MICROPROPAGATION AND SHORT TO MEDIUM TERM *IN VITRO* CONSERVATION OF TWO THREATENED ORCHIDS OF NORTH-EAST INDIA

BY

SUNGKUMLONG

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BOTANY

OF

DEPARTMENT OF BOTANY

NAGALAND UNIVERSITY, HEADQUARTERS: LUMAMI

MOKOKCHUNG-798 601, NAGALAND, INDIA

2008

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Dedicated

To

My Loving

Mom & Dad

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NAGALAND UNIVERSITY

December 2008

DECLARATION

I, **Sungkumlong,** bearing Ph. D. registration No. 197/2005 dated November 18, 2004 hereby, declare that the subject cited matter of my Ph. D. thesis entitled "**Micropropagation and Short to Medium-Term *In Vitro* Conservation of Two Threatened Orchids of North-East India**" is the record of work done by me, and that the contents of thesis did not form the basis for award of any degree to me or to anybody else to the best of my knowledge. The thesis has not been submitted by me for any research degree in any other University/Institute.

This thesis is being submitted to the Nagaland University for the degree of 'Doctor of Philosophy' in Botany.

(Sungkumlong)
Candidate

(Dr. Chitta Ranjan Deb)
Supervisor

Dr. Chitta Ranjan Deb
Faculty, Deptt of Botany
Nagaland University, HQ : Lumami
Mokokchung-798601, India

(Prof. N. S. Jamir)
Head of the Department

Head
Deptt. of Botany
Nagaland University
Hqs : Lumami

AGKNOWLEDGEMENT

1. I express my profound gratitude and happiness to my Dynamic and Loving Dear Teacher **Dr. Chitta Ranjan Deb**, Senior Lecturer, Department of Botany, Nagaland University, Headquarters: Lumami. Who always stand beside me and corrected my shortcomings with his Valuable and Unlimited Knowledge with his Enthusiastic Character, which I'll cherish forever and would always remain grateful for his able guidance.

"Sir, it was a Great Honor for me and Proud to say that you are my Teacher. Thank you, Sir"

2. I would like to thank the Hon'ble Vice-Chancellor, Prof. K. Kannan, Nagaland University, for giving valuable encouragement and allowing having access to the required University facility and Infrastructures throughout my research work.

3. I am also thankful to Prof. T. Lanusosang, Professor In-Charge, N.U. Hqrs: Lumami, Prof. Pradeshi Lal, Dean, School of Sciences, N.U. for giving me all the encouragement throughout my research work.

4. I am thankful to Prof. N. S. Jamir, Head, Department of Botany, Nagaland University for giving me constant correction, encouragement and allowing to have access to the required Department's facility.

5. I am also thankful to Dr. S. K. Chaturvedi, Dr. Neizo Puro, Dr. Talijungla, Dr. Limasenla and Mr. Sanjoy Kumar, Faculties, Department of Botany, N. U. for their valuable encouragement which I'll always cherish.

6. I offer my sincere thanks to G. B. Pant Institute of Himalayan Environment and Development, Kosi 263 43, Almora, Uttaranchal for JRF and SRF through a Research Project under my Supervisor Dr. Chitta Ranjan Deb, Department of Botany, Nagaland University, Lumami.

Contd...

7. I am thankful to Mrs. Madhabi Deb, for giving me endless support with her love, caring, cheerful and helpful characters throughout my study which I'll always cherished and remembered.

8. I am also thankful to my Lab-mate Dr. Temjensangba, who always advised me and do critical analysis in all my research works and giving valuable suggestion and endless support with his helpful character throughout my study.

9. I also like to thank my lab-mate Mr. Bendangchuchang, Ms. Aolemla and Ms. Arenmomgla, Ms. Bendangmenla and all my friends for their endless help in every possible way.

10. I also thank my Loving Sister and my two beloved Brothers who always encouraged me and support with their unlimited Prayers, Love and Care throughout my research work.

11. Lastly, I thank and offer all my credits to my beloved Mom & Dad, for their humble Prayers, Affection and Encouragement throughout my research work and never left me alone with their abundant Love, Care and Prayers.

(Sungkumlong)

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Chapter-1

Introduction

Biological diversity is the very basis of human survival and economic well-being as it provides food, clothing, shelter, medicine, biomass, energy and industrial raw materials, and yet there remains a great deal to or waiting to be discovered for human use. The word '***Biodiversity***' means the multiplicity of life forms including their genetic diversity and the assemblages they form, or in other words, the variety and variability in which they occur. The three different levels of biodiversity are: **1. *Diversity of ecosystem,* 2. *Diversity of species,* and 3. *Diversity of the genetic pool within species***. It is estimated that the species diversity living in our planet is about 5 to 50 million, out of which only 1,435,662 species have been described so far (Wilson, 1988). But, the rich biodiversity of the planet is under siege due to various factors. The human population has witnessed a three-fold increase in the last century and the rate of fossil fuel consumption has increased by 12-fold during the period. It is estimated that, the carrying capacity of earth would saturate by the middle of this 21st century (Myers, 1990). According to IUCN

(IUCN. 2000, *Red List of Threatened Species,* Switzerland: The World Conservation Union), plant species are declining in south and central America, Central and West Africa and Southeast Asia. Malaysia has the most threatened species (681) followed by Indonesia (384), and so on. Globally the number of threatened plant listed is 5,611, but this data is based on only 4% of described plant, which suggest that the percentage of threatened plant may be much higher. The recent report of IUCN brings out a list of 34 biodiversity hotspots region of the world; indicates an alarming situation the world is faced with, in terms of biodiversity resource *vis-a-vis* future of mankind. Biodiversity hotspots are geographical region which are extremely rich in species, have high endemism, and are under threat of destruction. The 34 biodiversity hotspots by region are: 4 in **North and Central America** (*California floristic province, Caribbean Island, Madrean pine-oak woodlands, Meso-America*); 5 in **South America** (*Atlantic Forest, Cerrado, Chilean Winter Rainfall-Valdivian Forest, Tumbes-Choco-Magdalena, Tropical Andes*); 4 in **Europe and Central Asia** (*Caucasus, Irano-Antolian, Mediterranean, Mountains of Central Asia*); 9 in **Africa** (*Cape Floristic Region, Coastal forest of eastern Africa, Eastern Afromontane, Guinean Forest of West Africa, Horn of Africa, Coastal Forest of Eastern Africa, Madagascar and the Indian Ocean Islands, Maputaland-Pondoland-Albany, Succuent Karoo*); 13 in **Asia-Pacific** (*East Melanesian Island, Himalaya, Indo-Burma, Japan, Mountains of Southwest China, New Caledonia, New Zealand, Philippines, Polynesia-Micronesia, Southwest Australia, Sundaland, Wallacea, Western Ghats and Sri Lanka*).

India is one of the 17 mega biodiversity countries, and has 26 recognized endemic centres that accounts for nearly a third of the flowering plants, though it constitute only

2.4% of land mass. Also, is a host of 3 biodiversity hotspots viz: Himalayas, Indo-Burma and Western Ghats and Sri Lanka. It is not only rich in biological diversity but is also an important centre of origin of agri-biodiversity. The Endemism of Indian biodiversity is impressively high, about 33% of the country's recorded flora are endemic to the country and are concentrated mainly in the North-East India, Western Ghats, North-West Himalayas and Andaman and Nicobar islands. India has a total of 89,451 animal species accounting for 7.31% of the faunal species in the world and the flora accounts for 10.78% of the global total (MoEF 1999). But according to State of Forest report 1999 (FSI, 2000), the forest cover in India is losing at an alarming rate coupled with various factors, which poses greater threat to the rich biodiversity of the country. The main causes of habitat loss are agricultural activities, extraction (including mining, logging and harvesting) and unplanned developmental works. The underlying causes of biodiversity loss, however, according to Wood *et al,* (2000) are poverty, macroeconomic policies, international trade factors, policy failures, poor environmental law/weak enforcement, unsustainable development projects and lack of local control over resources.

North-East India is equally rich in floral and faunal diversity and contains more than one-third of the country's total biodiversity. It lies between 22° 9′ - 29° 6′ N latitude and 89° 7′ E longitude, is known for its diverse and most extensive lush forest cover and species composition, but is one of the major regions facing severe deforestation. The region is one of the 18 hottest hotspots of the world, having at least 7, 500 flowering plants out of which 700 are orchids, 58 bamboos, 64 citrus, 28 conifers, 500 mosses, 700 ferns and 728 lichen species. The region is considered a meeting region of temperate east Himalayan flora, paleo-artic flora of Tibetan highland and wet evergreen flora of South

East Asia and Yunnan, forming a bowl of biodiversity. The region host a number of botanical curiosities like *Sapria himalayana, Nepenthes khasiana* and saprophytic orchids like species of *Epipogium and Galeola* and primitive angiosperm plants like *Exbucklandia, Manglietia, Holboellia,* etc. The rich presence of ancient plant like *Magnolia, Michelia, Camellia, Rhododendron,* orchids etc. in the region indicates that evolutionary development in wild and cultivated plants are continuously taking place (Choudhery, 2001). Takhtajan (1969) named the region "*The cradle of flowering plants*". The region is one of the richest and most interesting floristic regions of India, with orchids forming a prominent feature of the vegetation.

North-East region is endowed with almost all types of vegetation and has number of 'sacred groves or forest'. It is estimated that out of 1229 species of orchid known from India, about 750 to 800 species are found in North-East region of the country (King and Pantling, 1898; Pradhan, 1979; Kataki, 1986; Satish Kumar and Manilal, 1994; Chowdhery, 1998; Hynniewta *et al*, 2000; Deb *et al*, 2003; Deb and Imchen, 2008). A comparative analysis of distribution of orchid species within the region shows that maximum diversity is found in Arunachal Pradesh followed by Sikkim and the lowest in Tripura. The region has the highest number of monotypic orchid genera while large number of saprophytic orchid species belonging to the genera *Aphyllorchis, Cymbidium, Epipogium, Eulophia, Galeola, Gastrodia, Stereosandra*, etc. On the other hand, due to wide altitudinal variation and topographical features supported by favorable climatic conditions endowed the state with a rich floristic biodiversity including huge number of orchid species. The habitats of orchids are classified on the basis of different vegetation and forest types, they are: tropical moist evergreen and deciduous forest type (100- 1000

m), subtropical evergreen and semi evergreen forest type (1000- 2000 m), temperate and sub temperate forest type (2000- 3500 m) and alpine zone (3500- 5000 m).

Orchidaceae family is one of the largest among the flowering plants with a worldwide distribution comprising about 25,000-35,000 species in some 800 genera (Chowdhery, 2001; Deb *et al*, 2003; Deb and Imchen, 2008). More new species are being added every year. Orchids are distributed all over the world excepting Antarctica and are found in almost every colour except black. In Australia even two species are found in underground condition e.g. *Rhizanthella gardneri* and *R. slateri* and are extremely rare. They show an incredible range of diversity in shape, size, and colour of their flowers, and are highly valued for their beauty. This wondrous and beautiful plant has been attracting floriculturists since time immemorial and has led to 'Orchid Mania' throughout the world. They command a high market value due to their beautiful and long-lasting flowers. In addition to their commercial value, orchids are of considerable importance in medicines, food, and perfumes, while some are reported to have antibacterial activity.

Generally, orchids are classified into saprophytes (without leaves), terrestrial and epiphytes (stem/pseudobulb with leaves) on the basis of their habits. Majority of the orchids are epiphytes and generally found perched on tree trunk. Some grow as terrestrial on land, as lithophytes on rock/stones and as saprophytes on decaying organic materials. Roots help them in anchoring with the substratum of their habitat. Orchids exhibits amazing diversity in shape, size and flower colour. The smallest orchid measures only one mm across (*Bulbophyllum globuliforme*), while *Vanilla* species can climb the tallest tree in the forest and can be up to 20 m long whereas, *Grammatophyllum speciosum* is the biggest orchid in the world. Orchids are differentiated not only by their flowers but

also from the leaves and roots. Orchids are further divided into two types on the basis of vegetative structure and its growth: ***Monopodial orchids*** – they do not have rhizomes or pseudobulbs but grow from single vegetative apex continuously season after season e.g.: *Aerides, Rhynchostylis, and Vanda* and ***Sympodial orchids*** – they have number of vegetative apices situated in the rhizomes e.g.: *Coelogyne, Dendrobium, Bulbophyllum.* The rhizomes/bulb/pseudobulbs act as reserved organ and help the orchids to combat the extreme drought conditions faced by epiphytic orchids. Orchids stand distinct in having velamenous roots; zygomorphic flowers with well-developed gynostegium, compound pollen, elaborate perianth and resupinate ovaries; and microscopic and non-endospermic seeds with undifferentiated (reduced) embryos. The orchids are adapted to insect pollination; their flowers flaunt a variety of temptations, i.e. bright colours, a safe landing platform in the form of labellum (lip), a nourishing drink, tantalizing odours and even sex to the pollinators (insects).

Orchids are known to mankind for the last several centuries for their beautiful attractive flowers and as medicinal plants. One may find mentioned in the work "*Enquiry into Plants'* of Theophrastus, a Greek Philosopher. The Chinese who have been growing orchids for the last 500 years is the symbol of Scholar–unassuming enduring and ascetic, it also stood as a symbol of loves, beauty, grace nobility and elegance in a women. "*Paint bamboo when you are angry, orchids when you are happy*" is a well-known Chinese saying. Orchids have adapted themselves to extremes of the environmental conditions producing thereby great variations in vegetative forms and one may often find it difficult to identify them as orchids if they are not in the flower. Many orchid flowers resemble in shape of a slipper (*Paphiopedilum, Cypripedium*), dancing girl (*Oncidium, Renanthera*),

moth (*Phalaenopsis*), spider (*Brassia*), scorpion (*Arachnis*), bee (*Ophrys*), pineapple orchid (*Dendrobium densiflorum*), etc. Orchids are popular worldwide due to their marvellous flower architecture and the spectrum of colours, and comprise one of the most successful groups of plants- the Orchidaceae. (370-285 BC) (Chowdhery, 1998).

The North-East region of India is rich in biodiversity, which has played an important role in the economy of the region from ancient times. Unfortunately the plant genetic resources of the region in general and orchid diversity in particulars are fast depleting due to indiscriminate felling of forest trees including ground vegetations for 'slash and burn/shifting cultivation' together with ruthless exploitation of plants for trade and unplanned human activities. In the recent years, extinction has been the destiny of a great number of plant species including several unique and irreplaceable varieties, while many await a similar fate. Some of the rare and endemic, threatened, endangered orchids of the region are like *Arachnis flos-aeris, A. labrosa, Aerides odorata, Anoectochilus crispus, Bulbophyllum rotschialdianum, Calanthe ciciliae, Ceratostylis himalaica, Cleisostoma appendicutatum, C. filiforme, C. racimeferum, Coelogyne hitendrae, C. **suaveolens**, C. griffithii, Cymbidium iridioides, C. tigrinum, Dendrobium aggretum, D. chrysotoxum, D. densiflorum, D. devonianum, D. moschatum, D. nobile, D. williamsonii, Eria alba, Liparis bituberculata, Oberonia clarkii, O. denculata, O, orbicularis, Panisia apiculata, Paphiopedelium hirsutissimum, Peristylus mannii, Pholidota griffithii, Renanthera imschootiana, **Taenia latifolia,** T. viridi-fusca,., Vanda bicolor, V. coerulea,* and many more.

Orchids are an important ornamental crop in floriculture industry due to their beautiful foliage, colourful and fragrant flowers of varying shapes, and long vase life of

cut flowers. The amenability of these plants to hybridization has been successfully exploited by man to raise novel and striking hybrids in his horticultural pursuits (Vij, 2002). This fascinating feature has placed them at the top most position in aesthetic world. This group of plants is highly valued both in the national as well as in the international markets. However, unlike the south Asian countries, India has not been able to make inroads into this multibillion dollars business despite rich natural wealth of orchid diversity (Kumar and Manilal, 1994). So, commercial orchid growing is primarily in the hands of hobbyist and nurserymen, who collect orchids from naturally grown population, to meet their national and international commitments, adding to conservation related problems (Chadha, 1992).

Although, one of the largest families among flowering plants, the orchids are also probably among the most seriously threatened group of plants. Their vulnerability stems from two factors: the first being their highly specialized nature of germination and growth in association with a specific fungus and pollinator insects, and second being the attractive and beautiful flower of many species, making them so-sought after by man. In recent years though biotechnological means has been adopted for their multiplication in mass scale and a ban has been imposed for their wild collection for trade under *Convention on International Trades in Endangered Species* (CITES) regulations, through wild life conservation laws, still there are activities of collections from the wild. In developing countries like India, shifting cultivation and continual expansion of agricultural land coupled with deforestation for developmental activities have been a major threat to these plants. Each orchid species is adapted to life in a specialized environment. Because of their specialized requirements many orchids are very restricted

in distribution and endemism is high in many cases (Hegde and Sinha, 2002). Habitat destruction and disturbances coupled with lack of ecological awareness of people in general have driven some of the orchids from their natural niches to near extinction. Destruction and fragmentation of forest causes decrease in pollinator population. This results in the low frequency of pollinators visiting the orchid flowers. Nearly 98 per cent of flowering individual fail to set fruits under natural conditions due to lack of pollinators (Calvo, 1993). Mass propagation using conventional and tissue culture techniques thus seem to be the only strategy to commercialize orchids and conserve their natural populations from collection pressures (Vij, 2002).

The orchid seeds are microscopic and non-endospermous with undifferentiated embryos are produced in large numbers and their germination in nature is depend upon a suitable association with a mycorrhizal fungus to provide an essential physico-chemical stimulus for initiating germination (Harley, 1959). Ever since Knudson (1922) demonstrated the possibility of bypassing the fungal requirement of orchid seeds during germination *in vitro*, asymbiotic seed germination has been accepted as important tools for propagating orchids (Arditti *et al*, 1982). The orchids are primarily sexual but they also reproduce and propagate by vegetative means as well through seeds. The rate of vegetative propagation (i.e. keikis, back-bulbs, division of shoots etc.) is very slow in many orchid species and seed germination in nature is also very poor (~0.2-0.3%) because of their poorly organized and lack of an appropriate metabolic machinery to utilize their own lipidaceous food reserves, and required a fungal stimulus for germination in nature. The asymbiotic germination potential of fertilized ovules (seeds) has been positively tested in several commercially viable and or threatened Indian taxa

(Vij, 2002). But, not all the orchid needs the same nutrient composition but response of orchid seeds to physio-chemical factors differs from species to species.

Micropropagation, *In Vitro* Conservation of Rare and Threatened Orchids and Plant Tissue Culture: The Current Status

Orchids which were earlier thought to be parasites growing on trees are in fact the most advance group of flowering plants. The orchids are propagated by vegetative means as well through seeds. However, orchid seeds due to microscopic size i.e lack of endosperm require a special fungal association (mycorrhiza) to germinate in nature. The rate of seed germination, therefore, is very poor, i.e., 0.2-0.3% in nature. The mycorrhizal association is believed to help in the carbohydrate/auxin/vitamin transport. Knudson (1922) for the first time demonstrated the possibility of bypassing the fungal requirements during germination of *Cattleya* seeds *in vitro* with the supply of appropriate organic carbon in the medium, while Tsuchiya (1954) discussed the possibility of germinating orchid seeds from immature pods. The discovery of these two techniques led to the development of 'green pod culture' that enabled to rescue hybrid embryos from desired mating (Sagawa, 1963). However, it calls for devising protocols for rapid cloning for exploitation of elite hybrids. *In vitro* cloning of *Phalaenopsis* using uni-nodal floral stock cuttings was developed by Rotor (1949), whereas Thomale (1957) successfully cultured the shoot tips of *Orchis maculata,* but the possibility of using aerial roots for micropropagation was first suggested by Beechey (1970). Morel (1960) is credited for mass propagation of virus free *Cymbidium* clones from apical shoot meristem on Knudson 'C' medium. Shoot tips remain the most commonly used explants for

micropropagating *Cymbidiums* and other sympodial orchids but their utility is limited in monopodials as it involves the removal of the only growing apex, which endangers the survival of mother stock. Endeavors should therefore be made toward exploring an alternative but equally effective technique whose excision will not be detrimental to the survival of the mother plant. Different workers have reported regeneration of plants in cultures using different explant sources like shoots, roots, seeds, axillary buds, pseudobulbs, leaf (George and Ravishankar, 1997; Nayak *et al*, 1997; Sinha and Hegde, 1997; Prasad *et al*,2000; Vij and Pathak, 1999; Vij *et al*, 2000a, b; Deb and Temjensangba, 2007a) and through callus induction and somatic embryogenesis (Ishii *et al*, 1998). Biotechnological tools like plant tissue culture techniques have thus opened new possibilities in conservation.

Many orchid species have been propagated successfully through this technique particularly the threatened orchid species and re-introduced into the wild ameliorating their status in nature. Different explants sources like seeds, leaf, rhizome, roots, inflorescence, etc to propagate *in vitro* in various part of the world for conservation programme. Following are some of the works done by various workers: *Aerides multiflora* (seeds- Katiyar *et al*, 1987; foliar segment- Vij and Pathak, 1990; aerial roots- Vij- 1993); *Arachnis labrosa* (seeds- Temjensangba and Deb, 2005a, b; foliar segments- Deb and Temjensangba, 2007a; aerial roots- Deb and Temjensangba, 2006a); *Cleisostoma racemiferum* (seeds and leaf- Temjensangba and Deb, 2005a, 2006; aerial roots- Deb and Temjensangba, 2005); *Coelogyne porrecta* (seeds- Abdul and Hairani, 1990); *Cymbidium elegans* (seeds- Raghuvanshi *et al*, 1991); *C. Giganteum* and *C iridioedes* (seeds- Katiyar *et al*, 1991); *D. chrysanthum* (seeds- Raghuvanshi *et al*, 1986);

D. fimbriatum var. *oculatum* (seeds- Devi *et al*, 1990); *D. nobile* (seeds- Raghuvanshi *et al*, 1986); *E. hormusjii* (rhizome segments- Vij *et al*, 1989); *Luisia teretifolia* (foliar segments- Vij and Pathak, 1990); *Malaxis khasiana* (seeds- Deb and Temjensangba, 2006b); *Rhynchostylis retusa* (seeds- Nath *et al*, 1991; aerial roots- Chaturvedi and Sharma, 1986; Sood and Vij, 1986; foliar segments- Vij and Pathak, 1990); *Vanda cristata* (foliar segments- Vij and Pathak, 1990); *V. testaceae* (foliar segments- Vij and Pathak, 1990); *Vanda* Kasem's Delight 'Tom Boykin (aerial roots- Vij and Sharma, 1997).

A wide range of endangered plants including orchids has now been successfully propagated using *in vitro* techniques. There are many reports on *in vitro* multiplication of different types of orchids. Different workers have reported regeneration of plants in cultures using different explants sources like from shoots, roots, seeds, axillary buds, pseudobulbs and leaf.

Seed/Embryo Culture

The technique is invariably referred to as ovule/embryo/green pod/green fruit culture (Sagawa, 1963), which ensures better germination frequency and favors the production of virus free seedlings at a faster rate. Asymbiotic/non-symbiotic seed germination is the most common approach use in the propagation of tropical orchids, which tend to be easier to grow than their temperate relatives. The media used for asymbiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and organic carbon source must be in a form readily available to the orchid without the intermediary fungus (Mc Kendrick, 2000). The

technique involves an easy procedure for sterilization, ensures better frequency of germination, and reduces the time-lapse between pollination, sowing of seeds and production of virus free seedlings. Since all the seed/embryos are used in a single sowing in this technique, it is important to determine the harvest time of capsule or pod for getting optimal germination. The earliest stage at which the embryos can be cultured successfully varies with the orchid genotype and the local conditions. Very young ovules do not form suitable explants in orchids because the embryo sac development is a post pollination phenomenon and fertilization a prerequisite for obtaining seedlings. However as the ovules can be used for raising cultures immediately after fertilization, the importance of information on time interval between pollination and fertilization has often been stressed (Valmayor and Sagawa, 1967). *Doritis* ovules from pollinated ovaries germinated readily after getting fertilized *in vitro* (Yasugi, 1984) suggesting that fertilization is a pre-requisite for germination. Yam and Weatherhead (1988) also noted that immature embryos germinate better than the mature ones due to their distended testa cells and metabolically awakened embryos; they also lack dormancy or inhibitory factors. *Arachnis labrosa* and *Cleisostoma racemiferum* embryos obtained between 16 and 18, and 16 weeks after pollination respectively (WAP) (Temjensangba and Deb, 2005b; 2006), readily germinate but their germination frequency declines sharply, when obtained from beyond this window period. Likewise, *Satyrium nepalense, Nephalaphyllum cordifolium, Phaius tankervilliae* and *cymbidiums* germination frequency shows sharp decline when the embryos are collected 3-4 weeks prior to fruit dehiscence. The fruit/capsule that develops prominent ridges along the valves and ceases to grow in

diameter is considered a useful marker for selecting the right stage for embryo culture (Vij, 1995).

Meristem Culture

Resident Meristem: The embryo culture produces a great deal of heterozygosity in their progeny in orchids due to its out breeding characteristic. Because of this, it appears to be a disadvantageous proposition in cut-flower industry where pure lines of desired genotypes are preferred. The possibility of using excised shoot-meristem of *Cymbidiums* for regenerating complete plant from *in vitro* was first demonstrated by Morel (1960), whereas Wimber (1963) formulated, described and published a procedure for the purpose. This technique of using resident meristem (shoot-tips, axillary bud) has opened new vistas in orchid micropropagation (Arditti and Ernst, 1993; Deb and Temjensangba, 2005, 2006a; Temjensangba and Deb, 2005c). Through this technique, upto 200,000 plants can be regenerated from a single resident meristem within a year. However, it has limited utility in monopodial taxa as it involves the sacrifice of the growing tip thereby, endangers the survival of the mother plant.

Adventive meristems: The ability to use an adventives meristem is advantageous as it does not endanger the survival of mother plant. The regenerative competence or the proliferative potential has been positively tested in many orchid taxa, viz: leaf explants (Vij *et al*, 1984; Mathews and Rao, 1985; Chaturvedi and Sharma, 1986; Seeni, 1988; Vij and Pathak, 1988, 1990; Seeni and Latha, 1992; Temjensangba and Deb, 2005c; Deb and Temjensangba, 2007a; root (Chaturvedi and Sharma, 1986; Sood and Vij, 1986; Vij and Pathak, 1988; Vij, 1993; Deb and Temjensangba, 2005, 2006a), flower stalks (Singh and

Prakash, 1984; Kaur and Vij, 1995; Vij *et al*, 1997). The source, genetic constitution and physiological age of the explants are however, some of the important factor for regeneration. The juvenile tissues from green-house grown plants respond better than the mature ones grown outdoors. Generally, the proliferative loci get activated in the sub-epidermal cells and soon develop into somatic embryos and or protocorm-like-bodies. Somatic embryogenesis is either direct or callus mediated development, multiplication and differentiation of the PLBs is influence by the chemical stimulus present in the nutrient pool (Vij and Pathak, 1990; Seeni and Latha, 1992).

The advantages of leaf and root segment culture are apparent for more than one reason: they are easy to obtain, easier to disinfect, and their excision does not endanger the mother plant. Furthermore, as the regeneration occurs in the dermal cells, which is cytological more stable, mass production of genetically uniform plant from this is within the realm of reality (Vij, 2002).

Different species of orchids exhibits specific needs in respect to nutritional requirement and treatment with plant growth regulators (PGRs) for their growth and development. So, no standard media formulation can be prescribed for all the species. Most commonly employed basal medium for orchid tissue culture are Knudson 'C' (1946), Mitra *et al,* (1976), Murashige and Skoog (MS) (1962), Nitsch and Nitsch (1969), Vacin and Went (1949). The use of α-Naphthalene acetic acid (NAA) and one of the cytokinins like benzyladenine (BA) and kinetin (KN) yields a rich crop of PLBs in *Luisia trichorhiza, Satyrium nepalense, Vanda cristata* and *Vanda testaceae* leaf segment culture (Vij, 1995). Similarly, in *Rhynchostylis retusa,* a synergistic action of KN and indole 3-acetic acid (IAA) or NAA in peptone enriched medium favors enhanced

production of PLBs; while yeast extract (YE) is obligatory for regeneration in *Aerides multiflorum, Papilionanthe teres* and *Satyrium nepalense* foliar cultures and peptone in those of Vanda (Vij, 2002)

The phenolic exudation by the explants into the medium is a serious problem in orchid tissue culture as it impairs the growth of plant /regenerates considerably. But this can be offset by the use of activated charcoal (AC) in the medium. Activated Charcoal (AC) favors better health of the cultures because of its ability to absorb exudates/growth inhibitors, enhance medium aeration, and absorb light and provide enhanced quantum of energy per unit plant material (Yam *et al*, 1989).

A synthetic/artificial seed or synseeds, is analogous to the normal embryonic seed developed from fertilization of male and female gametes comprising a meristematic tissue (free from embryonic accessory structures) capable of differentiating into an entire plant body and enclosed in a hydrated gel capsule which permits development of the plant body (Redenbaugh, 1986). The prospect of encapsulating somatic embryos/regenerates in a nutritive gel, and using them for propagation purpose was first suggested by Murashige (1978). Since then the utility of encapsulated propagules (synthetic seeds or synseeds) as an efficient delivery unit is being increasingly realized. Encapsulation of somatic embryos using various gels has been reported (Redenbaugh *et al*, 1986; Sakamoto *et al*, 1995), but usually sodium alginate has been preferred for its ease of capsule formation and low toxicity to the embryo (Corrie and Tandon, 1993). Giant progress has been made in rapid mass multiplication of many orchid species due to great advancement in tissue culture technique. Despite this, appropriate delivery system of micropropagules is meagre and this limits the efficient application of tissue culture

technique in large scale. The use of synthetic seeds (encapsulated propagules/somatic seeds), thus, serve as an efficient delivery system, and ensure economy of space for storage and convenience of lab-to-land transfer of tissue culture raised propagules. Today, there are many reports of successful achievement in the preparation of synthetic seeds in several of rare and threatened orchid species.

The *in vitro* storage of germplasm and conservation is made possible by biotechnological tools like tissue culture. This can be achieved by slight manipulation of different physico-chemical conditions like light, reduced temperature or culture medium and it occupies less space. Seed storage is the most commonly used method for storage of plant genetic resources (Englemann, 1997). Seed storage is, however, prone to destruction by pathogen/pests; loss of viability while, cryopreservation of germplasm/somatic embryos/propagules is an expensive proposition. On the other hand, the technique of *in vitro* germplasm conservation or storage through slow-growth of culture is cost effective and does not interfere with the viability of regenerates, thus provides an efficient system, which has been applied to several plants including orchids (Aggrawal *et al*, 1992; Ichihashi and Hiraiwa, 1996; Chang and Chang, 1998; Vij *et al*, 2000a, b; Deb and Temjensangba, 2007b).

The slow growth technique is one of the techniques usually employed and provides a convenient option for *in vitro* germplasm conservation of short to medium term storage (Deb and Temjensangba, 2007b). A reduction in growth can be achieved in several ways: incubation of culture at low temperature, reduced light, lowering of the available oxygen levels, reduced nutritional status particularly carbon levels and by incorporation of osmoticum like mannitol, sub lethal level of growth inhibitor or

retardants in the medium (Englemann, 1997). The addition of AC to the culture medium is also been reported to be beneficial in minimal growth condition (Paul, 1999).

The minimal growth method is advantageous because the cultures can be maintained and subculture at an intervals ranging from 6 months or less to 2 years, and can be readily revert back to normal growth condition for resumption of growth to produce plants. However, it is not suited for long-term programmes, because of the risks of selection due to stresses imposed on the cultures during storage (Withers, 1991). There is also continued concern about the level of somaclonal variation under slow growth conditions (Jaret and Gawel, 1991). However, cassava could be stored for 10 years under slow growth condition and remained genetically stable (Angel *et al*, 1996).

Objectives of the Study

The loss of primary forest in North-Eastern part of India is primarily due to practice of primitive form of agriculture commonly called "Jhum", fragmentation of forest and unplanned developmental activities. As a result, many important plant species including orchids are under threat or on the verge of extinction before their commercial importance are being explored. Therefore it calls for an immediate intervention and develop an alternate route to preserve the threatened and endangered plant species particularly orchids. A biotechnological tool like tissue culture technique comes in handy for successful achievement of the objectives.

With a view to mass multiply and conserve two rare/ and threatened orchids of North-East India, I had proposed to work on the following two threatened Orchid species

Figure 1: a. *Coelogyne suaveolens* plants with flowers and green pods; b. Close up view of *C. suaveolens*; c. *Taenia latifolia* vegetative part showing habit; d. Flowers of *T. latifolia.*

Close up

owers of

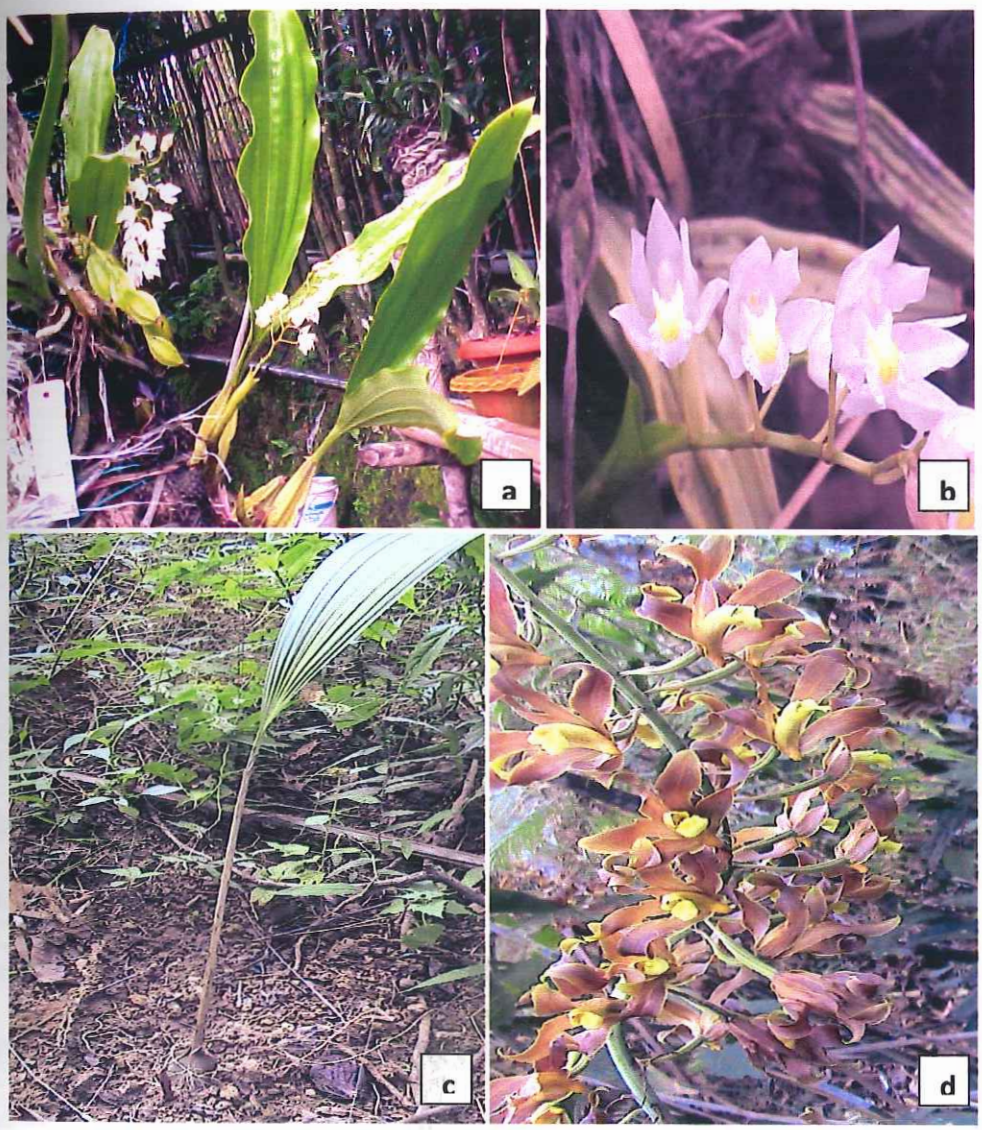

Figure-1

of North-East India for my Ph. D. degree, viz: ***Coelogyne suaveolens* (Lindl.) Hook.** and ***Taeinia latifolia* (Lindl.) Benth. Ex. Hook.** with the following objectives.

I) Micropropagation of above-mentioned two orchid species using various explants like immature embryos/seeds of various developmental stages, leaves, aerial roots and rhizome segments.

II) Preparation of synthetic seeds and regeneration of plantlets from the artificial seeds.

III) Short to medium-term *in vitro* storage of the selected plants using various growth retardants and manipulating the culture conditions.

IV) Reintroduction of *in vitro* raised plants in their natural habitats.

A Brief Account of the Two Selected Orchids

1. *Coelogyne suaveolens* (Lindl.) Hook. (Orchidaceae): This is an endemic orchid of North-East India. This is an epiphytic herb distributed in different parts of North-East India (between 1500 – 2000 m altitudes). Pseudobulbs distant on sheathed rhizomes. Leaves are elliptic, 10-35 x 3-7 cm, wavy at margins, sub-sessile. Inflorescence synanthous, 6-14 flowered racemes and up to 22 cm long. Flowers are white and about 2.5 cm across and are long lasting **(Fig. 1 a & b).** The flowering season is May. Due to destruction of primary forest cover for the purpose of timber and Jhum cultivation, this species is under threat.

2. *Taenia latifolia* (Lindl.) Benth. ex. Hook. (Orchidaceae): This is a terrestrial orchid found in North-East India, Sikkim. This orchid grows on leaf litter and humus in primary

forests. This is a glabrous herbs with wooly, rooted rhizomes and narrowly fusiform. The leaves are dark green, solitary and elliptic, size: 15-30 x 6.5–9 cm. Inflorescence purplish, many flowered racemes on scapes, twice as long as the leaves. It flowers in the month of March-April and it bears dark brown flowers which are up to 2 cm across **(Fig. 1 c & d).** This species is under threat as a result of indiscriminate destruction of forest cover to meet the demand of ever increasing populations and removal of ground vegetation and litters by forest fires for Jhum cultivation.

Chapter-2

Materials and Method

During the last thirty years, micropropagation and other *in vitro* techniques are being more widely used in commercial horticulture and agriculture for the mass propagation of crop plants and for conservation of genetic resources, particularly with those crops which are vegetatively propagated or have recalcitrant seeds which cannot be stored under conventional seed bank conditions (George and Sherrington, 1984). Likewise, *in vitro* culture is being used in an increasing number of threatened plant species. In addition to micropropagation in its strict sense, other techniques available include *in vitro* seed germination, regeneration of plants from callus cultures, dual culture with symbiotic fungi and micro grafting. The use of these techniques has allowed the propagation of many rare, threatened and endangered plant species which prove problematic with conventional horticultural methods and particularly useful with groups of plants which are difficult to propagate using conventional techniques.

The orchids are primarily sexual but they also reproduce through vegetative means. Their vegetative propagation through conventional means like *keikis*, back-bulb,

and division of shoots etc but are very slow and time consuming. Even in natural condition their regeneration through seeds is very limited; due to poorly organised/lack of endosperm, and only 0.2-0.3% of orchid seeds germinate under natural condition. Therefore, it is necessary to look for alternative technique for rapid mass multiplication and conservation of the germplasm. Tissue culture techniques provide means for rapid mass multiplication and *in vitro* conservation of plants (Lal *et al,* 1988; Tandon *et al,* 1990; Temjensangba and Deb, 2005a). In fact, orchid is the first floricultural crop to propagate successfully through this technique. The *in vitro* technique like micropropagation has opened a new route in conservation and commercialisation of orchids. Following this technique, round the year propagation of genetically uniform, disease free, fast maturing and high yielding plants are made possible; and enabled to exploit the regenerative competence more effectively than the conventional method.

With the discovery that orchid seeds germinate following fungal infection, only a practical seed germination method utilizing fungi was developed until Knudson in 1922 demonstrated the ability to by-passed the fungal requirements for germination of *Cattleya* seeds/embryos *in vitro* by using appropriate level of carbohydrates in the culture medium. Since then a remarkable progress has been made in micropropagation of orchids (Arditti *et al,* 1982; Mitra, 1986; Yam and Weatherhead, 1988; Vij and Pathak, 1990; Seeni and Latha, 1992; Arditti and Ernst, 1993; Bejoy *et al*, 2004; Chen *et al,* 2004; Temjensangba and Deb, 2005a, 2006). The immature embryos and the shoot meristem are the most commonly used explants for *in vitro* propagation of orchids. But the response of orchid seeds to physio-chemical factors differ from species to species (Arditti *et al,* 1982; Mitra, 1986; Yam and Weatherhead, 1988; Vij and Pathak, 1990; Seeni and Latha, 1992; Arditti

and Ernst, 1993; Bejoy *et al*, 2004; Chen *et al*, 2004; Temjensangba and Deb, 2005a).

Plant Collection and Maintenance of the Collected Plants

The Plants were collected from the different parts of the North-East India and extensive survey was made in different parts of Nagaland. The collected plants were maintained in the Departmental Botanical Garden, net house and adjoining forests. The Village Heads were informed about the species importance and status and needs for conservation. They were given the photos of the orchids both vegetative as well as flowering state. Regular survey was carried out in the same localities at regular interval to study their status. It was found that in some areas their populations were remain static while in some areas forest cover were removed and orchids could not be collected.

Explants Collection

When working with rare and endangered species, the amount of available plant material can be very small, and this can place restrictions on the choice of methods. Seeds are preferred to vegetative material as the source of propagation material as a wider genetic base can thus be maintained. However, in some species, seed is not readily available and therefore vegetative material has to be used. However, success to a great extent depends on the selection of right explants, physiological age, media composition, exogenous growth regulators and culture conditions.

Seeds: Immature seeds/green pods of different developmental ages [8-18 months after pollination (MAP)] were collected at one month interval for *Coelogyne suaveolens* and 3-12 week after pollination (WAP) in case of *Taenia latifolia* from the Departmental

Botanical Garden. The green pods were collected from the garden and used for the experimental purpose.

Leaf: The leaf explants (size: 1.0-2.0 cm) were collected from *in vivo* source of 5-6 wk after emergence. The leaves were also collected from 3-8 wk old from *in vitro*/axenic culture of both the selected orchids. The leaves were cut into 1-2 cm segments before inoculation after discarding the cut ends of the leaf which are exposed to the sterilizing agent. Whereas the leaf segments of size 0.8-1.5 cm were cut from *in vitro* source. The leaf from *in vitro* source that were taken out from the healthy plantlets inside the Laminar flow cabinet. The explants from *in vitro* source were rinse with double distilled water before cutting them into required size.

Rhizome/pseudobulb

***In vitro* source:** About 1 to 1.5 cm size pseudobulbs (about 3-4 wk old) were harvested from the *in vitro* raised culture and used for the purpose. The plantlets were carefully taken out inside the laminar flow cabinet and traces of agar were washed off with sterile distilled water. The explants were cut horizontally into two equal halves (i.e., proximal and distal part) and made segments of about 0.5 cm before inoculating on the nutrient medium.

***In vivo* source:** About 2.5 to 4.5 cm long freshly sprouted pseudobulb (6-7 wk after emergence) from green house grown plants were used as explants source. The greenish pseudobulb were first thoroughly surface sterilized with 'Extran' (a laboratory detergent, make: Merck) (1:10 ratio) (v/v) before washing under running tap water and successively sterilized with different sterilizing agents.

Sterilization of Plant Materials

Seeds: The green pods/capsule of different developmental stages were harvested and scrubbed cleaned with 'Extran' (1:10) (v/v) and rinsed them under running tap water. Pods were surface sterilized by 0.25 % (w/v) aqueous solution of Mercuric Chloride ($HgCl_2$) (w/v) for 5 min and subsequently rinsed 4-5 times with sterile doubled distilled water. Thereafter, the pods were dipped in 70% ethanol (v/v) for 30 sec and rinsed them 3-4 times with sterilized doubled distilled water. Prior to dissection of embryos the pods was dipped in alcohol and flamed.

Leaf: Leaf explants of both the species (*C. suaveolens* and *T. latifolia*) collected from plants grown out door were surface cleaned with 'Extran' (1:10) (v/v) and rinsed them under running tap water for 10 min. Explants were sterilized with 0.20% (w/v) aqueous solution of $HgCl_2$ for 5 min and washed 4-5 times with sterilized double distilled water. The explants collected from both the sources were soaked in sterile distilled water for 15-20 min before inoculation. The leaves were cut into segments of 0.5 to 1.0 cm and cultured on different initiation media. But, the leaves explants from *in vitro* source were soaked in double distilled water till used.

Rhizome/pseudobulb: Rhizome from both *in vitro* and *in vivo* sources of *T. latifolia* was cultured on different basal media.

***In vitro* source:** About 3-4 wk old pseudobulb/rhizome of *T. latifolia* (size: 1 to 1.5 cm) were harvested from the *in vitro* raised culture and used for the purpose. The plants were carefully taken out inside the laminar flow cabinet and the traces of agar were washed off with sterilized distilled water. The explants were cut horizontally into two equal halves

(i.e., proximal and distal part) and made segments of about 0.5 cm size before inoculating on the nutrient media.

***In vivo* source:** About 2.5 to 4.5 cm long freshly sprouted pseudobulb (6-7 wk after emergence) from green house grown plants were used as explants source. The greenish pseudobulb were first thoroughly surface sterilized with 'Extran' before washing under running tap water and successively sterilized with different sterilizing agents like 0.2% $HgCl_2$ (w/v) (~5 min) followed by 70 % ethanol (20-30 sec). The explants were subsequently rinsed repeatedly 4 or 5 times with double sterilized distilled water after every treatment. The cut-end of the explants exposed to sterilizing agents was sliced off before inoculation on culture media. The sterilized pseudobulbs were first cut horizontally into two equal halves from the centre (proximal and distal part) and they were further cut separately into pieces of ~0.5 cm size. Finally, the pseudobulb segments of both proximal and distal parts were inoculated separately.

Tissue Culture

Media: For initiation of embryo/seed clture, various media like Knudson 'C' (Knudson, 1946), Mitra *et al,* (Mitra *et al,* 1976) and Murashige and Skoog (MS) (Murashige and Skoog, 1962) media were used. For immature seed culture of both the species, the basal media were fortified with coconut milk (CW) (0-20%) (v/v), and sucrose 0- 4.0% (w/v) as carbon source along with different levels of NAA and BA (0-9 µM for *C. suaveolens* and 0-8 µM for *T. latifolia*) singly or in combination. For leaf culture of *C. suaveolens* and leaf and rhizome culture of *T. latifolia,* all the three basal media were fortified with sucrose (0-3%) (w/v), casein hydrolysate (CH) (0-0.15%) (w/v), AC (0-0.2%), and citric

acid 100 mgl^{-1} (as an antioxidant). The culture media were further supplemented with different levels of PGRs like IAA, NAA, BA and KN (0-9 µM) singly or in combination for leaf explants while IAA, NAA and BA for rhizome/pseudobulb culture.

Difco-bacto agar (0.8%) (Make: Hi-media) (w/v) was incorporated as gelling agent after adjusting the *p*H of the media to 5.6 using 0.1 N NaOH and 0.1 N HCl. About 15 ml medium was dispensed into each test tube (size 150 mm x 25 mm) and 30 ml into 400 ml culture bottle (diameter: 70 mm). The media were autoclaved at 1.05 Kg cm^{-2} pressures and at 121°C for 20 min.

Micropropagation

Initiation of Cultures

Immature embryos/green pods: The immature seeds/embryos of different developmental stages from both the species were dissected out from the sterilized green pods under a laminar flow chamber with the help of scalpel blade and cultured on different basal media fortified with different level of various adjuncts. For each treatment 20 numbers of culture vials were used and all the experiments were repeated at least thrice.

Leaf: The sterilized leaves from both *in vivo* and *in vitro* sources of the selected species were cut into 0.5 to 1 cm size and cultured on different initiation media. In each culture vial five numbers of leaf segments were cultured. To determine the effect of orientation, explants were placed in a slanted (~45°) and horizontal position, also position with upside and upside down. For each treatment 20 replicates were used and all the experiment was repeated thrice.

Rhizome/pseudobulb: Pseudobulbs of about 3-4 wk old from *in vitro* raised plants and about 2.5 to 4.5 cm long freshly sprouted pseudobulb (about 6-7 wk after emergence) from green house grown plants were used as explants. The rhizome/pseudobulb from both *in vitro* and *in vivo* sources were sterilized and first cut horizontally into two equal halve, which were further cut into 1 to 1.5 cm size source. The explants were inoculated separately on the nutrient media.

The cultured embryos were incubated under different light conditions viz. dark, diffused (20 µmol m^{-2} s^{-1}) and full light (40 µmol m^{-2} s^{-1}) at 12/12 h light/dark photo cycle. But the cultured leaf segments and rhizome were incubated in the dark for first 48 h followed by full laboratory light condition. All the cultures were maintained at 25±2°C and sub-cultured at 4-5 wk interval unless mentioned otherwise.

The PLBs/shoot buds developed from the germinated seeds, leaf and rhizome segments were maintained on the optimum initiation medium and condition for 2-3 passages for further differentiation.

Maintenance of Culture, Regeneration of Plantlets and Mass Multiplication

The young plantlets/shoot buds/advanced stage PLBs (with first set of leaflets) formed from the cultured immature embryos, leaf and rhizome/pseudobulbs segments were maintained further for 2 passages on the optimum initiation conditions for formation of tiny plantlets. The tiny plantlets so formed were separated from the clumps and transferred to different strengths of two different basal media (MS and Mitra *et al,*) containing sucrose (0-3%), AC (0-0.2%), CH (0-0.15%), citric acid (100 mgl^{-1}) as

antioxidant, different levels of plant growth regulators (PGRs) like IAA, NA, BA and KN (0- 9 µM) singly or in combination for regeneration of plantlets and mass multiplication. In every sub-culture the shoot buds formed were separated and transferred on fresh regeneration medium. The plantlets were maintained for 2-3 passages on regeneration medium before transferring them for hardening.

Artificial Seed Preparation

Uniform sizes of PLBs advance PLBs from different initiation medium of both the species were selected for preparation of artificial seeds. The selected PLBs were place on a sterile filter paper for mild dehydration inside the laminar airflow cabinet prior to encapsulation for one h. Sodium alginate solution (1-3%) (w/v) was prepared in liquid ½MS salt solution with 3% sucrose supplemented by NAA and BA (6 and 9 µM respectively) in combination for *C. Suaveolens*. While for *T. latifolia* MS salt solution was fortified with 3% sucrose, NAA and BA (3 and 9 µM respectively in combination). Besides the above, streptomycin (100 mgl^{-1}) was also incorporated. Different concentration of Calcium chloride ($CaCl_2$) (0.1, 0.15, 0.2 and 0.25 M) was prepared. All the solutions were autoclaved at 121°C and 1.05 $Kgcm^{-2}$ pressures for 20 min and allowed to cool. The PLBs were suspended into the sodium alginate solution and the PLBs along with suitable quantity of sodium alginate solution were seeded into the different concentration of $CaCl_2$ solution. The gel complexation was allowed for 30 min. The resultant beads/artificial seeds were washed with sterile water and bloated dry on sterile filter paper. One part of the beads was coated with water soluble capsule to make them user friendly for physical delivery. The empty capsule coatings were obtained from the pharmacology industry. The encapsulated beads were then cultured on respective

regeneration medium for seed germination and plantlet regeneration. The coated beads were transferred on seed bed made of washed and sterilized saw dust for germination. All the experiments were repeated at least thrice.

Short to Medium-Term *In Vitro* Storage

Attempt was made for *in vitro* short to medium-term storage of germplasm by slow growth of culture. The differentiated plantlets/tiny plantlets were subjected to *in vitro* conservation using slow growth method. The different types of propagules like tiny plantlets, shoot buds and advanced stage PLBs were cultured on different levels of MS medium (0, $1/4^{th}$, 1/2, $3/4^{th}$ and full strength) conjugated with varying concentrations of organic carbon sources like sucrose, mannitol, fructose, glucose and dextrose (0, 1, 2 and 3%) (w/v), containing $1/10^{th}$ strength of PGRs that present in regeneration medium (i.e. 0.6 µM NAA + 0.9 µM BA for *C. Suaveolens* and 0.3 µM NAA + 0.9 µM BA for *T. latifolia*). The cultures were maintained at different temperature (10, 20 and 25°C) and different light conditions [dark, diffused (20 µmol m^{-2} s^{-1}) and full light (40 µmol $m^{-2}s^{-1}$)]. The cultures are sub-cultured at 14-15 wk interval. The cultures are being maintained in the optimum conditions from last three years. The plants from the above conditions were transferred to normal regeneration medium for resumption of normal growth.

Hardening of Regenerated Plants

The well rooted plantlets from the regeneration medium with 3-4 roots (~ 3-4 cm long) of both the species were hardened for considerable period prior to transferring in the community potting mix. The tiny but well rooted plantlets were taken out from the regeneration medium and agar was washed out carefully using a soft brush and cultured

in culture vials containing $1/10^{th}$ MS salt solution with sucrose (2%) devoid of PGRs but containing small chips of charcoal, brick pieces and mosses in different combinations. Before using the MS salt solution and substrate (charcoal, chopped coconut husk and chopped litters) were autoclaved at 121°C of 1.05 kg cm^2 pressures for 30 min. Solution of basal medium was dispensed to the test tube (size 150 mm x 25 mm) of about 10 ml, to this added the chips of charcoal or chopped coconut husk or chopped litters. This operation was done under aseptic condition. The plantlets were maintained under the full light (40 µmol m^{-2} s^{-1}, provided by white cool fluorescent tube) condition for about 8-9 wk.

Potting Mix and Transplantation of Regenerates

For transplanting the hardened plantlets of both the orchid species (*C. suaveolens* and *T. latifolia*), the community potting mix were prepared by mixing different substrates like decayed wood, forest litter, charcoal pieces and coconut husk in different combinations in the ratio of 1:1:1:1 with a layer of moss. The hardened plants were transferred along with the content of the culture vials in the community potting mix. The potted plantlets were kept in the poly-house for acclimatization for about 7-8wk before transferring to the wild. The newly potted plants in the community potting mix were then fed with MS liquid salt solution ($1/10^{th}$ strength) at one-week interval during this period.

Chapter-3

Results

Plant Collection and Maintenance of the Collected Plants

The Plants were collected from the different parts of the North-East India and extensive survey was made in different parts of Nagaland. The collected plants were maintained in the Departmental Botanical Garden, net house and adjoining forests. The Village Heads were informed about the species importance and status and needs for conservation. They were given the photos of the orchids both vegetative as well as flowering state. Regular survey were carried out in the same localities at regular interval it study their status. It was found that in some areas their populations were remain static while in some areas forest cover were removed and orchids could not be collected.

Initiation of Aseptic Cultures from Different Explants

Immature seeds/embryos

The immature seeds/embryos of different developmental stages were cultured on different basal media containing different supplements. The green pod age, basal media

Figure 2: Different stages of immature seed germination of *Coelogyne suaveolens*. a. Germinating seeds shows the nodular swelling as a first sign of germination, b. Germinating seeds differentiating into PLBs, c. The differentiated PLBs converted into young plantlets and shoot buds.

aveolens. a.

ıination, b.

s converted

Figure-2

Figure 3: Different stages of immature seed germination of *Taenia latifolia*. a. Germinating seeds shows the nodular swelling as a first sign of germination, b. Germinating seeds differentiating into PLBs, c. The differentiated PLBs converted into young plantlets and shoot buds.

a latifolia. a.

rmination, b.

Bs converted

Figure-3

Table 1: Effect of green pod/seed age and media on immature seed culture of *C. suaveolens*[*]

Seed pod Age (MAP)	Germination time (Days)[**] 1	2	3	Germination % (±SE)[***] 1	2	3	Types of response	Remarks[#]
8	-	-	-	-	-	-	No response	-
9	82	-	-	50 (±0.5)	-	-	Nodular swelling followed by few PLBs	+
10	61	-	84	61 (±0.5)	-	43 (±1.0)	Nodular swelling followed by few PLBs	+
11	55	85	78	72 (±0.5)	50 (±1.0)	53 (±0.5)	Nodular swelling followed by few PLBs	++
12	45	75	72	80 (±1.0)	62 (±0.5)	56 (±0.5)	Nodular swelling followed by few PLBs	++
13	**40**	70	65	**93 (±1.0)**	64 (±0.5)	73(±0.5)	**Germinating embryos formed green PLBs followed by healthy plantlets**	+++
14	47	74	70	76 (±0.5)	57 (±0.5)	60(±0.5)	Nodular swelling followed by healthy plantlets	++
15	51	77	75	65 (±0.5)	52 (±0.5)	55(±0.5)	Nodular swelling followed by healthy plantlet formation	+
16	66	87	86	60 (±1.0)	45 (±0.5)	47(±1.5)	Nodular swelling followed by healthy plantlet formation	+
17	73	105	93	50 (±1.0)	33 (±1.5)	38(±1.0)	Nodular swelling followed by healthy plantlet formation	+
18	87	-	108	42 (±1.0)	-	34(±1.5)	Deformed and delayed germination	+

* On media containing sucrose (3%) (w/v); PGRs- 3 µM NAA and 9 µM BA in combination; CW (15%) (v/v); citric acid (100 mgl^{-1})

** Days taken to germinate on 1. MS medium; 2. Knudson 'C'; 3. Mitra *et al.*

*** ±SE Standard error

'-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response

Data represents the mean of three replicates.

Table 2: Effect of PGRs on non-symbiotic immature seed germination of *C. suaveolens*[*]

PGRs Conc. (µM) NAA	BA	Days taken to germinate	Germination % (±SE) [**]	Types of response	Remarks[#]
0	0	-	-	No response	-
3	-	68	47 (±1.5)	Few PLBs formed	+
6	-	63	48 (±1.0)	Few PLBs formed	+
9	-	60	45 (±1.5)	Few PLBs formed	+
-	3	57	50 (±1.0)	Green PLBs formed	+
-	6	55	60 (±1.5)	Green PLBs formed	++
-	9	55	65 (±1.5)	Green PLBs formed	++
3	3	53	70 (±1.5)	Green PLBs formed	++
3	6	46	76 (±2.0)	Green PLBs formed	++
3	**9**	**40**	**93 (±1.0)**	**All the embryo formed green PLBs followed by healthy plantlets**	**+++**
6	3	48	46 (±1.5)	Few PLBs formed	+
6	6	53	50 (±0.5)	Green PLBs formed	+
6	9	53	70 (±0.5)	Green PLBs formed	++
9	3	61	46 (±1.0)	Few PLBs formed	+
9	6	66	50 (±1.0)	Few PLBs formed	+
9	9	72	55 (±1.5)	Few PLBs formed	+

* Immature seeds of 13 MAP on MS medium containing Sucrose (3%) (w/v) and CW (15%) (v/v), and citric acid (100 mgl^{-1})

** ±SE-Standard error

'-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response

Data represents the mean of three replicates.

Table 3: Effect of sucrose and coconut water concentration on immature seed germination of *C. suaveolens*[*]

Sucrose Conc. (%)	CW Conc. (%)	Germination time (days)	Germination %) (±SE)[**]	Types of response	Remarks[#]
0	0	-	-	No response	-
1	-	92	23 (±1.5)	Few PLBs formed but subsequently degenerated	-
2	-	80	26 (±0.5)	Few PLBs formed	+
3	-	60	63 (±0.5)	Green PLBs formed but were growth Stunted	++
4	-	68	55 (±0.5)	Only few PLBs formed	+
1	5	83	23 (±1.5)	Few PLBs formed but subsequently degenerated	+
1	10	77	33 (±0.5)	Few PLBs formed but subsequently degenerated	+
1	15	75	38 (±1.5)	Few PLBs formed but subsequently degenerated	+
1	20	68	45 (±0.5)	Only few PLBs formed	+
2	5	80	30 (±1.5)	Formation of pale yellow PLBs	+
2	10	76	40 (±1.5)	Formation of pale yellow PLBs	+
2	15	72	45 (±0.5)	Formation of pale yellow PLBs	+
2	20	64	53 (±0.5)	Few green PLBs formed	+
3	5	60	72 (±0.5)	Few green PLBs formed	++
3	10	52	80 (±0.5)	Green and healthy PLBs formed	++
3	**15**	**40**	**93 (±1.0)**	**All the embryo formed green PLBs with one leaf each**	**+++**
3	20	47	66 (±0.5)	Green and healthy PLBs formed	++
4	5	58	65 (±0.5)	Green PLBs formed	+
4	10	67	56 (±0.5)	Few PLBs formed	+
4	15	72	45 (±1.0)	Only few PLBs formed	+
4	20	78	41 (±1.5)	Only few PLBs formed degenerated subsequently.	-

* Immature seed of 13 MAP; On MS medium containing 3 µM NAA & 9 µM BA in combination; and citric acid (100 mgl^{-1})

** Standard error

'-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response

Data represents the mean of three replicates.

Table 4: Effect of green pod age and media on immature seed culture of *T. latifolia*[*]

Seed pod age (WAP)	Germination time (Days)[**]			Germination % (±SE)[***]			Type of response	Remarks[#]
	1	2	3	1	2	3		
3	-	-	-	-	-	-	No germination	-
4	-	-	-	-	-	-	No germination	-
5	120	143	140	20 (±2.0)	20 (±1.0)	30 (±1.5)	Nodular swelling but no PLBs formation	+
6	97	134	130	30 (±1.5)	27 (±2.0)	33 (±2.0)	Nodular swelling but no PLBs formation	+
7	63	120	130	72 (±2.0)	32 (±1.5)	45 (±2.7)	Nodular swelling but no PLBs formation	++
8	**30**	105	120	**95 (±0.3)**	43 (±2.0)	**50** (±1.5)	**Healthy PLBs formation followed by healthy planets**	+++
9	54	110	120	80 (±2.0)	40 (±2.5)	30 (±2.0)	Healthy PLBs formation but failed to produce plantlets	++
10	86	130	130	40 (±1.0)	24 (±3.0)	15 (±2.0)	Deformed PLBs formation	+
11	-	-	-	-	-	-	No response	-
12	-	-	-	-	-	-	No response	-

* On MS medium containing 6 µM NAA, sucrose (3%) (w/v), CW (15%) (v/v) and citric acid (100 mgl-1)
** 1. MS medium, 2. Knudson 'C', 3. Mitra *et al.*
*** Standard error
# '-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three replicates.

composition, quantity of organic carbon, quality and quantity of PGRs were found to be crucial factors for successful culture initiation. Greening and nodular swelling was the first sign of germination after 20-25 days of culture in both the species (**Fig. 2 a, 3a**).

In ***Coelogyne suaveolens*** the nodular swelled seeds converted into PLBs after 40 days of culture initiation (**Table 1 and Fig. 2 b**). Amongst the three basal media studied MS medium exhibited better germination (93%) followed by Mitra *et al,* (73%) (**Table 1**). The cultured seeds on MS media supported formation of healthy PLBs, whereas Mitra *et al.* medium supported moderate green PLBs formation. But the cultures on Knudson 'C' medium after nodular swelling seeds the formation of PLBs was arrested and subsequently degenerated. Amongst the different developmental stages of immature embryos were used for the study, green pod age of <9 MAP failed to germinate. With increase in green pod age performance of seed germination improved substantially and optimum germination was registered from green pod age of 13 MAP where within 23 days of culture, the cultured seeds started greening about 93% germination was recorded after 40 days of culture (**Table 1**). Green pod age >14 MAP resulted impaired germination.

All the basal media were supplemented with different levels of various adjuncts like sucrose, CW and different PGRs. But optimum response was achieved on MS medium containing sucrose (3%), CW (15%) and NAA and BA (3 and 9 µM respectively in combination) (**Table 2 and 3**).

In the case of ***T. latifolia*** the optimum germination was achieved on MS medium (95%) followed by Mitra *et al,* and Knudson 'C' (**Table 4**). Healthy germination was

Table 5: Effect of PGRs on non-symbiotic immature seed germination of *T. latifolia*[*]

PGRs (µM) NAA	Conc. BA	Germination time (days)	Germination (%) (±SE)[**]	Types of response	Performance[#]
0	0	-	-	No response	-
2	-	35	80 (±0.5)	Nodular swelling and formation of green PLBs	++
4	-	30	90 (±0.5)	Nodular swelling and formation of green PLBs	++
6	**-**	**30**	**95 (±0.3)**	**Nodular swelling and later all the embryo formed green and healthy PLBs**	**+++**
8	-	32	85 (±0.5)	Nodular swelling and formed green PLBs	++
-	2	150	26 (±1.5)	Nodular swelling but only few PLBs formed	+
-	4	146	35 (±1.5)	Nodular swelling but only few PLBs formed	+
-	6	140	41 (±1.5)	Nodular swelling but only few PLBs formed	+
-	8	160	20 (±1.5)	Nodular swelling but only few PLBs formed	+
2	2	94	50 (±1.5)	Nodular swelling but only few PLBs formed	+
2	4	50	61 (±0.5)	Nodular swelling but formed few green PLBs	+
2	6	40	68 (±0.3)	Nodular swelling and formation of green PLBs	++
2	8	80	55 (±0.5)	Nodular swelling but formed few green PLBs	+
4	2	92	43 (±0.5)	Only few PLBs formed	+
4	4	90	48 (±0.3)	Only few PLBs formed	+
4	6	95	35 (±0.5)	Only few PLBs formed	+
4	8	98	30 (±0.5)	Only few PLBs formed	+
6	2	80	48 (±0.5)	Only few PLBs formed	+
6	4	82	43 (±0.5)	Only few PLBs formed	+
6	6	85	34 (±1.0)	Only few PLBs formed	+
6	8	90	30 (±1.0)	Only few PLBs formed	+
8	2	115	25 (±1.5)	Nodular swelling and gives deformed germination	-
8	4	120	20 (±1.5)	Nodular swelling and gives deformed germination	-
8	6	129	19 (±1.0)	Nodular swelling and gives deformed germination	-
8	8	145	15 (±1.0)	Nodular swelling and gives deformed germination	-

* Immature seed of 8 WAP on MS medium containing sucrose (3%) (w/v), CW (15%) (v/v), citric acid (100 mgl-1)

** Standard error

'-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response

Data represents the mean of three replicates.

Table 6: Effect of sucrose and coconut water on immature seed germination of *T. latifolia*[*]

Sucrose Conc. (%)	CW Conc. (%)	Germination time (days)	Germination (±SE)[**]	Type of response	Remarks[#]
0	0	-	-	No germination	-
1	-	94	26 (±1.0)	Only few PLBs formed but degenerated subsequently	-
2	-	86	45 (±1.0)	Only few PLBs formed	+
3	-	53	56 (±1.0)	Green PLBs formed	+
4	-	66	52 (±1.5)	Few PLBs formed	+
1	5	84	33 (±2.0)	Only few PLBs formed but degenerated subsequently	-
1	10	72	44 (±1.5)	Only few PLBs formed but degenerated subsequently	+
1	15	67	49 (±1.0)	Only few PLBs formed but degenerated subsequently	+
1	20	63	53 (±1.0)	Only few PLBs formed but degenerated subsequently	+
2	5	78	62 (±2.0)	Few PLBs formed	+
2	10	73	65 (±1.0)	Green PLBs formed	+
2	15	64	75 (±1.0)	Green PLBs formed	++
2	20	66	70 (±2.0)	Green PLBs formed	++
3	5	40	68 (±1.0)	Green PLBs formed	+
3	10	36	75 (±2.0)	Green and healthy PLBs formed	++
3	**15**	**30**	**95 (±0.3)**	**Healthy PLBs obtain followed by healthy plantlets formation**	**+++**
3	20	47	63 (±1.5)	Green PLBs formed	+
4	5	40	64 (±1.0)	Green PLBs formed	+
4	10	54	60 (±0.5)	Green PLBs developed but degenerated	-
4	15	52	54 (±1.0)	Green PLBs developed but degenerated	-
4	20	55	45 (±1.5)	Only few PLBs formed	-

* Immature seed of 8 WAP on MS medium containing 3 µM NAA & 9 µM BA in combination, sucrose (0-3%) (w/v), CW (0-20%) (v/v), citric acid (100 mgl-1)

** Standard error

'-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response

Data represents the mean of three replicates.

recorded on medium supplemented with sucrose (3%), CW (15%), citric acid (100 mgl^{-1}) and NAA 6 µM singly (**Table 5 and 6**). The green pod age or the developmental stage of the seeds was observed to be a deciding factor for successful culture initiation. Seed pod age up to 7 WAP did not support healthy seed germination and demanded longer duration for germination. The optimum and healthy germination was recorded with seeds pod age of 8 WAP where within 30 days of culture about 95% germination was recorded (**Table 4**). Seeds >8 WAP was found not suitable for culture initiation and as in most of the cases seeds either failed to germinate or culture degenerated after germination. The MS medium supported the optimum germination and formation of healthy PLBs, subsequently healthy plantlets with distinct pseudobulbs. The cultures raised on Mitra *et al.* medium results in formation of unhealthy green PLBs and the differentiated plantlets were etiolated without distinct pseudobulbs. Whereas, in Knudson 'C' medium PLBs formations were arrested right after nodular swelling of the embryos/seeds and remained pale yellow color and subsequently degenerated.

Besides the quality of the culture media and PGRs, the quality and quantity of other adjuvant also played a key role on successful culture initiation. In both the species incorporation of sucrose in the germination media was prerequisite. On media devoid of sucrose no germination was registered. At a concentration of 3% sucrose supported optimum germination in both the species. It was observed that the germination percentage declined considerable at lower as well as at higher concentration of sucrose. At lower concentration of sucrose (2%) produced unhealthy PLBs and germination duration prolonged while, medium with sucrose (4%) germination percentage decreases and results with stunted growth followed by browning of media and subsequently

degenerated. In both the species, though incorporation of CW in the germination medium was not prerequisite, incorporation of CW (15%) in the medium enhanced the early germination and differentiation of PLBs into healthy plantlets.

Amongst the different quality and quantity of PGRs tested for asymbiotic seed germination of *C. suaveolens* a combined treatment of NAA and BA was found to be most suitable over singly treatment. When used singly, they either delayed or lowered the germination percentage. Optimum germination was observed on medium containing NAA and BA (3 and 9 µM respectively in combination) followed by NAA and BA (3 and 6 µM respectively in combination) (**Table 2**). But, in case of *T. latifolia* the single treatment of NAA was found suitable over BA and the combined treatments. The seeds cultured on medium enriched either with BA singly or in combination with NAA delayed the germination and subsequently fewer PLBs differentiated. A concentration of 6 µM NAA in the medium supported optimum germination (95%) followed by 4 µM NAA (**Table 5**).

Three different light conditions were tested for *in vitro* seed germination for both the species. Of the three light conditions, diffused light (20 µmol m^{-2} s^{-1}) supported better germination followed by full light conditions (40 µ mol m^{-2} s^{-1}) in both the species. The cultures maintained in the dark either failed to germinate or callused and degenerated subsequently after it turns pale whitish-yellow.

The germinating seeds from both the species were subsequently converted into PLBs **(Fig. 2b and 3b)**. The resulted PLBs were maintained for another two passages where they started differentiating and release the first set of leaflets.

Figure 4: Initiation of cultures from *in vitro* raised foliar explants of *C. suaveolens* and *T. latifolia*. a. Cultured leaf of *C. suaveolens* curled and swelled (the sign of initiation of response), b. Cultured leaves of *T. latifolia* shows curling and swelling as they initiate the morphogenetic response, c. Multiple shoot buds developed from the cultured leaf of *C. suaveolens*, and d. Multiple shoot buds developed from the cultured leaf of *T. latifolia*.

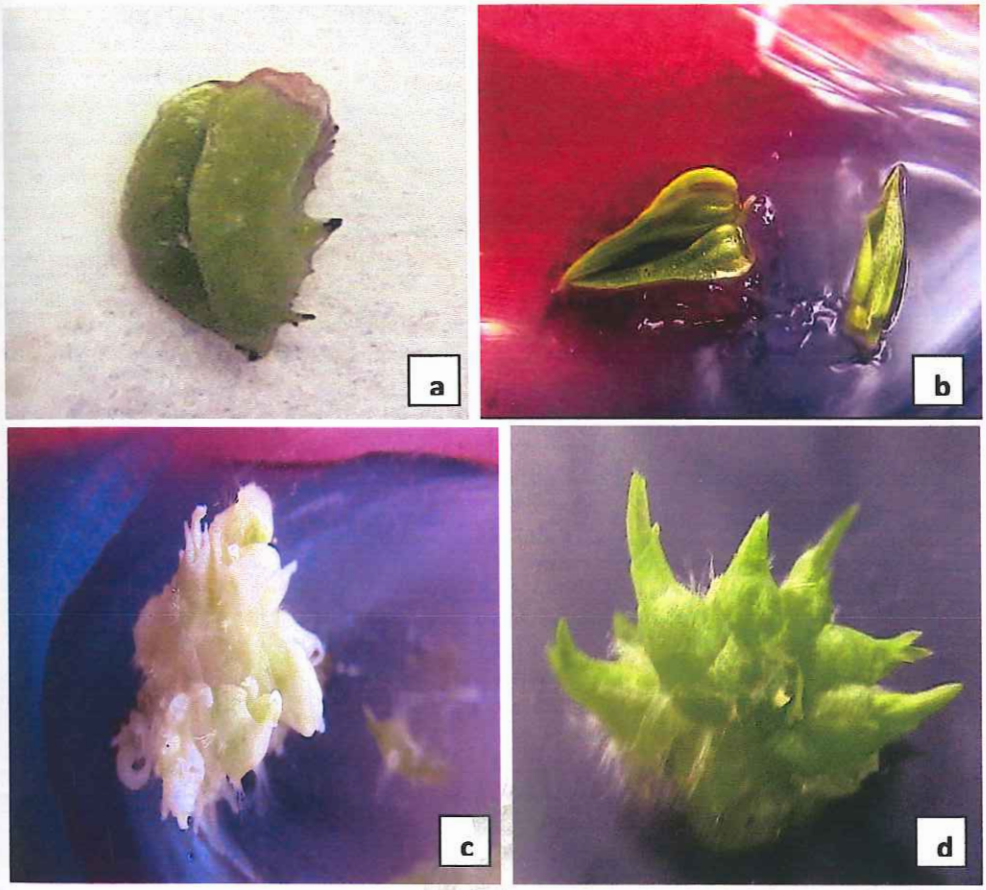

Figure-4

Table 7: Effect of PGRs on culture initiation from foliar explants of *in vitro* source of *C. suaveolens (5 wk old)**

PGRs Conc. (µM)@		% response (±SE)**	No. of regenerates per explant	Type of response	Performance#
0		-	-	No regeneration	-
NAA					
3		-	-	No regeneration	-
6		46 (±0.1)	3	Shoot bud formation	+
9		50 (±0.1)	5	Shoot bud formation but failed to developed plantlets	+
IAA					
3		-	-	No regeneration	-
6		30 (±0.1)	-	Swelling occurs but later	
9		34 (±0.1)	-	Swelling occurs but dies later	-
BA					
3		40 (±0.05)	2	Swelling followed by shoot bud development	+
6		54 (±0.1)	4	Swelling followed by shoot bud development	+
9		63(±0.1)	6	As above	++
KN					
3		33(±0.1)	-	Initial swelling but degenerated after browning	+
6		42(±0.1)	-	As above	+
9		45 (±0.1)	-	As above	+
NAA	**BA**				
6	6	54 (±0.1)	2	Shoot bud formation	+
6	9	66 (±0.1)	3	Shoot bud formation	+
9	**6**	**73 (±0.05)**	**9**	**Swelling and curling of leaf segment followed by shoot bud formation**	**+++**
9	9	61 (±0.1)	2	Shoot bud formation	+
NAA	**KN**				
6	6	39 (±0.1)	-	Initial swelling but degenerated subsequently	-
6	9	55 (±0.1)	-	As above	-
9	6	43 (±0.15)	-	As above	-
9	9	44 (±0.1)	-	As above	-
IAA	**BA**				
6	6	43 (±0.1)	-	As above	-
6	9	53 (±0.15)	-	As above	-
9	6	45 (±0.1)	-	As above	-
9	9	47 (±0.1)	-	As above	-
IAA	**KN**				
6	6	30 (±0.1)	-	As above	-
6	9	45 (±0.1)	1	Shoot bud formation but degenerated	-
9	6	30 (±0.1)	-	Degenerated after browning	-
9	9	42 (±0.1)	-	Degenerated after browning	-

* On MS medium containing sucrose (3%) (w/v), CH (0.1%) (w/v), AC (0.1%) (w/v), citric acid (100 mgl-1)
@ Only the significant treatments are computed
** Standard error
# '-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three replicates.

Table 8: Effect of PGRs on culture initiation from foliar explants of *in vitro* source of *T. latifolia (4 wk old)**

PGRs Conc. (µM)@		% response (±SE)**	No. of regenerates /explants	Type of response	Performance#
0		-	-	No response	-
NAA					
3		50 (±1.0)	1	Shoot buds formation	+
6		44 (±0.5)	-	Swelling occurs but no PLBs or shoot buds	-
9		40 (±0.5)	-	Swelling occurs but no PLBs or shoot buds	-
IAA					
3		48 (±0.5)	-	Swelling occurs but no PLBs or shoot buds	-
6		45 (±1.0)	-	Swelling occurs but no PLBs or shoot buds	-
9		37 (±1.0)	-	Swelling occurs but no PLBs or shoot buds	-
BA					
3		65 (±0.5)	1	Direct shoot buds	+
6		57 (±0.5)	1	Direct shoot buds	-
9		55 (±0.5)	1	Direct shoot buds	-
KN					
3		49 (±0.5)	-	No shoot buds or PLBs	-
6		54 (±0.5)	1	Direct shoot buds	+
9		56 (±0.5)	1	Direct shoot buds	+
IAA	**BA**				
3	3	57 (±0.5)	1	Swelling and curling occurs	-
3	**6**	**75 (±0.5)**	**5**	**Swelling and Shoot buds developed from the cut end of the leaf segment**	**+++**
3	9	66 (±0.5)	2	Swelling and curling occurs	++
6	3	45 (±1.0)	-	No shoot buds or PLBs	-
6	6	54 (±1.0)	1	Direct shoot bud	+
6	9	58 (±0.5)	1	Direct shoot bud	+
IAA	KN				
3	3	31 (±0.5)	-	Swelling occurs but no regeneration	-
3	6	40 (±1.0)	-	Swelling occurs but no regeneration	-
3	9	46 (±0.5)	1	Shoot bud formation	+
6	3	26 (±0.5)	-	Swelling occurs but later degenerated	-
6	6	30 (±0.5)	-	Swelling occurs but later degenerated	-
6	9	45 (±0.5)	-	Swelling occurs but no regeneration	-
NAA	BA				
3	3	37 (±0.5)	-	Swelling occurs but no regeneration	-
3	6	43 (±0.5)	-	Swelling occurs but no regeneration	-
3	9	48 (±0.5)	1	Shoot bud formation	+
6	3	27 (±0.5)	-	Swelling occurs but later degenerated	-
6	6	32 (±0.5)	-	Swelling occurs but later degenerated	-
6	9	43 (±0.5)	-	Swelling occurs but no regeneration	-
NAA	KN				
3	3	35 (±0.5)	-	Swelling occurs but no regeneration	-
3	6	41 (±0.5)	-	Swelling occurs but no regeneration	-
3	9	47 (±0.5)	-	Swelling occurs but no regeneration	-
6	3	25 (±0.5)	-	Swelling occurs but later degenerated	-
6	6	29 (±0.5)	-	Swelling occurs but no regeneration	-
6	9	40 (±0.5)	-	Swelling occurs but no regeneration	-

* On MS medium with source (3%) (w/v), AC (0.1%) (w/v), CH (0.1%) (w/v); citric acid (100 mgl-1)
@ Only the significant treatments are computed
** Standard error
# '-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three replicates.

Leaf explants

The cultures were also initiated from the foliar explants of *C. suaveolens* and *T. latifolia*. However, the foliar explants from *in vivo* source remained recalcitrant to *in vitro* morphogenetic response but in some cases slight swelling was observed at the basal part of the explants. The foliar explants of both the species from *in vitro* sources started swelling followed by curling within two wk of culture **(Fig. 4a and b)**. The responding foliar segments subsequently developed shoot buds from the upper surface of the leaf **(Fig. 4 c and d)**. Orientation of explants on medium exhibited a pronounced effect on morphogenetic response in both the species. Explants cultured at 45°C outperformed other orientation.

The foliar explants of *C. suaveolens* produced shoots/ buds after 6-7 wk of culture from the upper surface of the foliar segment. Amongst the different adjuvant incorporated, MS medium containing sucrose (3%), CH (0.1%), AC (0.1%), citric acid (100 mgl^{-1}) and NAA and BA (9 and 6 µM respectively in combination) supported optimum response where as many as 9 shoot/buds were developed in about 73% explants **(Table 7)**. While in the case of *T. latifolia* the first sign of response was marked by swelling and curling of the leaf segment after 15-20 days of culture. About 75% of explants responded positively on MS medium containing sucrose (3%), AC (0.1%), CH (0.1%), and IAA and BA (3 and 6 µM) after 5 wk of inoculation **(Table 8)**. Under optimum condition as many as 5 shoot buds were formed from the cut ends of the leaf segment.

Figure 5: Rhizome/pseudobulb culture of *Taenia latifolia* from *in vitro* and *in vivo* sources. a. Pseudobulb segment from *in vitro* source cultured on initiation medium showing the formation of meristematic loci, b. Pseudobulb segment from *in vivo* source cultured on initiation medium showing the formation of meristematic loci, c. Multiple shoot/buds formed from the pseudobulb segment of *in vitro* source, d. Shoot buds formed from the pseudobulb segments of *in vivo* source.

nd *in vivo*

n medium

vivo source

c. Multiple

hoot buds

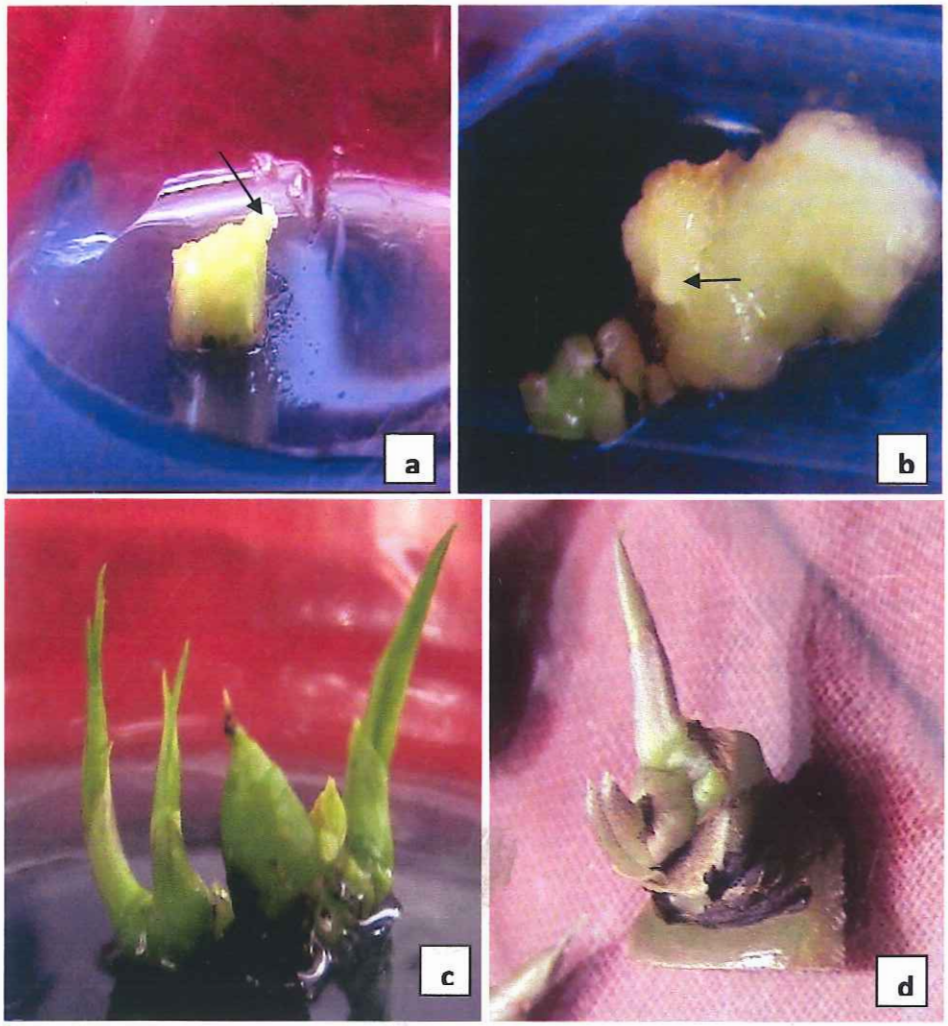

Figure-5

Table 9: *In vitro* regeneration response of *Taenia latifolia* pseudobulb segments procured from *in vivo* and *in vitro* source*

PGRs (Conc) (µM)	Response (%) (±SE)**	Regeneration pathway!	No. of regenerates per explants	Time taken for plantlet formation (wk)	Remarks!!
***In vitro* source explants**					
BA(3)	45±0.5	-	-	-	Regeneration response impaired
BA(6)	57±0.5	SB	1	8	Shoot buds formation from the proximal region and give raise to healthy plantlets
BA(9)	64±0.5	SB-PL	3	8	Multiple shoot buds formed
NAA(3) + BA(3)	57±0.2	SB-PL	1	9	Direct shoot buds from the proximal zone
NAA(3) + BA(6)	***78±0.2***	***SB/PL***	***12***	***7***	***Multiple shoot buds formed from the cut end of both distal and proximal zone***
NAA(3) + BA(9)	72±0.5	SB-PL	2	8	Direct shoot buds from the proximal zone
NAA(3) + BA(12)	72±0.5	SB	1	8	Proximal zone resulted shoot buds but distal segment failed to response
IAA(3) + BA(3)	52±0.5	SB	1	9	Shoot bud formation from the proximal part
IAA(3) + BA(6)	67±0.5	SB	1	9	Proximal region formed shoot bud
IAA(3) + BA(9)	70±0.5	SB	2	9	Proximal region shoot buds and subsequent healthy plantlets
***In vivo* source explants**					
NAA(3) + BA(3)	33±0.5	-	-	-	Regeneration response impaired
NAA(3) + BA(6)	40±0.5	SB	1	12	Direct shoot buds from the proximal region and distal part recalcitrant
NAA(3) + BA(9)	43±0.5	SB	3	9	Direct shoot buds develops from the proximal region but distal part recalcitrant
NAA(3) + BA(12)	***72±0.5***	***SB***	***4***	***10***	***As above***
IAA(3) + BA(3)	52±0.5	-	-	-	Regeneration response impaired
IAA(3) + BA(6)	67±0.5	SB	1	10	Shoot buds formed only from proximal region
IAA(3) + BA(9)	70±0.5	SB	-	10	Only proximal region responded through shoot bud development

* On MS medium containing sucrose (3%), AC (0.1%), CH (0.1%) and 100 mgl^{-1} citric acid as antioxidant.
** Standard error
! SB: Shoot buds, PL: Plantlets
!! Only the responding treatments are tabulated. Data represent the mean value of three replicates,

Rhizome/pseudobulbs

Cultures were also initiated from the rhizome/pseudobulbs segments of *T. latifolia* both from *in vitro* as well as *in vivo* sources. The explants from the two sources exhibited differential response. No meristematic loci invoked in the segments collected from the distal region of the *in vivo* sourced explants and shoot buds develops only from the proximal region of pseudobulbs explants. While, formation of meristematic loci were registered from the explants of both distal as well as proximal region of pseudobulbs of *in vitro* source **(Fig. 5a, b)**.

Within 7 wk of culture, the explants from the *in vitro* source developed multiple shoot buds from both surfaces of the explants. As many as 12 shoot buds developed in ~78% of culture on MS medium containing sucrose (3%), AC (0.1%), CH (0.1%), NAA and BA (3 and 6 µM respectively in combination) **(Fig. 5c and Table 9)**. Presence of AC (0.1%) in the medium proved beneficial by reducing the browning of media, but the medium with AC < 0.1% shows browning of media after 3-4 wks, whereas the explants remain recalcitrant and degenerated subsequently after 3-4 wk when AC concentration was > 0.1%. Presence of CH (0.1%) in the nutrient pool helped in early development of shoot buds and healthy plantlets.

After 10 wk of culture, swelling of explants was observed as the first sign of response in rhizome/pseudobulb culture from *in vivo* source. The optimum response was registered on MS medium fortified with sucrose (3%), AC (0.1%), CH (0.1%) and citric acid 100 mgl^{-1} (as anti oxidant) supplemented with NAA and BA (3 and 12 µM respectively in combination) **(Table 9)**. Under optimum condition as many as 4 shoot

buds developed where about 72% explants responded positively **(Fig. 5d)**. The PLBs and shoot buds formed from the cultured rhizome segments were maintained for another 2 passages for further differentiation on optimum growth conditions.

The advanced stage PLBs, shoot buds developed from the germinated seeds, foliar explants and rhizome segments were subjected to regeneration of plantlets and mass multiplication.

Maintenance of Culture, Regeneration of Plantlets and Mass Multiplication

The advance stage of PLBs (with first set of leaflets) developed from the cultured immature embryos/seeds, leaf of *C. suaveolens* and *T. latifolia* and, rhizome segment of *T. latifolia* were maintained for two more passages on the optimum initiation conditions for further differentiation (**Fig. 2c and 3c**). The PLBs/young plantlets from both the species were than transferred on different basal media for regeneration of plantlets and mass multiplication. All the media were supplemented with sucrose (0-3%), CH (0-0.2%) and various levels of PGRs [IAA, NAA, BA and KN (0-9 μM) singly or in combination].

Effects of basal media on plant regeneration and mass multiplication

The quality of different basal media exhibited a pronounced effect on plantlet regeneration and mass multiplication of both the species. The differentiated PLBs and young plantlets (~1.0-1.5 cm size) from different explants sources were then separated and were maintained on different levels of MS and Mitra *et al,* media. In the preliminary study it was observed that in both the species MS medium supported better differentiation

Figure 6: a. Plantlets of *C. suaveolens* on regeneration medium showing formation of multiple shoots/buds, b. Rooted plantlets of *C. suaveolens* ready for hardening, c. Multiple shoots/buds of *T. latifolia* on regeneration medium, and d. Rooted plantlets of *T. latifolia* ready for hardening.

nation of

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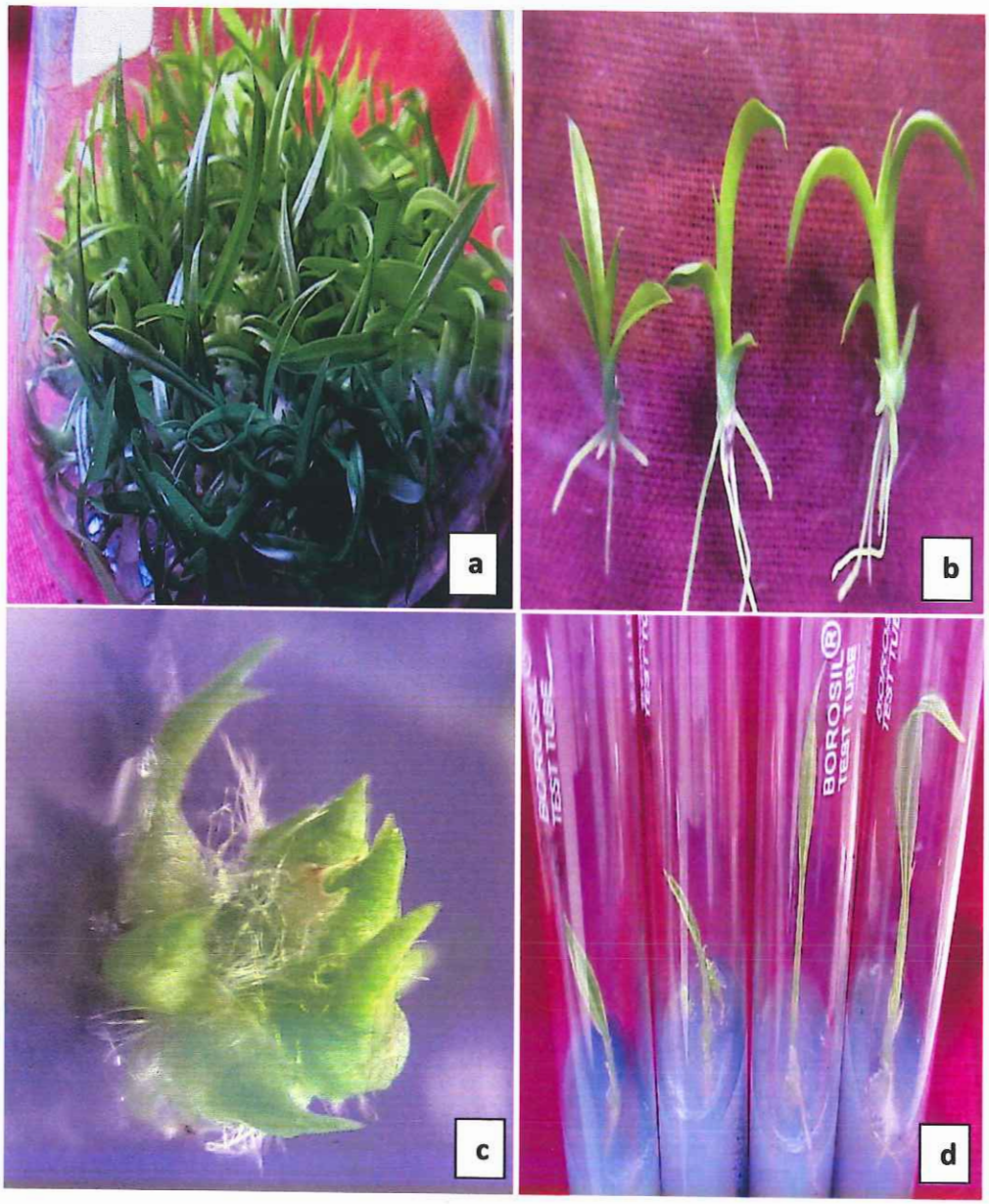

Figure-6

Table 10: Effect of different strength of MS medium for PLBs differentiation, plantlet regeneration and mass multiplication of *C. suaveolens*[*]

Strength of MS medium	Avg. plantlet height (cm)	No of roots/plantlet (±SE)[**]	No of shoot/bud formed per subculture	Type of response	Remarks[#]
0	-	-	-	All the plantlets degenerated after 1 wk of culture	-
1/4th	2(±0.1)	2 (±0.5)	3 (±0.2)	Plantlets exhibited stunted growth, poor roots development and shorter leaflets	++
1/2	**3 (±0.1)**	**4 (±0.2)**	**12 (±0.2)**	**Multiple shoot bud formation, well rooted healthy plantlets and with enlarge pseudobulbs**	+++
3/4th	3 (±0.5)	3 (±0.2)	9 (±0.5)	Multiple shoot bud formation, plantlets healthy but fewer roots and but retarded plantlet height	++
Full	2 (±0.5)	2 (±0.1)	6 (±0.5)	Plantlet healthy but fewer shoot buds and reduced pseudobulbs	++

* On different strength of MS medium containing sucrose (3%) (w/v), CH (0.1%) (w/v), AC (0.1%) (w/v); PGRs 6 µM NAA and 9 µM BA in combination, citric acid (100mgl-1)

** Standard error

'-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response

Data represents the mean of three replicates.

Table 11: Effect of different level of PGRs for regeneration and mass multiplication of *C. suaveolens*[*]

PGRs Conc. (µM)		No of shoot buds formed/explant	Type of response	Performance[#]
0		-	No regeneration, and subsequently degenerated	-
NAA				
3		1	Plantlet slender and etiolated	+
6		2	Shoot bud formation	++
9		1	Plantlet stunted growth	+
IAA				
3		1	Plantlet slender and etiolated	+
6		1	Plantlet slender and etiolated	+
9		1	Plantlet slender and etiolated	+
BA				
3		1	Plantlets healthy but no roots	+
6		2	Shoot bud formation	++
9		3	Shoot bud formation	++
KN				
3		-	Etiolated plantlets	-
6		-	Etiolated plantlets	-
9		-	Etiolated plantlets	-
NAA	BA			
3	3	1	Shoot bud formation	+
3	6	3	Shoot bud with few PLBs	+
3	9	5	shoot bud with PLBs formation	+
6	3	1	Plantlet slightly stunted growth	+
6	6	4	Shoot bud with few PLBs formation	++
6	**9**	**12**	**Multiple shoot bud and repetitive PLBs formation**	+++
9	3	1	Plantlet slightly stunted growth	+
9	6	1	Plantlet slightly stunted growth	+
9	9	2	Shoot bud formation	+
NAA	KN			
3	3	1	Plantlet slightly etiolated	+
3	6	1	Plantlet slightly etiolated	+
3	9	1	Plantlet slightly etiolated	+
6	3	1	Plantlet stunted growth	+
6	6	2	Plantlet healthy with shoot bud formation	++
6	9	1	Plantlet healthy	+
9	3	1	Etiolated plantlet	-
9	6	1	Etiolated plantlet	-
9	9	1	Etiolated plantlet	-
IAA	BA			
3	3	1	Plantlet slender and etiolated	-
3	6	2	Plantlet healthy with shoot bud formation	++
3	9	2	Plantlet healthy with shoot bud formation	++
6	3	-	Plantlet slender and etiolated	-
6	6	-	Plantlet slender and etiolated	-
6	9	-	Plantlet slender and etiolated	-
9	3	-	Plantlet slender and etiolated	-
9	6	-	Plantlet slender and etiolated	-
9	9	-	Plantlet slender and etiolated	-
IAA	KN			
3	3	-	Plantlet slender and etiolated	-
3	6	-	Plantlet healthy but slightly etiolated	-
3	9	1	Plantlet healthy but shoot no. less	+
6	3	-	Plantlet stunted growth	-
6	6	-	Plantlet stunted growth	-
6	9	-	Plantlet stunted growth	-
9	3	-	Plantlet stunted growth	-
9	6	-	Plantlet stunted growth and etiolated	-
9	9	-	Plantlet stunted growth	-

[*] On ½ MS medium with sucrose (3%) (w/v), CH (0.1%) (w/v), AC (0.1%) (w/v) and citric acid (100 mgl^{-1})
[#] '-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three replicates.

Table 13: Effect of different strength of MS medium for PLBs differentiation, plantlet regeneration and mass multiplication of *T. latifolia

Strength of MS medium	Avg. plantlet height (cm)	No of roots/plantlet (±SE)**	No of shoot/bud formed per subculture	Types of response	Remarks#
0	-	-	-	No regeneration, all the plantlets degenerated after 1 wk of culture	-
1/4th	-	-	-	No regeneration, plantlets growth stunted and subsequently degenerated	-
1/2	-	-	-	Plantlets etiolated and slender and elongated	+
3/4th	2 (±0.5)	2 (±0.5)	2 (±0.5)	Plantlets healthy but less distinct pseudobulb	++
Full	**5 (±0.5)**	**4 (±0.2)**	**18 (±0.5)**	**Multiple shoot buds and repetitive PLBs formation, distinct pseudobulb with well developed roots**	+++

* On different strength of MS medium containing sucrose (2%) (w/v), CH (0.1%) (w/v), AC (0.1%) (w/v); PGRs 3 µM NAA & 9 µM BA in combination, citric acid (100mgl-1)
**: Standard error
#: '-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three replicates.

and regeneration of plantlets over Mitra *et al*, medium. In *C. suaveolens* the culture on Mitra *et al,* medium exhibited etiolated and stunted plantlets formation accompanied by light green and thin leaves with poor rooting. While, the *T. latifolia* cultures registered a retarded plant growth and poor pseudobulb formation on Mitra *et al,* medium. On further study with the different strengths of MS medium it was recorded that 1/2 strength of MS medium supported better regeneration in *C. Suaveolens* while, in *T. latifolia* full strength MS medium supported optimum response (**Table 10 and 13**).

Effect of PGRs on regeneration and mass multiplication

***Coelogyne suaveolens*:** Amongst the different PGRs tested for plantlet regeneration and mass multiplication, a combination of NAA and BA (6 and 9 µM respectively in combination) resulted healthy plantlet regeneration and multiple shoot buds formation (**Table 11**) in *C. suaveolens*. Under optimum condition as many as 12 shoot buds/secondary PLBs per explants developed within 8-9 wk of culture (**Fig. 6a**). On this medium the PLBs differentiated into rooted plantlets and multiple shoot buds. Both auxin and cytokinin when used singly did not support healthy culture or differentiation. A singly treatment of IAA produced stunted growth and KN gives etiolated growth. The combine effect of auxin and cytokinin was found superior over singly treatment of both the PGRs. The regeneration media supplemented with NAA-KN supported the conversion of PLBs to plantlets but plantlets were slightly etiolated, and leaves were light green in colour. While medium enriched with IAA-KN did not support healthy culture growth and formed thin and etiolated plantlets accompanied by poor rooting. The proliferation rate was very poor and fewer repetitive PLBs and shoots developed. On the other hand the medium with IAA-BA combination, the optimum response was registered

Table 14: Effect of different levels of PGRs on regeneration and mass multiplication of *T. latifolia*[*]

PGRs Conc. (µM)			No. of shoot buds formed per explants	Types of response	Performance[#]
NAA					
0			-	No regeneration	-
3			3	Multiple shoot bud formation	++
6			1	Direct shoot bud formation	+
9			1	Direct shoot bud formation	+
IAA					
0			-	No regeneration	-
3			1	Few PLBs formation	+
6			1	Few PLBs and shoot bud formed	+
9			1	Only few PLBs formed	+
BA					
0			-	No regeneration	-
3			-	No shoot bud but remains healthy	+
6			1	Few PLBs formed	+
9			2	Shoot bud and PLBs formed	++
KN					
0			-	No regeneration	-
3			-	No shoot bud with etiolated plantlets	+
6			1	Plantlet elongated and no shoot bud	+
9			1	Few PLBs with etiolated plantlet	+
NAA	BA	KN			
3	3	-	2	Few PLBs Formed	+
3	6	-	6	Callus mediated PLBs formation	++
3	**9**	-	**18**	**Multiple PLBs and shoot bud formation**	+++
6	3	-	1	Only few PLBs formed	+
6	6	-	1	Only few PLBs formed	+
6	9	-	2	Shoot bud and few PLBs formed	++
9	3	-	-	No shoot bud or PLBs formed	-
9	6	-	1	Few PLBs formed	+
9	9	-	1	Few PLBs formed	+
3	-	3	1	Plantlets slender and etiolated	-
3	-	6	2	Plantlets slightly etiolated	-
3	-	9	2	Plantlets slightly etiolated	-
6	-	3	-	No shoot but plantlet healthy	-
6	-	6	-	No shoot but plantlet healthy	-
6	-	9	-	No shoot but plantlet healthy	-
9	-	3	1	Plantlet slender and etiolated	-
9	-	6	2	Plantlet slender and etiolated	-
9	-	9	-	Plantlet slender and etiolated	-
IAA	BA	KN			
3	3	-	-	Plantlet etiolated	-
3	6	-	3	Shoot bud formation	++
3	9	-	2	Shoot bud formation	+
6	3	-	1	Plantlet slightly etiolated	+
6	6	-	1	Plantlet slightly etiolated	+
6	9	-	1	Plantlet slightly etiolated	+
9	3	-	-	No response	-
9	6	-	1	Plantlet slightly etiolated	+
9	9	-	2	Shoot bud formed but	+
				Plantlets were slender and etiolated	+
3	-	3	-	No response	-
3	-	6	3	Repetitive PLBs formed	++
3	-	9	2	Few PLBs formed	+
6	-	3	1	Only few PLBs formed	+
6	-	6	1	Only few PLBs formed	+
6	-	9	2	Few PLBs formed	+
9	-	3	-	No response	-
9	-	6	1	Plantlet slightly etiolated	+
9	-	9	1	Plantlet stunted growth	+

[*] On ½ MS medium with sucrose (2%) (w/v), CH (0.1%) (w/v), AC (0.1%) (w/v) and citric acid (100 mgl-1)
'-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three replicates.

with 3 + 9 µM respectively. The plantlets on this medium exhibited slightly retarded growth, slender with small leaves and light green in colour. Under optimum condition within 8-9 wk of culture the plantlets attained a height of ~ 3.5 cm with 3-4 roots with multiple shoot buds.

***Taenia latifolia*:** Amongst the different PGRs combination tested for plant regeneration a combined treatment of NAA and BA (3 and 9 µM respectively in combination) supported better plantlet regeneration and multiple shoot buds/secondary PLBs formation. Optimum response was registered on MS medium enriched with sucrose (2%) along with the above mentioned PGRs where within 6 wk of culture as many as 18 shoot buds/PLBs developed per explants (**Table 14 and Fig. 6c**). The singly treatment of both auxin and cytokinin did not support healthy culture growth and differentiation. A singly treatment of KN produced etiolated plantlets and IAA produce stunted growth but BA containing medium supported secondary PLBs formation. The combine effect of NAA and BA (3+9 µM respectively in combination) exhibited the optimum result where multiple shoot buds/secondary PLBs formed in the culture.

Effect of AC and CH on plantlet regeneration and mass multiplication

One set of culture of both the species were also maintained on the optimum regeneration medium supplemented with optimum PGRs for regeneration and AC (0-0.2%) (w/v) and CH (0-0.15%) (w/v) to study their effect on morphogenetic response and mass multiplication. The propagules like PLBs shoot buds raised from different explants sources of both the species were maintained for 2-3 passages on these conditions. In

Table 12: Effect of AC and CH on plant regeneration and mass multiplication of *C. suaveolens*[*]

AC Conc. (%)	CH Conc. (%)	Types of response	Performance[#]
0	0	Plantlet stunted growth, browning occurs	-
0.05	-	Moderate plant growth	+
0.1	-	Plantlets healthy with few shoot buds	++
0.15	-	Growth retarded and fewer shoot buds formed	+
-	0.05	Plantlet growth moderate	+
-	0.1	Plantlet growth healthy with fewer shoot buds	++
-	0.15	Plantlet growth retarded	+
0.05	0.05	Growth retarded and pseudobulb size reduced	+
0.05	0.1	Plantlet slightly etiolated	+
0.05	0.15	Plantlet height reduced	+
0.1	0.05	Plantlet healthy but moderate pseudobulb size	++
0.1	***0.1***	***Healthy and well rooted plantlets, multiple Shoot buds with enlarged pseudobulb***	+++
0.1	0.15	Plantlet healthy but stunted	++
0.15	0.05	Plantlet stunted growth	+
0.15	0.1	Plantlet moderate growth, pseudobulb under developed	+
0.15	0.15	Plantlet growth retarded and roots poorly developed	+
0.2	0.05	As above	+
0.2	0.1	As above	+
0.2	0.15	As above	+

* On ½ MS medium containing sucrose (3%) (w/v), 6 µM NAA & 9 µM BA in combination, citric acid (100 mgl-1)
# '-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three replicates.

Table 15: Effect of AC and CH on plant regeneration and mass multiplication of *T. latifolia*[*]

AC Conc. (%)	CH Conc. (%)	Types of response	Performance[#]
0	0	Plantlet slow growth	-
0.05	-	Plantlets pale green color bur fewer shoot buds	+
0.1	-	Plantlet healthy and well rooted	++
0.15	-	Plantlet slightly etiolated and less no of roots	+
-	0.05	Plantlet growth moderate and less no of roots	+
-	0.1	Plantlet healthy but fewer shoot buds	++
-	0.15	Plantlet growth retarded	+
0.05	0.05	Plantlet slow growth, pseudobulb size reduced	+
0.05	0.1	Plantlet slightly etiolated	+
0.05	0.15	Plantlet height reduced, under developed shoot bud	+
0.1	0.05	Plantlet healthy and well developed pseudobulb but leaf and roots development takes time	++
0.1	0.1	Healthy and well rooted plantlets, multiple shoot bud with well developed pseudobulb	+++
0.1	0.15	Plantlet slow growth, leaves and roots reduced size	++
0.15	0.05	Plantlet stunted growth, pseudobulb size reduced	+
0.15	0.1	Plantlet moderate growths, distinct pseudobulb, but fewer leaves and roots	+
0.15	0.15	Plantlet growth stunted and less developed roots	+
0.2	0.05	As above	+
0.2	0.10	As above	+
0.2	0.15	Above	+

*** On MS medium with sucrose (2%) (w/v), 3 µM NAA & 9 µM BA in combination, citric acid (100 mgl^{-1})**
 # '-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three replicates.

general the AC rich medium promoted the healthy culture growth, induction of repetitive PLBs and early development of roots.

Coelogyne suaveolens: Of the different concentrations of AC tested, a concentration of AC (0.1%) outperformed the other concentration and resulted early differentiation of shoot, roots, and development of distinct pseudobulbs (**Table 12**). Presence of AC in the regeneration medium also helped in controlling media browning compare to AC free medium. On the other hand increase or decrease of AC (0.1%) in the regeneration medium exhibited slow growth and poor rooting. While presence CH (0.1%) in the medium enhanced the luxuriant growth of plants and early rooting. In absence of CH (0.1%) growth slowed down and delayed the response. At lower concentration of CH (<0.1%) exhibited stunted growth of culture accompanied by fewer shoot buds formation, while at higher concentration the effect negated and most of the cases culture degenerated.

Taenia latifolia: Incorporation of AC (0.1%) in the regeneration medium triggered secondary PLBs formation and pseudobulb enlargement. But at higher concentration (>0.15) inhibited differentiation of culture and culture degenerated subsequently (**Table 15**). The combined effect of AC (0.1%) and CH (0.1%) in the regeneration medium promoted healthy growth of plantlets and root formation.

Artificial or Synthetic Seeds

Though different concentrations of sodium alginate solution was used for encapsulation of PLBs, a concentration of 2.5% formed firm and spherical beads when seeds in $CaCl_2$ (0.2M) (**Table 16, 17**). The beads were well textured and spherical in both

Figure 7: a. Encapsulated PLBs in sodium alginate gelling matrix, b. The beads are coated with water soluble capsule coating to make them rigid for physical delivery, c. The beads are cultured for germination on regeneration medium, and d. A germinated bead on germination medium.

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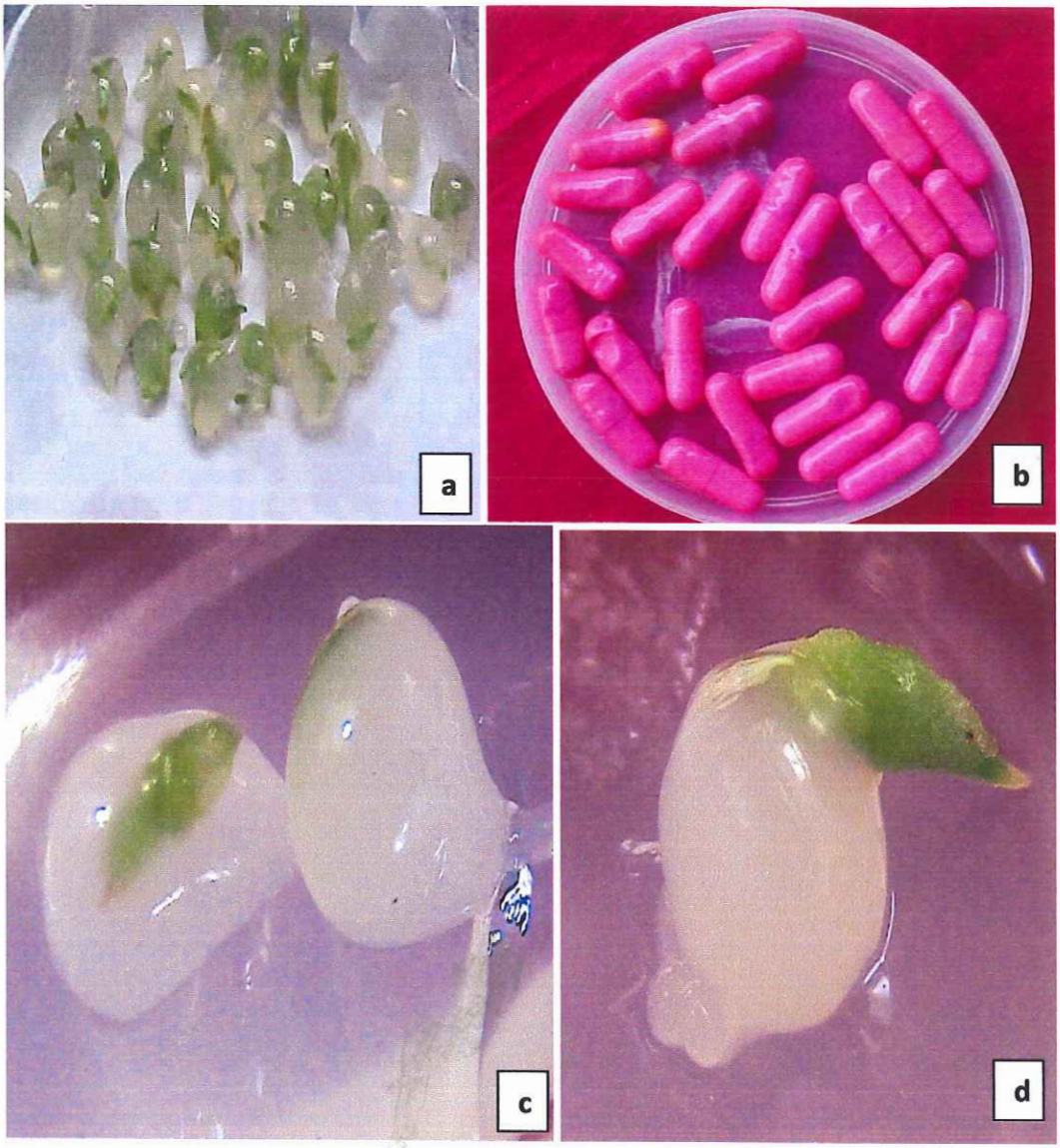

Figure-7

Figure 8: *In vitro* storage of germplasm through slow growth method. a. The Advanced stage PLBs and young plantlets of *C. suaveolens* under slow growth condition exhibiting retarded growth, b. The Advanced stage PLBs and shoot buds of *T. latifolia* slow growth condition exhibiting retarded growth.

Figure-8

Table 16: Effect of sodium alginate and calcium chloride ($CaCl_2$) concentration* on synthetic seeds formation of *C. suaveolens* using PLBs as explants

Sodium alginate Conc.) (%)	$CaCl_2$ Conc. (Mol)	Germination time (Days)**	Germination rate (%) (±SE)***	Types of response	Remarks#
0	0	-	-	-	-
1	0.1	-	-	Explant degenerated	-
1	0.15	-	-	Explant degenerated	-
1	0.2	-	-	Explant degenerated	-
1	0.25	-	-	Beads were very loose, irregular	-
1.5	0.1	88	20 (±1.0)	Beads were irregular in shape, explants degenerated before germination	-
1.5	0.15	86	20 (±1.0)	As above	-
1.5	0.2	83	24 (±0.5)	As above	-
1.5	0.25	76	30 (±1.0)	As above	-
2	0.1	64	33 (±1.0)	As above	-
2	0.15	57	40 (±1.0)	Beads were loose, irregular in shape and germination delayed	+
2	0.2	41	42 (±0.2)	Beads were loose, irregular in shape and germination delayed	+
2	0.25	28	54 (±0.2)	Beads were slightly loose but regular and germination period prolonged	+
2.5	0.1	26	68 (±0.3)	Beads were slightly loose but regular and plant growths moderate	+
2.5	0.15	20	84 (±0.2)	Beads were spherical, regular and plant growths moderate	++
2.5	**0.2**	**14**	**93 (±0.2)**	**Beads were spherical, regular, and gives healthy plantlets with shoot buds**	**+++**
2.5	0.25	18	78 (±0.2)	Beads were spherical, regular but were slightly harder than the above treatment	++
3.0	0.1	25	45 (±0.3)	Beads were spherical, regular but were hard and plantlets degenerated before germination	+
3.0	0.15	45	32 (±0.2)	Beads were spherical, regular but hard	-
3.0	0.2	57	25 (±0.2)	Beads were spherical, regular but hard	-
3.0	0.25	78	21 (±0.2)	Beads were spherical, regular but hard	-

* Sodium alginate is dissolved in ½MS medium containing sucrose (3%) (w/v), 6 µM NAA & 9 µM BA in combination, citric acid (100 mgl-1)

** Synthetic seeds cultured on ½MS medium (containing optimum adjuncts as in regeneration medium)

*** Standard error

'-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response

Data represents the mean of three replicates.

Table 17: Effect of sodium alginate and calcium chloride ($CaCl_2$) concentration* on synthetic seeds formation of *T. latifolia* using PLBs

Sodium alginate Conc.) (%)	$CaCl_2$ Conc. (Mol)	Germination time (Days)**	Germination rate (%) (±SE)***	Types of response	Remarks#
0	0	-	-	-	-
1	0.1	-	-	Explant degenerated	-
1	0.15	-	-	Explant degenerated	-
1	0.2	-	-	Explant degenerated	-
1	0.25	-	-	Beads were very loose, irregular	-
1.5	0.1	117	20 (±1.1)	Beads were very loose, irregular	-
1.5	0.15	94	20 (±1.0)	Beads were irregular in shape, explants degenerated before germination	-
1.5	0.2	88	24 (±0.5)	Beads were irregular in shape, explants degenerated before germination	-
1.5	0.25	86	30 (±1.0)	Beads were irregular in shape, explants degenerated before germination	-
2	0.1	63	33 (±1.0)	Beads were irregular in shape, explants degenerated before germination	-
2	0.15	47	31 (±1.0)	Beads were loose, irregular shape and delayed germination	+
2	0.2	41	32 (±0.1)	As above	+
2	0.25	37	44 (±0.1)	Beads were slightly loose but regular and Prolonged germination period	+
2.5	0.1	28	67 (±0.1)	Beads were slightly loose but regular and plant growths moderate	+
2.5	0.15	25	85 (±0.1)	Beads were spherical, regular and moderate plant growth	++
2.5	**0.2**	**21**	**95 (±0.1)**	**Beads were spherical, regular, develop multiple shoot buds, plantlets healthy**	**+++**
2.5	0.25	28	81 (±0.1)	Beads were spherical, regular but were slightly harder	++
3.0	0.1	55	55 (±0.1)	As above	+
3.0	0.15	64	32 (±0.1)	As above	-
3.0	0.2	76	23 (±0.1)	As above	-
3.0	0.25	88	20 (±0.2)	As above	-

* Sodium alginate is dissolved in MS medium containing sucrose (2%) (w/v), 3 µM NAA & 9 µM BA in combination, citric acid (100 mgl-1)

** Synthetic seeds cultured on MS medium (containing optimum adjuncts as in regeneration medium)

*** Standard error

'-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response

Data represents the mean of three replicates.

the species (**Fig. 7a**). A part of the beads from both the species were coated with water soluble capsule coating to make them rigid and user friendly for physical delivery (**Fig. 7b**). The uncoated beads were cultured on respective regeneration medium for germination (**Fig. 7c**). In both the species alginate beads prepared from 2.5% alginate solution supported highest conversion frequency. In *C. suaveolens* about 93% beads germinated after 14 days of culture, while in *T. latifolia* 95% beads germinated after 21 days of culture (**Table 16, 17 and Fig. 7d**). Beads prepared from higher concentration of alginate solution failed for support germination because of its hard textured and non breaking nature of the beads, and the failure of propagules to emerge out of the gel matrix. In contrast, in lower concentration of alginate solution, the beads were too soft, irregular shaped and propagules were not encapsulated properly. Synthetic seeds are sticky in nature and difficult to handle for physical delivery. To make the beads physically robust, the beads were coated with capsule coating. The coated beads were transferred on seed bed prepared from washed and autoclaved saw dust. A little success is being achieved till date and works are in progress to germinate the beads on seed bed.

In Vitro Short to Medium-Term Conservation

In the present study for *in vitro* storage of cultures of both the selected species, slow growth method was adopted. The young plantlets and advanced staged PLBs of both the species were collected from initiation media and cultured on different levels of MS medium (0, $1/4^{th}$, 1/2, $3/4^{th}$ and full strength) (**Fig. 8a, b**). The basal media were fortified with different levels of different organic carbon sources (0-3%) (viz. dextrose, fructose, glucose, mannitol and sucrose). All the propagules were cultured under different light and temperature. In general the cultures maintained in the dark mostly callused and failed to

Table 18: Effect of different organic carbon sources and media strength on *In vitro* conservation of *C. suaveolens through slow growth method**

Organic Carbon source	Conc. (%)	Strength of MS medium	Types of response	Performance#
	0	0	All the explants degenerated	-
Sucrose	1	1/4th	Plant growth highly stunted and subsequently degenerated	-
		1/2	Plant growth highly retarded and subsequently degenerated	-
		3/4th	Plant growth slightly retarded and degenerated	-
		Full	Plant growth stunted and retarded	+
	2	*1/4th*	***Plant growth retarded but without any gross anomaly***	+++
		1/2	Plant growth slightly retarded	++
		3/4th	Plant slightly retarded and growth slowed	+
		Full	Plant slightly retarded and etiolated	+
	3	1/4th	Plant growth slightly stunted but remains healthy	++
		1/2	Plant growth slightly retarded	+
		3/4th	No sign of slow growth and stunting	-
		Full	No sign of slow growth and stunting	-
Mannitol	1	1/4th	All the explant degenerated	-
		1/2	Plant growth highly etiolated and subsequently degenerated	-
		3/4th	Plant growth slightly retarded, etiolated and many degenerated	+
		Full	Plant growth slightly stunted but many degenerated subsequently	+
	2	1/4th	Plant growth slightly stunted and etiolated	+
		1/2	Plant growth retarded and stunted	++
		3/4th	Plant growth slightly stunted	+
		Full	Plant growth retarded and etiolated	+
	3	1/4th	Plant growth retarded but etiolated	+
		1/2	Plant growth retarded and remains stunted	++
		3/4th	Plant growth slightly etiolated	+
		Full	Plant normal growth or no sign of slow growth	-
Fructose	1	1/4th	All the explant degenerated	-
		1/2	Plant growth highly retarded and degenerated subsequently	-
		3/4th	Plant growth slightly retarded and stunted	+
		Full	Plant growth slightly retarded and stunted	++
	2	1/4th	All the explant degenerated	-
		1/2	Plant growth slightly retarded and degenerated subsequently	-
		3/4th	Plant growth slightly stunted but were etiolated	+
		Full	Plant growth slightly retarded and stunted	++
	3	1/4th	Plant growth retarded and stunted	++
		1/2	Plant growth slightly stunted	+
		3/4th	Plant growth normal and healthy	-
		Full	No sign of slow growth	-
Glucose	1	1/4th	All the plantlet degenerated subsequently	-
		1/2	All the plantlet degenerated subsequently	-
		3/4th	Plantlet highly stunted and many degenerated subsequently	-
		Full	Growth stunted and etiolated	-
	2	1/4th	Plantlet growth highly stunted and etiolated	+

Contd...

Organic Carbon source	Conc. (%)	Strength of MS medium	Types of response	Performance#
		1/2	Plantlet growth slightly retarded etiolated	+
		3/4th	Growth slightly retarded and remains etiolated	+
		Full	Growth slightly stunted and healthy	+
	3	1/4th	Plantlet growth slightly stunted and retarded	+
		1/2	Plantlet growth normal and healthy	-
		3/4th	Growth remains healthy and normal	-
		Full	Healthy plantlet growth or no sign of slow growth	-
Dextrose	1	1/4th	All the plantlet degenerated subsequently	-
		1/2	All the plantlet degenerated subsequently	-
		3/4th	Plantlet growth highly stunted and many degenerated subsequently	-
		Full	Plantlet growth slightly stunted and etiolated	+
	2	1/4th	Growth slightly stunted but etiolated and degenerated subsequently	+
		1/2	Plantlet growth slightly stunted and many degenerated subsequently	+
		3/4th	Plantlet growth retarded but half degenerated subsequently	+
		Full	Plantlet growth retarded but were slightly etiolated	+
	3	1/4th	Plantlet growth slightly stunted	+
		1/2	Growth healthy and normal	-
		3/4th	Plantlet growth healthy or no sign of slow growth	-
		Full	Plantlet remains health no sign of slow growth observed	-

* On different strength of MS medium with, 0.6 µM NAA & 0.9 µM BA in combination, citric acid (100 mgl-1)
# '-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three repeated experiments.

Table 19: Effect of different organic carbon sources and media strength on *in vitro* conservation of *T. latifolia through slow growth method**

Organic Carbon source	Conc. (%)	Strength of MS medium	Types of response	Performance#
	0	0	All the explants degenerated	-
Source	1	1/4th	All the plantlet degenerated subsequently	-
		1/2	All the plantlet degenerated subsequently	-
		3/4th	Plantlet growth stunted but many degenerated subsequently	-
		Full	Plantlet growth stunted but half degenerated subsequently	-
	2	1/4th	Plantlet growth highly retarded and stunted	++
		1/2	**Plantlet growth highly stunted, retarded but remains healthy with enlarge pseudobulb**	**+++**
		3/4th	Plantlet growth slightly stunted but healthy	++
		Full	Plantlet growth healthy though slightly stunted	+
	3	1/4th	Plantlet growth retarded but later many degenerated	-
		1/2	Plantlet growth remains healthy	-
		3/4th	Plantlet growth remains healthy	-
		Full	Plantlet growth remains healthy	-
Mannitol	1	1/4th	All the plantlet degenerated subsequently	-
		1/2	All the plantlet degenerated subsequently	-
		3/4th	Plantlet growth highly retarded and later degenerated	-
		Full	Plantlet growth highly retarded and many degenerated	-
	2	1/4th	Plantlet growth highly retarded and etiolated	+
		1/2	Plantlet growth highly retarded and etiolated	+
		3/4th	Plantlet growth slightly retarded but healthy	++
		Full	Plantlet growth slightly stunted and retarded but remains healthy	++
	3	1/4th	Plantlet growth slightly stunted and etiolated	+
		1/2	Growth slightly stunted but remains healthy	+
		3/4th	Growth healthy, no sign of slow growth	-
		Full	No sign of slow growth but remains healthy	-
Fructose	1	1/4th	All the plantlet degenerated subsequently	-
		½	All the plantlet degenerated subsequently	-
		3/4th	Plant growth highly stunted and half degenerated	-
		Full	Growth highly stunted and retarded, many degenerated	-
	2	1/4th	Plantlet growth highly stunted and many degenerated	-
		1/2	Plantlet growth highly retarded and etiolated	+
		3/4th	Growth slightly stunted and etiolated	+
		Full	Growth slightly stunted	+
	3	1/4th	Plantlet growth stunted and retarded	++
		1/2	Growth normal and remains healthy	-
		3/4th	Growth normal, no sign of slow growth	-
		Full	Growth normal, and healthy plantlet develop	-
Glucose	1	1/4th	All the plantlet degenerated subsequently	-
		1/2	All the plantlet degenerated subsequently	-
		3/4th	Plantlet highly stunted and many degenerated	-
		Full	Growth stunted and etiolated	-
	2	1/4th	Plantlet growth highly stunted and etiolated	+
		1/2	Plantlet growth highly retarded etiolated	+

Contd...

Organic Carbon source	Conc. (%)	Strength of MS medium	Types of response	Performance[#]
		3/4th	Growth slightly retarded and remains etiolated	+
		Full	Growth slightly stunted and retarded	+
	3	1/4th	Plantlet growth slightly stunted and retarded	+
		1/2	Plantlet growth normal and healthy	-
		3/4th	Growth remains healthy	-
		Full	Healthy plantlet growth or no sign of slow growth	-
Dextrose	1	1/4th	All the plantlet degenerated subsequently	-
		1/2	All the plantlet degenerated subsequently	-
		3/4th	Plantlet growth highly stunted and many degenerated	-
		Full	Plantlet growth slightly stunted and etiolated	+
	2	1/4th	Growth slightly stunted but etiolated and many degenerated	+
		1/2	Plantlet growth slightly stunted and many degenerated	+
		3/4th	Plantlet growth retarded but half degenerated	+
		Full	Plantlet growth retarded but were slightly etiolated	+
	3	1/4th	Plantlet growth slightly stunted	+
		1/2	Growth healthy and normal	-
		3/4th	Plantlet growth healthy or no sign of slow growth	-
		Full	Plantlet remains health, no sign of slow growth observed	-

* On different strength of MS medium with, 0.3 µM NAA & 0.9 µM BA in combination, citric acid (100 mgl^{-1})
# '-' No response; '+' Poor response; '++' Moderate response; '+++' Optimum response
Data represents the mean of three replicates.

regenerate on transferring to normal regeneration condition. While, the cultures maintained in normal laboratory light condition (40 µmol m^{-2} s^{-1}) and 25°C temperature maintained a healthy culture growth.

Diffused light (20 µmol m^{-2} s^{-1}) condition at 20°C was found to be suitable for both *C. suaveolens* and *T. latifolia* for slow growth method used for *in vitro* conservation. A $1/4^{th}$ strength of MS medium containing sucrose (2%) was found suitable over other concentrations for *C. suaveolens*, and ½MS medium containing sucrose (2%) for *T. latifolia*, where the plant growth retarded without any gross anomaly in normal physiological development of plant (**Table 18, 19**). The plantlets could be sub-cultured at 15-16 wk interval and normal growth resumed when they were transfer/sub-culture into optimum regeneration medium.

In *C. suaveolens* at lower level of MS medium ($1/4^{th}$ strength) the cultures degenerated on prolonged culture. The culture growth retarded on medium containing lower levels of mannitol (1 and 2%) and degenerated subsequently while, at higher level (3%) there was no adverse effect on culture growth and maintained normal growth. In general the medium containing fructose supported stunted growth but most of the culture degenerated in subsequent sub-cultures. But the cultures maintained on glucose rich medium supported retarded growth and could be retrieved the normal growth on normal regeneration. While, with dextrose all the plants remain stunted and degenerated later.

In *T. latifolia* ½MS medium performed optimally over other strength of MS medium. The different combinations of MS medium and organic carbon sources performed differently. Similar responses were registered with ½MS + sucrose (2%),

Figure 9: Hardening of regenerates and transplantation of hardened plants in community potting mix. a. *C. suaveolens* plantlets under hardening condition, b. *T. latifolia* plantlets under hardening condition, c. The hardened plants after transferring to community potting mix.

Figure-9

3/4thMS medium + mannitol (2%), 1/4th MS medium + glucose (3%) and 1/4thMS medium + fructose (1%) combinations. In these concentrations the culture growth arrested without causing any damage to the plants. At lower concentrations of most of the carbon sources the culture degenerated subsequently.

The cultures from both the species could be sub-cultured successfully at 15-16 wk interval. The cultures are being maintained from last three years. The cultures resumed their normal growth on transferring on normal regeneration medium.

Hardening of Regenerates and Field Establishment

Well rooted plantlets (~3-4 cm size with 3-4 roots) were taken out from the regeneration medium and washed off the traces of agar carefully from the roots (**Fig. 6b, d**). Plantlets were maintained for 8-9 wk in highly reduced level of liquid MS medium (1/10th strength) containing sucrose (2%) with small chips of charcoal, bricks or moss as a support to prevent the plantlets from submerging (**Fig. 9a, b**). The plantlets/seedlings were maintained in this medium under normal culture conditions and 12/12 h photoperiod. The hardened plants were then transferred to community potting mix containing charcoal pieces, brick pieces, coconut husk and decayed wood (1:1:1:1 ratio) with a layer of moss (**Fig. 9c**) and maintained for 7-8 wk in the poly-house. The newly transferred plantlets were fed with 1/10th Ms salt solution at one wk interval till transferring to the natural condition. The regenerates recorded survival rate of about 75-80% in *C. suaveolens* and 90% in the case of *T. latifolia* after 2 months of transfer. About 1100 regenerates of both the species are being transferred to the Poly-house/Botanical Garden/Wild.

Chapter-4

Discussion and Conclusion

Orchid seeds are minute with generally undifferentiated embryos and with little or no-endosperm. They depend upon a suitable mycorrhizal association, which provides an essential physio-chemical stimulus for germination. Orchids are propagated through vegetative means as well as seeds. However, the rate of vegetative propagation is very slow and the germination in nature is very poor (i.e. 0.2-0.3%). Their fungal requirement can, however, be compensated by a supply of sugars and the ingredients *in-vitro*. Several orchid species have responded to asymbiotic seed germination and has been positively tested in many threatened orchid taxa. However, they are greatly influenced by several factors, like seed age, different nutrient media and plant growth regulator and not a single nutrient media fulfils the requirements of the entire Orchidaceous group (Deb and Temjensangba, 2006a, 2007a, Temjensangba and Deb, 2005c). Knudson (1922) demonstrated the possibility of by passing the fungal requirement of orchid seeds during

in vitro germination and since then asymbiotic/non-symbiotic seed germination has emerged as an important tool for propagating a large number of orchid species and hybrids (Arditti *et al*, 1982). The fertilized ovules/immature embryos are used successfully for micropropagation and rapid mass multiplication of several commercially viable and or threatened orchids (Sharma and Tandon, 1990; Devi *et al*, 1998; Pathak *et al*, 2001; Temjensangba and Deb, 2005a, b).

The media used for asymbiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and sugars must be in a form readily available to the cultured immature embryos/seeds without the intermediary fungus (Mc Kendrick, 2000). But none of these basal nutrient media with different adjuvant fulfil the requirements of the entire orchidaceous group.

In the present study with *Coelogyne suaveolens* and *Taenia latifolia*, asymbiotic of seed/immature embryos were initiated on three different basal media viz: Knudson 'C', Mitra *et al,* and MS medium. Cultures were initiated from immature embryos/seeds representing different developmental of seed age (8-18 MAP) in *C. suaveolens* while in the case of *T. latifolia* 3-12 WAP were used. The first sign of seed germination in both the species was characterized by the nodular swelling of the immature embryos/seeds with a visible change in colour (circular yellowish embryos) due to physiological changes of cultured embryos followed by greening of the germinating embryos. The formations of nodular embryos were observed in both the selected species and these nodular embryos with the passages of time differentiated into PLBs. The relative time taken for the germination varied with the species. In *C. suaveolens* the first sign of germination was

observed after 20-25 days of culture while, a similar response in *T. latifolia* was registered only after 30 days of culture initiation.

Amongst the different media tested in *C. suaveolens* optimum germination was achieved on MS medium followed by Mitra *et al,* medium and Knudson 'C' medium and registered ~93%, 73% and 64% respectively. While, in case of *T. latifolia* ~95%, 50% and 43% germination was recorded on MS medium followed by Mitra *et al,* and Knudson 'C' medium. The nutrient requirements for asymbiotic seed germination of orchid is species specific and no single media can be prescribed for all the orchid taxa. Deb and Temjensangba (2006b) reported better seed germination of *Malaxis khasiana* on MS medium while, in *Cymbidium macrorhizon* on Mitra *et al,* medium (Vij and Pathak, 1988), *Dactylorhiza hatagirea* on Knudson 'C' medium (Vij *et al*, 1995), *Dendrobium chrysotoxum* on Vacin and Went (Rao *et al*, 1998), *Vanda coerulea* on Ichihashi & Yamashita (Rao *et al*, 1998) and VW medium (Devi *et al*, 1998), *Aerides rosea* on Knudson 'C', VW and MS media (Sinha *et al*, 1998), *Cymbidium iridioides* on Nitsch medium (Jamir *et al*, 2002). In the present study with the two species Knudson 'C' medium found to be least suitable for non-symbiotic seed germination. A similar response was also reported with other orchid species like *Renanthera imschootiana* (Laishram and Devi, 1999), *Geodorum densiflorum* (Sheela *et al,* 2000) and *Cymbidiums* (Nagaraju and Upadhyaya, 2001).

The influence of green pod developmental age varies with the genus, species within the genus. Different species of orchids exhibits a particular threshold, a factor genetically structured in the organism. The earliest stage at which the embryos can be cultured successfully varies within the orchid genotype and local conditions (Sauleda,

1976). Therefore it is desirable to determine the right stage to harvest the green capsule/pods to achieve an optimal germination. The culture of immature green pod/embryos ensures sterility but may require prolong period for germination as the seeds are immature. While, the culture of comparatively mature capsules/embryos before dehiscing may support better germination but the chances of contamination increases due to establishment of mycorrizal association (Mc Kendrick, 2000). Like other orchids in the present study also, the physiological age of the green pod found to be crucial factor for successful non-symbiotic seed germination in both the species. In the present investigation, the green pod age of 13 MAP supported optimum germination (93%) in *C. suaveolens*; while in the *T. latifolia* optimum germination was achieved from green pod of 8 WAP where about ~95% germination recorded. The green pod age <8 MAP in *C. suaveolens* and <4 WAP in *T. latifolia* either failed to germinate or germinated after prolonged period of culture. In *Malaxis khasiana* seeds of 8 WAP supported 75% germination (Deb and Temjensangba, 2006b) while, in *Dacylorrhiza hatagirea* seeds of 16 WAP exhibited better germination (Vij *et al*, 1995) but 12 WAP and 120 days old seeds were the ideal planting materials in *Cymbidium macrorhizon* and *C. iridioides* respectively (Vij and Pathak, 1988, Jamir *et al*, 2002). The ability of immature embryos to germinate better than the mature ones is due to their distended testa cells and metabolically awakened embryos and lack of dormancy and inhibitory factors (Yam and Weatherhead, 1988).

Presence of sucrose in the initiation medium showed a marked effect on seeds germination and was pre-requisite for seeds germination as there was no germination in both the species in the absence of sucrose. The different levels of sucrose showed

differential effect on seed germination in the present study. Sharma and Tandon (1990) reported that 2-3% sucrose; D-Fructose and D-Glucose were the suitable as organic carbon source for *in vitro* seed germination of *Cymbidium elegans* and *Coelogyne punctulata*. In the present investigation in both the species 3% sucrose supported optimum germination.

In the present study though the presence of CW in the germination medium was not pre-requisite for germination, but presence of CW in the germination promoted the early differentiation of germinated seeds into PLBs and shoot buds. A CW concentration of 15% proved to be most effective in both the species for seed germination as well as PLBs formation and differentiation. The role of CW in seed germination was reported in *Dendrobium aphyllum* (Talukdar, 2001), in *Vanda coerulea* (Devi *et al*, 1998), in *Cleisostoma racemiferum* (Temjensangba and Deb, 2006). Better seedling differentiation was achieved in *Peristeria elata* when medium supplemented with CW (10%) and proved to be advantageous over peptone (Bejoy *et al*, 2004) while, Sinha *et al*, (2002) and Vij and Aggarwal (2003) reported the enhanced repetitive PLbs formation in *Renades* and *Vanda coerulea* respectively when media were supplemented with CW (15%). However, Sheela *et al*, (2004) reported a rapid multiplication of PLBs as well as the development of shoots in *Dendrobium* CV Sonia when media is supplemented with 7.5% CW in the presence of BA (1.5 mgl^{-1}). Leetham (1974) reported that a plant growth regulator like substance (cytokinins) is present in coconut water whereas Lee *et al*, (1995) attributed its effect to its sugar and cytokinin content. But according to Jamir *et al*, (2002) the use of CW failed to elicit any effect on its growth in *Cymbidium iridioides* and *C. lowianum*.

In the present study three different light conditions were tested for *in vitro* seed germination for both the species. Of the three light conditions, diffused light (20 µmol m^{-2} s^{-1}) supported better germination followed by full light conditions (40 µ mol m^{-2} s^{-1}) in both the species. The cultures maintained in the dark either failed to germinate or callused and degenerated subsequently after it turns pale whitish-yellow. During the present investigation with the two selected orchids a diffused light (20 µmol m^{-2} s^{-1}) supported better germination followed by full light conditions (40 µ mol m^{-2} s^{-1}) at 12/12 h photoperiod. While, the cultures maintained in the dark degenerated mostly. Earlier Temjensangba and Deb (2005a) reported the effect of light on immature seed germination and observed that diffused light condition supported higher rate of germination in *Malaxis khasiana* while, Islam *et al*, (2003) reported that the *Phalaenopsis* callus growth and development of PLBs was better in illumination than in dark. The effect of light for embryogenic culture initiation in conifers is well documented (Von Arnold, 1987; Gupta and Grob, 1995, Deb and Tandon, 2004). In *Pinus kesiya*, light was reported inhibitory for initiation of embryogenic cultures and produced more non-embryogenic cultures. Dark was optimum while diffused light formed moderate embryogenic cultures (Deb and Tandon, 2004).

In both the species though there was nodular swelling of seeds even in the absence of PGRs in the germination medium but failed to differentiate into PLBs. The PGRs in the germinating medium showed a marked effect on the growth, differentiation and development of PLBs. In the present study seed germination of *C. suaveolens* was recorded as high as ~93% on MS medium containing NAA and BA (3 and 9 µM respectively) in combination but in *T. latifolia* higher seed germination was recorded

optimum on MS medium containing NAA (6 μM) singly. The spherules/nodular swelled embryos developed and differentiated into PLBs within 40 and 30 days respectively. A synergistic action of auxin and cytokinins was observed in asymbiotic seed germination in the present investigation with *C. Suaveolens* while, a singly treatment of auxin (NAA) proved to be effective in *T. latifolia*. Earlier Sharma and Tandon (1986) where they reported the stimulatory effect of NAA (0.1 mgl^{-1}) in conjunction with cytokinins (KN 1 mgl^{-1}) in *Coelogyne punctulata* for both seed germination and seedling growth. But Nagaraju *et al,* (2004) reported that in *Dendrobium* hybrid the single leaflet in the protocorm developed when basal medium was supplemented by BAP alone while, BA singly supported better germination in *Malaxis khasiana* (Temjensangba and Deb, 2005a; Deb and Temjensangba, 2006b).

In the current investigation cultures were also initiated from the foliar explants of both the species. Wimber (1965) successfully developed PLBs from the leaves of *Cymbidiums*, thus opened up an effective alternative to apical shoot meristem culture. Since then the regenerative competence of foliar explants were positively tested for more than 60 orchid species (Vij *et al*, 2000a; Temjensangba and Deb, 2005c; Deb and Temjensangba, 2007a). However, the success is restricted mostly with epiphytic orchids and only three species from terrestrial orchids suggesting thereby the ground orchids are less amenable to *in vitro* regeneration. In the present investigation, foliar explants of both the species from *in vivo* source however mostly remain recalcitrant though there were initial swellings at the basal part of the explants. The previous works suggest that the green house sourced explants are less responsive to *in vitro* regeneration due to profuse phenolic exudation from mature tissues, lose of chlorophylls, failure to developed

meristematic activity probably due to tissue damage during surface sterilization (Vij *et al*, 2000a) and present investigation is in agreement with this opinion. However, direct shoot buds/PLBs/young plantlets were induced from the basal part of the *in vitro* raised ~5 wk old foliar explants in *C. suaveolens*. A similar response was recorded from the foliar explants of *T. latifolia* (4 wk old). The better morphogenetic potential of the young tissue was attributed to the physiologically active state of the constituent cells owing to their less rigid cell walls (Misra and Bhatnagar, 1995). Vij *et al*, (1984) reported that the juvenile leaves proliferate along the surface where as the response is restricted to the basal region in relatively older explants. The importance of explants age is well documented in other orchid species, viz: in *Vanda coerulea* (Vij and Aggarwal, 2003), in *Aerides multiflora* (Vij *et al*, 2004), in *Cleisostoma racemiferum* (Temjensangba and Deb, 2005c), in *Arachnis labrosa* (Deb and Temjensangba, 2007a).

In both species the meristematic activity was restricted to the basal cut end of the foliar explants. The morphogenetic potential of leaf base has been reported in *Coelogyne, Dendrobium, Oncidium* and *Phalaenopsis* (Abdul Karim and Hairani, 1990); *Acampe praemorsa* (Nayak *et al*, 1997), in *V. coerulea* (Vij and Aggarwal, 2003), in *Arachnis labrosa* (Deb and Temjensangba, 2007a). Mathews and Rao (1985) considered the leaf base to be the decisive factor for culture initiation from foliar explants. Sinha and Hegde (1999) reported the development of meristematic activity along the entire in *Renades arunoday* Hybrid.

The incorporation of PGRs to the basal medium was obligatory for the initiation of culture. The explants of both the orchid species failed to respond, when cultured on PGRs free medium. The role of growth hormones in stimulating meristematic activity

and promoting proliferation in leaf explants is well documented in orchids (Abdul Karim, 1990; Vij and Pathak, 1990; Arditti and Ernst, 1993; Vij *et al*, 1994; Nayak *et al*, 1997; Temjensangba and Deb, 2005c; Deb and Temjensangba, 2007a). Murashige (1974) opined that *in vitro* plant regeneration occurs frequently through adventitious shoot formation and rarely through somatic embryogenesis. In the present study, NAA and BA (9 + 6 µM respectively) in combination produced as many as 9 regenerates per explants with 73% response in *C. suaveolens* after 6-7 wk of culture. A moderate response was registered in BA 9 µM singly containing medium. This result is in line with the earlier report that BAP was better used at 2 mgl^{-1}, which favoured development of multiple shoot buds (Vij *et al*, 2000b). Similarly, Saiprasad *et al,* (2003) reported BA enriched cultures produced more PLBs in *Dendrobium* 'Sonia'. However, in *T. latifolia* swelling and shoot buds development from the cut end was induced in IAA and BA (3+6 µM respectively) in combination. And 75% of the explants responded positively with as many as 5 regenerates forming per explants. However, combine treatment of BA + NAA, Kn + NAA showed little influence in stimulating the regeneration. Nagaraju *et al,* (2003) reported that supplementation of media with low concentration of BAP (0.5 mgl^{-1}) produced the maximum numbers of protocorm and shoots in *Cymbidium* and *Cattleya* culture. Cytokinins were successfully used to enhance the regeneration frequency and to multiply the cultures of several orchids (Vij and Pathak, 1990; Kaur, 1996; Temjensangba and Deb, 2005c; Deb and Temjensangba, 2007a). Earlier, Sinha and Hegde (1999) reported a 90% response in leaf explants containing 2 mgl^{-1} BA in a medium.

In the present study, the rhizome cultures from both *in vitro* and *in vivo* source were successfully established. The explants from the two sources exhibited differential response. The formations of meristematic loci were registered from both distal as well as proximal region of pseudobulbs of *in vitro* source. But, no meristematic loci invoked in the segments collected from the distal region of the *in vivo* sourced explants and, shoot buds develops only from the proximal region of pseudobulbs explants. The explants produced as many as 12 shoot buds after 7 wk of culture in medium containing NAA and BA (3+6 µM respectively in combination). While the explants from *in vivo* source, resulted 3-4 shoot buds after 10 wk of culture when enriched with NAA and BA (3 + 12 µM respectively in combination). Under optimum condition as many as 4 shoot buds developed where about 72% explants responded positively. No other PGRS except NAA could initiate morphogenetic response in the present study. The healthy shoot bud induction was registered on medium supplemented with both NAA and BA. The present study is in line with the reports of (Seeni and Latha, 1992; Vij and Aggarwal, 2003; Deb and Temjensangba, 2006a, b). Addition of AC proved to be beneficial in both explants. The positive effect of AC in shoot induction has also been reported in *Coelogne viscosa* (Vij *et al,* 1997) and *Malaxis khasiana* (Deb and Temjensangba, 2006b).

The differentiated PLBs and young plantlets (~1.0-1.5 cm size) from different propagules were then separated and were maintain on different levels of MS and Mitra *et al,* media. In the preliminary study it was observed that in both the species MS medium supported better differentiation and regeneration of plantlets over Mitra *et al,* medium. In *C. suaveolens* the culture on Mitra *et al,* medium exhibited etiolated and stunted plantlets formation accompanied by light green thin leaves with poor rooting. While, the *T.*

latifolia cultures registered a retarded plant growth and poor pseudobulbs formation on Mitra *et al,* medium. On further study with the different strengths of MS medium it was recorded that 1/2 strength of MS medium supported better regeneration in *C. suaveolens* while, in *T. latifolia* full strength MS medium supported optimum response. George and Sherrington (1984) and Chen *et al,* (2004) stated that the change in culture conditions and media could alter the pattern of organogenesis in orchids and such behaviour can be judiciously exploited to achieve desirable response in many orchid taxa by altering nutrient regimen.

In *C. suaveolens,* a combination of NAA and BA (6 and 9 µM respectively) resulted healthy plantlet regeneration and multiple shoot buds formation. Under optimum condition as many as 12 shoot buds/secondary PLBs per explants developed within 8-9 wk of culture but in *T. latifolia,* a combined treatment of NAA and BA (3 + 9 µM respectively in combination) supported better plantlet regeneration and multiple shoot buds/secondary PLBs formation. Optimum response was registered on MS medium enriched with sucrose (2%) along with the above mentioned PGRs where within 6 wk of culture as many as 18 shoot buds/PLBs developed per explants. Philip and Nainer (1988) observed that the requirement of exogenous supply of PGRs is species specific and it varies during initiation, multiplication and differentiation of cultures. In another report by Peres *et al,* (1999) observed that the exogenous PGRs markedly influenced the morphogenesis by alternation in the endogenous IAA/cytokinin balance. In the present study neither auxin nor cytokinin alone could results satisfactory regeneration of plantlets and multiple shoot buds formation in both the species. The present investigation is in agreement with the other reports where a synergistic action of auxin and cytokinin has

been reported with several other orchids (Vij and Pathak, 1990; Seeni and Latha, 1992). Earlier inhibitory effect of auxin on seedling development has been reported in *Orchis purpurella* (Hadley and Harvais, 1968), *Dactylorhiza purpurella, Coeloglossum viride* and *Platanthera bifolia* (Hadley, 1970). On the other hand, IAA at 0.1 mgl^{-1} is reported to promote seed germination and seedling development of *Cymbidium punctulata* (Sharma and Tandon, 1986). But Vij and Aggarwal (2003) reported that NAA favoured the development of multiple shoots/PLBs in *Vanda coerulea*. Bhadra and Hossain (2004) reported highest number of multiple shoot buds formation from nodal segment of *Micropera pallida* when medium was supplemented with 2.0 mgl^{-1} NAA and 2.0 mgl^{-1} BA. While the PGRs like BAP singly or in combination with IAA was best used for initiation of cultures and development of healthy plantlets there from leaf explants of *Saccolabium papillosum* (Kaur and Vij, 2000).

In the present study presence of AC (0.1%) and CH (0.1%) in the regeneration medium of both species resulted healthy and well rooted plantlets, multiple shoot bud formation with enlarged pseudobulbs. Presence of AC in the regeneration medium of both the species triggered the formation of repetitive PLBs, which added a new dimension in rapid mass multiplication of the selected species. Amongst the different concentrations of AC, a concentration of 0.1% in the regeneration medium was registered to be optimum for both the selected orchids. The AC concentration higher than 0.1% results in stunted growth plantlets and in most cases cultures were degenerated. The incorporation of CH to the basal medium was reported to enhance cell proliferation and rich crop of PLBs within 4 wk in pseudobulb segment culture of *Malaxis acuminata* (Vij and Kaur, 1998). Earlier Vij *et al,* (2000b) reported that the addition of AC to medium

promoted multiple shoot bud formation in *Bulbophyllum careyanum* culture with as many as 28 plantlets/explants and replacement of callus phase by direct development of shoot buds in the additional presence of AC. The benign effect of AC has been reported in many species (Chung *et al*, 1998; Paek *et al*, 1998; Chang and Chang, 2000; Nagaraju *et al*, 2004, Temjensangba and Deb, 2005c, Deb and Temjensangba, 2007a). All the workers reported that the incorporation of AC to the basal medium stimulated growth and produced healthy plantlets and significantly superior over the control. While, in *Cymbidium* hybrid culture presence of AC in the medium stimulated the early rooting (Nagaraju and Upadhyaya, 2001). However, AC in the regeneration medium proved inhibitory to regeneration in *Vanda* hybrid (Mathews and Rao, 1985), *Vanda teres* (Vij and Pathak, 1990) and *Vanda coerulea* (Vij and Aggarwal, 2003).

The artificial seeds/synthetic (Synseeds) seeds were first prepared by Redenbaugh (1986) for Alfalfa, since then synseeds have been prepared for number of plant taxa including orchids. According to Vij *et al,* (2001), synseeds have been prepared for more than 20 orchid species till date. In present study artificial seeds were prepared for both species and best beads were obtained in alginate solution concentration of 2.5% and $CaCl_2$ of 0.2M. Further, some of the beads were coated with water soluble capsule coating to make it rigid and user friendly for physical delivery. Further work on germination of these beads directly on the seed bed is in progress.

In *C. suaveolens* about 93% beads germinated after 14 days of culture, while in *T. latifolia* 95% beads germinated after 21 days of culture. Beads prepared from higher concentration of alginate solution failed for support germination because of its hard textured and non breaking nature of the beads, and due to the failure of propagules to

emerge out. While, in lower concentration of alginate solution, the beads formed were too soft, shape irregular and propagules were not encapsulated properly. Similarly, the concentration of alginate used for encapsulation, vary with the species and the type of propagules used (Redenbaugh *et al,* 1987; Ahuja *et al*, 1989; Datta *et al,* 1999; Nayak *et al*, 1998), also concentration requirements may vary depending upon the alginate quality which differ from brand to brand (Nayak *et al*, 1998) and batch to batch); likewise, effective concentration of $CaCl_2$ varies (Sakamoto *et al*, 1995 and Vij *et al*, 2001). The utility of synthetic seeds is highly advantageous and helpful in transplanting the regenerated plantlets to fields without undergoing the process of hardening as it is usually done in none encapsulated PLBs which is costly and labour intensive; also it greatly reduces time period required to obtain ready to transfer plantlet to field.

The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment. In other words the survival percentage is determined by the hardening of the plantlets. Losses of micropropagated plants after transferring to nature are attributed to low humidity, high levels of light and non-sterile condition of the *in vivo* environment (Paul, 1999). Conventionally the tissue-raised plants are hardened by transferring on a low nutrient medium having low organic carbon sources and maintained at high light intensity. Different matrix or substrates with manipulation in salt solution were employed for hardening of different angiospermic *in vitro* raised plants by various workers viz: soilrite for *Carica papaya* (Agnihotri *et al*, 2004), soaked cotton for *Saccharum offinarum* (Gill *et al*, 2004), etc. During the present investigation, the tiny plantlets of both the species were taken out from the regeneration medium and transferred in $1/10^{th}$ strength of MS salt solution containing sucrose (2%) and devoid of any PGRs.

In each culture tube small chips of charcoal, brick pieces and mosses were incorporated before the plantlets are transferred. Culture vials were maintained for ~8-9 wk under identical culture conditions. The hardened plants were then transferred to community potting mix-containing pieces of charcoal, brick, decayed wood (1:1:1) and moss. The potted plants were then transferred in poly-house and maintained for 7-8 wk. The plants were fed with $1/10^{th}$ MS salt solution at one wk interval. The acclimatized plants were transferred to Departmental botanical garden and the performances of these transplanted plants were monitored regular interval. Feeding the plantlets with nutrient salt solution has been reported to be beneficial for the promotion of orchid survival and growth (Mukherjee, 1983 and Kumaria and Tandon, 1994).

In the current investigation, it is interesting to note that during hardening process the newly developed roots get attached to the support medium with the passage of time and vigorous growth of the plantlets were observed. The attachment of roots to the support medium was mostly confined to the charcoal chips and moss, which strongly suggest the suitability of the material for the purpose. In other words, it resembles the velamenous root getting attached to the branch or the trunk of tree in natural habitat.

A survival frequency of about 75-80% was registered in the potting mix after about two months of transfer in both species. It was also observed that the transplanted regenerates were dark green and healthy and new roots and leaves started emerging after one month of transfer in the potting mix.

Attempt was made to conserve the germplasm of both the species *in vitro*. The propagules of both the selected species were subjected to *in vitro* short to medium term

storage using slow-growth method. The different types of propagules like advanced stage PLBs and young plantlets of both the species raised in culture were used for short to medium-term *in vitro* conservation. It was observed that among the different culture conditions studied, a diffused light (20 µmol m^{-2} s^{-1}) condition at 20°C was found to be suitable for both *C. suaveolens* and *T. latifolia*. The Cultures maintained in the dark mostly callused and in some cases cultures degenerated. The callused cultures failed to grow upon returning to normal light conditions and degenerated. While, the cultures maintained in normal light condition and 25°C temperature maintained a healthy culture growth and demanded rapid sub-culture.

Amongst the different levels of MS medium, a 1/4th strength of MS medium was found optimal for *C. suaveolens* and ½MS medium was superior of *T. latifoliu* over other concentrations when supplemented with sucrose (2%). Lower level of basal medium did not support plant growth and degenerated in subsequent subcultures. Other organic carbon sources like dextrose, glucose, mannitol etc though exhibited satisfactory result but failed to attain the optimal response compare to sucrose. In both the species the cultures could be successfully sub-cultured at 15-16 wk interval.

Earlier Deb and Temjensangba (2007b) also reported the similar response while working with *Arachnis labrosa* and *Cleisostoma racemiferum*. Withers (1991) reported that reducing the growth rate *in vitro* short to medium term storage provide a convenient option but not suited for long term programmes as it poses risk of selection due to stress imposed on the cultures during storage. Accordingly, the reduction of medium nutrient status had been reported by Malaurie *et al,* (1993) and the addition of activated charcoal to culture medium has also been reported as beneficial in minimal growth condition

(Paul, 1999). Slow growth method is a widely followed method but there is a continued concern about the level of somaclonal variation under slow growth conditions (Jaret and Gawel, 1991). In the present study the cultures were maintained under slow growth condition for more than 3 years and are still being maintained under slow-growth conditions. The normal plantlets were raised from the stored cultures when they were maintained on normal regeneration medium. No visible abnormality was registered in the regenerated plantlets and could be successfully potted in potting mix.

Conclusion

During the present study protocols are established for culture initiation from immature seeds/embryos, foliar explants of *Coelogyne suaveolens* and from immature seeds/embryos, foliar explants and rhizome segments *Taenia latifolia*, regeneration of plantlets and mass multiplication. These techniques open new routes for *in vitro* mass multiplication of these two rare and threatened orchids of North-East India. The use of *in vivo* sourced explants offers several advantages especially for monopodial orchids. The protocol established for culture initiation from *in vivo* sourced rhizome segments of *T. latifolia* indicates the possibility of using alternative explants for monopodial orchids and which demands further research to exploit this explants source. The techniques developed for *in vitro* short to medium-term storage of germplasm will ensure the presence of these species in nature in days to come. The protocols may be used by the local commercial orchid growers, NGOs and Government agencies associated with floriculture, which will add to economic development of the region.

Chapter-5

Summary

The North-Eastern region of India is endowed with great natural biotic resources including orchids. North-East India is represented by about 800 species including many endemic species. But these natural resources are fast depleting due to removal of forest cover and their natural habitat from unplanned developmental activities, logging and traditional agricultural practice "Slash and burn cultivation" prevailing in the region. As a result of these, many valuable floristic resources are lost, threatened and becoming extinct without realising their potential. So there is an urgent necessity to develop protocols for *in vitro* multiplication and conservation of the rare and threatened species. With a view to multiply and conserve two rare and threatened species (viz: ***Coelogyne suaveolens*** and ***Taenia latifolia***) of North East India the present study was undertaken. The cultures were initiated from different explants sources like immature embryos/seeds, foliar explants and rhizome/pseudobulbs of two selected rare and threatened orchids of North-East India.

In both the species cultures were initiated from immature embryos of different developmental stage [8-18 months after pollination, (MAP) for *C. suaveolens* and 3-12 week after pollination (WAP) for *T. latifolia*]. In *C. suaveolens* the seed cultured from green pod of 13 MAP resulted optimum germination while, in *T. latifolia* seeds of 8 WAP were found suitable for non-symbiotic germination. Seeds from <9 MAP and <7 WAP old pods of *C. suaveolens* and *T. latifolia* respectively showed delayed response and supported poor germination while the older seeds of both the species showed a decline in germination frequency and impaired germination. The first sign of successful germination was noted as nodular swelling of seeds after 20-25 days in both the species.

About 93% seed germination in *C. suaveolens* was achieved on MS medium containing sucrose (3%) (w/v), coconut water (CW) (15%) (v/v) followed by Mitra *et al,* (~73%) containing sucrose (3%) (w/v), CW (15%) (v/v). While in *T. latifolia* optimum seeds germination (~95%) was registered on MS medium containing sucrose (3%) (w/v), CW (15%) followed by on Mitra *et al,* medium (~50%) containing sucrose (3%) (w/v) and CW (15%) (v/v). Amongst the different plant growth regulators used in different combinations in *C. suaveolens* optimum germination was accomplished on medium containing NAA-BA (3 + 9 μM respectively in combination). But in *T. latifolia* a singly treatment of NAA (6 μM) was found optimum. With both the species cultures maintained in dark, diffused light condition (20 μmol m^{-2} s^{-1}) and full light condition (40 μmol m^{-2} s^{-1}) at 12/12 h photoperiod. But the optimum germination was registered in diffused light condition followed by full light condition (40 μmol m^{-2} s^{-1}) in both the species.

The regeneration from foliar explants of both the species from *in vivo* source remained recalcitrant to *in vitro* morphogenetic response though there were slight swellings in few cases. While ~5 and 4 wk old foliar explants of *C. suaveolens* and *T. latifolia* respectively from the *in vitro* source exhibited morphogenetic response by producing shoot buds and PLBs. About ~73% explants of *C. suaveolens* and 75% explants of *T. latifolia* responded positively where as many as 9 and 5 shoot buds/PLBs developed respectively. Amongst the adjuncts used, the optimum response was registered on MS medium containing sucrose (3%), casein hydrolysate (CH) (0.1%) (w/v), activated charcoal (AC) (0.1%) (w/v) and NAA + BA (9 + 6 µM in combination) in *C. suaveolens*, while, in the case of *T. latifolia* IAA + BA (3 + 6 µM in combination) was found suitable over other treatments. In *C. suaveolens* foliar explants produced direct shoot buds/PLBs within 6-7 wk while in *T. latifolia* within 5 wk a similar result was obtained. The explants were placed on the culture media in different orientation but, the foliar explants cultured at 45° outperformed explants cultured in other orientation.

Though the foliar explants from *in vivo* source remained recalcitrant, but the newly sprouted rhizome/pseudobulb (~6-7 wk old) segments (size: 0.5 cm) of *T. latifolia* resulted positively a *in vitro* morphogenetic response when cultured on MS medium containing sucrose (3%), AC (0.1%), CH (0.1%) and NAA + BA (3 + 12 µM respectively in combination). As many as 4 shoot buds resulted after 10 wk of culture. While the pseudobulbs harvested from *in vitro* grown plantlets (3-4 wk old) resulted a better crop. As many as 12 shoot/buds resulted after 7wk of culture on MS medium supplemented with sucrose (3%), AC (0.1%), CH (0.1%) and NAA + BA (3 + 6 µM respectively in combination). The explants cultured from *in vivo* source, the response

restricted only proximal region of the pseudobulb segments, while, response was registered from both distal as well as proximal region from the explants cultured from *in vitro* source.

The PLBs/shoot buds produced on initiation media were maintained for further development and differentiation. The PLBs/shoot buds/seedlings developed on initiation media from various explants sources of both the species were cultured on different levels of Mitra *et al,* and MS basal medium with different combinations of PGRs. In *C. suaveolens,* better growth, development and differentiation were recorded on ½MS medium containing sucrose (3%), CH (0.1%), AC (0.1%), and NAA and BA (6 + 9 µM respectively in combination) where as many as 12 shoot/buds/PLBs developed per explants/subculture. But in case of *T. latifolia* the optimum regeneration and culture multiplication was registered on MS medium containing sucrose (2%), CH (0.1), AC (0.1%) and NAA + BA (3 + 9 µM in combination) where 18 shoot/buds developed per explants per subculture.

The incorporation of activated charcoal in the regeneration medium showed a pronounced effect on mass multiplication by producing repetitive PLBs and multiple shoot buds. A concentration of AC (0.1%) was found optimum for both the species. In addition to AC incorporation of CH in the regeneration media in both the species enhanced the luxuriant growth of the plantlets and early rooting.

The regenerated rooted plantlets were maintained for ~2-3 passages for further growth followed by transferring them in hardening media. The well rooted plantlets (~3-4 cm long with 3-4 roots) were selected for hardening. The rooted plantlets were hardened

in 1/10th strength of MS salt solution containing sucrose (2%) devoid of any growth regulators with small chips of charcoal, bricks, or moss as a support to prevent the plantlets from submerging. The cultures were maintained in the normal laboratory light and temperature for 8-9 wk. The hardened plantlets were transferred to community potting mix along with the content of the hardening culture vials and maintained in the ploy-house for 7-8 wk before transferring to the wild. About 75-80% transplants survived after two months of transfer in case of *C. suaveolens* and 90% in case of *T. latifolia.*

The PLBs of both the species were encapsulated in sodium alginate. A concentration of 2.5% sodium alginate and 0.2 M $CaCl_2$ resulted firm beads which registered about 93% germination in *C. suaveolens* and 95% germination in case of *T. latifolia*. The beads were coated with water soluble capsule coating to make the robust for physical delivery and efforts are on to germinate the beads directly in the seed bed.

The *in vitro* short to medium term conservation of germplasm using slow growth method was registered better in diffused light (20 µmol m^{-2} s^{-1}) condition at 20°C temperature. The optimum slow-growth with *C. suaveolens* was achieved on ¼MS medium containing sucrose (2%). At this condition, slow and retarded growth of plants was observed without any gross abnormality in external morphology except the stunted growth. While, in *T. latifolia* a similar optimum slow-growth of culture were recorded on ½MS medium + sucrose (2%), 3/4thMS medium + mannitol (2%), 1/4thMS medium + glucose 3%) and 1/4thMS medium + fructose 1%). In general at lower concentration of organic carbon sources cultures degenerated while, at higher levels of organic carbon supported normal growth of the culture in both the species. The cultures could be

successfully at 15-16 wk interval. The cultures from slow-growth conditions resumed the normal growth on culturing on normal regeneration medium and conditions.

The protocols established opens new routes for *in vitro* mass multiplication of these two rare and threatened orchids of North-East India. The use of *in vivo* sourced explants offers several advantages especially for monopodial orchids. The protocol established for culture initiation from *in vivo* sourced rhizome/pseudobulbs of *T. latifolia* indicates the possibility of using *in vivo* source alternative explants for monopodial orchids and which demands further research to exploit this explants source. The protocol developed for artificial seeds and its coating tom make them physically robust will be of great value in the days to come. Though the present result is not very significant, but works are in progress to transfer the coated bead directly in the seed bed. The techniques developed for *in vitro* short to medium-term storage of germplasm will ensure the presence of these species in nature in days to come. The protocols may be used by the local commercial orchid growers, NGOs and Government agencies associated with floriculture, which will add to economic development of the region.

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6. **Sungkumlong and Deb C. R.** Rapid Mass Multiplication and Induction of Early *In Vitro* Flowering in *Dendrobium Primulinum* Lindl. *J Pl Biochem Biotech* (Communicated).

5. **Deb C. R., Sungkumlong and Temjensangba**. *Eulophia geniculata* King & Pantl.: A new record for Nagaland, India. *Rheedea* (In press).

4. **Deb C. R. and Sungkumlong.** On the regeneration competence of *Taenia latifolia* (Lindl.) Benth. Ex. Hook. Pseudobulb segments: A study *in* vitro. *Indian J Biotech*. (In press).

3. **Deb C. R. and Sungkumlong. 2008.** *In vitro* regeneration and mass multiplication of *Taenia latifolia* (Lindl.) using immature seeds: A threatened terrestrial orchid. *J Pl Biol*, 35: 1-6.

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1. **Deb C. R., Sungkumlong and Temjensangba. 2007**. A note on orchid additions to Nagaland flora. *J Orchid Soc* India, 21: 69-70.