In-vitro Conservation of Some Endangered Orchids of Himalayan Region

A

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DECLARATION

I hereby declare that the work presented in the thesis entitled ***In-vitro* Conservation of Some Endangered Orchids Of Himalayan Region** 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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CERTIFICATE

This is to certify that dissertation entitled ***In-vitro* Conservation of Some Endangered Orchids of Himalayan Region** submitted by **Miss Urvashi Pandita (Roll no. 602004021)** in the partial fulfilment of the requirements for the award of the degree of Masters in Technology in Biotechnology, Thapar Institute of Engineering and Technology, Patiala is a record of student's own 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

ABA	Abscisic acid
BAP	6-benzylaminopurine
CTAB	Cetyltrimethylammonium bromide
dNTPs	Deoxynucleotide triphosphates
GA_3	Gibberellic acid
HCl	Hydrochloric acid
Hr	Hour
IAA	Indole acetic acid
IBA	Indole 3 Butyric acid
ISSR	Inter-simple sequence Repeat
IUCN	International Union for Conservation of Nature and Natural Resources
Kn	Knudson
Min	Minutes
MS	Murashige and Skoog Medium
mM	Milli molar
NaOH	Sodium hydroxide
NAA	1-napthelene acetic acid
PBZ	Paclobutrazol
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGR	Plant Growth Regulatora
PLBs	Protocorm like bodies
PIC	Polymorphic information content
RAPD	Random amplified polymorphic DNA
SCoT	Start codon targeted marker
TDZ	Thidiazuron
VW	Vacin and Went
2,4-D	2,4-dichlorophenoxy acetic acid
%	Percentage
Ml	Microliter
µM	Micromolar
ng/µl	nano gram per microliter

SUMMARY

Orchids (28000 species belonging to 763 genera), family *Orchidaceae*, are well known for their colorful flowering, fragrance, ornamental and some medicinal uses (Pillon and Chase 2007). By the end of 2020, the IUCN Global Red List included assessments for 1641 orchid species, of which 0.30% are extinct, 45.52% are threatened, 12.00% are critically endangered, 21.63% are endangered, and 5.79% are vulnerable. IUCN Global Red List (IUCN 2020). Due to destruction of natural habitat and the damages caused by locals or tourists, the many orchid species are at verge of extinction. It is difficult to multiply orchids in their natural habitats as their propagation rate is very slow. So there is a felt need to develop faster the propagation techniques to prevent the orchids from their extinction. This study is focused to develop the methods of propagation and conservation of the orchids through artificial seed technology under *in-vitro* conditions.

In present study, the shoots of *Dendrobium nobile* Lindl., *Coelogyne flaccida* Lindl. and *Rhyncostylis retusa* (L.) were inoculated on MS medium supplemented with 87 mM sucrose and different concentrations of 6-benzylaminopurine (BAP-0, 1, 2.5 and 5 μM) and Indole acetic acid (IAA-0, 1, 2.5 and 5 μM). The highest number of shoots were recorded on medium containing 2.5 μM BAP and 5 μM IAA in all three orchids species under study. For the formation and multiplication of PLBs, the leaf explants were inoculated on MS medium supplemented with different concentrations of BAP (0, 1, 2.5 and 5 μM). The optimum result for both formation and multiplication of PLBs in all three orchid species were recorded on medium containing 2.5 μM BAP. After PLBs formation, the PLBs were inoculated on MS medium supplemented with different concentrations of sorbitol (0, 82.5, 165 and 247 mM) for maturation. Most PLBs of *D. nobile* (31%), *C. flaccida* (40%) and *R. retusa* (29%) matured on 247 mM sorbitol concentration followed by 165 mM sorbitol concentration. After maturation, the effect of plant growth regulators and sucrose on conversion of PLBs into plantlets was also studied. Mature PLBs were inoculated on MS medium supplemented with different concentrations and combinations of BA (0, 0.1 μM), GA3 (0, 1 μM) and sucrose (87, 174, 261 and 348 mM). The conversion rate was higher on MS medium supplemented with 0.1 μM BA, 1 μM GA_3 and 261 mM sucrose concentration. The matured PLBs were also encapsulated in sodium alginate-calcium chloride beads to produce artificial seeds. The effect of different concentrations of sodium alginate (1%, 2%, 3%, 4%) and calcium chloride (50 mM, 100 mM, 200 mM) was studied. The best bead formation was observed

in 3% sodium alginate and 100 mM calcium chloride. The storage effect on artificial seeds at two different temperatures 4 ℃ and 25 ℃ was also recorded at 10, 20, 30, 40, 50 and 60 days. It was observed that the artificial seeds of *Dendrobium nobile* were viable for longer period at 25 ^{o}C, whereas artificial seeds of *Coelogyne flaccida* and *Rhyncostylis retusa* were viable for longer period at 4 ^{o}C rather than 25 ^{o}C. The clonal fidelity of *in-vitro* germinated seeds of *D. nobile, C. flaccida* and *R. retusa* was also compared with that of mother plants using RAPD marker. All the marker scored using 26 RAPD primers were monomorphic showing clonal fidelity of the regenerated plants.

CHAPTER: 1

INTRODUCTION

Orchids, the indicators of a healthy ecosystem, belongs to the *Orchidaceae* family. It is one of the largest family of flowering plants with 28000 species belonging to 763 genera (Christenhusz and Byng 2016). Orchids are defined as a plant with showy 3 petalled complex flowers having a large specialized lip called labellum and frequently a spur. Usually grown as ornamental flowers, orchids are also valued for their beauty, fragrances, medicinal uses and also for flowers with long vase life (Chugh et al. 2009). *Orchidaceae*, being referred as cosmopolitan, occurs almost in all habitats and continents, excluding deserts, glaciers and Antarctica (Givnish et al. 2015). Orchids produce a wide variety of flowers consisting of different shapes, colors, scents and sizes (Murthy et al. 2018). The largest genera in the orchid family is *Bulbophyllum* (comprising of almost 2000 species) followed by *Epidendrum* (about 1500 species), *Dendrobium* (comprising about 1400 species) and then *Pleurothalis* (about 1000 species) (Pillon and Chase 2007). In India, the orchid family consists of about 1141 species out of which 657 species are epiphytic, 484 are terrestrial and very few are lithophytic (Barman et al. 2016). India, known for its diverse climatic conditions and topographic situations, provides a natural habitat for orchids to grow in its environmental conditions (Jalal and Jayanti 2013). Orchids are widely distributed over Himalayas and also in warm subtropical zones of India (Jalal and Jayanti 2013).

Scientific Classification

Kingdom: *Plantae*

Phylum: *Magnoliophyta*

Class: *Liliopsida*

Order: *Asparagales*

Family: *Orchidaceae*

Orchids are also known as perennial herbs (lacking wooden structure) which make them easily distinguishable from other flowering plants. The growth of orchids can be described in two patterns

- **Monopodial:** Orchids growing with only one stem in upward direction continuously that can reach several meters in length for example *Phalaenopsis* and *Vanda* etc.

- **Sympodial:** Orchids growing with a creeping stem in which roots grow in downward direction and flowers grow in upward direction for example *Cymbidium* etc.

There are three types of orchids

- **Epiphytic orchids:** The orchids that need support to grow i.e., they grow on trees. These plants take their nourishment from the debris on the tree bark and from the moisture in their environment for example *Vanila, Catasetum* and *Dendrobium* etc.
- **Terrestrial orchids:** Orchids growing on ground with moist and wet roots for example *Ludisia, Oncidium* and *Calypso* etc.
- **Lithophytic orchids:** Orchids growing on rocks for example *Brassavola, Vanda* and *Cymbidium* etc.

Orchidaceae is the second largest group in all the flowering plants (Chase et al. 2015). Besides using orchids for ornamental purposes or for fragrances and scents, they are also grown for traditional medicinal uses, food and horticulture (Hinsley et al. 2017).

Horticulture uses

Most of the orchid trade is of cut flowers. Orchids have been traded as ornamental cut flower plants in floriculture and horticulture industries. They can also be used in religious ceremonies (Hinsley et al. 2018). For example flowers of *Dendrobium* are used as offerings in temples of Srilanka (Duggal 1971).

Food industries

Some parts of wild orchids (*Habenaria acuminata* or *Microstylis wallachii*) like roots, pseudoblubs and rhizomes have been used as food and as nutritious health drink by ethnic groups of North Eastern India (Hinsley et al. 2018). For example stems of *Dendrobium* if given to cow can help increasing milk production. Vanilla is considered as edible orchid globally and is traded as flavoring agent (Lubinsky et al. 2008). 35 species of wild orchids are used to produce Salep from polysaccharide rich tubers that can be boiled in water or milk and used as nutritious drink for the betterment of the human health (Ghorbani et al. 2017; Tamer et al. 2006). Terrestrial orchids are also used in the production of Chikanda (served as dish in most parts of Africa) and baking soda (Kaputo 1996; Bingham 2009).

Medicinal uses

Phytochemicals like alkaloids, bi-benzyl derivatives, flavonoids and terpenoids have been isolated from orchids (especially from leaves and stem).These compounds attribute to pharmacological properties of orchids. (Gutierrez 2010). Orchids also play a vital role in anti-inflammatory, anti-diuretic, anti-cancer, anti-microbial and neuroprotective activities (Gantait et al. 2021). Some orchids can also be used for orthopedic purposes such as to treat fractures and also for bone regeneration purposes (Tsering et al. 2017). Preparation of orchids as medicines can be used to treat various ailments like chest pain, arthritis, earache, blood dysentery, malaria, wounds and eczema etc. (Hossain 2011). Hepatocosane from *Vanda roxburghii* show anti-inflammatory activity in rats and mice (Pant 2013). Some other compounds like 2, 6-Dimethyl-1,4-benzoquinone from *Cymbidium* sp. Show some allergic reactions in organisms (Hausen and Shoji 1984).

Anti-microbial activity: Some species of orchid family are proved to have antimicrobial activity (Singh et al. 2012) for example Gastrodianin from *Gastrodia elata* show activity against pathogenic fungi in plants. Methanolic extracts from orchids also tend to have antimicrobial activity (Matu and van Stedan 2003). Vanillin which is a compound present in vanilla extracted from *Vanilla plantifolia* has antimicrobial activity against *E. coli* and *Lactobaccilus* (Fitzgerald et al. 2004)

Anti-inflammatory activity: Few members of orchid species tend to have anti-inflammatory activity due to alkanes and alkanols, for example *Vanda roxburghii* show anti-inflammatory responses in carrageenan in mice model (Singh et al. 2012). A number of compounds like triterpenes and cyclophlidones are present in *Pholidota chinensis* which has strong inflammatory effect in RAW264.7 cell line (Wang et al. 2006).

Anti-oxidative property: Some orchid species also tend to have antioxidative properties like extract namely Ephemeranthone obtained from *Ephemerantha lonchophylla* has antioxidative activity against human lipoprotein (Chen et al. 1999). Cis-melilotoside and trans-melilotoside which were isolated from *Dendrobium aurantiacum* stem and alkyl ferulates that were obtained from *Dendrobium tosaense* are considered to be good antioxidants (Yang et al. 2007).

In general, orchids are not only known for their beautiful flowers, fragrances and scents but also for their great medicinal values and other horticultural and food needs.

In present study, the three orchid species namely *Dendrobium nobile* Lindl., *Coeloegyne flaccida* Lindl. and *Rhyncostylis retusa* (L.) were selected for *in-vitro* conservation developing strategies.

Dendrobium nobile Lindl.

Dendrobium belonging to Orchidaceae family, are predominantly epiphytic and lithophytic and is considered as the second largest genus in the family with about 1800-2000 species found in variety of habitats in Southeast Asia and various parts of Pacific Islands (da Silva et al. 2015). The roots and stems of *Dendrobium* grow over the tree trunks or rocks. Only few species of these orchids are terrestrial. The name *Dendrobium* came from two Greek words "*dendron*" which means "tree" and "*bios*" which means "life" (Cakova et al. 2017)

Dendrobium nobile
Source: https://i.pinimg.com

Being an epiphytic herb, *Dendrobium nobile* Lindl. is one of the main species belonging to genus *Dendrobium* orchids and is known for both ornamental as well as medicinal uses (Nie et al. 2020). It is of more significant use in China. Besides its ornamental and medicinal uses, it is also known for its antiaging, anti-mutagenic, immunostimulatory, anti-tumor and anti-oxidant properties (Nie et al. 2020, Miyazawa et al. 1997). According to Chinese medical theory, it is also used in keeping stomach healthy, body fluid secretion, preventing cataract and increasing immunity (Luo et al. 2010). Due to its enormous uses, these species are in more demand which lead to decline in its population. The species usually grow on tree trunks at a height of 800 to 1500 meters but unfortunately this habitat is more vulnerable to exploitation and degradation by human population (Bhattacharyya et al. 2016). That is why these species have become endemic and have been listed among the endangered species by IUCN (IUCN 2020).

Coelogyne flaccida Lindl.

With few exceptions, *Coelogyne* orchids predominantly blossoms in white or sometimes in brown flowers, which are particularly striking and gorgeous (Hartati et al. 2019).. Aside from their beauty, they have a very pleasant fragrance that attracts variety of pollinators. Being sympodial orchids, they can be found in variety of ecosystems ranging from Himalayan forests to tropical forests and are also found in some parts of India and China (Hartati et al. 2019). Their forms and environmental parameters are quite varied. The name *Coelogyne* came from ancient Greek words "*koilos*" which means "hollow" and "*gyne*" which means "female". Pseudobulbs with one or two folded leaves (usually close together or far apart) are found in *Coelogyne* which comes in a variety of shapes and sizes, particularly ovoid making them popular among the collections of orchids (Gravendeel 2000). They grow at intermediate temperature with good light and air and also need constant moisture for their growth and development (Abraham et al. 2012). *Coelogyne flaccida* Lindl. being an evergreen epiphytic orchid, is the most attractive species among the *Coelogyne* orchids with sweet aromatic flowers (Kaur and Bhutani 2013). Its populations stretches from India to China at altitudes ranging from 900 to 1400 meters (Wikipedia). In addition to being horticulturally important, it is also known for its medicinal use like in treating tuberculosis and headaches (Rajbhandari and Bhattarai 2001). Due to its sweet fragrance, it is also used for ornamental purpose (De and Sil 2015). Due to these uses, *Coelogyne flaccida* is being over-exploited and collected and their natural habitat is also destroyed because of various anthroprogenic activities. Because of these reasons, these species are on the verge of their extinction and have been listed among the endangered species by IUCN (IUCN 2020).

Coelogyne flaccida
Source: https://orchidroots.com

Rhyncostylis retusa (L.)

The name *Rhyncostylis* came from ancient Greek words "*rhyncos*" which means "beak" and "*stylis*" which means "column". These fleshy orchids having spicy aroma are native to Indian Subcontinent, China and Philippines (Saxena 2020). They tend to grow in warm environment with good air and no direct sunlight. *Rhyncostylis retusa* (L.) is a monopodial orchid having almost 25 cm long stem and fleshy leaves (Bhattacharjee and Islam 2015). They are also native to some parts of India: Assam, Kerala, Sikkim and Andhra Pradesh (Saxena 2020).

Rhyncostylis retusa
Source: https://upload.wikimedia.org

Apart from ornamental uses these orchids are known for their medicinal uses like anti-fungal, anti-microbial and anti-cancerous properties (Tsering et al. 2017). The juice from the roots of these orchids can be used to treat malaria (Tiwari et al. 2012), arthiritis (Panda and Mandal 2013), vertigo and menstrual troubles (Pant 2013). Due to over-exploitation of their natural habitat as well as the orchids itself for the needs and benefits, *Rhyncostylis retusa* have also become now endemic and have also been listed among the endangered species by IUCN (IUCN 2020).

In today's world, because of our day-to-day lifestyle needs and benefits, it has become a challenging task for scientists to conserve our biodiversity. For a healthy environment as well as healthy biodiversity, it is necessary for each species and organisms to survive (Kaur and Bhutani 2013). Orchids require favorable habitats in order to grow in a given environment. They effectively serve as a reliable marker for a healthy biodiversity. Due to several factors like over-exploitation, destruction of their natural habitat, deforestation, over-collection, increased pollution and illegal harvesting (Hinsley et al. 2018), the orchids are on their verge of extinction while some are listed among the endangered species (IUCN 2020). The growth of orchids is slow and the uncertain climate changes affecting the growth make them more vulnerable to destruction in their natural habitat (Lopez-Puc 2013). Previously ex-situ and in-situ techniques have been used to conserve the orchid species but due to some limitations they were considered unfavorable for their growth and yields (Pant 2013). So, to overcome these challenges the scientists and researchers have started using *in-vitro* methods that appeared to be a potential option rather than using ex-situ and in-situ methods of their conservation (Kumaria and Tandon 2007). As a result, tissue culture and micropropagation methods are now considered more suitable.

In-vitro propagation is defined as techniques and methods used for growing plant species on the nutrient rich medium with vitamins and plant growth regulators under aseptic conditions with proper temperature, light and air and no time limitations leading to increase in their yields (Pant 2013). As the growth rate of orchids is low resulting in their lowest yield, in their natural habitat, the *in-vitro* methods can be considered suitable for their conservation and highest yields (Behera et al. 2012). *In-vitro* propagation involves methods like shoot multiplication, rooting, induction of callus and formation of callus. The medium used for these tissue culture techniques should be nutrient rich containing macro-nutrients (nitrogen, calcium, phosphorous sulfur, and magnesium), micronutrients (boron, zinc, iron, copper), vitamins and plant growth regulators (Reed et al. 2011).

As we know for growth and development plants depends on basic environmental conditions but in addition to these, they also require some various substances like plant growth regulators (also known as plant growth hormones). These help in promoting plant growth, differentiation and organogenesis and also helps in inhibiting senescence and aging (Fay 1992).

Apart from micropropagation, there is another method called Artificial Seed Technology used for conservation of orchids. The synthesis of artificial seeds has opened new opportunities for the conservation of orchid species (Pradhan et al. 2016). This concept was first introduced by Murashige. Artificial seeds are also known as synthetic seeds (Khor and Loh 2005). Micropropagation methods will assure as the proper methods used for conserving and growing every plant species. But in some cases like plants with small size of seeds or no seeds, micropropagation methods are a limitation because of more time consumption and cost (Saiprasad 2001). So for these kind of plant species, artificial seed technology has been considered as the most effective method. It involves germination of new plants from any part of the mother plant like stem, leaves, roots or buds. In artificial seed technology, sodium alginate and calcium chloride are mainly used as encapsulation matrix (Gogoi et al. 2013). The explants are encapsulated in a protective coating and can be kept for storage at any suitable temperature for the further uses. These stored seeds can also be inoculated in the culture medium for germination purposes. The present study highlights the methods to conserve the orchids through artificial seed technology under *in-vitro* conditions through micro-propagation of PLBs.

OBJECTIVES

- Establishment of *in-vitro* cultures of endangered orchids namely *Dendrobium nobile* Lindl., *Coelogyne flaccida* Lindl., *Rhyncostylis retusa* (L.).
- Generation, multiplication and maturation of protocorm like bodies (PLBs).
- Encapsulation of PLBs to develop the synthetic seeds and to study the storage at two different temperatures.
- To check the clonal fidelity of regenerated plants.

CHAPTER: 2

REVIEW OF LITERATURE

Orchid conservation has now become an interested subject for many years. Apart from ex-situ and in-situ conservation techniques, researchers are now more focused on *in-vitro* techniques like micropropagation and artificial seed technology.

In-vitro propagation

In-vitro propagation has potential advantages over in-situ and ex-situ propagation for example large population of plants can be propagated simultaneously without any need of extensive labour at any time of year (Kumaria and Tandon 2007). Kalimuthu et al. (2007) developed a protocol for *in-vitro* seed germination of *Oncidium* sp. in which immature seeds were inoculated on MS medium supplemented with 2 mg/l of 6-benzylaminopurine (BAP). Abraham et al. (2012) studied the effect of various media formulations like Murashige and Skoog (MS), Knudson (KN) and Vacin and Went (VW) on seed germination of *Coelogyne nervosa*. The seeds were inoculated on ½ MS, MS, KN and VW supplemented with different plant growth regulators like BAP (0, 0.5 mg/l) and 1-napthelene acetic acid (NAA) along with different concentrations of coconut water (10, 20, 30 and 40%). 96% germination was recorded on MS medium supplemented with 30% coconut water, 3 mg/l BAP and 0.5 mg/l NAA and gave 91% success rate when transferred to soil.

Reddy (2008) also studied the effect of different media formulations on micropropagation of orchids and reported that MS medium was the best medium for micropropagation. Addition of plant growth regulators in MS medium helps in increasing the germination rate of seeds (Arya et al. 1999). In this study, the effect of different concentrations of BAP in MS medium was studied on seed germination of *Dendrocalamus asper*. 82% germination was recorded on MS medium supplemented with 2.5 μM BAP.

Asghar et al. (2011) also studied the effect of BAP, Kinetin (Kn) and Indole-3-Butyric acid (IBA) on *in-vitro* seed germination of *Dendrobium nobile*. It was observed that addition of 2 mg/l BAP in the medium resulted in the increase of number of shoots, while the addition of 1.5 mg/l Kinetin in the medium increased the shoot length and addition of 2 mg/l IBA in MS medium resulted in highest percentage (97.5%) of roots. Another experiment was also conducted which reported that the addition of chitosan in MS medium increased the seed germination rate in *Dendrobium* orchids (Pornpienpakdee et al. 2010).

Naing et al. (2011) conducted an experiment in which they studied the effect of BAP and NAA on the formation, multiplication and regeneration of PLBs of *Coelogyne cristata* through indirect somatic embryogenesis. Thidiazuron also played an important role in the formation and multiplication of protocorm like bodies in the orchids like *Vanda coerulea* (Malabadi et al. 2004). In this study, 95% survival rate of PLBs were recorded when Thidiazuron was added in VW medium.

Gnasekaran et al. (2010) also reported that adding organic additives in the medium increased the formation of PLBs in *Phalaenopsis violacea*. They conducted an experiment to study the effect of different organic additives (such as banana, tomato, papaya and coconut extracts) in different concentrations of MS medium on the formation of PLBs and observed that the addition of banana extract in ½ MS medium resulted in the highest PLBs formation. Novak et al. (2014) also reported the use of auxin in the medium to increase the PLB formation, maturation and germination. Apart from using tissue culture techniques for the production of PLBs, the scientists have focused on bioreactors like airlift or bubble column bioreactors for the mass production of PLBs to meet the large scale demands for orchids in the commercial market (Murthy et al. 2018). Kaur and Bhutani (2010) reported that addition of charcoal in the MS medium supplemented with BAP and NAA increased the multiplication of PLBs in *Malaxis acuminanata*.

Baker et al. (2014) had studied the effect of plant growth regulators such as BAP (0, 0.2, 0.5, 1, 1.5, 3 mg/l), IBA (0, 0.5 mg/l) and NAA (0, 0.5 mg/l) on the germination of PLBs of *Orchis calasetum*. It was observed that the highest number of leaves, roots and maximum height were recorded on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Sugar was also reported to play an important role in the germination of PLBs in *Oncidium* orchids (Jheng et al. 2006). Temjensangba and Deb (2005) conducted an experiment to study the effect of different concentrations of sucrose, coconut water in MS medium on germination of PLBs of *Arachnis labrosa*. They observed that addition of 3% sucrose in MS medium along with 15% coconut water and 10 μM NAA+8 μM BAP resulted in the highest germination (95%) of PLBs.

A slow growth protocol was also developed to study the effect of mannitol and sorbitol supplemented in MS medium for the germination of PLBs of *Epidendrum chlorocorymbos* Schltr.

and it was reported that the maximum number of PLBs germinated on ½ MS medium supplemented with 1% sorbitol (Lopez-Puc 2013). Utami and Hariyanto (2020) have also studied the effect of organic additives such as coconut water, peptone, potato extract etc. in the MS medium on the germination of PLBs.

Artificial seed formation, germination and conservation

Mohanty and Das (2013) studied the effect of various concentrations of sodium alginate (1%, 2%, 3%, 4% and 5%) and calcium chloride (50 mM, 75 mM and 100 mM) on artificial seed formation in *Dendrobium densiflorum*. 3% sodium alginate and 100 mM calcium chloride were recorded as the best gelling and complexing agents for encapsulating PLBs to form artificial seeds. Apart from using PLBs for artificial seed formation, leaf base or callus of orchids can also be used for seed formation (Sarmah et al. 2010). In this study, they had used 6 month old leaf base of *Vanda Coerulea* Grifft. Ex. Lindl. as explant for producing artificial seeds and also reported the importance of sodium alginate and calcium chloride on the germination percentage. Firm beads were formed at 3% sodium alginate and 100mM calcium chloride which showed 95% germination when directly inoculated in medium after artificial seed formation.

Datta et al. (1999) also developed a protocol for synthesis of artificial seeds by encapsulating PLBs of *Geodorum densiflorum.* They studied the effect of different concentrations of sodium alginate and calcium chloride on artificial seed formation and observed that the best artificial seeds were formed in 3% sodium alginate and 100 mM calcium chloride. After encapsulation, they inoculated the artificial seeds in the KN medium. 88% seeds germinated while only 28% germinated seeds survived when transferred directly to soil.

To check the viability of artificial seeds of *Vanda coerulea,* Devi et al. (2000) conducted a storage experiment in which the difference in germination rate of encapsulated PLBs and unencapsulated PLBs stored at 4^{o}C for 120 days and 30 days respectively was compared. 72% germination was recorded in encapsulated PLBs while 50% germination was recorded in unencapsulated PLBs. Orchid seed germination is entirely dependent on symbiotic relationships with fungi. In order to gain nutrition, fungi constantly attempts to penetrate into the cytoplasm of orchid cells. By limiting the infected hyphae’s growth, the orchid cells get their nutrients (Shimura et al. 2007).

Gantait et al. (2012) also conducted an experiment to study the effect of storage on germination of artificial seeds of *Aranda Wan Chark Kuan* 'Blue'× *Vanda coerulea* Grifft. ex. Lindl. The artificial seeds were stored in dark at 4 ^{o}C and 25 ^{o}C for different intervals of time i.e. 30, 60, 90, 120, 150 and 180 days. They reported that the artificial seeds were viable for more time at 25 ^{o}C as compared to 4 ^{o}C. At 4 ^{o}C, majority of encapsulated seeds turned brown which lead to their death. They also observed that germination percentage decreased with the increase in incubation time. Khor et al. (1998) developed a two-coating system protocol for encapsulating the PLBs of *Spathoglottis plicata*. Their study reported that encapsulated seeds of *Spathoglottis plicata* when treated with Abscisic acid (ABA) showed high viability.

Synthetic seeds has been widely used to preserve germplasm (Towill 1998). Previously seed banks were used to preserve the live plants but due to its limitation such as high maintenance and time consuming procedure, its use had become limited. As a result, maintaining live plants encapsulated in the gelling matrix as artificial seeds will aid in the retention of plants for long period of time in particular space under appropriate conditions. This particular method is very beneficial for the species lacking proper conservation measures for example *Vittis* spp. is conserved by this technique (Towill 1998).

Due to deterioration of the natural habitat, *Laelia anceps* has been listed among the endangered species (Ramirez-Mosqueda et al. 2018). Their study focused on the conservation of these orchid species. They studied the effect of different concentrations of MS medium (1/4, ½ and ¾) supplemented with different concentrations (0.5, 1, 2 mg/l) and combinations of Abscisic acid (ABA) + Paclobutrazol (PBZ) along with Polyethylen glycol (PEG-10, 20 and 30 g/l) on germination of PLBs and reported 89% germination on ½ MS medium supplemented with 2 mg/l PBZ and 30 g/l PEG. They also studied the effect of different concentrations (0, 0.5, 1, 2mg/l) of both Thidiazuron (TDZ) and BAP on germination of artificial seeds of *Laelia anceps*. 90% germination was recorded on MS medium supplemented with 2mg/l BAP.

Being the most popular and rare genus of orchids, *Paphiopedilum* are also on the verge of their extinction because of over-exploitation of their flowers (Zeng et al. 2015). In this study, they had focussed on improving the germination protocols to increase the propagation of *Paphiopedilum* by *in-vitro* techniques.

Pradhan et al. (2016) also developed a protocol for conservation of *Cymbidium aloifolium* using artificial seed technology. They studied the effect of different MS media formulations ($^{1}/_{4}$, ½ and full strength) on germination of artificial seeds and reported that the full strength MS medium was more suitable for germination protocols.

Clonal fidelity

Synthetic seeds are also known for maintaining true breeding lines because of their convenient storing, transportation, less variation and more viability (Ravi and Anand 2012).

Number of factors related with *in-vitro* culture techniques can cause clonal variations. Checking clonal fidelity of *in-vitro* grown PLBs is very important if true breeding plants are required (Singh et al. 2012). They developed a protocol for *in-vitro* propagation of *Eclipta alba* using thin cell layer culture technique and also checked their genetic fidelity using RAPD markers. It was observed that the plantlets germinated from PLBs inoculated on MS medium supplemented with BAP were genetically identical with the mother plants.

Another experiment was also conducted to check the clonal fidelity of *Dendrobium chrysotoxum* Lindl. (Golden orchid) using 12 RAPD and 11 ISSR markers (Tikendra et al. 2019). Their study reported 96.3% highest genetic similarity and 3.6% as lowest genetic similarity between *in-vitro* germinated PLBs and mother plants.

The *in-vitro* grown PLBs of *Rhyncostylis retusa* were also checked for their genetic similarity with their mother plants using 10 RAPD primers (Oliya et al. 2021). Their study reported 92% PLB germination on half strength MS medium and also observed that the *in-vitro* germinated PLBs were genetically similar to their mother plant.

Bhattacharyya et al. (2016) also analyzed the *in-vitro* grown seeds of *Dendrobium nobile* for their clonal fidelity using SCoT and RAPD primers. 97% genetic similarity with PIC values of 0.92 and 0.76 respectively was recorded using RAPD primers.

CHAPTER: 3

MATERIALS AND METHODS

Plant material, chemical and glasswares

The cultures of orchids such as *Dendrobium nobile* Lindl., *Coelogyne flaccida* Lindl. and *Rhyncostylis retusa* (L.) were already available at plant tissue culture laboratory of TIFAC CORE Thapar Institute of Engineering and Technology, Patiala. These cultures were maintained on MS medium (Murashige and Skoog, 1962) supplemented with (6-benzyl aminopurine) 2.5 μM BAP, 87mM sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.75 - 5.8 using 1N NaOH or 1N HCL and autoclaved at 121^{o}C, 15 psi for15 min. Cultures were subculture after regular interval of 21-30 days. The cultures were maintained in growth room under white fluorescent light (50 μmole m^{-2} sec^{-1}) at 25 ^{o}C and photoperiod of 16 hours of light and 8 hours dark. All the chemicals were procured from Himedia Laboratories Pvt Ltd. (Mumbai), Sigma Chemical Co. (St. Louis USA) and Thermofisher scientific. All the experiments were performed in culture bottles (300 ml) (Kasablanka Corporation, Mumbai). The other glassware like beakers and measuring cylinders were from Borosil Glass Works Ltd., Mumbai, India.

Medium Preparation

The medium for the culture experiments was prepared using the required volume of stock solutions as described by Murashige and Skoog (1962). 87mM sucrose and 0.8% Agar as gelling agent was also added. The total volume was made using distilled water. The pH of the medium was adjusted in the range of 5.8 using 1N NaOH or 1N HCl. The medium was transferred to culture bottles and autoclaved for 15 minutes at 121^{o}C and 15 psi. The bottles were then transferred to storage room under aseptic conditions till further use.

Effect of BAP and IAA on shoot multiplication

Small shoot clumps were inoculated on MS medium supplemented with different concentrations and combinations BAP (0, 1, 2.5 and 5 μM) and IAA (0, 1, 2.5 and 5 μM). The culture bottles were then incubated at 25 ^{o}C ± 1 ^{o}C under suitable conditions. Results were observed after 30 days of incubation and recorded as number of shoots per clump, average shoot length and number of leaves.

Effect of BAP on formation of PLBs

Approximately 30 leaves from culture bottle of each orchid were taken and cut in small sizes into 1-2 cm in size using scalpel and forceps on sterile glass plate. The leaf explants were then inoculated on MS medium supplemented different concentrations of BAP (0, 1, 2.5 and 5 µM). The culture bottles were sealed properly and incubated at 25 ^{o}C ± 1 ^{o}C. Results were observed after 2 months and recorded as total number of PLBs formed in all the three species.

Multiplication and Maturation of PLBs

For multiplication, PLBs of *D. nobile, C. flaccida* and *R. retusa* were cut into approximately 1-2 cm and inoculated on MS medium containing 2.5 µM BAP. For maturation, PLBs were inoculated on MS medium supplemented with different concentrations of Sorbitol (0, 82.5, 165 and 247 mM). All the cultures were incubated at 25 ^{o}C ± 1 ^{o}C. Results were recorded after 1 month as total number of PLBs multiplied and matured.

Conversion of PLBs into plantlets

After maturation of PLBs of *Dendrobium nobile*, *Coelogyne flaccida* and *Rhyncostylis retusa*, the matured PLBs were inoculated on MS medium supplemented with different concentrations of sucrose (87, 174, 261 and 348 mM), BAP (0. 0.1 µM) and Giberellic acid [GA_3 (0, 1 µM)] for conversion into plantlets. Cultures were incubated at 25 ^{o}C ± 1 ^{o}C. Results were recorded after 40 days as number of shoots per PLB, average shoot length and number of leaves.

Encapsulation of PLBs

Different concentrations of sodium alginate (1%, 2%, 3% and 4%) in combination with different concentrations of calcium chloride (50, 100 and 200 mM) was tested for encapsulation of PLBs Matured PLBs of each orchid (*D. nobile, C. flaccida* and *R. retusa*) were excised into small size approximately 5-6mm and mixed in sodium alginate solution. The solution is then dropped off in chilled calcium chloride solution using wide bore glass pipette. The beads formed were kept in calcium chloride solution for 15-20 minutes.

Storage and Germination of Synthetic Seeds

In order to check the effect of storage on viability and germination, the artificial seeds of each orchid (*D. nobile, C. flaccida* and *R. retusa)* were stored at 4°C (cold temperature) and 25°C (room temperature) for different intervals of time (10, 20, 30, 40, 50 and 60 days) under dark conditions. After the respective incubation time of storage, the seeds were inoculated on basal MS medium and incubated at 25 °C ±1 °C for germination. Results were noted within 2 months as percent of artificial seeds germinated. After germination, the plantlets of each orchid under study were multiplied for testing the clonal fidelity.

Clonal fidelity

Fresh and young shoots (2 g) were crushed in liquid nitrogen for DNA isolation using CTAB method (Doyle and Doyle 1990). The crushed samples were transferred to 50 ml Oakridge tube followed by addition of 10 ml prewarm CTAB buffer and 200 µl β-mercaptoethanol. The homogenate mixture was incubated at 60 °C for 1 hour. Equal volume of chloroform: isoamyl alcohol was added and centrifuged at 5000 rpm for 10 min. To aqueous phase 0.66 volume of cold isopropanol was added and incubated at -20 °C for 1 hr. Centrifugation was done at 10,000rpm for 15 min. To pellet 1ml TE buffer was added and dissolve completely. For DNA purification, 2 µl RNAase solution was added to above isolated DNA and incubated at 37 °C for 1 hr. After incubation equal volume of phenol: chloroform:isoamyl alcohol was added followed by centrifugation at 10,000rpm for 10 min. To aqueous layer 0.3 volume of sodium acetate and 0.6 volume of chilled isopropanol was added and incubated at -20 °C for 1 hr. Centrifugation was done at 10,000 rpm for 10 min and pellet was dissolved in 30 µl in TE buffer and stored at -20 °C. The quality of isolated DNA was checked on 0.8% agarose gel stained with 0.5 µg/ml ethidium bromide (EtBr) and concentration was checked using Nanodrop 1000™ UV/VIS spectrophotometer (Thermo Scientific). All DNA samples were diluted to 20 ng/ul concentration.

For RAPD analysis, a total of 26 RAPD primers were tested for each orchid species (Table 5.16). The primers producing clear and intact bands for *D. nobile* (Table 5.17), *C. flaccida* (Table 5.18) and *R. retusa* (Table 5.19) were selected for further amplifications. A typical 20 µl PCR reaction consisted of 2 µl of template DNA (20 ng/ul), 2 µl buffer (10x), 3 µl dNTPs (100 µM), 1.5 μl primer (10 μM), 0.2 μl Taq polymerase (1U) and final volume made upto 20 μl with Milli Q water. The given amplification conditions were followed; initial denaturation at 94 ^{o}C for 5 min, 41 cycles for denaturation at 94 ^{o}C for 1 min, annealing at 35 ^{o}C (*D. nobile*), 34 ^{o}C *(C. flaccida)* and 41 ^{o}C (*R. retusa*) for 45s, extension at 72 ^{o}C for 1.5 min and the final extension at 72 ^{o}C for 5 min. The amplified products were separated on 1.2% agarose gel stained with 0.5 µg/ml ethidium bromide (EtBr) and visualized under UV transilluminator (Gel Doc Mega: Biosystematics USA).

Statistical analysis

Unless otherwise mentioned, all plant tissue culture experiments were conducted thrice with three replicates having 5 explants per bottle. Statistical analyses were done using GraphPad Prism 5 software (GraphPad, San Diego, CA). One way analysis of variance (ANOVA) by t-test was applied and means were compared either by Ducan's or LSD test with significant level at $P<0.05$.

CHAPTER: 4

RESULTS

Shoot Multiplication

Multiplication of cultures of *Dendrobium nobile* Lindl., *Coelogyne flaccida* Lindl. and *Rhyncostylis retusa* (L.) was carried out on MS medium supplemented with 87 mM sucrose and different concentrations of BAP (0, 1, 2.5 and 5 µM) and IAA (0, 1, 2.5 and 5 µM). In case of ***D. nobile***, the highest shoot multiplication was recorded on MS medium supplemented with 2.5 µM BAP and 5 µM IAA with 23.63 ± 0.19 of shoots per clump. However, the lowest number (4.91 ± 0.97) of shoots per clump was recorded on MS medium with 5 µM BAP and 5 µM IAA after 35 days of inoculation. The highest average shoot length (4.27 ± 0.91) was recorded on MS medium with 0 µM BAP and 2.5 µM IAA, whereas the lowest average shoot length

Table 5.1: Effect of BAP and IAA on number of shoots per clump, average shoot length and number of leaves per clump in *Dendrobium nobile*,

BAP	IAA	No. of shoots per clump	Average shoot length (cm)	No. of leaves per clump
0	**0**	$6.08^{fg} \pm 0.26$	$2.35^{abc} \pm 0.34$	$19.30^{i} \pm 0.64$
1	**0**	$10.97^{d} \pm 0.83$	$1.57^{bc} \pm 0.66$	$27.80^{f} \pm 1.19$
2.5	**0**	$18.51^{b} \pm 0.94$	$1.36^{bc} \pm 0.10$	$32.36^{de} \pm 1.65$
5	**0**	$2.38^{h} \pm 0.79$	$0.58^{c} \pm 0.07$	$10.30^{j} \pm 0.55$
0	**1**	$8.72^{e} \pm 0.95$	$2.77^{abc} \pm 0.70$	$18.22^{i} \pm 0.33$
1	**1**	$11.38^{d} \pm 0.56$	$1.06^{bc} \pm 0.34$	$21.94^{h} \pm 0.15$
2.5	**1**	$15.61^{c} \pm 0.08$	$0.71^{c} \pm 0.23$	$33.69^{c} \pm 0.50$
5	**1**	$7.58^{ef} \pm 0.63$	$0.57^{c} \pm 0.20$	$23.75^{g} \pm 0.17$
0	**2.5**	$15.30^{c} \pm 0.51$	$4.27^{a} \pm 0.91$	$33.11^{cd} \pm 1.36$
1	**2.5**	$16.13^{c} \pm 0.36$	$2.15^{abc} \pm 0.49$	$35.19^{b} \pm 0.70$
2.5	**2.5**	$18.25^{b} \pm 0.22$	$1.75^{bc} \pm 0.40$	$36.16^{b} \pm 0.72$
5	**2.5**	$8.47^{e} \pm 0.60$	$0.93^{bc} \pm 0.33$	$22.52^{h} \pm 1.15$
0	**5**	$9.55^{de} \pm 0.54$	$3.18^{ab} \pm 0.84$	$27.11^{f} \pm 1.72$
1	**5**	$11.63^{d} \pm 0.60$	$1.58^{bc} \pm 0.33$	$31.19^{e} \pm 1.55$
2.5	**5**	$23.63^{a} \pm 0.19$	$1.11^{bc} \pm 0.37$	$37.38^{a} \pm 1.52$
5	**5**	$4.91^{g} \pm 0.97$	$0.48^{c} \pm 0.10$	$6.97^{k} \pm 0.16$

* Data were analyzed by one-way ANOVA using t test and means were compared by Ducan's at $P<0.05$.

(0.48 ± 0.10) was observed at 5 µM BAP and 5 µM IAA. The highest number of leaves (37.38 ± 1.52) per clump were recorded on MS medium with 2.5 µM BAP and 5 µM IAA and the lowest number of leaves (6.97 ± 0.16) per clump were recorded in 5 µM BAP and 5 µM IAA. The data were observed after regular time interval of 15 days.

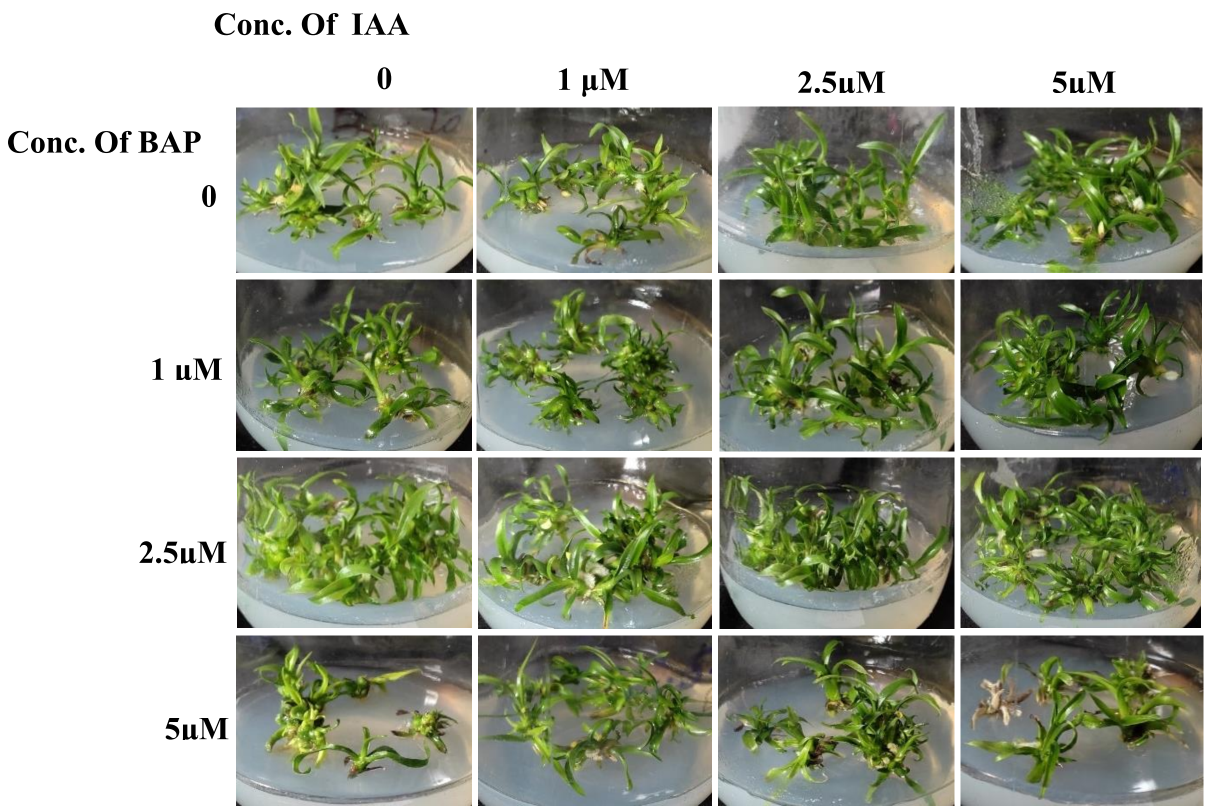

Figure 5.1: The effect of different concentrations of BAP and IAA on MS medium on shoot multiplication of *Dendrobium nobile*.

In case of ***C. flaccida***, the highest number of shoots per clump (22.56±0.42) was recorded on MS medium supplemented with 2.5 µM BAP and 5 µM IAA and the lowest number of shoots per clump (3.63±0.48) was recorded on MS medium with 5 µM BAP and 5 µM IAA after 30 days of inoculation. The highest average shoot length (3.42±0.07) was recorded on MS medium with 0 µM BAP and 2.5 µM IAA and the lowest average shoot length (1.35±0.70) was recorded in MS medium with 5 µM BAP and 5 µM IAA. The highest number of leaves per clump (35.66±1.78) were recorded on MS medium with 2.5 μM BAP and 5 μM IAA and the lowest number of leaves per clump (5.05±0.26) were recorded on MS medium supplemented with 5 μM BAP and 5 μM IAA. The data were observed after regular intervals of 15 days.

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* .

BAP	IAA	No. of shoots per clump	Average shoot length	No. of leaves per clump
0	0	$5.72^{g} \pm 0.22$	$2.94^{ab} \pm 0.66$	$13.13^{i} \pm 0.65$
1	0	$12.05^{d} \pm 0.36$	$2.27^{ab} \pm 0.66$	$21.94^{f} \pm 1.09$
2.5	0	$16.72^{b} \pm 0.61$	$2.07^{ab} \pm 0.62$	$30.16^{c} \pm 1.50$
5	0	$11.69^{d} \pm 0.20$	$1.86^{ab} \pm 0.15$	$20.52^{g} \pm 1.02$
0	1	$17.94^{b} \pm 0.36$	$2.50^{ab} \pm 0.73$	$30.58^{bc} \pm 1.52$
1	1	$18.11^{b} \pm 0.42$	$2.33^{ab} \pm 0.80$	$31.94^{b} \pm 1.59$
2.5	1	$22.32^{a} \pm 0.16$	$2.23^{ab} \pm 0.72$	$35.52^{a} \pm 1.77$
5	1	$9.88^{e} \pm 0.93$	$1.31^{b} \pm 0.32$	$12.00^{i} \pm 1.20$
0	2.5	$6.08^{g} \pm 0.21$	$3.42^{a} \pm 0.07$	$10.05^{j} \pm 0.50$
1	2.5	$12.31^{d} \pm 0.33$	$2.24^{ab} \pm 0.14$	$22.44^{ef} \pm 1.12$
2.5	2.5	$14.63^{c} \pm 0.07$	$1.66^{ab} \pm 0.49$	$26.08^{d} \pm 1.30$
5	2.5	$2.58^{h} \pm 0.33$	$1.58^{ab} \pm 0.60$	$8.61^{k} \pm 0.43$
0	5	$7.66^{f} \pm 0.34$	$2.87^{ab} \pm 0.79$	$14.80^{h} \pm 0.74$
1	5	$13^{d} \pm 0.41$	$2.14^{ab} \pm 0.25$	$23.66^{e} \pm 1.19$
2.5	5	$22.56^{a} \pm 0.42$	$1.42^{b} \pm 0.24$	$35.66^{a} \pm 1.78$
5	5	$3.63^{h} \pm 0.48$	$1.35^{b} \pm 0.70$	$5.05^{l} \pm 0.26$

* Data were analyzed by one-way ANOVA using t test and means were compared by Ducan's at $P<0.05$

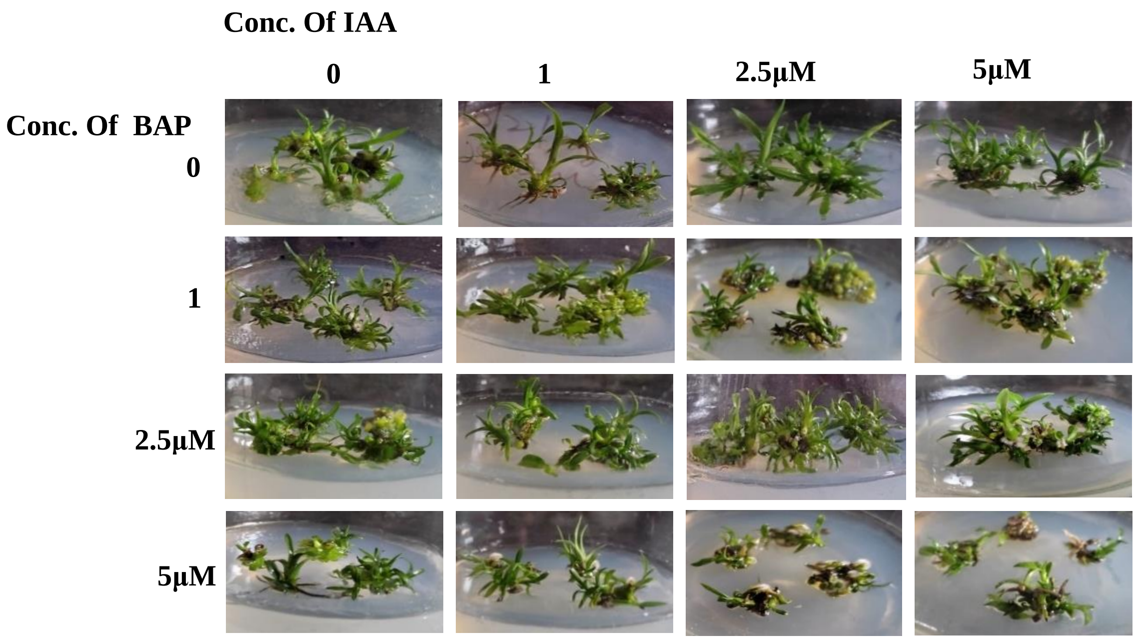

Figure 5.2: The effect of different concentrations of BAP and IAA on MS medium on shoot multiplication of *Coelogyne flaccida*.

In case of ***R. retusa,*** the highest number of shoots (17±0.28) per clump were recorded in MS medium with 2.5 µM BAP and 5 µM IAA and the lowest number of shoots per clump (4.5 ±1.20) were recorded on MS medium with 5 µM BAP and 5 µM IAA after 35 days of inoculation on the medium. The highest average shoot length (3.80±0.83) was recorded on MS medium with 0 µM BAP and 5 µM IAA and the lowest average shoot length (0.54±0.61) was recorded on MS medium with 5 µM BAP and 5 µM IAA. The highest number of leaves per clump (15.7 ±0.77) were recorded on MS medium with 2.5 µM BAP and 5 µM IAA and the lowest number of leaves per clump (5.25±0.92) were recorded on medium supplemented with 5 µM BAP and 5 µM IAA. The data were observed after regular interval of 15 days.

It was observed that with the increase in concentration of BAP upto 2.5 µM and 5 µM IAA, the number of shoots and of *D. nobile, C. flaccida* and *R. retusa* also increased.

Table 5.3: Effect of BAP and IAA on number of shoots per clump, average shoot length and number of leaves per clump in *Rhyncostylis retusa.*

BAP	IAA	No. of shoots per clump	Average shoot length	No. of leaves per clump
0	0	$7.41^{ef} \pm 0.41$	$2.32^{b} \pm 0.28$	$8.75^{efg} \pm 0.52$
1	0	$8.91^{de} \pm 0.30$	$1.23^{bc} \pm 0.21$	$11.91^{cde} \pm 0.59$
2.5	0	$13.16^{b} \pm 0.50$	$1.19^{bc} \pm 0.03$	$12.08^{cde} \pm 0.60$
5	0	$4.75^{g} \pm 0.57$	$1.16^{bc} \pm 0.31$	$6.66^{fg} \pm 0.59$
0	1	$10.91^{cd} \pm 0.31$	$2.35^{b} \pm 0.59$	$11.08^{de} \pm 0.33$
1	1	$12.33^{bc} \pm 0.44$	$1.45^{bc} \pm 0.17$	$13.25^{abcd} \pm 0.56$
2.5	1	$16.08^{a} \pm 0.44$	$1.23^{bc} \pm 0.15$	$15.08^{abc} \pm 0.45$
5	1	$5.66^{fg} \pm 0.74$	$1.05^{bc} \pm 0.23$	$6.75^{fg} \pm 0.67$
0	2.5	$8.08^{e} \pm 0.71$	$2.25^{b} \pm 0.11$	$9.25^{ef} \pm 0.83$
1	2.5	$15.75^{a} \pm 0.14$	$1.77^{bc} \pm 0.10$	$12.33^{bcde} \pm 0.22$
2.5	2.5	$13.41^{b} \pm 0.08$	$1.24^{bc} \pm 0.63$	$16^{a} \pm 0.07$
5	2.5	$8.5^{e} \pm 0.75$	$1.10^{bc} \pm 0.79$	$10.41^{de} \pm 0.20$
0	5	$9^{de} \pm 0.27$	$3.80^{a} \pm 0.83$	$11.33^{de} \pm 0.33$
1	5	$12.16^{bc} \pm 0.54$	$2.50^{ab} \pm 0.07$	$14.08^{abcd} \pm 0056$
2.5	5	$17^{a} \pm 0.28$	$2.44^{ab} \pm 0.06$	$15.75^{ab} \pm 0.77$
5	5	$4.5^{g} \pm 1.20$	$0.54^{c} \pm 0.61$	$5.25^{g} \pm 0.92$

*Data was analyzed by one-way ANOVA using t test and means were compared by Ducan's test at $P<0.05$

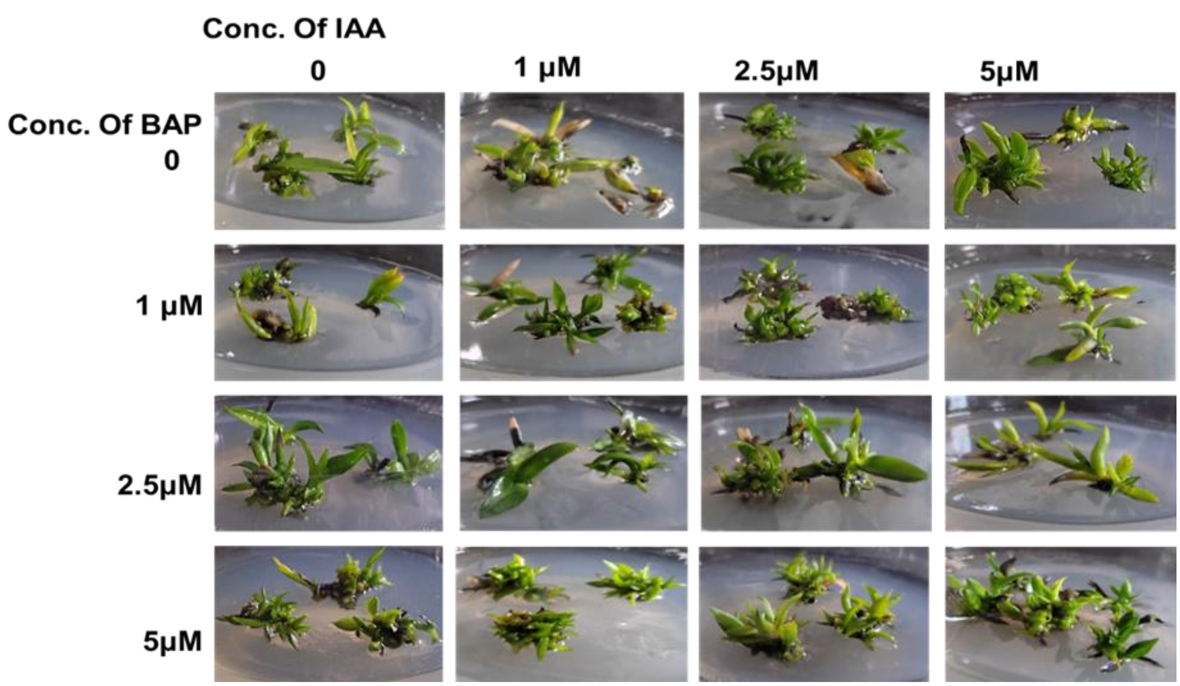

Figure 5.3: The effect of different concentrations of BAP and IAA on MS medium on shoot multiplication of *Rhyncostylis retusa*.

Effect of BAP on formation of PLBs

The formation of PLBs of *D. nobile, C. flaccida* and *R. retusa* was carried out using leaves of *in-vitro* grown cultures as explants. The leaves were cut into small size and inoculated on MS medium with different concentrations of BAP (0, 1, 2.5 and 5 μM). It took almost 50-60 days for the formation of PLBs after inoculation of explants.

D. nobile*:* The highest (95%) percentage of PLBs were formed on MS medium with 2.5 μM BAP after 60 days followed by MS medium with 5 μM BAP (77%). The lowest percentage (37%) of PLB formation was recorded in control i.e. MS medium with 0 μM BAP. The highest number of PLBs formed were 49.44 ±2.47 and the lowest number of PLBs formed were 17.44 ±0.87. The data were recorded after regular interval of 15 days from inoculation.

Table 5.4: Effect of BAP on formation of PLBs of *Dendrobium nobile* from leaf explants

BAP conc. (μM)	No. of leaves inoculated	No. of leaves responded (%)	Total PLBs formed
0	30	37.40^{d} ±1.87	17.44^{d} ±0.87
1	30	50.74^{c} ±2.53	23.66^{c} ±1.18
2.5	30	95.18^{a} ±4.75	49.44^{a} ±2.47
5	30	77.03^{b} ±3.85	36.66^{b} ±1.83

*Data were analyzed by one-way ANOVA using t test and means were compared by LSD at P<0.5.

C. flaccida: The highest (97%) percentage of PLBs were formed on MS medium with 2.5 μM BAP after 60 days followed by MS medium with 5 μM BAP (73%). The lowest percentage (17%) of PLBs were formed in control i.e. with 0 BAP. The highest number of PLBs formed were (52.77 ±2.63) and the lowest number of PLBs formed were (17.77 ±0.88). The data were observed after regular intervals of 15 days from inoculation.

Table 5.5: Effect of BAP on formation of PLBs of *Coelogyne flaccida* from leaf explants

BAP conc (μM)	No. of leaves inoculated	No. of leaves responded (%)	Total PLBs formed
0	30	39.25^{c} ±1.96	17.77^{d} ±0.88
1	30	72.59^{b} ±3.62	23^{c} ±1.15
2.5	30	97.77^{a} ±4.88	52.77^{a} ±2.63
5	30	73.70^{b} ±3.68	37.55^{b} ±1.87

* Data were analyzed by one-way ANOVA using t test and means were compared by LSD at P<0.5.

R. retusa: The highest (96%) percentage of PLB were formed on MS medium with 2.5 μM concentration of BAP after 60 days followed by MS medium with 5 μM BAP concentration(81%). The lowest (44%) percentage of PLBs formation was recorded in control i.e. with 0 μM BAP. The highest number of PLBs formed were (49.44 ± 2.42) and the lowest number of PLBs formed were (16.44 ± 0.82). The data were recorded after regular intervals of 15 days from inoculation.

Table 5.6: Effect of BAP on formation of PLBs of *Rhyncostylis retusa* from leaf explants

BAP conc (μM)	No. of leaves inoculated	No. of leaves responded (%)	Total PLBs formed
0	30	44.81^{d} ± 2.24	16.44^{d} ±0.82
1	30	72.59^{c} ± 3.62	26.44^{c} ± 1.29
2.5	30	96.29^{a} ±4.81	49.44^{a} ±2.42
5	30	81.11^{b} ± 4.05	31.11^{d} ±1.55

* Data were analyzed by one-way ANOVA using t test and means were compared by LSD at P<0.5.

Multiplication of PLBs

Multiplication of PLBs of *D. nobile*, *C. flaccida* and *R. retusa* was carried out in MS media supplemented with 2.5 μM concentration of BAP. The results for multiplication of PLBs of each orchid species under study were recorded within 25-30 days.

Maturation of PLBs

The maturation of PLBs was studied at different concentrations of sorbitol (0, 82.5, 165 and 247 mM) in MS medium with 87 mM sucrose. Shoot clumps of PLBs were inoculated in each culture bottle with each clump comprising of 10 PLBs.

***D. nobile*:** The highest percentage (31.44%) of PLBs matured was observed at 247 mM concentration of sorbitol after 40 days followed by 165mM concentration of sorbitol (27.11%). The lowest percentage (13.77%) of PLBs matured in control i.e. at 0 sorbitol concentration. It was observed that after 40-50 days there were no significant changes in maturation of PLBs. The data were recorded after regular interval of 10 days from inoculation.

Table 5.7: Percentage of matured PLBs of *Dendrobium nobile* inoculated on MS medium supplemented with different concentrations of sorbitol.

Sorbitol Conc.(mM)	Matured PLBs (%)
0	$13.77^{c} \pm 0.55$
82.5	$23.33^{b} \pm 0.45$
165	$27.11^{b} \pm 0.57$
247	$31.44^{a} \pm 0.66$

*Data were analyzed by one-way ANOVA using t test and means were compared by LSD at P<0.5.

C. flaccida: The highest percentage (40%) of PLBs matured were seen at 247 mM sorbitol concentration after 40 days followed by 165mM sorbitol concentration (30%). The lowest percentage (15%) of PLBs matured in control i.e. at 0 sorbitol concentration. It was observed that after 40-50 days there were no significant changes in maturation of PLBs. The data were recorded after regular interval of 10 days.

Table 5.8: Percentage of matured PLBs of *Coelogyne flaccida* inoculated on MS medium supplemented with different concentrations of sorbitol.

Sorbitol Conc.(mM)	Matured PLBs
0	$15^{d} \pm 0.68$
82.5	$26.77^{c} \pm 0.56$
165	$30.88^{b} \pm 0.61$
247	$40.22^{a} \pm 0.80$

*Data were analyzed by one-way ANOVA using t test and means were compared by LSD at P<0.5.

***R. retusa*:** The highest percentage (29%) of PLBs matured were seen at 247 mM sorbitol concentration after 40 days followed by 165mM sorbitol concentration (25%). The lowest percentage (14%) of PLBs matured in control i.e. at 0 sorbitol concentration. It was observed that after 40-50 days there were no significant changes in maturation of PLBs. The data were recorded after regular interval of 10 days.

Table 5.9: Percentage of matured PLBs of *Rhyncostylis retusa* inoculated on MS medium supplemented with different concentrations of sorbitol.

Sorbitol Conc.(mM)	Matured PLB (%)
0	14^{c} ±0.56
82.5	16.22^{c} ±0.40
165	25.55^{b} ±0.59
247	29.33^{a} ±0.65

*Data were analyzed by one-way ANOVA using t test and means were compared by LSD at $P<0.5$.

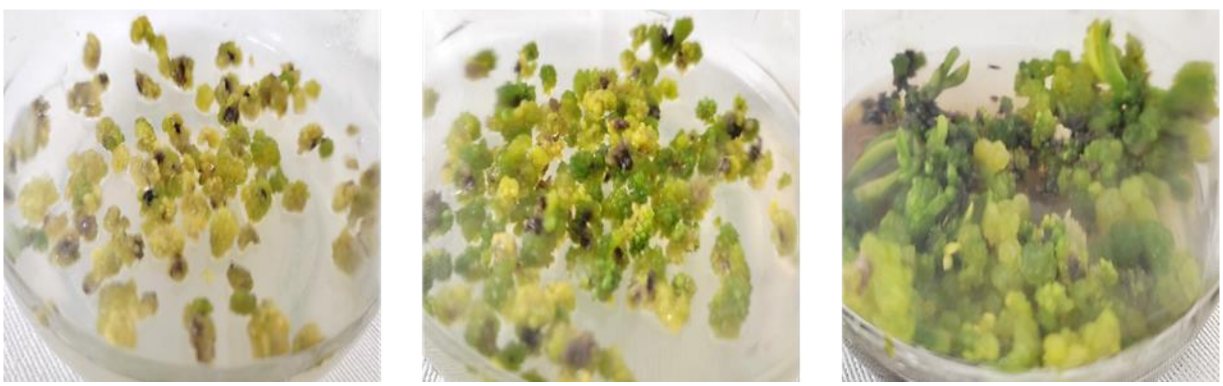

Figure 5.4: The effect of different concentrations of sorbitol on MS medium on maturation of PLBs of **(a)** *Dendrobium nobile* **(b)** *Coelogyne flaccida* **(c)** *Rhyncostylies retusa*

Conversion of PLBs into plantlets

The matured PLBs of *D. nobile, C. flaccida* and *R. retusa* were then inoculated on MS medium with different concentrations and combinations of Sucrose (87, 174, 261 and 348 mM), BAP (0, 0.1 µM) and GA_3 (0, 1 µM) for conversion into plantlets.

D. nobile**:** The effect of different concentrations of sucrose, GA_3 and BAP was studied on the matured PLBs in which highest number of shoots and leaves per PLB were seen at 261 mM concentration of Sucrose with 1 μM GA_3 and 0.1 μM BAP while the optimum results were recorded at 87 mM concentration of sucrose after 30-40 days from inoculation. The lowest number of shoots and leaves per PLB were observed at 348 mM concentration of sucrose followed by 174 mM concentration. The highest number of shoots per PLB (42.16±0.22) and leaves per PLB (42.47±0.24) were recorded in MS medium with 261 mM sucrose, 1 μM GA_3 and 0.1 μM BAP after 35 days from inoculation while the lowest number of shoots per PLB (7±0.17) and leaves (11.61±0.13) were recorded in MS medium with 348mM sucrose only i.e. with no BAP and GA_3. The highest average shoot length (4.44±0.05) was recorded at 261 mM concentration of Sucrose with 1 μM GA_3 and 0.1 μM BA while the lowest average shoot length (2.02 ±0.22) was recorded at 348 mM concentration of sucrose with no BA and GA3. The optimum number of shoot per PLB

Table 5.10: Effect of sucrose, BAP and GA_3 on number of shoots per PLB, average shoot length and number of leaves per PLB of *Dendrobium nobile*

Sucrose	GA_3	BAP	No. of shoots per PLB	Average Shoot Length (cm)	No. of leaves per PLB
87	**0**	**0**	22.63^{d} ±0.41	2.56^{abcd} ±0.60	24.66^{d} ±0.49
87	**0**	**0.1**	23.63^{d} ±0.64	3.22^{abcd} ±0.75	22.30^{f} ±0.66
87	**1**	**0**	26.80^{c} ±0.19	3.53^{abcd} ±0.84	31.83^{c} ±0.08
87	**1**	**0.1**	32.52^{b} ±0.87	4.36^{ab} ±0.05	33.16^{c} ±0.99
174	**0**	**0**	11.75^{g} ±0.04	2.24^{cd} ±0.01	16.66^{i} ±0.07
174	**0**	**0.1**	14.08^{g} ±0.39	2.28^{cd} ±0.64	22.63^{f} ±0.67
174	**1**	**0**	17.36^{f} ±0.23	2.33^{bcd} ±0.62	18.80^{h} ±0.99
174	**1**	**0.1**	18.80^{ef} ±0.36	3.02^{abcd} ±0.75	22.88^{ef} ±0.14
261	**0**	**0**	21.66^{d} ±0.43	2.54^{abcd} ±0.61	24.19^{de} ±0.39
261	**0**	**0.1**	29.30^{c} ±0.38	3.49^{abcd} ±0.92	32.38^{c} ±0.36
261	**1**	**0**	32.80^{b} ±0.65	4.24^{abc} ±0.05	39^{b} ±0.83
261	**1**	**0.1**	42.16^{a} ±0.22	4.44^{a} ±0.05	42.47^{a} ±0.24
348	**0**	**0**	7^{h} ±0.17	2.02^{d} ±0.22	11.61^{j} ±0.13
348	**0**	**0.1**	11.88^{g} ±0.12	2.11^{d} ±0.56	11.72^{j} ±0.28
348	**1**	**0**	20.97^{de} ±0.34	2.18^{d} ±0.60	20.30^{g} ±0.22
348	**1**	**0.1**	23.52^{d} ±0.10	2.40^{bcd} ±0.79	21.83^{f} ±0.31

*Data were analyzed by one-way ANOVA using t test and means were compared by Ducan's at $P<0.05$.

(32.52 ±0.87), optimum shoot length (4.36±0.05) and optimum number of leaves (33.16±0.99) were recorded in MS medium with 87 mM Sucrose, 1μM GA_3 and 0.1μM BAP after 30 days from inoculation. It was seen that there were no significant changes in shoots or leaves after 40-50 days from inoculation. The data were recorded after regular interval of 10 days.

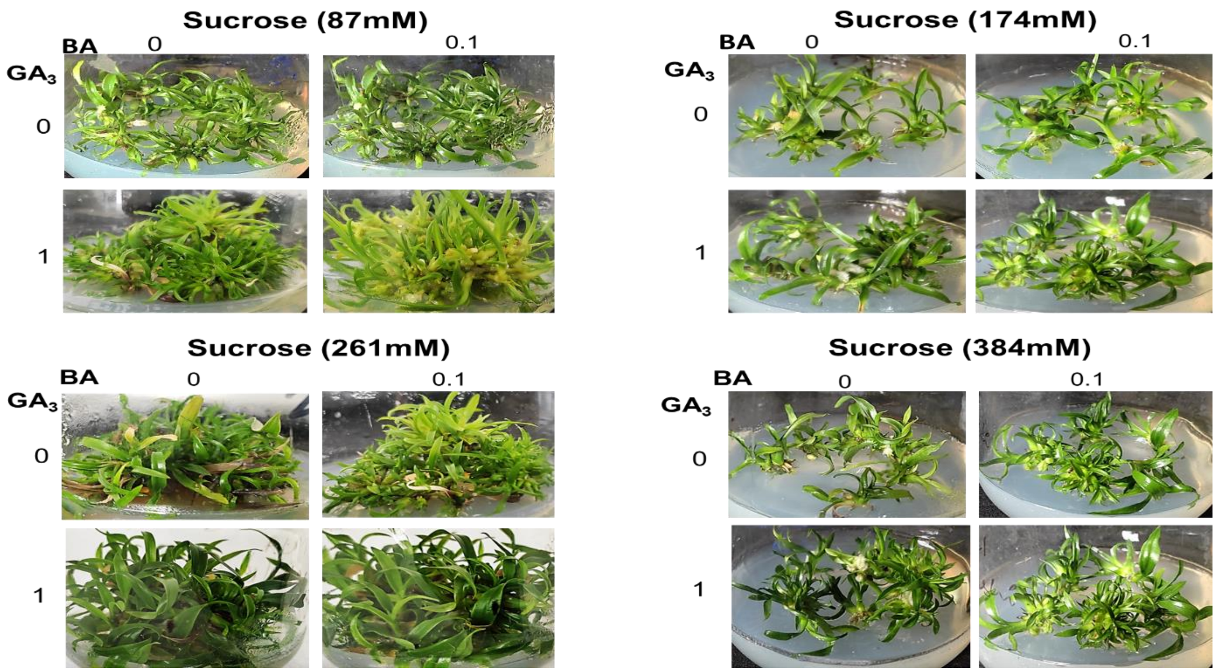

Figure 5.5*:* The effect of different concentrations of sucrose, BA and GA_3 on MS medium on germination of PLBs of *Dendrobium nobile*.

C. flaccida: The effect of different concentrations of sucrose, GA_3 and BAP was studied on the matured PLBs in which highest number of shoots and leaves were seen at 261 mM concentration of Sucrose with 1μM GA_3 and 0.1 μM BAP while the optimum results were recorded at 87 mM concentration of Sucrose after 30-40 days from inoculation. The lowest number of shoots and leaves were observed at 348mM concentration of sucrose followed by 174mM concentration. The highest number of shoots per PLB (35.16±0.30) and leaves (51.22±0.51) was recorded in MS medium with 261 mM Sucrose, 1 μM GA_3 and 0.1 μM BAP after 35 days from inoculation while the lowest number of shoots per PLB (8.83±0.44) and leaves (9.22±0.46) were recorded in MS medium with 348 mM Sucrose only i.e. with no BA and GA_3. The highest average shoot length (5.41±0.88) was recorded at 261 mM concentration of sucrose with 1 μM GA_3 and 0.1 μM BAP while the lowest average shoot length (1.39±0.67) was recorded at 348 mM concentration of sucrose with no BAP and GA_3. The optimum number of shoot per PLB (32.22±0.34), optimum shoot length (4.70±0.82) and optimum number of leaves (43.05±0.22) were recorded in MS medium with 87 mM Sucrose, 1 μM GA_3 and 0.1 μM BAP after 30 days from inoculation. It was observed that there were no significant changes in shoots or leaves after 40-50 days from inoculation. The data was recorded after regular interval of 10 days.

Table 5.11: Effect of sucrose, BAP and GA_3 on number of shoots per PLB, Average shoot length and number of leaves per PLB of *Coelogyne flaccida*

Sucrose	GA_3	BAP	No. of shoots per PLB	Average Shoot Length (cm)	No. of leaves per PLB
87	**0**	**0**	11.94^{f} ±0.34	2.26^{defg} ±0.03	15.55^{i} ±0.21
87	**0**	**0.1**	23.75^{d} ±0.37	3.70^{abcde} ±0.51	36.5^{e} ±0.12
87	**1**	**0**	31.16^{b} ±0.04	4.08^{abcd} ±0.07	38.30^{d} ±0.10
87	**1**	**0.1**	32.22^{b} ±0.34	4.70^{ab} ±0.82	43.05^{c} ±0.22
174	**0**	**0**	12.11^{f} ±0.18	2.01^{efg} ±0.54	12.80^{j} ±0.19
174	**0**	**0.1**	18.30^{e} ±0.20	2.78^{cdefg} ±0.04	25.27^{g} ±0.37
174	**1**	**0**	21.69^{d} ±0.10	3.39^{bcdef} ±0.50	34.77^{f} ±0.45
174	**1**	**0.1**	27.69^{c} ±0.14	3.55^{abcdef} ±0.71	38.5^{d} ±0.21
261	**0**	**0**	22.33^{d} ±0.12	3.34^{bcdef} ±0.40	36.91^{e} ±0.18
261	**0**	**0.1**	30.80^{b} ±0.12	4.32^{abc} ±0.83	43.22^{c} ±0.21
261	**1**	**0**	31.91^{b} ±0.55	4.93^{ab} ±0.93	45.05^{b} ±0.90
261	**1**	**0.1**	35.16^{a} ±0.30	5.41^{a} ±0.88	51.22^{a} ±0.51
348	**0**	**0**	8.83^{g} ±0.44	1.39^{g} ±0.67	9.22^{k} ±0.46
348	**0**	**0.1**	12.77^{f} ±0.22	1.72^{fg} ±0.04	13.44^{j} ±0.25
348	**1**	**0**	17.08^{e} ±0.26	2.23^{defg} ±0.68	16.83^{h} ±0.24
348	**1**	**0.1**	21.41^{d} ±0.17	2.69^{cdefg} ±0.48	26.11^{g} ±0.19

*Data were analyzed by one-way ANOVA using t test and means were compared by Ducan's at $P<0.05$.

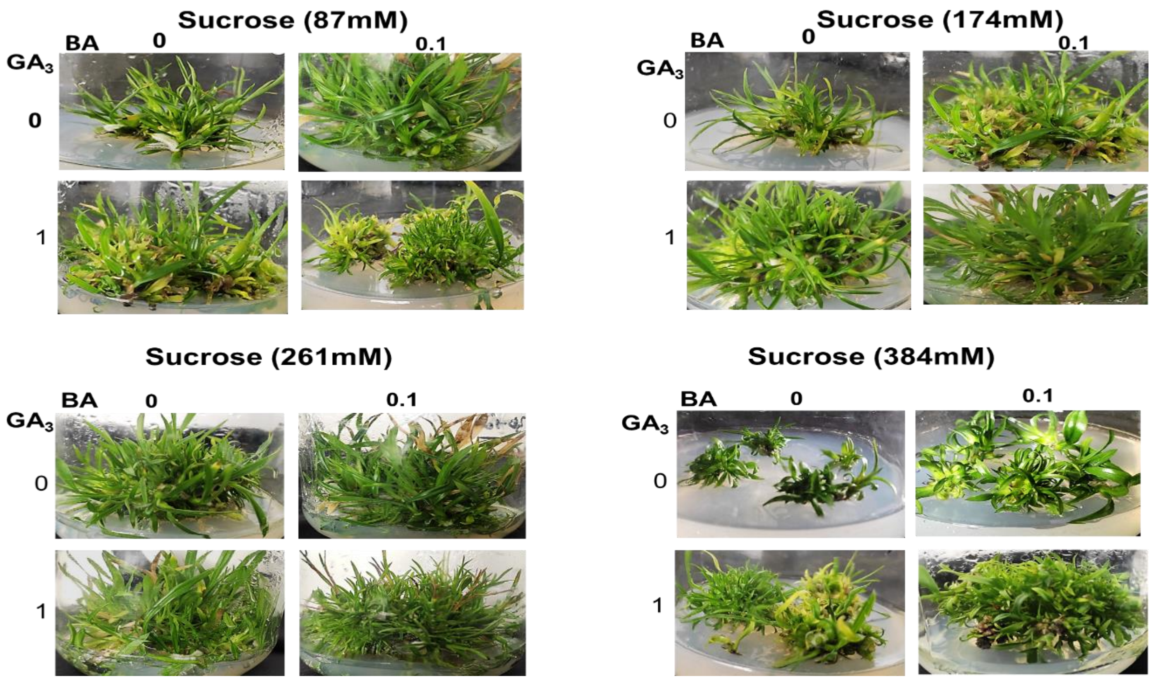

Figure 5.6: The effect of different concentrations of sucrose, BAP and GA_3 on MS medium on germination of PLBs of *Coelogyne flaccida*.

In case of ***R. retusa***: The effect of different concentrations of sucrose, GA_3 and BAP was studied on the matured PLBs in which highest number of shoots and leaves were seen at 261 mM concentration of Sucrose with 1 μM GA_3 and 0.1 μM BAP while the optimum results were recorded at 87 mM concentration of Sucrose after 30-40 days from inoculation. The lowest number of shoots and leaves were observed at 348 mM concentration of sucrose followed by 174 mM concentration. The highest number of shoots per PLB (18.97 ±0.48) and leaves (19.11 ±0.49) was recorded in MS medium with 261 mM Sucrose, 1 μM GA_3 and 0.1 μM BAP after 35 days from inoculation while the lowest number of shoots per PLB (5.08 ±0.37) and leaves (6.44 ±0.38) were recorded in MS medium with 348 mM Sucrose only i.e. with no BAP and GA_3. The highest average shoot length (3.25 ±0.62) was recorded at 261 mM concentration of Sucrose with 1 μM GA_3 and 0.1 μM BAP while the lowest average shoot length (1.03 ±0.54) was recorded at 348 mM concentration of sucrose with no BAP and GA_3. The optimum number of shoot per PLB (17.11 ±0.45), optimum shoot length (2.80 ±0.61) and optimum number of leaves (16.47 ±0.41) were recorded in MS medium with 87 mM Sucrose, 1μM GA_3 and 0.1 μM BA after 30 days from inoculation. It was seen that there were no significant changes in shoots or leaves after 40-50 days from inoculation. The data were recorded after regular interval of 10 days.

Table 5.12: Effect of sucrose, BAP and GA_3 on number of shoots per PLB, Average shoot length and number of leaves per PLB after conversion of PLBs of *Rhyncostylis retusa*

Sucrose	GA_3	BAP	No. of shoots per PLB	Average Shoot Length (cm)	No. of leaves per PLB
87	0	0	11.47^{h} ±0.11	1.01^{b} ±0.01	12.5^{gh} ±0.15
87	0	0.1	13.88^{g} ±0.16	2.22^{ab} ±0.63	15.19^{defg} ±0.18
87	1	0	14.94^{ef} ±0.26	2.52^{ab} ±0.67	14.74^{defg} ±0.29
87	1	0.1	17.11^{c} ±0.45	2.80^{ab} ±0.61	16.47^{abcde} ±0.41
174	0	0	12.41^{h} ±0.28	1.35^{ab} ±0.61	13.05^{fgh} ±0.32
174	0	0.1	13.63^{g} ±0.44	1.98^{ab} ±0.80	14.19^{efgh} ±0.49
174	1	0	14.22^{fg} ±0.47	2.14^{ab} ±0.56	15.13^{defg} ±0.52
174	1	0.1	18.41^{ab} ±0.20	2.5^{ab} ±0.73	19.5^{a} ±0.24
261	0	0	15.61^{de} ±0.16	1.11^{b} ±0.90	16.25^{bcde} ±0.16
261	0	0.1	16.02^{d} ±0.18	1.41^{ab} ±0.78	17^{abcde} ±0.19
261	1	0	17.69^{bc} ±0.36	2.36^{ab} ±0.58	18.61^{abc} ±0.37
261	1	0.1	18.97^{a} ±0.48	3.25^{a} ±0.62	19.11^{a} ±0.49
348	0	0	5.08^{i} ± 0.37	1.03^{b} ±0.54	6.44^{i} ± 0.38
348	0	0.1	12.36^{h} ±0.40	1.85^{ab} ±0.59	11.61^{h} ±0.42
348	1	0	13.94^{fg} ±0.27	1.55^{ab} ±0.60	15.63^{cdef} ±0.31
348	1	0.1	15.88^{de} ±0.05	1.35^{ab} ±0.03	17.5^{abcd} ±0.08

*Data were analyzed by one-way ANOVA using t test and means were compared by Ducan's at $P<0.05$.

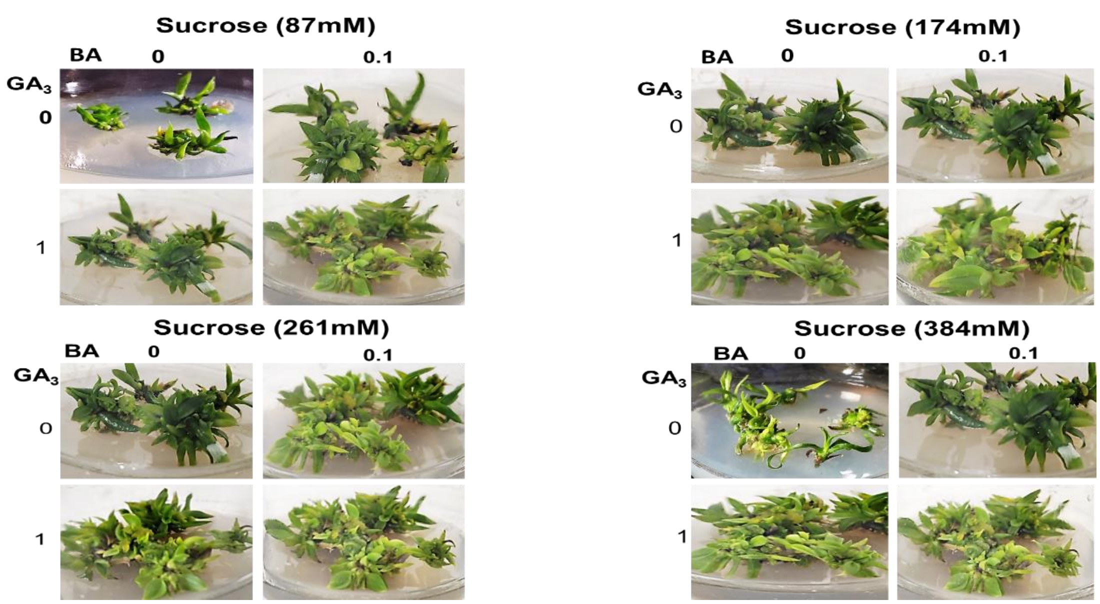

Figure 5.7: The effect of different concentrations of sucrose, BAP and GA_3 on MS medium on germination of PLBs of *Rhyncostylis retusa*.

Preparation and encapsulation of Synthetic seeds

The effect of different concentrations of Sodium Alginate (1%, 2%, 3% and 4%) and Calcium Chloride (50, 100 and 200mM) was studied on synthetic seed formation of *D. nobile, C. flaccida* and *R. retusa* for encapsulation and preparation of synthetic seeds. The matured PLBs were mixed in Sodium alginate and then dropped in chilled calcium chloride solution. After 15-20 minutes the extra calcium chloride solution was decanted off and the beads were stored in autoclaved bottles. The reactions between the bonds of sodium alginate and calcium chloride determines the forming of artificial beads. The best results of bead formation was recorded at 3% sodium alginate and 100mM calcium chloride as the beads were firm with proper shape and size and were considered best for the germination purposes. The artificial beads formed below 3% sodium alginate were not suitable for any purposes as they were totally disoriented in shape with no proper size and were not able to handle any temperature. Their gelling ability was very low. As the percentage of sodium alginate was increased above 3%, the beads became very rigid and opaque which were not considered suitable for germination purposes. The detailed observations of artificial seeds at each concentration are shown below in table:

The effect of sodium alginate and calcium chloride concentration on the morphology and texture of PLBs

		Calcium chloride		
		50mM	100mM	200mM
Sodium alginate	1%	Soft, no definite shape and size, transparent, fragile	Soft, no shape and size, transparent, fragile, no gelling ability	No proper shape and size, Beads not formed, transparent, fragile and soft.
	2%	Soft, very less gelling ability, no shape, Intolerance to temperatures	Little gelling ability but soft in nature, no shape, fragile	No proper shape and size, little transparent, fragile and soft while handling
	3%	Firm beads with proper shape and size, proper gelling ability.	Firm beads, spherical in shape with pointed tail, proper size, tolerate different temperatures, proper gelling ability	Little rigid beads, isodiametric in shape, proper gelling ability
	4%	Rigid beads, opaque, hard, little viscous	Rigid dark beads, opaque, spherical in shape with tail, hard for germination purposes.	Dark opaque beads, more viscous, rigid, not suitable for any process, spherical in shape.

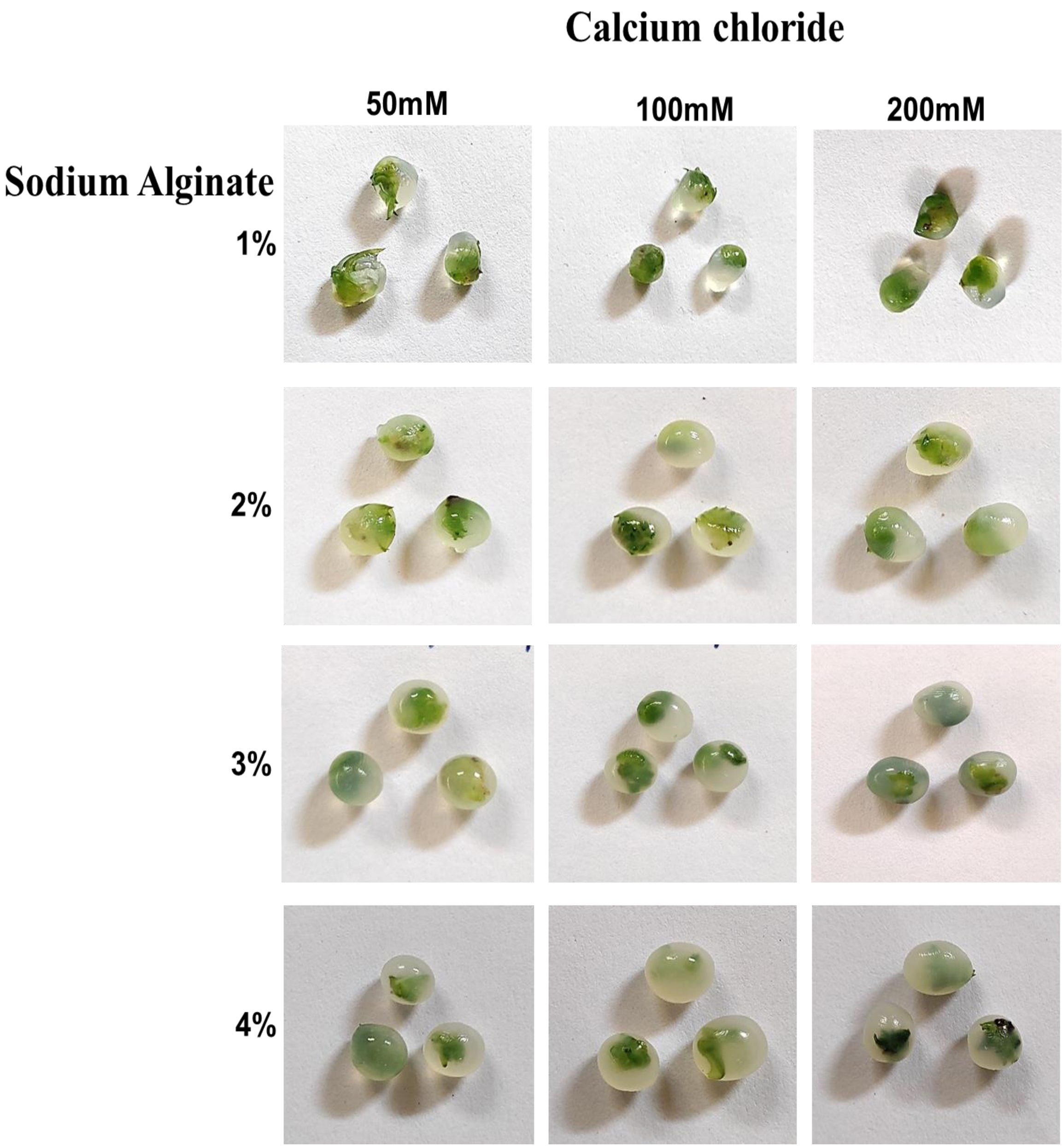

Figure 5.8*:* Effect of sodium alginate and calcium chloride on artificial seed synthesis

Storage and Germination of PLBs

The effect of storage on artificial seed was studied at two different temperatures (4ºC and 25ºC) at different time intervals (0, 10, 20, 30, 40, 50 days). After storage the seeds were inoculated on MS medium to observe the viability and germination percentage and results were recorded after 2 months. In case of ***D. nobile***, the highest germination percentage (87.44%) of artificial seeds was observed at 0-day storage period (seeds that were directly inoculated on MS medium after bead formation) followed by storage period of 10^{th} day at 25 ℃ temperature (Table 5.13). As compared to 4 ºC the germination was better at 25 ℃ temperature. It was noted that the germination percentage decreases with increase in incubation days. The lowest germination percentage (12.55%) of the artificial seeds was observed in seeds with storage period of 60^{th} day at temperature 25 ºC, whereas at 4 ºC, the germination was seen till 40^{th} day of storage period (4.32%). No germination was observed for the seeds stored at 4 ºC with storage period of 50^{th} and 60^{th} days.

Table 5.13: Effect of storage on germination percentage of artificial seeds of *Dendrobium nobile* in MS medium. Seeds were stored at 4ºC and 25ºC in dark for different intervals of time (10, 20, 30, 40, 50, 60 days).

Temperature	Days	Germination %	Temperature	Days	Germination %
Control	0	$87.44^{a} \pm 4.37$	**Control**	0	$87.44^{a} \pm 4.37$
4 ºC	10	$25.55^{b} \pm 1.27$	**25 ºC**	10	$78.66^{b} \pm 3.93$
4 ºC	20	$16.66^{c} \pm 0.83$	**25 ºC**	20	$67.66^{c} \pm 3.38$
4 ºC	30	$8.88^{d} \pm 0.41$	**25 ºC**	30	$57.77^{d} \pm 2.88$
4 ºC	40	$4.32^{de} \pm 0.21$	**25 ºC**	40	$35.77^{e} \pm 1.78$
4 ºC	50	$0.00^{e} \pm 0.00$	**25 ºC**	50	$20.34^{f} \pm 1.01$
4 ºC	60	$0.00^{e} \pm 0.00$	25 ºC	60	$12.55^{g} \pm 0.62$

*Data was analyzed by one-way ANOVA using t test and means were compared by Ducan's at $P<0.05$.

In case of ***C. flaccida***, the highest germination percentage (89.33%) of artificial seeds was observed at 0-day storage period (seeds that were directly inoculated on MS medium after bead formation) followed by storage period of 10^{th} day at 4 ℃ temperature (Table 5.14). As compared to 25 ºC the germination was better at 4 ℃ temperature. It was noted that the germination percentage decreases with increase in incubation days. The lowest germination percentage (13.33%) of the artificial seeds was observed in seeds with storage period of 60th day at temperature 4 ºC, whereas at 25 ºC, the germination was seen till 40th day of storage period (6.40%). No germination was observed for the seeds stored at 25 ºC with storage period of 50th and 60th days.

Table 5.14: Effect of storage on germination percentage of artificial seeds of *Coelogyne flaccida* in MS medium. Seeds were stored at 4ºC and 25ºC in dark for different intervals of time (10, 20, 30, 40, 50, 60 days).

Temperature	Days	Germination %	Temperature	Days	Germination %
4ºC	0	$89.33^{a} \pm 4.46$	**25ºC**	0	$89.33^{a} \pm 4.46$
4ºC	10	$73.33^{b} \pm 3.66$	**25ºC**	10	$38.88^{b} \pm 1.94$
4ºC	20	$64.33^{c} \pm 3.21$	**25ºC**	20	$22.22^{c} \pm 1.11$
4ºC	30	$55.33^{d} \pm 2.76$	**25ºC**	30	$10.66^{d} \pm 0.53$
4ºC	40	$32.33^{e} \pm 1.61$	**25ºC**	40	$6.40^{d} \pm 0.32$
4ºC	50	$24.44^{f} \pm 1.22$	**25ºC**	50	$0^{e} \pm 0$
4ºC	60	$13.33^{g} \pm 0.66$	**25ºC**	60	$0^{e} \pm 0$

*Data was analyzed by one-way ANOVA using t test and means were compared by Ducan's at $P<0.05$

In case of ***R. retusa***, the highest germination percentage (85.44%) of artificial seeds was observed at 0-day storage period (seeds that were directly inoculated on MS medium after bead formation) followed by storage period of 10th day at 4 ℃ temperature (Table 5.15). As compared to 25 ºC the germination was better at 4 ℃ temperature. It was noted that the germination percentage decreases with increase in incubation days. The lowest germination percentage (7.77%) of the artificial seeds was observed in seeds with storage period of 60th day at temperature 4 ºC, whereas at 25 ºC, the germination was seen till 40th day of storage period (5.55%). No germination was observed for the seeds stored at 25 ºC with storage period of 50th and 60th days.

Table 5.15: Effect of storage on germination percentage of artificial seeds of *Rhyncostylis retusa* in MS medium. Seeds were stored at 4ºC and 25ºC in dark for different intervals of time (10, 20, 30, 40, 50, 60 days).

Temperature	Days	Germination %	Temperature	Days	Germination %
4°C	0	85.44[a] ± 4.27	**25°C**	0	85.44[a] ± 4.27
4°C	10	77.77[b] ± 3.88	**25°C**	10	32.22[b] ± 1.61
4°C	20	68.88[c] ± 3.44	**25°C**	20	22.22[c] ± 1.11
4°C	30	41.11[d] ± 2.05	**25°C**	30	15.55[d] ± 0.77
4°C	40	31.11[e] ± 1.55	**25°C**	40	5.55[e] ± 0.27
4°C	50	27.77[f] ± 1.38	**25°C**	50	0[e] ±0
4°C	60	7.77[g] ± 0.38	**25°C**	60	0[e] ±0

*Data was analyzed by one-way ANOVA using t test and means were compared by Ducan's at P<0.05

Figure 5.9: Germination of shoots and leaves from the PLBs encapsulated in the gelling and complexing agent (sodium alginate and calcium chloride).

Clonal Fidelity

A total of 26 RAPD primers were tested for *Dendrobium nobile*, *Coelogyne flaccida* and *Rhyncostylis retusa*. Out of 26 primers, primers producing clear, intact and scorable bands were selected for analysis (Table 5.16).

Table 5.16: Screening of 30 RAPD primers for amplifying gDNA in *Dendrobium nobile, Coelogyne flaccida and Rhinchostylis retusa* mother plant.

S.No.	RAPD Primer	Primer Sequence	Result of DNA Amplification		
			Dendrobium nobile	***Coelogyne flaccida***	***Rhyncostylis retusa***
1	RAPD 1	AGCGCCATTG	Absent	Absent	Clear bands
2	RAPD 2	CTTCCCCAAG	Unclear bands	Absent	Absent
3	RAPD 3	AGGGCGTAAG	Absent	Absent	Absent
4	RAPD 4	CTGGGGGACT	Smear	Absent	Unclear bands
5	RAPD 5	ACCGCGAAGG	Smear	Absent	Absent
6	RAPD 6	GGACCCAACC	Clear bands	Absent	Clear bands
7	RAPD 7	GTCGCCGTCA	Clear bands	Absent	Unclear bands
8	RAPD 8	TCTGGTGAGG	Clear bands	Smear	Clear bands
9	RAPD 9	TGAGCGGACA	Absent	Smear	Unclear bands
10	RAPD 10	ACCTGAACGG	Smear	Smear	Clear bands
11	RAPD 11	TTGCACGGG	Unclear bands	Unclear bands	Clear bands
12	RAPD 12	GTGTGCCCCA	Smear	Clear bands	Clear bands
13	RAPD 13	CTCTGGAGAC	Absent	Unclear bands	Absent
14	RAPD 15	GGGGTGACGA	Absent	Smear	Absent
15	RAPD 18	GAGAGCCAAC	Absent	Unclear bands	Absent
16	RAPD 20	ACCCGGTCAC	Absent	Smear	Unclear bands
17	RAPD 21	CAGGCCCTTC	Clear bands	Clear bands	Smear
18	RAPD 22	TGCCGAGCTG	Unclear bands	Clear bands	Unclear bands
19	RAPD 23	AGTCAGCCAC	Clear bands	Absent	Clear bands
20	RAPD 24	AATCGGGCTG	Clear bands	Absent	Clear bands
21	RAPD 25	AGGGGTCTTG	Absent	Smear	Smear
22	RAPD 26	GGTCCCTGAC	Absent	Clear bands	Unclear bands
23	RAPD 27	GAAACGGGTG	Smear	Clear bands	Smear
24	RAPD 28	GTGACGTAGG	Unclear bands	Absent	Clear bands
25	RAPD 29	GGGTAACGCC	Clear bands	Clear bands	Clear bands
26	RAPD 30	GTGATCGCAG	Absent	Unclear bands	Absent

For RAPD analysis of ***D. nobile***, 4 RAPD primers (RAPD primer 6, 7, 23 and 24) were selected. The total number of 12 bands were scored from selected RAPD primers. Size of the bands ranges from 500-5000bp. RAPD primer 6 showed 3 bands, RAPD primer 7 showed 2 bands, RAPD primer 23 showed 5 bands and RAPD primer 24 showed 2 bands (Table 5.17). All the primers showed 100% monomorphic percentage which means the *in-vitro* germinated seeds from the PLBs of *D. nobile* showed clonal fidelity with their mother plants.

Table 5.17: RAPD primer name, total scorable band, monomorphism%, and size of the amplified fragments generated in the mother plant and in the in vitro regenerated plants of *Dendrobium nobile*.

SNo.	Primer	Total number of bands	Monomorphism %	Size (base pair)
1	RAPD 6	3	100%	500-750bp
2	RAPD 7	2	100%	1500-5000bp
3	RAPD 23	5	100%	750-4000bp
4	RAPD 24	2	100%	1000-2000bp

For RAPD analysis of ***C. flaccida*****:** 4 RAPD primers (RAPD primer 12, 22, 27 and 29) were selected. The total number of 13 bands were scored from selected RAPD primers. Size of the bands ranges from 250-3000bp. RAPD primer 12 showed 2 bands, RAPD primer 22 showed 3 bands, RAPD primer 27 showed 6 bands and RAPD primer 29 showed 2 bands (Table 5.18). All the primers showed 100% monomorphic percentage which means the *in-vitro* germinated seeds from the PLBs of *Coelogyne flaccida* showed clonal fidelity with their mother plants.

Table 5.18: RAPD primer name, total scorable band, monomorphism%, and size of the amplified fragments generated in the mother plant and in the in vitro regenerated plants of *Coelogyne flaccida*.

SNo.	Primer	Total number of bands	Monomorphism %	Size (base pair)
1	RAPD 12	2	100%	1500-3000bp
2	RAPD 22	3	100%	1500-3000bp
3	RAPD 27	6	100%	500-1500bp
4	RAPD 29	2	100%	250-750bp

For RAPD analysis of ***R. retusa:*** 4 RAPD primers (RAPD primer 8, 10, 24 and 27) were selected. The total number of 11 bands were scored from selected RAPD primers. Size of the bands ranges from 500-3000bp. RAPD primer 8 showed 3 bands, RAPD primer 10 showed 4 bands, RAPD primer 24 showed 2 bands and RAPD primer 29 showed 2 bands (Table 5.19). All the primers showed 100% monomorphic percentage which means the *in-vitro* propagated plants from the PLBs of *Rhyncostylis retusa* showed clonal fidelity with their mother plants.

Table 5.19: RAPD primer name, total scorable band, monomorphism% and size of the amplified fragments generated in the mother plant and in the in vitro regenerated plants of *Rhyncostylis retusa*.

SNo.	Primer	Total number of bands	Monomorphism %	Size (base pair)
1	RAPD 8	3	100%	500-1500bp
2	RAPD 10	4	100%	500-1500bp
3	RAPD 24	2	100%	750-3000bp
4	RAPD 29	2	100%	1000-2000bp

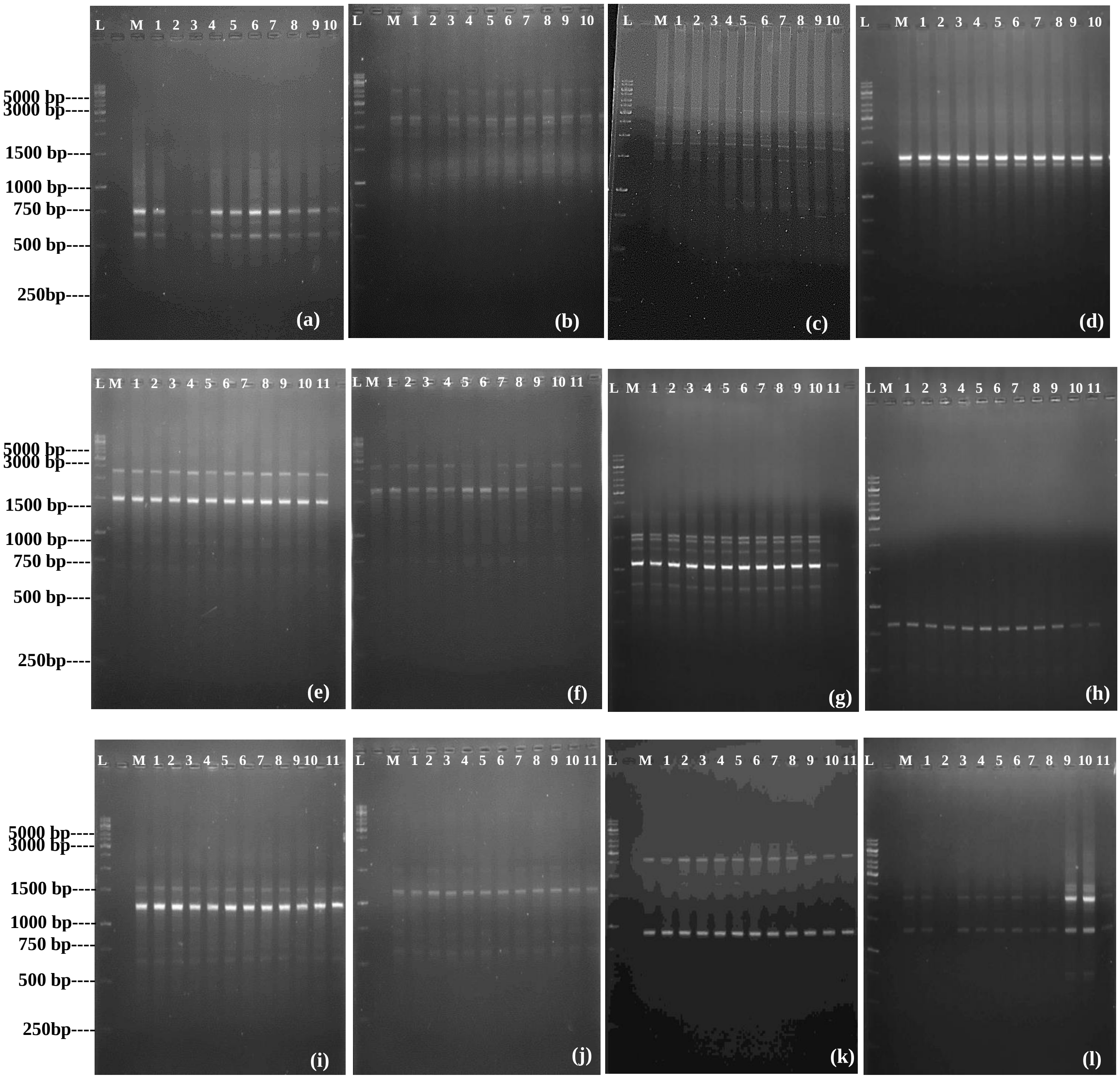

Figure 5.10: Clonal fidelity of *Dendrobium nobile* (**a-d)** *Coelogyne flaccida* **(e-h)** and *Rhyncostylis retusa* **(i-l)** using RAPD primers**. a-d =** banding pattern of RAPD primer 6, 7, 23, 24. **e-h =** banding pattern of RAPD primer 12, 22, 27, 29. **i-l =** banding pattern of RAPD primer 8, 10, 24, 29. **L=** 1kb DNA ladder, **M =** mother plant, **1-11 =** *in-vitro* propagated cultures.

CHAPTER: 5

DISCUSSION

For a healthy environment as well as healthy biodiversity, it is necessary for each species and organisms to survive (Kaur and Bhutani 2013). Orchids require favorable congenial habitats in order to grow under normal cinditions. They effectively serve as a reliable marker for a healthy biodiversity. Due to several factors like over-exploitation, destruction of their natural habitat, deforestation, over-collection, increased pollution or population and illegal harvesting (Hinsley et al. 2018), the orchids are on their verge of extinction while some are listed among the endangered species (IUCN 2020). To save the orchids from their complete extinction, scientists and researchers are now focusing on the *in-vitro* techniques which not only helped in saving the orchids but also increased their population. The effect of BAP and IAA had earlier been reported in many orchids like *Coelogyne nervosa* (Abraham et al. 2012), *Laelia anceps* (Ramirez-Mosqueda et al. 2018) and *Oncidium* spp. (Kalimuthu et al. 2007). MS medium is considered as the best medium for the *in-vitro* micropropagation of orchids (Reddy 2008; Arya et al. 1999). Addition of plant growth regulators like BAP to the medium especially MS medium gave the best results for the germination of seeds (Abraham et al. 2012).

The present study focused on the *in-vitro* conservation of some endangered Himalayan orchids like *Dendrobium nobile* Lindl., *Coelogyne flaccida* Lindl. and *Rhyncostylis retusa* (L.). The effect of BAP and IAA was studied on the shoot multiplication of *D. nobile, C. flaccida* and *R. retusa* (Table 5.1, 5.2 and 5.3). BAP is used as cytokinin in the experiments which helped in influencing the plant development and stimulating the cell division, while IAA is used as auxin which regulated plant development an elongation. The use of different concentrations and combinations of BAP, IAA and IBA had earlier been reported for rooting and plantlet development in *Dendrobium* orchids under *in-vitro* conditions (Khatun et al. 2010). In the present study highest shoot multiplication was recorded on MS medium supplemented with 2.5 μM BAP and 5 μM IAA. Similar results were seen in *Orchis catasetum* but with the combination of BAP and NAA (Baker et al. 2014). It was also noted that with the increase in concentration of IAA the number of shoots and leaves also increased while with the increase in concentration of BAP the average length of the shoots decreased. As the time increased, the leaves and shoots of these orchids started becoming brown in color leading to the death of these orchids. From 20-30 days, the leaves were green in color and the orchids were healthy. No significant changes were observed between 20-35 days. The importance of MS medium supplemented with BAP on shoot multiplication in *Vanilla planifolia* has also been studied by Kalimuthu et al. (2006).

For the rapid *in-vitro* germination of orchids, PLBs can be safely used as explants (Baker et al. 2014). As we know the seeds of the orchids lack endosperm so they need proper nutritional conditions (Arditti 1967). In the present study, the effect of different concentrations of BAP was studied on the formation of PLBs of *D. nobile*, *C. flaccida* and *R. retusa*. It was observed that 2.5 μM BAP was suitable for the formation of PLBs. Similar results were obtained by Naing (2011) for the formation of PLBs in *C. cristata.* Orchids reproduce through PLBs (protocorm like bodies) which are considered similar to somatic embryos (da Silva et al. 2006). It has already been studied that plant growth regulators play a vital role in the germination and multiplication of orchids as well as PLBs (Naing 2011). When the concentration of BAP was increased, no PLBs were formed as the explant turned black. It was noted that as the time increases the PLBs started turning brown and white resulting in their death. In contrary to this, Ng et al. (2010) studied the effect of BA, kinetin and coconut water for the formation of PLBs in *Paphiopedilum* and found that addition of coconut water in half strength MS medium gave the best results.

Sorbitol is used as a carbon source for the maturation of PLBs of orchids (Udomdee et al. 2015). In the present study, the effect of different concentrations of sorbitol was studied on the maturation of PLBs (Table 5.7, 5.8 5.9) and it was observed that 247 mM sorbitol concentration was optimum for the maturation. It was recorded earlier that sorbitol and glucose were much more effective than sucrose (Ruzic et al. 2008). For the conservation of *Epidendrum chlorocorymbos* Schltr, a slow protocol was developed to study the effect of mannitol and sorbitol supplemented in MS medium (Lopez-Puc 2013). Half strength MS medium supplemented with 1% sorbitol was declared as the best treatment for PLB formation. Among the different carbon sources like sucrose, mannitol and sorbitol, sorbitol was proved to be the best carbon source for the PLB development and plantlet development (Islam et al. 1998).

Plant growth regulators play a very critical role in the *in-vitro* propagation of orchids for their increase in shoot numbers, shoot length and rooting. The effect of BAP, Kinetin and IBA was studied for the *in-vitro* propagation of *Dendrobium nobile* (Asghar et al. 2011). It was seen that by addition of 2 mg/l BAP to the medium resulted in the high shoot number, while the addition of 1.5

mg/l Kinetin to the medium resulted in the highest shoot length. In the present study, the effect of different concentrations and combinations of plant growth regulators like BAP and GA_3 with respect to different concentrations of sucrose was studied on the germination of PLBs of *D. nobile*, *C. flacidda* and *R.* retusa (Table 5.10, 5.11 and 5.12). The highest germination was observed at 261 mM sucrose concentration with 1 μM GA_3 and 0.1 μM BAP. Similar results were obtained in *in-vitro* germination of *Oncidium* spp. (Kalimuthu et al. 2007). It was also noted that by increasing the sucrose concentration without any plant growth regulators, very few PLBs were able to germinate. As the time increased, the PLBs started turning brown in color with no plantlet formation resulting in their death. At 87 mM concentration of sucrose, optimum plantlet conversion was seen and the orchids were light green in color. The importance of MS medium supplemented with BAP on shoot multiplication in *Vanilla planifolia* was studied by Kalimuthu et al. 2006

Synthetic seeds also known as the encapsulating containing vegetative propagule, has been widely used to preserve germplasm (Towill 1998). Previously seed banks have been used to preserve the live plants but due to its limitation of high cost and time consuming procedure, its use had become limited. As a result, maintaining live plants encapsulated in the gelling matrix as artificial seeds will aid in the retention of plants for long period of time in particular space under appropriate conditions. This particular method is very beneficial for the species lacking proper conservation measures for example *Vittis* spp. is conserved by this technique (Towill 1998). Datta et al. (1999) reported the development of the protocol for synthesis of artificial seeds by encapsulating PLBs of *Geodorum densiflorum.* In the present study, the effect of different concentrations of sodium alginate and calcium chloride was studied on the formation of artificial seeds of *D. nobile, C. flacidda* and *R. retusa.* It was recorded that the best artificial seeds were produced at 3% sodium alginate and 100 mM calcium chloride. Lower concentrations of sodium alginate resulted in fragile, disoriented and transparent seeds because of low viscosity while concentration more than 3% resulted in hard, opaque and rigid seeds which were not considered suitable for storage or germination experiments because it might delay the initiation process for germination. The study of Mohanty and Das (2013) has also reported the formation of synthetic seeds of *Dendrobium densiflorum* using different sodium alginate concentrations (2, 3, 4 and 5%) and calcium chloride (50, 100 and 150mM).

Storage of artificial seeds is also done at various temperatures like 4 °C or 25 °C to check the viability of artificial seeds. Researchers can also compare the difference in germination of artificial seeds after storing at different temperatures for different time intervals. Artificial seeds of *Vanda coerulea* were stored at 4°C for 120 days (Devi et al. 2000). Gantait et al. (2012) conducted a storage experiment in which encapsulated seeds were stored in dark at 4 °C and 25 °C for different intervals of 30, 60, 90, 120, 150 and 180 days. In comparison with above study, the present study was also focused to conduct a storage experiment in which the artificial seeds were stored at 4 °C and 25 °C in dark for 0, 10, 20, 30, 40, 50 and 60 days to check the viability of seeds. For *D. nobile*, best results were recorded at 25 °C i.e. the seeds stored at 25 °C germinated more that the seeds stored at 4 °C (Table 5.13). For *C. flaccida* and *R. retusa*, the seeds stored at 4 °C germinated more than the seeds stored at 25 °C(Table 5.14 and Table 5.15) because *C. flaccida* and *R. retusa* are cool-growing orchids. Decline in regeneration rate was observed in artificial seeds stored for more than 40 days. This might be due to inhibition of respiration in PLBs because of sodium alginate covered around them.

Number of factors related with *in-vitro* culturing can cause clonal variations. Checking clonal fidelity of *in-vitro* grown plants is very important if true breeding plants are required. After the storage-germination experiments, genetic stability and similarity was checked between the *in-vitro* germinated cultures and mother plants of *D. nobile, C. flaccida* and *R. retusa.*. A total of 26 RAPD primers were tested for *D. nobile, C. flaccida* and *R. retusa*. Out of 26 primers, primers producing clear, intact and scorable bands were selected for analysis (Table 5.16). 100% monomorphism was recorded for each orchid which showed the genetic similarity between *in-vitro* germinated seeds and mother plants of *D. nobile, C. flaccida* and *R. retusa* (Table 5.17, 5.18 and 5.19). In contrary to it, Tikendra et al. (2019) also conducted an experiment to check the clonal fidelity of *Dendrobium chrysotoxum* Lindl. using 12 RAPD and 11 ISSR markers and reported 96% highest genetic similarity between *in-vitro* grown plants and mother plant.

CHAPTER: 6

CONCLUSION

CONCLUSION

Orchids, the indicators of a healthy ecosystem, belongs to the *Orchidaceae*, one of the largest family of flowering plants having more than 800 genera and 27800 species reported so far. In this research study, it was found that plant growth, sucrose and carbon sources play a vital role in the shoot-leaves multiplication of orchids, formation, multiplication, maturation and conversion of PLBs into plantlets respectively. It was also noticed that the storage temperature and time also affects the artificial seed germination in *Dendrobium nobile, Coelogyne flaccida* and *Rhyncostylis retusa.*

In present study can be concluded in following points

- Shoot multiplication of *Dendrobium nobile*, *Coelogyne flaccida* and *Rhyncostylis retusa* was observed best on MS medium supplemented with 2.5 µM BAP +5 µM IAA.
- The formation of PLBs from leaf explant were highest on MS medium +2.5 µM BAP
- The PLBs were successfully multiplied on MS medium +2.5 µM BAP.
- Best maturation was recorded on MS medium +247 mM sorbitol concentration.
- Maximum conversion of matured PLBs into plantlets was recorded on MS medium +1 µM GA3 +0.1 µM BA+261mM sucrose.
- The PLBs were successfully encapsulated in 3% sodium alginate and 100mM calcium chloride.
- Effect of storage of artificial seeds at 4 ^{o}C and 25 ^{o}C for different interval of time reveals that the seeds of *Dendrobium nobile* stored at 25 ^{o}C were viable for more time and also germinated well as compared to the seeds stored at 4 ^{o}C. On contrary, artificial seeds of *Coelogyne flaccida* and *Rhyncostylis retusa* stored at 4^{o}C were viable for more time and also germinated well as compared to the seeds stored at 25 ^{o}C.
- It was also observed germinated cultures of *Dendrobium nobile, Coelogyne flaccida* and *Rhyncostylis retusa* were genetically similar to their mother plants.

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ANNEXURE I

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

S.No	Composition	Amount (mg/l)
1.	**Macronutrients (Hi media)**	
	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2.2H_2O$	440
	$MgSO_4.7H_2O$	370
	KH2PO4	170
2.	**Micronutrients**	
	$MnSO_4.4H_20$	16.90
	$Fe_2EDTA.2H_2O$	27.80
	$ZnSO_4.7H_2O$	8.60
	H_3BO_4	6.20
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.25
	$CoCl_2.6H_20$	0.25
	$CuSO_4.5H_20$	0.025
3.	**Vitamins**	
	Myoinositol	100
	Glysine	2.0
	Nicotinic acid	0.5
	Pyridoxine HCl	0.5
	Thiamine HCl	0.1
4.	**Sugar**	**3%**
5.	**Agar**	**0.8% w/v**

ANNEXURE II

1. **6-benzyl amino purine (BAP): (**Stock of 2.5 mM BAP) 56 mg of BAP was dissolved in 500 µl HCl and the volume was made upto 100 ml using distilled water.
2. **Gibberellic Acid (GA_3): (**Stock of 2.5 mM GA_3) 86 mg of GA_3 was first dissolved in distilled water and then the total volume was made upto 100 ml using distilled water.
3. **Indole acetic acid (IAA):** (Stock of 2.5 mM IAA) 43.7 mg of IAA was dissolved in 500 µl KOH and the volume was made upto 100 ml using distilled water.
4. **CTAB buffer**

Ingredient	**Quantity**
CTAB	2.0% w/v
EDTA	20 mM
Tris-HCl (pH 8.0)	100 mM
NaCl	1.4 M
β-mercaptoethanol	0.2% w/v

20 g of CTAB was dissolved in 600 ml of warm distilled water followed by addition of 81.82 g of NaCl, 40 ml of 0.5 M EDTA (pH 8.0) and 100 ml of 1 M Tris HCl (pH 8.0). The total volume was made upto 1 L. 2 ml of β-mercaptoethanol was added before use.

5. **50X TAE Buffer:** Working (0.5x)

Ingredient	**Composition**
Tris base	24.2 g
Glacial acetic acid	5.71 ml
Disodium EDTA	3.72 g

24.2 g of Tris base was first dissolved in 5.71 ml of glacial acetic acid followed by addition of 3.72 g of disodium EDTA (pH was maintained at 8.0) and the volume was made up to 100 ml using distilled water. For working, 10 ml of TAE was dissolved in 1 L of distilled water.

6. **TE buffer:** 10 ml of 1 M Tris HCl (pH adjusted to 7.4) and 2 ml of 0.5 M EDTA (pH adjusted to 8.0) was mixed and the total volume was made upto 1 L using distilled water.

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