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

BY

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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.

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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
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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 (*Paphiopedillum*) 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 al.*, 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, Biolya, 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.*, 2005). 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