CHAPTER ONE

INTRODUCTION

1.1 Background

Nepal is rich in floral diversity with wide altitudinal variation and diverse climatic condition within a small geographical area. It is estimated that Nepal comprises more than 6,500 flowering plants with extreme diversity of floristic patterns ranging from low altitude of tropics to the highest altitude of alpine zone.

Orchids, one of the most fascinating creations of nature, are also one of the most widely distributed groups of flowering plants. The family Orchidaceae is one of the largest families of flowering plants comprising more than 19,505 spp. belonging to 803 genera (Dressler, 1993). Approximately 25 % of their spp. are terrestrial, 70 % are epiphytic and about 5 % are found on various other supports.

Nepal harbors about 377 spp. of native orchids belonging to 100 genera (Rajbhandari and Dahal, 2004), out of which 10 species are endemic to Nepal. Most of the Nepalese orchids have ornamental value. Some have medicinal as well as edible values. Orchidaceae is also known as "Sungava" and "Chandi gava" in Nepal.

Orchids are found in almost every type of habitats ranging in condition from arctic to tropical. Except in polar regions, orchids are notably diversified in the moist tropics of both hemispheres where the majorities are epiphytes in forest. Most of the temperate and all of the alpine genera are terrestrial. Numerous tropical species are grown around the world by florists and horticulturists, professionals and amateurs for their showy flowers of unusual structures and variety of colours. On the basis of

growth habit, orchids are of two types: Sympodial orchids and Monopodial orchids.

In sympodial orchids such as *Bulbophyllum, Cypripedium, Dendrobium* etc. the shoots are cultured together or spread out on a long rhizome and new shoots may arise from any part of the older shoots where there is an axillary's bud. Monopodial orchids such as *Aerides, Rhynchostylis, Vanda* etc. the shoots have the potentiality for indefinite apical growth but they do not bear rhizomes or pseudobulbs.

Most of the epiphytic orchids are common in eastern and central Nepal and terrestrial orchids (Ground orchids) are found more in western Nepal as climatic conditions are more suitable in those regions. The most important factor which determines distribution of orchids on Nepal is the monsoon.

Orchids are mostly used as ornamental and constitute a multi-million dollar industry in the world. Some orchids are also used for medicinal purpose. They have been highly exploited from their natural habitat although orchids included under CITES as cited in its Appendix-II. Orchids are highly exploited from their natural habitats. So, their conservation is needed. *Ex-situ* conservation is one of the most effective ways of conservation (Falke, 1990).

The most beautiful orchids of Nepal belong to the following genera: *Aerides, Ascocentrum, Arundina, Bulbophyllum,Calanthe, Coelogyne, Cymbidium, Dendrobium, Epigenecum, Eria, Esmeralda, Phaius, Phalaenopsis, Pleione, Rhynchostylis, Thunia, Vanda* and *Vandopsis* (Rajbhandari and Bhattarai, 2001). Some important medicinal orchids of Nepal are mentioned by Rajbhandari *et al*, (2000). They are *Brachycorythis obcorsata, Coelogyne flavida, Coeloghne stricta,*

Cymbidium aloifolium, Dactylorhiza hatgirea, Eulophia nuda, Flickingerea macrai, Pholidata imbricate, Luisia zeylanica and *Vanda tressellata.*

Due to these properties there is a serious threat to the conservation of orchids in Nepal. Their threat is associated with habitat loss, forest destruction, degradation and over exploitation of beautiful and medicinal orchids. Despite the ban imposed by the government of Nepal for collection and trade orchids are being collected illegally.

The orchid flowers are very appealing with long lasting quality and have significant contributed to the development of cut flower and pot plants as a profitable vocation. Several professionals and growers have taken to orchid cultivation. Orchids are widely propagated for horticultural purpose. Some of the countries like USA, UK, Indonesia, Singapore, Thailand, Norway and New Zealand are the commercial growers of the orchid.The orchids of Nepal having high expert potential are mainly *Cymbidium, Dendrobium, Calanthes* and *Coelogynes.*

Under natural condition, the seeds of orchid have only 5 % germination, because of particular fungal requirement (Rao, 1997). So, vegetative propagation is very slow process to propagate a large quality of clone orchids. Therefore it is better to use tissue culture for its propagation. Tissue culture is the technique of development of plant in an artificial nutrient media under aseptic condition. Explants can be a very small piece of plants such as embryos, seeds, stem, shoot tip, meristems, root tips, callus, single cell, protoplast and pollen grains. The plants developed by tissue culture are free from disease.

The tissue culture is also known as micropropagation. This technique can reduce the length of time required to introduce and conserve the new

varieties and is a labour intensive process. Orchid seeds are very small only 1-2 mm long and 0.5-1 mm width. They are produced in large numbers: 1,300 to 4,000,000 per capsule. The embryo has no endosperm, cotyledons and no root initials. Symbiotic (i.e. dixenic) seed germination was the first procedure to be developed by Bernard in 1909. Orchid seeds can germinate in the artificial nutrient media which sustain the seedlings so mycorrhizal fungus is not needed; it is therefore called asymbiotic germination.

Micropropagation 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 germ-plasm preservation. Nevertheless micropropagation technique has not been successful in commercial scale in Nepal yet due to lack of trained personnel and marketing facilities.

A number of works on micropropagation of orchid species have been carried out in Nepal. The main institutions are Central Department of Botany (CDB), Tribhuvan University (T.U.), National Herbarium and Plant Laboratories (NHPL) and Department of Plant Resources (DPR) under the government of Nepal. Some private organizations are also engaged in the micropropagation of orchid species in Nepal.

***Cymbidium elegans* Lindley.**

Cymbidium tends to grow more leaves than most orchids. There are 70 species of *Cymbidium* worldwide. The name *Cymbidium* derived from the Greckword 'kumbos', meaneing hole cavity. It refers to the form of the base of the lip. Ten species of *Cymbidium* are reported from Nepal (Press *et al*., 2000). Among them, one of the important species is *C. elegans* Lindley, syn: *C. longifolium* D. Don. found in Nepal, India, East

Himalaya and Bhutan. It is one of the popular and desirable orchids in the world because of the beautiful flowers. The colour of flowers ranges from olive-green to pale lemon-yellow, with parallel brownish streaks, 3.7-4.3 cm long and 1.5 cm across. It makes house plants and is also popular in floral arrangement. It used as demulcent and for stomach as a medicinal plant (Vaidya *et al.* 2000) It occurs as an epiphytic or a terrestrial herb in the temperate zone of Central and East Nepal at 2100-2500 m altitude. It has flowering period from September-November. In Nepal, it is popular with the name of Chandigava.

***Dendrobium densiflorum* Lindl.**

The genus *Dendrobium* has always been held in high esteem by orchidologist because of its varied morphological features and beautiful flowers. There are 1600 species distributed both in old and new world making the second largest genus in the orchid family (Williams, 1984). The name *Dendrobium* is derived from 'dendron' that means tree and 'bios' means life i.e. an epiphytic plant that exists by clinging to the branches and trunks of host trees. They inhibit in every type of environment from hot tropical forest to that snow line of the Himalayas. In Nepal, Hara *et al*. (1978) enumerated 26 species found at an elevation of 500-2900 m. *Dendrobium densiflorum* Lindl. is one of the important species called Sungava in Nepal due to it's golden colour flower. It is distributed in Central Nepal at an altitude of 900-2900m. It is kept in critically endangered category. It forms dense clumps of pseudobulbs. It prefers partly shaded intermediate conditions with plenty of water in summer and dry nest in winter. It has flowering period from April-June. The attractive, long lasting characteristics of the golden flowers (4-5 cm across) of *Dendrobium densiflorum* Lindl. have gathered considerable interest among horticulturist circle.

1.2 OBJECTIVES

Cymbidium elegans Lindley. and *Dendrobium densiflorum* Lindl. have high ornamental value. Their propagation and domestication are not considered as satisfactory. So, the present research work has following specific objectives:

- To compare and determine the effect of different growth hormones on *in vitro* seed germination of *Cymbidium elegans* Lindley. and *Dendrobium densiflorum* Lindl.
- To determine the appropriate condition for *in vitro* mass propagation of *D. densiflorum* Lindl.
- To acclimatize the *in vitro* grown plants of *D. densiflorum* Lindl and domestication (cultivation).

1.3 JUSTIFICATION

Orchids are the hidden heritage of Himalayan forest. They are under considerable threat due to habitat destruction and degradation. Weak implementation of conservation strategies is further adding the threatened number. The collection of orchid has been banned under the CITES in its Appendix-II. The implementation of regulation is poor and harvesting from wild sources still continues for commercial trade.

As orchids are being used for multipurpose, they have to be conserved for sustainable used. There are various methods of conservation of orchids. Among them, micropropagation is one of the important methods. A large number of plantlets can be produced in a very short span of time in small area through this method. Mass propagated threatened spp. can be used for the conservation as well as for the commercial purpose. There will be economic benefit to the local growers. There are ample opportunities of national economic gains if endogenous species are carefully selected and propagated *in vitro* for the commercial purpose (Pant 2002).

CHAPTER TWO

LITERATURE REVIEW

A lot of works have been done regarding the *in vitro* culture of orchids. The important literatures are reviewed here:

Bernard (1909) was the first to develop the procedure of symbiotic (i.e. dixenic) seed germination of orchids. He discovered the requirement of fungus infection for germination of orchid seeds. The fungus was found to be *Rhizoctonia* sp.which occurs in the roots of orchid plants.

Knudson (1922) developed a suitable nutrient media for the germination of the seeds of orchids without fungal infection (asymbiotic seed germination). The original method was modified as “Knudson C” media.

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

Murashize 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 Philips, 1995).

Raghavan and Torrey (1964) grew seeds of orchids *Cattleya in vitro* in a medium containing NH_4NO_3 as the sole source of nitrogen, germinated readily and preceeded to form small plantlets.

Kand (1967) germinated *Cymbidium viriscens* and *C. gyokuchin* in sterile culture, using one of the newly proposed media, the same media was useful 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 and the results suggested that the auxin and cytokinin ratio is important in controlling root and shoot initiation.

Vena and Torikata (1968) prepared protocorms by meristems culture, when subcultures *Cymbidium insigne* and *C. pumilum* developed plants in BM (Knudson C with Nitsch microelements) within 2 months. NAA, Bacto-trypton, -arginine and L-aspartic acid promoted growth and development while yeast extract was inhibitory.

Maitsui *et al.* (1970) studied the effect of BAP, NAA and their combined effects on the formation of PLB's and the development of shoots and roots meristem culture of *Cymbidium* NAA alone had no effect upon the formation of PLB's. BAP 0.1 ppm induced the greatest effect. NAA and BAP 10 ppm had marked effect on shoot formation.

Mitra (1971) reported that the seeds of orchid *Arundina bambusifolia* Lindl. germinated and developed into seedlings in Raghavan and Torrey (1964) medium. NH_4NO_3 was most suitable source of nitrogen for embryo development. The interesting findings were the formation of PLB's on the shoot tip culture in a medium supplemented with 0.1 X peptone. Higher number of shoots was produced when urea was supplemented and a complete plantlet was obtained from each shoot tip cultured in a medium containing coconut water (20 %). However medium with no coconut water produced numerous shoot buds only from the stem discs (6-8 mm long).

Fonnesbech (1972) studied the effect of auxins (IAA, NAA and 2,4-D), cytokinin (Kinetin and BA) and gibberellin (GA) alone or in combination

on the protocorm of *Cymbidium*. IAA alone had no effect and NAA inhibited chlorophyll synthesis at high cocentration. BA and Kinetin when used singly induced shoot formation in solid medium and callusing in liquid medium while GA induced shoots and leaf growth. NAA and Kinetin together resulted in maximum fresh weight.

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

Tanaka *et al.* (1975) formulated a medium for formation of PLBs when they obtained PLBs from the leaf tissues of *Phalaenopsis* and *Vanda* seedlings. Juvenile plants were developed from the PLBs on the medium employed for orchid seed germination.

Reinert and Hubert (1976) successfully propagated *Cattleya* orchid plants by tissue culture of lateral bud meristems 1st in a chemically defined liquid medium with constant agitation then later transferred to an agar medium. Addition of Kinetin after the 1st week of culturing favored the development of callus or protocorm like bodies, which produced plantlets.

Singh (1976) reported variation in the growth of meristem explants of *Dendrobium*. The meristems from the apical and axillary buds of single mother plant showed different response in the nutrient medium under same conditions. The varying age and size of the buds excised from the young shoots was responsible for such result.

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

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

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

Niroula and Rajbhandary (1985) established shoot proliferation and protocorms form the seedling tips of *Dendrobium fimbriatum* Hook. in MS medium supplemented with cytokinins. Complete plantlets were obtained when shoots and protocorms were transferred in Vacin and Went medium.

Raghuwansi *et al.* (1986) studied the germination and seedling growth in *Dendrobium nobile, D. chrysanthum* and *Sarcanthus pallidus* at different P^H (3,4,5,6,7,8 and 10) levels maintained on modified KnC medium. Optium results were obtained at 10 proved detrimental for germination and protocorm growth. The roots/absorbing hairs developed best at P^H 6, maximum leaves were produced at P^H 4 in *D. nobile* in contrast to *D.chrysanthum* and *S. pallidus* which showed more leaf primordial at P^H 5.

Bopaiah and Jorapur (1986) showed seeds of *Cymbidium aloifolium* Sw. on modified Knudson C (Kn C) medium (BM) supplemented with 100ml/l of coconut milk (CM) and 3 mg/l each of peptone (P) and Casein

hydrolysate (CH). The Plb's obtained from 8 week old cultures were subcultured on fresh medium containing all 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 aminoacid (glycine) in different concentration in the nutrient medium comprising BM + CM + P + CH + banana pulp along with 1mg/l each of thiamine HCl, niacin, glycine and kinetin was found most suitable for the normal and healthy seedling growth.

Mitra (1986) cultured orchid seeds which obtained from green pods after 8-12 weeks of culture, germinated readily in a large number of species. Of the various media tried for seed germination the one germinated by Mitra (1976) with lesser amounts of ammonia, and of nitrates of calcium and potassium and of phosphate ions along with several vitamins had been found to be most suitable for a large number of orchid species. The additional presence of amino acids, urea, peptone, casein hydrolysate, yeast extract, coconut milk, auxin, cytokinins, adenine and gibberellins in this medium had yielded a better germination of embryos and protocorm formation.

Muralidhar and Mehta (1986) germinated seeds of *Cymbidium longifolium* on three basal medium, K n C (knudson, 1946), VW (Vacin and Went, 1949), RT (Raghvan and Torrey, 1964) with or without various levels and combination of vitamins, hormones, amino acids, and micronutrients. The seed germination was assessed at up to 30 % on K n C, 60 % on VW, and 35 %on RT. The sequential steps of histomorphological changes from embryo to plb's were traced out.

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) 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 formed root meristem differentiated.

Shrestha and Rajbhandary (1988) regenerated plant through meristem of *Cymbidium giganteum* Wall ex. Lindl. The shoots were initiated on MS (1962) media, MS media, supplemented with BAP (5 mg/l), NAA (1 mg/l) and 10 % coconut milk. The proliferation continued survived in green house.

Yamamato *et al.,* (1991) observed that shoot primordial of *Calanthe siefoldii* were induced from the meristems in modified B_5 medium supplemented with 2 mg/l BA. Then PLBs were obtained from shoot primordial after transplanting onto agar medium. PLBs were regenerated into plantlets.

Inchihashi (1992) cultured lateral buds on young flower stalks of *Phalaenopsis* and rapid proliferation were attained without addition of hormones. Coconut water was both inhibitory and stimulatory depending on the cultivar. Bud growth was better on media solidified with gelrite. A medium for flower stalk bud culture of *Phalaenopsis* was developed.

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 the 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 (Vacin and Went, 1949) medium. The ground growing *Spalthaglottis plicata* responded better to MS medium and the epiphytic *Epidendrum radicans, Dendrobium crepidatum* and *Cymbidium aloifolium* to RL medium.

Shrestha and Rajbhandary (1993) regenerated plant through meristem culture of *Cymbidium grandiflorum.* It was carried out on MS medium supplemented with 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 greenhouse.

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

Vij (1993) studied the regeneration response of root explants, in two species and hybrids of orchids and found it to vary with its genetic constitution, physiological age and the chemical regime. Yeast extract proved obligatory for inducing proliferation in *Aerides multiflorum* and *Vanda teres* cultures and peptien in those of *Cymbidium pendulum, Vanda cristata and V. testacea.* The effect of plant growth hormones was species specific and varied during initiation, multiplication and differentiation of the cultures.

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

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, 1 mg/l NAA and 1 mg/l BA induced PLB formation in *Cymbidium.*

Yasugi *et al.* (1994) obtained the greatest number of shoots in *Dendronium* sp. on a medium containing 0.1 mg/l NAA and 0.1 mg/l BA. In both parts of segment culture a maximum of 5 multiple shoots were induced in the same medium after 8 work in MS medium + 2 % sucrose + 0.8 % agar at p^H 5.8. Multiple shoots were not obtained in NAA and BA free medium but roots were developed.

Hazarika and Sharma (1995) observed *in vitro* germination and regeneration of *Dendrobium transparens* Lindl. The seeds of *D. transparens* showed signs of swelling of embryos emerged out and

developed into distinct globular, yellowish green protocorms within 25days of culture in B_5 medium supplemented with 0.1 mg/l IBA, KN (0.1 mg/l), 1 mg/l NAA and 50 mg/l CH(Casein hydrolysate). The nodal culture was done on B_5 medium supplemented with different hormonal concentration of IBA, CH, KN, NAA and BAP. Shoot buds developed into healthy plantlets within 90days of inoculation after 2 subculture on medium supplemented with IBA (5 mg/l)), KN (1 mg/l), NAA (1 mg/l), BAP (1 mg/l) and CH (100 mg/l).

Pyati and Murthy (1995) studied *in vitro* seed germination and seedling development of *Dendrobium ovatum* (Wild.) Krunzl. The *in vitro* seed germination of *D. ovatum* was achieved on four basal media Knudson C (KC), Vacin and Went (VW), Murashige and Skoog (MS) and Burgeff (N_3f). Optimum germination occurred on 'KC' medium.The best result occurred on KC medium with 10 % coconut milk, 10% cane juice, 200 mg/l yeast extract, 200 mg/l casein hydrolysate, 200 mg/l peptone and 0.5 mg/l nicotinic acid.

Sharma and Chauhan (1995) established *in vitro* raised seedlings of *Dendrobium* and *Paphiopedilum.* The seedlings of *D. chrysanthum* and *P. spicerianum* different in their requirement of potting mixture. The potting mixture comprising brick, charcoal, tree fern, bark pieces, leaf mould and dry sphagnum in 1:1:1:1:1:2 ratios supported the maximum growth of *D. chrysanthum* whereas *P. spicerianum* seedlings required mixture of leaf mould, perlite, vermiculate and dry sphagnum in ratio of 1:1:1:2.

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 were obtained.

Devi *et al.* (1997) observed clonal propagation of *Dendrobium moschatum* and *Cymbidium aloifolium* through shoot tip culture. The shoot tips of both orchids were cultured in 5 different media viz. MS (1962), WI (Wimber, 1963), KC (Knudson, 1946), VW (1949) and NI (Nitsch and Nitsch, 1969). Nitsch and Nitsch (1969) medium were 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.

Nayak *et al.* (1997) induced shoot buds in basal parts of foliar explants of *Acampe praemorsa* on MS (1962) medium supplemented with BA, Kn or TDZ, the latter being most effective at 1 mg/l. Shoots regenerated from the foliar explants were rooted successfully on MS medium containing 1 mg/l IBA. The plants were acclimatized and transferred to a garden.

Liu and Zhang (1998) obtained a suitable medium for plantlet production of *Dendrobium candidium* in the period of strengthening plantlet method. The B_5 or ½ MS added with 10 % of banana aqueous extract and 2 mg/l NAA can be used as the best plantlet strengthening medium for *D. candidium.*

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 Paelobutrazol (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.

Banerjee and Mandal (1999) germinated immature *Cymbidium* seeds obtained from green capsules on defined orchid culture medium supplemented with folic acid, NAA and organic adjuvants (Casein hydrolysate, peptone, coconut water and tryptophan) at varying rates. 2-5 mg/l of folic acid in VW medium induced maximum 85 % germination while NAA 0.1 mg/l could induce 78 % 2,4-D, GA_3, BAP and Kinetin bore either null relationship or inhibitory effects on seed germination. NAA 2 mg/l in VW was most appropriate to induce 3-4 roots in two months.

Kamalakannan *et al.* (1999) regenerated the endemic orchid *Coelogyne odoritissima* var. *angustifolia* via encapsulation of protocorms in sodium alginate matrix. Encapsulation with sodium alginate and subsequent re-growth was compared to encapsulation with nutrient medium.

Shrestha (1999) obtained 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.

Murthy and Pyati (2001) conducted 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 2 mg/l BA gave best PLB formation (18 /leaf explants) and further subculture in basal MS medium resulted plantlets were successfully transferred to vermiculate initially and then to potting mixture 84 % of the plantlets survived after 3 months of transplantation.

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 and supplementation of 0.3 % activated charcoal brought about a marked effect on growth of shoots and roots.

Talukdar (2001) observed multiple shoot induction in *Dendrobium aphyllum* Roxb. The Knudson C medium supplemented with Kinetin (10 mg/l), coconut water (15 % w/v) and banana extract (6 %, w/v) within 13 weeks of culture regenerated maximum of seven shoots. Well developed shoots were developed in IAA and NAA treated cultures. Protocorms and callus formation was achieved in medium containing 2-4, D.

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 irridiodes* D.Don.

Karanjit (2002) cultured the seeds of *Coelogyne cristata* Lindl. and *Cymbidium irridiodes* D.Don . in MS medium and Gamborg B_5 (G-B_5) medium. The germination rate was noted vigorous in MS medium.

Roy and Banerjee (2002) studied the optimization of *in vitro* seed germination, protocorm growth and seedling proliferation of *Vanda tessellate.* Modified Kn C, 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 and Pant (2004) cultured the seeds of *Cymbidium irridiodes* D.Don. Growth and development of seedlings were favoured in MS medium supplemented with BAP (1 ppm) and NAA (1 ppm). The highest number of multiple shoots was obtained in MS medium supplemented with BAP (0.5 ppm). She also cultured shoot tip of *Coelogyne cristata* Lindl. and the highest number of multiple shoot was observed in MS medium with BAP (1 ppm) and NAA (1 ppm).

Wang *et al.* (2004) established an efficient and simple method of high protocorm regeneration. Pedicel axillary buds of *Phalaenopsis* 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 NAA and further subculture in the same medium gave shoots in 4 weeks. 0.1 mg/l IAA resulted root formation in 40 % of the shoots by subculture of protocorm.

Karki *et al.* (2005) micropropagated *Vanilla planifolia* from seeds by using Murashige and Skoog medium without growth hormones. The seedlings were subcultured in MS medium supplemented with 1.0 mg/Benzylaminopurine and 1.5 mg/l Kinetin with 10 % coconut milk.

Shrestha A. (2005) successfully achieved asymbiotic germination of *Coelogyne ovalis* Lindl. MS media supplemented with 1 ppm of NAA was the best medium for germination, growth and development of seedlings. The maximum number of shoot multiplication was observed in MS media with 1ppm of BAP (5.6 Shoots) while MS media with 2 ppm of IBA showed best rooting.

Sharma *et al.* (2005) cultured the immature seeds of *Dendrobium fimbriatum* Hook. asymbiotically in Vacin 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.

Gurung (2006) cultured the seeds of *Aerides odorata* Lour. in MS medium and hormonal MS medium under aseptic conditions. The best shoot multiplication (6.3 shoots/culture) was obtained in MS medium supplemented with 1.5 ppm BAP + 1 ppm NAA. 0.5 ppm of IAA formed the highest number of roots (4.7 roots/shoot).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

The materials used for the present experiment were the young capsules of *Cymbidium elegans* Lindley. and *Dendrobium densiflorum* Lindl. The materials were obtained from ICIMOD Conservation Demonstration, Garden, Lalitpur.

3.2 Methodology

The methodology applied for the seed germination and acclimatization are described under following headings:

3.2.1 Methods for seed germination

The methods for seed germination of *C. elegans* Lindley. and *D. densiflorum* Lindl. are described as under:

3.2.1.1 Preparation of stock solution

The Murashige and Skoog's (1962) medium (MS) was used as the basal medium for this experiment, along and in combination with different concentration of hormones (BAP, NAA, IAA and IBA). 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.	(100X)mg/100 ml stock Concentration	Volume to be taken for 1 litre medium
Micronutrients			
Manganese-sulphate ($MnSo_4.4H2O$)	22.3	2230	
Boric acid (H_3BO_3)			
Zinc sulphate ($ZnSO_4.7H_2O$)	6.2	620	
Potassium Iodine (KI)	8.6	860	
Sodium-molybdate ($Na_2MoO_4.2H_2O$)	0.83	83	
Cobalt Chloride ($CoCl_2.6H_2O$)	0.25	25	1 ml
Copper sulphate			
($CuSO_4.5H_2O$)	0.025	2.5	
	0.025	2.5	

C. Iron source (Fe, EDTA)

Components	Composition of MS (Final conc.) Mg/lit	(10X)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	(100X) mg/100ml stock Concentration	Volume to be taken for 1 litre medium
Glycine	2.0	200	1 ml
Nicotinic acid	0.5	50	
Pyridoxin HCL	0.5	50	
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 mesh upto 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 the refrigerator.

3.2.1.2 Hormones used for the experiments

a. ℑ-Naphthalene Acetic Acid (NAA)

b. 6-Benzyl Amino Purine (BAP)

c. Indole-3-Acetic Acid (IAA)

d. Indole-3-Butyric Acid (IBA)

3.2.1.3 Preparation of hormones

Auxins and Cytokinin are taken as growth hormones for the experiment. For the preparation of auxins (IAA, IBA and NAA), 10 mg of auxin was dissolved in 2.5 ml of 1 N NaOH and for the preparation of cytokinin, 10 mg of BAP was dissolved in 0.25 ml of 0.5 N NaOH. After that, the final volume was mesh upto 100 ml by the addition of sterilized water in each hormone separately. 1 ml of this hormone stock was used for 100 ml of media to make 1 mg/l of hormone concentration in the media.

3.2.1.4 Sterilization of Glassware's and Metal instruments

During the experiment, the necessary glasswares were subjected to dry heat sterilization before their use. Glasswares such as petridishes, culture tubes, pipettes, beaker, conical flask etc. were dipped in detergent solution for 24 hours and rinsed with tap water, and final rinse was done with distilled water. Glasswares and metal instruments were subjected to dry heat sterilization before their actual use in the process. They were sterilized in hot air oven at 150ºc for 2 hours. Metal instruments like forceps, scalpels and surgical blades were wrapped with aluminium foil before keeping inside the hot air oven for sterilization. Once they were sterilized in hot air oven, they were autoclaved at 121ºc for 20 min. at 15 lb/sq.inch.

3.2.1.5 Preparation of Media

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

- 1 litre sterile 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 sterile 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 media supplemented with different concentrations of hormones, 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 to 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 16 ml was dispensed in culture tubes. Then each tube was enclosed by aluminium foil cap.
- The tubes containing medium were subjected to 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 (25 $\pm$ 2°c).

3.2.1.6 Sterilization of plant material

The inoculation of seeds and explants was carried out in the laminar airflow chamber. Therefore before inoculation, the laminar airflow chamber was made sterile by cleaning it with spirit or cotton soaked with 70 % ethyl alcohol. The culture tubes containing media sterile instruments and glasswares were exposed under ultraviolet (UV) radiation for 45 minutes to remove the possible contaminants presenting and around the transfer area. After turning off the UV light, the blower was kept running during the process of inoculation.

The plant materials used for the experiment were green pods of *Cymbidium elegans* Lindley. and *Dendrobium densiflorum* Lindl. For sterilization of plant material, the *in vivo* grown orchid pods were washed in running tap water to remove the dust and soil remaining on their surface. The pods were dipped in detergent water for 15-20 minutes and then washed in running water for 1 hr. The pods were then rinsed with distilled water. The pods were made surface sterile in 70 % ethyl alcohol for 2 minutes and then dipped into 1 % sodium hypochlorite solution for 15-20 minutes. Finally, the pods were rinsed with sterile water for 5 times.

3.3 Inoculation of Seeds of *C. Elegans* Lindley. and *D. Densiflorum* Lindl.

Pods of both orchids were put on sterile petridish containing sterile filter paper for soaking the surface moisture of orchid pods. Then, each pod was cut longitudinally into two halves by sterile surgical blade. The very tiny seeds of orchids were scrapped with the help of sterile spatula and inoculated on the MS media alone and supplemented with different

growth hormones. After that all the cultures were maintained at 25ºC (± 2ºC) temperature about 12-15 hours photoperiods and observed regularly.

3.4 Histomorphological Study of *D. Densiflorum* Lindl.

For histomorphological study, mass of Plbs obtained from seed culture after 8 weeks of culture was fixed periodically in 1 % acetocarmine for 24 hrs. Small piece of stained mass was mounted on slide with few drops of acetocarmine solution. The slides were then examined under compound microscope. Photographs of the distinct stages were taken.

3.5 Inoculation Of Explant Of *Dendrobium Densiflorum* Lindl.

The inoculation of explants was done in laminar air-flow chamber under sterile condition. The explants used in this experiment were shoot and root tips of *D. densiflorum* Lindl. obtained from the *in vitro* grown plants. Small pieces of shoot tips and root tips (about 5 mm) were cut with the help of surgical blade and inoculated on the surface of media 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 25ºC (± 2ºC) under 12-15 hours photoperiod and observed regularly.

3.6 Shoot Multiplication

For shoot multiplication, individual microshoots of about 5 mm were cut and transferred to the most appropriate media for shoot multiplication and shoot growth was recorded in every 2 weeks.

3.7 Rooting Of Shoots

The microshoots obtained from the culture of shoot tips of *Dendrobium densiflorum* Lindl. were transferred to the media supplemented with

different concentrations of rooting hormones like IAA, IBA and NAA and recorded every 2 weeks.

3.8 Methods Of Acclimatization

The *in vitro* grown plantlets with well developed roots of *D. densiflorum* 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 bapistine (0.1 %) to minimize the chances of infection for 5 minutes.
- Washed the plantlets 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 5 % NPK solution was sprayed once a week regularly for fastening their growth.
- The plants were covered with transparent polythene sheets to control the humidity. The small holes were made into polythene sheet for aeration.

- The plants were kept in the greenhouse for several weeks and finally they were transferred to the natural environment to check their growth.

3.9 Stastical Analysis

Statistical analysis was done by using Analysis of Variance (ANOVA) one way classifiction system. The data obtained were analysed using application software-microsoft excel. The significant difference between the MS medium and MS medium supplemented with different growth hormones were analysed and used to compare the marginal means to obtain plausible discussions.

CHAPTER FOUR

RESULT

The results of *in vitro* seed germination of *Cymbidium elegans* Lindley. and *Dendrobium densiflorum* Lindl., culture of shoot tips and root tips, rooting of shoots and acclimatization of *D. densiflorum* Lindl. have been described under following headings:

4.1 *In vitro* culture of seeds of *Cymbidium elegans* Lindley. and *Dendrobium densiflorum* Lindl.

The immature seeds of *C. elegans* Lindley. and *D. densiflorum* Lindl. were cultured in MS basal and MS supplemented with various growth hormones. *C. elegans* Lindley. showed better germination in 1 mg/l BAP than rest of the other hormone supplemented media and hormone free MS media whereas *D. densiflorum* Lindl. showed quick response in hormone free MS media than in other hormone supplemented MS media. In *C. elegans* Lindley. seeds germinated in 9 weeks whereas in *D. densiflorum* Lindl. seeds germination started after 5 weeks of culture. The time taken for the seed germination was 4 weeks earlier than in *D. densiflorum* Lindl. then in *C. elegans* Lindley.

In vitro germination of immature seeds undergoes further differentiation to form seed clumping, protocorm like bodies (PLBs) and finally to the plantlet. All the protocorms formed in different media were chlorophyllous and globular. The observation of *in vitro* seed germination of *C. elegans* Lindley. and *D. densiflorum* Lindl. in MS media and MS supplemented with different concentration of hormones is summarized in the table 1 and 2 respectively.

Table 1

Effect of growth regulators supplemented in MS media on seed germination and seedling growth of *Cymbidium elegans* Lindley.

Media	Growth Hormones	Concentration of hormones (mg/l)	Observation taken in weeks				Remarks
			Initiation of Germination	Protocorm Formation	1st Shoot Formation	1st Root Formation	
MS	BM	-	14	17	39	Not observed	Good
"	BAP	0.5	15	18	38	"	Average
"	BAP	1.0	9	10	31	"	Best
"	BAP	1.5	17	22	40	"	Average
"	BAP	2.0	10	12	31	"	Good
"	NAA	0.5	18	24	42	"	Poor
"	BAP+NAA	0.5 + 0.5	10	14	36	"	Average
"	BAP+NAA	1.0 + 0.5	13	17	30	"	Good
"	BAP+NAA	1.5 + 0.5	11	18	33	"	Good
"	BAP+NAA	2.0 + 0.5	12	15	31	"	Good

Culture conditions: - MS medium, 25 ± 2°C, 42 weeks, 12-15 hrs photoperiod, 4 replicates were used in each combination.

Table 2

Effect of growth regulators supplemented in MS media on seed germination and seedling growth of *Dendrobium densiflorum* Lindl.

Media	Growth Hormones	Concentration of hormones (mg/l)	Observation taken in weeks				Remarks
			Initiation of Germination	Protocorm Formation	1st Shoot Formation	1st Root Formation	
MS	BM	-	5	6	8	19	Best
"	BAP	0.5	6	8	15	-	Poor
"	BAP	1.0	7	9	21	-	Poor
"	BAP	1.5	9	10	20	-	Poor
"	BAP	2.0	10	12	17	-	Poor
"	NAA	0.5	6	8	11	22	Good
"	BAP+NAA	0.5 + 0.5	6	10	15	-	Poor
"	BAP+NAA	1.0 + 0.5	7	11	15	-	Poor
"	BAP+NAA	1.5 + 0.5	8	12	18	-	Poor
"	BAP+NAA	2.0 + 0.5	8	13	20	-	Poor

Culture conditions: - MS medium, 25 ± 2°C, 32 weeks, 12-15 hrs photoperiod, 4 replicates were used in each combination.

4.1.1 *In vitro* germination of seeds

The inoculation of seeds of *Cymbidium elegans* Lindley. and *Dendrobium densiflorum* Lindl. in MS with different hormonal concentration of BAP and NAA showed following changes:

1. MS Basal Medium

For the seed germination *C. elegans* Lindley. required 14 weeks in MS basal medium. The protocorms were developed in 17th week. The shoot development was observed in 39th weeks.

In *D. densiflorum* Lindl., the ideal condition for seed germination was the MS basal medium. The germination was observed in 5th week. The protocorms were developed in 6th week. The initial shoot and roots were developed in 8th and 19th week respectively (Fig. SG 06).

2. MS + BAP 0.5 mg/l

In MS medium supplemented with 0.5mg/l BAP, the seeds of *C. elegans* Lindley. germinated in 15th week. The protocorm formation and initial shoot development were observed in 18th and 38th week respectively.

In *D. densiflorum* Lindl., the germination was observed in 6th week. The protocorms were formed in 8th week. The initial shoot development was observed in 15th week but root development was not observed till 32 weeks.

3. MS + BAP 1 mg/l

Seed culture in MS medium supplemented with BAP 1 mg/l required 9 weeks for germination of *C. elegans* Lindley. This medium was the ideal condition for the seed germination. The protocorms were developed in

10th week. The first shoot formation was observed in 31st week (Fig. SG07) .

In the same condition *Dendrobium densiflorum* Lindl. showed germination in 7th week. The protocorm and shoots were formed in 9th and 21st week. There was no development of roots till 32 weeks.

4. MS + BAP 1.5 mg/l

Seed culture in MS medium supplemented with BAP 1.5 mg/l required 17 weeks for seed germination in *Cymbidium elegans* Lindley. The protocorms were developed in 22 weeks and the shoots were developed in 40th week.

D. densiflorum Lindl. showed initiation of germination in 9th week. The protocorms and shoots were developed in 10th and 20th week respectively. Root primordia were not formed till 32 weeks.

5. MS + BAP 2 mg/l

C. elegans Lindley. showed initiation of germination in 10th week in MS medium supplemented with BAP 2 mg/l. The protocorms were developed in 12th week and the shoot development was observed in 31st week.

In *D. densiflorum* Lindl., the seed germination was observed in 10th week. The protocorms and shoots were developed in 12th and 17th week respectively but root primordia were not observed in this medium till 32 weeks.

6. MS + NAA 0.5 mg/l

Seed culture in MS medium supplemented with NAA 0.5 mg/l required 18 weeks for germination of *C. elegans* Lindley. The protocorms were

developed in 24th weeks. The primary shoot development was observed after 42 weeks.

In *Dendrobium densiflorum* Lindl., the initiation of seed germination was observed in 6th week. The protocorms and shoots were developed in 11th and 22nd week respectively.

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

In *Cymbidium elegans* Lindley., the initial germination of seed was observed in 10th week in MS medium supplemented with BAP 0.5 mg/l + NAA 0.5 mg/l. The protocorms were developed in 14th weeks and the shoot development was observed in 36th weeks.

In *D. densiflorum* Lindl., the initiation of seed germination was observed in 6th week in this medium. The protocorms and shoot development was observed in 10th and 15th week respectively. The pseudobulb of length about 0.4 cm was observed. Root primordia were not observed till 32 weeks.

8. MS + BAP 1.5 mg/l + NAA 0.5 mg/l

Cymbidium elegans Lindley., required 11 weeks for initiation of seed germination in this medium. The protocorms were developed in 18th week. The shoots were developed after 32 weeks of seed inoculation.

In *Dendrobium densiflorum* Lindl., the initial germination was observed in 8th week in this media. The protocorms and shoots were observed in 12th and 18th week respectively. Root primordia were not developed till 32 weeks of seed inoculation (Fig. SG09).

Flowers , Pods and Seed Germination of *Cymbidium elegans* Lindley. and *Dendrobium densiflorum* Lindl.

Fig. 01 Exotic flower of *Cymbidium elegans* Lindley.

Fig. 02 Exotic flower of *Dendrobium densiflorum* Lindl.

Fig. 03 *Dendrobium densiflorum Lindl.* with pods in its habitat.

Fig. 04 Longitudinal section of pod of *Cymbidium elegans* Lindley. showing yellowish tiny seeds.

Fig. 05 Longitudinal section of pod of *Dendrobium densiflorum* Lindl. showing yellowish tiny seeds.

Fig. SG 06 Seed cultured in hormone free MS basal medium after 6 weeks of culture showing whitish green globular mass of small protocorms of *Dendrobium densiflorum* Lindl.

Fig. SG 07 Seed cultured in MS medium supplemented with BAP 1 mg/l after 9 weeks of culture of *Cymbidium elegans* Lindley. showing browinish white seed clumping.

Fig. SG 08 Seed cultured in MS medium supplemented with BAP 0.5 mg/l + NAA 0.5 mg/l on 28th week of culture of *Cymbidium elegans* Lindley. showing green globular hairy mass of protocorm.

Fig. SG 09 Seed cultured in MS medium supplemented with BAP 1.5 mg/l + NAA 0.5 mg/l after 12 weeks of culture of *Dendrobium densiflorum* Lindl. showing the globular yellowish green mass of protocorm like bodies.

Fig. SG 10 Seed cultured in MS medium supplemented with BAP 1 mg/l + NAA 0.5 mg/l on 32nd week of culture of *Cymbidium elegans* Lindley. showing small plantlets developed from green globular protocorm like bodies.

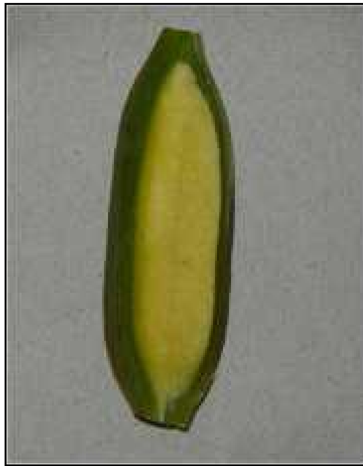
Fig. SG 11 Seed cultured in MS medium supplemented with BAP 2 mg/l + NAA 0.5 mg/l on 34th week of culture of *Cymbidium elegans* Lindley. showing small green plantlets with the formation of phenolic compound which affects the growth of seedlings.

Fig. SG 12 Seed cultured in MS basal medium after 20 weeks of culture of *Dendrobium densiflorum* Lindl. showing the many green plantlets with small roots.

01

02

03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SG 06

SG 07

SG 08

SG 09

SG 10

SG 11

SG 12

9. MS + BAP 1 mg/l + NAA 0.5 mg/l

Seed culture in MS medium supplemented witn BAP 1 mg/l + NAA 0.5 mg/l required 13 weeks for seed germination of *C. elegans* Lindley. The protocorms were developed in 17th week. The shoots were developed after 30th weeks (Fig. SG10).

In *D. densiflorum* Lindl., the initial seed germination was observed in 7th week. The protocorms and initial shoots were developed in 11th and 15th weeks respectively. Root primordia were not developed till 32 weeks.

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

The initial seed germination was observed in 15th week in *C. elegans* Lindley. The protocorms and initial shoots were developed in 15th and 31st weeks respectively (Fig. SG 11).

In *D. densiflorum* Lindl., the germination of seed was observed in 8th week. The protocorms and initial shoots were developed in 13th and 20th week respectively but root primordial not were developed till 32 weeks.

4.2 Histomorphological study of *D. densirflorum* Lindl.

After the seed germination, the protocorms were formed and finally developed into seedlings. In present investigation, the histomorphological study of protocorm like bodies was done after 8 weeks of seed germination in *D. densiflorum* Lindl. After 8 weeks, the embryos exhibited a prominent zone of promeristematic cells from which developed a pair of leaf primordia. Then the embryonic photosynthetic leaves were developed. Simultaneously with the development of embyonic leaves at the proximal end of the embryo, the marginal cells at

Histomorphological Study of *Dendrobium densiflorum* Lindl.

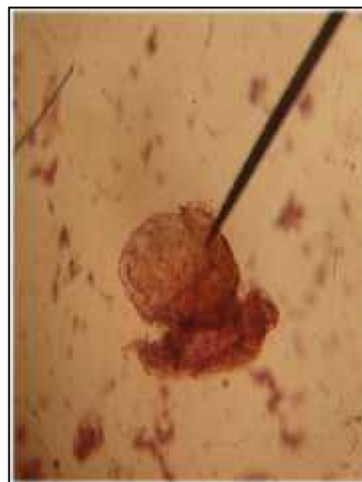
Fig. H 01	Seed with transparent coats with embryo inside it.
Fig. H 02 – H 03	Embryo emerging out of seed coat through ruptured terminal cells.
Fig. H 04 – H 06	Sequential changes of embryos proliferating to form PLBs and plantlets.
Fig. H 07	Leaf development from embryo.
Fig. H 08 – H 09	Bulbous embryos with rhizoids.

H 01

H 02

H 03

H 04



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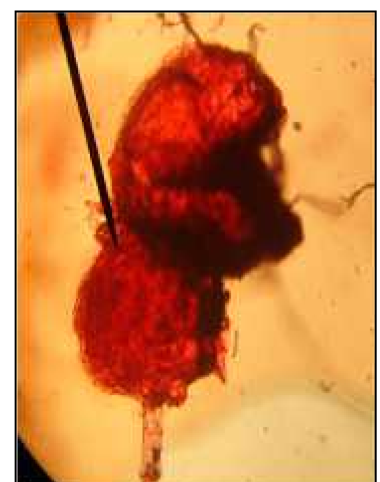

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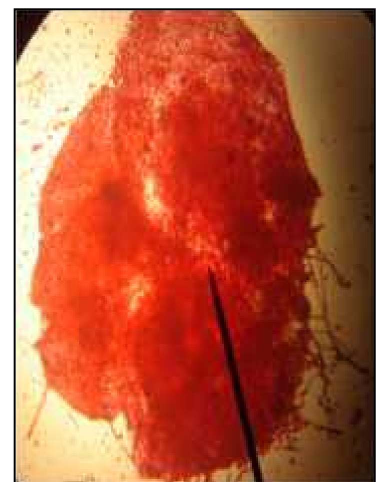


H 09

the distal end of the embryo started giving rise to tubular and unicellular rhizoids (Plate No.H01-H09).

4.3 Micropropagation of *Dendrobium densiflorum* Lindl.

4.3.1 Culture of Shoot tip of *D. densiflorum* Lindl.

The shoot tips of about 5 mm in size were cultured on MS basal medium and MS medium supplemented with various concentrations of NAA and BAP for inducing multiple shoots. The response of shoot tips in different hormones supplemented media and MS basal media after 12 weeks of culture of shoot tips have been given in table 3.

Table: 3

Effect of BAP and NAA on shoot tip culture of *Dendrobium densiflorum* Lindl.

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

Culture conditions: - MS medium, 25±2°C, 12 weeks, 12-15 hrs photoperiod, 4 replicates were used in each combination.

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

4.3.1.1 Development of Shoot tip explant of *Dendrobium densiflorum* Lindl.

Shoot tip explants developed well in hormone free MS medium as well as in MS medium supplemented with different concentration of auxin and cytokinin. The shoot tips of *D. densiflorum* Lindl. cultured in MS basal medium and MS supplemented with different concentration of BAP and NAA showed following changes:

1. MS Basal Medium

In MS basal medium, the shoot multiplication started after 6 weeks of culture of shoot tips. The elongation of shoots ranged from 2.4-3.4 cm. After 12 weeks, the explants were changed into multiple shoot with well developed roots and the average no. of shoots was 1.5 per culture. (Fig ST01).

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 5 weeks of culture of shoot tips. A well growth of two rooted shoots and other shoots without roots were observed after 12 weeks of culture. The shoot length range from 2-3 cm in this medium and the average no. of shoots was 2.25 per culture (Fig ST02).

3. MS + BAP 1 mg/l

MS medium supplemented with BAP 1 mg/l induced multiple shoots after 5 weeks of culture and pseudobulb was observed after 12 weeks of cultured of shoot tips. The shoot length ranged from 2.4-2.8 cm and the average no. of shoots was 1.5 per culture (Fig. ST03).

4. MS + BAP 1.5 mg/l

In MS medium supplemented with BAP 1.5 mg/l, the shoot multiplication started after 4 weeks of shoot tip cultured. The shoots were grown upto length of 3 cm. There was no root differentiation till 12 weeks of culture and the average no. of shoots was 2.75 per culture (Fig. ST04).

5. MS + BAP 2 mg/l

In MS medium supplemented with BAP 2 mg/l showed shoot differentiation after 5 weeks of culture of shoot tips. The shoots of length 2-3 cm were found upto 12 weeks of culture. There was no root differentiation and the average no. of shoots was 2 per culture (Fig ST05).

6. MS + NAA 0.5 mg/l

In MS medium supplemented with NAA 0.5 mg/l, the shoot multiplication started after 9 weeks. Three shoots and one rooted shoots were observed. The shoot length ranged from 2.1-2.3 cm till 12 weeks of culture of shoot tips and the average no. of shoots was 1 per culture (Fig. ST06)

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 8 weeks of culture. The shoots range with the length of 2.9-3.9 cm. There was callus formation and no root differentiation till 12 weeks of culture of shoot tips and the average no. of shoots was 1.75 per culture (Fig. ST07).

Development of Shoot Tip and Root Tip Explants of *Dendrobium densiflorum* Lindl.

Fig. ST — Shoot tips of Dendrobium densiflorum Lindl. on 12th week of seed culture in hormone free MS basal medium which were used as explant for the further proliferation of shoots.

Fig. ST 01 — Shoot tip cultured on hormone free MS basal medium on 10th week of culture of shoot tip showing elongation and multiplication of shoots.

Fig. ST 02 — Shoot tip cultured on MS medium supplemented with BAP 0.5 mg/l on 11th week of culture of shoot tip showing multiplication of shoots.

Fig. ST 03 — Shoot tip cultured on MS medium supplemented with BAP 1 mg/l on 7th week of culture of shoot tip showing multiplication of shoots.

Fig. ST 04 — Shoot tip cultured on MS medium supplemented with BAP 1.5 mg/l on 10th week of culture of shoot tip showing multiplication of shoots.

Fig. ST 05 — Shoot tip cultured on MS medium supplemented with BAP 2 mg/l after 12 weeks of culture of shoot tip showing multiplication of shoots without roots.

Fig. ST 06 — Shoot tip cultured on MS medium supplemented with NAA 0.5 mg/l after 12 weeks of culture of shoot tip showing multiplication of shoots with few roots.

Fig. ST 07 — Shoot tip cultured on MS medium supplemented with BAP 0.5 mg/l + NAA 0.5 mg/l after 12 weeks of culture of shoot tip showing multiplication of shoots without roots.

Fig. ST 08 — Shoot tip cultured on MS medium supplemented with BAP 1 mg/l + NAA 0.5 mg/l on 9th week of culture of shoot tip showing multiplication of shoots without roots.

Fig. ST 09 — Shoot tip cultured on MS medium supplemented with BAP 1.5 mg/l + NAA 0.5 mg/l after 12 weeks of culture of shoot tip showing multiplication of shoots with pseudobulb.

Fig. ST 10 — Shoot tip cultured on MS medium supplemented with BAP 2 mg/l + NAA 0.5 mg/l after 12 weeks of culture of shoot tip showing multiplication of shoots with pseudobulb.

Fig. RT 01 — Root tip cultured on MS basal medium showing multiple shoots and new roots after 24 weeks of culture.

ST

ST 01

ST 02

ST 03

ST 04

ST 05

ST 06

ST 07

ST 08

ST 09

ST 10

RT 01

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

In MS medium supplemented with BAP 1 mg/l +NAA 0.5 mg/l, multiple shoots were obtained after 4 weeks of culture of shoot tips. The shoot length ranged from 2.3-2.8 cm. One small pseudobulb was observed. There was no root differentiation upto 12 weeks of culture of shoot tips and the average no. of shoots was 2.25 per culture (Fig. ST08).

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 observed after 4 weeks of culture of shoot tips. Some shoots were found with multiple roots. The shoot length was ranged from 2.6-3.5 cm. Callus was induced in this medium after 12 weeks of culture of shoot tips and the average no. of shoots was 3.25 per culture (Fig. ST09).

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

Further increase in concentration of BAP 2 mg/l with NAA 0.5 mg/l induced the shoot multiplication after 3 weeks of culture of shoot tips. The highest number of multiple shoots were observed with one well developed rooted multiple shoot. A pseudobulb was also developed after 12 weeks of culture of shoot tips. The shoot length ranged from 2.2-3.5 cm was observed and the average no. of shoots was 4 per culture (Fig. ST10).

4.3.1.2 Shoot Multiplication

The maximum numbers of shoots were observed in both MS basal medium as well as in MS supplemented medium with different concentration of hormones after 12 weeks of culture of shoot tips. The

following diagrams showed that the multiplication of shoot varied with different combination of hormones.

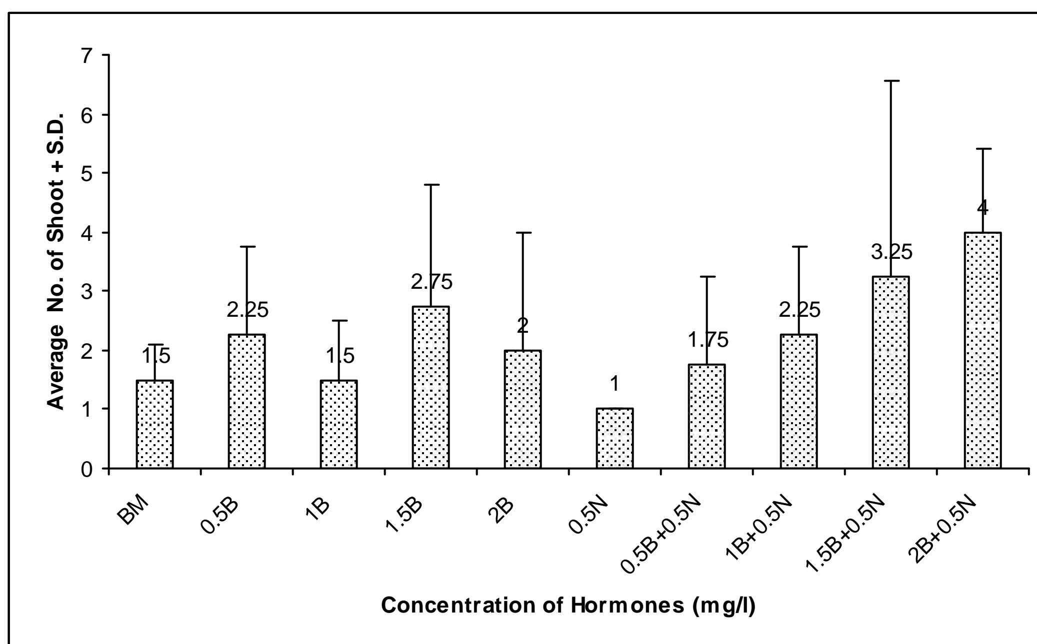


Diagram 1 : Average no. of shoots after 12 weeks of culture of shoot tip of *Dendrobium densiflorum* Lindl.

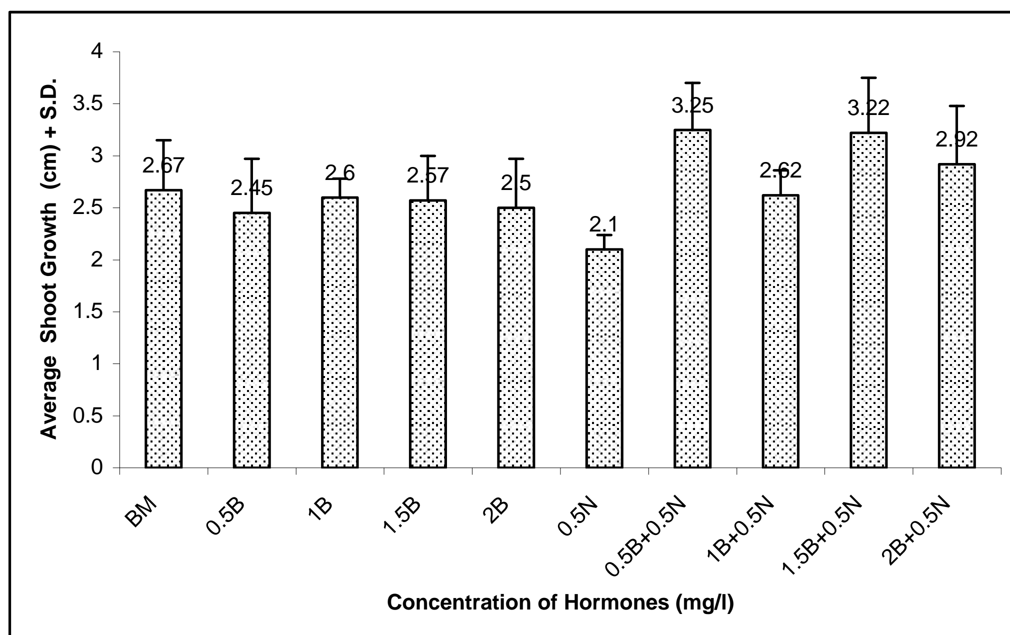


Diagram 2 : Average shoot growth after 12 weeks of culture of shoot tip of *D. densiflorum* Lindl.

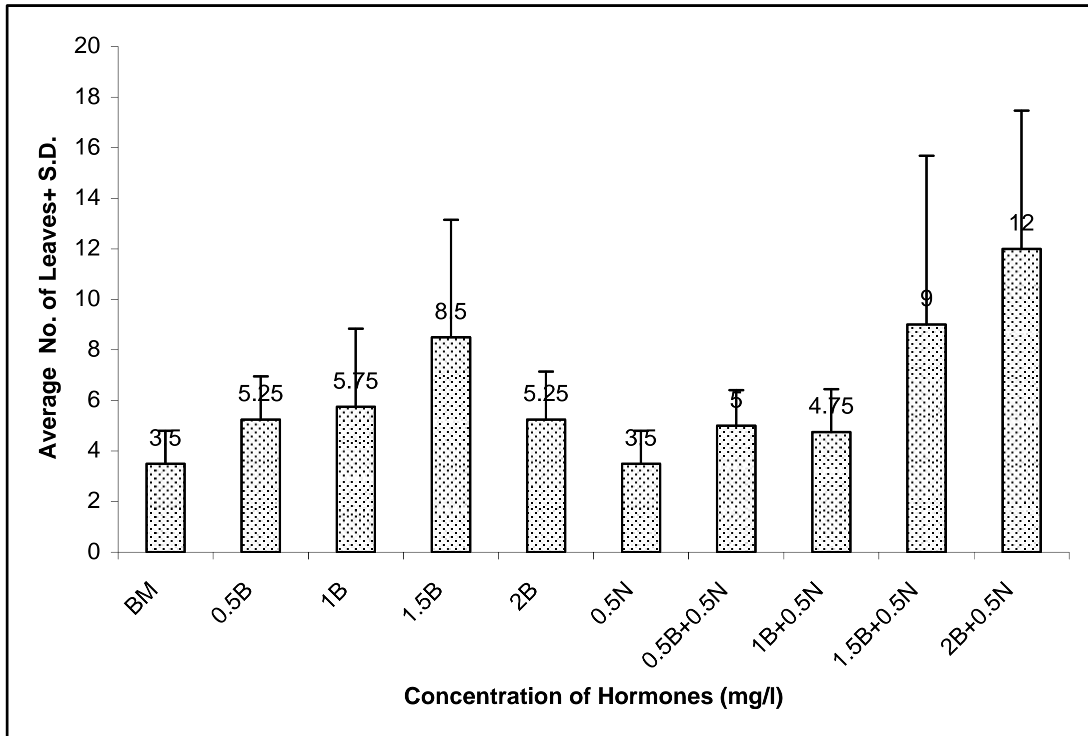


Diagram 3 :Average no. of leaves after 12 weeks of culture of shoot tip of *Dendrobium densiflorum* Lindl.

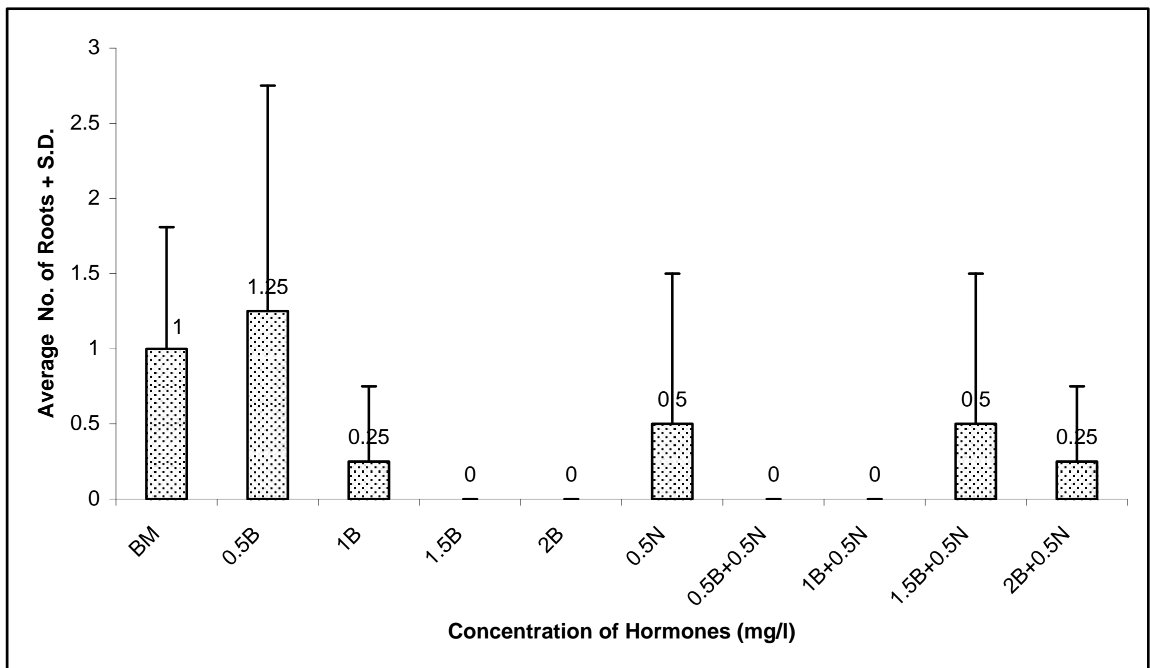


Diagram 4 : Average no. of roots after 12 weeks of culture of shoot tip of *D. densiflorum* Lindl.

The maximum number of shoots per culture was observed in MS medium supplemented with BAP 2 mg/l + NAA 0.5 mg/l after 3 weeks of shoot tip culture and the shoot length was found upto 2.92 cm per culture after 12 weeks of culture. The second highest number of shoot per culture was observed in MS medium supplemented with BAP 1.5 mg/l + NAA 0.5 mg/l. The MS medium supplemented with BAP 1.5 mg/l, BAP 1 mg/l + NAA 0.5mg/l, BAP 0.5 mg/l and BAP 2 mg/l also favoured the multiplication of shoot. The least multiplication of shoot was found in MS medium supplemented with NAA 0.5 mg/l. The maximum growth rate of shoot was observed in MS medium supplemented with BAP 0.5 mg/l + NAA 0.5 mg/l (3.25 cm/culture) but the least growth rate of shoot was observed in MS medium supplemented with NAA 0.5 mg/l in comparision to other combinations which was found to be only 2.1 cm per culture(shoot length).

The maximum numbers of leaves were observed in MS medium supplemented with BAP 2 mg/l + NAA 0.5 mg/l and least number of leaves was found in MS medium supplemented with NAA 0.5 mg/l and MS basal medium.

Similarly, the maximum number of roots were observed in MS medium supplemented with BAP 0.5 mg/l which mean number was found to be 1.25 per culture and least mean number of roots i.e. 0.25 per culture were found in MS medium supplemented with BAP 1 mg/l and BAP 2 mg/l + NAA 0.5 mg/l. In some combinations such as MS medium supplemented with BAP 1.5 mg/l, BAP 2 mg/l, BAP 0.5 mg/l + NAA 0.5 mg/l and BAP 1 mg/l + NAA 0.5 mg/l, roots were not observed.

4.3.2 Culture of Root tips of *Dendrobium densiflorum* Lindl.

The root tips obtained from *in vitro* seedling were cultured in MS basal medium and MS medium supplemented with different concentration of

BAP (0.5-2 mg/l) and NAA (0.5 mg/l). The changes in root tip explants have been presented in the table 4.

Table: 4

Effect of BAP and NAA on root tip culture of *Dendrobium densiflorum* Lindl.

BAP NAA (mg/l)	0	0.5	1	1.5	2
0	3MS, R 1MS, C	2MS, R 1MS, R, C 1MS, R, Pb	1MS, R 2S, R 1R	3MS, Pb, C 1MS, R	4MS, C
0.5	1MS, R 3MS	1S 3MS, R	1R	2MS, R 1S, R 1S	2MS, R, Pb 2MS, Pb

Culture conditions:- MS medium, 25±2°C, 24 weeks, 12-15 hrs photoperiod, 4 replicates were used in each combination.

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

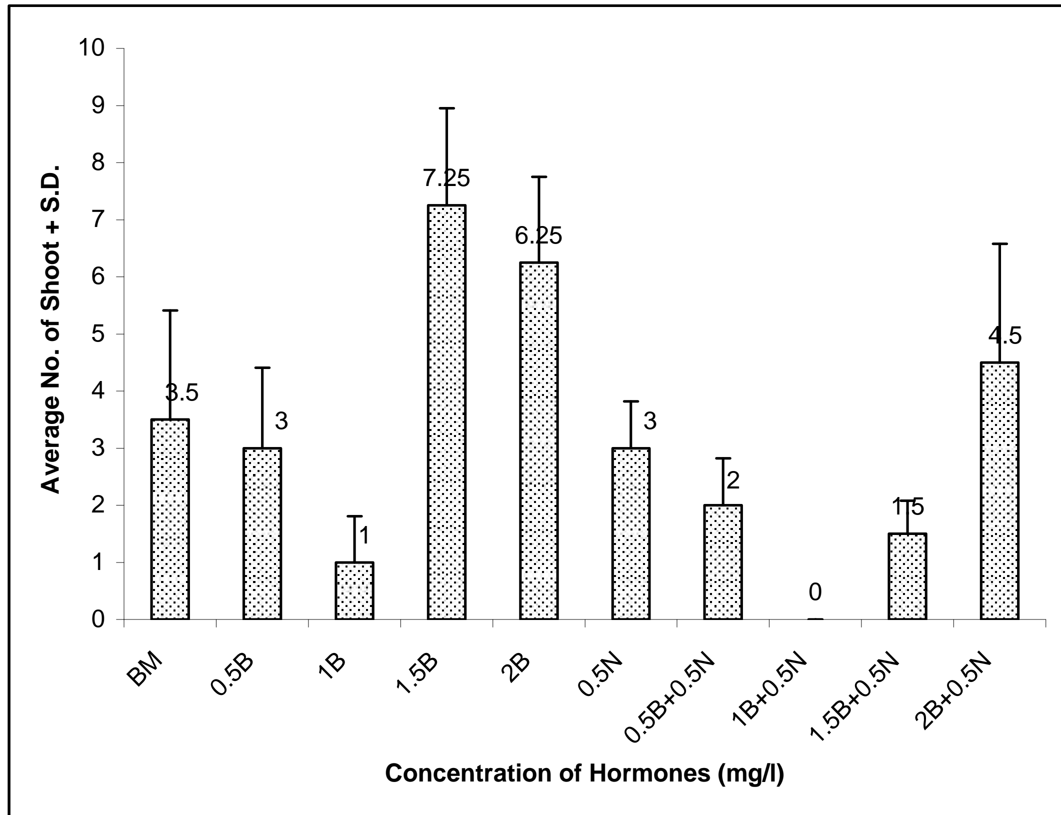


Diagram 5 : Average no. of shoot after 24 weeks of culture of root tip explant of *D. densiflorum* Lindl.

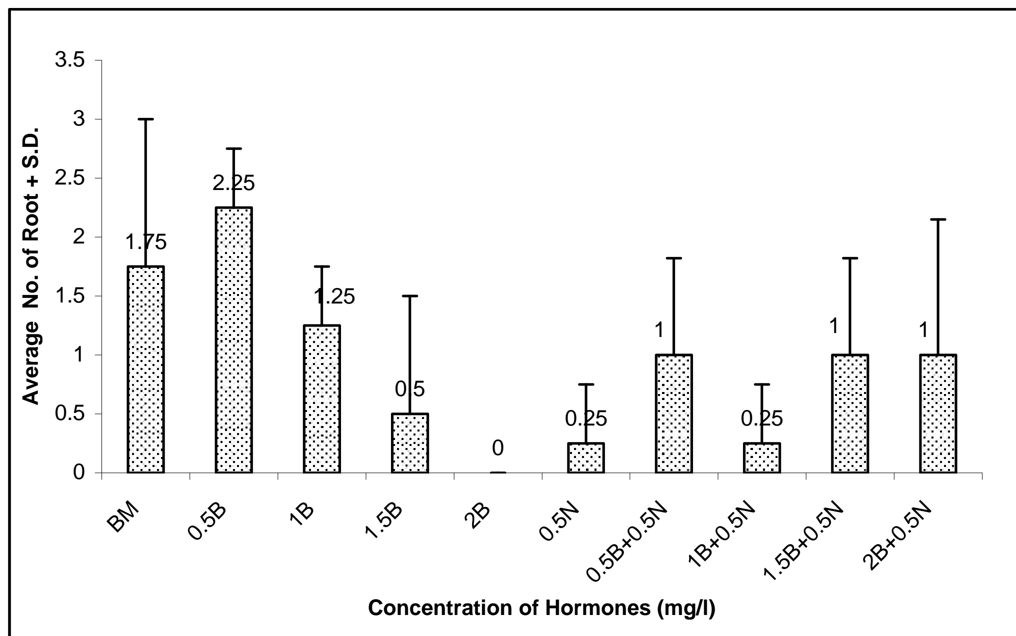


Diagram 6 : Average no. of Root after 24 weeks of culture of root tip explant of *D. densiflorum* Lindl.

4.3.2.1 Development of root tip explant of *Dendrobium densiflorum* Lindl.

After 24 weeks of culture of root tip explant obtained from *in vitro* grown shoots in MS basal medium amd MS medium supplemented with different concentrations of BAP and NAA, it showed following responses:

1. MS Basal Medium

The development of root tip explants started after 5 weeks of culture in MS basal medium. Multiple shoots were observed. Callus and some new roots formed after 24 weeks of culture and average no. of shoots was 3.5 per culture (Fig. RT01).

2. MS + BAP 0.5 mg/l

In MS medium supplemented with BAP 0.5 mg/l, the development of root tip explants initiated after 5 weeks of culture of root tips. Many multiple shoots were observed. Callus and new roots were obtained after 24 weeks of culture. Pseudobulb was also developed and average no. of shoots was 3 per culture (Fig. RT02).

3. MS + BAP 1 mg/l

The MS medium supplemented with BAP 1 mg/l showed root tip development after 10 weeks of culture. After 24 weeks of culture of root tips, many new roots were emerged and average no. of shoots was 1 per culture (Fig. RT03).

4. MS + BAP 1.5 mg/l

In MS medium supplemented with BAP 1.5 mg/l, the development of root tips started after 4 weeks of culture. After 24 weeks of culture of root

tips, multiple shoots were appeared. Callus, new roots and pseudobulbs were also developed and average no. of shoots was 7.25 per culture (Fig. RT04).

5. MS + BAP 2 mg/l

In MS medium supplemented with BAP 2 mg/l, the shoots developed after 4 weeks of culture of root tips. Callus and many multiple shoots were also observed after 24 weeks of culture of root tips and average no. of shoots was 6.25 per culture (Fig. RT05).

6. MS + NAA 0.5 mg/l

The MS medium supplemented with NAA 0.5 mg/l showed shoot growth after 5 weeks of culture of root tips. Many multiple shoots and few numbers of new roots were developed after 24 weeks of culture of root tips and average no. of shoots was 3 per culture (Fig. RT06).

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

The MS medium supplemented with BAP 0.5 mg/l + NAA 0.5 mg/l showed shoot initiation after 6 weeks of culture of root tips. Many multiple shoots and many new roots were also developed after 24 weeks of culture of root tips and average no. of shoots was 2 per culture (Fig. RT07).

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

In MS + BAP 1 mg/l + NAA 0.5 mg/l medium, shoots were not observed and roots were changed into brownish colour upto 24 weeks of culture of root tips (Fig. RT08).

Development of Multiple Shoots and New Roots after 24 weeks of Culture of Root Tips of *Dendrobium densiflorum* Lindl.

Fig. RT 02 Root tip cultured on MS medium supplemented with BAP 0.5 mg/l showing multiple shoots and few new roots with callus.

Fig. RT 03 Root tip cultured on MS medium supplemented with BAP 1 mg/l showing multiple shoots and few new roots.

Fig. RT 04 Root tip cultured on MS medium supplemented with BAP 1.5 mg/l showing multiple shoots and new roots.

Fig. RT 05 Root tip cultured on MS medium supplemented with BAP 2 mg/l showing callus and multiple shoots.

Fig. RT 06 Root tip cultured on MS medium supplemented with NAA 0.5 mg/l showing multiple shoots and one new root.

Fig. RT 07 Root tip cultured on MS medium supplemented with BAP 0.5 mg/l + NAA 0.5 mg/l showing multiple shoots and new roots.

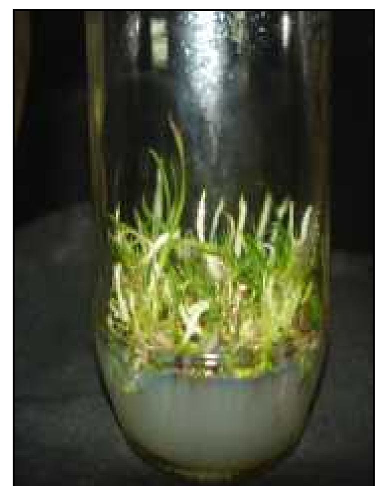
Fig. RT 08 Root tip cultured on MS medium supplemented with BAP 1 mg/l + NAA 0.5 mg/l showing small new root without shoot.

.Fig. RT 09 Root tip cultured on MS medium supplemented with BAP 1.5 mg/l + NAA 0.5 mg/l showing callus and new shoots after 20 weeks of culture.

Fig. RT 10 Root tip cultured on MS medium supplemented with BAP 2 mg/l + NAA 0.5 mg/l showing multiple shoots and new roots.

RT 02

RT 03



RT 04

RT 05

RT 06

RT 07

RT 08

RT 09

RT 10

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

In MS medium supplemented with BAP1.5 mg/l + NAA 0.5 mg/l, the shoots were started to develop after 8 weeks of culture of root tips. Well developed shoots with few new roots were observed after 24 weeks of culture of root tips and average no. of shoots was 1.5 per culture (Fig. RT09).

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 showed the shoot initiation after 5 weeks of culture of root tips. Many multiple shoots, pseudobulbs and well developed new roots were observed after 24 weeks of culture of root tips and average no. of shoots was 4.5 per culture (Fig. RT10).

4.4 Rooting of Shoots of *Dendrobium densiflorum* Lindl.

The shoots obtained from shoot tip explants were subcultured in MS medium with different auxins i.e. IAA, IBA and NAA in different concentrations (0.5 mg/l – 2 mg/l) for the root induction. The diagram 7 and 8 shows their different responses in various concentrations of auxins.

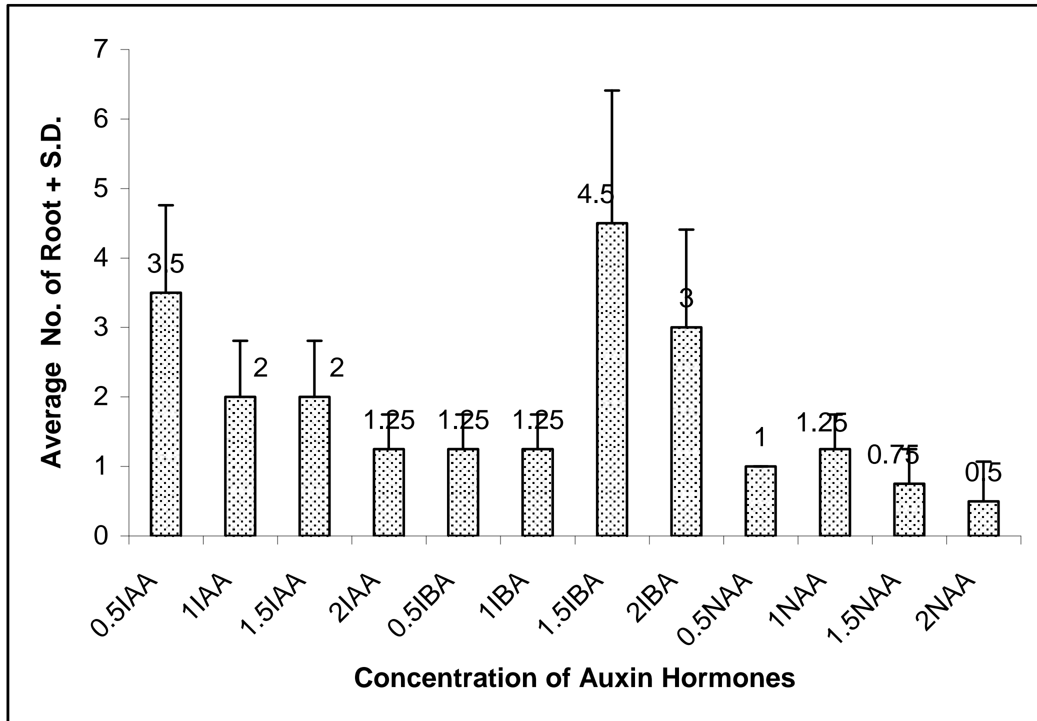


Diagram 7 : Average no. of root after 10 weeks of rooting of shoot tip explant of *D. densiflorum* Lindl.

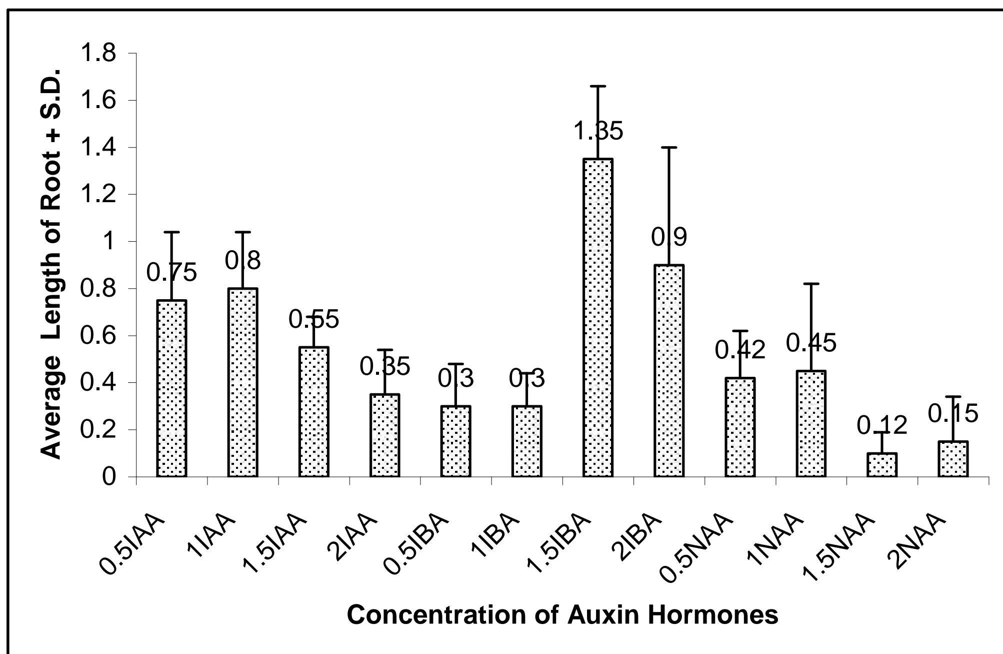


Diagram 8 : Average length of root after 10 weeks of rooting of shoot tip explant of *D. densiflorum* Lindl.

Development of Roots in Different Hormone Concentrations of Auxins after 10 weeks of Culture of Shoot Tip and Acclimatization of *Dendrobium densiflorum* Lindl.

Fig. RF 01	Root formation in MS medium supplemented with IAA 0.5 mg/l.
Fig. RF 02	Root formation in MS medium supplemented with IAA 1 mg/l.
Fig. RF 03	Root formation in MS medium supplemented with IAA 2 mg/l.
Fig. RF 04	Root formation in MS medium supplemented with IBA 0.5 mg/l.
Fig. RF 05	Root formation in MS medium supplemented with IBA 1 mg/l.
Fig. RF 06	Root formation in MS medium supplemented with IBA 1.5 mg/l.
Fig. RF 07	Root formation in MS medium supplemented with IBA 2 mg/l.
Fig. RF 08	Root formation in MS medium supplemented with NAA 0.5 mg/l.
Fig. RF 09	Root formation in MS medium supplemented with NAA 1 mg/l.
Fig. RF 10	Root formation in MS medium supplemented with NAA 2 mg/l.
Fig. A 01 - A02	Acclimatization of well developed *in vitro* rooted plantlet in pot containing only coco-peat and moss.

RF 01

RF 02

RF 03

RF 04

RF 05

RF 06

RF 07

RF 08

RF 09

RF 10

A 01

A 02

From the present study, the different concentrations of auxins were found to be effective for rooting of *D. densiflorum* Lindl. MS medium supplemented with IAA 0.5 mg/l was found to be better for rooting in comparison to other different concentrations of IAA supplemented with MS basal medium. In this medium the rooting was initiated after 2 weeks of culture. The root length ranged from 0.35 cm - 0.8 cm after 10 weeks of culture. The mean number of roots was found to be 3.25 per culture. The roots obtained were normal and short. On increasing the concentration of IAA, the number of roots was decreased (Fig. RF01-03).

Highest number of roots was obtained in MS medium supplemented with IBA 1.5 mg/l in comparision to other concentrations of auxins. The roots obtained in this medium were thick, healthy and long. The average no. of roots was 4.5 per culture and root length ranged from 0.3 cm - 1.35 cm after 10 weeks of culture (Fig. RF04-07).

Similarly in case of NAA, the maximum number of roots was found in MS supplemented with NAA 1 mg/l. These roots were thin and short. The mean number of roots was found to be 1.25 per culture and length of roots range from 0.12 cm - 0.45 cm. after 10 weeks of culture. It was found that increase in concentration of NAA by 1 mg/l had negative effect in rooting (Fig. RF08-10).

For rooting *in vivo*, microshoots were planted without using growth regulators. It didn't show effective result.

4.5 Acclimatization

The rooted plantlets measuring about 5.3 cm were transferred in pot containing mixture of coco-peat and moss for acclimatization. They were under the process of observation (Fig A01-08).

A03 - Chemicals used for Acclimatization

A04 - Shoot measurement before Acclimatization

A05 - Additiona of moss to the pot

A06 - Plantlet transfer in potting mixture

A07 - Covering the pot with white plastic cover

A08 - Acclimatized pots covered with white plastic cover

CHAPTER FIVE

DISCUSSION

5.1 *In vitro* seed germination of *Cymbidium elegans* Lindley. and *Dendrobium densiflorum* Lindl.

In the present study, two species of orchids were used for *in vitro* seed germination. The MS medium and MS supplemented with various growth hormones were found efficient for the germination of the seeds of *C. elegans* Lindley. and *D. densiflorum* Lindl. The most effective germination of *C. elegans* Lindley. was found to be in MS media supplemented with BAP (1 mg/l).

Whereas MS basal media was regarded as the appropriate for the germination of seeds of *D. densiflorum* Lindl. The most appropriate medium was selected on the basis of time taken for germination of seeds and their growth and development. Thus, the variation in seed germination with respect to protocorm, shoot and root formation were significantly superior in *D. densiflorum* Lindl. as compared to *C. elegans* Lindley. It might be due to the genetic constitution of the material and presence of different endogenous growth stimulating substances in the explants.

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. Antioxidants are widely used to overcome this problem (Areza *et al.*, 1993. Barbbar and Gupta, 1982, Sarathchancha *et al.*, 1990). However in present study the problem of phenolic compounds exudation was not occurred. Orchid seed

germination differs from that of other seeds because of the absence of an endosperm, radicle and leaf rudiments. Swelling of embryos followed by the formation of a round top shaped body called a protocorm. Other organs subsequently appear (Arditti, 1967, 1979).

Karanjit (2002) cultured the seeds of *Coelogyne cristata* Lindl. and *Cymbidium iridiodes* D.Don. in MS medium and G-B_5 medium. The germination rate was noted vigorous in MS medium. A total of 36 species of native and exotic species of orchids have been germinated *in vitro* in various media including MS (1962) medium by Shrestha and Rajbhandary (1994).

MS medium supplemented with 1 mg/l of BAP and 1 mg/l of NAA was the most effective medium for the germination of the immature of seeds of *Cymbidium iridiodes* D.Don. (Swar, 2003).

MS basal medium was found to be most effective for the germination of *Dendrobium densiflorum* Lindl. in the present investigation. Comparatively less effective response of hormones on germination and seedlings formation could probably be due to their effect on physiological processes or interaction between the hormones. This was supported by the findings made by 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*. Hoshi *et al.* (1994) also reported the similar findings in their study on the germination of four species of *Cyperidium*, Liu and Zhang (1998) studied the embryo germination and further proliferation of *Dendrobium candidum* and found ½MS basal medium best for it. Similarly, Pant and Gurung (2005) found

MS basal medium most effective for the *in vitro* seed germination of *Aerides odorata* Lour.

5.2 Shoot tip culture of *Dendrobium densiflorum* Lindl.

When the shoot tip explants of *Dendrobium densiflorum* Lindl. are inoculated in MS basal medium and MS supplemented with different hormonal concentrations, the explants developed into shoots and multiple shoots. Roots are also developed from some shoots in some culture conditions.

In present investigation, MS medium alone was not effective for induction of multiple shoots. Similar result was obtained by Yasugi *et al.* (1994) in *Dendrobium sp*.

Highest number of multiple shoots was obtained in MS medium supplemented with BAP (2 mg/l) and NAA (0.5 mg/l) whereas MS medium supplemented with NAA (0.5 mg/l) showed least number of shoot multiplication. The high concentration of BAP than NAA in shoot multiplication of shoots is supported by the work of different researchers. Swar and Pant (2004) obtained maximum number of shoots in MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) in *Coelogyne cristata* Lindl. Shrestha and Rajbhandary (1993) established the clonal propagation of *Dendrobium densiflorum* Lindl. by shoot tip culture. MS medium containing BAP (2.5 mg/l), NAA (1 mg/l), 15 % coconut milk and 1 g/l casein hydrolysate developed protocorms from the shoot tip explants.

Statistically, shoot length and leaf number of *D. densiflorum* Lindl. were found to be significant whereas shoot and root numbers were insignificant at 5 % level of significance.

Rajkarnikar and Niraula (1994) developed protocorms from the shoot tip explant of *D. fimbriatum* in the MS medium containing 5 ppm BAP, 1 ppm NAA and 10 % coconut milk. Multiple shoots were obtained by subculturing protocorms in MS medium supplemented with 1 ppm BAP, 1 ppm NAA and 10 % coconut milk.

Chung *et al.* (1998) studied the effect of medium composition on multiple shoot formation. Further growth of mericlone from rhizome of shoot tip culture of *Cymbidium* species. MS medium with BAP 3 mg/l and NAA 1 mg/l enhanced multiple shooting in *C. forrestii* and *C. kanran.*

5.3 Root tip culture of *Dendrobium densiflorum* Lindl.

After the inoculation of root tips of *Dendrobium densiflorum* Lindl. in MS basal medium and MS medium supplemented with different hormonal concentrations, the explants are changed into shoots, new roots and callus. Pseudobulbs were also observed.

The root tips of *Dendrobium densiflorum* Lindl. were cultured and complete plantlets were observed upto 24 weeks of it's culture. The most effective result was obtained in the MS medium supplemented with BAP 1.5 mg/l and least number of plantlets were obtained on the MS medium supplemented with BAP 1 mg/l and NAA 0.5 mg/l. So, a combined treatment with NAA and BAP induced direct or callus mediated PLB development and enhanced response frequency in root tip explant. Statistically, the shoot number was found to be significant and root number was found to be insignificant at 5 % level of significance.

In comparision to the regeneration of plantlets from shoot tip and root tip explants, root tip culture was found to be less effective. It may be due to the low growth rate and other physiological processes of root tip.

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*, *Vanda cristata* and *V. testacea*. The effect of plant growth hormones was species specific and varied during initiation, multiplication and differentiation of cultures.

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

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.

Chaturvedi and Sharma (1986) cultured the young roots (3 mm in length) of *in vitro* established *Vanda* hybrid to differentiate PLBs in a modified VW (Vacin and Went, 1949) medium supplemented with 1 mg/l BAP, 1 mg/l IAA and 200 mg/l Casein hydrolysate. The PLBs differentiated directly from the cut ends of explants without intervening callusing. The excised root tips turned green and increased in size before the differentiation of PLBs.

5.4 Rooting of Shoots of *Dendrobium densiflorum* Lindl.

The multiple shoots obtained from the culture of shoot tip in MS medium supplemented with different concentrations of NAA and BAP were

excised and subcultured on the root initiating media (NAA, IAA and IBA).

In MS basal medium, root induction was not effective. But Shrestha and Rajbhandari (1988) successfully rooted *Cymbidium giganteum* in MS control media in 2 months. Similar result was recorded in *Cymbidium longifolium* by Shrestha and Rajbhandari (1994).

In present experiment, the MS media supplemented with 1.5 mg/l IBA was found to be the most effective for rooting. It may be due to the presence of enhanced levels of auxin (IBA) and related compounds in the medium which has strong absorption power of inhibitory compounds. Statistically, both root number and root length of *Dendrobium densiflorum* Lindl. were found to be significant at 5 % level of significance.

Pant and Gurung (2005), observed the highest number of roots in *Aerides odorata* Lour. in 0.5 mg/l IAA.

Shrestha and Rajbhandary (1993) successfully rooted *Dendrobium densiflorum* Lindl. in MS control media in 2 months. Nayak *et al.* (1997) rooted the shoots on MS medium containing 1.0 mg/l IBA in *Acampe praemorsa.*

Swar and Pant (2004) used MS medium supplemented with different rooting hormones in different concentrations. MS + 1 ppm IBA was the best for rooting of *Cymbidium iridiodes* D.Don and *Coelogyne cristata* Lindl.

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

CHAPTER SIX

CONCLUSION

From the present investigation following conclusions have been made:

- MS medium supplemented with BAP (1 mg/l) was found to be comparatively better with respect to other concentrations of hormones for *in vitro* seed germination of *Cymbidium elegans* Lindley. whereas MS basal medium was found to be most effective for *in vitro* seed germination of *Dendrobium densiflorum* Lindl.

- MS medium supplemented with BAP 2 mg/l + NAA 0.5 mg/l was most effective for shoot multiplication with healthy roots of *D. densiflorum* Lindl.

- MS medium supplemented with BAP 1.5mg/l was found to be most effective for inducing the shoots from root tips of *D. densiflorum* Lindl.

- Shoot tip explants were found to be better than root tip explants for mass propagation of *D. densiflorum* Lindl.

- MS medium supplemented with IBA 1.5 mg/l favoured the development of maximum number of healthy, thick and long roots which was followed by MS medium supplemented with IAA 0.5 mg/l.

CHAPTER SEVEN

RECOMMENDATIONS

Following recommendations are made from the present research work:

- Establishment of orchidarium should be done for *Ex-situ* conservation of orchid.
- Educational and awareness programme should be launched to the local people for sustainable utilization of important orchids.
- The mass propagation of economically important orchids should be started by using shoot tip and root tip culture.
- Botany Department should launch awareness programme or workshop regarding the status, conservation and utilization of orchids from time to time.

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