CLONAL PROPAGATION AND CRYOPRESERVATION OF *DENDROBIUM LONGICORNU* LINDL. AND *DENDROBIUM FORMOSUM* ROXB.: TWO ENDANGERED ORCHIDS OF NORTH-EAST INDIA

BY

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DECLARATION

I do, hereby, declare that the thesis entitled 'Clonal propagation and cryopreservation of *Dendrobium longicornu* Lindl. and *Dendrobium formosum* Roxb.: Two endangered 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 Dr. Suman Kumaria and Prof. Pramod Tandon. The work 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 **'Clonal propagation and cryopreservation of *Dendrobium longicornu* Lindl. and *Dendrobium formosum* Roxb.: Two endangered orchids of North-East India',** submitted by Mr. Stadwelson Dohling 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 him under our supervision. He 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.

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(Stadwelson Dohling)

CONTENTS

CHAPTER I: GENERAL INTRODUCTION

Orchids belong to the family Orchidaceae, which is not only the most specialized families of the flowering plants, but also is one of the largest families. Orchidaceae includes 800 genera and between 25,000 to 30,000 species spread all over the world (Chowdhery, 2001). Orchids are the most fascinating and beautiful of all the flowers. They constitute an order of royalty in the world of ornamental plants and are of immense horticultural importance. They have attracted floriculturists since time immemorial due to their fads, fancies and fashions and have thus led to "orchid mania" throughout the world. The orchids are known to mankind for the last several centuries for their beautiful, attractive flowers and as medicinal plants. These plants have fascinated people even since their discovery by Theophrastus (370-285 BC) who referred to a group of curious plants called *Orchis* in his writing "Enquiry into Plants". The name 'orchid' is derived from the Greek word 'Orchis' meaning testis and refers to the roots of such plants. The paired tubers in the genus *Orchis* and other similar genera resemble human testicles which were considered of highly medicinal value. The orchids are cosmopolitan in distribution, occurring in almost all the parts of the world except the Antarctica. The great majority are to be found in the tropics, mostly Asia, South America and Central America. They are found above the Arctic Circle, in southern Patagonia and even on Macquarie Island close to Antarctica. Several species of orchids have been used in various indigenous systems of medicines since the Vedic periods (Kirtikar and Basu, 1935; Trivedi *et al.*, 1961; Kaushik, 1983; Handa, 1986; Vij *et al.*, 1997). The tubers and pseudobulbs of several orchids like *Orchis latifolia*, *O. mascula*, *Cymbidium aloifolium*, *Zeuxine strateumatica*, and some species of *Dendrobium*, *Eulophia* and *Habenaria* are used for preparing salep which is valued as a restorative and is used in the treatment of various diseases. Several orchids have been used as food in different parts of the world. *Anoectochilus* leaves are used in Indonesia and Malaysia as vegetables. Dried leaves of *Dendrobium salaccense* cooked with rice add delicate and exotic flovour. Pseudobulbs of *Cymbidium madidum* and *Dendrobium speciosum* are used as food. The famous vanillin used for flavouring is extracted from the green pods of *Vanilla planifolia.* Some orchids are glycosidal plants. Loroglossin glycosides, having fragrance of hay, is found in the genus *Loroglossum.* Coumarine glycosides are derived from *Angraecum fragrans*. *Paphiopedilum javanicum* has saponine glycosides which possess strong foaming properties. The vanilla glycosides and related aromatic compounds are found in several species of *Vanilla.*

The great geographic expanse of India encompassing a variety of bioclimatic zone and the enormous diversity of ecosystem accounts for the bewildering array of orchid species. The figure of orchid species occurring within the present political boundary of the country is at 1229 species belonging to 184 genera (Singh, 2001). The genus *Dendrobium* Sw. with about 104 species known to occur in the country is the largest orchid genus in India (Singh, 2001). The Indian orchids grow at altitudes as high as 5000m, and in areas having an annual rainfall of as low as 60mm and as high as 1100mm. The epiphytic orchids are abundant upto 1800m 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 Northeastern region of India with its wide ranging altitudinal variations from the foothills to the high Himalayan mountains and deep river valleys with high rainfall and high humidity, distinctive soil conditions, etc., have all played a significant role in the development of the highly rich orchid diversity. In spite of the rich vegetation, the flora of the Northeast India remains largely unexplored which hinders the full exploitation of the plant resources. A great number of species including several unique and irreplaceable varieties are getting extinct and many more are awaiting a similar fate. The disturbances in the flora of the region could be due to the following reasons (Tandon, 2004):

- Burning of the forests during the pre-monsoon months for the growth of grasss which is the secondary forest product for cattle rearing/dairy farming.
- Burning of the agricultural fields in the form of *Jhum* or shifting cultivation, *Bun* cultivation or burning of undergrowth.
- Excessive and unmindful collection of the rare and endangered plants.
- Cutting of the dense forests randomly for trade of timber.

About 50% of the country's orchid wealth harbouring 750-800 species is found in Northeastern India (Chowdhery, 2001). 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, Northeast India also hosts a large number of endemic, rare and threatened orchid species (Nayar and Sastry, 1997-98, 1999; Ahmedullah, 2000). Among the Northeastern 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). Unfortunately, the orchid diversity in the Northeast India and the country as a whole is being threatened for various reasons such as the increased biotic influences, socio-economic development and uncontrolled commercial exploitation of forest wealth. Almost all the epiphytes because of their habitat specificity and slow growing nature fail to withstand habitat destruction pressure and all of them figure prominently in the list of endangered plants (Rajeevan and Shobhana, 1993). The habitat destruction, which is occurring at an alarming rate due to deforestation and other unplanned human activities, has led to a considerable depletion of orchids in nature. Presently, all the orchid species are protected for the purpose of International Commerce under the Convention of International Trade for Endangered Species (CITES) as politically threatened or endangered in their natural habitat, with most of the species listed under Appendix II. Therefore, it has become necessary to propagate and conserve the orchids (Hegde *et al.,* 1992; Kataki, 1993). Biotechnological approaches of conservation are found to be complementary to conventional methods. These can directly assist plant conservation programmes through molecular marker technology, molecular diagnostics, *in vitro* technologies and cryopreservation (Tandon and Kumaria, 1998, 2005).

Biologically orchids are highly specialized and able to grow on a variety of substrata. The majority of orchids are epiphytes that prefer growing being perched on trees and sometimes even on moss covered boulders. However, lithophytes, terrestrials and saprophytes growing on rocks, ground and organic matter respectively are also found. Orchids are perennial plants blooming annually under favourable conditions of light, temperature and humidity. The flowers are borne on long, short or highly reduced floral axis in the leaf axil or opposite to leaf at the tip of the stem or from the base of the pseudobulb or from the body of pseudobulb; either solitary or 2-100 or even more in number. 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 monocots by the reproductive or sex organs (stamens and stigma) which are fused to form one structure called the "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. The seeds lack an endosperm and have 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. In majority of the orchids the embryo is few-celled at the time of seed maturation and its proper development takes place only during germination of seeds (Senthilkumar, 2001). However, as the seeds do not have sufficient reserve food materials to take care of the growth of embryo during germination (Richardson *et a*l., 1992), they have to depend on some external source for nutrients so as to enable 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.

Studies in orchids using tissue culture techniques are gaining wide importance (Charanasri, 1989). The application of these techniques to the production of quality orchids in large quantities by clonal multiplication, establishment of hybrid plants, improvements in 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, 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 the nature of 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 (Arditti, 1982; Arditti and Ernst, 1984; 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). Besides the selection of media, other physico-chemical factors for orchid seed germination have been investigated. Many scientists have reported the effect of both qualitative and quantitative light including photoperiod on orchid seed germination and growth (Zeigler *et al.*, 1967; Mitra, 1971, Ueda and Tarikata, 1972; Ernst, 1976, Hasegawa *et al.*, 1978). The optimum temperature for seed germination of most orchid species is reported to be between 20°C to 25°C (Grillo Mensa *et al.*, 1983). Several growth regulators have been incorporated in the media to promote orchid seed germination and seedling growth in many species (Pierik and Steegman, 1972; Strauss and Reisinger, 1976; Arditti, 1982; Nakamura, 1982; Sharma and Tandon 1986; Van Waes and Debergh, 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 - naphthaleneacetic 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 that incorporation of NAA in the medium inhibited both seed germination and seedling growth of *Dendrobium fimbriatum* var. *oculatum.* Addition of KN in medium containing NAA was found to be effective for subsequent growth and differentiation of seeds after germination in case of *Dendrobium transparens* (Hazarika and Sharma, 1995). Similarly, enhanced effect 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 the orchid *Phaius tankervilliae*. In plant tissue culture studies, 2, 4-dichlorophenoxy acetic acid (2,4-D) has been reported to induce callusing at very low concentrations (Negrutia *et al*., 1978; Cornijo-martin *et al*., 1979; Biondi and Thorpe, 1982). According to Mitra (1986), 2,4-D has been shown to either inhibit germination or stimulate callusing of seeds. Vasil (1982) reported that 2,4-D is more effective auxin to regenerate cell cultures via somatic embryogenesis. In case of orchids, it was reported to suppress rhizogenesis in *Aerides multiflorum* (Vij and Pathak, 1990) whereas in *Paphiopedilum* species it had been 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 had 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 in cultured leaf segments (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 or PLBs. Regular chopping of these PLBs and culturing them on to fresh medium resulted in their multiplication, but when left undisturbed the PLBs 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 1.0 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, 1979a, 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, 1986; Mathews and Rao, 1985; 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 meristems (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 Lokeswari, 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, 1979a, b; Yoneda and Momose, 1988).

There is a growing need for cryopreservation of plant genetic resources, which provides stable long-term storage in liquid nitrogen (LN) at -196°C (Tandon, 2000). Freezing at liquid nitrogen 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. In the past, mostly vegetatively propagated plants were cryopreserved, but with rapid progress in plant transformation, cryopreservation is widely used in preserving the experimental materials of primary transformed tissues, secondary cultures, etc. (Kendall *et al.*, 1993). Though cryopreservation has been carried out in almost all types of tissues and organs, shoot and embryo cultures are found to be more relevant to genetic conservation (Hatanaka *et al.*, 1994; Na and Kondo, 1996; Hirai and Sakai, 1999, 2001). Several reports are available on plant species whose embryo and/or embryonic axes have been successfully cryopreserved (Pence, 1995; Engelmann *et al.*, 1995; Kuranuki and Yoshida, 1996; Engelmann, 1997a, b). The different responses of desiccation of embryonic axes prior to immersion in liquid nitrogen are also available (Grout *et al.*, 1983; Normah *et al.*, 1986; Mycock *et al.*, 1995; Touchell *et al.*, 2002). Though shoot cultures have received as much attention as other cells and organs, they are more variable in response and generally are more difficult to cryopreserve (Kartha *et al.*, 1982a, b; Withers, 1987a, b). Subsequently, a lot of work has been carried out on shoot tips of various plants. The shoot tip of asparagus (Kumu *et al.*, 1983), brussel sprouts (Harada *et al.*, 1985), sweet potato (Pennycooke and Towill, 2000) were cryopreserved using 5-15% dimethyl sulfoxide (DMSO) as cryoprotectant. Shoot tips of sugar beet (Vandenbussche *et al.*, 2000), white poplar (Lambardi *et al.*, 2000), persimmon (Matsumoto *et al.*, 2001), shoot primordia of *Vanda pumila* (Na and Kondo, 1996), *Cattleya loddigesii*, *C. walkeriana*, *Dendrobium* cv. 'Yukidaruma'(Kondo *et al.*, 2001), root fragments of chicory (Demeulemeester *et al.*, 1993), PLBs of *Cymbidium* (Kondo *et al*., 2001) and cells of brume grass (Ishikawa *et al*., 1996) have been cryopreserved.

The process of cryopreservation involves various stages such as pregrowth, cryoprotection, cooling/freezing, storage, thawing and recovery. Pregrowth provides the opportunity for selecting or inducing the most freeze-tolerant growth phase. It covers the period of time in culture during which measures are taken to enhance freeze tolerance through influence on the growth and metabolism of the cultures and from which the most freeze tolerant stages of growth can be selected. Generally material in active growth is more resistant to freezing stress. For most cell suspension cultures, the late lag phase or the exponential phase is the most freeze-tolerant phase. To enhance freeze-tolerance the plant tissues are pre-grown in presence of substances such as amino acids, mannitol, sorbitol, sucrose, abscisic acid (ABA), DMSO, etc. The cultures become freeze-tolerant by changing their cell size, degree of vacuolation and cell wall flexibility. In case of shoot tips, pregrowth also provides opportunity to recover from dissection damage. The requirement for pregrowth varies from culture system to culture system and with species to species.

Cryoprotection which is the next step involves application of diverse compounds referred to as cryoprotectants during the period immediately preceding cooling. For successful cryopreservation this step is absolutely necessary. It gives a protection to the tissues against freeze damage. Application of cryoprotectants reduces the amount of freezing water from the cells, thereby giving less chance for ice formation and ice damage (Farrant, 1980; Meryman and Williams, 1982). Different hypertonic solutions are used as cryoprotectants. Glycerol was used for the first time in animal cells (Polge *et al.*, 1949). The most frequently used cryoprotectant chemical is DMSO singly or in combination with various sugars, sugar alcohols and other compounds, which may include mannitol, sorbitol, sucrose, glucose, etc. Sometimes a mixture of cryoprotectants shows better results than a single one (Finkle and Ulrich, 1979; Hauptmann and Widholm, 1982; Chen *et al.*, 1984). A considerable benefit of using a mixture of cryoprotectants involving polyethylene glycol (PEG), glucose and DMSO in sugarcane callus has been reported (Ulrich *et al.*, 1979; Finkle *et al.*, 1985). ABA has also been used in cultures of shoot primordia from shoot apices of cultured protocorms of *Vanda pumila* and others (Na and Kondo, 1996). These cryoprotective agents are usually with low molecular weight and are easily miscible, easily washable and can easily permeate the cells. Cryoprotectant mixtures are often prone to caramelisation during autoclaving and therefore must be sterilised by filtration. The concentration and the type of cryoprotectants suitable for successful cryopreservation vary for different plants. The duration of exposure of tissues to this cryoprotectant also varies. Generally a concentration of 5 to 10% of DMSO and 10 to 20% of glycerol is adequate for most experimental materials. During the cryoprotection step the materials are exposed to different cryoprotectant solutions and treated for different periods (for few minutes to hours) before cooling. The samples in cryoprotectants are next subjected to cooling/freezing.

The cooling rate has a very significant role in survival of tissues and organs. Cooling can be carried out either at a relative slow or rapid rate (Farrant, 1980; Meryman and Williams, 1982). Rapid freezing directly exposes the material to ultra low temperature at a rate of several hundred degrees centrigrade per minute. Rapid cooling causes relatively early intracellular freezing and little dehydration. Intracellular freezing leads to death of cells even in very hardy plants due to the mechanical destruction of biomembranes. In rapid freezing, innocuous intracellular freezing occurs. Cryopreservation by rapid freezing results in very low survival percentage of treated tissues/cells and sometimes even in complete loss of their viability. But it is effective in some organised tissues. Damage by intracellular freezing depends on the amount, crystal size and location of the ice. But in many cases slow freezing is found to be a better method. Slow cooling is carried out at a constant, linear rate, where there is progressive temperature reduction. At first the external medium supercools and then there is ice formation. So extracellular freezing occurs, which causes cellular dehydration. The cell wall acts as a barrier and prevents ice from forming inside the cell. Freezing injury is comparatively less and it brings an optimum situation between under- and over-dehydration. Slow cooling is usually carried out in a programmed freezer where the rate of cooling can be varied for different specimens.

Storage of the experimental materials is carried out in liquid nitrogen. The storage temperature should be low to prevent progressive deterioration resulting from ice recrystalization. Usually below -100°C is recommended which can be achieved by the use of liquid nitrogen cooled freezer in which the liquid phase is stable at -196°C.

Although metabolism is suspended at this temperature but molecular changes due to ionizing effects of radiation, and consequent free radical activity may lead to cumulative damage in long-term storage.

Warming or thawing is also as critical as cooling for the survival of specimens. It is essential to carry out thawing at a rate which prevents recrystalization of any ice that is present intracellularly. Warming is usually carried out rapidly by use of hot water bath (40°C) or warm medium (20°C or higher). The thawed specimens are cultured on fresh medium containing nutrients, vitamins and growth regulators for their recovery. Some times the composition of the recovery medium is modified depending on the regeneration of the species. Viability can be recorded based on the performance of the species on recovery medium or by some viability tests such as TTC (trichloro tetrazolium chloride) and FDA (fluorescein diacetate) staining.

Though rapid and slow freezing are the common methods used in cryopreservation, encapsulation-dehydration and vitrification methods developed recently are cheaper and easier to perform. Encapsulation-dehydration is a method based on successive osmotic and evaporative dehydration and has successfully been applied in shoot tips of pear (Dereuddre *et al.*, 1990), persimmon (Matsumoto *et al.*, 2001), somatic embryos of coffee (Hatanaka *et al.*, 1994) and citrus (Gonzalez-Arnao *et al.*, 2003). Vitrification, which is relatively a new method, enables cells and meristems to cool to temperature of liquid nitrogen without ice formation. It has become the preferred method for cryopreservation over the last two decades, with over 160 species and cultivars being successfully cryopreserved (Sakai, 2000; Sakai *et al.*, 2002). It is the most promising method for the cryopreservation of shoot tips (Matsumoto *et al.*, 1994; Hirai and Sakai, 1999, 2001; Pennycooke and Towill, 2000; Sharma and Sharma, 2003; Gagliardi *et al.*, 2003). The successful cryopreservation of orchid embryos in some cases has also been reported (Ishikawa *et al.*, 1997). To develop simpler techniques and to improve the survivability many vitrification solutions have been formulated (Sakai *et al.*, 1990; Matsumoto *et al.*, 1995; Tandon *et al.*, 2000).

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 shoots and roots during transplantations (Debergh 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 but the amount as well as the type varies from one orchid species to another.

The following are the two epiphytic orchids which were chosen for the present study:

Dendrobium longicornu Lindl., commonly known as 'Long-horned *Dendrobium*', has been reported to be an endemic species in Northeast India (Chowdhery, 2001). Its pseudobulb is long, sub erect, close, cylindric, gradually tapering towards the base. Leaves are sessile, few, linear-lanceolate, apex acutely bifid. It has 2-4 flowers on a short peduncle, which may be terminal or lateral, white in colour (Plate 1.1a, b). Its lip is white with a yellow patch in the middle; petals are ovate-oblong, acute, sub equal; dorsal sepals are ovate-oblong, while lateral sepals are ovate, acute, joined with the foot forming a long conical mentum. The flowering time of *D. longicornu* is September-November. It is found in Meghalaya, Nagaland, Sikkim and Bhutan (Kataki, 1986).

Dendrobium formosum Roxb., commonly known as 'Beautiful Giant-flowered *Dendrobium*', has erect or sub erect, stout, and cylindric stems. Leaves are broadly oblong, long, sub-amplexicaul, with young leaves pubescent on ventral side. Flowers are white, 3-5 in number, large and drooping (Plate 1.2a, b). The lip of the flower is white with a yellow patch from middle to the base; petals suborbicular, many nerved, margin undulate; sepals sub equal, oblong-lanceolate and acuminate. The flowering time of *D. formosum* is May-June. It is distributed in North-West and North-East Himalayas (Kataki, 1986).

The main objectives of the study were:

(I) Clonal propagation and establishment of *D. longicornu* and *D. formosum*.

(II) Cryopreservation of the selected dendrobes.

Plate 1.1

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

(b) A closer view of the flower


a
b

Plate 1.2

(c) *Dendrobium formosum* Roxb. blooming in natural habitat

(d) A closer view of the flower


a
b