COMPARATIVE STUDY ON IN VITRO GROWTH RATE OF TWO ORCHID SPECIES VIZ. *COELOGYNE OVALIS* LINDLEY AND *DENDROBIUM DENSIFLORUM* LINDLEY

A DISSERTATION
SUBMITTED TO CENTRAL DEPARTMENT OF BOTANY
IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR
MASTER OF SCIENCE IN BOTANY

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KATHMANDU, NEPAL
2010

CERTIFICATE

This is to certify that the work entitled "**COMPARATIVE STUDY ON IN VITRO GROWTH RATE OF TWO ORCIHD SPECIES VIZ. *COELOGYNE OVALIS* LINDLEY AND *DENDROBIUM DENSIFLORUM* LINDLEY"** has been carried out by Sarala Sitaula under my supervision. Her findings are based on the experiments carried out by her in our laboratory.

I have a pleasure in recommending the present study as dissertation work for the partial fulfillment of the requirement for degree in Botany. The results of the present work have not been previously submitted for any other degree.

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Date: 4th February, 2010

CHAPTER ONE
INTRODUCTION

1.1 Background of the Study

Nepal is situated in the Himalayan mountain ranges and hence it has a lot of variety of flora and fauna. One can find exquisite varieties of flowers in Nepal which are not found elsewhere in the world. Nepal comprises more than 6500 flowering plants with extreme diversities ranging from a low altitude of the tropics to the highest altitude of alpine zone of which about 250 species are endemic to Nepal (Chaudhary, 1998; Hara et al., 1978-1982; HMGN 2002)

Orchids the beautiful gifts of nature are one of the most widely distributed group of flowering plants. The family orchidaceae is one of the largest families of flowering plants comprising more than 19,505 species belonging to 803 genera (Dressler, 1993)

The distribution of orchids ranges from equator to Arctic Circle and low land upto the snow lines in mountain areas. They are found abundant in tropical conditions. There is great variation in habitat of orchid species. They may be terrestrial, epiphytic, lithophytic or even subterranean.

Nepal consists of about 384 species of native orchids belonging to 100 genera, (Rajbhandari and Dahal, 2004) out of which 11 species are endemic to Nepal. Most of the Nepalese orchids have ornamental values due to their shape, size, habitat, colourful flowers, shining green leaves and variously shaped pseudobulb. Some of them have medicinal as well as edible values. Orchids are known as “Sungava”, “Chandigava” and “Sunakhari” in Nepal.

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

The most beautiful orchids of Nepal belong to the following genera: *Aerides*, *Arundina*, *Bulbophyllum*, *Calanthe*, *Coelogyne*, *Cymbidium*, *Dendrobium*, *Epigenecum*, *Eria*, *Esmeralda*, *Phaius*, *Phalaenopsis*, *Pleione*, *rhinoshostylis*, *Thunia*, *Vanda and Vandopsis, (Rajbhandari and Bhattarai, 2001). Species cultivated for their ornamental purpose include: Aerides multiflora, Ascocentrum ampullaceum, Bulbophylum leopardinum, Calanthe masuca, C. plantagina, Coelogyne cristata, C. fuscecens, Cymbidum elegans, C. irridioides, Dendrobium densiflorum, D. nobile,* etc.

Some important medicinal orchids in Nepal are mentioned by Rajbhandari et al. (2000) are *Brachycorythis obcorsata, Coelogyne flavida, Coelogyne stricta, Cymbidium aloifolium, Dactylorhiza hatagirea, Eulophila nuda, Flickgeria macraie, Pholidota imbricata, Luisia zeylanica, Vanda tesselata*.

Some edible orchids are *Dactylorhiza hatagirea, Platanthera clavizera and Satyrium nepalensis* (Dahal and Shakya, 1998)

Species of orchids endemic to Nepal are *Bulbophyllum nepalensis, Eria baniaii, Eria nepalensis, Liparis olivecia, 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 of Nepal. (Subedi 2002)

Orchid seeds are very small; only 1.0-2.0mm long and about 0.5-1.0mm wide. They are produced in large numbers: 1300- 4,000,000 per capsule. The embryo has a rounded or spherical form without cotyledons, no radical and endosperm. In nature most orchids grow in association with fungi. This association is essential for germination of orchid seeds that lag their own internal food supplies (endosperm).

Orchids have been well known for their horticultural values due to various forms and colours of flowers with its long lasting properties. It has won the heart of flower lovers. Unfortunately many rare species of orchids are being extinct and threatened at present due to destruction of natural habitat for different purposes, lack of trained manpower and lack of knowledge and technology for their conservation.

In natural conditions less than 5 percent of orchid seeds get germinated because of particular fungal requirements (Rao, 1997) so vegetative propagation is very slow process to propagate a large quantity of clone orchids. Therefore tissue culture has become the standard method for the propagation of orchid.

Because of the continuous destruction of natural forests and ruthless collection by orchid lovers the natural population of our orchid species has been decreasing at a very high rate. From both commercial and conservation point of view it is important to develop quick methods of propagation of orchid species. The application of tissue culture technique for propagation of Orchids in a large quantity has been found more effective for many plant species.

Micropropagation is useful in mass propagation of specific clones, production of pathogen free plants, clonal propagation of parental stocks for hybrid seed production, year-round nursery production and

germplasm preservation. Nevertheless micropropagation technique has not been carried out in commercial scale in Nepal yet due to lack of capital, trained personnel and marketing facilities.

In Nepal number of works on micropropagation of orchid species has been carried out by Department of Plant Resources (DPR), Central Department of Botany (CDB), Tribhuwan University (T.U.). Few private organizations like Nepal Biotech Nursery, Parijat Nursery are also engaged in the commercial propagation of orchid species in Nepal.

1.2 *Coelogyne ovalis* Lindley

The genus *Coelogyne* is introduced by Dr. Lindley in 1825. 13 species of *Coelogyne* are reported from Nepal (Press et al., 2000). The genus includes many species of great horticultural merits.

C. ovalis Lindl, is one of the important ornamental species of the genera and desirable orchids in the world due to its beautiful flowers. It is an epiphyte with ovoid- fusiform to fusiform pseudobulbs. Leaves are about 9-15 cm long and 2.5-4 cm broad. They may be narrowly elliptic acute to acuminate. Flowers are pale brown with ovate – lanceolate sepals and linear petals.

Flowering Time: September to December.

Distribution: It is found in Nepal, India, Bhutan, China (southeast Tibet). In Nepal it occurs as an epiphytic herb in subtropical and temperate zones at 1300-2100 m altitude.

Uses: It has both horticultural and medicinal values. Pseudobulbs are used as an aphrodisiac.

Conservation Status: It has been illustrated as a seriously threatened species (Bails, 1985, TPC 1989, and Shrestha and Joshi, 1996)

1.3 *Dendrobium densiflorum* Lindley

The genus *Dendrobium* is the second largest genus in the orchid family (Williams, 1984). In Nepal, Hara et al. 1978 enumerated 26 species of *Dendrobium* at an elevation 500-2900m. *Dendrobium densiflorum* Lindley is one of the important evergreen epiphytic orchids with golden flower. It is also known as pineapple orchid. Its clubbed shaped pseudobulbs bear 4-5 leaves and are upto 40 cm tall. It has been highly esteem by orchidologists because of its attractive and characteristic golden flower.

Flowering Time:- April to June

Distribution: It is found in Nepal, Burma, Thailand, and Himalayas. In Nepal it is found as an evergreen epiphytic orchid at an altitude of 500-2900m.

Uses:- It has both horticultural and medicinal values.

Conservation status:- It has been listed as rare and critically endangered species(CITES, Appendix II)

1.4 Objectives

The specific objectives of this experiment are as follows:

1. To compare and investigate the effect of different phytohormones on *in vitro* shoot tip multiplication and rooting of shoot of *Coelogyne ovalis* Lindl. and *Dendrobium densiflorum* Lindl.
2. To develop the protocol for mass propagation of these orchids.

1.5 Justification of the Study

Orchids are the national treasure of Nepal .They are under considerable threat due to destruction and degradation of their natural habitat, and overexploitation for trade and commerce. The collection of orchid has been banned under the convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES). Due to the weak implementation of conservation strategies, harvesting of orchid from wild sources is still continuing for commercial trade.

Sustainable use of valuable orchids of Nepal will be helpful to upgrade country's economy. Micropropagation provides an exciting facet for the mass scale propagation of Nepalese orchids and holds much promise for the protection of rare and endangered germplasm of orchid species. This study is carried out as a small process for *ex-situ* conservation of the species having great commercial potential, for developing the protocol for mass propagation of these species and for comparing the growth rate of these species in the similar culture conditions.

CHAPTER TWO
LITERATURE REVIEW

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.

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

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

Raghuwansi *et al.* (1986) studied the germination and seedling growth on *Dendrobium nobile*, *D. chrysanthum* and *Sacanthus pallidus* at different pH (3, 4, 5, 6, 7, 8, and 10) levels maintained on modified KnC medium. Optimum results were obtained at 10 proved detrimental for germination and protocorm growth. The roots/absorbing haris 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

Liu et al,(1988) germinated seeds of *Dendrobium candidum* easily on medium (1/2 MS) without growth regulators. The embryo germination and the derivation of protocorm were promoted when NAA or potato extract was added to the medium. However the proliferation and growth of protocorms were inhibited when BA and NAA+BA was added in the medium. The aqueous extract of banana or potato and the alcoholic

extract of *Quercus glauca* bark apparently accelerated the plantlet regeneration and rooting (Swar 2003)

Shrestha and Rajbhandary (1988, 1993, 1994) regenerated plant through meristem of *Cymbidium giganteum, C. grandifolium* and *C.longiflorum*. Shoots of *C. giganteum* were initiated on MS (1962), MS supplemented with BAP ($5mg^{-l}$), NAA ($1mg^{-l}$) and 10% coconut milk. The proliferation continued on transfer to pots containing tree fern fiber and survived in green house. The culture of meristem of *C.grandiflorum* was carried out on MS media supplemented with BAP ($2.2mg^{-l}$), NAA($1.8mg^{-l}$) and 10% coconut milk. The protocorms were subcultured on the same medium .Shoots developed from protocorms rooted in the MS basal medium without growth hormones and coconut milk. Meristem culture of *C.longiflorum* initiated PLBs within six weeks after culturing in MS medium supplemented with BAP ($2mg^{-l}$), NAA ($1mg^{-l}$), 10%coconut milk and 3% sucrose. Rapid multiplication of shoots took place in MS medium containing $1mg^{-1}$ BAP,$1.5mg^{-1}$ kinetin and $10mg^{-l}$ Adenine Sulgfate. Rooting occurred on MS basal medium without growth hormones and coconut milk. They also propagated *Dendrobium densiflorum* Lindl. using shoot tip.

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 culture medium. MS (Murashige and Skoog,1962)and RL (Rosa and Laneri, 1977) mdia yieldedbetter results than KC (Knudson, 1946) and VW (VIacin and Went, 1949) medium. The ground growing *Spaltaglottis plicata* responded better to MS media

and the epiphytic, *Epidendrum radicans*, *Dendrobium cripidatum* and *Cymbidium alolifolium* to RL medium.

Shrestha and Rajbhandary (1993) established the clonal propagation of *Dendrobium densiflorum* by shoot tip culture. MS medium containing 2.5mg^{-1} BAP, 0.01mg^{-1} NAA,20mg^{-1} Adenine sulphate and 10% coconut milk. Roots were developed in Vacin and Went's (v/w) control medium. The rooted shoots were established in green house in the pots containing tree fern fibres.

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

Yasugi and Shinto (1994) observed the maximum number of plantlets per single segment of pseudobulb in the treatment of 1mg^{-1} NAA +1mg^{-1}BA of MS (1962) in *Dendrobium* (Noble type). The axilliary buds did not develop into a PLB but directly developed into shoots (Swar 2003).

Yasugi et al. (1994) obtained the greatest number of shoots in *Dendrobium sp.* on a medium containing 0.1mg^{-1} NAA and 0.1mg^{-1} BA.IN both parts of segment culture a maximum of 5 multiple shoots were induced in the same medium after 8 weeks in MS medium + 2% sucrose+ 0.8% agar at PH 5.8.Multiple shoots were not obtained in NAA and BA free medium but roots were developed (Aryal, 2004).

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

developed into distinct globular, yellowish green protocorms within 25 days of culture in B_5 medium 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 90 days of inoculation after 2 subculture on medium 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 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 (N3f).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 0.5mg^{-l} nicotinic acid.

Devi et al. (1997) observed clonal propagation of *Dendrobium moschantum* 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 formation and proliferation of PLBs. The duration for leaf and root differentiation from PLBs varied from 7-13 weeks in different media.

Liu and Zhang (1998) obtained a suitable medium for plantlet production of *Dendrobium candidum* in the period of strengthening plantlet method. the B_5 or 1/2 MS added with 10% of banana aqueos extract and 2mg^{-l}

NAA can be used as the best plantlet strengthening medium for *Dendrobium candidum.*

Pathenia et al. (1998) cultured the pseudobulbus 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 (1mg^{-l}) was found best for further multiplication of PLBs. The rooting was favored 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.

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.

Talukdar (2001) developed an in vitro method to regenerate multiple shoots of *Dendrobium aphyllum* Roxb. A maximum of 7shoots were regenerated in Knudson C medium supplemented with kinetin (10mg^{-l}), coconut water (15%v/V) and banana extract (6%w/v) within 13 weeks of culture. Well developed shoots were also developed in IAA and NAA treated cultures. Protocorms proliferated and callused in medium containing 2,4 D .

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

Nagarju,V.et al.,(2004) studied in vitro germination of seeds from unripened green capsules of two Dendrobium crosses(Dendrobium nobile xD.nobile var.alba and D.nobile xD. heterocarpum) which were tested on MS media and those from selfed capsules of D. chrysotoxum was tested on Kn C media supplemented with or without $3g^{-l}$ of activated charcoal and 0.5 mg^{-l} of BAP. The study showed that addition of growth regulators and activated charcoal in the medium delayed the onset of germination. Incorporation of BAP in the medium favoured rapid multiplication of protocorms. Activated charcoal proved beneficial for the seedling growth.The growth of seedling was better in MS, while presence of AC in the medium further improved the seedling growth.

Chung et al., (2005) showed the effect of four auxins viz. 2,4-D, IAA, IBA and NAA and five cytokinins viz. (N-6-[2-isopentyl] adenine [2iP], BA, kinetin, TDZ, and zeatin on direct embryo induction of leaf explants of Dendrobium cv. Chiengmai Pink cultured on 1/2 MS medium. Weather in light or darkness, explants easily became necrotic and no embryos were obtained on growth regulator free or auxins-containing media after 60 days of culture. By contrast, five cytokines tested induced direct embryo formation from explants, and explants cultured in light had a higher embryogenic response compared with those cultured in darkness. Regenerated plantlets were successfully transplanted and grown in a greenhouse environment.

Malabadi et al., (2005) developed a new method of propagation of Dendrobium nobile from shoot tip section. Successful shoot regeneration was achieved using thin shoot tip sections and triacontanol (TRIA). PLBs were observed when thin shoot tip sections were cultured on the basal medium of Mitra et al supplemented with 4.0 μg^{-l} TRIA. All the newly formed PLBs were survived and ultimately produced healthy shoots with

2-3 leaves. Shoots produced roots when cultured on basal medium supplemented with 2.0 μg^{-l} TRIA. The well-rooted shoots were transferred to pots containing charcoal chips. coconut husk and broken tiles (2:2:1), and a 92% survival rate was achieved.

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.

Shrestha A. (2005) successfully achieved asymbiotic germination *Coelgyne ovalis* Lindl. MS medium supplemented with 1 mg^{-1} of NAA was the best medium for the germination, growth and development of seedling. Germination starts after 8 weeks of inoculation. The maximum number of shoot multiplication was observed in MS media with 1 mg^{-1} of BAP singly and MS media in the combination of BAP 0.5 mg^{-1} with NAA 0.5 mg^{-1} showed callus induction in the base of some shoots. Best rooting was obtained in MS media with 3 mg^{-1} of IBA.

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 *Coelogyne fuscescens*, the best shoot multification (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})

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.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

The materials used for the present experiment were the *in vitro* grown shoot tips of *Coelogyne ovalis* Lindl. and *Dendrobium densiflorum* Lindl., which were obtained from the Tissue Culture Laboratory, Central Department of Botany, T.U.

3.2 Methodology

The Method used for the comparative study of *in vitro* growth of shoot tip and rooting of shoots of different orchid species is described under the following headings:

3.2.1 Preparation of stock solution

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

A. Macro-nutrients

Components	Composition of MS (Final Conc.) mg^{-l}	(10X)gm/lit Stock Concentration	Volume to be taken for 1litre medium
Macronutrients	1900	19.0	100ml
Potassium Nitrate (KNO_3)	1650	16.5	
Ammonium Nitrate (NH_4NO_3)	440	4.4	
Calcium Chloride ($CaCl_2.2H_2O$)	370	3.7	
Magnesium sulphate ($MgSO_4.7H_2O$) potassium dihydrogen phosphate (KH_2PO_4)	170	1.7	

B. Micro-nutrients

Components	Composition of MS (Final Conc.) mg^{-l}	(10X)gm^{-l} Stock Concentration	Volume to be taken for 1litre medium
Micronutrients			1ml
Manganese Sulphate ($MnSO_4.4H_20$)	22.3	2230	
Boric Acid (H_3BO_3)	6.2	620	
Zinc sulphate ($ZnSO_4.7H_2O$)	8.6	860	
Potassium Iodide (KI)	0.83	83	
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25	25	
Cobalt Chloride($CoCl_2.6H_2O$)	0.025	2.5	
Copper Sulphate ($CuSO_4.5H_2O$)	0.025	2.5	

C. Iron source (Fe, EDTA)

Components	Composition of MS (Final Conc.) mg^{-l}	(10X)gm^{-1} Stock Concentration	Volume to be taken for 1litre medium
Iron Source			10ml
Sodium ethylene diamine tetra acetate (Na_2EDT)	37.3	373	
Ferrous Sulphate ($FeSO_4$)	27.8	278	

D. Vitamins

Components	Composition of MS (Final Conc.) mg^{-l}	(10X)gm/lit Stock Concentration	Volume to be taken for 1litre medium
Glycine	2.0	200	1ml
Nicotinic Acid	0.5	50	
Pyrodoxin HCL	0.5	50	
Thiamin HCL	0.1	10	
Myo inositol	100.0	10,000	

(E) Carbon Source

Chemicals	**g^{-1}**
Sucrose	30g

(F) Gelling Agent

Chemicals	**g^{-1}**
Agar	8g

During the preparation of each stock solution, the above chemicals are 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 up to a litre for stock A, 100ml for stock B, C, D. Due to light sensitivity, all stock solutions were kept in clean brown bottles and preserved in the refrigerator.

3.2.2 Hormones used for 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

10 mg BAP was first dissolved in few drops of 0.5 N NaOH and final volume was made upto 10 ml by adding distilled water which is considered as the stock of 1000ppm. 1ml of this was used for 1000ml of medium to make 1ppm of hormone concentration in the medium ($1mg^{-1}$). Likewise NAA, IAA, and IBA were first dissolved in few drops of absolute ethyl alcohol and then prepared as BAP.

3.2.4 Sterilization of glassware and metal instruments

During the experiment, the necessary glass-wares were subjected to dry heat sterilization before their use. Glass-wares (Petridishes, culture tubes, pipettes, beaker, conical flask etc.) were dipped in detergent solution for 24 hours and rinsed with tap water; final rinse was made with distilled water. Glasswares were then sterilized in hot air oven at 150 degree Centigrade for two hours.

Glasswares and metal instruments were subjected to dry heat sterilization before their actual use in the process. Metal instruments like forceps, scalpels and surgical blades 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 media following protocol was applied:

- One litre sterilized conical flask was taken.
- 30 gm of sucrose was weighed and dissolved in about 400 ml distilled water in conical flask.
- 100 ml of stock A, 1ml of stock B, 10 ml of stock C and 1ml of stock D were added one by one into sugar solution of conical flask.
- The solution was stirred by magnetic stirrer in order to mix the salts more homogeneously. The solutions was mesh up to 1000 ml by adding distilled water.
- For the preparation of hormonal medium, hormone stocks was added according to the media requirement.
- pH of the solution was adjusted 5.8 with 0.1 N NaOH or HCL.
- Medium was solidified with by adding 8 gm (0.8%) Difco Bacto Agar and it was heated up to boiling to melt the agar. When the

solution became clear, about 20ml was dispensed into the sterilized culture tubes. Each tube was enclosed by aluminium foil cap.

- The tubes containing medium were sterilized in an autoclave at the temperature of 121^0 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.

3.2.6. Sterilization and use of inoculation chamber

For the process of inoculation, the laminar air-flow chamber was cleaned by cotton soaked with 70% alcohol or spirit. All the requirements for inoculation i.e. 70% ethyl alcohol, rubber bands, match box, sterile distilled water, sterile aluminium foils, sterile petri dishes, handled with surgical blade, spatula, beaker, forceps, tubes with media were exposed under ultraviolet(UV) radiation for 30 minutes to remove the possible contaminants present in and around the transfer area. After UV exposure, the blower was switched on for the inoculating. Then the laminar air flow chamber was ready for inoculation.

3.2.7 Inoculation of explant

For this experiment, shoot tip was taken as explants. Shoot tip explants 5-7mm were obtained from the *in-vitro* grown plants. The explants were inoculated on MS media alone and supplemented with different growth hormones. All the cultures were maintained at $25^0C \pm 2^0C$ under 16 hours photo period.

3.2.8 Shoot multiplication

For shoot multiplication individual micro shoots of about 5-7 mm were cut and transferred to the medium containing different combinations of NAA and BAP. Data was taken every 4 weekly.

3.2.9 Rooting of shoots

For *in vitro* rooting, the micro shoots 5-7mm obtained through the culture of shoot tip explants were transferred to the media containing different concentrations of various rooting hormones such as IAA, IBA and NAA.

CHAPTER FOUR

RESULT

The results of *in vitro* multiplication of shoot tips, rooting of shoots and comparative growth rate of *C. ovalis* Lindl. and D. *densiflorum* Lindl. has been described below under the following different headings:

4.1 Culture of shoot tip

The shoot tip of length 5-7mm with a very small portion were cultured on MS medium supplemented with various concentrations of BAP and NAA for inducing multiple shoots. The shoot tip cultured on MS basal and MS media supplemented with different hormones showed different responses. The effect of BAP and NAA on shoot tip culture has given on following tables:

RESULTS

Table 1

Effect of BAP and NAA on shoot tip culture of *C. ovalis* Lindl.

NAA (mg^{-l})	BAP				
	0	0.5	1	1.5	2
0	3S, Pb 1 RS, Pb	4MS	2MS, Pb, C 2MS	4MS, Pb	2MS 2S
0.5	2 RS, Pb 2RMS, Pb	2S, Pb 1MS 1RMS, Pb	1S 1MS, Pb 2RMS, Pb	2S 2MS, Pb	2S 1RMS 1MS

Culture conditions - MS medium, $25^0C \pm 2^0C$, 12 weeks, 12-15 hrs photo period,

4 replicates were used in each combination.

Table 2

Effect of BAP and NAA on shoot tip culture of *D. Densiflorum* Lindl.

NAA (mg^{-l})	BAP				
	0	0.5	1	1.5	2
0	1MS 2RMS 1RS	2S 2RMS	1MS 2RMS Pb 1S	1MS 3S	2MS 2S
0.5	2S 2RS	2S 2MS, C	2MS 2S	1S 2S 1RMS	2MS 2RMS Pb

Culture conditions-MS medium, $25^0C \pm 2^0C$, 12 weeks, 12-15 hrs photo period,

4 replicates were used in each combination.

Where,

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

Table 3

Effect of BAP and NAA on shoot tip culture of *C. ovalis* Lindl.

NAA (mg^{-l})	BAP				
	0	0.5	1	1.5	2
0	3RS, Pb 1 S, Pb	4MS	3MS, Pb 1MS	4MS, Pb	3MS 1S
0.5	4 RMS, Pb	2RS, Pb, C 2 RMS, Pb	4MS, Pb	1S 3MS, Pb	4MS , Pb

Culture conditions - MS medium, $25^0C \pm 2^0C$, 24 weeks, 12-15 hrs photo period,

4 replicates were used in each combination.

Table 4

Effect of BAP and NAA on shoot tip culture of *D. Densiflorum* Lindl.

NAA (mg^{-l})	BAP				
	0	0.5	1	1.5	2
0	3 RMS 1RS	2RS 2RMS	2RMS 2RMS Pb	3MS 1S	4MS
0.5	3RS 1S	3MS 2S, C	4MS	2S 1RMS	1MS 3RMS Pb

Culture conditions - MS medium, $25^0C \pm 2^0C$, 24 weeks, 12-15 hrs photo period, 4 replicates were used in each combination.

Where,

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

4.2 Development of shoot tip explant of *C.ovalis* Lindl. and *D.densiflorum* Lindl.

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

1. MS Basal Medium

In MS medium the shoot multiplication did not exhibit till 24 weeks of culture of shoot tip of *C.ovalis* Lindl. Pseudobulb was also formed in this medium.The elongation of shoot ranged from 1.6 cm -2.1cm with the average number of leaves and root 7.5 and 1.25 per culture respectively.

In *D.densiflorum* Lindl. the multiplication of shoot was not found in MS medium till 24 week of culture.The shoot length ranged from 1.5cm-1.6cm.The average number of shoot and leaves were found 1.75 and 5 per culture respectively.

2. MS+BAP 0.5 mg^{-l}

The shoot tip cultured in MS+BAP 0.5mg^{-l} multiplied after 5 weeks of culture in *C.ovalis* Lindl. 2-4 multiple shoots with average number of leaves 8.5 per culture was found.Root differentiation didn't exhibit till 24 weeks of culture. The shoot length ranged from 1.5 cm – 2.4cm.

In *D.densiflorum* Lindl. the shoot tip cultured in MS medium supplemented with BAP 0.5 mg^{-l} showed shoot differentiation after 4 weeks of culture of shoot tip. Well developed shoots of length 1.4cm –

1.5cm were observed in this medium. The average number of shoot and leaves were found 2.5 and 8.5 per culture respectively.

3. MS+BAP 1 mg $^{-l}$

MS medium supplemented with BAP 1mg^{-l} induced multiple shoots after 4 weeks of culture in *C. ovalis* Lindl. Root differentiation didn't exhibit up to 24 weeks of culture. The length of shoot varied from 2.6 cm – 3.5 cm with a pseudobulb. The average number of shoot and leaves were found 2.75 and 9 per culture respectively.

In *D.densiflorum* Lindl. MS medium supplemented with BAP 1 mg^{-l} induced multiple shoots after 4 weeks of culture and pseudobulbs were also observed after 10 weeks of culture of shoot tips. The shoot length ranged from 1.7cm – 1.9cm in this medium and the average number of shoot and leaves were found 1.77 and 9.0 per culture respectively. No roots were induced till 24 weeks of culture of shoot tip.

4. MS+BAP 1.5 mg $^{-1}$

MS medium supplemented with BAP 1.5 mg^{-l} induced multiple shoots after 4 weeks of culture in *C. ovalis* Lindl. Pseudobulb was also observed but the root was not differentiated in this medium. The shoot length ranged from 2.3 – 2.7 cm. The average number of shoot and leaves were found 3.5 and 8.7 per culture respectively till 24 weeks of culture of shoot tip.

In *D.densiflorum* Lindl. the shoot multiplication started after 4 weeks of shoot tip culture. The shoot length ranged 1.7 cm – 1.8 cm up to 24 weeks of culture. The average number of shoot and tip were found 3 and 6.5 per culture respectively. Root differentiation wasn't found in this medium.

5. MS + BAP 2 mg^{-1}

The shoot tip multiplied after 4 weeks of culture in MS supplemented with BAP 2 mg^{-l} in *C. ovalis* Lindl. Here also root differentiation was not found. The shoot length varied from 2.3 cm – 3.4 cm. The average number of shoot and leaves were found 3 and 8 per culture respectively till 24 weeks of shoot tip culture.

In *D.densiflorum* Lindl. MS medium supplemented with BAP 2 mg^{-l} showed shoot differentiation after 5 weeks of culture of shoot tip. The shoot of length 1.8 cm – 2.5 cm was found. The average number of shoot and leaves were found 3.75 and 7 per culture respectively. No root differentiation was found in this medium till 24 weeks of culture of shoot tip.

6. MS + NAA 0.5 mg^{-1}

In MS medium supplemented with NAA 0.5 mg^{-l}, the shoot multiplication started after 7 weeks of culture in *C. ovalis* Lindl. The shoot length varied from 1.6 cm – 2.6 cm till 24 weeks of culture. The well developed roots were observed. The average number of shoot and leaves were found 1 and 5.25 per culture respectively.

In *D.densiflorum* Lindl. the shoot tip cultured in MS medium supplemented with NAA 0.5 mg^{-l} showed shoot multiplication after 8 weeks of culture. The shoot length ranged from 2.1 – 2.4 cm with average number of root 1.75 per culture. The average number of shoot and leaves were found 3.75 and 6.25 per culture respectively.

7. MS + BAP 0.5 mg^{-1} + NAA 0.5 mg^{-1}

In MS medium supplemented with BAP 0.5 mg^{-l} + NAA 0.5 mg^{-l} showed shoot initiation after 5 weeks of culture in *C. ovalis* Lindl. the shoot

length varied from 2.8 cm – 3.7 cm with pseudobulb. The average number of shoot and leaves were found 3.5 and 10 per culture respectively. The roots were induced from some shoots.

In *D.densiflorum* Lindl. the MS medium supplemented with BAP 0.5 mg^{-l} + NAA 0.5 mg^{-l} showed shoot initiation after 7 weeks of culture. The shoots ranged with the length of 1.7 cm – 2.8 cm. No root differentiation was found till 24 weeks of culture of shoot tip and the average number of shoot and leaves were found 2.75 and 8 per culture respectively.

8. MS + BAP 1 mg^{-1} + NAA 0.5 mg^{-1}

In MS medium supplemented with BAP 1 mg^{-l} + NAA 0.5 mg^{-l} showed shoot initiation after 5 weeks of culture in *C. ovalis* Lindl. The shoot length varied from 0.8 to 1.8 cm with pseudobulb. The average number of root was found 2.25 per culture. The average number of shoot and leaves were 4.75 and 9.25 per culture respectively.

In *D.densiflorum* Lindl. the 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 ranged from 1.0 -1.6 cm. there was no root differentiation and the average number of shoot and leaves were 2.5 and 10.7 per culture respectively till 24 weeks of culture respectively.

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} showed shoot multiplication after 6 weeks of culture in *C. ovalis* Lindl. the shoot length varied from 1.1 cm -1.5 cm. The average number of shoot and leaves were found 3 and 12 respectively. No root differentiation was found till 24 weeks of culture of shoot tip.

In *D.densiflorum* Lindl. the MS medium supplemented with BAP 1.5 mg^{-l} + NAA 0.5 mg^{-l}, shoot multiplication was observed after 5 weeks of culture of shoot tip. Root development was observed with multiple shoots. The shoot length varied from 1.3 – 2.2 cm. Callus was induced after 11 weeks of culture of shoot tips and the average number of shoot and leaves were found 4.5 and 11.5 respectively.

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} showed shoot multiplication after 4 weeks of culture in *C. ovalis* Lindl. The shoot length varied from 1.1 cm – 2.2 cm. No root differentiation was found in this medium. The average number of shoot and leaves were found 3.25 and 8.75 per culture respectively.

In *D.densiflorum* Lindl. shoot multiplication was observed 4 weeks of culture of shoot tip. The root length varied from 1.5 cm – 2.3 cm. A pseudobulb was also developed after 10 weeks of culture of shoot tip. The average number of shoot and leaves were 4 and 7.5 per culture respectively.

4.3 Shoot Multiplication

After The culture of the shoot tip, regular observation was carried out. Proliferation of shoot along 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 12 weeks of culture.

Table 5

Effect of the BAP and NAA on multiple shoot formation, growth and root formation after 12 weeks of culture of *C.ovalis* lindl

Media	Growth hormones	Concentration of hormones (mg^{-1})	Parameters			
			Shoot proliferation (mean±SD)	Shoot growth (cm) (men±SD)	Number of Leaves (mean±SD)	Number of Roots (means±SD)
MS	BM	-	1.00 ± 0.00	1.30 ± 0.67	5.00 ± 0.81	0.75 ± 0.50
MS	BAP	0.5	1.75 ± 0.68	1.10 ± 0.08	7.00 ± 0.81	No Root
MS	BAP	1.0	3.25 ± 0.24	2.10 ± 0.28	6.75 ± 0.50	No Root
MS	BAP	1.5	2.50 ± 0.81	2.07 ± 0.28	6.50 ± 0.57	No Root
MS	BAP	2	2.25 ± 0.41	2.22 ± 0.17	6.25 ± 0.95	No Root
MS	NAA	0.5	1.50 ± 0.50	1.92 ± 0.41	5.70 ± 0.95	1.50 ± 0.57
MS	BAP+NAA	0.5+0.5	2.00 ± 0.81	1.22 ± 0.20	7.00 ± 1.40	1.50 ± 0.57
MS	BAP+NAA	1.0+0.5	1.75 ± 0.50	1.09 ± 0.09	8.00 ± 0.81	No Root
MS	BAP+NAA	1.5+0.5	1.25 ± 0.47	0.95 ± 0.05	8.50 ± 0.57	No Root
MS	BAP+NAA	2.0+0.5	2.25 ± 0.50	0.81 ± 0.33	6.25 ± 0.50	No Root

Culture conditions: MS medium, $25^0C \pm 2^0C$, 23 weeks of culture, 12 to 15 hours photo period 4 replicates were used in each condition.

Table 6

Effect of the BAP and NAA on multiple shoot formation, growth and root formation after 12 weeks of culture of *D. densiflorum* lindl

Media	Growth hormones	Concentration of hormones (mg^{-1})	Parameters			
			Shoot proliferation (mean±SD)	Shoot growth (cm) (men±SD)	Number of Leaves (mean±SD)	Number of Roots (means±SD)
MS	BM	-	1.00 ± 0.00	0.85 ± 0.05	3.25 ± 0.95	1.00 ± 0.00
MS	BAP	0.5	1.25 ± 0.57	1.00 ± 0.08	6.00 ± 0.00	1.25 ± 0.95
MS	BAP	1.0	1.50 ± 0.50	1.15 ± 0.05	5.75 ± 0.50	No Root
MS	BAP	1.5	2.00 ± 0.81	1.02 ± 0.09	5.50 ± 0.57	No Root
MS	BAP	2	2.25 ± 0.50	1.10 ± 0.08	5.25 ± 0.50	No Root
MS	NAA	0.5	1.00 ± 0.00	1.22 ± 0.18	4.00 ± 0.57	1.75 ± 0.50
MS	BAP+NAA	0.5+0.5	1.50 ± 0.57	0.97 ± 0.24	6.50 ± 0.00	No Root
MS	BAP+NAA	1.0+0.5	1.75 ± 0.50	0.87 ± 0.05	7.50 ± 0.95	No Root
MS	BAP+NAA	1.5+0.5	3.50 ± 0.57	0.85 ± 0.53	3.75 ± 0.50	1.75 ± 0.50
MS	BAP+NAA	2.0+0.5	3.00 ± 0.81	0.95 ± 0.09	6.00 ± 0.81	0.75 ± 0.50

Culture conditions: MS medium, $25^0C \pm 2^0C$, 12 weeks of culture, 12 to 15 hours photo period 4 replicates were used in each condition.

Table 7

Effect of BAP and NAA on multiple shoot formation, growth and root formation after 24 weeks of culture of *C.ovalis* Lindl.

Media	Growth hormones	Concentration of hormones (mg^{-1})	Parameters			
			Shoot proliferation (mean±SD)	Shoot growth (cm) (men±SD)	Number of Leaves (mean±SD)	Number of Roots (means±SD)
MS	BM	-	1.00 ± 0.00	1.60 ± 0.49	7.50 ± 2.00	1.25 ± 0.50
MS	BAP	0.5	2.50 ± 0.57	1.80 ± 0.84	8.50 ± 1.20	No Root
MS	BAP	1.0	5.50 ± 0.57	2.85 ± 0.20	7.75 ± 1.25	No Root
MS	BAP	1.5	3.25 ± 0.24	2.45 ± 0.36	8.75 ± 0.95	No Root
MS	BAP	2	3.00 ± 0.81	2.75 ± 0.36	8.00 ± 1.82	No Root
MS	NAA	0.5	1.50 ± 0.50	2.30 ± 0.18	8.00 ± 2.16	1.75 ± 0.50
MS	BAP+NAA	0.5+0.5	3.50 ± 0.57	2.75 ± 0.95	10.0 ± 1.29	2.25 ± 0.50
MS	BAP+NAA	1.0+0.5	4.75 ± 1.95	1.30 ± 0.47	9.25 ± 1.70	No Root
MS	BAP+NAA	1.5+0.5	3.00 ± 0.81	1.30 ± 0.14	12.0 ± 1.41	2.25 ± 0.95
MS	BAP+NAA	2.0+0.5	3.25 ± 0.0.5	1.57 ± 0.63	8.75 ± 1.95	2.25 ± 0.95

Culture condition: MS medium, $25^0C \pm 2^0C$, 24 weeks of culture, 12 to 15 hours photo period 4 replicates were used in each condition.

Table 8

Effect of the BAP and NAA on multiple shoot formation, growth and root formation after 24 weeks of culture of *D. densiflorum* Lindl.

Media	Growth hormones	Concentration of hormones (mg^{-1})	Parameters			
			Shoot proliferation (mean±SD)	Shoot growth (cm) (men±SD)	Number of Leaves (mean±SD)	Number of Roots (means±SD)
MS	BM	-	1.00 ± 0.00	0.52 ± 0.18	5.00 ± 1.15	1.75 ± 0.95
MS	BAP	0.5	2.50 ± 0.57	0.50 ± 0.05	8.50 ± 0.50	1.75 ± 0.50
MS	BAP	1.0	2.25 ± 0.41	0.79 ± 0.09	9.00 ± 0.81	No Root
MS	BAP	1.5	3.00 ± 0.81	1.82 ± 0.09	6.50 ± 0.57	No Root
MS	BAP	2	3.75 ± 0.95	1.97 ± 0.17	7.00 ± 0.81	No Root
MS	NAA	0.5	1.00 ± 0.00	1.77 ± 0.61	6.25 ± 0.50	2.25 ± 0.95
MS	BAP+NAA	0.5+0.5	2.75 ± 0.57	2.05 ± 0.61	8.00 ± 0.81	No Root
MS	BAP+NAA	1.0+0.5	2.50 ± 1.00	1.12 ± 0.46	10.7 ± 0.20	No Root
MS	BAP+NAA	1.5+0.5	4.50 ± 0.57	1.75 ± 0.63	11.5 ± 0.12	2.50 ± 0.557
MS	BAP+NAA	2.0+0.5	4.00 ± 0.81	1.75 ± 0.68	7.50 ± 0.16	2.00 ± 0.81

Culture condition: MS medium, $25^0C \pm 2^0C$, 24 weeks of culture, 12 to 15 hours photo period 4 replicates were used in each condition.

The mean number of shoots proliferation, the mean length of shoots, the mean number of leaves and mean number of roots and standard deviation in different combination of hormones used are presented in the table 5,6,7 and 8 the maximum number of shoots per culture was observed in MS medium supplemented with BAP 1.0mg^{-l} in *C.ovalis* Lindl. and the shoot length was found up to 1.3 cm per culture in this medium whereas in *D.densiflorum* Lindl. the maximum number of shoots per culture was observed in MS supplemented with BAP 1.5 mg^{-l} + NAA 0.5mg^{-l}. In *C.ovalis* Lindl. MS supplemented with Bap of Mg^{-l} + NAA 0.5 mg^{-l} MS supplemented with BAP 1.0 mg^{-l} + NAA 0.5 mg^{-l} also favoured multiplication of shoot where as in *D.densiflorum* Lindl. MS medium supplemented with BAP 2mg^{-l} and BAP 2.0 mg^{-l} and NAA 0.5mg^{-l} also favoured shoot shoot multiplication. The least growth of shoot was found in MS medium supplemented with BAP 1.5 mg^{-l} NAA 0.5 mg^{-l} (1.3 cm/ culture) was found in *C. ovalis* Lindl. Where as the least growth of shoot was found found in MS medium supplemented with BAP 0.5mg^{-l} in *D.densiflorum* Lindl.

In both the *C. ovalis* lindl. and *D.densiflorum* lindl. the maximum number of leaves in MS medium supplemented with BAP 1.5mg^{-l} + NAA 0.5 mg^{-l} and least number of leaves were found in MS basal medium.

Similarly the maximum number of roots were observed in MS medium supplemented with BAP 0.5mg^{-l} + NAA 0.5mg^{-l} which mean no was found to be 2.25per culture and the least number of roots was found in MS medium in case of *C.ovalis* Lindl. In *D.densiflorum* Lindl. the maximum no of roots i.e 2.50 per culture was found in MS supplemented with BAP 1.5 mg^{-l} + NAA 0.5 mg^{-l} where as in basal medium and BAP 0.5mg^{-l} 1.75 roots per culture were found. In some combinations such as MS medium supplemented with BAP 0.5mg^{-l}, BAP 1 mg^{-l}, BAP 1.5mg^{-l}, Bap 2mg^{-l}, BAP 1mg^{-l} + NAA 0.5mg^{-l}, BAP 1.5mg^{-l} + NAA 0.5mg^{-l}, BAP 2mg^{-l} + NAA 0.5mg^{-l} roots were not observed in *C. ovalis* Lindl. In *D.densiflorum* Lindl. the roots were not observed in MS medium supplemented with BAP 1mg^{-l}, BAP 1.5 mg^{-l}, BAP 2mg^{-l}, BAP 0.5mg^{-l} + NAA 0.5mg^{-l}, BAP 1mg^{-l} + NAA 0.5mg^{-l}.

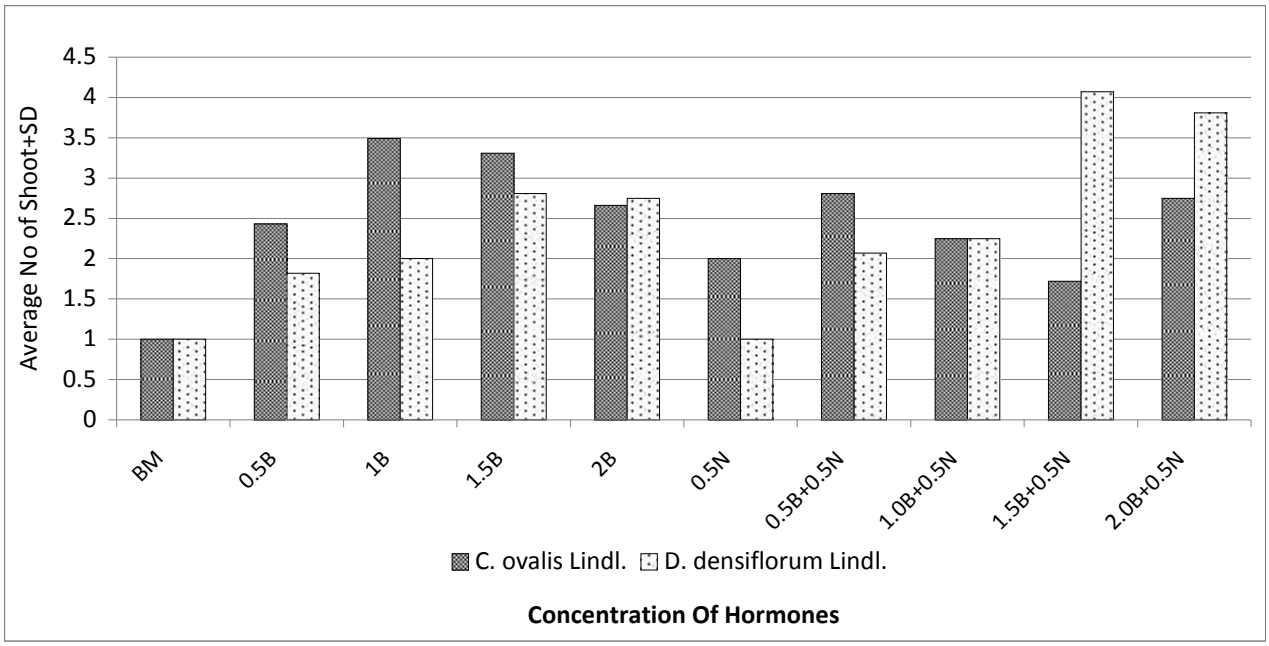

Figure 1: Number of shoots of C.ovalis Lindl. and D. densiflorum Lindl. after 12 weeks of culture

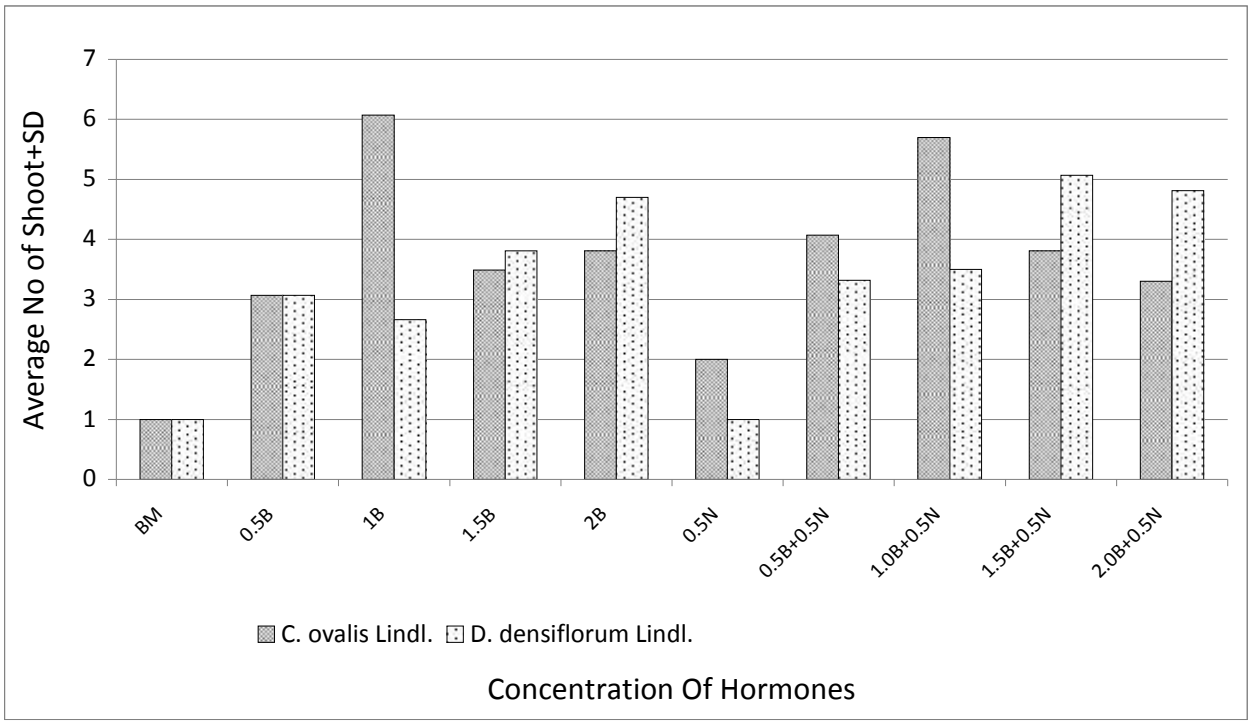

Figure 2: Number of shoots of C.ovalis Lindl. and D. densiflorum Lindl. after 24 weeks of culture

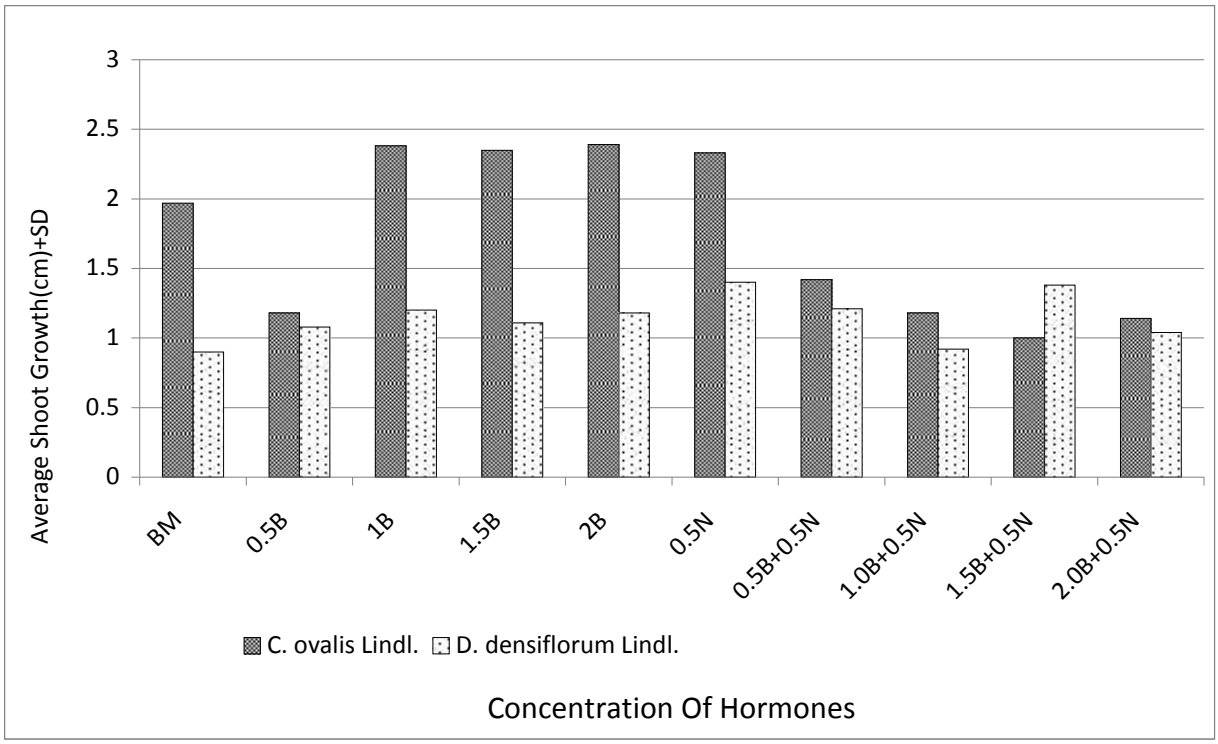

Figure 3 :Average Shoot Growth of C.ovalis Lindl. and D. densiflorum Lindl. after 12 weeks of culture

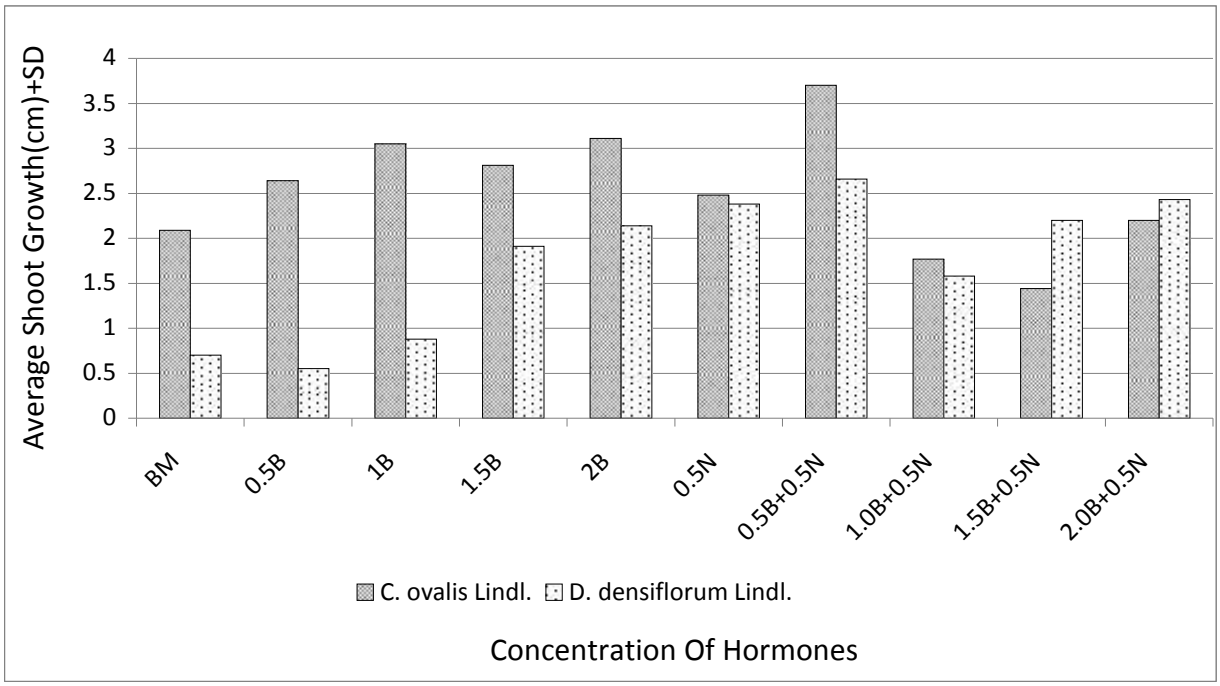

Figure 4: Average Shoot Growth of of C.ovalis Lindl. and D. densiflorum Lindl. after 24 weeks of culture

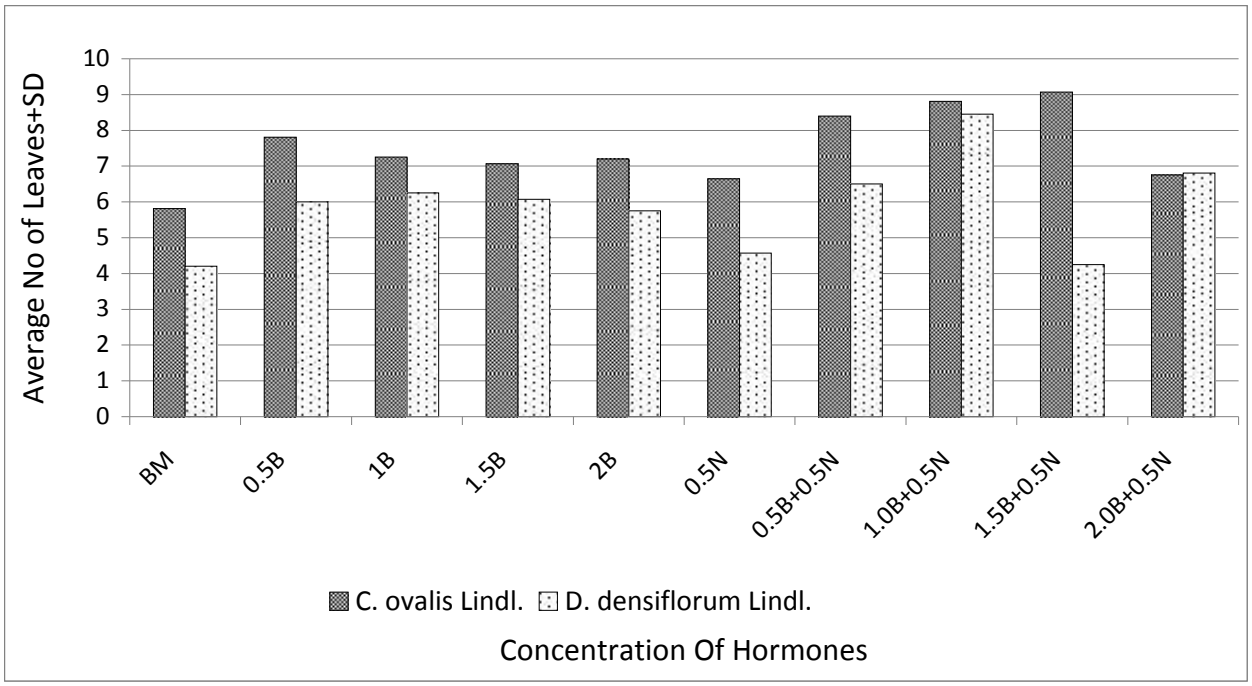

Figure 5: Average Number of Leaves of C.ovalis Lindl. and D. densiflorum Lindl. after 12 weeks of culture

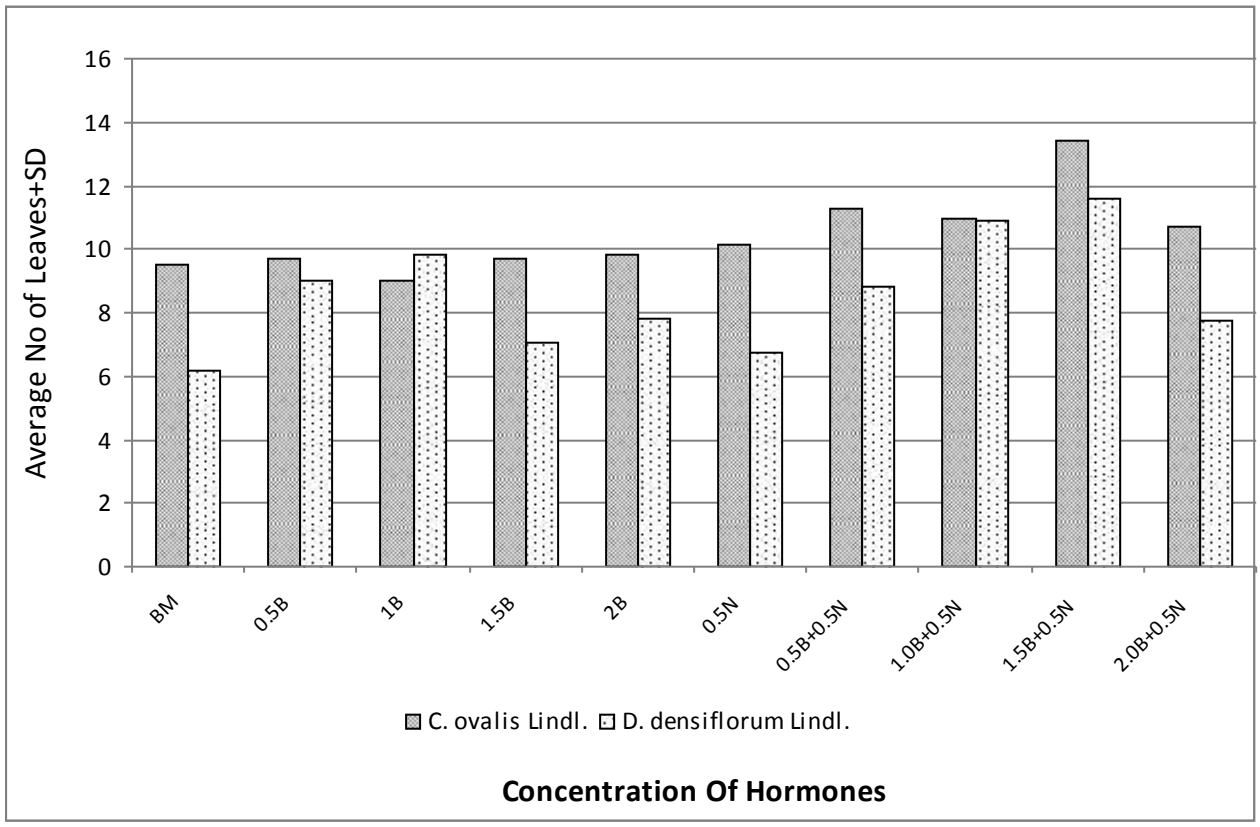

Figure6: Average Number of Leaves of C.ovalis Lindl. and D. densiflorum Lindl. after 24 weeks of culture

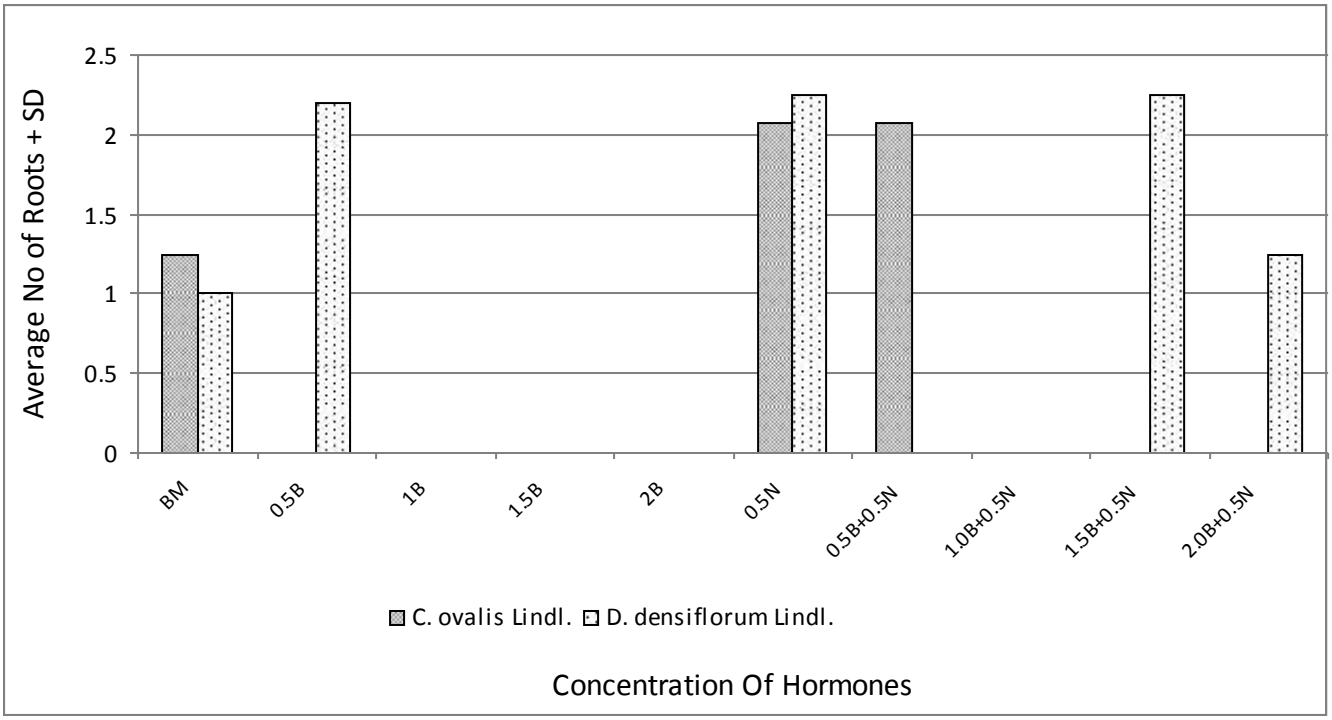

Figure 7: Average Number of Roots of C.ovalis Lindl. and D. densiflorum Lindl. after 12 weeks of culture

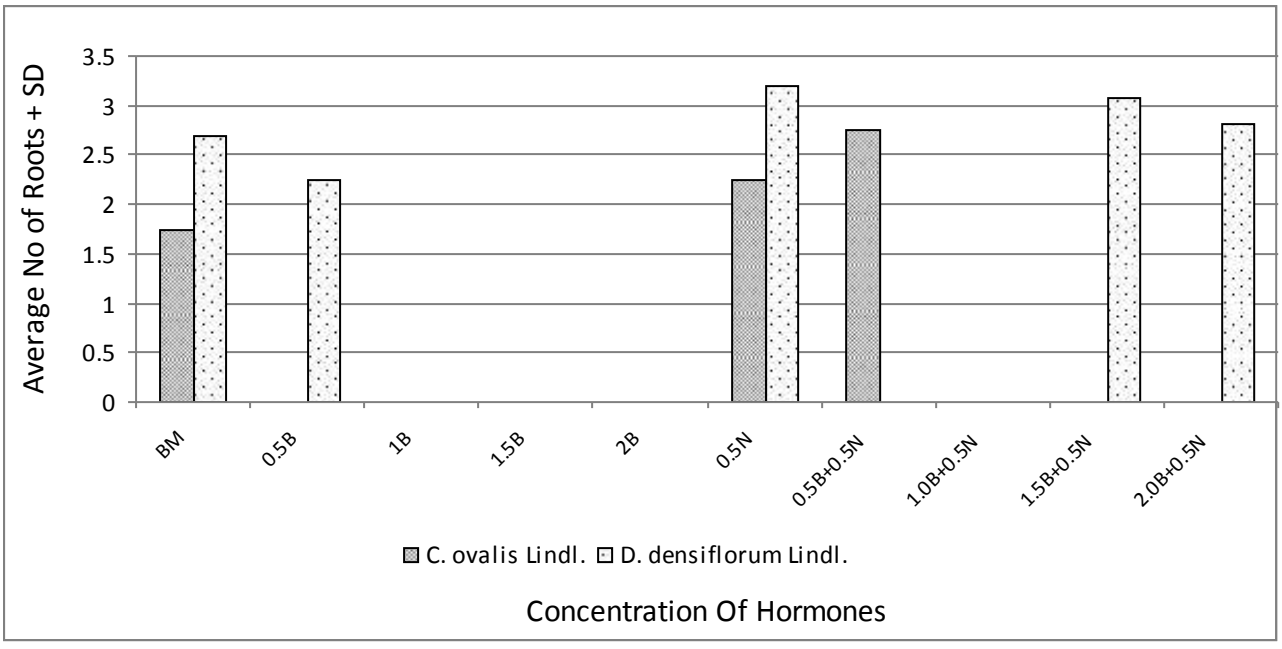

Figure 8: Average Number of Roots of C.ovalis Lindl. and D. densiflorum Lindl. after 24 weeks of culture

4.4 Rooting of shoots of *C. ovalis* Lindl. And *D. densiflorum* Lindl.

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

1. MS + IAA 0.5 mg^{-1}

In *C. ovalis* Lindl. The root formation started from 5 weeks of culture. The average number of root was found 2.75 per culture with the length varying from 0.8 to 0.9 cm till 12^{th} week of culture.

D. densiflorum Lindl. showed initiation of root after 3 weeks of culture with the average number of root 5.7 per culture. The length of root ranged from 0.6 to 0.8 cm till 12^{th} week.

2. MS + IAA 0.5mg^{-1}

Shoot tip cultured in MS supplemented with IAA 1mg^{-1} required about 3 weeks for root initiation in *C.ovalis* Lindl. The length of root was found between 0.8-0.9 cm with the average number of root 3.7 per culture.

In *D. densiflorum* Lindl. the root started to developed after 3 weeks of culture root elongation was found between 0.8 to 0.9 cm with the average number of root 3.75 per culture.

3. MS + IAA 1.5mg^{-1}

In *C. ovalis* Lindl. the root initiation started in about 4 weeks of shoot tip culture in MS supplemented with IAA 1.5 mg^{-1}. The length of root varied from 0.8 to 1.1 cm with the average root 3.07 per culture till 12^{th} week

In *D.densiflorum* Lindl. the root initiation started after 3 weeks of culture. The length of root varied form 0.5 to 0.9 cm with the average number of root 2.75 per culture till 12^{th} week.

4. MS + IAA $2mg^{-l}$

In *C. ovalis* Lindl. the root initiated after 4 weeks of culture in MS supplemented with IAA $2mg^{-l}$. The root length varied from 0.9 to 1.1 cm with the average number root 1.75 per culture.

In *D. densiflorum* Lindl. the root initiated after 4 weeks of culture in MS supplemented with IAA $2mg^{-l}$. The root length varied from 0.3 to 0.5 cm with the average number root 1.75 per culture.

5. MS + IBA $0.5mg^{-1}$

C. ovalis Lindl. showed the root initiation after 3 weeks of culture in MS supplemented with IBA $0.5mg^{-l}$. The length of root varied from 1.1 to 1.2 cm with the average number of root 2.5 per culture.

D. densiflorum Lindl. showed the root initiation after 4 weeks of culture in MS supplemented with IBA $0.5mg^{-l}$. The length of root varied from 0.3 to 0.7 cm with the average number of root per culture was 1.5

6. MS + IBA $1mg^{-l}$

In *C. ovalis* Lindl. the root initiation started after 5 weeks of culture of shoot tip in MS supplemented with IBA $1mg^{-1}$. The length of root varied from 1.1 to 1.3 cm with the average number of root 2.75 per culture.

D. densiflorum Lindl. showed the root initiation after 3 weeks of culture in MS supplemented with IBA $1mg^{-l}$. The length of root varied from 0.4 to 0.7 cm with the average number of root per culture was 2

7. MS + IBA 1.5mg^{-l}

In *C. ovalis* Lindl. the root initiation started after 4 weeks of culture of shoot tip in MS supplemented with IBA 1.5mg^{-l}. The length of root varied from 1.1 to 1.2 cm with the average number of root 3.5 per culture.

D. densiflorum Lindl. showed the root initiation after 4 weeks of culture in MS supplemented with IBA 1.5mg^{-l}. The length of root varied from 1 to 1.3 cm with the average number of root 5.75 per culture. This is the best rooting medium for the shoot tip of *D. densiflorum* Lindl.

8. MS + IBA 2mg^{-1}

In *C. ovalis* Lindl. the root initiation started in about 3 weeks of shoot tip culture in MS supplemented with IBA 2 mg^{-l}. The length of root varied from 1.1 to 1.5 cm with the average root 6.5 per culture till 12th week. This is the best rooting medium for the shoot tip of *C. ovalis* Lindl.

In *D.densiflorum* Lindl. the root initiation started after 4 weeks of culture in MS supplemented with IBA 2 mg^{-l}. The length of root varied form 0.8 to 1 cm with the average number of root 3.75 per culture till 12th week.

9. MS + NAA 0.5mg^{-1}

In *C. ovalis* Lindl. showed the root initiation after 3 weeks of culture in MS supplemented with NAA 0.5mg^{-l}. The length of root varied from 0.6 to 0.8 cm with the average number of root 3.5 per culture.

D. densiflorum Lindl. showed the root initiation after 4 weeks of culture in MS supplemented with NAA 0.5mg^{-l}. The length of root varied from 0.4 to 0.6 cm with the average number of root per culture was 1.75

10. MS + NAA 1mg^{-1}

In *C. ovalis* Lindl. the root initiation started after 5 weeks of culture of shoot tip in MS supplemented with NAA 1mg^{-1}. The length of root varied from 0.7 to 0.8 cm with the average number of root 2.5 per culture.

D. densiflorum Lindl. showed the root initiation after 5 weeks of culture in MS supplemented with NAA 1mg^{-1}. The length of root varied from 0.2 to 0.4 cm with the average number of root 1 per culture.

11. MS + NAA 1.5mg^{-1}

In *C. ovalis* Lindl. the root initiation started after 5 weeks of culture of shoot tip in MS supplemented with NAA 1.5mg^{-1}. The length of root varied from 0.6 to 0.8 cm with the average number of root 2.25 per culture.

D. densiflorum Lindl. showed the root initiation after 6 weeks of culture in MS supplemented with NAA 1.5mg^{-1}. The length of root varied from 0.1 to 0.2 cm with the average number of root 0.75 per culture.

12. MS + NAA 2mg^{-1}

In *C. ovalis* Lindl. the root initiation started after 6 weeks of culture of shoot tip in MS supplemented with NAA 2mg^{-1}. The length of root varied from 0.6 to 0.7 cm with the average number of root 1.75 per culture.

D. densiflorum Lindl. showed the root initiation after 6 weeks of culture in MS supplemented with NAA 2mg^{-1}. The length of root varied from 0.1 to 0.2 cm with the average number of root 0.5 per culture.

Table 9

Effect of different auxins on rooting of shoot tips of *D. densiflorum* Lindl.

After 12 weeks of culture.

Parameters	Concentration Of Different Auxins Hormones (mg/l)											
	IAA				IBA				NAA			
	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2
Numbers Of Roots (mean ± SD)	2.25 ± 0.5	2.5 ± 0.57	2.5 ± 0.57	1.75 ± 0.5	2.5 ± 0.57	2.75 ± 0.5	3.5 ± 0.57	6.5 ± 0.57	3.5 ± 0.57	2.5 ± 0.57	2.25 ± 0.95	1.75 ± 0.5
Length Of Roots (mean ± SD)	0.8 ± 0.08	0.87 ± 0.05	0.95 ± 0.12	0.97 ± 0.09	1.1 ± 0.08	1.12 ± 0.12	1.17 ± 0.05	1.3 ± 0.18	0.8 ± 0.08	0.67 ± 0.17	0.65 ± 0.12	0.62 ± 0.05

Culture conditions: MS medium, 25 ± 2^{oc} , 12 weeks of culture, 12 to 15 hours photo period

4 replicates were used in each condition

Table 10

Effect of different auxins on rooting of shoot tips of *C. ovalis* Lindl.

After 12 weeks of culture.

Parameters	Concentration Of Different Auxins Hormones (mg/l)											
	IAA				IBA				NAA			
	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2
Numbers Of Roots (mean ± SD)	4.5 ± 0.57	3.25 ± 0.5	2.75 ± 0.5	1.75 ± 0.5	1.5 ± 0.57	2 ± 0.81	5.75 ± 0.95	3.75 ± 0.95	1.75 ± 0.95	1 ± 0	0.75 ± 0.5	0.5 ± 0.57
Length Of Roots (mean ± SD)	0.7 ± 0.08	0.6 ± 0.08	0.67 ± 0.17	0.5 ± 0.21	0.42 ± 0.12	0.52 ± 0.15	1.2 ± 0.14	0.97 ± 0.09	0.45 ± 0.12	0.27 ± 0.09	0.2 ± 0.14	0.1 ± 0.08

Culture conditions: MS medium, 25 ± 2^{oc} , 12 weeks of culture, 12 to 15 hours photo period

4 replicates were used in each condition

after 12 weeks of culture. MS supplemented with IBA0.5mg^{-l} showed few numbers of roots than in the MS medium supplemented with IBA 2mg^{-l}. Thus the increase in the concentrations of IBA had positive effect in rooting. In MS medium supplemented with NAA 0.5mg^{-l} the average number of root was found 3.5 per culture. Increase in concentration of NAA has negative effect in rooting. In MS medium supplemented with IAA 0.5mg^{-l} the average number of root per culture was found to be 2.25 with the length of root 0.7cm-0.9cm.Further increase in the concentrations of IAA by 1mg^{-l} showed negative effect in rooting .The average number of root was found in the range of 0.9cm-1.1cm.

In *D.densiflorum* Lindl.MS medium supplemented with IBA 1.5mg^{-l} was found to be the best medium for rooting in comparison to other concentrations of IBA supplemented with MS basal medium. The root length varied from 1.1cm-1.2cm with the average number of root 5.75 per culture. On increasing the concentrations of IBA the number of root were decreased.

In MS medium supplemented with IAA 0.5mg^{-l} 4.5 roots per culture were obtained with the root length varying from 0.6cm-0.8cm after 12 weeks of culture. The roots obtained were thick and long.

Similarly in case of NAA, the maximum number of root was found in MS medium supplemented with NAA 0.5 mg^{-l}. The average number of roots were found to be 1.75per culture and the length of root ranged from 1.1`cm to 1.5 cm after 12 weeks of culture. It was found that increase in the concentration of NAA by 1mg^{-l} had negative effect in rooting of *D.densiflorum* Lindl.

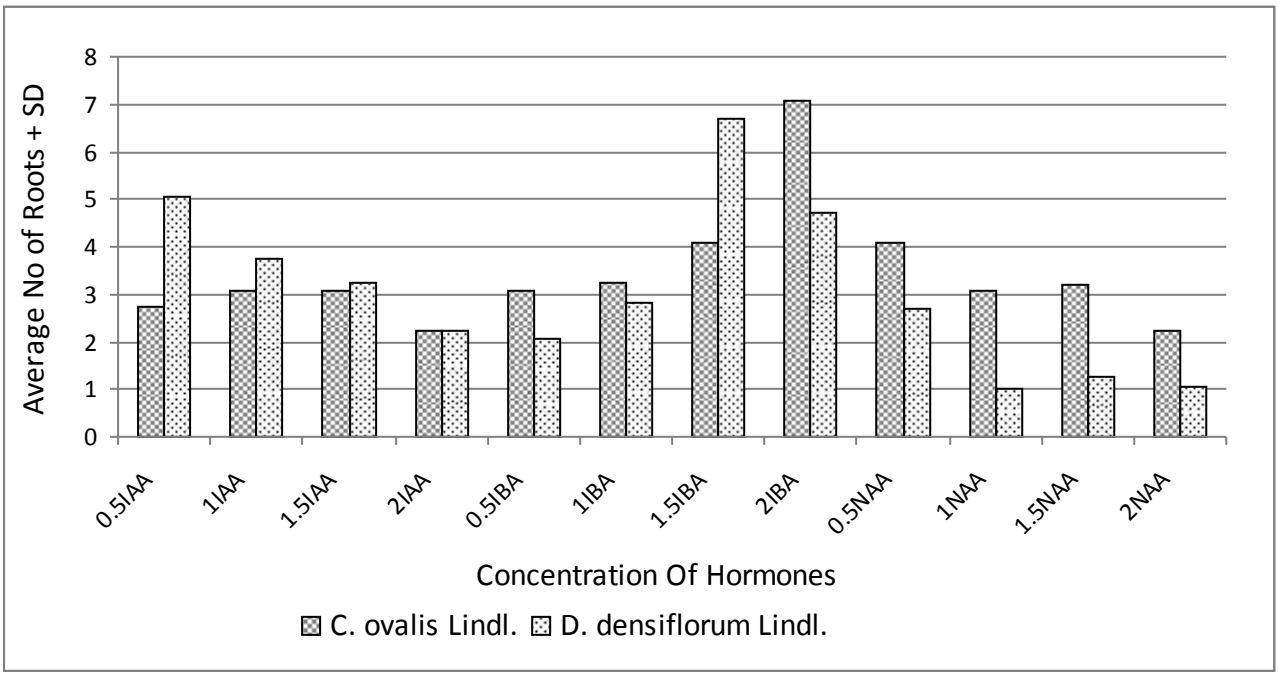

Figure 9: Average Number of Roots after 12 weeks of rooting shoot tip explants of C.ovalis Lindl. and D. densiflorum Lindl.

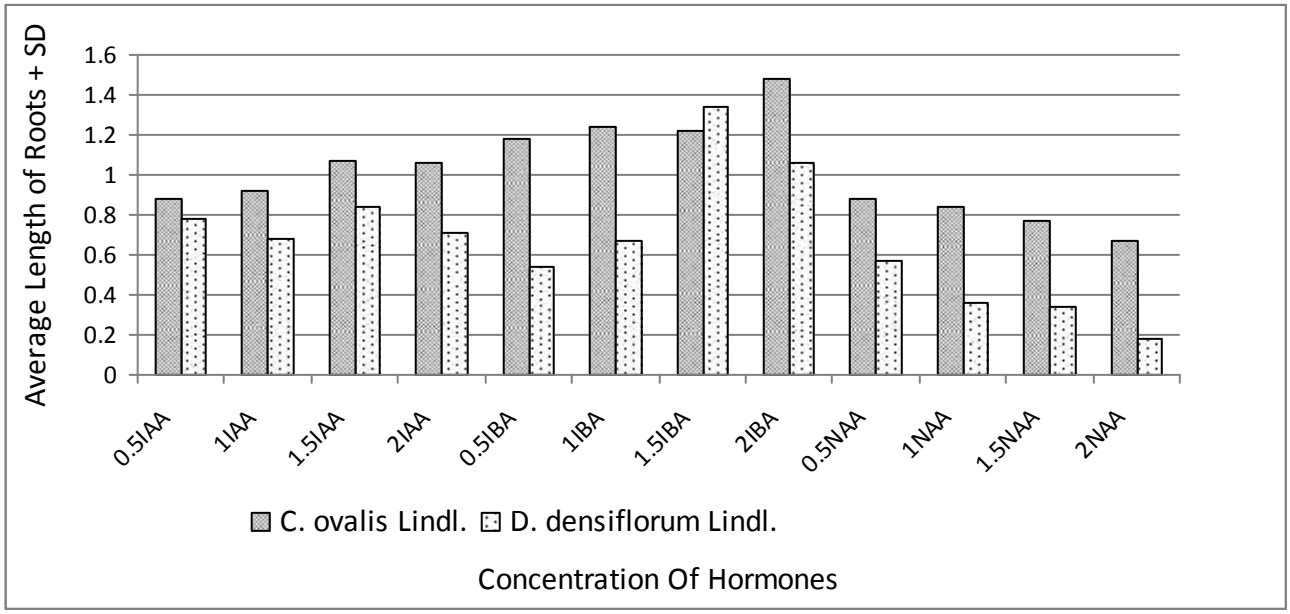

Figure 10: Average Length of Roots after 12 weeks of rooting shoot tip explants of C.ovalis Lindl. and D. densiflorum Lindl.

CHAPTER FIVE
DISCUSSION

In the present experiment when the shoot tip explants of of *C.ovalis* Lindl. and *D. densiflorum* Lindl. are inoculated in MS basal medium and MS medium supplemented with various concentrations of hormones explants develop into shoots and multiple shoots with well developed roots in some culture conditions.

In the present investigation, MS medium alone was not effective for induction of multiple shoots in both the orchid species *C.ovalis* Lindl. and *D. densiflorum* Lindl. similar result was obtained by Yasugi et al. (1994) in Dendrobium sp.

In case of *C.ovalis* Lindl. highest number of multiple shoots were obtained in MS medium supplemented with BAP $1mg^{-l}$ and less number of multiple shoots were obtained in MS supplemented with BAP $0.5mg^{-l}$. The differentiation of root was not obtained in MS medium supplemented in any concentration of BAP whereas in *D. densiflorum* Lindl. the highest number of multiple shoots (4.75 shoots per culture) were obtained in MS medium supplemented with BAP $1.5mg^{-1}$+ NAA 0.5mg-^{l}. The least number of shoots were obtained in MS medium supplemented with NAA $0.5mg^{-l}$. The higher concentration of BAP than NAA in shoot multiplication is supported by the work of different researchers.

Pant and Swar (2003) obtained maximum number of shoots in MS medium suplemented with $1mg^{-1}$ of BAP and $0.5mg^{-1}$ of NAA is *Coelogyne cristata* Lindl. They also obtained maximum number of multiple shoots in MS medium supplemented with $0.5mg^{-1}$ of BAP in *Cymbidium iridioides* D. Don.

Shrestha and Rajbhandary (1993) established clonal propagation of *D. densiflorum* Lindl. by shoot tip culture. MS medium containing BAP(2.5mg^{-l}),NAA(1mg^{-l}),15%coconut milk and 1g^{-l} casein hydrosylate developed protocorms from the shoot tip explants.

In case of *C.ovalis* Lindl. in combination, the highest multiplication was found in MS medium supplemented with BAP(1mg^{-l})+NAA(0.5mg^{-l}) whereas in *D. densiflorum* Lindl. the highest shoot multiplication was found in MS medium supplemented with BAP(1mg^{-1})+NAA(0.5mg^{-l}). Raj Karnikar and Niraula (1994)obtained multiple shoots in MS supplemented with BAP 1mg^{-1}, NAA 1mg^{-l} and 10% coconut milk in *Dendrobium fimbriatum*.

Chung et al 1998 studied the effect medium composition on multiple shoot formation. Further growth of Mericlone from rhizome of shoot tip culture of *Cymbidium* species. Ms medium with BAP 3mg^{-l} and NAA 1mg^{-l} enhanced multiple shooting in *C. forrestii* and *C. karan.*

The multiple shoots of *C. ovalis* Lindl. and *D. densiflorum* Lindl. obtained from the culture of shoot tip in MS medium supplemented with BAP were excised and each of them were subcultures on the root initiating media (IAA, IBA and NAA)

In MS basal medium the root induction was not found effective in both the *C.ovalis* Lindl. and *D. densiflorum* Lindl. (1998) successfully rooted *Cymbidium gigantum* in MS control media in 2 months. Similar result was recorded in *Cymbidium grandiflirum* and *Cymbidium longifolium* by Shrestha and Shrestha and Rajbhandary

In the present study MS media supplemented with 2mg^{-l} IBA was found to be the most effective for rooting of *C. ovalis* Lindl. and MS medium

supplemented with 1.5 mg-l of IBA was found to be the most effective condition for rooting of *D. densiflorum* Lindl. 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.

Swar (2003) used MS medium supplemented with different rooting hormones in different concentrations. MS + 1 ppm IBA was the most effective condition for rooting of *Cymbidium iridioides* D. Don and *Coelogyne cristata* Lindl.

CHAPTER SIX

CONCLUSIONS

From the present experiment following conclusions have been made:

- MS medium supplemented with BAP 1mg-l was found to be comparatively better with respect to other concentrations of hormones for shoot multiplication of C.ovalis Lindl.whereas MS basal medium supplemented with BAP1.5mg-1+NAA0.5mg-1 was found to be the most effective condition for the shoot multiplication of D.densiflorum Lindl.
- MS medium supplemented with BAP 0.5mg-1 +NAA0.5mg-1 was found the most effective conditon for shoot multiplication with healthy root of C.ovalis Lindl.
- MS medium supplemented with IBA2mg-l favoured the development of maximum number of healthy , thick and long roots which was followed by MS medium supplemented with NAA0.5mg-l for C.ovalis Lindl. Whereas MS medium supplemented with IBA1.5mg-l favoured the development of maximum number of healthy roots In case of D.densiflorum Lindl.
- Micropropagation is the most effective method For the conservation of threatened species like C. ovalis Lindl. and rare and critically endangered species like D.densiflorum Lindl.

RECOMMENDATIONS

Following recommendations are made from the present research work:

- The mass propagation of economically important orchids can be started using shoot tip and root tip culture in a commercial scale to raise the economic status of our country.
- The local people should be made aware of the conservation of orchids and their collection
- Additional research work should be started for the survival of in vitro grown plantlets in natural habitat.
- Tissue Culture Lab of Central Department of Botany should be well equipped and good facilities to carry out the research work effectively.

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