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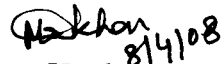
DECLARATION

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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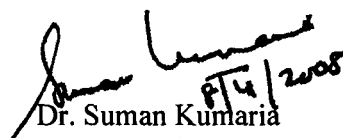


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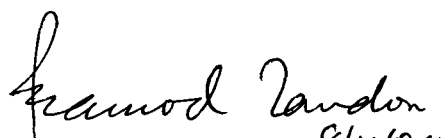


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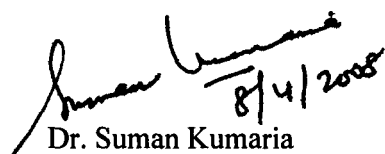
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CERTIFICATE

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)

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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 flavour. 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 grasses 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; Katakai, 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 al.*, 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 tankervilleae*. 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 (Kantha *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.

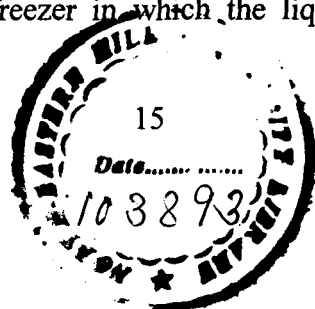
The process of cryopreservation involves various stages such as pregrowth, cryoprotection, cooling/freezing, storage, thawing and recovery. Pregrowth provides the opportunity for selecting or inducing the most freeze-tolerant growth phase. It covers the period of time in culture during which measures 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 centigrade 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 recrystallization. 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 recrystallization 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-Arno *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



Plate 1.2

- (c) *Dendrobium formosum* Roxb. blooming in natural habitat
- (d) A closer view of the flower



CHAPTER II: ASYMBIOTIC SEED GERMINATION AND SEEDLING GROWTH OF *DENDROBIUM LONGICORNU* AND *DENDROBIUM FORMOSUM*

INTRODUCTION

The most interesting and adaptive feature of the family Orchidaceae is the physiology of its seed germination. The orchid seeds although minute and produced in a large number i.e., 2-3 million per capsule, are non-endospermous and do not contain nutrients (Vu Quoc Luan *et al.*, 2006). Mycorrhizal 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. The application of *in vitro* seed propagation technique to rare orchid species which are suffering from over-collection and continuous loss of their natural habitats undoubtedly is a powerful tool for *ex situ* conservation (Buyun *et al.*,

2004). 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. 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 Knudson (1946), Murashige and Skoog (1962), Nitsch and Nitsch (1969), Gamborg *et al.* (1968), Mitra *et al.* (1976), Reddy *et al.* (1992), and Nagaraju *et al.* (2003). A better efficacy of these 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; Mukherjee *et al.*, 1974). But the success of *in vitro* orchid seed germination and seedling growth is not only restricted to nutrient supply but also to physical factors like temperature and light which greatly influence the physiology and development of orchid seedlings (Arditti and Ernst, 1984). However, the information on the influence of the physical factors on orchid seed germination is scanty. The influence of both qualitative and quantitative light including photoperiod on orchid seed germination has been studied to some extent (Withner, 1959; Ziegler *et al.*, 1967). Zettler and Mcinnis (1994) found that light increased the seed germination of *Platanthera integrilabia*. The same was found for *Dactylorhiza majalis* (Rasmussen and Rasmussen, 1991). However, Arditti *et al.* (1990) stated that 'light may inhibit the germination of some temperate terrestrials', as had been found by Stoutamire (1974) with seeds of *Cypripedium* species. Temperature is a major factor in seed germination. Temperature effects on germination are unclear as different species show differential responses to cold

and warm temperatures (Stoutamire, 1974; Ichihashi, 1989). Zettler and Mcinnis (1993) found that *Spiranthes cernua* responded favourably to chilling storage while *Goodyera pubescens* did not. The range between 20-25°C had been found suitable for most of the orchid species (Harvais, 1973; Stoutamire, 1974; Arditti, 1982; Van Weas and Debergh, 1986).

MATERIALS AND METHODS

Plants of *D. longicornu* and *D. formosum*, collected from the forests of Meghalaya, were maintained in the glass house of the Plant Biotechnology Laboratory, Botany Department, North-Eastern Hill University, Shillong. To obtain sufficient number of capsules the flowers were hand pollinated. About eight - months old unopened capsules were harvested from the plants. 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 30W 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 (27 ± 3 m/min) helped in preventing air-borne contamination. Forceps, needles, surgical blades etc. were sterilized by dipping in 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 aseptically after which the capsules

were rinsed five times with sterile distilled water and dried in a laminar airflow cabinet. The capsules were then dissected longitudinally with a surgical blade to expose the seeds. 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. The various media tried were Murashige and Skoog (MS, 1962), Gamborg *et al.* (B₅, 1968), Mitra *et al.* (Mitra, 1976) and Knudson C (KC, 1946). The pH of the media was adjusted to 5.8 prior to autoclaving. The culture tubes were incubated at 25 ± 2°C under 12-hour photoperiod of 150 μmol m⁻²s⁻¹ light intensity.

To study the optimal conditions required for asymbiotic seed germination of the dendrobes, the seeds were cultured onto the MS basal medium and subjected to different physical factors such as temperature (16, 20, 24 and 28°C), photoperiod (0, 8, 12, 16 and 20h day) and light intensity (0, 50 150, 300 and 450μmolm⁻²s⁻¹). The percentage germination and protocorm volume of germinated seeds on different media was determined by examining the seeds microscopically after 2 months. 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 oblate spheroid $\frac{4}{3} \pi a^2 b$, where 'a' and 'b' are minor and major semi-axes respectively.

For further studies on seedling growth and development, the orchid seedlings measuring 0.5 – 0.7 cm in size (bearing two leaf initials without any root) were used. The seedlings were subcultured onto the basal media viz., MS, B₅, Mitra and KC. Five

seedlings were cultured in each test tube. Observations of seedlings were made after 90 days of culture. Different growth parameters viz., shoot number and length, and root number and length were recorded. Five replicates of each treatment were taken and the experiments repeated twice. The data was subjected to statistical analysis.

RESULTS

Seed Germination

Initiation of seed germination and protocorm development varied with the medium employed for both *D. longicornu* and *D. formosum*.

The cultured seeds of *D. longicornu* started germinating on all the media tried (Table 2.1). The earliest visible response was greening of the embryos, which initiated at the end of the second week of culture on MS and B₅ media, and in 3-4 weeks the developing protocorms were formed onto these media. While on the other hand, greening of the embryos on other media (Mitra and KC) was initiated after the third week of culture and the time required for protocorm formation was longer. The germination percentage also varied significantly depending on the medium composition and among the different media tried maximum germination of 95.55% was recorded for *D. longicornu* on MS medium (Plate 2.1a) followed by B₅ (88.64%) (Plate 2.1b), Mitra (83.14%) (Plate 2.1c) and KC (77.42%) (Plate 2.1d). The size of the protocorms also varied in the different media. The protocorms volume was recorded to be the largest on MS (0.17 mm³) followed by Mitra (0.10 mm³), B₅ (0.072 mm³) and KC (0.067 mm³).

Similarly, in case of *D. formosum* the seed germination was observed on all the media tried (Table 2.2). The earliest visible response was greening of the embryos, which initiated at the end of the second week of seed culture. The highest germination of 84.90% was recorded on MS medium (Plate 2.2a) followed by Mitra (74.98%) (Plate 2.2b), B₅ (69.87%) (Plate 2.2c) and KC (57.88%) (Plate 2.2d). The size of the protocorms was also greatest i.e., 0.57 mm³ on MS medium, followed by that on Mitra (0.29 mm³), B₅ (0.14 mm³) and KC (0.13 mm³). The colour of the protocorms formed on different media varied from light green to green to dark green.

It was observed that the protocorms of *D. formosum* were nearly four times greater than those of the *D. longicornu*.

Seedling Growth and Development

The growth and development of seedlings in both the dendrobe species studied in terms of shoot number and length, root number and root length, was found to be the highest in MS medium as compared to the other media tried i.e., B₅, and Mitra. In KC medium, growth of seedlings was completely inhibited (Table 2.3, 2.4; Plate 2.3d, 2.4d). The seedlings turned brown after second week of subculture and died subsequently in both the cases.

In case of the cultured seedlings of *D. longicornu*, the shoot number (2.2), root number (3.7), shoot length (2.3cm) and root length (2.6cm) were found to be comparatively higher in MS medium after 90 days of culture (Table 2.3; Plate 2.3a). On

Table 2.1: Effect of different media on seed germination and growth of protocorms of *D. longicornu*

| Medium | Germination (%)* | Time taken for protocorm formation (in weeks) | Colour of protocorms | Volume (mm³) * |
|----------------|-------------------------|--|-----------------------------|----------------------------------|
| MS | 95.55 ± 1.18 | 3-4 | +++ | 0.171 ± 0.02 |
| B ₅ | 88.64 ± 1.28 | 3-4 | ++ | 0.100 ± 0.02 |
| Mitra | 83.14 ± 0.98 | 4-5 | + | 0.072 ± 0.01 |
| KC | 77.42 ± 2.25 | 4-5 | + | 0.067 ± 0.01 |

± S.D.

+ Light Green; ++ Green; +++ Dark Green

* Data recorded after 2 months

Plate 2.1

Asymbiotic seed germination of *D. longicornu* after 2 months:

Protocorms on MS (a), B₅ (b), Mitra (c) and KC (d) media

(bar = 1 mm)

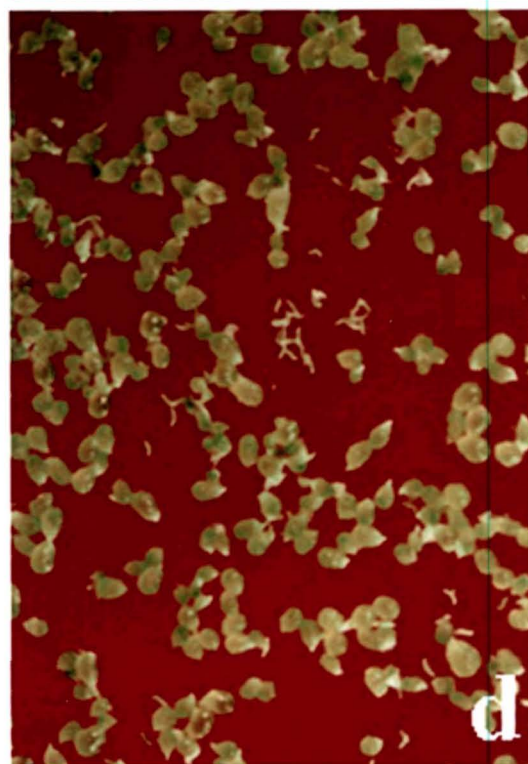
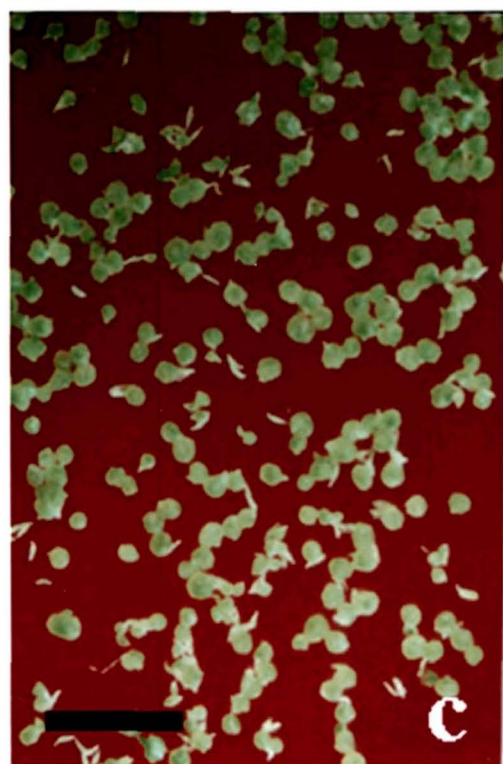
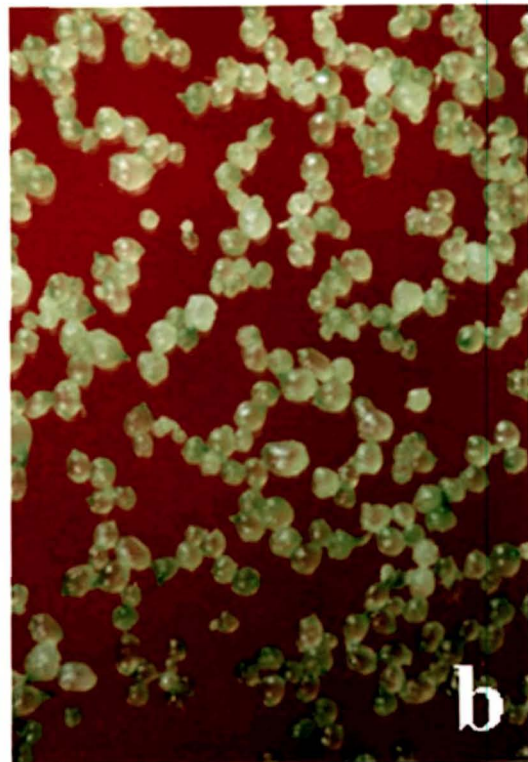
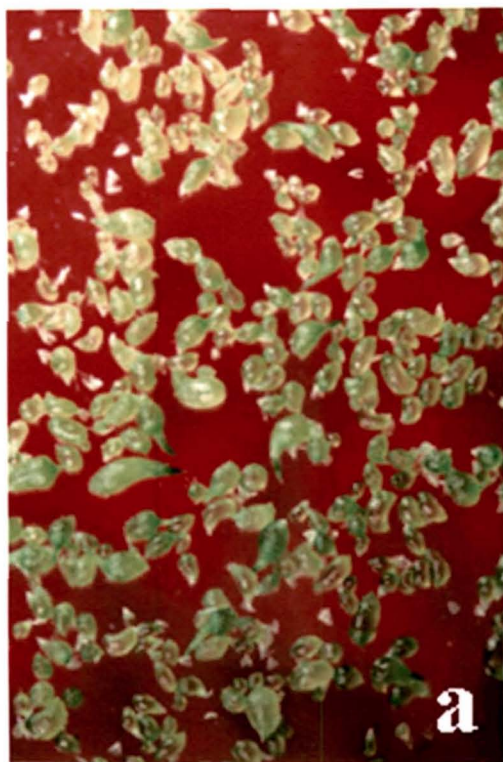


Table 2.2: Effect of different media on seed germination and growth of protocorms of

D. formosum

| Medium | Germination (%)* | Time taken for protocorm formation (in weeks) | Colour of protocorms | Volume (mm³) * |
|----------------|-------------------------|--|-----------------------------|----------------------------------|
| MS | 84.90 ± 1.42 | 3-4 | ++ | 0.57 ± 0.18 |
| B ₅ | 74.98 ± 2.58 | 3-4 | ++ | 0.29 ± 0.10 |
| Mitra | 69.87 ± 1.34 | 3-4 | + | 0.14 ± 0.01 |
| KC | 57.88 ± 1.66 | 3-4 | + | 0.13 ± 0.04 |

± S.D.

+ Light Green; ++ Green; +++ Dark Green

* Data recorded after 2 months

Plate 2.2

Asymbiotic seed germination of *D. formosum* after 2 months:

Protocorms on MS (a), Mitra (b), B₅ (c) and KC (d) media

(bar = 1 mm)

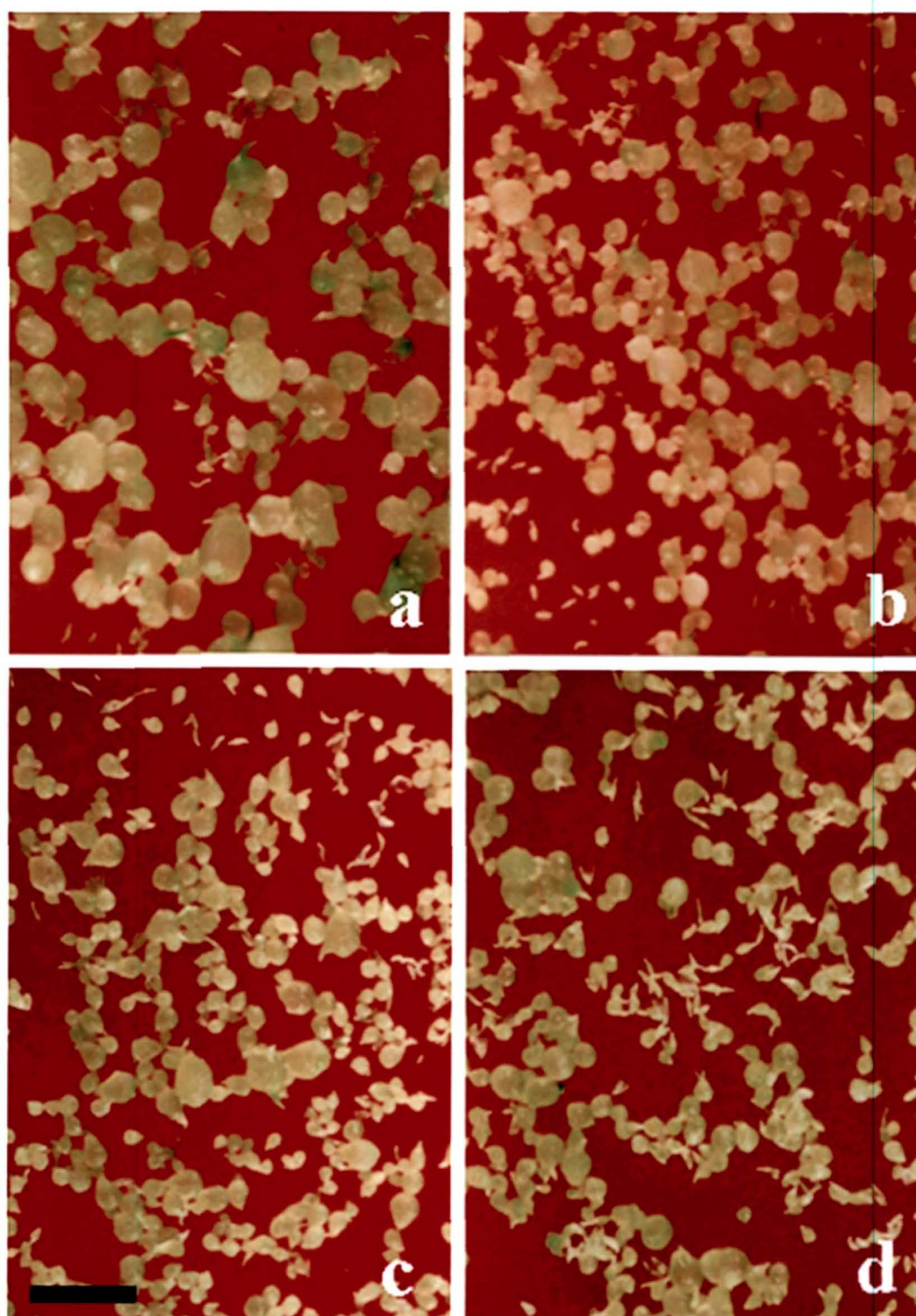


Table 2.3: Effect of different media on growth and development of *D. longicornu* seedlings after 90 days of culture

| Media | Shoot number * | Shoot length (cm) | Root number * | Root length (cm) |
|----------------|-----------------------|--------------------------|----------------------|-------------------------|
| MS | 2.20±0.12 (1-4) | 2.3±0.08 | 3.70±0.57 (2-5) | 2.63±0.25 |
| B ₅ | 1.25±0.01 (1-3) | 1.4±0.06 | 2.90±0.52 (2-4) | 1.92±0.05 |
| Mitra | 1.25±0.38 (1-3) | 1.45±0.12 | 2.65±0.38 (2-4) | 1.80±0.14 |
| KC | - | - | - | - |

± S.D.

- no response

* Average of five replicates

() indicates the minimum and maximum number of shoots/explant

Plate 2.3

Seedling growth of *D. longicornu* after 3 months of culture in

MS (a), Mitra (b), B₅ (c) and KC (d) media



Table 2.4: Effect of different media on growth and development of *D. formosum* seedlings after 90 days of culture

| Media | Shoot number * | Shoot length (cm) | Root number * | Root length (cm) |
|----------------|-----------------------|--------------------------|----------------------|-------------------------|
| MS | 2.42±0.12 (2-4) | 2.50±0.43 | 3.35±1.02 (2-4) | 1.45±0.05 |
| B ₅ | 1.50±0.12 (1-3) | 1.12±0.09 | 3.10±0.41 (2-4) | 1.17±0.18 |
| Mitra | 1.45±0.19 (1-2) | 1.02±0.05 | 2.50±0.12 (2-4) | 1.10±0.08 |
| KC | - | - | - | - |

± S.D.

- no response

* Average of five replicates

() indicates the minimum and maximum number of shoots/explant

Plate 2.4

Seedling growth of *D. formosum* after 3 months of culture in

MS (a), Mitra (b), B₅ (c) and KC (d) media



the other hand, no significant difference in these growth parameters was observed in other media tried. In B₅ medium, the cultured seedlings after 90 days of culture recorded average shoot number of only 1.25, root number of 2.9, shoot length of 1.4 cm and root length of 1.92 cm (Table 2.3; Plate 2.3b). Similarly, in Mitra medium, seedlings with shoot number of 1.25, root number of 2.65, shoot length of 1.45 cm and root length of 1.8 cm were observed to be growing after 90 days of culture (Table 2.3; Plate 2.3c).

The developing seedlings of *D. formosum* attained the maximum average shoot number (2.4), root number (3.3), shoot length (2.5cm) and root length (1.4cm) in MS medium (Table 2.4; Plate 2.4a) followed by that in B₅ with shoot number of 1.5, root number of 3.1, shoot length of 1.12 cm and root length of 1.17 cm (Table 2.4; Plate 2.4b), and Mitra with shoot number of 1.45, root number of 2.50, shoot length of 1.02 cm and root length of 1.10 cm (Table 2.4; Plate 2.4c).

Effect of Different Physical Factors on Seed Germination

Effect of Temperature

The maximum germination of seeds in case of *D. longicornu* was observed in seeds cultured at 24°C (87.25%), followed by 20°C (75.16%) and 28°C (68.16%). At 16°C, few seeds showed germination after 2 months of culture (Table 2.5).

In case of *D. formosum* also, the maximum germination of seeds was recorded at 24°C (83.01%), followed by 28°C (78.61%) and 20°C (72.92%) after 2 months of culture. No germination was observed in seeds cultured at 16°C (Table 2.6).

Effect of Light

i) Photoperiod

A wide range of photoperiod (8h-20h) was found to be suitable for seed germination of the dendrobes. For *D. longicornu*, the best germination of 93.81% was recorded at 12h photoperiod, while it decreased with increase or decrease in the photoperiod (Table 2.5). In case of seeds of *D. formosum*, the highest germination of 83.28% was also recorded at 12h photoperiod followed by that at 16h (81.06%), 20h (74.87%) and 8h (70.68%) (Table 2.6). No seed germination was recorded in complete darkness for both the dendrobes.

ii) Light Intensity

Light was promotory for asymbiotic seed germination of both the orchids. The maximum percentage of seed germination was recorded at $150 \mu\text{molm}^{-2}\text{s}^{-1}$ light intensity for both the species studied. In case *D. longicornu* the maximum seed germination of 89.74% was recorded at $150 \mu\text{molm}^{-2}\text{s}^{-1}$, while 69.63% germination at $50 \mu\text{molm}^{-2}\text{s}^{-1}$, 71.16% germination at $300 \mu\text{molm}^{-2}\text{s}^{-1}$ and 56.22% germination at $450 \mu\text{molm}^{-2}\text{s}^{-1}$ were recorded (Table 2.5).

In *D. formosum*, 81.83% of the seeds germinated at $150 \mu\text{molm}^{-2}\text{s}^{-1}$, followed by 71.16% at $50 \mu\text{molm}^{-2}\text{s}^{-1}$, 58.46% at $300 \mu\text{molm}^{-2}\text{s}^{-1}$ and 36.62% at $450 \mu\text{molm}^{-2}\text{s}^{-1}$ light intensity (Table 2.6). The seed germination declined at light intensities less or greater

Table 2.5: Effect of physical factors on seed germination of *D. longicornu*

| | Treatment | Germination (%)* |
|--|------------------|-------------------------|
| Temperature (°C) (12h, 150 $\mu\text{molm}^{-2}\text{s}^{-1}$) | 16 | 23.25 \pm 3.17 |
| | 20 | 75.16 \pm 3.01 |
| | 24 | 87.25 \pm 1.18 |
| | 28 | 68.16 \pm 3.12 |
| Photoperiod (h) (24°C, 150 $\mu\text{molm}^{-2}\text{s}^{-1}$) | 0 | - |
| | 8 | 83.16 \pm 1.51 |
| | 12 | 93.81 \pm 2.76 |
| | 16 | 86.13 \pm 2.45 |
| | 20 | 76.14 \pm 6.60 |
| Light intensity ($\mu\text{molm}^{-2}\text{s}^{-1}$) (24°C, 12h) | 0 | - |
| | 50 | 69.63 \pm 5.30 |
| | 150 | 89.74 \pm 1.47 |
| | 300 | 71.16 \pm 1.31 |
| | 450 | 56.22 \pm 2.90 |

\pm S.D.

- no response

* Data recorded after 2 months

Table 2.6: Effect of physical factors on seed germination of *D. formosum*

| | Treatment | Germination (%)* |
|--|------------------|-------------------------|
| Temperature (°C) (12h, 150 $\mu\text{molm}^{-2}\text{s}^{-1}$) | 16 | - |
| | 20 | 72.92±3.29 |
| | 24 | 83.01±3.62 |
| | 28 | 78.61±1.86 |
| Photoperiod (h) (24°C, 150 $\mu\text{molm}^{-2}\text{s}^{-1}$) | 0 | - |
| | 8 | 70.68±1.47 |
| | 12 | 83.28±2.03 |
| | 16 | 81.06±2.78 |
| | 20 | 74.87±1.74 |
| Light intensity ($\mu\text{molm}^{-2}\text{s}^{-1}$) (24°C, 12h) | 0 | - |
| | 50 | 71.16±1.31 |
| | 150 | 81.83±1.80 |
| | 300 | 58.46±1.55 |
| | 450 | 36.62±3.17 |

± S.D.

- no response

* Data recorded after 2 months

than $150 \mu\text{molm}^{-2}\text{s}^{-1}$. The seeds of both the species failed to germinate in complete darkness.

DISCUSSION

The orchid seeds responded differentially to different media used for germination. The development of orchid seeds requires a balanced supply of both organic and inorganic nutrients (Arditti, 1982; Zeigler *et al.*, 1985; Van Waes and Debergh, 1986). In the present study, seeds were found to have germinated 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, the presence of potassium nitrate and ammonium nitrate at high concentrations in MS medium could have been the most suitable form of nitrogen for seed germination of both *D. longicornu* and *D. formosum* respectively (Table 2.7, 2.8). The importance of ammonium or nitrate ions (individually or in combination) during *in vitro* germination of orchid seeds as source of nitrogen has been well established (Arditti and Ernst, 1984). Mitra and KC media both contain calcium nitrate and ammonium sulphate which might not have supported good growth in *D. longicornu*, while in *D. formosum* the presence of reduced nitrate in the form of ammonium sulphate in Mitra, B₅ and KC may have had some influence on the seed germination. The difference in response to ammonium sources used may be due to varied requirements of the species. The presence of vitamins in the above media, might have affected the germination of both the species by improving the

Table 2.7: Concentrations (mM) of major ions and vitamins in nutrient media

| Component | MS | B₅ | Mitra | KC |
|------------------------------------|-----------|----------------------|--------------|-----------|
| NO₃⁻ | 39.43 | 24.75 | 2.63 | 2.63 |
| NH₄⁺ | 20.62 | 1.01 | 0.76 | 0.76 |
| Total N | 60.05 | 25.76 | 3.39 | 3.39 |
| K⁺ | 20.06 | 24.76 | 1.78 | 2.93 |
| Ca₂⁺ | 2.99 | 1.02 | 0.85 | 0.85 |
| Mg₂⁺ | 1.50 | 1.01 | 0.81 | 1.02 |
| PO₄³⁻ | 1.25 | 0.96 | 1.60 | 1.10 |
| Cl⁻ | 5.99 | 1.02 | - | 0.02 |
| SO₄²⁻ | 1.73 | 2.19 | 0.86 | 0.84 |
| Thiamine HCL | 0.0003 | 0.03 | 0.0009 | 0.0009 |
| Nicotinic Acid | 0.004 | - | 0.01 | - |
| Pyridoxin HCL | 0.002 | - | 0.001 | 0.001 |
| Glycine | 0.03 | 0.03 | - | - |
| Riboflavin | - | - | 0.0001 | 0.0008 |
| Biotin | - | - | 0.002 | - |
| Folic Acid | - | - | 0.0007 | - |

Table 2.8: Inorganic, organic components and gelling agent of the media

| Costituents | MS | Mitra | B ₅ | KC |
|--|----------|----------|----------------|----------|
| Inorganic salts (mg/l) | | | | |
| NH ₄ NO ₃ | 1650.00 | - | - | - |
| KNO ₃ | 1900.00 | 180.00 | 2500.00 | 180.00 |
| KH ₂ PO ₃ | 170.00 | - | - | 150.00 |
| Ca(NO ₃) ₂ .4H ₂ O | - | 200.00 | - | 200.00 |
| MgSO ₄ .7H ₂ O | 370.00 | 200.00 | 250.00 | 250.00 |
| (NH ₄) ₂ SO ₄ | - | 100.00 | 134.00 | 100.00 |
| MgCl ₂ .6H ₂ O | - | - | - | - |
| KI | 0.83 | 0.03 | 0.75 | 80.00 |
| H ₃ BO ₃ | 6.20 | 0.06 | 3.00 | 6.20 |
| MnSO ₄ .4H ₂ O | 22.30 | 0.40 | 10.00 | 0.075 |
| ZnSO ₄ .7H ₂ O | 8.60 | 0.05 | 2.00 | - |
| Na ₂ MoO ₄ .2H ₂ O | 0.25 | 0.05 | 0.25 | 0.25 |
| CuSO ₄ .5H ₂ O | 0.25 | 0.05 | 0.025 | 0.025 |
| CoCl ₂ .6H ₂ O | 0.025 | - | 0.025 | 0.025 |
| FeSO ₄ .7H ₂ O | 27.80 | 27.80 | 27.80 | 27.30 |
| Na ₂ EDTA.2H ₂ O | 37.30 | 37.30 | 37.30 | 37.30 |
| CaCl ₂ .2H ₂ O | 440.00 | - | 150.00 | - |
| CO(NO ₃) ₂ .6H ₂ O | - | 0.05 | - | - |
| NaH ₂ PO ₄ .2H ₂ O | - | 250.00 | 150.00 | - |
| MnCl ₂ | - | - | - | 3.90 |
| Organic nutrients (mg/l) | | | | |
| Thiamine HCl | 0.10 | 0.30 | 10.00 | 0.30 |
| Nicotinic Acid | 0.50 | 1.25 | - | - |
| Pyridoxine HCl | 0.50 | 0.30 | - | 0.30 |
| Glycine | 2.00 | - | 2.00 | - |
| Riboflavin | - | 0.05 | - | 0.30 |
| Biotin | - | 0.50 | - | - |
| Folic Acid | - | 0.30 | - | - |
| Meso Inositol | 100.00 | - | 100.00 | - |
| Sucrose | 30000.00 | 20000.00 | 20000.00 | 20000.00 |
| Gelling agents (g/l) | | | | |
| Agar | 8.00 | 9.00 | 8.00 | 12.00 |
| pH | | | | |
| | 5.8 | 5.6 | 5.8 | 5.2 |

germination and growth (Kumaria and Tandon, 1991). Although there are reports of orchid seed germination in KC medium (Bopiah and Jorapur, 1986; Sharma and Tandon, 1987; Pyati and Murthy, 1995), in the present study it was found that seeds of *D. longicornu* and *D. formosum* germinated poorly on KC medium which is similar to the report of Clements (1981), wherein failure of seed germination of some epiphytic species on KC medium has been reported. Similarly, several Indian orchids including species of *Dendrobium* and *Cymbidium* are reported to have poor seed germination on KC medium (Chaturvedi *et al.*, 1987). Yam and Weatherhead (1988) had reported that the nutritional requirements of germinating orchid seeds vary due to their physiological state.

In vitro growth and development of seedlings 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). In the present study, the overall optimal growth of seedlings of both *D. longicornu* and *D. formosum* 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 of 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, as MS contains glycine as amino acid. Glycine could have provided nitrogen required for seedling growth. 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 on *D. longicornu* and *D. formosum* also

revealed that in KC medium seedling growth was inhibited. This could be due to the presence of calcium nitrate in the medium. Alan (1989) had reported that nitrate in the form of calcium nitrate generally increases all mineral contents except nitrogen in tissues, thereby reducing the nitrogen supply to the developing seedlings.

Environment exerts an important effect on the physiology and development of orchids (Arditti and Ernst, 1984). Factors such as light and temperature can also affect seed germination. The influence of light intensity and photoperiod seems to be related to species, with some benefiting from high intensities, other responding to intermediate levels, while others best cultured under low light or darkness (Thorpe and Murashige, 1970; Miller and Murashige, 1976; Papachatzki *et al.*, 1981). In the present study, the optimal seed germination in both the species was found to be at $150 \mu\text{molm}^{-2}\text{s}^{-1}$ light intensity. A decrease in the seed germination was noticed with increasing or decreasing light intensities. The range of photoperiod from 8h – 20h was found to be suitable for seed germination, though no significant variation was observed in germination percentage; however the highest percentage was recorded at 12h photoperiod. Arditti *et al.* (1981) suggested that germinating orchid seeds and developing seedlings vary in their tolerance, requirement and response to light. Stoutamire (1974) reported that seed germination in many orchids was affected by light which might be stimulatory or inhibitory depending on the wavelength and the plant species used. The influence of temperature on basic physiological processes such as respiration and on cell and organ formation is well known (Reed, 1992). In the present study the maximum germination was observed in seeds cultured at 24°C for both the species studied. The percentage of

germination declined by increasing or decreasing the temperature. The temperature range of 20-30°C has been reported to increase the seed germination and seedling growth in several orchids (Muire, 1982; Zeigler *et al.*, 1985).

The present study revealed that seeds of *D. longicornu* and *D. formosum* required a rich medium for their growth and differentiation. Kathyar *et al.* (1987) had suggested that the differentiation of organs in a particular species is a direct manifestation of its genetic and physiological features.

CHAPTER III: MICROPROPAGATION OF *DENDROBIUM LONGICORNU* AND *DENDROBIUM FORMOSUM*

INTRODUCTION

Clonal propagation of orchids by means of explants is gaining wide importance in tissue culture industry. For a long time, the major means for propagating orchids clonally was the division of plants but this was found to be a slow process for obtaining large number of plants. Plant tissue culture, the most successful commercial aspect of Plant Biotechnology has introduced an exciting new phase into plant propagation and breeding. The first horticultural plant to be cloned by tissue culture methods on a commercial scale are the orchids (Griesbach, 1986; Goh, 1990). Since Morel's successful experiment with *Cymbidium* (Morel, 1960), propagation by *in vitro* induction of adventitious buds on explants of shoot tips has become a widely accepted method for clonal propagation of orchids. Morel for the first time reported the formation of PLBs around the shoot tips of *Cymbidium* cultured *in vitro*. These PLBs when cut into small sections and subcultured onto 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 at a very high rate. Arditti and Ernst (1993) compiled micropropagation protocols of hybrids and varieties of orchid genera. Among the commercially important orchids, the genera *Dendrobium* and *Cymbidium* account for about 80% and 50% 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 subsequently (Withner, 1959; Butenko, 1968; Arditti, 1977). The media used is found to differ for 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) proved 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 and a number of media (Bergman, 1972a,b; Wang, 1989). *Cymbidium*, *Dendrobium*, *Vanda*, *Spathoglottis*, *Bulbophyllum*, *Saccolabium* and allied genera are widely propagated by tissue culture throughout 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 (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* orchids. 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 been published (Vij *et al.*, 1984; Tanaka, 1987; Vij and Pathak, 1990; Vij *et al.*, 1994; Pyati 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 dendrobates and cymbidiums (Morel, 1960; Wimber, 1963; Pathania *et al.*, 1998; Talukdar *et al.*, 2003). Chang and Chang (1998) reported initiation of calli from rhizomes or pseudobulb of *Cymbidium ensifolium var. misericors*. However, the techniques applied differ from species to species. 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). This present chapter deals with tissue culture of *D. longicornu* and *D. formosum* for mass propagation of identical genotypes of these orchids.

MATERIALS AND METHODS

Different explant sources such as leaf parts, root tips and axillary buds were obtained from the plants maintained in the net house of North-Eastern Hill University, Shillong. About 1-2 cm segments of the explants were cut and cleaned gently scrubbing with a soft brush and mild detergents. These were then washed in running tap water for about 15-20 min, and then rinsed with distilled water. The surface sterilization of the explants was done by 10% (v/v) sodium hypochlorite solution (4-6% available chlorine) for 10 min followed by 0.1% (w/v) HgCl₂ (2 min), and thoroughly washed with sterilized distilled water to remove the sterilants. Leaf bases, leaf tips and root tips measuring 2mm were cut from the larger section of the explants. The nodal sections containing the

axillary buds were also cut into 3-4 mm size after the removal of leaves, dry sheaths and other external tissues. These were then cultured on different media.

A number of media (MS, B5, Mitra and KC) were tried for the micropropagation of *D. longicornu* and *D. formosum*. The explant pieces were inoculated on these media aseptically. The cultures were maintained at $24 \pm 2^\circ\text{C}$ with 12h illumination of $150\mu\text{molesm}^{-2}\text{s}^{-1}$ light intensity. The pH of the medium was adjusted in accordance with the media prior to autoclaving. The optimum medium was supplemented with different growth regulators viz., IAA, NAA, 2,4-D, BAP and KN both separately and in combinations in a range of 0.0-50 μM so as to optimize the formation of PLBs and shoot buds on the cultured explants. The different combinations of growth regulators tried were BAP+IAA, BAP+NAA, BAP+2,4-D, KN+IAA, KN+NAA and KN+2,4-D. Ten replicates were maintained for each treatment and the experiment was repeated thrice. Observations were made after 45 days of culture of explants in the medium. The data was subjected to statistical analysis.

RESULTS

Out of all different explant sources tried, the axillary bud was found to be the most suitable explant for micropropagation of both *D. longicornu* and *D. formosum*

a) *Effect of Different Basal Media*

The axillary buds of *D. longicornu* cultured on different media showed response in the media tried i.e., MS, B₅, Mitra and KC (Table 3.1). Out of these four media on

Table 3.1: Effect of different media on the formation of shoots or PLBs from axillary bud of *D. longicornu* after 45 days of culture

| Treatments | Explant response (%) | Average number of shoots/explant | PLBs formed/explant | Remarks |
|-------------------|-----------------------------|---|----------------------------|--------------------------------|
| MS | 70.0 ± 10 | 1.71 ± 0.64 (1-3) | - | Healthy multiple shoots formed |
| B ₅ | 60.0 ± 10 | 1.12 ± 0.32 (1-2) | - | Around 2 shoots/explant |
| Mitra | 66.6 ± 5.70 | 1.10 ± 0.30 (1-2) | - | Around 2 shoots/explant |
| KC | 53.3 ± 5.4 | 1.00 ± 0.00 | - | Solitary shoot developed |

± S.D.

() indicates the minimum and maximum number of shoots/explant

Table 3.2: Effect of different media on the formation of shoots or PLBs from axillary bud of *D. formosum* after 45 days of culture

| Treatments | Explant response (%) | Average number of shoots/explant | PLBs formed/explant | Remarks |
|-------------------|-----------------------------|---|----------------------------|--------------------------------|
| MS | 73.3 ± 5.7 | 1.83 ± 0.48 (1-3) | - | Healthy multiple shoots formed |
| B ₅ | 60.0 ± 7.1 | 1.33 ± 0.48 (1-2) | - | Around 2 shoots/explant |
| Mitra | 60.0 ± 7.1 | 1.22 ± 0.42 (1-2) | - | Around 2 shoots/explant |
| KC | 36.6 ± 5.4 | 1.18 ± 0.40 (1-2) | - | Around 2 shoots/explant |

± S.D.

() indicates the minimum and maximum number of shoots/explant

which the axillary bud responded, MS medium was found to be the best. Highest response (70.0%) of the explant was recorded in this medium with 1-3 number of shoots arising from the single explant, followed by Mitra (66.6%) and B₅ (60.0%). A lower explant response of 53.3% was recorded on KC medium (Table 3.1). Solitary shoots were seen coming out from the explants cultured on KC medium. Hence, for further experiments on micropropagation of *D. longicornu* explants were cultured in MS medium supplemented with different growth regulators.

The axillary buds of *D. formosum* cultured in basal MS, KC, B₅ and Mitra media responded in terms of emergence of shoot buds (Table 3.2). Out of these four media, MS medium was found to be the optimum in bud response. Highest response (73.3%) of the explant was recorded in MS medium with 1-3 number of shoots arising from the single explant, followed by B₅ and Mitra wherein 60% response was observed. A lower explant response of 36.6% was recorded in KC medium (Table 3.2). Hence, for further experiments on micropropagation of *D. formosum*, explants were cultured in MS medium supplemented with different growth regulators.

b) Effect of Growth Regulators

Effect of IAA

The axillary buds of *D. longicornu* cultured in MS medium containing different concentrations of IAA showed no significant difference in response from the control. At 15µM of IAA in the medium the buds showed high percentage response (76.6%) with an

average number of 2.6 (2-4) shoots emerging from each explant (Table 3.3). However, it was observed that with increase in concentration of IAA in the medium, the responses of the explants also decreased.

On the other hand, the axillary buds of *D. formosum* responded well to IAA in the medium as compared to control. Good response of the buds was recorded in medium supplemented with 5 μ M, 15 μ M and 30 μ M of IAA, although highest response of explants (96.6%) with average number of 2.63 shoots/explant was recorded at 15 μ M of IAA in the medium. However, at 50 μ M IAA in the medium, there was a decrease in response of the buds as well as in the development of shoots from the explants (Table 3.4).

Effect of NAA

In case of the cultured axillary buds of *D. longicornu*, good response was observed in the medium containing different concentrations of NAA (Table 3.3). The highest percentage of response (86.6%) as well as the number of shoots i.e., 3.28 (3-5) from each explant was observed in the medium containing NAA at 30 μ M. Higher or lower than 30 μ M of NAA in the medium showed decrease in explant response and shoot number in each explant cultured.

The percentage response of explants of *D. formosum* cultured in NAA containing medium was much higher as compared to the control. However, the number of multiple shoots emerging was different at different concentrations. At 15 μ M NAA in the medium,

96.6% of bud response with an average of 4.02 number of shoots per explant was observed (Table 3.4).

Effect of 2, 4-D

The axillary buds of *D. longicornu* cultured in medium incorporated with 2,4-D showed differential results. In this treatment it was observed that the explants response in terms of shoot bud formation was low as compared to control but emergence of PLBs was a differential response. In medium containing 2,4-D at 30 μ M, 36.9% of the explants developed into PLBs, while in the medium containing 2,4-D at lower concentrations both PLBs as well as shoot development were recorded (Table 3.3). No response was observed at higher concentration of 2,4-D i.e., 50 μ M in the medium.

On the other hand, the axillary buds of *D. formosum* cultured in 2, 4-D containing medium showed both PLBs as well as shoot bud development. The treatments with 5 μ M and 15 μ M of 2,4-D resulted in higher PLBs emergence of 28.33% and 28.51% respectively from the explants. Highest percentage (75.0%) of explants responded in terms of both PLBs and shoot buds in the medium containing 5 μ M of 2, 4-D. With increase of 2, 4-D concentration in the medium a decrease in response of the explant was recorded and at 50 μ M of 2, 4-D in the medium, the explants failed to response (Table 3.4).

Effect of BAP

Among all the concentrations of BAP in the medium, the response of axillary buds of *D. longicornu* was low as compared to control. However, the maximum response was observed in medium supplemented with 15 μ M of BAP wherein a percentage response of 63.3% and average shoot number of 1.6 were observed (Table 3.3).

In case of *D. formosum*, the explants responded in all the concentrations of BAP in the medium. The highest response (83.3%) was found in the medium containing 5 μ M of BAP, while an increased shoot number of 2.48 (2-4) from each explant was found at the treatment of 15 μ M BAP. At 15 μ M and 30 μ M of BAP in the medium, PLB initiation was also observed in this case (Table 3.4).

Effect of KN

In case of cultured explants of *D. longicornu* there was an increase in response in medium containing KN at 5 μ M (76.6%) and 15 μ M (80.9%) as compared to the control. An average number of 2.16 and 2.34 shoots respectively were observed in these treatments. With increase in concentrations, the responses of explants decreased while there was no significant change in shoot number (Table 3.3).

As compared to control, the response of the explants of *D. formosum* was better at 5 μ M, 15 μ M and 30 μ M of KN in the medium wherein 96.6%, 83.3% and 79.2% of the explants responded respectively. The highest average number of 2.68 shoots/ explant was recorded at 5 μ M of KN in the medium (Table 3.4).

Table 3.3: Effect of growth regulators incorporated singly in MS medium on formation of shoot buds and PLBs from axillary bud of *D. longicornu* *

| Conc. (µM) | Explant response (%) | Average number of shoots / explant | Explant response into PLBs (%) |
|-----------------|----------------------|------------------------------------|--------------------------------|
| Control | 70.0 ± 10 | 1.71 ± 0.64 (1-3) | - |
| IAA | | | |
| 5 | 70.0 ± 7.1 | 1.55 ± 0.09 (1-3) | - |
| 15 | 76.6 ± 4.7 | 2.62 ± 0.20 (2-4) | - |
| 30 | 66.6 ± 4.7 | 1.78 ± 0.06 (1-3) | - |
| 50 | 66.6 ± 4.7 | 1.00 ± 0.00 (1) | - |
| NAA | | | |
| 5 | 70.0 ± 5.7 | 2.10 ± 0.09 (2-3) | - |
| 15 | 70.0 ± 7.1 | 2.23 ± 0.45 (2-4) | - |
| 30 | 86.6 ± 5.7 | 3.28 ± 0.49 (3-5) | - |
| 50 | 40.0 ± 8.1 | 1.55 ± 0.19 (1-3) | - |
| 2, 4 - D | | | |
| 5 | 66.6 ± 3.7 | 1.38 ± 0.34 (1-2) | 29.07 ± 9.5 |
| 15 | 65.4 ± 3.5 | 1.36 ± 0.12 (1-2) | 28.51 ± 5.7 |
| 30 | 36.9 ± 3.3 | - | 36.9 ± 3.3 |
| 50 | - | - | - |
| BAP | | | |
| 5 | 60.0 ± 0.0 | 1.44 ± 0.09 (1-3) | - |
| 15 | 63.3 ± 4.7 | 1.61 ± 0.24 (1-3) | - |
| 30 | 36.6 ± 9.4 | 1.41 ± 0.38(1-3) | - |
| 50 | 30.0 ± 0.0 | 1.00 ± 0.00(1) | - |
| KN | | | |
| 5 | 76.6 ± 5.7 | 2.16 ± 0.28 (2-3) | - |
| 15 | 80.9 ± 7.9 | 2.38 ± 0.20 (2-4) | - |
| 30 | 64.2 ± 5.2 | 1.86 ± 0.23 (1-3) | - |
| 50 | 55.2 ± 5.0 | 1.70 ± 0.23 (1-2) | - |

± S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Table 3.4: Effect of growth regulators incorporated singly in MS medium on formation of shoot buds and PLBs from axillary bud of *D. formosum* *

| Conc. (µM) | Explant response (%) | Average number of shoots / explant | Explant response into PLBs (%) |
|-----------------|----------------------|------------------------------------|--------------------------------|
| Control | 73.3 ± 5.7 | 1.66 ± 0.09 (1-3) | - |
| IAA | | | |
| 5 | 82.9 ± 5.1 | 2.12 ± 0.12 (2-3) | - |
| 15 | 96.6 ± 7.2 | 2.63 ± 0.28(2-4) | - |
| 30 | 73.3 ± 5.7 | 2.13 ± 0.18(2-3) | - |
| 50 | 55.2 ± 5.0 | 1.91 ± 0.12(1-3) | - |
| NAA | | | |
| 5 | 93.3 ± 7.2 | 2.93 ± 0.09(2-4) | - |
| 15 | 96.6 ± 5.7 | 4.02 ± 0.77(3-6) | - |
| 30 | 82.9 ± 5.1 | 2.28 ± 0.16(2-4) | - |
| 50 | 72.6 ± 4.4 | 1.91 ± 0.58(1-3) | - |
| 2, 4 - D | | | |
| 5 | 75.0 ± 5.0 | 1.60 ± 0.14(1-2) | 28.33 ± 2.8 |
| 15 | 74.8 ± 7.1 | 1.67 ± 0.24(1-2) | 28.51 ± 5.7 |
| 30 | 50.0 ± 10.0 | 1.55 ± 0.07(1-2) | 13.33 ± 5.7 |
| 50 | - | - | - |
| BAP | | | |
| 5 | 83.3 ± 5.7 | 1.90 ± 0.14(1-3) | - |
| 15 | 75.9 ± 5.2 | 2.48 ± 0.12(2-4) | 20.74 ± 1.3 |
| 30 | 80.0 ± 10.0 | 2.12 ± 0.17(1-3) | 13.33 ± 5.7 |
| 50 | 40.0 ± 10.0 | 1.49 ± 0.23(1-3) | - |
| KN | | | |
| 5 | 96.6 ± 5.7 | 2.68 ± 0.23 (2-4) | - |
| 15 | 83.3 ± 5.7 | 2.21 ± 0.16(1-4) | - |
| 30 | 79.2 ± 1.3 | 1.61 ± 0.30(1-3) | - |
| 50 | 65.5 ± 5.1 | 1.45 ± 0.28(1-3) | - |

± S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

c) Effect of Growth Regulators in Combination

Effect of BAP+ NAA

The axillary buds of *D. longicornu* showed varying responses in the medium containing BAP+NAA in combination. The lower concentration of 5 μ M BAP and 15 μ M NAA in combination in the medium enhanced percentage response and shoot number of the cultured explants. A high percentage of response (81.2%) of the explants was recorded at 15 μ M BAP and 5 μ M NAA in combination; while an increased number of shoots i.e., 4.42/explant was recorded in the treatment containing 5 μ M BAP and 15 μ M NAA (Table 3.5, Plate 3.1). The treatments containing 15 μ M each of BAP and NAA and 30 μ M each of BAP and NAA showed varying responses of the explants in terms of conversion of axillary buds into PLBs. The increase in the concentration of either BAP or NAA to 30 μ M or 50 μ M in combination showed decline in percentage response (Table 3.5).

As compared to control, the axillary buds of *D. formosum* showed highest response of 89.62% and shoot number of 3.84 (2-6) in the medium supplemented with 15 μ M each of BAP and NAA (Table 3.6). In the combinations of 15 μ M+15 μ M, 15 μ M+30 μ M and 30 μ M+30 μ M of BAP+NAA, the initiation of PLBs was recorded (Table 3.6). The average shoot number in all the cultured explants treated with different combinations of these growth regulators was slightly higher as compared to control.

Table 3.5: Effect of BAP and NAA in MS medium on the formation of shoots/PLBs from axillary bud of *D. longicornu* *

| Conc. (μ M) | Explant response (%) | Average number of shoots / explant | Explant response into PLBs (%) |
|---------------------|-------------------------|--|-----------------------------------|
| Control | 70.0 \pm 10 | 1.71 \pm 0.64 (1-3) | - |
| BAP+NAA | | | |
| 5 + 5 | 75.5 \pm 4.3 | 2.15 \pm 0.30(1-3) | - |
| 5 + 15 | 79.3 \pm 1.2 | 4.42 \pm 0.42 (2-6) | - |
| 5 + 30 | 57.1 \pm 2.5 | 2.22 \pm 0.19(1-3) | - |
| 5 + 50 | 56.1 \pm 3.7 | 1.75 \pm 0.08(1-3) | - |
| | | | |
| 15 + 5 | 81.2 \pm 4.1 | 3.20 \pm 0.17(2-5) | - |
| 15 + 15 | 80.8 \pm 6.2 | 2.85 \pm 0.24(2-4) | 38.75 \pm 1.7 |
| 15 + 30 | 59.7 \pm 6.0 | 2.27 \pm 0.19(2-3) | - |
| 15 + 50 | 39.2 \pm 1.4 | 1.44 \pm 0.09(1-3) | - |
| | | | |
| 30 + 5 | 56.6 \pm 5.7 | 2.85 \pm 0.48(2-4) | - |
| 30 + 15 | 62.2 \pm 3.8 | 2.61 \pm 0.24(2-4) | - |
| 30 + 30 | 53.3 \pm 5.7 | 2.43 \pm 0.20(2-4) | 22.40 \pm 2.5 |
| 30 + 50 | - | - | - |
| | | | |
| 50 + 5 | 33.6 \pm 3.7 | 2.33 \pm 0.28(1-4) | - |
| 50 + 15 | 32.2 \pm 1.9 | 1.55 \pm 0.19(1-3) | - |
| 50 + 30 | - | - | - |
| 50 + 50 | - | - | - |

\pm S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Plate 3.1

Complete development of plantlets from axillary bud of *D. longicornu* on

MS + 5 μ M BAP + 15 μ M NAA

- a. Initiation of shoots in 15 days time
- b. Proliferation of shoots in 30 days time
- c. Cluster of shoots in 60 days time
- d. Development of mature shoots after 90 days time

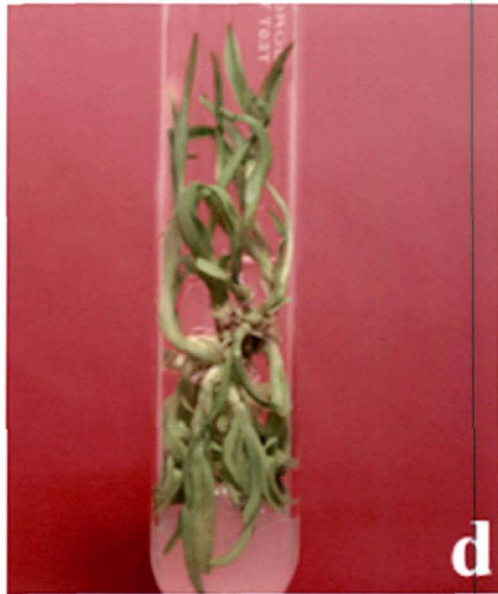
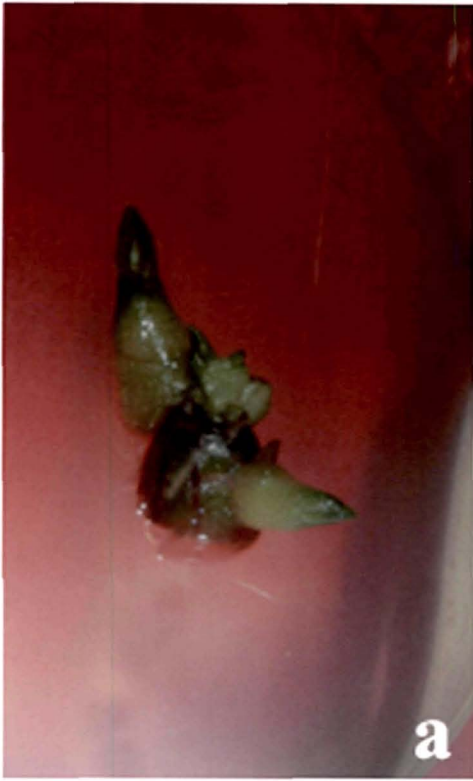


Table 3.6: Effect of BAP and NAA in MS medium on the formation of shoots/PLBs from axillary bud of *D. formosum* *

| Conc. (µM) | Explant response (%) | Average number of shoots / explant | Explant response into PLBs (%) |
|------------|----------------------|------------------------------------|--------------------------------|
| Control | 73.3 ± 5.7 | 1.66 ± 0.09 (1-3) | - |
| BAP+NAA | | | |
| 5 + 5 | 86.29 ± 5.4 | 3.43 ± 0.44(2-6) | - |
| 5 + 15 | 83.33 ± 5.7 | 3.37 ± 0.53(2-6) | - |
| 5 + 30 | 73.33 ± 5.7 | 2.25 ± 0.35(2-4) | - |
| 5 + 50 | 62.22 ± 3.8 | 2.01 ± 0.26(1-4) | - |
| | | | |
| 15 + 5 | 86.66 ± 5.7 | 2.54 ± 0.24(1-4) | - |
| 15 + 15 | 89.62 ± 0.64 | 3.84 ± 0.02(2-6) | 20.74 ± 1.2 |
| 15 + 30 | 82.96 ± 5.12 | 2.37 ± 0.17(2-4) | 31.11 ± 1.9 |
| 15 + 50 | 60.00 ± 10 | 2.33 ± 0.46(1-4) | - |
| | | | |
| 30 + 5 | 76.66 ± 5.7 | 2.54 ± 0.24(2-4) | - |
| 30 + 15 | 72.59 ± 4.4 | 2.14 ± 0.19(1-3) | - |
| 30 + 30 | 75.18 ± 4.4 | 2.1 ± 0.14(1-3) | 21.48 ± 1.3 |
| 30 + 50 | 40.00 ± 10 | 1.99 ± 0.47(1-3) | - |
| | | | |
| 50 + 5 | 66.66 ± 5.7 | 1.65 ± 0.21(1-3) | - |
| 50 + 15 | 65.55 ± 5.1 | 2.14 ± 0.19(1-3) | - |
| 50 + 30 | 40.00 ± 10 | 1.91 ± 0.82(1-3) | - |
| 50 + 50 | - | - | - |

± S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Effect of BAP + IAA

In different concentrations of BAP + IAA in the medium, the explants of *D. longicornu* showed a poor response except in the combinations of 5 μ M+5 μ M, 5 μ M+15 μ M and 5 μ M+30 μ M of BAP and IAA in the medium wherein an increased percentage response as well as shoot number were observed (Table 3.7). The other treatments tried were found less effective in terms of percentage response and shoot number conversion, though in a combination of 15 μ M BAP and 5 μ M IAA the PLBs conversion of axillary buds was observed (Table 3.7).

In case of *D. formosum*, different concentrations at 5 μ M, 15 μ M and 30 μ M of BAP + IAA in combination showed an increase in explant response as well as in number of shoots formed. The highest responses were observed at 15 μ M+15 μ M of BAP+IAA wherein 89.62% explants responded and an enhanced shoot number of 3.24/explant was recorded (Table 3.8). The explants treated with higher concentrations of 50 μ M of either BAP or IAA in the medium showed a decrease in percentage response as well as shoot number formation.

Effect of BAP and 2, 4-D

Induction of PLBs from axillary buds of *D. longicornu* was observed in medium containing different concentrations of BAP and 2, 4-D in combination; though the explant response was lower than the control. The highest percentage of explant response (72.59%) and PLBs conversion (41.48%) was observed at 15 μ M BAP and 15 μ M 2, 4-D

Table 3.7: Effect of BAP and IAA in MS medium on the formation of shoots/PLBs from axillary bud of *D. longicornu* *

| Conc. (μ M) | Explant response (%) | Average number of shoots / explant | Explant response into PLBs (%) |
|---------------------|-------------------------|--|-----------------------------------|
| Control | 70.00 \pm 10 | 1.71 \pm 0.64 (1-3) | - |
| BAP+IAA | | | |
| 5 + 5 | 82.96 \pm 5.1 | 2.22 \pm 0.48(1-3) | - |
| 5 + 15 | 80.92 \pm 7.8 | 2.22 \pm 0.48(1-3) | - |
| 5 + 30 | 73.33 \pm 5.7 | 1.77 \pm 0.19(1-3) | - |
| 5 + 50 | - | - | - |
| | | | |
| 15 + 5 | 66.38 \pm 3.7 | 1.68 \pm 0.24(1-2) | - |
| 15 + 15 | 66.66 \pm 5.7 | 1.78 \pm 0.06(1-3) | 23.33 \pm 5.7 |
| 15 + 30 | 53.33 \pm 5.7 | 1.38 \pm 0.23(1-2) | - |
| 15 + 50 | - | - | - |
| | | | |
| 30 + 5 | 44.81 \pm 5.0 | 1.58 \pm 0.38(1-3) | - |
| 30 + 15 | 43.33 \pm 5.7 | 2.13 \pm 0.33(1-3) | - |
| 30 + 30 | 32.22 \pm 1.9 | 1.16 \pm 0.28(1-2) | - |
| 30 + 50 | - | - | - |
| | | | |
| 50 + 5 | 33.33 \pm 5.7 | 1.36 \pm 0.23(1-2) | - |
| 50 + 15 | 23.33 \pm 5.7 | 1.33 \pm 0.57(1-2) | - |
| 50 + 30 | - | - | - |
| 50 + 50 | - | - | - |

\pm S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Table 3.8: Effect of BAP and IAA in MS medium on the formation of shoots/PLBs from axillary bud of *D. formosum* *

| Conc. (μM) | Explant response (%) | Average number of shoots / explant |
|--------------------------------------|---------------------------------|---|
| Control | 73.3 \pm 5.7 | 1.66 \pm 0.09 (1-3) |
| BAP+IAA | | |
| 5 + 5 | 76.66 \pm 5.7 | 2.38 \pm 0.07(1-4) |
| 5 + 15 | 86.66 \pm 5.7 | 2.09 \pm 0.33(1-3) |
| 5 + 30 | 78.51 \pm 1.2 | 1.90 \pm 0.14(1-3) |
| 5 + 50 | 52.38 \pm 4.1 | 1.47 \pm 0.13(1-3) |
| | | |
| 15 + 5 | 82.96 \pm 5.1 | 2.60 \pm 0.28 (2-4) |
| 15 + 15 | 89.62 \pm 0.64 | 3.24 \pm 0.17(2-5) |
| 15 + 30 | 83.33 \pm 5.7 | 1.56 \pm 0.33(1-3) |
| 15 + 50 | 32.22 \pm 1.9 | 1.35 \pm 0.28(1-3) |
| | | |
| 30 + 5 | 76.66 \pm 5.7 | 2.28 \pm 0.40(1-3) |
| 30 + 15 | 73.33 \pm 5.7 | 2.10 \pm 0.14(1-3) |
| 30 + 30 | 63.33 \pm 5.7 | 1.75 \pm 0.35(1-3) |
| 30 + 50 | 27.41 \pm 4.4 | 1.00 \pm 0.00(1) |
| | | |
| 50 + 5 | 66.66 \pm 5.7 | 1.66 \pm 0.47(1-3) |
| 50 + 15 | 40.00 \pm 10 | 1.75 \pm 0.35(1-3) |
| 50 + 30 | 33.33 \pm 5.7 | 1.38 \pm 0.09(1-2) |
| 50 + 50 | - | - |

\pm S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Table 3.9: Effect of BAP and 2,4-D in MS medium on the formation of shoots/PLBs from axillary bud of *D. longicornu* *

| Conc. (µM) | Explant response (%) | Average number of shoots / explant | Explant response into PLBs (%) |
|------------|----------------------|------------------------------------|--------------------------------|
| Control | 70.00 ± 10 | 1.71 ± 0.64 (1-3) | - |
| BAP+2,4-D | | | |
| 5 + 5 | 65.55 ± 5.0 | 1.55 ± 0.09(1-3) | 31.11 ± 1.9 |
| 5 + 15 | 63.33 ± 5.7 | 1.47 ± 0.24(1-3) | 30.00 ± 0.0 |
| 5 + 30 | 36.66 ± 5.7 | 1.00 ± 0.00(1) | 20.00 ± 0.0 |
| 5 + 50 | - | - | - |
| | | | |
| 15 + 5 | 67.77 ± 1.9 | 1.19 ± 0.17(1-2) | 24.81 ± 4.4 |
| 15 + 15 | 72.59 ± 4.4 | 1.00 ± 0.00(1) | 41.48 ± 2.5 |
| 15 + 30 | 33.33 ± 5.7 | - | 33.33 ± 5.7 |
| 15 + 50 | - | - | - |
| | | | |
| 30 + 5 | 30.00 ± 0.0 | 1.00 ± 0.00(1) | - |
| 30 + 15 | 23.33 ± 5.7 | 1.20 ± 0.44(1-2) | 10.00 ± 0.0 |
| 30 + 30 | 20.00 ± 0.0 | - | 20.00 ± 0.0 |
| 30 + 50 | - | - | - |
| | | | |
| 50 + 5 | - | - | - |
| 50 + 15 | - | - | - |
| 50 + 30 | - | - | - |
| 50 + 50 | - | - | - |

± S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Plate 3.2

Complete development of plantlets from plbs obtained from axillary buds of *D. longicornu* on MS + 15 μ M BAP + 15 μ M 2,4-D

- a. Initiation of plbs in 15 days time
- b. Proliferation of plbs in 30 days time
- c. Development of plantlets from plbs in 90 days time
- d. Development of mature shoots with roots after 120 days time

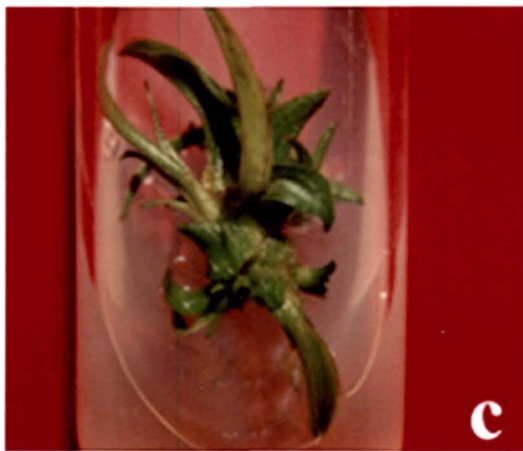
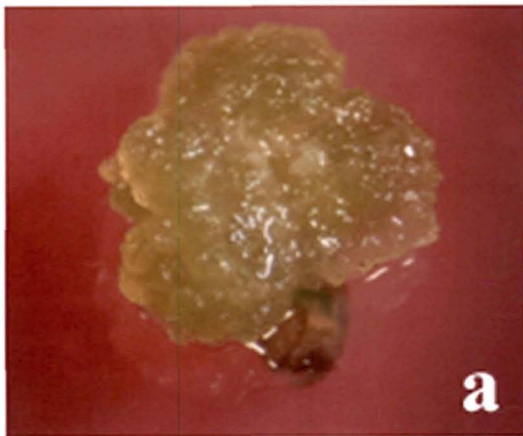


Table 3.10: Effect of BAP and 2,4-D in MS medium on the formation of shoots/PLBs from axillary bud of *D. formosum* *

| Conc. (µM) | Explant response (%) | Average number of shoots / explant | Explant response into PLBs (%) |
|------------|----------------------|------------------------------------|--------------------------------|
| Control | 73.30 ± 5.7 | 1.66 ± 0.09 (1-3) | - |
| BAP+2,4-D | | | |
| 5 + 5 | 78.51 ± 1.3 | 1.60 ± 0.14(1-2) | 36.66 ± 5.7 |
| 5 + 15 | 75.18 ± 4.4 | 1.35 ± 0.49(1-2) | 35.55 ± 3.8 |
| 5 + 30 | 40.00 ± 10 | 1.37 ± 0.17(1-2) | 13.33 ± 5.7 |
| 5 + 50 | - | - | - |
| | | | |
| 15 + 5 | 72.59 ± 4.4 | 1.85 ± 0.02(1-3) | - |
| 15 + 15 | 76.66 ± 5.7 | 1.24 ± 0.17(1-2) | 24.81 ± 4.5 |
| 15 + 30 | 30.00 ± 0.00 | 1.00 ± 0.00(1) | 10.00 ± 0.0 |
| 15 + 50 | - | - | - |
| | | | |
| 30 + 5 | 60.00 ± 10 | 1.45 ± 0.07(1-2) | - |
| 30 + 15 | 43.33 ± 5.7 | 1.33 ± 0.28(1-2) | - |
| 30 + 30 | 42.96 ± 2.5 | 1.00 ± 0.00(1) | 21.48 ± 1.2 |
| 30 + 50 | - | - | - |
| | | | |
| 50 + 5 | 26.66 ± 5.7 | 1.00 ± 0.00(1) | - |
| 50 + 15 | 26.66 ± 5.7 | 1.00 ± 0.00(1) | - |
| 50 + 30 | 10.00 ± 0.0 | 1.00 ± 0.00(1) | - |
| 50 + 50 | - | - | - |

± S.D.

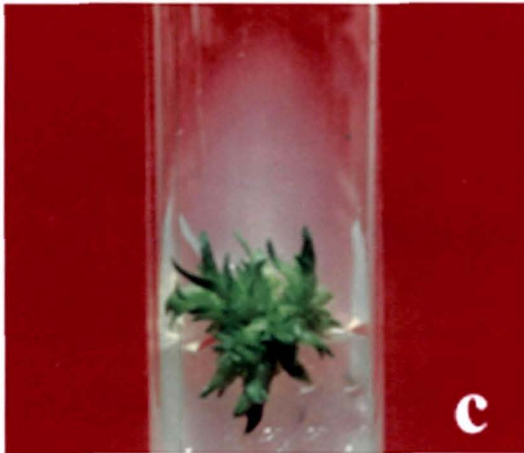
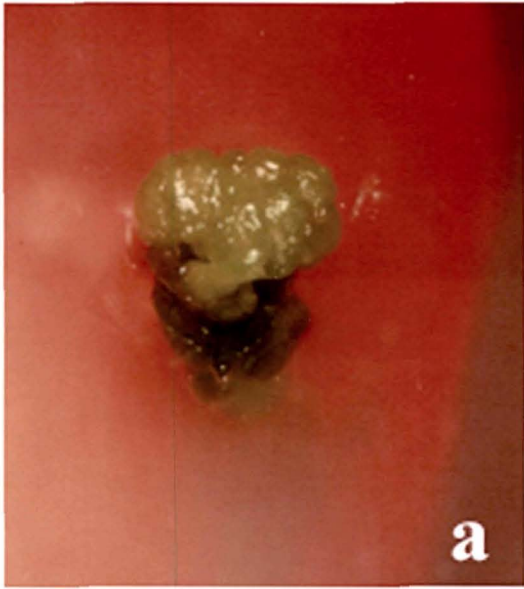
() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Plate 3.3

Complete development of plantlets from plbs obtained from axillary buds of *D. formosum* on MS + 5 μ M BAP + 5 μ M 2,4-D

- a. Initiation of plbs in 15 days time
- b. Proliferation of plbs in 30 days time
- c. Development of plantlets from plbs in 90 days time
- d. Development of mature shoots with roots after 120 days time



treatment (Table 3.9; Plate 3.2). A high concentration of 50 μ M of either BAP or 2, 4-D or both had an inhibitory effect and the explants failed to respond.

In case of *D. formosum*, the initiation of PLBs was observed in most of the treatments of both BAP and 2,4-D in the medium. The highest response of 78.51% and PLBs initiation of 36.66% was recorded at 5 μ M BAP and 5 μ M 2, 4-D in combination (Table 3.10; Plate 3.3). The average number of shoots decreased in almost all the concentrations of these growth regulators tried in combination. Both shoots as well as PLBs initiation were recorded in the combinations of BAP and 2, 4-D in the medium.

Effect of KN + NAA

In different combinations of KN and NAA tried in the medium, the cultured axillary buds of *D. longicornu* showed differential response. Lower concentration was found suitable for both percentage responses as well as shoots formation. At a concentration of 5 μ M KN and 15 μ M NAA in the medium, 96.66% of the explants responded with a formation of an average of 3.28 shoots/explant (Table 3.11). No formation of PLBs was recorded in this treatment of growth regulators.

On the other hand, the cultured axillary buds of *D. formosum* showed responses in all the concentrations of KN and NAA tried in the medium. At lower concentrations of KN and NAA in the medium the average response as well as shoot development was better as compared to the control. A treatment of 15 μ M of KN and 15 μ M of IAA in combination was found the most favourable as compared to other combinations tried, a

Table 3.11: Effect of KN and NAA in MS medium on the formation of shoots/PLBs from axillary bud of *D. longicornu* *

| Conc. (μ M) | Explant response (%) | Average number of shoots / explant |
|---------------------|-------------------------|---------------------------------------|
| Control | 70.00 \pm 10 | 1.71 \pm 0.64 (1-3) |
| KN+NAA | | |
| 5 + 5 | 92.12 \pm 6.8 | 2.83 \pm 0.05(2-4) |
| 5 + 15 | 96.66 \pm 5.7 | 3.28 \pm 0.36(2-5) |
| 5 + 30 | 66.38 \pm 3.7 | 2.61 \pm 0.24(2-4) |
| 5 + 50 | 57.50 \pm 6.6 | 2.31 \pm 0.15(2-4) |
| | | |
| 15 + 5 | 76.66 \pm 5.7 | 1.69 \pm 0.02(1-3) |
| 15 + 15 | 83.33 \pm 5.7 | 2.46 \pm 0.18(1-4) |
| 15 + 30 | 61.42 \pm 7.4 | 2.30 \pm 0.04(1-4) |
| 15 + 50 | 39.28 \pm 3.0 | 1.42 \pm 0.38(1-3) |
| | | |
| 30 + 5 | 76.66 \pm 5.7 | 1.69 \pm 0.02(1-3) |
| 30 + 15 | 64.44 \pm 3.8 | 2.05 \pm 0.19(1-3) |
| 30 + 30 | 62.22 \pm 3.8 | 1.75 \pm 0.28(1-3) |
| 30 + 50 | - | - |
| | | |
| 50 + 5 | 55.18 \pm 5.0 | 1.54 \pm 0.23(1-2) |
| 50 + 15 | 55.71 \pm 5.1 | 1.83 \pm 0.14(1-3) |
| 50 + 30 | - | - |
| 50 + 50 | - | - |

\pm S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Plate 3.4

Complete development of plantlets from axillary bud of *D. formosum* on

MS + 15 μ M KN + 15 μ M NAA

- a. Initiation of shoots in 15 days time
- b. Proliferation of shoots in 30 days time
- c. Cluster of shoots in 60 days time
- d. Development of mature shoots after 90 days time

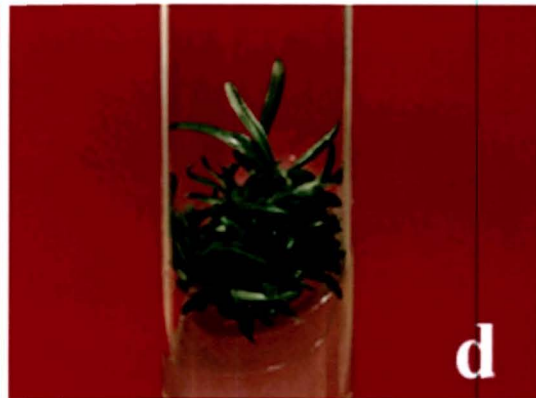
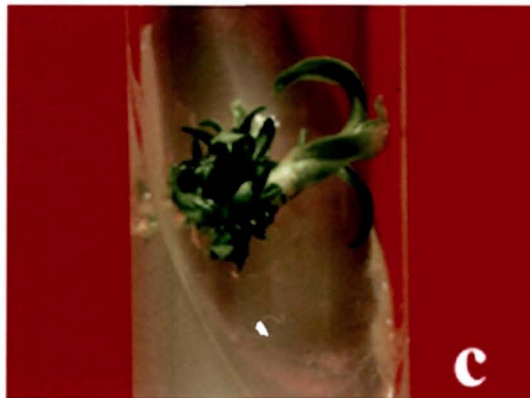
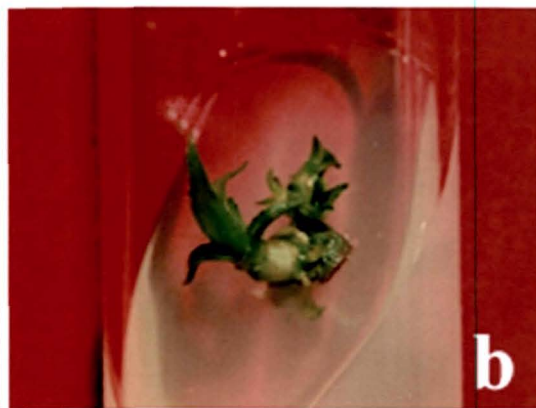
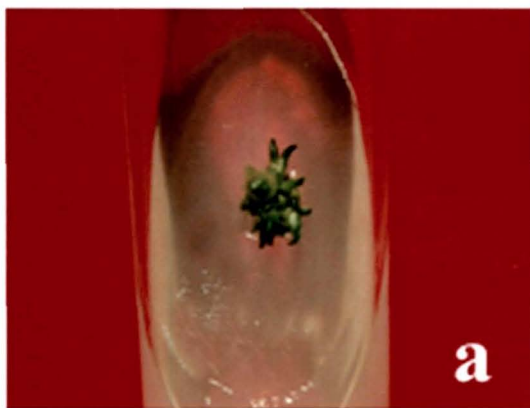


Table 3.12: Effect of KN and NAA in MS medium on the formation of shoots/PLBs from axillary bud of *D. formosum* *

| Conc. (μM) | Explant response (%) | Average number of shoots / explant |
|--------------------------------------|---------------------------------|---|
| Control | 73.30 \pm 5.7 | 1.66 \pm 0.09 (1-3) |
| KN+NAA | | |
| 5 + 5 | 73.25 \pm 1.28 | 2.91 \pm 0.12(2-4) |
| 5 + 15 | 85.92 \pm 5.1 | 2.08 \pm 0.11(1-3) |
| 5 + 30 | 67.77 \pm 1.9 | 2.10 \pm 0.47(1-3) |
| 5 + 50 | 56.66 \pm 5.7 | 1.90 \pm 0.14(1-3) |
| | | |
| 15 + 5 | 76.66 \pm 5.7 | 4.56 \pm 0.26(2-6) |
| 15 + 15 | 96.66 \pm 5.7 | 5.16 \pm 0.70(3-8) |
| 15 + 30 | 86.66 \pm 5.7 | 2.34 \pm 0.12(2-4) |
| 15 + 50 | 60.00 \pm 10 | 1.83 \pm 0.24(1-3) |
| | | |
| 30 + 5 | 96.66 \pm 5.7 | 5.03 \pm 1.06(3-7) |
| 30 + 15 | 83.33 \pm 5.7 | 2.95 \pm 0.53(2-4) |
| 30 + 30 | 70.00 \pm 10 | 2.41 \pm 0.35(2-4) |
| 30 + 50 | 56.66 \pm 5.7 | 1.91 \pm 0.12(1-4) |
| | | |
| 50 + 5 | 53.70 \pm 3.2 | 3.01 \pm 0.26(1-4) |
| 50 + 15 | 51.85 \pm 3.2 | 2.45 \pm 0.07(1-3) |
| 50 + 30 | 33.33 \pm 5.7 | 1.91 \pm 0.58(1-3) |
| 50 + 50 | - | - |

\pm S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

high percentage response of 96.66% and an average shoot number of 5.16 (3-8) was recorded in this treatment (Table 3.12; Plate 3.4). A high concentration of 50 μ M of either KN or NAA in combination showed decline in explant response, while there was an increase in the number of shoots formed.

Effect of KN + IAA

The axillary buds of *D. longicornu* showed a better response in lower concentrations of 5 μ M and 15 μ M in combination of both KN and IAA as compared to control. In the combinations of 5 μ M + 5 μ M and 15 μ M + 5 μ M of KN and NAA in the medium, the explants showed a highest response of 96.29% and 96.66% respectively and also a higher number of shoots/explant as compared to control (Table 3.13). A decrease in responses as well as in shoot number was recorded with increase in the concentrations of KN or NAA in the medium.

In case of *D. formosum*, the explant response of 96.66% was observed in the medium containing 15 μ M of KN with 15 μ M of IAA, while the highest average shoot number of 4.01 (2-6) was recorded at 15 μ M of KN in combination with 30 μ M of IAA (Table 3.14). The percentage response of the axillary buds in other combinations was at par with control though the average number of shoots was enhanced as compared to control.

Table 3.13: Effect of KN and IAA in MS medium on the formation of shoots/PLBs from axillary bud of *D. longicornu* *

| Conc. (μM) | Explant response (%) | Average number of shoots / explant |
|--------------------------------------|---------------------------------|---|
| Control | 70.00 \pm 10 | 1.71 \pm 0.64 (1-3) |
| KN + IAA | | |
| 5 + 5 | 96.29 \pm 6.4 | 3.27 \pm 0.02(2-6) |
| 5 + 15 | 75.47 \pm 4.3 | 1.75 \pm 0.13(1-4) |
| 5 + 30 | 60.00 \pm 10.0 | 1.72 \pm 0.12(1-3) |
| 5 + 50 | 31.11 \pm 1.9 | 1.44 \pm 0.09(1-2) |
| | | |
| 15 + 5 | 96.66 \pm 5.7 | 3.29 \pm 0.13(2-5) |
| 15 + 15 | 73.88 \pm 6.7 | 1.72 \pm 0.19(1-3) |
| 15 + 30 | 73.88 \pm 6.7 | 1.53 \pm 0.11(1-3) |
| 15 + 50 | 40.61 \pm 3.5 | 1.19 \pm 0.17(1-2) |
| | | |
| 30 + 5 | 72.14 \pm 2.5 | 2.72 \pm 0.48(2-3) |
| 30 + 15 | 62.19 \pm 4.6 | 1.60 \pm 0.19(1-3) |
| 30 + 30 | 41.90 \pm 1.6 | 1.55 \pm 0.19(1-3) |
| 30 + 50 | - | - |
| | | |
| 50 + 5 | 55.71 \pm 5.1 | 1.80 \pm 0.34(1-3) |
| 50 + 15 | 40.81 \pm 1.4 | 1.38 \pm 0.09(1-3) |
| 50 + 30 | - | - |
| 50 + 50 | - | - |

\pm S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Table 3.14: Effect of KN and IAA in MS medium on the formation of shoots/PLBs from axillary bud of *D. formosum* *

| Conc. (μ M) | Explant response (%) | Average number of shoots / explant |
|---------------------|-------------------------|---------------------------------------|
| Control | 73.30 \pm 5.7 | 1.66 \pm 0.09 (1-3) |
| KN + IAA | | |
| 5 + 5 | 76.66 \pm 5.7 | 3.40 \pm 0.24(2-4) |
| 5 + 15 | 75.18 \pm 4.4 | 2.14 \pm 0.19(1-3) |
| 5 + 30 | 73.33 \pm 5.7 | 2.20 \pm 0.28(1-3) |
| 5 + 50 | 51.85 \pm 3.2 | 1.52 \pm 0.41(1-3) |
| | | |
| 15 + 5 | 76.66 \pm 5.7 | 2.31 \pm 0.08(1-3) |
| 15 + 15 | 96.66 \pm 5.7 | 2.19 \pm 0.07(1-3) |
| 15 + 30 | 78.51 \pm 1.2 | 4.01 \pm 0.21(2-6) |
| 15 + 50 | 31.11 \pm 1.9 | 1.11 \pm 0.33(1-2) |
| | | |
| 30 + 5 | 75.18 \pm 4.5 | 3.03 \pm 0.65(2-4) |
| 30 + 15 | 71.47 \pm 5.7 | 2.22 \pm 0.08(1-4) |
| 30 + 30 | 70.00 \pm 10 | 2.30 \pm 0.03(1-4) |
| 30 + 50 | 40.00 \pm 10 | 1.91 \pm 0.12(1-3) |
| | | |
| 50 + 5 | 63.33 \pm 5.7 | 2.15 \pm 0.21(1-3) |
| 50 + 15 | 56.66 \pm 5.7 | 1.14 \pm 0.12(1-2) |
| 50 + 30 | 40.00 \pm 10 | 1.00 \pm 0.00(1) |
| 50 + 50 | - | - |

\pm S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Effect of KN + 2, 4-D

In case of the axillary buds of *D. longicornu* cultured in the medium supplemented with KN and 2,4-D in combination, a decrease in explant response was recorded in all the combinations. PLB initiation was recorded with the maximum response of 33.33% in the treatments containing 5 μ M+ 30 μ M and 15 μ M + 30 μ M of KN and 2, 4-D in the medium (Table 3.15).

The responses of the axillary buds of *D. formosum* was better as compared to control when cultured in the medium containing lower concentrations of both KN and 2,4-D. On the other hand, with the increase in the concentrations of these growth regulators the explant responses decreased. Initiation of PLBs was recorded at the treatments of 5 μ M of KN with 15 μ M and 30 μ M of 2,4-D in combination in the medium (Table 3.16). The maximum percentage response in terms of shoots/explant was recorded at 15 μ M each of KN and 2,4-D wherein 82.96% explants responded with the average shoot number of 2.08 (1-3). The average shoot number from the explants given different treatments of KN and 2, 4-D in the medium was found to be lower.

DISCUSSION

Tissue culture of orchids is a fast means of clonal propagation and also can be employed as a research tool for understanding the development process in basic research. The causative factors controlling morphogenesis and regeneration from various plant parts are diverse. Nutritional requirements for the optimal growth of the plants *in vitro*

Table 3.15: Effect of KN and 2,4-D in MS medium on the formation of shoots/PLBs from axillary bud of *D. longicornu* *

| Conc. (μ M) | Explant response (%) | Average number of shoots / explant | Explant response into PLBs (%) |
|------------------|----------------------|------------------------------------|--------------------------------|
| Control | 70.00 \pm 10 | 1.71 \pm 0.64 (1-3) | |
| KN+2,4-D | | | |
| 5 + 5 | 68.88 \pm 1.9 | 1.90 \pm 0.01(1-3) | 27.40 \pm 4.4 |
| 5 + 15 | 53.33 \pm 5.7 | 1.80 \pm 0.28(1-3) | 30.00 \pm 0.0 |
| 5 + 30 | 33.33 \pm 5.7 | - | 33.33 \pm 5.7 |
| 5 + 50 | - | | - |
| | | | |
| 15 + 5 | 63.33 \pm 5.7 | 2.20 \pm 0.06(1-4) | - |
| 15 + 15 | 56.66 \pm 5.7 | 1.70 \pm 0.42(1-3) | 20.00 \pm 0.0 |
| 15 + 30 | 33.33 \pm 5.7 | - | 33.33 \pm 5.7 |
| 15 + 50 | - | - | - |
| | | | |
| 30 + 5 | 60.00 \pm 0.0 | 1.60 \pm 0.34(1-3) | - |
| 30 + 15 | 41.48 \pm 2.5 | 1.75 \pm 0.35(1-3) | - |
| 30 + 30 | 36.66 \pm 5.7 | 1.00 \pm 0.00(1) | 26.66 \pm 5.7 |
| 30 + 50 | - | - | - |
| | | | |
| 50 + 5 | 53.33 \pm 5.7 | 1.20 \pm 0.20(1-2) | - |
| 50 + 15 | 46.66 \pm 5.7 | 1.00 \pm 0.00(1) | - |
| 50 + 30 | - | - | - |
| 50 + 50 | - | - | - |

\pm S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

Table 3.16: Effect of KN and 2,4-D in MS medium on the formation of shoots/PLBs from axillary bud of *D. formosum* *

| Conc. (µM) | Explant response (%) | Average number of shoots / explant | Explant response into PLBs (%) |
|------------|----------------------|------------------------------------|--------------------------------|
| Control | 73.30 ± 5.7 | 1.66 ± 0.09 (1-3) | - |
| KN+2,4-D | | | |
| 5 + 5 | 81.01 ± 5.6 | 2.01 ± 0.26(1-3) | - |
| 5 + 15 | 72.59 ± 4.5 | 1.91 ± 0.12(1-3) | 34.44 ± 5.1 |
| 5 + 30 | 66.66 ± 5.7 | 1.41 ± 0.12(1-2) | 33.33 ± 5.7 |
| 5 + 50 | - | - | - |
| | | | |
| 15 + 5 | 80.00 ± 0.0 | 1.78 ± 0.30(1-3) | - |
| 15 + 15 | 82.96 ± 5.1 | 2.08 ± 0.11(1-3) | - |
| 15 + 30 | 66.66 ± 5.7 | 1.16 ± 0.23(1-2) | - |
| 15 + 50 | 20.00 ± 0.0 | 1.00 ± 0.00(1) | - |
| | | | |
| 30 + 5 | 66.66 ± 5.7 | 1.85 ± 0.49(1-3) | - |
| 30 + 15 | 62.22 ± 3.8 | 1.79 ± 0.05(1-3) | - |
| 30 + 30 | 36.66 ± 5.7 | 1.33 ± 0.46(1-2) | - |
| 30 + 50 | - | - | - |
| | | | |
| 50 + 5 | 30.00 ± 0.0 | 1.83 ± 0.24(1-3) | - |
| 50 + 15 | 30.00 ± 0.0 | 1.58 ± 0.11(1-3) | - |
| 50 + 30 | - | - | - |
| 50 + 50 | - | - | - |

± S.D.

() indicates the minimum and maximum number of shoots/explant

*Data collected after 45 days of culture

vary from species to species. Different explants have been used for orchid micropropagation which include roots and rhizomes, pseudobulb segments, shoot meristems, stem explants, axillary buds, leaf explants, etc. (Arditti and Ernst, 1993; Kumaria and Tandon, 2001; Kumaria *et al.*, 2005). Explants employed for such purpose respond selectively depending upon their source, physiological age and nutrient environment (Vij *et al.*, 1983, 1984). Arditti (1977) reported variations in the media used for tissue culture of orchids and suggested that the composition of the medium changes with the same or different genera. Some media contain many components while others are simpler in their composition. The concentrations and chemical forms of the components are of defined chemical nature (salts, vitamins, hormones, amino acids, organic acids, nucleotides, nucleic acids and chelating agents). 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; Intuwong and Sagawa, 1973; Kako, 1973; Mosich *et al.*, 1974; Arditti, 1977).

In the present study, out of all different explant sources tried, axillary bud was found the most suitable explant for micropropagation of both *D. longicornu* and *D. formosum*. The response of the explants varied depending on the media used. Out of the four media tried, the cultured axillary bud responded best in MS medium with the emergence of 1-3 shoots per explant. However, no PLBs were formed from the explants in all the basal media tried. The perusal of literature revealed the development of only multiple shoots in basal medium from the base of the cultured growing shoot without the intervention of callus and PLBs in *Dendrobium* hybrids (Devi and Laishram, 1998).

Ichihashi (1992), however, obtained PLBs/ callus in *Phalaenopsis* in the medium bereft of growth hormones.

Auxins (IAA, NAA and 2,4-D) showed differential results in the responses of axillary buds of both *D. longicornu* and *D. formosum*. Shoot buds as well as PLBs were seen emerging in medium containing 2,4-D. The addition of growth regulators in the basal medium resulted in better response of the the axillary buds due to probably their habituated nature and juvenility. The juvenility of tissues 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 potencial of juvenile cells was earlier explained on the basis of their physiological and biochemical more active state due to their less rigid walls (Mishra and Bhatnagar, 1995). The axillary buds of both the species studied followed both shoot bud- and PLBs - mediated regeneration in 2, 4-D containing medium, while in IAA and NAA containing medium, the regeneration was mainly shoot bud - mediated. Such a varying response (shoot bud/PLB) has been attributed to the genetic and/or source related physiological intricacies (Vij *et al.*, 2000). In *D. longicornu*, 86.6% explant response with maximum number of shoots/explant was recorded in medium supplemented with NAA at 30 μ M, while incase of *D. formosum*, the explants cultured in medium containing lower concentration of NAA at 15 μ M showed maximum percentage response (96.66%) as well as increased shoot number. Vij and Kaur (1998) also reported similar results where NAA enriched medium favoured multiple shoot bud formation. Similarly, there are earlier reports on accentuated regeneration potential of *Dendrobium moschatum* pseudobulb

explants (Vij and Sood, 1982). The regeneration pathway of axillary buds cultured in MS medium supplemented with different concentrations of 2,4-D in case of both the dendrobe species was through both PLBs and shoot buds formation. In *D. longicornu*, maximum of 36.94% of explants response into PLBs was recorded at 30 μ M of 2,4-D in the medium, whereas in *D. formosum*, 28.51% of the explants responded into PLBs at 15 μ M 2,4-D in the medium. Sharon and Vasundhara (1990) reported PLB induction in *Dendrobium Joannie Ostenhault* in modified Vacin and Went medium supplemented with coconut water. Kim *et al.* (1970) also reported the formation of PLBs from bud explants as well as from leaf axils. Reports suggest that in 2,4-D activated charcoal enriched medium, the foliar explants respond but plantlets follow a callus-mediated development (Vij and Kaur, 1992). However, earlier study on *Rhynchostylis retusa* foliar segments suggested that 2,4-D favoured direct generation of PLBs (Vij *et al.*, 1984). Further, it had also been reported that 2,4-D was suitable for both callusing and PLB formation in leaf explants of *D. formosum*, and stem disc cultures of *D. moschatum* (Kanjilal *et al.*, 1999; Nasiruddin *et al.*, 2003). Therefore, it may be reasonably assumed that the regeneration pathway, under similar hormonal stimuli, vary with the nature and species of the explant used. In this connection it is worthwhile to mention that 2,4-D promoted the development of non-organogenetic callus in *Nicotiana tabaccum* epidermal peels (Tran Thanh Van *et al.*, 1974).

Cytokinins have been used for multiplication of 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). In the present study, the cytokinins (BAP/KN) promoted only shoot bud production or shoot bud and PLB formation from the cultured axillary bud. Only shoot bud induction was observed in *D. longicornu*, whereas both shoot buds as well as PLBs were induced in *D. formosum* as a result of BAP in the medium. Earlier studies with pseudobulb explants, Vij *et al.* (2000) reported that activation of meristematic activity in the *in vitro* explants was obligatory to the use of KN/BAP in the medium and development of multiple shoot buds was reported in the cultures treated with BAP/KN. Kosir *et al.* (2004) also reported direct shoot regeneration from nodes of *Phalaenopsis* when cultured in medium containing BAP.

The interactions of cytokinins and auxins in the medium bring different types of responses in the cultured explants. The cytokinins BAP/KN when combined with different auxins (IAA, NAA and 2,4-D) at different concentrations, brought varying results in the explants of both the species studied. Tokuhara and Mii (1993) also reported that the combination of hormones BAP and NAA in the cultures were of key importance for the micropropagation of *Phalaenopsis* on a commercial scale. Further, the stimulatory effect of BAP and NAA in the medium has been reported for certain species (Kosir *et al.*, 2004). Ernst (1994) reported that shoot and root development were reduced, while proliferation increased with increasing the concentration of the cytokinin, Thidiazuron. While some authors have reported reduced induction and regeneration in medium supplemented with NAA (Arditti and Ernst, 1993), others reported that an appropriate combination of NAA and BAP in the medium stimulated shoot formation (Tokuhara and Mii, 1993; Tisserat and Jones, 1999; Roy and Banerjee, 2003). Similar results were also

found in *D. longicornu* explants where in the medium containing a combination of BAP (5 μ M) and NAA (15 μ M), a maximum shoot number was recorded. On the other hand, for the axillary buds of *D. formosum*, a concentration of 15 μ M each of KN and NAA in the medium was found to be the most suitable. Several workers have reported the positive effects of NAA in combination with KN where both a rapid multiplication of PLBs and development of plantlets from foliar peels were observed in case of *R. retusa* (Vij and Kaur, 1992), and a fair response of explants in shoot bud formation was observed in pseudobulb segments of *Malaxis acuminata* (Vij and Kaur, 1998). The interactions of 2,4-D either with BAP or KN induced both PLBs and shoot buds from axillary buds of both *D. longicornu* as well as *D. formosum*. In the medium containing combinations of 15 μ M each of BAP and 2,4-D in case of *D. longicornu*, and 5 μ M each of BAP and 2,4-D in case of *D. formosum*, maximum PLBs were induced from the explants. It was observed that the explant response in terms of PLB induction was highest in the medium supplemented with BAP to 2,4-D at a ratio of 1.0. Ratios of these growth regulators below 1.0 though induced PLBs from the explant were not much effective. However, study of combination of KN and 2,4-D showed varying results in the formation of shoots and PLBs from axillary bud. In axillary buds of *D. longicornu* at ratios of KN to 2,4-D at 1.0 and below in the medium, both PLBs and shoots were induced, while ratios higher than 1.0 promoted only shoot induction. In the present study, in case of *D. formosum*, PLBs and shoot induction was seen only in the medium supplemented with 5 μ M KN in combination with either 15 μ M or 30 μ M of 2,4-D in the medium, while in other combinations only shoots were formed. Induction of multiple protocorms around the

apical and axillary meristem of *Cattleya* shoots with KN/BAP in combination with 2,4-D has been reported earlier (Kusumoto, 1979). Although some authors reported the induction of shoot buds without the intervention of callus and PLBs (Devi and Laishram, 1998), others have reported the formation of PLBs from different explants (Kim *et al.*, 1970; Sharon and Vasundhara, 1990). Since many orchid species require auxins and /or cytokinins for shoot and PLB formation (Arditti and Ernst, 1993), the combination, concentrations and the ratio between them are critically important. The ratio of auxin to cytokinin or vice versa for PLB formation varies from species to species and from explant to explant used (Teng *et al.*, 1997). Similarly, in the present study, for axillary buds of both the species high as well as low ratio of cytokinin to auxin in the medium proved beneficial for induction of PLBs, wherein BAP to 2,4-D ratio of 1.0 was best in explant response from the axillary buds. However, many inconsistencies were found in the results with KN and 2,4-D combination in the medium for both the orchid species studied. A high ratio (12.2) of NAA/BAP (i.e., low ratio of BAP/ NAA 0.5) in *Spathoglottis plicata* was reported best for induction of PLBs from nodal explants (Teng *et al.*, 1997). Although low ratio of 0.12 of NAA to BAP was employed for mature *Phalaenopsis amabilis* blume leaf cultures (Tanaka and Sakanishi, 1985), for axillary bud explants of *Dendrobium antennatum* Lindley the ratio of 0.42 was reported to be suitable (Kukulczanka and Wojciechowska, 1983). In several hybrid species of *Aranda*, the ratio of 1.23 of NAA to BAP was found to be most effective (Khaw *et al.*, 1978). Arditti and Ernst (1984) have given several possible reasons for inconsistencies found in the results

obtained by various workers in their experiments with plant growth regulators. These could be due to one or more of the following reasons:

- Interactions may have occurred between various combinations of hormones and the culture conditions
- Physiological responses and requirements of species and genera may vary
- Different forms and analogues of each hormone were used
- Culture conditions were different in each case
- A wide range of dosage concentrations was used
- The age of the plantlets used for experimentation may have varied

**CHAPTER IV: GROWTH AND DEVELOPMENT OF *IN VITRO* RAISED
PROTOCORM-LIKE BODIES (PLBs) OF *DENDROBIUM LONGICORNU* AND
*DENDROBIUM FORMOSUM***

INTRODUCTION

New prospects of propagation of orchids through tissue culture techniques were opened after Morel's observation of green, globular PLBs around the shoot tips of *Cymbidium* cultured *in vitro* (Morel, 1960, 1964). These PLBs when cut into smaller sections and transferred to fresh nutrient medium, multiplied and gave rise to secondary and tertiary PLBs. However, on leaving undisturbed, they developed into complete plantlets with roots and shoots. This technique of meristem culture has been used since, by many workers for virus elimination and production of asexual plantlets on a large scale (Arditti, 1977). Of the numerous factors affecting the successful induction of morphogenesis in plant tissue cultures, medium composition is one of the most important. Following Knudson's discovery (1922) that orchids could be grown on a medium supplemented with organic and inorganic nutrients, a number of media for tissue culture

of orchids have been used viz., Vacin and Went (1949), Murashige and Skoog (1962), Nitsch and Nitsch (1969), Mitra *et al.* (1976), etc. In general, plant tissue culture media consist of mineral salts, a carbon and energy source, vitamins and growth regulators, however, other organic compounds have often been included (Ozias-Akins and Vasil, 1985). The disaccharide sucrose, at a concentration of 2-3% is the most commonly used carbohydrate in plant tissue culture media. Homes and Vansverren-Van Espen (1973) while working on *Cymbidium* reported an enhancement in PLBs proliferation at supraoptimal levels of sucrose, and an increased organogenesis at suboptimal concentrations. A wide variety of complex natural extracts like yeast extract, coconut water, peptone, casein hydrolysate, fruit and vegetable homogenates, honey, fish emulsion, beef extracts, silkworm pupae and Malayasian beer have been used to supplement the orchid culture media (Arditti and Ernst, 1984). The beneficial effects of complex natural extracts and liquid endosperms are more pronounced in low salt media due to the contribution of inorganic as well as organic constituents, whereas their primary role in the high salt media is the supplementation of carbohydrates, plant growth regulators, vitamins and amino acids (Ozias-Akins and Vasil, 1985).

Growth and development of plants *in vitro* is largely due to the uptake of nutrients, including carbohydrates from the medium. Since plants are likely to differ in their responses to nutrients when grown *in vitro*, there is a clear need to understand the optimal nutrient requirements which would lead to increased growth rates and would also enable plant morphogenesis to be directed more efficiently. A number of reports on the nutritional requirements for *in vitro* growth and development of orchids have been made

(Prasad and Mitra, 1975; Stimart and Ascher, 1981; Arditti, 1982; Kukulczanka *et al.*, 1987; Pierik *et al.*, 1988; Soedjono, 1988; Tay *et al.*, 1988; Hew and Lim, 1989; Oyamada, 1989). However, nutrient and culture conditions found suitable for one orchid species may not be applicable to others (Arditti *et al.*, 1981).

Unlike other plant families, differences in requirements for *in vitro* propagation exist among the diverse genera, species and hybrids of the family Orchidaceae. Nutrient formulations and steps that are satisfactory for one plant type may not be applicable to another (Huang, 1988). Growth as well as differentiation can be controlled by various media components including mineral nutrition. Several plant tissue and cell culture media are in use including formulations devised by Murashige and Skoog (1962), Knudson (1946) and Gamborg *et al.* (1968). Composition and components of culture media have been investigated and reviewed by various workers (Gamborg *et al.*, 1976; Huang and Murashige, 1977). In the last three decades, many reports on the growth and development of various orchids as affected by different defined and undefined media have been made (Zeigler *et al.*, 1967; Fannesbech, 1972; Harvais, 1973; Mead and Bulard, 1979; Krishnan and Jorapur, 1984; Amaki and Higuchi, 1989).

Numerous factors affect the successful induction of morphogenesis in plant cell and tissue culture. Growth regulators markedly influence the growth and development of *in vitro* growing plantlets. Skoog and Miller (1957) indicated that auxin to cytokinin ratios determined the type and extent of organogenesis. While no universal ratio for root or shoot induction exists, both an auxin and a cytokinin are usually added to the medium

in order to obtain morphogenesis. Orchids have responded differentially to various auxins, cytokinins and gibberellins and the results have been inconsistent and consequently inconclusive (Arditti and Ernst, 1984). Auxins, mostly IAA, IBA and NAA have been reported to enhance the growth. Israel (1963) reported death of the excised *Dendrobium* ovaries in the absence of auxin. Enhanced growth and development of the protocorms of *Bletilla* when treated with NAA had been reported by Strauss and Reisinger (1976). The promotory effect of NAA on early organogenesis was observed by Vij and Pathak (1990). In addition, Kusumoto (1979) observed a stimulatory effect of 2,4-D on shoot formation, and NAA on shoot proliferation in *Cattleya*. An inhibitory effect of 2,4-D on *Cymbidium* cultures was, however, reported with the development of abnormal PLBs (Kusumoto, 1978; Harvais, 1982; Sharma and Tandon, 1986). A promotory influence of BAP on the proliferation of protocorms had been suggested by Pierik and Steegmans (1972). Also, Kusumoto (1979) reported an increased shoot formation in response to BAP in *Cattleya*. Retardation of root/root hair developmental and inhibition were, however, observed by Rucker (1974) in *Cymbidium* treated with BAP. The addition of KN in medium is reported to stimulate as well as inhibit the growth and development in different orchid species (Hadley, 1970; Harvais, 1973, 1982; Uesato, 1978; Nakamura, 1982).

MATERIALS AND METHODS

PLBs obtained from the explants (as described in chapter III) were multiplied by cutting into smaller pieces and culturing in MS medium devoid of growth regulators.

Each segment got converted into smaller clumps of PLBs and the process could be repeated to get a large number of PLBs. To study the effect of different growth regulators on growth and development of PLBs, well developed PLBs measuring around 5-7 mm in diameter grown on basal MS medium, were cultured aseptically on MS medium supplemented with different growth regulators viz., IAA, NAA, 2,4-D, BAP and KN both singly and in combination, in a range of 0.0-50.0 μ M. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C. The cultures were incubated at 24 \pm 2°C temperature and 12h photoperiod of 150 μ molm⁻²s⁻¹ light intensity. Fresh and dry weights of the PLBs were recorded after 30 and 60 days of culture. Ten replicates were maintained for each treatment and the experiment was repeated thrice. The data was subjected to statistical analysis.

RESULTS

The growth regulators incorporated in MS medium had differential effects on the growth and development of PLBs of the dendrobes studied.

Effect of Auxins

Effect of IAA

When compared to the control, IAA had stimulatory effect on PLB development of *D. longicornu* (Table 4.1), wherein an increase in both fresh and dry weights was recorded. In case of PLBs cultured in medium containing 15 μ M of IAA, the fresh weight was maximum after 30 days of culture. With increase in concentration of IAA in the medium, there was a decrease in the proliferation of cultured PLBs. The dry weight of the

Table 4.1: Effect of growth regulators on the growth and development of PLBs of *D. longicornu* cultured in MS medium supplemented with different growth regulators

| Growth regulator (μM) | Fresh weight (g) | | | Dry weight (g) | | |
|------------------------------------|------------------|------------------|-------------------|--------------------|-------------------|--------------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | | 0.35 \pm 0.01 | 0.610 \pm 0.03 | | 0.019 \pm 0.02 | 0.040 \pm 0.004 |
| IAA | | | | | | |
| 5 | | 0.93 \pm 0.02 | 1.681 \pm 0.17 | | 0.053 \pm 0.005 | 0.105 \pm 0.018 |
| 15 | | 1.09 \pm 0.16 | 1.652 \pm 0.39 | | 0.064 \pm 0.014 | 0.101 \pm 0.01 |
| 30 | | 0.68 \pm 0.05 | 1.212 \pm 0.007 | | 0.045 \pm 0.001 | 0.077 \pm 0.007 |
| 50 | | 0.67 \pm 0.14 | 1.151 \pm 0.024 | | 0.040 \pm 0.002 | 0.079 \pm 0.008 |
| NAA | | | | | | |
| 5 | | 0.53 \pm 0.09 | 0.841 \pm 0.16 | | 0.035 \pm 0.003 | 0.055 \pm 0.005 |
| 15 | | 0.30 \pm 0.07 | 0.494 \pm 0.19 | | 0.018 \pm 0.003 | 0.029 \pm 0.012 |
| 30 | 0.036 \pm 0.01 | 0.27 \pm 0.006 | 0.394 \pm 0.03 | 0.002 \pm 0.0006 | 0.016 \pm 0.002 | 0.023 \pm 0.002 |
| 50 | | 0.10 \pm 0.004 | 0.134 \pm 0.01 | | 0.006 \pm 0.001 | 0.007 \pm 0.0006 |
| BAP | | | | | | |
| 5 | | 1.49 \pm 0.08 | 2.402 \pm 0.07 | | 0.095 \pm 0.004 | 0.155 \pm 0.018 |
| 15 | | 0.74 \pm 0.14 | 1.323 \pm 0.27 | | 0.052 \pm 0.001 | 0.087 \pm 0.006 |
| 30 | | 0.49 \pm 0.15 | 0.971 \pm 0.06 | | 0.034 \pm 0.002 | 0.062 \pm 0.008 |
| 50 | | 0.28 \pm 0.07 | 0.591 \pm 0.03 | | 0.018 \pm 0.003 | 0.041 \pm 0.008 |
| KN | | | | | | |
| 5 | | 1.08 \pm 0.15 | 1.881 \pm 0.08 | | 0.078 \pm 0.003 | 0.120 \pm 0.009 |
| 15 | | 0.67 \pm 0.05 | 1.211 \pm 0.15 | | 0.040 \pm 0.004 | 0.074 \pm 0.003 |
| 30 | | 0.52 \pm 0.08 | 0.945 \pm 0.05 | | 0.035 \pm 0.001 | 0.059 \pm 0.003 |
| 50 | | 0.24 \pm 0.15 | 0.473 \pm 0.13 | | 0.015 \pm 0.002 | 0.021 \pm 0.009 |

\pm S.D.

- no response

Table 4.2: Effect of growth regulators on the growth and development of PLBs of *D. formosum* cultured in MS medium supplemented with different growth regulators

| Growth regulator (μM) | Fresh weight (g) | | | Dry weight (g) | | |
|------------------------------------|------------------|------------------|-------------------|--------------------|-------------------|-------------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | 0.027 \pm 0.01 | 0.151 \pm 0.01 | 0.21 \pm 0.01 | 0.002 \pm 0.0003 | 0.012 \pm 0.001 | 0.018 \pm 0.01 |
| IAA | | | | | | |
| 5 | | 0.337 \pm 0.07 | 0.52 \pm 0.08 | | 0.031 \pm 0.004 | 0.052 \pm 0.002 |
| 15 | | 0.595 \pm 0.02 | 0.98 \pm 0.02 | | 0.062 \pm 0.004 | 0.102 \pm 0.005 |
| 30 | | 0.474 \pm 0.03 | 0.74 \pm 0.01 | | 0.046 \pm 0.004 | 0.081 \pm 0.006 |
| 50 | | 0.206 \pm 0.02 | 0.33 \pm 0.007 | | 0.021 \pm 0.004 | 0.033 \pm 0.001 |
| NAA | | | | | | |
| 5 | | 0.562 \pm 0.08 | 0.95 \pm 0.05 | | 0.050 \pm 0.001 | 0.091 \pm 0.002 |
| 15 | | 0.682 \pm 0.03 | 1.01 \pm 0.09 | | 0.064 \pm 0.004 | 0.103 \pm 0.002 |
| 30 | | 0.655 \pm 0.03 | 0.91 \pm 0.10 | | 0.055 \pm 0.004 | 0.081 \pm 0.006 |
| 50 | | 0.37 \pm 0.008 | 0.46 \pm 0.03 | | 0.031 \pm 0.002 | 0.051 \pm 0.002 |
| BAP | | | | | | |
| 5 | | 0.701 \pm 0.11 | 1.16 \pm 0.07 | | 0.070 \pm 0.002 | 0.112 \pm 0.01 |
| 15 | | 0.501 \pm 0.03 | 0.91 \pm 0.11 | | 0.048 \pm 0.001 | 0.091 \pm 0.002 |
| 30 | | 0.295 \pm 0.02 | 0.52 \pm 0.06 | | 0.027 \pm 0.001 | 0.051 \pm 0.002 |
| 50 | | - | - | | - | - |
| KN | | | | | | |
| 5 | | 0.580 \pm 0.07 | 1.03 \pm 0.07 | | 0.062 \pm 0.002 | 0.114 \pm 0.009 |
| 15 | | 0.660 \pm 0.06 | 1.16 \pm 0.03 | | 0.066 \pm 0.005 | 0.123 \pm 0.005 |
| 30 | | 0.370 \pm 0.04 | 0.58 \pm 0.07 | | 0.034 \pm 0.004 | 0.063 \pm 0.005 |
| 50 | 0.190 \pm 0.03 | 0.26 \pm 0.03 | 0.023 \pm 0.002 | 0.021 \pm 0.002 | | |

\pm S.D.

- no response

PLBs in the medium supplemented with IAA was also found to be much higher as compared to the control (Table 4.1).

Similarly in *D. formosum*, IAA had stimulatory effect on PLB development (Table 4.2). An increase in fresh and dry weights was recorded in course of time. At 15 μ M of IAA in the medium, the PLBs showed maximum proliferation after 30 days wherein a fresh weight of 0.595g was recorded which increased to 0.979g after 60 days of culture which was significantly higher than those recorded for the control. Similarly the dry weight also increased as compared to the control. With increase in IAA concentration in the medium, there was a corresponding decrease in PLB proliferation (Table 4.2).

Effect of NAA

NAA in the medium had an inhibitory effect on the development of PLBs of *D. longicornu*. As compared to control, an increased response both in fresh and dry weights after 30 days and 60 days respectively was recorded only in the medium containing 5 μ M NAA (Table 4.1). An increase in NAA concentration in the medium proved inhibitory for the development and proliferation of PLBs (Table 4.1).

On the other hand, PLBs of *D. formosum* when cultured in NAA containing medium showed stimulatory effect as compared to *D. longicornu*, which implies species specificity in response. An increase in fresh and dry weights was recorded in all the concentrations of NAA after 30 and 60 days respectively as compared to the control. The

maximum fresh and dry weights were recorded at 15 μ M of NAA after 30 days and 60 days respectively (Table 4.2).

Effect of 2, 4-D

2,4-D in the medium had an inhibitory effect on the growth and development of PLBs of both *D. longicornu* and *D. formosum*. The PLBs cultured in the medium containing 2,4-D turned brown and ultimately died in course of time.

Effect of Cytokinins

Effect of BAP

The PLBs of *D. longicornu* when cultured on BAP containing medium showed an increase in fresh as well as dry weights after 30 and 60 days of culture respectively, the exception being the PLBs on medium containing 50 μ M of BAP wherein an inhibitory effect was observed (Table 4.1). The maximum increase in fresh and dry weights after 30 and 60 days of culture was recorded in the medium supplemented with BAP at 5 μ M (Table 4.1).

A similar trend was recorded in case of *D. formosum*, wherein the PLBs cultured in medium containing BAP showed an increase in fresh and dry weights after 30 and 60 days of culture as compared to the control, exception being the ones cultured in the medium containing higher concentration of BAP (50 μ M). In this concentration of BAP, the cultures turned brown and died subsequently (Table 4.2). The maximum fresh and dry weights in medium containing BAP were recorded at 5 μ M concentration. With the

increase in the concentrations of BAP in the medium, a decrease in proliferation of the PLBs was recorded (Table 4.2).

Effect of KN

KN in the medium showed an enhanced proliferation of the cultured PLBs of *D. longicornu* as compared to the control. With the exception of 50 μ M of KN in the medium, other concentrations of KN tried showed an increase in both fresh and dry weights after 30 and 60 days of culture respectively. The maximum increase was recorded at 5 μ M of KN in the medium (Table 4.1). With the increase in the concentrations of KN in the medium, a decrease in response of the PLBs was recorded.

A stimulatory effect of KN on the proliferation of PLBs was also recorded in case of *D. formosum*. As compared to the control, increased fresh and dry weights of PLBs after 30 and 60 days of culture was recorded in all the concentrations of KN tried, with the maximum being at 15 μ M of KN. With an increase in concentration of KN in the medium, a decrease in proliferation of PLBs was recorded (Table 4.2).

Effect of Cytokinins and Auxins in Combination

Effect of BAP+IAA

BAP and IAA in combination in the medium had an enhanced effect on the proliferation rate of PLBs of *D. longicornu* as compared to when added singly in the medium (Table 4.3). A lower concentration of both of these growth regulators was found to be beneficial as compared to the higher concentrations. The highest and the best proliferation and development was recorded at 15 μ M each of BAP and IAA in the

Table 4.3: Effect of growth regulators on the growth and development of PLBs of *D. longicornu* cultured in MS medium supplemented with BAP and IAA

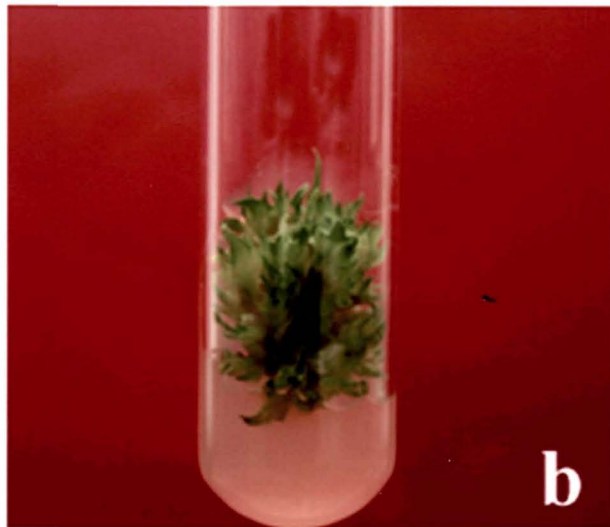
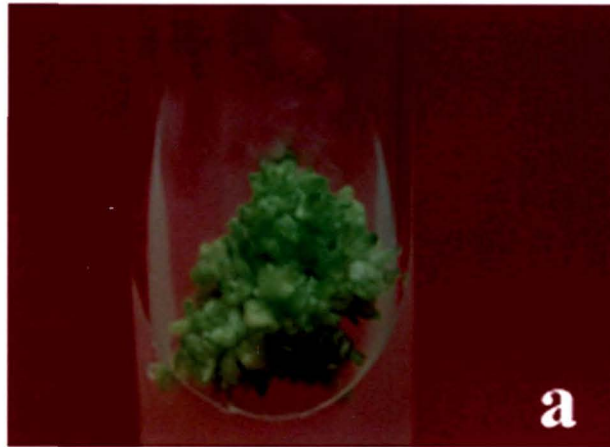
| Growth regulator (μM) | Fresh weight (g) | | | Dry weight (g) | | |
|------------------------------------|------------------|------------------|-------------------|--------------------|-------------------|-------------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | | 0.35 \pm 0.01 | 0.610 \pm 0.03 | | 0.019 \pm 0.02 | 0.040 \pm 0.004 |
| BAP+IAA | | | | | | |
| 5+5 | | 1.14 \pm 0.18 | 1.971 \pm 0.06 | | 0.081 \pm 0.006 | 0.127 \pm 0.04 |
| 5+15 | | 1.79 \pm 0.23 | 2.492 \pm 0.24 | | 0.130 \pm 0.012 | 0.136 \pm 0.018 |
| 5+30 | | 0.71 \pm 0.02 | 0.981 \pm 0.014 | | 0.051 \pm 0.006 | 0.063 \pm 0.005 |
| 5+50 | | 0.51 \pm 0.06 | 0.851 \pm 0.16 | | 0.041 \pm 0.006 | 0.046 \pm 0.003 |
| | | | | | | |
| 15+5 | | 0.98 \pm 0.02 | 1.553 \pm 0.10 | | 0.071 \pm 0.002 | 0.101 \pm 0.02 |
| 15+15 | 0.036 \pm 0.01 | 2.07 \pm 0.11 | 2.820 \pm 0.21 | 0.002 \pm 0.0006 | 0.151 \pm 0.014 | 0.156 \pm 0.018 |
| 15+30 | | 0.49 \pm 0.02 | 0.802 \pm 0.08 | | 0.021 \pm 0.004 | 0.053 \pm 0.002 |
| 15+50 | | 0.33 \pm 0.07 | 0.564 \pm 0.07 | | 0.022 \pm 0.005 | 0.038 \pm 0.007 |
| | | | | | | |
| 30+5 | | 0.61 \pm 0.007 | 1.053 \pm 0.07 | | 0.041 \pm 0.006 | 0.060 \pm 0.003 |
| 30+15 | | 0.87 \pm 0.04 | 1.452 \pm 0.31 | | 0.055 \pm 0.004 | 0.081 \pm 0.009 |
| 30+30 | | 0.27 \pm 0.012 | 0.371 \pm 0.08 | | 0.017 \pm 0.001 | 0.022 \pm 0.002 |
| 30+50 | | - | - | | - | - |
| | | | | | | |
| 50+5 | | 0.48 \pm 0.058 | 0.851 \pm 0.15 | | 0.032 \pm 0.003 | 0.054 \pm 0.004 |
| 50+15 | | 0.33 \pm 0.06 | 0.451 \pm 0.11 | | 0.016 \pm 0.004 | 0.026 \pm 0.006 |
| 50+30 | | - | - | | - | - |
| 50+50 | | - | - | | - | - |

\pm S.D.

- no response

Plate 4.1

Growth and development of PLBs of *D. longicornu* in MS + 15 μ M BAP + 15 μ M IAA after 30 days of culture (a), and 60 days of culture (b)



SSCL 5-each, 8x12, Jrs 027 0795 HD
456P 046 N N N-1 AC+02 AS+01 2419.4/100.0

Table 4.4: Effect of growth regulators on the growth and development of PLBs of *D. formosum* cultured in MS medium supplemented with BAP and IAA

| Growth regulator (μM) | Fresh weight (g) | | | Dry weight (g) | | |
|-----------------------|------------------|-------------|------------|----------------|--------------|-------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | | 0.151±0.01 | 0.21±0.01 | | 0.012±0.001 | 0.018±0.001 |
| BAP+IAA | | | | | | |
| 5+5 | | 0.721±0.05 | 1.14±0.03 | | 0.060±0.003 | 0.090±0.006 |
| 5+15 | | 0.655±0.03 | 0.95±0.02 | | 0.058±0.001 | 0.088±0.001 |
| 5+30 | | 0.676±0.05 | 1.16±0.07 | | 0.058±0.005 | 0.011±0.01 |
| 5+50 | | 0.295±0.02 | 0.54±0.008 | | 0.025±0.001 | 0.048±0.001 |
| | 0.027±0.005 | | | 0.0021±0.0003 | | |
| 15+5 | | 0.648±0.01 | 0.98±0.007 | | 0.058±0.001 | 0.088±0.003 |
| 15+15 | | 0.593±0.02 | 0.88±0.07 | | 0.055±0.004 | 0.086±0.001 |
| 15+30 | | 0.742±0.09 | 1.14±0.23 | | 0.067±0.001 | 0.095±0.003 |
| 15+50 | | 0.317±0.08 | 0.61±0.04 | | 0.0305±0.002 | 0.051±0.004 |
| | | | | | | |
| 30+5 | | 0.434±0.03 | 0.69±0.07 | | 0.041±0.004 | 0.059±0.002 |
| 30+15 | | 0.388±0.03 | 0.75±0.04 | | 0.036±0.002 | 0.067±0.006 |
| 30+30 | | 0.422±0.01 | 0.73±0.11 | | 0.035±0.001 | 0.064±0.005 |
| 30+50 | | 0.217±0.005 | 0.36±0.07 | | 0.021±0.001 | 0.324±0.001 |
| | | | | | | |
| 50+5 | | 0.226±0.02 | 0.38±0.07 | | 0.089±0.003 | 0.035±0.002 |
| 50+15 | | 0.165±0.03 | 0.22±0.03 | | 0.016±0.001 | 0.016±0.002 |
| 50+30 | | - | - | | - | - |
| 50+50 | | - | - | | - | - |

± S.D.

- no response

Plate 4.2

Development of complete plantlets from PLBs of *D. longicornu* after 150 days of culture on:

- a. MS + 15 μ M BAP + 15 μ M IAA
- b. MS + 5 μ M BAP + 5 μ M NAA
- c. MS + 5 μ M KN + 5 μ M IAA
- d. MS + 15 μ M KN + 5 μ M NAA



medium, wherein the maximum increase in fresh and dry weights was recorded after 30 and 60 days of culture (Table 4.3; Plate 4.1). A higher concentration of either BAP or IAA in the medium decreased the rate of proliferation and development of PLBs. Complete plantlets were obtained after 150 days of culture (Plate 4.2a).

Similarly in case of *D. formosum*, PLB proliferation and development was better and higher in medium supplemented with BAP and IAA in combination as compared to the control (Table 4.4). An increase in fresh and dry weights after 30 and 60 days of culture was recorded in most of the combinations tried as compared to control, with the highest proliferation observed at 15 μ M of BAP when combined with 30 μ M of IAA (Table 4.4). Concentrations lower than 30 μ M of either BAP or IAA in the medium showed enhanced effects, while higher at 50 μ M of BAP or IAA showed inhibitory responses. Complete plantlets were obtained after 150 days of culture of PLBs (Plate 4.3a).

Effect of BAP+NAA

When BAP and NAA were added in combination in the medium, a differential response of the PLBs was observed in *D. longicornu*. An increase in fresh and dry weights was recorded after 30 and 60 days of culture in the PLBs treated with a combination of 5 μ M of BAP and 5 μ M of NAA, 5 μ M of BAP and 15 μ M of NAA, and 15 μ M of BAP and 5 μ M of NAA. However, at other combinations of these growth regulators in the medium, a decrease in PLB proliferation was observed (Table 4.5). NAA when added singly in the medium inhibited PLB proliferation (Table 4.1).

Table 4.5: Effect of growth regulators on the growth and development of PLBs of *D. longicornu* cultured in MS medium supplemented with BAP and NAA

| Growth regulator (µM) | Fresh weight (g) | | | Dry weight (g) | | |
|-----------------------|------------------|------------|-------------|----------------|--------------|--------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | | 0.35±0.01 | 0.610±0.03 | | 0.019±0.02 | 0.040±0.004 |
| BAP+NAA | | | | | | |
| 5+5 | | 0.85±0.21 | 1.377±0.48 | | 0.059±0.009 | 0.082±0.02 |
| 5+15 | | 0.57±0.08 | 1.107±0.01 | | 0.036±0.007 | 0.063±0.005 |
| 5+30 | | 0.30±0.05 | 0.390±0.05 | | 0.019±0.002 | 0.0244±0.003 |
| 5+50 | | 0.17±0.002 | 0.217±0.005 | | 0.010±0.001 | 0.016±0.007 |
| | 0.036±0.01 | | | 0.002±0.0006 | | |
| 15+5 | | 0.51±0.17 | 0.770±0.28 | | 0.033±0.002 | 0.047±0.0016 |
| 15+15 | | 0.25±0.08 | 0.322±0.15 | | 0.017±0.0009 | 0.019±0.009 |
| 15+30 | | 0.12±0.06 | 0.185±0.004 | | 0.007±0.004 | 0.012±0.001 |
| 15+50 | | 0.08±0.003 | 0.129±0.003 | | 0.005±0.0001 | 0.007±0.0007 |
| | | | | | | |
| 30+5 | | 0.09±0.008 | 0.128±0.01 | | 0.006±0.0005 | 0.007±0.001 |
| 30+15 | | 0.11±0.036 | 0.161±0.03 | | 0.007±0.0006 | 0.009±0.002 |
| 30+30 | | 0.11±0.08 | 0.150±0.03 | | 0.006±0.0005 | 0.007±0.001 |
| 30+50 | | - | - | | - | - |
| | | | | | | |
| 50+5 | | 0.08±0.002 | - | | 0.005±0.0003 | - |
| 50+15 | | - | - | | - | - |
| 50+30 | | - | - | | - | - |
| 50+50 | | - | - | | - | - |

± S.D.

- no response

Table 4.6: Effect of growth regulators on the growth and development of PLBs of *D. formosum* cultured in MS medium supplemented with BAP and NAA

| Growth regulator (μM) | Fresh weight (g) | | | Dry weight (g) | | |
|------------------------------------|-------------------|-------------------|------------------|---------------------|-------------------|-------------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | | 0.151 \pm 0.01 | 0.21 \pm 0.01 | | 0.012 \pm 0.001 | 0.018 \pm 0.001 |
| BAP+NAA | | | | | | |
| 5+5 | | 0.751 \pm 0.16 | 1.21 \pm 0.31 | | 0.068 \pm 0.001 | 0.106 \pm 0.014 |
| 5+15 | | 0.681 \pm 0.07 | 1.12 \pm 0.12 | | 0.063 \pm 0.001 | 0.095 \pm 0.01 |
| 5+30 | | 0.709 \pm 0.001 | 1.18 \pm 0.06 | | 0.066 \pm 0.003 | 0.092 \pm 0.004 |
| 5+50 | | 0.501 \pm 0.04 | 0.79 \pm 0.07 | | 0.046 \pm 0.002 | 0.076 \pm 0.006 |
| | 0.027 \pm 0.005 | | | 0.0021 \pm 0.0003 | | |
| 15+5 | | 0.720 \pm 0.02 | 1.09 \pm 0.13 | | 0.068 \pm 0.001 | 0.087 \pm 0.004 |
| 15+15 | | 0.668 \pm 0.02 | 1.22 \pm 0.016 | | 0.065 \pm 0.001 | 0.092 \pm 0.007 |
| 15+30 | | 0.637 \pm 0.007 | 0.94 \pm 0.06 | | 0.066 \pm 0.005 | 0.081 \pm 0.002 |
| 15+50 | | 0.537 \pm 0.03 | 0.79 \pm 0.03 | | 0.048 \pm 0.002 | 0.068 \pm 0.007 |
| | | | | | | |
| 30+5 | | 0.650 \pm 0.02 | 0.99 \pm 0.01 | | 0.061 \pm 0.003 | 0.087 \pm 0.006 |
| 30+15 | | 0.380 \pm 0.04 | 0.71 \pm 0.03 | | 0.036 \pm 0.002 | 0.061 \pm 0.004 |
| 30+30 | | 0.355 \pm 0.06 | 0.63 \pm 0.02 | | 0.036 \pm 0.002 | 0.056 \pm 0.002 |
| 30+50 | | 0.205 \pm 0.01 | 0.34 \pm 0.04 | | 0.016 \pm 0.002 | 0.031 \pm 0.002 |
| | | | | | | |
| 50+5 | | 0.355 \pm 0.08 | 0.40 \pm 0.12 | | 0.033 \pm 0.002 | 0.031 \pm 0.005 |
| 50+15 | | 0.205 \pm 0.01 | 0.31 \pm 0.01 | | 0.018 \pm 0.001 | 0.025 \pm 0.003 |
| 50+30 | | 0.161 \pm 0.03 | 0.20 \pm 0.005 | | 0.015 \pm 0.001 | 0.018 \pm 0.001 |
| 50+50 | | - | - | | - | - |

\pm S.D.

- no response

Similarly, inhibitory effect of NAA in combination with BAP was also observed. The maximum proliferation in term of fresh and dry weight after 30 and 60 days was observed at 5 μ M each of BAP and NAA in the medium (Table 4.5). Complete plantlets were obtained after 150 days of culture (Plate 4.2b).

As compared to the control, increased PLB proliferation was observed in case of *D. formosum* when BAP and NAA were added in combination in the medium, which showed growth regulator specificity as compared to the results observed in *D. longicornu*. An increase in fresh and dry weights after 30 and 60 days was observed in all the combinations tried, with the maximum being recorded at a concentration of 5 μ M of BAP when added to 5 μ M of NAA in the medium (Table 4.6). Complete plantlets were obtained after 150 days of culture (Plate 4.3b).

Effect of KN+IAA

KN in combination with IAA in the medium brought about a differential response of development of PLBs of *D. longicornu*. An increase in fresh and dry weights of the cultured PLBs was observed after 30 and 60 days in the medium supplemented with either 30 μ M or less than 30 μ M of KN or IAA. A higher concentration of 30 μ M of either KN or IAA in the medium was inhibitory for the PLBs of *D. longicornu*. The maximum fresh and dry weights of the PLBs were observed in the medium containing 5 μ M each of KN and IAA in combination after 30 and 60 days of culture (Table 4.7). Complete plantlets were obtained after 150 days of culture (Plate 4.2c).

Table 4.7: Effect of growth regulators on the growth and development of PLBs of *D. longicornu* cultured in MS medium supplemented with KN and IAA

| Growth regulator (μM) | Fresh weight (g) | | | Dry weight (g) | | |
|------------------------------------|------------------|-----------------|-------------------|--------------------|-------------------|--------------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | | 0.35 \pm 0.01 | 0.610 \pm 0.03 | | 0.019 \pm 0.02 | 0.040 \pm 0.004 |
| KN+IAA | | | | | | |
| 5+5 | | 0.96 \pm 0.11 | 1.670 \pm 0.42 | | 0.064 \pm 0.004 | 0.098 \pm 0.027 |
| 5+15 | | 0.52 \pm 0.15 | 0.909 \pm 0.08 | | 0.034 \pm 0.002 | 0.0501 \pm 0.006 |
| 5+30 | | 0.42 \pm 0.17 | 0.682 \pm 0.29 | | 0.027 \pm 0.001 | 0.0404 \pm 0.018 |
| 5+50 | | - | - | | - | - |
| | 0.036 \pm 0.01 | | | 0.002 \pm 0.0006 | | |
| 15+5 | | 0.79 \pm 0.18 | 1.105 \pm 0.005 | | 0.052 \pm 0.005 | 0.0725 \pm 0.008 |
| 15+15 | | 0.36 \pm 0.02 | 0.583 \pm 0.05 | | 0.023 \pm 0.001 | 0.0396 \pm 0.003 |
| 15+30 | | 0.20 \pm 0.12 | 0.285 \pm 0.12 | | 0.013 \pm 0.001 | 0.0171 \pm 0.007 |
| 15+50 | | - | - | | - | - |
| | | | | | | |
| 30+5 | | 0.57 \pm 0.11 | 1.047 \pm 0.10 | | 0.037 \pm 0.001 | 0.059 \pm 0.008 |
| 30+15 | | 0.47 \pm 0.24 | 0.602 \pm 0.25 | | 0.029 \pm 0.002 | 0.040 \pm 0.002 |
| 30+30 | | - | - | | - | - |
| 30+50 | | - | - | | - | - |
| | | | | | | |
| 50+5 | | - | - | | - | - |
| 50+15 | | - | - | | - | - |
| 50+30 | | - | - | | - | - |
| 50+50 | | - | - | | - | - |

\pm S.D.

- no response

Table 4.8: Effect of growth regulators on the growth and development of PLBs of *D. formosum* cultured in MS medium supplemented with KN and IAA

| Growth regulator (µM) | Fresh weight (g) | | | Dry weight (g) | | |
|-----------------------|------------------|------------|-------------|----------------|-------------|--------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | 0.027±0.005 | 0.151±0.01 | 0.21±0.01 | 0.0021±0.0003 | 0.012±0.001 | 0.018±0.001 |
| KN+IAA | | | | | | |
| 5+5 | | 0.651±0.09 | 1.02±0.27 | | 0.056±0.005 | 0.091±0.004 |
| 5+15 | | 0.721±0.12 | 1.25±0.10 | | 0.066±0.002 | 0.124±0.01 |
| 5+30 | | 0.373±0.08 | 0.67±0.08 | | 0.035±0.002 | 0.063±0.002 |
| 5+50 | | 0.195±0.02 | 0.29±0.02 | | 0.016±0.001 | 0.027±0.001 |
| | | | | | | |
| 15+5 | | 0.854±0.02 | 1.43±0.07 | | 0.077±0.001 | 0.143±0.002 |
| 15+15 | | 0.886±0.04 | 1.42±0.01 | | 0.082±0.002 | 0.149±0.016 |
| 15+30 | | 0.307±0.04 | 0.53±0.14 | | 0.029±0.001 | 0.046±0.0018 |
| 15+50 | | 0.167±0.04 | 0.22±0.02 | | 0.015±0.001 | 0.019±0.001 |
| | | | | | | |
| 30+5 | | 0.396±0.03 | 0.57±0.07 | | 0.038±0.001 | 0.052±0.001 |
| 30+15 | | 0.365±0.02 | 0.49±0.01 | | 0.035±0.006 | 0.045±0.001 |
| 30+30 | | 0.337±0.03 | 0.47±0.02 | | 0.030±0.001 | 0.042±0.001 |
| 30+50 | | 0.187±0.01 | 0.25±0.01 | | 0.016±0.001 | 0.022±0.007 |
| | | | | | | |
| 50+5 | | 0.271±0.05 | 0.41±0.10 | | 0.026±0.002 | 0.038±0.001 |
| 50+15 | | 0.153±0.03 | 0.20±0.02 | | 0.016±0.001 | 0.017±0.001 |
| 50+30 | | 0.190±0.04 | 0.26±0.01 | | 0.018±0.001 | 0.024±0.001 |
| 50+50 | 0.157±0.03 | 0.24±0.02 | 0.015±0.001 | 0.022±0.001 | | |

± S.D.

- no response

Plate 4.3

Development of complete plantlets from PLBs of *D. formosum* after 150 days of culture on:

- a. MS + 15 μ M BAP + 30 μ M IAA
- b. MS + 5 μ M BAP + 5 μ M NAA
- c. MS + 15 μ M KN + 15 μ M IAA
- d. MS + 15 μ M KN + 5 μ M NAA

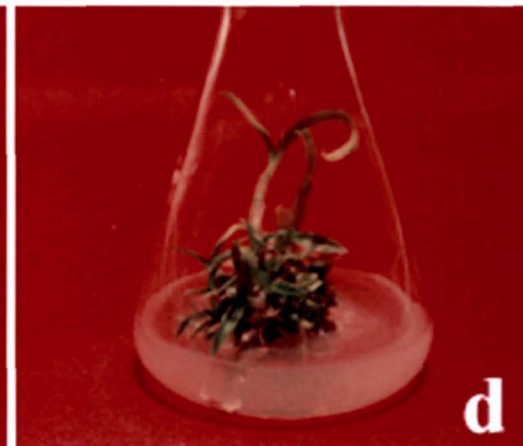
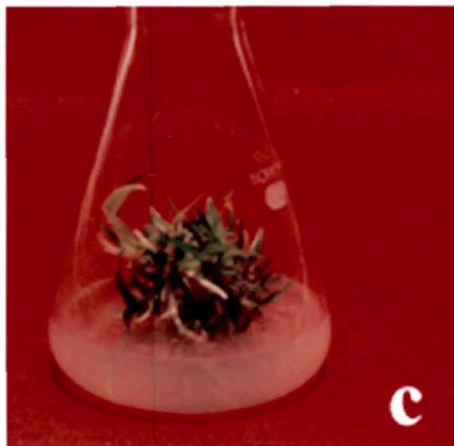
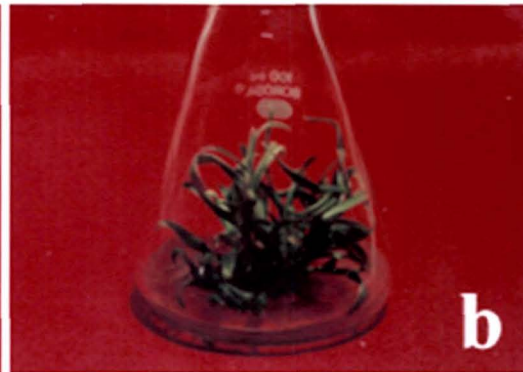
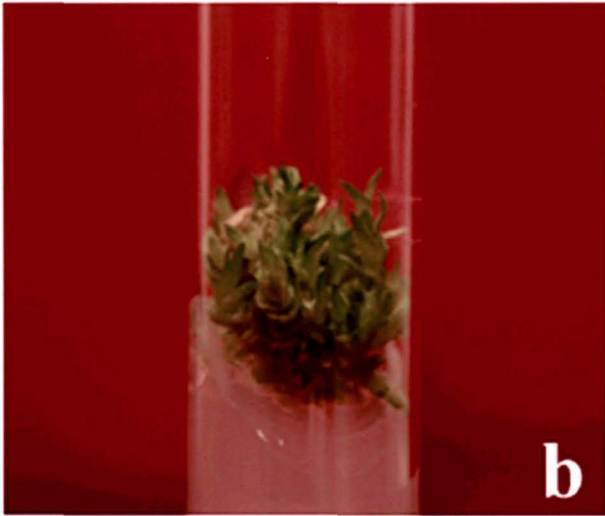
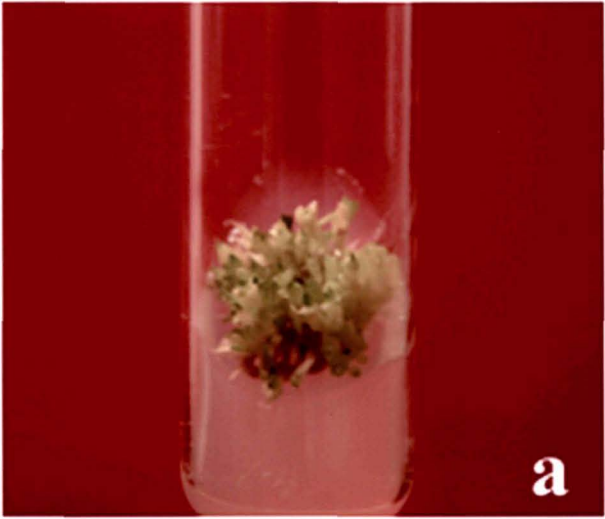


Plate 4.4

Growth and development of PLBs of *D. formosum* in MS + 15 μ M KN + 15 μ M IAA after 30 days of culture (a), and 60 days of culture (b)



In case of PLBs of *D. formosum*, a better response or proliferation of the cultured PLBs was observed in the medium containing KN and IAA in combination as compared to the control (Table 4.8). Increased fresh and dry weights of the PLBs were recorded after 30 and 60 days of culture in all the concentrations of these growth regulators tried, with the maximum being observed at 15 μ M each of KN and IAA in the medium (Table 4.8; Plate 4.4). Complete plantlets were obtained after 150 days of culture (Plate 4.3c). PLBs of *D. formosum* showed a better response to this treatment as compared to the PLBs of *D. longicornu*.

Effect of KN+NAA

In case of *D. longicornu* there was an enhanced proliferation of PLBs in the medium supplemented with KN and NAA in combination (Table 4.9). An increase in both fresh and dry weights was observed when KN or NAA was $\leq 30\mu$ M in the medium. An increase in the concentrations of these growth regulators inhibited PLB proliferation. The maximum fresh and dry weights after 30 and 60 days were observed at 15 μ M of KN and 5 μ M of NAA in the medium (Table 4.9). Complete plantlets were obtained after 150 days of culture (Plate 4.2d).

In the medium containing KN and NAA in combination, the cultured PLBs of *D. formosum* showed better responses or proliferations as compared to the control (Table 4.10). An increase in fresh and dry weights of the cultured PLBs was observed after 30 and 60 days of culture in all the concentrations tried, with the maximum being observed in those treated with the combination of 15 μ M of KN and 5 μ M of NAA (Table

Table 4.9: Effect of growth regulators on the growth and development of PLBs of *D. longicornu* cultured in MS medium supplemented with KN and NAA

| Growth regulator (μM) | Fresh weight (g) | | | Dry weight (g) | | |
|------------------------------------|------------------|------------------|-------------------|--------------------|-------------------|-------------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | | 0.35 \pm 0.01 | 0.610 \pm 0.03 | | 0.019 \pm 0.02 | 0.040 \pm 0.004 |
| KN+NAA | | | | | | |
| 5+5 | | 0.84 \pm 0.17 | 1.392 \pm 0.10 | | 0.055 \pm 0.005 | 0.086 \pm 0.006 |
| 5+15 | | 1.15 \pm 0.08 | 1.927 \pm 0.06 | | 0.086 \pm 0.007 | 0.094 \pm 0.003 |
| 5+30 | | 0.84 \pm 0.005 | 1.316 \pm 0.14 | | 0.055 \pm 0.002 | 0.078 \pm 0.007 |
| 5+50 | | - | - | | - | - |
| | 0.036 \pm 0.01 | | | 0.002 \pm 0.0006 | | |
| 15+5 | | 1.27 \pm 0.18 | 2.054 \pm 0.011 | | 0.080 \pm 0.011 | 0.107 \pm 0.016 |
| 15+15 | | 0.86 \pm 0.15 | 1.599 \pm 0.32 | | 0.062 \pm 0.007 | 0.081 \pm 0.01 |
| 15+30 | | 0.71 \pm 0.004 | 1.570 \pm 0.35 | | 0.046 \pm 0.006 | 0.092 \pm 0.007 |
| 15+50 | | - | - | | - | - |
| | | | | | | |
| 30+5 | | 0.84 \pm 0.08 | 1.519 \pm 0.15 | | 0.058 \pm 0.008 | 0.088 \pm 0.009 |
| 30+15 | | 0.50 \pm 0.05 | 0.884 \pm 0.13 | | 0.031 \pm 0.004 | 0.047 \pm 0.006 |
| 30+30 | | 0.33 \pm 0.05 | 0.605 \pm 0.17 | | 0.020 \pm 0.003 | 0.037 \pm 0.007 |
| 30+50 | | - | - | | - | - |
| | | | | | | |
| 50+5 | | - | - | | - | - |
| 50+15 | | - | - | | - | - |
| 50+30 | | - | - | | - | - |
| 50+50 | | - | - | | - | - |

\pm S.D.

- no response

Table 4.10: Effect of growth regulators on the growth and development of PLBs of *D. formosum* cultured in MS medium supplemented with KN and NAA

| Growth regulator (μM) | Fresh weight (g) | | | Dry weight (g) | | |
|------------------------------------|-------------------|-------------------|------------------|---------------------|--------------------|--------------------|
| | 0 days | 30 days | 60 days | 0 days | 30 days | 60 days |
| Control | | 0.151 \pm 0.01 | 0.21 \pm 0.01 | | 0.012 \pm 0.001 | 0.018 \pm 0.001 |
| KN+NAA | | | | | | |
| 5+5 | | 0.698 \pm 0.02 | 1.13 \pm 0.27 | | 0.066 \pm 0.005 | 0.105 \pm 0.01 |
| 5+15 | | 0.710 \pm 0.06 | 1.10 \pm 0.10 | | 0.066 \pm 0.002 | 0.101 \pm 0.006 |
| 5+30 | | 0.624 \pm 0.02 | 0.98 \pm 0.01 | | 0.057 \pm 0.002 | 0.095 \pm 0.003 |
| 5+50 | | 0.535 \pm 0.004 | 0.92 \pm 0.016 | | 0.052 \pm 0.001 | 0.086 \pm 0.005 |
| | 0.027 \pm 0.005 | | | 0.0021 \pm 0.0003 | | |
| 15+5 | | 0.869 \pm 0.07 | 1.39 \pm 0.03 | | 0.074 \pm 0.006 | 0.133 \pm 0.013 |
| 15+15 | | 0.775 \pm 0.08 | 1.23 \pm 0.07 | | 0.068 \pm 0.002 | 0.127 \pm 0.015 |
| 15+30 | | 0.606 \pm 0.03 | 1.01 \pm 0.24 | | 0.058 \pm 0.004 | 0.097 \pm 0.001 |
| 15+50 | | 0.456 \pm 0.03 | 0.78 \pm 0.06 | | 0.045 \pm 0.005 | 0.074 \pm 0.0018 |
| | | | | | | |
| 30+5 | | 0.690 \pm 0.028 | 0.95 \pm 0.02 | | 0.065 \pm 0.004 | 0.093 \pm 0.0018 |
| 30+15 | | 0.796 \pm 0.02 | 1.10 \pm 0.17 | | 0.069 \pm 0.0022 | 0.118 \pm 0.005 |
| 30+30 | | 0.585 \pm 0.07 | 0.93 \pm 0.06 | | 0.054 \pm 0.004 | 0.075 \pm 0.003 |
| 30+50 | | 0.272 \pm 0.019 | 0.41 \pm 0.03 | | 0.023 \pm 0.001 | 0.032 \pm 0.006 |
| | | | | | | |
| 50+5 | | 0.295 \pm 0.018 | 0.47 \pm 0.07 | | 0.022 \pm 0.001 | 0.041 \pm 0.001 |
| 50+15 | | 0.259 \pm 0.06 | 0.470 \pm 0.08 | | 0.022 \pm 0.002 | 0.038 \pm 0.001 |
| 50+30 | | 0.267 \pm 0.07 | 0.42 \pm 0.005 | | 0.023 \pm 0.006 | 0.042 \pm 0.001 |
| 50+50 | | 0.263 \pm 0.06 | 0.50 \pm 0.11 | | 0.023 \pm 0.002 | 0.045 \pm 0.001 |

\pm S.D.

- no response

4.10). Complete plantlets are obtained after 150 days of culture (Plate 4.3d). In this case too, PLBs of *D. formosum* responded better as compared to the PLBs of *D. longicornu*.

DISCUSSION

The results obtained by treating the PLBs with different growth regulators were found to be species specific. Following the transfer of the PLBs into plant growth regulator containing medium vigorous proliferation of the PLBs was exhibited. There was a significant difference between the number of PLBs produced in the medium containing growth regulators and the one devoid of growth regulators. In the present study, promotory effects of both NAA and IAA on PLB development were observed at lower concentrations in both the species of dendrobies studied. Kanjilal *et al.* (1999) observed the promotory effect of IAA in PLB production in *Dendrobium moschatum*, while NAA have little or no effect. However, Kusumoto (1979) found NAA to stimulate PLB proliferation. Hayes (1969), also, reported an increased differentiation of the protocorms in case of *Odontoglossum grande*, *O. schlieperianum* and *Miltonia spectabilis* in the medium containing IAA. Pages (1971) observed a growth enhancement in *Dendrobium* protocorms in response to IBA. On the other hand, a reduced percentage of plantlet development following IAA and NAA treatments was indicated in *Dendrobium* (Miyazaki and Nagamatsu, 1965). In concurrence with the present results with 2,4-D, inhibitory effect has also been elucidated by Goh (1971) in *Vanda* cv. Miss Joaquim. Also, Kusumoto (1978) had reported development of abnormal PLBs in *Cymbidium* subjected to different treatments of 2,4-D. Moreover, growth and

development in *Galeola septentrionalis* (Nakamura, 1982), *Cymbidium reginae* (Harvais, 1982) and *C. elegans* (Sharma and Tandon, 1986) have also been influenced negatively by 2,4-D. Inconsistent reports regarding auxin effects indicate that requirements of plants vary from species to species. Differential responses of the PLBs to cytokinins are well documented. In the present study, the cultured PLBs of both *D. longicornu* and *D. formosum* showed enhanced PLB production as well as proliferation in both BAP and KN containing medium. There are reports of BAP influencing the formation and proliferation of PLBs of *Cattleya aurantiaca* at low and high concentrations respectively (Pierik and Steegmans, 1972). However, following similar treatments retarded protocorm development was observed by Rucker (1974). Hadley and Harvais (1968) have reported a pronounced effect of kinetin on growth and development of *O. purpurella*. A stimulatory effect of the same has also been reported by Harvais (1982) and Nakamura (1982). Increased number of protocorms and shoots were indicated by Hadley (1970) and Uesato (1978) in *Platanthera bifolia* and *Phalenopsis* respectively on being treated with KN. In the present study, although BAP and KN had pronounced effects on PLB proliferation, the higher concentrations proved to be inhibitory resulting in lower proliferation rate. Teratogenic and toxic effects of KN at high concentrations have also been suggested by Rucker (1974).

The combination of auxins and cytokinins in the medium also had a pronounced effect on the growth and development of PLBs of both the species studied. The combination of auxin (IAA/NAA) with BAP resulted in differential responses in both the species. IAA/NAA with BAP in case of *D. longicornu* and *D. formosum* showed a better

and enhanced effect on PLB development. Similar report was observed in PLB development of *Zygopetalum intermedium* (Nagaraju and Mani, 2005). Kanjilal *et al.* (1999) also reported the sharp increase in PLB production in medium containing BAP and NAA in combination in the orchid *Dendrobium moschatum*. Roy and Banerjee (2003) observed high frequency of callusing at low ratios of NAA: BAP in the medium in *Dendrobium fimbriatum* var. *oculatum*. The supplementations of KN with IAA/NAA also had an influencing effect on both the species studied. In *D. longicornu*, a lower concentration of KN and IAA or KN and NAA in combinations showed rapid proliferation and development of PLBs, while higher concentrations showed inhibitory effects on the development of PLBs. In case of PLBs of *D. formosum*, a stimulatory effect of KN and IAA or KN and NAA in combination in the medium was observed. Similarly, Prasad and Mitra (1975) noted a high percentage of good growth of PLBs in case of *Cymbidium mastersii*. Also effects of NAA and KN on the proliferation of PLBs have been reported (Vij and Pathak, 1990; Vij and Kaur, 1992).

Thus, as observed from the present study, the dendrobes studied require supplementary cytokinins along with auxins for optimum response. This requirement of exogenous cytokinin could be related to the maintenance of a proper balance between auxins and cytokinin, which act synergistically to regulate cell division (Johri and Mitra, 2001). Studies have shown that incorporation of the growth regulators in the medium show pronounced effect on the growth at different stages of protocorm development *in vitro* and that growth regulators in the medium may act similar to the endophytic fungi, which bring about physiological changes in the developing protocorms in nature

(Kumaria *et al.*, 1990; Kumaria and Tandon, 2000, 2004). Hence, from the perspective of propagation, this continuous proliferation of PLBs along with regeneration shows great potential as an attractive method for large scale production of plantlets.

CHAPTER V: CRYOPRESERVATION OF *DENDROBIUM LONGICORNU* AND *DENDROBIUM FORMOSUM* THROUGH VITRIFICATION AND ENCAPSULATION-DEHYDRATION METHODS

INTRODUCTION

Germplasm preservation which is essential for the maintenance of biodiversity and the avoidance of genetic erosion is a costly activity for plant propagation enterprises. The preservation of germplasm is important for the economy and ecology of human life. But conservation of important germplasm in field gene banks in the form of orchards, plantations etc., will face serious problems in the near future due to the space availability and the high cost of maintenance. Moreover, there are high risks of diseases and damages by extreme weather conditions, fire and animals.

As most of the orchids are in danger of extinction due to extensive collection and disturbance of their natural habitats, it is important to have techniques to ensure their preservation. One of the methods of *ex situ* conservation of various plant species is the storage of germplasm under low temperatures and dry conditions. This method of

conservation is considered to be one of the most useful and easy ways for preserving genetic resources (Roos and Davidson, 1992).

Cryopreservation of plant cells, meristems and organs has become an important tool for the long-term preservation (usually in liquid nitrogen at -196°C) of germplasm and experimental material without genetic alteration (Kantha, 1985; Withers, 1985a). Through cryopreservation one can preserve or prevent variations not only in angiosperms (Kantha, 1987; Withers, 1985b) but also in gymnosperms (Kantha *et al.*, 1988). In the past, mostly vegetatively propagated plants were cryopreserved. But with rapid progress in plant transformations, 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 like cells, protoplasts, somatic and zygotic embryos, anthers, pollens and whole seeds (Kantha, 1985; Withers, 1985a, b), shoot and embryo cultures are found to be more relevant to genetic conservation. Successful cryopreservation has been reported from embryonic axes of some truly recalcitrant seeds e.g., *Hevea brasiliensis* (Normah *et al.*, 1986) and also from dissected embryos of seeds difficult to conserve like *Elaeis guineensis* (Grout *et al.*, 1983), *Veitchia* and *Howea* (Chin *et al.*, 1988). In these procedures, the technique involves desiccation of the explant to about 10-16% moisture level followed by immersion in liquid nitrogen. In most cases the zygotic embryos are the primary tissue in the transformations studied (Chibbar *et al.*, 1991; Vasil *et al.*, 1992). Cryopreservation of embryos in liquid nitrogen has been suggested as the most promising

method for storage in many woody species. The zygotic embryos and embryonic axes of tea and cocoa were successfully cryopreserved by Chandel *et al.* (1993). The embryonic axes of *Poncirus trifoliata* had been reported to have been preserved for one year by Radhamani and Chandel (1992). Different responses of embryonic axes and cotyledons of tea seeds to desiccation and cryoexposure have been also reported (Kuranuki and Yoshida, 1996). In most cases, desiccation of embryos is carried out by air flow in laminar flow cabinet which is followed by immersion in liquid nitrogen (Grout *et al.*, 1983; Normah *et al.*, 1986; Chin *et al.*, 1988; Abdelnour *et al.*, 1992).

Though shoot cultures have received much attention as compared to other cells and organs, they have proved to be far more variable in response and generally had been more difficult to cryopreserve (Kantha *et al.*, 1982a, b; Withers, 1987a, b). Over the years a thread of continuity and logic is seen in efforts to develop cryopreservation procedures for cell cultures and other organs. The shoot tips dissected from established plants or seedlings are more difficult to handle than those grown *in vitro*. But these are often chosen for cryopreservation as they are genetically stable intrinsically. The first report of successful cryopreservation of shoot tips was made by Seibert in the year 1976. Grout and Henshaw (1978) reported successful regrowth of shoot tips of *Solanum goniocalyx* after cryoprotection with 10% DMSO in liquid nitrogen. The shoot tips of asparagus (Kumu *et al.*, 1983), brussels sprouts (Harada *et al.*, 1985) and cold hardened woody species (Moriguchi *et al.*, 1985) were cryopreserved using 5 to 15% DMSO as cryoprotectant. Success of shoot tip cryopreservation of *in vitro* grown plantlets of potato (Towill, 1984) and apple (Kuo and Lineberger, 1985) had been demonstrated. Shoot tips

from *in vitro* grown plantlets of *Chamomilla recutita* (Diettrich *et al.*, 1990), root fragments of Chicory (Demeulemeester *et al.*, 1993), shoot primordia of *Vanda pumila* (Na and Kondo, 1996), shoot tips of Cassava (Escobar *et al.*, 1997) have also been cryopreserved.

Though numerous methods are available and used in cryopreservation studies, some new methods have been developed recently which are cheaper and easier to perform; these are encapsulation-dehydration and vitrification. Encapsulation-dehydration is a method based on successive osmotic and evaporative dehydration of the tissues (Dereuddre *et al.*, 1990). Here the material which is normally a shoot tip/somatic or zygotic embryo is encapsulated in sodium alginate beads. These encapsulated beads can be precultured in high osmoticum mainly in high sucrose solution and then air dried and plunged in liquid nitrogen. Encapsulation-dehydration procedure has been successfully applied to many temperate and tropical species (Dereuddre *et al.*, 1990). In orchids, encapsulation-dehydration methods have been successfully standardized for shoot tips of *Dendrobium Walter Oumae* (Lurswijidjarus and Thammasiri, 2004), seeds of *Dactylorhiza fuchsia* and *Anacamptis morio* (Wood *et al.*, 2000), *Vanda coerulea* (Jitsopakul and Thammasiri, 2005), seeds and protocorms of *Oncidium bifolium* (Flachsland *et al.*, 2006). On the other hand, vitrification enables cells and meristems to cool to the temperature of liquid nitrogen without ice formation. It is a physical process by which a highly concentrated solution is supercooled to very low temperature and finally solidifies into metastable glass without crystallisation. In this method, dehydration is induced by crystallisation of the external medium during the slow cooling steps.

Successful vitrification becomes difficult in non-hardy and less hardy cultured plant cells and organs without partial freeze dehydration in the presence of cryoprotectant prior to ultra rapid cooling (Sakai, 1986). The cultured cells and somatic embryos of asparagus were first cryopreserved by vitrification (Uragami *et al.*, 1989), but the survival rate was not satisfactory. To improve the survival and to develop simpler techniques, many vitrification solutions have been developed over a period of time (Sakai *et al.*, 1990; Towill, 1990; Matsumoto *et al.*, 1995). Good results were obtained with shoot tips of sweet potato when the vitrification solution (PVS2) was diluted and used at low concentration (Towill and Jarett, 1992). The nucellar cells of navel orange (*Citrus sinensi*) have been successfully cryopreserved for 40 days by vitrification (Sakai *et al.*, 1991). Vitrification have been successfully tried on zygotic embryo of *Bletilla striata* (Ishikawa *et al.*, 1997), seeds of *Bletilla striata* (Hirano *et al.*, 2005), *Doritis pulcherrima* (Thammasiri, 2000), *Ponerorchis graminifolia* (Hirano *et al.*, 2005), *Dendrobium cruentum* (Kagawa and Thammasiri, 2005), shoot primordia and shoot apices in *Vanda pumila* (Na and Kondo, 1996) and suspension culture cells of *Doritaenopsis* (Tsukazaki *et al.*, 2000).

MATERIALS AND METHODS

Experimental Material

The seeds from the unopened capsules (8 months after self pollination) of *D. longicornu* and *D. formosum* were used for cryopreservation through vitrification and

encapsulation-dehydration methods. The procedure of obtaining seeds from the capsules has been described in Chapter II.

Vitrification Method

A vitrification solution PVS2 (Sakai *et al.*, 1990) comprising 30% (w/v) glycerol, 15% (w/v) ethylene glycol (PEG), 15% (w/v) DMSO and 0.4M sucrose was prepared in basal MS medium. Approximately 2.0 mg of seeds were put in a 2.0 ml cryotubes. Then 0.5 ml PVS2 solution was added into the cryotubes which were then exposed for different periods of time ranging from 0 - 60 min at 25±2°C and then rapidly plunged in liquid nitrogen. The seeds were stored in liquid nitrogen for 1h, 24h and 7 days before thawing. The vitrified seeds in cryotubes were thawed immediately at varying temperatures (30-50°C) for 2 min. Soon after the ice melted the tubes were taken to laminar flow after being surface sterilized. The vitrified seeds were washed using 0.5 ml of 1.2M sterilized sucrose solution for 5 min. While adding the solution, the tubes were shaken slowly and the washing solution was decanted. The process was repeated thrice. The seeds together with the medium were taken out on a petri dish and then blot dried on filter paper before being cultured on MS basal medium.

The first sign of viability could be observed with the *in vitro* germination of the seeds and the survival was assessed by counting the number of seeds germinated after 2 months of culture.

Encapsulation-Dehydration Method

Approximately 2.0 mg of seeds were encapsulated in 3% Na-alginate matrix containing 3% sucrose under aseptic conditions. The encapsulated beads (3-5 mm diam.) were precultured for 48h in MS liquid medium supplemented with 0.3, 0.5, 0.7M sucrose. The precultured beads were then taken into the sterile laminar flow and gradually dehydrated over a period of time (0 - 8h). These dehydrated beads were cultured on regrowth medium to observe the germination in response to moisture content. Dehydrated beads around 10 in number were placed in each 2.0 ml cryotube and then directly plunged into liquid nitrogen. After 1h, the cryotubes were taken out and rapidly thawed at $40\pm 2^{\circ}\text{C}$ for 2 min. These cryopreserved beads were washed using 1.5ml of sucrose solution (1.2M) into the tube for 5min. While adding the solution the tubes were shaken slowly and the washing solution was decanted. The process was repeated thrice. The beads together with the medium were taken out on a petri dish and then blotted dried on filter paper before being cultured on MS liquid medium.

The moisture content of the beads was determined by drying in the oven at 105°C for 24h. The water content was expressed on fresh weight (FW) basis. After two months of culture, the germination rate was calculated on the basis of the emergence of the protocorms from the encapsulated beads.

RESULTS

Vitrification of Seeds

Effect of exposure time

The best exposure time of seeds of *D. longicornu* and *D. formosum* to vitrification solution PVS2 was found to be 10 min (Fig. 5.1) and 20 min (Fig. 5.2) resulting in maximum of 39.92% and 41.77% germination respectively. As the exposure time increased the germination rate decreased in both control as well as vitrified samples in both the orchid species.

Effect of volume of vitrification solution

Using 0.5 ml of vitrification solution a maximum of 40.02% of seed germination in *D. longicornu* (Fig. 5.3) and 42.73% germination in *D. formosum* (Fig. 5.4) were recorded after vitrification. The volume of the solution lower or higher than 0.5ml showed a slight decline in germination percentage of both the species studied.

Effect of thawing temperature

A thawing temperature of 40°C resulted in optimum germination in both the species studied. In *D. longicornu* a maximum of 39.73% germination was recorded when the seeds were thawed at 40°C (Fig. 5.5), while a maximum of 39.86% germination was recorded in case of *D. formosum* when thawed at 40°C (Fig. 5.6). A decrease in germination percentage was observed in both the species when thawed at temperatures lower or higher than 40°C.

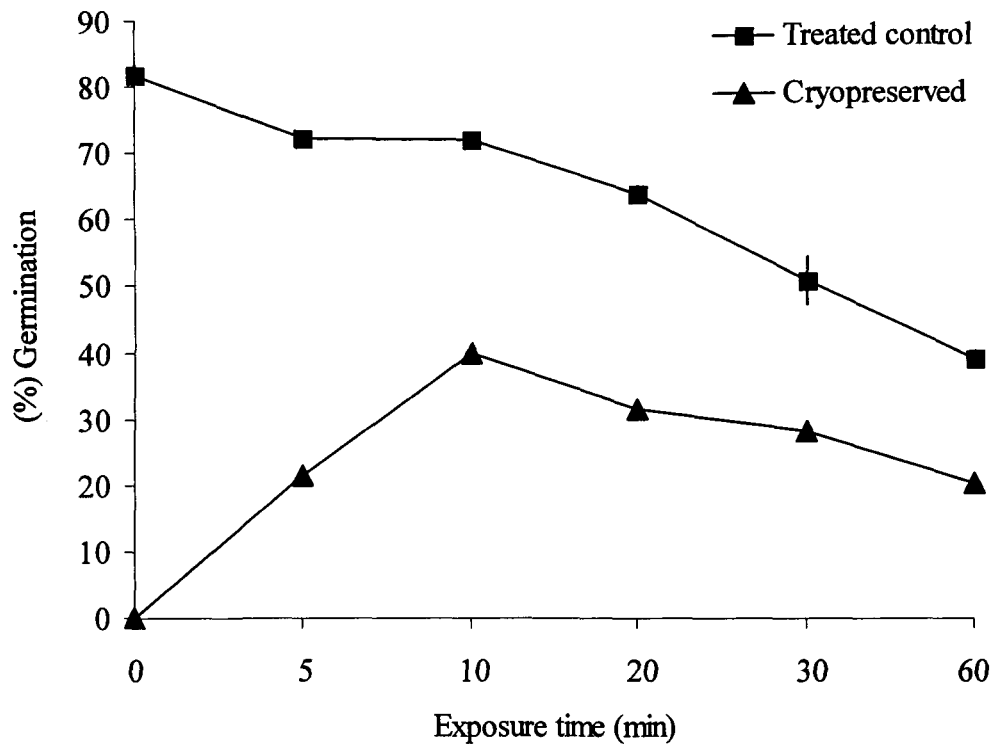


Fig. 5.1: Effect of different exposure time to vitrification solution on the % germination of seeds of *D. longicornu*

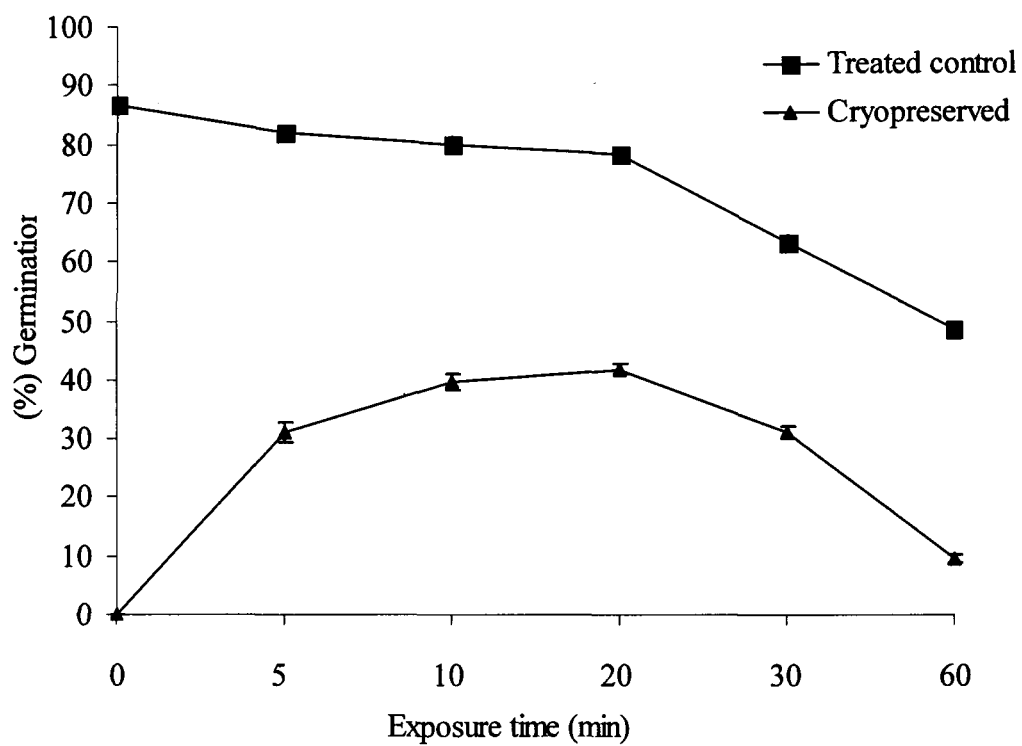


Fig. 5.2: Effect of different exposure time to vitrification solution on the % germination of seeds of *D. formosum*

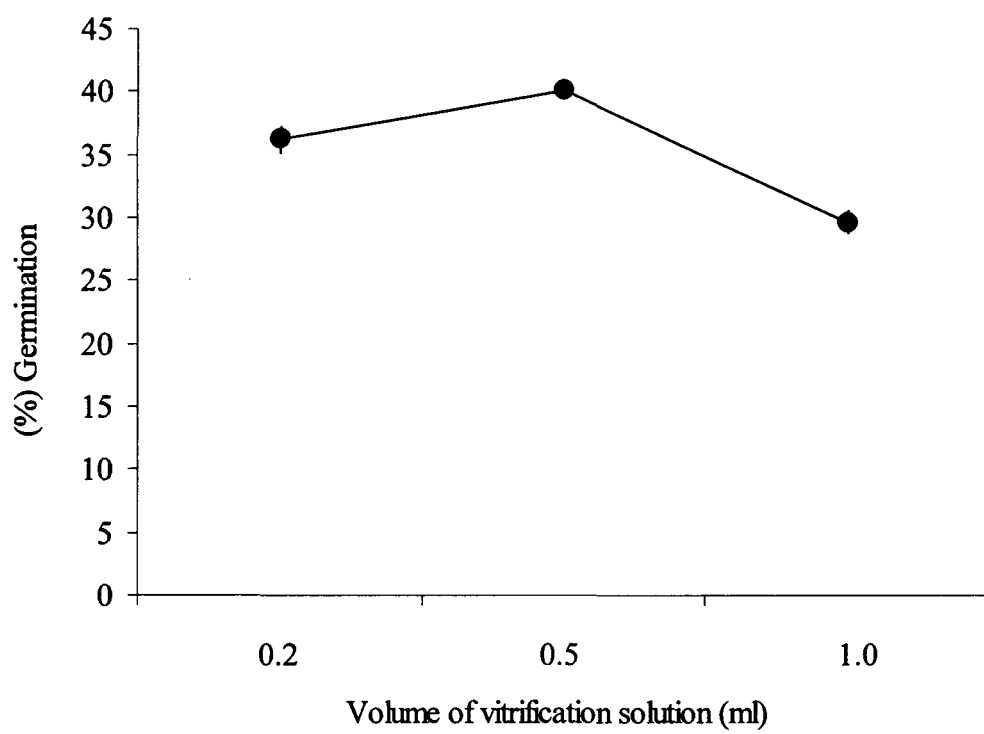


Fig. 5.3: Effect of volume of vitrification solution on % germination of cryopreserved seeds of *D. longicornu*

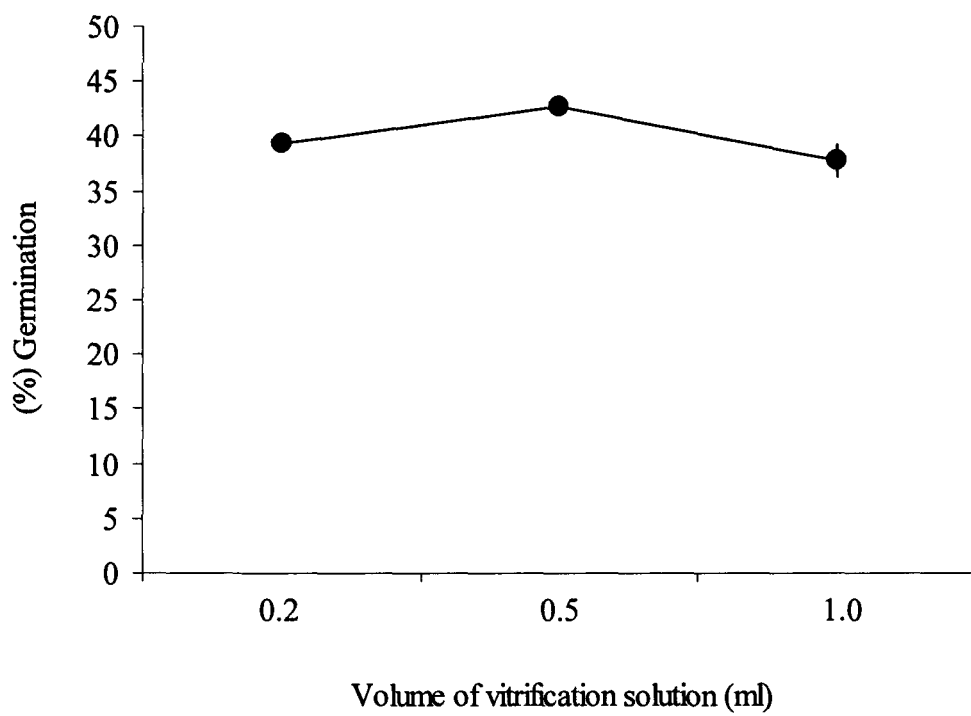


Fig. 5.4: Effect of volume of vitrification solution on % germination of cryopreserved seeds of *D. formosum*

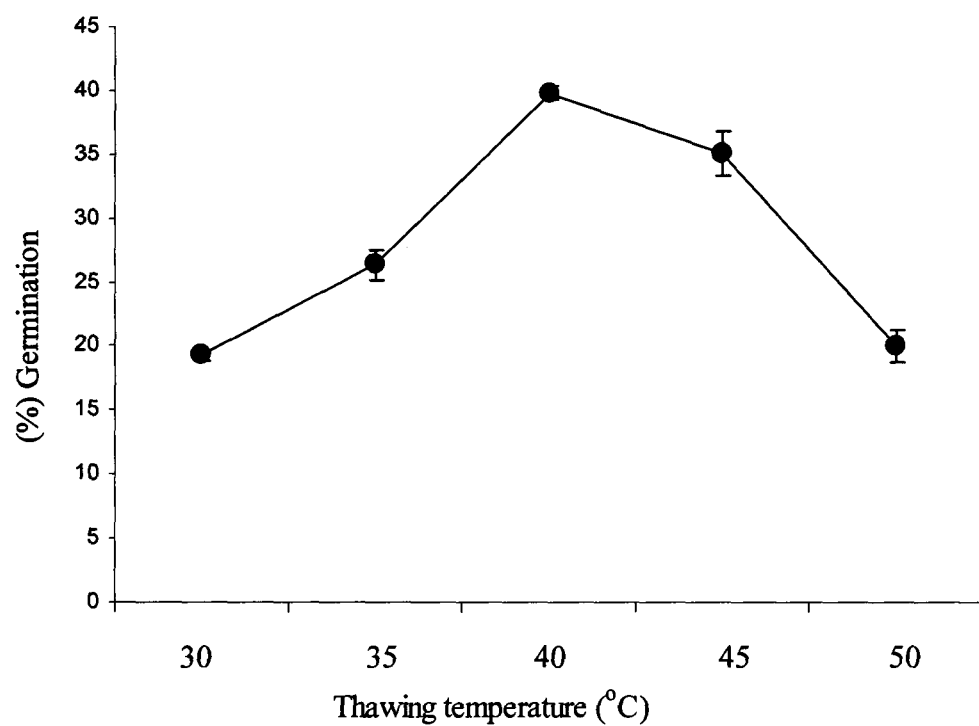


Fig. 5.5: Effect of thawing temperature on % germination of cryopreserved seeds of *D. longicornu*

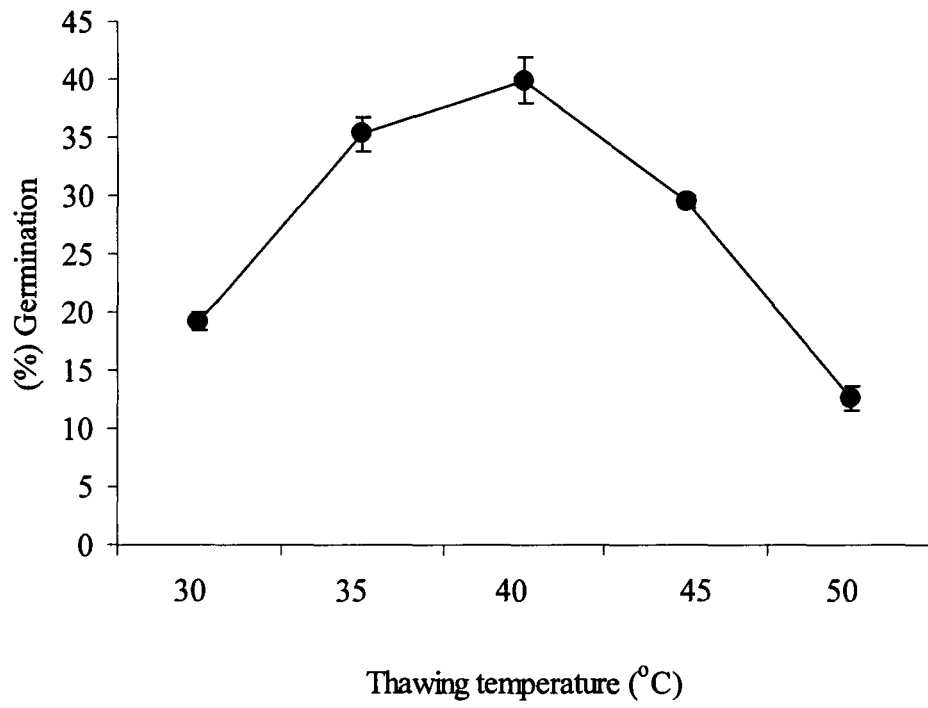


Fig. 5.6: Effect of thawing temperature on % germination of cryopreserved seeds of *D. formosum*

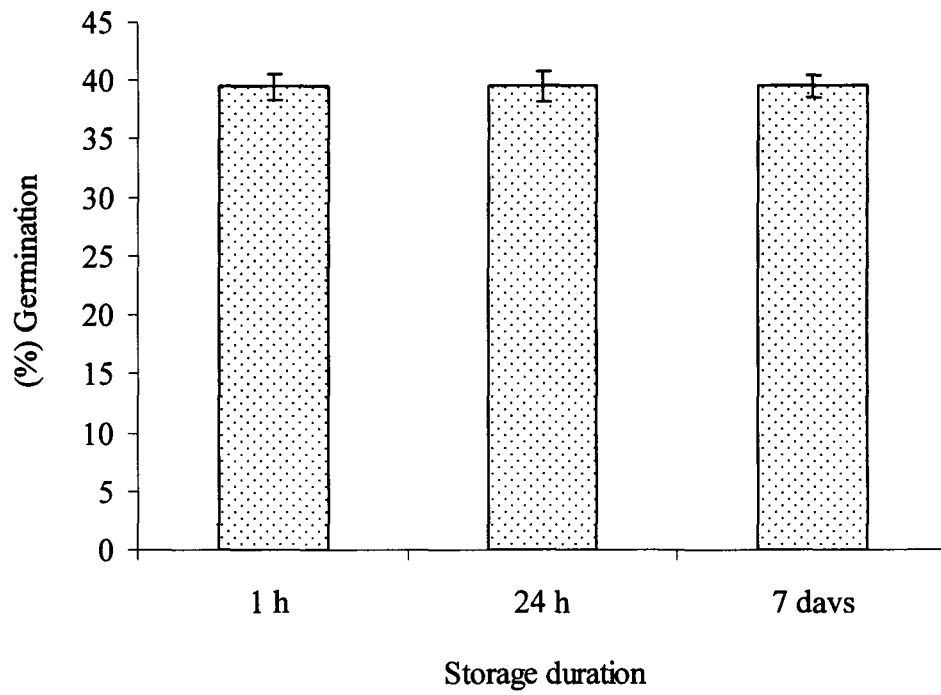


Fig. 5.7: Effect of different duration of storage in LN on % germination of seeds of *D. longicornu*

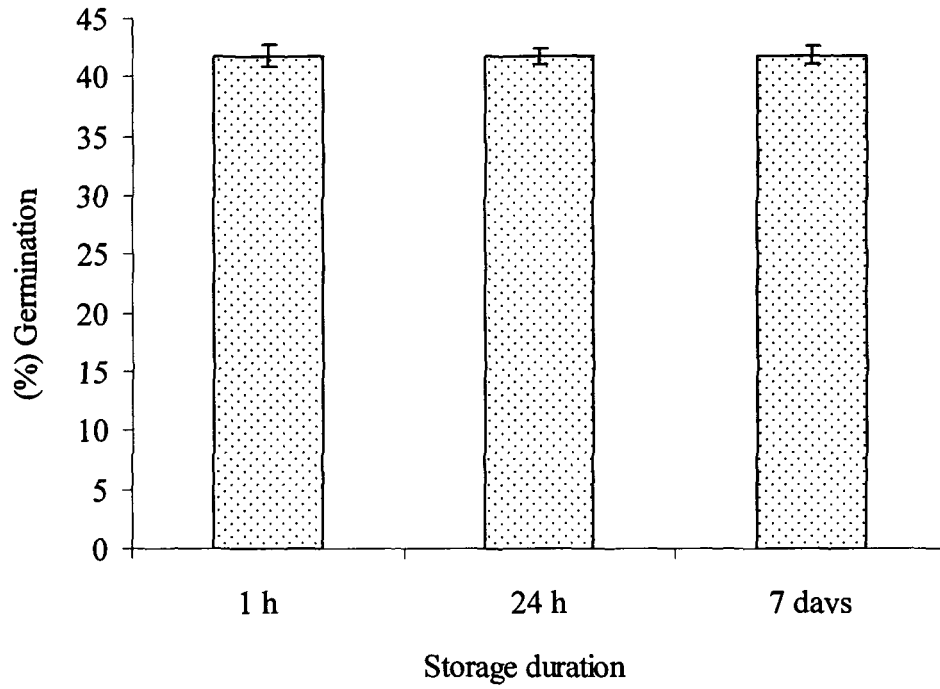


Fig. 5.8: Effect of different duration of storage in LN on % germination of seeds of *D. formosum*

Plate 5.1

Development of seedlings of *D. longicornu* from cryopreserved seeds through vitrification:

- a, b. Germinated seeds (protocorms) after 45 days (bar = 1 mm)
- c. Seedlings growth after 60 days
- d. Fully mature seedlings after 120 days

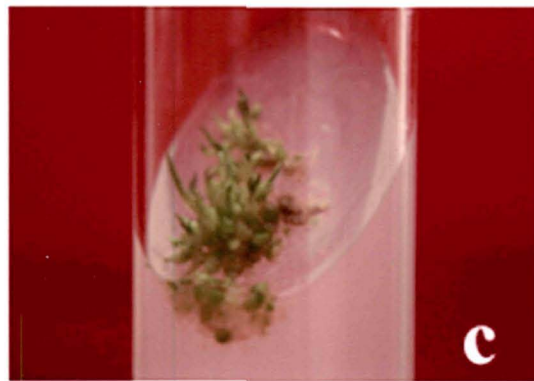
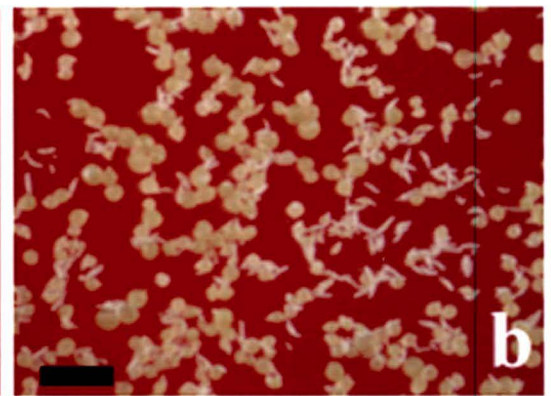
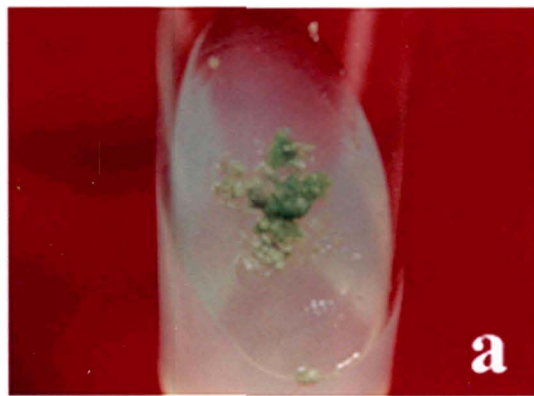


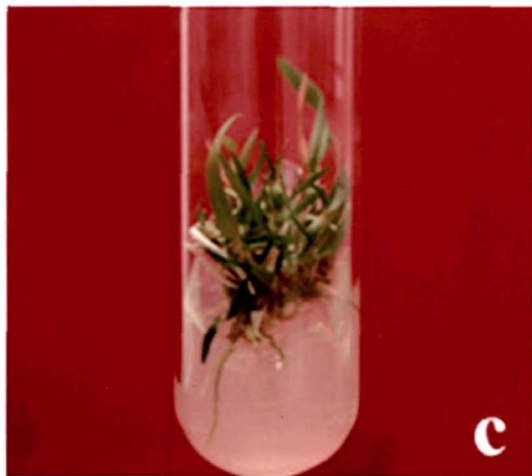
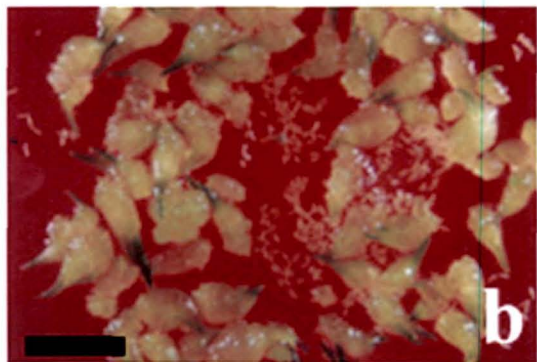
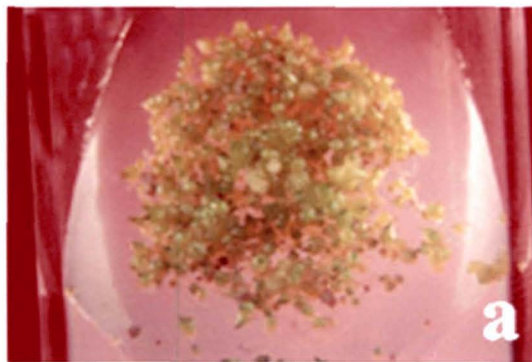
Plate 5.2

Development of seedlings of *D. formosum* from cryopreserved seeds through vitrification

a, b. Germinated seeds (protocorms) after 45 days (bar = 1 mm)

c. Seedlings growth after 60 days

d. Fully mature seedlings after 120 days



Effect of storage in liquid nitrogen

It was found that there was no decline in percent germination of the seeds when stored at 1h, 24h and 7 days in LN and the germination remained steady. After 1h storage, 39.44% of the seeds in case of *D. longicornu* (Fig. 5.7; Plate 5.1) and 40.77% in case of *D. formosum* (Fig. 5.8; Plate 5.2) germinated.

Encapsulation-Dehydration of Seeds

Effect of dehydration time on the water content and seed germination of *D. longicornu*

The water content of all the precultured beads continually decreased with increasing duration of air dehydration. The water content decreased from 92.08% at 0h to 24.62% at 8h in control (3% sucrose), 89.34% at 0h to 23.5% at 8h in beads pretreated with 0.3M sucrose, 79.15% at 0h to 15.5% at 8h in 0.5M sucrose pretreatment, and 72.28% at 0h to 15.3% at 8h in 0.7M sucrose pretreatment respectively (Fig. 5.9).

The germination of the precultured beads also decreased with increased dehydration time. The germination decreased from 87.25% at 0h to 31.27% at 8h in control, 76.1% at 0h to 28.68% at 8h in 0.3M sucrose, 77.08% at 0h to 39.75% at 6h in 0.5M sucrose and 75.16% at 0h to 11.28% at 6h in 0.7M sucrose pretreatment. No germination was observed after 8h dehydration time in case of beads pretreated with 0.5M and 0.7M sucrose (Fig. 5.9).

Effect of dehydration time on the water content and seed germination of *D. formosum*

The water content of all the precultured beads continually decreased with increasing duration of air dehydration. The water content decreased from 81.22% at 0h to 18.29% at 8h in control, 76.32% at 0h to 16.32% at 8h in 0.3M of sucrose pretreatment, 75.82% at 0h to 15.38% at 8h in case of 0.5M sucrose and 72.6% at 0h to 13.78% at 8h in 0.7M sucrose pretreatment (Fig. 5.10).

Similarly, the germination also decreased from 83.08% at 0h to 21.2% at 8h in control, 80.98% at 0h to 19.32% at 8h in 0.3M sucrose pretreatment, 78.55% at 0h to 15.45% at 8h in 0.5M sucrose pretreatment and 76.86% at 0h to 17.51% at 6h in 0.7M sucrose pretreatment. No germination was observed at 8h dehydration time in case of beads treated with 0.7M sucrose (Fig. 5.10).

Effect of liquid nitrogen on germination of dehydrated encapsulated beads

In case of *D. longicornu*, the germination of cryopreserved beads was detected in the beads given the treatment with 0.3M sucrose on being dehydrated for 6h (Plate 5.3a) and 8h, and in the beads treated with 0.5M sucrose dehydrated at 4h and 6h respectively (Fig. 5.11). No germination was observed in the beads treated for other dehydration times. The highest germination of 24.7% was observed in case of beads treated with 0.3M sucrose concentration and dehydrated for 6h with about 27.5% water content (Fig. 5.11). For *D. formosum* the germination of the beads was observed both in 0.3M and

Fig. 5.9: Effect of the duration of dehydration time(0-8 h) of *D. longicornu* on water content and germination of encapsulated seeds precultured on MS liquid medium supplemented with 0.3, 0.5, 0.7 M sucrose for 2 day (-LN)

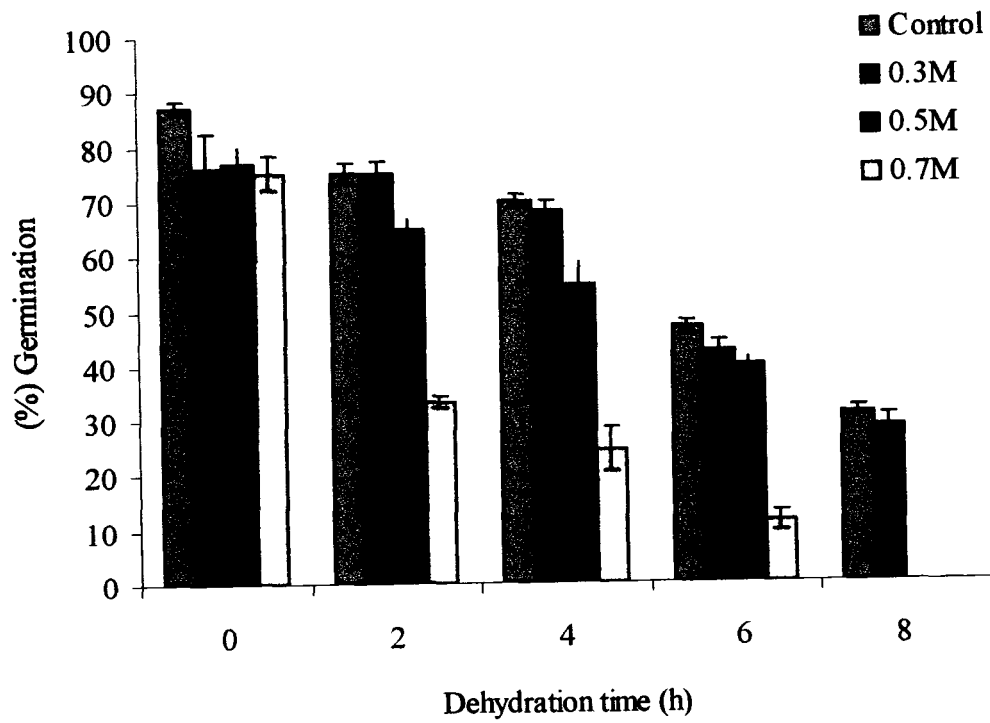
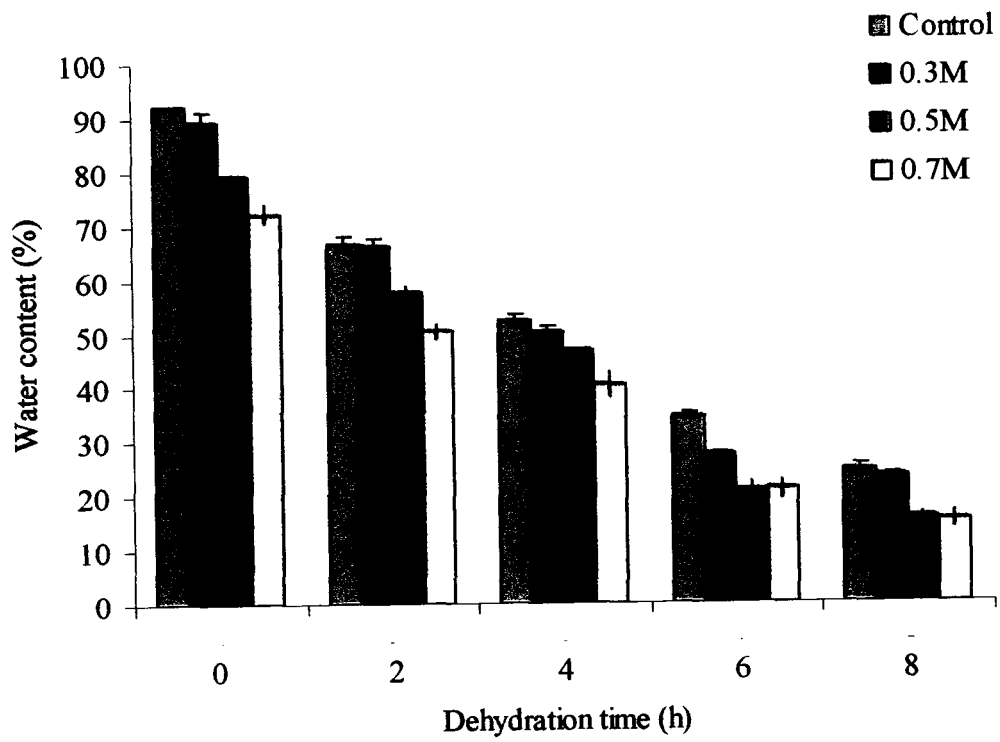


Fig. 5.10: Effect of the duration of dehydration time (0-8 h) of *D. formosum* on water content and germination of encapsulated seeds precultured in MS liquid medium supplemented with 0.3, 0.5, 0.7 M sucrose for 2 days (-LN)

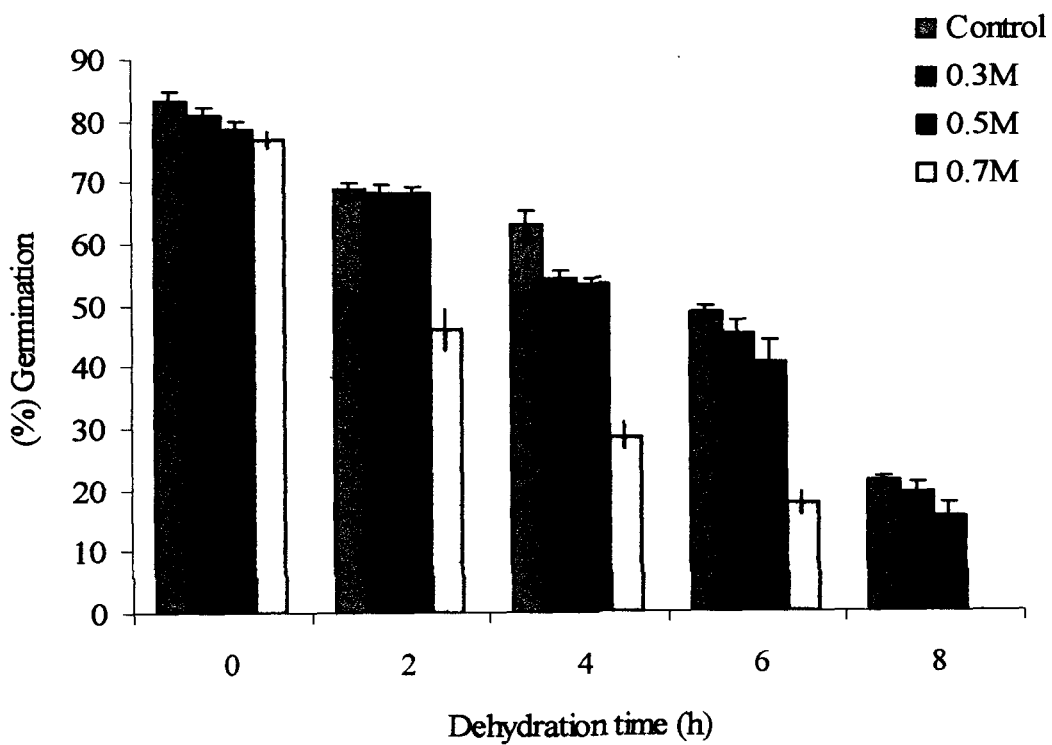
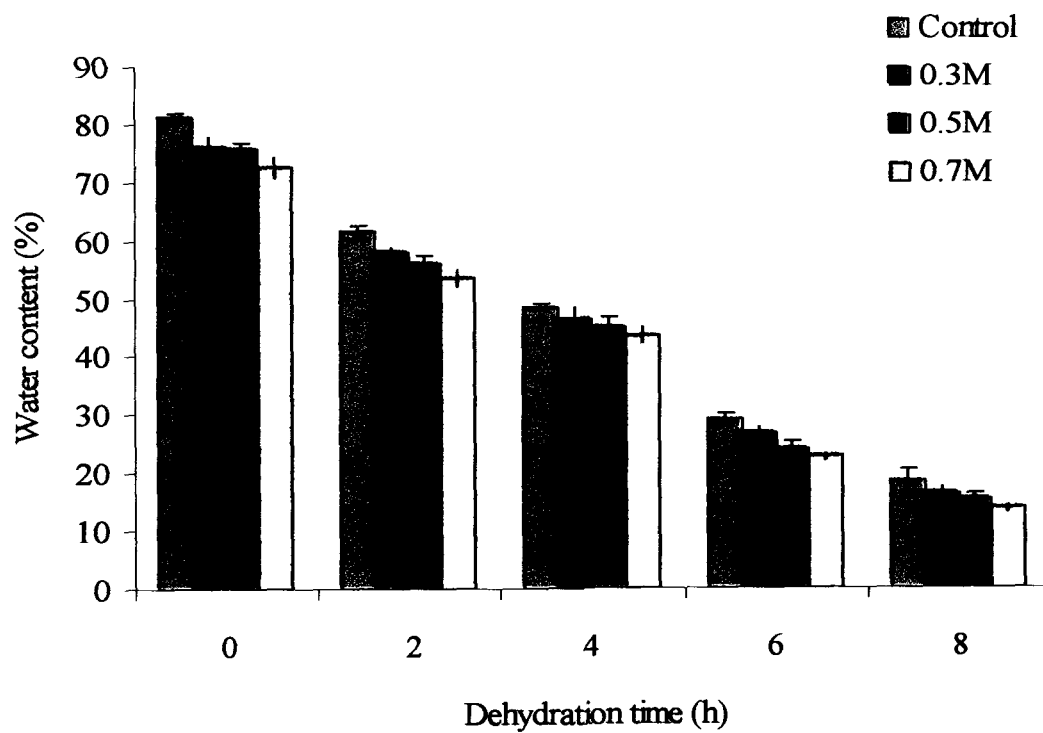


Fig. 5.11: Effect of liquid nitrogen (LN) treatment on *D. longicornu* for 1h on germination of encapsulated seeds precultured in MS liquid medium supplemented with 0.3, 0.5M sucrose for 2 days and dehydrated for 0-8 h.

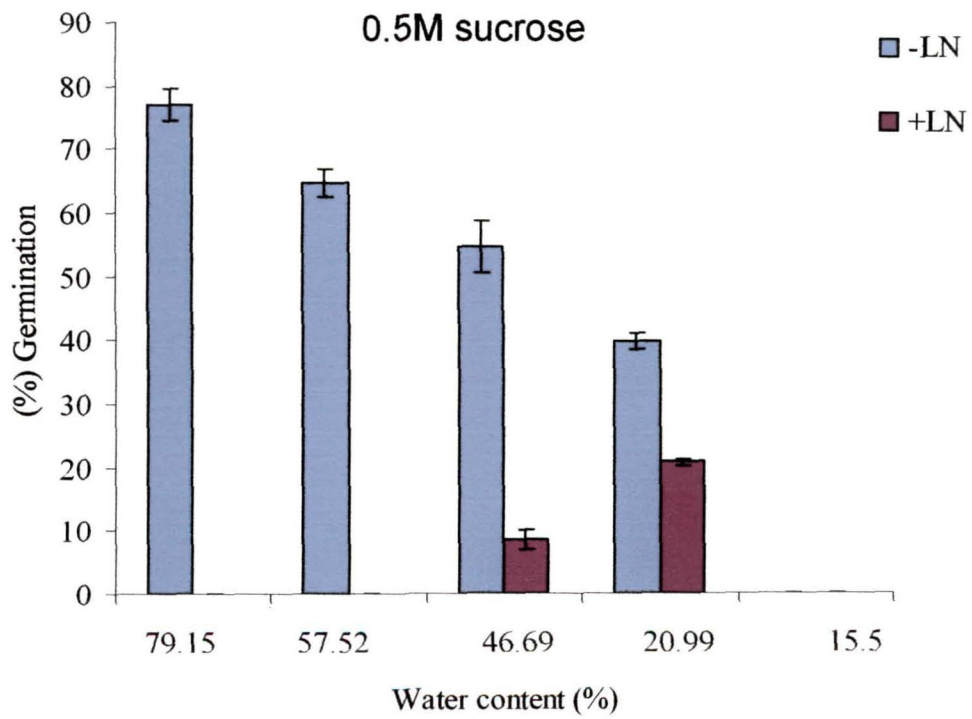
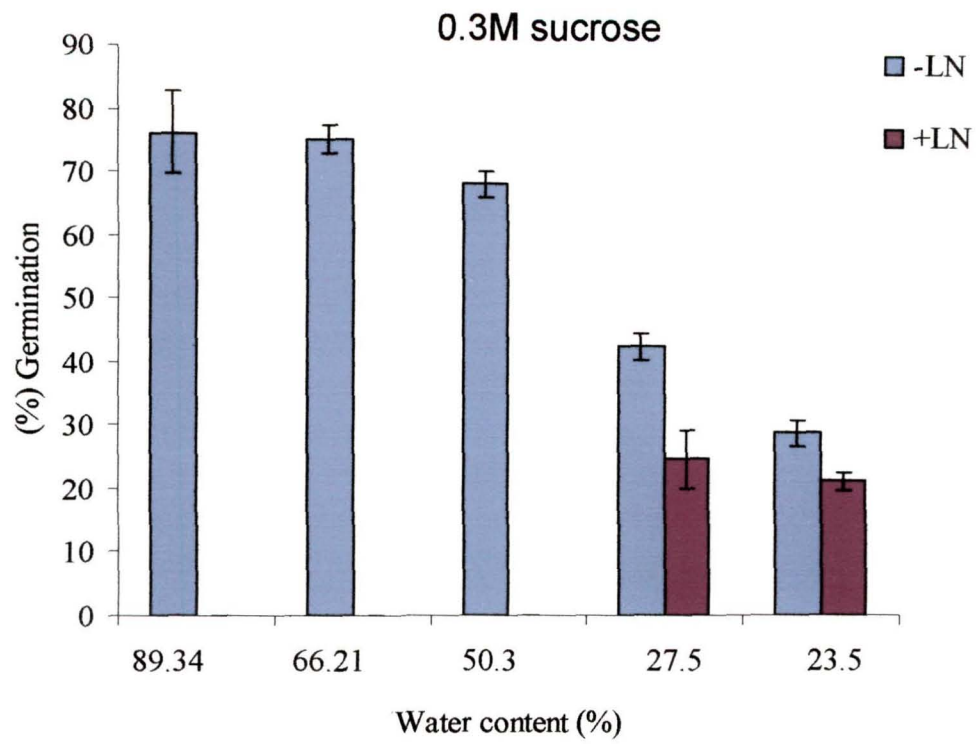


Fig. 5.12: Effect of liquid nitrogen (LN) treatment on *D. formosum* for 1h on germination of encapsulated seeds precultured in MS liquid medium supplemented with 0.3, 0.5M sucrose for 2 days and dehydrated for 0-8 h.

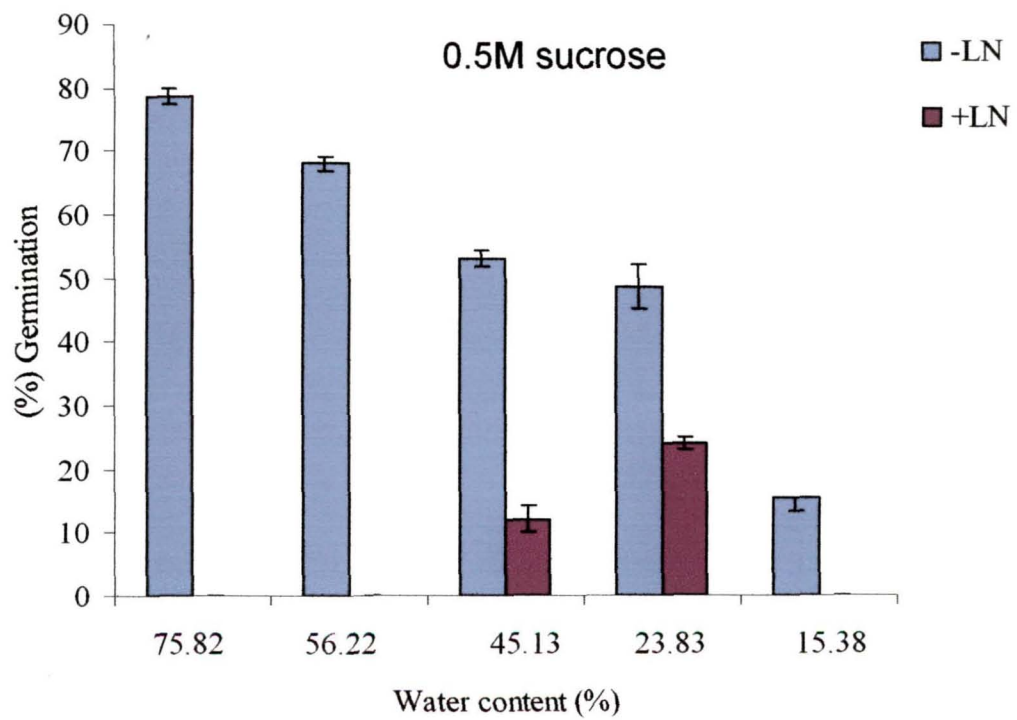
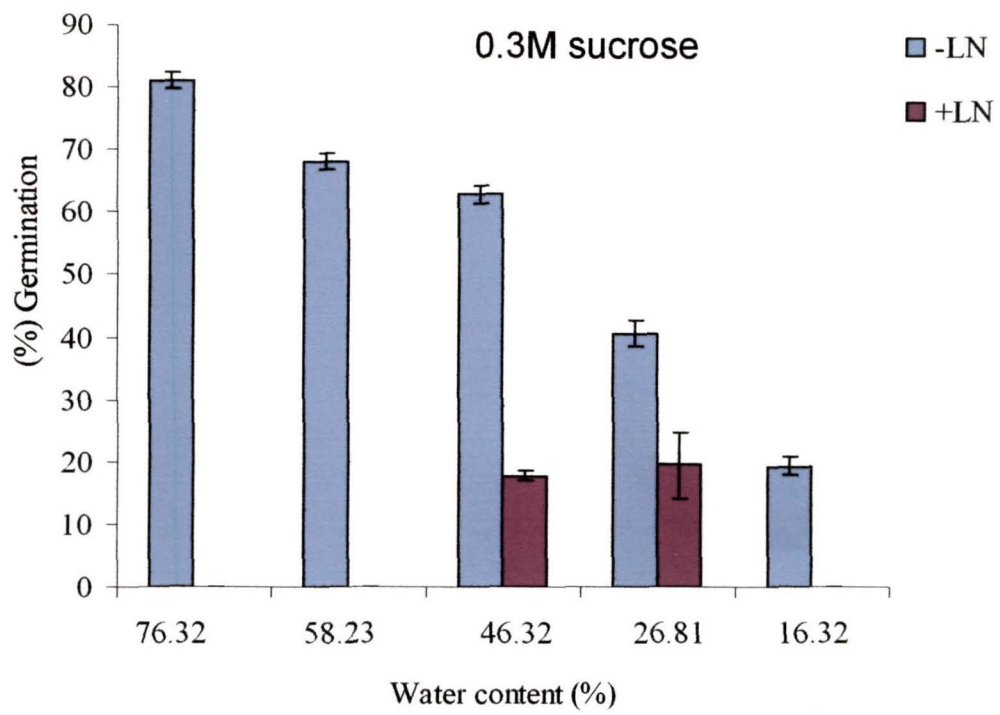
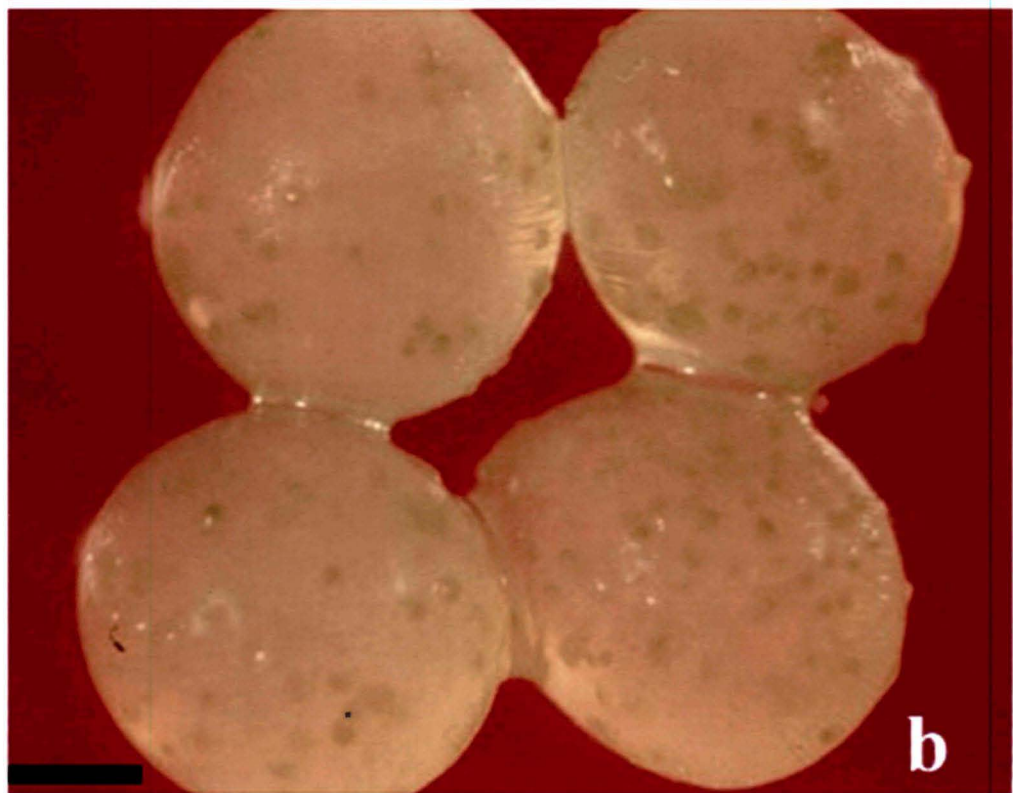
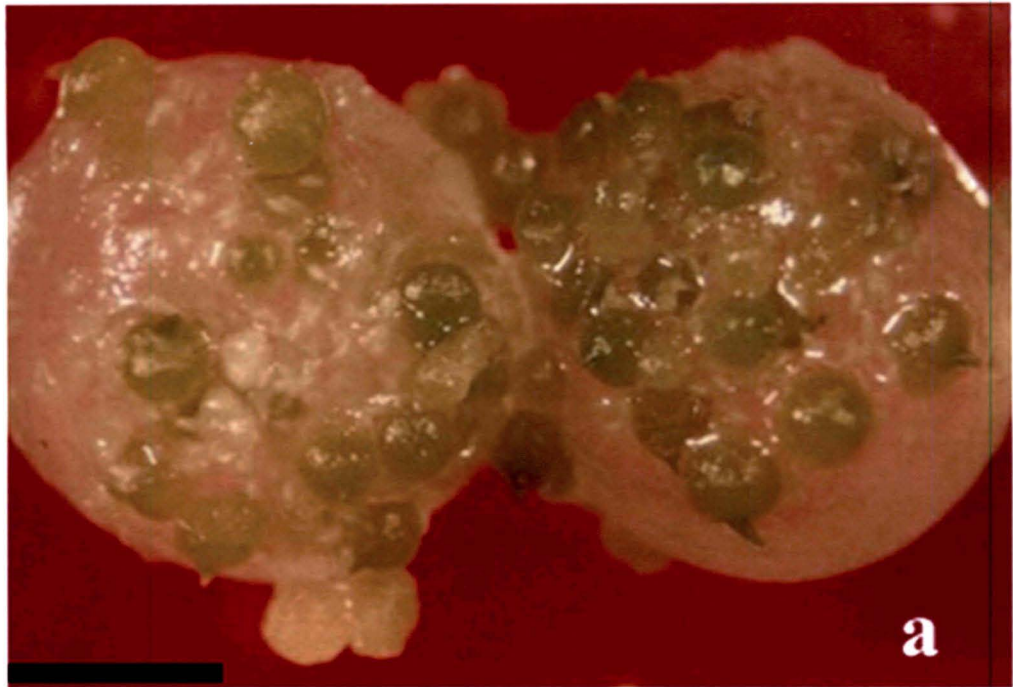


Plate 5.3

- a. Germinating seeds of *D. longicornu* in beads pretreated with 0.3M sucrose and on being dehydrated for 6h, after 5 weeks of culture
- b. Germinating seeds of *D. formosum* in beads pretreated with 0.5M sucrose and on being dehydrated for 6h, after 3 weeks of culture

(bar = 1 mm)



0.5M treatments of sucrose which were dehydrated for 4h and 6h respectively (Fig. 5.12; Plate 5.3b). The highest germination of 24.04% was observed for beads pretreated with 0.5M sucrose and dehydrated for 6h wherein the water content was reduced to 23.83%. No germination was recorded in other cases (Fig. 5.12).

DISCUSSION

Cryopreservation provides an important practical approach to orchid germplasm conservation as compared to *in vitro* culture. It is relatively inexpensive and requires less space and resources. The storage of seeds of many plant species in liquid nitrogen is now well developed and applied to agricultural crops and for the purpose of rescue of rare and endangered plant species. Though different tissues like PLBs, protocorms and seeds were tried for cryopreservation of *D. longicornu* and *D. formosum* for cryopreservation, the seeds were found to be the most suitable explant and were cryopreserved using both vitrification and encapsulation-dehydration methods.

Through vitrification method, seeds are dehydrated with highly concentrated vitrification solution. The vitrification solution needs to be a suitable solution that vitrifies during rapid freezing and which permeates the cells of the tissues to an extent that vitrification also occurs within the cells. In the present study, a vitrification solution termed PVS2 (Sakai *et al.*, 1990) comprising glycerol, DMSO, ethylene glycol with 0.4M sucrose was used. PVS2 has been widely used vitrification solution in case of seeds (Ishikawa *et al.*, 1997; Thammasiri, 2000; Hirano *et al.*, 2005 a, b; Kagawa and Thammasiri, 2005), suspension culture cells (Tsukazaki *et al.*, 2000), protocorms (Wang

et al., 1998), apical meristems (Thin and Takagi, 2000). In the present study, the solution PVS2 was found to be relatively toxic with increase in the exposure time both in control and cryopreserved seeds. A 10 min exposure time to PVS2 for *D. longicornu* and 20 min for *D. formosum* was found to be the optimum for both the species studied. However, Kagawa and Thammasiri (2005) reported the suitability of an exposure time of 30 min in case of *Dendrobium cruentum* seeds, and in case of *Doritis pulcherrima* 50 min exposure time was found to be the optimum (Thammasiri, 2000). The volume of vitrification solution used also is critical for the germination of the seeds. In the present study, 0.5 ml of the solution was found to be suitable for successful vitrification in both the species studied. A washing solution of 1.2M sucrose concentration used for both the species was found to be suitable for the vitrified seeds which is in consistent with the reports of Tsukazaki *et al.* (2000), Hirano *et al.* (2005) and Kagawa and Thammasiri (2005). A maximum of 40.02% of *D. longicornu* seeds and 42.73% of *D. formosum* seeds survived following the vitrification method. Kagawa and Thammasiri (2005) reported 34% survival in *Dendrobium cruentum* following vitrification, while Hirano *et al.* (2005) reported 78.7% survival in *Bletilla striata*. The cryopreserved seeds were later regrown in their respective regrowth medium and these produced plantlets identical to the untreated phenotypes. Fully developed plantlets were observed within 3 months of culture of the seeds. The first signs of viability of seeds could be seen after one week of culture when protocorms started to develop.

Encapsulation-dehydration technique is based on the technology developed for the production of synthetic seeds where somatic embryos are encapsulated in a bead of

hydrosoluble gel (Redenbaugh *et al.*, 1991). The procedure involves encapsulation of explants in alginate beads, pregrowth in liquid medium (containing higher osmoticum using sucrose), further desiccation with air in a laminar flow table and then plunging into liquid nitrogen. In the present study, the seeds of *D. longicornu* and *D. formosum* were subjected to various treatments to investigate the optimal duration of dehydration, the most favourable sucrose concentration and sucrose preculture for inducing cytoplasmic vitrification in order to avoid the formation of intracellular ice crystals during rapid cooling in liquid nitrogen. From the results obtained, the germination of the encapsulated seeds of *D. longicornu* and *D. formosum* decreased with the increase in the dehydration time. The germination of cryopreserved seeds was found to be the highest i.e., about 24.7% in case of *D. longicornu* in 0.3M sucrose pretreatment and dehydration for 6h. In this case the optimum sucrose concentration for cryopreservation of seeds was found to be 0.3M and 0.5M and the optimum water contents of the beads from 21 - 28% with the dehydration time of 6 - 8h. In case of *D. formosum* the highest germination of 24.04% was found at 0.5M sucrose pretreatment and dehydration for 6h. The optimum sucrose concentrations in this case were 0.3M and 0.5M, while the optimum water content ranged between 24 - 46% with the dehydration time of 4 - 6h. Flachland *et al.* (2006) reported that about 4.8% of cryopreserved seeds of *Oncidium bifolium* produced plantlets at 19.2% moisture content. On the other hand, 100% of *Vanda coerulea* seeds germinated at 25% and 22% water content with 0.5M and 0.7M sucrose preculture medium (Jitsopakul and Thammasiri, 2005). Lurswijidjarus and Thammasiri (2004) reported the highest regrowth of 13.33% in shoot tips of *Dendrobium* Walter Oumae after liquid nitrogen immersion.

Although cryopreservation of orthodox seed species is considered to be very straightforward methods, real data on amenability of seeds of many species to freeze preservation are not available (Mandal, 2000). Cryopreservation of seeds of many small seeded species is important, as it requires very little space and the seed can be conserved for indefinite periods. Earlier reports have shown that the germination rate of seeds of some tropical orchids significantly increased after cryopreservation (Nikishina *et al.*, 2001). This phenomenon could have been caused by damage to the seed coat during the freezing-thawing cycle (Pence, 1991; Tikhonova *et al.*, 1997). On the other hand, the germination rate of seeds of other species was reduced both at deep and nondeep (to -20°C) freezing (Tikhonova, 1999). Thus, it could be stated that a change in the germination rate of seed after the freezing-thawing cycle is probably species specific and could be related to orchid seeds as well.

From the present study on cryopreservation of the dendrobies, it has been found that vitrification method is the better and suitable method as compared to encapsulation-dehydration method. Thus, cryopreservation through vitrification of both *D. longicornu* and *D. formosum* is a viable long term strategy for their germplasm preservation and thus can open up promising opportunities of storing seeds of these valuable and endangered species of plants in cryobanks.

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

INTRODUCTION

The seedlings in culture are continuously exposed to an environment that provides minimal stress and optimal growth conditions. These seedlings when transferred from the culture vessels to the community pots require a careful, stepwise procedure which can cause the hardening of the seedlings and hence lead to better survival when transplanted to the pots. Studies have shown 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 changes (Brainerd and Fuchiagam, 1981; Roy, 1994; Baruah, 1996).

The correct method of potting in case of orchids is essential for rapid growth and development of the plantlets (Kang, 1979). 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).

Although different types of pots have been used for acclimatization of plantlets, but the 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 baskets or cradle for epiphytic orchids has been recommended. However, the use of plastic baskets or copper wire baskets as containers for orchids is also a common practice.

For the purpose of hardening, several composts have been proposed and their effects on growth have been evaluated in different species (Sharma and Chauhan 1995; Baruah 1996; Sharma and Roy 1996; Sharma and Kaur 1998; Saiprasad and Polisetty; 2003; Kumaria *et al.* 2005). In the past; the use of fern fibres as suitable compost had been a common practice (Meyer, 1951). Subsequently, 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*. 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). Malabadi *et al.* (2005) used caorcoal chips, coconut husk and broken tiles (2:2:1) for the compost in case of *Dendrobium nobile*. Therefore, an ideal compost which is inert, resistant to organic decomposition, porous to ensure adequate aeration for root respiration, less costly and easily available, is mostly supportive for acclimatization of the orchids in the glass house. 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 stakes (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 had been proven to be beneficial for healthy growth of transferred orchid seedlings. Addition of the nutrients to the compost varies with the composition of the potting materials and the type of orchid grown.

Apart from container types, compost mixture, fertilizer and humidity, temperature plays an important critical role in successful transplantation of orchid plantlets. The best temperature range is reported to be between 18.3°C to 29.4°C. Cribb (1990) reported 18°C and 23 – 25°C as the minimum and maximum temperatures respectively for better establishment of *Dendrobium alexandrae*. In case of

Phragmipedium bessae, 17°C and 30°C were the required minimum and maximum suitable temperatures respectively (Robbins, 1989). Similarly, Shiao *et al.* (2005) reported the mean day/night temperature of 24°C/18°C for maximum survivability of *Dendrobium candidum* plantlets.

The pretreatment of *in vitro* cultured plantlets before acclimatization to the field has been useful for successful acclimatization of plantlets. Preconditioning of the plantlets by growing them in the medium containing high concentrations of sucrose was reported to influence the *in vivo* rooting and establishment of cuttings (Wainwright and Scrace, 1989). Nagaraju and Mani (2005) reported an *in vitro* pre hardening of *Zygopetelum intermedium* in medium containing paclobutrazol and activated charcoal for its high rate of *ex vitro* survival and growth of plantlets. Hazarika *et al.* (2000, 2001) also reported that *in vitro* preconditioning of citrus microshoots with sucrose concentrations of 3% was optimum for subsequent *ex vitro* survival and growth. They also reported that preconditioning of citrus microshoots with paclobutrazol influences 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.

MATERIALS AND METHODS

In vitro raised complete plantlets of both *D. longicornu* and *D. formosum* were taken for hardening and establishment. Tiny plantlets measuring 2.0-3.0 cm in height were taken out 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 soft brush and washing with sterile water taking due care to avoid damage to the roots. The plantlets were then transferred to clean thermocol/plastic pots of 8 cm diameter containing different mixtures of composts viz., (i) brick pieces and charcoal chunks (1:1), (ii) brick pieces and charcoal chunks (1:1) + a top layer of moss, (iii) brick, charcoal chunks and decaying litter (1:1:1), (iv) brick pieces, charcoal chunks and decaying litter (1:1:1) + a top layer of moss, (v) brick pieces, charcoal and bark pieces (1:1:1), (vi) brick pieces, charcoal chunks and bark pieces (1:1:1) + a top layer of moss.

To minimize the spread of disease, thermocol pots were thoroughly washed with distilled water and dried. The pots were filled with 3/4th 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 along with the plantlets 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 the plantlets. The minimum and maximum temperatures of the glass house at the time of transplantation were 18°C and 25°C respectively. The relative humidity of the glass house was around 70-80%. The plantlets were watered in the evening on alternate days and fed with MS nutrient salt solutions (diluted 10 times) fortnightly for about a month. Readings were recorded after 90 days of hardening and subsequently the plantlets were transferred to larger earthen pots measuring 25 cm in diameter.

RESULTS

Of the various compost combinations used, the compost made up of brick pieces, charcoal chunks and bark pieces with a layer of moss on top was found to be the most suitable for the survival of transferred plantlets of *D. longicornu* wherein 68% survival of plantlets was recorded (Table 6.1). The compost having brick pieces, charcoal chunks and decaying litter with a layer of moss on top was also found to be a suitable substratum for transferred plantlets with a survival of 63%. The compost containing brick pieces, charcoal chunks without or with a layer of moss did not support good survival of the transferred plantlets. Also, the other composts used without moss did not support the survival of the plantlets. In the composts containing brick pieces, charcoal chunks and decaying litter, brick pieces, charcoal chunks and bark with a layer of moss on top, plantlets as tall as 4.10 cm and 4.00 cm respectively were obtained. The plantlets were hardened and established in about 50-60 days time (Plate 6.1a). Complete established plantlets were obtained after 90 days. Subsequently, these plantlets were transferred to earthen pots for further growth and development (Plate 6.1b).

In case of *D. formosum*, the composts containing brick pieces, charcoal chunks and decaying litter with a layer of moss on top was the best for high survivability (87%) and growth of the *in vitro* raised plantlets (Table 6.2). The compost containing only brick pieces and charcoal chunks showed the lowest survivability of the transferred plantlets. Other substrata used which contained moss as top layers were found to increase the survival of the transferred plantlets. After 90 days, it was found that the transferred

Table 6.1: Re-establishment of *Dendrobium longicornu* plantlets after 90 days of hardening

| Treatment | Survival % | Height (cm) |
|---|-------------------|--------------------|
| Brick + Charcoal (1:1) | 21 ± 1.4 | 3.44 ± 0.20 |
| Brick + Charcoal (1:1) + layer of moss | 38 ± 2.8 | 3.55 ± 0.25 |
| Brick + Charcoal + Decaying litter (1:1:1) | 32 ± 2.0 | 3.30 ± 0.11 |
| Brick + Charcoal +Decaying litter (1:1:1) + layer of moss | 63 ± 4.2 | 4.10 ± 0.30 |
| Brick + Charcoal + Bark (1:1:1) | 35 ± 1.4 | 3.05 ± 0.25 |
| Brick + Charcoal + Bark (1:1:1)+ layer of moss | 68 ± 2.8 | 4.00 ± 0.40 |

± S.D.

Plate 6.1

Hardened plants of *D. longicornu*

a. *In vitro* raised plantlets transferred to plastic pots (after 60 days)

b. Plantlets transferred to earthen pots (after 1 year)



Table 6.2: Re-establishment of *Dendrobium formosum* plantlets after 90 days of hardening

| Treatment | Survival % | Height (cm) |
|---|-------------------|--------------------|
| Brick + Charcoal (1:1) | 32 ± 2.8 | 2.45 ± 0.15 |
| Brick + Charcoal (1:1) + layer of moss | 65 ± 7.0 | 3.20 ± 0.10 |
| Brick + Charcoal + Decaying litter (1:1:1) | 53 ± 4.2 | 2.55 ± 0.15 |
| Brick + Charcoal +Decaying litter (1:1:1) + layer of moss | 87 ± 4.2 | 3.25 ± 0.35 |
| Brick + Charcoal + Bark (1:1:1) | 37 ± 4.2 | 2.70 ± 0.10 |
| Brick + Charcoal + Bark (1:1:1)+ layer of moss | 60 ± 2.8 | 2.55 ± 0.05 |

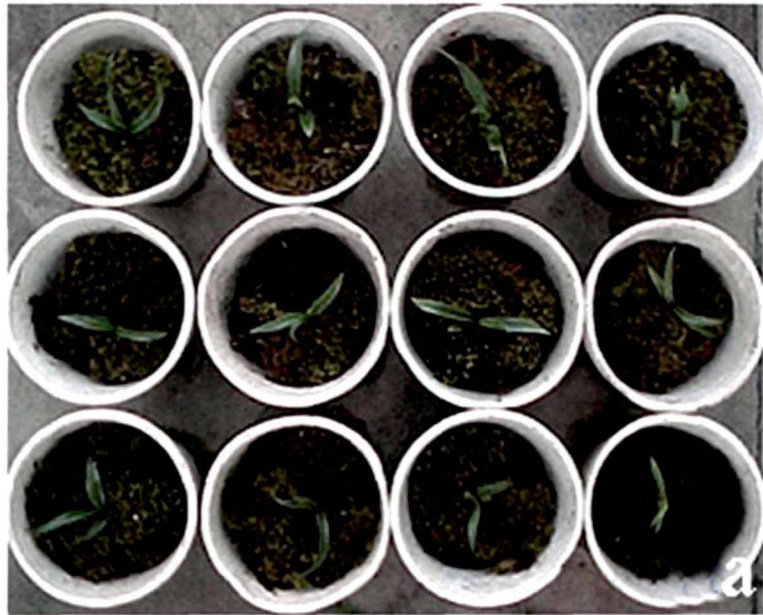
± S.D.

Plate 6.2

Hardened plants of *D. formosum*

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

b. Plantlets after 6 months of transfer



plantlets of *D. formosum* were more or less of the same height in all the substrata used. Feeding the plantlets initially with diluted MS nutrient salt solution for a fortnight proved to be beneficial for their healthy growth. The plantlets were hardened and established in 40 to 50 days time. Complete established plantlets were developed after 90 days (Plate 6.2a, b), and were subsequently transferred to earthen pots for further growth and development.

DISCUSSION

The transfer of plantlets from the culture vessels to the glasshouse conditions requires a careful and stepwise procedure. Successful transplantation also depends on suitable size of the plantlets and their state of growth *in vitro*. In the present study, healthy plantlets showing vigorous growth in the culture vessels were transferred to the pots. The hardiest and vigorous plants have been found to be easier to transplant as they are less susceptible to diseases and mechanical injuries. The plantlets transferred to the pots had healthy and vigorously growing root systems, which ensured better establishment and growth. The different composts used for both *D. longicornu* and *D. formosum* in this investigation were found to be satisfactory for survivability and normal growth of the transferred plantlets. The highest survivability of 68% in case of *D. longicornu* was obtained on substratum containing brick pieces, charcoal chunks and bark pieces (1:1:1) with a layer of moss on top, while a survivability of 87% in case of transferred plantlets of *D. formosum* was obtained on substratum containing brick pieces, charcoal chunks and decaying litter (1:1:1) with a top layer of moss. The substrata used

for both the dendrobies species were found to facilitate 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 periods of transfer (Bhojwai and Razdan, 1983). The rate of survival and growth of the plantlets of both the species studied was found to have reduced in a substratum containing only brick and charcoal. This might be due to the reason that the compost containing only brick pieces and charcoal chunks could not supply enough nutrients required for the growth of the transferred plantlets. Also, the compost was too porous and could have leached out the minimal of the nutrients available. However, the addition of litter to this substratum with a layer of moss was found to be beneficial for the survival of the transferred plantlets of both the species. Similar results where decaying litter had been found to be effective on growth and survivability of plantlets have been reported in *Vanda coerulea* (Kalita, 1999) and *Dendrobium fimbriatum* var. *oculatum* (Roy and Banerjee, 2003). Baruah (1996) achieved 90% survivability of plantlets of *D. transparens* on potting medium containing charcoal, brick pieces, chopped fern roots and farmyard manure. In the present study, bark pieces in the compost were found beneficial for survival and growth of plantlets of *D. longicornu*. Sharma and Chauhan (1995) reported 100% survivability of transferred plantlets of *D. chrysanthum* on potting media containing brick chips, charcoal, bark pieces, leaf mould, tree fern and dry sphagnum. The survivability of the micropropagated plantlets on being transferred to pots depends on proper acclimatization of plantlets. The best method to ensure optimal survival of cultured plantlets in natural conditions is to

expose them gradually to a relatively lower humidity, higher temperature and higher light intensity. Sharma and Chauhan (1995), Vij *et al.* (1995), and Nagaraju and Mani (2005) also suggested acclimatization of the *in vitro* raised plantlets prior to exposure to *ex vitro* environment. In the present study, feeding the plantlets with diluted MS nutrient salt solution was found to be beneficial for growth of the transferred plantlets. This is consistent with the earlier report of Kumaria and Tandon (1994) wherein in feeding the plantlets of *Dendrobium fimbriatum* var. *occulatum* with diluted MS nutrient salt solution for the initial period was beneficial as the supply of the essential nutrients to the transferred plantlets was not stopped abruptly. The promotion of orchid seedling growth by the nutrient solution for initial hardening had also been reported earlier in many other instances (Sander, 1979; Mukherjee, 1983).

The clonal propagation and cryopreservation of *Dendrobium longicornu* and *D. formosum*, two endangered orchids of Northeast India were accomplished successfully in the present study. Different media viz., MS, B₅, Mitra and KC were tried for asymbiotic seed germination and protocorm development of *D. longicornu* and *D. formosum* seeds. MS medium was found to be the best for seed germination in both the species studied. The presence of potassium nitrate and ammonium nitrate at high concentrations in MS medium could have been the most suitable form of nitrogen for seed germination of *D. longicornu* and *D. formosum*. The media, Mitra and KC both contained calcium nitrate and ammonium sulphate which might not have supported good growth in case of *D. longicornu*, while in *D. formosum* the presence of reduced nitrate in the form of ammonium sulphate in Mitra, B₅ and KC might have had some influence in the seed germination. The difference in response to ammonium sources used may be due to varied requirement of the species. The seeds were considered to have germinated upon emergence of the embryo from the testa. The volume of the protocorms recorded in the culture media also varied from medium to medium. The protocorms produced on MS

media were, however, quite large compared to those developed on other media. 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. The overall optimal growth of seedlings of both *D. longicornu* and *D. formosum* was enhanced in MS medium, while no seedling growth was observed in KC medium.

Environment exerts an important effect on the physiology and development of orchids. The optimal seed germination in both the species was found to be at $150\mu\text{molm}^{-2}\text{s}^{-1}$ light intensity. A decrease in the seed germination was noticed with increasing or decreasing light intensities. The range from 8 - 20h of photoperiod was found to be suitable for seed germination; though no significant variation was observed in germination percentage, the highest percentage was recorded at 12h photoperiod. The influence of temperature on basic physiological processes such as respiration and on cell and organ formation is well known. The maximum germination of seeds of both the species was observed at 24°C. The percentage of germination declined with increase or decrease in the temperature.

Micropropagation of *D. longicornu* and *D. formosum* was carried out keeping in mind the mass propagation of these rare and threatened plantlets. Out of all the different explant sources tried, axillary buds were found to be suitable for micropropagation for both *D. longicornu* and *D. formosum*. The response of these varied depending on the media used. Out of the four different media tried, the cultured axillary bud responded best in MS medium. However, no PLBs were formed from the explants in the basal media used. The incorporation of auxins (IAA, NAA and 2,4-D) in the medium showed

differential results in the response of cultured axillary bud of both *D. longicornu* and *D. formosum*. Shoot buds as well as PLBs were seen emerging in 2,4-D containing medium, while in IAA and NAA containing medium, it was shoot bud -mediated regeneration. In *D. longicornu*, NAA at 30 μ M in the medium induced 86.6% explant response with maximum number of shoots/explant, while in *D. formosum* maximum percentage response (96.66%) as well as shoot number were obtained in the medium containing 15 μ M of NAA. The regeneration pathway of axillary bud in both the species cultured in MS medium supplemented with 2,4-D was found to be through PLBs as well as shoot buds. In *D. longicornu*, maximum of 36.94% of explant response into PLBs was recorded at 30 μ M 2,4-D in medium, while in *D. formosum* 28.51% of explant response into PLBs was recorded at 15 μ M of 2,4-D in medium. With the addition of growth regulators to the basal medium the axillary buds responded readily due to probably habituated nature and juvenility of their tissues. The effect of cytokinins (BAP/KN) was on both shoot bud production and PLB formation from axillary buds. In *D. longicornu* only shoot bud induction was seen in cultured buds, while in *D. formosum* both shoot buds as well as PLBs were induced in BAP supplemented medium. BAP/KN when combined with auxins (IAA, NAA and 2,4-D) in the medium had varying responses of explants in both the dendrobes studied. In a combination of BAP (5 μ M) and NAA (15 μ M) in the medium, a maximum shoots were initiated in case of *D. longicornu*. On the other hand, KN and NAA at 15 μ M each in the medium were found to be suitable for optimal response of explants in *D. formosum*. Combination of 2,4-D either with BAP or KN in the medium induced both PLBs and shoot buds from cultured axillary buds of both *D. longicornu* as

well as *D. formosum*. Maximum PLBs were induced from the explants in the medium containing BAP and 2,4-D at 15 μ M each in combination for *D. longicornu*, and BAP and 2,4-D at lower concentrations (5 μ M) for *D. formosum*. The explant response in terms of PLB induction was highest in the medium supplemented with BAP and 2,4-D in the ratio of 1.

The transfer of the PLBs to the medium containing plant growth regulators exhibited vigorous proliferation of the PLBs. There was a significant difference between the number of PLBs produced in the medium containing plant growth regulators and the one devoid of these. The promotory influences of both NAA and IAA on PLB development were observed at lower concentrations of these auxins in the medium for both the dendrobes. 2,4-D in the medium, on the other hand, had a inhibitory effect on the growth and development of PLBs. Differential responses of the explants in media supplemented with cytokinins were seen; in both *D. longicornu* and *D. formosum* enhanced PLB production as well as proliferation in both BAP and KN containing medium were observed.

The combination of auxins and cytokinins in the medium also had a pronounced effect on the growth and development of PLBs of both the species. The combination of auxin (IAA/NAA) with BAP resulted in differential responses of the PLBs in both the species. IAA/NAA with BAP in *D. longicornu* and *D. formosum* showed a better and enhanced effect on the development of the PLBs. The supplementation of KN with IAA/NAA in the medium also had an influencing effect on both the species studied. In *D. longicornu*, rapid proliferation and development of PLBs was seen in the medium

containing lower concentrations of KN+IAA or KN+NAA, while higher concentrations of these growth regulators had an inhibitory effect on the development of PLBs. In *D. formosum*, a stimulatory effect of KN+IAA or KN+NAA was observed on the developing PLBs.

Cryopreservation provides an important practical approach to orchid germplasm conservation as compared to *in vitro* culture. Although different tissues like PLBs, protocorms and seeds of *D. longicornu* and *D. formosum* were tried for cryopreservation, seeds were to be the most suitable. Seeds of both the species were cryopreserved using vitrification and encapsulation-dehydration methods. With the use of vitrification methods, seeds are dehydrated with highly concentrated vitrification solutions. A vitrification solution termed PVS2 was used. A 10 min exposure time to this solution for *D. longicornu* and 20 min for *D. formosum* was found to be the optimum. The volume of vitrification solution used also had a critical role in growth of the seeds. 0.5ml of the solution was found to be optimum for successful vitrification in both the species. A washing solution of 1.2M sucrose concentration used for both the species was found to be suitable for the vitrified seeds. The cryopreserved seeds were later grown in their respective regrowth medium. These seeds produced plantlets upon culture in respective medium identical to the untreated phenotypes. On the other hand, encapsulation-dehydration technique was based on the technology where explants were encapsulated in the beads of hydrosoluble gel for the production of synthetic seeds and then dehydrated. Seeds of *D. longicornu* and *D. formosum* were given various treatments to investigate the optimal duration of dehydration, the most favourable sucrose concentration and sucrose

preculture for inducing cytoplasmic vitrification in order to avoid the formation of intracellular ice crystals during rapid cooling in liquid nitrogen. The germination of cryopreserved seeds was found to be the highest (24.7%) in case of *D. longicornu* at 0.3M sucrose concentration with dehydration time of 6h. Here, the optimum sucrose concentrations were found to be 0.3M and 0.5M and the optimum water contents of the beads in the range 21% - 28% with the dehydration time of 6-8h. In the case of *D. formosum* the highest germination of 24.04% was recorded at 0.5M sucrose concentration with 6h dehydration time. The optimum sucrose concentration in case of *D. formosum* was 0.3M and 0.5M, while the optimum water content was 24% - 46% with the dehydration time of 4-6 h.

The transfer of plantlets from the culture vessels to the glasshouse conditions requires a careful and stepwise procedure. Successful transplantation also depends on suitable size of the plantlets and their state of growth *in vitro*. Healthy plantlets showing vigorous growth in the culture vessels were transferred to the pots. The different composts used for both *D. longicornu* and *D. formosum* in this investigation were found to be satisfactory for survivability and normal growth of the plantlets. The highest survivability of 68% in case of *D. longicornu* was obtained on substratum containing brick pieces, charcoal chunks and bark pieces (1:1:1) with a layer of moss, while a survivability of 87% in *D. formosum* was obtained on substratum containing brick pieces, charcoal chunks and decaying litter (1:1:1) with a layer of moss, which facilitated proper drainage and aeration for root respiration for both the dendrobes. The layer of moss on top proved to be beneficial due to higher retention of moisture content. Bark pieces in the

compost were found beneficial for survival and growth of plantlets of *D. longicornu*. The survivability of the micropropagated plantlets on being transferred to pots depend on proper acclimatization of plantlets. The best method to ensure optimal survival of cultured plantlets in natural conditions was to expose them gradually to a relatively lower humidity, higher temperature and higher light intensity.

CHAPTER VIII:

REFERENCES

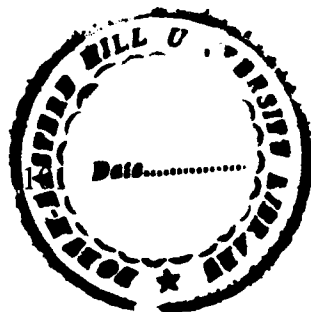
- Abdelnour-Esquivel, A., V. Villalobos and F. Engelmann. 1992. Cryopreservation of zygotic embryos of *Coffea* spp. *Crto-Letters* 13: 297-302.
- Abdul Karim, A. G. and H. Hairani. 1990. Leaf cultures of some Malaysian orchids. In: Proc. International Conference and Exhibition on orchids and ornamental plants. pp. 12. Kuala Lumpur, Malaysia
- 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.
- 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.
- 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 org. and tiss. Cult. pp. 203-293. In J. Arditti (ed.), *Orchid Biology, Reviews and perspectives Vol. 1*. Cornell Univ. Press Ithaca, New York.
- 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 (ed.). 1993. *Micropropagation of orchids*. John Wiley and Sons Inc. 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., 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.
- Arditti, J., Ernst, R., Yam, T. W. and Glabe C. 1990. The contributions of orchids mycorrhizal fungi to seed germination: a speculative review. *Lindleyana* 5: 249-255.
- Baruah, R. 1996. *In vitro* micropropagation of the species of *Calanthe*, *Phaius* and *Dendrobium*. Ph. D. Thess. G. U.
- Batygina, T. B. and E. V. Andronova. 1988. (In Russ.). Do Orchidaceae have cotyledons? *DOKL AKAD NAUK SSSR.* 302 (4): 1017-1019.

- Bergman, F. J. 1972 a. Shoot tip multiplication of orchid clones, Part 1. The status of "meristem" propagation through 1971. *Amer. Hort.* 51: 20-23.
- Bergman, F. J. 1972 b. Shoot tip multiplication of orchid clones, Part II. The status of "meristem" propagation through 1971. *Amer. Hort.* 51: 41-44.
- Bernard, N. 1909. L' evolution dans la symbiose. *Les Orchidales et leur champignons commensaux.* *Ann. Sci. Nat. Bot.* 9: 1-96.
- Bhojwani, S. S. and M. K. Razdan. 1983. *Plant Tiss. cult: Theory and practice.* Vol. 5. Elsevier Amsterdam-Oxford. New York, Tokyo.
- 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.
- Bopaiiah, A. K. and S. M. Jorapur. 1986. Studies on growth and development of *Cymbidium aloifolium* Sw. seedling *in vitro*. In: S. P. Vij (ed.), *Biol. Cons. and Cult. of orchid.* pp. 423-427. Affiliated East West Press.
- 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.
- Butenko, R. G. 1968. *Plant Tissue Culture and Plant Morphogenesis.* Translated from Russian. Israel programme for scientific Translation, Jerusalem.

- Buyun, L., A. Lavrentyeva, L. Kovalska, and R. Ivannikov. 2004. *In vitro* germination of seeds of some rare tropical orchids. *Acta Universitatis Latviensis, Biology*. 676: 159-162.
- Chandel, K. P. S., R. Chaudhury, J. Radhamani and S. K. Malik. 1993. Desiccation and freezing sensitivity in recalcitrant seed species of tea, cocoa and jackfruit. Presented at SLTB 93. Sussex, UK.
- Chang, C. and W. C. Chang. 1998. Plant regeneration from callus culture of *Cymbidium ensifolium* Var. *misericors*. *Plant Cell Rep.* 17: 251-255.
- 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.
- Chaturvedi, H. C. and A. K. Sharma. 1986. Mericlone 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, T. H. H., K. K. Kartha, F. Constabel and L. V. Gusta. 1984. Freezing characteristics of cultured *Catharanthus roseus* L. G. Don. Cells treated with dimethyl sulfoxide and sorbitol in relation to cryopreservation. *Plant Physiol.* 75: 720-725.

- 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.
- Chibbar, R. H., K. K. Kartha, N. Leung, J. A. Qureshi and K. Caswell. 1991. Transient expression of marker genes in immature zygotic embryos of spring wheat (*Triticum aestivum*) through microprojectile bombardment. *Genome*. 34: 453-460.
- Chin, H. F., B. Krishnapillay and Z. C. Alang. 1988. Cryopreservation of *Veitchia* and *Howea* palm embryos: Non-development of the haustorium. *Cryo-Letters*. 9; 372-379.
- Chowdhery, H. J. 2001. Orchid Diversity in North-East India. *J. Orchid Soc. India*. 15: 1-17.
- 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 orchid. *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 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 C₂H₂, CO₂ and cytokinins. *Z. Pflanzen Physiol*, 94: 117-123



- Cribb, P. 1990. *Dendrobium alexandrae*. The Kew Magazine. 7: 117-120.
- Das, A. and K. K. Ghosal. 1989. *In vitro* germination behavior of some orchid seeds developed in plains of W. Bengal. Indian Agric. 33: 103-109.
- Davidson, O. W. 1956. New orchid potting medium lowers cost of production. 38: 3-6.
- Deb, C. R., N. S. Jamir and Temjensangba. 2003. Orchid Diversity of Nagaland-A revised status. J. Orchid Soc. India. 17: 5-15.
- Debergh, P. C. and L. J. Maene. 1981. A scheme for commercial propagation of ornamental plants by tissue culture. Sci. Hortic., 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.
- Demeulemeester, M. A. C., B. Vandebussche and M. P. De Proft. 1993. Regeneration of chicory plants from cryopreserved *in vitro* shoot tips. Cryo-Letters. 14: 57-64.
- Dereuddre, J., C. Scottez, Y. Arnaud and M. Duron. 1990. Resistance of alginate-coated axillary shoots tips of pear (*Pyrus communis* L.cv.Beurre Hardy) *in vitro* plantlets to dehydration and subsequent freezing in liquid nitrogen. C.R.Acad.Sci.Paris. t. 310, Serie III,317-323.
- 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.

- Diettrich, B., P. Donath, A. S. Popov, R. G. Butenko and M. Luckner. 1990. Cryopreservation of *Chamomilla recutita* shoot tips. *Biochem. Physiol. Pflanzen.* 186: 63-67.
- Engelmann, F. 1997a. Importance of desiccation for the cryopreservation of recalcitrant seed and vegetatively propagated species. *Plant Genetic Resources News Letter*, 112: 9-18.
- Engelmann, F. 1997b. *In vitro* Conservation Methods. pp.119-162. In. J.A. Callow, B.V.Fort-Lloyd, and H.J. Newbury (eds.), *Biotechnology and Plant Genetic Resources: Conservation and Use*. CAB International, Wellingford.
- Engelmann, F., D. Dumet, N. Chabrillange, A. Abdelnour-Quivel, B. Assy -Bah, J. Dereuddre and Y. Duval. 1995. Cryopreservation of zygotic and somatic embryos from recalcitrant and intermediate-seed species. *Plant Genetic Resources News Letter*, 103: 27-31.
- Ernst, R. 1976. Charcoal or glass wool in asymbiotic culture of orchids, pp. 379-383. In *Proc. 8th world orchid conf., Frankfurt (1975)*.
- Ernst, R. 1994. Effect of thidiazuron on *in vitro* propagation of *Phalaenopsis* and *Doritaenopsis* (Orchidaceae). *Plant Cell Tiss. Org. Cult.* 39:273-275.
- Escobar, R. H., G. Mafla, and W. M. Roca. 1997. a methodology for recovering cassava plants from shoot tips maintained in liquid nitrogen. *Plant Cell Reports.* 16; 474-478.

- Farrant, J. 1980. General observations on cell preservation, pp 1-18. In: M. J. Ashwood Smith and J. Farrant (eds.), *Low Temperature Preservation in Medicine and Biology*. Pitman Medical, Tunbridge Wells.
- Finkle, B. J., and J. M. Ulrich. 1979. Effects of cryoprotectants in combination on the survival of frozen sugarcane cells. *Plant Physiol.* 63:598-604.
- Finkle, B. J., M. E. Zavala, and J. M. Ulrich. 1985. Cryoprotective compounds in the viable freezing of plants tissues, pp. 75-113. In: K. K.Kartha (ed.) *Cryopreservation of plant cells and organs*. CRC Press, Boca Raton, Florida.
- Flachsland, E., G. Terada, A. Scocchi, H. Rey, L. Mroginski and F. Engelmann. 2006. Cryopreservation of Seeds and *in vitro*-cultured protocorms of *Oncidium bifolium* Sims by Encapsulation-Dehydration. *Cryo-Letters.* 27: 235-242.
- 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.
- Gagliardi, R. F., G. P. Pacheco, L. A. Carneiro, J. F. M. Valls, M. L. C. Vieira and E. Mansur. 2003. Cryopreservation of *Arachis* species by Vitrification of *in vitro* grown shoot apices and genetic stability of recovered plant. *Cryo-Letters*, 24: 103-110.
- 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.
- Gamborg, O. L., T. Murashige, T. A. Torpe and I. K. Vasil. 1976. Plant Tissue Culture media. *In Vitro*, 12: 473-478.
- Geetha, S. and S. A. Shethy. 2000. *In vitro* propagation of *Vanilla planifolia*, a tropical orchid. *Curr. Sci.* 79: 886-890.
- George, E. F. and P. D. Sherrington. 1984. Plant propagation by tissue culture. Exegenetics 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.
- Goh, C. J. 1971. The influence of pH on orchid cultures. *Malays. Orchid Review*, 10: 32.
- Goh, C. J. 1990. Orchids-monopodials. In: *Handbook of plant cell culture*. Vol. 5. Ornamental species. (ed.), P. V. Ammirato, D. A. Evans, W. R. Sharp and Y. P. S. Bajaj), pp 598-637. Mc Graw-Hill. New York.
- Goh, C. J. and H. Tan. 1982. Clonal propagation from leaf explants in *Renantanda* orchid hybrid. *The Orchid Rev.*, 90: 295-296.
- Gonzalez-Arno, M. T., J. Juarez, C. Ortega, L. Navarro and N. Duran Vila. 2003. Cryopreservation of ovules and somatic embryos of citrus using the encapsulation-dehydration technique. *Cryo-Letters*, 24:85-94
- Griesbach, R. J. 1986. Orchid tissue culture. In: *Tissue culture as a plant production system for horticultural Crops* (ed.) R. H. Lawson. 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.
- Grillo Mensa, E., S. Pena and d. Perez. 1985. Mass *In vitro* germination of some species of orchids in the National Botanical Garden (Cuba). *Rev. Jard. Bot. Nac.* 6(2):95-100.
- 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. *Hortic. Res.*, 17: 1-7.
- Grout, B. W. W. and G. G. Henshaw. 1978. Freeze preservation of potato shoot tip cultures. *Annals of Bot.* 42: 1227-1229.
- Grout, B. W. W., K. Shelton and H. W. Pritchard. 1983. Orthodox behavior of oil palm seed and cryopreservation of excised embryos for genetic conservation. *Annals of Bot.* 52: 381-384.
- 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.
- Handa, S. S. 1986. Orchids for drugs and chemicals. In "Ecology, Conservation and Culture of Orchids" Ed., S. P. Vij, New Delhi.
- Handro, W., K. G. Hell and G. B. Kerbauy. 1977. Tissue culture of *Stevia rebaudiana* a sweetening plant. *Planta. Medica.* 32: 115-117.

- Harada, T., A. Inaba, T. Vakuwa, and T. Tamura. 1985. Freeze-Preservation of apices isolated from small heads of brussels sprouts. Hort. Sci. 20: 678-860.
- Harvais, G. 1973. Growth requirement and development of *Cypripedium reginae* 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.
- Hasegawa, A., M. Goi, M. Sato and Y. Ihara. 1978. Fundamental studies on the asymbiotic seed germination of *Calanthe*. Tech. Bull. Fac. Agric. Kagawa Univ. 29:251-259.
- Hatanaka, T., Y. Yasuda, T. Yamaguchi and A. Sakai. 1994. Direct regrowth of encapsulated somatic embryos of coffee (*Coffea canephora*) after cooling in liquid nitrogen. Cryo-Letters, 15: 47-52.
- Hauptmann, R. M., and J. M. Widholm. 1982. Cryopreservation of cloned amino acid analog-resistant carrot and tobacco suspension cultures. Plant Physiol. 70: 30-34.
- Hayes, A.B. 1969. Observations on orchid seed mycorrhizae. Mycopathologia et Mycologia Applicata. 30: 139-44.
- 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. 2001. Influence of *in vitro* preconditioning of citrus microshoots with paclobutrazol on *ex vitro* survival. Acta. Bot. Croat. 60: 25-29.

- Hazarika, R. and C. M. Sharma. 1995. *In vitro* germination and regeneration of *Dendrobium transparens* Lindl. J. Orchid. Soc. of India. 9: 69-74.
- Hedge, S. N. 1984. Orchids of Arunachal Pradesh. Published by the Forest Department, Arunachal Pradesh, Itanagar.
- Hedge, S N., A. N. Rao, and N. Ahmed. 1992. Further notes on Sessa orchid sanctuary, Arunachal Pradesh. J. Orchid Soc.India. 6 (1-2): 31-39.
- Hew, C. S.. and L. Y. Lim. 1989. Mineral uptake by orchid plantlets grown in agar culture medium. Soilless Culture, 5 (2): 23-24.
- Hirai, D and A. Sakai. 1999. Cryopreservation of in vitro-grown meristem of potato (*Solanum tuberosum* L.) by encapsulation -vitrification. Potato Res. 42: 153-160.
- Hirai, D and A. Sakai. 2001. Recovery growth of plants cryopreserved by encapsulation-vitrification. Bull. Hokkaido Agric. Expt Stn, 80: 55-64.
- Hirano, T., K. Ishikawa and M. Mii. 2005. Cryopreservation of immature seeds of *Poneorchis graminifoliai* var. *suzukiani* by vitrification. Cryo-Letters. 26: 139-146.
- Hirano, T., T. Godo, M. Mii and K. Ishikawa. 2005. Cryopreservation of immature seeds of *Betilla striata* by vitrification. Plant Cell Reports. 23: 534-539.
- 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.

- Homes, J. and N. Vanseveren-Van Espen. 1973. Effects du saccharose et de la lumidere sur le developement et la morphologie de protocormes d'orchidees cultives *in vitro*. Bull. Soc. Roy. Bot. Belg., 106: 117-121.
- 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.
- Huang, L and T. Murashige. 1977. Plant tissue culture media: Major constituents, their preparation and some application. Tissue Culture Association Manual, 3: 539-548.
- Huang, L. C. 1988. A procedure for asexual multiplication of *Paphiopedilums* *In vitro*. Am. Orchid Society. Bull., 57 (3) : 274-278.
- Ichihashi, S. 1989. Seed germination of *Ponerorchis graminifolia*. Lindleyana 4: 161-163.
- 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.
- Ishii, Y., T. Takamura, M. Goi and M. Tanaka. 1998. Callus induction and somatic embryogenesis of *Phalaenopsis*. Plant Cell Rep. 17: 466-470.
- Ishikawa, M., P. Tandon, M. Suzuki and A. Yamaguishi-Cimapi. 1996. Cryopreservation of brome grass (*Bromus inermis* Leyss) suspension cultured cells using slow freezing and vitrification procedures. Plant Science. 120: 81-88.
- Ishikawa, K., K. Harata, M. Mil, A. Sakai, K. Yohimatsu and K. Shimamura 1997. Cryopreservation of Zygotic embryos of a Japanese terrestrial orchid *Bletilla striata* by vitrification. Plant Cell Reports, 16 (11): 754-757.
- Israel, H. W. 1963. Production of *Dendrobium* seedlings by aseptic culture of excised ovaries. Am. Orchid. Soc. Bull. 32: 441-443.
- Jitsopakul, N. and K. Thammasiri. 2005. Cropreservation of seeds of *Vanda coerulea* Griff. Ex. Lindl. By Encapsulation-Dehydration. 31st Congress on Science and Technology of Thailand, Suranaree University of Technology.
- Johri, M. M. and D. Mitra. 2001. Action of Plant hormones. Curr. Sci. 80: 199-205.
- Kagawa, K. and K. Thammasiri. 2005. Cryopreservation of seeds of a Thai orchid (*Dendrobium cruentum* Rchb. f.) by vitrification. 31st Congress on Science and Technology of Thailand, Suranaree University of Technology.
- Kako, S. 1973. Clonal propagation of *Cattleya* through shoot meristem culture. Japan Agric. Res. Quart. 7: 109-115.

- Kalita, K. 1999. *In vitro* micropropagation of a few spectacular and endangered orchids species of North-East India. Ph.D. Thesis. Guwahati Univ.
- Kang, L. C. 1979. Orchids: Their cultivation and hybridization. Eastern Universities Press, SDN, BHD, Cingapore.
- 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.
- Kartha, K. K. 1985. Meristem culture and germplasm preservation, pp. 115-134. In: K. K. Kartha (ed.), *Cryopreservation of plant cells and organs*. CRC Press Inc, Boca Roton.
- Kartha, K. K. 1987. Advances in the cryopreservation technology of plants cells and organs, pp. 447-459. In: C. E. Grenn, D. A. Somers, W. P. Hackett and D. D. Biesboer (eds.), *Plant Tissue and Cell Culture*. Alan R. Liss Inc, New York.
- Kartha, K. K., N. L. Leung, P. Gaudet- Laprairie and Constabel. 1982a. Cryopreservation of Periwinkle (*Catharanthus rosells*) cells cultured in vitro. *Plant Cell Reports*, 1: 135-138.
- Kartha, K. K., N. L. Leung and L. A. Mroginski. 1982b. In vitro growth responses and plant regeneration from cryopreserved meristem of Cassava (*Manihot esculenta* cantz) *Z. Pflanzen Physiol.* 107: 133-140.
- Kartha, K. K., L. C. Fowke, N. L. Leung, K. L. Caswell and L. Hawkman. 1988. Induction of somatic embryos and plantlets from cryopreserved cell culture of white spruce (*Picea glauca*). *J. Plant Physiol.* 132: 529-539.

- Kataki, S. K. (ed.) 1986. Orchids of Meghalaya. Govt. of Meghalaya, Adland Publicity Pvt. Ltd., Calcutta, India.
- Kataki., S . K. 1993. The present status of some selected orchid genera in North Eastern India. J. Orchid Soc.India. 7 (1-2): 79-81.
- 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.
- 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. 1983. "Anatomical and Ecological marvels in Orchids," New Delhi.
- Kendall, E. J., K. K. Kartha, J. A. Qureshi and P. Chermak. 1993. Cryopreservation of immature spring wheat zygotic embryos using abscisic acid pretreatment. Plant Cell Reports, 12: 89-94.
- Khaw, C. H., H. T. Ong and H. Nair. 1978. Hormones in the nutrition of orchid tissues in mericlone. Malay Orchid. Rev. 13: 60-65.
- Kim, K. K., J. T. Kunisaki and Y. Sagawa. 1970. Shoot tip culture of *Dendrobium*. Amer. Orchid Soc. Bull. 39: 1077-1080.
- Kirtikar, P. K. and B. D. Basu. 1935. "Indian Medicinal Plants" 2nd Ed. (Reprinted, 1975), Dehra Dun.

- Knudson, C. 1946. A new nutrient solution for the germination of orchid seeds; Amer. Orch. Soc. Bull. 15: 214-217.
- 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, L. 1951. Nutrient solution for orchids. Bot. Gaz. 112: 528-532.
- Koch, L. 1974. Erbgleiche Vermehrung Von *Phalaenopsis in vitro* Gartenwelt. 74: 482-484.
- Kondo, K., I. V. Tatarenko, S. B. Varghese, Y. Iwai, and K. Matsumoto. 2001. Orchid Cryopreservation by using induced shoot primordia and protocormlike bodies of Tissue Culture. pp. 397-412. In: P. Pathak, R. N. Shekhar, M. Sharma and A. Sood (eds), Orchids: Science and Commerce, Bishen Singh Mahendra Pal Singh, New Connaught Place, Dehra Dun, India.
- 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.
- 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.
- Kukulczanka, K., B. Czastka and A. Marczonek. 1987. Effect of the substratum on the *In vitro* culture of *Cymbidium*. Acta Agrobot., 40 (1/2): 41-51.
- Kumaria, S. 1991. *In vitro* propagation of *Dendrobium fimbriatum* Var. *occulatum* KK. 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. 2000. Effects of growth regulators on peroxidase, polyphenol oxidase, IAA-oxidase and phenolic contents during protocorm development of *Dendrobium fimbriatum* var. *oculatum* Hk. f. Journal of Orchid Society of India 14: 27-39
- Kumaria, S. and P. Tandon 2001. Orchids: The world's most wondrous plant. In: P. Pathak, R. N. Shekhar, M. Sharma and A. Sood (eds.), Orchids: Science and Commerce, Bishen Singh MahendraPal Singh, New Connaught Place, Dehra Dun, India pp. 17-28

Kumaria, S. and P. Tandon. 2004. Nucleic acid and soluble protein contents at different stages of protocorm development of *Dendrobium fimbriatum* var. *oculatum* Hk. f. as influenced by growth regulators. Biosciences, Biotechnology Research Asia 2 (1): 65-70

Kumaria, S., N. K. Chrungoo and P. Tandon. 1990. Activities of some oxidative enzymes in axenic cultures of protocorms of *Cymbidium giganteum* Wall. as influenced by different growth regulators. J. Orchid Soc. India 4 (1-2): 37-44

Kumaria, S., S. Corrie., A. Sharma and P. Tandon. 2005. *In vitro* conservation of some commercially important orchids of North-East India. Int. J. For. Usuf. Mngt. 6(2): 36-40.

Kumu, Y., T. Harada and T. Yakuwa. 1983. Development of a whole plant from a shoot tip of *Asparagus officinalis* L. frozen down to -196°C . J. Fac. Agr. Hokaido Univ. 61; 285-294.

Kuo, C. C. and R. D. Lineberger. 1985. Survival of *in vitro* cultured tissues of "Jonathan" apples exposed to -196°C . Hort. Sci. 20: 764-767.

Kuranuki, Y and S. Yoshida. 1996. Different responses of embryonic axes and cotyledons from pea seeds to desiccation and cryoexposure. Breeding Sci., 46: 149-155.

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 S. Devi. 1999. Micropropagation of *Renanthera imschootiana* Rolfe through shoot-tip, axillary bud and leaf segment cultures. J. Orchid Soc. India. 13: 1-4.
- Lambardi, M., A. Fabbri and A. Caccavale. 2000. Cryopreservation of white poplar (*Populus alba* L) by vitrification of *in vitro* grown shoot tips. Plant Cell Reports, 19: 213-218.
- 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.
- 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 mycoheterotrophic 'Saprophytic' plants. New Phytol. 127: 171-216

- 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.
- 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.
- Lurswijidjarus, W. and K. Thammasiri. 2004. Cryopreservation of shoot tips of *Dendrobium* Walter Oumae by Encapsulation/Dehydration. *Sci. Asia.* 30:293-299.
- 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.
- Malabadi, R. B., G. S. Mulgund and N. Kallappa. 2005. Micropropagation of *Dendrobium nobile* shoot tip sections. *J. Plant Physiol.* Vol. 162 (4): 472-478.
- Mandal, B. B. 2000. Cryopreservation research in India: present status and future perspectives. *JIRCAS International Agriculture Series*, Vol. 8: 282-286.
- 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.

- Matsumoto, T., A. Sakai and K. Yamada. 1994. Cryopreservation of *in vitro* grown apical meristem of Wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regeneration. *Plant Cell Reports*, 13: 442-446.
- Matsumoto, T., A. Sakai and K. Yamada. 1995. Cryopreservation of *in vitro* grown apical meristem of Lily by vitrification. *Plant Cell, Tissue and Organ Culture*, 41: 237-241.
- Matsumoto, T., K. Mochida, H. Itamura, and A. Sakai. 2001. Cryopreservation of persimmon (*Diospyros kaki* Thunb) by vitification of dormant shoot tips. *Plant Cell Reports*, 20: 398-402.
- Mead, J. W and C. Bulard. 1979. Vitamins and nitrogen requirements of *Orchis laxiflora* Lamk. *New Phytol.*, 83: 129-136.
- Meryman, H. T., and R. J. Williams. 1982. Mechanisms of freezing injury and the principles of artificial cryoprotection, pp. 5-37. In: L. A. Withers and J. T. Williams (eds.), *Crop Genetic Resources. The conservation of difficult material.* IUBS Series B 42. IUBS/IBPGR/IGF, Paris.
- Meyer, J. R. 1951. Transplanting orchid seedlings from culture vessels to pots containing fern fibres. *Biologies*. 17: 99-102.
- 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.
- Miller, L. R. and T. Murashige. 1976. Tissue culture propagation of tropical foliage plants. *In Vitro*, 12: 797-813.

- 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.). *Biol. Conler and rchid Cult.* Affiliated East West prezss Ltd. pp. 401-412
- 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.
- Miyazaki, S. and T. Nagamatsu. 1965. Studies on the promotion of the early growth of *in vitro* orchid. Pt. I. *Agr. Bull. Saga. Pref. Univ.,* 21: 131-149.
- 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.
- Morel, G. M. 1960. Producing Virus-free *Cymbidium*. *Am. Orchid Soc. Bull.* 29: 495-497.
- Morel, G. 1964. Tissue culture-A new means of clonal propagation of orchids. *Amer. Orchid Soc. Bull.,* 33: 473-478.
- 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. 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., T. Akihawa and I. Kozaki. 1985. Freeze preservation of dormant pear shoot apices. *Japanese Journal of Breeding.* 35: 196-199.
- 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.
- Muire, Y. 1982. Establishment of orchid cultivation on the basis of photosynthetic properties of orchids. I. Influence of temperature, light intensity and air humidity on photosynthetic rate of orchids. *Bull. Kuregawa. Hort. Exptl. Stn.,* 28: 64-72.
- 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. *Biol. Cons 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*. 1978. 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.
- Mycock, D. J., J. Wesley-Smith and P. Berjak. 1995. Cryopreservation of somatic embryos of four species with and without cryoprotectant pretreatment. *Annals of Botany.* 75: 331-336.
- Na, H. and K Kondo. 1996. Cryopreservation of tissue cultured shoot primordial from shoot apices of cultured protocorms in *Vanda pumila* following ABA precultured and desiccation. *Plant Sci*, 118: 195-201.
- 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.
- Nagaraju, V. and S. K. Mani. 2005. Rapid *in vitro* propagation of orchid *Zygopetalum intermedium*. *J. Plant Biochem. and Biotech.*, vol. 14, 27-32.
- Nakamura, S. 1982. Nutritional conditions required for the non-symbiotic culture of an achlorophyllous orchid *Galeola septentrionalis*. *New Phytol.* 90: 701-715.
- 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.
- 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.

- Negrutiu, I., M. Jacob and D. Cachita. 1978. Some factors controlling *in vitro* morphogenesis of *Arabidopsis thaliana*. *Z. Pflanzen. Physiol.* 86: 113-124.
- Nikishina, T. V., A. S. Popov, G. L. Kolomeitseva, and B. N. Golovkin. 2001. Cryopreservation of seeds of some tropical orchids. *Doklady Biochem. and Biophysics.* Vol. 378: 231-233.
- Nitsch, J. P. 1969. Experimental androgenesis in *Nicotiana*: *Phythemorph.* 19: 389-404.
- Normah, M.N., H.F.Chin and Y.L. Hor. 1986. Desiccation and cryopreservation of embryogenic axes of *Hevea brasiliensis* Mull. Arg. *Pertanika*, 9: 299-303.
- 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. Az.* 145: 495-501.
- Oyamata, T. 1989. Effect of mineral nutrient composition on the proliferation of *Cymbidium* PLBs cultured *In vitro*. *Sci. Rep. Fac. Agr. Meijo Univ.*, 0 (25) 27-34.
- Ozias-Atkins, P. and I. K. Vasil. 1985. Nutrition of plant tissue cultures. In: I. K. Vasil (ed.), *cell culture and somatic cell genetics of plants*, Vol. II. Academic Press Inc., Harcourt Brace Jovanovich Publishers.
- Pages, P. G. 1971. Banana homogenate, coconut water, peptone and auxins as nutrient supplements in the *In vitro* culture of *Dendrobium* and *Phalaenopsis* ovules. *SERCA Bull No.2*, Coll. Lagnera, Phillipines.
- Papachatzi, M., P. A. Hammer and P.M. Hasegawa. 1981. *In vitro* propagation of *Hosta decorate* Thomas Hogg using cultured shoot tips. *J. Amer. Hortic. Sci.*, 106: 232-236.

- 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.
- 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.
- Pence, V. C. 1991. Cryopreservation of seeds of Ohio native plants and related species. *Seed Sci. Technol.*, Vol. 19: 235-251.
- Pence, V. C. 1995. Cryopreservation of recalcitrant seeds, pp. 29-52. In Y. P. S. Bajaj (ed.), *Biotechnology in Agriculture and Forestry* Vol. 32, Cryopreservation of plant germplasm I. Springer- Verlag, Berlin.
- Pennycooke, J. C and L. E. Towill. 2000. Cryopreservation of shoot tip from *in vitro* plant of sweet potato (*Ipomoea batatas* (L.) Lam.) by vitrification. *Plant Cell Reports*, 19: 733-737.
- 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.
- 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.
- Polge, C., A. U. Smith, and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperature. *Nature*. 164: 666-676.
- 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.
- Pyati, A. N. and H. N. Murthy. 1995. *In vitro* seed germination and seedling development of *Dendrobium ovatum*. (W) *Krunzbl. 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.
- Radhamani, J. and K. P. S. Chandel. 1992. Cryopreservation of embryonic axes of trifoliolate orange (*Poncirus trifoliata*). *Plant Cell Reports*. 11: 204-206.
- Raghavan, V. and J. G. Torrey. 1964. Inorganic nitrogen nutrition of the seedlings of the orchid *Cattleya*. *Amer. J. Bot.* 51: 264-274.
- Rajeevan, P. K and A. Sobhana. 1993. Performance of certain epiphytic species in Central Kerala. *J. Orchid Soc. India*, 7 (1-2): 31-35.

- Rao, A. N. 1977. Tissue culture in the orchid industry. pp. 44-69. In J. Reinert and Y. P. S. Bajaj (ed.) Applied and Fundamental aspects of plant cell, Tiss.Org.Cult. Springer-Verlag, Berlin.
- Rasmussen, H.N. and Rasmussen, R.N. 1991. Climatic and seasonal regulation of seed plant establishment *Dactylorhiza majalis* inferred from symbiotic experiments in vitro *Lindleyana* 5:221-227.
- 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., J. O. Fujii, and D. Slade. 1991. Synthetic seed technology. pp. 35-74. In: I. K. Vasil (ed.) Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, San Diego.
- Reed, P. E. 1992. Environmental and hormonal effects in micropropagation. In : K. Kurata and T. Kozai (eds.), Transplant Production Systems, pp. 231-246. Kluwer Academic Publishers, The Netherlands.
- Richardson, K. A., R. L. Peterson and R. S. Currah. 1992. Seed reserves and early symbiotic protocorm development of *Platanthera hyperborean* (Orchidaceae). *Can. J. Bot.* 70: 291-300.
- Robbins, 1989. *Phragmipedium bessae* (Orchidaceae). *The Kew Magazine.* Vol. 6: 156.
- Robbins, S. and S. Bell. 1990. *Clowesia rosea* (Orchidaceae). *The Kew Magazine.* Vol. 5: 51.
- Roos, E. E and D. A. Davidson. 1992. Record longevities of vegetable seeds in storage. *Hortic. Sci.* 27:393-396.

- 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.
- Roy, S. 1994. *In vitro* micropropagation of orchids and establishment of seedlings under natural conditions. Ph. D. Thess. G. U.
- Rucker, W. 1974. Einfluss von cytokininen anf wachstum und differenzierung *in vitro* Kultivierter protokorme von *Cymbidium*. Z. Pflanzen. Physiol. 72: 338-351.
- 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. 1986. cryopreservation of germplasm of woody plants, pp. 113-129. In: Y. P. S. Bajaj (ed.), Biotechnology in Agriculture and Forestry I. Springer-Verlag, Berlin, Heidelberg.
- Sakai, A, S. Kobayashi and I. Oiyama. 1990. Cryopreservation of nuceller cells of Navel orange (*Citrus sinensis* osb.var.*brasiliensis* Tanaka) by vitrification. Plant Cell Reports, 9: 30-33.
- Sakai, A, S. Kobayashi and I. Oiyama. 1991. Survival by vitrification of nuceller cells of Navel orange (*Citrus sinensis* osb.var.*brasiliensis* Tanaka) cooled to -196 °C. J. Plant Physiol. 137: 465-470.
- Sakai, A. 2000. Development of cryopreservation techniques. pp. 1-7. In: F. Engelmann and H. Takagi.(eds.). Cryopreservation of tropical plant germplasm. JIRCAS, Tsukuba, Japan.

- Sakai, A, T. Matsumoto, D. Hirai and R. Charoensub. 2002. Survival of tropical apices cooled to -196°C by vitrification-development of a potential cryogenic protocol of tropical plant by vitrification, pp. 109-119. In: P. H. Li and E. T. Paiva (eds.). Plant cold hardiness-gene regulation and genetic engineering. Kluwer/Plenum, New York.
- Sanchez, M. I. 1988. Micropropagation of *Cryptopodium* (orchidaceae) through root-tip culture. *Lindleyana* 3: 93-96.
- Sander, D. 1979. Orchids and their cultivation, Blandford Press, Dorset, U. K
- Seibert, M. 1976. Shoot initiation from carnation shoot apices frozen to -196°C . *Science*. 191: 1178-1179.
- Senthilkumar, S. 2001. Problems and prospects of orchid mycorrhizal Research. *J. Orchid Soc. India*. 15: 23-32.
- 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, 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

- 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.
- Smith, S. E. and D. J. Read. 1997. Mycorrhizal symbiosis. Academic press, London.
- Soedjono, S. 1988. Simple medium for regeneration of PLBs of *Dendrobium Walter Oumae*. Bulletin Penelitian Hortikultura, 16 (1): 73-78.
- 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.
- 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, 5(2): 51.
- Stimart, D. P. and P. D. Ascher. 1981. *In vitro* germination on a completely defined medium. Scientific Hort. 14: 165-170.
- Stoutamire WP. 1974. Terrestrial orchid seedlings. In: C. L. Whithner (ed.). The orchids: scientific studies pp 101-128. John Wiley and Sons, New York.
- 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 (ed.) E. H. Planton (Southfield : Michigan Orchid Society)

- 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, M. and S. Roy. 1996. Plantlet regeneration from seed culture of *Arundina graminifolia* (D. Don) Hochr. and establishment of plantlets under natural condition. In LK Pareek and PL Swarnakar (ed.), Trends in plant tissue culture and Biotechnology Agro. Bot. Publishers. pp. 248-252.
- Sharma, N. and B. Sharma. 2003. Cryopreservation of shoot tips of *Picrorhiza kurroa* Royle ex Benth., An Indigenous Endangered Medicinal Plant, through Vitrification. Cryo-Letters, 24: 181-190.
- Sharon, M. and G. Vasundhara. 1990. Micropropagation of *Dendrobium Joannie Ostenhault*. J. Orchid Soc. India. 4: 145-148.
- 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.
- 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.
- Shiau, Y., S. M. Nalwade., C. Hsia., V. Mulabagal and H. Tsay. 2005. *In vitro* propagation of the Chinese medicinal plant, *Dendrobium candidum* Wall. Ex Lindl., from axenic nodal segments. In Vitro Cell. Dev. Biol.-Plant, 41:666-670.
- Singh, D. K. 2001. Orchid Diversity in India: An Overview. In : Orchids: Science and Commerce (Pathak P, Shekhar R. N, Sharma M and Sood A eds.), Bishen Singh Mahendra Pal Singh, New Connaught Place, Dehra Dun, India pp. 35-65.

- 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, A. 2001. Multiple shoot induction in *Dendrobium aphyllum* Roxb. J. Orchid Soc. India. 15: 35-38.
- Talukdar, M., L. Paswan and S. Barna. 1988. Flowering behavior in relation to differential pot mixture treatment in orchids. In: S. P. Vij and S. P. Kullar (eds.). National seminar on current research trends in Indian orchids with special reference to tissue culture technology, pp. 34., Shillong.
- 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. 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., 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.
- Tandon, P. 2000. Role of Biotechnology in Conservation of plant Genetic Resources in the 21st century-An Indian Perspective. Pp.25-47. Platinum Jubilee Lectures Indian Science Congress, Kolkota.
- Tandon, P. 2004. Conservation and sustainable development of plant resources of North East India. *Man and Society* 1 (1): 49-59.
- Tandon, P. and S. Kumaria. 1998. Threats to plant diversity in high altitude of North-East India and conservation of rare and endangered plants using biotechnological approaches. In: S. Raha, P. K. Ray and B. Sinha (eds.), *Science at High Altitude. Proceeding of the National Symposium*. Allied Publishers Limited, India pp. 140-147.
- Tandon, P. and S. Kumaria. 2005. Prospects of plant conservation biotechnology in India with special reference to Northeastern region. In: P. Tandon, M. Sharma and R. Swarup (eds.), *Biodiversity: Status and Prospects*. Narosa Publishing House, New Delhi, India pp. 79-91.
- Tay, L. J., T. Kiyotoshi and Y. Hori. 1988. Culture conditions suitable for *In vitro* seed germination and development of seedlings in *Paphiopedilum*. J. Jpn. Soc. Hortic. Sci., 57 (2) : 243-249.

- 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.
- Thammasiri, K. 2000. Cryopreservation of seeds of a Thai orchid (*Doritis pulcherrima* Lindl.) by vitrification. Cryo-Letters. 21: 237-244.
- Thompson, R. P. 1971. Excision of a *Cymbidium* meristem: Photographed in colour. Am. Orchid Soc. Bull. 40: 580-584.
- Thorpe, T. A. and T. Murashige. 1970. Some histochemical changes underlying shoot initiation in tobacco callus cultures. Can. J. Bot., 48: 277-285.
- Tikhonova, V. A. 1999. Long-Term storage of seeds. Fiziol Rast. (Moscow), Vol. 46: 467-476.
- Tikhonova, V. L., N. I. Lysykh, and V. M. Firsanova. 1997. The effect of freezing on the viability of wild plant seeds. Byull. Gl. Bot. Sada (Moscow), 175: 83-90.
- 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, 127-134.
- 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.
- Touchel, D. H., V. L. Chiang and C. J. Tsai. 2002. Cryopreservation of embryogenic culture of *Picea mariana* (black spruce) using vitrification. *Plant Cell Reports*, 21: 118-124.
- Towill, L. E. 1984. Survival at ultra-low temperature of shoot tips from *Solanum tuberosum* groups Andigena, Phurreja, stenotomom, Tuberosum and other tuber-bearing *Solanum* species. *Cryo-Letters*. 5: 319-326.
- Towill, L. E. 1990. Cryopreservation of isolated mint shoot tips by vitrification. *Plant Cell Reports*. 9: 178-180.
- Towill, L. E. and L. Jarett. 1992. Cryopreservation of sweet potato (*Ipomoea batatas* L. Lam) shoot tips by vitrification. *Plant Cell Reports*. 11: 175-178.
- Tran Thanh Van, M., N. T. Dien, and A. Chlyah. 1974. Regulation of organogenesis in small explants of superficial tissue of *Nicotiana tabacum*. *Planta*, Vol. 119 (2): 149-159.
- Trivedi, K. P., D. S. Garg, J. P. Agarwal and D. D. Garg. 1961. Amarkand (*Eulophia nuda*). *Dhanvantri Vanausadhi Visheshank*, Part I: 201-203.
- Tsukazaki, H., M. Mii, K. Tokuhara and K. Ishikawa. 2000. Cryopreservation of *Doritaenopsis* suspension culture by vitrification. *Plant Cell Reports*. 19: 1160-1164.

- Ueda, H. and H. Torikata. 1972. Effect of light and culture medium on adventitious root formation by *Cymbidiums* in aseptic culture. Amer. Orch. Soc. Bull. 41:322-327.
- Uesato, K. 1978. Studies on the formation and development of protocorms in growth cycle of orchids. Bull. Coll. Agric. Univ. Ryukus. 25: 1-76.
- Ulrich, J. M., B. J. Finkle, P. H. Moore and H. Ginoza. 1979. Effect of a mixture of cryoprotectants in attaining liquid nitrogen survival of callus cultures of a tropical plant. Cryobiology 16: 550-556.
- Uragami, A., A. Sakai, M. Nagai, and T. A. Takahashi. 1989. Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. Plant Cell Reports. 8: 418-421.
- Vacin, E. and F. W. Went. 1949. Culture solution for orchid seedlings. Bot. Gaz. 110: 605-613.
- Van Waes, J. M. and P. C. Debergh. 1986. *In vitro* germination of some western European orchids. Physiol. Plant. 67: 253-261.
- Van, L. B., P. T. N. Hang, H. L. T. Anh and T. V. K. Tran. 1999. High frequency shoot regeneration from *Rhynchosstylis gigantea* (orchidaceae) using thin cell layers. Plant growth Reg. 28: 179-185.
- Vandenbussche, B., G. Weyens and M. De Proft. 2000. Cryopreservation of *in vitro* sugar beet (*Beta vulgaris*) shoot tip by vitrification technique. Plant Cell Reports, 19: 1064-1068.
- Varma, A. 1995. Arbuscular mycorrhizal fungi: State of Art. Crit. Rev. Biotechnol., 15: 179-200.

- Varma, A. 1998. Mycorrhizae: Their role in sustaining wasteland. In: Problems of Wasteland development and Role of Microbes (ed.) Mishra pp. 161-181. AMIFEM Publications.
- Vasil, K. 1982. Plant Cell Culture and somatic cell genetics of cereals and grasses. Vasil 1, Scowcraft W.R and K.J. Frey (ed.) PL. Imp. Somatic Cell. Gen. Academic Press, New York. pp: 179-203.
- Vasil, V., A. M. Castillo, M. E. Fromm and I. K. Vasil. 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. *Bio/Technology*. 10: 667-674.
- 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. 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 S. Kaur. 1992. Regeneration potential of foliar peels of *Rhynchostylis retusa*. J. Orchid Soc. India. 6: 71-74.
- Vij, S. P. and P. Kaur. 1994. Green pod culture in *Phaius tankervilleae*. 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 S. Kaur. 1998. Micropropagation of therapeutically important orchid: *Malaxis acuminata*. D. Don. J. Orchid Soc. India. 12: 89-93.
- 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. In: S. C. Verman (ed.). Contemporary trends in plant Science, Kalyani publisher, New Delhi. pp: 473-481
- 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 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., 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. 1997. On the regeneration competence of *Coelogyne viscosa* pseudobulb explants. In: proc. 5th National Seminar on Developmental Biology and Commercialization of Orchids (ed. S. P. Vij) pp. 88. TOSI, Chandigarh, India.
- 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., 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.
- Vu Quoc Luan, N., Q. Thien, D. V. Khiem and D. T. Nhut. 2006. *In vitro* germination capacity and plant recovery of some native and rare orchids. In: Proc. Of International Workshop on Biotechnology in Agriculture., Nong Lam University. pp. 175-177. Ho Chi Minh City.
- 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.
- Wang, P. J. 1989. Rapid clonal propagation of *Phalaenopsis* by tissue culture. *Acta Hort. Scien.* 16: 73-77.
- Wang, J. H., J. G. Ge, F. Liu, H. W. Bian and C. N. Huang. 1998. Cryopreservation of seeds and protocorms of *Dendrobium candidum*. *Cryo-Letters*. 19: 123-128.

- White, P. R. 1951. Nutritional requirements of isolated plant tissue and organs. *Ann. Rev. Plant Physiol.* 2: 231-244.
- Wimber, D. E. 1963. Clonal multiplication of *Cymbidium* through tissue culture of the shoot meristem. *Am. Orchid Soc. Bull.* 32: 105-107.
- Withner, C. L. 1959. Orchid Physiology, pp. 315-360. In: C. L. Withner (ed.), *The Orchids: A Scientific Survey*, Ronald Press, New York.
- Withers, L. A. 1985a. Cryopreservation of cultured cells and protoplasts, pp. 243-267. In: K. K. Kartha (ed.), *Cryopreservation of plant cells and organs*. CRC Press Inc, Boca Roton.
- Withers, L. A. 1985b. Cryopreservation of cultured cells and meristems, pp. 254-316. In: I. K. Vasil (ed.), *Cell Cultures and Somatic Cell Genetic Plants*. Vol.2. Acad. Press, New York.
- Withers, L. A. 1987a. *In vitro* methods for germplasm collection in the field. *Plant Genetic Resources Newsletter*, 69: 2-6.
- Withers, L. A. 1987b. Long term preservation of plant cells, tissues and organs. *Oxford Surveys of Plant Molecular and Cell Biology*, 4: 221-272.
- Wood, C. B., H. W. Pritchard and A. P. Miller. 2000. Simultaneous preservation of orchid seed and its fungal symbiont using encapsulation-dehydration is dependent on moisture content and storage temperature. *Cryo-Letters*. 21: 125-136.
- 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.

- Kullar (eds.). National 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., 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 propagation of *Phalaenopsis* by means of flower stalk, bud culture and shoot tip culture. *Bull. Coll. Agri and Vet. Med.* 40: 1-13.
- Zeigler, A. R., T. J. Sheehan and R. T. Poole. 1967. Influence of various media and photoperiod on growth and amino acid content of orchid seedlings. *A.O.S* 195-201.
- Zeigler, E., C. Grivet, S. M. Assmann, G. F. Deitzer and M. W. Hannegan. 1985. Stomatal limitation of carbon gain in *Paphiopedilum* sp. (Orchidaceae) and its reversal by blue light. *Plant Physiol. (Bethesda)* 77(2): 456-460.

Zettler, L.W. and T.M. Mcinnis. 1993. Symbiotic seed germination and development of *Spiranthes cernus* (L.) L.C. Rich, and *Goodyera pubescens* (Wild.) R. Br. (Orchidaceae: Spiranthoideae). *Lindleyana* 83:155-162.

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Ph.D. Thesis Title: Clonal propagation and cryopreservation of *Dendrobium longicornu* Lindl. and *Dendrobium formosum* Roxb.: Two endangered orchids of North-East India

Research Activities

Book (Chapter)

Dohling, S., M. C. Das, S. Kumaria and P. Tandon. Conservation of splendid orchids of North-East India. In: P. Tandon, Y. S. Abrol and S. Kumaria (ed.) Biodiversity and Significance. pp.354-365. I. K. International Publishing House Pvt. Ltd. New Delhi.

Poster presented in National Seminars/Conferences

Dohling, S., S. Kumaria and P. Tandon. *In vitro* propagation of some splendid dendrobes of North-East India. (Poster). T. N. Khoshoo Memorial Function, N.B.R.I. Lucknow. April 07-08, 2004

Dohling, S., S. Kumaria and P. Tandon. High frequency plantlets regeneration of *Dendrobium longicornu* and *Dendrobium formosum* through Asymbiotic seed culture. (Poster). 8th National Seminar on Orchid Conservation Improvement and Commercialization and Satellite Symposium on Orchids: Why and How? The Orchid Society of India (TOSI), Panjab University, Chandigarh, March 18-20, 2006.

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