INDUCTION OF HETEROPLOIDY IN *Dendrobium* sp.

REVATHI B S

(2019-11-115)

DEPARTMENT OF PLANT BREEDING AND GENETICS

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM - 695522

KERALA, INDIA

2021

INDUCTION OF HETEROPLOIDY IN *Dendrobium* sp.

by

REVATHI B. S

(2019-11-115)

THESIS

Submitted in partial fulfilment of the

requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BREEDING AND GENETICS

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM - 695522

KERALA, INDIA

2021

DECLARATION

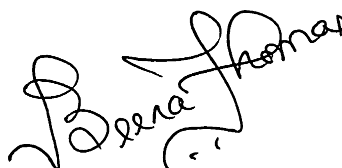
I, hereby declare that this thesis entitled **"Induction of heteroploidy in *Dendrobium* sp."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani
Date: 27|12|2021

Revathi B. S
(2019 - 11- 115)

CERTIFICATE

Certified that this thesis, entitled **"Induction of heteroploidy in *Dendrobium* sp."** is a record of research work done independently by **Ms. Revathi B. S (2019-11-115)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.



Vellayani

Date: 27/12/2021

Dr. Beena Thomas

(Chairman, Advisory Committee)

Assistant Professor

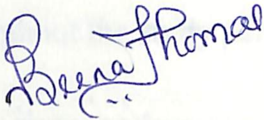
Department of Plant Breeding and Genetics

College of Agriculture, Vellayani

Thiruvananthapuram – 695 522

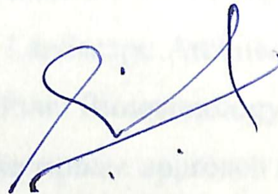
CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Revathi B S (2019-11-115)**, a candidate for the degree of **Master of Science in Agriculture** with major in Plant Breeding and Genetics, agree that the thesis **"Induction of heteroploidy in *Dendrobium* sp."** may be submitted by **Ms. Revathi B S (2019-11-115)**, in partial fulfilment of the requirement for the degree.



Dr. Beena Thomas
Assistant Professor
Department of Plant Breeding and Genetics
College of Agriculture, Vellayani
Thiruvananthapuram

Dr. Arya K.
Professor and Head
Department of Plant Breeding and Genetics
College of Agriculture, Vellayani
Thiruvananthapuram



Dr. Priyakumari. I
Assistant Professor
Department of Floriculture and
Landscape Architecture
College of Agriculture, Vellayani
Thiruvananthapuram

Ms. Sindura K.P.
Assistant Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram

DECLARATION

I, hereby declare that this thesis entitled **"Induction of heteroploidy in *Dendrobium* sp."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani **Revathi B. S**

Date: (**2019 - 11- 115**)

CERTIFICATE

Certified that this thesis, entitled **"Induction of heteroploidy in *Dendrobium* sp."** is a record of research work done independently by **Ms. Revathi B. S (2019-11-115)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellayani

Date:

Dr. Beena Thomas
(Chairman, Advisory Committee)
Assistant Professor
Department of Plant Breeding and Genetics
College of Agriculture, Vellayani
Thiruvananthapuram – 695 522

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Revathi B S (2019-11-115)**, a candidate for the degree of **Master of Science in Agriculture** with major in Plant Breeding and Genetics, agree that the thesis **"Induction of heteroploidy in *Dendrobium* sp."** may be submitted by **Ms. Revathi B S (2019-11-115)**, in partial fulfilment of the requirement for the degree.

Dr. Beena Thomas
Assistant Professor
Department of Plant Breeding and Genetics
College of Agriculture, Vellayani
Thiruvananthapuram

Dr. Arya K.
Professor and Head
Department of Plant Breeding and Genetics
College of Agriculture, Vellayani
Thiruvananthapuram

Dr. Priyakumari. I
Assistant Professor
Department of Floriculture and
Landscape Architecture
College of Agriculture, Vellayani
Thiruvananthapuram

Ms. Sindura K.P.
Assistant Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram

Acknowledgement

First and foremost, praises and thanks to the God Almighty, for everything that happens to me…

With great respect and devotion, I would like to record my deep sense of gratitude and indebtedness to my major advisor Dr. Beena Thomas, Assistant Professor, Department of Plant Breeding and Genetics, for her valuable guidance, constant inspiration, abundant encouragement, critical scrutiny of the manuscript and valuable suggestions which render me to accomplish the research work successfully. I extend my sincere gratitude for the care and affection bestowed on me throughout the study period.

It gives me immense pleasure to express my deep sense of gratitude to Dr. Arya K., Professor and Head, Department of Plant Breeding and Genetics, for her motherly approach, valuable advices and whole hearted approach right from the beginning, which helped me in the successful completion of the thesis.

I awe my heartfelt thanks to my advisory committee, Dr. Priyakumari. I, Assistant Professor, Department of Floriculture and Landscape Architecture and Ms. Sindura K.P., Assistant Professor, Department of Plant Biotechnology for their generous and timely help, support, suggestions and passionate approach rendered during the period of research work.

I consider it as my privilege to express my deep-felt gratitude to Dr. Jayalekshmi V. G., Professor (RC) and Head, Department of Seed Science and Technology, for her valuable suggestions, technical advices and incessant motivation throughout the research work.

I am deeply grateful to my beloved teachers, Dr. Lekha Rani. C, Dr. Mareen Abraham and Dr. Gayathri G for their generous support, timely advice and constant encouragement offered to me during the course of my study and research work.

I would like to express my sincere gratitude to Dr. Kiran A.G, Dr. V. L. Sheela for their whole hearted cooperation and support rendered throughout the course work and research programme.

My heartfelt thanks to Dr. M Saleem, Dr. Radhika B. J, Dr. Radha R. K, Lekshmi chechi, Shibin chettan, Aswathy chechi of Jawarharlal Nehru Tropical Botanical Garden and Research Institute, Palode for sharing their practical experience in tissue culture and flow cytometry analysis, which proved to be of immense help in successful completion of my research programme.

I extend my profound gratitude to Mrs. Indu Ramachandran of Rajiv Gandhi Institute for Biotechonology, Poojapura for her immense support in execution of flow cytometry analysis with utmost patience and valuable advice which helped in the analysis of the data and interpretation of results of the research work.

No choice of words will suffice to express thanks to my loving friends Arya, Anandhu, Midhu, Bindhya, Visakh, Akhil, Amrutha, Aravind, Priyanka, Priti for being with me from beginning of course study till now and played a big part in completion of the research programme. Without their persistent help, this would not have been possible.

Words are inadequate to express my thanks to Anandettan, Roshin chechi, Shahiba chechi, Chacko chettan for being the best seniors ever and for their unbound love, guidance and big-hearted support.

I wish to express my heartfelt thanks to my beloved seniors Niji chechi, Amrutha chechi, Arya chechi, Anju chechi, Bhaskar chettan, Smera chechi, Greeshma chechi, Reshma chechi, Mythri chechi, Darshana chechi, Thouseem chechi, Asoontha chechi, Stephan chettan, Bipin chettan for their love, care, support and timely guidance. My loving gratitude to Parvathy, Krishnendu, Akshay, Ninitha, KKP, Rabeen, Ashik, Aparna, Krishnasree, Sheethal, Arunima and to all other junior batch students. It is my pleasure to express my special thanks to office assistants and lab assistants of my department for their sincere cooperation and kindly approach offered during the study period.

I am thankful to Kerala Agricultural University for the technical and financial assistance for carrying out my study and research work. I would like to express my thanks to Librarian, library staffs, farm labourers and all other staffs of COA, Vellayani for their support and assistance during the study period.

It would be impossible to list out all those who have helped me in one way or another in successful completion of this work. I once again express my heartfelt thanks to all those who helped me in completing this research work in time.

Words are not enough to express my whole-hearted and affectionate gratitude to my Amma, Athira chechi and Achan for their unconditional love and support bestowed on me throughout my career.

Revathi B. S

CONTENTS

Sl. No.	CHAPTER	Page No.
1.	INTRODUCTION	
2.	REVIEW OF LITERATURE	
3.	MATERIALS AND METHODS	
4.	RESULTS	
5.	DISCUSSION	
6.	SUMMARY	
7.	REFERENCES	
	APPENDIX	
	ABSTRACT	

LIST OF TABLES

Table No.	Title	Page No.
1.	Treatments to assess the effect of different surface sterilization methods	
2.	Treatments to evaluate the effect of media and hormones on Callus induction	
3.	Treatments for induction of heteroploidy in *Dendrobium crumenatum* via *in vitro* technique	
4.	Treatments for induction of heteroploidy in *Dendrobium crumenatum* via *in vivo* technique	
5.	Effect of surface sterilization treatments on survival percentage of explant in *Dendrobium crumenatum*	
6.	Effect of hormones on callus induction of *Dendrobium crumenatum* (Callus induction percentage)	
7.	Effect of hormones on callus induction of *Dendrobium crumenatum* (Days to callus induction)	
8.	Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage survival of PLBs after colchicine treatment)	
9.	Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage of cultures showing response after colchicine treatment)	
10.	Effect of colchicine on PLBs of *Dendrobium crumenatum* (Days for shoot formation)	
11.	Effect of colchicine on PLBs of *Dendrobium crumenatum* (Days for shoot multiplication)	
12.	Effect of colchicine on PLBs of *Dendrobium crumenatum* (Number of shoots per culture)	

13.	Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage survival of cultures at multiplication stage)	
14.	Effect of colchicine treatments on *Dendrobium crumenatum* plantlets (Percentage survival of the plants after colchicine treatment)	
15.	Effect of colchicine treatments on *Dendrobium crumenatum* plantlets (Percentage of plants showing response after colchicine treatment.)	
16.	Analysis of variance of characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment	
17.	Mean performance of characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment	
18.	Phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) of seven characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment	
19a.	Phenotypic correlation of seven characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment.	
19b.	Genotypic correlation of seven characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment.	
20a.	Flow cytometry analysis of *in vitro* colchicine treated *Dendrobium crumenatum* samples	
20b.	Flow cytometry analysis of *in vivo* colchicine treated *Dendrobium crumenatum* samples	

21.	Common colchicine treatments from both *in vitro* and *in vivo* experiment	
22.	Comparison of the flow cytometry results of common treatments in *in vitro* and *in vivo* experiment for inducing heteroploidy in *Dendrobium crumenatum.*	

LIST OF FIGURES

Fig. No.	Title	Pages Between
1.	Effect of surface sterilization treatments on survival percentage of explant in *Dendrobium crumenatum*	
2.	Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage survival of PLBs after colchicine treatment)	
3.	Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage of cultures showing response after colchicine treatment)	
4.	Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage survival of cultures at multiplication stage)	
5.	Mean performance of shoot length (cm) after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment.	
6.	Mean performance of shoot diameter (cm) after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment	
7.	Mean performance of pseudobulb height (cm) after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment.	
8.	Mean performance of pseudobulb diameter after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment	
9.	Mean performance of number of leaves after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment	

10.	Mean performance of length of leaf after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment	
11.	Mean performance of width of leaf after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment	
12.	The flow cytometry histogram obtained for the control (diploid), mixoploid, and tetraploid sample.	
13.	Flow cytometry analysis of *in vitro* colchicine treated *Dendrobium crumenatum* samples	
14.	Flow cytometry analysis of *in vivo* colchicine treated *Dendrobium crumenatum* samples	
15a.	Flow cytometry results of percentage of mixoploids obtained from common treatments in *in vitro* and *in vivo* experiment for inducing heteroploidy in *Dendrobium crumenatum.*	
15b.	Flow cytometry results of percentage of tetraploids obtained from common treatments in *in vitro* and *in vivo* experiment for inducing heteroploidy in *Dendrobium crumenatum.*	

LIST OF PLATES

Fig. No.	Title	Pages Between
1.	*Dendrobium crumenatum*	
2.	*Dendrobium crumenatum in vivo* colchicine treatment	
3.	*In vivo* colchicine treated *Dendrobium crumenatum* plantlets	
4.	Inoculation of explant of *Dendrobium crumenatum* on culture medium	
5.	Initiation of bud of *Dendrobium crumenatum* on culture medium (4 weeks after inoculation)	
6.	Callus initiation from explant of *Dendrobium crumenatum* (8 weeks after inoculation of explant)	
7.	Callus proliferation from explant of *Dendrobium crumenatum* (10 weeks after inoculation of explant)	
8.	PLB from explant of *Dendrobium crumenatum* (12 weeks after inoculation of explant)	
9.	Shoot regeneration response of *Dendrobium crumenatum* after colchicine treatment (5 weeks after colchicine treatment of PLBs)	

LIST OF ABBREVIATIONS AND SYMBOLS USED

2,4-D	2,4-Dichlorophenoxyacetic acid
DAPI	4′,6-diamidino-2-phenylindole
AT	Adenine-Thymine
ANOVA	Analysis of Variance
et al.	And others
etc	And the rest
BA	Benzyl Adenine
BAP	Benzylaminopurine
cm	Centimetre
CRD	Completely Randomised Design
Con.	Concentration
CD	Critical Difference
Cv	Cultivar
°	Degree
°C	Degree Celsius
d.f	Degrees of freedom
D.	*Dendrobium*
DNA	Deoxyribonucleic acid

$Na_2HPO_4 . 12 H_2O$	Disodium hydrogen phosphate dodecahydrate
Fig.	Figure
FCM	Flow cytometry analysis
FACS	Fluorescence-activated single cell sorting
GCV	Genotypic coefficient of variation
g	Gram
$g\ L^{-1}$	Gram per litre
gL^{-1}	Gram per litre
>	Greater than
½ MS	Half strength Murashige and Skoog medium
hrs	Hours
HCl	Hydrochloric acid
IAA	Indole acetic acid
IBA	Indole butyric acid
KAU	Kerala Agricultural University
Kg	Kilo gram
$Kgcm^{-2}$	Kilogram per square centimetre
<	Less than
$MgCl_2$	Magnesium chloride

$HgCl_2$	Mercuric Chloride
µL	Microlitre
µm	Micrometre
µM	Micromolar
mg	Milligram
mg g^{-1}	Milligram per gram
mg L^{-1}	Milligram per litre
$mgmL^{-1}$	Milligram per millilitre
mL	Millilitre
mL L^{-1}	Millilitre per litre
mm	Millimetre
min	Minutes
M	Molar
MS	Murashige and Skoog medium
viz.	Namely
NAA	Naphthalene acetic acid
N	Normal
No.	Number
ppm	Parts per million

%	Per cent
g^{-1}	Per gram
L^{-1}	Per litre
PCV	Phenotypic coefficient of variation
PGR	Plant growth regulator
PI	Propidium iodide
PLB	Protocorm like bodies
RNA	Ribonucleic acid
Sl.	Serial
Sl. No.	Serial Number
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
sp. or spp.	Species (Singular and Plural)
S. E	Standard Error
i.e.	That is
Via	Through
Lux	Unit of illumination
VW	Vacin and Went medium
v/v	Volume / volume

Introduction

1. INTRODUCTION

Orchids belong to the Family Orchidaceae which is the largest and highly evolved family, accounting for almost 10% of flowering plants. This family comprises of about 35,000 species belonging to 850 genera. Orchids have a high economic value both in commercial horticulture, as potted plants or cut flowers, and in traditional Chinese medicine (Chase *et al.,* 2015; Grosso *et al*., 2017).

The Orchids especially *Dendrobium* occupies the foremost position in floriculture industry due to its beautiful colours, its ability to produce flowers continuously and a prolonged post-harvest life in comparison to other species. They have high potential to be used as cut flowers, potted plants and for landscaping purposes (Sugapriya *et al*., 2012).

*Dendrobium*s are usually propagated sexually by seeds and asexually by division of offshoots. Besides the low or no seed setting and germination, the seedling progenies are heterozygous, and do not warrant true-to-type plants of hybrid cultivars. Conventional vegetative propagation is beset with slow multiplication rate, and inadequate to provide sufficient clones within short timeframe (Martin and Madassery, 2006).

A large export market is available if novel varieties with commercial traits are developed and marketed properly. Although India has diversified climate and progressive farming technology, commercial orchid industry is in infancy. This is due to non-availability of suitable planting material for large scale cultivation, inadequate technology for commercial multiplication and lack of commercial approach in cultivation.

Orchid growing has now achieved the transition from a hobby to agri-business. It has created a huge impact in international market. However, the production volume is inadequate to meet the increasing demand. Therefore, development of novel varieties of orchids and their micropropagation will contribute to orchid industry.

Dendrobium crumenatum is a common epiphytic orchid, which is commonly known as ‘pigeon orchid’ or ‘sparrow orchid’. It is called so because, the white flower

with the tapering mentum resembles a bird's diminutive head, the dorsal sepal resembles the bird's tail, and the lateral sepals and lateral petals gives the impression of fluttering wings of pigeon in flight. (Leong and Wee, 2013).

It is a tropical epiphytic plant of 40-100 cm in size and usually grows on old trees under open conditions. The small flowers of 3.5-4.5 cm size are produced in clusters along the upper part of stalk-like plant body portions. The flower has a strong fragrance and is pure glittering white with the lip having a bright yellow disc (Meesawat and Kanchanapoom, 2007).

This orchid species has many uncommon features of flowering, for instance, gregarious flowering (Holttum, 1964; Bernier *et al*., 1993). The flowers bloom exactly 9 days after a rainfall event. All plants in a certain area start to flower at the same time and are at the same stage of floral development throughout the year, but the blooms last for only a full calendar day (Holttum, 1964; Goh *et al*., 1982). The time required to complete final floral development is constant in all individuals and development can be predicted to take place at a specific time (Endress, 1994).

Crop improvement can be done through, conventional, nonconventional or advanced breeding techniques and polyploidy breeding is one among them. Polyploidy can be induced in orchids without using antimitotic agents by exploiting the endopolyploidy cells. It can also be induced using antimitotic agents through *in vivo* and *in vitro* technique. *In vitro* colchicine induction can be done in cultures using explants viz., stem nodes, seeds, axillary buds, pseudobulb, transverse thin cell layer from protocorms, leaves etc. *In vivo* technique can be carried out by using small plantlets of orchids, plant cuttings etc.

Polyploid plants may exhibit increased vigor, increment in plant organs ("gigas" effect), higher yield level, product quality improvement, durability, improved resistance to both biotic and abiotic stresses, production of seedless varieties, and protection against deleterious mutations (Sattler *et al*., 2016; Huy *et al*., 2019).

Polyploidization has an important role in the hybridization, improvement and production of premium species and varieties in the orchid floriculture industry (Miguel and Leonhardt, 2011; Huy *et al*., 2019). Polyploid orchids had thicker stems, thicker

and wider leaves, and higher-quality flowers with intensified color patterns (Miguel and Leonhardt, 2011).

Orchid culture has been benefited with the fertility restoration of hybrids with problems of chromosome pairing and assortment during meiosis. Besides increasing genetic variability, the induced polyploidy results in flowers which are usually larger in size, with rounder conformation, and greater substance than the diploids (Wimber and Wimber, 1967). Dermen (1940) reported other changes, such as the intensification of color and fragrance. Because of these traits, breeders have selected and used polyploid forms as parents.

Chromosome doubling is an acknowledged mechanism to obtain different ploidy levels in plants, and it is usually achieved by chemical treatments using anti-microtubule agents such as colchicine or oryzalin. The development of a proper method for *in vitro* polyploidization requires the conduction of several tests to obtain the most suitable combination of antimitotic agent, concentration and exposure time for each species (Dhooghe *et al*., 2011). Clonal propagation using stem nodal explants from young shoots and kiekies (offshoots on spikes or stem), has been a reliable and efficient method for orchid micropropagation. This method utilizes the enhanced release of axillary buds for shoot multiplication without destroying the shoot tip and mother plants (Priyakumari and George, 2011).

For the identification of polyploid individuals, direct and indirect methods can be employed. Indirect methods involve the examination of physiological and/or morphological traits, especially those related with stomata. The direct procedures include chromosome counting and ploidy level determination by flow cytometry.

In this context, the present investigation was undertaken with the following objectives.

- To develop heteroploids in *Dendrobium crumenatum* using colchicine through *in vitro* and *in vivo* method.
- To confirm the ploidy change in *in vitro* and *in vivo* colchicine treated *Dendrobium crumenatum* using flow cytometry analysis.

Review of Literature

2. REVIEW OF LITERATURE

Orchids account for a large share in global floriculture trade both as cut flowers and potted plants. Among various orchids, *Dendrobium* has become increasingly popular due to its floriferous flower sprays, year-round availability, wide range of colours, sizes, shapes and long flowering life of several weeks to months.

Induction of polyploidy has been an important method for improvement of orchids to create increasing interest and demand in the floriculture market. The improvement of characters like colour, flower shape, longevity, fragrance, and the creation of novel variations in *Dendrobium* orchids through polyploidy can increase its commercial value and help produce outstanding hybrids. In light of this, the present investigation is formulated to induce heteroploidy in *Dendrobium crumenatum* through *in vitro and in vivo* techniques and the subsequent analysis using flow cytometry. A brief review of the works relevant to the study is presented below.

2.1 POLYPLOIDY IN ORCHIDS

There has been competition among growers to introduce new orchids that are made from interspecific and intergeneric hybrids. High infertility rates are common in the plants derived from these hybrids (Watrous and Wimber, 1988). Ketsa *et al*. (2001) observed that tetraploids showed an improved senescence in flowers and inflorescence compared to diploids of *Dendrobium* "Caesar". The rearrangement during chromosomal doubling might directly or indirectly affect the regeneration ability (Lin *et al*., 2001). Chromosome doubling can lead to chromosomal rearrangements, translocations, inversions and even loss or retention of the duplicated genes (Blanc and Wolfe, 2004).

Polyploidy induction can restore fertility, particularly in allodiploids since there is doubling of the chromosomal set and production of allotetraploid plants. This duplication allows pairing of homologous chromosomes to re-establish during gametogenesis (Ranney, 2006). Polyploidy plays a significant

role in breeding of orchids (Miguel and Leonhardt, 2011). Production of plant hybrids via polyploidization improves floral attributes, such as colour, flowering time, fragrance, and shelf life in the post-harvest period (Vichiato *et al*., 2014).

Eng and Ho (2019) reported that the efficiency of polyploidy induction system also depends on the advancement of propagation techniques of the species which consist of *in vitro*, and *in vivo* systems. It is believed that polyploidy induction under *in vitro* condition is more effective than that in *in vivo* condition (Huy *et al*. 2019).

2.2 *Dendrobium crumenatum*

Dendrobium crumenatum Swartz., the Pigeon orchid (2n=38), a common epiphytic, sympodial orchid widely distributed in South East Asia such as India, Sri Lanka, Myanmar, Indo-China, Malay Peninsula, Philippines and Taiwan, has very unique, attractive, white and fragrant flowers (Leong and Wee, 2013).

2.3 IN VITRO POLYPLOIDY INDUCTION

For induction of polyploidy, various plant parts such as apical meristem, flower bud, root tip and particularly seed can be used. In orchids, the most widely applied plant parts to induce polyploidy *in vitro* are protocorms and PLBs (Miguel and Leonhardt, 2011; Zakizadeh *et al*., 2020). Yenchon and Te-chato (2012) observed that both colchicine concentration and time of exposure influences the differentiation of PLBs. Protocorm is a small tuber-like body formed by germinating orchid seed. It has the potential to develop into a fully grown orchid plant. PLBs are structures similar to protocorms but are formed by tissue explants and/or callus *in vitro* (Zakizadeh *et al*., 2020).

2.3.1 Micropropagation

Prakash *et al*. (1996) reviewed the methods of tissue culture of orchids and recorded that the regeneration of orchids by tissue culture occurs through two main pathways (1) direct differentiation of protocorm like bodies (PLBs)

from cultured tissues and organs and their development into plantlets and (2) indirect differentiation of PLBs from explant tissue through an intermediary callus phase.

Meesawat and Kanjanapoom (2002) were successful enough in developing a protocol for micropropagation of *Dendrobium crumenatum* (pigeon orchid) via somatic organogenesis. Axillary bud-derived callus was induced on Vacin and Went (VW) solid medium (Vacin and Went, 1949) containing 0.1 mgL^{-1} NAA and 1 mgL^{-1} BA within 4 weeks of culture.

The induction of totipotent callus, somatic embryogenesis from callus, direct induction of axillary shoots (Pyati *et al*., 2002), direct somatic embryogenesis (Da Silva and Tanaka, 2006), and direct regeneration through shoot-bud formation is possible in *Dendrobium* from different explants *viz*. leaf, nodal, flower stalk, root, rhizome and pseudobulb segments, thin-sections of shoot tips, leaves, protocorms and roots (Martin and Madassery, 2006). The callus mediated regeneration is one of the favourite techniques for rapid *in vitro* propagation and for biotechnological applications that are widely used in many plants, including orchids (Tokuhara and Mill, 2001; Debnath *et al*., 2006).

In orchids, PLB regeneration from explants such as shoot tips, root tips and stem nodal segments is one of the accepted methods for *in vitro* propagation. (Dohling *et al*., 2012). The explants most commonly used in micropropagation of *Dendrobium*, include nodal segments (23.0 %), *in vitro*-derived PLBs (21.8 %), shoot tips (11.5%), protocorms (8.0 %), transverse thin cell layers from protocorms and young stems (8.0 %), leaves (5.7 %), pseudobulbs (4.6 %), *in vitro* seedlings (4.6 %), axillary buds (3.4 %), and callus (1.1 %) (Da Silva *et al*., 2015).

2.3.1.1 Sterilization

Bacterial and fungal contamination are serious problems that are to be overcome during micropropagation. Surface sterilizing agents like sodium hypochlorite is widely used for this purpose but sometimes efficiency of using single surface sterilant is doubtful (Hu and Wang, 1983). Stronger sterilizing

agents such as mercuric chloride can be used alone or in combination with other sterilizing agents inorder to obtain contamination free cultures.

Meesawat and Kanchanapoom (2002) used the nodal segments having an axillary bud as the explant for *Dendrobium crumenatum* micropropagation. These nodal segments (about 3-4 cm in length) were first washed with tap water containing few drops of detergent, followed by rinsing with water for 2-3 times. After removing their sheaths, they were surface sterilized with 20% Clorox containing 1-2 drops of Tween 20 for 20 minutes. Finally, the excised buds were washed with sterile distilled water for 2-3times.

Surface sterilization using the agents, 70% ethanol for 5 minutes and 1% NaOCl for 30 minutes followed by washing the explant with distilled water was found successful in *Dendrobium nobile* (Junior *et al*., 2011). Dohling *et al*. (2012) reported the use of 10 % NaOCl solution for 10 minutes followed by 0.1 % $HgCl_2$ treatment for 2 minutes for surface sterilization in *Dendrobium longicornu*. In a study conducted by Li *et al*. (2013), the combination of surface sterilization agents 70% ethanol for 30-45 seconds and 0.1 per cent $HgCl_2$ for 5-8 minutes were found to be effective in *Dendrobium fimbriatum*.

Sterilization of stem explants (1–2 cm long), each comprising a node and axillary bud, were carried out using mild detergent followed by washing in running tap water for 15–20 minutes and rinsing with distilled water was adopted by Bhattacharya *et al.* (2016). In the disinfection protocol followed by Riva *et al.* (2016), the explants were treated with 70% ethanol for 1- 2 minutes in an aseptic condition. They were then rinsed with sterile distilled water for 3-4 times. After ethanol treatment, they were immersed in 0.1% $HgCl_2$ and added 3-4 drops of Tween-20 for about 4-5 minutes with constant shaking. Then explants were further washed 3-4 times with sterile distilled water to make the materials free from chemical.

2.3.1.2 Media Components

2.3.1.2.1 Carbon

The exact requirement of sucrose (carbon) in orchid micropropagation varies from species to species. (Kano,1965). In orchid tissue culture, the most commonly used sucrose concentrations are 20.0 and 30.0 gL^{-1} (Arditti, 1977). Carbon is an essential component for plant tissue culture as it serves as the energy source to the plants particularly during the early stage of tissue culture when plantlets are not able to photosynthesize their own food (Al-Khateeb, 2008). The most preferred carbon source in plant tissue culture is sucrose. Sucrose is a major carbohydrate source which supply energy to culture cells and it is efficiently uptaken across the plasma membrane. (Kumaraswami *et al*., 2010).

2.3.1.2.2 Basal Medium

Sagawa and Shoji (1967) used modified VW medium for efficient protocorm development and vigorous plant growth in *Dendrobium*. Sudeep (1994) observed that, VW and half MS media are best for bud initiation in *Dendrobium*. High percentage of PLBs of *Aranda, Mokara* and *Dendrobium* were obtained when it was cultured in VW medium (Chan and Lee, 1996) Kuriakose (1997) observed that minimum number of days for bud initiation in *Dendrobium* cv. Sonia was recorded in VW basal medium.

Sivamani (2004) reported that among the various basal media tried for stem nodal segment explants of *Dendrobium* hybrids, early culture establishment and rapid growth recording minimum days for PLB initiation and greening and first leaf initiation was noticed in VW medium.

2.3.1.2.3 PGRs

The combination of kinetin at 1.0 mgL^{-1} and NAA at 0.1 $mgL^{-1}$was reported to produce vigorous and maximum shoot numbers in *Cymbidium pendulum* (Talukdar *et al*., 2002). Kim *et al.* (2003) reported that presence of BA in the culture medium is necessary for shoot regeneration. The highest

percentages of explants with PLBs were observed in Vacin and Went (VW) basal medium supplemented with 0.1mgL^{-1} NAA and 0.5 mgL^{-1} BA for 'Earsakul' (53.3%) and 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} BA for 'Omyapink' and 0.5 mgL^{-1} NAA (46.7%) for 'Semialba' (46.7%) varieties of *Dendrobium.*

Leaf explants of *Dendrobium formosum* showed early response for callus initiation and PLB formation in culture media supplemented with 0.5 mgL^{-1} 2,4-D (Nasiruddin *et al*., 2003). The combination of kinetin at 1.0 mgL^{-1} along with NAA at 0.1 mgL^{-1} induced highest number of multiple shoots in *Dendrobium* cv. Betty Ho. (Kurup *et al*., 2005).

Half strength MS medium fortified with kinetin (0.5 –5.0mgL^{-1}) singly and also in combination with NAA (0.1 -0.5 mgL^{-1}) induced bud break from flower stalk node explants of *Dendrobium* hybrids Sonia -17 and Sonia -28 (Martin and Madassery, 2006). Callus induction from protocorm segment was enhanced by growth hormone like BA. It was reported that BA succeeded in inducing callus on *D. fimbriatum* (Roy and Banarjee, 2003), and *D. candidum* (Zhao *et al*., 2007).

Das *et al*. (2008) reported that, the fastest initiation of protocorm like bodies (PLBs) were observed in cultures containing MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mgL^{-1} 2,4-D in *Dendrobium lituiflorum* using axillary bud as the explant. For multiple shooting in *Dendrobium* cv. Rungnappa Red, 2.0 mgL^{-1} kinetin and 2.0 mgL^{-1} NAA, gave early shoot multiplication, while 1.0 mgL^{-1} BA and 0.1-0.5 mgL^{-1} NAA gave maximum number of shoots. In cv. Miss Snow White, the combination of 1.0 mgL^{-1} kinetin and 0.1 mgL^{-1} NAA gave the earliest shoot multiplication response. In *Dendrobium* Miss Snow White, combination of kinetin 1.0 mgL^{-1}+ NAA 0.1 mgL^{-1} gave early bud initiation (Priyakumari, 2008).

The most commonly used plant growth regulators (PGRs) in *Dendrobium* micropropagation are BA (69.0 %) and NAA (65.5 %), and their use in combinations accounts for 56.9 % (Da Silva *et al*., 2015). Uddain *et al*. (2015) noticed that the percentage of PLBs induced was the highest in half

strength MS semi-solid medium supplemented with 1.0 mgL^{-1} BAP and 0.5 mgL^{-1} NAA in *Dendrobium* Broga Giant. MS medium supplemented with 2.0 mgL^{-1} BA was observed with best results for rapid shoot induction and regeneration of *D. bensoniae* using nodal explants (Riva *et al.,* 2016).

Refish *et al*. (2016) concluded that callus could be induced on the medium containing 2,4-D (0.5 and 1.0 mgL^{-1}) with high induction rate above 90% and supply of cytokinin could also benefit to the callus induction. Bhattacharyya *et al*. (2016) noticed that PLB formation at concentrations of 1mgL^{-1} BAP and 0.5 mgL^{-1} NAA produced 45 % PLBs in *Dendrobile nobile.*

2.3.1.3 Days for Bud Initiation

Initiation of axillary buds of *Dendrobium* sp. was observed within 4 weeks of culture in VW media by Meesawat and Kanchanapoom (2002). Priyakumari (2008) noticed that in *Dendrobium* cv. Miss Snow White, bud initiation occurred within 10.33 days from stem nodal explants, cultured in media supplemented with 1.0 mgL^{-1} kinetin and 0.1 mgL^{-1} NAA and the mean number of days for bud initiation ranged from 10.33-36 days. Also in *Dendrobium* cvs. Rungnappa and Earsakul, the mean number of days for initiation of bud varied from 7.33-30.0 and 9.67-22.50 days respectively.

2.3.1.4 Callus Induction

In a study conducted by Churchill *et al*. (1973) in *Laeliocattleya* plants, callus cultures were obtained when cultured in Heller's medium modified by 30 gL^{-1} sucrose which was supplemented with 1 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BAP.

The callus proliferation was observed after one month when cultures were transferred to VW media containing 1 mgL^{-1} BA and 0.1 mgL^{-1} NAA in *Dendrobium* sp. (Meesawat and Kanchanapoom, 2002). Initiation of callus from leaf explants of *Dendrobium formosum* using 0.5 mgL^{-1} 2,4-D after 9.74 days of inoculation was reported by Nasiruddin *et al*. (2003).

2.3.1.5 PLB Formation

The suitability of auxins for PLB induction was reported in a study conducted by Kanjilal *et al*. (1999) using stem nodal segments in *Dendrobium moschatum*. Meesawat and Kanchanapoom (2002) observed that PLB formation occurred within 10th week of culturing in *Dendrobium* sp. Das *et al*. (2008) noticed that fastest initiation of protocorm like bodies (PLBs) was observed within 13.3 days in cultures containing MS medium supplemented with auxin (2,4-D) in *Dendrobium lituiflorum* using auxiliary bud as the explant.

The highest proliferation rate of 8.7% of PLBs was obtained in cultures supplemented with 1.0 mgL^{-1} BAP and 0.5 mgL^{-1} NAA in *Dendrobium* Broga Giant (Uddain *et al*. 2015). Bhattacharyya *et al*. (2016) noticed that PLB formation at hormonal concentrations 1mgL^{-1} BAP and 0.5 NAA mgL^{-1} produced 45 % PLBs in *Dendrobile nobile.*

Significant difference was noticed in the days for PLB initiation, proliferation and regeneration in case of induced tetraploids and normal diploids. Response was found to be slower in case of tetraploids (17.4 days) when compared to diploids (13.4 days). (Pham *et al*., 2019).

2.3.1.6 Shoot Formation

Meesawat and Kanchanapoom (2002) reported that the shoot differentiation was observed after 16 weeks of the inoculation of explant in *Dendrobium* sp. Mean number of regenerated shoots was noted to be 6.13 when cultured in VW media with PGRs 1 mgL^{-1} BA and 0.1 mgL^{-1} NAA for *Dendrobium crumenatum.* Shoot proliferation is enhanced under the influence of BA by stimulation of quick cell divisions for the induction of large number of multiple shoots (Yakimova *et al*., 2000; Ronzhina, 2003; Hameed *et al*., 2006)

Among the combinations of cytokinin and auxins tried, BA at 0.5, 1.0 and 2.0mgL^{-1} along with NAA at 0.1, 0.5 and 1.0 mgL^{-1} produced more number of shoots. The maximum shoot numbers (3.67) were produced at combination of BA 1.0 mgL^{-1} with 0.1 mgL^{-1} or 0.5 mgL^{-1} NAA in *Dendrobium.* In

Dendrobium cv. Miss Snow White, the minimum days (9.3) for shoot initiation was obtained in cultures supplemented with kinetin 1.0 mgL^{-1} and NAA 0.1 mgL^{-1} (Priyakumari, 2008). The proliferated shoots ranged from 2.36±0.4 to 4.35±1.45 per explant for different concentrations of colchicine *viz*., 0.01%, 0.02%, 0.03% and 0.04% in *Dendrobium chrysotoxum* cultured in media containing 1 mgL^{-1} NAA and 0.5 mgL^{-1} BA. (Atichart, 2013).

The regeneration and proliferation of multiple shoots are closely related with the type and concentration of cytokinins used (Amoo *et al*., 2014). Bunnag and Hongthongkham (2015) noticed that when colchicine treatment at 0.05% concentration for 4 days were given, only 20% of protocorms survived and produced shoots. Meanwhile, treatment with colchicine at 0.10% concentration for 3 or 4 days resulted in protocorm death in *Dendrobium* orchid. Shoot formation ranged from 0-22% at 0.10% concentration of colchicine for 1-4 days.

Choopeng *et al*. (2019) reported that high concentration of colchicine with longer duration of application yielded low survival rate of cultured explant subsequent to shoot development. Shoot formation percentage ranged from 33.33%-100% for different doses of colchicine *viz*., 0.01%, 0.05%, 0.10%.

Pham *et al.* (2019) observed that shoot regeneration from PLBs showed difference in diploids and tetraploids Shoot initiation took nearly 14 days in diploids and about 16 days in the tetraploids The mean number of shoots regenerated from diploid PLBs (2.90) was significantly higher than that regenerated from tetraploid PLBs (1.20). The time required for shoot appearance from PLBs increased, but the number of shoots produced per PLB was reduced in tetraploid cultivars.

2.4 COLCHICINE

The method of artificial induction of polyploidy using colchicine was developed by Blakeslee and Avery (1937). The most commonly used chemical for polyploidy induction is colchicine and thus the method is referred as colchiploidization and the developed polyploids can be referred as colchiploids. Colchicine, a poisonous alkaloid extracted from the seeds and corms of

Colchicum autumnale, is used in various times and concentrations for ploidy manipulations. It is highly soluble in water and desirable target in plant cell is microtubule. At anaphase, chromatic fibre cannot split and its movement to polar end is restricted, so the chromosomes get increased from 2x to 4x, or become polyploid (Starr and Taggart, 1995).

High colchicine concentration and long duration of the treatment reduce the survival rate of PLBs and increase the percentage of polyploid plantlets (Griesbach, 1981; Atichart and Bunnag, 2007; Silva *et al.*, 2000). Sarathum *et al*. (2010) also observed that, high mortality rate in orchids occurred by application of high concentrations of colchicine.

Colchicine as an antimitotic agent has been applied for the improvement of horticultural characteristics for a variety of species by polyploidy induction (Dhooghe *et al*., 2011). The induction of polyploidy in orchid can produce large long-lasting flowers with more rounded floral shapes having intense colours (Miguel and Leonhardt, 2011). In the *Dendrobium* genus, autopolyploidy results in increased floral pieces with larger size and width of petals and sepals, as well as greater durability of flowering, greater fertility and a decreased number of flowers per pseudobulb (Chaicharoen 1995; Liao *et al*., 2012).

Vichiato *et al.* (2014) observed that the induced polyploidization resulted in the following morphological effects i.e., a) increased number of internodes (19.9%) and floral pieces, with greater height of the flower (4.5%) and width of the lip (18.5%) b) decreased number of flowers per pseudobulb (40.8%) and the diameter of the pseudobulb (64.9%) and c) delayed flowering.

Choopeng *et al*. (2019) concluded that the application of colchicine for polyploidy induction in orchid development is the important method for plant development in order to increase sizes of flower, pseudobulb or other desired characteristics. Zakizadeh *et al*. (2020) reported that when different concentrations of colchicine (0.05, 0.10 and 0.15% for 3 days) and oryzalin (14.40 and 28.90 µM for 4 days) was given as treatment to five-month-old plantlets derived from protocorm-like bodies produced *in vitro*, the highest

survival rate (95%) was found in plantlets treated with 0.05% colchicine. The highest percentage (53.33%) of polyploidy induction was achieved by exposure to 0.15% colchicine.

Mohammadi *et al*. (2021) concluded from their study that colchicine treatment on fully-developed plantlets derived from *in vitro* grown PLBs showed decreased survival percentage in line with increased colchicine concentration during *in vivo* induction.

2.4.1. Effective Colchicine Concentration for Heteroploidy Induction

The duration of exposure and concentration of colchicine treatment are the important factors that need to be determined for each type of material. (Dermen, 1940). Sanguthai *et al.* (1973) observed a higher production of hexaploid and mixoploid plants when *Dendrobium* PLBs were treated with 0.05, 0.10, and 0.15% of colchicine. Among these, the most effective concentration for ploidy change was found to be 0.10%.

Griesbach (1981) could obtain 50% of tetraploid *Phalaenopsis* using 0.05% of colchicine, using a more prolonged exposure time (10-14 days). Watrous and Wimber (1988) identified that 0.05% colchicine for 3 days was the best combination for development of tetraploids from shoots of *Vanda* Miss Joaquim and in *Paphiopedilum*, treating protocorms with 0.05% of colchicine produced more than 50% of tetraploid plants.

Silva *et al.* (2000) tried different concentrations of the colchicine (0.00, 0.05, 0.10 and 0.20%) as well as two treatment durations of exposure (4 or 8 days) in PLBs, to determine the best treatment for the induction of tetraploid plants in two *Cattleya intermedia* clones. Of the 3 colchicine levels applied, 0.05 and 0.10% of colchicine treatments for 4 days appeared to be effective on the production of mixoploids and tetraploids and the percentage of tetraploids obtained were in the range 11-75% and 20-60% respectively.

Petchang (2010) observed that treatment of protocorms with 0.10% colchicine for 4 days was found to be the best concentration to induce polyploidy in *Dendrobium draconis*. Sarathum *et al*. (2010) concluded that

treatment of colchicine at concentrations of 0.025 - 0.1% for 3–21 days produce tetraploids in *Dendrobium scabrilingue*. Tang *et al*. (2010) demonstrated that the highest tetraploid induction rate of 32% with death rate of 22% occurred when the protocorm of *D. candidum* was treated with 0.2% colchicine for 48 h.

Yenchon and Te-chato (2012) reported that when PLBs were treated with colchicine at different concentrations i.e., 0.05, 0.10, 0.15 and 0.20% in liquid MS medium for 24 and 48 hrs, the maximum polyploid plants were obtained with the application of 0.20% colchicine for 2 days in PLBs of *Dendrobium formosum.* Atichart (2013) observed that when PLBs of diploid *Dendrobium chrysotoxum* were treated with 0.01, 0.02, 0.03, 0.04, and 0.05% colchicine for 1, 2, 3, 4 and 5 days. The most effective treatment was noted in 0.04% colchicine for 1 day which resulted in about 84% surviving PLBs and with 47 % of tetraploid orchids, as measured by flow cytometry. At the same concentration of colchicine (0.04%) but when the time of exposure was increased to 2 days, 30% tetraploids and 46% mixoploids (2x+4x) were found among tested plantlets.

Successful tetraploid plants (17.85-31.49 %) with protocorm death rates of 17.3-64.16% were obtained, when *Dendrobium nobile* Lindl. were subjected to polyploidization (Xu *et al*., 2016). Grosso *et al*. (2017) reported that, using colchicine at a concentration of 0.025 and 0.075% for 3 days, a high percentage of polyploids, equal to 60 and 80%, respectively, were obtained and the lowest tetraploid percentage noticed was 20%. In addition, a high percentage of partially duplicated polyploids were also obtained in *Dendrobium* hybrids.

In general, the optimum colchicine concentration for polyploidy induction in orchids seems to be 0.01%-0.10%, while the optimum exposure time strongly depends on the type of explant and the method used for application (Sarathum *et al*., 2010; Huy *et al*., 2019). Huy *et al*. (2019) concluded that young tissues containing actively dividing cells are preferred to induce polyploidy.

Zakizadeh *et al*. (2020) observed that plantlets with changed chromosome number were obtained at 0.05–0.15% colchicine concentrations. The highest

percentage (26.60%) of polyploids were induced with 0.15% colchicine in *Dendrobium* 'Sonia'. The effectiveness of colchicine application and polyploid induction depends on some factors, especially the type of explant, exposure time and concentration.

2.4.2. Survival Rates After Colchicine Treatment

The mortality range of 0 to 44% was observed when PLBs of *Cattleya intermedia* clones were subjected to colchicine treatment (Silva *et al.,* 2000). Kim *et al*. (2003) observed the effect of different concentrations and treatment periods of colchicine on PLBs of *Cymbidium* orchid Showgirl 'Silky' and Mystery Island 'Silk Road' and reported that, at colchicine concentration of 50-500 mgL^{-1}, survival rates ranged from 63.3-97% and 23.3%-96.7% respectively.

Yenchon and Te-chato (2012) observed that at the concentration of 0.05%, survival rates of PLBs were approximately 80 and 67% after 24 and 48 h of treatment. The survival rates were below 50% when the concentration of colchicine reached 0.2% at both periods of treatment. Bunnag and Hongthongkham (2015) observed that survival rate ranged from 13.3 to 46 .67 % at 0.1% concentration of colchicine for a duration of 1-4 days and 56.67-90 % at 0.05% concentration of colchicine for a duration of 1-4 days.

Choopeng *et al.* (2019) noticed that when the nodal explants of *Dendrobium* hybrid were treated with 0.01%, 0.05% and 0.10% colchicine for 24 hours, it resulted in survival rate of 60.00%, 60.00%, and 13.33%, respectively. The explants treated with the same concentration of colchicine for 48 hours had the survival rate of 76.67% 16.67% and 6.67 %, respectively. When explants were treated with colchicine for 72 hours, the explants had lower survival rate of 13.33%, 6.67% and 16.67%, respectively.

In a study conducted by Zakizadeh *et al.* (2020), the mortality rate varied from 5.00 to 65.00% based on the dose of colchicine. Maximum mortality rate was observed with 0.15% colchicine. The highest survival rate (95%) was found in plantlets treated with 0.05% colchicine in *Dendrobium* orchid. Mohammadi *et al*. (2021) observed a survival percentage of range 50-

80 % when *P. amabilis* var. *grandiflora* Bateman was subjected to different doses of colchicine., *viz*., 0.05%, 0.01% and 0.15%.

2.4.3. Impact of Colchicine

Colchicine treated plants at an early stage exhibited slower rate of growth in *Phaius tankervillae* (Devi and Deka, 2000) and delayed organogenesis in *Rhododendron* hybrids (Vainola, 2000). Kermani *et al.* (2003) reported that size of stems, leaves and flowers of polyploid plants were larger than those observed for diploid plants. Thao *et al*. (2003) observed that while inducing polyploidy, although a number of cells in meristematic tissue became polyploid, many others might be unaffected and remain diploid. Thus, a mixture of normal and polyploidy cells can be found, and plantlets developed from both the cells were mixoploid.

In stem nodes of *Dendrobium* orchids, shoots regenerated from 0.1 per cent colchicine treated for 5 days, were found to be of unusual morphology characterized by shortened stem, reduced internodes, thicker and greener leaves and showed slow growth (Priyakumari, 2008; Bunnag and Hongthongkham, 2015).

High concentration of colchicine would be toxic to plant cell, which causes cellular imbalance and affect the internal process of cell causing plant death eventually. (Petchang, 2010; Blasco *et al*., 2015).

2.5 *IN VIVO* POLYPLOIDY INDUCTION

Ranney (2006) suggested that for the doubling of chromosome, seedlings can be soaked or the apical meristems can be submerged in different concentrations, frequencies or durations using a doubling agent. For the induction of polyploidy in *D. nobile*, Vichiato *et al*. (2007) used *D. nobile* diploid (2n = 38 chromosomes) plants of an average height of 5.0 cm and 3 leaves, which were then completely immersed in an aqueous colchicine solution. Constant air bubbling was provided using aquarium aerating pumps, to prevent plant damage caused by oxygen depletion.

Zakizadeh *et al*. (2020) reported a successful method for induction of polyploidy *in vivo* in *Dendrobium* 'Sonia' where the antimitotic agents colchicine and oryzalin were treated on five month old plantlets derived from protocorm like bodies produced *in vitro*. *In vivo* polyploidy induction of *Phalaenopsis amabilis* using colchicine was carried out using fully developed plantlets from *in vitro* grown PLBs was reported by Mohammadi *et al*. (2021).

2.5.1 Shoot Length

Vichiato *et al.* (2014) noticed that on colchicine treatment the tetraploid plants showed an increased plant height when compared to diploids. The average height of tetraploids was 26.42cm and that of diploids were 24.04cm. In a study conducted by Pham *et al.* (2019) in orchids, it was observed that, the average stem height of tetraploids was 4.20 cm while that of diploids were 5.43cm.

Zakizadeh *et al.* (2020) revealed that the growth of treated plantlets with colchicine was slower than that of control. The plantlets length decreased with increased colchicine concentration. The decreased growth was due to reduced rate of cell division due to colchicine. The plantlets length decreased with the increased colchicine concentration and the highest length was observed in diploids (Mohammadi *et al* 2021).

2.5.2 Shoot Diameter

Vichiato *et al.* (2014) observed a reduced value for stem diameter in case of tetraploids to that of diploids. Pham *et al.* (2019) noticed that stem diameter of tetraploids was greater than that of diploids. The average stem diameter of tetraploids was 0.42cm whereas for diploids it was 0.28cm. Choopeng *et al*. (2019) in *Dendrobium draconis* observed that stems of the colchicine treated groups were bigger than those of the control group.

2.5.3 Length and Diameter of Pseudobulb

Vichiato *et al.* (2014) observed that pseudobulb diameter of tetraploids were averaged to be 0.74cm and that of diploids were 1.22cm. Zakizadeh *et al.*

(2020) noticed that bulb length in tetraploids ranged from 17.84-19.84 mm whereas for diploids it was 20.10 mm. Bulb width in tetraploids was found to be 3.05mm whereas for diploids it was 2.33 ±0.12 mm in *Dendrobium* 'Sonia' treated with colchicine.

2.5.4 Leaf Number

Leaf number of putative tetraploid plantlets were 3.50 leaves per plant while in control diploid plantlets it was 2.80 leaves (Kerdsuwan and Te-chato, 2012). The average number of leaves in tetraploids were 4.53 whereas for diploids it was 4.93 when *Dendrobium nobile* was treated with colchicine (Vichiato *et al*., 2014).

Choopeng *et al*. (2019) in *Dendrobium draconis* observed that leaf number of the control and colchicine treated groups were not significantly different but the leaves were greener than the control group. Pham *et al*. (2019) noticed that leaf number of tetraploids were lesser than that of diploids. The average number of leaves in tetraploids were 5.40 whereas for diploids it was 5.70. Kazemi and Kaviani (2020) noticed an increase in leaf number after various doses of colchicine treatment when compared to untreated samples. Leaf number of treated samples was observed to be in the range of 3.66-5.33 cm, whereas incase of untreated sample, it was 4 cm. Mohammadi *et al*. (2021) observed an increase in leaf number in tetraploids when compared to diploids in *Phalaenopsis amabilis*.

2.5.5 Leaf Length and Width

Vichiato *et al*. (2014) observed that tetraploids showed decreased leaf length and width values than that of diploids. Kozemi and Kaviani (2020) noticed an increase in leaf length after various doses of colchicine treatment when compared to untreated samples. Leaf length of treated sample were observed to be in the range of 2.12-2.50 cm, whereas incase of untreated sample, it was 2.01 cm.

Zakizadeh *et al*. (2020) noticed that in antimitotic agent treated *Dendrobium* 'Sonia', leaf length and leaf width showed an increased value

when compared with diploids. Average leaf length of tetraploids was found to be 44.90mm while that of diploids were 39.10mm. Average leaf width of tetraploids was 8.43mm, while incase of diploids, it was 7.32mm. Mohammadi *et al*. (2021) observed an increase in leaf width in tetraploids when compared to diploids. The average width of leaves in tetraploid was 2.80 cm, while that of diploids was 1.87 cm.

2.6 STATISTICAL ANALYSIS

2.6.1 PCV and GCV

The characters such as length of cane, number of leaves/cane and width of leaf, obtained high PCV and GCV values and moderate PCV and GCV values were reported for the character length of leaf, in a study conducted by Nath (2003) in monopodial orchids. Moniruzzaman *et al*. (2012) observed high PCV and GCV for plant height (25.50 and 23.74 per cent) and diameter of pseudobulb (30.50 and 27.33 per cent). For the characters, leaf length and leaf width, high PCV and moderate GCV values were noticed. High PCV and GCV were obtained for the traits, plant height and leaf length in a study conducted by Vanlalruati *et al*. (2016).

High PCV and GCV value were obtained in the character plant height in a study conducted by Miano *et al*. (2016) in orchids. According to the study conducted by Thomas and Rani (2017), it was observed that the characters like length of shoot, number of leaves per shoot, length of leaf obtained high PCV and GCV, while the characters width of leaf and thickness of shoot recorded a low PCV and GCV.

According to Seeja (2018), high GCV and PCV values were noted for characters, plant height, shoot girth, number of leaves, leaf length, leaf width. In a study conducted by George (2020) in *Phalaenopsis*, it was observed that the characters, plant height and width of leaf obtained moderate PCV and GCV. The characters, number of leaves per plant and the length of leaf showed high PCV and moderate GCV values.

2.6.2 Correlation Studies

Kamemoto (1987) noticed a significant correlation of cane length with leaf number and cane diameter in a study conducted in *Dendrobium* orchids. Nagayoshi *et al*. (1996) reported positive comparative correlations between the characters, weight of the bulb and stem diameter, length of the stem and length of the leaf, length of the leaf and the number of leaves, length of the leaf and stem diameter, width of the leaf and stem diameter. Moniruzzaman *et al*. (2012) observed that, the trait length of leaves had negative association with leaf breadth, breadth of leaf had negative association with leaf number and number of spike but had positive association with the plant height and diameter of pseudobulb.

Islam *et al*. (2013) and Anna *et al*. (2015) observed a significant correlation between the characters plant height and leaf number. In a study conducted by Vanlalruati *et al.* (2015), it was observed that height of pseudobulb exhibited positive and highly significant association with leaf production (0.454 for genotypic and 0.449 for phenotypic), leaf length also had positive and significant correlation with leaf diameter (0.331 for genotypic and 0.325 for phenotypic). The traits, number of leaves/ plant and leaf length showed significant and negative phenotypic and genotypic correlation (Seeja, 2018).

2.7 FLOW CYTOMETRY

Among the methods for analysis of polyploidy, flow cytometry is the most efficient and precise method for detecting changes in ploidy level (Dolezel, 1997). The buffers that are most commonly used for flow cytometry analysis in plants are Galbraith's buffer (Galbraith *et al*., 1983), LB01 (Dolezel *et al*., 1989), Otto's buffer (Otto, 1990) and Tris.$MgCl_2$ (Pfosser *et al*., 1995). Ploidy screening using flow cytometry can be performed at an early ontogenetic stage, thus saving both time and space. (Vainola, 2000).

Otto's buffer obtained excellent results in many species, especially those with low nuclear DNA content. The citric acid in Otto's buffer improves the chromatin accessibility and help in homogenizing chromatin structure and thus eliminating the differences in staining intensity among nuclei with the same DNA content but different chromatin state (Louriero *et al.*, 2006). Fluorochromes are used for staining purpose. A broad range of fluorochromes are known to specifically bind to DNA, that includes propidium iodide (PI), 4,6-diamidino 2-phenylindole (DAPI), ethidium bromide etc.

The fluorochrome DAPI is base selective (i.e., AT -selective), while the fluorochrome propidium iodide and ethidium bromide intercalate into double-stranded DNA and RNA, without any base-dependent bias and thus RNase must be added prior to the analysis, inorder to remove the RNA (Dolezel *et al.,*1992; Greilhuber *et al*., 2007). The histogram peaks of the reference and test samples helps in interpretation as to diploid, mixoploid or higher ploidy levels as observed by Dolezel *et al.* (2007a). Flow cytometry is particularly well suited for the determination of ploidy variation in samples with low mitotic indices such as cultures obtained from plant cells *in vitro*, where chromosome counting cannot provide a representative picture of a heterogeneous population of cells (Dolezel *et al.,* 2007b).

Meesawat *et al.* (2008) studied the nuclear DNA content values for *D. crumenatum* using flow cytometry and confirmed the chromosome number, 2n=38 (diploid). Flow cytometry equipment presents important advantages like, samples are easily and rapidly prepared, there is no need to divide cells and only a few milligrams of tissue are needed, and it is a quick and reproducible method for determining the ploidy levels of large numbers of samples (Sakhanokho *et al*., 2009).

Flow cytometry is considered as an advanced analytical technique which has been used for the analysis of cells. The physical characteristics like internal complexity, relative size and relative fluorescence intensity are measured using a beam of laser light. The scattered and fluorescent wavelength are detected and analysed or used for sorting of cells (Givan, 2013).

Identification of polyploidy was carried out traditionally by chromosome counting (karyotype) using microscopy. This method is laborious and requires high skills (Chen and Tang, 2018). FCM helps to determine the nuclear DNA content, genome size determination and ploidy identification. (Miguel and Leonhardt, 2011; Eng and Ho, 2019; Hu *et al*., 2021).

Mohammadi *et al* (2021) concluded that in order to confirm the data obtained by flow cytometry, it is better to apply some morphological, anatomical and cytological parameters particularly stomata characteristics. Polyploidy levels can be precisely identified using a combination of these various methods.

Materials and Methods

3. MATERIALS AND METHODS

The present study was undertaken to induce heteroploidy in *Dendrobium* sp. using antimitotic agent colchicine through *in vitro* and *in vivo* technique and analyze them cytologically by using flow cytometry system. The investigation was carried out in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2019-21. The research programme involved 3 experiments; *viz.*,

Experiment 1: Development of heteroploids in *Dendrobium* sp. using colchicine *in vitro*

Experiment II: Development of heteroploids in *Dendrobium* sp. using colchicine *in vivo.*

Experiment III: Analysis of *in vitro* and *in vivo* induced heteroploidy.

3.1 EXPERIMENT 1

Development of Heteroploids in *Dendrobium* sp. Using Colchicine *In Vitro*

Dendrobium crumenatum (pigeon orchid), a sympodial orchid having strongly scented white flowers was chosen for the heteroploidy induction via *in vitro* propagation technique. The study was aimed at development of an effective protocol for *in vitro* heteroploidy induction in *Dendrobium crumenatum* using antimitotic agent colchicine in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2019-21.

Materials and methods utilized for the study are elaborated below:

3.1.1 Explant

Stem nodal segments containing an axillary bud were used as explant. Explants were obtained from healthy and actively growing mother plants.

3.1.2 Collection and Preparation of Explants

Main shoots (12-20 cm) from mother plant kept at net house were collected and was carefully wiped with 100% alcohol at the cut ends. The leaves were carefully removed and the shoot was cut into nodal segment of 2-3 cm bearing one axillary bud. These nodal segments were then soaked in one percent solution of 'Labolene' detergent in distilled water for 2 hours, followed by thorough washing with distilled water prior to surface sterilization. When the contamination in cultures were higher, in addition to detergent, explants were treated with 0.1% Bavistin for 30 minutes and repeated washing was carried out using distilled water before surface sterilization.

3.1.3 Surface Sterilization

Surface sterilization of explants were performed inside the laminar airflow chamber. The nodal explants bearing axillary buds were subjected to different combinations of surface sterilization agents, inorder to obtain the best treatment that results in the minimum contamination in culture bottles. The combinations of sterilization agents used is shown in Table 1. The best sterilization agent was identified based on the survival rates at culture initiation stage. The observation, percentage survival at culture initiation stage was noted and the sterilization treatment which recorded the maximum percentage was adopted for proceeding further with the experiment.

Table 1. Treatments to assess the effect of different surface sterilization methods

Sl.No	Treatment No.	Treatment	Duration
1	TS_1	10% sodium hypochlorite	10 minutes
2	TS_2	20% sodium hypochlorite	20 minutes
3	TS_3	0.1% mercuric chloride 70% alcohol	5 minutes 2 minutes
4	TS_4	20% sodium hypochlorite 0.1% mercuric chloride	20 minutes 5 minutes
5	TS_5	20% sodium hypochlorite 70% alcohol	25 minutes 2 minutes
6	TS_6	20% sodium hypochlorite 0.1% mercuric chloride 70% alcohol	20 minutes 5 minutes 2 minutes

3.1.4 Media

The basal media used for the study was Vacin and Went (VW) solid medium (Vacin and Went, 1949) prepared as per Appendix 1. For preparation of medium, standard procedures were followed (Thorpe, 1980).

Stock solutions of minor and major nutrients required for VW media were prepared by dissolving the adequate quantity of chemicals in exact volume of double distilled water. Stock solutions of plant growth regulators were prepared by dissolving the required quantity of hormones in 1N NaOH or 95% Ethanol and the volume was made up with double distilled water. Stock solutions were stored under refrigerated condition at 4° C. Stock solutions of minor and major nutrients were prepared and stored upto a period of one month, while that of plant growth regulators were stored only for a period of 1 week.

The glasswares used for preparation of culture medium were washed properly with tap water, after adding few drops of Labolene and then rinsed with double distilled water. Required quantities of stock solutions were pipetted into a 1000 mL beaker. Volume was made upto 950mL using double glass distilled water. For specific treatments, other required additives were added directly to medium during its preparation. Sucrose (20 g) was freshly added and dissolved. Once all the medium components were added the pH was adjusted between 5.3-5.8 using 0.1 N HCl or 0.1 N NaOH with an electronic pH meter. Agar, 6.4 gL^{-1} was added to medium and volume was made upto 1000mL using double glass distilled water. Then the medium was kept in a microwave oven followed by intermittent stirring using glassrod for melting of agar, till a clear solution was obtained.

Then the medium was quickly poured onto the sterilized test tubes and culture bottles. Soon after pouring, test tubes and culture bottles were closed tightly using presterilized cotton plugs and bottle caps respectively. Tissue culture bottles and test tubes containing the medium were then autoclaved at 121°C and 1.06 Kg cm^{-2} pressure for 20 minutes. After autoclaving the test tubes and culture bottles were allowed to cool and mouth of culture bottles were wiped with 70% alcohol and it was transferred to culture room. The media containing bottles and test tubes were kept for 5-7 days before using it for inoculation of explant. The culture bottles and test tubes showing contamination of medium were removed.

3.1.5 Inoculation

The inoculation operations were performed inside laminar air flow chamber. The materials required for inoculation were glasswares (Beakers, petriplate), screwcap bottle, and tools (forceps, blades, scalpels) which were autoclaved and kept ready before inoculation. This was done by thorough washing and rinsing with double glass distilled water, which were then dried properly and wrapped in plastic film. These were kept inside autoclavable polypropylene cover and autoclaved at 121°C and 1.06 Kg cm^{-2} pressure for 40 minutes.

Half an hour prior to inoculation, surface of laminar air hood was wiped using 95% alcohol and UV lights were put ON. All the glasswares, tools, and culture tubes, bottles that were required for the inoculation were kept inside the hood during the UV light sterilization.

After the surface sterilization of the explant, it was then rinsed thrice with sterile double distilled water and excess water adhered to the explant was removed using sterilized blotting paper kept over the petridish. The outer sheath of the explants were carefully removed using a blade and forceps to expose the axillary bud present inside. The bottom portion of explant was given a slant cut to increase the surface area of contact with the medium and top portion was also given a sharp cut to remove the tissues that might have become dead during the sterilization treatments.

During inoculation, the culture bottles and test tubes were carefully opened and closed in front of the spirit lamp flame and the explants were placed on to the medium. The inoculated culture bottles and test tubes after proper sealing using cling film were then incubated in the culture room. The observation, days for bud initiation was recorded. The cultures which showed response in bud initiation in VW media was transferred to callusing media.

3.1.6 Incubation

The buds which showed initiation were inoculated initially onto callusing medium, followed by callus proliferation, PLB formation, and plant regeneration medium. The inoculated culture bottles and test tubes were incubated at 25°C under dark during callus induction and proliferation stage and kept in light for subsequent stages. Subculturing was done once in every 2 weeks. Frequent spraying of 70% alcohol at an interval of 2 days in the racks, culture tubes, and culture bottles were carried out to avoid the entry of contaminants into culture test tubes and bottles.

3.1.7 Somatic Organogenesis

The explant which responded to bud initiation were subjected to different treatments for callus induction. The basal medium used was VW

medium. The effect of each treatment on callus initiation were studied. They were subcultured in the same medium which showed fast initial response among the different callusing medium after inoculation, for callus proliferation and PLB formation. It was followed by treatment of PLBs in the suspension media containing different doses of colchicine. After the colchicine treatment, it was then transferred to plantlet regeneration media.

3.1.7.1 Callus Induction

After the bud initiation, it was transferred to different callus induction media inorder to evaluate the effect of media and hormones on callus induction. Explants which showed response for bud initiation, were cultured in VW media with different hormonal concentration of BA (0.5,1 mgL^{-1}), 2,4-D (0.5 mgL^{-1}) and NAA (0.1 mgL^{1}) to obtain maximum callus induction with early response (Table 2).

Culture bottles were kept under temperature of 25°C and were subcultured every two weeks. Observations on the callus induction percentage and days to callus induction were recorded.

Table 2. Treatments to evaluate the effect of media and hormones on Callus induction

Sl No.	Treatment No.	Treatments
1	TC_1	VW+1 mgL^{-1} BA +0.1 mgL^{-1} NAA
2	TC_2	VW+1 mgL^{-1} BA +0.5 mgL^{-1} 2,4-D
3	TC_3	VW+1 mgL^{-1} BA +1 mgL^{-1} 2,4-D
4	TC_4	VW+0.5mgL^{-1} BA+ 0.5 mgL^{-1} 2,4-D

3.1.7.2 Callus Proliferation

For callus proliferation, the same callus induction medium was chosen which showed the fastest initial response in case of callus induction. The calli induced were subcultured for a period of 8 weeks for callus proliferation. Cultures were kept in dark condition during callus proliferation.

3.1.7.3 PLB Formation

The proliferated calluses were transferred to PLB formation medium. For PLB formation, the treatment which showed the fastest callus initiation response was chosen. The cultures were provided with white light (3000 lux) with a photoperiod of 16 hrs light and 8 hrs dark and incubated at 25°C. The observation on the days for PLB formation was recorded.

3.1.8 Induction of Heteroploidy

For induction of heteroploidy, antimitotic agent colchicine was used. The stock solution of colchicine at 0.5 per cent concentration was prepared by dissolving 0.5 g colchicine in 100 mL sterile distilled water. This solution was filter sterilized using syringe filter and stored in amber bottle under refrigerated condition. The required quantity of the chemical was pipetted using sterile microtips and transferred to liquid VW media for inducing heteroploidy. The addition of colchicine on to autoclaved suspension media were performed inside laminar airflow chamber. The PLBs were transferred to VW basal suspension medium containing colchicine. The treatments for induction of heteroploidy were based on colchicine concentration (0.05%, 0.10%, 0.15%, 0.20%) and the duration of exposure of PLBs when suspended in colchicine containing VW suspension media (24 & 48 hrs). (Table 3)

Design : Completely Randomized Design (CRD)
Treatments : 8
Replications : 12

Table 3. Treatments for induction of heteroploidy in *Dendrobium curmenatum* via *in vitro* technique

Sl.No.	Treatment	Colchicine concentration, duration of treatment
1	T_1	0.05%, 24 hrs
2	T_2	0.10%, 24 hrs
3	T_3	0.15%, 24 hrs
4	T_4	0.20%, 24 hrs
5	T_5	0.05%, 48 hrs
6	T_6	0.10%, 48 hrs
7	T_7	0.15%, 48 hrs
8	T_8	0.20%, 48 hrs

Each treatment was replicated 12 times and culture bottles were maintained at 26 ^{0}C and kept till their respective treatment durations under dark, since colchicine is photolabile. The treated cultures were then transferred to plantlet regeneration medium after proper washing in double distilled water inside laminar airflow chamber. The control for each treatment were the PLBs which were subjected to same conditions but without colchicine. Observations on percentage survival of PLBs after colchicine treatment were taken immediately after the transfer of treated PLBs on to plantlet regeneration medium.

3.1.9 Shoot Regeneration

The treated PLBs after colchicine treatment were transferred to plantlet regeneration medium i.e., VW basal media containing hormones BA (1mgL^{-1}) and NAA (0.1 mgL^{-1}) for shoot regeneration. Cultures were provided with white light (3000lux) with a photoperiod of 16 hrs light and 8 hrs dark and incubated at 25 ^{0}C. Observations on percentage of cultures showing response after colchicine treatment were recorded 1 week after the colchicine treatment. Days for shoot formation were also recorded for each of the 8 treatments of

heteroploidy induction, once it has been transferred to shoot regeneration medium.

For shoot proliferation and multiplication, the same shoot regeneration media were used. Cultures were provided with white light (3000lux) with a photoperiod of 16 hrs light and 8 hrs dark and incubated at 25 ^{0}C. The following observations like days for shoot multiplication, number of shoots per culture and percentage survival of cultures at multiplication stage were recorded.

3.2 EXPERIMENT II:

Development of Heteroploids in *Dendrobium* sp. Using Colchicine *In Vivo*.

Dendrobium crumenatum (pigeon orchid), a sympodial orchid was chosen for the heteroploidy induction via *in vivo* propagation technique. The study was aimed at inducing heteroploidy *in vivo* in *Dendrobium crumenetum* using antimitotic agent colchicine in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2019-21. Materials and methods utilized for the study are elaborated below.

3.2.1 Materials and Methods

The plantlets were subjected to different colchicine treatments. Treatments were based on colchicine concentration (0.05%, 0.1%) and the exposure of plants to these doses of colchicine (24 hrs,48 hrs, 72 hrs, 96 hrs). (Table 4)

Table 4. Treatments for induction of heteroploidy in *Dendrobium curmenatum* via *in vivo* technique

Sl. No.	Treatment	Colchicine concentration, duration of treatment
1	T_1	0.05%, 24 hrs
2	T_2	0.10%, 24 hrs
3	T_3	0.05%, 48 hrs
4	T_4	0.10%, 48 hrs
5	T_5	0.05%, 72 hrs
6	T_6	0.10%, 72 hrs
7	T_7	0.05%, 96 hrs
8	T_8	0.10%, 96 hrs

Colchicine solution for the treatment was prepared by dissolving 0.75 g in 1.5L of sterile distilled water, thus obtaining a concentration of 0.05%, and dissolving 1.5g in 1.5 L of sterile distilled water, obtaining a concentration of 0.1%. The plantlets for the experiment were subjected to 8 different treatments by completely immersing it in colchicine solution and were kept in dark till their respective treatment duration. Constant air bubbling was achieved with domestic aquarium aerating pumps inorder to prevent the damage caused by oxygen depletion. After each treatment, the treated plants were thoroughly washed in running water, followed by washing in sterile distilled water, to remove the excess of colchicine. The plants were then properly planted in black plastic pots using coconut husk chips, charcoal, brick pieces as substrate. For each treatment, 6 replications were carried out. Plants were maintained in the Department of Plant Breeding and Genetics. The control for each treatment of the experiment were plantlets which were subjected to same conditions but without colchicine. Management and fertilizer application were undertaken as per Package of Practices Recommendations of Kerala Agricultural University (KAU, 2016).

Design	:	Completely Randomized Design (CRD)
Treatments	:	8
Replications	:	6

3.2.2 Plant Protection

The major pest associated with the orchids were found to be snails and slugs. They were controlled by handpicking and also by the application of salt along the borders of the green house.

The observations recorded while carrying out the experiment were as follows:

3.2.3 Percentage Survival of the Plants After Colchicine Treatment

The number of survived plantlets were noted during potting of the plantlets after the colchicine treatments and were expressed in terms of percentage.

Percentage survival of the plants after colchicine treatment =

$$\frac{\text{Number of plants survived after colchicine treatment "X"}}{\text{Total number of plants subjected to colchicine treatment "X"}} \times 100$$

3.2.4 Percentage of Plants Showing Response After Colchicine Treatment

The observation on number of plants showing response after colchicine treatment were recorded 2 weeks after potting of the plants and were expressed in terms of percentage.

Percentage of plants showing response after colchicine treatment =

$$\frac{\text{Number of plants showing response after colchicine treatment "X"}}{\text{Total number of plants subjected to colchicine treatment "X"}} \times 100$$

Following observations were recorded after 6 months of colchicine treatment

3.2.5 Measurement of Shoot Length (cm)

Shoot length in centimetres was measured from the base of the plant to the tip of the top most leaf of the plant.

3.2.6 Diameter of Shoot (cm)

Diameter of shoot was measured in centimetres and recorded.

3.2.7 Height and Diameter of Pseudobulb (cm)

Height of pseudobulb in centimetres was measured from base of the pseudobulb to the top position till where it ends. For obtaining the diameter of pseudobulb in centimetres, measurement was taken by considering the broadest part of pseudobulb i.e., in a position on pseudobulb which recorded a maximum diameter value.

3.2.8 Number of Leaves.

The observation on number of leaves were also obtained 6 months after the colchicine treatment.

3.2.9 Length of Leaf (cm)

The length of leaf in centimetres was measured from base of the leaf to apex of the leaf.

3.2.10 Width of Leaf (cm)

The width of leaf in centimetres was measured and recorded.

3.2.11 Statistical Analysis

Statistical analysis of the recorded data of seven characters included in the experiment "Development of heteroploids in *Dendrobium* sp. using colchicine *in vivo*'' was performed. The characters were shoot length (cm), diameter of shoot (cm), height of pseudobulb (cm), diameter of pseudobulb (cm), number of leaves, length of leaf (cm), width of leaf (cm).

3.2.10.1 Analysis of Variance

The observations obtained from the experiments were subjected to analysis of variance. (ANOVA). Completely randomized design (CRD) was followed for performing statistical analysis wherever necessary as per Panse and Sukhatme (1985). Treatment means were compared by calculating the critical difference (CD) at 5 per cent level of significance. Statistical analysis of the data was performed using the softwares, GRAPES (Gopinath *et al.,* 2020) provided by Kerala Agricultural University and WASP 2.0 provided by Central Coastal Agricultural Research Institute.

3.2.10.2 Coefficient of Variance

Genotypic and phenotypic coefficients of variation were estimated using the formula proposed by Singh and Chaudhary (1977). The estimation of phenotypic and genotypic coefficients of Variance (PCV and GCV) for a character X is carried out using the formulae;

$$GCV = (\sigma_{gx} / M) * 100$$

$$PCV = (\sigma_{px} / M) * 100$$

Where,

σ_{gx} = genotypic standard deviation

σ_{px} = phenotypic standard deviation

M = mean of the character under study

According to classification of variation by Sivasubramanian and Menon (1973)

>20% - High PCV and GCV

10-20%- Moderate PCV and GCV

<10%- Low PCV and GCV

3.2.10.3 Correlation Analysis

The correlation coefficients namely phenotypic, genotypic, and environmental correlation coefficients between two characters denoted as 'X' and 'Y' were calculated as follows:

Genotype correlation (r_{gxy}) $= \sigma_{gxy} / (\sigma_{gx} \times \sigma_{gy})$

Phenotypic correlation (r_{pxy}) $= \sigma_{pxy} / (\sigma_{px} \times \sigma_{py})$.

Environmental correlation (r_{exy}) $= \sigma_{exy} / (\sigma_{ex} \times \sigma_{ey})$.

Where,

σ_{gxy}, σ_{pxy} and σ_{exy} are the genotypic, phenotypic and environmental co-variances between the characters 'X' and 'Y'.

σ_{gx}, σ_{px} and σ_{ex} are the genotypic, phenotypic and environmental standard deviations for the character 'X'.

σ_{gy}, σ_{py} and σ_{ey} are the genotypic, phenotypic and environmental standard deviations for the character 'Y'.

3.3 EXPERIMENT III

Analysis of *In Vitro* and *In Vivo* Induced Heteroploidy.

Analysis of *in vitro* and *in vivo* induced heteroploidy were performed using flow cytometry at Rajiv Gandhi Institute for Biotechnology, Poojapura. For carrying out flow cytometry analysis, leaves were collected both from *in vivo* and *in vitro* colchicine treated *Dendrobium crumenatum* plants. For the sample preparation for loading onto FACS machine for flow cytometry analysis, leaves were collected after 6 weeks of colchicine treatment and 6 months of colchicine treatment from *in vitro* experiment and *in vivo* experiment respectively. The same procedure was adopted irrespective of whether the leaves were obtained from *in vivo* or *in vitro* experiment for the analysis. The

flow cytometry analysis was performed based on the procedure by Dolezel *et al*. (2007a).

3.3.1 Materials, Chemicals and Methods

For performing flow cytometry analysis, the following buffers and fluorochrome were used;

- Otto I Buffer : 0.1M citric acid + 0.5 % Tween 20

For the preparation of Otto I Buffer, 1.0507 g citric acid + 250 µL Tween 20 was dissolved in 50 mL distilled water. The prepared solution was filtered using 0.22 µm filter and was stored at 4°C.

- Otto II Buffer: 0.4 M $Na_2HPO_4.12\ H_20$

For the preparation of Otto II Buffer 7.1628 g $Na_2HPO_4.12\ H_20$ was dissolved in 50 mL distilled water. The prepared solution was filtered using 0.22 µm filter and was stored at room temperature in a brown bottle. Dissolving was speeded up by heating.

- DNA fluorochrome used was, PI (Propidium iodide)

PI stock solution was prepared at a concentration of $1mgml^{-1}$. Preparation was carried out using double distilled water and was filtered using 0.22 µm filter and stored at -22^0C in 1mL aliquots. Refreezing again after thawing was avoided.

- RNase

RNase stock solution was prepared at a concentration of $1mgml^{-1}$. During preparation the solution was heated to 90^0C for 15 minutes to inactivate DNases. Double distilled water was used for preparing the solution and filtering was done using 0.22 µm filter and stored at -22^0C in 1mL aliquots. Refreezing again after thawing was avoided.

3.3.1.1 Sample Preparation for Flow Cytometry

Leaf sample of 30 mg was carefully weighed by avoiding the midrib and placed in the centre of plastic petridish. Petridish was placed above an ice pack to maintain the temperature below 4^0 C. 1ml of ice-cold Otto I buffer was added to one side of the petri dish. The tissue was immediately chopped in the buffer using the razor blade. The plant material was chopped into very fine slices. Homogenate was mixed by pipetting up and down for several times (air bubbles were avoided). It was then incubated for 15 minutes in ice (4^0 C). The homogenate was filtered using a 40 µm cell strainer into a labelled Eppendorf tube (2mL), so that the sample volume was above 500 µL. The tubes were centrifuged at 150g (g- relative centrifugal force) for 5 minutes at 4ºC. The supernatant was removed, leaving approximately 100 µl of the liquid above the pellet. The nuclei was resuspended by gentle shaking and 100 µl fresh ice cold Otto I buffer was added and stored at 4º C. After that, Otto II buffer was added to the nuclear suspension. 60 µl of RNase and PI was added, as the requirement is 50 µl mL^{-1}. It was incubated for 5 minutes and filtration using 40 µm cell strainer was done onto a labelled FACS tube, which were wrapped with an Aluminium foil. While loading onto FACS machine (BD FACSAria II) of Rajiv Gandhi Institute for Biotechnology, Poojapura, the Aluminium foil wrapped around the tube was removed.

The standardization regarding the initial weight of the leaf sample to be taken and the rpm of the centrifugation process for obtaining proper histogram was carried out using the untreated samples of *Dendrobium crumenatum*, which is the control (diploid). The diploid peak of the control sample was carefully noted. Flow cytometry histogram was obtained for each of the sample loaded. Chromosome number was interpreted by comparing the peaks obtained in control (diploid) and the treated samples.

Plate 1. ***Dendrobium crumenatum***

Plate 2a. 0.05 % colchicine treatment

Plate 2b. 0.10% colchicine treatment

Plate 2. ***Dendrobium crumenatum in vivo*** **colchicine treatment**

Plate 3. ***In vivo*** **colchicine treated** ***Dendrobium crumenatum*** **plantlets**

Results

4. RESULTS

The results of the three experiments of the present study are presented below, in three sections.

1. Development of heteroploids in *Dendrobium* sp. using colchicine *in vitro*
2. Development of heteroploids in *Dendrobium* sp. using colchicine *in vivo.*
3. Analysis of *in vitro* and *in vivo* induced heteroploidy.

4.1 DEVELOPMENT OF HETEROPLOIDS IN *DENDROBIUM* SP. USING COLCHICINE *IN VITRO*

Investigation was carried out in inducing heteroploidy in *Dendrobium crumenatum* using colchicine *in vitro*. The results of the investigation are presented in this chapter.

4.1.1 Surface sterilization

4.1.1.1 *Percentage survival at culture initiation stage*

Six treatments were compared to assess the effect of surface sterilization treatments on microbial contamination and percentage survival of the explant (Table 5) on VW medium. The treatment TS_6 (20 per cent sodium hypochlorite for 20 minutes + 0.1 per cent mercuric chloride for 5 minutes + 70 % alcohol for 2 minutes) was found to be the best treatment for surface sterilization, which was capable of producing 87.5 per cent explant survival at culture initiation stage followed by the treatment TS_4 (20 per cent sodium hypochlorite for 20 minutes + 0.1 per cent mercuric chloride for 5 minutes), obtained a survival percentage of 62.5 per cent. Among the six treatments, the treatment TS_1 (10 per cent sodium hypochlorite) which yielded a survival percentage of 12.5 per cent was found to be the least effective in producing contamination free cultures.

Table 5. Effect of surface sterilization treatments on survival percentage of explant in *Dendrobium crumenatum*

Sl.No	Treatments	Percentage survival at culture initiation stage
1	TS_1	12.5
2	TS_2	37.5
3	TS_3	25.0
4	TS_4	62.5
5	TS_5	50
6	TS_6	87.5

4.1.2 Days for Bud Initiation

The average number of days for initiation of axillary bud in *Dendrobium crumenatum* cultured in VW media was observed to be 29.25 days. The minimum days recorded for initiation of axillary bud was found to be 26 days and the maximum was 34 days.

4.1.3 Callus Induction Percentage

One month old growing stem nodal segment containing axillary bud was transferred to callus induction medium. The results observed from four callus induction treatments were converted into percentage response (Table 6). Among the four callus induction treatments performed, all the four treatments were found to be responsive towards callus induction and callus induction percentage ranged from 45.0 per cent to 70.0 per cent. The highest callus induction percentage of 70 percent was observed in the medium TC_2 (VW + 1 mgL^{-1}BA + 0.5 mgL^{-1} 2,4-D). The lowest callus induction percentage of 45.0 percent was observed in the medium TC_4 (VW + 0.5 mgL^{-1}BA + 0.5 mgL^{-1},2,4-D). Callus induction percentage of 60.0 percent in the medium TC_1 (VW + 1 mgL^{-1}BA + 0.1 mgL^{-1}NAA) and callus induction percentage of 55.0 percent in the medium TC_3 (VW + 1 mgL^{-1}BA +1 mgL^{-1} 2,4-D) were also obtained.

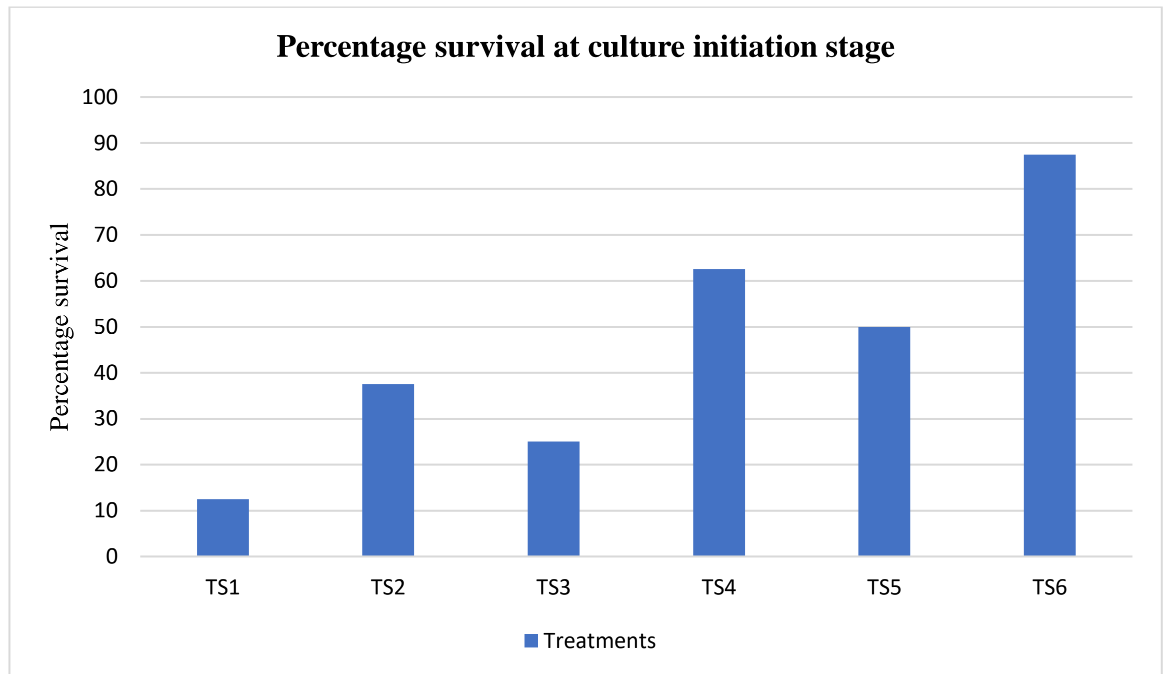


Fig. 1. Effect of surface sterilization treatments on survival percentage of explant in *Dendrobium crumenatum*

Plate 4. Inoculation of explant of *Dendrobium crumenatum* on culture medium

Plate 5. Initiation of bud of *Dendrobium crumenatum* on culture medium (4 weeks after inoculation)

Table 6. Effect of hormones on callus induction of *Dendrobium crumenatum* (Callus induction percentage)

Sl.No	Treatments	Callus induction percentage
1	TC_1	60.0
2	TC_2	70.0
3	TC_3	55.0
4	TC_4	45.0

4.1.4 Days for Callus Induction

On assessment of the observations made on callusing after transferring into the callus induction media (Table 7), the number of days taken for callus initiation varied from 27.33 to 37.33 days. The fastest response was observed in the medium TC_2 (VW + 1 mgL^{-1}BA + 0.5 mgL^{-1} 2,4-D) within 27.33 days and the slowest response was observed in the medium TC_4 (VW + 0.5 mgL^{-1}BA + 0.5 mgL^{-1},2,4-D) after 37.33 days. The medium TC_1 (VW + 1 mgL^{-1}BA + 0.1 mgL^{-1}NAA) and TC_3 (VW + 1 mgL^{-1}BA + 1 mgL^{-1}2,4-D) showed responses after 30.25 days and 33.67 days respectively. The media which showed the fastest response, TC_2 was chosen for subsequent subculturing and callus proliferation. The callus proliferation was seen within 17.67 days of subculturing.

Table 7. Effect of hormones on callus induction of *Dendrobium crumenatum* (Days to callus induction)

Sl.No	Treatments	Days for callus induction
1	TC_1	30.25
2	TC_2	27.33
3	TC_3	33.67
4	TC_4	37.33

Plate 6. Callus initiation from explant of *Dendrobium crumenatum*
(8 weeks after inoculation of axillary buds)

Plate 7. Callus proliferation from explant of *Dendrobium crumenatum*
(10 weeks after inoculation of axillary buds)

Plate 8. PLB from explant of *Dendrobium crumenatum* (12 weeks after inoculation of axillary bud.

4.1.5 Days for PLB Formation

The average number of days for PLB formation was noticed around 60.5 days after bud initiation stage. The minimum days for PLB formation was recorded 57 days and the maximum was 66 days. The treatment which showed fastest response for callus induction, i.e., TC_2 (VW + 1 mgL^{-1}BA + 0.5 mgL^{-1} 2,4-D) was used for PLB induction.

4.1.6 Percentage Survival of PLBs After Colchicine Treatment

The observed results of the two-week-old PLBs survived after eight different colchicine treatments and the control cultures for the treatment duration (24 and 48 hrs) were converted to percentage response (Table 8). The survival per cent of PLBs after colchicine treatment ranged from 91.67 to 41.67 per cent. The highest survival percentage, 91.67 per cent was observed in treatment T_1 (0.05% colchicine, 24 hrs) and lowest percentage, 41.67 per cent was observed in treatment T_8 (0.20% colchicine, 48hrs). The highest survival percentage of PLBs when the duration of colchicine treatment was 48 hrs, was observed to be 66.67 per cent in treatment T_5 (0.05% colchicine, 48 hrs) and the lowest, for the treatment T_8 (0.20% colchicine, 48hrs). Similarly, the highest survival percentage of PLBs when the duration of colchicine treatment was 24 hrs, was observed in treatment T_1 (0.05% colchicine, 24 hrs) and the lowest, for the treatment T_4 (0.20% colchicine, 24hrs) with 58.33 per cent. The survival percentage of PLBs of control i.e., for the treatment duration for 24 and 48 hrs were noted to be 100 and 91.67 per cent respectively.

Table 8. Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage survival of PLBs after colchicine treatment)

Sl.No	Treatments	Percentage survival of PLBs	Control
1	T_1	91.67	100
2	T_2	83.33	100
3	T_3	66.67	100
4	T_4	58.33	100
5	T_5	66.67	91.67
6	T_6	50	91.67
7	T_7	58.33	91.67
8	T_8	41.67	91.67

4.1.7 Percentage of Cultures Showing Response After Colchicine Treatment

Observations of the cultures showing response after colchicine treatment and the control cultures for the treatment duration (24 and 48 hrs) were recorded one week after the colchicine treatment and converted into percentage response (Table 9). The highest percentage of cultures showing response after colchicine treatment was found to be 83.33 per cent and was observed in treatments T_1 (0.05% colchicine, 24 hrs) and T_2 (0.10% colchicine, 24 hrs). The lowest percentage of cultures showing response after colchicine treatment was noted in treatment T_8 (0.20% colchicine, 48 hrs) with 33.33 per cent. The maximum survival percentage of cultures showing response after colchicine treatment for 48 hrs duration was obtained in treatment T_5 (0.05% colchicine, 48 hrs) with 66.67 per cent and the minimum in treatment T_8 (0.20% colchicine, 48 hrs). The control cultures of the observation for the duration 24 and 48 hrs noted a percentage value of 100 and 83.33 per cent respectively.

Table 9. Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage of cultures showing response after colchicine treatment)

Sl.No	Treatments	Percentage of cultures showing response after colchicine treatment	Control
1	T_1	83.33	100
2	T_2	83.33	100
3	T_3	66.67	100
4	T_4	58.33	100
5	T_5	66.67	83.33
6	T_6	50	83.33
7	T_7	50	83.33
8	T_8	33.33	83.33

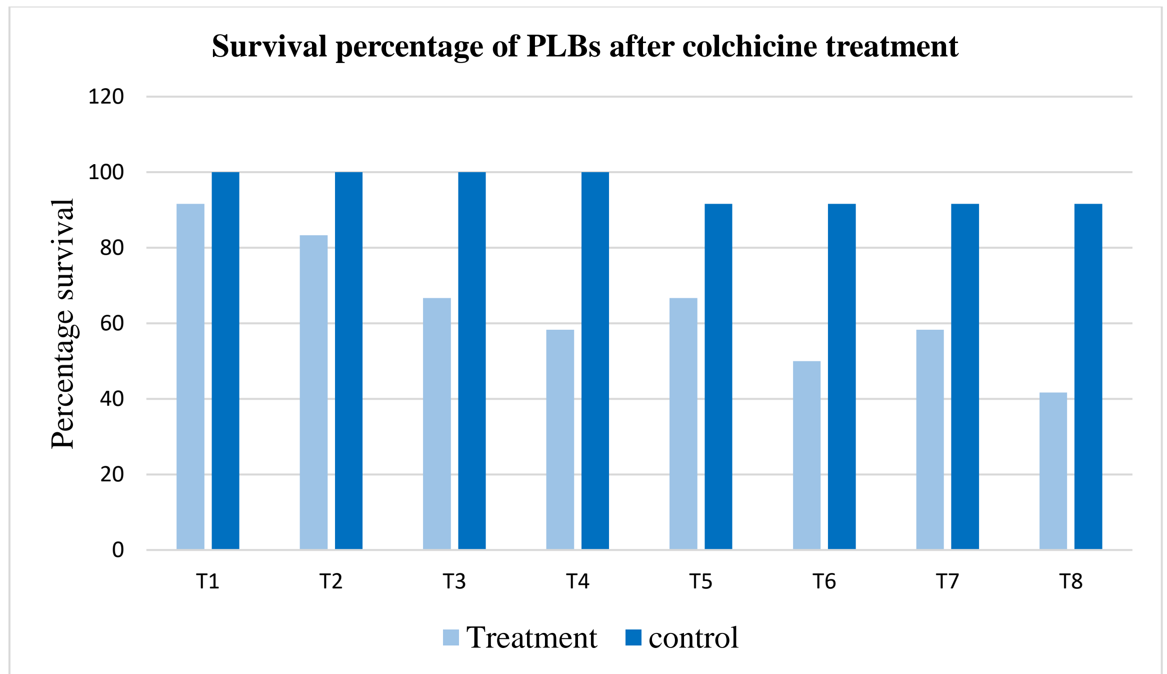


Fig. 2. Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage survival of PLBs after colchicine treatment)

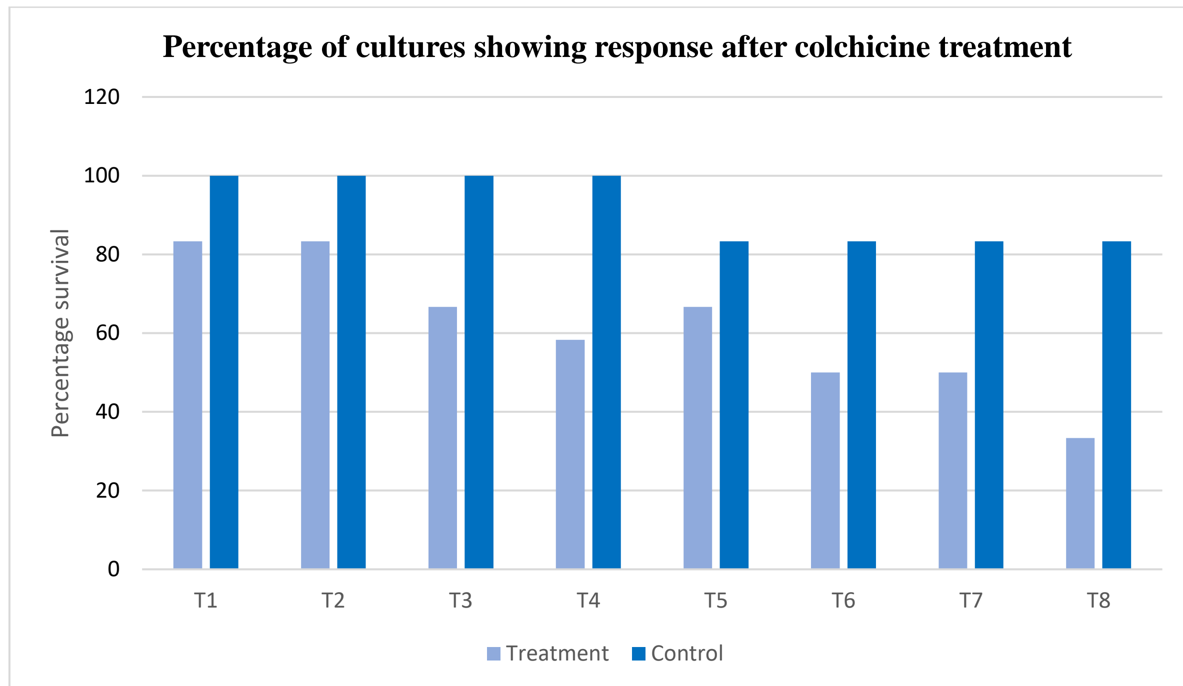


Fig. 3. Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage of cultures showing response after colchicine treatment)

4.1.8 Days for Shoot Formation

On assessment of the observations made on shoot formation after transferring the colchicine treated PLBs into the plantlet regeneration medium (VW media+ 1mgL^{-1}BA and 0.1 mgL^{-1}NAA) and the control cultures for each treatment duration (24 and 48 hrs), the number of days taken for shoot formation in colchicine treated PLBs varied from 32.75 to 46.20 days after PLB stage and in control cultures, it was observed after 30 (24hrs) and 34 days (48 hrs) (Table 10). Earliest response for shoot formation after transferring to regeneration medium was shown within 32.75 days in PLBs which were subjected to the treatment T_1 (0.05% colchicine, 24 hrs) and the slowest response (46.20 days) was noted in the PLBs which were subjected to the treatment T_8 (0.20% colchicine, 48 hrs). The control for the observation which was not subjected to any colchicine treatment obtained the response for shoot formation within 30 and 34 days respectively for the duration 24 and 48 hrs.

Table 10. Effect of colchicine on PLBs of *Dendrobium crumenatum* (Days for shoot formation)

Sl.No	Treatment	Days for shoot formation	Control
1	T_1	32.75	30
2	T_2	35.60	30
3	T_3	39.0	30
4	T_4	38.67	30
5	T_5	42.60	34
6	T_6	44.0	34
7	T_7	39..80	34
8	T_8	46.20	34

4.1.9. Days for Shoot Multiplication

Observations on the number of days of cultures showing multiplication of shoots after shoot formation and the control cultures without colchicine treatment has been recorded (Table 11). The fastest response for shoot multiplication within 11.40 days was shown by PLBs which were earlier subjected to treatment T_1 (0.05% colchicine, 24 hrs) and the slowest response (16.50 days) was recorded in the PLBs which were earlier subjected to the treatment T_8 (20% colchicine, 48 hrs). The control for the observation which was not subjected to any colchicine treatment obtained the response for shoot multiplication within 12 and 14.5 days respectively for the duration 24 and 48 hrs.

Table 11. Effect of colchicine on PLBs of *Dendrobium crumenatum* (Days for shoot multiplication)

Sl.No	Treatment	Days for shoot multiplication	Control
1	T_1	11.40	12
2	T_2	13.40	12
3	T_3	12.00	12
4	T_4	14.60	12
5	T_5	15.80	14.5
6	T_6	14.60	14.5
7	T_7	14.50	14.5
8	T_8	16.50	14.5

4.1.10. Number of Shoots Per Culture

Observations on the number of shoots per culture on the PLBs which were earlier subjected to different colchicine treatment and the control cultures without colchicine treatment has been recorded (Table 12). The maximum number of shoots per culture 5.82, was noticed in the culture, where the PLBs were earlier subjected to treatment T_1 (0.05% colchicine, 24 hrs) and the minimum number recorded was 2.85, which was obtained from the culture, where the PLBs were earlier subjected to treatment T_7 (0.15% colchicine, 48 hrs). The number of shoots per culture recorded for the control cultures were 6.20 and 5.75 for the duration 24 and 48 hrs respectively.

Table 12. Effect of colchicine on PLBs of *Dendrobium crumenatum* (Number of shoots per culture)

Sl.No	Treatment	Number of shoots per culture	Control
1	T_1	5.82	6.20
2	T_2	4.86	6.20
3	T_3	4.32	6.20
4	T_4	4.20	6.20
5	T_5	4.36	5.75
6	T_6	3.12	5.75
7	T_7	2.85	5.75
8	T_8	2.90	5.75

4.1.11 Percentage Survival of Cultures at Multiplication Stage

Observations on the survival percentage of cultures at multiplication stage on the PLBs which were earlier subjected to different colchicine treatment and the control cultures without colchicine treatment has been recorded. (Table 13). The maximum survival percentage of culture at multiplication stage was observed to be 83.33 per cent, which were earlier subjected to colchicine treatments T_1(0.05% colchicine, 24hrs and T_2 (0.10% colchicine, 48 hrs), and the minimum survival percentage of 25 per cent was obtained in cultures which were earlier subjected to colchicine treatment T_8 (0.20% colchicine,48 hrs). The control for the observation which was not subjected to any colchicine treatment recorded survival percentage of 91.67 and 83.33 per cent for the duration 24 and 48 hrs respectively.

Table 13. Effect of colchicine on PLBs of *Dendrobium crumenatum*

(Percentage survival of cultures at multiplication stage)

Sl.No	Treatments	Percentage survival of cultures at multiplication stage	Control
1	T_1	83.33	91.67
2	T_2	83.33	91.67
3	T_3	66.67	91.67
4	T_4	58.33	91. 67
5	T_5	66.67	83.33
6	T_6	50	83.33
7	T_7	33.33	83.33
8	T_8	25	83.33

Plate 9. Shoot regeneration response of *Dendrobium crumenatum* after colchicine treatment (5 weeks after colchicine treatment of PLBs)

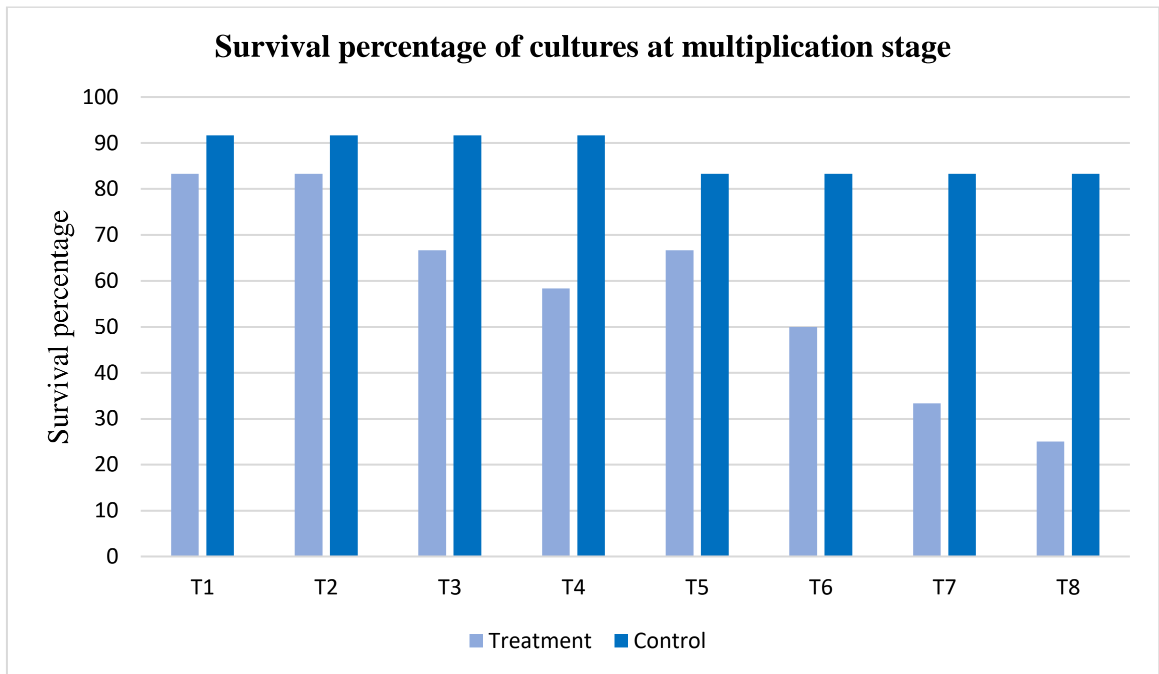


Fig. 4. Effect of colchicine on PLBs of *Dendrobium crumenatum* (Percentage survival of cultures at multiplication stage)

4.2 DEVELOPMENT OF HETEROPLOIDS IN *DENDROBIUM* SP. USING COLCHICINE *IN VIVO*.

Investigation was carried out in inducing heteroploidy in *Dendrobium crumenatum* using colchicine *in vivo*. Each colchicine treatment was replicated six times. The results of the investigation are presented in this chapter.

4.2.1 Percentage Survival of the Plants after Colchicine Treatment.

Observations on the survival of *Dendrobium crumenatum* plantlets immediately after colchicine treatment and the control plantlets without colchicine treatment were converted into percentage values. (Table 14). Maximum survival percentage (100 per cent) were noticed in the treatments T_1 (0.05% colchicine, 24 hrs), T_2 (0.10% colchicine, 24 hrs) and T_3 (0.01% colchicine, 48 hrs). Minimum survival percentage (66.67 per cent) were recorded in the treatments T_7 (0.05% colchicine, 96 hrs) and T_8 (0.10% colchicine, 96 hrs). Control plantlets which were subjected to same conditions, but without colchicine treatment recorded 100 per cent (for 24 hrs, 48 hrs, 72 hrs) and 83.33 per cent (96 hrs), survival percentage.

Table 14. Effect of colchicine treatments on *Dendrobium crumenatum* plantlets (Percentage survival of the plants after colchicine treatment)

Sl.No	Treatments	Percentage survival of plants after colchicine treatment	Control
1	T_1	100	100
2	T_2	100	100
3	T_3	100	100
4	T_4	83.33	100
5	T_5	83.33	100
6	T_6	83.33	100
7	T_7	66.67	83.33
8	T_8	66.67	83.33

4.2.2 Percentage of Plants Showing Response after Colchicine Treatment.

The observation on number of plants showing response after colchicine treatment and control plantlets without colchicine treatment recorded 2 weeks after potting of the plants were expressed in terms of percentage (Table 15). The highest percentage of plants showing response after colchicine treatment (83.33 per cent) were noticed in treatments T_1 (0.05% colchicine, 24 hrs) and T_3 (0.05 % colchicine, 48 hrs). The lowest percentage of plants showing response after colchicine treatment (16.67 per cent) was noticed in treatment T_8 (0.10% colchicine, 96 hrs). Control plantlets which were subjected to same conditions, but without colchicine treatment recorded 100 per cent (for 24 and 48 hrs) and 83.33 per cent (72 and 96 hrs) survival percentage.

Table 15 Effect of colchicine treatments on *Dendrobium crumenatum* plantlets (Percentage of plants showing response after colchicine treatment.)

Sl.No	Treatments	Percentage of plants showing response after colchicine treatment.	Control
1	T_1	83.33	100
2	T_2	66.67	100
3	T_3	83.33	100
4	T_4	50	100
5	T_5	66.67	83.33
6	T_6	50	83.33
7	T_7	33.34	83.33
8	T_8	16.67	83.33

4.2.3 The Statistical Analysis of the Recorded Data of the Characters; Shoot Length (cm), Diameter of Shoot (cm), Height of Pseudobulb (cm), Diameter of Pseudobulb (cm), Number of Leaves, Length of Leaf (cm), Width of Leaf (cm) were carried out and the results are under the following subheads.

4.2.3.1 Evaluation of Eight Different Colchicine Treatments

4.2.3.2 Estimation of Variability Components i.e., PCV And GCV

4.2.3.3 Correlation Analysis

4.2.3.1 Evaluation of Eight Different Colchicine Treatments

The seven characters recorded for eight different colchicine treatments were subjected to analysis of variance (Table 16) and significant variations were observed among all the treatments.

The mean performances of seven characters in eight different colchicine treatment and their corresponding control performance are represented in Table 17.

Table 16. Analysis of variance of characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment

Sl. No.	Observations	Mean square	
		Treatment	Error
1.	Shoot length (cm)	9.482	0.640
2.	Diameter of shoot (cm)	0.001	0.000
3.	Height of pseudobulb (cm)	0.102	0.037
4.	Diameter of pseudobulb (cm)	0.071	0.001
5.	Number of leaves	3.042	0.417
6.	Length of leaf (cm)	6.519	0.018
7.	Width of leaf (cm)	0.265	0.017

4.2.3.1.1 Measurement of Shoot Length (cm)

The treatment T_3 recorded the highest shoot length (17.8 cm) which was on par with treatment T_6 (17.50 cm). The lowest mean shoot length was exhibited by the treatment T_2 (13.00 cm) which was on par with T_4 (13.50 cm)

and T_7 (13.83 cm). The shoot length of control plantlets ranged from 12.50 - 14.00cm.

4.2.3.1.2 Diameter of Shoot (cm)

Shoot diameter of the treatment T_7 (0.233 cm) was found to be maximum which was on par with the treatments T_4 (0.226 cm), T_3 and T_6 (0.206 cm). Diameter of shoot was minimum for the treatment T_8 (0.180 cm) which was on par with the treatment T_5 (0.183 cm). Diameter of the shoot of the control plantlets ranged from 0.20-0.22 cm.

4.2.3.1.3 Height of Pseudobulb (cm)

Pseudobulb height was noticed to be highest in the treatment T_1 (7.20 cm) which was on par with the treatments T_3 (7.06 cm) and T_4 (7.03 cm). The lowest pseudobulb height was noticed in the treatment T_7 (6.66cm) which was on par with T_2 (6.73 cm), T_6 (6.76 cm) and T_8 (6.86cm). Pseudobulb height of control plantlets ranged from 6.60-7.20 cm.

4.2.3.1.4 Diameter of Pseudobulb (cm)

Pseudobulb diameter of the treatment T_7 (1.08 cm) was found to be the maximum followed by T_8 (0.95 cm), T_4 (0.75 cm). Diameter of pseudobulb was minimum for the treatment T_3(0.64 cm) which was on par with T_1(0.65 cm). Diameter of the pseudobulb of the control plantlets ranged from 0.57-0.72 cm.

4.2.3.1.5 Number of Leaves

The highest number of leaves was recorded in the treatments T_1 and T_2 (5.33) followed by treatments T_3 (3.67), T_4 (3.67), T_6 (3.33). The treatment T_8 (2.67) found to have the least number of leaves which was on par with the treatments T_5 (3.00) and T_7 (3.33). Number of leaves recorded in control plantlets ranged from 3-5.

4.2.3.1.6 Length of Leaf (cm)

The treatment T_2 (10.06 cm) recorded the highest length of leaf which was on par with the treatment T_5 (9.86 cm). The lowest length of leaf was observed

in treatment T_3 (5.73 cm). Length of leaf of the control plantlets ranged from 8.9-9.5 cm.

4.2.3.1.7 Width of Leaf (cm)

Width of the leaf was noticed to be highest in the treatment T_2 (2.56 cm) which was on par with the treatments T_7 (2.46 cm), T_1 (2.43 cm) and T_3 (2.36 cm). The lowest leaf width was observed in treatment T_8 (1.63 cm). Width of leaf of control plantlets ranged from 1.50-1.70 cm.

The characters length of shoot, diameter of pseudobulb, number of leaves and width of leaf showed an increased mean value than their corresponding control means. The mean values of the characters, diameter of shoot and length of leaves noted lower mean values than their control. The mean value for the character height of pseudobulb were found to be same for treated and control plantlets.

Table 17. Mean performance of characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment

Sl. No	Treatment	Shoot length (cm)	Control for shoot length (cm)	Diameter of shoot (cm)	Control for Diameter of shoot (cm	Height of pseudobulb (cm)	Control for Height of pseudobulb (cm)
1	T_1	15.16	12.5	0.20	0.20	7.2	7
2	T_2	13	12.5	0.203	0.20	6.73	7
3	T_3	17.8	14	0.206	0.22	7.06	7.2
4	T_4	13.5	14	0.226	0.22	7.03	7.2
5	T_5	15.66	14	0.183	0.21	6.86	6.6
6	T_6	17.5	14	0.206	0.21	6.76	6.6
7	T_7	13.83	13.5	0.233	0.20	6.66	6.8
8	T_8	14.66	13.5	0.18	0.20	6.86	6.8
	Mean	15.141	13.5	0.205	0.207	6.900	6.9
	SE	0.462		0.01		0.111	
	CD (5%)	1.385		0.029		0.331	

Sl. No	Treatment	Diameter of pseudobulb (cm)	Control for Diameter of pseudobulb (cm)	Number of leaves	Control for Number of leaves	Length of leaf (cm)	Control for Length of leaf (cm)
1	T_1	0.65	0.57	5.33	4	9.7	9.5
2	T_2	0.70	0.57	5.33	4	10.06	9.5
3	T_3	0.64	0.65	3.67	5	5.73	8.9
4	T_4	0.75	0.65	3.67	5	7.63	8.9
5	T_5	0.73	0.62	3	3	9.86	9.4
6	T_6	0.74	0.62	3.33	3	8	9.4
7	T_7	1.08	0.72	3.33	3	9.43	9
8	T_8	0.95	0.72	2.67	3	8.86	9

	Mean	0.783	0.64	3.791	3.75	8.662	9.2
	SE	0.017		0.373		0.076	
	CD (5%)	0.052		1.117		0.229	

Sl. No	**Treatment**	**Width of leaf (cm)**	**Control for Width of leaf (cm)**
1	T_1	2.43	1.6
2	T_2	2.56	1.6
3	T_3	2.36	1.5
4	T_4	2.06	1.5
5	T_5	2.2	1.5
6	T_6	2.33	1.5
7	T_7	2.46	1.7
8	T_8	1.63	1.7
	Mean	2.258	1.575
	SE	0.075	
	CD (5%)	0.223	

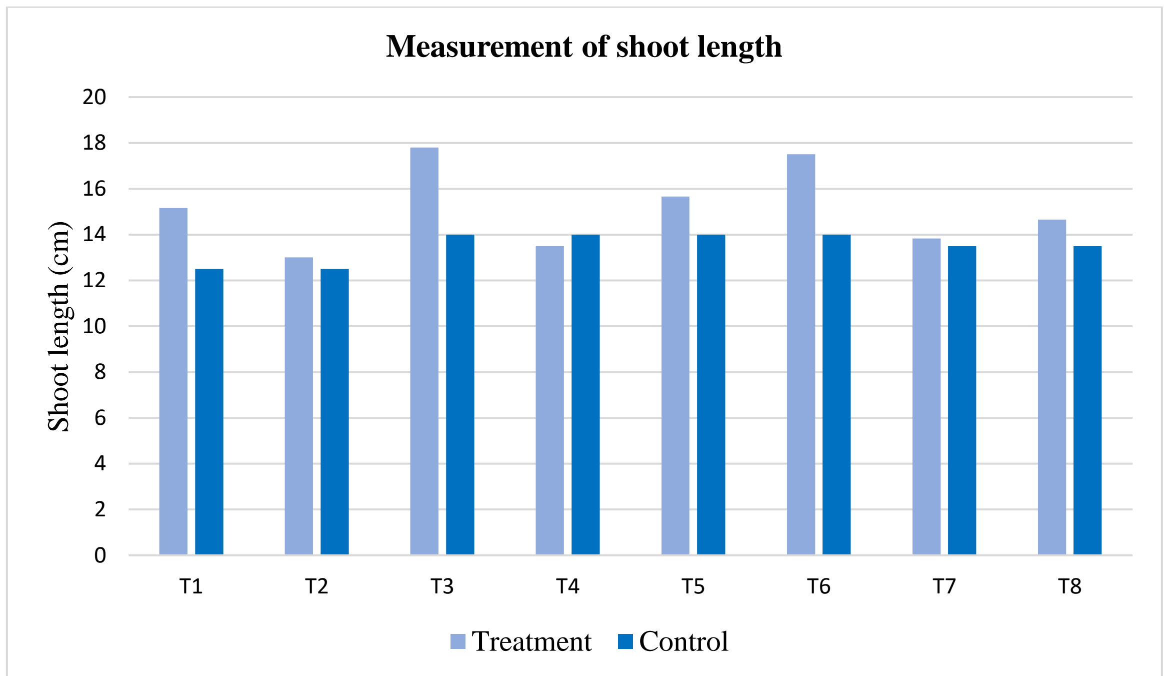


Fig. 5. Mean performance of shoot length (cm) after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment.

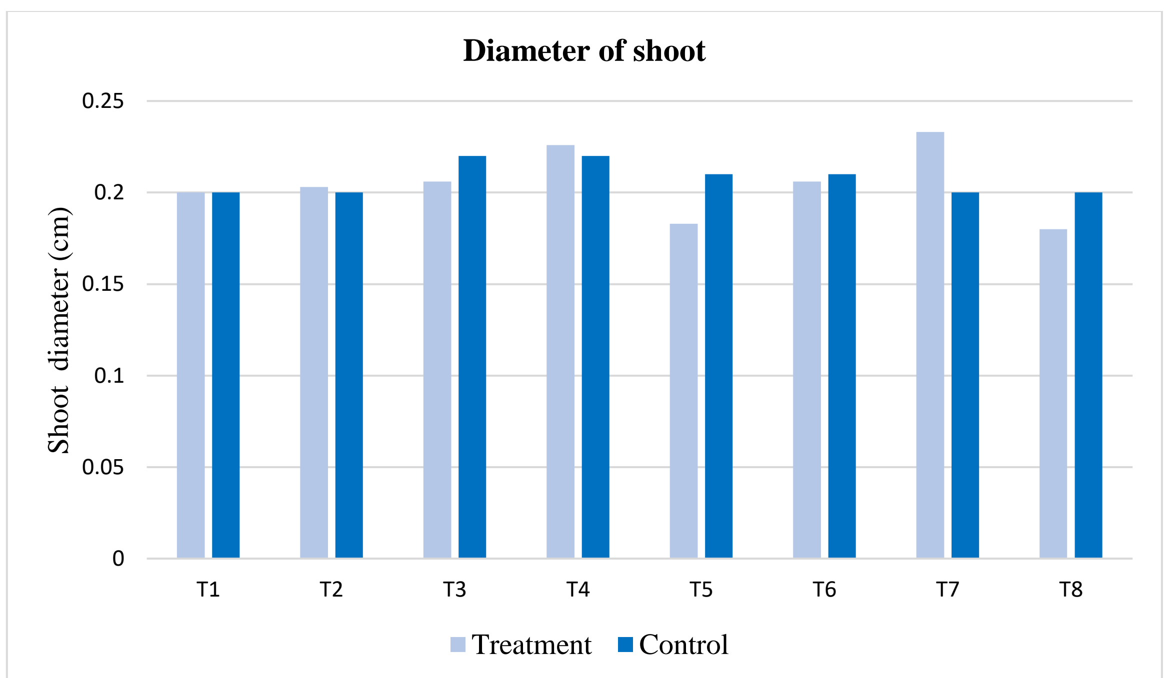


Fig. 6. Mean performance of shoot diameter (cm) after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment

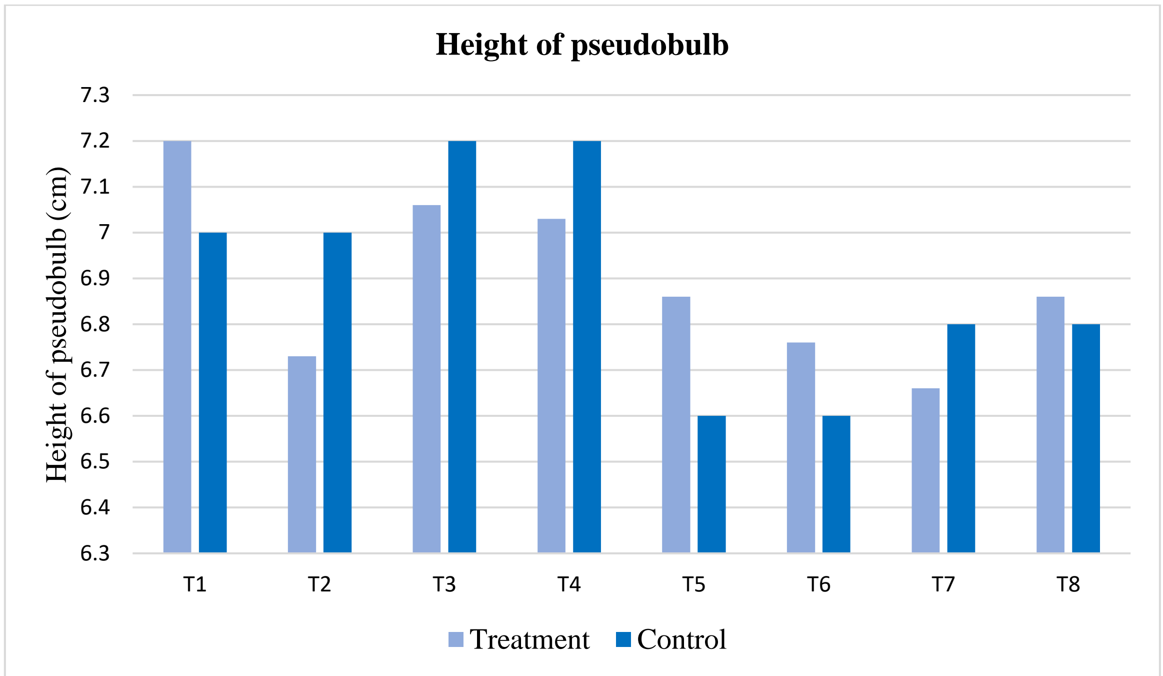


Fig. 7. Mean performance of pseudobulb height (cm) after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment.

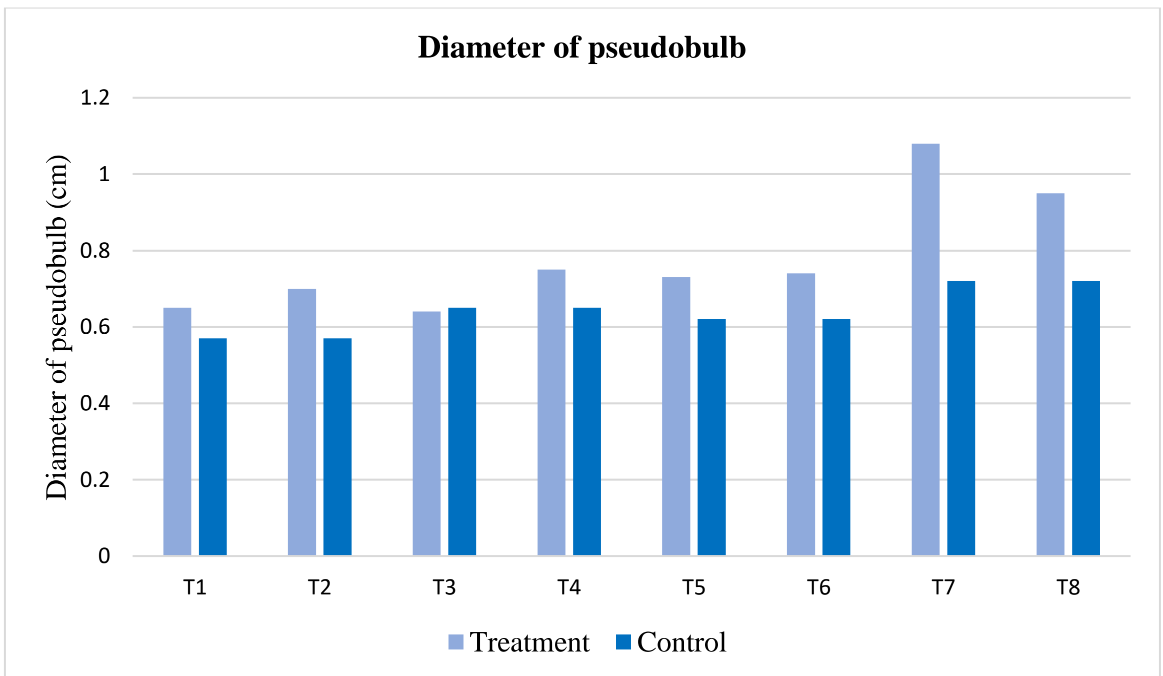


Fig. 8. Mean performance of pseudobulb diameter after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment

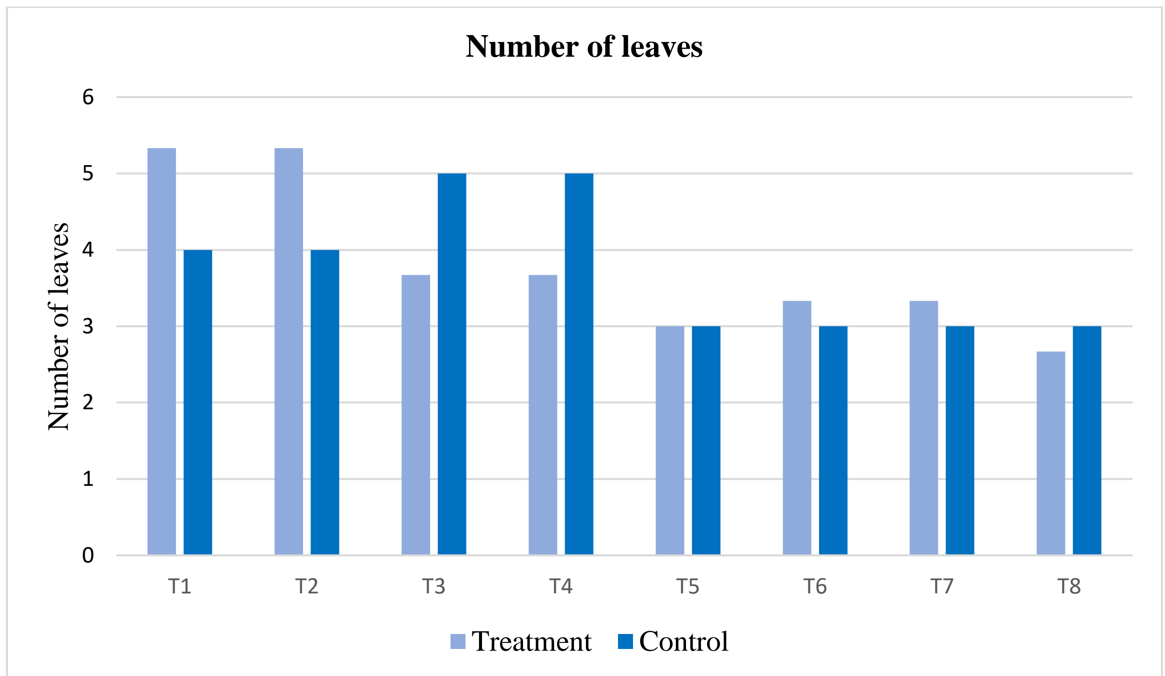


Fig. 9. Mean performance of number of leaves after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment

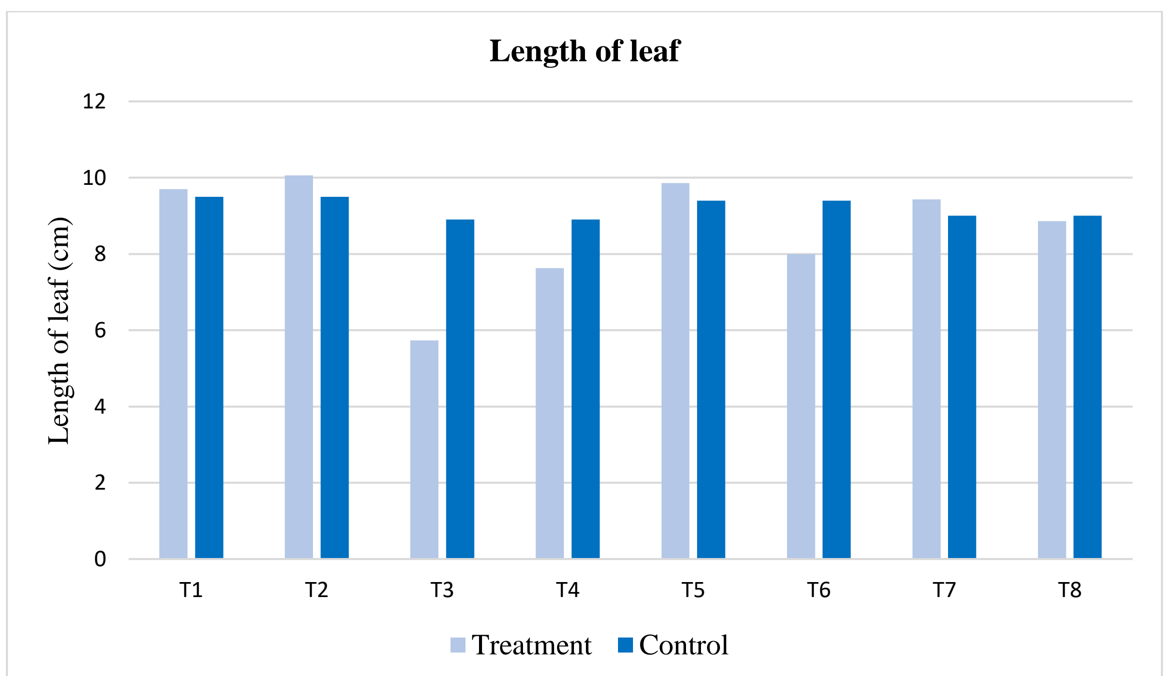


Fig. 10. Mean performance of length of leaf after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment

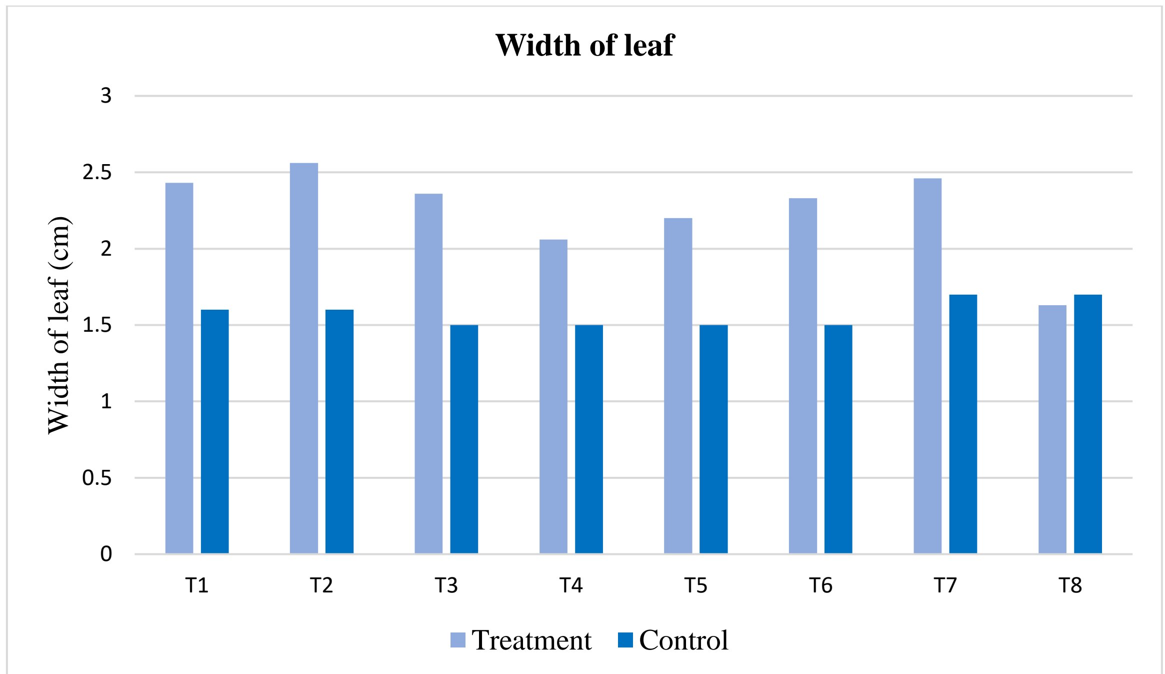


Fig. 11. Mean performance of width of leaf after colchicine treatment of *Dendrobium crumenatum* in *in vivo* experiment

4.2.3.2 Estimation of Variability Components i.e., PCV And GCV

Phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV), which are the relative measures of variation used for comparison among the characters and measured in different units were computed and presented in Table 18. Phenotypic coefficient of variation (PCV) was found to be greater in magnitude than genotypic coefficient of variation (GCV) for the following six characters *viz*., Shoot length, height of pseudobulb, diameter of pseudobulb, number of leaves, length of leaf, width of leaf and for the character, diameter of shoot, the magnitude of phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) obtained were same. This indicated that environment has significant role in expression of the characters.

Table 18. Phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) of seven characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment

Sl. No.	Observations	GCV (per cent)	PCV (per cent)
1	Shoot length (cm)	11.331	12.557
2	Diameter of shoot (cm)	8.906	8.906
3	Height of pseudobulb (cm)	2.067	3.589
4	Diameter of pseudobulb (cm)	19.500	19.914
5	Number of leaves	24.420	30.385
6	Length of leaf (cm)	17.007	17.038
7	Width of leaf (cm)	12.935	13.600

The character, number of leaves recorded high PCV and GCV. Moderate PCV and GCV were recorded for the characters *viz*., diameter of pseudobulb, length of shoot, length of leaf and width of leaf. The characters, diameter of shoot and height of pseudobulb obtained low PCV and GCV.

The maximum phenotypic (30.385 per cent) and genotypic (24.420 per cent) coefficients of variation was observed for number of leaves followed by diameter of pseudobulb (PCV 19.914 per cent and GCV 19.500 per cent), length of leaf (PCV 17.038 per cent and GCV 17.007 per cent) and length of shoot (PCV 12.557 per cent and GCV 11.331 per cent). The minimum phenotypic (3.589 per cent) and genotypic (2.067 per cent) coefficients of variation were observed for height of pseudobulb. For the character, diameter of shoot, both PCV and GCV obtained the same value (8.906). A small difference between GCV and PCV were observed for the characters, diameter of pseudobulb and length of leaf, which proves that environmental factors have negligible effect on these characters and their expression is mainly due to genetic factors.

4.2.3.3 Correlation Analysis

Correlation analysis helps in identification of correlated response among the characters studied. The results of correlation analysis are represented in Table 19.

4.2.3.3.1 Phenotypic Correlation

Table 19a. Phenotypic correlation of seven characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment.

	X_1	X_2	X_3	X_4	X_5	X_6	X_7
X_1	1.000						
X_2	-0.249	1.000					
X_3	0.206	-0.210	1.000				
X_4	-0.364*	0.254	-0.420*	1.000			
X_5	-0.283	0.224	0.221	-0.432*	1.000		
X_6	-0.303*	-0.462**	-0.214	-0.029	0.258	1.000	
X_7	0.013	0.431*	-0.160	-0.307*	0.528**	0.066	1.000

*Significant at 5 per cent level **Significant at 1 per cent level

X_1 Length of shoot X_5 Number of leaves
X_2 Diameter of shoot X_6 Leaf Length
X_3 Pseudobulb height X_7 Leaf width
X_4 Pseudobulb diameter

On studying phenotypic correlation (Table 19a), the character length of shoot showed significant and negative correlation with diameter of pseudobulb (0.364) and length of leaf (0.303).

Diameter of shoot recorded positive and significant correlation with width of leaf (0.431). Diameter of shoot obtained a significant negative correlation with length of leaf (0.462).

Height of pseudobulb was found to have negative significant correlation with diameter of pseudobulb (0.420).

Diameter of pseudobulb registered a negative significant correlation with length of shoot (0.364), height of pseudobulb (0.420), number of leaves (0.432) and width of leaf (0.307)

Number of leaves recorded positive and significant correlation with width of leaf (0.528) and showed a negative significant correlation with diameter of pseudobulb (0.432).

Length of leaf had significant and negative correlation with length of shoot (0.303), diameter of shoot (0.462).

Width of leaf registered a positive and significant correlation with diameter of shoot (0.431) and number of leaves (0.528) and showed negative significant correlation with diameter of pseudobulb (0.307).

4.2.3.3.2 Genotypic Correlation

On studying the genotypic correlation (Table 19b), the character length of shoot showed positive and significant correlation with height of pseudobulb (0.308) and recorded significant and negative correlation with diameter of shoot (0.298), diameter of pseudobulb (0.415), number of leaves (0.312) and length of leaf (0.329).

Diameter of shoot recorded positive and significant correlation with width of leaf (0.517). Diameter of shoot obtained a significant negative correlation with length of shoot (0.298) and length of leaf (0.469).

Height of pseudobulb was found to have positive and significant correlations with length of shoot (0.308), number of leaves (0.411) and showed negative significant correlation with diameter of pseudobulb (0.810) and length of leaf (0.369).

Diameter of pseudobulb registered negative significant correlation with length of shoot (0.415), height of pseudobulb (0.810), number of leaves (0.585) and width of leaf (0.325).

Number of leaves recorded positive and significant correlation with height of pseudobulb (0.411), length of leaf (0.315) and width of leaf (0.744) and showed a negative significant correlation with length of shoot (0.312) and diameter of pseudobulb (0.585).

Length of leaf had significant and positive correlation with number of leaves (0.315) and recorded significant and negative correlation with length of shoot (0.329), diameter of shoot (0.469), height of pseudobulb (0.369)

Width of leaf registered a positive and significant correlations with diameter of shoot (0.517) and number of leaves (0.744) and showed negative significant correlation with diameter of pseudobulb (0.325).

Table 19b. Genotypic correlation of seven characters in colchicine treated *Dendrobium crumenatum* in *in vivo* experiment.

	X_1	X_2	X_3	X_4	X_5	X_6	X_7
X_1	1.000						
X_2	-0.298*	1.000					
X_3	0.308*	-0.107	1.000				
X_4	-0.415*	0.285	-0.810**	1.000			
X_5	-0.312*	0.106	0.411*	-0.585**	1.000		
X_6	-0.329*	-0.469**	-0.369*	-0.032	0.315*	1.000	
X_7	0.042	0.517**	-0.072	-0.325*	0.744**	0.073	1.000

*Significant at 5 per cent level **Significant at 1 per cent level

X_1 Length of shoot X_5 Number of leaves
X_2 Diameter of shoot X_6 Leaf Length

X_3 Pseudobulb height X_7 Leaf width
X_4 Pseudobulb diameter

4.3 ANALYSIS OF *IN VITRO* AND *IN VIVO* INDUCED HETEROPLOIDY.

Flow cytometry analysis was performed for the colchicine induced *Dendrobium crumenatum* obtained from both *in vitro* and *in vivo* experiment at Rajiv Gandhi Institute for Biotechnology, Poojapura. The results of the investigation are presented below.

4.3.1 Flow Cytometry Histogram

The flow cytometry histogram obtained for the control (diploid), mixoploid, and tetraploid sample are shown in fig. 12.

4.3.2 Chromosome Number

For the samples subjected to flow cytometry analysis, flow cytometry histogram was obtained. From the flow cytometry histogram;

- Single major peak P2 was obtained at channel number 50 (X axis) indicating diploid condition (2n=38) when the control or untreated samples were analysed. (Fig. 12a.)
- Two peaks P2 and P3 were obtained at channel number 50 and 100 (X axis) indicating mixoploidy with both diploid and tetraploid condition. (Fig. 12b.)
- Single major peak P3 was obtained at channel number 100 (X axis) indicating the tetraploid condition (2n=76). (Fig. 12c.)

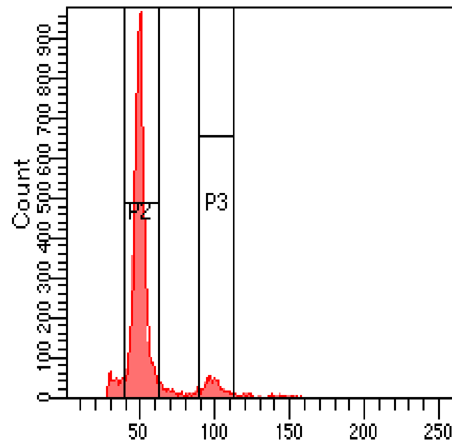


Fig. 12 a. Histogram of Diploid (control)

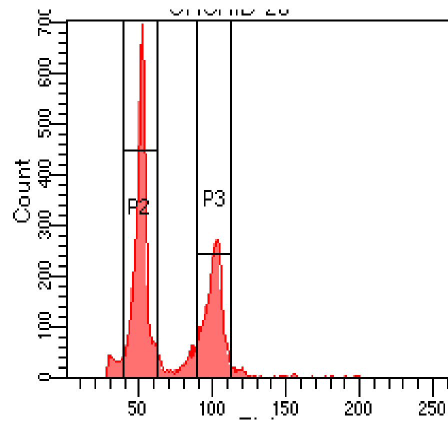


Fig. 12b. Histogram of mixoploid

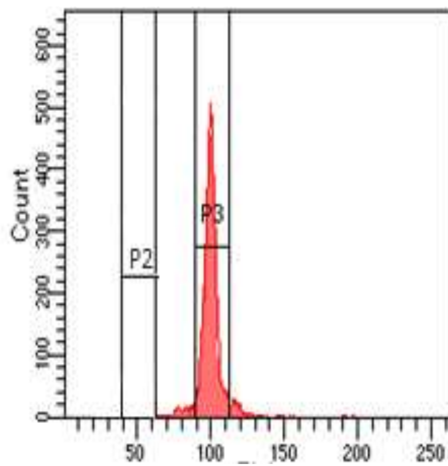


Fig. 12c. Histogram of tetraploids

Fig. 12. The flow cytometry histogram obtained for the control (diploid), mixoploid, and tetraploid sample.

The percentage of mixoploids and tetraploids obtained from *in vitro* and *in vivo* colchicine treated *Dendrobium crumenatum* after performing flow cytometry are presented below (Table 20).

4.3.3 Flow Cytometry Analysis of *In Vitro* Colchicine Treated *Dendrobium crumenatum* Samples

On analysing the histogram peaks of *in vitro* treated samples, the maximum tetraploids (66.67 %) were obtained from treatments T_3 and T_6 and treatment T_3 was successful in obtaining mixoploids (33.34 %) also. For the treatment T_8, the mixoploid and tetraploid percentage obtained was found to be 50%. The minimum tetraploids were recorded in treatment T_2 (25%). Maximum mixoploid (66.67%) was noticed in treatments T_1 and T_7. The treatments, T_8, T_4, T_3, T_2 and T_1 resulted in both mixoploids and tetraploids (Table 20a).

Table 20a. Flow cytometry analysis of *in vitro* colchicine treated *Dendrobium crumenatum* samples

Sl.No	Treatments	Mixoploid (%)	Tetraploid (%)
1	T_1	66.67	33.34
2	T_2	50.00	25.00
3	T_3	33.34	66.67
4	T_4	33.34	33.34
5	T_5	50.00	0
6	T_6	0	66.67
7	T_7	66.67	0
8	T_8	50.00	50.00

4.3.4 Flow Cytometry Analysis of *In Vivo* Colchicine Treated *Dendrobium crumenatum* Samples

On analysing the histogram peaks of *in vivo* treated samples, the highest tetraploid induction was noticed in treatment T_7 (50 %) followed by T_6 and T_5 (33.34%) and T_4 and T_2(25%). Maximum mixoploids were obtained from treatments T_5 and T_3 (66.67%). The treatments T_6, T_5, T_4, T_2 recorded both mixoploids and tetraploids. There was no tetraploid induction in the treatments T_1, T_3, T_8 (Table 20b).

Table 20b. Flow cytometry analysis of in *vivo* colchicine treated *Dendrobium crumenatum* samples

Sl.No	Treatments	Mixoploid (%)	Tetraploid (%)
1	T_1	25.00	0
2	T_2	25.00	25.00
3	T_3	66.67	0
4	T_4	50.00	25.00
5	T_5	66.67	33.34
6	T_6	33.34	33.34
7	T_7	0	50.00
8	T_8	50.00	0

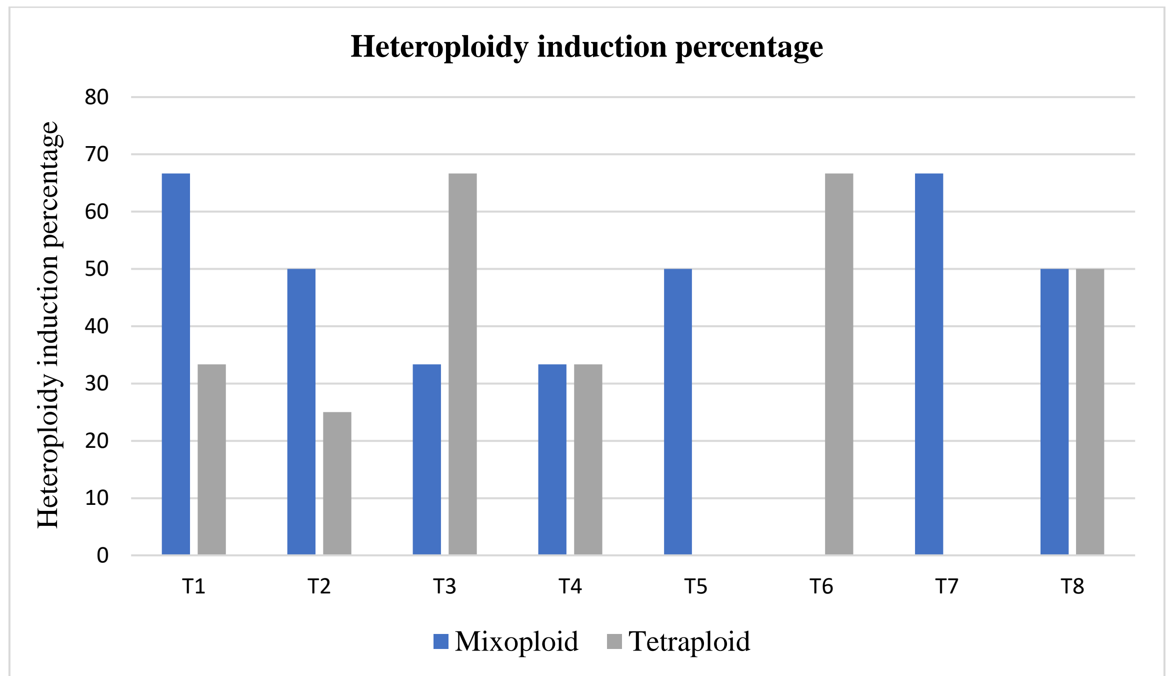


Fig. 13. Flow cytometry analysis of *in vitro* colchicine treated *Dendrobium crumenatum* samples

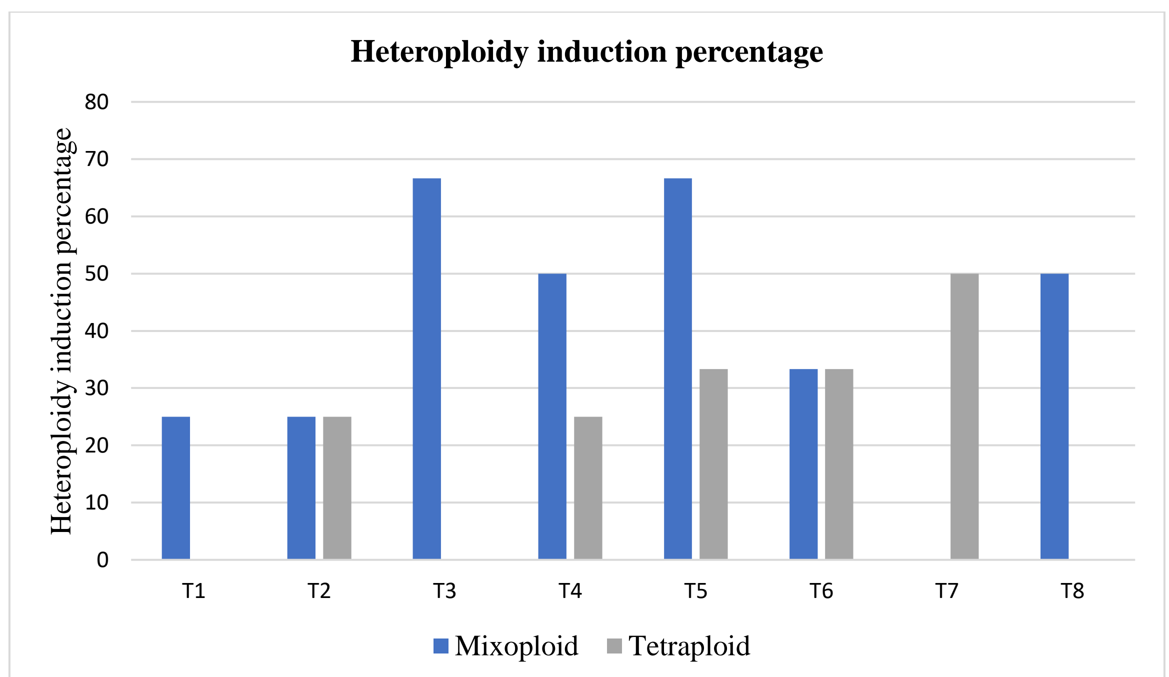


Fig. 14. Flow cytometry analysis of *in vivo* colchicine treated *Dendrobium crumenatum* samples

Following treatments were common in both *in vitro* and *in vivo* experiment for inducing heteroploidy in *Dendrobium crumenatum* (Table 21).

Table 21. Common colchicine treatments from both *in vitro* and *in vivo* experiment

Experiment 1	**Experiment 2**
T_1- 0.05% colchicine, 24 hrs	T_1- 0.05% colchicine, 24 hrs
T_2- 0.10% colchicine, 24 hrs	T_2- 0.10% colchicine, 24 hrs
T_5- 0.05% colchicine, 48 hrs	T_3- 0.05% colchicine, 48 hrs
T_6- 0.10% colchicine, 48 hrs	T_4- 0.10% colchicine, 48 hrs

On comparing the flow cytometry results obtained from the common treatments in experiment I and experiment II, the maximum tetraploid induction was noticed in treatment T_6 (66.67%) and treatments T_2 & T_4 (25%) respectively. In both these treatments, the PLBs or the plantlet of *Dendrobium crumenatum* were subjected to same dose of colchicine treatment i.e., 0.10% colchicine. The treatment T_1 and T_2 of experiment I and treatment T_2 and T_4 of experiment II recorded both mixoploids and tetraploids. In experiment I, the minimum tetraploid induction was noticed in treatment T_2 (25%). The highest mixoploid induction was noticed in treatment T_1 (66.67 %) of experiment I and treatment T_3 (66.67 %) of experiment II (Table 22).

Table 22. Comparison of the flow cytometry results of common treatments in *in vitro* and *in vivo* experiment for inducing heteroploidy in *Dendrobium crumenatum.*

Treatments	*In vitro*		*In vivo*	
	Mixoploid	**Tetraploid**	**Mixoploid**	**Tetraploid**
0.05% colchicine, 24 hrs	66.67	33.34	25	0
0.05% colchicine, 48 hrs	50	0	66.67	0
0.10% colchicine, 24 hrs	50	25	25	25
0.10% colchicine, 48 hrs	0	66.67	50	25

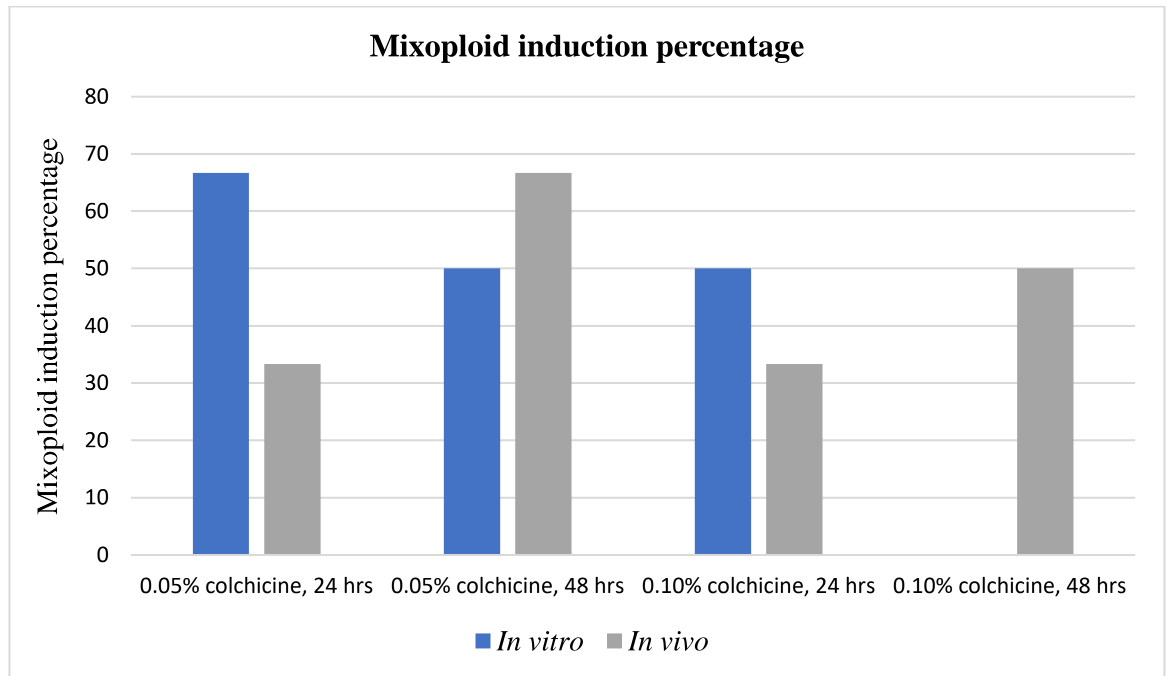


Fig. 15a. Flow cytometry results of percentage of mixoploids obtained from common treatments in *in vitro* and *in vivo* experiment for inducing heteroploidy in *Dendrobium crumenatum.*

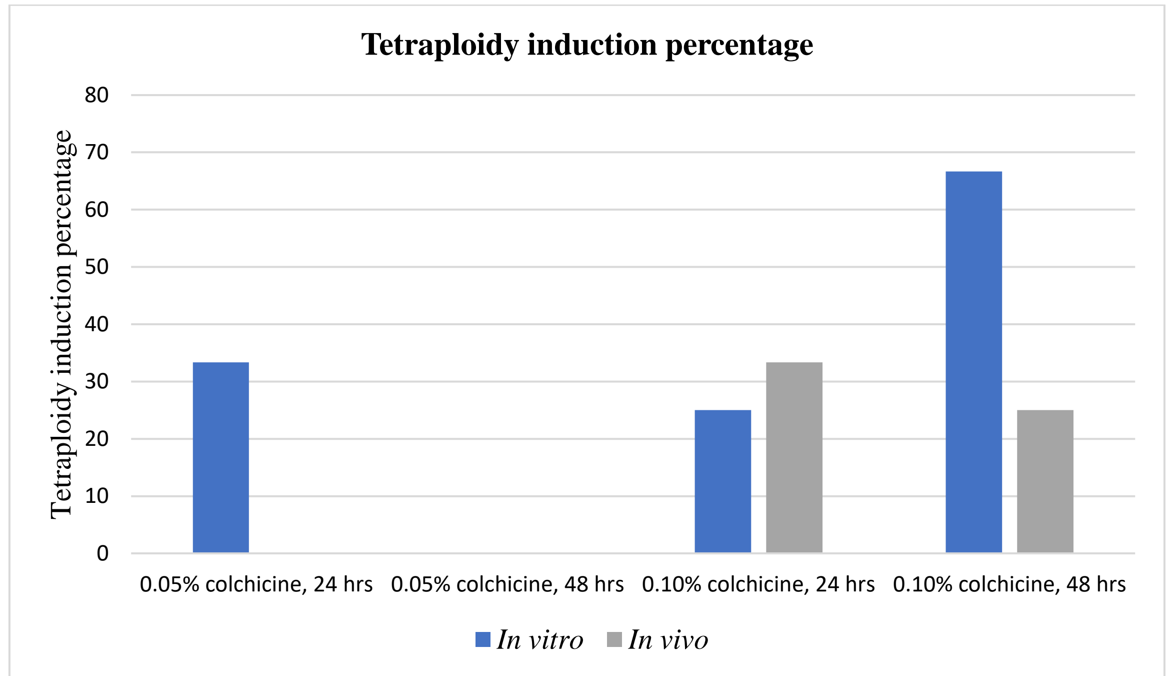


Fig. 15b. Flow cytometry results of percentage of tetraploids obtained from common treatments in *in vitro* and *in vivo* experiment for inducing heteroploidy in *Dendrobium crumenatum.*

Discussion

5. DISCUSSION

Orchids belongs to the largest family of angiosperms with 25,000 species belonging to 600-800 genera. They are prized for their vast diversity in the shape, size, colour and attractiveness of flowers and with good keeping qualities. Orchid generas mainly, *Cymbidium, Dendrobium, Phalaenopsis, Vanda, Cattleya, Paphiopedilum* and *Oncidium* command high value and great demand all over the world. Among these genera, *Dendrobiums* are the most popular tropical orchids getting fame as cut flowers in India as well as in the world.

Polyploidy induction plays a significant role in the hybridization and improvement of orchids (Orchidaceae). Induction of polyploidy can help to restore fertility by doubling of chromosomes and thus creating allotetraploids (Ranney, 2006). Polyploids can help breeders in developing improved hybrids and novel types by contributing beautiful floral or growth characteristics which is unobtainable from the diploid forms.

In this context, the present investigation was undertaken to induce heteroploidy in *Dendrobium crumenatum* via *in vitro and in vivo* technique. The findings of the *in vitro* and *in vivo* experiment for heteroploidy induction and the flow cytometry analysis are being discussed in this chapter.

5.1 *IN VITRO* HETEROPLOIDIZATION

Stem nodal segments containing an axillary bud was preferred as the explant for induction of heteroploidy in *Dendrobium crumenatum* via *in vitro* method. The basal medium used was VW medium. The basal medium and explant were chosen based on the study conducted by Meesawat and Kanchanapoom (2002) in *Dendrobium crumenatum.*

5.1.1 Surface Sterilization

Contamination caused by the fungi and bacteria were the major constraints faced during the establishment phase of tissue culture explants.

Mother plants can serve as hub for microbes since it is exposed to field conditions for longtime and thus the explants collected from these can act as source for systemic infection while culturing. High rates of fungal contaminations were observed in the initial stages of culture which imposed to experiment different sterilization treatments. To reduce the contamination and obtain contamination free cultures, in addition to the method adopted by Meesawat and Kanchanapoom (2002), 2 hours of external sterilization using detergent and several surface sterilization treatments were also carried out.

Among the six different treatments tried for surface sterilization in the present investigation, the percentage survival of axillary buds on VW medium was found to be the highest (87.5 percent) when surface sterilization was carried out using the combination of following sterilization agents i.e., 20 per cent sodium hypochlorite for 20 minutes followed by 0.1 per cent mercuric chloride treatment for 5 minutes and finally 70 % alcohol treatment for 2 minutes. This was in accordance to the findings of Da Silva *et al.* (2016). According to Da Silva *et al.* (2016), various combinations of different disinfectants increase the efficacy of the disinfection procedure. Cui *et al*. (2015) successfully surface sterilized capsules of *Dendrobium chrysotoxum* by disinfecting in 75% ethanol followed by 0.1% $HgCl_2$ treatment for 8 minutes and 10 minutes treatment of 10% NaOCl. Successful sterilization using different combination of sterilization agents viz., sodium hypochlorite, 70 per cent ethanol and 0.1 per cent mercuric chloride to obtain contamination free cultures were supported by the findings of Junior *et al.* (2011) in *Dendrobium nobile*, Dohling *et al.* (2012) in *Dendrobium longicornu*, and Li *et al.* (2013) in *Dendrobium fimbriatum*.

5.1.2 Days for Bud Initiation

In the present study, the days for bud initiation ranged from 26-43 days, with the average number of days for bud initiation as 29.25 days. Similar responses were observed in the studies conducted by Meesawat and Kanchanapoom (2002) and Priyakumari (2008) in *Dendrobium crumenatum.*

5.1.3 Callus Induction

Callusing response of a crop is highly influenced by the culture conditions, media composition and the explant used for *in vitro culture.* In the present study, young stem nodes containing the axillary buds from the growing proximal end showed better response than that from the distal end. This might be due to the increased number of dedifferentiating cells at the growing end. In the present study, callus induction percentage was found highest (70 per cent) when cultured in VW media with hormonal combination of 1 mgL^{-1} BA and 0.5 mgL^{-1} 2,4-D. Similar combination of hormones for induction of callus were also reported in the study conducted by Churchill *et al.* (1973) and Refish *et al.* (2016). The earliest response for callus induction in the present study was obtained within 27.33 days when cultured in VW medium containing hormonal combination of 1 mgL^{-1} BA and 0.5 mgL^{-1} 2,4-D. Similar response was shown in the study conducted by Meesawat and Kanchanapoom (2002) in *Dendrobium crumenatum*. In a study conducted by Nasiruddin *et al.* (2003), the fastest response for callus induction was obtained, when the culture media was supplemented with 0.5mgL^{-1} 2,4D. Roy and Banarjee (2003) and Zhao *et al*. (2007) concluded that the use of cytokinin (BA) in culture medium succeeded in inducing callus in *Dendrobium fimbriatum* and *Dendrobium candidum*. The calli obtained from the present study from the callus initiation medium were subcultured for further proliferation in the same medium itself which showed early callus response.

5.1.4 PLB Formation

In the present study, the average number of days for PLB formation was noted as 60.5 days after the bud initiation stage. Similar response was reported in the study conducted by Meesawat and Kanchanapoom (2002) in *Dendrobium crumenatum* and Das *et al.* (2008) in *Dendrobium lituiflorum*. The same medium which obtained the earliest response for callus induction was used for PLB formation. This was based on the findings by Nasiruddin *et al.* (2003) in *Dendrobium formosum* and Das *et al.* (2008).

5.1.5 Survival of PLBs after Colchicine Treatment

For the induction of heteroploidy, suspension culture medium (basal VW medium) containing different concentration of colchicine was used. This was in accordance with the study conducted by Yenchon and Te-chato (2012). In the present study, PLBs were chosen for induction of heteroploidy. In a study conducted by Miguel and Leonhardt (2011) and Zakizadeh *et al*. (2020), it was concluded that, in orchids, the most widely used plant part to induce polyploidy are protocorms and PLBs.

In the present study, as the concentration and exposure time of colchicine treatment increased, there observed a decrease in the survival percentage. The observation was in support of the findings of Griesbach (1981), Silva *et al.* (2000), Atichart and Bunnag,(2007), Sarathum *et al.* (2010), Choopeng *et al*. (2019). The survival percentage of PLBs immediately after colchicine treatment ranged from 91.67 to 41.67 percent. Similar results were obtained in a study conducted by Yenchon and Te-chato (2012) and Zakizadeh *et al.* (2020)

The percentage of cultures showing response to colchicine treatment which was recorded two weeks after colchicine treatment of PLBs showed further decrease in the survival percentage values ranging from 83.33 to 33.33 per cent. A pale-yellow colour change was observed in treated PLBs of some cultures initially, which turned to brown colour later. This might be due to the toxic effect of colchicine treatment which is causing cellular imbalance and may eventually lead to the plant death. Similar conclusions were also reported by Petchang (2010) in *Dendrobium draconis* and Blasco *et* al. (2015). Apart from the colour change, fungal contamination was also noticed in some of the treated cultures. The control cultures i.e., basal VW suspension medium without colchicine, corresponding to 48 hours treatment duration of colchicine also showed a decrease in the survival percentage. It was observed that this reduction was due to the fungal contamination in the control cultures.

5.1.6 Shoot Regeneration and Proliferation

In the present study, for inducing organogenesis, the colchicine treated PLBs were then transferred to shoot induction medium i.e., media containing hormones BA (1mgL^{-1}) and NAA (0.1 mgL^{-1}). The hormonal combination was adopted based on the study conducted by Meesawat and Kanchanapoom (2002) in *Dendrobium crumenatum*, Talukdar *et al.* (2002) in *Cymbidium pendulum*, Kurup *et al.* (2005) and Priyakumari (2008) in *Dendrobium* orchid. The minimum number of days for the induction of shoot was noted to be 32.75 days and the maximum was 46.20 days after the PLB stage. The corresponding control cultures for 24 and 48 hrs duration which were not subjected to colchicine treatment showed shoot induction after 30 and 34 days respectively, which was similar to the findings of Meesawat and Kanchanapoom (2002) in *Dendrobium crumenatum*. In the present investigation, as the exposure time and concentration of colchicine increased, it was observed that shoot induction was delayed. This might be due to the effect of colchicine which is causing a delay in the induction of organogenesis. The observation was in support to the findings by Pham *et al.* (2019) in *Dendrobium officinale*. The number of days for shoot multiplication ranged from 11.40-16.50 days and the control cultures took 12-14.5 days. It was observed that there was no much difference in the time taken for shoot multiplication when compared to the control cultures. In a study performed by Meesawat and Kanchanapoom (2002) in the same *Dendrobium sp*. the duration for shoot induction and multiplication were more or less the same as observed in the present study.

In the present study, cultures which were initially subjected to high concentration and exposure of colchicine treatments showed a decrease in the number of shoots, whereas the corresponding control cultures showed response in accordance with the findings of Meesawat and Kanchanapoom (2002). The number of shoots per culture (5.82) was noticed the highest in the cultures that were initially exposed to 24 hrs duration of 0.05 per cent colchicine and the lowest (2.90) was observed in the cultures that were initially subjected to 0.20 per cent colchicine treatment for 48 hrs. This decrease in the number of shoots

is observed to be the effect of antimitotic agent colchicine which indicates both concentration and duration of exposure of colchicine has a significant impact. The observation was in support of the findings of Priyakumari (2008), Atichart (2013) and Pham *et al.* (2019) in *Dendrobium* sp.

5.1.7 Percentage Survival of Cultures at Multiplication Stage

The percentage of cultures survived at multiplication stage in the present study were noticed to be the same as that obtained in the observation, 'percentage of cultures showing response after colchicine treatment' except for two treatments i.e., 0.15% and 0.20% of colchicine concentration and duration of exposure, 48 hrs. The further reduction in the survival per cent observed in the above mentioned two treatments might be due to the effect of colchicine, as there was a colour change observed from pale yellow to brown which then spread throughout the cultures in the culture bottles that were at shoot initiation stage, which was indeed accompanied by fungal contamination also. The reduction in the survival of cultures was in agreement with the findings of Silva *et al.* (2000) in in *Cattleya intermedia*, Zakizadeh *et al.* (2020) in *Dendrobium*, Mohammadi *et al*. (2021) in *Phalaenopsis amabilis*. The leaves collected from all the colchicine treated cultures were utilized for sample preparation for flow cytometry analysis.

5.2 *IN VIVO* HETEROPLOIDIZATION

The same species of *Dendrobium* i.e. *Dendrobium crumenatum* which was taken for *in vitro* heteroploidy induction study was chosen for induction of heteroploidy using colchicine via *in vivo* method also. *Dendrobium crumenatum* plantlets with height ranging from of 8-12 cm were used for the study for the colchicine treatment.

5.2.1 Survival Percentage of Plantlets after Colchicine Treatment

For the present investigation, *Dendrobium crumenatum* plantlets were subjected to colchicine treatment by completely immersing in colchicine solution and were maintained in dark conditions based on the colchicine treatment duration as the colchicine is light sensitive.

A decrease in the survival percentage was observed when the *D. crumenatum* plantlets subjected to colchicine treatment were potted. It was observed that, as the duration for exposure towards colchicine treatment increased, there was a reduction in the survival percentage of plantlets. In contradiction to the present study, Vichiato *et al*. (2007) observed 100 per cent survival in all the treatments for polyploidy induction in *Dendrobium nobile*. As the duration of exposure to colchicine treatment increased, the plantlets showed a difference in the leaf and stem colour. The leaf and stem colour changed from green to slight yellow and the texture of the leaf was also affected. Some of the plantlets showed signs of rotting also which accounted for the decrease in the survival percentage. The plantlets which expressed difference in leaf colour showed shedding of their leaves and remained as such without showing any response towards the colchicine treatments when the observation, 'percentage of plants showing response after colchicine treatment' was taken one month after colchicine treatment. The control for the experiment which was maintained in the same experimental condition but without colchicine also showed a difference from 100 per cent survival percentage in few cases. This indicated that, apart from the toxicity due to colchicine, the complete immersion of plantlets in water has got a significant impact on the survival of the plantlets, when the duration of immersion period is increased.

5.2.2 Shoot Length and Diameter

The mean length of shoot (15.141 cm) observed in different colchicine treated plantlets showed an increased value than that of control plantlets (13.5 cm).Significant variation was observed in shoot length which ranged from 13 cm in treatment T_2 to 17.8 cm in treatment T_3. The shoot length recorded in each treatment were greater than that observed in corresponding control plantlets except for treatment T_4. The effect of colchicine on increase in plant height was reported by Vichiato *et al.* (2014). The decrease in length of shoot as observed in one of the treatments when compared to control might be due to reduced rate of cell division due to the colchicine treatment. The observation was in line to

the findings by Pham *et al.* (2019), Zakizadeh, *et al.* (2020) and Mohammadi *et al.* (2021).

The mean diameter of shoot in treated plantlets (0.205 cm) were observed to be lesser than that recorded in control plantlets (0.207 cm). Except for the diameter of shoot noticed in treatments T_4 (0.226 cm) and T_7 (0.233 cm), all the other treatments showed either the same or lower value for shoot diameter when compared to corresponding controls. The study was in agreement with the findings by Vichiato *et al.* (2014) and Pham *et al.* (2019).

5.2.3 Pseudobulb Height and Diameter

The character pseudobulb height in present investigation ranged from 6.66 cm to 7.2 cm for eight different colchicine treatments. The mean height of pseudobulb in treated plantlets and the control were found to be same (6.9 cm). In treatments T_1, T_5, T_6 and T_8 the pseudobulb height were found to be higher than the corresponding controls. These observations were in support to the findings by Zakizadeh *et al.* (2020).

The colchicine treated *Dendrobium* plantlets showed an improved value for mean pseudobulb diameter (0.783 cm) than that of control plantlets (0.64 cm) maintained in the same experimental conditions. The diameter of pseudobulb in each treated plantlets recorded an increased value when compared to corresponding controls except for a single treatment T_3. The observations were in support of the findings by Zakizadeh *et al.* (2020). In contradiction to the present study, Vichiato *et al*. (2014) observed a decreased pseudobulb diameter in treated plants when compared to the control.

5.2.4 Number of Leaves, Leaf Length And Leaf Width

Number of leaves in treated plantlets ranged from 2.67 to 5.33 and in untreated control, it was noted to be 3 to 5. The mean number of leaves in eight treatments were observed to be 3.791 and in control, it was 3.75. The treatments T_1, T_2, T_6 and T_7 recorded an improvement in number of leaves than its corresponding control. This observation was in agreement with the findings of Mohammadi *et al.* (2021). The treatment T_5 did not show any difference in the

number of leaves when compared to the control plantlets. Similar observation was noticed in the study conducted by Choopeng *et al*., (2019).

The mean value for the character, length of leaf recorded a lower value in eight treatments (8.662 cm) when compared to the control (9.2 cm). The treatments T_1, T_2, T_5 and T_7 were observed to have increased leaf length when compared to its corresponding control. This observation was in line of the findings by Kazemi and Kaviani (2020) and Zakizadeh *et al.* (2020). The treatments T_3, T_4, T_6 and T_8 recorded decreased value for the character leaf length compared to its corresponding control plantlets. This observation was in agreement with the study conducted by Vichiato *et al*. (2014).

In the present investigation, it was observed that, the mean width of leaf in eight treatments (2.258 cm) was higher than that of the control plantlets (1.575 cm). The average width of leaf showed a trend of increased leaf width when compared to control except for treatment T_8. This observation was in agreement with the findings by Mohammadi *et al.* (2021) and Zakizadeh *et al.* (2020). In the present study, the treatment T_8 noted a reduced value for leaf width when compared to control. Similar finding was also observed in the study conducted by Vichiato *et al.* (2014).

5.2.5 Variability Components

Variability existing within a crop is considered as the criteria for effective selection. On computing phenotypic and genotypic coefficients of variability, absolute assessment of variability can be done, apart from the analysis of variance. Based on genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV), the environment influence on the character can be separated out from the total variability. In the present study, on analyzing the variability components, PCV obtained higher magnitude than GCV for all the characters analysed except for the character, diameter of shoot. The character, shoot diameter noted the same PCV and GCV values, indicating that environment influence is negligible. The character, number of leaves recorded high PCV and GCV values. Similar findings were also reported in the

study conducted by Nath (2003), Thomas and Rani (2017), Seeja (2018) and George (2020). In the present study, the character diameter of pseudobulb, length of shoot, length of leaf and width of leaf obtained moderate PCV and GCV values. High PCV and moderate GCV values were observed for the characters, leaf length and leaf width in a study conducted by Moniruzzaman *et al.* (2012). Moderate PCV and GCV values for plant height and width of leaf were also reported by George (2020). The characters, diameter of shoot and length of pseudobulb in the present study obtained low PCV and GCV values. The findings were similar to the observation made by Thomas and Rani (2017).

5.2.6 Correlation Studies

Correlation analyses the interrelationship and extent of association of various quantitative characters present in a population. When a selection is being carried out for a character of interest in a population, it is associated with the improvement of other character associated with the character of interest. In the present study, genotypic and phenotypic correlations were worked out for seven characters of the *in vivo* colchicine treated *Dendrobium crumenatum* plantlets.

On analysing the genotypic correlation among seven quantitative characters, the character, length of shoot showed significant positive correlation with the character height of pseudobulb. A significant correlation was noticed between the traits, length of shoot and the diameter of shoot. Similar result was also reported by Islam *et al*. (2013). The trait diameter of shoot was positively correlated with width of leaf. Similar findings were also reported by Nagayoshi *et al.* (1996).

The character number of leaves had significant positive correlation with height of pseudobulb, length of leaf and width of leaf. Similar correlations were also reported by Nagayoshi *et al.* (1996), Moniruzzaman *et al*. (2012) and Vanlalruati *et al.* (2015). Number of leaves showed a significant negative correlation with length of shoot. Similar findings were also reported by Islam *et al*. (2013) and Anna *et al*. (2015).

5.3 FLOW CYTOMETRY ANALYSIS

Flow cytometry is a simple, rapid, convenient and accurate technique in screening of the ploidy status and nuclear DNA content (Dolezel, 1991; Meesawat *et al.,*2008) The confirmation of the results obtained from flow cytometry analysis is carried out by chromosome counting.

Flow cytometry histogram obtained after performing flow cytometry analysis is used for determination of ploidy status. In the present study, the flow cytometry histogram was carefully analysed for categorizing the results obtained into diploids, mixoploids and higher ploidy levels. The colchicine treated *in vivo* and *in vitro Dendrobium crumenatum* samples when subjected to flow cytometry analysis, diploid, mixoploid and tetraploids were obtained. The interpretation of the flow cytometry histogram of treated samples were carried out by comparing the peak positions of diploid (control) and the treated samples. The interpretations were performed based on the findings of study conducted by Meesawat *et al.* (2008) and Dolezel *et al*. (2007b).

In the *in vitro* colchicine treated *Dendrobium crumenatum* samples, the maximum tetraploids (66.67%) were obtained from the colchicine concentration 0.15% and 0.10% for treatment duration 24 and 48 hrs respectively. The observations were in line with the findings by Sanguthai *et al.* (1973), Silva *et al*. (2000), Sarathum *et al*. (2010), Zakizadeh *et al*. (2020). Maximum mixoploids (66.67 %) in the present study were obtained from those samples which were subjected to 0.05% and 0.15% of colchicine concentration for treatment duration 24 and 48 hrs respectively. The observations were similar to the findings by Silva *et al*. (2000) and Thao *et al*. (2003). Yenchon and Te-chato (2012) and Atichart (2013) also obtained significant per cent of mixoploids in the polyploidy induction study in orchids using colchicine. In the present study, the minimum percentage of tetraploids were recorded in those samples which were subjected to colchicine concentration of 0.10% for treatment duration 24 hrs. In certain treatment, the percentage of induction of heteroploidy seemed to be zero. These observations were in agreement with findings of Yenchon and Te-chato (2012).

In the *in vivo* colchicine treated *Dendrobium crumenatum* samples, the maximum tetraploids (50%) were obtained from the colchicine concentration of 0.05% for the treatment duration 96 hrs. This result is comparable to the previous findings by Sanguthai *et al.* (1973), Watrous and Wimber (1988), Silva *et al*. (2000). In the present study, it was observed that the mixoploid induction was found to be more common than the tetraploid induction. It was also noted that, for the same exposure time i.e., 96 hrs and colchicine concentration 0.10%, only mixoploids were obtained. In a similar study conducted by Vichiato *et al.* (2007) in *Dendrobium nobile*, highest number of tetraploids (29.17%) were obtained by immersing the plants in 0.1% colchicine solution for 96 hrs.

On comparing the flow cytometry results obtained from the common treatments in experiment I and experiment II, the maximum tetraploid induction was noticed in treatment T_6 i.e., 0.10% colchicine, 48 hrs (66.67%) of experiment I and treatment T_2 & T_4 of experiment II (33.34%). The maximum mixoploid induction percentage (66.67%) was noticed in *in vitro* and *in vivo* experiment for the treatments 0.05% colchicine, 24 hrs and 0.05% colchicine, 48 hrs respectively. For the same dose and duration of exposure of colchicine in *in vivo* and *in vitro* experiment, i.e., 0.10% colchicine, 48 hrs, the *in vitro* experiment noted a higher tetraploid induction percentage (66.67%) than *in vivo* experiment (25%). Since, the maximum tetraploid induction was noticed in *in vitro* experiment than *in vivo* experiment, it was concluded from the present investigation that, for *Dendrobium crumenatum* which was subjected to both *in vitro* and *in vivo* colchicine treatments, *in vitro* colchicine treatment was found effective than *in vivo* colchicine treatment, when the concentration and duration of exposure of colchicine used for the study were same. This observation was in agreement with the findings by Vichiato *et al.* (2007).

Summary

6. SUMMARY

The present study entitled "Induction of heteroploidy in *Dendrobium* sp." was carried out at the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2019-21. The study was undertaken to induce heteroploidy in *Dendrobium crumenatum* using antimitotic agent colchicine. The investigation consisted of three experiments. Experiment I involved the induction of heteroploidy in *Dendrobium crumenatum* using colchicine via *in vitro tissue* culture technique. Experiment II involved the induction of heteroploidy in *Dendrobium crumenatum* using colchicine via *in vivo* method. Experiment III involved the flow cytometry analysis of the colchicine treated *Dendrobium crumenatum* from experiment I and II to confirm the heteroploidy induction.

In the Experiment I, induction of heteroploidy in *Dendrobium crumenatum* was carried out through tissue culture technique. The explant used in the micropropagation was stem nodal segments containing one axillary bud and the basal medium chosen was VW medium. Nodal segments of 1-2 cm length, cut from the main shoot were subjected to external sterilization in 1 per cent labolene detergent for 2hrs. When the cultures showed increased fungal contamination, external sterilization was supplemented with an additional fungicide treatment i.e., 0.1 per cent Bavistin for 30 minutes. Six treatments of surface sterilization were tried and compared in order to attain the maximum survival percentage at culture initiation stage and thus minimize the culture contamination. The treatment TS_6 (20 per cent sodium hypochlorite for 20 minutes + 0.1 per cent mercuric chloride for 5 minutes + 70 % alcohol for 2 minutes) was found to be the best treatment for surface sterilization, which obtained 87.5 per cent explant survival at culture initiation stage.

The explants were initially inoculated in the basal VW medium for initiation of bud. The average number of days observed for initiation of axillary bud cultured in VW media was observed to be 29.25 days. The cultures which showed bud initiation were then transferred to callus induction medium. For obtaining maximum callus induction with early response, VW media with

different hormonal concentration of BA (0.5,1 mgL^{-1}), 2,4-D (0.5 mgL^{-1}) and NAA (0.1 mgL^{-1}) were tried. Among the hormonal combinations, the highest callus induction percentage of 70 percent was observed in the medium TC_2 (VW + 1 mgL^{-1} BA + 0.5 mgL^{-1} 2,4-D) within 27.33 days. The inoculated culture bottles and testtubes were incubated at 25°C in dark during callus induction and proliferation stage. The same medium was used for further subculturing, callus proliferation and PLB induction. The average number of days for PLB formation was noticed within 60.5 days after bud initiation stage. The cultures were provided with white light (3000 lux) with a photoperiod of 16 hrs light and 8 hrs dark and incubated at 25 °C. For the induction of heteroploidy, PLBs were subjected to eight different colchicine treatments. The colchicine treatments were based on the concentration of colchicine (0.05%, 0.10%, 0.15%, 0.20%) and duration of exposure of PLBs to colchicine (24 and 48 hrs).

Basal VW suspension medium containing different doses of colchicine were used for inducing ploidy change in PLBs of *Dendrobium crumenatum*. The filter sterilized stock solution of colchicine was prepared prior and stored in amber bottle under refrigerated condition. The required quantity of colchicine was pipetted onto the autoclaved VW media inside laminar airflow chamber followed by transferring of PLBs onto the colchicine containing VW suspension media. The cultures were maintained in dark condition as colchicine is photolabile, till their respective treatment durations. The control for each treatment were the PLBs which were subjected to same conditions but without colchicine. After the treatment duration, the PLBs were washed in double distilled water and inoculated onto shoot induction medium.

Observations on percentage survival of PLBs after colchicine treatment were taken immediately after the transfer of treated PLBs on to plantlet regeneration medium i.e VW basal media containing hormones BA (1mgL^{-1}) and NAA (0.1 mgL^{-1}). The survival percent of PLBs after colchicine treatment ranged from 91.67 to 41.67 percent. The highest survival percent 91.67 was observed in treatment T_1 (0.05% colchicine, 24 hrs) and lowest percent 41.67 was observed in treatment T_8 (0.10% colchicine, 48hrs).

Observations of the cultures showing response after colchicine treatment and the control cultures for the treatment duration (24 and 48 hrs) were recorded one week after the colchicine treatment and converted into percentage. The percent of cultures showing response after colchicine treatment ranged from 33.33 to 83.33 percent. The highest percentage of cultures showing response after colchicine treatment was found to be 83.33 per cent and was observed in treatments T_1 (0.05% colchicine, 24 hrs) and T_2 (0.10% colchicine, 24 hrs). The lowest percentage of cultures showing response after colchicine treatment was noted in T_8 (0.20% colchicine, 48 hrs) with 33.33 per cent. On assessment of the observations made on shoot formation after transferring into the plantlet regeneration medium (VW media+ 1mgL^{-1} BA and 0.1 mgL^{-1} NAA) , the number of days taken for shoot formation in colchicine treated PLBs varied from 32.75 to 46.20 days after PLB stage, whereas in control cultures it was observed after 30 (24 hrs) and 34 (48 hrs) days.

The fastest response for shoot multiplication within 11.40 days was shown by PLBs which were earlier subjected to treatment T_1 (0.05% colchicine, 24 hrs) and the slowest response was recorded in the PLBs which were earlier subjected to the treatment T_8 (0.20% colchicine, 48 hrs). The maximum number of shoots per culture 5.82, was noticed in the culture, where the PLBs were earlier subjected to treatment T_1 (0.05% colchicine, 24 hrs) and the minimum number recorded was 2.85, which was obtained from the culture, where the PLBs were earlier subjected to treatment T_7 (0.15% colchicine, 48 hrs). The maximum survival percentage of culture at multiplication stage was observed to be 83.33 per cent, which were earlier subjected to colchicine treatments T_1 (0.05% colchicine, 24hrs and T_2 (0.10% colchicine, 48 hrs), and the minimum survival percentage of 25 per cent was obtained in cultures which were earlier subjected to colchicine treatment T_8 (0.20% colchicine,48 hrs).

In the Experiment II, induction of heteroploidy in *Dendrobium crumenatum* was carried out using *D.crumenatum* plantlets. The plantlets for the experiment were subjected to 8 different treatments by completely immersing it in colchicine solution. Treatments were based on colchicine

concentration (0.05%, 0.1%) and the exposure of plantlets to these doses of colchicine (24 hrs,48 hrs, 72 hrs, 96 hrs). The maximum survival percentage of 100 per cent (observation taken immediately after colchicine treatments) were noticed in the treatments T_1 (0.05% colchicine, 24 hrs), T_2 (0.10% colchicine,24 hrs) and T_3 (0.01% colchicine, 48 hrs). The minimum survival percentage (66.67 per cent) were recorded in the treatments T_7 (0.05% colchicine, 96 hrs) and T_8 (0.10% colchicine, 96 hrs).

The highest percentage of plants showing response after colchicine treatment of 83.33 per cent (observation recorded after 2 weeks of colchicine treatment) were noticed in treatments T_1 (0.05% colchicine, 24 hrs) and T_3 (0.05 % colchicine, 48 hrs). The lowest percentage of plants showing response after colchicine treatment (16.67 per cent) was noticed in treatment T_8 (0.10% colchicine, 96 hrs).

The observations shoot length (cm), diameter of shoot (cm), height of pseudobulb (cm), diameter of pseudobulb (cm), number of leaves, length of leaf (cm), width of leaf (cm) were recorded 6 months after colchicine treatment. The observations shoot length, diameter of pseudobulb, number of leaves and width of leaves showed mean values greater than their corresponding control means, whereas the observations, diameter of shoot and length of leaf noted lower mean values than corresponding control. The mean value for the character, height of pseudobulb were found to be same for treated and control plantlets.

The character, number of leaves recorded high PCV and GCV. Moderate PCV and GCV were recorded for the characters viz., diameter of pseudobulb, length of shoot, length of leaf and width of leaf. The characters, diameter of shoot and height of pseudobulb obtained low PCV and GCV. Significant positive genotypic correlation were observed between length of shoot and height of pseudobulb, diameter of shoot and width of leaf, length of pseudobulb and number of leaves, number of leaves and length of leaf, width of leaf and number of leaves. Significant negative genotypic correlation was noticed between length of shoot and diameter of shoot, diameter of shoot and length of leaf, length of pseudobulb and diameter of pseudobulb, length of leaf

and length of pseudobulb, diameter of pseudobulb and length of shoot, number of leaves and length of shoot, length of leaf and length of shoot, number of leaves and diameter of pseudobulb, width of leaf and diameter of pseudobulb.

In the Experiment III, flow cytometry analysis of colchicine treated *in vitro* samples from experiment I and experiment II were performed at Rajiv Gandhi Institute for Biotechnology, Poojapura. For the sample preparation, for loading on to FACS machine, leaves were collected from both *in vitro* and *in vivo* colchicine treated *Dendrobium crumenatum* cultures and plants. Otto buffers, fluorochrome propidium iodide and RNase were used for sample preparation. The histogram peaks of control (diploid) and treated samples were compared for interpretation of the results. In the *in vitro* treated samples, the maximum tetraploids (66.67 %) were obtained from treatments T_3 (0.15% colchicine, 24 hrs) and T_6 (0.10 %colchicine, 48 hrs). Maximum mixoploid (66.67%) was noticed in treatments T_1 (0.05% colchicine, 24 hrs) and T_7 (0.15% colchicine, 48 hrs).

On analyzing the histogram peaks of *in vivo* treated samples, the highest tetraploid induction (50%) was noticed in treatment T_7 (0.05% colchicine, 96 hrs) and the maximum mixoploids (66.67%) were obtained from treatments T_5 (0.05% colchicine, 72 hrs) and T_3 (0.05% colchicine, 48 hrs). Since, the maximum tetraploid induction was noticed in *in vitro* experiment than *in vivo* experiment, it was concluded from the present investigation that, for *Dendrobium crumenatum* which was subjected to both *in vitro* and *in vivo* colchicine treatments, *in vitro* colchicine treatment was found effective than *in vivo* colchicine treatment, when the concentration and duration of exposure to colchicine used for the study were same.

References

7. REFERENCES

Al-khateeb, A.A. 2008. Regulation of *in vitro* bud formation of date palm (*Phoenix dactylifera* L.) cv. *Khanezi* by different carbon sources. *Bioresour. Technol*. 99(14): 6550-6555.

Amoo, S.O., Aremu, A.O., Moyo, M., Szucova, L., Dolezal, K., and Staden, V. J. 2014. Physiological effects of a novel aromatic cytokinin analogue in micropropagated *Aloe arborescens* and *Harpagophytum procumbens*. *Plant Cell Tiss. Organ. Cult*.116(1): 17-26.

Anna, E., Vojtko, I.S., Balazs, A. L., and Attila, M.V. 2015. Factors affecting reproductive success in three entomophilous orchid species in Hungary. *Acta Biol. Hung*. 66(2): 231 -241.

Arditti, J. 1977. *Orchid Biology – Reviews and Perspectives*, Vol. I. Cornell University Press, Ithacca, London, 628p.

Atichart, P. 2013. Polyploid induction by colchicine treatments and plant regeneration of *Dendrobium chrysotoxum*. *Thai J. Agric. Sci*. 46(1): 59-63.

Atichart, P. and Bunnag, S. 2007. Polyploid Induction in *Dendrobium secundum* (Bl.) Lindl. by in vitro Techniques. *Thai J. Agric. Sci*. 40: 91-95.

Bernier, G., Havelange, A., Houssa, C., Petitjean, A., and Lejeune, P. 1993. Physiological signals that induce flowering. *The Plant Cell*. *5*(10): 1147.

Bhattacharyya, P., Kumaria, S., and Tandon, P. 2016. High frequency regeneration protocol for *Dendrobium nobile*: a model tissue culture approach for propagation of medicinally important orchid species. *S. Afr. J. Bot*. 104: 232-243.

Blakeslee, A.F. and Avery, A.G. 1937. Methods of inducing doubling of chromosomes in plants. *J. Heredity.* 28: 393 –411.

Blanc, G. and K.H. Wolfe. 2004. Functional divergence of duplicated genes formed by polyploidy during Arabidopsis evolution. *Plant Cell*. 16:1679–1691.

Blasco, M., Badenes, M.L., and Naval, M.D.M. 2015. Colchicine-induced polyploidy in loquat (*Eriobotrya japonica* (Thunb.) Lindl.). *Plant Cell Tiss. Organ Cult.* 120: 453–461.

Bunnag, S. and Hongthongkham, J., 2015. Polyploid induction of *Dendrobium draconis* Rchb. f. *Acta Hortic*., 1087: 445–451.

Chaicharoen, S. 1995. Studies on chromosome number, floral characteristics and fertility of diploid and allotetraploid *Dendrobium superbiens*. *Agric. Nat. Resour*. *29*(2): 150-157.

Chan, L.T.L. and Lee, S.M. 1996. Inflorescence culture of some tropical orchid varieties. *Singapore J. Primary Ind*. 24: 25-29.

Chase, M.W., Cameron. K. M., Freudenstein. J. V., Pridgeon. A.M., Salazar. G, Berg. C., and Schuiteman, A. 2015. An updated classification of Orchidaceae. *Bot. J. Linn. Soc.*177(2):151–174.

Chen, W.H and Tang, C.Y. 2018. A protocol for the induction of polyploids in *Phalaenopsis* orchids by *in vitro* method without using anti-microtubule agents. In: Lee, Y.I and Yeung, E.C.T. (eds). *Orchid Propagation: From Laboratories To Greenhouse - Methods And Protocols.* Humana Press, New York, pp.317-330.

Choopeng, S., Te-Chato, S., and Khawnium, T. 2019. Effect of colchicine on survival rate and ploidy level of hydrid between *Dendrobium santana* x *D. friedericksianum* orchid. *Int. J. Agric. Technol*. 15(2): 249-260.

Churchill, M.E., Ball, E.A., and Arditti, J. 1973. Tissue culture of orchids I. Methods for leaf tips. *New Phytol*. 72(1): 161-166.

Cui, B., Kang, Y.Y., Wang, J.Q., Jiang, S.H., Liang, F., and Liu, J. 2015. Research on the tissue culture and rapid propagation technology of *Dendrobium chrysotoxum* Lindl. *Chin. Agri. Sci. Bull.* 31(19): 111-121.

Da Silva, J. A. T and Tanaka, M. 2006. Multiple regeneration pathways via thin cell layers in hybrid *Cymbidium* (Orchidaceae). *J Plant Growth Regul.* 25:203–210.

Da Silva, J.A.T., Cardoso, J.C., Dobranszki, J., and Zeng, S. 2015. *Dendrobium* micropropagation: a review. *Plant Cell Rep*. 34(5): 671-704.

Da Silva, J.A.T., Winarto, B., Dobranszki, J., Cardoso, J.C., and Zeng, S. 2016. Tissue disinfection for preparation of *Dendrobium in vitro* culture. *Folia Hort*. 28(1): 57-75.

Das, M.C., Kumaria, S., and Tandon, P. 2008. *In vitro* propagation and conservation of *Dendrobium lituiflorum* Lindl through Protocorm-Like Bodies. *J. Plant Biochem. Biotechnol.* 17(2): 177-181.

Debnath, M., Malik, C. P., and Bisen, P. S. 2006 Micropropagation: a tool for the production of high quality plant-based medicines. *Curr. Pharm. Biotechnol.* 7:33–49.

Dermen, H. 1940. Colchicine polyploidy and technique. *Bot. Rev*. 6(11): 599-635.

Devi, J. and Deka, P.C. 2000. Induction of polyploidy in *Phaiustan kervilliae*. *J. Orchid Soc. India* 14: 1-5.

Dhooghe, E., Van Laere, K., Eeckhaut, T., Leus, L., and Van Huylenbroeck, J. 2011. Mitotic chromosome doubling of plant tissues in vitro. *Plant Cell Tiss. Org. Cult*. 104(3): 359-373.

Dohling, S., Kumaria, S., and Tandon, P. 2012. Multiple shoot induction from axillary bud cultures of the medicinal orchid, *Dendrobium longicornu*. *AoB Plants*, 2012: 1-7.

Dolezel, J. 1991. Flow cytometric analysis of nuclear DNA content in higher plants. *Phytochem. Anal.* 2(4): 143-154.

Dolezel, J. 1997. Application of flow cytometry for the study of plant genomes. *J Appl. Genet.* 38:285–302

Dolezel, J., Binarova, P., and Lucretti, S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. *Biol. Plant.* 31: 113–120.

Dolezel, J., Greilhuber, J., and Suda, J. 2007a. Estimation of nuclear DNA content in plants using flow cytometry. *Nat. Protoc*., *2*(9): 2233-2244.

Dolezel, J., Greilhuber, J., and Suda, J. 2007b. *Flow cytometry with plant cells: analysis of genes, chromosomes and genomes*. Wiley-VCH, Weinheim, Germany, 479p.

Dolezel, J., Sgorbati, S., and Lucretti, S. 1992. Comparison of three DNA fluorochromes for flow-cytometric estimation of nuclear DNA content in plants. *Physiol. Plant*. 85(4): 625–631.

Endress, P.K. 1994. *Diversity and Evolutionary Biology of Tropical Flowers*, Cambridge University Press, Cambridge, 511p.

Eng, W.H. and Ho, W.S. 2019. Polyploidization using colchicine in horticultural plants: A review. *Sci. Hortic*. 246: 604-617.

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., and Firoozabady, E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Sci. 220*(4601): 1049-1051.

George, R.M. 2020. Varietal evaluation and development of hybrids in *Phalaenopsis* orchids. M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 133p.

Givan, A.L. 2013. *Flow Cytometry: First Principles* (2nd Ed.). John Wiley & Sons, 296p.

Goh, C. J., Strauss, M. S., and Arditti, J. 1982. Floral Induction and Physiology in Orchids. [Includes ecology and genetics]. *Orchid Bio.: Rev. and Prospectives* 2: 213-241.

Gopinath, P. P., Parsad, R., Joseph, B., and Adarsh, V. S. 2020. GRAPES: General Rshiny Based Analysis Platform Empowered by Statistics. https://www.kaugrapes.com/home. version 1.0.0. DOI: 10.5281/zenodo.4923220.

Greilhuber, J., Temsch, E.M., and Loureiro, J.C. 2007. Nuclear DNA content measurement. In: Dolezel, J., Greilhuber, J., Suda, J. (eds.), *Flow Cytometry with Plant Cells: Analysis of Genes, Chromosomes And Genomes*, Wiley-VCH, Weinheim, Germany pp. 67-101.

Griesbach, R.J. 1981. Colchicine induced polyploidy in *Phalaenopsis* orchid. *Plant Cell Tiss. Org. Cult*. 1:103-107.

Grosso, V., Farina, A., Giorgi, D., Nardi, L., Diretto, G., and Lucretti, S. 2017. A high-throughput flow cytometry system for early screening of *in vitro* made polyploids in *Dendrobium* hybrids. *Plant Cell Tiss. Org. Cult*. 132(1): 57-70.

Hameed, N., Shabbir, A., Ali, A., and Bajwa, R. 2006. *In vitro* micropropagation of disease-free rose (*Rosa indica* L.). *Mycopath*, *4*(2): 35-38.

Holttum, R. E. 1964. The story of the pigeon orchid. In: Holttum, R.E. (ed.) *Plant Life in Malaya*, Longmans, UK, pp. 121-141.

Hu, C.Y. and Wang, P.J. 1983. Meristem, shoot tip and bud cultures. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (eds.), *Handbook Of Plant Cell Cultures: Techniques For Propagation And Breeding*. Macmillan Publishing Company, New York, pp. 177-277.

Hu, Y., Sun, D., Hu, H., Zuo, X., Xia, T., and Xie, J. 2021. A comparative study on morphological and fruit quality traits of diploid and polyploid carambola (*Averrhoa carambola* L.) genotypes. *Sci. Hortic*. 277: 109843.

Huy, N.P., Luan, V.Q., Tung, H.T., Hien, V.T., Ngan, H.T.M., Duy, P.N., and Nhut, D.T. 2019. *In vitro* polyploid induction of *Paphiopedilum villosum* using colchicine. *Sci. Hortic*. 252: 283-290.

Islam, M.S., Mehraj, H., Roni, M.Z.K., Shimasaki, K., and Jamal Uddin, A.F.M. 2013. Correlation between cane growth and flowering behaviour of *Dendrobium* orchid cultivars. *J. Bangladesh Acad. Sci. 37*(2): 205-209.

Junior, R.F.G., Neto, P.C., and Mantovani, C., 2011. *In vitro* growth of *Dendrobium nobile* Lindl. with the addition of activated carbon. *Sci*. 40: 28-34.

Kamemoto, H. 1987. Genome breeding in *Dendrobium* orchids. *Breed. Hortic. crops*. 35: 182–188.

Kanjilal, B., De Sarker, D., Mitra J., and Datta K.B. 1999. Stem disc culture: development of a rapid mass propagation method for *Dendrobium moschatum* (Buch. -Ham.) Swartz – an endangered orchid. *Curr. Sci.* 77: 497–500.

Kano, K.1965. Studies on media of orchid seed germination. *Mem. Fac. Agric. Kagawa Univ.* 22: 74.

KAU. 2016. Package of Practices Recommendations 'Crops'. Fourteenth edition. Directorate of Extension, Kerala Agricultural University, Thrissur, 278 p.

Kazemi, M. and Kaviani, B. 2020. Anatomical, morphological, and physiological changes in colchicine-treated protocorm-like bodies of *Catasetum pileatum* Rchb. f. *in vitro*. *Cogent Biol*. 6(1): 1840708.

Kerdsuwan, N. and Te-chato, S., 2012. Effects of colchicine on survival rate, morphological, physiological and cytological characters of chang daeng orchid (*Rhynchostylis gigantean* var. *rubrum* Sagarik) *in vitro*. *J. Agric. Sci. Technol.* 8(4): 1451-1460.

Kermani, M.J., Sarasan, V., Roberts, A.V., Yokoya, K., Wentworth, J., and Sieber, V.K. 2003. Oryzalin-induced chromosome doubling in Rosa and its effect on plant morphology and pollen viability. *Theor. Appl. Genet*. 107:1195-1200.

Ketsa, S., Uthairatanakij, A., and Prayurawong, A. 2001. Senescence of diploid and tetraploid cut inflorescences of *Dendrobium* "Caesar." *Sci. Hortic*. 91: 133–141.

Kim, M. S. Kim, J. Y., and Eun, J. S. 2003. Chromosome doubling of *Cymbidium* hybrid with colchicine treatment in meristem culture. *Amer. Orchid Soc. Bull.* 32: 885- 887.

Kumaraswami, M., Sudipta. K.M., Balasubramanya. S., and Anuradha, M. 2010. Effects of different carbon sources on *in vitro* morphogenesis response of patchouli (*Pogostemon cablin* Benth.). *J Phytol.* 2: 11–17.

Kuriakose, S .1997. Standardisation of *in vitro* techniques for mass multiplication of *Aranthera* and *Dendrobium.* Ph.D. (Ag) thesis, Kerala Agricultural University, Thrissur, 207 p.

Kurup, S.S., John, S., Rejimol, K. R., Mathew, J. C., and Rajmohan, K. 2005. In vitro propagation of *Dendrobium* var. Betty Ho through shoot tip culture. *Proceedings of National Symposium on Biotechnological Interventions for improvement of Horticultural Crops: Issues and Strategies,* Jan 10 -12, 2005. Kerala Agricultural University, Thrissur, pp. 195-196.

Leong, T. M. and Wee, Y. C. 2013. Observations of pollination in the pigeon orchid, *Dendrobium crumenatum* Swartz (Orchidaceae) in Singapore. *Nat. Singapore* 6:91–96

Li, C., Zhang, Z.J., Wei, Y., Li, L.X., Ling, Z.Z., and Wei, K.H. 2013. Study on tissue culture and rapid propagation of *Dendrobium fimbriatum* Hook. var. *oculatum*. Hook. *North Hortic*. 6: 105-107.

Liao, D.L., Xie, L., Zeng, R. Z., Li, Y.H., Yi, M. S., and Zhang, Z. S. 2012. Relationship between chromosome ploidy and morphology characters in *Dendrobium*. *Acta. Bot. Boreal*. *Occident*. *Sin.* 32(10):2023–2029.

Lin, H., de Carvalho, P., Kho, D., Tai, C.Y., Pierre, P., Fink, G.R., and Pellman, D. 2001. Polyploids require Bik1 for kinetochore–microtubule attachment. *Int. J. Cell Biol*. 155(7):1173-1184.

Loureiro, J., Rodriguez, E., Dolezel, J., and Santos, C. 2006. Comparison of four nuclear isolation buffers for plant DNA flow cytometry. *Ann. Bot. 98*(3): 679-689.

Martin K. P. and Madassery J. 2006. Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. *Sci. Hortic.* 108(1): 95–99.

Meesawat, U. and Kanchanapoom, K. 2002. *In vitro* plant regeneration through embryogenesis and organogenesis from callus culture of pigeon orchid (*Dendrobium crumenatum* Sw.). *Thammasat Int. J. Sc. Tech*. 7(2): 1-17.

Meesawat, U. and Kanchanapoom, K. 2007. Understanding the flowering behaviour of pigeon orchid (*Dendrobium crumenatum* Swartz). *Orchid Sci. Biotechnol*. 1: 6-14.

Meesawat, U., Srisawat, T., Eksomtramage, L., and Kanchanapoom, K. 2008. Nuclear DNA content of the pigeon orchid (*Dendrobium crumenatum* Sw.) with the analysis of flow cytometry. *Songklanakarin J. Sci. Technol*. 30(3): 277-280

Miano, T.F., Rabbani, M.G., and Memon, N.U.N. 2016. Assessment of genetic diversity among orchids. *Bangladesh J. Bot*. 45(5): 987-993.

Miguel, T.P. and Leonhardt, K.W. 2011. *In vitro* polyploid induction of orchids using oryzalin. *Sci. Hortic*. 130(1): 314-319.

Mohammadi, M., Kaviani, B., and Sedaghathoor, S. 2021. *In vivo* polyploidy induction of *Phalaenopsis amabilis* in a bubble bioreactor system using colchicine. *Ornam. Hortic*. 27: 204-212.

Moniruzzaman, M., Zaman, M.A., Hossain, M.E., Bhuiyan, M.M.H., and Rahman, M.Z. 2012. Genetic variability and character association in some native orchid species (*Dendrobium* spp.). *The Agric.* 10(1): 1-9.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays of tobacco tissue cultures. *Physiol. Pl.* 15:473-497.

Nagayoshi, T., Hatanaka, T., and Suzuki, T. 1996. Seed Propagation of *Habenaria radiata*. Morphological and physiological characteristics of plants derived from *in vitro* cultured seedlings. *Nat. Hum. Act*. 1: 67-81.

Nasiruddin, K.M., Begum, R., and Yasmin, S. 2003. Protocorm like Bodies and plantlet regeneration from *Demdrobium formosum* leaf callus. *Asian J. Plant Sci.* 13(2): 955-957.

Nath, C.N. 2003. Compatibility studies in monopodial orchids. M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 146p.

Otto, F. 1990. DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In *Methods in cell biology* (Vol. 33, pp. 105-110). Academic Press.

Panse, V.G. and Sukhathme, P.V. 1985. *Statistical Methods for Agricultural Workers* (2nd Ed.). Indian Council of Agricultural Research, New Delhi, 381p.

Petchang, R. 2010. The effects of colchicine concentration and treatment duration on growth and chromosome number in *Dendrobium draconis* Rchb. f. *J. Sci. Technol. Mahasarakham Univ.* 29(4): 413-419.

Pfosser, M., Heberle-Bors, E., Amon, A., and Lelley, T. 1995. Evaluation of sensitivity of flow cytometry in detecting aneuploidy in wheat using disomic and ditelosomic wheat–rye addition lines. *Cytometry: J. Int. Soc. Anal. Cytol.* 21(4): 387-393.

Pham, P.L., Li, Y.X., Guo, H.R., Zeng, R.Z., Xie, L., Zhang, Z.S., Chen, J., Su, Q.L., and Xia, Q. 2019. Changes in morphological characteristics, regeneration ability, and polysaccharide content in tetraploid *Dendrobium officinale*. *HortScience* 54(11): 79-86.

Prakash, L., Lee, C.L., Loh, C.S., and Goh, C.J. 1996. *In vitro* propagation of commercial orchids: An assessment of current methodologies and development of a novel approach – Thin section culture. *J. Orchid Soc. India* 10: 31-41.

Priyakumari, I. 2008. Enhancement of *in vitro* propagation efficiency and induction of polyploidy in orchids. Ph.D. thesis, Kerala Agricultural University, Thrissur, 190p.

Priyakumari, I. and George, T.S. 2011. *In vitro* clonal shoot morphogenesis of commercial Dendrobium orchid cultivars in polyamines supplemented medium. *J. Trop. Agric*. 49: 118-120.

Pyati, A. N., Murthy, H. N., Hahn, E. J., and Peak, K. Y. 2002. *In vitro* propagation of *Dendrobium macrostachym* Lindl.- a threatened orchid. *Indian J. Exp. Biol.* 40: 620–623.

Ranney, T. G. 2006. Polyploidy: from evolution to new plant development. *Proc. Int. Plant Propagators Soc*., 56: 137–142.

Refish, N.M.R., Wang, L., Fu, C., Xu, X., Jin, W., Li, M., and Yu, L. 2016. Establishment and optimization of high efficiency embryogenic callus induction system in *Dendrobium candidum*. *Afr. J. Plant Sci*. 10(4): 77-83.

Riva, S.S., Islam. A., and Hoque, M. E. 2016. *In vitro* regeneration and rapid multiplication of *Dendrobium bensoniae*, an indigenous ornamental orchid. *The Agric*. 14(2):24-31.

Ronzhina, E.S. 2003. Effect of 6-benzylaminopurine on the structure of photosynthetic apparatus of Faba bean (*Vicia faba* L.). *Appl. Biochem. Microbiol*. 39: 411-417.

Roy, J. and Banerjee, N. 2003. Induction of callus and plant regeneration from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum* Hk. f. *Sci. Hortic*. 97(3-4): 333-340.

Sagawa, Y. and Shoji, T. 1967. Clonal propagation of *Dendrobium* through meristem culture. *Am. Orchid Soc. Bull*. 36: 856-889.

Sakhanokho, H.F., Rajasekaran, K., Kelley, R.V., and Farjdj, N.I. 2009. Induced polyploidy in diploid ornamental ginger (*Hedychium muluense* R. M. Smith) using colchicine and oryzalin. *HortScience* 44(7): 1809–1814.

Sanguthai, O., Sanguthia, S., and Kamemoto, H. 1973. Chromosome doubling of a *Dendrobium* hybrid with colchicine in meristem culture. *Na Okika O Hawaii Hawaii Orchid J.* 12: 12–16

Sarathum, S., Hegele, M., Tantiviwat, S., and Nanakorn, M. 2010. Effect of concentration and duration of colchicine treatment on polyploidy induction in *Dendrobium scabrilingue* L. *Eur. J. Hortic. Sci*. *75*(3): 123-127.

Sattler, M.C., Carvalho, C.R., and Clarindo, W.R. 2016. The polyploidy and its key role in plant breeding. *Planta*. 243(2): 281-296.

Seeja, G. 2018. Distant hybridization and compatibility studies in wild orchids. Ph.D. thesis, Kerala Agricultural University, Thrissur, 201p.

Silva, P. A. K. X. M., Callegari-Jacques, S. and Bodanese-Zanettini, M.H. 2000. Induction and identification of polyploids in *Cattleya intermedia* Lindl.(Orchidaceae) by *in vitro* techniques. *Cienc. Rural 30*(1): 105-111.

Singh, R.K. and Chaudhary, B.D., 1977. *Biometrical Methods In Quantitative Genetic Analysis*. Kalyani Pub., Ludhiana, 78p.

Sivamani, S. 2004. Micropropagation of *Dendrobium* hybrids. M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 81p.

Sivasubramanian, S. and Menon, M. 1973. Heterosis and inbreeding depression in rice. *Madras Agric. J.* 60: 1139-1140.

Starr, C. and Taggart, R. 1995. *Cell Biology and Genetics,* (7th Ed). Brooks Cole, 300p.

Sudeep, R. 1994. Standardization of media supplements for shoot proliferation in *Dendrobium*. . M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 119 p.

Sugapriya, S., Mathad, J.C., Patil, A.A., Hegde, R.V., Lingaraju, S., and Biradar, M.S. 2012. Evaluation of *Dendrobium* orchids for growth and yield grown under greenhouse. *Karnataka J. Agric. Sci.* 25(1): 104-107.

Talukdar, M.C., Ahmed, H., and Mahanta, H. 2002. *In vitro* propagation and artificial seed production of *Cymbidium pendulum*. In: Misra, R.L. and Misra, S. (eds.), *Proceedings of National Symposium on Indian Floriculture in the New Millineum*, 25-27 February 2002, Lal Bagh, Banglore. pp.130-133.

Tang, Y.M., Zhang, C.L., Su, B., Cheng, Q., and Na, H.Y. 2010. Polyploid induction and identification of *Dendrobium candicum*. *North. Hortic.* 17.

Thao, N.T.O., Ureshino, K., Miyajima, I., Ozaki, Y., and Okubo, H. 2003. Induction of tetraploids in ornamental *Alocasia* through colchicine and oryzalin treatments. *Plant Cell Tiss. Organ Cult.* 72:19-25.

Thomas, B. and Rani, L. C. 2017. Analysis of genetic parameters in commercially important monopodial orchid genotypes. *Acta Hortic*. 1165: 101-106.

Thorpe, T.A., 1980. Organogenesis *in vitro*: structural, physiological, and biochemical aspects. *Int. Rev. Cytol.* 11:71-111.

Tokuhara, K. and Mill, M. 2001. Induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis* (Orchidaceae). *In Vitro Cell Dev. Biol. Plant* 37: 457–461.

Uddain, J., Gnasekaran, P., Zakaria, L., Lynn, C.B., and Subramaniam, S. 2015. The effect of different growth media, carbon source and PGRs on *Dendrobium broga* giant orchid's protocorm-like bodies (PLBs) proliferation supported with SEM and TEM analysis. *Pak. J. Bot*. 47(2): 587-593.

Vacin, F. and Went, F.W. 1949. Some pH changes in nutrient solutions. *Bot. Gaz.* 110: 605–613.

Vainola, A. 2000. Polyploidization and early screening of *Rhododendron* hybrids. *Euphytica* 112(3): 239-244.

Vanlalruati, T., Mandal, T., and Pradhan, S., 2016. Genetic variability, character association and path coefficient analysis in orchids (Orchid spp) of Mizoram, Sikkim and Darjeeling District of West Bengal for various quantitative traits. *Indian J. Agric. Sci*. 86(3): 385-90.

Vichiato, M.R.D.M., Vichiato, M., Pasqual, M., Rodrigues, F.A., and Castro, D.M.D. 2014. Morphological effects of induced polyploidy in *Dendrobium nobile* Lindl.(Orchidaceae). *Crop Breed. Appl. Biotechnol.*14(3): 154-159.

Vichiato, M.R.D.M., Vichiato, M., Pasqual, M., de Castro, D.M., and Dutra, L.F. 2007. Tetraploidy induction and identification in *Dendrobium nobile* Lindl. (Orchidaceae). *Rev. Cienc. Agron.* (Portuguese) 38 (4): 385.

Watrous, S.B. and Wimber, D.E. 1988. Artificial induction of polyploidy in *Paphiopedilum*. *Lindleyana* 3(4): 177-183.

Wimber, D.E. and Wimber, D.R. 1967. Floral characteristics of diploid and neotetraploid *Cymbidiuns. Amer. Orchid Soc. Bull*. 38: 572-576.

Xu, D., Chu, S., Zheng, M., Pan, Y., Qian, G., and Li, L. 2016. Creating novel tetraploid germplasm with high salt tolerance and ion utilization in *Dendrobium Nobile* Lindl. *Nanosci. Nanotechnol. Lett. 8*(12): 1127-1134.

Yakimova, E., Toteva, V.K., Groshkoff, I., and Ivanova, G. 2000. Effects of BA and CPPU on protease and α-amylase activity of in vitro cultured explants of *Rosa hybrida* L. *Bulg. J. Plant physiol.* 26: 39-47.

Yenchon, S. and Te-chato, S. 2012. Polyploidy induction of *Dendrobium formosum* by colchicine treatment *in vitro*. *Acta. Hort.* 1025: 81–88.

Zakizadeh, S., Kaviani, B., and Hashemabadi, D. 2020. *In vivo* induced polyploidy in *Dendrobium* 'Sonia'in a bubble bioreactor system using colchicine and oryzalin. *Rev. Bras. Bot*. *43*(4): 921-932.

Zhao, P., Wang, W., Feng, F.S., Wu, F., Yang, Z.Q., and Wang, W.J. 2007. High-frequency shoot regeneration through transverse thin cell layer culture in *Dendrobium candidum* Wall Ex Lindl. *Plant Cell Tiss. Org.* 90(2): 131-139.

Appendix

APPENDIX -I

Media composition of Vacin and Went media

Nutrient	Quantity (mgL^{-1})
Macronutrients	
$(NH_4)_2 SO_4$	500
KNO_3	525
KH_2PO_4	250
$MgSO_4 . 7H_2O$	250
$Ca_3(PO_4)_2$	200
Micronutrients	
$FeSO_4 . 7 H_2O$	560
$MnSO_4 . 4 H_2O$	150
Others	
Sucrose (gL^{-1})	20
Agar (gL^{-1})	6.4

INDUCTION OF HETEROPLOIDY IN *Dendrobium* sp.

by

REVATHI B. S

(2019-11-115)

Abstract of the thesis

Submitted in partial fulfilment of the

requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BREEDING AND GENETICS

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM - 695522

KERALA, INDIA

2021

ABSTRACT

The present study entitled "Induction of heteroploidy in *Dendrobium* sp." was carried out at the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2019-21. The study was undertaken to induce heteroploidy in *Dendrobium crumenatum* using antimitotic agent colchicine via *in vitro* and *in vivo* technique.

In *in vitro* induction of heteroploidy in *Dendrobium crumenatum*, the explant used in the micropropagation was stem nodal segments containing one axillary bud and the basal medium chosen was VW medium. The best combination of surface sterilization agents identified were, 20 per cent sodium hypochlorite for 20 minutes followed by 0.1 per cent mercuric chloride for 5 minutes and 70 % alcohol for 2 minutes. The medium VW+1 mgL-1 BA + 0.5 mgL-1 2,4-D was chosen for callus induction, callus proliferation and PLB formation. The explants were maintained in dark for callus induction and proliferation and for subsequent stages a photoperiod of 16 hours light and eight hours dark was provided. For the induction of heteroploidy, PLBs were subjected to eight different colchicine treatments in basal VW suspension medium. Colchicine treatments were based on the concentration of colchicine (0.05%, 0.10%, 0.15%, 0.20%) and duration of exposure of PLBs to colchicine (24 and 48 hrs). For shoot induction, VW basal medium supplemented with the hormones BA (1mgL-1) and NAA (0.1 mgL-1) was used. The maximum survival percentage of cultures at shoot multiplication stage after different colchicine treatments was observed to be 83.33 per cent, and the minimum survival percentage noted was 25 per cent.

In *in vivo* induction of heteroploidy in *Dendrobium crumenatum*, *D. crumenatum* plantlets were subjected to eight different colchicine treatments, based on colchicine concentration (0.05%, 0.1%) and the duration of exposure of plantlets to these doses of colchicine (24 hrs, 48 hrs, 72 hrs, 96 hrs). The highest percentage of plants showing response after colchicine treatment was

found to be 83.33 per cent (observation recorded after 2 weeks of colchicine treatment) and the lowest percentage recorded was 16.67 per cent.

The observations shoot length, diameter of pseudobulb, number of leaves and width of leaves showed mean values greater than their corresponding control means, whereas the observations, diameter of shoot and length of leaf noted lower mean values than corresponding control. The mean value for the character height of pseudobulb were found to be same for treated and control plantlets. The character, number of leaves recorded high PCV and GCV. Moderate PCV and GCV were recorded for the characters viz., diameter of pseudobulb, length of shoot, length of leaf and width of leaf. The characters, diameter of shoot and length of pseudobulb obtained low PCV and GCV. Significant positive genotypic correlation was observed between length of shoot and height of pseudobulb, diameter of shoot and width of leaf, length of pseudobulb and number of leaves, number of leaves and length of leaf and width of leaf and number of leaves.

Flow cytometry analysis of the colchicine treated *in vivo* and *in vitro* *D. crumenatum* samples were performed at Rajiv Gandhi Institute for Biotechnology, Poojapura. The histogram peaks obtained after the analysis of control (diploid) and treated samples were compared for interpretation of the results. In the *in vitro* treated samples, the maximum tetraploids (66.67 %) were obtained from treatments 0.15% colchicine, 24 hrs and 0.10 % colchicine, 48 hrs. On analyzing the histogram peaks of *in vivo* treated samples, the highest tetraploid induction (50%) was noticed in treatment 0.05% colchicine, 96 hrs.

It was concluded from the present investigation that, for *Dendrobium crumenatum* which was subjected to both *in vitro* and *in vivo* colchicine treatments, *in vitro* colchicine treatment was found effective than *in vivo* colchicine treatment, when the concentration and duration of exposure to colchicine used for the study were same.

സംഗ്രഹം

"ഡെൻഡ്രോബിയം സ്പീഷീസിൽ ഹെറ്ററോപ്ലോയിഡിയുടെ സൃഷ്ടിക്കൽ" എന്ന ഗവേഷണ പരിപാടി വെള്ളായണി കാർഷിക കോളേജിലെ സസ്യപ്രജനന ജനിതക ശാസ്ത്ര വിഭാഗത്തിൽ 2019-2021 കാലയളവിൽ നടത്തുകയുണ്ടായി. ആന്റിമൈറ്റോട്ടിക്ക് ഏജന്റ് 'കോൾചിസിൻ' ന്റെ സഹായത്തോടെ ഡെൻഡ്രോബിയം ക്രൂമെനേറ്റം ത്തിൽ ഇൻ വിട്രോ ഇൻ വിവോ വഴികളിലൂടെ ഹെറ്ററോപ്ലോയിഡി സൃഷ്ടിക്കുക എന്നായിരുന്നു പഠനത്തിന്റെ ലക്ഷ്യം.

ഇൻ വിട്രോ യിലൂടെ ഹെറ്ററോപ്ലോയ്ഡി സൃഷ്ടിക്കുന്നതിനായി ഡെൻഡ്രോബിയം ക്രൂമെനേറ്റത്തിന്റെ കക്ഷസ്കന്ധംമുള്ള സ്റ്റം നോട് ഭാഗവും അടിസ്ഥാന മാധ്യമമായി വി ഡബ്ല്യു മാധ്യമവും ഉപയോഗിച്ചു. ഏറ്റവും മികച്ച സംയോജിത ഉപരിതല അണുവിമുക്ത ഏജന്റായി 20 ശതമാനം സോഡിയം ഹൈപ്പോക്ലോറൈറ്റിനൊപ്പം (20 മിനിറ്റ്) 0.1 ശതമാനം മെർക്കുറിക് ക്ലോറൈഡും (5 മിനിറ്റ്) 70 ശതമാനം ആൽക്കഹോളും (2 മിനിറ്റ്) സ്ഥിതീകരിച്ചു. ക്യാലസ് രൂപീകരണത്തിനും വ്യാപനത്തിനും പി എൽ ബി രൂപീകരണത്തിനും 1 mgL^{-1} ബി. എ. യും 0.5 mgL^{-1} 2,4 -ഡി യും അടങ്ങിയ വി ഡബ്ല്യു മാധ്യമം ആണ് തിരഞ്ഞെടുത്തത്. ക്യാലസ് രൂപീകരണത്തിനും വ്യാപനത്തിനുമായി കൾച്ചർ കുപ്പികൾ അന്ധകാരത്തിൽ സൂക്ഷിച്ചിരുന്നു, അതിനു ശേഷമുള്ള ഘട്ടങ്ങളിൽ പ്രതിദിനം 16 മണിക്കൂർ പ്രകാശാവും 8 മണിക്കൂർ അന്ധകാരവും നൽകിയിരുന്നു. ഹെറ്ററോപ്ലോയ്ഡി സൃഷ്ടിക്കുന്നതിനായി പി എൽ ബി കൾ വി ഡബ്ല്യു അടിസ്ഥാന സസ്പെൻഷൻ മാധ്യമത്തിൽ 8 വ്യത്യസ്ത കോൾചിസിൻ ട്രീറ്റമെന്റസുകൾക്ക് വിധേയമാക്കി.

കോൾചിസിൻ ട്രീറ്റ്മെന്റസ് നിശ്ചയിച്ചത് കോൾചിസിന്റെ അളവും (0.05%, 0.10%, 0.15%, 0.20%) പി എൽ ബി കോൾചിസിൻ സമ്പർക്ക ദൈർഘ്യത്തിന്റെ (24 and 48 hrs) അടിസ്ഥാനത്തിലുമാണ്. ഷൂട്ട് രൂപീകരണത്തിനായി ബി എ യും (1 mgL^{-1}) എൻ എ എ (0.1 mgL^{-1}) യും

അടങ്ങിയ വി ഡബ്ലിയു മാധ്യമം ഉപയോഗിച്ചു. കോൾചിസിൻ ട്രീറ്റ്മെന്റസിനു ശേഷം ഷൂട്ട് വ്യാപന ഘട്ടത്തിൽ പരമാവധി അതിജീവന ശതമാനം 83.33 ശതമാനവും ഏറ്റവും കുറഞ്ഞ അതിജീവന ശതമാനം 25 ശതമാനവുംമായി രേഖപ്പെടുത്തി.

ഇൻ വിവോ യിലൂടെ ഹെറ്ററോപ്ലോയിഡി സൃഷ്ടിക്കുന്നതിനായി ഡെൻഡ്രോബിയം ക്രൂമെനേറ്റം ഓർക്കിഡുകളുടെ തൈകൾ വിവിധ കോൾചിസിൻ ട്രീറ്റ്മെന്റസിനായി വിധേയമാക്കി. കോൾചിസിൻ ട്രീറ്റ്മെന്റസ് നിശ്ചയിച്ചത് കോൾചിസിന്റെ അളവും (0.05%, 0.10%) തൈകളും കോൾചിസിനുമായിയുള്ള സമ്പർക്ക ദൈർഖ്യത്തിന്റെ (24 hrs, 48 hrs, 72 hrs, 96 hrs) അടിസ്ഥാനത്തിലുമാണ്. കോൾചിസിൻ ട്രീറ്റ്മെന്റസിനു ശേഷം (കോൾചിസിൻ ട്രീറ്റ്മെന്റിന്റെ രണ്ടു ആഴ്ചക്ക് ശേഷമുള്ള നിരീക്ഷണ അടിസ്ഥാനത്തിൽ) തൈകളിൽ പരമാവധി അതിജീവന ശതമാനം 83.33 ശതമാനവും ഏറ്റവും കുറഞ്ഞ അതിജീവന ശതമാനം 16.67 ശതമാനവുംമായി രേഖപ്പെടുത്തി.

ഷൂട്ടിന്റെ നീളം, സ്യുഡോബൾബിന്റെ വ്യാസം, ഇലകളുടെ എണ്ണം, ഇലകളുടെ വീതി എന്നിവ നിയന്ത്രിത ട്രീറ്റ്മെന്റസുകളെക്കാൾ കൂടിയതായി കാണാൻ സാധിച്ചു. ഷൂട്ടിന്റെ വ്യാസം, ഇലയുടെ വീതി എന്നിവ നിയന്ത്രിത ട്രീറ്റ്മെന്റ്സുകളെക്കാൾ കുറവായും കാണാൻ സാധിച്ചു. സ്യുഡോബൾബിന്റെ നീളം കോൾചിസിൻ ട്രീറ്റ്മെന്റ് സിലും നിയന്ത്രിത ട്രീറ്റമെന്റ്സിലും ഒരേമൂല്യം രേഖപ്പെടുത്തി. ഇലകളുടെ എണ്ണത്തിന് ഉയർന്ന പി. സി. വി. യും ജി. സി. വി. യും, സ്യുഡോബൾബിന്റെ വ്യാസത്തിനും, ഷൂട്ടിന്റെയും ഇലയുടെയും നീളത്തിനും ഇലയുടെ വീതിക്കും മിതമായ പി. സി. വി. യും ജി. സി. വി. യും, ഷൂട്ടിന്റെ വ്യാസത്തിനും സ്യുഡോബള്ബിന്റെ നീളത്തിനും താഴ്ന്ന പി. സി. വി. യും ജി. സി. വി. യും രേഖപ്പെടുത്തി. ഷൂട്ടിന്റെയും സ്യുഡോബൾബിന്റെയും നീളം തമ്മിലും, ഷൂട്ടിന്റെ വ്യാസവും ഇലയുടെ വീതി തമ്മിലും , സ്യുഡോബൾബിന്റെ നീളവും ഇലയുടെ എണ്ണം തമ്മിലും, ഇലയുടെ എണ്ണവും ഇലയുടെ നീളം തമ്മിലും. ഇലയുടെ

വീതിയും ഇലയുടെ എണ്ണം തമ്മിലും കാര്യമായ പോസിറ്റീവ് കോറിലേഷൻ രേഖപ്പെടുത്തി.

കോൾചിസിൻ ട്രീറ്റ്മെന്റ്സുകൾക്ക് വിധേയമാക്കിയ ഡി. ക്രൂമെനേറ്റം സാമ്പിളുകളുടെ ഫ്ളോ സൈറ്റോമെറ്ററി വിശകലനം രാജീവ് ഗാന്ധി ഇൻസ്റ്റിട്യൂട്ട് ഫോർ ബൈയോടെക്നോളജി, പൂജപ്പുര യിൽ നിർവഹിച്ചു. ഫല വ്യാഖ്യാനത്തിനായി നിയന്ത്രിതവും (ഡിപ്ലോയ്ഡ്) സാമ്പിളുകളുടെയും ട്രീറ്റഡ് സാമ്പിളുകളുടെയും ഹിസ്റ്റോഗ്രാം പീക്കുകൾ താരതമ്യം ചെയ്തു. ഇൻ വിട്രോ സാമ്പിളിൽ ഏറ്റവും ഉയർന്ന ടെട്രാപ്ലോയ്ഡ് ശതമാനം (66.67%), 0.15% കോൾചിസിൻ 24 മണിക്കൂർ, 0.10% കോൾചിസിൻ 48 മണിക്കൂർ എന്നീ ട്രീറ്റ്മെന്റസുകളിൽ നിന്ന് ലഭിച്ചു. ഇൻ വിവോ സാമ്പിളിൽ ഏറ്റവും ഉയർന്ന ടെട്രാപ്ലോയ്ഡ് ശതമാനം (50%) 0.05% കോൾചിസിൻ 96 മണിക്കൂറിലും രേഖപ്പെടുത്തി.

ഡെൻഡ്രോബിയം ക്രൂമെനേറ്റം ഓർക്കിടിൽ ഹെറ്ററോപ്ലോയിഡി സൃഷ്ടിക്കുന്നതിനായി ഇൻ വിവോ മാർഗ്ഗത്തേക്കാൾ ഇൻ വിട്രോ മാർഗമാണ് മികച്ചത് എന്നുള്ള നിഗമനം ഈ പഠനത്തോടുകൂടി മനസിലാക്കാൻ സാധിച്ചു.