IN VITRO REGENERATION OF ORCHID (*Dendrobium* sp.)

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CERTIFICATE

This is to certify that thesis entitled, ***"IN VITRO REGENERATION OF ORCHID (Dendrobium sp.)"*** *submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE*** *in* ***Biotechnology,*** *embodies the result of a piece of Bonafede research work carried out by* ***RAZIA SULTANA,*** *Registration* ***No.13-05542*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

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DEDICATED TO
MY
BELOVED PARENTS

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SAU, Dhaka **The Author**

IN VITRO REGENERATION OF ORCHID (*Dendrobium* sp.)[1]

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ABSTRACT

The experiment was conducted at the Biotechnology Laboratory of Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka from January 2021 to June 2021 to investigate the effect of different plant growth hormone for shoot proliferation and root regeneration of *Dendrobium* sp. where shoot tips were used as explant. The experimental data were taken on different parameters of shoot and root growth. Different doses of individual shooting hormone(BA), individual rooting hormone(NAA) and the combination of these two hormones were experimented here. The minimum days to shoot induction were recorded 15 days from treatment T_8 (2.0 mg/L BA + 1.50 mg/L NAA). Maximum percentage (73.4%) of shoot induction was noticed in treatment T_8 (2.0 mg/L BA + 1.50 mg/L NAA) .The maximum days to root induction was 44 days and it was recorded in control treatment .The minimum days to root induction was 17 and it was observed from treatment T_8 (2.0 mg/L BA + 1.50 mg/L NAA) days. In the following study significant variation was observed for the use of BA and NAA on the percent of shoot induction explants. Significant variation was observed for the use of BA and NAA on the percent of root induction of explants . The treatment of 2.0 mg/L BA response excellent in shoot induction and 1.0 mg/L NAA showed better performance in root development in *Dendrobium* sp. Combined effect of BA and NAA was very significant. Overall a reliable protocol was developed for shoot and root proliferation which can be used for further crop improvement program.

Keyword: In vitro, Growth hormones, Regeneration, Orchid, *Dendrobium sp.*, MS media.

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LIST OF ABBREVIATION AND ACRONYMS

AEZ	=	Agro-Ecological Zone
BARI	=	Bangladesh Agricultural Research Institute
BBS	=	Bangladesh Bureau of Statistics
FAO	=	Food and Agriculture Organization
et al.	=	And others
RCBD	=	Randomized Complete Block Design
DAI	=	Days after Inoculation
Mg	=	Milligram
kg	=	Kilogram
SAU	=	Sher-e-Bangla Agricultural University
SRDI	=	Soil Resources Development Institute
wt	=	Weight
LSD	=	Least Significant Difference
^{0}C	=	Degree Celsius
NS	=	Not significant
Max	=	Maximum
Min	=	Minimum
%	=	Percent
NPK	=	Nitrogen, Phosphorus and Potassium
N	=	Nitrogen
BA	=	Benzyl Adenine
NAA	=	1-Naphthaleneacetic acid
CV%	=	Percentage of Coefficient of Variance.

CHAPTER I

INTRODUCTION

Orchids are flowering plants and they are commercially grown worldwide as cut flower and potted plants in Floriculture trade. Orchids (Family *Orchidaceae*) represent one of the two largest plant families, including from 736 to 899 genera and 27,800 accepted species names and over 100,000 hybrids produced by artificial pollination (RHS, 2020). It included both terrestrial and epiphytic orchids. Among them *Dendrobium* is the largest genera. Different kinds of orchids are indigenous to Bangladesh.Based on popularity, some of those are *Pierardi* sp., *Arides* sp., *Dendrobium* sp. (3rd most popular), *Cymbidium* sp., *Arnada* sp., *Arathera* sp. etc are recognized to be represented in the flora by 72 genera and 188 species. Of these, 117 species belonging to 41 genera are epiphytic in nature and 71 species belonging to 33 genera are terrestrial. The genera *Cymbidium* Sw. and *Liparis* W.P. Rich. exhibit both epiphytic and terrestrial habit . The rich species diversity is found in the genera *Dendrobium* Sw. and *Bulbophyllum* Thou. represented by 27 and 12 species respectively. These orchids are found naturally growing or anchoring on the woody plants like mango tree, wood apple tree, tamarind tree, rain tree, sissoo tree etc. (Kabir, 2018).

Dendrobium is the most popular orchid in cut flower trade in Asia for its magnificent flowers of great delicacy and long vase life. The genus has second largest number of species about 1600 species in the *Orchidaceae* family. These are distributed in Bangladesh, India, Myanmar, Malaysia, Singapore, Indonesia, China and Japan. *Dendrobium* represents one of the most important orchid genera, ornamentally and medicinally.Plants of *Dendrobium* sp. are sympodial epiphytic plants, which is a name they are worthy of, the name coming from Greek origin: "dendros", tree, and "bios", life. *Dendrobium* species have been used for a thousand years as first-rate herbs in traditional Chinese medicine (TCM). They are source of tonic, astringent, analgesic, antipyretic, and anti-inflammatory substances, and have been traditionally used as medicinal herbs in the treatment

of a variety of disorders, such as, nourishing the stomach, enhancing production of body fluids or nourishing Yin. The Chinese consider *Dendrobium* as one of the fifty fundamental herbs used to treat all kinds of ailments and use *Dendrobium* tonic for longevity.

The cut flowers as well as pot plants of *Dendrobium* have high demand in USA, Japan, Italy, Europe and Germany (Prokash, 1994). Thailand alone exports *Dendrobium* worth more than US $12 million. However, developing countries like Malaysia, Singapore and Sri Lanka are contesting to catch up the international market and run the *Dendrobium* cut-flower industry on cooperative basis. Majority of *Dendrobium* orchids under cultivation are native of tropical countries. Bangladesh is bestowed with a wealth of orchid flora; more than 30 species of *Dendrobium* orchids are available, many of which are commercially important (Iqbal, 2006).

In Bangladesh production and demand of orchids increasing quite rapidly. Some NGOs including BRAC, PROSHIKA and private nursery namely Kingsuk, Dipta Orchids , Micro Orchids and Plants, OMNI Orchids are producing orchids for local market. But there is no systematic information about the amount of orchid sale in the local market. Only Hortex Foundation is started to export orchids in a small scale at Government level.

The cultivation of orchid in Bangladesh was first started in 1909. Mr. Norendra Narayan Roy, the land-lord of Baldha Garden , started collection of orchids in his garden . He collected different types of orchids from various countries of the world. In 1975, a booklet named “Baldha Garden” was published by Forest Department of Bangladesh where there is description of 26 species of orchids (Kabir, 2018). The climate of Bangladesh is suited for orchid cultivation. Orchid can be widely cultivated in Bangladesh and in this way, Bangladesh can earn a lot of foreign currency. It would be a great source of employment also (Kabir, 2018).

Plants of *Dendrobium* sp. are epiphytic, sympodial and herbs. Stems are nodose or of pseudo-bulbs, leaves are sessile and are never plicate. Leaves are variable with or without sheathing leaf bases. Different characteristics of *Dendrobium* such

as rapid growth, easiness of explants regeneration, beauty of the flower, and year-round production in control flowering and long lasting of the flower stalk are very advantageous of this genus. (Talukder *et al.,* 2003). Orchids can grow in nature through seeds but due to lack of suitable hosts they don't germinate in adequate numbers, so still now it considered as a rare species. It can also propagate through budding and grafting but this technique has limitation both qualitatively and quantitatively (Nilufar, 2000). However, that technique is not use for the fastest method of propagation. Seed germination is another method of propagation but through this method, genetically identical plants cannot be obtained. Also, as the orchids seeds are very small with small reserves and having no endosperm, they need to be germinated in a nutrient medium for best results.

Tissue culture has been extensively used for the mass rapid clonal propagation of commercial cut-flower cultivars of orchids. In the former case it resulted in reduced prices of desirable plants to levels within the reach of most growers. Tissue culture techniques or micropropagation have been widely used for the mass multiplication of several commercially important orchids like *Dendrobium* (Malabadi *et al.,* 2004). If a plant is attacked by a disease, it is possible in tissue culture technique, to take a very small piece of the apical meristem from a shoot, as meristem is virus free and culture it to create a disease-free plant. So, for large scale rapid propagation it is essential to go through micropropagation. Orchids can easily propagate through in Virto propagation or tissue culture technique by using different plant parts as explants such as seeds, shoot tip, flower bud segment, lateral bud, young inflorescence, inflorescence node and pseudobulb (Madassery, 2006). Other part, as leaf and root also propagated (Nilufar, 2000). Through micropropagation technique we can produce large number of disease-free explants at a very low cost. Though, there is often a preference for hybrids in commercial markets, but beauty and quality of many local species are unique and conspicuous which can compete with the best hybrids.

In vitro regeneration of plant is an essential element of plant biotechnology. The frequency of callus initiation and explants regeneration are influenced by many factors, such as genotypes, type of explants and composition of media. Different

culture media with different organic additives have been used for efficient plant regeneration in orchid tissue culture. MS medium was found to be most effective for PLBs (Protocorm like bodies) formation and explants regeneration of *Dendrobium* orchid when added with 2, 4-D, compared with other culture media (Nasiruddin *et al.,* 2003). Maximum increase in fresh weight and highest numbers of leaves and roots regenerated in NP medium (New Phalaenopsis), gave better results when mixed with high concentrations of BAP (Chowdhury *et al.,* 2003). The combination of BAP + NAA, BAP + IAA, BAP + IBA, and IAA + IBA at different concentrations with charcoal supplementation revealed that the use of different growth regulators had significant effect on different regeneration of *Dendrobium* orchid (Khatun *et al.,* 2010). Even suitable media and organic additives are needed for large-scale multiplication of orchid reported to be effective.

Therefore, the present study was undertaken to investigate which concentration and combination of growth regulators is the best for regeneration and also to get the subsequent growth and development of *Dendrobium* sp. The following objectives had been set up for availing the target;

Objectives:

1. To establish a reliable protocol for *in vitro* regeneration of orchid.
2. To identify the best hormonal effect for *in vitro* regeneration of orchid.
3. To investigate the combined effect of auxin and cytokinine group of plant hormone for regeneration of *Dendrobium* sp.

CHAPTER II

REVIEW OF LITERATURE

Orchids with their bewildering range of flowers and beautiful color combinations provide a source of profound aesthetic pleasure to both owners and visitors (Bose *el al.,* 2002). Orchids are the most alluring and fascinating flowering plants. Many investigations on the effect of different growth hormones in shoot proliferation and root induction in orchids are done previously. Therefore, information available in the literature pertaining to those aspects of orchids have been reviewed and briefly presented below:

2.1 Introductory concepts of *Dendrobium* sp. :

Dendrobium is one of the largest and most important (ornamentally and medicinally) Orchid genera. Tissue culture is now an established method for the effective propagation of members of this genus.*Dendrobium* sp. is an orchid found in almost every botanical garden and in many amateur collections.Among the largest orchid genera, today it contains almost about 1500 species. Genus name comes from the Greek words *dendron* meaning a tree and *bios* meaning life.

Scientific Classification:

Kingdom: Plantae

Division: Angiosperm

Class: Monocots

Order: Asparagales

Family: Orchidaceae

Subfamily: Epidendroideae

Tribe: Dendrobieae

Sub Tribe: Dendrobiinae

Genus: *Dendrobium*

Species: *Dendrobium* sp.

2.2 Botanical Aspects of *Dendrobium* sp.

Deciduous types lose their leaves during the dry season in nature. Withhold water in the late fall and early winter to mimic natural conditions and stimulate blooming for these types. During the summer plants should be kept fairly wet and may be placed outdoors, hanging under the shade of trees. There are hot, intermediate and cool-growing species, so consider temperature requirements to optimize growth and flowering. Most species can be grown in a bark-based medium containing horticultural charcoal, with some preferring mounting on a cork bark slab. *Dendrobium* orchids are primarily epiphytic, growing in nature on the branches and trunks of trees. They are sympodial, producing new stems (pseudobulbs) at the base of the previous year's stems. They have a wide distribution and great variation in growth characteristics and floral structure. They may be evergreen or deciduous, and both types may require a dormant rest phase in winter, or not. All are known for the profusion and delicate attractiveness of their flowers, which come in an array of pastel colors. Blooms occur along the full length of the stem in some species, or in sprays emanating from the apex of the stem in others.

2.3 Use of *Dendrobium* sp.

Orchids are widely used as medicine all around the world. Herbal extracts of orchids help to reduce or prevent diseases such as hypertension, migraine, allergies, headache, and cramps. Menashian *et al.* (1992) discovered that vanilla improves the capacity of food intake and reduces nausea and vomiting in patients given chemotherapy. Medicinal orchids belong mainly to genera: Anoctochilus, Bletilla, Calanthe, Coelogyne, Cymbidium, Cypipedium, Dendrobium, Ephemerantha, Eria, Galeola, Gastrodia, Gymnadenia, Habenaria, Ludisia, Luisia, Nevilia and Thunia etc (Szlachetko, 2001).

Dendrobium orchids have been considered to be an effective herbal treatment for a number of health problems. Practically, *Dendrobium* is used by the Chinese

people as an important fundamental herb for treatment of all kind of diseases. (Withner, 1959).

1. *Dendrobin*, a phenanthrene isolated from *Dendrobium moniliforme* and Nobile, seems to be an anti-cancerous potential.
2. Among its many uses, the Chinese use *Dendrobium* tonic for their longevity. It is believed that *Dendrobium* when mixed with licorice roots and made into a tea that transmits healing energy to different parts of the body.
3. *Dendrobium* helps to moisturize and nourish the skin which prevents dryness and flaky skin.
4. When lungs and air passages dry out by consuming smoke and polluted airs which increases thirst, *Dendrobium* can be used to get rid of these difficulties.
5. *Dendrobium* is used as an effective medicine for the treatment of diseases such as tuberculosis, flatulence, night sweats, anorexia, fever, and dyspepsia.
6. *Dendrobium* helps to improve the functioning of the lungs, kidneys, and stomach. It can reduce stomach pain and cramping and reduce vomiting.
7. It is believed that regular consumption of *Dendrobium* can also treat sexual impotency.
8. *Dendrobium* extract is used to relief pain in the feet and hands, lumbago, and arthralgia.
9. The immune system is improved with the use of *Dendrobium* which helps the body fight against infections.
10. Natives of the Eastern Himalayas use *Dendrobium* to heal problems related to eyes.

11. *Dendrobium* blossoms and canes are edible. The native people of Thailand and Singapore make delicious snacks from *Dendrobium* blossoms and canes.
12. Along with their beautiful attractive colors and decorative qualities, environment can be made pollution and toxin free by growing *Dendrobiums* at home.
13. In European countries, *Dendrobium* blossoms are used as edible cake decorations and as garnishes.
14. Besides *Dendrobium kingianum,* other orchids are also used as emergency bush-food by the aborigines in Australlia.
15. In Nepal, *Dendrobium* flowers are also used for making pickle.

2.4 Introductory Concepts of Orchid

Most Dendrobium species undergo cross-pollination (Niu *et al.,* 2017). According to differences in morphology, *Dendrobium* can be divided into dozens of varieties, including *Dendrobium officinale*, *Dendrobium nobile*, *Dendrobium huoshanense*, *Dendrobium chrysanthum*, *Dendrobium loddigesii*, *Dendrobium fimbriatum*, and so on. Among them, *D. officinale* is considered to be the most precious (Lin *et al.,* 2011). The wild herbal plant with the taxonomic name "*D. officinale* Kimura et Migo," called "Tie-Pi- Shi-Hu" in Chinese (Xing *et al.,* 2013).

Dendrobium genus accounts for 70-80 % of tropical orchid plant and flower trade. The main attraction of *Dendrobium* hybrid relative to other potted orchids is their floriferous flower sprays, wide range of colors, sizes and shapes, year-round availability and long flowering life of several weeks to months (Kuehnle, 2006).

2.5 Tissue Culture of Orchid

Among the *in vitro* cultivation techniques used for the *in vitro* seedling or explants production of orchids, it can be used the *in vitro* asymbiotic germination and

micropropagation techniques aiming at the large-scale production of clonal explants.

Chookoh *et al.,* (2019) discussed about one of the largest commercial applications is aimed at the mass propagation of clonal plants to meet the world's demanding flower production market, in which orchids play a significant part in both the pot and cut flower market. However, other applications such as for species conservation purposes and obtaining transgenic plants.

Asymbiotic germination involves the in vitro inoculation and germination of orchid seeds with the aid of a sucrose-containing culture medium, under conditions free of microorganisms; including those symbionts that assist in germination, especially under natural conditions, a technique known as symbiotic germination, which can be done *in vitro, ex vitro*, or *in situ* and which, unlike asymbiotic, considers the use of symbiotic microorganisms to assist in the germination and early development of newly germinated seedlings, and lacking nutritional reserves to support early seedling development (Li *et al*., 2018).

Huang *et al.,* (2018) *reported* that, *In vitro* germination of orchids makes it possible to increase the efficiency of conservation and breeding programs, since *in vitro* germination rates higher than 70% are commonly reported, while in *ex vitro* conditions under natural environmental conditions, these rates hardly exceed 5% germinated seeds. This is especially due to the fact that orchid seeds do not contain nutritional reserves, and the embryo and seedlings at early germination are highly dependent on symbiosis with microorganisms known as mycorrhizae, which nutritionally supply these plants during a long time until the complete establishment of the seedling in the natural environment. In *Serapias vomeracea* orchid, in symbiosis with *Tulasnella calospora* there was observed a differential gene expression related to organic nitrogen transport and metabolism, showing the nutritionally supply of fungus to orchids in early development of protocorms.

A characteristic of the *in vitro* asymbiotic germination of orchids is the formation of the so-called protocorms, prior to budding, mainly containing the first leaves and undeveloped stem, followed by the roots and later on with the development

of the leaf and pseudobulb. The term protocorm-like bodies (PLBs) are used as a reference to this type of protocorm-producing germination, characteristic of orchids. The main difference between the germination and the sexual reproduction process, which includes the fertilization process, zygotic embryogenesis, followed by the germination and formation of protocorms, is that PLBs comes from somatic tissues, therefore being considered a type of vegetative propagation (Huang *et al.*, 2018).

Cardoso (2017) discussed techniques involving the germination of orchid seeds under *in vitro* conditions are especially used in: Conservation and production of seedlings of native species; germination of seedlings from crosses aiming at genetic improvement and production of new orchid cultivars; aiming at the production of protocorms in order to study somatic embryogenesis *in vitro*, also known as protocorm-like bodies or simply PLBs. They can also be used for commercial propagation and seedlings production, but with high genetic variability inherent in the family *Orchidaceae,* including commercial groups used for flower production.

Fang *et al.*, (2016) reported the production of PLBs, therefore, can be compared to a specific type of somatic embryogenesis that occurs in orchids, and the anatomy, development and characteristics of cells and some cell wall markers at the beginning of PLB formation are similar to those in the development of protocorms in orchids. These authors observed that in non-embryogenic callus of Phalaenopsis orchids, the inability to synthesize some cell wall components such as the JIM11 and JIM20 epitopes resulted in loss of morphogenic capacity of these calli, and the correct formation of the cell wall is directly associated with the ability of cell division and elongation in these cell types. In contrast, embryogenic calli synthesized these components, similar to what occurred in zygotic embryogenesis.

Chen (2016) reported the limited efficiency of clonal multiplication by the induction of shoots from floral stems cultivated *in vitro* has been one of the main difficulties faced in micropropagation of Phalaenopsis, resulting in an increase in

the production cost of micropropagated explants and associated with falling prices in the international market place in vitro explants as the current major cost of producing Phalaenopsis. In this sense, the IPR–PLBs can be an important tool in the micropropagation of commercial hybrids of this genus aiming to increase the production efficiency, being necessary to know the main factors involved in each phase of explants from PLBs production, e.g., induction, proliferation, and regeneration, which result in efficient clonal and mass propagation techniques for Phalaenopsis.

Bose *et al.* (2002) reported that the international trade of cut-flower and pot plants of orchids are dominated by hybrids of *Aracnis, Aranda, A.scocenda, Caltleya, Cymbidium, Dendrobium, Milionia, Mokara, Odonlogfoxsum, Oncidium, Paphiopedilum, Phalaenopsis and Vanda.* They also reported about a new species, *Dendrobium prianganese* with white flowers, delicately scented which was found from Java. They observed that *D. precenganese* had white flowers which were medium delicately scented and drooping. Psudobulbs do not mature in one season but once matured they can continue to produce flowers for a long period, They noted that the width, thickness and length of capsules of *D. moniliforme* depicted a sigmoid pattern of development The most rapid growth of capsules took place 1-2 weeks after pollinations. Moreover, width, thickness and length of capsules reached their highest peaks at 3ul, 6ul and 9ul week, respectively. The optimum time for self-pollination was 2-7 days after flowering but achieved only 10% fruit set. The optimum time for cross pollination was 1-5 days after flowering and fruit set varied between 30% to 100%. 5 They again noted that keiki's are young platelets that develop either on a stem or old inflorescence. Keiki's tend to form on many monopodial orchids (Phalaenopsis) and also on some sympodial (*Oncidium, Dendrobium*) orchids.

Carr and Christenson (2001) studied the characteristics of a new orchid species *Cycnoches schmidlianum* and noticed that it had spindle shaped pseudobulbs; up to seven leaves which attenuated at the tapered bases; pendent lax racemes up to 25 cm long; lanceolate floret bracts, up to 12 fragrant flowers; pale green sepals;

petals covered with butterscotch; brown, oblong dorsal sepals; obliquely obovate petals and 16×0.6 cm unlobed clawed lip. Patil (2001) carried out an experiment in a greenhouse for characterization of 13 orchid species. He found that six species (*Dendrobium densiflorum, Ascoccnirum apallaceum, D. nobile, Phaius lankcrvi/leae, D. pierardii* and *Ascoccnirum cimpuUaccum* var *auraniicum*) and the other five species (*Spalhoglollis speciosa, Aeridcs mulliflorum, crepidalum, D. jeukinsii* and *D. primulinum*) flowered during October-December and January-April, respectively. Flowers of *Spalhogloilis speciosa* remained good for the longest duration (34 days) followed by *D. jenkinsii* (30 days). The largest flowers (13 x 13 cm) and the longest spikes (60 cm) were found in *P. lankervilleae*. Smallest flowers (2 x 2 cm) were recorded in Ascocetrum ampullaceum. *Ascocentrum ampallaceum* var. *auraniicum* had the highest number of flowers per plant (38) followed by *Aerides multiflorum* (35).

2.6 Effects of Different Culture Media

In orchids, PLBs are suggested to be somatic embryos due to the morphological similarity and developmental pattern observed between them and the zygotic embryos. Besides that, ontogenetic studies based on histological and histochemical methods developed by compared the early developmental pattern of zygotic embryos and PLBs, which led to the conclusion that cytological characteristics and cell wall markers were similar in the early developmental stages of both zygotic embryos and PLBs, which would justify saying that PLBs are somatic embryos. Still, histological analyses made by also showed that the formation of PLBs occurs directly on the epidermal surface of the leaf segment with a cluster of meristem cells in constant division and without connection with the leaf vascular system, which is interesting from a commercial point of view, since it ensures the health of plants obtained through PLBs and enable success of genetic transformation (Cardoso, 2017).

Cardoso (2017) also conducted the first studies involving clonal micropropagation of Phalaenopsis where it was conducted by using Phalaenopsis amabilis as a model. Soon after, concluded that leaf segments obtained from inflorescence buds

grown in vitro when grown in New Dogashima Medium (NDM) medium supplemented with 0.1 mg/l NAA (Naphthaleneacetic Acid) and 1.0 mg/l BA (6-Benzyladenine) could generate up to 10,000 PLBs within a year. Cardoso (2017) also reported PLB regeneration from a callus induction phase (indirect somatic embryogenesis) using Vacin and Went medium supplemented with 20% coconut water and 4% sucrose with the hybrid Phalaenopsis Richard Shaffer 'Santa Cruz'

A perusal of literature reveals that several hundred media compositions have been used. But the most commonly used media for the propagation of orchids are Murashige and Skoog (MS), Vacin and Went medium (Jayarama Reddy, 2016).

Jayarama Reddy (2016) reported a protocol for the economical micropropagation of Phalaenopsis Queen Emma using leaf explants. Multiple shoot induction from axillary bud cultures of the medicinal orchid, *Dendrobium longicornu* was reported by Dohling *et al.,* (2012).

For the propagation of orchids, the explants were cultured on MS nutrient medium added with different concentrations of BA and IBA. The findings of the combined effect of different concentrations of BA+IBA had been presented below (Sahida , 2016). the number of leaves also increased with days increased after inoculation. The highest number of leaves was obtained at 60 DAI from these treatments compared to control. The maximum number of leaves per explants (9.33) was noticed from 1.0 BA+2.0 IBA (mg/l), whereas the minimum was (1.23 ± 1.0) in control treatment of D. *bensoniae* (Sahida, 2016).

The length of leaves was varied due to the various concentrations of BA+IBA supplementations. The maximum length of leaves per explants (1.00 cm) was founded from 0.5 BA+1.0 IBA (mg/l), whereas the minimum was (0.75 cm) in control of *D. bensoniae* (Sahida, 2016). The highest root length (1.62cm) was observed in 0.5 mgL^{-1}NAA + 0.5 mgL^{-1} BAP and minimum root length (0.41cm) was found at 5 mgL^{-1}NAA + 5 mgL^{-1} BAP. That findings partially supported by K. Goswami *et al.,* 2015 who showed that 0.5 mgL^{-1} each of BAP and NAA performed better growth and development of orchid.

Teixeira da Silva *et al.* (2016) reported the most of results obtained with Phalaenopsis and Oncidium were similar to reported with other species of orchids of importance in floriculture, as Cymbidium and Dendrobium genera, such as the main PGRs used for IPR–PLBs. As example, the combination of cytokinin BA (5.0 mg/l) and auxin NAA (2.5 mg/l) were used to induce PLBs (20.55 PLB per primary protocorm) in Cymbidium mastersii protocorms. Thin cell layers (TCL) from different types of tissues were a technique used to improve the production of PLBs in Cymbidium, Dendrobium, Oncidium, and Phalaenopsis.

In Dendrobium, a wide and complete study about molecular research was exhaustively carried out by, and considered especially the identification, classification and breeding of Dendrobium. Similarly, other study with micropropagation of Dendrobium was realized by (Teixeira da Silva *et al.,* 2016) and concluded that PLBs were used as explants in 21.8% of studies, and together with nodal or nodal segments explants is one of the major method used for Dendrobium micropropagation. Thidiazuron was also an important PGR for induction of PLBs in Dendrobium orchids, but the response to different cytokinins depends on genotype (Teixeira da Silva *et al.,* 2016).

In Dendrobium aqueum, only the cytokinin 2iP [N-6-(2-isopentyl) adenine] at 1.5 mg/l proved it efficiency in production of PLBs (42.7 PLBs per explants) from callus, compared to other cytokinins BA, Kin and Zea, and cytokinin-like compound TDZ. These authors also observed that arginine at 25 mg/l increased direct somatic embryogenesis, instead of callus derived PLBs (Teixeira da Silva *et al.,* 2016). Meta-Topolins, a natural aromatic type of cytokinin, were also reported used in induction and regeneration of PLBs in D. nobile, which combined with 0.5 mg/l NAA resulted in best PLBs formation (92%) and shoots/explants (9.2). These same authors observed that addition of polyamines, such as spermidine and putrescine increased regeneration of shoots from PLBs and secondary PLB formation. (Teixeira da Silva *et al.,* 2016).

However, high concentrations of auxin inhibit root elongation instead of initiating adventitious root formation. Similarly, cytokinin also could not act properly in high concentration.

Goswami *et al.* (2015), also recorded that 0.5 mg each of BAP and NAA performed better growth and development of orchid. Initiation of shoot in MS medium supplemented with 0.5mgL^{-1} NAA+ 0.5 mgL^{-1} BAP, Proliferation of roots of Dendrobium sp. orchid on half strength MS medium after 60 days of culture and hardening of rooted explants. (K. Goswami *et al.,* 2015).

Yang *et al.* (2010) experimented the use of liquid medium, rather than semi-solidified with Agar, is also an alternative for in vitro PLB proliferation used 5 L balloon-type air-lift bioreactor to provide mass propagation of *Oncidium* 'Sweet Sugar', and show that this system provides 326.3 g PLBs and growth ratio of 10.2, and is more efficient than semi-solid (2.7 g PLBs and Growth ratio of 3.4) and liquid-agitated flask culture (3.5 g PLBs and growth ratio of 4.4). In bioreactor, the lag phase was observed in the first 10-d culture, accompanied by a sharp drop in pH (5.7 to 4.7) and EC (3.2 to 1.5 mS cm^{-1}) in the first 20-d of cultivation, followed by an intense mass growth from 10 to 40 days of cultivation, when the pH increased again to 5.9. An interesting fact was the dynamics of sugars in the culture medium, and a fast and drastic reduction of sucrose in the medium was observed, from 27 (day zero) to 5.5 (day five), 1.2 (day 10) and zero (day 20), associated with a substantial increase in glucose and fructose in the first 10 days of cultivation, with the exhaustion of these sugars at 40 days of cultivation, when the PLBs entered the stationary phase, demonstrating that during a certain period the PLBs release invertases in the culture medium to reduce sugars, and these are metabolized during the exponential phase of production of PLBs.

Wei, C.H. (2007) reported, Benzyladenine (BA) at 2.0 mgL^{-1} + 0.2 mgL^{-1} Naphthaleneacetic Acid (NAA) has been shown to be the most efficient treatment for inducing PLBs in *Oncidium* 'Sweet Sugar' apical and axillary buds and the combination of 0.1 mgL^{-1} BA + 0.2 mgL^{-1} ANA resulted in better response for *Oncidium Aloha* 'Iwanaga'. In this context, BA can be used efficiently to obtain

PLBs in *Oncidium* in 31.8% of the papers, and auxin NAA is the one most used along with BAP.

Hye (2003) investigated the effect of KC. VW and MS media in an experiment which supplemented with sabn banana pulp (Sg) 10% w/v, sagar banana pulp (Sg) 10% *v-!v*, tomato juice (Tj) 10% w/v, charcoal (C) 0.1% w/v, and coconut water (C) 15% *vlv* on growth,, development and multiplication of PLBs and PLB derived explants regeneration, shoot multiplication and rooting of a hybrid orchid He found that KC + Sb + Cw + Cm showed the maximum fresh weight (1.10 g/explants) and maximum number of PLBs (34.22 g/explants) at 90 DAI. On the other hand, the highest number of explants (22.11 g/explants) and rooted explants (10.67 *g/*explants) were found from VW + Sb + C among the VW media supplementation. This treatment combination was also the best for shoot number (50 *I* ex plant), shoot length (7.05 cm) and leaf number (5.50 g/explants), VW + Tj was found the best for fresh weight of root (0.80 g/explants), root number (4.56 *g/*explants) and root length (5 26 cm).

Saiprasad *et al.* (2003) reported that the effect of plant growth regulators on the production of protoconn like bodies and multiple shoots along with ethylene and methane function in *Dendrobium cv. Sonia.* The growth regulators were 6-benzyladenine (BA), kinetin (Kn), alphanapthalene acetic acid (NAA) indolyl-3-acetic acid (IAA), 2,4- dicholorophenoxy acetic acid (2 4-D) and gibberellic acid (GA), supplemented into MS basal medium each at 0 25, 0 5 and 1.0 mg per litter. The ex-plants used were fractionated protoconn like bodies. Ethylene and methane were determined at 20 and 40 days after inoculation (DAI) using Perkin-Elmer gas chromatogram along with data on protocorm like bodies and multiple shoot production. The maximum number of PLB was recorded with BA (1.0 mg/L) at 20 and 40 DAI Auxin (NAA and IAA) treatment at all concentrations produced significantly the highest number of multiple shoots at 20 and 40 DAT and no PLB at 20 DAI but a few PLB at 40 OAJ. GA and 2,4-D treatment did not produce any PLB and multiple shoots. The effects of culture media, i.e. Knudson C (KC). Vacin and Went (VW), Murashige and Skoog

(MS), and Rosa and Laneri, on the production of protoconn like bodies (PLBs) from shoot tip explantss, of *Dendrobium* cv *Sonia, Oncidium cv Gower Ramsay,* and *Cattleya Ieopoldii* were studied by Saiprasad *et al.* (2003). MS medium produced the greatest number of PLBs, multiple shoots, growth, and differentiation of explants of three orchid genera.

The effects of different culture media such as Murashige and Skoog (1962), plant growth regulators (NAA + benzyladcnine at various concentrations) on *in vitro* establishment and meristem differentiation and on subsequent growth and development of leaf meristems, were studied in *Dendrobium cv. sonia* (Prasad *et al.*, 2001).

The number of days required for meristem differentiation and first leaf initiation were the lowest (12.00 and 30 33, respectively) for MS medium. MS also gave higher leaves (4.00), number of multiple shoots (2.50) and roots (5.50), leaf area (5 27 cm^2), fresh weight (420.00 mg) and dry weight (32.00 mg). Meristem differentiation was the earliest (within 8.83days) with 0.1 mg benzyladenine + I 0 mg NAA. The highest number of multiple shoots production was observed with 1.0 benzylademne +1.0 mg NAA/L The earliest leaf initiation (within 21.83 days) with the greatest shoot (2.95 cm) and root (2.53 cm) length, number of leaves (5.83), leaf area (9.67 cm'), fresh weight (763.33 mg) and dry weight (64.17 mg) were recorded the best for MS medium supplemented with 0.1 mg BA+ 0.1 mg NAA/L The highest number of roots was obtained with NAA alone at 1.0 mg/L.

Saiprasad *et al.* (2002) reported that the effect of various plant growth regulators on PLB production was assessed m 3 orchid genera, i.e. *Dendrobium soma, Oncidium cv.* Gower rarnsay and *Cattleya leopoldii.* BAP, kinctin, NAA, IAA, 2,-l-D and GA3 were supplemented to MS medium at concentrations of 0 25, 0.5 and 1.0 mg/L. The number of days taken for initial changes was observed to be a function of growth regulator supplemented as well as orchid genera Maximum numbers of PLBs were produced m 1.0 mg BAP/L

CHAPTER III

MATERIALS AND METHODS

3.1 Time and location of the experiment

The experiment was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of July 2019 to June 2020.

3.2 Experimental materials

3.2.1 Source of material

The planting materials of *Dendrobium* sp. were collected from Agargaon Nursery, Sher-e-Bangla Nagor, Dhaka-1207.

3.2.2 Plant materials

Fresh, healthy and disease-free mother plant was selected as donor plant. Healthy disease-free shoot tip of Orchids of 2-3 cm were collected and used as explants. The final size of the explants were trimmed to size of 1 cm for further work and washed thoroughly with running tap water for several times for removing soil and other west material and so on.

Plate 1: Mother plant of *Dendrobium* sp. for tissue culture.

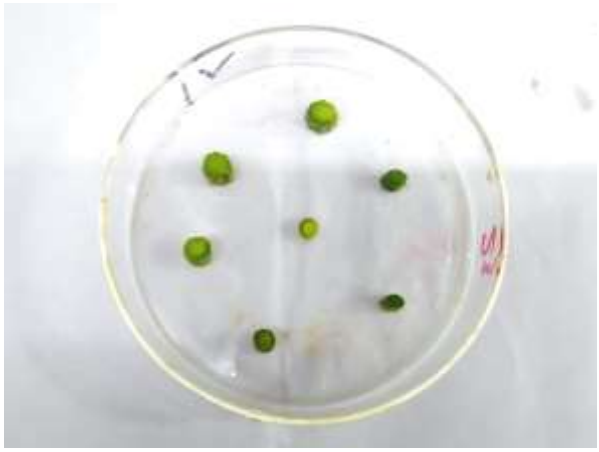

Plate 2: Shoot tips of *Dendrobium* sp. used as explants.

3.2.3 Instruments

Metal instruments viz., forceps, scalpels, needless, tissue, cotton, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121˚C for 45 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.2.4 Glass ware

The Pyrex glassware was used for all the experiments. Oven dried (250˚C) Erlen meyer flasks, culture bottles, flat bottom flasks, pipettes, Petri dishes, beaker and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) were used for media preparation. The glassware was first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water until the detergent was removed completely. Finally, they were rinsed with distilled water and sterilized in an autoclave at a temperature of 121˚C for 45 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.2.5 Culture medium

Explants were inoculated onto media composed of basal MS (Murashige and Skoog, 1962) medium supplemented with the plant growth regulators. Hormones were added separately to different media according to the requirements. To do so, stock solutions of hormones were prepared ahead of media preparation and stored at 4˚C temperature. The following components were used in culture media for *in vitro* regeneration of *Dendrobium* sp.

1. BA (1.0, 1.5 and 2.0 mg/L) was applied for shoot formation.
2. NAA (1.0, 1.5 and 2.0 mg/L) was applied for root formation.
3. BA (1.0, 1.5 and 2.0 mg/L) in combination with NAA (1.0, 1.5 and 2.0 mg/L), were used for shoot and root proliferation.
4. Sucrose (3%) was used as carbon source and media were solidified with agar (0.8%).
5. Coconut water (4%) as natural extract.
6. 0.1% Charcoal was added.

3.3 Preparation of the stock solution of hormones

To prepare these hormonal supplements, they were dissolved in proper solvent as shown against each of them below. Generally, cytokinine were dissolved in few drops of basic solutions (1N NaOH) and auxins were dissolved in few drops of ethanol.

Hormones (solute)	**Solvents used**
BA	1 N NaOH
NAA	96% ethyl alcohol

In present experiment, the stock solutions of hormones were prepared by following procedure. 100 mg of solid hormone was placed in a small beaker and then dissolved in 10 ml of 96% ethyl alcohol or 1 (N) NaOH solvent. Finally, the volume was made up to 100 ml by the addition of sterile distilled water using a measuring cylinder. The prepared hormone solution was then labeled and stored at 4±1˚C for use up to two months.

3.4 Preparation of culture media

To prepare 1000 ml of culture media the following steps were followed:

Step-1. 700 ml of sterile distilled water was poured into 1000 ml beaker.

Step-2. 5 gm of MS media and 30 gm of sucrose was added and gently stirred to dissolve these ingredients completely with the help of a hot plate magnetic stirrer.

Step-3. Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

Step-4.15% Coconut water was added to the mixture.

Step-5. 0.1% Charcoal was added to the mixture and the volume was made up to 1000 ml with addition of sterile distilled water.

Step-6. Finally, the pH was adjusted at 5.8.

Step-7. Eight (8) gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

Step-8. The prepared media was equally distributed to culture vials.

3.5 Steam heat sterilization of media (Autoclaving)

For sterilization 30 ml culture medium was poured in 200 ml culture bottles and then autoclaving was done at a temperature of 121˚C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure. After autoclaving the media were stored in at 21±2 °C for several hours to make it ready for inoculation with explants.

3.6 Preparation of explants

The trimmed shoot tips were washed thoroughly under running tap water and then with distilled water for 3-4 times. Subsequently the explants were transferred to laminar airflow cabinet and kept in a 250 ml sterilized beaker. The beaker with explants was constantly shaken during sterilization.

They were treated with 70% ethanol for 1-2 minute and rinsed with autoclave distilled water for 3-4 times. After treating with 70% ethanol, the explants were immersed in 0.1% $HgCl_2$ within a beaker and added 3-4 drops of Tween-20 for about 4-5 minutes with constant shaking in clockwise and anticlockwise direction. Then explants were washed 3-4 times with autoclaved distilled water to make the material free from chemical and ready for inoculation in culture vial.

3.7 Inoculation of culture

The sterilized explants were inoculated carefully through proper sterilization process within laminar airflow cabinet. Prior to use, the surface of the laminar flow bench was swabbed down with 70 % ethyl alcohol and the interior sprayed with the same alcohol. All glassware, instruments and media were steam-sterilized in an autoclave. During the course of the work, instruments in use were placed in a beaker containing 70 % ethanol and were flamed repeatedly using a spirit burner. The worker hands and forearms were washed thoroughly with soap and water and repeatedly sprayed with 70% alcohol during the period of work. The mouth of culture vial was flamed before and after positioning of the explants on the medium. For inoculation, explants were transferred to large sterile glass Petri dish

or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed with sterile scalpel blade to make suitable size. After cutting explants into suitable size explants is transferred to culture bottles containing MS medium with plant growth regulator (Plate 4). After vertically inoculating the explants singly in culture bottle, the mouth of bottle was quickly flamed and capped tightly. After proper labeling, mentioning media code, date of inoculation etc. the bottles were transferred to growth room.

Plate 3: Inoculation of explants in culture media.

3.8 Incubation

The bottles were kept to the culture racks and allowed to grow in controlled environment (Plate 3). The cultures were maintained at 21±2°C with light intensity varied from 3000–4000 lux (23 W white bulbs). White fluorescent lamps were used for growth of the culture. The photoperiod was generally 14 hours light and 10 hours dark having 70% relative humidity (RH)

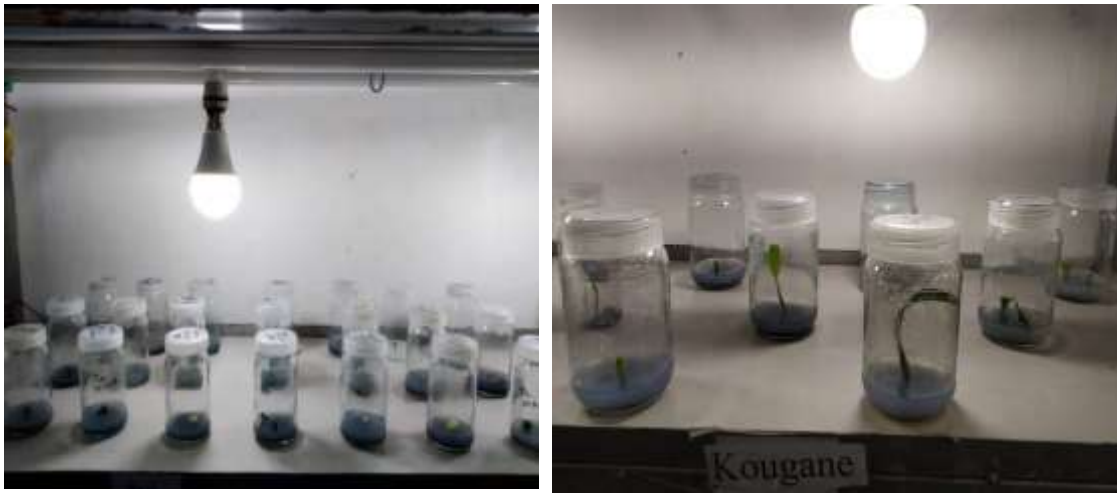

Plate 4: Bottles were kept on the culture racks.

3.9 Maintenance of proliferating shoot

The explants were cultured on MS nutrient medium supplemented with different concentration of BA alone or in combination of NAA. After successful shoot proliferation, subculture was done with newly formed shoot. Shoot were excised in aseptic condition with the help of sterile scalpel blade and sterile forceps and transferred to new MS media which was supplemented with same concentration of growth hormones in order to increase budding frequency. The observations on development pattern of shoot were made throughout the entire culture period. Data recording was started after 2 weeks from inoculation.

3.10 Root formation of regenerated shoot

Newly formed shoot with adequate length were excised individually from the culture vial and transferred to rooting media. Two types of growth regulators (BA and NAA) were used separately in different concentration (1.0, 1.5 and 2.0 mg/L) along with MS media. The observations on development pattern of root were made throughout the entire culture period. Data were recorded from 3rd week of inoculation.

3.11 Acclimatization

Acclimatization or "hardening-off' is a process by which in vitro propagated plants are made to adapt to an in vivo environment.

Step-1: After 35 days of culture on rooting media, the explants were taken out from culture vial with the help of forceps with utmost care to prevent any damage to newly formed root and dipped in gentle warm water to remove any traces of solidified agar media for acclimatization. Perforated Plastic pots (6×6 cm) were kept ready filled with coconut husk, small brick pieces and small coal pieces. Immediately after removing solidified agar media from newly formed root, the explants were then transplanted into the pots with special care.

Step-2: After planting, the explants were thoroughly watered and were kept at 25±2°C with light intensity varied from 2000–3000 lux. The photoperiod was generally 14 hours light and 10 hours dark and 70% RH for 7 days with consecutive irrigation.

Step-3: Then the plants were shifted to shade house with less humidity and indirect sunlight. The top of the pots was covered with transparent plastic sheet and grew at room temperature and 70% RH for 14 days with periodic irrigation (2 days interval).

Step-4: After 3 weeks, the plants were transferred to the bigger pots following de-potting and potting into different pot having bigger size. The plants were watered periodically.

3.12 Treatments

Four (4) sub-experiments were conducted to assess the effect of different concentrations of BA and NAA on shoot and root proliferation of *Dendrobium* sp. Proliferation.

Sub-experiment 1. Effect of BA on shoot induction potentiality in *Dendrobium* sp.

In this experiment, nodal segment of *Dendrobium* was used as sources of explants to investigate the effect of BA.

Treatments: Four (4) levels of BA (0.5,1.0, 1.5 and 2 mg/L) and control (0.0 mg/L).

The treatments were arranged in Completely Randomized Design (CRD) with 3 replications. Each replication contained 5 culture vials.

Sub-experiment 2. Effect of NAA on root induction potentiality in *Dendrobium* sp.

In this experiment, nodal segment of *Dendrobium* sp. was used as sources of explants to investigate the effect of NAA.

Treatments: Three (3) levels of NAA (1.0, 1.5 and 2 mg/L) and control (0.0 mg/L).

The treatments were arranged in Completely Randomized Design (CRD) with 3 replications. Each replication contained 5 culture vials.

Sub-experiment 3. Combined effect of BA and NAA on subsequent rooting of the multiplied shoot induction potentiality in *Dendrobium sp.*

In this experiment, nodal segment of *Dendrobium* was used as sources of explants to investigate the effect of BA and NAA.

Treatments: Three (3) levels of BA 1.0, 1.5 and 2 mg/L and Three (3) levels of NAA 1.0, 1.5 and 2 mg/L and control (0.0 mg/L).

Totals of 9 treatment combination were used. The treatments were arranged in Completely Randomized Design (CRD) with 3 replications. Each replication contained 5 culture vials.

Sub-experiment 4. Acclimatization and establishment of plants in environment.

In vitro propagated explants of *Dendrobium* sp. are made to adapt to natural environment in field condition.

After 60 days of culture on rooting media, the explants were taken out from culture vial with the help of forceps with utmost care to prevent any damage to newly formed roots and dipped in water to remove any traces of solidified agar media for acclimatization. Plastic pots (6×6 cm) were kept ready filled with coconut husk fiber, fir bark, hardwood charcoal in 4:1:1 proportion respectively. Immediately after removing solidified agar media from newly formed root, the explants were then transplanted into the pots with special care.

After planting, the explants were covered with plastic bags spraying water inside the plastic bags and were kept at 21±2°C with light intensity varied from 3000–4000 lux. The photoperiod was generally 14 hours light and 10 hours dark and 70% RH for 7 days with consecutive irrigation.

The plants were shifted to shade house with less humidity and indirect sunlight. The orchid pots were growing at room temperature.

3.13 Data recording

The observation on development pattern of shoot and root were made throughout the culture period. Three replicates each of them containing 4 bottles (single shoot per culture bottle) were used per treatment. Data were recorded after 21, 35 and 60 days of culture, starting from day of inoculation on culture media in case of shoot proliferation. In event of root formation, it was done every week starting from third week, fifth week and at 60 days of culture. The following observations were recorded in cases of shoot and root formation under in vitro condition.

1. Days for shoot induction
2. Days to root induction
3. Number of shoots per explants

4. Number of roots per explants
5. Number of leaves per explants
6. Average Length of shoot (cm)
7. Average length of root (cm)
8. Percentage of explants showing shoot induction
9. Percentage of explants showing root induction

3.13.1 Calculation of percent of shoot and root induction from culture:

Number of shoot and root were recorded and the percentage of shoot and root induction was calculated as:

Percentage (%) of shoot induction = $\frac{\textbf{\textit{Number of explant induced shoot}}}{\textbf{\textit{Number of explants incubated}}}$ x100

The percentage of root induction was calculated as:

Percentage (%) of root induction = $\frac{\textbf{\textit{Number of shoot induced root}}}{\textbf{\textit{Number of shoot incubated}}}$ x100

3.13.2 Calculation of days to shoot and root induction

Days to shoot and root induction were calculated by counting the days from explants inoculation to the first induction of shoot/root.

3.13.3 Calculation of number of shoot and root per explants

Number of shoot and root per explants was calculated by using the following formula,

Number of shoot / roots per explants=$\frac{\textbf{\textit{Number of shoot / root per explant}}}{\textbf{\textit{Number of observation}}}$

3.13.4 Calculation of shoot and root length (cm)

Shoot and root length were measured in centimeter (cm) from the base to the top of the explants by a measuring scale. Then the mean was calculated.

3.13.5 Calculation of diameter of root per explants

Root diameter was measured in centimeter (mm) with a screw micrometer by using the following formula,

Total reading = Linear scale reading (mm) + Circular scale reading (mm)

Then the mean was calculated.

3.13.6 Calculation of percent of plant establishment:

The percentage of established plants was calculated based on the number of explants placed in the pot and the number of plants finally established or survived by the following equation:

Percentage (%) of established explants = $\frac{\boldsymbol{Number\ of\ established\ plantlets}}{\boldsymbol{Total\ number\ of\ plantlets}} \times \mathbf{100}$

3.14 Statistical analysis

The experiment was one factorial set up in a completely randomized design (CRD) with 3 replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique and differences among treatment means were compared by using Duncan's multiple range test (DMRT) at 5% probability level using MSTAT-C (1990) program.

CHAPTER IV

RESULTS AND DISCUSSION

Three separate experiments were conducted to investigate the effect of different growth hormones in shoot proliferation and root induction in Orchid. The overall objective of the present study was to develop a protocol for the mass propagation of *Dendrobium*. These experiments were conducted at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University. The results of the experiments are presented and discussed in this chapter;

4.1 Sub-experiment 1. Effect of BA on shoot induction potentiality in *Dendrobium sp*.

Shoot tip of *Dendrobium* sp. orchid was used as sources of explants to investigate the effect of BA. The result of different concentrations of BA has been presented under following headings:

4.1.1 Days to shoot induction

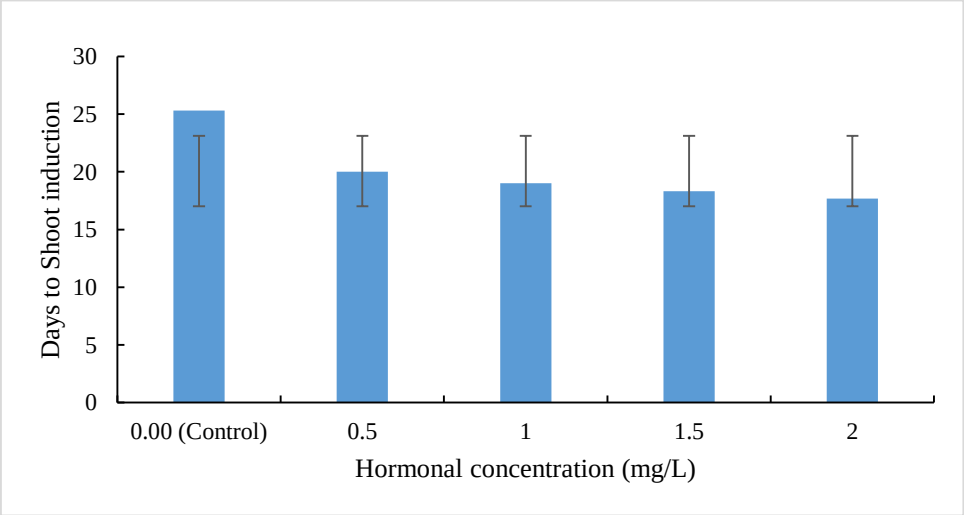

Figure 1: Effect of different hormonal concentration (mg/L) of BA in days to shoot induction of *Dendrobium* sp.

Significant variations were observed among different hormonal concentration (mg/L) of BA on days to shoot induction. The maximum days (25.3 days) to shoot induction were observed in 0.0 mg/L BA (Control) and minimum days (17.67 days) to shoot induction were recorded in 2.0 mg/L BA. Baksha *et al.* (2018) noticed that minimum of 24 days was required for shoot initiation in *Dendrobium moschatum* at 1.5 mg/l BA.

4.1.2 Percentage of explants showing shoot induction

Significant variation was observed for the use of BA on the percent of shoot induction explants. Maximum percentage (53.4 %) of shoot induction was noticed in treatment 2.0 mg/L BA and minimum in control (33.4 %). Baksha *et al.* (2018) reported the maximum 75 % in 1.5 mg/l BA in *Dendrobium moschatum*. There was an increasing trend of shooting in *Dendrobium moschatum* with the increasing concentration of BA.

Table 1. Percentage of shoot induction in *Dendrobium* sp.

Treatments BA (mg/L)	Shoot induction percent (%)
0.00 (Control)	33.40 c
0.5	40.00bc
1.50	46.60 bc
2.00	53.40 a
CV%	10.91
LSD (0.05)	0.85

4.1.3 Number of shoot per explants

There was significant influence of different hormonal concentration (mg/L) of BA on the number of shoots per explants. Data were recorded after 21, 35 and 42 days of inoculation on MS media. The highest no. of shoot per explants was obtained from 2.0 mg/L BA (2.3, 2.6, 3.0) and lowest in control treatment (1.0, 1.0, 1.6 at 21, 35 and 42 DAI (Table 2). This result was also supported by previous work of several researchers on *Dendrobium densiflorum* (Li *et al.*, 2018).

Table 2. Effect of different hormonal concentration (mg/L) of BA on number of shoots per explants of *Dendrobium* sp.

Treatments BA (mg/L)	Number of shoot per explants		
	21 DAI	35 DAI	42 DAI
0.00 (Control)	1.00 d	1.00 d	1.66 b
0.50	1.67bc	1.67bcd	2.00b
1.00	1.67 bc	2.00 abc	2.33 ab
1.50	2.00 ab	2.33 ab	2.33 ab
2.00	2.33 a	2.66 a	3.00 a
CV%	11.45	13.50	18.33
LSD (0.05)	0.66	0.76	0.75

4.1.4 Length of shoot (cm):

There was significant influence of different hormonal concentration (mg/L) of BA on the average length of shoot per explants. The maximum average length of shoot at 21 DAI was observed at 2.0 mg/L BA (1.00 cm) and the lowest in control treatment (0.16 cm). At 42 DAI maximum average length of shoot resulted from 2.0 mg/L BA (3.63 cm) and minimum from 0.00 mg/L BA (0.87 cm). (Table 3)

Table 3. Effect of different hormonal concentration (mg/L) of BA on length of shoot of *Dendrobium* sp.

Treatments BA (mg/L)	Length of shoot (cm)		
	21 DAI	35 DAI	42 DAI
0.00 (Control)	0.16 e	0.60 d	1.26 e
0.50	0.63d	0.73d	0.87f
1.00	0.73 c	1.40 c	2.53 c
1.50	0.73 c	1.40 c	2.60 c
2.00	1.00 a	2.00 a	3.63 a
CV%	7.48	6.66	7.14
LSD (0.05)	0.09	0.16	0.29

(A) **(B)**

(C)

Plate 5: Shoot proliferation of *Dendrobium on* MS media supplemented with 2 mg of BA (A)Induction of shoot in 21 DAI (B)Induction of shoot in 35 DAI (C) Induction of shoot in 42 DAI

4.1.5 Number of leaf per explants

There was significant influence of different hormonal concentration (mg/L) of BA on the number of leaf per explants. Data were recorded after 21, 35 and 42 days of inoculation on MS media. At 21 DAI the highest no. of leaf per explants was observed in 2.0 mg/L BA (2.33) and lowest in control (1.00). Similar result was found at 35 DAI also. But, for 42 DAI highest number of leaf per explants resulted from 2.0 mg/L BA (5.33) and the lowest from control treatment 0.50 mg/L BA (2.67). (Table 4) The maximum leaf number (8.66) was obtained for the treatment with 2.0 mg/l of BA in *Dendrobium pierardii* between fourth to fifth week (Kurup *et.al*, 2017).

Table 4. Effect of different hormonal concentration (mg/L) of BA in number leaf induction of *Dendrobium* sp.

Treatments BA (mg/L)	Number of leaf per explants		
	21 DAI	35 DAI	42 DAI
0.00 (Control)	1.00 b	2.00 b	3.00 c
0.50	1.33b	2.00b	2.67b
1.00	2.00 a	2.00 b	2.67 c
1.50	2.00 a	2.33 b	3.00 c
2.00	2.33 a	3.67 a	5.33 a
CV%	12.05	13.50	11.18
LSD (0.05)	0.66	0.54	0.66

In the present study, 2.00 mg/L BA performed best for *Dendrobium* sp. regeneration. This result shows consistency with other research works although some variations may occur due to the type of media used in respective research works. Similar findings were observed by Kobayashi *et al.* (1991) used healthy and sterilized protocorms of *Dendrobium* sp. as explants in his experiment. Among different concentrations of BA, 2.0 mg/L was found to be the most effective on enhancing the plant height. In another study, Kurup *et al.* (2005) reported that, the earliest bud initiation was observed in 4.0 mg/L BA (9.67) in *Dendrobium sonia*. Among different plant growth hormone so far studied, benzyl purine (BA) is considered to be more important for in vitro propagation of *Dendrobium* orchid. Use of diverse explants, medium and hormone combination may influence in vitro regeneration and multiplication efficiency of orchid. Orchids can be rapidly propagated through tissue culture techniques by using shoot tips, leaf and stem nodes (Pradhan *et al.*, 2013).

4.2 Sub-experiment 2. Effect of NAA on root induction potentiality in *Dendrobium* sp.

In this sub-experiment, shoot tip of *Dendrobium* orchid was used as sources of explants to investigate the effect of NAA.

4.2.1 Days to root induction

Significant variations were observed among different hormonal concentration (mg/L) of NAA on days to root induction. Maximum days to root induction were recorded in 0.0 mg/L NAA as control treatment (44.3) and minimum for 1.50 mg/L NAA (19 days) to root induction. (Table 5)

Table 5. Effect of NAA on days to root induction and root induction percentage in *Dendrobium* sp.

Treatments NAA (mg/L)	Days to root induction	Root induction (%)
0.00 (Control)	44.33 a	20.00 d
1.00	26.00 c	33.40 cd
1.50	19.00 e	60.00 a
2.00	22.00 d	46.60 ab
CV%	3.32	10.83
LSD (0.05)	1.75	0.76

4.2.2 Percentage of explants showing root induction

Significant variation was observed for the use of NAA on the percent of root induction explants. Maximum percentage (60%) of root induction was noticed in treatment of 1.50 mg/L NAA and minimum percentage (33.4) of root induction was noticed in control treatment. (Table 5) Chookoh *et al*. (2019) found a positive effect of NAA on rooting of multiplied shoots.

4.2.3 Number of root per explants

There was significant influence of different hormonal concentration (mg/L) of NAA on the number of root per explants. Data were recorded after 21, 35 and 42 days of inoculation on MS media. The maximum number of root was inducted in the treatment 1.0 mg/L NAA (3.00) at 21 DAI, it was minimum in control treatment at the same direction. At both 35 and 42 DAI similar results can be observed (Table 6).

Table 6. Effect of different hormonal concentration (mg/L) of NAA in days to root induction of *Dendrobium* sp.

Treatments NAA (mg/L)	Number of root per explants		
	21 DAI	35 DAI	42 DAI
0.00 (Control)	1.00 c	1.33 c	1.33 d
1.00	1.00 c	1.67 cd	5.33 a
1.50	3.00 a	4.00 a	2.33 cd
2.00	1.67 b	2.67 b	3.33 bc
CV%	12.05	13.39	12.29
LSD (0.05)	0.66	0.93	1.20

4.2.4 Length of root (cm)

The results of average length of root have been presented in Table 7. Data were recorded after 21, 35 and 60 days of inoculation on MS media. There was significant influence of different concentrations of NAA on the number of root per explants. The maximum average length of root (0.8 cm) at 21 DAI was observed from 1.0 mg/L NAA and minimum average length of root (0.13 cm) was observed from 0.0 mg/L NAA as control, at 42 DAI maximum average length of root (1.63 cm) was observed from 1.00 mg/L NAA.

Table 7. Effect of different concentrations of NAA in average length of root of *Dendrobium* sp.

Treatments NAA (mg/L)	Average length of root (cm)		
	21 DAI	35 DAI	42 DAI
0.00 (Control)	0.13 c	0.43 d	0.43 f
1.00	0.4 b	0.76 bc	1.63 a
1.50	0.8 a	1.4 a	0.96 cd
2.00	0.5 b	1 b	1.23 b
CV%	10.14	9.19	12.45
LSD (0.05)	0.15	0.25	0.23

4.3 Sub-experiment 3. Combined effect of BA & NAA on explants regeneration in *Dendrobium* sp.

In this sub-experiment, shoot tip of *Dendrobium* sp. was used as sources of explants to investigate the combined effect of BA and NAA. Treatments: three (3) levels of BA 1.0, 1.5 and 2 mg/L and three (3) levels of NAA 1.0, 1.5 and 2 mg/L totals of 9 treatment combination and control.

4.3.1 Days to shoot induction

Significant variations were observed among different hormonal concentration (mg/L) of BA and NAA on days to shoot induction. The maximum days to shoot induction were recorded in T_8 (2.0 mg/L BA + 1.50 mg/L NAA) 23.33 days and minimum was observed from treatment T_1 (1.0 mg/L BA + 1.0 mg/L NAA) 15.33 days. (Figure 2)

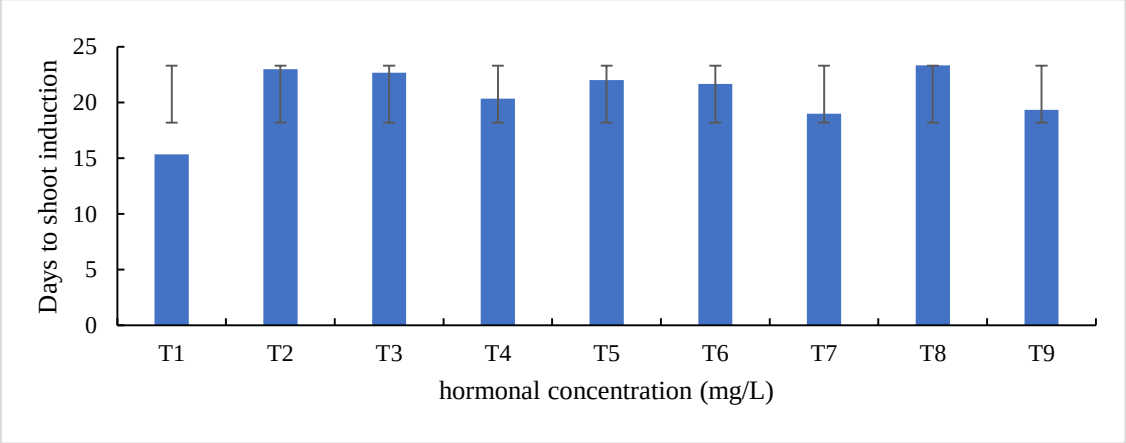

Figure 2: Combined effect of different hormonal concentration (mg/L) BA and NAA on days to shoot induction of *Dendrobium* sp.

4.3.2 Days to root induction

Significant variations were observed among different concentrations of BA and NAA on days to root induction. The maximum days to root induction was recorded in treatment T_5, 25 days and minimum days to root induction was observed from treatment T_8 ,17 days. (Table 8)

Table 8. Combined effect of BA and NAA on days to root induction of *Dendrobium* sp.

Treatments	Concentration BA+NAA (mg/L)	Days to root induction
T_1	1.00+1.00	20.67 ij
T_2	1.00+1.50	19.00 kl
T_3	1.00+2.00	19.67 k
T_4	1.50+1.00	23.67 e-g
T_5	1.50+1.50	25.00 a-d
T_6	1.50+2.00	23.33 de
T_7	2.00+1.00	21.00 g-i
T_8	2.00+1.50	17.00 m
T_9	2.00+2.00	19.67 k
CV%		3.80
LSD (0.05)		1.82

4.3.3 Number of shoot per explants

There was significant influence of different concentrations of BA and NAA on the number of shoot per explants (Table 9). Data were recorded after 21, 35 and 42 days of inoculation on MS media. At 21, 35 and 42 DAI lowest number of shoot per explants (1.0, 2.0 and 2.33) was observed for the treatment T_5 and highest number of shoot per explants(2.67, 3.67 and 5.33) was observed form treatment , T_8.

Table 9. Combined effect of BA and NAA on number to shoot induction of *Dendrobium* sp.

Treatments	Concentration BA+NAA (mg/L)	Number to shoot		
		21 DAI	35 DAI	42 DAI
T_1	1.00+1.00	1.67 bcd	2.67 cde	3.67 def
T_2	1.00+1.50	2.00 abc	3.33 bc	4.33 cde
T_3	1.00+2.00	1.33 cd	2.33 def	3.33 efg
T_4	1.50+1.00	2.00 abc	3.00 bcd	4.00 de
T_5	1.50+1.50	1.00 abc	2.00 bc	2.33 de
T_6	1.50+2.00	2.33 ab	3.33 bc	4.33 cde
T_7	2.00+1.00	2.00 abc	3.00 bcd	4.33 cde
T_8	2.00+1.50	2.67 a	3.67 a	5.33 a
T_9	2.00+2.00	2.00 abc	3.00 bcd	4.33 cde
CV%		16.67	19.23	16.89
LSD (0.05)		0.70	0.83	1.04

(A)

(B)

(C)

Plate 6:Shoot proliferation of Dendrobium sp. on MS medium with combined treatment T_8 of 2 mg/L of BA and 1.5 mg/L of NAA (A)Induction of shoot in 21 DAI (B)Induction of shoot in 35 DAI (C) Induction of shoot in 42 DAI

4.3.4 Number of leaf per explants

There was significant influence of different concentrations of BA and NAA on the number of leaf per explants. Data were recorded after 21, 35 and 42 days of inoculation on MS media. For all 21, 35 and 42 DAI lowest number of leaf per explants was observed for the treatment of T_1 (1.3, 2.3 and 3.0) and highest number of shoot per explants was observed from the treatment of 2.0 mg/L BA + 1.50 mg/L NAA (2.0,3.43 and 3.77). (Table 10)

Plate 8. Number of leaf per explants

Table 10. Combined effect of BA and NAA on number to leaf induction of *Dendrobium* sp.

Treatments	Concentration BA+NAA (mg/L)	Number to leaf		
		21 DAI	35 DAI	42 DAI
T_1	1.00+1.00	1.30 b	2.33 b	3.00 c
T_2	1.00+1.50	1.33 ab	3.00 ab	3.33 bcd
T_3	1.00+2.00	1.67 ab	3.00 ab	3.67 bcd
T_4	1.50+1.00	1.67 ab	3.00 ab	3.00 c
T_5	1.50+1.50	1.67 ab	3.00 ab	3.33 bcd
T_6	1.50+2.00	1.67 ab	3.00 ab	3.33 bcd
T_7	2.00+1.00	1.67 ab	3.33 ab	3.33 b
T_8	2.00+1.50	2.00 a	3.43 a	3.77 a
T_9	2.00+2.00	1.33 ab	3.33 ab	3.00 bc
CV%		13.36	18.61	11.67
LSD (0.05)		0.85	1.34	1.20

4.3.5 Number of root per explants

There was significant influence of different concentrations of BA and NAA on the number of root per explants. Data were recorded after 21, 35 and 42 days of inoculation on MS media. For 21, 35 and 42 DAI lowest number of root per explants was observed for the treatment of T_9 (2.0 mg/L BA + 2.0 Mg/L NAA) 2.30, 3.67 and 4.67. Highest number of root per explants was observed form all 21, 35 and 42 DAI for treatment of T_8 (2.0 mg/L BA + 1.50 mg/L NAA) 2.34, 3.45 and 4.87). (Table 11)

Table 11. Combined effect of BA and NAA on number to root induction of *Dendrobium* sp.

Treatments	Concentration BA+NAA (mg/L)	Number to root		
		21 DAI	35 DAI	42 DAI
T_1	1.00+1.00	2.00 b	3.00 b	3.00 bc
T_2	1.00+1.50	1.00 bc	2.67 bc	3.67 bcd
T_3	1.00+2.00	2.33 cd	2.67 bc	2.67 bcd
T_4	1.50+1.00	2.33 bc	2.33 bcd	3.67 bcd
T_5	1.50+1.50	2.00 bc	2.00 bcd	3.00 bcd
T_6	1.50+2.00	2.67 cd	2.00 bcd	3.00 bcd
T_7	2.00+1.00	3.00 bc	2.00 bcd	3.00 bcd
T_8	2.00+1.50	2.34 a	3.45 a	4.87 a
T_9	2.00+2.00	1.33 d	2.67 d	3.66 d
CV%		15.30	18.36	16.42
LSD (0.05)		1.13	1.31	1.45

4.3.6 Length of shoot per explants

There was significant influence of different concentrations of BA and NAA on the average length of shoot per explants (Table 12). Data were recorded after 21, 35 and 42 days of inoculation on MS media. For all 21, 35 and 42 DAI lowest average length of shoot per explants was observed for the treatment of T_1 0.43, 1.25 and 1.66 cm and highest average length of shoot per explants was observed form treatment of T_8 (2.0 mg/L BA + 1.50 mg/L NAA) 1.3, 2.27 and 4.33 cm. The previous work of several researchers also showed that the high concentration of BA and low concentration of IBA was favorable for the induction of multiple shoots. Bhadra and Hossain (2017) reported in *Dendrobium densiflorum*. Here, data was recorded after 30 days of culture.

Table 12. Combined effect of BA and NAA on average length of shoot of *Dendrobium* sp.

Treatments	Concentration BA+NAA (mg/L)	Length of shoot (cm)		
		21 DAI	35 DAI	42 DAI
T_1	1.00+1.00	0.43 f	1.25 h	1.66 m
T_2	1.00+1.50	0.57 d-h	1.30 c-h	1.80 j-m
T_3	1.00+2.00	0.57 d-h	1.27 d-h	1.73 klm
T_4	1.50+1.00	0.70 b-e	1.40 b-f	2.93 ef
T_5	1.50+1.50	0.53 d-i	1.53 b-e	3.03 de
T_6	1.50+2.00	0.73 bcd	1.53 b-e	2.47 hi
T_7	2.00+1.00	0.70 b-e	1.57 bcd	1.97 jk
T_8	2.00+1.50	1.30 a	2.27 a	4.33 a
T_9	2.00+2.00	0.80 bc	1.60 bc	1.83 j-m
CV%		15.14	15.27	7.61
LSD (0.05)		0.22	0.32	0.26

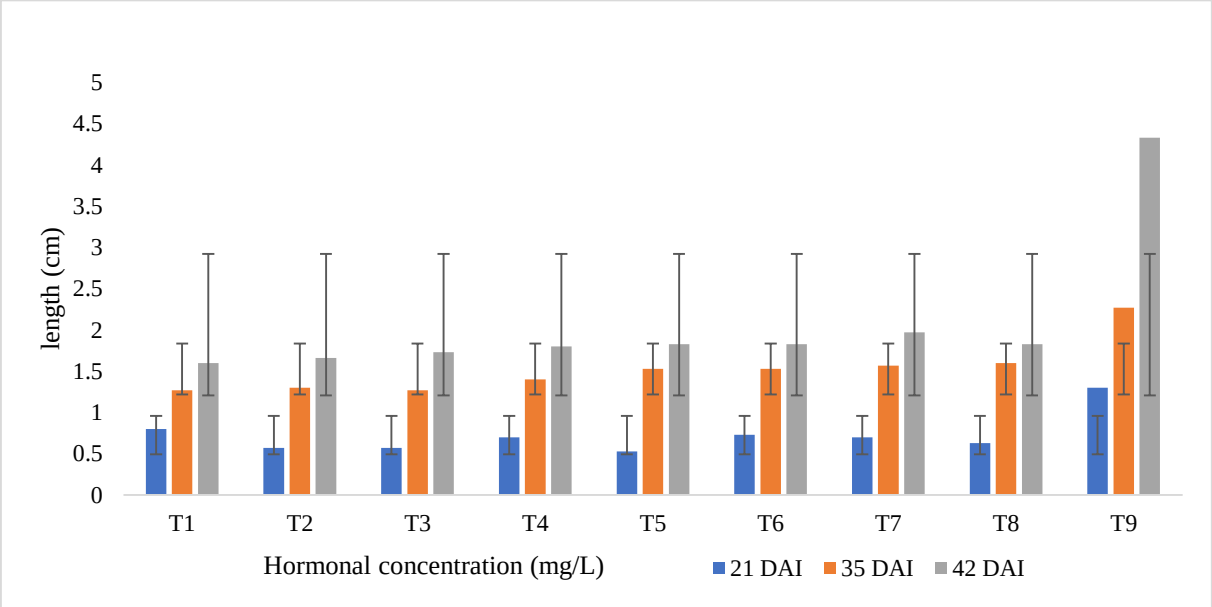

Figure 3: Combined effect of BA and NAA on average length of shoot induction of *Dendrobium* sp.

(A)

(B)

(C)

Plate 6: Root proliferation of *Dendrobium* sp. on MS medium with combined treatment T_8 of 2 mg/L of BA and 1.5 mg/L of NAA (A)Induction of root in 21 DAI (B)Induction of root in 35 DAI (C) Induction of root in 42 DAI

4.3.7 Percent of explants showing root induction

Significant variation was observed for the use of BA and NAA on the percent of root induction explants (Table 13). Maximum percentage (73.40%) of shoot induction was noticed in treatment T_8 (2.0 mg/L BA + mg/L 1.50 NAA). In this study, NAA yielded good results of root number because it is very effective to increase endogenous auxin contents and show higher stability against catabolism and in activation by conjugation with growth inhibitors (Hasan *et al.,* 2010).

Table 13. Combined effect of BA and NAA on percent shoot and root induction of *Dendrobium* sp

Treatments	Concentration BA and NAA (mg/L)	Percentage (%) of Shoot induction	Percentage (%) of root induction
T_1	1.00+1.00	46.00 c	40.00 c
T_2	1.00+1.50	53.40 ab	53.40 abc
T_3	1.00+2.00	46.60 b	53.40 abc
T_4	1.50+1.00	53.40 ab	53.40 abc
T_5	1.50+1.50	53.40 ab	46.60 bc
T_6	1.50+2.00	53.40 ab	53.40 abc
T_7	2.00+1.00	46.60 b	60.00 ab
T_8	2.00+1.50	73.40 a	73.40 a
T_9	2.00+2.00	53.40 ab	40.00 bc
CV%		13.50	15.09
LSD (0.05)		1.01	1.35

Similarly in the study of Kurup *et al*. (2017) early response was obtained in treatments with 2.0 or 4.0 mg/l kinetin alone. The highest shoot number (4.33 shoots) was obtained in the medium with 2.0 mg/l BA and 0.1 mg/l NAA. The combination of 1.0 and 2.0 mg/l kinetin along with 0.1 and 0.5 mg/l NAA also induced earliness in shoot multiplication. The maximum shoot number (4.66) was obtained for the treatment with 2.0 mg/l kinetin + 0.1 mg /l NAA (2017).

4.4 Sub-experiment 04: Acclimatization and establishment of explants in outer environment

After 42 DAI the explants with 3-4 roots were taken out from the culture flask to establish in pot. The planets were removed from vial carefully without any root damage. Culture medium adhered roots were washed with tap water to prevent

microbial infection. Then the culture vials were taken to grow cabinet for acclimatization and maintained for further observation under controlled condition with light, temperature and relative humidity. Then the explants were established in pot containing coconut fiber for the retention of available moisture. Pots were kept in a growth room for 7 days and then transferred in an outdoor area under 50% lighting condition. The growths of seedlings were normal. At first 20 plants were transplanted and 18 (90%) were survived in shade condition. Finally in normal atmospheric condition 18 plants were transplanted and among them all survived and survival rate is 100%

Plate 09. Acclimatization of explants under shade.

Plate 10. Acclimatization of explants under normal condition.

CHAPTER V

SUMMARY

The experiment entitled "*In vitro* regeneration of *Dendrobium* sp." was conducted at the Biotechnology Laboratory of Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka from July 2019 to June 2020 to investigate the effect of different plant growth regulator on shoot proliferation and root formation by conducting four separates' experiments of different growth hormones in shoot proliferation and root induction in *Dendrobium* sp. The overall objective of the present study was to develop a system for the mass propagation of *Dendrobium* sp.

Significant variations were observed among different hormonal concentration (mg/L) of BA on days to shoot induction. The maximum days (25.3 days) to shoot induction were observed in 0.0 mg/L BA (Control) and minimum days (17.67 days) to shoot induction were recorded in 2.0 mg/L BA. Maximum percentage (53.4 %) of shoot induction was noticed in treatment 2.0 mg/L BA and minimum in control (33.4 %). The highest no. of shoot per explants was obtained from 2.0 mg/L BA (2.3, 2.6, 3.0) and lowest in control treatment (1.0, 1.0, 1.6 at 21, 35 and 42 DAI (Table 2). The maximum average length of shoot at 21 DAI was observed at 2.0 mg/L BA (1.00 cm) and the lowest in control treatment (0.16 cm). At 42 DAI maximum average length of shoot resulted from 2.0 mg/L BA (3.63 cm) and minimum from 0.50 mg/L BA (0.87 cm).

Significant variations were observed among different hormonal concentration (mg/L) of NAA on days to root induction. Maximum days to root induction were recorded in 0.0 mg/L NAA as control treatment (44.3) and minimum for 1.50 mg/L NAA (19 days) to root induction. Maximum percentage (60%) of root induction was noticed in treatment of 1.0 mg/L NAA and minimum percentage (33.4) of root induction was noticed in control treatment. The maximum number of root was inducted in the treatment 1.0 mg/L NAA (3.00) at 21 DAI, it was minimum (1.00) in control treatment at the same direction. At both 35 and 42 DAI similar results can be observed. The maximum average length of root (0.8 cm) at

21 DAI was observed from 1.0 mg/L NAA and minimum average length of root (0.13 cm) was observed from 0.0 mg/L NAA as control, at 42 DAI maximum average length of root (1.63 cm) was observed from 1.00 mg/L NAA.

Significant variations were observed among different hormonal concentration (mg/L) of BA and NAA on days to shoot induction. The maximum days to shoot induction were recorded in T_8 (2.0 mg/L BA + 1.50 mg/L NAA) 23.33 days and minimum was observed from treatment T_1 (1.0 mg/L BA + 1.0 mg/L NAA) 15.33 days. The maximum days to root induction was recorded in treatment T_5 25 days and minimum days to root induction was observed from treatment (2.0 mg/L BA + 1.50 mg/L NAA) 17 days. At 21, 35 and 42 DAI lowest number of shoot per explants was observed for the treatment T_5 (1.0, 2.0 and 2.33) and highest number of shoot per explants was observed form treatment of 2.0 mg/ L BA + 1.50 mg/L NAA (2.67, 5.67 and 7.33). Highest number of root per explants was observed form all 21, 35 and 42 DAI for treatment of T_8 (2.0 mg/L BA + 1.50 mg/L NAA) 6.0, 8.0 and 10.0). For all 21, 35 and 42 DAI lowest average length of shoot per explants was observed for the treatment of T_1 0.43, 1.25 and 1.66 cm and highest average length of shoot per explants was observed form treatment of T_8 (2.0 mg/L BA + 1.50 mg/L NAA) 1.3, 2.27 and 4.33 cm.

CHAPTER VI

CONCLUSION

Following conclusions can be made from the present study:

i. A micropropagation protocol has been developed in regeneration of *Dendrobium sp.*

ii. The treatment of 2.0 mg/L BA responsed excellent in shoot induction and 1.0 mg/L NAA showed better performance in root development in *Dendrobium sp.*

iii. Combined effect of BA and NAA of individual effect of BA for shoot formation. The treatment 2.0 mg/L BA+ 1.50 mg/L NAA was revealed the best treatment for shoot induction and root regeneration.

RECOMMENDATIONS

Following recommendations could be addressed based on the present experiment:

i. Further research can be carried out on more specific hormonal combination of BA, NAA, IBA and Kinetin any other phytohormone.

ii. Callus induction could be practiced for more shoot proliferation.

iii. Future experiment should be carried on different type of genotypes of *Dendrobium* sp.

Iv. Fixed survival rate of *in vitro* plant should be studied and compared to normal planting.

v. Yield potentiality between micro propagated and vegetative propagated plants can be studied.

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