Development of *in-vitro* propagation protocol of *Coelogyne flaccida*

A thesis submitted in partial fulfillment of the degree of

Master of Science

in

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Under the guidance of

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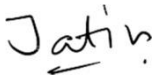
Declaration

I hereby declare that the work presented in the thesis entitled "**Development of in-vitro propagation protocol of *Coelogyne flaccida***" is a bonafide work under the supervision and guidance of **Dr. Anil Kumar,** Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala.

I also declare that this thesis or any other part of this thesis has never been submitted for any degree in this or any other university.



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Date: 17/07/2023

Certificate

This is to certify that the dissertation entitled **"Development of in-vitro propagation protocol of *Coelogyne flaccida*"** submitted by Mr. Jatin (Roll no. 302101012) in the partial fulfillment of the requirements for the award of the degree of Master of Science in Biotechnology, Thapar Institute of Engineering and Technology, Patiala is a record of student's work carried out under my guidance and supervision.

It is also certified that the matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree or diploma.

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Abbreviations

S.No.	Abbreviation	Full form
1	MS	Murashige and Skoog medium
2	PGR	Plant Growth Regulator
3	NAA	1-naphthalene acetic acid
4	BAP	6-Benzylaminopurine
5	GA_3	Gibberellic Acid
6	IBA	Indole-3-butyric acid
7	PLBs	Protocorm-like bodies
8	M	Molar
9	mM	Millimolar
10	µM	Micromolar
11	gm	Gram
12	L	Litre

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ABSTRACT

Abstract

Coelogyne flaccida (family *Orchidaceae*) is an important ornamental plant used as a pot and cut flower plant. The present study was focused to study the development of an in-vitro protocol for the regeneration of the orchid *Coelogyne flaccida* from Protocorm-like Bodies. Multiplication of the germinated culture of *Coelogyne flaccida* was achieved on MS medium supplemented with 2.5 µM BAP. The effect of BAP and NAA with different concentrations and combinations was observed on shoot formation and PLBs formation. The maximum number of shoot (5.22 ± 0.31) were recorded on medium supplemented with 2.5 µM BAP, and the maximum number of PLBs (14.77 ± 0.96) were formed at 1 µM each of BAP and NAA after 30 days of culture.

Maturation of the PLBs was attempted on basal MS medium supplemented with different concentrations of sucrose and mannitol to achieve efficient conversion of PLBs to plantlets. The highest maturation of PLBs was observed on an MS medium supplemented with 87 mM of sucrose. Matured PLBs were germinated on MS medium supplemented with BAP and GA_3. The maximum germination (18.32 ±0.44) was achieved at a concentration of 0.5 µM BAP and 1µM GA_3. Further, the germinated plantlets were acclimatized in invivo.

CHAPTER 1

INTRODUCTION

Introduction

Orchidaceae is one of the largest flowering plant families, with 28000 species divided into 763 genera (Christenhusz and Byng 2016). Orchids are widely grown as ornamental flowers, but they are also valued for their beauty, fragrances, medicinal properties, and long vase life (Chugh et al. 2009). Orchids exhibits a wide variety of flowers that vary in shape, colour, scent, and size (Murthy et al. 2018). Orchids can be found in almost every ecosystem and continent, with the exception of deserts, glaciers, and Antarctica (Givnish et al. 2015). In India, the diverse climatic and geographical conditions make it an ideal growing environment for orchids (Jalal and Jayanti 2013). There are around 1141 orchid species in India, with 657 being epiphytic, 484 being terrestrial, and only a few being lithophytic (Barman et al. 2016). Orchids can be found throughout the Himalayas and in the warm subtropical zones of India (Jalal and Jayanti 2013).

Coelogyne flaccida (family Orchidaceae) is also known as Loose Coelogyne and Bearded Coelogyne has the highest aesthetic value. It is an ornamental plant and can be found in its natural environments throughout Southeast and Northern South Asia. It is a warm-weather orchid brought from the eastern Himalayas, Nepal, North India, Bhutan, China, and Myanmar. It is regarded as an "easy" orchid to cultivate. This stunning species is recognized by its cone-shaped pseudobulb and green leaves. The flower spike begins to develop from the base of the mature pseudobulb. The plant can grow as an epiphyte on trees ranging in elevation from 1000 to 1800 meters above sea level. They thrive as hanging plants when planted in containers with medium pH, well-drained soil. The blossoms have a diameter of 3.5 to 4.0 cm and are stored in a cool, shady location to keep them fresh for as long as possible.

Fig 1: The picture represents (a) flower (b) pods containing seeds and (c) fully grown plant of *Coelogyne flaccida*

Table 1: Scientific Classification of *Coelogyne flaccida*

Kingdom	Plantae
Clade	Angiosperms
Order	Asparagales
Family	Orchidaceae
Genus	Coelogyne
Species	*C. flaccida*

Horticulture Use

Coelogyne flaccida is a significant horticultural species. Its white blossoms have a delicate fragrance that lasts for a long time. The beautiful blossoms of *C. flaccida* have a high decorative value as a cut flower, making it popular in the floriculture and horticulture industries.

Medicinal Use

In addition to its ornamental value, it has therapeutic potential also, as an extract of both the leaf and the pseudobulb can be used to treat a variety of ailments (Rajbhandari and Bhattarai 2001). The plant's pseudobulb paste was traditionally applied to the forehead to relieve migraines, and the plant's juice was used orally to treat indigestion (Pyakurel and Gurung 2008). The presence of secondary metabolites, flaccidinin, and oxoflaccidinin, in the plant's leaf provides antibacterial activity. The majority of its uses are for tuberculosis treatment. Furthermore, it contains Callosin, a functional constituent that corrects nutritional deficiencies (Majumdar and colleagues 1995).

Need of invitro propagation

Orchids require a favourable environment in order to grow. They effectively serve as a reliable indicator of biodiversity health. Orchids can reproduce vegetatively and sexually in their natural environment, via roots and seeds, respectively. But the rate of propagation and germination of seeds is very slow and poor. Therefore, scientists are facing a difficult problem in growing and conserving biodiversity of the orchids. Each species and organism must exist in order for the ecology and biodiversity to survive and prosper (Kaur and Bhutani 2013).

According to Bailes (1985) and Wu et al. (2009) *Coelogyne flaccida* is a rare and endangered orchid species. It is on the verge of extinction and has been listed as an endangered species by the IUCN (IUCN 2020). This is mainly due to the destruction of natural habitat by natural and human-caused disasters. Furthermore, the overharvesting of orchids by hunters, altered farming techniques, and the expansion of urban areas all contribute to the extinction of the species. Previously, ex-situ and in-situ conservation strategies were used to conserve orchid species, but they were deemed unfavourable for their growth and yields due to a variety of constraints (Pant 2013).

To overcome these challenges, scientists and researchers began using in-vitro procedures, which appeared to be a viable option, over ex-situ and in-situ conservation methods (Kumaria and Tandon 2007). In vitro germination of orchids is extremely beneficial in increasing the efficiency of conservation and breeding programmes, as in vitro germination rates of more than 70% are commonly reported, whereas in ex vitro conditions under natural environmental conditions, these rates rarely exceed 5% germinated seeds. This is due, in part, to the fact that orchid seeds lack nutritional reserves, and the embryo and seedlings at the early stages of germination are highly dependent on symbiosis with microorganisms known as mycorrhizae, which supply nutrition to these plants for an extended period of time until the seedling's complete establishment in the natural environment. As a result, tissue culture micropropagation methods are now deemed more appropriate. Therefore, it is crucial to develop a technique for rapid clonal propagation in order to revive the species in terms of conservation in its natural environment and eliminate the risk that such an important orchid for horticulture and medicine becoming extinct.

In-vitro propagation is defined as strategies and methods for growing plant species on nutrient-rich media with vitamins and plant growth regulators aseptically under optimum growth conditions (Pant 2013). Because orchids have a slow growth rate and poor yield in natural habitat therefore in-vitro procedures are best suited for their protection and maximum yield (Beheraet al. 2012). In-vitro propagation methods include callus induction, shoot and root multiplication. The medium used for in vitro propagation methods is nutrient-rich, providing macronutrients (nitrogen, calcium, phosphorous, sulfur, and magnesium), micronutrients (boron, zine, iron, copper), vitamins, and plant growth regulators (Reed et al. 2011). Zhao et al. (2008) claim that rapid in-vitro propagation techniques have become effective in the field of orchid propagation over the past few decades as a means of orchid multiplication for both conservation and commercial goals. Many researchers have used a variety of plant tissues including shoot tips, root tips, stem nodes, apical buds, protocorm-like bodies, calli, and immature and mature seeds for rapid in-vitro orchid propagation (Arditti and Ernst 1993, Morel 1960, Vajrabhaya 1978, Vij and Kaur 1998, Pyati et al. 2002, Basker and Narmatha 2006, Kaur and Bhutani 2010). One of the most favourable strategies for the mass multiplication of orchids in the international floricultural market is the process of induction, proliferation, and regeneration of protocorm-like bodies (PLBs). Furthermore, this strategy has been used to identify genes of interest connected with the generation of PLBs, as well as in breeding strategies that use biotechnology to develop new cultivars, such as transgenic plants. As a result, an effort is undertaken to develop an in vitro propagation method for *Coelogyne flaccida* that is as effective as possible by employing PLBs (Protocorm-like Bodies) as an explant.

The objective of the study:

- To establish the cultures of *Coelogyne flaccida* and study the effect of PGRs on PLB multiplication and formation of shoots.
- To examine the impact of sucrose or mannitol on the progression of the PLBs toward maturity and conversion.
- The acclimatization of plants into their respective pots.

CHAPTER 2

REVIEW OF LITERATURE

Coelogyne flaccida, native to Northern South Asia and Southeast Asia, is one of the most easily grown orchid species. These species can easily form an epiphyte on trees between 1000 and 1800 meters above sea level. It is easily recognizable by its pseudobulb and green leaves. It is an important plant in agriculture and horticulture due to its various features. It is not only valuable as an ornamental plant, but it also has medical and therapeutic properties. The leaves and pseudobulb extract can be used to cure a variety of diseases. It is frequently used to cure tuberculosis, and its pseudobulb paste is applied to the forehead to treat headaches. It is on the verge of extinction as a result of overexploitation. The conservation of the *Coelogyne flaccida* epiphytic orchid is essential for horticulture and medicine. Several studies have been conducted on the in-vitro germination and invitro propagation of several orchid species using various parts of the plant as explants.

Batygina et al. (2003) studied the reproductive system and germination of orchids, as well as the vegetative proliferation of orchids. The morphogenesis pathway that happens during the formation of the whole organism of orchids was studied in-vitro under normal conditions. The involvement of PLBs in orchid life cycles, polyembryony, and germination was also investigated.

Chen et al. (2004) propagated plant regeneration from *Paphiopedilum* orchid leaf culture using straight-shoot buds. The effect of 2,4-D and TDZ on straight shoot buds were investigated and evaluated.

Luthar et al. (2004) studied direct shoot regeneration in *Phalaenopsis* orchids by using nodes as an explant. In this experiment, six different culture mediums were used, each with different PGR in a different concentration and combination. The medium with no additional PGR

resulted shoot regeneration directly from dormant buds.

Silva and Tanaka (2006) developed a standard technique for orchid multiplication through production of PLBs, which are similar to somatic embryos.

Sungkumlong and Deb (2007) investigated the effect of various factors on the culture of immature embryos, the differentiation of PLBs, and the rapid proliferation of *Coelogyne suaveolens*. After 40 days of inoculation on MS medium containing 3% sucrose, 9 µM NAA, and 15% coconut water, exhibited a germination rate of 93%. The germinated shoots were subcultured on MS medium with 9 µM NAA, 3% casein hydrolysate, and 0.1% AC (activated charcoal), resulting in development of more than 12 shoots per explant. AC promoted the formation of a larger pseudobulb, which resulted in stronger plant roots. Plantlets that were hardened off in vitro and then transferred to pots had an 85% survival rate.

Kauth et al. (2008) studied the methods and applications of in-vitro seed germination of orchids. This article discusses the uses of asymbiotic and symbiotic orchid seed germination. The applications include photoperiod, nutrition, temperature, and fungal mycobiont.

Ng and Saleh (2010) propagated *Paphiopedilum* orchids in vitro by using PLBs. The highest number of PLBs were observed on a medium containing half MS supplemented with 4 µM kinetin. The secondary PLBs were sub cultured on a half MS medium supplemented with 60 g L^{-1} banana homogenate. This resulted tertiary PLBs, were then subcultured onto substrate containing various organic additives. Among them, the addition of 20% coconut water resulted in the highest average plantlet regeneration from the PLBs.

Naing et al. (2011) studied the role of different exogenous PGRs on different plant parts on the induction of shoots and also checked the ploidy level to ensure germplasm conservation. Micropropagation was accomplished by growing a culture of the shoot axis, an undamaged seedling, and pseudobulb segments from which the shoot axis had the best possible shoot response. These plant parts were grown on a half strength MS medium containing 0.44 mg L^{-1} of TDZ. A higher concentration of TDZ has been found to have inhibitory effects. The combination of 1 mg L^{-1} NAA and 1 mg L^{-1} BA in MS media produced the new shoots in entire seedlings. Pseudobulb segments were grown in two varieties: basal and apical. The basal type elicited a stronger response for the shoots.

With the use of PLBs, *Coelogyne cristata* can be produced in large amount. Growing PLBs in MS medium with NAA and coconut powder, dramatically impacted shoot regeneration and allowed new shoots to sprout from the PLBs section (Naing et al. 2011).

Dominguez et al. (2012) conducted a study for the preparation of artificial seeds of the orchid *Encyclia yucatanese* using a MS medium enriched with Abscisic acid, Proline, and Polyethylene glycol. They evaluated the germination and development of the PLBs, as well as their tolerance to desiccation.

Bustam et al. (2013) investigated plantlet regeneration from PLBs of varying sizes in *Dendrobium* 'Shaving White' by categorizing PLBs into different sizes. They determined that the size of the PLBs did not affect germination.

Plants can preserve their genetic homozygosity through a process known as regeneration, which also allow production of new plant species. Mohan and Das (2013) investigated the effect of medium strength and PGR on the regeneration of *Dendrobium densiflorum*, and their findings revealed that the MS full strength containing BAP provided the best results for both encapsulated and non-encapsulated PLBs, achieving a conversion rate of 100 %.

Kaur and Bhutani (2013) used a pseudobulb as an explant to develop an effective regeneration system for *Coelogyne flaccida*. The regeneration ability of MS and Mitra media supplemented with PGRs was investigated. In the MS and M medium, the apical and basal segments were treated with a 10% (v/v) mixture of BA, Kn, NAA, and CW. Throughout the trial, cultures inoculated on MS+ NAA or NAA+ BA developed a maximum of 10.25 and 10.00 shoots, respectively. Plantlets were produced from the shoots by rooting them in the same medium.

Parmar and Pant (2015) investigated in-vitro seed germination and growth of seedlings of the *Coelogyne flaccida*. It was carried out on an MS medium containing BAP and NAA, and the researchers discovered that a concentration of 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA was best for the full development of the seedling. According to findings, BAP and NAA are critical for the quick growth and development of in vitro seedlings.

Kalyan (2015) created a procedure for producing protocorm-like entities and plantlets from the immature leaves of *Coelogyne flaccida*. Young leaves were inoculated on MS

medium with different concentrations of NAA (0.5-2.0 mg L^{-1}), BAP (0.5-2.0 mg L^{-1}), and kinetin (0.5-2.0 mg L^{-1}). All procedures were carried out under sterile conditions. Direct PLB formation was observed from leaf without the callus formation. When NAA and kinetin (2.0 mg L^{-1} and 2.0 mg L^{-1}, respectively) were present in the medium, maximum plantlets (35) were formed.

Kundu and Gantait (2018) investigated the thidiazuron-induced regeneration of protocorm- like-bodies in orchids. They discovered that thidiazuron is the most active cytokinin-like PGR for in-vitro regeneration of orchid species, although it can have certain undesired side effects, such as the production of fascinated branches in some orchid species.

Utami and Hariyanto (2020) studied the influence of organic supplements on the germination of orchid seeds, the production of orchid PLBs, and the growth of orchid seedlings. Coconut water, banana homogenate, peptone, chitosan, potato homogenate, tomato juice, and yeast extract have been proven to promote orchid seedling development, seed germination, growth, and the formation of many shoots.

Table 2: Studies of regeneration of different species of *Coelogyne*.

S.no	Plant species	Explant	Medium composition	Response	References
1.	*Coelogyne flaccida*	Immature leaves	Step 1: Orchimax medium Step 2: MS medium +2 mg L^{-1} NAA + 2 mg L^{-1} Kinetin	The highest number of PLBs formed	De KK (2015)
2.	*Coelogyne cristata*	PLBs	Step 1: MS Media +0.5 mg L^{-1} BA Step 2: MS medium +30 g L^{-1} Banana powder + 30 g L^{-1} Coconut powder Step 3: MS media +0.5 mg L^{-1} IAA +2 mg L^{-1} IBA	The maximum number of shoots were developed from PLBs.	Naing et al. (2011)
3.	*Coelogyne cristata L*	PLBs	MS medium with TDZ +1 mg L^{-1} NAA +0.5 mg L^{-1} BA	Maximum number of PLBs developed shoots	Naing et al. (2011)
4.	*Coelogyne ovalis*	Nodal buds	KC medium + 10µM meta-topolin + 0.5 µM NAA	Maximum number of PLBs and shoots formation	Singh and Kumaria (2019)

The various studies on shoot regeneration of genus *Coelogyne* are given in Table 1. The studies are carried out by researchers using various explants, which are inoculated in the culture medium augmented with the different plant growth regulators in various concentrations and combinations.

The regeneration of *Coelogyne flaccida* is achieved with different explants. Our objective is to regenerate the plant using PLBs (Protocorm like bodies), which can be linked to the somatic embryo for the purpose of regeneration in orchids. This will be accomplished by inoculating the PLBs formed on the culture medium supplemented with various concentrations and combinations of PGRs, which are necessary for the healthy growth and development of the plant.

CHAPTER 3

MATERIAL AND METHODS

3.1 Media Preparation

The stock solutions of macronutrients, micronutrients, and vitamins were used to prepare MS medium as described by Murashige and Skoog (1962) containing 3 % (w/v) sucrose and 0.7 % (w/v) agar as a solidifying agent. The pH of the medium was adjusted in a range between 5.75 to 5.8 using 1 N NaOH or 1 N HCL. The Plant Growth Regulators (PGRs) were added as per the requirement after the pH adjustment. The medium was autoclaved for 15 min at 121 ºC and 15 psi followed by storage in a medium room for further use.

Chemicals, and Glassware

Plant tissue culture grade, molecular biology grade chemicals, plant growth regulators were purchased from HiMedia Laboratories Ltd, India. Glasswares such as test tubes, measuring cylinders, and beakers were purchased from Borosil Glass Works Limited, India. Tissue culture vessels (300 mL) were purchased from Kasablanka Corporation, India. Plasticwares such as pipette tis, measuring cylinder were purchased from Tarsons Products Private Limited, India.

Plant Material

The present study was performed on an orchid named *Coelogyne flaccida*. The pods containing the seeds of *Coelogyne flaccida* were collected from the Sikkim region. These pods were surface sterilized and germinated invitro for culture establishment of *Coelogyne flaccida* invitro at Plant Tissue Culture Laboratory of TIFAC-CORE, Thapar Institute of Engineering and Technology, Patiala (Punjab).

Surface Disinfection of Pods

Before inoculation, the pods were thoroughly washed with distilled water (dH_2O) for 15-20 min followed by treatment with Tween-20 detergent for 5 min. The pods were further disinfected with an antifungal agent, 0.2% (w/v) Bavistin for 15 min followed by washing with dH_2O (4-5 times). In the next step, the pods were transferred to Laminar Air Flow and treated with 70% ethanol for 1 min, and washed with autoclaved dH_2O (4-5 times). After washing, the pods were dipped in 0.2% Mercuric chloride for 5 min and then subsequently washed with autoclaved dH_2O.

Seed Germination and Multiplication of Culture

After surface disinfection, the pods were opened and seeds were collected aseptically and inoculated on a germination medium consisting of basal MS medium supplemented with 2.5 µM BAP, 87 mM sucrose, and 0.8% (w/v) agar. The culture bottles were incubated at 25 ± 1 ºC under a white fluorescent light (50 µmole m^{-2} sec^{-1}) at a photoperiod regime of 16 hours of light and 8 hours of dark. The seedlings were developed after 4 weeks. Fully grown seedlings were excised and inoculated on the above-mentioned medium for their multiplication. The developed cultures were repeatedly subcultured every 30 days of inoculation.

Effect of plant growth regulators on the development of protocorms like bodies (PLBs) or shoots

In this study, the effect of 6-Benzylaminopurine (BAP) and 1-naphthalene acetic acid (NAA) was studied on the development of PLBs and shoots. For that small shoot clumps of *Coelogyne flaccida* were inoculated on different concentrations and combinations of BAP (1 µM, 2.5, and 5 µM) and NAA (0µM, 1 µM, 2.5 µM, & 5 µM).

The culture bottles were incubated at 25 ± 1 ºC under a white fluorescent light (50 µmole m^{-2} sec^{-1}) at a photoperiod regime of 16 hours of light and 8 hours of dark. The results were recorded after 4-5 weeks as the average number of PLBs and shoot formation. The formulae used for the observed parameters are given below.

Table 3: Formulae for calculating average number of formation of PLBs/shoot

Parameter	**Formula**
Average no. of PLBs formed	$\sum A1 + A2 + ..An\ /n$
Average no. of shoots formed	$\sum A1 + A2 + ..An/n$
Where A1 represents the no. of PLBs/shoot formed per one shoot clump and n is the total no. of shoot clumps.	

Maturation of PLBs

After the development of PLBs, the effect of sucrose and mannitol was studied on the maturation of PLBs

Effect of Sucrose

Clustered PLBs were excised from growing PLBs cultures and inoculated on the basal MS medium supplemented with different concentrations of sucrose, i.e., 87 mM, 174 mM, 261 mM, and 348 mM. The cultured bottles were incubated at the above-mentioned growth conditions. The results were recorded after 4-5 weeks as an average no. of PLBs matured.

Table 4: Formula for calculating average number matured PLBs

Parameter	Formula
Average no. of PLBs matured	$\sum P1 + P2 + .. Pn/n$
Where P1 to Pn is the number of PLBs Inoculated.	and n is the number of clustered PLBs

Effect of mannitol

Clustered PLBs were excised from growing PLBs cultures and inoculated on the basal MS medium supplemented with different concentrations of mannitol, i.e., 0 mM, 82.5 mM, 165 mM, and 247 mM. The cultured bottles were incubated at the above-mentioned growth conditions and results were recorded after 4-5 weeks as an average number of PLBs matured.

Table 5: Formula for calculating average number matured PLBs

Parameter	Formula
Average no. of PLBs matured	$\sum P1 + P2 + .. Pn/n$
Where P1 to Pn is the number of PLBs Inoculated.	and n is the number of clustered PLBs

Germination of PLBs

After maturation, the PLBs were germinated for conversion into plantlets. For that, the matured PLBs were inoculated on basal MS medium supplemented with different concentrations and combinations of BAP (0 µM ,0.1 µM, 0.5µM) and GA_3 (0 µM , 0.5 µM, 1 µM, 2.5 µM). The cultured bottles were inoculated in above mentioned growth conditions for 4-5 weeks for germination. The germinated plantlets were then excised and multiplied on basal MS medium containing 2.5 µM BAP in the above-mentioned growth conditions.

Acclimatization of Plantlets

For acclimatization, the multiplied plantlets were incubated on basal MS medium containing 1 mg L^{-1}IBA for 15– 20 days for root induction. After root induction, the plantlets were then removed from the medium, and the roots were rinsed in distilled water to wash the attached medium. Further, the roots were dipped in 0.2% (w/v) bavistin for 1 min followed by 1 mg L^{-1} IBA. The treated plantlets were then transferred to pots already containing a mixture of bricks powder, coconut husk, and activated charcoal.

CHAPTER 4

RESULTS

Seed Germination

Seed inoculated on MS medium started to germinate after 4 weeks forming protocorms. In about 6-7 week about 100 percent seed germination was recorded

Multiplication

The cultures of *Coelogyne flaccida* were established in vitro through seed germination. The cultures were multiplied by inoculating in MS medium supplemented with 2.5µM BAP. The multiplied cultures were healthy and fully grown and these cultures were used to carry out further experiments.

Fig 2: Culture established in vitro of *Coelogyne flaccida* through seed germination.

Effect of PGRs on PLBs formation and shoot formation

(a) *Effect of BAP and GA_3 on shoot formation:* The effect of different concentrations and combinations of BAP and NAA was studied on shoot formation. In this study, the average number of shoots (5.22) were highest on medium containing 0 µM NAA and 2.5 µM BAP.

Table 6: Effect of BAP and NAA on formation of shoots per clump in *Coelogyne flaccida.*

S.No.	NAA (µM)	BAP (µM)	Average shoot formation
1	0	1	$2.05^{b} \pm 0.49$
2	1	1	$2.61^{ab} \pm 0.78$
3	2.5	1	$3.72^{ab} \pm 0.97$
4	5	1	$2.58^{ab} \pm 0.66$
5	0	2.5	$5.22^{a} \pm 0.31$
6	1	2.5	$2.11^{ab} \pm 0.43$
7	2.5	2.5	$2.25^{ab} \pm 0.04$
8	5	2.5	$2.37^{ab} \pm 0.54$
9	0	5	$2.52^{ab} \pm 1.39$
10	1	5	$2.33^{ab} \pm 0.03$
11	2.5	5	$2.55^{ab} \pm 0.63$
12	5	5	$2.02^{b} \pm 0.19$

*Data were analyzed by one-way ANOVA using t-test and means were compared by LSD at $P<0.05$.

Table 6 represents the average number of shoots produced after 30 days per shoot clump in different concentrations and combinations of PGRs. The lowest average number of shoots (2.02) per shoot clump were achieved on medium containing 5 µM BAP and 5 µM NAA.

(b) *Effect of BAP and GA_3 on PLBs formation*: Along with the shoots, the formation of PLBs (Protocorm like bodies) was also observed during incubation of the shoot clump on MS medium supplemented with different concentrations and combinations of BAP and NAA. The highest number of PLBs (14.77) were produced on medium containing 1 µM BAP and 1 µM NAA while the lowest number of PLBs (1.22) were produced on medium containing 2.5 µM BAP and 1 µM NA after 30 days of incubation. Table 7 represents the average number of PLBs formed after 30 days per shoot clump on medium containing different concentration and combinations of BAP and GA_3. The effect was statistically significant.

Table 7: Effect of BAP and NAA on formation of PLBs per clump in *Coelogyne flaccida*.

S.No.	NAA (µM)	BAP (µM)	Average PLBs formation
1	0	1	$6.77^{c} \pm 0.86$
2	1	1	$14.77^{a} \pm 0.96$
3	2.5	1	$10.22^{b} \pm 0.72$
4	5	1	$10.71^{b} \pm 0.41$
5	0	2.5	$11.35^{b} \pm 1.19$
6	1	2.5	$1.22^{e} \pm 0.36$
7	2.5	2.5	$3.07^{de} \pm 0.30$
8	5	2.5	$5.81^{cd} \pm 0.59$
9	0	5	$4.52^{cd} \pm 0.23$
10	1	5	$5.90^{cd} \pm 0.30$
11	2.5	5	$5.41^{cd} \pm 0.60$
12	5	5	$5.43^{cd} \pm 0.72$

*Data were analyzed by one-way ANOVA using t-test and means were compared by LSD at $P<0.05$.

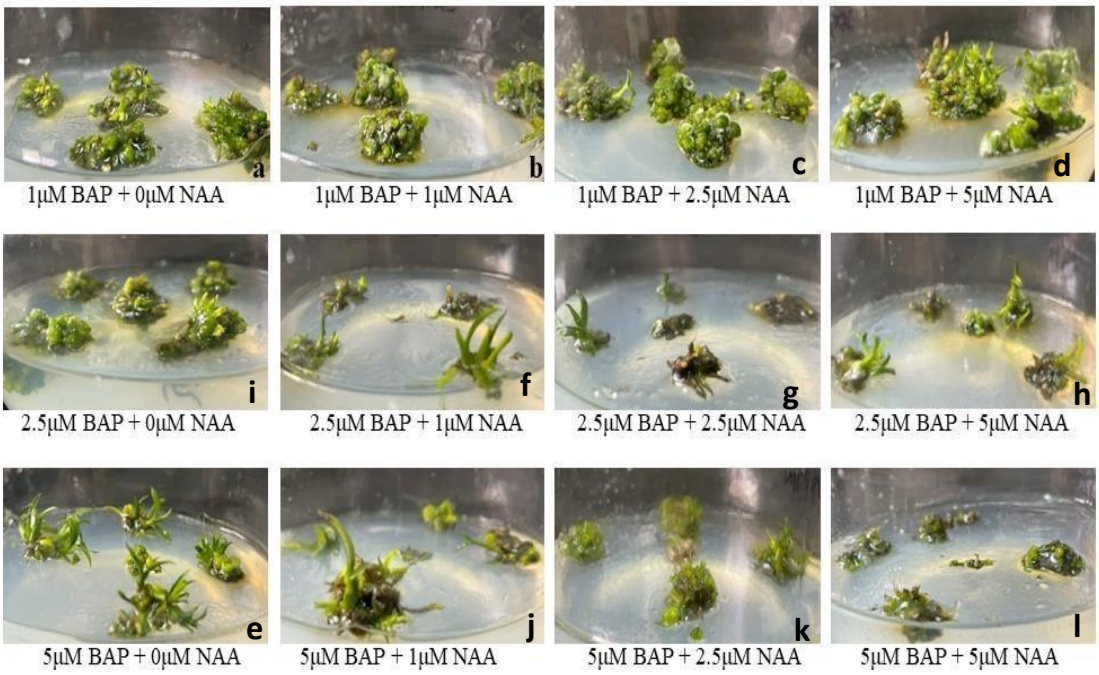

Fig 3: Shoot and PLBs formation on MS medium supplemented with different concentrations and combinations of BAP and NAA. Results were recorded after 30 days of culture.

Maturation of PLBs

For maturation of PLBs, effect of different concentrations and combinations of sucrose and mannitol was studied

(a) *The effect of sucrose*: The effect of different concentrations of sucrose was studied on the maturation of PLBs (cluster). The highest number of matured PLBs (6.97) were recorded on MS medium supplemented with 87 mM sucrose after 30 days of PLB cluster inoculation followed by 174 mM sucrose concentration (5.48). Minimum PLBs maturation (5.26) was recorded on MS medium supplemented with 348 mM sucrose after 30 days. (Table 8). It was observed that with increase in sucrose concentration the maturation of PLBs decreases. The effect of sucrose concentration from 174 mM to 348 mm was not statistically significant.

Table 8: Effect of different concentrations of sucrose on maturation of PLBs in *Coelogyne flaccida*.

S.No.	Sucrose Concentration (mM)	Average PLBs matured
1	87	$6.97^{a} \pm 0.38$
2	174	$5.48^{b} \pm 0.21$
3	261	$5.23^{b} \pm 0.17$
4	348	$5.26^{b} \pm 0.36$

*Data were analyzed by one-way ANOVA using t-test and means were compared by LSD at $P<0.05$.

b) *The effect of Mannitol*: The effect of different concentrations of mannitol was studied on the maturation of PLBs (cluster). The highest number of matured PLBs (6.73) were recorded on MS medium with 0 mM mannitol after 30 days of PLB cluster inoculation followed by 82.5 mM mannitol concentration. Minimum PLBs maturation was recorded on MS medium supplemented with 165 mM mannitol after 30 days. (Table 8).

The maximum number of matured PLBs was observed on MS medium supplemented with 87 mM sucrose. In comparison to sucrose and mannitol, sucrose is more efficient in the maturation of PLBs. The maturation of PLBs was not found much effective in mannitol with comparison of sucrose.

Table 9: Effect of different concentrations of Mannitol on maturation of PLBs *Coelogyne flaccida*.

S.No.	Mannitol Concentration (mM)	Average PLBs matured
1	0	$6.73^{a} \pm 0.34$
2	82.5	$5.66^{b} \pm 0.10$
3	165	$5.26^{b} \pm 0.20$
4	247	$5.53^{b} \pm 0.17$

*Data were analyzed by one-way ANOVA using t-test and means were compared by LSD at$P<0.05$.

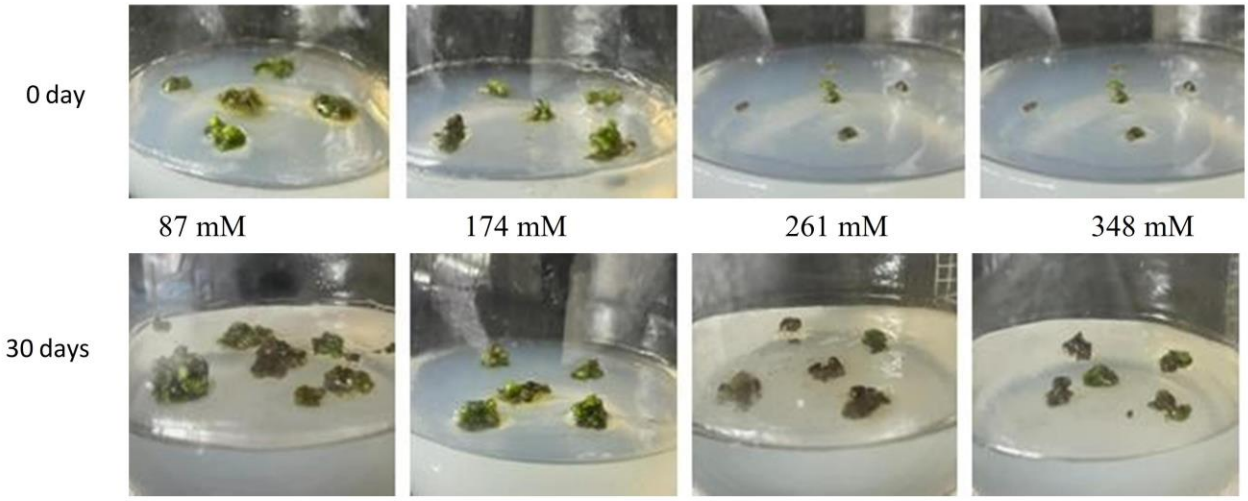

Fig 4: The picture representing the initial (0 day) and final stage of maturation (30 days) of the PLBs on MS medium supplemented with different concentrations of sucrose.

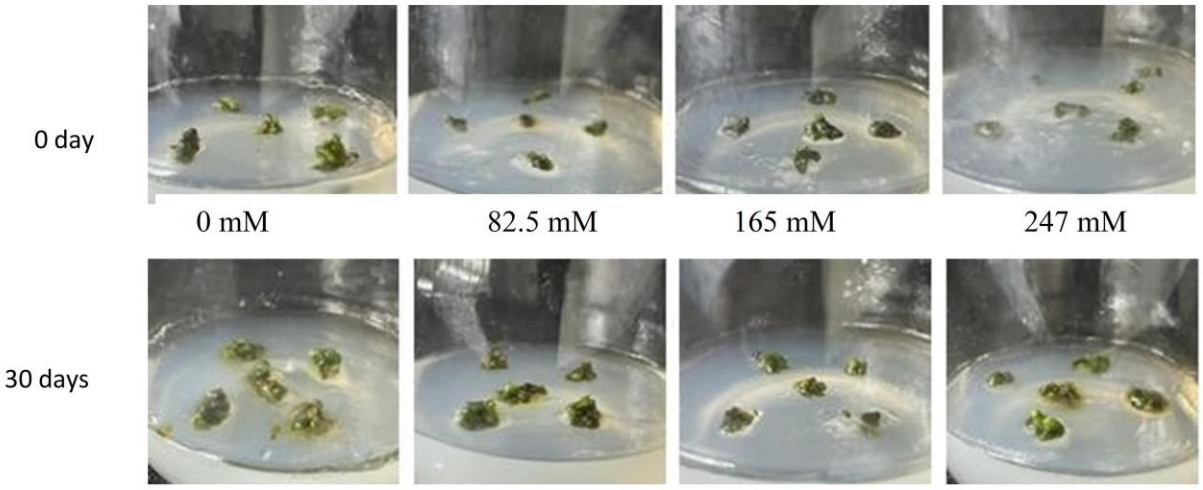

Fig 5: The picture representing the initial (0 day) and final stage of maturation (30 days) of the PLBs on MS medium supplemented with different concentrations of Mannitol.

Germination

The effect of different concentrations and combinations of BAP and GA_3 was studied on the germination of the matured PLBs. The germination rate (18.32) was recorded highest at 0.5µM BAP and 1µM GA_3. At concentration 0.5µM GA_3, lowest germination rate (4.32) of PLBs was recorded (Table 10). The effect of different concentrations and combinations of BAP and GA_3 on germination of PLBs was statistically significant (Table 10).

Table 10: The effect of BAP and GA_3 in basal MS medium on conversion of matured PLBs of *Coelogyne flaccida*.

S.No.	Concentration and Combination	Average germination
1	0 BAPµM + 0µM GA_3	$7.17^{\mathbf{def}} \pm 0.22$
2	0.1µM BAP + 0 µM GA_3	$8.18^{\mathbf{cde}} \pm 0.19$
3	0.5µM BAP + 0µM GA_3	$4.66^{\mathbf{fg}} \pm 0.59$
4	0µM BAP + 0.5µM GA_3	$4.30^{\mathbf{g}} \pm 0.30$
5	0.1µM BAP + 0.5µM GA_3	$7.23^{\mathbf{de}} \pm 0.13$
6	0.5µM BAP + 0.5µM GA_3	$5.81^{\mathbf{efg}} \pm 0.51$
7	0µM BAP+ 1µM GA_3	$9.27^{\mathbf{cd}} \pm 0.38$
8	0.1 µMBAP + 1µM GA_3	$9.86^{\mathbf{bc}} \pm 0.34$
9	0.5µM BAP + 1µM GA_3	$18.32^{\mathbf{a}} \pm 0.40$
10	0 µMBAP + 2.5µM GA_3	$10.66^{\mathbf{bc}} \pm 0.77$
11	0.1µM BAP + 2.5µM GA_3	$9.84^{\mathbf{bc}} \pm 0.39$
12	0.5µM BAP + 2.5 µM GA_3	$11.97^{\mathbf{b}} \pm 1.36$

*Data were analyzed by one-way ANOVA using t-test and means were compared by LSD at $P<0.05$.

Acclimatization of the plantlets

The regenerated plantlets cultured on MS medium supplemented with 1 mg L^{-1}IBA for root induction for 15-20 days and planted in thermocouple-based pots in a mixture consisting of bricks powder, charcoal powder, and coconut powder.

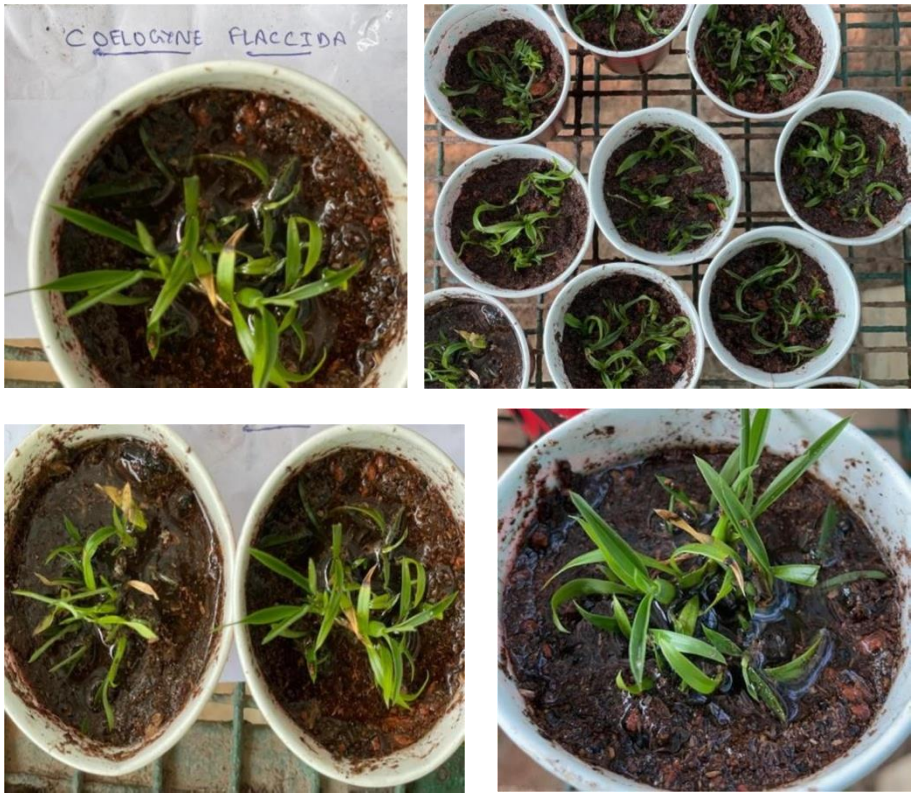

Fig 6: Establishment of the invitro-grown cultures in the pots.

CHAPTER – 5

DISCUSSION

The regenerative abilities of the orchid *Coelogyne flaccida,* as well as the effects of plant growth regulators (PGRs) on both multiplication and regeneration, was investigated in this study. Following the multiplication of the PLBs, an investigation into the effect of sucrose and mannitol concentration on the maturation process was carried out. After allowing the PLBs to grow, a germination experiments were performed on them using a variety of different PGRs in varying concentrations and combinations. After some time, the regenerated shoot cultures were transferred to an MS medium containing IBA to initiate root growth. After the plantlets developed roots, they were placed in soil in pots outside of the laboratory to mature into full plants.

In the beginning, the established cultures were multiplied on a basic MS medium that was augmented with 2.5 µM BAP. According to Kalimuthu et al. (2006), BAP is an essential complement to the process of multiplication. The shoot formation experiment was carried out using a clump of the culture that had been developed in vitro. These clumps were inoculated onto basal MS medium supplemented with various concentrations and combinations of BAP (1 µM, 2.5 µM, and 5 µM) and NAA (0 µM, 1 µM, 2.5 µM, and 5 µM). The study investigated how shoot formation was affected by the 12 different combinations of BAP and NAA. After 30 days of culture, the concentration and combination of 2.5 µM BAP with 0 µM NAA resulted in the highest shoot formation (Table 1). The immediate production of PLBs (Protocorm-like bodies) was observed with the shoot formation on a MS medium that was supplemented with varying concentrations of BAP and NAA in combination. The concentration and combination of 1 µM BAP and 1 µM NAA resulted in the highest number of PLBs formation (Table 2).

The PLBs that were produced were matured using clusters of PLBs. PLBs were inoculated on MS medium supplemented with varying concentrations of sucrose (87 mM, 174 mM, 261 mM, and 348 mM) or mannitol (0 mM, 82.5 mM, 165 mM, and 247 mM). After 30 days of culture, the PLBs appeared to have reached their full maturity when they were inoculated on a basal MS medium containing 87 mM sucrose (Table 3). At a concentration of 261 mM sucrose, the PLBs were green colour, but at a concentration of 348 mM sucrose, the PLBs changed their colour to brown, and PLBs died. However, at a concentration of 87 mM, the PLBs turned yellow in colour, which caused them to mature.

In a manner analogous to this, the PLBs were inoculated in clusters in mannitol-supplemented medium; however, the largest number of maturations was seen at 0 mM (Table 4). The maturation process went more smoothly in sucrose when compared to both mannitol and sucrose. The maturation of sucrose was successful, according to the findings of Trigiano and Grey (2010).

After the maturation, the conversion experiment was carried out using the matured PLBs (Protocorm like bodies). The 30 days matured PLBs were inoculated on the MS medium supplemented with different concentration and combinations of BAP and GA_3. Best conversion of matured PLBs was on MS medium supplemented with 0.5 µM BAP and 1µM GA_3. (Table 10)

The effect of 12 different combinations of BAP and GA_3 on conversion of matured PLBs was studied. Importance of GA_3 for shoot germination was reported in *Cajanus cajan* (Srivastava and Raghav 2014).

For acclimatization, the cultures were inoculated on MS medium supplemented with 1 mg L^{-1}IBA for root induction and planted in the pots under ex-vitro conditions. The plants were firstly washed and the medium was completely removed and were transferred to the thermoplastic pots containing a mixture, which consist of bricks powder, coconut powder and the activated charcoal and were kept in under shady region without direct contact of sunlight and were kept to grow as a full plant.

CHAPTER – 6

CONCLUSION

Coelogyne flaccida has been found to be one of the important ornamental plants. From the present research work it was found that the energy sources and PGRs plays an important role in shoot/PLBs formation, maturation, and germination of PLBs.

In the present study,

1. Cent per cent seed germination was achieved on MS medium after 7 weeks of culture.
2. Multiplication was successfully achieved on MS medium supplemented with 2.5 µM BAP.
3. Best PLBs multiplication was observed on MS medium supplemented with 1 µM each of BAP and NAA.
4. Best maturation of PLBs was observed on MS medium supplemented with 87 mM sucrose.
5. Maximum conversion of matured PLBs was achieved on MS medium supplemented with 0.5µM BAP and 1 µM GA_3.
6. The generated plants were successfully planted in ex vitro conditions in the pots.

References

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

2. Bailes C (1985) Orchids in Nepal, the conservation and development of a natural resource. Advisory report and recommendations. Royal Botanical Garden,UK.

3. Bustam SS (2013) Selection of optimal stage for protocorm-like bodies and production of artificial seeds for direct regeneration on different media and short-term storage of *Dendrobium* Shavin White. Plant Growth Regulation 69:215-224.

4. De KK, Sil S (2015) Protocorm-like bodies and plant regeneration from foliar explants of *Coelogyne flaccida*, A horticulturally and medicinally important endangered orchid of eastern Himalaya. Lankersteriana 15:151–158

5. Jheng F, Do Y, Liauh Y, Chung J, Huang P (2006) Enhancement of growth and regulation efficiency from embryogenic callus cultures of *Oncidium* 'Gower Ramsey' by adjusting carbohydrate sources. Plant Science 170:1133-1140.

6. Kaur S, Bhutani KK (2013) In vitro conservation and asymbiotic propagation of *Coelogyne flaccida* (Lindl.): a threatened orchid. Plant Biosystems 148:935-944.

7. Kauth (2006) In vitro seed culture and seedling development of *Calopogan tuberosus*. Plant Cell Tissue and Organ Culture 85:91-102.

8. Kumaria S, Singh N (2019) A combinational Phyto molecular-mediated assessment in micro propagated plantlets of *Coelogyne ovalis* Lindl. A horticultural and medicinal orchid. proceedings of the National Academy of Sciences India. Section B Biological Sciences 90:455-466

9. Majumdar P, Maiti D (1989) Flaccidin and oxoflaccidin, two phenanthrene derivatives of the orchids *Coelogyne flaccida*. Phytochemistry 28:887-890.

10. Majumdar PL, Banerjee S, Maiti DCS (1995) Stilbenoids from the orchids *Agrostophyllum callosum* and *Coelogyne flaccida.* Phytochemistry 39:649-653.

11. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

12. Naing AH, Chung JD, Park IS, Lim KB (2011) Efficient plant regeneration of the endangered medicinal orchid, *Coelogyne cristata* using protocorm-like bodies. Acta Physiologia Plantarum 33:659-666.

13. Ng C, Saleh N (2011) In vitro propagation of Paphiopedilum orchid through the formation of protocorm-like bodies. Plant Cell Tissue and Organ Culture 105:193-202.

14. Rajbhandari KPB (2001) Beautiful orchids of Nepal. Kishore Offset Press Pvt. Ltd.

15. Triango R, Gray D (2010) Plant tissue culture development and biotechnology (1st ed). CRC Press.

16. Vij S, Aggarwal S (2003) Regeneration competence of foliar explants: *Vanda coerulea* Griff. Journal of the Orchid Society of India 17:73-78.

17. Vij S, Pathak (1990) Micropropagation of orchids through leaf segments. Journal of the Orchid Society of India 4:69-88.

18. Vij S, Kaur P, Kondo K, Pathak P, Gupta A (2002) Regeneration competence of Orchid leaves: A study in vitro. In: Vij S, Kondo K, Sharma ML and Gupta A (Eds.), Plant Genetic Diversity: Exploration, Evaluation, Conservation (pp. 289-306) New Delhi: Affliated East-West Press

19. Wu ZY, Raven P, Hong D (2009) Flora of China. V*ol. 25 (Orchidaceae)*. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

20. Zhao P, Wu F, Feng FS, Wang WJ (2008) Protocorm-like body formation and plant regeneration from the callus culture of *Dendrobium candidum* Wall ex Lindl. In Vitro Cellular and Development Biology 44:178-185.

ANNEXURE 1

Composition of MS medium as described by Murashige and Skoog, 1962

Composition	Amount (mg/l)
1. Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$CaCl_2.7H_2O$	440
2. Micronutrients	
$MnSO_4.4H_2O$	16.90
$ZnSO_4.7H_2O$	08.60
H_3BO_4	06.20
$Fe_2EDTA.2H_2O$	27.80
KI	00.83
$Na_2Mo_4.2H_2O$	00.25
$CoCl_2.6H_2O$	00.25
$CuSO_4.5H_2O$	0.025
3. Vitamins	
Myoinositol	100
Nicotinic Acid	0.5
Glycine	2.0
Pyridoxine HCl	0.5
Thiamine HCl	0.1
4. **Sugar**	3%
5. **Agar**	8000

Annexure – 2

BAP

Stock of 2.5 mM BAP was prepared in HCl by dissolving 56 mg of BAP in 500 µL HCl and then the volume was made up to 100 mL by using distilled water.

NAA

2.5 mM stock solution was prepared by dissolving 0.4 gm NAA in 50 mL of distilled water and volume was up to 100 mL using distilled water.

GA_3

2.5 mM stock was prepared by dissolving 86.5 mg of GA_3 in 50 mL of distilled water and the volume was made up to 100 mL of distilled water.

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Findings Sources

1

Development of in-vitro propagation protocol of Coelogyne flaccida

A thesis submitted in partial fulfillment of the degree of
Master of Science
in
Biotechnology

Under the guidance of
Dr. Anil Kumar
(Professor)

Submitted By
Jatin
(302101012)

Department of Biotechnology
Thapar Institute of Engineering and Technology
(Deemed to be University)
Patiala-147004, Punjab, India
June 2023

2

Declaration
I hereby declare that the work presented in the thesis entitled "Development of in-vitro propagation protocol of Coelogyne flaccida " is a bonafide work under the supervision and
guidance of Dr. Anil Kumar, Professor,
Department of Biotechnology, Thapar

(...) Table 5.2
Effect of BAP and IAA on number of shoots per clump, average shoot length and number of leaves per clump in Coelogyne flaccida

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on
MS medium supplemented with 5 µM BAP and 5 µM NAA,
Table 3: The effect of BAP and NAA

number of leaves
(3.02 ± 0.27) per shoot were recorded on MS medium with 2.5µM BAP and 0µM NAA.
However the lowest number of

the matured PLBs were inoculated on ... MS medium supplemented with different concentrations of sucrose (87, 174, 261 and 348