CHAPTER ONE

INTRODUCTION

1.1 Background

Nepal, a Himalayan country has amazing extremes in ecology and biodiversity. This variation accounts for its high floral diversity. It is estimated that Nepal comprises about 7,000 flowering plants with extreme diversity ranging from low altitude of the tropics to the highest altitude of the alpine zone out of which about 252 species are endemic to Nepal. (Chaudhary, 1998; Hara *et al*, 1978 – 1982; HMGN, 2002; Press *et al*, 2000;).

Orchids belong to the family Orchidaceae. They are nature's most extravagant family of flowering plants and they exhibit incredible range of diversity in size, shape and color of their flower. Orchids get their name from the Greek orchis, meaning "testicle", from the appearance of subterranean tuberoids of the genus Orchis. The word "orchis" was first used by Theophrastus, father of Botany (372/371 – 287/286 BC), in his book "De Historia Plantarum" (The natural history of plants). Orchids, like grasses resembling in the form of their leaves—are monocotyledons. Being cosmopolitan in distribution, orchids occur in every habitat. Orchids, through their interactions with pollinators and their symbiosis with mycorrhizae fungi, are considered to be examples of the most advanced floral evolution.

Well documented reports estimate that there are 19,501 species of orchids belonging to 803 genera (Dressler,1993). So it is the second largest family of the flowering plant found throughout the world except the polar region.

Taxonomically, orchidaceae is the most evolved family among monocotyledons with 3 subfamilies, 6 tribes, 750-1000 genera and 25,000 -35,000 species. The Kew World Checklist of Orchids includes about 24,000 accepted species. About 800 new species are added each year. (www.wikepedia.com). Orchids can be classified as sympodials ("joint-footed") and monopodials ("one-footed") on the basis of habit while as epiphytes, terrestrial and lithophytes on the basis of habitat.

In sympodial orchids, the new shoots get arised from any axillary bud present in any part of older shoots that are spread out from a long rhizome. eg *Bulbophyllum, Cypripedium, Dendrobium* etc while in monopodial orchids the shoots undergo indifinite apical growth but lack rhizome or pseudobulb eg *Aerides, Rhynchostylis, Vanda* etc

Orchids are one of the most beautiful ornamental plants of Nepal. Nepalese orchids are very popular due to their shape, size, habit, habitat, colorful flowers, shining green leaves and variously shaped pseudobulbs, so are highly used for horticultural purpose. Beside the ornamental values of the orchids many orchid species have medicinal and edible values. Nepal is an abode to 377 species of native orchids belonging to 100 genera (Rajbhandary & Dahal, 2004). Out of 377 species of orchids 10 are endemic to Nepal. Orchidaceae has been recorded as the second largest plant family in Nepal in terms of number of species as shown by Rajbhandary (2004). Orchids are famous among Nepalese as "SUNGAVA", “CHANDIGAVA" & "SUNAKHARI".

Orchid diversity is rich in Central and Eastern Nepal from where 278 species and 230 species have been recorded respectively. In Western Nepal, orchid species are sparsely populated and represented by 103 species (Dahal and Shakya, 1989). It may be due to less orchid exploration, low amount of rainfall and abundance of coniferous forest. The distribution of orchids in different bioclimatic zones shows that subtropical and lower temperate zones are richest in orchid diversity. Orchid species of Nepal with spectacular beautiful flowers belong to the genera: *Aerides, Arundina, Ascocentrum, Bulbophyllum, Calanthe, Coelogyne, Cymbidium, Dendrobium, Epigeneium, Eria, Esmeralda, Phaius, Phalaenopsis, Pleione, Rhynchostylis, Thunia, Trudelia, Vanda & Vandopsis.* (Rajbhandari and Bhattarai, 2001).

Species cultivated mainly for their ornamental purpose include: *Aerides multiflora, Ascocentrum ampullaceum, Bulbophyllum leopardinum, Calanthe masuca, C.plantaginea, C.tricarinata, Coelogyne cristata, C. fuscescens, Cymbidium elegans, C. irridioides, Dendrobium densiflorum, D .moschalum, D. nobile, Phaiustancarvilliae,*

Pleione praecox, Rhynchostylis retusa and Vanda tesselata. Some important medicinal orchids as mentioned by Rajbhandari *et al.* (2000) are *Brachycorythis obcordata, Coelogyne flavida, Coelogyne stericta, Cymbidium aloifolium, Dactylorhiza hatagirea, Eulophia nuda, Flickingeria macraie, Pholidota imbricata, Luisia zeylanica and Vanda tessellata, Cypripedium cordygerum, Dactylorhiza hatagirea, Platanthera clavigera and Satyrium nepalensis* are edible orchids.

All orchid species are protected for the purpose of international commerce under Convention of International Trade in Endangered Species of Wild Fauna and flora (CITES) as potentially threatened or endangered in their natural habitat, with most species listed under Appendix II. A number of species and genera are afforded protection under Appendix I.

In spite of all the legal protection afforded to the orchids, due to their high global demand for ornamental and medicinal purposes, they have been highly exploited from their natural habitat.

Because of all the values that orchids have they are highly priced in the international market. Orchids have been well known for their horticultural values. So it is widely propagated for the horticultural purpose. The commercial growers of orchids are U.S.A, U.K, Indonesia, Singapore, Thailand, Norway and New Zealand. Thailand is the largest exporter. In the Year 1998 International trade on orchid amounted to more than 6 billion dollar of which Thailand shares 85 % of the total amount and annual increament rate is 7-10 %. (Swar & Pant, 2004)

Loss of habitat, deforestation, destructive collection technique and over exploitation of orchids with medicinal and ornamental values depleted the world orchid wealth. So effective strategies should be implemented to conserve these precious gems of nature. Propagation of the orchids can be carried out through seeds and vegetative parts. Seed grown plant progeny is not identical to the parent plant because they are heterozygous. Propagation through vegetative parts gives the identical progeny but it is a very slow process requiring ten years to obtain a clone of suitable size. In natural condition the majority of orchid flowers are not pollinated, their ovules are not fertilized and capsules are rarely formed.

The microscopic seeds of orchids are very numerous (over a million per capsule in most species). They blow off after ripening like dust particles or spores, barely visible to the human eye. The embryo has a rounded or spherical form without cotyledons, radicle and endosperm. Since they lack endosperm, they must enter symbiotic relationship with mycorrhizal fungi to germinate. These fungi provide the necessary nutrients to the seeds. All species rely upon mycorrhizal associations with various fungi, mostly genus *Rhizoctonia* (Class Basidiomycetes), for at least part of their life cycle. Of all the seeds released, only 5 % of total seeds grow into new orchids in natural condition (Rao, 1997). This process can take years; in some cases up to fifteen years.

Micropropagation is an *in vitro* propagation by which a large number of clones of the desired plant are produced in an artificial nutrient medium under aseptic condition in short time irrespective of physiological and climatic barriers. The explant may be a very small piece of plants such as embryos, seeds, stem, shoot tip, meristems, root tips, callus, single cell and pollen grains. Through micropropagation, from a very small part of a plant, numerous plantlets can be produced in very short duration. Moreover the plantlets obtained from this technique are disease free. Therefore the horticulturalists are implementing the tissue culture technique for mass propagation of economicaly valuable orchids all over the world. Orchids are the very first flowering plants of commercial value to be propagated *in vitro* both through seeds and tissue culture (Pant, 2006).

Bernard in 1909 for the very first time inoculated orchid seeds and fungi in the culture tubes and protocol for the symbiotic germination was developed. The second attempt was made by Knudson (1922). He developed a protocol for the asymbiotic germination of the orchid seeds in culture tubes eliminating the requirement of fungus for the germination. Since then a vigorous research is being carried out to improve the Knudson medium for better germination rate.

Tissue culture of orchids to obtain virus free *Cymbidiums* by meristem culture was first attempted by Morel (1960, 1962). He produced more than 4,000,000 plants of *Cymbidium in* one year from single shoot apex.

In Nepal, *ex situ* conservation of some of the Nepalese orchids is being carried out by National Herbarium and Plant Laboratory (NHPL), Godawari under Department of Plant Resource of Government of Nepal (DPR), Thapathali and Institutions like Tribhuwan University by tissue culture method. Some private organizations like Nepal Biotech Nursery, Standard Nursery (Kathmandu) are involved in the conservation of the orchids by cultivating them.

1.2 *Coelogyne fuscescens* Lindl.

Vernacular name: Bankera

C. fuscescens Lindl. (Fig 01) is one of the important ornamental species of the genera. It is found in Nepal, India, Sikkim and Bhutan as a medium to large sized, hot to warm growing, creeping perennial herb with erect, clustered, 7-9 cm long fusiform pseudobulbs carrying 2 apical, 22-27 cm long, 7-9cm broad oblanceolate to oblong-elliptic, acute leaves that blooms in the winter on an erect to suberect about 16 cm long, 2 to 7 flowered inflorescence that is subtended by 4 to 5 short sheaths.

Eleven species of *Coelogyne* are recorded from Nepal (Rajbhandari and Dahal, 2004). Flowers, apple green or light yellow or, lip ochraceous in colour, 3.5-5 cm in size, coconut smell in fragrance.

Commonly it is called the 'Ocher Yellow Coelogyne'. Its psedobulb looks like banana so the local people know it with the name of 'Bankera'. The beautiful flowers of the species blooms during October to November and the flowers persist for around 20 days.

In Nepal its distribution is confined in the Central Nepal in the altitutidinal range of 1200-1800m (Press *et al* 2000).

It has both horticultural and medicinal values. Local people of the area from where it was collected use it as a garden flower. Its leaves and pseudobulbs are used in therapeutic purpose. Fresh pseudobulbs is used for headache and fever. Paste of the pseudobulb is used over the burned skin.

1.3 *Cymbidium aloifolium* (L.) Sw.

Epidendrum aloifolium L.

Cymbidium pendulum (Roxb.) Sw.

Cymbidium simulans Rolfe.

Vernacular Name: Banharchuul, Kamaru

It is one of the most important ornamental and medicinal species of the genera (Fig 06). It is epiphytic in nature and it remains attached to the bark of old trees. It blooms in April to June and flower persists for about 20 days. The colour of flower is yellowish with central radial stripes. Thirteen species of *Cymbidium* have been reported from Nepal. It has wide range of distribution from 300m to 2900m (Rajbhandari and Dahal, 2004). The pod of the *Cymbidium aloifolium* (L.) Sw. is large and greenish. It remains hanging arising from a very small pseudobulb enveloped by leaf bases. It is locally named as Kamaru and Banharchhul in Kaski district from where it was collected. It is emetic and purgative. People use its pseudobulb and leaves for various medicinal purposes. Paste of its parts (pseudobulb and leaves) is used as tonic and used over fractured or dislocated bones. The leaves of *Cymbidium aloifolium* (L.) Sw. are used as fodder for cattle. The local people also use it in home gardens for ornamental purposes. (Paudyal & Subedi, 2001).

1.4 OBJECTIVES

The objectives of the present investigation are as follows.

1. To compare and determine the effect of different phytohormones combination on *in vitro* seed germination of *Coelogyne fuscescens* Lindl. and *Cymbidium aloifolium* (L.) Sw.

2. To determine the appropriate culture condition for *in vitro* propagation of *Coelogyne fuscecens* Lindl.

3. To acclimatize the *in vitro* grown plants of *Coelogyne fuscescens* Lindl. for domestication.

1.5 JUSTIFICATION OF THE STUDY.

Nepalese orchids are under considerable threat from continued habitat destruction, degradation and fragmentation, selective logging and timbering, illegal collection and trade and from road construction. Though the collection of orchid has been banned under the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) implementation of regulation is poor and harvesting from wild sources still continues for commercial trade. Annually many orchid plants gets exported to the International market.

Proper sustainable utilization of the Medicinal and Ornamental orchids of Nepal may be helpful in the economic upliftment of the country.

Micropropagation proves itself as an important tool in plant conservation. It is the only method that will not make any direct impact on biodiversity of wild population. Either seeds from wild or a meristem can be tissue cultured to carry out a mass propagation. Since micropropagation is carried out in aseptic condition a large number of pathogen free plants can be produced in very short time.

The species that are selected for the present study has great commercial potential but are commercially threatened (Bailes, 1985). Both the species are highly exploited for medicinal and ornamental purposes. They belong to the genera that are mostly exported to the International market. Their number in natual habitat is declining day by day. So conservation of these species should be carried out in time to save them from being extinct.

So this study is carried out as a small process for *ex situ* conservation of the species and to know the hormone concentration that fits well for the micropropagation of the species.

CHAPTER TWO

LITERATURE REVIEW

Bernard (1909) is the pioneer to develop the protocol of symbiotic (i.e dixenic) seed germination of orchids. The requirement of fungus for the germintation of Orchid seed was discovered and the fungus was found to be *Rhizoctonia* (Basidiomycetes), which remain in the orchid roots establising the symbiotic relationship.

Knudson (1922) became the first to develop a protocol of asymbiotic seed germinaion of orchid. He studied the germination of orchid seeds *in vitro* in the nutrient media containing protein, sugar without the fungal aid. He formulated the suitable medium and successfully germinated the seeds of *Cattaleya*, *Laelia* and *Epidendrum.* The media developed by him is popular now a days with the name of "Knudson C" media.

Ball (1946) for the first time demonstrated the possibility of regenerating whole plants *in vitro* from isolated explants of the shoot apex of certain angiosperm.

Morel (1960) attempted to obtain virus free *Cymbidium* by meristem culture. This attempt was the first important breakthrough in the clonal propagation of orchids. He discussed various aspects of meristem culture in detail. (Rao 1997)

Murashige and Skoog (1962) found striking four to five fold increase in yield with tobacco tissue when cultured on White's modified medium supplemented with kinetin and indoleacetic acid (IAA) within a three to four week growth period on addition of an aqueous extract of tobacco leaves.

Raghavan and Torrey (1964) grew seeds of orchids *Cattleya in vitro* in a medium containing ammonium nitrate as the sole source of nitrogen, germinated readily and proceeded to form small plantlets. Development of the embroys was accompanied by an increase in their total nitrogen and a decline in the present dry weight. When seedlings growing in ammonium nitrate for varying periods were transferred to sodium nitrate, it was found that those plants allowed to grow for 60 or more days in ammonium nitrate could resume normal growth thereafter in sodium nitrate.

Kand (1967) germinated the seeds of orchids like *Cymbidium viriscens* and *C. gyokuchin* which are commonly called as "Hard to germinate Orchids" in a sterile nutrient medium. The same media was used for the clonal propagation by meristem culture.

Hadley and Harvais (1968) studied the effect of growth substances on germination and development of orchid (*Dactylorchis purpurella*) seeds. He concluded that the gibberelic acid enhanced the protocorm survival but didn't influence the growth and size of protocorms. IAA imposed germination and caused some elongation of protocorms. Kinetin alone and with IAA had pronounced effect on growth and development.

Matsui *et al*. (1970) studied the effect of BAP, NAA and their combined effects on the formation of Protocorm like bodies and root and shoot meristems culture of *Cymbidium*. NAA had no influence on PLBs formation while BAP (0.1ppm) induced the highest effect.

Mitra (1971) reported that the seeds of orchid *Arundina bambusifolia* Lindl. germinated and developed into seedling in Raghavan and Torrey (RT) medium. Ammonium nitrate was found to be the most suitable form of nitrogen for the development of the embroys. Shoot tips (6-8mm long) and stem discs (1-2mm in diam.) grown in Raghvan and Torrey's medium supplemented with Urea, Peptone, Casein hydrolysate, Vitamin free, Casein hydrolysate with vitamins, Yeast extract, ribonucleic acid and coconut water were studied. PLB was formed in shoot–tips cultured in the nutrient medium containing 0.1x peptone and complete plants were formed in media containing coconut water (20 %) in shoot tip culture. Many shoot buds were formed in stem discs in each supplement except coconut water.

Fonnesbech (1972) studied the effect of auxins (IAA, NAA and 2,4-D), cytokinin (kinetin and BA) and gibberllin (GA_3) alone and in combination on the protocorm of *Cymbidium*. IAA alone had no effect and NAA inhibited chlorophyll synthesis at high concentration. BA and kinetin when used singly induced shoot formation in solid medium and callusing in liquid medium while GA_3 induced shoot and leaf growth.

Murashige and Skoog (1974) reported some 22 genera of orchids being propagated through tissue culture using shoot tip, root tip, young inflorescence, embryo, inflorescence, young leaf tip as explants.

Tanaka *et al*. (1975) obtained PLBs from leaf tissue from seedlings of *Phalaenopsis* and *Vanda*. A practical medium for PLBs formation was formulated. The PLBs formed on the explant of both *Phalaenopsis* and Vanda grew to juvenile plants on a medium suitable for orchid seed germination.

Reinert and Hubert (1976) first cultured the lateral bud meristems of *Cattleya* in a chemically defined liquid medium with constant agitation, then later they transferred it to the solid (agar) medium. They reported the development of callus or protocorm like bodies (PLBs) which ultimately developed into plantlets when kinetin was added after first week of culturing.

Singh (1976) reported variation in the growth of meristematic explants of *Dendrobium*. The meristem from the apical and axillary buds of single mother plant showed different response in the nutrient medium under same conditions.

Cheah and Sagawa (1978) obtained PLBs from the explants of apical and axillary buds of *Aranda* and *Aranthera* in Viacin and Went (VW) medium supplemented with 15 % coconut water. Multiplication of the PLBs was carried out in VW-2 % sucrose + 15 % coconut water. Shoots were induced in solid VW with 2 % sucrose for *Aranthera* and for *Aranda* solid VW with 2 % sucrose, 15 % coconut water and 5 % GB medium was used. In both the genera roots were induced in the solid VW with 15 % coconut water and 15 % GB medium.

Mathews and Rao (1980) established tissue culture procedure of rapid multiplication of three inter specific hybrids of *Vanda*. Germination frequency of seeds, protocorm development and seedling growth were qualitatively enhanced by suitably altering the composition of the medium. Yeast extract, pepton, urea and NAA markedly enhanced the growth and differentiation of protocorms. Coconut milk induced the formation of large size seedlings.

Kokonowicz and Jules (1984) achieved *in vitro* shoot proliferation of *Vanilla* by axillary branching in a basal medium (MS) with 0.5 mg/l 6-benzylaminopurine (per lit). While rooted multiple shoots are acclimatized in green house in organic medium.

Niroula and Rajbhandary (1985) established shoot proliferation and protocorms from the seedlings tips of *Dendrobium fimbriatum* Hook. in MS medium supplimented with cytokinins. Complete plantlets were obtained when roots and protocorms were transferred in VW medium.

Bopaiah and Jorapur (1986) sowed seeds of *Cymbidium aloifolium* (L.) Sw. on modified Knudson C (Kn C) medium (BM) supplemented with 100 ml/l of coconut milk (CM), and 3 mg/l each of peptone (P) and Casein hydrosylate (CH). The PLBs obtained from 8 week old cultures were subcultured on fresh medium containing all the above nutrients and 200 g/l of banana pulp. The additional presence of either of the growth regulators, i.e. Vitamins (thiamine HCL, niacin, pyridoxine), auxins (NAA, 2, 4 - D), cytokinin (kn), and amino acid (glycine), in different concentrations in the nutrient medium comprising BM + CM + P + CH + banana pulp along with 1 mg/l each of thiamine HCL, niacin, glycine, and kinetin was found most suitable for the normal and healthy seedling growth.

Muralidhar and Mehta (1986) germinated seeds of *Cymbidium longifolium* on three basal medium, Kn C (Knudson, 1946), VW (Vacin and Went, 1949) and RT (Raghvan and Torrey, 1964) with or without various levels and combinations of vitamins, hormones, amino acids, and micronutrients. The seed germination was assessed at up to 30 % on Kn C, 60 % on VW, and 35 % on RT. When supplemented with vitamins (2.0 mg/l each of thiamine HCl and pyridoxine HCl, 0.3 mg/l of biotin, and 0.03 mg/l of folic acid), hormones (2.0 mg/l each of IAA, 0.4mg/l of kinetin and aminoacids), the VW medium supported 80 % seed germination in 30 days. The sequential steps of histomorphological changes from embryo to PLBs were traced.

Raghuwanshi *et al*.(1986) carried out the study on germination and seedling growth in *Dendrobium nobile*, *D.chrysanthum* and *Sarcanthus pallidus* at different pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 10.0) levels maintained on modified Kn C medium. Optimum results were obtained at pH 4.0 and 5.0 whereas the medium with pH adjusted at 10.0

provided detrimental for germination and protocorm growth. While the roots/absorbing hairs were best found at pH 5.0. Maximum leaves were produced at pH 4.0 in *D. nobile* in contrast to *D.chrysanthum* and *S. pallidus* which showed more leaf promordia at pH 5.0. Significant variations were observed in seed germination and seedling growth at different pH levels.

Sharma and Tandon (1986) studied the influence of growth regulators on asymbiotic germination and early seedling development of *Coelogyne punctulata* Lindl. The effect of various growth regulators viz Indole acetic acid (IAA), Naphthalene acetic acid (NAA), Dichlorophenoxy acetic acid (2,4-D), 6-furfurylaminopurine (kinetin) and gibberellic acid (GA3), in different concentrations (0.1-10 mg/l)and combinations in Kn C medium were studied on seed germination and seedling growth of *Coelogyne punctulata*. Both germination and seedling growth were promoted by kinetin at lower concentration. The best results were obtained when kinetin (1 mg/l) was used in conjuction with NAA (0.1 mg/l)

Sood and Vij (1986) inoculated root segments on selective modifications of Mitra *et.al*. obtained from 6-month old seedlings of *Coleogyne cristata* in axenic cultures and found that they responded favourably. They generated PLBs or shoot buds at the cut or tip ends depending on the medium composition. Cell proliferations in certain combination failed to differentiate. Peptone in the medium promoted cell proliferation and kinetin was obligatory for plb's regeneration.

Philip and Nainar (1988) reported histogenesis and organogenesis during the process leading upto plantlet formation in tip cultures of aerial roots of *Vanilla planifolia* . Young root tips excised from aerial roots, cultured in liquid MS medium containing IAA and KN (Kinetin) differentiated into shoot meristem with leaf primordial. After few leaves were formed, root meristem was differentiated.

Shrestha and Rajbhandari (1988) obtained shoot proliferation through meristem of *Cymbidium giganteum* Wall ex.Lindl in the MS medium supplemented with BAP (5 mg/l), NAA(1 mg/l) and 10 % coconut milk. The shoots were transferred to pots containing the fern fiber and survived in the green house.

Yam *et al*. (1991) concluded that the Heller's medium containing BA (1 mg/l) and NAA (0.2 mg/l) was best for the leaf tip culture of *Pholidota chinensis*. The same medium supplemented with BA (1 mg/l) was found to be best for *Liparis viridiflora*. Similarly the most suitable medium for *Acampe rigida* was found to be Ichinashi and Jamashita medium containing BA (1 mg/l) and NAA (0.2 mg/l) and modified MS supplemented with BA (1 mg/l) was best for *Cleisostoma fordii*.

Yamamoto *et al*. (1991) observed that shoot primordia of *Calanthe siefoldii* were induced from the meristems in modified B5 medium supplemented with 2 mg/l BA. Then PLB's were obtained from shoot primordia after transplanting onto agar medium. PLB's were regenerated into plantlets.

Niraula and Rajbhandary (1992) observed PLBs formation when explants from *in vitro* grown seedling of *Vanda teres* were cultured in MS medium containing auxin and cytokinin. Complete plantlets were obtained when PLBs were grown in VW medium.

Reddy *et al*. (1992) studied *in vitro* seed germination and seedling development in four species of south Indian orchids and showed a significant interaction between the media and the orchids. The frequency of germination and protocorm development and the extent of seedlings growth varied with the species and the culture medium. MS (Murashige and Skoog, 1962) and RL (Rosa and Laneri, 1977) media yielded better results than KC (Knudson, 1946) and VW (Viacin and, 1949) medium. The ground growing *Spalthoglottis plicata* responded better to MS medium and the epiphytic *Epidendrum radicans*, *Dendrobium crepidatum* and *Cymbidium aloifolium* to RL medium.

Shrestha and Rajbhandary (1993) cultured the meristem of *Cymbidium grandiflorum* on MS medium containing BAP (2.2 mg/l), NAA (1.8 mg/l) and 10 % coconut milk. The protocorm developed gave shoots on subculture in the same medium but roots were developed when culture in the basal medium containing only coconut milk. The developed plantlets survived in the green house.

Shrestha and Rajbhandary (1993) carried out the clonal propagation of *Dendrobium densiflorum* Lindl. through shoot meristem culture. Protocorm were initiated from shoot tip explant in Murashige & Skoog's medium (MS) supplemented with 15 % coconut milk, 2.5 mg/l Benzylaminopurine (BAP), 1 mg/l Napthalene acetic acid (NAA) and Caseinhydrolysate 1 g/l. These protocorms were cultured in MS medium with BAP 0.25 mg/l, NAA 0.01mg/l, Adenine sulphate 20 mg/l and coconut milk 10 %. These shoots were transferred to Viacin & Went's (VW) control medium for rooting. The rooted shoots were transferred to community pots containing tree fern fibers and established well in the green house.

Vij (1993) studied the regeneration response of root explants of 21 species and hybrids of orchids and found it to vary with their genetic constitution, physiological age and the chemical regime. Root explants from mature plants failed to show any morphogenetic change when grown *in vivo* but root explants from *in vitro* raised culture responded favourly in selective nutrient combinations. The response was species specific and chemical stimulus present in the medium. Peptone was obligatory in inducing proliferations in *Cymbidium pendulum*, *Vanda cristata* and *V. testacea* while Yeast extract was found obligatory in case of *Aerides multiflorum* and *Vanda teres*.

Rajkarnikar and Niroula (1994) initiated protocorms of *Dendrobium fimbriatum* Hook from shoot tip explant in Murashige and Skoog's medium (1962) supplemented with 5 mg/l of Benzylaminopurine (BAP), 1mg/l of Napthaleneacetic acid (NAA) and 10 % coconut milk. Protocorms were subcultured in MS medium with 1 mg/l of BAP, 1mg/l of NAA and 10 % coconut milk for the multiple shoot production.
Roots were produced in shoots when transferred on MS medium with 0.5 ppm of NAA.

Shrestha and Rajbhandary (1994) studied the clonal multiplication of *Cymbidium longiflorum* D.Don by shoot tip culture. Clonal multiplication of *Cymbidium longiflorum* D.Don was achieved with shoot apical meristems cultured *in vitro*. Protocorm like bodies were initiated within six weeks after culturing on Murashige and Skoog medium (1962) supplemented with 2 mg/l Benzylaminopurine (BAP), 1mg/l Napthaleneaceticacid (NAA), 10 % coconut milk and 3 % sucrose. Rapid

multiplication of shoots occurred in MS medium containing 1 mg/l BAP, 1.5 mg/l kinetin and 10 mg/l Adenine sulfate. The proliferation continued on subculturing on same medium. Rooting occurred in MS basal medium without growth hormones.

Yasugi *et al*. (1994) observed that the root segment cultured in *Cymbidium* Kenny 'wine colour' proved as an useful method to induce PLB and plantlets and it is essential to culture the basal segments of root on a medium containing NAA and BA under light condition, 1 mg/l NAA and 1 mg/l BA induced PLB formation.

Vij *et al.* (1995) germinated immature seeds from unripe capsule of *Dactylorhiza hatagirea*, collected 16 weeks after pollination on agar modified Knudson 'C' medium supplemented with selective growth adjuncts. A combination containing yeast extract (YE, 1 mg/l), and 6-purfurylaminopurine (KN, 1 mg/l) proved very useful during germination and formation of complete seedling with leaf and tuberous roots.

Pant *et al*. (1996) induced multiple shoots from the apical domes of shoot tips of *Cnidium officinale* Makino (Apiaceae) by culturing them on Murashige and Skoog(MS) 1 static media solidified with 0.2 % glerite and supplemented with 6-benzylaminopurine (BAP) 10-6M. An average of 5.3 shoots per segment was obtained within 6 weeks and this ability did not decline even after two years of subculture. Subsequent transfer of these regenerated shoots on MS + NAA 10-7M + BAP 10-7M resulted in root formation. Rooted plantlets were able to grow in soil after a short period of acclimatization.

Devi *et al.* (1997) carried out clonal propagation of *Dendrobium moschatum* and *Cymbidium aloifolium* through shoot tip culture in five different media viz. MS (1962), WI (Wimber, 1963), KC (Knudson, 1946), VW (1949) and NI (Nitsch and Nitsch, 1969). Nitsch and Nitsch (1969) medium was found to be the best for formation and proliferation of PLBs. The duration for leaf and root differentiation from PLBs varied from 7-13 weeks in different media.

Chung *et al.* (1998) studied the effect of medium composition on multiple shooting, further growth of mericlone from rhizome of shoot tip culture of *Cymbidium* species.

MS medium with NAA (1 mg/l) and BA (3 mg/l) enhanced multiple shooting in *C. forrestii* and *C. kanran* while *C. forrestii* also gave high number of shoots in MS medium supplemented with NAA (1 mg/l) and BA (1 mg/l). *C. nishiuchianum* developed more shoots in MS medium supplemented with NAA (2 mg/l) and BA(1 mg/l). 5-6 cm long shoots of *C. forrestii* 'sojub' grew well further in the medium supplemented with 1 mg/l NAA and 1 mg/l BA and that of *C. forrestii* 'songmae' and *C. forrestti* 'yangshiso' grew well in medium containing NAA (2 mg/l) and BA(1 mg/l). 0.5mg/l NAA and BA containing medium resulted good shoot growth in *C. forrestti* 'changsoo'. *C. kanran* 'shoshim' and *C. niveo* 'marginatum'.

Liu *et al*. (1998) successfully germinated the *Dendrobium candidum* seeds in 0.5 MS medium without NAA and BA supplement. Addition of NAA or potato extract to the medium promoted embryo germination and protocorm development. BA alone as well as in combination with NAA inhibited the proliferation and growth of protocorm. The aqueous extract of banana, potato and alcoholic extract of *Quercus glauca* bark accelerated the plantlet regeneration and rooting.

Pathenia *et al.* (1998) cultured the pseudobulbs of *Dendrobium* cv Sonia by using four different culture media viz. MS (1962), Knudson C (Kc, 1946), Vacin and Went (VW, 1949) and Heller (1976) with or without growth regulators (NAA, BAP), Kc medium supplemented with BAP (1.5 mg/l), NAA (0.4 mg/l) and Paclobutrazol (1 mg/l) was found best for further multiplication of PLBs. The rooting was favoured in all media supplemented with IBA (1 mg/l). The potting mixture containing bark pieces, brick pieces, moss and charcoal pieces (1:1:1:1) proved suitable for the establishment of *in vitro* rooted plant.

Benerjee and Mandal (1999) germinated immature *Cymbidium* seeds on defined orchid culture medium containing folic acid. NAA and organic adjuvants viz. casein hydrolysate, peptone, coconut water and tryptophan at varying concentration i.e 2-5 ppm of folic acid in VW medium resulted highest germination rate (85 %) while 0.1ppm gave 78 % germination rate. 2 ppm NAA containing VW medium was found suitable for inducing 3-4 roots within 2 months.

Kamalakannan *et al*. (1999) regenerated the endemic orchid *Coelogyne odorittissima* var *angustifolia* via encapsulation of protocorms in solid alginate matrix..

Shrestha (1999) found that the half strength MS liquid medium containing IBA (1 ppm) was the best for *in vivo* rooting of *Guizotia abyssinica Cass.*

Chen *et al.* (2001) developed protocol for the propagation of *Onicidium.* It contained three steps viz. PLB induction, PLBs multiplications and PLBs shooting and rooting. Lateral buds of 10-15 cm long were developed. PLBs upto thousand in 9-12 months and then shoots and roots were induced.

Kabita and Sharma (2001) observed that cent percent germination was obtained on MS medium supplenented with NAA (0.1 mg/l) and Kn (1 mg/l) in *Acampe longifolia* Lindl. It was the lowest in B_5 medium.Formation of Spherules and development of distinct green PLBs occurred and leaves also took less time on MS medium than on other media tried.

Murthy and Pyati (2001) observed the clonal propagation of *Aerides maculosum* Lindl. MS medium supplemented with NAA, KN, BA and coconut liquid endosperm was used for the culture leaf explants from *in vivo* as well as *in vitro* grown plants. Explants from mature leaves showed no response while those from juvenile leaves formed PLBs in 4-8 weeks time depending on the growth medium. MS medium with 2 mg/l BA gave best PLB formation (18/leaf explants).

Nagaraju and Upadhyaya (2001) studied the *in vitro* morphogenetic response of *Cymbidium lunavian* Atlas. PLBs on three different basal media viz. KC (Knudson. 1946), MS (1962) and Nitsch (1969) media. Among the media, Nitsch was found to be best for growth of plantlets.

Jamir *et al*. (2002) obtained that asymbiotic germination was best in Nitsch medium supplemented with NAA and kinetin at 1 mg/l each in *Cymbidium iridioides* D.Don.

Karanjit (2002) cultured the seeds of *Coelogyne cristata* Lindl. and *Cymbidium iridioides* D.Don in MS medium and Gamborg B5 (G-B5) medium. The germination rate was noted vigorous in MS medium.

Rajkarnikar (2003) initiated the protocorms from shoot tip explant of *Dendrobium fimbriatum* Hook. in the MS (Murashige and Skoog's medium, 1962) medium fortified with 5 mg/l BAP (Benzyl amino purine), 1 mg /l NAA (Napthalene acetic acid) and 10 % coconut milk. Protocorms were subcultured in MS medium with 1 mg/l BAP, 1mg/l NAA and 10 % coconut milk for multiple shoots and protocorms production.
Micro shoots transferred on MS medium with 0.5 mg/l NAA produced roots.

Swar & Pant (2003) cultured the seeds of *Cymbidium iridioides* D.Don. Growth and development of seedlings were favoured in MS medium supplemented with BAP (1 ppm) and NAA (1 ppm). The multiple shoots were obtained through shoot tip culture The highest number of multiple shoots was obtained in MS medium supplemented with BAP (0.5 ppm). She also carried out the shoot tip culture of *Coelogyne cristata* Lindl. and the highest number of multiple shoot was observed in MS medium with BAP (1 ppm) and NAA(1 ppm).

Huan *et al*. (2004) recorded the callus induction from PLBs and plant regeneration in *Cymbidium* (Orchidaceae). They examined the embryogenic callus induction and plant regeneration in *Cymbidium*, Great flower "Rainbow Drop". Longitudinally bisected segments of protocorms like bodies (Plbs) formed calli within one month on modified Viacin and Went medium (1949) supplemented with 1-naphthaleneacetic acid (NAA) or 2, 4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with N-phenyl-N-1, 2, 3 - thiodiazol-5-yl, urea (TDZ). The combination of NAA (0.5 mg/l) and TDZ (0.1 mg/l) was found to be the most effective for the callus induction. The calli grew when subcultured every four weeks on the same medium. The calli easily formed PLBs after being transferred to media without plant growth regulators. Healthy plantlets without any phenotypic abnormality were obtained from callus derived PLBs.

Bhadra and Bhowmik (2005) carried out the axenic germination of seeds and rhizome based micropropagation of an orchid *Arundina graminifolia* (D.Don) Hochr. Seeds of *Arundina graminifolia* were aseptically cultured on MS, PM, and MVW basal media They germinated on MS and PM media but failed to germinate on MVW. The

rhizome segments developed on the plants from the seedlings were used for micropropagation and that produced highest number of multiple shoot buds on 0.8 % (w/v) agar solidified MS fortified with 3 % (w/v) sucrose + BAP (2.0 mg/l) + IAA (1.0 mg/l). The *in vitro* developed seedlings were finally transferred to pots by successive phases of acclimatization.

Hsia *et al*. (2005) carried out the mass propagation of Ornamental *Dendrobium* from protocorm like body (PLB). The PLBs of the ornamental *Dendrobium* were transversly cut and adopted as explants. They were cultured on the MS basal salt media containing 30 g/l of sucrose, 80 g/l of potato homogenate, 9 g/l of agar and in combination with 2 mg/l of different kinds of plant growth regulators such as NAA, IBA, BA, Kinetin, TDZ and Zeatin for PLB proliferation investigation. After 60 days PLBs from the Zeatin treatment, subcultured on the medium containing Yam bean had the highest shoot formation rate. The shoot formation rate of IBA-induced –PLBs was high, no matter they were cultured on Yam bean or potato homogenate containing media. The 1/4 diluted basal salt medium had the best root induction and growth rate.

J and Basavaraju (2005) obtained *in vitro* culture of *Vanilla*. Morphogenetic responses of nodal explants of *Vanilla* oil MS medium fortified with Auxins, Cytokinins individually and in combination were investigated to find effect of the above on the initiation of axillary bud and organogenesis of shoot. MS medium fortified with BAP revealed good initiation and formation of shoots when compared to others. The combination of BAP + IBA and BAP + NAA revealed good organogenesis in MS medium. In all multiplication medium the formation of multiple shoots were observed. Of these experiments the multiplication in MS medium with BAP + IBA produced good number of shoots/propagules by continous subculturing up to 40-50 in number, which is an advantageous feature for the micropropagation of *Vanilla.*

Karki *et al*. (2005) carried out the micropropagation of *Vanilla planifolia* Andrews. from seeds. They cultured the seeds of *Vanilla planifolia* Andrews. in Murashige and Skoog medium without growth hormones. The seeds germinated after 6-8 months. The seedlings were subcultured in Muradhige and Skoog medium supplemented with 1.0 mg/l Benzylaminopurine and 1.5 mg/l kinetin with 10 % coconut milk. The mature shoots were transferred in cocopit for rooting.

Pant & Gurung (2005) studied the influence of growth regulators on the germination and seedling growth of the orchid *Aerides odorata* Lour. MS media without any hormone supplement was found to be the best for the germination and seedling growth of the *Aerides odorata*. Germination of the seeds started first in the MS basal medium in 9 weeks of inoculation. MS medium supplemented with 1.5 ppm BAP + 1.0 ppm NAA was found effective in high number of healthy shoots with well developed roots. NAA induced callusing in shoot tip explants as well as caused brown discoloration of older tissues. IAA at low concentration (0.5 ppm) was highly effective for rooting of shoots and further increase in IAA concentration decreased the number of roots. IBA concentration above or below 1.0 ppm showed decreasing tendency in root formation. IAA (0.5 ppm) and IBA (1.0 ppm) were almost equally efficient for rooting but NAA showed no significant role in the rooting.

Rajbahak *et al*. (2005) carried out the *in vitro* multiplication of *Vanilla planifolia* Andrews. using axillary bud explants. The axillary buds (2-3cm in length) were collected from mother plants. The tip of the axillary bud about 0.5-1.0 cm long were cut off aseptically and cultured in MS medium (Murashige and Skoog 1962) supplemented with 1.0 mg/l Benzyl amino purine, 1.5mg/l kinetin along with 10 % coconut water. Two to three new shoots were developed after 4-6 weeks of culture. The micro shoots were subcultured in every 6-8 weeks in the same concentration of growth hormone along with coconut water. The mature shoots were transferred in cocopit or moss for rooting.

Sharma *et al*. (2005) demonstrated that immature seeds obtained from green pod of *Dendrobium fimbriatum* Hook. an endangered epiphytic forest orchid having horticultural importance can be germinated asymbiotically *in-vitro* for rapid micropropagation. Viacin and Went medium containing 0.1 mg/l NAA and 15 % coconut water was found most effective for high percentage (80-90 %) seed germination and seedling development.

Shrestha A. (2005) successfully achieved asymbiotic germination of *Coelogyne ovalis* Lindl. MS medium supplemented with 1 ppm of NAA was the best medium for germination, growth and development of seedlings. Germination started after 8 weeks

of inoculation. Complete plantlets were observed after 16 weeks of culture. The explant, shoot tip obtained from *in vitro* grown plant was cultured in MS hormonal free media and MS media supplemented with various combination of BAP and NAA. The maximum number of shoot multiplication was observed in MS media with 1 ppm of BAP singly and MS media in combination of BAP 0.5 ppm with NAA 0.5ppm showed callus induction in the base of some shoots. Best rooting was obtained in MS media with 2 ppm of IBA

Kuo *et al.* (2006) obtained the efficient plant regeneration through direct somatic embryogenesis from leaf explants of *Phalaenopsis* "Little steve".- Half strength MS medium supplemented with BA and TDZ was found to be the effective media for the induction of somatic embryogenesis from the adaxial surfaces near the wounded regions. Histological section revealed that somatic embryos mostly arose from epidermal cell layers of the explant .

Pradhan (2007) carried out the *Ex situ* conservation of two orchid species viz. *Cymbidium elegans* Lindley. and *Dendrobium densiflorum* Lindl. by tissue culture technique. MS + BAP (1 mg/l) and MS basal medium was found to be most effective for the seed germination of *C. elegans* Lindley and *D. densiflorum* Lindl. respectively. MS + BAP (2mg/l) + NAA (0.5mg/l) was found to be most favourable for multiplication of shoots from shoot tip culture where as MS + BAP (1.5mg/l) was found to be most effective for the regeneration of shoots from root tip culture of *D. densiflorum* Lindl. Rooting of *D. densiflorum* Lindl. was found to be most effective in MS + IBA (1.5 mg/l).

CHAPTER THREE

MATERIALS AND METHODS.

3.1 Materials

The materials used for the present experiment were the young capsules of *Coelogyne fuscescens* Lindl. and *Cymbidium aloifolium* (L.) Sw collected from the Thulswara V.D.C - 1 and Dhaulikuwa - 9 of Kaski district respectively. (Fig 04 & Fig 09).

3.2 Methodology

The methods for seed germination of *C. fuscecens* Lindl. and *C. aloifolium* (L.) Sw and acclimatization of *C. fuscecens* Lindl. are as follows.

3.2.1 Methods for seed germination.

The methods for the seed germination of *C. fuscecens* Lindl. and *C. aloifolium* (L.) Sw. are described under the following headings.

3.2.1.1 Preparation of stock solution

The Murashige and Skoog's medium (Murashige and Skoog, 1962) was used as the basal medium for this experiment, alone and in combination with different concentrations of hormones. The composition of the MS medium is as follows:

A. Macro-nutrients

Components	Composition of MS (Final conc.) mg/l	(10X) gm/l Stock Concentration	Volume to be taken for 1litre medium
Macronutrients			
Potassium nitrate (KNO_3)	1900	19.0	
Ammonium nitrate (NH_4NO_3)	1650	16.5	
Calcium chloride ($CaCl_2.2H_2O$)	440	4.4	100 ml
Magnesium sulphate ($MgSO_47H_2O$)	370	3.7	
Potassium dihydrogen phosphate (KH_2PO_4)	170	1.7	

B. Micro-nutrients

Components	Composition of MS (Final conc.) Mg/lit.	(1000X)mg/100ml stock Concentration	Volume to be taken for 1 litre medium
Micronutrients			
Manganese-sulphate ($MnSo_4$.4H2O)	22.3	2230	
Boric acid (H_3BO_3)	6.2	620	
Zinc sulphate ($ZnSO_4.7H_2O$)	8.6	860	
Potassium Iodine (KI)	0.83	83	
Sodium-molybdate ($Na_2MoO_4.2H_2O$)	0.25	25	1 ml
Cobalt Chloride ($CoCl_2.6H_2O$)	0.025	2.5	
Copper sulphate ($CuSO_4.5H_2O$)	0.025	2.5	

C. Iron source (Fe, EDTA)

Components	Composition of MS (Final conc.) Mg/lit	(100X)mg/100ml stock Concentration	Volume to be taken for 1 litre medium
Iron Source			
Sodium ethylene diamine tetra acetate (Na_2EDTA)	37.3	373	10 ml
Ferrous sulphate ($FeSO_4$)	27.8	278	

D. Vitamins

Components	Composition of MS (Final conc.) Mg/lit	(1000X) mg/100ml stock Concentration	Volume to be taken for 1 litre medium
Glycine	2.0	200	
Nicotinic acid	0.5	50	
Pyridoxin HCL	0.5	50	1ml
Thiamin HCL	0.1	10	
Myo inositol	100.0	10,000	

(5) Carbon source

Chemicals	g/l
Sucrose	30 g

(6) Gelling agent

Chemicals	g/l
Agar	8 g

During the preparation of each stock solution, the above chemicals were weighed accurately and dissolved completely in distilled water. To dissolve the chemicals more readily, the solutions were stirred with magnetic stirrer. The final volume was made a litre for stock A, 100 ml for stock B, C and D. Due to light sensitivity, all stock solutions were kept in clean brown bottles and preserved in refrigerator.

3.2.1.2 Hormones used for the experiments

a) Naphthalene acetic acid (NAA)
b) Benzyl aminopurine (BAP)
c) Indole-3-acetic acid (IAA)
d) Indole-3-butyric acid (IBA)

3.2.1.3 Preparation of hormones

The growth hormones taken for the germination of orchid seeds and development of their seedlings are auxins and cytokinins. For the preparation of auxins i.e Napthalene acetic acid (NAA), Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA), 10 mg of each was dissolved in 2.5 ml of 1N NaOH and for the preparation of cytokinin i.e BAP 10 mg of it was dissolved in 0.25 ml of 0.5 N NaOH. After that the final volume was made 100 ml by the addition of sterile water in each hormone separately.

3.2.1.4 Sterilization of Glassware's and Metal instruments.

During the experiment, the necessary glasswares and metal instruments were subjected to dry heat sterilization before their use. Glasswares such as petridishes, culture tubes, pipettes, beaker, conical flask etc and metal instruments like forceps, scapels and surgical blade's handle etc were dipped in detergent solution for 24 hours and rinsed with tap water, and final rinse was done with distilled water. Then the

glasswares and metal instruments were sterilized in hot air oven at 150°C for 2 hours. Metal instruments were wrapped with aluminium foil before keeping inside the hot air oven for sterilization.

3.2.1.5 Preparation of Media

For the preparation of 1litre medium, following protocol was applied:

- 1 litre sterilized conical flask was taken.
- 100 ml of stock A, 1 ml of stock B, 10 ml of stock C and 1ml of stock D were added one by one in about 400 ml distilled water in sterilized conical flask.
- 30 gm of sucrose was weighed and dissolved in stock solution.
- Distilled water was added up to 1000 ml. The solution was stirred by magnetic stirrer in order to mix the stock and sugar solution more homogeneously.
- For the preparation of hormonal medium, hormone stocks were added according to the media requirement in 10 separate beakers to make 100 ml media in it.
- pH of the solution was adjusted 5.8 with 0.1 N NaOH or HCl.
- The medium was solidified by adding 0.8 gm (0.8 %) agar in each beaker containing 100 ml media. It was heated with the help of heater upto boiling to melt the agar. When the solution become clear, about 16ml was poured in each of the six sterilized culture tubes. Then each tube was enclosed by aluminium foil cap.
- The tubes containing medium were sterilized in an autoclave at the temperature of 121 °C and pressure of 15 lb/sq inch for 20 minutes. After cooling down of autoclave tubes were taken out and kept in slanting position in culture room supplemented with air condition.

3.2.1.6 Sterilization and inoculation of seeds.

Before inoculation, the laminar airflow chamber was sterilized by cleaning it with spirit or cotton soaked with 70 % alcohol. All the requirements for inoculation i.e. 70 % ethyl alcohol, sodium hypochlorite solution, sterile distilled water, rubber bands, sterile aluminium foils, sterile petridishes, handle with surgical blade, spatuala, beaker, forceps, tubes with media were exposed under ultraviolet (UV) radidiation for

45 minutes to remove the possible contaminants presenting on them and around the transfer area. After UV exposure, the blower was switched on for 15 minutes. Then, the laminar air-flow chamber was ready for inoculation.

Since the immature capsules of the orchids were collected from the *in vivo* grown plants, they need proper sterilization before their culture. For their sterilization capsules were dipped in detergent water for few minutes and then washed in running water for 1 hour. The capsules were then rinsed with distilled water. Then, inside the laminar airflow, capsules were dipped in 70 % ethyl alcohol for 2 minutes and surface sterilized in 1 % sodium hypochlorite solution for 15 minutes. Finally, the capsules were washed with double distilled water for five times. The capsules were cut longitudinally into two equal halves (Fig 05 & 10) and the seeds were inoculated on MS medium alone and in media of different hormones in combination. Sterile spatula was used to spread the seed on agar medium and observed regularly. All the cultures were maintained at 25 °C (+2 °C) temperature and about 14 hours photoperiods.

3.3 Histomorphological study of *Cymbidium aloifolium* (L.) Sw.

To carry out the histomorphological study of the Protocorm like bodies (PLBs) obtained after the 10 weeks of culture, squash preparation of the material was done. For it the small mass of PLBs from a tube was taken out and fixed periodically in 1 % acetocaramine for 24 hours. Small piece of that was mounted on slide with few drops of acetocarmine solution. Finally the slides were examined under the microscope.

3.4 Inoculation of explants of *Coelogyne fuscescens* Lindl.

For this experiment, shoot tips and root tips were taken as explants. Explants were obtained from the *in vitro* grown plants. Since in this case the explants were obtained from *in vitro* grown plants, they didn't require sterilization. Inoculation of the seeds was carried out inside the laminar air flow. During explants isolation and inoculation the blades and the forceps used were flamed after dipping in spirit. All the cultures were maintained at 25 ºC (+2 ºC) under 14 hours photoperiod.

3.5 Shoot Multiplication

Micro shoots of 4-7 mm in length were subcultured in medium containing different combinations of NAA and BAP.

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3.6 Rooting of Shoots.

For *in vitro* rooting, the micro shoots obtained from shoot tip explants were transferred to media containing different concentrations of various rooting hormones such as IAA, IBA and NAA. To observe the combined effect of two rooting hormons namely IAA and NAA, MS media supplemented with various concentrations of the hormones in combination was prepared and micro shoots were transferred to the media.

3.8 Acclimatization

In vitro rooted plantlets were gradually acclimatized by gradually decreasing the humidity. The culture tubes containing rooted shoots were kept in the room temperature for about 2 weeks and then transferred to the pots with the epiphytic medium containing, coco-peat and sphagnum moss in the ratio of 2:1. The humidity of the pots were maintained high in the first few days by covering the pots with transparent polythene sheets with holes for aeration and after one week the polythene were removed thus lowering the humidity. 5 % NPK was sprayed once a week to the plants to facilitate their growth and after one week the polythenes were removed thus lowering the humidity.

3.8 Statistical Analysis

Statistical Analysis was carried out with the help of 12^{th} verson of SPSS program. One way ANOVA (Analysis of Variance) was used for the analysis of data. The results were used to compare the means so that effective discussion can be made.

CHAPTER FOUR

RESULTS

The results of *in vitro* seed germination of *Coelogyne fuscescens* Lindl. and *Cymbidium aloifolium* (L.) Sw., culture of shoot tips and root tips, and acclimatization of *C. fuscescens* Lindl. have been described below under different headings:

4.1 *In vitro* culture of seeds and germination:

The immature seeds of *C. fuscescens* Lindl. and *C. aloifolium* (L.) Sw. were cultured on MS medium supplemented with or without various concentrations of growth hormones NAA (Auxin) and BAP (Cytokinin). In *C. fuscescens* Lindl. fastest germination rate was observed in MS medium supplemented with BAP (1 mg/l) plus NAA (0.5 mg/l). In this medium germination was started after 6 weeks of culture and about 90 % of seeds were germinated. Complete plantlets were obtained after 20 weeks of culture. Seeds culture on MS basal medium supplemented with NAA (0.5 mg/l) also showed good germination. Germination and differentiation were satisfactory in hormone free MS media. On the other hand, the germination in *Cymbidium aloifolium* (L.) Sw. was most favoured in MS medium supplemented with BAP (2 mg/l) plus NAA (0.5 mg/l). Germination started after 8 weeks of culture, which was 2 weeks later than that in *C. fuscescens* Lindl.

After germination, the immature seeds underwent further differentation to form seed clumps, protocorm like bodies which finally gave rise to complete plantlets. The protocorm like bodies of *Cymbidium aloifolium* (L.) Sw. (Fig SG 07) were globular, hairy and chlorophyllus in all concentrations while the protocorm like bodies of *Coelogyne fuscescens* Lindl. (Fig SG 01) were found to be indistinct, grey in colour from which green plantlets were raised.

In vitro seed germination of *C. fuscescens* Lindl. and *C. aloifolium* (L.) Sw in MS alone and MS medium supplemented with different concentrations of different hormones is summarized in Table 1 and 2 respectively

Table1:

Effect of growth regulators supplemented in MS medium for seed germination and seedling growth of *C. fuscescens* Lindl.

Media	Growth Hormones	Concentration of hormones (mg/l)	Observation taken in weeks				Remarks
			Initiation of germination	Protocorm formation	Initiation of shoots	Initiation of roots	
MS	BM	-	10	13	17	-	Better
"	BAP	0.5	12	15	19	-	Good
"	BAP	1.0	15	18	21	-	Good
"	BAP	1.5	13	15	19	-	Better
"	BAP	2.0	18	22	28	-	Poor
"	NAA	0.5	9	12	16	23	Better
"	BAP+NAA	0.5 + 0.5	10	12	15	-	Better
"	BAP+NAA	1 + 0.5	6	10	13	-	Best
"	BAP+NAA	1.5 + 0.5	12	14	17	-	Better
"	BAP+NAA	2.0 + 0.5	17	20	22		Good

Culture conditions: - MS medium, 25 ± 2 °C, 44 weeks, 14 hrs photoperiod, 4 replicates were used in each combination.

Table 2:

Effect of growth regulators supplemented in MS medium for seed germination and seedling growth of *C. aloifolium* (L.) Sw.

Media	Growth Hormones	Concentration of hormones (mg/l)	Observation taken in weeks				Remarks
			Initiation of germination	Protocorm formation	Initiation of shoots	Initiation of roots	
MS	BM	-	19	23	39	-	Average
"	BAP	0.5	18	20	41	-	poor
"	BAP	1.0	16	19	28	-	Average
"	BAP	1.5	9	12	26	-	Good
"	BAP	2.0	12	13	26	-	Good
"	NAA	0.5	10	13	28	-	Average
"	BAP+NAA	0.5 + 0.5	9	11	25	-	Good
"	BAP+NAA	1 + 0.5	13	15	29	-	Average
"	BAP+NAA	1.5 + 0.5	13	15	28	-	Average
"	BAP+NAA	2.0 + 0.5	8	10	24	-	Best

Culture conditions: - MS medium, 25 ± 2 °C, 42 weeks, 14 hrs photoperiod, 4 replicates were used in each combination.

4.1.1 *In vitro* seed germination.

The pattern of seed germination and seedling growth of the orchids *C. fuscescens* Lindl. and *C. aloifolium* (L.) Sw. cultured on MS and MS supplemented with various hormonal concentration of BAP and NAA is summarised as under.

1. MS Basal Medium:

In MS Basal medium the germination in *C. fuscescens* Lindl. started in 10 weeks of culture. Protocorm formation was observed only in 13 weeks of culture. The protocorm were neither globular nor hairy. The protocorm were grey in colour. Initiation of green shoot from protocorm was observed in 17 weeks of culture. (Fig. SG 05)

In *Cymbidium aloifolium* (L.) Sw. germination in MS Basal medium was found to be rather late. The germination was observed only in 19 weeks of culture. Protocorm formation was observed in 23 weeks of culture. The protocorm were globular hairy and chlorophyllous. The initiation of shoots in this medium was observed only in 39 weeks of culture.

2. MS + BAP (0.5 mg/l).

Seeds of *C. fuscescens* Lindl. cultured in MS medium supplemented with BAP (0.5 mg/l) required 12 weeks for the initiation of the germination. Protocorm formation was initiated in 15 weeks. Initiation of shoot was observed in 19 weeks of culture. Root initiation was not observed till 44 weeks of culture.

In *C. aloifolium* (L.) Sw. the initiation of germination was observed in 18 weeks of culture. Protocorm formation was initiated in 20 weeks. Initiation of shoot formation was seen only in 41 weeks of culture.

1. MS + BAP (1 mg/l).

In MS medium supplemented with BAP (1 mg/l), the seeds of *C. fuscescens* Lindl. showed their response to germination only in 15 weeks of culture. While the protocorm formation was observed in 18 weeks of culture and shoot initiation was seen in 21 weeks of culture.

The seeds of *C. aloifolium* (L.) Sw. showed response to the germination in 16 weeks of culture. Protocorm formation was observed in 19 weeks of culture. The initiation of shoot was seen in 28 weeks of culture. Initiation of roots was not seen till 42 weeks of culture. (Fig. SG 06)

4. MS + BAP (1.5 mg/l).

Seeds of *C. fuscescens* Lindl. cultured in MS medium supplemented with BAP (1.5 mg/l) showed their germination in 13 weeks of culture. Protocorm were formed in 15 weeks of culture. Initiation of shoot was observed in 19 weeks of culture. No initiation of roots was observed till 44 weeks of culture. (Fig. SG 02)

Seeds of *C. aloifolium* (L.) Sw. cultured in the same culture condition germinated after 9 weeks of culture. Formation of protocorm was initiated after 12 weeks of culture. Shoot initiation started after 26 weeks of culture. (Fig. SG 10)

5. MS + BAP (2 mg/l).

In MS medium supplemented with BAP (2 mg/l), the germination of seeds of *C. fuscescens* Lindl. started after 18 weeks of culture. Formation of protocorm was started after 22 weeks of culture and initiation of shoot was observed after 28 weeks of culture.

In *Cymbidium aloifolium* (L.) Sw. the germination of seeds started after 12 weeks of culture. After 13 weeks of culture the initiation of protocorm formation started. Shoot initiation was started only after 26 weeks of culture. (Fig. SG 07)

6. MS + NAA (0.5 mg/l).

In MS medium supplemented with NAA (0.5 mg/l), the seeds of *C. fuscescens* Lindl. required 9 weeks for the germination. Initiation of the protocorm formation started after 12 weeks of culture and shoot initiation started only after 16 weeks of culture. Root primordia were seen in this condition after 23 weeks of culture.

Seeds of *Cymbidium aloifolium* (L.) Sw. cultured in the same condition germinated after 10 weeks of culture. Development of protocorm and shoot started after 13 and

24 weeks of culture respectively. No root primordia were seen in this culture condition till 42 weeks of culture.

7. MS + BAP (0.5 mg/l) + NAA (0.5 mg/l).

MS medium supplemented with BAP (0.5 mg/l) plus NAA (0.5 mg/l) was found to be good culture condition for the germination of seeds of *C. fuscescens* Lindl. In this condition the germination started after 10 weeks of culture. Development of protocorm was initiated after 12 weeks of culture. Initiation of shoots was started after 15 weeks of culture. (Fig.SG 01)

MS medium supplemented with the above mentioned concentration of BAP and NAA was also favourable for the seed germination of *C. aloifolium* (L.) Sw in which the germination of the seeds started after 9 weeks of culture. Protocorm development and shoot development was initiated after 11 and 25 weeks of culture respectively. (Fig. SG 09)

8. MS + BAP (1 mg/l) + NAA (0.5 mg/l).

MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) was found to be the most favourable condition for the germination of seeds of *C. fuscescens* Lindl. Protocorms were found to be developed after 4 weeks of germination i.e after 10 weeks of culture. Initiation of shoots was seen after 13 weeks of culure. Inspite of vigorious germination of the seeds the root primordia were not seen in this condition even after 43 weeks of culture. (Fig. SG 03)

The germination of seeds of *C. aloifolium* in this condition started after 13 weeks of culture. Protocorm formation was observed after 15 weeks of culture. Shoot development was seen after 29 weeks of culture.

9. MS + BAP (1.5 mg/l) + NAA (0.5 mg/l).

C. fuscescens Lindl required 12 weeks for the germination in this condition. Protocorm formation was initiated after 14 weeks. Development of shoots was started only after 17 weeks of culture. Root initiation was not observed in this condition. (Fig. SG 04)

Seeds of *C. aloifolium* (L.) Sw required 13 weeks for germination. Protocorms were developed after 15 weeks of culture. Shoot initiation was observed only after 28 weeks of culture. Root primordia was not seen in this culture condition.

10. MS + BAP (2 mg/l) + NAA (0.5 mg/l).

Initial germination of seeds of *C. fuscescens* Lindl was observed after 17 weeks of culture. Development of protocorm and shoot was observed after 20 and 22 weeks of culture respectively.

MS medium supplemented with BAP (2 mg/l) and NAA (0.5 mg/l) was found to be the most favourable condition for the germination of seeds of *C. aloifolium* (L.) Sw. Seed germination in this condition started after 8 weeks of culture. Development of protocorm and shoot started after 10 and 24 weeks of culture respectively. Althougth the germination of the seeds in this condition was earlier, the gap between the protocorm formation and initiation was rather longer. Initiation of the roots was not observed in this culture condition. (Fig. SG 08)

4.2 Histomorphological study:

The globular hairy protocorm like bodies were formed in *Cymbidium aloifolium* (L.) Sw. after 10 weeks of culture. After few weeks of formation of protocorm like bodies, single pair of leaf primordia emerged from the promeristematic cells of proximal part of embryo which finally developed into embryonic photosynthetic leaves. At the same time the cells of distal part of embryo gave rise to tubular and unicellular rhizoids. Some major stages of emergence of leaves and roots were captured in photographs which are illustrated in Fig. H 01 to H 08.

4.3 Micropropagation of *Coelogyne fuscescens* Lindl.

4.3.1 Culture of shoot tips of *C. fuscescens* Lindl.

The shoot tips of length 4 -7 mm with a very small portion of stem were cultured on MS Basal medium and MS medium supplemented with various concentrations of BAP and NAA for inducing multiple shoots. The shoot tip cultured on MS basal and MS medium supplemented with different hormones showed different responses. The effect of BAP and NAA on shoot tip cultures has been given in Table 3.

Table: 3

Effect of BAP and NAA on shoot tip culture of

***Coelogyne fuscescens* Lindl.**

BAP → NAA ↓ (mg/l)	0	0.5	1	1.5	2
0	2MS 1MS, Pb 1S	1MS, C, Pb 1S 2MS, C	3MS 1S	1S 2MS, Pb 1MS, C	2MS, Pb, C 2S
0.5	2RS 1MS, Pb 1S	1MS,Pb 1RMS, C 2MS	2MS, Pb 1S, Pb 1MS, C	3MS 1MS, Pb	1RMS, C 2MS, Pb 1MS

Culture condition: MS medium, 25+2 ºC, 16 weeks, 14 hrs photoperiod, 4 replicates were used in each combination.
Where,
S=Shoot, MS=Multiple shoot, RS = Rooted Shoots, RMS=Rooted multiple shoot, Pb=Pseudobulb, C=Callus.

4.3.1.1 Development of shoot tip explants of *C. fuscescens* Lindl.

The shoot tips cultured in MS Basal medium and MS medium supplemented with various concentration of BAP and NAA separately showed following changes.

1. MS Basal Medium

In hormone free MS medium the explants permitted average development of the shoots. In this condition the explant produced upto three shoots in 16 weeks of culture having maximum length of 3 cm and minimum length of 2 cm. Average no.of shoots per culture was found to be 2. Average number of leaves per culture was found to be 6.5. (Fig. ST 01)

2. MS + BAP (0.5 mg/l).

The shoot tips cultured on MS + BAP (0.5 mg/l) multiplied after 7 weeks of culture. Upto six shoots of length ranging from 1 to 2.2 cm were observed after 16 weeks. Roots differentiation did not occur upto 16 weeks. Average shoot number and leaf number was found to be 3.75 and 8.75 per culture respectively. (Fig ST 02 & 03).

3. MS + BAP (1 mg/l).

The shoot tip multiplied after 7 weeks of culture. Upto five shoots of length 2 to 4 cm were observed. Roots were not differentiated in this concentration. Average shoot number was found to be 3.25 per culture. Average leaf number per culture was found to be 8. (Fig. ST 04).

4. MS + BAP (1.5 mg/l).

Shoot tips multiplied after 8 weeks of culture. Upto four shoots of length 1.7 to 2.6 were observed. Pseudobulb was seen after 16 weeks of culture. Huge amount of green and black brownish callus was seen at the base of shoots after 16 weeks of culture. Average number of shoots was found to be 2.5 per culture and average number of leaves was found to be 7.5 per culture. (Fig. ST 05).

5. MS + BAP (2 mg/l).

The shoot tip multiplied after 8 weeks of culture in MS +BAP (2 mg/l) medium. The height of the plant found to be ranged from 1.4 to 2 cm. Pseudobulb and callus was found at the end of 16 weeks of culture. Average number of shoots and leaves was found to be 1.75 and 5.75 per culture respectively. (Fig. ST 06).

6. MS + NAA (0.5 mg/l).

In MS medium supplemented with NAA (0.5 mg/l), the multiplication of shoots started after 9 weeks of culture. Upto 2 shoots with pseudobulbs and 2 to3, short slender roots were observed. Shoot length ranged from 0.5 to 2.2 cm. Average shoot number and leaf number was found to be 1.25 and 5.25 per culture. (Fig. ST 07).

7. MS + BAP (0.5 mg/l) + NAA (0.5 mg/l).

In MS medium supplemented with BAP (0.5 mg/l) + NAA (0.5 mg/l), shoot multiplication started after 8 weeks of culture. 2 to 4 multiple shoots with pseudobulb

was observed after 17 weeks. Brownish and white callus was seen on the base of the shoots after 16 weeks of culture. Short roots were also seen in some culture. Average number of roots was found to be 1.25 per culture. Shoot length in this concentration varied from 1 to 2.8 cm. Average number of shoots was found to be 2.5 per culture and average number of leaves per culture was found to be 7.25 (Fig. ST 08)

8. MS + BAP (1 mg/l) + NAA (0.5 mg/l)

In MS medium supplemented with BAP (1 mg/l) plus NAA (0.5 mg/l) multiple shoots were seen after 7 weeks of culture. Upto 5 multiple shoots with pseudobulb and green callus were observed after 15 weeks of culture. Shoot length varied from 1 to 1.8 cm. Average number of shoots and leaves was found to be 3.25 and 6.25 per culture. (Fig. ST 09 & 10)

9. MS + BAP (1.5 mg/l) + NAA (0.5 mg/l)

MS medium supplemented with BAP (1.5 mg/l) plus NAA (0.5 mg/l) was found to be the most effective culture condition for the multipliation of shoots. Multiplication of shoots was started after 5 weeks of culture. 4 to 11 multiple shoots with pseudobulb were observed in 14 weeks of culture. Length of the shoot varied from 1.5 to 2.2 cm. Average shoot number was found to be 6 per culture and average number of leaves was found to be 12.25 per culture. (Fig. ST 11)

10. MS + BAP (2 mg/l) + NAA (0.5 mg/l)

In MS medium supplemented with BAP (2 mg/l) + NAA (0.5 mg/l), shoot multiplication was observed after 6 weeks of culture. Upto 8 multiple shoots were observed at the end of 16 weeks of culture. Rooted multiple shoot with pseudobulb were seen in 16 weeks of culture. The length of the shoots ranged from 1.2 to 1.8 cm. 1 to 2 roots were induced from the base of the shoots. Average root length was found to be 0.75 cm. Average shoot number and leaf number was found to be 4.25 and 9.0 respectively. (Fig. ST 12)

4.3.1.2 Shoot multiplication.

After the culture of the shoot tip regular observation was carried out .Proliferation of shoot along with shoot growth and root induction was observed and recorded.

Following table shows the effect of BAP and NAA on multiple shoot formation, growth and root formation after 16 weeks of culture.

Table: 4

Effect of BAP and NAA on multiple shoot formation, growth and root formation after 16 weeks of culture.

Media	Growth Hormones	Concentration of hormones (mg/l)	Parameters			
			Shoot Proliferation (mean±S.D)	Shoot Growth (cm) (mean±S.D)	Number of Leaves (mean±S.D)	Number of Root (mean±S.D)
MS	BM	-	2.00 ± 0.81	2.50 ± 0.47	6.50 ± 2.64	No root
"	BAP	0.5	3.75 ± 2.21	2.40 ± 0.93	8.75 ± 3.09	No root
"	BAP	1.0	3.25 ± 1.7	3.15 ± 0.83	8.00 ± 2.16	No root
"	BAP	1.5	2.50 ± 1.29	1.95 ± 0.47	7.50 ± 1.73	No root
"	BAP	2.0	1.75 ± 0.95	1.60 ± 0.28	5.75 ± 1.70	No root
"	NAA	0.5	1.25 ± 0.5	1.34 ± 0.69	5.25 ± 0.95	1.25 ± 1.5
"	BAP+NAA	0.5 + 0.5	2.50 ± 1.0	2.00 ± 0.74	7.25. ± 2.62	0.75 ± 1.5
"	BAP+NAA	1.0 + 0.5	3.25 ± 1.7	1.20 ± 0.51	6.25 ± 2.62	No root
"	BAP+NAA	1.5 + 0.5	6.00 ± 3.36	1.87 ± 0.29	12.25 ± 5.25	No root
"	BAP+NAA	2.0 + 0.5	4.25 ± 2.62	1.65 ± 0.34	10.25 ± 5.31	0.5 ± 1

Culture conditions: - MS medium, 25 ± 2 °C, 16 weeks of primary culture, 4 replicates were used in each combination.

Shoot proliferation in MS basal medium after 16 weeks of culture was found to be average. Highest multiplication of shoot was observed in MS + BAP (1.5 mg/l) + NAA (0.5 mg/l) which was 6 shoots per culture (Fig. ST 11). Decrease in concentration of the BAP resulted in the decrease of shoot proliferation. In the MS medium supplemented with different concentration of BAP alone, the differentiation of the roots was not observed. The highest shoot proliferation in MS + BAP (1.5 mg/l) + NAA (0.5 mg/l) was followed by MS + BAP (2 mg/l) + NAA (0.5 mg/l), MS + BAP (0.5mg/l), MS + BAP (1 mg/l).

Least multiplication of the shoots was observed in MS + NAA (0.5 mg/l) i.e 1.25 shoots per culture. The highest growth of shoots were observed in MS + BAP (1mg/l) i.e 3.15 cm which was followed by BM (2.5 cm) and BAP (0.5 mg/l) + NAA (0.5 mg/l) i.e 2.4 cm. Least shoot growth was observed in BAP (1 mg/l) + NAA (0.5 mg/l) i.e only 1.2 cm. In comparision to shooting, rooting of *C. fuscescens* Lindl. in MS medium supplemented with different concentration of hormones was not found

satisfactory. The highest number of the roots observed was in the MS medium supplemented with NAA (0.5 mg/l) which was just 1.25 roots per shoot. Highest number of leaves was observed in BAP (1.5 mg/l) + NAA (0.5 mg/l) i.e 12.25 leaves per shoot. Which was followed by BAP (2 mg/l) + NAA (0.5 mg/l) with 10.25 leaves per shoot. Least number of leaves per shoot was observed in NAA (0.5 mg/l) with 5.25 leaves per culture.

Table: 5

Mean Value of Shoot Number, Shoot growth, Leaf Number and Root Number after 16 weeks of culture of shoot tip explants

Treatments (mg/l)	Mean Value of			
	Shoot Number	Shoot Growth (cm)	Leaf Number	Root Number
BM	2.00^{a}	2.5^{bc}	6.50^{a}	0^{a}
0.5BAP	3.75^{ab}	1.60^{ab}	8.75^{ab}	0^{a}
1BAP	3.25^{ab}	3.15^{c}	8.00^{ab}	0^{a}
1.5BAP	2.50^{a}	1.95^{ab}	7.50^{ab}	0^{a}
2BAP	1.75^{a}	1.60^{ab}	5.75^{a}	0^{a}
0.5NAA	1.25^{a}	1.34^{a}	5.25^{a}	1.2500^{b}
0.5BAP+0.5NAA	2.50^{ab}	2.0^{ab}	7.25^{ab}	0.75^{ab}
1BAP+0.5NAA	3.25^{ab}	1.20^{a}	6.25^{a}	0^{a}
1.5BAP+0.5NAA	6.00^{b}	1.87^{ab}	12.25^{b}	0^{a}
2BAP+0.5NAA	4.25^{ab}	1.65^{ab}	10.25^{ab}	0.5^{ab}

Note:

- The values with same alphabets are not significantly different at 5 % level of significance.
- Four replicates were used in each concentration

.

Table 6 shows relationship between the mean values of a particular column. The mean values with same alphabets in a column are not significantly different though the numerical values are different.

4.3.2 Culture of root tips of *C. fuscescens* Lindl.

The root tips obtained from *in vitro* culture were cultured in MS basal medium and MS medium supplemented with different concentration of BAP and NAA showed following changes:

Table: 6

Effect of BAP and NAA on root tip culture of *C. fuscescens* Lindl

BAP → NAA↓ (mg/l)	0	0.5	1	1.5	2
0	2MS,R 1MS,C 1S	2MS,R,Pb 1S 1R	1MS,C,R 1MS,C 1S 1RS	2MS,C,R 1S,R 1R	1MS,C 1S 2S,C
0.5	1S 2S,R 1R	1MS,Pb 1R 1S 1S,C	1MS 1MS,R 1MS,Pb 1R	1S,R,Pb 1S 1R	1MS,Pb 1MS,C 1S 1R

Culture conditions: - MS medium, 25 ± 2 °C, 20weeks, 14 hrs photoperiod, 4 replicates were used in each combination.

Where, C=Callus, MS=Multiple Shoot, R=Root, Pb=Pseudobulb, S=Shoot, RS=Rooted Shoot.

Table: 8

Effect of BAP and NAA on root tip explants on multiple shoot formation and root formation after 20 weeks of culture.

Media	Growth Hormones	Concentration of hormones (mg/l)	Parameters	
			Number of Shoot (mean±S.D)	Number of Root (mean±S.D)
MS	BM	-	2.00 ± 1.63	0.75 ± 0.95
"	BAP	0.5	1.75 ± 1.7	1.00 ± 0.81
"	BAP	1.0	2.50 ± 1.73	0.75 ± 0.95
"	BAP	1.5	1.75 ± 1.7	1.50 ± 0.57
"	BAP	2.0	1.25 ± 0.5	0.00
"	NAA	0.5	0.75 ± 0.5	1.25 ± 0.95
"	BAP+NAA	0.5 + 0.5	1.25 ± 1.25	0.50 ± 1
"	BAP+NAA	1.0 + 0.5	2.25 ± 1.7	0.75 ± 0.95
"	BAP+NAA	1.5 + 0.5	0.50 ± 0.57	0.50 ± 0.57
"	BAP+NAA	2.0 + 0.5	2.00 ± 1.82	0.25 ± 0.5

Culture conditions: - MS medium, 25 ± 2 °C, 20 weeks of primary culture, 4 replicates were used in each combination.

4.3.2.1 Development of root tip explants of *C. fuscescens* Lindl.

The root tips obtained from the culture of shoot tips were cultured in the MS medium alone and MS medium supplemented with different hormones of different concentration. The so cultured root tips responded in various ways. Their responses upto the end of 20 weeks of culture is summarized as under.

1. MS Basal Medium.

The root tip explants cultured in the MS Basal medium showed response to regeneration after 6 weeks of culture. Multiple shoots were observed after 14 weeks of culture. Callus was seen in one replica. Root was also seen in some replica after 19 weeks of culture. Average number of shoots was 2 and average number of roots was 0.75. (Fig. RT 01)

2. MS + BAP (0.5 mg/l)

Regeneration of roottip explants cultured in MS medium supplemented with BAP (0.5 mg/l) started after 9 weeks of culture. Multiple shoots were seen by the end of 20 weeks. The multiple shoots were with pseudobulbs. In some replica only single shoots were seen. The average number of shoots in this concentration was found to be 1.75 and average number of roots was found to be 1 per culture. (Fig. RT 02)

3. MS + BAP (1 mg/l)

The root tips cultured in MS medium supplemented with BAP (1 mg/l) started to develop shoots after 7 weeks of culture. Multiple shoots were found to be seen after the completion of 15^{th} week of culture. Shoot generation and root generation were found to be good in this media. The multiple shoots were with callus and roots. The average number of shoots was found to be 2.50 per culture. The average number of roots was found to be 0.75 per culture. (Fig. RT 03)

4. MS + BAP (1.5 mg/l)

The root tips cultured in MS medium supplemented with BAP (1.5 mg/l) showed their response to regeneration on the completion of 7 weeks of culture. Multiple shoots were observed only after the completion of 18 weeks of culture. Root initiation was rather early. Roots were found to be emerged by the end of 16^{th} week of culture.

Average number of shoots and roots was found to be 1.75 and 1.5 per culture respectively. (Fig. RT 04)

5. MS + BAP (2 mg/l)

Regeneration of root tip explants of *C. fuscescens* Lindl. obtained from the *in vitro* grown plants cultured in this culture condition started after 10 weeks of culture. Multiple shoots were obtained by the end of 17 weeks of culture. Development of root was not seen in this condition till the end of 20 weeks of culture. Callus at the base of some shoots was seen at the end of 18 weeks of culture. The average number of shoots per culture was found to be 1.25. (Fig. RT 05)

6. MS + NAA (0.5 mg/l).

MS medium supplemented with NAA (0.5 mg/l) was found to be favourable condition for the regeneration of root tips into roots. Regeneration of root tips into roots started after 7 weeeks of culture while the regeneration into shoots started only after 9 weeks of culture. Multiple shoots were not seen even in the completion of 20 weeks of culture. Average number of shoots per culture in this condition was found to be 0.75. The average number of roots was found to be 1.25 per culture. (Fig. RT 06)

7. MS + BAP (0.5 mg/l) + NAA (0.5 mg/l).

The root tips cultured in MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l NAA showed their response to regeneration after 7 weeks of culture. Shoots were found to be arised early than roots. Multiple shoots were found to be arised after 15 weeks of culture. Average number of shoots was found to be 1.25 per culture. Average number of roots was found to be 0.5 per culture. (Fig. RT 07)

8. MS + BAP (1 mg/l) + NAA (0.5 mg/l)

Regeneration of root tips explants of *C. fuscescens* Lindl. obtained from the *in vitro* grown plants cultured in this condition started after 6 weeks of culture. Multiple shoots were formed by the end of 15 weeks of culture. The multiple shoots were with pseudobulbs. Average number of shoots arised per root tips cultured was found to be 2.25 and average number of roots per culture was found to be 0.75. (Fig. RT 08)

9. MS + BAP (1.5mg/l) + NAA (0.5mg/l)

MS medium supplemented with BAP (1.5 mg/l) + NAA (0.5 mg/l) was found to be average condition for the shoot regeneration from the roottips cultured. Regeneration of roottips into shoots was found to start after 11 weeks of culture. Multiple shoots were not found even on the completion of 20 weeks of culture. Shoot was with pseudobulb in one replica and with root as well. The average number of shoots per root tip cultured was found to be 0.5 and average number of roots per culture was found to be 0.5. (Fig. RT 09)

10. MS + BAP (2 mg/l) + NAA (0.5 mg/l)

The MS medium supplemented with BAP (2 mg/l) + NAA (0.5 mg/l) was found to be favourable culture condition for regeneration of shoots. The development of shoots from the root tips explants cultured was found to be started after 6 weeks of culture. Multiple shoots were found to be arised on the completion of 16^{th} week of culture. Development of roots in this condition was rather late. The average number of shoots on the completion of 20 weeks of culture was found to be 2 per culture. By the completion of 20 weeks of culture only 0.25 roots per culture was found. (Fig RT 10)

Table: 7
Mean Value of Shoot Number and Root Number after 20 weeks of culture of root tip explants

Treatments	Mean value of	
	Shoot number	Root number
BM	2.00^{a}	0.75^{ab}
0.5BAP	1.75^{a}	1.00^{ab}
1BAP	2.50^{a}	0.75^{ab}
1.5BAP	1.75^{a}	1.50^{b}
2BAP	1.25^{a}	0.00^{a}
0.5NAA	0.75^{a}	1.25^{ab}
0.5BAP+0.5NAA	1.25^{a}	0.50^{ab}
1BAP+0.5NAA	2.25^{a}	0.75^{ab}
1.5BAP+0.5NAA	0.50^{a}	0.50^{ab}
2BAP+0.5NAA	2.00^{a}	0.25^{ab}

Note:

- The values with same alphabets are not significantly different at 5 % level of significance.
- Four replications were used in each combination.

Table 7 shows the relationship between the mean values of shoot number at different concentration in a column and relationship between mean values of root number at different concentration in another column. The mean values with same alphabets in each column are not significantly different at 5 % level of significance.

4.3.3 Rooting of shoots of *C. fuscescens* Lindl.

The shoots of length 0.8 to 1 cm were used as explant for the rooting. Special rooting media was prepared for the rooting of the shoots. MS medium supplemented with different concentration of auxins (IBA, NAA and IAA) were used for rooting purpose.

Table: 8

Effect of different auxins on rooting of shoot tips of *Coelogyne fuscescens* Lindl after 12 weeks of culture.

Parameters	Concentration of different auxin hormones (mg/l)											
	IAA				IBA				NAA			
	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2
Number of Roots (mean±S.D)	1.0 ± 1.41	1.25 ± 0.5	0.75 ± 0.5	1.0 ± 0.81	1.25 ± 0.50	3.5 ± 1.91	1.5 ± 0.57	1.75 ± 0.25	1.25 ± 1.25	1.75 ± 0.95	1 ± 0.81	1.25 ± 0.95
Length of Roots (mean±S.D)	0.15 ± 0.19	0.32 ± 0.12	0.25 ± 0.05	0.3 ± 0.21	0.57 ± 0.17	1.57 ± 1.64	0.5 ± 0.08	0.63 ± 0.24	0.57 ± 0.41	0.52 ± 0.45	0.35 ± 0.25	0.43 ± 0.25

Culture conditions: - MS medium, 25 ± 2 °C, 12 weeks, 4 replicates were used in each combination.

From table 8 it can be seen that, MS + IBA (1 mg/l) was found to be the best condition for the rooting. The rooting in this condition started after 3 weeks of culture. The roots were hairy in nature and were greenish in colour. The mean number of the roots in this condition was 3.5. The length of the roots ranged from 0.6 to 4 cm after 12 weeks of culture. The increase or decrease of the concentration of IBA was not found to be effective for the rooting of the shoots. (Fig RF 01-RF 04).

MS medium supplemented with NAA (1 mg/l) was found to be average condition for rooting with 1.75 roots per culture, which vary in length from 0.2 to 1.2 cm while the

MS medium supplemented with other concentrations of NAA was found to be poor for rooting. (Fig RF 05-RF 08).

The rooting of shoot tip explants cultured in different concentrations of IAA was found to be further poor in comparison to the rooting in MS medium supplemented with various concentrations of IBA and NAA. Maximum number of roots was found in 1 mg/l IAA i.e 1.25 roots per shoot tip cultured. The increase or decrease of the concentration of IAA was not found to be supportive for the rooting of shoots.
The rooting in MS basal medium was not observed even after the completion of the 12 thweek of the culture. (Fig RF 09-RF 12)

Table: 9
Mean values of Root Number and Root growth after 12 weeks of culture of shoottip explants

Treatments	Mean value of	
	Root number	Root growth (cm)
0.5IBA	1.2500[a]	0.5750[a]
1IBA	3.5000[b]	1.5700[b]
1.5IBA	1.5000[a]	0.5000[a]
2IBA	1.7500[a]	0.6375[a]
0.5IAA	1.0000[a]	0.1500[a]
1IAA	1.2500[a]	0.3200[a]
1.5IAA	0.7500[a]	0.2500[a]
2IAA	1.0000[a]	0.3000[a]
0.5NAA	1.2500[a]	0.5750[a]
1NAA	1.7500[a]	0.5250[a]
1.5NAA	1.0000[a]	0.3500[a]
2NAA	1.2500[a]	0.4375[a]

Note:
- The values with same alphabets are not significantly different at 5 % level of significance.
- Four replicates were used in each combination.

Table 9 shows the relationship between the means of individual column. In the column of root number and root growth all the values are with same alphabets so even though the mean values are different, statistically there is no significant difference between the values of each column.Both the root number and root growth of 1mg/l IBA treatment were highest and significantly different from all other treatments.

4.3.4 Synergistic effect of auxins on rooting of shoot tips of *C. fuscescens* Lindl.

The rooting of the shoot tips cultured in MS medium supplemented with various concentrations of IBA was found to be satisfactory. While the rooting of the shoot tips cultured in MS medium supplemented with various concentrations of IAA and NAA was less in comparison to rooting in various concentrations of IBA. To find out the combined effect of the two rooting hormones in rooting, this experiment was carried out, where MS medium supplemented with various concentrations of IAA and NAA in combination were prepared and shoot tips were cultured. Diagram 9 and 10 shows the various responses of various concentrations of IAA and NAA in combination on rooting of shoot tips of *C. fuscescens* Lindl.

Table: 10

Synergistic effect of auxins on rooting of shoot tips of *C. fuscescens* Lindl.after 12 weeks of culture

Media	Hormones	Concentration of hormones (mg/l)	Parameters	
			Number of roots (mean ± S.D)	Length of roots. (mean ± S.D)
MS	IAA +NAA	0.5 + 0.5	2.75 ± 0.95	0.95 ± 0.42
"	"	1.0 + 0.5	1.50 ± 1.29	0.45 ± 0.33
"	"	1.5 + 0.5	1.50 ± 0.57	0.30 ± 0.14
"	"	0.5 + 1.0	1.75 ± 1.25	0.25 ± 0.18
"	"	1.0 + 1.0	2.25 ± 0.95	0.75 ± 0.26
"	"	1.5 +1.0	1.00 ± 0.81	0.30 ± 0.24

Culture conditions: - MS medium, 25 ± 2 °C, 12 weeks, 4 replicates were used in each combination.

Table 10 shows that the MS medium supplemented with two rooting hormones namely NAA and IAA in combination was effective for the rooting of shoots of *C. fuscescens* Lindl. MS + IAA (0.5 mg/l) + NAA (0.5 mg/l) was found to be the most favourable condition for rooting of shoots among the other media supplemented with various concentrations of IAA and NAA in combination. The average number of roots and average length of roots per culture in this condition was found to be 2.75 and 0.95 cm respectively. (Fig SRF 01 & SRF 02)

Second favourable media for rooting of shoots was found to be MS medium supplemented with equal concentration of NAA and IAA i.e 1mg/l. The average number of roots and average length of roots in this condition was found to be 2.25 and 0.75 cm per culture respectively. (Fig SRF 07 & SRF 08)

The rooting in MS + IAA (0.5 mg/l) + NAA (1 mg/l) was also good in terms of number of roots. The average number of roots and average length of roots in this condition was found to be 1.75 and 0.25 cm respectively. (Fig SRF 06).

In other remaining concentration also the rooting was found to be significantly effective. The average number of roots and average length of roots in MS + IAA (1 mg/l) + NAA (0.5 mg/l) was found to be 1.5 and 0.45 cm respectively per culture. (Fig SRF 03 & SRF 04)

Similarly the average number of roots and average length of roots in MS + IAA (1.5 mg/l) + NAA (0.5 mg/l) was found to be 1.5 and 0.3 cm per culture respectively. (Fig SRF 05).

The least rooting was found in MS + IAA (1.5 mg/l) + NAA (1mg/l) i.e the average number of roots and average length of roots was 1 and 0.3 cm per culture respectively. (Fig SRF 09).

Table:11
Mean value of Root Number and Root Growth.

Treatments	Mean value of	
	Root number	Root growth (cm)
1.5IAA+1NAA	1.0000^{a}	0.3000^{ab}
1IAA+0.5NAA	1.5000^{ab}	0.4500^{abc}
1.5IAA+0.5NAA	1.5000^{ab}	0.3000^{ab}
0.5IAA+1NAA	1.7500^{ab}	0.2500^{a}
1IAA+1NAA	2.2500^{ab}	0.7500^{bc}
0.5IAA+0.5NAA	2.7500^{b}	0.9000^{c}

Note:

- The values with same alphabets are not significantly different at 5 % level of significance.
- Four replicates were used in each combination.

Table 11 shows that there is significant difference between the mean values of the root number in MS +1.5IAA+1NAA and MS +0.5IAA+0.5NAA while there is no

significant difference between the mean values of root number in other concentration. Similarly in case of root growth there significant difference between the mean values of MS +0.5IAA+1NAA, MS +1IAA+1NAA and MS + 0.5IAA+0.5NAA. While in other concentration there is no significant difference between the mean values.

CHAPTER FIVE

DISCUSSIONS

5.1 *In vitro* seed germination of *Coelogyne fuscescens* Lindl. and *Cymbidium aloifolium* (L.) Sw.

The germination of the orchid seed is rather difficult in comparision to the gernimation of other seeds, because they lack endosperm, radicle and leaf rudiments. In nature they need the mycorrhizal symbiosis for the germination and which takes very long time. So tissue culture technique proves itself a reliable and promising method for the conservation of orchids.

Two orchid species namely, *Coelogyne fuscescens* Lindl. and *Cymbidium aloifolium* (L.) Sw. were used for the *in vitro* seed germination. Immature seeds of the two orchid species are cultured in MS basal and MS media supplemented with different hormones in different concentrations. MS medium supplemented with various concentration of BAP and NAA was efficient for the germination of seeds of *Coelogyne fuscecens* Lindl. and *Cymbidium aloifolium* (L.) Sw. The most effective culture condition for the gernmintion of *Coelogyne fuscescens* Lindl was MS medium supplemented with NAA (0.5 mg/l) and BAP (1 mg/l) and that for the *Cymbidium aloifolium* (L.) Sw. was MS medium supplemented with BAP (2 mg/l) and NAA (0.5 mg/l). In both the cases the MS medium supplemented with BAP and NAA in combination was the most appropriate condition. The most appropriate condition for the germination of the seeds was concluded on the basis of time taken for the germination, efficiency of germination and growth and development of the seedlings.

The germination of the *Coelogyne fuscescens* Lindl. was significantly superior to the germination of the *Cymbidium aloifolium* (L.) Sw. It might be due to the genetic constitution of the material and presence of different endogenous growth stimulating substances in the explants.

Exudation of the phenolic compounds from the seeds of *Cymbidium aloifolium* (L.) Sw was observed which was not found to hampere the germination of the seeds. Inspite of the exudation of the phenolic compounds, the germination of the seeds is found to be satisfactory, in terms of time taken for the germination and the efficiency of germination. So the use of antioxidants is denied for the germination of seeds of

Cymbidium aloifolium (L.) Sw. Some cultured tissue often exudes phenolic compounds which upon oxidation to quinines turn the media brown and also prove toxic to *in vitro* cultures.

Many similar works has been carried out in different parts of the world. Results of some works are found to be similar to our result while the results of other are found to vary.

Some works that support the present work are summarised as under.
Swar and Pant (2004) cultured the seeds of *Cymbidium iridiodes* in MS basal and MS medium supplemented with various concentration of BAP and NAA where MS medium supplemented with NAA (1 mg/l) and BAP (1 mg/l) was found to be the most effective condition for the germination.

Jamir *et al.* (2002) found that asymbiotic germination was best in Nitsch medium supplemented with NAA and Kinetin at 1 mg /l each.

Vij *et al.* (1995) concluded that immature seeds of *Dactylorrhiza hatagirea* germinated on agar modified Knudson's C medium and it's various combinations with 1 mg/l of growth regulators IAA, IBA, 2, 4-D, NAA, GA_3, Kn, Peptone, Yeast extract, Casein Hydrolysate, Urea etc

Hadley and Harvis (1968) concluded that auxin/cytokinin ratio was important in controling root and shoot initiation in *Orchis purpurellla* seeds during asymbiotic gernination.

Kabita and Sharma (2001) observed that cent percent germination was obtained on MS medium supplemented with NAA (0.1 mg/l) and Kn (1 mg/l) in *Acampe longifolia* Lindl. It was the lowest in B_5 medium. Formation of spherules and development of distinct green PLBs occurred and leaves also took less time on MS medium than on other media tried.

Some other works whose result are in sharp contrast to the result of present work are summarised as under.

Pradhan (2007) found that the germination of *Dendrobium densiflorum* Lindl. was most favoured on MS Basal medium. Similarly Pant and Gurung (2005) found that the germination of *Aerides odorata* Lour. was most favoured on MS Basal medium.

Reddy *et al.* (1992) in their study on the seed germination and seedling formation in four species of South Indian tropical orchids namely *Cymbidium aloifolium, Dendrobium crepidatum, Epidendrum radicans* and *Spathoglottis plicata.* found MS Basal medium to be the most fouvourable media for the germination.

Shrestha and Rajbhandari (1994) found that MS basal medium was the most favourable medium for the germination of 36 native and exotic orchid spp.

Shrestha A. (2005) successfully achieved asymbiotic germination of *Coelogyne Ovalis* Lindl. MS medium supplemented with NAA (1 mg/l) was the best condition for germination, growth and development of seedlings.

Comparatively less effective response of other hormonal condition on germination and seedlings formation could probably be due to their effect on physiological processes or interaction between the hormones.

5.2 Culture of Shoot tip explants.

The shoot tip explants of the *Coelogyne fuscescens* Lindl. cultured in MS basal medium and MS basal medium supplemented with different hormonal concentrations developed shoots and multiple shoots. In some culture conditions roots are also seen to be arised from some shoots. Pseudobulb were seen in most of the shoots while brown green, grey callus were seen in some culture conditions.

The result of the present study is consistent with result of the Yasugi *et al.* (1994), that MS medium alone was not effective for the induction of the multiple shoots.

The highest number of shoots was developed in MS + BAP (1.5 mg/l) + NAA (0.5 mg/l). Further increase or decrease of the concentration of BAP led to the decrease in shoot development. Least number of shoots was developed in MS + 0.5 mg/l NAA. Statistically the shoot number and shoot growth were significant while leaf number and root number were found to be not significant at 5 % level of significance.

Investigations carried out by various researcher show that the medium supplemented with the high concentration of BAP and low concentration of NAA is most favourable for the induction of the shoot multiplication. Similar result was obtained by Swar and Pant (2004). They obtained maximum number of shoots in MS medium supplemented with BAP (1mg/l) and NAA (0.5mg/l) in *Coelogyne cristata* Lindl. Pradhan (2007) obtained the similar results. She found that MS medium supplemented with BAP (2mg/l) and NAA (0.5 mg/l) was the most favourable culture condition for the shoot multiplication.

Shrestha and Rajbhandari (1988) obtained good shoot proliferation of *Cymbidium giganteum* Wall ex. Lindl. in the MS medium supplemented with BAP (5 mg/l), NAA (1 mg/l) and 10 % coconut milk.

Shrestha and Rajbhandary (1993) carried out the clonal propagation of *Dendrobium densiflorum* Lindl. through shoot meristem culture. Protocorm were initiated from shoot tip explant in Murashige & Skoog's medium (MS) supplemented with 15 % coconut milk, 2.5 mg/l Benzylaminopurine (BAP), 1 mg/l Napthalene acetic acid (NAA) and Casein hydrolysate 1 g/l.

Shrestha and Rajbhandary (1994) obtained the initiation of the potocorm like bodies within 6 weeks from shoot apical meristems of C*ymbidium longiflorum* cultured *in vitro* on Murashige and Skoog medium (1962) supplemented with 2 mg/l Benzylaminopurine (BAP), 1 mg/l Napthaleneaceticacid (NAA), 10 % coconut milk and 3 % sucrose. Rapid multiplication of shoots occurred in MS medium containing BAP (1 mg/l), kinetin (1.5 mg/l) and Adenine sulfate (10 ppm).

Protocorm were developed by Rajkarnikar and Niraula (1994) from the shoot tip explant of *D. fimbriatum* in the MS medium containing BAP (5 ppm), NAA (1 ppm)

and 10 % coconut milk. They obtained multiple shoots by subculturing protocorms in MS medium supplemented with BAP (1 ppm), NAA (1 ppm) and 10 % coconut milk.

Chung *et al*. (1998) obtained enhanced multiple shooting in *C. forrestii* and *C. kanran* on MS medium with NAA (1 mg/l) and BAP (3 mg/l).

The results obtained by various other researchers differ some what.

Pant & Gurung (2005) obtained highest number of shoots in MS medium supplemented with BAP (1.5 mg/l).

Shrestha A.(2005) obtained maximun number of shoots in the MS medium supplemented with BAP (1 mg/l) singly.

5.3. Root tip culture of *Coelogyne fuscescens* Lindl.

The roots generated on the shoot tip explants of *Coelogyne fuscescens* Lindl. cultured on different hormonal concentration were excised aseptically and cultured in fresh MS basal and MS medium supplemented with hormones of different concentrations. The so cultured roots turned brownish and finally regenerated into new roots and shoots. New roots and shoots were found to be arised from the excised end of the root tips. Regeneration of the roots started from 3 weeks of culture and complete plantlets were obtained by the end of 20 weeks. Some root tips developed green callus while other do not show any regeneration. The most fovourable condition of hormones for the regeneration of the root tips in terms of the number and length of new shoots, was MS medium supplemented with BAP (1 mg/l) followed by BAP (1.5 mg/l) + NAA (0.5 mg/l). While the most favourable condition for the formation of the new roots is found to be MS medium supplemented with BAP (1.5 mg/l) followed by NAA (0.5 mg/l).

Though the regeneration of the plantlets from the root tip culture was found to be satisfactory, it is found to be less effective in comparison to the regeneration from the culture of shoot tips. Statistically both shoot number and root number were found to be insignificant at 5 % level of significance.

Many similar works had been carried out by many researchers in which it can be seen that the regeneration from the root tip culture can be obtained from the culture of root

tips on different media supplemented with different concentration of growth hormones. Some of the works are summarized as under.

Sood and Vij (1986) cultured root segments of *Rhynchostylis retusa* Bl. on selective modification of Mitra *et al*. (1976) medium. They regenerated PLBs or shoot buds at the cut or tip ends depending on the medium composition.

Philip and Nainar (1988) cultured the root tips excised from the aerial roots of *Vanilla planifolia* in liquid MS medium supplemented with NA and KN. Shoot meristems with leaf primordia were obtained and root meristem was formed after the development of few more leaves.

Yam *et al*. (1991) observed that plantlets were produced from root tips of *Bletilla striata* and *Cleisostoma fordii* cultured on a modified MS medium containing NAA (1 mg/l) and BA (0.2 mg/l).

Vij (1993) studied the regeneration response of root explants of 21 species and hybrids of orchids and found it to vary with their genetic constitution, physiological age and the chemical regime. Root explants from mature plants failed to show any morphogenetic change when grown *in vivo* but root explants from *in vitro* raised culture responded favourly in selective nutrient combinations. The response was species specific and chemical stimulus present in the medium.

Yasugi *et al*. (1994) observed that the root segment culture in *Cymbidium*.Kenny 'wine colour' proved as an useful method to induce PLB and plantlets and it is essential to culture the basal segments of root on a medium containing NAA and BA under light condition, NAA (1 mg/l) and BA (1 mg/l) induced PLB formation in *Cymbidium.*

Similar work was carried out by Pradhan (2007) on *Dendrobium densiflorum* Lindl. According to her result the regenaration of shoots and roots from culture of root tips was most favoured on MS medium supplemented with BAP (1.5mg/l) singly.

5.4 Rooting of shoots of *Coelogyne fuscecens* Lindl.

The shoot tip explants cultured on MS basal medium and MS medium supplemented with different root initiating hormones (IAA, IBA and NAA), showed various responses on rooting.

Hormone free MS medium failed in inducing roots in shoot tip explant which is in sharp contrast with the findings of Shrestha and Rajbhandary (1988) in which MS basal medium induced good rooting in *Cymbidium giganteum* within two months. Shrestha and Rajbhandary again in 1994 gave similar results in *C .longifolium.*

Though the rooting in MS medium supplemented with (1mg/l) IBA was satisfactory, the rooting in the various concentrations of other two root initiating hormones i.e IAA and NAA was found to be poor. MS medium supplemented with various concentrations of IBA is the much better rooting condition for *in vitro* grown plants in comparision with the MS medium supplemented with IAA and NAA separately which has been proved by the works of various preceeding researchers.

Statistically root number and root length both were found to be insignificant at 5 % level of significance.

Pradhan (2007) observed that the rooting of *Dendrobium densiflorum* Lindl. was most favoured in MS medium supplemented with IBA (1.5 mg/l).

Shrestha A. (2005) used MS medium supplemented with different rooting hormones in different concentrations. MS + IBA (2 ppm) was found to be best for rooting of *Coelogyne ovalis* Lindl.

Similarly MS + IBA (1 mg/l) was the most favourable condition for the rooting of *Cymbidium iridiodes* D.Don and *Coelogyne cristata* Lindl. in the research work carried out by Pant and Swar (2004)

The rooting was favored in all media supplemented with IBA (1 mg/l) in the research work carried out by Pathenia *et al.* (1998) in *Dendrobium* sp.

These findings contrast with the findings of Banerjee and Mandal, (1999) who reported 2 mg/l of NAA best for the rooting of *Cymbidium.*

Similar works was carried out by Pant and Gurung (2005) on *Aerides odorata* Lour.but according to them the most favourable condition for rooting of *Aerides odorata* Lour.was, 0.5 ppm IAA. Which is in sharp contrast with the findings of the present study.

In the present research work rooting in MS media supplemented with various concentrations of IAA and NAA was found to be poor in comparision to the result given by various concentrations of IBA. So to find out whether the above two hormones give good results in combination. Experiment to find out synergestic effect of two hormones on rooting was carried out.

5.5 Synergistic effect of IAA and NAA on rooting of *C. fuscescens* Lindl.

The MS medium supplemented with two rooting hormones namely IAA and NAA was found to be more effective for the formation of roots in comparision to the MS medium supplemented with IAA and NAA individually. The rooting was found to be most favored in MS medium supplemented with IAA (0.5 mg/l) plus NAA (0.5 mg/l) (2.75 roots /culture) with respect to the rooting in MS medium supplemented with the individual concentration of IAA (0.5 mg/l) and NAA (0.5 mg/l) with 1 root/culture & 1.25 roots per culture in the respective concentration.

The present research work on synergistic effect of two rooting hormones (IAA and NAA) on rooting of *C. fuscescens* Lindl was found to be fruitful in comparison to the individual treatment of the respective hormones. The combined treatment of two rooting hormones strongly supported the formation of well developed roots.

Statistically, the root number of the synergistic effect of two rooting hormones (IAA and NAA) was found to be insignificant while the root length was found to be significant at 5 % level of significance.

CHAPTER SIX

CONCLUSION

Present study is focussed on the mass propagation of the two threatened species of orchids namely *C. fuscescens* Lindl. and *C. aloifolium* (L.) Sw. All the experiments were carried out in the lab of Central Department of Botany, T.U, Kirtipur. From the results obtained from the experiment following conclusions can be made.

- MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) was found to be most effective culture condition for the germination of *C. fuscescens* Lindl. Similarly MS medium supplemented with BAP (2mg/l) and NAA (0.5 mg/l) was found to be the most favourable condition for the germination of *C. aloifolium* (L.) Sw.

- MS medium supplemented with BAP (1.5 mg/l) + NAA (0.5 mg/l) was found to be the most effective media for the shoot multiplication of *C. fuscescens* Lindl.

- MS medium supplemented with BAP (1 mg/l) was found to be most favourable condition for the regeneration from root tips culture.

- For the mass propagation of *C. fuscescens* Lindl. culture of shoot tips is found to be more effective than that of root tips.

- IBA at 1 mg/l concentration was highly effective for the rooting of shoots. Increase or decrease in concentrtion of IBA decresed the number of roots. IAA and NAA were found to be less significant for rooting of the shoot explants of *C. fuscescens* Lindl.

- Though MS medium supplemented with IAA and NAA singly didn't give good results in rooting, their combined treatments gave satisfactory results. In the period of 12 weeks, rooting in all combintion was good. Among them MS medium supplemented with NAA (0.5 mg/l) and IAA (0.5 mg/l) was found to be the most effective condition for rooting.

CHAPTER SEVEN

RECOMMENDATIONS

Following recommendations have been outlined from the present investigation.

- Beside MS medium other culture media and tissue culture techniques can be examined so that more promising medium for the quick and efficient seed germination, seedling formation, shoot multiplication and rooting can be known and a reproducible protocol for the micro propagation of *Coelogyne fuscescens* Lindl. and *Cymbidium aloifolium* (L.) Sw. can be developed.

- Mass propagation of other commercially valuable orchids should be promoted for their conservation.

- People should be made aware for the conservation of the orchids.

- Additional investigation is disperately wanted to address the survival of the *in vitro* grown plantlets in natural habitat.

- Tissue culture lab of Central Department of Botany should be well equipped and well facilated to carry out the research work more efficiently which helps to develop effective protocol for the mass propagation of various orchids of multiple use.

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PHOTO PLATE I

Fig 01: Flower of *Coelogyne fuscescens* Lindl. in natural habitat.

Fig 02: Pseudobulbs, leaves and pods of *C. fuscescens* Lindl.

Fig 03: Epiphytic nature of *C.fuscescens* Lindl

Fig 04: Figure showing the pods of *C.fuscescens* Lindl.

Fig 05: Longitudinally transected pods of *C.fuscescens* Lindl. to

expose the microscopic seeds inside.

Fig 06: Flower of *Cymbidium aloifolium* (L.) Sw.

Fig 07: Pods of *C. aloifolium* (L.) Sw. drooping down being attached in natural habitat.

Fig 08: Pods of *C. aloifolium* (L.) Sw. dropping down from epiphytic environment

provided artificially in angle of Gulmohar tree.

Fig 09: Pods of *C. aloifolium* (L.) Sw.

Fig 10: Pods of *C. aloifolium* (L.) Sw. transected longitudinally to expose the yellow

microscopic seeds inside.

PHOTO PLATE II

Figures SG 01 - 05: Seed germination of *Coelogyne fuscescens* Lindl. in different culture conditions.

Fig SG 01 Seeds cultured in MS + 0.5 mg/l BAP +0.5 mg/l NAA showing protocorm formation after 12 weeks of culture.

Fig SG 02 Seeds cultured in MS + 1.5 mg/l BAP (17 weeks)

Fig SG 03 Seeds cultured in MS + 1 mg/l BAP + 0.5 mg/l NAA (15 weeks)

Fig SG 04 Seeds cultured in MS +1.5 mg/l BAP + 0.5 mg/l NAA (35 weeks)

Fig SG 05 Seeds cultured in MS Basal medium (42 weeks)

Figures SG 06 - 10: Seed germination of *Cymbidium aloifolium* (L.) Sw. in different culture conditions.

Fig SG 06 Seeds cultured in MS + 1 mg/l BAP.

Fig SG 07 Seeds cultured in MS + 2 mg/l BAP (22 weeks)

Fig SG 08 Seeds cultured in MS + 2 mg/l BAP + 0.5 mg/l NAA (25 weeks)

Fig SG 09 Seeds cultured in MS + 0.5 mg/l BAP+0.5 mg/l NAA (29 weeks)

Fig SG 10 Seeds cultured in MS +1.5 mg/l BAP (36 weeks).

PHOTO PLATE III

Figures H: 01- 08: Histomorphological study of Plbs of *Cymbidium aloifolium* L.

Fig H: 01 Seeds with transparent coat.

Fig H: 02 Embryo in initial stage of germination.

Fig H: 03 Embryo emerging out of seed coat through longitudinal splits.

Fig H: 04 Embryo in its initial stage of rupturing through terminal cells.

Fig H: 05 Figure showing the organogenesis from embryo.

Fig H: 06 Figure showing the initiation of the leaf primordia.

Fig H: 07 Figure showing the initial stage of emergence of rhizoids.

Fig H: 08 Figure showing the profuse development of rhizoids.

PHOTO PLATE IV

Figure ST: 01- 12 : Development of Shoot Tip Explants of *Coelogyne fuscescens* Lindl. in different culture conditions.

Fig ST 01 Explant of *C. fuscescens* Lindl. at Basal medium at 16 weeks of Culture.

Fig ST 02 Explant on MS + 0.5 mg/l BAP showing callus and Pseudobulb.

Fig ST 03 Explant on MS +0.5 mg/l BAP showing callus.

Fig ST 04 Explant on MS + 1mg/l BAP showing multiple shoots.

Fig ST 05 Explant on MS +1.5 mg/l BAP; multiple shoots with Pseudobulb.

Fig ST 06 Explant on MS + 2 mg/l BAP;multiple shoots with Pseudobulb and callus.

Fig ST 07 Explant on 0.5 mg/l NAA showing shoot with Pseudobulb and small roots arising from the base of the shoot.

Fig ST 08 Explant on 0.5 mg/l BAP + 0.5 mg/l NAA showing rooted multiple shoots with callus.

Fig ST 09 Explant on 1 mg/l BAP + 0.5 mg/l NAA showing multiple shoots with Pseudobulb.

Fig ST 10 Explant on 1 mg/l BAP + 0.5 mg/l NAA showing multiple shoots with callus.

Fig ST 11 Explant on 1.5 mg/l BAP +0.5 mg/l NAA showing multiple shoots with Pseudobulbs

Fig ST 12 Explant on 2 mg/l BAP +0.5 mg/l NAA showing rooted multiple shoots with callus.

PHOTO PLATE V

Figures RT: 01-10: Regeneration of root tip explant of *Coelogyne fuscescens* Lindl.

in different culture conditions.

Fig RT 01 Explant (root tip) of *Coelogyne fuscescens* Lindl. at 20 weeks of culture on

MS Basal medium.

Fig RT 02 Explant on MS with 0.5 mg/l BAP.

Fig RT 03 Explant on MS + 1mg/l BAP.

Fig RT 04 Explant on MS + 1.5 mg/l BAP.

Fig RT 05 Explant on MS + 2 mg/l BAP

Fig RT 06 Explant on MS + 0.5 mg/l NAA.

Fig RT 07 Explant on MS +0.5 mg/l BAP + 0.5 mg/l NAA.

Fig RT 08 Explant on MS +1 mg/l BAP +0.5 mg/l NAA.

Fig RT 09 Explant on MS +1.5 mg/l BAP + 0.5 mg/l NAA.

Fig RT 10 Explant on MS + 2 mg/l BAP + 0.5 mg/l NAA.

PHOTO PLATE VI

Figure RF: 01- 12 : Root formation of *Coelogyne fuscescens* Lindl. after 12 weeks of culture on different concentration of hormones.

Fig RF 01 Root formation of *Coelogyne fuscescens* Lindl. on MS + 0.5 mg/l IBA

Fig RF 02 Root formation on MS + 1 mg/l IBA.

Fig RF 03 Root formation on MS + 1.5 mg/l IBA.

Fig RF 04 Root formation on MS + 2 mg/l IBA

Fig RF 05 Root formation on MS + 0.5 mg/l NAA.

Fig RF 06 Root formation on MS + 1 mg/l NAA.

Fig RF 07 Root formation on MS + 1.5 mg/l NAA.

Fig RF 08 Root formation on MS + 2 mg/l NAA.

Fig RF 09 Root formation on MS + 0.5 mg/l IAA.

Fig RF 10 Root formation on MS + 1 mg/l IAA

Fig RF 11 Root formation on MS + 1.5 mg/l IAA.

Fig RF 12 Root formation on MS + 2 mg/l IAA.

PHOTO PLATE VII

Figures SRF: 01- 09: Synergistic effect of Auxins (IAA and NAA) on rooting of *Coelogyne fuscescens* Lindl. after 20 weeks of culture.

Fig SRF 01 Root formation on MS + 0.5 mg/l IAA +0.5 mg/l NAA.

Fig SRF 02 Root formation on MS + 0.5 mg/l IAA +0.5 mg/l NAA.

Fig SRF 03 Root formation on MS + 1 mg/l IAA + 0.5 mg/lNAA

Fig SRF 04 Root formation on MS + 1 mg/l IAA + 0.5 mg/lNAA

Fig SRF 05 Root formation on MS +1.5 mg/l IAA + 0.5 mg/l NAA.

Fig SRF 06 Root formation on MS + 0.5 mg/l IAA + 1 mg/l NAA.

Fig SRF 07 Root formation on MS + 1mg/l IAA +1 mg/l NAA.

Fig SRF 08 Root formation on MS + 1mg/l IAA +1 mg/l NAA.

Fig SRF 09 Root formation on MS + 1.5 mg/l IAA +1 NAA.

PHOTO PLATE VIII

Fig A 01- 04 Materials and Procedures of Acclimatization of *in vitro* grown plantlets of *Coelogyne fuscescens* Lindl.

Fig A 01 Fungicides and source of Nitrogen used during acclimatization.

Fig A 02 Measurement of the plantlets selected for the acclimatization.

Fig A 03 *In vitro* grown plantlets planted in pots containig coco – peat.

Fig A 04 Researcher adding club moss on the pots with plantlets.

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		Sum of squares	df	Mean Square	F	Sig.
Shoot Number	Between Groups	69.40	9	7.711	2.302	0.042
	Within Groups	100.5	30	3.35		
	Total	169.9	39			
Shoot growth	Between Groups	11.898	9	1.322	4.26	.001
	Within Groups	9.31	30	.310		
	Total	21.208	39			
Leaf Number	Between Groups	167.725	9	18.636	1.907	.090
	Within Groups	293.250	30	9.775		
	Total	460.975	39			
Root Number	Between Groups	7.000	9	.778	1.414	.226
	Within Groups	16.500	30	.550		
	Total	23.500	39			

2. Statistical Analysis

Analysis of Variance for Shoot Tip Culture.

Analysis of Variance for Root Tip Culture.

		Sum of squares	df	Mean Square	F	Sig.
Shoot Number	Between Groups	15.1	9	1.67	0.832	.593
	Within Groups	60.5	30	2.017		
	Total	75.6	39			
Root Number	Between Groups	7.225	9	0.803	1.284	0.286
	Within Groups	18.750	30	0.625		
	Total	25.975	39			

Analysis of variance for Rooting of Shoot Tips

		Sum of squares	df	Mean Square	F	Sig.
Root Number	Between Groups	22.563	11	2.051	1.982	.060
	Within Groups	37.250	36	1.035		
	Total	59.813	47			
Root length	Between Groups	5.860	11	.533	1.806	.090
	Within Groups	10.621	36	.295		
	Total	16.482	47			

Analysis of Variance for Synergistic effect of auxins on Rooting of Shoot Tips.

		Sum of Squares	df	Mean Square	F	Sig.
Root Number	Between Groups	7.708	5	1.542	1.521	.233
	Within Groups	18.250	18	1.014		
	Total	25.958	23			
Root Length	Between Groups	1.468	5	.294	3.335	.026
	Within Groups	1.585	18	.088		
	Total	3.053	23			

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