MICROPROPAGATION OF *DENDROBIUM* HYBRIDS

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DECLARATION

I hereby declare that this thesis entitled "**Micropropagation of *Dendrobium* hybrids** " 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 "**Micropropagation of *Dendrobium* hybrids** " is a record of research work done independently by Ms. S. Sivamani (2002-11-30) 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

2,4-D	–	2,4-dichlorophenoxy acetic acid
ABA	–	Abscisic acid
AC	–	Activated charcoal
BA	–	Benzyl adenine
BAP	–	Benzyl amino purine
CW	–	Coconut water
et al.	–	And others
Fig.	–	Figure
g	–	Gram
GA_3	–	Gibberellic acid
IAA	–	Indole-3-acetic acid
IBA	–	Indole-3-butyric acid
KC	–	Knudson C medium
KIN	–	Kinetin
l^{-1}	–	Per litre
mg	–	Milligram
ml	–	Millilitre
MS	–	Murashige and Skoog
NAA	–	Naphthalene acetic acid
PLB	–	Protocorm Like Body
VW	–	Vacin and Went medium

INTRODUCTION

1. INTRODUCTION

Tissue culture has a number of potential uses. Micropropagation represents the greatest use of tissue culture. It deals with aseptic vegetative multiplication of plants *in vitro*. Goal of micropropagation is to obtain a large number of genetically identical and physiologically normal plantlets, preferably with a high photosynthetic potential to survive in the harsh *ex vitro* condition, in a reduced time period at a lowered cost. The benefits provided by micropropagation include the rapid propagation of economically important plants like orchids and mass propagation of new cultivars and hybrids throughout the year.

Orchids are a group of plants belonging to the family Orchidaceae which is the largest family of flowering plants. They contribute significantly to the international trade in cut flowers and ornamental potted plants. The flowers of orchids in general have a longer vase life, which surpass that of any other cut flower. Orchid cut flower industry is currently a highly developed trade for local market and export. Development of new hybrids, mass multiplication through tissue culture and commercial cultivation of orchids has become a lucrative industry worldwide today.

The major reason for the dismal performance of floriculture industry in India is the non-availability of planting materials in the domestic market and the need to import the same at a very high price. There is a need to popularise our own varieties and hybrids in the international market and obtain license for the production of planting material by tissue culture. This will reduce the cost of the planting material and offer scope for utilising the existing micropropagation laboratories to their full capacity (Dhawan, 2002).

Tropical orchids are predominantly produced by tissue culture. They occupy 90 per cent of the total orchid tissue culture production. whereas temperate orchids take the rest. Among the tropical orchids. *Dendrobium* dominates with 30.2 million plantlets per year (80 per cent) followed by *Mokara* and *Aranda* (10 per cent), *Oncidium* (5 per cent), *Vanda, Cattleya* and *Ascocenda* (5 per cent) (Kumar and Sooch. 2002).

Dendrobiums are known to be 'splendid' among the orchids and form the second largest genera in the orchid family, with more than 1000 species. *Dendrobium* hybrids are the most popular of the sympodial orchids grown commercially at present. Flower spikes are medium sized with flowers numbering from 5 to 20, in colours such as white, mauve, pink, red, blue. purple and yellow (Mercy and Dale, 1997).

The problem in orchid production is that the seeds are notoriously difficult to germinate and maintain into seedling stage. Vegetative propagation through division of clumps or rhizome cuttings and separation of shoots and keikis produced from the stem or pseudobulbs is very slow and may not produce more than a few plants after 2-3 years (Devi and Deka, 2001). Micropropagation of orchids have proven to be a constant means of rapid clonal propagation (Dhawan, 2002).

The micropropagation procedures are specific for the different crop species for which separate protocols have to be standardised. Among the commercial orchids, *Dendrobium* hybrids are the most popular and of considerable export value but the breeding of dendrobiums is still in its infancy in India. Since the non-availability of locally adopted and reasonably priced novel varieties is the major bottleneck which restricts our commercial orchid cultivation development of new indigenous varieties is of utmost importance. As an initial step to solve this problem, a planned inter breeding programme involving commercial varieties, semi-commercial varieties and species of *Dendrobium* was undertaken in a DBT funded project in the Department of Plant Breeding and Genetics, College

of Agriculture, Vellayani from 1997 onwards. Several promising hybrids which are single superior plants exhibiting considerable novelty, distinctiveness and uniformity in floral characters were identified from the hybrid population, which have to be urgently multiplied. At this juncture, the present study was undertaken in the department to standardise the micropropagation protocol for these newly developed *Dendrobium* hybrids.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Clonal multiplication through tissue culture and micropropagation can be achieved in short time and space in vegetatively propagated plants. The benefits provided by this technique include rapid propagation of economically important flower crops, especially plants that are difficult to propagate conventionally such as orchids. Tissue culture methods have been employed as an important aid to conventional methods of plant improvement.

According to Murashige (1974) there are three possible routes available for *in vitro* propagule multiplication : (a) enhanced release of axillary buds (b) production of adventitious shoots through organogenesis and (c) somatic embryogenesis.

Callus mediated somatic organogenesis is not recommended for clonal propagation but may be ideal for recovery of useful variant lines. In shoot tip culture, genetic uniformity is favoured. Somatic embryogenesis is limited to a few species but results in the most rapid mode of plant regeneration (Evans *et al.*, 1981).

Orchids one of the major cut-flowers of Kerala, belongs to the family Orchidaceae. In sympodial orchid *Dendrobium* varieties as well as the monopodial *Aranthera* are of great demand in Kerala. Planting material is less and not sufficient to meet the demands of the market. Conventional methods of propagation are slow and time consuming.

Micropropagation of outstanding clones of orchids has been a standard horticultural practice since they have been cultivated for their high valued flowers. Orchids were the first group of plants to be commercially propagated through tissue culture. The major break through in the propagation of orchids is the *in vitro* germination of the orchid seed by Knudson (1922).

Since the pioneering work of Morel (1960), micropropagation of orchids has been the most actively researched area.

The available reports on the *in vitro* propagation of orchids have been reviewed here.

2.1 FACTORS INFLUENCING SUCCESS IN MICROPROPAGATION

2.1.1 Explants

The type, size and position of the explant and age of the mother plant used as source of explant has an important role in the success of micropropagation (Devi and Deka, 2001).

2.1.1.1 Type of Explant

Various plant parts like shoot tip or meristem, leaf and leaf segment, stem segments, pseudobulbs, floral parts, aerial roots, etc. have been successfully used for *in vitro* propagation of orchids (Devi and Deka, 2001).

Culture of shoot tip has become a well-established technique for orchid micropropagation since it is useful for maintaining uniformity of genotype. However, it requires the sacrifice of the entire new growth or the only growing point. So, as an alternative, culture of other parts of plant like leaf, inflorescence stalks, nodal sections, roots etc., is followed as a method of cloning of elite orchids (Devi and Deka, 2001).

2.1.1.1.1 Stem Node

Genera like *Cymbidium, Dendrobium, Epidendrum, Phalaenopsis*, etc. have been successfully cultured through stem nodal segments. When the stem segments with node are used to start cultures, dormant buds enlarge followed by leaf and root development (Devi and Deka, 2001).

When stem segments with a node were cultured for the *in vitro* propagation of *Phalaenopsis*, buds enlarged in two weeks followed by leaf and root development in 6-20 weeks and plantlets were ready for transfer

in 30 weeks (Sagawa, 1961). Sagawa and Sehgal (1967) reported development of plantlets in *Vanda* Miss Joaquim in 2-3 months by using similar technique. Mosich *et al.* (1974) used entire nodes of *Dendrobium* and obtained growth of bud after four weeks and development of plantlets in 45 days. Vij *et al.* (1994) reported higher number of healthy plantlets within six months *via* PLB multiplication using nodal explants of *Cymbidium pendulum*.

The top nodes of *Phalaenopsis* are used for cyclic propagation of new explants and middle nodes for producing shoots or adventitious buds (Duan *et al.*, 1996). Regeneration of PLB and subsequent plantlet development were higher from stem nodal explants than from leaf explants in *Spathoglottis plicata* (Teng *et al.*, 1997). Pathania *et al.* (1998) used stem explants procured from shoots emerging from pseudobulbs for successful micropropagation of *Dendrobium* cv. Sonia.

Kanjilal *et al.* (1999) reported PLB formation from stem discs of *Dendrobium moschatum* from third week of culturing onwards. Plantlets were formed in 10-12 weeks after subculturing of the PLBs. The same technique was reported successful in *Geodorum densiflorum* (Kanjilal and Datta, 2000). Isolated nodes of *Anoectochilus sikkimensis* and *A. regalis* cultured for 12 weeks produced maximum number of shoots (Gangaprasad *et al.*, 2000).

2.1.1.1.2 Leaf

Culture of leaf tip or whole leaf was a promising possibility without sacrificing the important portion of a plant as done in case of shoot tip culture (Arditti, 1967).

Tanaka *et al.* (1975) reported faster PLB formation from leaf segments of *Vanda* and *Phalaenopsis* than from the entire young leaves. In the studies with leaf explants of *Aranda*, *Cattleya*, *Dendrobium* and *Ascocenda* cultivars and hybrids leaf tips did not survive but the leaf parts

including the base proliferated and formed calli, which differentiated into plantlets depending on the cultivar (Lay, 1978). Seedling leaf segments of *Phalaenopsis* produced PLBs. Segments from dorsal parts of PLBs produced plantlets while segments from basal parts produced no plantlet (Amaki and Higuchi, 1989).

Lay (1979) observed that the entire young leaves were better than the leaf sections in respect to plantlet production. Kukulczanka and Wojciechowska (1983) tried leaf explants of *Dendrobium antennathum* and *D. phalaenopsis*. The leaves of *D. antennathum* dried in 2-3 weeks. In *D. phalaenopsis* discolouration and drying of leaves were observed in basal medium but remained green in medium enriched with peptone.

Tom and Weatherhead (1991) reported successful leaf tip culture in *Pholidota chinensis, Scampe rigide, Ckisostoma fordii* and *Cymbidium* hybrid. Direct regeneration of shoots were better from leaf explants than from root explants in *Cymbidium* orchids (Pindel and Miczynski, 1996).

Segments of young leaves of *Oncidium* cv. Gower Ramsey produced clusters of somatic embryos without intervening callus within one month. Subculturing of these embryos produced more embryos and subsequent plantlet formation (Paek *et al.*, 1996). Ramsundar *et al.* (2000) reported poor shootlet formation from leaf bits in *Dendrobium* Sonia.

Chen and Chang (2003) observed that one cm long leaf tip segments of *Oncidium* Gower Ramsey cultured *in vitro* on a modified half MS basal medium with paclobutrazol at 10 mg l^{-1} gave doubling of embryo numbers (193.2) per dish compared to the control treatment (89.4) after three weeks in culture.

2.1.1.1.3 Inflorescence

Griesbach (1983) used inflorescence nodal sections of *Phalaenopsis* Betty Hausermann for the production of vegetative shoots. These shoots were used to obtain leaf segments for culture, which were later induced to

form PLBs. A similar study was reported in *Phalaenopsis* hybrids, *P.* White. Falco x *P.* Persistent and *Phalaenopsis amabilis* hybrids (Tanaka *et al.*, 1988).

Inflorescence stalk with dormant bud can be induced to produce plantlets. Flower stalk sections were used for *in vitro* propagation of *Phalaenopsis* (Arditti *et al.*, 1977).

Goh and Wong (1990) observed PLB and plantlet formation using inflorescence tips of *Aranda* Deborah. Nuraini and Shaib (1992). while culturing scape nodes of *Oncidium* cv. Gower Ramsey, *Dendrobium* cv. Miss Hawaii and *Phalaenopsis* hybrids, observed shoot and PLB formation within two weeks in *Oncidium* cultures and from fourth week onwards in *Dendrobium* and *Phalaenopsis* cultures.

A high percentage of PLB formation was reported by using inflorescence tips in some tropical orchids *viz.*, *Aranda* Tay Swee Eng, *Mokara* Khaw Phaik Suan, *Dendrobium* cv. Sonia, *Aranthera* and *Renantanda* (Chan and Lee, 1996). Santana and Chapparro (1999) reported formation of PLBs from floral buds of immature inflorescence in *Oncidium* cv. Gower Ramsey. Among the shoot tip, axillary buds, leaf bits and inflorescence segments, the inflorescence segments produced more number of PLBs and shootlets in Dendrobium Sonia (Ramsundar *et al.*, 2000).

Indhumathi (2002) observed that the inflorescence nodal segments produced shoots faster than the stem nodal segments and shoot tips. Also the time taken for producing 0.5 cm long shoots was short for inflorescence nodal segments cultured in the KC medium supplemented with BAP 5.0 mg l^{-1} + NAA 3.0 mg l^{-1}.

2.1.1.2 Size of the Explant

Size of the explant is very important for the survival and further establishment of the explant. Kim *et al.* (1970) reported the highest

percentage of success with axillary buds weighing 2 g and measuring 4.9 cm. Tanaka *et al.* (1975) found earlier formation of PLBs from the leaf segments of 8 – 12 mm size in *Phalaenopsis* and *Vanda*. Griesbach (1983) used 2 cm sections, each containing a node from mature flowering inflorescence of *Phalaenopsis* Betty Hausermann.

Tanaka *et al.* (1988) reported maximum shoot formation (87 per cent) and minimum bud dormancy when 1 cm cuttings of flower stalks of *Phalaenopsis* hybrids were used. Vij and Pathak (1989) observed maximum number of shoots using pseudobulb segments of *Dendrobium* with a length of 0.5 – 1.0 cm. Scapes of *Oncidium*, *Dendrobium* and *Phalaenopsis* cut into single nodes, 4-5 mm away from the nodes were used by Nuraini and Shaib (1992).

Inflorescence tips were excised to 0.5 – 1.0 cm length for *in vitro* culture of *Aranda* Deborah (Goh and Wong, 1990). PLBs were obtained using 1-3 mm inflorescence buds in *Aranda, Dendrobium, Aranthera* and *Renantanda* (Chan and Lee, 1996).

2.1.1.3 Position of the Explant

In *Phalaenopsis* and *Vanda*, the proximal tissue of leaves showed better PLB formation than distal ones (Tanaka *et al.*, 1975). The best result in the *in vitro* culture of *Brassocattleya* was obtained by using the third bud in the new growing shoot of 15 cm length (Arditti *et al.*, 1977). Explants of *Rhynchostylis gigantea* from the distal region with undifferentiated buds regenerated better than those from the proximal part of the inflorescence (Vij *et al.*, 1984).

PLBs were formed on more than 50 per cent of the sections taken from top 3 cm of flower stalk of *Phalaenopsis* (Homma and Asahira, 1985). Sections from near the tip of the flower stalk gave best results for adventitious bud development in *Phalaenopsis* and *Dendrobium* (Lin,

1986). Pindel and Miczynski (1996) observed production of plantlets from basal parts of leaves of *Cymbidium* hybrids.

2.1.2 Composition of the Culture Medium

The organic and inorganic constituents of the medium are very important for the successful establishment of the explants used for *in vitro* culture and their further growth.

2.1.2.1 Nutrient Medium

Most orchids proliferate on very simple media such as Knudson C (1946) or the most stable Vacin and Went (1949) medium, while the genera *Cattleya* need a complex medium, Murashige and Skoog medium (1962).

Half strength MS and VW media were found best for bud initiation in *Dendrobium* (Sudeep, 1994). Multiple shoot formation was best in VW medium with NAA and BA when compared with MS medium in *Dendrobium nobile* (Lakshmidevi, 1992).

PLB initiation in *Aranda* Deborah was achieved in Liquid Knudson C medium, but liquid VW medium was the best for further proliferation and agar VW medium for faster plantlet development (Goh and Wong, 1990). Shoots formed in MS medium often produced necrotic leaves and in reducing the concentration of inorganic salts in the medium to half, the symptoms disappeared (Lakshmidevi, 1992). Santana and Chapparro (1999) used Knudson C medium to induce PLB formation and liquid MS for PLB multiplication and plantlet formation of *Oncidium* cv. Gower Ramsey.

Lakshmidevi (1992) reported that KC medium took the longest duration for culture establishment compared to MS and VW media in *Dendrobium* species. Sudeep (1994) obtained more shoots in half MS medium than with VW medium. MS basal medium supported rapid

proliferation of multiple shoots from stem node segments in *Vanilla walkeriae* (Agarwal *et al.*, 1992).

Best production of multiple shoots of six *Dendrobium* hybrids was observed in VW medium (Devi and Laishran, 1998). Both VW and KC media favoured formation of PLBs and subsequent development of plantlets of *Dendrobium* Sonia. Knudson C medium was the best for multiplication of PLBs (Pathania *et al.*, 1998). Chen *et al.* (1999a) reported direct somatic embryogenesis from leaf explants of *Oncidium* cv. Gower Ramsey in half MS medium.

Devi *et al.* (1997) reported that Nitsch medium was the best for formation and proliferation of PLBs. Park *et al.* (1998) observed the highest ratio of PLB multiplication in VW medium while shoot regeneration was the most effective on hyponex medium. Among the different strengths of MS media compared, full MS medium proved to be the best for multiple shoot induction in terms of number of shoots per culture, number of leaves per microshoot and shoot length in *Dendrobium* (Ganga *et al.*, 1999).

In *Dendrobium* Sonia the per cent of explants established, number of PLBs produced and number of shootlets per explant were high when cultured in KC medium (Ramasundar *et al.*, 2000).

According to Shimasaki and Uemoto (1991) MS medium with a concentration of 1/8 gave best results in axillary bud culture.

Irawati *et al.* (1977) reported that the best growth and survival rates were obtained in *Dendrobium* when cultured in modified KC medium based on studies with *Aranda, Cattleya, Dendrobium* and *Ascocenda* cv and hybrids in three different media. All *Dendrobium* explants cultured on modified KC, VW or modified VW appeared expanded, but only the ones on modified VW medium continued to grow (Fernando, 1979). Kuriakose (1997) observed that minimum number of days for bud initiation was

recorded by VW basal medium. Rani (2002) stated that the basal medium MS half strength was found to be the best for early germination and rapid *in vitro* development in *Dendrobium* as compared to MS quarter strength and MS full strength, KC and VW full strength.

2.1.2.2 Growth Regulators

Growth regulators are the vital supplements to the basal media, which determines the response of the explants cultured *in vitro*.

2.1.2.2.1 Auxins

Devi and Deka (1992) observed that the growth of hybrid seedlings of the cross *Dendrobium moschatum* x *D. amoenum* was enhanced by IAA and NAA at 1 mg l^{-1} each. The addition of 2 mg l^{-1} IBA to the medium was necessary for root formation of *Phalaenopsis* hybrid and *Dendrobium* Miss Hawaii (Nuraini and Shaib, 1992).

IBA at 0.1 mg l^{-1} was the best for producing many long rooted shoots in *Dendrobium* (Lim *et al.*, 1993). Mujib and Jana (1994) observed induction of roots in *Dendrobium* Madame Pompadour in the presence of NAA at 0.1 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 *Dendrobium antennathum* and *D. phalaenopsis*.

Fonnesbech (1972) observed that IAA had no effect on *Cymbidium* PLB formation when used alone. NAA results in optimal fresh weight at 10 µm and the protocorms were vigorous but lighter green.

Pathania *et al.* (1998) found that rooting of *Dendrobium* cv. Sonia was favoured by medium supplemented with IBA 1.0 mg l^{-1} or NAA 1.8 mg l^{-1}. Fang *et al.* (1999) reported effective rooting of *Anoectochilus formosanus* with the addition of NAA 0.5 mg l^{-1}. Significant increase in PLB production of *Dendrobium moschatum* was reported by Kanjilal *et al.* (1999) with 2,4-D at 1 mg l^{-1} and IAA at 2 mg l^{-1}.

Roots induced by NAA were shorter and thicker than those by IAA at 1.0 mg l^{-1} in *Ipsea malabarica* (Gangaprasad *et al.*, 1999).

Growth and plant differentiation were the best on medium containing 0.5 mg l^{-1} IBA from *Cymbidium longifolium* protocorms *in vitro* (Siddique and Paswan, 1998).

2.1.2.2.2 Cytokinins

Kinetin at 100 µm induced growth of shoots from protocorms of *Cymbidium*. There was no effect on fresh weight in solid medium but showed an increase in fresh weight in liquid medium. BA had similar effects but at lower concentrations (Fonnesbech, 1972). Kim and Kako (1982) found that the addition of BA encouraged PLB formation and shoot development. The greatest number of shoots in *Dendrobium antennathum* and *D. phalaenopsis* was obtained by enriching the medium with BA at 5 mg l^{-1} (Kukulczanka and Wojciechowska, 1983).

Paek *et al.* (1990) observed better shoot production from rhizomes of *Cymbidium* when exposed to BA at 10 mg l^{-1} for 10 to 20 days followed by BA at 0.5 mg l^{-1}, than transfer to medium without BA or exposure to 10 mg l^{-1} BA for 30 to 40 days. The leaves of *Laeliocattleya* cultured in medium with 0.5 – 1.0 mg l^{-1} BA gave higher yield of shoots (67 per explant) than medium without BA (Matos and Garcia, 1991).

Shoot growth of *Cymbidium* hybrid was the greatest when explants were cultured on medium containing BA at 10 mg l^{-1} for 10 days and then transferred to a medium containing 0.5 mg l^{-1} BA (Paek *et al.*, 1989). PLB and shoot formation from inflorescence tips of Aranda 'Deborah' were better when the medium was supplemented with KIN at 1 mg l^{-1} or combination of KIN and BA at 1 mg l^{-1} each (Goh and Wong, 1990).

BA caused marked acceleration in formation of PLB in *Catasetum fimbriatum* (Colli and Kerbauy, 1993). Vij and Dhiman (1997) reported that medium

supplemented with BA at 2 mg l^{-1} favoured development of multiple shoot buds in pseudobulbs of *Bletilla striata*.

2.1.2.2.3 Auxins and Cytokinins

The maximum fresh weight increased of protocorms of *Cymbidium* was observed when the kinetin concentration was one tenth of NAA concentration. The optimal growth and best development of protocorms occurred at 10 µm NAA and 1 µm kinetin combination (Fonnesbech, 1972). Kim *et al.* (1979) observed maximum growth of *Cymbidium kanran* rhizomes on adding NAA at 5 mg l^{-1} and BA at 0.5 mg l^{-1} to the basal medium.

High levels of cytokinin had deleterious effects on shoot growth. Auxin added to the medium nullified the suppressive effects of high cytokinin contents on axillary shoot growth (Lundergan and Janick, 1980). Lakshmidevi (1992) observed that shoots of *Dendrobium* on 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.

NAA 1.0 mg l^{-1} together with BA 3.0 mg l^{-1} was found to be the best for bud initiation in both half strength MS and VW media. Application of BA resulted in an increased shoot proliferation (Sudeep, 1994).

Ichihashi (1992) reported rapid proliferation of lateral buds on young flower stalks of four hybrids of *Phalaenopsis* cultivars in the absence of growth regulators.

A high concentration of NAA and BA was reported to induce maximum proliferation of shoots in *Cattleya* (Kusumoto, 1980). Successful growth of plantlets from internodal sections of flower stalk was

observed by the addition of 5 mg l^{-1} NAA and 20 mg l^{-1} BA (Choi *et al.*, 1989).

Phalaenopsis flower stalk segments formed adventitious buds (65 per cent) and PLBs (17 per cent) when the medium was supplemented with 5 mg l^{-1} BA and 1 mg l^{-1} NAA (Vij and Pathak, 1989). The best combination for leaf tip culture of *Pholidota chinensis* was BA and NAA each at 1 mg l^{-1} and for that of *Acampe rigide* was BA at 1 mg l^{-1} and NAA at 0.2 mg l^{-1} (Tom and Weatherhead, 1991).

Tokuhara and Mii (1993) reported that there was highest rate of PLB formation (60 per cent) in *Phaleanopsis* and *Doritaenopsis* with 20 mg l^{-1} BA and 0.1 mg l^{-1} NAA. 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}. Multiplication of PLB was reported in *Dendrobium* Sonia with BA at 1.5 mg l^{-1} and NAA at 0.4 mg l^{-1} (Pathania *et al.*, 1998).

Leaf explants of *Cymbidium* hybrid produced plantlets in the presence of BA and NAA at 22 µm each (Pindel and Miczynski, 1996). The NAA to BA ratio for PLB induction and plantlet development were 12.2 and 0.3 – 1.2 respectively. Maximum shoot proliferation was observed with kinetin 7.5 mg l^{-1} and NAA 2.0 mg l^{-1} (Kuriakose, 1997). Teng *et al.* (1997) showed that the best combination for PLB regeneration and plantlet development of *Spathoglottis plicata* was NAA at 2.69 – 10.74 µm and BA at 8.8 µm.

PLB multiplication and plantlet formation of *Oncidium* Gower Ramsey was the best in the presence of NAA at 0.5 mg l^{-1} and BA at 5.0 mg l^{-1} (Santana and Chapparro, 1999). Medium supplemented with BA at 1.0 mg l^{-1} and NAA at 0.1 mg l^{-1} was favourable for proliferation and differentiation of bulb explants of *Bletilla ochraceae* (Zhu and Wang, 1999).

PLB production was increased in *Geodorum densiflorum* by the addition of BA at 3 mg l^{-1} and NAA at 0.5 mg l^{-1}. Regeneration from PLBs of *Cymbidium aloifolium* showed well developed roots and a large number of shoot buds in the medium supplemented with kinetin, NAA and IBA at 5 mg l^{-1} each. The plants also exhibited healthy leaf growth (Buzarbarua, 1999).

The establishment of the explants, number of PLBs and shootlets per explant were higher in the combination of BAP 2.0 mg l^{-1} and NAA 1.0 mg l^{-1} in *Dendrobium* Sonia (Ramsundar *et al.*, 2000). Differentiation of shoots from the PLBs of orchid *Cymbidium* hybrid was the best in a medium supplemented with NAA at 4 mg l^{-1} and kinetin 0.05 mg l^{-1} (Prasad and Verma, 2001).

Combination of NAA at 0.5 mg l^{-1} and BAP at 0.5 mg l^{-1} inhibited root formation in *Cymbidium longifolium* but stimulated further proliferation and multiplication of protocorms. IBA and BAP each at 0.5 mg l^{-1} inhibited both shoot and root formation (Siddique and Paswan, 2001).

Talukdar *et al.* (2002) observed that the highest frequency of germination (78.33 per cent) and the shortest duration for protocorm development (7.33 weeks) was obtained with 1.0 mg IAA + 1.0 mg BA + CW 150 ml per litre treatment in *Cymbidium pendulum*.

Shoot tip explants of *Dendrobium fimbriatum* Lindl. Var. *oculatum* Hk. f. were cultured on modified nutrient solution of KC. Application of both plant growth regulators was essential for the induction of callus. Optimum callusing was recorded in the presence of 0.5 mg l^{-1} NAA and 1 mg l^{-1} BAP (66.7 per cent) (Roy and Banerjee, 2003).

2.1.2.3 Role of Additives

2.1.2.3.1 Coconut Water (CW)

The stimulatory effect of coconut water under *in vitro* conditions is due to the presence of growth regulator contents. Cytokinins are assumed to be the most important growth regulators present in CW.

Maximum PLB proliferation and the highest increase in fresh weight of PLBs was attained with 10 per cent CW in *Cattleya* (Kusumoto, 1980). For the initial culture of *Dendrobium*, supplementation of media with CW at 15 per cent is suitable (Soediono, 1983).

PLB formation and further multiplication were the best on addition of CW at 10 per cent in *Cymbidium ensifolium* and *C. goeringii* (Wang, 1988). Callus initiation was observed in shoot meristems of *Dendrobium* with CW 15 per cent (Sharon and Vasundhara, 1990). Inflorescence tips of *Mokara* Chark Kuan produced plantlets when CW 200 ml l^{-1} was added to liquid VW medium (Karim *et al.*, 1992).

Bagde and Sharon (1997) observed the best PLB formation and further multiplication in *Oncidium* when the medium was supplemented with 15 per cent CW.

Higher number of PLBs were formed from inflorescence tips of *Aranda* Deborah when the liquid KC medium was supplemented with 15 per cent CW (Goh and Wong, 1990). Addition of coconut water (15 per cent) significantly influenced the development of protocorm into plantlet in terms of early formation of leaf and more number of leaves in *Dendrobium* Sonia (Ramsundar *et al.*, 2000).

The optimum concentration of CW in the medium was 10-15 per cent and it was added before autoclaving (Intuwong and Sagawa, 1973). Half strength MS medium supplemented with coconut water in combination with NAA and BA was superior than using coconut water

alone. Here, half MS medium was better than VW medium in producing multiple shoots (Sudeep, 1994).

Buds excised from *Dendrobium* bulbs as well as those from leaf axils cultured on VW liquid medium supplemented with 15 per cent CW produced PLB's in 4-5 weeks and plantlets in eight weeks (Kim *et al.*, 1970). CW 15 per cent differentiated more number of plantlets within a short period from callus produced on the nodal explants of *Dendrobium* (Nair, 1982).

2.1.2.3.2 Activated Charcoal

Activated charcoal inhibited shoot formation in *Phalaenopsis* hybrids (Tanaka *et al.*, 1988). Villalobos *et al.* (1994) reported that the best plantlet development was obtained from protocorms of *Cymbidium aurantica, Epidendrum belizensis* sub sp. Paniflora and *Oncidium leucochilum* on the medium supplemented with activated carbon at 2 g l^{-1}.

AC was reported to be necessary for producing healthy plantlets and for stimulating shoot growth at a level of 0.1 - 0.3 per cent but reduced rhizome growth in *Cymbidium* (Peak and Kozai, 1998). Addition of AC 0.1 per cent to the MS medium promoted growth of protocorms of *Cymbidium sinense* (Chen *et al.*, 1999b). Rooting of plantlets of *Anoectochilus* was achieved by adding AC 0.2 per cent to the medium (Gangaprasad *et al.*, 2000).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present investigations were carried out in the Department of Plant Breeding and Genetics and Plant Molecular Biology and Biotechnology laboratory, College of Agriculture, Vellayani during 2002-2004, with the objective of developing a protocol for the rapid *in vitro* micropropagation of selected *Dendrobium* hybrids.

The experiment consisted of the following three major studies 1) Standardisation of explants in different basal media, 2) Standardisation of treatment from first experiment using plant growth substances and organic additive, 3) Standardisation of treatment for *in vitro* rooting of microshoots.

3.1 VARIETIES

The base material for the study consisted of the *Dendrobium* hybrids already developed under the DBT project "Breeding for commercial orchid hybrids" and maintained in the greenhouse.

3.2 EXPLANTS

The following explants were used.

3.2.1 Immature Leaf Segment

Leaf segments of 0.5 – 1.0 cm^2 size from fully opened, light green immature leaves with the mid vein were taken.

3.2.2 Stem Nodal Segment

Stem nodal segment of 1.0 – 1.5 cm length each, with one node were excised from keikis. The leaf sheath surrounding the node was removed to expose the dormant bud.

3.2.3 Inflorescence Axis

Inflorescence axis of 0.75 – 1.0 cm length each with one node were collected from the distal end of the developing flower stalks.

3.3 COLLECTION AND PREPARATION

The explants were collected from the *Dendrobium* hybrids raised in the greenhouse of the DBT project "Breeding for commercial orchid hybrids" under the Department of Plant Breeding and Genetics. The explants were washed thoroughly with tap water followed by a washing with Tween 20 emulsifier for 30 minutes. Then they were washed 2-3 times with double glass distilled water.

3.4 SURFACE STERILIZATION

Surface sterilization of the explant was carried out inside a laminar airflow chamber (Klenzaids; Horizontal model-1104) just before inoculation. The explant was transferred to a sterilized beaker and surface sterilized by soaking in freshly prepared 0.1 per cent mercuric chloride solution for 10 minutes with intermittent shaking. The solution was drained and the explant was washed 4-5 times with sterile double glass distilled water to remove all traces of the chemical. The explant was transferred carefully into a sterile petriplate.

3.5 INOCULATION AND INCUBATION

All inoculation operations were carried out inside a laminar airflow chamber. The vessels and tools (beakers, petriplates, blades, forceps etc.) required for inoculation were washed thoroughly, rinsed with glass distilled water, covered air tight with aluminium foil and autoclaved at 121°C temperature and 1.06 kg cm^{-2} pressure for 45 minutes. They were further flame sterilized just before inoculation using a spirit lamp inside the laminar air flow chamber.

To inoculate the explants into the culture medium the cotton plug of the culture vessel was removed and the mouth was flamed. The explant was inoculated into the medium using sterile forceps. The mouth of the culture vessel was flamed again and cotton plug was replaced.

The cultures were then incubated in a culture room with controlled conditions of light, temperature and humidity. Darkness was provided initially after inoculation by enclosing the culture racks in a black muslin cloth screen. Once response was initiated, a photoperoid of 15 hours light and nine hours darkness with a light intensity of 3000 lux under fluorescent tube light was provided. A uniform temperature of 26 ± 2°C and a relative humidity of 75 per cent was maintained in the culture room.

3.6 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 British Drug House (Mumbai), Sisco Research Laboratory (Mumbai) and Merck (Mumbai).

Standard procedures were followed for the preparation of media (Thorpe, 1980). Stock solution 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/alcohol and volume made up with double glass-distilled water. The stock solutions were stored under refrigerated condition (+4°C).

The culture vessels used were 'Borosil' brand test tubes (25 x 150 mm) and Erlenmeyer flasks (100 ml). They were washed with 1000 times diluted Labolene and tap water, rinsed with glass distilled water and kept over night in a hot air oven (60°C) for drying and pre-sterilization.

All items of glassware and vessels used for the preparation of culture media were washed thoroughly in 1000 times dilute Labolene and tap water and rinsed with glass distilled water. Specific quantities of stock solutions were pipetted out into a 1000 ml beaker. Sucrose and myo-inositol were added fresh and dissolved. Coconut water (CW) when used was collected from freshly harvested tender coconuts (8 months old) of the local 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 and an electronic pH meter (Global Electronic, model DPH 500). Agar was then added to the medium.

For the preparation of VW and KC media the chemicals were taken in required quantity and dissolved in double glass distilled water. Growth regulators, sucrose and inositol were added fresh and the volume was made up 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 or paper, labeled and autoclaved at 121°C temperature and 1.06 kg cm^{-2} pressure for 20 minutes. After sterilization, the culture vessels were transferred to the culture room.

3.7 EXPERIMENTAL DETAILS

3.7.1 Experiment (Ia)

Standardisation of explants in different basal media

Explants

1) Immature leaf segment
2) Stem nodal segment
3) Inflorescence axis

Basal media

1) MS (Full, half and quarter strength)
2) VW (Full strength)
3) KC (Full strength)

3.7.2 Experiment (Ib)

Standardisation of treatment from Experiment (Ia) using organic and inorganic growth adjuvants.

The most suitable treatment, stem nodal segment explant and VW medium identified from Experiment (Ia) was standardised for different plant growth substances (inorganic) and organic additives.

Independent experiments were conducted using the treatment combinations BA and NAA, BA and IAA, kinetin and NAA, kinetin and IAA and the organic additive coconut water at different levels, due to shortage of explants from the selected *Dendrobium* hybrids.

The objective was to arrive at the best treatment combination for the rapid micropropagation of selected *Dendrobium* hybrids with respect to the different sets of growth regulator tried.

The description of each experiment is given below.

3.7.2.1 Experiment (Ib-i)

Table 1. Different levels of BA and NAA tried for PLB differentiation and plantlet growth in stem nodal segment explant of *Dendrobium* hybrids

Medium : VW + sucrose 30 g l^{-1} + agar 6.0 g l^{-1}

Treatment	Plant growth substances (mg l^{-1})
T_1	BA 2.0 + NAA 2.0
T_2	BA 2.0 + NAA 4.0
T_3	BA 2.0 + NAA 6.0
T_4	BA 2.0 + NAA 8.0
T_5	BA 4.0 + NAA 2.0
T_6	BA 4.0 + NAA 4.0
T_7	BA 4.0 + NAA 6.0
T_8	BA 4.0 + NAA 8.0
T_9	BA 6.0 + NAA 2.0
T_{10}	BA 6.0 + NAA 4.0
T_{11}	BA 6.0 + NAA 6.0
T_{12}	BA 6.0 + NAA 8.0
T_{13}	BA 8.0 + NAA 2.0
T_{14}	BA 8.0 + NAA 4.0
T_{15}	BA 8.0 + NAA 6.0
T_{16}	BA 8.0 + NAA 8.0
Control	-

3.7.2.2 Experiment (Ib-ii)

Table 2. Different levels of BA and IAA tried for PLB differentiation and plantlet growth in stem nodal segment explant of *Dendrobium* hybrids

Medium : VW + sucrose 30 g l^{-1} + agar 6.0 g l^{-1}

Treatment	Plant growth substances (mg l^{-1})
T_1	BA 2.0 + IAA 2.0
T_2	BA 2.0 + IAA 4.0
T_3	BA 2.0 + IAA 6.0
T_4	BA 2.0 + IAA 8.0
T_5	BA 4.0 + IAA 2.0
T_6	BA 4.0 + IAA 4.0
T_7	BA 4.0 + IAA 6.0
T_8	BA 4.0 + IAA 8.0
T_9	BA 6.0 + IAA 2.0
T_{10}	BA 6.0 + IAA 4.0
T_{11}	BA 6.0 + IAA 6.0
T_{12}	BA 6.0 + IAA 8.0
T_{13}	BA 8.0 + IAA 2.0
T_{14}	BA 8.0 + IAA 4.0
T_{15}	BA 8.0 + IAA 6.0
T_{16}	BA 8.0 + IAA 8.0
Control	-

3.7.2.3 Experiment (Ib-iii)

Table 3. Different levels of kinetin and NAA tried for PLB differentiation and plantlet growth in stem nodal segment explant of *Dendrobium* hybrids

Medium : VW + sucrose 30 g l^{-1} + agar 6.0 g l^{-1}

Treatment	Plant growth substances (mg l^{-1})
T_1	Kinetin 2.0 + NAA 2.0
T_2	Kinetin 2.0 + NAA 4.0
T_3	Kinetin 2.0 + NAA 6.0
T_4	Kinetin 2.0 + NAA 8.0
T_5	Kinetin 4.0 + NAA 2.0
T_6	Kinetin 4.0 + NAA 4.0
T_7	Kinetin 4.0 + NAA 6.0
T_8	Kinetin 4.0 + NAA 8.0
T_9	Kinetin 6.0 + NAA 2.0
T_{10}	Kinetin 6.0 + NAA 4.0
T_{11}	Kinetin 6.0 + NAA 6.0
T_{12}	Kinetin 6.0 + NAA 8.0
T_{13}	Kinetin 8.0 + NAA 2.0
T_{14}	Kinetin 8.0 + NAA 4.0
T_{15}	Kinetin 8.0 + NAA 6.0
T_{16}	Kinetin 8.0 + NAA 8.0
Control	-

3.7.2.4 Experiment (Ib-iv)

Table 4. Different levels of kinetin and IAA tried for PLB differentiation and plantlet growth in stem nodal segment explant of *Dendrobium* hybrids

Medium : VW + sucrose 30 g l^{-1} + agar 6.0 g l^{-1}

Treatment	Plant growth substances (mg l^{-1})
T_1	Kinetin 2.0 + IAA 2.0
T_2	Kinetin 2.0 + IAA 4.0
T_3	Kinetin 2.0 + IAA 6.0
T_4	Kinetin 2.0 + IAA 8.0
T_5	Kinetin 4.0 + IAA 2.0
T_6	Kinetin 4.0 + IAA 4.0
T_7	Kinetin 4.0 + IAA 6.0
T_8	Kinetin 4.0 + IAA 8.0
T_9	Kinetin 6.0 + IAA 2.0
T_{10}	Kinetin 6.0 + IAA 4.0
T_{11}	Kinetin 6.0 + IAA 6.0
T_{12}	Kinetin 6.0 + IAA 8.0
T_{13}	Kinetin 8.0 + IAA 2.0
T_{14}	Kinetin 8.0 + IAA 4.0
T_{15}	Kinetin 8.0 + IAA 6.0
T_{16}	Kinetin 8.0 + IAA 8.0
Control	-

3.7.2.5 Experiment (Ib-b)

Table 5. Different levels of coconut water tried for PLB differentiation and plantlet growth in stem nodal segment explant of *Dendrobium* hybrids

Medium : VW + Kinetin 4.0 mg l^{-1} + IAA 4.0 mg l^{-1} + sucrose 30 g l^{-1} + agar 6.0 g l^{-1}

Treatment	Coconut water (ml l^{-1})
T_1	100
T_2	200
T_3	300
Control	-

3.7.3 Experiment (Ic)

Effect of growth substances and activated charcoal on rooting of microshoots.

Since rooting was not observed in Experiment (I b) further trials were done using AC and IBA. The medium used was ½ MS + sucrose 30 g l^{-1} + agar 6 g l^{-1} + CW 200 ml l^{-1}.

Table 6 Different levels of IBA and activated charcoal tried for *in vitro* rooting of microshoots of *Dendrobium* hybrids

Treatment	Plant growth substances (mg l^{-1})	
	Without AC	With AC (0.5 g l^{-1})
T_1	IBA 0.5	IBA 0.5
T_2	IBA 1.0	IBA 1.0
T_3	IBA 1.5	IBA 1.5
T_4	IBA 2.0	IBA 2.0
Control	-	-

3.8 OBSERVATIONS

3.8.1 Number of Days for Initiation of PLB's

Number of days from inoculation to the development of protocorm like bodies was observed.

3.8.2 Number of Days for Greening of PLB's

Number of days from inoculation to the development of chlorophyll in PLB's was recorded.

3.8.3 Number of Days for Initiation of First Leaf

Number of days from inoculation to the visible emergence of leaf was recorded.

3.8.4 Number of Days for Initiation of Shoot

Number of days from inoculation to visible differentiation of shoot was computed.

3.8.5 Number of Shoots at Subculture

Number of shoots at third subculture was counted.

3.8.6 Number of Days for Initiation of First Root

Number of days from inoculation to development of first root initial was recorded.

3.8.7 Number of Roots per Shoot

Number of roots produced per shoot was counted two weeks after root initiation.

3.8.8 Root Length

Length of root was recorded with the aid of graph paper two weeks after the root initiation.

3.8.9 Number of Days for Deflasking

Deflasking was done when the *in vitro* raised shoots attained sufficient growth for transplanting. Number of days taken from inoculation to deflasking was observed.

3.9 STATISTICAL ANALYSIS

Completely randomised design (CRD) and factorial completely randomised design (FCRD) wherever necessary as per Panse and Sukhatme (1978) were used.

RESULTS

4. RESULTS

Investigations were carried out for standardising *in vitro* techniques for the micropropagation of *Dendrobium* hybrids in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani, during 2002–2004. The *in vitro* experiments were carried out at the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani. The results obtained are presented in this chapter.

4.1 STANDARDISATION OF EXPLANTS IN DIFFERENT BASAL MEDIA

The response of immature leaf segment, stem nodal segment and inflorescence axis in MS (Full, half and quarter strength), VW (Full strength) and KC (Full strength) media were tried.

Among the three explants tried, immature leaf segment and inflorescence axis did not respond in these basal media. They remained green but did not show initiation of PLB's even after 45 days of inoculation. Hence they were abandoned and stem nodal segment showing speedy response alone was tried in the different basal media.

4.1.1 Effect of Different Basal Media on Culture Establishment in Stem Nodal Segment Explant

The effect of basal media on stem nodal segment for initiation of PLB's (Table 7) showed that VW medium took the minimum duration of 15.67 days which was significantly different from all other media tried. This was followed by half MS medium requiring 18.17 days. The maximum number of days for PLB initiation (23.67) was taken by full strength MS medium which was significantly different from all other treatments.

Plate 1 General view of mother plants of *Dendrobium* hybrids in pots

Plate 2. Explant investigated for micropropagation of *Dendrobium* hybrids

Immature leaf segment

Stem nodal segment

Inflorescence axis

Plate 3. General view of cultures of *Dendrobium* hybrids under incubation in the culture room

Plate 4. Secondary inflorescence formed from inflorescence axis

Plate 5. PLB initiation in stem nodal segment explant

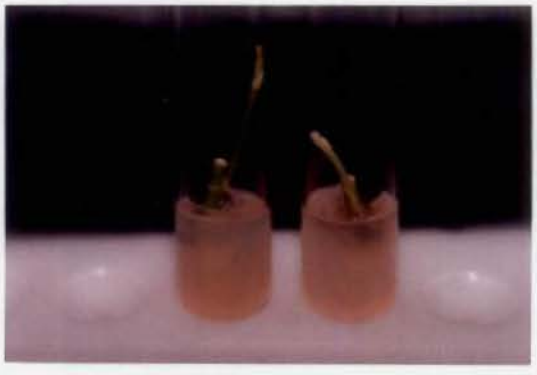
Plate 6. PLB greening in stem nodal segment explant

Plate 7. First leaf initiation in stem nodal segment explant

Plate 8. Shoot initiation in stem nodal segment explant

Table 7 Effect of different media on culture establishment in stem nodal segment explant of *Dendrobium* hybrids

Media	Number of days for initiation of PLB's	Number of days for greening of PBL's	Number of days for initiation of first leaf
MS full	23.67	31.50	40.83
Half MS	18.17	27.00	35.33
Quarter MS	21.17	28.00	38.17
VW	15.67	22.17	31.33
KC	21.17	28.83	38.50
Mean	19.97	27.50	36.83
F	83.66**	38.79**	18.45**
SE	0.338	0.549	0.848
CD	0.98	1.59	2.47



Number of days taken for greening of PLB's also differed significantly among the different basal media. VW medium took the minimum number of 22.17 days which was significantly different from all other media. It was followed by half MS taking 27.00 days for greening of PLB's. In full strength MS medium maximum duration (31.50 days) was taken for greening of PLB's

With respect to the number of days for initiation of first leaf, VW recorded the lowest value of 31.33 days followed by half MS taking 35.33 days. Maximum number of days was recorded by full strength MS medium, where 40.83 days was taken for first leaf initiation.

4.2 STANDARDISATION OF PLANT GROWTH SUBSTANCES AND ORGANIC ADDITIVE

4.2.1 Effect of BA and NAA on PLB Differentiation and Plantlet Growth

Effect of BA and NAA on PLB differentiation and plantlet growth was studied.

Regarding initiation of PLB's, BA and NAA applied in equal proportions at the lowest concentration, T_1 (BA 2 mg l^{-1} + NAA 2 mg l^{-1}) took the minimum duration of 8.33 days. This was on par with T_6 (BA 4 mg l^{-1} + NAA 4 mg l^{-1}) which took 9.17 days (Table 8a and Fig. 1a). They were followed by T_7 (10.00 days), T_2 (10.67 days) and T_5 (10.83 days) which were on par with each other. Control took the maximum number of days for initiation of PLB's (32.33 days) which was significantly higher from all other treatments.

With respect to greening of PLB's, T_1 (BA 2 mg l^{-1} + NAA 2 mg l^{-1} applied in 1 : 1 proportion) took the lowest duration of 14.83 days, which differed significantly from all other treatments (Table 8b and Fig. 1b). It was followed by T_5 which took 18.33 days for greening of PLB's.

Table 8a. Effect of BA and NAA on number of days for initiation of PLB's in stem nodal segment explant

BA (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	8.33 (T_1)	10.67 (T_2)	13.17 (T_3)	18.83 (T_4)
4	10.83 (T_5)	9.17 (T_6)	10.00 (T_7)	16.00 (T_8)
6	18.50 (T_9)	16.67 (T_{10})	17.50 (T_{11})	19.50 (T_{12})
8	15.83 (T_{13})	19.67 (T_{14})	23.17 (T_{15})	24.17 (T_{16})

Control	F	SE	CD
32.33	49.42**	0.308	0.855

**Significant at 1 per cent level

Table 8b. Effect of BA and NAA on number of days for greening of PLB's in stem nodal segment explant

BA (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	14.83 (T_1)	20.00 (T_2)	22.50 (T_3)	27.67 (T_4)
4	18.33 (T_5)	21.50 (T_6)	23.00 (T_7)	30.17 (T_8)
6	28.67 (T_9)	27.17 (T_{10})	29.83 (T_{11})	30.33 (T_{12})
8	30.50 (T_{13})	32.33 (T_{14})	33.33 (T_{15})	35.50 (T_{16})

Control	F	SE	CD
44.80	43.73**	0.346	0.958

**Significant at 1 per cent level

Table 8c. Effect of BA and NAA on number of days for initiation of first leaf in stem nodal segment explant

BA (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	27.67 (T_1)	35.00 (T_2)	37.00 (T_3)	39.67 (T_4)
4	36.67 (T_5)	33.17 (T_6)	36.17 (T_7)	39.17 (T_8)
6	40.00 (T_9)	38.00 (T_{10})	38.67 (T_{11})	52.50 (T_{12})
8	50.00 (T_{13})	40.67 (T_{14})	53.83 (T_{15})	60.83 (T_{16})

Control	F	SE	CD
59.50	272.93**	0.251	0.697

**Significant at 1 per cent level

Table 8d. Effect of BA and NAA on number of days for initiation of shoot in stem nodal segment explant

BA (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	41.67 (T_1)	45.67 (T_2)	47.50 (T_3)	50.50 (T_4)
4	46.00 (T_5)	42.83 (T_6)	46.00 (T_7)	51.50 (T_8)
6	51.83 (T_9)	48.17 (T_{10})	50.50 (T_{11})	62.17 (T_{12})
8	64.33 (T_{13})	54.17 (T_{14})	64.83 (T_{15})	70.00 (T_{16})

Control	F	SE	CD
79.00	82.74**	0.326	0.904

**Significant at 1 per cent level

Table 8e. Effect of BA and NAA on number of shoots in stem nodal segment explant

BA (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	1.50 (T_1)	1.83 (T_2)	2.83 (T_3)	3.50 (T_4)
4	3.00 (T_5)	3.50 (T_6)	4.33 (T_7)	4.33 (T_8)
6	3.67 (T_9)	4.00 (T_{10})	4.67 (T_{11})	3.17 (T_{12})
8	3.00 (T_{13})	3.83 (T_{14})	5.00 (T_{15})	8.33 (T_{16})

Control	F	SE	CD
1.00	3.79**	0.563	1.559

**Significant at 1 per cent level

Table 8f. Effect of BA and NAA on number of days for deflasking in stem nodal segment explant

BA (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	154.00 (T_1)	147.33 (T_2)	148.00 (T_3)	155.00 (T_4)
4	145.00 (T_5)	142.67 (T_6)	148.00 (T_7)	153.00 (T_8)
6	150.00 (T_9)	148.00 (T_{10})	166.00 (T_{11})	173.00 (T_{12})
8	170.33 (T_{13})	175.00 (T_{14})	160.00 (T_{15})	180.00 (T_{16})

Control	F	SE	CD
189.00	234.56**	0.344	0.953

**Significant at 1 per cent level

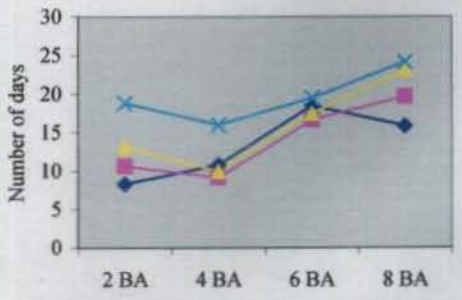


Fig. 1a Effect of BA and NAA on number of days for initiation of PLB's in stem nodal segment explant

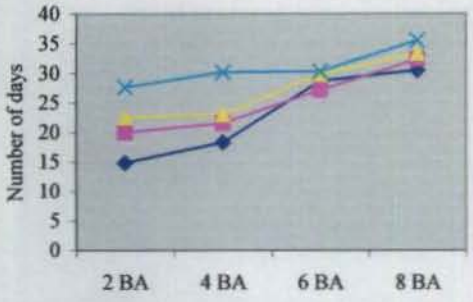


Fig. 1b Effect of BA and NAA on number of days for greening of PLB's in stem nodal segment explant

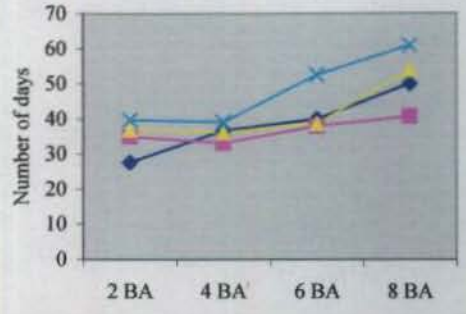


Fig. 1c Effect of BA and NAA on number of days for initiation of first leaf in stem nodal segment explant

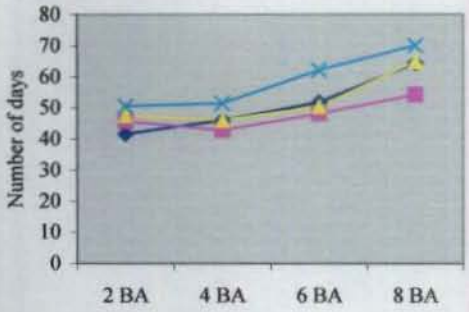


Fig. 1d Effect of BA and NAA on number of days for initiation of shoot in stem nodal segment explant

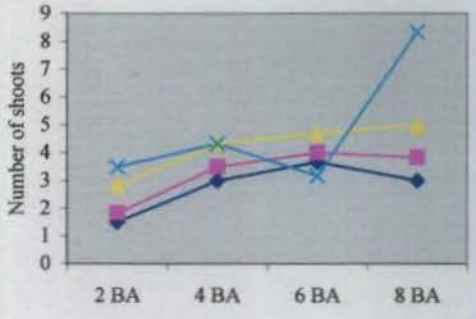


Fig. 1e Effect of BA and NAA on number of shoots in stem nodal segment explant

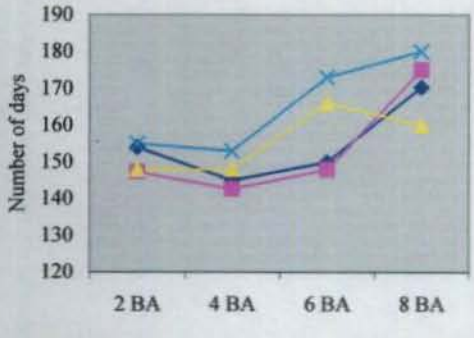


Fig. 1f Effect of BA and NAA on number of days for deflasking in stem nodal segment explant

Maximum number of days was taken by control (44.80 days) followed by T_{16} (35.50 days), T_{15} (33.33 days) and T_{14} (32.33 days).

T_1 (BA 2 mg l^{-1} + NAA 2 mg l^{-1}) recorded the minimum number of 27.67 days for initiation of first leaf and was significantly superior to all other treatments (Table 8c and Fig. 1c). It was closely followed by T_6 (33.17 days). T_{16} took the maximum duration of 60.83 days for initiation of first leaf which was on par with control (59.50 days).

Number of days for initiation of shoot was the lowest in T_1 (BA 2 mg l^{-1} + NAA 2 mg l^{-1}) being 41.67 days (Table 8d and Fig 1d). It was significantly lower in comparison to all other treatments. This was closely followed by T_6 (BA 4 mg l^{-1} + NAA 4 mg l^{-1}) taking 42.83 days. Control took the maximum number of 79.00 days for shoot initiation. It was preceded by T_{16} (70.00 days), T_{15} (64.83 days) and T_{13} (64.33 days).

With respect to number of shoots, T_{16} (BA 8 mg l^{-1} + NAA 8 mg l^{-1} applied in 1 : 1 proportion) recorded maximum number of shoots (8.33) and it was significantly different from all other treatments (Table 8e and Fig 1e). T_{16} was closely followed by T_{15} (5.00 shoots), T_{11} (4.67 shoots), T_7 (4.33 shoots) and T_8 (4.33 shoots). Minimum of one shoot was produced by control and was on par with T_1 and T_2 producing 1.50 and 1.83 shoots respectively.

Number of days for deflasking was the lowest in T_6 (BA 4 mg l^{-1} + NAA 4 mg l^{-1}) being 142.67 days which was significantly different from all other treatments (Table 8f and Fig. 1f). It was followed by T_5 (BA 4 mg l^{-1} + NAA 2 mg l^{-1}) taking 145 days and T_2 (BA 2 mg l^{-1} + NAA 4 mg l^{-1}) taking 147.33 days. T_{10}, T_3 and T_7 were deflasked in 148 days.

4.2.2 Effect of BA and IAA on PLB Differentiation and Plantlet Growth

Effect of BA and IAA on PLB differentiation and plantlet growth was recorded.

Table 9a. Effect of BA and IAA on number of days for initiation of PLB's in stem nodal segment explant

BA (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	11.33 (T_1)	9.00 (T_2)	14.33 (T_3)	15.00 (T_4)
4	11.00 (T_5)	11.00 (T_6)	14.00 (T_7)	16.83 (T_8)
6	15.17 (T_9)	19.17 (T_{10})	20.17 (T_{11})	21.83 (T_{12})
8	19.33 (T_{13})	19.83 (T_{14})	22.00 (T_{15})	24.00 (T_{16})

Control	F	SE	CD
32.33	853.66**	0.157	0.442

**Significant at 1 per cent level

Table 9b. Effect of BA and IAA on number of days for greening of PLB's in stem nodal segment explant

BA (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	18.67 (T_1)	20.17 (T_2)	23.83 (T_3)	27.00 (T_4)
4	22.33 (T_5)	21.83 (T_6)	25.83 (T_7)	28.67 (T_8)
6	26.83 (T_9)	28.67 (T_{10})	30.83 (T_{11})	32.17 (T_{12})
8	30.00 (T_{13})	30.50 (T_{14})	35.17 (T_{15})	36.83 (T_{16})

Control	F	SE	CD
44.80	579.16**	0.216	0.607

**Significant at 1 per cent level

Table 9c. Effect of BA and IAA on number of days for initiation of first leaf in stem nodal segment explant

BA (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	35.00 (T_1)	42.00 (T_2)	43.00 (T_3)	47.00 (T_4)
4	44.00 (T_5)	42.00 (T_6)	46.00 (T_7)	48.33 (T_8)
6	50.00 (T_9)	51.00 (T_{10})	52.00 (T_{11})	49.83 (T_{12})
8	51.00 (T_{13})	50.33 (T_{14})	56.00 (T_{15})	57.17 (T_{16})

Control	F	SE	CD
59.50	1849.16**	0.132	0.371

**Significant at 1 per cent level

Table 9d. Effect of BA and IAA on number of days for initiation of shoot in stem nodal segment explant

BA (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	57.00 (T_1)	61.83 (T_2)	68.00 (T_3)	70.00 (T_4)
4	62.17 (T_5)	62.00 (T_6)	64.00 (T_7)	68.00 (T_8)
6	64.00 (T_9)	65.00 (T_{10})	68.00 (T_{11})	72.67 (T_{12})
8	62.33 (T_{13})	65.00 (T_{14})	68.83 (T_{15})	73.00 (T_{16})

Control	F	SE	CD
79.00	805.69**	0.152	0.429

**Significant at 1 per cent level

Table 9e. Effect of BA and IAA on number of shoots in stem nodal segment explant

BA (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	1.33 (T_1)	1.83 (T_2)	3.00 (T_3)	3.17 (T_4)
4	2.83 (T_5)	2.83 (T_6)	4.33 (T_7)	4.83 (T_8)
6	3.33 (T_9)	2.83 (T_{10})	3.67 (T_{11})	2.67 (T_{12})
8	3.50 (T_{13})	4.00 (T_{14})	5.33 (T_{15})	8.33 (T_{16})

Control	F	SE	CD
1.00	41.99**	0.249	0.702

**Significant at 1 per cent level

Table 9f. Effect of BA and IAA on number of days for deflasking in stem nodal segment explant

BA (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	159.00 (T_1)	163.00 (T_2)	167.00 (T_3)	169.00 (T_4)
4	165.00 (T_5)	166.00 (T_6)	169.00 (T_7)	167.17 (T_8)
6	165.00 (T_9)	167.00 (T_{10})	168.00 (T_{11})	167.00 (T_{12})
8	169.00 (T_{13})	170.00 (T_{14})	175.00 (T_{15})	184.00 (T_{16})

Control	F	SE	CD
189.00	18876.44**	0.039	0.111

**Significant at 1 per cent level

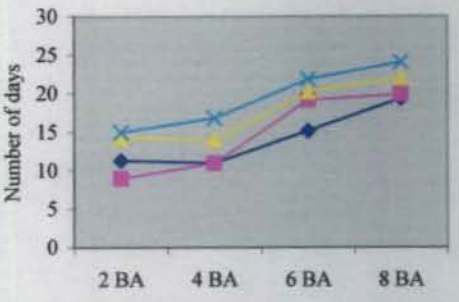


Fig. 2a Effect of BA and IAA on number of days for initiation of PLB's in stem nodal segment explant

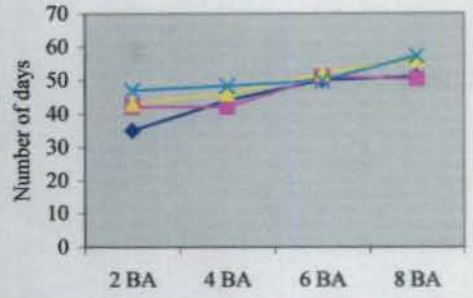


Fig. 2b Effect of BA and IAA on number of days for greening of PLB's in stem nodal segment explant

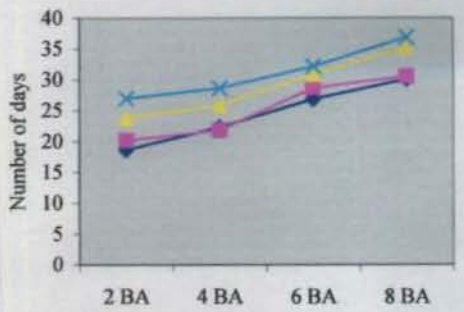


Fig. 2c Effect of BA and IAA on number of days for initiation of first leaf in stem nodal segment explant

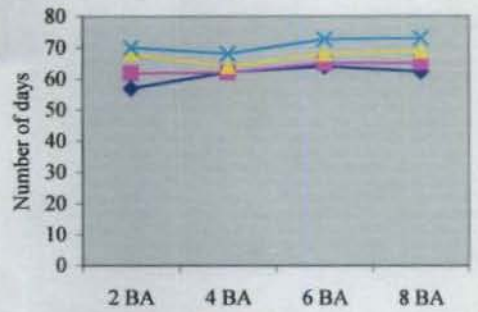


Fig. 2d Effect of BA and IAA on number of days for initiation of shoot in stem nodal segment explant

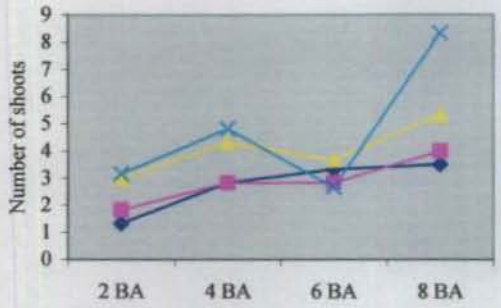


Fig. 2e Effect of BA and IAA on number of shoots in stem nodal segment explant

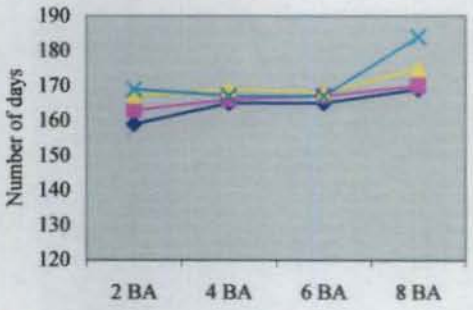


Fig. 2f Effect of BA and IAA on number of days for deflasking in stem nodal segment explant

Treatment T_2 (BA 2 mg l^{-1} + IAA 4 mg l^{-1} applied in 1: 2 proportion) recorded the minimum of 9.00 days for PLB initiation. It was significantly superior to all other treatments (Table 9a and Fig. 2a). This was followed by T_5 (11.00 days), T_6 (11.00 days) and T_1 (11.33 days). They were on par with each other. Control recorded the highest duration of 32.33 days for initiation of PLB's.

With respect to greening of PLB's, T_1 (BA 2 mg l^{-1} + IAA 2 mg l^{-1}) took the minimum duration of 18.67 days and it was significantly lower than that recorded by all other treatments (Table 9b and Fig. 2b). Control took the maximum duration of 44.80 days for greening of PLB's which was preceded by T_{16} needing 36.83 days.

For initiation of first leaf, T_1 (BA 2 mg l^{-1} + IAA 2 mg l^{-1} applied in 1 : 1 proportion) took the lowest duration being 35.00 days and was significantly different from all other treatments (Table 9c and Fig. 2c). Control took the highest number of days for initiation of first leaf being 59.50 days.

For initiation of shoot, T_1 (BA 2 mg l^{-1} + IAA 2 mg l^{-1} applied in 1 : 1 proportion) took the lowest number of days being 57.00 days and was significantly different from all other treatments (Table 9d and Fig. 2d). Control took the highest number of days for initiation of shoot being 79.00 days.

Treatment T_{16} (BA 8 mg l^{-1} + IAA 8 mg l^{-1}) recorded the highest shoot number of 8.33 which showed significant difference from all other treatments (Table 9e and Fig. 2e). This result was obtained at the higher dose of both BA and IAA in 1 : 1 ratio. Control recorded the lowest shoot number of one shoot which was on par with T_1 recording 1.33 shoots.

Regarding number of days for deflasking, T_1 (BA 2 mg l^{-1} + IAA 2 mg l^{-1}) took 159.00 days and was significantly lower from all other treatments (Table 9f and Fig. 2f). This was closely followed by T_2 taking

Table 10a. Effect of KIN and NAA on number of days for initiation of PLB's in stem nodal segment explant

KIN (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	9.33 (T_1)	13.33 (T_2)	15.33 (T_3)	18.00 (T_4)
4	14.00 (T_5)	15.17 (T_6)	16.00 (T_7)	17.33 (T_8)
6	20.83 (T_9)	19.00 (T_{10})	20.17 (T_{11})	21.83 (T_{12})
8	17.00 (T_{13})	20.00 (T_{14})	22.00 (T_{15})	25.00 (T_{16})

Control	F	SE	CD
32.33	501.95**	0.175	0.492

**Significant at 1 per cent level

Table 10b. Effect of KIN and NAA on number of days for greening of PLB's in stem nodal segment explant

KIN (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	21.00 (T_1)	23.50 (T_2)	26.83 (T_3)	27.33 (T_4)
4	25.83 (T_5)	26.83 (T_6)	30.17 (T_7)	35.17 (T_8)
6	31.00 (T_9)	30.67 (T_{10})	32.33 (T_{11})	34.00 (T_{12})
8	30.00 (T_{13})	35.17 (T_{14})	38.00 (T_{15})	37.67 (T_{16})

Control	F	SE	CD
44.80	654.80**	0.193	0.543

**Significant at 1 per cent level

Table 10c. Effect of KIN and NAA on number of days for initiation of first leaf in stem nodal segment explant

KIN (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	40.00 (T_1)	46.00 (T_2)	52.00 (T_3)	55.00 (T_4)
4	49.00 (T_5)	54.00 (T_6)	55.00 (T_7)	56.00 (T_8)
6	45.67 (T_9)	47.50 (T_{10})	50.00 (T_{11})	54.00 (T_{12})
8	50.33 (T_{13})	54.00 (T_{14})	56.00 (T_{15})	57.00 (T_{16})

Control	F	SE	CD
59.50	2579.18**	0.093	0.262

**Significant at 1 per cent level

Table 10d. Effect of KIN and NAA on number of days for initiation of shoot in stem nodal segment explant

KIN (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	59.00 (T_1)	63.00 (T_2)	64.00 (T_3)	67.00 (T_4)
4	64.00 (T_5)	65.00 (T_6)	69.00 (T_7)	70.00 (T_8)
6	63.00 (T_9)	64.00 (T_{10})	65.00 (T_{11})	69.00 (T_{12})
8	70.83 (T_{13})	70.50 (T_{14})	74.00 (T_{15})	75.00 (T_{16})

Control	F	SE	CD
79.00	1878.61**	0.101	0.283

**Significant at 1 per cent level

Table 10e. Effect of KIN and NAA on number of shoots in stem nodal segment explant

KIN (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	1.50 (T_1)	1.67 (T_2)	2.83 (T_3)	3.33 (T_4)
4	3.33 (T_5)	3.33 (T_6)	3.67 (T_7)	4.00 (T_8)
6	3.00 (T_9)	3.00 (T_{10})	4.50 (T_{11})	5.67 (T_{12})
8	4.33 (T_{13})	2.83 (T_{14})	5.50 (T_{15})	6.50 (T_{16})

Control	F	SE	CD
1.00	35.76**	0.228	0.642

**Significant at 1 per cent level

Table 10f. Effect of KIN and NAA on number of days for deflasking in stem nodal segment explant

KIN (mg l^{-1}) \ NAA (mg l^{-1})	2	4	6	8
2	158.00 (T_1)	162.00 (T_2)	165.00 (T_3)	165.83 (T_4)
4	163.67 (T_5)	163.50 (T_6)	166.00 (T_7)	168.67 (T_8)
6	164.83 (T_9)	166.50 (T_{10})	178.00 (T_{11})	180.00 (T_{12})
8	172.00 (T_{13})	180.00 (T_{14})	184.00 (T_{15})	185.00 (T_{16})

Control	F	SE	CD
189.00	4233.86**	0.131	0.369

**Significant at 1 per cent level

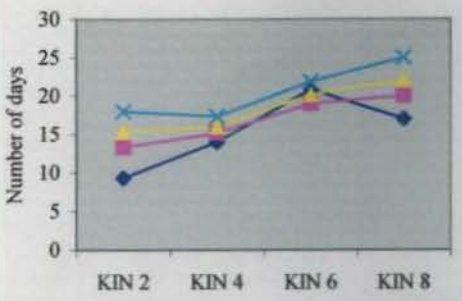


Fig. 3a Effect of KIN and NAA on number of days for initiation of PLB's in stem nodal segment explant

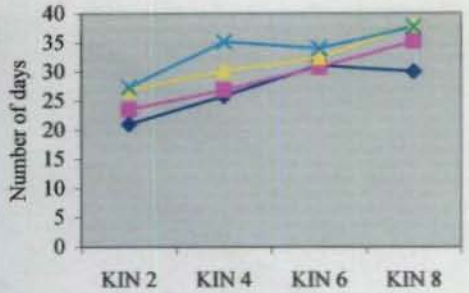


Fig. 3b Effect of KIN and NAA on number of days for greening of PLB's in stem nodal segment explant

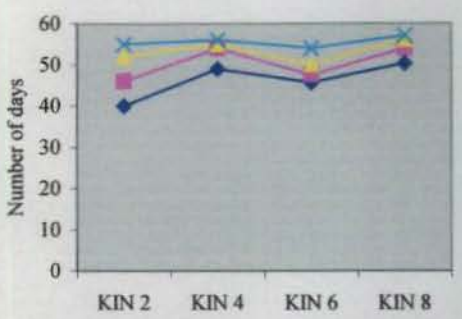


Fig. 3c Effect of KIN and NAA on number of days for initiation of first leaf in stem nodal segment explant

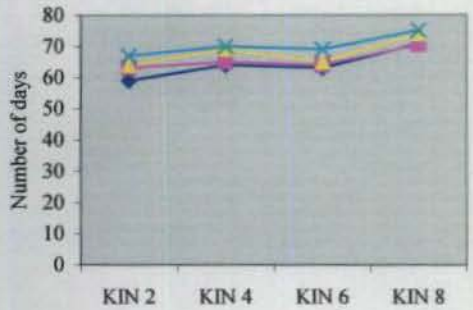


Fig. 3d Effect of KIN and NAA on number of days for initiation of shoot in stem nodal segment explant

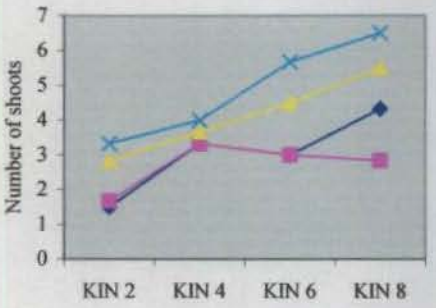


Fig. 3e Effect of KIN and NAA on number of shoots in stem nodal segment explant

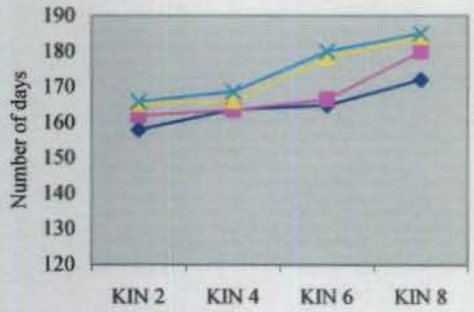


Fig. 3f Effect of KIN and NAA on number of days for deflasking in stem nodal segment explant

163 days for deflasking. The maximum duration of 189.00 days for deflasking was taken by control.

4.2.3 Effect of KIN and NAA on PLB differentiation and Plantlet Growth

Effect of KIN and NAA on PLB differentiation and plantlet growth was studied.

For initiation of PLB's, T_1 (KIN 2 mg l^{-1} + NAA 2 mg l^{-1}, applied in 1 : 1 ratio) took the minimum number of 9.33 days (Table 10a and Fig. 3a). This was significantly different from all other treatments. T_1 was followed by T_2 which took 13.33 days for PLB initiation Control took the highest number of days for PLB initiation being 32.33 days.

With respect to greening of PLB's, T_1 (KIN 2 mg l^{-1} + NAA 2 mg l^{-1} applied in 1 : 1 proportion) took the minimum number of 21.00 days (Table 10b and Fig. 3b). This was significantly different from all other treatments. T1 was followed by T2 taking 23.50 days for greening of PLB's. The highest number of days for greening of PLB's was taken by control being 44.80 days.

For initiation of first leaf, T_1 (KIN 2 mg l^{-1} + NAA 2 mg l^{-1}) recorded the lowest duration of 40.00 days followed by T9 taking 45.67 days (Table 10c and Fig. 3c). The maximum duration for initiation of first leaf was observed in control being 59.50 days. This was preceded by T_{16} taking 57.00 days.

Number of days taken for shoot initiation was the lowest in T_1 (KIN 2 mg l^{-1} + NAA 2 mg l^{-1}) recording 59.00 days (Table 10d and Fig. 3d). This was followed by T_2 & T_9 taking 63.00 days. Control took the maximum duration for initiation of shoot being 79.00 days. This was preceded by T_{16}, which took 75.00 days.

T_{16} (KIN 8 mg l^{-1} + NAA 8 mg l^{-1}) recorded the highest shoot number of 6.50 (Table 10e and Fig. 3e). In T_{16} KIN and NAA are applied in the highest concentration in 1 : 1 ratio. This was significantly different

Table 11a. Effect of KIN and IAA on number of days for initiation of PLB's in stem nodal segment explant

KIN (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	16.83 (T_1)	16.50 (T_2)	15.00 (T_3)	17.00 (T_4)
4	11.33 (T_5)	8.50 (T_6)	14.33 (T_7)	17.00 (T_8)
6	10.83 (T_9)	8.83 (T_{10})	13.50 (T_{11})	21.33 (T_{12})
8	9.17 (T_{13})	13.17 (T_{14})	11.83 (T_{15})	14.50 (T_{16})

Control	F	SE	CD
32.33	113.85**	0.254	0.705

**Significant at 1 per cent level

Table 11b. Effect of KIN and IAA on number of days for greening of PLB's in stem nodal segment explant

KIN (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	29.00 (T_1)	28.83 (T_2)	26.67 (T_3)	30.17 (T_4)
4	21.83 (T_5)	18.67 (T_6)	25.33 (T_7)	30.50 (T_8)
6	17.67 (T_9)	20.67 (T_{10})	18.50 (T_{11})	34.83 (T_{12})
8	17.50 (T_{13})	19.83 (T_{14})	25.00 (T_{15})	29.00 (T_{16})

Control	F	SE	CD
44.80	119.96**	0.333	0.924

**Significant at 1 per cent level

from all other treatments. T_{16} was followed by T_{12} (5.67 shots) and T_{15} (5.50 shoots) which were on par with each other . Shoot production was low in the lower concentrations of growth regulators being 1.50 in T_1 and 1.67 in T_2 which were on par with each other. Control recorded the lowest shoot number of 1.00.

T_1 took the lowest number of 158.00 days for deflasking which was significantly lower from all other treatments (Table 10f and Fig. 3f). This was followed by T_2 taking 162.00 days. Control took the maximum number of 189.00 days for deflasking.

4.2.4 Effect of KIN and IAA on PLB Differentiation and Plantlet Growth

Effect of KIN and IAA on PLB differentiation and plantlet growth was recorded.

This hormone group showed wide variation for the duration taken for the initiation of PLB's (Table 11a and Fig. 4a). Minimum number of 8.50 days for PLB initiation was recorded by T_6 (KIN 4 mg l^{-1} + IAA 4 mg l^{-1}) which was on par with T_{10} (KIN 6 mg l^{-1} + IAA 4 mg l^{-1}; 8.83 days) and T_{13} (KIN 8 mg l^{-1} + IAA 2 mg l^{-1}; 9.17 days). These were closely followed by T_9 (10.83 days), T_5 (11.33 days) and T_{15} (11.83 days). Control took the maximum number of 32.33 days for PLB initiation which was significantly different from all other treatments. This was preceded by T_{12} which recorded 21.33 days.

With respect to greening of PLB's, T_{13} (KIN 8 mg l^{-1} + IAA 2 mg l^{-1}, applied in 4 : 1 ratio) took the minimum duration of 17.50 days which was on par with T_9 (KIN 6 mg l^{-1} + IAA 2 mg l^{-1}) taking 17.67 days (Table 11b and Fig. 4b). These treatments were closely followed by T_{11} taking 18.50 days, T_6 taking 18.67 days and T_{14} taking 19.83 days. Maximum duration of 44.80 days for greening of PLB's was taken by control which was preceded by T_{12} taking 34.83 days.

Table 11c. Effect of KIN and IAA on number of days for initiation of first leaf in stem nodal segment explant

KIN (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	55.00 (T_1)	54.67 (T_2)	52.00 (T_3)	53.17 (T_4)
4	49.00 (T_5)	43.17 (T_6)	51.00 (T_7)	55.00 (T_8)
6	48.00 (T_9)	46.17 (T_{10})	50.00 (T_{11})	55.67 (T_{12})
8	34.83 (T_{13})	38.50 (T_{14})	44.67 (T_{15})	47.50 (T_{16})

Control	F	SE	CD
59.50	305.04**	0.195	0.542

**Significant at 1 per cent level

Table 11d. Effect of KIN and IAA on number of days for initiation of shoot in stem nodal segment explant

KIN (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	70.00 (T_1)	69.33 (T_2)	66.67 (T_3)	69.33 (T_4)
4	62.67 (T_5)	57.17 (T_6)	69.33 (T_7)	73.00 (T_8)
6	62.00 (T_9)	62.33 (T_{10})	69.50 (T_{11})	73.00 (T_{12})
8	46.16 (T_{13})	56.50 (T_{14})	58.83 (T_{15})	60.83 (T_{16})

Control	F	SE	CD
79.00	169.90**	0.33	0.915

**Significant at 1 per cent level

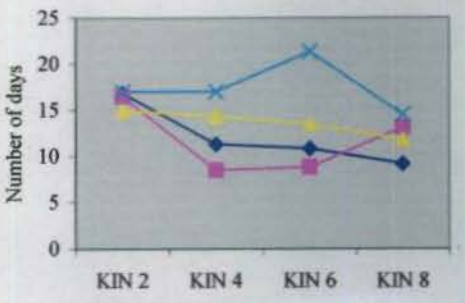


Fig. 4a Effect of KIN and IAA on number of days for initiation of PLB's in stem nodal segment explant

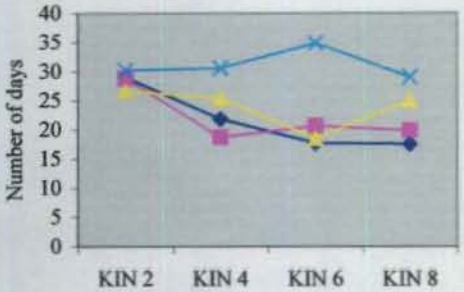


Fig. 4b Effect of KIN and IAA on number of days for greening of PLB's in stem nodal segment explant

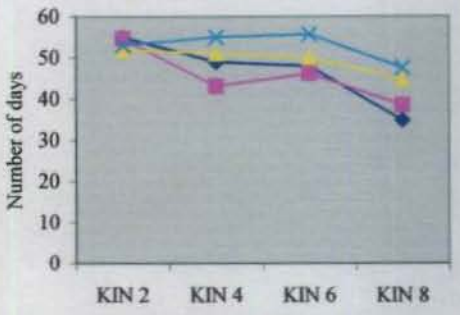


Fig. 4c Effect of KIN and IAA on number of days for initiation of first leaf in stem nodal segment explant

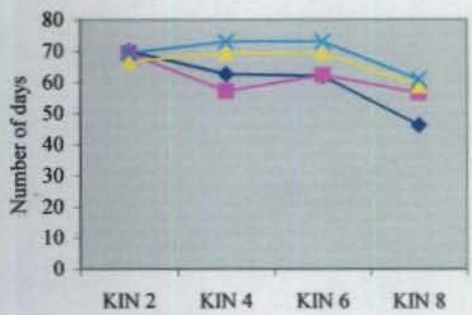


Fig. 4d Effect of KIN and IAA on number of days for initiation of shoot in stem nodal segment explant

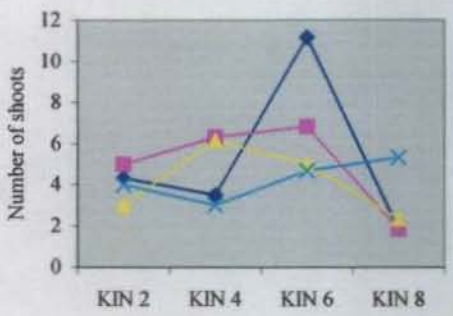


Fig. 4e Effect of KIN and IAA on number of shoots in stem nodal segment explant

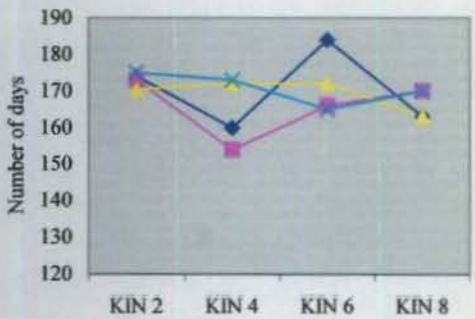


Fig. 4f Effect of KIN and IAA on number of days for deflasking in stem nodal segment explant

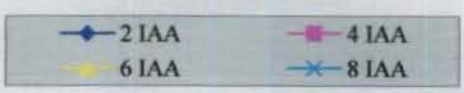


Table 11e. Effect of KIN and IAA on number of shoots in stem nodal segment explant

KIN (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	4.33 (T_1)	5.00 (T_2)	3.00 (T_3)	4.00 (T_4)
4	3.50 (T_5)	6.33 (T_6)	6.17 (T_7)	3.00 (T_8)
6	11.17 (T_9)	6.83 (T_{10})	5.00 (T_{11})	4.67 (T_{12})
8	1.83 (T_{13})	1.83 (T_{14})	2.33 (T_{15})	5.33 (T_{16})

Control	F	SE	CD
1.00	24.34**	0.443	1.229

**Significant at 1 per cent level

Table 11f. Effect of KIN and IAA on number of days for deflasking in stem nodal segment explant

KIN (mg l^{-1}) \ IAA (mg l^{-1})	2	4	6	8
2	174.00 (T_1)	173.00 (T_2)	170.33 (T_3)	175.00 (T_4)
4	160.00 (T_5)	154.00 (T_6)	172.00 (T_7)	173.00 (T_8)
6	184.00 (T_9)	166.00 (T_{10})	172.00 (T_{11})	165.00 (T_{12})
8	163.00 (T_{13})	170.00 (T_{14})	163.00 (T_{15})	170.00 (T_{16})

Control	F	SE	CD
189.00	7997.95**	0.082	0.229

**Significant at 1 per cent level

Plate 9 Multiple shoot formation during third subculture

Effect of BA 2 mg l^{-1} + NAA 2 mg l^{-1} on multiple shoot formation 96 days after inoculation

Effect of BA 2 mg l^{-1} + IAA 2 mg l^{-1} on multiple shoot formation 96 days after inoculation

Effect of BA 8 mg l^{-1} + NAA 8 mg l^{-1} on multiple shoot formation 96 days after inoculation

Effect of KIN 4 mg l^{-1} + IAA 4 mg l^{-1} on multiple shoot formation 102 days after inoculation

Effect of KIN 6 mg l^{-1} + IAA 2 mg l^{-1} on multiple shoot formation 96 days after inoculation

Effect of CW 200 ml l^{-1} on multiple shoot formation 80 days after inoculation

Plate 10. General view of rooting of microshoots

Plate 11. Effect of IBA 2 mg l^{-1} on rooting of microshoots four weeks after root initiation

Plate 12. Deflasked plantlet 150 days after inoculation









For initiation of first leaf, T_{13} (KIN 8 mg l^{-1} + IAA 2 mg l^{-1}) took the lowest number of days being 34.83 days which was significantly different from all other treatments and was followed by T_{14} taking 38.50 days (Table 11c and Fig. 4c). Control took the maximum number of 59.50 days for initiation of first leaf preceded by T_{12} (55.67 days).

Regarding number of days taken for shoot initiation, T_{13} (KIN 8 mg l^{-1} + IAA 2 mg l^{-1} applied in 4: 1 proportion) recorded the minimum duration of 46.16 days, which was significantly different from all other treatments(Table 11d and Fig. 4d). T_{13} was followed by T_{14} taking 56.50 days, T_{16} taking 57.17 days and T_{15} taking 58.83 days. Control took the highest number of 79.00 days for shoot initiation which showed significant difference from all other treatments. This was preceded by T_8 and T_{12} taking 73.00 days.

With respect to number of shoots, T_9 (KIN 6 mg l^{-1} + IAA 2 mg l^{-1}) produced the maximum number of 11.17 shoots and was significantly different from all other treatments (Table 11e and Fig. 4e). This was obtained from a 3 : 1 ratio of KIN and IAA. T_9 was followed by T_{10} with 6.83 shoots, T_6 with 6.33 shoots and T_7 with 6.17 shoots which were on par with each other. The lowest value of one shoot was produced by control and was on par with T_{14} and T_{13} producing 1.83 shoots.

Regarding number of days for deflasking, T_6 (KIN 4 mg l^{-1} + IAA 4 mg l^{-1} applied in 1 : 1 proportion) took 154.00 days and was significantly different from all other treatments (Table 11f and Fig. 4f). T_6 was followed by T_5 (160 days), T_{15} and T_{13} (163.00 days), T_{12} (165.00 days) and T_{10} (166.00 days) which were significantly different from each other. Control took the highest duration of 189 days for deflasking, which was closely preceded by T_9 (184.00 days).

Table 12 Effect of coconut water on *in vitro* PLB differentiation and development of plantlets in stem nodal segment explant of *Dendrobium* hybrids

Treatments	Number of days for initiation of PLB's	Number of days for greening of PLB's	Number of days for initiation of first leaf	Number of days for initiation of shoot	Number of shoots at subculture	Number of days for deflasking
T_1	9.00	16.50	26.50	41.00	7.50	143.33
T_2	6.50	15.67	25.00	39.67	10.50	140.00
T_3	14.50	28.83	39.50	51.50	5.83	150.00
Control	8.50	18.67	43.17	57.17	6.33	154.00
F	502.50**	345.15**	1907.50**	787.67**	64.53**	437.48**
SE	0.258	0.355	0.258	0.327	0.416	0.342
CD	0.550	0.756	0.550	0.696	0.887	0 728

Basal medium:

VW + KIN 4 mg l^{-1} + IAA 4 mg l^{-1} + sucrose 30.0 g l^{-1} + agar 6.0 g l^{-1}

4.2.5 Effect of CW on PLB Differentiation and Plant Growth

Effect of four different levels (0, 10, 20 and 30 per cent) of fresh coconut water on PLB differentiation and plantlet development of stem nodal segment was studied (Table 12).

The treatment, T_2 (CW 20 per cent) recorded the minimum of 6.50 days for initiation of PLB's which was significantly different from all the other three levels. The maximum duration of 14.50 days for PLB initiation was taken by T_3 (CW 30 per cent) which was significantly different from all other treatments. Control took 8.50 days for PLB initiation and was on par with T_1 (CW 10 per cent) taking 9.00 days.

The treatment, T_2 (CW 20 per cent) took the least number of 15.67 days for greening of PLB's which was followed by T_1 (CW 10 per cent) taking 16.50 days. Maximum number of 28.83 days was taken by T_3 (CW 30 per cent). Control recorded 18.67 days for greening of PLB's.

The treatment, T_2 (CW 20 per cent) took the lowest number of 25.00 days for initiation of first leaf which was followed by T_1 (CW 10 per cent) recording 26.50 days. Maximum number of days for initiation of first leaf was recorded by control (43.17 days) and was preceded by T_3 (CW 30 per cent) taking 39.50 days.

Number of days for initiation of shoot differed significantly between the treatments. T_2 (CW 20 per cent) recorded the minimum number of 39.67 days for initiation of shoot which was significantly different from all other treatments. Control took the maximum number of 57.17 days for shoot initiation. This was preceded by T_3 (CW 30 per cent) taking 51.50 days.

With respect to number of shoots, T_2 (CW 20 per cent) recorded the maximum number of 10.50 shoots which was significantly different from all the other three levels (Plate 9). T_2 was followed by T_1 (CW 10 per cent) recording 7.50 shoots. The minimum of 5.83 shoots was recorded

with T_3 (CW 30 per cent) which was on par with control recording 6.33 shoot.

For number of days for deflasking, T_2 (CW 20 per cent) took the minimum duration of 140.00 days which was significantly different from all the other three levels. T_2 was followed by T_1 (CW 10 per cent) taking 143.33 days. Maximum number of 154.00 days for deflasking, was taken by control which was preceded by T_3 (CW 30 per cent) taking 150 days.

4.3 STANDARDISATION OF TREATMENTS *IN VITRO* ROOTING OF MICROSHOOTS

The effect of different levels of IBA on *in vitro* root development in medium with (0.5 g l^{-1}) activated charcoal and without charcoal was studied. No significant difference was observed between treatments with and without activated charcoal. Hence different levels of IBA without activated charcoal alone were considered.

4.3.1 Number of Days taken for Rooting of Microshoots

Wide variation was noticed between treatments in the case of number of days taken for root initiation (Table 13and Fig. 5) Significantly early rooting was observed in T_4 (IBA 2 mg l^{-1}) taking 29.83 days followed by T_3 (IBA 1.5 mg l^{-1}) taking 33.67 days. The maximum number of 56.33 days for rooting was taken by control. All the different levels of IBA were found to be significantly superior to the control.

4.3.2 Number of Roots per Microshoot

With respect to the number of roots per microshoot, (Table 14 and Fig. 6), the highest number of 9.17 roots was recorded by T_4 (IBA 2 mg l^{-1}) which was on par with the number of roots recorded in T_3 (IBA 1.5 mg l^{-1}) being 9.00 roots. The minimum number of 1.33 roots was recorded by control which was on par with T_1 (IBA 0.5 mg l^{-1}) producing 1.50 roots.

Table 13 Effect of different levels of IBA and activated charcoal on initiation of roots in microshoots of *Dendrobium* hybrids

Treatment	Number of days for initiation of root	
	Without activated charcoal	With activated charcoal (0.5 g l^{-1})
T_1	40.08	39.67
T_2	38.67	37.67
T_3	33.67	34.67
T_4	29.83	30.92
Control	56.33	54.00
Mean	39.84	39.17
CD	1.079	0.683

Medium : Half MS + sucrose 30 g l^{-1} + agar 6.0 g l^{-1} + CW 200 ml l^{-1}

Table 14 Effect of different levels of IBA and activated charcoal on number of roots in micro shoot of *Dendrobium* hybrids

Treatment	Number of roots per microshoot	
	Without activated charcoal	With activated charcoal (0.5 g l^{-1})
T_1	1.50	1.50
T_2	6.17	4.83
T_3	9.00	8.33
T_4	9.17	9.50
Control	1.33	1.33
Mean	5.43	5.10
CD	0.477	0.753

Medium : Half MS + sucrose 30 g l^{-1} + agar 6.0 g l^{-1} + CW 200 ml l^{-1}

Table 15 Effect of different levels of IBA and activated charcoal on length of roots in microshoots of *Dendrobium* hybrids

Treatment	Root length (cm)	
	Without activated charcoal	With activated charcoal (0.5 g l^{-1})
T_1	1.17	1.17
T_2	1.33	1.67
T_3	1.83	1.83
T_4	2.67	3.00
Control	0.88	0.60
Mean	1.58	1.65
CD	0.3657	0.564

Medium : Half MS + sucrose 30 g l^{-1} + agar 6.0 g l^{-1} + CW 200 ml l^{-1}

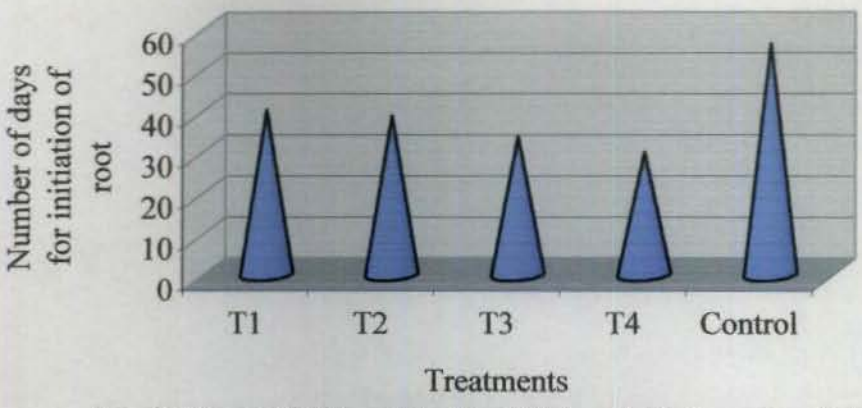


Fig. 5. Effect of different levels of IBA on initiation of roots in microshoots

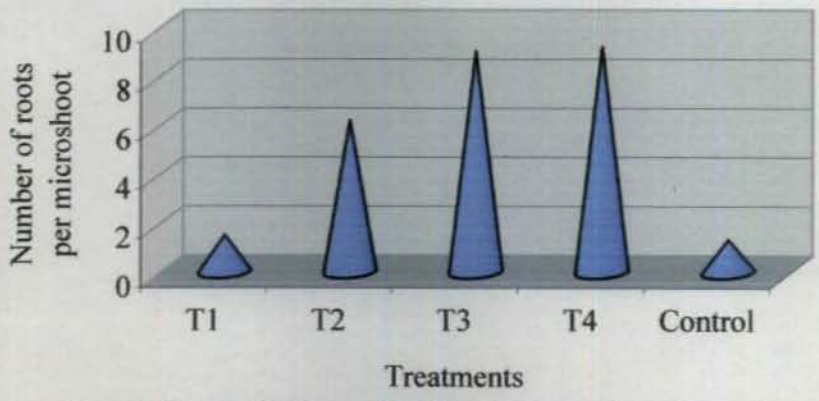


Fig. 6. Effect of different levels of IBA on of number of roots in micro shoot

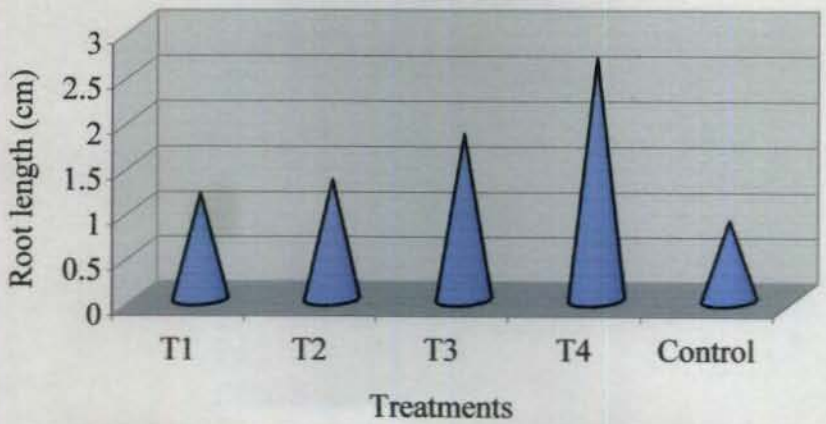


Fig. 7. Effect of different levels of IBA on length of roots in microshoots

4.3.3 Length of Roots

With respect to the length of roots (Table 15 and Fig. 7) T_4 (IBA 2.0 mg l^{-1}) recorded the maximum length of 2.67 cm which was significantly superior to all other treatments. The minimum number of 0.88 cm was recorded by control and was on par with T_1 (1.17 roots).

DISCUSSION

5. DISCUSSION

Micropropagation is an elite tool of biotechnology, which has revolutionized the commercial nursery industry, especially in the case of flower crops. Cut flowers are commercially propagated through tissue culture in recent years. Orchid is one among the most important cut flowers which has to be essentially multiplied on a large scale through tissue and seed culture. The high cost of orchid hybrids as potted plants and cut flowers justifies the high cost of micropropagation. The conventional propagation of orchids including dendrobiums is a slow process and the multiplication rate is also poor. At the same time the rapid propagation combined with a high multiplication rate of the promising *Dendrobium* from the DBT project becomes vital due to their commercial value and the urgent need for locally adapted indigenous hybrids. Therefore the present study was undertaken to standardise the protocol for the rapid micropropagation of these *Dendrobium* hybrids developed in the DBT project "Breeding for commercial orchid hybrids" in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani.

The results of the study are discussed below.

In the present study the experiments were subdivided into three phases (i) Standardisation of explants and basal media (ii) Standardisation of plant growth substances and organic additive (iii) I*n vitro* rooting of microshoots.

Explants and Media

Culture of shoot tip is a well established technique for orchid propagation since it is useful for maintaining uniformity of genotype in the progeny. This technique, however requires the sacrifice of the entire new growth or the only growing point. In the present study, the *Dendrobium* hybrids employed for micropropagation were single, superior

plants selected from the hybrid population based on novelty, distinctiveness and uniformity in floral characters. Hence shoot tip culture sacrificing the entire plant was ruled out and alternative explants were used from among the less vital plant parts. Culture of other parts of the plant like leaf, inflorescence stalks, nodal sections, roots etc. is fast emerging as a method for cloning elite orchids (Devi and Deka, 2001).

There are many reports on micropropagation of orchid species through stem nodal segments (Sagawa, 1961; Vij *et al.*, 1994; Duan *et al.*, 1996). Genera like *Dendrobium*, *Epidendrum* and *Phalaenopsis* have successfully been cultured through stem nodal segments. When the stem segments with node are used to initiate culture, the dormant buds swell and enlarge followed by leaf and root development (Devi and Deka, 2001).In the present study, the stem nodal segment with dormant buds cultured *in vitro* showed PLB formation and development of plantlet. Such plantlets are ideal for clonal propagation since the propgules are true to type. Similar results were obtained in *Dendrobium* by Mosich *et al.* (1974).

The inflorescence stalk segments with dormant buds can also be induced to form plantlets. Flower stalk sections were used by many workers for the *in vitro* propagation of orchids (Griesbach, 1983; Sagawa and Sehgal, 1967; Tanaka *et al.*, 1988). The use of flower stalks having well developed buds was reported to be economical and safe for the propagation of *Dendrobium* (Singh and Sagawa, 1972). Goh and Wong (1990) reported that developing inflorescence, even at the later phase of exponential growth, could be used as explants for the clonal propagation in monopodial orchids. They showed that as long as the inflorescence tips are still highly meristematic and with actively differentiating floral buds, they can be used as suitable explants. Excision of these developing inflorescence is simple and will in no way damage the mother plant. Intuwong and Sagawa (1973) showed that only young inflorescence buds

of *Ascofinetia* responded well. Nuraini and Shaib (1992) also reported that the percentage of shoot formation was higher with younger scapes compared to scapes from inflorescences in full bloom or scapes with intact flower bud. Full bloom inflorescence explants gave poor shoot formation.

According to Koch (1974) lateral buds on flower stalks cultured *in vitro* showed three growth patterns: they remained dormant or grew to form vegetative shoots or grew to form secondary flower stalks. In the present study, most of the flower stalk segments remained dormant in all the basal media, some swelled to form buds but later dried and some developed as secondary flower stalks (Plate 4). No vegetative shoots were produced from flower stalks during the trial period of six weeks in the media tried. Further trials with explants at different growth stages in different basal media changing the growth regulator concentrations may prove successful.

Investigations carried out by several workers indicated that the culture of leaf tip or whole leaf is a promising possibility without sacrificing the important portion of a plant, as done in the case of meristem culture. Tanaka *et al.* (1975) reported formation of PLB's in *Phalaenopsis* and *Vanda* by using leaf segments and young leaf. Lay (1978) reported that leaf tips of *Aranda*, *Cattleya*, *Dendrobium* and *Ascocenda* did not survive *in vitro* but leaf parts including leaf base proliferated and formed plantlets depending on cultivar. In the present study, there was no response of PLB formation from the leaf segment in the different media tried. They remained green in the culture medium throughout the trial period of six weeks and there was no organogenesis. Probably the catabolic enzymes in the leaf segments would have destroyed the cytokinin and auxin supplemented in the medium thereby arresting organogenesis. Similar results were reported by Kukulczanka and Wojciechowska (1983) in *Dendrobium* sp. and Kukulczanka (1980) in *Brassocattleya*.Teng *et al.* (1997) also observed poor response in the leaf

explants. However, better shoot induction from leaf explants of other orchid genera was reported by workers like Pindel and Miczynski (1996) in *Cymbidium* and Paek *et al.* (1996) in *Oncidium* cv. Gower Ramsey. Ramsundar *et al.* (2000) reported poor formation of PLB's and shoots from leaf tips in *Dendrobium* Sonia. In the present study, the poor response of leaf segments may be due to the fact that the medium and growth regulator combination tried may not have provided any positive signal for organogenesis from the leaf explants during the six weeks trial period. Detailed trials with leaf segment explant changing the growth regulators, their combinations and positioning of explant in the medium may yield response.

Among the different media tested in the present investigation stem nodal segment responded well in VW medium closely followed by half strength MS medium. MS and VW media were reported to be ideal for better establishment of certain species of orchids *in vitro* by many workers (Goh and Wong, 1990; Bagde and Sharon, 1997; Chen *et al.*, 1999b). Lakshmidevi (1992) observed that for multiple shoot formation in *Dendrobium nobile* VW medium was better than MS medium. This is in conformity with the finding of the present investigation. Best production of multiple shoots in six *Dendrobium* hybrids was observed in VW medium (Devi and Laishran, 1998). Park *et al.* (1998) observed the highest ratio of PLB multiplication in VW medium. A number of media have been reported to be ideal for the *in vitro* culture of different orchids. Vacin and Went medium (1949) was suggested to be the best for culturing different species of *Dendrobium* by many investigators (Kim *et al.*, 1970; Singh and Sagawa, 1972). Fernando (1979) also reported that the best medium for clonal propagation of *Dendrobium* Caesar Red lip was modified VW. In general, VW medium was found to have a better influence on the culture establishment in different orchid genera and is the best for *Dendrobium fimbriatum* and *D. moschatum* (Lakshmidevi, 1992). The success in the use of VW medium indicated that the requirement of

mineral elements for most of the orchids are relatively simple compared to those for higher plants.

Plant Growth Substances

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between substances produced endogenously by cultured cells and those supplied exogenously in the medium. The effect of growth regulators on tissue or organ culture may vary according to the particular compound used, the type of culture and the variety of plants from which it was derived. Cytokinins alone cannot favour better shoot production so auxins are to be added in the medium. Many aspects of cellular differentiation and organogenesis in tissue and organ cultures have been found to be controlled by 'an interaction between the concentrations of cytokinin and auxin (George and Sherrington, 1984). A balance between auxin and cytokinin is required for shoot production in orchids. The requisite concentration of each type of regulant differs greatly according to the kind of plant being cultured, the culture conditions and the compounds used.

In the present study, a comparison of treatment combinations under each group of growth regulators at different concentrations was worked out. It was observed that both the cytokinins tried (BA and KIN) generally differentiated PLBs and developed plantlets faster in lower concentration (2.0 mg l^{-1}) from stem nodal segment explant in *Dendrobium* hybrids. This is in agreement with the results reported by several workers. The shoot growth enhancing effect of BA at lower concentrations was reported by Fonnesbech (1972). Kim and Kako (1982) found that the addition of BA encouraged rapid PLB formation and shoot development. Enriching the *Dendrobium* culture medium with BA resulted in the greatest number of shoots (Kukulczanka and Wojciechowska, 1983). Paek *et al.* (1990) observed faster shoot production in *Cymbidium* in the presence of BA. PLB differentiation and

shoot formation in *Aranda* Deborah were better when the medium was supplemented with a low concentration of cytokinins, viz, KIN at 1.0 mg l^{-1} or a combination of KIN and BA at 1.0 mg l^{-1} each (Goh and Wong, 1990). Vij and Dhiman (1997) reported that medium supplemented with BA at 2.0 mg l^{-1} favoured rapid development of multiple shoot buds. In the present study, both the auxins tried (NAA and IAA) resulted in rapid PLB differentiation and plantlet development *in vitro* at low level (2.0 mg l^{-1}). This result is in conformity with the reports of many workers. Devi and Deka (1992) observed that *in vitro* plantlet growth in *Dendrobium* was enhanced by IAA and NAA at 1.0 mg l^{-1} each. Significant and rapid increase in PLB production in *Dendrobium* was reported by Kanjilal *et al.* (1999) in presence of IAA 2.0 mg l^{-1}.

Much stress has been given to the ratio of auxin to cytokinin rather than the absolute concentration for *in vitro* culture development. Skoog and Miller (1957) concluded that the ratio of auxin to cytokinin is critical for morphogenesis. Hadley and Harvais (1968) emphasized the importance of the ratio rather than the absolute concentration of auxin to cytokinin in maintaining proper shoot : root balance in orchids. In the present study from among the combinations of BA and NAA tried at different levels, both the growth regulators at 2.0 mg l^{-1} or in a 1:1 ratio was found to be the best for rapid *in vitro* plantlet development. The reports of Lakshmidevi (1992) that *Dendrobium* cultures showed rapid shoot growth in the presence of BA 3.0 mg l^{-1} and NAA 2.0 mg l^{-1}, a nearly 1:1 ratio supports this finding. Tom and Weatherhead (1991) reported that BA and NAA each at 1.0 mg l^{-1} (1:1 ratio) was the best for *in vitro* cultures of *Pholidota*. These reports are in support of the present findings. The growth promoting effects of a combination of BA and NAA on *in vitro* cultures of *Dendrobium* has been reported by Lakshmidevi (1992), Sounderrajan and Lokeshwari (1994), Pathania *et al.* (1998) and Sobhana and Rajeevan (2002). The beneficial effects of BA in combination with NAA on *in vitro* cultures of orchids have been reported by Kim *et al.*

(1979), Choi *et al.* (1989), Vij and Pathak (1989), Ichihashi (1992), Tokuhara and Mii (1993), Pindel and Miczynski (1996), Teng *et al.* (1997), Santana and Chapparro (1999), Buzarbarua (1999) and Prasad and Verma (2001). The effect of combinations of BA and IAA as well as KIN and NAA at different levels on rapid *in vitro* plantlet development observed in the present experiment revealed that a combination of 2.0 mg l^{-1} of cytokinin and auxin was the best in both cases.The effect of combination of KIN and IAA at different levels showed that KIN 4.0 mg l^{-1} along with IAA 4.0 mg l^{-1} was the best for rapid *in vitro* development. These are in conformity with the findings of Tom and Weatherhead (1991), Pindel and Miczynski (1996) and Devi and Laishran (1998). A 1:1 ratio of cytokinin: auxin was the best for the rapid development of *in vitro* cultures of orchids. The same result was reported by Sobhana and Rajeevan (2002) in *Dendrobium*.

In the present study, the comparison of treatment combinations on shoot production showed that higher doses of auxins and cytokinins generally in a 1:1 ratio resulted in more number of shoots per plant. It was further observed that shoots produced on lower levels of cytokinins (BA 2, 4 and KIN 2, 4 mg l^{-1}) had healthy appearance with well developed and expanded leaves. With increase in cytokinin concentration from 2.0 mg l^{-1} to 8.0 mg l^{-1} the shoot number increased, but the shoots were shorter with small sized leaves (Plate 9). The reports of Murashige (1974) that the application of BA at higher levels had a deleterious effect on shoot growth supports this observation. Lakshmidevi (1992) also reported the same in *Dendrobium*, stating that BA at 3.0 mg l^{-1} resulted in considerable vigour and rapid shoot growth with well expanded leaves, while BA at higher concentrations produced short shoots and small leaves, although the shoot production is increased. Higher levels of BA and NAA in 1:1 ratio was found suitable for plantlet development in *Cymbidium* leaf cultures as reported by Pindel and Miczynski (1996). Sobhana and Rajeevan (2002) observed that for improving *in vitro* plantlet characters in

Dendrobium BA or KIN at 4.0 mg l^{-1} combined with NAA or IBA at 4.0 mg l^{-1} was the best. The plantlet produced by KIN and IAA were longer, more healthy and vigourous compared to the other groups in the present investigation (Plate 9). In the present study a combinations of KIN 6.0 mg l^{-1} and IAA 2.0 mg l^{-1} in a 3:1 ratio produced the highest number of shoots (11.17) This is in accordance with the findings of Sudeep (1994) that a 3:1 ratio of cytokinin to auxin was the best for rapid bud initiation and shoot proliferation *in vitro*.

Plant extracts such as coconut milk of green or mature nuts has been widely used in orchid tissue culture and has been found to be very effective in providing an undefined mixture of organic nutrients and growth factors. The stimulatory effect of CW might be due to its growth regulator contents. Cytokinins were assumed to be the most important growth regulators present in coconut water (Letham, 1974).

In the present study, the initiation of PLBs was earlier with the addition of CW in the medium, compared to cultures without CW. This is in conformity with the findings of several workers who have pointed out the beneficial effects of CW in *in vitro* orchid cultures. According to Intuwong and Sagawa (1973) the liquid endosperm of coconut induces cell division in otherwise non-dividing cells and promotes morphogenesis in orchids. The early response on addition of CW in the present experiment may be attributed to this induction of cell division. Morel (1974) has enumerated the beneficial effects of CW in bringing about rapid protocorm multiplication of orchids. Maximum PLB proliferation and the highest increase in fresh weight of PLB's was attained with the addition of CW to the medium (Kusumoto, 1980).

Badge and Sharon (1997) observed the best PLB formation and further multiplication when the orchid culture medium was supplemented with CW 15 per cent. Goh and Wong (1990) observed enhanced PLB formation when the basal orchid medium was supplemented with CW.

The present investigation revealed that the time taken for shoot formulation decreased as the concentration of CW was increased upto 20 per cent. The number of shoots was also higher (10.50 vs 6.33 in control) in cultures with 20 per cent CW which was identified as the optimum concentration of CW in the medium. Karim *et al.* (1992) reported that plantlets were produced readily when CW 20 per cent was added to the basal orchid medium. They obtained PLB formation in shoot tip explant of *Renantanda* when cultured on medium supplemented with CW 20 per cent. Rani (2002) also reported that 20 per cent CW is the optimum for early morphogenesis, plantlet differentiation and development of vigourous plantlets in *in vitro* cultures of *Dendrobium* hybrids. The present study recorded a decrease in the number of shoots produced and a decrease in plantlet size and vigour in *Dendrobium* cultures when the concentration of CW was increased beyond 20 per cent.

The positive effect of CW on initial culture establishment and further plantlet development in *Dendrobium* meristem cultures have been emphasized by the present experiments. Several workers have reported similar beneficial effects of CW on *Dendrobium in vitro* cultures. Buds excised from bulbs and leaf axils of *Dendrobium* produced PLB's in 4-5 weeks and plantlets in eight weeks on CW enriched medium (Kim *et al.*, 1970). Nodal explants of *Dendrobium* differentiated more number of plantlets within a short period on culture medium supplemented with CW (Nair. 1982). Soediono (1983) has pointed out the fact that for initial culture of *Dendrobium*. supplementation of media with CW is suitable. Shoot meristem cultures of *Dendrobium* gave positive response on supplementation of culture medium with CW (Sharon and Vasundhara, 1990).

Devi and Deka (1992) observed the stimulatory effect of CW on the growth of shoots in cultures of *Dendrobium farmerii* and *D. primulinum* Sharon *et al.* (1992) observed the enhanced morphogenesis on addition of coconut water in *Dendrobium* Snow fire *in vitro* cultures. Addition of CW

significantly influenced the development of protocorm into plantlet in terms of early formation of leaf and more number of leaves in *Dendrobium* Sonia (Ramsundar *et al.*, 2000). Rani (2002) has reported similar growth enhancement following addition of CW to the medium in *Dendrobium* hybrid cultures. Coconut water is reported to contain a number of factors responsible for cell division including diphenyl urea (Shantz and Steward, 1952) and structures analogous with kinetin and 9-B-D-Ribofunanzol zeatin (Letham, 1974). Standen and Drewes (1971) also reported that CW contains zeatin and a compound, which co-chromatographs with zeatin riboside. Arditti (1979) made a similar conclusion pointing out the beneficial effects of CW. From the present investigations and previous works on similar lines, it may be concluded that no single ingredient as such stimulated PLB development or plant growth, but it was rather an interaction of the additives, components of media and the genotypes of explants.

***In vitro* Rooting of Microshoots**

The *in vitro* rooting of microshoots is done in preparation to withstand the shock on transplanting out of the aseptic protected environment of the test tube to the outdoor conditions of the greenhouse. Successful *in vitro* rooting of microshoots in orchids was reported by many workers (Kukulczanka and Wojciechowska, 1983; Nuraini and Shaib, 1992; Lim *et al.*, 1993; Mujib and Jana, 1994; Fang *et al.*, 1999).

In the present study, rooting was inhibited when the microshoots were grown on culture medium supplemented with auxins and cytokinins. This is not unusual, because root formation is frequently inhibited by the cytokinins that are used in the medium to induce shoot multiplications, so that shoots do not form roots *in vitro* until they are cultured on medium containing auxin alone (George and Sherrington, 1984). Accordingly the microshoots in the present experiment were transferred onto a medium with auxin alone and successful rooting resulted.

It is a general observation that *in vitro* culture medium having a high concentration of salts does not promote rooting. In the present experiment also the *in vitro* root induction in the microshoots was negligible in the basal full strength VW medium. For root induction the common practice is the transfer of shoots from high strength medium to a less concentrated one. Hence the microshoots were transferred to half strength MS medium supplemented with the auxin IBA and successful rooting followed. This is in conformity with the findings of Lakshmidevi (1992). She reported that half strength MS with the auxin IBA was the best with respect to root initiation within the shortest time in maximum number of cultures in *Dendrobium*. The transfer of shoots to half MS medium might have reduced vitrescene and enhanced the root promoting effect of auxins. She also observed very low per cent of *Dendrobium* cultures showing root initiation in VW medium.

In the present experiment the effect of auxin on root induction in combination with AC (0.5 g l^{-1}) was studied. Such a study was promoted by the reports of Gangaprasad *et al.* (2000) that rooting of plantlets of *Anoectochilus* was achieved by adding AC to the medium. The beneficial effects of AC on protocorm multiplication (Chen *et al.*, 1999b) and formation of healthy plantlets in *Cymbidium* (Villalobes *et al.*, 1994; Paek and Kozai, 1998) have also been reported. In the present study, no significant difference was observed between treatments without and with 0.5 g l^{-1} AC. Hence their mean was computed and employed for comparing the different levels (0.5, 1.0, 1,5 and 2.0 mg l^{-1}) of IBA.

In the present experiment, rooting was initiated by the addition of IBA in the medium. The beneficial effects of IBA on the induction of *in vitro* rooting has been pointed out by several workers (Kukulczanka and Wojciechowska, 1983; Nuraini and Shaib, 1992; Lakshmidevi, 1992; Lim *et al.*, 1993; Siddique and Paswan, 1998; Indhumathi, 2002). The particular effect of IBA on initiating rooting in *Dendrobium in vitro*

cultures has been reported by several investigators. Kukulczanka and Wojciechowska (1983) observed more number of roots in *in vitro* cultures of *D. antennathum* and *D. phalaenopsis* on addition of IBA. Nuraini and Shaib (1992) reported that the addition of IBA in the medium was necessary for root initiation in *Dendrobium* Miss Hawaii cultures.

The above discussions point out that the best *in vitro* culture medium for the rapid micropropagation of the selected *Dendrobium* hybrids is VW with KIN 4.0 mg l^{-1} + IAA 4.0 mg l^{-1} + CW 200 ml l^{-1}, using stem nodal segment explant. Findings of the present study will be highly useful for cloning the promising *Dendrobium* hybrids.

SUMMARY

6. SUMMARY

Attempts were made in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2002-2004 to develop a protocol for the rapid micropropagation of *Dendrobium* hybrids.

The results of the study are summarized below.

* Out of the different explants tried, stem nodal segment responded *in vitro*, showing early PLB development. Leaf segments and inflorescence axis did not respond even after 45 days of inoculation.

* Among the various basal media tried with stem nodal segment explants, VW medium showed early culture establishment and rapid growth, recording minimum number of days for PLB initiation, greening of PLB's and for first leaf initiation.

* Trial with different levels of BA and NAA combination showed that BA 2.0 mg l^{-1} + NAA 2.0 mg l^{-1} took the minimum number of days for plantlet development. Maximum number of 8.33 shoots was recorded by BA 8.0 mg l^{-1} + NAA 8.0 mg l^{-1}.

* Among the different levels of BA and IAA combination tried, BA 2.0 mg l^{-1} + IAA 4.0 mg l^{-1} took the lowest number of days for plantlet development. In this group, BA 8.0 mg l^{-1} and IAA 8.0 mg l^{-1} produced the maximum number of 8.33 shoots.

* Combination of KIN and NAA at different levels showed that the lowest number of days for plantlet development was observed in KIN 2.0 mg l^{-1} + NAA 2.0 mg l^{-1} and maximum shoot production of 6.50 was in KIN 8.0 mg l^{-1} + NAA 8.0 mg l^{-1}.

* Combination of KIN and IAA at different levels showed that minimum number of days for plantlet development was in KIN 4.0 mg l^{-1} + IAA

4.0 mg l^{-1}, but the highest number of 11.17 shoots was produced by KIN 6.0 mg l^{-1} + IAA 2.0 mg l^{-1}.

* Among the different groups of plant growth substances tried, KIN + IAA was found to be the best, producing more number of healthy and vigorous shoots compared to the other groups. In this group KIN 4.0 mg l^{-1} + IAA 4.0 mg l^{-1} produced more vigorous shoots and healthier leaves. Hence it was selected for standardising the different levels of CW.

* Among the different levels of CW tried CW 200 ml l^{-1} produced plantlets very early, and the number of shoots produced was also the highest, being 10.5 shoots.

* *In vitro* rooting of micro shoots of *Dendrobium* hybrids was best in half MS medium. Addition of AC did not evoke any significant effect.

* Among the different levels of IBA tried for *in vitro* rooting of microshoots, IBA 2.0 mg l^{-1} took the least number of 29.92 days for initiation of 9.33 roots and produced the highest number of roots with highest mean length of 2.83 cm.

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*Original not seen

MICROPROPAGATION OF *DENDROBIUM* HYBRIDS

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In the combination of KIN and NAA tried at different levels earlier plantlet development was observed in 2.0 mg l^{-1} each of the auxin and the cytokinin and the number of shoot produced was highest in 8.0 mg l^{-1} each of KIN and NAA. Among the different levels of KIN and IAA combination tried, 4.0 mg l^{-1} each of KIN and IAA was found to be the best for rapid plantlet development. Number of shoots produced was highest in the combination KIN 6.0 mg l^{-1} and IAA 2.0 mg l^{-1}.

Out of the three different levels of CW tried, CW 200 ml l^{-1} was observed to be the best in terms of early plantlet development and the production of more number of shoots. *In vitro* rooting of microshoots was the best in half strength MS medium supplemented with IBA 2.0 mg l^{-1}. Addition of AC did not produce any significant effect on root development.

ABSTRACT

Standardisation of protocol for the rapid *in vitro* propagation of *Dendrobium* hybrids was attempted. The studies were carried out in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2002-2004.

Different explants such as immature leaf segment, stem nodal segment and inflorescence axis were used. The effect of different basal media such as MS (full, half and quarter strength), VW (full strength) and KC (full strength) on these explants was studied.

The effect of different levels of plant growth substances such as IAA, NAA, Kinetin and BA and different levels of CW on PLB development and plantlet growth was studied. Plant growth substance IBA along with AC was tried for *in vitro* rooting of microshoots.

Among the various explants tried, stem nodal segment responded well showing early PLB differentiation. Since leaf segment and inflorescence axis did not respond even after 45 days of inoculation, they were abandoned and stem nodal segment explant alone was carried forward for further trials. Out of the different basal media tried with stem nodal segment explant, VW medium exhibited early culture establishment and rapid PLB development.

Different levels of the hormone combination, BA and NAA tried showed that BA 2.0 mg l^{-1} + NAA 2.0 mg l^{-1} responded with the earliest plantlet development, but number of shoots produced was more in BA 8.0 mg l^{-1} + NAA 8.0 mg l^{-1}. Combination of BA and IAA at different levels showed that BA 2.0 mg l^{-1} + IAA 4.0 mg l^{-1} produced plantlets in a minimum number of days. Number of shoots was maximum in BA and IAA each at 8.0 mg l^{-1}.