CHAPTER-ONE

INTRODUCTION

1.1 Background

Nepal, a Himalayan Country is rich in floral diversity with wide altitudinal variation, topography and diverse climatic condition. 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 alpine zone of which about 252 species are endemic to Nepal (Chaudhary, 1998; Hara *et al*. 1978-1982; HMGN, 2002).

Orchids belong to the family Orchidaceae. The word '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 Theophratus, father of Botany (370-285 BC), in his book "De Historia plantarum".

The family Orchidaceae is one of the largest families of flowering plants comprising more than 19,505 species belonging to 803 genera (Dressler, 1993). Nepal consists about 377 spp. of native orchids belonging to 100 genera (Rajbhandari and Dahal, 2004), out of which 10 species are endemic to Nepal. So, Orchidaceae has been recorded as the second largest plant family in Nepal in terms of number of species.

Orchids are the most fascinating groups of plant among the angiosperms. Nepalese orchids are very popular due to their shape, size, structure, fragrance, colorful flowers, shining green leaves, pseudobulbs etc, so it is used for horticulture purpose. Orchids, with their be wildering range of flowers in terms of exquisite colour combination, provides a source of profound aesthetic pleasure. Apart from the aesthetic and ornamental values, the orchids also have medicinal and edible values. Orchids are popularly known as “SUNGAVA”, “CHANDIGAVA”, and “SUNAKHARI” in Nepal.

The most beautiful orchids of Nepal belong to the following genera: *Aerides, Arundina, Bulbophyllum, Calanthe, Coelogyne, Cymbidium, Dendrobium, Epigenecum, Eria, Esmeralda, Phaius, Phalaenopsis, Pleione, Rhynochostylis, Thunia, Vanda* and *Vandopsis,* (Rajbhandari and Bhattarai, 2001). Species cultivated for their ornamental purpose include:

Aerides multiflora, Ascocentrum ampullaceum, Bulbophyllum leopardinum, Calanthe masuca, C. plantagina, Coelogyne cristata, C. fuscescens, Cymbidium elegans, C. irridioides, Dendrobium densiflorum, *D. nobile*, etc.

Some important medicinal orchids of Nepal are mentioned by Raj Bhandari *et al.* (2000) are *Brachycorythis obcorsata, Coelogyne flavida, Coelogyne stricta, Cymbidium aloifolium, Dactylorhiza hatgirea, Eulophia nuda, Flickingeria macraie, Pholidota imbricata, Luisia zeylanica, Vanda tessellata.*

Some edible orchids are *Dactylorhiza hatagirea, Platanthera clavigera* and *Satyrium nepalensis*, (Dahal and Shakya, 1989).

Endemic species of orchids of Nepal are *Bulbophyllum nepalensis, Eria baniaii, Eria nepalensis, Liparis olivacea, Listera nepalensis, Malaxis tamurensis, Oberonia nepalensis, Oreorchis porphyranthes, Pleione coronaria*.

Bulbophyllum moniliforme par et. Reichb. F. and *Liparis plantaginea* Lindl are newly recorded orchid flora to Nepal. (Subedi, 2002).

Due to their high global demand for ornamental and medicinal purposes, they have been highly exploited from their natural habitat.There is a serious threat to the conservation of orchids in Nepal. Loss of habitat, deforestation, destructive collection technique and over exploitation of orchids with medicinal and ornamental values has depleted the orchid wealth of Nepal.

Orchids have been well known for their horticultural values. The commercial growers of orchids are USA, UK, Indonesia, Singapore, Thailand, Norwey etc. The orchids of Nepal having high expert potential are mainly *Cymbidium, Dendrobium, Calanthes* and *Coelogyne.*

Orchids are regarded as one of the most vulnerable commodities of extinction due to trade and commerce. Wild orchids are the main sources for rare varieties and new cross breeds. So, 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.

Orchids seed, unlike the seeds of other flowering plants are extremely small, dust like, produce in large numbers (1,300-4,000,000 per capsule). Because of small size of the seed, the order to which orchids are placed has been named as *Microspermae*. The embryo has a rounded or spherical form without cotyledons, radicle and endosperm. In natural condition, the majority of orchid flowers are not pollinated, their ovules are not fertilized and capsule is rarely formed. 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 depend upon mycorrhizal associations with various fungi, mostly genus *Rhizoctonia* (Class Basidiomycetes), for at least part of their life cycle. So, under natural condition, the seeds of orchid have only 5 % germination, because of particular fungal requirement (Rao, 1997).

Vegetative Propagation is very slow process to propagate a large quality of clone orchids therefore, *ex-situ* conservation of highly exploited species can be done by tissue culture technique. Use of exogenous growth regulators in artificial media at suitable concentrations stimulates zygotic embryo to initiate protocorms that develop into plantlets.

Micropropagation is an *in vitro* propagation by which a large number of clones of desired plants are produced in an artificial nutrient medium under aseptic condition in short time irrespective of physiological and climatic barriers. Explants can be a very small piece of plants such as embryos, seeds, stem, shoot tip, meristems, root tips, single cell, protoplasts and pollen grains. Through this technique, from a very small part of plant, numerous plantlets can be produced in short duration but labour intensive process. It is useful in mass propagation of specific clones, production of pathogen free plants, clonal propagation of parental stock for hybrid seed production, year-round nursery production and germplasm preservation. Orchids are the very first flowering plants of commercial value to be propagated *in vitro* both through seeds and tissue culture (Pant, 2006).

Bernard, (1909) for the very first time inoculated orchid seeds and fungi in the culture tubes and developed protocol for the symbiotic germination. The second attempt was made by Knudson (1922) who developed a protocol for the asymbiotic germination of the orchid seeds in culture tubes eliminating the requirement of fungus for the germination. After this, followed a vigorous research to improve the Knudson medium for better germination rate.

Morel (1960) tried to develop virus free *Cymbidium* by meristem culture. He produced more than 4,000,000 plants of *Cymbidium* in one year from single shoot apex.

In Nepal, micropropagation of some of the Nepalese orchids is being done by National Herbarium and Plant Laboratory (NHPL), Godawari under Department of Plant Resources of Government of Nepal (DPR), Thapathali and institutions like Central Department of Botany, Tribhuvan University (T.U) and Kathmandu University. Some private organization like Nepal Biotech Nursery, Standard Nursery, Parijat Nursery etc are involved in the cultivation of endogenous orchid species in Nepal.

1.2 Cymbidium elegans Lindl.

The name *'Cymbidium'* derived from the Greek word 'Kumbos' meaning hole like appearance of the lip of flowers. Ten species of *Cymbidium* are reported from Nepal. (Press *et al*. 2000) It is a special orchid because of it's long lasting beautiful colour range characteristics.

Cymbidium elegans Lindl, syn, *C. longifolium* D. Don, a native Himalayan orchid from Nepal. It is one of the important ornamental species of the genera and desirable orchids in the world because of the beautiful flowers. The plant is epiphytic, pseudobulbs are short.The leaves are narrowly linear acuminate, slightly expanded and equitant at base, 45-60×1.5-1.8 cm. The flowers are olive green to pale lemon yellow with parallel brownish streaks, 3-4.5 cm long, 1.5 cm across. The lips are as long as petals, narrowly oblong, side lobes incurved rounded, disc between them with 2 narrow parallel ridges, united at the apex, mid lobe small, abcordate, undulate, incurved.The rostellum is beaked.

Flowering time: - September to November

Distribution; It is found in Nepal, India, Bhutan, China, Myanmar. In Nepal, it distribution is confined to Central and East Nepal in the altitudinal range of 2100–2500m.

It has both horticultural and medicinal values .The leaves and pseudobulbs are used for nerve tonic, hysteria, madness, epilepsy and rheumatism. It is used as demulcent and for stomach as a medicinal plant. (Vaidya *et al.* 2000).

1.3 OBJECTIVES

The objectives of the present investigations are as follows.

1. To determine the effect of different phytohormones on *in vitro* development and differentiation of protocorms like bodies of *Cymbidium elegans* Lindl.

2. To determine the appropriate culture condition for *in vitro* mass propagation of *C. elegans* Lindl.

3. To acclimatize the *in vitro* grown plants of *C. elegans* Lindl. for domestication.

1.4 JUSTIFICATION OF THE STUDY

Nepalese orchids are under considerable threat due to habitat destruction, degradation, trade and commerce. Weak implementation of conservation strategies is further adding to threatened problem. The collection of orchid has been banned under the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES). The implementation of regulation is poor and harvesting from wild sources still continues for commercial trade. So, effective strategies should be implemented to conserve these precious gems of nature.

Proper and sustainable use of valuable orchids of Nepal may be helpful to raise the economic status of our country. Micropropagation proves itself as an important technique in plant conservation, by which large number of plantlets can be produced in short period of time. *In vitro* grown plants are generally disease free and are used to preserve germplasm. There are ample opportunities of national economic gains if endogenous species are carefully selected and propagated *in vitro* for the commercial purpose (Pant, 2002).

The species that is selected for the present study has great commercial potential and they belong to the genera that are mostly exported to international market. So conservation of these species should be carried out in time to preserve them from being extinct. This study is carried out as a small process for *ex situ* conservation of the species and to develop the protocol for mass propagation of this species.

CHAPTER-TWO

LITERATURE REVIEW

Bernard (1909) was the first to develop protocol of Symbiotic (i.e. dixenic) seed germination of orchids. He discovered the requirement of fungal infection for germination of orchid seeds and the fungus was found to be *Rhizoctonia* species, which occurs in the roots of orchid plant.

Knudson (1922) was the first to develop asymbiotic seed germination of orchids. He developed a suitable nutrient media for the germination of the seeds without fungal infection. The original method was modified as "Knudson C'' media.

Morel (1960) attempted to obtain Virus free *Cymbidium* by meristem culture in detail (Rao, 1997).

Murashige and Skoog (1962) Or Linsmaier and Skoog (1965) are the most widely used salt compositions, especially in procedures where the plant regeneration is the objective (Gamborg and Phillips, 1995)

Kand (1967) germinated the seeds of orchids like *Cymbidium vijiscens* 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. They found that the auxin and cytokinin ratio is important in controlling root and shoot initiation.

Matsui *et al.* (1970) studied the effect of BAP, NAA and their combined effects on the formation of protocorm like bodies and the development of shoots and roots meristem cultures of *Cymbidium*. NAA alone had no effect upon the formation of PLBs, BAP (0.1 ppm) induced the greatest effect.

Fonnesbech (1972) studied the effect of auxins (IAA, NAA & 2, 4-D) Cytokinin (kinetin & 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 and embryos, inflorescence, young leaf tip as explants.

Arditti (1979) reviewed some 40 genera of orchid being propagated through shoot tip culture method.

Kononowicz and Jules (1984) achieved *in vitro* shoot proliferation of *Vanilla* by axillary branching in a basal medium (MS) with 0.5 mg BA (per liter). Rooted shoots were established in green house using organic medium.

Niroula and Rajbhandary (1985) established shoot proliferation and protocorms from the seedling tips of *Dendrobium fimbriatum* Hook. in MS medium supplemented with cytokinin. These shoots and protocorms when transferred in Vacin and Went medium grew into complete plantlets.

Sood and Vij (1986) inoculated root segments on selective modifications of Mitra *et al*. obtained from 6 months 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.

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 primodial. After few leaves formed root meristem differentiated.

Shrestha and Rajbhandary (1988) obtained shoot proliferation through meristem of *Cymbidium giganteum* Wall ex. Lindl in the MS (1962) medium, MS supplemented with BAP (5 mg /l), NAA (1 mg/l) and 10 % coconuts milk. The shoots were transferred to pots containing the fern fiber and survived in the green house.

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 with 1 mg/l NAA and 0.2 mg/l BA.

Niraula and Rajbhandary (1992) cultured explants of *Vanda teres* Lindl excised from aseptically grown seedlings on MS(1962)medium supplemented with auxin and cytokinin. PLBs were developed from the explants. These PLBs were transferred to a Vacin and Went medium and grew into complete plantlets.

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 orchids. The frequency of germination and protocorm development and the extent of seedling 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 Went, 1949) medium. The ground growing *Spalthaglottis plicata* responded better to MS media and the epiphytic, *Epidendrum radicans, Dendrobium crepidatum* & *Cymbidium aloifolium* to RL medium.

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

Shrestha and Rajbhandary (1993) established the clonal propagation of *Dendrobium densiflorum* by shoot tip culture. MS medium containing 2.5 mg/l BAP, 1 mg/l NAA, 15 % coconut milk and 1 g/l casein hydrolysate developed protocorms from the shoot tip explant and further growth was carried out in MS medium supplemented with 0.25 mg/l BAP, 0.01 mg/l NAA, 20 mg/l Adenine Sulphate and 10 % coconut milk. Roots were developed in VW medium. The rooted shoots were established in green house in the pots containing tree fern fibres.

Vij (1993) studies 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 favourably in selective nutrient combinations. The response was species specific and chemical stimulus present in the medium. Peptone was obligatory in inducing proliferation *in Cymbidium pendulum, Vanda cristata* and *V. testacea*. While yeast extract was found obligatory in case of *Aerides multiflorum* and *Vanda teres*.

Rajkarnikar and Niraula (1994) initiated protocorms of *Dendrobium fimbriatum* from shoot tip explants in MS medium supplemented with 5 mg/l BAP, 1 mg/l NAA and 10 % coconut milk for multiple shoot production .Microshoots produced roots when transferred on MS medium with 0.5 mg/l NAA.

Shrestha and Rajbhandary (1994) studied the clonal multiplication of *Cymbidium longiflorum* D. Don by shoot tip culture. Clonal multiplication of *Cymbidium longifolium* 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 BAP, 1 mg/l NAA, 10 % coconut milk & 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 sub culturing 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.

Devi *et al*. (1997) observed clonal propagation of *Dendrobium moschatum and Cymbidium aloifolium* through shoot tip culture in five different media Viz. MS (1962), WI (Wimber,1963) KnC (Knudson,1946) VW (1949) and NI (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,s further growth of mericlone from rhizome of shoot tip culture of *Cymbidium* species. MS medium with 1 mg/l NAA and 3 mg/l BA enhanced multiple shooting in *C. forrestii* and *C. kanran* while *C. forrestii* also gave high number of shoots in MS medium with 1 mg/l NAA and 1

mg/l BA. *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* 'Sangmae' and *C. forrestti* 'yangshiso' grew well in medium containing NAA (2 mg/l) and BA (1 mg/l). 0.5 mg/l NAA and BA containing medium resulted good shoot growth in *C. forrestti* 'Changsoo', *C. kanran* 'shoshim' and *C. niveo* 'marginatum'.

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 mg/l of folic acid in VW medium resulted highest germination rate (85 %) while 0.1 mg/l gave 78 % germination rate. 2 mg/l NAA containing VW medium was found suitable for inducing 3-4 roots within 2 months.

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

Chen *et al*. (2001) developed protocol for the propagation of *Onicidium*. It contained three steps PLB induction, PLBs multiplication and PLBs shooting and rooting. Lateral bud of 10-15 cm long were developed. PLBs upto 1000 in 9-12 months and then shoots and roots were induced.

Kabita and Sharma (2001) studied the *in vitro* germination and protocorm development of *Acampe longifolia* Lindl. by using MS, Knudson C ,VW and B_5 media supplemented with different concentrations of NAA, IBA and KN. MS medium containing 0.1 µg/ml NAA and 1 µg/ml KN resulted 100 % germination while B_5 medium showed low germination rate. Spherules formation and the development of green PLBs as well as leaves were faster in MS medium than in the other three medium used.

Nagaraju and Upadhyaya (2001) studied the *in vitro* morphogenetic response of *Cymbidium lunavian* Altas. 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 the asymbiotic germination was best in Nitsch medium supplemented with NAA and kinetin at 1 mg/l each in *Cymbidium iridioides* D.Don.

Roy and Banerjee (2002) studied the optimization of *in vitro* seed germination, growth and seedling proliferation of *Vanda tessellate*. Modified KnC, VW and 0.5 MS medium enriched with organic supplements were used for seed germination and all showed similar (66 %-73 %) response. However 0.5 % peptone showed inhibitory effect. NAA and BA together was the best for axillary shoot proliferation. BA was found to be essential for PLB formation and NAA only increased its frequency. High NAA: IAA ratio resulted in callusing and subsequent PLB formation.

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

Huan *et al*. (2004) observed the callus induction from PLBs and plant re-generation 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 1Naphthalene acetic acid (NAA) or 2,4 D alone or in combination with N-phenyl –N-1,2,3-thiodizol-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 callus induction. The calli easiely formed PLBs after being transferred to media without plant growth regulators. Healthy plantlets without any phenotypic abnormality were obtained from callus derived PLBs.

Wang *et al.* (2004) established an efficient and simple method of high protocorm regeneration. Pedicel axillary buds of P*haleonopsis* were cultured in MS medium containing 2-3 mg/l BA and 0.1-0.5 mg/l NAA. Highest protocorm formation (80 %) was obtained by using 3 mg/l BA and 0.1 mg/l BA and 0.1 mg/l NAA and further subculture in the same

medium gave shoots in 4 weeks time. 0.1 mg/l IAA resulted root formation in 40 % of the shoots obtained by subculture of protocorm.

Hsia *et al.* (2005) carried out mass propagation of *Dendrobium* from protocorm like body (PLBs). The PLBs of the *Dendrobium* were transversely cut and adopted as explants. They were cultured on MS basal salt media contaning 30 gm/l of sucrose, 80 gm/l of potato homogenate, 9 gm/l of agar and in combination with 2 mg/l of growth regulators such as NAA, IBA, BA, Kinetin, TDZ and Zeatin for PLB proliferation investigation. The shoot formation rate of IBA- induced PLBs was high.

J. and Basavaraju (2005) obtained *in vitro* culture of *Vanilla*. Morphogenetic responses of nodal explants of *Vanilla* on 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. The multiplication was observed in MS medium with BAP + IBA produced good number of shoots.

Karki *et al*. (2005) micropropagated *Vanilla planifolia* Andrews. from seeds using Murashige and Skoog medium without growth hormones. The seedlings were subcultured in MS medium supplemented with 1.0 mg/l BAP and 1.5 mg/l Kinetin with 10 % coconut milk. The mature shoots were transferred in cocopit for rooting.

Shrestha A. (2005) successfully achieved asymbiotic germination *Coelogyne ovalis* Lindl. MS medium supplemented with 1 mg/l of NAA was the best medium for the germination, growth, and development of seedlings. Germination starts after 8 weeks of inoculation. The maximum number of shoot multiplication was observed in MS media with 1 mg/l of BAP singly and MS media in combination of BAP 0.5 mg/l with NAA 0.5 mg/l showed callus induction in the base of some shoots. Best rooting was obtained in MS media with 2 mg/l of IBA.

Faria *et al.* (2006) carried out *in vitro* propagation of *Oncidium baueri* without the use of agar. The seeds were germinated in Murashige and Skoog medium (MS, 1962) modified with half of the macronutrients concentration. This was efficient and less costly alternative to replace the agar.

Pant and Gurung (2006) cultured the seeds of *Aerides odorata* Lour. in MS medium and hormonal MS medium under aseptic conditions. MS media without any hormone supplement was found to be the best for the germination and growth of seedling. The best shoot multiplication (6.3 shoots/ culture) was obtained in MS medium supplemented with 1.5 mg/l BAP and 1 mg/l NAA. The best rooting was observed on 0.5 mg/l IAA (4.7 roots/ shoot).

Silva *et al. (2*006) observed priming biotic factor for optimal protocorm like body and callus induction in hybrid *Cymbidium*. PLBs and callus were induced in hybrid *Cymbidium* Twilight moon 'Day light' where induction capacity was explant dependent. Among various explant (PLBs, leaf tip or base, root tip or base, cell and tissue suspension), highest PLBs formation and callus induction was observed, when they used whole PLBs, PLB segment or PLB trasverse thin cell layers or longitudinal TCLs. Acclimatization and survival rate was shown to be 100 % in the generated plants.

Zheng *et al.* (2006) carried out *in vitro* flowering of cultures from a hybrid of *Cymbidium goeringii* and *C. hybridium*. *Cymbidium goeringii*, wild type female spring orchid was crossed with male *Cymbidium hybridium* . Over 800 protocorms clones were obtained from hybrid offsprings. Among them, one protocorm clone was identified to differentiate visible floral buds in 2 months after subculture *in vitro*. The protocorms and shoot derived from these clone was further used in studying the effect of 6-Benzyleadenine on floral bud differentiation. The optimum combination of hormones in floral bud induction was 6-BA 10 mg/l and NAA 0.1 mg/l and total frequency of floral bud formation was up to 31 %.

Zhu *et al.* (2006) carried out the propagation of *Cymbidium kanran*. They cultured rhizome as explants to study the effects of growth regulators on the induction, multiplication of protocorm like bodies (PLBs) and the rooting. The result showed that the optimal medium for induction of PLBs was B-5 containing 0.5 mg/l TDZ and 0.25 mg/l NAA in which induction rate of PLBs was 98.3 %, for multiplication of PLBs, it was B-5 containing 1 mg/l uniconazole(S-3307) and 0.2 mg/l NAA and 3.5 % sucrose, the multiplication coefficient being 9.4.

Pradhan (2007) successfully achieved asymbiotic germination of *Cymbidium elegans* Lindl. and *Dendrobium densiflorum* Lindl. The best shoot multiplication from shoot tip culture was

found to be best on MS supplemented with BAP (2 mg/l) + NAA (0.5 mg/l) and best rooting was found to be effective in MS + IBA (1.5 mg/l) in *Dendrobium densiflorum* Lindl.

Koirala (2007) cultured the seeds of *Coelogyne fuscescens* Lindl. and *Cymbidium aloifolium* (L) Sw. in MS medium and hormonal MS medium under aseptic condition. In *C. fuscescens*, the best shoot multiplication (6 shoots/culture) was observed on MS media supplemented with BAP (1.5 mg/l) + NAA (0.5 mg/l) and best rooting (3.5 root/culture) was observed on MS media supplemented with IBA (1 mg/l).

CHAPTER – THREE

MATERIALS AND METHODS

3.1 Materials

The materials used for the present experiment were the protocorm like bodies (PLBs) of *Cymbidium elegans* Lindl. which were obtained form the Tissue culture laboratory, Central Department of Botany, T.U.

3.2 Methodology

The methods for the protocorm like bodies (PLBs) development and differentiation and acclimatization of *C. elegans* Lindl. are described under the following heading.

3.2.1. Preparation of stock solution

The Murashige and Skoog's medium (MS,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	
Magnesium sulphate ($MgSO_4 7H_2O$)	370	3.7	100 ml
Calcium chloride ($CaCl_2.2H_2O$)	440	4.4	
Potassium dihydrogen phosphate (KH_2PO_4)	170	1.7	

B. Micro-nutrients

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

C. Iron source (Fe, EDTA)

Components	Composition of MS (Final conc.) Mg/l	(100X)mg/100ml stock Concentration	Volume to be taken for 1 litre medium
Iron Source			
Sodium ethylene diamine	37.3	373	
tetra acetate (Na_2EDTA)			10 ml
Ferrous sulphate	27.8	278	
($FeSO_4.7H_2O$)			

D. Vitamins

Components	Composition of MS (Final conc.) Mg/l	(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	

(E) Carbon source

Chemicals	**g/l**
Sucrose	30 g

(F) 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 up to 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.

3.2.2 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)

3.2.3 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 up to 100 ml by the addition of sterile water in each hormone separately.

3.2.4 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.

3.2.5 Preparation of Media

- For the preparation of 1 litre 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.
- P^H 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, the tubes were taken out and kept in slanting position inside the culture room.

3.2.6 Sterilization and Use of inoculation chamber.

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, beaker, forceps, tubes with media except plant material were exposed under ultraviolet (UV) radiation for 45 minutes to remove the possible contaminants presenting on them and around the transfer area. Then by turning off the UV-light, air blower was switched on and after 15 minutes, the laminar air flow chamber was ready for inoculation.

3.3 Inoculation of single protocorm like bodies (PLBs) of *Cymbidium elegans* Lindl.

The small mass of PLBs from a tube were taken out and put on sterile petridish in a laminar air flow. Each PLBs was separated and cut at end by sterile surgical blade. Then single PLBs

was inoculated on the MS media alone and supplemented with different growth hormones. All the cultures were maintained at 25^0c ($\pm 2^0$c) under 14 hours photoperiod and observed regularly.

3.4 Inoculation of explant of *Cymbidium elegans* Lindl.

The inoculation of explants was done in laminar air flow under sterile condition for this experiment. Shoot tip and root tip were taken as explants which were obtained from the *in vitro* grown plants. Small piece of shoot tip and root tip (about 5-7 mm) were cut with the help of surgical blade and inoculated on the MS free media and MS media supplemented with various hormone concentrations of BAP (0.5 to 2 mg/l) and NAA (0.5 mg/l). All the cultures were maintained at 25^0c ($+2^0$ c) under 14 hours photoperiod and observed regularly.

3.5 Shoot multiplication

For shoot multiplication, individual microshoots of about 5-7 mm were cut and transferred to the medium containing different concentrations and combination of hormones (NAA, BAP) and shoot growth was recorded in every 2 weeks.

3.6 Rooting of Shoots

For *in vitro* rooting, the microshoots obtained from the culture of shoot tips explants were transferred to the media supplemented with different concentrations of rooting hormones like IAA, IBA and NAA and recorded every 2 weeks.

3.7 Methods of Acclimatization

The *in vitro* grown plantlets with well developed roots of *C. elegans* Lindl. were acclimatized. The following steps were carried out for acclimatization:-

- At first, the culture tubes containing rooted plantlets were opened and kept in room temperature for 1 week.
- The plantlets grown in cultured conditions were picked out and washing with running water to remove the entire agar attached with it.
- The plantlets were dipped in fungicide bavistine (0.1 %) to minimize the chances of infection for 5 minutes.

- The plantlets were washed in distilled water for few minutes and kept it in blotting paper for drying.
- The plantlets were then transferred to the cleaned earthen pots containing appropriate potting mixture. The potting mixture used was coco-peat along with sphagnum moss (2:1) to facilitate the holding of water.
- The plants were covered with transparent polythene sheets to control the humidity. The small holes were made into polythene sheet for aeration.
- The 5 % NPK solution was sprayed once a week regularly for fastening their growth.
- The plants were kept in the greenhouse for several weeks and finally they were transferred to the natural environment to check their growth.

3.8 Statistical Analysis

Statistical analysis was carried out with the help of 12^{th} version of SPSS program. One way ANOVA (Analysis of variance) was used for the analysis of data. Differences in means were tested by Duncan's Multiple Range Test. The results were used to compare the means so that effective discussion can be made.

CHAPTER – FOUR

RESULT

The result of *in vitro* development and differenciation of single protocorm like bodies, culture of shoot tips and root tips, rooting of shoot tips and acclimatization of *Cymbidium elegans* Lindl have been described below under different headings.

Micropropagation of *C. elegans* Lindl.

4.1.1 *In vitro* culture of single protocorm like bodies (PLBs) of *C. elegans* Lindl.

The immature seeds of *C. elegans* Lindl. took 14 weeks to germinate on MS basal media and PLBs formation was observed on 17 weeks. Protocorms are swelling of embryos that developed into green spherules.

Seventeen weeks old protocorms were inoculated singly by cutting on MS basal media and MS supplemented with different concentrations of hormones. The culture of single protocorm differenciated into embryogenic callus and later regeneration of seedling was observed in some culture tube. But in some culture tube, PLBs was differentiated but seedling was not developed. MS supplemented with BAP (1 mg/l) + NAA (0.5 mg/l) was appropriate condition for PLBs differentiation and elongation of mini-seedling. Differentiation of single protocorm on MS media and MS supplemented with different concentrations of hormones is summarized on Table 1.

Table: 1

Effect of BAP and NAA on *in vitro* development and differentiation of single protocorm like bodies (PLBs) of *C. elegans* Lindl.

Media	Treatment	Protocorm Culture	Callus formation	Number of PLBs	Observation taken in days		Seeding characteristics after 90 days		
					Ist leaf formation	Ist root formation	Mean length of Shoot	Mean no. of leaves	Mean no of roots
MS	MS	1	-	-	59.0	Not observed	1.5	1.5	Not observed
”	0.5 BAP	1	+	3.25	77.00	”	1.20	1.75	”
”	1 BAP	1	+	1.00	65.00	”	1.75	1.25	”
”	1.5 BAP	1	+	3.00	67.00	”	1.30	2.00	”
”	2 BAP	1	-	2.00	50.00	”	1.25	2.75	”
”	0.5 N	1	+	5.50	66.00	”	1.20	1.75	”
”	0.5 B + 0.5 N	1	+	4.00	54.00	67	1.30	1.25	2
”	1 B + 0.5 N	1	+	5.00	32.00	No	2.50	3.25	No
”	1.5 B + 0.5 N	1	_	-	57.00	”	1.12	1.25	”
”	2 B + 0.5 N	1	-	4.5	66.00	”	1.10	1.25	”

Culture Condition: MS medium, 25 ⨽ 2^0c, 90 days, 14 hours photoperiod, 4 replicates were used in each combination.

4.1.1.1 *In vitro* development and differentiation of single protocorm like bodies of *C. elegans* Lindl.

The effects of growth regulators supplemented in MS medium on single protocorm development and differentiation of *C. elegans* Lindl showed following response.

1. MS Basal medium

In MS Basal media, protocorm directly developed into seedling without multiplication of PLBs. In 59.0 days, first leaf was developed and root wasn’t observed. The average length of shoot was 1.5 cm till 90 days.

2. MS + BAP (0.5 mg/l)

In MS medium supplemented with BAP (0.5 mg/l), single protocorm differentiated into green cluster of numerous PLBs and developed into mini-seeding. First leaf was observed on 77 days and average length of shoot was 1.2 cm.

3. MS + BAP (1 mg/l)

In MS medium supplemented with BAP (1 mg/l), single protocorm did not differentiated into PLBs and directly developed into mini-seedling. First leaf formation was observed on 65 days and average length of shoot was 1.75 cm.

4. MS + BAP (1.5 mg/l)

In MS medium supplemented with BAP (1.5 mg/l), single protocorm differentiated into PLBs with callusing. First leaf formation was observed on 67.00 days and average length of shoot was 1.3 cm (Fig. 06).

5. MS + BAP (2 mg/l)

In MS medium supplemented with BAP (2 mg/l), single protocorm differentiated into shoot primordia without multiplication of PLBs. First leaf formation was observed on 50 days and average length of mini-seedling was 1.25 cm (Fig. 07).

6. MS + NAA (0.5 mg/l)

In MS medium supplemented with NAA (0.5 mg/l), single protocorm multiplied into green cluster of PLBs. First leaf formation was observed on 66 days and average length of shoot was 1.2 cm. This media was best for PLBs differentiation (Fig. 08-09).

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

In MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l), single prootocorm differentiated into PLBs with callusing. First leaf formation was observed on 54 days and average length of shoot was 1.3 cm and average length of root was 2 cm till 90 days (Fig.10).

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

In MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l), single protocorm differentiated into green clusters of PLBs with rapid elongation of miniseedling. This media was best for development of seedlings. First leaf formation was observed on 32.3 days and average length of seedling was 2.5 cm (Fig. 11).

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

In MS medium supplemented with BAP (1.5 mg/l) and NAA (0.5 mg/l), single protocorm differentiated directly into shoot primordia without multiplication of PLBs. First leaf formation was observed on 57 days and average length of shoot was 1.12 cm.

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

In MS medium supplemented with BAP (2 mg/l) and NAA (0.5 mg/l), single protocorm formed green clusters of PLBs. First leaf formation was observed on 66 days and average length of shoot was 1.1 cm.

4.1.2 Culture of shoot tips of *C. elegans* 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 2.

Table: 2

Effect of BAP and NAA on shoot tip culture of *Cymbidium elegans* Lindl.

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

Culture condition: MS medium, $25+2^0$C, 16 weeks 14 hrs photoperiod, 4 replicates were used in each combination.

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

4.1.2.1 Development of shoot tip explants of *C. elegans* Lindl.

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

1. MS Basal Medium.

In MS basal medium, the shoot multiplication started after 12 weeks of culture of shoot tips. In this condition, the explant produced only two shoots in 16 weeks of culture. Root multiplication started after 5 weeks and 3 roots were produced by single explant till 16 weeks. A well growth of two rooted shoot and one rooted multiple shoot was observed. The shoot length range from 2-4 cm in this medium and the average number of shoot and root were 1.25 and 3.0 per culture (Fig. 13).

2. MS + BAP (0.5 mg/l)

The shoot tip cultured on MS medium supplemented with BAP (0.5 mg/l) showed shoot differentiation after 7 weeks of culture of shoot tip. Maximum 4 shoots of length ranging from 1-4 cm was observed after 16 weeks of culture. PLBs was induced by shoot tip explants. Root differentiation did not occur upto 16 weeks. Average number of shoots was 2.5 per culture (Fig. 14)

3. MS + BAP (1 mg/l)

The shoot tip multipled after 8 weeks of culture in MS + BAP (1 mg/l) medium. Maximum four shoot of length 1.5-2.5 cm were observed. Root differentiation did not occur upto 16 weeks. The average number of shoots was 3 per culture. (Fig. 15).

4.MS + BAP (1.5 mg/l)

In MS medium supplemented with MS + BAP (1.5 mg/l), the shoot tips multiplication started after 8 weeks of culture. Maximum 3 shoots of length 1.1-2.8 cm was observed till 16 weeks of culture of shoot tip and average number of shoots was 2.75 per culture (Fig. 16).

5. MS + BAP (2 mg/l)

The shoot tip modified into embryogenic callus after 8 weeks of culture in MS + BAP (2 mg/l) medium. From embryogenic callus, many small plantlets of size 1-1.5 cm were

differentiated till 16 weeks. Root differentiation were not observed. Average number of shoot was found to be 2 per culture (Fig. 17-18).

6. MS + NAA (0.5 mg/l)

In MS medium supplemented with NAA (0.5 mg/l) the multiplication of shoots started after 13 weeks of culture. Maximum 2 shoots with short pseudobulb was observed. The shoot length ranged from 2.5 – 4.5 cm till 16 weeks of culture of shoot tips. Root multiplication was best which was differentiated after 5 weeks of culture. Maximum 2 roots of length 2.5-3.7 cm were observed. The average number of shoots was 1.25 per culture (Fig. 19).

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), well developed shoots were observed after 6 weeks of culture. The shoots range with the length of 1.2-2.1 cm. Small mass of protocorm like bodies was observed at the base of shoot and root differentiation was not observed till 16 weeks of culture of shoot tip. The average number of shoots was 3.5 per culture (Fig. 20).

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

MS medium supplemented with BAP (1 mg/l) + NAA (0.5 mg/l) was found to be most effective culture condition for the multiplication of shoots. Shoot proliferation was vigorous in this culture condition. Multiplication of shoots were started after 5 weeks of culture. Maximum 8 multiple shoots were observed in 12 weeks of culture. Length of shoot varied from 1.3-3 cm. Protocorm like bodies (PLBs) were induced by shoot tip explant. The average number of shoots was 6.75 per culture (Fig. 21).

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

In MS medium supplemented with BAP (1.5 mg/l) + NAA (0.5 mg/l), shoot multiplication was started after 7 weeks of culture of shoot tips. 3 to 5 multiple shoots were observed till 16 weeks of culture. The length of shoot ranged from 2.5 to 3.3 cm. Root differentiation was not observed. The average number of shoot was 2.75 per culture (Fig. 22).

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 8 weeks of culture. Maximum 4 multiple shoots were observed of length 2.3-2.8 cm till 16 weeks of culture of shoot tip. Short pseudobulb was observed while root differentiation was not observed. The average shoot number was found to be 3.0 per culture (Fig. 23).

4.1.2.2 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: 3

Effect of the BAP and NAA on multiple shoot formation, growth and root formation after 16 weeks of culture of *C. elegans* Lindl .

Media	Growth hormones	Concentration of hormones (mg/l)	Parameters			
			Shoot proliferation (mean±SD)	Shoot growth (cm) (mean±SD)	Number of Leaves (mean± SD)	Number of Root (mean± SD)
MS	BM	-	1.25±0.50	2.62±0.94	5.75±2.36	3.0±0.81
”	BAP	0.5	2.50±1.29	2.28±1.86	8.50±4.35	No root
”	BAP	1.0	3.00±0.81	1.93±0.42	7.00±2.16	”
”	BAP	1.5	2.75±0.50	1.70±0.79	6.50±1.29	”
”	BAP	2.0	2.00±0.81	1.31±0.47	5.50±1.91	”
”	NAA	0.5	1.25±0.50	2.00±1.29	4.00±0.81	1.75±0.57
”	BAP+NAA	0.5+0.5	3.50±0.57	1.83±0.49	9.00±1.15	No root
”	BAP+NAA	1.0+0.5	6.75±1.41	2.27±0.71	16.25±2.21	”
”	BAP+NAA	1.5+0.5	2.75±0.50	1.75±0.57	6.75±2.06	”
”	BAP+NAA	2.0+0.5	3.00±0.81	1.75±0.28	7.50±1.29	”

Culture conditions: MS medium, 25 ± 2^0c, 16 weeks of primary culture, 4 replicates were used in each combination.

The least multiplication of shoot was found in MS medium supplemented with NAA (0.5 mg/l) and MS basal media i.e. 1.25 shoot per culture in both condition. The highest growth of

shoots were observed in MS basal media i.e. 2.62 cm and BAP (0.5 mg/l) i.e. 2.28 cm and BAP (1 mg/l) + NAA (0.5 mg/l) i.e. 2.27 cm. Least shoot growth was observed in MS medium supplemented with BAP (2 mg/l) i.e. 1.31 cm (Fig. 17-18).

Shoot proliferation in MS basal medium after 16 weeks of culture was not effective. Highest multiplication of shoot was observed in MS medium supplemented with BAP (1 mg/l) + NAA (0.5 mg/l) which was 6.75 shoots per culture (Fig. 21) which was observed after 5 weeks of culture. The second highest shoot multiplication was observed in MS medium supplemented with BAP (0.5 mg/l) + NAA (0.5 mg/l) i.e. 3.5 shoots per culture. The MS medium supplemented with BAP (1 mg/l), BAP (2 mg/l) + NAA (0.5 mg/l) and BAP (1.5 mg/l) also favored the multiplication of shoots.

Similarly, the differentiation of roots were observed only on MS basal media and MS medium supplemented with NAA (0.5 mg/l). The maximum number of roots were observed in MS basal media i.e. 3 roots per shoot and growth rate of root was observed on MS basal media i.e. 3 cm. Highest number of leaves was observed in MS medium supplemented with BAP (1 mg/l) + NAA (0.5 mg/l) i.e. 16.25 leaves per culture which was followed by BAP (0.5 mg/l) + NAA (0.5 mg/l) i.e. 9.0 leaves per culture. Least number of leaves was observed in MS + NAA (0.5 mg/l) with 4.0 leaves per culture.

Table: 4

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	1.25^{a}	2.62^{ab}	5.75^{ab}	3.0^{b}
0.5 BAP	2.5^{abc}	2.28^{a}	8.5^{b}	0^{a}
1 BAP	3^{bc}	1.93^{a}	7^{ab}	0^{a}
1.5 BAP	2.75^{bc}	1.72^{a}	6.5^{ab}	0^{a}
2 BAP	2^{ab}	1.31^{a}	5.5^{ab}	0^{a}
0.5 NAA	1.25^{a}	2.20^{a}	4.0^{a}	1.75^{ab}
0.5 BAP + 0.5 NAA	3.5^{c}	1.83^{a}	9.0^{b}	0^{a}
1 BAP + 0.5 NAA	6.75^{d}	2.27^{a}	16.25^{c}	0^{a}
1.5 BAP + 0.5 NAA	2.75^{bc}	1.75^{a}	6.75^{ab}	0^{a}
2 BAP + 0.5 NAA	3^{bc}	1.75^{a}	7.5^{ab}	0^{a}

Note:

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

Table 4 shows relationship between the means values of a particular column. The means values with same alphabets in a column are not significantly different though the numerical values are different. From above, shoot number and leaf number are highly significiant and shoot growth and root number are insignificant at 5 % level of significance.

4.1.3 Culture of root tips of *Cymbidium elegans* 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. The changes in root tip explants have been presented in the table 5.

Table: 5

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

BAP → NAA ↓ (mg/l)	0	0.5	1.0	1.5	2.0
0	1S 1R	2MS, R 1MS	1S, R 2MS 1R	2S,R 1MS	1S, 1MS, R
0.5	2S, R 2S	2S,R 1MS, R	2S,R 1MS,	2S	1R

Culture condition: MS medium 25±2^{0}c, 24 weeks, 14hrs photoperiod, 4 replicates were used in each combination,

Where MS=Multiple shoot, R=Root, S=Shoot

Table: 6

Effect of BAP and NAA on root tip explants on multiple shoot formation and root formation after 24 weeks of culture of *C. elegans* Lindl.

Media	Growth hormones	Concentration of hormones (mg/l)	Parameters	
			Number of shoot (mean±SD)	Number of Root (mean±SD)
MS	BM	-	0.25±0.5	0.25±0.5
”	BAP	0.5	2.0±1.63	1±1.41
”	BAP	1.0	3±2.44	0.75±0.95
”	BAP	1.5	2.25±1.7	0.75±0.95
”	BAP	2.0	1.25±2.62	0.25±0.5
”	NAA	0.5	1.25±0.5	0.5±0.57
”	BAP+NAA	0.5+0.5	2.25±3.2	1.5±1.29
”	BAP+NAA	1.0+0.5	1.31±0.81	0.75±0.5
”	BAP+NAA	1.5+0.5	0.75±0.95	0±0
”	BAP+NAA	2.0+0.5	0±0	0.25±0.5

Culture condition: MS medium 25±2^{0}c, 24 weeks of primary culture, 4 replicates were used in each combination.

4.1.3.1 Development of root tip explants of *C. elegans* Lindl.

The root tips obtained from the cultured of shoot tip were culture in the MS medium alone and MS medium supplemented with different hormones of different concentration of BAP and NAA. Their responses upto the end of 24 weeks of culture is summarized as under.

1. MS Basal medium

The development of root tip explants started after 14 weeks of culture in MS basal medium. Multiple shoots were not formed and only single root and shoot was seen at the end of 22 weeks of culture. Average number of shoot and root are 0.25 and 0.25 respectively (Fig. 25).

2. MS+BAP (0.5 mg/l)

Regeneration of root tip explants cultured in MS medium supplemented with BAP (0.5 mg/l) started after 10 weeks of culture. Multiple shoots were seen. New roots were also obtained after 24 weeks of culture. The average number of shoots and roots were found to be 2.00 and 1.0 respectively (Fig. 26).

3. MS +BAP (1 mg/l)

The root tips cultured in MS medium supplemented with BAP (1 mg/l) develop shoots after 7 weeks of culture. Multiple shoots were found to be seen after the completion of 12^{th} week of culture. Regeneration of shoot was best among the other concentration. After 24 weeks of culture of roots tips, many new roots were emerged and average number of shoots and root were 3 and 0.75 per culture respectively (Fig. 27).

4. MS + BAP (1.5 mg/l)

The root tips cultured in MS medium supplemented with BAP (1.5 mg/l) showed regeneration on the completion of 9 weeks of culture. Multiple shoots and roots were observed at the end of 24 weeks of culture. Average number of shoots and roots were found to be 2.25 and 0.75 per culture respectively (Fig. 28).

5. MS + BAP (2 mg/l)

In MS medium supplemented with BAP (2 mg/l), the shoots developed after 11 weeks of culture. Multiple shoot with roots were observed after 24 weeks of culture of root tips and average number of shoots and roots were 1.25 and 0.25 per culture (Fig. 29).

6. MS + NAA (0.5 mg/l)

MS medium supplemented with NAA (0.5 mg/l) was found to be favorable condition for the regeneration of root tips into roots. Regeneration of root tips into roots started after 12 weeks of culture while the regeneration into shoots started only after 15 weeks of culture. Average number of shoot per culture was found to be 1.25 per culture and average number of roots per culture was found to be 0.5 (Fig. 30).

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

MS medium supplemented with BAP (0.5 mg/l) +NAA (0.5 mg/l) showed shoot initiation after 9 weeks of culture of root tips. Multiple shoots and many new roots were also developed after 24 weeks of culture of root tips and average number of shoots and roots were found to be 2.25 and 1.5 per culture respectively (Fig. 31).

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

The root tips cultured in MS medium supplemented with BAP (1 mg/l)+NAA (0.5 mg/l) showed their response to regeneration after 14 weeks of culture. Multiple shoot were seen in one replica and single shoot and root were seen in other replica after 24 weeks of culture. The average number of shoots and roots were found to be 1.31 and 0.75 per culture respectively (Fig. 32).

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

In MS medium supplemented with BAP (1.5 mg/l) +NAA (0.5 mg/l), the regeneration of shoot started to developed after 13 weeks of culture of root tips. Roots were not observed after 24 weeks of culture of root tips and average number of shoot was found to be 0.75 per culture (Fig. 33).

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), the regeneration of shoots were not observed upto 24 weeks of culture of roots tips. The average number of root was 0.25 per culture (Fig. 34).

Table: 7

Mean value of shoots number and root number after 24 weeks of culture of root tip explants of *C. elegans* Lindl.

Treatments	Mean value of	
	Shoot number	Root Number
BM	0.25^{ab}	0.25^{ab}
0.5 BAP	2.00^{ab}	1.00^{ab}
1 BAP	3.00^{b}	0.75^{ab}
1.5 BAP	2.25^{ab}	0.75^{ab}
2 BAP	1.25^{ab}	0.25^{ab}
0.5 NAA	1.25^{ab}	0.05^{ab}
0.5 BAP + 0.5 NAA	2.25^{ab}	1.50^{b}
1 BAP + 0.5 NAA	1.31^{ab}	0.75^{ab}
1.5 BAP + 0.5 NAA	0.75^{ab}	0.00^{a}
2 BAP + 0.5 NAA	0.00^{a}	0.25^{ab}

Note:

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

Table 7 shows 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 a column are not significantly different though the numerical values are different.

4.1.4 Rooting of shoots of *C. elegans* Lindl.

The shoots of length 0.8 to 1 cm were used as explants 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 *C. elegans* 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± SD)	1.5 ± 0.57	1.75 ± 0.95	1.5 ± 0.57	1.75 ± 0.5	3.25 ± 0.95	2.25 ± 0.57	1.75 ± 0.81	1.5 ± 0.57	2.25 ± 0.50	1.25 ± 0.50	1.5 ± 0.57	1.25 ± 0.50
Length of Roots (mean± SD)	1.31 ± 0.23	2.2 ± 0.47	2.53 ± 0.59	1.93 ± 0.42	2.73 ± 0.66	1.52 ± 0.16	2.26 ± 0.65	1.88 ± 1.03	2.12 ± 0.64	1.37 ± 0.47	0.42 ± 0.08	0.77 ± 0.24

Culture condition: - MS medium 25±2^{0}c, 12 weeks, 4 replicates were used in each combination.

From table 8, it can be seen that MS medium supplemented with IBA (0.5 mg/l) was found to be appropriate culture condition for the rooting. The rooting in this condition started after 4 weeks of culture. The roots were aerial, hairy in nature and were greenish in colour. The average number of root was 3.25 per culture and length of root ranged from 2.1-3.6 cm after

12 weeks of culture. It was observed that decrease in concentration of IBA was found to be effective for the rooting of the shoots (Fig. 35-38).

In case of IAA, maximum number of root was found in MS medium supplemented with 1 mg/l IAA and 2 mg/l IAA i.e 1.75 roots per shoot tip cultured in both. The increase or decrease of the concentration was not found to be supportive for the rooting of shoots (Fig. 39-42).

Similarly in case of NAA, MS medium supplemented with NAA (0.5 mg/l) was produced maximum number of roots i.e 2.25 roots per culture which vary in length from 2-3 cm while least growth of root was also observed on MS medium supplemented with NAA (1.5 mg/l) i.e. 0.42 roots (Fig. 43-46). Though, growth and length of root was poor on MS medium supplemented with NAA, development of short pseudobulb, thick aerial root and protocorm like bodies was good almost in different concentration of NAA in comparison to the MS medium supplemented with IAA & IBA.

Vigorous PLBs induction was observed on MS medium supplemented with NAA (1 mg/l) (Fig. 44).

Table: 9

Mean values of Root number and Root growth after 12 weeks of culture of shoot tip explants.

Treatments	Mean value of	
	Root number	Root growth (cm)
0.5 IBA	3.25^{c}	2.73^{f}
1 IBA	2.25^{bc}	1.52^{cd}
1.5 IBA	1.75^{ab}	2.26^{de}
2 IBA	1.50^{ab}	1.88^{cde}
0.5 IAA	1.50^{ab}	1.31^{bc}
1 IAA	1.75^{ab}	2.20^{cde}
1.5 IAA	1.50^{ab}	2.53^{e}
2 IAA	1.75^{ab}	1.93^{cde}
0.5 NAA	2.25^{ab}	2.12^{cde}
1 NAA	1.25^{a}	1.37^{bcd}
1.5 NAA	1.50^{ab}	0.42^{a}
2 NAA	1.25^{a}	0.77^{ab}

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 different. Statistically, root number and root growth are significant at 5 % level of significance. Both the root number and root growth of 0.5 mg/l IBA treatment were highest and significantly different from all other treatments.

4.1.5 Acclimatization

The rooted plantlets measuring about 4, 3.5 & 3.2 cm were transferred in pot containing mixture of coco-peat and sphagnum moss (2:1) to facilitate the holding of water. 5% NPK solution was sprayed every week to fasten their growth. They were under the process of observation (Fig. 47-55).

CHAPTER – FIVE

DISCUSSION

5.1 *In vitro* development and differenciation of single protocorm like bodies of *Cymbidium elegans* Lindl.

In the present study, 17 weeks old protocorms were cultured singly in MS basal and MS media supplemented with different hormones in different concentration. The earliest appearance of the first leaf was recorded in MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l). The same medium proved best for the formation of maximum number of leaves and maximum length of seedlings. So, the most effective culture condition for the *in vitro* development and differentiation of single protocorm like bodies of *C. elegans* Lindl. was MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l).It may be due to their effect on physiological processes or interaction between the hormones.

Tissue culture techniques have been extensively used for the mass propagation of orchids. The efficiency of this technique is markedly influenced by the medium formulation, but the cultured tissues often exude phenolic compounds which upon oxidation to quinines turn the media brown and also prove toxic to *in vitro* cultures. So, antioxidants are widely used to overcome this problem. In present study, some culture tubes exudes phenolic compounds. Each plant species has specific nutritional requriments for the plant regeneration *in vitro*. Basically *in vitro* grown tissues have more percentage of valuable phytochemical than *in vivo,* since *in vitro* grown cells have controlled environmental condition (Rao and Gupta, 1998).

This was supported by the findings made by Matsui *et al.* (1970). They found that NAA alone had no effect on development and differentiation of PLBs in *Cymbidium*. Combination of BAP + NAA induced the greatest effect.

In present study, the duration for first leaf and root differentiation from PLBs varied from 5-11 weeks on MS media. This was supported by Devi *et al*. (1997) who found that the duration for leaf and root differentiation from PLBs varied from 7-13 weeks in different media as MS, KC, VW, NI Media etc. Similarly Chen *et al*. (2001) developed protocol for PLBs induction, PLBs multiplication and PLBs shooting and rooting. Shoot and root were

induced from PLBs. Kabita and Sharma (2001) concluded that MS media was best for the development of PLBs than Knudson C, VW and BS media in *Acampe longifolia*.

In present study, the embryogenic callus was induced from wound portion of PLBs segments of *Cymbidium* and later average length of plantlets i.e. 2.5 cm was observed within 90 days on appropriate condition. Huan *et al.* (2004) observed the embroyogenic callus induction and plant regeneration in *Cymbidium* from longitudinally bisected segments of protocorm like bodies within one month on VW medium supplemented with 1-Naphthalene acetic acid. The calli easily formed PLBs after being transferred to media without hormones. Wang *et al.* (2004) observed that subculture of protocorm gave shoots on MS medium with BA (3 mg/l) and NAA (0.1 mg/l) in *Phalaenopsis.*

5.2 Shoot tip culture of *Cymbidium elegans* Lindl.

The shoot tip explants of *C. elegans* Lindl. are inoculated in MS basal medium and MS medium supplemented with different hormonal concentrations developed shoots and multiple shoots. In some culture conditions, roots are also seen to arise from some shoots while in some, protocorm like bodies were developed from shoot tip explants. The explant source varies with genotype, culture condition and also with the growth condition of the explant, the light. The hormone influenced on the proliferation, growth and differentiation of cultured plant parts.

In present investigation, MS medium alone was not effective for induction of multiple shoots. Similar result was obtained by Yasugi *et al*. (1994). The shoot explant of *C. elegans* was proliferated vigorously on MS medium supplemented with BAP (1 mg/l)+NAA (0.5 mg/l) whereas MS medium supplemented with NAA (0.5 mg/l) showed least number of shoot multiplication. It may be due to that the combined treatment with NAA and BAP induced direct or callus mediated PLBs development and enhanced response frequency in root tip explant.

Statistically, shoot number and leaf number were significant while shoot growth and root number were found to be not significant at 5 % level of significance.

Investigation carried out by various researcher shows that the medium supplemented with the high concentration of BAP and low concentration of the NAA is most favourable for the

induction of the shoot multiplication. Chung *et al*. (1998) obtained enhance multiple shooting in *Cymbidium forrestii* and *C. kanran* on MS medium with NAA (1 mg/l) and BAP (3 mg/l).

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. Similar result was obtained by Swar and Pant (2004). They obtained maximum number of shoots in MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) in *Coelogyne cristata* Lindl. Pradhan (2007) found that MS medium supplemented with BAP (2 mg/l) and NAA (0.5 mg/l) was the most favourable culture condition for the shoot multiplication of *Dendrobium densiflorum* Lindl. Koirala (2007) obtained maximum number of shoots in MS medium supplemented with BAP (1.5 mg/l) and NAA (0.5 mg/l) in *Coelogyne fuscescens* Lindl.

In the present investigation, the shoot tip explant turned into green callus and multiple shoot was obtained on MS media supplemented with BAP (2 mg/l). Shrestha and Rajbhandari (1994) obtained the initiation of the protocorm like bodies within 6 weeks from the shoot apical meristems of C*ymbidium longiflorum* cultured *in vitro* on Murashige and Skoog medium (1962) supplemented with 2 mg/l (BAP), 1 mg/l (NAA) 10 % coconut milk and 3 % sucrose.

The results obtained by various other researchers differ some what Pant and Gurung (2005) obtained highest number of shoots in MS medium supplemented with BAP (1.5 mg/l) in *Aerides odorata* Lour.

5.3 Root tip culture of *Cymbidium elegans* Lindl.

The root generated on the shoot tip explants of *C. elegans* Lindl. cultured on different hormonal concentration were excised aseptically and cultured in fresh MS basal and MS medium supplemented with hormones of different concentration. So cultured roots regenerated into new roots, new shoots and callus. Pseudobulb was not observed.

Regeneration of roots started from 8 weeks of culture and complete plantlets were obtained by the end of 24 weeks. The most effective result for the regeneration from the root tips was MS medium supplemented with BAP (1 mg/l). The most favorable condition for the

formation of the new roots is found to be MS medium supplemented with NAA (0.5 mg/l) and MS basal media.

Statistically, both shoot number and root number were found to be insignificant at 5 % level of significance but as compared to others, MS medium + BAP (1 mg/l) was appropriate condition for shoot regeneration from root tip culture. Though, the regeneration of the plantlets from the root tip culture was found to be satisfactory, but in comparison to the regeneration of plantlets from the shoot tip, it was found to be less effective. It may be due to the low growth rate and other physiological processes of root tip.

There are several reports regarding the regeneration of plant from root tip explant. Sood and Vij (1986) cultured root segments of *Rhynchostylis retusa* BL on selective modification of Mitra *et al.* (1975) 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 V*anilla 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 1 mg/l NAA and 0.2 mg/l BA. Vij (1993) studied the regeneration response of root explants in *Aerides multiflorum, Vanda teres, Cymbidium pendulum etc*. 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.The basal segment of root on a medium containing NAA (1 mg/l) and BA (1 mg/l) induced PLB formation in *Cymbidium*. Similar work was carried out by pradhan (2007). She found that the regeneration of shoots and roots from culture of root tips was most favoured on MS medium supplemented with BAP (1.5 mg/l). Koirala (2007) also found that the culture of root tips produced shoots and roots on MS medium supplemented with BAP (1 mg/l).

5.4 Rooting of shoots of the *Cymbidium elegans* 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.

In present investigation, MS basal medium was favoured for root induction. Similar result was obtained by Shrestha and Rajbhandari (1988) in which MS basal medium induced good rooting in *Cymbidium giganteum* within 2 months. Shrestha and Rajbhandari (1994), again gave similar results in *C. longifolium.*

In present investigation, the MS medium supplemented with IBA (0.5 mg/l) was found to be the most effective for rooting. Almost MS medium supplemented with various concentrations of IBA was the much better rooting condition for *in vitro* grown plant in comparision to MS medium with NAA and IAA. It may be due to the presence of enhanced level of auxin (IBA) and related compounds in the medium which has strong absorption power of inhibitory compounds.

Statistically, both root number and root length were found to be significant at 5 % level of significance. Statistically, it proves that MS media with IBA (0.5 mg/l) was highly significant at 5 % level of significance so it was most appropriate culture condition for rooting of *C. elegans* Lindl.

There are several reports that support the present work. Pant and Swar (2004) obtained the highest number of roots in MS medium supplemented with IBA (1 mg/l) in C*ymbidium irridiodes* D.Don. and *Coelogyne cristata* Lindl. Pradhan (2007) observed that the rooting of *Dendrobium densiflorum* Lindl was most favoured in MS medium supplemented with IBA (1.5 mg/l). Koirala (2007) found that MS medium supplemented with IBA (1 mg/l) was best for rooting in *Coelgyne fuscescens* Lindl.

This finding contrast with the findings of Pant and Gurung (2005), who reported highest number of roots in *Aerides odorata* Lour. in IAA (0.5 mg/l).

CHAPTER-SIX

CONCLUSION

The present study is focussed on the *in vitro* mass propagation of orchid namely *Cymbidium elegans* Lindl. All the experiments were carried out in the lab of Central Department of Botany, T.U., Kirtipur. From the present investigation, following conclusions have been made:

- MS media supplemented with BAP (1 mg/l) + NAA (0.5 mg/l) was found to be comparatively better with respect to other concentrations of hormones for *in vitro* development & differentiation of single protocorm like bodies of *C. elegans* Lindl.
- Callus induction from PLBs segment was vigorous on MS medium supplemented with BAP (1.5 mg/l).
- MS medium supplemented with BAP (1 mg/l) + NAA (0.5 mg/l) was found to be most effective media for shoot multiplication of *C. elegans* Lindl.
- MS medium supplemented with BAP (1 mg/l) was found to be most effective media for the regeneration from root tip explant.
- Shoot tip explants were proliferated vigorously than root tip explant for mass propagation of *C. elegans* Lindl.
- MS medium supplemented with IBA (0.5 mg/l) was appropriate culture condition for the development of maximum number of healthy root which was followed by MS basal medium in *C. elegans* Lindl.

CHAPTER-SEVEN

RECOMMENDATIONS

Following recommendations are made from the present research work:

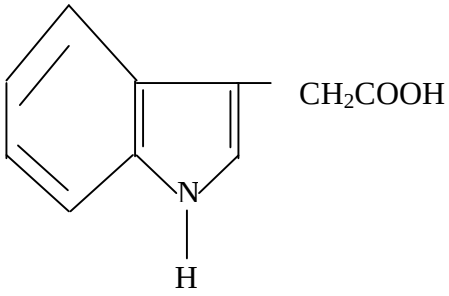
- Beside MS medium, other culture media and tissue culture technique can be examined so that effective protocol can be developed for the micropropagation of *Cymbidium elegans* Lindl.
- Mass propagation of economically important orchids should be started for their conservation.
- Additional research work should be started for the survival of *in vitro* grown plantlets in natural habitat.
- Researches in Botany, especially in plant biotechnology should be conducted to the farmers problems.
- Tissue Culture Lab of Central Department of Botany should be well equipped and well faciliated to carry out the research work effectively.

APPENDIX

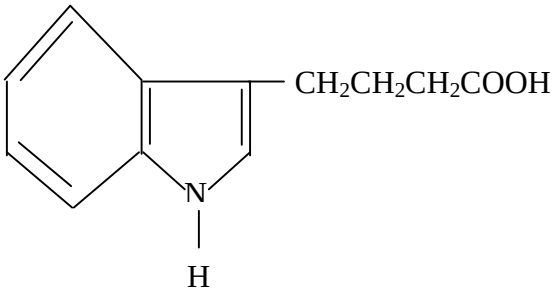
1. Hormone Used for investigation

Auxins:

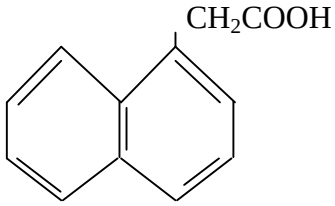
i. Indole-3-acetic acid (IAA)



ii. Indole-Butyric acid (IBA)

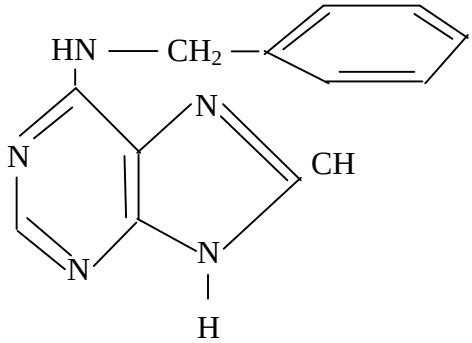


iii. Naphthalene acetic acid (NAA)



Cytokinins:

i. 6-Benzyl amino purine (BAP)



2. Statistical Analysis

Analysis of Variance for Shoot Tip Culture.

		Sum of Squares	df	Mean Square	F	Sig.
Shoot Number	Between Groups	94.600	9	10.511	15.016	.000
	Within Groups	21.000	30	.700		
	Total	115.600	39			
Shoot Growth	Between Groups	19.755	9	2.195	2.681	.020
	Within Groups	24.560	30	.819		
	Total	44.315	39			
Root Number	Between Groups	402.525	9	44.725	9.432	.000
	Within Groups	142.250	30	4.742		
	Total	544.775	39			
Leaf Number	Between Groups	3.125	1	3.125	6.818	.040
	Within Groups	2.750	6	.458		
	Total	5.875	7			

Analysis of Variance for Root Tip Culture

		Sum of Squares	df	Mean Square	F	Sig.
Shoot Number	Between Groups	33.100	9	3.678	1.219	.320
	Within Groups	90.500	30	3.017		
	Total	123.600	39			
Root Number	Between Groups	7.100	9	.789	1.154	.358
	Within Groups	20.500	30	.683		
	Total	27.600	39			

Analysis of Variance for Rooting of Shoot Tips.

		Sum of Squares	df	Mean Square	F	Sig.
Root Number	Between Groups	15.167	11	1.379	3.202	.004
	Within Groups	15.500	36	.431		
	Total	30.667	47			
Root Length	Between Groups	19.793	11	1.799	5.564	.000
	Within Groups	11.643	36	.323		
	Total	31.436	47			

REFERENCES

Arditti, J. 1979. Aspects of the physiology of orchids. Adv. in Bot. Research. 7: 136-150

Bailes, C.P. 1985. Orchids in Nepal, the conservation and development of natural resource. *Advisory report and recommendations*. Royal Botanical Garden, UK.

Bajaj, YPS amd J. Renert. 1978. Applied and fundamental aspect of plant cell, tissue and organ culture, Narosa Publishing House.

Bajracharya, D.M. & L.R. Shakya. 2002. Nepalese orchids & its status and systematic. *J. Nat. Mus*. **21**: 223-242.

Banerjee, B., and A.B. Mandal. 1999. *In vitro* germination of immature *Cymbidium* seeds for rapid propagation of plantlets .*Cell and Chromosome research*. **21** (1):1-5.

Banerjee, ML &P. Pradhan. 1999. *A synopsis of orchid flora*. Nepal Nature's Paradise, Published by M .Devi Gwalior, India, Hillside Press Ltd. Kathmandu.

Bernard, N. 1909. L. Evolution Dans La Symbios. Les Orchid et lur champignous. *Ann. Sc. Botany*. **9**: 1-196.

Chaudhary, R. P. 1998. Biodiversity in Nepal (Status and Conservation). S. Devi, Saharanpur (U.P.), India and Tecpress Books, Bangkok, Thailand.

Chen, Y. H., Y. S. Chang and W. H. Chen. 2002. Tissue culture advances for mass propagation of *Oncidium meri clones. Report of the Taiwan Sugar Research Institute* (172) September. 67-76.

Chung, Jae Dong., J. H. Leu, Sun-Ok Lee and Chang- Kil Kim. 1998. Effect of medium composition on multiple shooting and subsequent growth of mericlone from rhizome of shoot tip culture of temperate *Cymbidium* species. *In Biological Abstract* **105** (4): Ab-50.

Dahal, S. and P.R. Shakya. 1989. A Glimpse of orchid flora of Nepal. *In proceeding of National conference on Science and Technology*, April 24-29, 1988, Royal Nepal Academy of Science and Technology, Kathmandu, Nepal. 246-252.

Devi, J., B. Borthakur, and P.C. Deka. 1997. Clonal propagation of *Dendrobium maschatum* and *Cymbidium aloifolium* through shoot tip culture. *The Journal of orchid society of India*. Vol **II** No 1 & 2.

Dressler, R. L. 1993. *Phylogeny and Classification of the orchid family*. Harvard University Press. Cambridge.

Ernst, R. 1984. Seed and Clonal propagation of *Phalaenosis. In proceeding of the fifth Asian Orchid Congress seminar,* Singapur held on 1-3 August 1984, 31-41.

Faria, Tadeude., Ricardo, Ronaldo Jose, Purigan Dallo, Lilian, Keiko Unemato and Geraldo Lopes da Silva. 2006. *In vitro* orchid propagation of *Oncidium baueri* (Orchidaceae) without the use of agar. *Acta Scientiarum Agronomy*. **28** (1) Jan-Mar, 71-74.

Fonnesbech, M. 1972. Growth hormones and propagation of *Cymbidium in vitro. Physical. Plant*. **27**: 310-316.

Gurung, R. 2006. *In vitro* propagation *of Aerides odorata* Lour. by shoot tip culture. Insight into diverse facts of topographical flora and ecology. Hillside Press Ltd, Kathmandu. 226-234.

Hadley, G and G. Harvis. 1968. The effects of certain growth substances on asymbiotic germination and development of *Dactylorchis purpurella*. New Phytol**. 67** (2) 441-445 Illus.

Hara, H., W.T. Stearn and L. H. J. Williams. 1978-1982. An Enumeration of the flowering plants of Nepal, **1-3** British. Mus (Nat. His), London.

HMG Nepal, Ministry of Forest and Soil Conservation (HMGN). 2002. Nepal Biodiversity Strategy. Singh Durbar, Kathmandu.

Hsia, Chi-Ni, Shu-Ru Yang, Uei-Chen and Yih- Juh Shiau.2005. Mass Propagation of ornamental *Dendrobium* from protocorm like body. *Journal of Science Society for Horticultural Science*. **51**(3)Sept. 259-265.

Huan, Levan Tuong and M. Tanaka. 2004. Callus induction from Protocorm like body segments and plant regeneration in *Cymbidium* . *Journal of Horticulture Science and Biotechnology* **79** (3) May. 406-410.

J,vennel Raj and R. Basavaraju. 2005. *In vitro* culture of *Vanilla*. *Agricultural and Biological Research*. **21**.(1)Jan 59-75.

Jamir, C., J. Devi and P.C. Deka. 2002. *In vitro* propagation of *Cymbidium iridiodes* and *C. lowianum*, *J. Orchid. Soc India*, **16** (1-2): 83-89.

Kabita, M. and C. M. Sharma. 2001. Selection of suitable for *in vitro* germination and protocorm formation of *Acampe longifolia* Lindl. Advances in Plant sciences. **14** (1) June. 243-248.

Kamalakannan, R.,V. Narmantha and L. Jeyakodi. 1999. Plantlet regeneration from encapsulated protocorms of the endemic orchid *Coelogyne odoratissian* Var. *angustifolia* Lindl. *Journal of Phytological Research*. **12** (1-2): 21-23.

Kand, K. 1967. The nonsymbiotic germination of orchids and their clonal propagation by meristem culture. *In Advances in germfree research and gnotobiology* .Proceedings of international symposium on germ free life research. Nagoya and Inugama.

Karanjit, A. 2002. *In vitro* study of *Capsicum annum* Var. *grossum*, *Cymbidium irridiodes* D. Don. and *Coelogyne cristata* Lindl. M. Sc. Dissertation. Central Department of Botany, T.U., Kathmandu, Nepal.

Karki, A., S. Rajbahak and H. K. Saiju. 2005. Micropropagation of *Vanilla planifolia* from seeds. Nepal Journal of Plant Sciences. **1**: 42-44.

Knudson, L. 1922. Non symbiotic germination of orchid seeds. Botan. Gaz**. 73**: 1-25.

Knudson, L. 1951. Nutrient solution for orchids. Botan. Gaz. **112**: 528-532.

Kononowicz, H. and J. Janick. 1984. *In vitro* Propagation of *Vanilla planifolia* Hortscience **19**(1): 58-59.

Kuo, Huei-Lan, Jen-Tsung Chen and Wei-Chin Chang 2006. *In Vitro cellular and Developmental Biology Plant* **41**(4) Jul-Aug. 2005. 453-456.

Koirala, D. 2007. *In vitro* seed germination of *Cymbidium aloifolium* (L)sw and micropropagation of *Coelogyne fuscesecens* Lindl by tissue culture technique. M. Sc. Dissertation. Central Department of Botany, T. U., Kathmandu, Nepal.

Mathews, V. H. and P. S. Rao. 1980. *In vitro* multiplication *of Vanda* hybrids through tissue culture technique. *Plant Sci. lett*. **17**(3): 383-389.

Matsui, T.,K. lekokawai and S. Yoshihoko. 1970. Effect of Benzylaminopurine and L-Napethalene Acetic acid on organogenesis of *Cymbidium. Bul. Fac agrtamagawa univ*. **10:** 99-106.

Mitra, G. C. 1971. Studies on seeds, shoot tips and stem-discs of an orchid grown in aseptic culture. *Indian J. of exp. Bio***. 9**(1): 79-85

Mitra, G. C. 1986. *In vitro* culture of orchid seeds for obtaining seedlings. *Biology, Conservation and Culture of Orchids*. Ed. Vij, S. P., New Delhi: East- West Press, Pvt. Ltd. 401-409.

Morel, G. 1960. Producing virus free *Cymbidium*. *Am. Orchid Soc. Bull*. **29**: 495-497.

Muralidhar, C.E. and A. R. Tehta. 1986. Tissue culture studies on *Cymbidium longifolium* D. Don. *In vitro* seed germination and sequential stages of histomorphological changes from embryos to plbs. *Biology, Conservation, and Culture of Orchids*. 412-422.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assay with tobacco tissue culture *Physiol. Plant*. **15**: 473-497.

Murashige, T. and F. Skoog. 1974. Plant propagation through tissue culture. *Ann. Rev Plant physiology*. **25**: 135-166.

Nagaraju, V. and R. C. Updhayaya. 2001. *In vitro* morphogenetic response of C*ymbidium* PLBs to three basal media and activated charcoal. *J. Orchid Soc. India*. **15**(1-2).

Niroula, R. and S. B. Rajbhandary. 1985. Mass propagation of *Dendrodium fimbriatum* Hook. from seedling tips. *J. inst. Sc. Tech*. **8**: 7-18, 1985, T. U. Kath. Nepal.

Niroula, R. and S. B. Rajbhandary. 1992. Regeneration of plants from leaf explants in orchid *Vanda teres* Lindl. *Role of Biotechnology in Agriculture*, Ktm. Nepal. 103-107.

Philip, V. J. and S. A. J. Nainar. 1988. *In vitro* transformation of root meristem to shoot and plantlets in *Vanilla planifolia*. *Ann. Bot*. (Lond.) **61**(1): 193-200.

Pant, B. and R. Gurung. 2005. *In vitro* seed germination and seedling development in *Aerides odorata* Lour. *J. Orchid soc. India*, **19** (1-2): 51-55.

Pant, B., R.P. Chaudhary and A. Subedi. 2002. Orchids of Nepal Himalaya and their Conservation Strategies. *In Proceeding of International Seminar on Mountain.Kathmandu*.

Pant, B. 2006. Biotechnology in orchid conservation. *In Proceeding of Natural Resource Management*, Nepal Biological Society (NBS).

Paudyal, G. and A. Subedi. 2001. Study on orchids flora of Pokhara valley. NAHSON Bulletin Vol. **10-11**: 14-18.

Pradhan, S. 2007. *Ex situ* conservation of two orchid species viz. *Cymbidium elegans* Lindl. and *Dendrobium densiflorum* Lindl. by tissue culture technique. M. Sc. Dissertation, Central Department of Botany, T. U., Kathmandu, Nepal.

Press, J. R., K. K. Shrestha and D. A. Sutton. 2000. *Annotated checklist of the flowering plants of Nepal*. The natural history museum, London.

Rajbhahak S., A. Karki & H. K. Saiju. 2005. *In vitro* multiplication of *Vanilla planifolia* Andrews using axillary bud explants. *Plant resources*, An Occassional Publication of HMG, Department of Forests and Soil Conservation. 3-5.

Rajbhandary, K.R. 2004. Floral diversity (flowering plants) of Nepal. Earth preservation (Nepal), **1**(1): 12-18.

Rajbhandary, K.R. and S. Bhattarai. 2001. *Beautiful orchids of Nepal*. Kishor offset press,Kathmandu,Nepal.

Rajbhandary, K.R., S. Bhattrai and R. Joshi. 2000. Orchid diversity of Nepal and their conservation need. In: *Proceedings of the 8th International Workshop of BIO-REFOR,*, Kathmandu, Nepal, November 28-December 2, 1999, *BIO- REFOR*, Japan. 249-252.

Rajbhandary, K.R. and S. Dahal. 2004. Orchids of Nepal. A checklist. *Botanica Orientalis*, Vol **4**, Editorial Board of Botanica Orientalis, CDB, T.U. 89-106.

Rajkarnikar, K.M. and R. Niroula. 1994. Tissue culture of *Dendrobium fimbriatum* Hook. for mass production. *Second National Botanical Conferences*, Dec. 23, 1994, Kath. Nepal. 34.

Rao, A. N. 1997. Tissue culture in orchid Industry. In Reinert, J. and Bajaj, Y. P. S. (Edt). *Applied and fundamental aspects of plant cell, tissue and organ culture*. Narosa Publishing House, New Delhi. 46-69.

Rao, G. P. and V.N.P. Gupta. 1998. *Text book of botany* part II. Students friends 6, Allahabad, India.

Reddy, P. V., K. Nanjan and K. G. Shanmugavelu. 1992. *In vitro* studies in tropical orchids seed germination and seedling growth. *J. Orchid soc. India*. **6**(1,2): 75-78.

Roy, J and N. Banerjee. 2002. Optimization of *in vitro* seed germination, protocorm growth and seedling proliferation of *Vanda tessellate* (Roxb.) Hook. Ex. D. Don. *In Biological Abstract* **110**(3): Ab-31.

Shrestha, A. 2005. Ex situ conservation of *Ceologyne ovalis* Lindl. (Orchidaceae) through micropropagation. M. Sc. Dissertation, Central Department of Botany, T. U., Kathmandu, Nepal.

Shrestha, M. and S. B. Rajbhandary. 1988. Meristem culture of *Cymbidium giganteum* Wall ex.Lindl. *In Proceedings of National Conference on Science and Technology.* April 24-29, Kath, Nepal. 345-349.

Shrestha, M. and S. B. Rajbhandary. 1993. Meristem culture of *Cymbidium grandiflorum. First Regional Conference on Prospects of Biotechnology in Nepal.* April 11-13, 1993, Birgunj, Nepal Biotech and Biodiversity Society of Nepal. 38.

Shrestha, M. and S. B. Rajbhandary. 1993. Clonal propagation of *Dendrobium densiflorum* Lindl. through shoot merstem culture. *National Conference on Biotechnology*, April 29-30, 1993, Nepal Biotech Association. 25.

Shrestha, M. and S. B. Rajbhandary. 1994. Clonal propagation of *Cymbidium longiflorum* D. Don. by shoot tip culture, *IInd National Conference on Science and Technology*, June 8-11, RONAST, Kath, Nepal. 369-371.

Silva, Da., Jalme A. Teixeir, Nisha Singh and Michio Tanaka.2006. Priming biotic factors for optimal protocorm like body and callus induction in hybid *Cymbidium*. *Plant Cell and Organ Culture*. **84**(2)Feb.135-144.

Sood, A and S. P. Vij. 1986. *In vitro* root segment culture of *Rhynchostylis retusa Bl. Biology. Conservation and Culture of Orchids.* Ed. Vij, S. P., New Delhi. East- West Press. Pvt. Ltd. 463-468.

Swar, S. 2003. Micropropagation of *Cymbidium iridiodes* D. Don. and *Coelogyne cristata* Lindley. (Orchidaceae). M. Sc. Dissertation, Central Department of Botany, T. U., Kathmandu, Nepal.

Swar, S. and B. Pant. 2004. Micropropagation of *Cymbidium iridiodes* D. Don. In: *Proceeding 4th National Conference on Science and Technology* .RONAST, March 23-26 m, 2004, Kathmandu, Nepal.

Subedi, A. and G. Paudyal, 2001. Some notable orchids of Pokhara valley and their habitats, Botanica Orientalis, Annual Issue 2001, CDB, TU.

Vaidhya, B., M. Shrestha and N. Joshi. 2000. Report on Nepalese orchid species with medicinal properties. In: HMG Nepal (EDS). *Proceeding of Nepal Japan joint symposium on Conservation and Utilization of Himalayan medicinal resources*. Pp: 146-152.

Vena, H. and H. Torikata. 1968. Organogenesis in meristem culture of *Cymbidium* . I. Studies on the effect of growth substances added to culture media under Continuous illumination. *J. Jap. Soc. Hortsci.* **37**(3): 240-248 Illus.

Vij, S. P. 1993. Regeneration response of orchid roots. A study *in vitro. J. Orchid. Soc*. *India* **7**(1,2): 61-72.

Vij, S. P., P. Pathak and K. C. Mahut. 1995. Green Pod Culture of a therapeutically important species *Dactylorhiza hatagirea* (D. Don) Soo, *J.Orchid Soc. India*. **9**(1-2): 7-12.

Wang, Jing- Wens, Ming Feng, Dong, Yeming- Ming, Yu-Gurung, Liang Bin, Chenlong-ying and Shen, Da- Ling. 2004. A reliable protocol for plant regeneration from pedicel axillary bud of *Phalaenopsis in vitro. In Biological Abstract.* **111**(20): Ab-1033.

Yam, T. W. and M. A. Weatherhead. 1991. Root tip culture of several native orchids of Hong Kong. Lindleyana **6**(3): 151-153.

Yamamoto, M., K. Kondo, K. Taniguchi, K. Hshimota, and R. Tanaka. 1991. Studies on clonal mass propagation of *Calanthe sieboldii* by using tissue cultures shoot primordium method. *In Bulletin of Hiroshima Botanical Garden*, **13**: 1-15.

Yasugi, S., K. Sakamoto and T. Meishi. 1994. Plant regeneration in root segment culture of *Cymbidium* Kenny Wine colour. *Plant tissue culture letter*, **11**(2): 150-152.

Zheng, Li-ming and Ji-Liang pang.2006. *In vitro* flowering of cultures from a hybrid of *Cymbidium goeringii* and *C. hybridium*. *Plant Cell Tissue and Organ Culture*.**84** (2)feb .135-144.

Zhu, Guo-bing, Bai-Yung Yang, Qi- ying Cai, Li- ping Luo, and Bi-cai Guan. 2006.Rapid propagation of *Cymbidium kanran* 'Makino'. *Journal of Trophical and Subtrophical Botany*. **14** (2) March.151-156.