In Vitro Propagation of Three Horticultural Important Orchids of Nagaland and Assessment of Genetic Fidelity of Regenerates

by

Temjennokcha B. Longchar

Submitted to

NAGALAND UNIVERSITY

In Partial Fulfilment of the Requirements for Award of the Degree of

DOCTOR OF PHILOSOPHY IN BOTANY

DEPARTMENT OF BOTANY
NAGALAND UNIVERSITY
LUMAMI-798627
NAGALAND, INDIA

2022

नागालैण्ड विश्वविद्यालय

Nagaland University

(A Central University Estd. By the Act of Parliament No. 35 of 1989)
Lumami 798 627, Nagaland, India
Department of Botany

December 19, 2022

DECLARATION

I, Mr. Temjennokcha B. Longchar bearing Ph.D. Registration No. 780/2017 dated June 28, 2016 hereby declare that the subject matter of my Ph. D. thesis entitled **"*In Vitro* Propagation of Three Horticultural Important Orchids of Nagaland and Assessment of Genetic Fidelity of Regenerates"** is the record of work done by me, and that the contents of this thesis did not form the basis for award of any previous degree to me or to anybody else known to the best of my knowledge. This thesis has not been submitted by me for any other Research Degree in any other University/Institute.

This Ph. D. thesis is submitted in compliance with the UGC Regulation 2016 dated May 05, 2016 (Minimum Standard and Procedure for Award of M. Phil. /Ph. D. Degree). It is further certified that the content of the thesis is checked for 'Plagiarism' with licensed software 'URKUND' and satisfies with the norms of 'University Grants Commission, Govt. of India'. This thesis is being submitted to the degree of 'Doctor of Philosophy in Botany'.

(Mr. Temjennokcha B. Longchar)
Candidate

(Porf. Chitta Ranjan Deb) 19/12/22
Supervisor
Prof. Chitta Ranjan Deb
Department of Botany
Nagaland University
Lumami-798627. Nagaland, India

(Prof. N. S. Jamir)
Co-Supervisor

(Prof. Chitta Ranjan Deb) 19/12/22
Head, Department of Botany
Head
Department of Botany
Nagaland University
Hqs. Lumami.

Acknowledgement

With the completion of this thesis, I would like to foremost thank our almighty God for blessing me with wisdom, good health, and guidance throughout my research journey.

I would like to express my heartfelt gratitude to my supervisor, Professor Chitta Ranjan Deb, Supervisor and Head, Department of Botany for his unwavering guidance, support, patience, advice and faith in me.

I am also thankful to Professor N.S. Jamir, Co- supervisor for his support and encouragement as well as all of the faculty members for their constant encouragement, support and the necessary facilities provided by the department throughout my research.

I am extremely thankful to Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi for financial support through a research grant to C. R. Deb (File No.: DBT/NER/Agri/23/2013) and UGC for Non-Net fellowship for the financial support.

My gratitude to UGC-SAP(DRS-III), DST-FIST and DBT supported Advance Level Institutional Biotech Hub (File No. BT/22/NE/2011) programmes for the facilities provided

My heartfelt thanks Dr. Toshinungla Jamir and Dr Bendangnaro Jamir, for their support and research

I would like express my thanks to all of the non-teaching staffs for their immense support through my research period.

My greatest in debtedness goes to my labmates Dr. Aolemla pongener, Miss Bendangsenla Pongener, Miss Maman Megu, Miss Thejano Nugilli, Miss Mum Tatung, Mr. Thejavitsii Noah Viphru, Miss Ibeeka Sharma, Miss Roko Nagi for their unwavering support and encouragement throughout my work. My heartfelt thanks to all my research scholar friends for their wonderful friendship and encouragement.

I would like to give my heartfelt thanks to my family members and friends whose love, support, guidance and prayers have encouraged and pushed me to achieve where I have reached now.

Last but not the least, I want to acknowledge everyone who have directly or indirectly influence and contributed in furnishing to complete this research work.

Temjennokcha B. Longchar

NAGALAND UNIVERSITY

(A Central University Estd. By the Act of Parliament No. 35 of 1989)
Headquarters: Lumami – 798627, Nagaland, India

Ph.D. Thesis Certificate on Plagiarism check

Name of the Research Scholar	Temjennokcha B. Longchar
Ph. D. Registration Number	780/2017 dated June 28, 2016
Title of Ph. D. thesis	*In Vitro* Propagation of Three Horticultural Important Orchids of Nagaland and Assessment of Genetic Fidelity of Regenerates
Name & Institutional Address of the Supervisor	Nagaland University, Lumami-79862 Nagaland, India
Name of the Department and School	Department of Botany
Date of submission	2022-12-19
Date of plagiarism check	2022-12-14
Percentage of similarity detected by the **URKUND** software	2%

1. I hereby declare that/certify that the Ph.D. Thesis submitted by me is complete in all respect as per the guidelines of Nagaland University (NU) for this purpose. I also certify that the Thesis (soft copy) has been checked for plagiarism using **URKUND** similarity checked software. It is also certified that the contents of the electronic version of the thesis are the same as the final hardcopy of the thesis.
2. Copy of the report generated by the **URKUND** software is also enclosed.

Place: Lumami

Date: 19/12/22

Name & Signature of the Supervisor:
With seal

Prof. Chitta Ranjan Deb
Department of Botany
Nagaland University
Lumami-798627. Nagaland, India

Temjennokcha B. Longchar.
(Name & Signature of the Scholar)

Plagiarism report

Document Information

Analyzed document	Temjennokcha B Longchar Compliled thesis.docx (D153317043)
Submitted	2022-12-14 08:59:00
Submitted by	Prof. Chitta Ranjan Deb
Submitter email	debchitta@rediffmail.com
Similarity	2%
Analysis address	debchitta.naga@analysis.urkund.com

Sources included in the report

SA	**STUDIES ON IN VITRO PROPAGATION, CONSERVATION AND PHYTOCHEMICAL ANALYSIS OF SOME MEDICINALLY IMPORTANT ORCHIDS OF HIMACHAL PRADESH (Anamika_Kumari).pdf** Document STUDIES ON IN VITRO PROPAGATION, CONSERVATION AND PHYTOCHEMICAL ANALYSIS OF SOME MEDICINALLY IMPORTANT ORCHIDS OF HIMACHAL PRADESH (Anamika_Kumari).pdf (D145453861)	5
SA	**Dawande Varsha PhD Botany 20-1-17.pdf** Document Dawande Varsha PhD Botany 20-1-17.pdf (D25145849)	1
SA	**Tadu Yaniya dissertation.doc** Document Tadu Yaniya dissertation.doc (D80046498)	2
SA	**PPaulPhDBotany.pdf** Document PPaulPhDBotany.pdf (D47638807)	5
SA	**Taxonomic in vitro propagation and phytochemical studies in three medicinally important orchids of meghalaya by Gargi Prasad.pdf** Document Taxonomic in vitro propagation and phytochemical studies in three medicinally important orchids of meghalaya by Gargi Prasad.pdf (D141056387)	3
SA	**CONSERVATION FOR SUSTAINABLE ENVIRONMENT-Girish.docx** Document CONSERVATION FOR SUSTAINABLE ENVIRONMENT-Girish.docx (D97008585)	2
SA	**KU-TH-SSC-BIO-SAMEERA PARVEEN-18.pdf** Document KU-TH-SSC-BIO-SAMEERA PARVEEN-18.pdf (D41615872)	2
SA	**Nagaland University, Kohima / 20160616-Dendrobium_densiflorum_Lindl_paper.docx** Document 20160616-Dendrobium_densiflorum_Lindl_paper.docx (D21351461) Submitted by: t.temjen@gmail.com Receiver: t.temjen.naga@analysis.urkund.com	5
SA	**Clonal propagation germplasm conservation and DNA fingerprinting pattern in ... etc.pdf** Document Clonal propagation germplasm conservation and DNA fingerprinting pattern in ... etc.pdf (D34924813)	1
SA	**Tikendra_Biotech_Thesis_MU.pdf** Document Tikendra_Biotech_Thesis_MU.pdf (D56291781)	6

Sl. No. : 16- 62505

NAGALAND UNIVERSITY

STATEMENT OF MARKS

Ph. D COURSE WORK EXAMINATION 2016

DEPARTMENT OF BOTANY

The following are the marks secured by Mr. ***Temjennokcha B. Longchar***
Roll No. **04/16** *of Ph.D Course Work Examination held in* **2016**

Subject(s)/Paper(s)	Max. Marks	Minimun Qualifying Marks	Marks Secured
Paper No. B.Ph.D -1 Research Methodology	100	35	75
Paper No. B.Ph.D -02 Elective Paper	100	35	64
Paper No. B.Ph.D -03 Review of literature, Report Writing & Seminar	100	35	87
Total Aggregate Marks			226
Average Pass Mark – 55 %			

Result	Division	Percentage
Passed	**I Division**	**75.33 %**

NAGALAND UNIVERSITY
H.Q. LUMAMI

Marks compared by

COE/Dy. Reg./AR (Exams)

NAGALAND UNIVERSITY

HEAD QUARTERS : LUMAMI

Ph. D COURSE WORK EXAMINATION

This is to certify that Mr/Ms. **Temjennokcha B. Longchar**

of Nagaland University bearing Roll No. **04/16** *is qualified in the Ph.D Course Work Examination*

in the Department of **Botany**, *Nagaland University held in the Year 20* **16**

Head of Department
Head
Department of Botany
Nagaland University
Hqs. Lumami.

Dean
School of Sciences
Nagaland University
Hqs. Lumami Nagaland

List of Tables

List of figures

CONTENTS

Chapter - 1

Introduction

Orchids are one of the most beautiful, fascinating, and peculiar variety of angiosperms. Due to their numerous distinctive morpho-physiological physiognomies, including their various flower colours, sizes, and shapes, tiny non-endospermic seeds, pollination, obligate dependence on particular mycorrhizal fungi for their natural germination, and Crassulacean acid metabolism, orchids are a highly advanced group of plants (Zhang et al., 2016). Orchids are currently used in floriculture, pharmaceuticals, and the food business, generating millions of dollars in revenue. The orchid was first domesticated by the Chinese and called it the "King of Fragrant Plants". Theophrastus (370–287) named the orchids after the Greek word for testicles, orchis, since the underground tubers of many terrestrial orchids in Europe resemble a pair of testicles. He discussed orchids' therapeutic qualities in his book "Enquiry into Plants" (Bulpitt, 2005).

Orchids have been described as the ‘Royal Family’ of plants by those captivated by their exquisite flowers of myriad shapes, sizes and colours (Behera et al., 2013). Orchids are easily recognized by the distinctive structure of their flowers which have three petaloid sepals and three petals, one of which is modified into an often-extravagant form and colour *i.e.*, the labellum. The size of the flowers varies from the tiny 0.15 cm of the *Oberonia* genus, which are found in tropical areas of the southern hemisphere, to the 10 cm blooms of *Pecteilis susannae* in Thailand, the 12 cm blooms of *Coryanthes speciosa*, a type of bucket orchid, in Central and South America, and the extraordinarily long petals of *Paphiopedelum sanderiamun* in Borneo, which can reach over 1 m (Jones, 2006,

Ballantyne and Pickering, 2011). Orchids are classified as epiphytes, lithophytes, saprophytes, and terrestrial based on their habitat. They are further sub-divided as monopodial and sympodial based on their vegetative structures. According to the World Conservation Union, two-thirds of orchid's species are epiphytes and lithophytes with remaining third as terrestrial species (IUCN, 1999). The distribution of orchids is not uniform; however, they can be found in all parts of the world except in Polar Regions, deserts, aquatic and marine ecosystems. According to the mode of distribution, orchids could broadly be grouped as tropical and temperate (Behera et al., 2012). The distribution varies widely between continents and within regions even in the Tropics and areas of particular species abundance follow closely those of high plant diversity outlined by Myers et al., 2000. The Northern Andes of South America, the mountains of the narrow neck of meso-America, Madagascar, Indo-China and SW China, Sumatra, Borneo, New Guinea, and temperate South West Australia are all particularly rich in orchids (Wood et al., 1993). In India, Orchids are found in abundance in Peninsular India, especially Western Ghats, Palni Hills and Nilgiris, Eastern Himalayas and North Eastern region (Hedge, 1997).

Orchids belong to the family Orchidaceae, which is one of the largest vascular plants, with 736 genera and 28,000 species and the number of species in Orchidaceae is increasing with record of new species (Chase et al., 2015; Christenhusz and Byng, 2016). They are the most varied family of angiosperms, accounting up to about 10% of all blooming plants on Earth (Cribb et al., 2003). In India, the strength of the orchid-flora is estimated between 127 – 184 genera and 810-1229 species by various workers like Pardhan (1976, 1979), Bose and Bhattacherjee (1980), Kumar and Manilal (1994) and Karthikeyan (2000), but the latest report by Singh et al. (2019) have documented about 1256 taxa from 170 genera. North- eastern India is home to about 650 species of orchids and also records equally high endemic species (Hegde, 1997). According to Ninawe and

Swapna (2017), Arunachal Pradesh has the most orchid species in North East India, with 577 species across 147 genera, followed by Sikkim with 561 species across 144 genera, Meghalaya with 380 species in 113 genera, Nagaland with 387 species across 107 genera, Manipur with 314 species in 93 genera, Mizoram with 253 species across 86 genera, Assam with 231 species across 82 genera, and Tripura with 39 species of orchids. The history of orchid documentation of Nagaland starts with C.B. Clarke (1889) followed by J.D. Hooker (1890) and recent comprehensive accounts published by Chankija et al. (1992), Deorani and Naithani (1995), Hynniewta et al. (2000), Deb and Imchen (2008), Singh et al. (2019). The most recent update of orchid flora by Deb et al. (2021) in the state is represented by 423 species and one variety under 108 genera.

1.1 Orchids in floriculture

The incredible shapes and colour of flowers and their long vase life have made orchids a much sought-after plant all over the world (Joshi et al., 2009). There has been a significant increase in hybrid production since the creation of the first man-made hybrid, Calanthe Domyni, in 1856 (Nirmala et al., 2006). Hence, orchids are considered as 'Gems' of floriculture trade and over 85,000 man-made registered hybrids have been produced because of the ease in hybridizing orchids (Hedge, 1984). Orchid cultivation has been revolutionized with the discovery of tissue culture technology and meri-cloning making it a lucrative cut flower industry of considerable economic importance, elsewhere in the world today (Morel, 1960, 1964, Hedge, 1987). Orchids of both cut flowers and pot plants are now sold noticeably in international market. Its commercial demand has now made it an exclusive trade market. Thailand is the largest producer with an export destination to USA and Europe earning as high as 4 million US dollars by 1995 (Prasad and Prasad, 1998). Thailand is the largest producer of the tropical *Dendrobium*, *Vanda,* and *Paphiopedilum* cut flower, but significant quantities are now being produced throughout Asia mainly Sri

Lanka and Singapore. Italy is the single largest consumer of both tropical and temperate orchids in Europe. Italy imports nearly 63 million stems of tropical orchids from Thailand and 750 thousand stems from Singapore almost throughout the year. Germany is the second largest importer of orchids in Europe importing nearly 29.7 million stems annually from Netherlands (16.6 million stems), Thailand (11.7 million stems), and Singapore (996 thousand stems). Holland depends on Thailand for its requirement of tropical orchids. In total Holland imports nearly 17.5 million stems from Thailand (14 million stems), Singapore (1.9 million stems) and New Zealand (0.22 million stems). The United States of America is one of the major destinations for the orchid produced worldwide. It imports nearly 16.6 million stems of tropical orchids. *Dendrobium* from Thailand (14 million) followed by Singapore (9289 thousand stems), New Zealand (37,142 stems), Jamaica (44,611 stems), Costa Rica (23,660). Besides, USA also imports non-Dendrobiums mostly from Thailand (3.8 million stems), Holland (1.5 million stems) etc. So, a bird eye view of the present world market of orchids indicates that the demand for orchids is fast expanding (Sinha, 2000). Orchids now command a high price in world market of floriculture product emerging as a highly rewarding enterprise (Deb and Imchen, 2011). In India, the flower industry generates more than 300 million US dollars per year, of which a substantial income is contributed by orchids, including growing orchids through seeds and micropropagation (Bhattacharjee, 1995, Behera, 2013).

1.2 Orchids as food

Aromatic oil from the seed pod of *Vanilla* is the most famous orchid product used as flavouring agent. The Australian Aborigines and early settlers have used many orchids as emergency bush food, e.g., *Gastrodia sesamoides*, *Dendrobium speciosum* and *Caladenia* spp. Extract from dried tubers of *Orchis moculate* or *O. latifolia* called ‘Salep’ is used in greatest bulk in Turkey for making ice cream and beverages (Bulpitt, 2005). In

African region particularly in Zambia, Malawi and Tanzania a delicacy called Kinaka or Chikanda has been eaten by the people for hundreds of years. It is linked to a meatless sausage made by tubers belong to a variety of terrestrial orchid species mostly of the genera *Disa, Satyrium* and *Habenaria* (Davenport and Ndangalasi, 2003; Kaulo et al., 2009). Considering the valuable contribution of edible orchids to people's livelihood and the indications that its availability is decreasing while its consumption is increasing, several interventions focusing on their conservation is the need of the hour (Kasulo et al., 2009).

Orchids products like vanilla and '*Salep'* are now used widely, the former as delicious flavouring and wonderful perfume (Bulpitt, 2005). However, collection of orchids from the wild has threatened many species with extinction (Cribb et al., 2003). Approximately 10%of orchids are predisposed as vulnerable to extinction (Koopoowitz et al., 2003). Fragmentation of habitats, increased susceptibility of fire threats, pollinator decline, and introduction of feral animals are other noticeable reasons for drastic losses in orchid populations and diversity (Coates and Dixon, 2007).

1.3 Orchids in ethnomedicinal uses

Orchids have been used as a curative and for therapeutic purpose since ancient times (Handa, 1986). The Chinese were the first to mention using orchids as medicine (Bulpitt, 2005). Since at least 200 B.C., the *Dendrobium* orchids have served as a substantial source of tonic, analgesic, astringent, and anti-inflammatory chemicals, as shown by the Chinese pharmacopoeia "Sang Nueng Pen Tsao Ching" (Singh and Tiwari 2007). About 40 *Dendrobium* species have been used in the traditional Chinese medicine, five of which i.e., *D. chrysanthum, D. fimbriatum, D. loddigesii, D. nobile and D. officinale* are listed in the Chinese pharmacopoeia (Behera et al., 2013). *Anoectochilus roxburghii*, which is found in southern China, Japan, Sri Lanka, India, and Nepal (Li and

Zou, 1995), is also known as "King Medicine" in China. (Tseng et al., 2006; Pant 2013). In India, the record and the uses of orchids are found in Chaoka Samhita (A.D>100), which mentioned orchids under the name Vanda (Behera et al., 2013). The medicinal properties of orchids have been used since Vedic period. 'Ashtawarga' a group of 8 drugs in Ayurvedic system which are used for preparation of tonic, such as 'Chyavanprara', which consists of 4 orchid species, *viz*, *Malaxis muscifera*, *Malaxis acuminate*, *Habernaria intermedia* and *Habernaria edgeworthi* (Jalal, 2008). Salem (*Orchis latifolia* and *Eulophia latifolia*), jewanti (*Dendrobium alpestre*), shwethuli, and rasna (*Acampe papillosa* and *Vanda tessellata*) are additional extensively used orchid medications in the Ayurvedic system (Hossain, 2011). In western herbalism, *Cypripedium parviflora* is widely used as aphrodisiac and nerve tonic (Singh and Duggal, 2009). In Malawi (Africa), *Crytorchis arcuata* is used to treat diabetes or skin infections and *Eulophia cucullata* to prevent epilepsy. In Australia *Cymbedium canaliculatum* is used to cure dysentery. *Dendrobium teretifolium* bruised leaves is rubbed to relieve pain, bruised mature cane extract with spirit of *Dendrobium discolour* to cure ring worm etc. pseudobulb extract of *Malaxis acuminate* is used in tonic preparation and of *Pholidata imbricate* to treat rheumatic swellings (Bulpitt, 2005). About 20 genera and 49 native orchid species (mostly *Eulophia* species) are being traded and utilized in traditional South African medicine, particularly among the Zulu community for therapeutic and cultural purposes (Hutchings et al., 1996; Chinsamy et al., 2011).

A number of ethnic communities in India also use several species of orchids in their traditional system of medicines Roy et al. 2007. In Nagaland about fifteen species of orchid recorded are commonly used as medicine for diseases like rheumatism, cholera, nervous disorder, tuberculosis (Deb and Imchen, 2008).The majority of orchid species used in cultural rituals are used as emetics and it would be crucial to understand how these

medicines made from orchids affect the human body, especially with regard to their safety and toxicity (Chinsamy et al., 2011). It is well known that orchids have been utilised in traditional medical systems around the world for a range of maladies, understanding various ethno-pharmacological studies and connecting traditional knowledge of medicinal orchids with current research initiatives will offer a novel, trustworthy strategy that greatly increases the likelihood of medication discovery compared to random sampling.

1.4 Phytochemicals in orchids and *in vitro* propagation

Aside from the ornamental value, orchids are also known for their medicinal usage in traditional medicines which were recorded long back in many countries like Australia, China, Africa, India and in parts of Europe and America (Bulpitt, 2005). *Anoctochilus*, *Bletilla*, *Calanthe*, *Coelogyne*, *Cymbidium*, *Cypipedium*, *Dendrobium*, *Ephemerantha*, *Eria*, *Galeola*, *Gastrodia*, *Gymnadenia*, *Habenaria*, *Ludisia*, *Luisia*, *Nevilia*, and *Thunia* are the main genera that contain medicinal orchids (Szlachetko, 2001). Many orchids play significant role in traditional system of medicine because of their rich content in alkaloids, flavonoids, glycosides and phytochemicals (Jalal et al., 2008). In the therapy of various diseases, researchers have discovered a variety of activities for the metabolites and extracts of diverse orchid species. They have been used in a variety of ways to treat various illnesses, including as anti-rheumatic, anti-inflammatory, antiviral, anticarcinogenic, diuretic, neuroprotective, relaxation, anti-aging, wound healing, hypoglycaemic, antitumor and anticancer, as well as antimicrobial and antiviral agents, among many other functions (Ghanaksh and Kaushik, 1999; Shyur et al., 2004; Shimura et al., 2007; Wang et al., 2006; Prasad and Achari, 1966; Kumar et al., 2000; Zhao et al., 2003; Watanabe et al., 2007, Won et al., 2006; Lawler and Slaytor, 1970; Balzarini et al., 1992; Miyazawa et al., 1999; Jalal, et al.,2012; Pant, 2013; Chinsamy et al., 2014; Dileep, et al., 2014). Numerous constituents have been reported as a result of extensive investigation into the secondary

metabolites of orchidaceae plants (Kong et al., 2003), phenanthrene derivatives are the most frequently found in orchid plants (Jaime and da Silva, 2013). Da Silva et al. (1999) isolated a total of 28 compounds comprising 53, 74, and 86% of the head-space volatiles from *Bulbophyllum guttulatum*, *B. odororatissimum* and *B. triste*. 100 compounds, comprising of 32 alkaloids, 6 coumarins, 15 bibenzyls, 4 fluorenones, 22 phenanthrenes, and 7 sesquiterpenoids, were reviewed by Zhang et al. (2003) from 42 different Dendrobium species. Pant et al. (2021) have reported extracts of *Dendrobium densiflorum* showing higher IC_{50} values than that of cisplatin (a well-known chemotherapeutic drug), when tested against cervical cancer (HeLa) and glioblastoma (U251) cell line.

In vitro techniques have been found to perform a key role in the generation of secondary metabolites in addition to the creation of high-quality planting materials (Rout et al., 2000; Verpoorte et al., 2002; Shinde et al., 2010). Alkaloids, saponins, carotenoids, anthocyanins, polyphenols, and other chemical substances with pharmacological significance are produced and stored in cultivated plant tissues (Yesil-Celiktas et al., 2007; Shinde et al., 2010). The substantial role that polyphenols play in the treatment of a variety of degenerative and age-related disorders has made them a popular choice among them. Similar to how other bioactive substances like flavonoids, alkaloids, and tannins have also helped to treat a variety of chronic diseases. These bioactive compounds work in concert to produce powerful antioxidant activities in a variety of *in vitro* systems. They also consistently exhibit higher scavenging potentials for a variety of reactive oxygen species (ROS), such as the hydroxyl radical, hypochlorous acid, superoxide anion, and peroxynitrite (Bhattacharyya et al., 2015). Studies on the photochemical contents and antioxidant capacities reported significant increase in *in vitro* plantlets of *Coelogyne ovalis* (Singh and Kumaria, 2020). Prasad et al. (2021) when assessing phytomedicinal potential

of *Bulbophyllum odoratissimum* also stated that optimized *in vitro* protocol of orchids can promote the production of secondary metabolites.

1.5 Molecular characterization of orchids

Traditionally orchids are identified based on few selected floral traits that are frequently linked to interactions between the plants and their pollinators. There are significant morphological variations within the species as a result of parallelism and co-evolution of floral characteristics linked to pollinators and interspecific hybridization. Because of this, it is very challenging to identify and classify species just based on morphological characteristics (Dressler, 1993; Cameron et al., 1999; Parveen et al., 2017). DNA barcoding is a relatively recent technique that uses standardised DNA sequences as tags for the correct identification of plant species or genera, gaining popularity in botany (Hebert et al., 2003, 2004; Taberlet et al., 2007). Also, DNA sequences are now the primary source of new data for investigations of evolutionary and genetic links because of the developments in sequencing and computational tools (Hajibabaei et al., 2007; Galimberti et al., 2019; Saravanan et al., 2019). It is largely utilised in studies on local floras and plant communities for identifying specimens that are hard to recognise by morphological characters or that lack diagnostic floral characters viz, rarely blooming/ blooming period species and/or with brief juvenile stages, and identification of cryptic species (Xu et al., 2018; Aghayeva et al., 2021). The chloroplast DNA markers were created as plant DNA barcodes since all plants have chloroplasts, unlike animals whose DNA barcoding system is based on the mitochondrial DNA region (Cytochrome Oxidase I gene) (Ginibun et al., 2010). The coding regions *accD*, *matK*, *ndhJ*, *rbcL*, *rpoC1*, *rpoB*, and *ycf5*, as well as the noncoding areas *atpF-atpH*, *psbK-psbI*, *trnH-psbA*, and the *trnL* intron, have all been suggested for use as chloroplast barcodes (Gou et al., 2016). A multi-locus strategy is typically used for plant barcodes because animal mitochondrial genes are

more likely to experience mutation than plant chloroplast genes (Chase et al., 2005; Kress et al., 2007; Fazekas et al., 2008). The internal transcribed spacer (ITS) regions of the nuclear ribosomal cistron (18S-5.8S-26S) have been extremely successful at species-level discrimination across flowering plants because of their high rate of nucleotide substitution, their relatively simple amplification, and the large sequence data already available (Li D et al., 2011; Feng et al., 2016; Aghayeva et al., 2021). Therefore, one crucial area of research has been finding single or multiple sites in plant plastid and nuclear genomes that are suitable for DNA barcoding studies (Chen et al., 2010; Huda et al., 2017). Also, the Consortium for the Barcode of Life (CBOL) proposed a 2-core-locus made up of the coding regions *matK* and *rbcL* as a viable substitute for DNA barcoding after much discussion and testing (CBOL Plant Working Group, 2009). Several workers have worked on barcoding orchid to distinguish Orchid Species (Farrington et al., 2009; Chattopadhyay et al., 2017), study their Genetic diversity (Vu et al., 2019), endangered species (Flanagan et al., 2007; Parveen et al., 2012; Rajaram et al., 2019; Sherif et al., 2020; Srivastava and Manjunath, 2022) Medicinal (Huda et al., 2017; Raskoti and Ale, 2021; Yu et al., 2021) and edible orchids (Veldman et al., 2017; Veldman et al., 2018; Ghorbani et al., 2017). Thus, use of DNA barcoding has revolutionised current taxonomy, and most taxonomic investigations make use of at least one type of molecular analysis to assess and improve classificatory assumptions (Savolainen et al., 2005; Farrington et al., 2009).

1.6 Somaclonal variation and genetic fidelity assessment in *in vitro* cultures of orchids

Somaclonal variation is the term used to describe genetic changes seen in plants that have been regenerated from tissue or cell cultures (Larkin and Scowcroft, 1981; Evans et al., 1984). However, other names like protoclonal, gametoclonal, and mericlonal variation are frequently used to describe variants from protoplast, anther, and meristem

cultures, respectively. At the moment, the term somaclonal variation is universally used for all forms of tissue culture derived variants (Bajaj, 1990; Karp, 1994, 1995; Chen et al., 1998; Bairu et al., 2011). Genetic and epigenetic variants are the two forms of variations that are seen. Ploidy alterations or DNA mutations were the usual causes of genetic diversity and DNA methylation or histone modifications cause epigenetic changes, which then affect how genes are expressed (Bird, 2002; Chan et al., 2006; Kubis et al., 2003; Chen et al., 2008). Somaclonal variation can be detected through morphological studies (Israeli et al., 1991), Physiological/biochemical detection (Phinney, 1985; Israeli et al., 1995; Sandoval et al., 1995; Peyvandi et al., 2009), Molecular detection (Botstein et al., 1980), Cytological methods (Al-Zahim et al., 1999; Bogdanova, 2003; Nakano et al., 2006; Mujib et al., 2007) and through proteins and isozymes (Jarret and Litz, 1986; Bhat et al., 1992; Weising et al., 2005). Somaclonal variation may occur/can be induced due to number of cell divisions, use of mutagenic substances, response to stress, direct developmental changes in chromosomes and through cultured meristematic tissues (De Klerk, 1990). Induced somaclonal variation has been utilized in plant breeding projects to create new varieties of crops that may exhibit polygenic traits that benefit from genetic modification, such as disease resistance, quality improvement, and enhanced yield (Jain, 2001; Unai et al., 2004). However, the main goal of *in vitro* propagation is the ability to generate a large number of uniform and genetically stable plants under sterile conditions, the relative ease of managing the climatic and nutritional conditions, and the speedy development of novel types (Dobránzski and da Silva, 2010; Azman et al., 2014).

The preservation of any species' homogeneous genetic background over the course of its life is referred to as genetic fidelity (Joshi et al., 2013; Panwar et al., 2022). However, somaclonal variation in *in vitro* regenerants of orchid are likely to occur depending on number of variables, including the species, donor genotypes, explant types,

composition of the culture media, physical culture conditions, and time between subsequent subcultures (Kishor and Devi, 2009; Singh et al., 2012). Any genetic modifications brought about by the tissue culture environment will probably result in a plant with heritable traits (Jain, 2001; Soniya et al., 2001). Moreover, when working with plants vital to conservation, it is advisable to avoid techniques that could cause somaclonal variation (Fay, 1992). Thus, requires optimization of *in vitro* protocol that can produce plantlets that are genetically similar to the donor plant. Due to the fact that molecular approaches are not affected by environmental conditions and may produce precise and consistent results, they are preferred above other conventional methods for assessing the clonal stability of orchids (Oliya et al., 2021). In order to assess clonal variability in a variety of plant species, including orchids, molecular markers like Inter Simple Sequence Repeats (ISSR), Random Amplified Polymorphic DNA (RAPD), and Direct Amplification of Minisatellite DNA (DAMD) have been widely used (Devi et al., 2013; 2014; Bose et al., 2016; Bhattacharyya et al., 2016b; 2018). In addition, Start Codon Targeted (SCoT) Polymorphism, which typically relies on the tiny, conserved nucleotide area surrounding the ATG Commencement codon of plant genes, has been used (Collard and Mackill, 2009). Moreover, the gene-targeted molecular marker system (SCoT), has significantly surpassed the considerable limits of conventional markers like RAPD and ISSR, which focuses on and amplifies a specific region of the genome, usually non-coding regions (Bhattacharyya et al., 2018).

1.7 Status of orchids and threats

Orchid distribution and abundance are distinctly skewed towards the tropics and vary between continents and within regions, following hotspots of species richness and high angiosperm endemism as described by Myers et al. (2000). The continuous exploitation of several medicinal plant species from wild (Kala, 2003) and substantial loss

of their habitats during past two decades (FAQ, 2003) have resulted in population decline of many high value medicinal plant species over the years. Over-exploitation and continuous depletion of medicinal plants have not only affected their supply and loss of genetic diversity but have seriously affected the livelihoods of indigenous people living in the forest margins (Rao et al., 2004). Wild orchid collection and sale, particularly by rural communities, is a common practice. Uprooting the entire plant, which threatens the extinction of many species, results in the supply of a vast quantity of such orchids to both domestic and foreign traders (Kala, 2004). About 70% of orchid species listed by the Convention on the International Trade in Endangered Species (CITES) are traded illegally due to the demand in market (Hinsley et al., 2015). Orchidaceae, more than any other plants family, have a high proportion of threatened genera, with most containing threatened species. Two Australian examples are *Drakaea*, consisting of ten taxa, five of which are threatened and *Caladenia*, consisting of 243 taxa, 97 of which are threatened (Swarts and Dixon, 2009). It is apparent conundrums that while orchids are so numerous and widespread, many are rare or even threatened with extinction. Orchids of limited geographical range can be severely threatened, even if locally abundant. Many orchids, by the nature of their lifestyle, are naturally rare and confined to limited areas. For example, *Phragmipedium exstaminodium* and *Mexipedium xerophyticum* from Mexico are known from 30 and 11 wild plants respectively; the Australian *Calochilus richiae* is known from fewer than 20 plants in its native Victoria and only of which has recently been destroyed. Fortunately, although many orchids are known to be rare, relatively few are known to be extinct (Cribb et al., 2003)

A major threat to orchids worldwide is habitat loss due to clearing forests for timber, mining, agriculture and urban development (Brundett, 2007; Koopowitz et al., 2003; Swarts and Dixon, 2009). Orchids are also threatened by overgrazing of livestock,

construction of roads, dams, bridges, natural factors like forest fire; overexploitation etc. the changing pattern of rainfall and decrease in the forest cover has contributed to their decrease (Joshi et al., 2009). Although rarely discussed in the tourism literature, nature-based tourism is contributing to the decline of some wild orchid populations through direct collecting, habitat clearance and trampling and/ or indirectly by increasing the impact of other threats such as weeds, pathogens and climate change (Ballantyne and Pickering, 2011). Unscrupulous collection by enthusiastic students, taxonomist during field trips and illegal trade are some more reasons. Apart from *in situ* and *ex situ* measures, the preservation of orchids is a crucial issue that the government and private sector of the affected country should take very seriously. They should work with academic institutions, non-governmental organisations, community farmers, and other stakeholders.

Orchids have become an iconic plant in the world market with the increase in loyal customers for its flower a well as for its medicinal values. Understanding the nature value added properties through proper research in pharmaceutical studies; medicinal values and mass propagation can further help to increase its economic status. There is also a need of attention for scientific exploration of endemic and rare orchids with ethno-medicinal uses of human welfare. Commercial orchid farming whether in small or large scale is cost intensive and profitable at the same time with minimum space (Sinha, 2000). The number of linkages in medicinal plants supply, process, drug formulation, trade, transport, and the retail industry can be used to generate employment. The available rural technologies for farming of some valuable medicinal plant species can be made useful for their large-scale cultivation (Kala, 2006). However, with the issue of orchids being threatened and vulnerable to extinction in the wild due to over exploitation, destruction of habitat and change in climate, effective conservation steps need to be adopted.

1.8. Conservations prospects

Orchid conservation requires knowledge regarding its distribution range, habitat, species biology and the kind of pressure the orchid population is facing. Precise information on conservation status of native orchids can help to implement appropriate efforts to critical taxa. Biotechnology is also useful adjuvant technology in conservation by providing a means for protecting of rare plants that cannot be stored by seed or where the critically low number if plants predicate the need to store clonal copies of the last remaining genotypes (Coates and Dixon, 2007). Some important considerations in development of conservation programmes includes recognition of existing and future environmental threats, habitat specialization; reproductive biology; evolutionary processes influencing population structure, and ex situ conservation (Swarts and Dixon, 2009). The commercial collection pressure on the wild orchids can be diverted by developing new hybrids and mass production through conventional and tissue culture technique. International organizations like Convention of International Trade in Endangered Species (CITES), the orchid conservation committee (TOCC) and orchid conservation 97 are on the check to deal with orchid conservation. But the problems related to illegal trade of wild orchids still remains an issue in many nations. The government authorities responsible for the protection of wildlife also need to be advised by specialists to fulfil their responsibilities. The increase in human population and their impacts on the biosphere will however make conservation of orchids and wildlife a challenge.

Therefore, the usefulness of orchids to human race is inevitable. However, the knowledge to exploit the resources of orchids still needs thorough studies and scientific research. The job opportunities and income related to orchid cultivation can become a helpline for livelihoods of local people if properly established in today's constrain economy. With that on focus, the issues threatening the population and habitat of orchids must also be taken into concern. So, a sustainable and integrated conservation measures are

the need for orchid conservation. Developing new hybrids of orchids can help cultivars to produce flowers of interest in market and also divert the demand of wild orchid. Laws regarding illegal trade of orchids also need to be regulated and enforced to check the exploitation of orchids in the wild. Orchid conservations can be achieved only when the value of this exotic flower is realized by the people, for this educating the people starting from the rural area is needed.

1.9 *In vitro* propagation and mass multiplication of orchids

In the commercial world, micropropagation is a trusted technology used for large-scale plant reproduction, germplasm preservation, disease eradication, genetic manipulation, and the supply of certain species (Thorpe, 2007; Sornia et al., 2013). When a novel tissue culture/ *in vitro,* easy and practical method for vegetative/clonal propagation of *Phalaenopsis* orchid was established by Rotor (1949) at Cornell University, modern orchid *in vitro* propagation got its start (Arditti, 1996). The first technique for horticultural orchid seed germination by Moore (1849) was a significant innovation in horticulture and biology (Yam and Arditti, 2009). Later, Noel Bernard's discovery for the symbiotic germination of orchid seeds *in vitro,* provided valuable information for plant *in vitro* propagation (Yam et al., 2002). However, the history of orchid micropropagation by tissue culture is more complicated, fraught with debate, and filled with odd occurrences (Arditti, 1985, 2001; Arditti and Krikorian, 1996; Easton, 2001; Yam and Arditti, 2009).

One of the most noticeable features of the Orchidaceae is the structure and tiny size (0.250 to 1.2 mm in length and 0.090 to 0.270 mm in width) of the orchid seed (Arditti, 1967). Despite the fact that they produce many tiny seeds, orchids have a low rate of germination because their endosperms lack the nutrient stores needed for germination and their ovules are absent or underdeveloped at the time of anthesis (Paek et al., 2010). As part of their symbiotic relationship with host plants, fungus belonging to the *Rhizoctonia*

genus provides the nutrients that orchids need. The fungus grows into the seed, and their hyphae penetrate the cells' deeper layers. The developing protocorm or the undifferentiated parenchymatic cells that emerge during germination then receive nutrients from the decomposing hyphae (Pacek-Bieniek et al., 2010). Knudson (1922) conclude that tropical epiphytic orchids seeds germinate using certain sugars secreted by the fungus, which lead him to add sucrose in medium and succeeded in establishing *in vitro* asymbiotic germination of *Cattleya* seeds. Later, the Knudson C medium was published which is used till date (Arditti, 1990; Yeung, et al., 2018). Other basal medium used in *in vitro* propagation of orchids include Morel medium (1960), MS medium (Murashige and Skoog, 1962), Nitsch medium (Nitsch, 1969), Mitra et al. medium (1976) etc.

The outcome of *in vitro* propagation has been observed to be significantly influenced by the source chosen for the explants. Various workers have reported the propagation of orchids using different explants; some mentions are from shoot tips (Nagaraju and Parthasarathy, 1995; Devi et al., 1997), leaf (Abdul Ghani and Harris, 1992; Sheelavanthmath et al., 2005; Martin and Madassery, 2006), inflorescence (Goh and Wong, 1990; Chen and Chang, 2000; Mitsukuri et al., 2009), rhizome (Nayak et al., 1998; Bhadra and Hossain, 2003; Martin, 2003) and root (Vij, 1993; Park et al., 2003; Wu et al., 2004). The mass-market orchids of the future are thought to be explant propagated rather than seed propagated due to the extreme homogeneity in vegetatively propagated plants and the year-round availability of explants (Chugh et al., 2009). However, it can be observed that most explants used for propagation are *in vitro* plantlets or seed derived. According to Gupta (2016), asymbiotic seed germination offers an effective method for mass propagating orchids, the protocorms and seedlings developed can be used as explants for *in vitro* culture. Studies on the genesis of the explants source are thus vital, pointing the importance of seed germination. The idea that orchid seeds or embryos could sprout before

they were fully developed (Withner, 1953; Tsuchiya, 1954) led to the development of the "green pod" culture technique, which promises a higher germination rate and reduces the time between pollination and seed sowing (Sagawat, 1963; Kaur and Bhutani, 2011). Moreover, asymbiotic germination of orchid seed have been largely adopted with RET (Rare, endangered and threatened) status (Deb and Temjensangba, 2006; Thokchom et al., 2017; Diengdod et al., 2017; Kunakhonnuruk et al., 2018; Castillo-Perez et al., 2021), endemic (Abraham et al., 2012) medicinal value (Koirala et al., 2013; Devi et al., 2015; Bhowmik and Raham, 2017) and economic importance (Deb and Pongener, 2013; Jualang et al., 2014).

1.10 Scope of the study

In the present study, three orchids namely *Cymbidium bicolor* Lindl., *Dendrobium heterocarpum* Wall ex. Lindl and *Esmeralda clarkei* Rchb. f. found in Nagaland, India was selected. The selected species all fall under the RET status (Pamarthi et al., 2019). During the study, documentation on the use of the selected orchids species in enthomedicine in the state was not found. However, plant parts of *C. bicolor* is used among the Khamti tribe of Arunachal Pradesh for different ailments and injuries (Chowlu et al., 2017) and pseudobulb of *D. heterocarpum* is used in treating bone fractures (Subedi, 2011). The orchid species have breeding value and the fragrant in *D. heterocarpum* and *E. clarkei* can be exploited in perfumery. The primary issues in the development of orchid plants as a source of raw materials for medicine are the relatively difficult mass propagation technology, the vegetative phase of the plant's life cycle is prolonged and the plant's genetic stability **(**Utami et al., 2017**).** And the traditional methods of orchid propagation are quite slow and laborious, the reasons why orchid prices are so high (Bhadra and Hossain, 2003). An optimistic method of propagation for orchid conservation, asymbiotic seed germination, has been employed successfully in many endangered orchid species **(**Kunakhonnuruk et

al., 2019**)**. A constraint for orchid micropropagation is the definition of an adequate culture media (KIM et al., 2019). An adequate formulation of the nutrition media is essential to tissue culture, because media must supply the essential substances for in vitro growth development (Moraes et al., 2005). The green pod age, embryonic developmental stage, various nutritional media with adjuvant, and plant growth regulators are all significant determinants of orchid non-symbiotic seed germination (PGRs) (Deb and Pongener, 2011). Despite being touted as a fresh source of genetic variation that can provide better material, somaclonal variation is frequently undesirable when the goal is the micropropagation of elite genotypes (Khoddamzadeh et al., 2010). Although there are many documentation studies on medicinal orchids have but there is a lack of phytochemical and pharmacological studies (Tsering et al., 2017).

Therefore, the current research is aimed to encompass on studying the following objectives for the selected three orchids: -

- To authenticate the species collected both by morphological characteristics and molecular characterization using molecular markers (*matK* and ITS primers)
- Developing an optimized *in vitro* protocol for asymbiotic seed germination and plantlet development of the three species
- Assessment of genetic fidelity of the *in vitro* regenerants of the three orchids species studied using molecular markers viz., RAPD, DAMD and SCoT.
- Study on comparative assessment of secondary metabolites and antioxidant properties of the selected orchids and their regenerants.

Chapter - 2

Morphological and Molecular Characterization of the Three Selected Orchids

2.1. Introduction

Orchidaceae is the most diverse angiosperm family comprising about 736 genera and 28,000 species (Christenhusz and Bing, 2016) and accounts for ~10 % of the earth's flowering plants (Cribb et al., 2003). They have harboured recognition among researchers because of their distinctive flower structure, colour traits and medicinal properties which are found in many historical records. The distribution of orchids is known to be cosmopolitan and can be found in all parts of the world except in polar regions, aquatic and marine ecosystems, and are generally grouped as tropical and temperate orchids (Behera et al., 2013). Based on the nature of habitat, they are classified as epiphytic, lithophytic, saprophytic and terrestrial. The vegetative structures also grouped orchids as monopodial and sympodial. The status of wild orchids, unfortunately, over the years has been susceptible to environmental changes and human activities leading to threatening of many species and even extinction. Koopoowitz et al. (2003) have also stated that ~10 % of orchids are predisposed as vulnerable to extinction.

The first stages in conservation biology and the sustainable use of orchids as plant resources are the accurate identification of orchid species and comprehension of their evolutionary relationships (Sinclair et al., 2005; Gutierrez, 2010). However, a small number of floral traits, which are frequently linked to interactions between the plants and their pollinators, constitute the fundamental basis for the conventional classification of orchid species. It is challenging to identify and categorise an orchid species using morphological characters alone due to interspecific hybridization that frequently results in introgression and the occurrence of parallelism and convergence of the specialised floral structures linked to pollination systems, as well as the extensive morphological variation within species (Dressler, 1993; Chung et al., 2005; Devos et al., 2005; De Hert et al., 2011; Kim et al., 2014). Moreover, medicinal orchids typically are traded as dried or fresh plant parts such as pseudobulbs, tubers, leaves, stems, and flowers. These dried or fresh sections of several species have morphological characteristics that are difficult to distinguish from one another due to their similar appearance (Raskoti and Ale, 2021). Thus, the identification of species using varying DNA sequences/barcoding is excellent for differentiating orchids with similar physical features and a long vegetative growth stage since it is independent of morphology, developmental stage, tissue integrity, and environmental influences (Agarwal et al., 2008).

Though several loci from the plastid genome, including *rbcL, rpoB, rpoC1, psbA-trnH* spacer and *matK* have been suggested as potential barcodes in plants, there is still no such universal barcode that has been discovered (Praveen et al., 2012). Based on an analysis of more than 1000 plants, mostly orchids, Lahaye et al. (2008) suggested that a short portion of the plastid *matK* gene could be used as a universal barcode (Singh et al., 2012a). The 2-site combination of *rbcL+matK* has been recommended as a plant barcode by the CBOL Plant Working Group; after evaluating the recoverability, sequence quality, and level of species identification of seven leading candidate plasome DNA regions (*atpF-atpH* interval,

matK gene, *rbcL* gene, *rpoB* gene, *rpoC1* gene, *psbK-psbI* interval, and *trnH-psbA* interval) (CBOL, 2009; Li et al., 2021). Several groups have also proposed the nuclear ribosomal cistron's internal transcribed spacer (ITS) region (18S-5.8S-26S) as a potential plant barcode (Kress et al., 2005; Chase et al., 2005; Chen et al., 2010; Praveen et al., 2012; Vu et al., 2019). Kamba and Deb (2022) reported species resolution of 91.78%, on barcoding 31 vandaceous species from 15 genera using the barcode loci ITS. Therefore, the present study aimed to validate the identification of the selected orchids through morpho-taxonomic and by barcoding it using *matK*, *rbcL* and ITS primers.

2.2. Materials and Methods

2.2.1. Sample collection and morphological characterization

The selected orchid species were collected from different locations in Nagaland after a field survey. The collected orchids were maintained in the Orchidarium, Department of Botany, Nagaland University, Lumami. Morphological characterization of the species was done with special emphasis on the inflorescences.

2.2.2. DNA extraction and amplification

Genomic DNA was isolated using cetyl trimethyl ammonium bromide (CTAB) protocol with slight modifications (Doyle and Doyle, 1987). Molecular characterization of the species was done using universal primers *matK*, *rbcL* and ITS primers. The extracted DNA was checked for purity by running it in 1% (w/v) agarose gel electrophoresis. The PCR reaction mixture contained components as shown in Table 2.1. PCR reactions were performed with initial denaturation for 5 minutes at 95°C, followed by 35 cycles of final denaturation for 1 minute at 95°C, annealing at 56-58°C for 50 seconds, extension for 1 minute at 72°C and final extension for 10 minutes at 72°C.

Table 2.1: Components for PCR mixture and the final concentrations used

Components	Initial concentration	Volume for 25 µl	Final concentration
PCR buffer with 25 mM MgCl2	10X	2.5 µl	1X
dNTPs	10 mM	0.5 µl	0.2 mM
Forward primer	10 mM	0.5 µl	0.2 mM
Reverse primer	10 mM	0.5 µl	0.2 mM
Template DNA	>1000 ng	2 µl	50-100 ng
Taq polymerase	5U	0.2 µl	≤1U
De-ionized water		18.8 µl	-

2.2.3. Sequence and data analysis

By using a 100-base pair (bp) ladder (Genei) as the size standard, gel electrophoresis (1.2%, w/v) was used to confirm the PCR amplicons. After being amplified, the amplicons were forwarded to Chromos Biotech Pvt. Ltd. in Bangalore, India, 1st Base Laboratory in Singapore, and Eurofins Genomics Pvt. Ltd. in Bangalore, India for sequencing. Utilizing the BioEdit sequence alignment editor software and the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the quality of each sequence was checked and searched for the reference of each sequence similarity in NCBI GenBank. The suggested species' matK and ITS nucleotide sequences were uploaded to NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) to obtain unique accession numbers. Neighbor Joining (NJ) tree was constructed for each species with the sequences (*matK* and ITS) and those sequences showing similarity during BLAST (https://www.ncbi.nlm.nih.gov/genbank/samplerecord/) using MEGA software. The evolutionary history was inferred using the Neighbor-Joining method. The tree was drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were

computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

2.3. Results

Characterization of the *C. bicolor*, *D. heterocarpum* and *E. clarkei* were confirmed with both morphological characters and molecular tools (using universal primers of *matK* and ITS).

2.3.1. Morphological characterisation

Morphological characters of each species were noted and dissected floral parts were photographed to make photo plates/digital herbarium for future reference (Figure 2.1, 2.2, 2.3). Documentation details with taxonomic treatment for each species have been described below: -

2.3.1.1. *Cymbidium bicolor* Lindl.

Taxonomic Treatment

- *Cymbidium bicolor* Lindl., Gen. Sp. Orchid. Pl.: 164. 1833; Hook. f., Fl. Brit. India. 6: 11. 1890: H.J. Chowdhery, Orch. Arunachal Pradesh 253. fig. 150. 1998.
- Plants 20 – 70 cm tall. Pseudobulbs 6 – 10 × 2.5 – 3.5 cm, narrowly ovoid, usually enclosed in persistent, bladeless 4 or 5 sheaths (6 – 9 cm), scarious fibrous with age. Leaves 5 – 7, 40 – 70 × 2.5 – 3.5 cm, linear-oblong, stiff and arching; leaf base 3 – 10 cm long. Inflorescence arching to pendent, 10 – 15 flowered; peduncles 8 – 12 cm, sheathed; sheaths 5, 3 – 4.5 cm long, spreading, boat-shaped and overlapping; rachis 8 – 20 cm long; floral bracts 2 – 4 mm long. Flowers 2.5 – 4 cm across when open; sepals and petals pale yellow and striped brown-maroon; lip white to cream-yellow, with a broad, central maroon-brown stripe; lip white or cream-coloured, with a yellow patch at base, lateral lobes mottled with maroon or red, callus cream-yellow, column cream-coloured; pedicel and ovary 0.8 – 4 cm long; sepals 1.5 – 1.8 x 0.4 –

0.5cm, similar, narrowly oblong to obovate-oblong, obtuse to subacute, erect to spreading. Petals 1.4 – 1.8 × 0.4 – 0.6 cm, narrowly oblong-elliptic and spreading; lip 1.2 – 1.8 × 1 – 1.6 cm, saccate at base, papillose to shortly pubescent; lateral lobes clasping the column, erect, obtuse and recurved; mid-lobe 5 – 8 × 5.5 – 7 mm, broadly elliptic, rounded and mucronate at apex; callus 2 ridged, entire, minutely papillose-pubescent; column curved, winged at apex, 0.8 – 1 cm long; foot short. Anther cap ca 2.5 mm across; pollinia 2, ca 1 mm, pyramidal. Fruits 4 – 5 × 2 – 2.5 cm, cylindric-ellipsoid.

- Flowering: March – April.
- Altitude 782 m.
- Specimen collection site: Honito village, Kiphiri district, Nagaland.

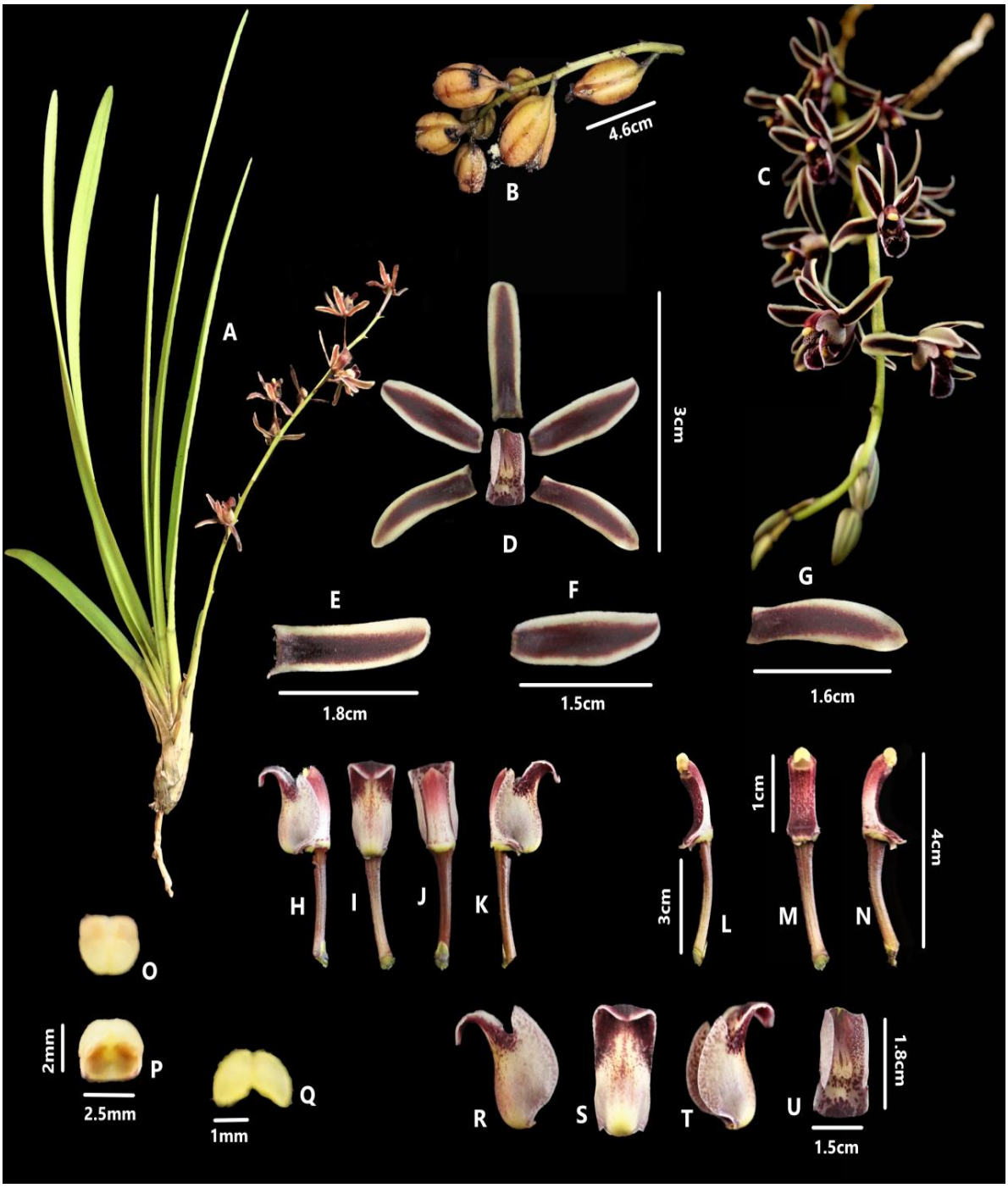

Figure 2.1: Photo plate showing whole plant of *Cymbidium bicolor* Lindl. and dissected parts of the flower. (A) Plant with flower; (B) Seeds; (C) Inflorescence; (D) Flower perigon with lip; (E) Dorsal sepal; (F) Petal; (G) Lateral sepal; (H-K) Anther with lip; (L-N) Column, pedicel, ovary and anther; (O-P) Anther, side and front views; (Q) Pollinia; (R-S) Lip dorsal, ventral and side views.

2.3.1.2. *Dendrobium heterocarpum* Wall ex. Lindl.

- *Dendrobium heterocarpum* Lindl., Sp. Orchid. Pl.: 78. 1830; Bot. Mag, t. 4708. 1853; Hook. f., FL. Brit. India 5: 737. 1890; King & Pantl. in Ann. Roy. Bot. Gard. Calc. 8: 53. T. 74. 1898; H.J. Chowdery, Orch. Fl. Arunachal Pradesh 302. 1998; Hynniewta, Kakati & Wadhwa, Orch. Nagaland, 148. 2000. *Callista heterocarpa* (Lindl.) Kuntze, *op. cit*. 654. 1891.
- Plant 15-30 cm tall. Roots clustered. Stems 8-12 cm, fusiform, erect or pendent, widening upwards, swollen at base, caespitose, yellow with age, bearing many, alternate leaves; internodes sheathed, 2-3 x 0.2-1.2 cm. Leaves 6-10 x 1.0-1.8 cm oblong-lanceolate to oblong, acute, many veined, sessile, jointed. Inflorescence lateral, arising from nodes once leaves have fallen, 2 or 3 flowered; peduncle short, sheathed, 2-3 mm long; sheaths scarious, tubular, ca 4 mm long; floral bracts scarious, oval retuse to emarginated or obtuse, 6-8 x 3-4 mm. Flowers fragrant, long-lived, 3.5-5 cm across, yellow, pale creamy-yellow to cream, lip cream or yellow with a central maroon to crimson blotch or striations, column cream with a yellow base; pedicel and ovary glabrous, slender, 2.0-3 cm long. Sepals oblong-lanceolate, obtuse to acute, 6-7 veined, 2.0-3.6 x 0.6-0.8 cm; lateral sepals adnate to form a mentum; mentum obtuse, 4-7mm long. Petals 2.2-3.4 x 0.7-1.2 cm, ovate, obtuse to acute, 7 veined. Lip obscurely 3-lobed, ovate-lanceolate, base wedge-shaped, convolute and hooded around the column, acute and reflexed at apex, margins minutely serrulate-fimbriate, pilose centrally, 2.4-3.7 x 1.0-1.8cm. Column 4-7 mm long; foot 0.6-0.8 mm long. Fruit 2.5-3.5 x 0.9-1.8 cm obconical.
- Flowering: March – April.
- Altitude: 650-1800 m.
- Specimen collection site: Sumi Settsu, Zunheboto district.

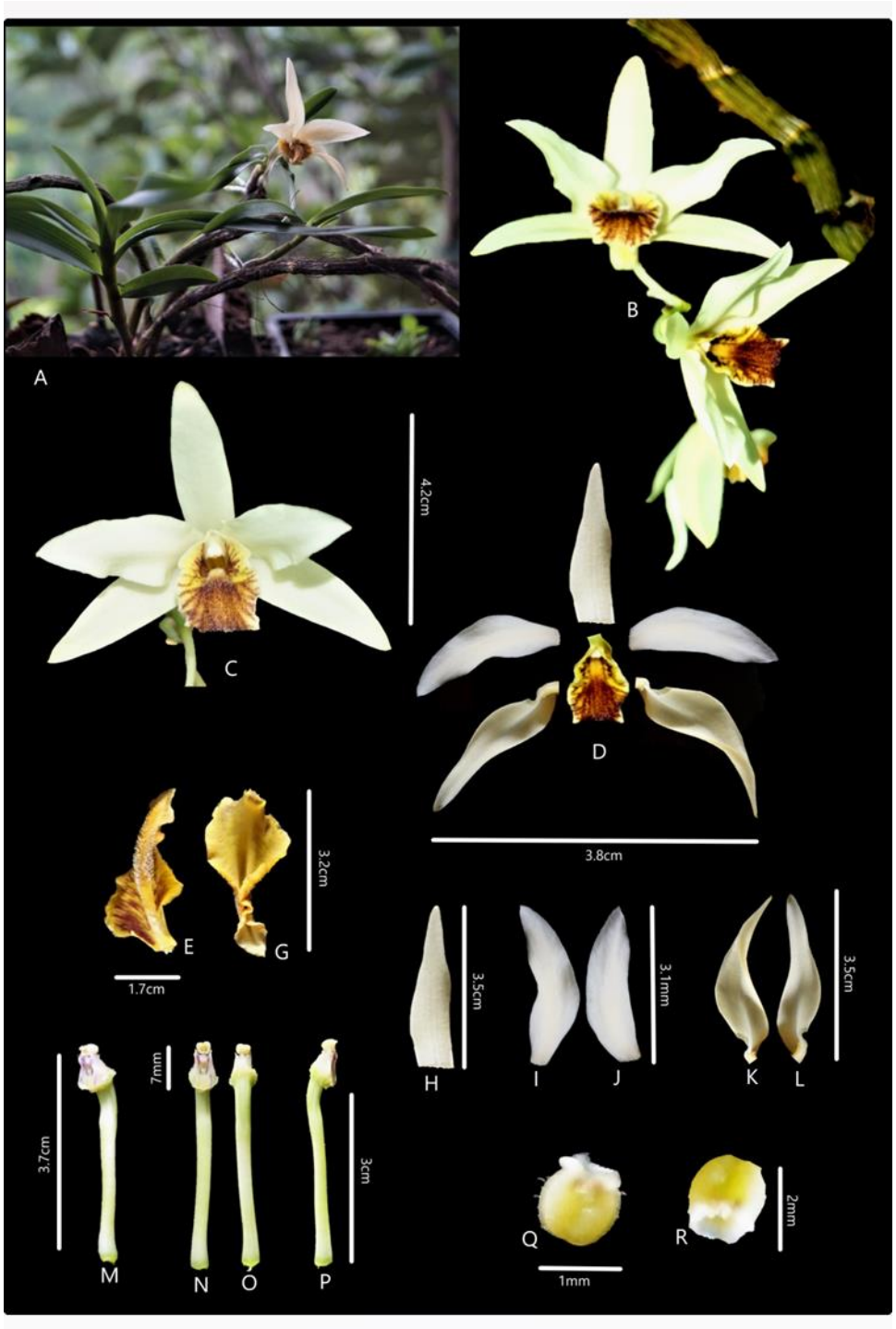

Figure 2.2: Photo plate showing Dendrobium heterocarpum Wall ex. Lindl. plant and dissected floral parts. (A) Plant with flower; (B-C) Inflorescence; (D) Flower perigon with lip; (E-G) Lip dorsal and ventral view; (H) Dorsal sepal; (I-J) Petals; (K-L) Lateral sepal; (M-P) column, pedicel, ovary and anther; (Q) Pollinia; (R) Anther.

2.3.1.3. *Esmeralda clarkei* Rchb. f.

- *Esmeralda clarkei* Reichenbach f. in Gard. Chron. n.s. 26: 552. 1886. Type: Indian Himalayas, Clarke s.n., cult. Low (Holo. W. Herb. No. 3921, 3922). Vanda clarkei (Reichenbach f.) Gard. Chron. n.s. 26: 552. 1886: 112. 1888.
- Plants 30 cm to 1 m tall. Stem covered with 3 – 4 cm long tubular leaf sheaths. Leaves many, distichous, oblong, 13 – 24 x 1.6 – 3.6 cm, unequally bilobed at apex, coriaceous. Inflorescence erect, lateral, up to 33 cm long; rachis 12-14 cm long, laxly 3 – 4 flowered. Flowers 5.5 – 7.6 cm in diameter, slightly fragrant, opening widely; sepals and petals yellow with red or brown transversal stripes on the inner surface and white externally. Dorsal sepal erect, oblong, obtuse, 3.3 – 3.9 x 0.9 – 1 cm; lateral sepal oblong, falcate, 2.5– 3 x 0.9 – 1.2 cm. Petals falcate, oblong, obtuse, 2.9 – 3.5 x 0.7 – 0.8 cm. Lip free, pendent, 3-lobed, 1.6-1.8 cm long with reniform midlobe and small erect side lobes, unspurred; disk with several longitudinal keels and 2 raised calli in the center of the lip. Column stout, clavate, 2 – 3.8 cm long.
- Flowering: December to February.
- Altitude 1969 m.
- Specimen collection site: Pangsa village, Tuensang district, Nagaland.

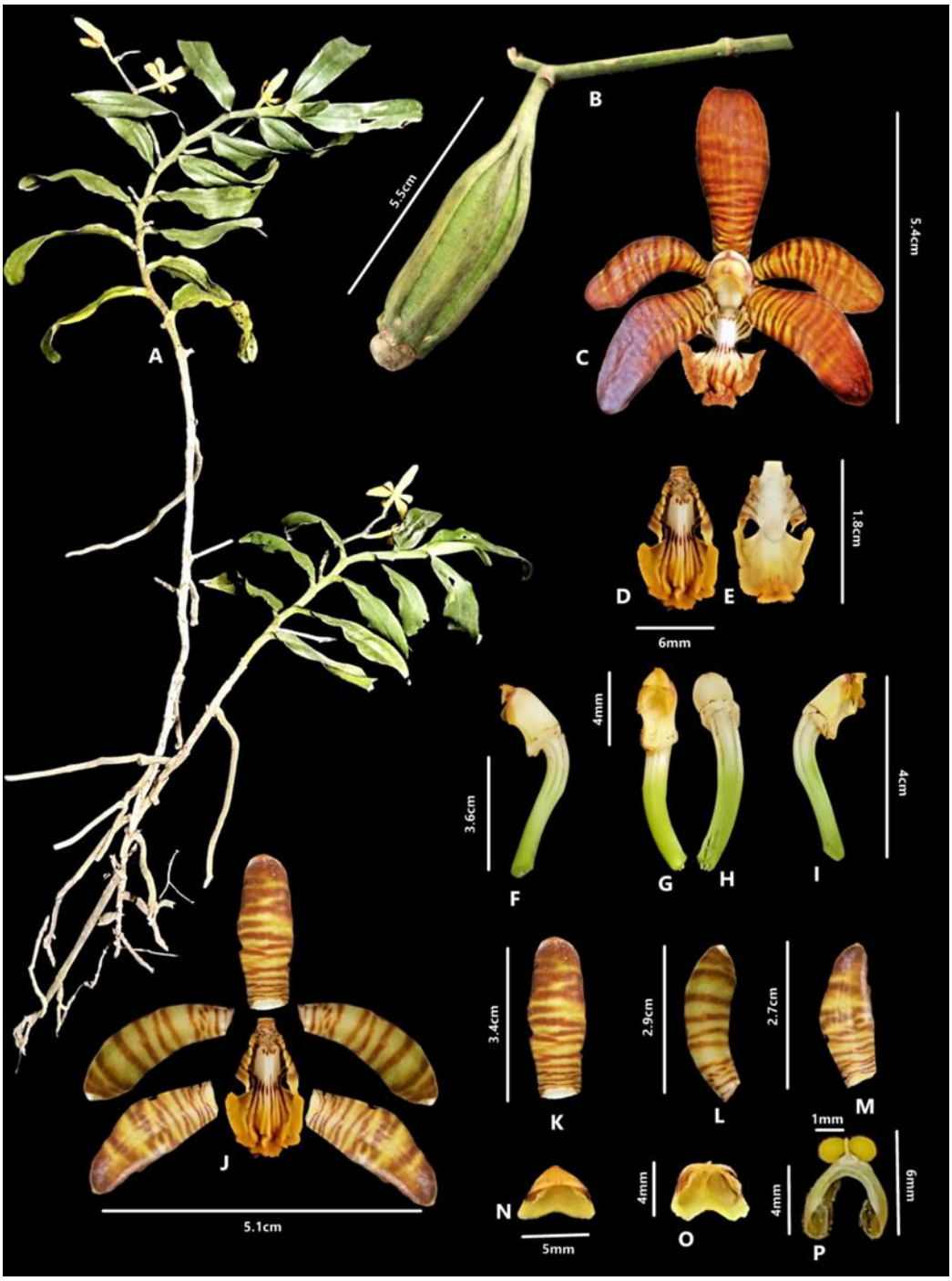

Figure 2.3: Photo plate showing whole plant of Esmeralda clarkei Rchb. f. and dissected parts of the flower. (A) Plant with flower; (B) Seeds; (C) Inflorescence; (D-E) Lip dorsal and ventral view; (F-I) Column, pedicel, ovary and anther; (K) Dorsal sepal; (L) Petals ; (M) Lateral sepal; (N-O) Anther, side and front views; (P) Pollinia.

2.3.2. Molecular characterization

Molecular characterization of the three selected orchid species was done based on possible barcode sites from the nuclear genome (ITS) and the chloroplast genome (*matK*) (Table 2.2). The amplified bands ranged from 800-900 bp in *matK* primers and 700-800 bp in ITS primers. The GC content of the submitted sequence amplified by *matK* and ITS were 31.1% and 60.3% for *C. bicolour*; 32.1% and 52.8% for *D. heterocarpum* and 30.9% and 59% for *E. clarkei* respectively (Table 2.2). The sequences were submitted to NCBI GenBank, and Table 2.2 lists their accession numbers. Using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/) at NCBI GenBank, highest blast hit was shown with 100% identity for *matK* in *D. heterocarpum* and 98.99% for ITS in *E. clarkei*. Neighbour joining (NJ) tree was constructed for three species using *matK* and ITS sequences (Figure 2.4, 2.5 and 2.6). In *Cymbidium bicolor*, the tree from *matK* region showed an interspecific divergence of 0.01 with *C. bicolor* subsp. *pubescens, C. goeringii* and *C. haematodes*, and zero interspecific divergence with *C. aloifolium* and *C. atropupureum*. Also, in ITS base tree, interspecific divergence within the species of *Cymbidium* was found to be 0.01 (Figure 2.4). In case of *Dendrobium heterocarpum*, tree based on *matK* sequence showed zero interspecific divergence with the sequence of other *Dendrobium* species and in ITS clade showing similarities with other submitted sequences of *D. heterocarpum* with low interspecific divergence of 0.01 (Figure 2.5). The tree for *Esmeralda clarkei* represented zero interspecific divergence with other submitted sequences of the same species in *matK*. However, ITS showed *E. clarkei* showing sharing interspecific divergence of 0.16 with sequences of other similar species (Figure 2.6).

Table 2.2: Sequence details and Gene Bank accession number of the three selected orchids

Sl. No	Species Name	Primer Name	Base pairs	Submitted bp length	Voucher No.	Accession no.
1.	*C. bicolor*	Matk-1RKIM-f Matk-3FKIM-R	5´ACCCAGTCCATCTGGAAATCTTGGTTC3´ 5´CGTACAGTACTTTTGTGTATACGAG3´	839	NU-BOT-TBL-CRD, MATKF	OM936982
2.	*C. bicolor*	ITS-1 ITS-4	5´TCCGTAGGTGAACCTGCGG3´ 5´TCCTCCGCTTATTGATATGC3´	494	NU-BOT-TBL-CRD-CB-011	OP108839
3.	*D. heterocarpum*	MatK-1-F MatK-1-R	5´ATCCATATGGAAATCTTGGTTC3´ 5´GTTCTAGCACACGAAAGTCG3´	780	NU-BOT-TBL-CRDEB-DH1F	MW000349
4.	*D. heterocarpum*	ITS 1 ITS2	5´TCCGTAGGTGAACCTGCGG3´ 5´GCTGCGTTCTTCATCGATGC3´	634	NU-BOT-TBL-CRDEB-ITS1	MW032194
5.	*E. clarkie*	Matk-1-F Matk-1-R	5´ATCCATATGGAAATCTTGGTTC3´ 5´GTTCTAGCACACGAAAGTCG3´	812	NU-BOT-TBL-CRD, matKf	OM959504
6.	*E. clarkie*	ITS 1 ITS 2	5´TCCGTAGGTGAACCTGCGG3´ 5´GCTGCGTTCTTCATCGATGC3´	441	NU-BOT-TBL-CRD-IT1	OM959360

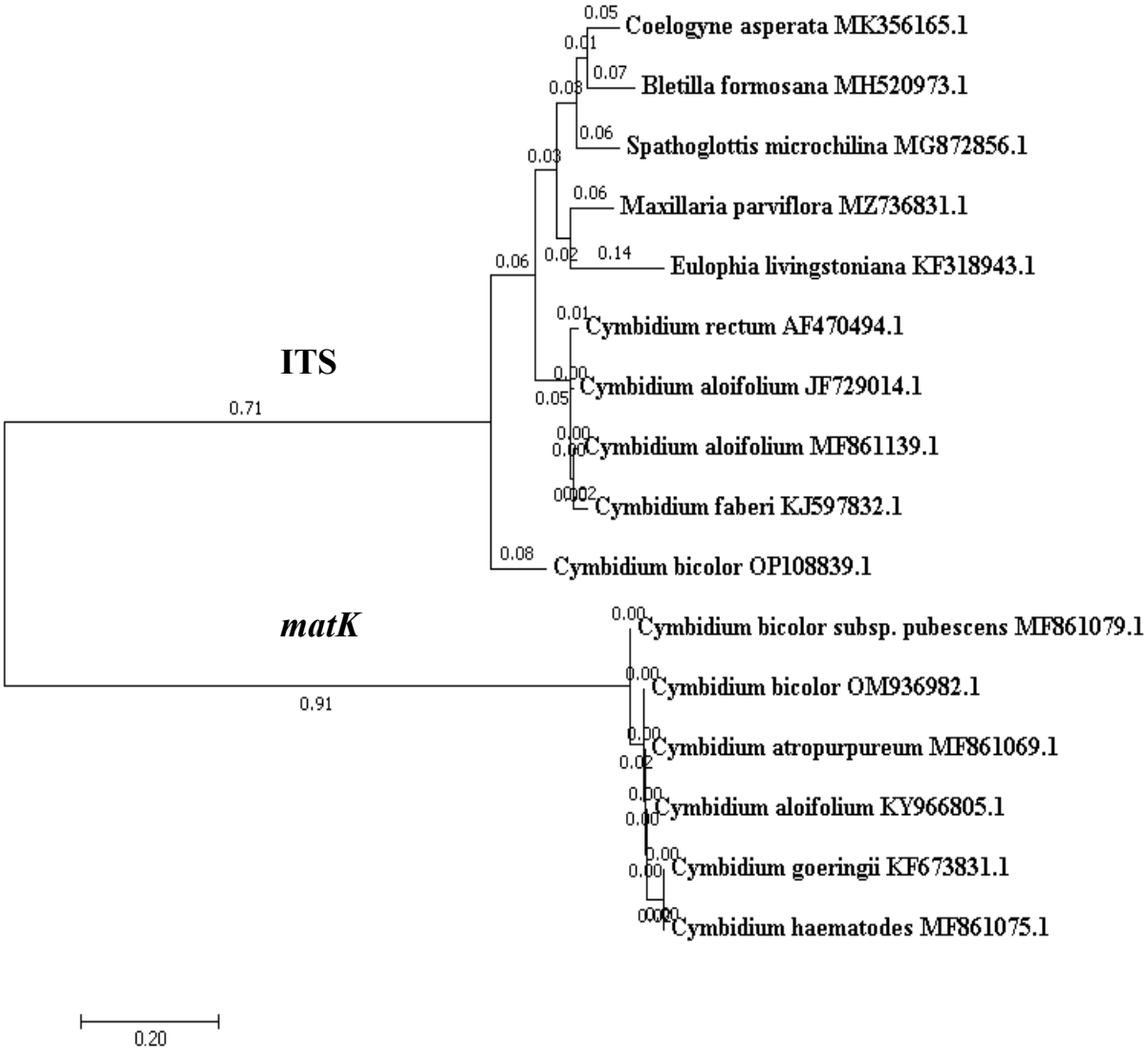

Figure 2.4: Neighbor joining tree constructed for *Cymbidium bicolor* based on chloroplast gene marker *matk* and nuclear region marker ITS.

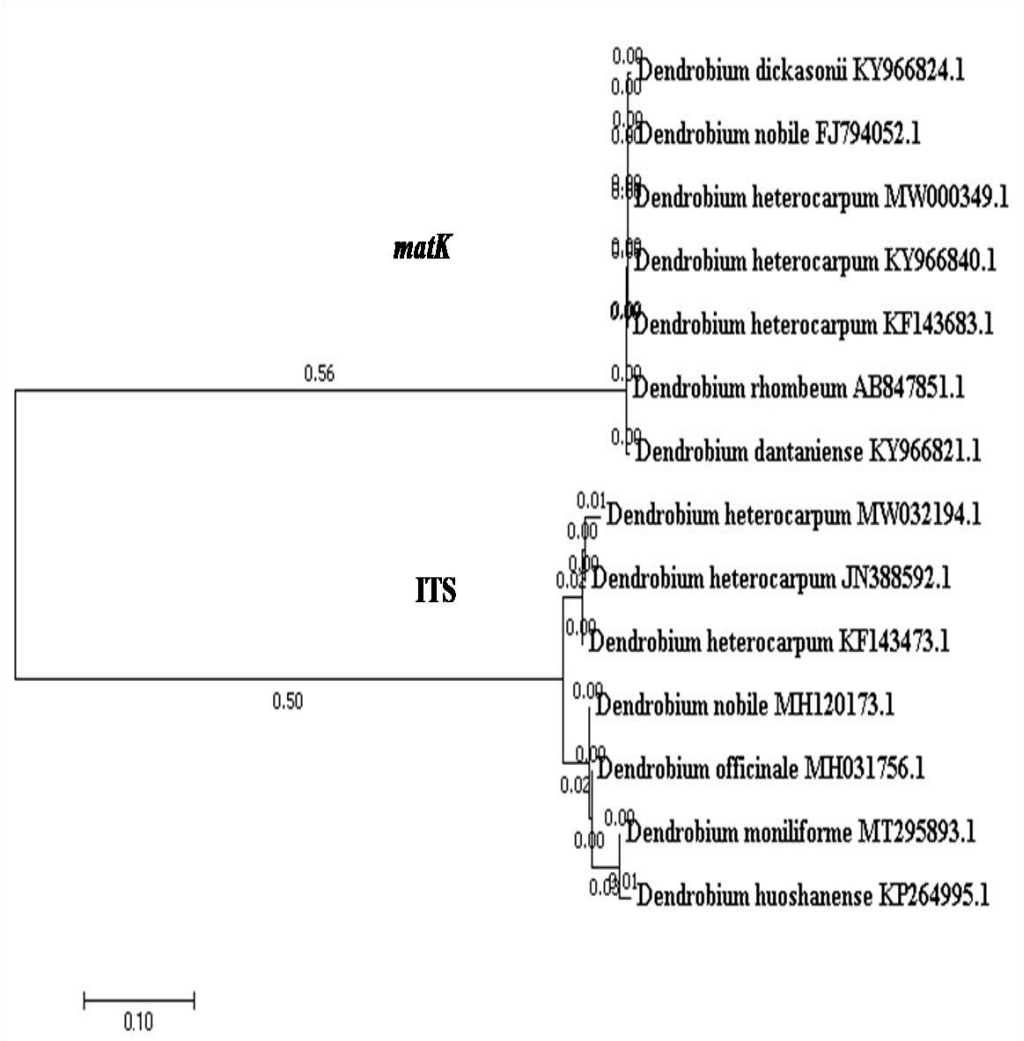

Figure 2.5: Neighbor joining tree constructed for *Dendrobium heterocarpum* based on chloroplast gene marker *matK* and nuclear region marker ITS.

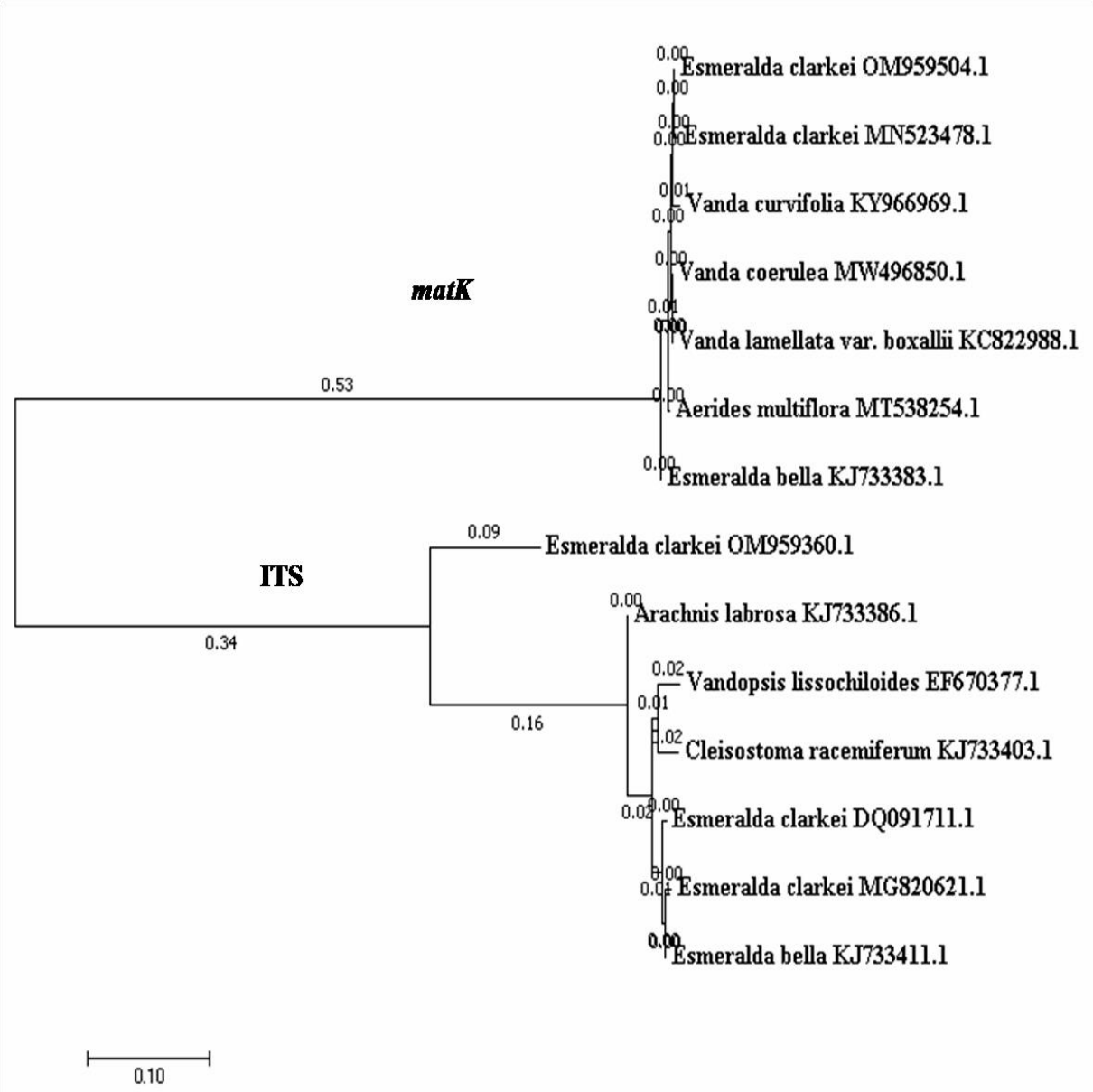

Figure 2.6: Neighbor joining tree constructed for *Esmeralda clarkei* based on chloroplast gene marker *matK* and nuclear region marker ITS**.**

2.4. Discussion

C. bicolor, *D. heterocarpum* and *E. clarkei* were collected from districts of Kiphire, Zunheboto and Tuensang of Nagaland. The morphological traits of the species were first documented for the initial species confirmation. To exclude the limitation of morphological approaches, molecular identification by DNA barcoding is important (Vu and Le, 2019). The fundamental principle of DNA barcoding is based on the finding of short DNA fragments

that only slightly vary within species, with far less variance than between species (Savolainen et al., 2005; Gigot et al., 2007). This method allows researchers to successfully identify species with just a small amount of sample, making it effective for plant conservation, particularly at biodiversity hotspots and for the differentiation of medicinal herbals from adulterants (Vu et al., 2019). Species identity with 99-100% blast hit was achieved in *matK* and 95-99% blast hit in ITS markers. The potential of barcoding orchids of genus *Masdevallia* using *matK* loci (Drever, 2018) and *Caladenia* species using ITS (Farrington, 2009) has been reported. The submitted sequence accession numbers will be a barcode of the species from the region. This information can be used to combat illegal trade and track indigenous plant species (Parveen et al., 2007; Hinsley et al., 2018).

2.5. Summary and Conclusion

In the present study, the selected orchids viz. *C. bicolor*, *D. heterocarpum* and E. *clarkei* were collected from different regions of the state (Nagaland, India) after a field survey. The identification of the plants was first done based on their morphological characteristics, especially considering their flowers. Later, in order to support their morphological identification, molecular characterization of the species was carried out. DNA-based technique was applied to barcode using marker sequences based on plastid (*matK*) and nuclear (ITS) region. Species identification was confirmed after BLAST in NCBI and sequences were submitted in GenBank, where sequences were provided with unique accession numbers. The Barcodes obtained for the species can be used in the future for species identification, diversity studies and monitoring illegal trade.

Apart of the chapter was published as:

Longchar, T.B., Deb, C.R., 2021. Comparative analysis of nutraceutical potential phytochemicals and antioxidant activities in different parts of wild and *in vitro* re generated plantlets of *Dendrobium heterocarpum* Wall. ex Lindl.: a medicinal orchid. Journal of Pharmacognosy and Phytochemistry. 10: 331–336. doi.org/10.22271/phyto.2021.v10.i4d.14169

Chapter - 3

In Vitro Propagation of *Cymbidium bicolor* Lindl.

3.1. Introduction

Cymbidium orchids commonly known as 'boat orchids', are highly valued as a genetic resource for breeding programmes, cut flowers, potted plants, hanging baskets, and herbal medicines (Sarmah et al., 2017). The taxa is well known for its exquisite hybrid and species-derived spikes, ranking first and making up 2.7% of all cut flower production in floricultural crops (Le and Singh, 2018). The conventional method of propagation involves the splitting of the pseudobulb, which stresses the plant and takes time to produce new shoots. The extremely tiny, abundant, and non-endospermic seeds of orchids are dependent on mycorrhizal interaction leading to poor seed germination rates in nature (Khamchatra et al., 2016; Thokchom et al., 2017; Decruse et al., 2018). The large number of seeds per capsule can be feasible through *in vitro* asymbiotic germination of seeds (Stewart and Kane, 2006; Hossain, 2008; Koirala et al., 2013). Thus, an effective technique for asymbiotic *in vitro* seed germination and propagation may be essential for mass reproduction and germplasm preservation of commercial and endangered orchid taxa (Nanekar et al., 2014). However, many parameters, most notably the make-up of germination media, have a big impact on how well asymbiotic germination works (Arditti and Ernst, 1993; Shekarriz et al., 2014; Kunakhonnuruk et al., 2018). Acclimatization is one of the most important and

crucial steps in the *in vitro* micropropagation process because so many plants die in this last stage (Teixeira da Silva et al., 2017; Castillo-Perez et al., 2021). *In vitro* acclimatisation hence is an important step in growing healthy plantlets before they are transferred to *ex vitro* environments (Cha-um et al., 2009).

Cymbidium bicolor Lindl. is an epiphytic orchid, typically seen growing on the main stem of trees with full exposure to sunlight. The species is well-known for its value in horticulture and ethnomedicine, as well as for its significance in floriculture. According to Chowlu (2017), the Khamti community in Arunachal Pradesh, India, uses various plant parts of the *C. bicolor* to treat various ailments. Whereas leaf paste is used to treat joint swelling, rheumatic pains, and skin inflammation; powdered roots are used to treat epilepsy and mental depression; and flower paste is used to treat burnt face and black spots on the skin. Deb et al. (2017) recorded the species as a new addition to the orchid flora of Nagaland, India. However, habitat loss brought on by urbanization, deforestation, and the practice of slash-and-burn cultivation has led to the rarity of the orchid in the state.

Given the significance of *C. bicolor* and its RET (Rare, Endangered and Threatened) status (Pamarthi et al., 2019), numerous researchers have sought to create effective *in vitro* production procedures. Plantlet regeneration of *C. bicolor* has been reported by Mahendran and Bai (2012) through direct somatic embryogenesis of seed protocorms; asymbiotic seed germination with mycorrhizal association by Mahendran et al. (2013) and from shoot tip by Malabali et al. (2008). However, to commercialise and conserve these orchids, cost-effective techniques for mass-propagating rare, threatened, and endangered orchids, new hybrids, and transgenic orchids must be devised (Chugh et al., 2009). Mass propagation of orchid like *Cymbidium aliofolium* (Deb and Pongener, 2012) and *Cymbidium iridioides* (Deb and Pongener, 2013) have been successfully achieved using low-cost substrata in place of agar. The present study, therefore, aims at developing an efficient and cost-effective *in vitro*

protocol for mass proliferation and plantlet regeneration of *C. bicolor* and evaluating the effect of nutrient medium, carbon source, plant growth regulators (PGRs) and developmental stage of seeds in *in vitro* cultures.

3.2. Materials and Methods

3.2.1. Sample collection and maintenance

C. bicolor Lindl. plants were collected from Honito village, Kiphiri, Nagaland, India and maintained in the Orchidarium, Department of Botany, Nagaland University, Lumami. Flowers were hand pollinated and tagged to record the seed age. For assessment of the effect of green pod age on asymbiotic seed germination, green seed pods of 8 to 11 months after pollination (MAP) was used in the present study.

3.2.2. Explant sterilization

Green seed pods (8-11 MAP) were used for sourcing immature embryos for culture. The collected seedpod was first checked for any surface tissue injury/infection and cut off with a sterile blade. Initial surface sterilization of the seed pod was done using diluted 'Labolene' (1:100, v/v, a commercial laboratory detergent) and scrubbed off to remove dirt with a soft brush. After washing thoroughly in running water, the seed pod was treated with 0.3% (w/v) aqueous solution of $HgCl_2$ for 3 minutes under aseptic conditions and rinsed 3-4 times using sterilised double distilled water. Finally, seed pods were dipped in 70% alcohol (v/v) for 30 seconds and flamed for 3-4 seconds. Seed pods were then split open to scoop out the immature embryos for culture on a fortified nutrient medium.

5.2.3. Culture media preparation

In the present study, three nutrient media viz. Murashige and Skoog (MS) (Murashige and Skoog, 1962), Mitra et al. (Mitra et al., 1976) and Knudson 'C' (Knudson, 1946) were used. All the inorganic and organic compounds including plant growth regulators (PGRs) used in the present investigation were obtained from HiMedia Laboratories Pvt. Ltd,

India unless mentioned otherwise. For assessment of asymbiotic seed germination the nutrient mediums were supplemented with different organic carbon sources *viz*., sucrose, fructose and glucose (0-3%, w/v) and further fortified with different PGRs viz, N_6–benzyl adenine (BA), kinetin (KN), α-naphthalene acetic acid (NAA) and Indole-3-acetic acid (IAA) (0-15 µM/L). For plant regeneration, MS medium with different combinations of cytokinins BA+NAA (0-15 µM/L) and KN+NAA (0-15 µM/L) were incorporated into the mediums. Media were gelled with 0.8% (w/v) tissue culture grade agar and *p*H was adjusted to 5.7 using 0.1N NaOH and 0.1N HCl.

5.2.4. Initiation of culture

The immature embryos/seeds at different stages of development (8-11 MAP) were scooped from surface sterilized seed pods in aseptic conditions and inoculated on the nutrient media fortified differently for *in vitro* germination (Figure 5.1 A). All cultures were maintained at 25±2ºC temperature and 40 µmolm^{-2}s^{-2} illuminations at 12/12 h light/dark photoperiods provided by white fluorescent tubes.

5.2.5. Plant regeneration and multiplication

The germinated seeds were allowed to differentiate into protocorms before sub-culturing on fresh and/or new nutrient medium fortified differently compared to initiation medium. The protocorms started releasing the first set of leaves which were considered for transferring on regeneration medium for plant regeneration. The shoot buds/young plantlets were separated and cultured on multiplication medium for culture proliferation. Well differentiated plantlets were allowed to grow on the same medium to experience nutrient stress and begin to get primarily acclimatized.

5.2.6. Primary acclimatization and transplantation

Well-developed rooted plantlets from regeneration medium were sourced and the roots were cleansed with a soft brush to remove the traces of agar and subjected to primary acclimatization. For this purpose, different substrata *viz*., chopped coconut husk (CH) (~0.5-1.0 cm), wood bark pieces (WB) (~0.5-1.0 cm), wood compost (WC), charcoal pieces (CP) and crushed bricks (CB) were used in different combination as agar alternative. For wood barks, any species of tree having more than 0.5 mm thickness was collected followed by soaking in water for 48 h and drying before use. All the substrata were first autoclaved at 1.05 kg cm^{-2} pressure and 121^{o}C for ~30 min. Appropriate quantities of the processed substrata were placed in culture vials in different ratios as below:

1. Coconut husk; 2. Wood bark; 3. Wood compost; 4. CH+WB (1:1 ratio); 5. CH+WB+CP (1:1:1 ratio); 6. CH+WB+CP+WB (1:1:1:1 ratio); 7. CH+WB+CP+WC+CB (1:1:1:1:1 ratio) and 8. CH+WB without any MS salt solution.

About 15-20 ml of 1/4th MS salt solution without any sucrose and PGRs were added to all the culture vials. The culture vials were autoclaved and cooled to room temperature before plantlets were cultured on the substrata and maintained under normal laboratory conditions as specified earlier for one week, under normal room temperature with indirect sunlight (~75% diffused light) for ~4 weeks. The primarily acclimatized plantlets were then transferred to a community potting mix prepared by mixing the same un-autoclaved substrata mixture as primary acclimatization conditions and covered with holed transparent plastic sheets and maintained for 2-3 weeks before transferring to normal field conditions. During this period the plants were fed with 1/10th MS salt solution at weekly interval.

3.3. Results

3.3.1. Effect of nutrient media, seed age and organic carbon source on asymbiotic germination

The germination of immature seeds of *C. bicolor* was observed after ~5 weeks of culture. Initial sign of germination was recorded as swelling of embryos followed by formation of protocorms (Figure 3.2 A, B, C, D). Of the three nutrient media tested in the present study with different levels of fortification, better germination was registered on MS medium (74%), followed by Mitra et al. (39%) while, Knudson C medium did not support germination (Table 3.1). Amongst the three different organic carbon sources used (1-3%), *C. bicolor* seeds germinated more readily in media fortified with sucrose and fructose compared to medium conjunct with glucose. Almost similar results of germination rate were recorded in sucrose 3% (74%), sucrose 2% (74%), fructose 3% (74%) and fructose 2% (73%) in MS media (Table 3.1). The relation of seed developmental stage with germination was evaluated in MS media fortified with sucrose (1-3%) and fructose (1-3%) (Figure 3.2). Across all media supplemented with various percentages of carbon sources, the seed pod age of 11 MAP relatively demonstrated the highest germination rate (67-78%), followed by 10 MAP (62-70%), 9 MAP (52-69%), and 8 MAP (48-67%).

Table 3.1: Effect of media and carbon source on germination of immature embryo of *Cymbidium bicolor* Lindl.

Culture medium	Organic carbon source	Concentration (%)	Germination rate (%) *
MS	Control	0	54.33 ±1.63^{f}
	Sucrose	1	69.63 ±2.42^{abcde}
		2	74.28 ±1.72^{abc}
		3	74.85 ±2.71^{a}
	Fructose	1	56.65 ±2.93^{ef}
		2	73.01 ±2.65^{abcd}
		3	74.89 ±0.87^{ab}
	Glucose	1-3	No response
Mitra et al.	Control	0	33.89 ±1.28^{ghij}
	Sucrose	1	34.53 ±1.48^{ghij}
		2	37.33 ±2.29^{ghi}
		3	39.26 ±3.32^{gh}
	Fructose	1	26.73 ±3.23^{ghijkl}
		2	32.51 ±2.35^{ghijk}
		3	39.54 ±1.74^{g}
	Glucose	1-3	No response
Knudson	Control	0	No response
	Sucrose	1-3	No response
	Fructose	1-3	No response
	Glucose	1-3	No response

Note:* ±SE: Standard error from mean. Mean with different superscripts within the same column differ significantly (P<0.05) by Tukey's HSD at 95% confidence level.

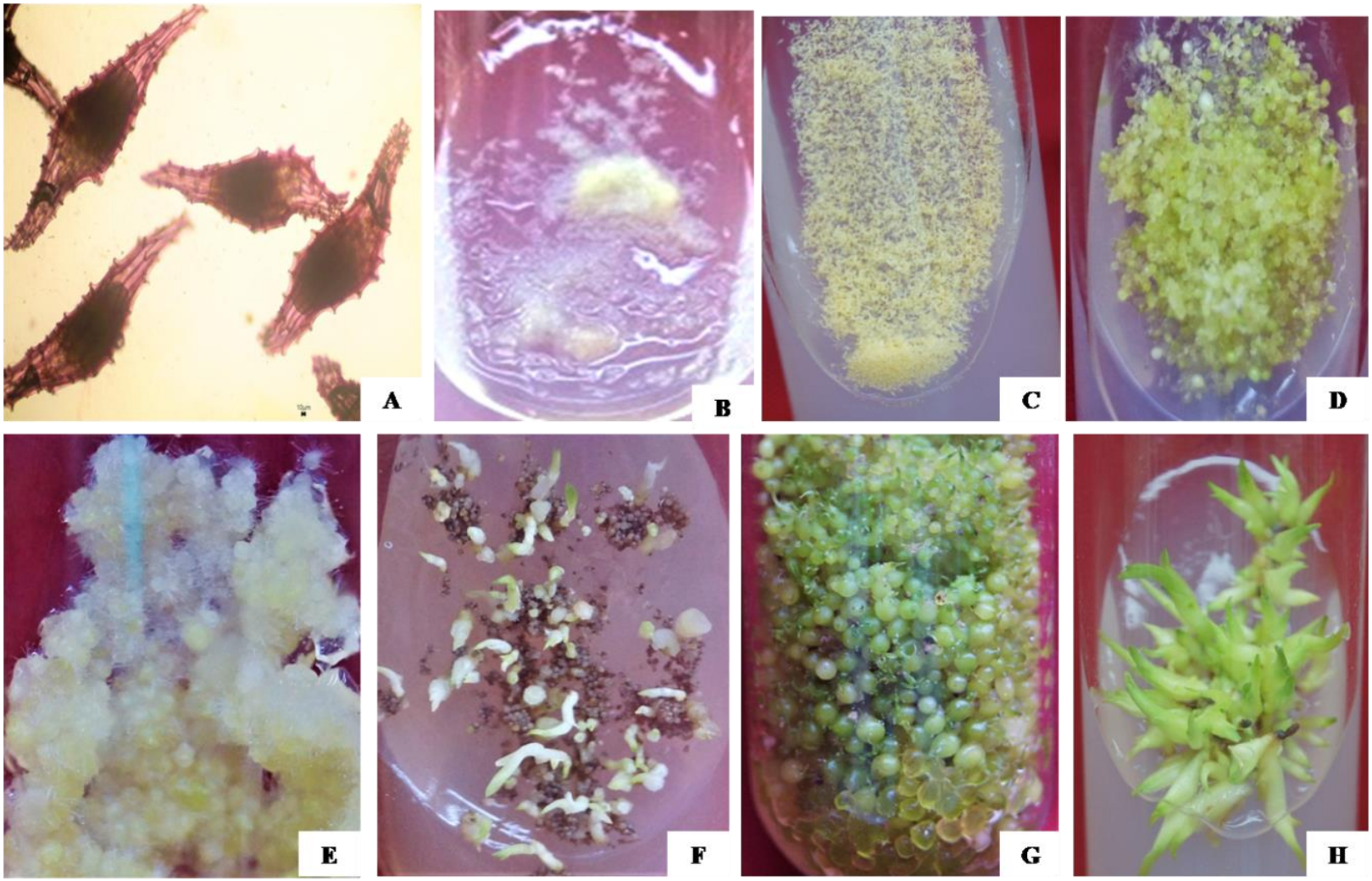

Figure 3.1: Showing asymbiotic germination of *C. bicolor* seeds and its stages on MS and Mitra et al. nutrient media. (A) Immature seeds; (B) Initial seed inoculation on MS medium; (C) Swelling of embryo on MS medium; (D) Initial stage of protocorms on MS medium; (E) Formation of hairy filaments in protocorms; (F) Germination on Mitra et al. medium; (G) Advance stage protocorms in MS medium (H) shoot formation from protocorm in MS medium.

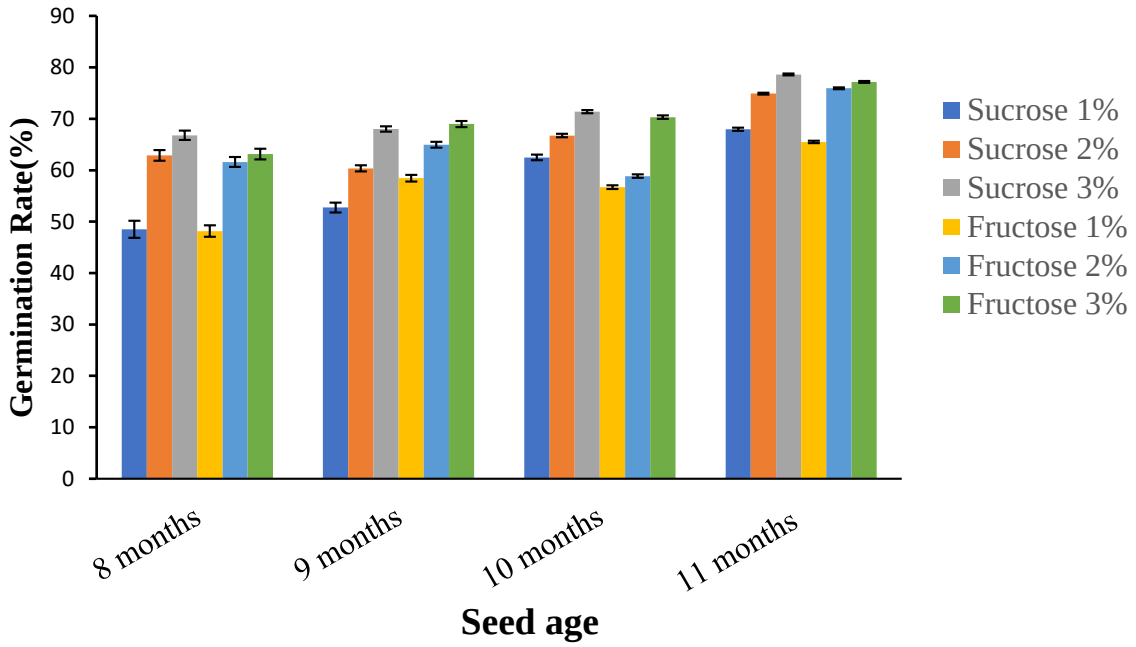

Figure 3.2: Effect of seed age and different carbon sources (1-3%, w/v) in MS medium on asymbiotic germination of immature *Cymbidium bicolor* embryos.

3.3.2: Effect of PGRs on asymbiotic germination

Murashige and Skoog, Mitra et al., and Knudson C media fortified with different carbon sources sucrose (0-3%) and fructose (0-3%) were supplemented with different concentrations of PGRs (BAP, KN, NAA, and IAA; 0-15 M/L) to study their effect on germination time and rate (Table 3.2). In the present study, no germination was recorded on Knudson medium, while, though seeds cultured on Mitra et al. medium supported moderate germination (~26-32%), protocorms were whitish and impaired subsequent regeneration (Figure 3.2 F). There was no significant difference in germination performance of seeds cultured on MS medium conject with both sucrose and fructose across the concentrations investigated. However, the type of PGRs (BAP, KN, NAA, IAA) and their concentrations in MS medium showed differences in the germination time and morphological response (Table 3.2). On MS medium (either sucrose 2-3% or fructose 2-3%) with IAA (6-9 µM/L) supported healthy germination and the growth of healthy green protocorms with hairy filaments (Figure 3.2 E). The shortest germination period of 29 days was recorded on MS

medium conjunct with either sucrose (3%) or fructose (3%) along with KN (3 μM/L), IAA (3 μM/L) and IAA (6 μM/L). Under the given conditions, optimum germination performance was registered on MS medium conjunct with sucrose (3%) + IAA (6 μM/L) and sucrose (2%) + IAA (9 μM/L) where ~95% germination rate was recorded. The protocorms developed on MS medium were green and healthy, and the advance stage of the protocorms when sub-cultured supported new shoot bud formation and leaf formation (Figure 3.1 G, H).

3.3.3. Plant regeneration and culture proliferation

Advance stage of protocorms was sub-cultured on MS medium with different carbon sources (sucrose 0-3%, fructose 0-3%) supplemented with BAP (0-15 μM/L), KN (0-15 μM/L) and NAA (0-15 μM/L) in combination for plantlet regeneration and multiplication (Table 3.3). MS medium fortified with different percentages of carbon source and combination of KN+NAA (3-9 μM/L) exhibited a significant increase in shoot length, leaf length and root length compared to when BAP and NAA (3-9 μM/L) were combined. MS medium containing sucrose (3%) and fructose (3%) supplemented with KN+NAA (6+6 μM/L) and KN+NAA (9+9 μM/L) observed the maximum average shoot length of ~8 cm, leaf length of ~8 cm, number of leaves ~5 and root length of ~3 cm. However, the plantlets produced in MS medium with KN+NAA exhibit etiolated growth with internodes, which is not a morphological feature of *C. bicolor* (Figure 3.3 A, B, C). Interestingly, it was noted that an increase in KN and NAA concentration caused globular formations in roots (Figure 3.3 D). Plantlets with identical morphologies to the mother plant were regenerated on MS medium with a combination of BAP and NAA (3-9 μM/L) (Figure 3.3 E, F). On MS medium fortified with sucrose/fructose (2-3%) with PGRs BAP+NAA, the combination of BAP+NAA (9+3-9 μM/L) and BAP+NAA (6+6 μM/L) showed optimum shoots length (~5cm), leaf length (~5cm), number of leaves (5) and root length (2 cm) (Table 3.3).

Table 3.2: Effect of PGRs and carbon source in asymbiotic germination time of *C. bicolor* Lindl.

Culture medium	PGRS conc. (µM/L)	Germination time				Germination rate				Morphological response
		Sucrose 3%*	Sucrose 2%*	Fructose 3%*	Fructose 2%*	Sucrose 3%*	Sucrose 2%*	Fructose 3%*	Fructose 2% *	
MS	BAP3	30.37 ± 0.49^{cdef}	30.82 ± 0.42^{abcde}	30.50 ± 0.27^{bcdefg}	30.65 ± 0.21^{d}	91.90± 1.25^{abcde}	90.19± 1.25^{abcde}	90.44± 0.56^{abcd}	87.32 ± 1.11^{bcdef}	Healthy and green PLBs
	BAP6	30.63 ± 0.08^{abcdef}	31.57 ± 0.12^{ab}	31.36 ± 0.07^{abc}	31.63 ± 0.09^{abc}	92.39± 1.51^{abcd}	93.52± 1.04^{abc}	90.40± 0.70^{abcde}	87.99 ± 0.25^{abcd}	‘’
	BAP9	31.68 ± 0.24^{ab}	31.90 ± 0.11^{a}	31.77 ± 0.06^{a}	31.82 ± 0.08^{a}	86.47± 1.36^{cdefghi}	89.05± 1.48^{bcdef}	88. 83± 0.48^{abcdef}	86.49 ± 0.46^{bcdefg}	,,
	KN3	29.78 ± 0.19^{cdef}	30.17 ± 0.49^{bcde}	29.90 ± 0.19^{defgh}	30.32 ± 0.30^{d}	88.99 ± 2.10^{abcdef}	84.12± 1.44^{fgh}	86.34± 0.80^{defghi}	85.98 ± 2.10^{cdefgh}	,,
	KN6	30.92 ± 0.23^{abcde}	30.59 ± 0.50^{abcde}	30.64 ± 0.07^{bcdef}	30.73 ± 0.15^{d}	87.73± 0.92^{cdefg}	87.08± 0.95^{defgh}	88.49± 0.80^{cdefgh}	84.72 ± 1.12^{defghi}	,,
	KN9	31.10 ± 0.35^{abcd}	30.98 ± 0.36^{abcd}	30.72 ± 0.49^{abcdef}	30.76 ± 0.05^{cd}	87.41± 0.95^{cdefgh}	88.55± 1.05^{bcdefg}	88.52± 0.13^{bcdefgh}	87.81 ± 1.25^{bcde}	,,
	NAA3	30.45 ± 0.11^{cdef}	30.29 ± 0.25^{bcde}	30.00 ± 0.42^{defgh}	30.25 ± 0.27^{d}	71.82± 1.88^{j}	81.72± 1.63^{hij}	66.07± 0.23^{j}	75.04 ± 1.99^{j}	Healthy PLBs with hairy filaments
	NAA6	31.47 ± 0.17^{abc}	30.88 ± 0.26^{abcde}	30.96 ± 0.22^{abcd}	30.98 ± 0.16^{abcd}	74.64± 0.70^{j}	78.70± 0.85^{ij}	68.40± 0.86^{j}	69.71 ± 0.43^{jk}	,,
	NAA9	31.76 ± 0.22^{a}	31.50 ± 0.04^{abc}	31.45 ± 0.25^{ab}	31.66 ± 0.10^{ab}	71.02± 1.33^{j}	78.06± 0.87^{j}	69.63± 1.42^{j}	68.12 ± 1.07^{k}	,,
	IAA3	29.07 ± 0.11^{f}	29.03 ± 0.22^{e}	29.25 ± 0.38^{h}	30.50 ± 0.13^{d}	92.79± 1.66^{abc}	92.49± 1.53^{abcd}	92.07± 1.22^{a}	92.39 ± 1.64^{abc}	,,
	IAA6	29.75 ± 0.05^{cdef}	30.10 ± 0.18^{bcde}	30.13 ± 0.18^{defgh}	30.44 ± 0.07^{d}	95.54± 0.50^{a}	94.32± 0.93^{ab}	91.77± 1.13^{abc}	92.86 ± 1.18^{ab}	,,
	IAA9	30.27 ± 0.19^{cdef}	30.46 ± 0.16^{abcde}	30.78 ± 0.13^{abcde}	30.94 ± 0.25^{abcd}	94.82± 0.93^{ab}	95.48± 0.44^{a}	92.07 ± 0.49^{ab}	94.54 ± 1.14^{a}	,,
Mitra	IAA9	35± .29^{g}	34.21 ± 0.56^{f}	34.88 ± 0.43^{i}	33.87 ± 0.25^{e}	30.42 ±1.28	26.54±0.72^{k}	32.43±2.69^{k}	29.69±1.74^{l}	Small protocorms + whitish colour + protocorm browning
Knudson	IAA9	No response								

Note: ∗ ±SE: Standard error from mean. ∗∗ Mean with different superscripts within the same column differ significantly (P<0.05) by Tukey’s HSD at 95% confidence level. Selection of organic carbon concentrations is based on the preliminary work as shown in table 3.1

Table 3.3: Effect of Plant growth regulators and carbon source in plant regeneration of *Cymbidium bicolor* Lindl.

MS media	Shoot length*				Leaf length*				No of leafs*				Root length*			
PGRs Conc. (µM/L)	S 3%	S 2%	F 3%	F 2%	S 3%	S 2%	F 3%	F 2%	S 3%	S 2%	F 3%	F 2%	S 3%	S 2%	F 3%	F 2%
BA3NAA3	4.90± 0.23^{d}	4.60± 0.10^{defg}	4.54± 0.18^{d}	4.57± 0.09^{f}	4.14± 0.36^{d}	4.25± 0.12^{de}	4.18± 0.15^{hi}	4.27± 0.06^{d}	4.76± 0.14^{d}	4.77± 0.10^{d}	4.72± 0.08^{a}	4.71± 0.08^{b}	2.21± 0.20^{bcd}	1.96± 0.29^{e}	2.25± 0.27^{b}	2.25± 0.19^{c}
BA3NAA6	4.34± 0.36^{d}	4.52± 0.12^{defg}	4.46± 0.14^{d}	4.54± 0.14^{f}	3.83± 0.06^{d}	4.14± 0.04^{de}	4.11± 0.15^{i}	4.27± 0.11^{d}	4.79± 0.10^{d}	4.70± 0.07^{d}	4.78± 0.02^{a}	4.70± 0.06^{b}	2.59± 0.22^{abcd}	2.55± 0.09^{abcde}	2.32± 0.18^{ab}	2.47± 0.10^{abc}
BA3NAA9	4.43± 0.35^{d}	4.26± 0.06^{g}	4.53± 0.08^{d}	4.59± 0.10^{f}	3.78± 0.02^{d}	3.89± 0.34^{e}	4.20± 0.06^{hi}	4.22± 0.12^{d}	4.63± 0.10^{d}	4.54± 0.09^{d}	4.71± 0.07^{a}	4.70± 0.11^{b}	2.84± 0.20^{abcd}	2.59± 0.10^{abcde}	2.66± 0.31^{ab}	2.40± 0.24^{abc}
BA6NAA3	4.68± 0.51^{d}	4.61± 0.12^{defg}	4.63± 0.19^{d}	4.66± 0.13^{ef}	4.09± 0.24^{d}	4.29± 0.11^{de}	4.40± 0.15^{hi}	4.25± 0.07^{d}	5.06± 0.20^{d}	4.60± 0.09^{d}	5.04± 0.35^{a}	4.65± 0.04^{b}	2.09± 0.17^{d}	2.35± 0.10^{bcde}	2.65± 0.11^{ab}	2.38± 0.09^{bc}
BA6NAA6	5.57± 0.11^{d}	5.30± 0.30^{de}	5.39± 0.11^{d}	5.33± 0.20^{def}	5.12± 0.09^{d}	4.82± 0.31^{de}	5.04± 0.15^{defg}	5.06± 0.14^{d}	5.14± 0.16^{d}	4.65± 0.14^{d}	5.08± 0.10^{a}	4.76± 0.04^{ab}	2.44± 0.30^{bcd}	2.38± 0.15^{bcde}	2.73± 0.07^{ab}	2.36± 0.10^{bc}
BA6NAA9	5.07± 0.33^{d}	5.20± 0.20^{def}	5.62± 0.10^{d}	5.76± 0.04^{d}	4.73± 0.29^{d}	4.93± 0.17^{de}	5.32± 0.09^{d}	5.49± 0.01^{bcd}	5.17± 0.12^{d}	4.70± 0.07^{d}	4.62± 0.10^{a}	4.72± 0.07^{b}	2.74± 0.06^{abcd}	2.55± 0.13^{abcde}	3.05± 0.30^{ab}	2.66± 0.24^{abc}
BA9NAA3	5.28± 0.25^{d}	5.05± 0.14^{defg}	5.19± 0.21^{d}	5.22± 0.26^{def}	5.28± 0.25^{d}	4.66± 0.24^{de}	4.72± 0.15^{efgh}	4.97± 0.22^{bcd}	4.88± 0.13^{d}	4.57± 0.11^{d}	4.77± 0.10^{a}	4.69± 0.05^{b}	2.08± 0.24^{d}	2.58± 0.09^{abcde}	2.29± 0.21^{b}	2.37± 0.10^{abc}
BA9NAA6	5.07± 0.43^{d}	5.02± 0.31^{defg}	5.41± 0.14^{d}	5.16± 0.13^{def}	4.70± 0.40^{d}	4.57± 0.16^{de}	5.27± 0.15^{def}	4.86± 0.10^{d}	4.81± 0.09^{d}	4.59± 0.11^{d}	4.63± 0.04^{a}	4.77± 0.06^{ab}	2.56± 0.12^{abcd}	2.64± 0.13^{abcde}	2.61± 0.09^{ab}	2.50± 0.08^{abc}
BA9NAA9	5.54± 0.10^{d}	5.43± 0.02^{cd}	5.62± 0.09^{d}	5.44± 0.03^{de}	5.12± 0.11^{d}	5.07± 0.09^{d}	5.30± 0.02^{de}	5.13± 0.07^{bcd}	4.78± 0.11^{d}	4.67± 0.08^{d}	4.72± 0.02^{a}	4.74± 0.03^{ab}	2.72± 0.29^{abcd}	3.06± 0.26^{abcd}	3.09± 0.12^{ab}	2.81± 0.18^{abc}
KN3NAA3	7.94± 0.31^{abc}	6.78± 0.27^{abc}	7.68± 0.09^{abc}	6.61± 0.19^{c}	7.37± 0.31^{abc}	6.53± 0.29^{bc}	7.36± 0.05^{c}	6.33± 0.18^{abc}	5.17± 0.21^{c}	5.04± 0.17^{c}	4.70± 0.02^{a}	5.14± 0.11^{a}	3.13± 0.18^{abc}	3.07± 0.15^{abc}	3.21± 0.03^{ab}	3.11± 0.19^{abc}
KN6NAA6	8.65± 0.50^{a}	7.79± 0.03^{ab}	8.71± 0.14^{a}	7.61± 0.16^{ab}	8.08± 0.46^{ab}	7.23± 0.16^{ab}	8.42± 0.09^{ab}	7.34± 0.12^{ab}	5.23± 0.22^{ab}	4.97± 0.23^{ab}	4.98± 0.22^{a}	5.01± 0.17^{ab}	3.16± 0.19^{ab}	3.21± 0.22^{ab}	3.26± 0.20^{ab}	3.20± 0.23^{ab}
KN9NAA9	8.65± 0.50^{ab}	7.88± 0.20^{a}	8.69± 0.05^{ab}	8.34± 0.28^{a}	8.09± 0.64^{a}	7.88± 0.20^{a}	8.46± 0.04^{a}	8.07± 0.28^{a}	5.27± 0.08^{a}	5.04± 0.14^{a}	5.02± 0.10^{a}	4.78± 0.05^{ab}	3.31± 0.14^{a}	3.31± 0.18^{a}	3.35± 0.28^{a}	3.51± 0.07^{a}

Note:∗ ±SE: Standard error from mean. ∗∗Only concentrations supported optimal response are computed. Mean with different superscripts within the same column differ significantly (P<0.05) by Tukey's HSD at 95% confidence level.

Formation of globular structure in roots was also observed with a higher concentration of BAP+NAA (9+9 μM/L) in MS medium (Figure 3.3 H). The ideal medium condition for plantlet regeneration from protocorms of *C. bicolor* was found on MS medium enriched with sucrose 3% or fructose 3% supplemented with BAP+NAA (6+6 μM/L), followed by BAP+NAA (6+9 μM/L), BAP+NAA (3+3 μM/L), BAP+NAA (6+3 μM/L). The proliferation of shoot was optimum on MS medium fortified with sucrose 3% and BAP+NAA (6+3 μM/L) followed by BAP+NAA (9+6μM/L), where an average multiple shoots of 7 and 6 were produced per protocorm (Table 3.4).

3.3.4. Acclimatization of regenerates

Well-developed plants with leaves and roots (Figure 3.4 A) were transferred to medium of different substrata with/without 1/4th MS salt solution devoid of any PGRs, to evaluate their response to primary acclimatization. During this period the plantlets were maintained in *in vitro* condition for one week before introducing to normal room conditions (Figure 3.4 B). Varied response of the regenerates to the acclimatization condition was observed (Table 3.5). Except for substrata of coconut husk supplemented with 1/4th MS salt solution, a survival rate of above 50% was recorded on other substrata/combinations supporting better acclimatization and establishment of plantlets and development of new leaves and roots (Table 3.5). Optimum acclimatization condition was provided with a combination of CH+WB+C+WB+CB with survival rate of 97% (Figure 3.4 C) followed by CH+WB+C+WC (94%) and WB (86%) respectively (Table 3.5). The transplants were fed with 1/10th MS nutrient solution at regular intervals, during this period the roots of the transplants adhered to the substrata (Figure 3.4 D). The primary acclimatized plants were then transferred to community potting mix with the substrata of the same combination as primary acclimatized (Figure 3.3 E).

Table 3.4: Effect of Plant growth regulators and carbon source in plant multiplication of *Cymbidium bicolor* Lindl.

PGRs Conc. (µM/L)	Number of shoots			
	S 3%	S 2%	F 3%	F 2%
BA3NAA3	3.98± 0.38^{cdefg}	5.15± 1.06^{abcdef}	3.85± 0.33^{cdefg}	3.91± 0.21^{bcdefg}
BA3NAA6	3.60± 0.62^{cdefg}	3.64± 0.57^{bcdefg}	3.79± 0.36^{cdefg}	4.02± 0.30^{bcdefg}
BA3NAA9	4.64± 0.29^{bcdef}	4.24± 0.36^{abcdefg}	3.72± 0.65^{cdefg}	3.75± 0.25^{bcdefg}
BA6NAA3	7.12± 0.28^{a}	6.56± 0.42^{a}	6.48± 0.55^{a}	6.08± 0.18^{a}
BA6NAA6	5.33± 0.29^{abcd}	5.16± 0.69^{abcde}	4.35± 0.25^{bcde}	4.40± 0. 63^{abcdef}
BA6NAA9	5.03± 0.74^{abcdef}	4.75± 0.33^{abcdefg}	5.14± 0.42^{abc}	4.83± 0.57^{abc}
BA9NAA3	5.35± 0.53^{abc}	5.48± 0.30^{abc}	4.31± 0.43^{bcdef}	4.57± 0.34^{abcde}
BA9NAA6	6.52± 0.44^{ab}	5.73± 0.48^{ab}	5.81± 0.32^{ab}	5.61± 0.29^{ab}
BA9NAA9	5.27± 0.42^{abcde}	5.37± 0.24^{abcd}	5.07± 0.34^{abcd}	4.59± 0.44^{abcd}
KN3NAA3	3.03± 0.36^{fg}	2.63± 0.08^{g}	2.39± 0.10^{g}	2.24± 0.10^{g}
KN6NAA6	2.66± 0.11^{g}	2.73± 0.10^{efg}	2.24±0.25^{g}	3.31±0.48^{cdefg}
KN9NAA9	2.77± 0.05^{g}	2.34± 0.13^{g}	2.29±0.10^{g}	2.32±0.06^{g}

Note: ∗ ±SE: Standard error from mean. ∗∗Only concentrations supported optimal response is computed. Mean with different superscripts within the same column differ significantly ($P<0.05$) by Tukey's HSD at 95% confidence level.

Well established plants were then transferred to green house and then to natural conditions (Figure 3.3 E). The objective of the current study was also to reduce the protocol's cost by substituting agar for the plantlet's acclimation. Comparison of the cost of tissue culture grade agar on the market today with the cost of low-cost substrata optimised in the current investigation revealed a significant difference in lowering the production cost for acclimation. Agar costs $9.6 to prepare 10 litres of medium, whereas reusable substrata like coconut husk, wood bark, charcoalwood compost and crushed bricks cost ~0.7 USD (Table 3.6). As a result, the cost-effectiveness of agar gel medium is reduced by about ~92.70% when agar gel and wood bark (in a 1:1 ratio) are used for the acclimation of regulators.

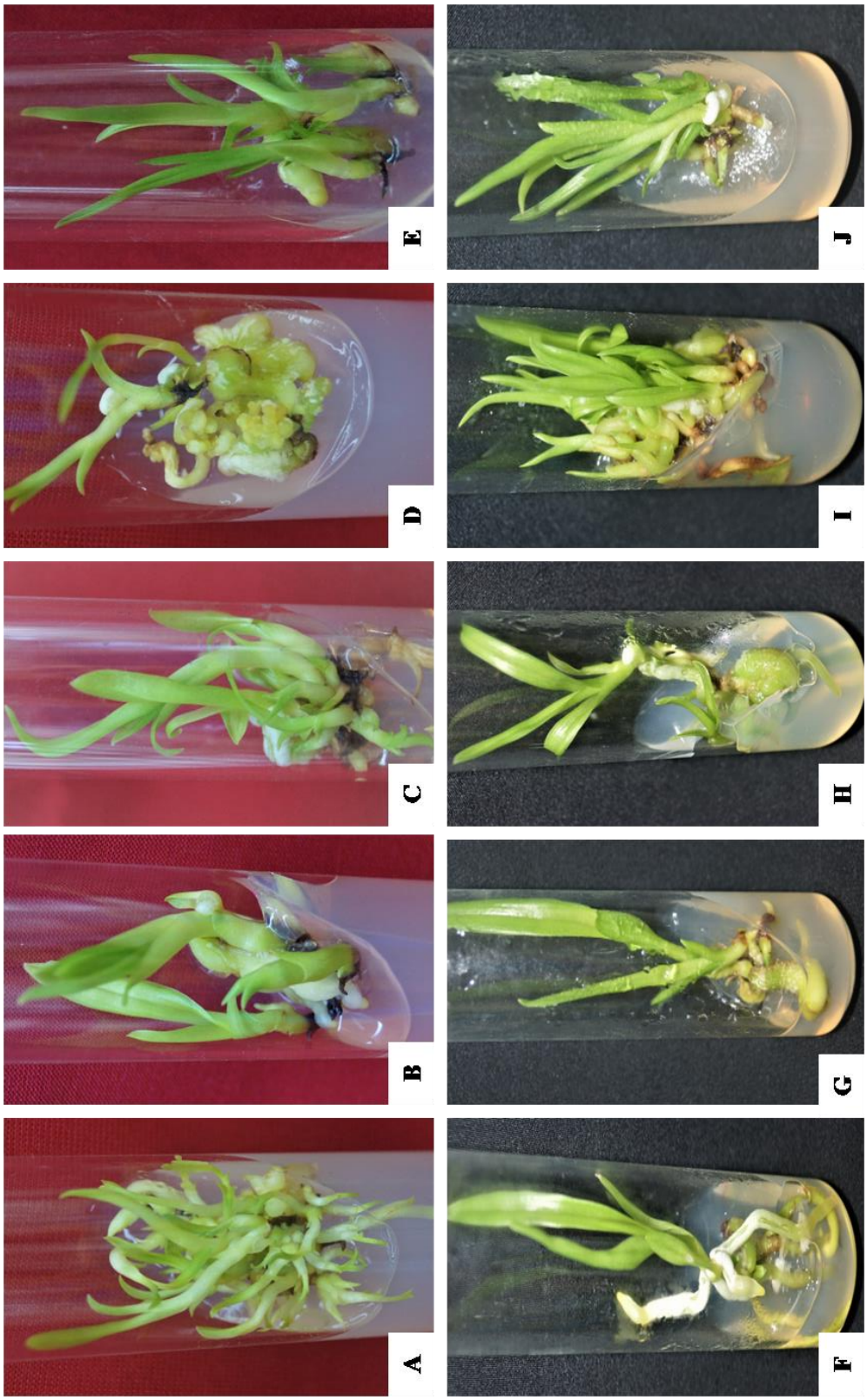

Figure 3.3: Plant regeneration and multiplication response on MS medium (Sucrose 3%) (A) Shoot etiolating with KN (B, C) Etiolated growth in KN3NAA3 and KA6NAA6, (D) Formation of globular tissues in root in KN9NAA9; (E) Healthy shoot and root formation in BA3NAA3; (F) Full developed plantlet in BAP6NAA6 (G, H) Globular tissues formation in roots with BAP9NAA9 and BAP6NAA9 (I, J) Shoot multiplication in sucrose 3% and fructose 3% (BAP6+NAA3)

Figure 3.4: Steps of acclimatization *in vitro* plants of *C. bicolor* before introducing to field conditions. (A) Stressing plantlets of nutrients in same exhausted nutrient medium; (B) Primary acclimation for 30 days in sterile substrata *in vitro*; (C) Introduction to normal room temperature; (D) Roots attaching to substrata (E) Potted *in vitro* plantlets (F) Plants fully acclimated for potting/filed transfer.

Table 3.5: Effect of acclimatization conditions for successful acclimatization and hardening of plantlets

Sl. No.	Acclimatization condition	Ratio of the substrata	Survival rate (%) (±SE)**
1	Coconut husk *	1	45.83 ± 2.31^{h}
2	Wood bark*	1	51.45 ± 2.40^{g}
3	Wood compost	1	86.74 ± 2.33^{c}
4	Coconut husk + Wood bark *	1:1	61.41 ± 1.15^{d}
5	Coconut husk + wood bark + charcoal *	1:1:1	51.67 ± 3.48^{f}
6	Coconut husk + wood bark + charcoal + wood compost	1:1:1:1	94.21 ± 2.40^{b}
7	Coconut husk + wood bark + charcoal + wood compost + crushed bricks	1:1:1:1:1	97.56 ± 0.58^{a}

Note: * Hardening substrata fortified with 1/4th MS nutrient solution except Sl. No. 6 and 7, **±SE: Standard error from mean. Mean with different superscripts within the same column differ significantly ($P<0.05$) by Tukey's HSD at 95% confidence level.

Table 3.6: Comparative analysis of agar gel medium Vs. low-cost substrata used in the present study

Sl. No.	Gelling agent/Substrata	Cost / per kg (US$)	Quantity to make 1 Lit. of medium (grams)**	Cost (US$)	Cost effectiveness of primary acclimatization against agar gel medium (%)
1	Agar	120.00	8	9.6	-
2	Coconut husk + wood bark + charcoal + wood compost + crushed bricks (1:1:1:1:1)	1.00/0.10/ 0.25/0.20 /0.10	500	0.70	92.70%

*Based on the current market price, ** One litre of medium can be dispensed in 70 test tubes (size: 25 mm diameter).

3.4. Discussion

The ideal culture medium for asymbiotic seed germination of each species of orchid should be investigated because each culture medium encouraged a different level of germination efficiency (Yam et al., 1989; Muthukrishnan et al., 2013; Kunakhonnuruk et al., 2019). In the present study three different media viz., MS, Mitra and Knudson 'C' fortified with three different organic carbon sources (sucrose, fructose and Glucose, 0-3%) were tested for immature seed germination and amongst these three media, MS medium was found to be most suitable as the medium resulting in early germination and formation of protocorms while other two media were found to be comparatively poorer. MS medium has been used for the germination and growth of several species of orchids, providing the best results because of the presence of inorganic salts, vitamins, amino acids, carbohydrates, and high concentrations of nutrients (Salazar and Cancino, 2012; Hunhoff et al., 2018; Mercado and Delgado, 2020). All of the media used in this experiment had distinct sources of nitrogen; the MS medium has NH_4 NO_3 and KNO_3 as sources of nitrogen, the Mitra medium has KNO_3 and $(NH_2)_4SO_4$, and the KC medium has $Ca(NO_3)_2$,$4H_2O$, KNO_3 and $(NH_4)_2$ SO_4. For the germination of seeds and the growth of seedlings of *C. bicolor*, ammonium nitrate in MS medium might be a practical source of nitrogen. This is in accordance to the results found by Mohanty et al. (2012) and Barman et al. (2020). Optimum germination was favoured in MS media supplemented with sucrose 3%, sucrose 2% and fructose 3% with germination rate of ~74% followed by fructose 2% (73%). Germination on Mitra et al. medium was found relatively lower with highest germination rate up to ~39% (Sucrose 3% and fructose 3%) only, and in Knudson C medium no germination was observed. From the different percentages of carbon sources added, germination rate was closely similar on MS media with sucrose and fructose (1-3%), but glucose failed to support any germination (1-3%). Seed ages of 8-11 MAP were taken to assess the relationship of the embryo

developmental stage and its response to asymbiotic germination *in vitro*. Where immature embryo development stage/green pod age was discovered to be an important determinant of effective asymbiotic seed germination. Varied response in germination rate was observed, where seed age of 11 months germinated best (up to 78%) in all media. Mayo-Mosqueda et al. (2020) also reported similar response where seed pods close to opening had the highest viability values. The underdeveloped embryo in the early seed capsules may be responsible for the low germination rate since it was unable to absorb nutrients from the medium (Long et al., 2010; Nanekar et al., 2014).

The germination of seeds devoid of PGRs suggests that sufficient levels of endogenous hormones were present in the seeds at the time of germination (Lo et al., 2001; Lan et al., 2010; Paul et al., 2012). However, addition of PGRs (BAP, KN, NAA, IAA: 3-9 µm/L) to the nutrient medium enriched with different carbon sources showed positive role of PGRs in germination time and morphological response of the immature embryos. Time taken for seeds to germinate was least in MS media fortified with sucrose 3% and fructose 3% (with KN 3 µm/L, IAA 3 µm/L and IAA 6 µm/L) and an average germination rate of 95% in IAA 3 µm/L (sucrose 3%). Development of healthy protocorms was observed in all mediums with PGRs; however hairy/filamentous protocorms were observed in medium supplemented with NAA and IAA. Protocorms, which are merely a transitional structure between the embryo and the plant, have been discovered to be an effective explant for regenerating pseudobulbs, i.e., organs that resemble protocorms and also trigger the development of plantlets in the same manner as protocorms do (Teixeira da Silva et al., 2006). The key benefits of direct pseudobulb creation, which does not involve a callus phase, creates the reduction of regeneration time and economic viability for effective mass communication (Roy et al., 2011). Plantlets regenerated from protocorms in MS medium enriched with different carbon percentages supplemented with a combination of KN+NAA

(3-9 µm/L) gave rise to etiolated plantlets with internodes, which are not morphological characteristic of *C. bicolor*. Thus, optimum medium condition for plantlet regeneration was observed in MS medium (Sucrose 3% and Fructose 3%) supplemented with BAP+NAA (6+6 µm/L, 6+9 µm/L, 3+3 µm/L and 6+3 µm/L). Additionally, it has been noted that BAP+NAA (6+3 µm/L) was the most efficient combination for protocorm multiplication and simultaneous shoot differentiation among the combinations and concentrations. However, it was observed that higher concentrations of BAP and KN (above 9 µm/L) when combined with NAA lead to formation of globular structures in roots. Such abnormal structures were borne to contamination and rotting during acclimatization causing loss of plants. Acclimatization is a crucial step in orchid *in vitro* propagation since when plants are transplanted to ex vitro settings, a substantial percentage of them are harmed or lost (Torres et al., 2006; Loeza et al., 2011). The ability to transfer plants out of culture on a large scale, inexpensively, and with good survival rates is crucial for the long-term viability of *in vitro* propagation on a commercial scale (Deb and Imchen, 2010; Diaz et al., 2010; Lesar et al., 2012). Substratum made from Coconut husk, wood bark, charcoal, wood compost and crushed bricks (1:1:1:1:1) had the highest survival rate over other substrata. Additionally, when the transplant was transferred with the substratum, the survival rate was significantly higher. The notably higher survival rate of above 70% in wood compost and substratum containing wood compost might be because it contains the required nutrients available for plantlet growth.

3.5: Summary and Conclusion

C. bicolor is a vulnerable orchid with floriculture, horticultural and medicinal values. The present research developed an optimised *in vitro* protocol for asymbiotic seed germination, plant regeneration and mass production. The seeds of the species were found to favour both sucrose and fructose (2-3%) as carbon source. From the three medium

employed *viz*, MS, Mitra et al. and Knudson C, optimum response was found on MS medium for seed germination, plant regeneration as well as plantlet multiplication over the latter two. The relationship of seeds' development stage with germination rate showed that seeds of 11 MAP gave the best response. *In vitro* acclimatization and the type of substratum the regenerates were transferred to was found to be very crucial for the survival of the plantlets in *ex vitro* condition. Thus, the finding of the study is in agreement that, the type of nutrient medium, carbon source, PGRs, seed age etc., for asymbiotic germination of seeds and plantlet regeneration in orchids is species dependent. Further research is required to understand the pseudobulbs' divergent reaction to regenerated plants. The results of this experiment could serve as a baseline for future studies on *in vitro* culture/clonal propagation.

Chapter - 4

In vitro propagation of *Dendrobium heterocarpum* Wall. ex. Lindl.

4.1. Introduction

Dendrobium is the second-largest genus in the Orchidaceae, with 1600 sympodial epiphytic species and is well-known for its floricultural and therapeutic uses (Tikendra et al., 2021a). Pseudobulbs, often known as "canes," which are modified versions of orchid stems sensitive to food and water reserves, are seen on *Dendrobium* orchids. Their most common habit is to creep over the substratum, such as tree trunks or branches. The majority of *Dendrobium* species thrive best in moderate climates, humid environments, and relatively high, hilly terrain between 1400 and 1600 metres above sea level. Their wide geographic distribution allows the species to grow into considerable diversities and produce a large number of interspecific hybrids with different morphological features (Lam et al., 2015).

In India, *Dendrobium* is the second-largest orchid genus in with 103 species found in the Andaman and Nicobar Islands, the Western Peninsula, the Eastern Himalayas, and the Himalayas (Singh et al., 2001). 82 species were found in north-east India according to a survey on the biodiversity of *Dendrobium* and its distribution (Lokho, 2013). In Nagaland, *Dendrobium* is the genus with the most species according to Deb et al., (2021), with 52 different species.

Dendrobium orchids are conventionally propagated asexually through keikes and by dividing or cutting pseudobulbs, but the rate of propagation is too low, and the process is time consuming to meet market demands. In addition, the seed heterozygosity of orchids, miniature size, lack of endosperm and requirement of mycorrhizal fungal association for

successful germination makes *in vivo* propagation difficult (Bhattacharjee and Islam, 2014); thus, making the conventional methods unreliable for mass production and commercialization. *In vitro* propagation is considered as a reliable technique for large scale culture of threatened and economically important plant species for recovering wild population and sustain market demands (Deb et al., 2018). Using *in vitro* techniques and various growth regulator studies, it has been made possible to maximise the seed germination and seedling development of rare and endangered orchid species (Adhikari and Pant, 2019). *Dendrobium* is micro propagated using a variety of basal medium, the most common of which are MS (Murashige and Skoog, 1962), KC (Knudson 1946), KC, VW (Vacin and Went, 1949), B5 (Gamborg et al., 1968), N6 (Chu et al., 1975), Phytotechnology medium, Mitra (Mitra et al., 1976) and modified RM medium (Kukulczanka and Wojciechowska, 1982; Jaime et al., 2015). However, the type of media used was greatly influenced by the species and explants selected.

Dendrobium heterocarpum Wall. ex. Lindl. with its attractive flowers have high potential in floriculture industry. The species is well known for its ethnomedicinal uses. In ethnomedical practices, the paste of its pseudobulb is also used to treat bone fractures (Subedi, 2011). Species of *Dendrobium* having therapeutic benefits have been widely harvested, rendering them scarce in their natural habitat (Teoh, 2016; Ninawe and Swapna, 2017). In addition, habitat loss, degradation, fragmentation, and illegal trafficking, classify majority of these species as severely endangered or threatened (Pant, 2013). Realizing the importance and threat of the species, Tham et al., (2018) established *in vitro* plant regeneration protocol with limited success through dormant bud culture of *D. heterocarpum*. Therefore, adoption of effective conservation measures and a rapid propagation of the species for reintroduction in its habitat and commercial exploitation are crucial.

Recognising the status of *D. heterocarpum* and having the potential of commercialisation for its flower and medicinal value, the present study was aimed at developing an efficient protocol for *in vitro* propagation through immature embryo culture and assess the genetic fidelity of the *in vitro* regenerants.

4.2. Materials and Methods

4.2.1. Sample collection and maintenance

Dendrobuim heterocarpum plants were collected from Sumi Setsü village, Zunheboto, Nagaland, India and maintained in Orchidarium, Department of Botany, Nagaland University, Lumami. Flowers were hand pollinated and tagged to record the seed age. For assessment of effect of green pod age on asymbiotic seed germination, green seed pods of 5 to 8 months after pollination (MAP) were used in the present study.

4.2.2. Explant sterilization

Green seed pods (5-8 MAP) were used for sourcing immature embryos for culture. Initial surface sterilization of the seed pod was done using diluted 'Labolene' (1:100, v/v, a commercial laboratory detergent) and scrubbed off to remove dirt with soft brush. After washing thoroughly in running water, seed pod was treated with 0.3% (w/v) aqueous solution of $HgCl_2$ for 3 min under aseptic condition and rinsed 3-4 times using sterilised pure water. Finally, seed pods were dipped in 70% alcohol (v/v) for 30 sec. and flamed for 3-4 sec. Seed pods were then split opened to scoop out the immature embryos for culture on fortified nutrient media.

4.2.3. Culture media preparation

For the study, three nutrient media viz. MS (Murashige and Skoog, 1962), Mitra et al. (Mitra et al., 1976) and Knudson 'C' (Knudson 1946) were used after different fortifications. All the inorganic and organic compound including plant growth regulators (PGRs) used in the present investigation was obtained from HiMedia Laboratories Pvt. Ltd,

India unless mentioned otherwise. The media were supplemented with different organic carbon sources *viz*., sucrose, fructose and glucose (0-3%, w/v) and further fortified with different PGRs like N_6–benzyl adenine (BA), kinetin (KN), α-naphthalene acetic acid (NAA) and Indole-3-acetic acid (IAA) (0-15 µM/L) for asymbiotic seed germination. For plant regeneration different concentration of cytokinins (BA and KN) and NAA were incorporated singly or in combination to the medium (0-12 µM/L). Media were gelled with 0.8% (w/v) tissue culture grade agar and *p*H was adjusted to 5.7 using 0.1N NaOH and 0.1N HCl.

4.2.4. Initiation of culture

The exalbuminous embryos at different stages of development (5-8 months) were scooped from surface sterilized seed pods and inoculated on the nutrient media fortified differently for *in vitro* germination (Figure 4.1 A,B). All cultures were maintained at 25±2ºC temperature and 40 $\mu molm^{-2}s^{-2}$ illuminations at 12/12 h light/dark photoperiods provided by white fluorescent tubes.

4.2.5. Plant regeneration and multiplication

The germinated seeds were allowed to differentiate into protocorms before sub-culturing on fresh and/or new nutrient medium fortified differently compared to initiation medium. The protocorms after releasing the first set of leaves was considered for transferring on regeneration medium for plant regeneration. The shoot buds/young plantlets were separated and cultured on multiplication medium for culture proliferation. Well differentiated plantlets were allowed to grow on the same medium to experience nutrient stress and let them begin to get primarily acclimatized.

4.2.6. Primary acclimatization and transplantation

Well-developed rooted plantlets from regeneration medium were sourced and the roots were cleansed with soft brush to remove the traces of agar and subjected to primary acclimatization. For the purpose different substrata *viz*., chopped coconut husk (CH) (~0.5-1.0 cm), wood bark pieces (WB) (~0.5-1.0 cm), wood compost (WC), charcoal pieces (CP) and crushed bricks (CB) were used in different combination as agar alternative. For wood barks, any species of tree having more than 0.5 mm thickness was collected followed by soaking in water for 48 h and dried before use. All the substrata were first autoclaved at 1.05 kg cm^{-2} pressure and 121°C for ~30 min. Appropriate quantity of the processed substrata were placed in culture vials in different ratios as below:

1. Coconut husk; 2. Wood bark; 3. Wood compost; 4. CH+WB (1:1 ratio); 5. CH+WB+CP (1:1:1 ratio); 6. CH+WB+CP+WB (1:1:1:1 ratio); 7. CH+WB+CP+WC+CB (1:1:1:1:1 ratio) and 8. CH+WB without any MS salt solution.

About 15-20 ml of 1/4th MS salt solution without any sucrose and PGRs were added in all the culture vials except combination where plain tap water was used instead of MS salt solution. The culture vials were autoclaved and cooled to room temperature before plantlets were cultured on the substrata and maintained under normal laboratory conditions as specified earlier for one week under normal room temperature with indirect sunlight (~75% diffused light) for ~4 week. The primarily acclimatized plantlets were then transferred to community potting mix prepared by mixing the same un-autoclaved substrata mixture as primary acclimatization conditions and covered with holed transparent plastic sheets and maintained for 2-3 week before transferring to normal field conditions. During this period the plants were fed with 1/10th MS salt solution at weekly interval.

4.2.7. Experimental design and statistical analysis

All experiments were designed randomly. All the experiments were repeated thrice with 10 replicates in each set and are presented in Mean ±SE. Statistical analysis was computed using SPSS (Version 2). Difference in plants response to experimental treatment were analysed using One-way Analysis of Variance (ANOVA) at 95% confidence level ($P<0.05$) followed by post hoc Tukey test to determine the mean significant difference between treatments.

4.3. Results

Successful *in vitro* germination of immature seeds was greatly influenced by different factors like, seed pod age, nutrient medium, quality and quantity of organic carbon in the nutrient medium, quality and quantity of PGRs, culture conditions etc. The influence of different factors on *in vitro* culture of immature embryos is presented below:

4.3.1. Effect of Nutrient media, seed age and carbon source on asymbiotic germination

Germination of immature cultured embryos was observed after 5 weeks of inoculation. The sign of successful germination was marked by nodular swelling (Figure 4.1 A, B, C) followed by embryos breaking off from testa (Figure 4.1D) and subsequent development of protocorms (Figure 4.1 E). The nutrient media for initiation of culture were supplemented with three different organic carbon sources (fructose, glucose and sucrose, 0-3%, w/v) to understand the effect of quality and quantity of organic carbon on immature seed germination of *D. heterocarpum* (7 MAP) and the result is shown in Table 4.1. Of the three organic carbon sources exogenously added in the initiation media at different concentrations, sucrose in general was found to be better across the concentrations in all the nutrient media. Nutrient media fortified with Sucrose 3% favoured higher germination rate in MS (85.48% ±0.61), Mitra et al. (72.44% ±2.51) and Knudson (69.79 ±2.38) compared to lower sucrose concentration. The fructose concentrations (0-3%) favoured moderate

germination of the embryos with highest on MS media with 3% fructose (67.41% ±2.63) and least on Knudson with 1% fructose (41.92% ±2.70). The germination of the embryos was found relatively lower in all the nutrient media containing glucose (0-3%) with highest germination rate up to 47.52% ±0.60 (MS, 3% glucose) only.

In order to assess the relationship between seed age and germination, MS with 3% sucrose, fructose, and glucose as the carbon source was taken since it demonstrated a higher germination rate. The age of green pods, which also determine the developmental stage of the growing embryos had accounted a significant impact on *in vitro* germination seeds. Immature embryos of lower age (5 MAP) registered comparatively poorer *in vitro* germination response which improved with increase of developmental age/green pod age. Under the given conditions, the optimum germination was achieved with immature seeds from green pods of 8 MAP where ~95% germination was recorded on nutrient medium containing sucrose (3%) followed by fructose (81.5%), and glucose (49.9%). The germination performance declined with lower seed age and least germination was recorded from seeds of 5 MAP where ~48% seeds germination on sucrose (3%) fortified medium followed by 30.2% in fructose (3%) and 22.67% in glucose (3%).

Table 4.1: Effect of nutrient medium and organic carbon source percentage in asymbiotic seed germination of *Dendrobium heterocarpum* Wall. ex Lindl

Carbon source	Nutrient medium		
	MS*	**Mitra et al.***	**Knudson***
Sucrose 1 %	62.57 ±2.48^{d}	45.98 ±1.39^{e}	40.50 ±2.64^{ef}
Sucrose 2%	81.64 ±2.22^{b}	64.16 ±2.33^{b}	50.33 ±1.83b^{c}
Sucrose 3 %	85.48 ±0.61^{a}	72.44 ±2.51^{a}	69.79 ±2.38^{a}
Fructose 1%	43.57 ±2.48^{g}	43.80±2.83^{ef}	41.92 ±2.70^{e}
Fructose 2%	57.17 ±1.46^{e}	51.99 ±1.21^{d}	48.29 ±2.38^{cd}
Fructose 3%	67.41 ±2.63^{c}	57.81±1.43^{c}	52.31 ±1.84^{b}
Glucose 1%	20.97 ±2.39^{i}	19.97 ±0.84^{i}	17.04 ±1.34^{h}
Glucose 2%	25.37 ±1.22^{h}	26.29 ±1.13^{gh}	22.28 ±1.16^{g}
Glucose 3%	47.52 ±0.60^{f}	27.42 ±1.56^{g}	25.14 ±1.49^{f}

Note: *±SE: Standard error from mean. Mean with different superscripts within the same column differ significantly (P<0.05) by Tukey's HSD at 95% confidence level.

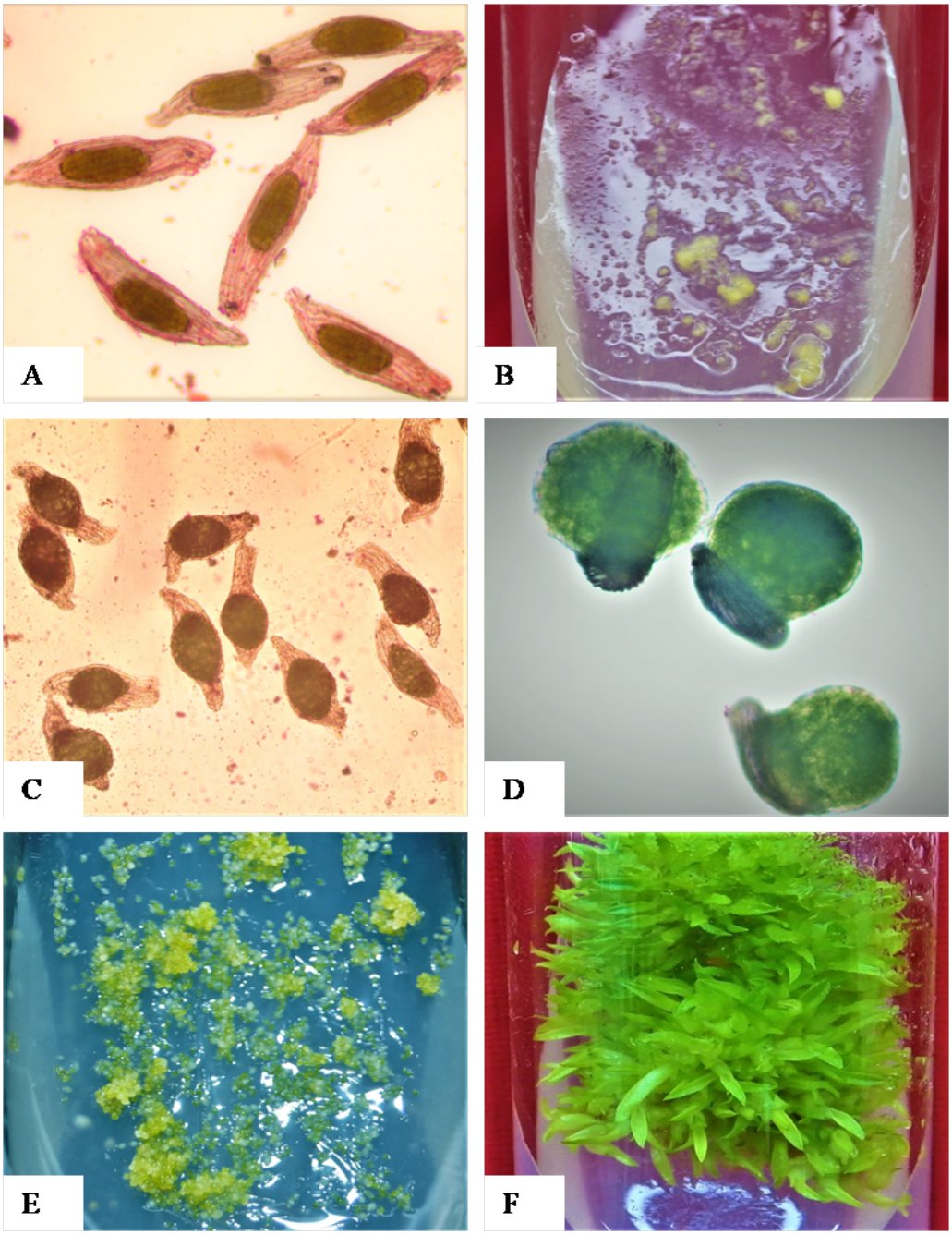

Figure 4.1: Different stages of *in vitro* asymbiotic immature seeds germination of *D. heterocarpum* on MS medium with sucrose 3%. **A**. Immature seeds; **B**. Cultured immature seeds on germination medium started swelling; **C**. Swollen embryos; **D**. Magnified image of nodular swelling and embryos breaking off from testa; **E**. Protocorms formation; **F**. Advance stage of protocorms differentiating into initial leaves.

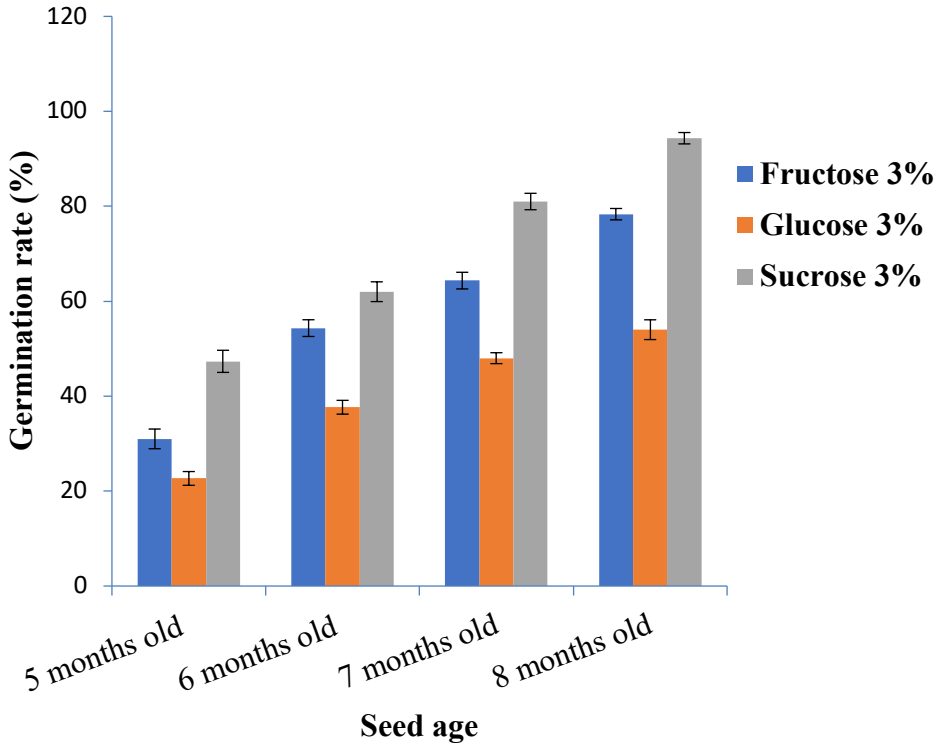

Figure 4.2: Effect of seed age and different organic carbon sources (3%, w/v) on asymbiotic germination of immature *D. heterocarpum* embryos.

4.3.2: Effect of nutrient media and PGRs on asymbiotic germination

Different nutrient media exhibited significant variations in germination and culture behaviours. The embryos cultured on water agar medium failed to invoke any germination; however, nutrient media devoid of any PGRs showed moderate response (90.65% germination) against PGRs fortified media. Of the three nutrient media tested for germination of immature seeds, MS medium was found to be better choice over Mitra et al. and Knudson 'C' media (Table 4.2). Seeds cultured on MS medium supported early germination and higher rate of germination than seeds cultured on other two nutrient media germination under otherwise identical conditions. On MS medium the germinating seeds produced green healthy protocorms (Figure 1 E); however, the protocorms developed on other two media were small and protocorms were devoid of chlorophyll on Knudson 'C' medium.

Besides nutrient media, the quality and quantity of exogenously added PGRs in the nutrient media exhibited pronounced effects on different aspects of seed germination. Of the four different PGRs incorporated at various levels, in general BA, KN and NAA were found to support better seed germination (Table 4.2). Under the given culture conditions, optimum germination was recorded on MS medium fortified with sucrose (3%) and BA (6 µM/L) (91.78%), KN (3 µM/L) (94.95%) and NAA (9 µM/L) (95.33%) after about 48, 38 and 45 days of culture initiation respectively. Though the germination rates were identical on KN and NAA enriched media, early germination response was observed on medium containing KN (3 µM/L) where germination resulted after 38 days against 45 days on NAA enriched medium. Further, comparatively better culture differentiation was recorded on MS medium fortified with KN (3 µM/L) where the protocorms differentiated to produce initial leaves (Figure 4.1 F). Besides these, it was observed that seeds cultured on IAA fortified medium, excessive formation of hairy filaments on the protocorms was recorded but failed to differentiate to plantlets. The resulted protocorms on different germination media were maintained on optimal initiation medium for 3-4 weeks to reach the advance protocorms stage before transferring on regeneration medium.

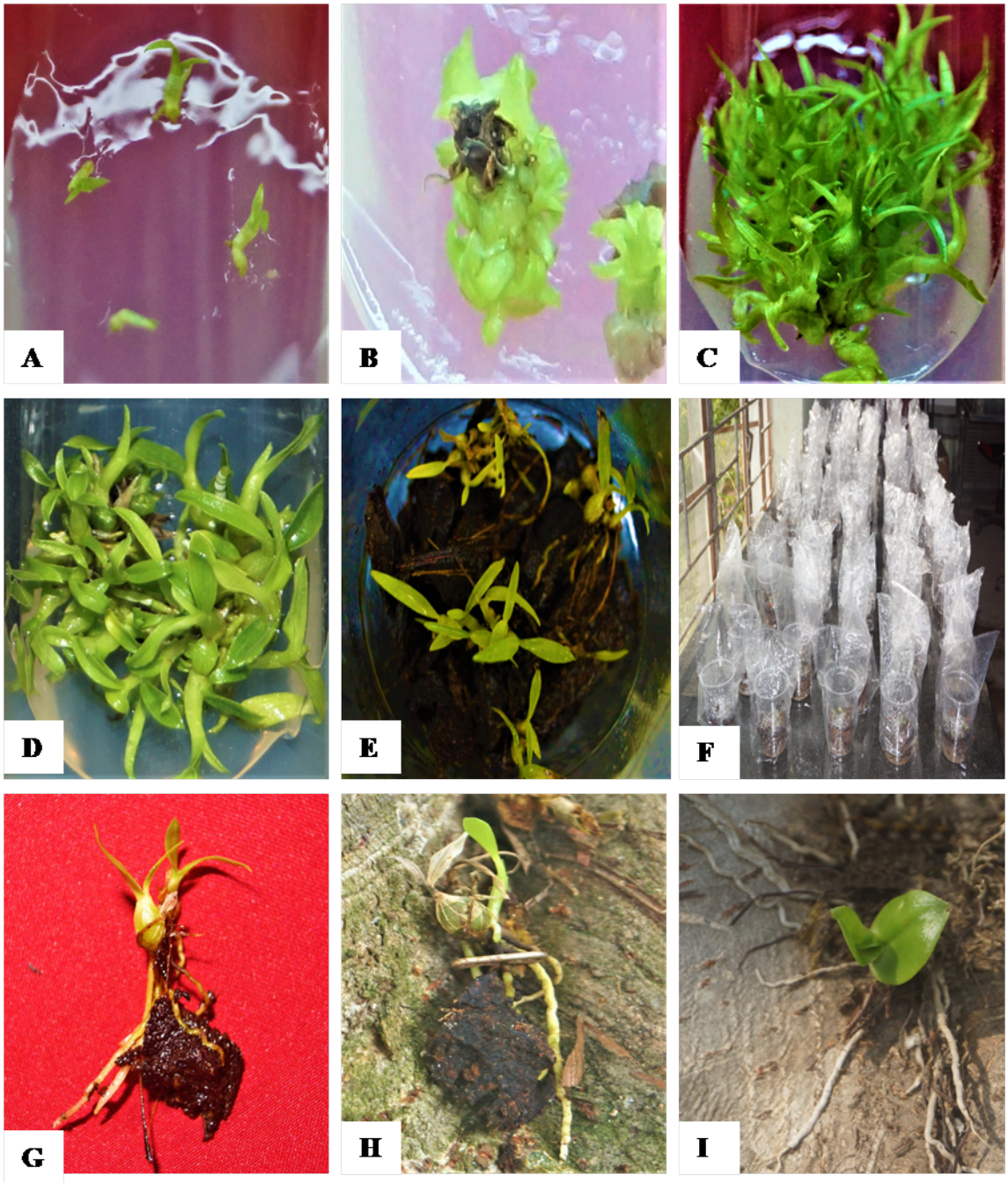

Figure 4.3: Plant regeneration and multiplication of *D. heterocarpum* on MS medium enriched with sucrose (3%) and KN+NAA (3+12 µM/L). **A**. Sub-culture of advance stage protocorms; **B**. Development of new protocorms; **C**. Multiple shoots formation with pseudobulbs on regeneration medium; **D**. Regenerated plants with well-developed shoots, psuedobulb and roots; **E.** Regenerates under primary acclimatization; **F & G**. Acclimatized plantlet with roots attached to substrata; **H**. Acclimatized plants transferred natural habitat conditions; **I**. Established plantlets in tree trunk.

Table 4.2: Effect of different culture media and concentrations of PGRs on *in vitro* germination of *D. heterocarpum* embryos

Nutrient Medium*	PGRs Conc.(µM/ml)	Time for Germination (days)(±SE)**	Germination rate (%)(±SE)**	Response type of protocorms
Water-agar	-	0	0	No response
MS	Control	44.36±0.54^{nopqrst}	90.65±0.64^{bcdefghi}	Formation of green healthy protocorms
	BA_3	43.65±0.26^{opqrstu}	90.81±0.61^{bcdefgh}	Formation of green healthy protocorms
	BA_6	47.75±0.48^{hijklmn}	91.78± 0.70^{abcdef}	Formation of green healthy protocorms
	BA_9	49.22±0.47^{fgh}	76.95±0.66^{qrs}	Formation of green healthy protocorms
	BA_{12}	49.50±0.36^{fg}	74.36±0.43^{rst}	Formation of green healthy protocorms
	BA_{15}	48.25±0.36^{fghi}	70.02±1.01^{v}	Formation of green healthy protocorms
	KN_3	37.75±0.53^{y}	95.65±0.36^{a}	Formation of green healthy protocorms
	KN_6	42.00±0.44^{stuvw}	94.41±0.57^{ab}	Formation of green healthy protocorms
	KN_9	43.50±0.88^{opqrstuv}	94.04±0.56^{abcd}	Formation of green healthy protocorms
	KN_{12}	45.21±0.52^{no}	88.48±1.06^{fghijkl}	Formation of green healthy protocorms
	KN_{15}	45.00±0.60^{nop}	73.78±0.70^{stuv}	Formation of green healthy protocorms
	NAA_3	41.25±0.28^{uvwx}	88.90±0.81^{efghijkl}	Formation of green healthy protocorms
	NAA_6	44.75±0.54^{nopqr}	94.28±0.62^{abc}	Formation of green healthy protocorms
	NAA_9	44.75±0.45^{nopqrs}	95.33±0.90^{a}	Formation of green healthy protocorms

	NAA_{12}	48.05 ± 0.14^{fghijkl}	78.4 ± 0.54^{qr}	Formation of green healthy protocorms
	NAA_{15}	44.75 ± 0.42^{nopq}	74.67 ± 0.54^{rst}	Formation of green healthy protocorms
	IAA_3	48.00 ± 0.41^{fghijklm}	90.41 ± 0.81^{bcdefghij}	Formation of green protocorms with hairy filaments
	IAA_6	48.35 ± 0.28^{fghijk}	92.36 ± 1.14^{abcde}	Formation of green protocorms with hairy filaments
	IAA_9	53.25 ± 0.15^{e}	86.28 ± 0.47^{jklmnop}	Formation of green protocorms with hairy filaments
	IAA_{12}	49.82 ± 0.49^{f}	79.67 ± 1.14^{q}	Formation of green protocorms with hairy filaments
	IAA_{15}	48.57 ± 0.46^{fghij}	$87.75\pm0.89^{fghijklmno}$	Formation of green protocorms with hairy filaments
Mitra***	BA_6	63 ± 0.39^{d}	88 ± 0.60^{fghijklm}	Small green protocorms
	KN_6	62 ± 0.37^{d}	91 ± 1.08^{bcdefg}	Small green protocorms
	NAA_6	66 ± 0.62^{nopqr}	89 ± 0.80^{cdefghijk}	Small green protocorms
Knudson 'C'***	BA_6	73 ± 0.90^{b}	45 ± 0.74^{w}	Small green protocorms + Colourless PLBs
	KN_6	84 ± 0.35^{ab}	47 ± 0.64^{w}	
	NAA_6	86 ± 0.59^{a}	39 ± 0.76^{x}	Small green protocorms + Colourless PLBs

Note: ∗ Nutrient medium contains 3% sucrose. ∗∗ ±SE: Standard error from mean. ∗∗∗ Only concentrations supported optimal response are computed. Mean with different superscripts

within the same column differ significantly (P<0.05) by Tukey's HSD at 95% confidence level.

4.3.3. Plant regeneration and culture proliferation

For differentiation of protocorms to plantlets and culture proliferation, three nutrient media were fortified with different concentrations of BA, KN and NAA (Table 4.3). Advance stage protocorms were subcultured, which proliferated giving rise to more protocorms from where multiple plantlets were formed (Figure 4.3 A, B, C). Among the three media tested, MS medium was found to support better differentiation of PLBs to rooted plantlets (Table 4.3). The three PGRs incorporated in the regeneration media exhibited varying regenerative response. Under ideal condition, protocorms differentiated to rooted plantlets with well expanded leaves (Figure 4.2 D). The average shoot length (1.53 cm) and pseudobulbs length (1.53 cm) were achieved on PGR free MS medium containing sucrose (3%) though leaf growth was impaired; while on medium fortified with one of the PGRs improved the leaf growth and shoot proliferation. For shoot proliferation, pseudobulbs, leaves and root formation BA in general was found to be inferior compared to KN and NAA fortified medium (Table 4.3). Optimum shoot length (1.62 cm) was achieved on MS medium supplemented with NAA (9 µM/L) followed by NAA (6 µM/L) (1.47 cm) and KN+NAA (6+12 µM/L) in combination (1.4 cm). Shoot length, pseudobulbs length, number of leaves and root length on MS medium enriched with sucrose (3%), and KN+NAA (3+12 µM/L) was found to be most suitable where average number of shoots per subculture, shoot length, pseudobulbs length, number of leaves and length and root length were 17.67, 1.17 cm, 0.77 cm, 4.67, 0.43 cm and 0.43 cm respectively followed by KN+NAA (9+12 µM/L). However, on Mitra et al. and Knudson C. nutrient medium plant regeneration from PLBs was delayed substantially.

Table 4.3: Effect of different basal medium and PGRs concentration on *in vitro* plant regeneration from protocorms

Culture medium	PGRs Conc. (µm/L) BAP	KN	NAA	Shoot length (cm) (±SE)*	Pseudobulb length (cm) (±SE)*	Leaf length (cm) (±SE)*	Root length (cm) (±SE)*	No. of leaves (±SE)*	No. of shoots (±SE)*
Control				-	-		-	-	-
MS			No PGR	1.53 ±0.08^{ab}	1.53 ±0.09^{a}	0.54±0.06^{abcd}	0.13±0.33^{k}	4.67±0.33^{abcd}	8.67±1.20^{defgh}
			BAP6	0.8 ±1.2^{fg}	0.32±0.75^{mnop}	0.62±0.06^{abcd}	0.13 ±0.89^{k}	4.44±0.24^{abcdefg}	4.42±0.78^{h}
			BAP9	0.97 ±0.72^{defg}	0.45±0.37^{hijklmn}	0.49 ±0.05^{cd}	0.19±0.72^{ijk}	4.21±0.15^{abcdefg}	5.26±1.45^{efgh}
			KN6	0.99 ±0.54^{defg}	0.58 ±0.27^{fhijk}	0.54 ±0.09^{abcd}	0.1 ±0.46^{k}	3.89±0.35^{abcdefg}	5.13±1.89^{fgh}
			KN9	1 ±0.95^{defg}	0.62 ±0.34^{fhij}	0.51 ±0.04^{cd}	0.15 ±0.16^{jk}	4.0±0.08^{abcdefg}	7.45±1.57^{efgh}
			NAA6	1.47±0.75^{abc}	0.92 ±0.62^{bcd}	0.6 ±0.18^{abcd}	0.25 ±0.64^{ij}	4.44±0.58^{abcdefg}	10.35±1.37^{cdefgh}
			NAA9	1.62 ±0.3^{a}	0.77 ±0.48^{cdefg}	0.74 ±0.05^{a}	0.37±0.25^{defgh}	4.67±0.95^{ab}	11.06±1.43^{bcdef}
			BAP3NAA3	0.83 ±0.47^{efg}	0.2 ±.04^{p}	0.68±0.21^{abc}	0.12 ±0.02^{k}	3.89±0.28^{abcdefg}	6.75±0.63^{efgh}
			BAP6NAA6	1.03 ±0.13^{cdefg}	0.23 ±0.15^{op}	0.50 ±.04^{cd}	0.13 ±0.25^{k}	4.68±0.25^{a}	14.20±0.88^{abcd}
			BAP6NAA9	1.3 ±0.15^{abcdef}	1.10 ±0.15^{b}	0.73 ±.03^{ab}	0.37 ±0.88^{defg}	4.57±0.02^{abcde}	11.33±1.00^{bcde}
			KN3NAA3	0.97 ±0.12^{defg}	0.67 ±0.57^{fghi}	0.67 ±.06^{abcd}	0.27 ±0.66^{fghi}	3.67±0.34^{efg}	5.00±0.33^{fgh}
			KN3NAA12	1.17 ±0.33^{abcdefg}	0.77 ±0.66^{cdef}	0.43 ±0.4^{d}	0.43 ±.67^{cd}	4.67±0.33^{abc}	17.67±1.85^{a}
			KN6NAA3	0.74 ±0.57^{g}	0.4 ±0.54^{klmno}	0.43 ±0.14^{d}	0.2 ±0.78^{ijk}	3.36±0.87^{g}	8.00±1.33^{efgh}
			KN6NAA12	1.4 ±0.1^{abcd}	0.87 ±0.1^{cde}	0.47 ±0.08^{d}	0.52 ±0.88^{bc}	4.08±0.58^{abcdefg}	16.28±0.58^{abc}
			KN9NAA3	0.95 ±0.49^{defg}	0.45 ±0.47^{hijklm}	0.67 ±0.33^{abcd}	0.38 ±0.35^{def}	3.84±0.67^{abcdefg}	9.02±0.55^{defgh}
			KN9NAA12	1.3 ±0.33^{abcde}	0.53 ±0.22^{fhijkl}	0.49 ±0.5^{cd}	0.63 ±0.57^{ab}	4.41±0.47^{abcdefg}	17.00±0.88^{ab}
			KN12NAA3	0.97 ±0.82^{defg}	0.67 ±0.87^{fgh}	0.50 ±0.1^{cd}	0.41 ±0.47^{cde}	3.67±0.74^{defg}	8.33±1.22^{defgh}
			KN12NAA12	1.2 ±0.5^{abcdefg}	0.93 ±0.28^{bc}	0.55 ±0.05^{abcd}	0.67 ±0.88^{a}	4.33±0.25^{abcdefg}	10.67±2.72^{cdefg}
Mitra	-	-	NAA6	Delay in formation of shoots / unhealthy shoot and leaves					
Knudson ‘C’	-	-	NAA6	Delay in formation of shoots					

Note: Nutrient medium fortified with sucrose (3%); Data harvested after 45 days of advance stage PLBs culture on regeneration medium. Only significant concentrations are computed; *±SE: Standard error from mean.

4.3.4. Acclimatization of regenerates

For primary acclimatization, the regenerants were maintained on different substrata with/without 1/4th MS salt solution and without any PGRs. On different substrata combinations the regenerants acclimatization response was different. Compared to CH+WB combination with plain water, other combinations (except WC and CH+WB+CP+WC) supported better acclimatization and establishment of plantlets and development of new leaves and shoot buds (Figure 4.3 E). Among the different combinations of substrata, a combination of coconut husk and wood bark was found to be most suitable. It was found that during the hardening process the roots of the regenerants adhered to the substrata (Figure 4.3 G). The primary acclimatized plants were transferred on community potting mix with the substrata of same combination as primary acclimatized (Figure 4.3 F). Of the different combinations about 87, 71, 69% transplants survived after transplantation on CH+WB, CH and CH+WB+CP substrate combination respectively (Table 3). The transplants were fed with 1/10th MS nutrient solution at regular intervals during this period. Well established plants were then transferred to green house and then to natural conditions, where they were attached to tree trunks with the help of a pin and monitored for growth response (Figure 4.3 H, I).

The present study also encompasses to minimise the cost of the protocol by substituting agar for acclimatization of the plantlet. When well-developed plantlets are sub-culture to acclimatise in agar gel medium, occurrence of contamination is high leading to loss of culture. Eliminating the step of using with low-cost substrata's fresh liquid nutrients in the culture vials can be added directly, reducing contamination and manpower cost. A comparative assessment of the present market cost of tissue culture grade agar to that of low-cost substrata cost optimised in the present study, showed a wide difference in reducing the production cost for acclimatization (Table 4.5). Preparing 10 litre of medium with agar

amount to ~9.6 USD compared to 2-6 USD for substrata like coconut husk, wood bark and charcoal, which can also be reused.

Table 4.4: Effect of acclimatization conditions for successful acclimatization and hardening of plantlets

Sl. No.	Hardening condition*	Ratio of the substrata	Survival rate (%) (±SE)**
1	Coconut husk	1	71.33 ± 1.65^{b}
2	Wood bark	1	61.65 ± 1.47^{d}
3	Wood compost	1	13.65 ± 1.73^{h}
4	Coconut husk + Wood bark	1:1	87.00 ± 1.65^{a}
5	Coconut husk +wood bark + charcoal pieces	1:1:1	69.33 ± 0.85^{bc}
6	Coconut husk + wood bark + charcoal pieces + wood compost	1:1:1:1	29.76 ± 1.11^{g}
7	Coconut husk + wood bark + charcoal pieces + wood compost + crushed bricks	1:1:1:1:1	43.33 ± 1.51^{e}
8	Coconut husk + wood bark without ¼th MS salt solution	1:1	35.67 ± 3.12^{f}

Note: *±SE: Standard error from mean. Mean with different superscripts within the same column differ significantly ($P<0.05$) by Tukey's HSD at 95% confidence level.

Table 4.5: Comparative analysis of agar gel medium Vs. low-cost substrata used in the present study

Sl. No.	Gelling agent/Substrata	Cost / per kg (US$)	Quantity to make 1 Lit. of medium (grams)**	Cost (US$)	Cost effectiveness of primary acclimatization against agar gel medium (%)
1	Agar	120.00	8	9.6	-
2	Coconut husk + Wood bark (1:1 ratio)	1.00	500	0.50	94.79%

4.4. Discussion

The developmental stage of immature embryos of orchids is crucial for successful *in vitro* germination and is considered to be species specific. Every species has a very small window period of developmental stage at which successful asymbiotic germination can be achieved (Deb and Jakha, 2019). Moreover, factors like seed age, nutrient medium, source of organic carbon, quality and quantity of plant growth regulators (PGRs) and light intensity have been associated with successful embryo germination and plant regeneration *in vitro* (Pongener and Deb, 2009). Thus, the present study emphasized on optimization of different factors like seed pod age, nutrient medium, quality and quantity of organic carbon, PGRs etc. for *in vitro* propagation of *Dendrobium heterocarpum* through seed culture and check the genetic uniformity of regenerates. Optimum germination of ~95% was achieved from seeds of 8 MAP when cultured on MS medium fortified with sucrose (3%) and KN (3 μM/L), however, seeds from 5-7 MAP did not support optimum germination. Findings of the present study with *D. heterocarpum* is in agreement with the past reports in *Cymbidium aloifolium,* (Deb and Pongener, 2011) and *Dendrobium nobile* hybrids (Udomdee et al., 2014) where age of seed was key factor for successful germination in asymbiotic conditions. Hossain et al., (2013) have reported that orchids exhibit specificity to nutrient requirements and even among species belonging to same genus. Past reports revealed the differential requirement of nutrient medium with the species; some of them are VW medium for *Dendrobium chrysanthum,* Nitsch and Nitsch for *D. fimbriatum* (Devi et al., 1990), Knudson 'C' for *Cymbidium elegans* (Sharma and Tandon, 1990), Mitra et al. medium for *Dendrobium moschatum* (Tikendra, 2019a), *Dendrobium palpebrae* in MS medium (Bhowmik and Rahman, 2020). In the present study three different media *viz.,* MS, Mitra et al. and Knudson 'C' fortified with three different organic carbon sources (sucrose, fructose and Glucose, 0-3%) were tested for immature seed germination and amongst these three media, MS medium

was found to be most suitable as the medium resulting in early germination and formation of protocorms while other two media were found to be comparatively poorer. Similar, responds of MS medium favouring protocorms formation have been recorded in *Dendrobium hookerianum* (Paul et al., 2012). Of the three carbon sources, sucrose (3%) was found most suitable for *in vitro* seed germination and plant regeneration compared to other two concentrations tested. Sucrose's positive influence is connected to the activation of seed metabolism, and it aids in the maintenance of an osmotic balance between both the seed and its aqueous environment, regulating solution absorption and minimising embryo damage (Mercado and Delgado, 2020).

Apart from the factors like seed age, nutrient medium and organic carbon sources, PGRs also played an important role on the overall germination performance. It was observed that media devoid of PGRs supported moderate seed germination and plant regeneration but failed to help in the process of better acclimatization. The MS medium supplemented with BA, KN, NAA supported growth differentially in different parts of the regenerate *viz.,* optimal phenotypic response like shoot length on medium enriched with NAA (9 µM/L), multiple shoot formation with KN+NAA (3+12 µM/L), pseudobulbs length with BA+NAA (6+9 µM/L), leaf length with NAA (9 µM/L), root length with KN+NAA (12+12 µM/L) etc. Pseudobulbs are important morphological character in the genus *Dendrobium* and are crucial in the survival of the regenerants. It was also observed that under higher concentration of auxin (NAA) and with decrease in cytokinin (KN) the number of multiple shoots and root length increased, which contradicts to the findings of Panwar et al. (2012) where it was found that increased KN favoured shoot multiplication. In, addition the histological studies of the different stages of PLBs carried out in the present study supported the better response in plant regeneration from advance stage of PLBs. This might be due to the fact that initial

differentiation of epidermal and sub-epidermal cells has already differentiated into apical cells.

The capacity to transfer plants from a controlled *in vitro* environment to nature at low cost and with significant survival rates is crucial for acclimatisation success (Deb and Imchen, 2010; Deb and Pongener, 2013; Mayo-Mosqueda et al., 2020). Well established plants with roots were transferred to different hardening medium in order to see their adaptability to the medium conditions and response (Table. 3). The substratum made from coconut husk + wood bark (1:1) resulted promising survival rate of regenerate over the other combinations. It was found that the regenerants adhered to the substrata during the hardening process and helped in establishment in potting mix as well as in nature. The result is in partial agreement with that of Muna et al. (2016), where *Dendrobium* plantlets tied to three pieces of coconut husk with coco peat was most suitable for hardening. It was also observed that prolonged acclimatization period under *in vitro* condition prompted fungal contamination in the substrate medium and the plantlets. Therefore, transferring the plants after one week to room temperature with indirect sunlight was found beneficial. Moreover, the use of low-cost substrata like wood bark and coconut husk which are relatively cheap can be considered to more economical production.

4.5. Summary and Conclusion

The present study succeeded in developing an efficient *in vitro* protocol for germination of immature embryo of *D. heterocarpum* and *ex vitro* transplantation of regenerated plantlets. The developmental stages of embryo were found responsible to the degree of success in *in vitro* seed germination. Seed of 8 MAP was found as potential explants for asymbiotic germination. In addition, with regard to germination, nutrient medium, carbon sources, PRGs concentrations and culture conditions were found crucial. Results showed seeds of *D. heterocarpum* germinated higher in MS media than in Mitra et

al. and Knudson C with carbon source as sucrose. Acclimatization have been described which was observed to be an important factor for *in vitro* culture of orchids. The type of substrata provided for acclimatization was found vital for the *in vitro* regenerants survival. Thus, it could be concluded that asymbiotic seed germination and plant regeneration of orchids is unambiguous of species and nutrients supplied. The differential response in the PLBs proliferation to regenerating plants needs further studies. The findings of the present investigation may provide base-line data for future *in vitro* culture research.

Chapter - 5

In vitro propagation of *Esmeralda clarkei* Rchb. f.

5.1. Introduction

The genus *Esmeralda* is represented by three species (*Esmeralda bella* Rchb.f., *Esmeralda cathcartii* (Lindl.) Rchb.f., *Esmeralda clarkei* Rchb.f.) in the world (http://www.theplantlist.org) and is distributed in Nepal, Northeast India, Thailand, Myanmar and South China. In India, two species represent the genus viz, *E. Cathcartii* (Lindl.) Rchb f. and *E. clarkei* Rchb f. distributed in North India (Rao, 2007). *Esmeralda clarkei* Rchb.f. is an epiphytic, monopodial orchid and grows on upper canopy of tree trunks or branches. Deb et al. (2014) reported the species as a new addition to orchid flora of Nagaland, India. The species is mostly collected for its ornamental value and have become rare in the State due to over collection and habitat disturbance. ICAR-NRC for orchids (Sikkim, India) have categorised *E. clarkei* under RET status, and have breeding value with economic importance for its fragrance (Pamarthi et al., 2019).

The orchid species being monopodial, the vegetative propagation is very slow. In their natural habitat, orchids frequently produce tiny, endosperm-free seeds and rely on mycorrhizal connections for the development of protocorms hence the germination rates are low (Arditti and Ernst, 1993; Vudala, 2019). Thus, large-scale orchid propagation has been

replaced by culture techniques since conventional orchid breeding is challenging (Sipayung et al., 2018). Moreover, instead of using clonal micropropagation, *in vitro* seed germination produces a greater variety of uncommon, native, and frequently over collected orchid species (da Silva, 2013). The age of seeds, culture media, source of carbohydrate, organic additives, plant growth regulators, culture conditions, genotype, and plant species all have an impact on how many asymbiotic orchid seeds germinate *in vitro* (Stewart and Kane, 2010; Park et al., 2018; Kim et al., 2019). Organic carbon sources, such as glucose, fructose, sorbitol, and maltose, are used in these culture media and sucrose is used the most frequently in orchid cultivation (Sopalun et al., 2010). Studies have also demonstrated that the optimum asymbiotic seed germination and seedling development of orchids require the addition of plant growth regulators to the culture medium (Kauth et al., 2008; Kim et al., 2017; Seon et al., 2018).

High death rate is often associated with a high output of phenolic compounds from the seedlings surface, which causes them to turn a medium pinkish-brown colour and is harmful to seedlings (Waes, 1978). Since activated charcoal has a huge inner surface area and a very thin network of pores, it is frequently utilised in culture medium to address these issues (Rittirat et al., 2012). The addition of activated charcoal (AC) to the media enhances growth of plant tissue cultures which can be mainly attributed to the adsorption of inhibitory compounds in the culture medium and significantly reducing the accumulation of toxic metabolites, phenolic exudation, and brown exudates (Wang and Huang, 1976; Fridborg et al., 1978). Although the impact of AC on the uptake of plant growth regulators (PGRs) is yet unknown, some experts claim that AC may gradually release some products that have been absorbed, such as nutrients and PGRs, in addition to the naturally occurring compounds in AC that encourage plant growth (Thomas, 2008). Many workers have concluded that addition of activated charcoal in culture media enhanced asymbiotic germination and plant

growth (Choi and Chung, 1989; Nagaraju et al., 2001; Kull and Arditti, 2002; Bhadra and Hossain, 2003; Moraes et al., 2003). However, the concentration of activated charcoal may vary in culture media depending on the plant species, medium, explants, purpose, etc. (Thomas, 2008; Chen et al., 2014). The present research aimed at establishing an effective *in vitro* method for *E. Clarkei*, on asymbiotic seed germination, seedling development and investigated the effects of AC, culture medium strength, and plant growth regulators.

5.2. Materials and Methods

5.2.1. Sample collection and maintenance

E. clarkei plants were collected from Pangsa village, Tuensang, Nagaland, India, and maintained in Orchidarium, Department of Botany, Nagaland University, Lumami. Flowers were hand pollinated and tagged to record the seed age. For assessment of effect of green pod age on asymbiotic seed germination, green seed pods of 10 to 11 months after pollination (MAP) was used in the present study.

5.2.2. Explant sterilization

Green seed pods (10-11 MAP) were used for sourcing immature embryos for culture. Collected seed pod was first check for any surface tissue injury/infection and cut off with sterile blade. Initial surface sterilization of the seed pod was done using diluted 'Labolene' (1:100, v/v, a commercial laboratory detergent) and scrubbed off to remove dirt with soft brush. After washing thoroughly in running water, seed pod was treated with 0.3% (w/v) aqueous solution of $HgCl_2$ for 3 minutes under aseptic condition and rinsed 3-4 times using sterilised double distilled water. Finally, seed pods were dipped in 70% alcohol (v/v) for 30 seconds and flamed for 3-4 seconds. Seed pods were then split open to scoop out the immature embryos for culture on fortified nutrient media.

5.2.3. Culture media preparation

In the present study, three nutrient media viz. MS (Murashige and Skoog, 1962), Mitra et al. (Mitra et al., 1976) and Knudson 'C' (Knudson, 1946) were used. All the inorganic and organic compounds including plant growth regulators (PGRs) used in the present investigation was obtained from HiMedia Laboratories Pvt. Ltd, India unless mentioned otherwise. For assessment of asymbiotic seed germination, firstly the media were supplemented with different carbon sources *viz*., sucrose, fructose and glucose (0-3%, w/v). Secondly, asymbiotic germination of seed pod (Age) was assessed on different strengths of MS media (full, 1/2, 1/4). Thirdly, half strength MS fortified with activated charcoal (0-0.5%) and different plant growth regulators (PGRs) like N_6–benzyl adenine (BA), kinetin (KN), α-naphthalene acetic acid (NAA) and Indole-3-acetic acid (IAA) (0-9 µM/L) singly and in combination was used. For plant regeneration, half strength MS medium with different concentrations of cytokinin (BA and KN) (0-12 µM/L) and NAA were incorporated singly or in combination to the medium (0-9 µM/L) along with activated charcoal (AC) (0-1%). Media were gelled with 0.8% (w/v) tissue culture grade agar and pH was adjusted to 5.7 using 0.1N NaOH and 0.1N HCl

5.2.4. Initiation of culture

The immature embryos/seeds at different stages of development (10-11 months) were scooped from surface sterilized seed pods and inoculated on the nutrient media fortified differently for *in vitro* germination (Figure 5.1 A). All cultures were maintained at 25±2ºC temperature and 40 $\mu molm^{-2}s^{-2}$ illuminations at 12/12 h light/dark photoperiods provided by white fluorescent tubes.

5.2.5. Plant regeneration and multiplication

The germinated seeds were allowed to differentiate into protocorms before sub-culturing on fresh and/or new nutrient medium fortified differently compared to initiation

medium. The protocorms which started releasing the first set of leaves were considered for transferring on regeneration medium for plant regeneration. The shoot buds/young plantlets were separated and cultured on multiplication medium for culture proliferation. Well differentiated plantlets were allowed to grow on the same medium to experience nutrient stress and begin to get primarily acclimatized.

5.2.6. Primary acclimatization and transplantation

Well-developed rooted plantlets from regeneration medium were sourced and the roots were cleaned with soft brush to remove the traces of agar and subjected to primary acclimatization. For this purpose, different substrata *viz*., chopped coconut husk (CH) (~0.5-1.0 cm), wood bark pieces (WB) (~0.5-1.0 cm), wood compost (WC), charcoal pieces (CP) and crushed bricks (CB) were used in different combination as agar alternative. For wood barks, any species of tree having more than 0.5 mm thickness was collected followed by soaked in water for 48 h and dried before use. All the substrata were first autoclaved at 1.05 kg cm^{-2} pressure and 121^{o}C for ~30 minutes. Appropriate quantity of the processed substrata were placed in culture vials in different ratios as below:

1. Coconut husk; 2. Wood bark; 3. Wood compost; 4. CH+WB (1:1 ratio); 5. CH+WB+CP (1:1:1 ratio); 6. CH+WB+CP+WB (1:1:1:1 ratio); 7. CH+WB+CP+WC+CB (1:1:1:1:1 ratio) and 8. CH+WB without any MS salt solution.

About 15-20 ml of ¼ strength MS salt solution without any sucrose and PGRs were added in all the culture vials. The culture vials were autoclaved and cooled to room temperature before plantlets were cultured on the substrata and maintained under normal laboratory conditions as specified earlier for one week under normal room temperature with indirect sunlight (~75% diffused light) for ~4 weeks. The primarily acclimatized plantlets were then transferred to community potting mix prepared by mixing the same un-autoclaved substrata mixture as primary acclimatization conditions and covered with holed transparent

plastic sheets and maintained for 2-3 weeks before transferring to normal field conditions. During this period the plants were fed with 1/10th MS salt solution at weekly interval.

5.3. Results

The present study observed that different parameters, such as seed pod age, nutrient medium, quality and quantity of organic carbon in the nutrient medium, quality and quantity of PGRs, culture conditions, activated charcoal etc., had a significant impact on the successful in vitro germination of immature seeds.

5.3.1. Effect of seed age and carbon source on asymbiotic germination

Asymbiotic germination response of the seed was studied using three nutrient media (MS, Mitra et al. and Knudson C) supplemented with three separate organic carbon sources (fructose, glucose and sucrose, 0-3%, w/v). After inoculation of immature embryos, the first indication of germination was seen as swelling of the embryos followed by a greenish nodular enlargement of seeds/protocorm development (Figure 5.1 A, B, C, D). In comparison to the other two media, full strength MS medium was shown to be the most successful for seed germination (Table 5.1). Amongst the carbon sources, 3% sucrose in MS medium was found to be significantly effective where 52.62% seeds germinated against 26.99% germination with fructose (3%) and glucose (3%) 15.33% germination (Table 5.1). Nutrient media like Mitra et al. (Figure 5.1, E) and Knudson C though supported initial swelling of the immature embryos, failed to support successful germination. Also, a decrease in sucrose concentration resulted in lower rate of germination. The efficacy of MS medium in conjunct with sucrose (3%) in germination of seeds was taken into consideration for further assessments. Therefore, MS medium of full strength, ½ strength and ¼ strengths enriched with sucrose 3% were used to assess the relationship between developmental stage of seeds (10-11 MAP) and germination rate. The optimum germination was achieved with green pods of 10 MAP, with germination rates of 68% on ½ MS strength medium, 51% on

¼ MS strength media and 47% on MS full strength media (Figure 5.2). However, the germination rate of seed pods of 11 MAP was found to be lower with 58% on ½ MS strength, 42% on ¼ MS strength and 35% on MS full strength (Figure 5.2).

5.3.2. Effect of nutrient media, PGRs and AC on asymbiotic germination

In addition to the age of the seed pod, the basal nutritional media, and the organic carbon source, the effect of PGRs and AC on the frequency of germination and related morphogenetic changes that result in seedling development were studied. Half strength MS medium with 3% sucrose and with or without AC (0.5% and 0.1%) were supplemented with different concentrations of BAP, KN, NAA and IAA (3-6 µM/L) singly or in combination, where varied response in germination time, rate and morphological development was observed (Table 5.2). The time taken to germinate was observed to have taken less time on ½ strength MS medium with addition of AC 0.5% (~21-28 days) and AC 0.1% (~22-30 days) in comparison to the media devoid of AC (~34-41 days) (Table 5.2). Medium containing 0.5% AC fortified with KN+NAA (3+3 and 6+6 µM/L) and NAA3 recorded the shortest germination time period of ~21 days in comparison to other concentrations of PGRs. Also, the average germination percentage was supported significantly higher with up to 88% in ½ strength MS (Sucrose 3%) with AC 0.5%, followed by 67% in medium with 0.1% AC and 64% in medium without AC (Table 5.3). Also, the type of PGRs and concentration present in the medium was found to influence both germination rate and morphological response of the protocorms developed. Highest germination rate was recorded in ½ strength MS medium (0.5%AC) with BAP+NAA (3+3 µM/L) (88.84%) followed by BAP+NAA (3+6 µM/L) (83.61%) and BAP (3 µM/L) (83.61%). The combination of BAP+IAA (3+6 µM/L) and KN+IAA (3+6 µM/L) supported the lowest germination rate in all the medium. Further, comparatively healthier differentiation of germinating seeds into protocorms was recorded on AC conjunct ½ strength MS medium when fortified with BAP+NAA (3-6

μM/L) and KN+NAA (3-6 μM/L). It was observed that in all the nutrient medium where PRGs (BAP, KN, NAA and IAA; 3-6 μM/L) when added singly, the protocorms tend to turn brown faster in comparison to when added in combination. However, after 30 days in the medium where the seeds germinated, the protocorms had to be transferred to fresh medium to develop to the advanced protocorm stage before being transferred to the regeneration medium.

Table 5.1: Effect of organic carbon source and culture medium on seed germination of immature embryo in *Esmeralda clarkei*

Culture medium	Organic carbon source	Concentration (%)	Germination rate (%)
MS	Control	0	25.87 ±2.13^{d}
	Sucrose	1	44.67 ±1.12^{c}
		2	49.48 ±1.95^{b}
		3	52.62 ±1.14^{a}
	Fructose	1	11.62 ±1.84^{g}
		2	18.58 ±1.60^{e}
		3	26.99 ±0.79^{d}
	Glucose	1	9.00 ±0.85^{i}
		2	10.91 ±01.95^{h}
		3	15.33 ±0.88^{f}
Mitra et al.	Control	0	No response
	Sucrose	1-3	No response
	Fructose	1-3	No response
	Glucose	1-3	No response
Knudson	Control	0	No response
	Sucrose	1-3	No response
	Fructose	1-3	No response
	Glucose	1-3	No response

Note: *± Standard error of replicates mean. Mean with different superscripts within the same column differ significantly (P<0.05) by Tukey's HSD at 95% confidence level.

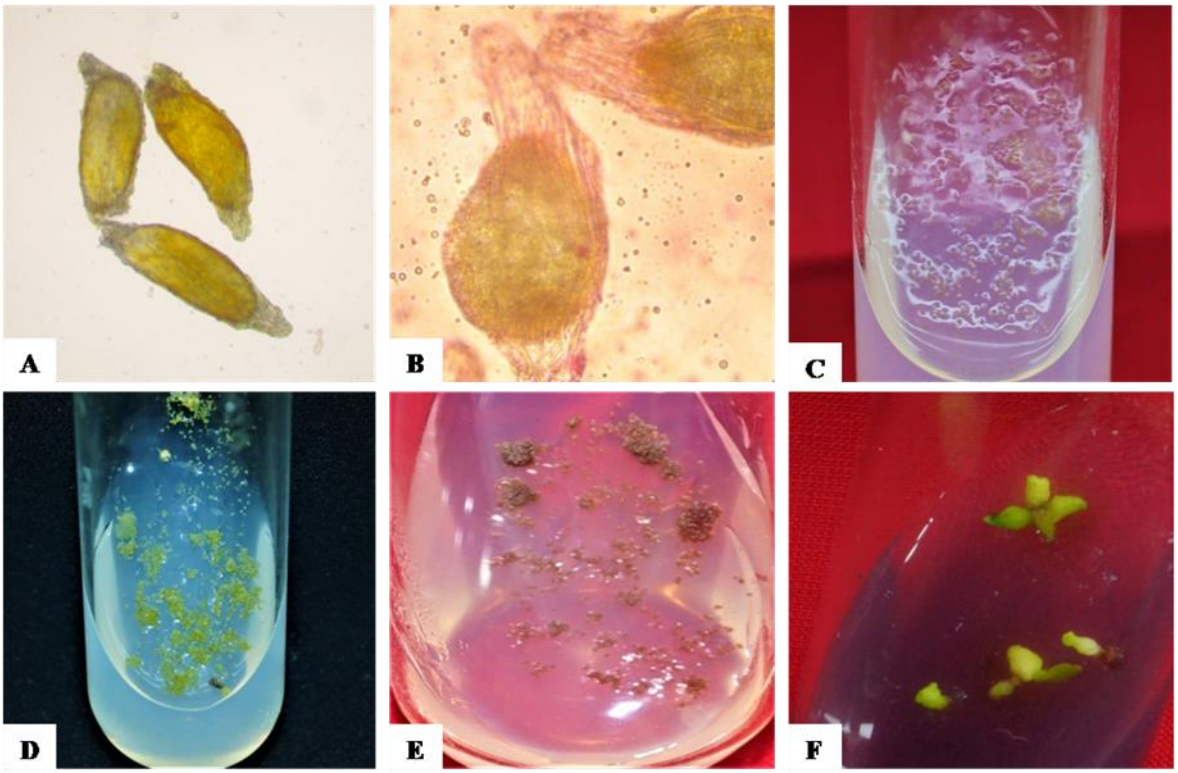

Figure 5.1: Showing asymbiotic seed germination of *E. clarkei* (A) Immature seeds (B) swelling of embryos (C) Initial seed inoculation in MS media (D) Browning of seeds in Mitra et al. medium (E) Seed germination in ½ strength MS media showing formation of protocorms (F) formation of advance stage protocorms.

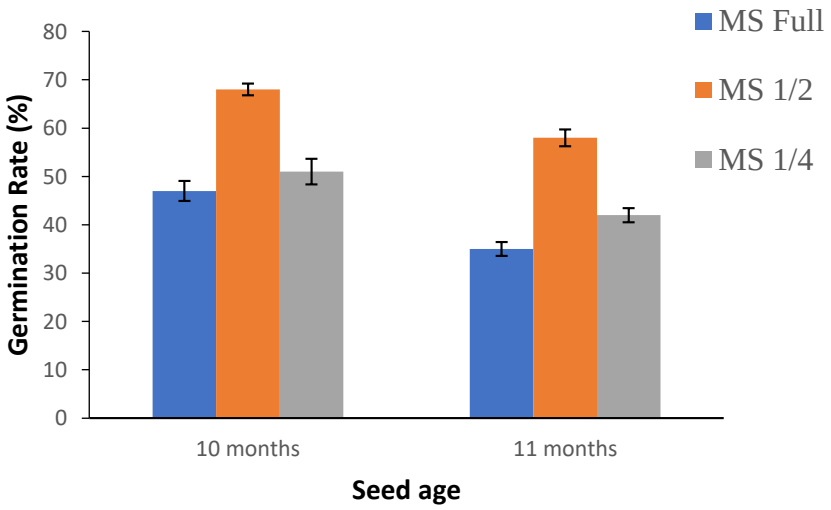

Figure 5.2: Effect of seed age and MS nutrient strength with 3% (w/v) sucrose on asymbiotic germination of immature *E. clarkei* embryos.

Table 5.2: Effect of PGRs concentration and activated carbon (%) in germination time of immature embryo in *Esmeralda clarkei*

PGRs	Germination Time (in days) **		
	Without AC	0.1%	0.5 %
BAP3	37.20 ±0.40^{defghij}	24.50 ±0.64^{de}	22.72 ±0.26^{hij}
BAP6	36.26 ±0.36^{ghijk}	25.36 ±0.49^{cde}	24.37 ±0.28^{cdefgh}
KN3	39.02 ±0.45^{abc}	26.24 ±1.12^{cde}	25.21 ±0.33^{cd}
KN6	37.69 ±0.49^{cdefgh}	26.13 ±0.81^{cde}	25.72 ±0.49^{bc}
NAA3	38.30 ±0.41^{bcde}	22.25 ±0.84^{e}	21.94 ±0.30^{j}
NAA6	38.52 ±0.13^{bcd}	23.02 ±0.85^{de}	22.80 ±0.55^{hij}
IAA3	36.35 ±0.61^{ghijk}	24.88 ±0.87^{de}	23.83 ±0.19^{defghi}
1AA6	34.88 ±0.26^{jk}	26.05 ±0.74^{cde}	25.16 ±0.12^{cde}
BAP3NAA3	34.31 ±0.29^{k}	22.22 ±0.98^{e}	22.03 ±0.25^{ij}
BAP3NAA6	35.32 ±0.44^{ghijk}	25.08 ±0.75^{cde}	24.86 ±0.52^{cdef}
BAP6NAA3	35.83 ±0.35^{ghijk}	26.57 ±0.51^{cd}	24.73 ±0.25^{cdefg}
BAP6NAA6	36.55 ±0.57^{ghijk}	29.21 ±0.54^{c}	27.18 ±0.35^{b}
KN3NAA3	36.08 ±0.38^{ghijk}	22.14 ±0.86^{e}	21.80 ±0.39^{j}
KN3NAA6	37.71 ±0.33^{cdefghi}	25.42 ±0.50^{cde}	22.53 ±0.44^{ij}
KN6NAA3	37.84 ±0.46^{cdef}	26.68 ±0.49^{cd}	22.35 ±0.33^{ij}
KN6NAA6	37.65 ±0.56^{cdefg}	23.40 ±0.59^{de}	21.15 ±0.19^{j}
BAP3IAA3	40.39 ±0.41^{ab}	30.14 ±0.78^{ab}	28.02 ±0.27^{a}
KN3IAA3	41.25 ±0.56^{a}	31.06 ±1.43^{a}	28.86 ±0.17^{a}

Note: * ½ strength MS media with sucrose (3%) ****± standard error of replicates mean. ** only concentrations supported optimal response are computed. Mean with different superscripts within the same column differ significantly (P<0.05) by Tukey's HSD at 95% confidence level.

Table 5.3: Effect of PGRs and activated carbon in germination rate of immature embryo in *Esmeralda clarkei*

PGRs	Without AC		AC 0.1%		AC 0.5%	
	Germination rate (%)*	Morphological response	Germination rate (%)*	Morphological response	Germination rate (%)*	Morphological response
BAP3	63.49 ±1.20^{abc}	Very small protocorms + browning	67.23 ±0.90^{ab}	Small protocorms + browning	83.61 ±2.60^{bc}	Very small protocorms + browning
BAP6	61.13 ±1.00^{abcd}	,,	65.72 ±0.73^{abc}	,,	71.98 ±1.25^{efg}	,,
KN3	55.86 ±1.08	,,	59.75 ±0.54^{defghijklm}	,,	74.40 ±1.01^{de}	,,
KN6	64.83 ±1.84^{a}	,,	64.22 ±0.90^{abcde}	,,	70.57 ±1.13^{efghijk}	,,
NAA3	52.72 ±1.43^{efghijk}		57.64 ±1.49^{hijklmn}		65.36 ±1.38^{ghijklmno}	
NAA6	57.29 ±0.55^{cdef}	,,	63.66 ±1.04^{abcdefg}	,,	71.21 ±1.35^{efghi}	,,
IAA3	42.90 ±1.67^{p}	,,	49.49 ±0.66^{p}	,,	68.27 ±0.95^{efghijklmn}	,,
1AA6	52.21 ±0.97^{efghijklm}	,,	59.90 ±1.04^{defghijkl}	,,	70.15 ±0.92^{efghijkl}	,,
BAP3NAA3	64.10 ±1.35^{ab}	green protocorms formation	68.23 ±0.95^{a}	,,	88.84 ±2.21^{a}	Healthy green protocorms
BAP3NAA6	57.13 ±0.76^{efghijkl}	,,	62.10 ±0.75^{bcdefgh}	,,	73.99 ±1.36^{def}	,,
BAP6NAA3	54.26 ±0.85^{defghij}	,,	60.34 ±0.97^{defghijk}	,,	71.47 ±1.39^{efgh}	,,
BAP6NAA6	52.46 ±0.80	,,	61.10 ±0.82		83.69 ±2.74	,,
KN3NAA3	55.40 ±1.03^{defghi}	,,	63.71 ±0.69^{abcdef}	,,	72.80 ±0.93^{def}	,,
KN3NAA6	51.34 ±1.19^{fghijklmn}	,,	57.77 ±0.57^{hijkmn}	,,	68.73 ±0.72^{efghijklm}	protocorms formation
KN6NAA3	57.93 ±1.09^{bcde}	,,	64.48 ±0.68^{abcd}	,,	70.61 ±1.24^{efghij}	,,
KN6NAA6	50.99 ±1.72^{fghijklmn}	,,	61.28 ±1.21^{cdefghi}	,,	80.02 ±0.80^{bcd}	,,
BAP3IAA3	28.76 ±0.87^{q}	,,	31.91 ±1.33^{q}	,,	44.24 ±1.97^{p}	,,
KN3IAA3	27.29 ±1.34^{q}	,,	28.53 ±1.70^{q}	,,	40.49 ±1.23^{q}	,,

Note: * MS1/2 media with sucrose (3%) **** ± standard error of replicates mean, ** only concentrations supported optimal response are computed. Mean with different superscripts within the same column differ significantly (P<0.05) by Tukey's HSD at 95% confidence level

5.3.3. Plant regeneration and culture proliferation

Morphological response and the percentage of plant regeneration was evaluated on half strength MS medium (3% sucrose) with and without AC (1, 0.5 and 0.1%) (Table 5.4). Higher plantlet regeneration was optimum on ½ MS medium with 0.5% AC (82%), followed by medium with 0.1% AC (64%), 1% AC (28±2.15) and without AC (8%). The protocorms deteriorated and turned brown or black in media without AC and with 1% AC (Figure 5.3 A), moderate plant regeneration was supported in media with 0.1% AC (Figure 5.3 B) and in media with AC 0.5% protocorms were green, increased in size and produced healthy leaves (Figure 5.3 C, D). Therefore, ½ strength MS with AC 0.5% supplemented with combination of BAP (3-12 µM/L), KN (3-12 µM/L) and NAA (3-9 µM/L) was taken to study the effect of PGRs in plant proliferation and multiplication (Table 5.5). The optimum condition for shoot length was found to be KN+NAA (9+9 µM/L) (0.87cm), followed by KN+NAA (6+6 µM/L) (0.83cm), KN+NAA (6+3 µM/L, 9+6 µM/L) and BAP+NAA (9+9 µM/L) (0.77cm) from the different combinations of PGRs employed (Figure 5.3 E, F, G). Leaf (1.33) and root (1.97cm) length was highest in media with KN9NAA6 (9+6 µM/L) (Figure 5.3 H), but no significant difference was observed in the number of leaves. For plantlets multiplication, the advanced stage of protocorms sub-culture differentiated, producing more protocorms from where new shoots were generated (Figure 5.4 A-C). In comparison to the combination of KN+NAA, it was discovered that the combination of BAP+NAA supported larger multiplication of shoots, producing shoots up to an average of ~10 in BAP+NAA (12+6 µM/L) and ~7 BAP+NAA (9+6, 12+3, 9+9 µM/L) (Table 5.5).

5.3.4. Acclimatization of regenerates

The regenerants were kept on various substrates with/without ¼ strength MS salt solution and without any PGRs for primary acclimation. The regenerants acclimation responses varied on various substrata combinations. Following transplantation onto the

different substrates, CH+WB, WB, and CH substrate, respectively, around 92.88, 78.75, and 72.18 % of the various combinations of transplants survived (Table 5.5). Throughout this time, the transplants were regularly fed with 1/10 strength MS medium solution. Then, well-established plants were moved to a greenhouse and finally to the outdoors, where they were affixed to tree trunks with the aid of a pin and their growth response was observed (Figure 5.4 D-F). In addition, the goal of the current study was to reduce the protocol's cost by substituting agar for the plantlet's acclimation. There is a considerable risk of contamination when well-developed plantlets are sub-cultured to acclimate in agar gel media, which can result in culture loss. By skipping the phase of acclimatization in agar basal media, adding new liquid nutrients to the culture vials with low-cost substrata, contamination and labor costs can be decreased. Comparison of the cost of tissue culture grade agar on the market today with the cost of low-cost substrata optimized in the current investigation revealed a significant difference in lowering the production cost for acclimation. Agar costs $9.6 to prepare 10 liters of medium, whereas reusable substrata like coconut husk, wood bark, and charcoal cost between 2-4 dollars.

Table 5.4: Effect of activated carbon (%) in plant regeneration from protocorms of *E. clarkei*

MS1/2*	**Plant regeneration (%)**	**Morphological response observed**
Without AC	8±2.15	The green protocorms slowly turned brown
1% AC	0	The protocorms degenerated and turned black
0.5% AC	82±3.21	Green protocorms increased in size and produced leaves
0.1% AC	54±2.54	Some protocorms developed well and some turned brown

Note: *Nutrient medium containing sucrose 3%

Figure 5.3: *In vitro* plant regeneration of *E. clarkei;* (A) Degeneration of protocorms in MS1/2 with 1% AC (B) Formation of initial leaves on ½ MS media with 0.5% AC (C) Formation of leafs and roots on ½ MS media with 0.5% AC (D Well developed plantlet in ½ MS BAP6NAA6 (E) Well developed plantlet in BAP9NAA9; (F&G) Well developed plantlet in KN6NAA6 AND KN9NAA9 (H) Plantlet in KN9NAA6

Figure 5.4: *In vitro* plantlet multiplication of *E. clarkei* on ½ MS medium with 0.5% activated carbon and sucrose 3%. (A&B). protocorm differentiation in B12NAA6, (C). Shoot and root formation from protocorms, (D). Primary acclimatization of plantlets in wood bark and coconut husk with ¼ MS liquid solution, (B). Plantlet attaching to wood substrata, (C). Transferring of plantlets to room temperature condition.

Table 5.5: Effect of **plant** growth regulators in plant regeneration and multiplication of *E. clarkei*

PGRS	Shoot length	Leaf length	Root length	No. of leafs	No. of shoots
BAP3NAA3	0.50±0.06^{bcdefghij}	0.42± 0.02^{lmn}	0.41± 0.02^{h}	4.20±0.15^{abcdefghijklm}	3.52±0.40^{l}
BAP3NAA6	0.47± 0.03^{j}	0.78± 0.08^{cdefgh}	0.57± 0.06^{cdefgh}	4.17± 0.12^{abcdefghijklm}	6.62±0.41^{bcdefghij}
BAP3NAA9	0.43± 0.03^{j}	0.74± 0.01^{cdefghijk}	0.59± 0.08^{cdefgh}	4.27± 0.15^{abcdefghijklm}	4.37±0.28^{efghijkl}
BAP6NAA3	0.57± 0.03^{abcdefghij}	0.66± 0.04^{cdefghijkl}	0.50± 0.02^{h}	4.47± 0.15^{abcdefghijkl}	5.88±0.68 ^{bcdefghijkl}
BAP6NAA6	0.63± 0.03^{abcdefghij}	0.65± 0.04^{cdefghijklm}	0.76± 0.04^{cdefgh}	4.37± 0.20^{abcdefghijklm}	5.93±0.71 ^{bcdefghijkl}
BAP6NAA9	0.60± 0.06^{abcdefghij}	0.77± 0.09^{cdefghi}	0.99± 0.13^{bcdefg}	4.50± 0.17^{abcdefghijk}	4.25±0.74^{efghijkl}
BAP9NAA3	0.57± 0.09^{abcdefghij}	0.78± 0.07^{cdefg}	0.82± 0.06^{cdefgh}	4.53± 0.09^{abcdefghi}	4.81±0.67 ^{cdefghijkl}
BAP9NAA6	0.70± 0.06^{abcdefghi}	0.56± 0.05^{efghijklmn}	0.73± 0.02^{cdefgh}	4.53± 0.13^{abcdefghij}	7.62±1.06^{bc}
BAP9NAA9	0.77± 0.09^{abc}	0.57± 0.04^{efghijklmn}	0.76± 0.05^{cdefgh}	4.57± 0.07^{abcedfg}	7.07±0.91^{bcde}
BAP12NAA3	0.73±0.07^{abcde}	0.54± 0.07^{efghijklmn}	0.63± 0.03^{cdefgh}	3.83± 0.24^{bcdefghijklm}	7.33±1.04^{bcd}
BAP12NAA6	0.63± 0.03^{abcdefghij}	0.38± 0.03^{lmn}	0.53± 0.08^{defgh}	3.57± 0.27^{m}	10.59±0.69^{a}
BAP12NAA9	0.37±0.03^{j}	0.55± 0.05^{efghijklmn}	0.73± 0.03^{cdefgh}	3.80± 0.25^{bcdefghijklm}	5.22±0.50 ^{bcdefghijkl}
KN3NAA3	0.60± 0.06^{abcdefghij}	0.34± 0.04^{n}	0.66± 0.06^{cdefgh}	4.37± 0.20^{abcdefghijklm}	3.99±0.85^{fghijkl}
KN3NAA6	0.67± 0.03^{abcdefghij}	0.76± 0.04^{cdefghij}	0.70±0.03^{cdefgh}	4.60± 0.06^{abcde}	4.92±0.25^{cdefghijkl}
KN3NAA9	0.70±0.06^{abcdefg}	0.48± 0.07^{hijklmn}	0.40± 0.04^{h}	4.57± 0.19^{abcdefgh}	6.56±0.74^{bcdefghijk}
KN6NNA3	0.77± 0.03^{abcd}	0.59± 0.02^{defghijklmn}	0.78± 0.02^{cdefgh}	4.40± 0.12^{abcdefghijklm}	6.37±1.23^{bcdefghijkl}
KN6NAA6	0.83±0.03^{ab}	0.87± 0.03^{bcd}	0.83± 0.02^{cdefgh}	4.77± 0.03^{a}	6.90±0.73^{bcdef}
KN6NAA9	0.70±0.03^{abcdefgh}	0.53± 0.06^{fghijklmn}	0.83± 0.08^{cdefgh}	4.60± 0.10^{abc}	6.30±1.14 ^{bcdefghijkl}
KN9NAA3	0.73±0.07^{abcdef}	0.49± 0.03^{ghijklmn}	1.79± 0.19^{ab}	4.60± 0.10^{abcd}	6.31±0.33 ^{bcdefghijkl}
KN9NAA6	0.77± 0.03^{abcd}	1.33± 0.09^{a}	1.97± 0.37^{a}	4.60± 0.06^{abcdef}	6.73±0.60^{bcdefghi}
KN9NAA9	0.87± 0.03^{a}	1.23± 0.04^{ab}	1.35± 0.62^{abcd}	4.67± 0.09^{ab}	6.87±0.35^{bcdefg}
KN12NAA3	0.63± 0.09^{abcdefghij}	0.94± 0.10^{bc}	1.52± 0.17^{abc}	3.77± 0.20^{cdefghijklm}	6.75±1.18^{bcdefgh}
KN12NAA6	0.60± 0.06^{abcdefghij}	0.80± 0.07^{cdef}	1.28± 0.02^{abcdef}	3.80± 0.23^{bcdefghijklm}	9.25±0.67^{ab}
KN12NAA9	0.57± 0.03^{abcdefghij}	0.83± 0.02^{cde}	1.31± 0.15^{abcde}	3.67± 0.18^{defghijklm}	4.90±0.68 ^{cdefghijkl}

Note:⁎ Nutrient medium- MS1/2 contains 3% sucrose. ⁎⁎ ±SE: Standard error from mean. ⁎⁎⁎ Only concentrations supported optimal response are computed. Mean with different superscripts within the same column differ significantly (P<0.05) by Tukey's HSD at 95% confidence level.

Table 5.6: Response of *in vitro* plantlets of E. clarkei to different acclimatization substrata

Sl. No.	Acclimatization condition*	Ratio of the substrata	Survival rate (%) (±SE)**
1	Coconut husk*	1	72.18 ±3.73
2	Wood bark*	1	78.75 ±2.34
3	Wood compost	1	7.50 ±0.83
4	Coconut husk + Wood bark*	1:1	92.88 ±2.09
5	Coconut husk +wood bark + charcoal*	1:1:1	50.93 ±4.37
6	Coconut husk + wood bark + charcoal + wood compost	1:1:1:1	11.58 ±2.01
7	Coconut husk + wood bark + charcoal + wood compost + crushed bricks	1:1:1:1:1	12.67 ±1.12

* Hardening substrata fortified with ¼ strength MS nutrient solution

**± SE: Standard error from mean.

Table 5.7: Comparative analysis of agar gel medium Vs. low-cost substrata used in the present study

Sl. No.	Gelling agent/Substrata	Cost / per kg (US$)	Quantity to make 1 Lit. of medium (grams)**	Cost (US$)	Cost effectiveness of primary acclimatization against agar gel medium (%)
1	Agar	120.00	8	9.6	-
2	Coconut husk + Wood bark (1:1 ratio)	1.00	500	0.50	94.79%

5.4. Discussion

In vitro propagation of *E. clarkei* through seed and shoot tip culture have been reported by Paudel and Pant (2012, 2013), but comprehensive analysis on nutrient media, carbon source, seed age etc. have not been covered. The percentage of seeds that germinate is the most crucial indicator of success when employing seeds as starting materials in tissue culture experiments. The seeds cultivated on the medium will receive water, swell, and absorb macro- and microelements from the medium, which will result in successful germination (Acemi and Ozen, 2019). However, factors like nutritional media, organic

carbon sources, quality and concentration of PGRs etc. influences culture initiation and plantlet regeneration (Deb and Pongener, 2012; Koirala et al., 2013). In the study, from the different media and carbon sources employed for asymbiotic germination of immature embryos of *E. clarkei* favoured MS medium with 3% sucrose (~52%), 2% sucrose (~49%) and 1% sucrose (~44%). Mitra and Knudson C media supplemented with different carbon sources (0-3%) supported initial swelling of the embryos but failed to produce protocorms. Such differences in germination response to different nutrient media have been reported in different orchid species (Sheelavantmath et al., 2000; Znaniecka et al., 2005; Wu et al., 2014). The success of *in vitro* germination is also influenced by seed maturity, although each species' appropriate developmental stage for excision must be determined (Thompson et al., 2006). Comparatively, germination rate of seed age of 10 MAP was higher with 68% (1/2 strength MS), 51% (1/4 strength MS) and 47% (MS full strength), than 58% (1/2 strength MS), 35% (1/4 strength MS) and 42% (MS full strength) in seed of 11 MAP. The superiority of ½ strength MS media (Sinha et al., 2009) and decrease in germination rate of older seeds has also been reported in asymbiotic germination of *Malaxis khasiana* (Deb and Temjensangba, 2006) and *Paphiopedilum villosum* var. *boxallii* (Deb and Jakha, 2019). However, it was observed that the media surface in contact with the germinated seeds/protocorms started to turn brown, leading to slow degeneration of the protocorms. The build-up of phenolic chemicals in the protocorm results in browning, which reduces the ability of the tissue to develop and kills it during culture (Rittirat et al., 2012). AC is frequently utilised in cultural media as their absorption property helps to absorb many substances enhancing cell growth and development (Pan and Staden, 1998). Therefore, ½ strength MS medium was chosen and fortified with different concentrations of PGRs (BAP, KN, NAA and IAA: 3-6 μM/L singly or in combination) and without AC (0.5% and 0.1%) to optimise medium and record their effect on germination and protocorm development.

Addition of PGRs in culture media was found ideal for optimum asymbiotic germination and development of seedlings (Kim et al., 2019). Significant decrease of germination time and increase in germination rate was observed in media fortified with AC 0.1% and 0.5%, compared to media devoid of AC. Similar response was observed in asymbiotic seed germination of *Cattleya crispate*, where with the addition of AC in media the germination time of the seeds reduced (Souza et al., 2021). Additionally, it was discovered that the type of PGRs and their concentrations had an impact on changes in germination time and rate in media containing AC. Combination of BAP+NAA (3+3 µM/L) recorded the shortest germination time of 22 days and germination rate up to 88%. The morphological response noted showed ½ strength MS media with 0.5% AC, supplemented with combinations of BAP+NAA (3-6 µM/L) and KN+NAA (3+3 µM/L) was found to be most optimum in generating green healthy protocorms. However, protocorms needed to be cultured to new medium after ~4 weeks for further proliferation of protocorms. The protocorms developed in 0.1% AC also showed promising results, but was followed by browning of the media in contact. Several orchid species asymbiotic *in vitro* seed germination frequently results in protocorm browning (Pimsen and Kanchanapoom, 2011; Hossain et al., 2013; Bustam et al., 2016; Magrini and Devitis, 2017; Kim et al., 2019).

To optimise plant regeneration medium, protocorms were cultured in half strength MS medium with/without AC (0-1%). Where ½ strength MS with AC 0.5% had the highest plant regeneration percentage (82%), while medium without AC caused protocorm degeneration. Reports of similar outcomes in the *in vitro* propagation of Brazilian orchids (Moraes et al., 2005) and *Zygostates grandiflora* (Pacek-Bieniek et al., 2010) have been noted, where ½ strength MS medium containing AC produces superior seedlings. Also, increase in AC resulted in drastic decrease in plantlet regeneration percent. It's possible that large levels of activated charcoal were a factor in the culture media's lack of several nutrients

(Buckseth et al., 2018) causing degeneration of protocorms. Overall, it was discovered that ½ strength MS medium containing AC 0.5%, sucrose 3% supplemented with combination of KN+NAA (6-9 μM/L) was preferable over combinations of BAP (3-12 μM/L) for plantlet regeneration, with optimum shoot length, leaf length, and root length in KN+NAA (6+6 μM/L) and KN+NAA (9+9 μM/L). However, BAP+NAA when combined was found to support shoot multiplication, with the best outcomes being seen in BAP+NAA (12+6 M/L) (10) and BAP+NAA (9+6 M/L) (9). This result of protocorm multiplication in BAP+NAA is consistent with some other results from orchid micropropagation (Seeni and Latha, 2000; Roy et al., 2011; Baker et al., 2014).

In order to assess how well-established plants with roots would adapt and respond to acclimatization, they were subjected to different hardening conditions. Over the other combinations, the substratum made from coconut husk with wood bark (1:1) produced a promising survival rate of regenerants. Studies showed that acclimatization of *in vitro* plantlets of *Geodorum densiflorum* (Sheelavantmath et al., 2000), *Laelia flava*, *Miltonia flavescens,* and *Oncidium trulliferum* (Moraes et al., 2005) grown on MS media containing AC enhanced the *in vitro* plant quality and survival rate. The regenerants were found to stick to the substrate during the hardening process and aid in its establishment in nature.

5.5. Summary and Conclusion

Successful asymbiotic germination of immature seeds of *E. clarkei* was established. The seeds responded differently depending on the PGR content, carbon source, and kind of nutritional media/strength. Additionally, it was shown that adding a certain amount of AC to the media was beneficial for seed germination and protocorm growth. It was observed that ½ strength MS with 0.5% AC was best for plantlet regeneration. Combination of PGRs KN+NAA (6+6 μM/L and 9+9 μM/L) in ½ strength MS with sucrose 3% was found to be most ideal for plantlet regeneration. Multiplication of plantlets from advance stage

protocorms culture in ½ strength MS with sucrose 3% and AC 0.5%, fortified with combination of BAP+NAA (12+6 M/L and 9+6 M/L) gave the optimum result. Acclimatization substrata combination was standardized using low-cost substrates, where combination of wood bark and coconut husk (1:1) had the highest survival rate of the *in vitro* regenerates. Therefore, future plant multiplication can employ the methodology that has been optimised for the species.

CHAPTER 6

Genetic Fidelity Assessment of the *In Vitro* Regenerants

6.1. Introduction

In vitro propagation is a very effective method for conserving orchids since it gets over issues like low seed germination from a lack of endosperm and the need for the right fungal interaction for germination (Tikendra et al., 2019a). These cryptic genetic effects can be influence of prolonged retention of cells and tissues in a micro environment, application of elevated concentration of plant growth regulators (PGR), and stress generated during by regular sub-culturing cultured cells and tissues (Bhattacharyya et al., 2015a, 2018). Despite the advantages of *in vitro* propagation, the emergence of hidden genetic flaws caused by somaclonal changes in the regenerants might significantly reduce the micropropagation system's overall usefulness (Salvi et al., 2001). Therefore, it has become obligatory to investigate the genetic variability and stability of in vitro plants (Butiuc-Keul et al., 2016).

Morphological markers are few in number and frequently do not indicate genetic links because of interactions with the environment, method of treating, and the generally unrecognised genetic regulation of the features (Smith and Smith, 1989). However, DNA markers are common and unaffected by a plant's environment or stage of growth, making them the perfect instrument for genetic relationship investigations (Kumar et al., 2013). Direct Amplification of Minisatellite DNA regions (DAMD), Inter Simple Sequence Repeats (ISSR), and Random Amplified Polymorphic DNA (RAPD) are PCR-based Single Primer Amplification Reaction (SPAR) methods that are gaining popularity as efficient tools for providing a comprehensive description of the nature and extent of the genetic diversity

in plants (Devi et al., 2014). In addition, the limitations of conventional DNA markers targeting a specific region is resolved by Start Codon Targeted (SCoT) markers (Collard and Mackill, 2009; Amom and Nongdam, 2017). Successful assessment of genetic fidelity in *in vitro* orchid regenerants have been reported using RAPD (Ferreira et al., 2006; Kishor and Devi, 2009; Oliya et al., 2021; Panwar et al., 2021), DAMD (Devi et al., 2013; Bose et al., 2016) and Start Codon Targeted (SCoT) (Gholami et al., 2021; Thakur et al., 2021).

The present study is aimed to access the genetic fidelity of the *in vitro* regenerants developed from the optimised *in vitro* propagation technique of *C. bicolor* (Chapter 3), *D. heterocarpum* (Chapter 4) and *E. clarkei* (Chapter 5) using RAPD, DAMD and SCoT.

6.2. Materials and Methods

6.2.1. Genomic DNA extraction

Leaves of the donor mother plant (MP) and well acclimatized ten *in vitro* plantlets (IP) were randomly selected for DNA extraction. Modified Cetyl Trimethyl Ammonium Bromide (CTAB) was used to isolate the genomic DNA (Doyle and Doyle, 1987). Purity of the DNA was checked by running it in 1% (w/v) agarose gel electrophoresis and quantified by Thermo Scientific Multiskan Go Spectrophotometer.

6.2.2. RAPD, DAMD and SCoT PCR amplification

All reaction mixture for PCR contained 1X PCR buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, 1.5 µM primer, 30 ng of DNA template, 1 U Taq Polymerase and de-ionized water. RAPD reaction was performed with initial denaturation of 94 °C for 3 min, followed by 45 cycles of denaturation at 94 °C for 45 s, annealing at 36 °C for 30 s, extension at 72 °C for 2 min and final extension at 72 °C for 7 min (Kumar et al., 2013); for DAMD denaturation of 94 °C for 2 min, followed by 40 cycles of denaturation at 92 °C for 1 min, annealing at 36 °C for 30 s, extension at 72 °C for 2 min and final extension at 72 °C for 5 min (Bhattacharyya et al., 2015a) and for SCoT primer denaturation of 94 °C for 4 min, followed

by 38 cycles of denaturation at 92 °C for 30 s, annealing at Tm -5 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 5 min (Thakur et al., 2021). The PCR amplicons were run in 1.5% (w/v) agarose gel to separate with 100 base pair (bp) ladder for size confirmation.

6.2.3. Data analysis

Data analysis of RAPD, DAMD and SCoT markers was done by scoring consistent, unambiguous and reproducible banding profiles. Bands were scored in binary matrix base on presence (1) and absence (0). Genetic similarity between the mother plant and the regenerates were estimated using SIMQUAL format to generate Jaccard's coefficient in the numerical taxonomy multivariate analysis system NTSYS-pc version 2.20 and dendrogram was constructed following UPGMA of the SAHN module (Rohlf, 2000).

6.3. Results

In the present study, the genetic fidelity of the donor plants and their regenerates were assessed using RAPD, DAMD and SCoT markers to check the genetic homogeneity. Preliminary screening of 45 RAPD primers, 24 DAMD primers and 15 ScoT was done from which, 15 RAPD primers, 10 DAMD primers and 10 SCoT primers each were selected for the three orchid species.

6.3.1. Genetic fidelity assessment of *Cymbidium bicolor* Lindl.

The 15 selected RAPD markers in *C. bicolor* scored Monomorphic bands of 100% in 15 primers and 1 polymorphic band in 1 primer with band size ranging from 120-6000 bp was recorded (Table 6.1, Figure 6.1 A-B). A maximum band of 11 was observed in OPG14 (Figure. 6.1 A) followed by 9 bands in OPBA7 and lowest of 4 bands each in OPA13 and OPB6 (Table 6.1). Polymorphic bands were observed in 2 loci of OPA01 and 1 locus of OPBA7 (Figure 6.1 B, C). In DAMD markers, the bands ranged from 300-600 bp with 7 primers showing 100% monomorphic bands and 3 primers having polymorphic bands (Table

6.2). Highest number of 9 bands each was produced in URP1F (Figure 6.2 D) and URP9F and least in URP6R with 5 bands (Table 6.2). Polymorphic bands in 1 locus each were observed in D6 and D18 and in 2 loci of URP9F (Figure 6.1 E-G). In case of SCoT, the amplified bands ranged from 330-2000 bp with 9 primers having 100% monomorphic bands and only 1 primer showing polymorphic band (Table 6.3). The number of bands was highest in S12 (Figure 6.1 H) with 12 bands and lowest in S7, S11, S9 and S34 with 5 bands each. Polymorphic band was observed only in 1 locus S6 primer (Figure 6.1 I). A total of 93 reproducible bands with average of 6.2 per primer was scored from RAPD, 70 bands with an average of 7 bands per primer from DAMD and SCoT (Table 6.4). Out of the three markers, RAPD showed the highest monomorphic degree of 98.18% and the least polymorphism degree with 1.81%, SCoT with monomorphic percentage of 96.77% and 3.22%. polymorphism and DAMD markers showed a monomorphic percentage of 94.29% and 5.71% polymorphism (Table. 6.4). The Jaccard's coefficient of the three markers ranged from 0.98-1.00 with a total monomorphism of 96.57% and 3.43% polymorphism with average band of 5.51 (Table. 6.4).

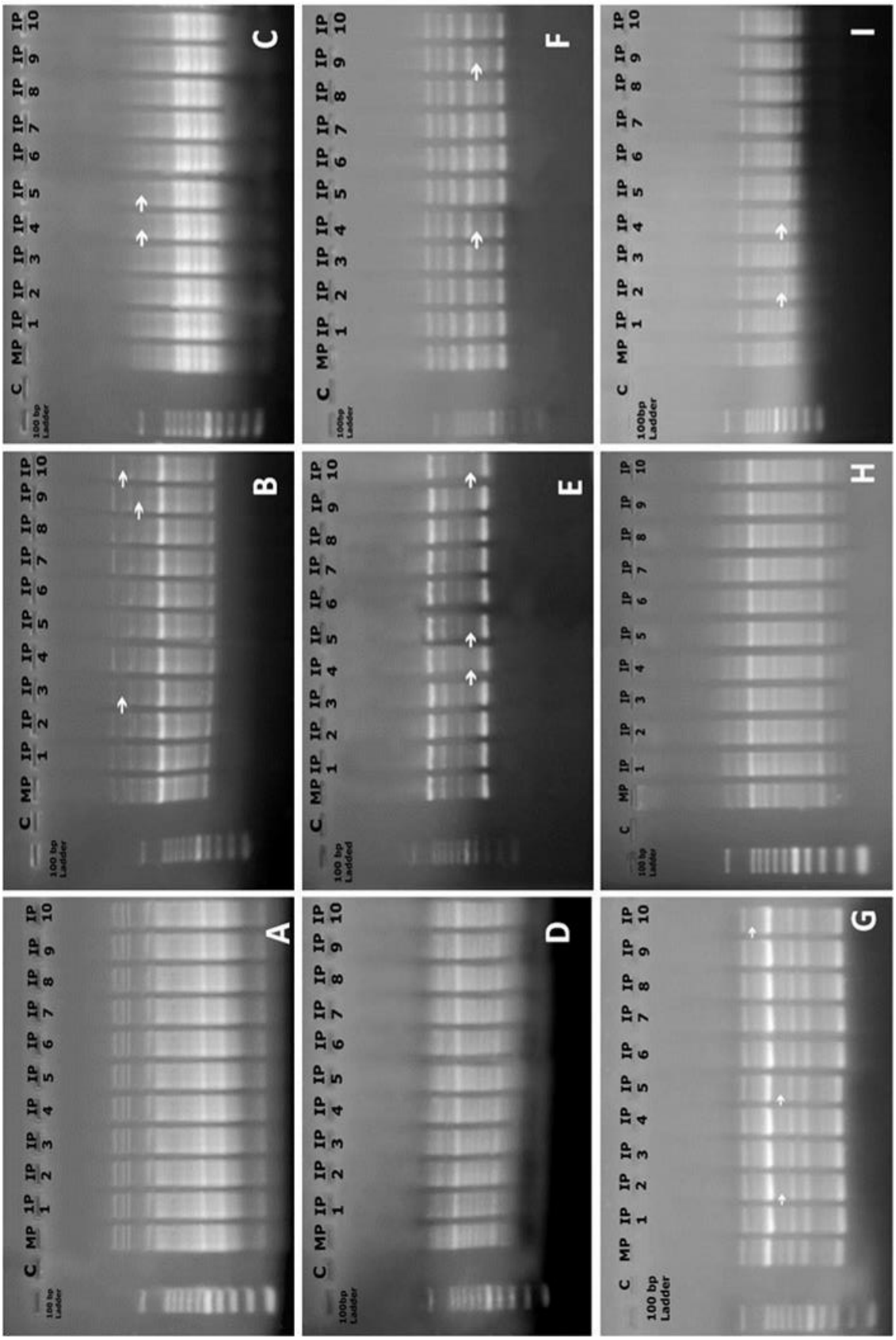

Figure. 6.1. Genetic fidelity assessment of *in vitro* regenerants of *D. heterocarpum*Wall ex. Lindl. using RAPD, DAMD and SCoT markers. Banding pattern of (A) OPG 14; (B)OPA 01; (C) OPBA 7 (D) URP1F; (E) D6; (F) D18; (G) URP9F; (H) S12 (I) S6. *C: control, MP: Mother plant, IP: in vitro regenerated plantlets

Table 6.1: Data of selected RAPD primers, total bands scored, monomorphism %, polymorphic % and size of amplified fragments of *C. bicolor* Lindl.

Sl.No	Primer code	Primer sequence (5'-3')	Total no. of bands	Monomorphic bands (mp)	% of mp	Polymorphic bands (pb)	% of pb	Range of amplification (bp)
1	OPA-01	5´CAGGCCCTTG3´	9	7	77.78	2	22.22	400-6000
2	OPA-05	5´AAAAGTCTTG3´	4	4	100			300-1000
3	OPA-11	5´CAATCGCCGT3´	5	5	100			200-1000
4	OPA-12	5´TCGGCGATAG3	5	5	100			250-900
5	OPA-13	5´CAGCACCCAC3´	4	4	100			200-800
6	OPB-1	5´GTTTCGCTCC3´	7	7	100			300-1200
7	OPAA-8	5´TCCGCAGTAG3´	5	5	100			250-1000
8	OPB-6	5´TGCTCTGCCC3´	4	4	100			300-900
9	OPBA-7	5´GGGTCGCATC3´	9	8	88.89	1	11.11	400-1700
10	OPG-14	5´GGATGAGACC3	11	11	100			120-5000
11	OPH-19	5´CTGACCACCC3´	5	5	100			400-1200
12	OPK-4	5´CCGCCCAAAC3´	6	6	100			500-1400
13	OPU-8	5´GGCGAAGGTT3	8	8	100			200-2000
14	OPU-10	5´ACCTCGGCAC3´	6	6	100			150-1000
15	OPU-12	5´TCACCGCGA3´	5	5	100			300-1000

Table 6.2: Data of selected DAMD primers, total bands scored, monomorphism %, polymorphic % and size of amplified fragments of *C. bicolor* Lindl.

Sl. No	Primer code	Primer sequence (5'-3')	Total no. of bands	Monomorphic bands (mp)	% ofmp	Polymorphic bands (pb)	% of pb	Range of amplification (bp)
1	D6	5´ATGTGTGCGATCAGTTGCTG3´	7	6	85.71	1	14.29	300-1100
2	D18	5´ACAGGGGTGTGGGG3´	7	6	85.71	1	14.29	400-1600
3	D19	5´CCCGTGGGGCCGCCG3´	7	7	100			400-3000
4	URP1F	5´ATCCAAGGTCCGAGACAACC3´	9	9	100			400-1000
5	URP9F	5´ATGTGTCGGATCAGTTGCTG3´	9	7	77.78	2	22.22	300-1000
6	UPR4R	5´AGGACTCGATAACAGGCTCC3´	6	6	100			500-3000
7	URP30F	5´GGACAAGAAGAGGATGTGGA3´	6	6	100			300-1200
8	URP6R	5´GGCAAGCTGGTGGGAGGTAC3´	5	5	100			400-1500
9	URP38F	5´AAGAGGCATTCTACCACCAC3´	6	6	100			300-1000
10	URP17R	5´AATGTGGGCAAGCTGGTGGT3´	8	8	100			650-6000

Table 6.3: Data of selected SCoT primers, total bands scored monomorphism %, polymorphic % and size of amplified fragments of *Cymbidium bicolor* Lindl.

Sl. No	Primer code	Primer sequence (5'-3')	Total no. of bands	Monomorphic bands (mp)	% of mp band	Polymorphic bands (pb)	% of pb band	Range of amplification (bp)
1	S4	5´CAACAATGG CTACCACCT3´	8	8				300-1000
2	S5	5´CAACAATGG CTACCACGA3´	6	6				300-900
3	S6	5´CAACAATGG CTAGCACGC3´	8	7	87.5	1	12.5	300-1200
4	S7	5´CAACAATGG CTACCACGG3´	5	5				300-1000
5	S9	5´CAACAATGG CTACCACGT3´	10	10				300-1000
6	S10	5´CAACAATGG CTACCAGCA3´	6	6				700-2000
7	S11	5´CAACAATGG CTACCAGCC3´	5	5				400-1000
8	S12	5´ACGACATGG CGACCAACG3´	12	12				200-1500
9	S17	5´ACGATGGCTA CCACCGAG3´	5	5				300-1000
10	S34	5´ACCATGGCTA CCACCGCA3´	5	5				300-1500

Table 6.4: RAPD, DAMD and SCoT markers comparison, individually/combination for measuring the extent of similarity and polymorphism in *C. bicolor* Lindl.

SRAP used	No of primers	Total bands	Average bands per primer	Total %		Distance range (Jaccard's coefficient)
				Monomorphism	Polymorphism	
RAPD	15	93	6.2	98.18	1.81	0.98 – 1.00
DAMD	10	70	7	94.29	5.71	0.96 – 1.00
SCoT	10	70	7	96.77	3.22	0.98 – 1.00
RAPD+DAMD+SCoT	35	233	6.66	96.57	3.43	0.98 – 1.00

The UGPMA based dendrogram obtained for RAPD markers represented four clusters, where MP, IP1, IP2, IP6, IP8, IP7 showed similarity as first group, IP9 in second, IP4i and IP5 in third and IP3 and IP10 the farthest from MP (Figure. 6.2). Dendrogram obtained from DAMD markers revealed two major clades having four sub-clades in 1st clade and two sub-clades in 2nd clade (Figure 6.3). Similarities with MP were shared with IP1, IP3, IP6, IP7, IP8, IP2 and slight deviation was shared with IP2, IP4 and IP9. The second clade consisting of IP5 and IP10 showed the farthest similarity with MP. In case of SCoT markers, the dendrogram revealed two clades with MP, IP1, IP3, IP10, IP8, IP7, IP6, IP5, IP9 showing similarity in 1st clade and IP2, IP4 in 2nd clades as farthest from MP (Figure 6.4).

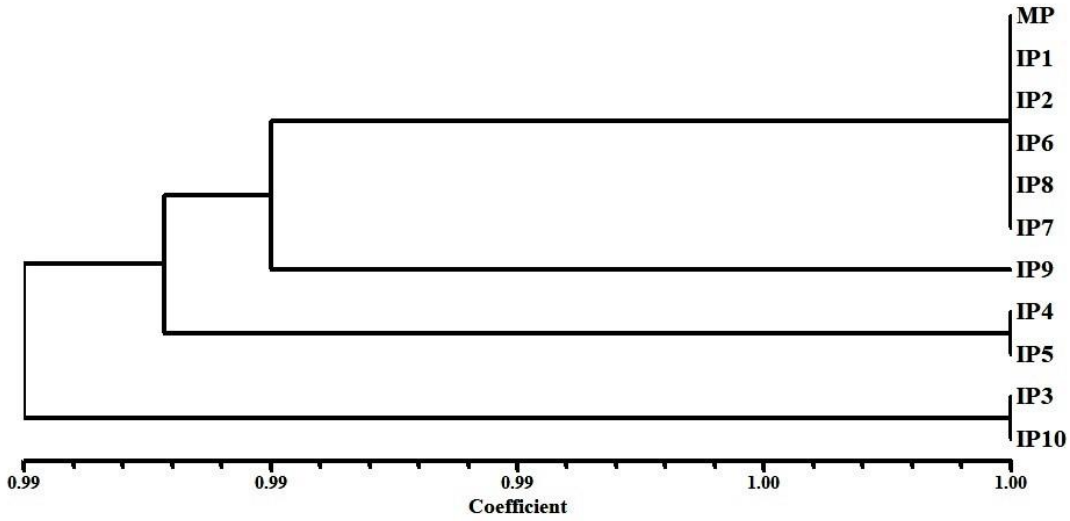

Figure 6.2: UPGMA (NTSYS-PC) dendrogram of RAPD analysis illustrating genetic relationship between MP (mother plant) and IP (*in vitro* regenerants – IP1 to IP 10) of *C. bicolor* Lindl.

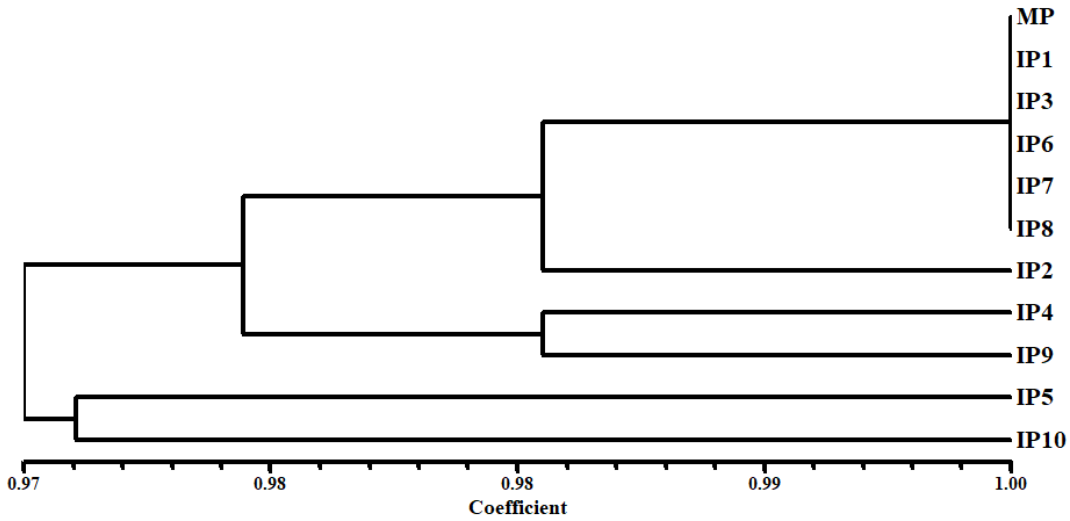

Figure 6.3: UPGMA (NTSYS-PC) dendrogram of DAMD analysis illustrating genetic relationship between MP (mother plant) and IP (*in vitro* regenerants – IP1 to IP 10) of *C. bicolor*Lindl.

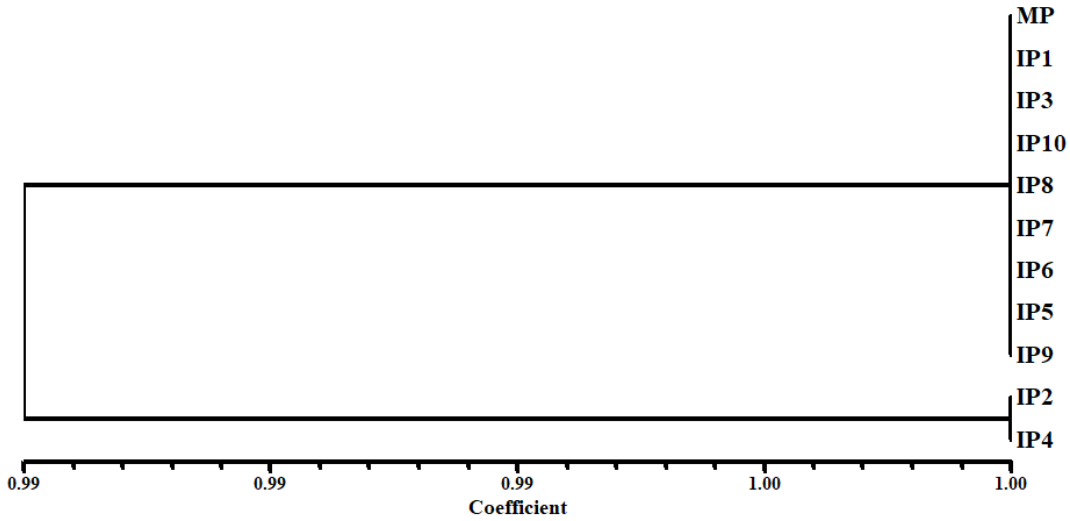

Figure 6.4: UPGMA (NTSYS-PC) dendrogram of SCoT analysis illustrating genetic relationship between MP (mother plant) and IP (*in vitro* regenerants – IP1 to IP 10) of *C. bicolor*Lindl

6.3.2. Genetic fidelity assessment of *D. heterocarpum* Wall ex. Lindl.

The selected 15 RAPD markers for *D. heterocarpum* produced amplified bands ranging from 200 to 2700 bp in size (Figure. 6.5 A–C). 12 primers recorded 100% monomorphic bands and only two primers produced polymorphic bands. A maximum scorable bands of 8 was produced in OPX10 and least of 3 in OPD18. Polymorphic bands were observed in 1 locus of OPD19 and in 2 loci of OPU10 (Table. 6.5, Figure 6.5 B, C). In case of DAMD markers, the amplified bands ranged from 200 to 2500 bp (Figure 6.5 D–F). Monomorphic bands of 100% were recorded in 8 DAMD primers except in D4 with two and URP6R with one polymorphic band (Table 6.6, Figure 6.5 E, F). Amplification of bands was highest in URP2F and URP9P (8 each) while the least was recorded in D14 (4). In addition, the selected SCoT markers amplified bands with size ranging from 200 to 3000 bp (Figure 6.5 G, H). SCoT primer S35 produced the maximum bands of 8 with one polymorphic band (Figure 6.5 H) and the least with 3 bands in S5 (Table 6.7). A total of 83 reproducible bands were scored with average of 5.4 bands per primer from RAPD, 61 bands with an average band of 6.1 per primer from DAMD and 51 bands with an average band of 5.1 per primer

from SCoT (Table. 6.8). Out of the three markers, SCoT showed the highest monomorphic degree of 98% and the least polymorphism degree with 2%, RAPD with monomorphic percentage of 96.38% and 3.61%. polymorphism and DAMD markers showed a monomorphic percentage of 95.08% and 4.92% polymorphism (Table. 6.8). The Jaccard's coefficient of the three markers ranged from 0.96 to 1.00 with a total monomorphism of 96.89% and 3.11% polymorphism with average band of 5.51 (Table. 6.8).

The UPGMA based dendrogram obtained for RAPD markers represented three clusters with MP, IP1, IP3, IP10, IP8, IP7, IP6, IP9 showing similarities in first group; IP4 and IP5 in second and IP2 showing least similarity (Figure 6.6). Dendrogram obtained from DAMD markers revealed two major clades having three sub-clades each. Similarities with MP were shared with IP4, IP5, IP8, while IP2, IP3 and IP7 shared with slight deviation. The second clade consisting of IP1, IP6, IP10 and IP9 showed the farthest similarity away from MP (Figure 6.7). SCoT marker base dendrogram acquired from UPGMA revealed genetic similarity of IP1, IP2, IP3, IP4, IP5, IP6, IP7, IP10 with MP. Only two samples IP8 and IP9 were clustered in a different group from MP (Figure 6.8)

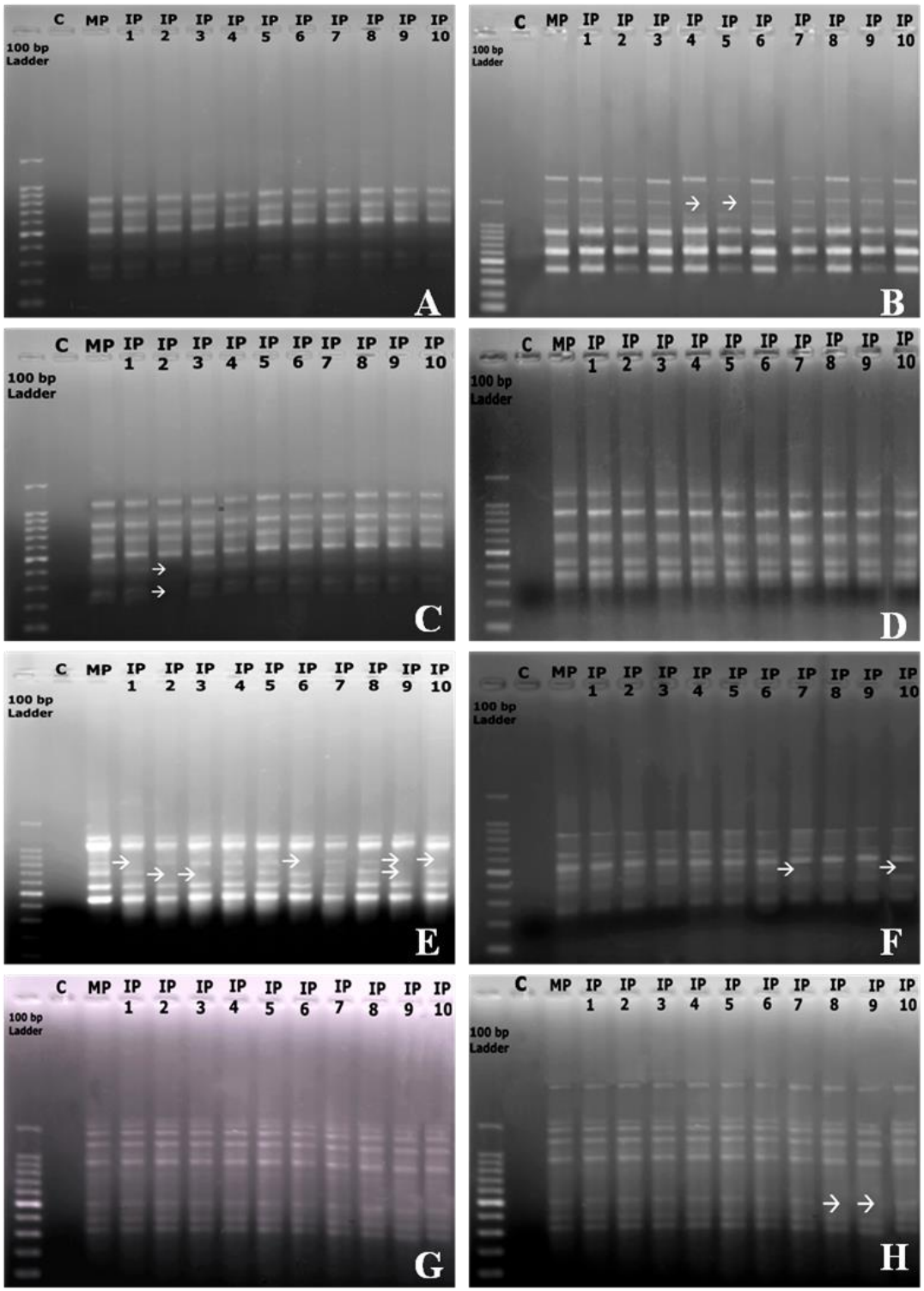

Figure 6.5: Genetic fidelity assessment of *in vitro* regenerants of *D. heterocarpum*Wall ex. Lindl. using RAPD, DAMD and SCoT markers. Banding pattern of (A) OPC 4; (B) OPD 19; (C) OPU 10 (D) URP13R; (E) D4; (F) URP6R; (G) S32; (H) S35. C: control, MP: Mother plant, IP: *in vitro* regenerated plantlets.

Table 6.5: Data of selected RAPD primers, total bands scored monomorphism %, polymorphic % and size of amplified fragments of *D. heterocarpum* Wall ex. Lindl.

Sl. No	Primer code	Primer sequence (5'-3')	Total no. of bands	Monomo-rphic bands (mp)	% of mp band	Polymor-phic bands (pb)	% of pb band	Range of amplification (bp)
1	OPA03	5'AGTCAG CCAC3'	5	5	100			300-1000
2	OPA06	5'GGTCCC TGAC3'	6	6	100			400-1300
3	OPA19	5'CAAACG TCGG3'	6	6	100			400-1000
4	OPAW17	5'TGCTGC TGCC3'	6	6	100			300-1500
5	OPC4	5'CCGCAT CTAC3'	6	6	100			200-800
6	OPD18	5'GAGAGC CAAC3'	3	3	100			400-800
7	OPD19	5'CTGGGG ACTT3'	6	5	83.33	1	16.67	400-3000
8	OPD20	5'ACCCGG TCAC3'	5	5	100			300-1000
9	OPK4	5'CCGCCC AAAC3'	6	6	100			300-1000
10	OPT6	5'CAAGGG CAGA3'	5	5	100			300-1000
11	OPU10	5'ACCTCG GCAC3'	6	4	66.66	2	33.33	200-1200
12	OPW2	5'ACCCCG CCAA3'	4	4	100			400-1200
13	OPX7	5'GAGCGA GGCT3'	6	6	100			300-2700
14	OPX10	5'CCCTAG ACTG3'	8	8	100			300-1100
15	OPG15	5'ACTGGG ACTC3'	5	5	100			300-900

Table 6.6: Data of selected DAMD primers, total bands scored monomorphism %, polymorphic % and size of amplified fragments of *D. heterocarpum* Wall ex. Lindl.

Sl. No	Primer code	Primer sequence (5'-3')	Total no. of bands	Monomor-phic bands (mp)	% of mp band	Polymor-phic bands (pb)	% of pb band	Range of amplification (bp)
1	D4	5'AGGACTCG ATAACAGGC TCC3'	6	4	66.66	2	33.33	400-2200
2	D10	5'GGACAAG AAGAGGATG TGGA3'	7	7	100			300-2500
3	D14	5'CCTCCTCC CTCCT3'	4	4	100			300-900
4	URP1F	5'ATCCAAGG TCCGAGACA ACC3'	5	5	100			200-1000
5	URP2F	5'GTGTGCGA TCAGTTGCT GGG3'	8	8	100			200-1200
6	URP4R	5'AGGACTCG ATAACAGGC TCC3'	5	5	100			300-1200
7	URP6R	5'GGCAAGCT GGTGGGAGG TAC3'	6	5	83.33	1	16.66	250-900
8	URP9P	5'ATGTGTCG GATCAGTTG CTG3'	8	8	100			200-1200
9	URP13R	5'TACATCGC AAGTGACAC AGG3'	6	6	100			300-1250
10	URP25F	5'GATGTGTT CTTGGAGCC TGT3'	6	6	100			270-1300

Table 6.7: Data of selected SCoT primers, total bands scored, monomorphism %, polymorphic % and size of amplified fragments of *D. heterocarpum* Wall ex. Lindl.

Sl. No	Primer code	Primer sequence (5'-3')	Total no. of bands	Monomorphic bands (mp)	% of mp band	Polymorphic bands (pb)	%of pb band	Range of amplification (bp)
1	S4	5'CAACAATGGCTACCACCT3'	5	5	100			200-1000
2	S5	5'CAACAATGGCTACCACGA3'	3	3	100			300-800
3	S6	5'CAACAATGGCTAGCACGC3'	4	4	100			450-1000
4	S7	5'CAACAATGGCTACCACGG3'	5	5	100			300-900
5	S12	5'ACGACATGGCGACCAACG3'	4	4	100			200-1000
6	S17	5'ACGATGGCTACCACCGAG3'	4	4	100			300-900
7	S25	5'ACCATGGCTACCACCGGG3'	6	6	100			300-1500
8	S32	5'CCATGGCTACCACCGCAC3'	7	7	100			300-1500
9	S34	5'ACCATGGCTACCACCGCA3'	4	4	100			400-1400
10	S35	5'CATGGCTACCACCCGCCC3'	8	7	87.5	1	12.5	300-3000

Table 6.8: RAPD, DAMD and SCoT markers comparison, individually/combination of *D. heterocarpum* Wall ex. Lindl.

Sl. No.	SRAP used	No of primers	Total bands	Average bands per primer	Total %		Distance range (Jaccard's coefficient)
					Monomorphism	Polymorphism	
1.	RAPD	15	83	5.4	96.38	3.61	0.96-1.00
2.	DAMD	10	61	6.1	95.08	4.92	0.96-1.00
3.	SCoT	10	51	5.1	98	2	0.98-1.00
4.	RAPD + DAMD+ SCoT	35	193	5.51	96.89	3.11	0.96-1.00

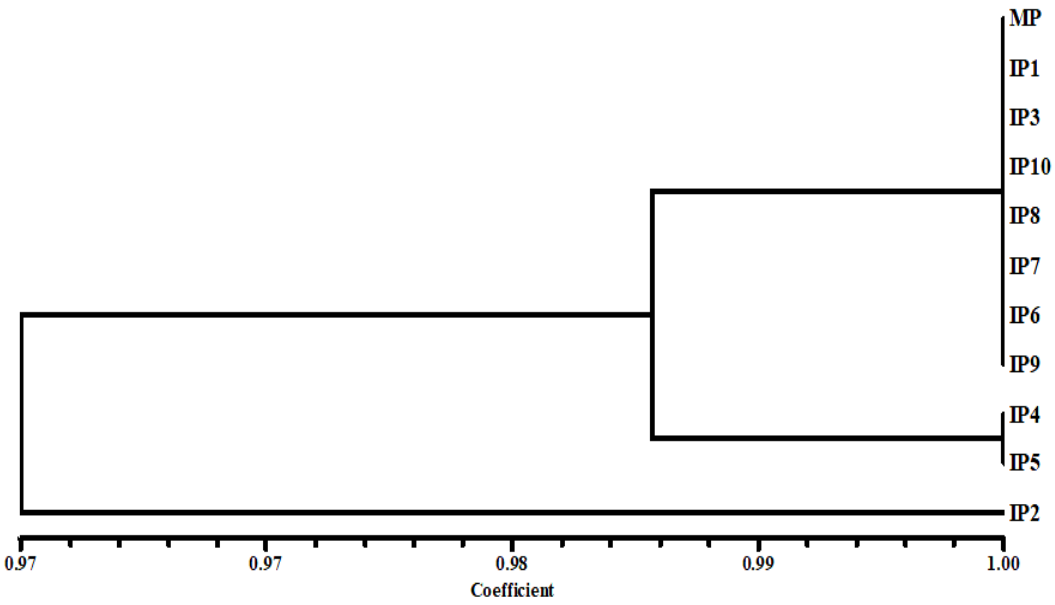

Figure 6.6: UPGMA (NTSYS-PC) dendrogram of RAPD analysis illustrating genetic relationship between MP (mother plant) and IP (*in vitro* regenerants – IP1 to IP 10) of *D. heterocarpum* Wall ex. Lindl.

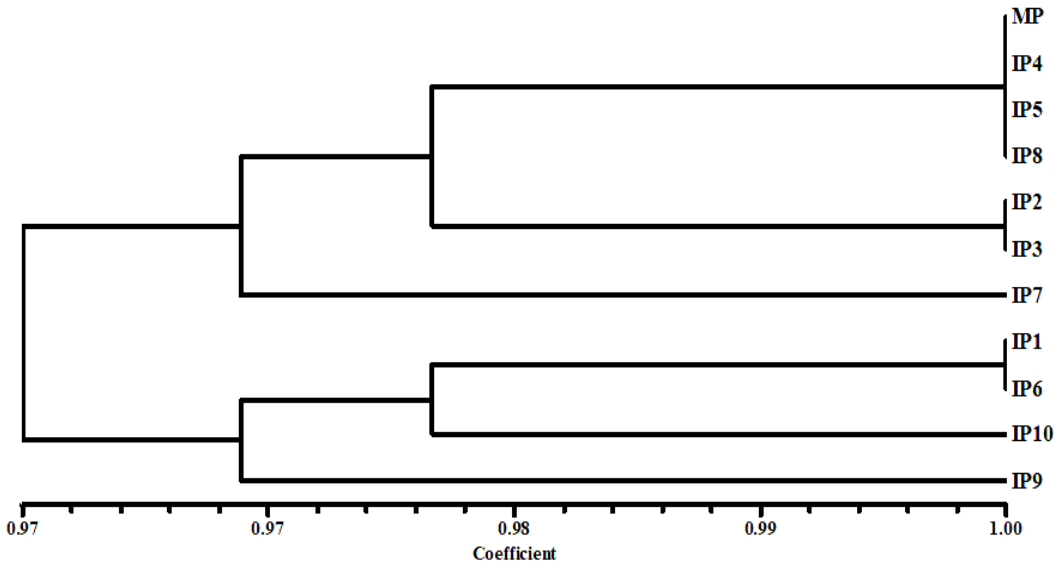

Figure 6.7: UPGMA (NTSYS-PC) dendrogram of DAMD analysis illustrating genetic relationship between MP (mother plant) and IP (*in vitro* regenerants – IP1 to IP 10) of *D. heterocarpum* Wall ex. Lindl.

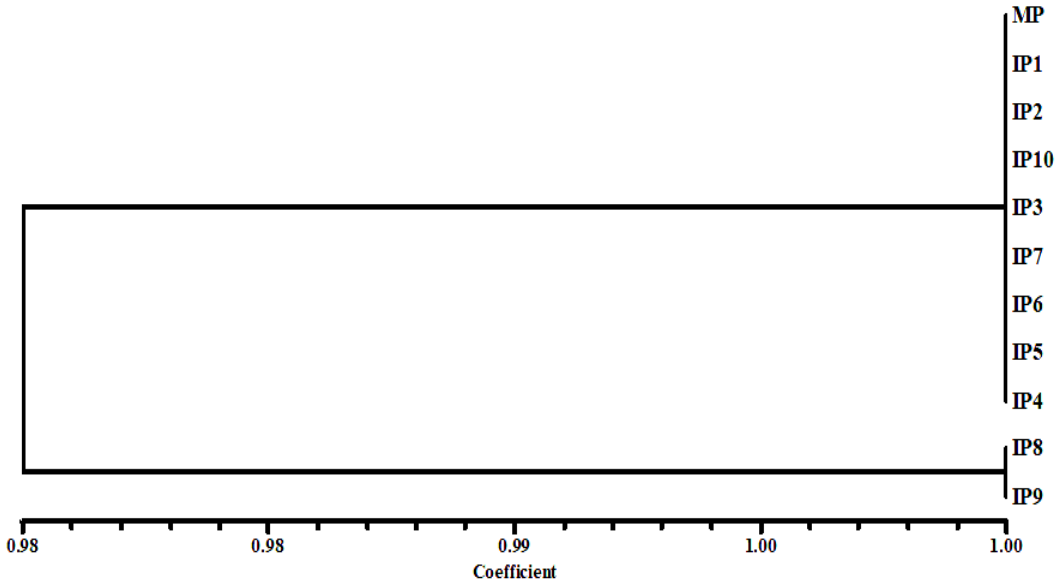

Figure 6.8: UPGMA (NTSYS-PC) dendrogram of SCoT analysis illustrating genetic relationship between MP (mother plant) and IP (*in vitro* regenerants – IP1 to IP 10) of *D. heterocarpum* Wall ex. Lindl.

6.3.3. Genetic fidelity assessment of *E. clarkei* Rchb. f.

In *E. Clarkei*, from the 15 selected RAPD primers monomorphic bands of 100% were recorded in 13 primers and polymorphic bands were observed in 2 primers (Table. 6.9). The amplified bands ranged from 150-1800 bp in size (Figure 6.9 A-C). Maximum scorable bands

of 10 was produced in OPU10 (Figure 6.9 A) and least of 5 bands was recorded in OPA04, OPA12, OPA20, OPAW8, OPG14 and OPU8 (Table. 6.9). Polymorphic bands were observed in 1 locus each of OPG14 and OPU8 (Figure. 6.9 B, C). In case of DAMD, Monomorphic bands of 100% were recorded in 7 primers and polymorphic bands in 3 primers from the selected 10 primers (Table 6.10). The size of the amplified bands ranged from 300-6000 bp. The maximum number of bands was scored 10 in both URP1F (Figure 6.9 D) and URP9F and the least of 3 bands in D6. Polymorphic bands of 5 was observed from DAMD markers, in 2 loci of URP1F and URP9F and in 1 locus of URP6R (Table 6.10). In addition, selected 10 SCoT primers reproduced monomorphic bands of 100% were produced in 9 primers and polymorphic band only in 1 primer (Table 6. 11). The amplified bands ranged from 250-1500 bp in size. A maximum of 8 bands were recorded in S4 and S10 (Figure 6.9 G, H) and least of 4 bands in S6 (Table 6.11). Polymorphic band was observed only in 1 locus of S10 (Figure 6.9 H). The total reproducible bands were 102 with an average of 6.8 bands per primer from RAPD, 67 scorable bands with an average of 6.7 bands from DAMD and 62 scorable bands with an average of 6.2 bands from SCoT (Table 6.12). Out of the three markers, SCoT showed the highest monomorphic degree of 98.39% and the least polymorphism degree with 1.61%, RAPD with monomorphic percentage of 98.04% and 1.96%. polymorphism and DAMD markers showed a monomorphic percentage of 92.54% and 7.46% polymorphism (Table. 6.12). The Jaccard's coefficient of the three markers ranged from 0.96 to 1.00 with a total monomorphism of 96.33% and 3.69% polymorphism with average band of 6.23 (Table. 6.12).

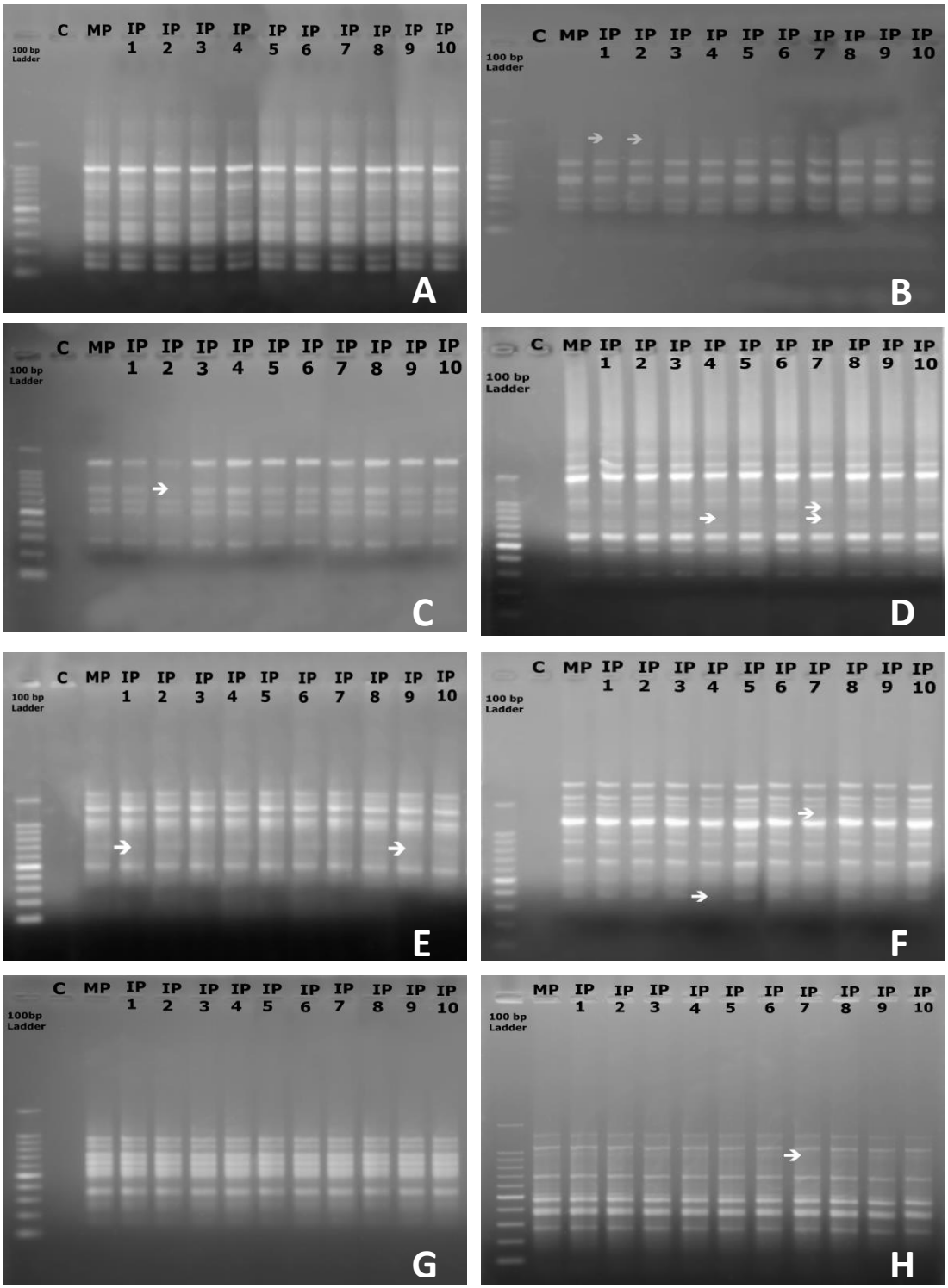

Figure 6.9. Genetic fidelity assessment of *in vitro* regenerants of *E. clarkei* Rchb. f. using RAPD, DAMD and SCoT markers. Banding pattern of (A) OPU 10; (B) OPG 14; (C) OPU 8; (D) URP1F; (E) URP6R; (F) URP9F; (G) S4; (H) S10. ***C**: control, MP: Mother plant, IP: *In vitro* regenerated plantlets.

Table 6.9: Data of selected RAPD primers, total bands scored monomorphism %, polymorphic % and size of amplified fragments *E. clarkei* Rchb. f.

Sl. No	Primer code	Primer sequence (5'-3')	Total no. of bands	Monomorphic bands (mp)	% of mp band	Polymorphic bands (pb)	% of pb band	Range of amplification (bp)
1	OPA-03	5´AGTCAGCCAC3´	9	9	100			200-1000
2	OPA-04	5´AATCGGGCTG3´	5	5	100			400-1000
3	OPA-11	5´CAATCGCCGT3´	9	9	100			300-900
4	OPA-12	5´TCGGCGATAG3´	5	5	100			300-1000
5	OPA-20	5´GTTGCGATCC3´	5	5	100			400-1000
6	OPAA-8	5´GTTGCGATCC3´	5	5	100			200-1200
7	OPAW-11	5´CTGCCACGAG3´	9	9	100			200-1500
8	OPAW-17	5´TGCTGCTGCC3´	7	7	100			150-1600
9	OPB-1	5´GTTTCGCTCC3´	7	7	100			200-1000
10	OPBA-7	5´GGGTCGCATC3´	7	7	100			200-1000
11	OPG-14	5´GGATGAGACC3´	5	4	80	1	20	200-1000
12	OPK-4	5´CCGCCCAAAC3´	8	8	100			300-1800
13	OPH-19	5´CTGACCACCC3´	6	6	100			300-1000
14	OPU-8	5´GGCGAAGGTT3´	5	4	80	1	20	300-1300
15	OPU-10	5´ACCTCGGCAC3´	10	10	100			150-1000

Table 6.10: Data of selected DAMD primers, total bands scored, monomorphism %, polymorphic % and size of amplified fragments of *E. clarkei* Rchb. f.

Sl. No	Primer code	Primer sequence (5'-3')	Total no. of bands	Monomorphic bands (mp)	% of mp band	Polymorphic bands (pb)	% of pb band	Range of amplification (bp)
1	D6	5´'ATGTGTG CGATCAGTT GCTG3 ´	3	3	100			400-1200
2	D18	5´ACAGGGG TGTGGGG3´	4	4	100			300-800
3	D19	5'CCCGTGG GGCCGCCG3 ,	6	6	100			400-1200
4	URP1F	5´ATCCAAG GTCCGAGA CAACC3´	10	8	80	2	20	300-3000
5	URP2F	5´GTGTGCG ATCAGTTGC TGGG3´	6	6	100			300-1500
6	URP4R	5´AGGACTC GATAACAG GCTCC3´	6	6	100			850-3000
7	URP6R	5´GGCAAGC TGGTGGGA GGTAC3´	6	5	88.33	1	16.66	500-1700
8	URP9F	5´ATGTGTC GGATCAGTT GCTG3´	10	8	80	2	20	400-3000
9	URP17R	5´AATGTGG GCAAGCTG GTGGT3´	9	9	100			450-6000
10	URP38F	5´AAGAGGC ATTCTACCA CCAC3´	7	7	100			400-1000

Table 6.11: Data of selected SCoT primers, total bands scored, monomorphism %, polymorphic % and size of amplified fragments of *E. clarkei* Rchb. f.

Sl. No	Primer code	Primer sequence (5'-3')	Total no. of bands	Monomorphic bands (mp)	% of mp band	Polymorphic bands (pb)	% of pb band	Range of amplification (bp)
1	S4	5''CAACAATGGC TACCACCT3 ´	8	8	100			250-1000
2	S5	5´CAACAATGGC TACCACGA3´	6	6	100			300-800
3	S6	5´CAACAATGGC TAGCACGC3´	4	4	100			450-1000
4	S7	5´CAACAATGGC TACCACGG3´	5	5	100			300-900
5	S10	5´ACGACATGGC GACCAACG3´	8	7	87.5	1	12.5	280-1300
6	S12	5´ACGATGGCTA CCACCGAG3´	5	5	100			300-900
7	S17	5´ACCATGGCTA CCACCGGG3´	6	6	100			300-1500
8	S25	5´CCATGGCTACC ACCGCAC3´	7	7	100			300-1500
9	S32	5´ACCATGGCTA CCACCGCA3´	5	5	100			400-1400
10	S35	5´CATGGCTACC ACCCGCCC3´	8	8	100			300-2500

Table 6.12: RAPD, DAMD and SCoT markers comparison, individually/combination *E. clarkei* Rchb. f.

Sl No.	SRAP used	No. of primers	Total bands	Average bands per primer	Total %		Distance range (Jaccard's coefficient)
					Monomorphism	Polymorphism	
1.	RAPD	15	102	6.8	98.04	1.96	0.98-1.00
2.	DAMD	10	67	6.7	92.54	7.46	0.94-1.00
3.	SCoT	10	62	6.2	98.39	1.61	0.98-1.00
4.	RAPD+ DAMD + SCoT	35	218	6.23	96.33	3.69	0.96-1.00

The UPGMA based dendrogram obtained for RAPD markers had two clades with MP, IP3, IP4, IP10, IP9, IP8, IP7, IP6, IP5 in first clade and the second clade had two sub-clades with IP1 and IP2 (Figure 6.10). DAMD marker based dendrogram revealed 2 clades with first clade further dividing into sub- clades. Similarities with MP were shared with IP2, IP3, IP10, IP8, IP5, IP6 and slight deviation was shared with IP1, IP9 and IP4. The *in vitro* plantlet sample IP7 shared the least similarity with MP (Figure 6.11). In SCoT marker based dendrogram acquired from UPGMA revealed genetic similarity MP with IP1, IP2, IP10, IP9, IP8, IP3, IP6, IP5, IP4 and one sample IP7 in different clade (Figure 6.12).

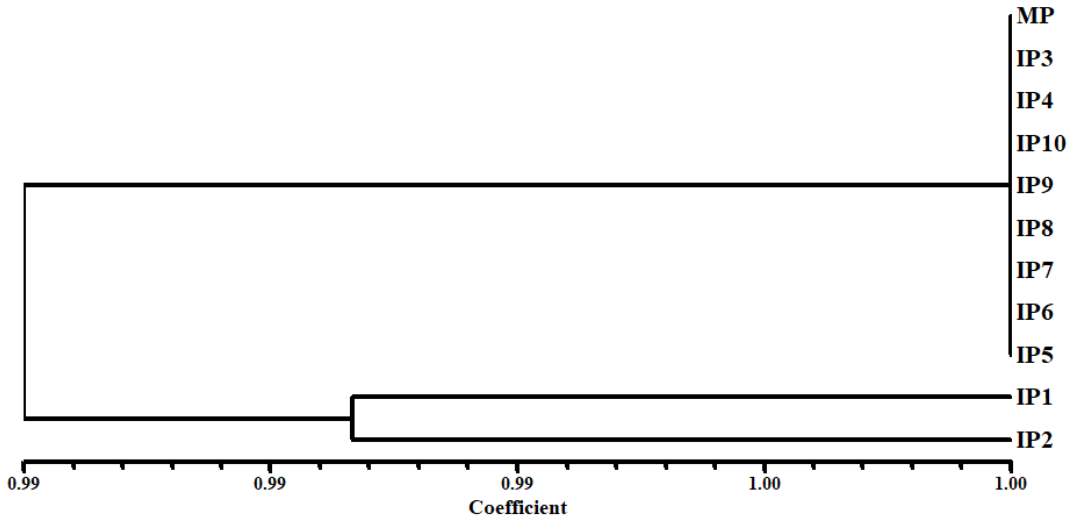

Figure 6.10: UPGMA (NTSYS-PC) dendrogram of RAPD analysis illustrating genetic relationship between MP (mother plant) and IP (*in vitro* regenerants – IP1 to IP 10) of *E. clarkei* Rchb. f.

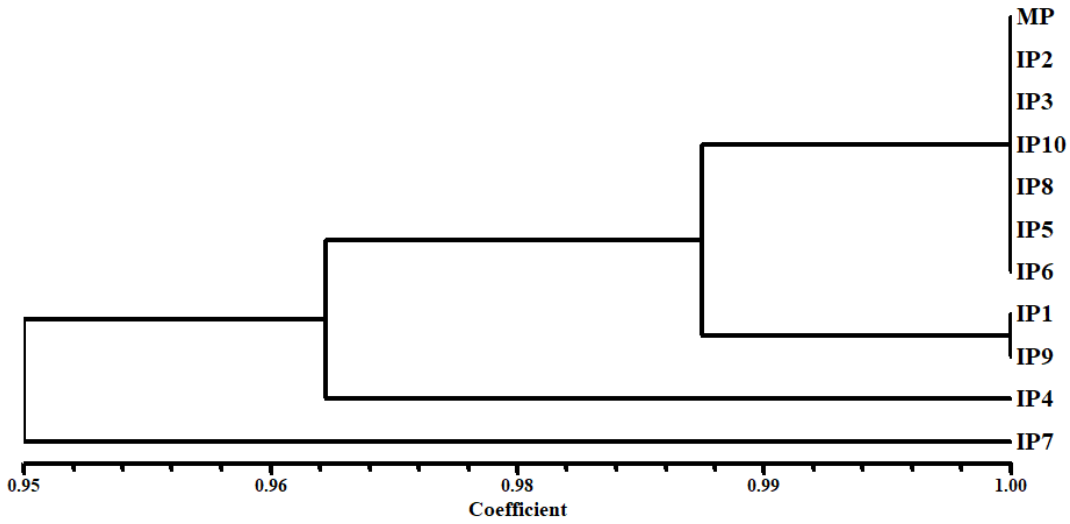

Figure 6.11: UPGMA (NTSYS-PC) dendrogram of DAMD analysis illustrating genetic relationship between MP (mother plant) and IP (*in vitro* regenerants – IP1 to IP 10) of *E. clarkei* Rchb. f.

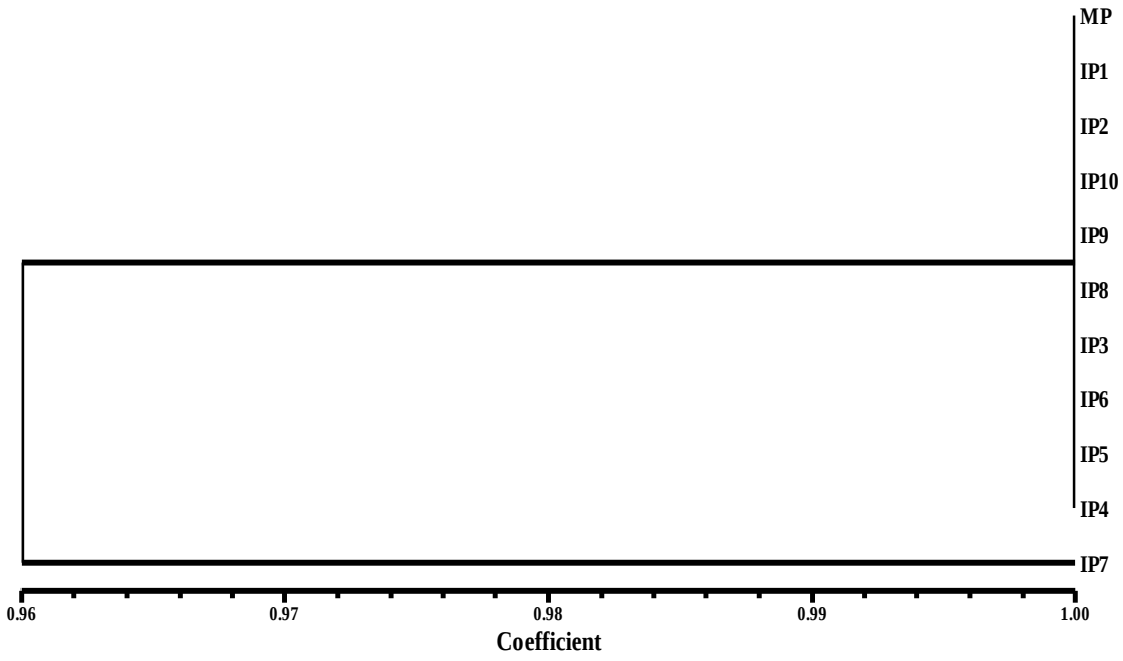

Figure 6.12: UPGMA (NTSYS-PC) dendrogram of SCoT analysis illustrating genetic relationship between MP (mother plant) and IP (*in vitro* regenerants – IP1 to IP 10) of *E. clarkei* Rchb. f.

6.4. Discussion

With the occurrence of soma clonal variation in *in vitro* regenerated plantlets, assessing genetic homogeneity has become a mandate (Tikendra et al., 2021a). Assessment of genetic variation through RAPD technique is favoured for its cheap, easy and rapid results but supplementing it with other advance markers substantiates its weakness (Tikendr et al., 2021b). Thus, the present study aimed to test the genetic uniformity of the donor plant and successfully raised and acclimatized *in vitro* plantlets of *C. bicolor*, *D. heterocarpum* and *E. clarkei* using RAPD, DAMD and SCoT markers.

RAPD markers revealed high genetic similarities between the donor plants (MP) and *in vitro* regenerated plantlets (IP) of the three orchids. High genetic similarity of 98.18% monomorphism and polymorphism of 1.81% in *C. bicolor*, 96.38% monomorphism and polymorphism of 3.61 in *D. heterocarpum* and 98.04% monomorphism and polymorphism of 1.96 in *E. clarkei* was observed between the MP and IP (Table 6.4, 6.8, 6.12). Reports of

such low genetic variation in vitro raised orchids using RAPD markers have been reported in *Paphiopedilum niveum* (Soonthornkalump et al., 2019) and *Dendrobium chrysotoxum* (Tikendra et al., 2019b).

RAPD markers may not be able to reveal the changes in repetitive regions in some plants' genomes (Palombi and Damiano, 2002). A DAMD marker which amplifies mini-satellite primers of core sequences and Variable Number of Tandem Repeats (VNTR) in parts of genome (Heath et al., 1993) was used to support RAPD analysis. The DAMD markers showed higher percentage of polymorphism with 5.71% in *C. bicolor,* 4.92% in *D. heterocarpum* and 7.46 in *E. clarkei* in comparison to RAPD and SCoT markers in all three species. The UPGMA based dendrograms in *C. bicolor* revealed that the number of IP sample deviating from MP were similar in RAPD (5) and DAMD (5) and least in SCoT (2) marker (Figure 6.2, 6.3, 6.4). In *D. heterocarpum,* the dendrogram obtained from DAMD marker showed higher IP samples (upto 7) deviating from MP compared to RAPD (3) and SCoT (1) (Figure 6.7, 6.6, 6.8). Similarly, in *E. clarkei* IP samples deviating from MP was higher in DAMD (5) followed by RAPD (2) and SCoT (1) (Figure 6.11, 6.10, 6.12). Such high percentage of polymorphism in DAMD from other markers has been observed in genetic uniformity study *in vitro* regenerates of *Nepenthes khasiana* (Devi et al., 2014) and *Dendrobium* Sabin Blue orchid (Chin et al., 2019).

In addition, SCoT markers which is significant in identifying genetic mutation of plant genes that targets conserved short regions by flanking ATG start codons (Sharma and Thakur, 2021) was used. The highest monomorphic and lowest polymorphic degree was resulted in SCoT in compared to the other two markers in all three species. Monomorphism of 96.57% and polymorphism of 3.22% in *C. bicolor* 98% monomorphism and 2% polymorphism in *D. heterocarpum* and 98.39% monomorphism and 1.61% polymorphism were recorded. Similar studies of genetic homogeneity between donor mother plant and *in*

vitro regenerates revealing genetic similarity of above 90% was also observed in *in vitro* raised orchids (Bhattacharyya et al., 2015a; Sherif et al., 2020).

6.5. Summary and Conclusion

Mass propagation of orchids through *in vitro* with economic importance has been considered as the novel means to sustain market demands and conserve wild populations. With target of creating clones with desirable features that are genetically identical. As environmental conditions affect how plants grow, it becomes more challenging to detect uniformity through morphological features. Therefore, it is crucial to verify genetic fidelity using molecular markers since it demonstrates the homogeneity of plants at the genetic level. In the current study, using RAPD, DAMD, and SCoT markers, the genetic stability of donor mother plants and in vitro regenerates was determined. Genetic homogeneity of MP with IP using RAPD, DAMD and SCoT levelled up to 96.57% in *C. bicolor*, 96.89% in *D. heterocarpum* and 96.33% in *E. clarkei*. This supports the validity of the study's optimised tissue culture procedure for producing authentic duplicates of *C. bicolor*, *D. heterocarpum* and *E. clarkei* for preservation and commercialization.

A part of the chapter was published as:

Longchar, T. B. and Deb, C. R. 2022. Optimization of *in vitro* propagation protocol of *Dendrobium heterocarpum* Wall. ex. Lindl. and clonal genetic fidelity assessment of the regenerates: an orchid of horticultural and medicinal importance, *South African Journal of Botany*, 149: 67-78. https://doi.org/10.1016/j.sajb.2022.05.058.

CHAPTER 7

Comparative Quantification of Nutraceutical Potential Phytochemicals and Assessment of Antioxidant Activity of the Selected Wild Orchid species and *In Vitro* Regenerants

7.1. Introduction

Orchids are generally farmed for their decorative value; however, many are also used as food, herbal medicines, and other cultural values by various nations and tribes around the world (Kasulo et al., 2009). Orchids, particularly *Dendrobium* species, have been used as herbal medicines by various cultures since ancient times. There are currently 40 or so *Dendrobium* species utilized in traditional Chinese medicine. Similarly, *Dendrobium* orchids have been used in China, Mongolia, Korea, and Japan for euphoria induction, blood cleansing, lung strengthening, and treatment of breast cancer, boils, abscesses, malignant swellings, ulcers, and pus (Zhang et al., 2006). *Acampe*, *Aerides*, *Coelogyne*, *Crepidiu*m, *Dactylorhiza*, *Gastrodia*, *Eulophia*, *Flickingeria*, *Otochilus*, *Pholidota*, *Satyrium*, and *Vanda* are other genera of medicinal orchids that are intensively exploited (Tsering et al., 2017). Ayurveda, an ancient Indian school of healing, includes orchids among its constituents. According to reports, Asthavarga, a key component of several traditional formulas like Chavyanprasa, contains 4 types of orchids: *Malaxis muscifrea*, *Malaxis acuminata*, *Habenaria intermedia* and *Habenaria edgeworthi* (Singh and Duggal, 2009).

The Trans-Himalayan region continues to use a variety of orchid species, including *Brachycorythis obcordata*, *Coelogyne cristata*, *Dactylorhiza hatagirea*, *Otochilus porrectus*, *Rhynchostylis retusa*, *Satyrium nepalense*, and *Vanda cristata*, while the Sowa-Rigpa system of traditional medicine frequently employs *Cypripedium himalaicum*, *Dendrobium densiflorum*, *Gymnadenia conopsea* and *G. Orchidis* (Dakpa, 2007). There are many orchid species which are used in ethno medicines, which are not well documented/not known and yet to be exploited. The species under study, especially *Cymbidium bicolor* Lind. and *Dendrobium heterocarpum* Wall. ex Lindl. has been reported to be used in treating ailments in tribal medicines (Pant, 2013; Chowlu et al., 2017).

Phytochemical studies of medicinal orchids have shown presences of major secondary metabolites like anthocyanins, bibenzyl derivatives, cypripedin, hircinol, jibantine, loroglossin, nidemin, orchinol, and phenantt (Gantait et al., 2021). It has been observed that orchids contain a variety of phytochemicals, including alkaloids, bibenzyl derivatives, flavonoids, and phenanthrenes. Secondary metabolites are low molecular weight organic substances. Despite not being the main drivers of growth and development, these chemicals are created under specific conditions like stress (Thakur et al., 2019). These phytochemicals have antibacterial, anticancer, anti-inflammatory, antiviral and antioxidant properties among other things (Singh et al., 2012). Many medicinal plants contain antioxidants, especially polyphenols and flavonoids, which have been associated to the protection of degenerative diseases like cancer, cardiovascular and neurological problems, and oxidative stress-related dysfunctions. Researchers have been searching for powerful antioxidants to lessen oxidative stress in cells and treat a number of human ailments for the last decade (Chimsook, 2016). However, there is a lack of knowledge on the therapeutic benefits of orchids in various parts of the world, information that is specifically tailored to

particular communities and places. The collection of such data is crucial for serving as a reference for the current generation of drugs for many serious disorders (Pant, 2013).

In vitro micro propagation technique offer a greater selectivity and yield in less time, ensuring the least possible harm to native environments. Additionally, plant tissue culture is effective enough to affect the production and build-up of secondary metabolites in newly grown plants. The use of varied medium strengths, plant growth regulators (PGRs), *elicitors*, and various additives to promote the formation of secondary metabolites are few of the methods used to achieve these phenomena (Prasad et al., 2021). Increase in secondary metabolite production in the *in vitro* produced plantlets of *Coelogyne ovalis* Lindl. (Singh and Kumaria, 2019), *Dendrobium nobile* Lindl. (Battacharyya et al., 2014b) have been reported.

Thus, in the current study a comparative quantification of certain potential secondary metabolites and antioxidant activity of different parts of *Cymbidium bicolor* Lind., *Dendrobium heterocarpum* Wall. ex Lindl. and *Esmeralda clarkei* Rchb. f. and their *in vitro* regenerants was assessed and aimed to identify the right stage for harvesting plant parts with secondary metabolites at their peak.

7.2. Materials and Methods

7.2.1. *In vitro* plant regeneration

Protocorms developed from immature embryos were cultured in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) fortified with 3% sucrose (v/w). Media were gelled with 0.8% (w/v) tissue culture grade agar and *p*H was adjusted to 5.7 using 0.1N NaOH and 0.1N HCl. Plant growth regulators (PGR) were added accordingly in combination/single of N6–benzyl adenine (BA, 6 µmol/L) and N^6 α-naphthalene acetic acid (NAA, 6 µmol/L) for *C. bicolor*, kinetin (KN, 3 µmol/L) and NAA (3 µmol/L) for *D. heterocarpum* and (KN, 9 µmol/L) and NAA (9 µmol/L) for *E. clarkei*. The cultures were

then kept at 25±2ºC temperature under fluorescent light (40 µmol m^{-2} s^{-1} illumination) at 12/12 h photo period. After the protocorms differentiated into young plantlets with 3 or 4 leaves, they were primarily hardened in the same growing media before assessment of phytochemicals.

7.2.2. Plant material and preparation of extract

The plant parts taken for phytochemical estimation were considered according to the morphology of the orchid species under study viz. *C. bicolor* (Figure 7.1 A), *D. heterocarpum* (Figure 7.1 D) and *E. clarkei* (Figure 7.1 E). Primarily hardened *in vitro* regenerants of the three species were taken. The plant samples were collected and oven dried at 60°C till the consistent weight of the samples was achieved. About 200 mg of each dry material was crushed and dissolved in 2, 3 and 5 ml of 80% (v/v) methanol followed by incubation in a water bath (60-70°C) for 30 min. The supernatant was filtered using Whatman's filter paper No.1 and the final volume was made to 10 ml with 80% methanol (v/v) after centrifugation at 10,000 rpm for 10 min. This methanol extract was used for analysis. Thermo Scientific UV-Visible Evolution 201 and Thermo Scientific Multiskan Spectrophotometer were used to determine phytochemicals and antioxidant activity respectively.

7.2.3. Biochemical analysis

7.2.3.1. Quantification of total phenol content

Modified Folin-Ciocalteu method (Genwali et al., 2013) was used to estimate total phenolic content. 100 µL of methanol extract was added to 2.9 ml of deionised water, 2 ml of 7% (w/v) sodium carbonate and 0.5 ml of 10% (v/v). Folin-Ciocalteu reagent were added in a test tube. The mixture solutions were incubated in dark room temperature for 90 min. The absorbance of the reactions was measured at 765 nm. Total phenolic content was quantified as mg of Gallic acid equivalent (GAE) per gram sample (dry weight basis, D/W).

7.2.3.2 Quantification of total flavonoid content

Total flavonoid was quantified following the method of Tan (Tan, 2018). For quantification, 50 µL of methanol extract was mixed with 0.15 ml of 0.5M sodium nitrate, 0.15 ml of 0.3M aluminium chloride and volume were adjusted to 4.0 ml with 30% methanol. After a gap of 5 min 1 ml of 1M sodium hydroxide was added and absorbance measured at 510 nm. The resultant absorbance was used to determine the concentration of flavonoid against standard quercetin, expressed as mg of quercetin equivalent (QE) per gram of sample (D/W).

7.2.3.3. Quantification of total tannin content

Folin-Dennis method (Bhattacharya et al., 2014) was used to estimate total tannin content. Reaction mixture was prepared by mixing 50 µL of methanol extract, 3.95 ml of deionised water, 0.5 ml each of Folin-Dennis reagent, 20% sodium carbonate and absorbance was measured at 775 nm. The total tannin content was obtained from calibration curve of tannic acid and expressed as mg of tannic acid equivalent (TAE) per gram sample (D/W).

7.2.3.4. Quantification of total alkaloid content

The total alkaloids were determined by following the protocol described by Patel et al. (2015) with minor modifications. In a Soxhlet apparatus, raw sample was filtered in 80% methanol and the extract was condensed and then evaporated to dryness. Residue obtained was dissolved adding 2N hydrochloric acid (HCl). In a separating funnel 1 ml of test solution was transferred, to which 5 ml phosphate buffer of *p*H 4.7 and 5 ml bromocresol green was added. Chloroform (1, 2, 3 and 4 ml) were added to the mixture and shaken and complex formed were collected in 10 ml volumetric flask, final volume was made to 10 ml with chloroform. The absorbance of complex was measured at 470 nm. Total alkaloids content was estimated as mg AE/g DW.

7.2.3.5. 2, 2-diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging assay

Radical scavenging activity of the sample extracts was determined by DPPH assay according to Brand-William et al. (2015). Sample extract at different concentrations were added to 3 ml of DPPH reagent (0.1mM in 80% methanol, v/v). Final volume was made to 4ml with 80% methanol and incubated for 30 min in dark and the absorbance of the mixture was measured at 517nm. The percentage inhibition of DPPH free radicals was calculated using the following equation:

$$\text{Free radical inhibition \%} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Abs}} \times 100$$

7.2.3.6. Ferric reducing antioxidant power (FRAP) assay

Antioxidant activity estimation through FRAP assay was determined based on ability of antioxidants to reduce ferric (Fe^{3+}) ion to ferrous (Fe^{2+}) ion in FRAP reagent with 2, 4, 6-tri-(2-pyridyl)-5-triazine (TPTZ) (Rakholiya et al., 2015). Freshly prepared 10 mM TPTZ solution, 20 mM ferric chloride and 300 mM sodium acetate buffer (pH 3.6) were added in the ratio of 10:1:1 at 37 °C water bath to prepare FRAP reagent. Sample extract and 3 ml of FRAP reagent was added to test tube and final volume made to 4 ml by double distilled water. After incubation of 30 minutes in dark at 37ºC, absorbance was measured at 593 nm. Standard curve was made with ferrous sulphate and sample absorbance was calculated to determine FRAP unit in mmol Fe^{2+} per gram of dry sample (DW).

7.2.3.7. 2, 2-Azino-(3-ethyl) benzothiazoline)-6-sulfonic acid diammonium salt (ABTS) radical cation scavenging assay

The antioxidant capacity of the sample extract was conducted by ABTS assay using modified Babbar et al. (2011) method. An ABTS stable stock solution incubated in dark room temperature for 16 h was made with 7 nmol/L aqueous ABTS solution and 2.45 mmol/L potassium persulfate. Absorbance of the solution was adjusted with ethanol to 0.70±

0.02 AU at 734 nm before use. Appropriate volume sample extract was added to 3 ml of ABTS working solution with control as methanol. After incubation in dark for 30 min, absorbance was taken at 734 nm.

7.3. Results

The present study evaluated certain secondary metabolites viz., total phenol, flavonoid, tannin, and alkaloid content from different parts of *C. bicolor* Lindl., *D. heterocarpum* Wall. ex. Lindl and *E. clarkei* Rchb. f. and there *in vitro* generated plantlets. The results of secondary metabolites contents revealed difference within the parts of orchids studied and also their regenerants.

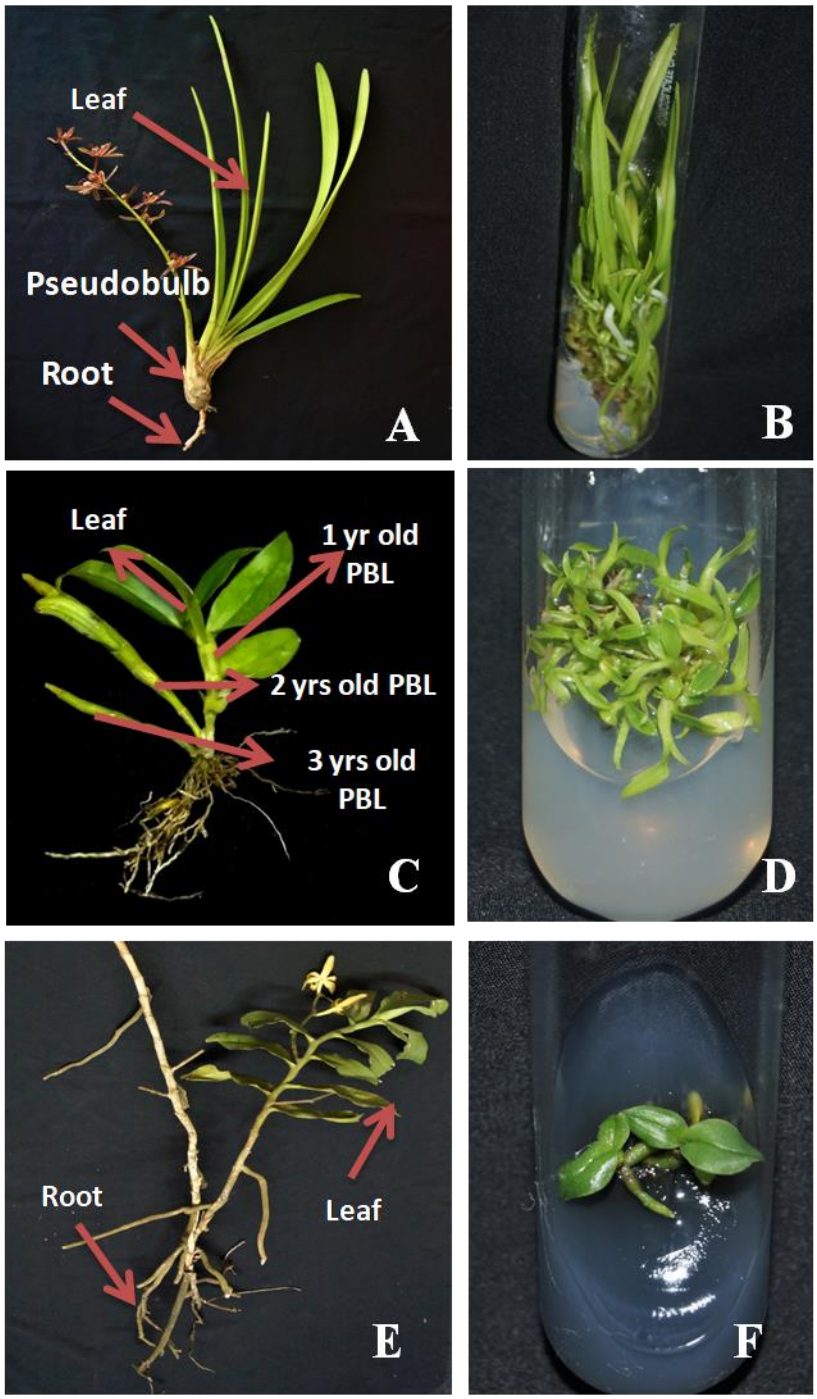

Figure 7.1: (A) *C. bicolor* Lindl. donor plant and different parts used for phytochemical analysis; (B) *In vitro* regenerants of *C. bicolor*; (C) *D. heterocarpum* Wall ex. Lindl. donor plant and different parts used for phytochemical analysis; (D) *In vitro* regenerants of *D. heterocarpum*; (E) *E. clarkei* Rchb. f. donor plant and different parts used for phytochemical analysis (F) *In vitro* regenerants of *E. clarkei*.

7.3.1. Phytochemical screening of *C. bicolor* Lindl.

In *C. bicolor* Lindl., the total phenolic concentration was found varied with highest in *in vitro* regenerants (8.67 mg GAE/g DW), followed by pseudobulb (6.18 mg GAE/g DW), leaf (5.34 mg GAE/g DW), and roots (4.47 mg GAE/g DW) (Table 7.1). Total flavonoid content from the extracts was observed much higher in *in vitro* regenerants of up to 27.73 mg QE/g DW, comparing to parts of mother plant ranging from 14.83 mg QE/ g DW in pseudobulb, 9.77 mg QE/g DW in leaf and 8.61 mg QE/g DW in root. Similar observation was also recorded for total tannin, with 11.95 mg TA/g DW in *in vitro* regenerants, 3.22 mg TA/g DW in pseudobulb, 3.06 mg TA/g DW in leaf and 2.37 mg TA/g DW in roots. The total alkaloid content was also found to be higher in *in vitro* regenerants (4.83 mg AE/g DW), followed by leaf (2.72 mg AE/g DW), pseudobulb (1.83 mg AE/g DW) and root (0.84 mg AE/g DW).

Table 7.1: Total phenolic, flavonoid, tannin and alkaloid contents quantified from different parts of donor plant and *in vitro* plantlets of *C. bicolor* Lindl.

Sample	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	Total Tannin (mg TA/g DW)	Total Alkaloids (mg AE/gDW)
Leaf	5.34 ±0.41	9.77 ±1.70	3.06 ±0.13	2.72±0.07
Pseudobulb	6.18 ±0.39	14.83 ±2.67	3.22 ±0.16	1.83±0.09
Root	4.47 ±0.32	8.61 ±0.63	2.37±0.35	0.84 ±0.19
In-vitro regenerants	8.67±0.26	27.73 ±1.09	11.95±0.56	4.83 ±0.04

Note: ±-standard error

7.3.2. Phytochemical screening of *D. heterocarpum* Wall ex. Lindl.

In *D. heterocarpum* Wall ex. Lindl., the total phenolic content was found to be highest in 3 years old pseudobulbs (15.98 mg GAE/g DW), followed by *in vitro* raised plants (14.18 mg GAE/g DW), and the least in 1 year old pseudo bulb (9.71 mg GAE/g DW) (Table 7.2). The total flavonoid of the extracts observed the highest concentration in *in vitro* grown

plants (38.38 mg QE/g DW) and comparatively 3 years old pseudobulbs (27.27±0.5 mg GAE/g DW) showed more TFC than other parts of the mother plant. Very high total tannin content was found in *in vitro* regenerated plantlets (41.48 mg TA/g DW) against significantly lower concentration in mother plant extract (highest in 3 years old psuedobulbs, 17.13 mg TA/g DW). Total alkaloid content was also highest in *in vitro* plantlets (8.78 mg AE/g DW) followed by 1 year old pseudobulb (7.57 mg AE/g DW), and least in young leaves (5.32 mg AE/g DW).

Table 7.2: Total phenolic, flavonoid, tannin and alkaloid contents quantified from different parts of donor plant and *in vitro* plantlets of *D. heterocarpum* Wall ex. Lindl.

Sample	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	Total Tannin (mg TA/g DW)	Total Alkaloids (mg AE/g DW)
Young leaf	13.93±0.3	25.2±1.4	12.64±0.6	5.32±0.05
1 yr old PBL*	9.71±0.3	11.6±0.6	12.03±0.3	7.57±0.12
2 yrs old PBL*	12.51±0.2	18.6±1.2	9.47±0.2	5.51±0.06
3 yrs old PBL*	15.98±0.5	27.27±0.5	17.13±0.7	6.83±0.06
In vitro regenerants	14.18±0.9	38.38±1.9	41.48±0.6	8.78±0.18

Note: * PBL-Pseudobulb, ±-standard error

7.3.3. Phytochemical screening of *E. clarkei* Rchb. f.

In case of *E. clarkei* Rchb. f. (table 7.3), total phenolic content in *in vitro* regenerants was higher with 13.93 mg GAE/g DW, leaf with 12.51 mg GAE/g DW and root 9.71 mg GAE/g DW. The total flavonoid content was also higher with 27.27 mg QE/g DW in *in vitro* regenerants, 18.6 mg QE/g DW in leaf and 11.6 mg QE/g DW in root. The total tannin was observed higher in *in vitro* regenerants (27.27±0.51 mg TA/g DW) followed by root (12.64±0.16mg TA/g DW) and leaf (10.03±0.31mg TA/g DW). The total alkaloids estimated show higher in *in vitro* regenerants (7.57 mg AE/g DW) followed by leaf (5.51 mg AE/g DW) and root (5.32 mg AE/g DW).

Table 7.3: Total phenolic, flavonoid, tannin and alkaloid contents quantified from different parts of donor plant and *in vitro* plantlets of *E. clarkei* Rchb. f.

Sample	TPC (mgGAE/g DW)	TFC (mg QE/g DW)	Total Tannin (mg TA/g DW)	Total Alkaloids (mg AE/g DW)
Leaf	12.51±0.24	11.6±0.61	10.03±0.31	5.51±0.06
Root	9.71 ±0.31	18.6±1.02	12.64±0.16	5.32±0.05
In vitro regenerants	13.93±0.32	27.27±0.51	19.47±0.24	7.57±0.12

Note: ±-standard error

7.3.4. Antioxidant capacities

The present study also determined the antioxidant capacity of the different parts of the three orchids donor plants and regenerates using DPPH, FRAP, and ABTS assays. Scavenging activity of the sample extracts by DPPH and FRAP assay was determined with lower absorbance of the reaction as higher scavenging activity of free radical. Antioxidant activity of the extracts was evaluated using the inhibition percentages to calculate IC_{50} value against Trolox as standard. Where, lower IC50 value denotes higher inhibition capacity of free radicals of the sample.

7.3.4.1. Scavenging activity in *Cymbidium bicolor* Lindl.

The inhibition percentage of extracts concentrations of *C. bicolor* using DPPH and ABTS methods were used to estimate the radical scavenging activity. The percentage of inhibition of the extracts was observed to be higher *in vitro* plantlets followed by pseudobulb, leaf and roots in both DPPH and ABTS method as shown in figure 7.2 and figure 7.3.

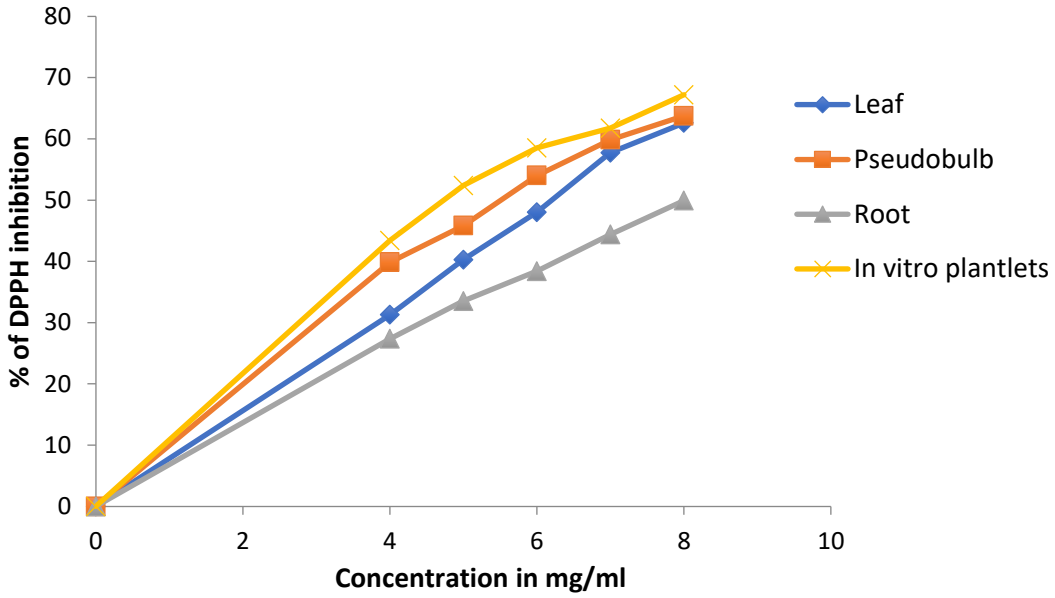

Figure 7.2: Scavenging activities of different parts of *C. bicolor* Lindl. and *in vitro* plantlets by DPPH assay

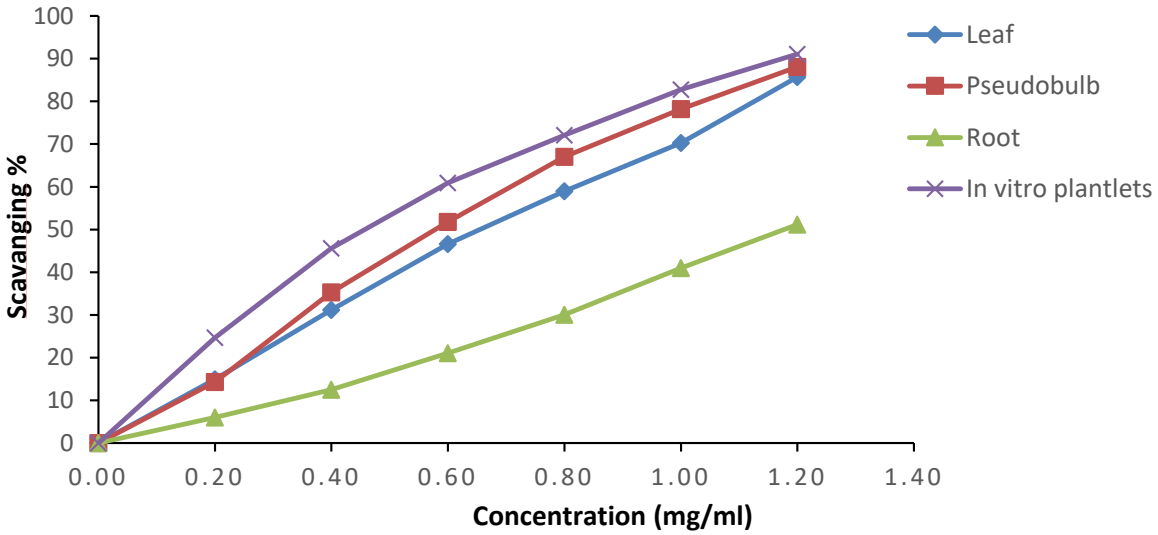

Figure 7.3: Scavenging activities of different parts of *C. bicolor* Lindl. and *in vitro* plantlets by ABTS assay

Table 7.4: Antioxidant activities of *C. bicolor* Lindl. extract in DPPH, ABTS and FRAP methods

Sample	DPPH IC_{50} value (mg/ml)	ABTS IC_{50} (mg/ml)	FRAP (mM Fe^{2+}/g)
Leaf	6.49 ±0.02	0.87 ±0.02	44.29 ±0.20
Pseudobulb	5.82 ±0.08	0.61 ±0.05	55.90 ±3.59
Root	8.11 ±0.06	1.01±0.03	35.71 ±7.69
In vitro regenerants	4.22 ±0.03	0.42 ±0.09	74.20 ±9.02
Trolox (1mg/ml)	0.01±0.04	0.008±0.0001	73.20±0.06

As given in table 7.4, the IC_{50} values were calculated from the inhibition percentage of DPPH to evaluate the antioxidant activity of the extracts which showed 8.11 ± 0.06 mg/ml in root, 6.49 ±0.02 mg/ml in leaf, 5.82 ± 0.08 mg/ml in pseudobulb and 4.22 ± 0.03 mg/ml in *in vitro* plantlets. Similarly, in ABTS method, IC_{50} ranged from 1.01± 0.03 mg/ml in roots to 0.87 ± 0.02 mg/ml in leaf, 0.61 ± 0.05 mg/ml in pseudobulb and 0.42 ± 0.09 mg/ml in *in vitro* plantlets. The ferric reducing assay revealed 74.20 ±9.02 mM Fe^{2+}/g in *in vitro* plantlets, followed by 44.29 ±.2 mM Fe^{2+}/g in leaf, 55.90 ±3.59 mM Fe^{2+}/g in pseudobulb and 35.51 ±7.69 mM Fe^{2+}/g in root.

7.3.4.2. Scavenging activity in *D. heterocarpum* Wall ex. Lindl.

The inhibition percentage of the extracts of *D. heterocarpum* using DPPH and ABTS method were taken to calculate the radical scavenging activity, as shown in Figure 7.4 and 7.5. In DPPH assay, the inhibition percentage was higher in 3 years old Pseudobulb followed by *in vitro* plantlets, leaf, 2 years old Pseudobulb and least in 1 year old Pseudobulb (Figure 7.4). The ABTS assay also revealed higher inhibition percentage in 3yrs old PBL followed by leaf, *in vitro* plantlets, 2 years old Pseudobulb and least in 1 year old Pseudobulb (Fig.7.5). The antioxidant activity of the extracts was evaluated using inhibition percentages of DPPH revealed IC_{50} of 1 year old Pseudobulb with 2.18±0.02 mg/ml, 2 years old Pseudobulb with 1.93±0.05 mg/ml, leaf with 1.41±0.01 mg/ml, in vitro plantlets with 1.39±0.01 mg/ml and 3

years old Pseudobulb with 1.18 mg/ml. Using the ABTS method, IC_{50} of 3 years old Pseudobulb had higher antioxidant activity with 0.24 ±0.02 mg/ml, leaf with 0.37±0.03 mg/ml, *in vitro* plantlets with 0.42±0.03 mg/ml, 2 years old Pseudobulb with 0.43±0.06 mg/ml and least in 1 year old Pseudobulb with 0.610 mg/ml. The ferric reducing assay revealed, 3 years old Pseudobulb with 290.15±1.7mM Fe^{2+}/g, *in vitro* plantlets with 165.09±1.4 mMFe^{2+}/g, leaf with 164.65±2.9 mM Fe^{2+}/g, 2 years old pseudobulbs with 107.76 mM Fe^{2+}/g and 1 year old pseudobulbs with 94.99 mM Fe^{2+}/g.

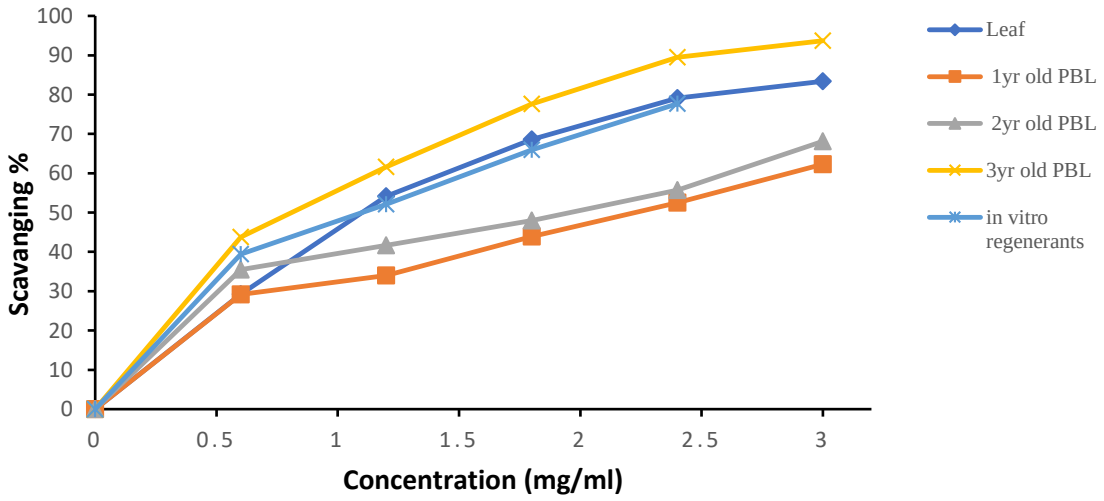

Figure 7.4: Scavenging activities of different parts of *D. heterocarpum* Wall ex. Lindl. and *in vitro* plantlets by DPPH assay. * PBL- pseudobulb

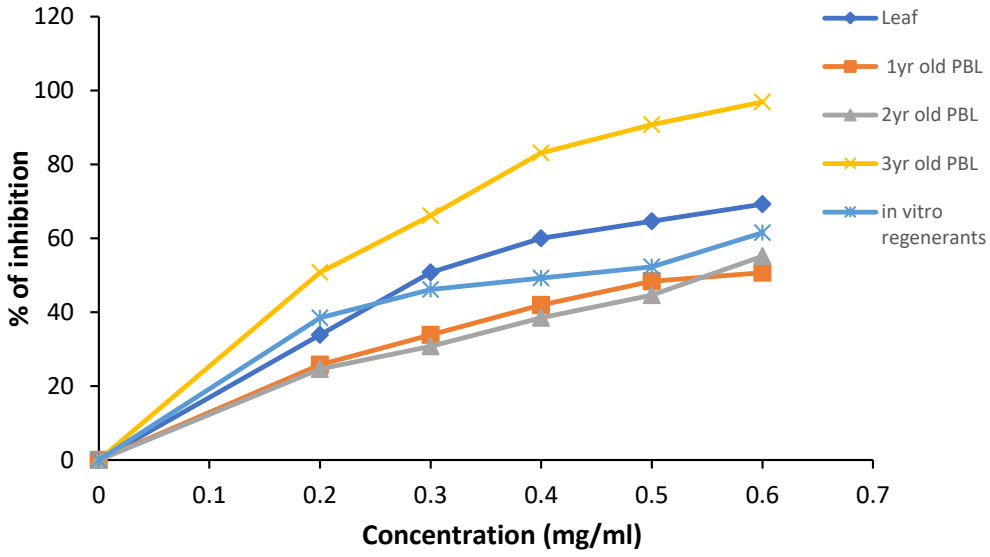

Figure 7.5: Scavenging activities of different parts of *D. heterocarpum* Wall ex. Lindl. and *in vitro* plantlets by ABTS assay. * PBL- pseudobulb

Table 7.5: Antioxidant activities of *D. heterocarpum* Wall ex. Lindl. extract in DPPH, ABTS and FRAP methods

Sample	DPPH IC_{50} value (mg/ml)	ABTS IC_{50} (mg/ml)	FRAP (mM Fe^{2+}/g)
Leaf	1.41±0.01	0.37±0.03	164.65±2.9
1 yr old PBL	2.18±0.02	0.61±0.08	94.99±1.6
2 yrs old PBL	1.93±0.05	0.43±0.06	107.76±1.1
3 yrs old PBL	1.18±0.02	0.24±0.02	290.15±1.7
In vitro regenerants	1.39±0.01	0.42±0.03	165.09±1.4
Trolox	0.01±0.04	0.008±0.0001	7.32±0.06

7.3.4.3. Scavenging activity in *E. clarkei* Rchb. f.

The inhibition percentage of extracts concentrations of *E. clarkei* using DPPH (Fig. 7.6) and ABTS (Fig. 7.7) methods were used to estimate the radical scavenging activity. In both DPPH and ABTS assay, *in vitro* plantlets extract showed higher inhibition percentage followed by root and leaf.

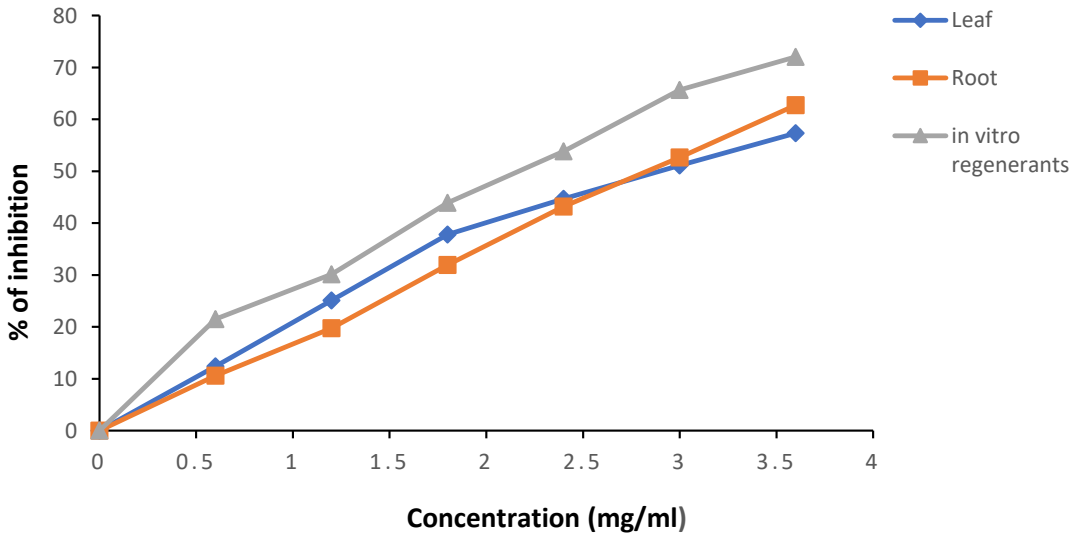

Figure 7.6: Scavenging activities of Leaf and roots of donor plant and *in vitro* plantlets of *E. clarkei* Rchb. f. by DPPH assay

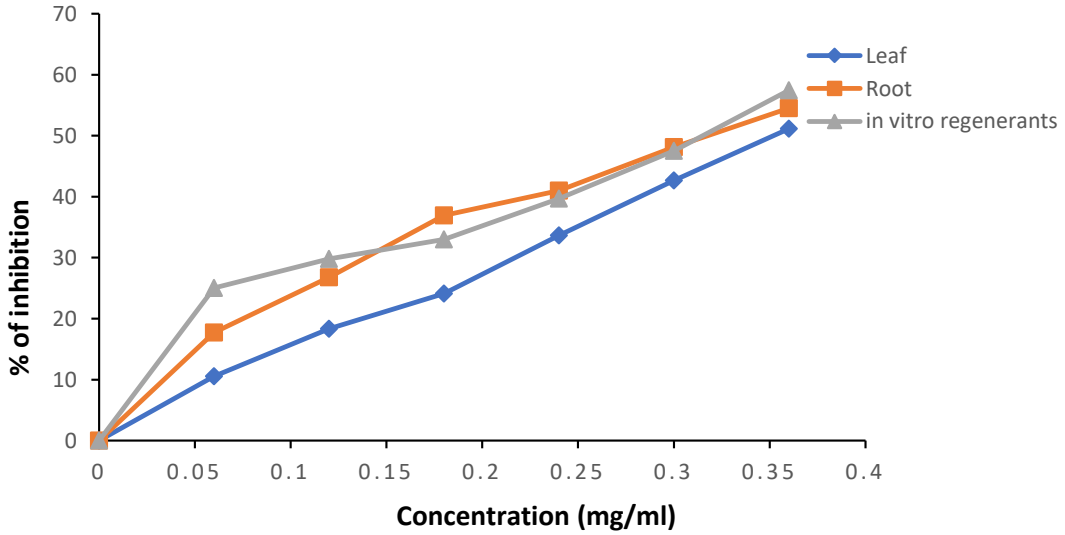

Figure 7.7: Scavenging activities of Leaf and roots of donor plant and *in vitro* plantlets of *E. clarkei* Rchb. f. by ABTS assay

The antioxidant activity evaluated (Table 7.6) using inhibition percentages of DPPH showed IC_{50} of 3.07 ±0.15 mg/ml in roots, 2.74 ±0.06 mg/ml in leaf and 2.27 ±0.01 mg/ml in *in vitro* plantlets. The ABTS method also revealed similar results with highest IC_{50} value of 0.35 ± 0.006 mg/ml in roots followed by 0.32 ± 0.009 mg/ml in leaf and 0.31 ± 0.003 mg/ml in *in vitro* plantlets. The ferric reducing assay showed concentrations ranging from 120.36 ± 16.76mM Fe^{2+}/g in *in vitro* plantlets to 92.71 ± 14.78mM Fe^{2+}/g in leaf and 73.50 ± 19.6 mM Fe^{2+}/g in roots.

Table 7.6: Antioxidant activities of *E. clarkei* Rchb. f. extract in DPPH, ABTS and FRAP methods

Sample	**DPPH** **IC_{50} value (mg/ml)**	**ABTS** **IC_{50} (mg/ml)**	**FRAP** **(mM Fe^{2+}/g)**
Leaf	3.07 ±0.15	0.35 ± 0.006	73.50 ± 19.6
Root	2.74 ±0.06	0.32 ± 0.009	92.71 ± 14.78
In vitro regenerants	2.27 ±0.01	0.31 ± 0.003	120.36 ± 16.76
Trolox (1mg/ml)	0.01±0.04	0.008±0.0001	7.32± 0.06

7.3.5. Correlation study of antioxidant activity with Total phenolic and flavonoid content

Analysis of correlation between reducing capacity of the sample extracts in DPPH, ABTS and FRAP methods of the three orchid species to that of total phenol and flavonoid was studied to evaluate its role in antioxidant activity. In *C. bicolor*, the total phenolic content showed significant linear relationship with DPPH, ABTS and FRAP scavenging assay. The strongest correlation was observed between TPC and FRAP ($y = 0.107x+0.513$, $R^2=0.983$) followed by TPC and DPPH ($y = -1.083+12.84$, $R^2=0.930$) and TPC and ABTS ($y = -6.564x+10.94$, $R^2=0.913$) (Table 7.7). Similar results of correlation with TFC was revealed and significant relation between TFC and FRAP ($y = 0.510x+11.61$, $R^2=0.948$) followed by TFC and DPPH ($y = -4.982x+45.92$, $R^2=0.841$) and TFC and ABTS ($y = -30.91x+37.27$, $R^2=0.866$) (Table 7.7).

Table 7.7: Linear correlation of antioxidant activity with total phenol and flavonoid content of *C. bicolor* Lindl.

	DPPH	**ABTS**	**FRAP**
TPC	$y = -1.083+12.84$, ($R^2=0.930$)	$y = -6.564x+10.94$ ($R^2=0.913$)	$y = 0.107x+0.513$ ($R^2=0.983$)
TFC	$y = -4.982x+45.92$ ($R^2=0.841$)	$y = -30.91x+37.27$ ($R^2=0.866$)	$y = 0.510x+11.61$ ($R^2=0.948$)

Table 7.8: Linear correlation of antioxidant activity with total phenol and flavonoid content of *D. heterocarpum*

	DPPH	ABTS	FRAP
TPC	y = -5.383x+21.97, (R^2=0.928)	y =-0.017x+20.29 (R^2=0.933)	y =0.026x+8.902 (R^2=0.765)
TFC	y =-19.44x+55.66 (R^2=0.659)	y =-38.95x+40.34 (R^2=0.323)	y =0.055x+15.04 (R^2=0.263)

In *D. heterocarpum*, the correlation analysis of total phenol and flavonoid with the scavenging assays showed TPC to have a significant linear relationship with the DPPH and ABTS scavenging assays. The strongest correlation was found between TPC and ABTS (y=-0.017x+20.29, R2=0.933); TPC and DPPH (y =- 5.383x+21.97, R2=0.928) and least in TPC and FRAP (y =0.026x+8.902, R^2=0.765) (Table 7.8). Total flavonoids in relation to scavenging activity as measured by DPPH, ABTS, and FRAP, on the other hand, revealed that flavonoid concentration had no/less influence on antioxidant activity.

Table 7.9: Linear correlation of antioxidant activity with total phenol and flavonoid content of *E. clarkei* Rchb. f.

	DPPH	ABTS	FRAP
TPC	y = -5.122x+25.84, (R^2=0.919)	y =-102.6x+45.59 (R^2=0.991)	y =0.087x+3.706 (R^2=0.918)
TFC	y =-19.44x+55.66 (R^2=0.998)	y =-355.1x+135.1 (R^2=0.887)	y =0.333x-12.64 (R^2=0.998)

In case of *E. clarkei*, the Analysis of correlation established between reducing capacity to that of total phenol and flavonoid content both revealed a strong linear relationship. Correlation of the total phenolic content was highest in TPC and ABTS (y =-102.6x+45.59, R^2=0.991) followed by TPC and DPPH (y = -5.122x+25.84, R^2=0.919) and TPC and FRAP (y =0.087x+3.706, R^2=0.918). The total flavonoids in relation to scavenging activity showed strongest relation between TFC and DPPH (y =-19.44x+55.66, R^2=0.998), TFC and FRAP

(y =0.333x-12.64, R^2=0.998) and least in TFC and ABTS (y =0.333x-12.64, R^2=0.998) (Table 7.9).

7.4. Discussion

Secondary metabolites found in medicinal orchids are extremely significant for pharmacological uses with each part of the orchid serving a distinct ethnomedical function (Gantait et al., 2021). As a result, identifying the appropriate developmental stage and parts of the plant in relation to the photochemical content can aid in exploiting its nutraceutical and medicinal aspect. In addition, employing variety of *in vitro* techniques can fulfil the gap between the demands for these species secondary metabolites and increasing their natural population. The present study evaluated certain secondary metabolites viz., total phenol, flavonoid, tannin, and alkaloid content from different parts of the plant of *C. bicolor* (Table 7.1), *D. heterocarpum* (Table 7.2) and *E. clarkei* (Table 7.3) and their corresponding *in vitro* generated plantlets. Results revealed that the secondary metabolites content varied significantly in different parts of the mother plant and *in vitro* plantlets.

In *C. bicolor*, *in vitro* regenerants exhibited higher TPC (8.67±0.26 mg GAE/g DW), TFC (27.73 ±1.09 mg QE/g DW), TTC (11.95 ±0.56 mg TA/g DW) and TAC (4.83 ±0.04 mg AE/g DW) comparing to the extracts of leaf, pseudobulb and roots of the mother plant (Table 7.1). Similarly, *in vitro* regenerants of *E. clarkei* had an increase in TPC (13.93±0.33 mg GAE/g DW), TFC (27.27±0.51 mg QE/g DW), TTC (19.47±0.24±0.25 mg TA/g DW) and TAC (7.57±0.12 mg AE/g DW) to that of leaf and root of mother plant (Table 7.3). Similar results of higher content of TPC, TFC, TTC and TAC in *in vitro* raised plantlet *Dendrobium crepidatum* (Bhattacharyya et al., 2016) and *Malaxis wallichii* (Bose et al., 2017) were observed. The levels of secondary metabolites in the *in vitro* cultivated plantlets have been demonstrated to considerably elevate when cytokinins were used during propagation (Bhattacharya et al., 2015b). Therefore, this effect might have attributed to the

higher deposition of total phenol, flavonoid, tannin and alkaloids in the *in vitro* propagated plantlets as compared to the various parts of the mother plant in the current study. In case of *D. heterocarpum*, it was observed that 3 years old pseudobulb contained higher TPC (15.98±0.5mg GAE/g DW) compared to *in vitro* regenerants (14.18±0.9mg GAE/g DW), young leaf (13.93±0.3 mg GAE/g DW)**,** 2 yrs old PBL (12.51±0.2 mg GAE/g DW) and 1 yr old PBL (9.71±0.3 mg GAE/g D/W. It is possible that variations in hormone levels, certain metabolic alterations, and a variety of endogenous physiological processes may be responsible for the variances in phenolic and flavonoid concentrations in different plant systems. (Baskaran et al., 2014). However, *in vitro* regenerants exhibited a significant increase in TFC (38.38±1.9mg QE/g DW), TTC (41.48±0.6mg TA/g DW) and TAC (8.78±0.18mg AE/g DW) to that of extracts of mother plant parts.

Due to the complex reactive nature of phytochemicals, determining antioxidant activity of extracts necessitates the use of multiple antioxidant techniques, as findings vary between tests (Chanda et al., 2013). As a result, the DPPH, FRAP, and ABTS technique were used in this study to evaluate scavenging activities of the methanolic extracts from the three orchids. In *C. bicolor*, the IC_{50} value calculated from the scavenging percentage revealed higher scavenging activity of *in vitro* regenerants in DPPH (4.22 ± 0.03mg/ml), ABTS (0.42 ± 0.09 mg/ml) and FRAP (74.30 ±9.02 mM Fe^{2+}/g) assays. IC_{50} value in *D. heterocarpum* exhibited higher activity in 3 yrs old PBL in DPPH (1.18±0.02mg/ml), ABTS (0.24±0.02 mg/ml) and FRAP (290.15±1.7 mM Fe^{2+}/g). In case of *E. clarkei*, IC_{50} value was recorded higher for in *vitro* regenerants in DPPH (2.27 ±0.01 mg/ml), ABTS (0.31 ± 0.003 mg/ml) and FRAP (120.36 ± 16.76 mM Fe^{2+}/g) assays. Antioxidants are important in protecting the human body against damage by reactive oxygen species molecule, as it prevents the oxidation of other molecules such as free radicals or reactive oxygen species (Tabaraki and Nateghi, 2011). Disorders like ageing, degenerative diseases and cancer

related to oxidative stress treatment have been reported to be effective with plant base natural antioxidants.

In the present study, analysis of reducing capacity to that of total phenol and flavonoid of the three orchids was done to establish its correlation. In *C. bicolor,* strong linear correlation of TPC with DPPH (y = -5.122x+25.84, R2=0.919), ABTS (y =102.6x+45.59, R2=0.991) and FRAP (y =0.087x+3.706, R2=0.918) was observed. Correlation between TFC with scavenging activity by DPPH, ABTS, and FRAP was found strongest with FRAP (y =0.333x-12.64, R^2=0.998). However, in *D. heterocarpum*, the total phenolic content was shown to have a significant linear relationship with the DPPH and ABTS scavenging assays. The strongest correlation was found between TPC and ABTS (y=-0.017x+20.29, R2=0.933); TPC and DPPH (y =- 5.383x+21.97, R2=0.928) (Table 7.8). Total flavonoids in relation to scavenging activity as measured by DPPH, ABTS, and FRAP, on the other hand, revealed that flavonoid concentration had less influence on antioxidant activity. In case of *E. clarkei*, TPC and TFC exhibited high linear correlation to the scavenging activity of DPPH, ABTS and FRAP. The strongest correlation was observed between TPC and ABTS (y =-102.6x+45.59, R^2=0.991), TFC and DPPH (y =-19.44x+55.66, R^2=0.998) and TFC and FRAP (y =0.333x-12.64, R^2=0.998). Many medicinal plants are known for its natural antioxidants, which have been linked to the content of total phenol and flavonoid (Tee et al., 2015). Association between higher antioxidant activities to their total phenolic compounds have been reported in many medicinal plants (Rakholiya et al., 2015). The findings confirm the existence of phyto-bioactive chemicals having antioxidant properties in *C. bicolor*, *D. heterocarpum* and *E. clarkei* and a link between increased of total phenol and flavonoid content with antioxidant activity of plant extracts.

7.5. Summary and Conclusion

In the present study, a comparative quantitative analysis of secondary metabolites viz. total phenol, flavonoid, tannin, alkaloids and antioxidant activity of different plant parts of *C. bicolor*, *D. heterocarpum*, *E. Clarkei* and their *in vitro* regenerants were done. Quantitative phytochemical estimation from the different methanolic extracts revealed the presence of different biologically important secondary metabolites in significant concentrations. Also, the antioxidant properties of the extracts were studied using DPPH, ABTS and FRAP methods which confirmed the presence of scavenging activity in the selected plant samples. Total phenolic and flavonoids which are considered to be responsible for the antioxidant properties in medicinal plants was also established through correlation of the TPC and TFC with the IC_{50} of the plant extracts. In addition, the potential plant parts and growth age at which the plant organ can be taken for separation of these metabolites instead of the entire plant was determined. The issue over exploitation in its natural habitat of such medicinal orchids has been a topic of concern. Thus, the finding in the present study where *in vitro* regenerants were found to exhibit higher secondary metabolites than their mother plant can be a viable alternative to large-scale production of medicinally important orchids. This will, in one hand relieve the pressure on the natural population and will aid in species conservation and on the other hand it will resolve the problem of contamination by different microbes from the naturally grown plants.

A part of the chapter was published as:

Longchar, T.B., Deb, C.R., 2021. Comparative analysis of nutraceutical potential phytochemicals and antioxidant activities in different parts of wild and in vitro re generated plantlets of *Dendrobium heterocarpum* Wall. ex Lindl.: a medicinal orchid. Journal of *Pharmacognosy and Phytochemistry*. 10: 331–336. doi.org/10.22271/phyto.2021.v10.i4d.14169s

CHAPTER 8

Summary and future scope

Orchids are known for their beautiful flowers, medicinal value, and food-flavouring products, which make them a resource of great economic importance in the global floriculture, horticulture and food industries. With an increase in the world population relying on traditional medicine, plant-based healthcare products, and cosmetics, thorough research in orchids for their medicinal value will help to exploit their uses to the fullest. However, the habitat and population of orchids are at threat because of human activities, climate cha,nge and unsupervised collection from the wild. So, a sustainable approach along with conservation through both *ex-situ* and *in-situ* must be employed for its conservation.

In the present study, three orchids were found in Nagaland, India, namely *Cymbidium bicolor* Lindl., *Dendrobium heterocarpum* Wall ex. Lindl. and *Esmeralda clarkei* Rchb., were selected to develop optimized *in vitro* protocol for each species, to assess their genetic fidelity, secondary metabolites, and antioxidant capacities of the regenerants. The selected species have ethnomedicinal and horticultural importance and fall under the RET status. The species identification of the collected species was authenticated with both morphological characteristics and molecular tools. For molecular identification, loci from the plastid genome (*matk*) and nuclear region (ITS) were used. The sequences were submitted to NCBI GenBank, after blast hit was checked using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/) at NCBI GenBank.

A comprehensive study on the effect of the seed development stage, nutrient medium, organic carbon source, plant growth regulators, and acclimatization protocol was studied to develop optimum and efficient *in vitro* protocols for the selected orchid species.

In *C. bicolor*, green seed pods of 8-10 MAP were used to study the relation of different developmental stages of immature seeds and their response to germination. The seeds of 11 MAP showed an optimum germination response of up to ~78%. Three nutrient media viz. MS, Mitra et al. and Knudson 'C' were used for asymbiotic seed germination, plant regeneration , as well as plantlet multiplication and best germination was registered on MS medium (~74%). The influence of organic carbon (sucrose, fructose and glucose, 0-3%) fortified in the culture medium on seed germination showed that in *C. bicolor* germination was favoured in sucrose 3% (~74%), sucrose 2% (~74%), fructose 3% (~74%) and fructose 2% (~73%) in MS media. The addition of PGRs in the culture medium was found to show significant differences in the germination time and morphological response of the immature seeds cultured. On MS medium fortified with sucrose 3% + IAA (6 µM/L) and sucrose 3% + IAA (9 µM/L) supported the highest germination rate of ~95%. The plantlet development from protocorms was optimum on MS medium (sucrose/fructose, 2-3%) fortified with BAP+NAA (9+3-9 µM/L) and BAP+NAA (6+6 µM/L) and proliferation of shoot was optimum on MS medium fortified with sucrose 3% and BAP+NAA (6+3 µM/L), followed by BAP+NAA (9+6 µM/L), where an average multiple shoots of 7 and 6 were produced per protocorm. The objective of the current study was also to reduce the protocol's cost by substituting agar with low cost substrata for the plantlet's acclimatization. An optimum acclimatization condition was provided with the combination of CH+WB+C+WB+CB where the survival rate of *in vitro* plantlets were as high as ~97%.

In the case of *D. heterocarpum*, green seed pods of 5-8 MAP were used to assess the relationship between seed age and germination, where ~95% germination rate was recorded

on MS medium containing sucrose (3%) from green pods of 8 MAP. Of the three organic carbon sources (0-3%) exogenously added, sucrose (~85-62%) in was found to be optimum. On nutrient media fortified with sucrose (3%) and PGRs, optimum germination of ~95.33% recorded on MS medium fortified with NAA (9 µM/L). Plantlet development from protocorms was optimum on MS medium enriched with sucrose (3%) and KN+NAA (3+12 µM/L). The combination of coconut husk and wood bark was determined to be the most effective substrata for acclimatization in *D.* heterocarpum with an ~87% success rate for in vitro regenerants.

Green seed pods of 10-11 MAP were taken for optimization of *in vitro* protocol for *E. clarkei,* where optimum germination was achieved with green pods of 10 MAP, with germination rates of 68% on ½ MS strength medium (sucrose 3%). Amongst the carbon sources, 3% sucrose in MS medium was found to be significantly effective. However, browning of media surface in contact with the germinated seeds/protocorms was observed in *E. clarkei* in comparison to protocorm development from seeds of *C. bicolor* and *D. heterocarpum* where the same was not observed. Therefore, ½ strength MS medium (sucrose 3%) with or without AC (0.5% and 0.1%) were supplemented with different concentrations of BAP, KN, NAA and IAA (3-6 µM/L) singly or in combination were employed to study the response in germination and morphological development. Half-strength MS medium (sucrose 3%) containing 0.5% AC fortified with KN+NAA (3+3 and 6+6 µM/L) and NAA3 recorded the shortest germination time period of ~21 days and highest germination rate of 88.84% was recorded in with BAP+NAA (3+3 µM/L). The optimum condition for plant regeneration from protocorms was observed in medium containing KN+NAA (9+9 µM/L) and KN+NAA (6+6 µM/L) and shoot multiplication was supported better in combination of BAP+NAA (12+6 µM/L), where up to an average of 10.59 shoots per protocorm was formed. Acclimatization of *in vitro* regenerants of *E. clarkei* showed optimum response in

substrata mixture of CH+WB (1:1) containing ¼ MS liquid medium with survival rate up to 92.88%.

The current research also aimed to assess the genetic fidelity of the *in vitro* regenerants of the three selected species using RAPD, DAMD and SCoT markers. For all the species 15 RAPD primers, 10 DAMD primers and 10 SCoT primers each were screened. In *C. bicolor*, RAPD showed the highest monomorphic degree of 98.18%, followed by the least polymorphism degree with 1.81%, SCoT with a monomorphism of 96.77%, and 3.22% polymorphism and DAMD markers showed a monomorphism of 94.29% and 5.71% polymorphism. A total monomorphism of 96.57%, 3.43% polymorphism, and Jaccard's coefficient ranged from 0.98-1.00 was revealed. In the case of *D. heterocarpum*, SCoT showed the highest monomorphism of 98% and the least polymorphism degree with 2%, RAPD with monomorphism of 96.38% and 3.61% polymorphism and DAMD markers showed a monomorphism of 95.08% and 4.92% polymorphism. The Jaccard's coefficient of the three markers ranged from 0.96 to 1.00 with a total monomorphism of 96.89% and 3.11% polymorphism. For *E. clarkei,* SCoT showed the highest monomorphic degree of 98.39% and the least polymorphism degree of 1.61%, followed by RAPD with monomorphic percentage of 98.04% and 1.96% polymorphism and DAMD markers showed a monomorphic percentage of 92.54% and 7.46% polymorphism. The Jaccard's coefficient of the three markers ranged from 0.96 to 1.00 with a total monomorphism of 96.33% and 3.69% polymorphism with an average band of 6.23. The low total polymorphic percent between the plantlets of *in vitro* regenerants and donor mother plants reveals that the optimised *in vitro* protocols for the orchid species supports the production of genetically stable regenerants.

Orchids have long been used in ethno-medicinal practices and research on their phytochemicals has been largely focused on over the years. The present study evaluated

a comparative assessment of some phytochemical and antioxidant capacities of the different plant parts of the selected species and the *in vitro* regenerants. In *C. bicolor, in vitro* regenerants exhibited higher TPC (8.67mg GAE/g DW), TFC (27.73 mg QE/g DW), TTC (11.95 mg TA/g DW) and TAC (4.83 mg AE/g DW) comparing to the extracts of leaf, pseudobulb and roots of the mother plant. Similarly, in *in vitro* regenerants of *E. clarkei* an increase in TPC (13.93 mg GAE/g DW), TFC (27.27 mg QE/g DW), TTC (19.47 mg TA/g DW) and TAC (7.57 mg AE/g DW) to that of leaf and root of mother plant was observed. In case of *D. heterocarpum*, it was found that 3 years old pseudobulb contained higher TPC (15.98 nmg GAE/g DW) compared to *in vitro* regenerants, young leaf**,** 2 years old pseudobulb and 1 year old pseudobulb. However, *in vitro* regenerants exhibited a significant increase in TFC (38.38 QE/g DW), TTC (41.48 TA/g DW) and TAC (8.78 AE/g DW) to that of extracts of mother plant parts.

Antioxidant capacity of the extracts of the selected orchid species and their *in vitro* regenerants were determined using the DPPH, FRAP, and ABTS methods. In *C. bicolor*, the IC_{50} value calculated from the scavenging percentage revealed higher scavenging activity of *in vitro* regenerants in DPPH (4.22 mg/ml), ABTS (0.42 mg/ml) and FRAP (74.30 mM Fe^{2+}/g) assays. In *D. heterocarpum* IC_{50} value exhibited higher activity in 3 years old pseudobulb in DPPH (1.18 mg/ml), ABTS (0.24 mg/ml) and FRAP (290.15 mM Fe^{2+}/g). In case of *E. clarkei*, IC_{50} value was recorded higher for in *vitro* regenerants in DPPH (2.27 mg/ml), ABTS (0.31 mg/ml) and FRAP (120.36 mM Fe^{2+}/g) assays.

In the current research, the barcoding of *C. bicolor*, *D. heterocarpum*, *E. Clarkei* can be used in future investigations of evolutionary and genetic links of the native species. Successful *in vitro* protocol for asymbiotic seed germination, plantlet production and multiplication of the selected orchids can be used to generate genetically stable plants for commercialization and conservation measures. Further research on the development of

plantlets using explants of different parts of the orchid species needs to be studied. The differences in secondary metabolite content in the different parts of plants from selected orchid species can be utilized to extract only the plant parts with higher phytochemical contents than the whole plants. Moreover, the study also revealed that optimization of *in vitro* protocol of the orchid species can result in an increase of secondary metabolites in the *in vitro* regenerated plantlets. This information can be used as a base for further studies to isolate and characterize important compounds with nutraceutical and pharmaceutical properties.

References

Abraham, S., Augustine, J., & Thomas, T. D. 2012. Asymbiotic seed germination and in vitro conservation of Coelogyne nervosa, a rich and endemic orchid to Western Ghats. Physiology and Molecular Biology of Plants, 18(3), 245-251.

Abdul Ghani, A.K. & Harris, H., 1992. Plantlet formation from young leaves of Mokara. Lindleyana 7, 11–12.

Acemi, A., & Ozen, F. 2019. Optimization of in vitro asymbiotic seed germination protocol for Serapias vomeracea. The EuroBiotech Journal, 3 (3): 143-151. DOI: 10.2478/ebtj-2019-0017

Adhikari, H., & Pant, B. 2019. Effect of 6-benzylaminopurine and α-naphthalene acetic acid hormonal supplements on the in vitro seed germination and seedling development of orchid Otochilus albus Lindl. African Journal of Biotechnology, 18(22), 472-477.

Agarwal, M., Shrivastava, N., & Padh, H. 2008. Advances in molecular marker techniques and their applications in plant sciences. Plant cell reports, 27(4), 617-631.

Aghayeva, P., Cozzolino, S., Cafasso, D., Ali-Zade, V., Fineschi, S., & Aghayeva, D. 2021. DNA barcoding of native Caucasus herbal plants: potentials and limitations in complex groups and implications for phylogeographic patterns. Biodiversity data journal, 9.

Al-Zahim, M. A., Ford-Lloyd, B. V., & Newbury, H. J. 1999. Detection of somaclonal variation in garlic (Allium sativum L.) using RAPD and cytological analysis. Plant Cell Reports, 18(6), 473-477.

Amom, T., & Nongdam, P. 2017. The use of molecular marker methods in plants: a review. International Journal of Current Research and Review, 9(17), 1-7.

Anvarkhah, S., Khajeh-Hosseini, M., Rashed-Mohassel, M. H., Panah, A. D. E., & Hashemi, H. 2013. Identification of three species of genus Allium using DNA barcoding. International Journal of Agriculture and Crop Sciences, 5(11), 1195.

Arditti, J. 1967. Factors affecting the germination of orchid seeds. The Botanical Review, 33(1), 1-97.

Arditti J. 1985. Plant pioneers. American Scientist. 73: 510.

Arditti, J. 1990. Lewis Knudson:(1884-1958): his science, his times, his legacy. American Orchids Society

Arditti, J., & Ernst, R. 1993. Micropropagation of orchids. John Willey & Sons. Inc., NY.

Arditti, J., & Krikorian, A. D. 1996. Orchid micropropagation: the path from laboratory to commercialization and an account of several unappreciated investigators. Botanical Journal of the Linnean Society, 122(3), 183-241.

Ashroff, S. M., & Bandara, N. L. 2021. DNA barcoding as a tool for conserving endemic orchids in Sri Lanka.

Aziz, N. A. A., Ahmad, M. I., & Naim, D. M. 2015. Molecular DNA identification of medicinal plants used by traditional healers in Malaysia. Genetics and Molecular Research, 14(4), 15937-15947.

Azman, A. S., Mhiri, C., & Tam, S. 2014. Transposable elements and the detection of somaclonal variation in plant tissue culture: a review. Malaysian Applied Biology, 43(1), 1-12.

Babbar, N., Oberoi, H. S., Uppal, D. S., & Patil, R. T. 2011. Total phenolic content and antioxidant capacity of extracts obtained from six important fruit residues. Food research international, 44(1), 391-396.

Bairu, M. W., Aremu, A. O., & Van Staden, J. 2011. Somaclonal variation in plants: causes and detection methods. Plant Growth Regulation, 63(2), 147-173.

Bajaj, Y. P. S. 1990. Somaclonal variation—origin, induction, cryopreservation, and implications in plant breeding. In Somaclonal variation in crop improvement I. Springer, Berlin, Heidelberg.3-48.

Baker, A., Kaviani, B., Nematzadeh, G., &Negahdar, N. 2014. Micropropagation of Orchis catasetum—A rare and endangered orchid. Acta Scientiarum Polonorum, Hortorum Cultus, 13, 197-205.

Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S., & Donoghue, M. J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri botanical garden, 247-277.

Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S., & Donoghue, M. J. 1996. Erratum: The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny (Annals of the Missouri Botanical Garden , Annals of the Missouri Botanical Garden, 83(1). 247-277.

Ballantyne, M.; Pickering, C. 2011. Ecotourism as a threatening process for wild orchids. Journal of Ecotourism. 11: 34-47.

Balzarini J, Neyts J, Schols D, Hosoya M, Van Damme E, Peumans W, De Clercq E (1992). The mannose-specific plant lectins from Cymbidium hybrid and Epipactis helleborine and the (Nacetylglucosamine) n-specific plant lectin from Urtica dioica are potent and selective inhibitors of human immunodeficiency virus and cytomegalovirus replication in vitro. Antiviral Res. 18:191-207.

Barman, B., Rao, S., & Banu, S. 2020. In vitro seed germination, protocorm and seedling development of Dendrobium jenkinsii Wall. Ex Lindl.- an ornamental, medicinal and threatened orchid, 11(3), 548-555.

Baskaran, P., Moyo, M., & Van Staden, J. 2014. In vitro plant regeneration, phenolic compound production and pharmacological activities of Coleonema pulchellum. South African Journal of Botany, 90, 74-79.

Behara, D.; Rath, C.C. & Mohapatra, U. 2013. Medicinal orchids in India and their conservation: A review. Floriculture and Ornamental Biotechnology, 7(1): 53-59.

Bhadra, S. K., & Hossain, M. M. 2003. In vitro germination and micropropagation of Geodorum densiflorum (Lam.) Schltr., an endangered orchid species. Plant Tissue Culture, 13(2), 165-171.

Bhat, K. V., Bhat, S. R., & Chandel, K. P. S. 1992. Survey of isozyme polymorphism for clonal identification in Musa.: I. Esterase, acid phosphatase and catalase. Journal of Horticultural Science, 67(4), 501-507.

Bhattacharjee, S.K. 1995. Cultural requirements of orchids In: Advance in Horticulture Ornamental Plants (Eds. Chadha, K.L. and Bhattacharjee, S.K.). Malhotra Publishing House, New Delhi, India. 673-701.

Bhattacharjee, B., & Islam, S. S. 2014. Effects of plant growth regulators on multiple shoot induction in Vanda tessellata (Roxb.) Hook. Ex G. Don an endangered medicinal orchid. International Journal of Science and Nature, 5(4), 707-712.

Bhattacharyya, P., Kumaria, S., Diengdoh, R., & Tandon, P. 2014. Genetic stability and phytochemical analysis of the in vitro regenerated plants of Dendrobium nobile Lindl., an endangered medicinal orchid. Meta gene, 2, 489-504.

Bhattacharyya, P., Kumaria, K., Tandon, P., 2015a. Applicability of ISSR and DAMD markers for phyto-molecular characterization and association with some important biochemical traits of Dendrobium nobile, an endangered medicinal orchid. Phytochemistry 117, 306–316. doi:10.1016/j.phytochem.2015.06.022.

Bhattacharyya, P., Kumaria, S., Job, N., & Tandon, P. 2015b. Phyto-molecular profiling and assessment of antioxidant activity within micropropagated plants of Dendrobium thyrsiflorum: a threatened, medicinal orchid. Plant Cell, Tissue and Organ Culture (PCTOC), 122(3), 535-550.

Bhattacharyya, P., Kumaria, S., Job, N., & Tandon, P. 2016. En-masse production of elite clones of Dendrobium crepidatum: A threatened, medicinal orchid used in Traditional Chinese Medicine (TCM). Journal of Applied Research on Medicinal and Aromatic Plants, 3(4), 168-176.

Bhattacharyya, P., Kumar, V., & Van Staden, J. (2018). In vitro encapsulation based short term storage and assessment of genetic homogeneity in regenerated Ansellia africana (Leopard orchid) using gene targeted molecular markers. Plant Cell, Tissue and Organ Culture (PCTOC), 133(2), 299-310.

Bhowmik, T. K., & Rahman, M. M. 2017. Effect of different basal media and PGRs on in vitro seed germination and seedling development of medicinally important orchid Cymbidium aloifolium (L.) Sw. Journal of Pharmacognosy and Phytochemistry, 6(1), 167-172.

Bhowmik, T. K., & Rahman, M. M. 2020. Micropropagation of commercially important orchid Dendrobium palpebrae Lindl. through in vitro developed pseudobulb culture. Journal of Advanced Biotechnology and Experimental Therapeutics, 3(3), 225-232.

Bird, A. 2002. DNA methylation patterns and epigenetic memory. Genes & development, 16(1), 6-21.

Brand-Williams, W., Cuvelier M. E. & Berset C. 1995. Use of a free-radical method to evaluate antioxidant activity. Food Science Technology. LWT. 28(1):25-30.

Bose, B., Kumaria, S., Choudhury, H., & Tandon, P. 2017. Insights into nuclear DNA content, hydrogen peroxide and antioxidative enzyme activities during transverse thin cell layer organogenesis and ex vitro acclimatization of Malaxis wallichii, a threatened medicinal orchid. Physiology and Molecular Biology of Plants, 23(4), 955-968.

Pant, B. 2013. Medicinal orchids and their uses: Tissue culture a potential alternative for conservation. African Journal of plant science, 7(10), 448-467. doi: 10.5897/AJPS2013.1031

Bogdanova, E. D. 2003. Epigenetic variation induced in Triticum aestivum L. by nicotinic acid. Russian Journal of Genetics, 39(9), 1029-1034.

Bose, T. K. & Bhattacharjee, S. K. 1980. Orchids of India. Naya Prokash, Calcutta.

Bose, T. K., Bhattacharjee, S. K., Das, P., & Basak, U. C. 1999. Orchids of India (No. Ed. 2). Naya Prokash.

Bose, B., Kumaria, S., Choudhury, H., & Tandon, P. 2016. Assessment of genetic homogeneity and analysis of phytomedicinal potential in micropropagated plants of Nardostachys jatamansi, a critically endangered, medicinal plant of alpine Himalayas. Plant Cell, Tissue and Organ Culture, 124(2), 331-349.

Bose, B., Kumaria, S., Choudhury, H., & Tandon, P. 2017. Insights into nuclear DNA content, hydrogen peroxide and antioxidative enzyme activities during transverse thin cell layer organogenesis and ex vitro acclimatization of Malaxis wallichii, a threatened medicinal orchid. Physiology and Molecular Biology of Plants, 23(4), 955-968.

Botstein, D., White, R. L., Skolnick, M., & Davis, R. W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American journal of human genetics, 32(3), 314.

Brundrett, M.C. 2007. Scientific approaches to Australian temperate terrestrial orchid conservation. Australia Journal of Botany, 55: 293-307.

Buckseth, T., Singh, R. K., Sharma, A. K., Sharma, S., Moudgil, V., & Saraswati, A. 2018. Optimization of activated charcoal on in vitro growth and development of potato (Solanum tuberosum L.). International Journal of Current Microbiology Application Science, 7:3543-3548.

Bulpitt, C.J. 2005. The uses and misuses of orchids in medicine. QJM: An International Journal of Medicine, 98: 625-631.

Bustam, B. M., Dixon, K., & Bunn, E. 2016. A cryopreservation protocol for ex situ conservation of terrestrial orchids using asymbiotic primary and secondary (adventitious) protocorms. In Vitro Cellular & Developmental Biology-Plant, 52(2): 185-195.

Butiuc-Keul, A., Farkas, A., & Cristea, V. 2016. Genetic Stability Assessment of in vitro Plants by Molecular Markers. Studia Universitatis Babes-Bolyai, Biologia, 61(1): 107–114.

Cahyaningsih, R., Compton, L. J., Rahayu, S., Magos Brehm, J., & Maxted, N. 2022. DNA Barcoding Medicinal Plant Species from Indonesia. Plants, 11(10), 1375.

Cameron, K. M. 2004. Utility of plastid psaB gene sequences for investigating intrafamilial relationships within Orchidaceae. Molecular phylogenetics and evolution, 31(3), 1157-1180.

Cameron K.M., Chase M.W., Whitten W.M., Kores P.J., Jarrel D.C. & Albert V.A., et al. 1999. A phylogenetic analysis of the Orchidaceae: evidence from rbcL nucleotide sequences. American Journal of Botany, 86: 208–224.

Castillo-Pérez, L. J., Martínez-Soto, D., Fortanelli-Martínez, J., & Carranza-Álvarez, C. (2021). Asymbiotic seed germination, in vitro seedling development, and symbiotic acclimatization of the Mexican threatened orchid Stanhopea tigrina. Plant Cell, Tissue and Organ Culture (PCTOC), 146(2): 249-257.

Chan, S. W. L., Henderson, I. R., Zhang, X., Shah, G., Chien, J. S. C., & Jacobsen, S. E. 2006. RNAi, DRD1, and histone methylation actively target developmentally important non-CG DNA methylation in Arabidopsis. PLoS genetics, 2(6):e83.

Chanda, S., Amrutiya, N., & Rakholiya, K. 2013. Evaluation of antioxidant properties of some Indian vegetable and fruit peels by decoction extraction method. American Journal of Food Technology, 8(3): 173-82.

Changkija, S., Kumar, Y., & Gurung, P. B. 1992. Orchids of Nagaland. Forest Department, Nagaland, India.

Cha-um, S., Puthea, O., & Kirdmanee, C. 2009. An effective in-vitro acclimatization using uniconazole treatments and ex-vitro adaptation of Phalaenopsis orchid. Scientia Horticulturae, 121(4): 468-473.

Chase, M. W., Salamin, N., Wilkinson, M., Dunwell, J. M., Kesanakurthi, R. P., Haidar, N., & Savolainen, V. 2005. Land plants and DNA barcodes: short-term and long-term goals. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462): 1889-1895.

Chase, M.W.; Cameron, K.M., Freudenstein, J. V., Pridgeon, A.M., Salazar, G., Van den Berg, C. & Schuiteman, A. 2015. An updated classification of orchidaceae. Botanical Journal of Linnaean Society, 177: 151-174.

Chen, W. H., Chen, T. M., Fu, Y. M., Hsieh, R. M., & Chen, W. S. 1998. Studies on somaclonal variation in Phalaenopsis. Plant Cell Reports, 18(1): 7-13.

Chen, J.T., Chang, W.C., 2000. Plant regeneration via embryo and shoot bud formation from flower-stalk explants of Oncidium Sweet Sugar. Plant Cell Tissue Organ Culture. 62: 95-100.

Chen, S., Song, J., Yao, H., Shi, L., Luo, K., & Han, J. 2009. Strategy and key technique of identification of Chinese herbal medicine using DNA barcoding. Chinese Journal of Natural Medicines, 7(5): 322-327.

Chen, F. C., Yu, J. Y., Chen, P. Y., & Huang, Y. W. 2008. Somaclonal variation in orchids and the application of biotechnology. Acta Horticulturae, 766:315-322.

Chen, S., Yao, H., Han, J., Liu, C., Song, J., Shi, L., & Leon, C. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PloS one, 5(1): e8613.

Chen, B., Trueman, S. J., Li, J., Li, Q., Fan, H., & Zhang, J. 2014. Micropropagation of the endangered medicinal orchid, Dendrobium officinale. Life Science Journal, 11(9), 526-530.

Chattopadhyay, P., Banerjee, G., & Banerjee, N. 2017. Distinguishing orchid species by DNA barcoding: Increasing the resolution of population studies in plant biology. OMICS: A Journal of Integrative Biology, 21(12): 711-720. DOI: 10.1089/omi.2017.0131

Chimsook, T. 2016. Phytochemical screening, Total phenolic content, antioxidant activities and cytotoxicity of Dendrobium signatum leaves. In MATEC Web of Conferences. EDP Sciences.62, 03005. doi: 10.1051/ matecconf/20166203005

Chin, C. K., Lee, Z. H., Mubbarakh, S. A., Antony, J. J. J., Chew, B. L., & Subramaniam, S. (2019). Effects of plant growth regulators and activated charcoal on somaclonal variations of protocorm-like bodies (PLBs) of Dendrobium Sabin Blue orchid. Biocatalysis and Agricultural Biotechnology, 22, 101426.

Chinsamy, M., Finnie, J. F., & Van Staden, J. 2011. The ethnobotany of South African medicinal orchids. South African Journal of Botany, 77(1), 2-9.

Chinsamy M, Finnie JF, Van Staden J; Anti-inflammatory, antioxidant, anti-cholinesterase activity and mutagenicity of South African medicinal orchids. South African Journal of Botany, 2014; 91: 88–98.

Christenhusz, M.J. M.;Bing, J.W.2016. The number of known plants species in the world and its annual increase. Phytotaxa, 216(3):201-217.

Choi, S. O., & Chung, J. D. 1989. Effects of media on multiplication of Rhizomes and growth of seedlings through asymbiotic seed germination of oriental Cymbidium. The Korean Society for Horticultural Science.30: 294-302.

Chowdhery, H. J. 1998. Orchid Flora of Arunachal Pradesh. Bishen Singh Mahendra Pal Singh, Dehradun. India.

Chowlu, K., Mahar, K. S., & Das, A. K. 2017. Ethnobotanical studies on orchids among the Khamti Community of Arunachal Pradesh, India. Indian Journal of Natural Products and Resources (IJNPR) [Formerly Natural Product Radiance (NPR)], 8(1), 89-93.

Chu, C., Chu, W. C., San, S. C., Chen, H., Chu, Y. K., & Yin, C. C. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Scientia Sinica, 18: 223-231.

Chung, M. Y., Nason, J. D., & Chung, M. G. 2005. Patterns of hybridization and population genetic structure in the terrestrial orchids Liparis kumokiri and Liparis makinoana (Orchidaceae) in sympatric populations. Molecular Ecology, 14(14):389-4402.

Chugh, S., Guha, S., & Rao, I. U. 2009. Micropropagation of orchids: a review on the potential of different explants. Scientia Horticulturae, 122(4): 507-520. doi:10.1016/j.scienta.2009.07.016

Clarke, C. 1889. An Abnormal Epigynous Cyperacea. Annals of Botany, (2): 267-268.

CBOL Plant Working Group A DNA barcode for land plants. Proc. Natl. Acad. Sci. USA 2009, 106, 12794–12797.

Coates, D.J.; Dixon, K.W. 2007. Current prospective in plant conservation biology. Australian Journal of Botany, 55: 187-193.

Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T.,& Willerslev, E. 2007. Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucleic Acids Research 3 (35).

Collard, B. C., & Mackill, D. J. 2009. Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. Plant molecular biology reporter, 27(1): 86-93.

Comber, J. 2001. Orchids of Sumatra. Natural History Publications, Kota Kinabalu, Sabah and Royal Botanic Gardens, Kew.

Cribb, P.J.; Kell, S.P., Dixon, K.W., Barrett, R. L., 2003. Orchid conservation: a global perspective Orchid Conservation Natural History Publications, Kota Kinabalu, Sabah. Pp.1-24.

Da Silva, U. F., Borba, E. L., Semir, J., & Marsaioli, A. J. 1999. A simple solid injection device for the analyses of Bulbophyllum (Orchidaceae) volatiles. Phytochemistry, 50(1), 31-34.

da Silva, J. T. 2013. Orchids: advances in tissue culture, genetics, phytochemistry and transgenic biotechnology. Floriculture and Ornamental Biotechnology, 7(1), 1-52.

Dakpa, T., & Sonam, B. D. 2007. Tibetan medicinal plants: An illustrated guide to identification and practical use. Paljor Publications Private Limited. 287.

Davenport, R.B.; Ndangalasi, H.J.2003. An escalating trade in orchid tubers across Tanzania's Southern highlands: assessments dynamics and conservation implications. The International Journal of Conservation-Oryx, 37(1): 55-61.

de Boer, H. J., Ichim, M. C., & Newmaster, S. G. 2015. DNA barcoding and pharmacovigilance of herbal medicines. Drug safety, 38(7): 611-620.

De Hert, K., Jacquemyn, H., Van Glabeke, S., Roldán‐Ruiz, I., Vandepitte, K., Leus, L., & Honnay, O. 2011. Patterns of hybridization between diploid and derived allotetraploid species of Dactylorhiza (Orchidaceae) co‐occurring in Belgium. American Journal of Botany, 98(6): 946-955.

De Klerk, G. J. 1990. How to measure somaclonal variation. Acta Botanica Neerlandica, 39(2), 129-144.

De, L. C., & Singh, R. 2018. Organic production of Cymbidium orchids. Acta Scientific Agriculture Volume, 2(4).

Deb, C. R. & Temjensangba. 2006. In vitro propagation of threatened terrestrial orchid, Malaxis khasiana Soland ex. Swartz through immature seed culture. Indian Journal of Experimental Biology. 44:762-766.

Deb, C.R.; Imchen, T. 2008. Orchid Diversity of Nagaland. SciChem Publishing House, Udaipur, Rajasthan India.

Deb, C.R.; Deb, M.S; Jamir, N.S. & Imchen, T. 2009. Orchids in indigenous system of medicine in Nagaland, India. Pleione, 3(2): 209-211.

Deb, C.R. & Imchen, T., 2010. An efficient in vitro hardening technique of tissue culture raised plants. Biotechnology 9 (1): 79–83. doi:10/10.3923/biotech.2010.79.83.

Deb, C.R. & Imchen, T. 2011. Orchids of Horticultural importance from Nagaland, India. Pleione 5(1): 44-48.

Deb, C. R., & Pongener, A. 2011. Asymbiotic seed germination and in vitro seedling development of Cymbidium aloifolium (L.) Sw.: a multipurpose orchid. Journal of Plant Biochemistry and Biotechnology, 20(1): 90-95. doi:10.1007/s13562-010-0031-4.

Deb, C. R., & Pongener, A. 2012. Development of a cost effective in vitro regenerative protocol of Cymbidium aloifolium (L.) Sw. using nodal segments as an explants source. International Journal of Chemical and Biochemical Sciences, 1: 77-84.

Deb, C. R., & Pongener, A. 2013. A study on the use of low cost substrata against agar for non-symbiotic seed culture of' Cymbidium iridioides D. Don. Australian Journal of Crop Science, 7(5): 642-649.

Deb, C. R., Jamir, N. S., Jakha, H. Y., Kikon, Z. P., & Dey, S. 2014. Esmeralda clarkei Reichenbach f.[Orchidaceae]: a new report for the flora of Nagaland, India. Pleione 8(1): 181 - 183.

Deb, C. R., Rout, G. R., Mao, A. A., Nandi, S. K., Singha, R. N., Vijayan, D., & Swain, D. 2018. In vitro propagation of some threatened plant species of India. Current Science, 567-575. doi:10.18520/cs/ v114/i03/567-575.

Deb, C. R., & Jakha, H. Y. 2019. In vitro immature embryo culture and propagation of Paphiopedilum villosum var. boxallii (Rchb. f.) Pfitzer. Biotechnology, 18(2): 77-88.

Deb, C. R., Kamba, J., Longchar, T. B., & Jakha, H. Y. 2017. Cymbidium bicolor Lindl. (orchidaceae): a new report for the orchid flora of Nagaland, India. Pleione, 11(2), 498-500.

Deb, C. R., Longchar, T. B., Kamba, J., & Jakha, H. Y. 2021. Wild orchid resources of Nagaland, India: updated status. Pleione, 15(2): 113-126. doi:10.26679/Pleione.15.2.2021.113-122.

Deb, C. R., & Kamba, J. 2022. Molecular characterization and study of phylogeny of some commercially important Vandaceous orchids (Orchidaceae) based on the internal transcribed spacer (ITS). South African Journal of Botany, 146: 875-882.

Decruse, S. W., Neethu, R. S., & Pradeep, N. S. 2018. Seed germination and seedling growth promoted by a Ceratobasidiaceae clone in Vanda thwaitesii Hook. f., an endangered orchid species endemic to South Western Ghats, India and Sri Lanka. South African Journal of Botany, 116: 222-229. https://doi.org/10.1016/j.sajb.2018.04.002.

Deorani, S. C., & Naithani, H. B. 1995. Orchids of Nagaland. Oriental Enterprises. Dehradun, India.

Devi, J., Nath, M., Devi, M., & Deka, P. C. 1990. Effect of different media on germination and growth of some north-east Indian species of Dendrobium. Journal of the Orchid Society of India, 4(1-2): 45-49.

Devi, J.B., Borthakur, B., Deka, P.C., 1997. Clonal propagation of Dendrobium moschatum and Cymbidium aloifolium through shoot tip culture. Journal of Orchid Society of India, 11: 19–21.

Devi, S. P., Kumaria, S., Rao, S. R., & Tandon, P. 2013. In vitro propagation and assessment of clonal fidelity of Nepenthes khasiana Hook. f.: a medicinal insectivorous plant of India. Acta physiologiae plantarum, 35(9): 2813-2820.

Devi, S. P., Kumaria, S., Rao, S. R., & Tandon, P. 2014. Single primer amplification reaction (SPAR) methods reveal subsequent increase in genetic variations in micropropagated plants of Nepenthes khasiana Hook. f. maintained for three consecutive regenerations. Gene, 538(1): 23-29. doi:10.1016/j.gene.2014.01.028

Devi, N. P., Lisipriya, B., & Bai, N. 2015. Asymbiotic seed germination and mass multiplication of Taprobanea spathulata (L.) Christenson (Asparagales: Orchidaceae): a medicinally important epiphytic orchid. Brazilian Journal of Biological Sciences, 2(4): 271-286.

Devos, N., Oh, S. H., Raspe, O., Jacquemart, A. L., Manos, P. S. 2005. Nuclear ribosomal DNA sequence variation and evolution of spotted marshorchids (Dactylorhiza maculata group). Molecular Phylogenetics and Evolution, 36: 568–580.

Dileep, N., Rakesh, K. N., Junaid, S., Kekuda, P. T. R., Vinayaka, K. S. & Raghavendra, H. L. 2014. Antibacterial and radical scavenging activity of selected plants of Western Ghats of Karnataka, India. Journal of Chemical and Pharmaceutical Sciences, 7(2): 79-84.

Diengdoh, R. V., Kumaria, S., Tandon, P. & Chettri, M. 2017. Asymbiotic germination and seed storage of Paphiopedilum insigne, an endangered lady's slipper orchid. South African Journal of Botany, 112: 215–224. http://dx.doi.org/10.1016/j.sajb.2017.05.028

Dobránszki, J., & da Silva, J. A. T. 2010. Micropropagation of apple—a review. Biotechnology Advances, 28(4): 462-488.

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bulletin; 19:11-15.

Dressler, R. L. 1993. Phylogeny and classification of the orchid family. Cambridge University Press.

Drever, J. 2018. DNA Barcoding of Masdevallia Orchids Using the matK Locus. Student Research Proceedings, 3(1).

Du Puy, D.; Cribb, P.J.; Bosser, J. & Hermans, J.G.1999. the orchids of Madagascar. Royal Botanic Gardens, Kew.

Emaldi, U., Trujillo, I., & De Garcia, E. 2004. Comparison of characteristics of bananas (Musa sp.) from the somaclone CIEN BTA-03 and its parental clone Williams. Fruits, 59(4), 257-263.

Easton, A. 2001. Profiles in orchid history: clonal multiplication. Orchids. 70:236–239.

Evans, D. A., Sharp, W. R., & Medina‐Filho, H. P. 1984. Somaclonal and gametoclonal variation. American Journal of Botany, 71(6): 759-774.

Fay, M. F., & Krauss, S. L. 2003. Orchid conservation genetics in the molecular age. Orchid conservation. Kota Kinabalu: Natural History Publications (Borneo), 91-112.

Farrington, L., MacGillivray, P., Faast, R., & Austin, A. 2009. Investigating DNA barcoding options for the identification of Caladenia (Orchidaceae) species. Australian Journal of Botany, 57(4): 276-286.

FAO. 2003. State of the world's forest. Food and Agricultural Organization, Rome.

Fay, M. F. 1992. Conservation of rare and endangered plants using in vitro methods. In Vitro Cellular & Developmental Biology-Plant, 28(1), 1-4.

Fazekas, A. J., Burgess, K. S., Kesanakurti, P. R., Graham, S. W., Newmaster, S. G., Husband, B. C., & Barrett, S. C. 2008. Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PloS one, 3(7): e2802.

Feng, S., Jiang, M., Shi, Y., Jiao, K., Shen, C., Lu, J., & Wang, H. 2016. Application of the ribosomal DNA ITS2 region of Physalis (Solanaceae): DNA barcoding and phylogenetic study. Frontiers in plant science, 7, 1047.

Ferreira, W. D. M., Kerbauy, G. B., & Costa, A. P. P. 2006. Micropropagation and genetic stability of a Dendrobium hybrid (Orchidaceae). In Vitro Cellular & Developmental Biology-Plant, 42(6): 568-571.

Flanagan, N., Peakall, R., Clements, M., & Otero, T. 2007. Molecular genetic diagnosis of the 'taxonomically difficult Australian endangered orchid, Microtis angusii: an evaluation of the utility of DNA barcoding. Lankesteriana. 7: 1-2.

Fridborg, G., Pedersen, M., Landstrom, L. E., & Eriksson, T. 1978. The effect of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morphogenesis. Physiologia plantarum, 43(2): 104-106.

Gao, T., Yao, H., Song, J., Liu, C., Zhu, Y., Ma, X., & Chen, S. 2010. Identification of medicinal plants in the family Fabaceae using a potential DNA barcode ITS2. Journal of ethnopharmacology, 130(1): 116-121.

Galimberti, A., Casiraghi, M., Bruni, I., Guzzetti, L., Cortis, P., Berterame, N. M., & Labra, M. 2019. From DNA barcoding to personalized nutrition: the evolution of food traceability. Current Opinion in Food Science, 28: 41-48.

Gamborg, O. L., Miller, R., & Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Experimental cell research, 50(1): 151-158.

Ganie, S. H., Upadhyay, P., Das, S., & Sharma, M. P. 2015. Authentication of medicinal plants by DNA markers. Plant Gene, 4: 83-99.

Gantait, S., Das, A., Mitra, M., & Chen, J. T. 2021. Secondary metabolites in orchids: Biosynthesis, medicinal uses, and biotechnology. South African Journal of Botany, 139: 338-351. https://doi.org/10.1016/j.sajb.2021.03.015.

Genwali, G. R., Acharya, P. P., & Rajbhandari, M. 2013. Isolation of gallic acid and estimation of total phenolic content in some medicinal plants and their antioxidant activity. Nepal Journal of Science and Technology, 14(1): 95-102.

Gigot, G., van Alphen-Stahl, J., Bogarín, D., Warner, J., Chase, M., & Savolainen, V. 2007. Finding a suitable DNA barcode for Mesoamerican orchids. Lankesteriana: International Journal on Orchidology. 7(1-2): 200-203.

Ghahremani, R., Daylami, S. D., Mirmasoumi, M., Askari, N., & Vahdati, K. 2021. Refining a protocol for somatic embryogenesis and plant regeneration of Phalaenopsis amabilis

cv. Jinan from mature tissues. Turkish Journal of Agriculture and Forestry, 45(3): 356-364.

Ghanaksh A, Kaushik P (1999). Antibacterial effect of Aerides multiflora Roxb.: a study in vitro. Journal of Orchid Society of India, 13:65-68

Gholami, S., Vafaee, Y., Nazari, F., & Ghorbani, A. 2021. Exploring genetic variations in threatened medicinal orchids using start codon targeted (SCoT) polymorphism and marker-association with seed morphometric traits. Physiology and Molecular Biology of Plants, 27(4): 769-785.

Ghorbani, A., Gravendeel, B., Selliah, S., Zarré, S., & De Boer, H. 2017. DNA barcoding of tuberous Orchidoideae: a resource for identification of orchids used in Salep. Molecular Ecology Resources, 17(2): 342-352.

Ginibun, F. C., Saad, M. R. M., Hong, T. L., Othman, R. Y., Khalid, N., & Bhassu, S. 2010. Chloroplast DNA barcoding of Spathoglottis species for genetic conservation. In I International Orchid Symposium, 878: 453-459.

Goh, C.J. & Wong, P.F., 1990. Micropropagation of the monopodial orchid hybrid Aranda Deborah using inflorescence explants. Scientia Horticulturae. 44: 315–321.

Gonçalves, L. D. M., Prizão, E. C., Milaneze Gutierre, M. A., Mangolin, C. A., & Machado, M. 2012. Use of complex supplements and light-differential effects for micropropagation of Hadrolaelia purpurata (= Laelia purpurata) and Encyclia randii orchids. Acta Scientiarum. Agronomy, 34: 459-463.

Guo, Y. Y., Huang, L. Q., Liu, Z. J., & Wang, X. Q. 2016. Promise and challenge of DNA barcoding in Venus Slipper (Paphiopedilum). PloS one, 11(1), e0146880.

Gu, W., Song, J., Cao, Y., Sun, Q., Yao, H., Wu, Q., & Duan, J. 2013. Application of the ITS2 region for barcoding medicinal plants of Selaginellaceae in Pteridophyta. PloS one, 8(6), e67818.

Gupta, A. 2016. Asymbiotic seed germination in orchids: role of organic additives. International Advanced Research Journal in Science, Engineering and Technology, 3(5): 5.

Gutiérrez, R. M. P. 2010. Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology. Journal of Medicinal Plants Research, 4(8): 592-638.

Hajibabaei, M., Singer, G. A., Hebert, P. D., & Hickey, D. A. 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends in Genetics, 23(4): 167-172.

Han, J. P., Shi, L. C., Chen, X. C., & Lin, Y. L. 2012. Comparison of four DNA barcodes in identifying certain medicinal plants of Lamiaceae. Journal of Systematics and Evolution, 50(3): 227-234.

Han, J., Pang, X., Liao, B., Yao, H., Song, J., & Chen, S. 2016. An authenticity survey of herbal medicines from markets in China using DNA barcoding. Scientific Reports, 6(1): 1-9.

Han, Y., Hui, Z., & Kan, D. 2020. A review of isolation methods, structure features and bioactivities of polysaccharides from Dendrobium species. Chinese Journal of Natural Medicines, 18(1): 1-27.

Handa, S.S.1986. Orchids for drugs and chemicals. In Biology, Conservation and Culture of Orchids (Ed Vij, S.P.), Affiliated East-West Press, New Delhi, India, Pp89-100.

Hazarika, B. N. 2003. Acclimatization of tissue-cultured plants. Current science, 1704-1712.

He, J., Wong, K. L., Shaw, P. C., Wang, H., & Li, D. Z. 2010. Identification of the medicinal plants in Aconitum L. by DNA barcoding technique. Planta medica, 76(14): 1622-1628.

Heath, D.D., Iwama, G.K., Devlin, R.H., 1993. PCR primed with VNTR core sequences yields species specific patterns and hypervariable probes. Nucleic Acids Res. 21 (24): 5782–5785. doi:10.1093/nar/21.24.5782.

Hebert, P. D., Cywinska, A., Ball, S. L., & DeWaard, J. R. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(1512): 313-321.

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. Proceedings of the National Academy of Sciences, 101(41): 14812-14817.

Hedge, S.N. 1984. Orchids of Arunachal Pradesh, Govt of AP, Itanagar, India.

Hedge, S.N. 1987. Development of orchidology as plant andcut-flower trade industry in North Eastern region vis-à-vis Arunachal Pradesh, Arunachal Forest News, 5: 52-56.

Hedge, S.N.1997. Orchids wealth of India. Proceedings of Indian National Science Academy, B63 No3: 229-244.

Hooker, J. D. 1890. Obituary: Mr. John Ball, FRS. In Proceedings of the Royal Geographical Society and Monthly Record of Geography, 99-108.

Hossain, M. M. 2008. Asymbiotic seed germination and in vitro seedling development of Epidendrum ibaguense Kunth.(Orchidaceae). African Journal of Biotechnology, 7(20).

Hossain, M. M. 2011. Therapeutic orchids: traditional uses and recent advances—an overview. Fitoterapia, 82(2): 102-140.

Hinsely, A.; Verissimo, D.; Roberts, D.L.2015. Heterogeneity in consumer preferences for orchids in international trade and the potential for the use of market research to study demand for wildlife. Biological Conservation. 190: 80-86.

Hinsley, A., De Boer, H. J., Fay, M. F., Gale, S. W., Gardiner, L. M., Gunasekara, R. S., & Phelps, J. 2018. A review of the trade in orchids and its implications for conservation. Botanical Journal of the Linnean Society, 186(4): 435-455.

Hira, A., & Bijaya, P. 2019. In vitro seed germination and seedling growth of the orchid Dendrobium primulinum Lindl. African Journal of Plant Science, 13(12): 324-331. doi: 10.5897/AJPS2019.1923Hossain, M. M. 2011. Therapeutic orchids: traditional uses and recent advances—an overview. Fitoterapia, 82(2): 102-140.

Hossain, M.M., Sharma, M., Pathak, P., 2013. In vitro propagation of Dendrobium aphyllum (Orchidaceae) seed germination to flowering. Journal of Plant Biochemistry and Biotechnology 22 (2): 157–167.

Hosseini-Mazinani, M., & Sheidai, M. 2009. Molecular analysis of genetic stability in long-term micropropagated shoots of Olea europaea L. (cv. Dezful). Asian Journal of Plant Sciences, 8(2): 146-152.

Huda, M. K., Price, A., & Wilcock, C. C. 2017. Identification of medicinal orchids of Bangladesh: DNA barcoding vs. traditional taxonomy. The Journal of the Orchid Society of India, 31, 33-40.

Hunhoff, V. L., Lage, L. A., Palú, E. G., Krause, W., & Silva, C. A. 2018. Nutritional requirements for germination and in vitro development of three Orchidaceceae species in the southern Brazilian Amazon. Ornamental Horticulture, 24: 87-94. https:// doi.org/10.14295/oh.v24i2.1130.

Hürkan, K., & TAŞKIN, K. M. 2021. Internal transcribed spacer (ITS) fails barcoding of the genus Neotinea Rchb. f.(Orchidaceae). Journal of Agricultural Sciences, 27(1): 69-75.

Hussain, A., Qarshi, I. A., Nazir, H., & Ullah, I. 2012. Plant tissue culture: current status and opportunities. Recent advances in plant in vitro culture, 6(10): 1-28.

Hutchings, A. 1996. Zulu medicinal plants: An inventory. University of Natal press.

Hynniewta, T. M., Kataki, S. K., Wadhwa, B. M., Hajra, P. K., & Chatterjee, U. 2000. Orchids of Nagaland.

Israeli, Y., Reuveni, O., & Lahav, E. 1991. Qualitative aspects of somaclonal variations in banana propagated by in vitro techniques. Scientia Horticulturae, 48(1-2): 71-88.

Israeli, Y., Lahav, E., & Reuveni, O. 1995. In vitro culture of bananas. In Bananas and plantains. Springer, Dordrecht. 147-178.

IUCN. 1999. IUCN guidelines for the prevention of biodiversity loss due to biological invasion. Species, 31: 28-42.

Jalal, J.S.; Kumar, P.; Pangtey, Y.P.S.2008. Ethnomedicinal orchids of Uttarakand, Western Himalaya. Ethnobotanical Leaflets, 12:1227-1230.

Jalal, J. S., Jayanthi, J. 2012 Endemic orchids of peninsular India: a review. Journal of Threatened Taxa, 4(15): 3415-3425.

Jain, M. S. 2001. Tissue culture-derived variation in crop improvement. Euphytica, 118(2): 153-166.

Jarret, R. L., & Litz, R. E. 1986. Isozymes as genetic markers in bananas and plantains. Euphytica, 35(2): 539-549.

Jaime A. T. da Silva, J. A., Cardoso, J. C., Dobránszki, J., & Zeng, S. (2015). Dendrobium micropropagation: a review. Plant cell reports, 34(5): 671-704.

Joongku, L. 2012. Assessing nrDNA ITS2 sequence based molecular signature of ginseng for potential use in quality control of drug. African Journal of Pharmacy and Pharmacology, 6(39): 2767-2774.

Jones, D.L. 2006. Native orchids of Australia. Sydney, Australia: New Holland Publishers

Joshi, G.C.; Tewari, L. M; Upreti, K.; Jalal, J. S.; Tewari, G. 2009. Diversity of orchids in Uttarakhand and their conservation strategy with special reference to their medicinal importance. Report and Opinion, 1(3): 47-52.

Joshi, R., Shukla, A., & Kumar, P. 2013. In vitro water deficit stress induced genotypic alterations in protein profile among aromatic rice varieties. Annual Plant Science, 2: 455-458.

Jualang, A. G., Devina, D., Hartinie, M., Sharon, J. S., & Roslina, J. 2014. Asymbiotic seed germination and seedling development of Vanda dearei. Malaysian Applied Biology, 43(2): 25-33.

Kaki, A., Vafaee, Y., & Khadivi, A. 2020. Genetic variation of Anacamptis coriophora, Dactylorhiza umbrosa, Himantoglossum affine, Orchis mascula, and Ophrys schulzei in the western parts of Iran. Industrial Crops and Products, 156, 112854.

Kakoti, M., Hazarika, D. J., Kumar, A., Barooah, M., Modi, M. K., Bhattacharyya, A., & Boro, R. C. 2021. Genetic diversity and DNA barcoding of wild mushrooms from Northeast India. Iranian Journal of Science and Technology, Transactions A: Science, 45(2): 469-479.

Kala, C. P. 2003. Commercial exploitation and conservation status of high value medicinal plants across the borderline of India and Nepal in Pithoragarh. The Indian Forester, 129: 80-84.

Kala, C. P. 2004. Assessment of species rarity. Current Science, 86(8): 1058-1059.

Kala, C.P. 2006. Medicinal plants potential for economic development in the state of Uttaranchal, India. International Journal of Sustainable Development and World Ecology, 13(6): 492-498.

Kalita, J., Singh, S. S., & Khan, M. L. 2022. Molecular Characterization and Phylogeny of Clerodendrum Species Occuring in North Eastern Region of India by Internal Transcribed Spacer 2 of Nuclear DNA. In Molecular Genetics and Genomics Tools in Biodiversity Conservation . 237-250.

Kamba, J., & Deb, C. R. 2018. A new simple and efficient DNA extraction protocol for orchid without liquid nitrogen and phenol. Plant Cell Biotechnology and Molecular Biology, 19(3-4): 143-147.

Kamba, J., & Deb, C. R. 2022. Molecular Decoding of Identity of Some Commercially Important Vandaceous Orchids (Orchidaceae) Based on the Sequences of the Internal Transcribed Spacer (ITS) Sequences and Their Phylogeny. https://doi.org/10.1016/j.sajb.2022.03.029

Kasulo, V., Mwabumba, L., Cry, M. 2009. A review of edible orchids in Malawi. Journal of Horticultural and Forestry, 1(7): 133-139.

Karp, A. 1994. Origins, causes and uses of variation in plant tissue cultures. In Plant cell and tissue culture .Springer, Dordrecht.139-151.

Karp, A. 1995. Somaclonal variation as a tool for crop improvement. Euphytica, 85(1), 295-302.

Karthikeyan, S. 2000. A statistical analysis of flowering plants of India. Flora of India introductory volume part-II, 201-217.

Kasulo, V.; Mwabumba, L.; Cry, M. 2009. A review of edible orchids in Malawi. Journal of Horticultural and Forestry, 1(7): 133-139.

Kaur, S. 2015. DNA barcoding and its applications. International Journal of Engineering Research and General Science, 3(2), 602-604.

Kaur, S., & Bhutani, K. K. 2011. In vitro propagation of Dendrobium chrysotoxum (Lindl.). Floriculture and Ornamental Biotechnology, 5(1), 50-56.

Kauth, P.J., Dutra, D., Johnson, T.R., Stewart, S.L., Kane, M.E., Vendrame, V., 2008. Techniques and applications of in vitro orchid seed germination. In: Teixeira da Silva, J. A. (Ed.), Floriculture, Ornamental and Plant Biotechnology: Advances and Topics Issues. Global Science Books, Isleworth, pp. 375–391

Khamchatra, N., Dixon, K. W., Tantiwiwat, S., & Piapukiew, J. 2016. Symbiotic seed germination of an endangered epiphytic slipper orchid, Paphiopedilum villosum (Lindl.) Stein. from Thailand. South African Journal of Botany, 104: 76-81. https://doi.org/ 10.1016/j.sajb.2015.11.012

Khoddamzadeh, A. A., Sinniah, U. R., Kadir, M. A., Kadzimin, S. B., Mahmood, M., & Sreeramanan, S. 2010. Detection of somaclonal variation by random amplified polymorphic DNA analysis during micropropagation of Phalaenopsis bellina (Rchb. f.) Christenson. African Journal of Biotechnology, 9(40), 6632-6639. DOI: 10.5897/AJB10.714

Kim, H. M., Oh, S. H., Bhandari, G. S., Kim, C. S., & Park, C. W. 2014. DNA barcoding of Orchidaceae in Korea. Molecular Ecology Resources, 14(3): 499-507. doi: 10.1111/1755-0998.12207

Kim, D.H., Kang, K.W., Enkhtaivan, G., Jan, U., Sivanesan, I., 2019. Impact of activated charcoal, culture medium strength and thidiazuron on non-symbiotic in vitro seed germination of Pecteilis radiate (Thunb.) Raf. South African Journal of Botany 124: 144–150. https://doi.org/10.1016/j.sajb.2019.04.015.

Kim, Y.-K., Kwak, M.H., Hong, J.-R., Kim, H.-W., Jo, S., Sohn, J.-Y., Cheon, S.-H., Kim, K.-J., 2017. The complete plastome sequence of the endangered orchid Habenaria radiata (Orchidaceae). Mitochondria DNA Part B 2: 704–706. https://doi.org/10.1080/23802359.2017.1390410.

Kishor, R., & Devi, H. S. 2009. Induction of multiple shoots in a monopodial orchid hybrid (Aerides vandarum Reichb. f× Vanda stangeana Reichb. f) using thidiazuron and analysis of their genetic stability. Plant Cell, Tissue and Organ Culture, 97(2): 121-129.

Khoddamzadeh, A. A., Sinniah, U. R., Kadir, M. A., Kadzimin, S. B., Mahmood, M., & Sreeramanan, S. 2010. Detection of somaclonal variation by random amplified polymorphic DNA analysis during micropropagation of Phalaenopsis bellina (Rchb. f.) Christenson. African Journal of Biotechnology, 9(40): 6632-6639.

Knudson, L. 1922. Nonsymbiotic germination of orchid seeds. Botanical gazette, 73(1): 1-25.

Knudson, L., 1946. A new nutrient solution for germination of orchid seeds. Am. Orchid Soc. Bull. 15, 214–217.

Koirala, D., Pradhan, S., & Pant, B. 2013. Asymbiotic seed germination and plantlet development of CoelogynefuscescensLindl., a medicinal orchid of Nepal. Scientific world, 11(11): 97-100.

Koopowitz, H., Lavarack, P.S. & Dixon, K.W. 2003. The nature of threats to orchid conservation. In Orchid conservation (Eds Dixon, K.W.; Kelly, S. P.; Barrett, R. L.; Cribb, P. J.). Kota Kinabalu, Sabah Natural History Publications, Pp, 25-42.

Larkin, P. J., & Scowcroft, W. R. 1981. Somaclonal variation—a novel source of variability from cell cultures for plant improvement. Theoretical and applied genetics, 60(4): 197-214.

Kool, A., de Boer, H. J., Krüger, Å., Rydberg, A., Abbad, A., Björk, L., & Martin, G. 2012. Molecular identification of commercialized medicinal plants in Southern Morocco. PloS one, 7(6), e39459.

Loeza, O. M. M., Salgado-Garciglia, R., Gómez-Alonso, C., & Ávila-Díaz, I. (2011). Acclimatization of the endangered Mexican epiphytic orchid, Laelia speciosa (HBK) Schltr. European Journal of Environmental Sciences, 1(2).

Kress, J. W., Wurdack, K. J., Zimmer, E. A., Weigt, L. A. & Janzen, D. H. 2005 Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences of USA, 102: 8369–8374.

Kress, W. J., & Erickson, D. L. 2007. A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. PLoS one, 2(6), e508.

Kubis, S. E., Castilho, A. M., Vershinin, A. V., & Heslop-Harrison, J. S. P. 2003. Retroelements, transposons and methylation status in the genome of oil palm (Elaeis guineensis) and the relationship to somaclonal variation. Plant molecular biology, 52(1): 69-79.

Kumar, C. S, & Manilal, K. S. 1994. Catalogue of Indian orchids. Bishen Singh Makendra Pal Singh.

Kunakhonnuruk, B., Inthima, P., & Kongbangkerd, A. 2018. In vitro propagation of Epipactis flava Seidenf., an endangered rheophytic orchid: a first study on factors affecting asymbiotic seed germination, seedling development and greenhouse acclimatization. Plant Cell, Tissue and Organ Culture (PCTOC), 135(3): 419-432.

Kunakhonnuruk, B., Inthima, P., & Kongbangkerd, A. 2018. The effect of culture media on asymbiotic seed germination in Epipactis flava, an endangered rheophytic orchid in Thailand. In III International Orchid Symposium 1262, 37-42.

Kunakhonnuruk, B., Inthima, P., & Kongbangkerd, A. (2019). In vitro propagation of rheophytic orchid, Epipactis flava Seidenf.—A comparison of semi-solid, continuous immersion and temporary immersion systems. Biology, 8(4), 72.

Kukulczanka, K., & Wojciechowska, U. 1982. Propagation of two Dendrobium species by in vitro culture. In Vitro Culture, XXI IHC, 131: 105-110.

Kull, T., & Arditti, J. (Eds.). 2002. Orchid Biology VIII: Reviews and Perspectives. Springer Science & Business Media. 8, 95.

Kumar, S.C.; Manilal, K.S. 1994. A Catalogue of Indian Orchids. Singh, B.; Singh, M. P. New Delhi, India.

Kumar, P. K. S., Subramoniam, A., Pushpangadan, P. 2000. Aphrodisiac activity of Vanda tessellata (Roxb.) Hook. ex Don extract in male mice. Indian Journal of Pharmaceutical Sciences. 32:300-304.

Kumar, S., Kumaria, S., & Tandon, P. 2013. SPAR methods coupled with seed-oil content revealed intra-specific natural variation in Jatropha curcas L. from Northeast India. biomass and bioenergy, 54: 100-106. doi:10.1016/J.BIOMBIOE.2013.03. 023.

Lahaye, R., Van der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., & Savolainen, V. 2008. DNA barcoding the floras of biodiversity hotspots. Proceedings of the National Academy of Sciences, 105(8): 2923-2928.

Lakshmanan, V., Reddampalli Venkataramareddy, S., & Neelwarne, B. 2007. Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. Electronic Journal of Biotechnology, 10(1): 106-113. doi:10.4067/ S0717-34582007000100010.

Lam, Y., Ng, T. B., Yao, R. M., Shi, J., Xu, K., Sze, S. C. W., & Zhang, K. Y. 2015. Evaluation of chemical constituents and important mechanism of pharmacological biology in Dendrobium plants. Evidence-Based Complementary and Alternative Medicine. http://dx.doi.org/10.1155/2015/841752

Lan, Y. T., Liu, S. Y., Luo, Y. T., Huang, L., Li, Z. L., & Wei, X. L. 2010. Sterile germination of Dendrobium chrysotoxum seeds and rapid propagation of its plantlets. Agricultural Science and Technology, 11: 89-91.

Lawler L, Slaytor M (1970). Uses of Australian orchids by Aborigines and early settlers. The Medical journal of Australia 2:1259.

Lee, Y. I., & Yeung, E. C. 2018. Orchid propagation: from laboratories to greenhouses-methods and protocols. Humana Press.

Li D, Gao L, Li H, Wang H, Ge X, Yang J, Liu J, Chen Z, Zhou S, Chen S, Jang J, Fu C, Zeng C, Yan H, YJ Z, Sun Y, Zhao, Wang K, Yang T, Duan,G. 2011. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proceedings of the National Academy of Sciences, 108 (49): 19641 19646. https://doi.org/10.1073/pnas.1104551108

Li, H., Xiao, W., Tong, T., Li, Y., Zhang, M., Lin, X., ... & Guo, X. 2021. The specific DNA barcodes based on chloroplast genes for species identification of Orchidaceae plants. Scientific Reports, 11(1): 1-15. https://doi.org/10.1038/s41598-021-81087-w

Li, M., Cao, H., BUT, P. P. H., & SHAW, P. C. 2011. Identification of herbal medicinal materials using DNA barcodes. Journal of Systematics and Evolution, 49(3): 271-283.

Li, X., Yang, Y., Henry, R. J., Rossetto, M., Wang, Y., & Chen, S. 2015. Plant DNA barcoding: from gene to genome. Biological Reviews, 90(1): 157-166.

Liu, J., Shi, L., Han, J., Li, G., Lu, H., Hou, J.,& Downie, S. R. 2014. Identification of species in the angiosperm family Apiaceae using DNA barcodes. Molecular ecology resources, 14(6): 1231-1238.

Liu, Y. C., Lin, B. Y., Lin, J. Y., Wu, W. L., & Chang, C. C. 2016. Evaluation of chloroplast DNA markers for intraspecific identification of Phalaenopsis equestris cultivars. Scientia Horticulturae, 203, 86-94. http://dx.doi.org/10.1016/j.scienta.2016.03.021

Lo, S. F., Nalawade, S. M., Kuo, C. L., Chen, C. L., & Tsay, H. S. 2004a. Asymbiotic germination of immature seeds, plantlet development and ex vitro establishment of plants of Dendrobium tosaense makino—A medicinally improrant orchid. In Vitro Cellular & Developmental Biology-Plant, 40(5): 528-535.

Lo, S. F., Nalawade, S. M., Mulabagal, V., Matthew, S., Chen, C. L., Kuo, C. L., & Tsay, H. S. 2004b. In vitro propagation by asymbiotic seed germination and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity studies of tissue culture raised plants of three medicinally important species of Dendrobium. Biological and Pharmaceutical Bulletin, 27(5): 731-735.

Loeza, O. M. M., Salgado-Garciglia, R., Gómez-Alonso, C., & Ávila-Díaz, I. 2011. Acclimatization of the endangered Mexican epiphytic orchid, Laelia speciosa (HBK) Schltr. European Journal of Environmental Sciences, 1(2).

Lokho, A. 2013. Diversity of Dendrobium Sw. its distributional patterns and present status in the Northeast India. International Journal of Scientific and Research Publications, 3(5): 1-9.

Long, B., Niemiera, A. X., Cheng, Z. Y., & Long, C. L. 2010. In vitro propagation of four threatened Paphiopedilum species (Orchidaceae). Plant Cell, Tissue and Organ Culture (PCTOC), 101(2): 151-162.

Mahadani, P., Sharma, G. D., & Ghosh, S. K. 2013. Identification of ethnomedicinal plants (Rauvolfioideae: Apocynaceae) through DNA barcoding from northeast India. Pharmacognosy Magazine, 9(35): 255.

Mahendran, G., & Bai, V. N. 2012. Direct somatic embryogenesis and plant regeneration from seed derived protocorms of Cymbidium bicolor Lindl. Scientia Horticulturae, 135: 40-44. doi:10.1016/j.scienta.2011.12.003

Mahendran, G., Muniappan, V., Ashwini, M., Muthukumar, T., & Narmatha Bai, V. 2013. Asymbiotic seed germination of Cymbidium bicolor Lindl. (Orchidaceae) and the influence of mycorrhizal fungus on seedling development. Acta Physiologiae Plantarum, 35(3): 829-840.

Magrini, S., & De Vitis, M. 2017. In vitro reproduction of three Limodorum species (Orchidaceae): impacts of scarification methods and nitrogen sources on mature seed germination and seedling development. Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology, 151(3): 419-428.

Malabadi, R. B., Teixeira da Silva, J. A., Nataraja, K., & Mulgund, G. S. 2008. Shoot tip transverse thin cell layers and 24-epibrassinolide in the micropropagation of Cymbidium bicolor Lindl. Floriculture and Ornamental Biotechnology, 2(2): 44-48.

Martin, K.P., 2003. Clonal propagation, encapsulation and reintroduction of Ipsea malabarica (Reichb. F.) J.D. Hook. An endangered orchid. In Vitro Cell. Dev. Biol. 39: 322–326

Martin, K.P., Madassery, J.P., 2006. Rapid in vitro propagation of Dendrobium hybrids through direct shoot formation from foliar explants and protocorm like bodies. Sci. Hortic. 108: 95–99.

Mayo-Mosqueda, A., Maceda-López, L. F., Andrade-Canto, S. B., Noguera-Savelli, E., Caamal-Velázquez, H., Cano-Sosa, J. D. S., & Alatorre-Cobos, F. 2020. Efficient protocol for in vitro propagation of Laelia rubescens Lindl. from asymbiotic seed germination. South African Journal of Botany, 133: 264-272. doi:10.1016/j.sajb.2020.07.030.

Mercado, S. A. S., & Delgado, E. A. B. 2020. Effect of the medium composition on the asymbiotic germination and in vitro development of the Laeliocattleya hybrid. South African Journal of Botany, 135: 80-86. doi:10.1016/j.sajb.2020.08.011.

Mitsukuri, K., Mori, G., Johkan, M., Shimada, Y., Mishiba, K.I., Morikawa, T. & Oda, M., 2009. Effects of explant position and dark treatment on bud formation in floret culture of Ponerorchis graminiflolia Rchb. f. Scientia Horticulturae. 121: 243–247.

Mishra, P., Kumar, A., Nagireddy, A., Mani, D. N., Shukla, A. K., Tiwari, R., & Sundaresan, V. 2016. DNA barcoding: an efficient tool to overcome authentication challenges in the herbal market. Plant biotechnology journal, 14(1): 8-21.

Mitra, G. C., Prasad, R. N., & Choudhury, A. R. 1976. Inorganic correlated salts and differentiation of protocorms in seed of an orchid (Dendrobium fimbriatum) and correlated changes in its free amino acid context. Indian Journal of Experimental Biology, 14: 350-351.

Miyazawa, M., Shimamura, H., Nakamura, S., Sugiura, W., Kosaka H., Kameoka, H. 1999. Moscatilin from Dendrobium nobile, a naturally occurring bibenzyl compound with potential antimutagenic activity. Journal of Agricultural and Food Chemistry. 47:2163-2167.

Mohanty, P., Paul, S., Das, M. C., Kumaria, S., & Tandon, P. 2012. A simple and efficient protocol for the mass propagation of Cymbidium mastersii: an ornamental orchid of Northeast India. AoB plants,

Moraes, L. M., Faria, R. T., & Cuquel, F. L. 2003. Activated charcoal for in vitro propagation of Brazilian orchids. In V International Symposium on New Floricultural Crops 683, 383-390.

Morel, G.M. 1960. Producing virus-free cymbidiums. American Orchids Society, 29: 495-497.

Morel, G. M. 1964. Tissue-culture. A new means of clonal propagation for orchids. American Orchid Society Bulletin, 33: 473-478.

Mujib, A., Banerjee, S., & Dev Ghosh, P. 2007. Callus induction, somatic embryogenesis and chromosomal instability in tissue culture raised hippeastrum (Hippeastrum hybridum cv. United Nations). Propagation of Ornamental Plants, 7: 169-174.

Muna, S. 2016. Standardization of Media Combinations for Hardening of Orchid Dendrobium Spp Cv. Sonia-17. International Journal of Agriculture Sciences, 0975-3710.

Murashige, T. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiology plant, 15, 473-497.

Muthukrishnan, S., Kumar, T. S., & Rao, M. V. 2013. Effects of different media and organic additives on seed germination of Geodorum densiflorum (Lam) Schltr.—an endangered orchid. International Journal of Scientific Research, 2(8): 23-26.

Mwabumba, L., Munthali, C. R. Y., & Kasulo, V. 2009. A review of edible orchids in Malawi.

Myers, N.; Mittermeier, R.A; Mittermeier, C. G.; da Fanseca, G. A. B.; Kent, J. 2000. Biodiversity hotspots for conservation priorities. Nature, 403: 853-858.

Nakano, M., Nomizu, T., Mizunashi, K., Suzuki, M., Mori, S., Kuwayama, S., & Amano, J. 2006. Somaclonal variation in Tricyrtis hirta plants regenerated from 1-year-old embryogenic callus cultures. Scientia Horticulturae, 110(4): 366-371.

Nagaraju, V., Parthasarathy, V.S., 1995. In vitro propagation of Phaius and bamboo orchid by shoot tip culture. Annals of Plant Physiology. 9: 102–104

Nagaraju, V., Das, S. P., Bhutia, P. C. & Upadhyaya, R. C. 2001. Morphogenetic response of Arundina graminifolia seeds: A study in vitro. Six National Seminar on Orchid Diversity in India, Science and Commerce and Orchid Show, held in Oct. 11-13, 2001 in the Institute of Himalayan Bioresource Technology, Palampur, Abstract No. 64, p. 94.

Nanekar, V., Shriram, V., Kumar, V., & Kishor, P. B. 2014. Asymbiotic in vitro seed germination and seedling development of Eulophia nuda Lindl., an endangered medicinal orchid. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 84(3): 837-846. DOI 10.1007/s40011-014-0353-4

Nayak, N.R., Chand, P.K., Rath, S.P., Patnaik, S., 1998. Influence of some plant growth regulators on the growth and organogenesis of Cymbidium aloifolium (L.) Sw. seed derived rhizomes in vitro. In Vitro Cellular & Developmental Biology - Plant , 34: 185–188.

Ninawe, A. S., & Swapna, T. S. 2017. Orchid diversity of Northeast India–traditional knowledge and strategic plan for conservation. Journal of Orchid Society of India, 31: 41-56.

Nirmala, C., Nongdam, P., & Tewari, R. 2006. Biotechnological and molecular approaches for improvement of orchids. Plant Cell Biotechnology and Molecular Biology, 7: 1-10.

Nitsch, J.P., 1969. Experimental androgenesis in Nicotiana. Phytomorphology. 19:389–404.

Oliya, B. K., Chand, K., Thakuri, L. S., Baniya, M. K., Sah, A. K., & Pant, B. 2021. Assessment of genetic stability of micropropagated plants of Rhynchostylis retusa (L.) using RAPD markers. Scientia Horticulturae, 281, 110008

Pacek-Bieniek, A., Dyduch-Siemińska, M., & Rudaś, M. 2010. Influence of activated charcoal on seed germination and seedling development by the asymbiotic method in Zygostates grandiflora (Lindl.) Mansf.(Orchidaceae). Folia Horticulturae, 22(2): 45-50.

Paek, K. Y., Hahn, E. J., & Park, S. Y. 2011. Micropropagation of Phalaenopsis orchids via protocorms and protocorm-like bodies. In Plant Embryo Culture. Humana Press. 293-306.

Palombi, M., & Damiano, C. (2002). Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (Actinidia deliciosa A. Chev). Plant Cell Reports, 20(11), 1061-1066. doi:10.1007/s00299-001-0430-z.

Pamarthi, R. K., Devadas, R., Kumar, R., Rai, D., Babu, P. K., Meitei, A. L., & Singh, D. R. 2019. PGR diversity and economic utilization of orchids. https://doi.org/10.20546/ijcmas.2019.810.217

Pang, X., Song, J., Zhu, Y., Xie, C., & Chen, S. 2010. Using DNA barcoding to identify species within Euphorbiaceae. Planta medica, 76(15): 1784-1786.

Pang, X., Song, J., Zhu, Y., Xu, H., Huang, L., & Chen, S. 2011. Applying plant DNA barcodes for Rosaceae species identification. Cladistics, 27(2): 165-170.

Pan, M.J. and Staden, J. 1998. The use of charcoal in in vitro culture-A review. Plant Growth Regulation, 26:155-163.

Pant, B. 2013. Medicinal orchids and their uses: Tissue culture a potential alternative for conservation. African Journal of plant science, 7(10): 448-467. doi:10.5897/AJPS2013. 1031.

Panwar, D., Ram, K., & Shekhawat, N. S. 2012. In vitro propagation of Eulophia nuda Lindl., an endangered orchid. Scientia Horticulturae, 139: 46-52.

Panwar, G. S., Joshi, B., & Joshi, R. 2022. Axenic rhizome culture and genetic fidelity assessment of Eulophia dabia (D. Don) Hochr: an endangered terrestrial orchid species. In Vitro Cellular & Developmental Biology-Plant, 1-10.

Patel, R. K., Patel, J. B. & Trivedi, P. D. 2015. Spectrophotometric method for the estimation of total alkaloids in the Tinospora cordifolia. and its herbal formulations. International Journal of Pharmacy and Pharmaceutical Sciences, 10:249-251.

Pradhan, U. C. 1976. Indian orchids: guide to identification & culture (Vol. 2). Pradhan. Kalimpong.

Park, S.Y., Murthy, H.N., Paek, K.Y., 2003. Protocorm-like body induction and subsequent plant regeneration from root tip cultures of Doritaenopsis. Plant Scence. 164: 919–923.

Panwar, G. S., Joshi, B., & Joshi, R. 2022. Axenic rhizome culture and genetic fidelity assessment of Eulophia dabia (D. Don) Hochr: an endangered terrestrial orchid species. In Vitro Cellular and Developmental Biology-Plant, 1-10.

Park, H. Y., Kang, K. W., Kim, D. H., & Sivanesan, I. 2018. In vitro propagation of Cymbidium goeringii Reichenbach fil. through direct adventitious shoot regeneration. Physiology and Molecular Biology of Plants, 24(2): 307-313.

Prasad, D. N., Achari, G. 1966. A study of anti-arthritic action of Vanda roxburghii in albino rats. Journal of Indian Medical Association. 46:234-237

Parveen, I., Singh, H. K., Raghuvanshi, S., Pradhan, U. C., & Babbar, S. B. 2012. DNA barcoding of endangered Indian Paphiopedilum species. Molecular Ecology Resources, 12(1): 82-90. Doi: 10.1111/j.1755-0998.2011.03071.x

Parveen, I., Gafner, S., Techen, N., Murch, S. J., & Khan, I. A. 2016. DNA barcoding for the identification of botanicals in herbal medicine and dietary supplements: strengths and limitations. Planta Medica, 82(14), 1225-1235.

Parveen, I., Singh, H. K., Malik, S., Raghuvanshi, S., & Babbar, S. B. 2017. Evaluating five different loci (rbcL, rpoB, rpoC1, mat K, and ITS) for DNA barcoding of Indian orchids. Genome, 60(8): 665-671.

Paudel, M. R., & Pant, B. 2012. In vitro micropropagation of rare orchid (Esmeralda clarkei Rchb. f.) from shoot tip section. International Journal of Biology, Pharmacy and Allied Sciences, 1(11): 1587-1597.

Paudel, M. R., & Pant, B. 2013. A reliable protocol for micropropagation of Esmeralda clarkeiRchb. f.(Orchidaceae). Asia-Paccific Journal of Molecular Biology and Biotechnology. 21(3): 114-120.

Paul, S., Kumaria, S., & Tandon, P. 2012. An effective nutrient medium for asymbiotic seed germination and large-scale in vitro regeneration of Dendrobium hookerianum, a threatened orchid of northeast India. AoB Plants,

Prasad, D.; Prasad, K.V. 1998. Orchids in world trade. In: International festival of orchids (ed. S.N. Hegde). Orchid Society of Arunachal, Arunachal Pradesh, India.

Pearce, N.R.; Cribb, P.J. 2002. Flora of Bhutan: including a record of plants from Sikkim and Darjeeling. Volume 3, Part 3. The Orchids of Bhutan Royal Botanic Garden, Edinburgh and Royal Government of Bhutan.

Phinney, B. O. 1985. Gibberellin A1 dwarfism and shoot elongation in higher plants. Biologia plantarum, 27(2): 172-179.

Pierik, R. L. M. 1997. In vitro culture of higher plants. Springer science & business media.

Peyvandi, F., Palla, R., Menegatti, M., & Mannucci, P. M. 2009. Introduction: rare bleeding disorders: general aspects of clinical features, diagnosis, and management. In Seminars in thrombosis and hemostasis, 35(4): 349-355.

Pimsen, M., Kanchanapoom, K., 2011. Effect of basal media and sugar types on in vitro regeneration of Grammatophyllums peciosum Blume. Notul. Scientific journal of biological sciences, 3: 101–104.

Pongener, A. & Deb, C. R. 2009. Asymbiotic culture of immature embryos, mass multiplication of Cymbidium iridioides D. Don. and the role of different factors. International Journal of Pharma and Bio Sciences, 1(1): 1-14.

Pornarong, S., Kobsukh, K., Piya, M., Tawatchai, T., Runglawan, S., & Arunrat, C. 2012. DNA barcoding of the Cymbidium species (Orchidaceae) in Thailand. African journal of agricultural research, 7(3): 393-404

Prasad, G., Seal, T., Mao, A. A., Vijayan, D., & Lokho, A. 2021. Assessment of clonal fidelity and phytomedicinal potential in micropropagated plants of Bulbophyllum odoratissimum-An endangered medicinal orchid of Indo Burma mega biodiversity hotspot. South African Journal of Botany, 141: 487-497.

Rajaram, M. C., Yong, C. S., Gansau, J. A., & Go, R. 2019. DNA barcoding of endangered Paphiopedilum species (Orchidaceae) of Peninsular Malaysia. Phytotaxa, 387(2): 94-104. https://doi.org/10.11646/phytotaxa.387.2.2

Rakholiya, K., Kaneria, M., Nagani, K., Patel, A., & Chanda, S. 2015. Comparative analysis and simultaneous quantification of antioxidant capacity of four Terminalia species using various photometric assays. World Journal of Pharmaceutical Research, 4: 1280-1296.

Rao, M. R.; Palada, M. C.; Becker, B.N. 2004. Medicinal and aromatic plants in agro-forestry system. Agro Forestry Systems, 61: 107-122.

Rao, A. N. 2007. Orchid Flora of north East India-An Update Analysis. Bulletin of Arunachal Forest Research, 23(1): 6-38.

Raskoti, B. B., & Ale, R. 2021. DNA barcoding of medicinal orchids in Asia. Scientific Reports, 11(1): 1-11. https://doi.org/10.1038/s41598-021-03025-0

Rasmussen, H. N. 1995. Terrestrial orchids: from seed to mycotrophic plant. Cambridge University Press.

Rittirat, S., Thammasiri, K., & Te-chato, S. 2012. Effect of media and sucrose concentrations with or without activated charcoal on the plantlet growth of P. cornu-cervi (Breda) Blume & Rchb. f. Journal of Agricutural Technology, 8(6): 2077-2087.

Rotor, G. 1949. A method of vegetative propagation of Phalaenopsis species and hybrids. American Orchid Society Bulletin. 18: 738-739.

Roy, A.R.; Patel, R.S.; Patel, V.V.; Yadav, D.S. 2007. Medicinal orchids of Meghalaya. J. Orchid Society of India, 21:15-17.

Roy, A. R., Patel, R .S., Sajeev, S., & Deka, C., 2011. Asymbiotic seed germination, mass propagation and seedling development of Vanda coerulea Griff ex. Lindle. (Blue Vanda): An in vitro protocol for an endangered orchid. Scientia Horticulturae. 128: 325–331.

Rohlf, F. J. 2000. NTSYSpc: Numerical taxonomy and multivariate system. Version 2.1 Exeter Software. Applied Biostatics Inc., New York.

Rout, G. R., Samantaray, S., & Das, P. 2000. In vitro manipulation and propagation of medicinal plants. Biotechnology advances, 18(2): 91-120.

Sagawa, Y. 1963. Green pod culture. Fla. Orchidist, 6: 296-297.

Sahare, P., & Srinivasu, T. 2012. Barcoding for authentic identification of medicinal plants. International Journal of Engineering and Science (IJES), 1(2), 33-36.

Salazar M, S. A., & Cancino, G. O. 2012. Evaluación del efecto de dos suplementos orgánicos en la germinación in vitro de orquídeas nativas de la provincia de Pamplona, Colombia. Revista Colombiana de Biotecnología, 14(1): 53-59.

Salvi, N. D., George, L., & Eapen, S. 2001. Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. Plant cell, tissue and organ culture, 66(2): 113-119.

Sandoval, J., Kerbellec, F., Cote, F., & Doumas, P. 1995. Distribution of endogenous gibberellins in dwarf and giant off-types banana (Musa AAA, cv.'Grand nain') plants from in vitro propagation. Plant Growth Regulation, 17(3): 219-224.

Saravanan, M., Mohanapriya, G., Laha, R., & Sathishkumar, R. 2019. DNA barcoding detects floral origin of Indian honey samples. Genome, 62(5):341-348.

Savolainen, V., Cowan, R. S., Vogler, A. P., Roderick, G. K., & Lane, R. 2005. Towards writing the encyclopaedia of life: an introduction to DNA barcoding. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462): 1805-1811.

Seeni, S., & Latha, P. G. 2000. In vitro multiplication and eco rehabilitation of the endangered Blue Vanda. Plant cell, tissue and organ culture, 61(1): 1-8.

Seon, K. M., Kim, D. H., Kang, K. W., & Sivanesan, I. 2018. Highly competent in vitro propagation of Thrixspermum japonicum (Miq.) Rchb. f., a rare epiphytic orchid. In Vitro Cellular & Developmental Biology-Plant, 54(3): 302-308.

Schori, M., & Showalter, A. M. 2011. DNA barcoding as a means for identifying medicinal plants of Pakistan. Pakistan Journal of Botany, 43: 1-4.

Sharma, S. K., & Tandon, P. 1990. Asymbiotic germination and seedling growth of Cymbidium elegans Lindl. and Coelogyne punctulata Lindl. as influenced by different carbon sources. Journal of the Orchid Society of India, 4(1-2): 149-159.

Sharma, V., & Thakur, M. 2021. Applicability of SCoT markers for detection of variations in Fusarium yellows resistant lines of ginger (Zingiber officinale Rosc.) induced through gamma irradiations. South African Journal of Botany, 140: 454-460. doi:10.1016/j. sajb.2021.01.021.

Sarmah, D., Kolukunde, S., Sutradhar, M., Singh, B. K., Mandal, T., & Mandal, N. 2017. A review on: in vitro cloning of orchids. International Journal of Current Microbiology and Applied Sciences, 6(9): 1909-27.

Sheelavantmath, S. S., Murthy, H. N., Pyati, A. N., Ashok Kumar, H. G., & Ravishankar, B. V. 2000. In vitro propagation of the endangered orchid, Geodorum densiflorum (Lam.) Schltr. through rhizome section culture. Plant Cell, Tissue and Organ Culture, 60(2): 151-154.

Shekarriz, P., Kafi, M., Deilamy, S. D., Mirmasoumi M 2014. Coconut water and peptone improve seed germination and protocorm like body formation of hybrid Phalaneopsis. Agriculture Sciences and Devlopment. 3(10):317–322.

Sherif, N. A., Senthil Kumar, T., & Rao, M. V. 2020. DNA barcoding and genetic fidelity assessment of micropropagated Aenhenrya rotundifolia (Blatt.) CS Kumar and FN Rasm.: a critically endangered jewel orchid. Physiology and Molecular Biology of Plants, 26(12): 2391-2405. doi:10.1007/s12298-020-00917-9.

Shimura, H., Matsuura, M., Takad,a N. & Koda Y (2007). An antifungal compound involved in symbiotic germination of Cypripedium macranthos var. rebunense (Orchidaceae). Phytochemistry, 68:1442-1447.

Shinwari, Z. K., Jan, S. A., Khalil, A. T., Khan, A. D. I. L., Ali, M., Qaiser, M., & Zahra, N. B. 2018. Identification and phylogenetic analysis of selected medicinal plant species from Pakistan: DNA barcoding approach. Pakistan Journal of Botany, 50(2): 553-560.

Shinde, A. N., Malpathak, N., & Fulzele, D. P. 2010. Determination of isoflavone content and antioxidant activity in Psoralea corylifolia L. callus cultures. Food Chemistry, 118(1): 128-132.

Shyur, L. F., Chen, C. H., Lo, C. P., Wang, S.Y., Kang, P. L., Sun, S. J., Chang, C. A., Tzeng, C.M. & Yang, N. S. 2004. Induction of apoptosis in MCF-7 human breast cancer cells

by phytochemicals from Anoectochilus formosanus. Journal of Biomedicine Sciences, 11:928-939.

Silva, J. A., Singh, N., & Tanaka, M. 2006. Priming biotic factors for optimal protocorm-like body and callus induction in hybrid Cymbidium (Orchidaceae), and assessment of cytogenetic stability in regenerated plantlets. Plant Cell, Tissue and Organ Culture, 84(2): 135-144. doi:10.1016/j.scienta.2011.01.023

Singh, N. P., & Singh, K. P. (Eds.). 1997. Floristic diversity and conservation strategies in India. Botanical Survey of India.

Singh, A.& Duggal, S. 2009. Medicinal orchids- An overview. Ethnobotanical Leaflets, 13: 399-412.

Singh, A. K. R., & Tiwari, C. 2007. Harnessing the economic potential of Orchids in Uttaranchal. ENVIS Bulletin Himalayan Ecology, 14: 1-3.

Singh, H. K., Parveen, I., Raghuvanshi, S., & Babbar, S. B. 2012. The loci recommended as universal barcodes for plants on the basis of floristic studies may not work with congeneric species as exemplified by DNA barcoding of Dendrobium species. BMC research notes, 5(1), 1-11.

Singh, S., Singh, A. K., Kumar, S., Kumar, M., Pandey, P. K., & Singh, M. C. K. 2012. Medicinal properties and uses of orchids: a concise review. Elixir Applied Botany, 52(2012): 11627-11634.

Singh, S. K., Rai, M. K., & Sahoo, L. 2012. An improved and efficient micropropagation of Eclipta alba through transverse thin cell layer culture and assessment of clonal fidelity using RAPD analysis. Industrial Crops and Products, 37(1): 328-333.

Singh, N., & Kumaria, S. 2019. A combinational phytomolecular-mediated assessment in micropropagated plantlets of Coelogyne ovalis Lindl.: a horticultural and medicinal Orchid. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 90(2): 455-466.

Sinclair, E. A., Pérez-Losada, M. A. R. C. O. S., & Crandall, K. A. 2005. Molecular phylogenetics for conservation biology. Phylogeny and conservation. Cambridge University Press, Cambridge, 19-56.

Sinha, S.K. 2000. Commercial cultivation of tropical orchids for income generation in Arunachal Pradesh. Arunachal Forest News, 18(1&2): 93-99.

Sinha, P., Alam, M. F., & Hakim, M. L. 2009. Micropropagation of Phalaenopsis Blume. Methods in Molecular Biology, 77–85. doi:10.1007/978-1-60327-114-1_8

Sipayung, P., Matanari, J., Lafau, M. B., Sulastri, Y. S., Ginting, B. B., Sihombing, D. R., & Giawa, T. 2018. The effect of activated charcoal dose and benzyl amino purine concentration on the growth of orchid plantlets in murashige and skoog media in vitro. In IOP Conference Series: Earth and Environmental Science, 205(1), 012025. doi:10.1088/1755-1315/205/1/012025

Smith, J. C., & Smith, O. S. 1989. The description and assessment of distances between inbred lines of maize. II: The utility of morphological biochemical, and genetic descriptors and a scheme for the testing of distinctiveness between inbred lines. Maydica, 34(2): 151-161.

Soonthornkalump, S., Nakkanong, K., & Meesawat, U. 2019. In vitro cloning via direct somatic embryogenesis and genetic stability assessment of Paphiopedilum niveum (Rchb. f.) Stein: the endangered Venus's slipper orchid. In Vitro Cellular & Developmental Biology-Plant, 55(3): 265-276. doi:10.1007/s11627-019-09981-7.

Soniya, E. V., Banerjee, N. S. and Das, M. R. 2001. Genetic analysis of somaclonal variation among callus-derived plants of tomato. Current Science, 80(9): 1213-1215.

Sopalun, K., Thammasiri, K., & Ishikawa, K. 2010. Micropropagation of the Thai orchid Grammatophyllum speciosum blume. Plant Cell, Tissue and Organ Culture, 101(2): 143-150.

Sorina, P., Maria, B. O., Lazăr, A., & Ursu, C. 2013. The assessment of the variability induced by tissue culture to Cymbidium sp. using RAPD markers. JOURNAL of Horticulture, Forestry and Biotechnology, 17(2): 273-276.

Souza, D. M. S. C., Fernandes, S. B., Molinari, L. V., Avelar, M. L. M., & Brondani, G. E. 2021. Activated charcoal application for the micropropagation of Cattleya crispate (Thunb.) Van den Berg. Nativa, 9(4): 352-358.DOI: https://doi.org/10.31413/nativa.v9i4.12164

Srivastava, D., & Manjunath, K. 2020. DNA barcoding of endemic and endangered orchids of India: A molecular method of species identification. Pharmacognosy Magazine, 16(70), 290.

Stewart, S. L., & Kane, M. E. 2006. Asymbiotic seed germination and in vitro seedling development of Habenaria macroceratitis (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell, Tissue and Organ Culture, 86(2): 147-158.

Stewart, S.L., Kane, M.E., 2010. Effects of carbohydrate source on the in vitro asymbiotic seed germination of the terrestrial orchid Habenaria macroceratitis. Journal of Plant Nutrition. 33: 1155–1165.

Subedi, A. 2011. New species, pollinator interactions and pharmaceutical potential of Himalayan orchids (Doctoral dissertation, Leiden University)

Subedi, A., Kunwar, B., Choi, Y., Dai, Y., van Andel, T., Chaudhary, R. P., & Gravendeel, B. 2013. Collection and trade of wild-harvested orchids in Nepal. Journal of ethnobiology and ethnomedicine, 9(1), 1-10.

Swarts, N. d. & Dixon, K.W. 2009. Terrestrial orchid conservation in the age of extinction. Annuals of Botany. 104:543-556.

Szlachetko, D. L. 2001. Genera et species Orchidalium. 1. Polish Botanical Journal, 46(1).

Tabaraki, R., & Nateghi, A. 2011. Optimization of ultrasonic-assisted extraction of natural antioxidants from rice bran using response surface methodology. Ultrasonics sonochemistry, 18(6): 1279-1286.

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L. & Miquel, C. 2007. "Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding." Nucleic acids research 35(3): e14-e14.

Tak, I., Mohiuddin, D., Ganai, B. A., Chishti, M. Z., Ahmad, F., & Dar, J. S. 2014. Phytochemical studies on the extract and essential oils of Artemisia dracunculus L. (Tarragon). African Journal of Plant Science, 8(1): 72-75.

Tan, V. P. 2018. The determination of total alkaloid, polyphenol, flavonoid and saponin contents of Pogang gan (Curcuma sp.). International Journal of Biology, 10(42): 10-5539.

Techen, N., Parveen, I., Pan, Z., & Khan, I. A. 2014. DNA barcoding of medicinal plant material for identification. Current opinion in Biotechnology, 25: 103-110.

Tee, L. H., Ramakrishnan, N. R., Tey, B. T., Chan, E. S., Abd-Aziz, A., Ismail, A., & Prasad, K. M. N. 2015. Phytochemicals and antioxidant capacities from Dacryodes rostrata fruits. Medicinal Chemistry, 5(1): 23-27.

Teixeira da Silva JA, Singh N, Tanaka M .2006 Priming biotic factors for optimal protocorm-like body and callus induction in hybrid Cymbidium (Orchidaceae), and assessment of cytogenetic stability in regenerated plantlets. Plant Cell, Tissue and Organ Culture, 84: 119-128.

Teixeira da Silva, J. A., Cardoso, J. C., Dobránszki, J., & Zeng, S. 2015. Dendrobium micropropagation: a review. Plant cell reports, 34(5): 671-704. doi 10.1007/s00299-015-1754-4.

Teixeira da Silva, J. A.., Hossain, M. M., Sharma, M., Dobránszki, J., Cardoso, J. C., & Songjun, Z. E. N. G. 2017. Acclimatization of in vitro-derived Dendrobium. Horticultural Plant Journal, 3(3): 110-124.

Teoh, E. S. 2016. Sources of medicinal orchids and conservation. Medicinal orchids of Asia. 691-727.

Thakur, M., Bhattacharya, S., Khosla, P. K., & Puri, S. 2019. Improving production of plant secondary metabolites through biotic and abiotic elicitation. Journal of Applied Research on Medicinal and Aromatic Plants, 12: 1-12.

Thakur, J., Dwivedi, M. D., Singh, N., Uniyal, P. L., Goel, S., & Pandey, A. K. 2021. Applicability of Start Codon Targeted (SCoT) and Inter Simple Sequence Repeat

(ISSR) markers in assessing genetic diversity in Crepidium acuminatum (D. Don) Szlach. Journal of Applied Research on Medicinal and Aromatic Plants, 23, 100310.

Tham, D. T., Hang, N. T. T., Van Khiem, D., Van Duy, N., Vinh, T. T., Van Hoi, Q., & Cong, V. K. 2018. Micropropagation of dendrobium heterocarpum lindl. Vietnam Journal of Biotechnology, 16(1): 127-135. doi:10.15625/1811-4989/16/1/13457

Thokchom, R., Maitra, S., & Sharma, S. 2017. In vitro Mass Propagation of Endangered Terrestrial Orchid Phaius tankervilliae (L'Her.) Blume through Green Seed Pod Culture. International Journal of Current Microbiology and Applied Science, 6(5): 722-728.

Thomas, T. D. 2008. The role of activated charcoal in plant tissue culture. Biotechnology advances, 26(6): 618-631.

Thompson, D. I., Edwards, T. J., & van Staden, J. 2006. Evaluating asymbiotic seed culture methods and establishing Disa (Orchidaceae) germinability in vitro: relationships, requirements and first-time reports. Plant Growth Regulation, 49(2): 269-284.DOI 10.1007/s10725-006-9137-z

Thorpe, T. A. 2007. History of plant tissue culture. Molecular biotechnology, 37(2): 169-180.

Thorpe, T. 2012. History of Plant Tissue Culture. Methods in Molecular Biology, 9–27. doi:10.1007/978-1-61779-818-4_2

Tikendra, L., Amom, T., & Nongdam, P. 2019a. Molecular genetic homogeneity assessment of micropropagated Dendrobium moschatum Sw.-A rare medicinal orchid, using RAPD and ISSR markers. Plant Gene, 19, 100196.

Tikendra, L., Koijam, A. S., & Nongdam, P. 2019b. Molecular markers based genetic fidelity assessment of micropropagated Dendrobium chrysotoxum Lindl. Meta Gene, 20, 100562.

Tikendra, L., Potshangbam, A. M., Amom, T., Dey, A., & Nongdam, P. 2021a. Understanding the genetic diversity and population structure of Dendrobium

chrysotoxum Lindl.-An endangered medicinal orchid and implication for its conservation. South African Journal of Botany, 138: 364-376. doi:10.1016/j.sajb.2021.01.002.

Tikendra, L., Potshangbam, A. M., Dey, A., Devi, T. R., Sahoo, M. R., & Nongdam, P. 2021b. RAPD, ISSR, and SCoT markers based genetic stability assessment of micropropagated Dendrobium fimbriatum Lindl. var. oculatum Hk. f.-an important endangered orchid. Physiology and Molecular Biology of Plants, 27(2): 341-357. doi:10.1007/ s12298-021-00939-x.

Tran, T. K. P., Vu, T. T. T., & Widiarsih, S. 2021. Comparison of matK and rbcL DNA barcodes for genetic classification of jewel orchid accessions in Vietnam. Journal of Genetic Engineering and Biotechnology, 19(1): 1-8.

Torres, J., Laskowski, L., Sanabria, M. 2006. Effect of the development environment on the anatomy of the foliar epidermis of Cattleya jenmanii Rolfe.Bioagro. 18(2):93-99.

Tseng, C. C., Shang, H. F., Wang, L. F., Su, B., Hsu, C. C., Kao, H, Y. & Cheng, K. T.

2006. Antitumor and immunostimulating effects of Anoectochilus formosanus Hayata. Phytomedicine. 13:366-370.

Tsering, J., Tam, N., Tag, H., Gogoi, B. J., & Apang, O. 2017. Medicinal orchids of Arunachal Pradesh: a review. Bulletin of Arunachal Forest Research, 32(1-2): 1-16.

Tsuchiya, I. 1954. Possibility of germination of orchid seeds from immature fruits. Na Pua Okika o Hawaii Nei, 4(1): 11-16.

Udomdee, W., Wen, P. J., Lee, C. Y., Chin, S. W., & Chen, F. C. 2014. Effect of sucrose concentration and seed maturity on in vitro germination of Dendrobium nobile hybrids. Plant growth regulation, 72(3): 249-255. doi:10.1007/s10725-013.9856-x.

Unai, E., Iselen, T., & de Garcia, E. 2004. Comparison of characteristics of bananas (Musa sp.) from the somaclone CIEN BTA-03 and its parental clone Williams. Fruits, 59:257–263

Utami, E. S. W., Hariyanto, S., & Manuhara, Y. S. W. 2017. In vitro propagation of the endangered medicinal orchid, Dendrobium lasianthera JJ Sm through mature seed

culture. Asian Pacific Journal of Tropical Biomedicine, 7(5): 406-410. http://dx.doi.org/10.1016/j.apjtb.2017.01.011

Vacin, E. & Went, F. W. 1949. Some pH changes in nutrient solutions. Botanical Gazette,110:605–613

Van Tan, P. 2018. The determination of total alkaloid, polyphenol, flavonoid and saponin contents of Pogang gan (Curcuma sp.). International Journal of Biology, 10(42), 5524-5539.

Van Waes, J. 1985. Effect of activated charcoal on in vitro propagation of Western European orchids. In Symposium on In Vitro Problems Related to Mass Propagation of Horticultural Plants 212 ,131-138.

Veldman, S., Otieno, J., Gravendeel, B., Andel, T. V., & Boer, H. D. 2014. Conservation of endangered wild harvested medicinal plants: use of DNA barcoding. Novel plant bioresources: applications in food, medicine and cosmetics, 81-88.

Veldman, S., Gravendeel, B., Otieno, J. N., Lammers, Y., Duijm, E., Nieman, A., & de Boer, H. J. 2017. High-throughput sequencing of African chikanda cake highlights conservation challenges in orchids. Biodiversity and Conservation, 26(9): 2029-2046. DOI 10.1007/s10531-017-1343-7

Veldman, S., Kim, S. J., Van Andel, T. R., Bello Font, M., Bone, R. E., Bytebier, B., & De Boer, H. J. 2018. Trade in Zambian Edible Orchids—DNA Barcoding Reveals the Use of Unexpected Orchid Taxa for Chikanda. Genes, 9(12), 595.

Verma, D., Barbhuiya, H. A., & Lavania, S. 2014. Bulbophyllum cherrapunjeensis (Orchidaceae), a new species from Meghalaya, India. Phytotaxa, 156(5): 298-300.

Verpoorte, R., Contin, A., & Memelink, J. 2002. Biotechnology for the production of plant secondary metabolites. Phytochemistry reviews, 1(1): 13-25.

Vij, S.P., 1993. Regeneration response of orchid roots: A study in vitro. Journal of Orchid Society of India, 7: 61–72.

Vu, T. H. T., Le, T. L., Nguyen, T. K., Tran, D. D., & Tran, H. D. 2017. Review on molecular markers for identification of orchids. Vietnam Journal of Science, Technology and Engineering, 59(2): 62-75.

Vu, H. T., Vu, Q. L., Nguyen, T. D., Tran, N., Nguyen, T. C., Luu, P. N., & Le, L. 2019. Genetic diversity and identification of Vietnamese Paphiopedilum species using DNA Sequences. Biology, 9(1), 9. doi:10.3390/biology9010009

Vudala, S. M., Padial, A. A., & Ribas, L. L. F. 2019. Micropropagation of Hadrolaelia grandis through transverse and longitudinal thin cell layer culture. South African Journal of Botany, 121: 76-82.https://doi.org/10.1016/j.sajb.2018.07.017

Waes, J. V., 1987. Effect of activated charcoal on in vitro propagation of Western European orchids. Acta horticulturae, 212: 131-138.

Wang, P. J., & Huang, L. C. 1976. Beneficial effects of activated charcoal on plant tissue and organ cultures. In vitro, 260-262.

Wang, J., Matsuzaki, K., & Kitanaka, S. 2006. Stilbene derivatives from Pholidota chinensis and their anti-inflammatory activity. Chemical and Pharmaceutical Bulletin, 54:1216-1218.

Watanabe, K., Tanaka, R., Sakurai, H., Iguchi, K., Yamada, Y., Hsu, C. S., Sakuma, C., Kikuchi, H., Shibayama, H., & Kawai, T. 2007. Structure of cymbidine A, a monomeric peptidoglycan-related compound with hypotensive and diuretic activities, isolated from a higher plant, Cymbidium goeringii (Orchidaceae). Chemical and Pharmaceutical Bulletin. 55:780- 783.

Wattoo, J. I., Saleem, M. Z., Shahzad, M. S., Arif, A., Hameed, A., & Saleem, M. A. 2016. DNA Barcoding: Amplification and sequence analysis of rbcl and matK genome regions in three divergent plant species. Advancements in Life Sciences, 4(1): 03-07.

Weising, K., Nybom, H., Pfenninger, M., Wolff, K., & Kahl, G. 2005. DNA fingerprinting in plants: principles, methods, and applications. CRC press.

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18(1): 315-322.

Withner, C. L. 1953. Germination of Cypripediums (Cyps). Orchid Journal, 2: 473-477.

Wood,J.J.; Beaman, J.; Beaman, R. 1993. The plants of mount Kinabalu 2. Orchids: Royal Botanic Gardens, Kew.

Won, J. H., Kim, J. Y., Yun, K. J., Lee, J.H., Back, N. I., Chung, H. G., Chung, S. A., Jeong, T. S., Choi, M. S., & Lee, K. T. 2006. Gigantol isolated from the whole plants of Cymbidium goeringii inhibits the LPS-induced iNOS and COX- 2 expression via NF-kappaB inactivation in RAW 264.7macrophages cells. Planta Medica.72:1181-1187

Worthy, S. J., Bucalo, K., Perry, E., Reynolds, A., Cruse-Sanders, J., Pérez, Á. J., & Burgess, K. S. 2022. Ability of rbcL and matK DNA barcodes to discriminate between montane forest orchids. Plant Systematics and Evolution, 308(3): 1-9.

Wu, I.F., Chen, J.T., & Chang, W.C., 2004. Effect of auxins and cytokinins on embryo formation from root-derived callus of Oncidium 'Gower Ramsey'. Plant Cell Tissue Organ Culture, 77: 107–109.

Wu, K., Zeng, S., Lin, D., Teixeira da Silva, J. A., Bu, Z., Zhang, J., & Duan, J. 2014. In vitro propagation and reintroduction of the endangered Renanthera imschootiana Rolfe. PloS one, 9(10), e110033.

XIANG, X. G., Hu, H. A. O., Wang, W. E. I., & JIN, X. H. 2011. DNA barcoding of the recently evolved genus Holcoglossum (Orchidaceae: Aeridinae): a test of DNA barcode candidates. Molecular ecology resources, 11(6): 1012-1021.

Xu, S., Li, D., Li, J., Xiang, X., Jin, W., Huang, W., & Huang, L. 2015. Evaluation of the DNA barcodes in Dendrobium (Orchidaceae) from mainland Asia. PloS one, 10(1), e0115168.

Xu, L., Gong, W., Cusack, S. A., Wu, H., Loovers, H. M., Zhang, H., & Jones, G. W. 2018. The β6/β7 region of the Hsp70 substrate-binding domain mediates heat-shock

response and prion propagation. Cellular and Molecular Life Sciences, 75(8): 1445-1459.

Yam, T. W., Arditti, J., & Weatherhead, M. A. 1989. The use of darkening agents in seed germination and tissue culture media for orchids: a review. Journal of Orchid Society of India, 3(1&2): 35-39.

Yam, T. W., & Arditti, J. 2009. History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnology Reports, 3(1), 1-56.

Yam, T. W., Nair, H., Hew, C. S., & Arditti, J. 2002. Orchid seeds and their germination: An historical account. Orchid biology: reviews and perspectives, VIII. Kluwer, Dordrecht, 387-504.

Yao, H., Song, J. Y., Ma, X. Y., Liu, C., Li, Y., Xu, H. X., & Chen, S. L. 2009. Identification of Dendrobium species by a candidate DNA barcode sequence: the chloroplast psbA-trnH intergenic region. Planta medica, 75(06): 667-669

Yao, H., Song, J., Liu, C., Luo, K., Han, J., Li, Y., & Chen, S. 2010. Use of ITS2 region as the universal DNA barcode for plants and animals. PloS one, 5(10), e13102.

Ya-Na, L. V., Chun-Yong, Y. A. N. G., Lin-Chun, S. H. I., Zhang, Z. L., An-Shun, X. U., Zhang, L. X., & Hai-Tao, L. I. 2020. Identification of medicinal plants within the Apocynaceae family using ITS2 and psbA-trnH barcodes. Chinese journal of natural medicines, 18(8): 594-605.

Yeung, E. C., Park, J., & Harry, I. S. 2018. Orchid seed germination and micropropagation I: background information and related protocols. In Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols. Humana Press, New York,101-125.

Yesil-Celiktas, O., Nartop, P., Gurel, A., Bedir, E., & Vardar-Sukan, F. 2007. Determination of phenolic content and antioxidant activity of extracts obtained from Rosmarinus officinalis' calli. Journal of plant physiology, 164(11): 1536-1542.

Yu, J., Wu, X., Liu, C., Newmaster, S., Ragupathy, S., & Kress, W. J. 2021. Progress in the use of DNA barcodes in the identification and classification of medicinal plants.

Ecotoxicology and Environmental Safety, 208, 111691. https://doi.org/10.1016/j.ecoenv.2020.111691

Zhang, H. J., J. J. Zhou, and S. S. Li. 2003. Study advance of Gastrodia elata b1. Amino Acids Biotic Resourse, 25: 17- 20.

Zhang L, Chen F, Zhang GQ, Zhang YQ, Niu S, Xiong JS, Lin Z, Cheng ZM, Liu ZJ . 2016 Origin and mechanism of crassulacean acid metabolism in orchids as implied by comparative transcriptomics and genomics of the carbon fixation pathway. Plant Journal, 86:175–185. https://doi.org/10.1111/tpj.13159

Zhang, D., Zhang, Y., Liu, G., & Zhang, J. 2006. Dactylorhin B reduces toxic effects of β-amyloid fragment (25–35) on neuron cells and isolated rat brain mitochondria. Naunyn-Schmiedeberg's, Archives of Pharmacology, 374(2): 117-125.

Zhao, C., Liu, Q., Halaweish, F., Shao, B., Ye, Y., & Zhao, W. 2003. Copacamphane, Picrotoxane, and Alloaromadendrane Sesquiterpene Glycosides and Phenolic Glycosides from Dendrobium moniliforme. Journal of Natural Products, 66:1140-1143.

Znaniecka, J. O. A. N. N. A., Królicka, A., Sidwa-Gorycka, M., Rybczynski, J. J., Szlachetko, D. L., & Lojkowska, E. 2005. Asymbiotic germination, seedling development and plantlet propagation of Encycliaaff. Oncidioides - an endangered orchid. Acta Societatis Botanicorum Poloniae, 74(3).

Zuo, Y., Chen, Z., Kondo, K., Funamoto, T., Wen, J., & Zhou, S. 2011. DNA barcoding of Panax species. Planta medica, 77(02): 182-187.

Zhu, Y., Chen, S. L., Yao, H., Tan, R., Song, J. Y., Luo, K., & Lu, J. 2010. DNA barcoding the medicinal plants of the genus Paris. Yao xue xue bao= Acta Pharmaceutica Sinica, 45(3): 376-382.

National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov

http://www.theplantlist.org

Appendix 1

List of Conferences/Seminars/Webinars/Workshops Attended

- National seminar on "Advance in Biological Science Research" held at Department of Botany, Nagaland University, Lumami, Nagaland, from 28th February –1st, March 2017.

- National seminar on 'Skill and Entrepreneurial Development of the Tribal Youth' with theme "Value-additions to Rich Bio-Resources with Special Reference to Medicinal and Aromatic Plants" held at Nagaland University Lumami, jointly organized by Biotech Park, Lucknow & Institutional Biotech Hub, Department of Botany, Nagaland University under the aegis of The National Academy of Sciences, India, from 25th- 28th July, 2018.

- National conference on "Stakeholders on Conservation, Resource Development and Sustainable Utilization of Medicinal Plants of North-Eastern India" held at Nagaland University Lumami, jointly organized by Department of Botany, Nagaland University, Lumami, Nagaland and Society for Conservation and Resource Development of Medicinal Plants (SMP), New Delhi, from 6th-7th March 2019.

- **Poster presentation** at Conference on "Trends in Plant Sciences and Agrobiotechnology 2019" held at Indian Institute of Technology Guwahati, Organized by Department of Biosciences & Bioengineering and Centre for Rural Technology, IIT Guwahati, India In association with Plant Tissue Culture Association- India (PTCA-I) from 14th-16th February 2019.

- Attended Hands on training of "Functional Genomics" Organized by Department of Biotechnology, Govt. of India sponsored, Institutional Biotech Hub, Nagaland University, Lumami & Department of Botany, Nagaland University, Sponsored by 'Institutional Biotech Hub', from 14th -21st November, 2017.

- Short-Term Skill Development Training Program on in Biotechnology for Students of North-East India on "Orchid Propagation". Sponsored by Institute of

Bioresources and Sustainable Development, Department of Biotechnology, Govt. of India, from16th November- 15th December, 2017.

- Hands on training on "Genomics and Gene Expression Analysis", Organized by Department of Biotechnology, Govt. of India sponsored, Advance Level Institutional Biotech Hub, Department of Botany, Nagaland University, Lumami, Nagaland, from 18th- 23rd July, 2018.

- Workshop on "Creation of Database" Organized by Bioinformatics Centre of Rajiv Gandhi University, from 1st- 2nd December 2018.

- Workshop on "Skill to Entrepreneurship- The Next Level" at St. Edmund's College, Shillong. Jointly organized by Biotech Park, Lucknow and Advance Level Institutional Biotech Hub, St. Edmund's College, Shillong, Meghalaya, from 12th -15th March 2019.

- Hands on training on "Molecular Taxonomy of Microbes and Higher Plants" Organized & sponsored by Department of Biotechnology, Govt. of India sponsored, Advance Level Institutional Biotech Hub, Nagaland University, Lumami. Jointly organized by Biotech Park, Lucknow and Institutional Biotech Hub, Nagaland University, from 17th-23rd July, 2019.

- **Oral presentation** at International conference on "Bioresources and Bioeconomy" (ICBB-2022). Organised by Department of Botany, Nagaland University, Lumami 798627, Nagaland. In collaboration with Nagaland Forest Management Project, Department of Environment, Forest and Climate Change, Govt. of Nagaland, India. September 19-21, 2022.

Appendix 2

List of publications

- Deb, C.R., Jamir, N.S., Dey, S., and Longchar, T.B. Addition Of *Bulleyia yunnanensis* Schltr., *Phalaenopsis yingjiangensis* (Z.H.Tsi) Kocyan & Schuit. And *Pholidota pygmaea* H.J. Chowdhery & G.D. Pal In The Orchid Flora Of Nagaland, India. *Indian Journal of Plant Sciences* 2016; 5 (3):63-65.
- Deb, C.R., Kamba, J., Longchar, T.B., and Jakha, H.Y. *Cymbidium bicolor* Lindl. (Orchidaceae): a new report for the orchid flora of Nagaland, India. *Pleione* 2017; 11(2): 498 - 500.
- Longchar, T.B. and Deb, C.R. Comparative analysis of nutraceutical potential phytochemicals and antioxidant activities in different parts of wild and in vitro regenerated plantlets of *Dendrobium heterocarpum* Wall. ex Lindl.: A medicinal orchid. *Journal of Pharmacognosy and Phytochemistry* 2021; 10(4): 331-336.
- Deb, C.R., Longchar, T.B., Kamba, J., and Jakha, H.Y. Wild orchid resources of Nagaland, India: updated status. Pleione. 2021;15(2): 113 – 122.
- Longchar, T.B., Deb, C.R. Optimization of *in vitro* propagation protocol of *Dendrobium heterocarpum* Wall. ex Lindl. and assessment of genetic fidelity of the regenerants: an orchid of horticultural and medicinal importance (under communication)
- Longchar, T.B. and Deb, C.R., Orchids of Nagaland and Scope in Horticulture. *Bioresources and Sustainable Livelihood of Rural India.* A Mittal Publication. 2021: 173-184. (Book chapter)