IN VITRO PROPAGATION AND CONSERVATION OF *CYMBIDIUM DEVONIANUM* PAXT. AND *DENDROBIUM LITUIFLORUM* LINDL., RARE AND THREATENED EPIPHYTIC ORCHIDS OF NORTH - EAST INDIA

BY

MEERA CHETTRI DAS

THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BOTANY

NORTH – EASTERN HILL UNIVERSITY
SHILLONG – 793022, INDIA
2007

2007

NORTH – EASTERN HILL UNIVERSITY

SHILLONG

JANUARY 2007

DECLARATION

I do hereby declare that the thesis entitled "*In vitro* propagation and conservation of *Cymbidium devonianum* Paxt. and *Dendrobium lituiflorum* Lindl., rare and threatened epiphytic orchids of North-East India" is a record of original and independent research work carried out by me in the Department of Botany, North-Eastern Hill University, Shillong under the supervision of Prof. Pramod Tandon and Dr. Suman Kumaria. The work done is original and no part of the thesis has been submitted for any other degree or diploma of any university.

Meera Chettri Das

North-Eastern Hill University
Shillong

Date 8-01-07.

Head

Supervisor 8/1/07

Joint Supervisor

Head
Department of Botany
School of Life Sciences
N.E.H.U., Shillong-22

Telefax Work: +91 364 255 0300 ☎Work: +91 364 272 2214/ 272 2244/ 255 0150
E-mail: tandon1@sancharnet.in / profptandon@yahoo.com

North-Eastern Hill University

Professor Pramod Tandon
Dr. Suman Kumaria

Plant Biotechnology Laboratory
Department of Botany
School of Life Sciences
Shillong 793 022, India

CERTIFICATE

We certify that the thesis entitled '***In vitro* propagation and conservation of *Cymbidium devonianum* Paxt. and *Dendrobium lituiflorum* Lindl., rare and threatened epiphytic orchids of North - East India'** submitted by Ms. Meera Chettri Das for the degree of Doctor of Philosophy in Botany Department of the North-Eastern Hill University, Shillong embodies the record of original investigation carried out by her under our supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph. D. Degree. This work has not been submitted for any degree of any other University.

Pramod Tandon 5/1/07
(Supervisor)

Suman Kumaria
(Joint Supervisor)

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to my Supervisor Prof. Pramod Tandon, Plant Biotechnology Laboratory, Botany Department North-Eastern Hill University, Shillong for being ever so supportive and motivating throughout the course of my Ph.D. programme. I am indebted to him for creating the environment conductive to labour with focus during my course of study.

I take this opportunity to express my deep sense of gratitude to my Joint Supervisor Dr. Suman Kumaria for her painstaking supervision, useful comments, valuable guidance and encouragement throughout the course.

I am also thankful to the Head of Botany Department, North-Eastern Hill University, Shillong for the necessary help extended during my research period.

I would like to thank CSIR, New Delhi for financial assistance bearing F. No.9/347(148)/2K3-EMR-I during the course of study.

I would like to express my sincere thanks to all the research scholars and staff members Iaibadaiahun Nongrum, Stadwelson Dohling, Sudipta Das Bhowmik, Jeremy Dkhar, Subarna Hajong, Shrawan Kumar, Ivan Lyngdoh, Manish Deb Nath, Nirmal Das, Pradeep Kar, Debendra Kumar, Tarionglin Mawblei, Dinesh Kumar of our Plant Biotechnology Laboratory and Bioinformatics Centre, North Eastern Hill University, Shillong for their full hearted support and co-operation.

I whole-heartedly thank my husband Mrinal Das, son Durlav Nandan Kashyap for their full support and encouragement.

Last but not the least, I thank my parents, brothers, sister, mother-in-law Gunnada Das, brother-in-laws Bhupen Das, Ramen Das, Ajay Das, Bharat Kalita, Bitupan Das. I also would like to remember my brother in law Late Purno Kalita who inspired me lot during my initial course of Ph.D programe but expired before my submission. I also owe a lot to my sister-in- laws Purnima Kalita, Usha Kalita, Rupjyoti Das and Bornalee Das. I would also like to thank all my nieces.

CONTENTS

CHAPTER I: GENERAL INTRODUCTION

Orchids belong to the family Orchidaceae, one of the largest families of the flowering plants. They are unique plants having a distinct mode of growth and reproduction, which make them incredible and fascinating. Orchids are considered to be the most evolved of the flowering plants. These plants have specialized requirements for habitat. Each orchid species grows only when these habitat requirements are optimal. Orchids have been attracting floriculturists since time immemorial due to their fads, fancies and fashion and this has led to "orchid mania" throughout the world. These are considered as luxury flowers because of their exotic prices. These beautiful and wondrous plants were thought to be parasites growing on trees but now it has been proved beyond doubt that the orchids are autotrophs, which use their hosts merely for anchorage. Orchids are economically important for their horticultural and floricultural appeal. These plants have fascinated people ever since their discovery by Theophrastus (370-285 BC) and they derive their name from the Greek word "Orchis" which means testicles and refers to the paired tubers of terrestrial orchids. The orchids can be found in almost all the parts of the world except the Antarctica.

Besides being commercially important in market, orchids are also important in medicines, food, perfumes etc. Several species of orchids for e.g. *Dendrobium macrae*, *Orchis longifolia*, *Vanda roxburghii* and *Pholidota pallida* are widely used in the

manufacture of Ayurvedic medicines, which help in the treatment of various human ailments (Withner *et al.*, 1974; Maheshwari *et al.*, 1978, Hedge, 1984; Kaushik and Kishore, 1991). The pseudobulbs of *Microstylis wallichi* are used in treatment of tuberculosis. The juice of entire plant of *Dendrobium ovatum* is helpful in all kinds of stomach aches, bile secretion and is also used as a laxative. In India, the orchids *Acampe* and *Vanda* are used for treating rheumatism (Kirtikar and Basu, 1918). The famous 'vanillin' used for flavouring is extracted from the green pods of *Vanilla planifolia.*

In nature, orchids are generally epiphytes growing on trees. However, lithophytes, terrestrials and saprophytes growing on rocks, grounds and organic matter respectively are also found. Orchids are perennial plants blooming annually under favourable conditions of light, temperature and humidity. The flowers are produced either singly or in a spray or balanced spike. Morphologically, the most colourful and showy part of orchid flowers are petals. There are three petals in the orchid flower and of these three petals; one is typically quite different from the others, forming the distinctive lip or the labellum. Orchids are also distinguished from other families by the fusion of their reproductive parts (stamen and pistil) into a column, found at the centre of the flower. These flowers are pollinated by different means, followed by fertilization, which results in the formation of minute seeds. These minute seeds lack an endosperm, resulting in a small embryo covered only by a thin protective wall. This lack of food reserves and protection makes the seeds extremely vulnerable to their environment, resulting in a high mortality rate unless optimum conditions are found for germination (Zeigler *et al.*, 1967). The seeds mature fully when the embryo is still undeveloped. According to Senthilkumar

(2001), in majority of the orchids the embryo are few-celled at the time of seed maturation and its proper development takes place only during germination of seeds. However, as the seeds do not have sufficient reserve food materials to take care of the growth of embryo during germination (Richardson *et al*., 1992), they have to depend on some external source for nutrients so as to make their undifferentiated embryo develop into a protocorm. The mycorrhizal fungi form the major external source of nutrients for the orchids. Consequently, under natural conditions, the orchids are heterotrophic and nourished by symbiotic fungi in the early stages of their establishment (Leake, 1994). Batygina and Adronova (1988) have reported the absence of cotyledons in seven out of the eight orchid species studied by them. It was Bernard (1909) who for the first time isolated the root infecting fungus, which helped orchid seed germination and paved the way for the development of *in vitro* asymbiotic germination of orchid seeds. Mycorrhiza represents ubiquitous associations (symbiotic) between the plant roots and soil-borne fungi (Smith and Read, 1997; Varma, 1998). The most common of these associations, involving arbuscular mycorrhizal fungi (AMF) plays an indispensable role in promoting growth, vigour and survival of plants by positively influencing their nutritional and hydratic status, improving the health of their rhizosphere for better root performance and providing a natural defense against the pests and pathogens.

The tissue culture studies on orchids are gaining wide importance (Charanasri, 1989). The application of tissue culture techniques to the production of quality orchids in large quantities by clonal multiplication, establishment of hybrid plants, improvements of orchid trade and industry are unlimited. The promotion of germination and stimulation of

protocorm growth in *Spiranthes sinensis* var. *amoena* have been reported when the seeds are grown in association with mycorrhizal fungi (Masuhara and Katseya, 1994; Linderman, 1994; Varma, 1995). However, the work of Knudson (1922, 1924 and 1925) suggested that the seed germination of orchids *in vitro* could be accomplished without fungal association by providing nutrient rich medium having balanced organic and inorganic nutrients for the developing embryos. A large number of orchids are propagated from seeds rather than vegetative means. Based on seed germination, the orchids can be divided into the following three categories: -

(i) Tropical epiphytes and lithophytes (*Cattleya, Phaius, Dendrobium* and *Cymbidium*) which germinate readily under asymbiotic conditions, (ii) Tropical terrestrials and lithophytes (*Paphiopedilum*) which are difficult to germinate asymbiotically and may require special media, and (iii) Temperate climate terrestrials which do not germinate under asymbiotic conditions and are solely dependant on their symbionts.

Different workers have suggested a number of media and their modifications for asymbiotic orchid seed germination (Vacin and Went, 1949; Zeigler *et al.*, 1967; Hadley and Harvais, 1968; Rao, 1977; Reyburn, 1978; Henrich *et al.*, 1981; Harvais, 1982; Nakamura, 1982; Krishnan and Jorapur, 1984; Oliva and Arditti, 1984; Pierik *et al.*, 1988; Yam and Weatherhead, 1988; Yam *et al.*, 1989; Kumaria and Tandon, 1991; Pathak *et al.*, 1992; Sharma, 1993; Vij *et al.*, 1995; Devi *et al.*, 1998; Nagaraju *et al.*, 2003). Several growth regulators have been incorporated in the media to promote orchid seed germination and seedling growth in different orchid species (Pierik and Steegman,

1972; Strauss and Reisinger, 1976; Arditti, 1982; Nakamura, 1982; Sharma and Tandon, 1986; Van Waes and Deberg, 1986; Kumaria, 1991; Talukdar, 2001; Nagaraju *et al.*, 2003). The response of orchid protocorms to different media and growth factors supplemented in the medium differ from one species to another (Arditti, 1982). Tamanaha *et al.* (1979) suggested that orchid seeds and seedlings do not require exogenous auxins in most cases. The effect of indole-3-acetic acid (IAA) on orchid culture has been established by many workers. Muralidhar and Mehta (1986) reported 80% germination of *Cymbidium longifolium* seeds in medium containing IAA in combination with Kinetin (KN), tryptophane and asparagine. Incorporation of IAA in the basal medium was also found effective in seed germination of *Cymbidium mastersii* and *Vandaceous* taxa (Prasad and Mitra, 1975; Vij *et al.*, 1981). The influencing effect of IAA on proliferation of protocorm like bodies (PLBs) and seedling growth of *Vanda* hybrids has also been reported (Chaturvedi *et al.*, 1987).

Various investigations regarding the effect of α - naphthalene acetic acid (NAA) on plant tissue culture established the fact that the hormone NAA stimulates growth of shoot, root and proliferation of tissue. Enhanced germination of seeds has been reported in medium containing NAA (Das and Ghosal, 1989). Seedling development of *Dendrobium transparens* was also enhanced in the medium supplemented with NAA (Hazarika and Sharma, 1995). However, Kumaria (1991) reported incorporation of NAA in the medium inhibited both seed germination and seedling growth of *Dendrobium fimbriatum* var *oculatum.* On the other hand, in other orchid species addition of KN in medium containing NAA was effective for subsequent growth and differentiation of

seeds after germination in *Dendrobium transparens* (Hazarika and Sharma, 1995). Similarly, enhanced affect on growth and development of seedlings of *D. fimbriatum* var *oculatum* was reported by Kumaria (1991) in the medium containing KN and NAA in combination. On the other hand, Vij and Kaur (1994) reported inhibitory affect of KN and NAA in combination while working with *Phaius tankervilliae*.

In the studies of plant tissue culture, 2,4-dichlorophenoxy acetic acid (2,4-D) has been reported to induce callusing at very low concentrations (Biondi and Thorpe, 1982; Negrutia *et al.*, 1978; Cornijo-martin *et al.*, 1979). It has been shown to either inhibit germination or stimulate callusing of seeds (Mitra, 1986). Vasil (1982) reported that 2,4-D is more effective auxin to regenerate cell cultures via somatic embryogenesis. In case of orchids, it suppressed rhizogenesis in *Aerides multiflorum* (Vij and Pathak, 1990) whereas in *Paphiopedilum* species it was used successfully (Morel, 1974; Stewart and Button, 1975).

The role of cytokinins in orchid cultures differs from species to species and on the genera studied. Although 6-benzyl amino purine (BAP) or benzyl adenine (BA) is reported to have stimulatory effect on shoot proliferation, leaf disc expansion and growth of stem (Handro *et al.*, 1977), it is reported to retard development and differentiation of cells and tissues of *Cymbidium* protocorms (Gailhofer and Thaler, 1975). KN has been reported to promote greening of protocorms and formation of plantlets leading to greater survival (Fonnesbech, 1972). Shoot bud multiplication through callusing, cell division and enlargement of plant tissue has been reported to be enhanced in the medium supplemented with KN (Miller *et al.*, 1956; Skoog and Miller, 1957). KN in the medium

increased shoot bud multiplication of *Dendrobium chrysanthum* cultures as reported by Vij and Pathak (1989). In case of *Rhynchostylis retusa* direct somatic embryogenesis was observed (Vij and Pathak, 1990).

Interactions between auxins (IAA, NAA and 2,4-D) and cytokinins (BAP and KN) may result in enhanced growth but the effects of these combinations vary with the hormones used, their concentrations and ratios and the orchid (Kusumoto, 1978, 1979a, b; Uesato, 1978).

In vitro multiplication of orchids is also an effective method of saving many species from extinction (Clements and Ellyard, 1979; Clements *et al.*, 1986). Morel (1960) observed that the shoot tips of *Cymbidium* cultured on a suitable medium formed a spherule-like body with rhizoids at the base. These structures resembled morphologically the protocorm developed from the embryo and were hence called Protocorm-like bodies (PLBs). Regular chopping of these PLBs and culturing them on to fresh medium resulted in their multiplication, but when left undisturbed developed into complete plantlets without addition of any growth adjuvants. Most of the economically important orchids, except *Paphiopedilum* are clonable *in vitro* (Murashige, 1978). Shoot tips measuring less than 1 mm can develop into a large number of PLBs and hence give rise to many plantlets (Morel, 1960, 1972). Different explants from orchid plants have been used for multiplication *in vitro*. Many studies have been conducted using shoot tips (Intuwong and Sagawa, 1974; Kusumoto, 1979 a, b; Arditti and Ernst, 1993; Devi *et al.*, 1998; Laishram and Devi, 1999), flower stalk nodes (Homma and Asahira, 1985), leaf segments (Tanaka *et al.*, 1975, 1989; Goh and Tan, 1982: Vij *et al.*, 1984; Mathews and Rao, 1985; Vij *et*

al., 1986; Vij and Pathak, 1990; Abdul Karim and Hairani, 1990; Vij and Aggarwal, 2003), root tips and root meristems (Chaturvedi and Sharma, 1986; Sood and Vij, 1986; Vij *et al.*, 2000), shoot meristem (Sharon and Vasundhara, 1990; Kumaria and Tandon, 1994; Laishram and Devi, 1999), stem explants (Prakash *et al.*, 1996; Pathania *et al.*, 1998; Kanjilal *et a*l., 1999; Van *et al.*, 1999), nodal explants (Teng *et al.*, 1997), axillary buds (Sounderrajan and Lokeshwari, 1994; George and Ravishankar, 1997; Laishram and Devi, 1999) and PLBs (Sheelavanthmath and Murthy, 2001). Large numbers of plants have been generated from stoloniferous stem explants (Latha, 1999). Calli regenerated somatic embryos and regeneration of orchids has also been reported (Ichihashi and Hiraiwa, 1996; Ishii *et al.*, 1998). The success of a particular species through tissue culture of explants largely depends on the medium and the explant source used and it differs from species to species. The incorporation of certain additives and growth factors into the media proves to be beneficial for tissue culture of many orchids (Kusumoto, 1979 a, b; Yoneda and Momose, 1988).

Artificial seed technology is an exciting and rapidly growing area of research in the delivery of propagules. It not only helps in easy handling and transportation of plantlets but also can be used for conservation of rare, endangered and desirable genotypes (Kumaria and Tandon, 2001). As propagation of many ornamental plant species is labour intensive, therefore integration of simple artificial seed system would dramatically reduce labour requirement thus lowering production costs (Gray and Compton, 1993). Moreover, the major aim in developing *in vitro* storage methods is to reduce the frequent demands of subculturing and preserving the unique genetic

constitution of the germplasm. Freezing at liquid nitrogen (LN_2) temperature tends to suppress cell division, arrests growth and retains the cells in metabolically inactive state which prevents the cells from ageing and provides indefinite life span with no genetic change. However, the technique is not yet applicable to many plant species. Hence, shoot cultures of many plant species have been stored under condition in which growth is slowed down by use of a reduced culture temperature or by the application of osmotica or growth retardants (Mix, 1982, 1985; Monette, 1986; Staritsky *et al.*, 1986; Love *et al.*, 1987). The inherent advantages of artificial seeds are the production of many somatic embryos and the use of conventional seed handling techniques for embryo delivery. Artificial seed production has been tried through encapsulation of seeds (Jha *et al.*, 1993; Khor *et al.*, 1998; Patel *et al.*, 2000), flower buds (Mitra and Chaturvedi, 1972), axillary buds (Bapat *et al.*, 1987; Bapat and Rao, 1988; Mathur *et al.*, 1989; Soneji *et al.*, 2002), shoot tips (Wang *et al.*, 2002), nodal explants (Rout *et al.*, 2001) and root (Micheli *et al.*, 1996; Picconi *et al.*, 1997). Sharma *et al.* (1992) and Sharma (1993) for the first time reported the regeneration of complete plantlets of *Dendrobium wardianum* from synthetic seeds. Subsequently, complete plantlets of *Cymbidium giganteum*, an endangered orchid, were obtained by the germination of artificial seeds (Corrie and Tandon, 1993; Corrie, 1994). Artificial seeds, consisting of somatic embryos and PLBs (orchid) enclosed in a protective coating have been proposed as a low-cost, high volume propagation system (Redenbaugh, 1990). Storage of alginate-encapsulated loblolly pine and Norway spruce somatic embryos has been reported by Gupta and Durzan (1986, 1987). Also, inhibited germination of alginate-encapsulated alfa-alfa somatic embryos stored for one week at

4°C was reported (Redenbaugh *et al.*, 1986a). Further, Fujii *et al.* (1989) arrested the germination of encapsulated alfa-alfa somatic embryos by treating them with abscissic acid (ABA), thus attaining maturation of the plantlets before transferring them to greenhouse conditions thereby enhancing the survival rate. Research on artificial seeds has increased significantly and various studies have been made (Kitto and Janick, 1985; Bapat *et al.*, 1987; Mathur *et al.*, 1989; Seneratna *et al.*, 1990; Fernandes *et al.*, 1992). However, the germplasm conservation reports in orchids remain scanty.

Plantlets developed *in vitro* wilt rapidly on transfer to normal green house or field conditions. Poor water uptake and excessive water loss (Grout and Aston, 1977) may lead to high rates of mortality unless plantlets are acclimatized by gradual stages to reduce humidity and increased light intensity (George and Sherrington, 1984). The problems of poor water relations are coupled by damage to shoot and roots during transplantations (Deberge and Maene, 1981). Thus, the establishment and healthy growth of *in vitro* raised plants in the glass house require suitable conditions of acclimatization and hardening. Different potting mixtures, containers and compost influence the growth of orchids extensively (Bose and Bhattacharjee, 1980; Stewart, 1988; Talukdar *et al.*, 1988; Yadav *et al.*, 1988; Cribb, 1990; Robbins and Bell, 1990). Water retaining capacity of sphagnum and osmunda moss helps in the initial establishment of the orchid plantlets in the pots. Addition of manure and fertilizers is considered beneficial and the amount as well as the type varies from one species to another.

There are about 30,000 species of orchids in about 800 genera distributed all over the world (Chowdhery, 2001). Orchids are found from sea level to snow covered alpine

regions but their number varies in different regions due to the prevailing climatic conditions. India is one of the richest reservoirs of orchids. It is estimated that about 1,300 species in 140 orchid genera are naturalized in India with Himalayas as their main home and the Eastern and Western Ghats as other localities (Chowdhery, 1998; Hynniewta, 2000). The Indian orchids grow at altitudes as high as 5,000 m, and in areas having an annual rainfall of as low as 60 cm and as high as 1,100 cm. The epiphytic orchids are abundant upto 1800 m and their frequencies progressively decrease with further increase in altitude. Several orchid genera including *Cryptochilus, Anthogonium, Risleya, Sirhookera* and *Cleistocentron* are endemic to India.

The North-Eastern region of India hosts a number of orchids. Out of 1,300 species of orchids recorded from India, 800 species are found in North-Eastern India (Deb *et al.*, 2003). This region has the highest concentration of monotypic orchid genera. It also harbours a large number of saprophytic orchid species belonging to the genera *Aphyllorchis, Cymbidium, Epipogium, Eulopia, Galeola, Gastrodia, Stereosandra*, etc. Besides, North-East India hosts a large number of endemic, rare and threatened orchid species (Nayar and Sastry, 1997-98, 1999; Ahmedullah, 2000). Among the North-Eastern states maximum diversity of orchids is found in Arunachal Pradesh (130 genera with 600 species), followed by Sikkim with 123 genera and 451 species, while it is lowest in Tripura with only 33 genera and 48 species (Deb *et al.*, 2003). Although the North-East India is reported to have the richest reservoir for rare ornamentals, the orchid resources of this region are fast depleting due to ruthless exploitation of orchid plants for commerce and trade and also due to increasing deforestation. On account of this, a few species are

extremely scarce or perhaps already extinct and many more are facing the danger of being wiped out. In this context, the natural population of *Cymbidium devonianum* and *Dendrobium lituiflorum* are on decline and has become rare and threatened in North-East India due to the loss of habitat (Chowdhery, 2001). Keeping in mind the conservation and protection of these orchids from extinction, work was undertaken for "*in vitro* propagation and conservation of these two orchids.

Cymbidium devonianum Paxt. is an epiphytic orchid of considerable ornamental and horticultural importance. Its pseudobulb is very short and has many leaves with long petioles. Flowers are with drooping scapes and pale yellowish in colour with purple dots (Plate 1a, b). Bracts are small, long, ovate and acute. The ovary is long, terete and pedicelled. Sepals are green in colour with three purple dotted lines, which are subequal, oblong and long. The flowering time of *C. devonianum* is April-July. It is found in Meghalaya, Arunachal Pradesh, Manipur, Mizoram and Nagaland (Kataki, 1986).

Dendrobium lituiflorum Lindl., is also an epiphytic orchid of North-East India. Its pseudobulb is long, pendulous, slender and gray in colour. The base is swollen and the upper part is terete. Leaves are narrow, deciduous, long, oblong-lanceolate and acutish. It bears 2-3 flowers per node, which are short and amethyst purple in colour (Plate 2a, b). Its lip is white with purple transverse stripes; sepals are linear-oblong, subacute, petals are broadly elliptic and mentum is short. The lip is trumpet in shape and puberulous. The flowering time is March-May. It is found in Assam and Manipur (Bose and Bhattacharjee, 1980).

Plate 1

(a) *Cymbidium devonianum* Paxt. blooming in natural habitat

(b) A closer view of the flowers


a


b

Plate 2

(a) *Dendrobium lituiflorum* Lindl. blooming in natural habitat

(b) A closer view of the flowers


a


b

CHAPTER II: ASYMBIOTIC SEED GERMINATION OF *CYMBIDIUM DEVONIANUM* AND *DENDROBIUM LITUIFLORUM*

INTRODUCTION

Orchid seeds are unique in several aspects (Arditti, 1979). They are minute and have limited food reserves (Singh, 1981; Smereciu and Currah, 1989). Lipids and small amounts of proteins are the storage materials as they lack endosperm (Savina, 1974) and cotyledons (Arditti and Ernst, 1984). Association with particular fungal species is necessary for early stages of development because of which less than 5% of the orchid seeds germinate in nature (Rao, 1977). On the other hand, a very high percentage of seed germination could be achieved asymbiotically in flasks or test tubes. Asymbiotic techniques of orchid seed culture are useful in tracing the seedling development from undifferentiated embryos. Seed culture can be applied not only to several artificial and natural hybrids, but also to certain desirable species and forms which are not available in quantity. Seedling production *in vitro* and further development is also an effective means of saving many orchid species from extinction. Using different nutrients and culture conditions, seed germination in a large number of orchid species has been accomplished. However, *in vitro* germination methods successful for one species are not always applicable to others and also procedures for orchids from one region may not be suitable for another (Arditti *et al.*, 1981).

MATERIAL AND METHODS

About nine - month old capsules of both *C. devonianum* and *D. lituiflorum* were collected from the plants growing in the glasshouse of the Department. All the experiments were carried out aseptically in the transfer table of the laminar flow. Before using the transfer table, the laminar flow cabinet was thoroughly scrubbed with cotton soaked in 70% ethanol. The ultra violet (UV) tube light of 30 watt was switched on for 10-15 min after which the transfer area was left as such for about 5 min with the flow of ultra filtered sterile air to strain out particles as small as 0.3 mm providing a sterile atmosphere for work. The velocity of the airflow which is 27 ± 3 m/min helped in preventing air-borne contamination. Forceps, needles, surgical blades etc. were sterilized by dipping in 70% alcohol and flamed before use for proper sterilization. The collected capsules were washed thoroughly with detergent under tap water and surface-disinfected in 70% ethanol for 30 sec followed by surface flaming in the transfer table. This process was repeated 3 times after which capsules were rinsed five times with sterile distilled water and dried in a laminar airflow cabinet before dissecting. The capsules were then dissected longitudinally with a surgical blade in the laminar airflow cabinet. The seeds were scooped out from sterilized capsules and inoculated spreading as thinly as possible over the surface of the culture medium in 25 x 150 mm glass test tubes each containing 15 ml of medium (Kumaria and Tandon, 1991). The media tried consisted of Murashige and Skoog (MS, 1962), Nitsch and Nitsch (NN, 1969), Gamborg *et al.,* (B_5, 1968), Mitra *et al.*, (Mitra, 1976) and Knudson C (KnC, 1946). The pH of the media was adjusted to 5.8 prior to autoclaving. The culture tubes were incubated at 25 ± 2°C under 16-hour

photoperiod of 150 μmol m^{-2} s^{-1} light intensity. The inoculated seeds were examined regularly every week. The percentage germination and protocorm volume of *C. devonianum* and *D. lituiflorum* seeds in different media was determined by examining the seeds microscopically after 3 and 2 months respectively. The seeds were considered to have germinated upon emergence of the embryo from the testa. The percentage germination was calculated using both green and white protocorms. The protocorm volume was determined using the formula given by Stoutamire (1981) for an oblete speroid 4/3 $\pi a^2 b$, where a and b are minor and major semi-axes respectively. For further studies of protocorm development, five different developmental stages were considered. These were as follows:

Stage I - Non-germinated seeds, embryo slightly swollen but still covered with its seed coat or testa

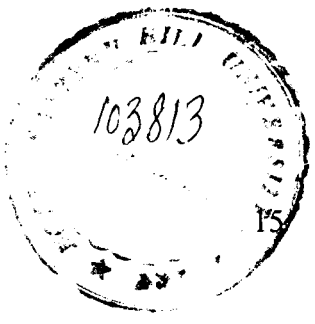
Stage II - Germinating seeds, embryo greatly swollen forming an ovoid tear-drop shaped protocorms without seed coat or testa

Stage III -Young protocorms showing pointed vegetative apex

Stage IV -Seedlings with 2-3 leaves

Stage V - Seedlings with roots

Ten replicates were used for each treatment and the experiments were repeated thrice.



RESULTS

C. DEVONIANUM

The best germination percentage for C. *devonianum* was recorded on B_5 medium wherein 92.82% of response was observed, whereas on NN and MS medium, 87.30 and 67.70% response was recorded respectively (Table 1; Plate 3). The seeds showed poor germination on Mitra medium with only 28.90% of germination. On KnC medium, slight swelling of seeds was observed which failed to germinate and turned brown in course of time (Plate 3). Protocorm volume recorded also varied from medium to medium (Table 1; Plate 3). The protocorm volume was recorded to be the largest (2.92 mm^3) on B_5 medium followed by NN (2.82 mm^3) and MS medium (2.14 mm^3). The size of the protocorms developing on Mitra medium was smaller (2.04 mm^3) as compared to those developing on B_5, NN and MS media. The time taken in attaining different stages of protocorm development on different media also varied (Table 2). On B_5 medium, germination was faster than that observed on other media. On the same medium, ungerminated seeds were observed for 5 weeks (Plate 4a), but the seeds started to germinate in 6-7 weeks time, after which transformation of seeds into protocorms was observed (Plate 4b). However, this transformation stage was delayed on other media studied. Stage II was attained within 7 weeks of culture on B_5 medium, whereas the same stage on NN was obtained in 9 weeks of culture. This stage was delayed on MS (11 weeks) and Mitra (12 weeks) media and was not obtained at all on KnC medium. Slight swelling of the seeds on KnC medium was recorded after 4 weeks of culture which remained as such for about 20 weeks after which the embryo inside the testa gradually turned brown and died. On the

Table 1: Effect of different media on seed germination and growth of protocorms of *C. devonianum*

Media	Germination (%) *	Volume (mm^3) *
MS	67.70±1.1	2.14±0.49
NN	87.30±3.0	2.82±0.28
B_5	92.82±1.7	2.92±0.33
Mitra	28.90±3.8	2.04±0.75
KnC	0	-

± SE

* Data recorded after 3 months

Plate 3

Asymbiotic seed germination of *C. devonianum* after 3 months

(bar = 1 mm)

Ungerminated seeds at 0 day (a), Protocorms on MS (b), NN (c),

B_5 (d), Mitra (e) and ungerminated seeds on KnC (f) media

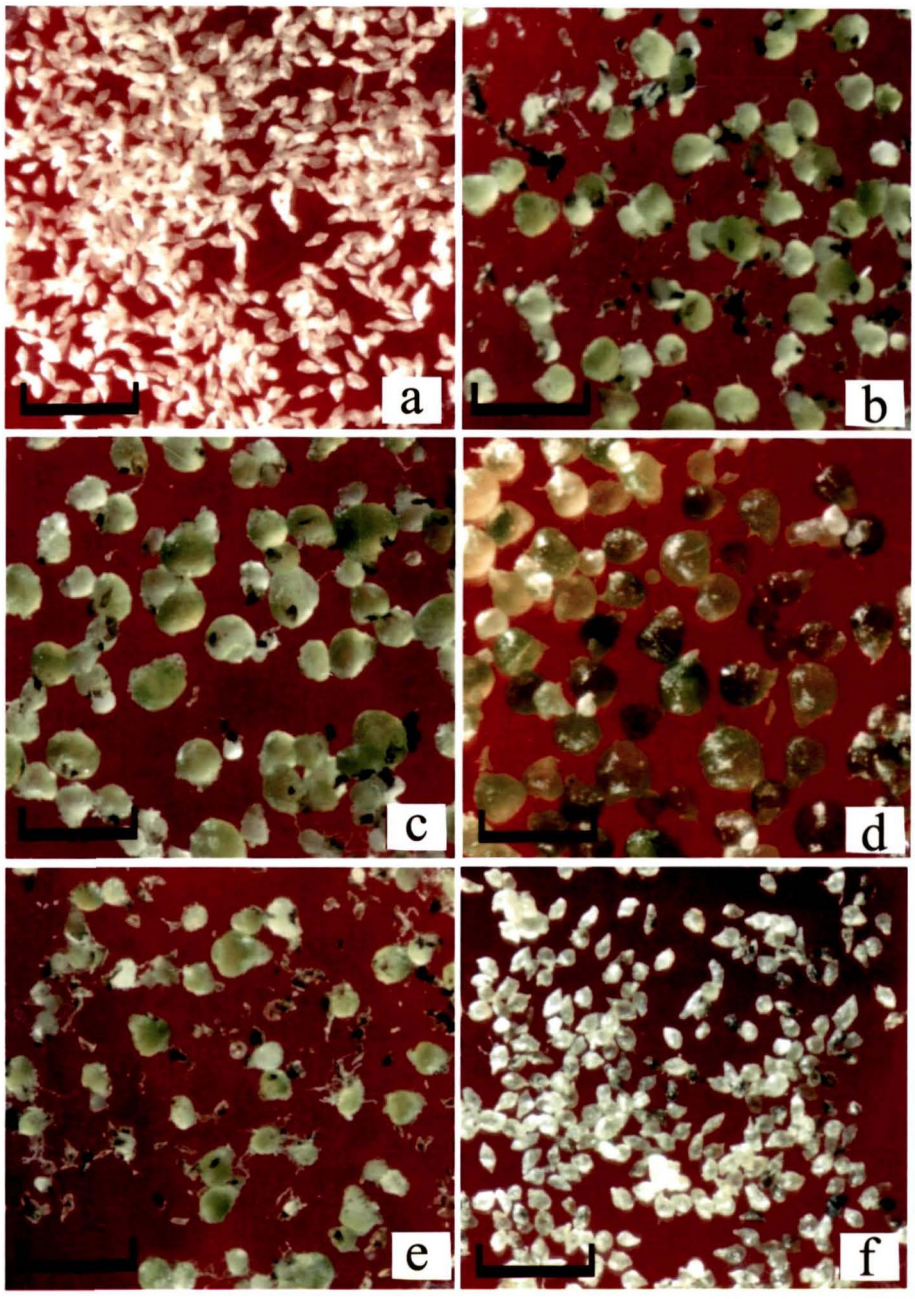

a
b
c
d
e
f

Table 2: Effect of different media on the developmental stages of protocorms of *C. devonianum*

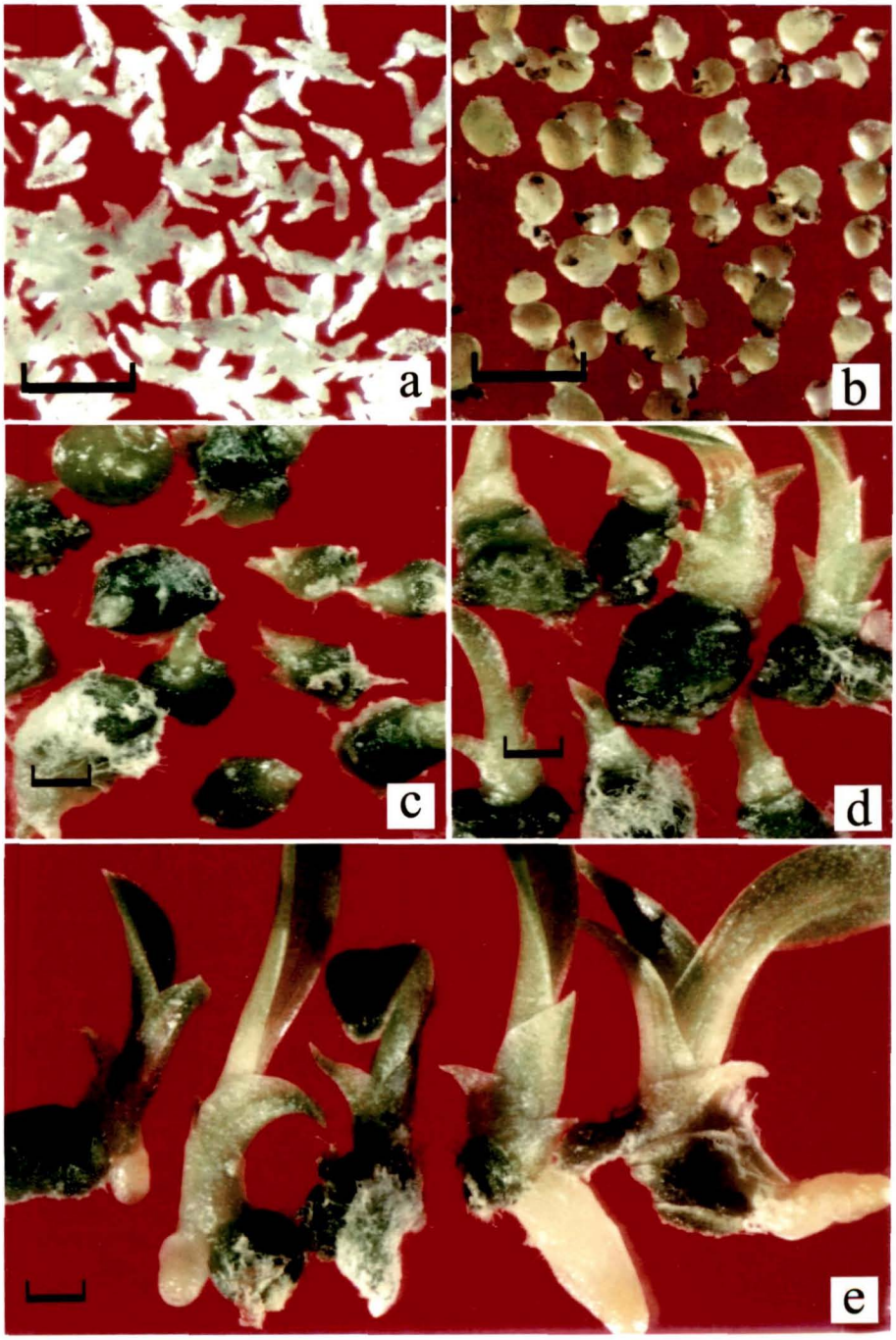
Media	Developmental Stages (weeks)					Remarks
	I	II	III	IV	V	
MS	7	11	13	15	17	Healthy complete plantlets developed
NN	6	9	14	17	20	Protocorms differentiated into healthy shoots and roots
B_5	5	7	12	15	19	Healthy plantlets differentiated into shoots and roots
Mitra	9	12	-	-	-	No differentiation beyond protocorm stage
KnC	20	-	-	-	-	Swelling of seeds; did not germinate, turned brown and died

± SE

Plate 4

Different developmental stages of *C. devonianum* in MS medium (bar = 1mm)

a. Stage I- Non-germinated seeds in 8 weeks
b. Stage II- Germinating seeds in 11 weeks
c. Stage III- Young protocorms with pointed vegetative apex in 13 weeks
d. Stage IV- Protocorms with 2-3 leaves in 15 weeks
e. Stage V- Development of shoots and roots in 17 weeks


a
b
c
d
e

other hand, the difference in time taken for development of protocorms from stage II to stage III was faster on MS medium which was attained in only 2 weeks (Plate 4). Development of the same stage was however delayed by 5 weeks in B_5 and NN media (Table 2). The protocorms on Mitra medium did not develop beyond stage II and eventually turned yellow after 12 weeks of culture and remained as such for several weeks without further development. The development of protocorms into stage IV and to stage V was also faster in MS media which took only 2 weeks each respectively. Similarly, stage IV was attained at the same time in both B_5 and NN media but further development into stage V was faster in NN medium when compared to B_5 medium (Table 2).

D. LITUIFLORUM

The seeds of *D. lituiflorum* germinated on all the five media tried. Maximum seed germination (99.2%) was recorded on MS medium followed by NN (97.2%) and B_5 (88.9%) media (Table 3). The percentage germination was comparatively reduced on Mitra (79.3%) and KnC media (65.4%). There was a large variation in the volume of protocorm developing on the different media (Table 3). The protocorm volume was recorded to be the largest (3.4 mm^3) on MS medium (Plate 5). Comparatively, the volume of the protocorms on the other media tried was much reduced. It was recorded to be as low as 1.1 mm^3 on NN medium. In B_5, Mitra and KnC media, the protocorm size was greatly reduced i.e. 0.3 mm^3, 0.4 mm^3 and 0.3 mm^3 respectively. Developmental stages of protocorms also differed with time in different media tried. The minimum time of two weeks was recorded to attain stage I on MS and NN media (Table 4). Further,

Table 3: Effect of different media on seed germination and growth of protocorms of *D. lituiflorum*

Media	Germination (%)*	Volume (mm^3) *
MS	99.2±0.6	3.4±1.70
NN	97.2±1.2	1.1±0.10
B_5	88.9±2.8	0.3±0.08
Mitra	79.3±4.9	0.4±0.17
KnC	65.4±5.6	0.3±0.04

± SE

* Data recorded after 2 months

development to stage II was faster in MS medium which took only a week, whereas in other media tried it was delayed by 2 weeks. That is, germination was faster in MS medium compared to other media. The transition period from stage II to stage III was also faster on MS medium which was attained in 3 weeks time (Table 4). The same transition period was delayed by 5 weeks on NN medium and by 6 and 8 weeks on B_5 and Mitra media respectively. However on KnC medium, stage III was not attained at all. Here, the protocorms turned brown after 10 weeks of culture and died subsequently. Out of all the five media tried, stage IV was obtained only on MS, NN and B_5 media (Table 4). Here also the transition period was faster on MS medium that is, 3 weeks time. It was observed that only on MS medium the protocorms developed into complete plantlets (Plate 6). The protocorms on NN and B_5 media failed to develop further, turned brown and died subsequently.

DISCUSSION

The orchid seeds responded differently to different media used for germination. The development of orchid seed requires a balanced supply of both organic and inorganic nutrients (Arditti, 1982; Zeigler *et al.*, 1985; Van Waes and Deberg, 1986). In the present study, seeds germinated asymbiotically on medium devoid of plant growth regulators. This could be due to the presence of sufficient endogenous growth regulator(s) required for the initial stages of germination (Lo *et al.*, 2001). The nutrient requirement of orchid seeds in terms of quantity as well as in form may vary at different stages of development (Arditti and Ernst, 1984). In the present study, seeds of *C. devonianum* germinated best in B_5 medium having optimum concentration of nitrogen (25.76 mM) (Table 5), whereas

Table 4: Effect of different media on the developmental stages of protocorms of *D. lituiflorum*

Media	Developmental stages (weeks)					Remarks
	I	II	III	IV	V	
MS	2	3	6	9	12	Protocorms differentiated into healthy shoots and roots
NN	2	4	9	13	-	Protocorms were differentiated only up to shoot stage
B_5	4	6	12	16	-	Protocorms did not differentiate into roots
Mitra	5	7	15	-	-	Protocorms differentiated only up to vegetative apex beyond which growth was stunted
KnC	6	8	-	-	-	Seeds germinated but failed to develop beyond protocorm stage

± SE

Table 5: Concentrations of major ions and vitamins in nutrient media (mM)					
Component	MS	B_5	NN	M	KnC
NO_3^-	39.43	24.75	18.39	2.63	2.63
NH_4^+	20.62	1.01	8.99	0.76	0.76
Total N	60.05	25.76	27.38	3.39	3.39
K^+	20.06	24.76	9.89	1.78	2.93
Ca_2^+	2.99	1.02	1.13	0.85	0.85
Mg_2^+	1.50	1.01	0.75	0.81	1.02
PO_4^{3-}	1.25	0.96	0.49	1.60	1.10
Cl^-	5.99	1.02	1.13	-	0.02
SO_4^{2-}	1.73	2.19	1.88	0.86	0.84
Thiamine HCL	0.0003	0.03	0.001	0.0009	0.0009
Nicotinic Acid	0.004	-	0.04	0.01	-
Pyridoxin HCL	0.002	-	0.002	0.001	0.001
Glycine	0.03	0.03	0.03	-	-
Riboflavin	-	-	-	0.0001	0.0008
Biotin	-	-	-	0.002	-
Folic Acid	-	-	-	0.0007	-

Plate 5

Asymbiotic seed germination of *D. lituiflorum* after 2 months

(bar = 1 mm)

Ungerminated seeds at 0 day (a), Protocorms on MS (b), NN (c),

B_5 (d), Mitra (e) and KnC (f) media

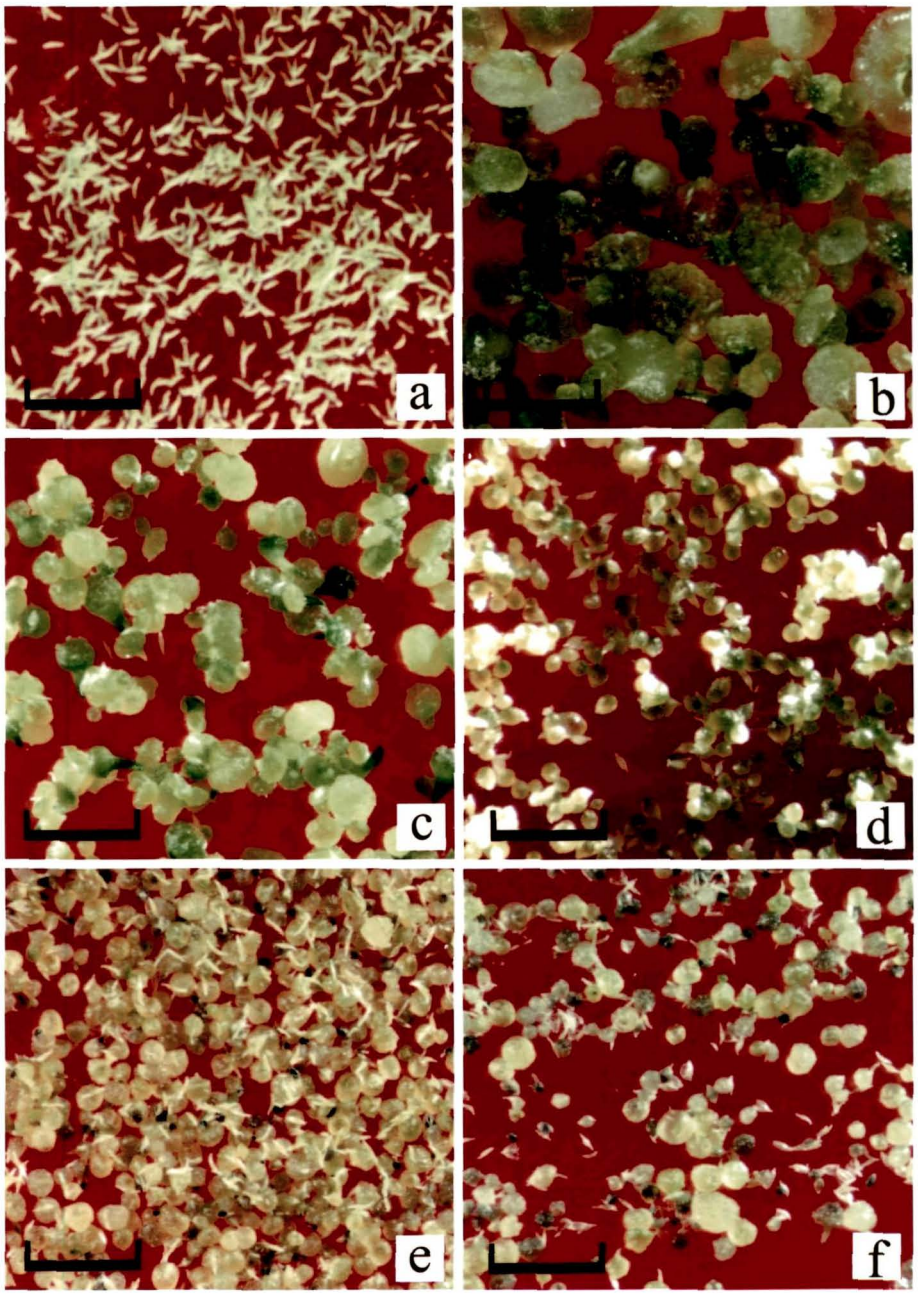
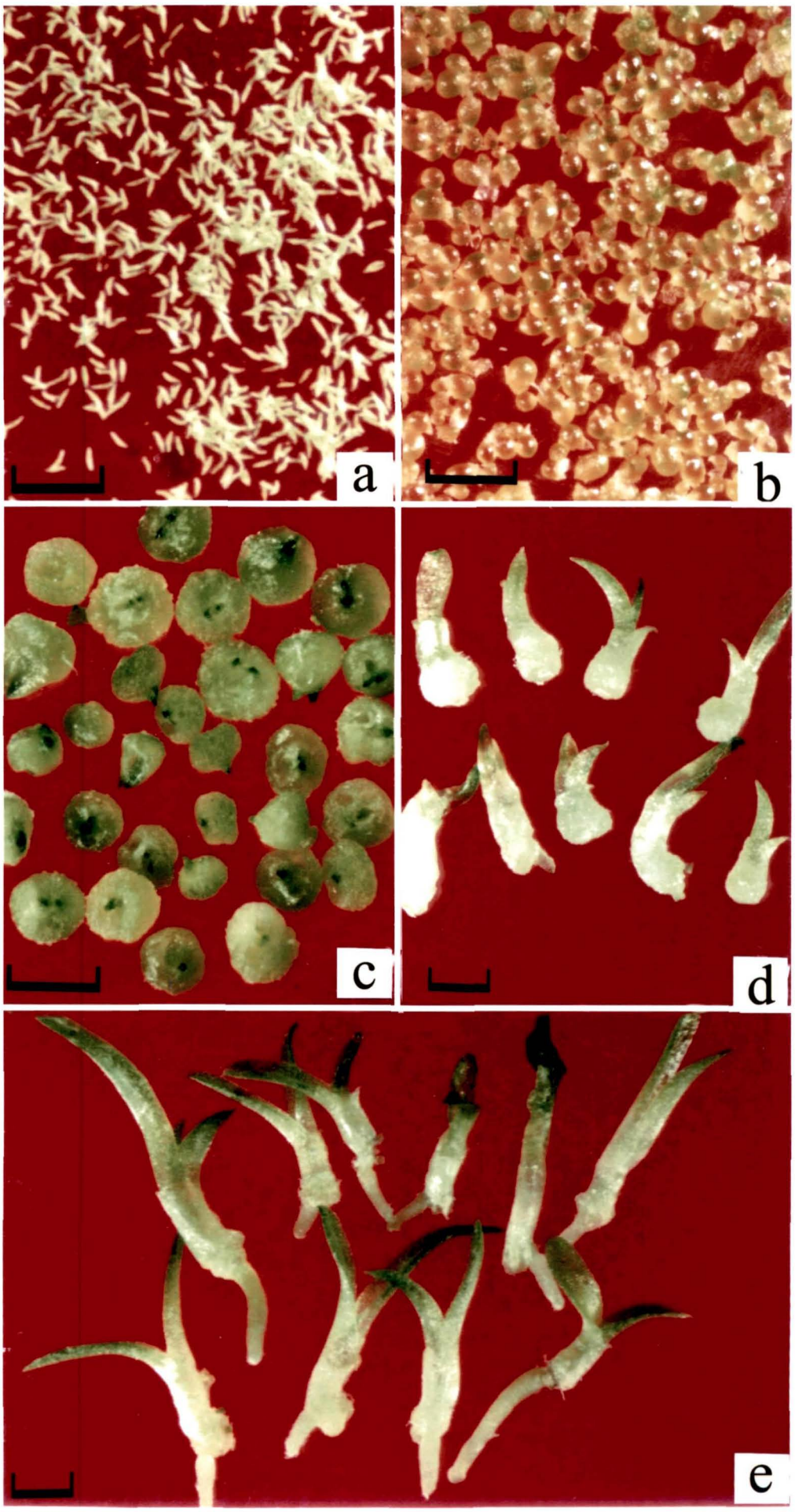

a
b
c
d
e
f

Plate 6

Different developmental stages of *D. lituiflorum* in MS medium (bar = 1mm)

a. Stage I- Non-germinated seeds, 0 Day
b. Stage II- Germinating seeds in 3 weeks
c. Stage III- Young protocorm with pointed vegetative apex in 6 weeks
d. Stage IV- Protocorms with 2-3 leaves in 9 weeks
e. Stage V- Development of shoots and roots in 12 weeks


a
b
c
d
e

high concentration of nitrogen (60.05 mM) present in MS medium was required for optimal germination of *D. lituiflorum* seeds. Also, presence of nitrates in the form of potassium nitrate in B_5 medium might have supported high germination of *C. devonianum* seeds, whereas in case of *D. lituiflorum*, MS medium proved more effective for inducing early and better germination of seeds. Presence of both ammonium nitrate and potassium nitrate in MS medium could have been the most suitable form of nitrogen for seed germination of *D. lituiflorum*. The importance of ammonium or nitrate ions (individually or in combination) during *in vitro* germination of orchid seeds as source of nitrogen is well established (Curtis and Spoerl, 1948; Withner, 1959). *Cymbidium* seeds are reported to germinate better in ammonium as compared to nitrate (Curtis and Spoerl, 1948). However in the present study, it was found that presence of nitrate in B_5 medium was a suitable source of nitrogen for seed germination of *C. devonianum*. Besides, presence of high amount of the vitamin, thiamine in B_5 medium might have affected the germination of *C. devonianum* seeds (Mead and Bulard, 1975; Ohira *et al.*, 1976; Sharma *et al.*, 1991). Both the seeds of *C. devonianum* and *D. lituiflorum* responded poorly on Mitra and KnC media. Mixtures of many vitamins present in both Mitra and KnC could have been inhibitory for germination of *C. devonianum* seeds. This is in agreement with the earlier report on orchids (Bahme, 1949). Although there are reports of orchid seed germination in KnC medium (Bopiah and Jorapur, 1986; Sharma and Tandon, 1987; Pyati and Murthy, 1995), in the present study it was found seeds of *C. devonianum* and *D. lituiflorum* germinated poorly/failed to germinate on KnC medium which is similar to the report of Clements (1981), wherein failure of seed germination of some epiphytic

species on KnC medium was observed. Likewise, several Indian orchids including species of *Dendrobium* and *Cymbidium* are reported to have poor seed germination on KnC medium (Chaturvedi *et al.*, 1987). Yam and Weatherhead (1988) reported that the nutritional requirements of germinating orchid seeds vary due to their physiological state. The protocorms of *C. devonianum* and *D. lituiflorum* produced on B_5 and MS media respectively were quite large compared to those developed on other media. Healthy growth of orchid protocorms in medium containing balanced supply of organic and inorganic nutrients has been reported (Arditti and Ernst, 1984). Initiation of seed germination, protocorm development and subsequent growth and development of seedlings vary with the species and the medium employed (Reddy *et al.*, 1992). In case of *C. devonianum*, seed germination was faster in B_5 medium than that observed on MS and Mitra media. However, further development of protocorms having vegetative apex, leaf initials, shoot and root initials was faster on MS medium as compared to other media. Incidentally, all the presently media employed differ from one another in their chemical composition. Nutritional requirement at different stages of development varied. Presence of optimum nitrogen salts and high amount of thiamine in B_5 medium might have promoted faster growth of seeds till protocorm stage, but presence of high nitrogen salts in MS medium was required for further growth and development of protocorms in *C. devonianum*. The difference in response to nitrogen sources used may be due to varied requirements of the species. Additional vitamins like folic acid, riboflavin in NN and Mitra media which may prove inhibitory in the later stages of development and might have affected further growth of the protocorms. Such negative results with vitamins for

seed germination/seedling growth have been reported in certain cases (Nakamura, 1982; Sharma *et al.*, 1991). The higher nitrogenous content and presence of optimal vitamins in MS medium might have led to further development of protocorms into plantlets. In case of *D. lituiflorum*, seed germination as well as protocorm growth and development was faster in MS medium. MS medium containing optimal macro and micro nutrients, vitamins, inositol, glysine etc. could have proved beneficial for seed germination as already suggested by Devi *et al.* (1999). Complete plantlets of *D. lituiflorum* were obtained only in MS medium. A better efficacy of MS medium in maintaining healthy growth of the seedlings may also be attributed to their Fe-EDTA contents; a growth promotory nature of Fe-EDTA is already on record (Lee *et al.*, 1983). Protocorms of *D. lituiflorum* differentiated only upto shoot stage on NN and B_5 media. Roots were not developed in these media which is in consistent with the literature of Nath *et al.*, (1991) who reported that the growth of the seedlings of *V. coerulea* was arrested prior to rooting in NN medium. In Mitra medium, the protocorms differentiated only upto vegetative apex beyond which growth was stunted. Whereas in case of KnC medium, only protocorm stage was developed after which the protocorm turned brown and died subsequently. Failure to develop beyond the protocorm stage in *V. coerulea* was also recorded in KnC medium (Nath *et al.*, 1991). A better response of *C. devonianum* seeds on B_5 and MS medium at different stages of development and *D. lituiflorum* seeds on MS medium suggest the importance of an interaction (compatibility) between the orchid genotype and the nutrient regime in the culture medium.

The present study revealed that the early stages of development (seed germination) of *C. devonianum* required optimum medium, whereas later stages of development (growth of vegetative apex, shoot and root development) required rich medium containing higher concentrations of nutrients and vitamins. The seeds of *D. lituiflorum* required a rich medium all through out its growth and differentiation. All the observations in the present study also suggest that differentiation of organ in a species is a direct manifestation of its genetic and physiological features as has been reported by Kathyar *et al.* (1987).

CHAPTER III: SEEDLING GROWTH AND DEVELOPMENT OF *CYMBIDIUM DEVONIANUM* AND *DENDROBIUM LITUIFLORUM*

INTRODUCTION

Orchid seedlings are very much adaptable to wide variations of inorganic salt combinations. They can grow well in a great variety of inorganic combinations and concentrations. The commonly used nutrient media for orchid seedlings are those proposed by Murashige and Skoog (1962), Reddy *et al.* (1992), Nitsch and Nitsch (1969), Gamborg *et al.* (1968) and Nagaraju *et al.* (2003). A better efficacy of MS, NN and B_5 media in maintaining healthy growth of the seedlings may be attributed to the presence of elements and vitamins in optimal quantities that promote seedling growth in orchids (Arditti, 1967, 1979; Harrison and Arditti, 1970; Harvais, 1973; Mukherjee *et al.*, 1974). Mitra *et al.* (1976) proposed a better medium for seed germination, growth and development of large number of orchid species. Poor seedling growth in KnC medium was recorded in *Cymbidium* and *Cattleya* species (Nagaraju *et al.*, 2003). The development of seedlings obtained from capsules of different ages was recorded poor in KnC medium. A similar result was reported where *Geodorum densiflorum* PLBs failed to develop into shoots in KnC medium (Sheelavantmath *et al.*, 2000).

Seedling growth can be manipulated by the addition of various growth regulators in the medium. *In vitro* seedling growth is influenced differentially by auxins, cytokinins and the results have been inconsistent and inconclusive (Arditti, 1967, 1979, 1982; Withner, 1959, 1974). Many workers have established the effect of IAA on orchid culture. This auxin was effective at 0.25 mg/l in V*andaceous* taxa (Vij *et al.*, 1981). However, inhibitory effect of IAA at higher concentrations for seedling growth of *Coelogyne punctulata* was recorded by Sharma and Tandon (1986). NAA in the medium have been reported to enhance germination/seedling growth to some extent (Arditti and Ernst, 1984; Sharon and Vasundhara, 1990; Talukdar, 2001). The auxin, 2,4-D generally used as weedicide is also used extensively for plant tissue culture by many workers (Vij *et al.*, 1984; Kanjilal *et al.*, 1999; Chen *et al.*, 2002). 2,4-D is reported to induce callusing in *Cymbidium ensifolium* var. *misericors* (Chang and Chang, 1998). It is more effective auxin to regenerate cell culture via somatic embryogenesis (Chen and Chang, 2000). It has been reported to suppress rhizogenesis in *Aerides multiflorum* (Vij and Pathak, 1990). Morel (1974), Stewart and Button (1975) used 2,4-D successfully in tissue culture studies of *Paphiopedilum* species. The inconsistent results indicate that in general the *in vitro* developing seedlings do not require an exogeneous source of auxins. The species, which require or benefit from an exogeneous source probably, derive them from their mycorrhizal fungus in nature (Arditti and Ernst, 1984). The growth of orchid seedlings *in vitro* is enhanced or inhibited/unaffected by cytokinins. Van Waes and Debergh (1986) reported the necessity of cytokinin for *Cypripedium calceolus* and *Epipactis hellebornie* but not for *Dactylorhiza musculata* and *Listera ovata.* Sheelavanthmath and Murthy

(2001) reported that multiple PLBs were developed from single protocorm cultured on the medium supplemented with BAP. A similar result was also reported where maximum number of protocorms, besides higher number of shoots was produced at low concentration of BAP (Nagaraju *et al.*, 2003). The inhibitory effect of BAP on the development and differentiation of *Cymbidium* protocorms has been reported (Gailhoper and Thaler, 1975). KN in medium has been found to stimulate seedling growth of *Orchis pupurella* (Harvais, 1973), *Cyprepedium reginae* (Harvais, 1982), *Dendrobium chrysanthum* (Vij and Pathak, 1989), *Dendrobium aphyllum* (Talukdar, 2001). An inhibitory role of KN in the medium was observed in *Dendrobium* and *Laeliocattleya* (Kano, 1965).

The information on the effects of hormonal interaction on the growth of orchid seedlings is scanty. However, combination of auxins and cytokinins may enhance growth but the effects of these combinations vary with the growth regulators used, their concentrations, ratios and the orchid species (Kusumoto, 1978). Pack *et al.* (1990) reported that higher concentration of auxin followed by lower concentrations of BA in the medium is beneficial for micropropagation of *Cymbidium* species. Vij *et al.* (1989) also observed that KN along with NAA in the medium was effective for complete development of plantlets. Chaturvedi *et al.* (1987) reported the influencing effect of IAA along with 2,4-D, BAP and casein hydrosylate on proliferation of PLBs and seedling growth of *Vanda* hybrids. On the other hand, the inhibitory effects of KN in combination with auxins on shoot/root balance in orchids are well documented (Hadley and Harvais, 1968; Rao, 1977; Harvais, 1982; Nayak *et al.*, 1997; Krapiec *et al.*, 2003).

MATERIAL AND METHODS

About 0.5–0.8 cm sized seedlings of *C. devonianum* and *D. lituiflorum* devoid of roots (both developed from protocorms on MS medium as reported in chapter II) were taken for experimental studies. To study the nutritional requirements of the developing seedlings, these seedlings were subcultured on 5 different basal media viz., MS, NN, B_5, Mitra and KnC. The optimum basal medium on which seedlings developed best was further considered for studying the effect of growth regulators viz, IAA, NAA, 2,4-D, BAP and KN incorporated both singly and in combination in the range of 0.0-10.0 mg/l in the medium. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C and 1.06 Kg cm^{-2} for 20 min. Five seedlings were cultured in each of the test tubes. The cultures were incubated at 25 ± 2°C temperatures and 16-hour photoperiod of 150 μmoles sec^{-1} m^{-2} light intensity. Observations of seedlings were made after 90 days of culture. Different growth parameters viz. fresh weight, shoot number and length and root number and length were recorded. Five replicates of each treatment were taken and the experiments repeated twice.

RESULTS

C. DEVONIANUM

a) Effect of Different Basal Media

Vigour as measured in terms of average fresh weight was high on MS medium (0.28 g) after 90 days of culture followed by B_5 and NN media wherein the fresh wt. of each seedling was recorded to be 0.09 g. The average shoot number (1.8) and length (2.0

cm) were also higher on MS medium followed by that on B_5 medium where 1.2 and 1.3 cm of shoot number and length were recorded respectively (Table 6; Plate 7 a, b, c). Solitary shoots (as in inoculation time) were seen in NN and Mitra media. At the time of culture the seedlings were devoid of roots, but after 15 days of culture it was observed that roots started developing and maximum average root number of 2.6 was recorded in MS medium followed by B_5 medium where root number of 1.8 averages was recorded. Maximum root length of 2.2 cm was observed on MS medium followed by Mitra medium wherein the average length of the roots was 1.8 cm (Plate 7 d). Considering all the growth parameters studied, the overall seedling growth was optimum on MS medium followed by B_5 medium. Seedlings cultured on KnC medium remained green for 2 weeks of subculture after which they started browning and died subsequently in about 90 days of culture (Plate 7 e).

b) *Effect of Growth Regulators*

Effect of IAA

As compared to control, seedlings developed in the medium containing IAA showed varying responses in terms of fresh weight, shoot number and length; root number and length. At low concentration of IAA in the medium, the growth in terms of fresh weight, shoot number and length was more or less similar to that of control whereas at higher concentration, these growth parameters were inhibited. However, the number of roots was higher at 0.5 mg/l of IAA, when compared to the control (Table 7). But with further increase in IAA concentrations (2.5, 5.0 and 10.0 mg/l) in the medium, there was decrease in root number as well. At 2.5 mg/l of IAA in the medium, an increase in root

Table 6: Effect of different basal media on growth and development of C. *devonianum* seedlings after 90 days of culture

Media	Fr. wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
MS	0.28±0.09	1.8±0.2(2-3)	2.0±0.5	2.6±0.5 (2-3)	2.2±0.2
NN	0.09±0.02	1.0±0.0	1.3±0.2	1.5±0.1 (1-2)	1.5±0.3
Mitra	0.06±0.03	1.0±.0.0	1.1±0.1	1.1±0.1 (1-2)	1.8±0.1
B_5	0.09±0.03	1.2±0.1 (1-2)	1.3±0.1	1.8±0.1(1-3)	1.6±0.2
KnC	-	-	-	-	-

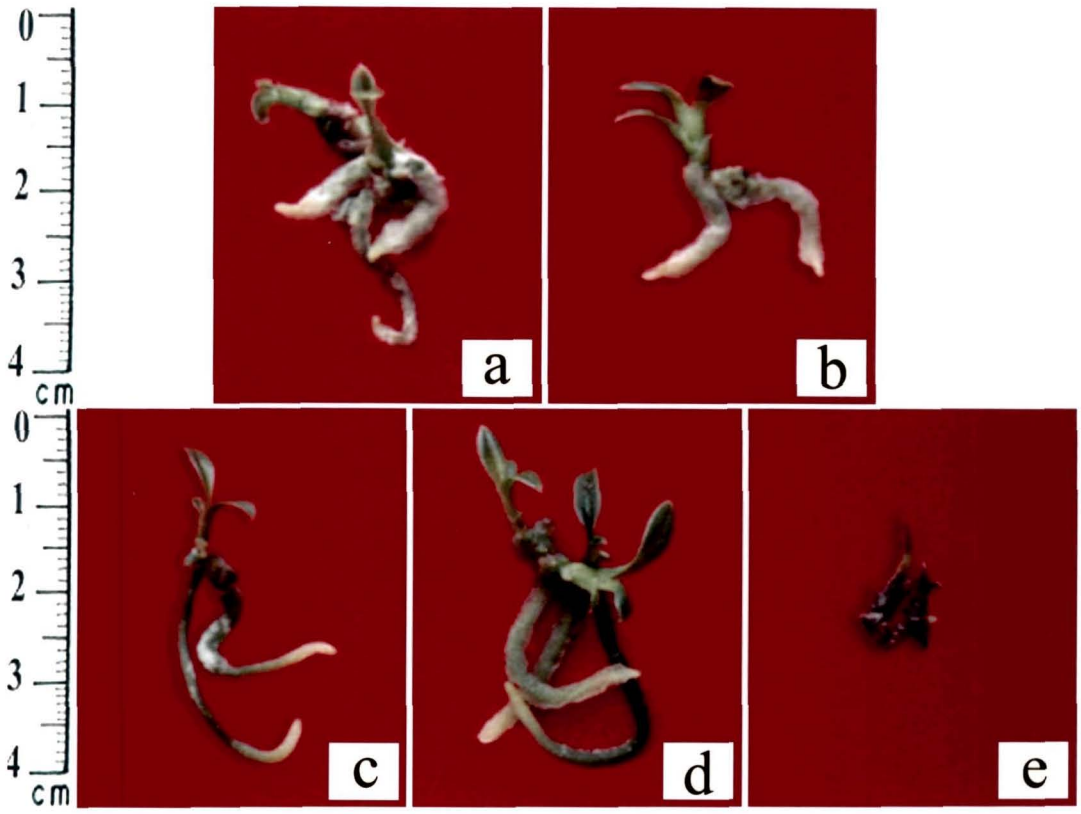
± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Plate 7

Seedling development of *C. devonianum* after 90 days of culture in MS (a), NN (b), B_5 (c), Mitra (d) and KnC (e) media


0
1
2
3
4
cm
a
b
0
1
2
3
4
cm
c
d
e

length (3.4 cm) was observed (Plate 8), but with high concentration of IAA in the medium (10.0 mg/l), decrease in root length was observed.

Effect of NAA

The seedling response was found inhibitory at higher concentrations of NAA tried in the medium (Table 7). At these concentrations of NAA (5.0, 10.0 mg/l) in the medium, seedlings turned brown and died subsequently (Plate 8). However at 0.5 mg/l NAA in the medium, the growth was more or less similar to that of the control except for shoot and root length and root number (Table 7).

Effect of 2,4-D

Exogenous supply of 2,4-D in the medium did not support growth of the seedlings (not shown in table). At low concentration i.e. 0.5 mg/l, the seedlings remained green, but no growth occurred (Plate 8). With increase in 2,4-D concentrations, the seedlings turned brown and died subsequently. Since seedlings responded poorly on all the concentrations of 2,4-D tried, no further experiments were set up in the medium containing 2,4-D in combination with BAP/KN.

Effect of BAP

BAP in the medium had a differential effect on the growth of the seedlings. The fresh weight of the seedlings was almost same when 0.5 mg/l and 2.5 mg/l of BAP were added in the medium but was less when compared to the control (Table 7). On the other hand, the average number of shoots was much higher at lower concentration of BAP (0.5 mg/l and 2.5 mg/l) in the medium (Table 7). With increase in BAP concentration to 5.0 mg/l in the medium, a decrease in shoot number was observed (Plate 8). Roots were

completely suppressed at 2.5 mg/l and at higher concentrations of BAP tried in the medium. The growth of the seedlings was greatly inhibited at 10.0 mg/l of BAP in the medium; the seedlings turned brown and died in course of time.

Effect of KN

As compared to the control, the growth of the seedlings (fresh weight, shoot number and length) was slightly enhanced at low concentrations of KN in the medium. The fresh weight, number and length of shoots were recorded to be higher at 2.5 mg/l of KN (Table 7; Plate 8). However as the concentration of KN was increased in the medium overall growth of the seedlings was comparatively inhibited.

c) *Effect of Growth Regulators in Combination*

Effect of BAP and IAA

As compared to the control, shoot number (5.1), root number (3.2) and fresh weight (0.33 g) was greatly enhanced at 0.5 mg/l each of BAP and IAA in the medium (Table 8; Plate 9 b). With increase in the concentrations of either BAP or IAA to 5.0 mg/l and 10.0 mg/l in the medium, the growth of the seedlings was slightly inhibited as compared to control. And at higher concentrations of both BAP and IAA in the medium, i.e., 10.0 mg/l BAP + 5.0 mg/l of IAA, 10.0 mg/l BAP + 10.0 mg/l IAA, seedlings did not response rather they turned brown and died.

Effect of BAP and NAA

The response of seedlings in the medium containing BAP and NAA in combination was overall inhibitory as compared to control (Table 9). However at low concentrations each of 0.5 mg/l of BAP and NAA in the medium, fresh weight (0.31 g)

Table 7: Effect of different growth regulators incorporated singly in MS medium on growth and development of *C. devonianum* seedlings after 90 days of culture

Conc. (mg/l)	Fresh wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.23±0.09	1.6±0.21(2-3)	2.1±0.48	2.8±0.48(2-3)	2.3±0.23
IAA					
0.5	0.25±0.01	1.4±0.14(1-3)	1.9±0.35	3.4±0.11(2-4)	2.9±0.21
2.5	0.24±0.01	1.2±0.20(1-2)	1.8±0.24	2.2±0.23(2-3)	3.4±0.39
5.0	0.17±0.01	1.1±0.24(1-2)	1.7±0.10	1.8±0.14(1-2)	2.5±0.08
10.0	0.12±0.03	1.0±0.18(1-2)	1.2 ±0.08	1.0±0.00	1.9±0.34
NAA					
0.5	0.20±0.12	1.5±0.10(2-3)	1.2±0.17	2.1±0.15(2-3)	1.1±0.16
2.5	0.15±0.01	1.1±0.07(1-2)	1.1±0.18	1.2± 0.15(1-2)	0.1±0.03
5.0	-	-	-	-	-
10.0	-	-	-	-	-
BAP					
0.5	0.18±0.18	3.4±0.07(2-4)	1.1±0.13	1.0±0.26(1-2)	1.2±0.74
2.5	0.18±0.04	4.1±0.42(3-5)	1.0±0.17	0	0
5.0	0.07±0.02	1.0± 0.81(1-2)	0.9±0.09	0	0
10.0	-	-	-	-	-
KN					
0.5	0.28±0.04	1.8±0.24 (1-2)	2.4±0.30	1.5±0.15(1-2)	0.9±0.33
2.5	0.37±0.04	2.6±0.48(2-4)	3.8±0.19	1.3±0.18(0-2)	0.7±0.38
5.0	0.06±0.02	2.4±0.35(2-3)	1.5±0.13	0	0
10.0	0.02±0.01	1.0±0.00 (1-1)	1.1 ±0.08	0	0

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Table 8: Effect of BAP and IAA incorporated in combinations in MS medium on growth and development of *C. devonianum* seedlings after 90 days of culture

Conc. (mg/l)	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.23±0.09	1.6±0.20(2-3)	2.1±0.48	2.8±0.21 (2-3)	2.3±0.23
BAP+IAA					
0.5+0.5	0.33±0.07	5.1±0.15(3-8)	2.4 ±0.05	3.2±0.34(2-4)	1.6±0.21
0.5+2.5	0.24±0.02	3.2±0.27(2-8)	2.2±0.02	2.9±0.14(1-4)	1.1±0.03
0.5+5.0	0.21±0.10	2.6±0.28(2-3)	1.1±0.13	1.3±0.11(1-2)	0.9±0.21
0.5+10.0	0.13±0.10	1.7±0.11(1-2)	0.9±0.22	0	
2.5+0.5	0.32±0.30	3.7±0.11(3-5)	1.3±0.20	2.1±0.53(1-2)	0.6±0.40
2.5+2.5	0.23±0.01	2.4±0.03(2-3)	1.1±0.10	1.6±0.32(1-2)	0.2±0.13
2.5+5.0	0.22±0.02	1.8±0.32(1-3)	1.0±0.13	0	0
2.5+10.0	0.11±0.02	1.0±0.00	0.8±0.12	0	0
5.0+0.5	0.28±0.02	2.5±0.50(2-4)	1.0±0.17	1.8±0.65(1-2)	0.3±0.11
5.0+2.5	0.21±0.03	1.9±0.30(1-2)	0.9±0.22	1.2±0.57(1-2)	0.1±0.02
5.0+5.0	0.14±0.02	1.2±0.80(2-3)	0.8±0.08	0	0
5.0+10.0	0.01±0.01	0.7±0.24(0-2)	0.6±0.06	0	0
10.0+0.5	0.05±0.01	1.2±0.07 (1-2)	0.7±0.06	0	0
10.0+2.5	0.01±0.01	1.0± 0.0	0.5±0.02	0	0
10.0+5.0	-	-	-	-	-
10.0+10.0	-	-	-	-	-

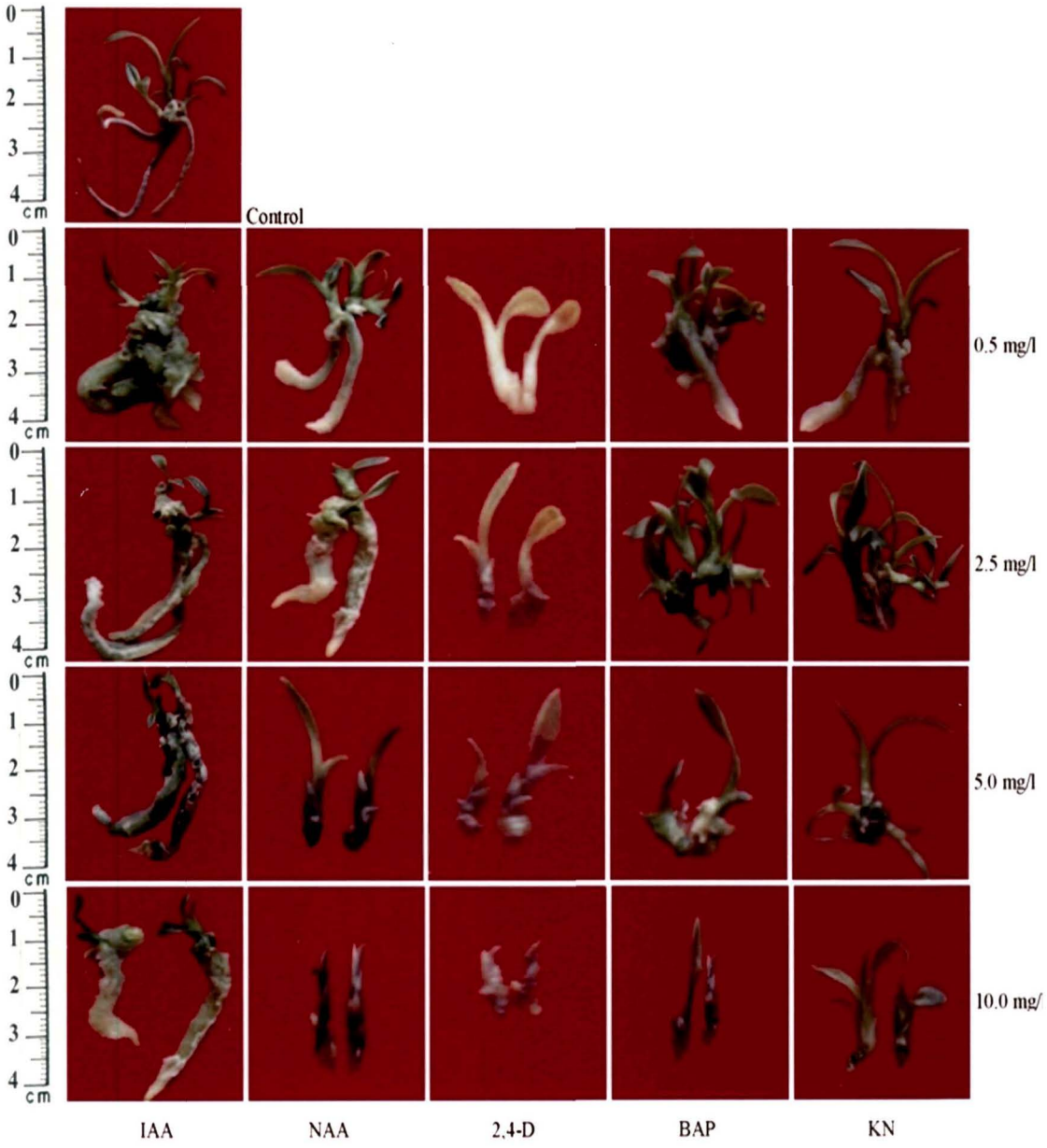
± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Plate 8

Seedling development of *C. devonianum* after 90 days of culture in MS medium incorporated with different concentrations of growth regulators singly


Control
0.5 mg/l
2.5 mg/l
5.0 mg/l
10.0 mg/
IAA
NAA
2,4-D
BAP
KN

and shoot number (3.5) were higher when compared to the control (Plate 9 c). Fresh weight and shoot numbers of seedlings were also enhanced at 0.5 mg/l BAP+2.5 mg/l NAA and also in 2.5 mg/l BAP+0.5 mg/l NAA when compared to control (Table 9). Higher concentrations of NAA and BAP in the medium were found to be inhibitory for the growth of the seedlings.

Effect of KN and IAA

As compared to the control, fresh weight (0.45 g), shoot number (2.3) and shoot length of the seedlings (3.0 cm) was high in the medium containing each of 0.5 mg/l of KN and IAA. However, fresh weight (0.52 g), shoot number (3.9), shoot length (2.6 cm) and root number (2.8) were enhanced in the seedlings cultured in 2.5 mg/l KN in combination with 0.5 mg/l IAA in the medium (Table 10; Plate 9 d). At high concentration of KN (5.0, 10.0 mg/l) in combination with high concentration of IAA (5.0, 10.0 mg/l) seedling growth was either inhibitory or could not be recorded because the seedlings turned brown and died subsequently.

Effect of KN and NAA

At low concentration each of 0.5 mg/l of KN and NAA in the medium, there was an increase in fresh weight (0.36 g) and shoot number (3.8) of the seedlings as compared to control (Table 11; Plate 9 e). However, the other growth parameters except for shoot length recorded in the same concentrations were more or less similar to that of control. With an increase in concentrations of both KN and NAA in the medium, the growth was inhibited. However, the shoot number was higher (3.1) at 2.5 mg/l of KN and 0.5 mg/l of

Table 9: Effect of BAP and NAA incorporated in combinations in MS medium on growth and development of *C. devonianum* seedlings after 90 days of culture

Conc. (mg/l)	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.23±0.09	1.6±0.20(2-3)	2.1±0.48	2.8±0.48(2-3)	2.3±0.23
BAP+NAA					
0.5+0.5	0.31±0.03	3.5±0.40(2-5)	1.0±0.33	1.8±0.16(1-2)	1.2±0.11
0.5+2.5	0.27±0.06	2.2±0.20(2-3)	0.9±0.25	1.5±0.39(0-2)	1.3±0.19
0.5+5.0	0.21±0.05	1.3±0.16(1-2)	0.7±0.25	1.3±0.23(0-2)	0.4±0.13
0.5+10.0	-	-	-	-	-
2.5+0.5	0.26±0.02	2.6±0.28(2-4)	0.8±0.31	1.4±0.34(1-2)	1.1±0.17
2.5+2.5	0.11±0.01	2.0±0.47(1-3)	0.7±0.12	1.2±0.32(0-2)	0.3±0.07
2.5+5.0	0.07±0.01	1.3±0.27(1-2)	0.5 ±0.10	0	0
2.5+10.0	-	-	-	-	-
5.0+0.5	0.20±0.04	1.8±0.24(1-3)	0.6±0.13	0	0
5.0+2.5	0.10±0.01	1.4±0.13(1-2)	0.5±0.11	0	0
5.0+5.0	-	-	-	-	-
5.0+10.0	-	-	-	-	-
10.0+0.5	-	-	-	-	-
10.0+2.5	-	-	-	-	-
10.0+5.0	-	-	-	-	-
10.0+10.0	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Table 10: Effect of KN and IAA incorporated in combinations in MS medium on growth and development of *C. devonianum* seedlings after 90 days of culture

Conc. (mg/l)	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.23±0.09	1.6±0.20(2-3)	2.1±0.48	2.8±0.48(2-3)	2.3±0.23
KN+IAA					
0.5+0.5	0.45±0.14	2.3±0.49(2-3)	3.0±0.40	1.8±0.23(1-2)	2.8±0.33
0.5+2.5	0.30±0.07	1.4±0.30(1-3)	1.7±0.13	1.0±0.00	1.3±0.29
0.5+5.0	0.10±0.03	1.2±0.23(1-2)	1.5±0.26	0.7±0.32(0-1)	1.0±0.15
0.5+10.0	-	-	-	-	-
2.5+0.5	0.52±0.16	3.9 ±0.80(3-4)	2.6±0.50	2.8±0.40(2-4)	1.8±0.34
2.5+2.5	0.38±0.10	2.2±0.90(1-4)	1.2±0.26	2.4±0.57(2-3)	1.3±0.08
2.5+5.0	0.16±0.01	1.0±0.00	0.9±0.26	1.6±0.21(0-2)	0.8±0.09
2.5+10.0	-	-	-	-	-
5.0+0.5	0.40±0.07	2.0±0.80(1-3)	1.6±0.15	1.2±0.16(1-2)	1.3±0.18
5.0+2.5	0.30±0.18	1.8±0.40(1-2)	0.9±0.14	1.0 ±0.00	1.1±0.15
5.0+5.0	0.20±0.08	1.6±0.70 (1-2)	0.6±0.22	0	0
5.0+10.0	-	-	-	-	-
10.0+0.5	0.20±0.05	1.5±0.40(1-2)	0.8±0.16	0	0
10.0+2.5	0.12±0.06	1.0±0.00	0.5 ±0.06	0	0
10.0+5.0	-	-	-	-	-
10.0+10.0	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Table 11: Effect of KN and NAA incorporated in combinations in MS medium on growth and development of *C. devonianum* seedlings after 90 days of culture

Conc. (mg/l)	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.23±0.09	1.6±0.20(2-3)	2.1±0.48	2.8±0.48(2-3)	2.3±0.23
KN+NAA					
0.5+0.5	0.36±0.04	3.8±0.40(2-4)	1.7±0.65	2.1±0.42(2-3)	2.2±0.29
0.5+2.5	0.19±0.05	2.9±0.48(2-3)	1.1±0.19	1.8±0.24(1-2)	1.8±0.24
0.5+5.0	0.06±0.03	1.8±0.26(1-3)	1.0±0.24	1.2±0.56(1-2)	1.0±0.40
0.5+10.0	-	-	-	-	-
2.5+0.5	0.22±0.13	3.1±0.38(2-3)	1.3±0.40	2.4±0.66(1-3)	1.7±0.32
2.5+2.5	0.16±0.04	1.7±0.35(1-3)	0.8±0.30	1.5±0.48(1-2)	1.3±0.45
2.5+5.0	0.10±0.04	1.0±0.00	0.5±0.33	1.0±0.00	0.9±0.23
2.5+10.0	-	-	-	-	-
5.0+0.5	0.14±0.04	2.2±0.29(1-3)	1.2±0.17	1.4±0.40	1.0±0.04
5.0+2.5	0.07±0.03	1.0±0.00	0.9±0.29	1.1±0.32	0.9±0.32
5.0+5.0	0.05±0.03	1.0±0.00	0.8±0.38	1.0±0.00	0.7±0.33
5.0+10.0	-	-	-	-	-
10.0+0.5	0.08±0.06	1.3±0.40(1-2)	0.7±0.03	0	0
10.0+2.5	0.02±0.01	1.0±0.00	0.5±0.05	0	0
10.0+5.0	-	-	-	-	-
10.0+10.0	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Plate 9

Seedling development of *C. devonianum* after 90 days of culture in medium incorporated with different concentrations of growth regulators in combination

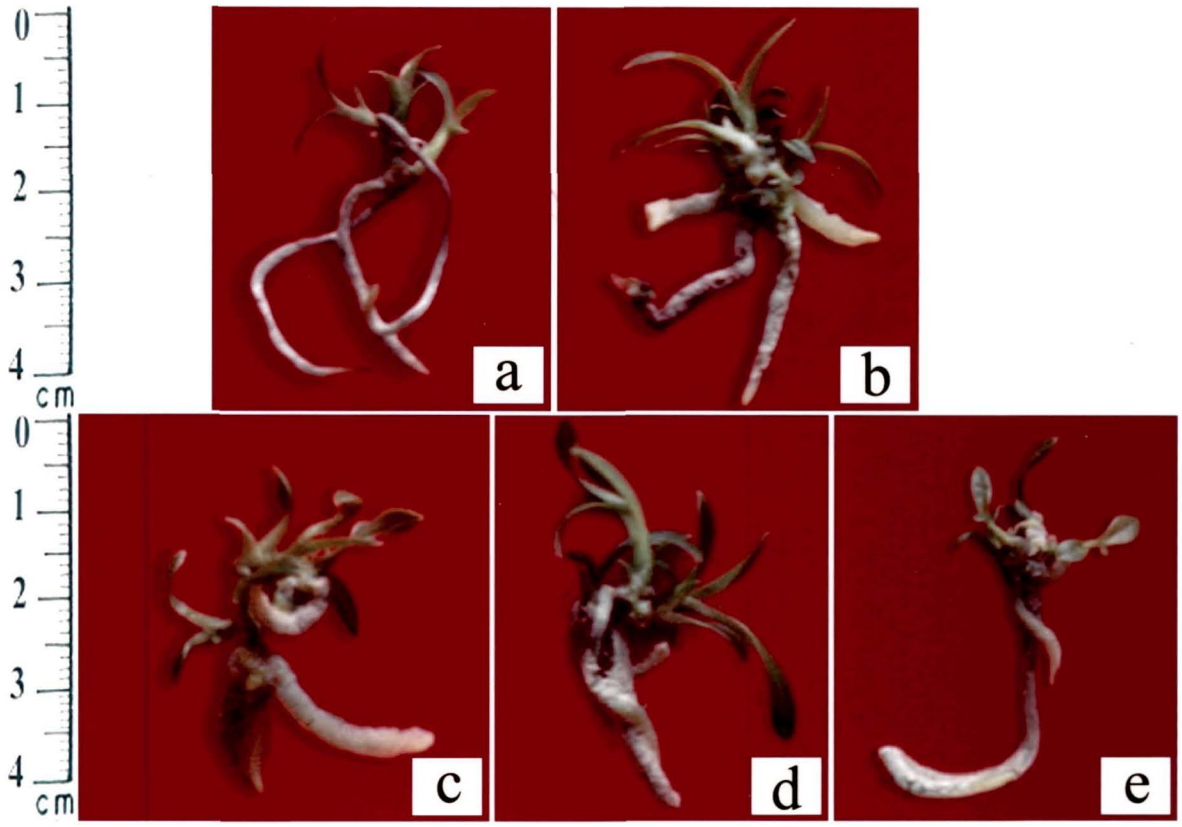
a. MS basal medium

b. MS + 0.5 mg/l BAP + 0.5 mg/l IAA

c. MS + 0.5 mg/l BAP + 0.5 mg/l NAA

d. MS + 2.5 mg/l KN + 0.5 mg/l IAA

e. MS + 0.5 mg/l KN + 0.5 mg/l NAA


0
1
2
3
4
cm
a
b
0
1
2
3
4
cm
c
d
e

NAA when compared to the control. High concentrations of NAA (10.0 mg/l) in combinations with different concentration of KN (0.5, 2.5, 5.0 and 10.0 mg/l) were inhibitory for the growth of the seedlings, which turned brown after 4 weeks of culture and died subsequently.

D. LITUIFLORUM

a) *Effect of Different Basal Media*

The overall growth of the developing seedlings in terms of fresh weight, shoot number, shoot length, root number and root length was highest in MS medium as compared to the other media tried i.e. NN, B_5, and Mitra (Table 12). Multiple shoots were seen to be emerging in all the other media but the maximum shoot number (3.6) was recorded in MS medium. The maximum shoot length of 2.8 cm, root number of 5.0 and length of 1.5 were recorded in the same medium (Plate 10 a). An average of 3.2 shoots was found to have initiated in both NN and B_5 media (Plate 10 b, c). The fresh weight and shoot length of the seedlings in both NN and B_5 media were more or less same. The average number of roots in B_5 medium was recorded to be 4.4 which were next highest to that in MS medium. Comparatively, the overall growth of the seedlings in Mitra medium was lower (Plate 10 d). In KnC medium, growth of seedlings was completely inhibited. The seedlings turned brown after 2nd week of subculture and died subsequently (Plate 10 e).

Table 12: Effect of different basal media on growth and development of *D. lituiflorum* seedlings after 90 days of culture

Media	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
MS	0.37±0.09	3.6±0.31(3-4)	2.8±0.11	5.0±1.63(4-6)	1.5±0.14
NN	0.11±0.07	3.2±0.48(2-4)	1.6±0.28	3.6±0.31(3-4)	0.8±0.09
B_5	0.10±0.03	3.0±0.00 (3-3)	1.6±0.09	4.4±0.57(4-5)	0.9±0.08
Mitra	0.05±0.02	2.8±0.16(2-3)	1.1±0.40	3.8±0.40(3-5)	0.9±0.18
KnC	-	-	-	-	-

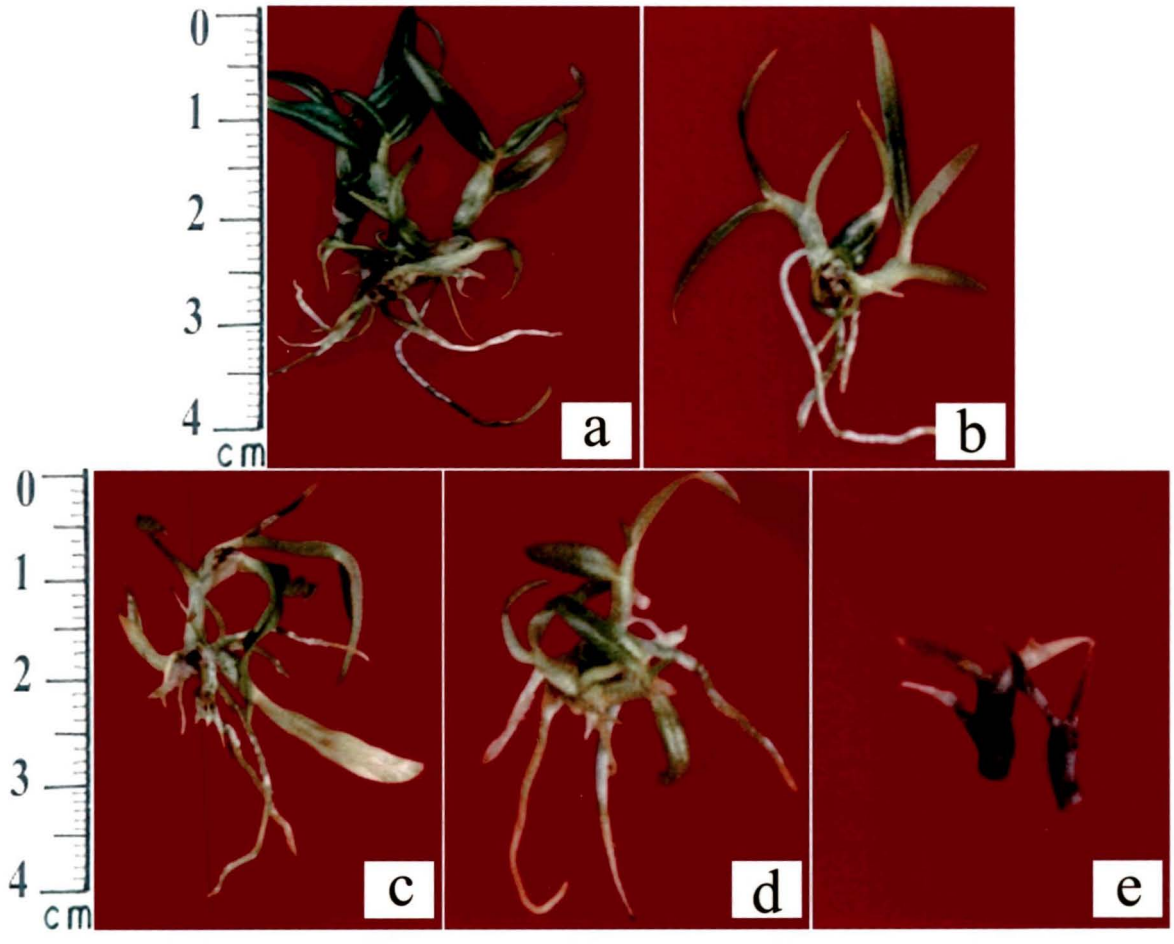
± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Plate 10

Seedling development of *D. lituiflorum* after 90 days of culture in MS (a), NN (b), B_5 (c), Mitra (d) and KnC (e) media


0
1
2
3
4
cm
a
b
0
1
2
3
4
cm
c
d
e

b) *Effect of Growth Regulators*

Effect of IAA

As compared to the control, there was slight increase in shoot number (3.9) of the seedlings at low concentration (0.5 mg/l) of IAA in the medium. The other growth parameters such as fresh weight and root length were close to the control whereas shoot length and root number were slightly inhibited in the same concentration. However, in all the other concentrations of IAA tried in the medium the growth of the seedlings was inhibited than that of the control (Table13; Plate 11).

Effect of NAA

When compared to control, fresh weight, shoot number and shoot length of the seedlings were higher at 2.5 mg/l of NAA in the medium (Table 13; Plate 11) and with increase in NAA concentration there was a decrease in both fresh weight and number of shoots. However, as compared to control, the number of roots initiating in NAA supplemented medium increased drastically at 0.5-5.0 mg/l concentrations. With increase in NAA concentration to 10.0 mg/l in the medium the number of roots also decreased.

Effect of BAP

As compared to control, overall growth of the seedlings showed poor response in medium containing different concentrations of BAP. However, number of shoots was slightly enhanced at 2.5 mg/l BAP in medium (Table 13; Plate 11). As compared to control poor roots were seen to be emerging at 0.5 mg/l of BAP in the medium. However, roots completely failed to develop at higher concentrations of BAP (i.e. 2.5, 5.0 and 10.0 mg/l).

Effect of KN

As compared to the control, slight enhancement in the shoot number (3.5) and roots (4.7) was observed in developing seedlings on 0.5 mg/l of KN in the medium (Table 13; Plate 11). However, the other growth parameters of the seedlings were by large inhibited in the KN containing medium.

Effect of 2,4-D

The seedlings showed a varying response in the medium containing different concentrations of 2,4-D (Table 14). At low concentration of 2,4-D (0.5 mg/l) in the medium around 3.8 numbers of shoots developed (Plate 11). As compared to control, both the shoot number and root number were increased at this concentration. At higher concentrations of 2.5 mg/l and 5.0 mg/l of 2,4-D in the medium, the seedlings developed into PLBs in 44% and 33% of cultures respectively (Plate 11). These PLBs when transferred to fresh MS medium without growth regulators further multiplied within four weeks of subculture and if left undisturbed formed complete plantlets within 8 weeks of subculture. The high concentration 10.0 mg/l 2,4-D in the medium was inhibitory for seedling growth. The seedlings turned brown and died in course of time

c) *Effect of Growth Regulators in Combination*

Effect of BAP and IAA

The seedling growth was more or less inhibited in the medium containing BAP in conjunction with IAA. However, the optimum seedling growth in terms of shoot number (4.0) was recorded at 2.5 mg/l BAP and 0.5 mg/l IAA together in the medium (Table 15;

Table 13: Effect of different growth regulators incorporated singly in MS medium on growth and development of *D. lituiflorum* seedlings after 90 days of culture

Conc. (mg/l)	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.36±0.07	3.1± 0.21(3-4)	3.0±0.16	4.3±1.2 0(4-6)	1.5±0.14
IAA					
0.5	0.34±0.06	3.9±0.55(3-5)	2.1±0.44	3.6±0.08(4-10)	1.6±0.08
2.5	0.21±0.07	2.4±0.38(2-4)	2.0±047	3.4±0.35(3-8)	1.0±0.13
5.0	0.18±0.03	2.3±0.51(2-4)	2.0±0.39	2.6±0.16(2-6)	0.9±0.40
10.0	0.17±0.04	2.2±0.53(2-3)	1.9±0.65	2.2±0.33(0-3)	0.6±0.14
NAA					
0.5	0.39±0.03	3.0±0.12(2-4)	2.4±0.26	6.7±0.42(3-15)	1.7±0.17
2.5	0.44±0.07	4.1± 0.12(3-5)	3.3±0.32	8.4±0.33(4-18)	2.6±0.22
5.0	0.24±0.03	2.2±0.42(1-5)	2.8±0.52	7.8±0.14(3-10)	2.4±0.28
10.0	0.17±0.01	1.4±0.19(1-3)	1.3±0.24	3.3± 0.31(2-7)	2.2±0.29
BAP					
0.5	0.13±0.01	3.8±0.08(2-4)	1.5±0.11	2.5±0.08(0-4)	0.5±0.07
2.5	0.11±0.05	4.1±0.32(3-6)	1.4±0.32	0	0
5.0	0.10±0.10	2.6±0.27(1-5)	1.1±0.08	0	0
10.0	0.09±0.02	2.5±0.35(1-4)	1.3±0.18	0	0
KN					
0.5	0.15±0.03	3.5±0.10(2-6)	1.8±0.08	4.7±0.11(3-8)	1.4±0.12
2.5	0.12±0.02	2.8±0.16(2-5)	1.3±0.12	3.5±0.07(0-6)	1.0±0.08
5.0	0.07±0.02	2.6±0.27(2-4)	1.3±0.13	2.9±0.19(0-5)	0.8±0.25
10.0	0.03±0.01	1.4±0.37(1-3)	1.2±0.15	0	0

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Table 14: Effect of 2,4-D incorporated singly in MS medium on growth and development of *D. lituiflorum* seedlings after 90 days of culture

		Shoot bud formation				PLB formation	
Conc. (mg/l)	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)	% response	PLB no.
Control	0.36±0.07	3.1±0.2(3-4)	3.0±0.16	4.3±1.2 (4-6)	1.5±0.14		
0.5	0.54±0.11	3.8±0.5(2-5)	1.9±0.10	6.9±0.1 (3-15)	1.1±0.15		
2.5	0.26±0.02					44.0±10.6	50.0±13
5.0	0.03±0.02					33.0±5.40	22.0±8.0
10.0	-	-	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Table 15: Effect of BAP and IAA incorporated in combinations in MS medium on growth and development of *D. lituiflorum* seedlings after 90 days of culture

Conc. (mg/l)	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.36±0.07	3.1±0.25(3-4)	3.0±0.16	4.3±1.2 (4-6)	1.5±0.14
BAP+IAA					
0.5+0.5	0.18±0.01	3.4±0.96(3-5)	2.0±0.30	1.9±0.23(1-3)	0.7±0.09
0.5+2.5	0.14±0.01	3.1±0.92(3-5)	1.8±0.20	2.1±0.30(2-4)	0.7±0.08
0.5+5.0	0.13±0.02	2.9±0.35(2-5)	1.8±0.09	2.4±0.41(2-4)	0.8±0.11
0.5+10.0	0.12±0.03	2.4±0.52(1-4)	1.5±0.35	1.7±0.35(1-3)	0.6±0.06
2.5+0.5	0.15±0.09	4.0±0.62(4-6)	1.8 ±0.11	0	0
2.5+2.5	0.13±0.01	3.8±0.08(3-6)	1.6±0.13	0	0
2.5+5.0	0.09±0.01	3.1±0.32(2-5)	1.3±0.12	0	0
2.5+10.0	0.06±0.02	1.2±0.23(1-3)	1.3±0.19	0	0
5.0+0.5	0.08±0.04	2.5±0.05(1-5)	1.2±0.12	0	0
5.0+2.5	0.07±0.01	2.2±0.25(1-4)	1.2±0.14	0	0
5.0+5.0	0.06±0.02	2.2±0.55(1-3)	1.1±0.09	0	0
5.0+10.0	0.06±0.01	1.4±0.02(1-2)	1.0±0.06	0	0
10.0+0.5	0.07±0.04	1.3±0.23(1-2)	1.1±0.14	0	0
10.0+2.5	0.05±0.02	1.2±0.01(1-2)	0.9±0.01	0	0
10.0+5.0	0.02±0.04	1.0±0	0.6±0.01	0	0
10.0+10.0	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Plate 11

Seedling development of *D. lituiflorum* after 90 days of culture in MS medium incorporated with different concentrations of growth regulators singly

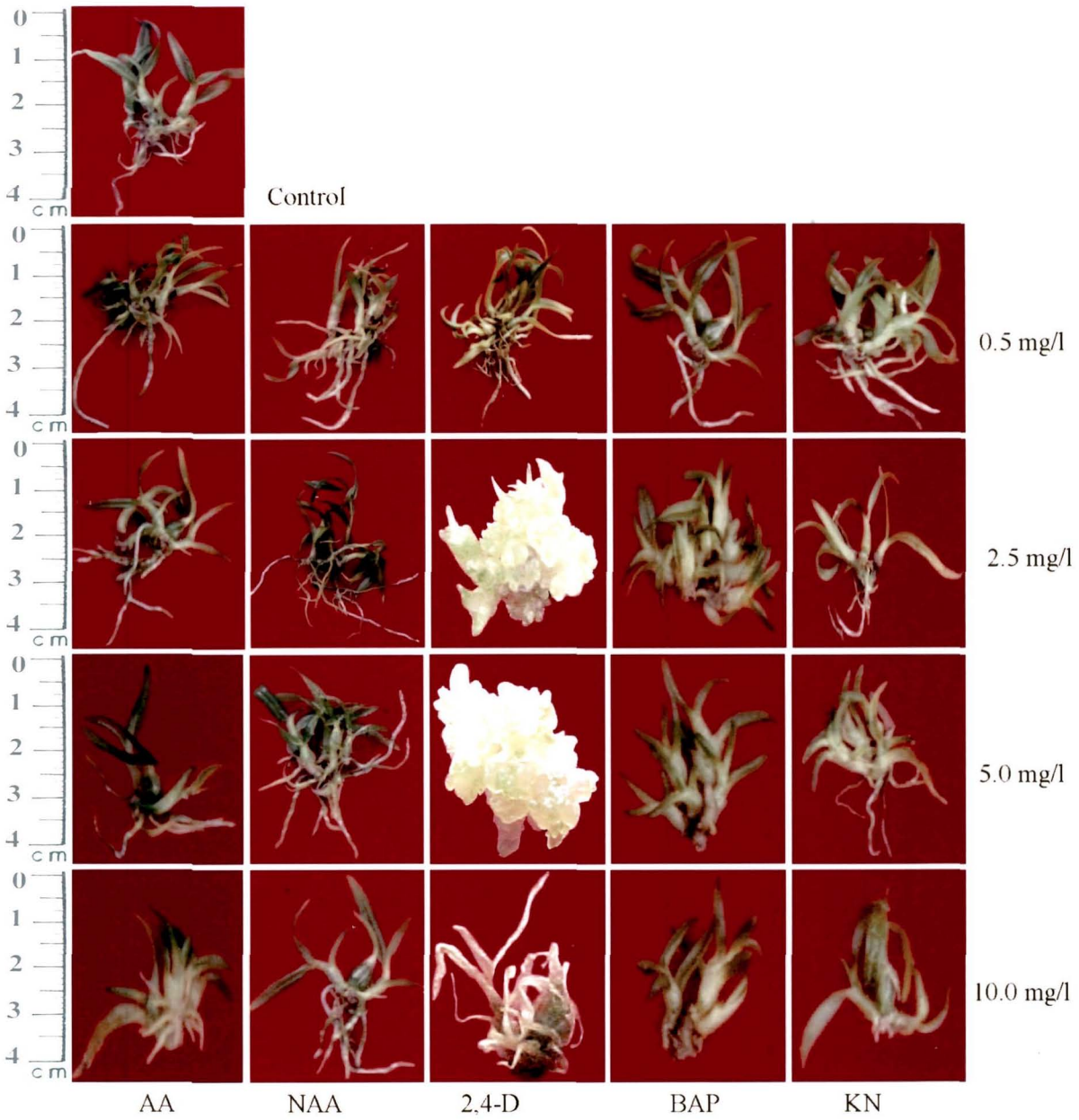

Control
0.5 mg/l
2.5 mg/l
5.0 mg/l
10.0 mg/l
AA
NAA
2,4-D
BAP
KN

Plate 12 b). Roots were completely suppressed at this and higher concentrations of the combinations of BAP and IAA in the medium.

Effect of BAP and NAA

The seedling growth was slightly enhanced in the medium containing BAP in combination with NAA. The optimum seedling growth in terms of fresh weight (0.40 g), shoot number (4.7) and root number (5.4) was recorded at 0.5 mg/l each of BAP and NAA in the medium (Table 16; Plate 12 c). The shoot and root number were higher as compared to control at this concentration. Healthy roots were initiated at low concentration of BAP (0.5 and 2.5 mg/l) in combination with different concentrations of NAA in the medium. However, in higher concentrations of BAP (i.e. 5.0 and 10.0 mg/l) with different concentrations of NAA (0.5, 2.5, 5.0 and 10.0 mg/l) in the medium total absence of roots was observed. At 10.0 mg/l each of BAP and NAA in medium growth of seedlings was totally suppressed. The seedlings after remaining green for some time died subsequently.

Effect of BAP and 2,4-D

Both PLB as well as multiple shoots were observed in seedlings cultured in medium containing 2,4-D in combination with BAP. At low concentrations each of 0.5 mg/l of BAP and 2,4-D, 2.5 mg/l BAP and 0.5 mg/l 2,4-D in medium, multiple shoots along with roots were observed. However, the overall seedling growth in terms of fresh weight, shoot number, and length, root number and length was lower as compared to control (Table 17). When different concentrations of BAP (i.e. 0.5 and 2.5 mg/l) is combined with increased concentrations of 2,4-D (2.5, 5.0 and 10.0 mg/l) the seedlings

Table 16: Effect of BAP and NAA incorporated in combinations in MS medium on growth and development of *D. lituiflorum* seedlings after 90 days of culture

Conc. (mg/l	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.36±0.07	3.1±0.23(3-4)	3.0±0.16	4.3±1.2(4-6)	1.5±0.14
BAP+NAA					
0.5+0.5	0.40±0.01	4.7±0.79(3-8)	2.6±0.31	5.4±1.1(4-8)	1.1±0.63
0.5+2.5	0.31±0.04	4.1±0.52(2-6)	2.3±0.30	5.1±1.6(3-6)	0.9±0.36
0.5+5.0	0.29±0.01	3.9±0.38(2-6)	2.1±0.29	5.0±1.6(1-5)	0.7±0.06
0.5+10.0	0.23±0.01	2.8±0.23(2-4)	1.9±0.24	4.5±1.7(1-4)	0.6±0.13
2.5+0.5	0.33±0.11	4.2±0.64(3-6)	1.5±0.31	5.0±0.6(2-7)	0.3±0.13
2.5+2.5	0.14±0.05	3.3±0.43(2-5)	1.3±0.21	4.2±0.6(2-5)	0.3±0.06
2.5+5.0	0.10±0.01	2.8±0.41(2-4)	0.9±0.24	2.6±0.4(2-3)	0.2±0.04
2.5+10.0	0.08±0.02	2.4±0.15(1-4)	0.9±0.16	2.0±0.0	0.1±0.02
5.0+0.5	0.14±0.01	3.1±0.22(2-4)	1.7±0.48	0	0
5.0+2.5	0.13±0.04	3.2±0.16(2-5)	1.2±0.18	0	0
5.0+5.0	0.10±0.03	2.4±0.25(1-4)	1.0±0.09	0	0
5.0+10.0	0.08±0.02	1.6±0.69(1-2)	0.7±0.07	0	0
10+0.5	0.12±0.04	2.1±0.27(2-3)	1.2±0.26	0	0
10+2.5	0.11±0.03	1.3±0.26(1-3)	0.84±0.06	0	0
10+5.0	0.09±0.01	1.0±0.00	0.5±0.02	0	0
10+10.0	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Table 17: Effect of BAP and 2,4-D incorporated in combinations in MS medium on growth and development of *D. lituiflorum* seedlings after 90 days of culture

Conc. (mg/l)	Fr.wt. (g)	Shoot no.	Shoot bud formation			PLB formation	
			Shoot length (cm)	Root no.	Root length (cm)	% response	PLB no.
Control	0.37±0.07	3.6±0.31(3-4)	2.8±0.11	5.0±1.63(4-6)	1.5±0.14		
BAP+2,4-D							
0.5+0.5	0.21±0.06	3.3±0.18(2-5)	1.5±0.24	4.1±0.44(3-5)	0.5±0.13		
0.5+2.5	0.26±0.01					92.0±6.6	138± 22.
0.5+5.0	0.07±0.01					44.0±5.7	38.0±5.7
0.5+10.0	0.02±0.01					36.0±6.3	24.0±.9.0
2.5+0.5	0.16±0.02	2.3±0.21(2-3)	1.2±0.21	2.5±0.54(1-4)	0.8±0.57		
2.5+2.5	0.26±0.12					88.0±6.5	129±13
2.5+5.0	0.09±0.01					36.0±11	31.0±11
2.5+10.0	0.03±0.02					20.0±6.0	14.0±9.5
5.0+0.5	0.16±0.09					52.0±14	52.0±6.6
5.0+2.5	0.19±0.06					38.0±12	24.0±7.2
5.0+5.0	0.09±0.05					20.0±7.1	10.0 ±4.3
5.0+10.0	-	-	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

turned into globular structure which then started dividing into smaller ball like structures (PLBs). Similarly when 5.0 mg/l of BAP is combined with different concentration of 2,4-D (0.5, 2.5 and 5.0 mg/l) PLBs were developed. These PLBs subsequently developed into complete plantlets when subcultured to MS medium. A high explant response (92%) and high number (138) was observed in the form of PLBs at 0.5 mg/l BAP in combination with 2.5 mg/l 2,4-D in the medium (Plate 12 d). However, with increase in 2,4-D concentration, there was decrease in both explant response and PLB numbers. Similarly, with an increase in concentration of BAP (2.5 mg/l) in combination with high concentrations of 2,4-D (5.0 and 10.0 mg/l), and in 5.0 mg/l each of BAP and 2,4-D in combination in the medium, corresponding decrease in both the explant response and PLB numbers was observed. At high concentration of BAP (5.0 mg/l) in combination with high concentration of 2,4-D (10.0 mg/l) the whole seedlings turned brown and died subsequently after 2 weeks of subculture. With increase in the concentration of BAP to 10.0 mg/l in the medium, the seedlings transformed into globular structure which failed to differentiate into plantlets.

Effect of KN and IAA

As compared to control, at 0.5 mg/l each of KN and IAA in medium, the seedling growth and development in terms of fresh weight and root length was more or less similar to the control (Table 18). However, there was drastic increase in root number at 0.5 mg/l of KN with 2.5 and 5.0 mg/l of IAA in the medium (Plate 12 e). At higher concentration of KN (10.0 mg/l) with different concentrations of IAA (0.5, 2.5 and 5.0 mg/l) in the medium, root formation was completely inhibited.

Table 18: Effect of KN and IAA incorporated in combinations in MS medium on growth and development of *D. lituiflorum* seedlings after 90 days of culture

Conc. (mg/l	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.36±0.07	3.1±0.23(3-4)	3.0±0.16	4.3 ± 1.20(4-6)	1.5±0.14
KN+IAA					
0.5+0.5	0.39±0.60	3.8±0.16(2-6)	2.1±0.20	6.4±0.50(3-8)	1.4±0.60
0.5+2.5	0.20±0.01	2.9±0.30(2-4)	1.8±0.23	8.2±0.60(6-12)	1.3±0.29
0.5+5.0	0.17±0.03	2.2±0.33(2-3)	1.8±0.10	8.0±0.16(4-11)	1.2±0.11
0.5+10.0	0.16±0.01	2.7±0.34(2-3)	1.8±0.12	6.2±0.43(2-10)	1.1±0.29
2.5+0.5	0.22±0.11	3.0±0.42(2-5)	2.0±0.65	5.9±1.05(2-6)	1.4±0.13
2.5+2.5	0.19±0.06	2.6±0.23(1-4)	1.8±0.15	6.2±0.14(2-8)	1.2±0.08
2.5+5.0	0.14±0.03	2.4±0.20(1-4)	1.6±0.17	5.3±0.95(2-4)	1.1±0.37
2.5+10.0	0.08±0.03	2.2±0.25(1-3)	1.5±0.12	3.6±0.60(1-3)	0.7±0.22
5.0+0.5	0.13±0.01	2.6±0.20(2-4)	1.8±0.32	3.2±0.30(1-4)	1.2±0.16
5.0+2.5	0.10±0.02	2.4±0.11(2-3)	1.6±0.34	3.3±0.69(1-6)	0.9±0.23
5.0+5.0	0.09±0.02	2.3±0.15(2-3)	1.6±0.71	2.8±0.53(1-3)	0.8±0.34
5.0+10.0	0.06±0.01	2.2±0.15(2-3)	1.4±0.08	2.3±0.26(0-3)	0.7±0.12
10.0+0.5	0.08±0.03	2.5±0.24(2-4)	1.5±0.29	0	0
10.0+2.5	0.07±0.02	1.6±0.17(1-4)	1.1±0.22	0	0
10.0+5.0	0.02± 0.01	1.1±0.02(1-2)	0.6±0.21	0	0
10.0+10.0	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Effect of KN and NAA

As compared to control, growth of the seedlings was more or less similar to that of the control in 0.5 mg/l each of KN and NAA in medium. However, poor response of all the growth parameters except root number and length was observed at other concentrations of KN and NAA in the medium. Root number (7.1) and root length (2.7 cm) were optimum at 0.5 mg/l KN in combination with 5.0 mg/l NAA in the medium (Table 19; Plate 12 f).

Effect of KN and 2,4-D

Both PLB formation as well as development of multiple shoots was observed from the seedlings cultured in the medium containing 2,4-D in combination with KN. At low concentration each of 0.5 mg/l and 2.5 mg/l of KN in combination with 0.5 mg/l of 2,4-D, multiple shoots were developed but the growth of the seedlings was comparatively inhibited as compared to control (Table 20). With increase in 2,4-D concentration (2.5 mg/l) in the KN (0.5 and 2.5 mg/l) containing medium, PLBs were initiated instead of multiple shoots. The optimal percent response of explants into PLB formation (34%) and number (23) were observed in the medium containing 0.5 mg/l KN in combination with 2.5 mg/l of 2,4-D. With increase in the concentrations of KN (2.5 mg/l) in combination with same concentration of 2,4-D (2.5 mg/l) in the medium, a decrease in both PLB formation and PLB number was recorded. At high concentrations of KN (2.5 mg/l) in combination with 2,4-D concentrations (5.0 and 10.0 mg/l) and 5.0 mg/l of KN in combinations with different concentrations (0.5, 2.5, 5.0 and 10.0 mg/l) of 2,4-D, no response on the seedling growth was observed. The seedlings remained green for 3 weeks

Table 19: Effect of KN and NAA incorporated in combinations in MS medium on growth and development of *D. lituiflorum* seedlings after 90 days of culture

Conc. (mg/l)	Fr.wt. (g)	Shoot no.	Shoot length (cm)	Root no.	Root length (cm)
Control	0.36±0.07	3.1±0.2 0(3-4)	3.0±0.16	4.3 ± 1.2(4-6)	1.5±0.14
KN+NAA					
0.5+0.5	0.33±0.14	2.8±0.37(2-4)	2.4±0.51	4.6±0.51(2-7)	1.9±0.41
0.5+2.5	0.23±0.04	2.6±0.19(2-4)	2.1±0.18	6.0±0.33(2-10)	2.6±0.29
0.5+5.0	0.23±0.10	2.4±0.12(2-3)	2.0±0.15	7.1±0.60(4-12)	2.7±0.46
0.5+10.0	0.18±0.04	1.9±0.56(1-3)	1.7±0.52	3.5±0.53(3-9)	1.7±0.48
2.5+0.5	0.17±0.08	2.4±0.44(2-4)	2.2±0.22	2.2±0.54(0-3)	1.2±0.18
2.5+2.5	0.15±0.04	2.3±0.43(1-4)	2.2±0.52	3.3±0.18(2-5)	1.6±0.30
2.5+5.0	0.14±0.05	2.2±0.58(1-3)	2.1±0.32	4.0±0.44(2-6)	1.1±0.80
2.5+10	0.11±0.05	2.1±0.42(1-3)	1.7±0.27	2.5±0.39(0-5)	0.7±0.40
5.0+0.5	0.08±0.01	1.8±0.33(1-2)	2.3±0.53	1.7±0.27(0-2)	0.8±0.33
5.0+2.5	0.05±0.01	1.4±0.16(1-2)	1.5±0.19	1.9±0.35(0-5)	1.0±0.11
5.0+5.0	0.02±0.01	1.0±0.00	1.5±0.07	3.7±0.34(2-12)	1.0±0.0.2
5.0+10.0	0.01±0.008	1.0±0.00	1.1±0.1	2.2±0.48(0-5)	0.6±0.0.2
10.0+0.5	0.06±0.01	1.0±0.00	0.9±0.11	1.0±0.00	0.7±0.03
10.0+2.5	0.01±0.004	1.0±0.00	0.5 ±0.06	1.0±0.43(0-2)	0.2±0.27
10.0+5.0	-	-	-	-	-
10.0+10.0	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Table 20: Effect of KN and 2,4-D incorporated in combinations in MS medium on growth and development of *D. lituiflorum* seedlings after 90 days of culture

Conc. (mg/l	Fr.wt. (g)	Shoot bud formation				PLB formation	
		Shoot no.	Shoot Length(cm)	Root no.	Root length(cm)	% Response	PLB no.
Control	0.37±0.07	3.6±0.31 (3-4)	2.8±0.11	5.0±1.63 (4-6)	1.5±0.14		
KN+2,4-D							
0.5+0.5	0.17±0.01	2.4±0.37 (1-4)	1.2±0.22	3.0±0.73 (1-5)	0.4±0.12		
0.5+2.5	0.22±0.11					34± 6.3	23± 11
0.5+5.0							
0.5+10							
2.5+0.5	0.13±0.01	2.0±0.00	1.0±0.07	2.3±1.32 (1-4)	0.7±0.11		
2.5+2.5	0.16±0.04					16±5.7	12±4
2.5+5.0	-	-	-	-	-	-	-
2.5+10.0	-	-	-	-	-	-	-
5.0+0.5	-	-	-	-	-	-	-
5.0+2.5	-	-	-	-	-	-	-
5.0+5.0	-	-	-	-	-	-	-
5.0+10.0	-	-	-	-	-	-	-

± SE

The numbers in bracket represents minimum and maximum observations made from 25 seedlings

- Seedlings turned brown

Plate 12

Seedling development of *C. devonianum* after 90 days of culture in medium incorporated with different concentrations of growth regulators in combination

a. MS basal medium
b. MS + 2.5 mg/l BAP + 0.5 mg/l IAA
c. MS + 0.5 mg/l BAP + 0.5 mg/l NAA
d. MS + 0.5 mg/l BAP + 2.5 mg/l 2,4-D
e. MS + 0.5 mg/l KN + 2.5 mg/l NAA
f. MS + 0.5 mg/l KN + 5.0 mg/l NAA


0
1
2
3
4
cm
a
b
c
0
1
2
3
4
cm
d
e
f

of subculture after which the seedlings started turning yellow, ultimately turned brown and died subsequently.

DISCUSSION

Growth and development of seedlings *in vitro* is largely due to the composition of the nutrient medium, which mainly differ from one another in the quantity of various salts and ions (White, 1951). This may account for the difference in growth behaviour of *C. devonianum* and *D. lituiflorum* seedlings. In the present study, the overall optimal growth of seedlings of both *C. devonianum* and *D. lituiflorum* was enhanced in MS medium. Kramer and Kozlowski (1979) reported that nitrogen present in MS medium greatly influences the growth and differentiation of cells. Besides, some amino acids in MS medium have been reported to replace ammonium nitrate in orchid cultures (Raghavan and Torrey, 1964; Van Waes and Debergh, 1986). This could be the reason for good growth of seedlings in MS medium, which contains glycine as amino acid. Glycine could have provided nitrogen required for seedling growth in this medium. Also presence of high nitrogen content in the form of ammonium nitrate in MS medium might have promoted the growth with NH_4^+ and NO_3^+ ions being readily assimilated during the initial and later stages of development respectively. The present study of *C. devonianum* and *D. lituiflorum* also reveals that in KnC medium seedling growth was inhibited. This could be due to the presence of calcium nitrate in the medium as it has been reported that nitrate in the form of calcium nitrate generally increases all mineral contents except nitrogen in tissues (Alan, 1989).

Growth regulators have profound effect on seedling growth of orchids (Fonnesbech, 1972a; Bose and Mukherjee, 1976; Kusumoto, 1978, Shiau *et al.*, 2002; Krapiec *et al.*, 2003; Talukdar *et al.*, 2003). Auxins have been reported to influence seedling growth. Harvais (1973) obtained inconclusive results with IAA in medium for *Dactylorhiza purpurella* seed and seedlings. In the present study it was found that incorporation of IAA in the medium, seedling growth of *C. devonianum* in terms of fresh weight, shoot number and length were either similar or inhibited when compared to control. However, root number and length was enhanced at 0.5 mg/l and 2.5 mg/l of IAA in the medium respectively. Inhibition of growth in the seedlings was recorded with further increase in IAA concentration in the medium. Inhibition of seedling growth as a result of higher concentrations of IAA is reported in *Coelogyne punctulata* (Sharma and Tandon, 1987). Auxins have been reported to enhance root formation in plants (Bhojwani and Razdan, 1983). Seedling growth of *C. devonianum* was inhibited by incorporation of NAA in the medium. The seedling response in terms of shoots and root number of *C. devonianum* at 0.5 mg/l NAA in the medium was similar to that of the control. However the growth of *C. devonianum* seedlings was poor at higher concentrations of NAA tried in the medium. At these concentrations the seedlings turned brown and died subsequently. Similar results were observed in seedlings of *Cattleya walkeriana* where NAA alone in the medium had no significant effect on multiple bud production (Krapiec *et al*, 2003). Similar results have been determined by Begum *et al.* (1994) in *Cymbidium* species. Addition of 2,4-D in the medium inhibited the seedling growth of *C. devonianum.* At low concentration i, e., 0.5 mg/l, the seedlings remained green but no

further growth occurred. At higher concentrations of 2,4-D in the medium, *C. devonianum* seedlings turned brown and died subsequently. The inhibitory effects of 2,4-D were also observed in some other orchids like *Vanda Miss Joaquin* (Goh, 1970), *Galeola septrentrionalis* (Nakamura, 1982), *Cypripedium reginae* (Harvais, 1982), *Cymbidium elegans* (Sharma and Tandon, 1987). Incorporation of 2,4-D in the medium is reported to adversely affect the germination and subsequent growth of saprophytic orchid *Cymbidium macrorhizon* (Vij and Pathak, 1988). The present study showed that the influence of cytokinins on seedling growth was quite pronounced. Maximum number of shoots of *C. devonianum* were induced at 0.5 mg/l and 2.5 mg/l of BAP incorporated in the medium. This is in consistent with the report that addition of 0.5 mg/l BAP in medium was effective in inducing higher frequency of shoot formation in *Habenaria crinifera* and was sufficient to sustain their growth (Latha, 1999b). Nagaraju *et al.* (2003) also reported that presence of BAP at low concentration induced production of maximum number of protocorms besides a higher number of shoots in *Cymbidium* and *Cattleya* species. With the increase in concentrations of BAP in the medium, a decrease in shoot number of *C. devonianum* was recorded. Similar results wherein increasing the concentration of BAP had no influence is indicated by inhibition of growth in *Cymbidium* and *Cattleya* species (Nagaraju *et al.*, 2003). These results also confirm the earlier findings of Nayak *et al.* (1997) and Nagaraju and Parthasarathy (1999). Roots of *C. devonianum* were completely suppressed at 2.5 mg/l and higher concentrations of BAP in the medium. Perusal of literature suggests that roots were not observed in higher concentration of BAP in case of *Phalaenopsis,* suggesting that the level of BAP is crucial

for vegetative growth (Duan and Yazawa, 1995). Similar reports exist where Shimura and Koda (2004) had reported root inhibition from PLBs of *Cypripedium macranthos* var. *rebunense* in 10.0 μM BAP singly in the medium within 3 months of culture. Developmental retardation and root inhibition was also observed by Rucker (1974) in *Cymbidium* treated with BAP in the medium.

KN is reported to cause cell division and enlargement of plant tissues in culture (Miller *et al.*, 1956; Skoog and Miller, 1957). The enhancement of seedling growth by KN in the medium was reported in *Cypripedium reginae* and *Galeola septentrionalis* (Harvais, 1982; Nakamura, 1982). Vij and Pathak (1989) reported that shoot bud multiplication of *Dendrobium chrysanthum* was enhanced by KN. The present study of *C. devonianum* also suggests that the growth of seedlings was slightly enhanced at low concentrations of KN in the medium. However, with increase in concentrations of KN in the medium overall growth was inhibited which is in contrast to the report of Talukdar (2001) who reported increase in multiple shoots of *Dendrobium aphyllum* with the relative increase of KN concentration in the medium.

The interacting effects of cytokinin and auxin on shoot/root balance in orchid are well documented (Fonnesbech, 1972a,b; Kusumoto, 1978). But the effects of these combinations are inconclusive as they vary with the growth regulators, their concentrations and the orchids used. In the present study, fresh weight was highest at 2.5 mg/l KN in combination with 0.5 mg/l of IAA. Maximum number of shoots was recorded in a combination of 0.5 mg/l each of BAP and IAA in the medium. Shoot length was higher on the medium containing 0.5 mg/l each of KN and IAA in the medium. Root

number observed in different combination of growth regulators tried in the medium was higher on the medium containing 0.5 mg/l each of BAP and IAA. Compared to control, root length observed was poor in all the combinations of growth regulators tried. Among the different auxins tried in the medium, IAA in combination with KN/BAP proved better in overall development of *C. devonianum* seedlings. These results are similar to that observed in seedlings of *Aerides williamsii* (Kalita, 1999) and *Calanthe*, *Phaius* and *Dendrobium* (Baruah, 1996). The response of seedlings of *C. devonianum* in the medium containing BAP and NAA was however inhibitory. Latha (1999) reported similar results wherein BAP and NAA in combination in the medium inhibited the growth of shoots in *Habeneria crinefera.*

In case of *D. lituiflorum* slight increase in shoot number of the seedlings was recorded at low concentrations of IAA in the medium. IAA has an influencing effect on growth of seedlings of the orchids, *Laeliocattleya* (Kano, 1965) and *Phalaenopsis* (Ernst, 1967). In the present study, it was found that the fresh weight, shoot number and shoot length of the seedlings increase at 2.5 mg/l of NAA in the medium. Various studies on the effect of NAA on plant tissue culture have established the fact that NAA stimulates growth of shoot and root system, and tissue proliferation. The enhancement of seedling growth by NAA has been reported in *Dendrobium* (Israel, 1963), *Cymbidium* (Torikata *et al.*, 1965), *Cattleya aurantiaca*, *Cymbidium madidum, Bletilla* species, *Chondrorhyncha discolor* (Strauss and Reisinger, 1976), *Cattleya* (Kusumoto, 1979) and *Galeola septentrionalis* (Nakamura 1982). The seedling development of *Dendrobium transparens* was also reported to be enhanced in media (B_5, MS) supplemented with NAA (Hazarika

and Sharma, 1995). The number of roots initiating in NAA supplemented medium was observed to be increased drastically. Talukdar (2001) reported similar results wherein high number of roots per plantlet was induced in the medium containing 2.0 mg/l of NAA in *Dendrobium aphyllum.* Handique and Talukdar (1998) also reported addition of 2.0 mg/l NAA in the medium resulted maximum number of roots. Addition of 2,4-D in the medium had a differential effect on the developing seedlings. At low concentration of 2,4-D (0.5 mg/l) in the medium, multiple shoots were developed where both shoot and root number were increased comparatively to control. However, 2,4-D in the medium inhibited the seedling growth of *Dendrobium fimbriatum* var. *occulatum* (Kumaria, 1991). Talukdar (2001) also reported the inhibitory effect of 2,4-D on plantlets of *Dendrobium aphyllum.* The present study showed that at higher concentrations of 2,4-D in the medium, the whole seedlings developed into PLBs, which multiplied profusely when transferred to fresh MS medium and developed completely into plantlets within 8 weeks of subculture. Though direct development of PLBs from orchid seedlings have not been reported, but formation of PLBs from different parts of orchid seedlings are reported. Vij *et al.* (1984) reported direct generation of PLBs in *Rhynchostylis retusa* leaf segments when 2,4-D is added in the medium. Kanjilal *et al.* (1999) also reported that 2,4-D at 1.0 mg/l in the medium showed a significant increase in PLB formation in liquid culture of stem disc of *Dendrobium moschatum.* In the present study high concentration of 10.0 mg/l 2,4-D in the medium was found to be inhibitory for seedling growth of *D. lituiflorum.* The seedlings turned brown and died in course of time. A similar result was also reported where incorporation of 10.0 mg/l 2,4-D in the medium retarded seedling

growth in *Dendrobium aphyllum* (Talukdar, 2001). Seedling growth of *D. lituiflorum* in terms of fresh weight, shoot length, root number and length showed poor response in medium containing BAP. However, the number of multiple shoots emerging increased at 2.5 mg/l BAP in the medium. Maximum number of PLBs that formed plantlets was reported in the medium containing 0.5 mg/l BAP in case of terrestrial orchid *Habenaria marginata* (Sheelavanthmath and Murthy, 2001). Similar results were obtained by Devi *et al.* (1998) where development of multiple PLBs from callus was observed in 2.5 mg/l BAP. Early studies on KN in medium reported to stimulate as well as inhibit growth and development in orchid species (Hadley and Harvais, 1968; Hadley, 1970; Harvais, 1973). In the present study, optimum development of seedlings of *D. lituiflorum* was recorded at low concentration of KN present in the medium when compared to control. Shoot bud multiplication of *Dendrobium chrysanthum* was reported to be enhanced by KN (Vij and Pathak, 1989).

The interaction of auxins and cytokinins in the medium produced differential response of the seedlings. The seedling growth of *D. lituiflorum* was more or less inhibited in the medium containing BAP in combination with IAA. However, optimum growth of seedling in terms of shoot number was recorded at 2.5 mg/l BAP and 0.5 mg/l IAA incorporated together in MS medium. Roots were completely suppressed in combinations containing higher concentrations of BAP and IAA in the medium. Baruah (1996) also reported that higher concentrations of auxin and cytokinin in the medium promoted shoot growth but delayed root development. The seedling growth of *D. lituiflorum* was enhanced slightly in the medium containing combinations of BAP and

NAA. The optimum seedling growth was recorded in the medium containing low concentration of BAP and NAA in combination. Similar results were obtained where combinations of both BAP and NAA proved beneficial for activating additional proliferative loci in some orchids (Sharma, 1996). Temjensangba and Deb (2005) also reported that as many as 20 shoot buds/ PLBs could be derived from immature seeds on medium supplemented with low concentrations of NAA and BAP in addition to coconut water. In the present study, an interesting feature was recorded in the medium containing 2,4-D in combination with BAP/KN, with both PLBs as well as multiple shoots developing. At low concentrations of BAP and 2,4-D in the medium, the overall growth of the seedling was inhibited when compared to control. With increase in concentration of 2,4-D and BAP in the medium, the cultured seedlings turned into PLBs. The present results are in line with earlier observations that KN (0.1-5.0 mg/l) with 2,4-D (0.5 mg/l) and BAP (0.5-5.0 mg/l) with 2,4-D (0.5-1.0 mg/l) induced multiple protocorms in *Cattleya* shoots (Kusumoto, 1979). In the present study, it was found that with an increase in concentrations of both BAP and 2,4-D in the medium there was a corresponding decrease in PLB formation/explant and with further increase the whole seedling turned brown and died subsequently. At 10.0 mg/l BAP in combination with low concentration of 2,4-D (0.5 mg/l) in the medium the seedlings transformed into a globular structure which failed to differentiate into plantlets. As compared to control, at each of 0.5 mg/l of KN and IAA in the medium, the seedling growth and development was more or less similar to the control. However, a drastic increase in root number at 0.5 mg/l KN with 2.5 mg/l IAA in the medium was recorded. Root number and length were also

optimum at 0.5 mg/l KN in combination with 5.0 mg/l NAA in the medium. The perusal of literature reveals that the incorporation of auxin IAA/NAA along with KN in the culture media proved to be highly satisfactory for the growth of root system (Baruah, 1996; Kalita, 1999).

The results on the effect of growth regulators on seedling growth of orchids are quite widely documented. The differential response of the growth regulators on the growth of the orchids could be attributed to various reasons. The possible reasons for the differential effect could be interactions occurring between different combinations of growth regulators, culture conditions and seedlings used, variations in the physiological responses of species and differences existing in the forms and analogues of hormones. The conditions of culture and the age of the seedlings of different orchids also greatly affect the response of seedlings to the growth regulators in the medium.

CHAPTER IV: MASS MICROPROPAGATION OF *CYMBIDIUM DEVONIANUM* AND *DENDROBIUM LITUIFLORUM*

INTRODUCTION

Plant tissue culture, the most successful commercial aspect of plant Biotechnology has introduced an exciting new phase into plant propagation and breeding. Micropropagation of orchids by means of explants is gaining wide importance in the tissue culture industry. Orchid was the first horticultural plant which was cloned by tissue culture methods on a commercial scale (Griesbach, 1986; Goh, 1990). Following Morel's successful demonstration of meristem culture in *Cymbidium* (Morel, 1960), tissue culture techniques have been extensively used for mass propagation of orchids. Morel for the first time reported the formation of PLBs around the shoot tips of *Cymbidium* which were cultured *in vitro*. These PLBs when cut into small sections and subcultured on fresh nutrient medium, multiplied and on being left undisturbed, developed into complete plantlets. This work led to the mass propagation of desirable virus-free varieties of orchids at a very high rate. Arditti and Ernst (1993) have compiled a large number of micropropagation protocols of hybrids and varieties of orchids. Among the commercially important orchids, the genera *Dendrobium* and *Cymbidium* account for about 80 and 50 percent respectively of the total micropropagated tropical orchids.

Many pioneer workers have applied the technique of meristem culture to either eliminate virus infection or for the production of asexual seedlings on a large scale (Ilsley, 1965; Taylor, 1971; Thompson, 1971). Tissue culture techniques have also been used to save many orchid species from extinction. Knudson's (1951) discovery that the developing orchid seedlings could be grown on a medium supplemented with organic and inorganic nutrients led to the formation and utilization of a number of media for tissue culture of orchids (Withner, 1959; Butenko, 1968; Arditti, 1977; Yam *et al.*, 1989; Devi *et a*l., 1998; Nagaraju *et al.*, 2003). The media used differs in obtaining tissue-culture raised seedlings of orchids from genus to genus and species to species. The incorporation of certain additives like coconut milk, tomato juice, banana homogenate, potato juice, (Talukdar, 2001; Shiau *et al.*, 2002; Decruse *et al.*, 2003; Temjensangba and Deb, 2005) and growth regulators (Arditti, 1977; Geetha and Shethy, 2000; Chen *et al.*, 2002; Malabadi *et al.*, 2004) proves to be beneficial for orchid tissue culture. However, the physiology of nutrition of orchids is difficult to explain and the available information is insufficient because the medium requirements of orchids vary; several media can be suitable for one genus and more than one genus can be cultured on a specific medium.

Tissue culture methods for the propagation of orchids were introduced by several workers using various plant parts as explants on a number of media (Bergman, 1972 ab; Wang, 1989). *Cymbidium*, *Dendrobium*, *Vanda*, *Spathoglottis*, *Bulbophyllum*, *Saccolabium* and allied genera are widely propagated by tissue culture through out the world (Vij, 1993; Vij and Sharma, 1996; Teng *et al.*, 1997; Pathania *et al.*, 1998; Vij *et al.*, 2000; Kaur and Vij, 2000). According to Murashige and Skoog (1978), important

orchids except *Paphiopedilum* are clonable *in vitro.* However, Chen *et al.* (2002) reported multiple shoot formation and plant regeneration from stem nodal explants of *Paphiopedilum.* Kusumoto (1979) used the shoot apices of *Cattleya* buds for the production of PLBs. Clonal propagation of *Phalaenopsis* by means of flower stalk, bud culture, shoot- tip culture, nodes with dormant buds from flower stalks, shoot- tip explants excised from flower stalks has been reported (Intuwong and Sagawa, 1974; Yoneda *et al.*, 1983; Tokuhara and Mii, 2001; Kosir *et al.*, 2004). Explants from the floral stalk have been used in studies on *in vitro* propagation of orchids (Koch, 1974; Lay, 1978; Tanaka and Sakanishi, 1978; Homma and Asahira, 1985; Yoneda, 1986; Momose and Yoneda, 1988; Park *et al.*, 1996; Vij *et al.*, 1997). Reports concerning the propagation of orchids through leaf segments have also been published (Vij *et al.*, 1984, 1994; Tanaka, 1987; Vij and Pathak, 1990; Payati and Murthy, 1999; Kaur and Vij, 2000). Phillip and Nainar (1988) reported the *in vitro* transformation of root meristem to shoot and plantlets in *Vanilla planifolia.* Sanchez (1988) carried out micropropagation of *Cyrtopodium punctatum* seedlings grown aseptically *in vitro*, through root tip culture. Yoneda and Momose (1988) and Vij (1993) reported PLBs and plantlet formation by root tip cultures in some orchids. *In vitro* shoot regeneration from root tips of an orchid *Mormodes histrio* has also been reported by Holters and Zimmer (1990).

Different tissue culture techniques have been developed to promote the selected clonal multiplication in dendrobes and Cymbidiums (Morel, 1960; Wimber, 1963; Vij and Sood, 1982; Vij and Pathak, 1989; Sharon and Vasundhara, 1990; Pathania *et al.*, 1998; Talukdar *et al.*, 2003). Chang and Chang (1998) reported initiation of calli from

rhizomes or pseudobulbs of *Cymbidium ensifolium* var. *misericors*. However, the techniques applied differ from species to species and the genera used. It has been reported that the explants employed for the tissue culture respond selectively depending on their source, physiological state and nutrient environment (Vij *et al.*, 1983, 1984). The following chapter deals with the micropropagation of *C. devonianum* and *D. lituiflorum* for mass propagation obtained from *in vitro* raised seedlings.

MATERIAL AND METHODS

Six months old *in vitro* grown seedlings of *C. devonianum* and *D. lituiflorum* obtained on basal MS medium were taken for setting up the experiments on micropropagation of these orchids. Different explants measuring about 1-2 cm in size, such as leaf parts (leaf tips, mid rib and leaf bases), root tips, axillary buds and apical meristems were excised from the *in vitro* growing plantlets. The apical meristem measuring about 1-2 mm in size was excised from the shoot tips aseptically and cultured on different media.

A number of media (MS, NN, B_5, Mitra and KnC) were tried for the micropropagation of *C. devonianum* and *D. lituiflorum.* About 15 ml of the media was dispensed in each test tube. The pH of the medium was adjusted in accord with the media prior to autoclaving. The explants pieces were inoculated on these media aseptically. The cultures were maintained at 24 ± 2°C with 16-hour illumination of 150 μ moles sec^{-1} m^{-2} light intensity. The optimum medium was supplemented with different growth regulators i.e. IAA, NAA, 2,4-D, BAP and KN both separately and in combinations so as to

optimize the formation of PLBs and shoot buds on the cultured explants. The different combination of growth regulators tried were BAP+IAA, BAP+NAA, BAP+2,4-D, KN+IAA, KN+NAA and KN+2,4-D in a range of 0.0-10.0 mg/l. Ten replicates were maintained for each treatment and the experiments were repeated thrice. Observations were made after 10 weeks of culture of explants in the medium.

RESULTS

C. DEVONIANUM

Out of the different explant sources tried, the apical meristem and the leaf bases were found the most suitable explants for micropropagation of *C. devonianum.*

A. Apical meristem

a) *Effect of Different Basal Media*

The apical meristem excised from the plantlets cultured on different basal media did not show any response in the form of PLBs or shoot buds. However, the explants remained green for long time i.e. 8 weeks of culture on MS medium (Table not shown). The explants cultured on NN, B_5 and Mitra media remained green for only 5 weeks after which they started turning brown and died subsequently. The apical meristems cultured on KnC medium were seen turning brown within a week of culture. Therefore, MS medium was used for micropropagation of *C. devonianum* through apical meristem culture.

b) *Effect of Growth Regulators*

Effect of IAA

The apical meristem cultured on MS medium containing different concentrations of IAA incorporated singly showed very poor response (Table 21). However, at 2.5 mg/l of IAA in the medium a low percentage response of apical meristem (10.0%) was recorded with 1-2 small green PLBs emerging from it. At other concentrations of IAA tried, apical meristem did not respond at all.

Effect of NAA

At different concentrations (0.5, 2.5 and 5.0 mg/l) of NAA in the medium, apical meristem responded differently. At low concentration of NAA i.e. 0.5 mg/l in the medium, a high percentage response (70.0%) of apical meristem in the form of PLBs was observed (Table 21). The apical meristem enlarged and produced globular green PLBs in 5-6 weeks time, with 3-6 clusters of PLBs developing in 10 weeks of culture (Plate 13 a). With increase of NAA concentration in the medium, a decrease in apical meristem response was observed. At 10.0 mg/l of NAA in the medium no response was observed. The explant remained green for some time and died subsequently.

Effect of 2,4-D

The apical meristem responded poorly in all the concentrations of 2,4-D tried in the medium (not shown in the table). Therefore, 2,4-D in combination with BAP/KN was not tried out for studying the response of apical meristem cultured in the medium.

Effect of BAP

Among all the concentrations of BAP tried in the medium, apical meristem responded best (50.0%) producing PLBs at 2.5 mg/l BAP in the medium (Table 21). In this concentration, 3-5 clusters of PLBs were observed in 10 weeks time of culture. At higher concentrations of BAP the response of the apical meristem decreased. At 10.0 mg/l of BAP in the medium, no response in the apical meristem was observed.

Effect of KN

Addition of KN at different concentrations in the medium led to good response of the cultured apical meristem. KN at 0.5 mg/l in the medium resulted in 80.0% response of apical meristem wherein PLBs initiation was observed (Table 21). The time taken for initiation of PLBs was 3-4 weeks of culture and at the end of 10 weeks time around 7-10 clusters of hairy, yellowish green and healthy PLBs were observed (Plate 13 b). The increase in KN concentration in the medium was directly proportional to the decrease in response of apical meristem.

c) *Effect of Growth Regulators in Combination*

Effect of BAP and IAA

Apical meristem responded poorly on the combinations of BAP+IAA tried in the medium. At 2.5 mg/l each of BAP and IAA a highest 43.3% conversion of apical meristem into PLBs was observed (Table 22). Here, 2-6 PLBs were obtained at 10 weeks of culture. The other treatments tried were found less effective in the conversion of apical meristem to PLBs or shoot buds.

Table 21: Effect of different growth regulators incorporated singly in MS medium on PLB formation from apical meristem of *C. devonianum*

Conc. (mg/l)	*Average % of explant response	*Average no. of PLBs/ explant	Remarks
Control	0	0	The tissue remained green
IAA			
0.5	0	0	-
2.5	10.0 ± 4.7	1.1 ± 0.5	1-2 small green PLBs
5.0	0	0	-
10.0	0	0	Explants dried
NAA			
0.5	70.0 ± 4.7	8.1 ± 0.2	3-6 clusters of green PLBs
2.5	56.6 ± 11.8	3.6 ± 0.0	3-4 pale yellow PLBs
5.0	36.6 ± 7.2	2.5 ± 0.1	2-3 smaller PLBs
10.0	0	0	-
BAP			
0.5	30.0 ± 9.4	2.6 ± 0.1	2-3 green PLBs
2.5	50.0 ± 4.7	3.9 ± 0.2	3-5 clusters of green PLBs
5.0	20.0 ± 4.7	2.1 ± 0.1	1-2 yellow PLBs
10.0	0	0	-
KN			
0.5	80.0 ± 9.4	9.7 ± 0.2	7-10 clusters of green, healthy PLBs
2.5	60.0 ± 4.7	5.1 ± 0.1	4-6 clusters of PLBs
5.0	26.6 ± 2.7	1.3 ± 0.1	3-4 PLBs
10.0	6.6 ± 2.7	1.0 ± 0.0	0-2 yellow and unhealthy PLBs

± SE; - no response

* Data recorded after 10 weeks

Table 22: Effect of BAP and IAA incorporated in combination in MS medium on PLB formation from apical meristem of *C. devonianum*			
Conc. (mg/l)	*Average % of explant response	*Average no. of PLBs/ explant	Remarks
Control	0	0	Tissue green, no response
BAP+IAA			
0.5+0.5	13.3±2.7	2.1±0.1	2-3 PLBs
0.5+2.5	26.6±3.2	2.8±0.1	2-4 green and healthy PLBs
0.5+5.0	0	0	-
0.5+10.0	0	0	-
2.5+0.5	20.0±4.7	2.9±0.3	2-4 PLBs
2.5+2.5	43.3±7.2	3.1±0.2	2-6 green and healthy PLBs
2.5+5.0	10.0±0.0	2.3±0.3	2-3 PLBs
2.5+10.0	0	0	-
5.0+0.5	3.3±2.7	1.0±0.0	Single PLB
5.0+2.5	23.3±2.7	2.6±0.3	2-4 PLBs
5.0+5.0	10.0±0.0	1.3±0.2	1-2 PLBs
5.0+10.0	0	0	-
			-
10.0+0.5	0	0	-
10.0+2.5	0	0	-
10.0+5.0	0	0	-
10.0 + 10.0	0	0	-

± SE; - no response

* Data recorded after 10 weeks

Effect of BAP and NAA

BAP in combination with NAA at different concentrations, showed varying response on the conversion of apical meristem into PLBs. A high response of 60.0% explants was observed from apical meristems cultured on medium containing 2.5 mg/l BAP in combination with 0.5 mg/l NAA (Table 23). About 4-8 green and healthy PLBs were observed after 10 weeks of culture. At 0.5 mg/l each of BAP and NAA in the medium, 56.6% of conversion of apical meristem into 3-6 PLBs was observed. The higher concentrations of BAP (5.0 and 10.0 mg/l) in combination with NAA (0.5, 2.5, 5.0 and 10.0 mg/l), apical meristem did not respond at all. In all these combinations tried, the explants remained green for 1-2 week's time and subsequently turned brown and died.

Effect of KN and IAA

Induction of PLBs from apical meristem was observed at low concentration of KN (0.5 mg/l) when combined with different concentrations of IAA (0.5, 2.5, 5.0 and 10.0 mg/l) and 2.5 mg/l of KN when combined with 0.5, 2.5, and 5.0 of IAA in the medium. PLBs were also seen emerging at high concentration of KN (5.0 mg/l) when combined with 0.5 mg/l of IAA in the medium. At 0.5 mg/l KN and 2.5 mg/l IAA, a high response (i.e. 83.3%) of apical meristem was recorded followed by 66.6% at 0.5 mg/l each of KN and IAA in the medium (Table 24). As many as 8-15 PLBs were seen to develop from each explants in the treatment containing 0.5 mg/l KN and 2.5 mg/l IAA in combination. The PLBs formed were green, diffused and healthy (Plate 13c). A low response of apical meristem (23.3%) was observed at high concentration of KN (5.0 mg/l) when combined with low concentrations of IAA (0.5 mg/l). However, at the same

Table 23: Effect of BAP and NAA incorporated in combination in MS medium on PLB formation from apical meristem of *C. devonianum*

Conc. (mg/l)	*Average % of explant response	*Average no. of PLBs/ explant	Remarks
Control	0	0	The tissue remained green
BAP+NAA			
0.5+0.5	56.6±5.4	4.6±0.1	3-6 PLBs
0.5+2.5	40.0±4.7	3.4±0.7	3-4 PLBs
0.5+5.0	0	0	-
0.5+10.0	0	0	-
2.5+0.5	60.0±4.7	5.9±0.36	4-8 green and healthy PLBs
2.5+2.5	46.6±2.7	3.6±0.07	3-4 green PLbs
2.5+5.0	10.0±0.0	2.0±0.0	2 number of PLBs
2.5+10.0	0	0	-
5.0+0.5	0	0	-
5.0+2.5	0	0	-
5.0+5.0	0	0	-
5.0+10.0	0	0	-
10.0+0.5	0	0	-
10.0+2.5	0	0	-
10.0+5.0	0	0	-
10.0+10.0	0	0	-

± SE; - no response

* Data recorded after 10 weeks

Table 24: Effect of KN and IAA incorporated in combination in MS medium on PLB formation from apical meristem of *C. devonianum*

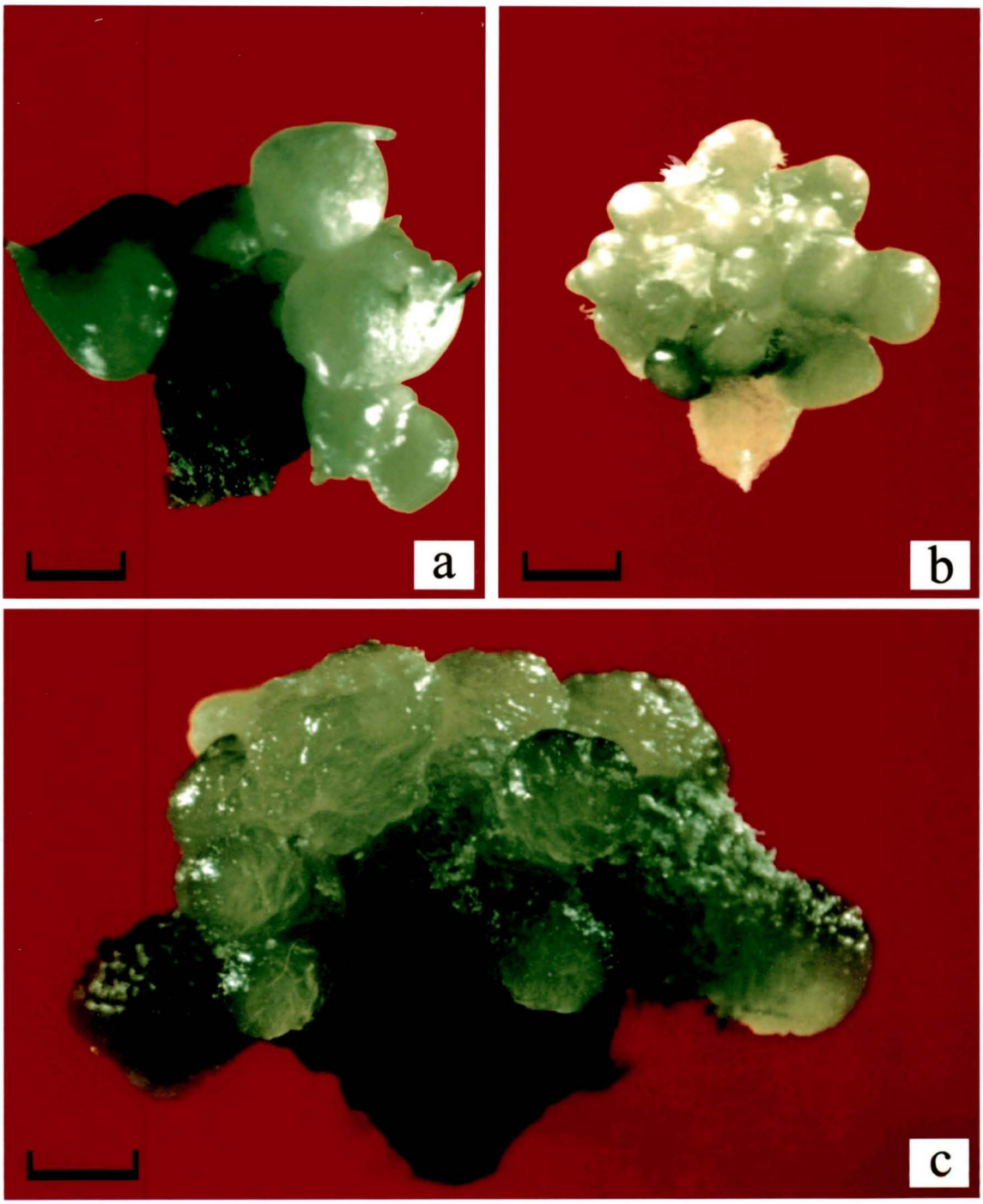
Conc. (mg/l)	*Average % of explant response	*Average no. of PLBs/ explant	Remarks
Control	0	0	The tissue remain green
KN+IAA			
0.5+0.5	66.6±2.7	8.9±0.3	7-13 clusters of PLBs
0.5+2.5	83.3±2.7	11.0±0.3	8-15 well developed PLBs
0.5+5.0	33.3±7.2	5.1±0.1	5-7 small PLBs
0.5+10.0	16.6±2.7	3.3±0.1	3-4 small, yellow PLBs
2.5+0.5	40.0±4.7	7.5±0.1	6-8 clusters PLBs
2.5+2.5	63.3±7.2	8.7±0.3	8-10 clusters PLBs
2.5+5.0	6.6±2.7	2.3±0.1	2-3 PLBs of smaller size
2.5+10.0	0	0	-
5.0+0.5	23.3±2.7	2.1±0.13	2-3 number of PLBs
5.0+2.5	0	0	-
5.0+5.0	0	0	-
5.0+10.0	0	0	-
10.0+0.5	0	0	-
10.0+2.5	0	0	-
10.0+5.0	0	0	-
10.0+10.0	0	0	-

± SE; - no response

* Data recorded after 10 weeks

Plate 13

Development of PLBs from apical meristem of *C. devonianum* in MS medium incorporated with 0.5 mg/l NAA (a), 0.5 mg/l KN (b) and 0.5 mg/l KN + 2.5 mg/l IAA (c) after 10 weeks of culture (bar = 1mm)


a
b
c

concentration of KN with 2.5, 5.0 and 10.0 mg/l of IAA in the medium, apical meristem failed to respond. In high concentration of KN i.e. 10.0 mg/l in combination with all concentrations of IAA, response of apical meristem was not observed at all.

Effect of KN and NAA

In some combinations of KN and NAA tried in the medium the cultured apical meristem showed response in some treatments. At low concentration of 0.5 mg/l each KN and NAA in the medium 60.0% of the explants responded leading to the formation of 3-5 PLBs (Table 25). With increase in KN (2.5 mg/l) in combination with the same concentration of NAA slight increase of response (66.6%) in apical meristem was recorded. The response of apical meristem decreased with increase in both concentrations of KN and NAA in the medium. At high concentrations of KN (5.0 and 10.0 mg/l) in combination with 2.5, 5.0 and 10.0 mg/l of NAA in the medium, there was no response of apical meristem.

PLBs obtained from the apical meristem given the above mentioned treatments were multiplied further by cutting and subculturing on fresh MS medium without growth regulators. The formation of complete plantlets from PLBs obtained from apical meristem took place in the same medium in about 16 weeks time on leaving the PLBs undisturbed (Plate 14).

B. Leaf base

Among the different parts of the leaf tried in the culture, a response was observed only with the leaf base. The other parts of leaf dried up in 2 week's time of culture.

Table 25: Effect of KN and NAA incorporated in combination in MS medium on PLB formation from apical meristem of *C. devonianum*

Conc. (mg/l)	*Average % of explant response	*Average no. of PLBs/ explant	Remarks
Control	0	0	Tissue remained green
KN+NAA			
0.5+0.5	60.0±9.4	3.9±0.15	3-5 number of PLBs
0.5+2.5	26.6±7.2	2.0±0.00	A constant number of PLBs (2)
0.5+5.0	0	0	-
0.5+10.0	0	0	-
2.5+0.5	66.6±7.2	5.0±0.2	4-6 green and healthy PLBs
2.5+2.5	46.0±2.7	3.5±0.1	3-4 PLBs
2.5+5.0	10.0±4.7	1.3±0.8	2-3 PLBs
2.5+10.0	0	0	-
5.0+0.5	40.0±2.7	2.5±0.3	2-4 PLBs
5.0+2.5	0	0	-
5.0+5.0	0	0	-
5.0+10.0	0	0	-
10.0+0.5	0	0	-
10.0+2.5	0	0	-
10.0+5.0	0	0	-
10.0+10.0	0	0	-

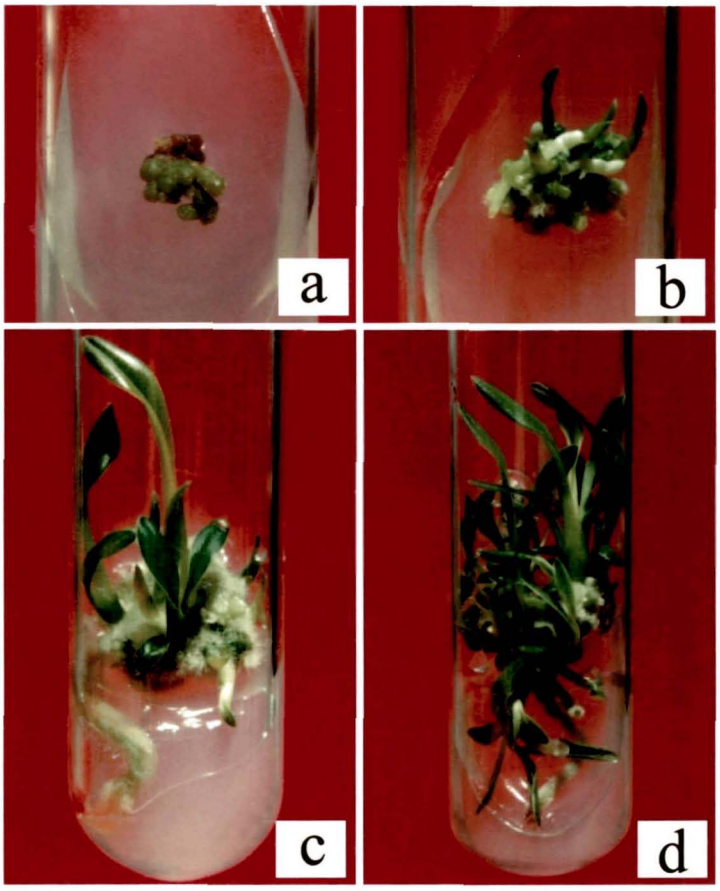
± SE; - no response

* Data recorded after 10 weeks

Plate 14

Development of complete plantlets from apical meristem of *C. devonianum* in MS + 0.5 mg/l KN + 2.5 mg/l IAA

a. Initiation of PLBs from apical meristem in 3 weeks of culture
b. Proliferating PLBs with initiation of shoots after 9 weeks culture
c. Shoots and PLBs along 1-2 roots after 12 weeks culture
d. Complete development of shoots and roots after 16 weeks culture


a
b
c
d

a) *Effect of Different Basal Media*

Leaf bases cultured on different media (MS, NN, B_5, Mitra and KnC) did not show any response. Among the various media tried leaf bases remained green for maximum period of 2 weeks of culture on MS medium (Table not shown) after which the explants turned yellow. In other media tried the explants turned brown and died subsequently. Hence, MS medium was tried for setting up the experiments for micropropagation of *C. devonianum* using leaf bases.

b) *Effect of Growth Regulators*

Effect of IAA

PLBs were observed emerging on the leaf bases cultured on all the concentrations of IAA tried in the medium. At low concentration of IAA (0.5 mg/l), only 16.6% leaf bases responded (Table 26). Increasing the concentration of IAA in the medium to 2.5 mg/l, 50.0% of the leaf bases responded wherein 3-5 PLBs were seen to have emerged from the leaf bases. However, with further increase of IAA to 5.0 and 10.0 mg/l in the medium a decrease in the response of leaf bases was recorded.

Effect of NAA

At 0.5 mg/l concentrations of NAA in the medium, only 20.0% of leaf base was observed (Table 26). In other concentrations of NAA tried, the explants failed to respond.

Effect of BAP

At low concentrations of BAP (0.5 and 2.5 mg/l), the leaf base responded producing PLBs in culture. A response of 33.3 and 10.0% of leaf bases was recorded at

0.5 and 2.5 mg/l of BAP respectively (Table 26). The other treatments were inhibitory for the cultured leaf bases.

Effect of KN

Leaf bases cultured in medium containing different concentrations of KN responded in the development of PLBs. At 0.5 mg/l of KN in the medium, 60.0% of response was observed in the cultured leaf base followed by drastic decrease (26.6%) on 2.5 mg/l of KN in the medium (Table 26). Average of 3-6 green hairy PLBs were observed from the leaf bases cultured at 0.5 mg/l of KN in the medium (Plate 15 a). The percentage of the explants response decreased to 16.6% at 5.0 mg/l of KN in the medium.

c) *Effect of Growth Regulators in Combination*

The leaf base responded poorly in the various combinations of growth regulators studied. However, a high percentage response (63.3%) of leaf base was observed on 0.5 mg/l BAP + 2.5 mg/l IAA wherein 6-15 clusters of well developed globular green PLBs were observed after 10 weeks of culture (Table 27; Plate 15 b). This was followed by 50.0 % response of apical meristem at 0.5 mg/l BAP + 5.0 mg/l of IAA in the medium with 3-8 numbers of PLBs initiating in the medium. The leaf bases failed to show any kind of response in all the treatments of BAP and NAA in combination. Fifty percent of response was also observed at 0.5 mg/l each of KN and IAA in medium with 3-4 numbers of PLBs emerging (Table 28). Leaf base responded poorly on the medium containing KN and IAA in all combinations. A similar response of leaf bases was observed in the medium containing KN and NAA. Only leaf bases treated with 0.5 mg/l

Table 26: Effect of growth regulators incorporated singly in MS medium on PLB formation from leaf bases of *C. devonianum*

Conc. (mg/l)	*Average % of explant response	*Average no. of PLBs / explant	Remarks
Control	0	0	Explant turned brown; died subsequently
IAA			
0.5	16.6±2.7	1.3±0.1	1-2 PLBs
2.5	50.0±4.7	4.8±0.3	3-5 green, healthy PLBs
5.0	20.0±4.7	3.1±0.1	2-4 PLBs
10.0	6.6 ±2.7	1.6±0.1	0-3 PLBs
NAA			
0.5	20.0±4.7	2.1±0.1	2 small hairy PLBs
2.5	0	0	Slight swelling of the explant
5.0	0	0	-
10.0	0	0	-
BAP			
0.5	33.3±2.7	2.4±0.1	1-3 green healthy PLBs
2.5	10.0±4.7	1.2±0.4	0-2 healthy PLBs
5.0	0	0	Explant remained green; no response
10.0	0	0	-
KN			
0.5	60.0±9.4	4.2±0.1	3-6 PLBs
2.5	26.6±7.2	3.1±0.1	2-4 PLBs
5.0	16.6±2.7	1.5±0.2	1-3 PLBs
10.0	0	0	-

± SE; - no response

* Data recorded after 10 weeks

Table 27: Effect of BAP and IAA incorporated in MS medium on PLB formation from leaf bases of *C. devonianum*

Conc. (mg/l)	*Average % of explant response	*Average no. of PLBs/ explant	Remarks
Control	0	0	Explant turned brown; died subsequently
BAP+IAA			
0.5+0.5	26.6±7.2	2.3±0.2	2-4 PLBs
0.5+2.5	63.3±7.2	10±0.4	Cluster of 6-15 green PLBs
0.5 5.0	50.0±4.7	5.2±0.4	3-8 number of PLBs
0.5+10.0	0	0	-
2.5+0.5	20.6±2.7	1.7±0.2	1-3 number of PLBs
2.5+2.5	0	0	-
2.5+5.0	0	0	-
2.5+10.0	0	0	-
5.0+0.5	0	0	-
5.0+2.5	0	0	-
5.0+5.0	0	0	-
5.0+10.0	0	0	-
10.0+0.5	0	0	-
10.0+2.5	0	0	-
10.0+5.0	0	0	-
10.0+10.0	0	0	-

± SE; - no response

* Data recorded after 10 weeks

Table 28: Effect of KN and IAA incorporated in combination in MS medium on PLB formation from leaf bases of *C. devonianum*

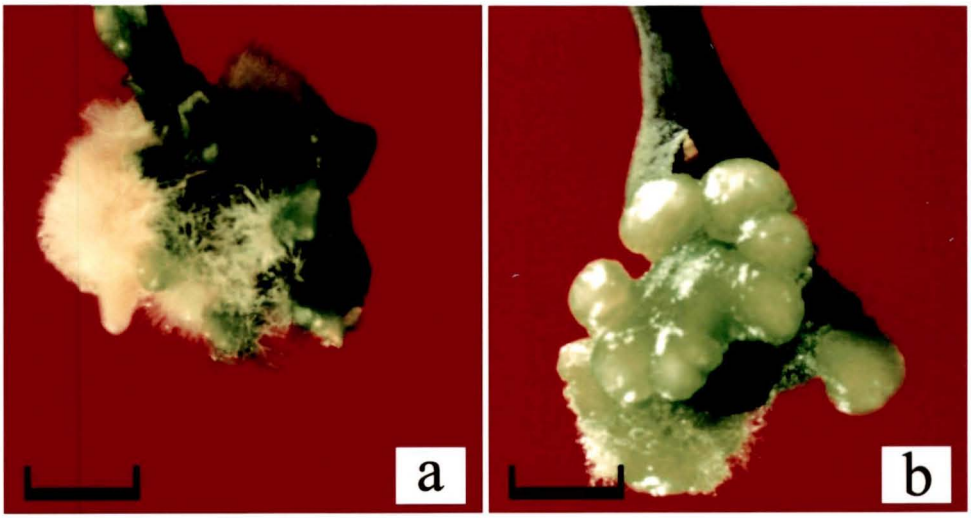
Conc. (mg/l)	*Average % of explant response	*Average no. of PLBs/ explant	Remarks
Control	0	0	Explants turned brown; died subsequently
KN+IAA			
0.5+0.5	50.0±4.7	3.4±0.09	3-4 number of PLBs
0.5+2.5	40.0±7.2	2.6±0.13	2-3 number PLBs
0.5+5.0	0	0	-
0.5+10.0	0	0	-
			-
2.5+0.5	20.0±4.7	2.0±0.00	2 PLBs in all
2.5+2.5	0	0	-
2.5+5.0	0	0	-
2.5+10.0	0	0	-
5.0+0.5	0	0	-
5.0+2.5	0	0	-
5.0+5.0	0	0	-
5.0+10.0	0	0	-
10.0+0.5	0	0	-
10.0+5.0	0	0	-
10.0+5.0	0	0	-
10.0+10.0	0	0	-

± SE; - no response

* Data recorded after 10 weeks

Plate 15

Developments of PLBs from leaf bases of *C. devonianum* in MS medium incorporated with 0.5 mg/l of KN (a), and 0.5 mg/l of BAP + 2.5 mg/l IAA (b), after 10 weeks of culture (bar = 1mm)


a
b

each of KN and NAA, 0.5 mg/l KN + 2.5 mg/l NAA and 2.5 mg/l KN + 0.5 mg/l NAA in the medium responded in initiation of PLBs (Table 29).

PLBs obtained from the leaf bases given the above mentioned treatments were multiplied further by cutting and subculturing on fresh MS medium without growth regulators. The formation of complete plantlets from PLBs obtained took place in about 18 weeks of culture time (Plate 16).

D. LITUIFLORUM

Out of all different explant sources tried, the axillary bud and apical meristem were found suitable explants for micropropagation of *D. lituiflorum*.

A. Axillary bud

a) *Effect of Different Basal Media*

The axillary buds cultured on different media showed response in MS, NN, B_5 and Mitra media. Out of these four media on which the axillary bud responded, MS medium was found the optimum wherein high response (90.0%) of the explant was recorded with 2-3 numbers of shoots arising from the single explant (Table 30; Plate 17a) followed by B_5 (86.6%) and Mitra (83.3%) media. A lower explant response of 66.6% was recorded on NN medium. Solitary shoots were seen coming out from the explants cultured on NN and Mitra media tried. However, no response was observed from the explants cultured on KnC medium. On this medium, the explants turned brown after 3 week of culture. Hence, for further experiments on micropropagation of *D. lituiflorum*

Table 29: Effect of KN and NAA incorporated in combination in MS medium on PLB formation from leaf bases of *C. devonianum*

Conc. (mg/l)	*Average % of explant response	*Average no. of PLBs/ explant	Remarks
Control	0	0	Explant turned brown; died subsequently
KN+NAA			
0.5+0.5	46.6±7.2	2.5±0.05	2-3 PLBs
0.5+2.5	20.0±4.7	2.0±0.00	2 PLBs
0.5+5.0	0	0	-
0.5+10.0	0	0	-
2.5+0.5	40.0±4.7	2.7±0.11	2-3 well developed PLBs
2.5+2.5	0	0	-
2.5+5.0	0	0	-
2.5+10.0	0	0	-
5.0+0.5	0	0	-
5.0+2.5	0	0	-
5.0+5.0	0	0	-
5.0+10.0	0	0	-
10.0+0.5	0	0	-
10.0+2.5	0	0	-
10.0+5.0	0	0	-
10.0+10.0	0	0	-

± SE; - no response

* Data recorded after 10 weeks

Plate 16

Complete development of plantlets from leaf base of *C. devonianum* in MS + 0.5 mg/l KN

a. Initiation of PLBs in 3 weeks of time (bar = 1mm)

b. Clusters of PLBs developed in 9 weeks time

c. Development of shoots and PLBs in 14 weeks

d. Development of shoots with few roots after 18 weeks

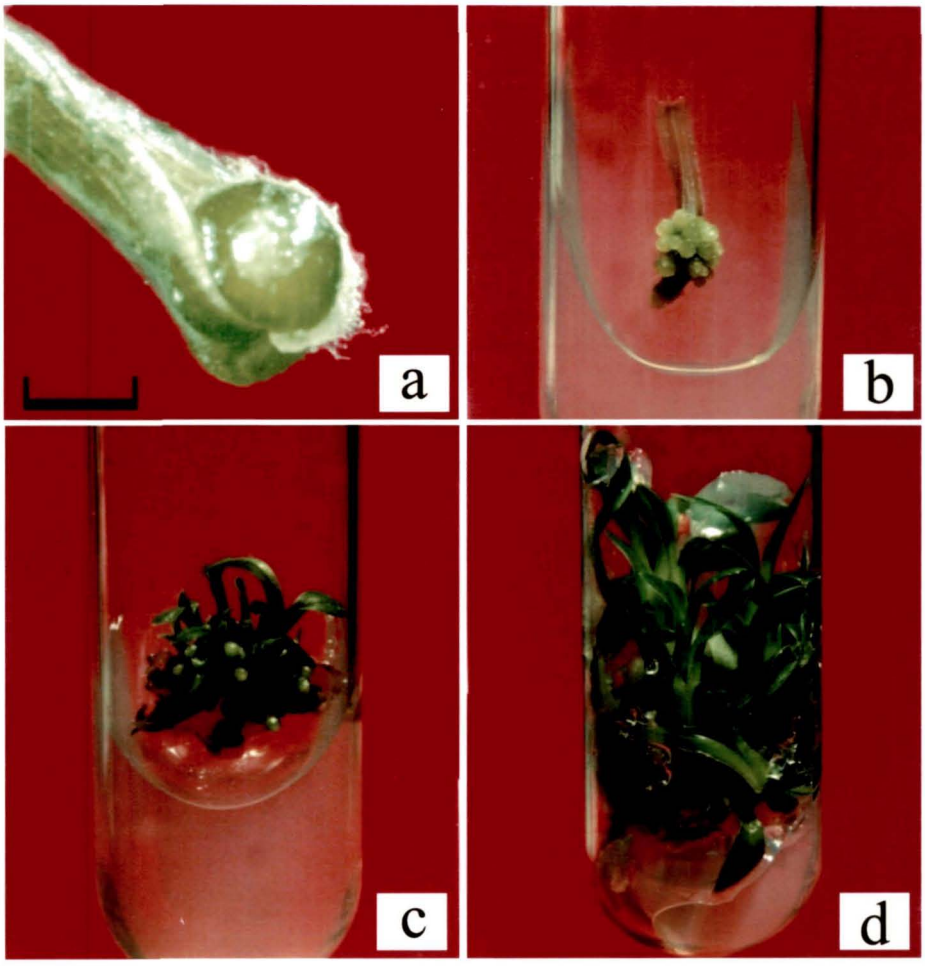

a
b
c
d

Table 30: Effect of different media on PLB and shoot formation and from axillary bud of *D. lituiflorum*

Media	*Average % of explant response	*Average no. of shoots/explant	Average % of PLBs formed/ explant	Remarks
MS	90.0±4.7	2.2±0.09 (2-3)	0	Healthy multiple shoots
NN	66.6±2.7	1.0±0.00	0	Solitary shoot
B_5	86.6±5.4	1.3±0.06 (1-2)	0	2 shoots
Mitra	83.3±4.2	1.0±0.00	0	Solitary shoot
KnC	0	0	0	Explants turned brown; Died

± SE

Numbers in bracket indicates the minimum and maximum shoots/explant

* Data recorded after 5 weeks

axillary buds were cultured on MS medium supplemented with different growth regulators.

b) *Effect of Growth Regulators*

Effect of IAA

Compared to control, axillary bud responded well in all the concentrations of IAA tried in the medium. High explants response (96.6%) with average number (1.7) of shoots was recorded at 0.5 mg/l concentration of IAA in the medium. However, with increase in IAA concentration (2.5 mg/l) in the medium, 100% explant response was recorded with an average number of 3.1 shoots per explant (Table 31). The percentage response of axillary bud decreased to 80.0 % at 5.0 mg/l of IAA and 63.3% at 10.0 mg/l of IAA in the medium. Besides shoot formation, PLBs were also observed in 40 and 20% of the explants cultured in medium containing 2.5 and 5.0 mg/l IAA respectively. PLBs were observed initiating at the base of the shoots (Table 31; Plate 17d). At higher concentrations of 10.0 mg/l of IAA in the medium, PLBs were not observed.

Effect of NAA

The percentage response of explants cultured in the medium containing 0.5 and 2.5 mg/l NAA was 100% which is much higher as compared to control. However, the number of multiple shoots emerging was different at these two concentrations. An average of 2.6 numbers of shoots with 2-5 denoting minimum and maximum range of shoots per explant was observed at 0.5 mg/l of NAA in the medium. Comparatively, a higher average number of 4.8 shoots with 3-8 minimum and maximum range of shoots was observed at 2.5 mg/l of NAA in the medium (Table 31; Plate17c). With increase in

concentrations of NAA in the medium, a decrease in both percentage response and in number of shoots developing from the axillary bud was recorded.

Effect of 2,4-D

The explants cultured on the medium containing 2,4-D responded in a different manner as compared to those given the other growth regulator treatments. In all the concentrations of 2,4-D tried in the MS medium, only PLBs were observed to have developed from all the cultured axillary buds. Highest percentage (83.3%) of explants responded in the medium containing 0.5 mg/l of 2,4-D (Table 31). Initially, the PLBs were small greenish globular structures (Plate 17b) but in course of time, these PLBs developed into plantlet when subcultured to fresh medium. With increase of 2,4-D concentration in the medium a decrease in response of the explant was recorded. However, PLBs developed on higher concentrations of 2,4-D (5.0 and 10.0 mg/l) in the medium did not multiply when subcultured to fresh medium; rather they turned brown and died in course of time.

Effect of BAP

The explants responded in all the concentrations of BAP tried in the medium. The highest response (93.3%) with an average 2.3 number of shoots was found best in the medium containing 2.5 mg/l of BAP; followed by 90.0% of response at 0.5 mg/l of BAP in the medium (Table 31). With increase in BAP concentration in the medium, there was decrease in response of the explant wherein a solitary shoot emerged.

Table 31: Effect of growth regulators incorporated singly in MS medium on shoot bud and PLB formation from axillary bud of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into shoots	*Average no. of shoots /explant	Average % of explant response into PLBs
Control	86.6±5.4	2.0±0.00 (2)	0
IAA			
0.5	96.6±2.7	1.7±0.01 (1-3)	0
2.5	100±0.0	3.1±0.08 (2-5)	40.0±4.7
5.0	80.0±4.7	1.8±0.07 (1-3)	20.0±4.7
10.0	63.3±7.2	1.2±0.02 (1-2)	0
NAA			
0.5	100.0±0.0	2.6±0.16 (2-5)	0
2.5	100.0±0.0	4.8±0.12 (3-8)	0
5.0	80.0±9.4	2.2±0.16 (1-4)	0
10.0	70.0±4.7	1.5±0.02 (1-3)	0
2,4-D			
0.5	0	0	83.3±12.4
2.5	0	0	56.6±4.7
5.0	0	0	23.3±12.4
10.0	0	0	10.0±4.7
BAP			
0.5	90.0±4.7	1.8±0.06 (1-3)	0
2.5	93.3±2.7	2.3±0.09 (1-4)	0
5.0	50.0±4.7	1.7±0.11 (1-3)	0
10.0	36.6±9.8	1.0±0.00	0
KN			
0.5	53.3±2.7	1.7±0.08 (1-3)	0
2.5	70.0±4.7	3.4±0.09 (2-5)	0
5.0	43.0±7.2	1.7±0.09(1-3)	0
10.0	36.6±7.2	1.3±0.12 (1-2)	0

± SE

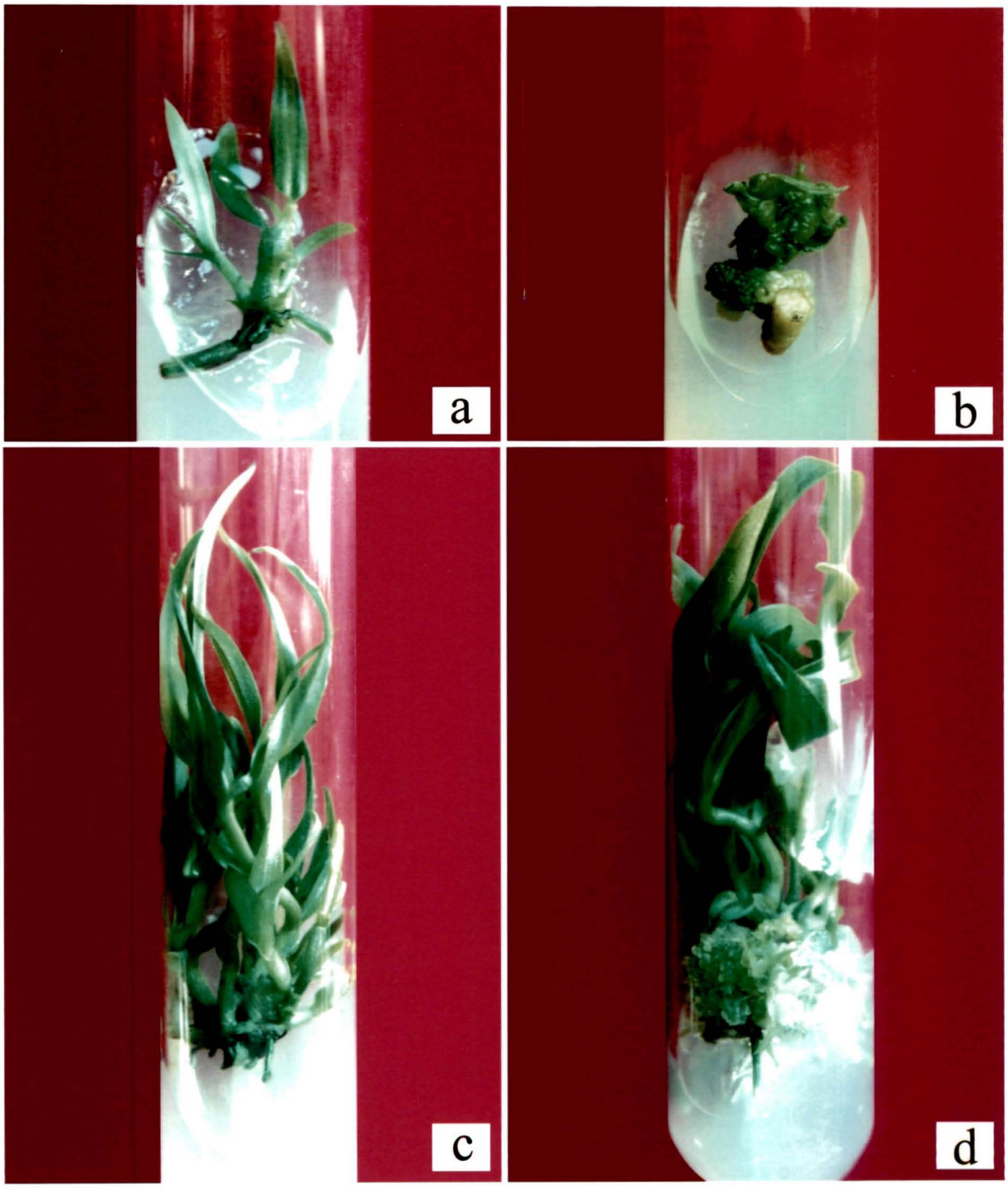
Numbers in bracket indicates the minimum and maximum shoots/explant

* Data recorded after 5 weeks

Plate 17

Development of shoots and PLBs from axillary bud of *D. lituiflorum* in

a. MS medium (multiple shoots); 10 weeks old culture
b. MS + 0.5 mg/l 2,4-D (PLBs only) in 2 weeks
c. MS + 2.5 mg/l NAA (multiple shoots only); 10 weeks old culture
d. MS + 2.5 mg/l IAA (multiple shoots + PLBs); 10 weeks old culture


a
b
c
d

Effect of KN

As compared to control, the exogenous supply of KN in the medium had an inhibitory effect on the response of the explants cultured. However, at 2.5 mg/l of KN in the medium higher response of 70.0% with an average of 3.4 number of shoots was recorded (Table 31). Both percentage and shoot number decreased with increase in KN concentration in the medium.

c) *Effect of Growth Regulators in Combination*

Effect of BAP and IAA

Different concentrations of BAP when combined with IAA at various concentrations in the medium had an influential effect on the cultured axillary buds. 100 % response of the explants was recorded at both 2.5 mg/l each of BAP and IAA, and 2.5 mg/l BAP + 5.0 mg/l IAA in the medium. However, an average shoot number of 4.2 were recorded in the former treatment (Table 32). Even at low concentration of BAP i.e. 0.5 mg/l in combination with 2.5 mg/l of IAA in the medium, comparatively higher response of 96.6% was recorded wherein an average of 2.7 (2-4) shoot buds was initiated.

Effect of BAP and NAA

As compared to control, 100% of explant response was recorded at 0.5 mg/l of BAP when combined with 2.5 mg/l of NAA followed by a percentage response 93.3% at 0.5 mg/l each of BAP and NAA in the medium (Table 33). Maximum number of 5.0 (4-8) shoots was also recorded highest at 0.5 mg/l of BAP with 2.5 mg/l of NAA in combination. However, in other concentrations of BAP and NAA tried in the medium, response in terms of percentage decreased when compared to control. However, in some

Table 32: Effect of BAP and IAA incorporated in combination in MS medium on the formation of shoots and PLBs from axillary buds of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response	*Average no. of shoots/explant
Control	86.6±5.4	2.0±0.00 (2)
BAP+IAA		
0.5+0.5	83.3±7.2	1.4±0.04 (1-2)
0.5+2.5	96.6±2.7	2.7±0.09 (2-4)
0.5+5.0	93.3±5.4	2.1±0.10 (1-3)
0.5+10.0	76.6±11.8	1.5±0.07 (1-2)
2.5+0.5	83.3±9.8	2.1±0.07 (1-3)
2.5+2.5	100±0.0	4.2±0.09 (3-7)
2.5+5.0	100±0.0	2.8±0.04 (1-4)
2.5+10.0	76.6±7.2	1.7±0.12 (1-3)
5.0+0.5	66.6±14.4	1.3±0.08 (1-2)
5.0+2.5	83.3±2.7	3.1±0.18 (2-5)
5.0+5.0	76.6±7.2	2.0±0.00 (1-4)
5.0+10.0	50.0±4.7	2.3±0.08 (1-4)
10.0+0.5	50.0±4.7	1.5±0.07 (1-2)
10.0+2.5	60.0±4.7	1.8±0.14 (1-3)
10.0+5.0	43.3±2.7	1.4±0.08 (1-2)
10.0+10.0	30.0±4.7	1.1±0.07 (1-2)

± SE

Numbers in bracket indicates the minimum and maximum shoots/explant

* Data recorded after 5 weeks

Table 33: Effect of BAP and NAA incorporated in combinations in MS medium on the formation of shoots and PLBs from axillary buds of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response	*Average no. of shoots/explant
Control	86.6±5.4	2.0±0.00 (2)
BAP+NAA		
0.5+0.5	93.3±7.2	2.7±0.08 (2-4)
0.5+2.5	100±0.0	5.0±0.19 (4-8)
0.5+5.0	76.6±9.8	2.9±0.19 (2-4)
0.5+10.0	46.6±7.2	1.2±0.09 (1-2)
2.5+0.5	80.0±4.7	2.2±0.04 (2-3)
2.5+2.5	86.0±10	2.5±0.20 (2-4)
2.5+5.0	70.0±4.7	1.8±0.13 (1-3)
2.5+10.0	43.3±7.2	1.2±0.03 (1-2)
5.0+0.5	63.3±7.2	1.7±0.04 (1-3)
5.0+2.5	73.3±7.2	2.3±0.06 (2-4)
5.0+5.0	43.3±7.2	1.2±0.03 (1-2)
5.0+10.0	36.6±7.2	1.1±0.02 (1-2)
10.0+0.5	56.6±7.2	1.5±0.06 (1-3)
10.0+2.5	43.3±7.2	1.0± .00
10.0+5.0	30.0±4.7	1.0±0.00
10.0+10.0	10.0±4.7	1.0±0.00

± SE

Numbers in bracket indicates the minimum and maximum shoots/explant

* Data recorded after 5 weeks

of the combinations, the average number of shoots was slightly higher when compared to control.

Effect of BAP and 2,4-D

As compared to control, the percentage response of the explants was lower in all the concentrations of both BAP and 2,4-D tried in the medium (Table 34). However, an increase in average number of shoots (3.4) with maximum of 4 shoots was recorded at 5.0 mg/l of BAP + 0.5 mg/l of 2,4-D in the medium. Low concentration of BAP i.e. 0.5 mg/l when combined with various concentrations of 2,4-D i.e. 0.5 and 2.5 mg/l in the medium PLBs were observed to be emerging on the explants cultured. Both shoots and PLBs were seen to have initiated on the medium containing 2.5 mg/l of BAP in combination with either 0.5 or 2.5 mg/l of 2,4-D and in 5.0 mg/l of BAP + 0.5 mg/l of 2,4-D.

Effect of KN and IAA

At high concentration of KN i.e. 5.0 mg/l when combined with 0.5 and 2.5 mg/l of IAA, the explant response observed was higher when as compared to control (Table 35). A high average number of shoots of around 4.0 (2-5) per explant was observed at 5.0 mg/l of KN + 2.5 mg/l of IAA followed by 2.5 mg/l each of KN and IAA (3.8) and 5.0 mg/l KN + 0.5 mg/l IAA (3.6). A higher average number of shoots (3.3) was also recorded in the medium containing 0.5 mg/l KN + 2.5 mg/l IAA. The percentage response observed on other combinations of KN and IAA was however lower when compared to control.

Table 34: Effect of BAP and 2,4-D incorporated in combination in MS medium on the formation of shoots and PLBs from axillary bud of *D. lituiflorum*

Conc. (mg/l)	*Average % of explants response into shoots	*Average no. of shoots/explant	*Average % of explant formed into PLBs
Control	86.6±5.4	2.0±0.00 (2)	-
BAP+2,4-D			
0.5+0.5	0	0	53.3±5.4
0.5+2.5	0	0	20.0±4.7
0.5+5.0	0	0	10.0±4.7
0.5+10.0	-	-	
2.5+0.5	63.3±7.2	2.3±0.01 (2-3)	20.0±4.7
2.5+2.5	30.0±4.7	1.5±0.02 (1-2)	16.6±7.2
2.5+5.0	10.0±0.0	1.0±0.00	0
2.5+10.0	-	-	-
5.0+0.5	70.0±8.1	3.4±0.04 (3-4)	16.6±2.72
5.0+2.5	43.3±2.7	2.4±0.06 (2-3)	0
5.0+5.0	33.3±7.2	1.0±0.0	0
5.0+10.0	-	-	-
10.0+0.5	23.3±7.2	1±0.0	0
10.0+2.5	10.0±0.0	1±0.0	0
10.0+5.0	-	-	-
10.0+10.0	-	-	-

± SE

Numbers in bracket indicates the minimum and maximum shoots/explant

* Data recorded after 5 weeks

Table 35: Effect of KN and IAA incorporated in combination in MS medium on the formation of shoots and PLBs from axillary bud of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response	*Average no. of shoots/explant
Control	86.6±5.4	2.0±0.00
KN+IAA		
0.5+0.5	56.6±11.8	1.7±0.11 (1-3)
0.5+2.5	66.6±7.2	3.3±0.14 (2-4)
0.5+5.0	43.3±7.2	1.4±0.07 (1-2)
0.5+10.0	33.3±7.2	1.2±0.11 (1-2)
2.5+0.5	80.0±4.7	2.3±0.10 (2-3)
2.5+2.5	86.6±5.4	3.8±0.06 (2-5)
2.5+5.0	56.6±7.2	2.1±0.06 (2-3)
2.5+10.0	23.3±7.2	2.0±0.00
5.0+0.5	90.0±4.7	3.6±0.12 (2-4)
5.0+2.5	93.3±2.7	4.0±0.09 (2-5)
5.0+5.0	30.0±4.7	2.5±0.00 (2-3)
5.0+10.0	16.6±7.2	1.0±0.00 (1-1)
10.0+0.5	50.0±9.4	1.4±0.09 (1-2)
10.0+2.5	20.0±4.7	2.0±0.00 (2-2)
10.0+5.0	13.3±2.7	1.0±0.0 0(1-1)
10.0+10.0	3.3±2.7	1.0±0.00 (1-1)

± SE

Numbers in bracket indicates the minimum and maximum shoots/explant

* Data recorded after 5 weeks

Effect of KN and NAA

As compared to control, average percentage response on all the concentrations of KN and NAA tried in the medium was lower (Table 36). However, the average number of shoots in some of the combinations of KN and NAA tried were higher when compared to control. A high average number (4.1) with maximum number (6) shoots were observed at 0.5 mg/l of KN in combination with 2.5 mg/l of NAA in the medium. At low concentration of KN i.e. 0.5 mg/l in combination with either 0.5 mg/l or 2.5 mg/l or 5.0 mg/l of NAA, PLBs along with the shoots were observed to have developed (Table 36).

Effect of KN and 2,4-D

As compared to control, the average percentage response both in PLB as well as shoot formation was much lower in the media containing KN and 2,4-D. Low concentration of 2,4-D (0.5 mg/l) when combined with either 0.5 or 2.5 or 5.0 mg/l of KN resulted in the formation of both shoots and PLBs (Table 37). However in other concentrations of KN and 2,4-D tried in the medium, only PLBs were developed from the explants cultured. Higher concentrations of both KN and 2,4-D in the medium were inhibitory for the cultured axillary buds.

A complete cycle of development of plantlets from culture of axillary buds of *D. lituiflorum* took place in about 12 weeks time (Plate 18). The PLBs obtained from axillary buds if left undisturbed developed into plantlets within 10 weeks time (Plate 19) whereas multiplied profusely when subcultured to fresh MS medium without growth regulators.

Table 36: Effect of KN and NAA incorporated in combination in MS medium on the formation of shoots and PLBs from axillary buds of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into shoots	*Average no. of shoots/explant	Average % of explants formed into PLBs
Control	86.6±5.4	2.0±0.00 (2)	0
KN+NAA			
0.5+0.5	70.0±9.4	2.8±0.05 (2-4)	10.0±4.7
0.5+2.5	80.0±9.4	4.1±0.2 0(3-6)	33.3±2.7
0.5+5.0	60.0±4.7	2.0±0.09 (1-3)	46.6±7.2
0.5+10.0	46.6±2.7	1.2±0.04 (1-2)	
2.5+0.5	60.0±14.1	2.5±0.04 (2-3)	0
2.5+2.5	80.0±8.1	3.1±0.00 (2-3)	0
2.5+5.0	50.0±14.1	1.5±0.04 (1-2)	0
2.5+10.0	33.0±5.4	1.0±0.00	0
5.0+0.5	60.0±4.7	2.1±0.07 (1-3)	0
5.0+2.5	70.0±9.4	2.8±0.15 (2-4)	0
5.0+5.0	40.0±4.7	2.1±0.06 (1-3)	0
5.0+10.0	30.0±9.7	1.7±0.10 (1-2)	0
10.0+0.5	40.0±9.4	2.2±0.10 (1-3)	0
10.0+2.5	30.0±9.4	1.5±0.17 (1-2)	0
10.0+5.0	10.0±4.7	1.0±0.00	0
10.0+10.0	-	-	-

± SE

Numbers in bracket indicates the minimum and maximum shoots/explant

* Data recorded after 5 weeks, - No response

Table 37: Effect of KN and 2,4-D incorporated in combination in MS medium on the formation of shoot and PLBs from axillary buds of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into shoots	*Average no. of shoots/explant	*Average % of explant formed into PLBs
Control	86.6±5.4	2.0±0.00 (2)	0
KN+2,4-D			
0.5+0.5	26.6±2.7	1.0±0.0	26.6±2.7
0.5+2.5	0	0	40.0±8.2
0.5+5.0	0	0	20.0±4.7
0.5+10.0	0	0	13.3±5.4
2.5+0.5	35.0±5.5	2.7±0.01(2-4)	35.0±5.5
2.5+2.5	0	0	66.7±7.2
2.5+5.0	0	0	56.3±2.7
2.5+10.0	0	0	30.0±4.7
5.0+0.5	23.3±7.2	1.7±0.12 (1-2)	23.3±7.2
5.0+2.5	0	0	20.0±4.7
5.0+5.0	0	0	13.3±7.2
5.0+10.0	-	-	-
10.0+0.5	20.0±8.1	1.0±0.00	0
10.0+2.5	-	-	-
10.0+5.0	-	-	-
10.0+10.0	-	-	-

± SE

Numbers in bracket indicates the minimum and maximum shoots/explant

* Data recorded after 5 weeks

- No response

Plate 18

Complete development of plantlets from axillary bud of *D. lituiflorum* in MS + 2.5 mg/l NAA

a. Initiation of PLBs in 3 weeks

b. Proliferation of PLBs in 5 weeks

c. Development of plantlets from PLBs in 7 weeks

d. Development of mature shoots with roots after 12 weeks

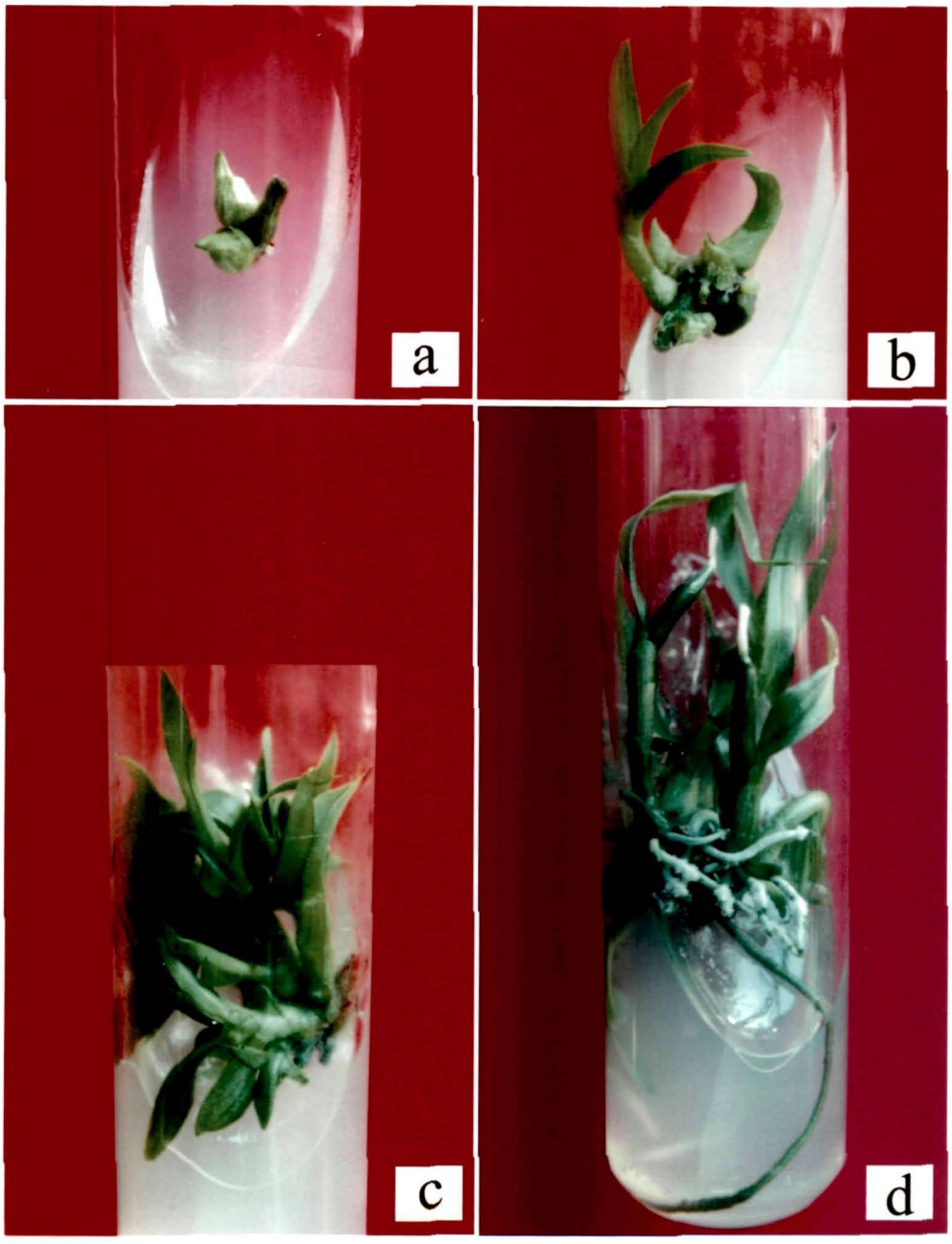
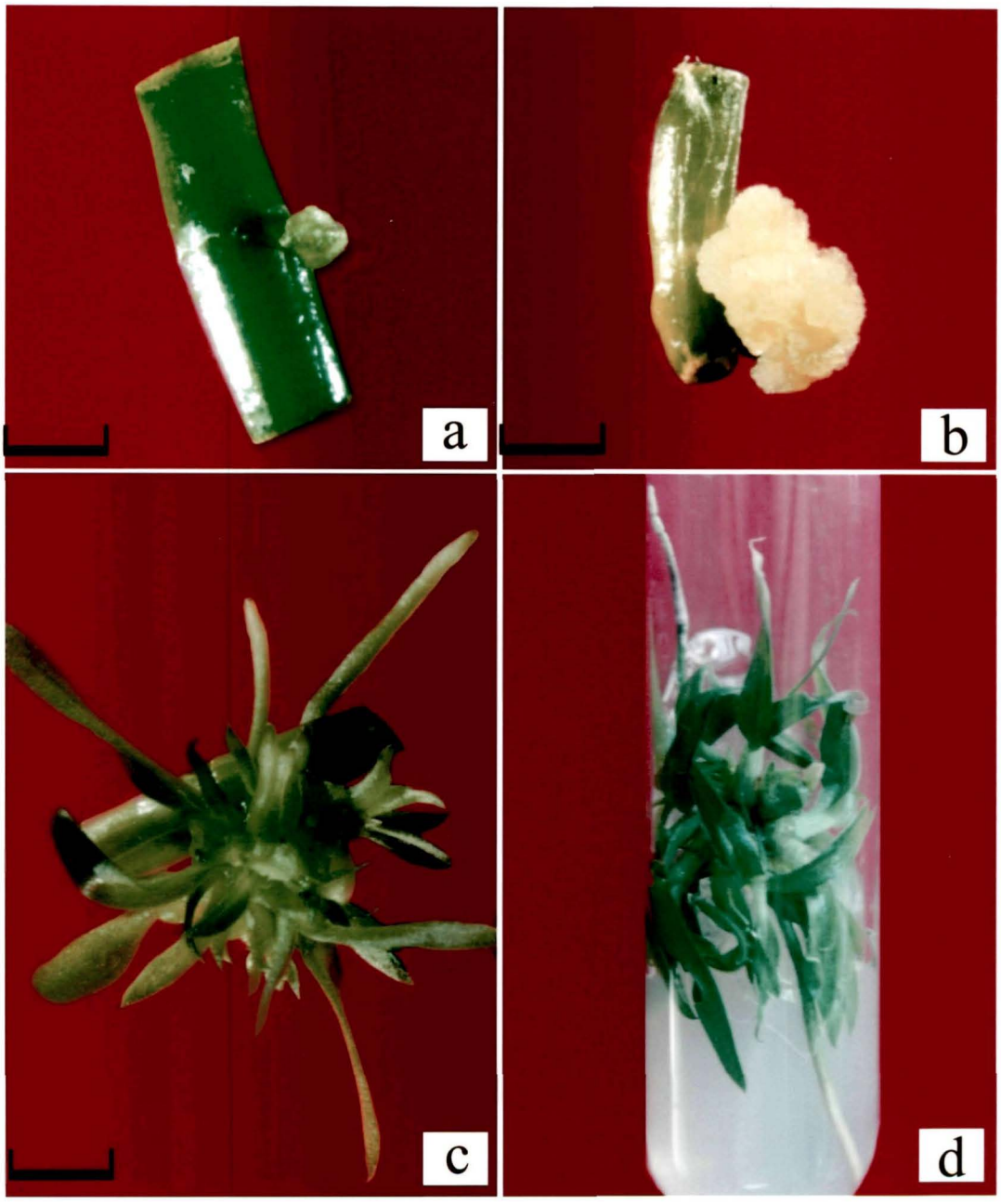

a
b
c
d

Plate 19

Complete development of plantlets of PLBs obtained from axillary buds of *D. lituiflorum* in MS + 0.5 mg/l 2,4-D (bar = 1mm)

a. Initiation of PLBs in 3 weeks
b. Proliferation of PLBs in 5 weeks
c. Development of plantlets from PLBs in 7 weeks
d. Development of plantlets with shoots and roots after 10 weeks


a
b
c
d

B. Apical meristem

a) *Effect of Different Basal Medium*

In all the media tried, green, globular PLBs were observed only on apical meristem cultured in MS medium with very low percentage response of 10.0% (Table 38). On NN and B_5 media, though the tissue remained green for sometime, no response of the explant was noticed. However, a change in colour of the explant was recorded on Mitra and KnC media tried. In KnC medium the explant cultured turned brown and died subsequently. Hence, further experiments were carried out with different growth regulators incorporated both singly and in combination in MS medium.

b) *Effect of Growth Regulators*

Effect of IAA

As compared to control, average percentage response of apical meristem into PLBs was enhanced in different concentration of IAA tried in the medium. At 2.5 mg/l of IAA in the medium, an average of 46.6% explant response was observed (Table 39). With increase in IAA concentration in the medium, there was a decrease of response in apical meristem. At 10.0 mg/l of IAA no response was observed in the apical meristem.

Effect of NAA

At low concentration (0.5 mg/l) of NAA in the medium 16.6% of response was observed in the apical meristem. However, at 2.5 mg/l of NAA in the medium, 30.0% of the apical meristems produced PLBs whereas only 10.0 % response was recorded with increase of NAA to 5.0 mg/l in the medium (Table 39). No response in the apical meristem was observed at high concentration i.e. 10 mg/l of NAA in the medium.

Table 38: Effect of different media on the formation of PLBs from apical meristem of *D. lituiflorum*

Media	*Average % of explant response	Remarks
MS	10 ± 4.7	Green, globular PLBs
NN	0	Explant remained green; no differentiation
B_5	0	Tissue remained green
Mitra	0	Tissue turned yellow; dried up
KnCS E	0	Tissue turned brown; died subsequently

* Data recorded after 5 weeks

Effect of 2,4-D

Apical meristem cultured in the medium did not respond to different concentrations of 2,4-D, except at 0.5 mg/l of 2,4-D an average of 33.3% of apical meristems responded leading to the formation of small light yellow unhealthy PLBs (Table 39).

Effect of BAP

As compared to control in all the concentrations of BAP tried in the medium, an increase in response of apical meristem was recorded. At 2.5 mg/l of BAP in the medium, 80.0% of the apical meristems produced PLBs followed by 53.3% at 5.0 mg/l concentration of BAP in the medium (Table 39). A cluster of diffused yellowish green PLBs were observed at 2.5 mg/l of BAP (Plate 20 b). A low response (26.5%) of apical meristem was observed at 10.0 mg/l of BAP tried in the medium.

Effect of KN

Apical meristem cultured on medium containing different concentrations of KN showed enhanced response when compared to control. Average percentage response of 46.6% was observed on 0.5 mg/l of KN contained in the medium. The percentage response of explant increased (66.6%) at 2.5 mg/l of KN in the medium (Table 39). In this concentration diffused green PLBs (Plate 20 c) were observed which on subculturing to fresh medium proliferated further and if left undisturbed plantlets were formed. With further increase in KN concentration (5.0 and 10.0 mg/l) in the medium, a decrease in the percentage response of apical meristem was observed.

Table 39: Effect of growth regulators incorporated singly in MS medium on PLB formation from apical meristem of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into PLBs	Remarks
Control	13.3±2.7	Light green diffused PLBs
IAA		
0.5	20.0±0.0	Few light green PLBs
2.5	46.6±2.7	Numerous healthy green PLBs
5.0	20.0±4.7	Small yellowish green PLBs
10.0	0	-
NAA		
0.5	16.6±2.7	Few healthy green PLBs
2.5	30.0±2.7	Globular green PLBs
5.0	10.0±4.7	Light brown PLBs; died subsequently
10.0	0	-
2,4-D		
0.5	33.3±5.4	Small light yellow unhealthy PLBs
2.5	0	-
5.0	0	-
10.0	0	-
BAP		
0.5	36.6±7.2	Few green PLBs
2.5	80.0±4.7	Cluster of numerous green globular PLBs
5.0	53.3±4.7	Green globular PLBs
10.0	26.5±3.4	Few light green PLBs
KN		
0.5	46.6±7.2	Globular light yellow PLBs
2.5	66.6±2.7	Diffused green mass of PLBs
5.0	33.3±7.2	Few mass of yellow PLBs
10.0	6.6±3.3	Very few brown PLBs

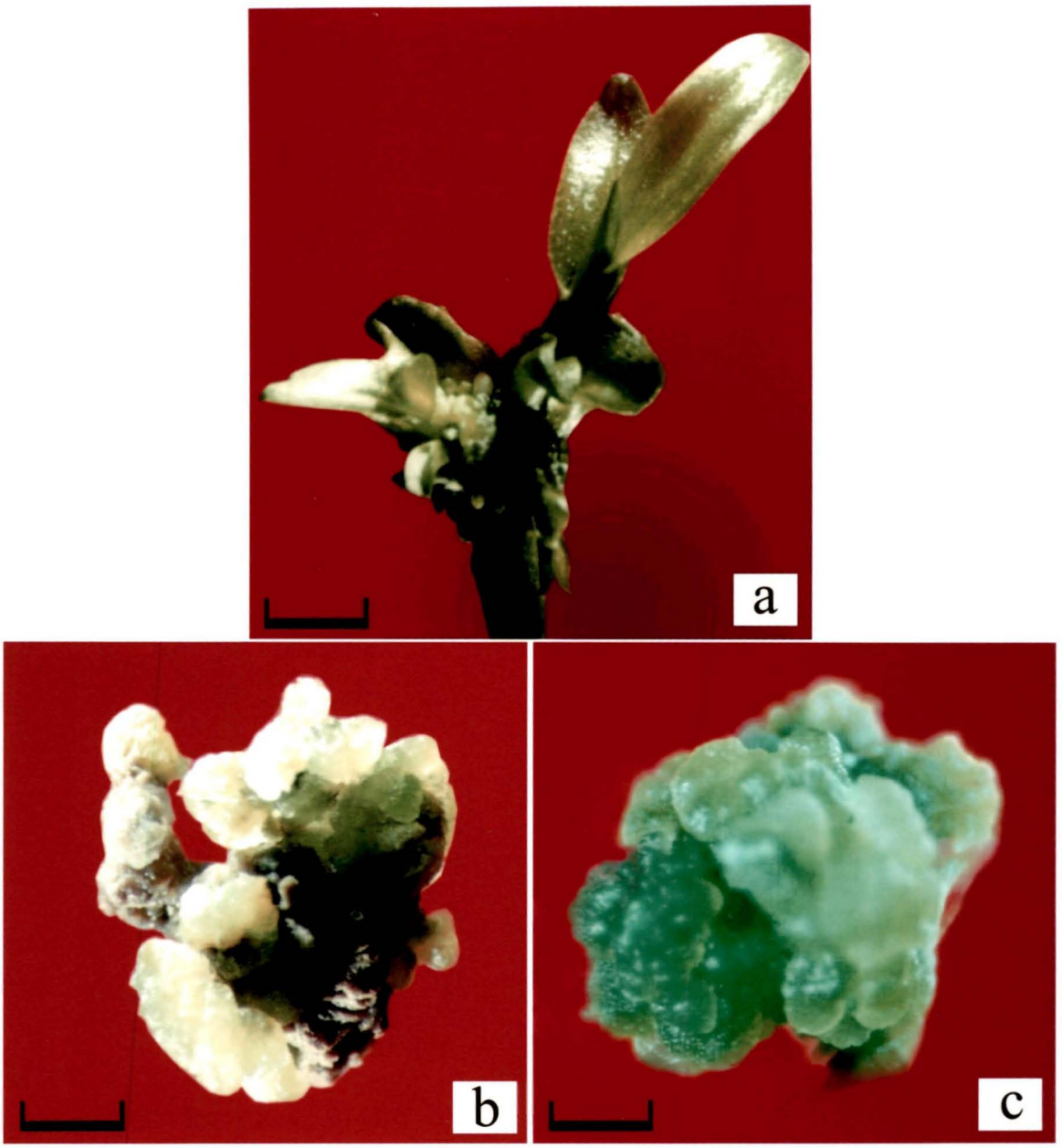
± SE

- No response

* Data recorded after 5 weeks

Plate 20

Development of PLBs and shoot buds from the apical meristem of *D. lituiflorum* after 10 weeks of culture in MS medium containing 2.5 mg/l BAP + 0.5 mg/l NAA (shoot buds) (a), 2.5 mg/l KN (PLBs) (b), and 2.5 mg/l KN (PLBs) (c) (bar = 1mm)


a
b
c

c) *Effect of Growth Regulators in Combination*

Effect of BAP and IAA

As compared to control, the combination of BAP and IAA in the medium enhanced the response of the explants. Among all the combinations tried, a high (63.3%) response of explant was observed at 2.5 mg/l each of BAP and IAA followed by an average response of 43.3% at 0.5 mg/l of BAP + 2.5 mg/l of IAA in the medium (Table 40). A reduced response in apical meristem was recorded at 10.0 mg/l of BAP + 0.5 mg/l of IAA and no response was observed at the same concentration of BAP with other concentrations of IAA in the medium.

Effect of BAP and NAA

A high average response of apical meristem was observed in the medium supplemented with both 2.5 mg/l and 5.0 mg/l of BAP in combination with 0.5 mg/l of NAA (Table 41). At 2.5 mg/l BAP + 0.5 mg/l NAA and 5.0 mg/l BAP + 0.5 mg/l NAA concentrations, shoots were observed (Plate 20 a).

Effect of BAP and 2,4-D

At low concentration of 0.5 mg/l each of BAP and 2,4-D in the medium an average of 60.0% of the explants responded in PLB formation, which was followed by a medium containing 2.5 mg/l of BAP + 0.5 mg/l with 53.3% of explants response (Table 42). Comparatively, response of the explants observed on other combinations of concentrations of BAP and 2,4-D was lower.

Table 40: Effect of BAP and IAA incorporated in combination in MS medium on the formation of PLBs from apical meristem of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into PLBs	Remarks
Control	13.3±2.7	Light green diffused PLBs
BAP+IAA		
0.5+0.5	26.6±5.4	Green, healthy PLBs
0.5+2.5	43.3±7.2	Healthy, green globular PLBs
0.5+5.0	13.6±2.7	Yellowish green PLBs
0.5+10.0	0	-
2.5+0.5	40.0±9.4	Green, globular PLBs
2.5+2.5	63.3±2.7	Healthy, green globular PLBs
2.5+5.0	36.6±8.1	Light green diffused PLBs
2.5+10.0	20.0±8.1	Yellowish green PLBs
5.0+0.5	30.0±5.7	Cluster of small green PLBs
5.0+2.5	10.0±4.7	Yellowish green PLBs
5.0+5.0	6.6±2.7	Light brown PLBs
5.0+10.0	0	-
10.0+0.5	16.6±5.4	Light yellow clusters of PLBs
10.0+2.5	0	-
10.0+5.0	0	-
10.0+10.0	0	-

±SE

- No response

* Data recorded after 5 weeks

Table 41: Effect of BAP and NAA incorporated in combination in MS medium on the formation of PLBs from apical meristem of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into PLBs	Remarks
Control	13.3±2.7	Light green diffused PLBs
BAP+NAA		
0.5+0.5	30.0±7.4	Few yellowish green PLBs
0.5+2.5	50.0±4.7	Healthy small green globular PLBs
0.5+5.0	20.0±4.7	Few unhealthy light brown PLBs
0.5+10.0	0	-
2.5+0.5	60.0±4.7	Both PLBs and shoots (2-3)
2.5+2.5	40.0±9.4	Small, green PLBs
2.5+5.0	23.3±7.2	Small globular unhealthy PLBs
2.5+10.0	0	-
5.0+0.5	43.3±2.7	Both PLBs and shoots (2)
5.0+2.5	23.3±5.4	Small globular unhealthy PLBs
5.0+5.0	13.3±5.4	Few light brown PLBs
5.0+10.0	0	
10.0+0.5	26.6±5.4	Small globular PLBs
10.0+2.5	10.0±4.7	Small brown PLBs; died subsequently
10.0+5.0	0	-
10.0+10.0	0	-

± SE, - No response

* Data recorded after 5 weeks

Table 42: Effect of BAP and 2,4-D incorporated in combination in MS medium on the formation of PLBs from apical meristem of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into PLBs	Remarks
Control	13.3 ± 2.7	Light green diffused PLBs
BAP+2,4-D		
0.5+0.5	60.0 ± 9.4	Numerous green healthy PLBs
0.5+2.5	40.0 ± 4.7	Few healthy PLBs
0.5+5.0	0	-
0.5+10.0	0	-
2.5+0.5	53.3 ± 7.2	Cluster of green healthy PLBs
2.5+2.5	26.6 ± 9.8	Few yellowish green PLBs
2.5+5.0	0	-
2.5+10.0	0	-
5.0+0.5	23.3 ± 7.2	Yellowish brown PLBs; died subsequently
5.0+2.5	0	-
5.0+5.0	0	-
5.0+10.0	0	-
10.0+0.5	0	-
10.0+2.5	0	-
10.0+5.0	0	-
10.0+10.0	0	-

±SE

- No response

* Data recorded after 5 weeks

Effect of KN and IAA

The response of apical meristem to different concentrations of KN and IAA in combination was higher as compared to control. A high response of 70.0% of apical meristems was recorded at 2.5 mg/l each of KN and IAA followed by 60.0% each at 5.0 mg/l KN + 2.5 mg/l of IAA and 2.5 mg/l KN + 0.5 mg/l IAA (Table 43). Average percent response on other concentrations tried was also higher than that of control except in the medium containing high concentrations of KN (5.0 mg/l) and IAA (5.0, 10.0 mg/l).

Effect of KN and NAA

As compared to control, a high average percentage response was obtained from apical meristem in most of the combinations of KN and NAA tried in the medium (Table 44). Seventy percent response was obtained in 0.5 mg/l of KN + 2.5 mg/l of NAA in the medium followed by 60.0% response in 2.5 mg/l of KN + 0.5 mg/l of NAA in the medium (Table 44). Both PLBs along with shoots were seen to be emerging on the explants cultured in the medium containing 5.0 mg/l KN and 0.5 mg/l NAA and 10.0 mg/l KN and 0.5 mg/l NAA in combination.

Effect of KN and 2,4-D

In a medium containing 2.5 mg/l each of KN and 2,4-D, 70.0% response of the apical meristems in the formation of PLBs was observed followed by 60.0% response in 0.5 mg/l of KN + 2.5 mg/l of 2,4-D in the medium (Table 45). In other treatments of KN and 2,4-D in combination in the medium the response recorded was reduced.

A complete cycle of development of complete plantlets from apical meristems took place in about 11 weeks (Plate 21).

Table 43: Effect of KN and IAA incorporated in combination in MS medium on the formation of PLBs from apical meristem of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into PLBs	Remarks
Control	13.3±2.7	Light green diffused PLBs
KN+IAA		
0.5+0.5	40.0±9.4	Green healthy PLBs
0.5+2.5	33.3±7.2	Few cluster of green PLBs
0.5+5.0	26.6±2.7	Few cluster of yellowish green PLBs
0.5+10.0	23.3±7.2	Cluster of yellow PLBs
2.5+0.5	60.0±4.7	PLBs developed are green and healthy
2.5+2.5	70.0±8.1	Numerous cluster of green PLBs
2.5+5.0	53.3±2.7	Green healthy PLBs
2.5+10.0	23.3±7.2	Few light yellow PLBs
5.0+0.5	53.3±2.7	Healthy green PLBs
5 .0+2.5	60.0±7.2	Cluster of green healthy PLBs
5 .0+5.0	33.3±7.2	Light yellow PLBs
5 .0+10.0	6.6±2.7	Brown PLBs ; died subsequently
10.0+0.5	36.6±7.2	Green PLBs
10.0+2.5	26.6±5.4	Unhealthy yellow PLBs observed
10.0+5.0	3.3±2.7	Brown PLBs; died in course of time
10.0+10.0	0	-

± SE, - No response

* Data recorded after 5 weeks

Table 44: Effect of KN and NAA incorporated in combination in MS medium on the formation of shoots and PLBs from apical meristem of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into PLBs	Remarks
Control	13.3±2.7	Light green diffused PLBs
KN+NAA		
0.5+0.5	23.3±7.2	Light green PLBs
0.5+2.5	70.0±4.7	Numerous cluster of healthy PLBs
0.5+5.0	53.3±7.2	Green healthy PLBs
0.5+10.0	33.3±7.2	Yellowish PLBs
2.5+0.5	60.0±7.4	Healthy PLBs
2.5+2.5	50.0±4.7	Healthy green PLBs
2.5+5.0	23.3±5.4	Light yellow PLBs
2.5+10.0	20.0±0.0	Light yellow PLBs
5.0+0.5	43.3±7.2	Both green PLBs and shoots (2-3)
5.0+2.5	36.6±2.7	Few yellow PLBs
5.0+5.0	10.0±4.7	Few brown PLBs
5.0+10.0	0	-
10.0+0.5	26.6±5.4	Both green PLBs and shoots (2-3)
10.0+2.5	10.0±4.7	Unhealthy brownish yellow PLBs
10.0+5.0	0	-
10.0+10.0	0	-

± SE, - No response

* Data recorded after 5 weeks

Table 45: Effect of KN and 2,4-D incorporated in combination in MS media on formation of PLBs from apical meristem of *D. lituiflorum*

Conc. (mg/l)	*Average % of explant response into PLBs	Remarks
Control	13.3±2.7	Light green diffused PLBs
KN+2,4-D		
0.5+0.5	36.6±2.7	Few green PLBs
0.5+2.5	60.0±4.7	Healthy green PLBs
0.5+5.0	23.3±7.2	Yellow PLBs
0.5+10.0	13.3±7.2	Few brown PLBs
2.5+0.5	53.3±2.7	Green healthy PLBs
2.5+2.5	70.0±4.7	Cluster of well developed PLBs
2.5+5.0	20.0±4.7	Yellow PLBs
2.5+10.0	3.3±2.7	Dark brown PLBs; died subsequently
5.0+0.5	36.6±7.2	Few cluster of green PLBs o
5.0+2.5	50.0±4.7	Numerous cluster of green PLBs
5.0+5.0	26.6±7.2	Few cluster of yellowish green PLBs
5.0+10.0	0	
10.0+0.5	16.6±2.7	Brownish yellow PLBs
10.0+2.5	0	-
10.0+5.0	0	-
10.0+10.0	0	-

± SE

- No response

* Data recorded after 5 weeks

Plate 21

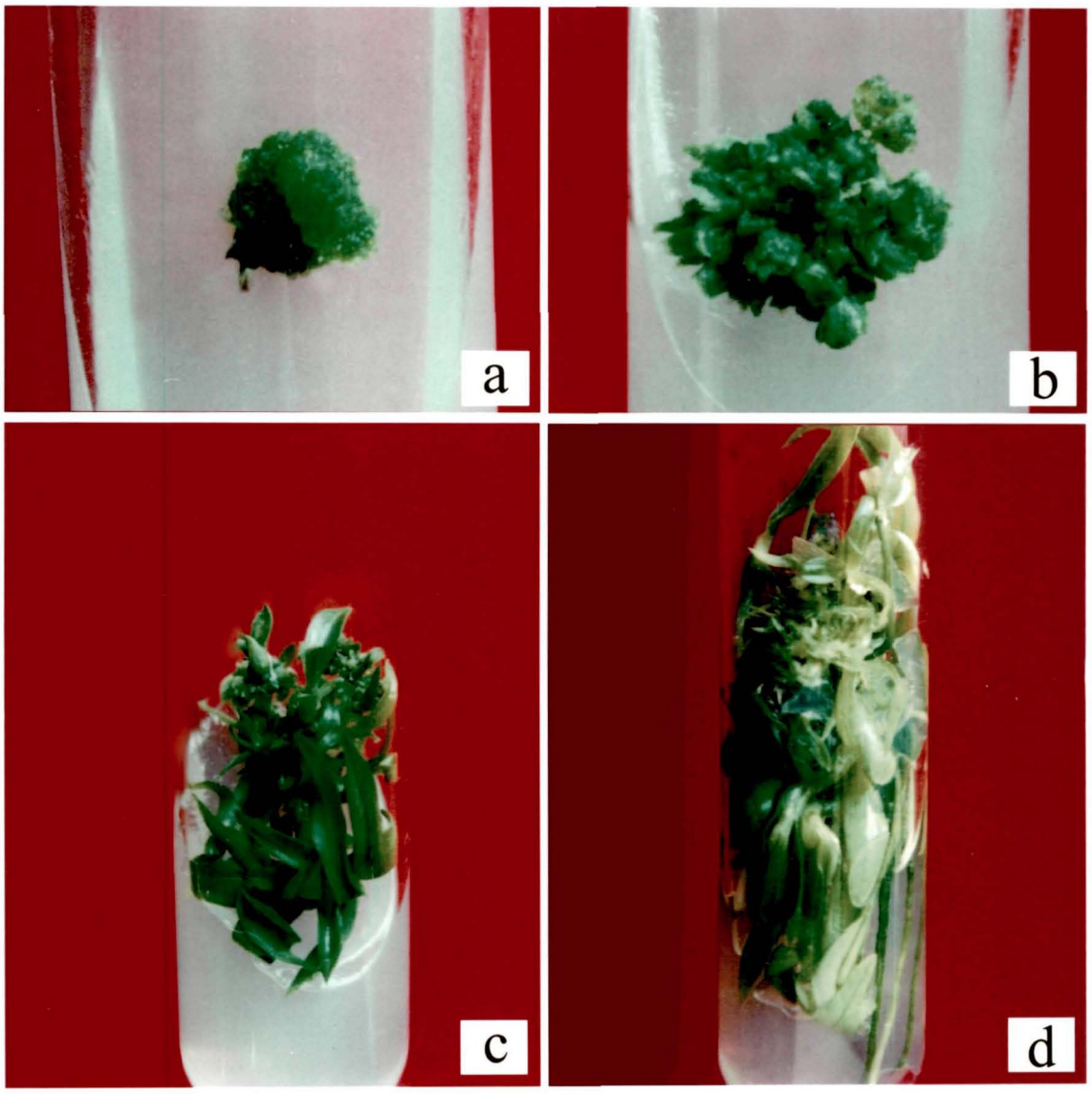
Complete development of plantlets from apical meristem of *D. lituiflorum* in MS + 2.5 mg/l KN

a. Initiation of PLBs in 4 weeks

b. Proliferation of PLBs in 5 weeks

c. Development of shoots from PLBs in 8 weeks

d. Development of plantlets with shoots and roots in 11 weeks


a
b
c
d

DISCUSSION

The mass propagation of economically viable and/or endangered orchid genotypes using tissue culture techniques which is a desirable proposition is fortunately gaining momentum. Explants employed for such purpose respond selectively depending upon their source, physiological age and nutrient environment (Vij *et al.*, 1983, 1984). Nutritional requirements for the optimal growth of the plants *in vitro* vary from species to species. Arditti (1977) reported the variation in the media used for tissue culture of orchids and suggested that the composition of the media changes with the same or different genera. Some media contain many components while others are simpler. Considerable variations are reported to exist between media used for the same or different genera of orchids (Morel, 1970, 1971; Mitra, 1971; Fonnesbech, 1972a,b; Intuwang and Sagawa, 1973; Kako, 1973; Mosich *et al.*, 1974; Arditti, 1977).

C. DEVONIANUM

Out the different explants sources tried, the apical meristem and leaf bases of *C. devonianum* were found suitable for micropropagation. The apical meristem and leaf bases of *C. devonianum* cultured on different media i.e., MS, NN, B_5, Mitra and KnC did not respond in the form of PLBs or shoot buds. However, both explants remained green for longer time in MS medium compared to other media tried. Similar results were observed by Vij and Kaur (1998) where explants did not show any signs of regeneration in the basal medium. In the present study, addition of growth adjuncts in the medium was obligatory for evoking responses in apical meristem and leaf bases of *C. devonianum.*

Micropropagation of *Cymbidium* through apical meristem, shoot meristem and leaf explants have been successfully accompanied in some other species of orchids (Morel, 1960; Wimber, 1965; Fonnesbech, 1972; Choi *et al.*, 1996). The other cultured explants of *C. devonianum* failed to respond. Vij (1993) had reported tissue culture of *Cymbidium* through root explants using purely synthetic media. The successful propagation through these explants has also been reported in other orchid species (Sagawa and Kunisaki, 1982; Philip and Nainar, 1986, 1988; Sanchez, 1988; Philip and Padikkala, 1989; Goh, 1990; Arditti and Ernst, 1993). The use of growth regulators in the medium is beneficial and it results in different morphogenetic responses. It has been reported that the tissues from different organs or/parts differ in their growth-substance requirements. In the present study, the apical meristem of *C. devonianum* cultured on MS medium containing different concentrations of IAA in the medium resulted in a low percentage response with 1-2 small green PLBs emerging from it. Similar results where percentage of the responding explants was significantly impaired in IAA treated cultures of foliar explants were observed in *Vanda coerulea* (Vij and Aggarwal, 2003). In the present study low concentration of NAA in the medium favoured high percentage response of apical meristem of *C. devonianum* in the form of PLBs. However, with increase in NAA concentration in the medium, a decrease in response was observed. The promotory effect of NAA on early organogenesis has been reported by Vij and Pathak (1990). Also the plantlet initiation in *Doritaenopsis* orchid was enhanced at 0.5 mg/l NAA whereas at the higher concentrations it was found to be inhibited (Chowdhery *et al.*, 2003). The apical meristem failed to respond in all the treatments of 2,4-D tried in the medium. Kalita

(1999) reported similar results where shoot tip of *Vanda coerulea* was completely inhibited in the medium containing 2,4-D. The inhibitory effect of auxins, IAA/2,4-D, may be attributed to the fact that meristems are known to be rich in endogenous auxin, hence exogenous addition of either IAA, or 2,4-D proved detrimental for inducing PLBs/shoot buds from the meristem.

Addition of cytokinin (BAP/KN) in the medium evoked a positive response of the apical meristem. At 2.5 mg/l BAP in the medium, 3-5 clusters of PLBs were observed to have emerged. BAP in the medium has been reported to be promotory for various explants of some orchids (Goh and Wong, 1990; Murthy and Payati, 2001). KN at 0.5 mg/l in the medium resulted in high response of cultured apical meristem wherein 7-10 clusters of hairy, yellowish green and healthy PLBs were observed. Mauro *et al.* (1994) observed similar results in *Cattleya aurantica* where BAP alone was necessary for shoot tip multiplication. Mathews and Rao (1985) also reported that the cytokinins alone favoured growth of *Vanda hybrid* shoot meristem. The stimulatory effect of KN where maximum explants (foliar peels) responded and generated a large number of PLBs each of which subsequently developed into a complete plantlet was recorded by Vij and Kaur (1992). A similar synergistic effect of this hormone on PLB proliferation is already on record (Vij and Pathak, 1990). As it is a known fact that meristem is poor in endogenous cytokinins and hence it may be that incorporation of cytokinin in the medium stimulated growth.

A combination of auxin (IAA/NAA) with BAP resulted in differential response. Apical meristem responded poorly to the BAP + IAA treatment in the medium. Only

43.0% conversion of apical meristem into PLBs was observed at 2.5 mg/l each of BAP and IAA in the medium. However, higher response of conversion (60.0 %) of apical meristem with 4-8 numbers of PLBs was observed at 2.5 mg/l BAP in combination with 0.5 mg/l NAA. Around 3-6 PLBs emerged from the cultured meristem at 0.5 mg/l each of BAP and NAA in the medium. Fonnesbech (1972a) reported that explants cultured in medium containing low concentration of auxin in combination with BAP formed PLBs and small shoots. Kumaria (1991) reported similar findings in *Dendrobium fimbriatum* var. *oculatum* where addition of NAA brought about the formation of PLBs both when added separately and in combination with BAP. The morphogenetic responses of the explants varied with the concentrations of the growth regulators used. The incorporation of NAA and BAP in addition to 10% coconut milk in the medium increased the rate of PLB formation in *Phalaenopsis*, (Yoneda *et al.*, 1983). In the present study, KN in combination with IAA or NAA resulted in the formation of PLBs from apical meristem. Similar effect of NAA and KN on PLB proliferation is already on record (Vij and Pathak, 1990; Vij and Kaur, 1992). However, in contrast to the present study, Vij and Aggarwal (2003) reported that KN in addition to IAA in the medium proved ineffective for PLB development on foliar explants of *Vanda coerulea*. Among the different parts of the leaf tried in the culture, response was observed only with the cultured leaf bases. The other parts of leaf dried up. Similarly, the greater proliferative potential of the leaf base has been reported in *Ascocenda*, *Vanda* (Fu, 1978, 1979), *Coelogyne*, *Dendrobium*, *Oncidium*, *Phalaenopsis* (Abdul Karim and Hairani, 1990), *Renanthera* (Seeni and Latha, 1992) and *Acampe* (Nayak *et al.*, 1997). The importance of leaf explants for

micropropagating orchids is being increasingly realized and offer exciting opportunities to raise a large number of true to type plants. Their regenerative potential has already been positively tested in several orchids representing diverse taxonomic affinities, habits and habitats (Laishram and Devi, 1997; Vij and Pathak, 1990, 1997; Vij *et al.*, 1994, 2002).

The role of growth adjuncts in activating meristematic loci in leaf explants and /or promoting proliferations thereof is well documented in orchids (Abdul Karim and Hairani, 1990; Vij and Pathak, 1990; Arditti and Ernst, 1993; Vij *et al.*, 1994). In the present study, leaf bases of *C. devonianum* responded to auxins IAA and NAA supplemented in the medium. However of the two above-mentioned auxins, leaf bases responded to all concentrations of IAA in the medium tried, the highest explants response being recorded at 2.5 mg/l of IAA where 3-5 green healthy PLBs developed. With increase in the concentration of IAA in the medium, a decrease in explant response and number of PLBs was recorded. This is in agreement with the report that IAA impaired the response frequency in the explants but its detrimental affect was more than compensated through its multiple PLB inducing affect (Vij and Aggarwal, 2003). Almost similar results were also reported with leaf segments of *Neofinetia falcate* and *Rhynchostylis retusa* (Vij and Pathak, 1990). At low concentration of NAA in the medium, low percentage response of leaf bases was observed. In higher concentration of NAA tried in the medium, the explant failed to respond. The inhibitory affect of NAA in foliar peels of *Rhynchostylis retusa* has been observed (Vij and Kaur, 1992). Lin (1986) also reported presence of NAA in the medium to be inhibitory to PLB formation in internodal segment

cultures of the flower stalk. Leaf base cultured in the medium containing 2,4-D failed to induce PLB formation . At lower concentration of 2,4-D, the explants remained green for 6-7 days while in higher concentrations the explants turned brown within 4-5 days of culture. This is in contrast to the findings that 2,4-D favoured both callus and PLB formation in foliar parts of *Rhynchostylis retusa* (Vij *et al.*, 1984; Vij and Kaur, 1992). It is reasonable to assume that under similar hormonal stimulus the response may vary with different genus or species of the explant.

Among the cytokinins (BAP/KN) tried singly in the medium, highest response of leaf base with 3-6 numbers of green PLBs emerging was observed at low concentration of KN in the medium. Decreased response of the explants was recorded with increase in KN concentration in the medium. Lower response of the explants cultured in the medium containing BAP was recorded. BAP at higher concentration in the medium was inhibitory for the cultured leaf bases of *C. devonianum*. PLB formation with maximum response of the explants has been recorded when cytokinins (BAP/KN) were present singly in the medium (Pyati and Murthy, 1999). The perusal of literature also reveals that KN in the medium yielded the best results where 75% explants responded and generated a large number of PLBs each of which subsequently developed into a complete plantlet (Vij and Kaur, 1992).

Of the various combinations of growth regulators studied in the medium, a high percentage response of leaf base was observed in 0.5 mg/l BAP + 2.5 mg/l IAA wherein 6-15 clusters of well developed globular green PLBs were observed. The other combinations where leaf bases responded was 0.5 mg/l BAP + 5.0 mg/l IAA and 0.5 mg/l

each of KN and IAA in the medium. Similar results were observed in the earlier findings where the combined effect of cytokinin and auxin proved useful in leaf cultures of *Vanda*, *Satyrium*, *Luisia* (Vij and Pathak, 1990), *Vanda coerulea* (Seeni, 1988), and hybrids of Vandas (Mathews and Rao, 1985). A combined treatment with IAA and BAP also proved highly productive where a large number of regenerants were obtained from leaf segments (Kaur and Vij, 2000). Earlier Yam and Weatherhead (1991) also reported a synergistic action of auxins and cytokinins in their orchid cultures.

D. LITUIFLORUM

In case of *D. lituiflorum,* out of all different explant sources tried, axillary bud and apical meristem were found suitable explants for micropropagation. The response of the explants varied depending on the media used. Out of the different media tried, the cultured axillary bud responded best on MS medium with 2-3 shoots emerging per explant. In KnC medium, the explants turned brown and died. Similar results were reported in KnC medium wherein the axillary bud turned brown in *Dendrobium* hybrids (Devi *et al.*, 1998). However, no PLBs were formed from the cultured explants in all the basal media tried. Devi and Laishram (1998) reported the development of only multiple shoots on basal medium at the base of the growing shoot without the intervention of callus and PLBs in *Dendrobium* hybrids. Ichihashi (1992), however, obtained PLBs/callus in *Phalaenopsis* in the medium bereft of growth hormones.

Both shoot buds and PLBs were seen emerging from the axillary bud of *D. lituiflorum* cultured at 2.5 and 5.0 mg/l of IAA present in the medium. The response of the axillary bud from *in vitro* grown plants was better in the basal medium supplemented

with different growth regulators due to probably habituated nature and juvenity of its tissues. The juvenity of tissue as an important factor controlling cell proliferations has already been indicated in several orchids (Vij and Kaur, 1989; Vij and Pathak, 1989; Arditti and Ernst, 1993; Vij *et al.*, 1997). A better morphogenetic potential of juvenile cells was earlier explained on the basis of their physiologically and biochemically more active state due to the presence of less rigid walls (Misra and Bhatnagar, 1995). Incidentally the response of axillary buds in medium containing IAA followed both shoot bud-mediated and PLB regeneration. However, response of axillary buds in NAA and 2,4-D containing medium followed a shoot bud and PLB mediated regeneration respectively. Such a differential response (shoot bud/PLB) mediated has been attributed to their genetic and/or source related physiological intricacies (Vij *et al.*, 2000). Presence of NAA (2.5 mg/l) in the medium induced 100% explant response and maximum number of shoots was also recorded in the same concentration of NAA. Vij and Kaur (1998) also reported similar results where NAA enriched medium favoured multiple shoot bud formation. The regeneration pathway of axillary bud cultured on MS medium supplemented with different concentration of 2,4-D was found to be through PLB formation. 83.3% of explant response in terms of PLBs was recorded at 0.5 mg/l of 2,4-D in the medium. However, with further increase in 2,4-D concentration a gradual decrease in the response of *D. lituiflorum* was recorded. Sharma and Vasundhara (1990) reported PLB induction in modified Vacin and Went medium supplemented with coconut water in *Dendrobium Joannie Ostenhault*. Kim *et al.* (1970) also reported the formation of PLBs from bud explants as well as from leaf axils of *Dendrobium* species. Various reports

suggest that 2,4-D and activated charcoal enriched medium lead to the response from foliar explants but plantlets followed a callus-mediated development (Vij and Kaur, 1992). The earlier study on culture of *Rhynchostylis retusa* foliar segments also confirmed that 2,4-D favoured direct regeneration of PLBs (Vij *et al.*, 1984). It has also been reported that both callusing and PLB formation in cultured leaf of *D. formosum* and stem disc cultures of *D. moschatum* is enhanced by 2,4-D in the medium (Kanjilal *et al.*, 1999; Nasiruddin *et al.*, 2003). Therefore, it is reasonable to assume the regeneration pathway, under similar hormonal stimulus, may vary with the nature and species of the explant. In this connection it is worthwhile to mention that the development of non-organogenetic callus in *Nicotiana tabaccum* epidermal peels was promoted by addition of 2,4-D in medium (Van *et al.*, 1974).

Cytokinins have been used to multiply cultures of several orchids (Vij and Pathak, 1990; Kaur, 1996). The cytokinins were successfully used to enhance the regeneration frequency and to multiply the regenerants through direct or callus mediated PLB multiplication (Vij and Aggarwal, 2003). However in the present study it was found that the effect of cytokinin (BAP/KN) on shoot bud production and PLB formation from axillary bud was promotory in case of *D. lituiflorum*. Vij *et al.* (2000) reported that meristematic activity in the *in vitro* obtained explants was enhanced by the use of KN/BAP in the medium. Kosir *et al.* (2004) reported direct shoot regeneration from nodes of *Phalaenopsis* when cultured in medium containing BAP.

Different concentrations of BAP in combination with auxins (IAA, NAA and 2,4-D) brought varying responses from the cultured explants. BAP in addition to either IAA

or NAA resulted in the formation of only shoot buds. Medium containing both BAP and IAA in combination showed 100% explant response at 2.5 mg/l each of BAP and IAA and at 2.5 mg/l BAP + 5.0 mg/l of IAA. However, highest number of shoots/explant was recorded in the former concentrations of growth regulators. A perusal of literature has shown that combination of cytokinin and auxin invariably induced multiple shoot formation to a variable extent. This confirmed with the report of Decruse *et al.* (2003) where combination of BAP and IAA induced maximum number of axillary shoots from the nodes in *Vanda spathulata.* Out of all combinations of BAP and NAA tried in the medium, 100% explant response with 5 shoots/explant was recorded in low concentration of BAP (0.5 mg/l) in combination with 2.5 mg/l of NAA, With further increase in both BAP and NAA in combination in the medium, a decrease in explants response and shoot number/explant was recorded. Tokuhara and Mii (1993) have reported that the hormones, BAP and NAA in combination were of key importance for micropropagation of *Phalaenopsis* on a commercial scale. Further, the stimulatory effect of BAP and NAA in orchid cultures have been reported (Kosir *et al.*, 2004). The addition of growth regulators in the medium evokes a differential response from the cultured explants. Ernst (1994) reported that shoot and root development were reduced, while proliferation increased with increasing concentration of cytokinin (thidiazuron), while addition of NAA reduced induction and regeneration of plantlets (Arditti and Ernst, 1993), and an appropriate combination of NAA and BAP stimulated shoot formation (Tokuhara and Mii, 1993; Tisserat and Jones, 1999; Roy and Banerjee, 2003). Combination of 2,4-D either with BAP/KN at certain concentrations induced both PLBs and shoot buds from axillary buds

of *D. lituiflorum*. Combination of BAP (0.5 mg/l) with different concentrations of 2,4-D (0.5, 2.5, 5.0 and 10.0 mg/l only PLBs were induced from the explants. However, with further increase i.e. 2.5 mg/l BAP in combination with different concentrations of 2,4-D (0.5 and 2.5 mg/l), and 5.0 mg/l BAP with 0.5 mg/l BAP, PLBs were induced along with the shoot buds from the explants. That is, it can be said that the explant response in terms of PLB induction was highest in concentrations containing BAP to 2,4-D ratio ≤ 1. However, PLBs from the explant cultured in BAP to 2,4-D ratio less than 1 were not much effective. The response of the explant was both in the form of PLB and shoots in the medium containing cytokinin to auxin ≤ 1. However, study of combination of KN and 2,4-D showed varying results in the formation of shoots and PLBs from axillary bud of *D. lituiflorum*. Here with all concentrations of KN (0.5, 2.5, 5.0 and 10.0 mg/l) in combination with 0.5 mg/l of 2,4-D both shoots and PLBs were formed, but BAP in combination with increased 2,4-D concentration (2.5, 5.0 and 10.0 mg/l) the explant developed into PLBs only. The highest explant response in terms of PLBs was observed at 2.5 mg/l each of BAP and 2,4-D (BAP to 2,4-D ratio of 1). Induction of multiple PLBs around the apical and axillary meristem of *Cattleya* shoots with KN/BAP in combination with 2,4-D has been reported (Kusumoto, 1979). Some authors have reported the direct induction of shoot buds without the intervention of callus and PLBs (Devi and Laishram, 1998). Many others reported the formation of PLBs from different explants used (Kim *et al.*, 1970; Sharon and Vasundhara, 1990). Since many orchid species require auxins/or cytokinins for shoot and PLB formation (Arditti and Ernst, 1993), the combination, concentrations and the ratio between them are usually critically important. The ratio of

auxin to cytokinin or vice versa for PLB formation varies from species to species and from explant to explant (Teng *et al.*, 1997). In the present study in axillary buds of *D. lituiflorum*, both high as well as low ratio of cytokinin to auxin proved beneficial for induction of PLB from axillary bud. However BAP to 2,4-D ratio of 1 was best in explant response from axillary buds of *D. lituiflorum.* This is in agreement with the report that high ratio of NAA/BAP 12.2 (i.e. low ratio of BAP/NAA 0.5) in *Spathoglottis plicata* was best in induction of PLBs from nodal explants (Teng *et al.*, 1997). Whereas low ratio of 0.12 of NAA to BAP was employed for mature *Phalaenopsis amabilis* blume leaf culture (Tanaka and Sakanishi, 1985). A ratio 0.42 of NAA/BA was reported for axillary bud explants of *Dendrobium antennatum* Lindl. (Kukulczanka and Wojciechowska, 1983) and 1.23 (NAA/BA) was effective in several hybrid species of Aranda (Khaw *et al.*, 1978).

Incorporation of KN in combination with IAA or NAA induced only shoot formation but at 0.5 mg/l KN in combination with different concentration of NAA (0.5, 2.5 and 5.0 mg/l), low induction of PLBs from axillary buds of *D. lituiflorum* was observed. Maximum explant response (93.3%) in terms of shoots/explants was recorded at 5.0 mg/l KN in combination with 2.5 mg/l IAA. Similar synergistic action of these hormones was reported in *D. chrysanthum* (Vij and Pathak, 1989) and in *Eulophia hormusjii* (Vij *et al.*, 1989).

Success in propagating orchids with shoot tip culture has been reported in *Aranda* (Goh *et al.*, 1975), *Aranthera* (Irawati *et al.*, 1977), *Dendrobium* (Kim *et al.*, 1970; Morel, 1970; Sharon and Vasundhara, 1990), *Vanda* (Goh, 1970), *Cymbidium* (Wimber

1963, Fonnesbech, 1972). Among all the basal media tried (MS, NN, B_5, Mitra and KnC) for induction of PLBs from apical meristem of *D. lituiflorum*, 10% explant response in terms of PLBs was recorded in apical meristem in MS medium only. In this medium green globular PLBs could be seen developing from apical meristem. Presence of rich nutrients (both macro and micro) in MS medium especially presence of high Nitrogen content might have attributed for its response. Nitrogen is supplied in the form of ammonium along with nitrate in MS medium. It is reported that reduced form of nitrogen is beneficial for certain plant tissue culture (Skirvin, 1981). However in orchids, no such generalizations can be made as different orchids have been found to have nitrogen requirements in different forms (Arditti and Ernst, 1984).

The apical meristem of *D. lituiflorum* cultured in the medium containing auxins (IAA, NAA, 2,4-D) and cytokinins (BAP, KN) responded differently when tested individually. Among the auxins, IAA at 2.5 mg/l in the medium was best for explant response in the form of PLBs. A better response of apical meristem was also recorded at 2.5 mg/l NAA and 0.5 mg/l of 2,4-D incorporated singly in the medium. However, as the concentrations of auxin in the medium increased, a decreased in explant response was recorded. Inhibitory effect of auxins in PLB formation from different explants has been reported (Latha and Seeni, 1991; Vij *et al.*, 2000). PLB initiation was enhanced at 2.5 mg/l BAP followed by 2.5 mg/l of KN when added singly in the medium. However, with further increase in BAP or KN concentration in the medium a decrease in explant response was recorded. Parallel to the present report, BAP alone was also found promotory for PLB proliferation by Goh and Wong (1990) in their studies with *Aranda*

Deberoh. Moreover, the need of BAP concentration to be equivalent or higher to that of auxin for development of PLBs from *Phalaenopsis* inflorescence tips has been reported (Yoneda, 1986). In contrast to the present study, increased frequency and number of PLBs in flower stalk cultures of orchids was obtained in high concentrations (5.0-10 mg/l) of BAP (Tanaka and Sakanishi, 1978; Ernst, 1985; Latha and Seeni, 1991). Similar to the present report of *D. lituiflorum*, presence of KN at 2.0 mg/l in the medium was reported to induce highest explant response in the forms of PLBs from leaf epidermal peels (Vij and Kaur, 1992). Addition of BAP in combination with IAA/NAA or 2,4-D in the medium, resulted in lower explant response in terms of PLBs when compared to BAP alone. In contrast to the present report, some studies suggest that NAA and BAP when used independently had very little or no effect on PLB formation and survival percentage of the explants (Kanjilal *et al.*, 1999; Van *et al.*, 1999). However, their combination resulted in a sharp increase in PLB production. The positive effect of cytokinins and auxins is also reported in many orchid species (Valmayor *et al.*, 1986; Goh and Wong, 1990; Kaur and Vij, 2000). This morphogenetic potential of BAP alone does not rule out auxin involvement, as there might be some endogenous auxin formation at the organ forming loci. However, combinations with auxin concentrations found unfavourable for PLB differentiation could be attributed to high endogenous auxin level in the explants manifesting apical dominance (Sharma, 1993).

Supplementation of KN in combination with different auxins (IAA, NAA and 2,4-D) in the medium showed varying results in the explant response. 2.5 mg/l each of KN and IAA and 5.0 mg/l KN + 2.5 mg/l IAA showed better explant response in terms of

PLB induction when compared to KN singly in the medium. The incorporation of KN in combination with IAA is less documented in orchid study. However, Van *et al.* (1974) reported that increase in the level of KN in combinations with IAA modified the developmental pathway; differentiation of further buds was completely replaced by that of vegetative buds in *Nicotiana tabacum* dermal peel cultures. Several workers have reported the positive effect of NAA in combination with KN for rapid multiplication of PLBs and development of plantlets from foliar peels of *Rhynchostylis retusa* (Vij and Kaur, 1992) and fair response of explants in shoot bud formation was also observed in combinations of same hormone in pseudobulb segments of *Malaxis acuminata* (Vij and Kaur, 1998).

CHAPTER V: OPTIMISATION OF ENCAPSULATING MATRIX FOR BEAD FORMATION

INTRODUCTION

Artificial seed technology involves the production of tissue culture derived somatic embryos/PLBs encased in a protective coating. Artificial seed is an analog to a natural seed and the encapsulating matrix is expected to play the role of a seed coat, endosperm and cotyledons. An appropriate matrix for somatic embryos/PLBs is provided after considering diverse factors. The encapsulating matrix should be pliable enough to cushion and protect the embryos, allow germination, supply nourishment and yet be sufficiently rigid to allow rough handling of the capsules (during manufacture, transportation and planting). The matrix should be able to contain and deliver sufficient nutrients, growth and developmental control agents, and other biological or chemical components necessary for the developing embryo.

Several gels like agar, alginate, carboxy methylcellulose, silica gel, gelatin etc. have been tested for synthetic seed production. Alginate encapsulation is found to be more suitable and practicable for synthetic seed production in many cases. Alginate has a variety of properties that makes it amenable to somatic embryos/PLB encapsulation. Sodium alginate solubilizes at room temperature (25°C), does not require heat to produce

a gel and gels upon contact with relatively non-toxic divalent metal salts. The divalent metal salts cause complexation by forming ionic bonds between carboxylic acid groups on the glucoronic acid molecules of the alginate polymers to form metal alginate gels. The hardiness of the capsule is a function of the glucoronic-mannuronic acid ratio, the cation and the complexing time. The nutrient retaining power of the alginate has been found to be very poor. To overcome these problems two approaches have been used: (i) micro-encapsulation of nutrients to provide their controlled release inside the synthetic seeds, and (ii) addition of complex carbohydrate to the gel capsule to slow leaching.

Redenbaugh *et al.* (1986, 1988) reported conversion frequency of synthetic seeds to be as high as 86.0% (*in vitro*) for alfalfa embryos. Several workers have used Redenbaugh' s technique for encapsulating somatic embryos/axillary buds using sodium alginate and have obtained *in vitro* and *in vivo* conversion in many plants (Hama, 1986; Gupta and Durzan, 1987; Bapat and Rao, 1988; Fernandes *et al.*, 1992; Sarkar and Naik, 1997; Adriani *et al.*, 2000; Nandina *et al.*, 2003; Chand and Singh, 2004 and Gangopadhyay *et al.*, 2005)

Kitto and Janick (1985) reported only 3.0% conversion of coated carrot somatic embryos in polyoxyethylene gel by drying the embryo/polyox mixture. Embryo survival was found to be increased with high sucrose, high inoculum density or chilling treatment, and with incorporation of high osmoticum (sucrose, mannitol) and growth retardant (ABA).

In the present study the utility of various gels has been assessed using various complexing agents with affect to time and the percentage survival of *C. devonianum* and *D. lituiflorum* encapsulated PLBs have been evaluated.

MATERIAL AND METHODS

A number of gels and complexing agents were tested for encapsulating PLBs. Various concentrations (2-8%) of sodium alginate, silica gel, gelatin and carboxy-methyl cellulose were prepared separately in 100 ml of distilled water. The mixture was stirred constantly so as to avoid lump formation. Different concentrations (50-150 mM) of complexing agents each of $Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSO_4.7H_2O$, $Al(NO_3)_2.H_2O$ were prepared separately in 100 ml water. Beads were formed by dropping the solutions of sodium alginate, silica gel, gelatin and carboxy-methyl cellulose separately in various concentrations of the complexing agents with the help of microspoon. The complexation was allowed to take place for 5-30 min. The quality of beads i.e. shapes, firmness and ease of handling was noted for each concentration of the gelling solutions with that of different concentrations of complexing agents. The quality of beads as a function of time was also recorded. The concentration of sodium alginate, silica gel, gelatin and carboxy-methyl cellulose and that of complexing agents in which the best beads obtained were selected for subsequent experiments.

To record the percentage survival of the PLBs encapsulated in the synthetic beads, 100 ml solution of sodium alginate (best gelling agent) and the optimal concentrations of complexing agents {100 mM of $Ca(NO_3).2H_2O$, $Ca(OH)_2$,

$ZnSO_4.7H_2O$ and $Al(NO_3)_2.H_2O$} were prepared separately in MS liquid medium and autoclaved for 15 min at 121°C after adjusting the pH to 5.8. Ninety days old single PLBs of *C. devonianum* and *D. lituiflorum* which were 3-4 mm in size were mixed in sodium alginate solution and dropped in the complexing solutions. The gel complexation was allowed to take place for 20-30 min and the beads were strained, washed in sterile water and plated out on MS medium. Twenty encapsulated beads were placed in each petriplates (measuring 60 x 15 mm) for all treatments and the whole experiment was repeated thrice. The cultures were maintained at 25 ± 2°C with 16-hour illumination of 150 μmoles sec^{-1} m^{-2} light intensity. PLB survival (emergence of PLBs from beads) was recorded after a week on subculture to regrowth medium (MS medium).

RESULTS

No proper beads could be formed with all the concentrations of silica gel, gelatin and carboxy-methyl cellulose solutions tried. Therefore, these solutions were not used subsequently for PLB encapsulation experiments. An assessment of various concentrations of sodium alginate {2, 4, 6 and 8% (w/v)} with $Ca(NO_3).2H_2O$ (50, 100 and 150 mM) as complexing agent for the formation of beads at different incubation time (5, 10, 20 and 30 min) is presented in Table 46. Four percent solution of sodium alginate upon complexation with 100 and 150 mM solution of $Ca(NO_3).2H_2O$, gave excellent round beads with ion-exchange duration of 20-30 min (Table 46). Lower concentrations of sodium alginate (2%) resulted in the formation of very fragile ill formed beads which were flattened with trailing ends in all the concentrations of $Ca(NO_3).2H_2O$ with respect to all the complexation time studied. On the other hand, higher concentration (6%) of

Table 46: Effect of different concentrations of sodium alginate and calcium nitrate and effect of incubation time on quality of beads

Complexing agent	Concentrations (mM)	Incubation time (min)	Sodium alginate % (w/v)			
			2	4	6	8
$Ca(NO_3).2H_2O$	50	5	+	+	+	-
		10	+	+	+	-
		20	+	++	++	-
		30	+	++	++	-
	100	5	+	+	+	-
		10	+	++	+	-
		20	+	+++	-	-
		30	++	+++	-	-
	150	5	+	+	++	-
		10	+	++	-	-
		20	+	+++	-	-
		30	+	+++	-	-

Quality of beads with reference to shape, size and firmness, indicated as follows

\+ Flat shapeless mass with trailing ends

++ Round, not firm, fragile

+++ Round, firm beads

sodium alginate when complexed with 50 mM of $Ca(NO_3).2H_2O$ formed fragile, flat, shapeless beads with trailing ends in 5-30 min. When the same concentration of sodium alginate (6%) was complexed with 100 and 150 mM of $Ca(NO_3).2H_2O$ for 5-10 min, fragile ill formed beads were observed. And on increase in the time 20-30 min, round but hard and viscous beads were formed (Table 46). Further increase in concentration of sodium alginate to 8% resulted in hard and viscous beads in all $Ca(NO_3).2H_2O$ concentrations with ion-exchange duration of 5-30 min. The effect with different complexing agents showed that when 4% of sodium alginate was complexed with different concentrations (50, 100 and 150 mM) of $Ca(OH)_2$, round firm beads were formed only in 100 and 150 mM of $Ca(OH)_2$ in 20 min (Table 47). Similar results were obtained with same concentrations of $ZnSO_4.H_2O$ (100 and 150 mM) and $Al(NO_3)_2.H_2O$ when complexed with 4% sodium alginate (Table 48, 49). The PLBs were encapsulated in 4% sodium alginate [prepared in liquid MS nutrients devoid of $Ca(NO_3).2H_2O$)] and complexed with 100 mM $Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSO_4.7H_2O$ and $Al(NO_3)_2.H_2O$ (also prepared in liquid MS nutrients devoid of their respective nutrients). The gel complexation was allowed to take place for 20 min for both $Ca(NO_3).2H_2O$ and $Ca(OH)_2$ (Table 46, 47) whereas 30 min for $ZnSO_4.7H_2O$ and $Al(NO_3)_2.H_2O$ (Table 48, 49).

Firm, round beads with PLBs of both *C. devonianum* (Plate 22 a) and *D. lituiflorum* (Plate 23 a) were obtained in 4% sodium alginate with 100 mM $Ca(NO_3).2H_2O$ (in MS medium). Hundred percent survivals of encapsulated PLBs of both *C. devonianum* and *D. lituiflorum* were obtained when cultured in MS medium devoid of growth regulators (Table 50). Emergence of PLBs from encapsulated PLB of

Table 47: Effect of different concentrations of sodium alginate and calcium hydroxide and effect of incubation time on quality of beads

Complexing agent	Concentration mM	Incubation time (min)	Sodium alginate % (w/v)			
			2	4	6	8
$Ca(OH)_2$	50	5	+	+	-	-
		10	+	+	-	-
		20	+	++	-	-
		30	+	++	-	-
	100	5	+	+	-	-
		10	+	++	-	-
		20	+	+++	-	-
		30	++	+++	-	-
	150	5	+	+	-	-
		10	+	++	-	-
		20	+	++	-	-
		30	+	++	-	-

Quality of beads with reference to shape, size and firmness, indicated as follows

+ Flat shapeless mass with trailing ends

++ Round, not firm, fragile

+++ Round, firm beads

Table 48: Effect of different concentrations of sodium alginate and zinc sulphate and effect of incubation time on quality of beads

Complexing agent	Concentration mM	Incubation time (min)	Sodium alginate % (w/v)			
			2	4	6	8
$ZnSO_4.7H_2O$	50	5	+	+	-	-
		10	+	+	-	-
		20	+	++	-	-
		30	+	++	-	-
	100	5	+	+	-	-
		10	+	++	-	-
		20	+	++	-	-
		30	++	+++	-	-
	150	5	+	+	-	-
		10	+	++	-	-
		20	+	++	-	-
		30	+	++	-	-

Quality of beads with reference to shape, size and firmness, indicated as follows

+ Flat shapeless mass with trailing ends

++ Round, not firm, fragile

+++ Round, firm beads

Table 49: Effect of different concentrations of sodium alginate and aluminium nitrate and effect of incubation time on quality of beads

Complexing agent	Concentration mM	Incubation time (min)	Sodium alginate % (w/v)			
			2	4	6	8
$Al(NO_3)_2.H_2O$	50	5	+	+	-	-
		10	+	+	-	-
		20	+	++	-	-
		30	+	++	-	-
	100	5	+	+	-	-
		10	+	++	-	-
		20	+	++	+	-
		30	++	+++	+	-
	150	5	+	+	-	-
		10	+	++	-	-
		20	+	++	-	-
		30	+	++	-	-

Quality of beads with reference to shape, size and firmness, indicated as follows

+ Flat shapeless mass with trailing ends

++ Round, not firm, fragile

+++ Round, firm beads

Table 50: Effect of sodium alginate and different complexing agents incubated at different time on PLB survival in 10 weeks of culture in MS medium at RT

Sodium alginate (%)	Complexing agents (100 mM)	Incubation time (min)	% Survival	
			C. *devonianum*	*D. lituiflorum*
4	$Ca(NO_3).2H_2O$	20	100	100
	$Ca(OH)_2$	20	-	-
	$ZnSO_4.7H_2O$	30	-	-
	$Al(NO_3)_2 H_2O$	30	-	-

- No response

C. devonianum was obtained in 3 week's time and by 4 weeks time of culture clusters of PLBs were seen emerging around the beads (Plate 22 b). On the other hand, it took only 2 weeks for emergence of PLBs from synthetic seeds of *D. lituiflorum* and cluster of PLBs was observed in 3 week of culture (Plate 23 b). Shoots with 2-3 leaves started to develop in 5 and 4 weeks time in *C. devonianum* (Plate 22 c) and *D. lituiflorum* (Plate 23 c) respectively on regrowth medium. Development of roots in *C. devonianum* and *D. lituiflorum* was obtained in 8 and 6 week's time respectively. Well-developed mature plantlets of both *C. devonianum* and *D. lituiflorum* were obtained in 10 and 8 weeks respectively (Plates 22d, 23d). Encapsulated PLBs in other complexing agents {$Ca(OH)_2$, $ZnSO_4.7H_2O$ and $Al(NO_3)_2.H_2O$} and 4% sodium alginate did not survive and as such no emergence of PLBs could be observed. Therefore, 4% sodium alginate complexed with 100 mM of $Ca(NO_3).2H_2O$ was found to be optimal for studies on storage of *C. devonianum* and *D. lituiflorum* PLBs.

DISCUSSION

Studies on encapsulation have shown that alginate hydrogel is suitable matrix for synthetic seed production. This is due to moderate viscocity and low spinnability of alginate in solution, its low toxicity for explants and quick gellation, low cost and biocompatibility characteristics (Saiprasad, 2001). Alginates are produced by brown seaweeds (Phaeophyceae, mainly *Laminaria*) and are linear unbranched polymers containing β (1-4) linked D-mannuronic acid and α (1-4) linked L-guluronic acid residues. The major principle involved in the alginate encapsulation process is that the sodium alginate droplets containing the explants when dropped into the complexing

agents such as $Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSo_4.7H_2O$ and $Al(NO_3)_2$ formed round and firm beads due to ion exchange between the Na^+ in sodium alginate with Ca^{2+} in $Ca(NO_3).2H_2O$ and $Ca(OH)_2$; Zn^{2+} in $ZnSo_4.7H_2O$ and Al^{2+} in $Al(NO_3)_2$ solution. The hardiness or rigidity of the capsule mainly depends upon the number of sodium ions exchanged with the corresponding ions. Therefore, it has been suggested that proper concentrations of the gelling agents i.e., sodium alginate and complexing agents [$Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSO_4.7H_2O$ and $Al(NO_3)_2$], and the complexation time should be optimized so as to obtain optimum bead hardness and rigidity (Saiprasad, 2001).

The present study showed that out of the various gels tried 4 % Na-alginate appeared to be the most promising for formation of round firm beads when complexed with 100 mM solutions of $Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSO_4.7H_2O$ and $Al(NO_3)_2$ with ion exchange duration of 20 and 30 min. Lower concentrations of sodium alginate resulted in the formation of very fragile and ill formed beads. In the present study, 4% sodium alginate with 100 mM of $Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSO_4.7H_2O$ and $Al(NO_3)_2$ was recorded best for formation of round, firm beads as has been reported earlier in *Dendrobium wardianum* (Sharma *et al.*, 1992) and *Cymbidium giganteum* (Corrie, 1994). This may be due to an increased concentration of sodium alginate (4%) which resulted in a lower absorbance of calcium nitrate. Hence, toxicity due to calcium nitrate may be less with 4% of $Ca(NO_3).2H_2O$. These findings are in agreement with Redenbaugh *et al.*, (1987), Ghosh and Sen, (1994) and Malabadi and Nataraja, (2002 b). However, concentrations higher than 4% proved to be too viscous for the formation of beads. In

Plate 22

Complete development of plantlets cultured in MS medium from encapsulated PLBs of *C. devonianum* prepared in 4% sodium alginate and 100 mM calcium nitrate at RT (bar = 1 mm)

a. Encapsulated PLBs

b. Germinating encapsulated PLBs after 4 weeks

c. Developing shoots after 5 weeks

d. Developing roots after 8 weeks

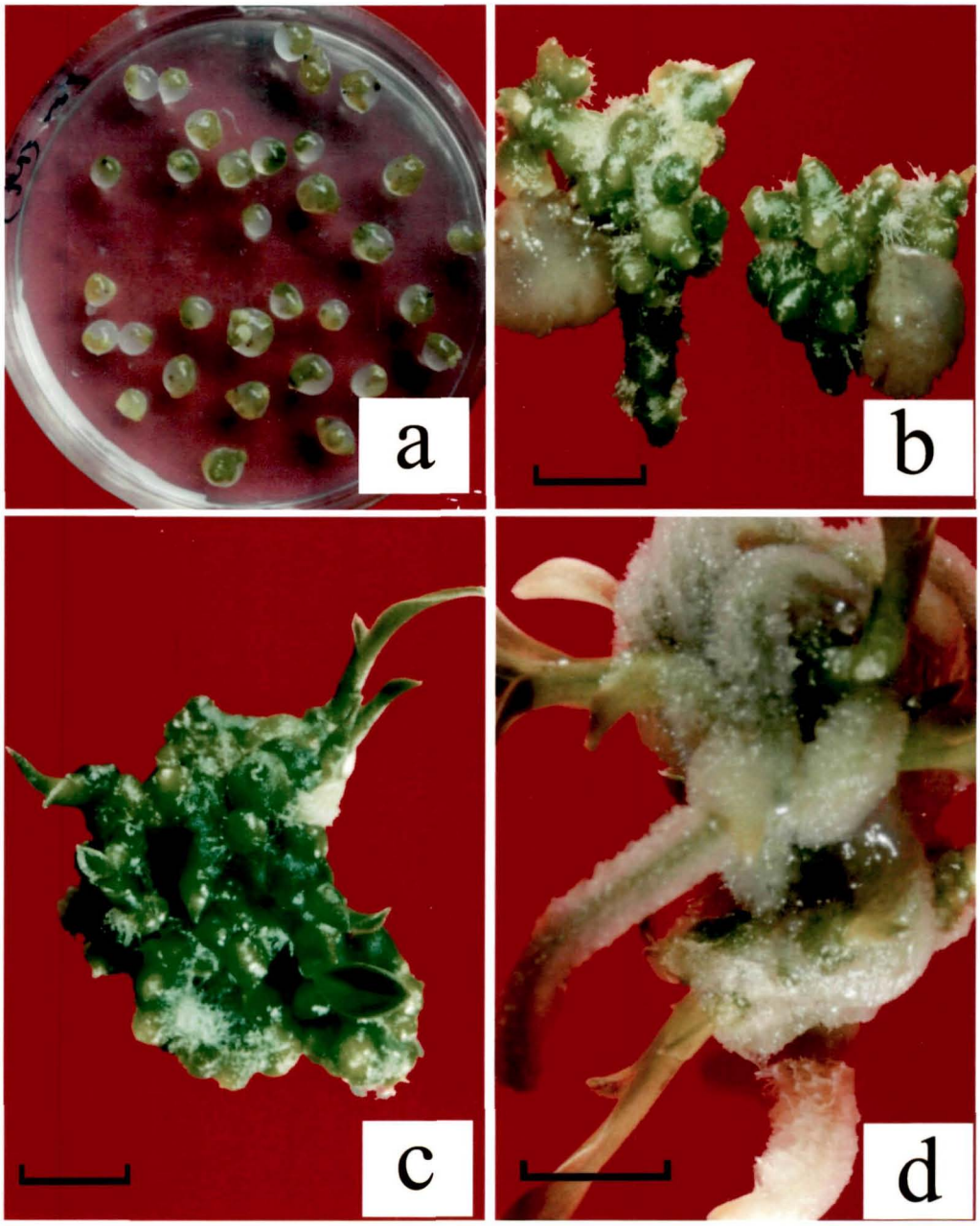

a
b
c
d

Plate 23

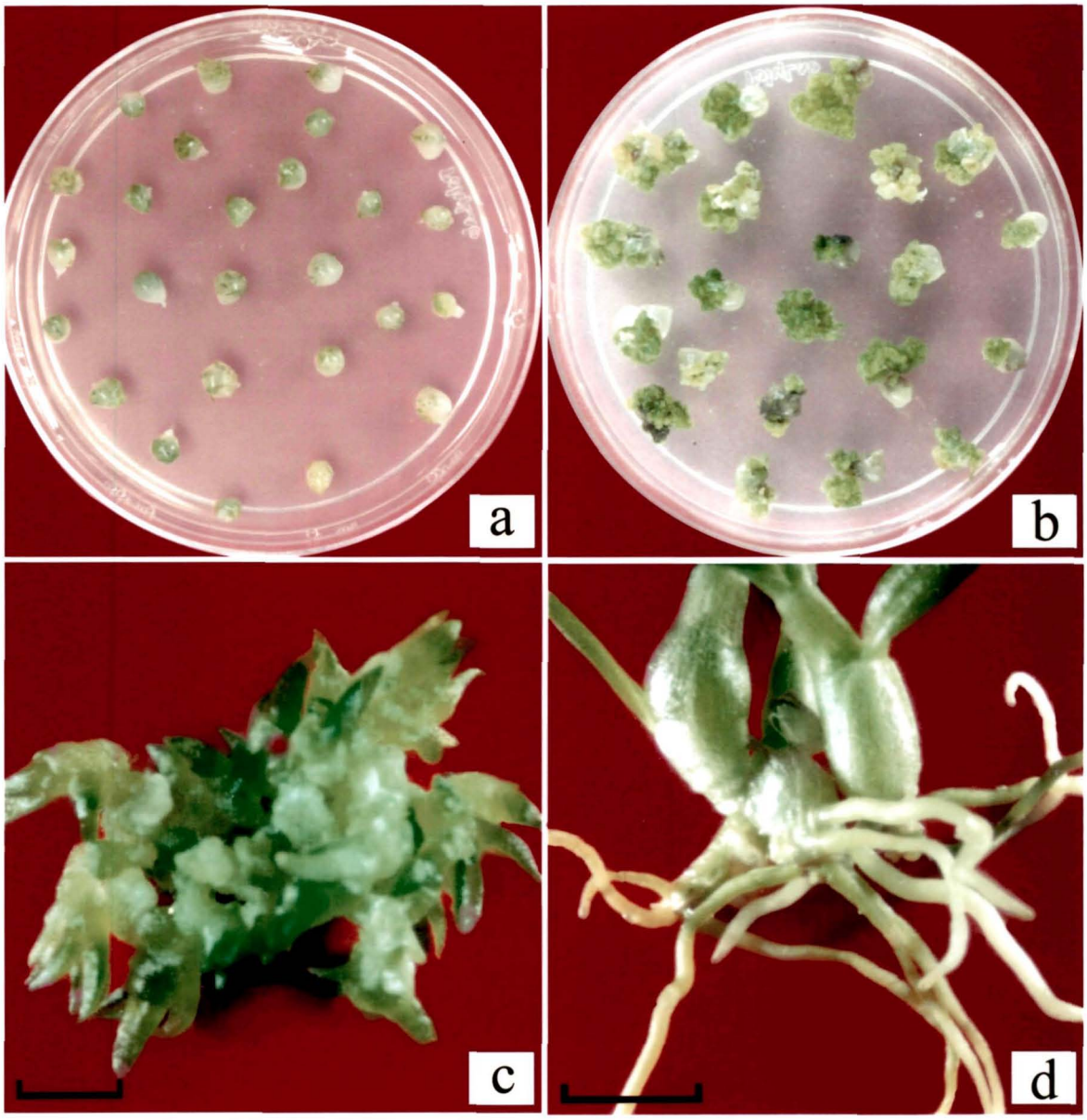
Complete development of plantlets in basal MS medium from encapsulated PLBs of *D. lituiflorum* prepared in 4% sodium alginate and 100 mM calcium nitrate at RT (bar = 1 mm)

a. Encapsulated PLBs

b. Germinating encapsulated PLBs after 3 weeks

c. Developing shoots after 4 weeks

d. Developing pseudobulbs and roots after 6 weeks


a
b
c
d

case of *Clitoria ternatea* and *Vigna aconitifolia*, lower concentrations of sodium alginate resulted in a lower percentage germination (Malabadi and Nataraja, 2002 ab). This may be due to $Ca(NO_3).2H_2O$ toxicity in beads formed with lower concentrations of sodium alginate. Beads with less percentage of sodium alginate exposed for a longer period to $Ca(NO_3).2H_2O$ may adsorb a larger quantity of calcium nitrate (Malabadi and Nataraja, 2002 b). Adsorption is a surface phenomenon and therefore $Ca(NO_3).2H_2O$ may inhibit the emergence of PLBs of *C. devonianum* and *D. lituiflorum*. Malabadi and Staden (2005) reported 2% sodium alginate to be the best for somatic embryo encapsulation, but Redenbaugh (1986a) found 2.5% of alginate to be the best for encapsulation of meristematic tissue. Redenbaugh (1993) and Redenbaugh *et al.* (1987) noted that variables related to the encapsulation method, including alginate type and concentration, medium and methods used to produce synthetic seeds were responsible for significant variation in conversion percentage in alfalfa, carrot and celery. In the present study, the duration of exposure of sodium alginate in different complexing agents ($Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSO_4.7H_2O$ and $Al(NO_3)_2$ solutions affected the hardiness of beads during hardening process. The present study showed 20 and 30 min exposure of sodium alginate in complexing agent was best for the formation of round and firm beads. However, Sharma *et al.* (1992) and Corrie and Tandon (1993) reported exposure to 30 and 40 min respectively to complexing agent $Ca(NO_3).2\ H_2O$ as optimum for best bead formation. Malabadi and Staden, (2005) also reported that duration of exposure to $CaCl_2$ played an important role in germination of *Pinus patula*. However, in contrast to our results he reported that beads formed after 5 min was best for germination in *Pinus patulata* but 30

min of exposure to 100 mM to complexing agent resulted in hard bead with low percentage of germination.

In the present study, both the encapsulated PLBs prepared in MS liquid medium of *C. devonianum* and *D. lituiflorum* showed 100% survival. In order to prevent the embryo from desiccation and mechanical injury, nutrients may be incorporated into the encapsulation matrix. Moreover, PLBs lack seed coat and endosperm that provide protection and nutrition for zygotic embryo in developing seeds. To augment these deficiencies, addition of nutrients to the encapsulation matrix is desired, which serves as an artificial endosperm. Such addition of nutrients to the encapsulation matrix results in increase efficiency of germination and viability of encapsulated PLBs. On the other hand, PLBs encapsulated in 4% sodium alginate and 100 mM of either $Ca(OH)_2$ /$ZnSO_4.7H_2O$ or $Al(NO_3)_2.H_2O$ in MS liquid medium did not show any response; rather they turned brown and died. Datta *et al.* (1999) also suggested that the most suitable encapsulating agent for orchid PLBs to be sodium alginate due to its solubility at room temperature and its ability to form completely permeable gel with $Ca(NO_3).2H_2O$. Their findings have also revealed that the method provides an efficient mechanism for handling and storage of orchid PLBs.

CHAPTER VI: STORAGE OF SYNTHETIC SEEDS USING REDUCED NUTRIENTS, LOW TEMPERATURE, GROWTH RETARDANT (ABA) AND DIFFERENT OSMOTICA (SUCROSE AND MANNITOL)

INTRODUCTION

Threats to biodiversity posed by the destruction of natural habitat notably by urbanization have led to an extinction of a large number of plant species (Ford-Lloyd and Jackson, 1986; Pearce, 1989; Wilkins, 1991). Preservation of any such endangered/rare plant species *in vitro* can be carried out in two steps:

(i) by use of micropropagation technique for rapid mass propagation, and (ii) by preservation of germplasm either using slow growth approaches (limiting the multiplication rate) or by cryo-exposure (arresting the metabolic activities).

Plant tissues cultured *in vitro* are generally maintained by periodically transferring them to fresh medium. However, genetic, physiological or biochemical variations are often observed during serial subcultures (Withers, 1991). To diminish such variations and to save expense and time for routine culture maintenance, the preservation of germplasm in the inactive state has been emphasized (Henshaw, 1975; Withers, 1991). Extensive investigation has been carried out over the years into increasing or decreasing culture medium components and modifying the physical environment to reduce growth rates

(Withers, 1992). Artificial seeds, which are usually clonal propagules of the donor plants, could allow storage of a unique genotype for prolonged periods of time. However, if artificial seed could be manipulated to reduced nutrient content typically found in true seeds, then it would have the advantage of serving as a germplasm storage system. In this state the propagules could be maintained in a quiescent state for extended periods of time. It would also provide a more efficient usage of space and labour in a commercial production system. A common approach is also to reduce the temperature at which cultures are maintained. Artificial seeds have more advantage on storage at reduced temperature over naked explants because the explants are well protected. Banerjee and Langhe (1985) reported a temperature of 15°C to be optimal for the storage of *Musa* species. On the other hand, a temperature of 9°C was reported optimal to store *Colocasia esculenta* shoot cultures (Zandvoort and Staritsky, 1986). Slow growth storage by a reduction in incubation temperature has also been reported in *Dioscorea* species (Ng and Hahn, 1985), *Eucalyptus* species (Aitken-Christie and Singh, 1987) and *Pyrus* species (Wanas *et al.*, 1986; Wilkins *et al.*, 1988). Besides, limitation of growth has been achieved by using different osmotica (Ng and Hahn, 1985; Espinoza *et al.*, 1986; Staritsky *et al.*, 1986; Love *et al.*, 1987), natural or synthetic hormonal inhibitors (Mix, 1982, 1985; Ng and Ng, 1991; Wilkins, 1991), mineral oil overlay (Druart, 1985; Moriguchi *et al.*, 1988; Crane and Hughes, 1990; Mathur *et al.*, 1991), reduced oxygen tension (Bridgen and Staby, 1981; Engelmann, 1990) and defoliation of shoots (Withers, 1987). Singh (1988) made use of organic solvents for the preservation of orchid seeds and reported petroleum ether effective to store seeds of *Spathoglottis plicata* 'Alba' for a

considerable period of time. Recent technique of artificial seed production has opened up new possibilities for preservation by storing the encapsulated tissues at low temperature (Bapat and Rao, 1988; Datta *et al.*, 1999; Lisek and Orlikowka, 2003; Malabadi and Staden, 2005). Artificial seeds, consisting of somatic embryos enclosed in a protective coating have been proposed as a low cost, high-volume propagation system (Redenbaugh *et al.*, 1986). These provide a potential method to deliver plant material produced from tissue culture directly to the greenhouse or field. The direct planting of the somatic embryos into soil, thus bypasses *in vitro* plant production steps that require high labour effort and expense (Fujii *et al.*, 1989). Insufficient and inconsistent information of artificial seeds for different slow growth approaches, however, has made it difficult to recommend any one method suitable for a group of plants. In an attempt to preserve the germplasm of threatened and endangered *C. devonianum* and *D. lituiflorum,* different approaches to slow growth storage viz., reduced nutrients, reduced temperatures, different osmotica and growth retardant have been studied.

MATERIAL AND METHODS

a) *Reduced nutrients and temperature*

Well developed 90 days-old PLBs (3-4 mm) of both *C. devonianum* and *D. lituiflorum* obtained from *in vitro* grown seedlings were taken for experiments after subculturing in hormone-free MS medium for three times. The selected PLBs were separated into single PLB and blot dried. Encapsulation of PLBs was carried out by mixing them in 4% sodium alginate [prepared in different strengths of MS medium i.e. full, ½, ¼ and 1/8 MS medium devoid of $Ca(NO_3).2H_2O$]. These were then dropped in

100 mM $Ca(NO_3).2H_2O$ solution [also prepared in the above given strengths of MS medium and devoid of $Ca(NO_3).2H_2O$]. The reduced strength of MS medium contained reduced major ad minor salts whereas the concentrations of vitamins and sucrose were kept the same as that in full MS medium. Round and firm beads with encapsulated PLBs were obtained in 4% calcium alginate when dropped and stirred continuously in 100 mM $Ca(NO_3)$ $2H_2O$ solution for 20 min. The synthetic seeds so obtained were washed 3-4 times with MS liquid solution and blot dried with sterilized filter paper. Twenty synthetic seeds per petriplates (12 petriplates for each treatment) were plated out and stored for varying duration in the dark at RT (25 ± 2°C). For studies on storage at reduced temperature each set of encapsulated PLBs was kept at 0, 4 and 8°C in dark and one set of encapsulated petriplates which serve as control was kept at RT also in dark. The cultures were incubated at 25 ± 2°C temperatures and 16-hour photoperiod of 150 μmoles sec^{-1} m^{-2} light intensity. Each petriplate was taken out regularly at 30 days of time interval, subcultured onto fresh basal MS medium (regrowth medium) contained in the flasks. The survival percentage (emergence of PLBs from beads) of stored synthetic beads after subculturing to regrowth medium was recorded after 10 weeks of culture. The time taken in initiation of PLBs from beads and subsequent emergence of shoots and roots were also recorded and the experiment was repeated thrice.

b) *Osmotica (sucrose and mannitol) and growth retardant (ABA)*

Ninety-days old PLBs (3-4 mm) of both *C. devonianum* and *D. lituiflorum* were encapsulated in 4% sodium alginate [dissolved in MS liquid medium containing different concentrations of osmotica (sucrose and mannitol) in a range of 0.0 - 12.5% (w/v)] and

100 mM $Ca(NO_3).2H_2O$, [also dissolved in MS liquid medium containing different concentrations of sucrose and mannitol in the same range as for sodium alginate). Synthetic beads containing PLBs of both *C. devonianum* and *D. lituiflorum* were prepared in the same method as described earlier. Another set of experiment was set up where encapsulated PLBs of both *C. devonianum* and *D. lituiflorum* were prepared in 4% sodium alginate and 100 mM $Ca(NO_3).2H_2O$ dissolved separately in MS liquid medium containing different concentrations of ABA, a hormonal growth retardant, at a range of 0.0-5.0 mg/l. The procedure for preparation of synthetic beads containing PLBs was same as described earlier. Twenty synthetic seeds per petriplates were plated for each treatment (5 petriplates for each treatment) and kept in dark at RT. Petriplates were taken out after 90 days and subcultured on regrowth medium. The culture conditions were same as described earlier. The survival percentage (emergence of PLBs from beads) of stored synthetic beads was recorded after 10 weeks of subculture in the regrowth medium. The experiment was repeated thrice.

For further studies of PLB development, three different developmental stages were considered. These were as follows:

Stage I - Emergence of PLBs from encapsulating PLB

Stage II – Shoot initiation

Stage III – Root initiation

RESULTS

C. DEVONIANUM

a) *Effect of different salt/nutrient strengths of MS medium*

Encapsulated PLBs of *C. devonianum* stored at RT showed varying results on full-strength MS medium after 10 weeks of culture in the regrowth medium. After 30 days of storage, 100% survival was recorded in the regrowth medium (Fig.1). With increase in storage time, stored encapsulated PLBs started drying. Only 67 and 30% survival was recorded after 60 and 90 days of storage respectively (Fig. 1). After 120 days of storage, further drying of stored synthetic seeds was recorded resulting in less survival percentage (17%). With further increase of storage time to 150 days all the stored synthetic seeds dried and did not survive on regrowth medium. PLBs of *C. devonianum* encapsulated in ½ MS medium could also be stored for 120 days with 20% survival when stored at RT (Fig. 1). High survival percentages of 100 and 70% were recorded after 30 and 60 days of storage respectively in beads encapsulated with ½ MS medium. Forty percent survival was recorded after 90 days of storage. No survival was recorded beyond 120 days of storage. Hundred percent survivals of stored encapsulated PLBs of *C. devonianum* were recorded after 30 days storage at RT on ¼ MS medium. Though decrease in survival percentage was recorded with increase in storage time, a higher survival percentage (74%) was recorded after 60 days of storage while the remaining stored encapsulated PLBs dried. Storage of synthetic seeds for 90 and 120 days resulted in 40 and 23% of survival respectively. Survival was not recorded on ¼ MS medium with further increase in storage time. A drastic decrease in survival of stored

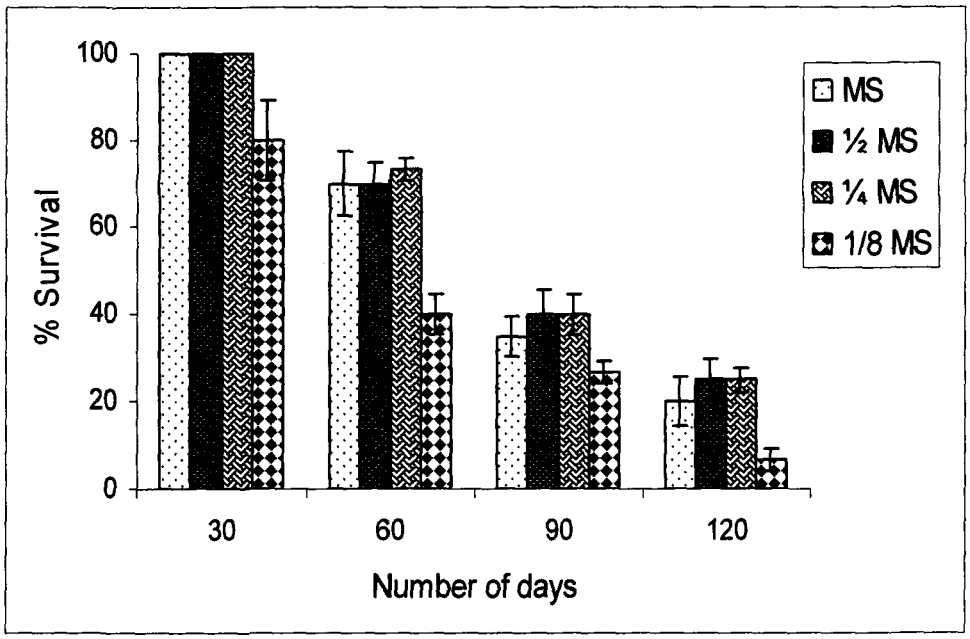


Fig 1: Effect of different salt strengths of MS medium on survival of encapsulated PLBs of *C. devonianum* after10 weeks of subculture in MS medium

encapsulated PLBs of *C. devonianum* on 1/8 MS medium with increase of storage time was recorded. Only 80% survival was recorded after storing for 30 days and the survivability of stored encapsulated PLBs decreased to 40% after 60 days (Fig. 1). Still lower survival (27% and 7%) was recorded after 90 and 120 days of storage respectively.

The time taken to attain different developmental stages of stored encapsulated PLBs of *C. devonianum* was inversely proportional with decrease in MS strength in the encapsulating matrix. However, the time taken to attain these stages within the same strength was directly proportional to increase in storage time (Table 51). With 30 days difference in storage time, a delay in PLB emergence, shoot and root initiation by 1-2 weeks was recorded. Among all the strengths of MS medium studied in the encapsulating matrix for storage, time taken in emergence of PLB, shoot and root initiation was least in full-strength MS medium. In this medium, emergence of PLBs from beads stored for 30 days was recorded within a week on subculturing to regrowth medium (Table 51). However emergence of PLBs, shoot and root initiation stored for same time was delayed in the encapsulating matrix containing 1/8 strength of nutrients in MS medium (Table 51).

Encapsulated PLBs of *C. devonianum* could be stored for 60 days in ½ and ¼ MS medium with maximum 70 and 74% survival respectively. That is, stored encapsulated PLBs of *C. devonianum* in different strengths of MS medium (½ and ¼) showed more or less similar results but due to lesser content of nutrients and slightly better results in ¼ MS medium, for further experiments on storage, ¼ MS medium was used as encapsulating matrix.

Table 51: Effect of different strengths of MS medium on developmental stages of encapsulated PLBs of *C. devonianum* stored at RT after subculturing toms medium

Treatments	Storage time (days)	Stages (weeks)		
		I	II	III
MS	30	1	3	7
	60	2	5	8
	90	4	6	9
	120	5	7	10
½ MS	30	3	5	7
	60	4	6	9
	90	5	7	10
	120	6	8	10
¼ MS	30	3	6	8
	60	4	8	11
	90	5	10	13
	120	6	11	14
1/8 MS	30	5	8	12
	60	7	10	13
	90	8	11	14
	120	10	12	15

Stage I - Emergence of PLBs from encapsulated PLB
Stage II - Shoot initiation
Stage III - Root initiation

b) ***Effect of different temperatures on storage of encapsulated PLBs.***

Encapsulated PLBs responded varyingly to the stored temperature. Though 100% survival of stored encapsulated PLBs of *C. devonianum* was recorded after 30 days of storage at room temperature but survival percentage decreased with further increase in storage time and the encapsulated PLBs could be stored for only 120 days with 23% survival (Fig. 2). Encapsulated PLBs stored at 0°C did not survive. Instead they turned brown and died subsequently on regrowth medium (not shown in Fig. 2). Encapsulated PLBs of *C. devonianum* stored at 4°C could be stored for longer time when compared to control (encapsulated PLBs stored on ¼ MS medium at culture RT). Till 120 days of storage, 100% synthetic seeds survived. However, decrease in survivability was recorded with further increase in storage time. Seventy percent encapsulated PLBs survived when stored for 150 days (Fig. 2). Further decrease in survival percentage (40 and 20%) was recorded after 180 and 210 days respectively. Encapsulated PLBs could be stored till 240 days at 4°C but only with 5 % of survival. Longer storage of encapsulated PLBs at 8°C was recorded when compared to control and encapsulated PLBs at 4°C. Hundred percent survival of encapsulated PLBs stored for 180 days was recorded on regrowth medium after 10 weeks of subculture. But decrease in survival of encapsulated PLBs was recorded with further increase in storage time. Seventy percent survival of encapsulated PLBs was recorded after 210 days of storage (Fig. 2). The survival reduced to 62 and 50% after 240 and 270 days of storage respectively at 8°C. Encapsulated PLBs could be stored further to 300 days with 30% survival only. However, with further increase in storage time to 330 days, the encapsulated PLBs showed only 16.6% survival on regrowth medium, after

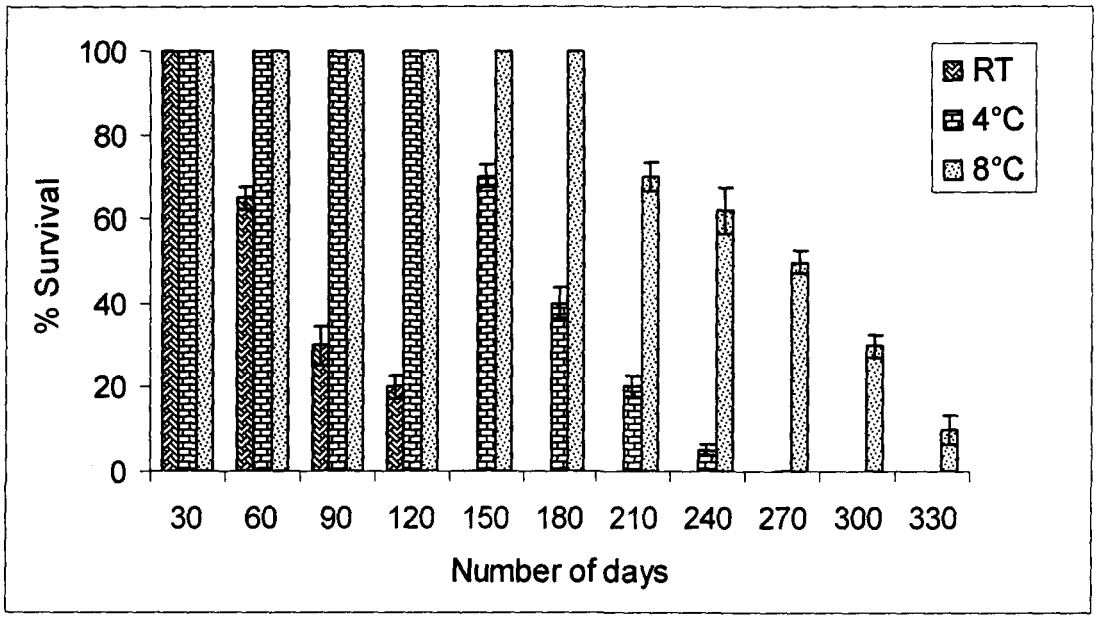


Fig 2: Effect of different storage temperatures on survival of encapsulated PLBs of *C. devonianum* enclosed in ¼ MS medium after 10 weeks of subculture in MS medium

which all the encapsulated PLBs though green while storage, turned brown and died when subcultured on regrowth medium.

Emergence of PLBs and subsequent development of shoots and roots in encapsulated PLBs stored at 8°C was observed after subculturing on MS medium (Plate 24 a, b, c). It was found that with decrease in storage temperature, a corresponding increase in time for all the developmental stages of the cultured encapsulated PLBs was recorded (Table 52). Encapsulated PLBs stored for 30 days at RT showed emergence of PLBs in 3 weeks of subculture. However, encapsulated PLBs stored at 4 and 8°C showed emergence of PLBs only at 5 and 4 weeks of subculture respectively when stored for same time. The shoot and root development was also delayed accordingly.

PLBs encapsulated in ¼ MS medium could be stored longer with maximum survival at 8°C when compared to control and encapsulated PLBs at 4°C. At 8°C encapsulated PLBs could be stored for 180 days with 100% survival after 10 weeks of subculture on regrowth medium. Although survival percentage of encapsulated PLBs decreased with further increase in storage time but these could be stored for 330 days with 16.6% survival.

c) *Effect of different concentrations of osmotica (sucrose, mannitol) and ABA in ¼ MS medium on storage of encapsulated PLBs at RT*

Storage of encapsulated PLBs in ¼ MS medium devoid of sugars showed 50% survival on regrowth medium after 90 days of storage (Fig. 3). The remaining 50% encapsulated PLBs stored in the same medium dried during storage.

Plate 24

Complete development of plantlets from encapsulated PLBs of *C. devonianum* stored for 150 days at 8°C after subculturing to MS medium

a. Germinated encapsulated PLBs after 7 weeks of culture
b. Prolifering PLBs with shoot initiation after 9 weeks of culture
c. Well developed shoots and roots after 13 weeks of culture

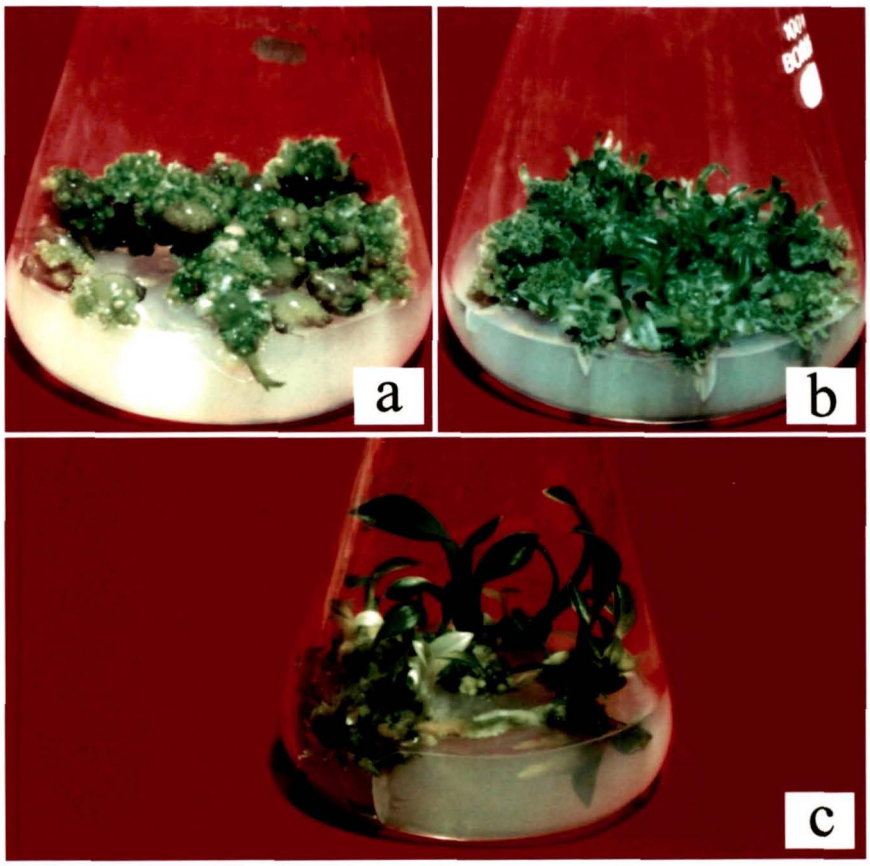

a
b
c

Table 52: Effect of different temperatures on developmental stages of stored encapsulated PLBs of *C. devonianum* in ¼ MS medium after subculturing to MS medium

Treatments	Storage time (days)	Stages (weeks)		
		I	II	III
RT	30	3	6	8
	60	4	8	11
	90	5	10	13
	120	5	11	14
4°C	30	5	8	13
	90	7	10	15
	150	8	11	16
	210	9	13	19
8°C	30	4	7	10
	90	6	8	11
	150	7	9	13
	210	8	10	16
	270	9	12	18
	330	10	14	19

Stage I - Emergence of PLBs from encapsulated PLB
Stage II - Shoot initiation
Stage III - Root initiation

Sucrose

Incorporation of different concentrations of sucrose in the encapsulating matrix showed varying results. With incorporation of 2.5 and 5% sucrose in the encapsulating matrix, 60 and 75% survival of synthetic seeds was recorded respectively after 90 days of storage (Fig. 3). However, with increase in sucrose concentration to 7.5% in the encapsulating matrix, high percentage survival (83%) was recorded. With further increase in sucrose concentrations (10% and 12.5%) in the encapsulating matrix, a decrease in survival percentage of stored encapsulated PLBs was recorded (Fig. 3).

Mannitol

Incorporation of different concentrations of mannitol in the encapsulating matrix showed similar pattern of survival as that for sucrose incorporated matrix (Fig. 3). The highest survival percentage (90%) of encapsulated PLBs was recorded after 90 days of storage when 7.5% of mannitol was incorporated in the encapsulating matrix whereas with further increase in concentrations of mannitol to 10 and 12.5% in the encapsulating matrix, decrease in survival to 70 and 45% was recorded respectively after 90 days of storage (Fig. 3).

A varied difference in the developmental stages of encapsulated PLBs enclosed in different concentrations of sucrose and mannitol was observed on regrowth medium after 90 days of storage (Table 53). Emergence of PLB and subsequent initiation of shoots and roots was delayed in the encapsulated PLBs without incorporation of any sugars on regrowth medium. Emergence of PLBs was slightly faster in all the encapsulated PLBs with all concentrations of sucrose compared to encapsulated PLBs

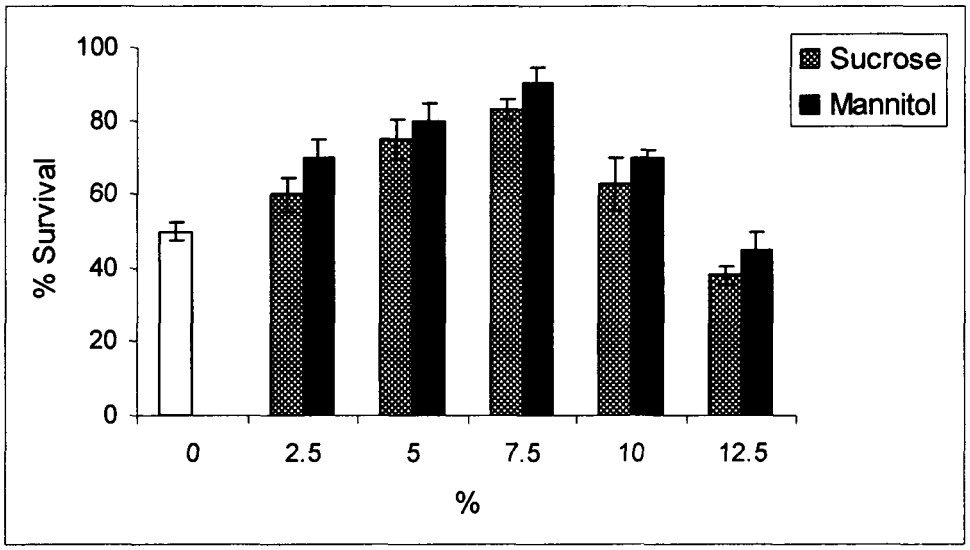


Fig 3: Effect of different concentrations of sucrose and mannitol incorporated separately in ¼ MS medium on survival of encapsulated PLBs of *C. devonianum* stored for 90 days at RT. Data recorded after 10 weeks subculture in MS medium

Table 53: Different developmental stages of encapsulated PLBs* of *C. devonianum* (in ¼ MS containing different concentrations of sucrose and mannitol separately) obtained after subculturing to MS medium

Treatments (%)	Stages (weeks)					
	I		II		III	
0	10		15		20	
	Sucrose	Mannitol	Sucrose	Mannitol	Sucrose	Mannitol
2.5	5	6	9	7	13	15
5.0	5	6	13	8	18	17
7.5	6	7	16	9	20	18
10.0	7	8	-	10	-	18
12.5	7	9	-	12	-	19

Stage I - Emergence of PLBs from encapsulated PLB
Stage II - Shoot initiation
Stage III - Root initiation
* Stored for 90 days

enclosed with different concentrations of mannitol. The time taken in emergence of PLBS from the encapsulating matrix containing 2.5 and 5.0% of sucrose and mannitol was same in both the cases. But with increase in both sucrose and mannitol concentrations to 7.5, 10 and 12.5% in the encapsulating matrix, delay in emergence of PLBs by a week was recorded (Table 53). However, subsequent initiation of shoots was much delayed in the encapsulating PLBs incorporated with 2.5-7.5% of sucrose in the encapsulating matrix. Though shoot and root development (Plate 25a) was observed in lower concentrations of sucrose (2.5-7.5%) but the shoots and roots were completely inhibited in high concentrations (10 and 12.5%) of sucrose in the encapsulating matrix (Table 53). In these high concentrations of sucrose clusters of small protuberances of PLBs could be observed (Plate 25b). Shoots developed on mannitol containing matrix were morphologically different from that formed in encapsulation matrix containing sucrose. Here the shoots were thin, long and slender and aroused while still embedded in the beads (Plate 25c). Once the shoots were formed, the time taken in root development was however faster in PLBs encapsulated with all concentrations of sucrose when compared to PLBs encapsulated with mannitol on regrowth medium (Table 53).

ABA

Without incorporation of ABA in ¼ MS medium, 45% survival of encapsulated PLBs was recorded after 90 days of storage (Fig. 4). With incorporation of 0.5 and 1.0 mg/l of ABA in the encapsulating matrix high survival percentage of 87 and 90% of the stored encapsulated PLBs was recorded respectively after 90 days of storage (Fig. 4). Hundred percent survival was recorded when 2.0 mg/l of ABA was incorporated in the

encapsulating matrix. However, with an increase in ABA concentration to 5.0 mg/l in the encapsulating matrix, a reduction in survival percentage on regrowth medium was recorded after 10 weeks of subculture when stored for 90 days (Fig. 4).

The time taken in emergence of PLBs and subsequent shoot and root initiation was delayed in control (i.e. without incorporation of ABA in the encapsulating medium) when compared with the encapsulating PLBs contained with lower concentrations of ABA (0.5 - 2.0 mg/l) in the encapsulating medium (Table 54). PLBs which emerged from encapsulated matrix containing all concentrations of ABA were morphologically different with many hair-like structures all over it (Plate 25d). However, with high concentration of ABA (5.0 mg/l) in the encapsulating medium, a delay in PLB emergence and subsequent shoot and root development were recorded (Table 54).

Highest survival percentage of encapsulated PLBs stored in ¼ strength MS medium with 2.0 mg/l ABA for 90 days was recorded after 10 weeks of subculture on regrowth medium. On the other hand, incorporation of both sucrose and mannitol separately in ¼ MS of encapsulating matrix showed poorer survival percentage of encapsulated PLBs.

D. LITUIFLORUM

a) *Effect of different salt/nutrient strengths of MS medium*

Only 10% survival of encapsulated PLBs of *D. lituiflorum* in full-strength MS medium was recorded on regrowth medium after 90 days of storage at RT (Fig. 5). PLBs encapsulated in ½ strength MS medium showed 100% survival after 30 days of storage

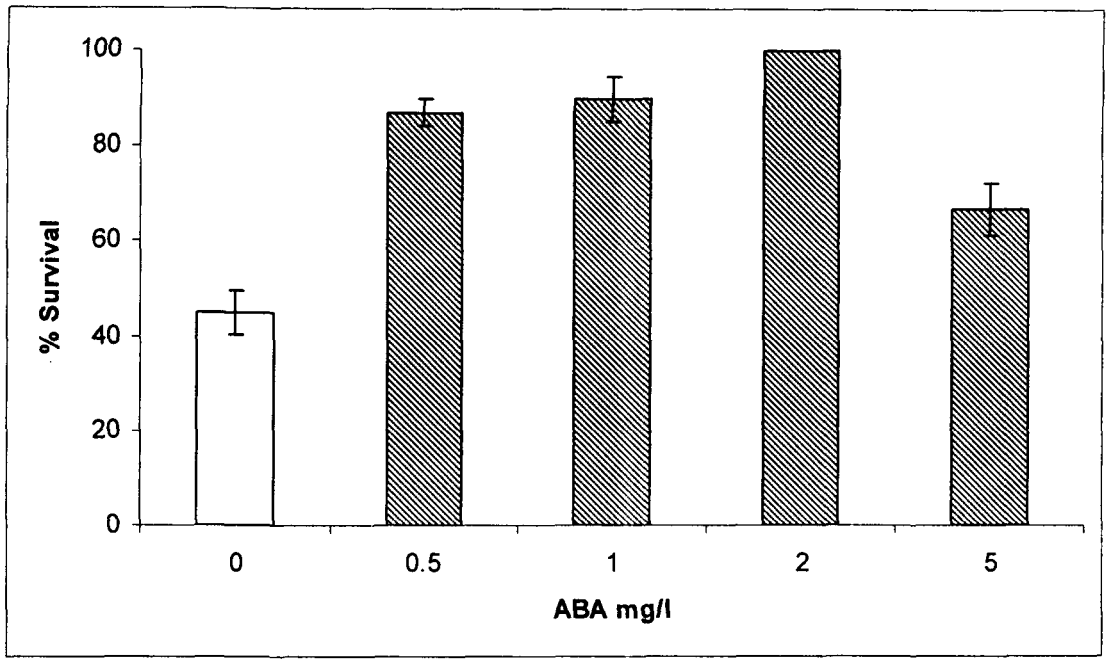


Fig 4: Effect of different concentrations of ABA incorporated in ¼ MS medium on survival of encapsulated PLBs of *C. devonianum* stored for 90 days at RT. Data recorded after 10 week subculture in MS medium

Table 54: Different developmental stages of encapsulated PLBs* of *C. devonianum* (in ¼ MS containing different concentrations of ABA) obtained after subculturing to MS medium

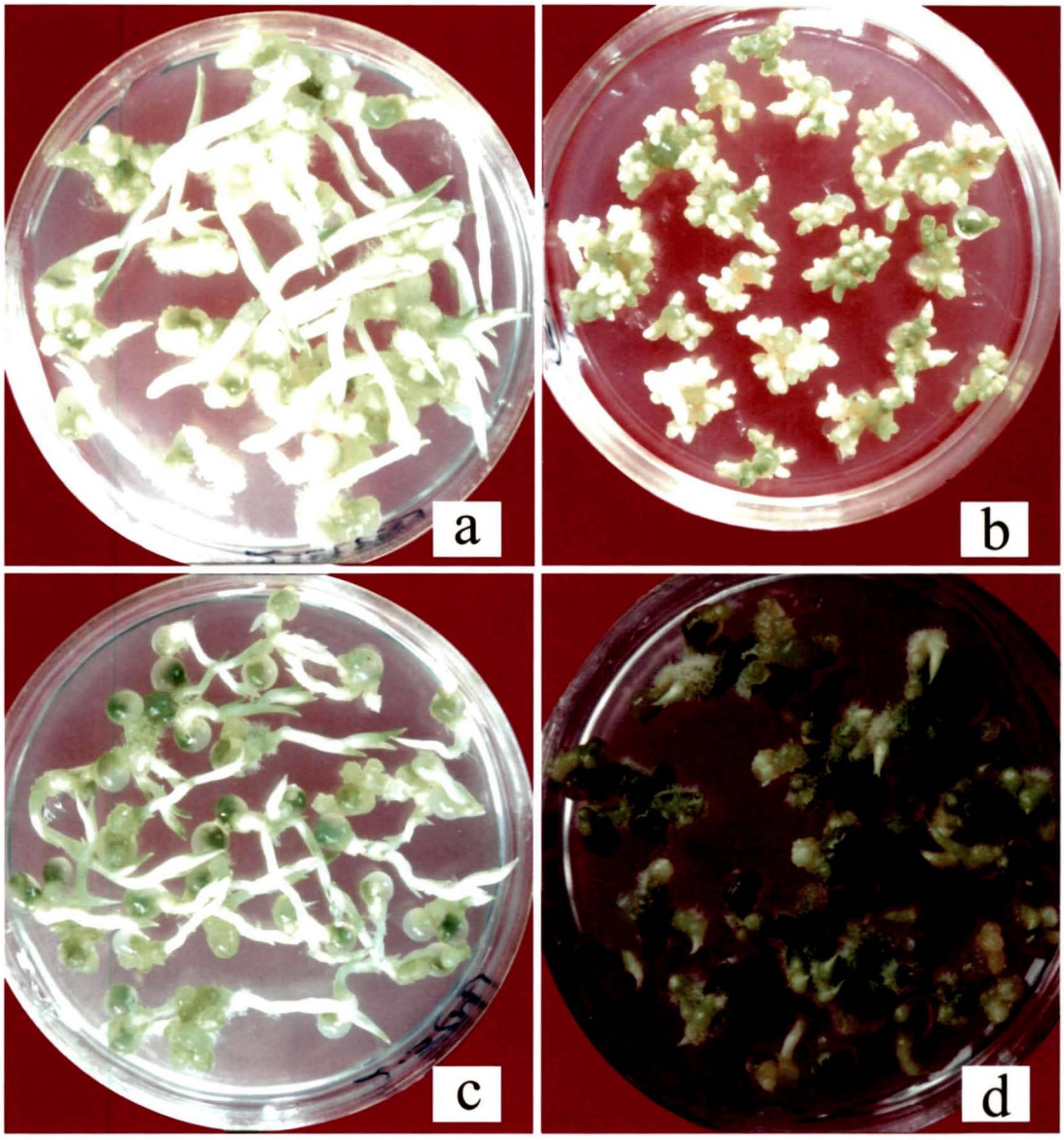
Treatments (ABA mg/l)	Stages (weeks)		
	I	II	III
0	5	10	13
0.5	3	5	7
1.0	3	6	9
2.0	4	9	12
5.0	6	11	15

Stage I - Emergence of PLBs from encapsulated PLB
Stage II - Shoot initiation
Stage III - Root initiation
* Stored for 90 days

Plate 25

Different stages of development of encapsulated PLBs of *C. devonianum* in MS medium after 90 days of storage

a. Developing shoots and roots from encapsulated PLBs containing 7.5 % sucrose after 16 weeks of culture
b. Clusters of protuberances of small PLBs from encapsulated PLBs containing 12.5 % sucrose after 20 weeks of culture
c. Developing shoots from encapsulated PLBs containing 7.5% mannitol after 20 weeks of culture
d. Germinating PLBs with shoot initiation from encapsulated PLBs containing 2.0 mg/l ABA after 9 weeks of culture


a
b
c
d

on regrowth medium (Fig. 5). However, with increase in storage time, a decrease in survival percentage of encapsulated PLBs was recorded. Seventy percent survival of stored encapsulated PLBs was recorded after 60 days of storage, which further decreased to 40 and 16% after 90 and 120 days of storage respectively (Fig. 5). PLBs encapsulated in the encapsulating matrix with ¼ MS medium also showed 100% survival after 30 days of storage on regrowth medium. But, a drop of survival (60%) was recorded after 60 days of storage (Fig. 5). The survival percentage further reduced to 20% and 3.0% after 90 and 120 days of storage respectively (Fig. 5). Sixty percent survival of stored encapsulated PLBs was recorded after 30 days of storage in 1/8 MS medium (Fig. 5). With further increase in storage time to 60 days, lower survival percentage (20%) was recorded. Further increase in storage time resulted in drying up of stored encapsulated PLBs.

PLBs encapsulated in different strengths of MS medium (full, ½, ¼) for 30 days of storage emerged faster when subcultured on regrowth medium (Table 55). Emergence of PLBs was attained within a week or two from encapsulated matrix containing full, ½ and ¼ MS medium. However, PLBs encapsulated in 1/8 MS medium showed delayed emergence (4 week) after 30 days of storage (Table 55). Correspondingly, an increase in emergence time by a week or two was recorded with increase in storage time within the same strengths. But PLBs encapsulated in full MS medium emerged within the same time after 30 and 60 days of storage (Table 55). The time taken between stages 1 to stage II development in full MS medium encapsulated PLBs was 2-3 weeks and between stages II to stage III development was 1-2 weeks at different storage time. On the other hand, the time taken between stage 1 to stage II development of encapsulated PLBs in ½ and ¼ MS

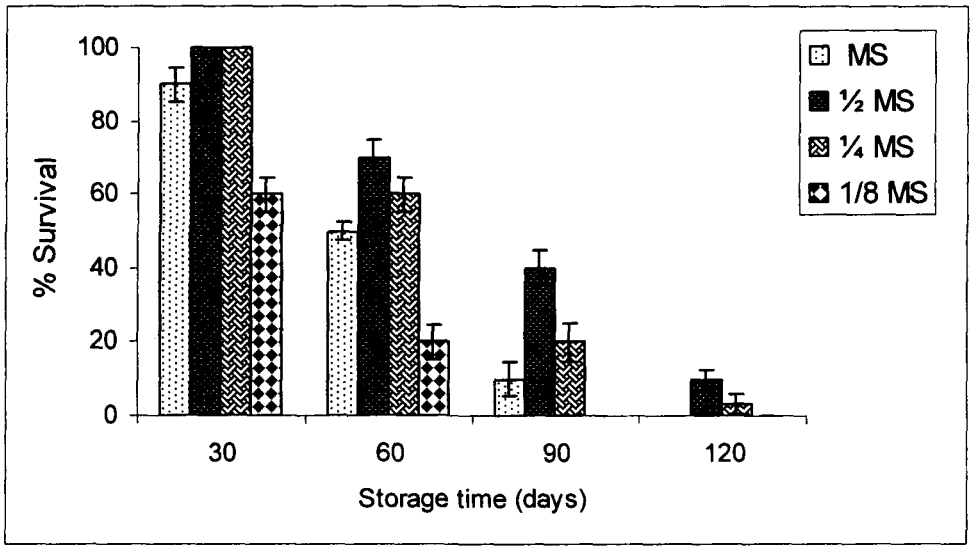


Fig 5: Effect of different strengths of MS medium on survival of stored encapsulated PLBs of *D. lituiflorum* after 10 weeks subculture in MS medium

Table 55: Effect of different strengths of MS medium on developmental stages of stored encapsulated PLBs of *D. lituiflorum* at RT after subculturing to MS medium

Treatments	Storage time (days)	Stages (weeks)		
		I	II	III
Full MS	30	1	3	4
	60	1	3	5
	90	2	5	7
	120	4	6	8
½ MS	30	1	4	6
	60	2	6	8
	90	3	7	10
	120	4	8	11
¼ MS	30	2	5	8
	60	3	7	10
	90	5	9	11
1/8 MS	30	4	6	8
	60	5	8	10
	90	5	9	11

Stage I - Emergence of PLBs from encapsulated PLB
Stage II - Shoot initiation
Stage III - Root initiation

medium was 3-4 weeks and the development between stages II to III was 2-3 weeks in all the strengths of medium tried. The difference in time taken of PLBs encapsulated in 1/8 medium between stages 1 to II development was 2-3 weeks after 30 and 60 days of storage. However, a difference of 4 weeks was recorded after 90 days of storage.

Since PLBs encapsulated in ½ MS medium could be stored for longer time with better survival percentage, therefore PLBs encapsulated in ½ strength MS medium were used for further experiments on storage of *D. lituiflorum*.

b) *Effect of different temperatures on stored encapsulated PLBs*

Temperature had a significant effect on the stored encapsulated PLBs of *D. lituiflorum*. PLBs encapsulated in ½ MS medium at RT though showed 100% survival after 30 days of storage but with further increase in storage time, a decrease in survival percentage of encapsulated PLBs was recorded on being subcultured to MS medium (Fig. 6). Only 10% survival of stored encapsulated PLBs at RT was recorded after 120 days of storage (Fig. 6). All the encapsulated PLBs of *D. lituiflorum* stored at 0°C did not survive. Instead they all turned brown and died subsequently on regrowth medium (not shown in Fig. 6). Hundred percent survival of encapsulated PLBs stored at 4°C was recorded after 30 days of storage on being subcultured to regrowth medium (Fig. 6). However, with further increase in storage time to 60 days, a reduction in survival percentage (80%) of stored encapsulated PLBs was recorded. Further, decrease in survival percentage (50%) was recorded after 90 days of storage. The survival percentage reduced to 20% after 120 days of storage and beyond which survival of encapsulated PLBs was not recorded. At 8°C, till 60 days of storage 100% survival of encapsulated

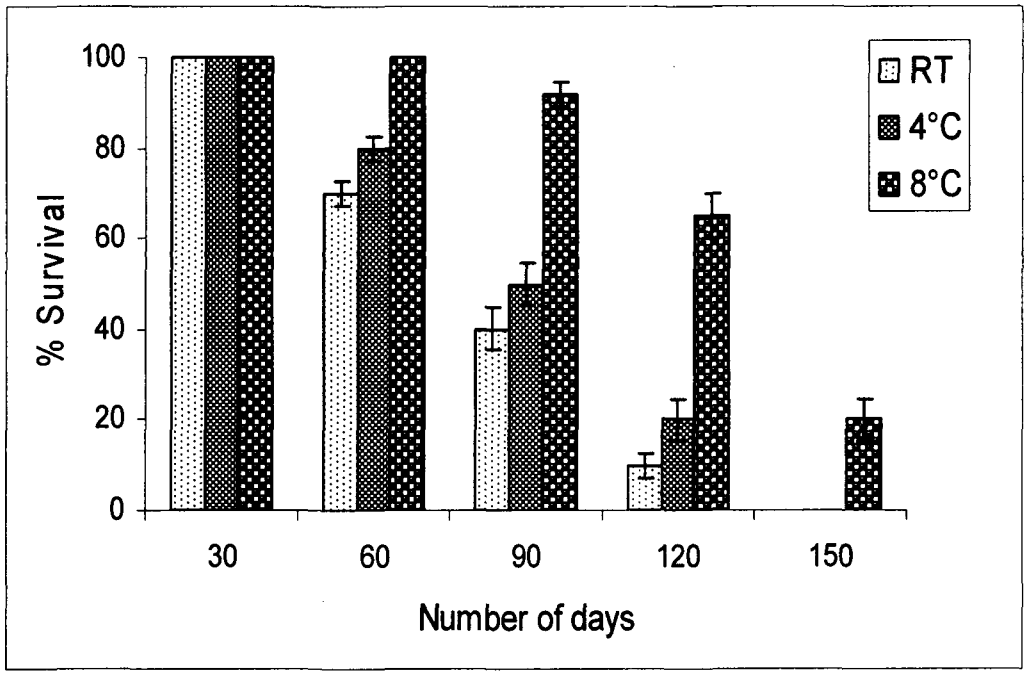


Fig 6: Effect of different temperatures on survival of stored encapsulated PLBs of *D. lituiflorum* in ½ MS medium after 10 weeks subculture in MS medium

PLBs was recorded. Thereafter, a gradual decrease in survival percentage of stored encapsulated PLBs was recorded with increase in storage time (Fig. 6). Encapsulated PLBs of *D. lituiflorum* stored at 8°C could be stored till 150 days but with a low survival percentage of 20%.

Emergence of PLBs and subsequent initiation of shoot and root of stored encapsulated PLBs (Plate 26 a, b, c) at 8°C followed a similar trend as that of *C. devonianum*. However, time taken for emergence of PLBs of *D. lituiflorum* was much faster compared to that of C. *devonianum* (Table 56). Emergence of PLBs was observed within a week after 30 days of storage at RT. But with an increase in storage time of encapsulated PLBs, emergence of PLBs was delayed by 1-2 weeks. Once the PLBs emerged (stage I), it took 3-5 weeks to develop into the next stage (stage II) in all the treatments (Table 56). On the other hand, it took another 2-3 weeks for stage III to be developed.

Longer storage of encapsulated PLBs in ½ strength of MS medium at 8°C was recorded when subcultured to regrowth medium with 20% survival after 150 days of storage.

c) *Effect of different concentrations of osmotica (sucrose, mannitol) and ABA in ½ MS medium on storage of encapsulated PLBs at RT*

Storage of encapsulating PLBs in MS medium devoid of sugars resulted in poor survival percentage (10%) on regrowth medium after 90 days of storage (Fig. 7).

Plate 26

Complete development of plantlets from encapsulated PLBs of *D. lituiflorum* stored for 90 days at 8°C after subculturing to MS medium

a. Germinated encapsulated PLBs after 4 weeks of subculture
b. Development of shoots after 9 weeks of culture
c. Development of roots after 12 weeks of culture

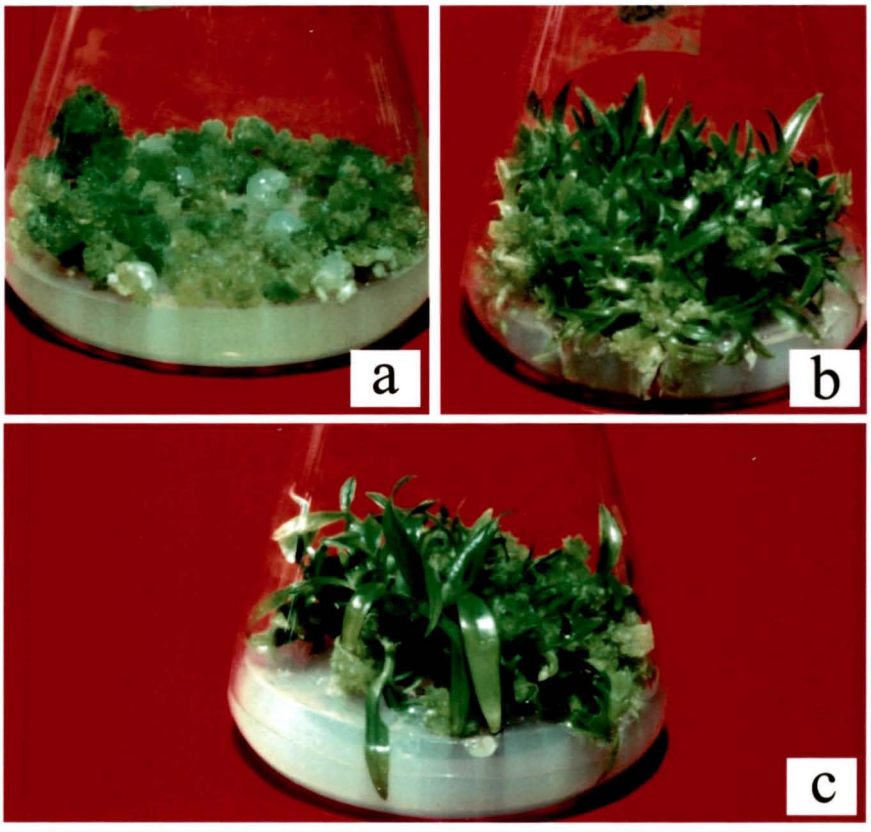

a
b
c

Table 56: Effect of different temperatures on developmental stages of stored encapsulated PLBs in ½ MS medium of *D. lituiflorum* after subculturing to MS medium

Treatments	Storage time (days)	Stages (weeks)		
		I	II	III
RT	30	1	4	6
	60	2	6	8
	90	3	7	10
	120	4	8	11
4°C	30	3	6	8
	60	5	8	10
	90	7	11	14
8°C	30	2	5	7
	60	3	8	11
	90	4	9	12
	120	5	10	12
	150	6	10	13

Stage I - Emergence of PLBs from encapsulated PLB
Stage II - Shoot initiation
Stage III - Root initiation

Sucrose

Addition of sucrose in the encapsulating matrix showed varying results. Incorporation of 2.5 and 5.0% sucrose in the encapsulating matrix resulted in slight increase in survival percentage (35 and 44%) respectively after 90 days of storage. With further increase in sucrose concentration to 7.5% in the encapsulating matrix, increase in survival to 57% was recorded on regrowth medium. However, a decrease in survival percentage of encapsulated PLBs was recorded with further increase in sucrose concentration in the matrix.

Mannitol

Incorporation of different concentrations of mannitol in the encapsulating matrix resulted in better survival response of encapsulated PLBs (Fig. 7) When 2.5% mannitol was incorporated in the encapsulating matrix, 53% survival of encapsulated PLBs was recorded after 90 days of storage. With increase in mannitol concentration to 5.0%, drastic increase in survival percentage (83%) of encapsulated PLBs was recorded and slight increase in survival percentage to 90% was recorded with increase in mannitol to 7.5% in the encapsulating matrix. However, with further increase in mannitol concentration to 10.0 and 12.5% in the encapsulating matrix a decrease in survival percentage of PLBs to 60 and 30% respectively was recorded.

On being subcultured to regrowth medium, the time taken to attain different stages of development of PLBs was delayed in encapsulated PLBs without sugars in the matrix. It took 18 weeks to attain complete plantlets from the encapsulated PLB (Table 57). PLBs encapsulated in medium containing different concentrations of sucrose and

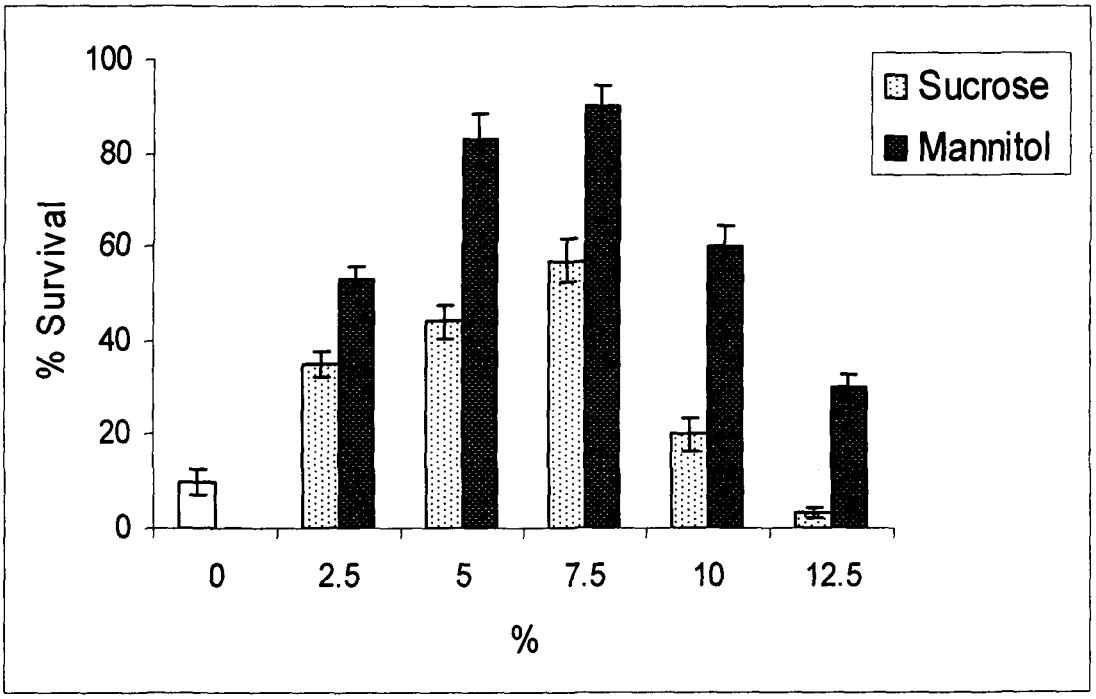


Fig 7: Effect of different concentrations of sucrose and mannitol in ½ MS medium on survival of encapsulated PLBs of *D. lituiflorum* stored for 90 days at RT. Data recorded after 10 weeks subculture in MS medium

Table 57: Different developmental stages of encapsulated PLBs* of *D. lituiflorum* (in ¼ MS medium containing different concentrations of sucrose and mannitol separately) obtained after subculturing to MS medium

Treatments (%)	Stages (weeks)					
	I		II		III	
0	9		13		18	
	Sucrose	Mannitol	Sucrose	Mannitol	Sucrose	Mannitol
2.5	3	-	7	3	10	7
5.0	4	-	10	3	13	9
7.5	6	-	-	4	-	10
10	6	-	-	6	-	13
12.5	9	-	-	8	-	15

Stage I - Emergence of PLBs from encapsulated PLB
Stage II - PLBs with initiation of shoots
Stage III - PLBs with initiation of roots
* Stored for 90 days

mannitol showed different developmental pathway. All the three stages of development were observed in low concentrations (2.5 and 5.0%) of sucrose (Plate 27 a), whereas small unhealthy PLBs were developed in encapsulated PLBs containing high percentage of sucrose (7.5%, 10% and 12.5%; Plate 27 b). A delay in emergence of encapsulated PLBs was recorded with gradual increase in sucrose concentrations (Table 57). PLBs encapsulated in matrix with high concentrations of sucrose turned brown and died after 3 months of culture. PLBs encapsulated in different concentrations of mannitol bypassed stage I development. Instead, the whole PLB inside the encapsulating matrix got converted into small seedlings (Plate 27 c). Therefore, complete development of plantlet was observed faster in mannitol encapsulated PLBs when subcultured to MS medium. Time of initiation (3 weeks) of shoots from encapsulating PLBs was same in 2.5 and 5.0% of mannitol contained in encapsulating matrix but time taken in initiation of shoots was directly proportional with further increase in mannitol concentration in the encapsulating matrix. Development of roots was however delayed with increase in mannitol concentrations in MS medium of encapsulated PLBs.

ABA

With absence of ABA in ½ MS medium, 43% of encapsulated PLBs survived after 90 days of storage (Fig. 8). Incorporation of ABA in the encapsulating matrix did not support the survival of stored encapsulated PLBs. Only 45% and 50% survival of encapsulated PLBs was recorded with incorporation of 0.5 and 1.0 mg/l of ABA respectively in the matrix. Slight increase in survival percentage to 66% was recorded with 2.0 mg/l of ABA in the encapsulating matrix (Fig. 8). However, with an increase in

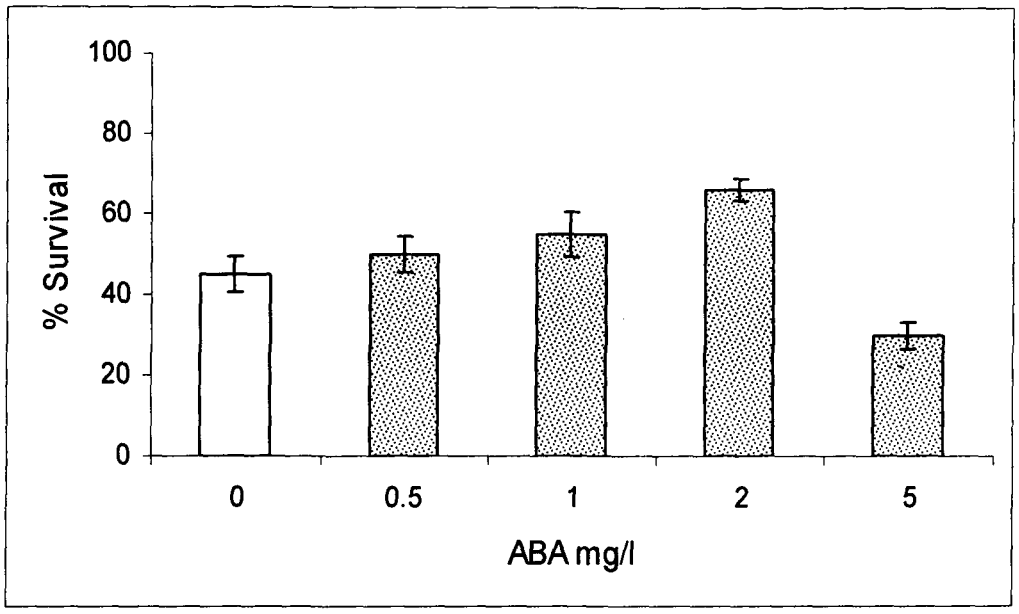


Fig 8: Effect of different concentrations of ABA incorporated in ½ MS medium on survival of encapsulated PLBs of *D. lituiflorum* stored for 90 days at RT. Data recorded after 10 week subculture in MS medium

ABA concentration to 5.0 mg/l, reduction in survival percentage to 30% was recorded after 90 days of storage.

The time taken to attain different stages of development in encapsulated PLBs was faster in control when compared to the encapsulated PLBs in medium containing different concentrations of ABA. The time taken for germination of PLBs encapsulated in 0.5 mg/l and 1.0 mg/l ABA was same as that of control (Table 58). However, with further increase in concentration of ABA in the encapsulating medium, a delay in emergence of PLBs was recorded. Shoot initiation was delayed by 9-10 weeks in all the concentrations of ABA tried in the encapsulating medium. Incorporation of ABA at 5.0 mg/l in MS medium inhibited both shoots and root development on regrowth medium (Table 58). On subculturing to fresh medium, only emergence of PLB was recorded incase of 5.0 mg/l ABA contained in encapsulating matrix (Plate 27 d).

Highest survival percentage of encapsulated PLBs stored in ½ strength of MS medium with incorporation of 7.5% mannitol was recorded after 90 days of storage. On the other hand, incorporation of both sucrose and ABA separately in encapsulating matrix resulted in poorer survival percentage of encapsulated PLBs after 90 days of storage when compared to PLBs encapsulated in medium containing mannitol.

DISCUSSION

As for most *in vitro* techniques, the successful implementation of minimal growth technology requires the establishment of specific protocols for each type of explant and species under consideration (Paula *et al.*, 2000). Slow growth by modifying the culture

Table 58: Different developmental stages of encapsulated PLBs* of *D. lituiflorum* (in ¼ MS containing different concentrations of ABA) obtained after subculturing to MS medium

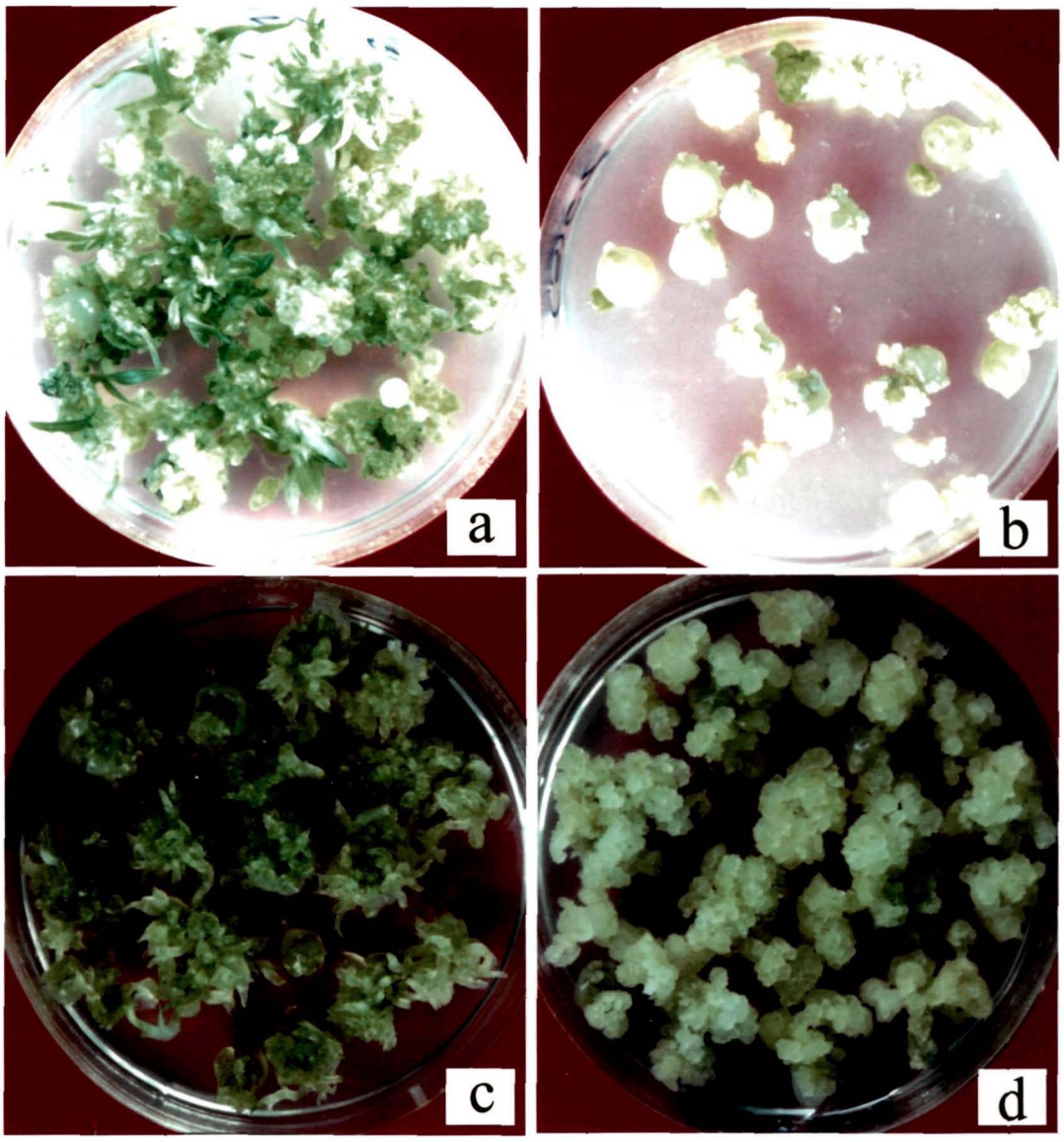
Treatments (mg/l)	Stages (weeks)		
	I	II	III
0.0	2	7	10
0.5	2	11	12
1.0	2	11	13
2.0	4	13	16
5.0	5	-	-

Stage I - Emergence of PLBs from encapsulated PLB
Stage II - PLBs with initiation of shoots
Stage III - PLBs with initiation of roots
* Stored for 90 days

Plate 27

Different stages of development of encapsulated PLBs of *D. lituiflorum* stored for 90 days

a. Developing PLBs and shoots from encapsulated PLBs containing 5% sucrose after 12 weeks of culture
b. Clusters of small unhealthy PLBs from encapsulated PLBs containing 10% sucrose after 12 weeks of culture
c. Direct shoot regeneration from encapsulated PLBs containing 7.5% mannitol after 4 weeks of culture
d. Germinating healthy PLBs from encapsulated PLBs containing 5.0 mg/l ABA after 10 weeks of culture


a
b
c
d

medium has been reported (Withers, 1987; Paula *et al.*, 2000). No work has been reported till date on storage of orchid explants on reduced nutrient medium in the encapsulating matrix. In the present study, PLBs of *C. devonianum* contained in encapsulating matrix prepared in full , ½ and ¼ MS medium showed almost same survival percentage after 120 days of storage. However, ½ MS medium contained in the encapsulating matrix was found best for storage of PLBs of *D. lituiflorum* that could be stored for 120 days with 10 percent survival. The varying requirement of different strengths of MS medium for storage of *C. devonianum* and *D. lituiflorum* may be attributed to their nature of growth. In nature and *in vitro* grown cultures, *C. devonianum* plants grow very slowly. It takes a much longer time to complete its life cycle. Irrespective of presence of different strengths (full, ½ and ¼) of MS nutrient medium in the encapsulating matrix, same rate of growth of PLBs inside the encapsulating matrix was observed. But, reducing the strength of MS medium to 1/8 further in the encapsulating matrix failed to sustain the growth of encapsulated PLBs of *C. devonianum.* Hence, very low survival percentage (6.6%) of *C. devonianum* PLBs was recorded after 120 days of storage. On the other hand, growth of *D. lituiflorum* is very fast in tissue cultured plants. Therefore, with full-strength MS medium in the encapsulating matrix, fast growing PLBs of *D. lituiflorum* absorbed nutrients very fast and as such grew very fast so that storage for only 90 days with 10 percent survival could be attained. PLBs encapsulated in matrix containing full-strength MS medium did not survive after 120 days of storage and dried up when subcultured to regrowth medium. However, addition of ½ MS medium in encapsulating matrix limited the fast growth of *D. lituiflorum* PLBs inside the capsule. Therefore, presence of ½ MS

medium in the encapsulating medium was found optimum for storage of synthetic seeds of *D. lituiflorum*, which could be stored for 120 days with 10% survival. However, with further decrease in MS strength to ¼ and 1/8, storage time of encapsulated PLBs of *D. lituiflorum* was also decreased. This could be due to low availability of MS nutrients, which is below the optimum requirement. The relative growth rate of plants typically increases with resource availability and becomes saturated at some maximum as reported by Grover (1997). Plants that are adapted to low or alternatively to high resource availability responded differentially to changes in resource availability. Plants with low resource requirements usually have a low growth rate and get saturated at low resource availabilities. If resources are moderately available they only respond weakly to a surplus of resources. Plants with high resource requirements respond strongly to additional resources (Chaplin, 1991).

In vitro conservation refers to maintenance of germplasm in a relatively stable form under more or less defined nutrient conditions in an artificial environment. The major aim in developing *in vitro* storage methods is to reduce the frequent demands of subculturing and preserving the unique genetic constitution of germplasm. The most successful and widely applied approach to reduce the rate of growth and thereby extend the interval between transfers to fresh medium for convenient storage involves reducing the growth temperature (Smith *et al.*, 1982; Monette, 1986; Aitken-Christie and Singh, 1987; Evers *et al.*, 1988; Arora and Bhojwani, 1989; Withers, 1991; Ghosh and Sen, 1994; Onay *et al.*, 1996; Antonietta *et al.*, 1999; Chand and Singh, 2004). Moriguchi *et al.* (1988) reported that moderate depression of growth, which is important for minimum

growth during the storage period, could be obtained by reduction of temperature. In the present study, encapsulated PLBs of both *C. devonianum* and *D. lituiflorum* could be stored for 210 and 120 days respectively at 4°C. Encapsulated PLBs of *C. devonianum* and *D. lituiflorum* could be stored further for 330 and 150 days respectively at 8°C. The low but consistent survival of encapsulated cold stored PLBs emphasize the potential of calcium alginate as a coating compound which seems to provide protection, perhaps preventing lethal embryo desiccation. Encapsulated PLBs stored at temperature lower than 4°C did not survive when subcultured to fresh medium rather they turned brown and died. Poor performance in terms of growth rate and secondary product synthesis in certain cases after low temperature treatment has been reported earlier by Hiraoka and Kodama (1984) and Withers (1986). It is thought that the decline in the emergence frequency observed among encapsulated propagules stored at low temperatures may be due to inhibited respiration of plant tissues by alginate (Redenbaugh *et al.*, 1987).

The present experiment using different concentrations of sucrose, mannitol and ABA revealed that different concentrations of sucrose, mannitol and ABA considerably delayed PLB proliferation and growth. In case of *C. devonianum* incorporation of 7.5% of both sucrose and mannitol in the encapsulating matrix resulted in high percent survival after 90 days of storage. However, with incorporation of 2.0 mg/l ABA in the encapsulating matrix, 100% survival of the encapsulated PLBs of *C. devonianum* was recorded after 90 days of storage. In case of *D. lituiflorum* different concentrations of sucrose and ABA in the matrix were less effective in storage of encapsulated PLBs and resulted in lower survival percentage after 90 days of storage. However, encapsulated

PLBs of *D. lituiflorum* could be stored for 90 days when 7.5% mannitol was incorporated in the matrix. In this concentration of mannitol a high percent survival of 90% was recorded. Higher survival percentage recorded in the same concentration of mannitol than that of sucrose could be due to inert nature of mannitol whereas sucrose is easily metabolized. Choi and Jeong (2002) reported that high osmotic treatment might stimulate somatic embryos of *E. senticosus* into dormancy. Choi *et al.* (1999) observed a similar dormancy phenomenon after applying a high sucrose treatment to somatic embryos of *Panax ginseng*. Ammirato and Steward (1971) reported inhibition of precocious germination of somatic embryos of carrot and parshup by raising the osmotic concentration of the culture medium. Sucrose plays two important roles *in vitro* as a carbon source and as an osmotic agent. Increased osmotic stress has been associated with cell plasmolysis (Loveys *et al.*, 1975), cell growth inhibition (Wong and Sussex, 1980) and elevation of endogenous ABA level (Loveys *et al.*, 1975). Exposure of embryo to high sucrose concentration has been linked to maturation (Drew, 1979), inhibition of precocious germination (Ammirato and Steward, 1971) and acquisition of desiccation tolerance (Norstog, 1966). The somatic embryo of alfalfa was tolerant to drying with sucrose treatment (Anandarajah and Kersie, 1990), thus, mimicking the *in vivo* osmotic environment of developing zygotic embryo in controlling embryo development and maturation (Morris *et al.*, 1988). The alginate matrix supplemented with sucrose serves as an artificial endosperm, thereby providing nutrients to the encapsulated explants for plant regrowth (Bapat and Rao, 1992; Nieves *et al.*, 1998). Sucrose is known to provide a carbon source for *in vitro* propagules and its inclusion in the alginate matrix enhanced

plant recovery. However, relatively concentrations of sucrose in the alginate matrix significantly decreased plant development, especially root formation. High levels of sucrose have been found to have adverse effects on shoot and root morphogenesis (George, 1993; Panis, 1995). However, an alginate matrix that contains a high level of sucrose is necessary for cryogenic storage through encapsulation dehydration as sucrose is known to prevent ice nucleation through vitrification (Bryant *et al.*, 2001) and the stability of membranes under severe dehydration (Sakai, 1997).

The sugar alcohol, mannitol is most widely employed as pregrowth media additive for preservation studies (Withers and King, 1980; Ng and Hahn, 1985; Pritchard *et al.*, 1986; Love *et al.*, 1987). However, variations exist between plants in their physiological and structural responses to osmotically active compounds. Pritchard *et al.* (1986) reported growth rate reductions and cell wall thinning in Sycamore and Soyabean cells following osmotic stress but emphasized that cells display a different capacity for osmotic adjustment and alteration in their cytoplasmic complement. Incorporation of 7.5% mannitol in the encapsulation medium was found to be optimum in the present study for the dormancy of encapsulated PLB followed by 5.0% mannitol in the medium. Dehydration of the PLBs at these concentrations seems to be at a critical level, thus resulting in the suspension of growth. Espinoza *et al.* (1986) have also reported that mannitol in the medium exerts an osmotic stress that leads to reduction in the growth rate at 25°C. For successful freeze hardening of maize embryos, a high concentration of osmoticum (mannitol) was reported to result in an increased survival rate (Delvalle *et al.*, 1989). Further, it was observed that the cultures of excised embryos at high osmoticum

induced water stress thus achieving freezing tolerance by low water content. However, a very high osmoticum resulted in a decrease in embryo survival thereby emphasizing that the water stress, apart from the protective action also had a detrimental effect.

An increased endogenous ABA level is associated with reduction in precocious germination of the embryo produced *in vivo* (Santos and Yamaguchi, 1979; Robichaud *et al.*, 1980) and *in vitro* (Ammirato, 1974) which suggests that ABA induces embryo quiescence. ABA-treated embryos while quiescent continue to synthesize specific proteins associated with embryonic growth and seed maturation (Crouch and Sussex, 1981). Ammirato (1974) observed restriction of cell expansion preventing premature cell expansion and precocious germination by ABA in carrot cells. Increase survival of encapsulated ABA-treated carrot embryos may be due to imposition of a developmental arrest (quiescence) during which time embryos mature and develop desiccation tolerance. ABA when added at concentration that did not totally inhibit growth and development could selectively inhibit certain aspects of somatic embryo development (Ammirato, 1974). The inhibitory effect of ABA on the growth of roots, shoots and leaves has been reported in many plant systems (Davies *et al.*, 1980; Bornmann, 1983). Wakabayashi *et al.* (1991) suggested that suppression of growth by ABA application could be the result of inhibition of cell elongation as well as cell wall synthesis (in particular, the synthesis of cellulose and hemi-cellulose polysaccharides) and that ABA-mediated inhibition of cell wall synthesis occurs prior to the suppression of growth of the explants. An ABA concentration, in the present study seems to affect the PLB response greatly at 2.0 mg/l suppressing the growth temporarily but maximum survival was recorded when

subcultured to fresh medium. Higher ABA concentration increases the cytotoxicity possibly, with maximum cultures turning brown by the end of 90 days. Fujii *et al.* (1989) have shown ABA induced arrest in the germination of *alfa alfa* somatic embryos. They have reported that an arrest in the germination of the somatic embryos using ABA leads to their maturation, which is beneficial for direct germination of the same under non-sterile soil conditions. Further, they suggested that the increased conversion frequency of somatic embryos in soil might be due to ABA promoted storage of carbohydrates, proteins and other nutrients within the embryo. Jarret and Gawel (1991) reported that slow growth of *in vitro* cultures could be achieved by adding growth retardants (ABA). Similar results were also reported by Paula *et al.* (2000) where shoot survival in storage was longest in cultures treated with ABA. Slow growth storage methods that are reproducible and are widely applicable among different crops and among genotypes of a crop could be used for the conservation of the germplasm especially the rare and endangered species (Withers, 1991). However, germplasm storage techniques developed should reduce the detrimental effects of growth inhibiting treatment while retaining the desired retardation of growth. No reports on slow growth storage of encapsulated PLBs of orchids *in vitro* have been made so far. Dubus (1980 a, b) reported preservation of *Cymbidium* protocorms by increasing the sucrose concentration, however many other authors have reported maintaining the cultures at low temperatures for storage and preservation (Sharma *et al.*, 1992; Corrie, 1994; Datta *et al.*, 1999; Saiprasad and Polisetty, 2003). The present studies suggest effective storage of encapsulated PLBs at

low temperatures and the suppression of the growth of encapsulated PLB by sucrose, mannitol and ABA at culture room temperature (25 ± 2°C).

CHAPTER VII: HARDENING AND ESTABLISHMENT OF THE *IN VITRO* RAISED PLANTLETS

INTRODUCTION

The transfer of the *in vitro* raised orchid seedlings from the culture vessels to the community pots requires a careful, stepwise procedure which can cause the hardening of the seedlings and hence lead to better survival when transplanted to the pots. The survey of literature shows that the direct transfer of *in vitro* raised plantlets to *in vivo* environment is restricted (Corner and Thomas, 1981; Griffis *et al.*, 1983). However, acclimatization of *in vitro* raised plantlets prior to transfer helps the plants to adapt to the environmental change (Brainerd and Fuchiagam, 1981; Mohammad and Vidaver, 1988; Roy, 1994; Baruah, 1996).

As orchids are delicate plants, they find it difficult to withstand sudden change of relative humidity during *in vitro* to *in vivo* transfer. Moreover, *in vitro* raised plantlets fail to withstand direct exposure to harsher environment outside the *in vitro* regimes due to poorly developed cuticle, stomatal apparatus, photosynthetic ability and conducting tissues (Vij, 1998). Therefore, the first and foremost requirement for successful transplantation is the maintenance of seedlings under very high humidity conditions (90-

100%) for the first 10-15 days (Bhojwani and Razdan, 1983) after which gradual reduction of humidity (70-60%) and temperature (28-38°C) is required (Vij *et al.*, 1995).

Different types of pots have been used for acclimatization of plantlets. Glazed pots are not suitable, as they do not allow sufficient aeration of the roots and the compost. Mukherjee (1983) suggested the use of clay pots for many epiphytic orchids like *Cattleya, Epidendrum, Dendrobium* etc. To facilitate drainage and aeration, the plastic pots are poked for small holes. According to Hedge (1984) four types of containers can be used for orchids viz., (i) pots (ii) baskets or cradles (iii) wooden logs and (iv) tree fern blocks. The use of wooden or bamboo basket or cradle for epiphytic orchids has been recommended. The use of plastic baskets or copper wire baskets as containers for orchid is also common.

Several composts have been proposed for the purpose of hardening and their effect on growth evaluated in different species by Davidson (1956), Arora *et al.* (1975), Hazarika and Sharma (1995), Sharma and Chauhan (1995), Baruah (1996), Sharma and Roy (1996), Sharma and Kaur (1998). Davidson (1956) proposed a mixture of coarse peat moss, dried oak leaves, red wood bark fiber (in equal parts) for profitable cultivation of epiphytic *Cattleya*. Brick, charcoal, bark, leaf-mould, tree-fern, dry sphagnum in 1:1:1:1:1:2 ratio proposed by Sharma and Chauhan (1995) showed 100% survivability in *Dendrobium chrysanthum*. Therefore, an ideal compost which is inert, resistant to organic decomposition, porous to ensure adequate aeration for root respiration, less costly and easily available are mostly supportive for acclimatization of the orchids in the glass house. Meyer (1951) used fern fibres for the compost. Bark based compost have been

used for the cultivation of *Clowesia rosea*, *Dendrobium alexandrae* and *Lemboglossum cervantesii* (Stewart, 1988; Cribb, 1990; Robbins and Bell, 1990). Terrestrial orchids like *Cymbidium*, *Paphiopedilum*, *Phaius*, etc., are generally potted in a porous media containing loamy soil and adequate organic matter but epiphytic orchids are held in position by using stake (Bose and Bhattacharjee, 1980). A majority of workers have used mixtures of equal parts of chopped tree-fern fiber, chopped sphagnum moss and crushed bark preparation. Use of fertilizers proves to be beneficial for healthy growth of orchid seedlings. Addition of the nutrients to the compost varies with the composition of the potting materials and the type of orchids grown.

Besides, the humidity, container, compost, fertilizer, temperature also play an important role in successful transplantation of orchid plantlets. The best temperature range is 18.3°C to 29.4°C. Cribb (1990) reported 18°C and 23°C - 25°C as the minimum and maximum temperatures respectively for better establishment of *Dendrobium alexandrae.* In case of *Phragmipedilum hesseae* 17°C and 30°C were the required minimum and maximum suitable temperatures respectively (Robbins, 1989).

Pretreatment of *in vitro* cultured plantlets before acclimatization to the field have been useful for successful acclimatization of plantlets. Preconditioning by addition of high concentration of sucrose was reported to influence the *in vivo* rooting and establishment of cuttings (Wainwright and Scrace, 1989). Hazarika *et al.* (2000, 2001) also reported that *in vitro* preconditioning of citrus microshoots with sucrose concentrations of 3% was found optimum for subsequent *ex vitro* survival and growth.

They also reported that preconditioning of citrus microshoots with paclobutrazol influence higher *ex vitro* survival by intensifying internode length, thickening of root and reducing leaf dehydration, by regulating the stomatal function and increasing epicuticular wax per unit area of leaf, besides chlorophyll synthesis. In this chapter, successful hardening of the plantlets of *C. devonianum* and *D. lituiflorum*, their transfer and establishment are studied.

MATERIAL AND METHODS

Complete plantlets regenerated *in vitro* from seeds, seedlings and PLBs derived either from apical meristem, axillary buds, leaf bases and from stored synthetic PLBs of both *C. devonianum* and *D. lituiflorum* using the experimental protocols were taken for experiments. Tiny plantlets measuring 2.5-3.0 cm in height were removed from the culture tubes/flasks by means of long handled spoon along with a small amount of the adhering agar. The agar medium sticking to the roots was removed slowly with a brush and care was taken to avoid damage. The plantlets were then transferred to clean thermocol pots of 8 cm diameter containing different mixtures of compost viz., (i) brick and charcoal pieces (1:1), (ii) brick and charcoal pieces (1:1) + moss, (iii) brick, charcoal and decaying litter (1:1:1), (iv) brick, charcoal and decaying litter (1:1:1) + moss, (v) brick, charcoal, decaying litter and saw dust (1:1:1:1), (vi) brick, charcoal, decaying litter and saw dust (1:1:1:1) + moss, (vii) brick, charcoal, decaying litter and cowdung (1:1:1:1:1), (viii) brick, charcoal, decaying litter, and cowdung (1:1:1:1) + moss, (ix) brick, charcoal, decaying litter and coconut husks (1:1:1:1) and (x) brick, charcoal, decaying litter and coconut husks (1:1:1:1) + moss.

To minimize the spread of disease, thermocol pots were thoroughly washed and dried. They were then tightly filled with packed composts. The pots were filled with $3/4^{th}$ compost and watered as planting in the moistened compost is easier. The washed plantlets were picked up with the help of forceps and the roots were carefully placed into the crevices of the compost. Single plantlet was potted in each pot. The pots were covered with holed polythene bags for about 2-3 weeks and were carefully sprayed with water and shifted to the glass house for hardening of plantlets. The minimum and maximum temperatures of the glass house at the time of transplantation were 18°C –25°C respectively. The relative humidity of the glass house was around 70-80%. The plantlets were watered alternately in the evening and fed with MS nutrient salt solutions (diluted 10 times) fortnightly for about a month. Readings were recorded after 90 days of hardening after which plantlets were transferred to larger earthen pots measuring 25 cm in diameter.

RESULTS

C. DEVONIANUM

Of the various composts tried, the combination of brick, charcoal, decaying litter (1:1:1) with a layer of moss on top was found to be the best substratum with 90% survival of the hardened plantlets (Table 59). The same compost without a layer of moss resulted in 80% survival. The compost having only brick and charcoal did not support good survival and growth of the hardened plantlets. Addition of moss on top of the compost improved survivability of transferred plantlets to some extent in all cases (Table

Table 59: Re-establishment of *Cymbidium devonianum* plantlets after 90 days of hardening		
Treatment	Survival %	Height (cm)
Brick + charcoal(1:1)	50 ± 7.1	2.1 ± 0.6
Brick + charcoal(1:1) + moss	70 ± 4.7	2.9 ± 0.9
Brick + charcoal + decaying litter (1:1:1)	80 ± 4.7	5.3 ± 0.9
Brick + charcoal + decaying litter (1:1:1) + moss	90 ± 4.1	5.8 ± 0.7
Brick + charcoal + decaying litter + saw dust (1:1:1:1)	41 ± 3.6	3.9 ± 0.6
Brick + charcoal + decaying litter + saw dust (1:1:1:1) + moss	55 ± 4.7	4.6 ± 0.9
Brick + charcoal + decaying litter + cow dung (1:1:1:1)	67 ± 1.3	2.8 ± 0.8
Brick + charcoal + decaying litter + cow dung (1:1:1:1) + moss	72 ± 1.3	3.6 ± 1.0
Brick + charcoal + decaying litter + coconut husk (1:1:1:1)	35 ± 4.7	3.1 ± 0.2
Brick + charcoal + decaying litter + coconut husk (1:1:1:1) + moss	45 ± 3.6	3.7 ± 0.5

± SE

59). The compost comprising brick + charcoal + decaying litter with addition of saw dust and coconut husk did not support good survival but as mentioned earlier with addition of moss on top improved survival percentage. Feeding the plantlets with diluted MS nutrient salt solution fortnightly proved to be beneficial for the healthy growth of hardened plantlets. In 40-50 days of time the plantlets were hardened and by 90 days established healthy growth of plantlets were observed (Plate 28).

D. LITUIFLORUM

The compost containing brick + charcoal + decaying litter with a layer of moss was best for survivability percentage (77%) and growth of the plantlets (Table 60). As tall as 6.6 cm hardened plantlets were obtained in this mixture of compost (Plate 29). The compost containing brick + charcoal + decaying litter without addition of layer of moss resulted in reduced survival of the transferred plants (Plate 29). The compost comprising brick + charcoal + decaying litter + cowdung (1:1:1:1) with a layer of moss was also a suitable substratum and resulted in 65% survival. Addition of moss layer to the entire substratum proved beneficial for both survival and height of the hardened plants (Table 60). On the other hand, the composts containing brick + charcoal brick + charcoal + decaying litter + sawdust, did not support good survivability and growth of the hardened plants. The plantlets were hardened and established after 40-50 days time. Complete established plantlets were developed after 90 days (Plate 29) after which they were transferred to earthen pots for further growth and development (Plate 29). The *in vitro* raised hardened plants of about 2 ½ years age flowered in the glass house in the months of March and April (Plate 29).

Plate 28

Hardened plants of *C. devonianum*

a. *In vitro* raised plantlets transferred to thermocol pots after 90 days

b. Plantlets transferred to earthen pots after 180 days


a
b

Plate 29

Hardened plants of *D. lituiflorum*

a. *In vitro* raised plantlets transferred to thermocol pots after 90 days

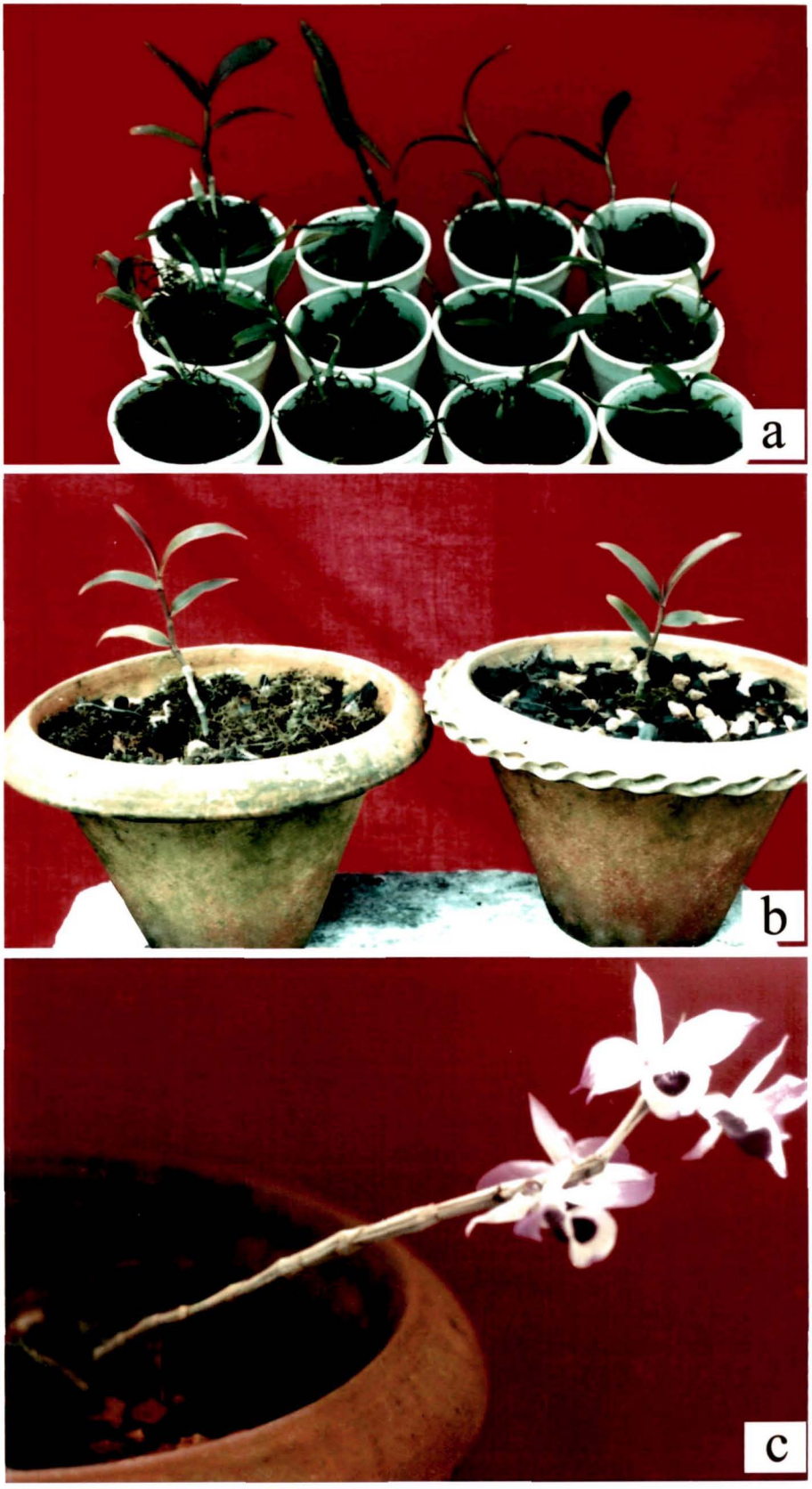
b. Plantlets transferred to earthen pots after 180 days

c. Flowers of tissue culture raised plants after 2 ½ years

Table 60: Re-establishment of *Dendrobium lituiflorum* plantlet after 90 days of hardening

Treatments	Survival %	Height (cm)
Brick + charcoal (1:1)	40 ± 2.3	3.2 ± 1.3
Brick + charcoal (1:1) + moss	47 ± 2.7	3.4 ± 0.8
Brick + charcoal + decaying litter (1:1:1)	60 ± 4.7	4.5 ± 1.2
Brick + charcoal + decaying litter (1:1:1) + moss	77 ± 4.9	6.6 ± 2.4
Brick + charcoal + decaying litter + saw dust (1:1:1:1)	43 ± 2.7	3.6 ± 0.9
Brick + charcoal + decaying litter + saw dust (1:1:1:1) + moss	55 ± 2.3	4.5 ± 0.9
Brick + charcoal + decaying litter + cow dung (1:1:1:1)	59 ± 3.6	4.9 ± 0.5
Brick + charcoal + decaying litter + cow dung (1:1:1:1) + moss	65 ± 3.6	5.6 ± 1.3
Brick + charcoal + decaying litter + coconut husk (1:1:1:1)	30 ± 2.5	2.8 ± 0.6
Brick + charcoal + decaying litter + coconut husk (1:1:1:1) + moss	37 ± 3.6	3.2 ± 0.4

± SE


a
b
c

DISCUSSION

The successful transplantation of the *in vitro* grown plantlets depends on the suitable size and the compost used for potting. Healthy plantlets showing vigorous growth in the culture vessels were transferred to the pots. It is a well-established fact that the hardiest and vigorous plants are easier to transplant and these are less susceptible to diseases and mechanical injuries. The transferred plantlets had a healthy and vigorously growing root system, which ensured higher establishment and growth. The different composts used in this investigation for both *C. devonianum* and *D. lituiflorum* were found to be satisfactory for survivability and normal growth of the plantlets. However, percentage survivability (90% and 77%) respectively of *C. devonianum* and *D. lituiflorum* were obtained on substratum containing brick, charcoal, decaying litter (1:1:1) with a layer of moss on top as this compost may have facilitated proper drainage and aeration for root respiration. The layer of moss on top proved to be beneficial due to higher retention of moisture content. It has been reported that successful transplantation requires the maintenance of seedlings under high humidity for initial period of transfer (Bhojwani and Razdan, 1983). The rate of survival and growth of the plantlets was reduced on a substratum containing brick, charcoal and decaying litter with either sawdust or coconut husk in *C. devonianum*. However, presence of only brick and charcoal without addition of decaying litter also reduced the rate of survival and height of the plantlets. Addition of cowdung to the compost brick, charcoal and decayng litter improved the survival rate and heights of the plantlets in both *C. devonianum* and *D. lituiflorum*. Similar results where decaying litter is found to be effective on growth and

survivability have been reported in *Vanda coerulea* plantlets (Kalita, 1999). Baruah (1996) achieved 90% survivability of *D. transparens* on potting medium containing charcoal, brick pieces, chopped fern roots and farmyard manure whereas Sharma and Chauhan (1995) reported 100% survivability of *D. chrysanthum* on potting media containing brick chips leaf mould, tree fern and dry sphagnum. The survivability of the micropropagated plantlets depends on proper acclimatization. The best method to ensure survival of cultured plantlets under natural condition is to expose them gradually to a relatively lower humidity, higher temperature and higher light intensity. Sharma and Chauhan (1995) and Vij *et al.* (1995) also suggest acclimatization prior to *ex vitro* environment. Feeding the plantlets with diluted MS nutrient salt solution was found to be beneficial as it supplied the essential nutrients to the developing plantlets. The promotion of orchid seedling growth by the nutrient solution for initial hardening has been reported in many instances (Sheehan, 1960; Sander, 1969, 1979; Mukherjee, 1983; Kumaria and Tandon, 1994). The procedure described for the hardening and transplanting of the plantlets of C. *devonianum* and *D. lituiflorum* results in 90% and 80% survivability respectively and healthy growth of the plantlets. The compost found most suitable (brick + charcoal + decaying litter with a layer of moss) is easily available, porous and convenient for the transfer and establishment of these *in vitro* grown plantlets.

CHAPTER VIII: SUMMARY

Different media viz., MS, NN, B_5, Mitra and KnC were tried for asymbiotic seed germination and protocorm development of *C. devonianum* and *D. lituiflorum* seeds. The orchid seeds of these two orchids responded differentially to different media used for germination. The best germination percentage for C. *devonianum* was recorded on B_5 medium followed by NN and MS medium. The seeds showed poor germination on Mitra medium. On KnC medium, slight swelling of seeds was observed which failed to germinate and turned brown in course of time. The seeds were considered to have germinated upon emergence of the embryo from the testa. Protocorm volume recorded also varied from medium to medium and the protocorm volume was determined using the formula given by Stoutamire (1981) for an oblete speroid $4/3\ \pi\ a^2\ b$, where a and b are minor and major semi-axes respectively. The protocorm volume was recorded to be the largest on B_5 medium followed by NN and MS medium. The time taken in attaining different stages of protocorm development on different media also varied. On B_5 medium, germination was faster than that observed on other media whereas, further development of protocorms into shoots and roots was faster on MS medium. Development of the same stage was however delayed in B_5 and NN media. The protocorms on Mitra medium did not develop into shoots and roots and eventually turned yellow and died whereas seeds of

D. lituiflorum germinated on all the five media tried and maximum germination were recorded on MS medium followed by NN and B_5 media. The percentage germination was comparatively reduced on Mitra and KnC media. There was a large variation in the volume of protocorm of *D. lituiflorum* developing on the different media. The protocorm volume was also recorded the largest on MS medium. The development of all stages of protocorms of *D. lituiflorum* was also faster in MS medium.

Among the five different media (MS, NN, B_5, Mitra and KnC) tried for the seedling growth of *C. devonianum*, fresh weight, shoot number and length was high on MS medium whereas maximum root length was observed on B_5 medium followed by Mitra medium. Seedlings cultured on KnC medium remained green for 2 weeks of subculture after which they started browning and died subsequently. Growth regulators have differential effect on seedling growth of *C. devonianum* and *D. lituiflorum*. Among the auxins (IAA, NAA and 2,4-D) tried in the medium, it was found that incorporation of 0.5 mg/l and 2.5 mg/l of IAA singly in the medium enhanced root number and length respectively of *C. devonianum* seedlings. The response of *C. devonianum* seedlings on medium containing other auxins was, however, poor. Of the various growth parameters tried in the medium, shoot number was recorded highest at 0.5 mg/l BAP. However, with increase in BAP concentration to 5.0 mg/l in the medium, a decrease in shoot number was observed. Roots were completely suppressed at 2.5 mg/l and at higher concentrations of BAP tried in the medium. As compared to the control, the growth of the seedlings (fresh weight, shoot number and length) was slightly enhanced at low concentrations of KN in the medium. The fresh weight, number and length of shoots were recorded to be

higher at 2.5 mg/l of KN. However as the concentration of KN was increased in the medium overall growth of the seedlings was comparatively inhibited. The interacting effects of cytokinin and auxin resulted in higher fresh weight at 2.5 mg/l KN in combination with 0.5 mg/l of IAA. Maximum number of shoots was recorded in a combination of 0.5 mg/l each of BAP and IAA in the medium. Shoot length was higher on the medium containing 0.5 mg/l each of KN and IAA in the medium. Root number observed in different combination of growth regulators tried in the medium was higher on the medium containing 0.5 mg/l each of BAP and IAA. Compared to control, root length observed was poor in all the combinations of growth regulators tried. Among the different combinations of auxins tried in the medium, IAA in combination with KN/BAP proved better in overall development of *C. devonianum* seedlings. In case of *D. lituiflorum* seedlings, slight increase in shoot number was recorded at 0.5 mg/l of IAA in the medium whereas the fresh weight, shoot number and shoot length of the seedlings increased at 2.5 mg/l of NAA in the medium. Similarly, the number of roots initiating in *D. lituiflorum* seedlings in 2.5 mg/l NAA supplemented medium was observed to be increased drastically. Addition of 2,4-D in the medium had a differential effect on the developing seedlings. At low concentration of 2,4-D (0.5 mg/l) in the medium, multiple shoots were developed where both shoot and root number were increased comparatively to control. However, at higher concentrations (2.5 and 5.0 mg/l) of 2,4-D in the medium, the whole seedlings developed into PLBs. At high concentration (10.0 mg/l) of 2,4-D in the medium, the seedlings turned brown and died. Seedling growth of *D. lituiflorum* in terms of fresh weight, shoot length, root number and length showed poor response in

medium containing BAP. However, the number of multiple shoots emerging increased at 2.5 mg/l BAP in the medium. In the present study, optimum development of seedlings of *D. lituiflorum* was recorded at low concentration of KN present in the medium when compared to control. The interaction of auxin and cytokinin in the medium produced differential response of *D. lituiflorum* seedlings. The seedling growth was more or less inhibited in the medium containing BAP in combination with IAA. However, optimum growth of seedling in terms of shoot number was recorded at 2.5 mg/l BAP and 0.5 mg/l IAA incorporated together in MS medium. Roots were completely suppressed in combinations containing higher concentrations of BAP and IAA in the medium. The seedling growth of *D. lituiflorum* was enhanced slightly in the medium containing combinations of BAP and NAA. In the present study, an interesting feature was recorded in the medium containing 2,4-D in combination with BAP/KN, with both PLBs as well as multiple shoots developing. In a combination of 0.5mg/l each of BAP and 2,4-D and 2.5 mg/l BAP + 0.5 mg/l IAA in the medium, multiple shoots were developed from the seedling however, the overall growth of the seedlings in these concentrations was inhibited when compared to control. Whereas 0.5 and 2.5 mg/l BAP when combined separately with different concentrations (2.5, 5.0 and 10.0 mg/l) of 2,4-D; and combinations of 5.0 mg/l of BAP with (0.5, 2.5 and 5.0 mg/l) of 2,4-D also separately in the medium resulted in the formation of PLBs. A high explant response (92%) and high number (138) was observed in the form of PLBs at 0.5 mg/l BAP in combination with 2.5 mg/l 2,4-D in the medium. These PLBs multiplied profusely when transferred to fresh MS medium and developed into complete plantlets within 8 weeks of subculture.

Micropropagation of *C. devonianum* and *D. lituiflorum* was carried out keeping in mind the mass propagation of these plantlets. Nutritional requirements for the optimal growth of the explants *in vitro* vary from species to species. Out the different explants sources tried, the apical meristem and leaf bases of *C. devonianum* were found suitable for micropropagation. The apical meristem and leaf bases of *C. devonianum* cultured on different media i.e., MS, NN, B_5, Mitra and KnC did not respond in the form of PLBs or shoot buds however, both explants remained green for longer time in MS medium. Therefore, addition of growth adjuncts in the medium was obligatory for evoking responses in apical meristem and leaf bases of *C. devonianum*. The apical meristem of *C. devonianum* cultured on MS medium containing different concentrations of IAA in the medium was poor. However, low concentration of NAA (0.5 mg/l) in the medium favoured high percentage response of apical meristem of *C. devonianum* in the form of PLBs whereas the explant failed to respond in all the treatments of 2,4-D tried in the medium. Addition of cytokinin (BAP/KN) in the medium evoked a positive response of the apical meristem. At 2.5 mg/l BAP in the medium, 3-5 clusters of PLBs were observed to have emerged. KN at 0.5 mg/l in the medium resulted in high response of cultured apical meristem wherein 7-10 clusters of hairy, yellowish green and healthy PLBs were observed.

A combination of auxin (IAA/NAA) with BAP resulted in differential response. Apical meristem responded poorly to the BAP + IAA combination treatment in the medium, however, higher response of conversion (60%) of apical meristem with 4-8 numbers of PLBs was observed at 2.5 mg/l BAP in combination with 0.5 mg/l NAA.

Around 3-6 PLBs also emerged from the cultured meristem at 0.5 mg/l each of BAP and NAA in the medium. Among the different parts of the leaf tried in the culture, response was observed only with the cultured leaf bases. The other parts of leaf dried up. In the present study, leaf bases of *C. devonianum* responded to auxins IAA and NAA supplemented in the medium. However of the two above-mentioned auxins, leaf bases responded better in IAA containing medium, the highest explants response being recorded at 2.5 mg/l of IAA where 3-5 green healthy PLBs developed. With increase in the concentration of IAA in the medium, a decrease in explant response and number of PLBs was recorded. Leaf bases cultured in the medium containing 2,4-D failed to induce PLB formation .

Among the cytokinins (BAP/KN) tried singly in the medium, highest response of leaf base with 3-6 numbers of green PLBs emerging was observed at low concentration (0.5 mg/l) of KN in the medium. Of the various combinations of growth regulators studied in the medium, a high percentage response of leaf base was observed in 0.5 mg/l BAP + 2.5 mg/l IAA wherein 6-15 clusters of well developed globular green PLBs were observed. In case of *D. lituiflorum,* out of all different explant sources tried, axillary bud and apical meristem were found suitable for micropropagation. Out of the different media tried, the cultured axillary bud responded best on MS medium with 2-3 shoots per explant emerging. Both shoot buds and PLBs were seen emerging from the axillary bud of *D. lituiflorum* cultured at 2.5 and 5.0 mg/l of IAA in the medium. However, response of axillary buds in NAA containing medium followed only shoot bud regeneration. Presence of NAA (2.5 mg/l) in the medium induced 100% explant response and maximum number

of shoots was also recorded in the same concentration of NAA. The regeneration pathway of axillary bud cultured on MS medium supplemented with different concentration of 2,4-D was found to be through PLB formation. About 83.3% of explant response in terms of PLBs was recorded at 0.5 mg/l of 2,4-D in the medium. Cytokinins have been used to multiply cultures of several orchids. In the present study, it was found that the effect of cytokinin (BAP/KN) on shoot bud production and PLB formation from axillary bud was promotory in case of *D. lituiflorum*. Different concentrations of BAP in combination with auxins (IAA, NAA and 2,4-D) brought varying responses from the cultured explants. BAP in addition to either IAA or NAA resulted in the formation of only shoot buds. Medium containing both BAP and IAA in combination showed 100% explant response at 2.5 mg/l each of BAP and IAA and at 2.5 mg/l BAP + 5.0 mg/l of IAA. However, highest number of shoots/explant was recorded in the former concentrations of growth regulators. Out of all combinations of BAP and NAA tried in the medium, 100% explant response with 5 shoots/explant was recorded in low concentration of BAP (0.5 mg/l) in combination with 2.5 mg/l of NAA. Combination of 2,4-D either with BAP/KN at certain concentrations induced both PLBs and shoot buds from axillary buds of *D. lituiflorum*. Combination of BAP (0.5 mg/l) with different concentrations of 2,4-D (0.5, 2.5, 5.0 and 10.0 mg/l) only PLBs were induced from the explants. However, with further increase in BAP concentration (2.5 mg/l) in combination with different concentrations of 2,4-D (0.5 and 2.5 mg/l), and in combination of 5.0 mg/l BAP with 0.5 mg/l 2,4-D both PLBs and shoot buds were induced from the explants. Study of combination of KN and 2,4-D showed varying results in the formation of shoots and PLBs from axillary bud of *D.*

lituiflorum. Here with all concentrations of KN (0.5, 2.5, 5.0 and 10.0 mg/l) in combination with 0.5 mg/l of 2,4-D both shoots and PLBs were formed, but BAP in combination with increased 2,4-D concentrations (2.5, 5.0 and 10.0 mg/l) the explant developed into PLBs only. The highest explant response in terms of PLBs was observed at 2.5 mg/l each of BAP and 2,4-D. Incorporation of KN in combination with IAA or NAA induced only shoot formation but at 0.5 mg/l KN in combination with different concentration (0.5, 2.5 and 5.0 mg/l) of NAA in the medium, low induction of PLBs from axillary buds of *D. lituiflorum* was observed. Maximum explant response (93.3%) in terms of shoots/explants was recorded at 5.0 mg/l KN in combination with 2.5 mg/l IAA.

Among all the basal media tried (MS, NN, B_5, Mitra and KnC) for induction of PLBs/ shoot buds from apical meristem of *D. lituiflorum*, explant response of 10% in terms of PLBs was recorded in apical meristem in MS medium only. In this medium green globular PLBs could be seen developing from apical meristem. The apical meristem of *D. lituiflorum* cultured in the medium containing auxins (IAA, NAA, 2,4-D) and cytokinins (BAP, KN) responded differently when tested individually and in combination. Among the auxins, IAA at 2.5 mg/l in the medium was best for explant response in the form of PLBs. A better response of apical meristem was also recorded at 2.5 mg/l NAA and 0.5 mg/l of 2,4-D incorporated singly in the medium. PLB initiation was enhanced at 2.5 mg/l BAP followed by 2.5 mg/l of KN when added singly in the medium. Addition of BAP in combination with IAA/NAA or 2,4-D in the medium, resulted in lower explant response in terms of PLBs when compared to BAP alone. Supplementation of KN in combination with different auxins (IAA, NAA and 2,4-D)

showed varying results in the explant response. At 2.5 mg/l each of KN and IAA and 5.0 mg/l KN + 2.5 mg/l IAA showed better explant response in terms of PLB induction.

Artificial seed is an analog to a natural seed and the encapsulating matrix is expected to play the role of a seed coat, endosperm and cotyledons. Studies on encapsulation have shown that alginate hydrogel is suitable matrix for synthetic seed production. The major principle involved in the alginate encapsulation process is that the sodium alginate droplets containing the explants when dropped into the complexing agents [$Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSO_4.7H_2O$ and $Al(NO_3)_2$] formed round and firm beads due to ion exchange between the Na^+ in sodium alginate with Ca^{2+} in $Ca(NO_3).2H_2O$ and $Ca(OH)_2$; Zn^{2+} in $ZnSO_4.7H_2O$ and Al^{2+} in $Al(NO_3)_2$ solution. The hardiness or rigidity of the capsule mainly depends upon the number of sodium ions exchanged with the corresponding ions. Therefore, it has been suggested that proper concentrations of the gelling agents i.e., sodium alginate and complexing agents [$Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSO_4.7H_2O$ and $Al(NO_3)_2$], and the complexation time should be optimized so as to obtain optimum bead hardness and rigidity.

The present study showed that out of the various gels tried 4% Na-alginate appeared to be the most promising for formation of round firm beads when complexed with 100 mM solutions of $Ca(NO_3).2H_2O$, $Ca(OH)_2$, $ZnSO_4.7H_2O$ and $Al(NO_3)_2$ with ion exchange duration of 20 - 30 min. Lower concentrations of sodium alginate resulted in the formation of very fragile and ill formed beads. Whereas, concentrations higher than 4% proved to be too viscous for the formation of beads. PLBs of both *C. devonianum* and *D. lituiflorum* encapsulated in calcium alginate [prepared in 4% sodium alginate and

complexed with 100 mM $Ca(NO_3).2H_2O$ for 20 - 30 min duration] showed 100% survival on subculture to regrowth medium. On the other hand, PLBs contained in 4% sodium alginate when complexed with 100 mM of either $Ca(OH)_2$ /$ZnSo_4.7H_2O$ or $Al(NO_3)_2.H_2O$ in MS liquid medium did not show any response when subcultured to regrowth medium; rather they turned brown and died.

In vitro conservation refers to maintenance of germplasm in a relatively stable form under more or less defined nutrient conditions in an artificial environment. The major aim in developing *in vitro* storage methods is to reduce the frequent demands of subculturing and preserving the unique genetic constitution of germplasm. The most successful and widely applied approach to reduce the rate of growth and thereby extend the interval between transfers to fresh medium for convenient storage involves modifying the culture medium, reducing the growth temperature, exposing the explants to higher osmotica such as sucrose and mannitol and to growth retardant such as ABA in the medium. In the present study, encapsulated PLBs of *C. devonianum* prepared in full, ½ and ¼ MS medium showed almost same survival percentage after 120 days of storage irrespective of presence of different strengths (full, ½ and ¼) of MS medium in the encapsulating matrix. But, further reducing the strength of MS medium to 1/8 in the encapsulating matrix failed to sustain the growth of encapsulated PLBs of *C. devonianum.* Hence, very low survival percentage (6.6%) of *C. devonianum* PLBs was recorded after 120 days of storage. On the other hand, encapsulated PLBs of *D. lituiflorum* in ½ MS medium was found optimum for storage of synthetic seeds, which could be stored for 120 days with 10% survival. However, with further decrease in MS

strength to ¼ and 1/8, survival of encapsulated PLBs of *D. lituiflorum* was also decreased. The time taken to attain different developmental stages of stored encapsulated PLBs of *C. devonianum* was inversely proportional to decrease in strength of MS medium tried. However, the time taken to attain these stages within the same strength was directly proportional with increase to storage time. Among all the strengths of MS medium studied in the encapsulating matrix for storage, time taken in emergence of PLB, shoot and root initiation was least in full-strength MS medium. However emergence of PLBs, shoot and root initiation stored for same time was delayed in the encapsulating matrix containing 1/8 strength of nutrients in MS medium.

In the present study, encapsulated PLBs of both *C. devonianum* and *D. lituiflorum* could be stored for 210 and 120 days respectively at 4°C. However, encapsulated PLBs *C. devonianum* and *D. lituiflorum* could be stored further for 330 and 150 days respectively at 8°C. Encapsulated PLBs stored at temperature lower than 4°C did not survive when subcultured to fresh MS medium, rather, turned brown and died.

Emergence of PLBs and subsequent development of shoots and roots in encapsulated PLBs stored in different temperatures was observed after subculturing on MS medium. It was found that with decrease in storage temperature, a corresponding increase in time for all the developmental stages of the cultured encapsulated PLBs was recorded. Encapsulated PLBs stored for 30 days at RT showed emergence of PLBs in 3rd week of subculture. However, encapsulated PLBs stored at 4 and 8°C showed emergence of PLBs only at 5th and 4th week of subculture respectively when stored for same time. The shoot and root development was also delayed accordingly.

The present experiment using different concentrations of sucrose, mannitol and ABA revealed that different concentrations of sucrose, mannitol and ABA considerably delayed PLB proliferation and growth. In case of *C. devonianum* incorporation of 7.5% of both sucrose and mannitol in the encapsulating matrix resulted in high percent survival after 90 days of storage. However, incorporation of 2.0 mg/l of ABA in the encapsulating matrix, 100% survival of the encapsulated PLBs of *C. devonianum* was recorded after 90 days of storage. Whereas, encapsulated PLBs of *D. lituiflorum* could be stored for 90 days with 90% survival when 7.5% of mannitol was incorporated in the matrix.

A varied difference in the developmental stages of encapsulated PLBs of both *C. devonianum* and *D. lituiflorum* enclosed in different concentrations of sucrose and mannitol was observed on regrowth medium after 90 days of storage. Emergence of PLBs and subsequent initiation of shoots and roots was delayed in the encapsulated PLBs of *C. devonianum* and *D. lituiflorum* without incorporation of any sugars in the encapsulating matrix. In case of *C. devonianum* emergence of PLBs was slightly faster in all the encapsulated PLBs with all concentrations of sucrose compared to encapsulated PLBs enclosed with different concentrations of mannitol. However, subsequent initiation of shoots was much delayed in the encapsulating PLBs incorporated with 2.5 - 7.5% of sucrose in the encapsulating matrix. Though shoot and root development was observed in lower concentrations of sucrose (2.5 - 7.5%) but the shoots and roots were completely inhibited in high concentrations (10 and 12.5%) of sucrose in the encapsulating matrix. Shoots developed on mannitol containing matrix were morphologically different from that formed in encapsulation matrix containing sucrose. Here the shoots were thin, long

and slender and aroused while still embedded in the beads. Once the shoots were formed, the time taken in root development was however faster in PLBs encapsulated with all concentrations of sucrose when compared to PLBs encapsulated with mannitol on subculture to regrowth medium whereas in case of *D. lituiflorum* PLBs encapsulated in different concentrations of mannitol bypassed PLB proliferation stage when subcultured to regrowth medium. Here, the whole PLBs inside the encapsulating matrix got converted into small seedlings. Therefore, in *D. lituiflorum* complete development of plantlet was observed faster in mannitol encapsulated PLBs when subcultured to MS medium. Development of roots was however delayed with increase in concentrations of encapsulated mannitol in MS medium.

The successful transplantation of the *in vitro* grown plantlets depends on the suitable size and the compost used for potting. Healthy plantlets showing vigorous growth in the culture vessels were transferred to the pots. The different composts used in this investigation for both *C. devonianum* and *D. lituiflorum* were found to be satisfactory for survivability and normal growth of the plantlets. However, percentage survivability (90% and 80%) respectively of *C. devonianum* and *D. lituiflorum* were obtained on substratum containing brick, charcoal, decaying litter (1:1:1) with a layer of moss on top. The rate of survival and growth of the plantlets was reduced on a substratum containing brick, charcoal and decaying litter with either sawdust or coconut husk in *C. devonianum*. However, presence of only brick and charcoal without addition of decaying litter also reduced the rate of survival and height of the plantlets. Addition of cowdung to the compost brick, charcoal and decayng litter improved the survival rate and heights of the

plantlets in both *C. devonianum* and *D. lituiflorum*. The survivability of the micropropagated plantlets depends on proper acclimatization. The best method to ensure survival of cultured plantlets under natural condition is to expose them gradually to a relatively lower humidity, higher temperature and higher light intensity.

CHAPTER 1X: REFERENCES

Abdul Karim, A. G. and H. Hairani. 1990. Leaf cultures of some Malayasian orchids. In: Proc. International Conference and Exhibition on Orchids and Ornamental Plants. pp. 12. Kualalumpur, Malayasia

Adriani, M., E. Piccioni and A. Standardi. 2000. Effects of different treatment on the conversion of 'Hayward' Kiwi fruit synthetic seeds to whole plants following encapsulation of *in vitro* derived buds. NZJ crop Hortic. Sci. 28: 59-67.

Ahmedullah, M. 2000. Endemism in the Indian Flora. In: Flora of India-Introductory Vol. Part II (ed. N.P. Singh) pp. 246-65. Bot. Survey of India, Calcutta.

Aitken-Christie, J. and A. P. Singh. 1987. Cold storage of tissue cultures. In: J. M. Bonga and D. J. Durjan (eds.), Tree and Tissue Culture in Forestry, Vol. 2. Specific Principles and Methods, Growth and Development, Martinus, Nijhoff, Dordrecht., pp. 285-304.

Alan, R. 1989. The effect of nitrogen nutrition on growth, chemical composition and response of cucumbers (*Cucumis sativus* L.) to nitrogen forms in solution culture. J. Hort. Sci. 64: 467-474.

Amaki, W. and H. Higuchi. 1989. Effects of dividing on the growth and organogenesis of protocorm like bodies in *Doritaenopsis*. Sci. Hort. 39: 63-72.

Ammirato, P. V. 1974. The effects of ABA on the development of somatic embryos from cells of caraway. Bot. Gaz. 135: 328-329.

Ammirato, P. V. and F. C. Steward. 1971. Some effects of the environment on embryos from cultured free cells. Bot. Gaz. 132: 149-158.

Anandarajah, K. and B. D. Mc Kersie. 1990a. Manipulating the desiccation tolerance and vigour of dry somatic embryos of *Medicago sativa* L. with sucrose, heat shock and abscisic acid. Plant Cell Rep. 9: 451-455.

Anandarajah, K. and B. D. Mc Kersie. 1990b. Enhanced vigour of dry somatic embryos of *medicago sativa* L. with increased sucrose. Plant Sci. 71: 261-266.

Antonietta, G. M., E. Piccioni and A. Standardi. 1999. Effects of encapsulation on *Citrus reticulata* Blanco somatic embryo conversion. Plant Cell Tiss. Org. Cult. 55: 235-237.

Arditti, J. 1967. Factors affecting the germination of orchid seeds. Bot. Res. 7: 422-638.

Arditti, J. 1977. Clonal propagation of orchids by means of organ and tissue culture, pp. 203- 293. In J. Arditti (ed.), Orchid Biology, Reviews and Perspectives Vol. 1. Cornell Univ. Press Ithaca, NewYork.

Arditti, J. 1979. Aspects of the physiology of orchids. Adv. Bot. Res. 7: 241-665.

Arditti, J. 1982. Orchid seed germination and seedling culture - A manual, pp. 244-370. In J. Arditti (ed.), Orchid Biology, Reviews and Perspectives II. Cornell University Press Ithaca, New York.

Arditti, J. and R. Ernst. 1984. Physiology of germinating Orchid seeds. pp. 177-222. In J. Arditti (ed.), Orchid Biology, reviews and perspectives III. Cornell University Press, Ithaca, New York

Arditti, J. and R. Ernst (ed.). 1993. Micropropagation of orchids. John Wiley and Sons Inc. New York.

Arditti, J., J. D. Michaud and A. P. Oliva. 1981. Seed germination of North American Orchids. I. Native California and related species of *Calypso*, *Epicactis*, *Goodyera*, *Piperia* and *Platanthera*. Bot. Gaz. 142 (4): 442-453.

Arora, Y. K., A. S. Rao and A. Mukherjee. 1975. Fancy *Cymbidium* in North East India. Indian Hort. 20: 23-27.

Arora, R. and S. S. Bhojwani. 1989. *In vitro* propagation and low temperature storage of *Sassaurea lappa* C. B. Clarke-an endangered medicinal plant. Plant Cell Rep. 8: 44-47.

Bahme, R. 1949. Nicotinic acid as a growth factor for certain orchid embryos. Sci. 109: 522-23.

Banerjee, N. and E. A. L. De Langhe. 1985. A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (Banana and Plaintain). Plant Cell Rep. 4: 351-354.

Bapat, V. A., M. Mhatre and P. S. Rao. 1987. Propagation of *Morus indica* L. by encapsulated shoot buds. Plant Cell Rep. 6: 393-395.

Bapat, V. A. and P. S. Rao. 1988. Sandalwood plantlets from Synthetic seeds. Plant Cell Rep. 7: 434-436.

Bapat, V. A. and P. S. Rao. 1992. Plantlet regeneration from encapsulated and non-encapsulated desiccated somatic embryos of forest tree: Sandalwood (*Santalum album* L). J. Plant Biochem. Biotech. 1: 109-113.

Baruah, R. 1996. *In vitro* micropropagation of the species of *Calanthe, Phaius* and *Dendrobium.* Ph. D. Thesis. G. U.

Batygina, T. B. and E. V. Andronova. 1988. (In Russ.). Do Orchidaceae have cotyledons? DOKL AKAD NAUK SSSR. 302 (4): 1017-1019.

Begum, A. A., M. Tamaki, M. Takahara and S. Kato. 1994. Somatic embryogenesis in *Cymbidium* through *in vitro* culture of inner tissue of protocorm-like bodies. J. Jpn. Soc. Hort Sci. 63: 419-427.

Bergman, F. J. 1972a. Shoot tip multiplication of orchid clones, Part 1. The status of "meristem" propagation through 1971. Amer. Hort. 51: 20-23.

Bergman, F. J. 1972b. Shoot tip multiplication of orchid clones, Part I1. The status of "meristem" propagation through 1971. Amer. Hort. 51: 41-44.

Bernard, N. 1909. L' evolution dams la symbiose. Les Orchidales *et* leur champagnons conmousaux. Ann. Sci. Nat. Bot. 9: 1-96.

Bhojwani, S. S. and M. K. Razdan. 1983. Plant Tissue Culture: Theory and Practices. Vol. 5. Elsevier Amsterdam-Oxford. New York, Tokyo.

Bhuyan, J. and P. C. Deka. Seed Culture of *Phaius tankervilliae* BL. The Indian Forrester. 125 (9): 910-912.

Biondi, S. and T. A. Thorpe. 1982. Growth regulator effects, metabolite changes and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata.* Bot. Gaz. 143: 20-25.

Bopaiah, A. K. and S. M. Jorapur. 1986. Studies on growth and development of *Cymbidium aloifolium* Sw. seedling *in vitro*. In: S. P. Vij (ed.), Biology Conservation and Culture of Orchids. pp. 423-427, Affiliated East West Press, New Delhi.

Bornmann, C. H. 1983. Morphological, anatomical and structural responses to abscissic acid. In: F. T. Addicott (ed.), Abscissic acid. CBS Inc., New York. pp. 523 – 555.

Bose, T. K. and T. P. Mukherjee. 1976. Effect of growth substances on growth of protocorm and plantlet formation in seedlings of *Cymbidium giganteum*. Orchid Rev. 84: 107-108.

Bose, T. K. and S. K. Bhattacharjee (ed.). 1980. Orchids of India. Published by M. Mitra, Naya Prakash, Calcutta, India.

Brainerd, K. E. and L. H. Fuchiagam. 1981. Acclimatization of aseptically cultured apple plant to low relative humidity. J. Amer. Soc. Hort. Sci. 106: 515-518.

Bridgen, M. P. and G. L. Staby. 1981. Low pressure and low oxygen storage of *Nicotiana tobacum* and *Chrysanthemum morofolium* through tissue culture. Plant Sci. Lett. 22: 177-186.

Bryant, G., L. K. Koster and J. Wolfe. 2001. Membrane behaviour in seeds and other systems at low water content; the various effects of solutes. Seed Science Research. 11: 17-25.

Butenko, R. G. 1968. Plant Tissue Culture and Plant Morphogenesis. Translated from Russian. Israel programe for scientific Translation, Jerusalem.

Carnijo-Martin, M. J., A. M. Mingocastel and E. Primo-Millo. 1979. Organ re-differentiation in rice callus: Effects of C_2H_4, CO_2 and Cytokinins. Z. Pflanzen. Physiol. 94: 117-123.

Chand, S. and A. K. Singh. 2004. Plant regeneration from encapsulated nodal segments of *Dalbergia sissoo* Roxb., a timber-yielding leguminous tree species. Journal of Plant Physiol. 161: 237-243.

Chang, C. and W. C. Chang. 1998. Plant regeneration from callus culture of *Cymbidium ensifolium* var. misericors. Plant Cell Rep. 17: 251-255.

Chang, C. and W. C. Chang. 2000. Micropropagation of *Cymbidium ensifolium* var. misericors through callus-derieved rhizomes. *In vitro* Cell. Dev. Biol.-Plant. 36: 517-520

Charanasri, U. 1989. Tissue culture of orchids in Thailand, pp. 245-248. In V. Dhawan (ed.). Applications of Biotechnology in Forestry and Horticulture. Plenum Publishing Corp., New York.

Chaplin III F. S. 1991. Effects of multiple environmental stresses on nutrient availability. In: Mooney, M. A., Winner, W. E. and Pell E. J. (eds.), Response of Plants to Multiple Stresses, Academic Press, London pp. 67-88.

Chaturvedi, H. C. and A. K. Sharma. 1986. Mericloning of orchids through culture of the tips of leaves and roots. In: S. P. Vij (ed.), Biology, Conservation and Culture of Orchids. pp. 469-472. Affiliated East-West Press, New Delhi.

Chaturvedi, H. C., A. K. Sharma, R. N. Prasad and M. C. Sharma. 1987. Tissue culture of some ornamentals. In proc. National Seminar on Plant Tissue culture. pp: 76-90, ICAR, New Delhi.

Chen, J. T. and W. C. Chang. 2000. Efficient plant regeneration through somatic embryogenesis from callus cultures of *Oncidium* (Orchidaceae). Plant Sci. 160: 87-93

Chen, T. Y., J. T. Chen and W. C. Chang. 2002. Multiple shoot formation and plant regeneration from stem nodal explants of *Paphiopedilum* orchids. *In vitro* Cell. Dev. Biol.- Plant 38: 595-597.

Cheye, V. A. and P. J. Dale. 1980. Shoot tip culture in forage legumes. Plant Sci. Lett. 19: 303-309.

Choi, Y. E. and J. H. Jeong. 2002. Dormancy induction of somatic embryos of Siberian ginseng by high sucrose concentrations enhanced the conservation of hydrated artificial seeds and dehydration resistance. Plant Cell Rep. 20: 1112-1116.

Choi, S., J. D. Chung and J. H. Lee. 1996. Effect of culture medium on rhizome formation and its subsequent growth from shoot-tip culture of temperate *Cymbidium* species. Kor. J. Plant Tiss. Cult. 23: 167-172.

Choi, Y. E., D. C. Yang, E. S. Yoon and K. T. Choi. 1999. High efficiency plant production via direct somatic single embryogenesis from preplasmolysed cotyledons of *Panax ginseng* and possible dormancy of somatic embryos. Plant Cell Rep. 18: 493- 499.

Chowdhery, H. J. 1998. (ed.) Orchid flora of Arunachal pradesh. Bishen Singh, Mahendra Pal Singh, Dehra Dun.

Chowdhery, H. J. 2001. Orchid Diversity in North-East India. J. Orchid Soc. India. 15: 1-17.

Chowdhury, I., A. R. M. Rahman, M. O. Islam and S. Matsui. 2003. Effects of plant growth regulators on callus proliferation, plantlets regeneration and growth of plantlets of *Doritaenopsis* orchid. Biotechnol. 2: 214-221.

Clements, M. A. 1981. The germination of Australian orchid seeds. Paper presented 13th International Botanical Congress Satellite Orchid Symposium.

Clements, M. A. and R. K. Ellyard. 1979. The symbiotic germination of Australian terrestrial orchids. Amer. Orchid. Soc. Bull. 48: 810-816.

Clements, M. A., H. Muir and P. J. Cribb. 1986. A preliminary report on the symbiotic germination of European terrestrial orchids. Kew. Bull. 41: 437-497.

Corner, A. J. and M. B. Thomas. 1981. Re-establishing of plantlets from tissue culture: A Review. Comp. Proc. Intern. Plant Prop. Soc. 31: 342-357.

Cornijo-Martin, M. T., A. M. Mingocastel and E. Primo-millo. 1979. Organ re-differentiation in rice callus, effects of C_2H_2, CO_2 and cytokinins. Z. Pflanzen Physiol. 94: 117-123.

Corrie, S. 1994. Studies on encapsulation of protocorm like bodies of *Cymbidium giganteum* Wall. with special reference to storage, germination and growth behavior. Ph. D. Thesis. NEHU, Shillong.

Corrie, S. and P. Tandon. 1993. Propagation of *Cymbidium giganteum* Wall. through high frequency conversion of encapsulated protocorms under *in vivo* and *in vitro* conditions. Indian J. Exptl. Biol. 31: 61-64.

Crane, J. and H. Hughes. 1990. Medium overlays for improved hardening of micropropagated potatoes. Hort. Sci. 25: 794-795.

Cribb, P. 1990. *Dendrobium alexandrae*. The Kew Magazine. 7: 117-120.

Crouch, M. L. and I. M. Sussex. 1981. Development and storage protein synthesis in *Brassica napus* L. embryos *in vivo* and *in vitro*. Planta 153: 64-74.

Curtis, J. T. and E. Spoerl. 1948. Studies on the nitrogen nutrition of orchid embryos. II. Comparative utilization of nitrate and ammonium nitrogen. Amer. Orchid Soc. Bull. 17: 111-114.

Das, A. and K. K. Ghosal. 1989. *In vitro* germination behavior of some orchid seeds developed in plains of West Bengal. Indian Agric. 33: 103-109.

Datta, K. B., B. Kanjilal and De. D. Sarker. 1999. Artificial seed technology: development of a protocol in *Geodurum densiflorum* (Lam) Schltr.- An endangered orchid. Curr. Sci. 76: 1142-1145.

Davidson, O. W. 1956. New orchid potting medium lowers cost of production. 38: 3-6.

Davies, W. J., T. A. Mansfield and R. R. Wellburn. 1980. A role for abscissic acid in drought endurance and drought avoidance. In : Plant Growth Sustances. pp. 242-253. Springer Verlag, Berlin.

Deb, C. R., N. S. Jamir and Temjensangba. 2003. Orchid Diversity of Nagaland-A revised status. J. Orchid Soc. India. 17: 5-15.

Deberge, P. C. and L. J. Maene. 1981. A scheme for commercial propagation of ornamental plants by tissue culture. Sci. Hort. 14: 335-345.

Decruse, S. W., A. Gangaprasad, S. Seeni and V. S. Menon. 2003. Micropropagation and ecorestoration of *Vanda spathulatha*, an exquisite orchid. Plant Cell Tiss. Org. Cult. 72: 199-202.

Delvallee, I., J. Gullaud, M. Beckert and C. Dumas. 1989. Cryopreservation of immature maize embryos after freeze-hardening in the ear and *in vitro*. Plant Sci. 60: 129-136.

Devi, S., Y. Sunitabala and J. M. Laishram. 1998. *In vitro* propagation of *Dendrobium* hybrids through shoot tip and axillary bud culture. J. Orchid Soc. India. 12: 79-81.

Devi, Ch. G., M. Damayanti and J. Sharma. 1999. Aseptic embryo culture of *Vanda coerulea* Griff. J. Orchid Soc. India. 12: 83-87.

Drew, R. 1979. The development of carrot (*Daucus carota* L.) embroids (derived from cell suspension culture) into plantlets on a sugar free basal medium. Hort. Res. 19: 79.

Druart, P. H. 1985. *In vitro* germplasm preservation technique for fruit trees. In: A. Schafer Menuhr (ed.), pp. 167-171. *In vitro* techniques-propagation and long term storage. Nijhoff Junk for CEC, Dordrecht.

Duan, J. and H. Hu. 1989. Clonal propagation of *Vanilla planifolia.* Acta Bot. Yunn. 11: 107-109.

Duan, J. X. and S. Yazawa. 1995. Floral induction and development in *Phalaenopsis in vitro*. Plant Cell Tiss. Org. Cult., 43: 71-74.

Dubus, F. 1980a. Limites de la resistance au froid de protocorms d' orchidus cultiverdans conditions simples *in vitro*. Bull. Soc. Roy. Bot. Belg. 113: 63-65.

Dubus, F. 1980b. Emploi du saccharose pur en mélange avec du glucose ou du glycerol comme Crysprotecteurs pour la Conglelation de protocorms de *Cymbidium* en culture *in vitro*. Bull. Soc. Roy. Bot. Belg. 113: 599-562.

Engelmann, F. 1990. Utilsation d' atmospheres a teneur en Oxygene reduite pour la conservaton d' embryos somatiques Jacq.) apres Comgletion dans l' agote liquide. Completes Rendus del. Acad. des Sci. 301: 111-116.

Ernst, R. 1967. Effect of selected organic nutrient additives on growth *in vitro* of *Phalaenopsis* seedling. Am. Orchid Soc. Bull. 36: 694-904.

Ernst, R. 1985. Seed and clonal propagation of *Phalaenopsis*. In: Proc. 5th Asian orchid Congress (ed. A. N. Rao), pp. 31-41. Ministry of National Development, Singapore.

Ernst, R. 1994. Effect of thidiazuron on *in vitro* propagation of P*halaenopsis* and *Doritaenopsis* (Orchidaceae). Plant Cell Tiss. Org. Cult. 39:273-275.

Espinoza, N., R. Estrada, P. Tovar, J. Bryan and J. H. Dodds. 1986. Tissue culture micropropagation, conservation and export of potato germplasm. Specialized technology document I. International potato center, Lima, Peru. pp. 1-20.

Evers, P. W., J. Donkers, A. Prat and E. Vermeer. 1988. Micropropagation of forest trees through tissue culture. Pudoc, Wageningen.

Fernandes, P. C., V. A. Bapat and P. S. Rao. 1992. *In vivo* germination of encapsulated somatic embryos of *Santalum album* Lindl. J. Exptl. Biol. 30: 839-841.

Fonnesbech, M. 1972a. Growth hormone and propagation of *Cymbidium in vitro.* Physiol. Plant. 27: 310-315.

Fonnesbech, M. 1972b. Organic nutrients in the media for propagation of *Cymbidium in vitro*. Physiol. Plant. 27: 360-364.

Ford-Lloyd, B. and M. Jackson. 1986. Plant genetic resources: An introduction to their conservation and use. Edward Arnold, New York.

Fu, F. M. L. 1978. Clonal propagation of *Aranda*, *Ascocenda* and *Cattleya* by leaf tissue culture. Gard. Bull. 31: 132-138.

Fu, F. M. L. 1979. Studies on the tissue culture of orchids. Clonal propagation of *Aranda, Ascocenda* and *Cattleya* by leaf tissue culture. Orchid Rev. 87: 343-346.

Fujii, J. A. A., D. Slade and K. Redenbaugh. 1989. Maturation and Green house planting of alfa alfa artificial seeds. *In vitro* Cell. Dev. Biol. 25: 1179-1182.

Gailhoper, M. and I. Thaler. 1975. Einfluss Von 6-benzyl-aminopurine auf die Feinstructure Von mitochondrien undplastiden *in vitro* Kultivierten Protokorme Von *Cymbidium*. Phyton. 17: 159-165.

Gamborg, O. L., A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of Soyabean root cells. Exp. Cell Res. 50: 151-158.

Gangaprasad, A. T., P. G. Latha and S. Seeni. 2000. Micropropagation of terrestrial orchids *Anoetochilus sikkimensis* and *Anoetochilus regalis*. Indian J. Exp. Biol. 38 149-154.

Gangopadhyay, G., T. Bandyopadhyay, R. Podder, S. B. Gangopadhyay and K. K. Mukherjee. 2005. Encapsulation of Pineapple microshoots in alginate beads for temporary storage. Curr. Sci. 88: 972-977.

Geetha, S. and S. A. Shethy. 2000. *In vitro* propagation of *Vanilla planifolia*, a tropical orchid. Curr. Sci. 79: 886-890.

George, E. F. 1993. Plant propagation by tissue culture. 2nd (ed.). Exegetics, Edington. pp. 322-326.

George, E. F. and P. D. Sherrington. 1984. Plant propagation by tissue culture. Exegetics Ltd. Basingstoke. U. K.

George, P. S. and G. A. Ravishanker. 1997. *In vitro* multiplication of *Vanilla planifolia* using axillary bud explants. Plant Cell Rep. 17: 490-494.

Ghosh, B. and S. Sen. 1994. Plant regeneration from alginate encapsulated somatic embryos of *Asparagus coopri* Baker. Plant Cell Rep. 13: 381-385.

Goh, C. J. 1970. Some effects of auxin on orchid seed germination and seedling growth. Malayan Orchid Rev. 9: 115-118.

Goh, C. J. 1983. Asexual mass propagation of orchids and its commercialization: A review of the present status. Life-Sci. 22: 319-336.

Goh, C. J. 1990. Orchids- Monopodials. In: Handbook of Plant Cell Culture, Vol. 5. Ornamental Species. pp. 598-637. P. V. Ammirato, D. A. Evams. W. R. Sharp and Y. P. S. Bajaj (eds.), Mc Graw-Hill, New York.

Goh, C. J. and H. Tan. 1982. Clonal propagation from leaf explants in *Renanthera* orchid hybrid. Orchid Rev. 90: 295-296.

Goh, C. J. and P. F. Wong. 1990. Micropropagation of the monopodial orchid hybrid *Aranda Deberoh* using inflorescence explants. Sci. Hort. 44: 315-321.

Goh, C. J, C. S. Loh and A. N. Rao. 1975. Clonal propagation of *Aranda* hybrids through shoot meristem culture. Proc. of seminar Biology in Society, pp. 26-35.

Gray, D. and M. Compton. 1993. Use of somatic embryos as synthetic seeds for reducing micropropagation costs. Hort. Sci. 28: 515.

Griesbach, R. J. 1986. Orchid tissue culture. In: Tissue culture as a plant production system for horticultural crops. Lawson R. H. (ed.). pp. 343-349. Martinus Nijhoff Publishers, Dordrecht.

Griffis, J. L., G. Hennen and R. P. Oglisbby. 1983. Establishing tissue cultured plants in soil. Comp. Proc. Intern. Plant Prop. Soc. 33: 618-622.

Grout, B. W. W. and M. J. Aston. 1977. Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hort. Res. 17: 1-7.

Grover, J. P. 1997. Resource competition. Chapman and Hall, London.

Gupta, P. and D. Durzan. 1986. Plantlet regeneration via somatic embryogenesis from sub-cultured callus of mature embryos of *Picea abies* (Norway spruce). *In vitro* Cell. Dev. Biol.-Plant. 22: 685-688.

Gupta, P. and D. Durzan. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblollypine. Bio-technol. 5: 147.

Hadley, G. 1970. The interaction of kinetin, auxin and other factors in the development of North Temperate Orchids. New Phytol. 69: 549-555.

Hadley, G. and G. Harvais. 1968. The effect of certain growth substances on asymbiotic germination and development of *Orchis purpurella*. New Phytol. 67: 441-445.

Hama, I. 1986. Artificial seeds. Japanese Patent application No. 40708/ 1986.

Hanako, S. and Y. Koda. 2004. Micropropagation of *Cypripedium macranthos* var. *rebunense* through protocorm like bodies derived from mature seeds. Plant Cell Tiss. Org. Cult. 78: 273-276.

Handique, A. K. and A. Talukdar. 1998. Phytohormone induced variation *in vitro* seed culture of *Dendrobium* Roxb. J. Phyt. Res. 11: 19-22.

Handro, W., K. G. Hell and G. B. Kerbauy. 1977. Tissue culture of *Stevia rebaudiana*, a sweeting plant. Planta. Medica. 32: 115-117.

Harrison, C. R. and J. Arditti. 1970. Growing orchids from seeds. Orchid Digest 34: 199-204.

Harvais, G. 1973. Growth requirements and development of *Dactylorhiza* (orchis) *purpurella* in axenic culture. Can. J. Bot. 57: 327-332.

Harvais, G. 1982. An improved culture medium for growing the orchid *Cypripedium reginae* axenically. Can. J. Bot. 60: 2547-2555.

Hasan, S. M. Z. and H. Takagi. 1995. Alginate coated nodal segments of yam (*Dioscorea sp.*) for germplasm exchange and distribution. Plant Genetic Resource. Newslett. 103: 32-35.

Hayes, A.B. 1969. Observations on orchid seed mycorrhizae. Mycopathologia *et* Mycologia Applicata. 30: 139-44.

Hazarika, R. and C. M. Sharma. 1995. *In vitro* germination and regeneration of *Dendrobium transparens* Lindl. J. Orchid. Soc. India. 9: 69-74.

Hazarika, B. N., V. A. Parthasarathy, V. Nagaraju and G. Bhowmik. 2000. Sucrose induced biochemical changes in *in vitro* microshoots of *Citrus* species. Indian J. Hort. 57: 27-31.

Hazarika, B. N., V. A. Parthasarathy and V. Nagaraju. 2000. Paclobutrazol induced biochemical changes in microshoots of *Citrus* species. Folia. Hort. 12: 69-77.

Hazarika, B. N., V. A. Parthasarathy and V. Nagaraju. 2001. Influence of *in vitro* preconditioning of *Citrus* microshoots with paclobutrazol on *ex vitro* survival. Acta. Bot. Croat. 60: 25-29.

Hedge, S. N. 1984. A note on medicinal usage of some orchids. Arunachal Forest News, 6: 11-18.

Hedge, S. N. 1984. Orchids of Arunachal Pradesh. Published by the Forest Department, Arunachal Pradesh, Itanagar.

Henrich, J. E., D. P. Stimart and P. D. Ascher. 1981. Terrestrial orchid seed germination *in vitro* on defined medium. J. Amer. Soc. Hort. Sci. 106: 193-196.

Henshaw, G. G. 1975. Technical aspects of tissue culture storage for genetic conservation. In: O. H. Frankel and J. G. Hawkers (eds.), Crop Genetic Resources for Today and Tomorrow. pp. 349-358. Cambridge University press, Cambridge.

Hiraoka, N. and T. Kodama. 1984. Effects of non-frozen cold storage on the growth, organogenesis and secondary metabolism of callus cultures. Plant Cell Tiss. Org. Cult. 3: 349-357.

Holters, J. and K. Zimmer. 1990. Shoot regeneration from root tips of orchids *in vitro*. IV. Organogenesis on roots of *Mormodes histrio* (Ger., Engl. Summ.). Gartebauwesseschaft 55: 264-267.

Homma, Y. and T. Asahira. 1985. New means of *Phalaenopsis* propagation with internodal sections of flower stalk. J. Japan Soc. Hort. Sci. 54: 379-387.

Hynniewta, T. M. 2000. Orchids of Nagaland. Bot. Survey of India, Calcutta.

Ichihashi, S. 1992. Micropropagation of *Phalaenopsis* through the cultures of lateral buds from young flower stalks. Lindleyana. 7: 208-215.

Ichihashi, S. and H. Hiraiwa. 1996. Effect of solidifier, coconut water and carbohydrate source on growth of embryogenic callus in *Phalaenopsis* and allied genera. J. Orchid Soc. India. 10: 81-88.

Ilsley, P. 1965. Meristem tissue propagation techniques and potentials. Orchid Rev. 73: 371-376.

Intuwong, O. and Y. Sagawa. 1973. Clonal propagation of Sarcantine orchids by aseptic culture of inflorescence. Am. Orchid Soc. Bull. 42: 209-215.

Intuwong, O. and Y. Sagawa. 1974. Clonal propagation of *Phalaenopsis* by shoot tip culture. Amer. Orchid Soc. Bull. Oct. 893-895.

Irawati, H. S. S., H. Suseno and S. Idris. 1977. Tissue culture of *Aranthera* James storie Orchid Rev. 85: 138-142.

Ishii, Y., T. Takamura, M. Goi and M. Tanaka. 1998. Callus induction and somatic embryogenesis of *Phalaenopsis*. Plant Cell Rep. 17: 466-470.

Israel, H. W. 1963. Production of *Dendrobium* seedlings by aseptic culture of excised ovularies. Am. Orchid. Soc. Bull. 32: 441-443.

Jarret, R. L. and N. Gawel. 1991. Abscisic acid-induced growth inhibition of sweet potato (*Ipomoea batatas* L.) *in vitro*. Plant Cell Tiss. Org. Cult. 24: 13-18.

Jian – Xiong, D and Y. Susumu. 1995. Floral induction and development in *Phalaenopsis in vitro*. Plant Cell Tiss. Org. Cult. 43: 71-74.

Jha, P., S. Nair and C. R. Babu. 1993. Encapsulation of seeds of *Sesbania sesban* with polyacrylamide and alginate gel entrapped rhizobia beads to effective symbiotic nitrogen fixation. Indian J. Exptl. Biol. 31: 161-167.

Kalita, K. 1999. *In vitro* micropropagation of a few spectacular and endangered orchid species of North-East India. Ph.D. Thesis. Guwahati Univ.

Kanjilal, B., D. De. Sarkar, J. Mitra and K. B. Dutta. 1999. Stem disc culture: Development of a rapid mass propagation method of *Dendrobium moschatum* (Buch-Ham) Swartz-an endangered orchid. Curr. Sci. 77: 497-500.

Kano, K. 1965. Studies on the media for orchid seed germination. Mem. Fac. Agr. Kagawa Univ. 20: 74.

Kaur, P. 1996. Micropropagation of some endangered and economically important orchids. Ph.D. Thesis. Punjab University, Chandigarh, India.

Kaur, S. and S. P. Vij. 2000. Regeneration potential of *Saccolabium papillosum* leaf segments. J. Orchid Soc. India. 14: 67-73.

Kaushik, P. and N. Kishore. 1991. Antibacterial potential of *Pholidota articulata* Lindl- A study *in vitro*. J. Orchid Soc. India. 5: 93-96.

Kataki, S.K. 1986. Orchids of Meghalaya. Forest Department, Shillong, Meghalaya

Kathyar, R. S., G. D. Sharma and R. R. Mishra. 1987. Asymbiotic seed germination and seedling development in *Coelogyne punctulata* and *Aerides multiflorum*. Indian Forrester, 113: 574-577.

Kako, S. 1973. Clonal propagation of *Cattleya* through shoot meristem culture. Japan Agric. Res. Quart. 7: 109-115.

Khaw, C. H., H. T. Ong and H. Nair. 1978. Hormones in the nutrition of orchid tissues in mericloning. Malay Orchid Rev. 13: 60-65.

Khor, E., W. E. Ng and C. S. Loh. 1998. Two coat system for encapsulation of *Spathoglottis plicata* (Orchidaceae). Biotechnol. Bioeng. 59: 635-639.

Kim, K. K., J. T. Kunisaki and Y. Sagawa. 1970. Shoot tip culture of *Dendrobium*. Amer. Orchid Soc. Bull. 39: 1077-1080.

Kirtikar, K. R. and B. D. Basu. 1918. Indian medicinal plants. Vol. 4. Lalit Mohan Basu. Allahabad.

Kitto, S. K. and J. Janick. 1985. Production of synthetic seeds by encapsulating asexual embryos of carrot. J. Amer. Soc. Hort. Sci. 110: 277-282.

Knudson, L. 1922. Non-symbiotic germination of orchid seeds. Bot. Gaz. 73: 1-25.

Knudson, L. 1924. Further observations on non-symbiotic germination of orchid seeds. Bot. Gaz. 77: 212-219.

Knudson, L. 1925. Physiological study of the symbiotic germination of orchid seeds. Bot. Gaz. 79: 345-379.

Knudson, C. 1946. A new nutrient solution for the germination of orchid seeds; Amer. Orch. Soc. Bull. 15: 214-217.

Knudson, L. 1951. Nutrient solution for orchids. Bot. Gaz. 112: 528-532.

Koch, L. 1974. Erbgleiche Vermehrug Von *Phalaenopsis in vitro* Gartenwelt. 74: 482-484.

Kosir P., S. Suzana and L. Zlata. 2004. Direct shoot regeneration from nodes of phalaenopsis orchids. Acta Agric. Sloven. 83: 233-242.

Kramer, P. J. and T. T. Kozlowski. 1979. Physiology of woody plants. Academic Press, New York, San Francisco, London.

Krapiec, P. V., A. Milaneze and M. De. F. P. S. Machado. 2003. Effects of different combinations of growth regulators for bud induction from seedlings of *Cattleya walkeriana* Gardner (Orchidaceae). Acta. Scien. Biol. Sci. 25: 179-182.

Kraus, J. E. and W. R. Moneiro. 1989. Formation of protocorm like bodies from root apices of *Catasetum pileatum* (orchidaceae) cultivated *in vitro*. I. Morphological aspects. Amer. Bot. (Lond.) 6): 491-498.

Krishnan, M. P. T. and S. M. Jorapur. 1984. *In vitro* seed germination of *Calanthe masuca* (D. Don) Lindl. (Orchidaceae). J. Indian Bot. Soc. 63: 121-123.

Kukulczanka, K. and U. Wojciechowska. 1983. Propagation of two *Dendrobium* species by *in vitro* culture. Acta Hort. 131: 105-110.

Kumaria, S. 1991. *In vitro* propagation of *Dendrobium fimbriatum* var. occulatum HK. F: Some functional and biochemical aspects of its growth. Ph.D. Thesis. North Eastern Hill University, Shillong, India.

Kumaria, S. and P. Tandon. 1991. Asymbiotic germination of *Dendrobium fimbriatum* var. *oculatum* Hk. F. seeds on different media. Proc. Indian Natn. Sci. Acad. 57: 277-279.

Kumaria, S. and P. Tandon. 1994. Clonal propagation and establishment of *Dendrobium fimbriatum* var. oculatum HK. F. pp. 21-231. In P. Tandon (ed.), Advances in Plant Tissue culture in India. Pragati Prakashan, India.

Kumaria, S. and P. Tandon. 2001. Orchids, the world's most wondrous plants. pp. 17-28. In P. Pathak, R. N. Sehgal, N. Shekhar, M. Sharma and A. Sood (ed.), Orchids; Science and Commerce, Bishen Sinh Mahendra Pal Singh, New Connaught Place, Dehra Dun, India.

Kusumoto, M. 1978. Effects of combinations of growth regulating substances and of organic matter on the proliferations and organogenesis of *Cymbidium* protocorm cultured *in vitro*. J. Jap. Soc. Hort. Sci. 47: 391-400.

Kusumoto, M. 1979a. Effects of combinations of growth regulators and some supplements on the growth of *Cattleya* plantlets cultured *in vitro*. J. Jap. Soc. Hort. Sci. 47: 492-501.

Kusumoto, M. 1979b. Effects of combinations of growth regulators and of organic supplements on the proliferation and organogenesis of *Cattleya* protocorm-like bodies cultured *in vitro*. J. Jap. Soc. Hort. Sci. 47: 502-510.

Laishram, J. M. and Y. S. Devi. 1997. Micropropagation of *Renanthera imshootiana* through shoot tip, axillary bud and leaf segment cultures. In: Proc. 5th National Seminar on Developmental Biology and Commercialisation of Orchids and Orchid Show. pp. 87. Gangtok, Sikkim, India.

Laishram J. M. and S. Devi. 1999. Micropropagation of *Renanthera imschootiana* Rolfe through shoot-tip, axillary bud and leaf segment cultures. J. Orchid Soc. India. 13: 1-4.

Latha, P. G. 1999a. *In vitro* culture of the regionally endangered medicinal orchid, *Nervilea aragoana* Gaud. J. Orchid Soc. India. 13: 59-64.

Latha, P. G. 1999b. Micropropagation and *in vitro* flower induction in *Habenaria crinifera* Lindl. J. Orchid. Soc. India. 13: 47-53.

Latha, P. G. and S. Seeni. 1991. Rapid multiplication of *Phalaenopsis* from leaf and root issues cultured *in vitro*. J. Orchid Soc. India. 5: 1-8.

Lay, F. F. M. 1978. Studies on the tissue culture of orchids 1: Clonal propagation of *Phalaenopsis* by lateral buds from flower stems. Amer. Orchid Soc. Bull. 86: 308 -310.

Leake, J. R. 1994. The biology of myceoheterotrophic 'Saprophytic' plants. New Phytol. 127: 171-216.

Lee, H. S., K. Y. Peak and J. K. Lee. 1983. Studies on the non symbiotic germination of seeds of *Laelia briegeri*. II. Effect of anionic and cationic composition, nitrogen and iron nutrition on the growth of seedlings *in vitro*. J. Korean. Soc. Hort. Sci. 24: 169-174.

Lin, C. C. 1986. *In vitro* cultures of flower stalk internodes of *Phalaenopsis* and *Doritaenopsis*. Lindleyana. 1: 158-163.

Linderman, R. G. 1994. Mycorrhizae for plant health. In: symposium series (ed. F.L. Fleges and R. G. Linderman) pp. 1-25. APS Press St. Paul, Minnesota.

Lisek, A. and T. Orlikowka. 2003. *In vitro* storage of strawberry and raspberry in calcium -alginate beads at 4°C. Plant Cell Tiss. Org. Cult. 75: 1-6.

Lo, S. H., S. M. Nulawade, C. L. Kuo, C. L. Chen and H. S. Tsay. 2001. Asymbiotic germination of immature seeds, plantlet development and *ex vitro* establishment of plants of *Dendrobium tosaenso* makino-a medicinally important orchid. *In vitro* Cell. Dev. Biol. Plant. 10: 528-535.

Love, S. L., B. B. Rhodes and J. W. Scowcroft. 1987. Meristem-tip culture and virus indexing of sweet potatoes. Practical manuals for handling crop germplasm *in vitro*. IBPGR, Rome.

Loveys, B. R., C. J. Brien and P. E. Kriedemann. 1975. Biosynthesis of ABA under osmotic stress. Studies based on a dual labeling technique. Physiol. Plant. 33: 166-170.

Maheswari, U. H., D. S. Garg., J. P. Agrawal and D. D. Garg. 1978. Salam Misri (*Orchis latifolia*). Dhanvanri Vaausadhi Vishishank, 6: 329-339.

Malabadi, R. B. and K. Nataraja. 2002a. *In vitro* storage of synthetic seeds in *Clitoria ternatea* Linn. Phytomorph. 52: 231-237.

Malabadi, R. B. and K. Nataraja. 2002b. Large scale production and storability of encapsulated somatic embryos of mothbean (*Vigna aconitifolia* Jacq.). J. Plant Biochem. Biotechnol. 11: 61-64.

Malabadi, R. B. and J. V. Staden. 2005. Storability and germination of sodium alginate encapsulated somatic embryos derived from the vegetative shoot apices of mature *Pinus patula* trees. Plant Cell Tiss. Org. Cult. 82: 259-265.

Malabadi, R. B., G. S. Mulgund and K. Nataraja. 2004. Efficient regeneration of *Vanda coerulea*, an endangered orchid using thidiazuron. Plant Cell Tiss. Org. Cult. 76: 289-293.

Marino, G., P. Rosati and F. Sagrati. 1985. Storage of *in vitro* cultures of *Prunus* root stocks. Plant Cell Tiss. Org. Cult. 5: 73-78.

Masuhara, G. and K. Katseya. 1994. *In situ* and *in vitro* specificity between *Rhizoctonia* spp. and *Spiranthes Sinensis* (persoon) Ames. var. amoena (M. Biebersein) Hara (Orchidaceae). New Phytol. 127: 711-718.

Mathews, V. H and P. S. Rao. 1985. *In vitro* culture of *Vanda* hybrid (*Vanda* TMA × *Vanda* Miss Joaquim) I. Studies on protocorm explants. Proc. Indian Natn. Sci. Acad. 51: 96-103.

Mathur, J., P. S. Ahuja, N. Lal and A. K. Mathur. 1989. Propagation of *Valeriana Wallichi* using encapsulated apical and axial shoot buds. Plant Sci. 69: 111-116.

Mathur, J., S. Mukunthakumar, S. N. Gupta and S. N. Mathur. 1991. Growth and morphogenesis of plant tissue cultures under mineral oil. Plant Sci. 74: 249-254.

Mauro, M. D., A. Sabapatty and R.A. Smith. 1994. Influence of benzyl aminopurine and α- naphthalene acetic acid on multiplication and biomass production of *Cattleya aurantiaca* shoot explants. Lindleyana, 9:169-173.

Mead, J. W. and C. Bulard. 1975. Effect of vitamins and nitrogen sources on asymbiotic germination and development of *Orchis laxiflora* and *Ophyrs sphaegodes*. New Phytol. 74: 33-40.

Meyer, J. R. 1951. Transplanting orchid seedlings from culture vessels to pots containing fern fibres. Biologies. 17: 99-102.

Micheli, M., E. Piccioni and A. Standardi. 1996. Conversion of M. 26 apple root stock on different substrata. *In vitro*. 32: 92A - 93A.

Miller, C. and F. Skoog. 1953. Chemical control of bud formation in tobacco stems segments. Am. J. Bot. 40: 768-773.

Miller, C., F. Skoog, F. S. Okumera, M. H. V. Saltza and A. M. Strong. 1956. Isolation, structure, synthesis of kinetin, a substance promoting cell division. J. Amer. Chem. Soc. 78: 1375-1380.

Mishra, A. K. and S. P. Bhatnagar. 1995. Direct shoot regeneration from the leaf explants of cucumber (*Cucumis sativus* L.). Phytol. Morph. 45: 47-55.

Mitra, G. C. 1971. Studies on seeds shoot tips and stem discs of an orchid grown in aseptic culture. Indian J. Expt. Biol. 9: 79-85.

Mitra, G.C. 1986. *In vitro* culture of orchid seeds for obtaining seedlings. In: S.P. Vij (ed.). Biology, Conservation and Orchid Culture. Affiliated East West Press, pp. 401-412.

Mitra, G. and H. Chaturvedi. 1972. Embroids and complete plants from unpollinated ovaries and from ovules of *in vitro* grown encapsulated flower buds of *Citrus* spp. Bull Tor. Bot. Club. 99: 184-189.

Mitra, G. C., R. N. Prasad and A. Roychoudhury. 1976. Inorganic salts and differentiation of protocorms in seed callus of an orchid (*Dendrobium fimbriatum*) and correlated changes in its free amino acid content. Indian J. Exp. Biol. 14: 350 -351.

Mix, G. 1982. *In vitro* preservation of potato materials. Plant genetic Resources. Newslett. 51: 6-8.

Mix, G. 1985. *In vitro* preservation of potato genotypes. In: Lange, A., C. Steven and N. G. Hogeeboom (eds.), Efficiency in Plant Breeding. Pudoc, Wagenigen. pp. 194-195.

Mohammad, H. G. and W. E. Vidaver. 1988. Root production and plantlet development in tissue cultured conifers. Plant Cell Tiss. Org. Cult. 14: 107-110.

Momose, H. and K. Yoneda. 1988. Protocorm like body (PLB) formation by flower stalk node bud culture by means of cutting off the top of inflorescence of *Phalaenopsis*. Bull. Coll. Agric. Vet. Med. Nihon Univ. 45: 197-202.

Monette, P. L. 1986. Cold storage of Kiwi fruit shoot tips *in vitro*. Hort. Sci. 21: 1203-1205.

Morel, G. M. 1960. Producing Virus-free *Cymbidiums*. Am. Orchid Soc. Bull. 29: 495-497.

Morel, G. 1970. Neuces auf dem Gebiet der meristem Forschung Die Orchidee. 20: 433-443.

Morel, G. 1971. The principles of clonal propagation of orchid. Proc. 6th World Orchid Conf., pp. 101-106.

Morel, G. 1972. Morphogenesis of stem apical meristem cultivated *in vitro*: application to clonal propagation. Phytomorph. 22: 265-277.

Morel, G. M. 1974. Clonal multiplication of orchids In: C.L Wither (ed.). The Orchid: Scientific Studies. pp: 169-222. John Wiley, New York.

Moriguchi, T., J. Kozaki, N. Matsuta and S. Yanaki. 1988. Plant regeneration from grape callus stored under a combination of low temperature and silicone treatment. Plant Cell Tiss. Org. Cult. 15: 67-71.

Morris, P, E. Weiler, S. Maddock. 1988. Determination of endogenous abscisic acid levels in immature cereal embryos during *in vitro* culture. Planta. 173: 110-116.

Mosich, S. K., E. A. Ball and J. Arditti. 1974. Clonal propagation of *Dendrobium* by means of node cultures. Amer. Orchid Soc. Bull. 43: 1055-1061.

Mukherjee, S. K. 1983. Orchids. Published by P. C. Bedi, Under-Secretary for the ICAR, New Delhi.

Mukherjee, T. P., S. Roy and T. K. Bose. 1974. Seed germination and propagation of orchids. Indian Hort. 18: 21-24.

Muralidhar, C. E. and Mehta, A. R. 1986. Tissues culture studies on *Cymbidium longifolium* D. Don: In: S. P. Vij (ed.). *In vitro* seed germination and sequential

stages of histo morphological change from embryo to PLBs. Biology, Conservation and Culture of Orchids. Affiliated East West Press, pp. 413-422.

Murashige, T. 1978. The impact of plant tissue culture on agriculture, pp. 15-26, 518-524. In T. A. Thorpe (ed.), Frontiers of Plant Tissue Culture. University of Calgary Press, Canada.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Murthy, H.N., A.N. Pyati. 2001. Micropropagation of *Aerides maculosum* Lindl. (Orchidaceae). *In vitro* Cell Dev. Biol. Plant. 37: 223-226.

Nagaraju, V. and V. A. Parthasarathy. 1999. *In vitro* micropropagation of *Cymbidium giganteum*. Indian J. Hort. 56: 270-273.

Nagaraju, P., S. P. Das, P. C. Bhutia and R. C. Upadhyaya. 2003. Effect of media and BAP on protocorms of *Cymbidium* and *Cattleya*. J. Orchid Soc. India. 17: 67-71.

Nakamura, S. 1982. Nutritional conditions required for the non-symbiotic culture of an achlorophyllous orchid *Galeola septentrionalis*. New Phytol. 90: 701-715.

Nandina, N., Y. Zambrano, R. Tapia, M. Cio, D. Pina and R. Castillo. 2003. Field performance of artificial seed derived sugarcane plants. Plant Tiss. Org. Cult. 75: 279-282.

Nasiruddin, K. M., R. Begum and S. Yasmin. 2003. Protocorm like bodies and plantlet regeneration from *Dendrobium formosum* leaf callus. Asian J. Plant Sci. 2: 955-957.

Nath, M., J. Devi, B. Borthakur, J. Sharma and P. C. Deka. 1991. Embryo Culture of *Rhynchostylis retusa* and *Vanda coerulea*. J. Orchid Soc. India. 5: 97-101.

Nayak, N. R., S. Patnaik and S. P. Rath. 1997. Direct shoot regeneration from foliar explants of epiphytic orchid *Acampe praemorsa* (Roxb.) Blatter and Mc. Cann. Plant Cell Rep. 16: 583-586.

Nayar, M. P. and A. R. K. Sastry. 1997-98, 1999. Red Data Book of Indian plants. Vol. 1-3. Bot. Survey of India, Calcutta.

Nazarov, V. V. 1988. Determination of actual seed productivity in *Dactylorhiza romana* and *Dactylorhiza incarnata* (Orchidaceae). Bot. ZH (Leningkr.) 73: 231-233.

Negrutiu, I., M. Jacob and D. Cachita. 1978. Some factors controlling *in vitro* morphogenesis of *Arabidopsis thaliana*. Z. Pflanzen. Physiol. 86: 113-124.

Ng, S. Y. C. and K. Hahn. 1985. Application of tissue culture to tuber crops at IITA Biotechnology in International Agricultural Research. International Agricultural Rice Research Institute, Manila. pp. 29-40.

Ng, S. Y. C. and N. Q. Ng. 1991. Reduced growth storage of germplasm. I: J. H. Dodds (ed.), *In vitro* methods for conservation of plant genetic Resources. Chapman and Hall, London. pp. 11-39.

Nieves, N., J. C. Lorenzo, M. A. Blanco, J. Gonzalez, H. Perralta, M, Hermandey, R. Santos, O. Concepcion, C. G. Borroto, E, Borroto, R. Tapia, M. F. Martinez, G. A. Fundora. 1998. Artificial endosperm of *Cleopatra tangerine* zygotic embryos: a model for somatic embryo encapsulation. Plant Cell Tiss. Org. Cult. 54: 77-83.

Nitsch, J. P. 1969. Experimental androgenesis in *Nicotiana*: Phythomorph. 19: 389-404.

Norstog, K. 1966. Influence of nutritional and physical factors on growth cultures of barley embryos. Amer. J. Bot. 53: 613-614.

Nshimura, G. 1982. Japanese Orchids. (J. Arditti. ed.) Orchid Biology-Reviews and Perspective II. Cornell University Press, Ithaca. pp. 331-346.

Ohira, K. I., M. Ikeda and K. Ojima. 1976. Thiamine requirements of various plant cells in suspension culture. Plant Cell Physiol. 17: 583.

Oliva, A. P. and J. Arditti. 1984. Seed germination of North-American Orchids II. Native California and related species of *Aplectrum*, *Cypripedium* and *Spiranthes*. Bot. Gaz. 145: 495-501.

Onay, A., C. E. Jeffree and M. M. Yeoman. 1996. Plant regeneration from encapsulated embroids and an embryogenic mass of Pistachio, *Pistacia vera* L. Plant Cell Rep. 15: 723-726.

Pack, K. Y., G. B. Shim and J. J. Kim. 1990. Exploitation of temperate *Cymbidium* and establishment of a natural products and BAP exposure period on organogenesis from rhizomes of temperate *Cymbidium*. J. Kor. Soc. Hort. Sci. 31: 74-80.

Panis, B. 1995. Cryopreservation of banana (*Musa* spp.) germplasm. Ph.D thesis. Catholic University, Leuven, Belgium.

Park, Y. S., S. Kakati, A. Kend and M. Okabe. 1996. Efficient propagation of Protocorm like bodies of *Phalaenopsis* in liquid medium. Plant Cell Tiss. Org. Cult. 45: 79-85.

Patel, A. V., I. Pusch, G. M. Wagner and K. D. Vorlop. 2000. A novel encapsulation technique for production of artificial seeds. Plant Cell Rep. 19: 868-874.

Pathak, P., S. P. Vij and K. C. Mahant. 1992. Ovule culture in *Goodyera biflore* (L) HKF: A Study *in vitro*. J. Orchid Soc. India. 6: 49-53.

Pathania, N. S., O. P. Seghal, D. Paul and B. S. Dilta. 1998. Studies on micropropagation in *Dendrobium* cv. Sonia. J. Orchid Soc. India. 12: 35-38.

Paula, W. M., N. L. Thokoane, D. M. Cock and F. Blakeway. 2000. *In vitro* storage of *Eucalyptus grandis* germplasm under minimal growth conditions. Plant Cell Tiss. Org. Cult. 61: 161-164.

Pearce, F. 1989. Kill or Cure? Remedies for the rain forest. New Scient. 123: 40-43.

Pershina, L. A and Isaeva. 1982. Callus formation and features of organogenesis in tissue culture of interspecific and intergeneric hybrids of barley. Fiziol. Rast. 29 (3): 557 – 563.

Philip, V. J. and S. A. Z. Nainar. 1986. Clonal propagation of *Vanilla planifolia* (Solisb.) Ames using tissue culture. J. Plant Physiol. 122: 211-215.

Philip, V. J. and S. A. Z. Nainar. 1988. *In vitro* transformation of root meristem to shoot and plantlets in *Vanilla planifolia.* Ann. Bot. 61: 193-199.

Philip, V. J. and J. Padikkala. 1989. The role of IAA in the conversion of root meristem to shoot meristem in *Vanilla planifolia.* J. Plant Physiol. 135: 233-236.

Picconi, E., G. Capuano and A. Standardi. 1997. Conversion of M. 26 apple root stock synthetic seeds on perlite. *In vitro.* 33 Pt: 2A-51A.

Pierik, R. L .M. and H. H. M. Steegmans. 1972. The effect of 6-benzylamino purine on growth and development of *Cattleya* seedlings grown from unripe seed. Z. Pflanzen. Physiol. 68: 228-234.

Pierik, R. L. M., P. A. Sprenkels, B. Vander Harst and Q. G. Van Der Meys. 1988. Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. *In vitro* Scient. Hort. 34: 139-153.

Prakash, L., C. L. Lee and G. H. Goh. 1996. *In vitro* propagation of commercial orchids: An assessment of current methodology and development of a novel approach, Thin section culture. J. Orchid Soc. India. 10: 31-41.

Prasad, R. N. and G. C. Mitra. 1975. Nutrient requirement for germination of seeds and development of protocorms and seedlings of *Cymbidium* in aseptic culture: Ind. J. Exp. Biol. 13: 123-126.

Pritchard, H. W., B. W. W. Grout and K. C. Short. 1986. Osmotic stress as pre-growth procedures for cryopreservation. 3. Cryobiology of Sycamore and soyabean cell suspension. Ann. Bot. 57: 379-387.

Pyati, A. N. and H. N. Murthy. 1995. *In vitro* seed germination and seedling development of *Dendrobium ovatum*. (W) Krunzl. J. Orchid Soc. of India. 9: 69-74.

Pyati, A. N. and H. N. Murthy. 1999. *In vitro* propagation of *Acampe praemorsa* (Roxb.) Blatt. & Mc. Cann. from leaf explants. J. Orchid Soc. India. 13: 33-35.

Pochet, M., D. Beck and A. Poupet. 1985. *In vitro* shoot proliferation for mass propagation of two species of *Serapias: Serapias olbia* and *serapias pseudocordigera* (Orchidaceae). Bull. Soc. Bot. Fr. Lett : Bot. 132: 289-300.

Polonca, K., S. Suzana and Z. Luthar. 2004. Direct shoot regeneration from nodes of *Phalaenopsis* orchids. Acta agric. Sloven. 83: 233-242.

Raghavan, V. and J. G. Torrey. 1964. Inorganic nitrogen nutrition of the seedlings of the orchid *Cattleya.* Amer. J. Bot. 51: 264-274.

Rao, A. N. 1977. Tissue culture in the orchid industry. pp. 44-69. In J. Reinert and Y. P. S. Bajaj (eds.) Applied and fundamental aspects of plant cell, tissue and organ culture. Springer-Verlag, Berlin.

Reddy, V. P., K. Nanjan and K. G. Shanmugavelu. 1992. *In vitro* studies in tropical orchids: Seed germination and seedling growth. J. Orchid Soc. India. 6: 75-78.

Redenbaugh, K. 1986a. Delivery system for meristematic tissue. U. S. Patent 4: 320-583.

Redenbaugh, K. 1986b. Analogs of Botanic seed. U. S. Patent. 4: 562-663.

Redenbaugh, K. 1990. Application of artificial seed to tropical crops. Hort. Sci. 25: 251-255.

Redenbaugh, K., J. Fujii, D. Slade, D. Viss, M. Kossler. 1986a. Synthetic seeds-encapsulated somatic embryos "78th Annual meeting of the American Society of Agronomy".

Redenbaugh, K., B. Paasch, J. Nichols, M. Kossler, P. Viss and K. Walkar. 1986b. Somatic seeds: Encapsulation of asexual plant embryos. Bio. Tech. 4: 797-801.

Redenbaugh, K., D. Slade, P. R. Viss and J. A. Fujii. 1987. Encapsulation of somatic embryos in synthetic seed coats. Hort. Sci. 22: 803-809.

Reisinger, D. M., E. A. Ball and J. Arditti. 1976. Clonal propagation of *Phalaenopsis* by means of flower-stalk node cultures. Orchid Rev. 84: 45-52.

Reyburn, A. N. 1978. The effects of pH on the expression of darkness-requiring dormancy in seeds of *Cypripedium reginae* Walt. Amer. Orchid Soc. Bull. 47: 798 -802.

Richardson, K. A., R. L. Peterson and R. S. Currah. 1992. Seed reserves and early symbiotic protocorm development of *Planthera hyperborean* (Orchidaceae). Can. J. Bot. 70: 291-300.

Robichand, C. S., J. Wong and I. M. Sussex. 1980. Control of *in vitro* growth of viviparous embryo mutants of maize by abscissic acid. Develop. Gen. 1: 325-330.

Robbins, 1989. *Phragmipedium bessae* (Orchidaceae). The Kew Magagine. Vol. 6: 156.

Robbins, S. and S. Bell. 1990. *Clowesia rosea* (Orchidaceae). The Kew Magagine. Vol. 5: 51.

Rout, G. R., G. Das, S. Samantary and P. Das. 2001. Micropropagation of *Plumbago zeylanica* L. by encapsulated nodal explant. J. Hort. Sci. Biotechnol. 76: 24-29.

Roy, S. 1994. *In vitro* micropropagation of orchids and establishment of seedlings under natural conditions. Ph. D. Thess. G. U.

Roy, J. and N. Banerjee. 2003. Induction of callus and plant regeneration from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum* Hk. F. Sci. Hort. 97: 333-340.

Rucker, W. 1974. Einfluss von cytokininen anf wachstum und differenzierung *in vitro* Kultivierter protokorme von *Cymbidium*.Z. Pflanzen. Physiol. 72: 338-351.

Sagawa, Y. and J. T. Kunisaki. 1982. Clonal propagation of orchids by tissue culture. In: Plant Tissue culture (ed. A. Fujiwara) pp. 683-684. Maruzen, Tokyo.

Saiprasad, G. V. S. 2001. Artificial seeds and their applications. Resonance. 39-47.

Saiprasad, G. V. S. and R. Polisetty. 2003. Propagation of three orchid genera using encapsulated protocorm-like bodies. *In vitro* Cell. Develop. Biol. Plant. 39: 42-48.

Sakai, A. 1997. Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. pp. 53-66 in Conservation of Plant Genetic Resources In Vitro Volume 1: General Aspects. M. K. Razdan and E. C. cocking (eds.). Science Publishers Inc., Enfield USA.

Sanchez, M. I. 1988. Micropropagation of *Crytopodium* (orchidaceae) through root-tip culture. Lindleyana 3: 93-96.

Sander, D. 1969. Orchids and their cultivation, Great Britain.

Sander, D. 1979. Orchids and their cultivation, Blandford Press, Dorset, U. K.

Santos, D. D. and M. Yamaguchi. 1979. Seed sprouting in tomato fruits. Sci. Hort. 11: 131-139.

Saranjeet, K. and S. P. Vij. 2000. Regeneration potential of *Saccolabium papillosum* leaf segments. J. Orchid Soc. India. 14: 67-73.

Sarkar, D. and P. S. Naik. 1997. Synseeds in potato; an investigation using nutrient-encapsulated *in vitro* nodal cutting segments. Sci. Hort. 73: 179-184.

Savina, G. I. 1974. Fertilization in orchids. In: Linskens, H. F., (ed.) Fertilization in higher plants. pp. 197-204. New York: American Elsevier Co.

Seeni, S. 1988. Micropropagation of blue *Vanda* using leaf bases. In: Proc. Natl. Seminar on Current Research trends in Indian Orchids with special reference to Tissue Culture Technology. pp. 22. North Eastern Hill University, Shillong. India.

Seeni, S. and P. G. Latha. 1992. Foliar regeneration of the endangered red *Vanda, Renanthera imschootiana* Rolfe (Orchidaceae). Plant Cell Tiss. Org. Cult. 29: 167-172.

Seneratna, T., B. D. Mc Kersie and S. R. Bowley. 1990. Artificial seeds of alfa alfa (Medicago Sativa L.) induction of desiccation tolerance in somatic embryo. *In Vitro* Cell. Dev. Biol. 26: 85-90.

Senthilkumar, S. 2001. Problems and prospects of orchid mycorrhizal research. J. Orchid Soc. India. 15: 23-32.

Sharma, A. 1993. Clonal multiplication and establishment of *Dendrobium wardianum* Warner. Some aspects of physico-chemical requirements, nitrogen metabolism and preservation. Ph.D Thesis. NEHU, Shillong

Sharma, S. K. and P. Tandon. 1986. Influence of growth regulators on asymbiotic germination and early seedling development of *Coelogyne punctulata* Lindl. pp. 441-451. In S.P. Vij. (ed.), Biology, Conservation and Culture of Orchids. Affiliated East-West Press Pvt. Ltd., New Delhi.

Sharma, S. K. and P. Tandon. 1987. Axenic germination of some epiphytic orchid of Meghalaya, India. J. Orchid Soc. India. 1: 85-90.

Sharma, S. K, P. Tandon and R. R. Mishra. 1991. Vitamins as related to axenic seed germination and seedling growth of *Cymbidium elegans* Lindl. and *Coelogynae punctulata* Lindl. J. Orchid Soc. India 5: 25-28.

Sharma, A., P. Tandon and A. Kumar. 1992. Regeneration of *Dendrobium wardianum* Warner (Orchidaceae) from synthetic seeds. Indian J. Exptl. Biol. 30: 747-748.

Sharma, J. and Y. S. Chauhan. 1995. Establishment of *in vitro* raised seedling of *Dendrobium* and *Paphiopedilum*. J. Orchid Soc. India 9: 37-41.

Sharma, V. 1996. Orchid propagation for conservation and commercialization. A study *in vitro*. Ph.D Thesis. Punjab Univ. Chandigarh, India.

Sharma, M. and S. Roy. 1996. Plantlet regeneration from seed culture of *Arundina graminifolia* (D. Don) Hochr. and establishment of plantlets under natural condition. In L. K. Pareek and P. L. Swarnakar (eds.), Trends in Plant Tissue Culture and Biotechnology. pp. 248-252. Agro. Bot. Publishers.

Sharma, C. M. and S. Kaur. 1998. Effect of different media on regeneration and establishment of plantlets of *Arudina graminifolia* orchids (D. Don) Hochr. Intn. fast of orchid. Abs. pp. 99.

Sharon, M. and G. Vasundhara. 1990. Micropropagation of *Dendrobium Joannie Ostenhault*. J. Orchid Soc. India. 4: 145-148.

Sheehan, T. J. 1960. Effect of nutrition and potting media on growth and flowering of certain epiphytic orchids. Proc. 2nd World orchid Conf., London. pp. 211-218.

Sheelavanthmath, S. S. and H. N. Murthy. 2001. *In vitro* propagation of a terrestrial orchid *Habenaria marginata* Coleb. J. Orchid Soc. India 15: 85-88.

Sheelavanthmath, S. S., H. N. Murthy, A. N. Pyati, H. G. K. Ashok, B. V. Ravishankar. 2000. *In vitro* propagation of the endangered orchid *Geodorum densiflorum* Lam. Schltr. through rhizome section culture. Plant Cell Tiss. Org. Cult. 60: 151-154.

Shiau, Y. J., A. P. Sagare, U. C. Chen, S. R. Yang and H. S. Tsay. 2002. Conservation of *Anoectochilus formosanus* Hayata by artificial cross-pollination and *in vitro* culture of seeds. Bot. Bull. Acad. Sin. 43: 123-130.

Shimura, H. and Y. Koda. 2004. Micropropagation of *Cypripedium macranthos* var. rebunense through protocorm-like bodies derieved from mature seeds. Plant Cell Tiss. Org. Cult. 78: 273-276.

Singh, F. 1981. Differential staining of orchid seeds for viability testing. Am. Orchid Soc. Bull. 50: 418.

Singh, F. 1988. Storage of orchid seeds in organic solvents. Gartenbauwissenschaft. 53: 122-124.

Skirvin, R. M. 1981. Fruit Crops. I. In: B. V. Conger (ed.), Cloning of Agricultural plants via *in vitro* Techniques. CRC press, Boca Rato, Florida.

Skoog, F. and C. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultivated *in vitro*. In: Bio action of growth substances. 11th Symp. Soc. Exp. Biol. 11: 118-131.

Smereciu, E. A. and R. S. Currah. 1989. Symbiotic germination of seeds of terrestrial orchids of North America and Europe. Lindleyana. 1: 6-15.

Smith, S. E. and D. J. Read. 1997. Mycorrhizal Symbiosis. Academic press, London.

Smith, D. R., K. J. Horgan and J. Aiken-Christie. 1982. Micropropagation of *Pinus radiata* for afforestation. In: A. Fujiwara (ed.), Plant Tiss. Cult. Marizen, Tokyo. pp. 723-724.

Soneji, J. R., P. S. Rao and M. Mhatre. 2002. Germination of synthetic seeds of pineapple (*Annanus*). Plant Cell Rep. 20: 891-894.

Sood, A. and S. P. Vij. 1986. *In vitro* root segment culture of *Rhynchostylis reusa* BL. pp. 463-468. In S. P. Vij (ed.), Biology, Conservation and Culture of Orchids. Affiliated East-West Press Pvt. Ltd., New Delhi.

Sounderrajan, T. and T. S. Lokeshwari. 1994. Multiplication of orchid protocorms in liquid medium. J. Orchid Soc. India. 8: 47-51.

Staritsky, G., A. J. Dekkers, N. P. Louwaars and E. A. Zandvoort. 1986. *In vitro* conservation of aroid germplasm at reduced temperatures and under osmotic stress. In: L. A. Withers and P. G. Alderson (eds.), Plant Tissue Culture and its agricultural applications. Butterworth, London. pp. 277-286.

Steward, J. and J. Button. 1975. Tissue culture studies of *Paphiopedilum*. Am. Orchid. Soc. Bull. 49: 591-598.

Stewart, J. 1988. *Lemboglossum Cervantesii* (Orchidaceae). The Kew magazine. Vol. 5: 51.

Stoutamire, W. P. 1981. Early growth in North American terrestrial orchid seedlings. In: Proc. Symp. II and Lectures, North American Terrestrial Orchids. pp. 14-24. E. H. Planton (ed.), Michigan Orchid Society, Southfield.

Strauss, M. S. and D. M. Reisinger. 1976. Effects of α-Naphthaleneacetic acid on different orchid seed germination. Amer. Orchid. Soc. Bull. 45: 722-723.

Talukdar, M., L. Paswan and S. Barma. 1988. Flowering behaviour in relation to differential pot mixture treatment in orchids. In S. P. Vij and S. P. Khuller (eds.), Natl. Seminar on Current Research Trends in Indian Orchids with special reference to Tissue Culture Technology, Shillong, pp. 34.

Talukdar, A. 2001. Multiple shoot induction in *Dendrobium aphyllum* Roxb. J. Orchid Soc. India. 15: 35-38.

Talukdar, S. K., K. M. Nasiruddin, S. Yasmin, L. Hassan and R. Begum. 2003. Shoot proliferation of *Dendrobium* orchid with BAP and NAA. J. Biol. Sci. 3: 1058-1062.

Tamanaha, L. R., C. G. Shimizu and J. Arditti. 1979. The effects of ethephon on *Cattleya aurantiaca* (Orchidaceae) seedlings. Bot. Gaz. 140: 25-28.

Tanaka, M. 1987. Studies on the clonal propagation of *Phalaeopsis* through *in vitro* culture. Mem. Fac. Agric. Kagawa Univ. 49:81-85.

Tanaka, M., A. Hasegawa and M. Goi. 1975. Studies of the Clonal propagation of the monopodial orchids by tissue culture I. Formation of protocorm-like bodies from leaf tissue in *Phalaenopsis* and *Vanda*. J. Japan Soc. Hort. Sci. 44: 47-58.

Tanaka, M. and O. Y. Sakanishi. 1978. Factors affecting the growth of *in vitro* cultured lateral buds from *Phalaenopsis* flower stalks. Sci. Hort: 169-178.

Tanaka, M. and Y. Sakanishi. 1985. Regeneration capacity of *in vitro* cultured leaf segments excised from mature *Phalaenopsis* plants. Bull Univ. Osaka prefect Ser. 37: 1-4.

Tanaka, M., J. Yayakawa, M. Nishimura and M. Goi. 1989. Micropropagation of *Phalaenopsis* by leaf segment culture. pp. 6-13. In: R. Shooter (ed.) Proc. of 3rd Asia Pacific orchid Conference, Lave Bros. Master Printers Pvt. Ltd., Adelaide, South Australia.

Taylor, R. H. 1971. Are virus diseased plants really necessary? pp. 63-66. In: M. J. G. Corrigan (ed.), proc. 6th World Orchid Conf. Sydney.

Temjensangba and C. R. Deb. 2005. Regeneration and mass multiplication of *Arachnis labrosa* (Lindl. ex Paxt.) Rechb. A rare and threatened orchid. Curr. Sci. 88: 1966 1968.

Teng, W. L., L. Nicholson and M. C. Teng. 1997. Micropropagation of *Spathoglottis plicata*. Plant Cell Rep. 16: 831-835.

Thompson, R. P. 1971. Excision of a *Cymbidium* meristem: Photographed in colour. Am. Orchid Soc. Bull. 40: 580-584.

Tisserat, B. and D. Jones. 1999. Clonal propagation of orchids. In: Hall, R. D. (ed.), Plant Cell Culture protocols, Methods in Molecular Biology, III. Humana Press Inc., Totowa, NY, USA, pp. 127-134.

Torikata, H., Y. Sawa and M. Sisa. 1965. Non-symbiotic germination and growth of the orchid seeds. Pt. I. Studies on the medium and additive for germination of seed in *Cymbidium*. J. Jap. Soc. Hort. Sci. 34: 63-70.

Tokuhara, K. and M. Mii. 1993. Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Rep. 13: 7-11.

Tokuhara, K. and M. Mii. 2001. Induction of embryogenesis callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis* (Orchidaceae). *In Vitro* Cell. Dev. Biol. Plant 37: 457-461.

Uesato, K. 1978. Studies on the formation and development of protocorms in growth cycle of orchids. Bull. Coll. Agric. Univ. Ryukus. 25: 1-76.

Vacin, E. and F. W. Went. 1949. Culture solution for orchid seedlings. Bot. Gaz. 110: 605-613.

Valmayor, H. L., M. L. Pimentel and M. T. Martinez. 1986. Callus formation and plantlet morphogenesis in *Vanda.* Malay. Orchid Rev. 20: 22-30.

Van Waes, J. M. and P. C. Debergh. 1986. *In vitro* germination of some western European orchids. Physiol. Plant. 67: 253-261.

Van, T. T., M. N. T. Dien and A. Chlyah. 1974. Regulation of organogenesis in small explants of *Nicotiana tobacum*. L. Planta. 119: 149-159.

Van, L. B., P. T. N. Hang, H. L. T. Anh and T. V. K. Tran. 1999. High frequency shoot regeneration from *Rhynchostylis gigantea* (orchidaceae) using thin cell layers. Plant growth Reg. 28: 179-185.

Varma, A. 1995. Arbuscular mycorrhizal fungi: State of Art. Crit. Rev. Biotechol., 15: 179-200.

Varma, A. 1998. Mycorrhizae: Their role in sustaining wasteland. In: R. R. Mishra (ed.) Problems of Wasteland Development and Role of Microbes. pp. 161-181. AMIFEM Publications.

Vasil, K. 1982. Plant Cell Culture and somatic cell genetics of cereals and grasses. In: 1. Vasil, W. R. Scowcraft and K.J. Frey (eds.) PL. Imp. Somatic Cell. Gen. Academic Press, New York. pp: 179-203.

Vij, S. P. 1993. Regeneration response of orchid roots: A study *in vitro*. J. Orchid Soc. India. 5: 61-67.

Vij, S. P. 1998. Orchidology of India. Current Status: International festival of orchids. pp. 1-11.

Vij, S. P. and A. Sood. 1982. *In vitro* pseudobulb segment culture-a means for rapid clonal propagation of *Dendrobium moschatum* (orchidaceae). In: Natl. Sym. on development and comparative aspects of plant structure and function, Hyderabad. pp.18-21.

Vij, S. P. and P. Pathak. 1988. Asymbiotic germination of the saprophytic orchid, *Cymbidium macrorhizon*. A study *in vitro*. J. Orchid Soc. India. 2: 25-32.

Vij, S. P. and P. Pathak. 1989. Micropropagation of *Dendrobium chrysanthum* W. through pseudobulb segments. J. Orchid. Soc. of India. 3: 25-28.

Vij, S. P. and S. Kaur. 1989. Micropropagation of therapeutically important orchids: *Malaxis acuminata*. J. Orchid. Soc. of India. 3: 25-28.

Vij, S. P. and P. Pathak. 1990. Micropropagation of orchids through leaf segments. J. Orchid. Soc. of India. 4: 69-88.

Vij, S. P. and P. Kaur. 1994. Green pod culture in *Phaius tankervillae*. Orchid News 10: 10-11.

Vij, S. P. and V. Sharma. 1996. Regeneration competence of *Bulbophyllum careyanum* Hook Spreng. Pseudobulb segments. A study *in vitro*. J. Orchid Soc. India. 10: 25-29.

Vij, S. P. and P. Pathak. 1997. Micropropagation of *Luisia trichorhiza* (Hook.) Bl. using foliar explants. In: Proc. 5th National Seminar on Developmental Biology and Commercialization of orchids and orchid show. pp. 85. TOSI, Chandigarh, India.

Vij, S. P. and S. Kaur. 1998. Micropropaation of therapeutically important orchid: *Malaxis acuminata*. D. Don. J. Orchid Soc. India. 12: 89-93.

Vij, S. P. and S. Kaur. 1992. Regeneration potential of foliar peels of *Rhynchostylis retusa*. J. Orchid Soc. India. 6: 71-74.

Vij, S. P. and S. Aggarwal. 2003. Regeneration competence of foliar explants: *Vanda coerulea* Griff. J. Orchid Soc. India. 17: 73-78.

Vij, S. P., A. Sood and K. K. Plaha. 1981. *In vitro* seed germination of some epiphytic orchids. pp: 473-481. In: S. C. Verman (ed.), Contemporary Trends in Plant Science, Kalyani Publisher, New Delhi.

Vij, S.P., M. Sharma and S.S. Datta. 1983. Mycorrhizal association in North Indian Orchidaceae: A morphological study. Bib. Mycol. 91: 467-73.

Vij, S. P., A. Sood and K. K. Plaha. 1984. Propagation of *Rhynchostylis retusa* BL. (Orchidaceae) by direct organogenesis from leaf segment cultures. Bot. Gaz., 145: 210-214.

Vij, S. P., A. Sood and M. Sharma. 1986. *In vitro* leaf segment culture of *Vanda testacea* (Lindl.) Reichb f. (*V. Parviflora* Lindl.) (Orchidaceae). Curr. Sci., 55: 1100-1101.

Vij, S. P., P. Pathak and M. Sharma. 1987. On the regeneration potential of *Rhynchostylis retusa* root segments. A study *in vitro*. J. Orchid Soc. India. 1: 71-74.

Vij, S. P., A. Sood and P. Pathak. 1989. On the utility of rhizome segments in micropropagating *Eulophia hormusjii* Duth. J. Orchid Soc. India. 3: 41-45.

Vij, S. P., V. Sharma and S. Kaur. 1994. Foliar explant and orchid micropropagation: *Vanda* Kasem Delight "Tom Boykin". J. Orchid Soc. India. 8: 79-83.

Vij, S. P., S. Aggarwal and Kanika. 1995. Pots and mixes for orchid culture. Orchid News. 11: 1-2.

Vij, S. P., P. Pathak and K. C. Mahant. 1995. Green Pod culture of a therapeutically important species *Dactylorhiza hetagirea* (D. Don) Soo. J. Orchid Soc. India. 9: 7-12.

Vij, S. P., P. Pathak and K. C. Mahant. 1997. On the regeneration competence of *Coelogyne viscosa* pseudobulb explants. In: proc. 5th National Seminar on Developmental Biology and Commercializaton of Orchids (ed. S. P. Vij) pp. 88. TOSI, Chandigargh, India.

Vij, S. P., P. Pathak and A. Kher. 1997. Regeneration response of *Rhynchostylis gigantea* inflorescence segments: A study *in vitro*. J. Orchid Soc. India. 11: 75-78.

Vij S. P., A. Kher and P. Pathak. 2000. Regeneration competence of *Bulbophyllum careyanum* (Hook.) spreng. pseudobulb segments. J. Orchid Soc. India. 14: 47-55.

Vij, S. P., P. Kaur, K. Konda, P. Pathak and A. Gupta. 2002. Regeneration competence of orchid leaves: A study *in vitro*. In: S. P. Vij, Ko Kondo, M. L. Sharma and A.

Gupta (eds.) Plant Genetic Diversity Exploration, Evaluation and Conservation pp. 289-306. Affiliated East-West Press, New Delhi, India.

Wainwright, H. and J. Scrace. 1989. Influence of *in vitro* preconditioning with carbohydrates during the rooting of microcuttings on *in vivo* establishment. Sci. Hort. 38: 261-267.

Wakabayashi, K., N. Sakurai and S. Kuraishi. 1991. Effects of abscissic acid on the synthesis of cell wall polysaccharides in segments of etiolated squash hypocotyls II. Levels of UDP neutral sugars. Plant Cell Physol. 32: 427-432.

Wanas, W. H., J. A. Callow and L. A. Withers. 1986. Growth limitations for the conservation of pear genotypes. In: L. A. Withers and P. G. Alderson (eds.), Plant Tissue Culture and its Agricultural application. pp. 285-289. Butterworths, London.

Wang, P. J. 1989. Rapid clonal propagation of *Phalaenopsis* by tissue culture. Acta Hort. Sci. 16: 73-77.

Wang, Q. C., O. Batterman, P. Li, B. Joseph and R. Gafny. 2002. Cyropreservation of *in vitro* grown shoot tips of royer citrange (*Poncirus trifoliata*) (L) Raf × Citrus Sinensus (L) Osbeck by encapsulation - dehydration. Plant Cell Rep. 20: 901-906.

White, P. R. 1951. Nutritional requirements of isolated plant tissue and organs. Ann. Rev. Plant Physiol. 2: 231-244.

Wiegel, R. C. and K. W. Hughes. 1983. Morphogenesis n barley tissue cultures. *In vitro*. 19: 248.

Wilkins, C. P., H. J. Newbury and J. H. Dodds. 1988. Tissue culture conservation of fruit trees. FAO/ IBPGR Plant Genetic Resources. Newsletter pp.73-74.

Wilkins, C. P. 1991. Conservation of tree crops. In: J. H. Dodds (ed.), *In vitro* methods for conservation of plant genetic Resources, pp. 151-237. Chapman and Hall, London.

Wimber, D. E. 1963. Clonal multiplication of *Cymbidium* through tissue culture of the shoot meristem. Am. Orchid Soc. Bull. 32: 105-107.

Wimber, D. E. 1965. Additional observation on clonal multiplication of *Cymbidium* through culture of shoot meristem. Cymb. Soc. News. 20: 7-10.

Withers, L. A. 1986. Cryopreservation and genebanks. In: M. M. Yeoman (ed.), Plant Cell Culture Technology. Blackwell Scientific, Oxford. pp. 96-140.

Withers, L. A. 1987. Long-term preservation of plant cell tissue and organs. Oxford surveys of plant molecular and cell biology. pp. 221-272.

Withers, L. A. 1991. *In vitro* conservation. Biological Journal of the Linnean Society. 43: 31-42.

Withers, L. A. 1992. Preservation of plant tissue cultures. In: B. E. Kirsop and J. J. S. Snell (eds.), Maintenance of microorganisms. Academic press, London.

Withers, L. A. and P. J. King. 1980. A simple freezing unit and cyropreservation method for plant cell suspensions. Cryoletters. 1: 213-220.

Withner, C. L. 1959. Orchid Physiology. In: The Orchids. A Scientific Survey (ed. C. L. Withner), pp. 315-360. Ronald Press, New York.

Withner, C. L. 1974. Development in orchid physiology. In: C. L. Withner (ed.), The Orchids-Scienific Studies. Wiley, Interscience, New York.

Withner, C. L., P. K. Nelson and Wejksonra. 1974. The anatomy of orchids. In: C.L Withners (ed.), The Orchids Scientific Studies. John Wiley and Sons, New York. pp: 267-347.

Wong, J. R. and I. M. Sussex. 1980. Isolation of ABA resistant variants from tobacco cell cultures. I. Physiological basis for selection. Plant. 148: 97-102.

Wynd. F. L. 1933. Sources of carbohydrate for germination and growth of orchid seedling. Ann. Missouri. Bot. Gard. 20: 569-581.

Yadav, L. P., R. Chatterjee, A. Roy and D. Sengupta. 1988. Effect of potting media on growth and flowering in some orchids of commercial value. In S. P. Vij and S. P. Khuller (ed.), Natl. Seminar on Current Research Trends in Indian orchids with special reference to Tissue Culture Technology. pp. 31. Shillong.

Yam, T. W. and M. A. Weatherhead. 1988. Germination and seedling development of some Hong Kong orchids. Lindleyana 3: 156-160.

Yam, T. W. and M. A. Weatherhead. 1991. Leaf tip culture of several native orchids of Hongkong. Lindleyana, 6: 151-53.

Yam, T. W., J. Arditti and M. A. Weatherhead. 1989. The use of darkening agents in seed germination and tissue culture media for orchids: A Review. J. Orchid Soc. India. 3: 35-39.

Yoneda, K. 1986. A study on the culture of the inflorescence tip of the *Phalaenopsis*. Bull. Coll. Agri and Vet. Med. Nihon Univ. 43: 124-127.

Yoneda, K. and H. Momose. 1988. PLB and plantlet formation by root-tip culture in *Phalaenopsis*. Bull Coll. Agric. Vet. Med. Nihon. Univ. 45: 191-196.

Yoneda, K., T. Sakamoto and H. Sasaki. 1983. Studies on Mericlone of Orchids III. Clonal propaation of *Phalaenopsis* by means of flower stalk, bud culture and shoot tip culture. Bull. Coll. Agri and Vet. Med. 40: 1-13.

Zandvoort, E. A. and G. Staritsky. 1986. *In vitro* genebanks of tropical aroids-research of storage conditions. VI. International Congress of Plant Tissue Cell Culture pp. 426. Minneapolis (Abs.)

Zeigler, A. R., T. J. Sheehan and R. T. Poole. 1967. Influence of various media and photo period on growth and amino acid content of orchid seedlings. A.O.S 195-201.

Zhou, T. S. 1998. Propagation of *Phalaenopsis* orchids. Hort. Sci. 33: 595.

CURRICULUM VITAE

Name : Meera Chettri Das

Husband's Name : Mrinal Kumar Das

Address : Mendipathar, East Garo Hills, Meghalaya

Date and Place of Birth : November 14, 1973

Mendipather, East Garo Hills (Meghalaya)

Sex and Maritial Status : Female (Married)

Educational Qualifications

Examination Passed	Board/University	Year	Div./ Percentage
H.S.L.C	M.B.O.S.E	1990	1st, 62.5%
P.U. (Science)	N.E.H.U.	1992	2nd, 54.1%
B.Sc (Pass)	-do-	1994	2nd, 56.0%
B.Sc (Botany Honours)	-do-	1995	1st, 60.7%
M.Sc	-do-	1998	1st, 68.2%
N.E.T.	C.S.I.R.	2002	-

Ph. D. Title : *In vitro* propagation and conservation of *Cymbidium devonianum* Paxt. and *Dendrobium lituiflorum* Lindl., rare and threatened epiphytic orchids of Northeast India

Professional Experience

Teaching : Taught on leave vacancy and as substitute at graduate level

Research : Research experience of five years in plant tissue culture

Fellowships

Research Fellow	2001-2002	DSA sponsored project (*In vitro* conservation of *Coelogyne* spp. of Northeast India)
J.R.F. (C.S.I.R.)	2002-2004	C.S.I.R. sponsored fellowship
S.R.F. (C.S.I.R.)	2004-2006	- do-

Academic Awards

Secured second letter mark in Arithmetic in H.S.L.C.

Secured second position in B. Sc (Hons) and third position in M.Sc.

Research Activities

Publications

Chettri Das, M., S. Kumaria and P. Tandon. Protocorm regeneration, seedling growth and establishment of *Cymbidium devonianum* Paxt., rare and threatened orchid species of Northeast India (Asian Journal of Plant Sciences) Accepted

Chettri Das, M., S. Kumaria and P. Tandon. *In vitro* propagation and conservation of *Dendrobium lituiflorum* Lindl, through protocorm like bodies (Indian Journal of Plant Biochemistry and Biotechnology) Communicated

Chettri Das, M., S. Kumaria and P. Tandon. Storage and conservation of protocorm like bodies (PLBs) of *Cymbidium devonianum* Paxt., rare and threatened orchid of Northeast India through encapsulation. (Biodiversity and Conservation). Communicated

Chettri Das, M., S. Kumaria and P. Tandon. Mass micropropagation of *Dendrobium lituiflorum* Lindl.: rare and threatened epiphytic orchid species of Northeast India (The Orchid Society of India). Communicated

Books (Chapter)

Dohling, S, M. Chettri Das, S. Kumaria and P. Tandon. Conservation of splendid orchids of North- East India. (Chapter): In press, Book: Biodiversity and Significance (eds.) P. Tandon, Y. P. Abrol and S. Kumaria. Publishers I. K. International Publishing House Pvt. Ltd. New Delhi. Accepted

Posters presented in National Seminars/Conferences

Chettri Das, M., S. Kumaria and P. Tandon. High frequency plantlet regeneration of *Dendrobium lituiflorum* through asymbiotic seed germination (Poster). T. N. Khoshoo Memorial Function N.B.R.I. Lucknow. April 07-08, 2004.

Chettri Das, M., S. Kumaria and P. Tandon, Mass Micropropagation of *Dendrobium lituiflorum* Lindl : A rare and threatened epiphytic orchid species of Northeast India. Abstract: 8th National Seminar on Orchid conservation improvement and commercialization and satellite symposium on Orchids. Why and How? The Orchid Society of India (TOSI. March 18-20, 2006. p 41).

Chettri Das, M., S. Kumaria and P. Tandon. Conservation of *Cymbidium devonianum* Paxt through artificial seed technology November 2-3, 2006. ABS no. 74. page 56

Short Term Course Training

Participated in

- The training course on " Application of Basic I.T. Resources" during 20th - 23rd June 2001 conducted by the Bio-Information Centre, N.E.H.U.
- Attended the 72th Annual Session of the National Academy of Sciences held on October 25-27, 2002.
- Workshop on Conservation and Sustainable Utilization of Medicinal Plants of North-East India. Sponsored by Ministry of Environment & Forests, Govt. of India. May 27-28, 2004
- The training course on "Computer Basic Applications" Bio-informatics (Internet & Database) April 18-20, 2005