ENHANCEMENT OF *IN VITRO* PROPAGATION EFFICIENCY AND INDUCTION OF POLYPLOIDY IN ORCHIDS

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DECLARATION

I hereby declare that this thesis entitled 'Enhancement of *in vitro* propagation efficiency and induction of polyploidy in orchids' is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled 'Enhancement of *in vitro* propagation efficiency and induction of polyploidy in orchids' is a record of research work done independently by Ms. Priya Kumari, I. (2004 - 22 - 02) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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LIST OF ABBREVIATIONS

AC	Activated Charcoal
BA	Benzyl Adenine
BAP	Benzyl Amino Purine
CD	Critical Difference
CH	Casein Hydrolysate
CS	Copper Sulphate
CW	Coconut Water
cv.	Cultivar
cvs.	Cultivars
EDTA	Ethylene Diamino Tetra Acetic acid disodium salt
et al	And others
Fig.	Figure
GA_3	Gibberellic Acid
g	Gram
HCl	Hydrochloric acid
IAA	3 - indole acetic acid
IBA	3 - indole butyric acid
KC	Knudson C medium
l^{-1}	per litre
M	Molar

mg	milligram
ml	milliliter
mM	millimolar
MS	Murashige and Skoog medium
NAA	1-Naphthyl Acetic Acid
NaOH	Sodium hydroxide
PA	Polyamines
PLB	protocorm like body
pH	potential hydrogen
viz.	Namely
VW	Vacin and Went medium
^{0}C	Degree celsius
28- HBL	28 - homobrassinolide
½ MS	Half strength Murashige and Skoog medium
2, 4 – D	2, 4 – dichlorophenoxy acetic acid

Introduction

1. INTRODUCTION

Orchids represent aristocracy in floriculture. Renowned for their spectacular flowers with brilliant colors, myriad sizes, shapes and forms, orchids rank second among the top selling cut flowers in the world. The demand for orchids at domestic level as well as for export market is steadily increasing in India. Presently, world floriculture trade involving orchids is worth 2,000 million US $ (Hegde, 2001).

Orchids are marketed both as potted plants and as cut flowers. The major orchid plant exporters are Taiwan, Thailand, United Kingdom, Italy, Japan, New Zealand, Brazil, India, Malaysia, the Ivory Coast, Sri Lanka, Peru, Mauritius, Singapore, Philippines, Madagascar and China. The largest orchid plant importer in the world is the United States. The major orchid cut flower exporters are Thailand, Singapore, Malaysia, New Zealand and Italy. The major orchid cut flower importers in the world are Japan, Italy, France, Germany, United States, United Kingdom, Netherlands, Austria, Belgium and Greece. The orchid genera *Arachnis*, *Cattleya*, *Cymbidium*, *Dendrobium*, *Phaphiopedilum* and *Phalaenopsis* are important in international trade. *Dendrobium* alone accounts for 85 % of the total trade in tropical cut flowers followed by *Mokara*, *Aranda* and *Oncidium* (Baker and Baker, 1996).

Besides having commercial importance and aesthetic value as cut flowers and potted plants, orchids have many other useful attributes. In tropical and temperate forests they play a very useful role in balancing the ecosystem. Many orchids have been used as spice, flavouring agent and food in different parts of the world and their therapeutic uses are also well known. Over 3000 types of glycosides and 60 different types of alkaloids are reported to have been isolated from orchids. Orchid flowers directly or cast to moulds are used as jewellery. In several countries, orchid species or hybrids are given the status of national flower.

Orchids belong to the largest family of angiosperms, Orchidaceae comprising of over 800 genera and 35,000 species. The family in addition has more than over one lakh hybrids. In India a total of about 200 genera and 1,700 species have been reported. There are about 60 genera and 250 species of orchids in Kerala.

The important orchid genera cultivated in Kerala are monopodials such as *Arachnis*, *Aranthera*, *Vanda*, *Mokara*, *Aranda* and sympodials such as *Oncidium* and *Dendrobium.* The varietal wealth of these genera available in Kerala is considerable. However, commercial cultivation is restricted to hybrids having demand in internal and export markets. The planting materials of these hybrids are mostly imported from leading countries in orchid trade such as Thailand, Singapore, Malaysia, New Zealand, Japan, Australia and Hawaii. The high prices of such imported planting materials limit their availability on a large scale for commercial cultivation. Apart from the exorbitant price the hybrids, whose planting materials are imported, would usually have lost their competitive value in international markets. Thus growers are forced to content with the available varieties rather than authoritatively demand for the commercially prefered varieties that have demand and value in international markets.

In India as well as in Kerala, *in vitro* propagation protocols are commercially available only for few varieties. Though a few private enterprises are involved in the commercial *in vitro* propagation of orchids, the *in vitro* protocols they apply are kept highly confidential. Also bulk of the quality plantlets developed using these protocols are exported to their parent companies who are the actual winners in international orchid markets. This highlights the fact that though private enterprises, are involved in the commercial *in vitro* propagation of orchids in India, most of them serve as out-sourcing agents of foreign companies. This necessitates the need for developing indigenous *in vitro* protocols for large scale propagation of commercially important varieties. Only then the quality planting materials can be made available to small scale orchid growers who serve as the backbone of orchid industry in Kerala.

Standardization of viable *in vitro* propagation protocol specific for varieties in demand is hence indispensable for the large-scale multiplication of disease free true-to-type plants. Together with this, improvement of their propagation efficiency can help to define protocols.

Induction of polyploidy under *in vitro* condition offers wider possibilities for crop improvement. It is a quick and reliable method of improving the cut flower attributes of cultivars and ensures the large-scale multiplication of desired cultivars and their polyploids. Induced polyploids in orchids were reported to be of better flower size, form, floriferousness, coloration and resistance to mosaic virus.

Considering the above aspects the present investigation was undertaken with the following objectives.

(i) To evolve *in vitro* propagation protocols for selected orchid cultivars

(ii) To enhance their propagation efficiency

To induce polyploidy under *in vitro* condition

Review of Literature

2. REVIEW OF LITERATURE

Orchids are the first horticultural plants cloned by tissue culture technique on a commercial scale. The major break through in the propagation of orchids is the *in vitro* germination of the orchid seed by Knudson (1922). Morel (1960) for the first time successfully demonstrated the technique of mericloning in orchids. Later Wimber (1965) modified the technique and several workers successfully followed it for the mass multiplication of desired plants.

According to Murashige (1974) there are three possible routes for *in vitro* regeneration:

(a) enhanced release of axillary buds

(b) production of adventitious shoots through somatic organogenesis and

(c) somatic embryogenesis

Prakash *et al*. (1996) reviewing the methods of tissue culture of orchids recorded that the regeneration of orchids by tissue culture occurs through two main pathways (1) direct differentiation of protocorm like bodies (PLBs) from cultured tissues and organs and their subsequent development into plantlets and (2) indirect differentiation of PLBs from explant tissue through an intermediary callus phase.

2.1. *IN VITRO* PROPAGATION

The key factors that influence *in vitro* clonal propagation include the explant, culture medium, plant growth substances, media supplements, mode of culture and culture conditions.

2. 1. 1. Explant

Explant refers to a piece of tissue consisting of non-dividing, differentiated, quiescent cells which when grown on a nutrient medium undergoes dedifferentiation and redifferentiation to a whole plant or a plant organ (Chawla, 2002). The type, size and position of the explant and the physiological age of the explants have an important role in the success of micropropagation (Devi and Deka, 2001).

Arditti and Ernst (1993) reported that i*n vitro* sourced explants regenerated rather slowly. On the other hand, Kaur and Vij (2000) reported that *in vitro* sourced leaf tissues responded far more frequently to a wide range of stimuli in comparison to greenhouse sourced tissue in *Saccolabium papillosum.* Martin et al., (2006) reported direct organogenesis from *in vitro* derived foliar explants of *Dendrobium* hybrids Sonia - 17 and 28 by the induction of PLBs and its regeneration to shoots. No significant difference was observed in the induction of direct shoots, shoot multiplication, PLB formation and subsequent development and rooting of shoots between the two cultivars.

2. 1. 1. 1. Age

Juvenility of explants is an important factor that influences the regeneration response in orchid tissue culture. This is because juvenile cells do not have rigid cell walls and hence physiologically and biochemically more active (Kaur and Vij, 2000). In addition, the use of pre-existing meristems as explant is of great importance in ensuring genetic stability of the micropropagated plants (Das and Bhattacharjee, 2006).

Leaf segments from young seedling tissue were reported to yield better PLBs than that formed by mature tissue in *Phalaenopsis* (Tanaka et al., 1988). Mature tissues required etiolation to induce primodia, which subsequently developed into PLBs.

2. 1. 1. 2. Type

The type of explant as well as the orchid species and nutritional regime in the culture medium greatly influence the time required for PLB or callus formation (Arditti and Ernst, 1993).

2. 1. 1. 2 .1. Meristem or shoot tip culture

Shoot tip culture is a well established technique for orchid micropropagation since it is useful for maintaining uniformity of genotype (Devi and Deka, 2001). Meristem or shoot tip remains the first choice as explant for commercial orchid micropropagation. However, shoot tip culture is labour intensive and hence expensive. Its application is recommended for those species for which the economic returns are high (Rajmohan et al., 2004)

Shoot tips measuring 0.3 - 0.5 cm size excised from six month old *in vitro* established *Dendrobium* cv. Betty Ho were reported to produce multiple shoots in a period of 25 - 35 days (Kurup et al., 2005). There are a number of other explants which were used successfully by different workers. These include leaf or leaf segments, roots, inflorescence and floral parts, pseudobulbs, stem or nodal segments etc. (Das and Bhattacharjee, 2006).

2. 1. 1. 2. 2. Stem Node

The use of stem nodal segments as explants for the successful micropropagation of orchids has been reported by several workers.

Stem nodal segments of *Phalaenopsis* cultured under *in vitro* condition resulted in bud enlargement within two weeks followed by leaf and root development in 6 - 20 weeks. The plantlets were ready for transfer in 30 weeks (Sagawa, 1961). Duan *et al*. (1996) used the top nodes of *Phalaenopsis* for cyclic propagation of new explants and middle nodes for producing shoots or adventitious buds. Jimenez and Guevara (1996) successfully regenerated complete

rooted plants from the stem nodal cuttings of commercial hybrids of *Phalaenopsis,* which were ready for transfer within six months of culturing *in vitro.*

Mosich et al. (1974) used entire nodes of *Dendrobium* and obtained bud growth after four weeks and plantlets developed in 45 days. Successful micropropagation using stem nodes as explants was achieved in *D. aduncum*, *D. lodigesii* and *D. transparens* (Yam and Weatherhead, 1990). Nayak et al. (1997) induced high frequency shoot proliferation from shoot segments of *D. aphyllum* and *D. moschatum* on MS medium containing thidiazuron. Pathania et al. (1998) used stem explants procured from shoots emerging from pseudobulbs for the successful micropropagation of *Dendrobium* cv. Sonia. Stem nodal segment of 1.0 - 1.5 cm length, with one node each from kiekies were used for culture initiation in *Dendrobium* hybrids (Sivamani, 2004).

Kiekies are produced from the nodal region of the stem or inflorescence axis. These tiny plantlets are not desirable on an orchid, as kiekies indicate that the plant is becoming old or it is not healthy and usually formation of kiekies suppress flower production. However, they can become effective means for multiplication (Das and Bhattacharjee, 2006).

2. 1. 1. 2. 3. Leaf

Meristem culture, though widely practiced has its limitation in the fact that it requires sacrifice of an entire new growth. Attempts were therefore made to use other plant parts like leaf, root, inflorescence etc. as explants in orchid micropropagation. Leaf segments or entire leaf as explant source to produce large number of identical clones through direct or callus mediated organogenesis was emphasized by Arditti (1967).

Regeneration potential of leaf tissues was demonstrated for the first time by Wimber (1965), who was able to produce PLBs from leaf tissues in *Cymbidium*. Pindel and Miczynski (1996) observed production of plantlets from basal part of leaf explants which were found as better explants than root explants in terms of direct shoot regeneration in *Cymbidium* hybrids.

Latha and Seeni (1991) reported the initiation of PLBs near the tip region and its quick spread in a sporadic fashion all over the sides of the leaves in *Phalaenopsis* indicating that the entire surface of the organ was potentially meristematic and regenerative. Seeni and Latha (1992) observed that cultured leaf base produced more uniform plantlets in *Renanthera.*

Park et al. (1996) produced clusters of somatic embryos without intervening callus within one month using segments of young leaves of *Oncidium* cv. Gower Ramsey. When these embryos were subcultured more embryos were produced followed by plantlets formation. Chen et al. (1999) reported direct somatic embryogenesis from leaf explants of *Oncidium* cv. Gower Ramsey in ½ MS medium. Chen and Chang (2003) observed that one cm long leaf tip segments of *Oncidium* Gower Ramsey cultured *in vitro* on a modified half MS medium with paclobutrazol at 10.0 mg l^{-1} gave doubling of embryo numbers (193.2) per dish compared to the control treatment (89.40) after three weeks in culture.

Ramsunder et al. (2000) reported poor shootlet formation from leaf bits in *Dendrobium* cv.Sonia.

Martin et al. (2006) reported rapid *in vitro* propagation of *Dendrobium* hybrids Sonia- 17 and 18 using *in vitro* derived leaf explants. Plantlets derived exhibited more than 80 per cent *ex vitro* establishment.

2. 1. 1. 2. 4. Inflorescence

Arditti et al. (1977) reported that inflorescence with dormant bud can be induced to produce plantlets in *Phalaenopsis.* Griesbach (1983) successfully produced vegetative shoots from inflorescence nodal sections in *Phalaenopsis* cv. Betty Hausermann, the leaf segments of which were later induced to form PLBs. A similar result was reported in *Phalaenopsis* hybrids, *P*. White Falco x *P*. Persistent and *P. amabilis* hybrids by Tanaka et al. (1988). Jimenez and Guevara (1996) reported successful *in vitro* regeneration from nodal cutting of senescent flowering stems of commercial hybrids of *Phalaenopsis.* Explants were cultured on modified ½ MS containing 2.9 mg l^{-1} calcium supplemented with vitamins, adenine, coconut milk,

activated charcoal, growth regulators, sucrose and glucose. The best results were given by larger stem segments. Park et al. (1998) obtained highest frequency of PLB multiplication in *Phalaenopsis* using lateral buds from flower stalks on VW medium.

Santana et al. (1999) reported formation of PLBs from floral buds of immature inflorescence in *Oncidium* cv. Gower Ramsey.

Among the shoot tips, axillary buds, leaf bits and inflorescence segments tested for regeneration the inflorescence segments produced more number of PLBs and shootlets in *Dendrobium* cv. Sonia (Ramsundar et al., 2000). Indhumathi (2002) observed that the inflorescence nodal segments produced shoots faster than the nodal segments and shoot tips in *Dendrobium* cv. Sonia.

In vitro culture of scape nodes of *Oncidium* cv. Gower Ramsey, *Dendrobium* cv. Miss Hawaii and *Phalaenopsis* hybrids, produced shoots and PLBs within two weeks in *Oncidium* and from fourth week onwards in *Dendrobium* and *Phalaenopsis* cultures (Nuraini and Shaib,1992). Chan and Lee (1996) obtained a high percentage of PLB formation using inflorescence tips in some tropical orchids viz., *Aranda* cv. Tay Swee Eng, *Mokara* cv. Khaw Phaik Suan, *Dendrobium* cv. Sonia, *Aranthera* and *Renantanda.*

2. 1. 1. 2. 5. Pseudobulb

In *Dendrobium moschatum* NAA accentuated the regeneration potential of pseudobulb explants via PLB/shoot bud formation (Vij and Sood, 1982). Kishor et al. (2007) obtained plantlet regeneration through shoot bud initiation in three year old pseudobulbs from *in vitro* culture of *Dendrobium* hybrid (*D. nobile* L. x *D. chrysotoxum* L.).

In vitro grown pseudobulb segments of *Coelogyne stricta* were successfully used for induction of shoot buds in half MS medium, supplemented with NAA 1.0 mg l^{-1} + BA 2.0 mg l^{-1} and BA individually (Basker and Bai, 2006).

2. 1. 1. 2. 6. Thin section (TS) cultures

TS cultures consists of explants of small size (0.3-1 mm) from different plant organs such as stems, leaves, inflorescences, flower primordia or floral organs, cotyledons, hypo-/epicotyl, apical zone or embryo. TS can be either longitudinal cell layer where single tissue is used as explant or transversal cell layer where cells from different tissue-types are used as explants (Teixeira da Silva, 2003).

Lekshmanan et al. (1995) used 0.6-0.7 mm thin sections from a single shoot tip (6-7 mm) of *Aranda* hybrid Deborah and obtained 13.6 PLBs per thin section and 2.7 PLBs per shoot tip.

Kanjilal et al. (1999) reported PLB formation from thin stem discs (1 - 1.5 mm) of *D. moschatum* from third week of culturing onwards. Plantlets were formed in 10 - 12 weeks after subculturing of the PLBs.

Prakash et al. (1996) reported that thin section explants of *Aranda*, *Cattleya*, *Dendrobium*, *Mokara*, *Oncidium*, *Spathoglottis* and *Vanda* hybrids showed high regenerative rate of 40 - 60 per cent.

2. 1. 2. Culture Medium

Growth and morphogenesis of plant tissues under *in vitro* is largely dependant on the composition of the culture medium. A proper tissue culture medium generally consists of both major and minor inorganic nutrients, energy sources such as carbohydrates, organic compounds like vitamins, amino acids and plant growth regulators.

In some cases complex organic additives like yeast extract, coconut milk, casein hydrolysate, corn milk, malt extract and tomato juice are used to support plant tissue growth. The composition of the growth medium is designed to sustain the plant cells, encourage cell

division, and control development of either an undifferentiated cell mass, or particular plant organs.

A number of chemically defined media and their modifications have been used successfully for orchid micropropagation. Commonly used media are Knudson C, 1946 (KC), Vacin and Went, 1949 (VW), Murashige and Skoog, 1962 (MS), Heller's (1953), Rosa and Laneri, 1977 (RL), Ichihashi, 1979 (I) and Nitsh and Nitsch, 1969 (NI). Other media like Hyponex, Knop's and White's have also been reported for orchid micropropagation. However, Knudson C (1946), Vacin and Went (1949) and Murashige and Skoog (1962) are the most commonly used media (Das and Bhattacharjee, 2006).

Sagawa and Shoji (1967) used modified VW medium for efficient protocorm development and vigorous plant growth in *Dendrobium*. Soediono (1983) also found that VW medium containing 15 per cent (by volume) coconut water was suitable for initial culture of *Dendrobium* Jacqueline Thomas. Chan and Lee (1996) obtained high percentage of PLBs in *Aranda*, *Mokara* and *Dendrobium* when cultured in VW medium. Kuriakose (1997) observed that minimum number of days for bud initiation in *Dendrobium* cv. Sonia was recorded in VW basal medium. Sivamani (2004) reported that among the various basal media tried for stem nodal segment explants of *Dendrobium* hybrids, VW medium showed early culture establishment and rapid growth recording minimum days for PLB initiation and greening and first leaf initiation.

Shylaraj et al. (2005) noted that agitated liquid VW medium could induce a large number of protocorms within two weeks, where as agitated liquid MS medium could result in the rapid multiplication of the protocorms from the shoot tips of *Dendrobium* cv. Sonia. They reported that MS solid medium was better when compared to VW medium for multiple shoot formation.

Irawati et al. (1977) reported KC medium as the best with respect to growth and survival rates in *Dendrobium*. Of the different media *viz*., MS, KC, VW and Heller's (1953) tried for culture establishment with or without growth regulators; KC medium was proved best with

respect to PLB development as well as multiple shoot development in *Dendrobium* cv. Sonia. Ramsunder *et al*. (2000) found that KC medium was the best for *Dendrobium* Sonia in terms of maximum percent explants established, number of PLBs produced and number of shootlets per explant.

Sudeep (1994) reported half MS and VW media as the best for bud initiation in *Dendrobium*. Pathania et al. (1998) obtained more shoots in ½ MS medium than with VW medium. Kishor et al. (2007) found that the regeneration capacity of pseudobulb segments of a hybrid (*D. nobile* L. x *D. chrysotaxum* L.) *in vitro* was better in half MS medium than on full MS.

Among the different strengths of MS media compared, full MS medium proved to be the best for multiple shoot induction with respect to the number of shoots per culture, number of leaves per microshoot and shoot length in *Dendrobium* (Ganga et al. 1999). In a study on the effect of various nutrient media, MS medium was found the best for *in vitro* establishment, differentiation, subsequent growth and development of mericlones of *Dendrobium* cv. Sonia (Saiprasad et al. 2001). Lekha Rani (2002) stated that half MS basal medium was best for early germination and rapid *in vitro* development in *Dendrobium* hybrids as compared to ¼ MS, full MS, KC and VW full strength.

Devi et al. (1997) reported that out of the five different semi-solid media viz. MS, WI, KC, VW and NI tested, for the clonal propagation using shoot tips of *Dendrobium moschatum and Cymbidium aloifolium,* NI medium was found best for the induction and proliferation of PLBs.

Santana et al. (1999) used KC medium for PLB induction and liquid MS for PLB multiplication and plantlet formation of *Oncidium* cv. Gower Ramsey.

Mitra's medium supplemented with 2 .0 per cent sucrose, peptone 1.0 g l^{-1}, BA 1.0 mg l^{-1}, 3 per cent guargum or 3 per cent isubgol was reported successful for the PLB initiation and shoot regeneration from leaf explants of *D. chrysotaxum* (Jain and Babbar, 2005).

2. 1. 3. Growth Regulators

The type of morphogenesis could be influenced by the concentration of plant growth regulators such as auxin and cytokinin in the medium (Skoog and Miller, 1957). A higher cytokinin to auxin ratio induces shoot morphogenesis, while higher auxin to cytokinin ratio induces root morphogenesis. The intermediate concentrations of these produce unorganized tissue.

The most commonly used growth regulators in orchid tissue culture are indole 3-butyric acid, naphthalene acetic acid, indole- 3- aceticacid, 2,4-dichlorophenoxyacetic acid , benzyl amino purine and kinetin. Growth promoting effect of these growth adjuncts was reported to be orchid specific and therefore, cannot be generalized; rather they need to be empirically determined for each species and hybrid (Prakash et al., 1996).

2. 1. 3. 1. Cytokinins

Cytokinins are adenine derivatives, mainly concerned with cell division. At higher concentrations, cytokinins induce adventitious shoot formation, but inhibit root formation. They promote axillary shoot formation by decreasing the apical dominance.

Kukulczanka and Wojciechowska (1983) obtained greatest number of shoots in *D. antennathum* and *D. phalaenopsis* by enriching the medium with BA 5.0 mg l^{-1}.

Paek et al. (1989) observed that shoot growth of *Cymbidium* hybrid was the greatest when explants were cultured on medium containing BA at 10.0 mg l^{-1} for 10 days and then transferred to a medium containing BA at 0.5 mg l^{-1}.

2. 1. 3. 2. Auxin

Auxins induce cell division and cell elongation. It often inhibits adventitious and axillary shoot formation. Generally at low auxin concentrations, adventitious root formation predominate where as at high concentrations, callus formation occurs.

Soediono (1983) found that addition of 10.0 mg l^{-1} NAA to VW medium led to rapid proliferation of PLBs in *Dendrobium* Jacqueline Thomas. Devi and Deka (1992) observed that the growth of hybrid seedlings of the cross D. *moschatum* X *D*. *amoenum* was enhanced by IAA and NAA at 1.0 mg l^{-1}. Significant increase in PLB production of *Dendrobium moschatum* was reported by Kanjilal et al. (1999) with 2, 4- D at 1.0 mg l^{-1} and IAA 2.0 mg l^{-1}. Nuraini and Shaib (1992) noted that the addition of 2.0 mg l^{-1} IBA to the medium was necessary for rooting of *Phalaenopsis* hybrid and *Phalaenopsis* cv. Miss. Hawai.

Saiprasad and Raghuveer (2002b) reported that maximum number of multiple shoots was observed in MS medium supplemented with NAA 0.5 mg l^{-1} in *Dendrobium,* NAA 1.0 mg l^{-1} in *Cattleya* and IAA 0.25 mg l^{-1} in *Oncidium.*

2.1. 3. 3. *Auxins and Cytokinins*

Lundergan and Janick (1980) noticed that the deleterious effects of high levels of cytokinin on axillary shoot growth were nullified when auxin was added to the medium.

Phalaenopsis flower stalk segments formed adventitious buds (65 per cent) and PLBs (17 per cent) when the medium was supplemented with BA 5.0 mg l^{-1} and NAA 1.0 mg l^{-1} (Vij and Pathak, 1989). Lakshmidevi (1992) observed that shoots of *Dendrobium* cultured with 2.0 mg l^{-1} NAA and 3.0 mg l^{-1} BA had a healthy appearance, exhibiting considerable vigour, rapid shoot growth and well expanded leaves. With 5.0 mg l^{-1} BA, shoot production was found to increase but short shoots with small leaves were produced.

A combination of BA 20.0 mg l^{-1} and NAA 0.1 mg l^{-1} resulted in highest rate of PLB formation (60 per cent) in *Phalaenopsis* and *Doritaenopsis* (Tokuhara and Mii, 1993).

Sounderrajan and Lokeswari (1994) observed better shoot multiplication of *Dendrobium* Madame Pompadour when the medium was supplemented with BA at 0.5 mg l^{-1} and NAA 0.1 mg l^{-1}. Kuriakose (1997) observed maximum number of vigorous shoots in ½ MS with BA 2.5 mg l^{-1} and NAA 1.0 mg l^{-1} in *Dendrobium* cv. Sonia, where as for the same variety , Pathania et al. (1998) obtained maximum number of shoots in KC medium fortified with BAP 1.0 mg l^{-1} and NAA 0.4 mg l^{-1}.

Sunithibala and Laishram (1998) induced multiple shoots from basal cut portion of shoot tip explants of six *Dendrobium* hybrids namely *D*. Kasem Gold X *D*. Thed Takiguchi, *D*. Sonia No.28, *D*. New Sabin Red, *D*. Ekapol Panda No.1, *D*. Sakura Pink and *D*. Banyad Pink, cultured in modified VW medium, supplemented with NAA (0.1 mg l^{-1}), BA (3.0 mg l^{-1}) and CW (20.0 per cent).

Santana et al. (1999) observed that PLB multiplication and plantlet formation of *Oncidium* was the best in the presence of NAA at 0.5 mg l^{-1} and BA at 5.0 mg l^{-1}.

In *Dendrobium* cv. Sonia, the combination of BAP 2.0 mg l^{-1} and NAA 1.0 mg l^{-1} in KC medium gave higher rate of explant establishment, more number of PLBs and shootlets per explant (Ramsunder et al., 2000), where as in the same variety the combination BAP 0.1 mg $l^{-1}$and NAA 1.0 mg l^{-1} in MS medium gave early meristem differentiation and the combination BAP 1.0 mg l^{-1} and NAA 1.0 mg l^{-1} produced maximum number of multiple shoots (Saiprasad et al. 2001).

Shoot tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum* Hk.f. cultured on modified KC medium produced optimum callusing in the presence of 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} BAP (66.7 per cent) (Roy and Banerjee, 2003).

Kurup et al. (2005) reported that LS medium supplemented with kinetin 1.0 mg l^{-1} and NAA 0.1 mg l^{-1} induced highest number of multiple shoots in *Dendrobium* cv. 'Betty Ho' while Shylaraj et al. (2005) observed that the combination of kinetin 2.5 mg l^{-1} and NAA 0.75 mg l^{-1} was good for direct multiple shoot formation of *Dendrobium* cv Sonia in solid MS medium. Kumaria et al. (2005) reported that in *D. wardianum* leaf explants cultured on MS medium supplemented with 2.5 µM NAA and 10.0 µM BA gave the optimum PLB formation. The nodal buds started developing in approximately 3 weeks of culture in MS medium supplemented with BA alone or in combination with IAA or NAA. Martin et al. (2005) observed that *in vitro* derived leaf explants from *Dendrobium* hybrids Sonia -17 and 28 when cultured on ½ MS medium supplemented with 44.4 µM BA developed more than seven shoots per explant.

In a comparative study on the effect of various plant growth regulators *viz*. BAP, kinetin, NAA, IAA, 2, 4 - D and GA_3 at concentrations 0.25 -1.0 mg l^{-1}. Saiprasad and Raghuveer (2002b) observed that BAP at 1.0 mg l^{-1} produced the maximum number of PLB's in *Dendrobium* cv. Sonia, *Oncidium* cv. Gower Ramsey and *Cattleya leopoldii.*

2. 1. 4. Steroid Plant Growth Regulators

A group of naturally occurring polyhydroxy steroidal substances first isolated from the pollen of rape plant (*Brassica napus* L.) was categorized as the sixth group of phytohormones, brassinosteroids. Brassinosteroids, ubiquitous in plant kingdom are reported to have pleotropic effects. (Clausse and Sasse, 1998).

They affect cell elongation, cell division and differentiation, splitting of internode (Mandava, 1988; Sakurai and Fuijoka, 1993, 1997; Altmann, 1999; Khripach et al. 2000) enhance trachiery element differentiation, act synergistically with cytokinins in the growth of cell cultures, promote ethylene biosynthesis, control microtubule orientation, alter mechanical properties of cell walls as well as other developmental processes (Mayumi and Shibaoka, 1995; Dhaubhadel et al. 1999).

Mitra et al. (1976) medium supplemented with 4.0 µM epibrassinolide resulted in successful PLB initiation and *in vitro* regeneration from shoot tip sections of *Cymbidium elegans* (Malabadi and Nataraja, 2007).

2. 1. 5. Media supplements

2. 1. 5. 1. Coconut Water (CW)

Coconut water contains inorganic ions, nitrogenous compounds, vitamins, sugars, aminoacids and related substances besides being rich in plant hormones such as auxin, 1, 3 - diphenyl urea, cytokinins and gibberellins (Lawrence and Arditti, 1984). The myo-inositol content in the coconut water is responsible for shoot emergence and its further growth (Taniguchi *et al*., 1987).

Intuwong and Sagawa (1973) suggested that the optimum concentration of CW for orchids in the medium was 10 - 15 per cent and it was added before autoclaving. CW induces division in other wise non-dividing cells and thus enhances mass multiplication of PLBs (Sagawa and Kunisaki, 1982). The efficacy of coconut water on *in vitro* multiplication was studied in *Cymbidium pendulum, Paphiopedilum spicerianum and Thunia alba* (Kanika, 1998), *Dendrobium* sp. (Devi et al., 1990), *Rhynchostylis retusa* (Nath et al., 1991) and *Dendrobium chrysotoxum* (Kher, 1999).

Kim et al. (1970) noticed that the buds excised from bulbs and leaf axils of *Dendrobium* cultured on VW liquid medium supplemented with 15 per cent CW produced PLBs in 4-5 weeks and plantlets in 8 weeks. Soediono (1983) also reported that for the initial culture of *Dendrobium* Jacqueline Thomas supplementation of media with CW at 15 per cent is suitable. While the same concentration was reported to initiate callusing in shoot meristems of *Dendrobium joanie* Ostenhault (Sharon and Vasundhara, 1990). Shylaraj et al. (2005) found that for the fast morphogenesis from protocorms of *Dendrobium* cv. Sonia, MS solid medium supplemented with 20 - 25 per cent coconut milk was most effective.

CW at 15 per cent resulted in best PLB formation and further multiplication in *Oncidium* (Bagde and Sharon, 1997).

Nagaraju and Mani (2007) reported that in *Cymbidium* cv. Showgirl Cooks Bridge, maximum number of PLB's and shoot and root differentiation was obtained in KC medium supplemented with 200 ml l^{-1} of CW.

2. 1. 5. 2. Aminoacids

Cultured tissues are normally capable of synthesizing the aminoacids necessary for various metabolic processes. The addition of aminoacids to the media is important for stimulating cell growth. Unlike inorganic nitrogen, aminoacids are taken up more rapidly by cells. Casein hydrolysate, CH (0.05 – 0.1 per cent), L - Glutamine (8 mmol l^{-1}), L - asparagine (100 mmol l^{-1}), L - glycine (2 mmol l^{-1}), L - arginine and L - cysteine (10 mmol l^{-1}) are common sources of organic nitrogen used in culture media (Razdan, 1993).

In *Dendrobium* cv. Sonia, CH at 100 mg l^{-1} registered maximum number of shoots. It was also observed that the shoot proliferation rate decreased on increasing the level of CH (Kuriakose, 1997). Kurup et al. (2005) reported that MS basal medium supplemented with BA 5.0 mg l^{-1} along with casein hydrolysate 1.0 - 2.0 g l^{-1} produced maximum number of multiple shoots in *Dendrobium* cv. Betty Ho, indicating that cytokinin in combination with CH is beneficial for multiple shooting.

Saiprasad and Raghuveer (2002a) reported that addition of aminoacids viz. glutamine, asparagine and phenyl alanine (1.0 - 5.0 mg l^{-1}) produced more number of shoots than their respective controls in the three orchid genera such as *Dendrobium* cv. Sonia, *Oncidium* cv. Gower Ramsey and *Cattleya leopoldii.* In *Dendrobium* cv. Sonia, glutamine at 1.0 mg l^{-1} resulted in 7 and 3 fold increase in PLB and multiple shoot production respectively.

2. 1. 5. 3. Carbon sources

Plant cells and tissues in the culture medium lack autotrophic ability and therefore need external carbon source for energy. The addition of external carbon source enhances proliferation of cells and regeneration of green shoots. They also act as osmoticum that can stimulate and regulate morphogenesis (Wethrell, 1984). Carbon sources such as sucrose, glucose and fructose are very important components in *in vitro* culture media. Glucose and fructose differ from sucrose in their energy potential and ability to modify medium osmoticum. An addition of 10.0 g l^{-1} glucose/ fructose is osmotically equivalent to that of 20.0 g l^{-1} sucrose and the molar equivalents of monosaccharides yield half the energy of disaccharides (Ichihasi and Hiraiwa, 1996).

The exact requirement of sucrose in orchids varied with the species (Kano, 1965) and 2.0 per cent sucrose was most suitable for *Cymbidium* hybrid and *Dendrobium* while for *Brassavola*, *Brassolaeliocattleya* and *Paphiopedilum* 4.0 per cent sucrose was found optimum. Sucrose concentrations of 20.0 and 30.0 g l^{-1} are the most commonly used in orchid tissue culture studies (Arditti, 1974).

Ishii et al. (1998) observed that in *Phalaenopsis* the presence of sucrose in the culture medium caused protocorm formation and its absence caused callus formation. Bhattacharjee et al. (1999) demonstrated that sucrose at 20.0 g l^{-1} was optimum for PLB formation and leaf development in *Phalaenopsis*.

Madhuri et al. (1990) reported that the best culture medium combination for the micropropagation of *Dendrobium joannie* Ostenhault was 3.0 per cent sucrose supplemented with 1.0 mg l^{-1} of IAA, IBA and NAA. Wang et al. (2000) found that in *D. officinale* sucrose level of 20.0 - 40.0 g l^{-1} was ideal for multiplication and *in vitro* conservation. Faria et al. (2004) verified a positive relationship between increase in sucrose concentration and increase in plant height and concluded that sucrose at a concentration of 60.0 g l^{-1} in half MS basal medium was the most effective for increasing height and fresh weight of *in vitro* raised *D. nobile* plantlets. High values of growth parameters *viz.* crop growth rate, net assimilation rate

and relative growth rate as well as maximum survival percentage was observed for *in vitro* plants of *Dendrobium* cv. Sonia - 17 grown in rooting media supplemented with 40.0 g l^{-1} of sucrose (Samasya, 2007).

According to Vij et al. (1996) *Vanda cristata* Lindl. plantlets obtained from protocorms cultured in culture medium with a reduced sucrose concentration from 0.5 to 2.0 g l^{-1} showed a success rate of 80 per cent when transferred to green house conditions.

Nagaraju (2007) concluded that in *Cymbidium* hybrids, MS medium supplemented with 40.0 g l^{-1} sucrose is optimum for PLB multiplication, plantlet differentiation and conservation of PLBs and plantlets for over 15 months.

2. 1. 5. 4. Polyamines (PA)

Polyamines are low molecular weight nitrogen-containing molecules, that affect the cell activity and modulate the plant development (Bais and Ravishankar, 2002; Mendoza and Rocha, 2002). They have been shown to be associated with induction of cell division, growth and differentiation of plant cells (Tran Thanh Van and Trinh, 1990). It is known that application of PA to abiotically stressed plants can increase their growth and yield, and minimize the damage caused by the abiotic stress (Montenegro, 1995; Norato and Romero, 1995; Mendoza et al., 2000; Navakoudis et al., 2003). In addition, pathogen attack in plants leads to a defense response that includes several metabolic changes like accumulation of PA and variation in its content (Greenland and Lewis, 1984; Legaz et al., 2002; Walters, 2000).

Saiprasad (2004) reported maximum number of PLB's in *Dendrobium* cv. Sonia when MS basal medium having 4.44 μ*m* BAP was supplemented with putrescine 0.4 mM.

Wei *et al*. (2007) reported that in suspension cultures of PLBs from *Dendrobium huoshanense* spermine at 0.6 m mol L^{-1} was the most effective in increasing the cell growth and polysaccharide synthesis by 1.32 fold and 1.31 fold respectively, than that of the control on day 30. They observed that invertase and nitrate reductase activities were found to increase

significantly in the cultured cells treated with spermine, which was beneficial to the utilization of carbon and nitrogen source.

2. 1. 5. 5. Activated Charcoal (AC)

Effect of adding AC to plant tissue culture medium depends upon the species, the stage of development and the concentration. The addition of AC is beneficial for those species with a high release of phenolic compounds in the culture medium such as *Phalaenopsis* (Ernst, 1975). AC can adsorb phenolic compounds and also stabilize pH. Tanaka *et al*. (1988) reported that activated charcoal inhibited shoot formation in *Phalaenopsis* hybrids.

In *Cymbidium* AC at 0.1 - 0.3 per cent was reported to be necessary for producing healthy plantlets and for stimulating shoot growth, but reduced the rhizome growth (Peak and Kozai, 1998).

2. 1. 5. 6. Solidifying agents

Solidifying or gelling agents are commonly used for preparing semi-solid or solid tissue culture media. They support the tissues growing in static conditions. A change in the agar concentration affects the nutrients in it as well as the overall nutrient concentration of the media (Razdan, 1993).

Agar and gelrite are the commonly used solidifying agents for orchid *in vitro* culture. Agar is obtained from red purple algae of *Rhodophyceae* and is a polysaccharide of galactose. Gelrite is obtained from *Pseudomonas elodea* and it is an extra cellular polysaccharide of glucose, rhamnose and glucoronic acid (Oohashi et al., 1986).

Gelrite at 4.0 g l^{-1} was reported to give better growth than agar, after 8 and 12 weeks of callus culture from flower stalks of *Phalaenopsis* and *Doritaenopsis* hybrids (Ichihashi and Hiraiwa, 1996). Though gelrite has many desirable qualities, it is reported to cause hyperhydricity and vitrification (Pasqualetto et al., 1998).

2. 2. *In vitro* rooting

Roots may develop in shoots in the shoot multiplication media itself. However, in many cases separate rooting phase is needed to develop plantlets with strong and functional root system. Generally there are three stages for rooting-induction, initiation and elongation.

2. 2. 1. Plant growth substances

Among various auxins, IBA and NAA are the most effective for root induction (Ancora et al., 1981).

Sunithibala and Laishram (1998) observed development of 2-3 roots during the fourth and fifth subculture of shoots of *Dendrobium* hybrids in shoot multiplication medium supplemented with NAA 0.1 mg l^{-1} and BAP 3.0 mg l^{-1}.

Kukulczanka and Wojciechowska (1983) reported more number of roots in medium supplemented with 1.75 mg l^{-1} NAA and 1.75 mg l^{-1} IBA in *D. antennathum* and *D. phalaenopsis.* Sharon and Vasundhara (1990) observed that NAA 5.62 μM favoured rooting in *Dendrobium joanie* Ostenhault. Lim et al. (1993) opinioned that IBA at 0.1 mg l^{-1} was the best for producing many long rooted shoots in *Dendrobium,* where as Mujib and Jana (1994) induced roots in *Dendrobium* cv. Madame Pompadour in the presence of NAA 0.1 mg l^{-1} .

Kuriakose (1997) found ½ MS with NAA 1.0 mg l^{-1} best for root regeneration in *Dendrobium* cv. Sonia - 17. Pathania *et al*. (1998) reported that in *Dendrobium* cv. Sonia, KC medium supplemented with IBA 2.0 mg l^{-1} favoured maximum rooting (98 per cent) as well as number of roots per culture (3.20) after 30 days of subculture. Martin et al. (2005) reported that ½ MS with AC 2.0 g l^{-1} was the best for rooting of shoots of *Dendrobium* cvs. Sonia - 17 and Sonia - 28.

Sunithibala and Laishram (1998) induced rooting in *in vitro* shoots of six *Dendrobium* hybrids namely *D*. Kasem Gold X *D*. Thed Takiguchi, *D*. Sonia No.28, *D*. New Sabin Red, *D*. Ekapol Panda No.1, *D*. Sakura Pink and *D*. Banyad Pink, cultured in modified VW medium, supplemented with IBA and IAA together at a concentration of 2.0 mg l^{-1} each. Kurup *et al.* (2005) rooted 70 day old *in vitro* shoots of *Dendrobium* cv. Betty Ho in MS medium containing BA 0.1 mg l^{-1} and NAA 0.5 mg l^{-1}.

2. 3. Hardening and planting out

The environment inside a tissue culture container is one with high humidity, low light levels and usually constant temperature. The plantlets coming out of such an environment as a result will be very poorly adapted to resist the low relative humidity, high light levels and more variable temperature found *in vivo* (Wainwright, 1988). The tissue cultured plants are characterized by physiological peculiarities such as heterotrophic nature, defective epicuticular wax formation, less number of mesophyll and palisade cells in the leaves, inability of stomata to close under stress condition, poor development of vascular connections between the roots and the shoots, which affect the water economy of the plants. So to improve the survivability of plantlets, light, temperature and relative humidity are to be controlled during acclimatization. Humidity acclimatization of transferred plantlets is done through polythene tent, misting and fogging. In polythene tent, as the aerial environment is closed, it is possible to take advantage of carbon dioxide enrichment during hardening (Lakso et al., 1986).

The media for planting out is also an important factor that determines the success rate of hardened plants. The media should provide good drainage, sufficient moisture retention and also good aeration. Under hot tropical climatic conditions, the best potting material is a combination of charcoal, detanned coconut husk and brick pieces or polystyrene lumps (Mercy and Dale, 1997).

Kuriakose (1997) obtained 100 percent survival rate of hardened plantlets of *Dendrobium* cv. Sonia transferred to coconut husk. Pathania et al. (1998) reported that

combination of bark pieces, brick pieces, moss and charcoal pieces (1:1:1:1) proved to be promising for successful establishment of *in vitro* rooted plantlets of *Dendrobium* cv. Sonia. Shobhana (2000) obtained a survival rate of 94 per cent in selfed hybrids of *Dendrobium* cv. New Pink, planted out in a medium with equal proportion of charcoal and brick pieces. Saiprasad et al. (2001) obtained hundred per cent survival and better growth of *in vitro* derived plantlets of *Dendrobium* cv. Sonia in either wood charcoal pieces alone or crushed tree bark alone. Shylaraj et al. (2005) reported that keeping rooted *in vitro* plantlets in sterile coconut husk fibre supplemented with liquid ½ MS without sucrose for two weeks was ideal for hardening. Such hardened plants kept in moisture chamber in small pots filled with coconut husk fibre, charcoal and brick pieces covered with polythene sheets and irrigated with tap water resulted in 80 per cent survival rate.

2. 4. *In vitro* polyploidisation

In vitro polyploidisation is an alternative tool to obtain polyploid plants (Murashige and Nakano, 1966). It is reported to offer a quick and reliable method of improving the cut flower attributes and at the same time ensure the large-scale multiplication of desired cultivars and their polyploids.

In vitro induction of tetraploids was first achieved in tobacco by Murashige and Nakano (1966). Unlike i*n vivo* polyploidisation which results in mixed population of diploids, tetraploid, aneuploids and chimeras, *in vitro* induction of polyploidy offers wider possibilities to polyploidy breeding. It helps in achieving rapid homozygosity in one-step in case of polyploidisation of haploids. It speeds up the heterosis breeding of ornamentals, reduces the number of aberrant plants and reduces the time span required for polyploid production. Moreover, *in vitro* method offers the possibility of utilizing approaches such as polyploidization of somatic embryos (Eeckhaut et al., 2004) and the domestication and early exploration of wild germplasm (Escandon et al., 2005). *In vitro* polyploidization has been realized in several ornamental crops such as *Rosa* (Roberts et al., 1990), *Cyclamen persicum* (Takamura and Miyajima, 1996), *Alstroemeria* (Ishikawa et al., 1999), *Rhododendron*

(Vainola, 2000; Eeckhaut et al., 2001), *Syringa* (Rose et al., 2000 a), *Buddleia* (Rose et al., 2000 b), *Alocasia* (Thao et al., 2003) and *Bacopa monnieri* (Escandon, 2006).

In orchids, *in vitro* induction of polyploidy using colchicine was reported in *Dendrobium* (Sanguthai *et al.*, 1973), *Phalaenopsis* (Griesbach, 1981), *Paphiopedilum* (Watrous and Wimber, 1988), *Cymbidium* (Wimber and Van Cott, 1966; Kim et al., 1997; Silva et al., 2000) and *Phaius tankervilliae* (Devi and Deka, 2000).

Tetraploid *Cymbidium* plants are reported to be more superior to their diploid counterparts with respect to spike habit, color, substance, size and shape of flowers (Dunn, 1979). Tetraploid florets of *Dendrobium* 'Caesar' were observed to have longer life than the diploid florets due to the lower rate of respiration by thicker petals and higher fresh and dry weights (Ketsa et al., 2001).

Induced polyploids in orchids were reported to be of larger flower size with rounder conformation and greater substance than the diploids (Wimber and Wimber, 1967). Griesbach (1985) achieved perfection in size, substance and form as a result of autopolyloidy in white *Phalaenopsis* orchids.

The tetraploid *Ionocidium* (hybrid cross of *Oncidium flexuosum* and *Ionopsis utricullariosides*) cv. Popcorn produced by colchicine treatment of PLBs were reported to be larger than diploids with respect to flower size, floral parts, pedicel, stomatal apparatus size, epidermal size, leaf thickness and cell size (Fan et al., 2003). The overall flower size and weight of colchicine induced tetraploids of *Spathoglottis* 'Lion' was found to be significantly improved over that of diploids (Zong et al*.,* 2003).

2. 4. 1. Polyploidy inducing agents

The commonly used method for inducing polyploidy is by using colchicine. The method is referred as colchiploidization and the induced polyploids as colchiploids. This method of artificial induction of polyploidy using colchicine was developed by Blakeslee and Avery

(1937). Since then it had become an important tool for the experimental study of polyploidy and its induction in plants.

Colchicine is an alkaloid extracted from meadow saffron (*Colchicum autumnale*) which inhibits mitosis at anaphase by hampering the development of nuclear spindle. Mitosis that takes place after treatment with colchicine is called C-mitosis. The chromosomes form x-shaped structure, since chromatids are connected at centromeres. They repel each other; the chromatids finally part, but do not segregate. They become enclosed by a new nuclear membrane and proceed to interphase state. Thus the chromosomes are doubled.

Ever since Blakeslee and Avery (1937) discovered the polyploidization potential of colchicine, many other compounds have been experimented for the same. Of these, oryzalin is found to be more efficient and less phytotoxic compared to colchicine and is preferred over colchicine for induction of polyploidy in many crop plants (Van Tuyl et al., 1992, Tosca et al., 1995, Takamura et al., 2002).

In orchids the protocorms of *Cymbidium* (Wimber and Van Cott, 1966), *Dendrobium* (Sanghuthai et al., 1973), *Phalaenopsis* (Griesbach, 1981; 1985) and *Paphiopedilum* (Watrous and Wimber, 1988) were induced to double their chromosome number in liquid culture medium containing colchicine and thereby regenerated tetraploid plants. The concentrations of colchicine as well as the duration of treatment are important factors that should be determined for each type of material (Dermen, 1940). Higher concentrations or prolonged treatments were reported to be lethal to sensitive plant tissue and in some cases show undesirable mutagenic activity (Van Tuyl et al., 1992).

The other polyploidy inducing agents include nitrous oxide (Taylor et al., 1976), carbamate herbicides such as chlorpropham and propham (Vaughn and Lehnen, 1991), dinitroaniline herbicides such as oryzalin (Bouvier et al., 1994; Van Tuyl et al., 1992) and trifluralin (Hansen and Andersen, 1996) and phosphoric amide such as amiprophos-methyl (Hansen et al., 2000).

Watrous and Wimber (1988) obtained more than 50 per cent of tetraploids when *Paphiopedilum* cultures in liquid medium were treated using colchicine 0.05 per cent for 3 to 10 days.

Kim et al. (1997) reported induction rates of tetraploids as 5.2, 4.5 and 6.7 per cent in colchicine treatments of 0.05 per cent for 1 week, 0.01 per cent for 2 weeks and 0.1 per cent for 1 week respectively, using rhizomes of *Cymbidium kanran.*

Silva et al. (2000) produced mixoploids and tetraploids of *Cattleya intermedia* by treating PLBs with 0.05 and 0.1 per cent colchicine.

Devi and Deka (2000) reported 40 per cent polyploid regenerants in *Phaius tankervilliae*, a therapeutically important orchid, when the PLBs were treated with 50.0 mg l^{-1} colchicine for 5 days.

2. 4. 2. Estimation of polyploidy

Chromosome counting in mitotic cells of root tips is an accurate procedure to determine the ploidy, but it is time consuming and requires much experience.

The area of stomata has been used to differentiate diploid and tetraploid regenerants of orchids (Watrous and Wimber, 1988), rye-grasses (Speckmann *et al*., 1965), daylilies (Arisumi, 1972; Chen and Goeden- Kallemeyen, 1979), barley (Borrino and Powell, 1988) and banana (Hamill et al., 1992; Vandenhout et al., 1995; Van Duren *et al*., 1996). The number of chloroplasts per guard cell pair (Jacobs and Yoder, 1989; Singsit and Veilleux, 1991; Compton et al., 1996) and differences in stomata density Vandenhout et al., 1995; Van Duren et al., 1996) have also been used as criteria in the distinction between diploid and tetraploid plants.

The increase in stomatal density (Silva et al., 2000) and the increase in stomatal size (Dunn, 1979; Pandita, 1986; Kim et al*.,* 1997; Devi and Deka, 2000) of tetraploids over diploids were reported as preliminary identification criteria for tetraploids in many orchids.

Another simple and rapid method of estimating ploidy level is by using spectrophotometry. This method relies on the fact that the DNA content increases proportionately with increase of ploidy level (Raza et al., 2003).

Flow cytometry (FCM) offers a valuable, rapid, simple, accurate and less expensive alternative for the estimation of ploidy levels in tissues. It involves the analysis of fluorescence and light-scattering properties of single particles during their passage within a narrow, precisely defined, liquid stream (Galbraith et al., 1983; Dolezel et al., 1989; Dolezel, 1991).

Heller (1973) was the first to use flow cytometry for DNA analysis in plant cells; subsequently it was used for different plant species (Galbraith, 1990; Arumuganathan and Earle, 1991). In most plants the analysis of relative DNA content of nuclei isolated from young leaf tissue yields a histogram showing a dominant peak corresponding to nuclei at the G_1 phase of the cell cycle (Dolezel, 1991). Ploidy level can be deduced by comparing peak position of G_1 nuclei of a plant with known ploidy with that of unknown sample. FCM does not require dividing cells; it is non-destructive and can detect mixoploidy (Dolezel, 1997).

Materials and Methods

3. MATERIALS AND METHODS

The present investigations were carried out in the Department of Pomology and Floriculture and the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2005 - 2008. The objective of the study was to standardize protocols for *in vitro* propagation, enhancement of its efficiency and induction of polyploidy in selected varieties of orchid genera *Dendrobium*, *Phalaenopsis* and *Oncidium.*

The materials and methods utilized for the study are described in this chapter.

3.1. *IN VITRO* PROPAGATION

The study was mainly focused on *in vitro* propagation which was done through enhanced release of axillary buds.

3. 1. 1. Varieties

In *Dendrobium,* varieties which are important as commercial cut flowers were selected. In the genera *Phalaenopsis* and *Oncidium*, which are commonly grown as potted plants, popular varieties were selected.

Dendrobium: Earsakul, Rungnappa Red, Sheriffa Fatimah, Miss Snow White, Burana Jade, *D. philippica*

Phalaenopsis: Happy Valentine x Zaeuberrot, Red Jewel, White Cygnus, Leopard King x Brother Stripe, Terrapot Blue x White Cygnus

Oncidium: Golden Shower, Gower Ramsey, Sharry Baby

3. 1. 2. Explant

Stem nodal segments of 1.0 - 2.0 cm length and with atleast one node were excised from shoots of 2 - 3 weeks old age for use as explants .

3. 1. 2. 1. Collection and preparation of explants

The stem nodal explants were collected from basal shoots of mother plants procured from commercial orchid growers. The shoots with 8-12 cm length having 3-5 nodes were harvested and wiped with 100 per cent alcohol. The basal root portion and leaves were removed and shoot portion cut to small pieces with single node was immersed in 'Labolene' solution diluted 1000 times for 30 minutes, washed thoroughly in running tap water and then in glass distilled water. They were transferred to distilled water in clean beakers until surface sterilization and inoculation were carried out.

3. 1. 2. 2. Surface sterilization

Surface sterilization of the explants was carried out inside a laminar air flow chamber just before inoculation. The explants were transferred to sterilized beaker and surface sterilized using mercuric chloride. The solution was drained and the explants were washed four to five times with sterile distilled water. The explants were transferred to sterile petri plate.

3. 1. 2. 3. Inoculation and incubation

All the culture operations were carried out inside a laminar air flow chamber. The vessels and tools (beakers, petri plates, blades, forceps etc.) required for culture operations were washed thoroughly, rinsed with glass distilled water, covered with polypropylene cover and autoclaved at 121^{o} C temperature and 1.06 kg cm^{-2} pressure for 45 minutes. They were further flame sterilized with alcohol prior to inoculation inside the laminar air flow chamber. To inoculate the explants on the culture medium, the cotton plugs of the culture vessels were removed and the mouth was flamed. The explants were inoculated into the medium using

sterile forceps. The mouth of the culture vessels was flamed again and cotton plugs were replaced.

The cultures were then transferred to the culture rooms provided with controlled conditions of light, temperature and humidity. A photoperiod of 15 hours light and 9 hours darkness with a light intensity of 3000 lux under fluorescent light was provided. A uniform temperature of 26 +/- 2^{o} C and a relative humidity of 75 per cent were maintained in the culture room.

3. 1. 3. Culture Media

The basal media used for the study were MS (Murashige and Skoog, 1962), VW (Vacin and Went, 1949) and KC (Knudson C, 1946). The chemicals used for the preparation of the culture media were of analytical grade obtained from Sisco Research Laboratory (Mumbai) and Merck (Mumbai).

3. 1. 3. 1. Media preparation

Standard procedures were followed for the preparation of media (Thorpe, 1980). Stock solutions of major and minor nutrients were prepared by dissolving the required quantity of chemicals in specified volume of double glass distilled water. Plant growth substances were first dissolved in dilute acid/ alkali and volume made up with double glass distilled water. The stock solutions were stored under refrigerated condition (4 ^{0} C).

The culture vessels used were 'Borosil' brand test tubes (25 x 150 mm) and Erlenmeyer flasks (100 ml). They were washed with Labolene diluted 1000 times and tap water, rinsed with glass distilled water and autoclaved at 121 ^{0} C temperature and 1.06 kg cm^{-2} pressure for 45 minutes.

All items of glassware and vessels used for the preparation of culture media were washed thoroughly in 'Labolene' diluted 1000 times and tap water and then rinsed with glass distilled water. Specific quantities of stock solutions were pipetted out into a 1000 ml beaker. Sucrose, myo-inositol and copper sulphate were added fresh and dissolved. Coconut water when used was collected from freshly harvested tender coconut (6 months old) of West Coast Tall variety, filtered and added to the medium. The volume was made up to 1000 ml using glass distilled water. The pH of the medium was adjusted between 5.6 – 5.8 using 0.1 N NaOH or 0.1 N HCl with the aid of an electronic pH meter.

For the preparation of VW and KC media the chemicals were taken in required quantity and dissolved in double distilled water. Growth regulators, sucrose and myo-inositol were added fresh and the volume was made to 1000 ml.

The medium was heated by placing the vessels on a heating mantle with constant stirring using a glass rod till the agar melted. Activated charcoal (AC) when used in the medium was added at this stage and stirred well for uniform distribution. The medium was then poured into the pre-sterilized culture vessels at the rate of 15 ml for test tubes and 40 ml for Erlenmeyer flasks.

The mouth of the culture vessels were plugged tightly with sterilized cotton, covered with aluminium foil and paper, labeled and autoclaved at 121 ^{0} C temperature and 1.06 kg cm^{-2} pressure for 20 minutes. After sterilization, the culture vessels were transfered to the culture room.

Steroid plant growth regulators and polyamines were filter sterilized and stored in sterilized bottles in refrigerator. The required quantities were added prior to subculturing to the cool melted media.

3. 1. 4. Culture establishment

3. 1. 4. 1. Standardization of explants for culture establishment

Explants used were:

Young leaves
Stem nodes
Inflorescence stalk
Roots (*in vitro*)
Thin sections (*in vitro*)

3. 1. 4. 2. Standardization of surface sterilants for culture establishment

Surface sterilants used were:

Mercuric chloride
Ethyl alcohol

The percentage of surviving explants, percentage of explants which initiated buds, number of shoots regenerated and general appearance of regenerated shoots was recorded. Based on these results, the best responding explant and the most effective sterilant were identified and selected for the further treatments of culture establishment stage.

3. 1. 4. 3. Standardization of plant growth regulators for culture establishment

The treatments for standardization involved different levels of cytokinins (BA and kinetin) alone and in combination with auxins (NAA and IAA). The treatments were replicated three times. Each replication had single explant. The treatments tried to assess the effect of plant growth regulators on culture establishment of *Dendrobium* cvs. were given in Tables 1, 2, 3 and 4.

Observations were recorded on the percentage of surviving cultures, number of days for bud initiation, number of shoots per culture four weeks after inoculation and nature of shoots developed.

Table 1. Treatments tried to assess the effect of plant growth regulators on culture establishment of *Dendrobium* cv. Rungnappa Red

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Plant growth regulators (mg l^{-1})
CRT 0	CONTROL
CRT 1	BA 0.5
CRT 2	BA 1.0
CRT 3	BA 2.0
CRT 4	BA 4.0
CRT 5	kinetin 0.1
CRT 6	kinetin 0.5
CRT 7	kinetin 1.0
CRT 8	kinetin 2.0
CRT 9	kinetin 4.0
CRT10	NAA 0.1
CRT 11	NAA 0.5
CRT 12	NAA 1.0
CRT 13	NAA 2.0
CRT 14	IAA 1.0
CRT 15	IAA 2.0
CRT 16	BA 0.5 + NAA 0.5
CRT 17	BA 1.0 + NAA 0.5
CRT 18	BA 2.0 + NAA 0.5
CRT 19	BA 4.0 + NAA 0.5
CRT 20	BA 0.5 + NAA 1.0
CRT 21	BA 1.0 + NAA 1.0
CRT 22	BA 2.0 + NAA 1.0
CRT 23	BA 4.0 + NAA 1.0
CRT 24	BA 0.5 + NAA 2.0

CRT 25	BA 1.0 + NAA 2.0
CRT 26	BA 2.0 + NAA 2.0
CRT 27	BA 4.0 + NAA 2.0
CRT 28	kinetin 0.5 + NAA 0.1
CRT 29	kinetin 1.0 + NAA 0.1
CRT 30	kinetin 2.0 + NAA 0.1
CRT 31	kinetin 0.5 + NAA 0.5
CRT 32	kinetin 1.0 + NAA 0.5
CRT 33	kinetin 2.0 + NAA 0.5
CRT 34	kinetin 4.0 + NAA 0.5
CRT 35	kinetin 0.5 + NAA 1.0
CRT 36	kinetin 1.0 + NAA 1.0
CRT 37	kinetin 2.0 + NAA 1.0
CRT 38	kinetin 4.0 + NAA 1.0
CRT 39	kinetin 0.5 + NAA 2.0
CRT 40	kinetin 1.0 + NAA 2.0
CRT 41	kinetin 2.0 + NAA 2.0
CRT 42	kinetin 4.0 + NAA 2.0
CRT 43	BA 0.5 + IAA 1.0
CRT 44	BA 1.0 + IAA 1.0
CRT 45	BA 2.0 + IAA 1.0
CRT 46	BA 4.0 + IAA 1.0
CRT 47	BA 0.5 + IAA 2.0
CRT 48	BA 1.0 + IAA 2.0
CRT 49	BA 2.0 + IAA 2.0
CRT 50	BA 4.0 + IAA 2.0
CRT 51	kinetin 0.5 + IAA 1.0
CRT 52	kinetin 1.0 + IAA 1.0
CRT 53	kinetin 2.0 + IAA 1.0
CRT 54	kinetin 4.0 + IAA 1.0
CRT 55	kinetin 0.5 + IAA 2.0
CRT 56	kinetin 1.0 + IAA 2.0
CRT 57	kinetin 2.0 + IAA 2.0
CRT 58	kinetin 4.0 + IAA 2.0

Table 2. **Treatments tried to assess the effect of plant growth regulators on culture establishment of *Dendrobium* cv. Miss Snow White**

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Plant growth regulators (mg l^{-1})
CST 0	CONTROL
CST 1	BA 0.5
CST 2	BA 1.0
CST 3	BA 2.0
CST 4	BA 4.0
CST 5	kinetin 0.1
CST 6	kinetin 0.5
CST 7	kinetin 1.0
CST 8	kinetin 2.0
CST 9	kinetin 4.0
CST10	NAA 0.5
CST 11	NAA 1.0
CST 12	IAA 1.0
CST 13	IAA 2.0
CST 14	BA 0.5 + NAA 0.5
CST 15	BA 1.0 + NAA 0.5
CST 16	BA 2.0 + NAA 0.5
CST 17	BA 4.0 + NAA 0.5
CST 18	BA 0.5 + NAA 1.0
CST 19	BA 1.0 + NAA 1.0
CST 20	BA 2.0 + NAA 1.0
CST 21	BA 4.0 + NAA 1.0
CST 22	BA 0.5 + NAA 2.0
CST 23	BA 1.0 + NAA 2.0
CST 24	BA 2.0 + NAA 2.0

CST 25	BA 4.0 + NAA 2.0
CST 26	kinetin 0.5 + NAA 0.1
CST 27	kinetin 1.0 + NAA 0.1
CST 28	kinetin 2.0 + NAA 0.1
CST 29	kinetin 0.5 + NAA 0.5
CST 30	kinetin 1.0 + NAA 0.5
CST 31	kinetin 2.0 + NAA 0.5
CST 32	kinetin 4.0 + NAA 0.5
CST 33	kinetin 0.5 + NAA 1.0
CST 34	kinetin 1.0 + NAA 1.0
CST 35	kinetin 2.0 + NAA 1.0
CST 36	kinetin 4.0 + NAA 1.0
CST 37	kinetin 0.5 + NAA 2.0
CST 38	kinetin 1.0 + NAA 2.0
CST 39	kinetin 2.0 + NAA 2.0
CST 40	kinetin 4.0 + NAA 2.0
CST 41	BA 0.5 + IAA 1.0
CST 42	BA 1.0 + IAA 1.0
CST 43	BA 2.0 + IAA 1.0
CST 44	BA 4.0 + IAA 1.0
CST 45	BA 0.5 + IAA 2.0
CST 46	BA 1.0 + IAA 2.0
CST 47	BA 2.0 + IAA 2.0
CST 48	BA 4.0 + IAA 2.0
CST 49	kinetin 0.5 + IAA 1.0
CST 50	kinetin 1.0 + IAA 1.0
CST 51	kinetin 2.0 + IAA 1.0
CST 52	kinetin 4.0 + IAA 1.0
CST 53	kinetin 0.5 + IAA 2.0
CST 54	kinetin 1.0 + IAA 2.0
CST 55	kinetin 2.0 + IAA 2.0
CST 56	kinetin 4.0 + IAA 2.0

Table 3. Treatments tried to assess the effect of plant growth regulators on culture establishment of *Dendrobium* cv. Earsakul

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Plant growth regulators (mg l^{-1})
CET 0	CONTROL
CET 1	BA 1.0
CET 2	BA 2.0
CET 3	BA 4.0
CET 4	kinetin 1.0
CET 5	kinetin 2.0
CET 6	kinetin 4.0
CET 7	NAA 0.1
CET 8	NAA 0.5
CET 9	IAA 1.0
CET10	IAA 2.0
CET 11	BA 1.0 + NAA 0.1
CET 12	BA 2.0 + NAA 0.1
CET 13	BA 4.0 + NAA 0.1
CET 14	BA 1.0 + NAA 0.5
CET 15	BA 2.0 + NAA 0.5
CET 16	BA 4.0 + NAA 0.5
CET 17	kinetin 1.0 + NAA 0.1
CET 18	kinetin 2.0 + NAA 0.1
CET 19	kinetin 4.0 + NAA 0.1
CET 20	kinetin 1.0 + NAA 0.5
CET 21	kinetin 2.0 + NAA 0.5
CET 22	kinetin 4.0 + NAA 0.5
CET 23	BA 1.0 + IAA 1.0
CET 24	BA 2.0 + IAA 1.0

CET 25	BA 4.0 + IAA 1.0
CET 26	BA 1.0 + IAA 2.0
CET 27	BA 2.0 + IAA 2.0
CET 28	BA 4.0 + IAA 2.0
CET 29	kinetin 1.0 + IAA 1.0
CET 30	kinetin 2.0 + IAA 1.0
CET 31	kinetin 4.0 + IAA 1.0
CET 32	kinetin 1.0 + IAA 2.0
CET 33	kinetin 2.0 + IAA 2.0
CET 34	kinetin 4.0 + IAA 2.0

Table 4. Treatments tried to assess the effect of plant growth regulators on culture establishment of *Dendrobium philippica*

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	**Plant growth regulators (mg l^{-1})**
CPT 0	CONTROL
CPT 1	BA 0.5
CPT 2	BA 1.0
CPT 3	BA 2.0
CPT 4	BA 4.0
CPT 5	BA 0.5 + NAA 0.5
CPT 6	BA 1.0 + NAA 0.5
CPT 7	BA 2.0 + NAA 0.5
CPT 8	BA 4.0 + NAA 0.5
CPT 9	BA 0.5 + NAA 1.0
CPT10	BA 1.0 + NAA 1.0
CPT 11	BA 2.0 + NAA 1.0
CPT 12	BA 4.0 + NAA 1.0
CPT 13	BA 0.5 + NAA 2.0
CPT 14	BA 1.0 + NAA 2.0
CPT 15	BA 2.0 + NAA 2.0
CPT 16	BA 4.0 + NAA 2.0

3. 1. 4. 4. Standardization of basal media for culture establishment

Different basal media such as full MS, ½ MS, VW and KC were tried for culture establishment stage (Table 5).

Table 5. Treatments tried to assess the effect of basal media on multiple shooting of *Dendrobium* cvs.

Medium: Medium composition given in Appendix I

Treatment	Basal Media
SFM	Full MS
SHM	Half MS
SKC	KC
SVW	VW

3. 1. 5. Multiple shooting

The elongated shoots of 2 - 3 cm having 3 - 4 leaves from the culture establishment phase were subjected to treatments for multiple shooting. The selected shoots were transferred to shooting media. Different combinations of cytokinins alone and in combination with auxins were tried for multiple shooting (Table 2). Of these, the best treatment combination was tried for the standardization of other media components. Six replications per treatment were provided for the standardization of steroid plant growth regulators and media supplements.

Observations were recorded on the number of days for shoot initiation, number of shoots per culture four weeks after subculture and nature of the shoots developed

3. 1. 5. 1. Standardization of plant growth regulators for multiple shooting

The different levels of cytokinins (BA and kinetin) alone and in combination with auxins (NAA and IAA) were tried for the standardization of plant growth regulators for multiple shooting. The treatments were replicated three times. Each replication had one explant.

Table 6. Treatments tried to assess the effect of plant growth regulators on multiple shooting of *Dendrobium* cv. Rungnappa Red

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Plant growth regulators (mg l^{-1})
MSR 0	CONTROL
MSR 1	BA 0.5
MSR 2	BA 1.0
MSR 3	BA 2.0
MSR 4	kinetin 0.5
MSR 5	kinetin 1.0
MSR 6	kinetin 2.0
MSR 7	NAA 0.1
MSR 8	NAA 0.5
MSR 9	NAA 1.0
MSR 10	NAA 2.0
MSR 11	BA 0.5 + NAA 0.1
MSR 12	BA 1.0 + NAA 0.1
MSR 13	BA 2.0 + NAA 0.1
MSR 14	BA 0.5 + NAA 0.5
MSR 15	BA 1.0 + NAA 0.5
MSR 16	BA 2.0 + NAA 0.5

MSR 17	BA 0.5 + NAA 1.0
MSR 18	BA 1.0 + NAA 1.0
MSR 19	BA 2.0 + NAA 1.0
MSR 20	kinetin 0.5 + NAA 0.1
MSR 21	kinetin 1.0 + NAA 0.1
MSR 22	kinetin 2.0 + NAA 0.1
MSR 23	kinetin 0.5 + NAA 0.5
MSR 24	kinetin 1.0 + NAA 0.5
MSR 25	kinetin 2.0 + NAA 0.5
MSR 26	kinetin 0.5 + NAA 1.0
MSR 27	kinetin 1.0 + NAA 1.0
MSR 28	kinetin 2.0 + NAA 1.0
MSR 29	kinetin 0.5 + NAA 2.0
MSR 30	kinetin 1.0 + NAA 2.0
MSR 31	kinetin 2.0 + NAA 2.0

Table 7. Treatments tried to assess the effect of plant growth regulators on multiple shooting of *Dendrobium cv.* Miss Snow White

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2g l^{-1} + AC 0.5 g l^{-1}

Treatment	Plant growth regulators (mg l^{-1})
MSS 0	CONTROL
MSS1	BA 0.5
MSS 2	BA 1.0
MSS 3	BA 2.0
MSS 4	kinetin 0.1
MSS 5	kinetin 0.5
MSS 6	kinetin 1.0
MSS 7	NAA 0.1
MSS 8	NAA 0.5
MSS 9	BA 0.5 + NAA 0.1
MSS 10	BA 1.0 + NAA 0.1
MSS 11	BA 2.0 + NAA 0.1
MSS12	BA 0.5 + NAA 0.5
MSS 13	BA 1.0 + NAA 0.5
MSS14	BA 2.0 + NAA 0.5
MSS15	kinetin 0.5 + NAA 0.1
MSS 16	kinetin 1.0 + NAA 0.1
MSS 17	kinetin 2.0 + NAA 0.1
MSS18	kinetin 0.5 + NAA 0.5
MSS 19	kinetin 1.0 + NAA 0.5
MSS 20	kinetin 2.0 + NAA 0.5
MSS 21	kinetin 0.5 + IAA 1.0
MSS 22	kinetin 1.0 + IAA 1.0
MSS23	kinetin 0.5 + IAA 2.0
MSS 24	kinetin 1.0 + IAA 2.0

Table 8. Treatments tried to assess the effect of plant growth regulators on multiple shooting of *Dendrobium* cv. Earsakul

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2g l^{-1} +AC 0.5 g l^{-1}

Treatment	Plant growth regulators (mg l^{-1})
MSE 0	CONTROL
MSE 1	BA 0.5
MSE 2	BA 1.0
MSE 3	BA 2.0
MSE 4	kinetin 0.5
MSE 5	kinetin 1.0
MSE 6	kinetin 2.0
MSE 7	NAA 0.1
MSE 8	NAA 0.5
MSE 9	IAA 1.0
MSE 10	BA 0.5 + NAA 0.1
MSE 11	BA 1.0 + NAA 0.1
MSE12	BA 2.0 + NAA 0.1
MSE 13	BA 0.5 + NAA 0.5
MSE 14	BA 1.0 + NAA 0.5
MSE 15	BA 2.0 + NAA 0.5
MSE 16	BA 0.5 + NAA 1.0
MSE 17	BA 1.0 + NAA 1.0
MSE 18	BA 2.0 + NAA 1.0
MSE 19	kinetin 0.5 + NAA 0.1

MSE 20	kinetin 1.0 + NAA 0.1
MSE 21	kinetin 2.0 + NAA 0.1
MSE 22	kinetin 0.5 + NAA 0.5
MSE23	kinetin 1.0 + NAA 0.5
MSE 24	kinetin 2.0 + NAA 0.5
MSE 25	BA 0.5 + IAA 1.0
MSE 26	BA 1.0 + IAA 1.0
MSE 27	BA 2.0 + IAA 1.0
MSE 28	BA 0.5 + IAA 2.0
MSE 29	BA 1.0 + IAA 2.0
MSE 30	BA 2.0 + IAA 2.0
MSE 31	kinetin 0.5 + IAA 1.0
MSE 32	kinetin 1.0 + IAA 1.0
MSE 33	kinetin 2.0 + IAA 1.0
MSE 34	kinetin 0.5 + IAA 2.0
MSE 35	kinetin 1.0 + IAA 2.0
MSE 36	kinetin 2.0 + IAA 2.0

3. 1. 5. 2. Standardization of media supplements for multiple shooting

3. 1. 5. 2. 1. Coconut water

Different levels of coconut water (0, 25, 50, 150 and 200 ml l^{-1}) were tried to assess their effect on multiple shooting of *Dendrobium* cvs. (Table 9). Six replications were provided for each treatment.

3. 1. 5. 2. 2. Aminoacids

3. 1. 5. 2. 2. 1. Casein hydrolysate

Casein hydrolysate at different levels (0, 250, 500 and 1000 mg l^{-1}) were tried to assess their effect on multiple shooting of *Dendrobium* cvs. (Table 10). Six replications were provided for each treatment.

3. 1. 5. 2. 2. 2. Glutamine

Glutamine at varying levels (0, 1.25, 2.5 and 5.0 mg l^{-1}) were tried to assess their effect on multiple shooting of *Dendrobium* cvs. (Table 11). Six replications were provided for each treatment.

3. 1. 5. 2. 3. Carbon source, sucrose

Varying concentrations of the carbon source, sucrose (15, 30, 45 and 60 g l^{-1}) were tried to assess their effect on multiple shooting of *Dendrobium* cvs. (Table 12). Six replications were provided for each treatment.

Table 9. Treatments tried to assess the effect of coconut water on multiple shooting of *Dendrobium* cvs.

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CS 15.0 g l^{-1} + kinetin 1.0/ 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Coconut water (ml l^{-1})
CONTROL	0
SCW 1	25
SCW 2	50
SCW 3	150
SCW 4	200

Table 10. Treatments tried to assess the effect of aminoacid, casein hydrolysate on multiple shooting of *Dendrobium* cvs.

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + kinetin 1.0/ 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Casein hydrolysate (mg l^{-1})
CONTROL	0
SCH 1	250
SCH 2	500
SCH 3	1000

Table 11. Treatments tried to assess the effect of amino acid, glutamine on multiple shooting of *Dendrobium* cvs.

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + kinetin 1.0/ 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Glutamine (mg l^{-1})
CONTROL	0
SGL 1	1.25
SGL 2	2.5
SGL 3	5.0

3. 1. 5. 2. 4. Polyamines

3. 1. 5. 2. 4. 1. Spermine

In order to assess the effect of polyamine, spermine on multiple shooting of *Dendrobium* cvs , varying levels were tried (0, 0.25, 0.5 and 1mM) (Table 13). Six replications were provided for each treatment.

3. 1. 5. 2. 4. 2. Spermidine

Polyamine, spermidine at different concentrations (0, 0.25, 0.5 and 1mM) were tried to assess their effect on multiple shooting of *Dendrobium* cvs. (Table 14). Six replications were provided for each treatment.

3. 1. 5. 2. 5. Activated charcoal

The different levels of activated charcoal (0, 0.5, 1.0 and 2.0 g l^{-1}) were tried to assess their effect on multiple shooting of *Dendrobium* cvs. (Table 15). Six replications were provided for each treatment.

3. 1. 5. 3. Standardization of steroid plant growth regulator for multiple shooting

The effect of steroid plant growth regulators on multiple shooting of *Dendrobium* cvs. were assessed by trying different concentrations of steroid plant growth regulator, 28- homobrassinolide (0, 0.5, 1.0. 2.0 and 4.0 mg l^{-1}). (Table 16). Six replications were provided for each treatment.

Table 12. Treatments tried to assess the effect of carbon source, sucrose on multiple shooting of *Dendrobium* varieties

Medium: ½ MS + inositol 100.0 mg l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 g l^{-1} + kinetin 1.0/ 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Sucrose (g l^{-1})
SPS 1	15
SPS 2	30
SPS 3	45
SPS 4	60

Table 13. **Treatments tried to assess the effect of polyamine, spermine on multiple shooting of *Dendrobium* varieties**

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + kinetin 1.0/ 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Spermine (mM)
CONTROL	0.0
SSM 1	0.25
SSM 2	0.5
SSM 3	1.0

Table 14. Treatments tried to assess the effect of polyamine, spermidine on multiple shooting of *Dendrobium* cvs.

Medium**:** ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + kinetin 1.0/ 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Treatment	Spermidine (mM)
SSD 1	0.25
SSD 2	0.5
SSD 3	1.0

Table 15. Treatments tried to assess the effect of activated charcoal on multiple shooting of *Dendrobium* cvs.

Medium**:** ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + kinetin 1.0/ 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} + agar 6.2 g l^{-1}

Treatment	Activated charcoal (g l^{-1})
CONTROL	0.0
SAC 1	0.5
SAC 2	1.0
SAC 3	2.0

Table 16. Treatments tried to assess the effect of steroid plant growth regulators on multiple shooting of *Dendrobium* cvs.

Medium**:** ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1}

Treatment	28 –homobrassinolide (mg l^{-1})
CONTROL	0.0
SHB 1	0.5
SHB 2	1.0
SHB 3	2.0
SHB 4	4.0

3. 1. 6. *In vitro* rooting

Healthy single shoots measuring 2.0 – 3.0 cm were subcultured to rooting medium. Nine treatments were tried to assesss the effect of auxins ((IBA, NAA and IAA) on rooting (Table 17). Six replications were provided for each treatment.

Table 17. Treatments tried to assess the effect of plant growth regulators on *in vitro* rooting of *Dendrobium* varieties

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 1.0 g l^{-1}

Treatment	Plant growth regulators (mg l^{-1})
RT 0	CONTROL
RT 1	IBA 0.5
RT 2	IBA 1.0
RT 3	IBA 2.0
RT 4	NAA 0.5
RT 5	NAA 1.0
RT 6	NAA 2.0
RT 7	IAA 0.5
RT 8	IAA 1.0
RT 9	IAA 2.0

3. 1. 7. Hardening and planting out

The culture vessels with rooted plantlets were transferred to room temperature and kept near window to get diffused sunlight for two weeks. The cotton plugs were removed, sterile water added to the vessel and kept for 10 to 15 minutes. The plantlets were taken out carefully from culture vessels using forceps. The agar adhering to the roots were completely removed by thorough washing with running tap water.

The plantlets were treated with Carbendazim (Bavistin 50 WP), 0.1 % solution for 30 minutes before planting. The planting media was autoclaved and transferred to perforated plastic containers. The different media used were soilrite, charcoal pieces + brick pieces and charcoal pieces + brick pieces + soilrite. Polythene covers finely perforated were tied over each individual container to maintain humidity around the plants. Mist spraying with water was done daily. Weekly spraying of plants with ½ MS was also done.

Observations were made on the survival rate of plantlets after one month.

3. 2. *IN VITRO* POLYPLOIDISATION

3. 2. 1. Explant

Studies on *in vitro* polyploidisation were carried out on PLBs and stem nodes of *Dendrobium* cv. Miss Snow White. PLBs were taken from actively growing cultures prior to attaining green stage. Clumps consisting of 8 -10 PLBs were chosen and three such clumps were transferred to a single flask. Each treatment was replicated thrice. Nodes with axillary buds were taken from the basal part of *in vitro* growing shoots and transferred to culture tubes. Nine replications were maintained for each treatment.

3. 2. 2. Polyploidy induction

Colchicine (Sisco Research Laboratory Chemicals, Mumbai) was used for inducing polyploidy in all the treatments. The stock solution of colchicine at 5.0 per cent concentration was prepared by dissolving 5.0 g in 1.0 per cent dimethyl sulphoxide (DMSO) and made up the volume to 100 ml using sterile distilled water. This solution was filter sterilized using syringe filter (Axivia) and stored in amber bottle under refrigerated condition. The required quantity of the chemical was pipetted using sterile microtips and transferred to liquid ½ MS for inducing polyploidy. Different concentration and duration of the treatments were tried (Table 18 and 19).

Table 18. Treatments tried to assess the effect of colchicine on plb's of *Dendrobium* cv. Miss Snow White

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1}

Treatment	Concentration (%)	Duration (days)
PPC 1	½ MS	2
PPC 2	½ MS + 1 % DMSO	"
PPT 1	Colchicine 0.05	"
PPT 2	" 0.1	"
PPT 3	" 0.2	"
PPC 3	½ MS	5
PPC 4	½ MS + 1 % DMSO	"
PPT 4	Colchicine 0.05	"
PPT 5	" 0.1	"
PPT 6	" 0.2	"
PPC 5	½ MS	8
PPC 6	½ MS + 1 % DMSO	"
PPT 7	Colchicine 0.05	"
PPT 8	" 0.1	"
PPT 9	" 0.2	"

Table 19. Treatments tried to assess the effect of colchicine on stem nodes of *Dendrobium* cv. Miss Snow White

Medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1}

Treatment	Concentration (%)	Duration (Days)
NPC 1	½ MS	2
NPC 2	½ MS + 1 % DMSO	”
NPT 1	Colchicine 0.05	”
NPT 2	“ 0.1	”
NPT 3	“ 0.2	”
NPC 3	½ MS	5
NPC 4	½ MS + 1 % DMSO	”
NPT 4	Colchicine 0.05	”
NPT 5	“ 0.1	”
NPT 6	“ 0.2	”
NPC 5	½ MS	8
NPC 6	½ MS + 1 % DMSO	”
NPT 7	Colchicine 0.05	”
NPT 8	“ 0.1	”
NPT 9	“ 0.2	”

3. 2. 2. 1. Media

In vitro polyploidisation was carried out in the following two stages.

1. Induction stage - In this stage the explants were treated with colchicine added to liquid ½ MS medium. The liquid cultures were kept in rotary shaker (100 rpm). The temperature was maintained at 26 ^{0}C and cultures were kept in dark since colchicine is photolabile. After the required treatment duration, the explants were transferred to ½ MS without colchicine for two days to remove the residues of the same.

2. Multiplication stage - After the initial induction stage, the cultures were transferred to solid multiplication medium in the case of PLBs and culture establishment medium in the case of nodes. The regenerated shoots were subcultured every three weeks to fresh medium. Four subculturings were done prior to carrying out the stomata studies.

Observations were recorded on percent survival of explants after colchicine treatment, percentage of cultures showing response, the nature of response and morphological features of regenerated shoots.

3. 2. 3. Stomatal study

Stomatal number and stomata size were determined for the preliminary assessment of ploidy status of the shoots. The procedure reported by Silva *et al.* (2000) was followed for the determination of stomatal number and stomata size. The young leaves of regenerated shoots fixed in formaldehyde - acetic acid - 50 % ethanol (1:1:18) solution. The lower leaf surface is peeled off and the imprint laid on microscopic slide with a drop of water. Stomata count was noted under 400 X magnification. The epidermal cell size and stomatal size were measured using Micrometer under 225 X magnification.

3.3. STATISTICAL ANALYSIS

Completely Randomised Design was adopted for all the experiments. The data was analysed using ANOVA (Analysis of Variance) as per Panse and Sukhatme (1985).

Result

4. RESULTS

Investigations were carried out on enhancement of *in vitro* propagation efficiency and induction of polyploidy in orchid varieties at the Department of Pomology and Floriculture. The *in vitro* experiments were done at the Department of Plant Biotechnology. The results of the investigations are presented in this chapter.

4. 1. *IN VITRO* PROPAGATION

The initial screening for *in vitro* response was done in 14 varieties belonging to three genera namely *Dendrobium*, *Oncidium* and *Phalaenopsis*. In the 8 varieties of genera *Oncidium* and *Phalaenopsis,* initial response and survival of cultures was poor. The *Dendrobium* cultivars that showed good response with respect to culture establishment and bud initiation were selected for further studies.

4. 1. 1. CULTURE ESTABLISHMENT

4. 1. 1. 1. Surface sterilization

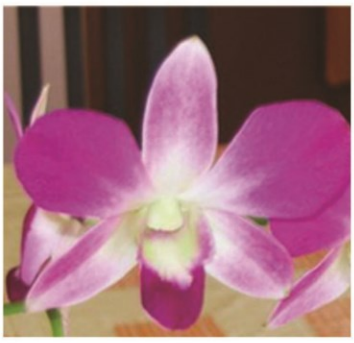
Mercuric chloride at concentrations 0.08 per cent and 0.10 per cent were tried for 5 and 10 minutes to assess their effect on survival percentage and bud initiation from different explants of *Dendrobium* cultivars (Table 20).

In cv. Rungnappa Red, the stem node segments treated with 0.10 per cent mercuric chloride for 5 minutes gave maximum survival rate of 75.00 per cent over the other treatments. In all the surviving explants, swelling followed by bud initiation was noticed.

The cv. Miss Snow White, gave maximum survival rate of 88.89 per cent and further 100.00 per cent bud initiation when the stem nodal segments were treated with mercuric chloride at 0.08 per cent for 10 minutes.

Plate 1. Cultivars of Dendrobium selected for culture establishment phase

Dendrobium cv.
Rungnappa Red



Dendrobium cv.
Earsakul

Dendrobium cv.
Miss.Snow White

Dendrobium philippica

Table 20. Effect of mercuric chloride on survival of explants and bud initiation in *Dendrobium* cvs.

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + agar 6.2 g l^{-1} + BA 2.0 mg l^{-1}

Cultivar and explant	Concentration % (w/v)	Duration Minutes	Survival %	Bud initiation %	Pre treatment with absolute ethanol	
					Survival %	Bud initiation %
Rungnappa Red						
Stem node	0.08	10	62.50	100.00	80.00	100.00
	0.10	5	75.00	100.00		
	”	10	25.00	100.00		
Miss Snow White						
Stem node	0.08	10	88.89	100.00	90.00	100.00
	0.10	5	55.55	80.00		
	”	10	44.44	100.00		
Earsakul						
Stem node	0.08	10	37.50	100.00	80.00	100.00
	0.10	5	62.50	100.00		
	”	10	50.00	50.00		
Tender leaves	0.08	10	100.00	-		
	0.10	5	60.00	-		
	0.10	10	100.00	-		
Spent inflorescence stalk	0.10	10	40.00	100.00		
D.philippica						
Stem node	0.08	10	75.00	100.00	90.00	100.00
	0.10	5	50.00	100.00		
	”	10	75.00	66.67		
Tender leaves	0.08	10	66.67	-		
	0.10	5	66.67	-		
	0.10	10	55.56	-		
Young inflorescence	0.05	5	100.00	100.00		

In cv. Earsakul, as in Rungnappa Red, the stem nodal segments treated with 0.10 per cent mercuric chloride for 5 minutes gave maximum survival rate of 62.5 per cent. All the surviving explants resulted in bud initiation. The tender leaf segments treated with mercuric chloride at 0.08 – 0.10 per cent for 10 minutes gave 100.00 per cent survival rate, but no bud initiation was obtained. At 0.10 per cent mercuric chloride treatment for 5 minutes, however the survival rate was found low. The spent inflorescence segments treated with mercuric chloride at 0.10 per cent for 5 minutes duration resulted in survival rate of 40.00 per cent. Bud initiation occurred from flower nodes, but failed to develop into shoots.

The stem nodal segments of *Dendrobium philippica* treated with 0.08 and 0.10 per cent, mercuric chloride for 10 minutes gave maximum survival rates of 75.00 per cent, and bud initiation of 100.00 per cent and 66.67 per cent respectively. In the case of tender leaf segments the maximum survival rate of 66.67 per cent was obtained at treatments of mercuric chloride at 0.08 per cent for 10 minutes and at 0.10 per cent for 5 minutes. At 0.10 per cent, when duration is increased to 10 minutes, survival rate slightly declined to 55.56 per cent. The young inflorescence segments treated at 0.05 per cent for 5 minutes gave 100 per cent survival rate and 100 per cent bud initiation subsequently.

Pre- treatment of explants with absolute ethanol was found to enhance the survival rate and subsequent bud initiation from stem nodal explants in all the varieties. The increase in survival rate was 80.00 per cent in Rungnappa Red and Earsakul, and 90.00 per cent in Miss Snow White and *D. philippica.* All the pre-treated explants showed bud initiation and subsequent shoot growth.

4. 1. 1. 2. Explants

Leaf and root explants of the four *Dendrobium* cultivars were tried for culture establishment to assess their *in vitro* response (Table 21) and further regeneration potential.

In cv. Rungnappa Red, callusing was initiated in 33.33 per cent of *in vitro* leaf explants inoculated in establishment medium supplemented with BA 2.0 mg l^{-1} and NAA 0.5 - 1.0 mg l^{-1}.

At higher level of either BA or NAA at 4.0 mg l^{-1}, the explants dried. In other treatments the explants inoculated expanded in the first week, remained green upto four weeks and dried. All the root explants inoculated dried in the first week itself.

In cv. Miss Snow White, in all the treatments involving BA and NAA, the *in vitro* leaf explants expanded and remained green up to 6 weeks. Callus was produced in 66.67 per cent of explants inoculated in the treatment with BA 4.0 mg $l^{-1}$and NAA 4.0 mg l^{-1} (Plate 2a). All the root explants in treatment combination of BA and NAA resulted in expansion. But callusing (66.67 per cent) occurred only at combination of BA 2.0 mg $l^{-1}$and NAA 0.5 mg l^{-1}.

The *in vitro* leaf explants of cv. Earsakul showed expansion and greening up to four weeks in treatment combinations of BA and NAA at 0.5 - 2.0 mg l^{-1}. However, in control and at higher dose of BA at 4.0 mg l^{-1}along with NAA at 1.0 and 4.0 mg l^{-1} drying of explants resulted. None of the root explants survived in any of the treatments.

In *D. philippica*, the leaf explants inoculated in treatment combinations of BA 0.5 – 4.0 mg l^{-1} along with NAA at 0.5 - 2.0 mg l^{-1} showed expansion and greening upto four weeks. Direct shoot regeneration (33.33 per cent) from cut end of leaf was obtained for the treatment with BA 4.0 mg l^{-1} and NAA 1.0 mg l^{-1} (Plate 2b). At higher levels of NAA (4.0 mg l^{-1}) all the explants dried. None of the root explants survived in the establishment medium.

The nodal explants from young inflorescence stalk showed survival rate of 66.67 per cent to 100.0 per cent in kinetin supplemented media. At kinetin 2.0 mg l^{-1} alone and in combination with IAA (1.00 to 4.00 mg l^{-1}) the explants developed callus from cut ends. Shoot buds developed when kinetin concentration was increased to 4.0 mg l^{-1} (Table 22).

The thin section explants of approximately 1mm from *in vitro* shoots of three *Dendrobium* cvs. were tried. How ever, these cultures were heavily bacterial contaminated.

Table 21. Effect of plant growth regulators on regeneration from various explants in *Dendrobium* cvs.

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + agar 6.2 g l^{-1}

Treatment		Rungnappa Red		Miss Snow White		Earsakul		*D.philippica*	
BA + NAA		Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
0	0	E	D	E	D	D	D	D	D
0.5	0.5	E,G	D	E,G	E	E,G	D	E,G	D
1	0.5	E,G	D	E,G	C(66.67%)	E,G	D	E,G	D
2	0.5	C (33.33%)	D	E,G	E	E,G	D	E,G	D
4	0.5	D	D	E,G	E	E,G	D	E,G	D
0.5	1	E	D	E,G	E	E,G	D	E,G	D
1	1	E	D	E,G	E	E,G	D	E,G	D
2	1	C (33.33%)	D	E,G	E	E,G	D	E,G	D
4	1	D	D	E,G	E	D	D	S (33.33%)	D
0.5	2	E,G	D	E,G	E	E,G	D	E,G	D
1	2	E,G	D	E,G	E	E,G	D	E,G	D
2	2	E,G	D	E,G	E	E,G	D	E,G	D
4	2	D	D	E,G	E	D	D	E,G	D
0.5	4	D	D	E,G	E	D	D	D	D
1	4	D	D	E,G	E	D	D	D	D
2	4	D	D	E,G	E	D	D	D	D
4	4	D	D	C (66.67%)	E	D	D	D	D

E – Expanded; E, G - Expanded, green; C- Callus initiation; D- Dried; S-Shoot regeneration

Table 22. Effect of plant growth regulators on regeneration from young inflorescence stalk in *Dendrobium philippica*

Kinetin	IAA	Response	Rate of Response (%)	Days for callus/bud initiation
2.0	0	Callusing	66.67	42.0
2.0	1.0	”	100.00	44.6
2.0	2.0	”	100.00	34.0
2.0	4.0	”	100.00	59.6
4.0	0	Shoot bud initiation	66.67	37.5
4.0	4.0	”	66.67	35.0
F		-	-	6.14**
£CD (2,2)				14.58
(2,3)				13.31
(3,3)				11.90

£ Since the effective number of replications varies, separate CD values were computed

4. 1. 1. 3. Plant growth regulators

The culture establishment phase was initiated using nodal explants in ½ MS medium supplemented with cytokinins (BA and kinetin) alone and in combination with auxins (NAA and IAA). (Plate 2c, 2d, 2e and 2f). Difference in responses was noticed among the four varieties tried for culture establishment phase.

Fifty eight treatments were tried to assess the effect of plant growth substances on culture establishment of *Dendrobium* cv. Rungnappa Red. Analysis of variance showed significant difference among these treatments (Table 23). The mean number of days for bud initiation varied from 7.33 to 30.0 (Plate 2g). The treatment CRT 30 (kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}) took minimum number of days (7.33) for bud initiation. This was found statistically on par with CRT 5, CRT 57, CRT 42, CRT 37, CRT 76, CRT 21, CRT 51, CRT 34, CRT 22, CRT 26, CRT 37, CRT 16, CRT 29, CRT 33, CRT 36, CRT 58, CRT 18, CRT 9, CRT 17, CRT 38, CRT 54, CRT 3, CRT 44, CRT 8 and CRT 32. The maximum number of days (30.0) for bud initiation was obtained in the treatment CRT 19 which was statistically on par with control, CRT 24, CRT 4, CRT 13, CRT 55, CRT 25 and CRT - 12.

The number of shoots produced showed significant difference. The mean number of shoots produced at fourth week varied from 1.00 to 3.00. The highest number of shoots (3.00) was obtained in CRT 30 and CRT 17 which were statistically on par with CRT 2, CRT 43, CRT 54, CRT 22 and CRT 27.

Statistically significant difference was observed among the 56 treatments experimented to find the effect of plant growth substances on culture establishment of *Dendrobium* cv. Miss Snow White (Table 24). The mean number of days for bud initiation showed wide variation ranging from 10.33 to 36.00 (Plate 2h). The minimum number of days (10.33) for bud initiation was recorded for the treatment CST 27 (kinetin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}). This was found statistically on par with CST 6, CST 5, CST 29, CST 28, CST 7, CST 15, CST 32, CST 26, CST 31, CST 37, CST 40, CST 44, CST 21, CST 35, CST 36, CST 38, CST 39, CST 34 and CST 33. The maximum number of days (36.0) for bud initiation was obtained in the

treatment CST 42 which was statistically on par with CST 19, CST 22, CST 41, CST 45 and CST 23.

In the case of number of shoots produced at fourth week, significant difference was noticed among the treatments tried. The maximum numbers of shoots were obtained in treatments CST 21 (5.00) and CST 25 (4.00).

In *Dendrobium* cv. Earsakul, the 34 treatments tried to assess the effect of plant growth substances showed significant difference (Table 25). The mean number of days for bud initiation varied from 9.67 to 22.50 days. The minimum number of days for bud initiation was observed in CET 3 (9.67). This was statistically on par with CET 18, CET 28, CET 6, CET 19, CET 15, CET 11, CET 25, CET 5, CET 12, CET 31 and CET 17. The maximum number of days for bud initiation was obtained in the treatment CET 10 (22.50), which was found on par with control, CET 29, CET 9 and CET 32.

With respect to the number of shoots produced at fourth week, significant difference was found among the treatments. The mean shoot number varied from 1.00 to 4.33, the maximum shoot number (4.33) was obtained in the treatment CET 12 (BA 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}) which was superior to other treatments.

Significant difference was noticed among the treatments tried for *D. philippica* with respect to the number of days for bud initiation, the mean of which varied from 10.66 to 41.33 days (Table 26). The minimum number of days (10.66) for bud initiation was observed in the treatment CPT 8 (BA 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}). This was found on par with CPT 3, CPT 4, CPT 9, CPT 12, CPT 10, CPT 15, CPT 16, CPT 6 and CPT 7. The maximum number of days (41.33) for bud initiation was obtained in control which was on par with CPT 1 and CPT 14.

In all the treatments single shoots were produced at fourth week stage.

Plate 2.

Initial culture establishment of *Dendrobium* cultivars

a. Callus initiation from leaf explant of cv. Miss. Snow White

b. Shoot bud regeneration from leaf explant of *Dendrobium philipica*

c. Bud initiation from stem node explant of *Dendrobium* cv. Rungnappa Red

d. Bud initiation from stem node explant of *Dendrobium* cv. Miss. Snow White

e. Bud initiation from stem node explant of *Dendrobium* cv. Earsakul

f. Bud initiation from stem node explant of *Dendrobium philipica*

g. Bud initiation from different treatments of plant growth regulators at fourth week in *Dendrobium* cv. Rungnappa Red

1. ½ MS

2. Kinetin 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}

3. Kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}

4. Kinetin 2.0 mg l^{-1} + NAA 2.0 mg l^{-1}

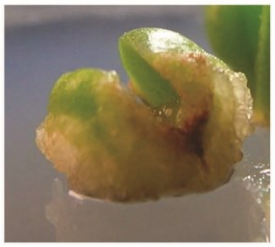
h. Bud initiation from different treatments of plant growth regulators at fourth week in *Dendrobium* cv. Miss. Snow White

1. ½ MS

2. Kinetin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}

3. Kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}

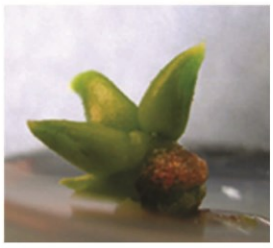
a.



b.

c.

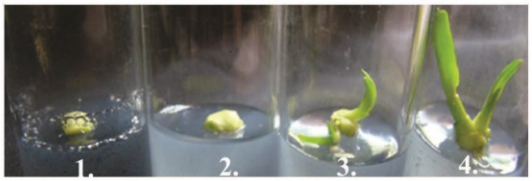
d.



e.

f.

g.



h.

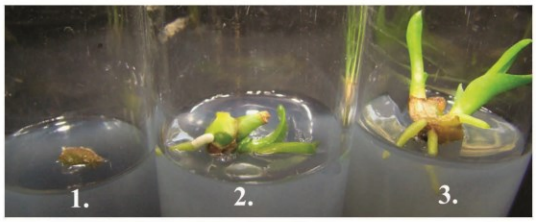


Table 23. Effect of plant growth regulators on culture establishment of *Dendrobium cv.* Rungnappa Red

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Culture period: 4 weeks

♦Treatments (mg l^{-1})	Survival %	Days for bud initiation	No: of shoots
CRT 0	66.67	25.00	1.00
CRT 1	100.00	19.67	1.00
CRT 2	66.67	17.50	2.50
CRT 3	100.00	13.00	1.33
CRT 4	66.67	8.00	1.00
CRT 5	66.67	10.33	1.50
CRT 6	100.00	10.00	1.00
CRT 7	100.00	13.30	1.30
CRT 8	100.00	12.67	1.67
CRT 9	100.00	15.00	1.67
CRT 10	66.67	15.33	1.00
CRT 11	100.00	22.00	1.00
CRT 12	100.00	23.50	1.00
CRT 13	66.67	20.67	1.00
CRT 14	100.00	20.50	1.00
CRT 15	66.67	24.00	1.00
CRT 16	100.00	12.00	1.33
CRT 17	100.00	12.67	3.00
CRT 18	100.00	12.30	1.33
CRT 19	66.67	30.00	1.00
CRT 20	100.00	17.67	1.33
CRT 21	100.00	10.30	1.33

CRT 22	100.00	11.30	2.00
CRT 23	66.67	21.50	1.00
CRT 24	66.67	24.00	2.00
CRT 25	100.00	22.00	1.33
CRT 26	100.00	11.30	1.33
CRT 27	66.67	14.00	2.00
CRT 28	66.67	21.00	1.00
CRT 29	66.67	12.00	1.00
CRT 30	100.00	7.33	3.00
CRT 31	100.00	10.00	1.00
CRT 32	100.00	13.67	1.33
CRT 33	100.00	12.00	1.60
CRT 34	100.00	10.67	1.33
CRT 35	100.00	18.67	1.33
CRT 36	100.00	12.00	1.00
CRT 37	100.00	11.30	1.00
CRT 38	100.00	12.67	1.00
CRT 39	100.00	15.30	1.00
CRT 40	100.00	19.00	1.00
CRT 41	100.00	20.00	1.00
CRT 42	100.00	10.00	1.33
CRT 43	66.67	15.50	2.50
CRT 44	100.00	13.00	1.00
CRT 45	100.00	14.00	1.00
CRT 46	100.00	21.00	1.00
CRT 47	100.00	21.00	1.00
CRT 48	100.00	16.30	1.00
CRT 49	100.00	21.30	1.00
CRT 50	100.00	15.67	1.00
CRT 51	100.00	10.30	1.00

CRT 52	100.00	14.00	1.00
CRT 53	100.00	16.30	1.67
CRT 54	100.00	12.67	2.33
CRT 55	100.00	22.00	1.00
CRT 56	100.00	19.30	1.33
CRT 57	100.00	10.00	1.00
CRT 58	100.00	12.00	1.33
F		3.96 * *	1.49 * *
£CD (2,2)		8.00	1.33
(2,3)		7.31	1.21
(3,3)		6.53	1.08

♦Treatment details are given in the Table 1

£Since the effective number of replications varies, separate CD values were computed

* * Significant at 1 % level

Table 24. Effect of plant growth regulators on culture establishment of *Dendrobium* cv. Miss Snow White

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Culture period: 4 weeks

♦ Treatments (mg l^{-1})	Survival %	Days for bud initiation	No: of shoots
CST 0	66.67	21.00	1.00
CST 1	100.00	27.50	1.00
CST 2	100.00	17.67	1.67
CST 3	100.00	19.00	1.00
CST 4	100.00	17.67	1.67
CST 5	100.00	11.00	1.33
CST 6	100.00	10.67	1.33
CST 7	66.67	12.00	1.33
CST 8	100.00	21.67	1.33
CST 9	66.67	16.50	2.50
CST 10	66.67	18.67	1.00
CST 11	66.67	23.50	1.00
CST 12	66.67	19.50	1.00
CST 13	100.00	25.00	1.00
CST 14	100.00	26.67	1.67
CST 15	66.67	12.67	1.67
CST 16	100.00	27.50	1.00
CST 17	100.00	24.00	1.00
CST 18	66.67	21.67	1.00
CST 19	66.67	33.00	1.00
CST 20	100.00	23.00	1.00
CST 21	100.00	14.33	5.00

CST 22	66.67	32.67	1.00
CST 23	66.67	31.00	1.00
CST 24	100.00	27.00	1.00
CST 25	66.67	18.67	4.00
CST 26	100.00	13.50	1.50
CST 27	100.00	10.33	1.67
CST 28	100.00	11.67	2.67
CST 29	100.00	11.33	1.67
CST 30	100.00	10.67	1.33
CST 31	100.00	13.67	1.67
CST 32	100.00	13.00	2.67
CST 33	100.00	16.00	1.33
CST 34	100.00	15.67	1.33
CST 35	100.00	14.67	1.67
CST 36	100.00	14.67	2.00
CST 37	100.00	13.67	1.67
CST 38	100.00	15.00	1.33
CST 39	100.00	15.33	1.33
CST 40	66.67	13.67	2.33
CST 41	100.00	31.00	1.00
CST 42	100.00	36.00	1.00
CST 43	100.00	26.00	1.00
CST 44	66.67	14.33	1.00
CST 45	66.67	31.00	1.50
CST 46	100.00	28.00	1.00
CST 47	100.00	24.67	1.00
CST 48	66.67	22.00	1.67
CST 49	100.00	23.00	1.00
CST 50	100.00	21.33	1.00
CST 51	100.00	18.00	1.33

CST 52	100.00	17.33	1.33
CST 53	100.00	26.00	1.00
CST 54	100.00	18.00	1.00
CST 55	100.00	16.67	1.00
CST 56	100.00	19.33	1.00
F		9.33 * *	2.10 * *
£CD (2,2)		7.00	1.71
(2,3)		6.39	1.57
(3,3)		5.73	1.40

♦ Treatment details are given in the Table 2

£ Since the effective number of replications varies, separate CD values were computed

* * Significant at 1 % level

Table 25. Effect of plant growth regulators on culture establishment of *Dendrobium* cv. Earsakul

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Culture period: 4 weeks

♦ Treatments (mg l^{-1})	Survival %	Days for bud initiation	No: of shoots
CET 0	100.00	22.33	1.33
CET 1	100.00	17.33	1.33
CET 2	100.00	13.67	1.67
CET 3	100.00	9.67	2.33
CET 4	100.00	14.67	1.00
CET 5	100.00	12.33	1.33
CET 6	66.67	11.33	1.67
CET 7	66.67	16.00	1.00
CET 8	66.67	17.50	1.00
CET 9	66.67	21.00	1.00
CET 10	66.67	22.50	1.00
CET 11	100.00	12.00	1.50
CET 12	100.00	12.67	4.33
CET 13	100.00	15.33	1.33
CET 14	100.00	14.33	1.67
CET 15	66.67	11.67	1.50
CET 16	100.00	19.50	1.00
CET 17	100.00	13.00	2.67
CET 18	100.00	10.00	3.00
CET 19	66.67	11.33	2.67
CET 20	66.67	16.50	1.50

CET 21	100.00	15.00	2.00
CET 22	100.00	13.33	2.00
CET 23	100.00	17.33	1.00
CET 24	100.00	14.00	1.33
CET 25	66.67	12.33	2.33
CET 26	100.00	13.50	1.00
CET 27	100.00	13.33	1.00
CET 28	66.67	10.67	2.33
CET 29	100.00	22.00	1.50
CET 30	100.00	16.00	1.33
CET 31	66.67	12.67	1.67
CET 32	66.67	20.00	1.00
CET 33	100.00	17.50	1.50
CET 34	100.00	14.00	2.00
F		6.49 * *	2.61 * *
[£] CD (2,2)		4.39	1.49
(2,3)		4.00	1.37
(3,3)		3.58	1.22

♦ Treatment details are given in the Table 3

[£] Since the effective number of replications varies, separate CD values were computed

* * Significant at 1 % level

Table 26. Effect of plant growth regulators on culture establishment of *Dendrobium philippica*

Media: ½ MS + inositol 100.0 mg l^{-1}+ sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Culture period: 4 weeks

♦ Treatments (mg l^{-1})	Survival %	Days for bud initiation	No: of shoots
CPT 0	100.00	41.33	1.00
CPT 1	66.67	36.00	1.00
CPT 2	66.67	29.50	1.00
CPT 3	100.00	13.00	1.00
CPT 4	100.00	15.00	1.00
CPT 5	100.00	26.00	1.00
CPT 6	66.67	20.00	1.00
CPT 7	66.67	20.50	1.00
CPT 8	100.00	10.66	1.00
CPT 9	66.67	15.50	1.00
CPT10	100.00	18.00	1.00
CPT 11	66.67	22.50	1.00
CPT 12	100.00	16.66	1.00
CPT 13	100.00	27.33	1.00
CPT 14	66.67	35.50	1.00
CPT 15	100.00	18.33	1.00
CPT 16	100.00	18.33	1.00
F		5.59* *	NS
£ CD(2,2)		12.22	
(2,3)		11.16	
(3,3)		9.98	

♦ Treatment details are given in the Table 4

£ Since the effective number of replications varies, separate CD values were computed

* * Significant at 1 % level

4. 1. 1. 4. Basal media

Among the four different basal media viz., full MS, half MS, VW and KC tried, ½ MS was found to give 100 per cent survival rate, minimum days for bud initiation and maximum number of shoots in the three *Dendrobium* cultivars (Table 27). In cv. Rungnappa Red, ½ MS registered the minimum number of days (22.00) for bud initiation and maximum number (1.33) of shoots. In cv. Miss Snow White, the minimum days (20.17) for bud initiation were noticed in ½ MS, which was found statistically on par with VW and full MS. The maximum number of shoots (1.16) was found in full as well as in ½ MS. The cv. Earsakul, also took minimum number (23.50) of days for bud initiation in ½ MS which was found on par with full MS. The maximum number of shoots (1.33) was obtained in ½ MS.

4. 1. 2. MULTIPLE SHOOTING

In the second phase of *in vitro* propagation namely multiple shooting, single healthy shoots of 2 - 3 cm having 3 - 4 leaves were subcultured to fresh media of various treatments of plant growth regulators and other media supplements.

4. 1. 2. 1. Plant growth regulators

The multiplication rate and nature of shoots varied with the plant growth regulators tried alone and in combinations, in the three varieties.

In *Dendrobium* cv. Rungnappa Red, the 32 treatments showed significant difference (Table 28). The mean number of days for shoots initiation varied from 10.33 to 20.5. The minimum number of days (10.33) for shoot multiplication was noticed in the treatment MSR 5 (kinetin 1.0 mg l^{-1}) and MSR 31 (kinetin 2.0 mg l^{-1} + NAA 2.0 mg l^{-1}), which were statistically on par with MSR 25, MSR 20, MSR 12, MSR 11, MSR 30, MSR 17, MSR 21, MSR 22, MSR 24, MSR 29, MSR 19, MSR 2, MSR 26, MSR 5, MSR 10 and MSR 28. The treatment MSR 4 (NAA 2.0 mg l^{-1}) took maximum days (20.5) for shoot multiplication. This was followed by treatments MSR 7, control, MSR 16, MSR 15,

MSR 14, MSR 11, MSR 13, MSR 6, MSR 3, MSR 12 and MSR 28 which were statistically on par.

Table 27. Effect of basal media on survival of explants and bud initiation in *Dendrobium* cvs.

Media: As given in the Appendix I

Culture period: 6 weeks

Basal media	Rungnappa Red			Miss Snow White			Earsakul		
	Survival %	DBI	NOS	Survival %	DBI	NOS	Survival %	DBI	NOS
SFM	83.33	26.40	1.00	100.00	23.50	1.16	100.00	26.33	1.00
SHM	100.00	22.00	1.33	100.00	20.17	1.16	100.00	23.50	1.33
SKC	66.67	26.75	1.00	100.00	26.33	1.00	83.33	29.00	1.00
SVW	66.67	23.75	1.00	100.00	21.83	1.00	66.67	31.00	1.00
F	-	0.53	1.71	-	3.45*	0.67	-	5.17*	2.02
£CD		NS	NS		4.18	NS		(6,6)-4.45 (6,5)-4.67 (6,4)-4.98 (5,4)-5.17	NS

♦ Treatment details are given in the Table 5

£ Since the effective number of replications varies, separate CD values were computed

In the case of shoot numbers produced at fourth week, significant difference was noticed among the treatments. The maximum shoot numbers (3.67) was obtained for the treatment MSR 15 (BA 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and MSR 12 (BA 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}). These were found to be on par with MSR 14, MSR 16, MSR 11, MSR 13, MSR 23, MSR 18, MSR 21, MSR 22, MSR 4, MSR 5, MSR 6, MSR 9, MSR 17, MSR 24, MSR 27 and MSR 30.

In the cv. Miss Snow White, significant difference was noticed among the 24 treatments tried for multiple shooting with respect to the number of days for shoot multiplication (Table 29). The mean days for shoot multiplication varied from 9.33 to 21.33. The minimum days for shoot initiation were obtained in treatment MSS 16 (kinetin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}) which recorded 9.33 days. This was statistically on par with MSS 18, MSS 17, MSS 6, MSS 14, MSS 4, MSS 15, MSS 20, MSS 10 and MSS 11. The maximum number of days for shoot initiation was noticed in control (21.33), which was found statistically on par with MSS 3, MSS 21, MSS 1 and MSS 23.

Significant difference was obtained for the number of shoots produced at fourth week. The average shoot number varied from 1.00 to 5.33. The maximum shoot numbers was noticed in treatment MSS 12 (BA 0.5 mg l^{-1} + NAA 0.5 mg l^{-1}) which was found on par with MSS 9.

The 36 treatments of plant growth regulators tried for multiple shooting in *Dendrobium* cv. Earsakul showed significant difference (Table 30). The mean number of days varied from 11.00 to 22.50. The minimum number of days (11.00) for shoot initiation was noticed in the treatment MSE 21 (kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}). This was found statistically on par with MSE 6, MSE 22, MSE 24, MSE 15, MSE 20, MSE 18, MSE 3, MSE 14, MSE 5, MSE 12, MSE 4, MSE 19, MSE 17, MSE 23 and MSE 32. The maximum number of days for shoot initiation was obtained for the treatment MSE 9 (IAA 1.0 mg l^{-1}).

Table 28. Effect of plant growth regulators on multiple shooting of *Dendrobium* cv. Rungnappa Red

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Culture period: 4 weeks

♦ Treatments (mg l^{-1})	Survival %	Days for bud initiation	No: of shoots
MSR 0	100.00	20.00	1.00
MSR 1	100.00	14.33	2.00
MSR 2	100.00	17.33	1.33
MSR 3	100.00	20.00	1.00
MSR 4	66.67	11.67	2.33
MSR 5	100.00	10.33	2.33
MSR 6	100.00	12.33	2.33
MSR 7	66.67	13.33	1.33
MSR 8	100.00	14.00	1.67
MSR 9	100.00	17.00	2.33
MSR 10	100.00	20.50	1.00
MSR 11	100.00	11.00	3.00
MSR 12	100.00	11.00	3.67
MSR 13	100.00	14.33	3.00
MSR 14	100.00	18.67	3.33
MSR 15	100.00	15.67	3.67
MSR 16	66.67	18.33	3.33
MSR 17	100.00	18.67	2.33
MSR 18	100.00	19.00	2.67
MSR 19	100.00	19.50	1.50
MSR 20	100.00	10.67	1.67
MSR 21	100.00	12.00	2.67

MSR 22	100.00	12.00	2.67
MSR 23	100.00	13.33	3.00
MSR 24	100.00	12.33	2.33
MSR 25	100.00	10.67	2.00
MSR 26	100.00	14.00	2.00
MSR 27	100.00	13.67	2.33
MSR 28	100.00	14.67	2.00
MSR 29	100.00	12.33	2.00
MSR 30	100.00	11.33	2.33
MSR 31	100.00	10.33	2.00
F		3.96 * *	2.00 *
£CD (3 3)		4.52	1.41
(3 2)		5.05	1.57
(2 2)		5.53	1.72

♦ Treatment details are given in the Table 6

£ Since the effective number of replications varies, separate CD values were computed

* * Significant at 1 % level
* Significant at 5 % level

Table 29. Effect of plant growth regulators on multiple shooting of *Dendrobium* cv. Miss Snow White

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Culture period: 4 weeks

♦ Treatments (mg l^{-1})	Survival %	Days for shoot initiation	No: of shoots
MSS 0	100.00	21.33	1.00
MSS1	100.00	18.33	1.67
MSS 2	100.00	16.00	1.67
MSS 3	100.00	20.67	4.00
MSS 4	100.00	11.67	1.67
MSS 5	100.00	14.33	1.33
MSS 6	100.00	11.33	2.00
MSS 7	66.67	14.00	1.00
MSS 8	100.00	15.00	1.00
MSS 9	100.00	16.00	5.33
MSS 10	100.00	13.33	2.33
MSS 11	100.00	13.33	3.00
MSS 12	100.00	16.33	5.67
MSS 13	100.00	14.00	3.67
MSS 14	100.00	11.67	3.00
MSS 15	100.00	12.33	2.00
MSS 16	100.00	9.33	2.67
MSS 17	100.00	11.00	2.33
MSS 18	100.00	10.67	1.33
MSS 19	100.00	13.00	2.33
MSS 20	100.00	12.33	2.00
MSS 21	100.00	19.33	1.00

MSS 22	66.67	16.00	1.50
MSS 23	66.67	17.50	2.50
MSS 24	66.67	17.00	3.00
F		5.00 * *	5.13 **
£ CD (2,2)		4.93	1.91
(2,3)		4.50	1.75
(3,3)		4.03	1.56

♦ Treatment details are given in the Table 7

£ Since the effective number of replications varies, separate CD values were computed

* * Significant at 1 % level

Table 30. Effect of plant growth regulators on multiple shooting of *Dendrobium* cv. Earsakul

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Culture period: 4 weeks

♦ Treatments (mg l^{-1})	Survival %	Days for shoot initiation	No: of shoots
MSE 0	100.00	20.67	1.33
MSE 1	100.00	16.33	1.00
MSE 2	100.00	15.67	1.33
MSE 3	100.00	12.33	1.67
MSE 4	100.00	14.00	1.33
MSE 5	100.00	13.67	1.33
MSE 6	100.00	11.33	2.00
MSE 7	66.67	17.00	1.00
MSE 8	66.67	21.00	1.50
MSE 9	66.67	22.50	1.00
MSE 10	100.00	16.67	1.33
MSE 11	100.00	15.67	1.67
MSE 12	100.00	13.67	1.67
MSE 13	66.67	16.00	1.50
MSE 14	100.00	13.00	1.00
MSE 15	100.00	11.67	2.66
MSE 16	66.67	17.00	2.00
MSE 17	100.00	14.33	1.67
MSE 18	66.67	12.00	2.00
MSE 19	66.67	14.00	1.50
MSE 20	100.00	11.67	2.66
MSE 21	100.00	11.00	4.66
MSE 22	100.00	11.33	3.33

MSE 23	100.00	14.33	2.66
MSE 24	100.00	11.33	2.33
MSE 25	66.67	19.00	1.50
MSE 26	66.67	17.50	1.00
MSE 27	66.67	17.00	1.50
MSE 28	66.67	19.50	1.00
MSE 29	66.67	18.50	1.00
MSE 30	66.67	22.50	2.00
MSE 31	66.67	18.00	1.00
MSE 32	66.67	14.50	1.00
MSE 33	66.67	16.00	1.50
MSE 34	66.67	22.00	1.00
MSE 35	66.67	21.00	1.00
MSE 36	66.67	19.50	1.00
F		5.90* *	2.04 *
£ CD (2,2)		4.46	1.81
(2,3)		4.06	1.66
(3,3)		3.64	1.48

♦ Treatment details are given in the Table 8

£Since the effective number of replications varies, separate CD values were computed

* * Significant at 1 % level

* Significant at 5 % level

In the cv. Earsakul also, significant difference was obtained for the number of shoots produced at fourth week. The mean shoot number varied from 1.00 to 4.66. The maximum shoot number (4.66) was obtained for MSE 21 (kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}) followed by MSE 22 (kinetin 0.5 mg l^{-1} + NAA 0.5 mg l^{-1}).

Considering the nature of shoots produced in all the three varieties, it was noticed that those produced in treatments involving both cytokinins and auxins were better than in treatments with either auxin or cytokinin alone. In treatments involving combinations of BA (0.5 - 2.0 mg l^{-1}) and NAA (0.5 -1.0 mg l^{-1}), the shoot buds were proliferating, short, non-distinct and pale green with delayed leaf emergence. On the other hand, in treatments involving combinations of kinetin (0.5 - 4.0 mg l^{-1}) and NAA (0.5 -1.0 mg l^{-1}), the shoot buds were non- proliferating, few in number, distinct, dark green, vigorous, healthy with fully emerged leaves. (Plate 3).

4. 1. 2. 2. Coconut water

In all the three varieties, CW at 200 ml l^{-1} was found superior with respect to early bud initiation and maximum shoot production. With increase in CW there was gradual decrease in days for bud initiation in all the three varieties. Similarly an increase in multiple shoots was also observed in cultivars Miss Snow White and Earsakul (Table 31).

Significant difference was observed among the treatments with respect to the number of days for shoot multiplication and the number of shoots produced in cv. Rungnappa Red (Plate 4). The mean numbers of days for shoot multiplication varied from 12.33 to 25.50. The treatment SCW 4 with 200 ml l^{-1} CW registered earliest shoots multiplication of 12.33 days which was found on par with SCW 3 (18.67 days). The maximum days for shoot multiplication were observed in control lacking CW, which was statistically on par with SCW1 and SCW 2. The mean number of shoots produced varied between 1.33 and 2.67. The treatment SCW 4 recorded maximum numbers of shoots of 2.66, while SCW 2 produced minimum numbers of shoots of 1.33.

Plate 3.

Multiple shooting in treatments of plant growth regulators in *Dendrobium* cvs.

Rungnappa Red

a. Kinetin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}

b. BA 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}

Miss. Snow White

c. Kinetin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}

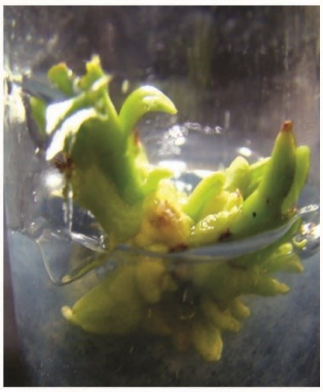
d. BA 0.5 mg l^{-1} + NAA 0.5 mg l^{-1}

Earsakul

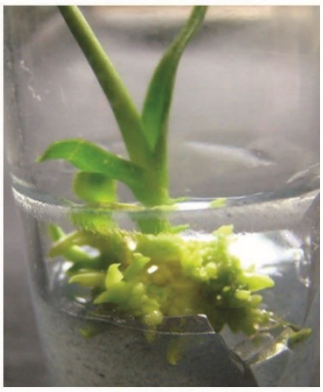
e. Kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}

f. BA 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}

a. 

b. 

c. 

d. 

e. 

f. 

In cv. Miss Snow White, the mean number of days for shoots multiplication varied from 10.67 to 19.33. In this case also SCW 4 registered minimum days (10.67) for shoot multiplication. The maximum days for shoot multiplication were found in control (19.33) which was on par with SCW1 and SCW 2. The mean number of shoots produced was found non significant and varied between 1.33 and 2.67. The maximum number of shoots produced was found in SCW 4 and minimum in control.

The mean number of days for shoots multiplication varied from 11.67 to 18.67 in cv. Earsakul. The minimum days (11.67) for shoot multiplication were obtained in SCW 4 which was found on par with SCW 3, having 150 ml l^{-1} CW. The treatment SCW 2 took maximum days (18.67) for shoot multiplication which was on par with control and SCW 1. The number of shoots produced at fourth week showed significant difference. The mean shoot numbers varied between 1.00 and 4.67. SCW 4 recorded maximum shoot numbers (4.67) which was significantly superior to other treatments. The minimum shoot number (1.00) was obtained in control which was on par with SCW 1, SCW 2 and SCW 3.

In all the three varieties, in control and the treatments with 50.00 ml l^{-1} and 100.00 ml l^{-1} CW, the early senescing of lower leaves were observed.

4. 1. 2. 3. Amino acids

4. 1. 2. 3. 1. Casein Hydrolysate

The response to CH was varying in all the three *Dendrobium* varieties (Table 32) (Plate 4). In cv. Rungnappa Red, supplementing the multiplication media with CH at 250-1000 mg l^{-1} was found to delay shoot multiplication. The minimum days (12.33) for shoot multiplication were noticed in control which was on par with SCH 3, while maximum number of days for shoot multiplication was found in SCH 1 and SCH 2 (18.33). Significant difference was noticed for the number of shoots produced. The maximum number of shoots (2.67) was produced in control which was superior over the other treatments.

Table 31. Effect of coconut water on multiple shooting in *Dendrobium* cvs

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1} + NAA 0.1 mg l^{-1} + kinetin 2.0 mg l^{-1} (kinetin 1.0 mg l^{-1} for cv. Miss Snow White)

Culture period: 4 weeks

♦ Treatments (mg l^{-1})	Rungnappa Red		Miss Snow White		Earsakul	
	DSI	NOS	DSI	NOS	DSI	NOS
CONTROL	26.33	1.50	19.33	1.33	18.00	1.00
SCW 1	22.50	1.50	17.00	1.50	18.67	1.30
SCW 2	24.00	1.33	16.83	1.50	17.33	1.67
SCW 3	18.67 [1]	1.67	14.83	1.83	13.67[1]	1.67
SCW 4	12.33[1]	2.67[1]	10.67[1]	2.67	11.67[1]	3.83[1]
F	4.19**	3.90*	10.98**	NS	9.17**	24.63**
CD	7.51	0.79	2.86		2.92	0.87

♦ Treatment details are given in the Table 9
The data represents mean value of six replications

Table 32. Effect of casein hydrolysate on multiple shooting in *Dendrobium* cvs.

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1} + NAA 0.1 mg l^{-1} + kinetin 2.0 mg l^{-1} (kinetin 1.0 mg l^{-1} for cv. Miss Snow White)

Culture period: 4 weeks

♦ Treatments (mg l^{-1})	Rungnappa Red		Miss Snow White		Earsakul	
	DSI	NOS	DSI	NOS	DSI	NOS
CONTROL	12.30[1]	2.67[1]	10.67[1]	2.67	11.67[1]	3.83
SCH 1	18.33	1.33	13.17[1]	2.33	25.67	3.17
SCH 2	18.33	1.33	16.67	1.67	17.50	5.17
SCH 3	15.00[1]	1.50	20.67	1.17	16.00	6.33
F	4.74 **	6.63**	21.23**	NS	25.36**	NS
CD (0.01)	3.94	0.74	2.78		3.43	

♦ Treatment details are given in the Table 10
The data represents mean value of six replications

In cv. Miss Snow White also, addition of CH at 250, 500 and 1000 mg l^{-1} delayed shoot multiplication. The control recorded minimum number of days (10.67) for shoot multiplication which was on par with SCH 1. As CH concentration increased from 250 to 1000 mg l^{-1} the number of days for shoot multiplication was also found increasing gradually. The maximum number of days (20.67) for shoot multiplication was recorded in SCH 3. The mean number of shoots produced varied from 1.17 to 2.67 and was found non significant statistically.

In cv. Earsakul, the days for shoot multiplication observed for the different treatment levels were found significant. The minimum number of days (11.67) for shoot multiplication was observed in control. The maximum number of days (25.67) for shoots multiplication was observed in SCH 1. Unlike the other varieties the shoot number decreased as CH concentration increased .The maximum shoot number (6.33) was obtained in SCH 3 and the minimum (3.17) in SCH 1, however the results were statistically non significant.

4. 1. 2. 3. 2. Glutamine

In cv. Rungnappa Red no significant difference was noticed with respect to the number of days for shoot multiplication (Table 33). The minimum days (12.33) for shoot multiplication were obtained in control. Significant difference was observed among the number of shoots produced which varied from 1.17 to 2.67. The control registered maximum number of shoots (2.67) produced which was statistically on par with SGL 1 and SGL 2. The treatment SGL 3 produced minimum number of shoots (1.17).

Significant difference was observed with respect to the number of days for shoot multiplication in *Dendrobium* cv. Earsakul. As in cv. Rungnappa Red, the minimum days (11. 67) for shoot multiplication were seen in control. This was on par with SGL 2. The maximum days (17.33) for shoot multiplication were found in SGL 3, which was on par with SGL 1. The shoot numbers produced at fourth week was found non significant.

In cv. Miss Snow White, the treatments involving different doses of glutamine failed to induce multiple shooting. The sub cultured shoots remained healthy and green at fourth week

without producing axillary shoots. Shoots subcultured in SGL 2 and SGL 3 became stouter with bright green and wider leaves (Plate 4).

4. 1. 2. 4. Sucrose

In cv. Rungnappa Red, the different doses of sucrose tried for multiple shooting were found significant for the number of days for shoot multiplication and the number of shoots produced (Table 34). The minimum number of days (12.17) for shoot multiplication was seen in SPS 4 which was on par with SPS 2 and SPS 3. The treatment supplemented with sucrose at 15.0 g l^{-1} took maximum number of days (17.00) for shoot multiplication. The shoot numbers obtained varied from 1.17 to 2.83. The maximum number of shoots (2.83) was obtained with SPS 4, which was on par with SPS 2 and SPS 3. The treatment SPS 1 gave minimum number of shoots (1.17).

In cv. Miss Snow White, significant difference was noticed with respect to the number of days for shoot multiplication, the mean of which varied from 10.67 to 19.83. The minimum days (10.67) for shoot multiplication were shown in SPS 2 (sucrose 30.0 g l^{-1}). The treatment SPS 4 having 60.0 g l^{-1} sucrose took maximum days for shoots multiplication. This was found on par with SPS 1 with 15.0 g l^{-1}. The number of shoots obtained with different doses of sucrose at 15.0 - 60.0 g l^{-1} was found non significant.

The treatments involving different concentrations of sucrose at 15.0, 45.0 and 60.0 g l^{-1} failed to produce multiple shoots in cv. Earsakul.

Though the effect of sucrose was found varying among the three varieties, it was seen that at higher levels of 45.0 g l^{-1} and 60.0 g l^{-1}, active rooting was noticed in the three varieties. Also at higher doses of sucrose the shoots were found to be luxuriant, with wider and greener leaves (Plate 5).

Plate 4.

Multiple shooting in treatments of coconut water in *Dendrobium* cvs.

1. Rungnappa Red **2. Miss. Snow White**

a. control

b. 50 ml l^{-1}

c. 100 ml l^{-1}

d. 150 ml l^{-1}

e. 200 ml l^{-1}

Multiple shooting in treatments of casein hydrolysate in *Dendrobium* cvs.

3. Rungnappa Red **4. Earsakul**

a. control

b. 250 mg l^{-1}

c. 500 mg l^{-1}

d. 1000 mg l^{-1}

Multiple shooting in treatments of glutamine in *Dendrobium* cvs.

5. Rungnappa Red **6. Miss. Snow White**

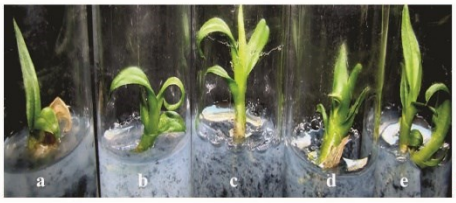
7. Earsakul

a. control

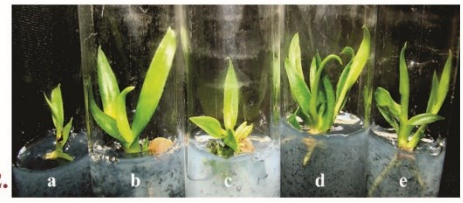
b. 1.25 mg l^{-1}

c. 2.5 mg l^{-1}

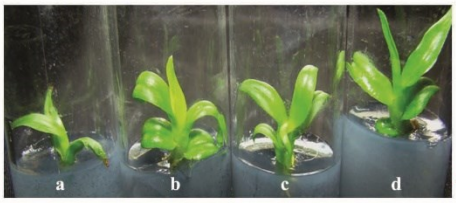
d. 5.0 mg l^{-1}

1. 

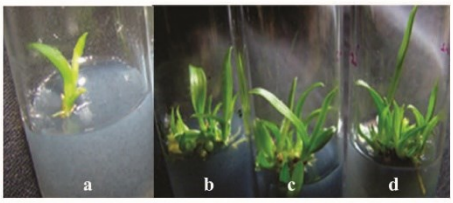
Dendrobium cv. Rungnappa Red

2. 

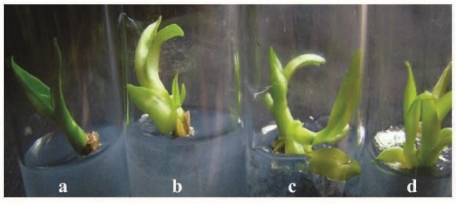
Dendrobium cv. Miss.Snow White

3. 

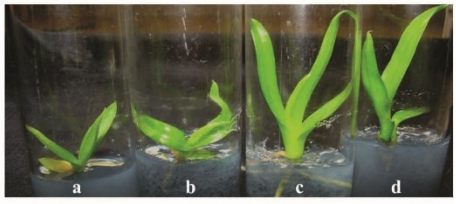
Dendrobium cv. Rungnappa Red

4. 

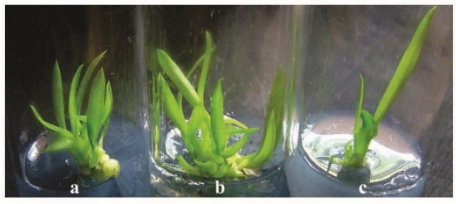
Dendrobium cv. Earsakul

5. 

Dendrobium cv. Rungnappa Red

6. 

Dendrobium cv. Miss. Snow White

7. 

Dendrobium cv. Earsakul

Table 33. Effect of glutamine on multiple shooting in *Dendrobium* cvs

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30 g l^{-1} + CS 15.0 g l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1} + NAA 0.1 mg l^{-1} + kinetin 2.0 mg l^{-1}

Culture period: 4 weeks

♦Treatments (mg l^{-1})	Rungnappa Red		Earsakul	
	DSI	NOS	DSI	NOS
CONTROL	12.33	2.67[1]	11. 67[1]	3.83
SGL 1	15. 83	1.83	16.00	2.33
SGL 2	13.00	2.50	13.50[1]	3.83
SGL 3	15.83	1.17	17.33	2.83
F	NS	4.51**	10.37**	NS
CD (0.01)	-	0.95	2.32	-

♦ Treatment details are given in the Table 11

The data represents mean value of six replications

Table 34. Effect of sucrose on multiple shooting in *Dendrobium* cvs.

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1} + NAA 0.1 mg l^{-1} + kinetin 2.0 mg l^{-1} (kinetin 1.0 mg l^{-1} for Miss Snow White)

Culture period: 4 weeks

♦Treatments (g l^{-1})	Rungnappa Red		Miss Snow White	
	DSI	NOS	DSI	NOS
SPS 1	17.00	1.17	18.17	1.83
SPS 2	12.33[1]	2.67[1]	10.67[1]	2.67
SPS 3	12.50[1]	2.67[1]	15.50	2.00
SPS 4	12.17[1]	2.83[1]	19.83	2.67
F	4.83 *	4.00*	11.69 *	NS
CD (0.05)	3.14	1.15	3.45	-

♦ Treatment details are given in the Table 12

The data represents mean value of six replications

4. 1. 2. 5. Polyamines

4. 1. 2. 5. 1. Spermine

The response to polyamine, spermine was different in three varieties (Table 35)**.** Significant difference was observed among the treatments in the case of days for bud initiation as well as the number of shoots produced at fourth week in cv. Rungnappa Red. The presence of spermine in general was found to speed up the days for shoot multiplication, the mean of which varied from 11.00 to 16.67. The treatment SSM 3 with 1.0 mM spermine induced early shoot multiplication in11.00 days. The maximum days (16.67) for shoot multiplication were observed in control, which was on par with SSM 1. The mean shoot numbers produced were between 1.16 and 2.50. The maximum shoot production (2.50) was found in SSM 3. The minimum shoot numbers (1.16) were obtained from control.

In cv. Miss Snow White the days for shoot multiplication as well as the number of shoots produced at fourth week were found significantly different. The treatment SSM 1 gave minimum number of days (15.00) for shoot multiplication which was statistically on par with SSM 3. The maximum number of days (23.50) for shoot multiplication was obtained in control which was on par with SSM 2. The mean number of shoots produced at fourth week was 1.00 - 3.00. The maximum shoot production (3.00) was found in SSM 2; where as the minimum number of shoots (1.00 and 1.67) were obtained from control and SSM 3.

The cv. Earsakul showed significant difference to different doses of spermine for the number of days for shoot multiplication. Similar to cv. Rungnappa Red, the minimum number of days (14.17) for shoot multiplication was recorded for the treatment SSM 3 which was on par with SSM 2. The maximum days (19.67) for shoot multiplication were observed in control which was on par with SSM 1. The data on number of shoots were found non significant. The shoot numbers varied from 1.67 to 3.83.

4. 1. 2. 5. 2. Spermidine

Significant difference was shown to different doses of spermidine for the number of days for shoot multiplication and the number of shoots produced at fourth week in cv. Rungnappa Red (Table 36).

The mean numbers of days for shoot multiplication varied between 10.00 and 19.50. The treatment SSP 3 took minimum days (10.00) for shoot multiplication. The treatment SSP 1 recorded maximum number of days (19.50) which was on par with SSP 2. Considering the number of shoots produced, treatment SSP 3 recorded the maximum value (2.33). The minimum number of shoots (1.16) was obtained in control.

The different treatment concentrations of spermidine were significant for the number of days for shoot multiplication in cv. Miss Snow White. The minimum number of days (18.83) for shoot multiplication was noted in SSP 3; however this was significantly on par with SSP2 and SSP 1. The control registered maximum days for shoot multiplication. The shoot numbers obtained were found non significant.

In cv. Earsakul the effect of spermidine at 0.25 to 1.0 mM concentrations were found non significant for the number of days for shoot multiplication and the number of shoots produced at fourth week.

In all the cultures supplemented with polyamines, the shoots appeared healthier with increased number of leaves and roots. Unlike the other cultures where phenolic browning of media was observed in the absence of activated charcoal, the polyamines supplemented cultures were free from the problem (Plate 5).

Plate 5.

Multiple shooting in treatments of sucrose in *Dendrobium* cvs.

1. Rungnappa Red **2. Miss. Snow White**

3. Earsakul

a. 15 g l^{-1}

b. 30 g l^{-1}

c. 45 g l^{-1}

d. 60 g l^{-1}

Multiple shooting in treatments of polyamine, spermine in *Dendrobium* cvs.

4. Rungnappa Red **5. Miss. Snow White**

a. control

b. 0.25 mg l^{-1}

c. 0.5 mg l^{-1}

d. 1.0 mg l^{-1}

Multiple shooting in treatments of polyamine, spermidine in *Dendrobium* cvs.

6. Rungnappa Red **7. Miss. Snow White**

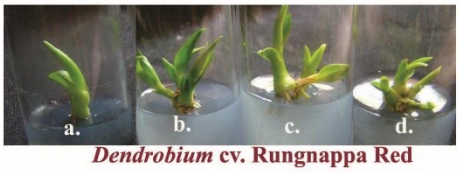
8. Earsakul

a. control

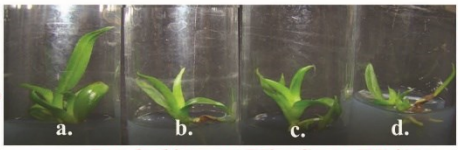
b. 0.25 mg l^{-1}

c. 0.5 mg l^{-1}

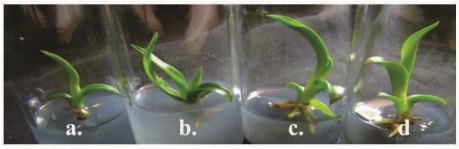
d. 1.0 mg l^{-1}

1. 

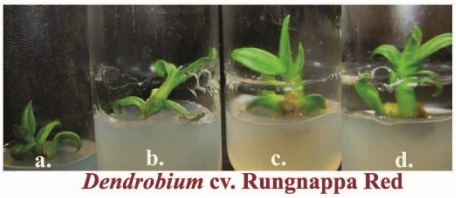
Dendrobium cv. Rungnappa Red

2. 

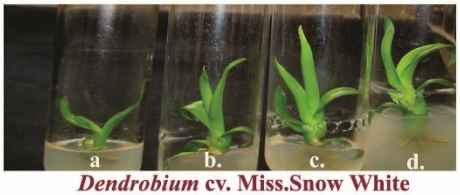
Dendrobium cv. Miss.Snow White

3. 

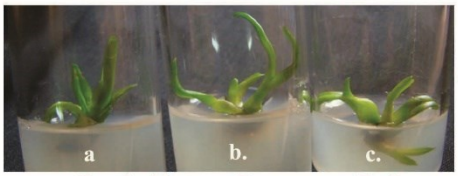
Dendrobium cv. Earsakul

4. 

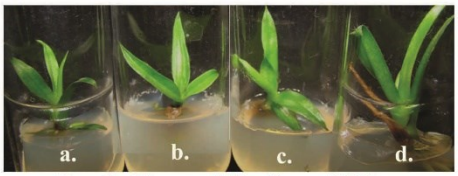
Dendrobium cv. Rungnappa Red

5. 

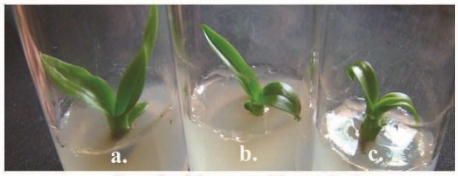
Dendrobium cv. Miss.Snow White

6. 

Dendrobium cv. Rungnappa Red

7. 

Dendrobium cv. Miss.Snow White

8. 

Dendrobium cv. Earsakul

Table 35. Effect of polyamine, spermine on multiple shooting in *Dendrobium* cvs.

Media: ½ MS + inositol 100 mg l^{-1} + sucrose 30.0 g l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + NAA 0.1 mg l^{-1} + kinetin 2.0 mg l^{-1} (kinetin 1.0 mg l^{-1} for Miss Snow White)

Culture period: 4 weeks

♦Treatments (mM)	Rungnappa Red		Miss Snow White		Earsakul	
	DSI	NOS	DSI	NOS	DSI	NOS
CONTROL	16.67	1.16	23.50	1.00	19.67	2.17
SSM 1	15.50	1.67	15.00[1]	2.17	17.67	3.83
SSM 2	14.00	2.00[1]	20.17	3.00	16.67[1]	2.17
SSM 3	11.00[1]	2.50[1]	17.00 [1]	1.67	14.17[1]	1.67
F	7.44**	8.72**	10.12**	13.83**	7.06	NS
CD (0.01)	2.65	0.64	3.45	0.67	2.53	-

♦ Treatment details are given in the Table 13

The data represents mean value of six replications

Table 36. Effect of polyamine, spermidine on multiple shooting in *Dendrobium* cvs

Media: ½ MS + inositol 100 mg l^{-1} + sucrose 30.0 g l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + NAA 0.1 mg l^{-1} + kinetin 2.0 mg l^{-1} (kinetin 1.0 mg l^{-1} for Miss Snow White)

Culture period: 4 weeks

♦Treatments (mM)	Rungnappa Red		Miss Snow White		Earsakul	
	DSI	NOS	DSI	NOS	DSI	NOS
CONTROL	16.67	1.16	23.50	1.00	19.67	2.17
SSP 1	19.50	1.20	20.50[1]	1.33	18.33	1.83
SSP 2	17.80	1.30	19.67[1]	1.33	18.50	1.83
SSP 3	10.00[1]	2.33[1]	18.83[1]	1.50	18.67	2.00
F	38.79**	8.72**	5.22**	NS	NS	NS
CD(0.01)	1.97	0.56	2.63	-	-	-

Treatment details are given in the Table 14

♦ The data represents mean value of six replications

4. 1. 2. 6. Activated charcoal

Significant difference was noticed among the different levels of AC for the number of days for shoot multiplication and the number of shoots produced at fourth week in cv. Rungnappa Red (Table 37). The mean number of days for bud initiation varied from 11.66 to 18.50. The minimum number of days (11.67) for shoot multiplication was obtained in the treatment SAC 1. The treatment SAC 2 took maximum number of days (18.50) for shoot multiplication, which was on par with SAC 3 and control. The average shoot numbers produced varied from 1.20 to 2.81. The minimum number of shoots (1.20) was produced in control, which was on par with SAC 3. The treatment SAC 2 produced maximum shoot numbers (2.81) and was followed by SAC 1 (2.71).

In cv. Miss Snow White, significant difference was noticed with respect to the number of days for shoot multiplication as well as for the number of shoots produced. The mean numbers of days for shoot multiplication varied between 10.67 and 23.50. The treatment SAC 1 registered minimum days (10.67) for shoot multiplication. The maximum days (23.50) for shoot multiplication occurred in control, which was on par with SAC 3. The mean number of shoots produced varied from 1.00 to 2.71. The minimum shoot numbers was recorded in control and maximum in SAC 1 which was on par with SAC 2 and SAC 3.

In cv. Earsakul, the mean number of days for shoot multiplication varied significantly among the different treatment levels of activated charcoal. The mean number of days for shoot multiplication was from 11.67 to 25.10. The minimum number of days (11.67) for shoot multiplication happened in SAC 1, which was superior over the other treatment levels. The maximum number of days (25.10) was noted in SAC 3. The shoot numbers produced were found non significant (Plate 6).

Plate 6.

Multiple shooting in treatments of activated charcoal in *Dendrobium* cvs.

1. Rungnappa Red **2. Miss. Snow White**

3. Earsakul

a. **control**
b. **0.5 g l^{-1}**
c. **1.0 g l^{-1}**
d. **2.0 g l^{-1}**

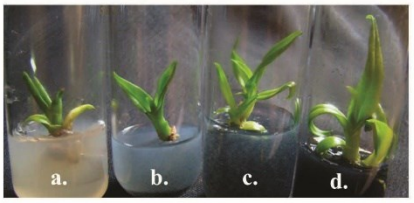
***In vitro* rooting in treatments of plant growth regulators in *Dendrobium* cvs.**

4. Rungnappa Red **5. Earsakul** **6. Miss. Snow White**

7. Hardening of plantlets of *Dendrobium* cvs.

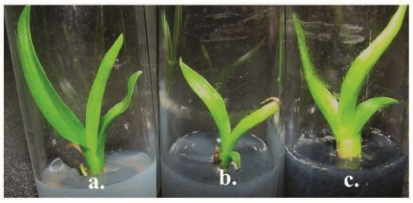
8. *Ex vitro* established plantlet of 2 months old

1.



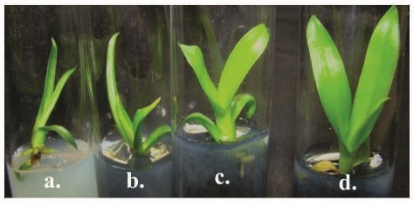
Dendrobium cv. Rungnappa Red

2.



Dendrobium cv. Miss.Snow White

3.



Dendrobium cv. Earsakul

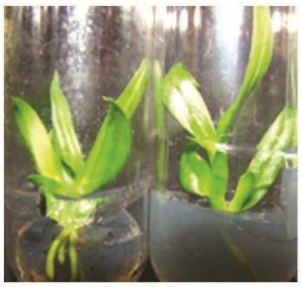
4.

Dendrobium
cv. Rungnappa Red

5.

Dendrobium
cv. Earsakul

6.



Dendrobium
cv. Miss.Snow White

7.

a.

b.

4. 1. 2. 7. Steroid plant growth regulator

The response to steroid plant growth regulator, 28 - homobrassinolide showed wide variation among the varieties (Table 38)**.**

In cv. Rungnappa Red, the mean days for shoot multiplication varied from11.00 to 21.67 days. The treatment with SHB 4 (4.0 mg l^{-1}) showed earliest shoot multiplication of 11.00 days. This was found on par with treatments SHB 3, SHB 2 and SHB 1. The maximum days (21.67) for shoot multiplication were observed in control. The maximum number of shoots (4.00) was produced in SHB 1, whereas the minimum shoot numbers (1.00) were produced in SHB 4 and control. SHB 1 was statistically found to be on par with SHB 2 and SHB 3.

In cv. Miss Snow White, the minimum days (19.00) for shoot multiplication were seen in SHB 1. The treatment SHB 4 recorded maximum days (24.50) for shoot multiplication. The maximum numbers of shoots (3.00) were produced in SHB 3, which was found on par with SHB 4, SHB 1 and SHB 2. The minimum number of shoots (1.00) was produced in control.

The mean number of days for shoot multiplication in cv. Earsakul varied from 21.00 to 28.33. The treatment SHB 4 recorded minimum days (21.00) for shoot multiplication. This was found on par with SHB 3, which recorded 23.33 days. The maximum days (28.33) for shoot multiplication were observed in control as well as in SHB 1. The shoot numbers produced increased with the concentration of 28 - HBL. The maximum shoot numbers (7.00) were obtained in SHB 4, which was found statistically superior over the other treatments. The minimum number of days (1.00) was noted in SHB 1, with which the treatment SHB 2 and control were found on par.

Table 37. Effect of activated charcoal on multiple shooting in *Dendrobium* cvs.

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + NAA 0.1 mg l^{-1} + kinetin 2.0 mg l^{-1} (kinetin 1.0 mg l^{-1} for Miss Snow White)

Culture period: 4 weeks

♦Treatments (g l^{-1})	Rungnappa Red		Miss Snow White		Earsakul	
	DSI	NOS	DSI	NOS	DSI	NOS
CONTROL	16.67	1.20	23.50	1.00	19.67	2.16
SAC 1	11.67 [1]	2.71[1]	10.67 [1]	2.71[1]	11.67 [1]	3.83
SAC 2	18.50	2.81[1]	18.00	2.31[1]	22.17	2.50
SAC 3	17.00	1.67	20.20	2.11[1]	25.10	1.33
F	5.76 **	9.19**	34.89**	4.07**	32.33 **	NS
CD(0.01)	3.34	0.78	2.72	1.06	3.00	-

♦ Treatment details are given in the Table 15

The data represents mean value of six replications

Table 38. Effect of 28- homobrassinolide on multiple shooting in *Dendrobium* cvs.

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1}

Culture period: 4 weeks

♦Treatments (mg l^{-1})	Rungnappa Red		Miss Snow White		Earsakul	
	DSI	NOS	DSI	NOS	DSI	NOS
CONTROL	21.67	1.00	24.00	1.00	28.33	2.33
SHB 1	12.50[1]	4.00	19.00[1]	2.33[1]	28.33	1.00
SHB 2	14.00[1]	3.00[1]	22.00	2.00[1]	26.00	2.00
SHB 3	14.00[1]	3.00[1]	23.00	3.00[1]	23.50[1]	4.50
SHB 4	11.00[1]	1.00	24.50	2.50[1]	21.00[1]	7.00[1]
F	11.01**	3.97*	NS	3.11*	6.49**	8.74**
CD	3.82	1.96		1.23	3.67	2.36
General observation	Rooting more prominent		Rooting more prominent		Multiple shooting more prominent	

♦ Treatment details are given in the Table 16

The data represents mean value of six replications

4. 1. 3. *IN VITRO* ROOTING

The *in vitro* rooting response varied in the three *Dendrobium* varieties (Plate 6).The mean number of days for root initiation in cv. Rungnappa Red was from 13.67 to 22.40. The minimum duration (13.67) for rooting was seen in RT 1 (IBA 0.5 mg l^{-1}) which was statistically on par with RT 5 (14.00) and RT 4 (16.00). No significant difference was noted for the number of roots produced at fourth week (Table 39).

In cv. Miss Snow White, the number of days for root initiation as well as the number of roots produced at fourth week showed significant difference among the treatments and control. The treatment RT 1 showed minimum number of days (18.17) for rooting which was statistically on par with RT 6 (18.83) and RT 7 (19.17). The maximum days for root initiation were noticed in RT 4 (28.00). The root numbers produced were found significant with the maximum root numbers being obtained in RT 9 (4.83), which was found on par with RT 6 (4.33), RT 0 (3.67), RT 2 (3.67) and RT 1 (3.50).

Significant difference was noticed for the mean number of days for root initiation in cv. Earsakul. The minimum days for root initiation was recorded in RT 4 (19.60) which was on par with RT 1, RT 8, RT 5, RT 7 and RT 2. As in cv. Rungnappa Red no significant difference was noted for the number of roots produced at fourth week.

4. 1. 4. HARDENING AND PLANTING OUT

The survival rate of plantlets was highly dependant on the media used. (Table 40). None of the plantlets survived in media with soilrite alone. In cv. Rungnappa Red, the plantlets transferred to charcoal and brick pieces media showed survival rate of 83.33 per cent. In. cv. Miss Snow White plantlets survived in soilrite added charcoal and brick pieces media. But the survival rate was only 14.29 per cent. A survival rate of 75.00 per cent was obtained in charcoal and brick pieces media (1:1). The cv. Earsakul also showed survival rate of 66.67 per cent in charcoal and brick pieces media.

Table 39. Effect of plant growth regulators on *in vitro* rooting of *Dendrobium* cvs.

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1}

Culture period: 4 weeks

♦Treatments (mg l^{-1})	Rungnappa Red		Miss Snow White		Earsakul	
	DRI	NOR	DRI	NOR	DRI	NOR
RT 0	22.40	2.20	25.67	3.67[1]	26.40	2.00
RT 1	13.17[1]	2.33	18.17[1]	3.50[1]	20.75[1]	2.25
RT 2	17.17	2.67	20.67	3.67[1]	22.00[1]	3.00
RT 3	16.33	3.17	21.00	2.00	23.60	3.20
RT 4	16.00[1]	3.75	28.00	2.00	19.60[1]	2.60
RT 5	14.00[1]	3.00	23.75	2.00	21.33[1]	3.00
RT 6	17.00	2.50	18.83[1]	4.33[1]	23.17	2.50
RT 7	18.00	3.00	19.17[1]	1.33	21.75[1]	1.75
RT 8	20.25	3.00	21.17	2.50	20.75[1]	2.50
RT 9	21.40	2.80	21.00	4.83[1]	23.17	1.67
F	19.54 * *	NS	12.47 * *	6.19 * *	3.97 * *	NS
£CD	(6,6) - 1.78		(6,6) - 2.07	1.28	(6,6) - 2.50	
	(6,5) - 1.86		(6,4) - 2.32	1.43	(6,5) - 2.63	
	(6,4) - 1.99		(6,2) - 2.93	1.81	(6,4) - 2.79	
	(5,5) - 1.95		(2,4) - 3.12	1.92	(5,5) - 2.74	
	(5,4) - 2.06				(5,4) - 2.91	
	(4,4) - 2.18				(4,4) - 3.07	

♦Treatment details are given in the Table 17

£ Since the effective number of replications varies, separate CD values were computed

Table 40. Survival rate of plantlets after 1 month

Media	Survival Rate (%)		
	Rungnappa Red	Miss Snow White	Earsakul
Soilrite	-	-	-
Charcoal + Brick pieces + soilrite	-	14.29	-
Charcoal + Brick pieces	83.33	75.00	66.67

4. 2. *IN VITRO* POLYPLOIDISATION

4. 2. 1. Explant

The explants such as PLBs and *in vitro* stem nodes were subjected to colchicine treatments.

The survival rate of colchicine treated PLBs varied from 0.00 to 100.00 per cent. The rate of shoot regeneration was from 40.00 to 100.00 per cent. It was seen that as the concentration of colchicines or duration of treatment increased the survival rate was found to be low (Table 41). At concentration 0.10 per cent for 8 days and at 0.20 per cent for 5 and 8 days, none of the treated explants survived.

The mean days for shoot regeneration showed wide variation of 10.00 to 24.00 days. The minimum days of 10.00 for shoot regeneration were observed in PPC 1, PPT 5 and PPC 5, which were found statistically on par with PPT 4, PPC 2, PPC 6 and PPT 1. The maximum days (24.00) for shoot regeneration were seen in PPT 7. The mean number of shoots also showed wide variation of 2.00 to 6.00, the minimum being produced in PPC 1 and PPC 6. The maximum number of shoots was obtained in treatment PPT 4 (6.50), which was found on par with PPT 5 (6.00) and PPT 7 (5.67). All the regenerated plants were found to be having normal morphology.

The colchicine treated stem nodes had survival rate of 0 to 100.00 per cent. The shoot regeneration rate showed wide variation from 33.33 to 100.0 per cent (Table 42). Unlike the PLBs treated at higher colchicine concentration or longer duration, the stem nodes treated with higher colchicine concentration or longer duration survived and shoots were regenerated (Plate 7a and 7b). The rate of shoot regeneration varied from 20.00 to 71.43 per cent.

The mean number of days for shoots regeneration varied from 8.50 to 17.67 which was found statistically non significant. The minimum days (8.50) for shoot regeneration were noticed in NPT 7. The maximum days (17.67) for shoot regeneration were obtained in NPT 4.

The mean number of shoots regenerated varied from 1.00 to 5.00. The maximum number of shoots was obtained in treatment NPT 9, which was statistically superior over other treatments and the minimum was obtained from NPT 3.

The shoots regenerated from NPT 5 were different in morphology when compared to the other regenerated shoots. The shoot buds developed were found slow growing. They were distinct in morphology with shortened internodes, thicker and greener leaves (Plate 7c).

4. 2. 2. Stomatal study

Stomata number, stomata size and epidermal cell size were measured using Micrometer (Plate 7d, 7e, 7f and 7g).

The stomata number of leaves of regenerated shoots from PLBs varied from 4.00 to 9.00 (Table 43). The highest value was noticed in shoots regenerated from treatments PPT 5 and PPT 7 which were found statistically on par with PPT 3. The stomatal diameter was around 7.0-8.0 µ. The epidermal cell size varied from 61.66 to 81.00 μ^2 which was found statistically non significant.

In the case of shoots regenerated from stem nodes, the stomata number was between 3.00 and 11.00 (Table 44). The maximum numbers of stomata were noticed in NPC 1, NPC 2 and NPT 9 which were on par with NPC 3. The lowest stomata number (3.00) was observed in NPT 6 which was found on par with NPT 4.

The stomatal size varied from 6.8 to 8.0 µ. The largest stomata size was obtained from treatments NPC 1, NPC 2, NPT 1, NPT 3, NPC 3, NPC 6 and NPT 9.The smallest stomata size was noticed with treatments NPT 2 and NPT 4.

The epidermal cell size varied from 61.66 to 998.33 μ^2. The slow growing shoots with large epidermal cell size which were supposed to be polyploids were characterized by absence of stomata and large sized epidermal cells.

Table 41. Effect of colchicine on survival and shoot regeneration from PLBs of cv. Miss Snow White

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1}

Explant: Three clumps of 8 -12 plbs/ tube

♦Treatments	Survival rate (%)	Shoot regeneration rate	Days for shoot regeneration	No: of shoots
PPC 1	100.00	77.78	10.00[1]	2.00
PPC 2	100.00	77.78	10.43[1]	3.14
PPT 1	100.00	66.67	11.17[1]	3.00
PPT 2	100.00	55.56	11.67[1]	2.20
PPT 3	77.78	50.00	14.00	3.25
PPC 3	100.00	100.00	19.89	2.56
PPC 4	100.00	88.89	13.00	2.50
PPT 4	22.22	100.00	10.50[1]	6.50[1]
PPT 5	55.56	40.00	10.00[1]	6.00[1]
PPC 5	55.56	100.00	10.00[1]	2.60
PPC 6	66.67	66.67	11.00[1]	2.00
PPT 7	55.56	60.00	25.00	5.67[1]
F	-	-	22.91**	6.86**

[£]CD values for comparing treatment means for 'Days for shoot regeneration':

(PPT5,PPC2) - 3.36	(PPC5,PPC2) - 2.24	(PPC1,PPC2) - 2.24
(PPT5,PPT4) - 4.19	(PPC5,PPT4) - 3.50	(PPC1,PPT4) - 3.36
(PPT5,PPC6) - 3.63	(PPC5,PPC6) - 2.81	(PPC1,PPC6) - 2.62
(PPT5,PPT1) - 3.42	(PPC5,PPT1) - 2.54	(PPC1,PPT1) - 2.33
(PPT5,PPT2) - 3.42	(PPC5,PPT2) - 2.54	(PPC1,PPT2) - 2.33

[£]CD values for comparing treatment means for 'No: of shoots':

(PPT4,PPT5) - 2.11 (PPT4,PPT7) - 1.20

♦ Treatment details are given in the Table 18

[£] Since the effective number of replications varies, separate CD values were computed

Table 42. Effect of colchicine on survival and shoot regeneration from stem nodes of cv. Miss Snow White

Media: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1}

♦Treatments	Survival rate	Shoot regeneration rate	Days for shoot regeneration	No: of shoots (10 th week)
NPC 1	66.67	50.00	13.00	2.33
NPC 2	66.67	66.67	12.75	1.75
NPT 1	77.78	71.43	13.40	2.20
NPT 2	77.78	57.14	13.50	2.25
NPT 3	55.56	40.00	13.50	1.00
NPC 3	88.89	62.50	14.20	2.00
NPC 4	66.67	66. 67	13.50	2.50
NPT 4	55.56	60.00	17.67	3.00
NPT 5	66.67	50.00	17.33	2.67 ●
NPT 6	33.33	33.33	16.00	2.00
NPC 5	100.00	55.56	15.00	1.60
NPC 6	100.00	33.33	8.67	2.33
NPT 7	100.00	22.22	8.50	3.00
NPT 8	33.33	66.67	11.00	1.00
NPT 9	55.56	20.00	9.00	5.00[1]
F	-	-	NS	2.32*
CD				1.89

● Shoots were slow growing, reduced internodes with thicker and greener leaves

♦ Treatment details are given in the Table 19

Table 43. Stomata number, stomata size and epidermal cell size of regenerated shoots from PLBs

♦Treatments	Stomata number (Mean of 3 fields of view)	Stomata size Diameter in µ (Mean of 3 stomata)	Epidermal cell size(μ^2) (Mean of 3 epidermal cells)
PPC 1	4.00	8	66.00
PPC 2	4.00	8	61.66
PPT 1	4.00	8	72.33
PPT 2	4.00	7	78.33
PPT 3	7.67	8	78.33
PPC 3	4.00	8	77.67
PPC 4	4.33	8	77.67
PPT 4	6.33	7	66.66
PPT 5	9.00	8	66.66
PPT 6	4.33	8	81.00
PPC 5	4.00	8	77.67
PPT 7	9.00	8	61.66
F	28.20	-	NS
CD	1.12		

♦ Treatment details are given in the Table 18

Table 44. Stomata number, stomata size and epidermal cell size of regenerated shoots from stem nodes

Treatments	Stomata number (Mean of 3 fields of view)	Stomata size – Diameter in µ (Mean of 3 stomata)	Epidermal cell size (Mean of 3 epidermal cells)
NPC 1	11.00	8.00	68.33
NPC 2	11.00	8.00	66.66
NPT 1	6.00	8.00	78.33
NPT 2	9.00	6.30	72.33
NPT 3	9.00	8.00	77.67
NPC 3	10.33	8.00	68.33
NPC 4	6.00	7.00	72.33
NPT 4	4.00	6.30	66.66
NPT 5	-	-	998.33
NPT 6	3.00	7.00	72.33
NPC 5	6.66	7.00	61.66
NPC 6	9.00	8.00	77.67
NPT 7	6.66	7.00	78.33
NPT 8	9.00	7.00	61.66
NPT 9	11.00	8.00	68.33
F	24.48**	27.11**	8.68**
CD	2.00	0.47	234.93

♦ Treatment details are given in the Table 19

Plate 7.

a. Regenerating shoots from colchicine treated plbs

b. Regenerating shoots from colchicine treated stem nodes

c. Putative polyploidy shoot

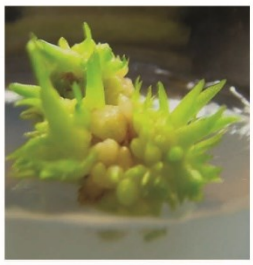
Microscopical view of epidermal cell layer of leaves

d. Shoot regenerated from treated stem node

e. Shoot regenerated from treated plb

f. Untreated shoot

g. Epidermal section of putative polyploid

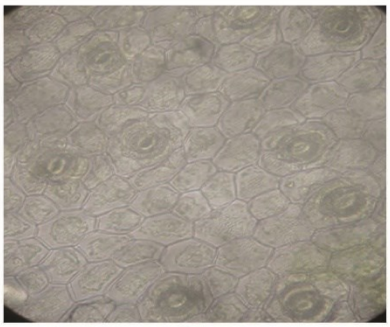


a.

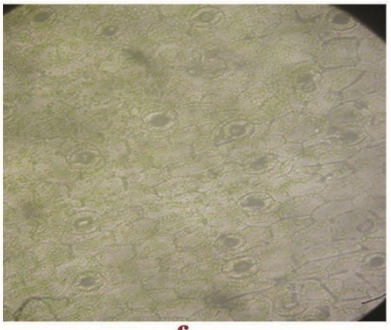
b.

c.

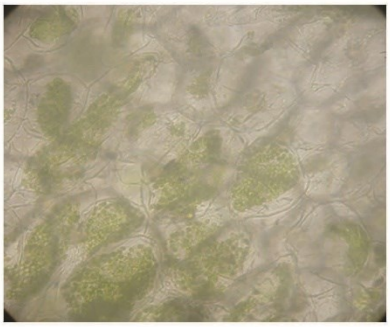
d.



e.



f.



g.

Discussion

5. DISCUSSION

Orchids rank second among the top selling cut flowers in the world. Orchid generas such as *Aranda, Cymbidium, Dendrobium, Oncidiums* and *Paphiopedilum* command high value and great demand world over. Of these *Dendrobium* alone commands 85 per cent of the total trade in tropical orchid cut flowers. *Dendrobium* being the second largest genus in the family Orchidaceae with 1340 species and over one lakh man-made hybrids provides ample scope for crop improvement (Baker and Baker, 1996).

Micro propagation is the sole technique that enables large scale production of orchid plants to meet their increasing global as well as local demand. The standardization of *in vitro* propagation protocol is essential to establish a viable regeneration system that produces healthy, vigorous and true-to-type plantlets of popular cultivars. Such a regeneration protocol is specific for each cultivar. The specificity could be so precise that even among hybrids of same parentage, the *in vitro* response was found to be varied (Rahana, 2006).

Induction of polyploidy using colchicine is an established procedure for crop improvement. In orchids improvement in flower size, colour, flower form and floriferousness as a result of induced polyploidy have been reported. The incorporation of this method to *in vitro* system has got added advantage of mass propagation of uniform desirable polyploids.

Three popular *Dendrobium* cultivars were selected for the present study of enhancement of *in vitro* propagation and induction of polyploidy in orchids. The outcome of the study is discussed in this chapter.

Surface sterilization of explants using mercuric chloride was reported effective in initiating aseptic cultures in many orchid varieties. In the present study the most effective concentration and duration of treatment with mercuric chloride was found to vary slightly in the four varieties tried. The survival rate was highly dependant on type of explants, concentration of mercuric chloride and duration of the treatment.

In cv. Rungnappa Red and Earsakul stem nodal explants of keikies treated with 0.1 per cent mercuric chloride for 5 minutes produced maximum survival rate of 75.0 per cent and 62.5 per cent, respectively. An increase in duration was found to lower the survival rate indicating the lethality of mercuric chloride above a critical dose, while decrease in concentration also lowered the survival rate due to increased instance of microbial contamination. In cv. Miss Snow White, maximum survival rate of 88.9 per cent was obtained for stem nodal explants treated with 0.08 per cent mercuric chloride solution for 10 minutes. In *D. philippica*, mercuric chloride at 0.08-0.1 per cent for 10 minutes gave maximum survival rate of 75.0 per cent for the stem nodal explants. A survival rate of 80.0 per cent was obtained in *Dendrobium* cv. Sonia – 17, when keikies were surface sterilized with 0.1 per cent mercuric chloride solution for 10 minutes (Kuriakose, 1997). Surface sterilization with 0.1 per cent mercuric chloride solution for 10-12 minutes in shoot tips and axillary buds of *Dendrobium* hybrids was found effective by Devi and Laishram, 1998.

The tender leaf segments of cv. Earsakul treated with mercuric chloride 0.08-0.1 per cent for 10 minutes produced the maximum survival rate of 100.00 per cent; however no callusing or bud regeneration occurred from the treated explants. In the treatment with 0.1 per cent mercuric chloride solution for 5 minutes the survival rate decreased to 60.00 per cent due to microbial contamination. On the other hand in *D. philippica* the same treatment gave maximum survival rate of 66.67 per cent along with the treatment of 0.08 per cent for 10 minutes. None of the treated explants of both varieties produced callus or shoot buds.

Spent inflorescence stalk of cv. Earsakul treated with 0.10 per cent mercuric chloride solution for 10 minutes gave 40 per cent survival rate and 100 per cent subsequent bud regeneration, whereas in cv. *D. philippica,* nodal segments of young inflorescence gave 100.00 per cent survival rate and subsequent bud regeneration. Surface sterilization with 0.10 per cent mercuric chloride solution for 9 -12 minutes was reported effective for treating the flower stalk explants of Sonia -17 and 28 (Martin et al., 2005).

Stem nodal segments from kiekies are ideal explants for clonal propagation since it does not involve the sacrifice of entire mother plant. The successful plantlet regeneration from stem nodal segments has been reported by many workers (Sagawa, 1961; Duan et al., 1996; Teng et al., 1997; Devi and Deka, 2001). Sivamani (2004) reported PLB formation and plantlet development when the stem nodal segment with dormant buds of *Dendrobium* hybrids was cultured.

The response of various explants was found to be influenced by cultivar and growth regulator combination in basal medium. Callusing was initiated in cv. Rungnappa Red at BA 2.0 mg l^{-1} and NAA 0.5 - 1.0 mg l^{-1}. Increasing either BA or NAA concentration to 4.0 mg l^{-1} caused drying of leaves, indicating the sensitiveness of leaves to higher dose of BA. On the other hand lower level of hormones failed to induce callusing. A similar response wherein at low concentration of hormone, the leaf explants failed to show prominent callusing and at higher concentration caused the necrosis of explants was reported in *Dendrobium malones* 'Victory' (Anjum et al., 2006). The failure of root explants to produce any *in vitro* response indicates their poor regenerative ability. Teng et al. (1997) reported that root segments dissected from *in vitro* plantlets of *Spathoglottis plicata* were able to regenerate only occasionally.

The leaf explants of cv. Miss Snow White showed expansion and greening in all the treatments, but initiated callus only at higher levels of both BA and NAA (4.0 mg l^{-1}). Unlike the root explants of cv. Rungnappa Red, the root explants of cv. Miss Snow White showed expansion in all the hormone supplemented media, but callus initiation occurred in treatment having BA 2.0 mg l^{-1} and NAA 0.5 mg l^{-1}. This may be the indication of specificity of explants to hormonal combinations, but further trials need to be done to conclude on this aspect. The low regeneration capacity of buds, from root derived callus, induced in basal medium with NAA 1.25 mg l^{-1}, as a result of incomplete cell dedifferentiation possibly due to high cytokinin levels either in the explant or in callus was reported in *Oncidium varicosum* (Kerbauy, 1984).

The *in vitro* response of leaf and root explants of cv. Earsakul was found more or less similar to that of cv. Rungnappa Red however callus was not initiated.

Similar response was noticed in *D. philippica* also. Unlike the other cultivars direct shoot regeneration was obtained from leaf explants inoculated to ½ MS supplemented with BA 4.0 mg $l^{-1}$and NAA 1.0 mg l^{-1}. Direct organogenesis from *in vitro* leaf explants cultured under darkness in 1/2 MS supplemented with BA 1.0 mg $l^{-1}$and NAA 1.5 mg l^{-1} was obtained in *Dendrobium* Sonia-17 (Kuriakose, 1997). Good PLB regeneration and subsequent plantlet development from juvenile leaf segments excised from three to four months old *in vitro* established *Dendrobium* hybrids was reported by Rahana (2006). The direct shoot organogenesis from thin leaf explants was reported in *D. malones* (Anjum et al., 2006).

The regenerative potential of flower stalks was demonstrated for the first time by Rotor (1949) in *Phalaenopsis.* In the present study the spent inflorescence of cv. Earsakul developed shoot buds in half MS supplemented with BA 2.0 mg l^{-1}, but the shoot buds failed to elongate in further subcultures. In *Dendrobium philippica* callus developed from the nodal segments of young inflorescence at lower concentration of kinetin 2.0 mg l^{-1} alone and in combination with IAA 1.0 - 4.0 mg l^{-1}. Shoot buds developed at higher concentration of kinetin 4.0 mg l^{-1} and its combinations with IAA 1.0 - 4.0 mg l^{-1}. Shoot bud regeneration from flower-stalk internodes of *Oncidium* cv. Sweet Sugar in half MS basal medium supplemented with thidiazuron (0.1 - 3.0 mg l^{-1}) was reported by Chen and Chang (2000). Half strength MS medium fortified with kinetin (0.5 – 5.0 mg l^{-1}) singly and also in combination with NAA (0.1 - 0.5 mg l^{-1}) induced bud break from flower stalk node explants of *Dendrobium* hybrids Sonia -17 and Sonia -28 (Martin et al., 2005).

The *in vitro* response to plant growth regulators in culture establishment medium varied among the four *Dendrobium* cutivars tried. Out of the 58 treatments tried for cv. Rungnappa Red, the earliest bud initiation (7.33) from stem nodal explants was obtained in CRT 30 having kinetin 2.0 mg l^{-1} and NAA 0.1 mg l^{-1}. Generally from the results of treatment combinations it can be inferred that kinetin alone at 0.1, 0.5, 1.0, 2.0 and 4.0 mg l^{-1}, and in combination with NAA at 0.1, 0.5, 1.0 and 2.0 mg l^{-1} in a wider proportion of 1 -20: 1, could induce relatively early bud break within two weeks. The proportion of BA and NAA that could induce early bud break was only at a narrow range of 1- 4: 1. Direct axillary shoot induction from nodal explants of *Dendrobium macrostachyum* in MS medium with BA at 0.5, 1.0 and 2.0 mg l^{-1} in

combination with NAA at 0.5 mg l^{-1} (Pyati et al., 2002), a proportion of 1- 4: 1, supports the present result. Early bud break was noticed in kinetin and IAA combinations of relative proportion 0.5-4.0 : 1, and BA and IAA combinations of relative proportion 1: 1. The maximum number of days (30.0) for bud initiation was obtained in the treatment with BA 4.0 mg l^{-1} and NAA 0.5 mg l^{-1}. The higher concentration of BA at 4.0 mg l^{-1} and that of auxins at 1.0 mg l^{-1} and 2.0 mg l^{-1} were found to delay bud initiation beyond 20 days. The delay in bud initiation induced by auxins at higher levels was also pronounced when they were in combination with relatively lower levels of cytokinins.

The maximum number of shoots (3.00) was also obtained in treatments with kinetin 2.0 mg l^{-1} and NAA 0.1 mg l^{-1} as well as in BA 1.0 mg l^{-1} and NAA 0.5 mg l^{-1}. A comparatively higher level of cytokinin in combination with auxin was found to produce maximum shoot numbers in cv .Rungnappa Red. Generally the combinations of BA and NAA at 2 : 1 proportion was found to favor the production of maximum number of shoot buds at culture establishment stage. Sivamani (2004) reported a 1:1 ratio of higher doses of auxins and cytokinins as best for the production of more number of shoots in *Dendrobium* hybrids.

A similar observation as in the case of cv. Rungnappa Red was evident among the 56 treatments of plant growth regulators for the culture establishment medium of cv. Miss Snow White. In this case the earliest bud initiation (10.33) from stem nodal explants was observed in treatment with kinetin 1.0 mg l^{-1} and NAA 0.1 mg l^{-1}. The relative proportion of kinetin and NAA at 1 - 20: 1 and that of BA and NAA at 2 - 4: 1 was found to induce early bud break of less than 16 days. The combinations of BA at 0.5 and 1.0 mg l^{-1} along with higher doses of NAA and IAA (1.0 and 2.0 mg l^{-1}) delayed bud initiation over the control. However higher level of BA (4.0 mg l^{-1}) along with higher level of IAA and NAA (1.0 and 2.0 mg l^{-1}) induced early bud break. In *Dendrobium* cv. Sonia, the culture establishment medium supplemented with BA 1.0 mg $l^{-1}$and NAA 1.5 mg l^{-1} was found most effective in early bud initiation (Kuriakose, 1997).

The maximum shoot numbers during establishment phase were produced in treatments with higher levels of BA (4.0 mg l^{-1}) along with higher levels of NAA (1.0 and 2.0 mg l^{-1}). But

the shoots produced were shorter, pale and without fully emerged leaves. Such a deleterious effect on shoot growth at higher levels of BA was noted by Murashige (1974), Lekhsmidevi (1992) and Sivamani (2004) thus confirming the present inference.

In cv. Earsakul, early bud break was obtained in treatment with BA 4.0 mg l^{-1}. Similar early response was obtained in treatments with kinetin 2.0 and 4.0 mg l^{-1} alone. The combinations of kinetin at 1.0, 2.0 and 4.0 mg l^{-1} along with NAA 0.1 mg l^{-1} also induced early bud break less than 13 days. The combinations of BA at 1.0 and 2.0 mg l^{-1} along with NAA at 0.1 and 0.5 mg l^{-1} also induced early bud initiation. Kinetin and BA at higher levels of 4.0 mg l^{-1} along with IAA 1.0 mg l^{-1} too produced early bud break. On the other hand, presence of IAA at higher levels of 1.0 and 2.0 mg l^{-1} alone and in combination with kinetin 1.0 mg l^{-1} delayed bud break. The maximum shoot number (4.33) was obtained in the treatment having BA 2.0 mg l^{-1} and NAA 0.1 mg l^{-1}.

Early bud break in *D. philippica* was noticed in treatment combination of BA 4.0 mg l^{-1} and NAA 0.5 mg l^{-1}. The concentration of BA at higher levels of 2.0 and 4.0 mg l^{-1} alone and in combination with NAA (0.5, 1.0 and 2.0 mg l^{-1}) was found to induce early shoot bud initiation.

Among the four different basal media tried for culture establishment half MS was found better than MS, VW and KC. This indicates that the presence of major nutrients at low dose along with that of the minor nutrients is beneficial for shoot bud induction and development. A similar response showing the superiority of half MS over full MS in shoot bud regeneration from pseudobulb segments of *Dendrobium* hybrid was reported by Kishor et al. (2007). The superiority of MS over the other media in orchid culture has been established by many researchers (Kaur and Sarma, 1997, Saiprasad et al. 2001, Saiprasad and Raghuveer, 2002 and Alam et al., 2002).

The optimum shoot multiplication and regeneration of healthy, vigorous shoots is dependant on plant growth regulators and media supplements used. In addition, the interaction and balance between the growth regulators in the medium and the growth substances produced

endogenously by cultured cells influence growth and morphogenesis *in vitro* (Krikorian, 1982). In general, the balance between auxins and cytokinins is critical for shoot proliferation (Skoog and Miller, 1957). Cytokinins at higher concentration have deleterious effect on shoot growth, while the presence of auxins can nullify the suppressive effect of cytokinins on shoot growth (Lindergan and Janick, 1980). The three *Dendrobium* cultivars used for shoot multiplication trials varied differently in their response to growth regulators and media supplements.

The minimum number of days for shoot multiplication in cv. Rungnappa Red was noticed in treatments of kinetin at 0.5, 1.0 and 2.0 mg l^{-1}, BA at 0.5 mg l^{-1}, NAA at 0.1 and 0.5 mg l^{-1}, combination of BA at 0.5, 1.0 and 2.0 mg l^{-1} along with NAA at 0.1 mg l^{-1} and combination of kinetin at 0.5, 1.0 and 2.0 mg l^{-1} along with NAA at 0.1, 0.5, 1.0 and 2.0 mg l^{-1} (Fig.1). The concentration of BA and NAA at higher levels of 1.0 and 2.0 mg l^{-1} were found to delay shoot multiplication. Also the treatment combinations of BA at 0.5, 1.0 and 2.0 mg l^{-1} along with NAA at 0.5 and 1.0 mg l^{-1} were found to delay the shoot multiplication. The delay in shoot multiplication might be the indication of inhibitory effect of relatively higher level of growth regulators on shoot morphogenesis. A similar effect of higher dose of NAA being found inhibitory on shoot morphogenesis was reported by Fonnesbech (1972) and Kim et al. (1988).

The maximum shoot numbers (3.67) were produced at combination of BA 1.0 mg l^{-1} with NAA 0.1 mg l^{-1} or 0.5 mg l^{-1}. It was observed that maximum number of shoots was obtained in treatments of kinetin at 0.5, 1.0 and 2.0 mg l^{-1} and NAA at 1.0 mg l^{-1}. Among the combinations of cytokinin and auxins tried, BA at 0.5, 1.0 and 2.0 mg l^{-1} along with NAA at 0.1, 0.5 and 1.0 mg l^{-1} and the combination of kinetin at 0.5, 1.0 and 2.0 mg l^{-1} along with NAA at 0.1, 0.5, 1.0 and 2.0 mg l^{-1} produced more number of shoots. The combination of kinetin at 1.0 mg l^{-1} along with NAA at 0.1 mg l^{-1} induced highest number of multiple shoots in *Dendrobium* cv. Betty Ho (Kurup et al., 2005) while the combination of BA 2.0 mg l^{-1} and NAA 1.0 mg l^{-1} was found most effective for maximum shoot number production of *Coelogyne stricta* (Basker and Bai, 2006).

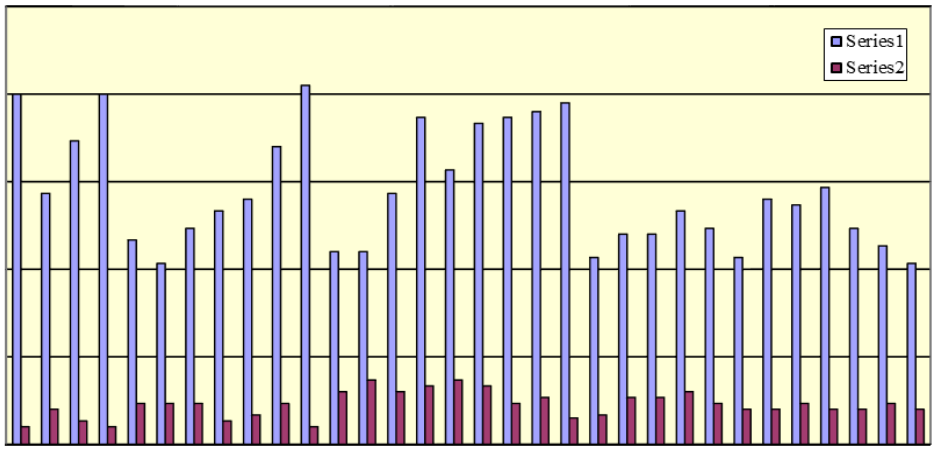


Fig. 1. Effect of plant growth regulators on multiple shooting in *Dendrobium* cv. Rungnappa Red

In *Dendrobium* cv. Miss Snow White, the minimum days (9.3) for shoot initiation were obtained in treatment with kinetin 1.0 mg l^{-1} and NAA 0.1 mg l^{-1}. Comparing the various treatments tried for multiple shooting, it was found that earliness in shoot multiplication was attained with kinetin 1.0 mg l^{-1} and combinations of kinetin at 0.5, 1.0 and 2.0 mg l^{-1} with NAA at 0.1 and 0.5 mg l^{-1} (Fig. 2). The treatments involving BA alone delayed shoot multiplication while their combination with auxin, NAA enhanced shoot multiplication. This might be due to synergistic effect of cytokinins and auxins. Similar result was obtained in *Dendrobium* cv. Sonia (Saiprasad et al., 2001).

The maximum shoot numbers (5.67) in *Dendrobium* cv. Miss Snow White was noticed in treatment with BA 2.0 mg l^{-1} and NAA 0.1 mg l^{-1}. Such a wide proportion of BA and NAA favouring shoot production could be found contrary to other reports in *Cymbidium* leaf cultures (Pindel and Miczynski, 1996) and *Dendrobium* (Sobhana and Rajeevan, 2002) wherein the lower proportion (1:1) of BA and NAA was found suitable for shoot multiplication and plantlet development .

Comparatively early shoot multiplication (11.00 days) was initiated in cv. Earsakul in treatment combination of kinetin 2.0 mg l^{-1} and NAA 0.1 mg l^{-1}. Among the various treatments tried, the early shoot multiplication was noticed in BA at 2.0 mg l^{-1}, kinetin at 0.5, 1.0 and 2.0 mg l^{-1} and combination of BA at 1.0 and 2.0 mg l^{-1} along with NAA at 0.1 and 0.5 mg l^{-1}. The combination of kinetin at 0.5, 1.0 and 2.0 mg l^{-1} along with NAA at 0.1 and 0.5 mg l^{-1} also induced earliness in shoot multiplication. The maximum shoot number (4.66) was also reported in treatment combinations of kinetin 2.0 mg l^{-1} and NAA 0.1 mg l^{-1} followed by kinetin 0.5 mg l^{-1} and NAA 0.5 mg l^{-1} (Fig. 3). The combination of kinetin at 1.0 mg l^{-1} and NAA at 0.1 mg l^{-1} was reported to produce vigorous and maximum shoot numbers in *Cymbidium pendulum* (Talukdar et al. 2002)

In all the three cultivars it was noted that the shoot buds produced in treatment combination of BA and NAA were proliferating, very small, non- distinct and pale green. The shoots obtained from treatment combinations of kinetin and NAA were non-proliferating, distinct with fully emerged leaves, dark green, vigorous and healthy. Similar effect of kinetin

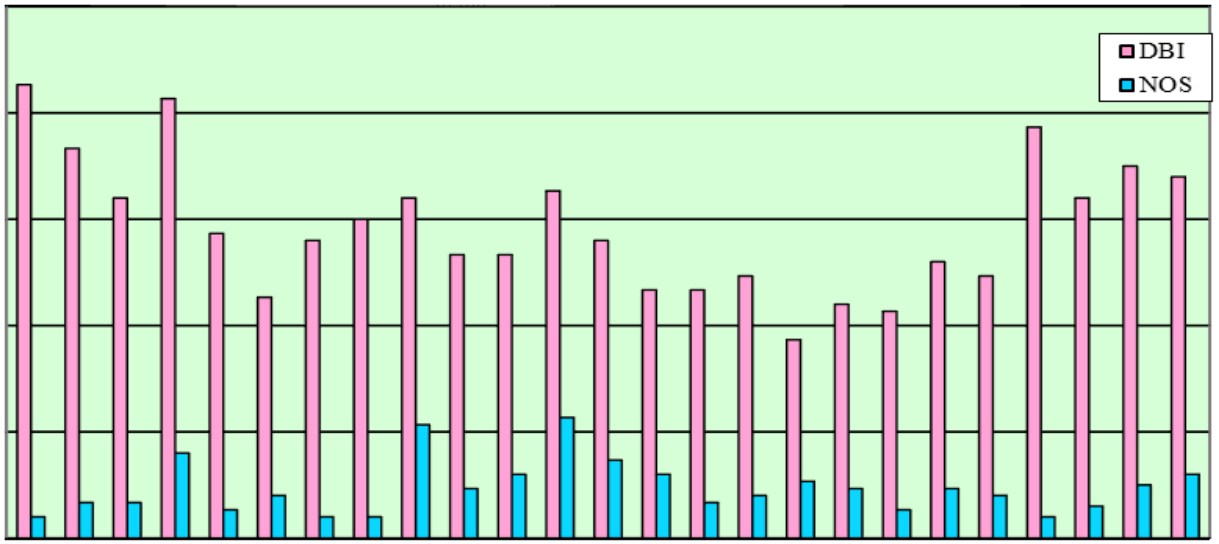
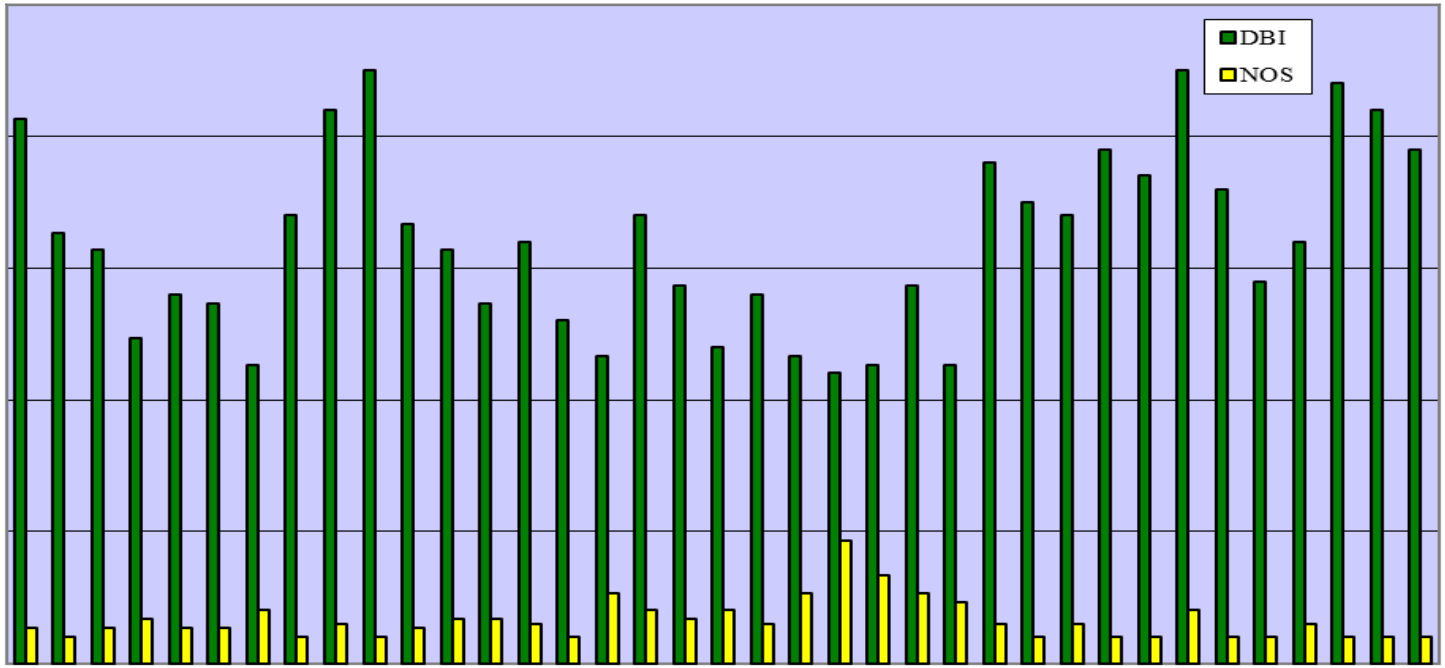


Fig. 2. Effect of plant growth regulators on multiple shooting in *Dendrobium* cv. Miss Snow White

added media producing better shoot growth (>2.0 cm) and BA added media producing short (<1.5 cm) shoots was reported during the *in vitro* propagation of *Dendrobium* hybrids Sonia- 17 and 28 (Martin et al., 2005).

In the present study CW at 200 ml l^{-1} was found superior with respect to early bud initiation and maximum shoot production in all the three varieties (Fig. 4). With increase in CW concentration from 0 to 200 ml l^{-1} there was gradual decrease in days for bud initiation. Similarly an increase in multiple shoot production was also noticed in cultivars Miss Snow White and Earsakul. CW at 200 ml l^{-1} could be the optimum dose that could influence many metabolic processes mainly by cell division and cell enlargement effected by the inherent presence of cytokinin like compounds, which is a pre requisite for multiplication (Sagawa and Kunisaki, 1982; Kher, 1999; Kanika, 1998). Kuriakose (1997) obtained maximum shoot proliferation rate in multiplication media supplemented with 100 ml l^{-1} CW in *Dendrobium* cv. Sonia. Sivamani (2004) observed early shoot initiation and maximum shoot numbers in 200 ml l^{-1} CW supplemented media in *Dendrobium* hybrids thus supporting the present study. Nagaraju and Mani (2007) also obtained early shoot emergence and maximum number of shoots in basal medium supplemented with CW 200 ml l^{-1}.

The amino acids, CH and glutamine exerted varying effects in the three varieties. In cv. Rungnappa Red and Miss Snow White presence of CH and increase of its concentration delayed shoot multiplication. In cv. Earsakul though the presence of CH delayed shoot multiplication, the increase in concentration from 250 to 1000 mg l^{-1} shortened the duration for shoot multiplication (Fig. 5). On the contrary, Rahana (2006) observed significantly early shoot initiation in *Dendrobium* hybrids in shoot multiplication medium supplemented with CH 250 mg l^{-1}. The shoot multiplication medium with CH at 250, 500 and 1000 mg l^{-1} exerted no effect on increase in shoot number in cv. Rungnappa Red and Miss Snow White. In cv. Earsakul, CH at 500 and 1000 mg l^{-1} increased the shoot numbers over that of control, but was found statistically non significant. A lower dose of CH at 100 mg l^{-1} registering maximum number of shoots was reported in *Dendrobium* cv. Sonia (Kuriakose, 1997).



Treatments

Fig. 3. Effect of plant growth regulators on multiple shooting in *Dendrobium* cv. Earsakul

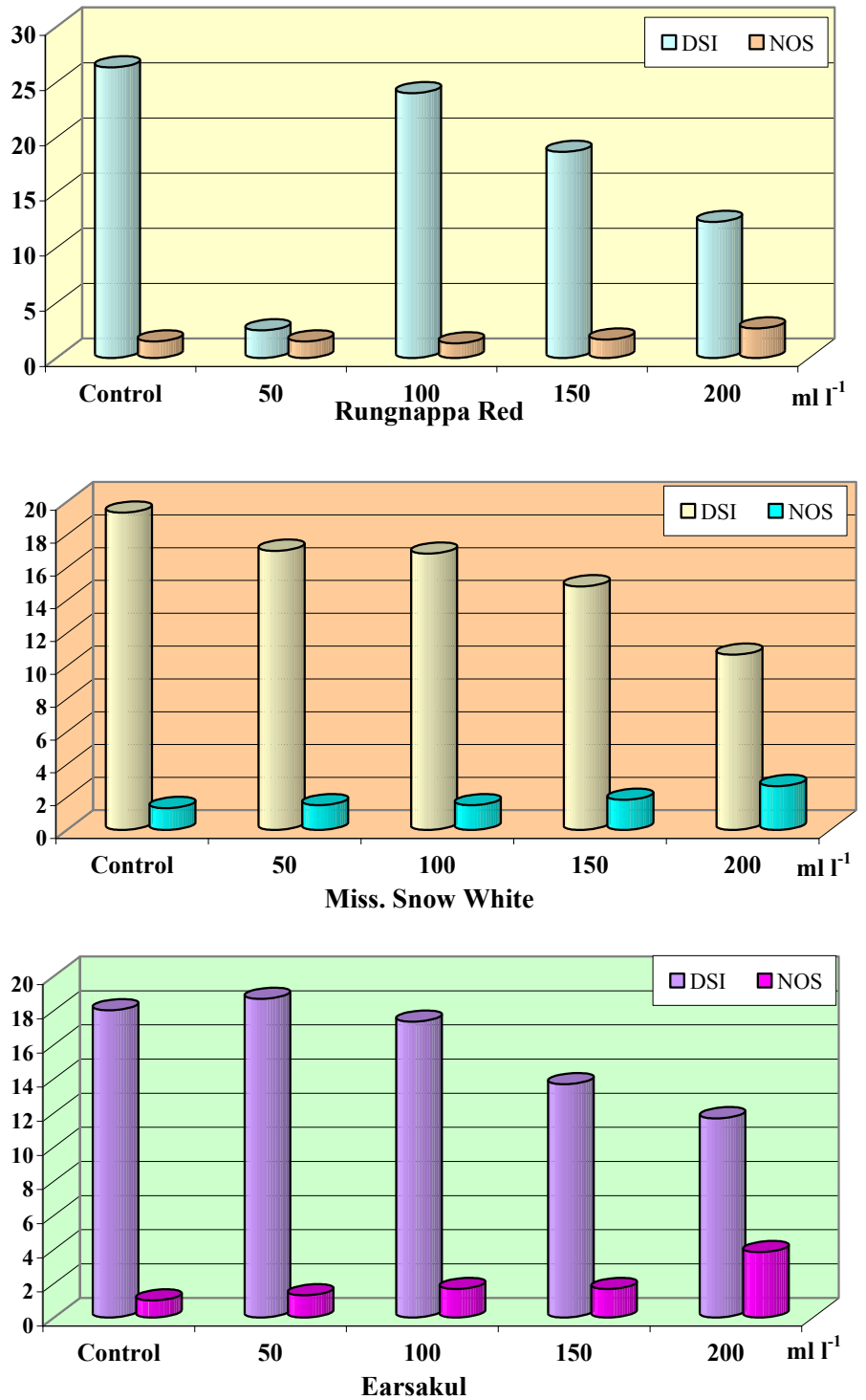


Fig. 4. Effect of coconut water on multiple shooting in *Dendrobium* cvs.

The delay in shoot multiplication was noted in glutamine (1.25, 2.5 and 5.0 mg l^{-1}) supplemented media in cvs. Rungnappa Red and Earsakul. No increase in shoot production over the control was exerted with glutamine at 1.25, 2.5 and 5.0 mg l^{-1}. Kuriakose (1997) also reported that glutamine exerted no significant effect on shoot proliferation in *Dendrobium* cv. Sonia; however Saiprasad and Raghuveer (2002a) obtained a 3 fold increase in multiple shoot production with glutamine at 1.0 mg l^{-1} in the same variety.

Carbon source such as sucrose enhances cell proliferation and regeneration of green shoots. Low concentration of sucrose at 15.0 g l^{-1} delayed shoot development and also reduced the shoot number in cv. Rungnappa Red. A positive effect of earlier shoot multiplication and increased shoot numbers were noticed as the concentration increased to 60.0 g l^{-1}. This increase may be attributed to the enhanced uptake of nitrate and ammonium in the presence of higher levels of sucrose. In *D. nobile* also ½ MS supplemented with 60.0 g l^{-1} proved to be the most efficient treatment for plantlet growth (Faria et al., 2004). Nagaraju (2007) also emphasized the positive effect of higher level of sucrose at 40.0 g l^{-1} on shoot numbers in *Cymbidium* hybrids.

On the other hand, sucrose at higher levels of 45.0 and 60.0 g l^{-1} delayed shoot development in cv. Miss Snow White. In cv. Earsakul no multiple shooting was noticed in various treatments of sucrose, except at 30.0 g l^{-1} which served as control. The differential response of cultivars to sucrose could be due to their differential content of endogenous carbohydrates and growth regulators which influence the response to exogenously supplied sucrose.

The response to polyamines showed variation among the varieties. The polyamine spermine supplemented in multiplication media induced earliness in shoot multiplication in all the three varieties tried (Fig. 7). It was also noticed that as concentration of spermine increased from 0.25 to 1.00 mM, days for shoot multiplication also lowered. Considering the maximum number of shoots produced, spermine at 0.50 mM concentration could be adjudged as beneficial in cvs. Rungnappa Red and Miss Snow White. In cv. Earsakul spermine at lower level of 0.25 mM induced maximum shoot production, how ever it was non significant statistically.

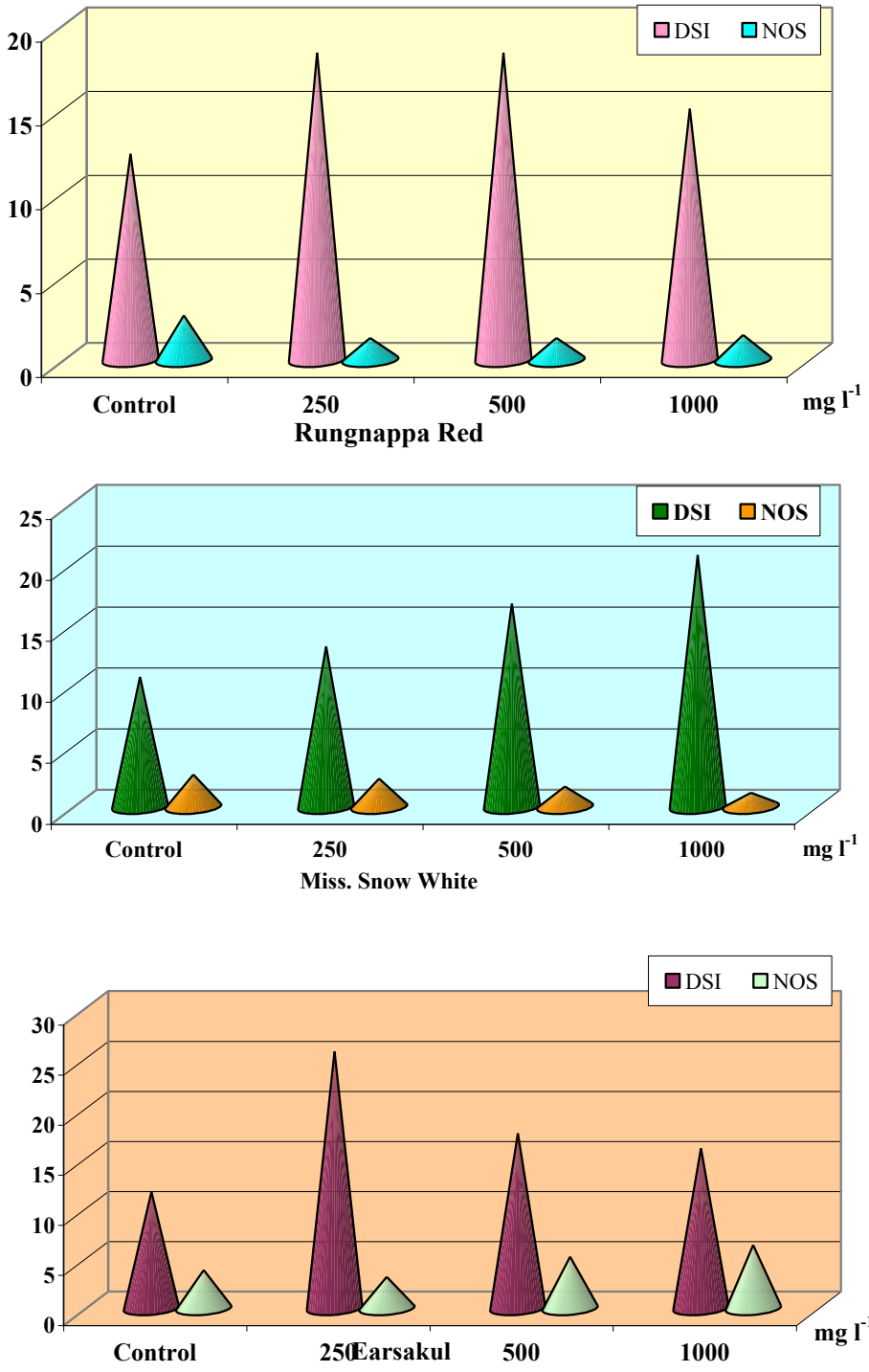


Fig. 5. Effect of casein hydrolysate on multiple shooting in *Dendrobium* cvs.

Similar to spermine, the polyamine spermidine induced earliness in shoot multiplication in all the three varieties tried (Fig. 8). In this case the maximum shoot numbers were produced at spermidine 1.0 mM. Putrescine at 0.25, 0.50 and 1.00 mM failed to induce shoot multiplication in the three varieties tried. Saiprasad et al. (2004) observed that the polyamines, spermine, spermidine and putrescine at 0.2, 0.4 and 1.0 mM, resulted in the production of PLBs, the maximum being with putrescine 0.4 mM in *Dendrobium* cv. Sonia. Polyamines were found inhibitory to direct embryo formation from leaf explants of *Phalaenopsis* species (Gow et al*.,* 2008). Scholten (1998) found that in *Syringa,* putrscine and spermidine at 0.1 - 1.0 mM could partly compensate for the cytokinin, 2- iP stimulated stem elongation.

Activated charcoal is added in the medium to adsorb the phenolic compounds which are otherwise inhibitory to tissues. In the present study AC at 0.5 g l^{-1} was found beneficial in all the three varieties with respect to earliness in shoot multiplication, maximum number of shoots and general vigour of shoots (Fig. 6). The beneficial effect of adding 0.5 per cent activated charcoal for maintaining the explants green and for the luxuriant growth of shoots was reported by many workers (Vij and Pathak, 1989; Shylaraj et al., 2005) thus supporting the present investigation.

Varietal difference in response was evident in the case of treatments of 28- HBL. In cv. Rungnappa Red, as the 28- HBL concentration increased from 0.5 to 4.0 mg l^{-1} the mean days for shoot multiplication decreased. The shoot number produced also decreased as the 28-HBL concentration increased. In cv. Miss Snow White, how ever, as concentration increased from 0.5 to 4.0 mg l^{-1}, the mean days for shoot multiplication increased. More number of shoots was produced at higher doses of 28- HBL at 2.0 and 4.0 mg l^{-1}. In both these varieties rooting of shoots was found more prominent. In cv. Earsakul, 28-HBL at 4.0 mg l^{-1} induced earliness in shoot multiplication as well as maximum shoot production. The varietal difference might be the result of interaction of 28-HBL with the endogenous growth regulators in the shoots, which vary with the varieties.

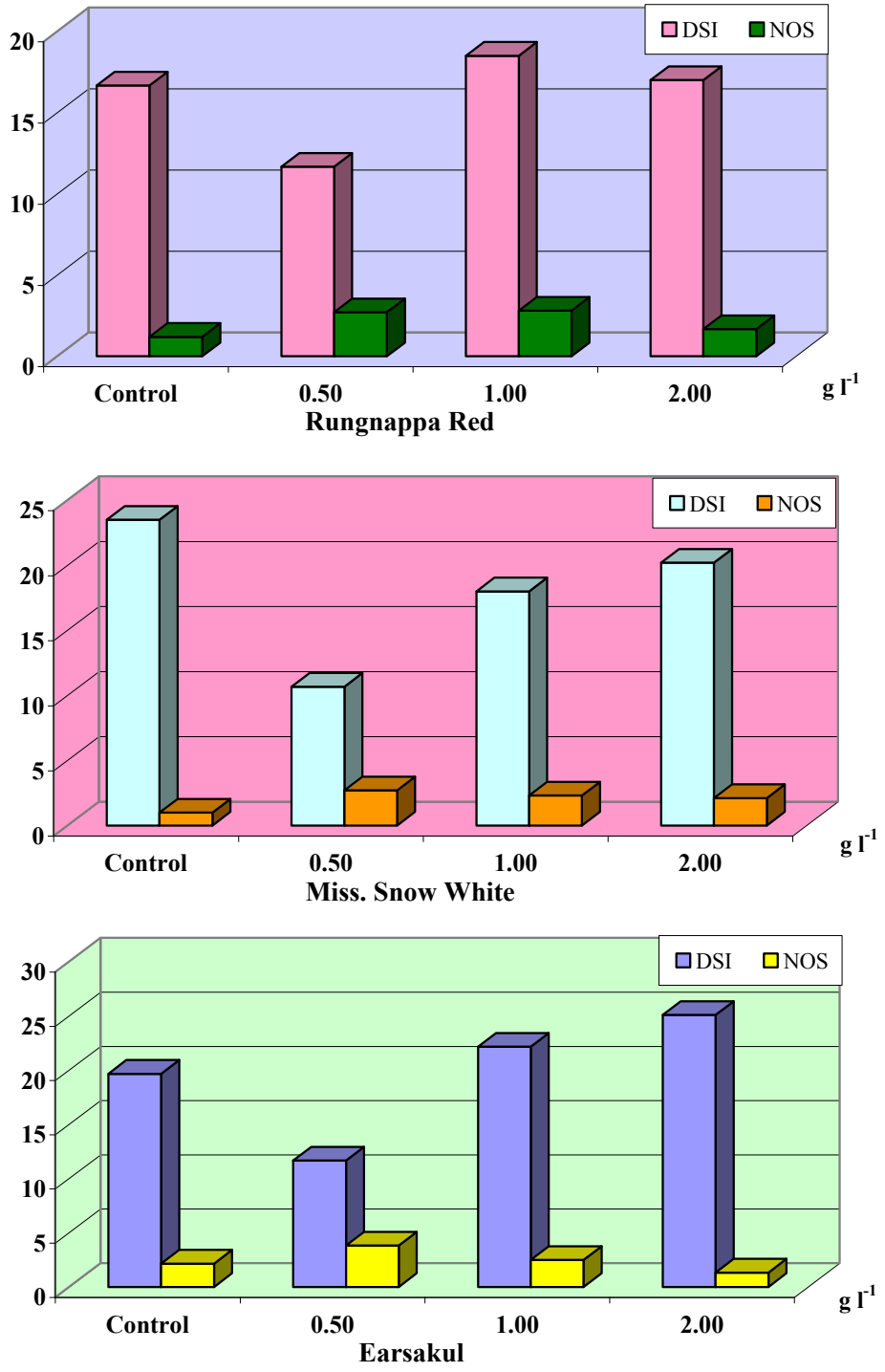


Fig. 6. Effect of activated charcoal on multiple shooting in *Dendrobium* cvs.

In *Cymbidium elegans,* 24- epibrasinolide at 3.0 - 4.0 µ M was found to produce luxuriant growth of PLBs or proliferating shoot buds (Malabadi and Nataraja, 2007). The increase in shoot production in cv. Earsakul as 28 - HBL increased might be the indication of promoting cell division thereby substituting cytokinin in culture. Similar effects were reported by Hu et al. (2000) who showed that 24- epibrassinolide can substitute cytokinin in *Arabidopsis* callus and suspension cultures. In the protoplast cultures of *Petunia hybrida* (Oh and Clouse, 1998) and Chinese cabbage (Nakajima, et al. 1996) brassinolides promoted cell division.

The optimum dose of growth regulator requirement for rooting varied among the varieties (Table 39). In cv. Rungnappa Red and Miss Snow White minimum days for root initiation was noticed in rooting media supplemented with IBA 0.5 mg l^{-1}, whereas in cv. Earsakul the same was noticed with NAA 0.5 mg $l^{-1.}$ The rooting media supplemented with NAA 1.0 mg l^{-1} was found better in *Dendrobium* Sonia by Kuriakose (1997) and Saiprasad et al. (2001). A general trend of delay in root emergence was noticed in the three varieties as the concentration of auxins increased except the concentrations of NAA in cv. Miss Snow White. The root numbers produced was found non significant in Rungnappa Red and Earsakul. In cv. Miss Snow White, maximum number of roots was produced at IAA 2.0 mg l^{-1}. The combination of NAA at 0.5 mg l^{-1} and BA at 0.1 mg l^{-1} was found effective for rooting of microshoots of *Dendrobium* cv. Betty Ho (Kurup et al., 2005).

The composition of the media into which *in vitro* rooted plantlets are transplanted is important for their survival (Jones, 1982). In the present study the maximum survival rate of rooted plantlets of the three varieties were obtained in media with charcoal and brick pieces in equal proportion. This combination might have provided the ambient condition of good drainage, sufficient moisture retention and also good aeration, which are very much essential for orchid growth. A survival rate of 94 per cent in selfed hybrids of *Dendrobium* cv. New Pink, planted out in a medium with equal proportion of charcoal and brick pieces was reported by Shobhana (2000). The combination of charcoal and brick pieces along with bark pieces and moss (1:1:1:1) proved to be promising for successful establishment of *in vitro* rooted plantlets of *Dendrobium* cv. Sonia (Pathania et al. 1998).

The regeneration potential of colchicine treated explants depended on the concentration and duration of treatment and nature of explant. The increase in concentration or the duration of the treatment increased the mortality rate of the explants (Table 41 and Table 42). Such an increase in mortality of explants with increase in colchicine concentration was observed in *Dendrobium* (Sanguthai, *et al*., 1973) and *Cymbidium* (Silva *et al*., 2000). In the present study all the shoots regenerated from colchicine treated PLBs were found to be of normal morphology. In stem nodes, shoots regenerated from 0.1 per cent colchicine treated for 5 days, explants were found to be of unusual morphology characterized by shortened stem, reduced internodes, thicker and greener leaves and slow growth. The slow rate of growth in colchicine treated plants at an early stage was observed in *Phaius tankervillae* (Devi and Deka, 2000). Similar slower growth rates and delayed organogenesis were also reported by Vainola (2000) and Jaskani (2004). Successful induction of *in vitro* polyploidy using stem nodal segments was reported in *Scoperia montevidiensis* (Escandon, 2005) and *Bacopa monnieri* (Escandon, 2006).

The difference in stomatal density (Silva et al., 2000) and stomatal size (Dunn, 1979; Pandita, 1986; Kim et al. 1997; Devi and Deka, 2000) were reported as preliminary identification criteria for polyploidy in many orchids. In the case of shoots regenerated from colchicine treated plbs no variation was noted among the shoots for the stomatal number, size and epidermal cell size (Table 43). While the shoots regenerated from the treatment of stem nodes by colchicine at 0.5 per cent for 5 days duration, were distinct by the absence of stomata and large sized epidermal cells (Table 44). The increase in stomatal size of polyploids over that of diploids was noticed in *Phaius tankervillae* (Devi and Deka, 2000) and *Orchis latifolia* (Pandita, 1986).

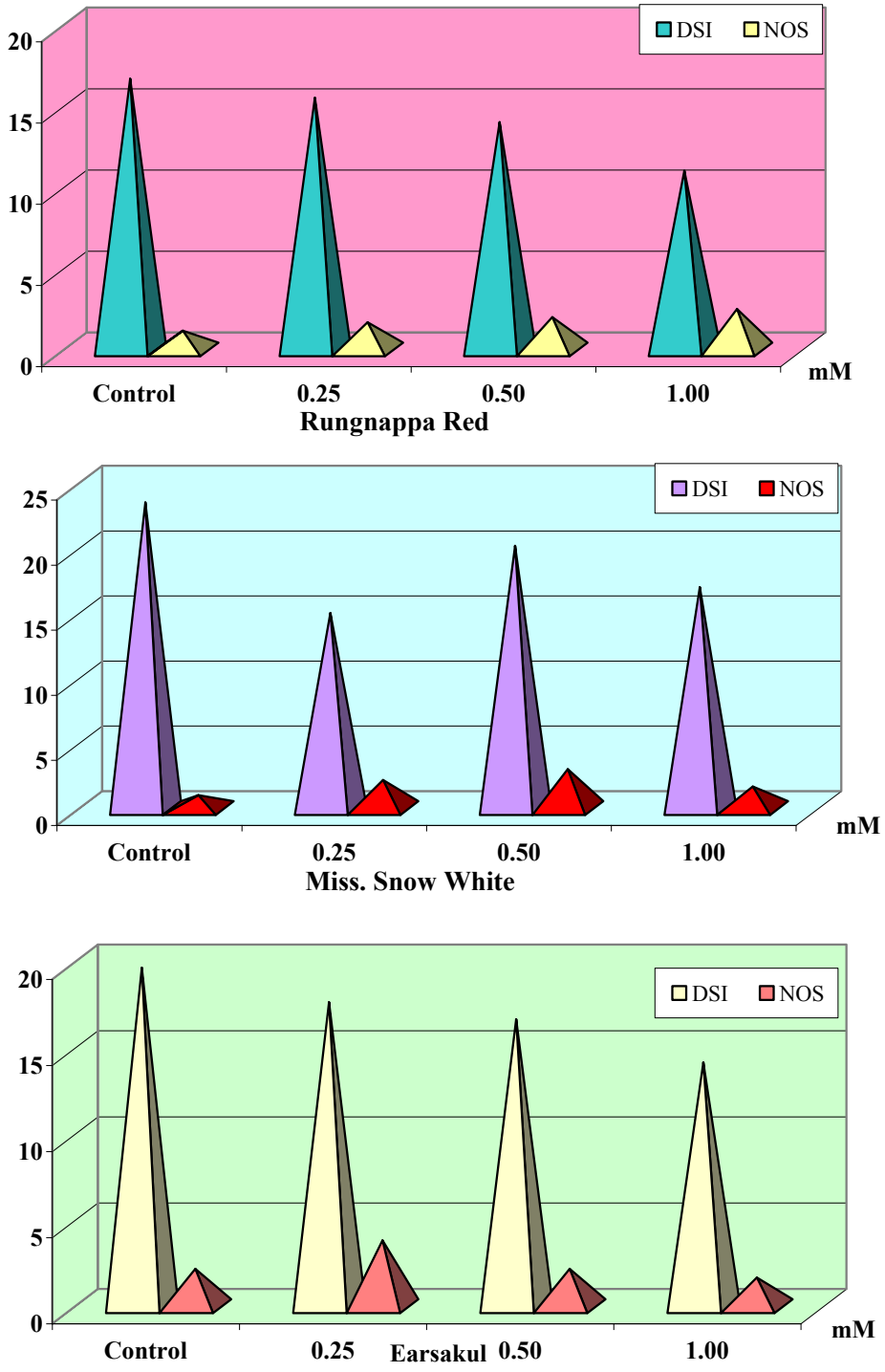


Fig. 7. Effect of polyamine, spermine on multiple shooting in *Dendrobium* cvs.

The present investigation has evolved *in vitro* protocols for clonal propagation of three commercially important *Dendrobium* varieties namely, Rungnappa Red, Miss Snow White and Earsakul. The *ex vitro* establishment studies need to be refined so as to improve the survival rate of plantlets. The preliminary results obtained in *in vitro* polyploidisation studies are helpful in further improvement of the cultivar.

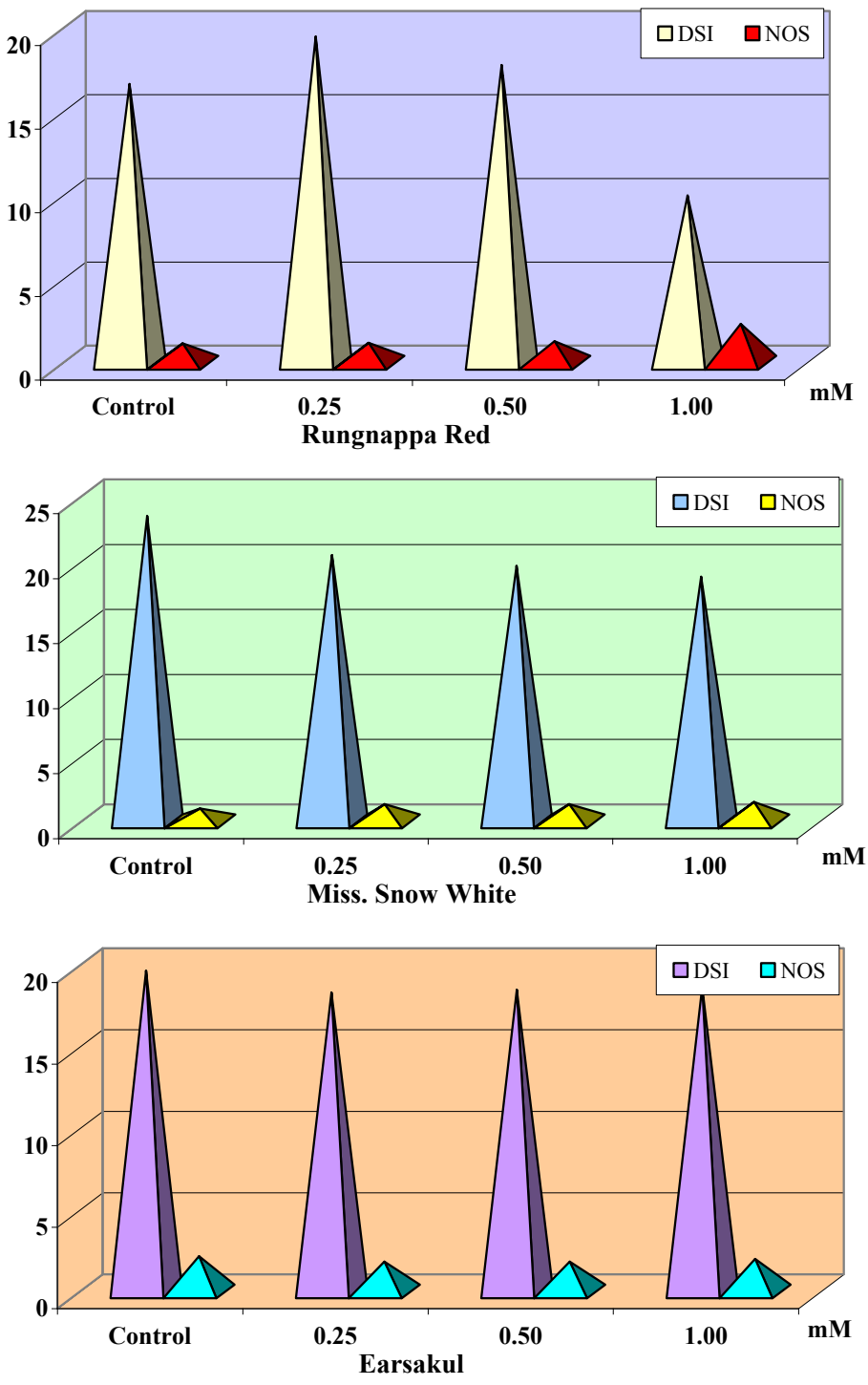


Fig. 8. Effect of polyamine, spermidine on multiple shooting in *Dendrobium* cvs.

Summary

6. SUMMARY

Attempts were made at the Department of Pomology and Floriculture and Department of Plant Biotechnology, College of Agriculture, Vellayani during 2005 – 2008 to enhance the *in vitro* propagation efficiency and induction of polyploidy in orchids. Commercially important *Dendrobium* varieties were selected for the present study.

The effect of surface sterilant, explant, plant growth regulators and basal media on culture establishment, effect of plant growth regulators and media supplements on multiple shooting and the effect of plant growth regulators on rooting were studied. The effect of colchicine on induction of polyploidy in PLBs and stem nodes of *Dendrobium* cultivar Miss Snow White was also assessed.

The salient findings of the study are summarized below:

1. Mercuric chloride at 0.1 % for 10 minutes gave maximum survival rate of stem node explants of *Dendrobium* cvs. Rungnappa Red and Earsakul, while at 0.08 % for 10 minutes gave maximum survival rate of stem node explants of cultivars Miss Snow White and *D. philippica*.

2. Callusing of *in vitro* leaf explants occurred in establishment medium with BA 2.0 mg l^{-1} + NAA 0.5 - 1.0 mg l^{-1} in cv. Rungnappa Red and with BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1} in cv. Miss Snow White. Direct shoot regeneration from leaf explants of *D. philippica* was obtained in establishment medium with BA 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}.

3. The nodal explants from young inflorescence stalk of *D. philippica* produced callus in establishment medium with kinetin 2.0 mg l^{-1} alone and combination with IAA at 1.0 to 4.0 mg l^{-1}. Shoot buds were obtained at kinetin 4.0 mg l^{-1} alone and at kinetin 4.0 mg l^{-1}+ IAA 4.0 mg l^{-1}.

4. Among the four different basal media tried for culture establishment viz., full MS, half MS, VW and KC, ½ MS was found to give 100 per cent survival rate, minimum days

for bud initiation and maximum number of shoots in the cultivars Rungnappa Red, Miss Snow White and Earsakul.

5. For the culture establishment in cv. Rungnappa Red the plant growth regulator combination of kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} gave early bud initiation and maximum number of shoots, where as in cv. Miss Snow White, combination of kinetin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1} gave early bud initiation and BA 4.0 mg l^{-1} + NAA 1.0 mg l^{-1} produced maximum number of shoots. In cv. Earsakul, BA 4.0 mg l^{-1} gave early bud initiation and BA 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} produced maximum number of shoots.

6. For multiple shooting in *Dendrobium* cv. Rungnappa Red, kinetin 2.0 mg l^{-1} + NAA 2.0 mg l^{-1}, gave early shoot multiplication, while BA 1.0 mg l^{-1} + NAA 0.1 - 0.5 mg l^{-1} gave maximum number of shoots. In cv. Miss Snow White the combination of kinetin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1} gave earliest shoot multiplication and BA 0.5 mg l^{-1} + NAA 0.5 mg l^{-1} gave maximum number of shoots. In cv. Earsakul the early shoot multiplication as well as the maximum number of shoots was noted in kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}.

7. In all the three varieties, CW at 200 ml l^{-1} was found superior with respect to early bud initiation and maximum shoot production.

8. CH at 250, 500 and 1000 mg l^{-1} delayed shoot multiplication in cv. Rungnappa Red and Miss Snow White. In cv. Earsakul, CH at 1000 mg l^{-1} gave maximum shoot production.

9. Glutamine at 1.25, 2.5 and 5.0 mg l^{-1} failed to give any beneficial effect on the cultivars.

10. Sucrose at 60.0 g l^{-1} recorded minimum number of days for shoot multiplication as well as maximum number of shoots in cv. Rungnappa Red while sucrose at 30.0 g l^{-1} recorded minimum number of days for shoot multiplication as well as maximum number of shoots in cv. Miss Snow White.

11. AC at 0.5 g l^{-1} was found beneficial in all the three varieties with respect to earliness in shoot multiplication, maximum number of shoots and general vigour of shoots.

12. The polyamine, spermine at 1.0 mM induced early shoot multiplication and maximum shoot numbers in cv. Rungnappa Red. In cv. Miss Snow White, spermine at 0.25 mM induced early shoot multiplication and at 0.5 mM gave maximum shoot numbers. In cv. Earsakul, spermine at 1.0 mM induced early shoot multiplication and at 0.25 mM gave maximum shoot numbers.

13. Spermidine at 1.0 mM induced early shoot multiplication and maximum shoot numbers in cv. Rungnappa Red as well as in cv. Miss Snow White.

14. In cv. Rungnappa Red, 28- HBL at 4.0 mg l^{-1} showed earliest shoot multiplication and at 0.5 mg l^{-1} gave maximum shoot production. 28- HBL at 0.5 mg l^{-1} showed earliest shoot multiplication and at 2.0 mg l^{-1} gave maximum shoot production in cv. Miss Snow White. In cv. Earsakul, 28- HBL at 4.0 mg l^{-1} induced earliness in shoot multiplication as well as maximum shoot production.

15. The *in vitro* rooting response also varied among the three *Dendrobium* varieties. IBA at 0.5 mg l^{-1} induced early root initiation in cv. Rungnappa Red as well as in cv. Miss Snow White. In cv. Miss Snow White, the maximum number of roots was produced at IAA 2.0 mg l^{-1}. In cv. Earsakul, NAA at 0.5 mg l^{-1} induced early rooting.

16. The planting out media comprising charcoal and brick pieces (1:1) gave maximum survival rate in all the three varieties.

17. Shoots regenerated from 0.1 % colchicine treated stem nodes of Miss Snow White for 5 days, were found to be of unusual morphology typical to polyploids characterized by slow growth, shortened stem, reduced internodes, thicker and greener leaves, lack of stomata at early stage and increased epidermal cell size.

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* Originals not seen

Appendices

APPENDIX -I

Composition of MS, VW and KC media

Nutrient (mg l^{-1})	Quantity (mg l^{-1})		
	MS	VW	KC
Macronutrients			
NH_4NO_3	1650.00	-	-
$(NH_4)_2 SO_4$	-	500.00	250.00
$NH_4H_2PO_4$	-	-	-
KNO_3	1900.00	525.00	-
KH_2PO_4	170.00	250.00	250.00
$MgSO_4. 7H_2O$	370.00	250.00	250.00
$NaH_2PO_4. H_2O$	-	-	-
$CaCl_2. 2H_2O$	440.00	-	-
$Ca (NO_3)_2. 4H_2O$	-	-	1000.00
$Ca_3(PO_4)_2$	-	200.00	-
Micronutrients			
H_3BO_3	6.20	-	-
$MnSO_4. 4 H_2O$	22.30	7.00	7.50
$ZnSO_4. 7 H_2O$	8.60	-	-
KI	0.83	-	-
$Na_2MoO_4. 2 H_2O$	0.25	-	-
$CuSO_4. 5 H_2O$	0.025	-	-
$CoCl_2. 6 H_2O$	0.025	-	-
$FeSO_4. 7 H_2O$	27.85	-	25.00
$Fe(C_6H_5O_7) \cdot H_2O$	-	28.00	-
$Na_2EDTA. 2 H_2O$	37.25	-	-

Nutrient	Quantity (mg l^{-1})		
	MS	VW	KC
Vitamins			
Thiamine, HCl	0.1	-	-
Pyridoxine, HCl	0.5	-	-
Nicotinic acid	0.5	-	-
Aminoacid			
Glycine	-	-	-
Others			
Inositol	100	-	-
Sucrose (g l^{-1})	30	20	20
Agar (g l^{-1})	6.4	6.4	6.4

APPENDIX -II a

In vitro clonal propagation protocol for *Dendrobium* cv. Rungnappa Red

Explant: Stem nodes from kiekies

Basal medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Surface sterilization

(Pre- treatment with absolute ethanol,
$HgCl_2$ at 0.1 % for 10 minutes)

↓

Culture establishment

(kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1})

(1-2 weeks) ↓

Multiple shooting

(kinetin 0.5 mg l^{-1} + NAA 0.5 mg l^{-1}) /
(kinetin 1.0 mg l^{-1} + NAA 0.1mg l^{-1})

Sucrose at 60 mg l^{-1} ←
Spermine/Spermidine at 1.0mM

(4-6 weeks) ↓

***In vitro* rooting**

(IBA 0.5 mg l^{-1})

(3 weeks) ↓

Planting out

APPENDIX -II b

In vitro clonal propagation protocol for *Dendrobium* cv. Miss Snow White

Explant: Stem nodes from kiekies

Basal medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Surface sterilization

(Pre- treatment with absolute ethanol,
$HgCl_2$ at 0.08 % for 10 minutes)

↓

Culture establishment

(kinetin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1})

(1-2 weeks) ↓

Multiple shooting

(kinetin 1.0 mg l^{-1} + NAA 0.1mg l^{-1})

Spermine at 0.5 mM / Spermidine at 1.0mM ←

(4-6 weeks) ↓

***In vitro* rooting**

(IBA 0.5 mg l^{-1})

(3 weeks) ↓

Planting out

APPENDIX -II c

In vitro clonal propagation protocol for *Dendrobium* cv. Earsakul

Explant: Stem nodes from kiekies

Basal medium: ½ MS + inositol 100.0 mg l^{-1} + sucrose 30.0 g l^{-1} + CW 200.0 ml l^{-1} + CS 15.0 mg l^{-1} + agar 6.2 g l^{-1} + AC 0.5 g l^{-1}

Surface sterilization

(Pre- treatment with absolute ethanol,

Hg Cl_2 at 0.1 % for 10 minutes)

↓

Culture establishment

(BA 4.0 mg l^{-1})

(1-2 weeks) ↓

Multiple shooting

(kinetin 2.0 mg l^{-1} + NAA 0.1mg l^{-1})

Spermine at 0.25 mM ←

(4-6 weeks) ↓

***In vitro* rooting**

(NAA 0.5 mg l^{-1})

(3 weeks) ↓

Planting out

APPENDIX -III

Abstract of analysis of variance for the effect of different treatments

Sl. No.	Character	Treatment mean squares			Error mean squares		
		Rungnappa Red	Miss Snow White	Earsakul	Rungnappa Red	Miss Snow White	Earsakul
I	**Culture establishment**						
1.	**Effect of plant growth regulators**						
	1.Days for bud initiation	64.04	115.67	31.26	16.17	12.40	4.81
	2. No: of shoots	0.66	1.56	1.45	0.44	0.74	0.56
2.	**Effect of basal media**						
	1.Days for bud initiation	7.92	41.49	68.99	15.07	12.03	13.34
	2. No: of shoots	0.15	5.56	0.16	0.09	0.08	0.08
II.	**Multiple shooting**						
1.	**Effect of plant growth regulators**						
	1.Days for shoot initiation	30.27	29.63	29.13	7.65	5.92	4.94
	2. No: of shoots	1.48	4.57	1.67	0.74	0.89	0.82
2.	**Effect of coconut water**						
	1.Days for shoot initiation	166.88	63.38	55.53	39.83	5.77	6.05
	2. No: of shoots	1.72	1.72	13.13	0.44	0.82	0.53
3.	**Effect of casein hydrolysate**						
	1. Days for shoot initiation	50.67	113.38	205.59	10.7	5.34	8.11
	2. No: of shoots	2.49	2.71	11.93	0.38	0.94	4.39
4.	**Effect of glutamine**						
	1. Days for shoot initiation	20.5	-	38.49	9.65	-	3.71
	2. No: of shoots	2.82	-	3.37	0.63	-	2.39
5.	**Effect of sucrose**						
	1. Days for shoot initiation	32.78	96.15	-	6.78	8.23	-
	2. No: of shoots	3.67	1.15	-	0.92	0.88	-

6.	**Effect of spermine**						
	1. Days for shoot initiation	36.04	83.17	31.38	4.84	8.22	4.44
	2. No: of shoots	1.89	4.26	5.38	0.28	0.31	1.79
7.	**Effect of spermidine**						
	1. Days for shoot initiation	104.11	24.82	2.15	2.68	4.76	6.08
	2. No: of shoots	1.89	0.26	0.15	0.22	0.21	0.83
8.	**Effect of activated charcoal**						
	1. Days for shoot initiation	44.28	177.39	201	7.68	5.08	6.22
	2. No: of shoots	3.83	3.15	6.49	0.42	0.78	2.13
9.	**Effect of 28- homobrassinolide**						
	1. Days for shoot initiation						
	2. No: of shoots	113.37	28.5	61.79	10.30	29.74	9.52
		10.8	3.33	34.53	2.72	1.07	3.95
III	***In vitro* rooting**						
	Effect of plant growth regulators						
	1. Days for root initiation	45.32	39.33	18.28	2.32	3.15	4.61
	2. No: of roots	0.94	7.49	1.49	1.49	1.21	1.20
IV	***In vitro* polyploidisation**						
1.	**Effect of colchicine on PLBs**						
	1. Days for shoot initiation	99.38			4.34		
	2. No: of shoots	6.86			1.00		
	3. Stomata number	12.53			0.44		
	4. Stomata size	0.37			0		
	5. Epidermal cell size	108.07			182.25		
2.	**Effect of colchicine on stem nodes**						
	1. Days for shoot initiation	20.27			13.81		
	2. No: of shoots	1.48			0.64		
	3. Stomata number	20.98			0.86		
	4. Stomata size	1.29			0.05		
	5. Epidermal cell size	172275.10			19854.95		

APPENDIX - IV

a. Anticipated production of *in vitro* plantlets per explant as per the evolved protocol with plant growth regulators

Media composition - As in Appendix II

Stages of micropropagation	**Shoots per stemnode**		
	Rungnappa Red	**Miss Snow White**	**Earsakul**
1. Culture establishment Duration- 2 weeks	3	5	4
2. Multiple shooting Duration- 6 weeks of 2 cycles of subculture	3x4 =12 12x2=24	5x6=30 30x2=60	4x5=20 20x2=40
3. Rooting Duration **-** 3 weeks	24	60	40
4. Hardening (Charcoal +Brick pieces) Duration **-** 4 weeks	@83.33% Survival rate **20**	@75% Survival rate **45**	@66.67% Survival rate **27**

b. Anticipated production of *in vitro* plantlets per explant as per the evolved protocol with media supplements for the enhancement of propagation efficiency

Stages of micropropagation	**No: of plantlets:**		
	Rungnappa Red	**Miss Snow White**	**Earsakul**
Multiple shooting (2 nd cycle) Duration: 3 weeks Spermine@0.5 mM Spermine@ 1 mM	3x4 =12 - 12x3=36	5x6=30 30x3=90 -	4x5=20 - -
Hardening Duration: 4 weeks	@83.33 % Survival rate 30	@75 % Survival rate 68	
Multiple shooting Duration: 6 weeks 28 - HBL@ 0.5 mg l^{-1} 28 - HBL@ 2.0 mg l^{-1} 28 - HBL@ 4.0 mg l^{-1}	3x4 =12 12x4=48 - -	5x6=30 - 30x3=90 -	4x5=20 - - 20x7=140
Hardening Duration: 4 weeks	@83.33 % Survival rate **40**	@75 % Survival rate **68**	@66.67 % Survival rate **93**

ENHANCEMENT OF *IN VITRO* PROPAGATION EFFICIENCY AND INDUCTION OF POLYPLOIDY IN ORCHIDS

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

DOCTOR OF PHILOSOPHY
IN
HORTICULTURE

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ABSTRACT

Studies were conducted at the Department of Pomology and Floriculture and theDepartment of Plant Biotechnology, College of Agriculture, Vellayani during 2005 - 2008 to enhance the *in vitro* propagation efficiency and induction of polyploidy in orchids.

Commercially important *Dendrobium* cultivars such as Rungnappa Red, Miss Snow White and Earsakul were selected for the present study. The effects of explant, plant growth regulators, basal culture medium and media supplements during various stages of *in vitro* propagation were studied. The effect of colchicine in inducing polyploidy under *in vitro* condition was also studied.

Stem nodal explants from kiekies were identified as the best explant for the initial culture establishment of the selected *Dendrobium* cultivars. Varietal difference in response to plant growth regulators and media supplements was significant among the three cultivars.

Half MS medium was found the best basal medium with respect to earliness in bud initiation as well as maximum number of shoots produced.

Half MS medium supplemented with kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1} gave early bud initiation in cv. Rungnappa Red where as the combination of kinetin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1} gave early bud initiation in cv. Miss Snow White. In cv. Earsakul, BA at 4.0 mg l^{-1} gave early bud initiation.

In multiple shoot production stage, in cv. Rungnappa Red, BA 1.0 mg l^{-1} + NAA 0.1 - 0.5 mg l^{-1} gave maximum number of shoots. In cv. Miss Snow White maximum number of shoots were observed in BA 0.5 mg l^{-1} + NAA 0.5 mg l^{-1} and in cv. Earsakul maximum number of shoots were observed in kinetin 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}.

CW at 200 ml l^{-1} was found superior with respect to early bud initiation and maximum shoot production in all the three varieties. CH at 1000 mg l^{-1} gave maximum shoot production in cv. Earsakul. Sucrose at 60.0 g l^{-1} recorded maximum number of shoots in cv. Rungnappa Red while sucrose at 30.0 g l^{-1} recorded maximum number of shoots in cv. Miss Snow White. Activated charcoal at 0.5 g l^{-1} was found beneficial in all the three varieties. The effective concentration of polyamine, spermine for maximum shoot production was at 1.0 mM, 0.5 mM and 1.0 mM in cvs. Rungnappa Red, Miss Snow White and Earsakul respectively. The effective concentration of polyamine, spermidine for maximum shoot production was at 1.0 mM in cvs. Rungnappa Red and Miss Snow White. The optimum concentration of steroid plant growth regulator 28-HBL, that gave maximum shoot numbers in cvs. Rungnappa Red, Miss Snow White and Earsakul were 0.5 mg l^{-1}, 2.0 mg l^{-1} and 4.0 mg l^{-1} respectively.

Early root initiation in cv. Rungnappa Red as well as in cv. Miss Snow White was observed at 0.5 mg l^{-1} IBA, and in cv. Earsakul, at 0.5 mg l^{-1} NAA.

The planting out media comprising charcoal and brick pieces in equal proportion gave maximum survival rate in all the three varieties.

Shoots regenerated from 0.1 % colchicine treated stem nodes of Miss. Snow White for 5 days were observed to have unusual morphology characterized by slow growing shoots with reduced internodes and thicker and greener leaves.