 25°C started emerging out of the beads after 30 days of storage.

** : Non encapsulated protocorms stored at 25/4/0°C temperature did not show regeneration capacity.

protocorms inside the beads were frozen at $0^{o}C$ temperature.

Regeneration capacity of non-encapsulated and encapsulated plant material of Saintpaulia ionantha

a. Petiole sections

The petiole sections from in vitro plantlets were cut into pieces and were encapsulated and their regeneration capacity after storage at different temperature were tried on solid MS medium supplemented with 1 mgl^{-1} IBA + 1 mgl^{-1} BA (Table 40).

The non-encapsulated petiole showed a regeneration capacity to an extent of 70 per cent when cultured on medium. The non-encapsulatd petiole sections stored in different temperatures failed to show any response.

The encapsulated petiole sections when cultured on medium soon after encapsulation responded to an extent of 65 per cent. The callusing was observed after 4 weeks of culture which burst open the beads and started proliferating in the medium. These callus ultimately differentiated giving many plantlets.

The encapsulated petole sections stored at $25^{o}C$ did not show any response either during storage or after culture. The beads stored at $25^{o}C$ started drying after 4 weeks due to dessication. Similarly, beads stored in $0^{o}C$

Table 40. Regeneration capacity of encapsulated and non encapsulated petiole sections of Saintpaulia ionantha.

Treatment	Storage temperature (^{o}C)	Storage time (weeks)						
		0	1	2	3	4	5	6
Encapsulated petiole	NS	65						
	25		-	-	-	-	-	-
	4		50	45	30	30	-	-
	0		-	-	-	-	-	-
Non encapsulated petiole	NS	70						
	25/4/0*		-	-	-	-	-	-

NS : No storage; Petiole sections were cultured before and after encapsulation without any storage.

* : Non encapsulated petiole sections stored at 25/4/0^{o}C temperature did not show regeneration capacity.

g. 35. Saintpaulia ionantha : Encapsulated beads of petiole sections

g. 36. Saintpaulia ionantha : Encapsulated beads of callus piece

g. 37. Saintpaulia ionantha : Encapsulated beads of axenic shoot buds

temperature did not show any regenerative response after culturing.

Encapsulated petiole sections showed regenerative response to different storage timings at $4^{o}C$ (Fig.38). However, regenerative capacity decreased to 30 per cent at 4 weeks of storage. Stored beads at $4^{o}C$ temperature did not show any response when cultured after 4 weeks of storage.

b. Callus pieces

Yellowish green friable pieces of callus obtained during *in vitro* cultures of *Saintpaulia ionantha* were encapsulated using 2.5 per cent sodium alginate prepared in MS basal medium and their regeneration capacity was recorded after different storage timings at different temperatures (Table 41).

The non-encapsulated callus pieces without any storage proliferated showing a response of 70 per cent when cultured on MS medium supplemented with IBA 1 mgl^{-1} + BA 1 mgl^{-1}. The non-encapsulated callus pieces stored in different temperatures and storage time failed to show any response after culturing.

The encapsulated pieces of friable green callus turned brownish but regenerated when cultured on medium. The encapsulated callus beads which were stored in $25^{o}C$

Table 41. Regeneration capacity of encapsulated and non encapsulated callus pieces of Saintpaulia ionantha.

Treatment	Storage temperature (°C)	Storage time (weeks)						
		0	1	2	3	4	5	6
Encapsulated callus pieces	NS	70						
	25			*				
	4		60	45	30	30	20	-
	0		-	-	-	-	-	-
Non encapsulated callus pieces	NS	70						
	25/4/0**		-	-	-	-	-	-

NS : No storage; callus pieces were cultured before and after encapsulation without any storage.

* : Encapsulated beads of callus showed proliferation after 2 weeks of storage at 25°C without culturing.

** : Non encapsulated callus pieces stored at 25/4/0°C temperature did not show regeneration capacity.

Fig. 38. Saintpaulia ionantha : Regeneration of encapsulated petiole section after 4 weeks of storage at 4°C

Fig. 39. Saintpaulia ionantha : Proliferation of callus from encapsulated callus piece after 5 weeks of storage at 4°C

Fig. 40. Saintpaulia ionantha : Regeneration of encapsulated shoot buds after 8 weeks of storage at 4°C

produced hairy rhizoids which burst open the matrix after 2 weeks. These beads when cultured on appropriate medium regenerated ultimately giving rise to plantlets. However, these beads stored in 25°C started dessicating after 3-4 weeks losing the regeneration capacity of callus beads.

Encapsulatd callus beads showed regenerative response at different storage time at 4°C temperature when cultured on medium. However, regeneration capacity decreased to 20 per cent at 5 weeks of storage at 4°C. There was a maximum of 60 per cent regeneration after 1 week of storage at 4°C (Fig.39).

The encapsulated beads of callus did not regenerate when stored in 0°C temperature and cultured on appropriate medium.

c. **Shoot buds**

Shoot buds obtained from initial cultures of Saintpaulia ionantha were encapsulated and their regenerative was capacity after different storage time and temperature were recorded (Table 42).

The non-encapsulated shoot buds without any storage treatment when cultured on MS medium supplemented with IBA 1 mgl^{-1} + BA 1 mgl^{-1} responded (100%) showing multiple plantlet formation and rooting. However, the non-encapsulated shoot buds stored in different

Table 42. Regeneration capacity of encapsulated and non encapsulated shoot buds of Saintpaulia ionantha.

Treatment	Storage temperature (^{o}C)	Storage time (weeks)					
		0	2	4	6	8	10
Encapsulated shoot buds	NS	100					
	25		*				
	4		85	70	50	40	-
	0		-	-	-	-	-
Non encapsulated shoot buds	NS	100					
	25/4/0**		-	-	-	-	-

NS : No storage; shoot buds were cultured before and after encapsulation without any storage.

* : Shoot buds emerged out of beads in stored condition after 3 weeks of storage at 25^{o}C resulting in growth and root initiation (without culturing).

** : Non encapsulated shoot buds stored at 25/4/0^{o}C temperature did not show regeneration capacity.

temperatures and time failed to show any response after culturing.

The encapsulated shoot buds when cultured on medium soon after encapsulation showed 100 per cent response which ultimately formed multiple plantlets after 12 weeks. The beads of shoot buds when stored in 25°C temperature started root initiation and growth of the shoot bud in petridishe itself after 3 weeks. After 4 weeks these beads in storage dessicated.

Encapsulated shoot buds stored in 4°C showed a response of 85 per cent regeneration after 2 weeks of storage and culturing. However, the regeneration capacity decreased to 40 per cent at 8 weeks of storage (Fig.40). After 8 weeks the beads of shoot buds failed to regenerate when cultured on appropriate medium.

Encapsulated shoot buds stored in 0°C temperature did not respond when beads were culturerd on medium.

4.3 ANTHER CULTURE

Effect of media and growth regulator on anthers of Saintpaulia ionantha

The anthers were cultured on Blaydes and MS medium supplemented with various growth regulator combinations to

Table 43. Effect of media and growth regulators on anthers of Saintpaulia ionantha.

Treatment (mgl^{-1})	NR	%R	Callus				Differentiation		
			DI	EX	CC	NC	DD	PL	R
Blaydes + IBA $5mgl^{-1}$+Kn $0.5mgl^{-1}$	C+SB+R	40	27	++	C	F	85	6	+
Blaydes + IBA $1mgl^{-1}$+Kn $1mgl^{-1}$	-	-	-	-	-	-	-	-	-
Blaydes + IBA $2mgl^{-1}$+Kn $0.5mgl^{-1}$	-	-	-	-	-	-	-	-	-
MS + NAA $0.5mgl^{-1}$ + BA $0.5mgl^{-1}$	-	-	-	-	-	-	-	-	-
MS + NAA $1mgl^{-1}$ + BA $1mgl^{-1}$	C+SB	50	38	++	YG	F	125	4	-
MS + NAA $2mgl^{-1}$ + BA $2mgl^{-1}$	C	30	41	+	DB	G	-	-	-
MS + NAA $1mgl^{-1}$ + BA $2mgl^{-1}$	C	30	40.5	+	DB	G	-	-	-
MS + IBA $1mgl^{-1}$ + BA $1mgl^{-1}$	C+SB	60	30	+++	C	F	110	3	-
MS + IBA $2mgl^{-1}$ + BA $2mgl^{-1}$	-	-	-	-	-	-	-	-	-
MS + IBA $2mgl^{-1}$ + BA $0.2mgl^{-1}$	-	-	-	-	-	-	-	-	-

NR : Nature of response; %R : Percentage response; DI : Days taken for initiation; EX : Extent of callusing; NC : Nature of callus; DD : Days taken for differentiation; PL : Plantlets; R : Roots.
C : Cream; YG : Yellow green; DB : Dark brown; F : Friable; G : Globular.

-: Nil; + : Low; ++ : Moderate; +++ : High.

Fig. 41. Saintpaulia ionantha : Callus from anther cultured on Blaydes medium with IBA (5 mgl^{-1}) + kinetin (0.5 mgl^{-1})

Fig. 42. Saintpaulia ionantha : Differentiation of callus into plantlets on Blaydes medium with IBA (5 mgl^{-1}) + kinetin (0.5 mgl^{-1})

study the extent of callusing and differentiation (Table 43).

Anthers cultured on Blaydes medium supplemented with IBA 5 mgl^{-1} + kinetin 0.5 mgl^{-1} showed 40 per cent response. The callus from the interior of anthers started on 27th day after culturing (Fig.41). This callus proliferated to a moderate extent giving friable callus. However, callusing was not observed from the antherwall. The callus obtained from anther interior differentiated on 85th day after culturing giving shoot buds (Fig.42) which developed into 6 plantlets. There was no root initiation from the clump of callus and plantlets. The plantlet that was differentiated was subcultured on same medium which resulted in multiplication of 8 plantlets in 35 days which initiated few roots.

MS medium supplemented with NAA 2 mgl^{-1} + BA 2 mgl^{-1} and NAA 1 mgl^{-1} + BA 2 mgl^{-1} resulted only in callus to a lower extent from the anther interior. This callus turned dark and globular which ultimately failed to differentiate.

Anthers cultured on MS + NAA 1 mgl^{-1} + BA 1 mgl^{-1} resulted in initiation of callus on 38th day giving moderate intensity of creamy friable callus. This callus differentiated giving shoot buds which developed into 4 plantlets. However the differentiation took long time

Fig. 43. Saintpaulia ionantha : Proliferation of callus and differentiation from anther cultured on MS medium with IBA (1 mgl^{-1}) + BA 1 (mgl^{-1})

Fig. 44. Saintpaulia ionantha : Production of haploid plantlets from anther callus subcultured on MS medium with IBA (1 mgl^{-1}) + BA (1 mgl^{-1})

(125 days). MS + IBA 1 mgl^{-1} + BA 1 mgl^{-1} resulted in intense callusing which started after 30 days of culturing. The creamy friable callus took 110 days to differentiate after culturing the anthers. Though there was good callusing, the differentiation was poor leading to formations of 3 plantlets without any roots (Fig.43). However, this callus when subcultured on same medium (MS + IBA 1 mgl^{-1} + BA 1 mgl^{-1}) resulted in moderate proliferation of callus and differentiation leading to production of 23 plantlets at the end of 8 weeks (Fig.44). The callus cultured on either MS basal medium or MS with various combination of IBA (1 and 2 mgl^{-1}) + BA (1 and 2 mgl^{-1}) there was only proliferation of callus to a little extent without any differentiation. However, there was production of few hairy like rhizoids on callus cultured on MS + IBA 1 mgl^{-1} medium. The haploid chromosome number (n = 14) was confirmed by cytological evaluation.

4.4 *PROTOPLAST ISOLATION*

To determine a method suitable for the isolation of protoplasts from leaf of Dendrobium 'Jaquelyn Thomas', few preliminary experiments were conducted. Leaf material released mesophyll protoplasts after enzymatic treatment (Table 44).

600 mg leaf material was preplasmolysed in 20 ml of 13 per cent mannitol solution for 2 hrs. This leaf

Table 44 Influence of cellulase and macerozyme concentrations at different incubation periods on release of protoplasts of Dendrobium 'Jaquelyn Thomas'

Concentration (%)		Incubation time (hours)			
Cellulase	Macerozyme	8	12	16	20
0.5	0.2	-	-	-	-
0.5	0.4	-	-	-	-
0.75	0.2	-	-	+	+
0.75	0.4	-	+	+++	-
1.0	0.2	-	+	-	-
1.0	0.4	-	+	-	-
2.0	0.2	-	-	-	-
2.0	0.4	-	-	-	-

-: Nil; + : Low; ++ : Moderate; +++ : High.

Fig. 45. Isolation of protoplasts (x 1610) from leaf of Dendrobium 'Jaquelyn Thomas'. note, the fast disintegrating cell wall

material was then cut into small pieces and incubated in 5 ml of enzyme solution in dark for different periods to isolate the protoplasts.

The enzyme solution had cellulase, pectinase and macerozyme for digestion of cell wall, whereas MES, a biological buffer was used to maintain to pH. The osmoticum used in enzyme solution was sorbitol at a concentration of 5 mM.

Leaf pieces were incubated in 5 ml of enzyme solution having different concentration of cellulase and macerozyme. After regular time interval of 8, 12, 16 and 20 hours the leaf tissues treated with enzyme solution were observed for the release of protoplats. At lower concentration of cellulase (0.5%) there was no release of protoplast at different time interval. At 0.75 per cent cellulase and 0.4 per cent macerozyme in the enzyme solution incubated for 16 hrs there was a good release of viable intact protoplasts which floated on the surface of enzyme solution (Fig.45). At this enzye concentration the protoplast released were spherical in shape with lower number of ruptured unviable protoplasts. As the time of incubation increased after 16 hrs, the protoplasts ruptured showing irregular shapes. At higher concentration, there was a release of protoplast to some extent which was unviable.

DISCUSSION

V. DISCUSSION

Plant tissue culture which originated as a botanical discovery, has evolved into a major commercial tool for horticulture and other segments of biotechnology. Large scale multiplication of ornamental plants for the horticultural industry is one of the major practical application of the science of plant tissue culture. It is well known that *in vitro* techniques can overcome many problems faced in conventional vegetative propagation. Apart from micropropagation, this technique offers excellent opportunities in obtaining haploid plants from anthers, isolation and culture of protoplasts for genetic manipulations and encapsulation methods for efficient storage and delivery system.

5.1 MICROPROPAGATION

The most extensive and visible application of plant tissue culture has been in rapid clonal multiplication, which is identified as micropropagation. Micropropagation allows for the production of large number of plants in a relatively smaller space or growing area and in a shorter time. Further, the production cycle can be continued throughout the year irrespective of the season and even in double shifts for maximum output.

5.1.1 *Saintpaulia ionantha* Wendl.

Saintpaulia ionantha is conventionally propagated by leaf cuttings. Though this technique is relatively easier, it requires more space and is prone to diseases during multiplication. In the recent years there has been an increasing interest in the application of tisue culture technique as a rapid means of clonal multiplication. The method requires less space needed for mass multiplication and avoids diseases that spreads during multiplication. In view of this, therer has been an upsurge of interest in rapid *in vitro* multiplication of *Saintpaulia ionantha* (Flores *et al.*, 1976; Start and Cumming, 1976; Cooke, 1977; Vazquez *et al.*, 1977; Cassells and Plunkett, 1984, 1986 and Molgaard *et al.*, 1991).

In vitro propagation rate of *Saintpaulia* is controlled by many factores which include type of explants, genotype, media components, growth regulators and number of subcultures (Schulze, 1988). Experiments were carried out to standardise the overall protocols for faster *in vitro* multiplications of *Saintpaulia ionantha*.

Explants

Results have shown that leaf discs are superior source of explants than petiole sections, as they gave a better callusing and differentiation. A perusal of

earlier literature reveals that leaf sections were preferred as a source of explants by many earlier workers (Kukulczanka and Suszyńska, 1972; Flores et al., 1976; Grunewaldt, 1976; Start and Cumming, 1976; Cooke, 1977; Cassells and Plunkett, 1984, 1986).

Media

The leaf disc and petiole section explants were cultured on various media such as MS, 1/2 strength MS B_5, Margara N_5K and Margara $N_{45}K$, each supplemented with 0.5 mgl^{-1} NAA and BA. Out of 5 media, MS medium gave the best results in form of callusing and differentiation of shoot buds from leaf disc explants. Response of petiole sections was also similar to that of leaf explants, but with a reduced intensity. However, all other medium tried showed lesser degree of callusing and differentiation. The MS medium characterised by high concentration of mineral salts helped in induction of callus and then differentiation to a greater extent. Similarly, Saintpaulia explants have been used on MS medium for better results in several cases (Bilkey et al., 1978; Vazquez and Short, 1978; Hanney and Knap, 1979; Jacob et al., 1980; Chang, 1985 and Ioannou, 1987). However, media such as Margara (Margara and Piollart, 1985) and B_5 (Flores et al., 1976), which contain low salt concentrations were also used for successful regeneration of Saintpaulia explants.

Growth regulators

Synthetic growth rgulators function in plants by magnifying, inhibiting or interacting with endogenous growth regulators. Shifts in hormonal balance caused by a synthetic growth regulator are long lasting and produce a much stronger response in plants. Leaf disc explants of Saintpaulia did not respond either to growth regulator free medium or medium with individual auxin and cytokinin. Morphogenic response was observed in equal ratio (0.5: 0.5; 1:1; 2:2) of NAA and BA, resulting in callusing and differentiation. This suggests that for callusing and differentiation, auxin and cytokinin at equal proportions are necessary. At 0.5 mgl^{-1} NAA and BA the callus mass which was proliferated having green in colour and friable in nature, differentiated into highest number of shoot buds. Petiole sections responded to all the NAA and BA combinations. As the concentrations of NAA and BA combinations increased there was a inverse relation in extent of callusing and differentiation. The results indicate that probabaly NAA and BA at equal proportions provide stimulas for the explants for callusing and differentiation. Similarly, Schlegel (1983) obtained callusing and shoot buds from leaf stalks of Saintpaulia when cultured on MS supplemented with NAA and BA at 1 mgl^{-1} combination. The unequal ratio of NAA and BA, resulted

in callusing and differentiation of only petiole sections. However, there was a delay in time taken for response. This result may be due to the endogenous level of auxin and cytokinin in petiole sections which might have helped in responding even at unequal ratio of NAA and BA. A balance between auxin and cytokinin growth regulator is most often required for the induction of callus and its differentiation (George and Sherrington, 1984). The requisite concentration of each type of regulant differ greately according to the kind of plant part, culture conditions and the compounds used.

When kinetin was used as a source of cytokinin along with NAA, the explants induced only callus. The callus mass proliferated to a considerable extent giving brownish nodular callus. Continued cultures of this callus only resulted in induction of rhizoids and roots in some cases which failed to induce shoot buds. In line with this result, Vazquez et al. (1977) and Vazquez and Short (1978) obtained colourless nodules, which eventually grew into roots without any shoot bud differentiation from floral and leaf explants when cultured on medium supplemented with NAA and kinetin combinations. The role of cytokinin in morphogenesis in cultured Saintpaulia tissue was critical. The addition of BA to medium containing NAA promoted shoot bud development from callus, whereras the substitution of kinetin resulted in nodular

rhizoids. It is probable that the efficiency with which these compounds were metabolised was responsible for the variation in organogenesis. One more possible explantation is that BA is a more potent cytokinin than kinetin (Skoog et al., 1967). Among the different IBA and BA combinations, 1 mgl^{-1} each of these regulants induced moderate callusing and differentiation.

Among the different growth rergulator combinations, it was evident from the results that NAA and BA at 0.5 mgl^{-1} combination resulted in better callusing and differentiation from both the explants during initiation of cultures. Similarly, several workers obtained better callusing and differentiation from explants on medium having NAA and BA combinations (Flores et al., 1976; Vazquez and Short, 1978; Bilkey and Cocking, 1981)

Axenic leaf culture

In an attempt to recycle its stored axenic material, leaf explants from in vitro raised axenic plant material were cultured. This method avoids the cost and space for maintaining mother plants and also avoids the risk of disease outbreaks in the stock. Axenic leaf explants cultured on MS supplemented with BA, induced direct morphogenesis without any intervention of callus. BA at 0.5 mgl^{-1} was found to be optimum in induction of shoot buds to a greater extent from axenic leaf explant.

BA, a cytokinin which is known for induction of shoots, has helped in enhanced development of adventitious shoot buds directly without any auxins in the culture. Vazquez et al. (1977) obtained considerable number of shoot buds from leaf explants when cultured on medium enriched with 1 mgl^{-1} BA. NAA and BA combinations resulted in direct and indirect morphogenesis from axenic leaf explants. Axinic leaf cultured on medium having 0.5 mgl^{-1} NAA and BA initiated callus followed by direct differentiation from the explant. The callus mass also differentiated with a lower intensity. Cassells and Plunkett (1984) obtained shoot buds from axenic leaves cultured on medium having NAA and BA combinations.

At higher concentrations of NAA with lower or higher concentrations of BA, axenic leaf explants induced only brownish nodular callus without any differentiation. This confirms that NAA and BA at equal proportions are necessary for differentiation in case of Saintpaulia.

Axenic leaf explants cultured on medium supplemented with either IBA alone or in combination with BA induced callusing and shoot bud differentiation. So far there was no report of using IBA as an auxin source in Saintpaulia ionantha. Interestingly, IBA as auxin source along with BA resulted in better differentiation of adventitious shoot buds both directly and indirectly. Unlike NAA, IBA at higher concentrations with BA resulted

in induction of friable callus which differentiated into shoot buds. Axenic leaf explants on medium having 2,4-D at lower concentration and 2,4-D + BA combintions resulted only in induction of brownish nodular callus which failed to differentiate. The results of present investigations revealed that IBA and BA at 1 mgl^{-1} induced maximum number of plantlets from axenic leaf explant, thus helping in rapid clonal multiplication of Saintpaulia.

The axenic leaf cultures responded better in induction of shoot buds either directly or indirectly when compared to leaf disc explants obtained from mother stock. The leaf explants from mother stock only differentiated via callus and there was no direct morphogenesis. The juvenile state of axenic leaf explants and due to endogenous growth regulator levels in these explants there might have been a response of direct organogenesis and also, the time taken for response was faster when compared to explants taken from mother stock. Although growth regulators may also help in induceing direct morphogenesis, cells of the explant in many instances appears to be partially predetermined to a particular morphogenic pathway so that it takes only a slight change in environment to induce them. The extent of predetermination varies between differnt tissues in the whole plant (George and Sherrington, 1984).

Callus culture

During differentiation, storage products typically found in resting cells tend to disappear and new meristems arise producing undifferentiated parenchymatous cells without any of the structural order that was characteristic of the organ or tissue from which they were derived. Experiments were carried out to assess the proliferation of callus and its differentiation capacity when subcultured on medium enriched with different growth regulator combinations.

Callus cultured on growth regulator free medium differentiated into few shoot buds with intense rooting. At combination of NAA and BA (0.5 mgl^{-1} each). there was further proliferation of callus to a greater extent but with a moderate differentiation. Cytokinin alone in the form of BA (0.5 and 1.0 mgl^{-1}) resulted in greater differentiation of shoot buds. During callus culture, IBA (1 mgl^{-1}) as a source of auxin along with BA (1 mgl^{-1}) faired better in induction of shoot buds from the callus. Kinetin as a source of cytokinin resulted in shoot bud differentiation when used alone (1 mgl^{-1}) or along with lower concentration of auxin. However, kinetin at equal proportion with auxin proliferated callus which became recalcitrant or produced roots in some instances after continued cultures. This trend was observed by Vazquez et

al. (1977) in Saintpaulia cultures. Bilkey et al. (1978), obtained greater callus growth and differentiation from BA when compared to kinetin as a cytokinin source along with auxin.

Subculturing

For rapid multiplication, subculturing of plantlets to obtain highly proliferative shoot mass was tried using various combinations of growth regulators. Axenic plantlets cultured on medium enriched with combination of auxins and cytokinins induced adventitious shoot buds directly on leaf and axils of plantlet. Though NAA and BA combination resulted in slight callusing at the base of the plantlet, there was a direct morphogenesis to a greater extent from plantlets. IBA and BA at 1 mgl^{-1} combination resulted in maximum multiplication followed by NAA (1 mgl^{-1}) and BA (1 mgl^{-1}) combination. However, increased concentration of NAA (2 mgl^{-1} and 4 mgl^{-1}) along with BA (0.5and 2 mgl^{-1}) resulted in dedifferentiation of whole plantlets which turned recalcitrant. The plantlets differentiated in medium enriched with auxin and cytokinin (BA) were found to be smaller in size and with nil to low intensity of rooting. Start and Cumming (1976) subcultured plantlets on a medium enriched with auxin and cytokinin to obtain increased multiplication rate. Similarly, recurrent propagation method was adopted by

Schulze (1988) in Saintpaulia ionantha for rapid multiplication.

When kinetin was substituted with BA as a cytokinin source, there was a lower rate of multiplication. This indicates the suitability of BA in multiplication steps when compared to kinetin as a cytokinin source. Bilkey et al. (1978) preferred BA over kinetin for callus growth and subsequent differentiation of Saintpaulia plantlets.

Appropriate rate of multiplication was assessed over 8 passages each at 10 week interval. It was observed that axenic plantlets can give a multiplication rate of 51.56 per culture without much variation in the 8 subcultures. Similarly, Reist and Le (1987) used shoot clusters obtained by in vitro clusters for further multiplication by series of subculturing for several months.

Variegation and albino type of plantlets

Continued in vitro cultures produced some of the plantlets with variegated or albino leaves. The question of variegtion in phenotype that may arise following cultures in vitro has been raised in Saintpaulia (Grunewaldt, 1980). Some of the variations like fluted leaf were reported in Saintpaulia in vitro cultures (Cassells and Plunkett, 1986). They nave also noticed

variation in flower colour, leaf shape and altered pigmentation which varieng from leaf flecking to an almost completely albino appearence. In the present study the regenerants from this cultured variegated leaf were normal without showing any signs of variegation or albino type of plantlets. Majority of variegated leaf are known to be periclinal chimeras and owe their existance to the outer zone tunica and the inner zone corpus. Two and three layered chimeras are formed in many monocot families. However the pattern of this variegation in present case may be due to mixing of two plastid types within the layers L1, L2 and L3 and are termed as sandwitch chimeras. In some cases all the three layers may be genetically distinct and the pattern in subsequent development depends upon from which layer the new leaf has come. These type of chimeras are called trichimeras or genetic chimeras. A perusal of earlier literature reveals that in tissue culture plants all plants derived from green L2, will be green and white, when L2 is white, however, in either case the chimeral structure is lost (Tilney Besset, 1991).

Rooting

Plantlets cultured on growth regulator free medium initiated roots slowly, but with a higher intensity of rooting. In this case the growth of the plantlet was maximum which attained a transplantable size after 6 weeks

in culture. Though the individual auxins induced roots at a faster rate, the intensity was lower. Moreover, there was further differentiation of shoot buds which grew into smaller plantlets. For good growth of plantlet and rooting, subculturing of plantlets on growth regulator free medium has been suggested by many workers (Cooke, 1977; Jacob et al., 1980 and Grout, 1990).

Hardening and planting out

Other determinants in the establishment of Saintpaulia plantlets like age of plantlets, maintenance of humidity and potting mixture were optimised in further experiments. Plantlets after 3 weeks of root initiation survived to a greater extent (85%) when transferred to appropriate potting mixture with maintenance of humidity. The plantlets at root initiation stage (without any roots) did not survive in the pots. To achieve optimum transplanting success, Grout (1990) slected plantlets after a growth period of 4 weeks in growth regulator free medium which had atleast three healthy leaves and good root system.

Hardening of plantlets to make them adapt to the outside environment is a critical process due to the anatomical and physiological pecularities of the plantlets. Water loss from the plants had been recorded which was attributed to the improper development of the

cuticle and slowness of stomatal response to water stress (Brainerd and Fuchigami, 1981; Fabbri et al., 1984). The problem may be aggravated if the vascular connection between root and shoot is improper. A period of humidity acclimatization was considered necessary for the newly transferred plantlets to adapt to the outside environment, during which the plantlets undergo morphological and physiological adaptation enabling them to develop typical terrestrial plant-water control mechanisms (Grout and Aston, 1977; Sutter et al., 1985). High relative humidity (90-95%) was maintained during the initial period of planting out with the help of polythene covers and intermittent water sprays. After 3 weeks, the polythene covers were removed and the intermittent sprays of water was reduced to make the plantlets hardened at lower relative humidity. The maximum survivality of 90 per cent was achieved by following this method. Jacob et al. (1980) obtained a 100 per cent success in survivality of plantlets when they were hardened at 20-25^{o}C and high relative humidity.

Transfer to pots

A potting mixture containing soilrite, sand and compost (2:1:1 V/V) favoured the maximum survivality (85%) of plantlets. The best results from this mixture (soilrite + sand + compost) in the present studies are apparantly related to the retention of optimum moisture

and good aeration in the root zone. Higher rate of success was achieved by Flores et al.(1976) in survivality of transplanted plantlets in 1:1 mixture of peat moss and soil. In the present study pure sand as a medium showed poor survivality due to lower retention of water leading to quick dessication of plantlets.

5.1.2 Begonia 'Lucerna'

Cane type of begonias are conventionally propagated by stem cuttings. Many attempts have been made to grow Begonia plants by tissue culture (Ringe and Nitsch, 1967; Wealander, 1977; Bigot, 1981). However most workers studied the effect of plant growth regulators or physiochemical conditions on differentiation and growth (Takayama, 1990). In the present study, an attempt was made to assess the regenerative capacity of Begonia 'Lucerna' explants on different growth regulator combinations.

Explants

Different explants such as petiole sections, leaf and stem segments were cultured on MS medium supplemented with various combinations of growth regulators. Petiole explants gave better response for callusing and differentiation. Leaf sections with a vein resulted in response in few cases. Whereas stem segments did not

respond either for callusing or for differentiation. Ringe and Nitsch (1967) cultured petiole sections of Begonia 'Lucerna' for obtaining vegetative buds. Similarly, the morphogenic response was better when petioles were used as explants in many cases (Wealander, 1977 and Bigot, 1981). The differences in the degree of response in the same plant may be related to the difference in the endogenous hormone level in the plant parts used (George and Sherrington, 1984).

Effect of growth regulators

Petiole sections cultured on various NAA and BA concentrations and combinations responded to only a few treatments. BA alone at higher concentration (5 mgl^{-1}) or with lower concentration of NAA (1 and 2 mgl^{-1}) resulted in callusing of petiole sections. However, the callus obtained from these treatments turned dark brown which did not differentiate. NAA 1 mgl^{-1} + BA 2 mgl^{-1} combination resulted in higher intensity of callusing which differentiated to maximum number (18) of plantlets. When these plantlets were subcultured on the same medium there was greater proliferation of plantlets. NAA 2 mgl^{-1} + BA 5 mgl^{-1} resulted in callusing and differentiation to a lower extent. Ringe and Nitsch (1967) obtained vegetative buds from petiole explants of Begonia 'Lucerna' on MS medium supplemented with NAA and BA along with adenine. Similarly, callusing and differentiation was observed in

higher concentration of BA with lower concentration of NAA in case of Begonia feastii (Li, 1983).

When IBA was used as an auxin source along with BA, only the combination of IBA 2 mgl^{-1} + BA 2 mgl^{-1} induced callusing and differentiation to a lower extent. When 2,4-D was used, there was callusing with either 2,4-D alone (1 mgl^{-1}) or with combination of 1 mgl^{-1} BA. But the callus failed to differentiate. The callus initiation was observed when Begonia fimbristipula leaf explants cultured on medium having 2,4-D and kinetin (Guang et al., 1983). It is evident from the results that, for callusing and differentiation of petiole explants a 1:2 ratio of NAA: BA is necessary. The induction of callus and differen-tiation seem to be related to concentration and balance of internalphytohormones (Heide, 1967; Heide and Skoog, 1967). However, as indicated in the results, supplementation of growth rgulators to the medium also play a major role in different responses. Similarly, many workers have reported regeneration of Begonia petioles in various combinations of auxin and cytokinin (Takayama and Misawa, 1981, 1982; Reuther and Bhandari, 1981; Bigot, 1981).

Leaf sections with a major vein, when cultured on MS medium having NAA and BA combination resulted in callusing and differentiation in only afew cases. NAA at

1 mgl^{-1} along with BA (1 and 2 mgl^{-1}) resulted in callusing and differentiation. The callus was restricted to main vein area of the leaf explants. NAA at 1 mgl^{-1} along with increased level of BA (5 mgl^{-1}) resulted in moderate callusing and differentiation forming 11.5 plantlets per culture. Peck and Cumming (1984) obtained callus and shoot buds from leaf explant having a major vein of Begonia tuberhybrida when cultured on MS supplemented with 1 mgl^{-1} NAA + 5 mgl^{-1} BA. Similar effect of auxin and cytokinin have been reported by many workers in Begonia (Reuter, 1980; Takayama and Misawa, 1981; Takayama, 1990).

5.1.3 Cymbidium aloifolium Sw.

Though this species is found wild in Western Ghats, its population is shrinking due to over collection, disease outbreaks and change in the environment of the habitat. The common method of propagating cymbidiums is by division. Recently there has been an upsurge of interest in vitro propagation either by using seeds or other plant material such as rhizome tip. The in vitro cultured seeds absorbs the nutrients, thus farming a protocorm. These protocorms could be used either to differentiate to form shoots or to induce proliferation of protocorm like bodies (PLB's) which inturn differntiate.

Protocorm culture

In the present study an attempt was made to proliferate PLB's from the cultured protocorms. The protocorms culturd on liquid and solid medium resulted in proliferation of PLB's which differentiated in to multiple shoots. However, the liquid medium kept in rotary shaker was found to be more effective in increasing PLB's and multiple shoots. The closer contact of the tissues with the liquid medium may greatly facilitate the uptake of nutrients and growth regulators, which must be the reason for the efficient proliferation and differentiation of Cymbidium aloifolium protocorms. Wimbler (1963) introduced liquid shake cultures for Cymbidium, who obtained increased differentiation and growth of the plantlets. The results of present study is in line with those of Gu et al. (1987) and Stewart (1989) who recommended liquid shake cultures for Cymbidium multiplication.

Media and growth regulators

All the growth regulator concentrations and combinations resulted in proliferation of PLB's. However, Vacin and Went liquid medium (1949) supplemented with 0.4 mgl^{-1} BA resulted in intense proliferation and differentiation of PLB's. The effect of BA in increased proliferation and differentiation of PLB's was reported in

Cymbidium by Gu et al. (1987). Among the 2 media used, Vacin and Went (1949) medium responded in a better way when compared with MS (1962) medium either with agar or without agar. Vacin and Went medium was preferred in many cases for Cymbidium cultures (Sagawa et al., 1966; Gu et al., 1987).

Nodal segment culture

The nodal segments obtained from in vitro shoots were cultured on liquid and solid media to assess their effects on multiplication of shoots. In the case of liquid Vacin and Went medium supplemented with 0.4 mgl^{-1} BA there was an enhanced production of multiple shoots indicating a faster rate of clonal multiplication. Matsuri et al. (1970) obtained increased number of shoots with BA added to the medium. MS solid medium without any growth regulator or with IBA 1 mgl^{-1} resulted in growth of the plantlet which induced moderate roots.

The nodal segments were subcultured serially at 8 weeks interval for 6 subcultures. The mean rate of multiplication per culture was 30.66 at the end of each subculture of 8 weeks. This suggests that liquid Vacin and Went medium supplemented with lower (0.4 mgl^{-1}) BA helps in faster clonal multiplication of Cymbidium aloifolium.

5.1.4 Anthurium scherzerianum Schott.

Anthurium is coming up as one of the most important cut flower of this century. The area under Anthurium is fast increasing. However, the availability of planting material is a major bottleneck as vegetative propagation of Anthurium by terminal cutting and stem sections results in multiplication rates which is insufficient for mass propagation. Hence, the recent development of efficient micropropagation technique which has opened up new and promising prospects for Anthurium breeding and improvement (Geier, 1990).

Explants

In the present study, an attempt was made to induce callusing from Anthurium scherzerianum explants. The leaf explants with a vein resulted in callusing in few treatments. As in other monocotyledon plants the potentiality for dedifferentiation was restricted to immature leaves. Geier (1986) successfully used young leaf explants with midrib for multiplication of Anthurium scherzerianum. In the present study, the callusing was initiated on the middle vein of young leaf explants which then spread to other parts of lamina. The petiole explants did not respond for callusing in different media supplemented with various growth regulators and light conditions. The regeneration of callus from leaf explants have bee: reported in Anthurium scherzerianum by Peirik and

Steegmans (1975, 1976), Fersing and Lutz (1977) and Geier (1986). They also observed lower potentiality of leaf explants of Anthurium scherzerianum for callusing when compared to Anthurium andreanum.

Media, growth regulator and culture conditions

One of the factor which influenced the callus formation on leaf section was the extent of illumination. In the present study, callus was observed in both the illumination conditions. However, the callus proliferation was better in the case of light/dark (16/18 hrs) condition on Vacin and Went medium supplemented with 1 mgl^{-1} 2,4-D + 1 mgl^{-1} BA. This callus continued to proliferate even after 5 months without any differentiation. Nitsch medium did not induce callus in light/dark (16/8 hrs) condition. However, Nitsch medium resulted in slight callusing when incubated in continuous dark conditions. Optimum callus formation was observed in continuous darkness in several cases (Pierik et al., 1974; Singh and Sangama, 1991) in Anthurium andreanum. However, Finnic and Staden (1986) reported better callus production in a light/dark environment. Apparently Anthurium scherzerianum has no specific light requirement, since successful culturing has been performed in continuous darkness (Geier and Reuther, 1981; Geier, 1987) as well as under 16/8 hrs light/dark regimes (Zens and Zimmer, 1986).

This was confirmed in the present study in which callusing was observed in both the conditions.

In dark conditions, Vacin and Went medium supplemented with 2,4-D 0.2 mgl^{-1} + BA 0.2 mgl^{-1} and modified Nitsch medium with 500 mgl^{-1} ammonium nitrate + 1 mgl^{-1} BA and 0.1 mgl^{-1} 2,4-D resulted in slight callusing which failed to proliferate further. However, Nitsch medium supplemented with very low (200 mgl^{-1}) and normal concentrations (720 mgl^{-1}) of ammonium nitrate failed to respond. Investigation by Geier (1986) revealed the beneficial effects of low ammonium nitrate levels on regeneration from Anthurium scherzerianum leaf explants. This medium was found to be an absolute requirement for both callus and shoot induction in a number of genotypes. In line with the present study Geier (1987) reported the induction of callus from Anthurium scherzerianum explants from modified Nitsch medium supplemented with 1 mgl^{-1} BA and 0.1 mgl^{-1} 2,4-D.

5.2 ENCAPSULATION

Encapsulation of cells, somatic tissue and somatic embryos have been attempted in several plants and is also termed as synthetic seed technology. This technique has become very popular as a simple way of handling cells, tissues and protecting them against strain and strong external gradients and as an efficient delivery system.

Encapsulation of somatic embryos to be used as a artificial seeds have been described by many workers (Kitto and Janick, 1985; Redenbaugh et al. (1984, 1986, 1987; Bapat and Rao, 1988). Other than somatic embryos, plant material such as axillary buds (Bapat et al., 1987; Bapat and Rao, 1990) protocorms (Singh, 1991) and cell suspensions (Bapat and Rao, 1988) were also used for encapsulation, efficient storage and delivery systems. This technique offers an excellent procedure for nonfrozen storage under limited refrigeration conditions.

Sodium alginate as a gelling material

In the present study, each unit of plant material was encapsulated using a matrix and the size of the beads were 5-6 mm in diameter. Cymbidium and Saintpaulia plant material were encapsulated and were assessed for their efficacy in encapsulation using sodium alginate as a gelling agent. Sodium alginate formed a bead or capsule around the plant material which was pliable enough to cushion and protect the plant material and allow regeneration, yet sufficiently rigid to allow rough handling of the capsule during production, storge and culturing. The sodium alginate matrix was prepared using MS basal medium which provided moisture and nutrients necessary for survivality of plant material. Among the different concentrations, sodium alginate at 2.5 per cent

(W/V) found to be suitable for encapsulation. Similarly, Singh (1991) used sodium alginate at 2.5 per cent concentration for prepring beads of Spathoglottis plicata protocorms.

Among the different plant material, protocorms of Cymbidium aloifolium; petiole section, callus pieces and shoot buds of Saintpaulia ionantha formed proper beads and were found suitable for encapsulation.

Regeneration capacity of encapsulated Cymbidium aloifolium protocorms

The protocorms without encapsulation did not survive during storge. The encapsulated protocoms stored in $4^{o}C$ temperature when cultured on liquid Vacin and Went medium supplemented with 0.4 mgl^{-1} BA the regeneration waas observed. But the regeneration capacity reduced to 20 per cent at 10 weeks of storage at $4^{o}C$ temperature. Singh (1991) obtained a regeneration of encapsulated Spathoglottis plicata protocorms even after 180 days of storge in $4^{o}C$ temperature. The regeneration capacity of encapsulated Cymbidium protocorms after 10 weeks storage at $4^{o}C$ suggests that this method can be used to conserve desirable genotypes and also these beads can be used in an efficient delivery system.

Regeneration capacity of encapsulated Saintpaulia ionantha plant material

In Saintpaulia, there has been no report of using this systm for storage studies. Plant material such as petiole sections, callus piece and shoot buds obtained from in vitro cultures were used for encapsulation.

Petiole sections

The encapsulated petiole sections regenerated when cultured on MS medium supplementd with IBA and BA 1 mgl^{-1}. However, the regeneration capacity rduced to 30 per cent at 4 weeks of storage ($4^{o}C$). The callus mass which was initiated from petiole burst open the beads and there was a differentiation of shoot buds. So far therer has been no report of encapsulating the explant material. The present study suggests the suitability of encapsulated explants for storage and also for efficient transportation from one place to another at $4^{o}C$ temperature with a limited refrigeration space.

Callus piece

The callus pieces which were encapsulated could survive after 5 weeks in storage at $4^{o}C$. However, the regeneration capacity was reduced to 20 per cent at 5 weeks storage. Similarly, Bapat and Rao (1988) obtained regeneration from encapsulated sandal wood cell suspensions stcred in $4^{o}C$ for 45 days. In the present

study the non-encapsulated callus did not survive at $4^{o}C$ temperature.

Shoot buds

The encapsulated shoot buds stored in $4^{o}C$ temperature regenerated when cultured on MS medium supplemented with 1 mgl^{-1} IBA and BA. However, the regeneration capacity decreased to 40 per cent at 8 weeks of storge. The encapsulated shoot buds stored in $25^{o}C$ grew in the beads leading to desiccation of shoot buds after 4-5 weeks of storage. Bapat et al. (1987) and Bapat and Rao (1990) successfully stored encapsulated mulberry axillary buds for 45 days at $4^{o}C$ temperature. Similarly, encapsulated somatic embryos have been shown to regenerate after storage at $4^{o}C$ temperature in sandal wood (Bapat and Rao, 1988) and barley (Datta and Potrykus, 1989).

The regeneration capacity of encapsulated plant material stored at $4^{o}C$ temperature indicate the suitability of this method in efficient delivery systems and also in preserving the genotypes of plant species.

5.3 ANTHER CULTURE

Anther culture offers a tremendous potentialities as a model system for assessing the value of haploid plants in crop improvement programmes. Furthermore, the availability of homozygous plants could lead to the

production of new commercial cultivars. Anthers of Saintpaulia ionantha have been successfully cultured in many cases (Hughes et al., 1975; Smith et al., 1981; Weatherhead et al., 1982; Norris et al., 1982).

In the present study anther cultured on Blaydes medium supplemented with IBA 5 mgl^{-1} + kinetin 0.5 mgl^{-1} resulted in moderate callusing leading to formation of 6 plantlets. These results are in accordance with those of Hughes et al. (1975) who obtained 1-5 plantlets from each anther cultured on Blaydes medium. Production of plantlets directly from anther cultured on Blaydes medium have also been reported by Smith et al. (1981) and Norris et al. (1982). The anthers cultured on MS medium supplemented with either NAA 1 mgl^{-1} + BA 1 mgl^{-1} or IBA 1mgl^{-1} + BA 1 mgl^{-1} resulted in callusing and differentiation. The callusing was initiated from the anther interior. In some cases, higher concentration of BA (2 mgl^{-1}) with NAA (1 or 2 mgl^{-1}) resulted in dark brown callus which turned recalcitrant. MS supplemented with NAA 1 mgl^{-1} + BA 1 mgl^{-1} resulted in formation of 4 plantlets. Whereas, IBA 1 mgl^{-1} + BA 1 mgl^{-1} combination resulted in higher intensity of callusing but with a lower extent of differentiation. When this callus was subcultured on same medium there was higher intensity of differentiation leading to formation of 23 plantlets after 8 weeks of culture. Weatherhead et al. (1982) obtained

callus from anther interior which differentiated in MS medium supplemented with NAA 1 mgl^{-1} + BA 0.5 mgl^{-1}. It was evident from the results that the plantlets obtained from callus is lower in number, but by subculturing of these plantlets or callus, considerable numbers of plantlets could be obtained. Though there are reports of direct morphogenesis on anthers (Hughes *et al.*, 1975), in the present study differentiation was observed indirctly via callus. Callus initiated was restricted to anther interior and not anther wall. In the present study, the response of anther was found to be sporadic. In many plants, the response has been shown to be strongly influenced by several factors including genotype, growth conditions of the donor plant, temperature stress, media components and culture conditions (Maheshwari *et al.*, 1980; Chaleff and Stolarz, 1981).

5.4 PROTOPLAST ISOLATION

Plant protoplasts represent the finest single cell system and offer exciting possibilities in the field of somatic cell genetics and crop improvement. Despite the current awareness, the magnitude of the efforts to develop protoplast technology as applicable to orchid is small and quantum of information available on protoplast isolation is scarce. An attempt to isolate protoplasts of *Dendrobium* 'Jaquelyn Thomas' was made in the present

study. The integrity of the outer plasma membrane and the maintenance of the stability of the protoplasts are important for getting large number of viable protoplasts. Sorbitol and mannitol were used as a osmoticum in the present study. It was found that 13 per cent mannitol was better for maintaining the integrity of leaf mesophyll protoplasts. Therefore this concentration of mannitol was used for preplasmolysis for 2 hours. Okuno and Furusawa (1977) found mannitol as a good osmoticum for the isolation of mesophyll protoplasts. To facilitate the penetration of enzyme solution into the intercellulr spaces, the leaf material was cut into small pieces and were incubated in enzyme solution. This was essential for effective digestion and thus, for getting high yield of protoplasts. Digestion of 600 mg leaf tissues for 16 hours in 5 ml of enzyme solution having 0.75 per cent cellulase + 0.4 per cent Onozuka R-10 macerozyme, pectinase (0.5%) facilitated isolation of viable protoplasts. MES which is a biological buffer was used to maintain the pH of the enzyme at 5.5. Seeni and Abraham (1984) obtained protoplasts from several species and hybrids of orchids by using enzyme mixture containing cellulase (2%) macerozyme (0.5%) which was incubated for 6 hours. In the present study, digestion after 16 hours resulted in obtaining ruptured protoplasts. Variation in the concentration and duration of enzyme treatment affected the isolation and viability of the protoplasts.

SUMMARY

VI. SUMMARY

The commercial production of ornamental plants greatly depend on availability of elite planting material throughout the year. Though the conventional vegetative propagational methods could be used for multiplication, they have got several constraints resulting in low volume of planting material. Similarly, the conventional approaches employed in improving the plants are time consuming. In these aspects, cell and tissue culture offer excellent technique for rapid multiplication of elite types of plants and their improvement. Keeping this in view, the present studies were carried out in an attempt to develop suitable tissue culture techniques for rapid micropropagation, anther culture, encapsulation and isolation of protoplast for some important ornamental plants.

6.1 MICROPROPAGATION

6.1.1 Saintpaulia ionantha Wendl.

Culture initiation using explants such as leaf discs and petiole sections from stock plant demonstrated the morphogenetic ability. Leaf discs were found to be a superior source of explants than petiole sections as they gave a better result in callusing and differentiation. However, the axenic leaves when used as explants during recurrent cultures, responded to both direct and indirect

morphogenesis, and there was a reduction in time taken for differentiation into shoot buds.

Among the five different media viz., MS, 1/2 MS, B_5, Margara N_5K and Margara $N_{45}K$ there was a better response for callusing and differentiation from explants cultured on MS medium.

NAA and BA at 0.5 mgl^{-1} combination were found to be optimum in callusing and differentiation of shoot buds from explants obtained from mother stock. When kinetin at 0.5 and 2 mgl^{-1} was substituted with BA as a cytokinin source along with auxins, it resulted in induction of callus from the explants which later turned recalcitrant or gave some roots after continued cultures.

The axenic leaf obtained from in vitro grown plantlets responded to growth regulator combination resulting direct and indirect morphogenesis. BA at 0.5 mgl^{-1} was found to be optimum in direct morphogenesis from axenic leaf material. However, growth regulator combination of IBA and BA at 1 mgl^{-1} resulted in maximum number of plantlets production through direct and indirect morphogenesis.

Various growth regulator concentrations and combinations either proliferated or differentiated when callus was subcultured. NAA and BA at 0.5 mgl^{-1}

combinations proliferated callus to a greater extent. However, the differentiation from callus cultures was maximum in either BA alone (0.5 and 1 mgl^{-1}) or with IBA and BA at 1.0 mgl^{-1} combination.

The axenic plantlets showed induction of multiple plantlets when subcultured on MS medium supplementd with auxin and cytokinin concentrations and combinations. NAA and BA or IBA and BA at 1 mgl^{-1} combination resulted in maximum number of plantlet production during subcultures. There was an average multiplication rate of 51.56 plantlets per culture from IBA and BA at 1 mgl^{-1} combination evenafter series of subcultures at 10 weeks interval.

The higher intensity of rooting and better growth of the plantlet occured in MS basal medium without any growth regulators. The plantlets when transferred after three weeks of root initiation faired better in survivality of plantlets in pots. Covering the plantlets with polythene covers for three weeks with intermittent watering to maintain the humidity resulted in good survivality of plantlets. Potting mixture of soilrite + sand + compost (2:1:1 V/V) recorded the highest survival of plantlets in pots.

6.1.2 Begonia 'Lucerna'

The petiole and leaf explants showed sporadic responses for callusing and differentiation. Stem segments did not respond for morphogenesis. Petiole explants were however, found to be a superior source of explants for obtaining plantlets.

Petiole explants cultured on MS medium supplemented with NAA 1 mgl^{-1} + BA 2 mgl^{-1} resulted in higher intensity of callusing and differentiation producing 18 plantlets per culture. When this plantlets were subcultured on the same medium, there was a faster multiplication of plantlets with moderate rooting.

Leaf segments when culturerd on MS medium supplemented with NAA 1 mgl^{-1} + BA 5 mgl^{-1}, there was an induction of callus from main vein area which resulted in differentiation of 11.5 plantlets per culture.

6.1.3 Cymbidium aloifolium Sw.

The protocorm obtained from in vitro seed cultures, resulted in proliferation of PLB's and differentiation into multiple shoots in liquid and solid Vacin and Went medium. However, liquid medium kept in a rotary shaker was found to be having greater influence on proliferation and differentiation. Vacin and Went medium

faired better in induction of multiple shoots when compared to MS medium with or without agar.

Liquid Vacin and Went medium having 0.4 mgl^{-1} BA induced maximum proliferation of PLB's resulting in formation of higher number of multiple shoots.

Nodal segments obtained from axenic shoots, induced maximum number of multiple shoots in liquid Vacin and Went medium supplemented with 0.4 mgl^{-1} BA. Nodal segments induced 30.66 multile shoots per culture during series of subcultures at 8 weeks interval in the same medium indicating a faster rate of multiplication of Cymbidium aloifolium.

The multiple shoots induced, when separated and cultured on solid MS medium without any growth regulator or with 1 mgl^{-1} BA, there was a better growth of shoots with moderate intensity of rooting.

6.1.4 Anthurium scherzerianum Schott.

The leaf explants with vein responded for higher callusing in few cases. Wherreas, petiole segments did not respond when cultured on different nutrient medium.

In leaf sections, the callus started from the vein and then spread to other part of lamina. The callusing was observed in few cases when cultured on modified Vacin and Went medium supplemented with 2,4-D and BA at 1 mgl^{-1}

combination and incubated in light/dark (16/8 hours) cycle. Incubation in continued dark condition resulted in slight callusing from leaf explants cultured on Vacin and Went medium supplemented with 2,4-D and BA t 0.2 mgl^{-1} combination and modified Nitsch medium with reduced concentration of ammonium nitrate (500 mgl^{-1}) + 1 mgl^{-1} BA + 0.1 mgl^{-1} 2,4-D

6.2 ENCAPSULATION

Plant material such as protocorms of Cymbidium aloifolium; petiole sections, callus pieces and axenic shoot buds of Saintpaulia ionantha were found suitable for encapsulation. Sodium alginate (Sigma A. 7128) at 2.5 per cent (W/V) concentration prepared in MS basal liquid medium gave proper consistency of beads which were found suitable for storage studies.

Encapsulated protocorms of Cymbidium aloifolium showed regenerative capacity upto 10 weeks of storage at $4^{o}C$. All the encapsulated material of Saintpaulia ionantha were found suitable for storage at $4^{o}C$ temperature. The encapsulated petiole sections showed regeneration capacity upto four weeks of storage. The encapsulated callus pieces could store upto five weeks at $4^{o}C$ temperature. Axenic shoot buds had a maximum storage ability of 8 weeks when encapsulated and stored at $4^{o}C$ temperature.

The ability of these plant materials for encapsulation and their storage at $4^{o}C$ temperature suggests the suitability of encapsulated material for efficient delivery system, storage and effective transport from one place to other under limited refrigeration conditions.

6.3 ANTHER CULTURE

Anthers from unopened flower buds of Saintpaulia ionantha when cultured on medium showed responses in few cases. Cultured anthers initiated callus from anther interior and in some cases there was differentiation into shoot buds. Blaydes medium supplemented with IBA 5 mgl^{-1} + kinetin 0.5 mgl^{-1} resulted in moderate callusing and differentiation leading to production of 6 plantlets. Anthers cultured on MS + IBA 1 mgl^{-1} + BA 1 mgl^{-1} resulted in higher callusing and lower differentiation. However, the plantlets and callus when subcultured on the same medium resulted in higher differentiation and production of 23 haploid plantlets.

6.4 PROTOPLAST ISOLATION

Leaf mesophyll protoplast were isolated from Dendrobium 'Jaquelyn Thomas' following suitable methods. Preplasmolysis of leaf material in 13 per cent mannitol for 2 hours was necessary. The 600 mg of leaf material

was cut into pieces and incubated in dark in 5 ml enzyme solution having 0.75 per cent cellulase, 0.4 per cent Onozuka R-10 macerozyme. The enzyme solution also contained pectinase (0.5%) and MES (5 mM). The time for incubation for release of viable protoplasts was found to be 16 hours. This method greately facilitated the release of viable protoplasts.

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VII. REFERENCES

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* Original not seen

APPENDIX

APPENDIX

ABBREVIATIONS/ACRONYMS

2,4-D	2,4-dichlorophenoxy acetic acid
% R	per cent of response
BA	6-benzyladenine or 6-benzylamino purine
B_5	Gamborg *et al.*, medium (1968)
C	callus
^oC	degree celsius
CC	colour of callus
cm	centimetre(s)
DM	direct morphogenesis
EDTA	ethylene diamine tetraacetic acid
h/hrs	hour(s)
IBA	indole-butyric acid
IM	indirect morphogenesis
Kg	kilogram
Kn	kinetin
l	litre
M	molar
mg	milligram(s)
mgl^{-1}	milligram(s) per one litre
mm	millimetre
mM	millimolar
MSh	multiple shoots
MS	Murashige and Skoog basal medium (1962)
NAA	2-naphthalene acetic acid

NC	nature of callus
NR	nature of response
pH	hydrogen ion concentration
PL	plantlet(s)
PLB	protocorm like body(s)
R	root(s)
SB	shoot bud(s)
µM	micro molar
VW	Vacin and Went basal medium (1949)