IN VITRO STUDY OF *DENDROBIUM CREPIDATUM* LINDL. & PAXTON AND ANALYSIS FOR GENETIC HOMOGENEITY BY USING RAPD MARKER

A DISSERTATION
SUBMITTED FOR THE PARTIAL FULFILLMENT
OF THE REQUIREMENT OF MASTER'S DEGREE IN BOTANY

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RECOMMENDATION

9th February , 2015

This is certify that the dissertation entitled " ***In vitro*** **study of** ***Dendrobium crepidatum*** **Lindl. & Paxton and analysis for genetic homogeneity by using RAPD markers"** submitted by Ms. Savita Dhungana for the partial fulfillment of the requirement of Master's Degree in Botany has been carried out under my supervision and guidance. The result of this work has not yet been submitted for any other degree.

Therefore, I recommend her work for approval and acceptance.

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APPORVAL LETTER

This dissertation work submitted by **Ms. Savita Dhungana**, entitle," ***In vitro* study of *Dendrobium crepidatum* Lindl. & Paxton and analysis for genetic homogeneity by using RAPD marker.** has been accepted as a partial fulfillment of M.Sc. Degree in Botany.

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ABSTRACT

Dendrobium crepidatum Lindl. & Paxton is a highly medicinal and threatened epiphytic orchid of Nepal, listed under the Appendix II of CITES. The main purpose of this study was to carry out *in vitro* shoot multiplication and use RAPD technique to study the variation among the wild population (mother plant) and the *in vitro* regenerated of *Dendrobium crepidatum.* Shoot tips obtained from *in vitro* culture of seeds were inoculated on MS (Murashige and Skoog 1962) medium with different growth regulators, Benzyl Amino Purine (BAP), Naphthalene Acetic Acid (NAA), Gibberellic Acid (GA_3) and coconut water supplement. It was found that the most effective medium for shoot multiplication was MS medium supplemented with 10% coconut milk, 2mg/l BAP and 0.5mg/l NAA, which regenerated healthy greenish shoots. In molecular investigation, 4 arbitrary primers were used for RAPD analysis of the *in vitro* regenerated normal shoots, callus regenerated shoots and wild sample. As such, in the study of *in vitro* regenerated shoots formed through callus showed polymorphism while the remaining normal shoots showed genetic homogeneity. The possible cause of the polymorphism may be due to the production and abnormal growth on the shoots (possible somaclonal variation).

ABBREVATIONS

BAP	Benzyl Amino-Purine
BM	Basal Media
DB	Central Department of Botany
EDTA	Ethylene Diamino Tetra Acetate
IAA	Indole-3-Acetic Acid
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
PLBs	Protocom like bodies
GA_3	Gibberellic acid
TU	Tribhuvan University
WWF	World Wide Fund for Nature
RAPD	Random Amplified Polymorphic DNA

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CHAPTER ONE

1.1 Introduction

Orchids belong to family orchidaceae, which is a highly evolved and diverse form of monocotyledons. Orchidaceae is the second largest family compressing 25,000-35,000 species and 800-1000 genera, distributed through the world (Yonzone *et al*., 2011). It is the most extravagant family of flowering plants. The word orchid is derived from Greek, meaning 'testicles' as a similar appearance of tuberous of orchid. Firstly the name was used by Theophrastus, the father of Botany. Orchids are perennial herbs of varying habitat. They can be classified as epiphytic, terrestrial, lithophytic. Vegetatively, orchids are grouped under two groups: sympodial (Joint footed) and monopodial (One footed).

Nepal is endowed with rich orchid flora due to wide geographical distribution, altitude variation and varied climatic condition. In Nepal wild orchids are popularly known by Sungava or Sunakhari, which refers to their shiny yellow pseudo bulbs (Subedi *et al.,* 2013). In Nepal there are 458 taxa of orchids are estimated including 104 genera, 437 species, 16 varieties, 3 subspecies and 2 forma (Rokaya *et al*., 2013). Orchids are mostly confined in eastern and central Nepal in comparison to western Nepal. It is reported that 47 species of orchids were listed from eastern Nepal, 81 species were listed from central Nepal, 69 species were found eastern and central Nepal, 9 species were found throughout eastern to western Nepal (Ghimire, 2008). The distribution in different bio-climate zone shows that subtropical and lower temperate zone are rich in orchid diversity. According to elevation maximum orchids can be observed at 1700 meter above the sea level in Nepal (Acharya *et al.,* 2010).

Mostly orchids have prominent value in medicine, ornamental as well as also in food. Most of the ornamental orchids has been cultivated which are highly prized. Some species of orchids are cultivated for ornamental purpose are *Aerides multiflora, Ascocentrum ampullaceum, Bulbophyllum leopardinum, Calanthe masuca, Calanthe plantaginea, Calanthe tricarinata, Coelogyne cristata, Cymbidium elegans, Cymbidium iridioides, Dendrobium densiflorum, Dendrobium moschatum, Dendrobium nobile, Phaius tankervilleae, Pleione praecox, Rhynchostylis retusa* and *Vanda tessellata* (Rajbhandari and Bhattarai, 2001). Presence of alkaloids, saponin,

glycosides, tannins, phenol, flavonoids steroids and reducing sugar in stem and leaves of orchid gives wide scope for pharmacological and clinical values (Harshitha *et al*., 2013). Some medicinally important orchids listed by Rajbhandari *et al* (2000) are *Brachycorythis obcordata, Coelogyne stricta, Cymbidium aloifolium, Dactylorhiza hatagirea, Eulophia nuda, Flickingeria macraei, Pholidota imbricata, Luisia zeylanica and Vanda tessellata.* Some edible orchids are *Disa englerina, Disa robusta, Habana clavata, Satyrium cursonii* (Kasulo *et al*., 2009).

Due to the large number of beneficial properties of orchids, there is indiscriminately harvesting which cause to serious threat to conservation of orchids in Nepal. Loss of orchids is due to the habitat loss, forest destruction, degradation, destructive collection and over exploitation of orchids (Chaudhari *et al*., 2002). Thus effective strategies should be implemented to conserve the precious gems of nature.

Orchid seeds are numerous like dust (million per capsule) very small 1.0-2.0 mm and 0.5-1.0 mm wide. They blow off after the ripening like the dust particles, barely visible by naked eyes (Mitra, 1971). The embryo has round spherical form, without cotyledons, radical and endosperm. Thus orchids require fungi for their germination and nutrition support. So that only 5% seed germinate successfully in nature (Rao, 1997).

Vegetative propagation is the common method for large scale production of orchids, but the method takes too long time and it is difficult to preserve and propagate. Thus micropropagation technique is the best alternative method for the propagation and conservation of orchids. This technique propagates large number of clone on the artificial nutrient media with use of exogenous hormones which initiate the explants to develop complete plantlet under aseptic condition in short time period (Pant and Gurung, 2005). Through the micropropagtion many disease free plantlets can be obtained successfully (Pant, 2006).

Somaclonal variation is the common phenomenon in plant species propagated through *in vitro*. The species are more valuable for plant breeders as their variation has greatest potential for the plant improvement with the selection of desirable character at cellular level (Nwauzoma, 2013). Somaclonal variant tissue produce phenotypic as well as genotypic variation, but the technique is more accepted for clonal propagation

of germplasm. The variation is most effective due to the mutation on the cultured plant. (Ngezahayo, 2007).

Somaclonal variation depend on the genotype of the material, duration of culture, types of regeneration, stress of the plant growth regulators, heat, osmotic stress and nutrient media concentration (Ngezahayo *et al*., 2007; Sun *et al*., 2013). Somaclonal variation also shows the morphological variation with the parental plant (control) material, such as length of leaf, sheath, shoot tips, internode, stolen, width of leaf, thickness of node, and number of inflorescence. In plant *Triticum durum* also observed deformed leaf, albino seedling chimerical plantlet with leaves produced through the somaclonal variation (Bouiamrine, 2012). Similarly tulips grown *in vitro* shows variation in the flower and leaf (Podwyszynska, 2005), production of Glabrous rice is also the effect of somaclonal variation (Yamamoto *et al.,* 1994) and *in vitro* grown *Populous deltoids* also shows partial resistant with leaf rust diseases (Prakash, 1998) which are more beneficial parameters for the cultivation (Li *et al*., 2010). Also some somaclonal variation is documented in *citrus limon* (Orbovic, *et al*. 2008), rice (salehian *et al*., 2013), *Phalaenopsis bellina* (rchb.f.) (Khoddamzadeh, 2010.), *Allium sativum* L, (Saker and sawahel, 1998.) by using the molecular markers.

Cytological studies can also help to detect the somaclonal variation by chromosomal studies, growth rates of plants and cell division (Raha and Ray, 2003; Ekanema and Osuji, 2006). Some genetic changes like polyploidy, aneuploidy, chromosomal breakdown, deletion, translocation, gene amplification and mutation are some causes of somaclonal variation observed in barley and oat plants (Jain, 2003; Satyanaryan, 2011).

The techniques used to study the genetic variation in plant species are Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), Microsatellite etc are frequently used method for the plant diversity (Mondini *et al.,* 2009).

Rapid Amplified Polymorphic DNA molecular marker is highly applied tools for the detection of the genetic variation. The technique is simple, cheap, not require for sequence information, small amount of DNA sample is sufficient to yield quick result

(Micheli *et al.*, 1994; Pathak and Jaroli, 2012). The marker is cost effective in methods, assessing genetic diversity of plant varieties but the reproducibility is affected by several factors (Ngam and Lakote, 2008). RAPD technique is based on PCR reaction, requires single short arbitrary oligonucleotides primer which anneals with multiple locations on genomic DNA and amplified randomly (Kumar and Gurusubuamanian, 2011; Wiliam *et al*., 1990). It is most sensitive method for the identification of medicinal plant, and quality of herbal drugs (Khan *et al*., 2009). RAPD is the effective tool for the molecular studies (Padmalaha and Prasad, 2006), which easily detect the variation within population, relationship, contrasting level of genetic diversity among population and also polymorphism in many plant species (Tivang *et al*., 1996), (Xue *et al*., 2010). Most of the genetics information is achieved in inter-specific and intra-specific variation by the RAPD markers, (Narendrula and Nkongolo, 2012). The method is also applied for the detection of genetic variation on the plant species in the continental level distribution i.e. - Europe, Asia, and Australlia (Martin *et al*., 2003). Thus markers are applied in gene mapping, population genetic, molecular evolution and plant and animal breeding (Kumar and Ghurusubramian, 2011.)

1.1.1 *Dendrobium crepidatum*

Dendrobium is the second largest genus of orchidaceae. Approximately 1500 species are recorded all over the world. The species has ornamental as well as medicinal value (Chen and Ji, 1998). In Nepal 29 species are well recognized (Raskoti, 2009.). *Dendrobium crepidatum* is epiphytic plant confined an altitude range 600-1400m (Raskoti *et al.,* 2013). Plant is terete which bear lanceolate acute apex leaf and flower with white pinkish color blooming March to April (Pant and Raskoti, 2013). Mostly, stem of the *Dendrobium* species are used for stomach upset and promoting the body fluid (Takamiya *et al*., 2011). Likely, older stem of *Dendrobium crepidatum* is used for neuroprotective activities (Cheng *et al*., 2013).

1.2 Research Questions

- *In vitro* propagation of highly medicinal and threatened species, *Dendrobium crepidatum* is possible or not?

- *In vitro* and *In vivo* (mother) plant of *Dendrobium* is genetically identical (homogenous) or not?

1.3 Objectives

The objective of present research work is to develop the protocol for *in vitro* propagation of *Dendrobium crepidatum* and observe the genetic homogeneity / variation among *in vitro* and *in vivo* plant materials.

Specific objectives of research are:

1. To propagate *Dendrobium crepidatum* by shoot tip culture.

2. To analyze the genetic homogeneity between in *vitro* and *in vivo* grown plant of *Dendrobium crepidatum*.

1.4 Justification of Study

Nepal is a small country but gifted in the orchid diversity, orchids are most important medicinal plants as used by number of people for a long time ago. The various parameters of continuous habitat destruction, degradation fragmentation, timbering, illegal collection, trade and construction of road make the orchids threatened (Chaudhary *et al*., 2002). Thus collection of orchids has been banded under the convention of international trade in endangered species of Wild Fauna and Flora.

For the sustainable utilization of the medicinal and ornamental value it is necessary to cultivate the orchids, which may help to build the economy of national and achieve the goal of sustainable use of medicinal orchids. In this context, tissue culture technique is more reliable tool for propagation of medicinal and ornamental orchids. Thus it can meet to reach for the commercialization of the orchids for international trade to generate large income and conservation of orchids in nature.

Genetic stability is important for the conservation of the threatened and rare orchid for the long term. Genetic variation may occur due to several environmental factors. Thus by using different molecular techniques; RAPD, AFLP, ISSR etc genetic relationship, and genetic variation can be analyze.

CHAPTER TWO

2.1 Literature Review

Knudson (1922) firstly reported asymbiotic seed germination of orchids in the nutrient media. The medium was successful for the germination and now a day the media is popular as "knudson C". Raghavan and Turrey (1964) also grew the seeds of Cattleya *in vitro* medium containing NH_4NO_3 as the sole source of nitrogen. The medium was best for germination of seeds and development to small plantlets. Similarly, Mitra (1971) reported the seeds germination and development of orchid *Arundina bambusifolia* Lindl. in Raghavan and Tarrey (RT) medium. Medium Raghavan and Tarrey (RT) was supplemented with urea, peptone, casein hydrolysate without vitamin, and casein hydrolysate with vitamin, yeast extract, ribonucleic acid and coconut water. Likewise, Mularidhar and Mehta (1986) studied on the three basal media KnC (Knudson 1946), VW (Vacin and Went, 1949) and RT (Raghavn and Torrrey, 1964) with or without various concentration and combination of vitamins, hormones, amino acids and micronutrient for the seed germination of *Cymbidium longifolium*. The basal medium KnC assessed at up to 30% germination, VW 65% and RT assessed at up to 35% germination. Raghuvansi *et al*. (1986) germinated and grew seeds of *Dendrobium nobile, D. chrysanthum* and *Sarcanth pallidus* at different pH (3, 4, 5, 6, 7, 8, and 10) in modified KnC medium where roots were observed best at pH 6, maximum leaves were observed at pH 4 in *D. nobile* in contrast to *D. chrysanthum* and *S.pallidus* which showed more leaf primordia at pH 5. Reddy *et al*. (1992) also studied *in vitro* seed germination and seedling development in four species of south Indian orchids and reported significant effect between the media and orchids. MS and RT media yielded best result than KnC and VW medium. Ground orchid *Spathoglottis plicata* gave best response in MS medium while epiphytic *Epidendrum radicans, Dendrobium crepidatum* and *Cymbidium aloifolium* gave better result on RT medium. Yamamoto *et al*., (1991) modified B_5 medium and supplemented cytokinin 2mg/l BAP for the production of the shoot primordial of *Calanthe sieboldii*. Faria (2005) also cultured *Catasetum fimbriatum* and *Cyrtopodium paranaense* on MS medium, MS modified half strength macronutrient, MS modified one of fourth strength macronutrient, Vacin and Went and knudson C.

The best result observed for development of *C. fimbriatum* was on MS medium, and for *C. paranaensis* was on MS modified with half strength of macronutrient.

Likely, hormonal effect was studied by Fonnesbech (1972) on auxin (IAA, NAA and 2,4-D), cytokinin (kinetin and BA) and gibberellic acid along and combination which was treated on the protocorm of *Cymbidium*. IAA alone had no effect and NAA inhibited chlorophyll synthesis at high concentration. BA and kinetin were used singly to induce shoot formation in solid medium and formation of callus in liquid medium while gibberellic acid induced shoot and leaf growth. Fujieda *et al*. (1977) used various level of shoot tips of welsh onion on various level of kinetin and NAA for the production of adventitious shoot. Murashige and Skoog medium containing 2mg/l kinetin and 0.5 mg/l IAA found to be best shoot multiplication medium for onion. Devi *et al*. (1997) observed clonal propagation of *Dendrobium moschatum* and *Cymbidium aloifolium* through shoot tip culture by using 5 different medium i.e MS (1962), WI (Wimber, 1963), KnC (1946), VW (1949), and NI (Nitsch and Nitsch 1969). NI (Nitsch 1969) medium found to be the best for the formation and proliferation of PLBs. Swar and Pant (2004) found MS basal medium supplemented with BAP 1ppm and NAA 1PPM was best medium for seedling development of *Cymbidium iridioides* and for the multiplication MS medium supplemented with 0.5ppm BAP found to be best. In *Coelogyne cristata* they used MS medium supplemented with 1ppm BAP and 1ppm NAA to obtain multiple number of shoots.

Likewise, Kisor *et al.* (2004) also studied hormonal effect on *Phalaenopsis* orchid using MS medium supplemented with 2BAP and 0.5 NAA which was found the most appropriate medium for the production of large number of vegetative shoots without callus formation, but high hormonal condition 4.41mg/l BAP and 1mg/l NAA was found less appropriate for the vegetative shoot production of *Phalaenopsis*. Sheela *et al.* (2006) also used cytokinin BA 1mg/l and auxin 1mg/l NAA on half MS for the production of PLBs of mutant *Dendrobium* species. Kongbangkerd and Wongsa (2007) cultured 1.5cm-2cm young shoot of *Dendrobium* hybrid on semisolid Vacin and Went medium supplemented with various types of cytokinin BA, TDZ (Thidiazuron), zip, and zeatin at different concentration for production of number of shoots. Highest shoot regeneration was observed on 5mg/l zeatin and root in 2.5mg/l zeatin. Sunitibala and Kishor (2009) found the best germination of immature embryos

of *Dendrobium* tr*ansparens* on half strength of MS medium supplemented with 1mg/l NAA and 2mg/l BAP and for shoot multiplication found MS medium supplemented with 1mg/l NAA and 2mg/l BAP was best. Rahman *et al.* (2009) achieved maximum number of shoot on MS medium supplemented with 0.5mg/l NAA and 1mg/l BAP and for induction of maximum number of root used MS medium supplemented with 1.5 mg/l NAA and 1mg/l BAP on *Vanda tessellata.* Julkiflee *et al*. (2014) studied effect of Phytohormones for the production of protocorm like bodies of *Dendrobium Sonia*-28, half strength MS medium produced highest PLBs compared to full and double strength MS medium in semisolid culture while the combination of BAP (4.411 µM or 8.88µM) and NAA (8.88 µM) increased PLBs growth rate to 14% .

Pant and Pradhan (2010) reported best medium for the germination of seeds of *Cymbidium elegens* on MS basal medium supplement with 1mg/lBAP and reported maximum number of shoots on MS medium supplemented with BAP (1mg/l) and NAA(0.5mg/l). Similarly maximum number of roots was reported on MS medium supplemented with IBA (0.5mg/l). Niknejad *et al*. (2011) used leaf section of *in vitro Phalaenopsis gigantea* and cultured on Dogashima medium (NDM) supplemented with cytokinin (BAP), TDZ (Thidiazuron) and kinetin (kin), along with NAA. TDZ with combination of auxin found to be the best for induction of callus and PLBs on *Phalaenopsis gigantea*.

Similarly, Pant and Shrestha (2011) also obtained maximum number of shoot by using shoot tips of *Phaius tankervilleae*. Shoot tips were cultured on MS medium with various concentrations of NAA and BAP to achieve good result. Maximum number of shoot was reported on MS medium supplemented with 1mg/l BAP and for rooting MS with 0.5mg/l NAA found to be best medium. Pant and Thapa (2012) used shoot tips explants of *Dendrobium primulinum* for the production of number of shoots and roots. They found that MS medium supplemented with 1.5mg/l BAP and 0.5mg/l NAA for the maximum shooting and for the rooting reported MS medium supplemented with 0.5mg/l IAA to be the best medium. Pradhan *et al.* (2013) cultured shoot tips of *Dendrobium densiflorum* on MS medium supplemented with different combination of BAP and NAA for shooting and rooting. Maximum number of shoots were found on MS medium supplemented with BAP 2mg/l and 0.5mg/l NAA and maximum number of roots were found on MS medium supplemented with IBA 1.5mg/l. Kabita and

Sharma (2001) germinated and developed seeds of *Acampe longifolia* Lind. *In vitro* by using MS, KnC, VW, and $B_{5,}$ media supplement with different concentrations of NAA, IBA, and KN. Best germination was found on MS medium containing 0.1mg/l NAA and 1mg/l Kn while low germination was found on B_5 medium.

Aktar *et al*., (2008) used banana pulp for the growth and development of protocorm like bodies of *Dendrobium*, half strength of MS medium with sabri pulp was found best for the length of shoots and leaves of *Dendrobium*. While shoot elongation of *Dendrobium* was found on KC medium with sabri banana pulp. Likely, Cheah and Sagawa (1978) obtained PLBs from the explants with apical and auxiliary buds of *Aranda* and *Aranthera* in Vacin and Went (VW) medium supplemented with 15% coconut water and multiplication of the PLBs was found on VW medium with 2% sucrose and supplemented with 15 % coconut water. Liu and Zhang (1988) germinated seeds of *Dendrobium candidum* on half strength of MS medium and developed protocorm on MS medium with the potato extract. Pyati and Murthy (1995) germinated seeds of *Dendrobium ovatum* in medium Knudson C, Vacin and Went, Murashige and skoog, and Burgeff medium. Among the four media, Knudson C was found to be suitable medium. The was supplemented with 10% coconut milk, 10% cane juice, 200mg/l yeast extract, 200mg/l casein hydrolysate, 200mg/l peptone and 0.5mg/l nicotinic acid gave best result on *Dendrobium ovatum* .

Pyati *et al*. (2002) used nodal explants for culture of *Dendrobium macrostachyum* on the MS basal medium with BA, kinetin and coconut water. Most shoot induction was observed on the MS medium supplemented with coconut water 15%. Chen *et al*. (2014) used half strength of MS medium supplemented with 2mg/l BAP, 0.1mg/l NAA and 100g/l potato extract for the mass production of *Dendrobium officinale* and for root proliferation used half strength of MS medium supplemented with 0.2 mg/l BA and 1mg/l NAA.

CHAPTER THREE

3.1 Materials and Methods

3.2 Plant Material

The material used for the present experiment were small shoot tips of *Dendrobium crepidatum* obtained from *in vitro* germination of seeds.

3.3 Methods

The methods applied for the *in vitro* multiplication of orchid were under following headings:

3. 3.1 Preparation of stock solution

The MS (Murashige and Skoog 1962) supplemented with the growth regulators in different concentrations was used in the present research work. The chemical composition of the MS medium is as follows:

3.3.1.1 Macro-nutrients (Stock-A)

Components	Composition of MS (final conc.)mg/l	(10X) gm/l stock concentration	Volume to be taken for 1liter medium
Potassium Nitrate (KNO_3)	1900	19	
Ammonium Nitrate ($N H_4NO_3$)	1650	16.5	
Calcium Chloride($CaCl_2H_2O$)	440	4.4	
Magnesium Sulphate ($MgSO_4$,7 H_2O)	370	3.7	
Potassium Dihydrogenphosphate (KH_2PO_4)	170	1.7	

3.3.1.2 Micro-nutrients (Stock-B)

Components	Composition of MS(final conc) mg/l	100Xmg/100ml stock concentration	Volume to be taken1liter medium
Magnesium Sulphate($MnSO_4.4H_2O$)	22.3	2230	
Boric Acid (H_3BO_3)	6.2	620	
Zinc Sulphate ($ZnSO_4.7H_2O$)	8.6	860	
Potassium Iodite (KI)	0.83	83	
Sodium Molybdate ($Na_2MoO_4.2H_20$)	0.25	25	
Cobalt chloride ($COCl_2.6H_20$)	0.025	2.5	
Copper Sulphate ($CuSO_4.5H_2O$)	0.025	2.5	

3.3.1.3 Iron source (Fe, EDTA) (Stock-C)

Components	Composition of MS (final conc.)mg/l	10X mg/100ml Stock concentration	Volume to be taken for 1 liter medium
Sodium ethylene diamine tetra acetate(Na_2EDTA)	37.3	373	
Ferrous Sulphate ($FeS0_4.7H_2O$)	27.8	278	

3.3.1.4 Vitamins (Stock-D)

Components	Composition of MS (final conc.)mg/l	10Xmg/100ml stock concentration	Volume to be taken
Glycine	2.0	200	
Nicotinic acid	0.5	50	
Pyrodoxin HCl	0.5	50	
Thamine HCl	0.1	10	
Myo-inositol	100	10,000	

3.3.1.5 Carbon source

Sucrose 30g/l

3.3.1.6 Gelling agents

Agar 8g/l

The stock solution of A (Macro nutrient), B (micro nutrient), C (iron sources) and D (vitamins) were prepared. The stock contains macronutrients micronutrients iron, and vitamins as Murashige and Skoog (1962) media.

3.3.2 Hormones used for the experiments

Cytokinin

A. 6-Benzylaminopurine (BAP) (HIMEDIA Mumbai, India)

Auxin

B. Naphthalene Acetic Acid (NAA) (HIMEDIA Mumbai, India)

Gibberellic acid

A. (GA_3) (HIMEDIA Mumbai, India)

3.3.2.1 Preparation of hormone stock solution

Cytokinin (BAP) hormome (10mg) was dissolved in 2.5ml of 0.5N NaOH and sterile water was added to make final volume of 100ml. From this concentration 1ml was used for 100ml volume of medium or 100ml of medium to make 1mg/l of hormone concentration in the medium.

Similarly, 10mg of NAA was dissolved in 2.5ml of 0.5N NaOH and sterile water was added to make final volume of 100ml.This was used as 1ml for 100 ml of medium to make 1mg/l of hormone concentration in medium.

Further, 10mg of gibberellic acid was dissolved in 2.5ml of absolute alcohol then the final volume was made to 100ml.

3.3.2.2 Preparation of Coconut water

Healthy coconut was drilled and coconut water was used after autoclaving at 120ºC for15 minutes. The milk was used directly on the hormone supplemented basal MS medium as 5% and 10%.

3.3.3 Sterilization of glassware and metal instruments

During the experiment sterilized glassware were used. All the glassware (Petridish, culture tubes, pipettes, beaker, conical flask, measuring cylinder, and metal like forceps, scalpels and blade handle etc.) were dipped in detergent solution for 24 hours and washed with tap water. Then, the glasswares except pipettes and measuring cylinder were autoclaved at 120ºC for 15 minutes. Metal instruments were wrapped in aluminum foil before keeping inside the autoclave for sterilization.

3.3.4 Preparation of Media

For the preparation of 1liter of MS media following protocol was applied.

A. 1 liter sterilized conical was taken
B. Different stock solution were added in the conical flask as follows
    I. Sock A (Macronutrients)-100ml
    II. Stock B (micronutrients)-1ml

III. Stock C (Fe,EDTA)-10ml

IV. Stock D (vitamins)-1ml

C. Then, 100mg of Myoinositol was added.

D. Then, 30gm sucrose was added and it was dissolved by adding sterile water and the final volume was made 1000ml.

E. For the preparation of hormonal medium, hormone was added according to media requirements in separate beakers.

F. The media was solidified by adding the agar 0.8% in separated beakers, boiled and dispensed in the culture tubes.

G. The culture tubes containing media was sterilized in autoclaved for 120ºCfor 15 minutes.

3.3.5 Inoculation of explants

Shoot tips of 0.2-0.3cm were taken as explants for this experiment. Shoot tips were obtained from the in vitro grown seedling, Explants were inoculated on the MS medium supplemented with various concentration of hormones ($GA_{3,}$ BAP, NAA and coconut water). All the culture tubes were transferred to room where temperature was maintained at 25^0C (±5).

3.3.6 Hormone concentrations used for shoot elongation

1. MS
2. MS + 0.1mg/lGA_3
3. MS + 0.2mg/lGA_3
4. MS + 0.25mg/lGA_3
5. MS + 0.5mg/lGA_3
6. MS + 1mg/lGA_3

3.3.7 Hormone concentrations used for shoot multiplication

(I) Low hormone concentration

BAP →	0.1mg/l	0.2mg/l	0.25mg/l	0.5mg/l	1mg/l
NAA 0.1mg/l ↓	(0.1B+0.1N)mg/l	(0.2B +0.1N) mg/l	(0.25B+ 0.1N) mg/l	(0.5B+ 0.1N) mg/l	(1B+ 0.1N) mg/l
0.2mg/l ↓	(0.1B+0.2N)mg/l	(0.2B+ 0.2N) mg/l	(0.25B+ 0.2N) mg/l	(0.5B+ 0.2N) mg/l	(1B+ 0.2N) mg/l

(II) High hormone concentration

BAP →	0 mg/l	0.5mg/l	1mg/l	1.5mg/l	2mg/l
NAA ↓	0	(0.5mg/l B)	(1mg/l B)	(1.5mg/l B)	(2mg/l B)
0.5mg/l ↓	(0.5mg/l N) mg/l	(0.5B+0.5N) mg/l	(1B+0.5N) mg/l	(1.5B +0.5N) mg/l	(2B+ 0.5N) mg/l
1mg/l	(1mg/l N) mg/l	(0.5B+ 1N) mg/l	(1B+ 1N) mg/l	(1.5B+1N) mg/l	(2B+1N) mg/l

(III) High hormone with coconut water

1. MS+ 1mg/l BAP+ 0.5mg/l NAA + 5%
2. MS+ 1.5mg/l BAP + 0.5mg/l NAA+ 5%
3. MS+ 2mg/l BAP + 0.5mg/l NAA + 5 %
4. MS+ 1mg/l BAP+ 0.5mg/l NAA + 10%
5. MS+ 1.5mg/l BAP+ 0.5mg/l NAA + 10%
6. MS+ 2mg/l BAP+ 0.5mg/l NAA + 10%

3.3.8 Preparation of reagents for molecular studies

3.3.8.1 Preparation of TBE buffer 5X-1000ml

Tris base 54gm was dissolved in sterile water after dissolved that, 27.5gm Boric acid was dissolved in 20ml of 0.5M EDTA (ph-8) and it was mixed to the Tris base solution then made up final volume 1000 ml by adding sterile water.

3.3.8.2 Preparation of agarose gel

Agarose, (Fisher Scientific, Mumbai, India) 1% was dissolve on 1X TBE buffer. After boiling the solution 1µl Ethidium bromide (10mg/ml) was added and solution was finally poured on the gel tray which bear comb.

3.3.8.3 Extraction of genomic DNA by using Kit (Promega)

Genomic DNA of plant was extracted by using kit (Promega, Madison, USA). Samples (normal shoots, callus regenerating shoots and wild leaf) were weighed approximately 50mg which were washed with ice cold sterile water and absolute ethanol. The materials were grinded in presence of liquid nitrogen to obtained fine powder then 600µl nuclei lysis solution was added to make paste and transferred to sterile microcentrifuge tube (vol-2ml). These samples were incubated in the 65°C for 15 minutes in hot water bath. After the incubation, 3µl of RNase solution was added and inverted 2-5 times. Then, the tubes were again incubated at 37ºC for 15 minutes in hot water bath. After the incubation the microcentrifuge tubes was cool at room temperature for 5 minutes. After cooling the samples, 200µl protein precipitation solution was added and vortex the samples on the high speed for 20 seconds and centrifuged at 13000-16000xg for 3 minutes. Then supernatant was transferred to clean sterile microcentrifuge tube containing 600µl isopropanol, then it was mixed by inversion and again samples were centrifuged at 13000-16000x g for1minutes. Then added 600µl ethanol 70% for wash DNA pellets. The samples were again centrifuged at 13000-16000x g for 1 minute after that aspired supernatant from the tube and pellets were dried in room temperature for 15 minutes. After drying, about100µl of DNA rehydration solution was added to the tube containing pellets and store at 4ºC for overnight.

3.3.8.4 DNA amplification

DNA amplification was done by using 4 different RAPD primers (MACRO GEN, Korea) i.e. G04 (-5□ ACGACCGACA-3□), G10 (5□-AGGGCCGTCT- 3□) G17 (5□-ACGACCGAC -3□) and G18 (5□- GGCTCATGTG- 3□). Reaction was performed in 25µl volume containing 1µl of 50ng DNA (concentration of DNA sample was estimated by gel electrophoresis and using spectrophotometer at 260nm thermo scientific Genesys 10), master mix (Thermosceientific, Dreama taq pcr, Waltham, MA, USA) 12.5µl, primer(MACRO GEN, Korea) 20 picmol / µl of 1µl, and nuclease free water 10.5 µl. PCR cycling was programmed as preheat lid at 106°C for 5 minutes, initial denaturation at 94°C for 3 µl minutes, denaturation at 94°C for 1 minutes, annealing at 36°C for 1 minutes, extension at 72°C for 2 minutes followed by 40 cycle and final extension was performed by at 72°C for 10 minutes. DNA amplification was performed in thermal cycler (GeNei, India).

3.3.8.5 Gel electrophoresis

PCR amplified DNA product were separated by electrophoresis 1.5 w/v agarose gel in 1X TBE buffer which was stained by Ethidium bromide (10mg/ml) of 1µl volume at 70 volt for 55 minutes using EMBI Tec (Santiago,CA) gel tank. Following the electrophoresis the gel tank was filled with 0.5X TBE buffer of 1000ml volume. The gel was then viewed under ultraviolet light and photographed in UV trans-illuminator (UVITEC, Cambridge).

3.3.9 Data analysis

Data of micropropagation was carried out with six replicate per treatment and experiment was carried out only one time. The result expressed as mean ± SD of all experiments. Variation analyzed by general linear model and means were compared by Dunnett multiple range test at $P < 0.05$ using SPSS ver16.0 for the low hormone treatment and high hormone treatment. Reaming (gibberellic acid treatment and high hormone with coconut water treatment) was analyzed by General linear model multivariate analysis. Molecular data were observed directly using gel documentation photograph.

CHAPTER FOUR

4.1 Results

Present study summarized about regeneration potential of explants (shoot tips) by their physiological status, chemical stimulus, genetic homogeneity and variation among mother (wild) material and *in vitro* material with callus regenerated *in vitro* material o f *Dendrobium crepidatum*, has been described under different heading.

4.1.1 Gibberellic acid Treatment

Shoot tips were tested in different hormonal treatment of gibberellic acid to achieve efficient shoot length of shoot.

1. MS basal medium

In MS medium the shoots were small as well not shoot multiplication. The shoots were very small average length was recorded 0.43 cm within 24 weeks of the culture. Shoots were not healthy and observed pale greenish color.

2. MS + 0.1 mg/l GA_3

The shoot tip culture in MS medium supplemented with 0.1mg/l GA_3 result average shoot elongation 0.44cm within 24 weeks of the culture. The shoots were turned pale greenish color after 5 weeks of culture and after 9 weeks started to be yellowish in color. The yellowish shoots were retard to grow.

3. MS + 0.2 mg/l GA_3

In this condition, the shoot tips observed average length 0.47cm within 24 weeks of culture, which was good among the tested hormonal medium, although the shoots were not healthy leaves were pale greenish color.

4. MS + 0.25 mg/l GA_3

MS supplement with 0.25 mg/l GA_3 was found better cultured medium for shoot elongation within 24 weeks of cultured, average length of shoot was 0.46cm. The shoots were not healthy they were pale yellowish.

5. MS + o.5 mg/l GA_3

Shoot tip culture in MS medium supplemented with 0.5 mg/l GA_3 result most efficient culture condition for shoot elongation among tested gibberellic acid treatment. After 11 weeks medium resulted multiplication which gave 1.02 shoot per culture and average length of shoot length was 0.55cm best for the shoot elongation.

6. MS +1 mg/l GA_3

The medium MS medium supplemented with 1 mg/l GA_3 result highest shoot multiplication 1.3 shoot per culture. The average length of shoot resulted was 4.16 cm at the 24 week of the culture. The shoots observed pale greenish color after 8 weeks 0f culture and after 11 weeks some leaves observed faint blackish color.

TABLE-1 *In vitro* regeneration from shoot tips explants on MS medium with different concentration of Gibberellic acid.

Media	no of shoot(mean) ± sd	length of shoot(mean) cm ± sd	no of leaf(mean) ± sd	length of leaf(mean)cm ± sd
MS	1±0	0.43±.05	1.68± 0.33	0.25± .07
0.1 mg/l GA_3	1±0	0.44±.02	1.61± .31	0.39± .41
0.2 mg/l GA_3	1±0	0.47± .04	2.07± .08	0.28± .07
0.25 mg/l GA_3	1±0	0.46± .03	2.22± .23	0.19± .00
0.5 mg/l GA_3	1.02± 0.12	0.55± .03	2.25± .37	0.23± .05
1 mg/l GA_3	1.3±0.28	0.416± 0.05	2.12± .41	0.2± .00

Culture condition: 25± 5ºC, 24 weeks days of culture, 6 replicates were used in each combination.Sd- standard deviation.

Fig 1- Showing average length ofshoot and length of leaf using gibberellic acid on MS basal medium.

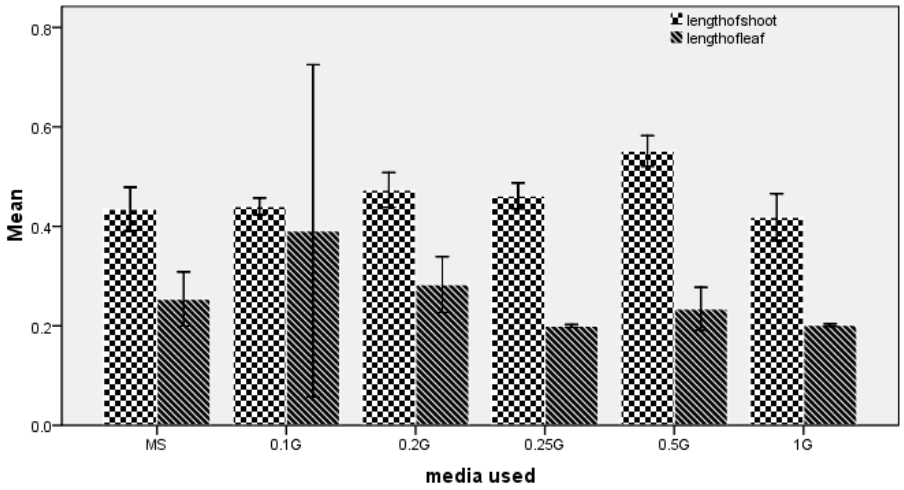
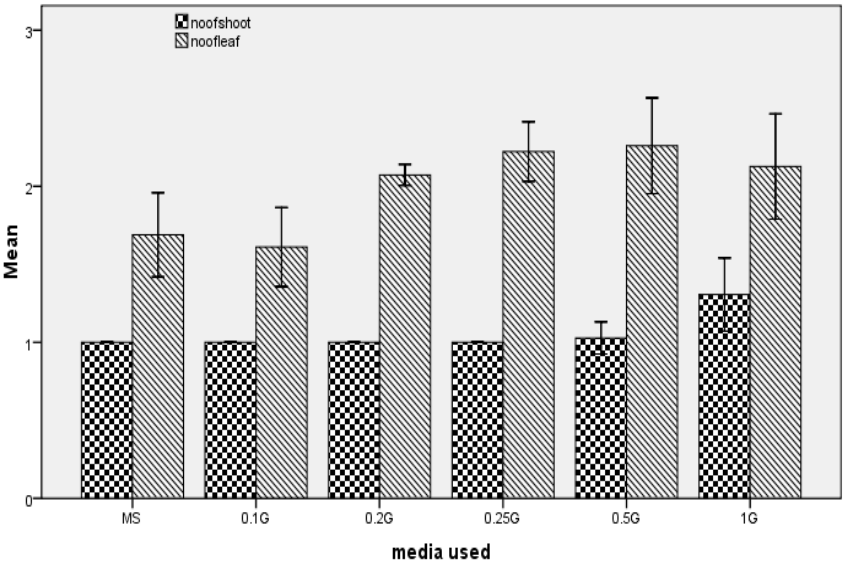


Fig 2- showing average no of shoot and leaf on MS basal medium with gibberellic acid in different condition.



4.1.2 Low hormone treatment

For shoot multiplication, shoot tips were inoculated in different concentration of hormonal medium BAP and NAA. The effect of BAP and NAA on the shoot tip culture of *Dendrobium crepidatum* described under different heading.

1. MS basal medium

In MS medium, the shoot resulted small shoot elongation but not shoot multiplication. The shoots were very small. Average length was recorded 0.43 cm within 24 weeks of the culture. Shoots were not healthy and observed pale greenish color.

2. MS + 0.1mg/l BAP + o.1mg/l NAA

The shoot tips cultured in MS medium supplemented with 0.1mg/l BAP and 0.1mg/l NAA result multiplication after 3 weeks of culture. The average length was 0.36cm and 1.33 shoot per culture.

3. MS + 0.2mg/l BAP + 0.1mg/l NAA

In this condition, the shoot tips were multiplied after 3 weeks of culture. The average number was 1.33 and average length of shoot was 0.44 cm. The shoot observed small leaves with achlorophyllous.

4. MS + 0.25mg/l BAP + 0.1mg/l NAA

MS medium supplemented with 0.25mg/l BAP + 0.1mg/l NAA, the shoot tip was multiplied after 3 weeks of culture, the condition provided 1.56 shoot per culture and 0.41 cm average length. The shoots were not healthy which bear more than 2 leaves per shoots.

5. MS +0.5mg/l BAP +0.1mg/l NAA

In this condition shoot tips were multiplied after 4 weeks of culture, maximum 3 shoots were observed and average length was 0.4cm and 1.94 shoot per culture. The shoots observed were greenish color than other treatment.

6. MS + 1mg/l BAP + 0.1 mg/l NAA

MS medium supplement with 1mg/l BAP and 0.1 mg/l NAA was found to be the most efficient cultured condition for shoot multiplication which gave 2.11 shoot per culture and 0.4 cm average length of shoot. The shoots were greenish and healthy.

7. MS + 0.1mg/l + 0.2mg/l NAA

The medium provided lowest number of shoots, it started shoots multiplication after 6 weeks of culture and shoots were yellowish color. After 11weeks of culture, shoots started to die. The average length of shoot was 0.4 cm and shoot per culture was 1.04. Some shoots were resulted small tiny roots also.

8. MS + 0.2mg/l BAP + 0.2NAA

In this medium the shoot tips multiplied after the 6 weeks of culture. The shoots were pale yellowish color which provided 1.11 shoot per culture and length of shoot was 0.4cm. The shoots were not healthy but presence of small roots.

9. MS + 0.25 mg/l BAP + 0.2 mg/l NAA

In this condition the shoot tips result multiplication after 2 weeks of inoculation, but few shoots turned to yellowish in color the shoot gained 1.77 shoot per culture and 0.38cm average length. The shoots observed 1-2 number of shoot with small length.

10. MS +1mg/l BAP +0.2 mg/l NAA

The medium which resulted fast multiplication among the all treated hormone, it started multiplication within 2 weeks of culture. The shoot tips observed healthy and greenish with average length 0.38cm and 2 shoot per culture.

TABLE-2 In *vitro* plant regeneration from shoot tips explants cultured from on Murashige and Skoog (MS) medium with different concentration of 6-Benzyl aminopurine (BAP) and Naphthalene acetic acid (NAA).

BAP mg/l	NAAm g/l	no of shoot± sd	length of shoot± sd (cm)	no of leaf± sd	length of leaf ± sd (cm)	no of root± sd	length of root± sd (cm)
0	0	1± 0.52[b]	0.4± 0.01[a]	1.68± 0.33[a]	0.253± 0.667[a]	-	-
0.1	0.1	1.33± 0.52[a]	0.36± 0.07[a]	1.89± 0.10[a]	0.22± 0.046[a]	-	-
0.2	0.1	1.38± 0.34[a]	0.44± 0.05[a]	1.93± 0.20[a]	0.259± 0.058[a]	-	-
0.25	0.1	1.56± 0.77[a]	0.41± 0.08[a]	2.14± 0.41[a]	0.247± 0.054[a]	-	-
0.5	0.1	1.94± 0.57[a]	0.4± 0.02[a]	1.84± 0.28[a]	0.223± 0.027[a]	-	-
1	0.1	2.11± 0.32[a]	0.4± 0.02[a]	2.15± 0.20[a]	0.249± 0.056[a]	-	-
0.1	0.2	1.04± 0.46[b]	0.4± 0.12[a]	1.42± 0.33[b]	0.229± 0.027[a]	0.5± 0.547[a]	0.05± 0.054[a]
0.2	0.2	1.11± 0.19[b]	0.4± 0.06[a]	1.64± 0.40[b]	0.261± 0.013[a]	0.666± 0.516[a]	0.066± 0.052[a]
0.25	0.2	1.77± 0.75[a]	0.38± 0.07[a]	1.86± 0.23[a]	0.216± 0.030[a]	0.5± 0.548[a]	0.05± 0.055[a]
0.5	0.2	1.59± 0.45[a]	0.4± 0.03[a]	1.77± 0.17[a]	0.203± 0.019[a]	0.5± 0.548[a]	0.05± 0.055[a]
1	0.2	2± 0.09[a]	0.38± 0.03[a]	2.11± 0.15[a]	0.22± 0.018[a]	0.666± 0.516[a]	0.066± 0.052[a]
χ^2		166.727	51.09	1.795	10.364	19.63	21.879
Df		19	45	36	55	1	1

Culture condition: 25± 5ºC, 24 weeks of culture, 6 replicates were used in each combination. The value with same superscript in each column and it is not significantly different at $p \leq 0.05$.

Fig 3- Showing average number of shoot, leaf and root in MS medium supplement with BAP and NAA in different hormone condition.

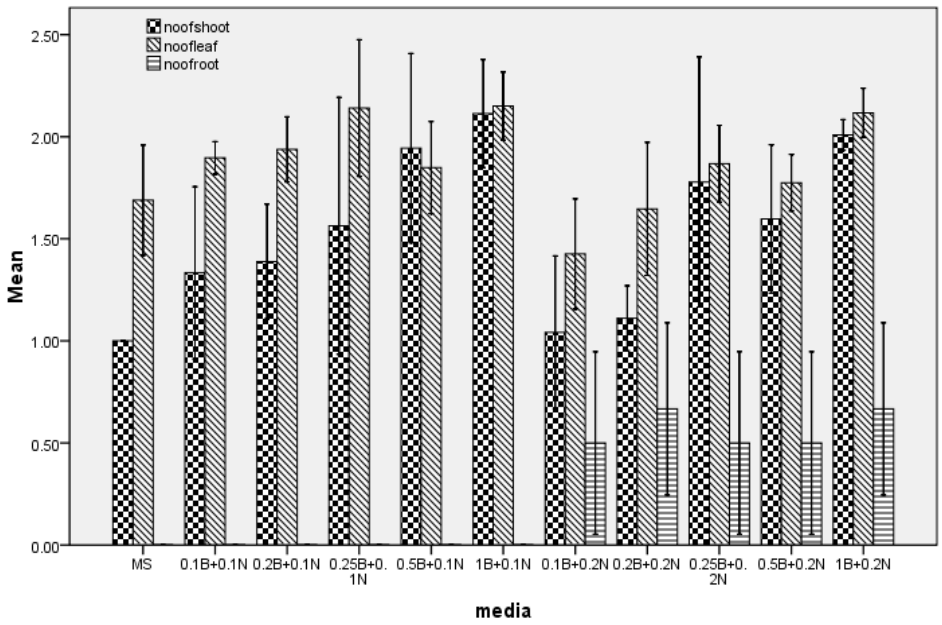
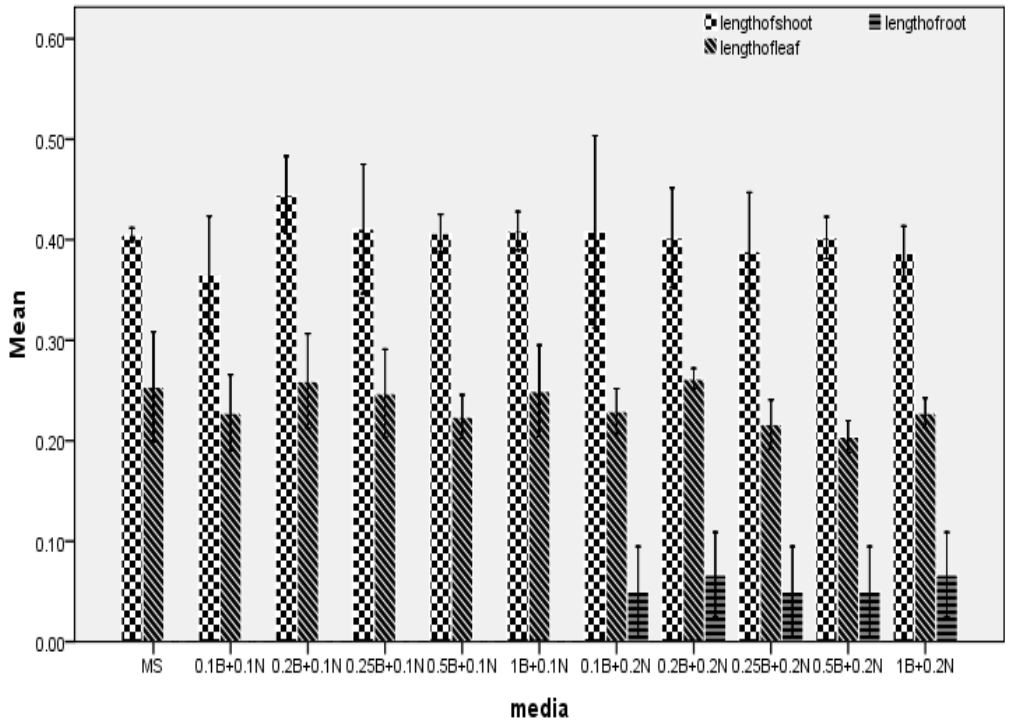


Fig 4- Showing the average length of shoot, leaf and root in MS medium suppliment with BAP and NAA indifferent concentration.



4.1.3 High hormone treatment

To achieve good number of shoots further shoots were inoculated in high hormonal condition. The significant regeneration capacity of hormonal concentration of Dendrobium *crepidatum* described following heading.

1. MS Basal medium

In MS basal medium the shoot multiplication started after 9 weeks of culture of shoot tip. In this condition the explants produce maximum 2 shoot up to the 24 weeks of culture. The average length of shoot was 0.49 cm and 1.08 shoot per culture.

2. MS + 0.5mg/l BAP

The shoot tips culture in MS medium supplemented with 0.5mg/l BAP result multiplication after 4 weeks of cultured. The average number of shoot was 1.42 and average length of shoot was 0.51cm. The shoots were not healthy and greenish.

3. MS + 1mg/l BAP

In this medium the shoot tips observed greenish which multiplied after 3 weeks of culture and average length was 0.49cm. The shoot multiplied maximum 3 number of shoot. The medium resulted in 1.58 shoots per culture.

4. MS + 1.5mg/l BAP

The MS basal medium supplemented with 1.5mg/l BAP was started multiplication after 3 weeks of culture. The average length of shoot was 0.56cm and 1.24 shoot per culture were observed. The observed shoot tips were not healthy and attractive.

5. MS + 2mg/l BAP

In this medium, shoot tips were multiplied within 3 weeks of culture and observed pale greenish leaves. It result1.56 shoot per culture and 0.54cm average length of shoot.

6. MS + 0.5mg/l NAA

In this condition, shoots started to multiply after 11 weeks after the culture which produce maximum 2 shoot with in the 24 weeks of culture. Medium resulted in 1.16 shoot per culture and 0.62 cm average length.

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

In this condition the shoots started multiplication after 6 weeks of culture which turned pale yellowish after 16 weeks of culture and shoot resulted 1.27shoot per culture and average length 0.51cm.

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

The medium Supplemented with 1mg/l BAP and 0.5mg/l NAA which multiplied within 3 weeks of cultured and the shoots were greenish healthy and attractive which bear 2 leaves. The average shoot was 1.63 per culture and average length 0.54cm.

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

The medium MS Supplemented with 1.5mg/l BAP and 0.5mg/l NAA resulted multiplication within 3 weeks of culture and observed 2.19 shoot per culture and average length 0.62cm. The shoots were greenish and healthy but the older leaf observed pale yellowish color.

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

The medium MS medium supplemented with 2mg/l BAP and 0.5mg/l NAA was found the most effective culture condition for the shoot multiplication. In this condition shoot proliferation rate was highest compared to other culture. The shoots were 2.99 per culture and average length 0.59cm. Among the tested on the high hormone the medium was most effective for greenish and attractive shoot.

11. MS + 1mg/l NAA

The condition MS Supplemented with 1mg/l NAA resulted multiplied after 12 weeks of culture and the shoots were yellowish and unhealthy. The average length of shoot was o.56cm and 1.28 shoot per culture.

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

In MS medium Supplemented with 0.5mg/l BAP and 1mg/l NAA started multiplication after 7 weeks of culture. Observed shoots were unhealthy with pale yellowish color. The average shoot length was 0.56cm and 1.16 shoot per culture were observed.

13. MS+ 1mg/l BAP + 1mg/l NAA

In MS medium Supplemented with 1mg/l BAP and 1mg/l NAA the shoot started multiplication after 8weeks of culture. The average shoot length was 0.54 cm and1.16 per culture was observed.

14. MS + 1.5mg/l BAP +1mg/l NAA

In MS medium supplement with 1.5mg/l BAP and 1mg/l NAA, the shoots were multiplied within 4 weeks of cultured and observed 1.51 shoot per culture and average length 0.47cm. The shoots were yellowish in color.

15. MS + 2mg/l BAP +1mg/l NAA

In this condition the shoot was resulted multiplication after 4weeks of inoculation and record 1.32 shoot per culture and 0.46cm average length. The shoot was not healthy and greenish.

TABLE -3 *In vitro* plant regeneration from shoot tips explants cultured from on Murashige and Skoog (MS) medium with different concentration of 6-Benzyl aminopurine (BAP) and Naphthalene acetic acid (NAA).

BAP mg/l	NAA mg/l	no of shoot± sd	length of shoot(cm) ± sd	no of leaf± sd	length of ± sd leaf(cm)	no of root ± sd	length of root(cm) ± sd
	0	1.08±0.13^{a}	0.49± 0.10^{a}	2.22± 0.32^{a}	0.25± 0.04^{a}	0.66± 0.52^{a}	0.06± 0.05^{a}
0.5	0	1.42±0.59^{a}	0.51± 0.12^{a}	2.22± 0.27^{a}	0.29± 0.02^{a}	0.66±0.52^{a}	0.06± 0.05^{a}
1	0	1.58±0.07^{a}	0.49± 0.09^{a}	2.18± 0.26^{a}	0.21± 0.02^{a}	0.83± 0.41^{a}	0.09± 00^{a}
1.5	0	1.24±0.21^{a}	0.56± 0.06^{a}	2.2± 0.43^{a}	0.27± 0.05^{a}	0.5± 0.55^{a}	0.05± 0.05^{a}
2	0	1.56±0.06^{a}	0.54± 0.07^{a}	2.68± 0.38^{a}	0.4± 0.25^{b}	0.5± 0.55^{a}	0.05± 0.05^{a}
0	0.5	1.16±0.41^{a}	0.62± 0.02^{b}	2.02± 0.07^{a}	0.21± 0.03^{a}	1.0± 0^{a}	0.15± 0.05^{a}
0.5	0.5	1.27±0.23^{a}	0.51± 0.10^{a}	2.03± 0.11^{a}	0.25± 0.05^{a}	0.66± 0.52 ^{a}	0.06± 0.05^{a}
1	0.5	1.63±0.43^{a}	0.54± 0.03^{a}	3.21± 0.40^{b}	0.25± 0,05^{a}	0.83± 0.41^{a}	0.833± 0.04^{a}
1.5	0.5	2.19±0.31^{b}	0.62± 0.15^{b}	3.3± 0.37^{b}	0.22±0.04^{a}	1.1± 0.13^{a}	0.163± 0.04^{a}
2	0.5	2.99±0.95^{b}	0.59± 0.08^{a}	4.5± 0.86^{b}	0.23± 0.05^{a}	1.0± 0^{a}	0.13±0.04^{a}
0	1	1.28±0.36^{a}	0.56± 0.06^{a}	2.0± 00^{a}	0.22± 0.02^{a}	1.18± 0.22^{a}	0.2± 0.04^{b}
0.5	1	1.16± 0.30^{a}	0.56± 0.08^{a}	2.055± 0.09^{a}	0.2± 0.01^{a}	1.83± 0.41^{a}	0.18± 0.05^{b}
1	1	1.16± 0.42^{a}	0.54± 0.09^{a}	2.05±0.12^{a}	0.23± 0.02^{a}	0.83± 0.41^{a}	0.11± 0.06^{a}
1.5	1	1.51± 0.30^{a}	0.47± 0.05^{a}	3.16± 0.41^{b}	0.21± 0.01^{a}	0.5± 0.55^{a}	0.05± 0.05^{a}
2	1	1.32± 0.15^{a}	0.46± 0.04^{a}	2.53± 0.59^{a}	0.2±0.01^{a}	1.16± 0.75^{a}	0.089± 0.05^{a}
χ^2		250	39.67	489.35	251.46	288	206
Df		35	65	30	38	8	9

Culture condition: 25± 5ºC, 24 weeks of culture, 6 replicates were used in each combination. The value with same superscript in each column and it is not significantly different at $p \leq 0.05$.

Fig 5- Showing number of shoot, leaf and root in MS medium with supplement of BAP and NAA, in different conditions.

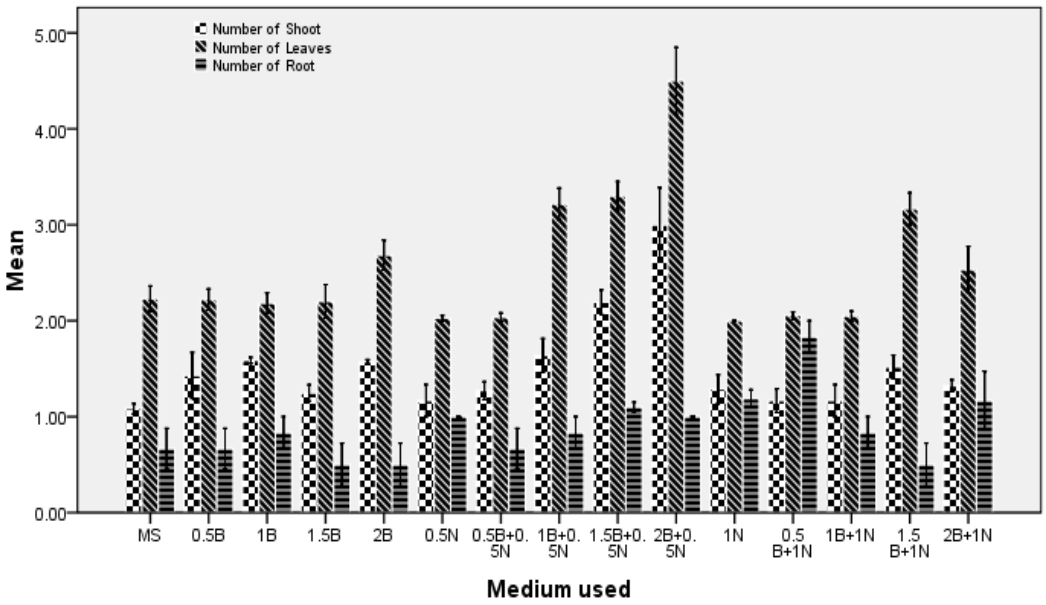


Fig 5-Showing length of shoot, leaf and root in MS medium with different condition of BAP and NAA.

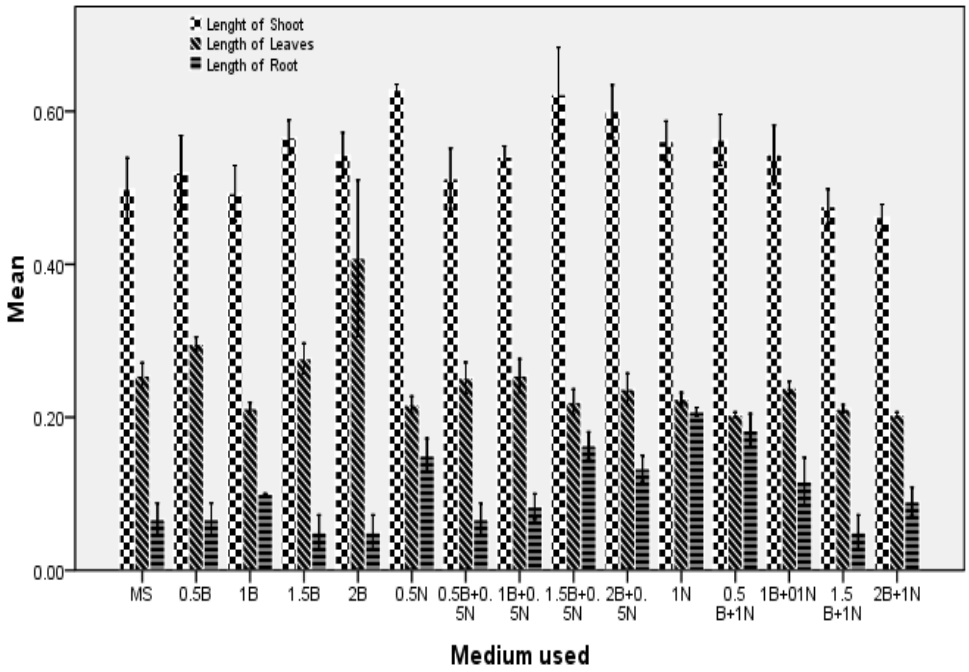
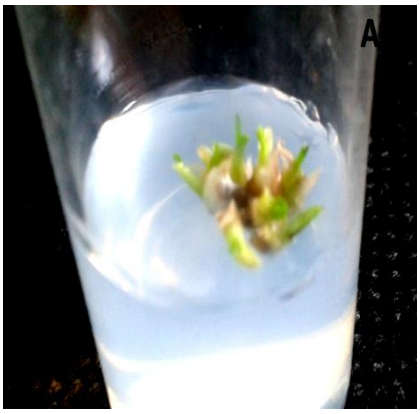
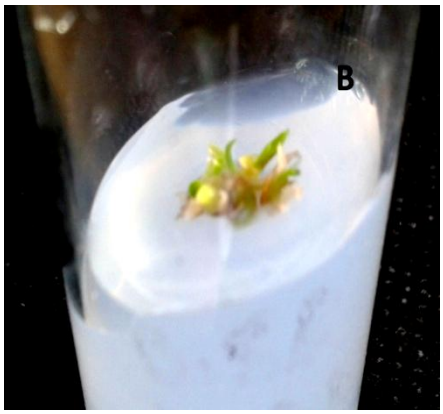


PHOTO PLATES





A: MS Medium with 2BAP mg/l+0.5 mg/l NAA (shoot multiplying), **B**: MS Medium with 1.5 mg/l BAP+ 0.5 mg/l NAA (shoot multiplying), **C**: MS Medium with 1 mg/l BAP+0.5 mg/l NAA shoot multiplying, **D**:MS Medium with 1.5 mg/l BAP (shoot elongation).

4.1.4 High hormone treatment with coconut water

To produce healthy, greenish and multiple number of shoot tips of *Dendrobium crepidatum* was again inoculated on the different hormonal treatment with coconut water.

1. MS + 1mg/l BAP + 0.5mg/l NAA +5%

The medium supplemented with 2mg/l BAP, 0.5mg/l NAA and 5% started shoot multiplication after 3weeks of culture. It produced multiple number of small protocorm like bodies with small leaves. The medium gave 2.35 shoot per culture and average length 0.64cm. The shoots were greenish with good number of shoot.

2. MS + 1.5mg/l BAP + 0.5mg/l NAA +5%

The medium supplemented with 1.5mg/l BAP, 0.5mg/l NAA and 5% coconut milk result shoot multiplication after 3 weeks of inoculation, observed shoot were small with small greenish leaf. The shoot per culture was 2.3 and average length was 0.64cm.

3. MS + 2mg/l BAP +0.5mg/l NAA +5%

In this medium shoot tips started to multiplication after 3 weeks of culture and maximum 2-5 number of shoot were observed. The shoots were healthy and greenish, average length of shoot was 0.72cm and 2. 3 shoot per culture.

4. MS + 1mg/l BAP +0.5mg/l NAA +10%

In this medium shoot tips responded with in 3 weeks of culture. The shoots were 2 per culture and average length 1.35cm which was most efficient medium for elongation than multiplication. Shoots were healthy with long and greenish leaf.

5. MS + 1.5mg/l BAP +0.5mg/l NAA +10%

In this medium shoot tips multiplied after 3 weeks of culture, shoots were complete differentiated with long and attractive leaf. Shoot per culture was 2.53 and average length was 1.3cm.

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

The shoot tips started multiplication within 3 weeks of culture. Medium was most efficient of shoot multiplication as well as shoot elongation. The medium was best for the production of healthy and attractive shoot .The shoot per culture was 3.08 and average length was 1.10cm at 16 weeks of culture.

TABLE-4 *In vitro* plant regeneration from shoot tips explants cultured from on Murashige and Skoog (MS) medium with different concentration of 6-Benzyl amino purine (BAP) and Naphthalene acetic acid (NAA).

BAP mg/l	NAA mg/l	CW %	No of shoot± sd	length of shoot ± sd (cm)	no of leaf ± SE	length of leaf ± sd (cm)
1	0.5	5%	2.35± 0.26	0.64± 0.07	3.51± 0.37	0.33± 0.07
1.5	0.5	5%	2.3± 0.40	0.67±0.12	3.41± 0.64	0.33± 0.05
2	0.5	5%	2.3± .21	0.72± 0.07	3.46± 0.39	0.37± 0.05
1	0.5	10 %	2.00± 0.34	1.35± 0.13	3.8± 0.74	0.49± 0.08
1.5	0.5	10 %	2.53± .30	1.13± 0.19	4.33± 0.24	0.43± 0.08
2	0.5	10 %	3.08± .23	1.10± 0.15	5.43± 0.63	0.44± 0.08

Culture condition: 25± 5ºC, 16 weeks of culture, 6 replicates were used in each combination. sd -standard deviation.

Fig 6-Showing average number of shoot, leaf, in MS basal medium with BAP and NAA suppliment with coconut water iin 5%and 10%.

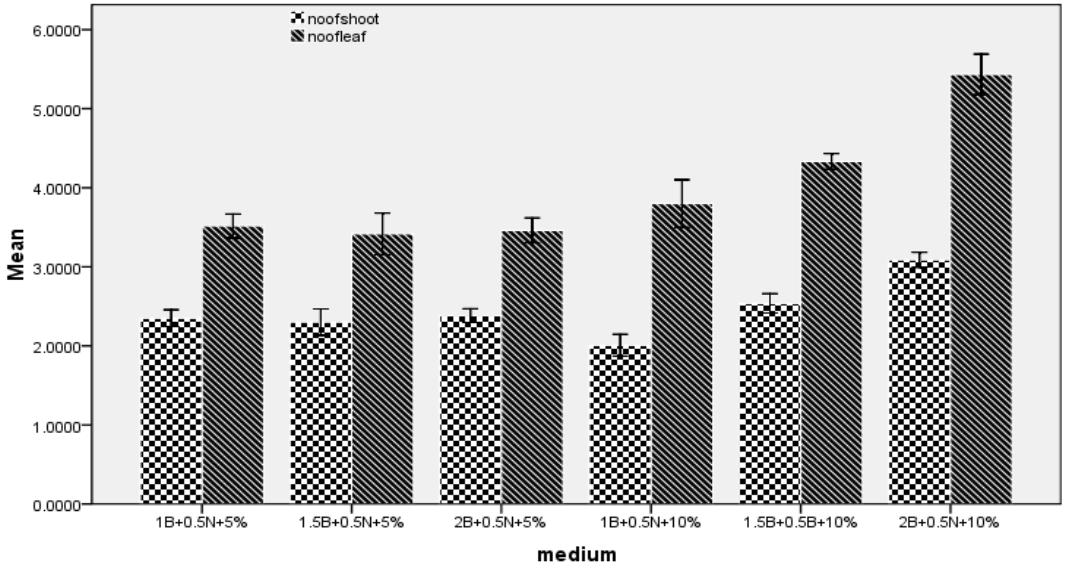


Fig 7- showing length of shoot and length of leaf in MS medium withBAP and BAP suppliment with 5% and 10% coconut water.

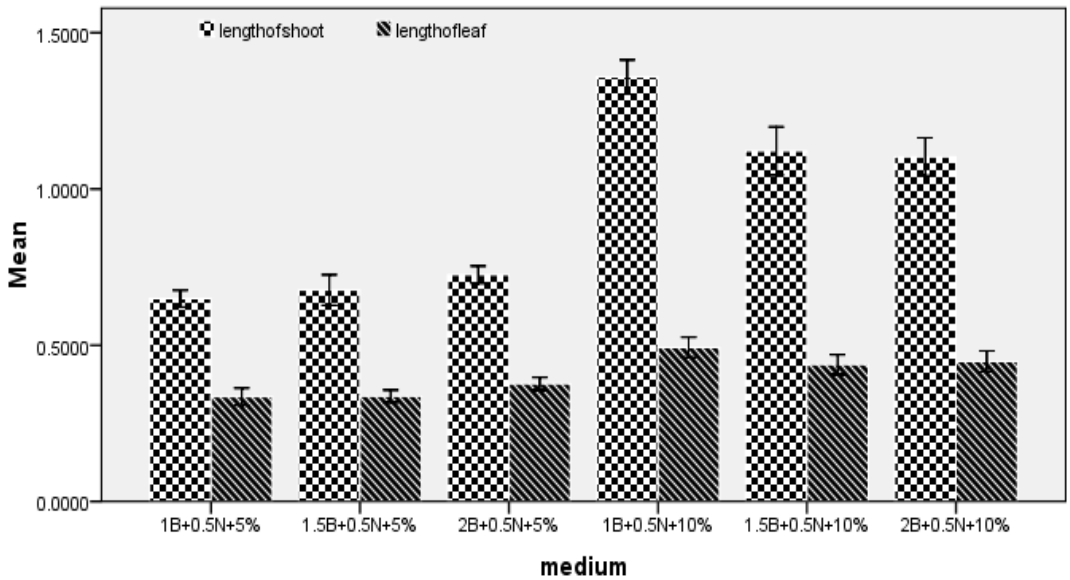
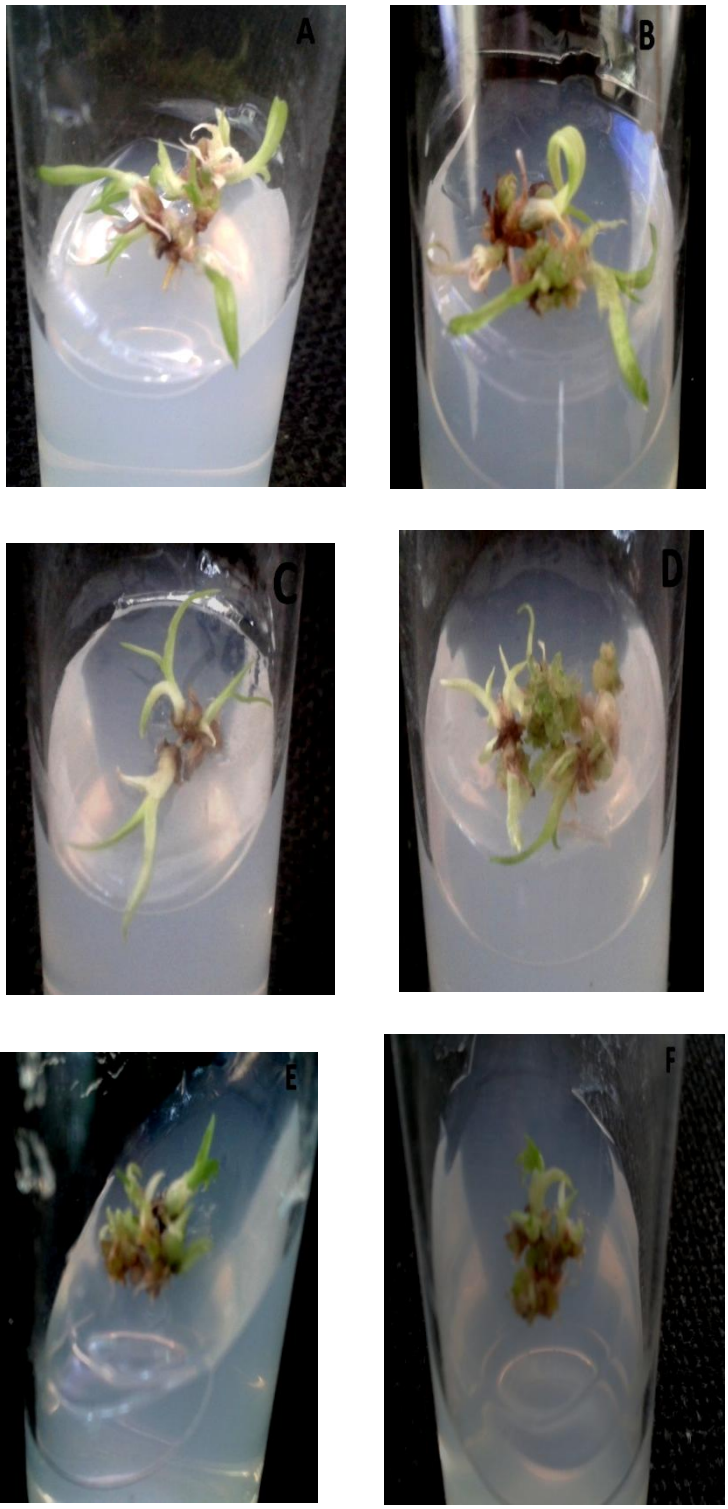


Photo plates



A: MS medium with 2mg/l BAP+ 0.5mg/lNAA+10%, **B**: MS medium with 1.5mg/lBAP+ 0.5mg/lNAA +10%, **C:** :MS medium with 1mg/lBAP+ 0.5mg/lNAA+10%, **D**: MS medium with 2mg/lBAP+ 0.5mg/lNAA+5%. E: MS+1.5mg/l BAP+ 0.5mg/lNAA+5%. F: MS+1mg/lBAP +0.5mg/lNAA+5%.S

Photo plates

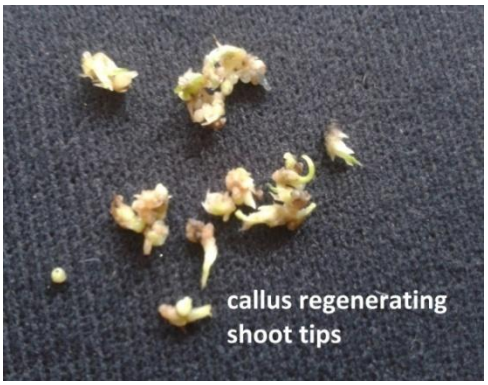
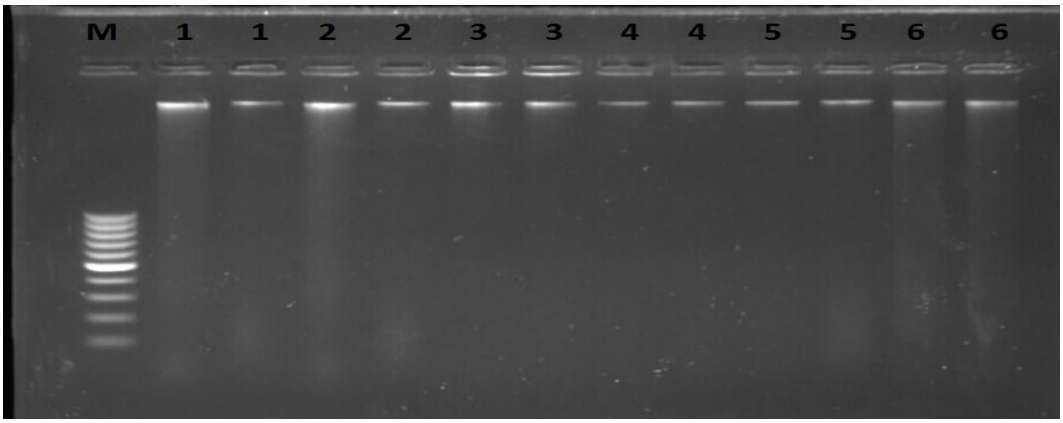


Fig 8- Bands of DNA extraction using kit. M-1oobp DNA ladder, 6- DNA extracted from callus regenerating shoots, 5- DNA extraction from mother sample (*in vivo*),1 ,2, 3, 4 DNA extracted from normal shoots (*in vitro*).



4.1.5 Testing for Homogeneity

Fig 9-Amplified genomic DNA by PCR reaction using primer G04

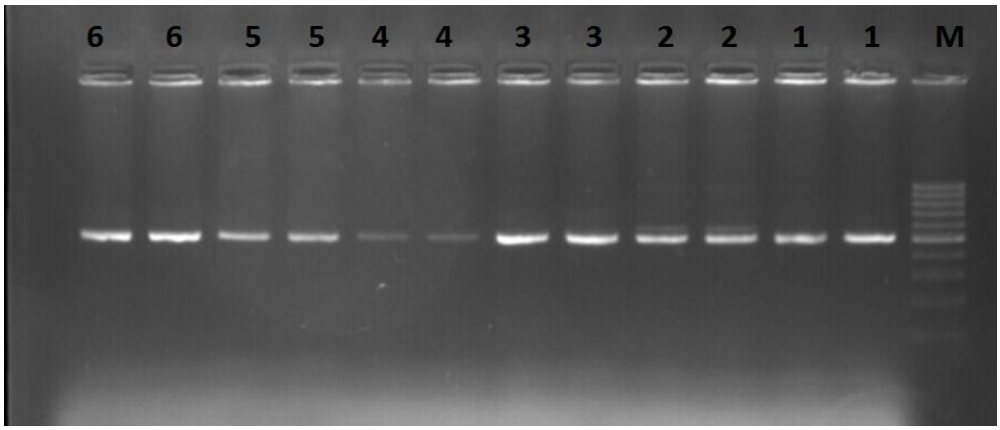


Fig 9- M-1oobp DNA ladder, 6- DNA extracted from callus regenerating shoots, 5- DNA extraction from mother sample (*in vivo*), 1, 2, 3, 4, DNA extracted from normal shoots (*in vitro*).

Table -5 Bands observed after the amplification of genomic DNA by using PCR reaction using G04 primer.

SN	Samples	Total no of bands	Homogeneity band compare with mother	Polymorphic band compare with mother
1	Normal shoot-1	1	1	-
2	Normal shoot-2	1	1	-
3	Normal shoot-3	1	1	-
4	Normal shoot -4	1	1	-
5	Callus regenerated shoots	1	1	-

Fig 10-Amplification of genomic DNA by PCR reaction using G10 primer.

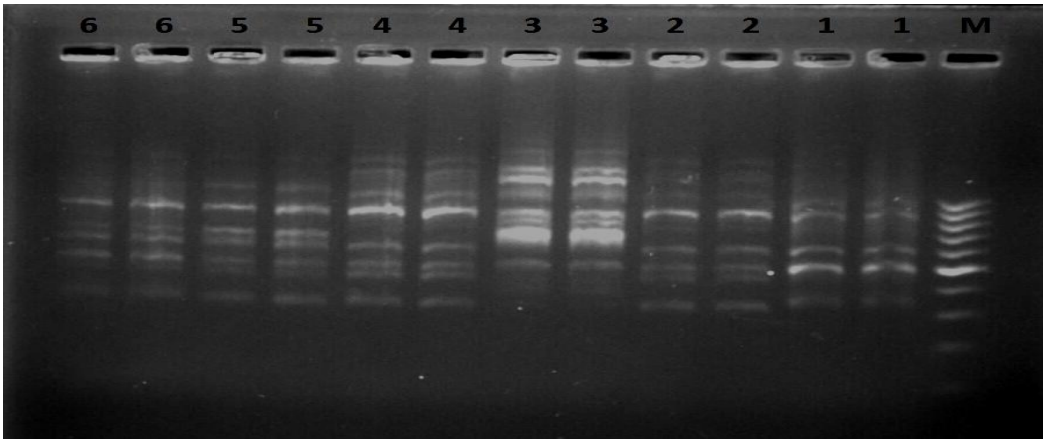


Fig 10- M-1oobp DNA ladder, 3- DNA extracted from callus regenerating shoots, 5-DNA extraction from mother sample (*in vivo*), 1, 2, 4, 6 DNA extracted from normal shoots (*in vitro*).

Table -6 Bands observed after the amplification of genomic DNA by using PCR reaction using G10 primer.

SN	Samples	Total no of bands	Homogeneity band compare with mother	Polymorphic band compare with mother
1	Normal shoot -1	4	4	-
2	Normal shoot -2	4	4	-
3	Normal shoot -4	4	4	-
4	Normal shoot -6	4	4	-
5	Callus regenerated shoot	5	2	3

Fig 11 -Amplification of genomic DNA by PCR techniques using G17 primer

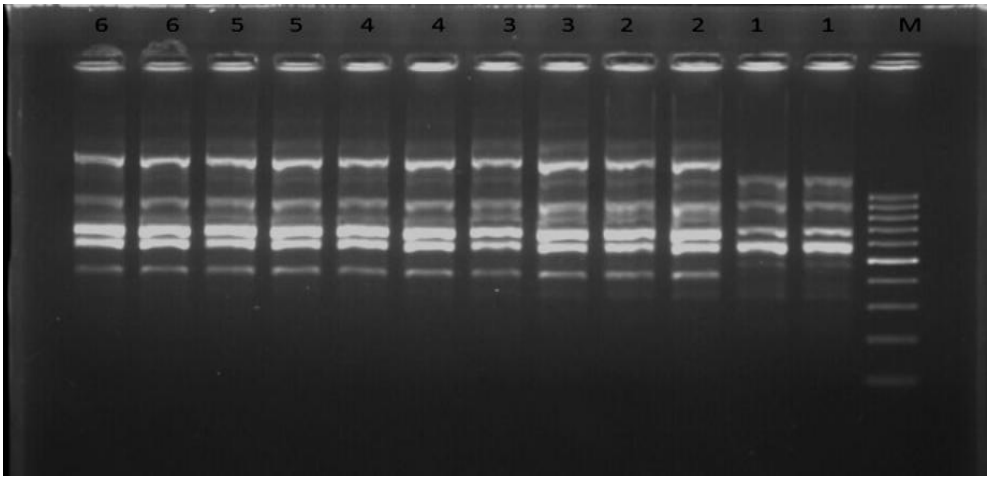


Fig 11- M-100bp marker, 1- callus induced shoots, 6-mother leaf sample (*in vivo*), 2, 3, 4, 5, - other normal shoots regenerated in different hormonal concentration (*in vitro*).

Table -7 Bands observed after the amplification of genomic DNA by using PCR reaction using G17 primer.

SN	Samples	Total no of bands	Homogeneity band compare with mother	Polymorphic band compare with mother
1	Normal shoot -2	5	5	-
2	Normal shoot -3	5	5	-
3	Normal shoot -4	5	5	-
4	Normal shoot -5	5	5	-
5	Callus regenerated shoot	4	3	1

Fig 12-Amplification of genomic DNA by PCR techniques using G18 primer.

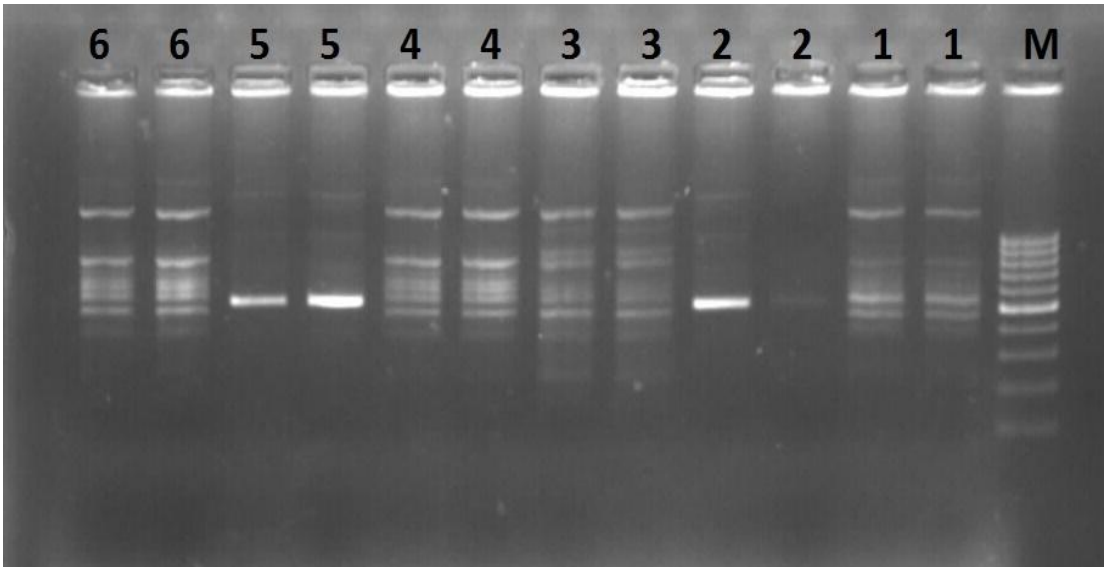


Fig 12-M- 100bp DNA ladder, 1, 3, 4,- normal shoots DNA ladder(*in vitro*), 2 and 5-callus induced shoots, 6- mother leaf sample(*in vivo*).

Table -8 Bands observed after the amplification of genomic DNA by using PCR reaction using G18 primer.

SN	Samples	Total no of bands	Homogeneity band compare with mother	Polymorphic band compare with mother
1	Normal shoot-1	4	4	-
2	Normal shoot -3	4	4	-
3	Normal shoot -4	4	4	-
4	Callus regenerated shoot	1	1	1

For the analysis of the genetic homogeneity, 4 primers were used to obtain scorable bands. Among the 4 primer (G04, G10, G17, G18), G04 primer could not amplify the genomic DNA of callus regenerating shoots, normal shoots and wild sample (mother). Remaining three primers amplified genomic DNA of required sample (callus regenerating shoot tips, normal shoot tips and wild sample). Largest number of scoroable band was observed on G17, i.e – 58 number of band where 5 bands were

homogeneity with mother or wild samples to the normal shoots and single polymorphic band on the callus regenerating shoot tips. In G10 primer which gave 49 numbers of bands 4 numbers of bands were observed from normal shoots sample and 5 numbers of bands form callus regenerating shoots. This primer gave 4 number of homogametic band with mother and normal shoots and 3 polymorphic bands with callus regenerating shoots. Primer G18 gave total 36 numbers of bands. Normal shoots and mother (wild) material shows 4 homogametic bands and callus regenerating shoots shows 1 polymorphic band.

CHAPTER FIVE

5.1 Discussion

Shoot tips obtained from *in vitro* culture of seeds, were culture on MS basal medium and MS medium supplemented with different concentration of BAP, NAA, Gibberellic acid and coconut water to obtain large number of shoot.

5.1.1 Gibberellic acid treatment

In vitro propagation of *Dendrobium crepidatum* was carried out by using single shoot tip. The average length of 20 weeks culture of shoot tips was found (0.2-0.3cm) and number of leaf was (1-2) during the period.

In vitro propagated shoot tips were inoculated on MS basal medium and MS medium supplement with various concentrations of gibberellic acid, (0.1 mg/l GA_3, 0.2 mg/l GA_3, 0.25 mg/l GA_3, 0.5 mg/lGA_3, 1mg/lGA_3). Among the concentration MS+ 0.5mg/l GA_3 showed good shoot elongation compare to other concentrations whose average length was 0.55 cm. The result is followed by MS medium supplemented with 0.2mg/l GA_3 whose length of shoot was 0.47cm. Lowest shoot elongation was observed on MS medium supplemented with 1.0mg/l GA_3 and MS medium without gibberellic acid had no effect on the shoot elongation. Many researchers also found that gibberellic acid enhanced the shoot elongation, Sultana *at el*., 2013 reported that 0.5µM concentration of gibberellic acid was found to be the best for shoot elongation on *Phaius tankervilleae* .Similarly (Gabryszewska, 2010) treated gibberellic acid singly to stimulate shoot and leaf growth and inhibit the bud formation on *Paeonia lactiflora*. In *Gihngamukungu* 2.5µM gibberellic acid produce longest microshoots (Ndirigw *et al*., 2014).

For the shoot multiplication 1.0mg/l GA_3 was found to be effective condition for multiplication of shoot tip, which gave average number of shoot 1.3 greater than other concentrations of medium. Shoot tips treated with MS supplemented with gibberellic acid were not greenish they were pale yellowish shoot without root and leaf much smaller. Similarly gibberellic acid gave negative result on the germination of *Galax urceolata* seed, which reduce the germination 49% to 30% (Yang *et al*., 2013).

5.1.2 Low hormone treatment

In vitro propagation of *Dendrobium crepidatum* was carried out using single shoot tip explants. The average length of explants (shoot tips) of 20 week was (0.2-0.3cm) completely differentiated into leaf and number of leaf was (1-2) during the period of inoculation.

In vitro Shoot tips were sub culture on MS medium alone and MS medium supplemented with different hormonal concentration of BAP (0.1- 1mg/l) and NAA (0.1- 0.2) mg/l (**Table-2**). Each single explants shows significant regeneration capacity depending up on the plant growth regulators. In the present study, primary shoot regenerated from shoot tip was first observed on MS medium supplement with BAP 1mg/l and 0.2mg/l NAA, gave first shoot within 2 weeks of inoculation but the maximum number of the shoot (2.11) was observed on MS medium supplement with BAP 1mg/l and 0.1mg/l NAA. The result was followed by BAP (1.0mg/l) and (0.2mg/l) NAA, the average value was 2.0 and it was followed by BAP (0.5mg/l) and NAA (0.1mg/l), whose average value was 1.94. The result was also supported by previous several researchers(1mg/l) BAP and(0.5 mg/l) picloram was the effective condition for *Dendrobium fimbriatum* (Kabir *et al.*,2013), similarly (Shrestha, 2005) found that multiple number of shoot on BAP (1mg/l) on *coelogyne ovalis*, (Paudel and Pant , 2010) also found MS medium with BAP 1mg/l was favorable for *Esmeraulda clarkei*. (Nagarju *et al*., 2004) reported that 0.5mg/l BAP was effective medium for multiplication of *Cymbidium* and *Cattleya* species.

In present study MS medium supplemented with BAP 1mg/l and 0.1mg/l NAA found best medium for the greenish healthy leaves. For the root inductionMS medium supplemented with BAP 0.2mg/l and 0.2mg/l NAA found the best medium among the various hormonal treatments.

Present study showed that higher hormonal concentration differentiated the explants into plantlets, while the lower hormone regenerated callus. The callus was yellow greenish with small shoot tip like appearance and very few were regenerated after 16 weeks. In orchid callus regenerated was found in protocorm segment on orchid *Cymbidium elegaens* by (Pant and Pradhan, 2010).

5. 1.3 High Hormone Treatment

In vitro propagation of *Dendrobium crepidatum* was carried out using single micro shoot of (0.3-0.5cm), cultured on MS medium supplemented with different hormonal combination of BAP (0.5- 2mg/l) and NAA (0.5-1) mg/l (**Table 3**). Among the hormonal treatment MS medium supplemented with BAP (2mg/l) and NAA (0.5 mg/l) found the effective medium for shoot multiplication containing average shoot number 2.99 with healthy greenish leaf. It was also the same medium for production of first shoot within 15 days of culture. The result was followed by MS medium supplemented with BAP (1.5 mg/l) and NAA (0.5mg/l) whose average shoot number was 2.19 with highest shoot length. This result was supported by Kosir *et al*., (2004) who repored that MS medium supplemented with BAP (2mg/l) and NAA (0.5mg/l) condition was effective for multiplication in *Phalaenopsis* species, similarly Bhadra and Hossin, (2003) also recorded BAP 2mg/l is suitable medium for multiplication of *Geodorum densiflorum*. Asghar *et al*., (2011) also reported hormonal condition BAP 2mg/l was the best medium for the *Dendrobium nobile*. Sunitibala and Kisor, (2009) also achieve good result of shoot multiplication on MS medium supplement with BAP (2mg/l) and NAA (1mg/l) in *Dendrobium transparens* .

Increase in the concentration of hormone showed promoting effect on number of shoot and length of shoot up to certain level in comparison to MS basal medium. Healthy and large number of shoot as well as number of leaf was found on the high concentration of BAP treatment hormonal condition and large number and length of root was observed on NAA. This was supported by Yasugi et al., (1994) on *Dendrobium* species.

Number of root observed on the MS medium supplemented with BAP (0.5mg/l) and NAA (1mg/l) was larger and it was followed by NAA (1mg/l) hormonal combination. Similarly highest length of root also observed on MS supplemented with NAA (1mg/l) which was followed by the MS medium supplemented with BAP (0.5mg/l) and NAA (1mg/l). Basker and Narmatha, (2006) also obtained same result on development of healthy root on BAP (1mg/l) and NAA (2mg/l) gave large and good length of root.

5.1.4 High hormone with Coconut water treatment

In vitro propagation of *Dendrobium crepidatum* was successfully carried out using single shoot tip (0.3-0.5cm) obtained from *in vitro* culture of seed. The shoot was introduced in high hormonal condition where good multiplication was observed in previous work. The concentration of BAP was varied to achieve best result in shoot multiplication but the concentration on NAA was constant. Coconut water 5% and 10% was supplemented on BAP (1, 1.5, 2 mg/l) with NAA 0.5mg/l. Best result for multiplication of shoot was found on coconut water 10% with 2mg/l BAP and 0.5mg/l NAA. The average shoot number was 3.08 within 16weeks of culture. It was followed by MS medium supplemented with BAP (1.5mg/l), NAA (0.5mg/l) and 10% coconut water for the shoot multiplication with average number of shoot tips 2.53. Similarly highest length of shoot and highest length of leaf was found on MS medium supplemented with1mg/l BAP, 0.5NAA and 10% coconut water (**Table-4**). From the present study it showed that multiplication of shoot tips was good in all hormonal medium supplements with 5% and 10% coconut water. But it showed that development of length of shoot, number of leaf and length of leaf was found more effective on 10% coconut water treatment as compare to 5% coconut water. Ali *et al*., (2011) reported that higher concentration of coconut water (15%) influence 90% of seed germination in *Dendrobium tetrachromum.*

The present study showed that 5% coconut water treatment gave large number of shoot like but they were not completely differentiated with clear shoot like appearance (greenish protocorm like with small leaf). Shoot tips sub cultured on MS medium supplement with Coconut water 10% found the effective for the induction of large number of healthy and greenish shoot, shoot length and leaf number. Several researchers proved that 15% coconut water supplement with hormonal treatment gave good shoot induction in *Dendrobium macrostachyum* (Pyati *et al*., 2002). Similarly large and good elongation of shoot found on 100ml/l coconut water on *Dendrobium nobile* treatment with BAP 1.5mg/l found best shoot elongation, (Asghar *et al*., 2011) they reported coconut water is effective for the shoot multiplication as well as shoot elongation.

5.1.5 Extraction of DNA

In vivo (wild) grown young and healthy leaf of Dendrobium *crepidatum* and small shoots obtained from tissue culture were material was used as the plant sample for DNA extraction. Extraction of genomic DNA of *in vivo* and *in vitro* plant was carried out by using plant DNA extraction Kit (Promega). Kit was effective means of production of high amount of DNA (50ng) which was found by using spectrophotometer and electrophoresis in 1% agarose gel. Thus the obtained DNA was stored at -4ºC and directly used for PCR reaction.

5.1.6 RAPD – PCR of *In vitro* and *In vivo* species

Molecular marker has been used for evaluating the genetic variation within the species which may affected by environmental factors. Random amplified polymorphic DNA is easy and effective process for detection of polymorphism. This technique is based on PCR amplified of random DNA segment by using small fragment of oligonucleotide (-10bp) primer. RAPD is useful tools for identify genetic homogeneity and variation among population and within the same population. Soliman *et al*., (2012) used RAPD marker for detection of genetic stability on the same species of *Moricandia nitens* obtained from tissue culture and *in vivo* mother plant. Similarly (Saha *et al*., 2011) also evaluated the genetic fidelity on in vitro propagated *Ocimum gratissimum*. Pathak and Jaroti, (2012) found genetic variation within eight species of *Dendrobium* by using five RAPD primers and four ISSR markers. Likely Nosrati *et al*., (2012) also detected genetic variation in population of *Capparis spinosa* by RAPD markers.

The present work showed that good DNA amplification was found on G17 primer which produce 58 scorable band with single polymorphic band and 2 absence band on DNA achieved from callus regenerating shoots (*in vitro*), scorable band shows best homogeneity with compare to mother plant (*in vivo*). The result was supported by Saha *et al*., (2012) who found not polymorphism that *in vitro* and *in vivo* (mother) of *Ocimum gratissimum* , Soliman *et al*., (2012) also found the same result and reported genetic stability on *in vitro* and *in vivo* plant *Moricandia nitens*. Effat and Mabrouk, (2000) found variation on callus regenerated plant and mother plant of potato. Primer G18 gave 36 number of scorable band and G10 gave 50 bands with single

polymorphic band and 3 missing band. G18 primer also resulted the variation with the mother (*in vivo*) and callus regenerating shoots and other reaming (normal shoots obtained from tissue culture) *in vitro* were stable with mother (*in vivo*). Larger number of polymorphism was detected by G10 primer which gave three number of polymorphism band with callus regenerated sample. Remaining primer G04 produced lowest number of bands i.e. 12 which amplified was not good as compare to other primers which had used. It did not give genetic polymorphism with mother sample and callus formation shoot tips.

From the present study it was found that differential hormonal treatment shoots which were used for genetic test gave genetic homogeneity to compare mother sample but the callus regeneration shoots which were not totally differentiated with perfect leaf and shoot does not gave genetic homogeneity with mother plant. Callus affect the genetic makeup of species which may be the somaclonal variation, Mahmoud *et al*., (1998) detect 20% Polymorphism on garlic by using 15 RAPD primers. Ruben *et al*., (2010), reported that RAPD technology was effective for analyzing the genetic stability in among regenerated plant of *Centaurea ultreia*. Similarly result supported Latto *et al*., (2006) and suggest RAPD the best tool for analyze somaclonal variation and also by Khosravi *et al., (*2009) on *Dendrobium* which was treated with colchicines.

CHAPTER SIX

6.1 Conclusion

Shoot multiplication of *Dendrobium crepidatum* was carried out on MS basal medium and MS medium supplemented with different hormonal treatment of BAP and NAA, Gibberellic acid alone and coconut water with hormonal supplement of BAP and NAA. Regeneration potential of shoot tips depends on the growth regulators which gives significant shoot multiplication. MS medium supplement with Gibberellic acid 1mg/l GA_3 found the best medium for shoot multiplication and 0.5mg/l GA_3 found shoot elongation. In lower hormonal concentration MS medium supplement with BAP (1mg/l) and NAA (0.1mg/l) found the most effective for shoot multiplication but which was not satisfactory thus again subculture on high hormonal concentration where MS medium supplemented with BAP (2mg/l) and NAA(0.5mg/l) found the most effective medium for shoot multiplication. MS medium supplemented with 2mg/l BAP, 0.5mg/l NAA and 10% coconut water was found to be the most prominent result for the *Dendrobium crepidatum* to achieve good number, greenish and healthy shoot. It is found that this could be standardized protocol for mass propagation of this medicinally important and threatened orchid *Dendrobium crepidatum* and will significantly contribute to conservation as well meet the commercial demand of orchid.

Normal shoots regenerated from different hormonal growth are genetically similar with the mother analyzed by RAPD primer but variation achieved on callus regenerated shoots which may cause by somaclonal variation. Thus genetic homogeneity with *in vivo* and *in vitro* observed and it can say that tissue culture technique is important technique for production of large number of clone of threatened and medicinal orchid for their *ex-situ* and germplasm preservation.

CHAPTER SEVEN

7.1 Recommendation

From the above study following recommendation are made-

- The mass propagation using shoot tip culture can be started in commercial scale to conserve the orchid so that it is lightens the pressure on nature and also upgrade economic status by commercial cultivation of such horticultural important orchid.
- *In vitro* techniques are best method for preservation of germ of nature without disturbing the genetic character of species. So method should be promoted for conservation of rare and endangered plant species.
- Somaclonal variation product may be fruitful for the horticulture to achieve new character in the species which may beneficial in floriculture. Thus some work should be done for production of ideal character on commercial plant.
- Tissue culture laboratory of CDB should be well equipped and facilities to carry out the research work more efficient in molecular.

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