STANDARDISATION OF EXPLANT FOR *in vitro* PROPAGATION IN *Dendrobium spp.*

By

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THESIS

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requirement for the degree of

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DECLARATION

I hereby declare that this thesis entitled "**Standardisation of explant for in vitro propagation in Dendrobium spp.**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Certified that this thesis entitled "Standardisation of explant for in vitro propagation in Dendrobium spp." is a record of research work done independently by Miss.S. Lakshmi Devi 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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We, the undersigned members of the Advisory Committee of Miss.S. Lakshmi Devi, a candidate for the degree of Master of Science in Horticulture, agree that the thesis entitled "Standardisation of explant for in vitro propagation in Dendrobium spp." may be submitted by Miss.S. Lakshmi Devi in partial fulfilment of the requirements for the degree.

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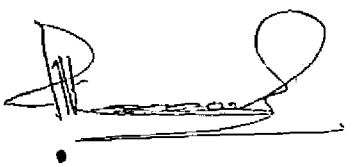

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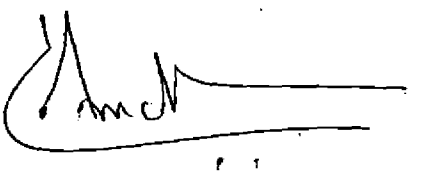

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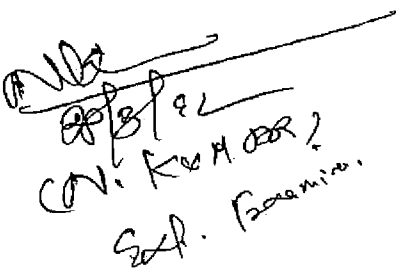

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S. LAKSHMI DEVI

ABBREVIATIONS

AC	activated charcoal
BA	benzyladenine
BAP	benzylamino purine
cv	cultivar
CW	coconut water
2,4-D	2,4-dichlorophenoxyacetic acid
Fe EDTA	ferric salt of ethylenediaminetetra acetic acid
GA/GA_3	gibberellic acid
IAA	indole - 3 - acetic acid
IBA	indole - 3 - butyric acid
KC	Knudson's C (1946) medium
KIN	kinetin; N^6-furfuryladenine
MS	Murashige and Skoog's (1962) medium
NAA	α - naphthalene acetic acid
ppm	parts per million; mg/l
VW	Vacin and Went's (1949) medium
v/v	volume in volume
w/v	weight in volume

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

To my parents

C O N T E N T S

Introduction

INTRODUCTION

Orchids belong to the family Orchidaceae which is considered as the largest family of flowering plants, comprising about 600-800 genera and 25,000 - 35,000 species. They exhibit an incredible range of diversity in size, shape and colour of their flowers and occupy prime position among all the flowering plants. They are known for their longer lasting and bewitchingly beautiful flowers which fetch a very high price in the international market.

Majority of the cultivated orchids are native of tropical countries and occur in their greatest diversity in humid tropical forests of South and Central America, Mexico, India, Ceylon, Burma, South China, Thailand, Malayasia, Phillipines, New Guinea and Australia. From India about 1300 species have been reported (Maheshwari, 1980), scattered all over N.E. Himalayas (600 species), N.W. Himalayas (300 species), Maharashtra (130 species), Andaman and Nicobar islands (70 species) and Western Ghats (200 species).

In spite of being very rich in orchid wealth, the orchid industry in India is still in its infancy. The tropical orchid production in Thailand was 83.0 per cent during 1987-88, while India, though endowed with a varied agro-climatic conditions, rich manpower and technological advancement, has made only a marginal progress in exploiting the potential international market.

Kerala is one of the leading States in the cultivation of orchids in India. The climatic conditions of Kerala enable the cultivation of some important tropical orchids like Arachnis, Dendrobium, Cymbidium, Cattleya, Phalaenopsis and Vanda. One of the largest and most diverse genera of orchids, with nearly a thousand different species, Dendrobium is the most challenging to the beginner. It is also one of the important genera widely exploited in orchid cut flower industry. Though new crosses and clones are introduced annually, there is a considerable demand for them.

The length of time taken for clonal propagation by the conventional method of back-bulb culture has been a serious drawback in the orchid industry. Therefore, there must be a rapid clonal propagation technique to meet the market demands and to optimise the income for the growers. The technique of meristem culture makes it possible to produce quality plants in large quantities by clonal multiplication, establishment of hybrid plants and improvement of orchid trade.

Success in clonal propagation of Dendrobium through meristem culture has been reported by Morel (1965a, 1965b). Though in vitro propagation technique has long been in vogue, several minor factors are found to influence the percentage of success, with special reference to shoot or root formation, as well as the performance of plantlets in vitro and in vivo. The rate of growth of the plant, especially

in the case of wild species, is extremely slow and the explants available per plant will also be low. Hence it would be desirable to examine the possibility of using all plant parts like shoot tips, axillary buds, nodal sections and flower-stalk cuttings as explants.

The present investigation is aimed at standardising the explants for in vitro propagation of Dendrobium spp. with the following objectives.

i) To study the response of different explants

ii) To identify the best explant source

Review of Literature

REVIEW OF LITERATURE

The standardisation of *in vitro* cloning techniques of orchids in 1960's has made a big impact on their cultivation, and tissue culture techniques are now widely used throughout the world for orchid propagation. The use of tissue culture technique in orchids has enabled the multiplication of quality plants in large numbers, establishment of hybrid plants and improvement of orchid trade. Examining the suitability of different plant parts for use as explants has an added value in the context of slow growth of orchids.

2.1. Advances in orchidology

The role of mycorrhizae in orchid seed germination was discovered by Noel Bernard in 1899 (Bernard, 1909) and it formed the basis of *in vitro* symbiotic seed germination procedure. Later on, a non-symbiotic culture method was developed by Knudson (1922) who showed that orchid seeds could germinate on a relatively simple medium containing sucrose. This became the standard procedure for germinating orchid seeds. Thus *in vitro* procedure for seed germination of orchids has been in practice since the turn of the century.

The earliest report of using tissue culture technique in the clonal propagation of orchids was that of Rotor (1949). Observing that plantlets could develop from buds of inflorescence in *Phalaenopsis*, he cultured flower stalk nodes *in vitro* and obtained some plantlets.

Very successful results have already been obtained in the meristem culture of carnations, dahlias and potatoes (Morel and Martin, 1952, 1955). However, the credit for the initiation of meristem culture techniques in orchids goes to Dr.G. Morel of INRA, Versailles, France (Morel, 1960, 1964). Morel (1965a) outlined various aspects of meristem culture in detail. Successful results were obtained in Cattleya, Dendrobium, Lycaste, Miltonia, Odontoglossum and Phiaus. Morel (1965a) suggested that, by using the techniques, large scale orchid plant production could be possible, as in roses or carnations. The techniques and details involved in meristem tissue propagation in vitro were further explained by Isley (1965) and Russon (1965).

The benefits of meristem culture attracted the attention of many researchers and work on various valuable orchid hybrids was initiated in different laboratories. Details were discussed regarding the nature of propagation material, method of obtaining sterile meristem, size of the explant, pH of the medium and different environmental conditions suitable for cultures (Bertsch, 1966; Marston, 1966, 1967, 1969; Marston and Voraurai, 1967; Goh, 1971). The major development in the cultivation of orchids in the world has been due to modern scientific tehnology which has been suitably used in orchid seed germination and meristem culture (Chadha and Singh, 1985).

Of the different explants tried in the meristem culture of orchids, in different genera, apical shoots or axillary buds proved to be the best source of explants for successful clonal propagation, followed by inflorescence, leaves and roots. Meristem cultures were

also used for induction of polyploidy in Cymbidium, Dendrobium and Vanda hybrids. The cultures were treated with colchicine (0.1-0.5 per cent) and the tetraploid and hexaploid plantlets that resulted were grown to maturity (Wimber, 1963; Wimber and Van Colt, 1966; Vajrabhaya and Vajrabhaya, 1970; Sanguthai et al., 1973; Sanguthai and Sagawa, 1973; Vajrabhaya, 1975). Further works are necessary in the rapid clonal multiplication of orchids making use of all the available plant parts.

2.2. Selection of explant

It has been fairly well documented that tissues taken from field grown plants round the year are not equally amenable to tissue culture conditions. From different studies it has been found that the time of the year the cultures were established and the cultural conditions in the field were crucial for the success of the tissue culture technique.

Hasegawa and Goi (1987) reported that the rhizome formation from shoot tip cultures in Cymbidium goeringii Reihenbachfil was maximum in April when cultured in MS medium and in June with modified Vacin and Went medium.

Sterilization of the explants is an important procedure in tissue culture. The aim in surface sterilization is to remove all of the microorganisms present on the explant, with a minimum of damage

to the plant part to be cultured. The standard practice is to use a strong oxidant, either calcium hypochlorite or sodium hypochlorite (in the form of commercially prepared household bleach, eg., Chlorox or other brand name) to kill the microorganisms. The strength of sterilization solution and the time period required for decontamination depend on the cleanliness of the explant material. The strength of calcium hypochlorite solution used by various workers ranged from 0.4 to 8.0 per cent (80 g/l, which is already a saturated solution), whereas that of commercial bleach ranged from 5 to 20 per cent, with the extreme of 50 per cent used by Ball et al. (1975) for Phalaenopsis flower stalks. For relatively clean plant organs, a general cleaning followed by a short period exposure (5 to 15 min.) in commercial bleach solution is sufficient. With difficult material, usually a two-stage procedure, a stronger solution followed by a more dilute solution, is recommended. Very often, either the organ is cleaned first with ethanol, or a wetting agent such as Tween 20 is added to the sterilization solution to achieve better results. Raskauskas et al. (1989) suggested that the best sterilization sequence for Cymbidium corm meristems was treatment in 70 per cent ethyl alcohol for 10 minutes, in 0.1 per cent diacid for 5 minutes and finally in 10 per cent Chloramine B for 10 minutes.

In sympodial orchids like Cattleya, Cymbidium and Dendrobium, a young shoot arising from back-bulb provides suitable material for tissue culture. On longer shoots, there may be several axillary buds

as well as apical buds, while in monopodial orchids like Vanda, Aerides and Phalaenopsis nodal sections, shoot apices, keikis or off shoots and even flower stalk cuttings could be used. The other parts of the plant like leaf and root have also been used by various workers.

The use of in vitro techniques for clonal or asexual mass propagation is the most advanced application of plant tissue culture (Thorpe and Patel, 1984). Rapid asexual multiplication can be achieved by either enhancing axillary bud breaking or by producing adventitious shoots through organogenesis or through somatic embryogenesis (Murashige, 1974; Thorpe and Patel, 1984).

2.2.1. Enhanced release of axillary buds

Axillary buds have been used together with shoot tips in most studies with orchids. These buds are present in axillary position and they normally develop into inflorescence. However, in most orchids, not every axillary bud develops into an inflorescence. Furthermore, because of the apical dominance effect atleast a certain number of buds below the apex remain quiescent. These buds may, however, be very poorly developed (Morel, 1974). In many studies axillary bud explants were as responsive as shoot tips. Loh et al. (1978) were of the opinion that there was generally a decrease in the generative capacity when the buds were derived further away from the apex.

Johanson (1967) used both micro and macro meristems (apical and lateral buds) of Cymbidium for culture. He found that the macro-meristems were better than the micro meristems. Sagawa and Kunisaki (1969) suggested that the best explants for tissue culture for Cymbidium, Dendrobium and Cattleya were apical and axillary buds. In another study Kim et al. (1970) observed that the percentage of survival of axillary buds of Dendrobium phalaenopsis was higher than terminal bud explants, but the survival diminished with increasing height of nodes from which axillary buds were taken.

Schmude (1984) reported that fully grown plants could be obtained from meristems in 2-3 years, with the first flowering 4-6 months after removal from cultural flasks. Successful and rapid multiplication was observed by Wu et al. (1987) in different species of Cymbidium through shoot meristem culture. Sagawa and Shoji (1967) opined that shoot tip cultures necessiated the sacrifice of the entire new growth or a whole plant for a procedure which at best might be successful with 66.7 per cent of the explants.

As with Cymbidium and other sympodials, shoot tips remained the most commonly used explant for monopodials (Goh, 1970; Vajrabhaya and Vajrabhaya, 1970; Kunisaki et al., 1972; Goh, 1973; Teo et al., 1973; Intuwong and Sagawa, 1974; Goh et al., 1975; Irawati et al., 1977; Lim-Ho, 1981). Stewart and Button (1975) reported that plantlets and callus which subsequently gave rise to plantlets, could

be differentiated from a single Paphiopedilum stem apex if bacterial free cultures could be obtained. In a study by Wu et al. (1987) on shoot tip cultures of oriental orchids, successful and rapid multiplication was achieved with 20 out of 45 species and cultivars tested. These included Cymbidium goeringii and C. goeringii var. longibracteatum.

Stem segments of Phalaenopsis (3.0-4.5 cm long) with a node were used to start the cultures, by Sagawa (1961) and Scully (1965, 1966). Buds enlarged in two weeks followed by leaf and root development in 6-20 weeks. Sagawa and Sehgal (1967) reported that stem segments of Vanda Miss Joaquim could be used as explant to obtain plantlets in a period of 2-3 weeks. Intuwong and Sagawa (1973) were able to produce plantlets directly in a period of 40-55 days from nodal sections of Calanthe masuca, Dendrobium and Epidendrum radicans. Sagawa and Shoji (1967) reported that some cultures of Dendrobium took a period of two years for plantlet formation and flowering. Node cultures as a mean of clonal propagation for Dendrobium was reported by Ball and Arditti (1975). Slow shoot multiplication was noticed in Dendrobium transperensis when nodal sections were used as explants (Honmode and Sehgal, 1991).

2.2.2. Somatic organogenesis (callus mediated)

When tissues other than meristems are inoculated in vitro, somatic organogensis is observed either directly or through callus. The

growing potential of seedling leaves was demonstrated and they could thus be used to obtain plantlets in great numbers. Champagnat et al. (1970) used seedling leaves of Cattleya hybrid to induce further growth. Young leaf tips of Dendrobium, Epidendrum and Laeliocattleya were used by Churchill et al. (1970, 1971) to induce regeneration.

Loh et al. (1975) were able to obtain plantlets in 4 months from leaf tips of Aranda hybrids. By using leaves as explants the damage to the clonal plant could be minimised or avoided. Another study by Vij et al. (1984) using leaf segments as a source of explants in Rhyncostylis retusa showed that mature leaves did not grow and became necrotic while those obtained from juvenile leaves of 6 month old plantlets growing in vitro developed PLBs in 4-7 weeks depending on the growth medium. Mathews and Rao (1985) observed leaf tip regeneration when whole leaf explants were used and among the isolated leaf parts, the leaf base showed proliferation. The ability of the epidermal cells to regenerate in vitro offered exciting opportunities to use epidermal peel for micropropagating orchids (Vij and Pathak, 1990).

Most orchids produce racemose inflorescence and generally buds in the basal region of the axis remain dormant. Rotor (1949) observed that flower stalks with buds obtained from the middle portion of an inflorescence served as useful material in Phalaenopsis. Tse et al. (1971) recorded poor growth in flower stalks of Phalaenopsis as

compared with Cattleya and Cymbidium. Intuwong and Sagawa (1973) inoculated the inflorescence primordia (1.5 cm long) of Ascofinetia, Neostylis and Vascostylis on liquid medium after removing the bracts. In a few weeks abundant proliferations were formed from hypodermal tissues of the axis. Tissues were transferred in agar medium after 4 months and seedlings ready for transplanting were developed in 15 months. When primordia from larger inflorescence were used (3.0 cm or more) the axis elongation was evident and buds developed into small flowers. By a similar technique plantlets were obtained within five months in Dendrobium, Phalaenopsis and Vanda (Intuwong et al., 1972; Singh and Sagawa, 1972 and Intuwong and Sagawa, 1974).

In Paphiopedilum protocorms have been reported to be formed sometimes on the callus which developed on young inflorescences, but plantlets have been derived from them only infrequently (Stewart and Button, 1975). Yoneda (1986) was able to obtain plantlets from both primary and secondary inflorescence of Phalaenopsis hybrids. Singh and Prakash (1984) successfully propagated Thunia alba through the use of flower stalk cuttings. Lin (1986) recommended that the best time of collecting the explant for culture was following stalk formation and before the appearance of the first flower. Section from near and tip of the flower stalk gave best results.

Attempts to culture root or rhizome tips or sections have yielded a low rate of success. Beechay (1970) discussed the possibilities of propagation from aerial roots and pointed out that difficulties might

arise because of their special anatomical structures. Explants of root tips from Vanda seedlings in vitro (Goh, 1970) and mycorrhizae-free root tips (5 mm) from plants growing outdoors (Churchill et al., 1972) developed into thin etiolated structures. Tanaka et al. (1976) were able to obtain callus, PLBs and plantlets from root tip explants of 194 - and 349 - day old seedlings of Phalaenopsis grown in vitro. Mathews and Rao (1985) conducted experiments for induction of organogenesis in Vanda root explants. Churchill et al. (1972) reported that Epidendrum root segments grew into thin etiolated elongated structures without producing any calli or plantlets.

2.3. Media and media supplements

2.3.1. Media

The type of medium used was a crucial factor in determining success (Churchill et al., 1971, 1972, 1973). The important media used for orchid tissue culture are Knudson C (Knudson, 1946), Murashige and Skoog (1962), Morel (1965a) Vacin and Went (1949) and White (1943). Of these, Vacin and Went and Knudson C are the popular media used. However, there are reports that Murashige and Skoog's medium was more effective than Knudson C medium (Murashige and Skoog, 1962).

Different species of Dendrobium were successfully cultured on various media. Vacin and Went medium was found the best by many investigators (Gilliland, 1958; Nimote and Sagawa, 1961; Sagawa and Valmayor, 1966 ; Sagawa et al., 1967; Kim et al., 1970; Singh and

Sagawa, 1972; Sanguthai and Sagawa, 1973 and Valmayor, 1974). Clonal propagation of Dendrobium through meristem culture by Sagawa and Shoji (1967) showed that explants measuring 2-4 cubic millimetre from terminal or axillary buds of D. phalaenopsis Jacquelyn Thomas and other evergreen types, when cultured on Knudson's C media proliferated and produced numerous additional PLBs, but when left undisturbed plantlets were formed. Irawati et al. (1977) reported that the best growth and survival rates were obtained when the apical and axillary bud sections of Dendrobium James Storie were cultured in modified Knudson's C medium.

Studies by Fernando (1979) on clonal propagation of Dendrobium Caeser Red Lip through meristem culture revealed that the best medium was modified Vacin and Went (+4 ppm NAA) and the following observations were made on the explants: a) swelling of the explants gave rise directly to a single shoot (b) the explant expanded into a green mass which subsequently grew into PLBs; which, when fractioned, gave more similar bodies or (c) the explants developed into a single shoot at the base of which PLBs developed later and gave rise to plantlets. According to Soedjono (1988) Bayfolan was a new medium for Dendrobium Walter Oumae and was cheaper than Vacin and Went medium. Apical meristems of Cymbidium were excised from young shoots and inoculated on a liquid medium. Cultures were agitated for four weeks. PLBs were produced within 2½ months. Many shoots

developed when they were transferred to the solid medium (Wimber, 1963, 1965).

Wilfert (1966) suggested that transversely cut sectors produced more PLBs than the vertically cut sectors when cultured in liquid medium initially and then transferred to an agar medium. This was later confirmed by Sagawa et al. (1966). According to Bivins and Hackett (1969) Knudson C medium was found to be best for PLB formation and subsequent plantlet development in Cymbidium shoot tip culture. Ichihaushi and Kako (1977) reported that Cattleya shoot tips when cultured on a solid medium turned brown and died eventually due to polyphenoloxidase activity, which was highest at pH 6.5 and inhibited at lower pH. Seedling tip cultures of Dendrobium phalaenopsis produced better results on MS medium supplemented with both nitrate and ammonium than nitrate alone (Gandawidjaja, 1980).

Hasegawa and Goi (1987) reported that shoot tip cultures of Cymbidium kanran Makino, produced rhizomes better when cultured in Linsmaier and Skoog medium containing organic nutrients at 1/5th of standard concentration plus 10 per cent coconut water. According to Scully (1967) axillary buds from pseudobulbs of Cattleya first cultured in liquid medium when transferred to agar medium after 2-5 weeks showed increase in size in 4-10 weeks. The axillary buds were subdivided into smaller units and inoculated in fresh medium. Two months after subculturing plantlets were formed.

Kunisaki et al. (1972) reported increased proliferation of the axillary buds of Vanda Miss Joaquim and three other hybrids when cultured in a medium without sucrose. After trying different strength of MS medium, Shimasaki and Uemoto (1987) reported that the medium with a dilution of 1/8th gave best results in the axillary bud culture of Calanthe.

The technique of culturing leaves from mature plants in shaken liquid medium which produced rooted plantlets or PLBs initially and then plantlets on transfer to solid medium was refined by Churchill et al. (1973). Microinflorescence of Oncidium varicosum (obtained from a natural mutant, Baldin) when cultured on liquid Knudson medium, supplemented with 0.5 mg/l NAA under 16 h photoperiod and 25°C, produced PLBs (Kerbauy, 1984b). Plant regeneration studies on O. varicosum by root tip culture indicated that the buds produced from PLBs in VW medium with 15 per cent CW when transferred to Knudson C medium resulted in root regeneration. The modified MS medium containing lesser amounts of KNO_3 and NH_4NO_3 than those of the original MS medium was optimal for the production of plantlets from rhizomes of C. kanran without auxins and cytokinins (Shimasaki and Uemoto, 1990).

2.3.2. Growth regulators

Subsequent to the development of different media for orchid tissue culture, several modifications were made to the media by

changing the ingredients and their quality and quantity. The most important development in the culture media was the incorporation of growth substances which include auxin, giberellins, cytokinins etc.

When excised apices of Rhyncostylis gigantea were cultured on a composite agar medium supplemented with NAA and coconut milk, plantlets could be produced in 3½ months but for callus production about 7 months were required (Vajrabhaya and Vajrabhaya, 1970). According to Steward annd Mapes (1971) shoot apex of Cymbidium hybrids when grown in agar medium supplemented with coconut milk and NAA developed callus which was transferred to a liquid medium of same composition. Free cell production was better when NAA was replaced by 2,4-D. Studies by Kukulczanka (1976) on effect of cytokinins and hormones on Cymbidium shoot tip culture showed that a phytohormonal complex (S-161) prevented proliferation of PLBs at low concentration (0.2 - 10.0 ppm) but there was 100 per cent plantlet formation at 100 - 1000 ppm. Kim and Kako (1984) reported that shoot apices of Cymbidium x Sazanami (cv. Harunoumi) when cultured on solid MS medium with BA 0.1 ppm, enhanced and NAA at 0.5 ppm inhibited leaf elongation, differentiation and formation of PLBs.

Kukulczanka (1985) studied the effect of Biostinin on the growth of meristematic tissue and protocorm formation of Dendrobium and Cymbidium. He found that addition of Biostinin resulted in good growth of the meristematic tissue of an intense green colour and marked increase in fresh and dry weights. The Biostinin, like BA, increased

the number of protocorms and like auxins, stimulated the formation of leafy shoots and roots.

Gu et al. (1987) were of the opinion that PLBs, and eventually plantlets, could be produced when shoot tips from lateral buds of Cymbidium pseudobulbs were cultured in a modified VW medium containing BA while the addition of 2,4-D or IAA had little or no effect. Honmode and Sehgal (1991) were able to produce multiple shoots from shoot tips of Rhyncostylis retusa when cultured in MS medium supplemented with NAA (5 μ M) and Kinetin (20 μ M). In order to establish the asexual propagation time of Peristeria elata through tissue culture technique, the shoot apices with 2-3 internodes from in vitro raised seedlings were cultured on MS medium with supplements like BAP and 4-phenylurea to induce multiple shoots. During enhanced axillary branching, nearly 15-20 shoots were produced in 40-45 days (Gayatri and Mahesha, 1991).

Kukulczanka and Wojcicchowska (1983) obtained good results on the development of lateral buds on isolated shoots of Dendrobium antennathum and D. phalaenopsis by treating the culturing medium with BA and peptone. Shimasaki and Uemoto (1987) found that application of BA to explants of axillary buds of Calanthe promoted shoot growth.

Under the influence of orthochlorophenoxy acetic acid, callus was obtained from Epidendrum leaf tips and at optimal concentration (2 mg/l) growth improved, as evidenced by an increase in the dry weight (Rudolph et al., 1972). Tanaka and Sakanishi (1977) were able

to produce PLBs, and eventually plantlets, from leaf segments excised from young shoots developing on nodal flower stem cutttings of a Phalaenopsis amabilis hybrid, cultured in MS medium, supplemented with NAA 1 ppm, BA 10 ppm and adenine 10 ppm. For meristem tissue development of Cymbidium corm, Reinert-Mohr or Knudson C medium was most suitable and protocorms formed best on Prasad-Mitra medium containing kinetin, adenine, vitamins B_1, B_6 and PP, IAA and NAA stimulated plant regeneration (Raskaukas et al., 1989).

Vij et al. (1991a) made attempts to assess the regenerative potential of leaf epidermis (peels) of Rhyncostylis retusa. The explants comprising exclusively of epidermal cells perished within 2 weeks of inoculation when cultured in Mitra et al (1976) medium supplemented with auxins (IAA, IBA or 2,4-D) and cytokinins (KIN) and GA_3. The explants with 2-3 underlying layers of cells proliferated in 3 weeks. The callus produced were irregularly compact with several growing points, yellow and caulogenic, when 2,4-D was supplemented in the medium. With kinetin as the medium supplement, somatic embryos were produced, which developed into PLBs and later into plantlets with 2-3 leaves and 1-2 roots.

Transcinnamic acid in Knop's medium stimulated the development of dormant buds on Phalaenopsis flower stalks (Ball and Arditti, 1975). According to Jambor-Benezer et al. (1986), buds from inflorescence stem of P. amabilis soaked in 100 ppm BA for 1-3 hours and cultured in vitro gave best results. Vij et al. (1991b) reported the morphogenetic

response of floral buds of Dendrobium crepidatum and D. pierardii in vitro. When cultured in Mitra et al. medium supplemented with 2 g/l peptone/yeast extract, 1.0 - 2.5 mg/l IAA/NAA and 1.0 mg/l BAP/kinetin, the older buds matured into a flower, whereas the younger ones reverted to vegetative growth. Flower stalk sections of Phalaenopsis and Doritaenopsis, when cultured on modified VW medium with BA 1 or 5 mg/l, resulted in formation of PLBs on epidermis and cut surface in 30 days. Adventitious buds were formed and grew into plantlets after 60 days of culture (Lin, 1986). In another study Kim and Kako (1984) cultured floral parts of Cymbidium x Sazanami cv. Harunoumi on MS medium (solid or liquid) supplemented with NAA and BA.

Sagawa et al. (1966) could successfully produce plantlets by culturing root-tips of Dendrobium Lady Hay and Dendrobium Jacquelyn Thomas on modified VW medium supplemented with 2,4-D. Stewart and Button (1978) opined that callus could be obtained from root tips from plantlets of Epidendrum Obrienianum grown in vitro when 2,4-D was added to the medium. Vij et al. (1987) reported that root segments of Rhyncostylis retusa (obtained from 38 week old axenic cultures) when cultured in MS medium, supplemented with NAA or BA (1 mg/l) and 0.2 per cent AC, gave complete plantlets with 2-3 leaves in 17-32 weeks. Plantlets were obtained at a faster rate (in 12 weeks) if NAA alone was supplemented in the medium.

Rhizome tips of Cymbidium goeringii and C. pumilum inoculated on agar medium sulpplemented with KIN (10 mg/l) and maintained in the dark gave rise to masses of rhizomes without shoot formation, but when L-arginine (10^{-3}M) was added, shoots developed (Ueda and Torikata, 1972). Lee et al. (1986) reported that rhizomes of Cymbidium kanran obtained from seedlings raised from surface sterilized seeds, when cultured in M5 medium containing high NAA : BAP ratios, the growth rate was enhanced.

Shimasaki and Uemoto (1990) reported that only vegetative shoots resulted from rhizomes cultured in vitro when lower NAA : BAP ratio was used. The rhizomes were induced from the axils of leaves when shoots were explanted to medium containing high concentrations of NAA. Root formation of C. kanran was inhibited by the addition of either auxins or cytokinins to the culture media.

2.3.3. Media supplements

Orchid growers add to the orchid media an extraordinary variety of ingredients either for seed germination or for the further growth of the plantlets. The effect is real but most difficult to study, due to the chemical complexity of these ingredients.

Soediono (1983a) suggested a method for saving contaminated tissue culture of Dendrobium. Callus, plantlets and PLBs of Dendrobium (cv. Tay Sweekeng) contaminated with unidentified fungi and bacteria were disinfected successfully by repeatedly transferring them to 50 ml

of culture medium containing 2.5 mg Benlate (50% Benomyl , 5000 units of nystain, 8000 units Penicillin G (Benzylpenicillin) and 2.5 mg Gentamycin.

Soedjono (1988) reported that in Dendrobium Walter Oumae best root and shoot growth were obtained when Bayfolan medium was supplemented with coconut water, sugar, charcoal and fish emulsion. The shoot meristems of Cymbidium spp when cultured on MS or White media, supplemented with 10 per cent coconut water and NAA 5 mg/l, produced PLBs in 4-6 months. When these were divided and subcultured in liquid medium containing same additives they formed new bright green rosette of PLBs within a month. The rosette PLBs formed plantlets on White, MS or VW basic liquid media with 10 per cent CW and cultured in a rotary shaker. This suggested the possibility of repetitive mass of clonal propagation (Wang, 1988).

Steward and Mapes (1971) were able to produce callus from shoot apex of Cymbidium hybrids by culturing them in a medium supplemented with coconut milk. Soediono (1983b) suggested that VW medium with 15 per cent CW was suitable for initial culture of shoot tips of Dendrobium cv. Jacqueline Thomas 'White', and also for rapid proliferation of PLBs and plantlet formation. According to Kim et al. (1970) buds excised from Dendrobium bulbs, as well as those from leaf axils cultured on VW liquid medium, supplemented with 15 per cent coconut water, produced PLBs in 4-5 weeks and plantlets in 8 weeks.

The PLBs obtained through in vitro culture of seedling leaf segments of Phalaenopsis spp were cultured on media containing peptone or tryptone of various concentrations. Segments from dorsal parts of PLBs, formed plantlets while those from basal parts produced no plantlets (Amaki and Higuchi, 1989).

A study was conducted on clonal propagation of sarcanthine orchids by aseptic culture in inflorescence by Intuwong and Sagawa (1973). This study has shown that inflorescence 1.5 cm or less from sarcanthine orchids could be successfully induced in aseptic culture to proliferate and differentiate into plantlets. Modified VW medium with sucrose, CW, potato extract and homogenized banana were used for control of growth and development. The PLBs obtained from micro-inflorescence of Oncidium varicosum when cultured in Knudson's medium containing 60 g/l banana, 10 g/l sucrose, 0.8 per cent agar and 1 g/l AC and 27.8 mg/l Fe EDTA, flowering occurred after 8-9 months of culture, compared with plants growing under natural conditions (Kerbauy, 1984b) In another study by Kerbauy (1984a) on plant regeneration of O. varicosum by means of root tip culture indicated that callus proliferation was highest (16 per cent) in root tips cultured on VW medium supplemented with 15 per cent coconut water.

2.4. In vitro rooting

Although a number of plants root spontaneously in culture, shoots of most species multiplied in vitro lack a root system. Rooting

can be achieved either by subculturing to a medium lacking cytokinin, with or without a rooting hormone or by treating the shoots as conventional cuttings after removal from sterile cultures (Yeoman, 1986). All cytokinins inhibit rooting, and BA which is widely used for shoot multiplication, does so particularly strongly, even after transfer to cytokinin free medium. Since auxin is essential for root initiation, majority of stage III media contains auxin as a supplement. Several researchers have shown that *in vitro* rooting can successfully be achieved by reducing salt concentrations in the media, particularly MS, which contain high salt concentrations. Honmode and Sehgal (1991) reported good rooting in shoots obtained *in vitro* from nodal sections of *Dendrobium transperensis* in MS medium containing IBA (5 µ M). NAA favoured root induction in half strength MS medium in *Peristeria elata* Hook (Gayatri and Mahesha, 1991).

2.5. Planting out and acclimatization

Acclimatization is necessary in the case of micropropagated plants because *in vitro* plant material is not adopted for *in vivo* conditions (Brainerd and Fuchigami, 1981). The success in acclimatization of micropropagated plants is largely dependant upon not only the post-transfer growth conditions, but also upon the pre-transfer culture condition (Ziv, 1986). Tissue cultured plants are very poorly adapted to resist the low relative humidity, higher light levels and more variable temperatures prevailing outside (Wainwright, 1988).

Light, temperature and relative humidity are the three major factors to be controlled during acclimatization. Hu and Wang (1983) suggested a period of humidity acclimatization for the newly transferred plantlets. Rajmohan (1985) reported the use of plastic microscope covers for maintaining 90-100 per cent relative humidity and obtained 55-60 per cent survival of in vitro produced jack plantlets.

Thorough washing of the plantlets to remove the traces of nutrient medium and sterilising the potting mixture eliminated serious problems of fungal infection (Anderson, 1980). Physical, chemical and biological properties of the potting mixture is also important in the establishment of in vitro regenerated plantlets when planted out.

Orchids are not grown in ordinary soil mixtures as other plants. Since their roots need plenty of air around them at all times, special orchid mixes are usually used which would provide support to the plant, supply water and nutrients to the roots and provide enough air for the roots to breathe. The commonly used materials for potting orchids are fir and red wood bark, pieces of corkbark, tree fern, osmunda ferr root, sphagnum moss, gravel and coconut fibre. Charcoal, tree fern fibre and red burnt bricks are the main potting media used for orchid growing in Kerala. In a study on post-transplantation growth of Dendrobium hybrid seedlings in community pots, Kumar (1991) has reported that maximum survival and optimal growth of shoots and roots were in a media having rubber-seed husks, coconut-shell

pieces and gravel. The growth was as good as seen in tree fern, charcoal and burnt brick pieces. The mortality rate was high and poor overall growth of seedlings was recorded when grass root moss and wood shavings were used as the potting medium. Coconut husk, though recommended by orchid growers in Kerala (Abraham and Vatsala, 1981) being very cheap and found in plenty in South India, was found to be unsatisfactory because of the poor adhering capacity of the roots to the husk.

Materials and Methods

MATERIALS AND METHODS

The present investigations on the standardisation of explants for *in vitro* propagation in *Dendrobium* spp. were carried out at the Plant Tissue Culture Laboratory, attached to the All India Co-ordinated Floriculture Improvement Project (AICFIP), College of Horticulture, Vellanikkara, during 1990-'91. The details regarding the experimental method and analytical techniques adopted are presented step-wise in this chapter.

In the present study *in vitro* propagation through enhanced release of axillary buds and somatic organogenesis (callus mediated) (Murashige, 1974) were attempted using different explants. The different stages involved are illustrated in Fig. 1.

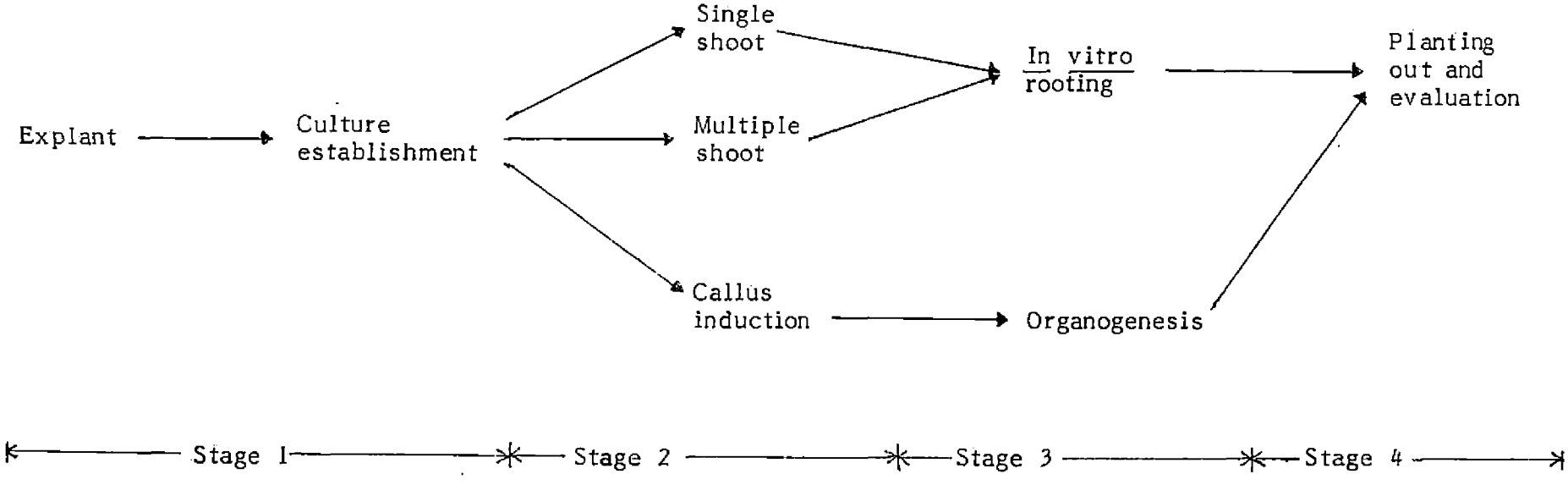
2.1. The species

The explants for the study were collected from three important species of *Dendrobium*, viz., *D. fimbriatum*, *D. moschatum* and *D. nobile*. The salient features of these species are presented in Table 1.

2.2. The explants

The details of different types of explants used for the study are given in Table 2.

Fig. 1. In vitro cloning procedure in orchids



Stage 1 - Physiological preconditioning of the explant and explant establishment

Stage 2 - Induction of axillary shoots/callus mediated organogenesis and rapid multiplication

Stage 3 - In vitro rooting and acclimatization

Stage 4 - Planting out and evaluation

Table 1. Salient features of the three Dendrobium spp. utilised for in vitro culture studies

Character	D. fimbriatum Lindl. oculata Hook f.	D. moschatum Sw.	D. nobile Lindl.
Pseudobulbs	75.0 - 150.0 cm long, tapering towards apex	90.0 - 180.0 cm x 1.0 - 1.2 cm, terete, striate, pointed towards apex	30.0 - 60.0 cm long, turning yellow on maturity, laterally compressed, narrow at the base
Leaves	Several, 10.0 - 15.0 cm x 1.5 - 2.8 cm size, oblong lanceolate, acuminate in shape	Several, alternate 10.0 - 15.0 cm x 3.5 cm, acute or faintly notched	Several, 8.0 - 12.0 cm x 2.5 - 3.0 cm oblong, apex unequally lobed
Inflorescence	Produced on leafy or leafless pseudobulbs, lateral, pendulous, 7-12 flowered	10.0 - 30.0 cm long, 15 flowered	Fascicles of 1-4 flowers
Flowers	5.0 - 7.5 cm across, bright yellow, sepals broadly oblong, rounded entire, petals broader, lip orbicular, fimbriate pubescent and having large orbicular patch of dark reddish brown at the base.	5.0 - 7.0 cm across, orange yellow colored and fragrant. Sepals 3.0 cm long, broadly ovate, obtuse, lip lanceolate, Anterior part very hairy inside and on the outer surface. Base with two dark maroon blotches.	5.0 - 7.0 cm across, white with deep purple tinge, highly variable, rarely pure white. Lip transversely ovate - oblong, pubescent, with a central blotch of very deep purple, surrounded by broad margin of yellow or white
Flowering time	April-May	May-June	April

Source: Pradhan (1979)

Table 2. Explants used for *in vitro* culture studies of *Dendrobium* spp.

Route	Explant
*Enhanced release of axillary buds	Shoot tip
	Axillary bud
	Inflorescence stalk
**Somatic organogenesis (callus mediated)	Shoot tip
	Axillary bud
	Leaf (tip portion, middle portion, base with sheath)
	Inflorescence stalk
	Root segments (from keikis)
	Whole leaf (from culture)
	Root (from culture)

*All the species of Dendrobium were used
**Only *D. moschatum* was used

2.2.1. Collection and preparation of explants

The explants for culture were collected from healthy plants growing in the orchidarium attached to the AICFIP, College of Horticulture, Vellanikkara. The explants were excised from the plants using surgical blades.

For shoot tip and axillary bud explants, the leaves were removed and the stem washed with running tap water. The stem was initially cleaned with teepol solution followed by a treatment with Bavistin 0.2 per cent for half an hour. It was then washed thoroughly with distilled water and cut into sections having two to three nodes each.

In the case of leaves, the individual whole leaf was washed with teepol solution followed by washings with distilled water. After blotting the leaves between folds of tissue paper, they were scrubbed with cotton wool wetted with alcohol.

Aerial roots from keikis were excised for root culture. They were cleaned thoroughly with teepol solution, followed by washings with distilled water.

The inflorescence stalks after excising from the plants were initially cleaned under running tap water. After draining, they were scrubbed with a cheese cloth wetted with 95 per cent ethyl alcohol. The bracts were removed and the nodes exposed.

2.2.2. Standardisation of surface sterilization methods

The details of the chemicals used for the surface sterilization of the explants are given in the Table 3. Observations were made on the percentage contamination and survival on ten cultures each, after three weeks of culturing.

2.3. The media

The culture media used for the study were KC (Knudson, 1946), MS (Murashige and Skoog, 1962) and VW (Vacin and Went, 1949). The chemical composition of the media are given in Table 4.

The chemicals used for preparing the culture media were of analytical grade from British Drug House (BDH), Sisco Research Laboratories (SRL), Merck or Sigma.

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of MS media. Stock solutions of major and minor nutrients were prepared first by dissolving the required quantity of chemicals in double glass distilled water and were stored under refrigerated conditions in amber colored bottles. The stock solution of nutrients were prepared fresh every four weeks and that of vitamins, aminoacids and phytohormones were prepared fresh every week.

Specific quantities of the stock solutions of chemicals and phytohormones were pipetted out into a 1000 ml beaker. Sucrose and

Table 3. Standardisation of surface sterilization of explants in Dendrobium spp.

Explant	Sterilant	Concenration (%)	Duration (min)
*Shoot tip	Bleaching powder (Cl)	4.00	10
	Mercuric chloride	0.05	10
	,,	0.10	10
	,,	0.20	10
	Sodium hypochlorite	4.00	20
	,,	4.00	30
*Axillary bud	Bleaching powder (Cl)	4.00	10
	Mercuric chloride	0.05	10
	,,	0.10	10
	,,	0.20	10
	Sodium hypochlorite	4.00	20
	,,	4.00	30
**Leaf segments	Bleaching powder (Cl)	4.00	10
	Mercuric chloride	0.05	3
	,,	0.10	3
	,,	0.20	3
	Sodium hypochlorite	4.00	20
	,,	4.00	30
**Root segments	Bleaching powder (Cl)	4.00	10
	Mercuric chloride	0.05	10
	,,	0.10	10
	,,	0.20	10
	Sodium hypochlorite	4.00	20
	,,	4.00	30
**Inflorescence stalk	Bleaching powder (Cl)	4.00	10
	Mercuric chloride	0.05	10
	,,	0.10	10
	,,	0.20	10
	Sodium hypochlorite	4.00	20
	,,	4.00	30

*All the three species were used
**Only D. moschatum was used

Table 4. Chemical composition of the media

Chemical	Quantity (mg/l)		
	KC	MS	VW
Major elements			
$Ca(PO_4)_2$	-	-	200
$CaCl_2.2H_2O$	-	440	-
$Ca(NO_3)_2.4H_2O$	1000	-	-
Ferric citrate	-	-	28
$FeSO_4.H_2O$	25	27.8	-
KNO_3	-	1900	525
KH_2PO_4	250	170	250
$MgSO_4.7H_2O$	250	370	250
$(NH_4)_2SO_4$	250	-	500
NH_4NO_3	-	1650	-
Na_2.EDTA	-	37.3	-
Minor elements			
$CoCl_2.6H_2O$	-	0.025	-
$CuSO_4.5H_2O$	-	0.025	-
H_3BO_3	-	6.2	-
KI	-	0.83	-
$MnSO_4$	7.5	22.3	7
$Na_2MoO_4.2H_2O$	-	0.25	-
$ZnSO_4$	-	8.6	-
Organic constituents			
Glycine	-	2.0	-
Myo-inositol	-	100	-
Nicotinic acid	-	0.5	-
Pyridoxine HCl	-	0.5	-
Thiamine HCl	-	0.1	-
Sucrose	20.00 g	30.00 g	20.00 g
Agar	9.00 g	6.00 g	9.00 g

inositol were added fresh and dissolved. The volume was then made upto about 1000 ml by adding double glass distilled water. The pH of the solution was adjusted using an electronic pH meter with 0.1 N HCl or 0.1 N NaOH. Agar was then added to the medium and the final volume was made up exactly to 1000 ml.

The solution was then melted by keeping in a water bath, maintained at a temperature of 90-95°C. The medium (@ 15 ml) was poured hot to the oven sterillized culture vessels which were previously rinsed twice with double glass distilled water. The containers with the medium were then tightly plugged with non-absorbent cotton wool plugs. Corning brand test tubes and conical flasks were the containers used.

For the preparation of VW and KC media, fresh weights of the chemicals were taken in required quantity and dissolved in double distilled water. After addition of sucrose and phytohormones, the volume was made up.

In order to ensure aseptic condition of the medium, the containers plugged with cotton were autoclaved for 15-20 min at 15 psi pressure and 121°C temperature (Dodds and Robert, 1982). After sterilization, the culture vessels were immediately transferred to the culture room.

2.4. Preparation and inoculation of explants

All the inoculation operations were carried out under perfect aseptic conditions in a 'Klenzaids' laminar air flow cabinet.

The explants after surface sterilization were rinsed four to five times with sterile distilled water. They were then transferred to sterile filter paper placed over sterile petridish in order to blot the excess moisture. The sterilized explants were further reduced in size using sterilized scalpel and forceps.

The explants prepared for inoculation are shown in Plate 1. For shoot tip, about 3-5 mm long apex portion was excised. To get the axillary bud, two oblique incisions were made into the stem, one above and one below the axillary bud, so as to meet at about one-third to one-half of the stem. For culturing of leaves, sections of about 5 mm of the tip, middle portion and base with sheath were made. For inflorescence culture, about 40 mm long sections, including a node was excised. For roots, 5-10 mm long tips were used. The explants were then inoculated into the culture medium.

The cotton wool plug of the culture vessel was removed and the vessel neck was first flamed over a gas burner kept in the chamber. The sterile explants, were quickly transferred into the medium, using sterilized forceps. The neck of the culture vessel was once again flamed and the cotton wool plug quickly replaced.

Plate 1. Explants prepared for inoculation

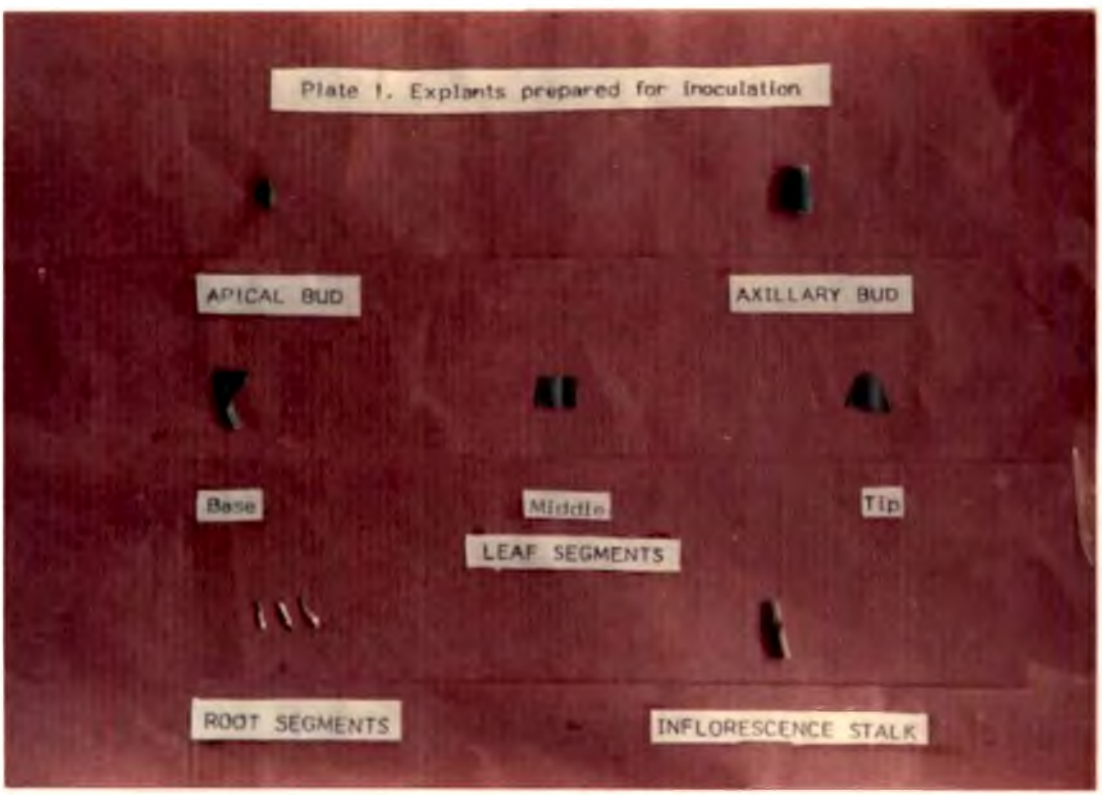


Plate 1. Explants prepared for inoculation
APICAL BUD
AXILLARY BUD
Base
Middle
Tip
LEAF SEGMENTS
ROOT SEGMENTS
INFLORESCENCE STALK

The culture vessels were then transferred to a culture room where they were incubated at a temperature of 27 ± 2°C. Artificial illumination was provided using cool white fluorescent lamps. The light intensity was maintained at 2000 lux. Photoperiod was fixed as 16 h per day which was regulated by a diurnal timer.

2.5. Season of explant collection on culture establishment

The establishment of explants collected from field grown plants varied with the season of their collection. An experiment was therefore conducted to standardise the best season of explant collection in which culture establishment was more and contamination rate the minimum. Explants were collected for this purpose from January-December 1990. Data pertaining to the meteorological parameters during this period are presented in Table 5.

Shoot tip, axillary bud and leaves were collected for this purpose from uniform sized field grown plants during each month and cultured in VW medium. Observations on the percentage of cultures contaminated and percentage of cultures survived were recorded after three weeks of culturing. As the inflorescence stalk was not available in all the seasons, standardisation of the season of collection was not attempted in this explant.

Table 5. Meteorological parameters of the experimental site at the College of Horticulture, Vellanikkara for the period from January to December, 1990

Month	Mean temperature (°C)		Mean relative humidity (%)	Rainfall (mm)	Number of rainy days	Mean sunshine (hours)
	Maximum	Minimum				
January	33.5	20.8	50	3.5	0	9.0
February	34.9	21.9	58	0.0	0	10.0
March	36.0	23.8	64	4.4	1	9.7
April	35.8	25.4	68	38.8	2	8.3
May	31.5	24.1	82	583.9	18	4.5
June	29.7	23.3	85	467.3	25	3.4
July	28.4	22.5	88	759.3	28	2.4
August	29.0	23.0	85	356.4	22	2.5
September	30.7	23.4	79	87.5	8	6.2
October	31.9	23.2	80	313.3	12	6.5
November	31.2	22.6	74	69.8	3	6.0
December	32.3	23.1	59	1.8	0	10.2

2.6. The routes

2.6.1. Enhanced release of axillary buds

2.6.1.1. Explant choice

The explants used for the enhanced release of axillary buds are given in Table 2. A trial was conducted for screening various explants of Dendrobium for initiating enhanced release of axillary buds. Shoot tips, axillary buds and inflorescence stalks were cultured in VW basal medium. Observations were recorded on the percentage of response from ten cultures after three weeks of culturing. Based on the observations, further studies were conducted using axillary bud as the explant.

Culture establishment (Stage 1)

The trials for the culture establishment were carried out using axillary buds of D. fimbriatum, D. moschatum and D. nobile in KC, MS and VW media supplemented with auxin (NAA 0.5, 1.0, 1.5 ppm) and cytokinin (BA 0.5, 1.0, 1.5 ppm) in all possible combinations, replicated five times.

Observations on number of days taken for bud initiation (Plate 2) and bud elongation (Plate 3) were recorded after three weeks of culturing.

2.6.1.2. Shoot proliferation (Stage 2)

All the trials in Stage 2 were conducted on MS and VW media.

Plate 2. Bud initiation

Plate 3. Bud elongation

Explants used for the induction of axillary shoots were elongated shoots (1-2 cm in length) from Stage 1. Details of the standardisation of basic proliferation medium utilizing auxins (NAA) and cytokinins (BA, KIN) are presented in Table 6.

Observations on the percentage of cultures developing shoots and number of shoots produced per culture were recorded on five replications after three weeks of culturing.

Standardisation of medium supplements

Studies were conducted to determine the effect of CW on proliferation of shoots from axillary buds of three Dendrobium spp. by adding CW at the rate of 0, 5, 10 and 15 per cent. VW medium containing NAA 2 ppm and BA 3 ppm was used for the study.

Observations on percentage of culture survival and number of shoots produced per culture from five replications were recorded after three weeks of culturing.

Effect of continuous subculturing on the multiplication rate

Regular subculturing of the proliferated shoots on the medium containing high concentration of BA was found to increase the number of shoots per culture. Therefore a trial was conducted to assess the multiplication rate of shoots on continuous subculturing at three weeks interval in ten serial subcultures.

Table 6. Standardisation of basal proliferation media

Mediaum	Explant	Treatment
MS	Axillary bud	5 levels of BA (1, 2, 3, 4, 5 ppm) and 3 x 3 combinations of NAA (1, 2, 3 ppm) and BA (3, 4, 5 ppm)
VW	,,	-do-
VW	,,	5 levels of KIN (1, 2, 3, 4, 5 ppm) and 2 x 4 combinations of NAA (1, 2 ppm) and KIN (2, 3, 4, 5 ppm)

VW medium containing NAA 2 ppm + BA 3 ppm + CW 15 per cent was used for the study. Cultures derived from axillary buds of three Dendrobium spp. were used:

Observations were recorded on the number of shoots produced per culture and the percentage increase in the number of shoots over the initial culture in four culture tubes per subculture.

2.6.1.3. In vitro rooting (Stage 3)

Trials on the in vitro rooting were conducted in VW medium as well as in half and full strength MS medium. Individual shoots having 2-3 cm length excised from shoot proliferating cultures of D. fimbriatum and D. moschatum were utilised as explants for these trials. Two auxins (IBA and NAA) at two levels (2 and 4 ppm) each, were tried independently for rooting.

Observations on the percentage of cultures showing root initiation, number of days required for root initiation, number of roots produced per shoot and nature of roots were recorded on five cultures after three weeks of culturing.

Standardisation of medium supplements

Effect of various medium supplements on rooting of Dendrobium fimbriatum excised from shoot proliferation cultures was studied in the best medium identified. The details of various compounds and their levels tested are as follows.

Medium supplement	Level (%)
Sucrose	1.5 3.0, 4.5, 6.0
AC	0.10, 0.25, 0.50, 1.00

Observations were recorded on five replications after three weeks of culturing on the percentage of cultures initiating roots, number of days required for root initiation, number of roots produced per shoot and nature of roots

The following observations were recorded on the growth parameters of D. fimbriatum and D. moschatum at the time of planting out.

Plant height

Length from the collar region to the tip of the plantlet was measured and expressed in cm.

Number of leaves per plant

The total number of leaves borne by a plantlet was counted and recorded.

Length of the longest leaf

The length was taken from the base to the tip of the leaf and expressed in cm.

Number of roots per plant

The total number of roots per plantlet was counted and recorded.

Average length of the root

Length of the root was measured from the collar region to the tip and the mean length was expressed in cm.

2.6.1.4. Planting out and acclimatization (Stage 4)

The plug of the culture vessel was removed and a weak solution of Bavistin (0.05 per cent) was added to the culture vessel, shaken gently and allowed to stand for 10-15 minutes. Then the rooted plantlets with 5-8 leaves and 15-20 roots were taken out from culture vessels with the help of forceps. The agar adhering to the roots were completely removed by thorough washing with distilled water.

In order to study the effect of the potting media on the growth of plants the plantlets were planted in potting media:

1. Coconut husk
2. Brick : charcoal (1:1 v/v)
3. Charcoal alone

The coconut husk was beaten well to remove the hard outer layer and to loosen the fibrous material. The husk was then soaked in water for 24 hours. They were then squeezed well to remove any excess water and arranged in two layers in a tray. A hole was then made in the husk with a glass rod and the plantlets were planted.

The brick and charcoal pieces used were of the size between 2 and 4 mm.

The potting media after wetting were autoclaved at 15 psi for 20 min. to make it free from soil borne pathogens. Small clay pots (diameter 8 cm) or a small plastic bowl with sufficient holes for drainage were used for filling the media. The containers were drenched with a weak solution (0.05 per cent) of Bavistin. The plantlets were then planted in the potting media and subjected to post-planting treatments

Standardisation of hardening treatments

In order to acclimatize the plantlets produced in vitro, a trial to standardise the hardening treatments was carried out. The plantlets were subjected to the following post-transfer treatments.

1. Keeping in open
2. Keeping in the culture room
3. Covering the plants and pots with plastic cover with holes for two weeks
4. Covering the plants and pots with a microscope cover for two weeks (Rajmohan, 1985 and Reghunath, 1989)
5. Covering the plants with plastic cover for three days

The covers were removed and the plantlets were kept under partial shade conditions for a longer period. The plantlets were exposed to the sunlight gradually. Water was sprayed frequently to prevent

the plantlets from drying. Observations were made on percentage of plantlet survival after a month of planting out.

2.6.2. Somatic organogenesis (callus mediated)

2.6.2.1. Explant choice

The explants used for initiating callus are given in Table 2.

Culture establishment (Stage 1)

Addition of CW to the basal medium had a desirable effect on the proliferation of callus (Goh, 1970, 1973; Goh et al, 1975). Therefore the explants were cultured in Vacin and Went medium modified with 150 per cent CW supplemented with 3 x 2 levels of NAA (1, 2, 4 ppm) and BA (1, 2 ppm).

Observations were recorded on the percentage of culture establishment from ten cultures after four weeks of culturing.

2.6.2.2. Callus initiation and organogenesis (Stage 2)

The trials were conducted in modified VW medium supplemented with 2,4-D at four levels (0.5, 1.0, 2.0 and 4.0 ppm). The explants showing response in the culture establishment were used for the study.

The following observations were recorded.

Percentage of cultures initiating callus (P)

After four weeks of culturing, the number of cultures showing response out of ten cultures was noted and recorded as percentage.

Growth of the callus (G)

It was assessed based on a visual rating (with score 1 to the smallest and score 4 to the largest callus). The mean score was expressed as the growth score.

Callus Index (CI)

It was computed by multiplying per cent explants initiating callus (P) with the growth score (G).

Nature of the callus

The colour and the nature (friable, brittle, loose, compact etc.) of the callus were noted and recorded.

As there was no further differentiation of the callus due to drying, the study was not pursued.

2.6.3. Statistical analysis

The data generated from the various experiments were subjected to statistical analysis in completely randomised design, wherever necessary, following Panse and Sukhatme (1985).

Results

RESULTS

The results generated from the *in vitro* propagation studies of *Dendrobium* spp. through enhanced release of axillary buds and somatic organogenesis (callus mediated) conducted at the Plant Tissue Culture Laboratory attached to the All India Co-ordinated Floriculture Improvement Project, College of Horticulture, Vellanikkara, are presented in this chapter.

4.1. Surface sterilization

The results of the trial on the standardisation of surface sterilization of various explants used for the study are presented in Table 7.

Of the various sterilants tried, mercuric chloride (all levels) gave better results than sodium hypochlorite and bleaching powder. The lowest rate of contamination and the highest percentage of explant survival was observed when the explants were treated with 0.1 per cent mercuric chloride for 10 minutes. The rate of contamination at this level was only 10 per cent, each, in shoot tip, axillary bud and leaf explants. The inflorescence stalk and root segments, on the other hand showed 20 and 40 per cent contamination, respectively. The culture survival was 50, 80, 20, 40 and 40 per cent, respectively in shoot tip, axillary bud, inflorescence stalk, leaf segments and root segments.

Table 7. Standardisation of surface sterilization in various explants of Dendrobium

Basal medium - VW

Explant	Sterilant	Concentration*** (%)	Duration (min)	Contamination (%)	Survival (% culture alive)
*Shoot tip	Bleaching powder (CI)	4.00	10	100	0
	Mercuric chloride	0.05	10	50	10
	,,	0.10	10	10	50
	,,	0.20	10	50	10
	Sodium hypochlorite	4.00	20	70	0
	,,	4.00	30	80	0
*Axillary bud	Bleaching powder (CI)	4.00	10	100	0
	Mercuric chloride	0.05	10	60	20
	,,	0.10	10	10	80
	,,	0.20	10	40	30
	Sodium hypochlorite	4.00	20	80	10
	,,	4.00	30	80	0
**Inflorescence stalk	Bleaching powder (CI)	4.00	10	100	10
	Mercuric chloride	0.05	10	60	20
	,,	0.10	10	20	20
	,,	0.20	10	50	10
	Sodium hypochlorite	4.00	20	80	0
	,,	4.00	30	80	0
**Leaf segments	Bleaching powder (CI)	4.00	10	100	0
	Mercuric chloride	0.05	3	40	10
	,,	0.10	3	10	40
	,,	0.20	3	20	20
	Sodium hypochlorite	4.00	20	50	0
	,,	4.00	30	50	0
**Root	Bleaching powder (CI)	4.00	10	100	0
	Mercuric chloride	0.05	10	60	20
	,,	0.10	10	40	40
	,,	0.20	10	50	30
	Sodium hypochlorite	4.00	20	70	0
	,,	4.00	30	70	0

Culture period - three weeks

*Average of ten observations each of D. fimbriatum, D. moschatum and D. nobile

**Average of ten replications of D. moschatum

***Mercuric chloride on w/v basis
Sodium hypochlorite on v/v basis

Use of sodium hypochlorite and bleaching powder resulted in very high contamination rate and low survival percentage. Bleaching or discoloration of the explants also resulted when sodium hypochlorite or bleaching powder was used.

4.2. Season of explant collection on culture establishment

Data pertaining to the influence of season on explant collection and culture establishment of the three species of Dendrobium in VW medium are presented in Table 8 and Fig. 2.

The results indicated that during the period from January to June the contamination rate was the lowest and the explant survival was the highest, compared to the period from July to December in all the explants tried. The explant collection during April resulted in the lowest contamination rate and the highest explant survival in shoot tip (40 and 30 per cent, respectively), axillary bud (10 and 90 per cent, respectively) and leaf segments (10 and 20 per cent, respectively).

4.3. Enhanced release of axillary buds

4.3.1. Explant choice

Data recorded from the trial conducted for screening the various explants of Dendrobium for initiating enhanced release of axillary buds are presented in Table 9.

Table 8. Influence of season of collection of explants on establishment of culture in Dendrobium

Basal medium : VW

Month	*Shoot tip		*Axillary bud		**Leaf segments	
	Contamination (%)	Survival (% cultures alive)	Contamination (%)	Survival (% cultures alive)	Contamination (%)	Survival (% cultures alive)
1990						
January	60	10	40	60	40	0
February	60	10	40	60	50	0
March	50	10	40	60	20	20
April	40	30	10	90	10	20
May	50	20	20	80	70	20
June	60	10	30	70	60	10
July	70	10	60	40	100	0
August	70	0	50	50	100	0
September	70	10	40	60	70	10
October	80	10	50	50	80	0
November	100	0	90	10	100	0
December	100	0	90	10	100	0

Culture period - Three weeks

* Average of ten replications each of D. fimbriatum, D. moschatum and D. nobile

**Average of ten replications of D. moschatum

Fig. 2. Influence of season of collection of explants on culture establishment in Dendrobium spp.

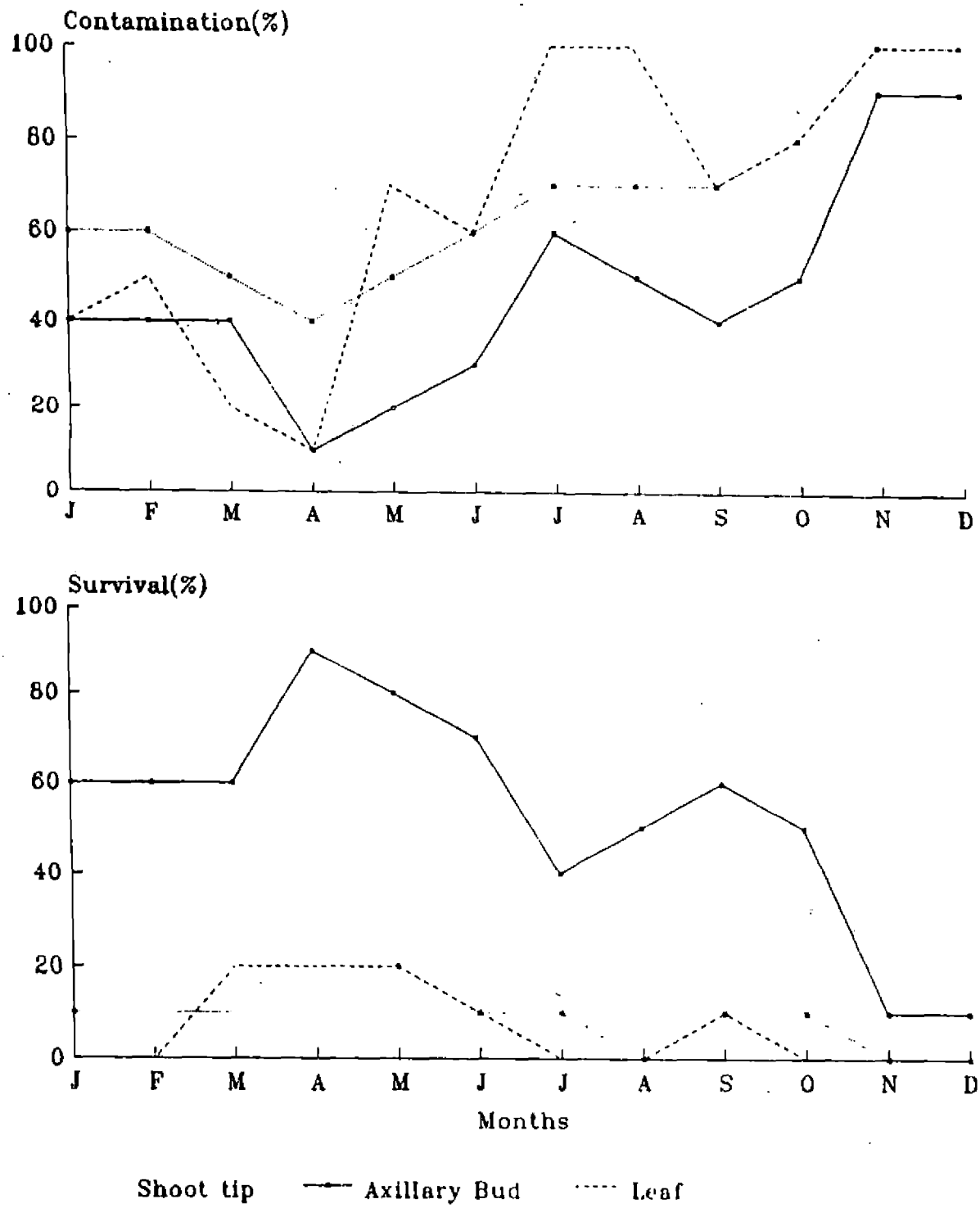


Table 9. Response of various explants of Dendrobium on initiating axillary bud release

Basal medium : VW

Explant	Cultures exhibiting shoot proliferation*
Shoot tip	10
Axillary bud	100
Inflorescence stalk**	0

* Average of ten replications
**Only one species (D. moschatum) was tried
0 - No response
Culture period - three weeks

The results indicated that the axillary bud explant was the most suitable wherein all the cultures initiated shoot proliferation, as compared to shoot tip (10 per cent) and inflorescence stalk (0 per cent). Therefore further trials were conducted using axillary buds as the explant.

4.3.2. Culture establishment (Stage 1)

Data pertaining to the effect of different levels of NAA and BA in the KC, MS and VW media in the three species of Dendrobium are furnished in Tables 10, 11 and 12.

All the surviving explants started swelling of the buds (bud initiation) within a period ranging from 9 to 50 days, depending on the media and the combination of growth regulators used.

4.3.2.1. Effect of KC medium

All the combinations of NAA and BA recorded bud initiation and elongation in this medium (Table 10). Significant difference could be noticed between different combinations tried in all the species of Dendrobium, except for bud elongation in D. nobile.

In D. fimbriatum. T_8 (NAA 1.5 ppm + BA 1.0 ppm) recorded minimum number of days for bud initiation (31) which was on par with T_3 (NAA 0.5 ppm + BA 1.5 ppm), T_6 (NAA 1.0 ppm + BA 1.5 ppm) and T_9 (NAA 1.5 ppm + BA 1.5 ppm) and significantly superior to all other treatments. T_2 (NAA 0.5 ppm + BA 1.0 ppm) recorded

Table 10. *Effect of different levels of NAA and BA on the culture establishment in* Dendrobium spp.

Basal medium - KC
Explant - Axillary bud

Treatment	Period (days) for culture establishment*					
	D. fimbriatum		D. moschatum		D. nobile	
	Bud initiation	Bud elongation	Bud initiation	Bud elongation	Bud initiation	Bud elongation
1. NAA 0.5 ppm + BA 0.5 ppm	49	59	48	48	37	52
2. ,, + BA 1.0 ppm	55	58	47	50	51	48
3. ,, + BA 1.5 ppm	38	54	43	48	46	50
4. NAA 1.0 ppm + BA 0.5 ppm	46	48	37	41	48	42
5. ,, + BA 1.0 ppm	47	68	33	39	50	46
6. ,, + BA 1.5 ppm	35	62	37	45	39	43
7. NAA 1.5 ppm + BA 0.5 ppm	44	49	32	36	46	47
8. ,, + BA 1.0 ppm	31	38	31	35	31	39
9. ,, + BA 1.5 ppm	39	40	34	39	43	49
CD (0.05)	8.70	4.50	2.22	1.10	6.78	NS
SEm±	3.03	1.60	0.80	0.37	2.40	2.90

*Values taken as average of five replications

maximum number of days (55) for bud initiation which was on par with T_1 (NAA 0.5 ppm + BA 0.5 ppm) and T_5 (NAA 1.0 ppm + BA 1.0 ppm). For bud elongation also T_8 (NAA 1.5 ppm + BA 1.0 ppm) recorded the minimum number of days (38) which was on par with T_9 (NAA 1.5 ppm + BA 1.5 ppm). T_5 (NAA 1.0 ppm + BA 1.0 ppm) recorded the maximum number of days (68) which was significantly different from all other treatments.

For bud initiation in D. moschatum, T_8 (NAA 1.5 ppm + BA 1.0 ppm) took minimum number of days (31) and was on par with T_5 (NAA 1.0 ppm + BA 1.0 ppm) and T_7 (NAA 1.5 ppm + BA 0.5 ppm). Maximum duration (48 days) was observed in T_1 (NAA 0.5 ppm + BA 0.5 ppm) which was on par with T_2 (NAA 0.5 ppm + BA 1.0 ppm). In the case of bud elongation, T_8 (NAA 1.5 ppm + BA 1.0 ppm) was significantly superior to other treatments and was on par with T_7 (NAA 1.5 ppm + BA 0.5 ppm). T_2 (NAA 0.5 ppm + BA 1.0 ppm) recorded maximum number of days and was significantly different from all other treatments.

With regard to D. nobile T_8 (NAA 1.5 ppm + BA 1.0 ppm) took the shortest duration (31 days) for bud initiation which was significantly superior to all other treatments. T_2 (NAA 0.5 ppm + BA 1.0 ppm) recorded 51 days which was the longest period for bud initiation and was on par with all others except T_6 (NAA 1.0 ppm + BA 1.5 ppm) T_8 (NAA 1.5 ppm + BA 1.0 ppm) and T_9 (NAA 1.5 ppm + BA 1.5 ppm). There was no significant difference between the treatments tried for bud elongation.

4.3.2.2. Effect of MS medium

Data pertaining to the culture establishment in MS medium are presented in Table 11. Significant differences were obtained in all the three species with respect to both bud initiation and bud elongation.

In the case of bud initiation in D. fimbriatum. minimum number of days (25) was recorded by T_8 (NAA 1.5 ppm + BA 1.0 ppm) which was on par with T_3 (NAA 0.5 ppm + BA 1.5 ppm), T_4 (NAA 1.0 ppm + BA 0.5 ppm), T_6 (NAA 1.0 ppm + BA 1.5 ppm), T_7 (NAA 1.5 ppm + BA 0.5 ppm) and T_9 (NAA 1.5 ppm + BA 1.5 ppm) and maximum by T_5 (NAA 1.0 ppm + BA 1.0 ppm) (46 days) which was on par with T_1 (NAA 0.5 ppm + BA 0.5 ppm) and T_2 (NAA 0.5 ppm + BA 1.0 ppm). T_8 (NAA 1.5 ppm + BA 1.0 ppm) recorded the minimum number of days (38) for bud elongation, was on par with T_4 (NAA 1.0 ppm + BA 0.5 ppm) and significantly superior to all other treatments. Maximum number of days (69) was recorded by T_1 (NAA 0.5 ppm + BA 0.5 ppm) which was on par with T_2 (NAA 0.5 ppm + BA 1.0 ppm) and T_5 (NAA 1.0 ppm + BA 1.0 ppm).

In D. moschatum, T_8 (NAA 1.5 ppm + BA 1.0 ppm) recorded minimum number of days for both bud initiation and elongation (9 and 30 days, respectively) and was significantly superior to all other treatments. Longest duration was recorded by T_1 (NAA 0.5 ppm + BA 0.5 ppm) for bud initiation (48 days) and by T_4 (NAA 1.0 ppm + BA 0.5 ppm) for bud elongation (76 days), which were significantly different from all other treatments.

Table 11. Effect of different levels of NAA and BA on the culture establishment in Dendrobium spp.
Basal medium - MS
Explant - Axillary bud

Treatment	Period (days) for culture establishment*					
	D. fimbriatum		D. moschatum		D. nobile	
	Bud initiation	Bud elongation	Bud initiation	Bud elongation	Bud initiation	Bud elongation
1. NAA 0.5 ppm + BA 0.5 ppm	45	69	48	64	44	47
2. ,, + BA 1.0 ppm	40	63	42	48	37	63
3. ,, + BA 1.5 ppm	32	54	42	50	42	53
4. NAA 1.0 ppm + BA 0.5 ppm	25	47	37	76	41	64
5. ,, + BA 1.0 ppm	46	67	32	45	34	73
6. ,, + BA 1.5 ppm	30	53	23	51	33	51
7. NAA 1.5 ppm + BA 0.5 ppm	32	57	25	48	45	67
8. ,, + BA 1.0 ppm	25	38	9	30	27	33
9. ,, + BA 1.5 ppm	30	53	26	45	33	45
CD (0.05)	9.20	10.69	5.40	7.97	2.60	8.10
SEm±	3.21	3.73	1.90	2.78	0.91	2.83

*Values taken as average of five replications

In D. nobiie also, T_8 (NAA 1.5 ppm + BA 1.0 ppm) was significantly superior to all other treatments and took only 27 and 33 days, respectively for bud initiation and elongation. Maximum number of days for bud initiation (45) was recorded by T_7 (NAA 1.5 ppm + BA 0.5 ppm) which was on par with T_1 (NAA 0.5 ppm + BA 0.5 ppm). For bud elongation T_5 (NAA 1.0 ppm + BA 1.0 ppm) recorded the longest duration (73 days) which was on par with T_7 (NAA 1.5 ppm + BA 0.5 ppm).

4.3.2.3. Effect of VW medium

Establishment of cultures occurred in all the nine combinations of NAA and BA tried for all the species of Dendrobium in this medium. Data pertaining to the observations recorded are presented in Table 12.

For bud initiation in D. fimbriatum T_8 (NAA 1.5 ppm + BA 1.0 ppm) recorded the minimum number of days (24) and was on par with T_6 (NAA 1.0 ppm + BA 1.5 ppm). Maximum number of days (50) for bud initiation was recorded by T_2 (NAA 0.5 ppm + BA 1.0 ppm) which was on par with T_1 (NAA 0.5 ppm + BA 0.5 ppm) and T_5 (NAA 1.0 ppm + BA 1.0 ppm). With respect to the period taken for bud elongation also T_8 (NAA 1.5 ppm + BA 1.0 ppm) was superior to other treatments (33 days) and was on par with T_9 (NAA 1.5 ppm + BA 1.5 ppm). Maximum number of days (63) was taken by T_5 (NAA 1.0 ppm + BA 1.0 ppm) which was significantly different from all other treatments.

Table 12. Effect of different levels of NAA and BA on the culture establishment in Dendrobium spp.

Basal medium - VW
Explant - Axillary bud

Treatment	Period (days) for culture establishment*					
	D. fimbriatum		D. moschatum		D. nobile	
	Bud initiation	Bud elongation	Bud initiation	Bud elongation	Bud initiation	Bud elongation
1. NAA 0.5 ppm + BA 0.5 ppm	45	54	46	45	42	35
2. ,, + BA 1.0 ppm	50	53	43	50	46	43
3. ,, + BA 1.5 ppm	33	49	37	40	41	45
4. NAA 1.0 ppm + BA 0.5 ppm	41	43	47	35	43	34
5. ,, + BA 1.0 ppm	42	63	50	45	45	41
6. ,, + BA 1.5 ppm	26	57	31	47	34	38
7. NAA 1.5 ppm + BA 0.5 ppm	39	44	37	48	41	42
8. ,, + BA 1.0 ppm	24	33	21	31	28	32
9. ,, + BA 1.5 ppm	34	35	29	45	38	44
CD (0.05)	8.62	4.50	9.72	6.01	7.07	7.80
SEm±	3.01	1.57	3.39	2.10	2.50	2.72

*Values taken as average of five replications

With regard to D. moschatum minimum number of days (21) was recorded by T_8 (NAA 1.5 ppm + BA 1.0 ppm) for bud initiation which was on par with T_4 (NAA 1.0 ppm + BA 0.5 ppm). For bud initiation the maximum number of days (50) was recorded by T_5 (NAA 1.0 ppm + BA 1.0 ppm) which was on par with T_1 (NAA 0.5 ppm + BA 0.5 ppm), T_2 (NAA 0.5 ppm + BA 1.0 ppm) and T_4 (NAA 1.0 ppm + BA 0.5 ppm). T_2 (NAA 0.5 ppm + BA 1.0 ppm) recorded the maximum number of days (50) for bud elongation and was significantly different from T_3 (NAA 0.5 ppm + BA 1.5 ppm), T_4 (NAA 1.0 ppm + BA 0.5 ppm) and T_8 (NAA 1.5 ppm + BA 1.0 ppm).

In the case of D. nobile, T_8 (NAA 1.5 ppm + BA 1.0 ppm) recorded the minimum number of days for bud initiation and elongation (28 and 32, respectively). It was on par with T_6 (NAA 1.0 ppm + BA 1.5 ppm) for bud initiation and with T_1 (NAA 0.5 ppm + BA 0.5 ppm), T_4 (NAA 1.0 ppm + BA 0.5 ppm) and T_6 (NAA 1.0 ppm + BA 1.5 ppm) for bud elongation. Maximum number of days (46) was recorded by T_2 (NAA 0.5 ppm + BA 1.0 ppm) for bud initiation and T_3 (NAA 0.5 ppm + BA 1.5 ppm) (45) for bud elongation.

4.3.2.4. Comparative performance of Dendrobium spp. on different culture media

Data pertaining to the performance of the three species of Dendrobium (D. frimbriatum, D. moschatum and D. nobile) on the three culture media (KC, MS and VW) with respect to the culture establishment of the axillary bud explant are presented in Table 13.

Table 13. Comparative performance of Dendrobium spp. on different culture media

Explant - Axillary bud
NAA 1.5 ppm + BA 1.0 ppm

Medium/species	Period (days) for culture establishment*	
	Bud initiation	Bud elongation
KC		
D. fimbriatum	31	38
D. moschatum	31	35
D. nobile	31	42
MS		
D. fimbriatum	25	38
D. moschatum	9	30
D. nobile	27	33
VW		
D. fimbriatum	24	33
D. moschatum	21	31
D. nobile	28	32
CD (0.05)	3.61	7.14
SEm±	1.26	2.50

*Values taken as average of five replications

D. moschatum in MS medium was found to be significantly superior to all other treatments with respect to the duration for bud initiation (9 days). This was followed by D. fimbriatum and D. moschatum in VW medium which were on par with each other, recording a duration of 24 and 21 days, respectively. The longest period for bud initiation (31 days, each) was taken by all the three species on KC medium.

Bud elongation occurred within the minimum number of days (30) in D. moschatum in MS medium, which was on par with all the three species tried in VW medium, with D. moschatum in KC medium and with D. nobile in MS medium. Maximum number of days (42) was recorded by D. nobile in KC medium, which was on par with D. fimbriatum and D. moschatum in KC medium and with D. fimbriatum in MS medium.

4.3.3. Shoot proliferation (Stage 2)

Data pertaining to the observations recorded on the standardisation of basal proliferation medium utilising auxins and cytokinins are presented in Tables 14 and 15.

4.3.3.1. Effect of MS (half strength of inorganic salts) medium

In the trials conducted during the initial cultures, leaf tip necrosis was observed when the medium had full strength of both inorganic salts and organic growth factors (Plate 4). Therefore, for further subcultures MS medium containing half the strength inorganic salts and full strength of organic growth factors was used.

Plate 4. Comparison of full strength (left) and half strength (right) MS on shoot proliferation, showing leaf tip necrosis in the former

Plate 5. Effect of NAA 2 ppm + BA 5 ppm (left) and NAA 2 ppm + BA 3 ppm (right) on shoot proliferation of D. fimbriatum in $\frac{1}{2}$ MS

The effect of BA alone or in combination with NAA was significant on the induction of multiple shoots, in all the three species of Dendrobium tried (Table 14).

In D. fimbriatum, T_{12} (NAA 2 ppm + BA 5 ppm) was significantly superior to all other treatments producing 5.6 shoots on average, but was on par with T_{10} (NAA 2 ppm + BA 3 ppm), T_{11} (NAA 2 ppm + BA 4 ppm) and T_{15} (NAA 3 ppm + BA 5 ppm). Minimum number of shoots (1.0) was recorded by T_1 (control) which was on par with T_2 (BA 1 ppm), T_3 (BA 2 ppm) and T_5 (BA 4 ppm).

In D. moschatum, maximum number of shoots (5.8) was recorded by T_{12} (NAA 2 ppm + BA 5 ppm) which was on par with T_{10} (NAA 2 ppm + BA 3 ppm) and significantly superior to all other treatments. The minimum number of shoots (1.0) was recorded by T_1 (control) which was on par with T_3 (BA 2 ppm) and T_5 (BA 4 ppm).

In D. nobile, maximum number of shoots (5.0) was produced by T_{12} (NAA 2 ppm + BA 5 ppm) which was on par with T_{10} (NAA 2 ppm + BA 3 ppm), T_{11} (NAA 2 ppm + BA 4 ppm) and T_{14} (NAA 3 ppm + BA 4 ppm) and significantly superior to all other treatments. T_1 (control) recorded the minimum number of shoots (1.0) per culture.

Though maximum number of shoots in all the three species was produced by T_{12} (NAA 2 ppm + BA 5 ppm), the shoots were compressed and often malformed in appearance (Plate 5). Though T_{10} (NAA 2 ppm + BA 3 ppm) was on par with T_{12} (NAA 2 ppm + BA

Table 14. Effect of BA alone and in combination with NAA on multiple shoot formation in Dendrobium spp.

Basal medium - ½ MS
Explant - Axillary bud

Treatment	D. fimbriatum		D. moschatum		D. nobile	
	Percentage of cultures developing shoots	Shoots/ culture	Percentage of cultures developing shoots	Shoots/ culture	Percentage of cultures developing shoots	Shoots/ culture
1. Control	100	1.0	100	1.0	100	1.0
2. BA 1 ppm	100	1.2	100	2.4	100	2.0
3. BA 2 ppm	100	2.0	100	2.0	100	2.0
4. BA 3 ppm	100	2.8	100	2.6	100	3.0
5. BA 4 ppm	100	2.2	100	1.2	100	2.4
6. BA 5 ppm	100	2.8	100	3.0	100	3.0
7. NAA 1 ppm + BA 3 ppm	100	3.8	100	2.4	100	2.0
8. ,, + BA 4 ppm	100	3.2	100	2.8	100	2.4
9. ,, + BA 5 ppm	100	3.8	100	3.0	100	3.0
10. NAA 2 ppm + BA 3 ppm	100	5.4	100	4.8	100	4.2
11. ,, + BA 4 ppm	100	5.0	100	4.0	100	4.0
12. ,, + BA 5 ppm	100	5.6	100	5.8	100	5.0
13. NAA 3 ppm + BA 3 ppm	100	3.0	100	3.0	100	3.4
14. ,, + BA 4 ppm	100	3.0	100	3.4	100	3.8
15. ,, + BA 5 ppm	100	4.4	100	4.0	100	3.2
CD (0.05)		1.30		1.30		1.50
SEm±		0.45		0.50		0.52

Values taken as average of five replications
Culture period - three weeks

5 ppm), the shoots produced were normal and healthy. The number of shoots produced by this treatment in D. fimbriatum, D. moschatum and D. nobile were 5.4, 4.8 and 4.2, respectively. All the 15 treatments recorded cent per cent culture proliferation.

4.3.3.2. Effect of VW medium

In the trial conducted using VW as the basal medium the effect of BA alone or in combination with NAA was significant on the induction of multiple shoots in all the three species, as evidenced from Table 15.

In D. fimbriatum maximum number of shoots (27.8) was produced by T_{14} (NAA 2 ppm + BA 5 ppm) which was significantly superior to all other treatments. The minimum number of shoots (1.0) was recorded by T_1 (control) which was on par with T_2 (BA 1 ppm), T_3 (BA 2 ppm), T_5 (BA 4 ppm), T_7 (NAA 1 ppm + BA 2 ppm), T_9 (NAA 1 ppm + BA 4 ppm), T_{10} (NAA 1 ppm + BA 5 ppm) and T_{11} (NAA 2 ppm + BA 2 ppm).

In D. moschatum, T_{14} (NAA 2 ppm + BA 5 ppm) recorded maximum number of shoots (13.0) per culture which was on par with T_{12} (NAA 2 ppm + BA 3 ppm). T_1 (control) produced minimum number of shoots (1.0) per culture and was on par with all other treatments, except T_4 (BA 3 ppm), T_6 (BA 5 ppm), T_{12} (NAA 2 ppm + BA 3 ppm), T_{13} (NAA 2 ppm + BA 4 ppm) and T_{14} (NAA 2 ppm + BA 5 ppm).

Table 15. Effect of BA alone and in combination with NAA on multiple shoot formation in *Dendrobium* spp.

Basal medium - VW
Explant - Axillary bud

Treatment	*D. fimbriatum*		*D. moschatum*		*D. nobile*	
	Percentage of cultures developing shoots	Shoots/ culture	Percentage of cultures developing shoots	Shoots/ culture	Percentage of cultures developing shoots	Shoots/ culture
1. Control	100	1.0	100	1.0	100	1.0
2. BA 1 ppm	100	2.2	100	2.4	100	3.0
3. BA 2 ppm	100	3.4	100	1.8	100	2.8
4. BA 3 ppm	100	8.4	100	5.2	100	5.6
5. BA 4 ppm	100	3.0	100	3.2	100	2.6
6. BA 5 ppm	100	8.4	100	5.4	100	4.6
7. NAA 1 ppm + BA 2 ppm	100	2.0	100	1.4	100	2.8
8. ,, + BA 3 ppm	100	10.2	100	4.0	100	6.4
9. ,, + BA 4 ppm	100	3.2	100	2.4	100	3.0
10. ,, + BA 5 ppm	100	2.8	100	2.8	100	3.0
11. NAA 2 ppm + BA 2 ppm	100	3.0	100	2.2	100	6.0
12. ,, + BA 3 ppm	100	18.8	100	10.8	100	7.6
13. ,, + BA 4 ppm	100	16.0	100	6.4	100	5.2
14. ,, + BA 5 ppm	100	27.8	100	13.0	100	10.2
CD (0.05)		6.30		3.98		2.03
SEm±		2.23		1.42		0.73

Values taken as average of five replications
Culture period - three weeks

Table 16. Comparative performance of Dendrobium spp. on multiple shoot formation

Treatment/species	Explant - Axillary bud NAA 2 ppm + BA 3 ppm Percentage of cultures developing shoots	Shoots/ culture*
MS		
D. fimbriatum	100	5.4
D. moschatum	100	4.8
D. nobile	100	4.2
VW		
D. fimbriatum	100	18.8
D. moschatum	100	10.8
D. nobile	100	7.6
CD (0.05)		3.60
SEm±		1.23

*Values taken as average of five replications
Culture period - three weeks

In the case of D. nobile, T_{14} (NAA 2 ppm + BA 5 ppm) recorded maximum number of shoots (10.2) and was significantly superior to all other treatments. T_1 (control) recorded lowest number of shoots (1.0) per culture which was on par with T_2 (BA 1 ppm), T_3 (BA 2 ppm), T_5 (BA 4 ppm), T_7 (NAA 1 ppm + BA 2 ppm), T_9 (NAA 1 ppm + BA 4 ppm) and T_{10} (NAA 1 ppm + BA 5 ppm).

The shoots produced by T_{14} (NAA 2 ppm + BA 5 ppm) were compressed and unhealthy. T_{12} (NAA 2 ppm + BA 3 ppm), which was on par with T_{14} (NAA 2 ppm + BA 5 ppm) in D. moschatum and was next to T_{14} (NAA 2 ppm + BA 5 ppm) in D. fimbriatum and D. nobile produced normal and healthy shoots. D. fimbriatum, D. moschatum and D. nobile recorded 18.8, 10.8 and 7.6 shoots per culture, respectively, as influenced by T_{12} (NAA 2 ppm + BA 3 ppm).

4.3.3.3. Comparative performance of Dendrobium spp in different media

Data pertaining to the comparative performance of the three species of Dendrobium on multiple shoot production in the two media are given in Table 16. When the number of healthy shoots produced in MS (half strength) and VW media were considered, a treatment combination of NAA 2 ppm + BA 3 ppm proved to be the best for the three species of Dendrobium.

Table 16. Comparative performance of Dendrobium spp. on multiple shoot formation

Treatment/species	Explant - Axillary bud NAA 2 ppm + BA 3 ppm Percentage of cultures developing shoots	Shoots/ culture*
MS		
D. fimbriatum	100	5.4
D. moschatum	100	4.8
D. nobile	100	4.2
VW		
D. fimbriatum	100	18.8
D. moschatum	100	10.8
D. nobile	100	7.6
CD (0.05)		3.60
SEm±		1.23

*Values taken as average of five replications
Culture period - three weeks

Maximum number of shoots (18.8) was produced by D. fimbriatum in VW medium, which was significantly superior to all other treatments tried. D. nobile in MS (half strength) medium recorded minimum number of shoots (4.2) which was on par with the shoots produced by D. fimbriatum and D. moschatum in MS (half strength) medium and D. nobile in VW medium.

The results of the study conducted on the proliferation of shoots revealed that the performance of all the three species was better in VW medium. Hence for further trials VW was used as the basal proliferation medium.

4.3.3.4. Effect of KIN alone and in combination with NAA

Data on the independent influence of KIN and that in combination with NAA on the number of shoots produced per culture and the percentage of cultures developing shoots are presented in Table 17.

In D. fimbriatum, T_{14} (NAA 2 ppm + KIN 5 ppm) recorded the maximum number of shoots (6.4) which was on par with T_{12} (NAA 2 ppm + KIN 3 ppm) and was significantly superior to all other treatments. Minimum number of shoots (1.0) was produced by T_1 (control) which was on par with T_2 (KIN 1 ppm).

In the case of D. moschatum, T_{14} (NAA 2 ppm + KIN 5 ppm) recorded a maximum of 5.6 shoots per culture and was on par with T_{10} (NAA 1 ppm + KIN 5 ppm). T_1 (control) recorded the lowest

Table 17. Effect of KIN alone and in combination with NAA on multiple shoot formation in Dendrobium spp.

Basal medium - VW
Explant - Axillary bud

Treatment	D. fimbriatum		D. moschatum		D. nobile	
	Percentage of cultures developing shoots	Shoots/ culture	Percentage of cultures developing shoots	Shoots/ culture	Percentage of cultures developing shoots	Shoots/ culture
1. Control	100	1.0	100	1.0	100	1.0
2. KIN 1 ppm	100	1.8	100	1.2	100	1.2
3. KIN 2 ppm	100	2.8	100	2.4	100	2.0
4. KIN 3 ppm	100	3.6	100	2.8	100	2.4
5. KIN 4 ppm	100	3.2	100	2.4	100	2.2
6. KIN 5 ppm	100	4.2	100	3.2	100	3.0
7. NAA 1 ppm + KIN 2 ppm	100	3.0	100	2.6	100	2.6
8. ,, + KIN 3 ppm	100	3.0	100	2.4	100	4.4
9. ,, + KIN 4 ppm	100	3.0	100	3.0	100	2.6
10. ,, + KIN 5 ppm	100	3.2	100	4.8	100	3.4
11. NAA 2 ppm + KIN 2 ppm	100	4.0	100	5.0	100	4.0
12. ,, + KIN 3 ppm	100	6.0	100	5.2	100	4.6
13. ,, + KIN 4 ppm	100	3.8	100	4.4	100	3.0
14. ,, + KIN 5 ppm	100	6.4	100	5.6	100	5.8
CD (0.05)		1.70		1.40		1.22
SEm±		0.60		0.49		0.43

Values taken as average of five replications
Culture period - three weeks

number of shoots (1.0) which was on par with T_2 (KIN 1 ppm), T_3 (KIN 2 ppm), T_5 (KIN 4 ppm) and T_8 (NAA 1 ppm + KIN 3 ppm).

With respect to D. nobile, T_{14} (NAA 2 ppm + KIN 5 ppm) recorded maximum number of shoots (5.8), which was on par with T_{12} (NAA 2 ppm + KIN 3 ppm) and was significantly superior to all other treatments. T_1 (control) produced only 1.0 shoot per culture which was on par with T_2 (KIN 1 ppm), T_3 (KIN 2 ppm) and T_5 (KIN 4 ppm).

All the 14 treatments showed cent per cent culture proliferation for all the three species.

A comparative effect of different sources of cytokinins (BA and KIN) on the number of shoots per culture in Dendrobium spp is presented in Fig. 3.

4.3.3.5. Effect of medium supplement

Data on the percentage of cultures developing shoots and number of shoots per culture, as influenced by the levels of CW are presented in Table 18.

Of the various levels of CW tried (0, 5, 10 and 15%), that at 15 per cent recorded the highest values in all the three species which was on par with CW at 10 per cent in D. fimbriatum and D. nobile. In D. moschatum it was significantly superior to all other

Fig. 3. Effect of sources of cytokinins on the number of shoots per culture in Dendrobium spp.

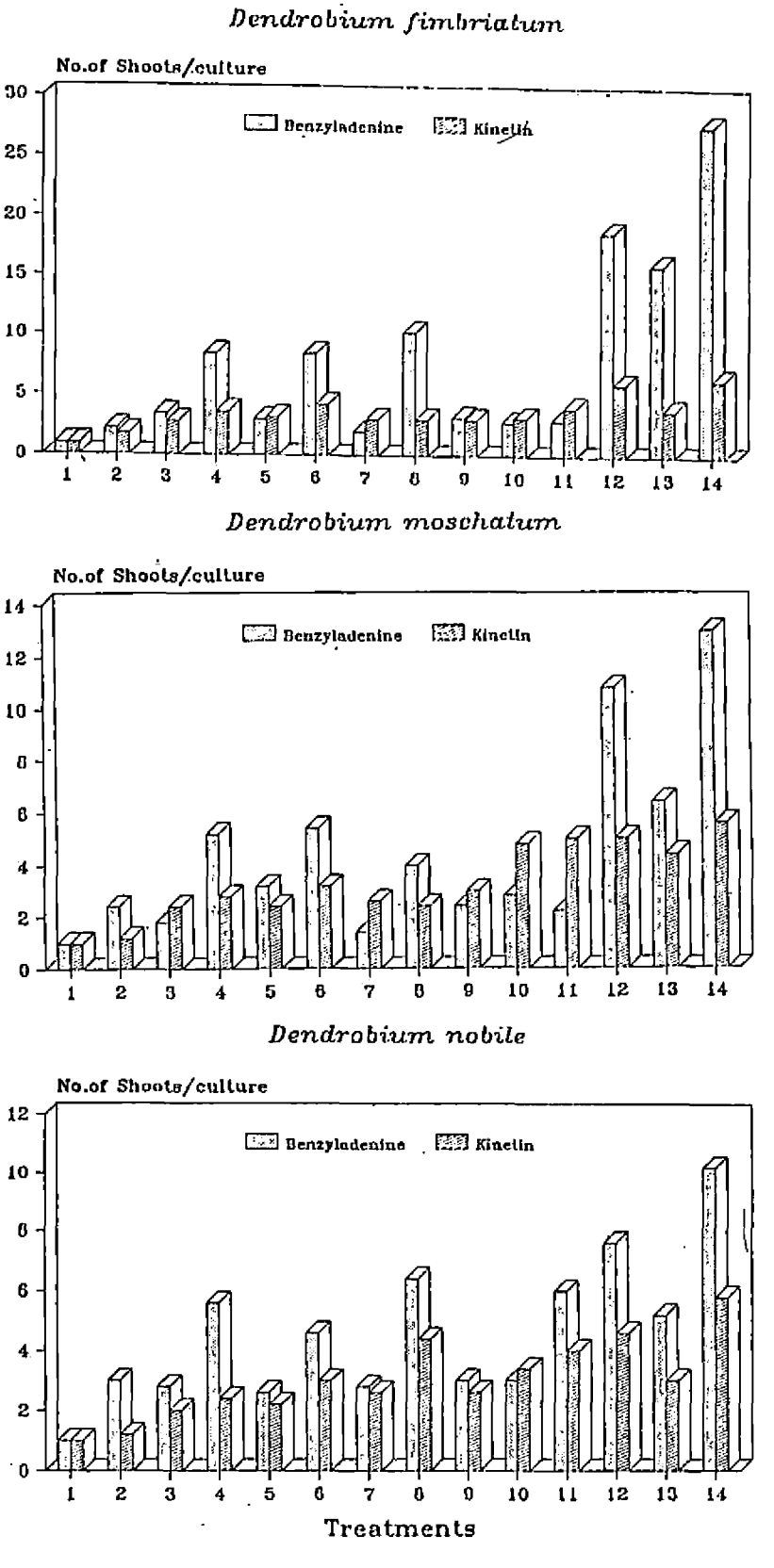


Table 18. Effect of coconut water on shoot proliferation in Dendrobium spp.

Medium - VW + NAA 2 ppm + BA 3 ppm
Explant - Axillary bud

Treatment	D. fimbriatum		D. moschatum		D. nobile	
	Percentage of cultures developing shoots	Shoots/ culture	Percentage of cultures developing shoots	Shoots/ culture	Percentage of cultures developing shoots	Shoots/ culture
CW 0 %	100	12.0	100	8.8	100	4.6
,, 5 %	100	12.6	100	9.4	100	5.8
,, 10 %	100	15.6	100	13.4	100	7.0
,, 15 %	100	19.0	100	18.0	100	8.0
CD (0.05)		4.62		4.21		2.17
SEm±		1.54		1.40		0.73

Values taken as average of five replications
Culture period - three weeks

treatments. The lowest number of shoots was produced by CW at 0 level which was on par with CW at 5 per cent in all the three species of Dendrobium.

The number of shoots produced per culture by CW at 15 per cent in D. fimbriatum, D. moschatum and D. nobile were 19.0, 18.0 and 8.0, respectively. The proliferation of cultures in all the three species of Dendrobium was cent per cent.

4.3.3.6. Effect of continuous subculturing

In a trial conducted to assess the rate of increase or decrease in the multiplication of shoots in axillary bud explants of D. fibriatum, D. moschatum and D. nobile consequent on continuous subculturing at three week interval it was found that the rate of multiplication increased in all the subcultures starting from the third subculture (Table 19 and Fig. 4).

The percentage of increase in number of shoots over the initial culture also increased and the maximum values (44.44, 29.63 and 26.10, respectively) were obtained at the seventh and eighth subcultures for D. fimbriatum, at the eighth subculture for D. moschatum and at the seventh and eighth subcultures for D. nobile. During the course of the ten subcultures, number of shoots with reference to the initial culture increased from 6.75 to 9.75 at the seventh subculture in D. fimbriatum, from 6.75 to 8.75 at the eighth

Table 19. Influence of continuous sub-culturing on the multiplication rate of shoot explants excised from the initial shoot proliferating cultures

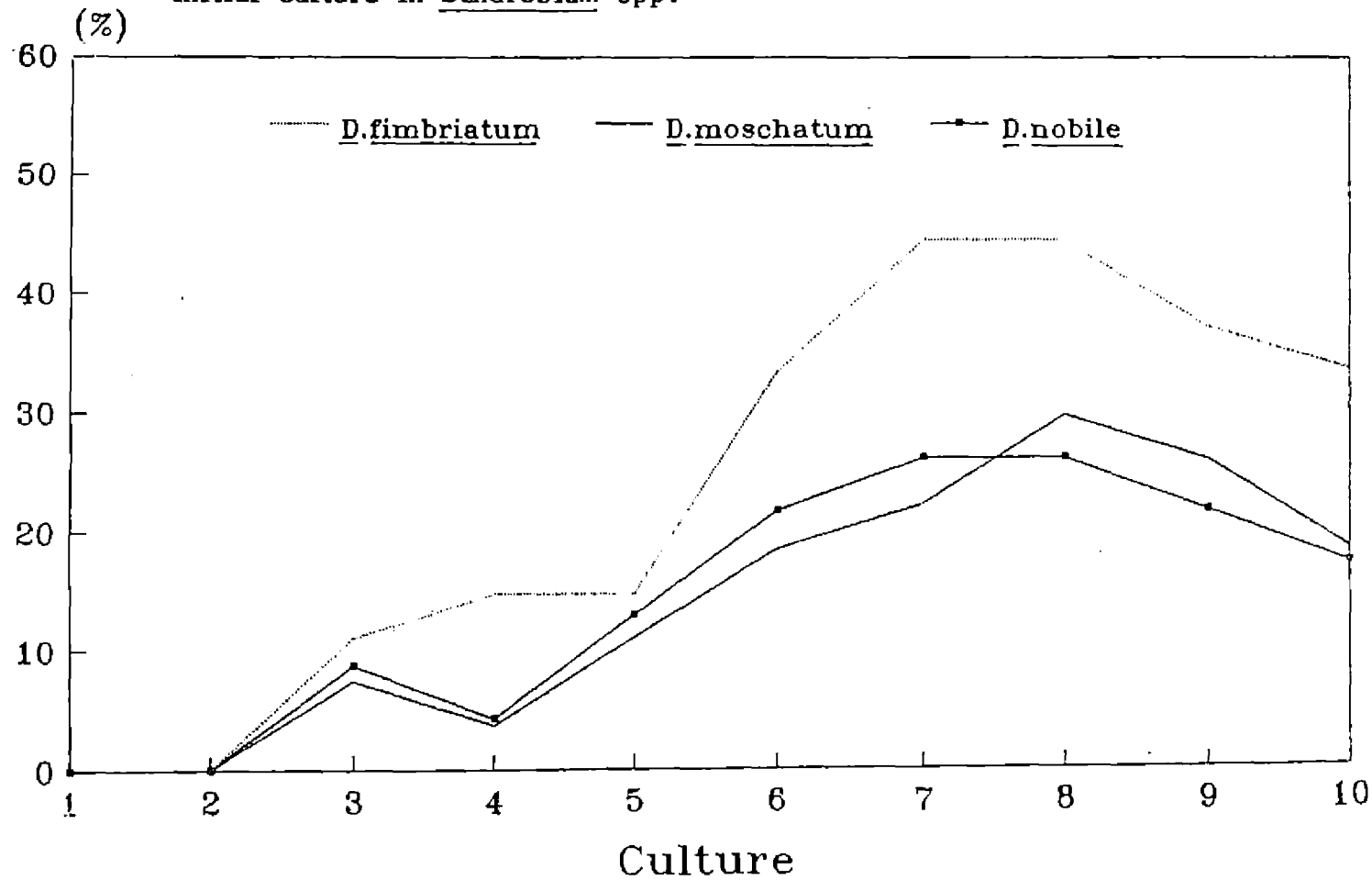
Medium - VW + NAA 2 ppm + BA 3 ppm + CW 15%
Explant - Axillary bud

Culture	Time	Shoots/culture*		
		D. fimbriatum	D. moschatum	D. nobile
1	0	6.75 (-)	6.75 (-)	5.75 (-)
2	3	6.75 (0.0)	6.75 (0.0)	5.75 (0.0)
3	6	7.50 (11.11)	7.25 (7.41)	6.25 (8.70)
4	9	7.75 (14.81)	7.00 (3.70)	6.00 (4.35)
5	12	7.75 (14.81)	7.50 (11.11)	6.50 (13.04)
6	15	9.00 (33.33)	8.00 (18.52)	7.00 (21.74)
7	18	9.75 (44.44)	8.25 (22.22)	7.25 (26.10)
8	21	9.75 (44.44)	8.75 (29.63)	7.25 (26.10)
9	24	9.25 (37.03)	8.50 (25.93)	7.00 (21.74)
10	27	9.00 (33.33)	8.00 (18.52)	6.75 (17.39)
Mean		8.33	7.70	6.60
CD (0.05)		NS	NS	NS
SEm±		0.71	0.67	0.60

* Values taken as average of four replications

Values in parenthesis indicate the percentage of increase of shoots over the initial culture

Fig. 4. Effect of continuous subculturing on the percentage increase of shoots over the initial culture in Dendrobium spp.



subculture in D. moschatum and from 5.75 to 7.25 at the seventh subculture in D. nobile. The variations in the multiplication rate among subcultures were not significant.

From the various trials pursued on the proliferation of shoots in axillary bud explant of the three species of Dendrobium, D. fimbriatum was found to respond well compared to D. moschatum and D. nobile (Plate 6). In D. nobile, the rate of multiple shoot formation was low. The shoots produced were also short and did not have a healthy appearance. Therefore D. nobile was not used for further studies.

4.3.4. In vitro rooting (Stage 3)

Data pertaining to the results of the trials conducted to standardise the media, concentration of auxins (NAA , IBA) and medium supplements for rooting of shoots produced in vitro in the two species of Dendrobium are presented in Tables 20, 21, 22 and 23.

4.3.4.1. Effect of auxins and media

The results of the trial conducted using two auxins (NAA, IBA) at two levels (2 and 4 ppm) in three media (MS half strength, MS full strength and VW) are presented in Table 20. The treatments differed significantly in respect of the rooting of shoots produced in vitro.

Plate 6. Comparative performance of the three species of Dendrobium (from left to right) - D. fimbriatum, D. moschatum and D. nobile

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Table 20. Effect of auxins (NAA and IBA) and media on the rooting of Dendrobium shoots produced in vitro

Treatment	D. fimbriatum			D. moschatum			Nature of roots
	Root initiation (%)	Days for root initiation	Roots/ shoot	Root initiation (%)	Days for root initiation	Roots/ shoot	
1. MS[a] + NAA 2 ppm	100	27	5.0	100	28	3.2	Very short, thick roots
2. MS[a] + NAA 4 ppm	100	27	5.2	100	25	5.0	Short, thick roots
3. MS[a] + IBA 2 ppm	100	28	6.0	100	28	5.8	Short, thin long roots
4. MS[a] + IBA 4 ppm	100	27	7.2	100	28	6.6	Long, thin, fibrous roots
5. MS[b] + NAA 2 ppm	100	27	6.2	100	26	4.2	Short, thick roots
6. MS[b] + NAA 4 ppm	100	25	6.6	100	24	5.0	Thick, long roots
7. MS[b] + IBA 2 ppm	100	24	7.8	100	25	6.0	Short, thin roots
8. MS[b] + IBA 4 ppm	100	21	8.2	100	24	7.4	Long, fibrous roots
9. VW + NAA 2 ppm	30	37	2.8	30	34	3.2	Very short, thick roots
10. VW + NAA 4 ppm	35	40	3.6	40	35	3.0	Short, thick roots
11. VW + IBA 2 ppm	33	46	2.4	20	33	2.0	Short, thin, fibrous roots
12. VW + IBA 4 ppm	30	35	2.4	30	30	2.0	Long, thin, fibrous roots
CD (0.05)		3.92	1.75		3.21	1.80	
SEm±		1.40	0.62		1.14	0.64	

Values taken as average of five replications
a - Medium containing full concentration of both inorganic salts and organic growth factors
b - Medium containing half concentration of inorganic salts and full concentration of organic growth factors

With regard to the days taken for initiation of roots in D. fimbriatum, T_8 (MS^b + IBA 4 ppm) recorded the minimum number of days (21) which was on par with T_7 (MS^b + IBA 2 ppm) and significantly superior to others. T_{11} (VW + IBA 2 ppm) took the maximum number of days (46) and was on par with T_9 (VW + NAA 2 ppm).

In D. moschatum also, T_8 (MS^b + IBA 4 ppm) recorded the shortest duration (24 days) for root initiation which was on par with T_2 (MS^a + NAA 4 ppm), T_5 (MS^b + NAA 2 ppm), T_6 (MS^b + NAA 4 ppm) and T_7 (MS^b + IBA 2 ppm) and significantly superior to others. T_{10} (VW + NAA 4 ppm) recorded the maximum duration of 35 days which was on par with T_9 (VW + NAA 2 ppm) and T_{11} (VW + IBA 2 ppm).

The cultures initiating roots were cent per cent in MS medium (both full and half strength). In VW medium, it was only 20-40 per cent. The roots produced were, in general, thin, long, white and fibrous in the medium containing IBA compared to NAA, which produced short thick roots.

In D. fimbriatum T_8 (MS^b + IBA 4 ppm) produced maximum number of roots (8.2) which was on par with T_4 (MS^a + IBA 4 ppm), T_6 (MS^b + NAA 4 ppm) and T_7 (MS^b + IBA 2 ppm) and significantly superior to all other treatments. T_{11} (VW + IBA 2 ppm) produced the minimum number of roots (2.4) and was on par with T_9 (VW +

NAA 2 ppm), T_{10} (VW + NAA 4 ppm) and T_{12} (VW + IBA 4 ppm). Comparative performance of NAA and IBA at two levels each on ½ MS medium is shown in Plates 7 and 8.

In D. moschatum also T_8 (MS^b + IBA 4 ppm) recorded maximum number of roots (7.4) which was on par with T_3 (MS^a + IBA 2 ppm), T_4 (MS^a + IBA 4 ppm) and T_7 (MS^b + IBA 2 ppm) and significantly superior to all other treatments. T_{11} (VW + IBA 2 ppm) recorded the minimum number of roots (2.0) and was on par with T_1 (MS^a + NAA 2 ppm), T_9 (VW + NAA 2 ppm), T_{10} (VW + NAA 4 ppm) and T_{12} (VW + IBA 2 ppm).

From the studies it was found that T_8 (MS^b + IBA 4 ppm) excelled all other treatments in respect of number of roots produced per shoot and the number of days taken for root initiation in both the species. This treatment was closely followed by, and was on par with T_7 (MS^b + IBA 2 ppm). At T_8 (MS^b + IBA 4 ppm), D. fimbriatum recorded the maximum number of roots (8.2) in minimum number of days (21) compared to D. moschatum which recorded only 7.4 roots per culture in 24 days.

Observations were made on the growth parameters of D. fimbriatum and D. moschatum at the time of planting out and the data are presented in Table 21.

Plate 7. Effect of NAA 2 ppm (left) and 4 ppm (right) on the *in vitro* rooting of shoots of *D. fimbriatum* in ½ MS

Plate 8. Effect of IBA 2 ppm (left) and 4 ppm (right) on the *in vitro* rooting of shoots of *D. fimbriatum* in ½ MS

Table 21. Effect of auxins (NAA and IAA) and media on the growth parameters of Dendrobium spp.

Explant: Axillary bud

Treatment	D. fimbriatum					D. moschatum				
	Plant height (cm)	No. of leaves/ shoot	Length of the longest leaf (cm)	Roots/ shoot	Average length of the root (cm)	Plant height (cm)	No. of leaves/ shoot	Length of the longest leaf (cm)	Roots/ shoot	Average length of the root (cm)
1. MS[a]+NAA 2 ppm	2.60	6.6	1.40	8.4	0.70	2.22	5.0	0.96	5.2	0.60
2. " + NAA 4 ppm	3.00	8.2	1.70	10.0	0.80	3.08	5.8	1.00	10.6	0.62
3. " + IBA 2 ppm	3.64	10.2	2.04	16.0	1.00	3.30	5.0	1.20	16.4	1.14
4. " + IBA 4 ppm	5.96	10.4	2.66	18.6	1.80	5.72	7.2	1.98	21.0	1.54
5. MS[b]+NAA 2 ppm	2.50	6.8	1.28	8.0	0.80	2.98	5.0	0.82	10.6	0.56
6. " +NAA 4 ppm	2.96	8.2	2.02	10.2	1.04	2.66	7.4	1.08	11.0	0.74
7. " +IBA 2 ppm	5.94	11.0	2.54	21.4	1.92	3.36	9.0	1.92	18.2	1.24
8. " +IBA 4 ppm	7.00	16.0	2.98	36.2	2.82	5.84	12.0	2.72	22.0	1.99
CD (0.05)	0.43	1.90	0.22	2.50	0.22	0.64	0.68	0.41	1.60	0.22
SEm±	0.05	0.92	0.01	1.65	0.01	0.04	0.13	0.04	0.80	0.01

Values taken as average of five replications at the time of planting out
Culture period: Six weeks
a - Medium containing full strength of both inorganic salts and organic growth factors
b - Medium containing half strength of inorganic salts and full strength of organic growth factors

Plant height

The treatments exerted significant influence on the growth parameters studied in both the species.

In D. fimbriatum T_8 (MS^b + IBA 4 ppm) recorded the maximum plant height (7.00 cm) and was significantly superior to all other treatments. The lowest height (2.50 cm) was recorded by T_5 (MS^b + NAA 2 ppm) which was on par with T_1 (MS^a + NAA 2 ppm).

With respect to D. moschatum, the tallest plants (5.84 cm) were observed in T_8 (MS^b + IBA 4 ppm) which was on par with T_4 (MS^a + IBA 4 ppm) and significantly superior to all other treatments. The minimum plant height (2.22 cm) was recorded by T_1 (MS^a + NAA 2 ppm). This was on par with T_6 (MS^b + NAA 4 ppm) and significantly different from all other treatments.

Number of leaves per shoot

The number of leaves produced per shoot also differed significantly as influenced by the treatments. In D. fimbriatum it was the maximum (16.0) at T_8 (MS^b + IBA 4 ppm) which was significantly superior to all other treatments and the minimum number of leaves (6.6) was recorded at T_1 (MS^a + NAA 2 ppm) which was on par with T_2 (MS^a + NAA 4 ppm), T_5 (MS^b + NAA 2 ppm) and T_6 (MS^b + NAA 4 ppm).

In D. moschatum, T_8 (MS^b + IBA 4 ppm) recorded the maximum number of leaves per shoot (12.0) which was significantly superior to all other treatments. Minimum number of leaves (5.0) per shoot was recorded by T_1 (MS^a + NAA 2 ppm) which was on par with T_3 (MS^a + IBA 2 ppm) and T_5 (MS^b + NAA 2 ppm).

Length of the longest leaf

The treatment differences were significant in influencing the length of the longest leaf. In D. fimbriatum T_8 (MS^b + IBA 4 ppm) recorded the maximum leaf length (2.98 cm) and was significantly superior to all other treatments. T_5 (MS^b + NAA 2 ppm), recording the minimum leaf length of 1.28 cm, stood on par with T_1 (MS^a + NAA 2 ppm).

D. moschatum recorded the maximum leaf length of 2.72 cm in T_8 (MS^b + IBA 4 ppm) which was significantly superior to all other treatments. Minimum leaf length (0.82 cm) was recorded by T_5 (MS^b + NAA 2 ppm) which was on par with T_1 (MS^a + NAA 2 ppm), T_2 (MS^a + NAA 4 ppm), T_3 (MS^a + IBA 2 ppm) and T_6 (MS^b + NAA 4 ppm).

Number of roots per shoot

The number of roots produced also differed significantly among the treatments. The maximum number of roots (36.2) in D. fimbriatum was recorded by T_8 (MS^b + IBA 4 ppm) which was significantly superior to all other treatments. Only 8.0 roots per

shoot was recorded by T_5 (MS^b + NAA 2 ppm) which was on par with T_1 (MS^a + NAA 2 ppm), T_2 (MS^a + NAA 4 ppm) and T_6 (MS^b + NAA 4 ppm) and significantly lower than other treatments.

In D. moschatum, T_8 (MS^b + IBA 4 ppm) recorded the maximum number of 22.0 roots per shoot and was on par with T_4 (MS^a + IBA 4 ppm). The minimum number (5.2) of roots per shoot was recorded by T_1 (MS^a + NAA 2 ppm) which was significantly different from all other treatments.

Average length of the roots

The average length of roots differed significantly as influenced by the treatments. In D. fimbriatum, T_8 (MS^b + IBA 4 ppm) was significantly superior to all other treatments, recording a root length of 2.82 cm. T_1 (MS^a + NAA 2 ppm) recorded the minimum root length (0.70 cm) and was on par with T_2 (MS^a + NAA 4 ppm) and T_5 (MS^b + NAA 2 ppm).

In D. moschatum, maximum root length (1.99 cm) was observed in T_8 (MS^b + IBA 4 ppm) which was significantly superior to all other treatments. T_5 (MS^b + NAA 2 ppm) recorded the minimum root length of 0.56 cm and was on par with T_1 (MS^a + NAA 2 ppm), T_2 (MS^a + NAA 4 ppm) and T_6 (MS^b + NAA 4 ppm)

The above studies on rooting indicated that D. fimbriatum responded better than D. moschatum in respect of the growth

parameters of the plantlet. Hence further studies were restricted to D. fimbriatum.

4.3.4.2. Effect of medium supplements

Data pertaining to the effect of various compounds other than growth regulators on the rooting of shoots produced in vitro in D. fimbriatum are presented in Tables 22 and 23.

Effect of sucrose

Data pertaining to the results of the trials conducted to study the effect of sucrose on the number of days taken for root initiation and the number of roots produced per shoot in vitro are presented in Table 22.

The treatments differed significantly with respect to the days taken for root initiation. Among the four levels of sucrose tried, that at 1.5 per cent recorded the minimum number of days (20) for root initiation and was on par with sucorse at 3.0 and 4.5 per cent. Maximum days (26) for root initiation was taken by sucrose at 6.0 per cent.

With regard to number of roots produced per shoot also the treatments differed significantly. Sucrose at 1.5 per cent recorded the highest value (9.4) which was significantly superior to all other treatments (Plate 9). Sucrose at 6.0 per cent recorded the least value (4.6) and was on par with that at 4.5 per cent.

Table 22. Effect of sucrose on the rooting of shoots produced *in vitro* in *Dendrobium fimbriatum*

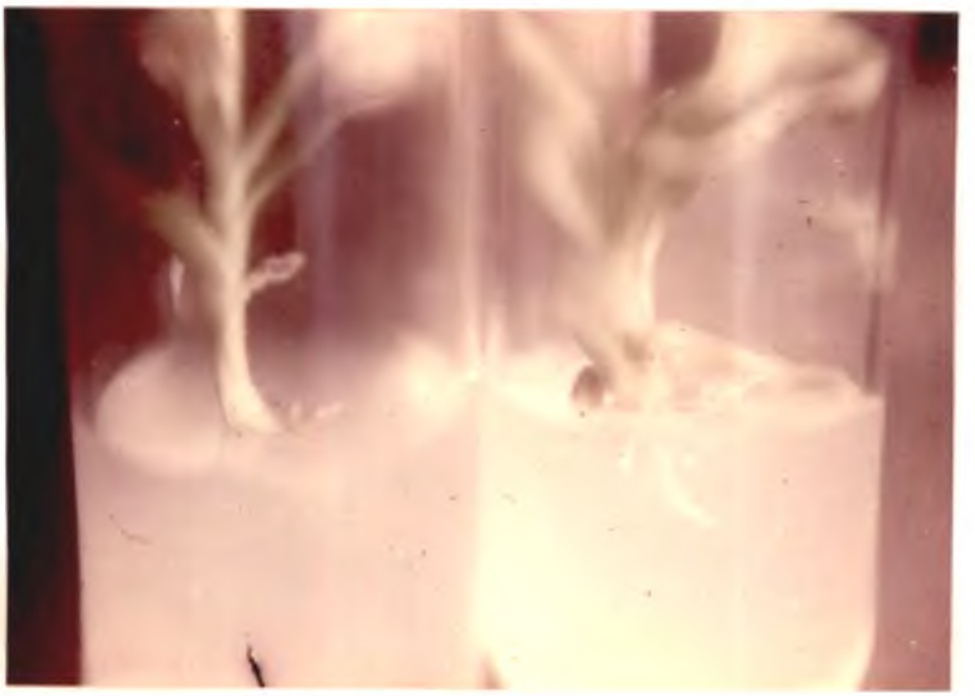
Medium : ½ MS + BA 4 ppm

Treatment	Root initiation (%)	Days for root initiation	Roots/shoot	Nature of roots
Sucrose 1.5%	100	20	9.4	Long, thin, white roots
,, 3.0%	100	21	6.4	Long, thin, white roots
,, 4.5%	100	24	5.6	Short, thin, white roots
,, 6.0%	100	26	4.6	Very short thin, white roots
CD (0.05)		4.00	1.60	
SEm±		1.33	0.53	

Values taken as average of five replications
Culture period - three weeks

Plate 9. Effect of sucrose 1.5 per cent (right) and 6.0 per cent (Left) on in vitro rooting of shoots of D. fimbriatum in ½ MS

Plate 10. Effect of activated charcoal 1.0 per cent (left) and 0.1 per cent (right) on in vitro rooting of shoots of D. fimbriatum in ½ MS



Cent per cent rooting was observed at all the levels of sucrose tried.

Effect of AC

The data resulting from the trial conducted employing AC as a medium supplement for rooting of shoots produced in vitro are presented in Table 23.

The treatments exhibited significant differences with respect to the period taken for root initiation as well as the number of roots produced. In respect of the period taken for root initiation, minimum days (19) was recorded by AC at 0.10 per cent which was significantly superior to all other treatments. AC at 1.00 per cent took the maximum number of days (28) for root initiation, which was significantly different from all other treatments.

As regards the number of roots produced per shoot (Plate 10), AC at 0.10 per cent recorded the highest value (9.6) which was on par with that at 0.25 per cent. Minimum number of roots was recorded by AC at 1.00 per cent (6.6) which was on par with that at 0.50 per cent.

All the four levels of AC recorded cent per cent root initiation.

The effect of sucrose and AC on root initiation and number of roots produced in D. fimbriatum is shown in Fig. 5.

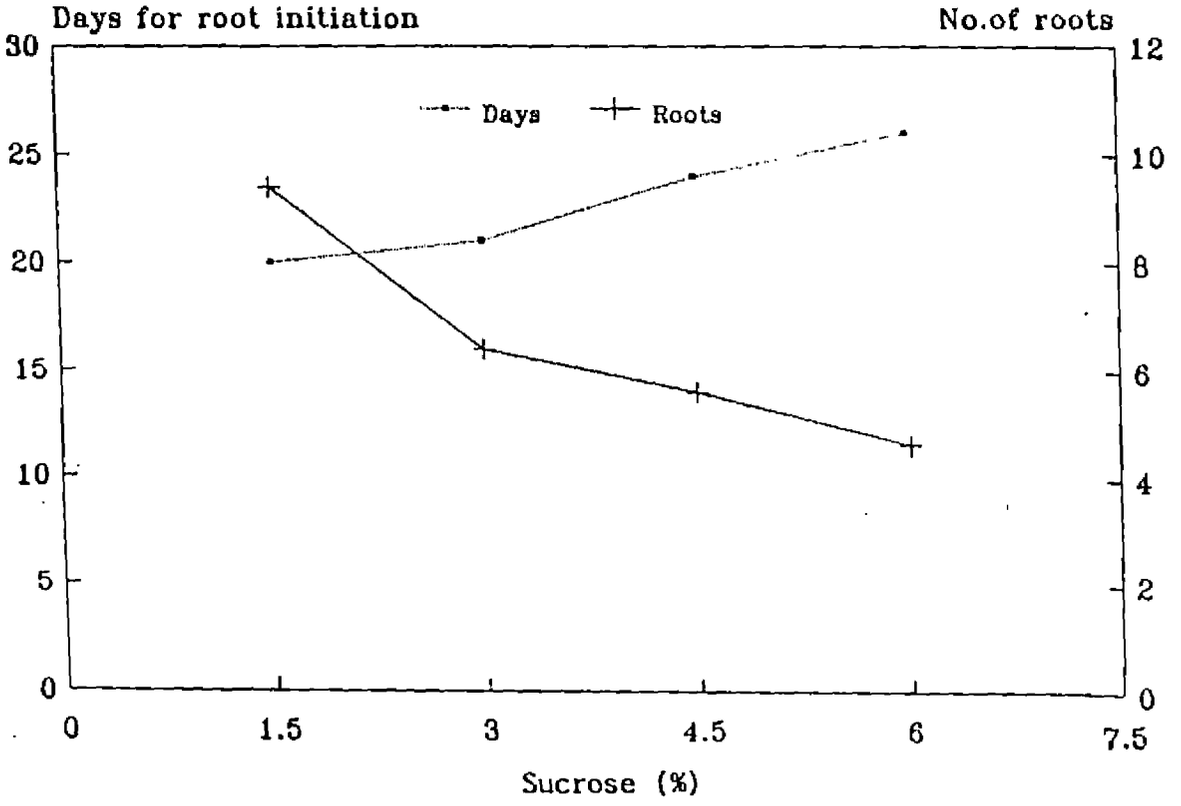
Table 23. Effect of activated charcoal on the rooting of shoots produced *in vitro* in *D. fimbriatum*

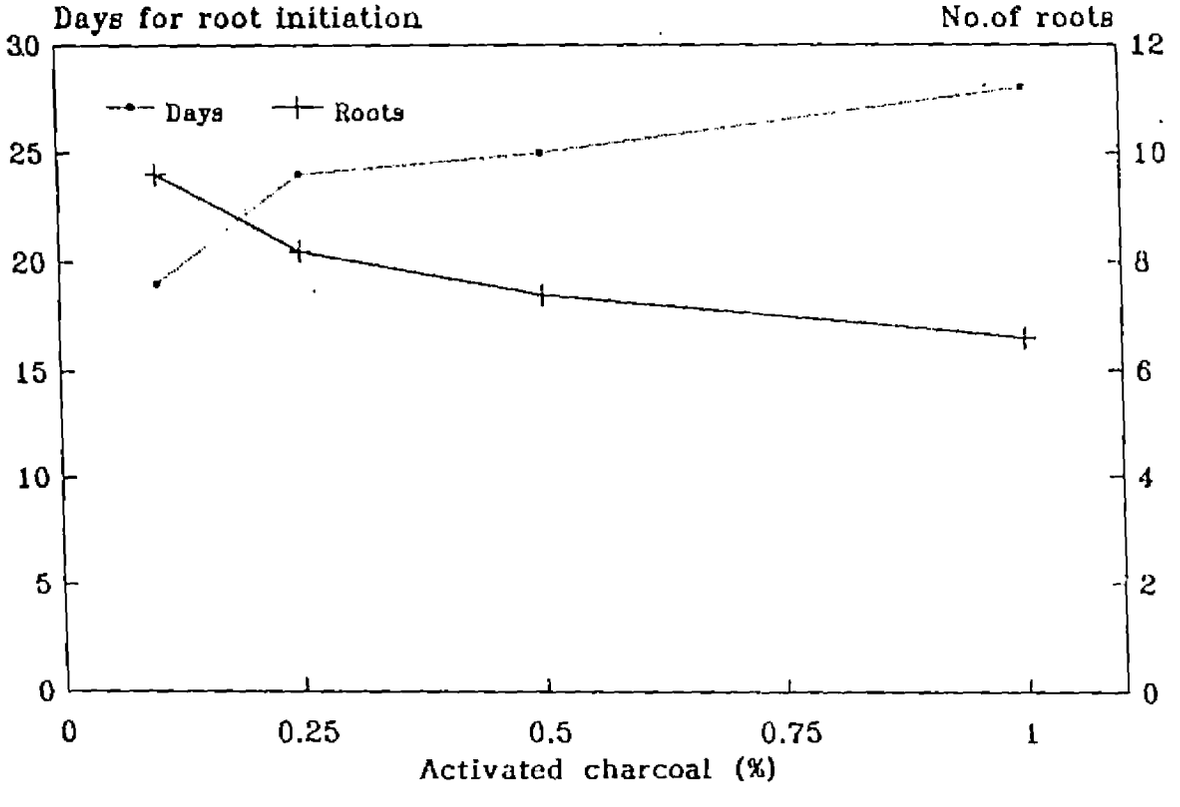
Medium : ½ MS + BA 4 ppm

Treatment	Root initiation (%)	Days for root initiation	Roots/shoot	Nature of roots
AC 0.10%	100	19	9.6	Short, thick, white roots
,, 0.25%	100	24	8.2	Short, thick, greenish white roots
,, 0.50%	100	25	7.4	Short, thick, greenish white roots
,, 1.00%	100	28	6.6	Short, thick, greenish white roots
CD (0.05)		2.82	1.53	
SEm±		0.94	0.51	

Values taken as average of five replications
Culture period - three weeks

Fig. 5. Effect of sucrose and activated charcoal on root initiation and number of roots produced in D. fimbriatum





Planting out and acclimatization

The size of the plantlets that were ready for planting out in Dendrobium fimbriatum and D. moschatum is shown in Plates 11 and 12. Both the potting media and the hardening (post-transfer) treatments were found to influence the survival of the plantlets of both the species.

Potting media

The most suitable potting medium was found to be 1:1 (v/v) mixture of brick and charcoal pieces (Table 24). It recorded maximum survival of plantlets in both D. fimbriatum (70 per cent) and D. moschatum (40 per cent). Charcoal registered minimum percentage of survival in both D. fimbriatum and D. moschatum (20 per cent, each). The plantlets did not survive in coconut husk in both the species.

Hardening treatments

The results of the trial conducted to study the effect of post-transfer hardening treatments on the survival of plantlets are presented in Tables 25 and Plates 13, 14 and 15.

Covering the plants with plastic sheet for three days and keeping in shade recorded the highest survival percentage in D. fimbriatum (60 per cent) and D. moschatum (40 per cent). This was followed by covering of plants with plastic sheet with holes and

Plate 11. Plantlet ready for planting out - D. fimbriatum

Plate 12. Plantlet ready for planting out - D. moschatum

Table 24. Influence of potting media on the survival of the *in vitro* plantlets of *Dendrobium*

Potting medium	Survival of plantlets (%)	
	D. fimbriatum	*D. moschatum*
Coconut husk	0	0
Brick : Charcoal (1:1)	70	40
Charcoal	20	20

Table 25. Influence of hardening treatments on the survival of the *in vitro* plantlets of *Dendrobium*

Post-transfer treatment	Survival of plantlets (%)	
	D. fimbriatum	*D. moschatum*
Keeping in open	0	0
Keeping in culture room	20	0
Covering the plants and pots with plastic sheet with holes for two weeks	30	30
Covering the plants and pots with a microscope cover for two weeks	30	20
Covering the plants with plastic sheet for three days and keeping in the shade	60	40

Plate 13. Effect of post-transfer hardening treatments - kept in open

Plate 14. Effect of post-transfer hardening treatments - covering with plastic cover

Plate 15. Effect of post-transfer hardening treatments - covering with microscope cover

covering with a microscope cover for two weeks. In both the treatments, D. fimbriatum recorded 30 per cent survival of the plantlets, each. D. moschatum recorded 30 and 20 per cent, respectively. The plantlets did not survive when kept in the open.

4.4. Somatic organogenesis

4.4.1. Explant choice

The results of the trial conducted for screening of various explants of Dendrobium spp. for initiating callus are presented in Table 26.

Of the various explants tried, root (aerial as well as from culture) segments showed the maximum response (60 and 50 per cent, respectively). This was followed by leaf (middle portion of mature leaf as well as whole leaf from culture, recorded 40 and 20 per cent, respectively) and inflorescence stalk (20 per cent). The shoot tip and axillary bud did not respond. The base with sheath of the leaf explants, though recorded 10 per cent response, showed necrosis and gradually dried.

4.4.2. Culture establishment (Stage 1)

The results of the trials conducted to study the culture establishment as influenced by the combination of auxin (NAA) and cytokinin (BA) in modified VW medium in Dendrobium moschatum are presented in Table 27.

Table 26. Response of various explants of Dendrobium moschatum on initiating callus

Basal medium: VW

Explant	Response (%)*
Shoot tip**	0
Axillary bud**	0
Inflorescence stalk	20
Leaf	
Tip portion	0
Middle portion	40
Base portion (with sheath,	10
Whole leaf (from culture)	20
Root	
Aerial	60
From culture	50

* Average of ten replications

**All the three species were used

Table 27. Effect of different levels of NAA and BA on the culture establishment of various explants of Dendrobium moschatum

Basal medium - VW

Treatment	Culture establishment (%)*				
	Leaf		Root		Infloresence stalk
	Middle portion of mature leaf	Whole leaf from culture	Aerial	From culture	
NAA 1 ppm + BA 1 ppm	10	0	20	0	0
,, + BA 2 ppm	10	0	10	0	0
NAA 2 ppm + BA 1 ppm	20	10	40	10	20
,, + BA 2 ppm	10	10	20	0	10
NAA 4 ppm + BA 1 ppm	30	10	50	30	20
,, + BA 2 ppm	10	10	20	20	20

* Values taken as average of ten replications
Culture period - four weeks

4.4.2.1. Leaf segments

Middle portion of mature leaf

Increase in size (swelling) of the explant was observed at all the treatment combinations. At NAA 4 ppm + BA 1 ppm, the establishment of cultures was 30 per cent.

Leaf from culture

The explants exhibited swelling at the treatment combinations involving NAA 2 or 4 ppm with BA 1 or 2 ppm. The establishment of culture recorded at these four combinations was 10 per cent, each.

4.4.2.2. Root segments

Aerial root

The maximum percentage of culture establishment recorded was 50 per cent at the treatment combination of NAA 4 ppm + BA 1 ppm. Thickening of the explant at the tip was observed at this level. Culture establishment was the minimum (10 per cent) at the combination of NAA 1 ppm + BA 2 ppm.

Root from culture

The roots obtained from culture were short and thick. Further thickening of the root segments resulted in the treatment combination of NAA 4 ppm + BA 1 ppm. Establishment of cultures at this level was recorded as 30 per cent. At the treatment combinations of NAA

Plate 16. Enlargement of the middle portion of the mature leaf of D. moschatum

Plate 17. Callus formation in in vitro leaf (whole) of D. moschatum

1 ppm + BA 1 ppm, NAA 1 ppm + BA 2 ppm and NAA 2 ppm + BA 2 ppm, culture establishment could not be obtained.

4.4.2.3. Inflorescence stalk

Culture establishment was the maximum at the combination of NAA 2 ppm + BA 1 ppm, NAA 4 ppm + BA 1 ppm and NAA 4 ppm + BA 2 ppm. The percentage of response at these levels was recorded as 20 per cent, each. Treatment combinations having NAA 1 ppm + BA 1 ppm and NAA 1 ppm + BA 2 ppm recorded the minimum value (0 per cent).

4.4.3. Callus induction and organogenesis (Stage 2)

The explants from the culture establishment were tried for callus induction and organogenesis in modified VW medium supplemented with 2,4-D at various levels (0.5, 1.0, 2.0 and 4.0 ppm). The results are presented in Table 28 and Fig. 6.

4.4.3.1. Leaf segments

Middle portion of the mature leaf

Thickening of the explant was observed, especially at the base. They exhibited water-soaked appearance (Plate 16). Maximum percentage of cultures initiating callus (40) was recorded at 2,4-D 2.0 ppm. The percentage of cultures initiating callus at 2,4-D 1.0 ppm was the minimum (0 per cent). Further differentiation of the callus was not observed.

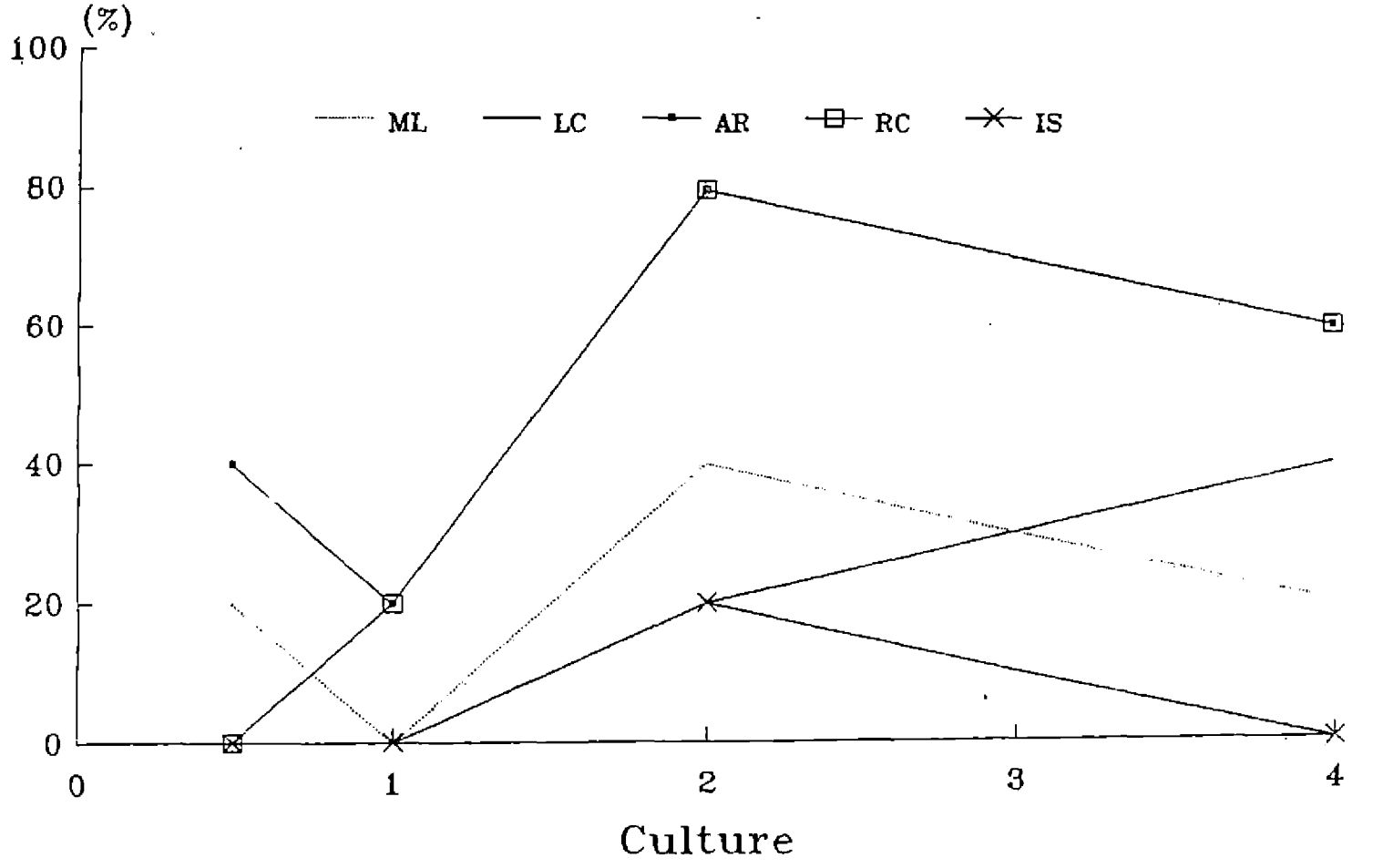
Table 28. Effect of 2,4-D on the induction and growth of callus in various explants of Dendrobium moschatum

Basal medium - VW

Treatment	Leaf						Root						Infloresence stalk		
	Middle portion of mature leaf			Whole leaf from culture			Aerial			From culture					
	Cultures initiating callus (%)	Growth score (G)	Callus index (CI)	Cultures initiating callus (%)	Growth score (G)	Callus index (CI)	Cultures initiating callus (%)	Growth score (G)	Callus index (CI)	Cultures initiating callus (%)	Growth score (G)	Callus index (CI)	Cultures initiating callus (%)	Growth score (G)	Callus index (CI)
2,4-D 0.5 ppm	20	0	0	0	0	0	40	1	40	0	0	0	0	0	0
,, 1.0 ppm	0	0	0	0	0	0	20	1	20	20	1	20	0	0	0
,, 2.0 ppm	40	0	0	20	1	20	80	1	80	80	3	240	20	1	20
,, 4.0 ppm	20	0	0	40	1	40	60	1	60	60	1	60	0	0	0

Values taken as average of ten replications
Culture period - four weeks

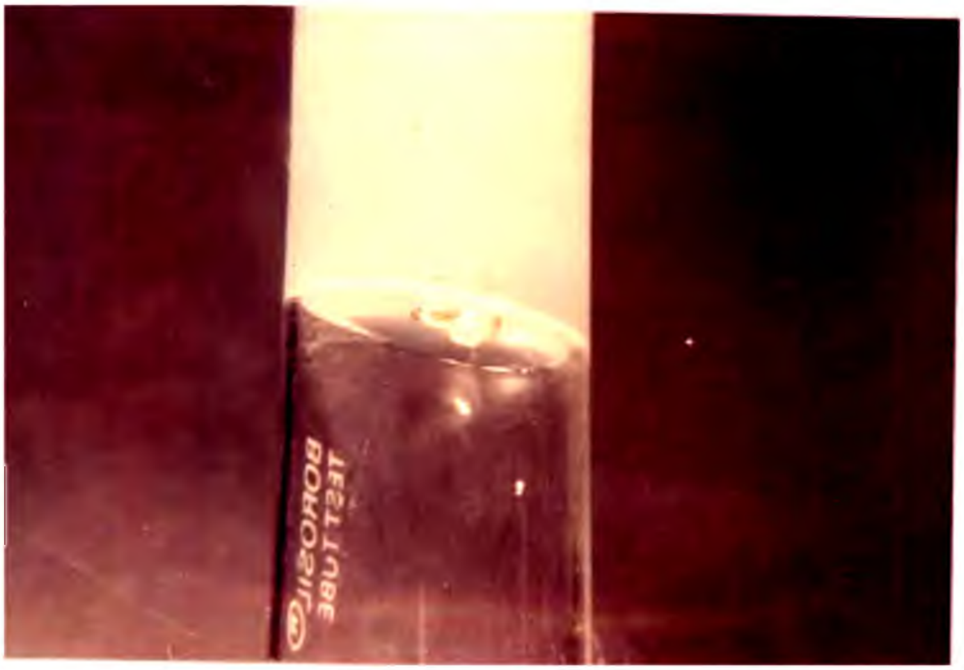
Fig. 6. Effect of 2,4-D on callus induction in various explants of D. moschatum



ML - Mature Leaf
LC - Leaf from Culture
AR - Aerial Root
RC - Roots from Culture
IS - Inflorescence Stalk

Plate 16. Enlargement of the middle portion of the mature leaf of D. moschatum

Plate 17. Callus formation in in vitro leaf (whole) of D. moschatum


TEST TUBE
BOROSIL

Leaf from culture

Maximum percentage of cultures initiating callus (Plate 17) was recorded at 2,4-D 4.0 ppm (CI = 40). 2,4-D at 0.5 ppm and 1.0 ppm recorded no callus initiation.

4.4.3.2. Root

Aerial root

Maximum callusing (CI = 80) was recorded at 2,4-D 2.0 ppm. The callus produced was greenish yellow in colour and poorly attached (Plate 18). Minimum callusing (CI = 20) was recorded at 2,4-D 1.0 ppm.

Root from culture

The treatment having 2,4-D 4.0 ppm resulted in maximum callusing (CI = 240) of the explant. The callus produced was light green in colour and compact (Plate 19). The callusing was absent at 2,4-D 0.5 ppm.

4.4.3.3. Inflorescence stalk

In general, the response of the explant to callus induction was poor. 2,4-D at 2.0 ppm alone exhibited callusing of the explant (Plate 20), which was 20 per cent (CI = 20).

Plate 18. Callus formation in aerial root segment of D. moschatum

Plate 19. Callus formation in root segment from culture of D. moschatum

Plate 20. Callus formation in the infloresence stalk of D. moschatum

Discussion

DISCUSSION

The present investigations on the standardisation of explants for *in vitro* propagation in *Dendrobium* spp. were carried out at the Plant Tissue Culture Laboratory, attached to the All India Co-ordinated Floriculture Improvement Project (AICFIP), College of Horticulture, Vellanikkara, during 1990-91. The results of the study are discussed in this chapter.

Orchids comprise an unique group of plants. Taxonomically they represent the most highly evolved family among monocotyledons with 600-800 genera and 25,000-35,000 species (Garay, 1960). They exhibit an incredible range of diversity in size, shape and colour of their flowers. The long life span of orchid flowers, sometimes upto several months, also contributes to the popularity of the flowers.

The length of time taken for clonal propagation by using back-bulbs or top cuttings has been a serious drawback in the orchid industry. According to Blowers (1964), depending on the kind, it takes about ten years to cultivate from six to a dozen good-sized propagules. In addition to this, all the cultivated orchids, being complex hybrids, are highly heterozygous and there is very little hope of breeding pure lines out of them. On the other hand, the new technique of tissue culture makes it possible to multiply unlimited number of desirable clones as is done for carnation, roses and

chrysanthemum. When there is a need to mass-propagate a new hybrid or variety within a short period of time, tissue culture is the only method currently available. Apart from the need for mass propagation of desired clones, tissue culture technique is used to free existing hybrids or clones from disease.

The present investigations carried out were mainly aimed at to study the response of different explants to the enhanced release of axillary buds. Attempts were also made for standardising techniques for inducing somatic organogenesis (callus mediated) from meristematic parts of clonal plant material.

Of the various species grown in the orchidarium at the College of Horticulture, Vellanikkara, D. fimbriatum, D. moschatum and D. nobile established well and were available in plenty. Hence explants for the present investigations were taken from these three species.

India has a very large variety of orchids and majority of them are native of tropical region. They are found in abundance in the States of Assam, Meghalaya, West Bengal, Karnataka and Kerala. In Kerala, some of the orchid genera which are of high ornamental values are Arachnis, Cattleya, Cymbidium, Dendrobium, Oncidium, Paphiopedilum, Phaelaenopsis and Vanda. The genus Dendrobium occupies the major area under orchid cultivation. It is an important genus for the orchid cut flower industry and is found to perform well under the climatic conditions of Kerala.

Orchids are one of the few flowering plants of commercial value to be propagated in vitro, both through seed and tissue culture. Micropropagation requires that the organs and tissues be carried through a sequence of steps in which differential cultural and environmental conditions are provided. These steps have been indicated in Fig. 1 as different Stages with Stage 1 being physiological preconditioning of the explant and culture establishment, Stage 2 rapid multiplication of shoots through increased axillary branching, Stage 3 in vitro rooting and hardening of in vitro raised plants and Stage 4 planting out and acclimatization and transplantation to field conditions (Murashige, 1974).

The plants grown under field conditions accumulate lot of soil and dust. This necessitates a thorough and effective surface sterilization of explants before culturing. In the present studies, after detailed surface sterilization experiments, it was found necessary to standardise the chemicals, duration and procedure of treatment, separately for each explant.

Of the various sterilants tried, mercuric chloride at all the levels (0.05, 0.10 and 0.2 per cent) was found to give better sterilization of explants than sodium hypochlorite or bleaching powder. Though in literature use of sodium hypochlorite is more common (Reghunath, 1989) in the present studies it was found to be less efficient than mercuric chloride. The least rate of contamination and the maximum percentage of explant survival were obtained,

when treated with mercuric chloride 0.1 per cent, for all the explants tried.

The month or season of explant collection was found to have some influence on the percentage contamination of the culture and rate of survival of the explants. This is largely because of the availability of congenial conditions for the growth of certain micro-organisms. A preliminary study was made in this regard and was found that the period from January to June recorded least contamination rate and maximum survival. This may be because of lesser rainfall and humidity conditions prevailed during this period which were not congenial for the growth of microorganisms.

Among the different explants tried for initiating enhanced release of axillary buds, axillary bud explant was found to give maximum response (100 per cent) under culture conditions. Though the shoot tip recorded 10 per cent survival, the shoots originating from them gradually dried up and failed to survive when subcultured. In a similar study, Kim et al. (1970) reported that the percentage of survival of axillary buds was higher than terminal buds in Dendrobium phalaenopsis. Node cultures as a means of clonal propagation for Dendrobium was also reported by Ball and Arditti (1975). These reports and the present study indicate that for the production of clonal plantlets from axillary buds are more suitable than from shoot tip.

In general, VW medium was found to have a better influence on the culture establishment. It was found to be the best in Dendrobium fimbriatum and D. moschatum The VW medium for culturing of different species of Dendrobium was suggested to be the best by many investigators (Gilliland, 1958; Nimote and Sagawa, 1961; Sagawa and Valmayor, 1966; Sagawa et al., 1967; Kim et al., 1970; Singh and Sagawa, 1972; Sanguthai and Sagawa, 1973 and Valmayor, 1974). Fernando (1979) also reported that the best medium for the clonal propagation of Dendrobium Caeser Red Lip was modified VW. The success in the use of VW medium indicated that requirements of mineral elements for most of the orchids are relatively simple compared to those for higher plants.

However, Murashige and Skoog (1962) were of the opinion that MS medium was more effective than KC medium for orchid tissue culture. This was found to be in line with respect to the culture establishment of the axillary bud of D. moschatum in the present investigation.

Though KC medium was also suggested by various workers (Sagawa and Shoji, 1967; Irawati et al., 1977) for clonal propagation of Dendrobium, in the present study, it was not found to be effective. KC medium took the longest duration for culture establishment, compared to MS and VW media.

The favourable effects of axillary bud bursting and multiple shoot production by cytokinins had been reported by Murashige (1974). In the present studies also, an increase in the production of shoots was noticed due to the application of BA in all the three species of Dendrobium tried. The reports of Kukulczanka and Wojcicchowska (1983) are also on similar lines. They observed that application of BA to the axillary buds increased the shoot proliferation in D. antennathum and D. phalaenopsis. Similar results were obtained in Calanthe by Shimasaki and Uemoto (1987) too.

Murashige (1974) also reported that application of BA at higher level had a deleterious effect on shoot growth. This was found to be true in the present investigation. Shoots on 2 ppm NAA and 3 ppm BA had a healthy appearance, exhibiting considerable vigour, rapid shoot growth and well expanded leaves. When 5 ppm BA was added to the medium, shoot production was further enhanced, but the shoots were short and possessed small leaves. Similar observations were recorded in Halesia carolina on shoot proliferation as influenced by higher levels of BA (Brand and Lineberger, 1986).

In the present study, addition of KIN was found to favour multiple shoot production particularly at higher levels. Honmode and Sehgal (1991) also opined that addition of KIN to the culture medium enhanced multiple shoot formation in shoot tips of Rhyncostylis retusa. Higher levels of KIN however, did not affect the growth of shoots.

The effect of cytokinin on tissue or organ culture may vary according to the particular compound used, the type of culture and the variety of plant from which it was derived (George and Sherrington, 1984). A specific cytokinin is frequently observed to be more effective in shoot tip cultures of many species. Axillary bud proliferation of Castanea was promoted by BAP whereas KIN had no effect (George and Sherrington, 1984).

In the present studies, though addition of KIN increased the production of multiple shoots, the rate of multiplication compared to BA was low. Increase in the concentration of BA/KIN resulted in corresponding increase in the number of shoots produced, although the growth of shoots was inhibited at higher levels of BA.

Auxin added to the medium helps to nullify the suppressive effect of high cytokinin concentration on axillary shoot growth (Lundergan and Janick, 1980). In the present studies also addition of NAA to the medium containing BA or KIN was found to influence the axillary shoot growth. Matsui et al. (1970) reported that increase in number of shoots in Cymbidium was observed when cultured in a medium containing NAA and BA. A higher concentration of NAA and BA induced maximum proliferation of shoots in Cattleya (Kusumoto, 1979). Honmode and Sehgal (1991) were able to produce multiple shoots from shoot tips of Rhyncostylis retusa when cultured in MS medium containing NAA (5 ppm) and KIN (20 ppm).

It was noticed during the course of the study that the shoots formed in MS medium often produced necrotic leaves. The leaves started drying from the distal end which gradually spreaded down through the leaf margins. It was suspected that the leaf tip drying was caused by certain inorganic components of MS medium which were present either at a toxic level or deficient level. On reducing the concentration of inorganic salts in the medium to half, the symptoms disappeared, indicating the level of inorganic salts in MS medium to be toxic. Similar observations were made by several other workers and they have recommended the use of half or one fourth strength of MS inorganic salts (Anderson, 1980; Mikami et al., 1985; Ilahi and Jabeen, 1987).

Addition of CW to the culture medium was found to have a beneficial effect in the production of PLBs and plantlet formation in Dendrobium (Soediono, 1983b; Kim et al., 1970). In the present investigation, CW at four different levels were used and that at 15 per cent was found to be more effective. The beneficial effect of CW is undoubtedly related to its growth regulator content, the most important being the cytokinins (Letham, 1974; Vanstaden and Drewes, 1974).

Effect of serial subculturing of an explant stock continuously for a longer period of time was also tried and it was found that when subculturing was done at a frequency of once in three weeks, the number of shoots produced per culture vessel increased at a

mean rate of 8.3, 7.7 and 6.6, in D. fimbriatum, D. moschatum and D. nobile, respectively. Increase in multiple shoot production continued from the first subculture onwards. Several workers had reported increased shoot multiplication rate achieved through continuous subculturing. It is supposed that continuous subculturing modifies the physiological state of the plant in such a way that it favours revitalisation of innate dormant vegetative buds (Litz and Conover, 1978; Franclet, 1979; David, 1982).

Stage 3 involves de novo regeneration of adventitious roots from the shoots produced in Stage 2. In the present studies, various factors of rooting, viz., basal medium, auxins and other medium supplements were standardised. MS (half strength) medium having IBA 4 ppm was the best with respect to root initiation in maximum number of cultures within the shortest time. On an average 8 and 7 roots were produced in 21 and 24 days in D. fimbriatum and D. moschatum, respectively. The findings of Honmode and Sehgal (1991) also agree with this.

Sucrose had already been recognised (Chong and Pua, 1985) as a source of energy as well as a factor for osmoregulation for optimising the rooting response. Based on this, trials for in vitro rooting was conducted and it was found that sucrose at lesser concentration (1.5 per cent) gave maximum number of roots (9.4) in minimum number of days (20) in D. fimbriatum. Advantage of lower concentration of sucrose in rooting is in confirmity with the findings of Mateille and Foncelle (1988).

The favourable effect of AC in rooting had been reported by many workers (Wang and Huang, 1976; Banks and Hackett, 1978; Reghunath, 1989). In the present study, AC at 0.1 per cent produced maximum number of roots (9.7) in minimum number of days (19). Stimulation of root growth has been ascribed to charcoal absorbing inhibitory substances (preventing tissue blackening), absorbing auxins or to its effect in darkening the culture medium.

In vitro rooting of shoots (Stage 4) was the most labour intensive part of micropropagation (Debergh and Maene, 1981). During the course of present study too it was found that the in vitro rooting of individual shoots after separation from the multiple shoot cluster was both time and labour consuming. Keeping the plantlet in the same medium for another two weeks after initiation of roots resulted in better survival percentage than transferring of the plantlets into the potting medium soon after the production of all roots. Similar observations were made in banana by Bhaskhar (1991).

The potting medium having charcoal and brick pieces was found to be better compared to coconut husk. The plantlets when planted in the coconut husk medium showed wilting. Kumar (1991) also reported about a similar observation. He was of the opinion that the failure of the plantlets to survive may be due to poor adhering capacity of roots to the husk.

Hardening the in vitro raised plantlets so as to make them adapt to the outside environment is a critical process due to their anatomical and physiological peculiarities. Of the various hardening methods tried in the present studies, covering the plantlets for three days with plastic sheet and then keeping under partial shade was found to be effective. Rajmohan (1985) and Reghunath (1989) reported that covering the plantlets with a transparent microscope cover with intermittent cold water sprays was also effective. But in the present investigations, it was found that, hardening of plantlets by covering with microscope cover and then gradually exposing to natural conditions was not effective in the establishment of the plantlet.

The most remarkable advantage of the method of producing shoots/plantlets through enhanced release of axillary buds is the genetic stability of the plantlets produced. But when the rate of plant multiplication is concerned, somatic organogenesis (callus mediated/direct) and embryogenesis are reported to have greater potentialities than axillary bud release method. However, the principal disadvantage of this method, compared to clonal propagation, is the genetic variation developing in many of its component cells. In the present studies attempts were made to induce somatic organogenesis (callus mediated) in Dendrobium moschatum. Of the various explants, viz., leaf segments (mature leaf, and from culture) root segments (aerial and from culture) inflorescence stalk, shoot tip and axillary bud tried for initiating callusing, root segments gave maximum

response. Though promising results were obtained in callus or calloid formation, subsequent plant regeneration could not be obtained.

After standardising the explant, the basal medium for callusing was standardised. VW medium modified by the addition of CW was found to be ideal. CW was commonly added to stimulate callus or protocorm formation (Goh, 1970, 1973; Goh et al., 1975). Taking VW as the basal medium, detailed trials were conducted and levels of auxins (NAA, 2,4-D) and cytokinins (BA) were standardised. As a result, a basal callus proliferation medium was formulated. In the present studies, 2,4-D was found to be the best for callus induction, particularly at 2.0 ppm. The use of 2,4-D in callus induction by culturing of root tips have been reported by Sagawa et al. (1966) in Dendrobium and Stewart and Button (1978) in Epidendrum.

Response of the explants to callus formation was generally poor. Out of the total callus index assigned (CI=400) none of the explants could record a CI value higher than 240. In the present studies it was noticed that though treatments involving 2,4-D had produced profuse, greenish white, friable callus, they gradually dried up and failed to survive when subcultured. Initiation of organised development in the callus involves a shift in metabolism. Identification of the areas of metabolism involved in this shift as well as determination of the role of various phytohormones and other interacting metabolites may help to further increase the rate of callusing and subsequent organogenesis (Thorpe, 1980).

As somatic organogenesis is useful to generate variants, further efforts may be made to improve the callus production, to increase the rate of organogenesis and to achieve better survival rates when planted out.

The current use of tissue culture technique in other crops is a consequence of the success in orchids. The success of cultures on relatively simple media indicated a greater degree of adaptability, as well as an ability to synthesize their own needs, in orchids through evolution, which is manifested in the poorly developed seeds. With further experiments, many tissues, including young aerial roots, could be used as explant materials. Much need to be done, on anther (haploid) and protoplast culture, which are of not only academic interest but also of practical importance. Many orchid hybrids, particularly intergeneric hybrids, are infertile. Establishment of haploid cultures, followed by diploidization, would not only restore fertility but also would segregate the various characteristics which may be selected for further hybridization programmes. The success of protoplast culture could lead to somatic hybridization which would create new forms not seen in nature.

There is need in orchid tissue culture for research which spans explant to mature plant. To produce large number of plantlets which mature at the same time, direct embryogenesis or embryogenesis from callus on suspension cultures may be a viable

path way. The rapid destruction of natural habitats of orchid species as well as heterozygosity and high cost of maintenance of breeding stock make it important to develop reliable methods for germplasm storage too.

Summary

SUMMARY

Investigations were carried out at the Plant Tissue Culture Laboratory, attached to the All India Co-ordinated Floriculture Improvement Project (AICFIP), College of Horticulture, Vellanikkara to standardise the explants for *in vitro* propagation in *Dendrobium* spp. The salient findings of the study are presented in this chapter.

Of the various sterilants tried, mercuric chloride gave better results than sodium hypochlorite or bleaching powder. Dipping for 10 minutes in mercuric chloride at 0.1 per cent gave the lowest rate of contamination and the highest percentage of explant survival.

The most ideal period for collection of shoot tip, axillary bud and leaf explants was April, during which period the rate of culture contamination was the lowest and the explant survival the highest.

The best suited explant for initiating enhanced release of axillary buds was axillary bud which gave cent per cent shoot proliferation, compared to shoot tip and inflorescence stalk.

The explants which were not contaminated, started swelling of the buds (bud initiation) within a period ranging from 9 to 50 days, depending on the media and the combination of growth regulators.

In the culture establishment, the KC medium supplemented with NAA 1.5 ppm + BA 1.0 ppm, took the minimum number of days

for bud initiation and bud elongation, compared to all other treatment combinations. The periods taken for bud initiation and bud elongation at this level were 31 and 38, 31 and 35 and 31 and 39 days, respectively, in Dendrobium fimbriatum, D. moschatum and D. nobile.

In the case of MS medium also, the treatment combination of NAA 1.5 ppm + BA 1.0 ppm recorded the minimum number of days for bud initiation and bud elongation among all the treatment combinations. D. fimbriatum, D. moschatum and D. nobile recorded 25 and 38, 9 and 30 and 27 and 33 days, respectively, for bud initiation and elongation.

With respect to VW medium, for the bud initiation and bud elongation, NAA 1.5 ppm + BA 1.0 ppm took the shortest duration. The days taken for bud initiation and bud elongation at this level in D. fimbriatum, D. moschatum and D. nobile were 24 and 33, 21 and 31 and 28 and 32 days, respectively.

In a comparative study on the performance of the three species of Dendrobium in different culture media, D. moschatum in MS medium exhibited bud initiation and bud elongation within the shortest period of 9 and 30 days, respectively. This was followed by D. fimbriatum and D. moschatum in VW medium for bud initiation and all the three species of Dendrobium in VW medium for bud elongation. The longest duration for bud initiation and elongation in all the three species of Dendrobium was registered in KC medium.

The elongated buds from the established cultures were excised and cultured for shoot proliferation in different media, supplemented with NAA and BA/KIN at various levels. Wide variation in the proliferation of shoots was noticed.

In MS (half strength) medium, the effect of BA alone and in combination with NAA was significant in the induction of multiple shoots, in all the three species of Dendrobium. Maximum number of shoots was recorded in the treatment combination of NAA 2 ppm + BA 5 ppm in all the species. The number of shoots produced at this level in D. fimbriatum, D. moschatum and D. nobile were 5.6, 5.8 and 5.0, respectively. Though the maximum number of shoots was produced at this level, the shoots were compressed and often malformed in appearance. The treatment combination of NAA 2 ppm + BA 3 ppm, which was on par with NAA 2 ppm + BA 5 ppm, on the other hand, produced normal and healthy shoots. The number of shoots produced by this treatment in D. fimbriatum, D. moschatum and D. nobile were 5.4, 4.8 and 4.2, respectively. The lowest number of shoots (1.0) was recorded by the control in all the three species.

In VW medium also the treatment combination of NAA 2 ppm + BA 5 ppm recorded the maximum number of shoots per culture in all the three species of Dendrobium. The shoots produced at this treatment combination were 27.8, 13.0 and 10.2, respectively, in D. fimbriatum, D. moschatum and D. nobile. Though the number of shoots

produced at this level was the highest, they were compressed and malformed in appearance, compared to the shoots produced at NAA 2 ppm + BA 3 ppm, which was on par with NAA 2 ppm + BA 5 ppm. The number of shoots produced in D. fimbriatum, D. moschatum and D. nobile at this level (NAA 2 ppm + BA 3 ppm) was 18.8, 10.8 and 7.6, respectively. Minimum number of shoots (1.0) was produced by the control treatment in all the three species.

In a comparative study on the performance of the three species of Dendrobium in different culture media on shoot proliferation, D. fimbriatum in VW medium was significantly superior to all other treatments, recording the maximum of 18.8 shoots per culture. D. nobile in MS (half strength) medium recorded the minimum number of shoots (4.2).

The study on the effect of KIN alone or in combination with NAA in VW medium, on the number of shoots produced per culture, revealed that the combination of NAA 2 ppm + BA 5 ppm was the most effective treatment. The number of shoots produced at this level was 6.4, 5.6 and 5.8, respectively, in D. fimbriatum, D. moschatum and D. nobile. In all the three species the minimum number of shoots (1.0) was recorded by the control.

Addition of CW at 15 per cent was found to be beneficial on the proliferation of shoots in all the three species. D. fimbriatum, D. moschatum and D. nobile recorded 19.0, 18.0 and 8.0 shoots per culture, respectively, at this level.

From the study conducted to assess the rate of growth in the multiplication of shoots in axillary bud explants of the three species of Dendrobium, consequent on continuous subculturing at three week interval, it was found that there was increase in all the subcultures, starting from the third subculture. The number of shoots produced per culture with reference to the initial culture increased from 6.75 to 9.75 in D. fimbriatum, in seventh subculture, from 6.75 to 8.75 in D. moschatum, in eighth subculture and from 5.75 to 7.25 in D. nobile in eighth subculture.

In all the trials conducted on shoot proliferation of the axillary bud explant, D. fimbriatum was found to respond well, followed by D. moschatum. D. nobile produced the lowest number of shoots. The shoots produced were short and unhealthy too.

The results of the trials conducted on the in vitro rooting of shoots in D. fimbriatum and D. moschatum, employing MS (full and half strength) and VW media, supplemented with auxins (NAA, IBA) at two levels (2, 4 ppm), revealed that MS (half strength) with IBA 4 ppm performed better compared to all other treatments. At this treatment level, cent per cent rooting of cultures was recorded. The minimum number of days (21 and 24, in D. fimbriatum and D. moschatum, respectively) for root initiation and the maximum number of roots (8.2 and 7.4, in D. fimbriatum and D. moschatum, respectively) was recorded at this level.

With respect to the growth parameters, viz., plant height, number of leaves produced per shoot, length of the longest leaf, number of roots per shoot and average length of the root, MS (half strength) + IBA 4 ppm gave better response compared to all other treatments. The response of D. fimbriatum in MS (half strength) + IBA 4 ppm was better than D. moschatum.

Addition of sucrose to the medium for inducing rooting of the shoots produced in vitro was found to be effective, especially at the lower levels. Sucrose at 1.5 per cent took the minimum number of days (20) for root initiation and recorded the maximum number of roots per shoot (9.4) in D. fimbriatum.

Activated charcoal was found to influence the in vitro rooting of shoots in D. fimbriatum. AC at lower level was more effective than that at higher level. AC 0.1 per cent recorded the minimum number of days (19) for root initiation and produced maximum number of roots (9.6) per shoot compared to AC at 1.00 per cent.

Of the various explants tried for somatic organogenesis (callus mediated) root segments (aerial and from culture) recorded the maximum percentage of response (60 and 50 per cent, respectively) compared to all other explants tried.

In the culture establishment, the maximum percentage of response in different explants was recorded by the treatment combination of NAA 4 ppm + BA 1 ppm. At this level, the root segments (aerial

as well as from culture) exhibited the highest value (50 and 30 per cent, respectively) followed by leaf (middle portion of mature leaf), where the response was 30 per cent.

With regard to callus induction, the medium containing 2,4-D at 2.0 ppm was found to be the best among the four levels tried. Maximum callusing (CI = 240) was recorded for root segments (from culture) at this level, followed by aerial root (CI = 80). The response of inflorescence stalk to callus was, in general, poor.

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* Original not seen

Appendix

APPENDIX

Abstract of analysis of variance for the effect of different treatments

Sl. No.	Character	Treatment MSS	Error MSS	Level of significance
1	2	3	4	5
	Enhanced release of axillary buds			
1	Period (days) for culture establishment			
a	KC medium			
i	Bud initiation			
	D. fimbriatum	289.76	45.87	1%
	D. moschatum	217.90	3.00	1%
	D. nobile	199.96	27.93	1%
ii	Bud elongation			
	D. fimbriatum	486.90	12.32	1%
	D. moschatum	149.83	0.68	1%
	D. nobile	69.14	40.69	NS
b	MS medium			
i	Bud initiation			
	D. fimbriatum	321.00	51.41	1%
	D. moschatum	731.77	17.74	1%
	D. nobile	191.61	4.12	5%
ii	Bud elongation			
	D. fimbriatum	488.74	69.42	1%
	D. moschatum	826.51	38.59	1%
	D. nobile	803.85	40.00	1%

Contd.

1	2	3	4	5
c	VW medium			
i	Bud initiation			
	D. fimbriatum	381.99	45.17	1%
	D. moschatum	463.27	57.40	1%
	D. nobile	167.40	30.38	1%
ii	Bud elongation			
	D. fimbriatum	486.90	12.32	1%
	D. moschatum	193.99	21.94	1%
	D. nobile	116.06	36.87	1%
d	Comparative performance of Dendrobium spp on different culture media			
i	Bud initiation	237.27	7.91	1%
ii	Bud elongation	82.56	30.96	1%
2	Shoot proliferation			
a	MS (half strength) medium			
	Number of shoots/culture			
	D. fimbriatum	9.85	1.02	1%
	D. moschatum	7.99	1.20	1%
	D. nobile	5.35	1.37	1%
b	VW medium			
	Number of shoots/culture			
	D. fimbriatum	314.42	25.01	1%
	D. moschatum	45.23	10.10	1%
	D. nobile	30.01	2.63	1%
c	Comparative performance of Dendrobium spp in different media			
	Number of shoots/culture	153.92	7.51	1%

Contd.

1	2	3	4	5
d	KIN alone and in combination with NAA			
	Number of shoots/culture			
	D. fimbriatum	10.02	1.80	1%
	D. moschatum	9.90	1.20	1%
	D. nobile	8.20	0.99	1%
e	Medium supplement (CW)			
	Number of shoots/culture			
	D. fimbriatum	51.60	11.90	5%
	D. moschatum	90.47	9.84	1%
	D. nobile	10.85	2.63	5%
f	Continuous subculturing in VW medium			
	Number of shoots/culture			
	D. fimbriatum	5.40	2.54	NS
	D. moschatum	2.11	2.26	NS
	D. nobile	1.40	1.58	NS
3	In vitro rooting			
a	Media and auxins			
i.	Days for root initiation			
	D. fimbriatum	178.38	9.70	1%
	D. moschatum	73.83	6.51	1%
ii	Number of roots/culture			
	D. fimbriatum	21.51	1.93	1%
	D. moschatum	16.11	2.03	1%
iii	Growth parameters			
	Plant height			
	D. fimbriatum	16.40	1.93	1%
	D. moschatum	9.35	0.25	1%

Contd.

1	2	3	4	5
	Number of leaves/shoot			
	D. fimbriatum	46.17	2.05	1%
	D. moschatum	128.80	0.28	1%
	Length of the longest leaf			
	D. fimbriatum	1.87	0.03	1%
	D. moschatum	2.25	0.10	1%
	Number of roots/culture			
	D. fimbriatum	453.77	3.70	1%
	D. moschatum	174.88	1.72	1%
	Average length of the root			
	D. fimbriatum	2.83	0.03	1%
	D. moschatum	1.30	0.03	1%
b	Medium supplements			
i	Sucrose			
	Days for root initiation	37.92	8.90	5%
	Number of roots/culture	21.40	1.43	1%
ii	AC			
	Days for root initiation	79.67	4.43	1%
	Number of roots/culture	8.20	1.30	1%

STANDARDISATION OF EXPLANT FOR *in vitro* PROPAGATION IN *Dendrobium spp.*

By

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ABSTRACT OF A THESIS

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requirement for the degree of

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ABSTRACT

Investigations were carried out at the Plant Tissue Culture Laboratory attached to All India Co-ordinated Floriculture Improvement Project (AICFIP), College of Horticulture, Vellanikkara, during 1990-91 to standardise the explants for *in vitro* propagation in *Dendrobium* spp.

The explants used for the study were shoot tip, axillary bud, inflorescence stalk, leaf segments and root segments. The species of *Dendrobium* used were *D. fimbriatum*, *D. moschatum* and *D. nobile*. For culture establishment, proliferation of shoot/callus and *in vitro* rooting studies, different sources of auxins (NAA , IBA, 2,4-D) and cytokinins (BA, KIN) were used with KC, MS and/or VW media.

In all the explants, surface sterilization using 0.1 per cent mercuric chloride for 10 minutes was found to be the best. The explants collected during April recorded the minimum rate of contamination and the maximum survival percentage. Axillary bud was found to be the ideal explant for enhanced release of axillary buds.

For *Dendrobium moschatum*, MS medium with the treatment combination of NAA 1.5 ppm + BA 1.0 ppm was found to be effective in influencing early bud initiation and elongation, but for *D. fimbriatum* and *D. nobile*, VW medium containing NAA 1.5 ppm + BA 1.0 ppm was found to be the best.

When the cultures were subjected to shoot proliferation, VW medium containing NAA 2 ppm + BA 3 ppm gave maximum number of shoots in D. fimbriatum (18.8), D. moschatum (10.8) and D. nobile (7.6). The shoots produced at this level were well elongated and healthy. The proliferation of shoots also increased when NAA 2 ppm + KIN 3 ppm were used in VW medium, but the number of shoots produced was low, compared to NAA 2 ppm + BA 3 ppm. BA was found to be more efficient than KIN for the induction of axillary shoots.

Addition of CW (15%) into the basal proliferation medium increased the number of shoots in all the three species of Dendrobium. Axillary shoots produced per culture vessel increased at a mean rate of 8.3, 7.7 and 6.6 shoots per subculture in D. fimbriatum, D. moschatum and D. nobile, respectively.

For in vitro rooting, MS (half strength) medium containing IBA 4 ppm was found to be the best. Addition of sucrose at 1.5 per cent and AC at 0.10 per cent enhanced the rooting of the shoots produced in vitro.

Of the various explants tried for somatic organogenesis (callus mediated), root segments (aerial as well as from culture) was found to be the best explant in initiating callus. In the culture establishment, swelling of the explant was observed in modified VW medium containing NAA 4 ppm + BA 1 ppm. As regards callus induction, 2,4-D at 2.0 ppm in modified VW medium was found to be better for all the explants. Maximum callusing (CI = 240) was observed in root from culture. Efforts to induce organogenesis were not successful.