MICROPROPAGATION OF SOME ORCHIDS OF DARJEELING AND SIKKIM HIMALAYAS

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ABBREVIATIONS

KnC	Knudson 'C'
MS	Murashige & Skoog
OMM	Orchid Maintenance Medium
VW	Vacin & Went
IAA	Indole-3-acetic acid
IBA	Indole butyric acid
NAA	α-Naphthalene acetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
CW	Coconut water
BA	N^6-benzyladenine
BAP	6-Benzyl amino purine
DMAP	Dimethyl amino purine
EDTA	Ethylene Diamine Tetracetic Acid
TDZ	Thidiazuron
PLB	Protocorm-like bodies
MW	moleculat weight
L	Litre
mm	milimeter
cm	centimeter
mg	milligram
gm	gram
°C	Degree Celsius
lb psi	Pounds per square inch
CITES	Convention on International trade on endangered species on wild flora and fauna

Contents

Introduction

1.INTRODUCTION

Orchids comprise a unique and distinctive group of plants. Taxonomically, these plants belong to the most highly evolved family orchidaceae in the plant group monocotyledons with 750 genera and 18000 species (Heywood, 1993) or 788 genera and 18500 species (Mabberley, 1998) constituting second largest family of flowering plants in the world. Orchid species are found in almost all regions of the world but their greatest diversity occurs in the tropical and subtropical climate where positive factors for growth, thick vegetation and high humidity prevail. India accounts for nearly 7% of the world's orchid genetic diversity contributed 1300 species in 184 genera. Out of 1300 species known to exist in India, 675 species are known to occur in north east and 730 species in eastern Himalaya. Pradhan (1976, 1979) described 130 genera and 810 species of orchids form India. In Sikkim and Darjeeling Himalaya, total number of orchid genus is more or less 115 and 496 species. Out of 675 species of north eastern India, 76 species are endemic, 18 are extinct or nearly extinct and 105 species are endangered.

Orchids occupy a special position in the plant kingdom due to its specialization and modification in their vegetative and floral characters. They are perennial, herbaceous epiphytic and occasionally terrestrial plants with long lasting flowers in myriad varieties of colors, shapes, sizes, architecture and fragrance. Approximately 60% of the orchids of Indian origin are epiphytic while the rest are terrestrial. However, certain genera like *Cymbidium* and *Liparis* have both epiphytic and terrestrial species. Amongst the terrestrial orchid species some are saprophytic in nature but few species are also adapted to saprophytic mode of life. The genus *Dendrobium* with 104 known species constitute largest genus of orchid in India. The endemicity in Indian orchids is very high and nearly 29% recorded taxa are endemic. Economically, orchids can be divided into two major categories under one category most of the orchids are horticultural and floricultural important plants that can spin cash in the international market, generate jobs and create an eco-friendly industry while medicinally important group of orchids having inherent therapeutic value constitute the second category and are used to cure many simple and complex diseases. Raj Bhandari & Bhattarai (2001) documented 53 species having medicinal value from north eastern region of India. The most significant use of orchid is ornamental because of their exquisitely attractive flowers or inflorescences remain fresh for longer period of time in comparison to other flowers. These qualities have made

orchid growing a high profitable industry all over the world. So, orchids are cultivated in a large scale for cut flowers and potted plants. On the other hand a large number of species bear attractive flowers and their capacity for inter specific as well as intergeneric breeding has generated tremendous possibilities producing hybrids or diverse floral characters. There are about 70 species of Indian orchids that have been used in breeding programs for producing hybrids in and outside the country for example *Dendrobium nobile*, an Indian orchid species extensively used in hybridizing over 77 hybrids registered so far in which it was parent. Today more than 1.2 lakh hybrids are known all over the world. The wild native orchids are likely to play a unique role in the development of new cultivars or hybrids and also restructing or existing with one or more attributes. India constitutes invaluable reservoir of these genes or germplasm of orchids that are needed for the development of new varieties.

The therapeutic value of orchids is related to variety of phytochemical constitutes including alkaloids, flavonoids, tarpens, carbohydrates and glycosides present in them. The ethno botanic studies carried out in the country showed that many orchids were used in medicines, so; the Indian orchids have also been used in various indigenous system of medicines since time immemorial. In the ayurvedic system of medicines, Indian orchids are used in the preparation of various rejuvenating formulations and tonics. Orchids also show immense evolutionary diversity in a response to different conditions particularly in the tropics like India. Some species are epiphyte growing on trees and others grow on rocks, while the majority of the temperate species are terrestrial. Many orchids perform a vital role in natural eco-systems and have complex interactions with insects and other animals which act as pollinators. In some cases the orchid provides a source of food or shelter for insects, most species also form relationships with mycorrhizal fungi which enable their tiny seeds to germinate.

In the present study a preliminary survey of Himalayan orchid biodiversity was done on the basis of their importance and status of availability in Darjeeling and Sikkim Himalaya. The list of total orchids has been presented in Table-1. List was made from the collection of orchids from the orchid growers of different places of the present study area.

Table 1- List of orchids found in Darjeeling and Sikkim Himalaya

Name of the orchids	Habitat	Status
Acampe papillosa	Epiphyte	Medicinal/Ornamental
Acanthephippium striatumu	Terrestrial	Ornamental/Horticultural
Acanthe phipiure	Terrestrial	Ornamental/Horticultural
Acrochaene punctata	Terrestrial	Ornamental/Horticultural
Acanthophippium striatumu	Terrestrial	Ornamental/Horticultural
Aerides biswasiana	Terrestrial	Ornamental/Horticultural
A.odorata	Epiphyte	Medicinal/Ornamental
A.vandara	Terrestrial	Ornamental/Horticultural
Anoectochilus brevilabris	Terrestrial	Vulnerable/Endangered
A.crispus	Terrestrial	Ornamental/Horticultural
A.elwesis	Terrestrial	Ornamental/Horticultural
A.grandiflora	Terrestrial	Ornamental/Horticultural
A.lanceolatus	Terrestrial	Ornamental/Horticultural
A.parviflora	Epiphyte	Scarce/Rare
A.regalis	Terrestrial	Medicinal/Scarce/Rare
A.roxburghii	Terrestrial	Scarce/Rare
A.tetrapterus	Terrestrial	Ornamental/Horticultural
A.tortus	Epiphyte	Ornamental/Horticultural
Anthogonium gracile	Lithophyte	Ornamental/Horticultural
Arachnanthe cathcartii	Terrestrial	Ornamental/Horticultural
Arundina graminifolia	Terrestrial	Medicinal/Ornamental
Ascocerntrum ampullaceum	Epiphyte	Ornamental/Horticultural
Biermannia bimacuiata	Terrestrial	Ornamental/Horticultural
Bulbophylum affine	Epiphyte	Ornamental/Horticultural
B.cylindraceum	Terrestrial	Ornamental/Horticultural
B.ebulbum	Epiphyte	Ornamental/Horticultural
B.helenae	Epiphyte	Ornamental/Horticultural
B.hirtum	Epiphyte	Ornamental/Horticultural
B.polyrizum	Terrestrial	Ornamental/Horticultural
B.putidum	Terrestrial	Ornamental/Horticultural
B.sikkimense	Terrestrial	Ornamental/Horticultural
C.labrosa	Terrestrial	Ornamental/Horticultural
C.keshabii	Terrestrial	Ornamental/Horticultural
C.mannii	Terrestrial	Ornamental/Horticultural
C.masuca	Terrestrial	Ornamental/Horticultural

Contd...

Table 1- (Contd.) List of orchids found in Darjeeling and Sikkim Himalaya.

C.plantaginea	Terrestrial	Ornamental/Horticultural
C.puberula	Terrestrial	Ornamental/Horticultural
C.tricarinata	Terrestrial	Ornamental/Horticultural
C.trulliformis	Terrestrial	Ornamental/Horticultural
C.whiteana	Terrestrial	Ornamental/Horticultural
C.yuksomensis	Terrestrial	Ornamental/Horticultural
Chilochista lunifera	Epiphyte	Ornamental/Horticultural
Cirrhopetalum ornaticimum	Lithophyte	Ornamental/Horticultural
C.walichii	Lithophyte	Ornamental/Horticultural
Cleisoscentron trichromum	Epiphyte	Ornamental/Horticultural
Cleisostoma filiforme	Epiphyte	Ornamental/Horticultural
Coelogyne barbata	Epiphyte	Ornamental/Horticultural
C.corymbosa	Epiphyte	Medicinal/Ornamental
C.cristata	Epiphyte	Medicinal/Ornamental
C.elata	Lithophyte	Medicinal/Ornamental
C.flaccida	Epiphyte	Medicinal/Ornamental
C.flavida	Epiphyte	Ornamental/Horticultural
C.fuscescens	Epiphyte	Ornamental/Horticultural
C.graminifolia	Lithophyte	Ornamental/Horticultural
C.hookerianum	Epiphyte	Ornamental/Horticultural
C.nitida	Epiphyte	Ornamental/Horticultural
C.occulata	Epiphyte	Ornamental/Horticultural
C.ochracea	Epiphyte	Ornamental/Horticultural
C.oracea	Epiphyte	Ornamental/Horticultural
C.ovalis	Epiphyte	Medicinal/Rare
C.punctulata	Lithophyte	Ornamental/Horticultural
C.rossiana	Epiphyte	Vulnerable/Endangered
C.treutleri	Epiphyte	Ornamental/Horticultural
Cremastra appendiculata	Terrestrial	Medicinal/Ornamental
C.wallichiana	Lithophyte	Ornamental/Horticultural
Cryptochilus lutea	Terrestrial	Ornamental/Horticultural
C.sanguinea	Epiphyte	Ornamental/Horticultural
C.cochleare	Epiphyte	Ornamental/Horticultural
C.devonianum	Epi/Lithophyte	Ornamental/Horticultural
Cymbidium aloifolium	Epiphyte	Medicinal/Ornamental

Contd...

Table 1- (Contd.) List of orchids found in Darjeeling and Sikkim Himalaya.

C. euburneum	Epi/Lithophyte	Ornamental/Horticultural
C.elegans	Terrestrial	Scarce/Rare
C.ensifolium	Epiphyte	Ornamental/Horticultural
C.hookerianum	Epiphyte	Medicinal/Rare/Ornamental
C.nitida	Epiphyte	Ornamental/Horticultural
C.occulata	Epiphyte	Ornamental/Horticultural
C.ochracea	Epiphyte	Ornamental/Horticultural
C.oracea	Epiphyte	Ornamental/Horticultural
C.ovalis	Epiphyte	Medicinal/Rare
C.punctulata	Lithophyte	Ornamental/Horticultural
C.rossiana	Epiphyte	Vulnerable/Endangered
C.treutleri	Epiphyte	Ornamental/Horticultural
Cremastra appendiculata	Terrestrial	Medicinal/Ornamental
C.wallichiana	Lithophyte	Ornamental/Horticultural
Cryptochilus lutea	Terrestrial	Ornamental/Horticultural
C.sanguinea	Epiphyte	Ornamental/Horticultural
C.cochleare	Epiphyte	Ornamental/Horticultural
C.devonianum	Epi/Lithophyte	Ornamental/Horticultural
Cymbidium aloifolium	Epiphyte	Medicinal/Ornamental
C. euburneum	Epi/Lithophyte	Ornamental/Horticultural
C.elegans	Terrestrial	Scarce/Rare
C.ensifolium	Epiphyte	Ornamental/Horticultural
C.hookerianum	Epiphyte	Medicinal/Rare/Ornamental
C. insigne	Terrestrial	Ornamental/Horticultural
C.lancifolium	Terrestrial	Ornamental/Horticultural
C.longifolium	Epiphyte	Ornamental/Horticultural
C.lowianum	Lithophyte	Ornamental/Horticultural
C.tigrinum	Epiphyte	Ornamental/Horticultural
C.traceyanum	Lithophyte	Ornamental/Horticultural
C.whiteae	Epiphyte	Vulnerable/Endangered
Cypripedium elegans	Terrestrial	Ornamental/Horticultural
C.himaliacum	Terrestrial	Scarce/Rare
Dediciea cunning hamii	Terrestrial	Vulnerable/Endangered
Dendrobium alba	Lithophyte	Ornamental/Horticultural
D. arachnites	Epiphyte	Ornamental/Horticultural

Contd...

Table 1- (Contd.) List of orchids found in Darjeeling and Sikkim Himalaya.

D. aurantiacum	Epiphyte	Ornamental/Horticultural
D.cathcartii	Epiphyte	Ornamental/Horticultural
D.crepidatum	Epiphyte	Ornamental/Horticultural
D.chrysanthum	Epiphyte	Medicinal/Ornamental
D. chryxotoxum	Lithophyte	Ornamental/Horticultural
D. crassinod	Epiphyte	Ornamental/Horticultural
D.denudans	Epiphyte	Ornamental/Horticultural
D.densiflorum	Epiphyte	Medicinal/Rare/Ornamental
D.falcorni	Epiphyte	Scarce/Rare
D.farmeri	Epiphyte	Scarce/Rare
D.fimbriatum	Epi/lithophyte	Scarce/Rare
D. formosum	Epiphyte	Scarce/Rare
D.gibsoni	Lithophyte	Ornamental/Horticultural
D.hirsute	Lithophyte	Ornamental/Horticultural
D.jenkinsii	Epiphyte	Medicinal/Ornamental
D.longicornu	Epiphyte	Medicinal /Ornamental
D.moschatum	Epiphyte	Ornamental/Horticultural
D.nobile	Epi/Lithophyte	Medicinal/Rare
D.primulinum	Epiphyte	Ornamental/Horticultural
D.rotundatum	Lithophyte	Ornamental/Horticultural
Diplomeris hirsuta	Lithophyte	Vulnerable/Endangered
D.pulchella	Lithophyte	Vulnerable/Endangered
Doritis tatenialis	Lithophyte	Ornamental/Horticultural
Eria bambusifolia	Epiphyte	Ornamental/Horticultural
E. conboleris	Lithophyte	Ornamental/Horticultural
E.convallarioides	Epiphyte	Ornamental/Horticultural
E. coronaria	Epiphyte	Ornamental/Horticultural
E.paniculata	Epiphyte	Ornamental/Horticultural
E. spicata	Epiphyte	Ornamental/Horticultural
E. stricata	Lithophyte	Ornamental/Horticultural
Gastrochilus acutifolius	Epiphyte	Ornamental/Horticultural
G.affinis	Lithophyte	Ornamental/Horticultural
G.dasypogon	Epiphyte	Ornamental/Horticultural
G. pseudodisticus	Epiphyte	Ornamental/Horticultural
Goodyera hemsleyana	Terrestrial	Ornamental/Horticultural
G. hispida	Terrestrial	Ornamental/Horticultural
G.schlechtendaliana	Terrestrial	Medicinal/Ornamental
G. vitata	Epiphyte	Ornamental/Horticultural
Habenaria arietina	Terrestrial	Ornamental/Horticultural

Contd...

Table 1- (Contd.) List of orchids found in Darjeeling and Sikkim Himalaya.

H. pectinata	Terrestrial	Ornamental/Horticultural
Herpysma longicaulis	Lithophyte	Ornamental/Horticultural
Hetaeria rubens	Lithophyte	Ornamental/Horticultural
Liparis dongchenii	Epiphyte	Ornamental/Horticultural
L.resupinata	Lithophyte	Ornamental/Horticultural
Luisia filiformis	Epiphyte	Ornamental/Horticultural
Malaxis calophylla	Terrestrial	Ornamental/Horticultural
M.saprophyllum	Epiphyte	Ornamental/Horticultural
Masdevalla herisonii	Epiphyte	Ornamental/Horticultural
Monomeria barbata	Epiphyte	Ornamental/Horticultural
Nephelaphyllum pulchrum var.sikkimensis	Terrestrial	Scarce/Rare
Nervilia macroglossa	Lithophyte	Ornamental/Horticultural
Oberonia micrantha	Terrestrial	Ornamental/Horticultural
Octochilus albus	Epiphyte	Ornamental/Horticultural
O. fuscus	Epiphyte	Ornamental/Horticultural
Paphiopedilum farrieanum	Terrestrial	Scarce/Rare/Medicinal
P. insigne	Terrestrial	Vulnerable/Endangered
P.maculates	Epiphyte	Ornamental/Horticultural
P. spicerianum	Terrestrial	Vulnerable/Endangered
P. teres	Epiphyte	Ornamental/Horticultural
P. venustum	Terrestrial	Vulnerable/Endangered
P.villosum	Terrestrial	Vulnerable/Endangered
P.wallichi	Epiphyte	Ornamental/Horticultural
P.wardii	Lithophyte	Vulnerable/Endangered
Papilionanthe biswasiana	Lithophyte	Ornamental/Horticultural
P. subulata	Epiphyte	Ornamental/Horticultural
P.teres	Epiphyte	Medicinal/Ornamental
P. uniflora	Epiphyte	Ornamental/Horticultural
P. vandarum	Terrestrial	Ornamental/Horticultural
Peristylus constrictus	Terrestrial	Ornamental/Horticultural
Phaius flavus	Terrestrial	Scarce/Rare
Phalaenopsis decumbens	Epiphyte	Ornamental/Horticultural
P. lobbii	Epiphyte	Ornamental/Horticultural

Contd....

Table 1- (Contd.) List of orchids found in Darjeeling and Sikkim Himalaya.

P. manii	Epiphyte	Ornamental/Horticultural
P.parishii	Epiphyte	Scarce/Rare
Pholidata watti	Epiphyte	Ornamental/Horticultural
Phreatia elegans	Epiphyte	Ornamental/Horticultural
Pleione hookeriana	Epiphyte	Ornamental/Horticultural
P. humilis	Epiphyte	Medicinal/Ornamental
P. lagenaria	Epiphyte	Scarce/Rare
P. maculata	Epiphyte	Medicinal/Rare/Ornamental
P. praecox	Epi/Lithophyte	Ornamental/Horticultural
Renanthera imschootiana	Epiphyte	Ornamental/Horticultural
Rhynchostylis retusa	Epiphyte	Medicinal/Ornamental
Risleya atro purpurea	Lithophyte	Ornamental/Horticultural
Ritia himalaica	Terrestrial	Ornamental/Horticultural
Sarcanhus pallidus	Epiphyte	Ornamental/Horticultural
Saturopsis undulates	Epiphyte	Ornamental/Horticultural
Satyrium alba	Terrestrial	Ornamental/Horticultural
S.nepalense	Lithophyte	Medicinal/Ornamental
Sobralia amesiana	Epiphyte	Ornamental/Horticultural
Spathoglottis ixioides	Terrestrial	Ornamental/Horticultural
Spiranthes sinensis	Epiphyte	Ornamental/Horticultural
T. cepidiforme	Epiphyte	Ornamental/Horticultural
Thrixpermum pygamaeum	Epiphyte	Ornamental/Horticultural
Thunia alba	Epiphyte	Ornamental/Horticultural
T. marshalliana	Epiphyte	Ornamental/Horticultural
T. venosa	Epiphyte	Ornamental/Horticultural
Trudelia cristata	Epiphyte	Ornamental/Horticultural
Uncifera lancifolia	Lithophyte	Ornamental/Horticultural
Vanda alpine	Epiphyte	Scarce/Rare
V.corulea	Epiphyte	Medicinal/Ornamental/Rare
V. cristata	Epiphyte	Medicinal/Ornamental
V. odoratum	Epiphyte	Ornamental/Horticultural
V passiflora	Epiphyte	Ornamental/Horticultural
V. pumila	Epiphyte	Ornamental/Horticultural
V. roxbugi	Lithophyte	Ornamental/Horticultural
V. stangeana	Epiphyte	Ornamental/Horticultural
V. suruila	Epiphyte	Scarce/Rare
V. testacea	Epiphyte	Ornamental/Horticultural
V. teres	Epiphyte	Ornamental/Horticultural
Vandopsis undulate	Epiphyte	Ornamental/Horticultural
Zeuxine goodyeroides	Epiphyte	Ornamental/Horticultural
Z.pulchra	Lithophyte	Ornamental/Horticultural

*List has been made from the collection of orchids from the orchid growers of several places of the present study area.

OBJECTIVES:

The orchid seeds generally do not germinate in nature and the common practice is to reproduce asexually by pseudobulbs or cuttings. Thus, huge commercial demand of planting materials is difficult to achieve. The plantlets developed through embryo culture or seed culture is generally subjected to micropropagation for rapid multiplication. This technique is helpful for commercialization of orchids. Micropropagation when done from a plantlet developed through any technique of plant tissue culture, it is called clonal micropropagation. Clonal micropropagation is very popular in commercial horticultural plants and orchids. The major advantages of micropropagation are i) propagation can be carried out under disease free conditions throughout the year, ii) sub-culturing of *in vitro* plantlets can be done after a defined intervals and each time one plantlet can give at least 3 to 4 nodal cuttings. This was necessary for meeting the huge demand of planting materials and also for survival of some wild orchids *in vitro* and for mass multiplication. After the survey of the orchids in the present study area twenty plants were selected for seed culture and also for micropropagation. Hence the objectives of the present study were set forth as:

- Production of plants in large scale through *in vitro* seed culture.
- Micropropagation of the plants developed through *in vitro* seed culture.

Literature Review

2. LITERATURE REVIEW

Besides medicinal importance, orchids are regularly used in the breeding program to develop new varieties and hybrids both at inter generic and inter specific level to improve their horticultural aspects. The major breeding criteria for improving orchids are suitable colors as per consumer's demand flower shape, fragrance, increase flower size, increase number of flower etc. the success of breeding process depends upon the fact that in most of the orchid genera and species there is no genetic barrier and they cross freely with each other. Once a hybrid is made, the only major problem is the large scale propagation because the seed setting capacity of the important hybrid is very poor along with its slow growing vegetative propagation. Other than all above mentioned economic consideration, orchids have some inherent capacities for drought resistance, nutrient conservations and long shelf-life of the flowers etc. These characters are very peculiar and not present in common crops. So, it is believed that orchids can contribute many genes to other crops just as went on creation of 'golden rice'. Therefore, the germplasm of orchids should be conserved because they may act as important gene donor in future for the improvement of other crops through genetic manipulation.

Although orchids belong to one of the largest families, they are also perhaps most seriously threatened plants on the globe. Their vulnerability depends upon following reasons:

1. Orchids have specialized life cycle. The vegetative propagation and multiplication of orchids is very slow process and it takes a long duration. In case of vegetative propagation through separation of stem and pseudobulbs one may not get more than a few plants after 4-5 years.
2. The pollination of orchid flower depends on the pollinators.
3. In the seeds of orchid, there is lack of reserve food material i.e., absence of endosperm, cotyledon etc.
4. The seed germination of orchid is always dependent on the association of mycorrhizal fungi, in some cases they are species specific. Therefore, only less than 1% of seed germinate in their natural environment due to non availability of specific fungi, so slow vegetative propagation through asexual method and the

need of mycorrhizal fungi for seed germination make their life cycle more vulnerable.

5. Owing to ornamental and therapeutic values which they possess has made them so sought after the man.

Each species is adapted to live in a specialized environment because of their specialized requirement and many species are restricted to distribution and so endemism is very high. Any destruction or degradation of their natural habitat beyond a tolerable limit causes threat for the survival. Due to human pressure on land and developmental activities, orchid wealth is depleted sadly and seriously. The epiphytic species of orchids are faced with the maximum danger due to cutting of host tree serving as a substrate. The natural process of extinction of orchids is also accelerated by the global warming as a result due to increasing aridity or decreasing moisture in the climate by deforestation and global warming cause a serious threat to the survivality of epiphytic orchids. In north eastern hill, tribal people are habituated to cultivate following shifting cultivation, this practice results to an ecological imbalance which reduces indirectly the orchid biodiversity and their habitat.

With the advancement of human civilization and exploration of orchid's world over for medicinal, ornamental and scientific purpose, the actual depletion of orchid began from there. During the early phase of exploitation many valuable orchids were exported from India to European destination and there was no attention towards their conservation. Any biological wealth of a country is its valuable heritage which is the product of million year's evolution, so, exploitation without conservation made continuous decrease of wealth. For orchid flora it is estimated that 250 species of native orchid are now under the threats of various categories. Therefore, other than economic consideration it is better to protect and conserve them for generation after generation before they reach the point of extinction. Protection can resist the depletion of orchid wealth in their natural habitats and the biopiracy, while the conservation is also a type of protection but is related with the propagation. Orchids are generally propagated by vegetative methods but vegetative method of propagation is very slow process which is not suitable for their rapid conservation. The slow rate of vegetative reproduction pointed to need for an alternative rapid propagation system. The alternative methods of orchid propagation are from seeds. In their natural condition, the majority of orchid flowers are

not pollinated, so their ovules are not fertilized and capsules are rarely formed. In case of successful pollination, fertilization of orchid ovule takes place 50-80 days after pollination and their seeds are formed within the fruit or pod. Orchid seeds are unique in several respects.

They are extremely small and usually undifferentiated. They are produced in large numbers ranging from 1300-40000 per capsule. Each seed contains, an undifferentiated embryo composed of 80-100 cells without any functional endosperms. The entire embryo is covered by a membranous transparent loose air filled seed coat or testa. The embryos are situated in the middle of the testa being attached to it by few fine strands. Testa cells are dead, vary in size and have longitudinal and transverse walls of different thickness which gives them a net like appearance, this is a marked character and is species specific. Since there is no cotyledon and no endospermic nutrient reserve for the embryo, the seed germination and the subsequent germination of the seedlings in orchids are extremely low. This process is activated in the presence of suitable fungus in nature. The fungus digests the seed coat while breaking the starch and cellulose into usable sugar for the embryo to ensure germination. Under natural condition, the orchid seed germinate in association with fungus (orchidomychorrhiza). Most of the orchidomychorrhizal fungi of orchids fall into groups like *Rhizoctonia*, of late; however, 54 different fungi have been isolated from 20 different orchid species. When an orchid seed comes in contact with specific mycorrhizal fungus, then they start to germinate and produce into plantlets. Stoutamire (1974) suggested that the fungus probably provides the growth substance to activate their enzyme system. On the basis of dependency on fungi for seed germination, orchid can be divided into two categories. In one group, the fungi invade the embryonic tissue during germination and such fungi remain associated with the orchid throughout its life in the root system. The orchid root has a partnership in which the fungus penetrates its thread like body or mycelium into the cortical cells of the root tissue. This relationship is of mutual benefit to the orchid to obtain nutrition and to the fungus as assured habitat. Seed of these orchids however germinate without the requirement of fungus when they fall into substratum. On the other hand, most of the orchid cannot germinate in nature without a fungus, because the mature plants do not allow the fungus to remain as orchidomychorrhiza, and they may be less dependent on this relationship. In absence of mycorrhizal association the seeds are not able to geminate in nature and as a result thousands of seeds perish every year. Until the middle of 19th

century, there was no method for germination of orchid seeds, therefore, neither vegetative nor seeds are suitable for rapid mass propagation of orchid by conventional way, but if the vegetative parts or seeds could be propagated by any alternative method, it would provide the basis for a technology to overcome limitation for conventional method of propagation for orchids. Since then plant scientists, made an intelligent attempt to exploit the plant tissue culture technique for the rapid mass propagation of the orchid. Plant tissue culture has opened a number of possibilities and, the literature on this aspect has accumulated rapidly more than thirty years. The application of tissue culture technique for the mass propagation of orchids and the benefits derived are marvelous for the production of orchid plants in large quantities which in time improves the orchid trade and industry.

The pioneer work of Bernard (1909) is important in the development of *in vitro* culture techniques of orchids. He successfully isolated the root infecting fungi helpful in orchid seed germination. Comparative germination studies were made of infected and non infected seeds when sterilized seeds were grown in association with the fungus, the percentage of germination improved considerably. Subsequently, the work of Knudson (1922, 1924, 1925) clarify many important points regarding the formation of seedling and organogenesis. Fungi were chiefly responsible for breaking down starch into simple sugars into germinating seeds. Thus Knudson's work showed for the first time that germination of orchid seeds could be possible *in vitro* without fungal association, Knudson (1951) also suggested a medium that provided balanced organic and inorganic nutrition for the developing seedlings. The medium proposed by Vacin and Went (1949) is widely used for germination of hybrid seeds. The credit for the initiation of meristem culture technique goes to Morel (1960). Therefore, vegetative propagation of orchids is also possible by *in vitro* technique. After Knudson, the seeds of different orchid varieties have been successfully germinated asymbiotically *in vitro* by many workers. The germination potential of embryo varies with their developmental stages. The immature embryos in general germinate readily and much better than the mature ones. Successful germination of immature embryos can depend on selecting the correct time after pollination at which to harvest the pods. The optimal harvest time for immature seed is therefore not easy to guess. In general terms, seeds are removed from the capsule which have progressed approximately ½ to ⅔ in their development from pollination to maturity. There are several advantages in using unripe immature seeds for *in vitro* culture.

The advantages are as follows:

1. It increases the rate of orchid seed germination.
2. Seedlings are given an earlier start by green pod culture and this can reduce the time from seed to flowers.
3. Immature seed culture can assist in obtaining seedlings from wide crosses where embryos often abort before reaching maturity.
4. In many species it is easy to miss the point at which capsules become ripe and start to shed seeds. Culturing the immature seeds can lessen the chances of seed loss by natural dehiscence.
5. The advantage of using seeds from capsules or pods for micropropagation is that, virus diseases etc. present in the capsule tissue may be eliminated when they are transferred to culture Mitchell (1989).
6. Large numbers of seedlings are produced to enable reintroduction, reinforcement, habitat restoration or improvement in native and alien forest segments.
7. Sustainable utilization of the commercially important taxa selection is possible; contributing to local nursery trade.
8. These provide an alternative source of species and take the pressure of waning wild population without harming the mother plants thereby, guaranteeing the survival of the species in the wild.
9. These contribute to maintaining a variable gene pool in contrast to clonal propagation. In fact, genetic diversity enhanced since most, if not all the seeds, including those that would not have germinated in the wild, are induced into seedling *in vitro*.
10. Genetic improvement of orchid is possible by culturing inter-specific/inter-generic hybrid seeds.
11. The length of reproductive cycle is shortening thereby achieving considerable saving in time by using immature seeds to produce plants.
12. Flasked orchid species are easier to establish than the wild-collected plants.
13. International transfer of flasked species is easily done avoiding CITES (convention on International trade on endangered species on wild flora and fauna)

Bhojwani and Razdan (1983) also reported that the nutritional requirements of the younger embryos are complex as compared to matured ones. Yam and Witherhead (1988) suggested that better germination response is related to their distending testa cells, metabolically awakened embryos and lack of dormancy and/or inhibitory factors. The embryo germinates just when procured from the capsules which have ceased to grow in diameter and develop deep ridges along the valve (Light 1990, Viz 1995).

The germination potential of immature seed was found to be directly co-related with their physiological age. The mature seeds, on the other hand either fail to germinate or germinate very poorly due probably to loss of growth promoting factors and accumulation of inhibitory and other dormancy factor, besides a change in the quality of food reserves, (Burgeff 1959, Kano 1965, Stoutamire 1974, Withner 1959). Besides the difference in age, and response in culture, the mature and immature seeds are morphologically more or less indistinct.

An analysis of morphogenetic changes in the orchid embryo prior to the development into seedlings *in vitro* indicate that orchid seed germination and subsequent development into seedlings is usually accompanied by an intervening protocorm stage or callus stage. The intervening protocorm stage develops directly from the embryo whereas the intervening callus tissue also develops directly from embryo and such callus tissue further develops into protocorm. In general direct germination related events include swelling of embryos and their emergence by bursting the seed coat as globular or elongated spherules which subsequently form chlorophyllus hairy and pear shaped protocorms. The development of protocorms into seedling is in general monopolar i.e., seedling development takes place from one pole of the protocorm. The protocorms develop directly into seedling in most of the species where their development of seedling is associated with intervening rhizomatous stage.

Transformation of the seedling in case of orchid from heterotrophic to autotrophic mode during early stage of development is an unusual aspect of differentiation, because a reverse trend characterizes most of the other symbiotic systems. The orchid protocorm has been variously considered as an undifferentiated (callus) or a differentiated (shoot primodia) tissue (Ariditti and Ernst 1984, Kanase *et al.*, 1993), whereas, functionally it is believed to behave as a cotyledon for supplying

nutrients during development of embryo and its subsequent growth into seedlings (Lee 1987).

Since the germinating entities (spherule, protocorms) profusely develop epidermal hairs and penetrate into the medium, it indicates their absorptive function. Therefore, the orchid seed germination with the development of rhizoids during early stage of *in vitro* germination is another unusual aspect because symbiotic seed germination has been changed into independent asymbiotic condition by the development of epidermal hairs. The protocorm budding characterizes orchid and it leads to the development of multiple plantlets. Depending upon the genotype and nutritional environment majority of the orchid species either follow the direct protocorm formation or callus mediated multiplication. The immature condition of the seed and the undifferentiated embryos are probably factors for callusing of the embryos and the callus develop from the undifferentiated embryo has been considered to be analogous to highly proliferated protocorm. Since the callus can be maintained or differentiated into multiple seedling at any time by varying chemical stimulus, so the callus mediated development is highly beneficial where the seeds are limited in number or their germination frequency is very low. It is also useful where the capsule otherwise take several months to develop. But only limitation is that numerical chromosomal abnormalities may occur in callus tissue after prolong culture and such cultures are not suitable for differentiation into multiple seedling. The germination responses of seed and their subsequent development into seedlings have been tested in a variety of standardizes nutritional media.

Since, the seedlings or plants obtained from seeds are genetically different, so, clonal propagation is not possible from seeds. This type of propagation only becomes possible on large scale by using the meristem or shoot tip explant of an orchid. This is also termed as vegetative micropropagation; the culture of an orchid meristem or shoot tip explant usually results in either the direct formation of protocorms or to the formation of callus from which protocorms can be regenerated. Thus the callus formed from orchid explant could be considered to be embryogenic callus in that protocorms are regenerated from it. The work mericloning is also adopted for the production of clones from meristem. Therefore, plant produce through the tissue culture of vegetative orchid tissue containing meristems are often called as mericlones. Besides terminal or axillary bud, the other parts of the plant were also used as explant. Among the other parts of the plant, young leaves, leaf tips, nodal sections, inflorescence stalk, young inflorescence and roots

have been used for *in vitro* culture of orchids. But in general, immature seeds and shoot tips containing meristems are commonly used for rapid mass propagation in terms of either conservation or commercial exploitation for orchid industry.

Conservation of orchid is not only linked with the large scale propagation but it is also related to commercialization. In order to truly conserve orchids, a large market must be created to promote and propagate orchids extensively by growers specifically for the economic development in the hill and foot hill regions of North Eastern India and elsewhere. When a large market condition is guaranteed, automatically the growers will be interested. To meet the large demand and commercialization of orchids, the large scale propagation can only be handled by tissue culture methods.

The seedlings when they are sufficiently large within the culture vessels then they are easier to handle and have better chances of survival. Generally, the flask seedlings are useless until and unless they are transferred to the community pots where the plantlets resume its growth like natural wild plant. The number of plants within the culture further maintained exploitation. The process, by which the seedlings are made to form independently from *in vitro* condition to *in situ* condition, is called hardening. In this process, seedlings are generally placed into the substratum in a group or singly. During removal from the flask seedlings are thoroughly washed in warm water in order to remove the agar and finally they are placed into a pot containing hardening medium. Mixtures of charcoal, brick pieces, pit moss etc. are generally used as hardening media. The seedlings are kept into shady place for few initial days and then gradually they are shifted to normal natural condition. On the basis of their habitat, the plantlets are reintroduced either as epiphyte or as terrestrial plant in the nature for enriching the declining population particularly in case of endangered and rare orchids.

Orchids are well known for their economic importance and widely cultivated for ornamental purposes as because of their beautiful long lasting flowers; but less for their medicinal value. History of alternative system of medicine, ayurveda and traditional chinese medicine has been found to utilize orchids in the preparation of medicines. Ayurvedic formulations like Chavyanprasa consists of eight ashtavarga (group of eight medicinal plants) and out of which four have been reported to be orchids.

In India work has been carried out on chemical analysis of some medicinally useful orchids; many of them are reported to contain alkaloids, triterpenoids, flavonoids

and stilbenoids. Recently there has been tremendous progress in medicinal plants research; however, orchids have not been exploited fully for their medicinal application. Habitat loss, forest destruction and degradation and over exploitation has posed threats to the conservation of orchids in local area.

Different orchids have been categorized in different headings below in seven different tables. Twenty five species of medicinally important orchids along with their habitat, flowering time, medicinal usage have been listed in (table-2). Seven ornamental orchids have been presented below for their commercial importance in cut flower nurseries (table-3). Forty one species have been found suitable as potted ornamental varieties (table-4) and twelve as hanging varieties (table-5). Fifty three species have been listed in (table-6) suitable for inter-specific breeding. Considering the cost effectiveness several species (table-7) have been suggested for trade by the orchid growers in Darjeeling and Sikkim Himalayas (Pal and Nagrare, 2006).

Table-2: List of medicinally important orchids of Darjeeling and Sikkim Himalaya.

Name of orchids (Habitat)	Flowering time	Medicinal uses (References)
Acampe papillosa (Epiphyte)	Sept-Dec	Roots used for rheumatism, sciatica, neuralgia, syphilis and uterine diseases. (Rao , 2004)
Aerides odorata Lour. (Epiphyte)	May-July	Fruits used for healing wounds, juice of leaves to heal boils in ears and nose. (Rao , 2004)
Anoectochilus regalis Bl. (Epiphyte)	June- August	Stems and leaves are used to prepare medicinal oils. (Rao , 2004)
Arundina graminifolia (Terrestrial)	July- August	Scrapped bulbous stem applied on heels to treat cracks, anti bacterial. (Singh, 2001)
**Coelogyne corymbosa* Lindl. (Epiphyte)	April-May	Pseudo-bulb A paste of the pseudo bulb is applied to the forehead to relieve headache. (Rao , 2004)
**Coelogyne cristata* Lindl. (Epiphyte)	March-April	Juice of the pseudobulb is applied to boils Pseudo-bulb and wounds for cooling and soothing effect, also used as diuretic. (Rao , 2004)
Coelogyne punctulata Lindl.(Epiphyte)	April-June	Pseudo-bulb dried, crushed and used in burn injuries and healing of wounds. (Rao , 2004)
**Coelogyne ovalis* Lindley (Epiphyte)	April- June	Aptly called as Jeevanti, the whole plant is used for cough, urinary infections and eye disorders. (Rao , 2004)
Cremestra appendiculata (Terrestrial)	March-April	Roots paste used for toothache and as emollient. Tuber used for abscesses, scrofula, and freckles and as an antidote to snakebite. (Rao , 2004)
Cymbidium aloifolium (L) Sw. (Epiphyte)	April- May	Whole plant used as an emetic, to induce vomiting and diarrhoea, to cure chronic illness, weakness of the eyes, vertigo and paralysis, to cure benign and malignant tumors. (Rao , 2004)
Cymbidium hookerianum (Epiphyte)	Jan-April	Seeds applied on cuts and injuries as hemostatic. (Rao , 2004)
**Dendrobium chrysanthum Lindl. Ex Wall.* (Litho-Epiphyte)	July – October	Stem is powdered used to replenish body fluids, tonic to moisten the stomach, lungs, enhance skin quality. As an aphrodisiac. (Singh, 2001)
Dendrobium jenkinsii Wall. Ex Lindl(Epiphyte)	March-April	Fresh and dried stems used in preparation of Chinese drug Shih-hu as a tonic. (Rao, 2004)
**Dendrobium densiflorum Lindl. Ex Wall. (Epiphyte)*	April- May	Leaves crushed to make paste with salt and applied on fractured area to set the bones. Pulp of pseudo bulb is applied to boils and pimples. (Rao, 2004)

Contd...

Table-2: (Contd.) List of medicinally important orchids of Darjeeling and Sikkim Himalaya.

**Dendrobium longicornu* Lindl. (Epiphyte)	Oct- Nov	Juice of the plant is used to relieve fever. Root is fed to livestock suffering from cough.(Rao, 2004)
**Dendrobium nobile* Lindl. (Epiphyte)	April - May	Stems used in preparation of Chinese drug Shih-hu for longevity and as an aphrodisiac, stomachic, analgesi (Rao, 2004). Plant is used in the treatment of pulmonary tuberculosis, general debility, flatulence, dyspepsia, reduced salivation, night sweats, fever and anorexia. (Singh, 2001)
Goodyera schlechtendaliana (Terrestrial)	March –June	Tincture of the plant in rice wine is used as a tonic for internal injuries and to improve circulation. (Rao, 2004)
Papilionanthe teres (Epiphyte)	March –June	Whole plant Used for remission of bodily heat in fever. (Rao, 2004)
Phaius tankervilliae Bl. (Terrestrial)	Sept-Oct	Paste of Pseudo bulbs is used to heal swelling of hands & legs, poultice to soothe pain of abscess. (Rao , 2004)
**Pleione humilis* (Smith) D.Don (Litho/Epiphyte)	Feb- March	Paste of the pseudo bulb is applied to the cuts and wounds. (Singh,2001)
**Pleione maculate* Lindl (Epiphyte)	October-Nov	Pseudo bulb is used in liver complaints and headache. (Rao , 2004)
**Rynchostylis retusa* Bl. (Epiphyte)	July - Sept	Roots are used for rheumatism, asthma, tuberculosis, nervous twitching, cramps, infantile epilepsy, vertigo, palpitation, kidney stone, and menstrual disorders. (Rao , 2004)
Satyrium nepalense (Epiphyte)	October- Dec	Tubers eaten for Malaria, dysentery, also used as aphrodisiac. (Rao , 2004)
Vanda corulea Griffex Lindl. (Epiphyte)	Sept-October	Leaf juice used for diarrhoea, dysentery and also used as aphrodisiac. (Rao, 2004)
Vanda tessellata (Roxb.) Hook. Ex Don (Epiphyte)	April - July	A paste of the plant is applied to cuts and wounds. The root is used as antidote against scorpion sting and remedy for bronchitis. (Singh, 2001).

* Indicates materials used in the present investigation

Table 3: Ornamental orchids suitable for cut flowers from the present study area.

1. Cymbidium eubrneum	*5. Paphiopedilum venustum*
2. Paphiopedilum insigne	*6. Vanda coerulea*
3. Paphiopedilum farrieanum	*7. Renanthera imschootiana*
4. Paphiopedilum villosum	

Table 4: List showing orchid species suitable as potted ornamentals

1. Anoectochilus brevilabris	*22.Goodyera hemsleyana*
2. Ascocentrum ampullaceum	*23.Goodyera hispida*
3. Bulbophyllum putidum	*24. Malaxis calophylla*
4. Calanthe chloroleuca	*25. Paphiopedilum farrieanum*
5. Calanthe masuca	*26. Paphiopedilum villosum*
6. Calanthe plantaginea	*27. Paphiopedilum venustum*
7. Coelogyne corymbosa	*28. Paphiopedilum insigne*
8. Coelogyne cristata	*29. Phaius flavus*
9. Coelogyne nitida	*30. Phaius tankarvillae*
10. Coelogyne ochracea	*31. Phalaenopsis lobbii*
11. Cymbidium aloifolium	*32. Phalaenopsis mannii*
12. Cymbidium eubrneum	*33. Pleione hookeriana*
13. Cymbidium lancifolium	*34. Pleione humilis*
14. Cymbidium mastersii	*35. Pleione maculata*
15. Dendrobium chrysotoxum	*36. Pleione praecox*
16. Dendrobium densiflorum	*37. Rynanthera imschootiana*
17. Dendrobium fimbriatum	*38. Vanda coerulea*
18. Dendrobium moschatum	*39. Vanda cristata*
19.Dendrobium nobile	*40. Vanda stangeana*
20. Eria bambusifolia	*41. Vandopsis undulata*
21. Eria coronaria	

Table 5: List of hanging species of orchids

1. Aerides odorata	*7. Dendrobium farmeri*
2. Cymbidium devonianum	*8. Dendrobium pendulum*
3. Dendrobium chrysanthum	*9. Dendrobium primulinum*
4. Dendrobium crepidatum	*10. Gastrochilus acutifolius*
5. Dendrobium desiflorum	*11. Gastrochilus dasypogon*
6. Dendrobium falconeri	*12. Rhynchostylis retusa*

Table 6: List showing species of orchids used for inter-specific breeding

1. Ascocentrum ampullaceum	*28. Dendrobium primulinum*
2. Bulbophyllum leopardinum	*29. Paphiopedilum farrieanum*
3. Bulbophyllum putidum	*30. Paphiopedilum insigne*
4. Calanthe chloroleuca	*31. Paphiopedilum spicerianum*
5. Calanthe masuca	*32. Paphiopedilum villosum*
6. Calanthe plantaginea	*33. Paphiopedilum venustum*
7. Coelogyne barbata	*34. Papilionanthe teres*
8. Coelogyne corymbosa	*35. Phaius flavus*
9. Coelogyne cristata	*36. Phaius tankervillae*
10.Coelogyne fuscescens	*37. Phalaenopsis decumbens*
11. Coelogyne nitida	*38. Phalaenopsis lobbii*
12. Coelogyne ochracea	*39.Halaenopsis mannii*
13. Cymbidium devonianum	*40. Pleione hookeriana*
14. Cymbidium eubrneum	*41. Pleione humilis*
15. Cymbidium hookerianum	*42. Pleione maculata*
16. Cymbidium lancifolium	*43. Pleione praecox*
17. Cymbidium longifolium	*44. Rynanthera imschootiana*
18. Cymbidium tigrinum	*45. Thunia alba*
19. Cymbidium tracyanum	*46. Thunia marshalliana*
20. Cymbidium whiteae	*47. Thunia venosa*
21. Dendrobium densiflorum	*48. Vanda corulea*
22. Dendrobium farmeri	*49. Vanda cristata*
23. Dendrobium formosum	*50. Vanda pumila*
24. Dendrobium gibsoni	*51. Vanda stangeana*
25. Dendrobium nobile	*52. Vanda tessellate*
26. Dendrobium parishi	*53. Vandopsis undulata*
27. Dendrobium pendulum	

Table 7: List of Orchid species suitable for market trade

1. Anoectochilus brevilabris	*20. Paphiopedilum fairrieanum*
2. Ascocentrum ampullaceum	*21. Paphiopedilum insigne*
3. Calanthe masuca	*22. Paphiopedilum spicerianum*
4. Coelogyne cristata	*23. Paphiopedilum villosum*
5. Coelotyne nitida	*24. Paphiopedilum venustum*
6. Coelogyne ochracea	*25. Phaius flavus*
7. Cymbidium devonianum	*26. Phaius tankervillae*
8. Cymbidium eubrneum	*27. Halaenopsis decumbens*
9. Cymbidium lancifolium	*28. Phalaenopsis lobbii*
10. Cymbidium mastersii	*29. Phalaenopsis mannii*
11. Cymbidium tigrinum	*30. Pleione hookeriana*
12. Cymbidium whiteae	*31. Pleione humilis*
13. Dendrobium chrysotoxum	*32. Pleione maculata*
14. Dendrobium densiflorum	*33. Pleione praecox*
15. Dendrobium falconeri	*34. Rynanthera imschootiana*
16. Dendrobium farmeri	*35. Vanda corulea*
17. Dendrobium nobile	*36. Vanda coerulescends*
18. Dendrobium pendulum	*37. Vanda cristata*
19. Dendrobium primulinum	*38. Vanda stangeana*

Materials and Methods

3. MATERIALS AND METHODS

3.1 Materials selected for micropropagation and sources of explants:

The total number of orchids according to the survey list was found to be 218 species available in Darjeeling and Sikkim Himalayas, out of which 20 different species have been taken into consideration for their micropropagation in the present study. The selection was based on their horticultural or medicinal importance. All the 20 species along with their status have been shown in Table-8 and the places of collection have been shown in the Map (Fig. 1).

Table 8: Orchid species used for micropropagation in the present study.

Names	Status
Bulbophyllum ornaticimum (Rchb.f.) J.J.Sm.	Ornamental and Horticultural
Coelogyne corymbosa Lindl.	Medicinal and Ornamental
Coelogyne cristata Lindl.	Medicinal and Ornamental
Coelogyne elata Lindley.	Ornamental and Horticultural
Coelogyne flavida Wall. Ex. Lindl.	Ornamental and Horticultural
Coelogyne nitida (Wall.) Lindley.	Ornamental and Horticultural
Coelogyne ovalis Lindley.	Medicinal and Ornamental
Cymbidium elegans Lindley.	Ornamental and Rare sp.
Dendrobium chrysanthum Lindl ExWall.	Medicinal and Ornamental
Dendrobium crepidatum Lindl.	Ornamental and Horticultural
Dendrobium densiflorum Lindl. Ex wall.	Medicinal and Ornamental
Dendrobium fimbriatum Lindl.	Rare and Endangered sp.
Dendrobium longicornu, Lindley	Medicinal and Ornamental
Dendrobium nobile Lindl.	Medicinal and Ornamental
Dendrobium primulinum Lindl.	Ornamental and Horticultural
Pleione humilis (Smith) D.Don	Medicinal and Ornamental
Pleione maculate (Lindl.) Lindl.	Medicinal and Ornamental
Pleione praecox (Smith) D.Don.	Ornamental and Horticultural
Rhynchostylis retusa Bl.	Medicinal and Rare sp.
Thunia alba Reichenbach fil.	Ornamental and Horticultural

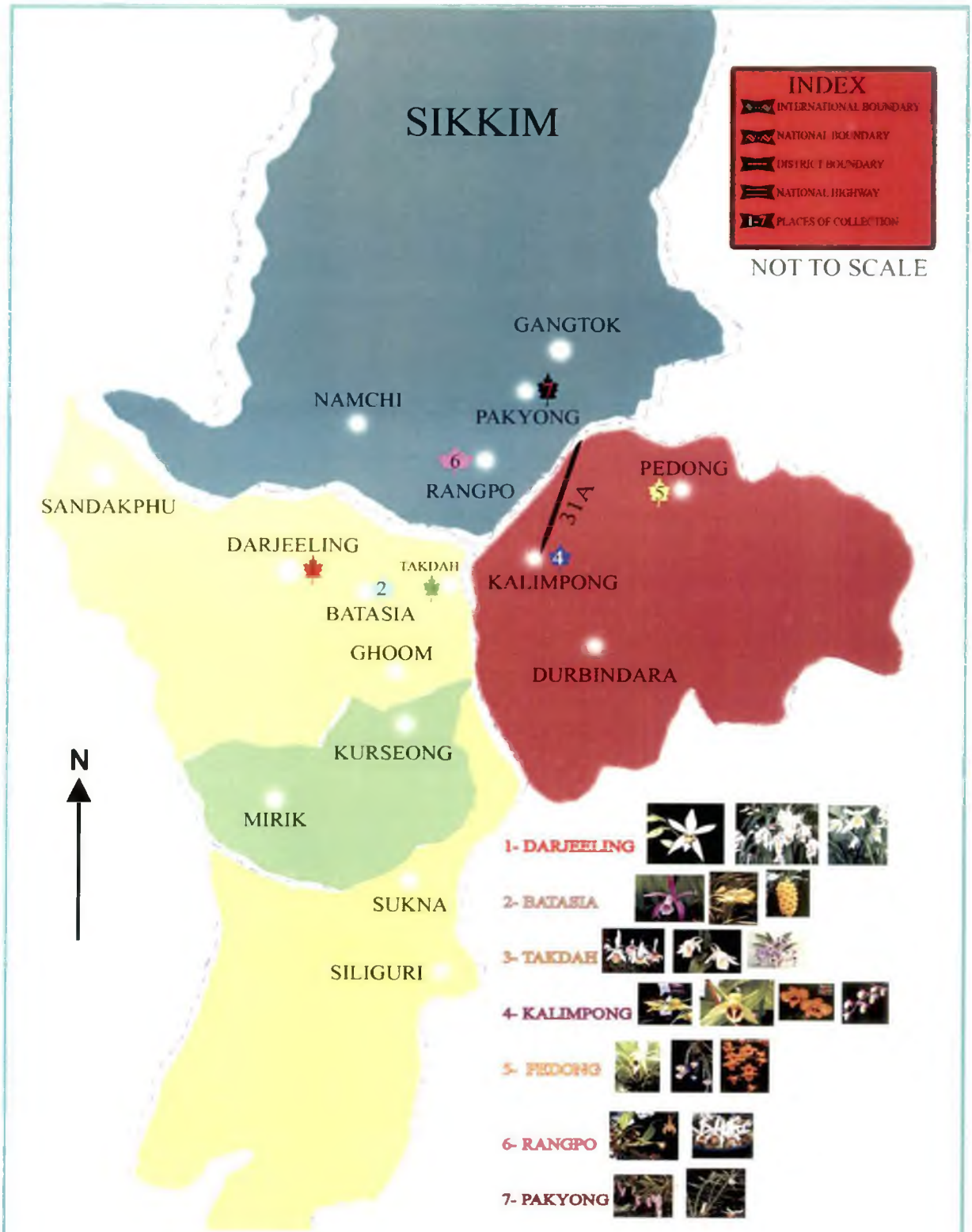

Fig.1: Map showing the places of collection.

Sources of explant: The source of explant was undehised fruits of the selected plant. Collection of undehised fruit or seed pod started from the natural habitat without damaging the mother plant on the basis of flowering and fruiting calendar. The total area covered for the collection of explant for tissue culture in the laboratory is being shown on the map (Fig.1). Main area includes different forest locations of Batasia, Ghoom, 6th Mile, Darjeeling (Latitude 26°31′ - 27°13′ N, Longitude 87°59′ - 88°53′ E), Kalimpong (Latitude 27.073610 N, Longitude 88.477859 E) and Sikkim (Latitude 27.20 N, Longitude 88.40). Approximate harvesting period of the pods has been shown in the Table-9.

Table 9: Seed harvesting time of orchids used in the present study.

Names of the orchids	Time of harvesting (Days)
Bulbophyllum ornatissimum (Rchb.f.) J.J.Sm	120
Coelogyne corymbosa Lindl.	170
Coelogyne cristata Lindl.	270
Coelogyne elata Lindley	150
Coelogyne flavida Wall. Ex. Lindl.	170
Coelogyne nitida (Wall.) Lindley	240
Coelogyne ovalis Lindley	300
Cymbidium elegans Lindley	210
Dendrobium chrysanthum Lindl. Ex Wall.	275
Dendrobium crepidatum Lindl.	215
Dendrobium densiflorum Lindl. Ex wall.	221
Dendrobium fimbriatum Lindl.	120
Dendrobium longicornu, Lindley	201
Dendrobium nobile Lindl.	108
Dendrobium primulinum Lindl.	217
Pleione humilis (Smith) D.Don	248
Pleione maculate (Lindl.) Lindl.	270
Pleione praecox (Smith) D.Don	247
Rhynchostylis retusa Bl.	300
Thunia alba Reichenbach fil.	218

3.2 Seed Culture Technique:

The seed culture technique helps in germination of plants where the normal growth is difficult. This technique ensures much better germination particularly in the genera where germination is not possible due to some abnormality within the seeds (Arditti *et al.*, 1982; Linden, 1980; McIntyre *et al.*, 1972; Pathak, 1989; Pathak *et al.*, 1992; Vij and Pathak, 1988; Vij *et al.*, 1995; Yam and Weatherhead, 1988). The orchid seeds are minute and exhibit very poor level of differentiation. The development of the embryo is suppressed as such that the embryo is arrested at a bulbous stage. In spite of being insubstantial embryo,

they can germinate in nature if infected with a suitable fungus (Bernard, 1904). However, the germination can bypass the fungal requirement by supplying an appropriate sugar rich nutrient medium *in vitro* (Knudson, 1925). Significantly, the orchid ovules are also capable of development *in vitro*. Prior to being fully ripe the capacity to germinate drops progressively (Arditti, 1967; Withner, 1943).

3.3 Micropropagation Technique:

The technique of orchid micropropagation has been originated since many years, its origin lies in several lines of research and came from the work of many well known scientists, (Arditti and Krikorian, 1996). Method for the *in vitro* culture of isolated plant cells, leaves and organs or seeds are not difficult or complex but they do require some equipment and certain skills and knowledge. More than one hundred years ago the method of propagating *Phalaenopsis* was viewed by simply placing an explant on a medium and cultured until it produced a plantlet or died.

The modern history or orchid micropropagation started when a new (*in vitro*), simple and practical method for vegetative (clonal) propagation of *Phalaenopsis* was developed (Rotor, 1949). The nutrient medium used to culture the *Phalaenopsis* nodes was Knudson "C" (KnC) formulated for the asymbiotic germination of orchid seeds by Lewis Knudson. The solution was improved and published as Knudson C, "KC" reasonably a good medium for orchid seed germination (Knudson, 1946). This medium is used very widely for orchid seed germination (Arditti *et al.*, 1982) and the propagation of some orchids.

3.4 Media Components:

The germination response of seeds, multiplication of shoots tips directly or indirectly and their subsequent development into seedling has been tested in a variety of chemically defined medium. Among them Vacin & Went (1949) [VW], Knudson 'C' (1946) [KnC], Murashige & Skoog 1962 [MS], Orchimax and orchid maintenance media were selected and used for the present work. The detail compositions of the media are given in Table-10.

Table 10: Showing the basic components of media

COMPONENTS	Vacin & Went (1949)	Knudson C (1946)	MS (1962)	Orchimax	OMM
Macronutrients (mg/lt)					
$Cacl_2$	-	-	332.02	166	166.08
$Ca(NO_3)_2$	-	241.30	-	-	-
$Ca_3(PO4)_2$	200	-	-	-	-
KCl	-	250	-	-	-
KH_2PO_4	250	250	170	85	85.00
KNO_3	525	-	1900	950	950.00
$MgSO_4$	122	122.15	180.54	90.35	90.34
NH_4NO_3	-	500	1650	825	825.00
$(NH_4)_2SO_4$	500	500	-	-	-
Micronutrients (mg/lt)					
$COCl_2.6H_2O$	-	-	0.025	0.0125	0.0125
$CuSO_4.5H_2O$	-	-	0.025	0.0125	0.0125
$Fe_2(C_4H_4O_6)_3$	23.13	-	-	-	-
FeNaEDTA	-	-	36.70	36.7	37.30
$FeSO_4.7H_2O$	-	25	-	-	27.80
H_3BO_3	-	-	6.20	3.1	3.10
KI	-	-	0.83	0.415	0.42
$MnSO_4.H_2O$	5.68	5.68	16.90	8.45	8.45
$Na_2MO_4.2H_2O$	-	-	0.25	0.125	0.12
$ZnSO_4.7H_2O$	-	-	8.60	5.3	5.30
Vitamins (mg/lt)					
myo-inositol	-	-	100	100	100
Glycine	-	-	2	-	-
Nicotinic acid	-	-	0.50	1.00	1.00
Pyridoxin HCl	-	-	0.50	1.00	1.00
Thiamine HCL	-	-	0.10	10.00	10.00
Buffers (mg/lt)					
MES(Morpholino ethane sulfonic acid)	-	-	500	1000	1000
Organics (gm/lt)					
Sucrose	20	20	-	20	20
Tryptone	-	-	-	2	2
Activated Charcoal	-	-	-	2	2

In the orchid tissue culture media different macro and micro elements were mixed together. All the chemicals were stored in accordance with instructions on the package or in the refrigerator. Some chemicals may absorb water from air and either solidify or liquefy. Such chemicals were stored under vacuum in refrigerator.

Macro elements:

Macro elements are the major elements or nutrients that are needed in relatively larger amounts. They include calcium (Ca), magnesium (Mg), nitrogen (N), phosphorus (P), potassium (K) and sulfur(S). Depending on the medium used, several salts were used to supply each mineral in that medium.

Stock solution of macro elements (except those containing nitrogen) could be stored at room temperature (usually about 22°C). Nitrogen containing stock solutions may be contaminated even in the refrigerator and therefore, stored in a freezer, within a container that is capped tightly to prevent evaporation. After mixing in the medium, all macro element salts were sterilized by autoclaving.

Micro elements:

Culture media vary widely in the use and content of micro- or minor elements or nutrients. Several micro elements were used in the present study. Micro element stock solution was stored in the freezer. Microelements were mixed in the medium, as and when required. Finally the medium was sterilized by autoclaving.

Iron:

Many iron salts are mixed in the medium. Some are soluble initially, but in solution the iron is oxidized to an insoluble or sparingly soluble form. Ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), and a number of other can be used in the preparation of media. Instead of using iron compounds directly, certain alternative compounds may be used. These are ethylene diamine tetracetic acid (EDTA,MW 292.25), disodium salt (Na_2EDTA,MW 336.02) and disodium dehydrate ($Na_2EDTA.2H_2O$,MW272.24), these compounds are used widely in the culture media including Murashige-Skoog (MS) medium (Murashige and Skoog, 1962).

3.5 Plant hormones and propagation additives:

Plant hormones are the substances of critical importance to *in vitro* culture of the plant cells, tissues as well as to the differentiation of cultured plantlets.

A century ago the existences of plant hormones was only being suggested but at present, the plant hormones are included in the culture media for tissue culture, to control development and also in differentiation during *in vitro* propagation.

Auxin:

Gottlieb Haberlandt, was the first to propose the existence of plant hormones by stating that pollen tubes affect ovary growth through the release of substances called as *Wuchsenzyme* ("growth enzymes") (Haberlandt, 1902; Arditti and Krikorian, 1996; Laimer and Röcker,2003). Hans Fitting for the first time showed, after working with *Phalaenopsis* pollinia, the pollen tubes do indeed release a substance which brings about post-pollination phenomena and ovule development in orchids (Fitting, 1909a, 1909b, 1910, 1911, 1921). He named the substance as *Pollenhormon*.

The first intimation that *Pollenhormon* contains auxin was by Fredrich Laibach and reported that the active substances can be extracted with diethyl ether (Laibach, 1932).

Auxin was discovered in Holland by Frits W. Went (Went 1926, 1990; Went and Thimann, 1937) before Laibach extracted it from *Pollenhormon*. It was identified as indole-3-acetic acid (IAA) in 1934 (Went and Thimann, 1937; Haagen-Smith, 1951) and made successful use in tissue culture (Gautheret, 1935, 1937, 1983, 1985; Loo 1945a, 1945b). At present IAA and a number of synthetic auxins are used in orchid micropropagation.

The most commonly used auxins in orchid tissue culture media are the naturally occurring auxins, indole acetic acid (IAA), and the synthetics naphthalene acetic acid (NAA), indole butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Other auxins and occasionally auxin-amino acid conjugates are also used in some media. The result of auxin may differ from each other qualitatively and quantitatively i.e., the effect of one auxin on a certain species may be different from those of other auxins, and may differ with the orchid species. The effects of different concentrations of the same auxin may differ in respect to one species and may not be the same with another orchid species.

Most auxins are not destroyed by autoclaving at 110-120°C for 50-60 minutes especially if the pH is non-acidic. However, autoclaving at low pH and in the presence of other factors may destroy IAA (Posthumus, 1973). It has been confirmed in a study that IBA is more stable than IAA (Nissen and Sutter, 1990). In the present study different concentrations of NAA were used.

Cytokinin:

The effects of cytokinin differ like those of other hormones. The synthetic kinetin (6-furfuryl aminopurine), benzyladenine (N^6-benzylaminopurine, N^6-benzyladenine, BA, BAP) dimethylaminopurine (DMAP), thidiazuron (TDZ), and the naturally occurring zeatin are used most commonly in orchid culture media. Experiments with aqueous solution of kinetin, zeatin and isopentenyladenosine have shown that they are not broken down when autoclaved for 1h at 120°C (Dekhuijzen, 1971). Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea, TDZ) is a later addition to the list of cytokinin which are used in orchids, specially *Phalaenopsis* (Ernst,1994). It has been proved to be very potent cytokinin (Mok *et al.*, 1982; Mok and Mok 1985). BAP in different concentrations were used in the present study.

Gibberellins:

Gibberellins are used very seldom in the culture media for orchids. When used GA_3 is the most common form. Autoclaving reduces gibberellin's activity by more than 90% (Van Bragt and Pierik, 1971). Therefore, gibberellins must be cold-sterilized through filtration or by dissolving them in ethanol. Gibberellin was not used in the present study in any form.

Amino acids:

The most commonly used amino acid in orchid culture media is glycine because it is a component of the MS medium (Murashige and Skoog, 1962). Other amino acids are also used in some media.

Polyols:

The only polyols used in orchid culture media are *myo*-inositol, *meso*-inositol, *i*-inositol, inositol, cyclohexitol, inosite, meat sugar and bios I; chemically it is hexahydroxycyclohexane ($C_6H_{12}O_6$) and it is a part of the MS medium (Murashige and Skoog 1962). Hexitols in coconut water have also been shown to have growth promoting effects on plant embryos (Van Overbeek *et al.*, 1941; Shantz and Steward, 1952, 1955). However these substances were not added to culture media other than as a part of coconut water.

Coconut water:

Haberlandt suggested the utilization of embryo sac fluids (Haberlandt, 1902; Krikorian and Berquam, 1969; Laimer and Rücker, 2003). Then there was the use of liquid endosperm of coconut i.e., coconut water as an additive to the medium. Consequently, at present coconut water is widely used in micropropagation of many plants.

The substances like cytokinin, zeatin was isolated from coconut water (Letham, 1968) some suggested that the presence of such agents explained the reasons for its activity (Galston, 1969; Skoog, 1994).

F. Mariat may have been the first to publish the use of coconut water also referring to it as milk and copra extract as additive to media employed for orchid seed germination. When added at concentration of 2% it did not inhibit germination and development but the seedlings were yellowish green. At higher levels coconut water was inhibitory to germination and development and the seedlings died (Mariat, 1951; Arditti and Ernst, 1984). Experiments with *Phalaenopsis* seedlings showed that coconut water can induce proliferation of protocorms (Ernst, 1967b). In the present study coconut water was used in different concentrations.

Hydrolysates and Autolysates:

Peptone, tryptone (both hydrolysates), yeast extracts (an autolysate), and a number of other complex additives are used in some orchid tissue culture and micropropagation media. In the present study yeast extract was used.

Charcoal:

Activated charcoal or carbon is extensively used for the absorption of substances. Both animal and vegetable charcoals are available, but the later are preferable for culture media.

The first attempt to darken a culture medium used for orchid seed germination was made in an effort to germinate American *Cypripedium* sp. (Curtis, 1947). Lampblack had no effect of any kind. The addition of animal charcoal to a culture medium used for *Cymbidium* plantlets improved differentiation and plantlet growth but reduced the proliferation of protocorm-like bodies (PLBs) and the formation of aerial roots (Werkmeister, 1970a,

1970b, 1971). Werkmeister was the first to use charcoal to darken orchid culture media. He did it to study the effect of dark media on root growth and development.

Activated vegetable charcoal improved seedling growth of the terrestrial *Paphiopedilum* (Ernst, 1974) and the epiphytic *Phalaenopsis* (Ernst, 1975, 1976) orchids. One possible explanation of the effects of charcoal on orchid seedlings or tissue culture derived plantlets is that it improves aeration. Growth similar to that on charcoal containing media was also observed when seedlings of *Paphiopedilum* and *Phalaenopsis ambionensis* were grown on Pyrex glass wool alone or in combination with Nuchar C vegetable charcoal (Ernst, 1974, 1975, 1976; Arditti, 1979; Arditti and Ernst, 1984). One possible explanation of the effects of charcoal on orchid seedlings or tissue cultured derived plantlets is that it improves aeration. A second possibility is that charcoal adsorbs ethylene (Ernst, 1975) and may also adsorb plant hormones and vitamins and this may explain the fact that it can also be inhibitory to growth (Fridborg and Eriksson, 1975; Weatherhead *et al.*, 1979).

3.6 Solvents:

Distilled water has been used as a solvent in the preparation of culture media. Ethanol (ethyl alcohol, 70% in distilled water) has been used as a sterilizing solvent for substances that cannot withstand autoclaving.

3.7 pH:

The term pH is indicative of the alkalinity of acidity (i.e., hydrogen ion concentration) of a medium. It is defined as the logarithm of the reciprocal of the hydrogen ion concentration. The pH is important because it affects the availability of nutrients.

The pH of culture media has been adjusted to 5. Growth may be inhibited if the pH is lower or higher than the appropriate for the plants under culture.

3.8 Solidifiers:

Agar was the major solidifier used for orchid seed germination and micropropagation media for several decades and hence has been used in these experiments also. It is widely used where it is added slowly with continuous stirring to the medium and then to bring the mixture to boil. Once agar gets dissolved, medium has to be autoclaved after dispensing into culture vessels.

Gellan gum (phytagel, Gelrite) is an agar substitute consisting of glucuronic acid, rhamnose and glucose produced through fermentation by *Pseudomonas elodea*. It was discovered by Kelco Division of Merck and Co., in San Diego, California. The gum produces a high strength, colorless and clear gel. While using gellan gum, there should be continuous stirring to avoid the formation of lumps in the media.

3.9 Media preparation:

The media preparation was required immediately after the collection was made and that required very careful and systematic steps. It can be sequenced as follows:

1. The required amount of the desired media was dissolved in little amount of the distilled water, dissolved properly and carefully.
2. All the additives and desired hormones were incorporated carefully and slowly.
3. The volume was made up to the required total volume by the addition of distilled water.
4. pH was adjusted prior to autoclave as 5.00. If the pH of the media indicates below 5.00, it was balanced with the help of few drops of 1(N) NaOH and if it exceeds 5.00, then was balanced with few drops of 1(N) HCl.
5. To solidify the medium required amount of agar-agar powder (8gms/L) was added and brought to boil with continuous stirring so as to avoid the formation of lumps in the medium.
6. The proper amount of the medium was dispensed in the culture tubes, vessels and Erlenmeyer flasks.
7. Tubes, flasks and vessels were adequately capped and covered with the help of brown paper and rubber bands.
8. The prepared culture medium was sterilized under high temperature and pressure in an autoclave maintaining the pressure at 121°C and 15 lb psi for 15minutes.
9. After sterilization, they were taken out from the autoclave, cooled at room temperature and kept at the working table of laminar air flow and further sterilized by spraying 70% ethanol.

3.10 Sterilization procedure:

The capsule or the green pod collected from the experimental garden is usually contaminated with micro organisms and other contaminants, thus needs surface sterilization.

The pod was first cleaned thoroughly with the help of a soft brush, scraping slowly and washing under running tap water followed by submerging with 5% Tween 20 (liquid detergent) for 10 minutes and subsequently rinsed with distilled water until the forth is totally washed away. Further, the pod was taken to the laminar air flow and on that table once again it was surface sterilized by freshly prepared 0.1% mercuric chloride solution for 15 minutes with occasional agitation and was subsequently rinsed three times with autoclaved distilled water. Now, the explant was ready for the inoculation.

3.11 Inoculation:

After surface sterilization, the explants were taken in a sterile petridish containing filter paper to soak the surface water of the fruit or capsule. The capsules were cut longitudinally with the help of sterilized surgical blade and forceps. Mass of tiny seeds was inoculated on the slant surface of the desired media in the culture tubes.

3.12 Culture conditions:

The culture conditions under which the explants, seeds, PLBs and callus masses are maintained can determine the success and failure of culture and further affects the growth and development of the *in vitro* plantlets. The seeds are placed on a solid medium establishing a good contact between them and the medium with even distribution. The seeds are not totally buried in the agar solidified medium in order to prevent death from improper gas exchange.

After inoculation of the seeds, the culture tubes were kept at 22 ±1°C under 16 hours photoperiod from cool white light giving 2659 lux.

Results

4. RESULTS

4.1 Morphological description of the plants used in the present study.

At the onset of the experimental studies all the twenty plants were studied morphologically. This was very important for proper identification of the plants used in the present study. Vegetative and the floral features of orchids have distinct morphological characters. These morphological characters become important in identification of the species. Hence, all the plants used in the present study have been described below along with their distribution, habitat and flowering time.

4.1.1 *Bulbophyllum ornatissimum* (Rchb.f.) J.J.Sm (Fig. 2-A)

Epiphytic in habitat, the species is distributed in Darjeeling and Sikkim at an altitude range of 500-600 m. The plant shows pseudobulbs 25mm long, ellipsoid and spaced 20-25mm apart on creeping rhizomes; leaf 5.5-10cm x 18-22 cm, oblong, sessile, fleshy; scape 7.5cm long, bearing a solitary flower at apex; flower 17.5-20cm long, yellowish-green with purple markings, the lip and paleae being deep purple, fetid smelling; dorsal sepal with paleae 15mm long; the lateral sepals 10-17.5cm long, with twisted filiform tips free at their apices; lips oblong, obtuse, edges upturned and enclosing three raised lines; column very broad at the middle and bearing hooded, decurved apical processes with dentate bases. Their flowering time is October-November.

4.1.2 *Coelogyne corymbosa* Lindl. (Fig. 2-B)

Beautiful species found in wet temperate evergreen forests as epiphytes on moss, laden trees with pseudobulbs embedded in the moss, distributed throughout Bhutan, Sikkim, Darjeeling, Meghalaya and Nepal at an elevation of 2000-2800m having pseudobulbs oblong, terete, crowded 2.5 - 4cm long, ovoid and bearing dark green oblong lance-shaped leaves 10-15cm long 2-3cm broad. Inflorescence raceme 2-4 flowered, pendulous, flowers large 6-7 cm across, pure white and fragrant. Lip white with two bright yellow ocellate

Bulbophyllum ornatissimum

Coelogyne corymbosa

Coelogyne cristata

Coelogyne elata

Fig. 2: Orchids
A: *Bulbophyllum ornatissimum* (Rchb.f.) J.J. Sm
B: *Coelogyne corymbosa* Lindl.
C: *Coelogyne cristata* Lindl.
D: *Coelogyne elata* Lindley.

spots, anteriorly with 4 yellow blotches in the middle and surrounded by brown margins. The plants flower during April-May.

4.1.3 *Coelogyne cristata* Lindl. (Fig. 2-C)

It is a magnificent orchid, dwarf and evergreen in habit. It is found as epiphyte on trees and rocks in temperate forests forming large impressive masses. The plants are distributed over Bhutan, Uttar Pradesh, Himachal Pradesh, Sikkim, Darjeeling Meghalaya and Nepal at an elevation of 1600-2600 m. Morphology shows pseudobulbs 5-7.5 cm long at distance of 5-6 cm, arising from stout sheathed rhizomes. Leaves about 6 inches long, lance-shaped. The flowers, six or eight together, proceed from the base of the oblong angulated pseudobulbs in a drooping raceme, each flower being 3 or 4 inches across, with charmingly undulated lanceolate sepals and petals; the colour pure white, with a prominent blotch of yellow cristate keels. This plant, which is largely grown for cut flowers, is the finest of the genus and in fact one of the finest of all orchids of. In this study flowering time was recorded from March-April in Darjeeling and Sikkim.

4.1.4 *Coelogyne elata* Lindley (Fig. 2-D)

This species occurs as an epiphytic herb in the subtropical and temperate zones of central and east Nepal, India, Bhutan and North Myanmar at an elevation of 1400-2100 m. Description explains pseudobulbs are oblong-cylindric, polished, sheathed at the base, 7.5-12.5 cm long, 2.5-5 cm in diameter, rising at distances of about 5 cm from a stout sheathed rhizome. Leaves in pairs, thinly coriaceous, 17.5-35 cm long, 3.5-7 cm broad, narrowly elliptic-oblong, acute, narrowed at the base to the long petiole; petiole 3.2-7.5 cm long. Inflorescence recemose from between the pair of leaves on the apex of pseudobulb, peduncle naked below, bearing many short imbricate sheaths, raceme 10-15 cm long, distichous, many flowered; floral bract sub-rhomboid, acute, conduplicate, slightly longer than the stalked ovary, caduceus. Flowers are 3.2-3.5 cm across, ochraceous white. Sepals are sub-equal, oblong, sub-acute, spreading. Petals are as long as the sepals but much narrower, sub-acute. Lip elongate, having a broad spot of yellow and pink on the lamellae on its mid-lobe, the lower part of the lamellae being yellow, the lower part oblong and with narrow entire side lobes, separated from the sub-orbicular med-lobe by an erose-edged

sinus; mid-lobe irregularly erose, undulate, obtuse; the disc with two erose-crenulate lamellae from the base to near the apex. Column is white, winged in its upper half, the wing erose at the apex. The flowering time was noted is April– June in the present study area.

4.1.5 *Coelogyne flavida* Wall. Ex. Lindl. (Fig. 3-A)

This species are found on exposed rocks and trees often forming large masses in the Sub-tropical zone of Bhutan, Sikkim, Meghalaya, Arunachal Pradesh and Nepal at an elevation of 1500-1700 m. Morphological description shows that plant bears a height of 15-25 cm erect with conical, grooved pseudobulbs borne on sheathed rhizomes. Leaves 2, 10-20 cm x 2-3 cm. Flower spikes arising from the middle of the leaves and elongating every year and varying from 10-25 cm, 5-6 flowered. Flower 12mm across, bright uniform yellow. The flowering time of this species was seen from May- August.

4.1.6 *Coelogyne nitida* (Wall.) Lindley (Fig. 3-B)

It is an epiphytic herb on exposed rocks and trees, distributed in India, Nepal, Bhutan, Bangladesh, Myanmar, China (Yunnan), Thailand and Laos at 1300-2400m altitude. The plant shows pseudobulbs 3-10 cm long, 1.3 cm. in diameter, ovoid to conical, 1- or 2- leaved at apex. Leaves 8-25 cm long, 2-6 cm broad, and narrowly elliptic-lanceolate, acute, narrowed to the channeled petiole. Inflorescence racemose from the base of the pseudobulb, 3- to 6- flowered, proteranthous, erect or drooping, 18-20 cm long. Flowers are fragrant, 3.5 - 4cm across and white. Sepals are narrowly oblong, subacute to obtuse. Petals narrowly oblong-lanceolate, subacute, lip oblong or almost ovate, constricted about the middle, upper surface of lip with two elongated blotches of yellow margined with red on its side lobes and two united orbicular spots at the base of the mid-lobe, with reddish oblique lines on the outer surface of the side lobes; side lobes oblong to rounded, incurved, the edges serrulate; mid-lobe rotund to oblong, blunt, entire or very slightly notched at the apex, the disc with two smooth ridges. Column found narrowly clavate with vertical yellowish-brown band in front. Flowering time was found to be from April – June.

4.1.7 Coelogyne *ovalis* Lindley (Fig. 3-C)

Found as epiphytic in habitat distributed in India, Nepal, Bhutan, and China at an elevation of 1300-2100 m altitude. Plant shows pseudobulbs ovoid-fusiform to fusiform, 3-8 cm long, 1.2-1.5 cm broad, borne somewhat distant on a creeping rhizome, 2-leaved at apex. Leaves 9-15 cm long, 2.5-4 cm broad, narrowly elliptic, acute to acuminate, inflorescence racemose from between the pair of leaves on the apex of pseudobulb; 1-3 flowered, 12 cm long, subtended at base 3 sheaths; floral bracts coriaceous, ovate-lanceolate, convolute, caduceus, Flowers pale brown, 3-5.5 cm across. Sepals are ovate-lanceolate, acute, spreading. Petals are linear, acute, spreading or relaxed. Lip oblong, 3 lobed in middle; brown having blackish-brown markings and hairs; the side lobes narrow, recurved; the mid lobe broadly oblong, its edges and upper surface with stiff black hairs; the disc with two crisped lamellae from base to apex. Column curved, broadly winged in its upper half, of light colour than the rest of the flower. The flowering time was observed in the month of September – December.

4.1.8 Cymbidium *elegans* Lindley (Fig. 3-D)

A rare and curious species, endemic to Sikkim found on exposed rocks and trees as epiphytes as well as terrestrial, distributed in Darjeeling, Sikkim, Nepal, Bhutan, Khasia and Naga Hills at elevations of 1600-2000 m altitude. The pseudobulbs are 5-7cm long, with linear-ensiform, many leaves and producing many nodding racemes of cylindraceous, pale tawny-yellow flowers, which are spotted inside the lip with blood red and have the peculiarity of remaining half closed, labellum 3 lobed, lateral lobes narrow, rounded, erect, incurved; apical lobe small, orbicular-oblong, undulate, the margins incurve; disc between lateral lobes with 2 narrow parallel keels united at apex and with a large pubescent callus at base. It is worthy of cultivation on account of the distinct colour of its flowers. Flowering time was recorded in the month of October-November.

Coelogyne flavida

Coelogyne nitida

Coelogyne ovalis

Cymbidium elegans

Fig. 3: Orchids
A: *Coelogyne flavida* Wall.Ex.Lindl.
B: *Coelogyne nitida* (Wall.) Lindley
C: *Coelogyne ovalis* Lindley
D: *Coelogyne elegans* Lindley

4.1.9 *Dendrobium chrysanthum* Lindl. Ex Wall. (Fig. 4-A)

This orchid is epiphytic and lithophytic on trees and rocks in the subtropical and temperate zone of Bhutan, Sikkim, Darjeeling, Meghalaya to Arunachal Pradesh and Nepal at elevations of 1700-2000m. Pendulous deciduous stems with 60-120 cm long fleshy pseudobulbs with sheathing leaf bases, 3 to 4 long. Furnished with twisted ovate-lanceolate acuminate leaves 10-15 cm x 2.5 - 4 cm. Flowers are in clusters of 2-4 along the stem at the time of its growth; they are produced at different times of the year and grow in pairs or in clusters of three. The flowers are of an intense deep yellow fleshy with oblong sepals and obovate petals and a cucullate denticulate lip of the same colour, fimbriate at the margin and marked at the centre with a large double spot of purple-red. Flowering time was July to October.

4.1.10 *Dendrobium crepidatum* Lindl. (Fig. 4-B)

This species are epiphytic and lithophytic on trees and rocks, widely distributed from Bhutan, Sikkim, Darjeeling, Khasia Hills, Manipur, Bihar, Orissa and Nepal at an elevations of 1500-2100m. The pseudobulb stretches 15-45cm in length, with sheaths having distinct white stripes; leaves 5-7.5cm X 1.5-2.5 cm linear-lanceolate, acute; flowers borne on purple pedicels, 2.5-3.5cm across, waxy; sepals oblong, obtuse; petals broadly-oblong, spreading, wavy; sepals and petals white tinged lilac; lip orbicular-oblong with convolute base, white with yellow base and tipped purple. Flowering time recorded as March-April

4.1.11 *Dendrobium densiflorum* Lindl. Ex Wall. (Fig. 4-C)

Flowering evergreen, epiphytic orchid found on trees of *Schima wallichii* and distributed in Bhutan, Sikkim, Darjeeling, Meghalaya, Arunachal Pradesh and Nepal at elevations of 800-1500m with erect and having 4 angled, clavate, 30-40 cm pseudobulbs that appears yellow- green and shiny. Leaves 3-5 clustered near the apex, 10- 15 cm x. 2.5 -5 cm oblong-lance-shaped, darker green in colour. The densely flowered inflorescence is produced in graceful arches from the bud eyes located between the leaves. The ovate

spreading sepals and petals are rich bright yellow and the broad rhomboid serrulate retuse lip is of a deep orange colour. Flowers are golden yellow, 3.5-4 cm across and last for about a week. This species bears nearly one hundred flower spikes at one time. Flowering time recorded was April- May.

4.1.12 *Dendrobium fimbriatum* Lindl. (Fig. 4-D)

Evergreen species found as epiphytes and lithophytes in the tropical and sub-tropical zone of Bhutan, Uttar Pradesh, Sikkim, Darjeeling, Meghalaya, Assam, Arunachal Pradesh and Nepal at elevations of 500-1000m. The plants are with stout terete erect stems 2 or 3 feet long, producing its drooping racemes from near the apex of the last year's stem; has 75-150 cm tall, cane type having erect pseudobulbs tapering towards the apex, and bearing several 10-15 cm x 15-28 mm oblong to lance-shaped leaves. 1-3 pendulous spikes arise from the apical part of pseudobulbs. Each spike bears 7-12 flowers, 5-7.5 cm across and pure orange-yellow in colour. The lip is also orange and beautifully fringed with lacerate fimbriae at the edges. Flowering time noted April – May

4.1.13 *Dendrobium longicornu*, Lindley (Fig. 5-A)

A distinct and pretty species found as epiphytes on trees in the areas of Sikkim, Darjeeling, Bhutan, Khasia and Naga Hills at elevations of 1500- 2500 m. The pseudobulbs are 15-30cm x 5-6mm covered with short black hairs; several lanceolate obliquely pointed apex unequally lobed leaves, and terminal long spurred flowers in 1-3 racemes, solitary or in fascicles, which are 2-2.5cm across, 4.5-5 cm long white except the dentate lip, which has a yellow centre, lip 3-lobed, lateral lobes rounded, nerved, emarginated at the apex; apical lobes small, orbicular-rhomboid with fimbriate margins; broad keel running from base and branching at the apex. It produces its blossoms from the top of the stem. Flowering time recorded is October-November.

Dendrobium chrysanthum

Dendrobium crepidatum

Dendrobium densiflorum

Dendrobium fimbriatum

Fig. 4: Orchids
A: *Dendrobium chrysanthum* Lindl.Ex.Wall
B: *Dendrobium crepidatum* Lindl.
C: *Dendrobium densiflorum* Lindl.Ex.Wall
D: *Dendrobium fimbriatum* Lindl.

4.1.14 *Dendrobium nobile* Lindl. (Fig. 5-B)

A beautiful evergreen species grows as epiphyte on rocks and trees in Bhutan, Sikkim, Darjeeling, Meghalaya, Arunachal Pradesh and Nepal at an elevation of 1000- 1700 m. This plant grows to a height of 30-60 cm and has tufted yellow, erect pseudobulbs that are slightly compressed. Leaves are shiny, several, persistent, 8-12 cm X 2.5-3cm oblong, ovate-lanceolate, unequally lobed at the apex; the blossoms are rather large and very showy, with oval sepals and much broader undulated petals, Flowers arise in fascicles of 2-4 from the nodes of the pseudobulbs and measures 5-7cm across. The highly ornamental flowers are white merging to deep purple towards the tips of sepals, petals and lip. The lip is ovate, oblong, and shortly hairy, with a very dark purple central blotch. Flowering time is April – May.

4.1.15 *Dendrobium primulinum* Lindl. (Fig. 5-C)

A deciduous species found as epiphytes on trees in the areas including Bhutan, Sikkim, Darjeeling, Meghalaya, Arunachal Pradesh and Nepal at an elevations of 800-1500 m. The plant shows pendulous growth with the stems terete and clustered, furrowed, a foot or more in length, the young ones leafy at the top, the few leaves being oblong obtuse and obliquely emarginated; have rather stout, pendulous pseudobulbs 24-45 cm x 10-15 mm covered with white leaf sheaths. Leaves are lance-shaped, deciduous, 10-12 cm x 2.5-3 cm. The flowers arise in bunches of 1-2 and are 5-7.5 cm across and fragrant. The sepals and petals are pale rose-purple with deeper colored veins, while the nearly orbicular lip is of pale primrose colour run by purple streaks. The flowering time noted in the month of March–April.

4.1.16 *Pleione humilis* (Smith) D.Don (Fig. 5-D)

A dwarf species of great beauty found on steep moss covered embankments, rocks and trees in Bhutan, Sikkim, Darjeeling, Meghalaya, Arunachal Pradesh and Nepal at elevations of 2000-2800 m. They have dark green flask-shaped pseudobulbs clothed with fibrous scales with 4-4.5 cm length, crowned with a lanceolate acuminate dark green solitary membranous

Dendrobium longicornu

Dendrobium nobile

Dendrobium primulinum

Pleione humilis

Fig. 5: Orchids
A: *Dendrobium longicornu* Lindley
B: *Dendrobium nobile* Lindl.
C: *Dendrobium primulinum* Lindl.
D: *Pleione humilis* (Smith) D.Don

narrowly-elliptic, plicate leaf measuring 7.5–12 cm. x 3-4 cm. Scape 1-2 flowered, appearing from base of the pseudobulb before the leaf. Flowers are 5-6 cm across, white with an elliptical funnel shaped lip. The lip has a central yellow disc carrying fimbriate keels, and mottled with brown spots and blotches, the sepals and petals are linear-lanceolate, emarginated and fimbriate in front is also bluish-white, traversed by six parallel fringed veins with alternating stripes of rich purplish-crimson. Flowers present in the month of February-March.

4.1.17 *Pleione maculate* (Lindl.) Lindl. (Fig. 6-A)

Dwarf epiphytic orchids growing on trees in the area of Bhutan, Sikkim, Darjeeling, Meghalaya and Eastern Nepal at elevations of 700-1500 m. The plant shows depressed pseudobulbs of 3-3.5 cm x 2-2.5 cm, tuberculate at the base, dark green glossy and bearing brown imbricating scales; the leaves produced before the flowers are lanceolate plicate, 6 inches long; and the flowers are solitary, 5-7cm across, on short peduncles which spring from the base of the bulbs; the sepals and petals are narrow lanceolate, spreading white, the lip also white, oblong, the basal part incurved, marked with oblique purple lines the front lobe ovate, retuse, wavy, the disk yellow, with five elevated fringed veins having purple lines between, the margins wavy and boldly cross-barred with crimson-purple. Flowering time was noted in the month of October-November.

4.1.18 *Pleione praecox* (Smith) D. Don (Fig. 6-B)

A most distinct and beautiful species found on steep moss covered embankments, rocks and trees as epiphytes in Sikkim, Darjeeling, Meghalaya, Arunachal Pradesh and Nepal at elevations of 1800-2300 m. The plants are with pale mottled brownish purple turbinate pseudobulbs 3-4.5cm x 2.5 – 3.5 cm green, mottled with purplish-brown, ending in a short conical point, sparsely covered by loose grayish-brown fibres, covered with a strong veining of loose network and surmounted by a solitary broadly lanceolate membranaceous plaited leaf which decays before the appearance of the scape; the later springs from the side of the

pseudobulb and bears one showy flower, which is fully 3 inches across, and saccate at the base; the lanceolate sepals and the smaller petals are deep rose colour, and the lip, which is prominent and 2 inches long, is bluish-white, the basal part connivent over the column, the front portion beautifully fringe-toothed and the disk yellow furnished with five toothed keels or crests reaching nearly to the apex and a few yellow and rose-coloured spots. Flowering time was in the months of October – November.

4.1.19 *Rhynchostylis retusa* Bl. (Fig. 6-C)

A free-growing form and more robust in habit, short, stout, erect plant covered as epiphytic on trees in Sikkim, Darjeeling, Meghalaya and Nepal at elevations of 1000-1500 m. The sheaths of old leaves bears 3-5 pairs of thick fleshy, strap like leaves crowded on the upper part of the stem. Leaves arching was 15-45 cm long x 2.5-3 cm striated, unequally bilobed at the apex. Inflorescence was arching, 25-45 cm long, densely flowered. Each flower measures 18-20 mm across, white or light purple-pink marked and spotted purple. Flowering time is in between July – September.

4.1.20. *Thunia alba* Reichenbach fil. (Fig. 6-D)

This species covers steep moss embankments, rocks and trees in the Temperate zone distributed in the area of Bhutan, Sikkim, Darjeeling, Arunachal Pradesh and Nepal at elevations of 1500- 2100 m. The stems of this handsome species are terete, usually about 2 feet high, clothed throughout with leaves, the lower ones, produced when the stem first begins to lengthen, broad, round, amplexicaul and scale like, the upper ones also stem-clasping, oblong-lanceolate acute, some 6 or 8 inches long, glaucous beneath, falling during the resting season so as the leave the slender terete stems naked; the flowers are produced on a short dense terminal nodding raceme just as growth is finished; the sepals and petals are pure white, oblong-lanceolate, acute and nearly equal; and the lip is oblong-cucullate, rounded at the apex, where it is denticulate, white, beautifully penciled over the disk and front portion with purple and lilac. Flowering period observed was from April-June.

Pleione maculata

Pleione praecox

Rhynchostylis restusa

Thunia alba

Fig. 6: Orchids
A: *Pleione maculata* (Lindl.) Lindl.
B: *Pleione praecox* (Smith) D.Don
C: *Rhynchostylis restusa* Bl.
D: *Thunia alba* Reichenbach fil.

4.2 *In vitro* Seed culture, Micropropagation and observation.

The seeds of selected plants procured from the orchid growing nurseries and/or forest of the present study area. Then the seeds were used for seed culture studies. Once the seeds germinated in aseptic tissue culture media it was allowed to grow up to 5-7cms. Finally micropropagation was done with the explants cut from the plants grown in seed culture. Details of the seed culture technique and micropropagation have been discussed in the sections 3.2 and 3.3. The experimental details including results of seed culture and micropropagation of the different orchids have been mentioned below.

Observations: The first noticeable changes observed after inoculation was swelling and glistering of the embryos, the swelling of the embryo was followed by breaking up of the seed coat and formed rounded corm like bodies termed as Protocorm like bodies (PLBs). Normally, PLBs are covered with fine rhizoids and differentiation leads to the plantlets formation which can be observed in the following Fig. 7.

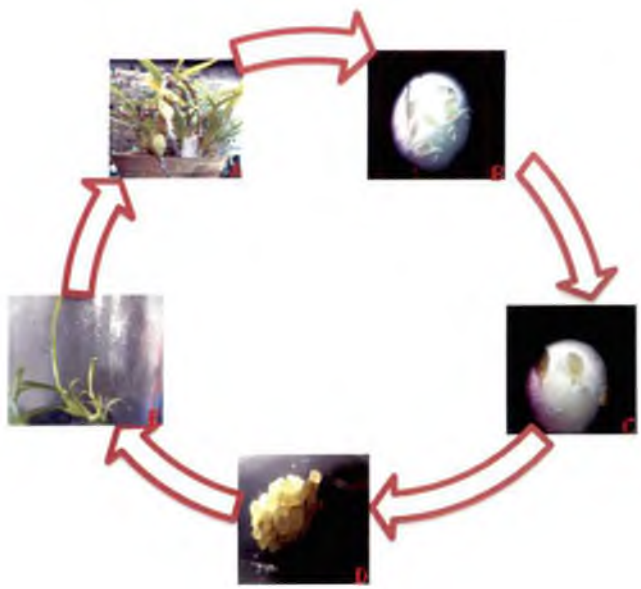

Fig. 7: A=Seed pod; B=Seeds; C=Spherule stage; D=PLBs, E= Growing plantlets.

4.2.1 Studies on *Bulbophyllum ornatissimum* (Rchb.f.) J.J.Sm

The explant was seeds from a green pod collected from Rangpo, Sikkim. The seed pod (Fig. 8-A) was pale yellow in colour, 4.5 cm long and 1.6 cm wide. The explant was sterilized before inoculation. The process for surface sterilization of the explant, media preparation and inoculation technique have been discussed under in the sections 3.9, 3.10, and 3.11 of Materials and Methods. Three different basal media (Knudson "C", Orchimax and Vacin & Went) were selected for seed culture. In each type of basal medium 20% coconut water and 1gm/L yeast extract was used as additives. For the initiation of *in vitro* germination four different concentrations of NAA have been used as growth regulators. The results of the culture have been presented in Table-11. After 35 days of inoculation the seeds started swelling in case of charcoal added orchimax medium. The sign of germination was observed after 50 days when charcoal free orchimax media was used. The seeds of the charcoal added medium formed globular rounded protocorm like bodies (PLBs) (Fig. 8-B & C) after 51 days and after 70 days plantlets were formed (Fig.8-D&E). The plantlets were grown up to 5cm after 24 weeks.

When the seeds were allowed to germinate in Knudson C media the seeds swelled up after 45 days then turned to PLBs after 53 days and formed plantlets after 71 days.

In case of the third media (Vacin & Went) showed swelling of seeds after 65 days and after 80 days they formed the PLBs but further growth was not seen as the plantlets became brown and could not survive even after sub-culturing.

Among the four concentrations of NAA supplemented in the above three different media 3mg/L concentration was found to be best.

Table 11: Percent germination of seeds of *Bulbophyllum ornatissimum* in different culture medium

Basal media with 20% CW and 1gm/L YE	Concentration of NAA (mg/L)	Percent germinated (with charcoal)	Percent germinated (without charcoal)
Knudson "C"	0.5	20%	10%
	1.0	30%	10%
	2.0	50%	20%
	3.0	75%	40%
Orchimax	0.5	25%	15%
	1.0	50%	30 %
	2.0	80%	50%
	3.0	99%	70%
Vacin & Went	0.5	10%	10%
	1.0	25%	20%
	2.0	45%	30%
	3.0	45%	30%

NAA =α-Naphthalene acetic acid; YE= Yeast extract; CW= coconut water

Micropropagation was carried out in Orchimax medium (half strength) supplemented with 1mg/L NAA and different concentrations of BAP and two different concentrations of coconut water. It was found that the quality and survivality of the regenerated plantlets (Fig.8-F&G) were best when 15% coconut water and 5mg/L BAP was supplemented. Details of the results after 13 weeks have been presented in Table -12.

Hardening process was started after separation of the plantlets from the flask; they were washed with luke warm water very gently and were planted in small pots (10 cm X 10 cm in size) with intensive care. Pieces of rotten wood and brick chips along with sand covered with sphagnum moss were used as potting materials.

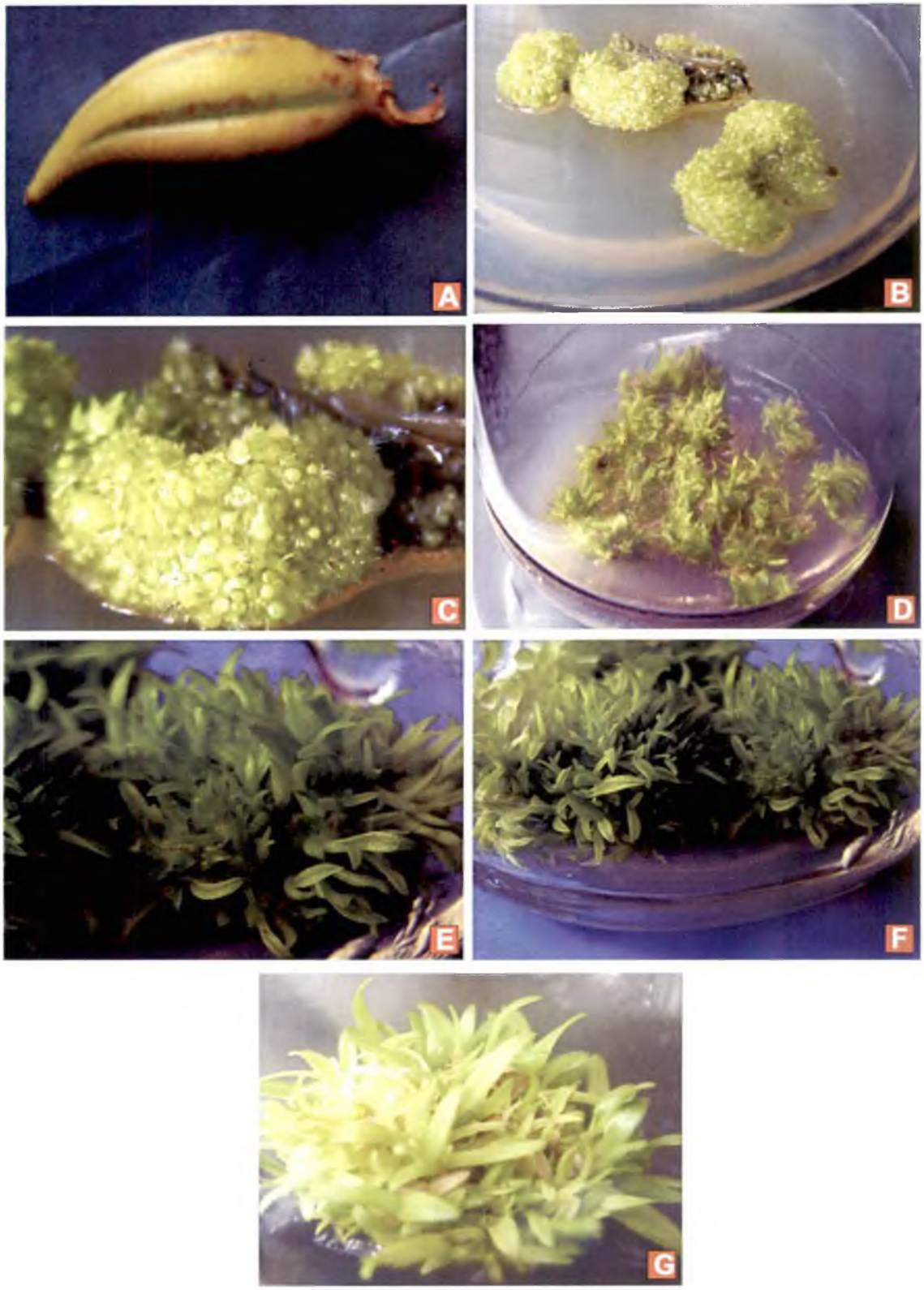

Fig.8: Different stages of seed culture and micropropagation of *Bulbopyllum ornatissimum*. A: Seed pod of *B.ornatissimum*; B&C:PLBs of *B.ornatissimum*; D&E: Growing plantlets of *B.ornatissimum*; F& G: Regenerated plantlets of *B. ornatissimum*

Table 12: Percent regenerated plantlets of *Bulbophyllum ornatissimum* and their morphological observations:

Basal media	Hormone & Supplements	Percent regenerated plants	Observation after 36 weeks
Orchimax (half strength)	1mg/LNAA +2mg/LBAP+15%CW	60%	The plantlets are thin and weak.
Orchimax (half strength)	1mg/LNAA+ 3mg/LBAP+7.5%CW	78%	Plantlets are short in height.
Orchimax (half strength)	1mg/LNAA+ 3mg/LBAP+15%CW	80%	Plantlets are healthy.
Orchimax (half strength)	1mg/LNAA+ 4mg/LBAP+7.5%CW	95%	Plantlets have increased in height up to 4cm.
Orchimax (half strength)	1mg/LNAA+ 4mg/LBAP+15%CW	98%	Regenerated plantlets are of 4.5 cm high and 1.2 cm in cross section.
Orchimax (half strength)	1mg/LNAA+ 5mg/LBAP+7.5%CW	99%	Plantlets could survive very easily after hardening.
Orchimax (half strength)	1mg/LNAA+ 5mg/LBAP+15%CW	99%	

NAA =α-Naphthalene acetic acid; CW= coconut water, BAP= 6 Benzyl amino purine.

4.2.2 *Coelogyne* species:

The genus *Coelogyne* is a medicinally important orchid and some species shown to possess some medicinally important compounds (Majumder *et al.*, 2002). *Coelogyne* comprises over 200 species distributed throughout Southeast Asia with main diversity in Himalayas. Most species are epiphytes and occur in primary forests. They have a fairly large number of medium sized to large flowers with delicate colours and sweet scent. Joseph Hooker collected orchids and other plants in 1848-1850, recorded that "On the ascent from Darjeeling the straight shafts of many of the timber trees are literally clothed with a continuous garment of white-flowered Coelogynes, which bloom in a profuse manner, whitening their trunks like snow." These are also called as cool growing Coelogynes. In the present study, seven *Coelogyne* species were studied for seed culture and micropropagation. All the species are commercially important. The seed culture followed by micropropagation will help in producing huge planting materials for the orchid industry in Darjeeling and Sikkim.

4.2.2.1. Studies on *Coelogyne corymbosa* Lindl.

Coelogyne corymbosa, is the most common species of Darjeeling hills, during the flowering season it gives snowy appearance in the hill covering trunks of the trees. The undehised seed pod (Fig. 9-A) was collected from Darjeeling, brought in the laboratory, length and breadth was measured (average length-3.02 cm and average breadt-1.9 cm).

It was sterilized and seed culture was done taking three different basal media i.e., Orchimax, Murashige & Skoog and Vacin & Went, in which two different concentrations of NAA and three different percentages of coconut water were used as growth regulators. The details of observation have been given in the Table-13. The seeds (Fig. 9-B) or the embryo (Fig. 9-C) inoculated in Orchimax medium, swelled up after 25 days of inoculation and formed spherule stage (Fig. 9-D&E) after 40 days. They started forming plantlets (Fig.9-F&G) after 95 days. After attaining a height of 4-5 cm the plantlets were used for micropropagation to increase the number of plantlets.

Table 13: Different culture media and percent germination of seeds of *Coelogyne corymbosa*.

Basal media	NAA mg/L + % of CW	Percent germination
Orchimax	0.5 + 10%	20%
	0.5 + 15%	50%
	0.5 + 20%	85%
	1 + 10%	90%
	1 + 15%	90%
	1 + 20%	95%
Murashige and Skoog	0.5 + 10%	40%
	0.5 + 15%	40%
	0.5 + 20%	70%
	1 + 10%	75%
	1 + 15%	90%
	1 + 20%	90%
Vacin and Went	0.5 + 10%	20%
	0.5 + 15%	20%
	0.5 + 20%	20%
	1 + 10%	30%
	1 + 15%	30%
	1 + 20%	40%

NAA =α-Naphthalene acetic acid; CW= coconut water.

The seeds inoculated in Murashige & Skoog medium formed PLBs after 60 days of inoculation and further grew into healthy plantlets after 120 days. In third set of medium (Vacin & Went), the bursting out of the seed coat and forming PLBs took 85 days and further development to a plantlet took more than 20 weeks.

Among the two concentrations of NAA and three percentages of coconut water, 1mg/L NAA and 15% CW was found to be the best supplement.

Micropropagation was carried out taking the healthy plantlets of approximately 5cm height in Murashige & Skoog (half strength) medium added with 1mg/L IAA plus four different concentrations of BAP and two different percentages of coconut water. The observations made to find the percentage of regeneration of plants and their weight after 20, 28, 36 weeks have been shown in Table-14. It was found that when 5mg/L BAP and 15% of CW was added in the basal medium all the plantlets regenerated grown healthy. The healthy plantlets could survive after hardening.

Table 14: Percent regenerated plantlets after micropropagation of *Coelogyne corymbosa* and fresh weight of plantlets after different periods of growth.

Basal media (½MS) + 1mg/L IAA +	Percent germination	Weight of the plantlets in grams after		
		20 Weeks	28 Weeks	36 Weeks
BAP 2mg/L + 15%CW	15%	0.165	1.349	1.789
BAP 3mg/L + 7.5%CW	20%	0.154	1.376	1.897
BAP 3mg/L + 15%CW	40%	0.176	1.685	1.923
BAP 4mg/L + 7.5%CW	50%	0.188	1.710	2.087
BAP 4mg/L + 15%CW	65%	1.243	2.835	3.510
BAP 5mg/L + 7.5%CW	87%	1.267	2.853	3.615
BAP 5mg/L + 15%CW	91%	1.845	2.924	4.025

MS = Murashige&Skoog; IAA= Indole acetic acid; BAP= 6 Benzyl amino purine; CW= Coconut water

4.2.2.2 Studies on *Coelogyne cristata* Lindl.

The seed pod of *C. cristata* was collected from the botanical garden of Darjeeling, the length and breadth was measured as 3.9 cm 1.9 cm respectively. Seed pod (Fig. 10-A) was surface sterilized and inoculated in three different basal media Orchimax, Knudson"C" and Vacin &Went added with 1mg/L NAA and four different concentrations of coconut water.

The sign of germination i.e., swelling of the seeds (Fig. 10-B &C), elongation of shoot (Fig. 10-D), and plantlet development up to 2.5cm height was first observed in the Orchimax medium, secondly in Knudson"C" medium and then in Vacin & Went medium (Table-15). Basal media added with 20% coconut water, 1mg/L NAA and 3mg/L BAP produced healthy plantlets (Fig. 10-E). The seeds germinated to a healthy plantlets was maximum (99%) in case of Orchimax medium. But healthy plantlet development was 60% in Knudson"C" medium and 10% in Vacin & Went medium.

Micropropagation of these cultured plantlets was carried on in Orchimax (half strength) media with 20% coconut water and five different concentrations of BAP. The observations indicated that the micropropagated plantlets (Fig. 10-F & G), used for hardening was best grown in media combined with 20% CW and 5mg/L BAP (Table-16).

Table 15: Seed culture of *Coelogyne cristata* in three different media with different concentrations of BAP and time required for swelling of embryo, formation of spherule stage, PLB & plantlet formation, and percent of germination

Basal media with 20% CW + 1mg/L NAA	Conc. of BAP mg/L	Percent germination	Number of days taken for the initiation of			
			Swelling of embryo	Spherule stage	PLB formation	Plantlet formation
Knudson "C"	0.5	10%	79	112	129	234
	1.0	15%	66	102	116	210
	2.0	42%	50	82	98	195
	3.0	60%	42	75	87	180
Orchimax	0.5	25%	55	99	109	200
	1.0	55%	55	90	93	188
	2.0	82%	45	77	82	180
	3.0	99%	32	64	71	169
Vacin & Went	0.5	0%	115	169	No growth	No growth
	1.0	5%	112	166	No growth	No growth
	2.0	10%	105	No growth	No growth	No growth
	3.0	10%	70	No growth	No growth	No growth

NAA =α-Naphthalene acetic acid; BAP= 6 Benzyl amino purine; CW= coconut water.

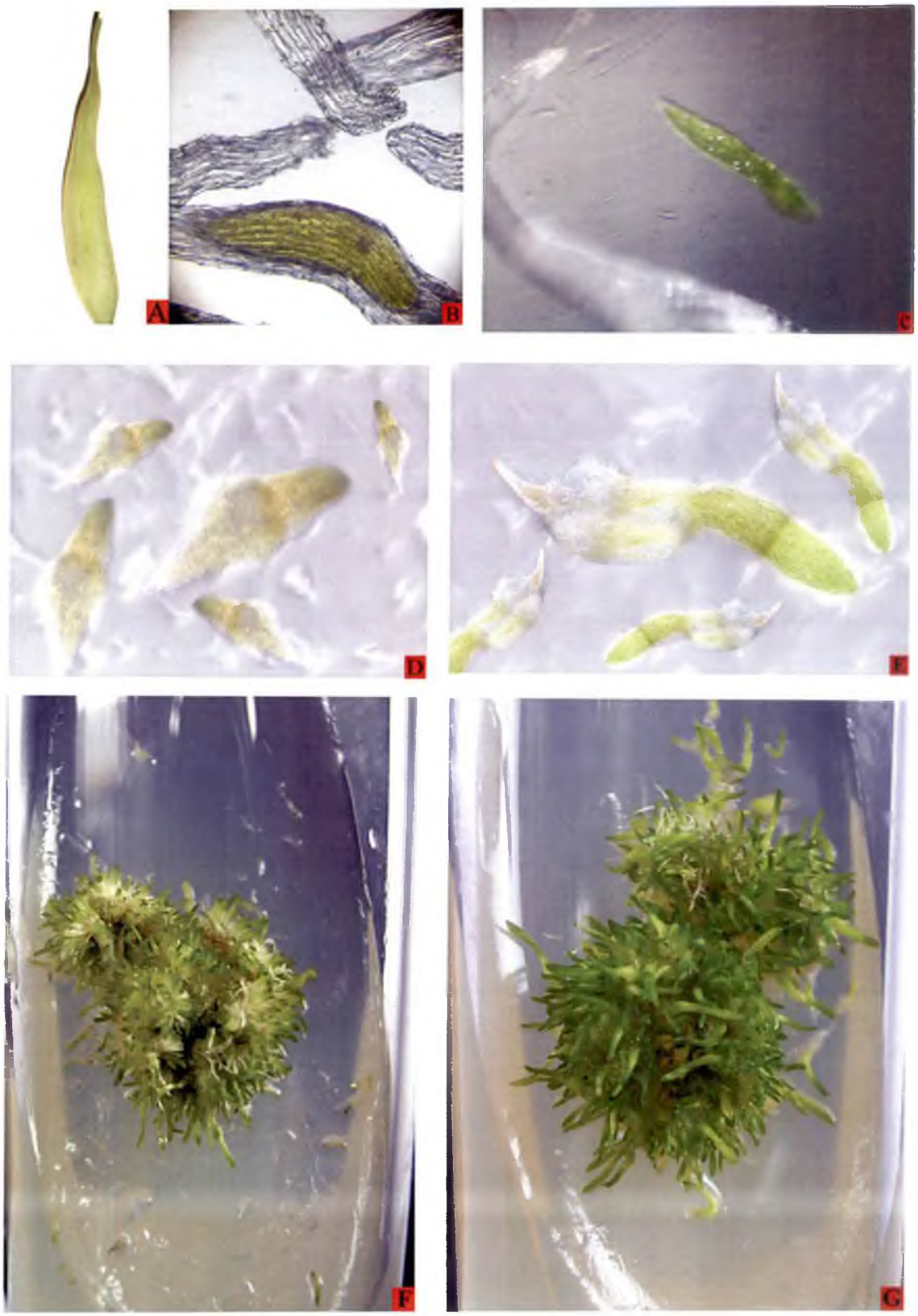

Fig. 9: Different stages of seed culture and micropropagation of *Coelogyne corymbosa*. A: Seed pod of *C. corymbosa*; B&C: Seeds of *C. corymbosa;* D&E: Spherule stage of *C. corymbosa*; F&G: Plantles of *C. corymbosa.*

Table-16: Percent of regenerated plantlets after micropropagation of *C. cristata*

Basal media	Hormone & Supplements	Percent regenerated plantlets
Orchimax (Half strength)	1mg/LNAA+1mg/LBAP+20%CW	50%
Orchimax (Half strength)	1mg/LNAA+2mg/LBAP+20%CW	66%
Orchimax (Half strength)	1mg/LNAA+3mg/LBAP+20%CW	75%
Orchimax (Half strength)	1mg/LNAA+4mg/LBAP+20%CW	95%
Orchimax (Half strength)	1mg/LNAA+5mg/LBAP+20%CW	99%

NAA =α-Naphthalene acetic acid; BAP= 6 Benzyl amino purine; CW= coconut water

4.2.2.3 Studies on *Coelogyne elata* Lindley.

The experimental material seed pod (Fig. 11-A) was collected from Pedong, brought to the laboratory and the measurement recorded as length 2.5cm and breadth 1.3 cm. Three different basal media, Knudson"C", Orchimax, and Murashige & Skoog along with the growth regulators, 15% coconut water, 1mg/L NAA and three different concentrations of BAP was used for the initiation of germination and further development of healthy plantlets.

Germination of the seeds (Fig. 11-B) started with swelling and bursting of the seed coat i.e., the spherule stage (Fig. 11-C); however the number of days observed were different in three different media combined along with growth regulators, as shown in Table-17. In later stages of development they formed globular PLBs (Fig. 11-D&E) which protruded the shoot system (Fig. 11-F&G) and further grown into the healthy plantlets. The percent germination of seeds of *C. elata* (98%) was found to be best in Orchimax medium supplemented with 1mg/L NAA, 3mg/L BAP and 15%CW. However, in the same combination of growth regulators 80% seeds germinated in Knudson"C" medium but 86% seed germination was recorded in Murashige & Skoog medium.

Micropropagation of these cultured plantlets was carried on in Orchimax (half strength) media with 20% coconut water and five different concentrations of BAP. The details of observation indicated that the micropropagated plantlets (Fig. 11-H), used for hardening was best when grown in media combined with 20% CW plus 5mg/L BAP (Table-18).

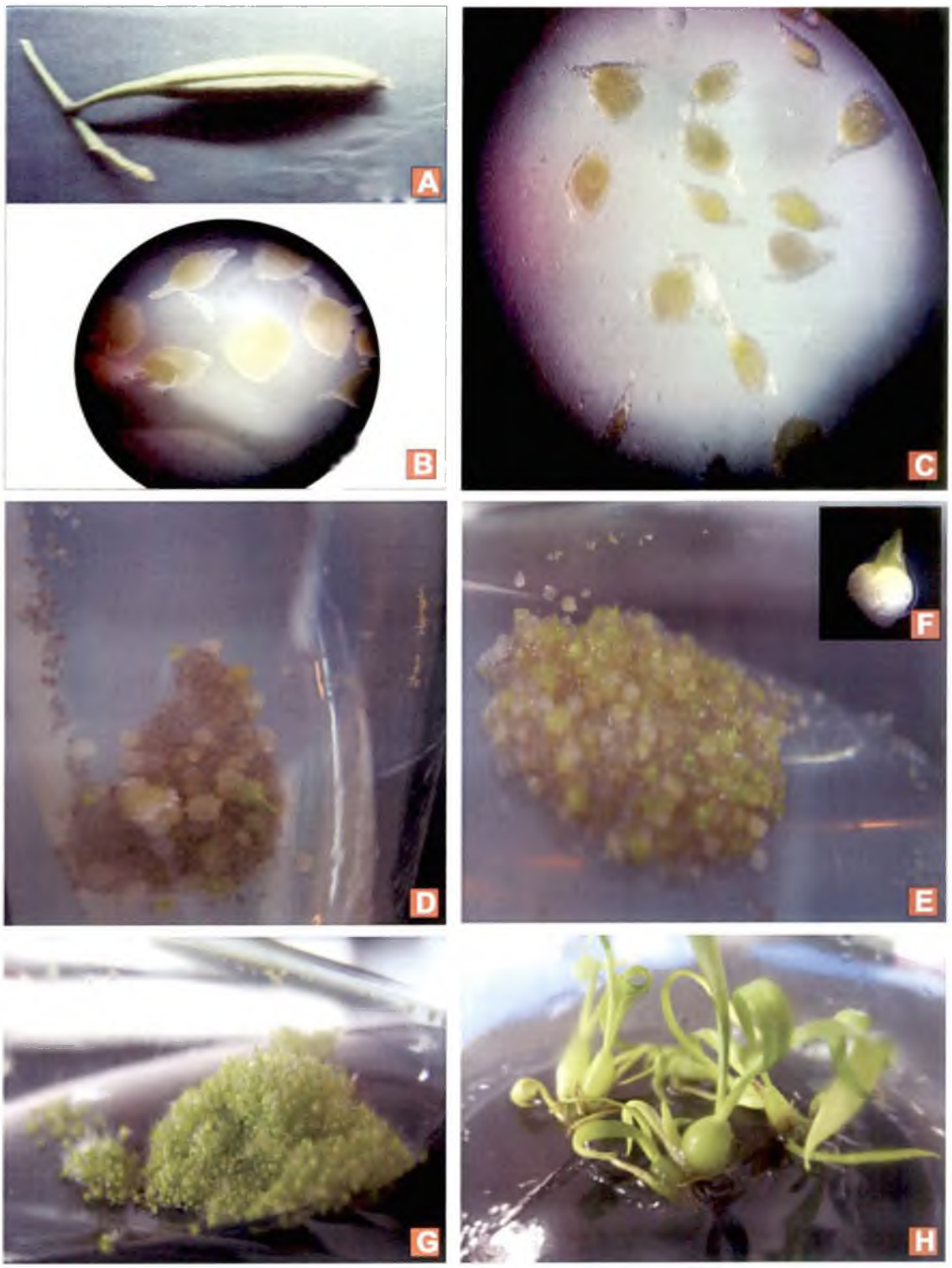

Fig 11 : Different stages of seed culture and micropropagation of *Coelogyne elata*. A: Seed pod of *C.elata*; B: Seeds of *C.elata*; C: Spherule stage of *C.elata;* D,E,F,G: Protuding shoots from PLBs of *C.elata*; H: regenerated plantlets of *C.elata*.

Table 17: Seed culture of *Coelogyne elata* in three different media with different concentrations of BAP and time required for swelling of embryo, formation of spherule stage, PLB formation and plantlet formation, and percent of germination.

Media	Combined adjuncts in the media	Percent germina -tion	Number of days taken for the initiation of			
			Swelling of embryo	Spherule stage	PLB formation	Plantlet formation
Knudson "C"	1mg/L NAA+1mg/L BAP+15%CW	50%	60	70	140	201
	1mg/L NAA+2mg/L BAP+15%CW	68%	55	70	115	193
	1mg/L NAA+3mg/L BAP+15%CW	80%	50	66	99	177
Orchimax	1mg/L NAA+1mg/L BAP+15%CW	71%	46	65	100	189
	1mg/L NAA+2mg/L BAP+15%CW	84%	44	60	89	160
	1mg/L NAA+3mg/L BAP+15%CW	98%	40	60	72	149
Murashige & Skoog	1mg/L NAA+1mg/L BAP+15%CW	52%	55	78	170	No Growth
	1mg/L NAA+2mg/L BAP+15%CW	74%	59	78	135	220
	1mg/L NAA+3mg/L BAP+15%CW	86%	50	76	120	197

NAA =α-Naphthalene acetic acid; BAP= 6 Benzyl amino purine; CW= Coconut water.

Table-18: Percent of regenerated plantlets after micropropagation of *C. elata*

Basal media	Hormone & Supplements	Percent regenerated plantlets
Orchimax (Half strength)	1mg/L NAA+1mg/L BAP+20%CW	50%
Orchimax (Half strength)	1mg/L NAA+2mg/L BAP+20%CW	66%
Orchimax (Half strength)	1mg/L NAA+3mg/L BAP+20%CW	75%
Orchimax (Half strength)	1mg/L NAA+4mg/L BAP+20%CW	95%
Orchimax (Half strength)	1mg/L NAA+5mg/L BAP+20%CW	99%

NAA =α-Naphthalene acetic acid; BAP= 6 Benzyl amino purine; CW= coconut water

4.2.2.4 Studies on *Coelogyne flavida* Wall. Ex. Lindl.

The seed pod (Fig.12-A) of *C. flavida* was collected from Kalimpong. The measurement of the pod was found as length 1.8cm and breadth 0.46 cm. For the experimental work, seed pod was surface sterilized and after opening it aseptically the seeds (Fig.12-B) were taken out and dusted over the culture media in the culture tubes.

Three different basal media Orchimax, Knudson "C" and Vacin &Went added with two different concentrations of NAA and three different percentages of coconut water as growth regulators. The sign of germination was swelling of the embryo after 40days of inoculation in Orchimax media, and then they formed rounded PLBs after 58 days. Initiation of root hairs and shoot system (Fig.12-C&D) was observed after 74 days and the plantlets achieved height of 3cm after 148 days. The best combination of growth regulators with Orchimax media was found to be 1 mg/L NAA and 20% coconut water.

Although the seeds were germinated in all three different types of media, the percent germination of the seeds was different as evident from Table-19. The response of germination in case of Knudson"C" medium was positive. Bursting of the seed coat took 59 days, PLB formation took 87 days and plantlets formed after 175 days of inoculation. Germination was positive in case of Vacin & Went medium supplemented with the same growth regulators but PLBs formed after 95days. Although 50% of seeds germinated up to a plantlet after 190 days but they could not survive even after sub culturing.

Micropropagation of these *in vitro* cultured plantlets was carried on with Orchimax (half strength) medium supplemented with four concentrations of BAP and 10%, 15% and 0% (in absence of) coconut water. The result and percent response of regenerated plantlets (Fig.12-E&F) and the best combination of growth regulators have been presented in Table-20. NAA (1mg/L), 5mg/L BAP and 15% coconut water when supplemented in Orchimax media 98% plantlets regenerated with vigorous growth.

Table 19: Percent germination of *C. flavida* seeds in different media with growth regulators.

Basal media	NAA(mg/L) + % of CW	Percent germination
Orchimax	0.5 + 10%	70%
	0.5 + 15%	70%
	0.5 + 20%	70%
	1 + 10%	70%
	1 + 15%	90%
	1 + 20%	95%
Knudson"C"	0.5 + 10%	50%
	0.5 + 15%	50%
	0.5 + 20%	50%
	1 + 10%	50%
	1 + 15%	80%
	1 + 20%	80%
Vacin & Went	0.5 + 10%	30%
	0.5 + 15%	20%
	0.5 + 20%	30%
	1 + 10%	30%
	1 + 15%	50%
	1 + 20%	50%

NAA =α-Naphthalene acetic acid; CW= Coconut water.

Table 20: Percent regenerated plantlets with different concentrations of BAP of *C. flavida*

Basal Media	% of C.W.	Percent regenerated plantlets with different concentrations of BAP (mg/L)			
		2(mg/L)	3(mg/L)	4(mg/L)	5(mg/L)
Orchimax	0%	10%	19%	25%	39%
With 1mg/L NAA	10%	30%	45%	60%	80%
	15%	20%	40%	80%	98%

NAA =α-Naphthalene acetic acid; BAP= 6 Benzyl amino purine; CW= Coconut water.

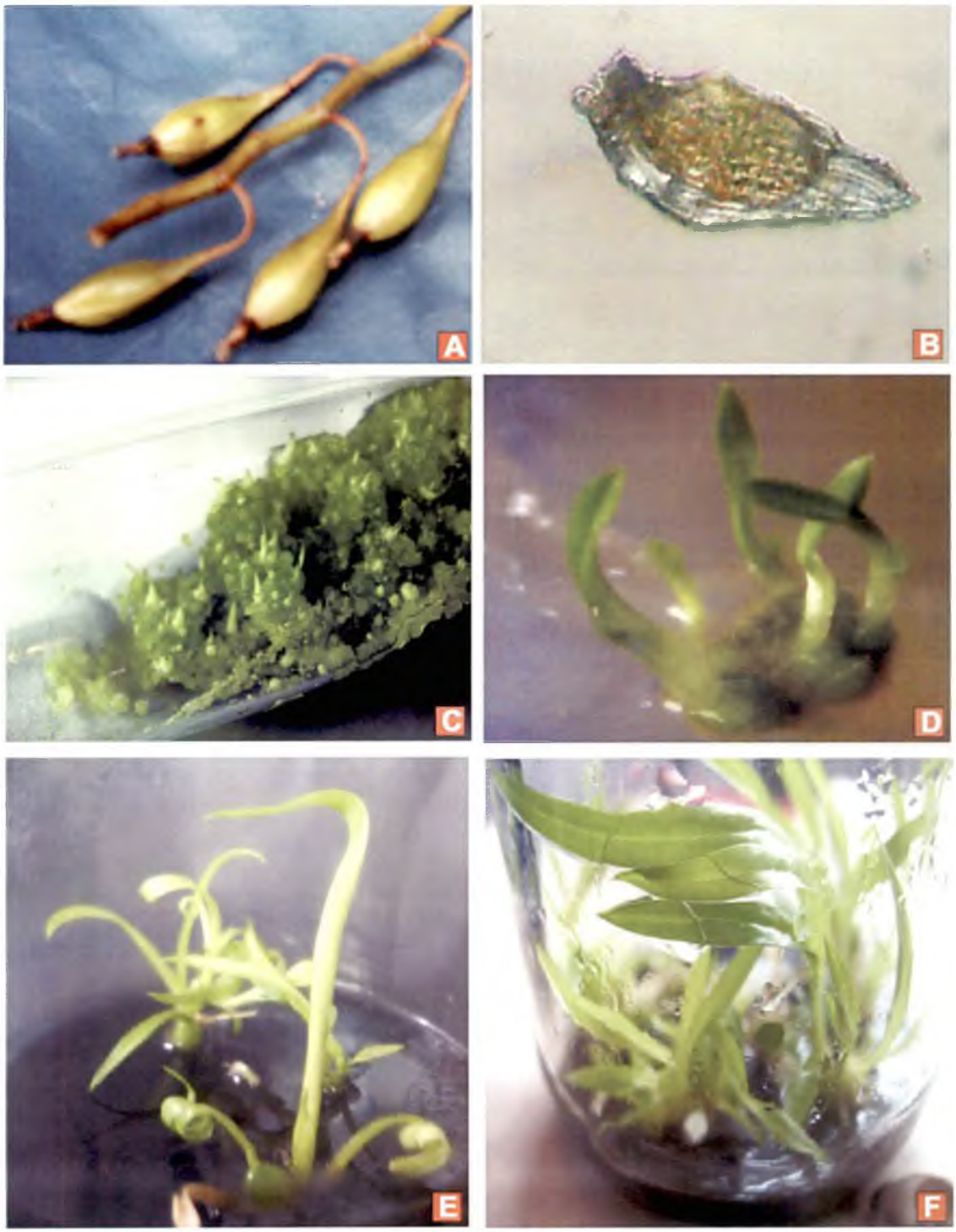

Plate 12: Different stages of seed culture and micropropagation of *Coelogyne flavida*. A: Seed pod of *C.flavida*; B: Seeds of *C.flavida*; C&D: Plantlets development from PLBs of *C.flavida*; E&F: Regenerated plantlets of *C.flavida*.

4.2.2.5 Studies on *Coelogyne nitida* (Wall.) Lindley:

The experimental material seed pod was collected from Jalapahar area of Darjeeling. The seed pod (Fig. 13-A) was inoculated in Basal media Knudson "C", Orchimax and Vacin & Went, supplemented with different growth regulators i.e., 1gm/L Yeast extract, 20% coconut water and four concentrations of NAA has been used for the initiation of germination and development of plantlets.

The first sign of germination was the swelling of the seeds (Fig.13-B) after 23 days of inoculation in case of Orchimax medium with 3mg/L NAA, after 30 days the swollen seeds bursted into spherule stage, later after 45 days they elongated to form plantlets (Fig.13-C) and consecutively after 68 days of inoculation rhizoids are coming out from the plantlets (Fig.13-D). However with the same concentration of NAA, in Knudson "C" and Vacin & Went media the onset of germination took 28days and 38 days respectively. The number of days taken for the development of different stages in different media is given in Table-21. The highest percent of germination was 99% in Orchimax medium and at the same time period with same growth regulators it was 75% in Knudson"C" medium and 45% in Vacin & Went medium.

Micropropagation of *C. nitida* plantlet (Fig.13-E&F) developed through seed culture was carried on in Orchimax medium (half strength) supplemented with only coconut water in absence of any hormone, without coconut water and only with 2mg/L NAA, and third sets with three different concentrations of NAA and three different percentages of coconut water. The best combination recorded for the vigorous regeneration of plantlets (Fig.13-G&H) was 2.5mg/L NAA with 25% coconut water. Results have been presented in Table-22. The process of hardening was followed in the similar way as discussed earlier. Plantlets after hardening were healthy.

Table 21: *In vitro* germination of seeds and formation of different developmental stages of *C. nitida*

Basal media with 20% CW + 1gm/L YE	Conc. of NAA mg/L	Percent germination	Number of days taken for the initiation of			
			Swelling of embryo	Spherule stage	PLB formation	Plantlet formation
Knudson "C"	0.5	15%	41	55	71	135
	1.0	20%	41	63	86	130
	2.0	50%	28	36	58	98
	3.0	75%	28	34	50	76
Orchimax	0.5	15%	38	45	62	119
	1.0	50%	34	60	75	112
	2.0	80%	25	32	50	88
	3.0	99%	23	30	45	68
Vacin & Went	0.5	10%	58	69	No growth	No growth
	1.0	20%	55	72	92	No growth
	2.0	40%	40	54	79	110
	3.0	45%	38	49	65	98

NAA =α-Naphthalene acetic acid; YE= Yeast extract; CW= coconut water

Table 22: No. of days taken for micropropagation and percent regenerated platelets of *C. nitida*.

Orchimax . (half strength) and growth regulators	No. of days taken for the development of a plantlet	Percent regenerated plantlets	Observations /Comments
+ 20% CW	55	40%	Plantlets could not survive after 50 weeks.
+ 2mg/L NAA	65	20%	Plantlets could not survive.
+ 20% CW + 2mg/L NAA	59	100%	Healthy plantlets.
+0.5mg/L NAA+ 10% CW	210	20%	Plantlets could not survive after 52 weeks.
+0.5mg/L NAA+ 15% CW	190	60%	Regenerated plantlets are very thin.
+0.5mg/L NAA+ 25% CW	140	40%	Plantlets are shorter but thicker.
+ 1mg/L NAA + 10% CW	70	40%	Plantlets could not survive.
+ 1mg/L NAA + 15% CW	63	80%	Less number of plantlets regenerated.
+ 1mg/L NAA + 25% CW	45	100%	Plantlets reached a height up to 1.5inches.
+ 2.5mg/L NAA+ 10% CW	68	80%	Plantlets are healthy but took time for hardening
+ 2.5mg/L NAA+ 15% CW	50	100%	Vigorous regenerated plantlets with a height up to 3inches.
+ 2.5mg/L NAA+ 25% CW	40	100%	Vigorous regenerated plantlets with a height up to 5inches.

NAA =α-Naphthalene acetic acid; CW= coconut water.

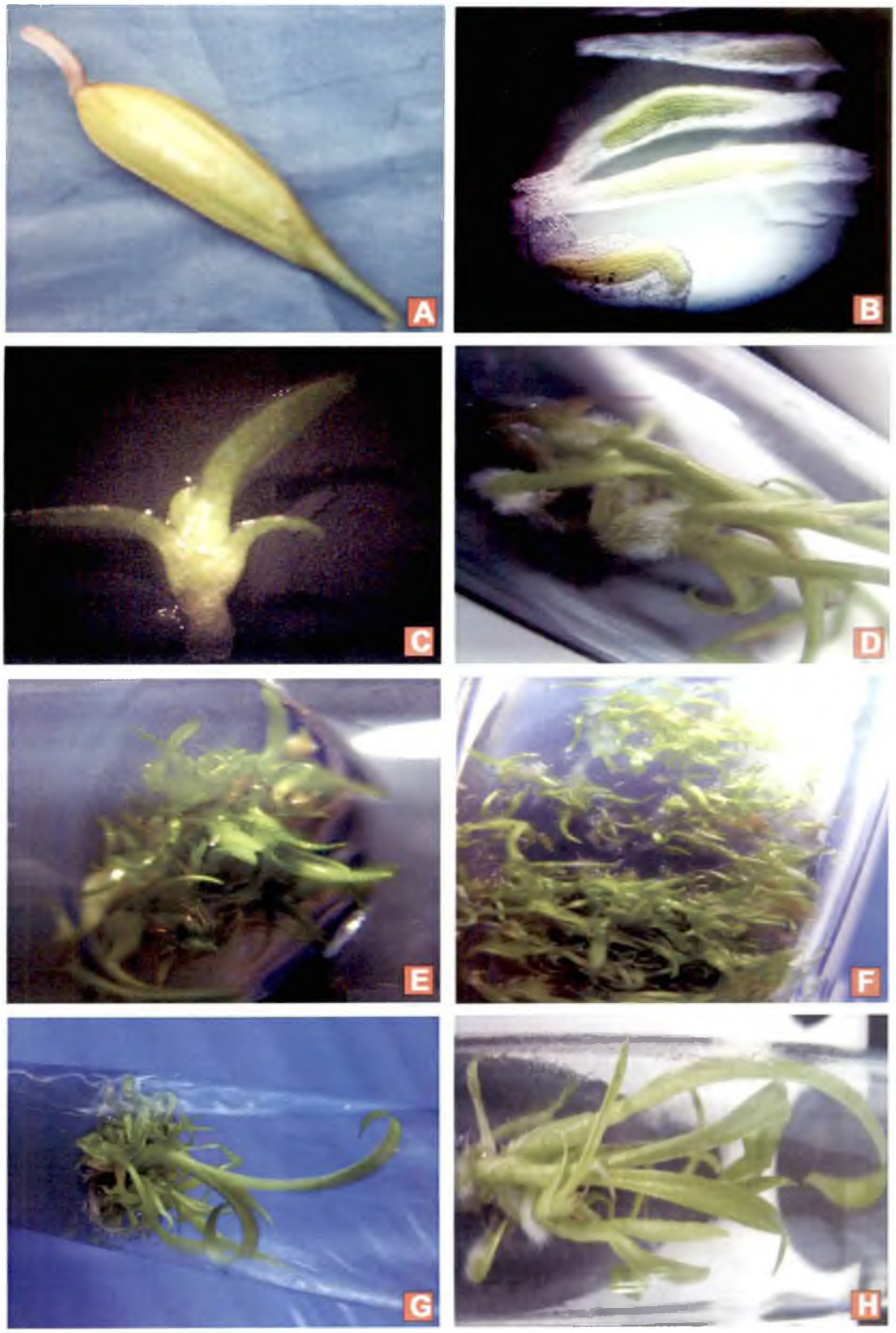

Plate 13: Different stages of seed culture and micropropagation of *Coelogyne nitida*. A: Seed pod of *C.nitida*; B: Seeds of *C.nitida*; C: Newly formed plantlets of *C.nitida*; D: Rhizoids from growing plantlets of *C.nitida*; E&F:Development of the plantlets of *C.nitida*; G&H: Regenerated plantlets of *C.nitida*.

4.2.2.6 Studies on *Coelogyne ovalis* Lindley

The seed pod of *C. ovalis* was collected from the forest area of Kalimpong. The seed pod (Fig.14-A) was 2.07cm long and 0.9 cm broad. Basal media Orchimax along with three concentrations of NAA and 10-20% of coconut water was used for the initiation of germination. The first sign of germination was the swelling of the seeds (FIG.14-B&C) was evident after 35 days of inoculation in the culture media with 2mg/L NAA and 20% coconut water. After 46 days they formed globular protocorm like bodies (FIG.14-D), and after 52 days PLBs started producing the shoots (FIG.14-E&F) and after 84 days formed the healthy plantlets (FIG.14-G). The best combination of growth regulators found to be 2gm/L yeast extract, 2mg/L NAA, 20% CW. The incubation period for germination and for sequential developments has been recorded in Table-23. The healthy plantlets were taken for micropropagation in the Orchimax (half strength) media with 1mg/L NAA, 10-15% coconut water and four different concentrations of BAP (Table-24). The plantlets (Fig.14-H,I&J) developed in orchimax media combined with 1mg/L NAA, 15% CW and 5mg/L BAP could produce healthy regenerated plantlets which survived well after hardening.

Table 23: No. of days taken for swelling of embryo, spherule stage, PLB formation and plantlet formation and percent germination of *C. ovalis* seeds:

Orchimax With 2gm/L YE	Days taken for the initiation of				Percent Germination
	Swelling of embryo	Spherule stage	PLB formation	Plantlet formation	
0.5mg/L NAA+ 10% CW	54	71	90	No further growth	10%
0.5mg/L NAA+ 20% CW	49	68	74	120	30%
1mg/L NAA+ 10% CW	40	76	86	No further growth	15%
1mg/L NAA + 20% CW	35	48	63	92	50%
2mg/L NAA+ 10% CW	28	39	47	88	65%
2mg/L NAA+ 20% CW	15	28	36	84	85%

NAA =α-Naphthalene acetic acid; CW= coconut water, YE = Yeast extract.

Fig.14 : Digfferent stages of seed culture and micropropagation of *Coelogyne ovalis*; A: Seed pod of *C.ovalis*; B & C swollen seed of *C.ovalis*; D: PLBs of *C.ovalis*; E & F Shoots coming from PLB of *C.ovalis*;G: development of plantlets of *C.ovalis*; H,I &J: Regenerated plantlets of *C.ovalis*.

Table 24: Percent germination of micropropagation of *C. ovalis* in the orchimax (half strength) media

Basal media (half strength)	Concentration of BAP	% of CW	Percent Regenerated plantlets
Orchimax +1mg/L NAA	2mg/L	10	10%
Orchimax +1mg/L NAA	2mg/L	15	10%
Orchimax + 1mg/L NAA	3mg/L	10	40%
Orchimax +1mg/L NAA	3mg/L	15	40%
Orchimax + 1mg/L NAA	4mg/L	10	72%
Orchimax + 1mg/L NAA	4mg/L	15	75%
Orchimax + 1mg/L NAA	5mg/L	10	92%
Orchimax + 1mg/L NAA	5mg/L	15	98%

NAA =α-Naphthalene acetic acid; BAP= 6 Benzyl amino purine; CW= Coconut water.

4.2.3. *Cymbidium elegans* Lindley

Experimental capsule or the seed pod of *C. elegans* was collected from Batasia. The seed pod (Fig. 15-A) showed the length of 3.1cm and breadth of 1.8 cm. For the germination of the seeds (Fig. 15-B), basal media Orchimax along with 2gm/L yeast extract, 10%, 15% and 25% coconut water and three different concentrations of NAA has been used for the initiation of germination and for the development of plantlets.

After 30 days of inoculation the swelling of embryo was observed and immediately formed the spherule stage (Fig.15-C) by bursting the seed coat. Globular protocorm like bodies (FIG.15-D) was found after 52 days of inoculation. The globular rounded structures produced the rhizoids (FIG.15-D). Development of shoots started from PLBs and after 120 days and there were 3.5 centimeter high healthy plantlets (FIG.15-E&F). For the fastest response and healthy growth of the plantlets of *C. elegans*, the best combination of supplements were NAA (2.5mg/L), yeast extract (2gm/L) and 25% coconut water Table-25.

The regenerated plantlets were healthy and further micropropagated in Orchimax (half strength) media supplemented with 1mg/L NAA, 10-15% coconut water and four different concentrations of BAP. Results of the experiment have been presented in Table-26. It was

found that 1mg/L NAA, 5mg/L BAP and 15% CW combined with Orchimax (half strength) medium produced healthy plantlets (FIG.15-G&H). The healthy plantlets were used for hardening experiment.

Table 25: *In vitro* germination of *C. elegans* seeds and plantlet formation in different media.

Orchimax With 2gm/L YE	Days taken for the initiation of				Percent Germination of seeds
	Swelling of embryo	Spherule stage	PLB formation	Plantlet formation	
0.5mg/L NAA + 10% CW	54	62	89	No growth	15%
0.5mg/L NAA + 15% CW	54	62	89	200	30%
0.5mg/L NAA + 25% CW	52	62	87	165	20%
1mg/L NAA + 10% CW	45	53	69	No growth	15%
1mg/L NAA + 15% CW	45	53	69	210	45%
1mg/L NAA + 25% CW	45	53	65	159	60%
2.5mg/L NAA + 10% CW	32	45	56	143	75%
2.5mg/L NAA + 15% CW	30	45	56	138	99%
2.5mg/L NAA + 25% CW	30	45	52	120	99%

NAA =α-Naphthalene acetic acid; CW= coconut water; YE =Yeast extract

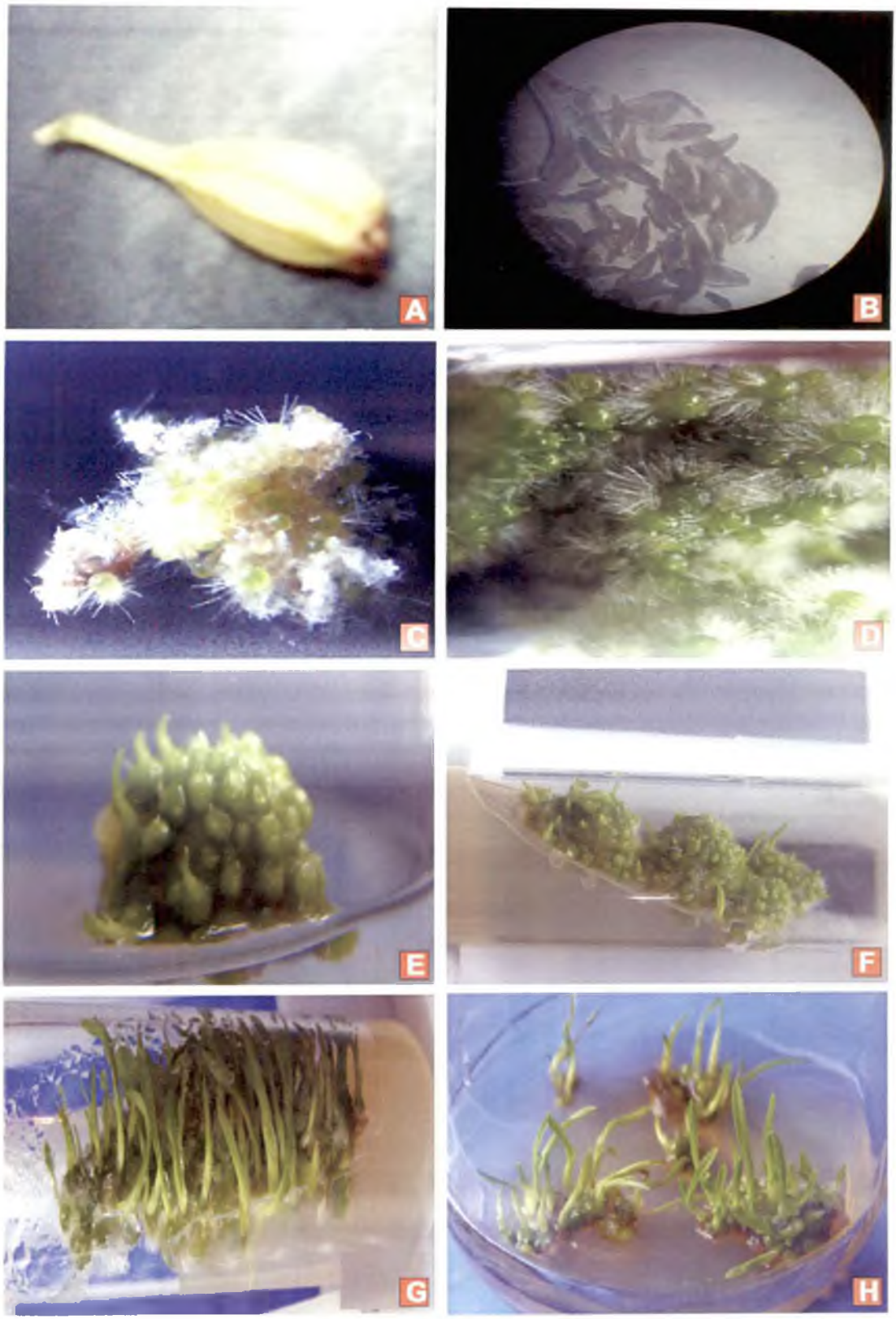

Fig. 15: Different stages of seed culture and micropropagation of *Cymbidium elegans*. A: Seed pod of *C.elegans*; B: Seeds of *C.elegans*; C: Spherule stage of *C.elegans*; D: PLBs of *C.elegans*; E & F: Producing shoots from PLBs of *C.elegans*; G & H: Regenerated plantlets of *C.elegans*.

Table 26: Percent germination of *C. elegans* in the orchimax (half strength) media with NAA, BAP and CW.

Basal media	Concentration of BAP	% of CW	Percent regenerated plantlets
Orchimax (half strength)+ 1mg/L NAA	2mg/L	10	45%
Orchimax (half strength)+ 1mg/L NAA	2mg/L	15	60%
Orchimax (half strength)+ 1mg/L NAA	3mg/L	10	69%
Orchimax (half strength)+ 1mg/L NAA	3mg/L	15	75%
Orchimax (half strength)+ 1mg/L NAA	4mg/L	10	80%
Orchimax (half strength)+ 1mg/L NAA	4mg/L	15	99%
Orchimax (half strength)+ 1mg/L NAA	5mg/L	15	99%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= 6 Benzyl animo purine

4.2.4 Dendrobiums

Dendrobiums are among the top in the commercial market due to its beautiful flowers. The genus *Dendrobium* consists of large number of species (about 1500) and forms one of the important groups of commercially valuable orchids with scientific importance. *Dendrobium* species have some medicinal uses also. The species of *Dendrobium* were in abundance in the forest area of Darjeeling and Kalimpong. In the present experiment, seven different species of *Dendrobium* have been taken into consideration as they are declining in nature drastically.

4.2.4.1. *Dendrobium chrysanthum* Lindl Ex Wall.

Capsule or the seed pod was collected from the forest of Kalimpong. It measured 3.2 cm in length and 1.9 cm in breadth. For the germination of seed three different nutrient media, i.e., Knudson"C", Murashige & Skoog and Vaccin & Went was used added with growth regulators. Each media was added with 1mg/L NAA and 15% coconut water. Four

different concentrations of BAP were tested as for best growth of the plantlets. The results have been shown in Table-27. Among the three media taken, seeds (Fig.16-A) in Knudson"C" media showed the first sign of germination after 45 days, then they formed the PLBs (Fig. 16-B) after 70 days and finally after 102 days they turned into healthy plantlets (Fig. 16-C&D). It was observed that 98% germination was possible in Knudson"C" media in combination with 1mg/L NAA, 15% coconut water and 3mg/L BAP.

When the same combination of growth regulators was added with Murashige & Skoog media, swelling of the seed coat occurred after 75 days, PLBs formed after after 98 days and the plantlets formed after 138 days. It was observed that germination was 69% in comparison to 98% in Knudson"C" medium. However, in case of Vacin & Went media the response of germination was after 92 days and after 159 days it changed into round PLBs and 52% plantlets were formed after 192 days. Moreover the plantlets were not suitable for further micropropagation.

Micropropagation was carried out with the help of meristems and nodal buds from the plantlets grown up to a height of 5 cm. Knudson"C" (half strength) was added with some growth regulators for the regeneration of plantlets (Fig.16-E&F). Six different concentrations of NAA, along with 3mg/L BAP and 20% coconut water were supplemented in the media. The results have been shown in the Table-28. BAP (3mg/L), 20% CW and 5 mg/L NAA was the best combination to regenerate the plantlets up to 89% from the meristem and 65% from the nodal buds.

Table 27: Percent of seed germination of *D. chrysanthum* seeds:

Basal media with	Concentration of BAP (mg/L)	Percent germination of seeds
Knudson"C" 15% CW and 1gm/L NAA	0.5	59%
	1.0	65%
	2.0	74%
	3.0	98%
Murashige &Skoog 15% CW and 1gm/L NAA	0.5	50%
	1.0	52%
	2.0	60%
	3.0	69%
Vacin & Went 15% CW and 1gm/L NAA	0.5	No growth
	1.0	18%
	2.0	35%
	3.0	52%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= 6Benzyl animo purine

Table 28: Percent of micropropagated plantlets from meristems and nodal buds of *D. chrysanthum*

Selected explant	Basal Media	Percent regenerated plantlets in different concentrations of NAA (mg/L)					
		0.5	1	2	3	4	5
Meristem	Knudson"C"(halfstrength) +3mg/L BAP +20% CW	65%	68%	70%	79%	81%	89%
Nodal buds	Knudson"C"(half strength) + 3mg/L BAP +20% CW	43%	48%	51%	59%	62%	65%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= 6 Benzyl animo purine

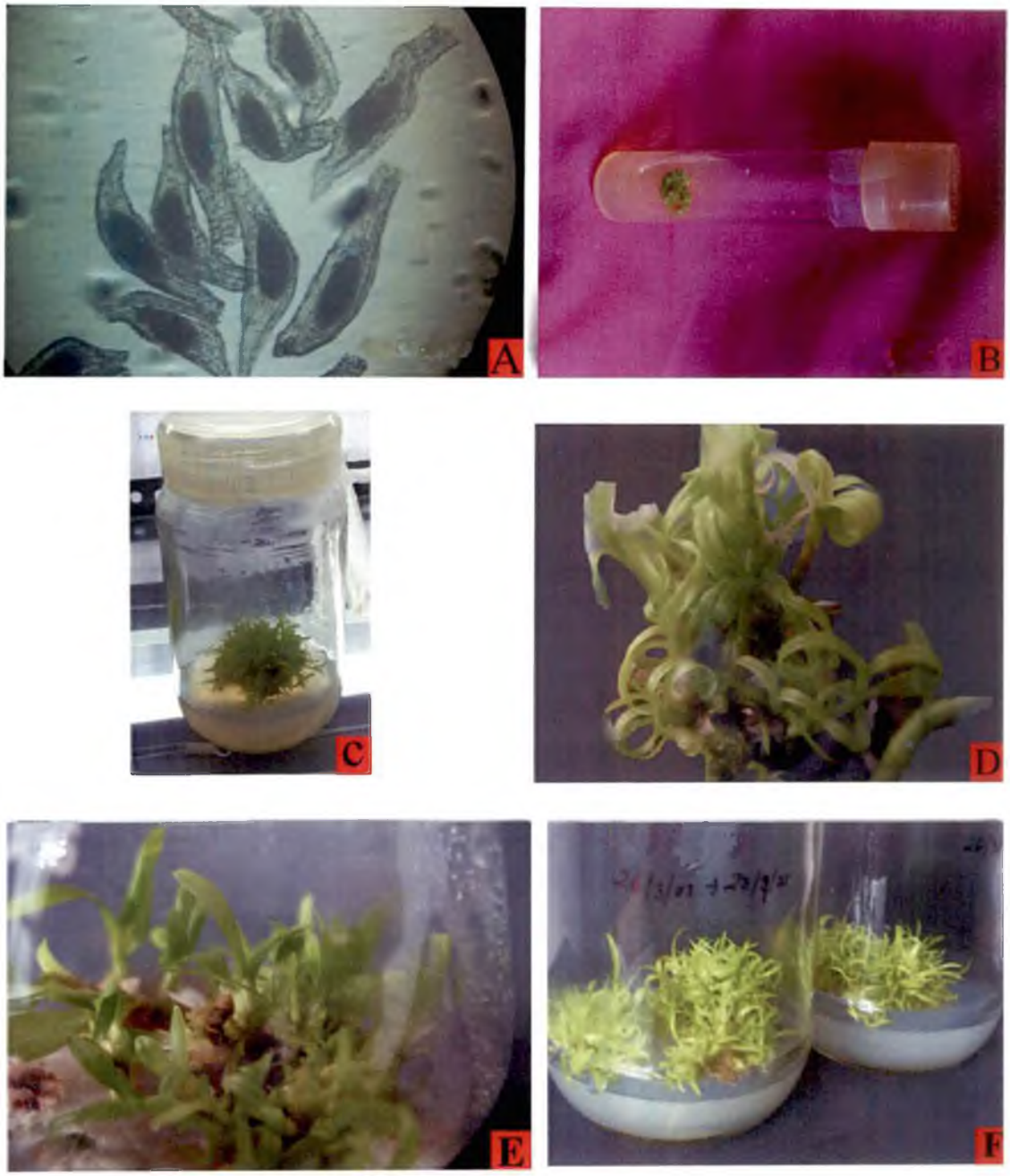

Fig. 16: Different stages of seed culture and micropropagation of *Dendrobium chrysanthum*. A: Seeds of *D.chrysanthum*; B: PLBs of *D.chrysanthum*; C&D: Growing plantlets of *D.chrysanthum*; E&F: Regenerated plantlets of *D.chrysanthum*.

4.2.4.2. Studies on *Dendrobium crepidatum* Lindl.

The experimental material is the green seed pod (Fig.17-A) collected from Pedong, Kaimpong. It measured 2.01cm in length and 0.6 cm in breadth. For *in vitro* seed germination three different nutrient media, i.e., Knudson"C", Murashige & Skoog and Vacin & Went was used in combination with some growth regulators such as 1mg/L NAA, 15% coconut water and either of four different concentrations of BAP. The percent germination was computed in Table-29.

After 52 days the seeds (Fig.17-B) in Knudson"C" media swelled up and after 74 days they formed the PLBs (Fig.17- C). PLBs transformed into plantlets (Fig.17-D) after 105 days. Highest 98% germination was recorded in Knudson"C" media combined with 1mg/L NAA, 15% coconut water and 3mg/L BAP.

Maximum 75% germination was observed when Murashige & Skoog media supplemented with similar growth regulators and additives. Seed coat swelled after 65 days, PLBs formed after 81 days and finally the plantlets formed after 112 days. However, in case of Vacin & Went media the seed swelling, PLB formation and plantlet formation was found to occur after 75 days, 97 days and 135 days respectively. Plantlets were not suitable for micropropagation although 49% seeds found to germinate.

Micropropagation was carried out from plantlets grown up to the height of 5cm. Knudson"C" (half strength) was added with growth regulators 1mg/L NAA, five different concentrations of BAP and 15% coconut water for the regeneration of plantlets. The results of micropropagation have been incorporated in Table-30 (Fig.17-E).

Table 29: Record showing percent of seed germination of *D. crepidatum* seeds

Basal media with	Concentration of BAP mg/L	Percent germination of seeds
Knudson"C" + 15% CW + 1gm/L NAA	1.0	62%
	2.0	62%
	3.0	72%
	4.0	75%
	5.0	98%
Murashige &Skoog + 15% CW + 1gm/L NAA	1.0	49%
	2.0	55%
	3.0	55%
	4.0	61%
	5.0	75%
Vacin & Went + 15% CW + 1gm/L NAA	1.0	No growth
	2.0	No growth
	3.0	20%
	4.0	31%
	5.0	49%

NAA =α-Naphthalene acetic acid; CW= coconut water;
BAP= 6Benzyl animo purine

Table 30: Showing the percent of micropropagated plantlets of *D. crepidatum*

Basal media	Concentration of BAP	Percent regenerated plantlets
Knudson"C"(half strength)+1mg/L NAA	1mg/L	75%
Knudson"C"(half strength)+ 1mg/L NAA	2mg/L	79%
Knudson"C"(half strength)+ 1mg/L NAA	3mg/L	84%
Knudson"C"(half strength)+ 1mg/L NAA	4mg/L	86%
Knudson"C"(half strength)+1mg/L NAA	5mg/L	99%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= Benzyl animo purine

Fig. 17: Different stages of seed culture and micropropagation of *Dendrobium crepidatum*. A: Seed pod of *D. crepidatum*; B: Seeds of *D.crepidatum*; C: PLBs of *D.crepidatum*; D: Growing plantlets of *D.crepidatum*; E: Regenerated plantlets of *D.crepidatum*.

4.2.4.3 Studies on *Dendrobium densiflorum* Lindl Ex wall.

Seed pod of this exotic species was collected from Batasia, Darjeeling. The seed pod (fig.18-A) was quite big in size measuring 9cm length and 4cm breadth. For the seed germnation the Orchimax medium was chosen. Three different concentrations of BAP and five different conentrations of NAA with 20% coconut water was supplemented with the medium. Details of the experimental combinations have been presented in the Table-31. The seeds (Fig.18-B) came out of its seed coat (Fig.18-C) after 40 days of inoculation and after 55 days globular PLBs formed. Development of shoot from PLBs took place after 98 days (Fig.18-D&E), finally it grown into a plantlet (Fig.18-F&G). The combination of 20% CW, 3mg/L BAP and 4 mg/L NAA with orchimax medium was the best and it produced 92% seed germination. The result have been illustrated clearly in Table-31. Plantlets (Fig.18-H). were very strong and numerous and they were taken for further micropropagaion in Orchimax (half strength) media supplemented with different concentrations of BAP. The results of micropropagation have been tabulated in Table-32. The best result of micropropagation was found in Orchimax (half strength) with 5mg/L BAP ,1mg/L NAA and 20% CW. Maximum 97% plantlets (Fig.18-I) were formed. The plantlets were taken to a glass house for hardening.

Table 31: Percent germination in *D. densiflorum* seeds in coconut water supplemented Orchimax medium in different concentrations of BAP and NAA.

Basal Media	BAP mg/L	Percent germination of seed in different concentrations of NAA (mg/L)				
		0.5	1	2	3	4
Orchimax +20% CW	1.0.	55%	59%	62%	65%	65%
Orchimax+20% CW	2.0	71%	82%	82%	85%	85%
Orchimax+20% CW	3.0	85%	85%	87%	90%	92%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= Benzyl animo purine

Fig.18 : Different stages of seed culture and micropropagatio of *Dendrobium densiflorum*; A:Seed pod of *D.densiflorum*; B: Seeds of *D.densiflorum*;C: Sperule stage of *D. densiflorum*; D & E: Shoot and root coming out of PLBs of *D. densiflorum*; F & G : Growing of PLBs into plantlets of *D.densiflorum*; H: Developing plantlets of *D. densiflorum*;.I: Regenerated plantlets of *D. densiflorum.*

Table 32: Percent germination of *D. densiflorum* in the orchimax (half strength) media with NAA, BAP and CW.

Basal media	Concen-tration of BAP	Percent regenerated plantlets
Orchimax (half strength)+1mg/LNAA+20% CW	2mg/L	41%
Orchimax (half strength)+1mg/L NAA+20%CW	2mg/L	52%
Orchimax (half strength)+1mg/L NAA+20%CW	3mg/L	65%
Orchimax (half strength)+1mg/L NAA+20%CW	3mg/L	73%
Orchimax (half strength)+ 1mg/LNAA+20%CW	4mg/L	82%
Orchimax (half strength)+ 1mg/LNAA+20%CW	4mg/L	91%
Orchimax (half strength)+1mg/L NAA+20%CW	5mg/L	97%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= 6 Benzyl animo purine

4.2.4.4 Studies on *Dendrobium fimbriatum* Lindl.

Capsule or the seed pod was collected from Pedong, Kalimpong. The germination of seed (Fig. 19-A) was tested in three different nutrient media, i.e., Knudson 'C', 'Murashige & Skoog' and 'Vacin & Went' added with the growth regulators 1mg/L NAA, 25% coconut water and four different concentrations of BAP. The results of the experiment have been noted in Table-33.

Among the three media tested Knudson 'C' medium showed the first sign of germination after 49 days. Spherule stage, PLBs (Fig. 19-B) and healthy plantlets (Fig.19-C) were formed after 52, 71 and 121 days respectively. It was found that 98% seeds germinated in Knudson"C" medium in combination with 1mg/L NAA, 25% coconut water and 3mg/L BAP. In Murashige & Skoog media (with similar supplements) seeds swelled after 65 days, PLBs formed after 83 days and the plantlets formed after 143 days. Seed germination was 62%. However, in case of Vacin & Went media the response of germination, spherule formation and plantlet formation were respectively after 86, 112, and 207 days. Only 32% plantlets were formed and that too were unsuitable for micropropagation.

Micropropagation was carried out with meristems and nodal buds of the plantlets grown up to a height of 5cm. Knudson"C" (half strength) was added with other growth regulators for the regeneration of plantlets. Six different concentrations of NAA, 3mg/L of BAP and 25% coconut water were added. The percent of regenerated plantlets (Fig.19-D) through micropropagation was recorded in Table-34. From the results it was evident that 3mg/L BAP, 25% CW and 5 mg/L NAA supplemented in Knudson"C" (half strength) medium gave best results. In the above mentioned medium 94% and 79% plantlets regenerated from the meristem and nodal bud explants respectively.

Table 33: Percent of seed germination of *D. fimbriatum* seeds

Basal media With NAA and CW	Concentration of BAP mg/L	Percent germination of seeds
Knudson"C" +25% CW and 1gm/L NAA	0.5	61%
	1.0	62%
	2.0	84%
	3.0	98%
Murashige & Skoog +25% CW and 1gm/L NAA	0.5	40%
	1.0	43%
	2.0	55%
	3.0	62%
Vacin & Went +25% CW and 1gm/L NAA	0.5	No growth
	1.0	No growth
	2.0	17%
	3.0	32%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= 6 Benzyl animo purine

Table 34: Percent of micropropagated plantlets from meristems and nodal buds of *D. fimbriatum*

Selected explant	Basal Media	Percent regenerated plantlets in different concentrations of NAA (mg/L)					
		0.5	1	2	3	4	5
Meristem	Knudson"C"(half strength) + 3mg/L BAP +25% CW	69%	69%	74%	75%	91%	94%
Nodal buds	Knudson"C"(half strength) + 3mg/L BAP +25% CW	42%	42%	42%	50%	60%	79%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= 6 Benzyl animo purine

4.2.4.5 Studies on *Dendrobium longicornu* Lindley

The experimental material is the green seed pod (Fig. 20-A) collected from Takdah. It measured 2.03cm in length and 0.8 cm in breadth. For the seed germination, Murashige & Skoog medium was used with growth regulators, 1mg/L NAA, 25% coconut water and four different concentrations of BAP. The percent germination was recorded in Table-35.

After 43 days the seeds (Fig. 20-B) in Knudson"C" media swelled up and then they formed the PLBs after 67 days. PLBs started producing shoots(Fig. 20-C & D) after 95 days and then turned into healthy plantlets (Fig. 20-E&F) media combined with 1mg/L NAA, 25% coconut water and 3mg/L BAP showed 98% germination.

Micropropagation was carried out in Murashige & Skoog (half strength) with the explants cut from the plantlets and grown through seed culture. The MS half strength was supplemented with growth regulators. Six different concentrations of NAA, 3mg/L of BAP and 25% coconut water were added. The percent of micropropagated plantlets (Fig. 20-G&H) was recorded in Table-36. MS half strength in combination with 3mg/L BAP, 25% CW and 5 mg/L NAA was the best combination to regenerate the plantlets up to 88% from the meristem and 69% from the nodal buds.

Fig. 19: Different stages of seed culture and micropropagation of *Dendrobium fimbriatum*. A: Seeds of *D.fimbriatum*; B: PLBs of *D.fimbriatum*; C: Growing plantlets of *D.fimbriatum*; D: Regenerated plantlets of *D.fimbriatum*.

Table 35: Percent seed germination of *D. longicornu* in MS medium + 1mg/L NAA+ 25% CW supplemented with four different concentrations of BAP

Basal media	BAP mg/L	Percent germination of seeds
Murashige and Skoog+ 1mg/L NAA+ 25% CW	0.5	40%
Murashige and Skoog+ 1mg/L NAA+ 25% CW	1.0	68%
Murashige and Skoog+ 1mg/L NAA+ 25% CW	2.0	85%
Murashige and Skoog+ 1mg/L NAA+ 25% CW	3.0	98%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= Benzyl animo purine

Table 36: Percent plantlets regenerated from 'meristem explants' and 'nodal-bud explants' in micropropagation of *D. longicornu.*

Selected explant	Basal Media	Percent regenerated plantlets in different concentrations of NAA (mg/L)					
		0.5	1	2	3	4	5
Meristem	Murashige & Skoog (half strength) + 3mg/L BAP +25% CW	45%	45%	69%	75%	81%	88%
Nodal buds	Murashige & Skoog (half strength) + 3mg/L BAP +25% CW	39%	41%	59%	60%	66%	69%

NAA =α-Naphthalene acetic acid; CW= coconut water; BAP= 6 Benzyl animo purine

4.2.4.6 Studies on *Dendrobium nobile* Lindl.

This is the most common medicinal species of Darjeeling hills and the seed pods were collected from Takdah. The length of the seed pod was 9 cm and breadth 3cm. Three different nutrient media, i.e., Orchid maintenance medium, Murashige & Skoog and Vacin & Went added with the growth regulators 1mg/L NAA, 25% coconut water and four

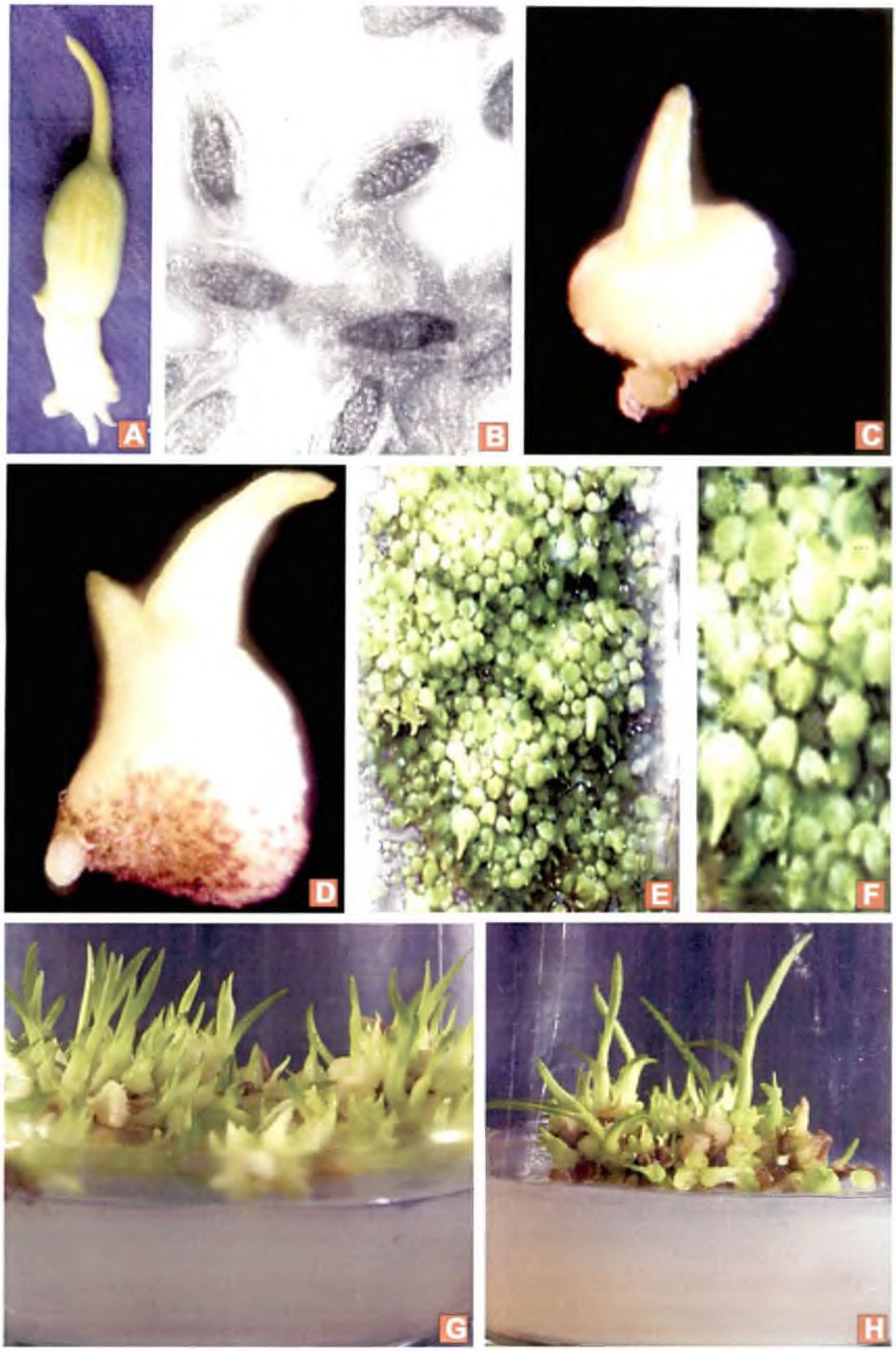

Fig. 20: Different stages of seed culture and micropropagation of *Dendrobium longicornu*; A: Seed pod of *D. longicornu*; B: Seeds of *D. longicornu*; C & D: Shoot & root coming from PLBs; E & F: PLBs developing into plantlets of *D.longicornu*; G&H:Regenerated plantlets of *D. longicornu.*

different concentrations of BAP. Percent germination of seeds was calculated and was noted in Table-37.

After 48 days of seed inoculation, the first sign of germination was evident (Fig. 21-A) in Orchid maintenance medium. Spherule stage (Fig. 21-B), PLBs (Fig. 21-C), and healthy plantlets (Fig. 21-D) were formed after 59 days, 81 days and 120 days respectively. It was observed that 85% germination could occur in Orchid maintenance medium combined with 4mg/L NAA and 25% coconut water.

The same combination of growth regulators when added with Murashige & Skoog medium it was noted that swelling of the seeds occurred after 59 days. Subsequent development of PLBs happened after 75 days and the plantlets formed after 133 days. Total seed germination was 63%. In case of Vacin & Went medium the response of germination, PLB formation and plantlet formation was recorded after 89 days, 102 days and 214 days respectively. Only 29% plantlets were formed but the plantlets were not suitable for further micropropagation.

Micropropagation was carried out from the plantlets grown through seed culture (Fig. 21-E), in Orchid maintenance medium (half strength) combined with 1mg/L NAA, 10% coconut water and five different concentrations of BAP. The best composition for the regeneration of the plantlets (Fig. 21-F), was found to be 1mg/L NAA, 10% CW and 5mg/L BAP where the 99% regenerated plantlets could survive very easily after hardening. The results have been tabulated in Table-38.

Table 37: Percent of seed germination of *D. nobile*

Basal media with NAA & CW	Concentration of BAP mg/L	Percent germination of seeds
OMM + 1mg/LNAA + 10% CW	1.0	59%
	2.0	71%
	3.0	84%
	4.0	85%
Murashige & Skoog + 1mg/LNAA + 25% CW	1.0	45%
	2.0	47%
	3.0	65%
	4.0	63%
Vacin & Went + 1mg/LNAA + 25% CW	1.0	No growth
	2.0	No growth
	3.0	15%
	4.0	29%

OMM=Orchid maintenance medium; NAA=α-Naphthalene acetic acid; CW= coconut water; BAP= 6 Benzyl animo purine

Table 38: Percent of regenerated plantlets in Orchimax (half strength) media of *D. nobile*

Basal media	BAP mg/L	Percent germination
OMM (half strength)+1mg/LNAA+ 10%CW	1.0	60%
OMM (half strength)+1mg/LNAA+ 10%CW	2.0	72%
OMM (half strength)+ 1mg/LNAA+10%CW	3.0	88%
OMM (half strength)+1mg/LNAA+ 10%CW	4.0	98%
OMM (half strength)+1mg/LNAA+10% CW	5.0	99%

OMM=Orchid maintenance medium; NAA=α-Naphthalene acetic acid; CW= coconut water; BAP= 6 Benzyl animo purine

4.2.4.7 Studies on *Dendrobium primulinum* Lindl.

The seed pod of *D. primulinum* was collected from Kalimpong, and the length of the pod (Fig. 22-A) was 3.5 cm long and breadth was 1.5cm. After surface sterilization, seed pod cut wide open and the seeds (Fig. 22-B) were inoculated in Orchid Maintenance medium supplemented with 1mg/L NAA, 20% coconut water and five different

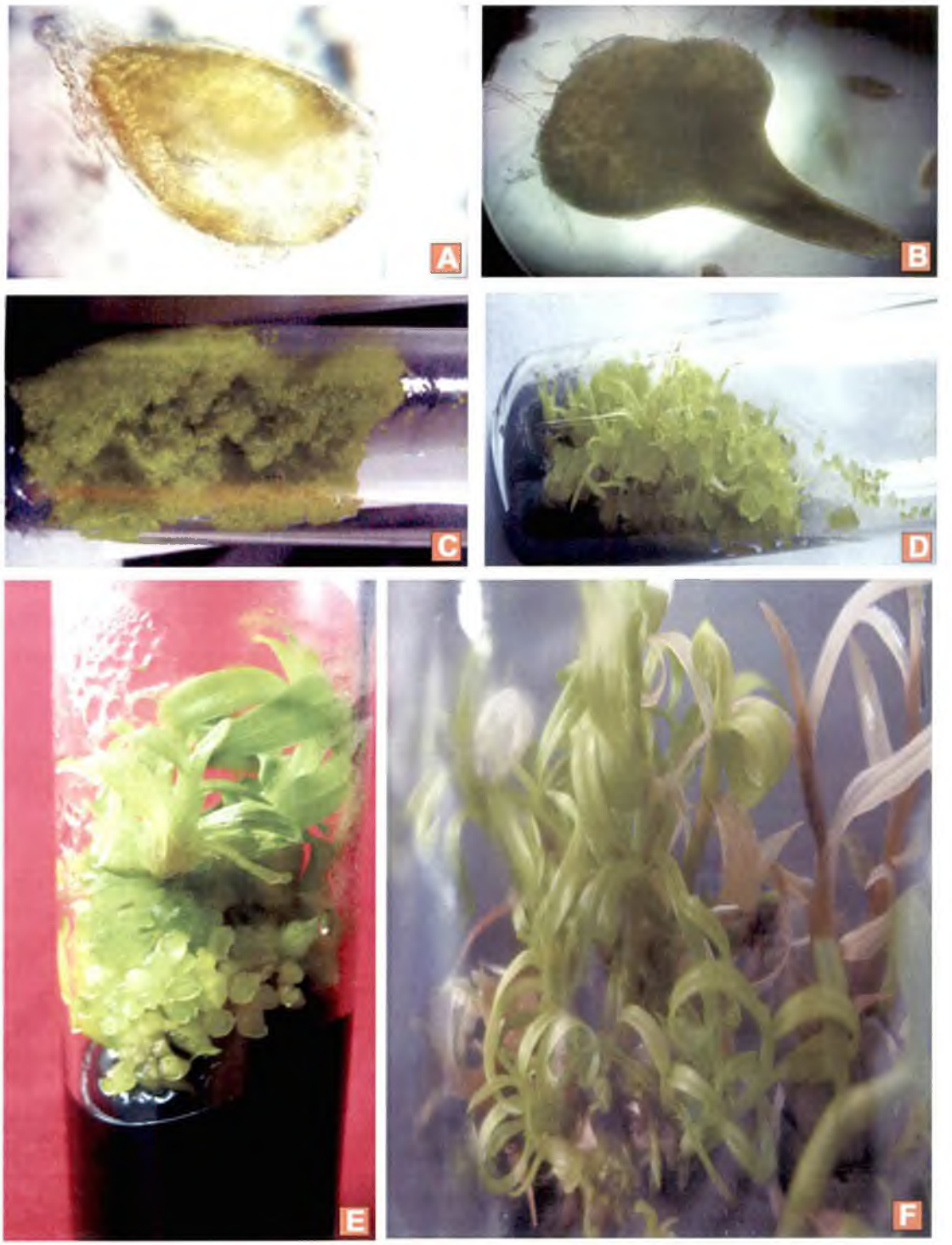

Fig.21: Different stages of seed culture and micropropagation of *Dendrobium nobile*; A: Seed of *D. nobile*; B: Spherule stage of *D. nobile*; C: PLBs of *D. nobile*; D & E: PLBs producing plantlets of *D.nobile*; F:Regenerated plantlets of *D. nobile*.

concentrations of BAP. The best combination of growth regulators for the development of the plantlets from the seeds was 1mg/L NAA, 20% CW and 5mg/L BAP. The number of days taken to form the spherule stage (Fig.22-C), PLBs and plantlets (Fig.22-D) were 58 days, 72 days and 132 days respectively and tabulated in Table-39. The germinated plantlets were healthy and vigorous. After attaining a height of 5.5 cm, plantlets (Fig.22-E) were cut and were used as explants for micropropagation in Orchid maintenance medium (half strength) with/without BAP. Three different concentrations of BAP were used. In micropropagation 99% healthy plantlets were produced (Fig.22-F) when Orchid maintenance medium (half strength) was combined with 5mg/L BAP. The results of micropropagation were recorded in Table-40. Finally, the plantlets were transferred to the glass house for hardening.

Table 39: Percent germination of *D. primulinum* seeds

Basal media	BAP (mg/L)	Percent germination
OMM + 1mg/L NAA + 10%CW	1.0	75%
OMM+ 1mg/L NAA + 10% CW	2.0	82%
OMM+ 1mg/L NAA + 10% CW	3.0	88%
OMM+ 1mg/L NAA+ 10% CW	4.0	98%
OMM+ 1mg/L NAA+ 10% CW	5.0	99%

OMM=Orchid maintenance medium;NAA=α-Naphthalene acetic acid;
CW= coconut water; BAP= 6 Benzyl animo purine

Table 40: Percent germination of micropropagation of *D. primulinum*

Basal media	BAP (mg/L)	Percent germination
OMM (half strength)	0.0	79%
OMM (half strength)	1.0	86%
OMM (half strength)	3.0	89%
OMM (half strength)	5.0	99%

OMM=Orchid maintenance medium; BAP= 6 Benzyl animo purine

Fig. 22: Different stages of seed culture and micropropagation of *Dendrobium primulinum*; A: Seed pod of *D. primulinum* B: Seeds of *D. primulinum*; C: Spherule stage of *D.primulinum*; D: PLBs with shoots of *D. primulinum*. E: Growing plantlets from PLBs of *D. primulinum*; F: Regenerated plantlets of *D. primulinum*.

4.2.5.1 Studies on *Pleione humilis* (Smith) D.Don.

The seed pod was collected from Takdah. Length and breadth was measured as 1.9 cm and 0.6 cm respectively. After surface sterilization the seeds (Fig.23-A) were inoculated in Orchid maintenance medium supplemented with 20% coconut water, three different concentrations of NAA and four different concentrations of BAP. Developments like germination of seeds, formation of spherule stage (Fig. 23-B), and formation of plantlets (Fig. 23-C & D) were found to occur sequentially.

The percent germination recorded was 95% in media containing 4mg/L BAP and 5mg/L NAA but it reduced to 70% when 4mg/L BAP combined with 3mg/L NAA. The germination rate further reduced to 50% when 4mg/L BAP with 1mg/L NAA was used. Results of the seed culture were recorded in Table-41.

Table 41: Percent germination of *Pleione humilis* seeds

Basal medium	NAA	BAP mg/L	Percent germination
OMM+20% CW	1 mg/L	1	46%
OMM+20% CW		2	46%
OMM+20% CW		3	48%
OMM+20% CW		4	50%
OMM+20% CW	3 mg/L	1	59%
OMM+20% CW		2	62%
OMM+20% CW		3	62%
OMM+20% CW		4	70%
OMM+20% CW	5mg/L	1	82%
OMM+20% CW		2	88%
OMM+20% CW		3	93%
OMM+20% CW		4	95%

OMM=Orchid maintenance medium;NAA=α-Naphthalene acetic acid;
CW= coconut water; BAP= Benzyl animo purine

Micropropagation was performed in Orchid maintenance medium (half strength) without BAP, and with three different concentrations of BAP. Although the micropropagation was successful in each set of media but 96% healthy plantlets regenerated (Fig. 23-E&F) when medium was combined with 5mg/L BAP. The results of the micropropagation were recorded in Table-42. The regenerated plantlets were then transferred carefully to the glass house for hardening as in other cases of the present study.

Table 42: Percent germination of micropropagation of *Pleione humilis*

Basal media	BAP mg/L	Percent germination
OMM (half strength)	0.0	82%
OMM (half strength)	1.0	90%
OMM (half strength)	3.0	91%
OMM (half strength)	5.0	96%

OMM=Orchid maintenance medium; BAP= 6 Benzyl animo purine

4.2.5.2 Studies on *Pleione maculate* (Lindl) Lindl.

The seed pods (Fig. 24-A) were collected from Rangpo. The length and breadth of the seed pods were 1.4 cm and 0.3 cm respectively. After surface sterilization seeds (Fig. 24-B) were inoculated in three different media such as Orchid maintenance media, Murashige & Skoog and Vacin & Went, supplemented with 20% coconut water, 2mg/L NAA and four different concentrations of BAP. The seeds were found to swollen after 49 days in OMM with 20% coconut water, 2mg/L NAA and 4mg/L BAP. Globular PLBs (Fig. 24-C) formed after 58 days and ultimately after 103 days plantlets developed (Fig. 24-D & E). The percent germination was recorded as 98% in Orchid maintenance medium containing 4mg/L BAP but same combination of growth regulators in Murashige & Skoog medium produced 75% of the plantlets. Only 45% seed germination was recorded in Vacin & Went medium (Table-43). The plantlets were very healthy and explants were cut from the plantlets raised through seed culture and was used for micropropagation studies. Orchid maintenance media (half strength) without any growth regulators was used for

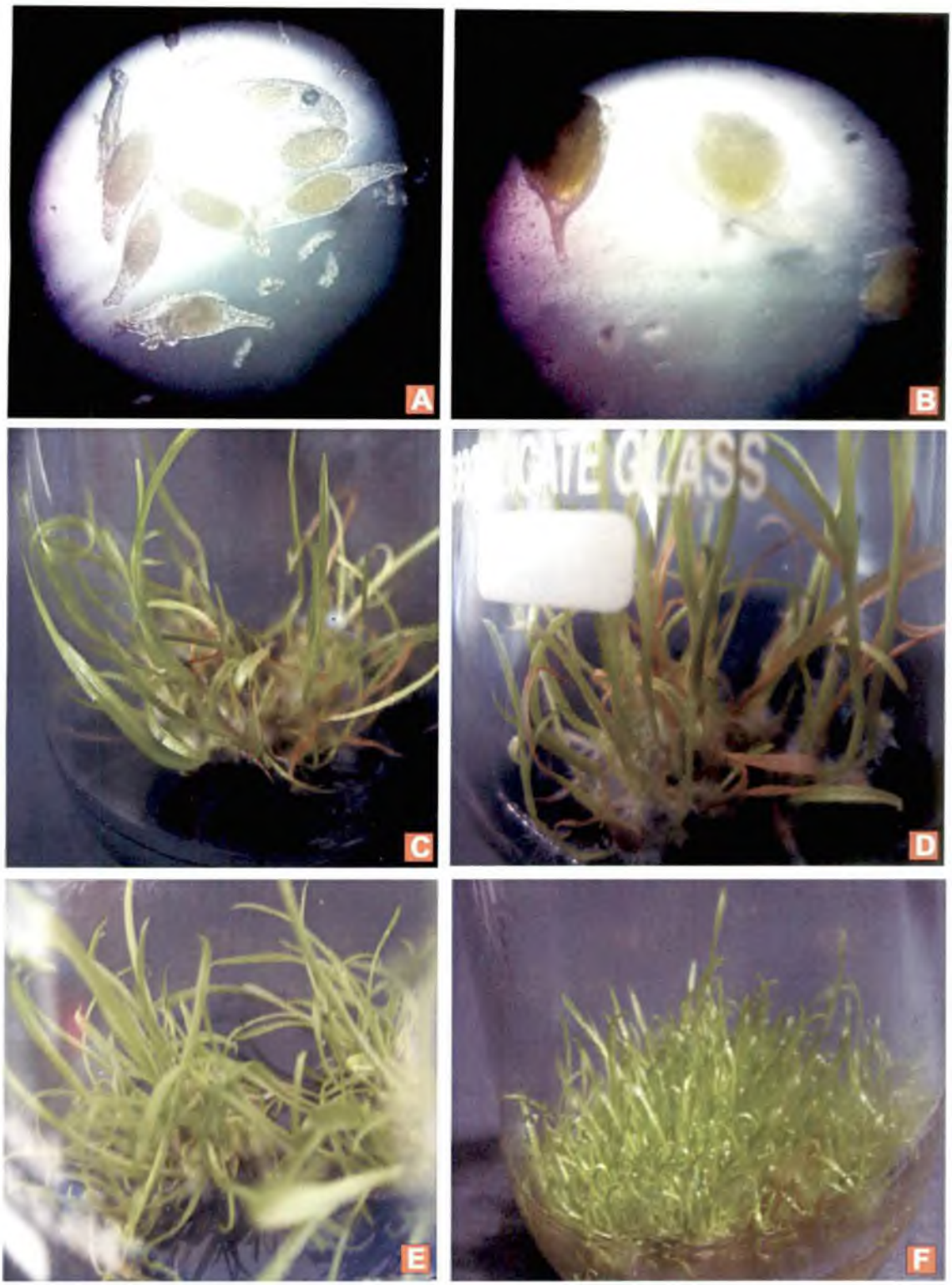

Fig.23: Different stages of seed culture and micropropagation of *Pleione humilis*. A:Seeds of *P.humilis*; B: Spherule stage of *P.humilis*; C& D: Developing plantlets of *P.humilis*; E & F: Regenerated plantlets of *P. humilis*.

micropropagation. Finally the regenerated plantlets (Fig. 24-F&G) were transferred to a glasshouse for hardening.

Table 43: Percent germination of *P. maculate* seeds and time required for plantlet formation.

Basal media	BAP mg/L	No. of days taken for the development of a plantlet	Percent regenerated plantlets
OMM + 2mg/L NAA + 20% CW	1.0	57	65%
	2.0	57	65%
	3.0	57	97%
	4.0	49	98%
Murashige&Skoog + 2mg/L NAA + 20% CW	1.0	60	45%
	2.0	60	55%
	3.0	62	55%
	4.0	72	75%
Vacin &Went + 2mg/L NAA + 20% CW	1.0	No growth	0%
	2.0	No growth	0%
	3.0	131	10%
	4.0	93	45%

OMM=Orchid maintenance medium;NAA=α-Naphthalene acetic acid;
CW= coconut water; BAP= 6 Benzyl animo purine

4.2.5.2 Studies on *Pleione precox* (Smith) D.Don.

The seed pod (Fig.25-A) was collected from Batasia. After surface sterilization the seeds were inoculated in Orchid maintenance media, supplemented with 20% coconut water, 2mg/L NAA and five different concentrations of BAP. In OMM with 20% coconut water, 2mg/L NAA and 4mg/L BAP, the seeds (Fig.25-B) were found to swell after 52 days. The seeds turned to globular PLBs (Fig.25-C&D) after 69 days. Shoots and rhizoids (Fig.25-E) developed after 102 days and finally plantlets formed (Fig.25-F). The percent germination was recorded as 96% in OMM medium containing 4mg/L BAP. Table-44 shows the results of seed culture. Micropropagation with Orchid maintenance media (half strength) without BAP and with three different concentrations of BAP was tested. Although the micropropagation was successful in each set of media but 98% healthy regenerated plantlets (Fig.25-G) were obtained in the media combined with 5mg/L BAP. Results (Table-45)

Fig. 24: Different stages of seed culture and micropropagation of *Pleione maculata*; A: Seed pod of *P. maculata*; B: Seed of *P. maculata* C: PLBs of *P. maculata*; D & E : PLBs growing into plantlets of *P. maculata*; F & G: Regenerated plantlets of *P. maculata*.

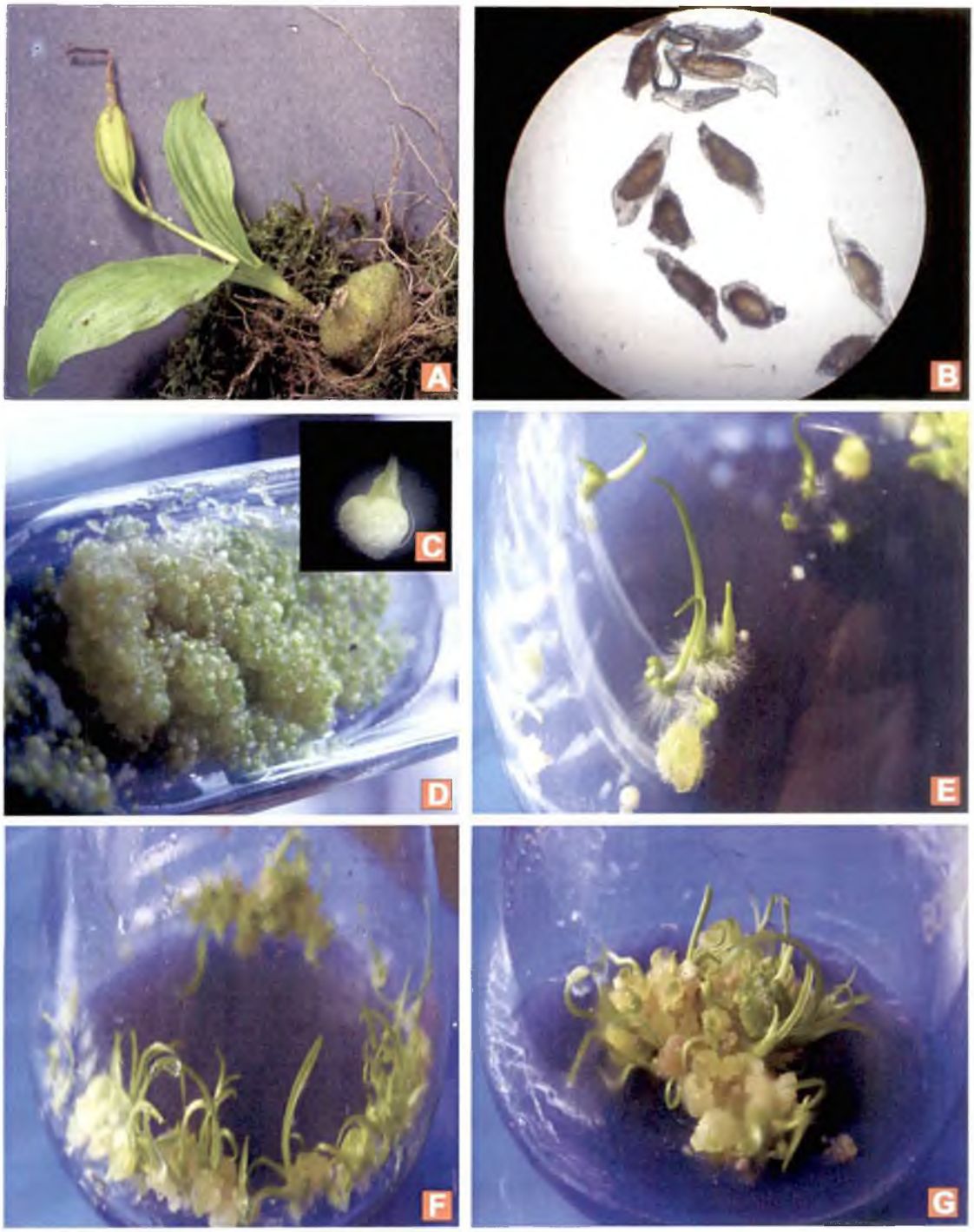

Fig .25: Different stages of seed culture and micropropagation of *Pleione praecox*. A: Seed pod of *P. praecox*; B: Seeds of *P. praecox*;C & D: PLBs of *P. praecox* ;E & F: PLBs with rhizoids and shoot of *P. praecox*; G: Regenerated plantlets of *P. praecox*.

reveal that 98% plantlets could be produced. Healthy plantlets were then transferred carefully to the glass house for hardening.

Table 44: Percent seed germination of *P. precox*

Basal medium	BAP mg/L	Percent germination
OMM+20% CW + 2mg/L NAA	0.5	70%
OMM+20% CW +2mg/L NAA	1.0	78%
OMM+20% CW +2mg/L NAA	2.0	89%
OMM+20% CW +2mg/L NAA	3.0	89%
OMM+20% CW +2mg/L NAA	4.0	96%

OMM=Orchid maintenance medium;NAA=α-Naphthalene acetic acid; CW= coconut water; BAP= 6 Benzyl animo purine

Table 45: Percent regeneration of micropropagated plantlets of *P. precox*

Basal media	BAP mg/L	Percent germination
OMM (half strength)	0.0	84%
OMM (half strength)	1.0	85%
OMM (half strength)	3.0	90%
OMM (half strength)	5.0	98%

OMM=Orchid maintenance medium; BAP= 6 Benzyl animo purine

4.2.6 Studies on *Ryncostylis retusa* Bl.

The seed pod of *R. retusa* was collected from the forest area of Pakyong. After surface sterilization the seed pod was cut aseptically and the seeds (Fig. 26-A) were inoculated in three different media, Orchimax, Knudson 'C', and Vacin & Went. The media was added with the growth regulators 10-20% coconut water and three different concentrations of NAA. The seeds started swelling after 21 days in Orchimax media, after 49 days turned into globular PLBs (Fig. 26-B) and after 126 days the seeds germinated into

plantlet (Fig. 26-C&D). Orchimax media combined with 2mg/L NAA and 20% coconut water was found to be the best for seed germination *in vitro*. Results of the growth and plantlet formation have been described in Table-46. It was evident that Orchimax medium was best among the three different media tested. The healthy plantlets (Fig.26- E&F) when attained the height of 5.5 cm were taken for hardening.

Table 46: No. of days taken for swelling of embryo, sperule stage, PLB and plantlet formation and percent of germination of *R. retusa* in Orchimax, Knudson "C" and Vacin & Went medium.

Media	Growth regulators	Number of days for the formation of				Percent germination
		Swelling of embryo	Spherule stage	PLB stage	Plantlets	
Orchimax	0.5mg/L NAA +10%CW	28	63	84	229	50%
	0.5mg/L NAA +20%CW	28	56	77	217	50%
	1.0 mg/L NAA +10%CW	28	60	84	203	59%
	1.0mg/L NAA +20%CW	28	56	82	210	60%
	2.0mg/L NAA +10%CW	28	63	79	196	78%
	2.0mg/L NAA +20%CW	21	35	49	126	92%
Knudson "C"	0.5mg/L NAA +10%CW	63	87	94	269	55%
	0.5mg/L NAA +20%CW	63	77	98	252	55%
	1.0 mg/L NAA +10%CW	49	77	91	245	59%
	1.0mg/L NAA +20%CW	49	70	84	245	61%
	2.0mg/L NAA +10%CW	49	70	77	154	80%
	2.0mg/L NAA +20%CW	49	63	66	140	82%
Vacin & Went	0.5mg/L NAA +10%CW	No sign of germination				
	0.5mg/L NAA +20%CW	No sign of germination				
	1.0 mg/L NAA +10%CW	63	84	No growth	No growth	0%
	1.0mg/L NAA +20%CW	63	84	No growth	No growth	0%
	2.0mg/L NAA +10%CW	49	91	126	No growth	0%
	2.0mg/L NAA +20%CW	42	91	119	280	25%

NAA =α-Naphthalene acetic acid; CW= coconut water;

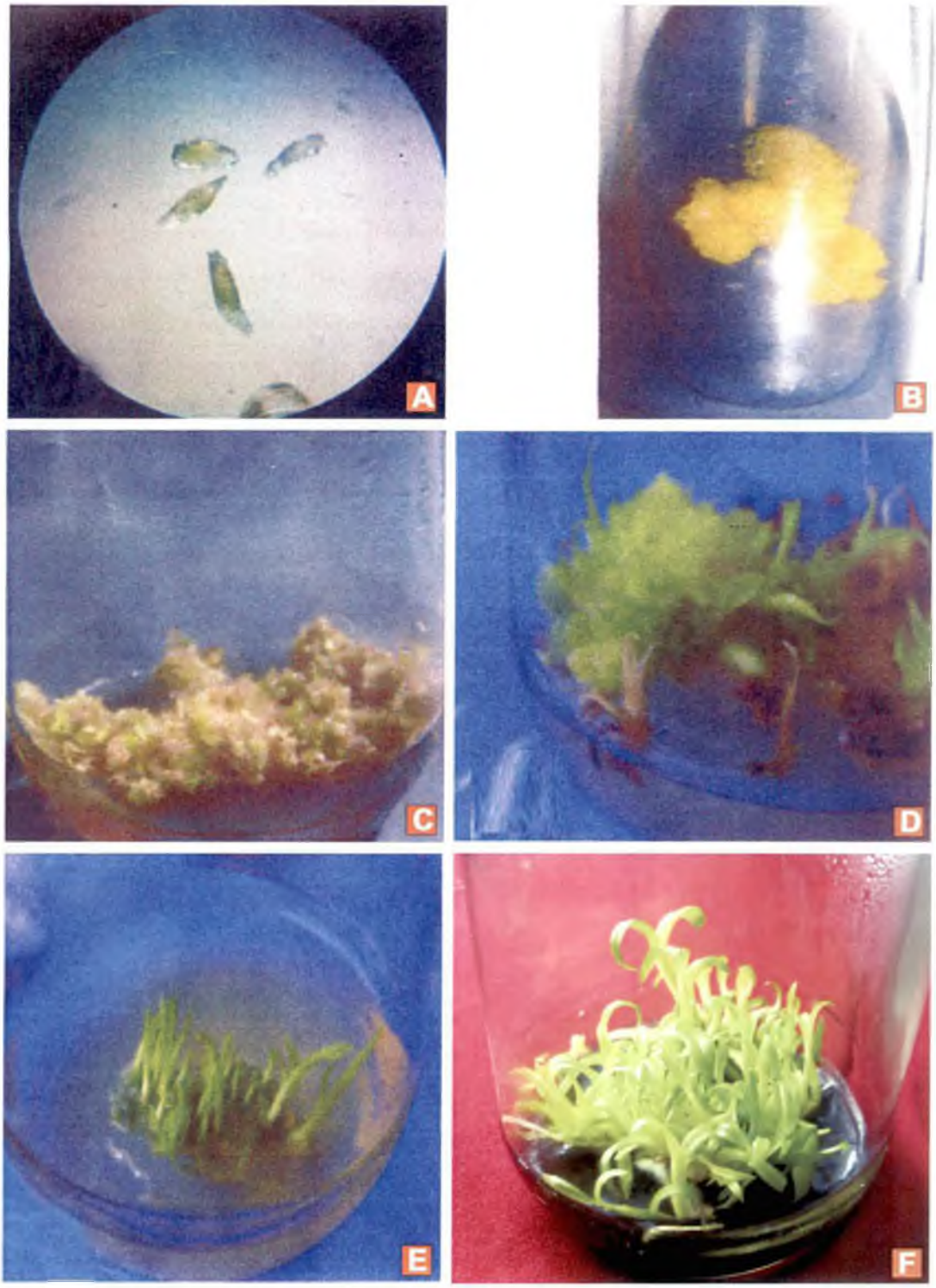

Fig. 26: Different stages of seed culture and micropropagation of *Rhynchostylis retusa*; A: Seeds of *R.retusa*; B: PLBs of *R.retusa*; C & D: PLBs producing plantlets of *R.retusa*; E & F: Regenerated plantlets of *R.retusa*.

4.2.7 Studies on *Thunia alba* Reichebach fil.

The seed pod (Fig. 27-A) of the plant was collected from the forest of Pakyong, Sikkim. After surface sterilization the seeds (Fig. 27-B) were inoculated in Murashige & Skoog media supplemented with the growth regulators and three different percentages (10%, 15%, 20%) of coconut water and three different concentrations of NAA. The seeds started swelling after 35 days in the media and the germinated seeds turned into globular PLBs (Fig. 27-C) after 48 days. Plantlets from PLBs (Fig. 27-D) formed after 122 days. Murashige and Skoog in combination with 5mg/L NAA and 20% coconut water were proved to be the best combination for germination. Results of the experiment have been presented in Table-47.

The healthy plantlets were used as explants for micropropagation which was performed in Murashige & Skoog (half strength) medium combined with 1mg/L NAA, different concentrations of BAP and coconut water. Plantlets were found to grow up to a height of 6.5 cm. The best combination of supplements was recorded to be 1mg/L NAA plus 5mg/L BAP plus 15% coconut water. The percent of germination and plantlet development have been described in Table-48. Healthy plantlets (Fig. 27-E&F) were used for hardening in nature.

Table 47: Percent germination in Murashige & Skoog media of *T.alba* seeds

Basal media	NAA mg/L	Percent germination
Murashige&Skoog + 10% CW	1.0	65%
	3.0	65%
	5.0	67%
Murashige&Skoog + 15% CW	1.0	81%
	3.0	81%
	5.0	85%
Murashige&Skoog + 20% CW	1.0	92%
	3.0	92%
	5.0	98%

NAA =α-Naphthalene acetic acid; CW= coconut water;

Table 48: Showing the percentage of germination and remarks of *T. alba* regenerated plantlets

Basal media	Percent of germination	Observation/ Comments
MS(half strength)+ 1mg/L NAA +2mg/L BAP+ 15% CW	65%	Regenerated plantlets could not survive after 40 weeks of hardening.
MS(half strength)+ 1mg/L NAA +3mg/L BAP +7.5% CW	58%	Plantlets survived only up to 42 weeks after hardening.
MS(half strength)+ 1mg/L NAA +3mg/L BAP +15% CW	79%	Regenerated plantlets are of short height and thin.
MS(half strength)+ 1mg/L NAA +4mg/L BAP +7.5% CW	79%	Plantlets are shorter but healthy.
MS(half strength)+ 1mg/L NAA +4mg/L BAP +15% CW	85%	Plantlet regeneration is very healthy.
MS(half strength)+ 1mg/L NAA +5mg/L BAP +7.5% CW	89%	Regenerated plantlets survived very well after hardening.
MS(half strength)+ 1mg/L NAA +5mg/L BAP +15% CW	95%	

MS= Murashige&Skoog media; BAP=6 Benzyl amino purine; NAA =α-Naphthalene acetic acid; CW= coconut water;

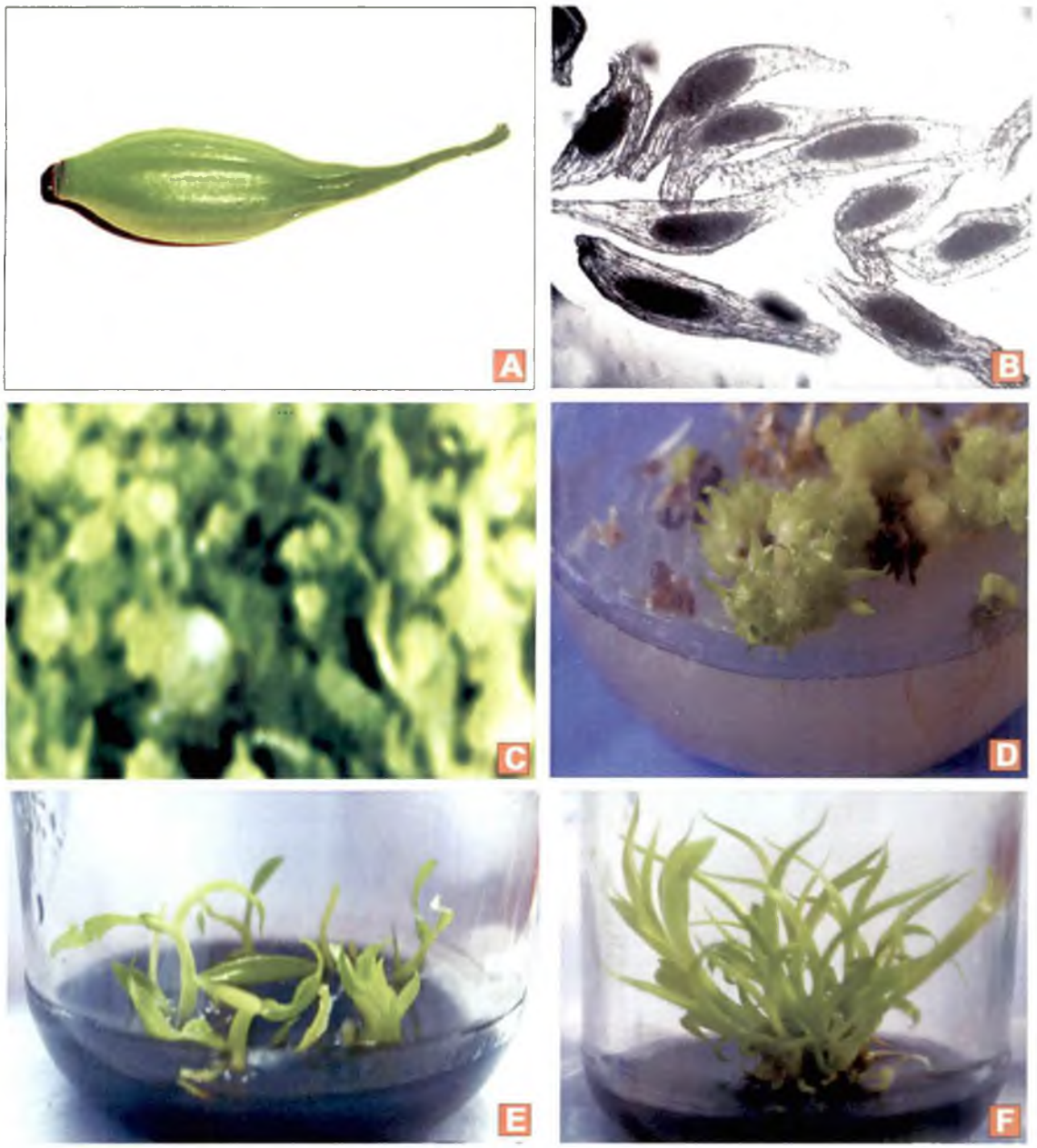

Fig. 27: Different stages of seed culture and micropropagation of *Thunia alba*; A: Seed pod of *T.alba*; B: Seeds of *T.alba*; C: PLBs of *T.alba*; D: PLBs producing plantlets of *T.alba*; E & F: Regenerated plantlets of *T.alba*.

Discussion

5. DISCUSSION

Each fruit of an orchid plant develops several thousand tiny seeds which contain morphologically undifferentiated embryos. These embryos are the spherical mass of tissue lacking both radical and plumule. There is no storage tissue in the seeds and seed coat is reduced to a membranous structure. For this reason, the entire seed of orchid containing undifferentiated embryo which if cultured is treated as embryo culture. *In vitro* culture of orchid seeds is routinely employed for orchid propagation (De, 1992). In the present study several orchids of the Darjeeling and Sikkim Himalayas were subjected to seed culture in artificial media for standardization of media. The plantlets developed through seed culture (or embryo culture) were subjected to micropropagation for rapid multiplication.

Micropropagation is a technique, helpful for commercialization of orchids. Micropropagation when done from a plantlet, developed through any technique of plant tissue culture, is called clonal micropropagation. Clonal micropropagation is very popular in commercial horticultural plants and orchids. The major advantages of micropropagation are i) propagation can be carried out under disease free conditions throughout the year, ii) sub culturing of *in vitro* plantlets can be done after a defined intervals and each time one plantlet can give at least 3 to 4 nodal cuttings. In the present study several media with different concentrations of additives and supplements were tested for development of orchid plants of Darjeeling and Sikkim Himalayas. The best media with optimum additives and supplements for each of the 20 orchid species were recorded. The 20 orchids include six species of *Coelogyne*, seven species of *Dendrobium*, three species of *Pleione*, one species each of *Bulbophyllum*, *Cymbidium*, *Thunia*, and *Rhynchostylis*.

The present investigation deals with initiation of germination and developmental biology from some medicinal and horticultural important orchids. The seeds are unique and poorly developed even on maturity. They lack endosperm and require a suitable fungal association for the natural germination and the fungus is believed to enhance the carbohydrate, vitamin and auxin transport in orchids (Arditti *et al.* 1982). As seeds of the twenty selected orchids are minute, fragile, undifferentiated and possess inherent problems in natural germination it was considered worthwhile to study *in vitro*

germination of immature seeds from green capsule in different media. In nature only 0.2% to 0.3% of the seeds germinate (Singh, 1992).

A systematic investigation was undertaken for initiation of germination and growth of immature seeds in at least three different modified media out of the five different media tested in the present study. The five different media were Murashige & Skoog, Knudson "C", Orchimax, Orchid maintenance medium and Vacin & Went medium. Although the incidence of callus in seed culture were observed by several workers (Curtis 1947; Curtis and Nichols 1948; Rao 1963; Rao and Avadhani 1964; Mitra *et al.*, 1976; Vij *et al.*, 1981), practically no such detailed investigations have been carried out on the differentiation of plantlets from such callus.

In the study of *Bulbophyllum ornatissimum* it was evident from the results that the earliest seed germination observed in charcoal added orchimax medium. In each type of basal medium 20% coconut water and 1gm/L yeast extract was used along with four different concentrations of NAA. Among the four concentrations of NAA supplemented in three different media, 3mg/L concentration was found to be best. It took 35 days for the seeds to swell in case of charcoal added orchimax medium. The sign of germination was observed after 50 days in charcoal free Orchimax media. The seeds in charcoal added medium formed PLBs after 51 days and after 70 days plantlets were formed. When the seeds were allowed to germinate in Knudson "C" media the seeds swelled up after 45 days then turned to PLBs after 53 days and formed plantlets after 71 days.

However, in Vacin & Went media swelling of seeds observed after 65 days and after 80 days they formed the PLBs but further they could not survive. It was clearly observed that Orchimax media with the presence of charcoal produced healthy plantlets in a very short time period. One possible explanation of the effects of charcoal on shoot proliferation was that it improved aeration (Ernst 1974). A second possibility was that the charcoal adsorbed ethylene (Ernst 1975), which could inhibit growth and proliferation.

Further, in half strength Orchimax medium micropropagation was carried out in presence of 1mg/L NAA, BAP (in different concentrations) and coconut water (two different concentrations). It was found that 15% coconut water and 5mg/L BAP regenerated best healthy plantlets. The occurrence of germination in presence of charcoal

which binds toxic polyphenolics was suggested by several authors (Van Waes and Debergh 1986a; Waterman and Mole 1994; Miyoshi and Mii 1995).

Six *Coelogyne* species were considered for *in vitro* propagation in four different media i.e., Orchimax, Murashige & Skoog, Knudson"C" and Vacin & Went. Several workers have tried various growth regulators and various concentrations of growth hormones used in an attempt to promote seed germination and seedling growth (Arditti, 1979). Similar observation was found in the present study. The Orchimax medium produced 95% to 99% of healthy plantlets in each species of *Coelogyne*. In the similar condition, seed germination of *Coelogyne corymbosa* and *Coelogyne elata* produced 90% and 86% plantlets in Murashige & Skoog. Only 80% plantlets of *Coelogyne elata* and *Coelogyne flavida* were produced in Knudson"C" medium. *In vitro* germination was fairly successful in the present study in comparison to the other workers. Several authors have suggested different nutrient solution suitable for different stages of growth for various species (Arditti, 1979; Ernst, 1974; Jonn. 1988; Shobhana and Rajeevan, 1993; Nagaraju and Parthasarathi, 1995a; Talukdar, 2001; Temjensangba and Chitta, 2005).

In, Vacin & Went medium *Coelogyne flavida, C. nitida, C. corymbosa and C. cristata* showed 50%, 45%, 40% and 10% germinations respectively. Among the four media chosen in present investigation for *Coelogyne* species, Orchimax was the best medium and Vacin & Went was not very much successful. In the present study, Murashige & Skoog medium produced 86% and 90% germination in case of *Coelogyne elata* and *Coelogyne corymbosa* respectively and at the same time Knudson"C" media produced 60%, 75% and 80% germination in case of *Coelogyne cristata, C. nitida* and *C. elata* respectively. From the results it may be concluded that Orchimax medium was the best for orchids in Darjeeling-Sikkim environmental condition.

Auxin was the first plant growth hormone added to the seed culture. In majority of the cases auxins (mostly NAA and IAA) enhanced the germination and seedling growth (Arditti, 1979). In the present study BAP and NAA stimulated shoot and root growth in *Coelogyne* species as also reported in *Epidendrum nocturunum* (Yates and Curtis, 1949). Concentrations of BAP above 2 mg/L were found to inhibit protocorm multiplication but increases rapid shoot growth (Hasegawa *et al.*, 1965; Shimasaki & Uemoto, 1990; Chang & Chang, 2000). In comparison to previous studies, the micropropagated plantlets were very vigorous and survive well when they were

germinated in combination with 20% coconut water, 5mg/L BAP and 1mg/L NAA. In contrast to previous studies (Harvais 1972, 1973, 1982; Clements 1982; Arditti and Ernst 1984; Van Waes and Debergh 1986b; Van Waes 1987; Yam et al. 1990; Chu and Mudge 1994; Rasmussen 1995; Wodrich 1997; Michel 2002), plant growth regulators as well as coconut water promoted germination. It was also found that coconut water could produce healthier plantlets with its increasing concentration up to a certain level.

Cymbidium orchids are commercially significant in cut flower and potted plant industries. *Cymbidium elagans* is an extraordinary species in which inflorescences carries a dense cluster of attractive yellow funnel-shaped blossoms. *In vitro* regeneration of *Cymbidiums* have been reported by Wimber (1963), Morel (1964), Sagawa *et al.* (1966), Ueda and Torikata (1968), Steward and Mapes (1971), Wang (1988), Begum *et al.* (1994), Ichihashi (1997), Chang and Chang (1998), Phukan and Mao (1999), Nayak *et al.* (1997, 2002) and Huan *et al.* (2004).

In vitro regeneration of *Cymbidium elegans* was achieved using axenic seeds germination (Sharma *et al.*, 1991; Sharma and Tandon, 1990). In the present study the seeds germinated forming PLBs within 52 days and plantlets (approximate of 1.2cms high) after 120 days of inoculation in modified basal media Orchimax with 2gm/L yeast extract, 25% coconut water and 2.5 mg/L of NAA. A comparison was observed combining the medium with 10% to 15% coconut water and 0.5mg/L to 1mg/L NAA. Although seeds germinated in all the sets of medium but 99% production was recorded in 25% coconut water and 2.5mg/L NAA. Lower NAA ratios in the culture medium resulted in the formation of many protocorms with the rhizomes but these protocorm shoots never developed to plantlets, but higher concentration of NAA regenerated plantlets. Terrestrial *Cymbidium* seeds formed protocorms after germination which develop into rhizomes *in vitro* (Hagiya & Fujita ,1968; Kako, 1968; Kano K, 1965; Sawa Y, 1969; Sawa Y & Nanba M, 1976; Sawa Y & Torikata H,1976). In support to this concept, it was observed in the present study that the seeds produced rhizomes (Fig. 15-C & D) within 69 days. Russian researchers considered the protocorm of orchids to be a seedling (Batygina and Vasilyeva, 1980, 1983a,b; Batygina and Shevtsovag, 1985; Batygina and Andronovae, 1991; Vakhra-meeva *et al.*, 1991; Vinogradova and Filin, 1993; Kulikov, 1995; Vinogradova, 1996, 1999; Tatarenko, 1996; Batalov, 1998). The uses of cytokinins for regeneration of PLBs and multiplication thereof were reported earlier in orchids (Vij *et al.* 2000).

A synergistic action of cytokinins and auxins was reported in several orchid species viz. *Phalaenopsis* (Tanaka and Sakanishi 1977), *Vanda* hybrid (Mathewa and Rao 1985 a, b), *Vanda criststa* (Vij *et al.* 1994), *Saccolabium papillosum* (Kaur and Vij 2000) *Cymbidium* hybrid (Vij and Aggarwal 2003). After sub culturing the plantlets found to be very strong. These were further micropropagated in half strength Orchimax medium supplemented with 1mg/L NAA, and different concentrations of BAP. However, BAP and NAA were two most commonly used cytokinin and auxin respectively known to be important for shoot induction and elongation of many plant species *in vitro* (George, 1993). Similarly, in the present study, 5mg/L BAP, 1mg/L NAA and 15% CW was best suited for the elongation and regeneration of numerous plantlets.

The genus *Dendrobium* is a highly evolved and diversified group (Puchooa 2004; Xu *et al.* 2006). Among the commercially important orchids, *Dendrobium* accounts for about 80% of the total of micropropagated orchids (Deberg & Zimmerman, 1991). In the present experimental study, seeds of seven *Dendrobium* species germinated in different media i.e., Knudson"C", Orchimax, Murashige & skoog, Orchid maintenance medium and Vacin & Went medium. *Dendrobium* plants were used for the production of orchid flowers (Ketsa *et al.* 1995) and in traditional Chinese medicine (Fan *et al.*, 2001). *Dendrobium* is micropropagated in tissue culture by protocorm-like bodies (PLBs) but the growth is very slow (Hawkes, 1970).

However, in the present study, seed germination of seven species was of rapid nature. The swelling of seeds started between 49-52 days and that formed PLBs after 59-69 days. The PLBs, then, turned into a plantlet (of around 1.2cm high) after 121-143 days. The best medium confirmed in case of seven species of *Dendrobium* was Knudson"C" where the percent germination was 98% in *Dendrobium chrysanthum, D. crepidatum* and *D fimbriatum.* Murashige & Skoog was the second type of medium [redacted]sen in the present study. Different species showed different percent of seed [redacted]nation (75% of *Dendrobium crepidatum*, 69% of *D. chrysanthum*, 63% of *D.* [redacted] and 62% of *D. fimbriatum*) in MS. In order to stimulate efficient [redacted]pagation of PLB, much effort has been directed to modify the culture media, [redacted]y.inclusion of plant growth regulators (Prakash *et al.*, 1996; Nayak *et al.* 2002) [redacted]6-benzylamino purine and naphthalene acetic acid (Roy & Banerjee, 2003). In [redacted]sent investigation on the whole, 0.5-5mg/L concentration of NAA, 1mg to 3mg/L

BAP and 15% to 20% coconut water was used as growth regulators. It was evident from the results that higher percentage of coconut water produced PLBs in a short period of time. The same action of coconut water enhancing vigorous growth of *Dendrobium* was found in another orchid (De *et al.*, 2006).

As huge planting materials are required for commercial production, plantlets were further micropropagated in a half strength media best suited for micropropagation. Generally the medium which showed highest germination of seeds in seed culture of a species was selected for the purpose. Further study of micropropagation was carried out in media supplemented with different concentrations of BAP and NAA. It was clear from the observations of the results that 5mg/L was the highest concentration of BAP which produced the plantlets with high vitality and existence potential for hardening. These results suggest a critical role of BAP in the formation of plantlets in *Dendrobium* species. Similar efficacy of BAP alone in PLB formation and plantlet development was also shown in *Dendrobium* hybrid "Sonia" and *D. nobile* (Nayak *et al.*, 2002). In the present study BAP promoted the development of shoots from meristem up to 88% in *D. longicornu* and 89% in *D. chrysanthum* and at the same time from the nodes up to 65% in *D. chrysanthum* and 69% in *D. longicornu*, similar findings was also reported by Kukulczanca (1983) in shoot development study from axillary buds in *D. antennathum*.

Pleione is one of the genuses with beautiful flowers with high demand for hybridization. *P. humilis*, *P. maculata* and *P. praecox* were the three species considered in the present study for *in vitro* germination. In case of *P. humilis* 5mg/L NAA, 20% coconut water and 4mg/L BAP was found to be best among the different combinations of growth regulators and additives supplemented in the OMM medium. In case of *P. maculata*, Orchid maintenance medium was found best (98% plantlets generated) in combination with 20% CW, 2mg/L NAA and 4mg/L BAP in comparison to other two media (Murachige & Skoog and Vacin & Went). Addition of growth regulators, coconut water, NAA and BAP enhanced the germination of the seeds and it was observed that higher concentration of BAP raised the production up to 98%. Jonojit & Nirmalya (2001) also showed larger production of micropropagated plantlets in increased BAP concentrations in the medium. They also observed that addition of higher concentrations of BAP in the medium stimulated protocorm development and shoot bud initiation in *Geodorum densiflorum*.

It was also evident that addition of coconut water was conducive for the seed germination of three species of *Pleione* and this observation was supported by the fact that coconut water is generally added in orchid seed culture as a source of sugar, natural cytokinins and vitamins (Mathews and Rao, 1980). Orchid maintenance medium used in the present study contained charcoal and the medium produced very healthy plantlets. This was supported by the fact that addition of charcoal in the medium of some orchid culture was very effective for growth and development (Heberele-Bors, 1980). Possible account to use activated charcoal was that it absorbs many inhibitory fatty acids present in the agar solidified medium and a phenolic substance secreted by the explants as well as it promotes the growth of the plantlets.

Ryncostylis retusa, although collected from the forest area of Pakyong, it is a state flower of Arunachal Pradesh. The present investigation deals with the seed germination and growth of *R. retusa* plantlets *in vitro* in three different media and different concentrations of NAA. This material revealed optimal growth of 92% in orchimax medium within 126 days. In case of Knudson"C medium 82% of seed germinated and in Vacin & Went medium the percent germination was 25%. The growth regulators added were 10% to 20% coconut water and 0.5 mg/L to 2mg/L NAA. The highest percent of germination was obtained when combined with 20% coconut water and 2mg/L NAA. The percent germination of seed varied in different media although all the basal media supplemented with the same growth regulators. The findings indicated that the differential growth of a species was evident in different media. Such differential response of a species to various media was also reported by several workers (Baker *et al.* 1987; Roy *et al.* 1993; Hoque *et al.* 1994; Oddie *et al.* 1994; Gupta and Bhadra, 1998 and Barua and Bhadra, 1999).

Thunia alba seeds were the last chosen experimental material in the present investigation. For this species Murashige and Skoog medium was selected in combination with 1mg/L - 5mg/L NAA and 10% - 20% coconut water. It was observed that coconut water helped in growth of seed germination. From the results, it was also evident that increase in coconut water and NAA produced maximum number of plantlets with strong existence in the nature after hardening and the best combination was considered 5mg/L NAA and 20% coconut water. Inhibitory effects of NAA on seed germination were reported in few orchids (Hadley and Harvais, 1968; Hadley, 1970). Experiments with auxin either individually or in combination with cytokinin on orchid

seed germination and seedling growth have given inconsistent and inconclusive results (Arditti, 1979).

However, findings of the present investigation in *T. alba* did not match the earlier statement. In the present study it was observed that the plant growth regulators markedly influenced seed germination and subsequent stages of growth and regeneration of plantlets. Earlier reports suggested that auxin promoted seed germination in many orchids (Arditti 1967; Blowers 1958; Ernst 1967b; Kano 1968; Mathews and Rao 1980; Straus and Reisinger 1976). Murashige & Skoog (half strength) medium combined with 15% coconut water, 1mg/L NAA and 5mg/L BAP was a combined medium for the micropropagation of *in vitro* germinated plantlets of *T. alba*. The decrease of NAA was practiced to gain the gradual independence of the plantlets from growth regulators before hardening. In the present study seed-raised plants were used for conservation programs such as re-introduction because each seedling is genetically unique, greater genetic diversity in a population allows more flexibility, enabling it to adapt to changing environmental conditions. For *ex situ* conservation, seed raised plants are of course desirable to conserve a greater range of genetic diversity. Conversely, vegetative micropropagation in which small pieces of plants were used provided uniform plants that were genetically identical. Such plants are useful for scientific research including molecular studies and to preserve desirable trades. The horticultural trade may use this method to multiply new introductions quickly.

Seed maturity also influenced success of *in vitro* germination. Immature seed ('green-podding') was widely used for germinating terrestrial orchids (Stoutamire 1964, 1974; Borris 1969; Linde'n 1980, 1992; Vogelpoel 1980, 1987; Arditti 1984; Clements 1982; Fast 1982; Oliva and Arditti 1984; Pritchard 1989; Anderson1990, 1991; Rasmussen et al. 1991; Rasmussen and Rasmussen 1991; Zettler and McInnis 1992; Rasmussen 1995; LaCroix and LaCroix 1997; Wodrich 1997; Michel 2002) However, the appropriate developmental stage for excision must be assessed for each species. Michel (2002) reported optimal harvest time (in the range 21–360 days after pollination) of different terrestrial tropical orchid species.

An interesting phenomenon has also been noted in the developmental stages of plants from embryo under *in vitro* conditions. It was observed that the germinating entities such as spherule stage of most of the genus profusely developed epidermal hairs

and penetrated into the medium. It indicated their absorptive function. A similar absorptive function was also noted in the protocorm stage. Rhizoids were produced from the basal part of the protocorm while a leafy shoot was formed the growing point of the upper surface of the protocorm. The absorptive potential of the protocorm appeared to vary with the chemical stimulus in the nutrient medium. The use of BAP and/or NAA in the nutrient mixture was often inhibitory in absorbing hair development (Dhiman, 1999). The development of the lower half of the protocorm into fleshy rhizomatous structure and the upper half into an embryonal shoot was to support the nutritional function of the protocorm (Salwan, 1992). Therefore, greening of embryo and the formation of green protocorm with profuse rhizoids under *in vitro* condition made them heterotrophic rather than autotrophic. When the roots came out between the junction of the seedling and protocorm, then gradually they became autotrophic, transformation of seedlings from heterotrophic to autotrophic mode during early stage of development was an unusual differentiation in case of orchids as they gradually became an autotrophic, the nutritional requirement could be reduced for their further growth and development and they were able to grow in the low profile of nutrient indicating their independent autotrophic nature. The development of chlorophyll at the pre protocorm stage was almost universal in orchids which appeared to be related to genetic or ecological factors.

The present study was initiated based on the need for providing simple protocols for large scale regeneration, establishment and conservation of Darjeeling and Sikkim Himalayan orchids through asymbiotic seed germination. Although during many decades tissue culture techniques was widely exploited for micropropagation of orchids (Prakash *et al.* 1996; Perk *et al.* 1996) and also a number of literatures was accumulated, a number of possibilities were also on the way to obtain for optimum germination, better growth, development and regeneration of the orchid plantlets *in vitro*. The results of the present study were encouraging since several plants could be regenerated in comparatively simpler media. In the course of this investigation certain new facts of fundamental importance have been revealed. All other results are almost in conformity with those obtained by previous workers. Thus in general this work have confirmed and extended some of the findings of the earlier workers. It dealt with collection and identification of 20 orchids of Darjeeling and Sikkim Himalayas. The most potential media for each of the 20 orchids were optimized. Micropropagation media of the plantlets were also optimized. All the information of the present study may be integrated in the commercial

tissue culture techniques being followed in different tissue culture farms. The relatively simpler cost effective media may boost the orchid tissue culture industries of Sikkim and Darjeeling Himalayas. The extended knowledge of the present study may be helpful to the orchid scientists and growers of different parts of the world.

Summary

6. SUMMARY

1. The present study deals with "Micropropagation of some medicinal and horticultural orchids of Darjeeling and Sikkim Himalayas."
2. An introduction has been presented to the work of tissue culture with special reference to orchids of Darjeeling and Sikkim.
3. A brief review of literature related to tissue culture has been presented. This section deals with observations of the previous workers in concord with the present line of investigation. The review is in selective manner rather than comprehensive one. This study involves investigations to find out the best media with optimum additives and supplements for orchid species.
4. After the survey of the orchids in the present study area twenty plants were selected for seed culture and also for micropropagation. The 20 orchids include six species of *Coelogyne*, seven species of *Dendrobium*, three species of *Pleione*, one species each of *Bulbophyllum*, *Cymbidium*, *Rhynchostylis* and *Thunia*. The objectives of the present study were set forth as: (i) Production of plants in large scale through *in vitro* seed culture and (ii) Micropropagation of the plants developed through *in vitro* seed culture.
5. A detailed description of different experimental procedures and techniques used during the present study has been given in different sections of materials and methods.
6. The present investigation deals with initiation of germination and developmental biology from some medicinal and horticultural important orchids. The seeds were unique and poorly developed even on maturity. As seeds of the twenty selected orchids are minute, fragile, undifferentiated and possess inherent problems in natural germination it was considered worthwhile to study *in vitro* germination of immature seeds from green capsule in different media.
7. A systematic investigation was undertaken for initiation of germination and growth of immature seeds in at least three different modified media out of the five different media tested in the present study. The five different media were Murashige & Skoog, Knudson "C", Orchimax, Orchid maintenance medium and Vacin & Went medium.
8. Earliest seed germination of *Bulbophyllum ornatissimum* was observed in charcoal added orchimax medium. Presence of charcoal significantly produced healthy plantlets. In micropropagation studies half strength Orchimax in presence of 1mg/L NAA, 5mg/L BAP and 15% coconut water regenerated best healthy plantlets.

9. Six *Coelogyne* species were considered for *in vitro* propagation in three different media i.e., Orchimax, Murashige & Skoog, Knudson"C" and Vacin & Went. Orchimax medium produced 95% to 99% of healthy plantlets of each species of *Coelogyne*. In the similar condition other two media showed much lower plantlet formation.
10. In the present study BAP and NAA stimulated shoot and root growth in *Coelogyne* species. In comparison to previous studies, the micropropagated plantlets were very vigorous and survived well when they were germinated in combination with 20% coconut water, 5mg/L BAP and 1mg/L NAA.
11. In the present study the seeds of *Cymbidium elegans* germinated forming PLBs within 52 days and plantlets (approximate of 1.2cms high) after 120 days of inoculation in modified basal media Orchimax with 2gm/L yeast extract, 25% coconut water and 2.5 mg/L of NAA. A comparison was observed combining the medium with 10% to 15% coconut water and 0.5mg/L to 1mg/L NAA. Although seeds germinated in all the sets of medium but 99% production was recorded in 25% coconut water and 2.5mg/L NAA. These were further micropropagated in half strength Orchimax medium supplemented with 1mg/L NAA, and different concentrations of BAP.
12. Seed germination of seven species of *Dendrobium* was performed rapid nature in Knudson"C". The swelling of seeds started between 49-52 days and that formed PLBs after 59-69 days. The PLBs, then, turned into a plantlet (of around 1.2cm high) after 121-143 days. The best medium confirmed in case of seven species of *Dendrobium* was Knudson"C". The percent germination was 98% in *Dendrobium chrysanthum, D. crepidatum* and *D fimbriatum.* Murashige & Skoog was the second type of medium which showed much lower percent of seed germination. It was evident from the results that higher percentage of coconut water produced PLBs in a short period of time. A half strength medium of the same medium which showed highest germination of seeds in seed culture was used for the purpose of micropropagation. Further study was carried out in media supplemented with different concentrations of BAP and NAA. It was clear from the observations of the results that 5mg/L was the highest concentration of BAP which produced the plantlets with high vitality and existence potential for hardening.
13. *P. humilis, P. maculata* and *P. praecox* were the three *Pleione* species considered in the present study for *in vitro* germination. In case of *P. humilis* 5mg/L NAA, 20% coconut water and 4mg/L BAP was found to be best among the different combinations of growth regulators and additives supplemented in the OMM medium. In case of P.

maculate, 'Orchid maintenance medium' was found best (98% plantlet generated) in combination with 20% CW, 2mg/L NAA and 4mg/L BAP. It was also evident that addition of coconut water was conducive for the seed germination of three species of *Pleione*.

14. *Rhynhcostylis retusa*, although collected from the forest area of Pakyong, it is a state flower of Arunachal Pradesh. The present investigation deals with the seed germination and growth of *R. retusa* plantlets ***in vitro*** in three different media and different concentrations of NAA. This material revealed optimal growth of 92% in orchimax medium within 126 days. In case of Knudson"C medium 82% of seed germinated and in Vacin & Went medium the percent germination was 25%. The growth regulators added were 10% to 20% coconut water and 0.5 mg/L to 2mg/L NAA. The highest percent of germination was obtained when combined with 20% coconut water and 2mg/L NAA. The percent germination of seed varied in different media although all the basal media supplemented with the same growth regulators. The findings indicated that the differential growth of a species was evident in different media. Such differential response of a species to various media was also reported by several workers.

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Erratum

Heading	Page No.	Line No	Word	Correct form
Abbreviations	-	17	moleculat	molecular
Results	36	18	cylindric	cylindrical
	39	14	elevations	elevation
	42	25	fibres	fibers
	61	11	animo	amino
	63	15,21		
	65	21,29		
	66	26		
	67	13		
	68	24		
	69	8		
	70	10,18		
	72	17,28		
	73	20		
	74	28		
	75	13		
	76	20		
	77	12,20		

Additional parts of Contents to be included

Kalyan Kumar De

Additional portion of discussion to be considered with original discussion

Modern orchid micropropagation began in 1949, when tissue culture or *in vitro* method for clonal propagation of *Phalaenopsis* was developed at Cornell University (Rotor, 1949). Since orchid seeds are unique due to the presence of an unorganized embryo and the absence of a functional endosperm, usually asymbiotic methods are used for easy germination (Ernst, 1975; Yam & Weatherhead, 1988; Hoshi *et al.*, 1994). In many terrestrial orchids like *Cypripedium reginae* (Harvis, 1982), *Cypripedium candidum* (De Pauw *et al.*, 1995) and *Cypripedium calceolus* (van Waes & Debergh, 1986) *in vitro* seed culture has been reported.

The nutrient medium used for these cultures was 'Knudson C' formulated for the asymbiotic germination of orchid seeds by Lewis Knudson (1884–1958) Professor of Plant Physiology at Cornell University. There are many reports of species-specific media utilized for germination of orchid seeds (Arditti and Ernst, 1984). Among the four media chosen in present investigation for *Coelogyne* species, Orchimax was the best medium and Vacin & Went was not very much successful.

Orchid seed germination and micropropagation media are often darkened with activated charcoal because the plants grow better in the presence of this additive. John T. Curtis was the first to darken a nutrient medium for orchid seedlings *in vitro*. He did it in an unsuccessful effort to simulate natural conditions and thereby bring about the germination of *Cypripedium reginae, C. pubescens, C. parviflorum, C. candidum* and *C. acaule* seeds (Curtis, 1943). Later on, a number of investigations favoured charcoal in asymbiotic medium (Ernst, 1974; Yam et al.,1989). The beneficial effects of charcoal in culture media are: i) adsorption of unidentified morphogeneticaly active or toxic substances (Klein and Bopp, 1971); ii) adsorption of 5-hydroxymethylfurfural which was produced by

the dehydration of sucrose during autoclaving (Weatherhead et al., 1978); iii) adsorption of inhibitory phenolics and carboxylic compounds produced by the tissues (Fridborg et al., 1978); iv) adsorption of excessive hormones and vitamins in the media (Fridborg and Eriksson, 1975); and v) create dark condition that favoured rooting (Bhadra and Hossain, 2003).

The positive effect of activated charcoal can be considered in at least two aspects, reduction of light at the base of the shoots, thus providing an environment conductive to the accumulation of auxin or cofactors (Druart and Wulf 1993) and absorption of inhibitory substances such as polyphenols from the media (Fridborg and Eriksson 1975). Activated charcoal has not only good adsorption properties but also creates partial darkness. Probably the activated charcoal-fortified media enhanced induction of roots. Such stimulation by activated charcoal in the medium was also reported by Nagaraju et al. (2001). In the present study most of the media contained activated charcoal which released nutrients slowly in the media. Thus response of charcoal was positive in the present study like the previous workers.

Organic compounds in the form of carbohydrates, vitamins, amino acids and growth regulators may be incorporated into media directly, or through undefined additives such as coconut milk, banana homogenate or potato extract (Arditti 1982; Arditti and Ernst 1984; Chu and Mudge 1994) resulting in increasingly complex media in which no single factor can be identified as the germination-stimulating agent. The use of coconut water in tissue culture media has been widely reported, especially in the micropropagation of orchids (Arditti and Ernst, 1993). It was evident from the results of present study that higher percentage of coconut water produced PLBs in a short period of time in case of *Dendrobium* spp. It was also evident in the present study that addition of coconut water was

conducive for the seed germination of three species of *Pleione* and this observation was supported by the fact that coconut water is generally added in orchid seed culture as a source of sugar, natural cytokinins and vitamins (Mathews and Rao, 1980).

A synergistic action of cytokinins and auxins is reported in several orchid species e.g. *Phalaenopsis* (Tanaka and Sakanishi 1977), *Vanda* hybrid (Mathewa and Rao 1985 a, b), *Vanda criststa* (Vij et al. 1994), *Saccolabium papillosum* (Kaur and Vij 2000) *Cymbidium* hybrid (Vij and Aggarwal 2003). The liquid endosperm of coconut contains a range of biochemicals, which can act as growth factors synergistically (Shantz and Steward, 1952). One active ingredient that has been isolated is 1,3-diphenylurea, which shows cytokinin-like activity (Shantz and Steward, 1955). However, because coconut endosperm is a natural product, its activity may depend on different factors like age, season, source etc and its activity cannot be guaranteed.

In the present study BAP and NAA has been utilized in different concentrations to analyze the combined effects as well as the production of number of plantlets. Although BAP in combination with NAA had been suggested by some investigations to obtain the maximum number of PLBs and the highest frequency of responsive explants with PLB formation (Puchooa 2004; Kim and Kim 2003), NAA added to the medium containing optimal BAP did not significantly improve response of explants in *D. densiflorum* and even decreased production of PLBs at higher concentration. Individual treatment with BAP and combined treatment with NAA resulted in production of direct development of shoot buds of *Coelogyne* spp. Auxin was the first plant growth hormone added to the seed culture. In majority of the cases auxins (mostly NAA and IAA) enhanced the germination and seedling growth (Arditti, 1979). In the present study BAP and NAA

stimulated shoot and root growth in *Coelogyne* species. The results were in agreement with the results of *Cattleya* (Mauro *et al.,* 1994); shoot tips and flower stalk buds of *Phalaenopsis* and *Doritaenopsis* (Tokuhara and Mii, 1993) and foliar meristems of *Vanda coerulea* (Seeni and Latha, 2000).

Though orchid micropropagation has shown spectacular development in the recent years, the wide spread use of micropropagation is believed to be still limited due to problems like exudation of phenolics from explants, transplantation to field, somaclonal variation etc. Scientists endeavor to include the major investigations on explant-based orchid tissue culture starting from the pioneering works of Rotor (1949) followed by Morel (1960) and Wimber (1963).

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