Micropropagation and Assessment of Genetic Homogeneity in *Dendrobium chryseum*

A Dissertation submitted for the partial fulfillment of the requirement of Master's Degree in Botany

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RECOMMENDATION

This is to certify that the dissertation entitled "**Micropropagation and Assessment of Genetic Homogeneity in *Dendrobium chryseum***" submitted by Ms. Manju Kanu Baniya for the partial fulfillment of the requirement of Master's Degree in Botany has been carried out under my supervision and guidance. The entire work is based on the information collected by her in the laboratory and the results of this work has not been submitted for any other degree.

Therefore, I recommend this dissertation work to be accepted as a partial fulfillment of Master's Degree of Science Botany (Plant Biotechnology Unit)

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LETTER OF APPROVAL

The dissertation work submitted by Ms. Manju Kanu Baniya entitled "**Micropropagation and Assessment of Genetic Homogeneity in *Dendrobium chryseum*"** has been accepted for the partial fulfillment of requirement for Masters in Science Botany (Plant Biotechnology Unit).

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ABSTRACT

Dendrobium chryseum, an epiphytic orchid has high horticultural and medicinal value. The present study was carried out to develop the efficient propagation technique of *D. chryseum,* from *in vitro* culture microshoots and to evaluate the genetic homogeneity between *in vitro* regenerated plantlets using a molecular markers system i.e., RAPD and ISSR. In present investigation, *in vitro* raised micro shoots were used as explants (approximately 0.5-1 cm) induced from protocorm on half strength MS media fortified with 2 mg/L of Kn and 10% CW. Different strength of agar solidified MS medium with or without fungal elicitors [*Coniochaeta africana* (R3) and *Coniochaeta dendrobiicola* (R7)], adenine sulphate, coconut water, and hormones like BAP (6-Benzyl Amino Purine), NAA (α-Naphthalene Acetic Acid), IAA (Indole-3-Acetic Acid), Kn (Knetin) and IBA (Indole-3-Butyric Acid) were used for induction of shoots and roots. Young, healthy leaves derived from wild plants (same plant capsule was used for *in vitro* culture) and *in vitro* raised plantlets were used as explants for DNA isolation**.** Among the tested medium, the best condition for *D. chryseum* for shoot number was on full strength MS medium fortified with 15% CW (6.25 ± 0.50) per microshoot which is followed by half strength MS medium fortified with fungal elicitor 0.5% R3 (5.75 ± 0.50) per microshoot. Similarly the most suitable medium for *D. chryseum* for root number was on full strength MS medium fortified with 15% CW (9.75 ± 0.50) per shoot which is followed by half strength MS medium fortified with fungal elicitor 0.5% R3 (8.25 ± 0.50) per shoot. The most appropriate medium for shoot length of for *D. chryseum* was on full strength MS medium fortified with 15% CW (6.7 ± 0.14 cm) which is followed by full strength MS medium fortified with fungal elicitor 1% R3 (5.37 ± 0.15cm). Similarly the most appropriate medium for root length of for *D. chryseum* was on full strength MS medium fortified with 15% CW (6.3 ± 0.11cm) which is followed by full strength MS medium fortified with fungal elicitor 1% R3 (5.55 ± 0.20cm). Similarly high degree genetic homogeneity was found among and within the mother and *in vitro* regenerated plantlets of *D. chryseum* analyzed by using RAPD and ISSR markers. Hence, the present research showed no genomic alternation in regenerated plantlets supporting the efficiency of the protocol for obtaining clonally stable true-to-type plantlets and conservation of a threatened medicinal orchid.

ABBERVIATION AND ACRONYMS

°C	Degree Celsius
Mm	Micromolar
2,4-D	2,4- Dichlorophenoxyacetic acid
asl	Above sea level
Ads	Adenine sulphate
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
BA	Benzyl Adenine
BAP	6-Benzylaminopurine
CDB	Central Department of Botany
CITES	Convention on of International Trade in Endangered Species of Wild Fauna and Fauna
CW	Coconut water
cm	Centimeter
CTAB	Hexadecyltrimethyl Ammonium Bromide
Df	Degrees of freedom
DNA	Deoxyribonucleic acid
DPR	Department of Plant Resources
DSO	Direct shoot organogenesis
EDTA	Ethylene Diamino Tetra Acetate
et al.	and others
etc	Etcetera
eg	as an example
Fig.	Figure
g	Gram
GA3	Gibberellic acid
HCl	Hydrochloric acid
Hrs	Hours
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IRAP	Inter-Retrotransposon Amplified Polymorphism
ISO	Indirect shoot organogenesis

ISSR	Inter Simple Sequence Repeats
IUCN	International Union for Conservation of Nature
Kn	Kinetin
Lindl.	John Lindley
mg	Milligram
ml	Milliliter
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
PCR	Polymerase Chain Reaction
PIC	Polymorphic Information Content
PLBs	Protocorm-like bodies
ppm	Parts per million
psi	Pound per square inch
R3	*Coniochaeta Africana*
R7	*Coniochaeta Dendrobiicola*
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
Rp	Resolving power
Rpm	Revolutions per minute
RT	Room temperature
Sig.	Significance level
ScoT	Start Codon Targeted
SPAR	Single Primer Amplification Reaction
SPSS	Statistical Package for Social Sciences
SSR	Simple Sequence Repeats
TDZ	Thidiazuron
UV	Ultra violet
V	Volt
Vol.	Volume
VW	Vacin and Went

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CHAPTER I

INTRODUCTION

1.1 Background

Orchids one of the largest and successful plant groups of monocotyledons belonging to the second largest family Orchidaceae which shows a floral diversity throughout the world in all continents except Antarctica and their greatest presence occurs in tropical and sub-tropical regions (Tsering *et al.,* 2017). The name 'Orchid' is derived from a Greek word 'Orkhis' which was firstly coined by Theophrastus "The father of Botany" which literally means testicles simulating the morphology of underground tubers of the orchid plant (Steward and Griffith, 1995).

Orchids consists of unique, fascinating and gorgeous flowers that ranges a diversity of colors, sizes, form and shapes that make them an order of royalty in the world of ornamental flower (Kalimuthu *et al.,* 2006). Orchids are one of the most pampered plants and occupy a top position amongst all the flowering plants valued for cut flower and potted plants, fetching a very high price in the international market (Bhattacharyya *et al.,* 2017). The world consumption of orchids was reported to be valued at more than $500 million in 2000 (Hew and Yong, 2004; Wang, 2004).

Apart from economic importance i.e., ornamental value, orchids are also known for their medicinal importance especially in the traditional systems of medicine. It is believed that the Chinese were the first to cultivate and also to describe orchids with respect to nature, beauty and the basic botanical aspects but almost certainly they were also the first to describe orchids for their medicinal use (Bulpitt, 2005). Orchids are also ingredient of Indian Ayurveda which is based on a group of 8 medicinal plants called the '*Ashtvarga'*. Out of these, four plants are orchids namely, four species Jivak (*Malaxis muscifera*), Rishbhak (*Malaxis acuminata*), Ridhi (*Habenaria intermedia*) and Vridhi (*Habenaria edgeworthii).*They were known to impart Jeevaniya (Vitality), Vayashapan (revival of youthful conditions) body nourishment and antioxidant effects (Arora *et al.,* 2017). Orchids are also used in pharmacological treatments as anti-inflammatory, anti- viral, anti- carcinogenic, anti- convulsive diuretic, neuro- protective , anti- ageing, wound healing, antitumor and anti-

rheumatic (Pant, 2013) Due to bioactive compounds of orchids their demand in pharmaceutical industries and cosmetic industries are high for the drug development of many problematic diseases at present(Pant *et al.,* 2016).

A recent tentative checklist indicates nearly 25,500 known orchids species in the world (Govaerts *et al.,* 2003) and the genus *Dendrobium* is one of the largest genera in Orchidaceae, comprising approximately 1,500-2000 species distributed in the tropical and subtropical regions of Asia and North Australia (Hou *et al.,* 2012, 2017) having both ornamentally and medicinally value (Da Silva *et al.,* 2015). As early as 200 BC the Chinese pharmacopoeia - "the Sang Nung Pen Tsao Ching", mentions *Dendrobium* as a source of tonic, astringent, analgesic, anti-inflammatory substances (Singh *et al.,* 2007). *Dendrobium* species have been used as medicinal herbs in different countries for centuries. The fresh or dried stem of many *Dendrobium* species are regarded as “superior grade” tonic for its traditional properties of nourishing the kidney, moisturizing the lung, benefiting the stomach, promoting the production of body fluids and clearing heat (Deng *et al.,*2001; Zhang *et al.,*2006). The pharmacological activities examined include antioxidant, anti-inflammatory, immunomodulatory, antitumor, antimicrobial/antifungal, antimutagenic activities, and antiplatelet aggregation activities (Song *et al.,* 2012; Lau *et al.,* 2011; Hwang *et al.,* 2010; Yang *et al.,* 2014; Pan *et al.,* 2014; Chen *et al.,* 2008, 2008a, 2008b; Paudel and Pant, 2017; Paudel *et al.,* 2019).

In Nepal, 450 species belonging to 107 genera of orchids were recorded (Rajbhandari, 2014). Pant and Raskoti, (2013) have reported 98 species of orchids having medicinal value in Nepal but now, more than 100 species of Nepalese orchids are recorded as medicinal (Rajbhandari, 2014). More than 80 species of Nepalese orchids are ornamental (Rajbhandari, 2014).Wild Nepalese orchids are popularly known by their vernacular names: Sunakhari, Sungava, Jiwanti, Bankera, Thur or Thurjo.

The latest update of the IUCN Global Red list (IUCN, 2020), released in July 2020, included 88 new assessments for orchids, and this brings the total number of orchids species that have been assessed to 1641 (nearly 6 % of orchids). Five of these are Extinct and 747 are threatened: 197 Critically Endangered (CR), 355 Endangered (EN) and 195 Vulnerable (VU). Eightyseven and 575 species have been assessed as Near Threatened (NT) and Least Concern (LC), respectively. Accounting for the 227

Data Deficient (DD) species, for which threat status is unknown. Also, the whole family Orchidaceae is included in Appendix-II of Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES). However, Paphilopedilum is listed in the Appendix-I (Shrestha *et al.,* 2018). Due to heavy rate of deforestation and anthropogenic pressures, the natural populations of many splendid orchids are depleting fast from their natural habitats at an alarming rate and their conservation is becoming a matter of global concern.

1.2. Tissue culture and its importance in orchids

Plant tissue culture is the science or art of growing plant cells, tissues or organs isolated from the mother plant on artificial media (Kyesmu *et al.,* 2004). Plant tissue culture was first proposed by the German Botanist Golliob Haberlandt in 1902. He is regarded as the father of plant tissues culture. In 1950s tissue culture was first used on large scale by the orchid industry (Gaikwad *et al.,* 2017).

Tissue culture is one of the most important part of applied biotechnology. A whole plant can be regenerated from a small tissue or plant cells in a suitable culture medium under controlled environment. The plantlets thus produced are called tissue culture raised plants. “Tissue and organism are grown in the artificial prepared nutrient medium static or liquid under aseptic conditions” (Gaikwad *et al.,* 2017).

Disease elimination, quick international distribution of genetic resources, germplasm conservation and reduction in quarantine requirements, time and space of regenerations are the positive gains of plant tissue culture practices. Sterile condition is a pertinent precaution necessary in the preparation of a tissue culture media. Crop improvement can be done through any of the plant tissue culture technique such as meristem culture, shoot tip or shoot culture, nodal culture, protoplast culture and somatic embryogenesis (Ikenganyia *et al.,* 2017).

Since long tissue culture techniques have been used for propagation of rare, endangered and threatened orchids (Arditti and Ernst, 1993). A single capsule contains millions of microscopic seeds. If all these had the ability to develop onto plants, entire world would have been full of orchids (Singh, 1986) but the irony is that the seeds of orchid have less than 3% germination because of particular fungal

requirement (Papenfus *et al.,*2016). Besides, a skinny cuticle around a small embryo is not adequate to protect it against desiccation (Lee *et al.,* 2006).

Multiplication by means of vegetative propagation in natural habitat is extremely slow and time consuming to generate large quantity of orchids replica (Pradhan and Pant, 2009). Its slow growing properties hardly fulfill the need of people, market and various pharmaceutical companies (Basker and Bai, 2010). Long maturation process even reduces its market value. *In vitro* methodology can reduce the length of time needed for germination and large scale multiplication (Pradhan and Pant, 2009). Hence, tissue culture is the alternative for mass scale propagation and conservation of rare, endangered and threatened orchids (Pant and Gurung, 2005).

In plant tissue culture techniques, protocol are developed according to the need of the plant growth. Modified forms of media and different chemical compounds, gelling agent, organic and inorganic salts and cofactors are used to propagate orchids *in vitro* (Deb, 2008; Hossain, 2009; Pant and Thapa, 2012; Pradhan *et al.,* 2014, 2016). The recent development in the tissue culture is the use of elicitors in culture medium have opened a new avenue for the production of secondary metabolites compounds and induction of plants in *in vitro* condition (Naik and Al-Khayri, 2016). Elicitors can be defines as signaling molecules triggering the formation of secondary metabolites in cell cultures by inducing plant defence, hypersensitive response and/or pathogenesis related proteins. Among different types of elicitors, fungal elicitors have been most widely studied for the synthesis of commercially important compounds and induction of plants in plant tissue culture system (Baldi *et al.,* 2009).

Fungal elicitors are a group of extractions and mixtures that include fungal organisms, filtrate concentrate, extracts of mycelia and filtrate, soluble components of fungal mycelia with high temperature treatment, hydrolytic products of cell walls, peptides and proteins (Smith *et al.,* 1996). Fungal elicitors stimulate multiplication of plant cell and secondary metabolite production in orchids (Zhang *et al.,* 2008). Fungal elicitors of orchid mycorrhiza were effective in promoting the development of protocorms and seedling growth of orchids (Guo and Xu, 1991; Gao and Guo, 2001; Chen *et al.,* 2008; Dong *et al.,* 2008; Zhao and Liu, 2008). Although the mechanism is still unclear, the effects of fungal elicitors on stimulating seedling growth were confirmed. Endophytic fungi like *Mycenia* sp, *Tunasinella* sp, *Epulorhiza and P. indica* were

isolated from different parts of medicinal orchids plays significant role in germination and overall growth of plant by elicitor (Liu *et al.,* 2010).

Mass propagation of medicinal orchids using *in vitro* culture technique has been successfully reported in many orchids. Some of them are *Dendrobium chrysanthum* (Vij and Pathak, 1989), *Dendrobile formosum* (Nasiruddin *et al.,* 2003), *Coelogyne Cristata* (Pant *et al.,* 2008), *Cymbidium mastersii* (Mohanty *et al.,* 2012), *Dendrobium candidum* (Zhao *et al.,* 2008), *Dendrobium densiflorum* (Pradhan and Pant, 2009), *Dendrobium transparens* (Sunitibala and Kishor, 2009) *Cymbidium elegans* (Pant and Pradhan, 2010), *Phaius tancarvilleae* (Pant and Shrestha, 2011), *Dendrobium primulinum* (Pant and Thapa, 2012), *Cymbidium bicolor* and *Dendrobium lituiflorum* (Vyas *et al.,* 2012), *Esmeralda clarkei* (Paudel and Pant, 2012), *Dendrobium thyrisumflorum* (Bharrachary *et al.,* 2015), *Dendrobium chryseum* (Maharjan *et al.,* 2020). However, for large-scale propagation, efficiency of propagation methods along with genetic stability of the regenerated plants is of paramount importance for many orchids which is yet to be developed (Haisel *et al.,* 2001).

1.3. Molecular characterization and its importance in orchids

In order to conserve orchids, plant tissue culture techniques have been successfully applied for their clonal propagation and conservation (Tandon and Kumaria, 1998). However, for large-scale propagation, efficiency of propagation methods along with genetic stability of the regenerated plants is of paramount importance (Haisel *et al.,* 2001). Reports have shown that the regenerated plants might not always be clonal copies of their mother plant when passed through micropropagation pathways (Devi *et al.,* 2014). The presence of cryptic genetic defects occurring due to somaclonal variations can deregulate the broader utility of the *in vitro* propagation system (Salvi *et al.,* 2001). The occurrence of clonal variability is due to various causes of which explant source and types of plant growth regulators (PGRs) used plays a pivotal role (Devi *et al.,* 2014). Thus understanding the reason and mechanisms involved in the development of clonal variability at an early stage is important for controlling the resultant impact of somaclonal variations.

Keeping a perspective view of the various advantages, molecular markers are considered much more efficient tools in detection of clonal variability, primarily as

they are not influenced by any environmental factors and also because of their high reproducibility. Traditionally, various conventional molecular markers like random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), and simple sequence repeats (SSR) are used extensively in the assessment of clonal fidelity in a wide range of plants species including orchids (Bhattacharyya *et. al.,* 2014, 2014a, 2015; Devi *et al.,* 2014). Moreover it is advocated to use more than one molecular marker in order to evaluate clonal fidelity of tissue culture-raised plants (Palombi and Damiano, 2002).

RAPD technique is simple, easy, cost effective, does not require sequence information and a small amount of DNA sample is sufficient to yield quick result (Micheli *et al.,* 1994; Pathak and Jaroli, 2012). The marker is highly used for assessing genetic diversity of plant varieties but their producibility is affected by several factors (Kate-ngam and Lakote, 2008). RAPD technique is based on PCR reaction, require single short arbitrary oligonucleotides primer which anneals with multiple locations on genomic DNA and amplified randomly (Kumar and Gurusubramanian, 2011). RAPD is one of the effective tools for the molecular studies 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). ISSR primers are designed from simple sequence repeats (SSR). SSR are highly scattered throughout the eukaryotic genomes. They use a single 16-20 bp long primer composed of a repeated sequence anchored at the 3‘ or 5‘ end of 2-4 arbitrary nucleotides (Zietkiewicz *et al.,* 1994; Javan *et al.,* 2012). ISSR markers have been used to generate gene-specific or trait-specific markers and for investigating variability at intraspecific level, genetic diversity studies and cultivar identification (Khattab *et al.,* 2014). They also requires small amount of DNA and doesn‘t require information on DNA sequence. ISSR markers have higher reproducibility than RAPD.

Thus, in the present research, RAPD and ISSR were used to evaluate the clonal fidelity of *D. chryseum* regenerates, where the former detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence and the latter permits detection of polymorphism in inter-microsatellite loci using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Parab and Krishnan, 2008).

1.4. Plant Description

***Dendrobium chryseum* Rolfe.**

Synonyms:-

Aporum rivesii (Gagnepain) Rauschert; Callista aurantiaca Kuntze; Dendrobium aurantiacum H. G. Reichenbach (1887), not (F. Mueller) F. Mueller (1870); D. aurantiacum var. zhaojuense (S. C. Sun & L. G. Xu) Z. H. Tsi; D. chryseum var. bulangense G. X. Ma & J. Xu; D. clavatum Wallich ex Lindley var. aurantiacum Tang & F. T. Wang; D. flaviflorum Hayata; D. rivesii Gagnepain; D. rolfei A. D. Hawkes & A. H. Heller; D. tibeticum Schlechter; D. zhaojuense S. C. Sun & L. G. Xu.

Common name: "The Yellow Flowered Dendrobium"

Figure 1: Image of *Dendrobium chryseum.* (source:-www.google.com)

Status of occurrence: CITIES (Appendix II)

Habit: Epiphytic herb

Habitat: Shady places, tree trunks

Flowering time: May–June

Distribution: C Nepal (1000–2100 m), Bhutan, China (C Sichuan-Ebian, Emei Shan, Taiwan, NW to SE Yunnan), NE India (Assam, Sikkim), Myanmar, Thailand, Vietnam (Sinh *et al.,* 2016)

Chromosome number: 2n =38 (Jones *et al.,* 1982)

Taxonomic description: Stems cylindric, usually 25-35 cm, slender, 2-4 mm in diam., unbranched, with many nodes, internodes 2.5-4 cm, pale yellow or yellowish brown when dry. Leaves linear or narrowly oblong, 8-10 × 0.4-1.4 cm, leathery, base sheathing, apex obtuse and emarginated or sometimes subacute and obliquely hooked. Inflorescence lateral on old leafless stem, ca. 1 cm, usually 1 or 2(or 3)-flowered; peduncle sub erect, ca. 0.5 cm; basal sheaths 3 or 4, overlapping, pale white, cupular or tubular, papery, basal ones short, distally elongate, 5-20 mm; floral bracts pale white, cymbiform, 12-13 × ca. 5 mm, membranous, apex obtuse. Flowers spreading, ca. 6.5 cm wide, concolorous yellow, center a darker shade, occasionally with large

purple spots on sepals and petals and a purple spot on lip disk, inner surface of lip sometimes with several red stripes.

Traditional medicinal and others use of *Dendrobium chryseum* Rolfe

Dendrobium chryseum Rolfe. is valued greatly for its attractive yellow flowers and is an immensely popular ornamental orchid and it's very important in Chinese herb and remedies where it is used for medicine for its antipyretic, eye benefitting and immune-modulatory effects (Yang *et al.,* 2007). Polysaccharides, alkaloids, dendrobine and bibenzy (scoparone and gigantol) are active compounds of the *D. chryseum* (Gu *et al.,* 2017), where the extract of the dried stem of *D. Chryseum* contain nine chemical compounds that are coumarin (1), alkyl 4´- hydroxy-trans-cinnamates (2), campesterol (3), stigmasterol (4), β-sitosterol (5), aliphatic alcohols (6), alkyl trans-ferulates (7), stigmast-4-en-3-one (8), β-aliphatic acids (9) (Chang *et al.,* 2001). *D.chryseum* consists of gigantol which has inhibitory properties against the growth of human leukemia cell lines K562 and HL-60, human lung adenocarcinoma A549, human hepatoma BEL-7402, and human stomach cancer SGC-7901 (Chen *et al.,* 2008a.). Pharmacological experiments showed that it possessed tumor inhibitory and blood glucose reducing effect and antioxidant activity in vivo (Yang *et al.,* 2007; Ying *et al.,* 2009). *D. chryseum* have the medicinal values and they could be used in the market of medicinal *Dendrobium* (Gu *et al.,* 2017)

In spite of the fact that most species of Orchidaceae including *Dendrobium chryseum* are listed in Appendix-II of CITES where the collection from the wild is completely prohibited, still illegal harvesting of useful orchids from the wild continues and *Dendrobium chryseum* is no exception (Shrestha *et al.,* 2018). This has resulted in a decrease in population size in natural habitats, thereby makng the species rare. *D. chryseum* is localized and species is threatened due to deforestation and overexploitation for ornamental purposes (Joshi *et al.,* 2017).

1.5. Research Questions

1. Does the true to type *in vitro* propagation of *Dendrobium chryseum* is possible?
2. Do fungal elicitors have role in growth and development of *Dendrobium chryseum* in *in vitro* culture?

3. What is the percentage of genetic similarity in between *in vivo* and *in vitro* plantlets of *Dendrobium chryseum* ?

1.6. Research Objectives

Dendrobium chryseum, an epiphytic orchid which has high horticultural and medicinal value. Due to various anthropogenic pressures the natural populations of this important orchid species are presently facing threats of extinction. So, the present research work was carried out with the following objectives.

1.6.1. General Objectives

To establish a fast and cost effective regeneration protocol for *Dendrobium chryseum* with reproducible molecular detection for obtaining clonally stable true-to-type plantlets.

1.6.2. Specific objectives

1. To access the effect on *in vitro* plantlets development of *Dendrobium chryseum* among the following culture medium used like growth hormones (auxin and cytokinin), coconut water, fungal elicitors and adenine sulphate.

2. To assess the genetic stability of regenerated plants (*in vitro* plantlets) and *in vivo* plantlets (mother plant) of *Dendrobium chryseum* using random amplified polymorphic DNA (RAPD) and inter – simple sequence repeat (ISSR) markers.

1.7. Justification

Considering the rare and threatened status of the *Dendrobium chryseum*, the *in vitro* mass propagation is an alternative tool for its commercial production and conservation. This species is high in demand for commercial cultivation due its attractive flower and aesthetic value. This research can help to develop standard protocol that will be a fast and cost effective for *in vitro* mass propagation through different culture techniques with reproducible molecular detection system for obtaining clonally stable true-to-type plantlets. Thus, efficient plant regeneration system developed in this study will be useful for conservation and homogenous controlled mass production of this important orchid species which may help to meet

the present market demand. The present approach is novel and cost effective so it can be used for conservation and large scale homogenous *in vitro* production of other endangered orchids as well as other plants to meet their medicinal and industrial needs too.

Furthermore, an effort concerning the genetic stability of micropropagated *Dendrobium chryseum* orchids has been made for the first time.

CHAPTER II

LITERATURE REVIEW

2.1. Tissue culture research in *Dendrobium* species

There are a large number of studies dedicated to the tissue culture and micropropagation of *Dendrobium* from around the world. The explants most commonly used in micropropagation, derived from greenhouse-grown or *in vitro* *Dendrobium* plants, include nodal segments, *in vitro*-derived PLBs, shoot tips, protocorms, transverse thin cell layers (tTCLs) from protocorms and young stems, leaves, pseudobulbs, *in vitro* seedlings, axillary buds, and callus, the *in vitro*-derived material not requiring a disinfection step (Sagawa and Shoji, 1967; Kim *et al.,* 1970; Kukulczanka and Wojciechowska, 1982; Vij and Pathak, 1989; Gao *et al.,* 1994; Mei *et al.,* 2012; Hossain, 2013; Da Silva *et al.,* 2015)

Martin and Madassery (2006) found that half-strength Murashige and Skoog (MS) medium containing 6.97 mM kinetin (Kn) facilitated conversion of more than 90% PLBs to shoots, half-strength MS medium supplemented with 44.4 mM N6-benzyladenine (BAP) developed more than seven shoots per explant, half-strength MS medium with 2 g/l activated charcoal was the best for *in vitro* rooting of *Dendrobium* hybrids Sonia 17 and 28. Sharma *et al.,* (2007) reported that maximum number of shoots per explant (38.6±3.9) were obtained in a medium containing 2.0 mg/L BAP along with highest shoot elongation (2.9 ± 0 .3cm) in the same media and basal medium containing Knop salts with MS vitamins and MS iron salts without auxin favoured root initiation in the elongated shoots after 2 weeks of transfer and developed (5.3±0.4) number of roots per shoot of *Dendrobium microbulbon.* Zhao *et al.,* (2008) reported that highest frequency of callus induction (82%) from explants was found half strengrh MS basal medium with 8.8µM BAP, highest length of shoot (2.01±0.34 cm) and root number (4.15±0.98) per shoot were found in same media, half MS basal medium with 1.08µM NAA and highest root length (2.73±0.61cm) was found in half MS basal medium with 2.7µM NAA of *Dendrobium candidum.*

Sunitibala and Kishor (2009) reported that the combination of BAP (2 mg/L) and NAA (1 mg/L) showed development of highest shoot number (7.5±0.7) of shoots and highest number of roots per pseudobulb (9.8±0.63). However, development of

maximum mean root length (4.1±0.12 cm) was favored by 1 mg/L IBA of *Dendrobium transparens*. Maridass *et al.,* (2010) reported that the maximum shoot induction (15.78 ± 0.37mm) was observed on basal medium supplemented with BAP (0.5µM/l-1) and roots was developed better on MS basal media of *Dendrobium nanum.* Dutta *et al.,* (2011) reported the maximum seed germination of *Dendrobium aphyllum* (Roxb.) on MS basal medium. The PLBs of *D. aphyllum* showed better germination and developed into multiple roots and shoots, when supplemented with IAA & mixture of IAA & Kn compared to the media supplemented with Kn only. The highest average length of shoots & roots are found on MS supplemented with 50µg/100mL IAA and 50µg/100mL Kn (0.50 cm & 0.78 cm) respectively. Kabir *et al.,* (2013) reported the rate of elongation reached highest (7.56cm) of *Dendrobium fimbriatum* when the germinated seedlings were exposed to liquid MS containing 2.0 mg/L BAP and 0.01 mg/L IBA. Asghar *et al.,* (2011) reported that *Dendrobium nobile,* maximum number of shoots (4.33), was obtain at 2 mg/L BAP, while 1.5 mg/L of Kn exhibited the highest shoot length (4.18 cm) and the highest number (4.70) and length (3.47 cm) of roots was observed at 2 mg/L IBA.

Pant and Thapa (2012) found that the maximum numbers of shoots and its length were observed on MS medium fortified with BAP 1.5 mg/1 with an average value of 4.5 shoots per culture and 1.62 ± 0.10 cm respectively, MS medium with BAP (1.5 mg /L) and NAA (0.5 mg/L) were most effective for the shoot multiplication and the best rooting response was observed on MS medium with IAA(0.5 mg/L) with highest root number (3.5 ±0.21) per shoot and root length (1.18±0.05cm) of *Dendrobium primulinum* . Hossain, (2013) reported that the highest number of shoot buds (5.42 ± 0.15) and there elongation (3.35 ± 0.08cm) was on same media MS + 1.0 mg/L BAP + 1.0 mg/L NAA of *Dendrobium aggregatum* and half strength MS + 1.5% (w/v) sucrose + 0.5 mg/L IAA was proved to be effective for roots number (3.50 ± 0.16) per shoot and root length (3.65 ± 0.18cm). Pradhan *et al.,* (2013) reported that the maximum elongation of shoot (3.25cm) was observed on MS medium supplemented with BAP (0.5 mg/L) and NAA (0.5 mg/L), the maximum number of healthy shoot was observed on MS+ BAP (2 mg/L) + NAA (0.5 mg/L) (40.3 shoots/ culture).and the maximum number of roots (4.5 per culture) and root length (1.35cm) was found on MS+ IBA (1.5 mg/L) of *Dendrobium densiflorum.*

Rao and Barman (2014) reported that percentage of seed germination was maximum in MS (98± 0.48) % and highest secondary protocorms (21.25±0.63) were observed in MS medium containing 4.0 µM BAP. MS medium supplemented with 8µM IBA induced the maximum roots (6.84 ± 0.05) per shoot and highest root length (3.64 ± 0.05cm) was found on MS medium supplemented with 12µM IBA of *Dendrobium chrysanthum*. Nongdam and Tikendra (2014) reported that MS + 2mg/L BAP + 2 mg/L IAA produced highest seed germination percentage (98.1± 3.9), maximum seedling conversion (92.66 ± 3.2)% from germinated seeds was recorded in medium incorporated with 1.5mg/L Kn and 1.5mg/L IBA, The medium augmented with combination of 2.0mg/L Kn and 0.5mg/L NAA induced maximum leaf formation (4.0 ± 1.4) and also leaf production, and the highest root number (6.1 ±1.4) was recorded in medium supplemented with 3.0 mg/L Kn and 1.5mg/L NAA of in *Dendrobium chrysotoxum.* Bhattacharyya *et al.,* (2015) reported that the highest regeneration frequency (96 %) from explant along with an average of 17.7 shoots proliferating with a mean length of 3.5 cm was recorded in MS medium supplemented with 2 mg/L of TDZ and 0.5 mg/L of NAA. Highest rooting frequency of 85.2 % was reported in 2 mg/L IBA supplemented half-strength MS media with an average of 3.5 roots per shoot was produced with a mean length of 2.7 cm of *Dendrobium thyrsiflorum*. Sagaya and Divakar (2015) found that MS medium supplemented with 2mg/L of BAP+5mg/L NAA gave excellent growth for plantlet formation of *Dendrobium peguanum* and MS medium for fortified with 2mg/L of BAP+1.5mg/L of IAA was best for *in-vitro* rooting. Similarly MS medium supplemented with 1 mg/L BAP and 0.5 mg/L Picloram was proven to be the best for multiple shoot formation (4.35 shoots per explant) and average length of induced roots reached highest (5.2 cm) with the average number of root per shoot was 9.30 on the half strength MS medium supplemented with 1 mg/L IBA.

Maharjan *et al.,* (2020) reported the media that was best for shoot multiplication (18.75 ± 0.48 shoots per culture) was half strength MS medium fortified with 2.0 mg/L Kn and 10% CW, whereas the media that supported the most extended shoots (2 ± 0.20 cm) per culture and roots (4.5 ± 0.65) per culture was half strength MS medium fortified with 1.0mg/L GA3 and 10% CW The longest roots (1.28 ± 0.14 cm) were observed on half strength MS medium supplemented with 0.5 mg/L GA3 and 10% for *Dendrobium chryseum.*

Several studies have shown the importance of endophtyes in the germination of orchid seeds and the growth of protocorms, seedlings and adult plants. In a report Hou and Guo (2009) have identified fungal species, *Leptodontidium* from *Dendrobium nobile Lindl.*, have shown its role in plant growth and development. Similar type of study has been carried out by Chen *et al.,* (2012) in which they have isolated and identified endophytic fungus from seeds and roots of *D. nobile* and *D. chrysanthum.* They have also reported the role fungus in seed germination and development. Xu and Guo (1989) reported the association of two different species of fungi with *Gastrodia elata* at different stages of the plant life cycle, while Leake (1994) suggested that more than 100 species of orchids depend on endophytic fungi throughout their lifetime. *Rhizoctonia zeae* isolated from *Vanda coerulea* have showed it plays a significant role in promoting *in vitro* seed germination and seedling growth as well as in increasing the survival rate during acclimatization (Aggarwal *et al.,* 2012). Orchid seed germination and protocorms development is also dependent on the effects of *Piriformospora indica* (Varma *et al.,* 2013).

Gong *et al.,* (2018) reported that the fungal elicitor isolated from *Cymbidium georingii* promoted the germination of *Cymbidium georingii* seeds and its acclimatization. Shah *et al.,* (2019) reported that the endophytic fungi isolated from the roots of *Dendrobium moniliforme* showed significant growth-promoting effects on the protocorms stage of *Rhynchostylis retusa* that developed into a plantlet. Chand *et al.,* (2020) reported that the all the endophytic fungi isolated from *Vanda cristata* showed significant growth-promoting effects on the protocorms stage of *Cymbidium aloifolium* that developed into a plantlet. All the isolated endophytic fungi showed growth-promoting activity by auxin production, ammonia synthesis, and phosphate solubilization (Shah *et al.,* 2019; Chand *et al.,* 2020). A significantly higher number of roots and shoots, as well as increased root/shoot length when compared to the controls was found. It is found that different fungal elicitors increased the rate of growth and regulate the metabolism by secreting different chemical compounds which is not provided in growth medium (Yamaguchi and Huffaker, 2011). Patel *et al.,* (2013) also reported that fungal elicitors used in tissue culture causes enhancement of *in vitro* plant growth. Hou and Guo (2009) reported growth promoting effect of the dark septate endophyte, *Leptodontidium* on seedlings of *Dendrobium nobile* and found the heights of shoots, number of new buds, number of roots, stem diameters

and dry weights of fungal-colonized plantlets were all greater than those of uncolonized plantlets. Similarly, Warcup (1981) reported that endophytes such as *Sebacina vermifera, Tulasnella calospora, T. asymmetrica, T.cruciata, T. irregularis, T.violea* and *T. allantospora* are involved in seed germination in different orchid species

2.2 Analysis of genetic homogeneity in micropropagated orchids

Production of true-to-type plant is one of the most important goals in mass propagation of endangered plants like orchids because, during the *in vitro* development, plant tissue undergoes several morpho-physiological changes, genetic alterations with chromosome abnormality, doubling of genetic elements like aneuploidy and polyploidy which is caused by exogenous plant growth regulators (PGRs) and other intrinsic factors originated gene mutation (Saker *et al.,* 2000; Takagi *et al.,* 2011). Genetic instability or somaclonal variation of a genotype affects long-term preservation of germplasm *in vitro.* Molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSRs) and start codon targeted polymorphism (SCoT) were extensively used to evaluate genetic homogeneity of *in vitro* besides *ex vivo* plants (Milella *et al.,* 2011; Bhattacharyya and Kumaria 2014; Khilwani *et al*., 2016; Seth *et al.,* 2017). Genetically stable plants are important for clonal propagation, plant conservation studies, secondary metabolite production at *in vitro* and also for the preservation of genetic resources of rare and endangered plants. Thus, the analysis of genetic characters or stability of *in vitro* derived plants through molecular markers are invitetable (Sherif *et al.,* 2018). Clonal fidelity of in vitro regenerated plants is another aspect of plant propagation which is imperative for their commercial utilization. However, only few investigations exist on genetic fidelity of the *in vitro* regenerated orchids with special reference to dendrobes (Bhattacharyya *et al.,* 2014).

Ferreira *et al.,* (2006) found no variation in RAPD profiles among in *in vitro* clones of orchid hybrid *Dendrobium* Second Love *(D. Peace ×D. Awayuki)* derived through TDZ treatment. Giri *et al.,* (2012) found no variation in ISSR profiles among the *in vitro* regenerated plantlets of *Habenaria edgeworthii* (Terrestrial orchids) derived through 6-benzyladenine (BAP) and a-naphthalene acetic acid (NAA) treatments. But

Roy *et al.,* (2012) found 5.81% variation in RAPD profile among the *in vitro* regenerants of *Cymbidium giganteum* derived through thidiazuron (TDZ) treatment.

Samarfard *et al.,* (2013) reported that secondary PLBs of *Phalaenopsis gigantea* produced during sub-culturing process of chitosan treated liquid culture were genetically uniform and similar to mother plant through ISSR profiling, no somaclonal variation in *in vitro* raised PLBs. Bhattacharyya *et al.,* (2014a) revealed 97% of genetic fidelity in RAPD and SCoT profile among the regenerants of *Dendrobium nobile* derived through TDZ treatment. Bhattacharyya *et al.,* (2015) revealed 3.22 and 8 % clonal varibality within the direct shoot organogenesis (DSO) and indirect shoot organogenesis (ISO) of *Dendrobium thyrsiflorum* through SCoT markers. On the other hand, with ISSR marker a variability of 4.76 % was detected in the ISO derived plants and no variability within the DSO derived plants. A cumulative SPAR approach revealed 1.88 % variability within the DSO generated plants and 6.52 % variability amongst the ISO propagated plants. Bhattacharyya *et al.,* (2018) revealed a high degree of genetic homogenity in inter-retrotransposon amplified polymorphism (IRAP) and start codon targeted (SCoT) profie among the *in vitro* regenerated plantlets of *Ansellia africana* (Leopard orchid) derived through TDZ and NAA treatments. Sherif *et al.,* (2018) found that the homogeneity is comparatively higher in direct somatic embryo regenerated plants (94.22%) as compared to plants elevated from an indirect somatic embryo (93.05%) in ISSR profile of *in vitro* regenerated plantlets of *Anoectochilus elatus* derived through treatment with TDZ, morel vitamins and NAA. Tikendra *et al.,* (2019) found 95.2% monomorphism among the *in vitro* raised plant and mother plant source of *Dendrobium moschatum* using RAPD primers.

Globally, very few works has been carried out in genetic homogeneity of *in vitro* plantlets especially in orchids and no work related to this has been reported for *Dendrobium chryseum.* Thus, the analysis of genetic characters or stability of *in vitro* derived plants through molecular markers is invited for large *in vitro* production of genetic homogeneous plants which is important for conservation of this species.

CHAPTER III

MATERIALS AND METHODS

3.1. Materials

The explants used in the present research were *in vitro* micro shoots (0.5-1cm) of *Dendrobium chryseum* induced from capsule on half strength MS media fortified with 2 mg/L of Kn and 10%CW in Plant Biotechnology Laboratory of Central Department of Botany, TU, Kirtipur (Maharjan *et al.,*2020).

Approximately 0.5-1 cm *in vitro* raised micro shoots of *Dendrobium chryseum,* were selected as plant materials for induction of shoots and roots in fungal elicitors (*Coniochaeta africana* (R3) and *Coniochaeta Dendrobiicola* (R7)), in hormones like auxin or cytoknin alone and in combination and adenine sulphate containing media alone or with auxin or cytoknin. Young, healthy leaves derived from wild plants (same plant capsule was used for *in vitro* culture) and tissue cultured plants were used as explants for DNA isolation**.**

3.2. Methods

The methods applied for the *in vitro* micropropagation and test of their genetic homogeneity of *D. chryseum* are described under following headings:

3.2.1. *In vitro* propagation of *D. chryseum* plantlets:

The method for *in vitro* micropropagation is described as under:

3.2.1.1. Preparation of stock solution

3.2.1.1.1. Preparation of Murashige and Skoog's medium (MS)

The Murashige and Skoog, (1962) medium consist of macronutrients (stock A), micronutrients (stock B), iron source (stock C), vitamins (stock D), sucrose and agar (as gelling agent). The composition of the MS medium is as follows:

Table 1: Preparation of stock solution for MS medium

Stock A (Macronutrient)-10 X (g/l)

S.N.	Components	10X gm/l stock concentration	Composition of MS (final conc.) mg/L	Volume to be taken for 1 lit. Medium
1.	Potassium nitrate	19.0	1900	100ml
2.	Ammonium nitrate	16.5	1650	
3.	Calcium chloride	4.4	440	
4.	Magnesium Sulphate	3.7	370	
5.	Potassiumdihyrogen phosphate	1.7	170	

Stock B (micro-nutrient)-100X (mg/100 ml)

S N.	Components	100X mg/100ml stock con.	Composition of MS(final conc.) mg/L	Volume to be taken for 1 lit medium
1.	Boric acid	62	6.2	1ml
2.	Manganese sulphate	223	22.3	
3.	Zinc sulphate	86	8.6	
4.	Sodium molybdate	2.5	0.25	
5.	Cobalt sulphate	0.25	0.025	
6.	Copper sulphate	0.25	0.025	
7.	Potassium Iodide * (KI)	8.3	8.3	

*100ml stock solution of potassium Iodide should be made separately and used 1ml/l in MS media

Stock C (Iron Source)

S.N.	Component	(10 X)mg/100ml stock concentration	Composition of MS(Final conc.) mg/L	Volume to be taken for 1 lit. Medium
1.	Sodium ethylene diamine tetra acetate	37.3	37.3	10 ml
2.	Ferrous sulphate	27.8	27.8	

Stock D (Vitamin and amino acids)

S.N.	Component	(100X)mg/100ml stock concentration	Composition of MS (final conc.)	Volume to be taken for 1 lit medium
1.	Glycine	20	2.0	1ml
2.	Nicotine acid	5	0.5	
3.	Pyridoxin Hcl	5	0.5	
4.	Thiamine-HCL	1	0.1	
5.	Myo-inositol**	1,000	100	

* Myo-inositol was freshly made at the time of media preparation (100 mg/L).

30 gm of sucrose is required as carbon source and 0.8 gm of agar is required as gelling agent. The pH of the media was maintained at 5.8 with the help of 0.1 N Hydrochloric acids HCl and 0.1N sodium hydroxide (NaOH).

During the preparation of each stock solution, the above chemicals were weighed accurately and dissolved completely in distilled water. Myo-inositol (which is considered as vitamin) was added directly while preparing medium (100 mg/L). To dissolve the chemicals more readily, the solutions were stirred with magnetic stirrer. The final volume was mesh upto 1 litre for stock A and 100 ml for stock B, C and D.

Due to light sensitivity, all stock solutions were kept in clean brown bottles and stored.

3.2.1.1.2. Preparation of hormones used in the experiment

In the present investigation, two plant growth hormones, cytokinin and auxins were used. For the preparation of cytoknin, i.e., [BAP (6-Benzyl Amino Purine)], 10 mg of it was dissolved in 2.5 ml of 0.5N NaOH and for the preparation of auxins, i.e., [NAA (α-Naphthalene Acetic Acid), IAA (Indole-3-Acetic Acid), Kn (Kinetin) and IBA (Indole-3-Butyric Acid)], 10 mg of each was dissolved in 2.5 ml of 1N NaOH. After that the final volume was made 100 ml by addition of sterile distilled water in each hormone separately. 1 ml of this hormone stock was used for 100 ml of media to make 1 mg/L of hormone concentration in the media.

3.2.1.1.3. Preparation of fungal elicitors used in the experiment

A fungal elicitors *Coniochaeta africana* (R3) and *Coniochaeta Dendrobiicola* (R7) was prepared from the supernatant of 10-days old fungus which was extracted from *Dendrobium longicornu* Lindl., roots and leaves was inoculated in czapek broth medium(Shah *et al.,*2019). The entire broth was centrifuged at 5000 rpm, and the supernatant was filtered through Whatman filter paper Grade 1:11 µm (medium flow filter paper). The flow-through was used as the fungal elicitor solution. MS media supplemented with fungal elicitor was prepared in 0.5%, 1% and 2 % i.e., 0.5ml, 1ml and 2ml of fungal extract in 100ml. The media was than autoclaved at 121 °C for 20 min (Shah *et al.,* 2019).

3.2.1.1.4. Preparation of one litre MS media

Sterilization of media under preparation was done and all the stock solution were kept aside. One litre of sterilized conical flask was taken; 400ml of double distilled water was added. 100Ml of stock A, 1 ml of stock B, 10 ml of stock C and 1ml stock D are added to the flask. Solution was mixed well with magnetic starrier and then 100 mg per litre of myo-inosital was added. 30 mg of sucrose was added as a carbon source. Then, final volume of one litre was made by adding required distilled water. PH was maintained to 5.6 by adjusting with 0.1NaOH or HCl. The medium was solidified with 0.8% agar and boiled on heater until agar was completely dissolved. Hormone containing media was prepared by adding the required concentration of

phytohormones in 100 ml medium. It was heated with the help of heated to melt agar. When solution become clear about 12-16ml of media was poured in each of the 8 to 9 sterilized tubes. Then, each tube was enclosed by aluminium foil cap and autoclaved at temperature of 121°C and pressure of 15lb/sq for 20 minutes. After autoclave, tubes were allow to cool at room temperature and then kept in slanting position (for solid media) to make enough surface area.

Half strength concentrations of media were prepared by taking, equal volume of media solution and sterile distilled water, i.e.,1:1 ratio. But half strength concentration of media required 0.8% agar for its solidification. Media of Adenine sulphate (Ads) was made by adding 0.1gram of Ads in 100ml (1g/L) of MS media i.e., 0.1%. 10% ,15% and 20% coconut water supplemented Ms Medium was also prepared.For the hormones supplemented medium the hormones stock solution were added using volumetric analytical formula S1V1 =S2V2, WhereS1= Initial strength of hormone stock (mg/L),V1= Initial volume of stock to be taken,S2= Required strength of hormone stock (mg/L) and V2= Final volume of stock to be made.

3.2.1.1.5. Methods of sterilization

3.2.1.1.5. a. Sterilization of glassware's and metal instruments

While preparing the media and working with laminar air flow chamber, the necessary glassware‘s and metal instruments were subjected to wet and dry heat sterilization before their use. Glassware‘s such as beaker, petridish, culture tubes, pipettes, conical flasks, glass rod, measuring cylinder and metal instruments like forceps, scalpels, needle and scissors were dipped in detergent solution for 24 hours and washed with tap water. They were then steam sterilization with autoclave at 121ºC for 20 minutes at 15 lb. pressure and dried with hot air oven at 150ºC for 2 hours. Metal instruments were wrapped in aluminum foil before keeping inside the hot air oven for sterilization. Finally, flaming sterilization was done before using in a laminar air flow.

3.2.1.1.5. b. Sterilization of inoculation chamber

Before starting the inoculation of explant, the laminar air flow cabinet was sterilized by 70% ethanol soaked by sterilized cotton. Sterilized necessary instrument like forceps, scalpel, surgical blade, petridish, filter paper, beaker conical flask, sprit, double distilled water, culture tubes with medium were exposed under the UV

radiation for 45 minutes inside the laminar air flow cabinet except explants. After 45 minutes laminar air flow cabinet was turn off and blower was turn on and chamber was ready for inoculation**.**

3.2.1.1.6. Inoculation of explants

The explants used in present research were *in vitro* micro shoots (0.5-1cm) of *D. chryseum* derived from protocorms culture in Plant Biotechnology Laboratory of Central Department of Botany. The appropriate developmental stage of micro shoots, obtained from *in vitro* culture of protocorm were aseptically taken out from the culture vessels and cultured individually on different strength of MS media supplemented with or without hormones, fungal elicitors and adenine sulphate respectively.

In vitro grown shoots, were cultured on half strength MS medium and full strength MS media as control, with auxin and cytoknin combination i.e., BAP (0.5,1 and 2 mg/L), with NAA(0.5mg/L), IAA (0.5mg/L), and Kn(0.5mg/L) and also with 10%, 15% and 20% CW and *in vitro* grown shoot of were also culture on different strength (0.5%-2%) of fungal elicitors (R3 and R7), rooting hormones like were IAA (1 and 1.5 mg/L), IBA (1 and 1.5 mg/L) and NAA (1 and 1.5mg/L) on both half and full MS Medium and also 10% CW with 1mg/L IBA. Similarly, in case of MS medium fortified with 0.1% of adenine sulphate *in vitro* grown shoots were cultured on half and full strength MS medium alone (control) and with 0.1% Ads and with different concentration of hormones like BAP (0.5 and 1 mg/L), NAA (0.5 and 1mg/L), Kn (0.5 and 1 mg/L) alone and in combination. All the cultures were maintained at 25±2ºC and 16/8 hrs photoperiod. They were observed at different intervals.

3.2.3. Marker analysis for genetic homogeneity

3.2.3.1. Extraction and quantification of genomic DNA

Young and healthy leaves harvested from wild (mother plant collected from natural habitat) and four explants each set derived from *in vitro* culture of micro shoots grown on MS media supplemented with Fungal elicitors (R3 and R7), CW, and hormones and protocorm raised on half strength MS medium were used as explants for DNA extraction of *D. chryseum* (Appendix 1). Young leaves collected from wild mother plant were washed under running tap water and rinsed with 70% ethyl alcohol

for surface sterilization. Leaves and protocorm derived from *in vitro* culture were not subjected for surface sterilization. Total genomic DNA of *D. chryseum* leaf and protocorm (100 mg) was extracted using Hexadecyltrimethyl Ammonium Bromide (CTAB). The quality and quantity of extracted DNA were determined by gel electrophoresis on 1% agarose gel and UV spectrophotometer. The DNA content of the isolate was adjusted to 30 µl by TE buffer or DNA rehydration solution and stored at -20°C for further study.

3.2.3.1.1. Extraction of DNA from CTAB method

Genomic DNA both *in vitro* and *in vivo* samples of *Dendrobium chryseum* were extracted by using CTAB method (Doyle, 1991) with some modifications described as follow. Approximately 0.1 gm of leaf and protocorm samples was taken and grinded to fine powder by using motor and pestle in liquid nitrogen. 500 µl of CTAB buffer (2% CTAB, 0.5M EDTA, 5M NaCl, 1M Tris-HCl, pH 8.0, 0.2% β-mercaptoethanol) was added to make the fine paste and transferred into clean sterilized micro-centrifuge tube (vol. 2.0 ml). Samples were incubated at 65˚C for 45 minutes in a recirculating water bath. After incubation, samples were centrifuged at 10,000 rpm for 5 minutes to spin down cell debris. Then supernatant were transferred to clean sterilized micro-centrifuge tube and equal volume of Chloroform: Isoamyl alcohol in the ratio of 24:1 was added and mixed gently by inversion for several times (5-8 minutes). It was again centrifuged for 10 minute in 12,000 rpm and upper aqueous phase was transferred to sterilize micro-centrifuge tube which was stored at 20˚C for one hour.

After that, DNA was spinned at 12,000 rpm for 5 minute to form the pellet. Supernatant was discarded and pellet was washed with ice cold 70% ethanol (500 µl volumes). Again, it was spinned at 10,000 rpm for 5 minute to get rid of salt. Then, ethanol was pipetted out and pellet was left to dry for 15 minutes. Finally, it was re-suspended in TE buffer (30 µl) and stored at -20˚C. The quality and quantity of extracted DNA was estimated by electrophoresis on agarose gel (1%) and by UV spectrophotometry respectively. The concentration of extracted DNA was adjusted to 30 ng and finally DNA samples were stored at – 20ºC.

3.2.3.2. DNA amplification

Out of 10 RAPD and two ISSR primers (Appendix 2 & 3) only 4 RAPD markers and 1 ISSR marker showed a clear and reproducible banding pattern, which were used for further analysis (Appendix 2 and 3). PCR reaction for RAPD and ISSR assays were conducted in 15 µl reaction volume with varying concentrations of nuclease free water(4.5 µl), master mix (7µl) primers (1.5 µl) and DNA template (2 µl). Cycling condition followed by Cerasela and Lazar, (2009) was also optimized for identifying best cycling conditions (45 cycles) for RAPD and (35 cycles) for ISSR. DNA amplifications were performed in thermal cycler (BioRad) with the following PCR program: 5 min. of initial denaturation step at 92°C, 45 cycles comprising denaturation at 92°C for 1 min., annealing at 44.2°C for 1 min, and extension at 72°C for 1 min; followed by a final extension step of 5 min at 72°C (OPA 10) and for rest RAPD, DNA amplification were performed with following PCR program: as 5 min. of initial denaturation step at 94°C, 45 cycles comprising denaturation at 94°C for 1 min., annealing at 37°C for 1 min, and extension at 72°C for 2 min; followed by a final extension step of 10 min at 72°C and for ISSR , 95˚C for 5 min followed by 35 cycles of 94˚C for 1 min, 52.3˚C of 1 min, 72˚C of 2 min and final extension of 7 min at 72˚C. The amplified products were separated on an agarose gel (1 %) electrophoresis and stained with ethidium bromide.

3.2.3.3 Gel electrophoresis

The PCR amplified fragments were separated on 1% agarose gel using 1X TBE (Tris base, boric acid and EDTA) buffer and stained by ethidium bromide (1 µg/ml). The gel was running at 90 V constant for 1.5 to 2 hours. and then visualized and photographed under UV light in a gel documentation system (UVITEC, Cambridge). The sizes of the amplification fragments were determined by comparisons with the 100bp DNA ladder marker (BioLabs). The PCR reactions were repeated thrice and only the clear bands were considered.

3.2.4. Data recording

After the inoculation, the culture tubes were transferred to the culture room and they were regularly monitored for shoot and root length and numbers. The data was taken

at regular interval of 4 weeks. Data was recorded upto 24 weeks of culture for shoot and root length and numbers.

3.2.5. Statistical analysis

Data on shooting and rooting were presented as mean and standard error. The significant difference between the MS medium and MS medium supplemented with different growth hormones and fungal elicitors were analyzed by using one - way analysis of variance (ANOVA) with F-statistics at a 95% confidence interval using SPSS version 20.

The banding pattern in genetic fidelity analysis using RAPD and ISSR markers were scored manually. On the basis of presence (1) or absence (0) of individual amplified DNA bands. Clear and reproducible band was considered for counting allele. The data were pooled into the binary matrix based on presence (1) and absence (0) of the selected bands. The number of allele and allele size per primer was recorded and compared with its mother plant (wild type). The polymorphic information content value for each primer was calculated using the formula, PIC= 2*fi *(1-fi) where, fi is the frequency of amplified allele (allele present), 1-fi is the frequency of null allele (allele absent) (Powell *et al.,* 1996). The amplified allele size was recorded. The data for PIC value, number of scorable bands, amplified allele size range and the monomorphism percentage were recorded. By comparing the banding patterns of all the plant accessions, specific bands were identified and a marker system for genetic purity *Dendrobium chryseum* was established.

CHAPTER IV

RESULT

The result of *in vitro* micro propagated *Dendrobium chryseum* in MS medium supplemented with different combination (Fungal elicitors, CW, auxin and cytoknin hormones and adenine sulphate) and its genetic homogeneity have been described under the following headings:

4.1. *In vitro* development of *Dendrobium chryseum*

4.1.1. Effect of fungal elicitors (R3 and R7) in shoots and roots development of *D. chryseum*

The young and healthy microshoots of 0.5-1 cm derived from protocorm culture of *D. chryseum* were sub cultured on half and full strength MS medium alone (control) and with different concentration of fungal elicitors for shoot and root development. Concentration used for *in vitro* development were 0.5%, 1% and 2% of each fungal elicitors (R3 and R7). One way ANOVA showed that value of all growth parameters varied significantly among the tested used condition of MS media ($p \leq 0.05$) with different concentration of elicitors supplemented medium (Appendix 4 and 5).

The following responses were obtained of *D. chryseum* after 24 weeks of culture.

4.1.1.1. Effect of fungal elicitor (R3) in shoots and roots number

Among different tested condition of fungal elicitor (R3), the maximum number of shoots was obtained on half strength of MS medium supplemented with 0.5% R3 which was found (5.75 ± 0.50) per microshoot. This number was followed by half strength of MS medium supplemented with 2% R3 where the shoot number was found (4.75 ± 0.50) per microshoot. However the least shoot number was observed on full strength of MS medium supplemented with 0.5% R3 which was found (1.755 ± 0.50) per microshoot (Figure 2 and 4).

Similarly, the maximum number of roots was obtained on half strength of MS medium supplemented with 0.5% R3 which was found (8.25 ± 0.50) per shoot. This number was followed by full strength of MS medium supplemented with 2% R3

where the root number was found (7.75 ± 0.50) per shoot. However the least root number was observed to be on half strength of MS medium which was found (0.75 ± 0.50) per shoot. Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of fungal elicitors R3 (Figure 2 and 4).

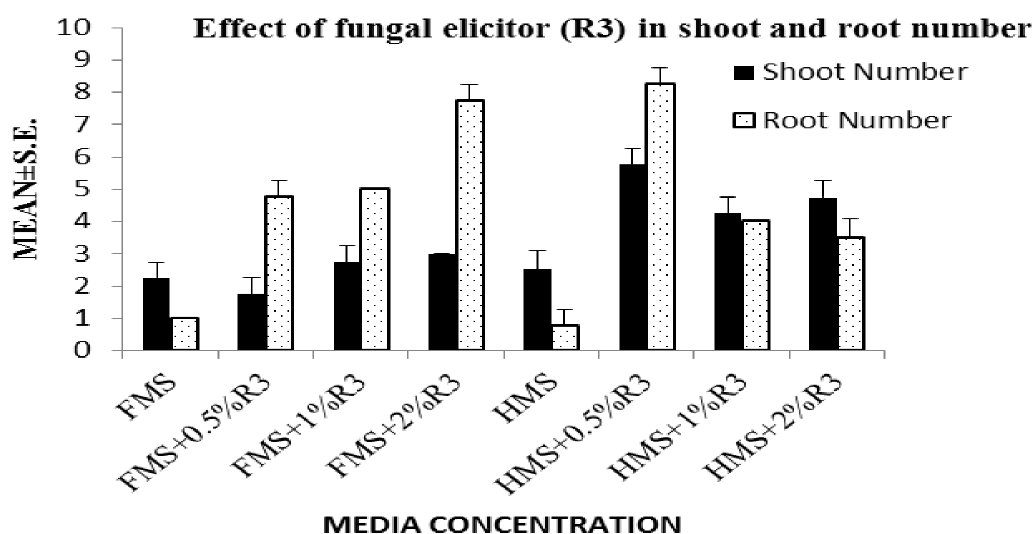

Figure 2: Average number of shoots and roots of *D. chryseum* on HMS and FMS medium supplemented with different concentration 0.5%, 1% and 2% of fungal elicitor (R3). (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C. Four replicated were used for each combination.

4.1.1.2. Effect of fungal elicitor (R3) in shoots and roots length

Among different tested condition of fungal elicitor (R3), the maximum length of shoots was obtained on full strength of MS medium supplemented with 1% R3 which was found (5.37 ± 0.15 cm). This number was followed by full strength of MS medium supplemented with 0.5% R3 where the shoot length was found (4.37 ± 0.09 cm). However the least shoot length was observed to be on half strength of MS medium which was found (1.32 ± 0.05cm) (figure 3 and 4).

Similarly, the maximum length of roots was obtained on full strength of MS medium supplemented with 1% R3 which was found (5.5 ± 0.20 cm). This number was followed by full strength of MS medium supplemented with 0.5 % R3 where the root number was found (5.12 ± 0.25cm). However the least root length was observed to be on half strength of MS medium which was found (1.35 ± 01cm). Statistically, all the

values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of fungal elicitors R3 (Figure 3 and 4).

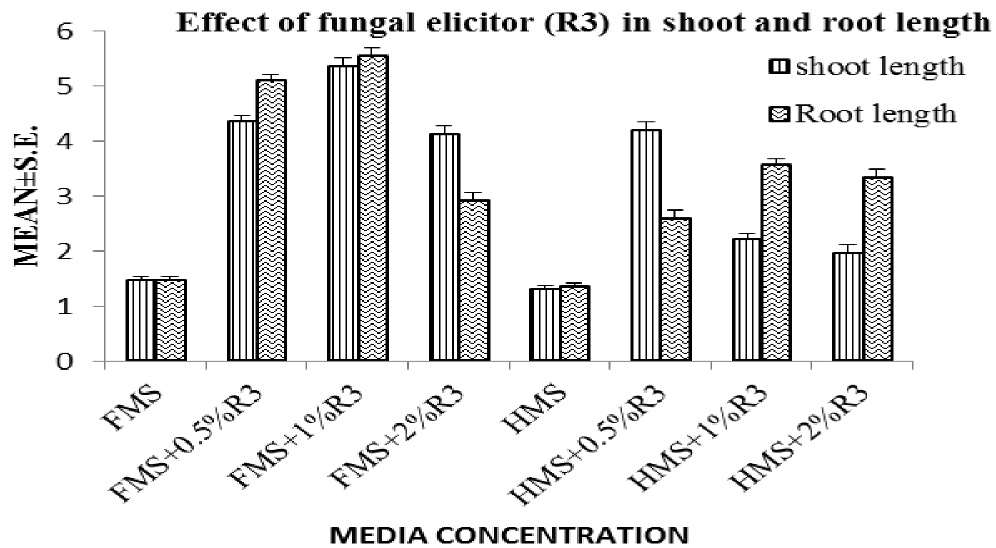

Figure 3: Average length of shoots and roots of *D. chryseum* on HMS and FMS medium supplemented with different concentration 0.5%, 1% and 2% of fungal elicitor (R3). (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C). Four replicated were used for each combination.

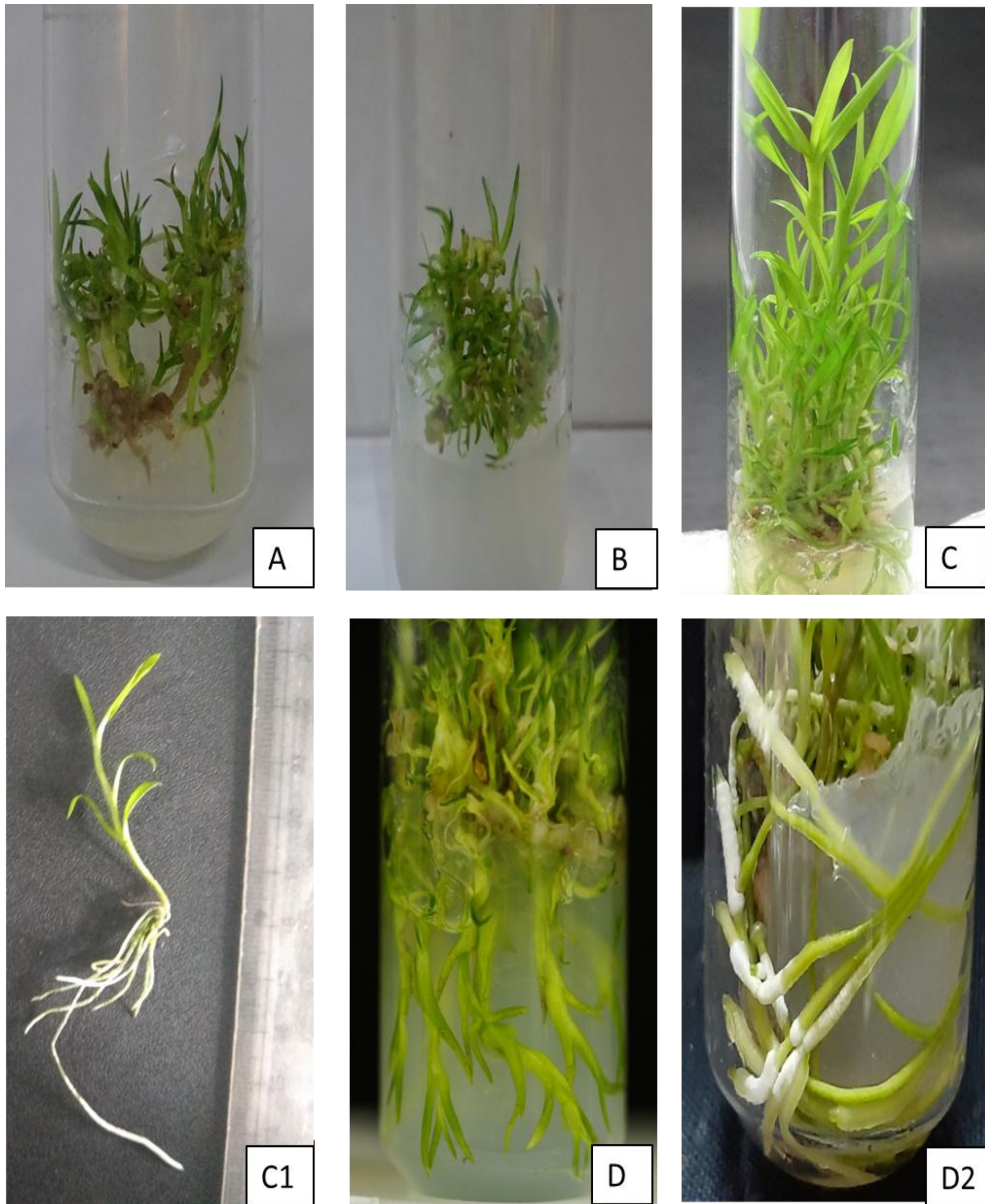

Figure 4: *In vitro* development of *Dendrobium chryseum* on solid MS media fortified with different concentration of fungal elicitor R3. A = Development of plantlets on half strength of MS medium (control), B = Development of plantlets on full strength of MS medium (control) C = Development of plantlets with roots on full strength of MS medium fortified with 1% R3, C1=Development of plantlets with roots on full strength of MS medium fortified with 1% R3 (shoot and root length), D=Development of plantlets with roots on half strength of MS medium fortified with 0.5% R3 (shoots number), D1= Development of plantlets with roots on half strength of MS medium fortified with 0.5 % R3 (root number).

4.1.1.3. Effect of fungal elicitor (R7) in shoots and roots number

Among different tested condition of fungal elicitor (R7), the maximum number of shoots was obtained on full strength of MS medium supplemented with 2% R7 which was found (4.5 ± 0.58) per microshoot. This number was followed by full strength of MS medium supplemented with 1% R7 where the shoot number was found (4.25 ± 0.50) per microshoot. However the least shoot number was observed to be on full strength of MS medium supplemented with 0.5% R7 which was found (1.75 ± 0.50) per microshoots (Figure 5 and 7).

Similarly, the maximum number of roots was obtained on full strength of MS medium supplemented with 2% R7 which was found (7.75 ± 0.50) per shoot. This number was followed by full strength of MS medium supplemented with 0.5% R7 where the root number was found (5.00 ± 0.00) per shoot. However the least root number was observed to be on half strength of MS medium which was found (0.75 ± 0.5) per shoot. Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of fungal elicitors R7 (Figure 5 and 7).

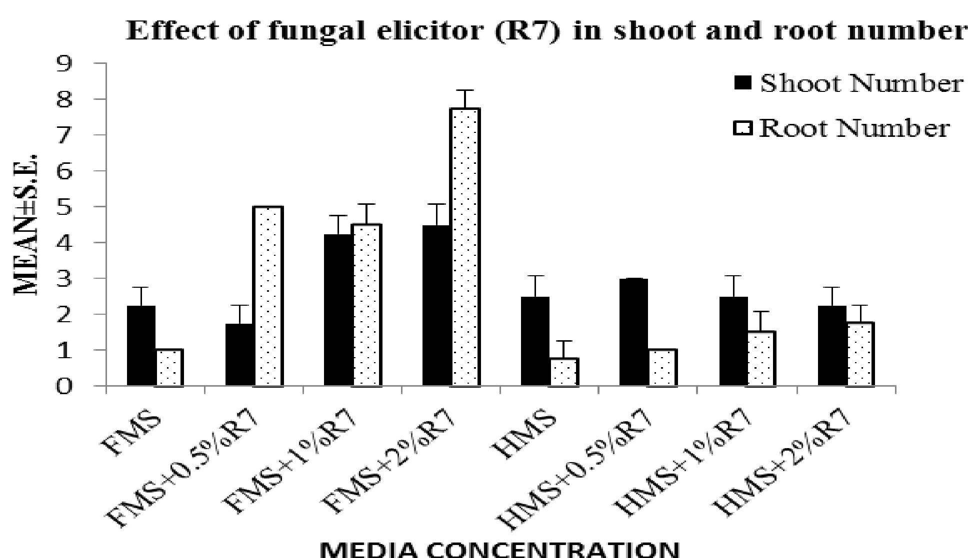

Figure 5: Average number of shoots and roots of *D. chryseum* on HMS and FMS medium supplemented with different concentration 0.5%, 1% and 2% of fungal elicitor (R7). (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C). Four replicated were used for each combination.

4.1.1.4. Effect of fungal elicitor (R7) in shoots and roots length

Among different tested condition of fungal elicitor (R7), the maximum length of shoots was obtained on full strength of MS medium supplemented with 2% R7 which was found (5.05 ± 0.10 cm). This number was followed by full strength of MS medium supplemented with 1% R7 where the shoot length was found (4.42 ± 0.09 cm). However the least shoot length was observed to be on half strength of MS medium which was found (1.32 ± 0.05cm) (Figure 6 and 7).

Similarly, the maximum length of roots was obtained on full strength of MS medium supplemented with 0.5% R7 which was found (4.82 ± 0.12 cm). This number was followed by full strength of MS medium supplemented with 2% R3 where the root number was found (3.9 ± 0.11cm). However the least root length was observed to be on half strength of MS medium supplemented with 1% R7 which was found (0.85 ± 0.05 cm). Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of fungal elicitors R3 (Figure 6 and 7).

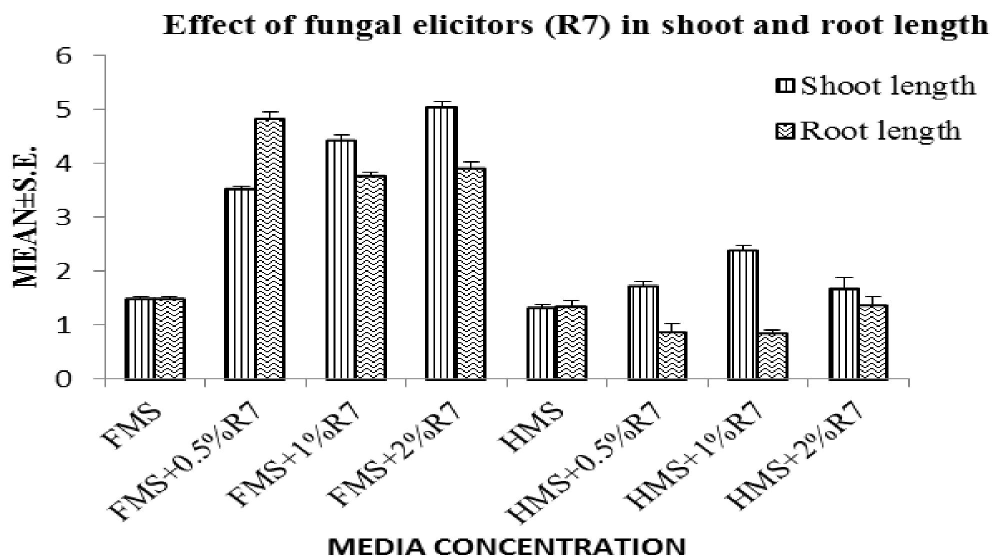

Figure 6: Average lengths of shoots and roots of *D. chryseum* on HMS and FMS medium supplemented with different concentration 0.5%, 1% and 2% of fungal elicitor (R7). (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C). Four replicated were used for each combination.

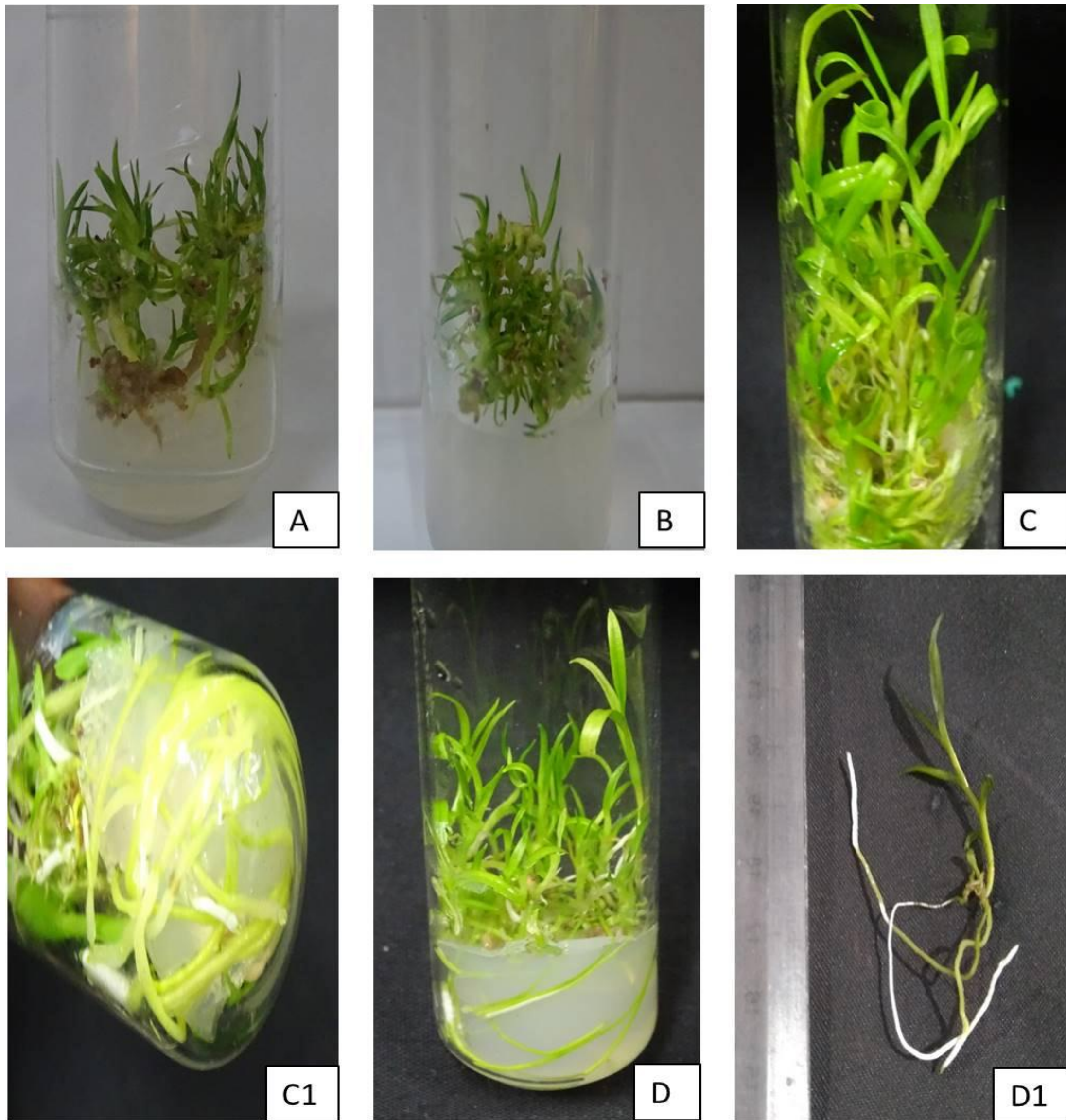

Figure 7: *In vitro* development of *Dendrobium chryseum* on solid MS media fortified with different concentration of fungal elicitor R7. A = Development of plantlets on half strength of MS medium (control), B = Development of plantlets on full strength of MS medium (control), C = Development of plantlets with roots on full strength of MS medium fortified with 2% R7 (shoot length and shoot number), C1 = Development of plantlets with roots on full strength of MS medium fortified with 2% R7 (root number), D = Development of plantlets with roots on full strength of MS medium fortified with 0.5% R7, D1. = Development of plantlets with roots on full strength of MS medium fortified with 0.5 % R7 (root length).

4.1.2. Effect of different concentration and combination of auxin and cytoknin hormones alone and with CW in shoots and roots development of *D. chryseum*

In vitro grown micro shoots of 0.5-1cm, were cultured on full strength MS media alone (control), with 10%, 15% and 20% CW and with auxin and cytoknin combination i.e., BAP (0.5,1 and 2 mg/L), with NAA(0.5mg/L), IAA (0.5mg/L), and Kn(0.5mg/L) and also with 10%, 15% and 20% CW. However, half strength MS hormone combination with same concentration of hormone and CW didn't respond well. So, the data of FMS were only included in research work. One way ANOVA showed that value of all growth parameters varied significantly among the tested used condition of MS media ($p \leq 0.05$) with different concentration of hormones supplemented medium (Appendix 6).

The following responses were obtained of *D. chryseum* after 24 weeks of culture.

4.1.2.1 Effect of different hormones combination alone and with CW in shoots and roots number

Among different tested conditions of hormones, the maximum number of shoots was obtained on full strength MS medium supplemented with 15% CW which was found (6.25 ± 0.50) per microshoot. This condition was followed by full strength MS medium supplemented with 0.5 mg/L BAP, 0.5mg/L NAA and 15% CW where the number was found (4.75 ± 0.50) per microshoot. However the least development of shoots was observed on full strength of MS medium supplemented with 0.5 mg/L BAP, 0.5mg/L Kn and 15% CW which was found (1± 0.00) per microshoot.

Similarly, the maximum number of roots was also obtained on full strength MS medium supplemented with 15% CW which was found (9.75 ± 0.50) per shoot. This condition was followed by full strength MS medium supplemented with 0.5mg/L BAP, 0.5mg/L IAA and 20 % CW where the number was found (4.75 ± 0.50) per shoot. However the least shoots number was observed on full strength of MS medium supplemented with 0.5mg/L BAP and 0.5mg/L Kn which was found (0.25 ± 0.50) per shoot .Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of hormones (Figure 8 and 10).

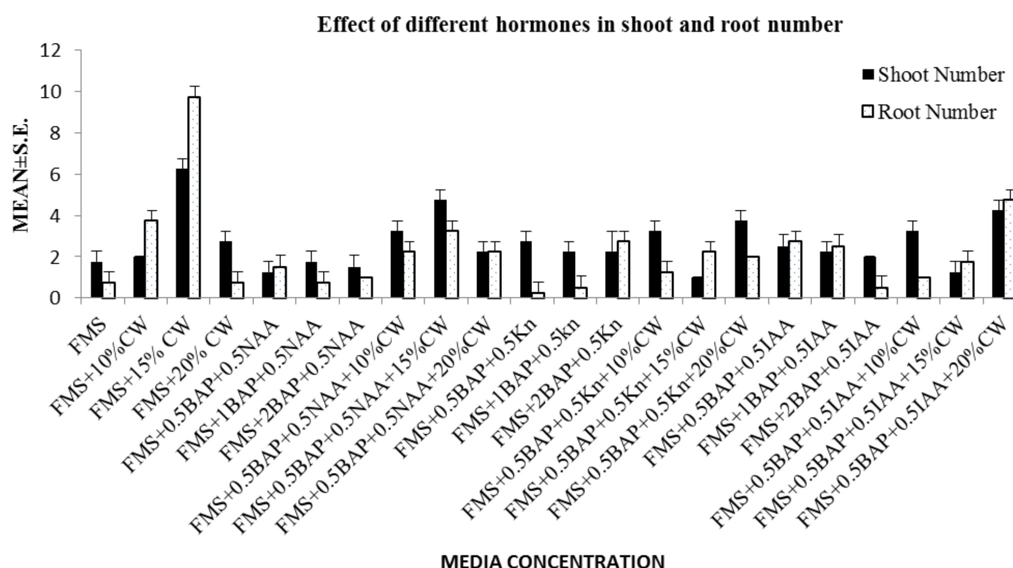

Figure 8: Average number of shoots and roots of *D. chryseum* on full strength MS medium supplemented BAP, NAA, Kn, IAA and CW with different concentration. (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C). Four replicated were used for each combination.

4.1.2.2. Effect of different hormones combination alone and with CW in shoots and roots length

Among different tested conditions of hormones, the maximum length of shoots was obtained on full strength MS medium supplemented with 15% CW which was found (6.7 ± 0.14 cm). This condition was followed by full strength MS medium supplemented with 1 mg/L BAP and 0.5mg/L IAA where the number was found (3.02 ± 0.05cm). However the least development of shoots length was observed on full strength of MS medium supplemented with 2 mg/L BAP and 0.5mg/L NAA which was found (0.8 ± 0.14 cm).

Similarly, the maximum length of roots was also obtained on full strength MS medium supplemented with 15% CW which was found (6.3 ± 0.11cm). This condition was followed by full strength MS medium supplemented with 0.5mg/L BAP, 0.5mg/L NAA and 10% CW where the number was found (4.2 ± 0.24cm). However the least development of roots length was observed on full strength of MS medium supplemented with 1 mg/L BAP and 0.5mg/L Kn which was found (0.57 ± 0.09 cm). Statistically, all the values were varied significantly among different tested

conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of hormones (Figure 9 and 10).

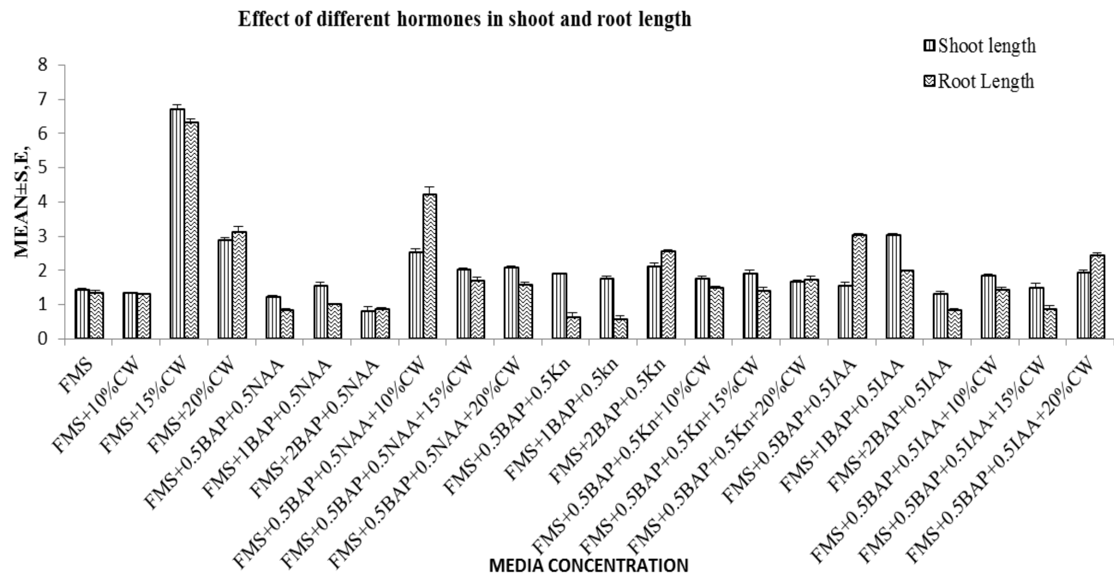

Figure 9: Average length of shoots and roots of *D. chryseum* on full strength MS medium supplemented BAP, NAA, Kn, IAA and CW with different concentration. (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C). Four replicated were used for each combination.

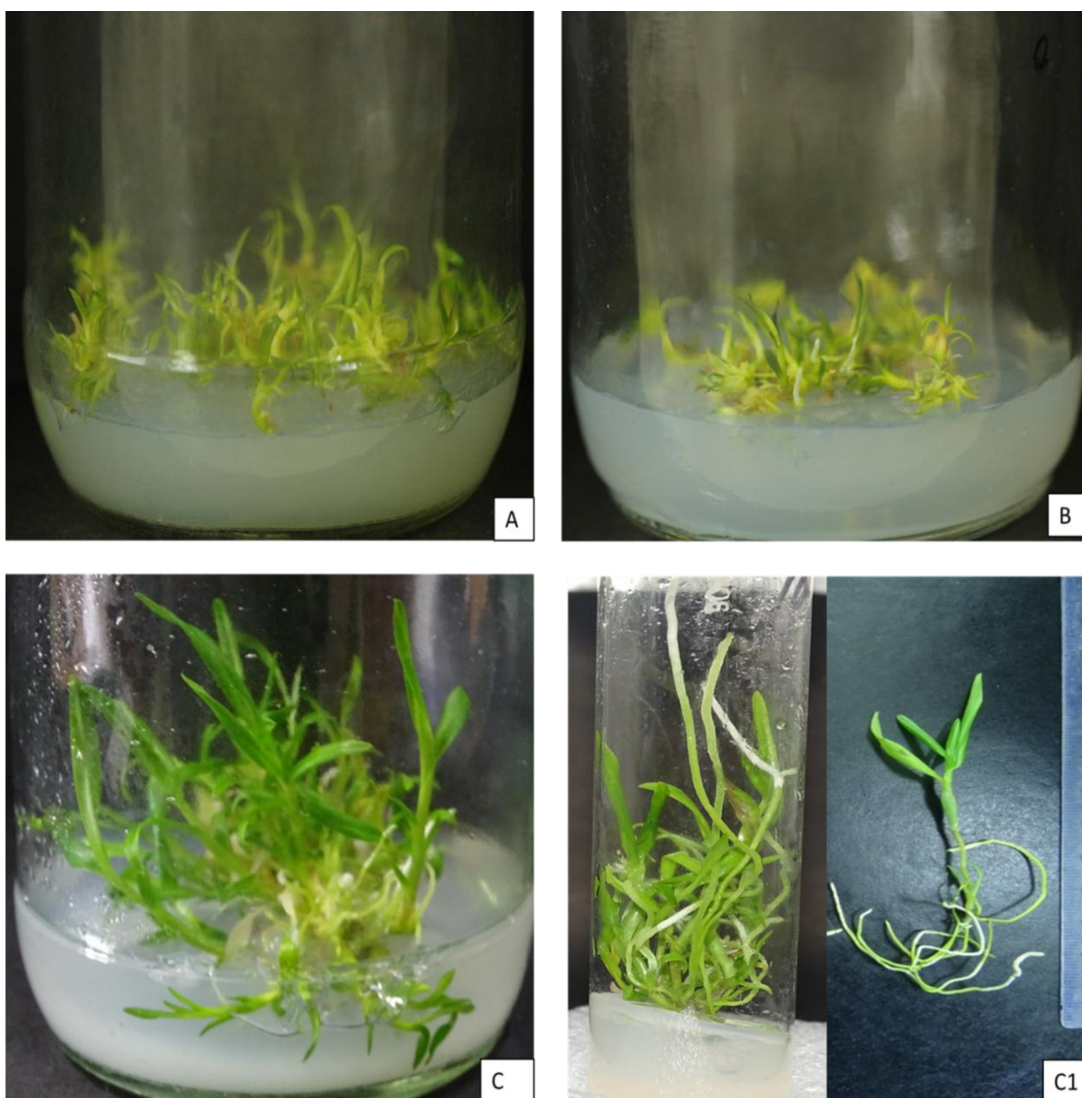

Figure 10: *In vitro* development of *Dendrobium chryseum* on solid MS media fortified with different concentration of auxin and cytoknin alone or with and without CW. A = Development of plantlets on full strength of MS medium (control), B = Development of plantlets on full strength of MS medium fortified with 10% CW, C = Development of plantlets with roots on full strength of MS medium fortified with 15% CW (shoot number and length), C1 = Development of plantlets with roots on full strength of MS medium fortified with 15% CW (root and shoot length)

4.1.3. Effect of different concentration of auxin in roots development of *D. chryseum*

Young healthy shoot of about 0.5 - 1 cm microshoots derived from protocorm culture of *D. chryseum* on full strength MS medium fortified with 10% coconut water were subcultured on half and full strength of MS medium fortified with different concentration of auxin for rooting. Rooting hormones used for *in vitro* rooting were IAA (1 and 1.5 mg/L), IBA (1 and 1.5 mg/L) and NAA (1 and 1.5mg/L) on both half and full MS Medium and also 10% CW with 1mg/L IBA. Half and full strength MS medium were used as a control. One way ANOVA showed that value of all growth parameters varied significantly among the tested used condition of MS media ($p \leq 0.05$) with different concentration of hormones supplemented medium (Appendix 7).

The following responses were obtained of *D. chryseum* after 24 weeks of culture.

4.1.3.1 Effect different concentration of auxins in shoots and roots number

Among different tested conditions of rooting hormones, the maximum number of shoots was obtained on half strength MS medium supplemented with 1 mg/L IBA which was found (4 ± 00) per microshoot. This condition was followed by full strength MS medium supplemented with 1 mg/L IAA where the number was found (3.5 ± 0.58) per microshoot. However the least development of shoots was observed on full strength of MS medium supplemented with 1 mg/L IBA, which was found (1.25 ± 0.50) per microshoot (Figure 11 and 13).

Similarly, the maximum number of roots was also obtained on half strength MS medium supplemented with 1 mg/L IBA which was found (7.75 ± 0.00) per shoot. This condition was followed by half strength MS medium supplemented with 1.5 mg/L IBA, where the number was found (7.5 ± 0.50) per shoot. However the least shoots number was observed on half strength of MS medium which was found (0.75 ± 0.5) per shoot .Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of auxins hormones (Figure 11 and 13).

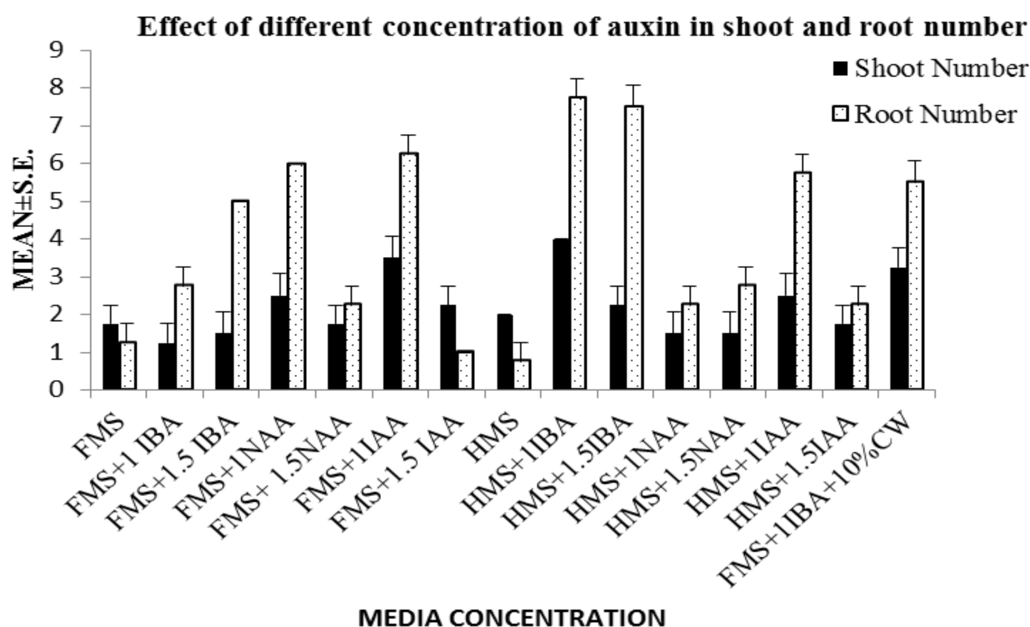

Figure 11: Average number of shoots and roots of *D. chryseum* on full and half strength of MS medium supplemented IBA, NAA, and IAA with different concentration. (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C). Four replicated were used for each combination.

4.1.3.2. Effect of different concentration of auxins in shoots and roots length

Among different tested conditions of rooting hormones, the maximum length of shoots was obtained on half strength MS medium supplemented with 1 mg/L NAA which was found (4.95 ± 0.1cm). This condition was followed by full strength MS medium supplemented with 1 mg/L IBA where the number was found (4.87 ± 0.05cm). However the least development of shoots length was observed on half strength of MS medium which was found (1.32 ± 0.05 cm).

Similarly, the maximum length of roots was also obtained on full strength MS medium supplemented with 1.5 mg/L IBA which was found (5 ± 0.00cm). This condition was followed by half strength MS medium supplemented with 1.5 mg/L IBA, where the number was found (3.8 ± 0.00cm). However the least development of roots length was observed on half strength of MS medium which was found (1.30 ± 0.00cm). Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of hormones (Figure 12 and 13).

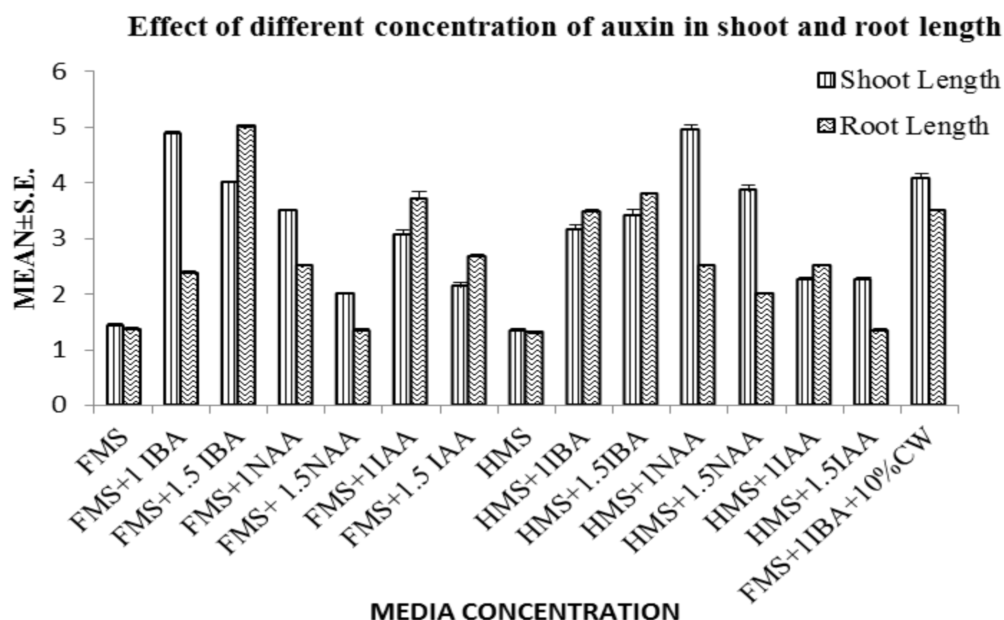

Figure 12: Average length of shoots and roots of *D. chryseum* on full and half strength of MS medium supplemented IBA, NAA, and IAA with different concentration. (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C). Four replicated were used for each combination.

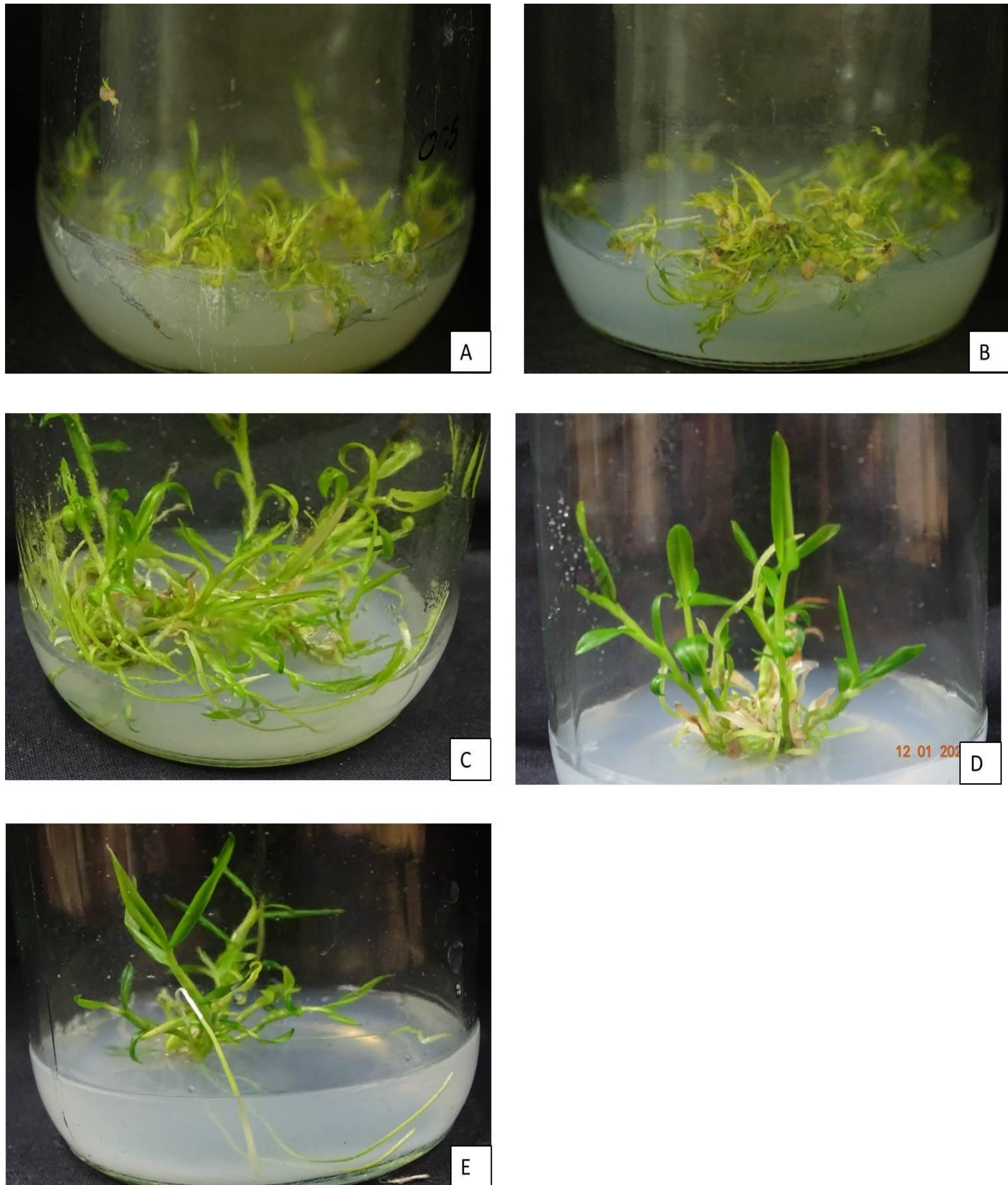

Figure 13: *In vitro* development of *Dendrobium chryseum* on solid MS media fortified with different concentration of auxin. A = Development of plantlets on half strength of MS medium (control), B = Development of plantlets on full strength of MS medium (control), C = Development of plantlets with roots on half strength of MS medium fortified with 1mg/L IBA (shoot and root number), D = Development of plantlets with roots on half strength of MS medium fortified with 1mg/L NAA (Shoot length), E= Development of plantlets with roots on half strength of MS medium fortified with 01.5mg/L IBA (root length).

4.1.4. Effect of adenine sulphate in shoots and roots development of D. chryseum

Young healthy microshoots of about 0.5 - 1 cm derived from protocorm culture of *D. chryseum* on full strength MS medium fortified with 10% coconut water were subcultured on full strength of MS medium fortified with 0.1% of adenine sulphate and different concentration of hormones like BAP, NAA and Kn. Hormones combination which were used with full strength MS with 0.1% Ads were BAP (0.5 and 1 mg/L), NAA (0.5 and 1mg/L), Kn (0.5 and 1 mg/L) and combination of BAP (0.5 and 1 mg/L) with NAA (0.5 mg/L) and Kn (0.5mg/L). Half and full strength MS medium were used as a control. The same combination were used for half strength MS but there result were not efficient. One way ANOVA showed that value of all growth parameters varied significantly among the tested used condition of MS media ($p \leq 0.05$) with different concentration of hormones supplemented medium (Appendix 8).

4.1.4.1. Effect of 0.1% adenine sulphate shoots and roots number

Among different tested conditions of hormones with 0.1% of Ads, the maximum number of shoots was obtained on full strength MS medium supplemented with 0.5 mg/L NAA with 0.1% Ads which was found (2.5 ± 0.57) per microshoot. This condition was followed by full strength MS medium where the number was found (2.00 ± 0.00) per microshoot. However the least development of shoots was observed on full strength of MS medium supplemented with 1 mg/L Kn and 0.1% Ads, which was found (0.5 ± 0.57) per microshoot (Figure 14 and 16).

Similarly, the maximum number of roots was also obtained on full strength MS medium supplemented with 0.5mg/L NAA with 0.1% Ads which was found (6.5 ± 0.57) per shoot. This condition was followed by full strength MS medium supplemented with 0.1% of Ads where the number was found (1.60 ± 0.57) per shoot. However the least shoots number was observed on full strength of MS medium supplement with 1 mg/L Kn and 0.1% Ads which was found (0.25 ± 0.50) per shoot. Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of auxins hormones (Figure 14 and 16).

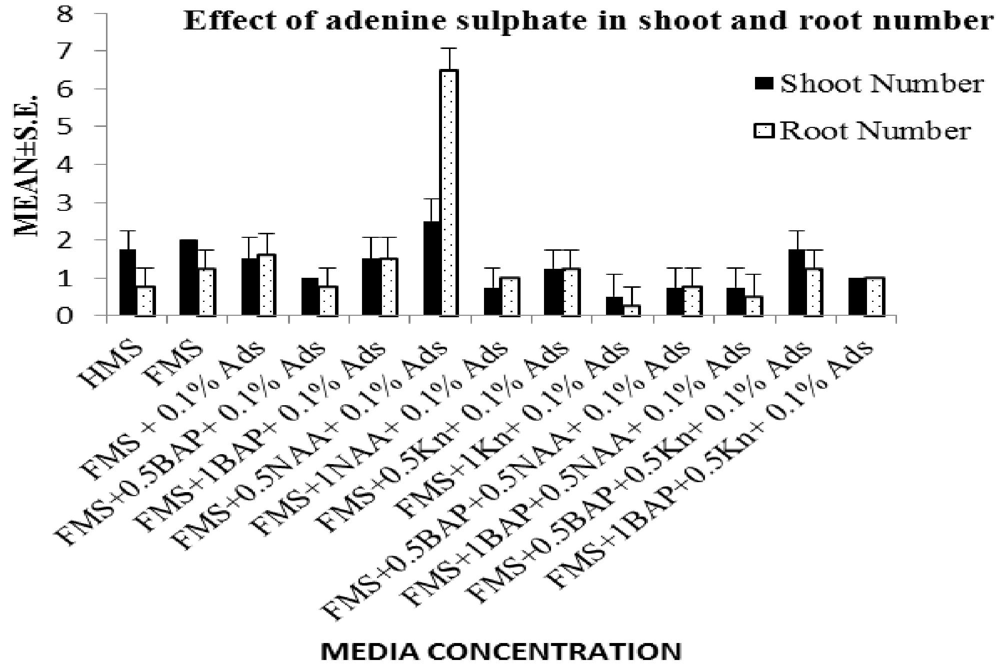

Figure 14: Average number of shoots and roots of *D. chryseum* on full strength of MS medium supplemented with 0.1% Ads and BAP, NAA, and Kn with different concentration. (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C. Four replicated were used for each combination.

4.1.4.2. Effect of 0.1% adenine sulphate in shoots and roots length

Among different tested conditions of hormones with 0.1% Ads, the maximum length of shoot was obtained on full strength MS medium supplemented with 0.5 mg/L NAA with 0.1% Ads which was found (4.87 ± 0.09cm). This condition was followed by full strength MS medium supplemented with 0.5 mg/L BAP, 0.5 mg/L Kn and 0.1% Ads where the number was found (4.12 ± 0.15cm). However the least development of shoots length was observed on half strength of MS medium which was found (1.37 ± 0.05 cm) (Figure 15 and 16).

Similarly, the maximum length of roots was also obtained on full strength MS medium supplemented with 0.5 mg/L NAA and 0.1% Ads which was found (2.07 ± 0.05cm). This condition was followed by full strength MS medium supplemented with 0.5 mg/L BAP, 0.5mg/L Kn and 0.1% Ads where the number was found (1.85 ± 0.05cm). However the least development of roots length was observed on full strength of MS medium supplemented with 1 mg/L Kn and 0.1% Ads which was found (0.35 ±

0.05cm). Statistically, all the values were varied significantly among different tested conditions of MS medium ($p \leq 0.05$) supplemented with different concentration of hormones (Figure 15 and 16).

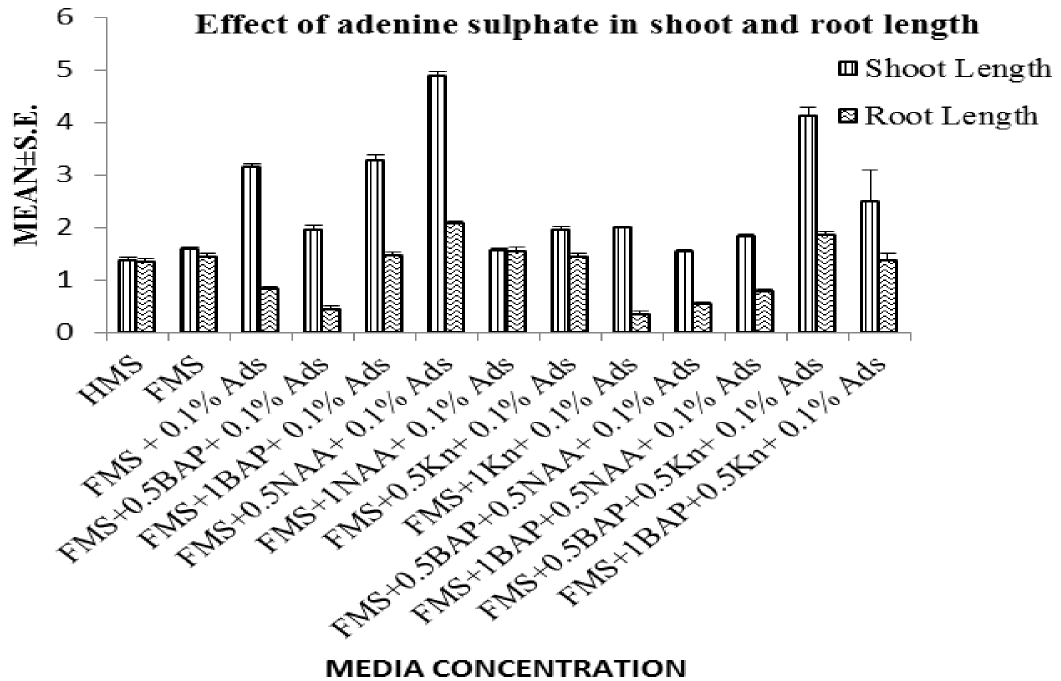

Figure 15: Average length of shoots and roots of *D. chryseum* on full strength of MS medium supplemented with 0.1% Ads and BAP, NAA, and Kn with different concentration. (Condition: artifical light (fluroscent light) with a light/ dark cycle of 16/8 hours at 25 ± 2 ˚C. Four replicated were used for each combination

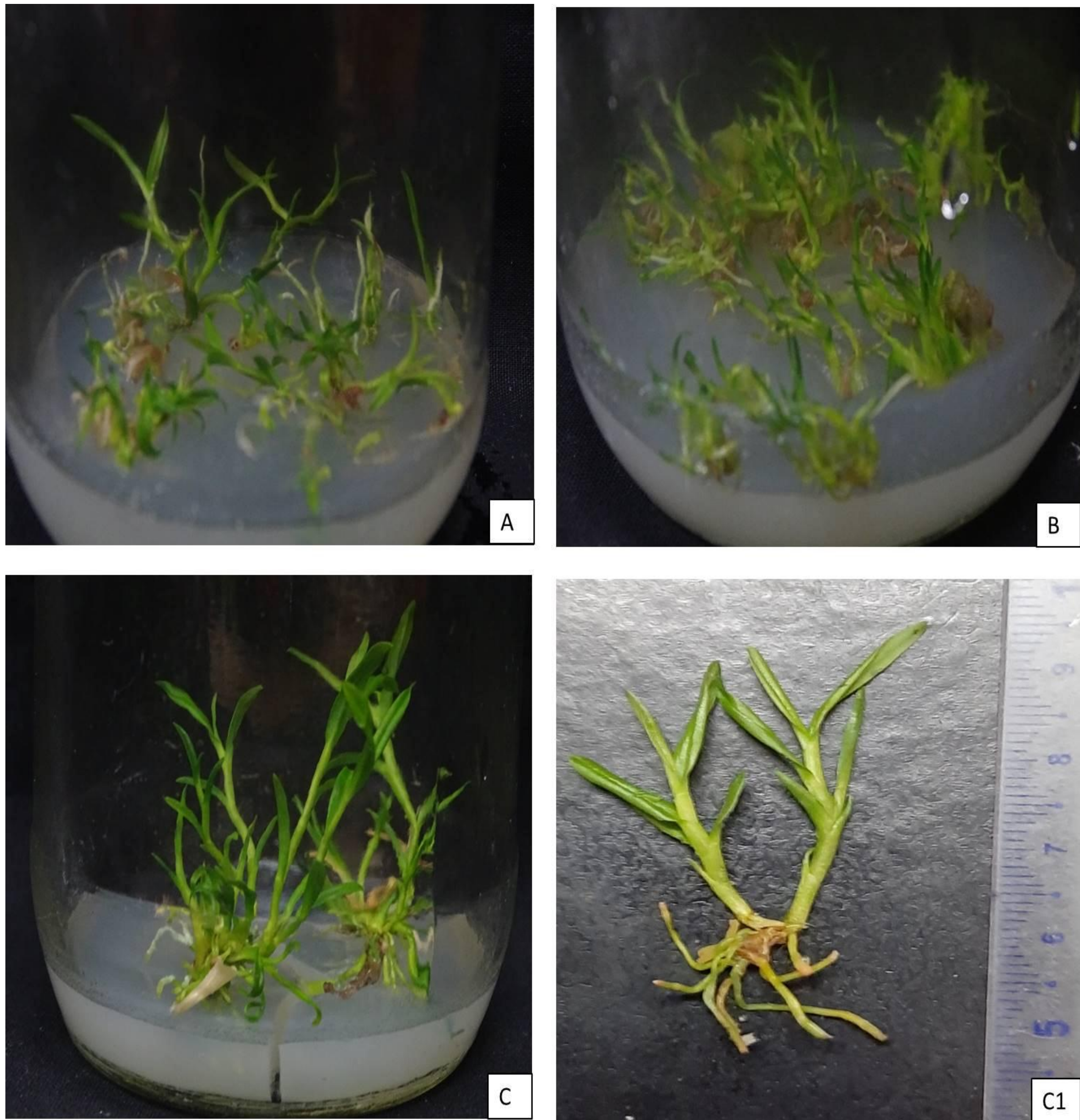

Figure 16: *In vitro* development of *Dendrobium chryseum* on solid MS media fortified with 0.1% Ads along with different concentration of auxin and cytoknin alone or with combination. A = Development of plantlets on half strength of MS medium (control), B. = Development of plantlets on full strength of MS medium (control), C = Development of plantlets with roots on full strength of MS medium fortified with 0.5% Ads along with 0.5 mg/L NAA (Shoots number, length), C1 = Development of plantlets with roots on full strength of MS medium fortified with 0.5% Ads along with 0.5mg/L NAA (Shoot and root length).

4.2. Genetic homogeneity (Molecular analysis)

4.2.1. RAPD analysis

RAPD primers are still widely used in the genetic homogeniety of many micropropagated orchids (Bhattacharyya *et al.,* 2014; Samarfard *et al.,* 2013; Tikendra *et al.,* 2019). In the present study, ten polymorphic RAPD primers (OPA 01, OPA 02, OPA 03, OPA 06, OPA 10, OPP 01, OPP 02, OPP 03, OPP 04 and OPP 05) were used to evaluate the genetic profile of tissue cultured plantlets developed from various media treatments such as hormones, CW, fungal elicitor and protocorm and compared with its mother plant (Appendix 1 and 2). All the analyzed *in vitro* samples showed genetic uniformity among themselves and with the mother plant.

Clear and reproducible band was considered for counting allele. The data were pooled into the binary matrix based on presence (1) and absence (0) of the selected bands. The number of allele and allele size per primer is recorded and compared with its mother plant (wild type). The polymorphic information content value for each primer was calculated using the formula, PIC= 2*fi *(1-fi) where, fi is the frequency of amplified allele (allele present), 1-fi is the frequency of null allele (allele absent) (Powell *et al.,* 1996). Out of 10 RAPD primers only 4 RAPD primers showed (OPA 01, OPA 03, OPA 06, OPA 10), clear and reproducible bands. A total of 14 bands were scored from 6 individuals (one mother plant, four rooted plantlets in *in vitro* condition and one protocorm) of *D. chryseum.* The number of scorable band varied from 2 to 5 within the approximate size range of 200-1300 bp while compare to Ladder marker (100bp sized). The amplified allele size of all *in vitro* samples were monomorphic among themselves and with their mother plant. The PIC value for *D. chryseum* ranged from 0.28 (OPA-3 & OPA-10) to 0.44 (OPA-1 & OPA-6). (Table 2, Figure 18. B and C)

4.2.2. ISSR analysis

ISSRs marker is frequently used to analyse the genetic homogeneity or variation studies in plant biology, because of its high reproducibility (Dangi *et al.,* 2014). In the present study, two ISSR primers (HB8 and HB9) were used to evaluate the genetic profile of tissue cultured plantlets developed from various media treatments such as hormones, CW, fungal elicitor and protocorm and compared with its mother plant

(Appendix 1 and 3). All the analyzed *in vitro* samples showed genetic uniformity among themselves and with the mother plant.

Clear and reproducible band was considered for counting allele. The data were pooled into the binary matrix based on presence (1) and absence (0) of the selected bands. The number of allele and allele size per primer is recorded and compared with its mother plant (wild type). The polymorphic information content value for each primer was calculated using the formula, PIC= 2*fi *(1-fi) where, fi is the frequency of amplified allele (allele present), 1-fi is the frequency of null allele (allele absent) (Powell *et al.,* 1996). Out of two ISSR markers only one ISSR marker showed clear and reproducible bands. A total of 1 band were scored from 6 individuals (one mother plant, four rooted plantlets in *in vitro* condition and one protocorm) of *D. chryseum* . The number of scorable band is 1 within the approximate size range of 1000 bp while compare to Ladder marker (100bp sized). The amplified allele size of all *in vitro* samples were monomorphic among themselves and with their mother plant. The PIC value for *D. chryseum* is 0.94 (HB9). (Table 2, Figure 18.D)

Table 2: List of RAPD and ISSR primers used in the study for confirming the genetic fidelity of *D. chryseum* plantlets and total number of bands amplified, scorable band, monomorphism % PIC value and size of the amplified fragments generated in the mother plant and the *in vitro* regenerated plants of *D. chryseum.*

Species name and markers used	Primers name	Primer sequence	Total number of scorable band	Monomorphism %	PIC value	Amplified size range
Dendrbium chryseum (RAPD)	OPA-1	5'CAGGCCCTTC3'	2	100	0.44	490-700
	OPA-3	5'AGTCAGCCAC3'	5	100	0.28	500-1210
	OPA-6	5'GGTCCCTGAC3'	2	100	0.44	990-1100
	OPA-10	5'GTGATCGCAG3'	5	100	0.28	250-1300
Dendrbium chryseum (ISSR)	HB9	5'GTGTGTGTGTGTGG3'	1	100	0.94	~1000

Figure 17: Samples and instrument for DNA extraction and PCR. A = *In vivo* sample of *D. chryseum,* B = *In vitro* sample of *D. chryseum* (protocorm), C = *In vitro* sample of *D. chryseum* (plantlets), D = Gel electrophoresis, E = PCR machine, F= Laminar air flow (Preparation of samples for PCR).

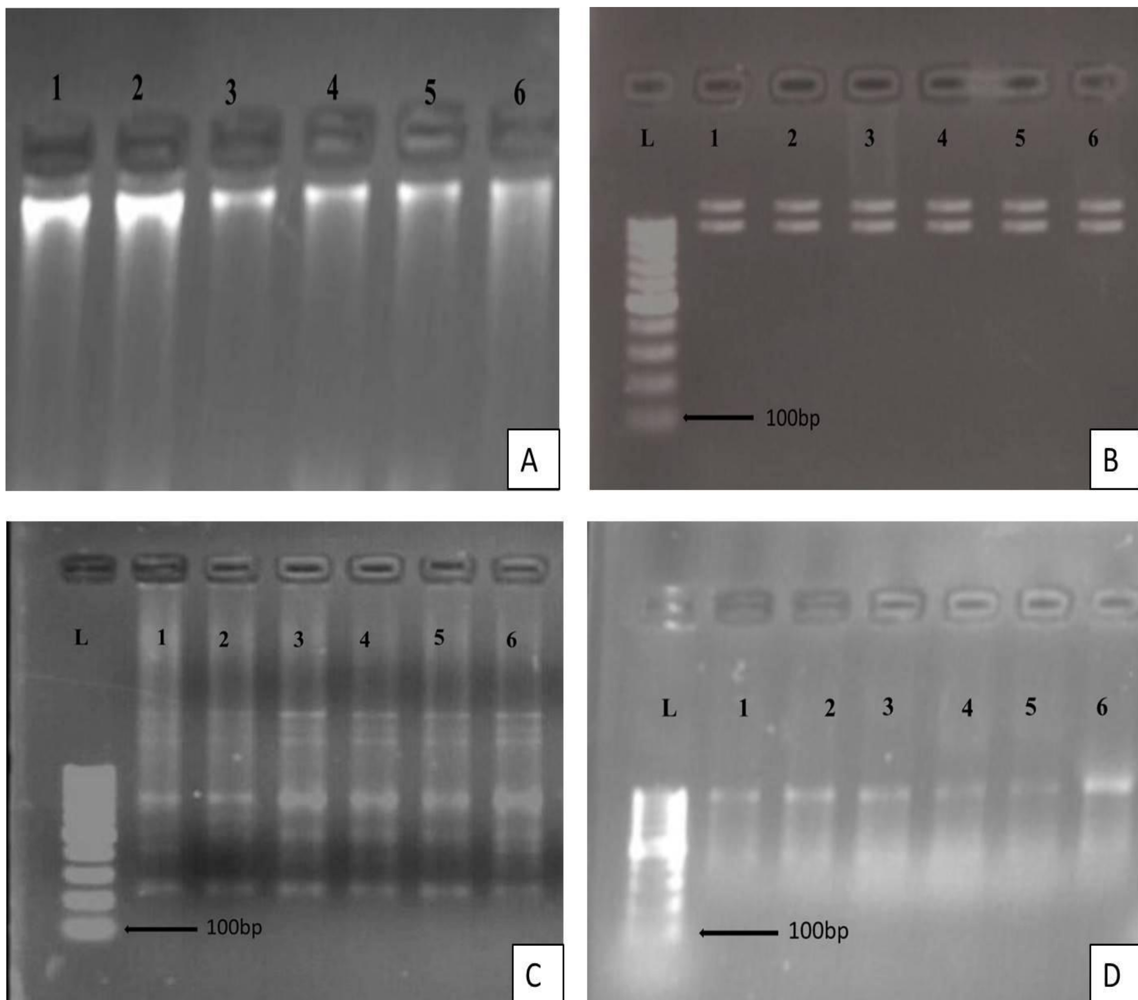

Figure 18: DNA bands and PCR bands of *in vivo* (wild) and *in vitro* plant samples of *D.chryseum* by using RAPD and ISSR primers (Genetic homogeneity assessment), A = DNA bands (1= *in vivo* samples 2-5= *in vitro* raised plant developed in MS medium supplemented by rooting hormonal combination, fungal elicitor R3, coconut water 15% and fungal elicitor R7 respectively. 6= *in vitro* raised protocorm developed in HMS medium, 1.5% Agarose gel was used, the PCR product was run with the constant voltage of 70V for 1.5 hr.) (Appendix 1), B = Banding profile of *Dendrobium chryseum* amplified by RAPD primer OPA6, C = Banding profile of *Dendrobium chryseum* amplified by RAPD primer OPA10, D = Banding profile of *Dendrobium chryseum* amplified by ISSR primer HB9.

CHAPTER V

DISCUSSION

5.1. Effect of elicitors on *in vitro* growth of *Dendrobium chryseum*

Fungal elicitors are a group of extractions and mixtures that include fungal organisms, filtrate concentrate, extracts of mycelia, and soluble components of fungal mycelia with high temperature treatment, hydrolytic products of cell walls, peptides and proteins (Smith *et al.,* 1996). Fungal elicitors are rich in specific chemical signal substances such as peptides, proteins, polysaccharides, chitin, fatty acids, oligosaccharides, glycolipids, glycoprotein and other substances (Yamaguchi and Huffaker, 2011). Different fungal elicitorshelps in increasing the biomass and secondary metabolites of plant. A fungal elicitors *Coniochaeta dendrobiicola* (R7) and *Coniochaeta africana* (R3) extracted from endophytic fungi of *Dendrobium longicornu* Lindl (Shah *et al.,* 2019) showed significant growth on root and shoot of *Dendrobium chryseum* on *in vitro* growth of plantlet.

In case of fungal elicitor (R3), microshoots of 0.5 - 1 cm were inoculated on the half and full strength MS medium supplemented with different concentration of 0.5%, 1% and 2 % shows various response. The highest number of shoots (5.75 ± 0.50 per microshoot) and roots (8.25 ± 0.50 per shoot) was obtained on half strength MS medium with 0.5% R3 while the maximum length of shoot (5.37 ± 0.15 cm) and root (5.55 ± 0.20 cm) was obtained on full strength MS medium with 1% R3.

Similarly in case of fungal elicitors (R7), microshoots of 0.5- 1cm were inoculated on the half and full strength MS medium supplemented with different concentration of 0.5%, 1% and 2 % shows various response. The highest number of shoots (4.5 ± 0.58 per microshoot) and roots (7.75 ± 0.50 per shoot) and shoot length (5.05 ± 0.1cm) was obtained on full strength MS medium with 2% R7 while the maximum length of root (4.82 ± 0.12 cm) was obtained on full strength MS medium with 0.5% R7. Similarly Full strength MS medium fortified with 0.5%, 1% and 2% of R7 was found effective in all aspects than half strength MS medium fortified with 0.5%, 1% and 2% of R7.

There are several reports that support the present work. Chand *et al.,* (2020) reported that the all the endophytic fungi isolated from *Vanda cristata* showed showed

significant growth-promoting effects on the protocorms stage of *Cymbidium aloifolium* that developed into a plantlet. All the isolated endophytic fungi showed growth-promoting activity by auxin production, ammonia synthesis, and phosphate solubilization. Shah *et al.,* (2019) reported that the endophytic fungi isolated from the roots of *Dendrobium moniliforme* showed significant growth-promoting effects on the protocorms stage of *Rhynchostylis retusa* that developed into a plantlet. A significantly higher number of roots and shoots, as well as increased root/shoot length when compared to the controls was found. Gong *et al.,* (2018) reported that the fungal elicitor isolated from *Cymbidium georingii* promoted the germination of *Cymbidium georingii* seeds and its acclimatization. It is found that different fungal elicitors increased the rate of growth and regulate the metabolism by secreting different chemical compounds which is not provided in growth medium (Yamaguchi and Huffaker, 2011). Patel *et al.* (2013) also reported that fungal elicitors used in tissue culture causes enhancement of *in vitro* plant growth. Hou and Guo (2009) reported growth promoting effect of the dark septate endophyte, *Leptodontidium* on seedlings of *D. nobile* and found the heights of shoots, number of new buds, number of roots, stem diameters and dry weights of fungal-colonized plantlets were all greater than those of uncolonized plantlets. Similarly, Warcup (1981), reported that endophytes such as *Sebacina vermifera, Tulasnella calospora, T. asymmetrica, T.cruciata, T. irregularis, T.violea* and *T. allantospora* are involved in seed germination in different orchid species. However, the use of fungal elicitors in *Dendrobium* species are new so very few reports have been reported.

However, both elicitors were found effective in comparison to control i.e, full and half strength MS medium but the comparison between both R3 and R7 shows R3 more effective in *in vitro* culture of *D. chryseum*. All the different concentration used of both elicitors gave positive respond.

Elicitor prepared by using endophytic fungi isolated from *Dendrobium longicornu* showed significant growth of *Dendrobium chryseum* in *in vitro* growth which suggested that endophytic fungi can be used as elicitor like hormones in *in vitro* conditions in the future.

5.2. Effect of different hormones, CW and Ads on *in vitro* growth of *Dendrobium chryseum*

In vitro grown shoots of about 0.5-1 cm, were cultured full strength MS media alone and with 10%, 15% and 20% CW and with auxin and cytoknin combination i.e., BAP (0.5,1 and 2 mg/L), with NAA(0.5mg/L), IAA (0.5mg/L), and Kn (0.5mg/L) and also with 10%, 15% and 20% CW. Plantlets were response variously and the result with half strength MS was not efficient in compare to full strength MS. Thus the work was continue with full strength MS medium combination. The highest number of shoots (6.25 ± 0.50 per microshoot), roots(9.75 ± 0.50 per shoot) and length of shoot (6.7 ± 0.14 cm) and root (6.3 ± 0.11cm) was found on full strength MS with 15% CW.

The coconut water is the liquid endosperm containing amino acids, organic acids, nucleic acids, vitamins, carbohydrates, growth hormone (auxin and cytoknins), minerals and other substances that can improve the quality of growth plantlets (Molnar *et al.,* 2011). Hence in the present investigation, on the basis of development of strong shoot and root system, MS medium supplemented with 15% CW was considered as the most effective condition for root and shoot development of *D. chryseum.*

The present result was supported by Utami *et al.,* (2017) who reported that VW medium with 15% CW was found effective in shoot and root development compared to other treatments in *Dendrobium lasianthera.* Similarly Utami and Hariyanto (2019) reported that 15% (v/v) coconut water added together with banana homogenate (10 g/L) to the VW medium, showed plantlets with highest length in root and shoot and their numbers incompare to other treatments in *Phalaenopsis amboinensis.* Al – khayri *et al.,* (1992) also reported that MS medium supplemented with 15% CW significantly improved callus growth, shoot-regenerative capacity, and shoot growth in *Spinacia oleracea* L. Inpeuy *et al.,* (2011) reported that BAP and other cytoknins were not effective in induction of multiple shoots whereas CW at 15% (v/v) promoted a healthy development of shoots with normal leaves in oil palm. The present is partially supported by Hartati *et al.,* (2017). They reported MS medium supplemented with 0.1 mg/ L of BAP, 1.0 mg/ L of NAA and 15% CW is an important factor in the propagation of *Dendrobium* 'Sonia'. Romeida *et al.,* (2018) reported that MS medium supplemented with 10% CW showed dominant responses on all PLBs development

becoming plantlets variables of *Eulophia graminea*, including plantlet height, leaf number, root number and root length than those of banana or potato. Baque *et al.,* (2011) reported that Hyponex media with 5% coconut water is beneficial for *in vitro* propagation of Calanthe hybrid and *Anoectochilus formosanus*.

Rooting of *in vitro* regenerated shoots is a critical step in any micropropagation protocol. The roots developed should be hardy enough to support the plantlets on being transferred from *in vitro* conditions to ambient ones. Generally, an auxin or rarely a combination of auxins is used for the rooting of shoots of orchids.

In case of rooting hormones *in vitro* grown shoots of about 0.5 - 1 cm were cultured on half and full strength MS medium alone and with rooting hormones like were IAA (1 and 1.5 mg/L), IBA (1 and 1.5 mg/L) and NAA (1 and 1.5mg/L) on both half and full MS Medium and also 10% CW with 1mg/L IBA. Plantlets were response variously for the root development in different concentration of auxin. The highest number of shoots (4.00 ± 0.00) per microshoot and roots (7.75 ± 0.00) per shoot was found on half strength 1mg/L IBA but the maximum length of shoot (4.95 ± 0.1cm) was obtained on half strength MS medium supplemented with 1 mg/L NAA and the maximum length of roots (5 ± 0.00cm) was also obtained on full strength MS medium supplemented with 1.5 mg/L IBA. Hossain *et al.,* (2010) reported development of solid root system from PLBs and shoot buds of *Cymbidium giganteum* inoculated on half strength of Phytamax or Mitra‘s medium supplemented with 0.5mg/l IAA which partially support our result.

In *Vanilla planifolia*, IBA alone at 0.5mg/l proved to be the best in inducing the highest number of roots along with good length in small time (Philip and Nainar, 1986). The efficiency of IBA in root induction has also been observed in *Cymbidium pendulum* (Nongdam *et al.,* 2006). These results are also consistent with the findings of Mohanty *et al.,* (2012), who successfully rooted regenerated shoots of *Dendrobium nobile* by transferring them to MS medium containing 1.5 mg/l IBA. Likewise, *in vitro* shoots of *Dendrobium thyrsiflorum* rooted best on 1/2 MS medium supplemented with 1 mg/l IBA and 0.5 mg/l phloroglucinol (Bhattacharya *et al.,* 2015). However, IAA and IBA were not always effective in inducing roots in many species of orchids. Sheelavanthmath *et al.,* (2000) reported ineffectiveness of IAA and

IBA in induction of roots from shoots of *Geodorum densiflorum* , 100% of which developed shoots on medium containing NAA (1µM).

There are more reports that support the present work. Pant and Swar (2011) obtained the highest number of roots on MS medium supplemented with IBA (1 mg/L) in *Cymbidium iridioides*. Pradhan and Pant (2009) reported that MS medium supplemented with IBA (1.5 mg/L) favoured the rooting of *Dendrobium densiflorum.* Koirala, (2007) found that MS medium supplemented with IBA (1 mg/L) was best for rooting in *Coelogyne fuscescens*. Asghar *et al.,* (2011) obtained maximum number of roots on modified MS medium fortified with various concentration of IBA than in NAA in *Dendrobium nobile*. Nayak *et al.,* (1997) reported that MS medium supplemented with 10.8 µM IBA was most effective for inducing strong root from *Cymbidium aloifolium, Dendrobium aphyllum and Dendrobium moschatum.* Nongdam and Chongtham (2011) found that MS basal medium alone and MS medium supplemented with 0.5 mg/L IBA or NAA was effective for maximum development of roots in *C. aloifolium.*

Similarly, in case of MS medium fortified with 0.1% of adenine sulphate *in vitro* grown micro shoots of about 0.5 - 1 cm were cultured on half and full strength MS medium alone and with 0.1% Ads and with different concentration of hormones like BAP (0.5 and 1 mg/L), NAA (0.5 and 1mg/L), Kn (0.5 and 1 mg/L) alone and in combination. The best result was on full strength MS medium supplemented with 0.5 mg/L NAA with 0.1% Ads with highest shoot number (2.5 ± 0.57 per microshoot), root number (6.55 ± 0.57 per shoot), maximum length of shoots (4.87 ± 0.09cm) and root (2.0 ± 0.05cm). However, all of the shoots could not attain considerable height to be utilized for root initiation for complete plantlet production.

According to Murashige, (1974) adenine in the form of adenine sulphate can stimulate cell growth and greatly enhance the shoot formation. It is apparent from the result that Ads was found to reinforce the effect of other PGRs as implied by the enhancement in shoot proliferation (Gatica Arias *et al.,* 2010). This may be due to adenine sulphate acting as precursor for cytoknin synthesis or enhancing the biosynthesis of natural cytoknins. Alternatively, adenine sulphate may act synergistically as a cytoknin such as Kn or zeatin (Bantawa *et al.,* 2009). Ads also provides an additional source of

nitrogen to cells, which can be taken up more rapidly than inorganic nitrogen (Hussain *et al.,* 2006).

The present result is partially supported by Sharma and Vimala, (2010) in which they reported use of Ads with BAP enhances shoot multiplication in *Centella asistica*. Similarly, Jana and Shekhawat, (2011)) also reported multiple shoot induction using BAP and Ads in *Anethum graveolens*. However Naaz *et al.,* (2014) also reported the best combination for multiple shoots was BAP with GA3 and Ads in *Syzygium cumini.* However the use of Ads in orchid tissue culture is very new to know its overall effect in shoot and root. The present result suggest that in *D. chryseum* use of auxin with Ads is effective for both shoot and root number and their length and the combination of auxin alone BAP and with cytoknin is not that effective as compare to auxin alone i.e NAA. In case of *D. chryseum* the use of Ads was found least effective in compare to hormones and elicitors.

Maharjan *et al.,* (2020) reported the media that was best for shoot multiplication (18.75 ± 0.48 shoots per culture) was half strength MS medium fortified with 2.0 mg/L Kn and 10% CW, whereas the media that supported the most extended shoots (2 ± 0.20 cm) per culture and roots (4.5 ± 0.65) per culture was half strength MS medium fortified with 1.0mg/L GA3 and 10% CW The longest roots (1.28 ± 0.14 cm) were observed on ½ MS medium supplemented with 0.5 mg/L GA3 and 10% for *D. chryseum.* However, in present investigation we have better result in all aspect of shoot, root number and their length. The media that was best for maximum shoot number (6.25 ± 0.50 per microshoot) and root number (9.75 ± 0.50 per shoot) was full strength MS medium fortified with 15% CW along with longest shoot (6.7 ± 0.14cm) and root (6.3 ± 0.11cm) also in it. The reason behind the difference in result may be the use of explant as Maharjan *et al.,* (2020) used protocorm and many combination of plant growth regulators where not used. In present research we worked with microshoots as explant as well with wide range of PGRs and their combination alone and with and without CW which helped to developed effective propagation strategy for *D. chryseum.*

5.3. Molecular analysis (genetic fidelity)

In order to conserve orchids, plant tissue culture techniques have been successfully applied for their clonal propagation and conservation (Tandon and Kumaria, 1998).

However, for large-scale propagation, efficiency of propagation methods along with genetic stability of the regenerated plants is of paramount importance (Haisel *et al.,* 2001). Reports have shown that the regenerated plants might not always be clonal copies of their mother plant when passed through micropropagation pathways (Devi *et al.,* 2014). The presence of cryptic genetic defects occurring due to somaclonal variations can deregulate the broader utility of the *in vitro* propagation system (Salvi *et al.,* 2001). The occurrence of clonal variability is due to various causes of which explant source and types of plant growth regulators (PGRs) used plays a pivotal role (Devi *et al.,* 2014).

Conventional molecular markers like random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), and simple sequence repeats (SSR) are used extensively and as efficient tool in the assessment of clonal fidelity in a wide range of plant including orchids because of their high reproducibility (Bhattacharyya *et al.,* 2014; Devi *et al.,* 2014). Moreover it is advocated to use more than one molecular marker in order to evaluate clonal fidelity of tissue culture-raised plants (Palombi and Damiano, 2002). Thus, in the present research, RAPD and ISSR will be used to evaluate the clonal fidelity of the regenerates, where the former detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence and the latter permits detection of polymorphism in inter-microsatellite loci using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Parab and Krishnan, 2008).

In the present study, a set of 10 RAPD and two ISSR primers were used to evaluate genetic profile of tissue cultured plantlets of *D. chryseum* developed from various media treatments such as hormones, CW, fungal elicitors(R3 and R7) and protocorm are compared with its mother plant. *D.chryseum* showed fair genetic uniformity. The high homogeneity obtained among the *in vitro* regenerates and mother plant of medicinal orchid *D. chryseum* in our study could be attributed to the proper supplement of growth hormones and additives, regular monotoring of *in vitro* raised plants (such as age of subculture, number of subculture passage), avoidance of callus formation and use of the same mother plant sample from which the pod was obtained for seed culture as explant.

Greater polymorphisms are indicative of greater genetic variations. However the present investigation revealed the presence of higher genetic similarity than polymorphism in *D. chryseum*. RAPD and ISSR markers showed 100% of monomorphism among and within the *in vitro* cultured plants. The present result is supported by Giri *et al.,* (2012) who found no variation in ISSR profiles among the *in vitro* regenerated plantlets of *Habenaria edgeworthii* (Terrestrial orchids) derived through 6-benzyladenine (BAP) and a-naphthalene acetic acid (NAA) treatments. Bhattacharyya *et al.,* (2018) revealed a high degree of genetic homogenity in inter-retrotransposon amplified polymorphism (IRAP) and start codon targeted (SCoT) profie among the *in vitro* regenerated plantlets of *Ansellia africana* (Leopard orchid) derived through TDZ and NAA treatments. Similarly Roy *et al.,* (2012) found 5.81% variation in RAPD profile among the *in vitro* regenerants of *Cymbidium giganteum* derived through thidiazuron (TDZ) treatment. Sherif *et al.,* (2018) found that the homogeneity is comparatively higher in direct somatic embryo regenerated plants (94.22%) as compared to plants elevated from an indirect somatic embryo (93.05%) in ISSR profile of *in vitro* regenerated plantlets of *Anoectochilus elatus* derived through treatment with TDZ, morel vitamins and NAA. Bhattacharyya *et al.,*(2014a) revealed 97% of genetic fidelity in RAPD and SCoT profile among the regenerants of *Dendrobium nobile* derived through TDZ treatment.

Sometimes RAPD markers fail to reveal changes in the repetitive regions of the genome of some species (Palombi & Damiano, 2002). Therefore, the two marker systems employed in the present investigation is used to crosscheck and validate the degree of genetic variability of test genotypes (Bhattacharyya & Kumaria, 2015). The PIC values further determine the efficacy of the markers used in the genetic fingerprinting assay. PIC provides an estimate of the discriminatory power of a gene or locus in a population (Smith *et al.,* 1997). The average PIC value of RAPD and ISSR was found 0.47 demonstrating the low polymorphism. The PIC values of the primers used in present investigation strengthen the significance of present findings. Hence, the data reported here based on the two marker systems was found almost similar and revealed the existence of no genetic variation among and within the mother and *in vitro* regenerated plants of *D. chryseum*

The high level of genetic homogeneity obtained among *in vitro* regenerants and mother plant of medicinal orchid *D. chryseum* in the present study fairly support the

applicability of tissue culture protocol for true to type plant production and conservation of the endangered medicinal orchid. However, few investigation have been conducted to identify genetic variations in the *in vitro* generated plants of orchids and to our knowledge; no such report is available on *Dendrobium chryseum.*

CHAPTER VI

CONCLUSION

In vitro mass propagation of *Dendrobium chryseum* was done by using different culture techniques and RAPD and ISSR markers were used to access its genetic homogeneity.

The most suitable medium for *D. chryseum* for shoot number was on full strength MS medium fortified with 15% CW (6.25 ± 0.50) per microshoot which is followed by half strength MS medium fortified with fungal elicitor 0.5% R3 (5.75 ± 0.50) per microshoot. Similarly the most suitable medium for *D. chryseum* for root number was on full strength MS medium fortified with 15% CW (9.75 ± 0.50) per shoot which is followed by half strength MS medium fortified with fungal elicitor 0.5% R3 (8.25 ± 0.5) per shoot.

The most appropriate medium for shoot length of for *D. chryseum* was on full strength MS medium fortified with 15% CW (6.7 ± 0.14 cm) which is followed by full strength MS medium fortified with fungal elicitor 1% R3 (5.37 ± 0.15cm). Similarly the most appropriate medium for root length of for *D. chryseum* was on full strength MS medium fortified with 15% CW (6.3 ± 0.11cm) which is followed by full strength MS medium fortified with fungal elicitor 1% R3 (5.55 ± 0.20cm).

High genetic homogeneity (100%) monomorphism was found among and within the mother and *in vitro* regenerated plantlets of *D. chryseum* and analyzed by using RAPD and ISSR markers.

Thus, the efficient plant regeneration system developed in this study might be useful for conservation and homogenous controlled production of this important ornamental orchid species. The current approach appears to be a promising approach for germplasm conservation and large clonal homogenous micropropagation of *D. chryseum.* However, few investigations have been conducted to identify genetic variations in the *in vitro* generated plants of orchids and to our knowledge; no such report is available on *D. chryseum.*

CHAPTER VII

RECOMMENDATION

Some recommendation can be outlined from this present investigation, which are as follows:

- Technology developed from this research findings can be transformed for its commercialization and *ex situ* conservation which will be helpful to upgrade the economic status of rural people by cultivation of tissue cultured raised *D. chryseum* and reintroduction from lab to land (nature) can be done for species restoration programme.
- Comparison between different explants (stem, leaves, infloresences) could be tested for proper assessment of *in vitro* conservation scope.
- Molecular characterization of acclimatized plants can be carried out as the plants were not transplanted in their natural sites or acclimatized in this research work.
- Extensive phytochemistry and biological activity research can be carried out as these species can be proved medicinal.
- Isolation and identification of fungi from roots, stem and leaves of *D. chryseum* on different media will be a novel work.
- Treatment of *in-vitro* orchid plantlets with fungal elicitor prepared from isolated fungi of *D. chryseum* on *in vitro* culture and acclimatization could be tested for proper assessment of *in vitro* conservation scope.
- Molecular work like protein coding and gene sequencing of isolated fungi from *D. chryseum* can reveal even greater knowledge to study this species.

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APPENDIX

Appendix 1: Plant samples used for DNA extraction of *Dendrobium chryseum.*

Plant	No. of samples	Sample source
	1	*In vivo* (wild) plant, Natural habitat
	2	*In vitro* plant regenerated on MS solid medium supplemented with rooting hormone
Dendrobium chryseum	3	*In vitro* plant regenerated on MS solid medium supplemented with fungal elicitors R3
	4	*In vitro* plant regenerated on MS solid medium supplemented with 15%CW
	5	*In vitro* plant regenerated on MS solid medium supplemented with fungal elicitors R7
	6	*In vitro* raised protocorm on half strength MS solid medium

Appendix 2: The sequences of oligonucleotides primers used for RAPD analysis and PCR amplicons obtaines from *in vivo* and *in vitro* grown *Dendrobium chryseum.*

S.N.	Primer name	Primer sequence	Result of DNA Amplification
1	OPA-01	5'CAGGCCCTTC3'	Clear band/s
2	OPA-02	5'TGCCGAGCTG3'	Absent
3	OPA-03	5'AGTCAGCCAC3'	Clear band/s
4	OPA-06	5'GGTCCCTGAC3'	Clear band/s
5	OPA-10	5'GTGATCGCAG3'	Clear band/s
6	OPP-01	5'GTAGCACTCC3'	Absent
7	OPP-02	5'TCGGCACGAC3'	Absent
8	0PP-03	5'GTGATACGCC3'	Absent
9	OPP-04	5'GTGTCTCAGG3'	Absent
10	OPP-05	5'CCCCGGTAAG3'	Absent

Appendix 3: The sequences of oligonucleotides primers used for ISSR analysis and PCR amplicons obtaines from *in vivo* and *in vitro* grown *Dendrobium chryseum.*

S.N.	Primer name	Primer sequence (5' to 3')	Result of DNA Amplification
1	HB-8	GAGAGAGAGAGAGG	Absent
2	HB-9	GTGTGTGTGTGTGG	Clear band/s

Appendix 4: One Way ANOVA by Tukey test for *in vitro* development of shoots and roots of *Dendrobium chryseum* on full and half strength MS medium alone and with different concentration of fungal elicitor (R3).

Growth parameters (shooting)		**Sum of Square**	**Df**	**Mean square**	**F**	**Sig.**
Shoot	Between Groups	182.469	7	26.067	147.202	0.000
number	Within Groups	4.250	24	0.177		
	Total	186.719	31			
Root	Between Groups	209.500	7	29.929	176.571	
number	Within Groups	4.00	24	0.167		
	Total	213.500	31			
Shoot	Between Groups	67.5	7	9.643	740.568	0.000
length	Within Groups	0.312	24	0.013		
	Total	67.812	31			
Root	Between Groups	64.839	7	9.263	444.609	0.000
length	Within Groups	0.5	24	0.021		
	Total	65.339	31			

Appendix 5: One Way ANOVA by Tukey test for *in vitro* development of shoots and roots of *Dendrobium chryseum* on full and half strength MS medium alone and with different concentration of fungal elicitor (R7).

Growth parameters (shooting)		**Sum of Square**	**Df**	**Mean square**	**F**	**Sig.**
Shoot	Between Groups	27.500	7	3.929	15.714	0.000
number	Within Groups	6.00	24	0.250		
	Total	33.500	31			
Root	Between Groups	182.469	7	26.067	147.202	0.000
number	Within Groups	4.250	24	0.177		
	Total	186.719	31			
Shoot	Between Groups	58.707	7	8.387	766.788	0.000
length	Within Groups	0.263	24	0.011		
	Total	58.97	31			
Root	Between Groups	70.732	7	10.105	873.911	0.000
length	Within Groups	0.277	24	0.012		
	Total	71.01	31			

Appendix 6: One Way ANOVA by Tukey test for *in vitro* development of shoots and roots of *Dendrobium chryseum.*on full and half strength MS medium alone and with different concentration of auxin and cytoknin alone and with CW.

Growth parameters (shooting)		**Sum of Square**	**Df**	**Mean square**	**F**	**Sig.**
Shoot	Between Groups	133.330	21	6.349	25.017	0.000
number	Within Groups	16.750	66	0.254		
	Total	150.080	87			
Root	Between Groups	349.818	21	16.658	75.823	0.000
number	Within Groups	14.500	66	0.220		
	Total	364.318	87			
Shoot	Between Groups	113.802	21	5.419	647.354	0.000
length	Within Groups	0.553	66	0.008		
	Total	114.354	87			
Root	Between Groups	153.023	21	7.287	728.682	0.000
length	Within Groups	0.66	66	0.01		
	Total	153.683	87			

Appendix 7: One Way ANOVA by Tukey test for *in vitro* development of shoots and roots of *Dendrobium chryseum* on full and half strength MS medium alone and with different concentration of auxin

Growth parameters (shooting)		**Sum of Square**	**Df**	**Mean square**	**F**	**Sig.**
Shoot number	Between Groups	36.933	14	2.638	10.552	0.000
	Within Groups	11.250	45	0.250		
	Total	48.183	59			
Root number	Between Groups	394.233	14	28.160	144.820	0.000
	Within Groups	8.750	45	0.194		
	Total	402.983	59			
Shoot length	Between Groups	74.832	14	5.345	934.107	0.000
	Within Groups	0.258	45	0.006		
	Total	75.09	59			
Root length	Between Groups	67.714	14	4.837	2024.681	0.000
	Within Groups	0.107	45	0.002		
	Total	67.822	59			

Appendix 8: One Way ANOVA by Tukey test for *in vitro* development of shoots and roots of *Dendrobium chryseum* on full and half strength MS medium alone and with 0.1% of Ads along with different concentration of auxin and cytoknin.

Growth parameters (shooting)		**Sum of Square**	**Df**	**Mean square**	**F**	**Sig.**
Shoot number	Between Groups	16.577	12	1.381	6.338	0.000
	Within Groups	8.500	39	0.218		
	Total	25.077	51			
Root number	Between Groups	119.269	12	9.939	41.905	0.000
	Within Groups	9.520	39	0.237		
	Total	128.519	51			
Shoot length	Between Groups	58.148	12	4.846	155.861	0.000
	Within Groups	1.213	39	0.031		
	Total	59.361	51			
Root length	Between Groups	14.433	12	1.203	271.92	0.000
	Within Groups	0.173	39	0.004		
	Total	14.605	51			