In Vitro Regeneration and Rapid Multiplication of Two Orchid Varieties of *Dendrobium bensoniae* and *Dendrobium aphyllum*

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Dedicated

To

My beloved parents

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis entitled **"*In vitro* Regeneration and Rapid Multiplication of *Dendrobium bensoniae* and *Dendrobium aphyllum*"** submitted by the undersigned have been carried out under supervision of Dr. Aparna Islam, Professor, Department of Mathematics and Natural Sciences, BRAC University, Dhaka and co-supervision of Dr. Md. Ekramul Hoque, Professor, Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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Abstract

The effect of different concentrations of BA and IBA on *in vitro* plant regeneration was studied in both varieties of *Dendrobium bensoniae* and *Dendrobium aphyllum*. The composition of the culture medium using different hormone along or in combination affected the induction, regeneration and multiplication of both varieties of *Dendrobium* orchid. BA with 0.5, 1.0, 1.5 and 2.0 mg/l and IBA with 0.5, 1.0, 1.5 and 2.0 mg/l were used in both varieties. Combined hormonal concentrations were also used. Shoot regeneration using nodal explants was found to be best in 2.0 mg/l BA supplementation on MS medium, which gives better responses than all other combinations of BA and BA+IBA concentrations under study in both *D. bensoniae* and *D. aphyllum*. In hormonal combination, highest number of shoots and leaves were found using 1.0 mg/l BA and 1.5 mg/l IBA along with MS medium in both varieties. The highest number and maximum length of roots were noticed using 1.5 mg/l IBA in *D. bensoniae.* Similarly, highest number and maximum length of roots were found in concentration of 2.0 mg/l in case of *D. aphyllum*. In hormonal combination, 0.5 mg/l BA and 1.0 mg/l IBA with MS medium found to be most effective for root induction in both varieties of *D. bensoniae* and *D. aphyllum*. The well-rooted plantlets were successfully acclimatized, planted in pots and transferred to the shade house for establishment. So MS medium supplemented with 2.0 mg/l BA may be used for rapid shoot induction, regeneration and multiplication and 0.5 BA+1.0 IBA (mg/l) supplemented with MS medium may be used for rapid induction of root in *D. bensoniae* and *D. aphyllum*.

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Abbreviations

BRAC	**Bangladesh Rural Advancement Committee**
BARI	**Bangladesh Agricultural Research Institute**
SAU	**Sher-e-Bangla Agricultural University**
BA	**Benzyl adenine**
IBA	**Indole-3-butyric acid**
HRC	**Horticulture Research Institute**
MS	**Murashige and Skoog (1962) medium**
mg/l	**Milligram/liter**
ml	**Milliliter**
NAOH	**Sodium Hydroxide**
cm	**Centimeter**
HCL	**Hydrochloric Acid**
$HgCl_2$	**Mercury (II) chloride**
%	**Percentage**

Introduction

Chapter I

Introduction

Orchid represents the most evolved and one of the largest groups among the angiosperm. Orchidaceae is the largest and most diverse family of flowering plants, consisting of 30,000 - 35,000 species belonging to 600 - 800 genera (Freudenstein and Rasmussen, 1999; Hajra, 2001; Singh *et al*., 2007; Bektas *et al*., 2013). It has both terrestrial and epiphytic members (Karasawa, 1996). Its name has been derived from the word "*Orchis"* which means testicles (Royer, 2003). They play a very useful role to balance the forest ecosystems. Demand for high quality of orchids has been increasing day by day due to their popularity in horticulture industry. Because of their medicinal importance they demand a very high price in the international market. There are some indigenous orchids in some countries. Bangladesh is one of them. Indigenous orchids of Bangladesh are *Rhyncostylis* sp., *Pierardi* sp., *Arides* sp., *Dendrobium* sp., *Cymbidium* sp., *Arnada* sp., *Arathera* sp., *Bokthara* sp., *Eridis* sp., *Miltonia* sp., *Hoya* sp., *Vanda* sp. These orchids are found naturally occurring on the mango tree, wood apple tree, tamarind tree, rain tree, sissoo etc (Kabir, 2007). Exotic orchids of Bangladesh are *Dendrobium* sp., *Oncidium* sp., *Phalaenopsis* sp., *Cattleya* sp., *Vanda* sp., *Ascocenda* sp., *Brassavola* sp., *Mokara* sp., *Paphiopedilum* sp. (Kabir, 2007). The genus *Dendrobium* is the third largest in the family of Orchidaceae comprising of about 1184 species around worldwide (Leitch *et al*, 2009). Now-a-days, in our country and also the other countries of the world, orchids are widely used as cut flowers and indoor decorations. Among the orchid genera, *Dendrobium* is one of the most popular orchids all over the world including Bangladesh. Rapid growth, easiness of plantlet regeneration, beauty of the flower, year round production in control flowering and long lasting of the flower stalk are the advantages of *Dendrobium* (Talukder *et al.,* 2002). *Dendrobium* hybrid is the most popular orchid for cut flower trade in Asia. Orchid cultivation is considered as a cottage industry in Sri-Lanka (Islam, 1985). According to a report of 2004, about 70% of total orchid exports of Singapore were *Dendrobium* (Singapore Orchid Industry, 2004). Orchids are currently the second most valuable poted crop in the United States with a total wholesale value of US $144 million in 2005 (U.S Department of Agriculture, 2006).

1.1 Distribution of orchid

The great majority of orchids are found in the tropics, mostly in Asia, South America and Central America (Chakrabarti, 1986). According to Mark W. Chase *et al.* (2001), the overall biogeography and phylogenetic patterns of Orchidaceae show that they are even older and may go back roughly 100 million years. The orchid was one of the first plants to evolve on the earth. It was around in the time of the dinosaur, over 120 million years ago (Anonymous, 2003). Of the different medicinal species, *Dendrobium* species reported from Chinese Pharmacopoeia, namely *D. aphyllum, D. bellatulum, D. densiflorum, D. fimbriatum* and *D. nobile* are the native species found in India (Singh *et al*, 2001). Orchids are usually grown in the tropical regions of different countries of Asia like Nepal, Bhutan, India, Thailand, Bangladesh etc. In Asia, a special type of orchid named "Butterfly orchid" is commonly available in Chaina. The word "Butterfly orchid" is used by Chinese people to describe the beauty of this orchid. In addition to this, there is also another type of orchid known as "Phalaenopsis". It derives from a Greek word where “phalaina” and “opsis” means "moth like". This type of orchid is generally found to grow on the branches of trees and between rocks, usually near a source of water for moisture. They also grow naturally in grassland areas where they adapted to many different types of environments. A huge number of wide varieties of orchids are seen in the forest and hilly areas. These are grown in almost all countries of the world except Antarctica. Countries like Bangladesh, Bhutan, India, America, Australia, New-Zealand and Bangkok have their own type of native species of orchids having own characteristics (Kabir, 2007)**.** The number of orchid species is rapidly and steadily declining because of their low rate of natural propagation and the ongoing collection from nature (Bhadra *et al.*, 2002; Bektas *et al*., 2013). Due to random and careless collection of these sorts of orchids, many have already been listed as endangered species (Ozhatay, 2000; Clemenets, 2003; Machaka-Houri *et al*., 2012).

1.2 Types of orchid

Orchids are the perennial epiphytic or terrestrial or saprophytic herbs. They come with an infinite variety of colors, shapes and sizes and many make great indoor house plants (Royer, 2003). They range a size from a 2 inch. plant that can sit in the palm of one’s hand to 5 feet giants that need a tub to grow in (Anonymous, 2003). Scientists are always trying to invent

new hybrids of orchids and about 800 new species are being added each year (Anonymous, 2005). Orchids are mainly of 2 types:

(a) Terrestrial orchids: They grow on the ground, usually in marshland. *Paphiopedilums* and *Cymbidiums* are terrestrial orchids.

(b) Epiphytic orchids: They grow mainly on rocks and trees, where they hold on with thin or thick roots and take nutrients that fall to them. They also absorb sunlight that reaches to them.

(c) There is one another type of orchid which is few in number. They do not have any green leaves and live on decayed vegetation. These are called saprophytes.

1.3 Plant description

Orchids can be easily identified by its leaves, stems and roots, flowers, fruits and seeds. Description about orchid leaves, stems and roots, flowers, fruits and seeds are given below:

1.3.1 Leaves

Orchids generally have simple leaves with parallel veins. They may be ovate, lanceolate or orbiculate and variable in size. The structure of leaves corresponds to the specific habitat of the plant. Species, which grow in sunlight or in site that can be occasionally dry, have thick, leathery leaves. On the contrary, species growing in shade have long, thin leaves. The leaves of most orchids are perennial and in some orchids they are considered as ornamental (Royer, 2003).

1.3.2 Stems and roots

All orchids are perennial herbs and they lack any permanent woody structure. Terrestrial orchids may be rhizomatous or form tubers. The root caps of terrestrial orchids are smooth and white. Epiphytic orchids have their modified aerial roots that can sometimes be a few meters long (Amin *et al*, 2004).

1.3.3 Flowers

Orchidaceae are well known for their structural variations in their flowers. Orchid flowers are beautiful and have many shapes, sizes and brilliant attractive colors. They have two whorls of

sterile elements. The outer whorl has three sepals and the inner whorl has three petals (Royer, 2003).

1.3.4 Pollination

The way in which the orchid is so diverse is its ability to reproduce. Some orchids rely on flying insects, others rely on crawling insects. The rest are dependent on the wind for pollination (Sinha, 2004). Pollinators are often visually attracted by the shape and color of orchid flowers. The flowers may also produce attractive odor. On the other hand, some orchids mainly or totally rely on self-pollination, especially in colder regions where pollinators are particularly rare **(**Sinha, 2004). After pollination, the sepals and petals fade and wilt but they usually remain attached to the ovary (Sinha, 2004).

1.3.5 Fruits and seeds

The ovary typically develops into a capsule. The ripening of a capsule usually takes time of 2 to 18 months. The seeds are generally almost microscopic and very numerous and in some species it is over a million per capsule. After ripening they blow off like dust particles or spores (Alam, 2002).

1.4 Uses of orchid

Besides there attractive beauty and wonderful color, orchids have many other uses which are given below:

1.4.1 Medicine

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). Also, *Orchis latifolia, Eulophia campestris, Vanda tessellate,* and *Vanda roxburghii* have certain antibacterial substances and phytochemical activity that helps in the treatment of certain illnesses. Recently, more species belonging to different genera have been reported to have medicinal properties and in future

more will be added in the list (Gutiérrez, 2010; Pant *et al.,* 2011)**.** Orchids are also effective in curing sore throat, digestive problems, diarrhea, and gum disease.

1.4.2 Flavouring agent

The orchid *Vanilla planifolia*, which is native to Central America, is cultivated in the West Indies and Java for its vanillin flavor (Pant *et al.*, 2011). The dried seed pods of one orchid genus, Vanilla (especially *Vanilla planifolia*), are commercially important as a flavouring agent in baking, for perfume manufacturing and aromatherapy. For flavoring, both Vanilla and Salep are well known and widely used from long time ago, the former is used as a delicious flavoring and wonderful perfume (Bechtel *et al.,* 1992). Both are used in making ice-cream and beverages (Bulpitt, 2005). Salep is also effective in curing sore throat, digestive problems, diarrhea, and gum disease.

1.4.3 Food

There are also some saprophytic orchid species in the group of *Gastrodia* which produces potato-like tubers. They are consumed as food by native peoples in Australia. The underground tubers of terrestrial orchids mainly *Orchis mascula* (early purple orchid) are ground to a powder and used for cooking (Bulpitt, 2005). Many orchid blooms are used in salads. They are considered safe for consumption, but there are also some reports that somewhat they are bitter and some species may irritate the stomach.

1.4.4 Aroma therapy

Orchid essential oil is very popular in aroma therapy. It has the ability to nourish and rehydrate the skin and it diminishes the signs of aging. Its antioxidant and protective properties help reduce the appearance of fine lines which makes the skin looks younger and radiant.

1.4.5 Perfume industry

The scent of orchids is frequently analysed by perfumers using headspace technology and gas-liquid chromatography to identify potential fragrance chemicals.

1.4.6 Beverage

The dried leaves of *Jumellea fragrans* are used to flavour rum on Reunion Island.

1.5 Health benefits and therapeutic uses of *Dendrobium* orchids

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.

- Dendrobin, a phenanthrene isolated from *Dendrobium moniliforme* and *Nobile*, seems to be an anti-cancerous potential.
- 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.
- *Dendrobium* helps to moisturize and nourish the skin which prevents dryness and flaky skin.
- 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 difficulities.
- *Dendrobium* is used as an effective medicine for the treatment of diseases such as tuberculosis, flatulence, night sweats, anorexia, fever, and dyspepsia.
- *Dendrobium* helps to improve the functioning of the lungs, kidneys, and stomach. It can reduce stomach pain and cramping and reduce vomiting.
- It is believed that regular consumption of *Dendrobium* can also treat sexual impotency.
- *Dendrobium* extract is used to relief pain in the feet and hands, lumbago, and arthralgia.
- The immune system is improved with the use of *Dendrobium* which helps the body fight against infections.
- Natives of the Eastern Himalayas use *Dendrobium* to heal problems related to eyes.
- *Cymbidium canaliculatum* and *Cymbidium madidum* are used to cure of dysentery.
- *D. aphyllum* dried stems commonly used to support the immune system which gives longevity.

- Dried *Dendrobium chrysotoxum* stems and *Dendrobium cathenatum* canes are used as herbal tea to regain strength after illness.
- *Schoenorchis fragrans* is very good for flue and bronchitis (Anonymous, 2005).

1.5.1 Other uses of *Dendrobium*

- *Dendrobium* blossoms and canes are edible. The native people of Thailand and Singapore make delicious snacks from *Dendrobium* blossoms and canes.
- Along with their beautiful attractive colors and decorative qualities, environment can be made pollution and toxin free by growing *Dendrobiums* at home.
- In European countries, *Dendrobium* blossoms are used as edible cake decorations and as garnishes.
- Besides *Dendrobium kingianum,* other orchids are also used as emergency bush-food by the aborigines in Australlia.
- In Nepal, *Dendrobium* flowers are also used for making pickle.
- *Anoectochilus* leaves are used in Indonesia and Malaysia as vegetable (Withner, 1959).

1.6 Orchid cultivation in Bangladesh

The cultivation of orchid was first started in 1909. Mr. Norendra Narayan Roy, the land-lord of Baldha, started collection of orchids in his garden which is popularly known as “Baldha 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, 2007). 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 by exporting orchids to different countries, also it would be a great source of employment (Kabir, 2007). In Bangladesh, three NGO's are planting orchids in a large scale. These are BRAC, Wonderland Toys and Proshika (Anonymous, 2007). There is a tissue culture laboratory of BRAC at Joydebpur in Gazipur. At Fulpur BRAC has a farm of orchid multiplication. BRAC

imported orchids from Thailand and after multiplication here sold in market. Proshika has a well-established tissue culture laboratory at Koitta in Manikganj. At present they are working on micro propagation of ornamental plants along with orchids. They sold their orchid seedling to different nursery. There are many more plant tissue culture laboratories doing the same but in a smaller scale. As orchids has a huge potential to earn currency as well as creating employment opportunity, country like Bangladesh should give emphasis on the management and cultivation techniques of orchids to hold this sector. On the other hand, the lack of knowledge about orchid management, cultivation techniques and usefulness of orchid is an obstacle to develop this sector into an exportable industry (Jalal *et al.,* 2008).

1.7 Scientific classification of *Dendrobium bensoniae*

Scientific Classification

Kingdom: Plantae

Division: Magnoliophyte

Class: Liliopsida

Order: Asparagales

Family: Orchidaceae

Subfamily: Epidendroideae

Tribe: Dendrobieae

Sub Tribe: Dendrobiinae

Genus: *Dendrobium*

Species: *Dendrobium bensoniae*

Bionomial Name

Dendrobium bensoniae

1.8 Plant description of *Dendrobium bensoniae*

Dendrobium bensoniae also known as Lady Benson's dendrobium (Sinha, 2004). *In Greek, "Dendron" means "tree" and "Bios" means "life". This refers to the epiphytic mode of the plant genus. It* is an epiphytic species found on tree trunks in lowland and submontane moist forests. It is a popular ornamental orchid which mainly grows in the forests of India, Burma and possibly Thailand. It has large flowers with a lip which has a characteristic golden disk and two large, purple spots. Plant requires warm to hot temperatures and medium amounts of light to grow well. Plant should keep moist and fertilize during growth season. It is also essential to slowly reduce watering rate as winter approaches and should continue until the new shoots appear. *Dendrobium bensoniae* grows well in a drain mix of sphagnum moss or medium fir bark. There is no particular threat associated with this species. Lady Benson's *Dendrobium* is cultivated as an ornamental plant.

1.9 Scientific classification of *Dendrobium aphyllum*

Scientific Classification

Kingdom: Plantae

Division: Angiosperm

Class: Monocots

Order: Asparagales

Family: Orchidaceae

Subfamily: Epidendroideae

Tribe: Dendrobieae

Sub Tribe: Dendrobiinae

Genus: *Dendrobium*

Species: *Dendrobium aphyllum*

Bionomial Name

Dendrobium aphyllum

1.10 Plant description of *Dendrobium aphyllum*

Dendrobium aphyllum is an orchid found in almost every botanical garden and in many amateur collections. It is attractive and easily cultivated with long, pendulous stems. In the resting period it becomes leafless. During the spring season it carries numerous, pinkish violet, fragrant flowers with a pale yellow or whitish lip. *Dendrobium* is a huge genus of orchids. Among the largest orchid genera, today it contains almost about1500 species. Many of these are prettier than *D. aphyllum*, few are as rewarding and persistent in cultivation. The correct name of *D. aphyllum* only came into general use after 1985, when the influential Danish botanist Gunnar Seidenfaden confirmed, although with some reservations, that this was the same species that people were growing as *D. pierardii* (Kabir, 2007). Gradually, the name *D. aphyllum* has come back into use. *D. aphyllum* dried stems commonly used to support the immune system. Latest research has proven their improving effects on the immune system, and these effects may give clues into the key for longevity (Talukder *et al.*, 2002)

1.11 Constrains of orchid cultivation

1.11.1 Diseases and pests

As orchids are attacked by various types of diseases and pests, so careful attempts should be taken for the protection of orchid plants. This plant is a very propitious one for disease infection. So far, more than 30 different viruses are found to attack the orchids in various countries of the world. Among them, *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CyMV) are considered the most important for damaging orchid plants and flowers. Flower necrosis can be caused by CyMV and by fungi such as *Botrytis cinerea*. In *Cattleya* plants, flower color break can be caused by ORSV and ORSV-infected *Cymbidium* can exhibit a mild mosaic which causes nutrient deficiency. These are the most common orchid virus around the world. CymMV was reported for the first time by Jenson (1950) to induce mosaic or black streak in *Cymbidium*. The reverse situation may also occur in some infected plants. When two different viruses produce two different diseases, they may induce the same lesions when they occur in the same plant (Thornberry and Philippe 1964; Wisler *et al*., 1979). Abnormal nutrition and fungal infections also produce

virus-like symptoms (Pataky, 1990). Bacterial diseases include soft and brown rots (Erwinia), bacterial brown spot (Acidovorax), black rot (Pythium and Phytophthora). If unchecked the bacterial soft and brown rot diseases, the infection will rapidly rot the leaves and roots. *Dendrobium* leaves appear yellow and water-soaked and become black and sunken. Black rots infection usually starts from the leaves or roots, though all plant parts are susceptible. If untreated, the disease spreads rapidly and will kill the plants. Fungal Disease includes fusarium wilt (Fusarium) and root rot (Rhizoctonia).

Common orchid pests are of 2 types.

(a) Plant sap feeding insects: It commonly includes scale, mealybugs, aphids, thrips, whiteflies, spider mites.

(b) Chewing pests: It includes anails and slugs, caterpillars, roaches and grasshoppers symptom.

These insect pests are harmful for plants in many ways. They feed on tender young shoot, suck the sap and damage the young bud and shoots that act as the carrier of different diseases.

1.11.2 Implication of diseases

These mentioned diseases are greatly hampering the net production of orchids. They are susceptible to multiple viruses which may cause serious economic losses. At the same time, infected plant material may not be acceptable for export (Loebenstein *et al.,* 1995). Reports that there are more than 50 viruses infecting orchids, *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) are reported to be the most prevalent and economically important (Zettler *et al.,* 1990). In Singapore, the occurrence of *Cymbidium mosaic virus* (CymMV) infection in orchids is higher than that of *Odontoglossum ringspot virus* (ORSV) (Wong *et al.,* 1994).

1.12 Orchid propagation

Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Micropropagation is a modern plant tissue culture method. Tissue culture

is an important tool of biotechnology, which can be used to improve productivity of crop via rapid availability of superior planting stock (Bhatia *et al.*, 2004). It can also be used to produce disease free plants. Various parts of orchids are used as explants for micropropagation. These parts include shoot tip, leaf segment, stem nodal segment, rhizome segment, root segment, flower bud segment, etc. Orchids can be propagated by several methods including, stem and rhizome cuttings. These methods are beneficial because they produce exact clones unlike sexual reproduction. However, it is not the fastest method of production. Seed germination is another method of propagation but this is not genetically identical to the parent. Also, as the seeds are very small with small reserves, they need to be germinated in a nutrient medium for best results (White, 1939). Tissue culture is also used for some special cases of orchid propagation. If a plant is attacked by a disease, it is possible to take a very small piece of the apical meristem from a shoot and culture it to create a disease free plant (Murashige, 1974). Tissue culture techniques for micropropagation of orchids are well known for their exploitation as a major trade in recent years in developed countries (Sagawa and Kunisaki, 1982). Mass propagation of orchid in commercial exploitation, millions of plantlets is produced by tissue culture techniques (Lim-Ho *et al.*, 1985). The environmental conditions required for the growth, development and culture of orchids are adequately available throughout the year in Bangladesh.

Many studies on micropropagation of orchids have been carried out (Fu, 1978; Lin, 1986; Tanaka, 1987; Kobayashi *et al.*, 1991; Ichihashi, 1992). Tokuhara and Mu (1993) reported that the appropriate combination and concentrations of hormones, organic additives and the composition of macro and micro elements in the culture medium were of key importance for micropropagation of *Dendrobium* on commercial scale. Kobayashi *et al.* (1991) used healthy and sterilized protocorms of *Dendrobium transparens* as explants in his experiment. A combination of 0.5 mg/l BA and 0.5 mg/l NAA induced maximum protocorm like bodies (20.40 × plantlet-1). Among all treatments of BA, highest protocorm like bodies (12.30 × plantlet-1) was obtained in medium containing 0.2 mg /l BA. Among different concentrations of BA, 0.2 mg/l was found to be the most effective on enhancing the plant height. Explants cultured in the presence of 0.5 mg/l BA along with 0.5 mg/l NAA contained the largest number of leaf.

In another paper, Kurup *et al*. (2005) reported that, the earliest bud initiation was observed in 4.0 mg/l BA (9.67d) in *Dendrobium sonia*. Similar 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 0.5, 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.

Among different plant growth hormone so far studied, benzyl amino purine (BAP) and benzyl purine (BA) are considered to be more important for *in vitro* propagation of 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 (Pradhan *et al*., 2013; Saiprasad *et al.*, 2002), leaf (Chen *et al.*, 2001), and stem nodes (Pathania *et al.*, 1998). Pradhan *et al.* (2013) carried out their experiment using shoot tips about 5 mm length. Explants were excised for *in vitro* shoot developed and inoculated on MS basal medium with or without supplement with various combination and concentration of BAP and NAA for inducing multiple shoots. Among different concentration of BAP alone, MS + BAP (1.5 mg/l) was most effective for shoot proliferation which took only four weeks of culture to start multiplication. Here, combine treatment of BAP with NAA on MS medium gave synergistic effect on shoot growth and multiplication. MS medium supplemented with BAP (2.0 mg/l) and NAA (0.5 mg/l) was the most effective condition for shoot proliferation in *D. desnsiflorum* which took three weeks for induction of shoots. These findings were supported by the work of different researchers. Swar and Pant (2004) obtained maximum number of shoots on MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) in *Coelogyne cristata* Lindl.

Vegetative propagation through tissue culture is important for multiplication of orchids. In the culturing of plant cells, plant growth regulators are used to produce callus growth, multiplication and rooting. Also, the mode of morphogenetic differentiation depends on a number of factors such as type and source of explants, orientation of explants in the culture medium, concentration and combinations of plant growth regulators, state of the culture medium, culture conditions, and even culture period (Seeni and Latha 2000; Zhao *et al.* 2008). Like other countries, appropriate propagation technique for large scale production of

hybrid orchids would be a profitable source of earning foreign exchange in Bangladesh. Also, to develop protocols for an efficient regeneration, it is important to investigate the roles and interactions of different genotypes, explant sources, and hormonal effects. Therefore, the present experiment was planned to investigate the effect of different plant growth regulators like BA and IBA at different concentrations and combinations using stem nodal explants growth and development, shoot multiplication, root formation and plant regeneration.

1.13 Objectives of this study

Considering the above impressions in mind, the present study was undertaken with the following objectives:

1. To investigate the effect of different concentrations of BA on *in vitro* shoot initiation.
2. To investigate the effect of different concentrations of BA+IBA combinations on *in vitro* shoot initiation.
3. To investigate the effect of different concentrations of IBA on *in vitro* root initiation.
4. To investigate the effect of different concentrations of BA+IBA combinations on *in vitro* root initiation.
5. Acclimatization of the regenerated plantlets.

Materials and Methods

Chapter II

Materials and Methods

An experiment on *in vitro* regeneration of *D. bensoniae* and *D. aphyllum* were conducted in the Tissue Culture Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Sher-e-Bangla Nagar, Dhaka-1207, during the period of June, 2014 to August, 2015. Five experiments were conducted to fulfil the objectives of the present study.

Experiment 1: Effect of BA on *in vitro* shoot initiation of *D. bensoniae* and *D. aphyllum.*

Experiment 2: Multiple shoot proliferation in *D. bensoniae* and *D. aphyllum*.

Experiment 3: Effect of IBA on *in vitro* root initiation of *D. bensoniae* and *D. aphyllum.*

Experiment 4: Combined effect of different concentrations of BA+IBA combination on *in vitro* root initiation of *D. bensoniae* and *D. aphyllum.*

Experiment 5: Acclimatization and establishment of plants in natural field condition.

2.1 Experimental materials

2.1.1 Plant materials

Disease free shoot nodes of *D. bensoniae* and *D. aphyllum* were used as explant in this experiment.

2.1.2 Sources of plant materials:

The materials were collected from Horticulture Research Centre (HRC), Bangladesh Agricultural Research Institute, Gazipur (BARI) (Plate 2.1).

Plate 2.1: Plant materials of orchid collected from HRC, BARI

a) *D. bensoniae* b) *D. aphyllum*

2.2 Culture media

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^{o}C temperature.

1. BA (0.5, 1.0, 1.5 and 2.0 mg/l) alone were used for shoot proliferation.
2. IBA (0.5, 1.0, 1.5 and 2.0 mg/l) were applied for root formation.
3. Sucrose (3%) was used as carbon source and media were solidified with agar (0.8%).
4. The pH was adjusted to pH 5.8 prior to autoclaving at a temperature of 121^{o}C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

2.3 Preparations of the stock solutions

To prepare these hormonal supplements, they were dissolved in proper solvent as shown against each of them below.

Hormone (Solute)	**Solvents used**
BA	1 N NaOH
IBA	70% ethanol

In present experiment, the stock solution of hormones was prepared by the following procedure. 100 mg of powder was placed in a small beaker and then dissolved with few drops of 70% ethyl alcohol or 1 (N) NaOH solvent. Finally, the volume was made upto 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^{o}C for use up to two month. Growth regulators were purchased from Sigma, USA.

2.4 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. 5gm 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 and mixed well.

Step-4. The volume was made up to 1000 ml with addition of sterile distilled water.

Step-5. The pH was adjusted at 5.8.

Step-6. Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

2.5 Steam heat sterilization of media (Autoclaving)

For sterilization the culture medium was poured in 200 ml culture bottles and then autoclaved at a temperature of 121 ^{o}C for 60 minutes at 1.06 kg/cm^{2} (15 PSI) pressure. After autoclaving the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explant.

2.6 Instruments

Metal instruments *viz*., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave.

2.7 Preparation of explants

The trimmed shoot nodes were washed thoroughly under running tap water and then with sterilized distilled water for several times. Subsequently the explants were transferred to laminar airflow cabinet and kept in a 250 ml sterilized beaker. The beaker with explant 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 media.

2.8 Inoculation of culture

The sterilized explants were inoculated carefully following proper sterilization process within laminar air flow cabinet. Prior to use, the surface of the laminar air flow bench was swabbed down with 70% ethyl alcohol and the interior sprayed with the same. 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 using a spirit burner. 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 in to suitable size (0.5-1 cm), explants are transferred to culture bottles containing MS medium with plant growth regulator. 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 was transferred to growth room.

2.9 Incubation

The bottles were kept to the culture racks and allowed to grow in controlled environment. The cultures were maintained at 25±2 °C with light intensity varied from 2000–3000 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).

2.10 Maintainance of proliferating shoots

The explants were cultured on MS nutrient medium supplemented with different concentrations of BA and IBA. After successful shoot proliferation, subculture was done with newly formed shoot. Shoot ware 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 15 days after inoculation.

2.11 Root formation on regenerated shoot

Newly formed shoot with adequate length were excised individually from the culture vial and transferred to rooting media. The growth regulator IBA was used in different concentration (0.5, 1.0, 1.5, 2.0 mg/l) along with MS media. The observations on development pattern of root were made throughout the entire culture period.

2.12 Acclimatization

In vitro propagated plants are made to adapt to natural environment in field condition.

Step-1: After 60 days of culture on rooting media, the plantlets 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 plantlets were then transplanted into the pots with special care.

Step-2: After planting, the plantlets were covered with plastic bags spraying water inside the plastic bags 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: The plants were shifted to shade house with less humidity and indirect sunlight. The orchid pots were grew at room temperature.

2.13 Data recording

The observation on development pattern of shoot and root were made throughout the culture period. Five replicates each of them containing 4 bottles (single shoot per culture bottle) were used per treatment. Data were recorded after 15, 30 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 15 days till 60 days of culture.

The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

1. Percent age of explant showing shoot induction
2. Number of shoots per explant
3. Average length of shoots (cm)
4. Days to leaf induction
5. Number of leaves per explant
6. Length of leaves per explant
7. Days for root induction
8. Number of roots per explant
9. Average length of roots (cm)

2.14 Statistical analysis

The experiment was one factorial set up in a completely randomized design (CRD) with five replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique and at 5% probability level using MSTAT-C (1990) program.

Results and Discussion

Chapter III

Results and Discussion

Five separate experiments were performed to establish a rapid micropropagation protocol of the orchid varieties *Dendrobium bensoniae* and *Dendrobium aphyllum*.

3.1 Experiment 1: Effect of BA on *in vitro* shoot initiation of *D. bensoniae* and *D. aphyllum*

3.1.1 Effect of BA on multiple shoot proliferation in *D. bensoniae*

The results of the effect of different concentrations of BA have been presented under following headings with Table 1 and Plate 3.1.

3.1.1.1 Percentage of explant showing shoot induction

There was a significant variation on percentage of explant showing shoot induction in presence of various concentrations of BA supplementations. The highest percentage (80%) of shoot induction was in treatment with 2.0 mg/l BA and the lowest percentage (20%) was induced in hormone free media in *D. bensoniae* (Table 1). Baksha *et al.* (2005) reported the maximum 80% shoot induction in 2.0 mg/l BA and 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. This increase is also supported by Hashemabadi and Kaviani (2008). According to Tan *et al.* (2011), the highest number of shoots per explant was observed from medium supplemented with 1.0 mg/l BAP; the shoot induction rate decreased when BAP concentrations exceeded 3.0 mg/l. The same trend was observed by Mackay *et al.* (1995) in *Cercis canadensis*. George et al. (2008) noted that elevated high level of cytokinin caused many small shoots to be produced, but such shoots typically failed to elongate. Jaramillo *et al.* (2008) reported that presence of BA in the culture medium is necessary for shoot regeneration. Furthermore, higher concentration reported to increase the shoot regeneration frequency in *Dendrobium bensoniae* and *Dendrobium macrostachyum.* This is in agreement with the present experiment data.

3.1.1.2 Days to shoot initiation

Shoot initiation period was recorded on day basis. Variations were observed among different concentrations of BA on days to shoot induction. The highest number of days to shoot induction was recorded in control of *D. bensoniae* (25 days) and 2.0 mg/l BA required lowest for *D. bensoniae* (17 days) (Table 1). Baksha *et al.* (2005) noticed that minimum of 24 days were required for shoot initiation in *Dendrobium moschatum* at 1.5 mg/l BA.

3.1.1.3 Average number of shoots per explants

In this experiment, all the media tested alone or with BA, MS medium with 2.0 mg/1 BA was found to be most effective for shoot multiplication which indicates that MS medium with BA might be suitable for shoot proliferation. This result was also supported by previous work of several researchers on *Dendrobium densiflorum* (Luo *et al*., 2006), *Geodorum densiflorum* (Bhadra and Hossain, 2003), Cymbidium and Cattleya (Nagarju *et al*., 2003). There was a significant influence of different concentrations of BA on the number of shoots per explant in this experiment. It was observed that MS media supplemented with 2.0 mg/l BA showed highest number (4.66 ± 0.57) of shoot induction at 30 days after inoculation, whereas the lowest number of shoots (0.49 ± 0.57) at 30 days was found with hormone free media in *D. bensoniae* (Table 1 and Plate 3.1). The importance of BA in stimulating shoot elongation has been highlighted in *Vanilla planifolia, Dendrobium aphyllum* (Geetha and Shetty, 2000), *Dendrobium formosum* (Nasiruddin *et al*., 2003) and *Achilleamillefolium* (Shatnawi, 2013). Similar effect of kinetin added media producing better shoot growth (>2.0 cm) and BA added media producing less (<1.5 cm) shoots was reported during the *in vitro* propagation of *Dendrobium Sonia* 'BOM17' and 'BOM28' (Martin *et al*., 2005). But this experiment showed that, BA added media was found to be most effective in multiple shoot proliferation in both *D. bensoniae* and *D. aphyllum*.

3.1.1.4 Days to leaf induction

Leaf initiation period was recorded on day basis. The mean value of the data provided the days to leaf initiation. Significant variations were observed among different concentrations of BA on the days to leaf induction. The highest number of days to leaf induction was recorded

in hormone free MS media of *D. bensoniae* (41.12 days), whereas it was lowest in the concentration of 2.0 mg/l BA (15 days) (Table 1).

3.1.1.5 Average number of leaves per explant

Cytokinin level, in this case BA produced a significant response upon the number of leaves per explants. The number of leaves was recorded at 60 days after inoculation. The number of leaves per explant was significantly different due to the different concentrations of BA supplementations. The highest number of leaves per explant (9.33 ± 1.15) was noticed from 2.0 mg/l BA in *D. bensoniae*, whereas the lowest was (1.40 ± 0.0) in control treatment (Table 1). The mean leaf number varied from 4.00 to 6.85 produced after four week in *D. bensoniae* culture. The maximum leaf number (8.66) was obtained for the treatment with 1.0 mg/l of BA in *Dendrobium pierardii* between fourth to fifth week (Kurupet *et.al*, 2005).

3.1.1.6 Average length of leaves/plantlet

The length of leaves was recorded at 60 days after inoculation. The mean value of the data provided the length of leaves/plantlet. The highest length of leaf was found (1.16 ± 0.21 cm) at 60 DAI in the treatment with MS+1.5 mg/l BA. The lowest length of leaf was found (0.40 ± 0.0 cm) at 60 DAI in control treatment. These findings are in agreement with the investigation of Manik (2009), where the highest length of leaf (1.29 ± 0.16 cm) was obtained in the treatment with 1.5 mg/L BA in *Dendrobium barisanum*.

Table 1: Efficacy of different BA concentrations on induction of shoots and leaves in *D. bensoniae.*

Hormonal (BA) concentration (mg/l)	Number of explants initiated shoot	% of explants showing shoot induction	Initiation of regeneration (Days)	Average number of shoots per explants ± SD (30 DAI)	Days to leaf induction	Average number of leaves per explants ± SD (60 DAI)	Average length (cm) of leaves per explants ± SD (60 DAI)
MS (Control)	4	20	25	0.49±0.57	41.12	1.40±0.00	0.40±0.00
0.5	10	50	21	4.00± 0.57	26.67	6.33±1.00	0.96±0.21
1.0	13	65	18	4.33±1.00	25.33	8.00±0.57	0.96±0.38
1.5	15	75	22	3.00±1.00	17.00	8.33±0.57	1.16±0.21
2.0	16	80	17	4.66±0.57	15.00	9.33±1.15	1.13±0.10
SE				1.20		0.60	0.04
LSD				1.99		1.40	0.38
Level of Significance (5%)				*		*	*

DAI= Days after inoculation

* = presence of level of significance

*20 explants were taken for each treatment

3.1.2 Effect of BA on multiple shoot proliferation in *D. aphyllum*

Different results were found with different concentrations of BA supplementations which have been presented under following headings with Table 2 and Plate 3.1.

3.1.2.1 Percentage of explant showing shoot induction

In the present study, MS medium alone was not so effective for induction of multiple shoots. Similar result was obtained in *Dendrobium* species (Yasugi *et al*., 1994). This revealed that the addition of plant growth regulators in nutrient medium is essential for growth, development and proliferation of shoot. Here, in this experiment, more or less similar result was noticed in case of *D. aphyllum* comparing with *D. bensoniae*. The highest percentage (85%) of shoot induction was noticed in presence of 2.0 mg/l BA and the lowest percentage (25%) was induced in hormone free MS media (Table 2). Singh *et al*., (2007) reported the maximum 90% shoot induction was noticed in 1.5 mg/l BA and minimum 35% in case of 0.5 mg/l BA in *Dendrobium macrostachyum*. Jaramillo *et al.* (2008) recommended that presence of BA and BAP in the culture medium is necessary for shoot regeneration frequency.

3.1.2.2 Days to shoot initiation

Variations were observed among different concentrations of BA on days needed to shoot induction. The highest number of days to shoot induction was recorded in control of *D. aphyllum* (27 days) and 1.5 mg/l BA was required lowest for *D. aphyllum* (16 days) (Table 2). Shatnawi (2013) reported that minimum of 23 days were required for shoot initiation in *Dendrobium acianthum* at 1.5 mg/l BA. Ganesh *et al.* (1996) investigated the effects of culture media type and he used BA and BAP on shoot proliferation *in vitro* of *Dendrobium piradeii*; quick and good shoot proliferation was observed only in the presence of BA.

3.1.2.3 Average number of shoots per explants

Among the different combinations tested in this study, BA with 2.0 mg/1 concentration was found to be effective for the shoot multiplication. The obtained result showed that MS medium supplemented with 2.0 mg/l BA is suitable for shoot multiplication. The previous

work of several researchers also showed that the high concentration of BA was favorable for the induction of multiple shoots. Similar results were obtained by Talukdar *et al.* (2003) in *Dendrobium* orchid, and also Sunitibala and Kishor (2009) found in *Dendrobium transparens*. In this experiment, there was a significant influence of different concentrations of BA on the number of shoots per explant. It was observed that MS media supplemented with 2.0 mg/l BA showed highest number (3.66 ± 0.57) of shoot induction at 30 days after inoculation, whereas the lowest number of shoots (0.50 ± 0.57) at 30 days was found with hormone free media in *D. aphyllum* (Table 2 and Plate 3.1). Variations in shoot proliferation due to BA concentrations were also reported by Bhandari *et al*. (2010) and Gantait *et al.* (2010). Baksha *et al.* (2005) noticed that 5.2 shoots per explant were induced in media supplemented with 2.0 mg/l BA in *Dendrobium aphyllum* at 30 DAI. This is in agreement with the present experiment data.

3.1.2.4 Days to leaf induction

Leaf induction period was recorded on day basis. Significant variations were noticed among different concentrations of BA on days to leaf induction. The maximum number of days to leaf induction was recorded in control treatment of *D. aphyllum* (39.30 days), whereas it was lowest in presence of 2.0 mg/l BA (15.67 days) (Table 2).

3.1.2.5 Average number of leaves per explant

The number of leaves was recorded at 60 days after inoculation. The number of leaves per explant was significantly different due to the different concentrations of BA supplementations. The highest number of leaves per explant (9.66 ± 1.15) was noticed from 1.5 mg/l BA, whereas the lowest was (1.33 ± 0.57) in control treatment of *D. aphyllum* (Table 2).

3.1.2.6 Average length of leaves/plantlet

The length of leaves was recorded at 60 days after inoculation. The mean value of the data provided the length of leaves/plantlet. The highest length of leaf was found in the variety of *D. aphyllum* (1.29 ± 0.21 cm) at 60 DAI in the treatment with MS+1.0 mg/l BA (Table 2). The lowest length of leaf was found (0.60 ± 0.05 cm) at 60 days after inoculation in control

treatment. These findings are in agreement with the investigation of Wang *et al.*, (2006), where the highest average length of leaf (1.35±0.19 cm) was obtained in the treatment with 1.5 mg/l BA in *Dendrobium barbatum* at 60 DAI.

Table 2: Efficacy of different BA concentrations in induction of shoots and leaves in *D. aphyllum*.

Hormonal (BA) concentration (mg/l)	Number of explants initiated shoot	% of explants showing shoot induction	Initiation of regeneration (Days)	Average number of shoots per explants ± SD (30 DAI)	Days to leaf induction	Average number of leaves per explants ± SD (60 DAI)	Average length (cm) of leaves per explants ± SD (60 DAI)
MS (Control)	5	25	27	0.50±0.57	39.30	1.33 ±0.57	0.60±0.05
0.5	9	45	18	3.00±1.00	26.00	7.00 ±0.57	1.23±0.29
1.0	13	65	22	3.36±1.15	24.00	8.66 ±0.00	1.29±0.21
1.5	16	80	16	2.60±0.57	16.33	9.66±1.15	1.16±0.31
2.0	17	85	19	3.66±0.57	15.67	9.00±0.57	1.09±0.21
SE				1.00		1.62	0.09
LSD				1.81		2.33	0.39
Level of Significance (5%)				*		*	*

DAI= Days after inoculation

* = presence of level of significance

*20 explants were taken for each treatment

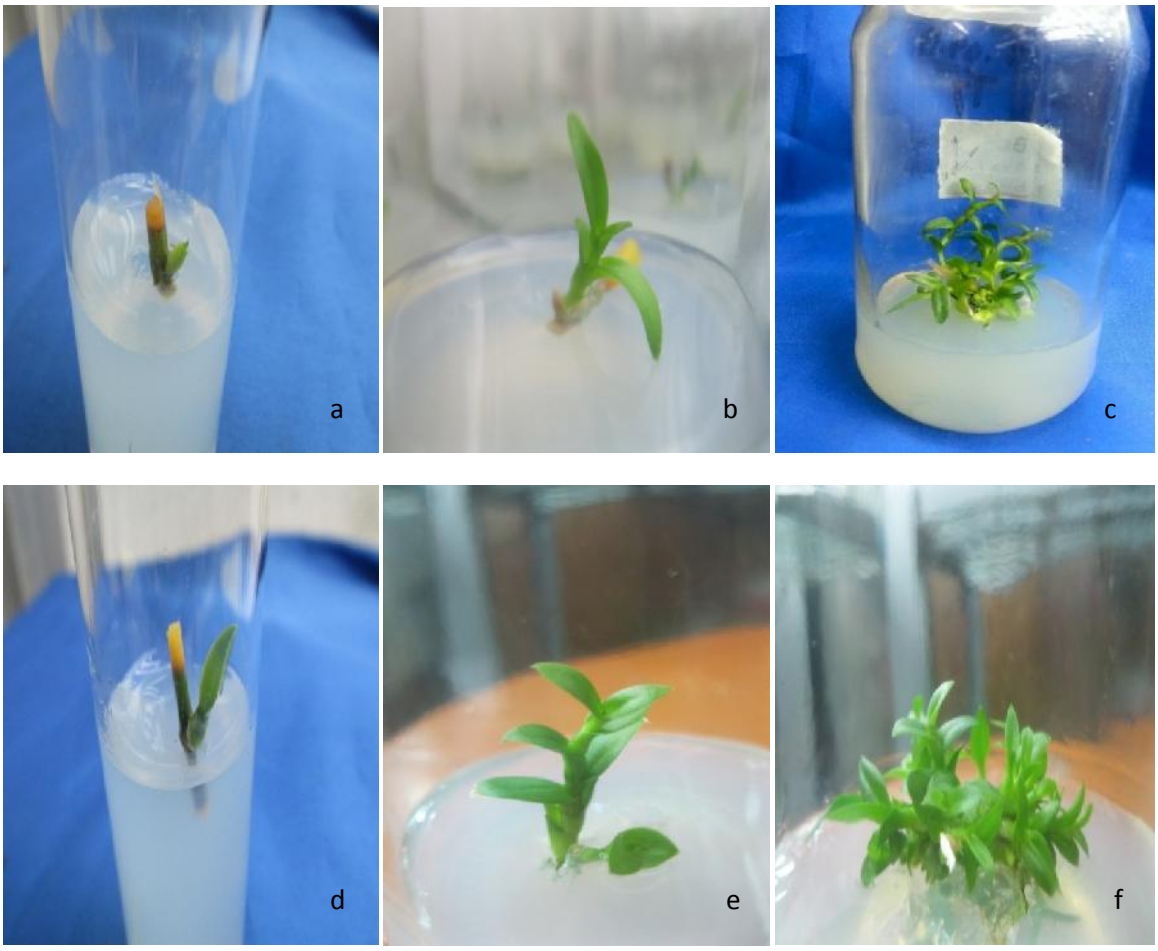

Plate 3.1 Shoot induction, regeneration and multiplication of *D. bensoniae* and *D. aphyllum* on MS media supplemented with 2.0 mg/l BA.

D. bensoniae at a) 15 DAI b) 30 DAI c) 45 DAI and *D. aphyllum* at d) 15 DAI e) 30 DAI f) 45 DAI.

3.2 Experiment 2: Multiple shoot proliferation in *D. bensoniae* and *D. aphyllum*

The results are presented separately under different headings below.

3.2.1 Combined effect of BA+IBA combinations in *D. bensoniae*

The results of the combined effect of different concentrations of BA+IBA have been presented under following headings.

3.2.1.1 Percentage of explant showing shoot induction

Significant variations were observed on percentage of explant showing shoot induction in presence of various concentrations of cytokinin and auxin supplementations. The highest percentage (90%) of shoot induction was observed in the treatment with 1.0 BA+2.0 IBA (mg/l) and the lowest percentage (25%) was induced in hormone free media in case of *D. bensoniae* (Table 3). Some authors have reported that the addition of IBA along with BA reduced induction and regeneration (Arditti and Ernst, 1993), whereas, others reported that an appropriate combination of BA and IBA stimulated shoot formation (Tokuhara and Mii, 1993; Tisserat and Jones, 1999; Roy and Banerjee, 2003).

3.2.1.2 Days to shoot initiation

The initiation of regeneration frequency was late in the control treatment. The highest number of days to shoot induction was recorded in the control treatment of *D. bensoniae* (26 days). Here, 1.0 BA + 1.5 IBA (mg/l) were required lowest for *D. bensoniae* (15 days) (Table 3).

3.2.1.3 Average number of shoots per explant

Among the different combinations tested in this study, BA (1.0 mg/1) and IBA (1.5 mg/l) was found to be effective for the shoot multiplication. The obtained result showed that combination of BA and IBA is also suitable for shoot multiplication. 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 (2003) reported in *Dendrobium densiflorum.* Here, data was recorded after 30 days of culture. There was significant influence of different concentrations of BA+IBA on the number of shoots per

explant after 30 days of inoculation. The results have been presented in Table 3. Here 1.0 BA + 1.5 IBA (mg/l) gave the highest number of shoots (3.67 ± 0.57), whereas the lowest number of shoots (0.95 ± 0.0) was found with hormone free MS media in *D. bensoniae* (Table 3). Vij and Kaur (1998) also reported similar results where BA-enriched medium in combination with IBA favoured multiple shoot bud formation in *Dendrobium bensoniae*. Similarly, there are earlier reports on accentuated regeneration potential of *Dendrobium moschatum* pseudobulb explants (Vij and Sood 1982). In the present study, the regeneration pathway to shoot formation was directly through the shoot bud of orchid.

3.2.1.4 Days to leaf initiation

The highest number of days to leaf initiation was recorded in control of *D. bensoniae* (37.21 days) and 1.0 BA+1.5 IBA (mg/l) was recorded lowest for *D. bensoniae* (17.95 days) (Table 3). These findings are in agreement with the investigation of Hossain (2013), where the minimum days to leaf induction was obtained in the treatment with 1.0 BA+ 2.0 IBA (mg/l) in *Dendrobium barisanum.*

3.2.1.5 Average number of leaves per explant

The number of leaves also increased with days after inoculation. Maximum number of leaves was obtained at 60 DAI from these treatments compared to control. The highest number of leaves per explant (9.33 ± 0.57) was noticed from 1.0 BA+2.0 IBA (mg/l), whereas the lowest were (1.23 ± 1.0) in control treatment of *D. bensoniae*. Gantait *et al.* (2010) noticed that 11.23 leaves per explant were induced in media supplemented with 0.5 BA+ 1.0 IBA (mg/l) in *Dendrobium longicornu.* This is also in agreement with the present experiment data.

3.2.1.6 Average length of leaves per explant

The length of leaves was varied due to the different concentrations of BA+IBA supplementations. The highest length of leaves per explant (1.00 ± 0.20 cm) was noticed from 0.5 BA+1.0 IBA (mg/l), whereas the lowest was (0.75 ± 0.0 cm) in control of *D. bensoniae* (Table 3).

Table 3: Efficacy of BA+IBA combinations in induction of shoots and leaves in *D. bensoniae.*

Hormonal (BA+IBA) concentration (mg/l)	Number of explants initiated shoot	% of explants showing shoot induction	Initiation of regeneration (Days)	Average number of shoots per explants ±SD (30 DAI)	Days to leaf induction	Average number of leaves per explants ± SD (60 DAI)	Average length (cm) of leaves per explants ± SD (60 DAI)
MS (Control)	5	25	26	0.95±0.0	37.21	1.23±1.00	0.75±0.00
0.5 + 0.5	11	55	20	3.00±1.15	25.15	6.35±0.00	0.90±0.10
0.5 + 1.0	12	60	19	3.34±1.00	27.30	8.00±0.57	1.00±0.20
1.0 + 1.5	15	75	15	3.67±0.57	17.95	8.25±0.57	0.85±0.10
1.0+ 2.0	18	90	21	2.65±0.57	19.85	9.33±0.57	0.80±0.20
SE				0.60		0.60	0.10
LSD				1.40		1.41	0.25
Level of Significance (5%)				*		*	*

DAI= Days after inoculation

* = presence of level of significance

*20 explants were taken for each treatment

3.2.2 Combined effect of BA+IBA combination in *D. aphyllum*

The results of the combined effect of different concentrations of BA+IBA combination have been presented under following headings.

3.2.2.1 Percentage of explant showing shoot induction

There was a significant variation on percentage of explant showing shoot induction in presence of various concentrations of BA+IBA supplementations. The highest percentage (90%) of shoot induction was induced in presence of 1.0 BA +2.0 IBA (mg/l) and the lowest percentage (20%) was induced in hormone free media in case of *D. aphyllum* (Table 4). Development of shoot buds and formation of multiple shoots from explants were noticed in all the experimental conditions.

3.2.2.2 Days to shoot initiation

Shoot induction period was recorded in day basis. The highest number of days to shoot induction was recorded in the control treatment (25 days), whereas 1.0 BA + 1.5 IBA (mg/l) was required lowest for *D. aphyllum* (16 days) (Table 4).

3.2.2.3 Average number of shoots per explant

Data was recorded after 30 days of culture. There was significant influence of different concentrations of BA+IBA on the number of shoots per explant after 30 days of inoculation. 1.0 BA+1.5 IBA (mg/l) gave the highest number of shoots (3.34 ± 0.57), whereas the lowest number of shoots (0.80 ± 0.0) was found with hormone free media in *D. aphyllum.* Here, quick responses of regenerated shoots were observed and all the regenerated shoots were healthy. Tokuhara and Mii (1993) reported that the combination of hormones was of key importance for the micropropagation of *Dendrobium*. A stimulatory effect of BA and IBA together in the medium has been reported for certain species of orchids before (Kosir *et al*. 2004). Some authors have reported reduced induction and regeneration in medium supplemented with BA and IBA (Arditti and Ernst 1993), others reported that an appropriate combination of BA and IBA stimulated shoot formation (Tokuhara and Mii 1993; Tisserat and Jones 1999; Roy and Banerjee 2003). Similar results were also obtained in this

experiment where a maximum number of shoots were recorded in medium containing a combination of BA (1.0 mg/l) and IBA (1.5 mg/l).

3.2.2.4 Days to leaf initiation

The highest number of days for leaf initiation was recorded in control (40.34 days), whereas 1.0 BA+1.5 IBA (mg/l) was recorded lowest for *D. aphyllum* (18.95 days) (Table 4). Each new shoot initials continued to grow separately and develop leafy shoots.

3.2.2.5 Average number of leaves per explant

Among the different combinations tested in this study, 1.0 BA+ 1.5 IBA (mg/1) were found to be effective for induction of highest number of leaves. The previous work of several researchers also showed that the high concentration of BA and IBA was favorable for the induction of highest number of leaves. The number of leaves was recorded after 60 days of culture. The highest number of leaves per explant (9.85 ± 1.0) was noticed from 1.0 BA+1.5 IBA (mg/l), whereas the lowest were (1.42 ± 1.0) in control of *D. aphyllum*. Similar results were obtained by Sunitibala and Kishor (2009) in *Dendrobium transparens*.

3.2.2.6 Average length of leaves per explant

The length of leaves was varied due to the different concentrations of BA+IBA supplementation. The highest length of leaves per explant (0.88 ± 0.20 cm) was noticed from 1.0 BA+2.0 IBA (mg/l), whereas the lowest was (0.68 ± 0.05 cm) in control treatment of *D. aphyllum* (Table 4).

Table 4: Efficacy of BA+IBA combination on induction of shoots and leaves in *D. aphyllum*.

Hormonal (BA+IBA) concentration (mg/l)	Number of explants initiated shoot	% of explants showing shoot induction	Initiation of regeneration (Days)	Average number of shoots per explants ± SD (30 DAI)	Days to leaf induction	Average number of leaves per explants ± SD (60 DAI)	Average length (cm) of leaves per explants ± SD (60 DAI)
MS(Control)	4	20	25	0.80±0.00	40.34	1.42±1.0	0.68±0.05
0.5 + 0.5	9	45	17	2.67±0.57	27.13	7.00±0.00	0.73±0.21
0.5 + 1.0	12	60	21	2.35±1.15	24.25	5.58±0.57	0.79±0.15
1.0 + 1.5	15	75	16	3.34±0.57	18.95	9.85±1.00	0.83±0.15
1.0 + 2.0	18	90	20	2.65±1.15	19.21	9.00±0.57	0.88±0.20
SE				0.66		1.59	0.95
LSD				1.48		2.31	0.29
Level of Significance (5%)				*		*	*

DAI= Days after inoculation

* = presence of level of significance

*20 explants were taken for each treatment

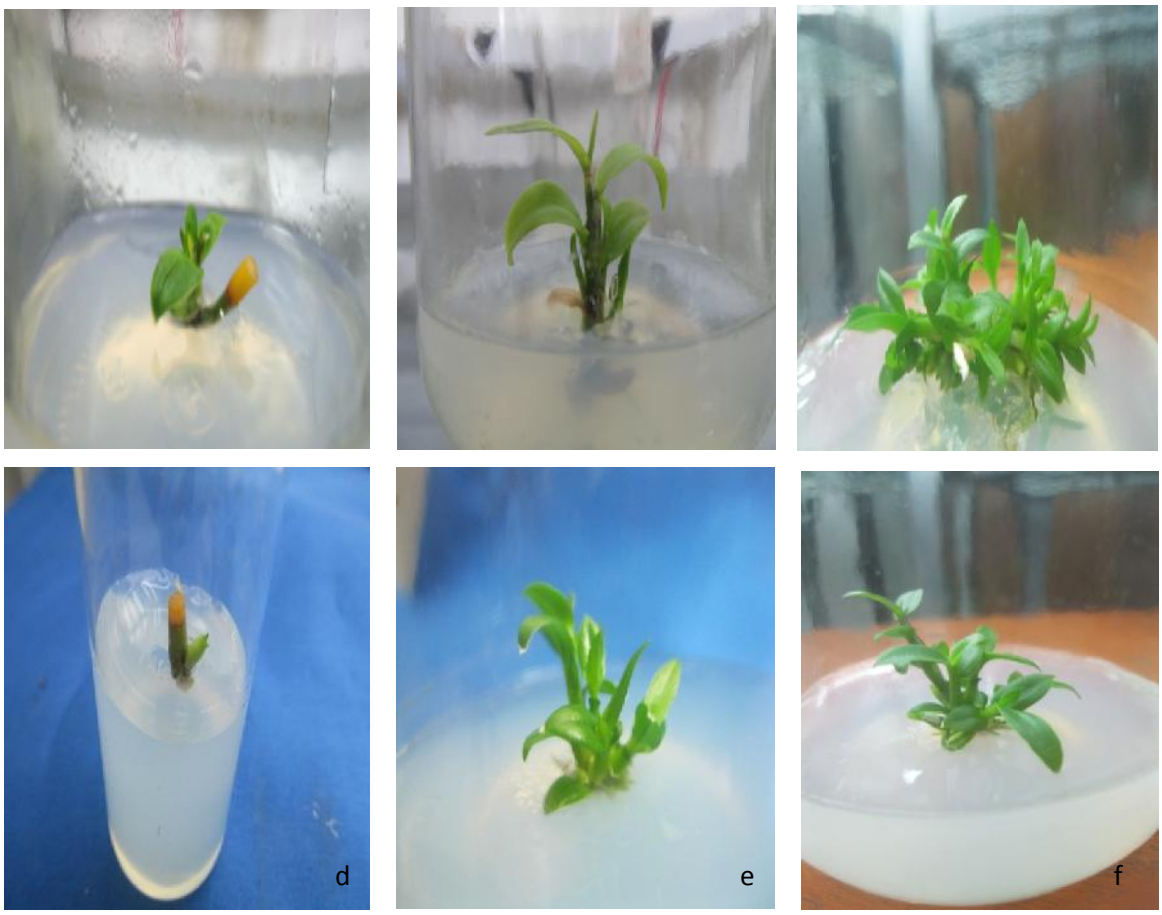

Plate 3.2: Shoot induction, regeneration and multiplication of *D. bensoniae* and *D. aphyllum* on MS media supplemented with 1.0 mg/l BA + 1.5 mg/l IBA.

D. bensoniae at a) 15 DAI b) 30 DAI c) 45 DAI and *D. aphyllum* at d) 15 DAI e) 30 DAI f) 45 DAI.

3.2.3 Final comparison of hormonal treatment

The nodal segments of *D. bensoniae and D. aphyllum* showed various responses on MS medium supplemented with different plant growth regulators either separately or in combinations. These results showed that, 2.0 mg/l BA supplementation on MS medium gives better response than all other combinations of BA and BA + IBA concentrations under study in both *Dendrobium bensoniae* and *Dendrobium aphyllum*. The difference in result varies into species to species. This might be due to a different genus of orchids in the present study. Variations in species to species due to genotypic and phenotypic differences were also reported by Gregor Johann Mendel (1900), Wilhelm Johannsen (1908), Thomas Hunt Morgan (1915) and Ronald Fisher (1930).

3.2.4 Regeneration capacity using different hormone

Among the two varieties of *D. bensoniae* and *D. aphyllum*, MS medium supplemented with 2.0 mg/l BA showed highest number (4.66 ± 0.57) of shoot induction at 30 days after inoculation in *D. bensoniae*. This experiment showed that, without any nutrient rich mixture such as, coconut water, banana extract or activated charcoal, MS+2.0 mg/l BA gives better number of shoots in *D. bensoniae*. Variations in shoot proliferation due to BA concentrations were also reported by Bhandari *et al*. (2010) and Gantait *et al.* (2010). Baksha *et al.* (2005) noticed that 5.2 shoots per explant were induced in media supplemented with 2.0 mg/l BA in *Dendrobium aphyllum* at 30 DAI. Similar results were obtained by Talukdar *et al.* (2003) in Denrobium orchid, Sunitibala and Kishor (2009) in *Dendrobium transparens*. In this experiment, cytokinin along with MS medium gives the satisfactory number of shoots per explants. Other scientists also used different auxin and cytokinin combinations in their experiments and got different results of their experimental data. George and Kumari (2013) used different concentrations of auxin and cytokinin combination. Half MS (Murashige and Skoog, 1962) fortified with 3% sucrose, 200.0 ml/l coconut water (CW), 0.5 g/l activated charcoal (AC) and 6.2 % agar was used as the basal media**.** BA 4.0 + IAA 1.0 (mg/l) showed the highest days for shoot bud induction. Maximum number of shoots found 2.33 after 4 week in *Dendrobium nobile*. The maximum shoot number (4.66) was obtained for the treatment with 2.0 mg/l kinetin + 0.1 mg/l NAA (George and Kumari, 2013). Such optimum combination of kinetin and NAA producing highest number of multiple shoots of Dendrobium var. Betty Ho was reported by Kurupet *et al.* (2005), supporting the present study. Bhattacharjee and Islam (2014) reported that, shoot differentiation was first observed after 28 days of culture on MS media fortified with BAP, NAA, IAA and Kinetin at the concentration of (0.5-1.5) mg/l. The highest rate of shoot induction (7.52) per explants was observed in MS medium fortified with1.0 mg/l BAP and 1.0 mg/l NAA in *Dendrobium calcaratum* after 7 week. Multiple shoots were produced in combination of BAP and NAA medium reported previously in various orchid species by different researcher (Nhat and Dung, 2006; Rahman *et al*., 2009; Long *et al*., 2010; Pant and Shresta, 2011). In this experiment, MS +2.0 mg/l BA gives the better number of shoots (4.66 ± 0.57) at 30 days after inoculation without using any coconut water, banana extracts or activated charcoal.

3.3. Experiment 3. Effect of IBA on *in vitro* root initiation of *D. bensoniae* and *D. aphyllum*

3.3.1 Root formation of *D. bensoniae* using different concentrations of IBA

Newly formed shoot with adequate length were excised individually from the culture vial and transferred to the rooting media. The growth regulator IBA was used in different concentration (0.5, 1.0, 1.5, 2.0 mg/l) along with MS media. The observations on development pattern of root were made throughout the entire culture period. The results of experiment have been presented under different heading utilizing Table 5 and Plate 3.3.

3.3.1.1 Percentage of explants showing root induction

The application of auxins to micropropagated shoots seems to intensify the root number by mounting the endogenous contents of enzymes (Asghar *et al.,* 2011). Liu *et al*. (2002) reported that auxin induces the complicated process of lateral root formation through repetitive cell division. George *et al*. (2008) suggested that auxins are essential for the maintenance of polarity of the plants. There was a significant variation of IBA concentrations on percentage of explants showing root induction. The highest percentage (90%) of root induction was induced in treatment with 2.0 mg/l IBA and the lowest percentage (40%) was induced in hormone free media in case of *D. bensoniae* (Table 5). Auxins have been reported to enhance root formation in plants by Bhojwani and Razdan (1983). In this study, IBA 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 (George *et al*., 2008; Hasan *et al*., 2010).

3.3.1.2 Days to root induction

Hormonal concentration has significant level of variation on days for root induction. The maximum 41.21 days to root induction was observed in media lack of growth regulator. Minimum 23.33 days is required in case of 2.0 mg/l of IBA for *D. bensoniae* (Table 5). The mean number of days for root initiation from microshoots exhibited significant variation. There was a declining trend of rooting in *Dendrobium nobile* with increasing concentration of IBA. The minimum days (19.60) for root initiation were noticed in rooting media supplemented with 0.5 mg/l NAA. It was observed that the root initiation was delayed when

the concentration of auxins was increased from 0.5 to 2.0 mg/l (George *et al*, 2013). In this experiment, the mean number of days to root initiation from microshoots showed increasing trend when the concentration of auxin was increased.

3.3.1.3 Number of roots per explants

The highest number of roots (9.33±0.19) per explant was recorded in 1.5 mg/l IBA at 60 DAI and the lowest number of roots (4.33±0.10) was obtained in control in *D. bensoniae* (Table 5). Dwivedi *et al.* (2014) found 10 roots in medium with IBA (0.5 mg/l) in 8 weeks of time in *Dendrobium speciosum*. Bhandari *et al.* (2010) reported 12.6 roots per explants using IAA with 1.0 mg/l after 60 days of culture in *Dendrobium nobile*. Sunitibala and Kishor (2009) obtained highest level of rooting response (11.80) of *Dendrobium transparens* on half strength MS medium with exogenous supply of IAA 1.0 mg/l. George *et al.* (2013) reported that 3.20 roots per explants using IBA with 2.0 mg/l after after 4 week of culture in *Dendrobium sonia.* These results are in agreement with those obtained in MS media supplemented with 1.5 mg/l of IBA in this experiment.

3.3.1.4 Length of root (cm)

The length of roots per explant (cm) was regulated by the different concentrations of IBA. The maximum average root length (1.40±0.25 cm) was obtained from 1.5 mg/l IBA (Table 5) and the minimum (0.80±0.05 cm) length of root was in control in *D. bensoniae.* The longest root observed was about 0.4 to 1.5 cm in MS medium supplemented with IBA 1.5 mg/l. These results are also supported by Akter *et al.* (2010) where they found highest length of root of *Dendrobium* species at 1 mg/l IBA in MS medium. Jafari and Hamidoghli (2009) explained that concentration of 2.0 mg/l IBA has given a bigger number of roots and the maximum root length in *Dendrobium nobile*. Dwivedi *et al.* (2014) found length 1.30 cm was obtained in medium with IBA 1.5 mg/l in 8 weeks of time in *Dendrobium speciosum.*

Table 5: Efficacy of different IBA concentrations on induction of roots in *D. bensoniae.*

Hormonal (IBA) concentrations (mg/l)	Number of explants initiated root	% of explants showing root induction	Days to root initiation	Average number of roots per explants ±SD	Average length (cm) of roots per explants ±SD
				60 DAI	60 DAI
MS (Control)	4	40	41.21	4.33±0.10	0.80±0.05
0.5	8	80	35.33	5.35±0.16	1.10±0.06
1.0	8	80	33.00	7.39±0.26	1.20±0.15
1.5	7	70	25.00	9.33±0.19	1.40±0.25
2.0	9	90	23.33	8.67±0.21	1.00±0.15
SE				1.06	0.10
LSD				1.88	0.28
Level of significance (5%)				*	*

DAI= Days after inoculation

* = presence of level of significance

*10 explants were taken for each treatment

3.3.2 Root formation of *D. aphyllum* using different concentrations of IBA

Auxins promote rooting in the plants through changes in the biochemical systems of the plants (Henrique *et al.,* 2006). Among various auxin, IBA is known to stimulate rooting more efficiently due to its weak toxicity and greater stability for induction of roots (Han *et al.,* 2009). Liu *et al*., (2002) described that IBA is physiologically a more active auxin than NAA and IAA in promoting the root initiation as it acts as a precursor for endogenous IAA. The results of experiment have been presented under different heading utilizing Table 6 and Plate 3.3.

3.3.2.1 Percentage of explants showing root induction

Application of auxin to microshoots is stated to intensify the number of adventitious roots. The regenerated shoots of *D. chrysanthum* responded best in terms of rooting, number of roots/shoot, and average length of roots in MS medium fortified with 0.5 mg/l NAA (George *et al.,* 2013). There was a significant variation of IBA concentrations on percentage of explants showing root induction. The highest percentage (90%) of root induction was induced in treatment with 1.5 mg/l IBA and the lowest percentage (50%) was induced in hormone free media in case of *D. aphyllum* (Table 6). The role of IBA in stimulating root formation has been illustrated in *Dactylorhiza* species, *Dendrobium candidum*, *Geidorum densiflorum*, *Vinca minor* and *Vanilla planifolia* (Sheelavantmath *et al*., 2000; Shiau *et al*., 2005; Wotavova-Novotna *et al*.,2007; Raouf Fard *et al*., 2008; Tan *et al*.,2011).

3.3.2.2 Days to root induction

Application of auxin to microshoots is stated to intensify the number of adventitious roots. Hormonal concentration has significant level of variation on days for root induction. The maximum 37.23 days to root induction was required in control treatment and minimum 22 days is required in case of 2.0 IBA (mg/l) for *D. aphyllum* (Table 6). Similar results were reported by Talukder *et al*. (2002), where they observed that days to root formation were the minimum (19.2 days) at 1.0 mg/l IBA, while the maximum days (28.5 days) were required at 2.5 mg/l IBA in *Dendrobium* orchid.

3.3.2.3 Number of roots per explants

To evaluate the response and effectiveness of IBA on the number of roots, a range of treatment (0.5, 1.0, 1.5 and 2.0) was applied and significant variations were observed during data recording at 60 DAI. The highest number of roots (9.67±0.41) per explant was recorded in 2.0 mg/l IBA at 60 DAI and the minimum number of roots (4.35±0.10) was obtained in control in *D. aphyllum* (Table 6). These results were partially supported by Talukder *et al*. (2002), where they found 1.62 roots per plantlet from 2.0 mg/l IBA with MS media at 30 DAI in *D. nobile*. Similar result was obtained by the Martin (2006). In this investigation, maximum number (10.35±0.41) was observed on MS medium with the addition of IBA 1.5 mg/l in *D. densiflorum.* Asghar *et al.* (2011) also obtained maximum number of roots on modified MS medium fortified with various concentration of IBA than in NAA in *Dendrobium nobile.* Ahmed (2010) also reported that, the highest number (4.70) and length (3.47 cm) of roots was observed at 2.0 mg/l of IBA in *Dendrobium nobile.* Sunitibala and Kishor (2009) obtained that, the regenerated shoots cultured in MS medium supplemented with NAA resulted in 100% shoots forming roots in MS+1.5 mg/l NAA. Maximum root number/shoot (11.26±0.32) with 2.45±0.043 cm average length of roots was obtained in the same medium. At the lowest level of NAA (0.5 mg/l), the percentage of shoots forming roots, number of roots/shoot, and the average length of roots declined.

3.3.2.4 Length of root (cm)

The length (cm) of root per explant was regulated by the concentrations of IBA. The maximum average root length (1.30±0.06 cm) was obtained from 2.0 mg/l IBA and the minimum (0.75±0.05 cm) average length of root was in control in *D. aphyllum* (Table 6). Daneshvar *et al.* (2013) noticed (1.20±0.05 cm) root length in 1.0 mg/l IBA in *Dendrobium densiflorum.* The result of the present study was supported by Talukder *et al*. (2002), where they obtained 0.51 cm root with 1.0 mg/l IBA in MS medium. Nasiruddin *et al*. (2003) found the highest length of root of *Dendrobium formosum* at 0.5 mg/l 2, 4-D. Lim *et al*. (1985) observed that IBA at 0.1 mg/l was the best for producing tall roots in *D. moniliforms.* The effect of IAA or IBA on induction of roots in other orchid species like *Dendrobium formosum* was also reported by several authors (Malabadi *et al*., 2004, William *et al.*, 2003, Mitra *et al.,* 1976). These results are in agreement with the present study.

Table 6: Efficacy of different IBA concentrations on induction of roots in *D. aphyllum*.

Hormonal (IBA) concentrations (mg/l)	**Number of explants initiated root**	**% of explants showing root induction**	**Days to root initiation**	**Average number of roots per explants ±SD**	**Average length (cm) of roots per explants ±SD**
				60 DAI	**60 DAI**
MS (Control)	5	50	37.23	4.35±0.10	0.75±0.05
0.5	7	70	36.67	4.67±0.21	1.10±0.16
1.0	8	80	32.40	5.66±0.21	1.13±0.15
1.5	9	90	25	6.81±0.38	1.03±0.24
2.0	8	80	22	9.67±0.41	1.30±0.06
SE				1.80	0.53
LSD				2.44	0.41
Level of significance (5%)				*	*

DAI= Days after inoculation

* = presence of level of significance

*10 explants were taken for each treatment

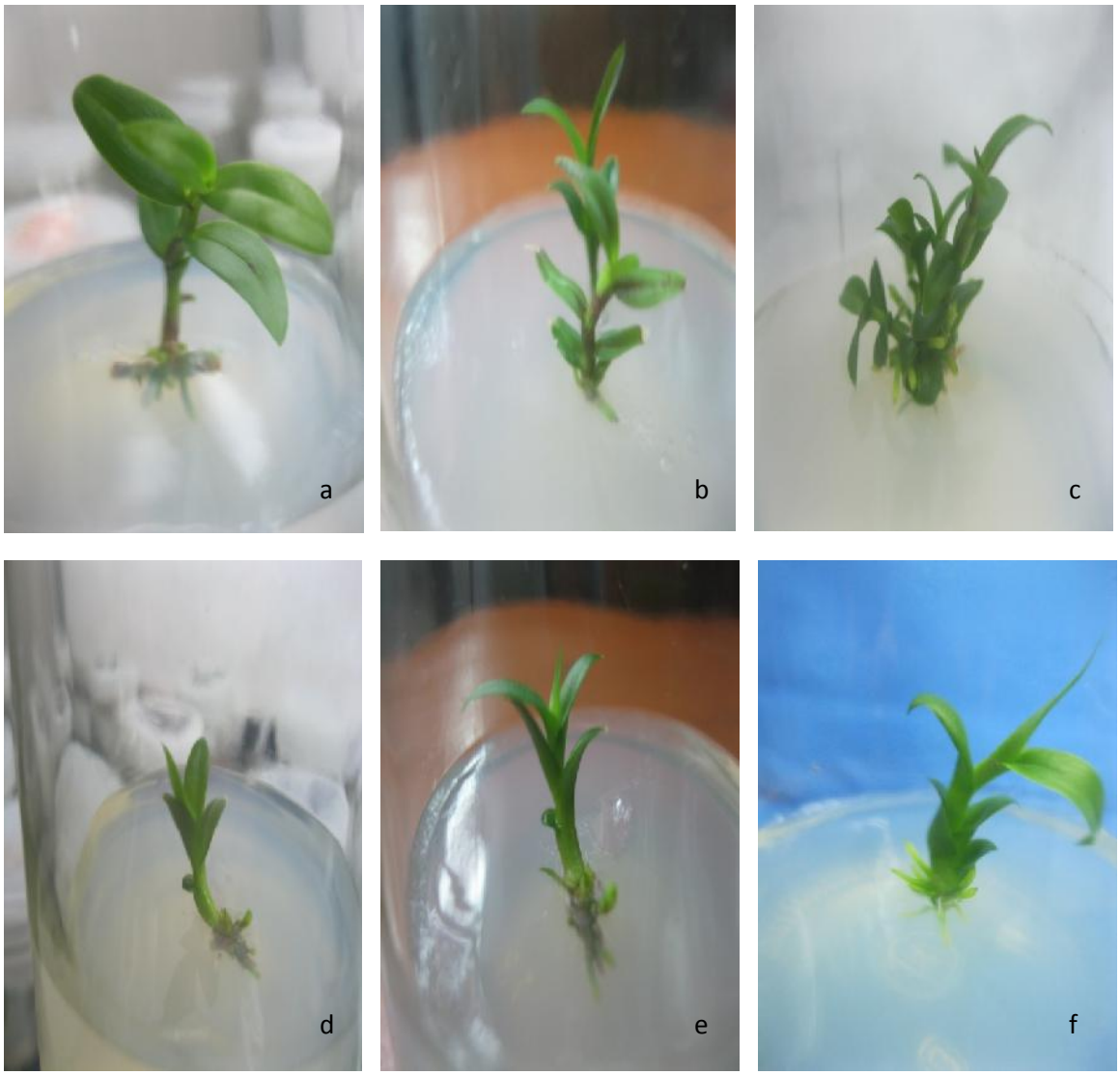

Plate 3.3: Root formation, induction and elongation of *D. bensoniae* and *D. aphyllum* on MS media supplemented with 1.5 mg/l IBA.

D. bensoniae at a) 30 DAI b) 45 DAI c) 60 DAI and *D. aphyllum* at d) 15 DAI e) 30 DAI f) 45 DAI.

3.4. Experiment 4. Combined effect of different concentrations of BA+IBA combination on *in vitro* root initiation of *D. bensoniae* and *D. aphyllum*

3.4.1 Root formation of *D. bensoniae* using different concentrations of BA+IBA combination

Auxins and cytokinins may not have direct effect on the development of shoots, but may be effective mostly through induction of roots (Husen and Pal, 2007). The results of experiment have been presented under different heading utilizing Table 7 and Plate 3.4.

3.4.1.1 Percentage of explants showing root induction

The minimum rooting percentage was recorded in the control as compared to other treatments containing different levels of auxins. There was a significant variation of BA+IBA concentrations on percentage of explants showing root induction. The highest percentage (90%) of root induction was induced in treatment with 1.0 BA+1.5 IBA (mg/l) and the lowest percentage (50%) was induced in hormone free media in case of *D. bensoniae* (Table 7).

3.4.1.2 Days to root induction

Hormonal concentration has significant level of variation on days for root induction. The maximum 40.33 days to root induction was observed in media lack of growth regulator. Minimum 21 days is required in case of 0.5 BA+1.0 IBA (mg/l) for *D. bensoniae* (Table 7).

3.4.1.3 Number of roots per explants

To evaluate the response and effectiveness of BA+IBA combination on the number of roots, a range of different concentrations of treatment was applied and significant variations were observed during data recording at 60 DAI. The highest number of roots (10.35±0.07) per explant was recorded in 0.5 BA+ 1.0 IBA at 60 DAI and the minimum number of roots (3.95±0.08) were obtained in control in *D. bensoniae* (Table 7). Dohling *et al*. (2007) showed that the 1.93 per explant root of the orchid at 2.5 mg/l BAP + 0.5 mg/l NAA at 30 DAI. Doods (1991) found that shoots of *Dendrobium* hybrids rooted on VW medium supplemented with 2.0 mg/l IBA and IAA.

3.4.1.4 Length of root (cm)

Root length was significantly influenced by different levels of growth regulators. The length of roots per explant (cm) was regulated by the different concentrations of IBA. The maximum average root length (1.35±0.15 cm) was obtained from 0.5 BA+ 1.0 IBA (mg/l) and the minimum (0.63±0.07 cm) length of root was in control in *D. bensoniae* (Table 7). Khatun (2010) reported that, the root length was significantly high (0.916 cm) at 2.0 mg/l BAP + 1.0 mg/l IBA after 6 week of culture.

Table 7: Efficacy of different concentrations of BA+IBA on induction of roots in *D. bensoniae.*

Hormonal (IBA) concentrations (mg/l)	Number of explants initiated root	% of explants showing root induction	Days to root initiation	Average number of roots per explants ±SD	Average length (cm) of roots per explants ±SD
				60 DAI	60 DAI
MS (Control)	5	50	40.33	3.95±0.08	0.63±0.07
0.5	7	70	33.33	6.68±0.15	0.83±0.08
1.0	8	80	21.00	10.35±0.07	1.35±0.15
1.5	9	90	27.00	7.00±0.10	1.20±0.25
2.0	7	70	23.33	9.35±0.20	1.19±0.15
SE				0.80	0.20
LSD				1.62	0.27
Level of significance (5%)				*	*

DAI= Days after inoculation

* = presence of level of significance

*10 explants were taken for each treatment

3.4.2 Root formation of *D. aphyllum* using different concentrations of BA+IBA combination

The results of experiment have been presented under different heading utilizing Table 8 and Plate 3.4.

3.4.2.1 Percentage of explants showing root induction

There was a significant variation of BA+IBA concentrations on percentage of explants showing root induction. The highest percentage (90%) of root induction was induced in treatment with 1.0 BA+1.5 IBA (mg/l) and the lowest percentage (30%) was induced in hormone free media in case of *D. aphyllum* (Table 8). Kurup*et et al*. (2005) reported that roots produced in treatments involving both cytokinins and auxins were healthier than in treatments with either auxin or cytokinin.

3.4.2.2 Days to root induction

Hormonal concentration has significant level of variation on days for root induction. The maximum 39.21 days to root induction was observed in media lack of growth regulator. Minimum 22 days is required in case of 0.5 BA+1.0 IBA (mg/l) for *D. aphyllum* (Table 8).

3.4.2.3 Number of roots per explants

Root number was affected by the presence of BA and IBA in MS medium. The effect of BA and IBA in combination with each other, are shown in Table 8. To evaluate the response and effectiveness of BA+IBA on the number of roots, a range of different concentrations of treatment was applied and significant variations were observed during data recording at 60 DAI. The highest number of roots (9.35±0.06) per explant was recorded in 0.5 BA+ 1.0 IBA at 60 DAI and the minimum number of roots (4.71±0.10) was obtained in control in *D. aphyllum* (Table 8). Khatun (2010) reported that, the highest number of roots (2.583) was observed is 2.0 mg/l BAP + 1.0 mg/l IBA at 120 DAI.

3.4.2.4 Length of root (cm)

The length (cm) of roots per explant was regulated by the different concentrations of BA+IBA supplementations. The maximum average root length (1.50±0.15 cm) was obtained

from 0.5 BA+ 1.0 IBA (mg/l) and the minimum (0.65±0.05 cm) length of root was in control (mg/l) in *D. aphyllum* (Table 8).

Table 8: Efficacy of different concentrations of BA+IBA combination in induction of roots in *D. aphyllum*.

Hormonal (IBA) concentrations (mg/l)	Number of explants initiated root	% of explants showing root induction	Days to root initiation	Average number of roots per explants ±SD	Average length (cm) of roots per explants ±SD
				60 DAI	60 DAI
MS (Control)	3	30	39.21	4.71±0.10	0.65±0.05
0.5	7	70	34.33	6.10±0.16	0.80±0.06
1.0	7	70	22	9.35±0.06	1.50±0.15
1.5	9	90	25.39	5.80±0.10	1.31±0.25
2.0	8	80	24.50	7.95±0.21	1.19±0.15
SE				1.20	0.20
LSD				1.95	0.27
Level of significance (5%)				*	*

DAI= Days after inoculation

* = presence of level of significance

*10 explants were taken for each treatment

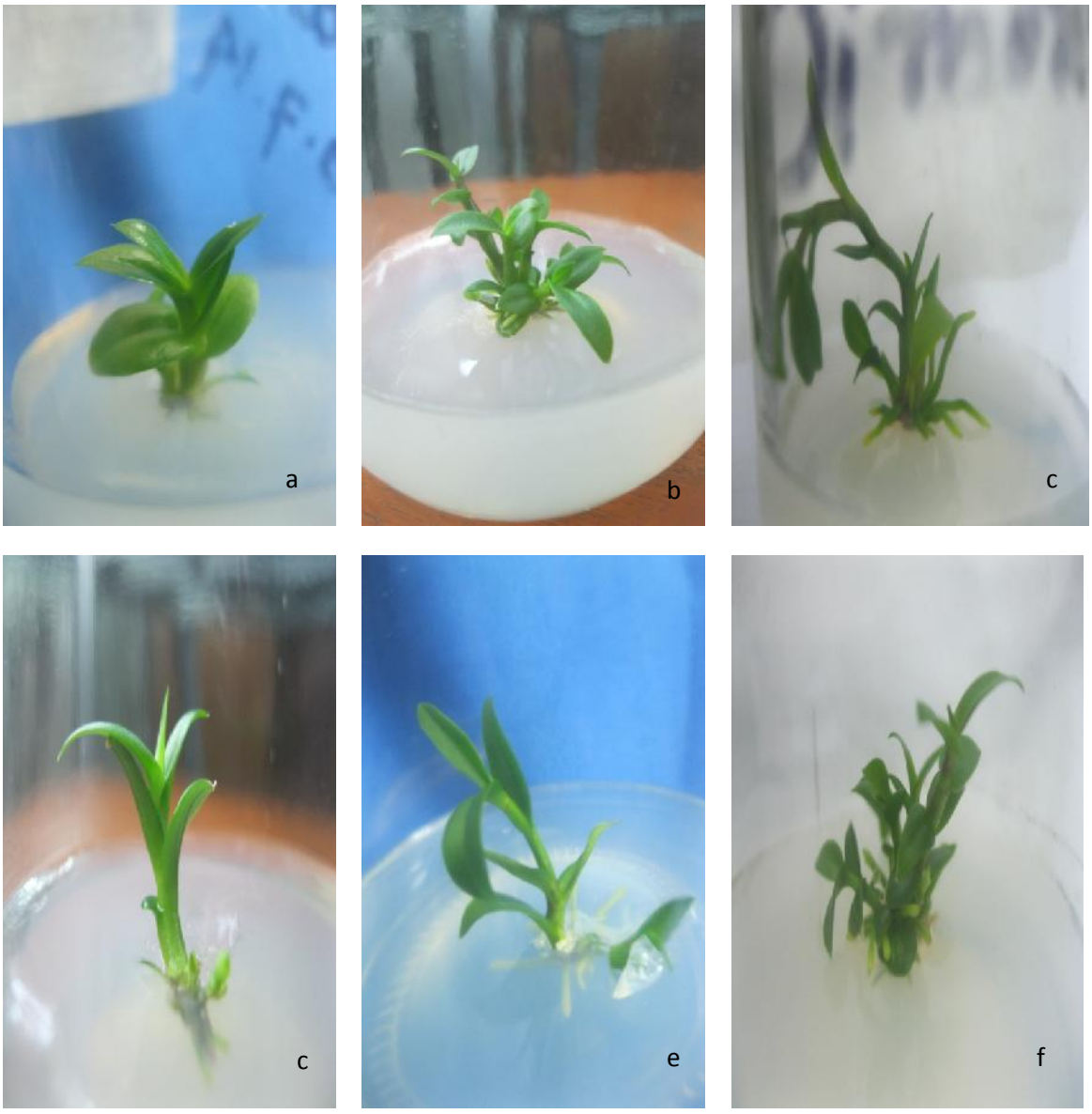

Plate 3.4: Root formation, induction and elongation of *D. bensoniae* and *D. aphyllum* on MS media supplemented with 0.5 mg/l BA + 1.0 mg/l IBA.

D. bensoniae at a) 30 DAI b) 45 DAI c) 60 DAI and *D. aphyllum* at d) 15 DAI e) 30 DAI f) 45 DAI.

3.4.3 Final comparison of hormonal treatment

The nodal segments of *D. bensoniae and D. aphyllum* showed various responses on MS medium supplemented with different plant growth regulators either separately or in combination. These results showed that, 0.5 mg/l BA and 1.0 mg/l IBA in combination with MS medium gave better response than all other combinations of BA+IBA concentration under study in *D. bensoniae* and *D. aphyllum*.

3.4.4 Regeneration capacity using different hormone

In this experiment, the highest number of roots (10.35±0.07) per explant was recorded in 0.5 BA+ 1.0 IBA at 60 DAI in *Dendrobium bensoniae*. Kim *et al*. (2003) showed that the 1.93 per explant root of the orchid at 2.5 mg/l BAP + 0.5 mg/l NAA after 3 week. Khatun *et al*. (2010) reported that, the highest number of roots (2.583) was observed is 2.0 mg/l BAP + 1.0 mg/l IBA at 120 DAI. It was noticed from this experiment, roots formed from microshoots produced in treatments involving both cytokinins and auxins were healthier than in treatments with either auxin or cytokinine and also the number of roots per explant was satisfactory enough compared with other experiments of other scientists. The enrichment of medium with higher concentration of IBA along with low cytokinin content induced excellent rooting response in the culture and gives a better number of roots in this experiment. Similar observations were also reported by Khatun and Al-Amin (2006).

3.5. Experiment 5. Acclimatization of plantlets

3.5.1 After culture on rooting media, the plantlets were taken for acclimatization. The results of acclimatization or hardening have been presented in Table 9 and 10 and Plate 3.5.

A micropropagation system can be deemed beneficial only by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro* (Hazarika, 2003). A significant number of micropropagated plants do not survive when transferred from *in vitro* conditions to greenhouse or field environment which have substantially lower relative humidity, higher light and septic environment compared to the *in vitro* conditions. However, Pospisilova *et al*. (1999), Hazarika (2003), and Deb and Imchen (2010) reported that *in vitro* acclimatization of plantlets prior to their *ex vitro* transplantation is important in producing healthy plantlets. The composition of the media into which in vitro rooted plantlets are transplanted is important for their survival (Jones, 1982).

Table 9. Survial rate of *in vitro* regenerated plantlet of *D.bensoniae*

Acclimatization	No. of plants transplanted	Duration of observation	No. of plants survived	Survival rate (%)
In culture room	20	7days	14	70
In shade house	14	14 days	13	65

Table 10. Survial rate of *in vitro* regenerated plantlet of *D.aphyllum*

Acclimatization	No. of plants transplanted	Duration of observation	No. of plants survived	Survival rate (%)
In culture room	20	7days	14	70
In shade house	14	14 days	14	100

The results of acclimatization showed that 70% of plantlets were survived to the culture room in case of *D. bensoniae* and *D. aphyllum* (Table 9, Table 10 and Plate 3.5). Here, in the culture room, the top of the pots were covered with transparent plastic sheet and grew at

room temperature with periodic irrigation. *In vitro* grown plantlets were gradually shifted to shade house from poly-bag house containing high humidity and low temperature. In these conditions, the 65% and 100% of the plantlets survived in both species of *D. bensoniae* and *D. aphyllum* (Table 9 and Table 10). It was also revealed that regenerated plants were morphologically similar to the mother plant.

Plate 3.5: Acclimatization of regenerated planted of *D. bensoniae* a) in culture room covered with transparent plastic sheet b) in culture room without transparent plastic sheet c) in shade house and *D. aphyllum* d) in culture room covered with transparent plastic sheet e) in culture room without transparent plastic sheet and f) in shade house.

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