

**RAPID DEVELOPMENT OF *IN VITRO* GERMINATION,
MICROPROPAGATION AND ANTIMICROBIAL
ACTIVITIES OF INDIGENOUS ORCHID
SPECIES IN BANGLADESH**



**THESIS SUBMITTED FOR THE DEGREE
OF
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**BY
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DECLARATION

I hereby declare that the research work embodied in this thesis entitled “**Rapid Development of *in vitro* Germination, Micropropagation and Antimicrobial Activities of Indigenous Orchid Species in Bangladesh**” has been carried out by me for the degree of Doctor of Philosophy under the supervision of Dr. S. M.. Shahinul Islam, Institute of Biological Sciences, University of Rajshahi, Bangladesh and I also declare that the result of my own investigation. The thesis or any part of it has not been submitted to any other University or elsewhere for any degree or diploma or for other similar purpose.

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ABSTRACT

In vitro micropropagation, hybridization and cryopreservation techniques are essential tools for orchid development. Considering the importance of rapid and mass propagation and medicinal value of indigenous orchids in Bangladesh this research programme has been undertaken with a major view to develop a reproducible micropropagation technique, to estimate antimicrobial activity and to analyze of bioactive compounds of indigenous orchids. For this research five different orchid species were selected. It was observed that immature capsule showed better performance for germination (100%) and development of PLBs (90%). The highest number (90%) and weight of PLBs (5.53g), best plant regeneration (16.50) and shoot length (3.25 cm) were recorded when the medium supplemented with BAP (1.0 mg l^{-1}) and NAA (1.5 mg l^{-1}). On the other hand, somatic embryos were induced from leaf and shoot that cultured on MS medium supplemented with BAP, NAA and kinetin. Among them BAP (0.5 mg l^{-1}) and kinetin (1.0 mg l^{-1}) showed better performance on somatic embryogenesis. Germination and regeneration efficiency of somatic embryos were recorded as highest 60.83% in $\frac{1}{2}$ MS medium supplemented with 1.0 mg l^{-1} BAP. Maximum number of multiple shoots (10.8 ± 0.22) was recorded in MS medium supplemented with 1.5 mg l^{-1} BAP and 1.0 mg l^{-1} NAA. So far as we know there is no report regarding the plant regeneration through somatic embryogenesis from leaf and stem explants of *V. tessellata*. In case of multiple shoot induction and subsequent development, twenty different plant growth regulators (PGRs) were tested. It was revealed that the combination of 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} BAP was proved to be the best combination for multiple shoot development and elongation.

Another experiment were conducted using different PGRs were applied for *in vitro* development of protocorms, regeneration and mass multiplication derived from immature seeds of *Rhynchosstylis retusa* (L.) Blume. The maximum percentage of seed germination (72.60%) was recorded after 7-8 weeks of culture initiation. The highest numbers of secondary PLBs (16.0) were obtained from each of primary protocorms in MS medium supplemented with 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA. On the other hand, the somatic embryo induction was highest in leaf (75%) and root (50.2%) when 1.0 mg l^{-1} BAP + 1.5 mg l^{-1} kinetin was used. Another investigation for *in vitro* germination and enhancing PLBs development were carried out in three indigenous orchids. Under this study three basal media were tested and among them PM was found to be the best because of the highest rate of seed germination

(98.20%), PLBs development and plant formation. The MS with 1.0 mg l⁻¹ BAP and 0.1 mg l⁻¹ picloram was the most efficient medium for seedling growth of *A. premorsa* and *A. khasianum*. But in the case of *P. cornorerris* the best seedling growth was observed using medium supplemented with 0.5 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA.

In case of antimicrobial activity and photochemical screening the whole plant extracts of *Vanda tessellata* and *Rhynchostylis retusa* were studied. The various solvent extracts such as chloroform, methanol, ethanol and hexane of this orchid were considered for *in vitro* antimicrobial activity against five clinical pathogenic bacteria and three fungi by disc diffusion method. A preliminary phytochemical analysis was performed for the detection of alkaloid, terpenoids, flavonoids, phenols, tannins, steroids and glycosides etc. The antibacterial activity against all bacteria with the zone of inhibition ranging from 5 - 15 mm were observed and found that highest inhibition zone (14 - 15 mm) with the concentration of 10.0 mg l⁻¹ of chloroform extract. Chloroform extract showed significant antifungal activity against *Penicillium* sp., *Rhizopus* sp. and *Aspergillus niger* with the highest zone (16-17 mm) of inhibition. In *R. retusa*, the antibacterial activity against all bacteria with the zone of inhibition ranging from 5 - 12 mm were observed and found that highest inhibition zone (10 - 12 mm) with the concentration of 10.0 mg l⁻¹ of chloroform extract. Chloroform extract also showed significant antifungal activity against *Penicillium*, *Rhizopus* and *Aspergillus niger* with the highest zone (12-15 mm) of inhibition.

Another attempt of this study was taken to enhance anther culture response of *Vanda tessellata* by applying physical and chemical pre-treatment factors including with different media. It was observed that 3 days cold pretreatment at 4°C and ½MS medium slightly modified by adding CuSO₄.5H₂O (2.2 mg l⁻¹) with BAP (10 mg l⁻¹), NAA (1.5 mg l⁻¹), peptone (2.0 mg l⁻¹) and 3% sucrose as carbon sources was the best medium for callus induction. From the experimental findings it might be concluded that the cold pretreatment, different media composition and carbon sources and their interaction clearly influence the anther culture ability of *V. tessellata* and this protocol will be helpful to commercial growers for mass multiplication of orchids.

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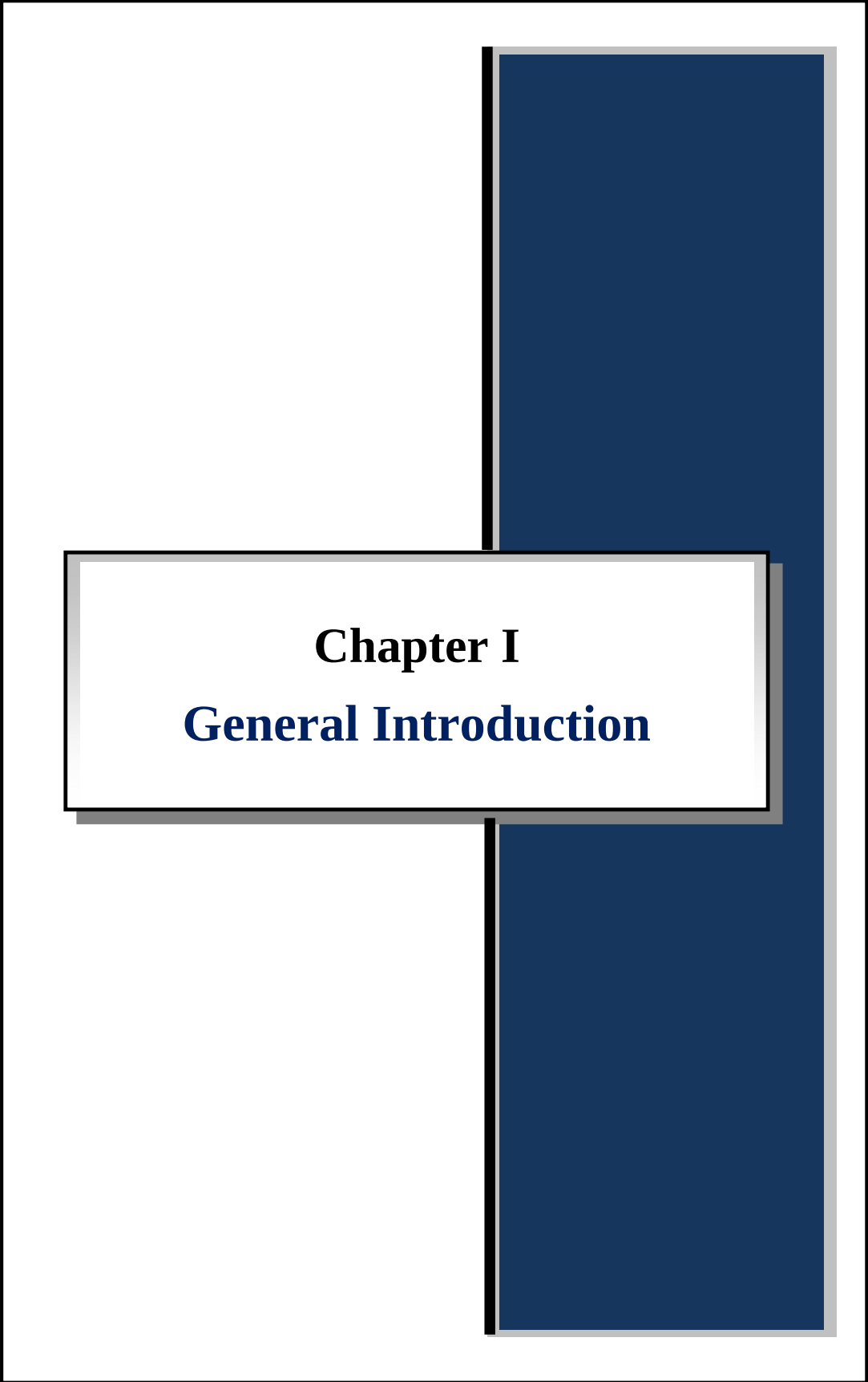
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List of Abbreviations

The following abbreviations have been used through the text:

BA	:	Benzyl adenine
BAP	:	6-Benzyl amino purine
B ₅	:	Gamborg et al. (1968) medium
°C	:	Celsius
CH	:	Casein hydrolysate
cm	:	Centimetre
d	:	Days
DW	:	Distilled water
et al.	:	Et alia = and others
Fig.	:	Figure
GA ₃	:	Gibberellic acid
g	:	Gram
gl ⁻¹	:	Gram per litre
h	:	Hour (s)
HCl	:	Hydrochloric acid
IAA	:	Indole- 3-acetic acid
IBA	:	Indole-3- butyric acid
i.e	:	That is (to say)
Kin.	:	Kinetin
Kg	:	Kilogram
KC	:	Knudson C medium
Mg	:	Milligram
mg ^l ⁻¹	:	Milligram per litre
MVW	:	Modified Vacin and Went medium
MS	:	Murashige and Skoog (1962) medium

PM	:	Phytamax ®™ medium
NaOH	:	Sodium hydroxide
NAA	:	α-naphthalene acetic acid
PGRs	:	Plant growth regulators
PLBs	:	Protocorm like bodies
ppm	:	Parts per million
sp.	:	Species
via	:	By the way
2,4-D	:	2,4-dichloro phenoxy acetic acid
%	:	Percent
w/v	:	Weight by volume
v/v	:	Volume by volume
VC	:	Vacin and Went (1949) medium
viz.	:	Videlicet (L.); namely
2ip	:	6-(y,y-dimethylallylamino) purine (2ip)
0.1N	:	0.1 Normal
ANOVA	:	Analysis of variance
DMSO	:	Dimethylsulfooxide
EDTA	:	Ethylenedinitrilo tetra acetic acid
LSD	:	Least significant difference
p ^H	:	Negative logarithm of hydrogen ion (H ⁺) concentration
S.E	:	Standard error



Chapter I

General Introduction

1. General Introduction

Orchids belong to family of angiosperms called Orchidaceae. It includes 800 genera and between 30,000 to 35,000 species spread all over the world (Singh et al., 2007; Chugh et al., 2009; Nongdam and Chongtham, 2011; Bektas et al., 2013). Many orchid species have floricultural importance because of their attractive and long-lived flowers. Due to their popularity, orchids now occupy the top position among all flowering plants for cut flower production and as potted ornamentals. Orchids are highly valued for their fantastic range of variation in their colors, forms and long lasting flowers properties (Paudel and Pant, 2012). Many of the commercially important orchids are now artificially grown for their beautiful flowers. This group of plants is valued highly both in the national as well as in the international markets (Kumar et al., 2002).

Bangladesh is in the subtropical zone and quite a large number of orchid species abundantly in the forests of Chittagong, Sylhet, Rangamati and the Sundarbans (Khulna, Bagerhat and Patuakhali). About 1,250 species of orchids were first reported from the British India and of them at least 72 species were recorded from Bangladesh part of the British India (Hooker, 1894). Taxonomical studies revealed that there are about 115 different varieties of local orchids in Bangladesh (Bhadra et al., 2002). Some of these species such as *Cymbidium grandiflorum* Griseb., *C. eburneum* Lindl., *Eulophia geniculata* King and Pantl., *E. mackinnonii* Duthiei, *Spathoglottis plicata* Bl., *Rhyncostylis retusa* (L.) Bl., *Dendrobium plapebrae* Lindl., *D. fimbriatum* var *occulata* HK., *D. crepidatum* Lindl. and *Vanda parishii* Reichb have very attractive flowers and are used as ornamental plants. Unfortunately, many orchids are threatened by extinction because of environmental degradation, human occupation of natural habitats, and overexploitation for horticultural and ethno-botanical reasons (Coates and Dixon, 2007). Habitat destruction due to road construction, fire, urbanization, drainage and other anthropogenic influences can also directly affect (Cribb et al., 2003). The number of orchid species is rapidly and steadily declining now because of their low rate of propagation in nature and

the ongoing collection from nature (Bhadra et al., 2002; Bektas et al., 2013). Careless collection of these species has led to serious genetic and ecological erosion; many have already been listed as endangered species (Clements, 2003; Machaka-Houri et al., 2012). But the demand of these orchids is increasing day by day both in local and foreign markets. In this context, it is important to develop rapid propagation of economically important orchids in Bangladesh. Development of such propagation technique is also important for conservation of this group of plant species. However, research on the production of unique and improved orchids may help create a competitive national and international floriculture market.

Orchids are not only important for floricultural value but also some of them are used as medicine and food (Bose and Bhattacharjee, 1980). *Vanda tessellata* (Roxb.) Hook. Ex. G. is an epiphytic orchid, 30-60 cm high, with leafy stem. This plant has been used as indigenous medicine sources such as Ayurveda and local traditional medical practices (Chopra et al., 1956). The roots of *Vanda tessellate* are antipyretic; useful in dyspepsia, bronchitis, inflammations, piles and hiccup. Externally the root is used in rheumatism and allied disorders and diseases of the nervous system. It is also remedy for secondary syphilis and scorpion-sting. Juice of the leaves is given in otitis and the paste as febrifuge. The roots possess significant anti-inflammatory activity. The plant contains an alkaloid, glucoside, tannins, β -sitosterol, γ -sitosterol and a long chain aliphatic compound, fatty oils, resins and colouring matters. Roots contain tetracosyl ferrulate and β -sitosterol-D-glycoside (Ghani et al., 1990). A novel aphrodisiac compound isolated from the flowers of *Vanda tessellata* (Roxb.) Ex. Don which activates neuronal and endothelial, but not inducible (Subramoniam et al., 2013). Dried leaves of *Dendrobium salaceense* when cooked with rice impart a delicate and exotic flavour and are used in some areas. The leaf extract of *Cymbidium aloifolium* SW. has a unique blood clotting property. It is also emetic and purgative. A paste made from an orchid of the genus *Grammatophyllum* is used for curing sores. *Dendrobium teretifolium* is used externally by removing the leaves for curing headache and pain.

The whole plant preparations of *Rhynchosyilis retusa* is used to treat rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases and external inflammations (Kumar et al., 2012). It is also used as an emollient and in the treatment of throat inflammation (Shanavaskhan et al., 2012). Leaf juice of this plant and aerial roots are used in ear pain and cleaning (Basumatary et al., 2004). *Rhynchosyilis retusa* roots are also used to cure malarial fever (Tiwari et al., 2012; Radhika and Murthy, 2013). In Kurigram district of Bangladesh, people use the leaves of this plant to cure rheumatic pain (Das et al., 2012). Juice of roots applied to cuts and wounds and dried flowers are used as insect repellent and to induce vomiting (Subedi et al., 2013).

Orchids are also important for their glycosidal value. Four types of glycosides have been isolated from the member of the family, Orchidaceae. Loroglosine glycosides are derived from *Loroglossum* sp. and are used as fragrance in Europe. Comarine glycosides available in *Angraecium* used as popular beverage. The saponine glycosides found in *Paphiopedilum* posses strong foaming properties. The vanilla glycosides and related aromatic compounds are found in many species of *Vanilla* and in other orchid genera like *Nigritella*, *Listera* and *Gymnadenia*. *Vanilla planifolia* is the original source of commercial vanilla extract, which is used for flavouring chocolates, biscuits and confectionary commodities, and also in perfumes.

Some orchids contain economically important alkaloids. The presence of this biologically active compound was first reported in several species of *Dendrobium*. Dendrobine and nobiline are the two alkaloids isolated from *Dendrobium nobile*. The tribes - Neottinae, Liparidinae, *Phajinae*, *Cryptopodinae*, *Catasetinae*, *Dendrobiinae* and *Sarcanthinae* of the family, orchidaceae are very rich in alkaloids.

The bioactive compounds such as alkaloid, terpenoids, flavonoids, phenols, tannins, steroids and glycosides were present in the whole plant extracts of *V. tessellata* (Bhattacharjee et al., 2015). Besides this, the secondary metabolites like alkaloids, terpenoids, flavonoids, tannins, coumerins, steroids, amino acids and carbohydrates were

also present in trace amount in the whole plant extract of *R. retusa* (Bhattacharjee and Islam, 2015b).

Orchids are highly cross pollinated and the gene flow among their different species is generally uninterrupted which helps in spontaneous development of new hybrid varieties. Such hybrids can easily be developed by artificial crossing. About 75,000 desirable orchid hybrids have been produced so far which are commercially used. Orchids are generally epiphytic or terrestrial in nature. A few lithophytic, saprophytic or hydrophytic orchids have also been reported. Epiphytic groups are pre-dominating in the tropical and sub tropical regions; the terrestrials are in the temperate zones. Orchids are distributed throughout the world. Most of the species grow luxuriantly in hot and steaming forests.

One of the special features of this family is the production of a large number of minute seeds with only minimal reserves of nutrients (Arditti and Ghani, 2000). In spite of a very large number of seeds produced, only 0.2 - 0.3% germinates in nature (Singh, 1992). Because of this feature, orchids depend upon mycorrhizal fungi for the carbon resources necessary for the germination and subsequent growth (Rasmussen, 1995). Many orchids are threatened by extinction because of environmental disruption, human succession of natural habitats, medicinal properties and overexploitation for horticulture and ethno-botanical reasons (Coates and Dixon, 2007). This necessitates the development of rapid propagation techniques for conservation of orchids. *In vivo* vegetative propagation is undesirable due to the heterozygosity of seed, minute seed size, presence of reduced endosperm, and the requirement of an association with mycorrhizal fungi (Saiprasad and Polisetty, 2003). This necessitates the application of *in vitro* seed propagation technique for orchid conservation (Stenberg and Kane, 1998; Gangaprasad et al., 1999).

Thus *in vitro* cultural techniques are now adopted for quick propagation of commercially important orchid species. Orchid seeds are artificially germinated for commercial purpose and seedlings are raised in large scale (Gayatri et al., 2006). The orchids were the first one to be propagated through tissue culture for the commercial use (Arditti and Ernst, 1993). Regeneration from seeds via protocorm-like bodies (PLBs) has become the

preferential method for the production of orchids (Bhattacharjee and Islam, 2014; Das et al., 2007). Micropropagation of orchid seedlings can also be done with the use of the aseptically grown seedlings (Hoque et al., 1994; Jawan et al., 2010). In this context, it is important to develop rapid propagation of economically important orchids. Development of such propagation technique is also important for conservation of this group of endangered plant species. The most commonly used micropropagation techniques include direct and indirect shoot organogenesis and somatic embryogenesis (Antony et al., 2014). Somatic embryogenesis is one of the techniques that emerged in recent years with the potential of providing an efficient clonal propagation system. Somatic embryogenesis is more necessary biotechnological technique for large-scale clonal propagation of elite genotypes, development of synthetic seed technology, plant transformation (Bomal and Tremblay, 2000; Bandyopadhyay and Hamill, 2000) and conservation of threatened plant species. Plant regeneration through somatic embryogenesis is one of the main prerequisites for the potential applications of clonal propagation, genetic transformation and *in vitro* conservation of germplasm of woody plants (Handley, 1995; Minocha and Jain, 2000; Park et al., 1998). The phenomenon of somatic embryogenesis via callus is rather rare as success of callus formation in orchids has limitations due to slow growth and a tendency to become necrotic (Kerbaui, 1984). Especially, callus formation from leaf and stem segment of orchids has been and is the rarest even now. Even when plant regeneration from callus of orchids was achieved, it usually occurred via protocorm like bodies (Begum et al., 1994; Huana et al., 2004; Roy and Banerjee, 2003). Therefore, success of somatic embryogenesis via callus culture is very important and interesting in plant regeneration of orchids.

Morel (1960) developed the technique of shoot apex culture in *Cymbidium* sp., a common orchid species. He further (Morel, 1964) improved the technique of meristem culture and raised several hundred plants from a single bud. Subsequently Ito (1960), Sagawa et al. (1966), Isreal (1963), Bhattacharjee and Islam (2014) demonstrated that orchid seeds could be germinated and used in micropropagation through *in vitro* culture techniques. Ultimately, many *in vitro* cultured techniques have been developed for quick propagation

of different commercially important orchids. Different explants including shoot tip (Kim et al., 1972; Choi et al., 1980; Sharon and Vasundhara, 1990; Yoneda et al., 1983; Hasegawa, 1992), shoot segment (Gupta et al., 1998; Bhattacharjee and Islam, 2014), axillary bud (Mosich et al., 1974; Wang, 1988). Leaf-tip (Yam and Weatherhead, 1991a), leaf base (Loh et al., 1975) , leaf stalk (Kim et al., 1970), leaf segment (Vij et al., 1984), young leaves (Matos and Garcia, 1991), flower stalk (Arditti, 1977; Singh and Prakash, 1984; Tanaka et al., 1988), inflorescence (Deb and Pongener, 2013; Intuwong and Sagawa, 1973), root-tip (Churchill et al., 1972; Yam and Watherhead, 1991b), rhizome segment (Kim et al., 1988; Vij and Patak, 1989) have been successfully used for commercial propagation of orchids.

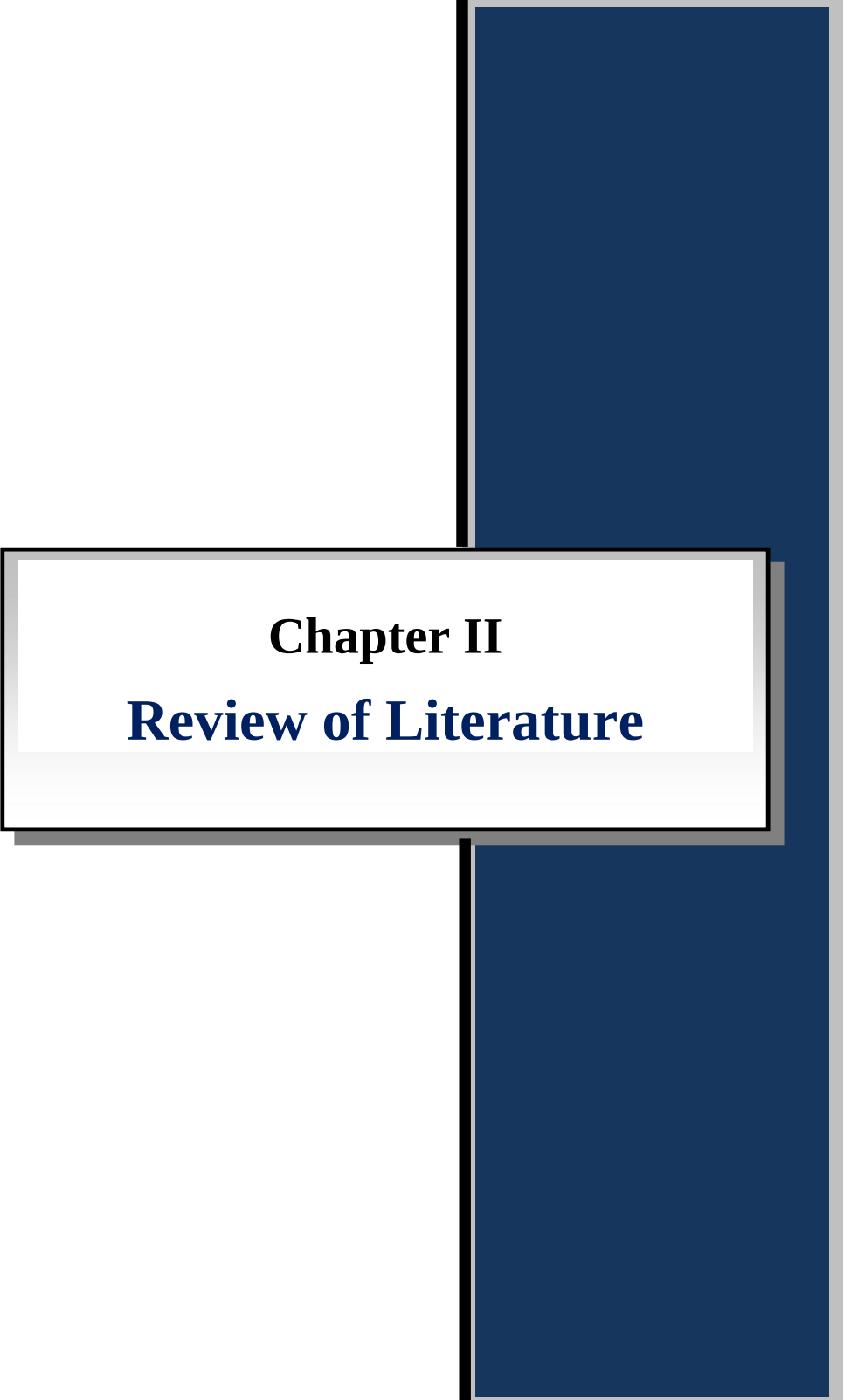
Micropropagation protocols have been developed for *Vanda tessellata* (Rahman et al., 2009; Bhattacharjee and Islam, 2014), *Rhynchostylis retusa* (Bhattacharjee and Islam, 2015a) and many *Dendrobium* hybrids using shoot tips and axillary buds (Sagawa and Shoji, 1967; Kim et al., 1970; Arditti et al., 1973; Sharma and Tandon, 1991). It is pertinent to mention here that since the early work of Morel (1964) on *Cymbidium*, many orchid genera have been successfully cloned by this tissue culture techniques, Murashige (1974) listed 22 genera, whereas Arditti (1977) reported that 35 including some intergeneric hybrids. Further success in other orchid species and their hybrids have been reported for *Aranthera* (Irawati et al., 1978), *Anoectochillus* (Ho et al., 1987), *Holtuara* (Teo and Wong, 1978), *Iris* (Yabuya et al., 1991), *Laeliocattleya* (Matos and Garcia, 1991), *Microstylis* (Sood, 1985), *Paulownia* (Rosu and Grigorescu, 1984), *Renantanda* (Goh and Tan, 1979), *Spathoglottis* (Singh, 1991) and *Thunia* (Singh and Prakash, 1984). In most cases the cultural techniques and media composition have been found to be specific in individual species and organ. Therefore, it is necessary to develop reliable culture technique and formulate media for individual species separately. The orchids represent the first floricultural crop successfully mass propagated through tissue culture technique and the commercial application of micropropagation is being increasingly realized in this group of great ornamentals.

Androgenesis in flowering plants is a unique biological phenomenon. The principle of androgenesis is to arrest the development of the pollen grains and to force them towards a somatic pathway. Anther culture is the main technique for haploid induction in crop improvement (Sharma, 2012). Since Guha and Maheshwari (1964, 1966; 1967) reported that the induction of haploid plants from *Datura innoxia*, this technique became important for plant breeding and crop improvement (Nitsh and Nitsh, 1969). Anther culture has become a powerful tool for the rapid production of haploid and inbred lines used for obtaining hybrid cultivars (Sopory and Munshi, 1996) and it has reduced the time required for breeding new cultivars by at least 3 to 5 years (Tai, 2003). Hence, this system provides an unparalleled opportunity to shorten the breeding cycle and fix agronomic traits in the homozygous state and provides excellent material for research, plant breeding and plant transformation (Datta, 2005). Production of haploids plants has been useful in providing access to recessive genes and for biotechnological manipulations, while using as a tool for cultivar development. Therefore, *in vitro* techniques are considered to be alternative tools of conventional method of plants improvement (Sayem et al., 2010). It is particularly useful in out breeders like orchids which generate a great deal of heterozygosity in the progenies.

Till now there is two report on anther culture in Orchids (Sharma, 2012), but attempts to assess a similar competence of anther culture in monocots including orchidaceae, have remained almost negligible because of the following reasons: (a) little success in inducing callus and maintaining proper growth, (b) difficult to obtain suitable size of homogenous tissue from monocotyledonous plants. Considering the importance of rapid propagation and medicinal value of indigenous orchids of Bangladesh this research programme has been undertaken with a major view to develop a reproducible micropropagation technique for rapid multiplication, to estimate antimicrobial activity, and to analyses of bioactive compounds of indigenous orchids.

Therefore, the main objectives of the present study was –

1. To develop reproducible and an efficient *in vitro* cultural techniques for germination of indigenous orchids.
2. To compare the efficiency of different media during organogenesis of the species.
3. To develop protocorm like bodies derived from immature seeds and subsequent plant regeneration and mass multiplication of orchids.
4. Plant regeneration through somatic embryogenesis from different explants of orchids.
5. To observe the effects of plant growth regulators on multiple shoot induction and direct shoot regeneration from different explants of orchids.
6. To evaluate the antimicrobial activity and phytochemical screening of indigenous orchids using plant extracts.



Chapter II

Review of Literature

2. Review of Literature

2.1 Geographical distribution

Orchids are nature's most extravagant group of flowering plants distributed throughout the world from tropics to high alpine (Pant, 2013). They exhibit incredible range of diversity in size, shape and color of their flowers. Orchids are distributed all over the world except in the Antarctica. They are abundantly found in tropical countries in South East Asia, South and Central America and South Africa. Also found distributed in China, Japan, Philippines, Europe and Australia (Rao, 1979). Epiphytic orchids are mainly distributed in the northern Andes of South America, Madagascar, Sumatra and Borneo while, Indo-China harbor both epiphytic and terrestrial species and South Western Australia believed to be the centre for terrestrial orchids (Cribb et al., 2003).

Bangladesh is in the subtropical zone and quite a large number of orchid species abundantly in the forests of Chittagong, Sylhet, Chittagong Hill Tracts and the Sundarbans. About a century back Hooker (1894) described that the orchids of India including those present in Bangladesh. About 1,250 species of orchids were first reported from the British India (Hooker, 1894) and of them at least 72 species were recorded from Bangladesh part of the British India. Some of these species such as *Cymbidium grandiflorum* Griseb., *C. eburneum* Lindl., *Eulophia geniculata* King and Pantl., *E. mackinnonii* Duthiei, *Spathoglottis plicata* Bl., *Rhyncostylis retusa* (L.) Bl., *Dendrobium plapebrae* Lindl., *D. fimbriatum* var *occulata* HK., *D. crepidatum* Lindl. and *Vanda parishii* Reichenb. have very attractive flowers and are used as ornamental plants.

2.2 Economic importance and uses

Orchids are important aesthetically, medicinally and also regarded as ecological indicators (Joshi et al., 2009). Several orchid species are cultivated for their various economic uses especially in floriculture. Orchids are grown primarily as ornamentals and are valued as cut flowers because of their exotic beauty and their long lasting blooming

period (Hew et al., 1997). Many of them are used as herbal medicines, food, and other have cultural value by different cultures and tribes in different parts of the world (Pant, 2013).

Orchids are unique group of plants that exhibit an incredible range of diversity in size, shape, structure, colour and fragrance of the flower (Thomas and Michael, 2007). Many orchids are useful for small scale industries. Fresh bulbs of *Coelogyne asperata* are used as black board erasers in Sumatra (Withner, 1959). Glue prepared from the species of *Cryptopodium*, *Geodorum nutans* and *Bletilla* are utilized for the manufacture of musical instruments. Yellow pseudo-stems of *Dendrobium utile* are used as ornaments in New Guinea by tribal people (Bose et al., 1999). *Dendrobium* stems are used in making baskets in the Philippines, Indonesia and New Guinea (Yadav and Basak, 1998). In several parts of the world, orchids are used as food. Roasted bulbs of *Gastrodia sesamoides* were used as food during emergency situation (Lawler and Slaytor, 1970). Dried leaves of *Dendrobium salaccense* are used as flavoring agent during cooking of rice. In islands of Cyprus, milk custard drink is prepared from dried ground tubers of *Orchis anatolica*. Pseudobulbs of *Cymbidium madidum* and *Dendrobium speciosum* are also reported to be used as food (Bose et al., 1999). Root tubers of *Disa*, *Habenaria* and *Satyrium* are used to make food dish called Chikanda or Kinaka in Zambia (Davenport et al., 2004). Powdered roots of *Vanda tessellata* are used as antidotes of poisoning, rheumatic pain and abdominal complaints (Amin et al., 2004). Different parts of orchid are used in treatment of various diseases. Juice obtained from the leaves of *Cymbidium aloifolium* are used for the treatment of otitis and other inflammatory conditions. In Malaysia, the decoction of leaves prepared from *Nervilia aragoana* is used as a protective medicine after childbirth. Roots of *Vanda tessellata* which forms constituents of medicated oils and used for rheumatic swellings and nervous disorders (Yoganarasimhan, 1996). Medicinal orchids belong mainly to genera: *Anoctochilus*, *Bletilla*, *Calanthe*, *Coelogyne*, *Cymbidium*, *Cypripedium*, *Dendrobium*, *Ephemerantha*, *Eria*, *Galeola*, *Gastrodia*, *Gymnadenia*, *Habenaria*, *Ludisia*, *Luisia*, *Nevilia* and *Thunia* (Szlachetko, 2001). 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). We have listed ninety species of orchids from Nepal with their medicinal uses (Pant and Shrestha, 2011). In the Khasi hills, juice prepared from the leaves of *Cymbidium giganteum* is used to prevent blood flow in wound, while powdered flower of *Vanda spathulata* is effective against hysteria. *Dendrobium fimbriatum* is used against liver upsets and nervous debility (Bose et al., 1999). Stem paste of *Vanilla walkeriae* is given orally along with feed to treat fever in cattle and also used as nutritive supplement for cattle (Balasubramanian et al., 2000). *Cyrtorchis arcuata* is employed in the treatment of diabetes (Morris, 2003). Gastrodin is active ingredient of *Gastrodia elata*, lowers blood pressure and protects the central nervous system due to its anti-delirium and anti-convulsive activity (Sun et al., 2004). Paste made from pseudobulbs of *Ansiella africana* used as a contraceptive in Africa (Bulpitt, 2005). *Bletilla striata* is used as anti-haemorrhage agent in Chinese medicine (Bulpitt et al., 2007). Orchids are valued for cut flowers due their wonderful nature of flowers and extended keeping qualities (Amin et al., 2004). Orchids are mostly grown in parks, gardens and urban areas (Borys et al., 1999; Lopez and Runkle, 2005). Some orchids, flowers are long lasting for one to three months when remain attached to the plant while, cut flowers remains fresh for one to four weeks. It was reported that flowers of *Dendrobium crumenatum* last for a day, while, the flowers of *Grammatophyllum multiflorum* last for about 270 days (Avadhani et al., 1994). Attri et al. (2008) reported that un-pollinated flowers of *Aerides multiflora* and *Rhynchostylis retusa* can remain fresh for 17 and 24 days, respectively. Commercialization of orchids has taken place in many countries such as Thailand, Singapore, Malaysia, Japan, Korea and Taiwan for both plant sale and cut flowers (Gavinlertvatana and Prutpongse, 1991). In India, commercial orchid growing is limited, as it is in the hands of dealers who collect orchids from the wild populations (Rao, 1977). Many of the Indian orchids such as *Cymbidium*, *Dendrobium*, *Paphiopedilum*, *Renanthera* and *Vanda* are currently in the International market. It was reported that about 70% of orchid plants are illegally imported by USA from India and Thailand

(Chadha, 1992). They earn a lot of foreign exchange by exporting different attractive orchid flowers to developed countries.

2.3 Orchid flora

Although there are several characteristics that vary among the flowers of various orchids, all orchid flowers have some common characteristics including three sepals and three petals. Another characteristic of most orchidaceae plants is their elongated leaves with parallel veins, which have varying shapes and sizes and are considered either thin or thick-leaved (Hew et al. 1997). Orchidaceae are characterized by a diverse range of life histories, reproductive biology and distributions which reflects varieties of patterns of orchid populations (Scacchi et al., 1990; Peakall and Beattie, 1996; Sun, 1996; Gustafsson, 2000; Tremblay and Ackerman, 2001; Wallace, 2002; Forrest et al., 2004). It consists of five sub-families (Chase et al., 2003) and about 35,000 species belong to 800 genera distributed throughout the world (Singh et al., 2007). Two third of orchids are epiphytes and lithophytes, while, one third contributed by terrestrial orchids (IUCN, 1999). The family orchidaceae is one of the largest families of flowering plants. Approximately 25% of their species are terrestrial, 70% are epiphytic and about 5% are found on various other supports (lithophytes, saprophytes).

2.4 Growth habits

Orchids can be broadly divided into three growth habits. It is important to know to which group a plant belongs, because they each have different requirements. Orchids have varying habit to adjust different environmental conditions in which they grow. They include terrestrial, epiphytic and saprophytic forms (Jonathan and Raju, 2005).

2.4.1 Terrestrial orchids

Terrestrial orchids grow on the ground, with their roots in the topmost layer of humus. Most orchids in this category are from temperate regions. Although it grows in the tropics, the *Paphiopedilum* genus belongs in this group. These orchids grow on land and do not require a host plant for their survival. The orchids which grow in cooler regions

are remains dormant during the dry season and produces new shoots during wet season (Freudenstein, 1994; Bose et al., 1999). Orchids in the tropical and sub tropical regions are generally evergreen and possess pseudobulbs (Jonathan and Raju, 2005).

2.4.2 Epiphytic orchids

Epiphytic orchids grow on other plants, although they are not parasitic. They are mostly native to the tropics. The *Phalaenopsis* and *Cattleya* genera belong to this group. Epiphytic orchids are entirely depending on trees for structural and nutritional support. They are not parasitic and do not feed on the host plants (Dematte and Dematte, 1996; Jonathan and Raju, 2005). Orchids obtain nutrients from rains and debris which accumulated on the barks of host plant. They have adapted to have aerial roots and do not have the advantage of absorption of water from the soil. Usually in orchids, water is absorbed by a spongy structure called "velamen tissue" which formed by dead cells layers in the roots. Orchids withstand severe drought, however, they die if there is inadequate drainage (Bomba, 1975; Batchelor, 1981; Dematte and Dematte, 1996).

2.4.3 Saprophytes

A very small number of orchids have peculiar characteristic that they live in symbiosis with particular fungi which provide the food for them. Thus saprophytes have no need for chlorophyll; they do not photosynthesize. They are mostly lacking colour, although at times they can be pale pink, lavender or soft brown. They have no leaves, only vestigial scales along their glassy stems. They possess mycorrhizal fungus in their root for the supply nutrients. These plants do not have chlorophyll and reduced in size with bunch of coralloid roots. They are of underground and emerged out of the ground only during flowering. Fischer (1928), reported that three saprophytic species such as *Didymoplexis pallens*, *Epipogum nutans* and *Aphyllorchis montana* from South India. Varjavelu and Radhakrishnan (1968) have reported fourth saprophytic species namely, *Aphyllorchis prainii* from the forest of Attapadi. Taxonomic review of saprophytic orchid species was reported from Arunachal Pradesh (Rao, 1988; Hegde, 2001). Total of 42 saprophytic species belong to 18 genera were reported from Indian regions (Pradhan, 2004). A rare

and giant saprophytic orchid *Galeola falconeri* Hook. F. was reported from lower Subansiri district of Arunachal Pradesh (Das and Khumbongmayum, 2006). One of these saprophytic orchids is the rarely seen Phantom orchid (*Cephalanthera austiniiae*); others are the coral root orchids (*Corallorhiza* sp.) whose roots really do look like white coral, forever underground.

2.5 Characteristics of orchids

2.5.1 Leaves

The characteristic of most orchidaceae plants is their elongated leaves with parallel veins, which have varying shapes and sizes and are considered either thin or thick-leaved (Hew et al., 1997). Orchids have typical monocotyledonous pattern of leaves which are generally green in colour. Sometimes they have golden or silvery veins that provide attractive ornamentation as in *Anoectochilus* species. Leaves are long, plicate or short, elliptic oblong or linear ovate and always with sheathing bases in terrestrial orchids. In epiphytic species, leaves are thick and coriaceous. In species such as *Vanda*, the leaves act as a storage organ. Leaves are laterally compressed and succulent in *Oberonia* and *Podochilus* (Bose et al., 1999).

2.5.2 Inflorescences

The origin of inflorescence is specific to each genera and species. They are either terminal or lateral in terrestrial orchids while, in epiphytic orchids inflorescences are lateral. Exceptionally, in few epiphytic orchids like *Porpax* and *Polystachya* inflorescence is terminal. Usually, inflorescences are developed from a leafy shoot, but in some cases leaves are shed before flowering like in *Bulbophyllum fimbriatum*. Only leafless flowering shoot appears in saprophytes (Bose et al., 1999).

2.5.3 Flower

Although there are several characteristics that vary among the flowers of various orchids, all orchid flowers have some common characteristics including three sepals and three

petals. Orchid flowers have tremendous diversity in morphology (Mondrago'n-Palomino and Theiben, 2008). The orchid *Sorbralia macrantha*, known for largest flower sized 15 to 30 cm across while, *Bulbophyllum minutissimum* possess tiniest flower of pin head size. The principal shades are white, yellow, green and purple of in every possible combination of colours. The important characteristic of orchid flowers is zygomorphic nature of flowers (Bose et al., 1999). Orchids flowers usually composed of three outer tepals (sepals) in the first floral whorl, two lateral inner tepals (petals) and a median inner tepal termed as the labellum or lip in the second whorl (Rudall and Bateman, 2002; Mondrago'n-Palomino and Theiben, 2008). The primary characteristics, of orchid flowers are the fusion of male and female organs within a single structure located at the centre of the flower, pollinia and zygomorphic nature of flowers (Montalvo and Ackerman, 1987; Proctor and Harder, 1994; Nazarov and Gerlach, 1997; Freudenstein and Rasmussen, 1997; Pacini and Hesse, 2002). The labellum is morphologically complex structure in orchids which primarily attracts the pollinators such as insects (Nilsson, 1992; Rudall and Bateman, 2002). Labellar structure consists of fused style and stamens of the gynostemium which is diagnostic character for orchids (Bateman and Rudall, 2006).

2.6 Orchid fruits/capsules

The orchid fruit is a capsule and it takes a long time to mature. Each ripened capsule contains numerous dust-like seeds (about 4 million) (Arditti and Ghani, 2000). The orchid seeds are the smallest among the flowering plants (Arditti et al., 1980). They vary in size, morphology, structures and colour. Orchid seeds vary in size from 150 to 6000 μm , whereas in most of the taxa it ranges from 300 - 800 μm (Molvray and Kores, 1995). Shape of the seed varies from filiform to fusiform, clavate to ellipsoidal and rarely winged. In general seed coat is papery and loosely surrounds the embryo and rarely seeds are covered with hard coat (Molvray and Kores, 1995). Usually harvesting of the capsules is done before the dehiscence and normally it takes about 4-10 months to mature and ripen. However, time taken for ripening of capsules varies among the species.

2.7 Cultivation of orchids

2.7.1 In shade house

Generally two different types of greenhouses (un-cooled and controlled) are used as orchid shade house. Tropical warm orchids are mostly grown in un-cooled greenhouse which protects from direct sun light which reduces the inside temperature and increases humidity and favoring luxuriant growth and flowering of orchids (Bose et al., 1999). In controlled greenhouse, temperature, light and humidity are controlled which provides favorable environment for growing high value orchids.

2.7.2 Growing orchids

Different types of container are used for growing orchids. Most commonly used are clay pot, wooden and galvanized wire baskets, tree fern blocks and logs of woods. The containers such as clay pots have several cuts and holes to facilitate adequate aeration and proper drainage are routinely used. In the recent times, plastic containers are gaining importance due to their less weight, easy to transport, free from deposition of algae or salts on their sides and longer moisture retaining capacity than clay pots.

2.7.3 Potting medium

A suitable growing media for orchids is necessary for the plant growth and development and flowering. Ideal medium should support the plant, supply water and nutrients to the roots and provide excellent drainage and aeration. For several years the *Osmunda* fiber was used as potting medium, either alone or in combination with moss which increases water holding capacity. Recent years, orchids have been successfully grown in various potting medium (Tan and Lee, 2001; Lauzer et al., 2007). Use of broken tiles along with charcoal chips (1:2) was reported in blue *Vanda* (Seeni and Latha, 2000). Temjensangba and Deb (2005) used mixture of charcoal pieces, brick pieces, coconut husk and decayed powder (1:1:1:1) with a layer of moss to achieve 60% survival of hardened plants. Requirement of pre-hardening medium was reported for *Zygopetalum intermedium* (Nagaraju and Mani, 2005). The rooted plantlets were cultured on pre-hardening medium

containing paclobutrazol (0.25 mg l^{-1}) and activated charcoal (1.5 mg l^{-1}) for a period of two months. Subsequently, after pre-hardening, the plants were transferred to community pots containing mixture of sterilized cocco peat and tree fern (1:1). Chen et al. (2005) successfully utilized mixture of perlite, sand, charcoal and soil (3:2:2:3) for hardening *Cymbidium faberi* plants. Regenerated plants of *Oncidium* sp. (dancing doll) were grown in the greenhouse after transferring to perforated plastic pots containing mixture of charcoal and brick pieces (1:1) (Kalimuthu et al., 2007). Roy et al. (2007) reported that in *Dendrobium chrysotoxum*, 80% survival rate using dried coconut husk, brick pieces and charcoal in the ratio of (1:1:1). Bhattacharjee and Islam (2014) reported that about 65-80% of *in vitro* grown *Acampe premorsa*, *Agrostophyllum khasianum* and *Phalaenopsis cornorerris* seedlings were successfully grown in pots when used brick pieces, charcoal pieces and moss (1:1:1).

2.7. 4 Nutritional requirements

Harvais (1973) first reported that the nutritional requirements for *in vitro* growth and development of a terrestrial orchid, *Cypripedium reginae* in axenic culture. This orchid species showed the greatest promise for investigation of the effects of temperature, light and nutrients. Seedlings were grown from seeds in sterile cultures on agar slopes of media consisting of various combinations of minerals, sugars, casein hydrolysate, yeast extract, the vitamins- thiamine, pantothenic acid and pyridoxine and the aminopurines- kinetin, kinetin riboside, 6- γ , γ -dimethylallylamino purine and zeatin. Better germination and growth occurred at 25°C vs 15°C . Germination was better in the dark than in the light. The young protocorms were adversely affected by light until a crucial stage of development reached. Premature exposure to light, even at the low intensity of 70 lux/ft^2 , caused mortality. There was no germination on sterile distilled water agar or on mineral media alone. Mineral- sugar media produced fairly healthy plantlets. Better results were obtained with sucrose, dextrose and fructose, respectively. The orchid species was highly intolerant to the casein hydrolysate and yeast extract at 10% at the original concentration was very beneficial. The presence of ammonium ions could be responsible for a marked

enhancement of growth. The nitrate- ammonium ratio might be critical. Low levels of nitrate or high levels of chloride or both hindered response to iron supplements. The effects of the three vitamins were restricted to the leaves causing them to broaden to natural proportions; while those in the controls remained spindly. Thus *C. reginae* may be heterotrophic for those vitamins. There was no morphogenetic response to the four aminopurines. It impeded growth equally in the light as in the dark.

The supply of nutrients in liquid forms improves the growth and flowering of orchids. In nature, orchids get their nutrients from the decayed organic matter and soil on which they are grown and also from the atmosphere by the rains (Bose et al., 1999). During cultivation all the nutrients are required to be supplied to the orchids regularly for their growth and development and flowering. Several fertilizer solutions have been recommended by various workers. Kumaria and Tandon (1994) reported that in *Dendrobium fimbriatum* var. *oculatum* that feeding the plantlets with MS liquid medium (10 times diluted) was found beneficial to the developing hardened plantlets. Similar recommendation of use of MS medium (10 times diluted) was made in *Arachnis labrosa* (Temjensangba and Deb, 2005), *Cymbidium devonianum* (Das et al., 2007) and *Vanilla planifolia*, (Janarthanam and Seshadri, 2008). Seeni and Latha (2000) used foliar spray of Vijay complex, NPK 17:17:17 (Madras Fertilizer Co., Madras) at weekly intervals in case of blue *Vanda*. Park et al. (2002) used hyponex solution (6.5N:4.5P:19K) at 15 day intervals in case of *Phalaenopsis*. A mixture of two commercial fertilizer i.e DAP (Diammonium phosphate) and NPK (20:10:10) was used at weekly intervals for hardening of *Vanda coerulea* (Malabadi et al., 2004). Thomas and Michael (2007) used full strength macro elements of MS medium for *in vitro* regenerated plants of *Rhynchostylis retusa* during hardening. 150 ppm of N:P:K balanced liquid fertilizer (Peter's 20:20:20, The Scott's Company, Marysville, OH) was used weekly as a source of nutrient for greenhouse acclimation of *Bletia purpurea* and *Cyrtopodium punctatum* (Dutra et al., 2008; Dutra et al., 2009).

2.7.5 Water supply

The proper frequency of watering will depend on the climatic conditions. In orchid cultivation, time and quantity of water is very important for the survival of plant. It is difficult to prescribe a schedule of watering for orchids, since the number of watering necessary to keep the plants alive depends on the climatic conditions, type of media used and type and size of the pots used during the cultivation. For most of the orchids watering should be done only when the medium completely dry, however, in case of *Paphiopedium* and *Cymbidium*, require water around their roots at all times. Over watering should be avoided for wild orchid species such as *Vanda* which requires minimal supply of water usually absorbed through the velamen roots (Bose et al., 1999). Over watering blocks the air passages in the roots and asphyxiated. Wrinkling of pseudobulbs and yellowing of leaves are the initial sign of over watering, at this point of time watering should be stopped immediately and confine to aerial spraying. In general, watering twice in a day would be more suitable (Seeni and Latha, 2000; Chen et al., 2005), however, there are reports of watering once in day (Dutra et al., 2009). Bhattacharjee and Islam (2015a) reported that the plantlets were watered alternately in the evening. In general, water once a week during the winter and twice a week when the weather turns warm and dry.

2.8 Conservation of orchids

Swarts and Dixon (2009) reported that orchidaceae consist of a high number of threatened species. Orchid species are under major threat worldwide than any other plant family due to over exploitation by collectors and enthusiasts (IUCN, 1999). Coates and Dixon (2007) reported that the major threat to orchid populations are human activities, land clearing for agriculture purposes, mining and urban development, weed invasion, grazing and collection of plants for medicinal, horticulture and ethnobotanical reasons. During the formulation policies for conservation programmes, several aspects such as existing and future environmental threats, taxonomic distinctiveness, geographic distribution, habitat specialization, reproductive biology, evolutionary processes

influencing population structure and *ex situ* conservation methods should be taken in to consideration (Swarts and Dixon, 2009). *Ex situ* conservation is often taken as the key conservation aspects, but preservation of germplasm off-site should be considered as an 'emergency ward' targeting at extinction of those species under the immediate threat. Despite of off-site conservation such as seed and germplasm banks and *in vitro* propagation, the *in situ* conservation and conservation via assisted migration are the premier approaches for global biodiversity conservation. They also reported that the effective conservation strategies must be developed to avoid further loss of essential species and ecosystem. Bormann et al. (2007) reported that the adaptive management approach was one of the useful methods for linking research to operational practicality in management and restoration of species and ecosystems.

2.9 Tissue culture of orchids

Plant tissue culture plays an important role of mass multiplication and conservation of endangered plant species (Grell et al., 1988; Rubluo et al., 1989; Gangaprasad et al., 1999; Seeni and Latha, 2000; Decruse et al., 2003). Morel (1960) successfully cultured *Cymbidium* shoot apices on Knudson C agar medium. Later, this method of clonal propagation technique created interest among orchid grower and nurseries.

2.9.1 Explants used for micropropagation

Different explants such as shoot tip, stem, leaf, roots and immature seeds were used for *in vitro* micropropagation.

2.9.1.1 Leaf

Wimber (1965) at first reported the formation of protocorm-like bodies (PLBs) from orchid leaf tissue. Churchill et al. (1971) studied the tip of young leaves from mature plant of *Cattelya* was able to proliferate into protocorm-like bodies. This method was developed to limit the need to sacrifice entire plant or shoots and to provide a simple procedure for clonal propagation of *Cattleya*. Tanaka and Sakanishi (1977) induced the formation of PLBs on leaf segments excised from the shoots originated from a flower

stalk by aseptic culture. Churchill et al. (1973) used different explants (leaf tip, petioles, various section of leaf blade) from *Epidendrum* and *Laeliocattleya*. However, he was successful to get PLBs only from leaf tip, which produced callus initially. According to him, the production of plantlets from leaf tips is interesting because whole plant originates from a region which is destined to mature into foliar mesophyll and epidermis of the plant, thereby losing all meristematic capacity. Tanaka (1987) conducted some experiments on clonal propagation of *Phalaenopsis*. *Phalaenopsis* being a monopodial, rarely produces vegetative off shoots. Therefore, it was considered desirable to use leaf tissue as explants. Till nineteen hybrids were successfully propagated. Of the 6 hybrids which failed to respond, 3 hybrids produced no shoots from the flower stalk cuttings, while for the remaining 3, the leaf segments did not form PLBs. There were however, differences between various hybrids in the number of PLBs formed per leaf segment, in that it ranged from 1 to 1.54 PLBs. 18 *Phalaenopsis* species (including one *Doritis* sp.) were also used to test this method propagation. 12 species were successfully propagated using this method. The species were: *P. amabilis*, *P. aphrodite* (Philippine), *P. aphrodite* (Taiwan), *P. sanderana*, *P. sanderana alba*, *P. stuartiana*, *P. amboinensis*, *P. fasciata*, *P. luddemanniana*, *P. intermedia* and *D. pukherrima*. Of the 6 species which did not produce shoots from the flower stalk cuttings, whereas the other 2 species did not form PLBs in leaf segments. Of the species in which the leaf segment culture was successful, the number of PLBs formed per leaf segment varied from 1 to 6.5 PLBs depending on the species concerned. The final section of this study was to determine whether the regenerated plants express the original mother plant characters such as flower colour and shape which is important for practical application. When the employment of a clonal propagation gave a high frequency of variation of plants, this method would not be useful. It took 5-7 months 70 plantlets obtained from the PLBs stage in the flask. All these plantlets were planted using a conventional way in the greenhouse. In 1983, after 22 months of growth, many plants were similar to their respective mother plants. These results confirmed that the clonal propagation method of *Phalaenopsis* mentioned above would have a commercial value.

Vij et al. (1984) were able to micropropagate *Rhyncostylis retusa* by direct organogenesis from leaf segment cultures. Young leaf segments of plants growing both *in vivo* and *in vitro* were cultured by Mitra et al. (1976) and the medium was supplemented with various auxins (LAA, NAA, 2,4-D), kinetin, GA₃ and organic supplements (peptone and Yeast extract). The explants from nature leaves did not show any growth and turned necrotic. While those obtained from Juvenile leaves of 6 month old plantlets growing *in vitro* developed PLBs at their cut surfaces within 4-7 weeks, depending on the growth medium. Formation of PLBs subsequently spread all along the explants and suggested a meristematic /generative potential of the epidermal walls in immature leaves. Upon sub-culture in basal medium, the PLBs differentiated into plantlets within 4-5 weeks. Although regeneration of PLBs was achieved in BM and the auxins or GA₃, kinetin in combination with IAA/NAA in the presence of peptone accelerated their formation. Regeneration of large number of plantlets from leaf explants of *Acampe praemorsa* was reported by Nayak et al. (1997), which could be used for both large-scale propagation and *ex situ* conservation of this important orchid species. Murthy and Pyati (2001) developed an *in vitro* propagation system for the rare/endemic orchid species *Aerides maculosa* using juvenile leaf explants.

2.9.1.2 Shoot tips

Cymbidium was the first orchid genus which was propagated by shoot tip culture (Morel, 1960). Sagawa et al. (1966) also developed technique in *Cymbidium* using shoot tip, which can also produce plantlets quickly. Mitra (1971) reported that tissue culture method in *Arundina bambusifolia* using shoot tips from mature plant. Kunisaki et al. (1972) propagated *Vanda* Miss Joaquim by shoot tip culture. Strap leafed *Vanda* was successfully propagated by shoot tip culture by Teo et al. (1973). In hybrid *Aranda* 'Deborah' noticeable swelling was observed from all the shoot tip explants after 3-4 weeks in culture (Goh, 1973). Intuwong and Sagawa (1974) successfully developed method for the culture of *Phalaenopsis* shoot tips.

Choi et al. (1980) succeeded in regeneration of plants and rhizomes from shoot tip culture of *Cymbidium* sp. Shoot tip or auxiliary bud cultures of 13 cultivars of *C. kanran* and 17 cultivars of *C. forrestii* were established. In 12 cultivars, rhizomes (pseudobulbs) were formed but later died. Twelve of those in which rhizomes survived growth in sub-culture was vigorous. Culture of 12 cultivars of 5 other species (*C. goeringii*, *C. sinensis*, *C. sinense*, *C. niveo-marginatum*, *C. fobrigemmum* and *C. faberi*) were also successful. In *C. goeringii* survival increased with decreasing efficiency of disinfection. Rhizome formation in shoot tip culture of *C. goeringii* was best with at 1.0 mg l^{-1} kinetin + 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin + 1.0 mg l^{-1} NAA. Devi and Laishram (1998) cultured shoot tip and auxiliary bud from young shoot of six *Dendrobium* hybrids. *In vitro* clonal multiplication of *Dendrobium* cv. Sonia was investigated using offset segments (Pathania and Sehgal, 1999). Gangaprasad et al. (2000) successfully micropropagated *Anoectochilus regalis* using shoot tip. Development of plantlets from shoot tip derived suspension cultures of *Phalaenopsis* was reported by Tokuhara and Mii (2003). A rapid and efficient procedure for *in vitro* clonal propagation of an elite cultivar of jewel orchid *Anoectochilus formosanus* was reported by Ket et al. (2004). Shoot regeneration of *Vanda coerulea*, an endangered epiphytic orchid was reported by using thin shoot tip sections (Malabadi et al., 2004). Successful initiation of PLBs and *in vitro* regeneration of *Cymbidium elegans* was achieved using shoot tip in presence of an endogenous plant growth regulator epi-brassinolide (Malabadi and Nataraja, 2007). Rahman et al. (2009) reported that *in vitro* micropropagation of *Vanda tessellata* from shoot tip explants.

2.9.1.3 Inflorescence

Flower stalks of *Dendrobium* having well developed buds were used for the multiplication (Singh and Sagawa, 1972). Two stage sterilization procedure using chlorax was developed by Intuwong et al. (1972) for *Phalaenopsis* using flower stalk cuttings. Intuwong and Sagawa (1973) developed effective clonal propagation method without sacrificing a mother plant in *Ascofinetia* using inflorescence.

Griesbach (1983) conducted some experiments on the use of indoleacetyl amino acids in the *in vitro* propagation of *Phalaenopsis*. This orchid could be propagated through *in vitro* culture of inflorescence nodal buds. A procedure was developed whereby nodal buds were included to grow into shoots. The basal section of the shoots was cut off and placed on a medium, which induced formation of protocorm-like structures. The effects of 4 indoleacetyl amino acids on proliferation of those structures were examined.

Chen and Chang (2000) were successful in producing somatic embryos from the segments of flower stalk inter-nodes of *Oncidium* cultivar Sweet Sugar. Tokuhara and Mii (2001) reported that micropropagation method of *Phalaenopsis* using shoot tip explants excised from flower stalk buds. High proliferation rate of both the callus and cell suspension culture without loss of embryogenic potential for at least 5 years makes this method highly efficient. An efficient and rapid *in vitro* method for regeneration of *Phalaenopsis* using flower stalk nodes was developed by Park et al. (2002). Role of thidiazuron (TDZ) in direct PLBs formation from flower stalk internodes of *Epidendrum radicans* was reported by Chen et al. (2002). Thin sections of inflorescence axis were used for the micropropagation of *Phalaenopsis amabilis* cv. Cool Breeze (Sinha et al., 2007).

Sharma (2012) became successful in regeneration competence of anther culture of *Rhynchostylis retusa* (Orchidaceae). The possibility of using anthers for initiating *in vitro* cultures of *Rhynchostylis retusa*, has been positively tested. The study was designed to study the effects of physiological status, stage of microspore development and cold pretreatment on androgenic response in *R. retusa*. The anthers from open flowers (2 days of anthesis) in tetrad stage failed to respond despite variations in the chemical regime; whereas those from unopened buds (1.25 - 1.35 cm long) with an intact operculum and cold pre-treatment with microspores in early and late-uninucleate decussate regenerated provided their nutritional complexities were satisfied through an exogeneous supply of peptone (2 mg l⁻¹) in the BAP (10 mg l⁻¹) and NAA (1 mg l⁻¹) enriched Sharma medium (Sharma, 2012). The best response was obtained when a 24 h cold pre-treatment was

employed at 4°C in darkness. The complete plantlet (2-3 leaves and 1-2 roots) was formed in 24 weeks.

2.9.1.4 Immature seed

The horticultural trade currently depends on wild orchid populations as a source of stock plant (Kalimuthu et al., 2007). Although many commercially exploited orchid species are common, no sufficient artificial propagation methods are developed to reduce collection pressure. Orchid capsule contains millions of seeds however, it lacks metabolic machinery and do not have any endosperm. Despite the production of large number of seeds in orchids, only 0.2 - 0.3% of seeds germinates in nature (Singh, 1992; Murthy and Pyati, 2001). Knudson (1951) successfully germinated *Cattelya* seeds without any symbiotic association of fungus on nutrient medium. Since then vast extensive work using mature seeds have been reported by Buyun et al. (2004), Sinha and Roy (2004), Kauth et al. (2006), Manrique and Gutie (2006), Johnson and Kane (2007), Johnson et al. (2007), Dutra et al. (2008), Dutra et al. (2009). Sharma et al. (2004) reported that slow growth and extended time taken for flowering are the limitations for the use of mature seeds as explants for micropropagation. The technique of culturing of orchid seeds prior to maturity is referred as ovule/embryo/green pod/green fruit culture (Sagawa, 1963). Sauleda (1976) reported that the optimal time for harvesting of capsules of a number of orchid species and hybrids. In green pod culture, the immature seeds are obtained from early stages of development starting from fertilization to maturity (De et al., 2006). Micropropagation methods for several orchids using immature seed have reported earlier. Mitra et al. (1976) was successful in achieving the differentiation of protocorms in seed callus of *Dendrobium fimbriatum* in absence of any kind of auxin or cytokinin. Seedling from immature seed of *Vanda coerulea* (four month after self pollination) was used by Seenii and Latha (2000) and achieved rapid multiplication for horticultural use. *Arachnis labrosa* a rare and threatened monopodial epiphytic orchid was successfully regenerated by culturing immature seeds 16-18 week after pollination (Temjensangaba and Deb, 2005). In China many elite varieties of *Cymbidium faberi* has been cultivated with a very

limited number of plants for more than 100 years, but all the cultivars were developed from its natural variants and its cross breeding has not been recorded (Wu, 1992). Chen et al. (2005) found that cross breeding was mainly due to absence of efficient protocol of plant regeneration from the seeds. They further established *in vitro* plant regeneration protocol for immature seeds of *Cymbidium faberi*. Sharma et al. (2005) developed *in vitro* protocol for rapid micropropagation and conservation of *Dendrobium fimbriatum* using immature seeds (70 days old). *In vitro* regeneration, multiplication and rooting of plantlets were achieved from immature seed of *Oncidium* sp. cultured on MS medium (Kalimuthu et al., 2007). Thompson et al. (2007) developed dual phase protocol for ten *Disa* species. Dual phase cultures were initiated using immature seed from six week old capsule, two weeks prior to dehiscence. This novel system was adapted to test the germination response. Mahendran and Bai (2009) became successful in direct somatic embryogenesis and plant regeneration from seed derived protocorms of *Cymbidium bicolor*. A protocol for the induction of direct somatic embryogenesis from seed derived protocorms was developed in *Cymbidium bicolor*. Ninety-day-old protocorms cultured on half strength MS medium alone or supplemented with auxin and cytokinin induced direct somatic embryogenesis. The best response was observed in protocorms cultured half strength MS medium supplemented with BAP (1.0 mg l^{-1}) and 2-4,D (2.0 mg l^{-1}). Both epidermal and sub-epidermal cells were involved in the formation of embryos. The pro-embryos developed into globular stage and subsequently developed into protocorms. Complete plantlets were formed after 60 days culture. The plantlets were acclimatized in plastic pots containing sterilized vermiculite. Survival rate was 90% when maintained in culture room condition ($25 \pm 2^\circ\text{C}$). Bhattacharjee and Islam (2014) reported that an efficient protocol for *in vitro* seed germination and for growth and development of protocorm like bodies (PLBs) from immature seeds of three indigenous orchid species namely *Acampe premorsa* (Roxb.), *Agrostophyllum khasianum* L. and *Phalaenopsis cornorerris*. Bhattacharjee and Islam (2015a) were successful in *in vitro* development of protocorm, regeneration and mass multiplication of *Rhynchostylis retusa* by culturing immature seeds.

2.9.1.5 Stem

First stem culture was noticed in *Arundina* stem around 1966 by Arditti (1977). An efficient protocol for regeneration of endangered medicinal orchid of *Vanda tessellata* via multiple shoot has been established using shoot segments as explants (Bhattacharjee and Islam, 2014). Under this study four culture media viz. PM, MS, ½MS and B5 were used. Twenty different in combinations of plant growth regulators (PGRs) were tested for multiple shoot induction via stem culture.

2.9.1.6 Root

The first printed suggestion to culture roots was published appeared in a theoretical article in 1970 (Arditti and Krikorian, 1996). Churchill et al. (1972) cultured epidendrum root tips, but never obtained plantlets. Following the standard protocol (Churchill et al. 1972) other orchid roots have been cultured in *Phalaenopsis*, *Catasetum*, *Cattleya*, *Crimean* orchids, *Cymbidium*, *Cypripedium yatabeanum*, *Cyrtopodium*, *Doritaenopsis* and *Rhynchostylis* rhizome tips by Ueda and Torikata (1972). Other orchid propagated from rhizome explants such as *Cymbidium ensifolium*, *Cymbidium ensifolium* × *Cymbidium kanran* and *Cymbidium kanran* Jeju × *Cymbidium goeringii*) and (*Cymbidium aloifolium*, *Cymbidium goeringii*, *Cymbidium niveo-marginatum*, *Cymbidium sinense* and *Geodorum densiflorum*).

3. Study of antimicrobial activity and phytochemical analysis

Various medicinal plants have always had important properties in the therapeutic armory of mankind (Velanganni et al., 2011). Medicinal plants would be the best source of drugs and therefore such plants should be investigated to understand better about their properties, safety and efficacy and major sources of obtaining antimicrobial drugs (Nascimento et al., 2000; Sofowora, 1986). Kalaiaarasan et al., (2012) reported that the antimicrobial activity of *Bulbophyllum kaitense* Rechib stem of eastern peninsular flora in India. An attempt has been made together for the information about the traditional use of herbs from the local healers. *Bulbophyllum kaitense* stem is being used for the treatment

of certain anticancer. The plant parts of stem are collected and phytochemicals present in them are analyzed.

Mazumder et al. (2010) studied *in vitro* propagation and phytochemical screening of *Papilionanthe teres* (Roxb.) Schltr. For phytochemical screening qualitative tests were performed from the dried powder of the plants. From the qualitative test it was observed that the plant extracts contain alkaloid, flavonoid and tannin in petroleum ether extract and alkaloid, tannin and saponin were found in ethyl acetate extract. Steroid was found present in methanol and benzene extract. Flavonoid was found only in acetone extract.

Marasini and Joshi (2012) studied antibacterial activities of different extracts of epiphytic orchids were tested against 5 species of bacteria and antifungal activities were tested against 3 species of fungi. All orchid extracts showed good bacterial zone of inhibition (ZOI) against *Staphylococcus aureus*. The crude extracts of *Pholidota imbricata* and *Coelogyne cristata* were shown highest activity against *Vibrio cholerae* and *Staphylococcus aureus* respectively. The minimum inhibitory concentration (MIC) and maximum bacteriocidal concentration (MBC) value of the extracts of *Pholidota imbricata* and *Coelogyne cristata* were found to be 62.5 mg/ml, 31.25 mg/ml and 125 mg/ml, 250 mg/ml against *Vibrio cholerae* and *Staphylococcus aureus* respectively. Only *Pholidota imbricata* and *Pholidota articulata* extracts were shown fairly good activity but all others extracts were shown very less activity or even failed to show any activity against fungal organisms.

Chaitanya et al. (2013) reported that phytochemical and antimicrobial screening of the polar and non-polar solvent root extracts of *Vanda tessellate* (Roxb.) Hook. Ex. G. Don. Bhattacharjee and Islam (2014) reported that on antimicrobial activity and phytochemical screening of whole plant extracts of *Vanda tessellata* (Roxb.) Hook. Ex. G. Don. The purpose of this study was to determine phytochemical constitutes and antimicrobial activity using extracts from whole plant of *Vanda tessellata*. The various solvent extracts such as chloroform, methanol, ethanol and hexane of this orchid were considered for *in vitro* antimicrobial activity against five clinical pathogenic bacteria viz. *Staphylococcus*

aureus, *Bacillus subtilis*, *Vibrio cholerae*, *Escherichia coli*, *Klebsiella pneumonia* and three fungi viz. *Penicillium* sp. *Rhizopus* sp. *Aspergillus niger* by disc diffusion method. A preliminary phytochemical analysis was performed for the detection of alkaloid, terpenoids, flavonoids, phenols, tannins, steroids and glycosides etc. All the extracts showed different degree of inhibitory that potential against tested bacteria under this study. Gupta and Katewa (2014) studied *in vitro* evaluation of the antimicrobial activity of different solvent extracts of roots of *Vanda tessellata* (Roxb.) In the experiment bioassay (petroleum ether, chloroform, ethyl acetate, acetone, methanol and hexane) of various root extracts were investigated for an *in vitro* antimicrobial activity by agar-well diffusion method. These were tested against six bacteria: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Bacillus subtilis* and two fungi *Candida albicans* and *Aspergillus niger*. Bhattacharjee and Islam (2015b) assess the antibacterial and antifungal activities of the extracts of *Rhynchosyilis retusa* Blume. In the experiment different extract (methanol, ethanol, chloroform and hexane) of *Rhynchosyilis retusa* was applied on five different bacteria (gram positive: *Staphylococcus aureus* and *Bacillus subtilis*; gram negative: *Vibrio cholerae*, *Escherichia coli* and *Klebsiella pneumonia*) and three fungus species (*Penicillium* sp., *Rhyzopus* sp. and *Aspergillus niger*). The antmicrobial activity was assessed using disc diffusion method. A preliminary phytochemical screening was performed on the basis of detection of alkaloid, terpenoids, flavonoids, tannins, steroids, coumerins, amino acids and carbohydrates.

4. Androgenetic study

Guha and Maheshwari (1964, 1966) reported that the induction of haploid plants from *Datura innoxia*, this technique became important for plant breeding and crop improvement (Clapham, 1977). Anther culture has become a powerful tool for the rapid production of haploid and inbred lines used for obtaining hybrid cultivars and it has reduced the time required for breeding new cultivars by at least 3 to 5 years (Tai, 2003). Hence, this system provides an unparalleled opportunity to shorten the breeding cycle

and fix agronomic traits in the homozygous state and provides excellent material for research, plant breeding and plant transformation (Datta, 2005). Production of haploids plants has been useful in providing access to recessive genes and for biotechnological manipulations, while using as a tool for cultivar development (Tai, 2003). Therefore, *in vitro* techniques are considered to be alternative tools of conventional method of plants improvement (Sayem et al., 2010). It is particularly useful in out breeders like orchids which generate a great deal of heterozygosity in the progenies. Sharma (2012) reported that the possibility of using anthers for initiating *in vitro* cultures of *Rhynchostylis retusa* Bl. (Orchidaceae), a genus of fox tail orchid, is an important stem herb.

Chapter III
Materials and Methods
(General)

3. Materials and Methods (General)

3.1 Plant materials

Five different orchid species namely *Vanda tessellata* (Roxb.), *Rhynchosstylis retusa* (Blume), *Acampe premorsa* (Roxb.) Blatter., *Agrostophyllum khasianum* L., and *Phalaenopsis cornorerris* were selected for the study (**Fig.1**). The morphological characteristics and capsules of these species are shown in **Figs. 3-8**. Experiments were carried out for all the species on *in vitro* germination, micropropagation, antimicrobial activity and phytochemical analysis. At a glance brief research plan were describes in **Fig. 2**. The fruits of the selected orchid species were collected from naturally grown plants and used as the source of seeds for *in vitro* culture.

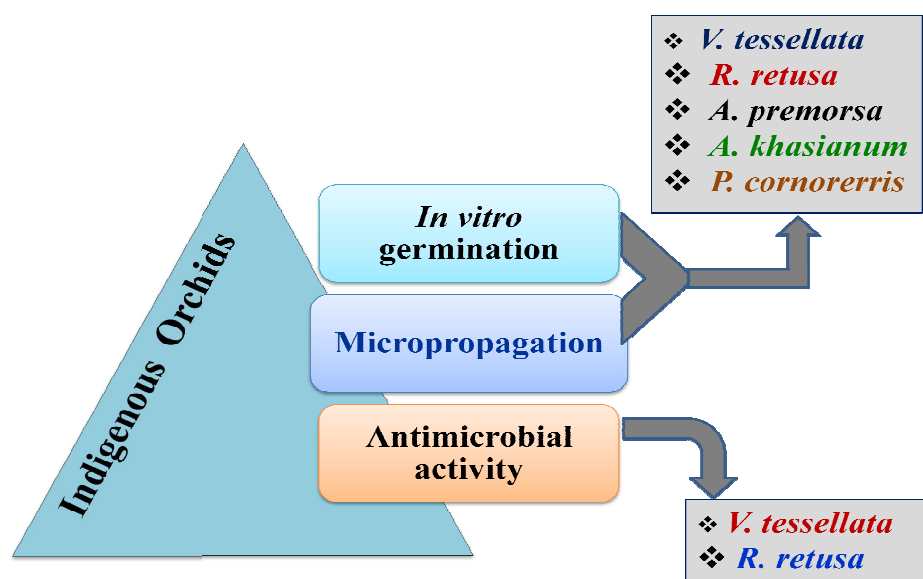


Fig.1. Plant materials and their uses at a glance.

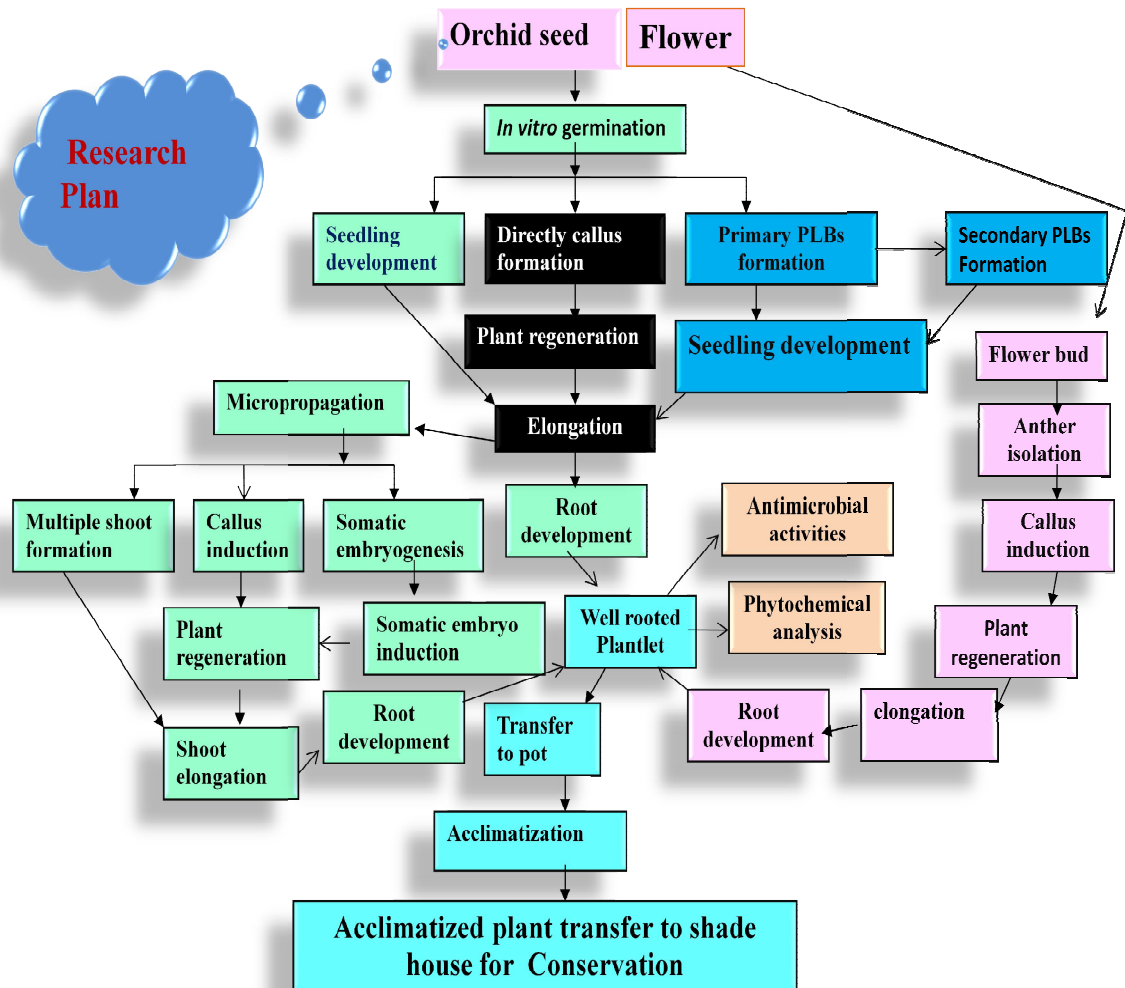


Fig. 2: Flow chart is showing details the research activities at a glance.



Fig. 3. Morphology of *V. tessellata*: a) acclimatized plant of *V. tessellata*, b) inflorescence of *V. tessellata*, c) single flower, and d) seed pod.

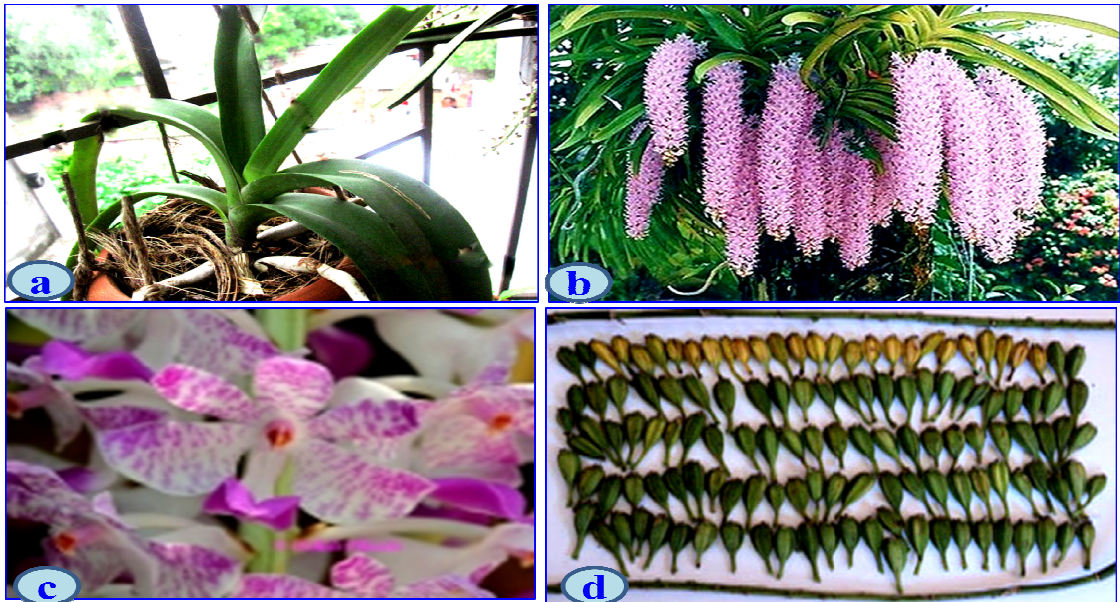


Fig. 4. Morphology of *Rhynchostylis retusa*: a) *Rhynchostylis retusa* in pot, b) inflorescence of *Rhynchostylis retusa*, c) individual flower, and d) seed pods of *Rhynchostylis retusa*.



Fig. 5. Morphology of *Acampe premorsa*: a) *A. premorsa* in natural habitat, b) inflorescence of *A. premorsa*, and c) seed pod with flower in natural habitat.



Fig. 6. Morphology of *Agrostophyllum khasianum*: a) *A. khasianum* in pot, b) inflorescence of *A. khasianum*, c) individual flower, and d) seed pod of *A. khasianum*.

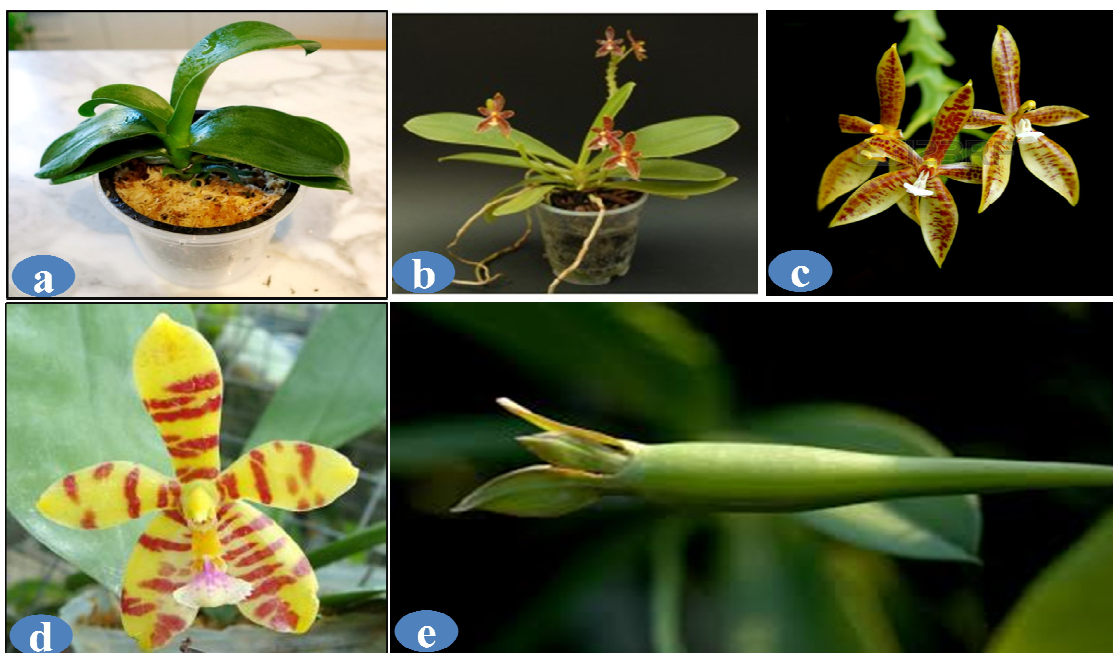


Fig. 7. Morphology of *P. cornorerris*: a) acclimatized plant of *Phalaenopsis cornorerris*, b) flowering stage in acclimatization condition, c) inflorescence of *P. cornorerris*, d) single flower and e) seed pod of *P. cornorerris*.

3.2 Methods

3.2.1 Media preparation and sterilization

The macro and micronutrients, vitamins and myo-inositol were taken from the stock solutions according to the requirement of plant tissue culture medium. Sucrose (30 g/l) was added and mixed well. The growth regulators at required concentrations were added. The pH of the media was adjusted to 5.6-5.8 using 0.1N NaOH or 0.1N HCl. 0.8% of the solidifying or gelling agent (agar) was added to the media and steamed to melt the agar. It was then dispensed in culture bottles (25 ml per bottle) and autoclaved at 15 lbs pressure at 121°C for 20 minutes.

3.2.2 Culture media

Full strength and half strength of MS (Murashige and Skoog, 1962) medium, Phytamax, Vacin and Went media were used for all the experiments with certain modifications.

Table 1. Composition of three major orchid's media

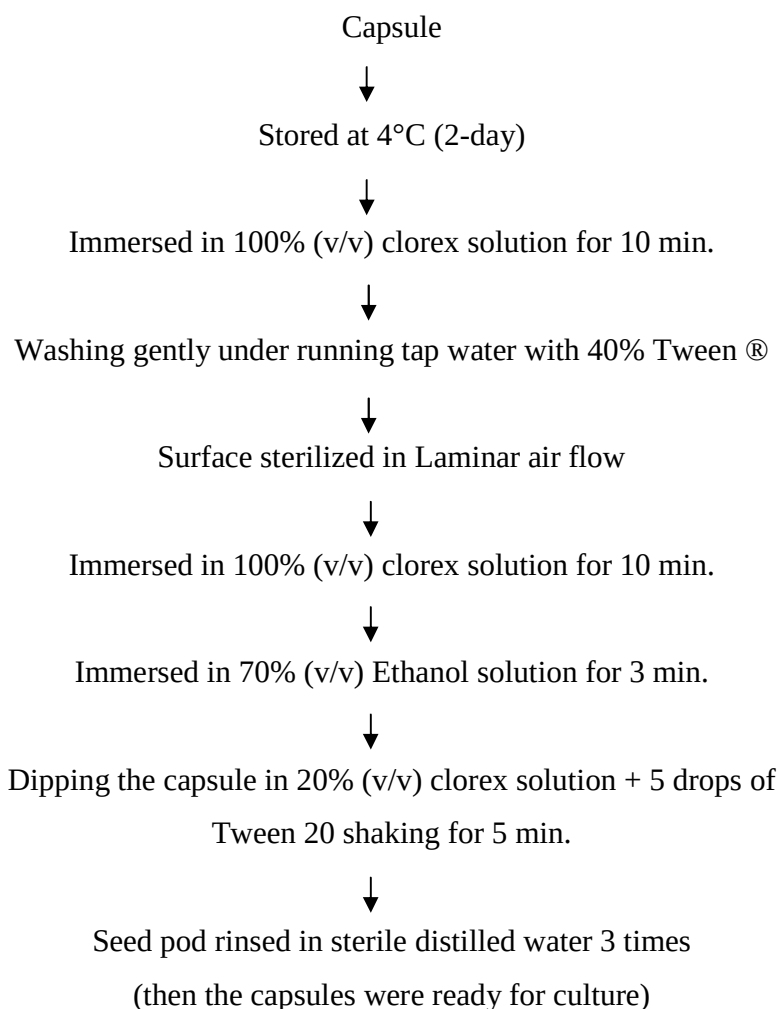
Category	Constituents	Media (mg l ⁻¹)		
		MS	VW	PM
A. Macronutrients	KNO ₃	1900.00	525.00	950.00
	NH ₄ NO ₃	1650.00	-	825.00
	KH ₂ PO ₄	170.00	250.00	85.00
	Ca ₂ PO ₄	-	200.00	-
	CaCl ₂ .2H ₂ O	440.00	-	166.00
	MgSO ₄ .7H ₂ O	370.00	122.09	90.00
	NH ₄ SO ₄	-	500.00	-
B. Micro-nutrients	MnSO ₄ .4H ₂ O	22.30	5.68	8.45
	H ₃ BO ₃	6.20	-	3.10
	ZnSO ₄ .7H ₂ O	8.60	-	5.30
	KI	0.83	-	0.45
	Na ₂ MoO ₄ .2H ₂ O	0.25	-	0.125
	CuSO ₄ .5H ₂ O	0.025	-	0.0125
	CoCl ₂ .6H ₂ O	0.025	-	0.0125
C. Micro-nutrients (Iron)	FeSO ₄ .7H ₂ O	27.80	27.80	27.85
	Na ₂ -EDTA	37.30	37.30	37.24
D. Vitamins-I	Glycine	2.00	-	-
	Nicotinic acid	0.50	-	0.50
	Pyridoxine HCl	0.50	-	0.50
	Thiamine-HCl	0.10	0.40	1.00
E. Vitamin-II	Myo- inositol	100.00	-	100.00
	Peptone	-	-	2000.00
F. Carbon source	Sucrose	30,000.00	20,000.00	20,000.00
p ^H	-	5.8	5.4	5.2

Table 2. Various treatments based on PGRs in three media

No.	Media	Media combination (supplement)
A. Germination of seeds		
1.	MS	3% sucrose
2.	PM	2% sucrose
3.	VW	3% sucrose
B. Multiplication of Shoots		
4.	MS	3% (w/v) sucrose + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA
5.	MS	3% (w/v) sucrose + 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA
6.	MS	3% (w/v) sucrose + 1.5 mg l^{-1} BAP + 1.0 mg l^{-1} NAA
7.	MS	3% (w/v) sucrose + 2.0 mg l^{-1} BAP + 1.5 mg l^{-1} NAA
8.	MS	3% (w/v) sucrose + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} KIN
9.	MS	3% (w/v) sucrose + 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} KIN
10.	MS	3% (w/v) sucrose + 1.5 mg l^{-1} BAP + 1.0 mg l^{-1} KIN
11.	MS	3% (w/v) sucrose + 2.0 mg l^{-1} BAP + 1.5 mg l^{-1} KIN
12.	MS	3% (w/v) sucrose + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} IAA
13.	MS	3% (w/v) sucrose + 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} IAA
14.	MS	3% (w/v) sucrose + 1.5 mg l^{-1} BAP + 1.0 mg l^{-1} IAA
15.	MS	3% (w/v) sucrose + 2.0 mg l^{-1} BAP + 1.5 mg l^{-1} IAA
C. Elongation of shoots		
16.	MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} NAA
17.	MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} IAA
18.	MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} 2,4-D
19.	MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} GA ₃
20.	MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} IBA
21.	MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} KIN
22.	MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} picloram
D. Rooting media		
23.	½ MS	1.5% (w/v) sucrose
24.	½ MS	1.5% (w/v) sucrose + 0.5 mg/l IAA
25.	½ MS	1.5% (w/v) sucrose + 0.5 mg/l IBA
26.	½ MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} KIN
27.	½ MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} NAA
28.	½ MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} BAP + 0.5 - 2.0 mg l^{-1} IAA
29.	½ MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} NAA
30.	½ MS	3% (w/v) sucrose + 0.5 - 2.0 mg l^{-1} IAA

3.2.3 In vitro germination of seeds

3.2.3.1 Steps of sterilization procedure of seed pod/capsules



3.2.3.2 Inoculation of seeds

The working area of the laminar airflow chamber was first surface sterilized with 70% ethanol. The petri dishes and tools which were used during inoculation of the seeds (forceps, scalpel, sterile cotton, and paper towels) were sterilized in autoclave at 15 lbs 121°C for 20 min. and kept them in the laminar airflow cabinet. The ultra violet (UV) light was switched on for 20 min. before inoculation. The laminar airflow was put and hands were sterilized with 70 percent alcohol, forceps were dipped in alcohol, flamed,

cooled and used for the inoculation. The surface sterilized seeds were carefully inoculated into various media as mentioned in **Table 1** for germination. The effectiveness of each treatment and different modes of incubation was assessed based on the germination percentage using the following formula.

$$\text{Germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of inoculated seeds}} \times 100$$

3.2.4 *In vitro* Micropropagation

3.2.4.1 Explants

Different explants (shoot tip, nodal segment, leaves, roots, etc.) from the *in vitro* germinated seedlings and anthers from the naturally grown mature plants were used as the source for multiple shoot induction.

3.2.4.2 Initiation of multiple shoots

The explants were carefully excised and inoculated on MS basal medium supplemented with various concentrations and combinations of PGRs (**Table 2**).

3.2.4.3 Multiplication of shoots

Forty five days after inoculation, for multiplication the initiated multiple buds were separated and sub cultured into respective fresh medium with the same concentrations of growth regulators as that of the induction medium.

3.2.4.4 Elongation of shoots

Fifteen days after sub culturing, the well-grown shoots of uniform height were transferred to the elongation medium and maintained for 30 days (**Table 2**).

3.2.4.5 Root induction

The elongated plantlets were transferred to rooting medium (**Table 2**) for root induction and maintenance.

3.2.4.6 Hardening

The well-rooted plants were transferred from *in vitro* condition for hardening. It was planted on different mixture viz., brick and charcoal pieces, decaying litter, saw dust and coconut husk. All the substrate covered with a layer of 10.0 gm sphagnum moss (**Table 3**). The plantlets were watered alternately in the every evening and fed with MS nutrient salt solutions (diluted 10 times) fortnightly for about a month and eventually established under natural condition following the protocols of Das et al. (2007).

Table 3. Different potting mixture used for acclimatization

Category	Composition of substratum
I	a) Brick + charcoal (1:1) + moss (10.0 gm/pot)
	b) Brick + charcoal + decaying litter (1:1:1) + moss (10.0 gm/pot)
II	a) Brick + charcoal + decaying litter + saw dust (1:1:1:1) + moss (10.0 gm/ pot)
	b) Brick + charcoal + decaying litter + coconut husk (1:1:1:1) + moss (10.0 gm/ pot)
	c) Brick + charcoal + decaying litter + cow dung (1:1:1:1) + moss (10.0 gm/ pot)

Table 4: Various plant growth regulators (PGRs) and its solubility

PGRs			
Types	Name	Solubility	Molecular weight (g/mol)
Auxin	NAA	1N NaOH	186.21
	IAA	1N NaOH	175.19
	Picloram	Ethanol	241.46
	2,4-D	Ethanol	221.04
	IBA	1N NaOH	203.24
Cytokinin	BAP	1N NaOH	225.25
	Kin	1N NaOH	215.22
	Zeatin	1N HCl	219.20
Gibberellins	GA ₃	1N KOH	346.38

3.2.5 Anther culture

3.2.5.1 Plant materials

Flower buds of *V. tessellata* were collected with anthers in different stages of development in the morning before 10:00 AM were used as explants (anther) source. The developmental stages of the microspores were determined by studying microscopic slides prepared according to the acetocarmine squash method.

3.2.5.2 Cold pre-treatment, surface sterilization and inoculation

Collected flower buds were wrapped in aluminum foil and kept in a refrigerator at 4°C for 0-72 hours to investigate the effect of cold as a pretreatment. Before culture, surface sterilization of flower buds was carried out in the laminar air flow cabinet. They were

immersed in 0.1% HgCl₂ for one minute, then thoroughly washed with sterilized distilled water for three times. After being removed aseptically, anthers from each bud were inoculated on induction medium.

3.2.5.3 Culture media

Full strength and half strength of MS (Murashige and Skoog, 1962), N₆ and B₅ media were used for the experiments with certain modifications. The media supplemented with different carbon sources such as sucrose (0-5%), maltose (0-5%) and glucose (0-5%) along with BAP (1-10 mg l⁻¹), NAA (1-1.5 mg l⁻¹), kinetin (1.0 mg l⁻¹), 2,4-D (1.0 mg l⁻¹), peptone (2.0 mg l⁻¹) and CuSO₄.5H₂O (2.2 mg l⁻¹). Plated anthers were incubated at 25°C ± 1°C chamber in dark for three to four weeks for callus induction and checked to record the response.

3.2.5.4 Transfer of the callus for shoot regeneration

After the development of callus which were transferred to semi-solid ½ MS medium with different carbon sources containing BAP (1.0 mg l⁻¹) and NAA (1.0 mg l⁻¹) and placed them in growth chamber of 16/8h light/dark regimes for shoot regeneration.

3.2.5.5. Subculture of the regenerated shoot for root induction

The sub-cultured callus contained to proliferate and differentiate into shoots when these grew 2-3 cm in length. They were rescued aseptically from the cultured vials and were separated from each other and again cultured on vials with freshly prepared three kinds of root induction medium such as i) T₁ = ½ MS + 0.5 mg l⁻¹NAA + 0.5 mg l⁻¹ IAA, ii) T₂ = ½ MS + 0.5 mg l⁻¹ NAA + 1.0 mg l⁻¹ IAA, and iii) T₃ = ½ MS + 0.5 mg l⁻¹ NAA + 1.5 mg l⁻¹ IAA. The vials containing plantlet were incubated under continuous light. Observations were carried out to note the response of the growing plantlets.

3.2.6 Antimicrobial study

3.2.6.1 Plant materials and their processing

As plant materials the epiphytic orchid *Vanda tessellata* and *Rhynchostylis retusa* was collected from Rajshahi, Bangladesh. The whole plant parts were used as plant materials for the purpose. Different solvents such as methanol, ethanol, chloroform and hexane used were of analytical grade and obtained commercially from Merck- Limited, Mumbai, India.

For extract preparation, different parts of orchid plants were washed by running tap water and after excess water absorbing by tissue paper chopped it and made small pieces and then dried in incubator at 28°C. Then they were coarse powdered by using a pulverizor. The coarse powders were then subjected to successive extraction with organic solvents such as methanol, ethanol, chloroform and hexane by sonication method. The extracts were then collected and distilled off on a water bath at atmospheric pressure and the last trace of the solvents was removed in vacuo and stored at 4°C and used it for antimicrobial activity and phytochemical analysis.

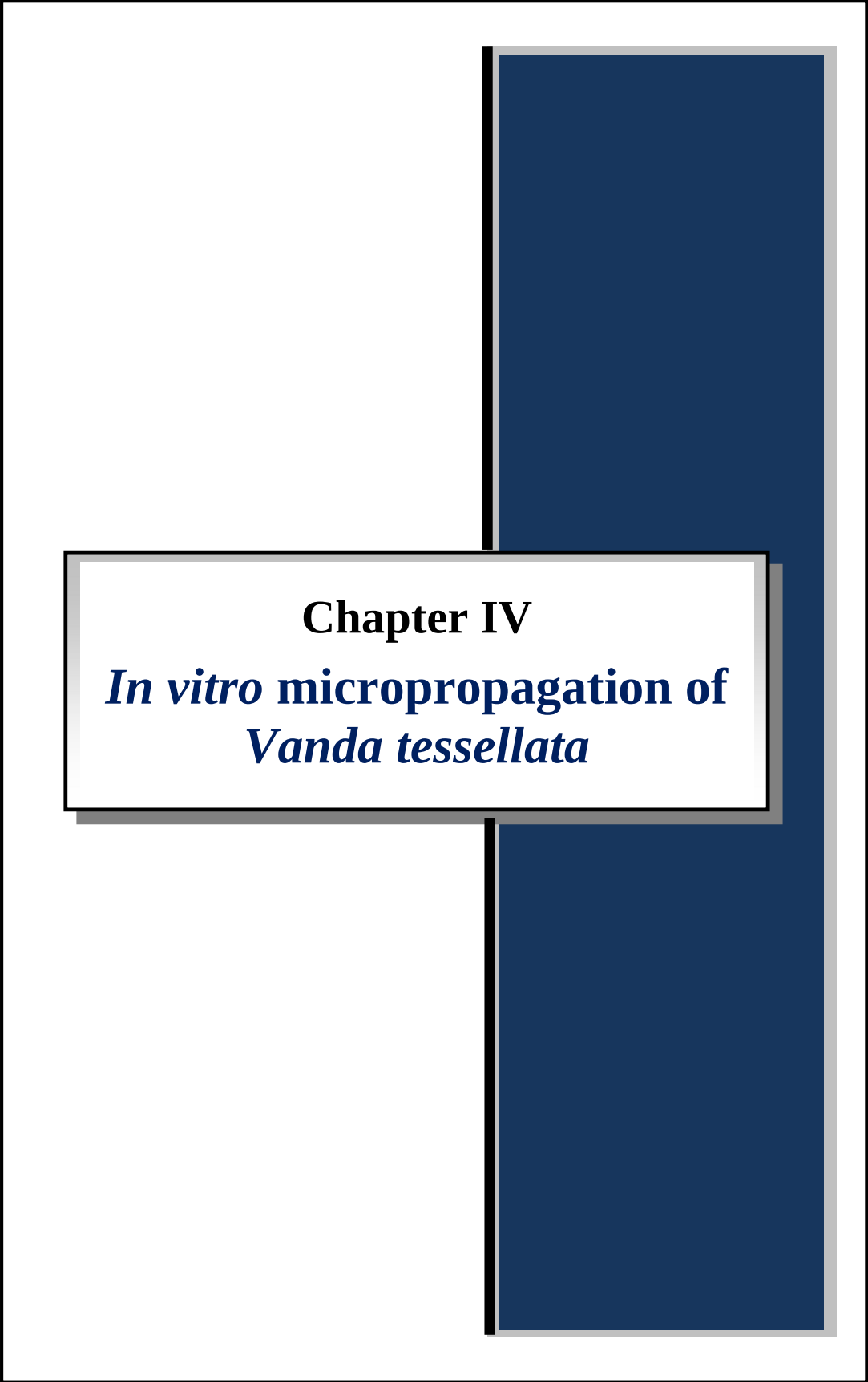
3.2.6.2 Collection of microorganism as materials

Clinical strains of bacteria isolated from patient sample were collected from the Popular Diagnostic Centre (Pvt.) Ltd. Rajshahi, Bangladesh. In the present study includes five different species of bacteria including both gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram negative (*Vibrio cholerae*, *Escherichia coli* and *Klebsiella pneumonia*) were evaluated. Cultures of these bacteria were grown in a nutrient broth (liquid media) at 37°C for 24 hours. Pure culture of these bacteria was maintained at 4°C on nutrient agar medium. The fungal organisms which used for the present study were namely *Penicillium* sp., *Rhyzopus* sp. and *Aspergillus niger*.

3.2.7 Precautions to ensure aseptic condition

All the inoculations and aseptic manipulations were carried out in a laminar air flow cabinet. The cabinet was switched on half an hour before starting the work and UV

switch was also kept on for 20-30 minutes before starting of work. The bench of the laminar flow cabinet was cleaned. With rectified spirit for reducing the chance of contamination. All instruments such as scalpels forceps and aluminium slabs were sterilized earlier by autoclaving. During operation these were further sterilized by an alcoholic dip and flaming over a spirit lamp taken inside the laminar flow cabinet, In order to ensure aseptic condition hands were also cleaned with rectified spirit. Surgical operations were undertaken very carefully to maintain contamination free condition.



Chapter IV

In vitro micropropagation of *Vanda tessellata*

4.1 Rapid Development of PLBs Derived from Immature Seeds and Mass Multiplication of *Vanda tessellata* (Roxb.) Hook. Ex. G. Don a Threatened Orchid in Bangladesh

4.1.1 Introduction

V*anda tessellata* (Roxb.) Hook. Ex.G. is an epiphytic endangered medicinal and floricultural important orchid in Bangladesh. It is also a threatened medicinal plant belongs to the family of Orchidaceae includes 800 genera spread all over the world (Singh et al., 2007; Chugh et al., 2009, Nongdam and Chongtham 2011). The plant contains an alkaloid, a glucoside, tannins, β -sitosterol, γ -sitosterol and a long chain aliphatic compound, fatty oils, resins and colouring matters. The roots of *Vanda tessellata* are antipyretic; useful in dyspepsia, bronchitis, inflammations, piles and hiccup and contain tetracosyl ferrulate and β -sitosterol-D-glucoside (Ghani et al., 1990). A novel aphrodisiac compound isolated from the flowers of *Vanda tessellata* (Roxb.), which activates neuronal and endothelial, but not inducible (Subramaniam et al., 2013). The agro-climatic conditions of Bangladesh are congenial for natural orchid vegetation in different regions especially in Chittagong Hills tracks, Rangamati, Sylhet and Sundarbans. There are about 115 different varieties of orchids are available in Bangladesh (Bhadra et al., 2002). But habitat destruction, deforestation, climate change, indiscriminate collection by hobbyists and traders are some reasons for the extinction of local orchids. Now orchid is belong to endangered species threatened with extinction and due to uncontrolled collection and habitat destruction have led also to drastic reduction in number of orchids in Indian sub continent (Pradhan, 1985, Kalimuthu, 2007). It is very important to collect our local orchids and to find out better solution for its improvement and conservation systems. Since 1970's, different biotechnological approaches e.g. *in vitro* micropropagation, genetic transformation, cryopreservation and hybridization techniques are widely using for orchid improvement (Antony et al., 2014). Those techniques are essential tools to meet the commercial demand and conservation of wild, rare and useful orchid species in the world. Pierik (1987) reported that orchid produced

1,300 to 4 million seeds per capsules and only 0.2 - 0.3% germinates in nature (Singh, 1992). Orchid seeds are very small and do not have sufficient reserve food material (lacks endosperm) to take care of the growth of embryo during germination they have to depend on some external source for nutrients so as to make their undifferentiated embryo to develop into a protocorm (Bindiya et al., 2012). The present work has been considered to study the effects of various media for seed germination, development of protocorm like bodies, their regeneration, mass multiplication for their conservation and advance level of biotechnological research in Bangladesh.

4.1.2 Materials and Methods

The immature capsule of *Vanda tessellate* used as seed source that collected from Rajshahi, Bangladesh. At first, the capsules were washed by running tap water and surface sterilized by detergent. Then capsules were treated with 0.2% (w/v) HgCl_2 solution for 10 minutes and finally dip in 70% ethanol for 10-12 seconds. Then washed them with sterile distilled water and sterilized capsules were cut longitudinally by a sterile surgical blade (**Fig. 8a**). Around 200 mg seeds were cultured per vessel (**Fig. 8b**). Various media viz. MS (Murashige and Skoog, 1962), B_5 (Gamborg et al., 1968) and PM (PhytamaxTM, Sigma, USA) were used under this study. The basal media (MS and B_5) amended with 3% (w/v) sucrose and the PM was amended with 2% (w/v) sucrose. The pH for all medium was adjusted at 5.6 - 5.8. Inoculated vessels were maintained in the culture room under a period of 16 hrs light and dark for 8 hrs at $25 \pm 2^\circ\text{C}$. After two weeks of inoculation, some of the seeds were taken out and dispersed in one drop of water on a glass slide and examined its developments under a light microscope (**Fig.8 c,d**). Germination percentages were calculated employing the following formula:

$$\text{Germination (\%)} = \frac{\text{No. of seeds showing swelling of the embryo}}{\text{No. of total seeds}} \times 100$$

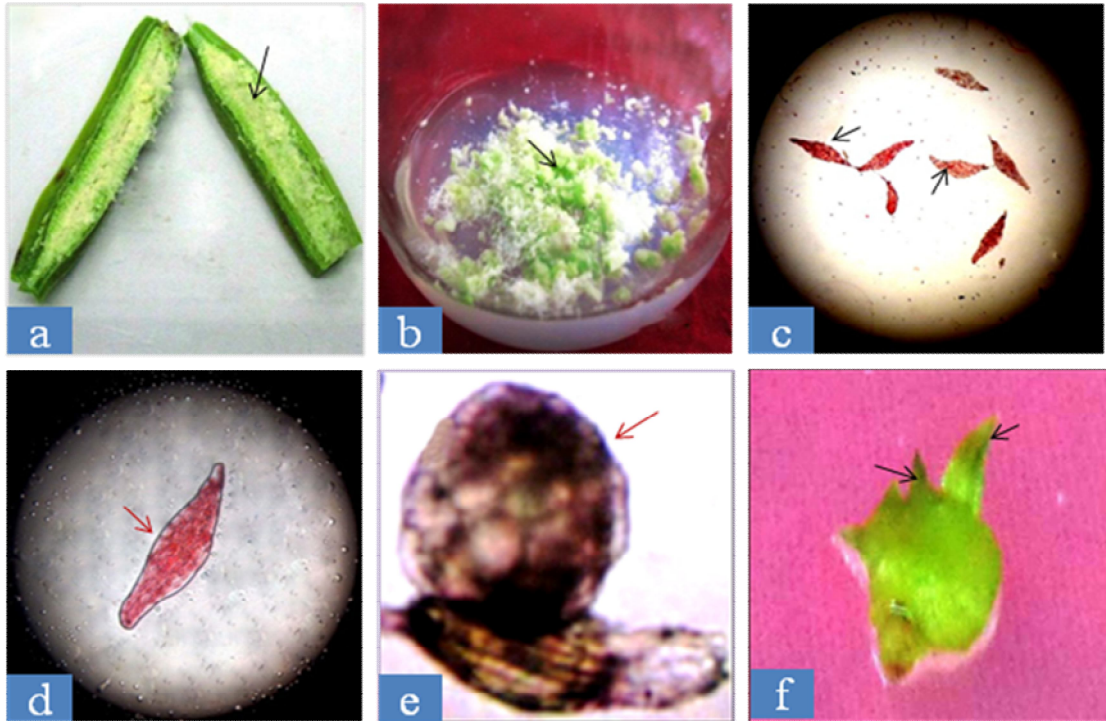


Fig. 8 (a-f): Seed germination of *Vanda tessellata*. (a) Longitudinal dissection of immature capsule; (b) Inoculated seeds into MS Medium; (c) Early stage of seed germination under light microscope (10 × 10); (d) Single seed after one week of culture initiation that showing swelling of embryo; (e) Parenchymatous cell mass coming out by rupturing testa; (f) A protocorm with leaf primordia.

Once the spherules were formed, then protocorms developmental stages were recorded in every week upto 8-10 weeks. Then PLBs were transferred to $\frac{1}{2}$ MS medium supplemented with different concentration of auxins and cytokinin. Further, regenerated plants were transplanted and grown in the outside environment for *ex-vitro* establishment and complete plantlets were recorded within 60-70 days of culture initiation. The well rooted plants were removed from the culture bottle and hardened successfully in clean plastic pots of 8.0 cm diameter containing different mixtures of compost viz., (i) brick

and charcoal pieces (1:1), (ii) brick, charcoal and decaying litter (1:1:1), (iii) brick, charcoal, decaying litter and saw dust (1:1:1:1), (iv) brick, charcoal, decaying litter and coconut husk (1:1:1:1). All the substrate covered with a layer of 10.0 gm sphagnum moss. The plantlets were watered alternately in the every evening and fed with MS nutrient salt solutions (diluted 10 times) fortnightly for about a month and eventually established under natural condition following the protocols of Das et al. (2007). On the basis of seed germination and its developmental stages, PLBs formation, regeneration, shoot and root developments were recorded. Each value represents an average of 10 replicates. Data were subjected to analysis of variance (ANOVA) and means were separated by Duncan's Multiple Range Test (DMRT).

4.1.3 Results and Discussion

Orchid seeds do not possess endosperm and that limits their germination in nature. Usually orchid seeds germinate in symbiotic association with some species - specific mycorrhiza (a kind of symbiotic fungus), which supplies food to the germinating undifferentiated orchid embryo (Rahman et al., 2009). In the present study, immature green pods were considered and four types of basal media (MS, $\frac{1}{2}$ MS, PM and B₅) without PGRs were used to assess their effect on seed germination and PLBs formation. Among the basal media tested, MS was the most effective for germination and protocorms development (**Fig. 8f**). Highest percentage of seed germination was recorded with MS medium (100.0%) followed by $\frac{1}{2}$ MS (90.0%), PM (60%) and B₅ (45.0%). It was observed that the germinated seeds increased in size and highest protocorms (90.0%) was recorded in MS medium (**Fig. 9a; Table 5**).

Table 5. Effects of various media and different strength of growth regulators on seed germination and PLBs development in *V. tessellata*.

Media	Amount of seeds per culture vessel	Seed germination		PLBs development	
		Days	% of germination	Days	% of Protocorms
MS	200 mg	30 - 35	100.0	35 - 40	90.0
½MS	200 mg	34 - 40	90.0	40 - 50	75.0
PM	200 mg	50 - 55	60.0	60 – 65	50.0
B ₅	200 mg	45 - 50	45.0	50 - 55	30.0

MS = Murashige and Skoog (1962); PM = PhytamaxTM, P-1056, Sigma, USA; B₅ = Gamborg et al. (1968).

After 25 days the protocorms were sub-cultured in the same medium. It was observed that in every sub-culture stages the protocorms vigorously grown and number increased but their individual growth and development was not so satisfactory (**Fig. 9b**). In order to induce rapid growth and elongation, the protocorms were sub-cultured on ½MS medium supplemented with different concentrations of BAP, Kin., NAA and IAA. To promote the normal growth of the seedlings, the combination of NAA and BAP was better than other combinations of auxin and cytokinin (**Table 6; Fig. 9c, d**). Significant variation in weight of PLBs was recorded with various combinations of plant growth regulators of BAP + Kin, BAP + NAA and BAP + IAA. In case of BAP + Kin, the highest weight (4.08g) of PLBs found in BAP (2.0 mg l⁻¹) + Kin (2.0 mg l⁻¹) and lowest (2.56g) from 0.5 mg l⁻¹BAP + 0.5 mg l⁻¹ Kin. In case of BAP + NAA, the highest weight (5.53g) of PLBs was obtained from 1.0 mg l⁻¹BAP +1.5 mg l⁻¹ NAA and lowest (3.74g).

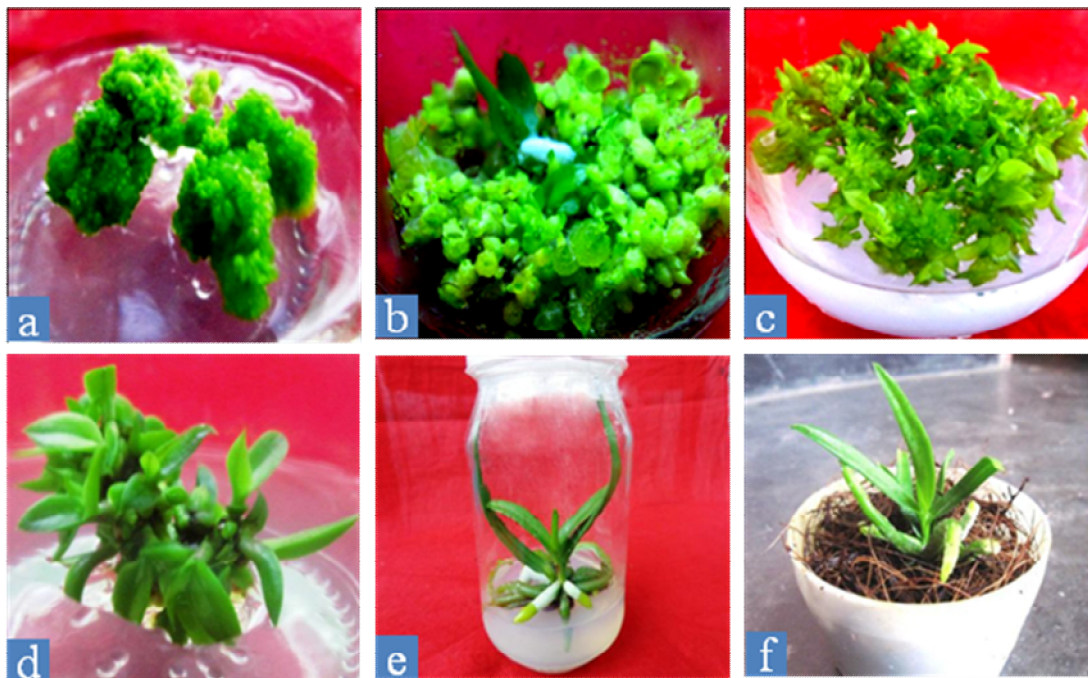


Fig. 9 (a-f): Protocorm development and mass multiplication of *Vanda tessellata*. (a) Proliferation of protocorm after 5-6 weeks; (b-c) Secondary protocorms derived from primary PLBs; (d) Plantlets development from PLBs; (e) Well rooted plants; (f) Plant transferred to pot after 90 days.

Table 6: Effect of growth regulators on the development of PLBs of *V. tessellata* 60 days after inoculation in ½MS medium.

PGRs	Concentration (mg l ⁻¹)	Wt. of PLBs (g)	Regenerated plantlets	Length of shoot (cm)
BAP + Kin.	0.5 + 0.5	2.56 ^b	11.33 ^{ab}	1.17 ^b
	1.0 + 1.5	3.72 ^a	12.33 ^a	2.39 ^a
	1.5 + 1.0	4.01 ^a	12.67 ^a	1.14 ^b
	2.0 + 2.0	4.08 ^a	10.33 ^b	1.36 ^b
BAP + NAA	0.5 + 0.5	3.74 ^c	12.6 ^b	1.63 ^b
	1.0 + 1.5	5.53 ^a	16.50 ^a	3.25 ^a
	1.5 + 1.0	4.94 ^b	13.88 ^b	2.00 ^b
	2.0 + 2.0	5.38 ^a	12.75 ^b	2.20 ^b
BAP + IAA	0.5 + 0.5	2.78 ^a	11.43 ^b	1.75 ^a
	1.0 + 1.5	3.33 ^{ab}	13.14 ^a	2.27 ^a
	1.5 + 1.0	3.68 ^b	13.00 ^a	2.10 ^a
	2.0 + 2.0	4.10 ^c	11.71 ^{ab}	2.10 ^a

Means in a column with the different letter (superscript) are significantly different according to Duncan's Multiple Range Test (DMRT) at P < 0.05 levels.

In the combination of BAP + IAA, the highest weight (4.10g) of PLBs was obtained from 2.0 mg l⁻¹ BAP + 2.0 mg l⁻¹ IAA and lowest (2.78g) from 0.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ IAA. Among all combinations of growth regulators, the highest weight of PLBs (5.53 g) recorded from 1.0 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA. Results indicated that out of all combinations 1.0 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA showed suitable results on proliferation of PLBs. This result is in agreement with the findings of Khatun et al. (2010) where they were reported that 1.0 mg l⁻¹ BAP + IBA are the best for PLB's proliferation. In case of

protocorms development, the highest number of regenerated plantlets (16.50) was obtained in 1.0 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA followed by BAP + NAA (13.88) with the concentration of (1.5 + 1.0) mg l⁻¹ (**Table 6**). On the other hand the lowest number (10.33) of regenerated plantlets obtained from 2.0 mg l⁻¹ BAP + 2.0 mg l⁻¹ Kin. Such results are in agreement with the findings of Khatun et al. (2010) where they reported the highest number of regenerated plantlet was found in 0.5 mg l⁻¹ IAA + 1.0 mg l⁻¹ IBA. The combined effect of different combinations and concentrations of growth regulators on shoot height showed significant difference. The highest shoot height (3.25 cm) was obtain from 1.0 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA followed by 1.0 mg l⁻¹ BAP + 1.5 mg l⁻¹ Kin (**Table 6**). Similar type of observation was done by Prasad et al. (2001). They found that the highest mean shoot height was obtained from 1.0 mg l⁻¹ each of BAP + NAA on MS medium.

The number of leaves produced per plantlet showed significant variation at various combinations of growth regulators. The highest number (3.33) of leaves produced in the combination of 1.5 mg l⁻¹ BAP + 1.0 mg l⁻¹ IAA and lowest (1.25) number was recorded on 0.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ Kin (**Fig. 10**). Similarly, the highest length of leaves (2.59 cm) obtained in 2.0 mg l⁻¹ each of BAP + IAA and lowest (0.96 cm) was recorded on 0.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ Kin (**Fig. 11**). A significant difference found on number of rooted plantlets among the combined effect of different combination and concentration of PGRs. The highest number (3.75) of rooted plantlets was observed from 1.5 mg l⁻¹ BAP + 1.0 mg l⁻¹ IAA and the lowest (0.67) was recorded in 2.0 mg l⁻¹ BAP + 2.0 mg l⁻¹ Kin (**Fig. 12**). On the other hand, the highest number of root (5.00) was obtained in 1.5 mg l⁻¹ BAP + 1.0 mg l⁻¹ IAA and the highest length (3.22) of root was found in 1.5 mg l⁻¹ BAP + 1.0 mg l⁻¹ IAA (**Fig. 13 and 14**). Bektas et al. (2013) reported that 0.25 mg l⁻¹ 6-BA is suitable for shoot length and number and lengths of root development. Whereas, Erisen et al. (2011) reported that TDZ was the most effective plant growth regulator for *in vitro* shoot formation. The findings from the present study suggest that the combination of BAP + NAA is the best for protocorm regeneration and for shoot elongation as well as mass multiplication. They reported that single uses of NAA had no effect but the combination

of BAP + NAA induced greatest effect on development and differentiation of PLBs in *Cymbidium*. It might be due to their effect on physiological processes or interaction between the two hormones. On the other hand, the combined effect of BAP + IAA is found suitable for root development (**Fig. 9e**). The different composts used for hardening of *in vitro* grown plantlets of *V. tessellata* were found to be satisfactory for survivability and normal growth of the plantlets.

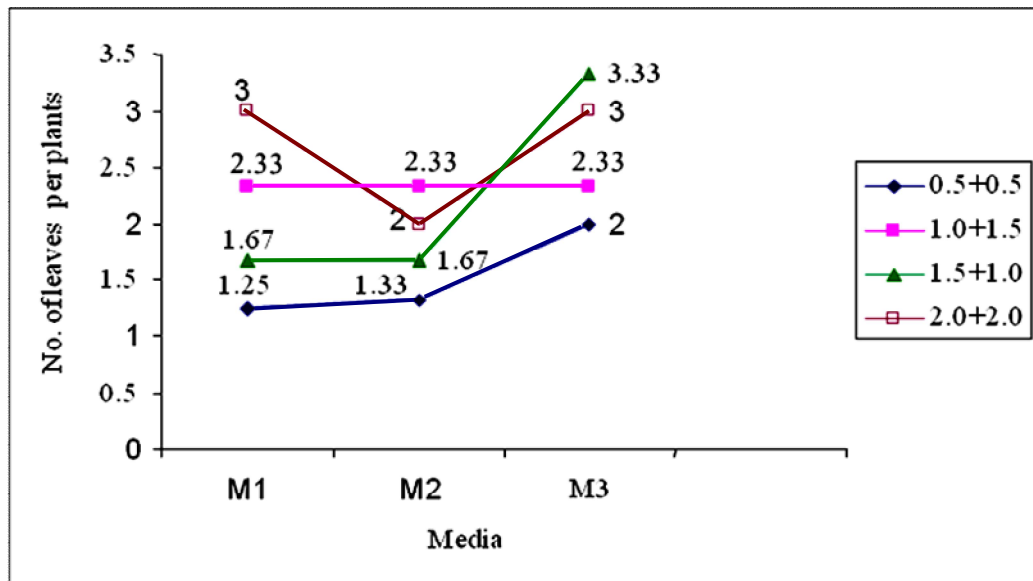


Fig. 10. Leaves per plants using various concentrations of BAP + Kin. (M_1), BAP + NAA (M_2) and BAP + IAA (M_3) in $\frac{1}{2}$ MS medium.

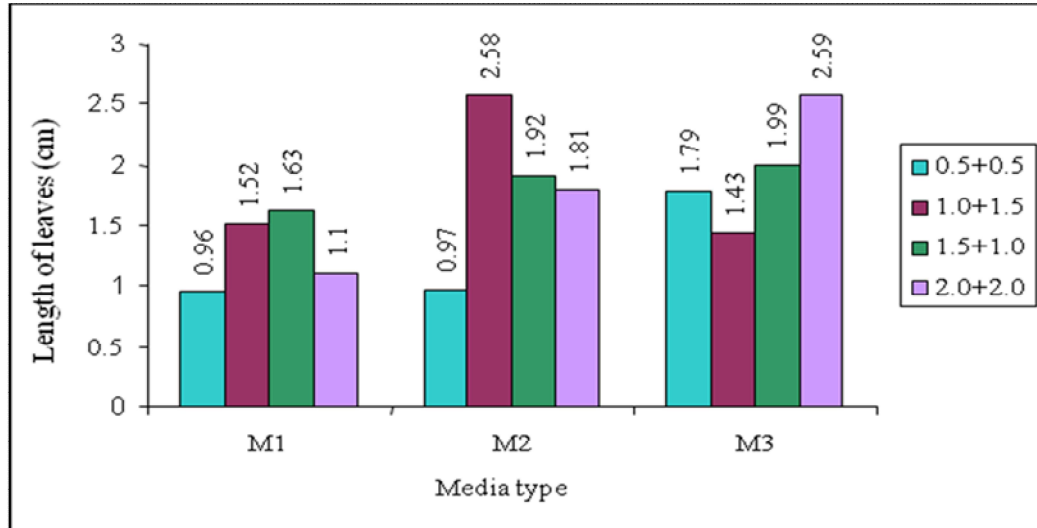


Fig. 11. Evaluation of length of leaves (cm) by the effect of different concentrations of BAP + Kin. (M₁), BAP + NAA (M₂) and BAP + IAA (M₃) in ½MS medium.

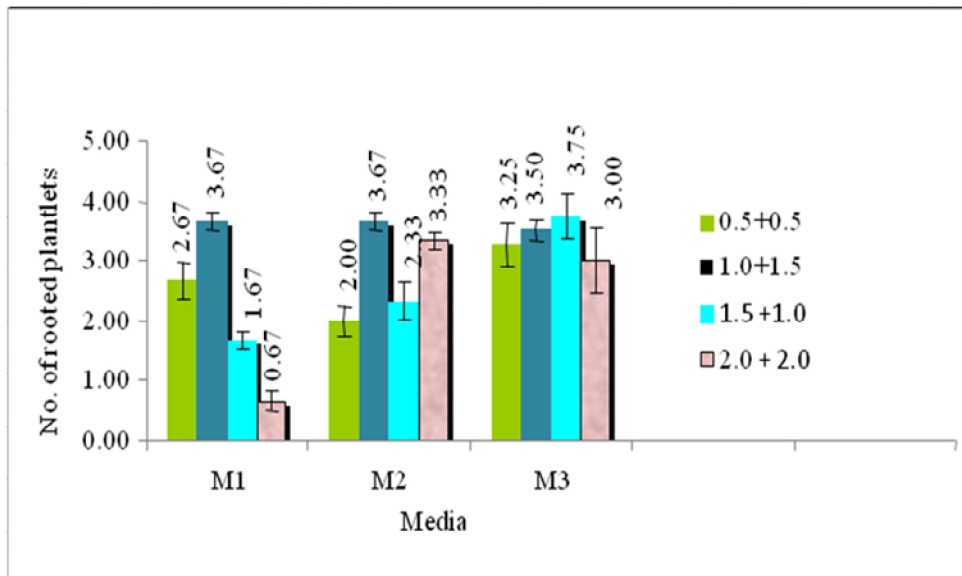


Fig. 12. Effect of different concentrations of BAP + Kin. (M₁), BAP + NAA (M₂) and BAP + IAA (M₃) on well rooted plantlets.

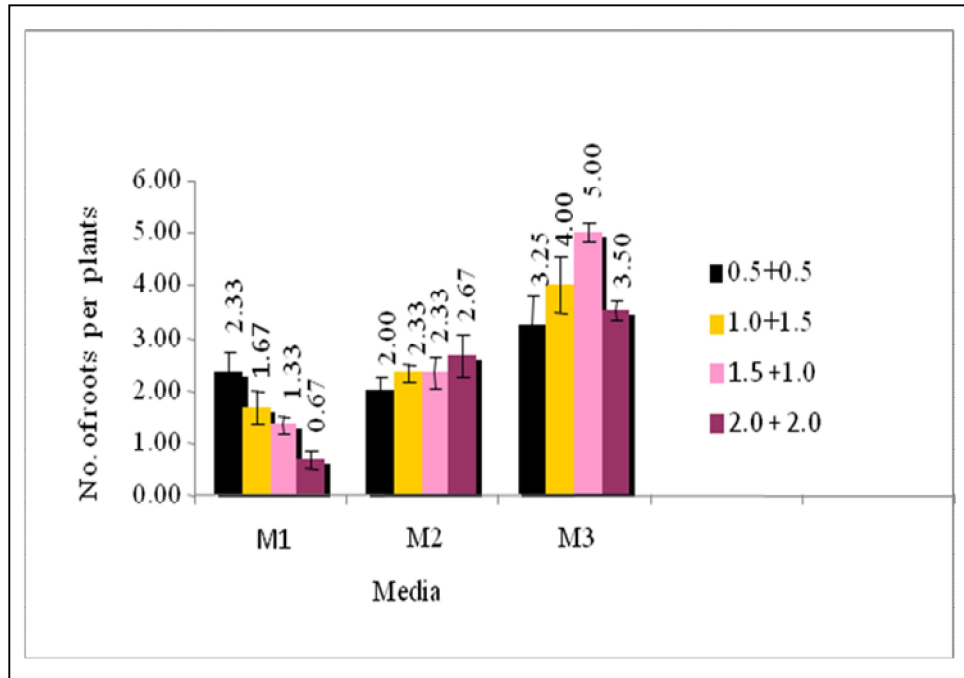


Fig. 13. Number of roots per plants using different concentrations of BAP + Kin. (M₁), BAP + NAA (M₂) and BAP + IAA (M₃) in ½MS medium.

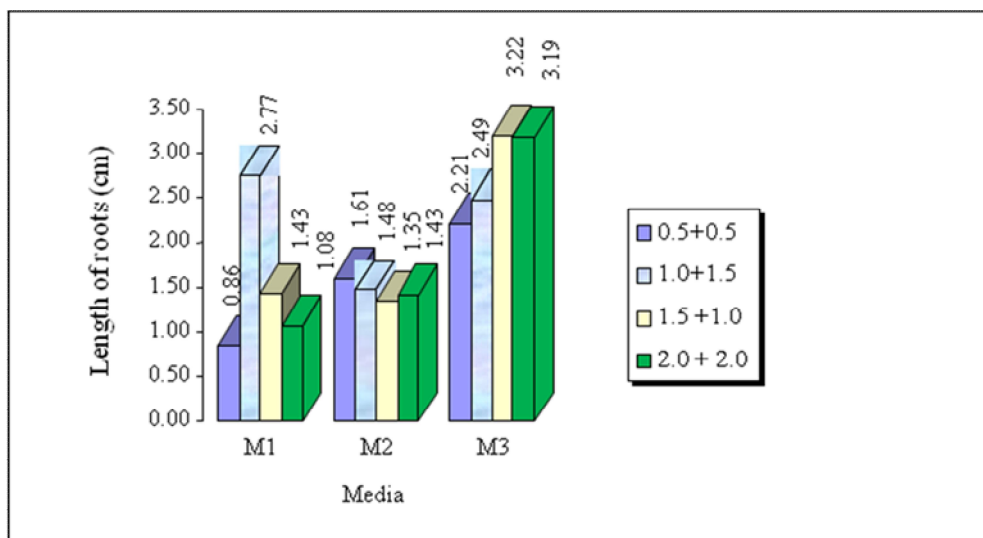


Fig. 14. Development of roots using various concentrations of BAP + Kin. (M₁), BAP + NAA (M₂) and BAP + IAA (M₃) in ½MS medium.

Table 7: Establishment of plants after 90 days in pots.

Category	Composition of substratum	No. of plants	Survival rate (%)	Plant height (cm)
I	a) Brick + charcoal (1:1) + moss (10.0 gm/pot)	50	50.0	2.6
	b) Brick + charcoal + decaying litter (1:1:1) + moss (10.0 gm/pot)	40	70.0	4.5
II	a) Brick + charcoal + decaying litter + saw dust (1:1:1:1) + moss (10.0 gm/pot)	50	52.0	3.2
	b) Brick + charcoal + decaying litter + coconut husk (1:1:1:1) + moss (10.0 gm/pot)	45	80.0	5.2
	c) Brick + charcoal + decaying litter + cow dung (1:1:1:1) + moss (10.0 gm/pot)	40	60.0	3.5

Each pot diameter was 8.0 cm.

However, highest percentage survivability (80.0%) with maximum length (5.2 cm) of *V. tessellata* hardened plants was obtained on substratum containing in category-II: brick, charcoal, decaying litter, coconut husk (1:1:1:1) with a layer of moss (**Table 7; Fig. 9f**). The layer of moss on top proved to be beneficial to higher retention of moisture content. Feeding the plantlets with dilute MS medium found beneficial to the developing hardened plantlets of *V. tessellata*. This protocol of protocorm regeneration, shoot formation and *ex-vitro* establishment of *V. tessellata* can be effectively used for its mass multiplication and hence conservation of this endangered epiphytic orchid.

4.1.4 Conclusion

The above findings showed that MS medium is the best for seed germination and protocorm formation. Similarly MS medium supplemented with BAP (1.0 mg l^{-1}) and NAA (1.5 mg l^{-1}) was found to be the most suitable for PLBs development and plantlet formation. On the other hand, the combined effect of BAP and IAA was the suitable hormonal combination for root development. Thus, the results clearly demonstrated that above culture condition was the most effective for mass multiplication of *Vanda tessellata* and this protocol will be useful to commercial growers for mass multiplication. For further research on biochemical compound isolation for medicine, genetic transformation and conservation of endangered orchids this techniques and protocol will be helpful for orchid development in Bangladesh.

4.2 Mass Propagation of PLBs Derived from Leaf and Shoots of *Vanda tessellata* (Roxb.) Hook. an Endangered Medicinal Orchid in Bangladesh

4.2.1 Introduction

Orchidaceae, one of the large angiosperm families and it includes 800 genera and between 25,000 to 30,000 species spread all over the world. Many of them have floricultural importance because of their beautiful flowers. Orchids are highly valued for their fantastic range of variation in their colors, forms and long lasting flowers properties (Paudel and Pant, 2012). Many of the commercially important orchids are now artificially grown for their beautiful flowers. But some orchids are threatened by extinction because of environmental disruption, human succession of natural habitats, medicinal properties and overexploitation for horticulture and ethno botanical reasons (Coates and Dixon, 2007). Due to lack of proper cultivation practices, destruction of plant habitats and illegal and indiscriminate collection of plants from natural habitats, many medicinal plants are severely threatened in the world (Devendra et al., 2011). *Vanda tessellata* is one that is now listed as endangered in Bangladesh. But the demand of these orchids is increasing day by day. *Vanda tessellata* (Roxb.) Hook. Ex.G. is an epiphytic orchid. It has a lot of economic importance such as medicinal, glycosidal, alkaloidal etc. The roots of *Vanda tessellata* are antipyretic; useful in dyspepsia, bronchitis, inflammations, piles and hiccup. Externally the root is used in rheumatism and allied disorders and diseases of the nervous system. It is also remedy for secondary syphilis and scorpion-sting. Juice of the leaves is given in otitis and the paste as febrifuge. The roots possess significant anti-inflammatory activity (Ghani, 2003). The plant contains an alkaloid, a glucoside, tannins, β -sitosterol, γ -sitosterol and a long chain aliphatic compound, fatty oils, resins and colouring matters. Roots contain tetracosyl ferrulate and β -sitosterol-D-glucoside (Ghani et al. 1990). A novel aphrodisiac compound isolated from the flowers of *Vanda tessellata* (Roxb.) Ex

Don, which activates neuronal and endothelial, but not inducible, nitric oxide synthases (Subramoniam et al., 2013).

One of the special features of this family is the production of a large number of minute seeds with only minimal reserves of nutrients (Arditti and Ghani, 2000). In spite of a very large number of seeds produced, only 0.2 - 0.3% germinate in nature (Singh, 1992). Because of this feature, orchids depend upon mycorrhizal fungi for the carbon resources necessary for the germination and subsequent growth. This necessitates the development of rapid propagation techniques for conservation of orchids. *In vivo* vegetative propagation is undesirable due to the heterozygosity of seed, minute seed size, presence of reduced endosperm, and the requirement of an association with mycorrhizal fungi (Saiprasad and Polisetty, 2003). The concept of *in situ* conservation of orchids is wrought with many difficulties. This necessitates the application of *in vitro* seed propagation technique for orchid conservation (Stenberg and Kane, 1998; Gangaprasad et al., 1999). Thus *in vitro* cultural techniques are now adopted for quick propagation of commercially important orchid species. Orchid seeds are artificially germinated for commercial purpose and seedlings are raised in large scale. Micropropagation of orchid seedlings can also be done with the use of the aseptically grown seedlings. In this context, it is important to develop rapid propagation of economically important orchids. Development of such propagation technique is also important for conservation of this group of endangered plant species. The most commonly used micropropagation techniques include direct and indirect shoot organogenesis and somatic embryogenesis (Antony et al., 2014). Somatic embryogenesis is one of the techniques that emerged in recent years with the potential of providing an efficient clonal propagation system. Somatic embryogenesis is more necessary biotechnological technique for large-scale clonal propagation of elite genotypes, development of synthetic seed technology, plant transformation (Bomal and Tremblay, 2000; Bandyopadhyay and Hamill, 2000) and conservation of threatened plant species. Plant regeneration through somatic embryogenesis is one of the main

prerequisites for the potential applications of clonal propagation, genetic transformation and *in vitro* conservation of germplasm of woody plants (Handley, 1995; Minocha and Jain 2000; Park et al., 1998). The phenomenon of somatic embryogenesis via callus is rather rare as success of callus formation in orchids has limitations due to slow growth and a tendency to become necrotic (Kerbaui, 1984). Especially, callus formation from leaf and stem explants of orchids has been and is the rarest even now. Even when plant regeneration from callus of orchids was achieved, it usually occurred via protocorm like bodies (PLBs) (Begum et al., 1994; Huana et al., 2004; Roy and Banerjee, 2003). Therefore, success of somatic embryogenesis via callus culture is very important and interesting in plant regeneration of orchids. To the best of my knowledge, till there is no report on plant regeneration through somatic embryogenesis from leaf and stem explants in *V. tessellata* in Bangladesh as well as world. Under this study the induction of somatic embryos from leaf and stem explants of *V. tessellata* and their regeneration into complete plantlets has been successfully established and optimized media and regeneration protocols.

4.2.2 Materials and Methods

4.2.2.1. Plant materials

Vanda tessellata were collected from Rajshahi, Bangladesh as the source of explants for this experiment. As explants of young leaves and stems were used that grown *in vitro* tissue culture systems in the Plant Genetic Engineering Lab., University of Rajshahi, Bangladesh.

4.2.2.2 Methods

Young leaves and stems of *in vitro* grown plants were cut into small pieces (approximately 1-2 cm) and the explants were inoculated in glass culture bottles containing 30 ml MS (Murashige and Skoog, 1962) medium. MS medium was fortified with 3% (w/v) sucrose, 0.8% agar and various combination and concentrations of BAP,

2, 4-D, NAA and kinetin (**Table 8**). The pH of the culture medium was adjusted to 5.8 ± 0.2 before autoclaving at 120°C and at 15 lb pressure.

4.2.2.3 Maintain cultures and temperature

Culture bottles were maintained at $25 \pm 2^\circ\text{C}$, 16/8 hours photoperiod using cool white florescent light in growth chamber. Sub-culturing was done after every 6 weeks of culture initiation. Observations were recorded regularly after 1 week interval. Each experiment was replicated for three times. Leaf and stem induced direct somatic embryos were multiplied and maintained on same medium in glass culture bottle and were sub-cultured monthly.

4.2.2.4 Plant regeneration

Somatic embryos were isolated and then transferred to MS and $\frac{1}{2}$ MS basal medium with various concentrations of BAP for germination and cultures were incubated at 16h photoperiod. After 6 weeks somatic embryogenesis or adventitious shoot formation was investigated. Plant regeneration rate was calculated from number of plantlets obtained from three replicates of each treatment.

4.2.2.5 Multiple shoots formation

For multiple shoot formation, the induced adventitious shoot were transferred to MS basal media containing with different concentration of BAP and sub-cultured every month. After five subcultures, the average propagation coefficient was calculated. Cultures were maintained at $25 \pm 2^\circ\text{C}$, with 16 h photoperiod.

4.2.2.6 Rooting and plant transferred to pots

To induce rooting, single shoots were isolated from multiple shoot clusters and transferred to six kinds of half strength of MS agar medium viz. (i) $\frac{1}{2}$ MS + 0.5 mg l^{-1} IAA (T_1), ii) $\frac{1}{2}$ MS + 0.5 mg l^{-1} NAA (T_2), iii) $\frac{1}{2}$ MS + 0.5 mg l^{-1} IBA (T_3), iv) $\frac{1}{2}$ MS + 0.5

mg l^{-1} IAA + 0.5 mg l^{-1} NAA (T_4), v) $\frac{1}{2}$ MS + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA (T_5) and vi) $\frac{1}{2}$ MS + 0.5 mg l^{-1} IAA + 0.5 mg l^{-1} IBA (T_6).

After one month of incubation the plantlets were removed from the culture bottles, roots were washed of agar residue and root percentage and growth were recorded. Well shoot and rooted plants were acclimatized in plastic pots containing coconut husk, charcoal, brick pieces in the ratio of 2:1:1 and eventually established under natural condition.

4.2.2.7 Statistical analysis

Each treatment had three replicates containing five explants in each culture vessel. Data collected on different parameters were subjected to analysis of variance (ANOVA) and mean values of treatments were compared by Least Significant Difference (LSD) test were calculated at the confidence level of $P < 0.05$ (Gomez and Gomez, 1976) using SPSS (version 16.0) for Windows 7.

4.2.3 Results

4.2.3.1 Induction of direct somatic embryos

Young leaf and stem explants of *in vitro* grown *V. tessellata* were cultured on MS medium supplemented with $0.5 - 2.0 \text{ mg l}^{-1}$ BAP, $1.0 - 2.5 \text{ mg l}^{-1}$ 2,4-D, $1.0 - 2.5 \text{ mg l}^{-1}$ NAA and $1.0 - 2.5 \text{ mg l}^{-1}$ kinetin. At first the leaf and stem explants showed swelling. The induction of embryos was visible within 6-7 weeks. Somatic embryos were induced directly at the cut edges or surfaces of leaf explants (**Fig. 15 b,c**) and embryos were green, small and globular, arising individually or in group both the cases of leaf (**Fig. 15 b,d**) and stem explants (**Fig. 16 b,c**). MS medium fortified with 0.5 mg l^{-1} BAP and 1.0 mg l^{-1} kinetin showed faster response to induce direct somatic embryos as compared to other combination and concentrations (**Table 8**). The induction frequency of embryos was significantly high at lower concentration of BAP and kinetin. The frequency of direct somatic embryo induction was highest in the leaf explants (25.00 ± 0.15) as compared to stem explants (16.20 ± 0.39) on 0.5 mg l^{-1} BAP and 1.0 mg l^{-1} kinetin (**Table 1**). These

somatic embryos formed either individually or in groups, directly on the explants without callus formation. Most of the globular embryos gradually developed as bipolar structures resembling heart, torpedo and cotyledon-stage embryos (**Fig.15e** and **16d**). After 6-7 weeks of culture initiation the cultures exhibited near-confluent growth, which turned green with patches of developing embryos.



Fig. 15: Direct somatic embryogenesis from leaf explants of *Vanda tessellata* and subsequent plant regeneration, a) inoculation of leaf explants on the media, b) somatic embryos directly formed from near the cut end region of leaf, c)) somatic embryos directly formed from the leaf surface, d) a cluster of embryos directly formed from the leaf base region, e) a cluster of leaf derived embryos, f-g) embryos formed shoots, h) regenerated plantlet, and i) acclimatized plantlets.

4.2.3.2 Germination of embryos

Germination of embryos was occurred within 3 weeks of transfer of the embryos onto fresh medium with the same composition. The embryo germination are displayed by (**Fig.**

15 f, g) for the cases of leaf and (Fig.16 e,f) for stem explants. Highest regeneration frequency (60.83 ± 0.96) of the somatic embryos was noticed in embryos generated from leaf explants (Fig. 17) as compared to stem explants (53.67 ± 0.74) (Fig. 18), when cultured on $\frac{1}{2}$ MS medium supplemented with 3% sucrose and 1.0 mg l^{-1} BAP. After 3 weeks somatic embryogenesis or adventitious shoot formation was investigated.

Table 8: Effects of different concentration and combination of PGRs with MS medium on induction of embryos from leaf and stem explants of *V. tessellata*.

Combination of PGRs	Concentrations of PGRs (mg l^{-1})	% of embryo induction	
		Leaf	Stem
BAP + 2,4-D	0.5 + 1.0	$10.00^b \pm 0.33$	$7.33^{cd} \pm 0.45$
	1.0 + 1.5	$10.67^{bc} \pm 0.48$	$6.67^{abc} \pm 0.42$
	1.5 + 2.0	$11.83^{bc} \pm 0.68$	$6.83^{bc} \pm 0.41$
	2.0 + 2.5	$6.83^a \pm 0.38$	$5.33^{ab} \pm 0.45$
BAP + NAA	0.5 + 1.0	$15.60^d \pm 0.23$	$12.40^e \pm 0.43$
	1.0 + 1.5	$13.60^{cd} \pm 0.47$	$11.80^e \pm 0.34$
	1.5 + 2.0	$10.20^b \pm 0.38$	$7.40^{cd} \pm 0.39$
	2.0 + 2.5	$6.00^a \pm 0.26$	$5.00^a \pm 0.26$
BAP + Kinetin	0.5 + 1.0	$25.00^f \pm 0.15$	$16.20^f \pm 0.39$
	1.0 + 1.5	$18.80^e \pm 0.15$	$12.00^e \pm 0.30$
	1.5 + 2.0	$13.00^{bcd} \pm 0.26$	$11.80^e \pm 0.39$
	2.0 + 2.5	$10.40^b \pm 0.75$	$8.80^d \pm 0.52$

Values represent mean $\pm$ S.E. (Standard Error). Each treatment was repeated thrice and each treatment counted of 5 replicate culture vessels. Means in a column with the different letter (superscript) are significantly different according to Least Significant Difference (LSD Test) ($P < 0.05$).

For multiple shoot formation, the induced adventitious shoots were transferred to propagation media containing different combinations of plant growth regulators. Maximum number of multiple shoots (10.80 ± 0.22) with highest length of shoots was observed on MS medium fortified with 1.5 mg l^{-1} BAP + 1.0 mg l^{-1} NAA in comparison to all other combination and concentrations of BAP, NAA, IAA and kinetin (**Table 9**).



Fig. 16: Direct somatic embryogenesis from stem explants of *Vanda tessellata* and subsequent plant regeneration, a) inoculation of stem explants on the media, b) somatic embryos directly formed near the cut end region of stem, c)) somatic embryos directly

formed vigorously from the stem surface, d) a cluster of stem derived embryos, e) regeneration of stem derived embryos, f) a cluster of multiple shoots, g) individual shoot isolated from multiple shoot clusters, h) root induction in individual shoot, and i) acclimatized plantlets.

Table 9: Effects of different concentration and combination of PGRs with MS medium on multiple shoot formation and individual shoot length of *V. tessellata*.

PGRs	Conc. of PGRs (mg l ⁻¹)	Average no. of multiple shoots/explants ± S.E	Average length of shoots (cm) ± S.E
BAP + NAA	0.5 + 0.5	4.80 ^b ± 0.34	1.60 ^b ± 0.23
	1.0 + 0.5	8.40 ^f ± 0.14	2.05 ^d ± 0.03
	1.5 + 1.0	10.80 ^g ± 0.22	3.10 ^f ± 0.14
	2.0 + 1.5	6.40 ^d ± 0.39	1.90 ^{cd} ± 0.23
BAP + Kinetin	0.5 + 0.5	3.80 ^a ± 0.28	1.30 ^a ± 0.15
	1.0 + 0.5	7.80 ^e ± 0.22	1.63 ^b ± 0.13
	1.5 + 1.0	8.80 ^f ± 0.22	2.05 ^d ± 0.10
	2.0 + 1.5	4.80 ^b ± 0.28	1.25 ^a ± 0.11
BAP + IAA	0.5 + 0.5	3.60 ^a ± 0.30	1.20 ^a ± 0.20
	1.0 + 0.5	5.60 ^c ± 0.39	1.80 ^c ± 0.26
	1.5 + 1.0	7.80 ^e ± 0.26	2.30 ^e ± 0.13
	2.0 + 1.5	4.40 ^b ± 0.15	1.60 ^b ± 0.07

Values represent mean ± S.E. (Standard Error). Each treatment was repeated thrice and each treatment counted of 5 replicate culture vessels. Means in a column with the different letter (superscript) are significantly different according to Least Significant Difference (LSD Test) ($P < 0.05$).

4.2.3.3 In vitro rooting and plantlet establishment

To induce rooting, single shoots were isolated from multiple shoot clusters (**Fig. 16 g**) and transferred to rooting media. Half strength of MS media that supplemented with 3% (w/v) sucrose + 0.5 mg l⁻¹ IAA + 0.5 mg l⁻¹ NAA) showed the best performance on root formation (**Table 10**). The induction of strong and stout root system is displayed by **Fig. 15 (h)** and **2 (h)**.

Well rooted shoots were taken out and washed with sterile distilled water to remove the traces of agar. The plants were then shifted to micro pots (plastic pots) containing coconut husk, charcoal, brick pieces in the ratio of 2:1:1 and eventually established under natural condition (**Fig. 15i** and **16i**). It was observed that around 60% plants survived when temperature has been maintained in room at 26 ± 2°C.

Table 10: Effects of different combination and concentration of PGRs with ½MS medium on induction of roots and root lengths in individual shoot from multiple shoot clusters of *V. tessellata*.

Treatment	PGRs			Number of roots/shoot (M ± S.E)	Length (cm) of roots after 30 days of culture on rooting medium (M ± S.E)
	IAA	NAA	IBA		
T ₁	0.5	0	0	2.40 ^a ± 0.14	2.00 ^a ± 0.17
T ₂	0	0.5	0	3.60 ^b ± 0.29	2.40 ^a ± 0.11
T ₃	0	0	0.5	2.42 ^a ± 0.38	2.30 ^a ± 0.12
T ₄	0.5	0.5	0	5.20 ^c ± 0.38	3.38 ^b ± 0.11
T ₅	0	0.5	0.5	3.80 ^b ± 0.22	2.76 ^{ab} ± 0.21

T ₆	0.5	0	0.5	4.00 ^b ± 0.42	2.60 ^{ab} ± 0.25
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Values represent mean ±S.E. (Standard Error). Each treatment was repeated thrice and each treatment counted of 5 replicate culture vessels. Means in a column with the different letter (superscript) are significantly different according to Least Significant Difference (LSD Test) ($P < 0.05$).

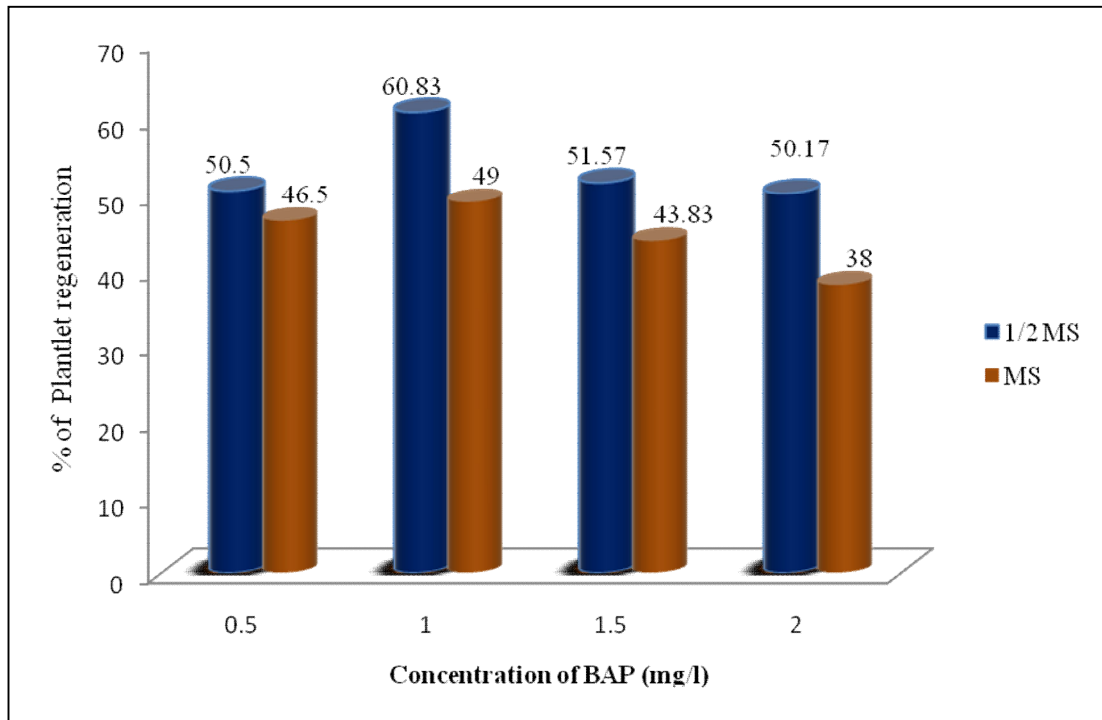


Fig. 17: Effects of BAP on regeneration of somatic embryos into plantlets derived from leaf explants.

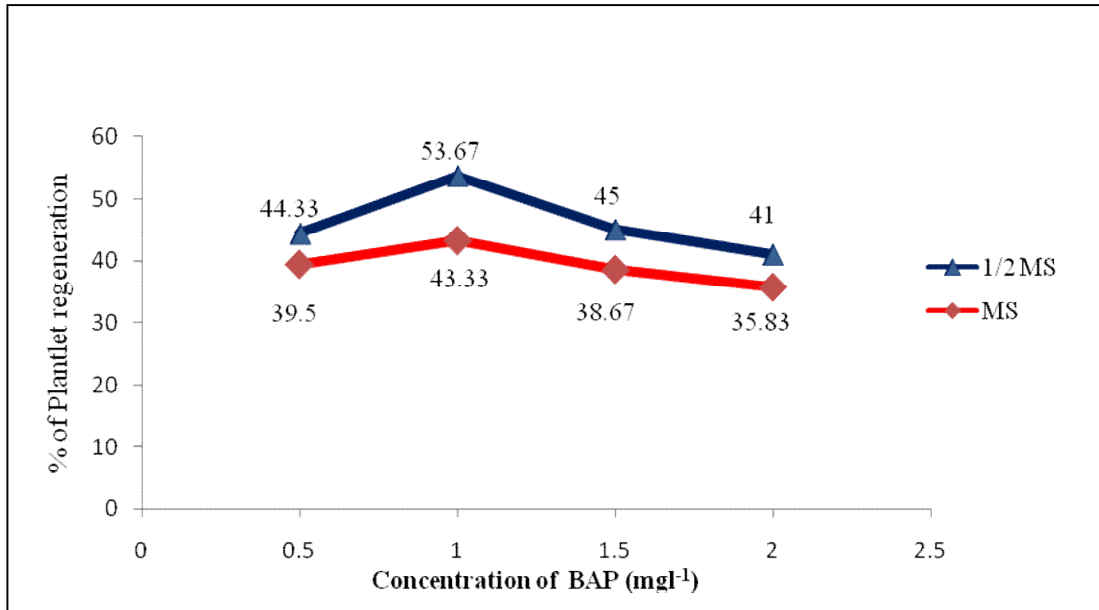


Fig. 18: Effects of BAP on regeneration of somatic embryos into plantlets derived from stem explants.

4.2.4 Discussion

In this case the direct somatic embryogenesis was induced from leaf and stem explants of *V. tessellata*. To the best of our knowledge, this is the first report of direct somatic embryogenesis from leaf and stem explants and subsequent plant regeneration of *V. tessellata*. However, the similar results were obtained in different plant species previously (Khadke and Kuvalekar, 2013; Devaraju and Reddy, 2013). Here shows, somatic embryos formed directly at cut edges of leaf sections, around cut ends of stem explants. In another orchid, *Oncidium*, generally, leaf tips had the highest ability to form embryos (Chen et al., 1999, Chen and Chang, 2001). Somatic embryos germinated and developed on the induction medium. Embryogenesis was slower and asynchronous in leaf explants. Small, round, individual somatic embryos appeared 6-7 wks after culture. The formation of somatic embryos around stem segments was slower and more asynchronous. In order to adjust a protocol of somatic embryogenesis sections young leaves of *Phytolacca tetramera* obtained *in vitro* were placed in MS medium that supplemented

with 2,4-D acid at different concentrations. Somatic embryos were obtained directly from the cutting edge of the blade and embryogenic callus (indirect way) from the midrib (Cordal et al., 2014). In this experiment, MS medium fortified with 0.5 mg l⁻¹ BAP and 1.0 mg l⁻¹ kinetin showed faster response to induce direct somatic embryos. On the other hand, thidiazuron (TDZ) at 0.3, 1 and 3 mg dm⁻³ induced 5-25% of leaf tip segments of *in vitro* grown plants to directly form embryos after 60 d of culture, and 1 mg dm⁻³ TDZ was the best treatment reported by Chung et al. (2007). In general, leaf explants were found to produce significantly more embryos than stem explants (**Table 1, Fig. 1**). In the present study, maximum regeneration frequency of the somatic embryos was noticed on ½MS medium containing 1.0 mg l⁻¹ BAP supplemented with 3% sucrose. Although plant regeneration with direct and indirect organogenesis from various explants sources of *N. foetida* species have already been reported (Thengane et al., 2001; Rai, 2002). Khadke and Kuvalekar (2013) reported that similar results with the medicinal plant *Nothapodytes foetida*, a critically endangered plant species, and suggest that this system may prove to be beneficial for *ex situ* conservation as well as for amenability of this endangered plant for genetic manipulation. In this present communication, we developed a simple and efficient protocol for induction of direct somatic embryogenesis of *V. tessellata* and successfully obtained plantlets from regenerated embryos. Regeneration system through direct somatic embryogenesis is suitable for further studying on physiology and morphology status of embryo development, mass propagation and genetic transformation (Chung et al., 2007).

4.2.5 Conclusion

The regeneration method standardized in the present investigation relies on the fact that it is efficient, quick and highly reproducible method which might be useful for genetic transformation studies. These reliable protocols also useful for *V. tessellata* mass propagation through somatic embryos formation had been established and verified. Plant regeneration through somatic embryogenesis has several advantages over other routes to

in vitro plant production and appears that most promising area of research for large scale production, rapid plant propagation and support of conservation strategies. This system is potentially useful for the micropropagation of this species. The results obtained in this study could have enormous significance, since this is the first report of somatic embryogenesis for this endangered medicinal important orchid. As per our knowledge, no such reports have been discussed regarding the efficient plant regeneration through somatic embryogenesis from leaf and stem explants of *V. tessellata*.

4.3 Effects of Plant Growth Regulators on Multiple Shoot Induction in *Vanda tessellata* (Roxb.) Hook. Ex. G. Don- an Endangered Medicinal Orchid

4.3.1 Introduction

Orchids, the doyens among ornamentals, are one of the most important global cut flower and pot plants and their sheer beauty has enchanted and fascinated people since early times (Teixeira da Silva JA, 2013). Orchid family Orchidaceae is a morphologically diverse and widespread family of monocots. Many of the commercially important orchids are now artificially grown for beautiful flowers. Some orchids are also threatened by extinction because of environmental disruption, human succession of natural habitats, medicinal properties and over exploitation for horticulture and ethno botanical reasons (Coates and Dixon, 2007). The agro-climatic conditions of Bangladesh are congenial for natural orchid vegetation in different regions especially in Chittagong Hills tracks, Rangamati, Sylhet and Sundarbans. Taxonomical studies revealed that there are about 115 different varieties of local orchids in Bangladesh (Bhadra et al., 2002). But habitat

destruction, deforestation, climate change, indiscriminate collection by hobbyists and traders are some reasons for the extinction of local orchids. Therefore, it is very important to collect and conserve the local orchid varieties that would be useful for the improvement of orchids in Bangladesh.

However, due to lack of proper cultivation practices, destruction of plant habitats and illegal and indiscriminate collection of plants from natural habitats, many medicinal plants are severely threatened (Devendra et al., 2011). *Vanda tessellata* (Roxb.) Hook. is an epiphytic orchid and it is now listed as endangered in Bangladesh. It has a lot of economic importance such as medicinal, glycosidal, alkaloidal etc. The roots of *Vanda tessellata* are antipyretic; useful in dyspepsia, bronchitis, inflammations, piles and hiccup. Externally the root is used in rheumatism and allied disorders and diseases of the nervous system. It is also remedy for secondary syphilis and scorpion-sting. Juice of the leaves is given in otitis and the paste as febrifuge. The roots possess significant anti-inflammatory activity and the plant contains an alkaloid, a glucoside, tannins, β -sitosterol, γ -sitosterol and a long chain aliphatic compound, fatty oils, resins and colouring matters. Roots contain tetracosyl ferrulate and β -sitosterol-D-glucoside (Ghani et al., 1990). A novel aphrodisiac compound isolated from the flowers of *Vanda tessellata* (Roxb.) Ex. Don, which activates neuronal and endothelial (Subramoniam et al., 2013). One of the special features of this family is the production of a large number of minute seeds with only minimal reserves of nutrients (Arditti and Ghani, 2000). In spite of a very large number of seeds produced, only 0.2 - 0.3% germinates in nature (Singh, 1992). Because of this feature, orchids depend upon mycorrhizal fungi for the carbon resources necessary for the germination and subsequent growth (Rasmussen, 1995). This necessitates the development of rapid propagation techniques for conservation of orchids. *In vivo* vegetative propagation is undesirable due to the heterozygosity of seed, minute seed size, presence of reduced endosperm and the requirement of an association with mycorrhizal fungi (Saiprasad and Polisetty, 2003). The concept of *in situ* conservation of orchids is wrought with many difficulties. This necessitates the application of *in vitro* seed

propagation technique for orchid conservation (Stenberg and Kane, 1998, Gangaprasad et al., 1999).

Thus *in vitro* cultural techniques are now adopted for quick propagation of commercially important orchid species. Orchid seeds are artificially germinated for commercial purpose and seedlings are raised in large scale. Micropropagation of orchid seedlings can also be done with the use of the aseptically grown seedlings. In this context, it is important to develop rapid propagation of economically important orchids. Development of such propagation technique is also important for conservation of this group of endangered plant species. The present investigation was undertaken to establish a suitable protocol for *in vitro* germination, micropropagation and root induction. The effects of plant growth regulators on the development of multiple shoot formation and elongation have been investigated for this species also. In the present studies we have developed a very successful and efficient micropropagation method that can be successfully employed in breeding of *Vanda tessellata* for commercial purposes.

4.3.2 Materials and Methods

4.3.2.1 Plant materials

The immature capsule of *Vanda tessellata* was used as seed source which was collected from Rajshahi, Bangladesh. The plantlets of *Vanda tessellata* were grown through *in vitro* condition for further used as the source of explants for experiment purpose.

4.3.2.2 Seed sterilization and *in vitro* seed germination

At first, the capsules were washed by running tap water and surface sterilized by detergent. Then capsules were treated with 0.2% (w/v) HgCl_2 solution for 10 minutes and finally dip in 70% ethanol for 10-12 seconds. Then washed them with sterile distilled water and sterilized capsules were cut longitudinally by a sterile surgical blade. Around 100 mg seeds were cultured per vessel (**Fig. 1a**). Various media viz. MS (Murashige and Skoog, 1962), B₅ (Gamborg et al., 1968) and PM (PhytamaxTM, Sigma, USA) were used

under this study. The basal media (MS and B₅) amended with 3% (w/v) sucrose and the PM was amended with 2% (w/v) sucrose. The pH for all medium was adjusted at 5.6 - 5.8. Inoculated vessels were maintained in the culture room under a period of 16 hrs light and dark for 8 hrs at 25±2°C. After two weeks of inoculation, some of the seeds were taken out and dispersed in one drop of water on a glass slide and examined its developments under a light microscope. Once the spherules were formed, then protocorm like bodies (PLBs) developmental stages were recorded in every week up to 8-10 weeks. Germination percentages were calculated employing the following formula:

$$\text{Germination (\%)} = \frac{\text{No. of seeds showing swelling of the embryo}}{\text{No. of total seeds}} \times 100$$

4.3.2.3 PLBs development and seedlings elongation

To evaluate the regeneration efficiency of protocorms, the PLBs were transferred to half strength MS medium supplemented with different concentration and combination of auxins and cytokinins. The protocorms, which developed from the germinated orchid seeds were isolated aseptically and transferred into fresh culture vessels containing the same germinating medium. Further subculture of the protocorm was done at an interval of 15-20 days. Prior to each subculture the density of seedlings per vessel was reduced. For estimating rapid elongation of shoots, germinating seedlings were transferred into different types of shoot elongation media. The elongation media were based on agar solidified MS medium supplemented with BAP (0.5-2.0 mg l⁻¹), NAA (0.5 mg l⁻¹), kinetin (0.5 mg l⁻¹) and IAA (1.0 mg l⁻¹).

4.3.2.4 Multiple shoots induction

For multiple shoot development the *in vitro* growing seedlings were used when it raised around 4-5 cm in height. These shoot segments were transferred into shoot induction and elongation media for development of adventitious shoots. Subculture of these explants was done at an interval of 4 weeks.

4.3.2.5 Rooting and acclimatization

Newly developed adventitious shoots when reached a height of 2-3 cm were transferred into rooting medium for root development. For efficient root induction we used three plant growth regulators viz. IAA, IBA and NAA in $\frac{1}{2}$ MS medium. The well rooted *in vitro* grown plantlets were hardened successfully in the potting mixture containing coconut husk, charcoal, brick pieces in the ratio of 2:1:1 and eventually established under natural condition following by the methods of Rahman et al. (2009).

4.3.3 Results

4.3.3.1 *In vitro* seed germination and seedling elongation

Seed germination occurred after four weeks of culture. We have investigated four different agar solidified media. The highest percentage of seed germination (80.0%) obtained with MS medium, whereas the lowest (45.0%) was recorded in B₅ medium (Fig. 19 b and Table 11).

Table 11: Effect of different media on *in vitro* germination of *V. tessellata* seeds

Media	Amount of seeds per culture vessel (mg)	Seed germination		Mean time of germination
		Days	% of germination	Days
MS	100	28 - 35	80.0	31.5
$\frac{1}{2}$ MS	100	34 - 40	65.0	37.0
PM	100	50 - 55	50.0	52.5
B ₅	100	45 - 50	45.0	47.5

MS = Murashige and Skoog (1962); PM = PhytamaxTM, P-1056, Sigma, USA; B₅ = Gamborg et al. (1968).

It was also reveals that the period of germination on MS medium was comparatively shorter (average germination time 31.5 days) than that observed on $\frac{1}{2}$ MS, PM and B₅ media. On the other hand, the highest rate of shoot elongation (5.0 cm) was achieved in MS medium fortified with 1.5 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA (**Table 12**).

Table 12: Effect of different concentrations and combinations of PGRs on seedlings development with MS medium.

PGRs (mg l ⁻¹)	Initial length (cm) Mean	Length (cm) after 30 days of culture initiation (Mean)	Seedling growth (cm) (M ± S.E)
BAP + NAA			
0.5 + 0.5	3.10	4.00	0.95 ^c ± 0.03
1.0 + 0.5	3.20	4.32	1.12 ^c ± 0.09
1.5 + 0.5	3.00	8.00	5.00 ^a ± 0.54
2.0 + 0.5	3.00	3.85	0.89 ^c ± 0.04
BAP + Kinetin			
0.5 + 0.5	2.75	3.50	0.90 ^c ± 0.04
1.0 + 0.5	2.80	3.80	1.02 ^c ± 0.12
1.5 + 0.5	3.42	6.50	3.12 ^b ± 0.24
2.0 + 0.5	3.00	3.95	0.95 ^c ± 0.03
BAP + IAA			
0.5 + 1.0	2.00	2.65	0.68 ^c ± 0.10
1.0 + 1.0	2.70	3.65	0.95 ^c ± 0.12
1.5 + 1.0	2.85	3.90	1.07 ^c ± 0.14
2.0 + 1.0	2.40	3.20	0.88 ^c ± 0.08

PGRs = Plant growth regulators (PGRs), values represent mean ± S.E (standard error). Each treatment was repeated thrice. Means in a column with the different letter (superscript) are significantly different according to least significant difference (LSD) at P < 0.05 levels.

4.3.3.2 Multiple shoot induction and individual shoot elongation

Differentiation of shoot buds was observed to the shoot segments which was isolated from the shoots elongated *in vitro* from the germinated seedlings (**Fig. 19c**). Shoot differentiation was first observed after 28 days of culture on MS media fortified with BAP, NAA, IAA and Kin at the concentration of (0.5 - 1.5 mg l⁻¹). The highest rate of shoot induction (7.52) per explants was observed in MS medium fortified with 1.0 mg l⁻¹ BAP and 1.0 mg l⁻¹ NAA (**Fig. 19e** and **Table 13**). The lowest multiple shoot (2.81) was also observed in MS with 0.5 mg l⁻¹ BAP and 0.5 mg l⁻¹ kinetin. Results indicated that out of 20 treatment combinations, 1.0 mg l⁻¹ BAP + 1.0 mg l⁻¹ NAA is found suitable for multiple shoot induction (**Table 13**).



Fig. 19 (a-h): Developmental stages of multiple shoot buds in *V. tessellata*. (a) *In vitro* seed germination; (b-c) Seedlings is undergoing for elongation; (d-e) multiple shoot induction from single shoot segment; (f) single shoot buds; (g) Elongated shoot bud is going to develop roots on agar solidified medium; (h) plantlets acclimatized in a pot.

Table 13: Effect of different concentrations and combinations of plant growth regulators (PGRs) with MS medium on the development of multiple shoot buds from shoot segments.

PGRs	Concentrations (mg l ⁻¹)	Average no. of shoot buds sprouting in each shoot segments	Time required (days)
BAP + NAA	0.5 + 0.5	3.07 ^{abc} ± 0.26	28-35
	1.0 + 0.5	3.28 ^{abcd} ± 0.24	
	0.5 + 1.0	4.38 ^{efgh} ± 0.19	
	1.0 + 1.0	7.52 ⁱ ± 0.28	
	1.0 + 1.5	5.06 ^h ± 0.82	
	1.5 + 1.0	4.86 ^{fgh} ± 0.24	
	1.5 + 1.5	4.00 ^{cdefg} ± 0.54	
BAP + IAA	0.5 + 0.5	2.87 ^{ab} ± 0.24	35-42
	1.0 + 0.5	3.87 ^{bcdef} ± 0.09	
	0.5 + 1.0	4.28 ^{defgh} ± 0.35	
	1.0 + 1.0	4.37 ^{efgh} ± 0.29	
	1.0 + 1.5	3.00 ^{abc} ± 0.54	
	1.5 + 1.0	4.00 ^{cdefg} ± 0.35	
	1.5 + 1.5	2.86 ^a ± 0.09	
BAP + Kinetin	0.5 + 0.5	2.81 ^a ± 0.28	42-49
	1.0 + 0.5	5.00 ^{gh} ± 0.54	
	0.5 + 1.0	3.20 ^{abc} ± 0.20	
	1.0 + 1.0	5.12 ^h ± 0.24	
	1.5 + 1.0	4.24 ^{defgh} ± 0.25	
	1.5 + 1.5	3.76 ^{abcde} ± 0.37	

Values represent mean $\pm$ S.E. (standard error). Each treatment was repeated thrice. Means in a column with the different letter (superscript) are significantly different according to least significant difference (LSD Test) ($P < 0.05$).

On the other hand, the individual shoots were separated and sub-cultured them into the fresh elongation medium. After 30 days of culture the highest shoot elongation (3.75 cm) was observed in MS medium fortified with 1.5 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA. The minimum elongation (0.75 cm) was recorded from MS with 2.0 mg l⁻¹ BAP + 0.5 mg l⁻¹ kinetin (**Table 14**).

Table 14: Effect of different concentrations and combinations of PGRs with MS medium on the elongation of individual shoot.

PGRs (mg l ⁻¹)	Initial length (cm)	Length (cm) of shoot after 30 days of culture	Increased length (cm) M $\pm$ S.E
BAP + NAA			
0.5 + 0.5	2.00	3.05	1.04 ^c $\pm$ 0.22
1.0 + 0.5	2.75	4.05	1.36 ^c $\pm$ 0.29
1.5 + 0.5	3.25	7.00	3.75 ^a $\pm$ 0.37
2.0 + 0.5	2.05	4.25	2.26 ^b $\pm$ 0.39
BAP + IAA			
0.5 + 0.5	1.95	2.90	0.99 ^c $\pm$ 0.15
1.0 + 0.5	2.80	4.32	1.50 ^{bc} $\pm$ 0.22
1.5 + 0.5	2.85	5.55	2.68 ^a $\pm$ 0.09
2.0 + 0.5	3.00	4.85	1.87 ^b $\pm$ 0.09
BAP + Kinetin			
0.5 + 0.5	2.50	4.06	1.55 ^b $\pm$ 0.20
1.0 + 0.5	2.70	4.70	2.00 ^{ab} $\pm$ 0.27
1.5 + 0.5	2.30	4.50	2.28 ^a $\pm$ 0.18
2.0 + 0.5	2.25	3.00	0.75 ^c $\pm$ 0.07

Values represent mean $\pm$ S.E. (standard error). Each treatment was repeated thrice and each treatment counted of 5 replicate culture vessels. Means in a column with the different letter (superscript) are significantly different according to least significant difference (LSD Test) ($P < 0.05$ levels).

4.3.3.3 Root induction, elongation and acclimatization

All elongated shoots were transferred into rooting medium for induction of roots. Results indicate that the average length of induced roots reached highest (4.0 cm) when the shoots were cultured on half strength MS medium supplemented with 1 mg l⁻¹ IAA (Fig. 20). In addition, this combination of medium was also effective for developing adventitious roots. In this medium the average number of root per shoot was 6.10 (Fig. 21). The well rooted micropropagated plantlets were hardened successfully in the potting mixture containing coconut husk, charcoal, brick pieces in the ratio of 2:1:1 and eventually established under natural condition following by the methods of Rahman et al. (2009). In my investigation 80 % plantlets survived acclimatization process and grew to a normal flowering plant under field condition (Fig. 19h).

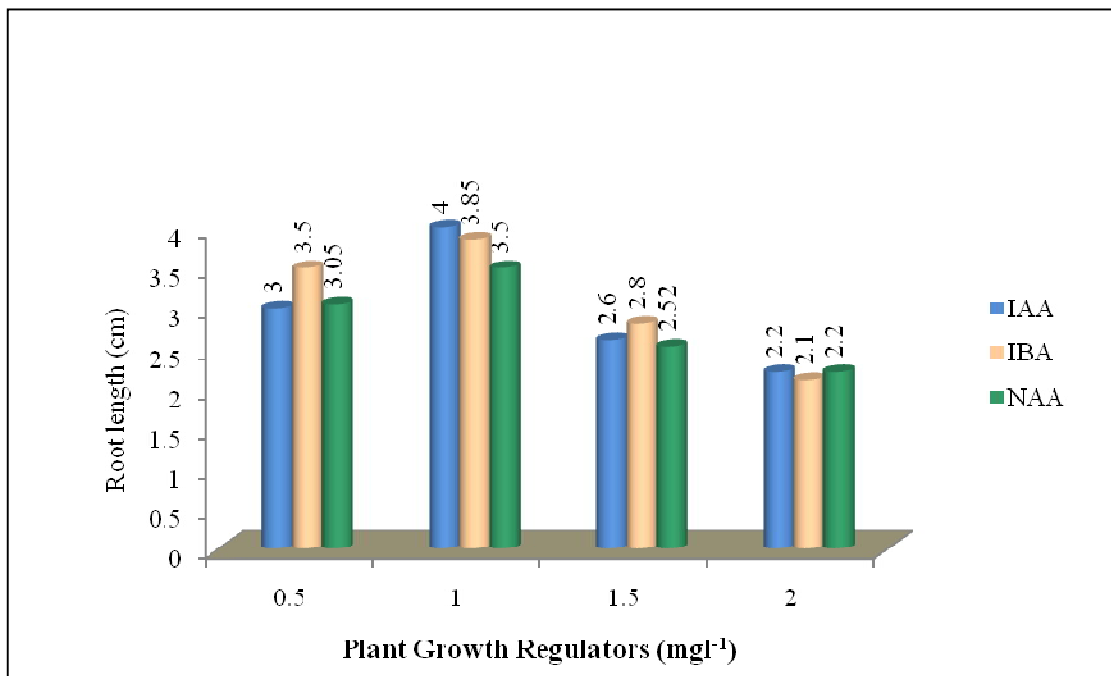


Fig. 20: Effects of different auxins on root development with ½MS medium.

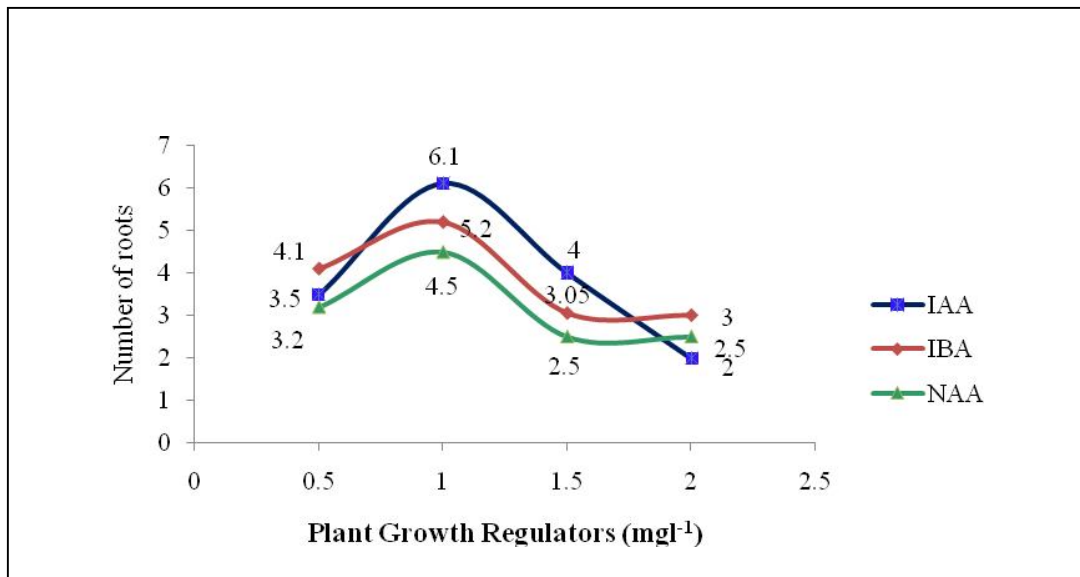


Fig. 21: Effects of different auxins with $\frac{1}{2}$ MS medium on number of root per plant.

4.3.4 Discussion

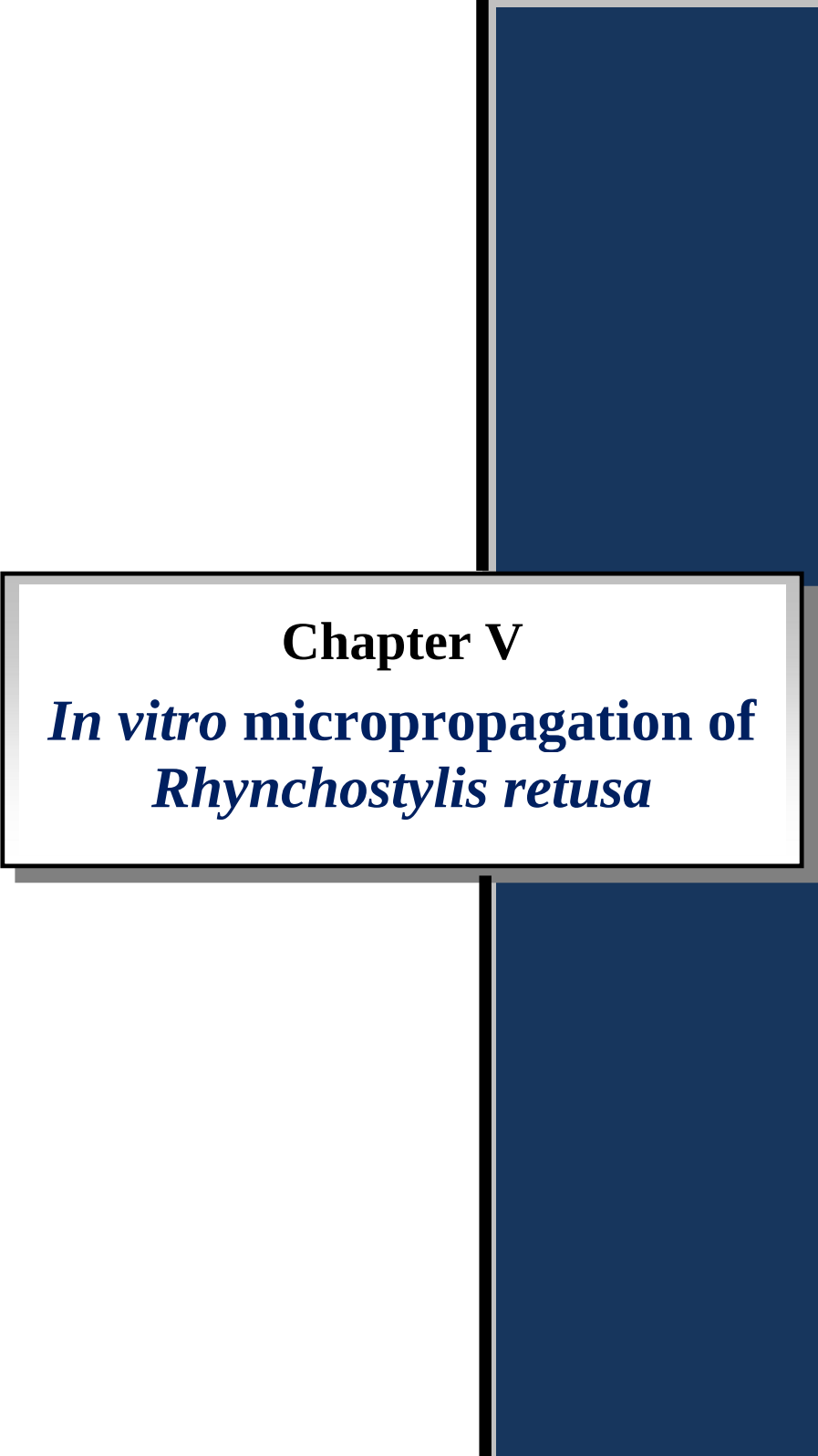
We have developed necessary parameters that are involved in the tissue culture processes. For germination of seeds among the four investigated basal media we found MS medium was the most suitable one. In MS medium the exposed seeds (80.0%) started to germinate (**Table 1**). In addition to germination rate, the time of germination was found to be the shortest also in this MS medium. Considering these two features, rate and time of germination we suggest that MS medium can be effectively used for *in vitro* germination of orchid seeds. Mohanty et al. (2012) reported that MS medium was equally effective also for germination of *Cymbidium mastersii* species. One of the key steps of *in vitro* propagation is the elongation of the donor plant materials that can be used as the source for regenerating multiple shoots. To investigate this feature we have exposed the germinated orchid seedlings to various elongation media containing different plant

growth regulators in different combinations and concentrations. We found that the rate of elongation reached highest when the germinated seedlings were exposed to MS containing 1.5 mg l^{-1} BAP and 0.5 mg l^{-1} NAA. In this medium the average length of germinated seedlings resulted about 5.00 cm (**Table 2**). Such type of positive effect of this culture condition for elongation of germinated seedlings was reported previously by Pradhan and Pant (2009). Analyzing the results obtained in micropropagation of the induced shoots we found that the media containing BAP and NAA developed the highest number of adventitious shoots (in average 7.52 shoots per explants). Furthermore, in this medium the time of shoot initiation was the shortest (in average 28-35 days). It was observed that in addition of NAA and BAP in the MS medium lead to optimum micropropagation of *Vanda tessellata* species and this combination can be successfully employed by the orchid breeders. Multiple shoots were produced in combination of BAP and NAA medium reported previously in various orchid species by different researcher (Rahman et al., 2009; Pant and Shresta, 2011). One of the major problems for orchid breeders is to generate a well developed root system facilitating adaptation of *in vitro* grown plantlets to field conditions. For investigating this problem we tested three potential plant growth regulators of IBA, IAA and NAA. Shoots when exposed to half strength MS medium supplemented with 1 mg l^{-1} IAA developed the highest number of adventitious roots (**Fig. 3**). The length of these roots was also highest in this medium (**Fig. 2**). The effect of IAA or IBA on induction of roots in other orchid species like *Vanda coerulea* was also reported by several authors (Malabadi et al., 2004, William et al., 2003, Mitra et al., 1976). These results are in complete agreement with those we obtained in half strength MS supplemented with 1 mg l^{-1} of IAA.

4.3.5 Conclusion

The results suggest that combined effect of BAP and NAA is more effective for multiple shoot induction from shoot segments of *Vanda tessellata*. On the other hand, single effect of IAA with $\frac{1}{2}$ MS medium was proved the best for root induction from the individual elongated shoot. Under this study an efficient regeneration protocol for *in vitro*

micropropagation in *V. tessellata* through shoot segment has been established. For advance level of biotechnological research and orchid conservation those experimental findings can be helped to plant breeder and scientist.



Chapter V

In vitro* micropropagation of *Rhynchosyilis retusa

5.1 Effect of PGRs on *In Vitro* Development of Protocorms, Regeneration and Mass Multiplication Derived from Immature Seeds of *Rhynchostylis retusa* (L.) Blume

5.1.1 Introduction

Orchids are important in the floriculture industry due to beautiful foliage, colourful and fragrant flowers of varying shapes, and long vase life of cut flowers and also having medicinal properties (Bhattacharjee et al., 2014). This group of plants is valued highly both in the national as well as in the international markets (Kumar et al., 2002). *Rhynchostylis retusa* is a monopodial, epiphytic orchid species with beautiful flowers arranged in racemose inflorescence. It is an endangered orchid species that grows in moist areas and blossoms during monsoon. This plant having a robust stem of about 25 cm long and its leaves are strap-shaped and about 25 cm long and the pendulous inflorescences are about 60 cm long which densely flowered. Flowering sits in summer to autumn, especially in May - June (Sinha and Jahan, 2012). The plant is found in semi-deciduous and deciduous dry lowland forests woodlands at elevations of sea level to 1200 m (3900 ft.). This orchid is found in Bangladesh, Benin, Burma, Cambodia, China, India, Indonesia, Laos, Malaysia, Nepal, Philippines, Singapore, Sri Lanka, Thailand and Vietnam.

The whole plant preparations of *Rhynchostylis retusa* is used to treat rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases and external inflammations (Kumar et al., 2012). It is also used as an emollient and in the treatment of throat inflammation (Shanavaskhan et al., 2012). The plant leaf juice and aerial roots are also used in ear pain and cleaning (Basumatary et al., 2004). In the Kurigram district of Bangladesh, people use the leaves of this plant to cure rheumatic pain (Das et al., 2012). *Rhynchostylis retusa* roots are also used to cure malarial fever (Tiwari et al., 2012, Radhika and Murthy, 2013). Juice of roots applied to cuts and wounds. Dried flowers are used as insect repellent and to induce vomiting (Subedi et al., 2013). It is also reported that the plant showed significant antibacterial activity against *Bacillus subtilis* and *Escherichia coli* (Hossain, 2011).

Many attractive *Rhynchosstylis* orchids have become commercially important in potted plant industries. These species needed to be protected from the danger of extermination through deforestation in Bangladesh and also in the world. Therefore, a rapid multiplication in commercial scale by micropropagation of this species is required. The main objective of the study was to develop *in vitro* micropropagation methods for multiplication of this rare orchid and strengthen the resource base through restoration.

5.1.2 Materials and Methods

5.1.2.1 Plant materials

The immature capsule of *Rhynchosstylis retusa* was used as seed source which was collected from Rajshahi, Bangladesh (Fig. 22a).

5.1.2.2 Methods

5.1.2.2.1 Sterilization of capsule and inoculation

The capsules were surface sterilized by submerging them in 0.2% (w/v) HgCl₂ solution for 10 minutes with occasional agitation followed by a dip in 70% ethanol for 25-30 seconds. The sterilized capsules were washed with sterile distilled water for 5-6 times and then cut them longitudinally with a sterile surgical blade in the laminar air flow cabinet. The feathery seeds were then taken out with the help of a sterile forceps and were inoculated to the media taken in culture vessels. The lid of culture vessels was closed tightly and finally sealed with parafilm very carefully.

5.1.2.2.2 Media and culture condition

Four basal media viz. MS and ½MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968) and PM (PhytamaxTM, P-1056, Sigma, USA) amended with 3% sucrose and 0.8% agar were used on germination and for protocorm (PLBs) development. One set of each seed culture was maintained on amended with 2% (w/v) sucrose for each medium. The pH for all media was adjusted at 5.6-5.8. The media was autoclaved at a temperature of

121°C at pressure of 15 psi for 15 minutes. The culture vessels with inoculated seeds were maintained in the culture room under a photo period of 16h light and 8h dark at 25±2°C. Seed germination data were recorded after 50 days of inoculation. Different developmental stages of PLBs (i.e. percentage of protocorms with vegetative apex, plantlets with 2-3 leaves) were recorded in every 30 days of culture initiation.

5.1.2.2.3 Plant growth regulators

For secondary protocorm development different concentrations of 6-benzylaminopurine (BAP), kinetin (Kin), α -naphthalene acetic acid (NAA), picloram, indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) were used in MS medium. Four concentration of plant growth regulators (0.5, 1.0, 1.5, 2.0 mg l⁻¹) in combination were used for this study to know about their effectiveness in promoting proliferation of protocorms and subsequent plantlet development. Sixty days old primary protocorms (one protocorm per test tube) were used for the production of secondary protocorm and its subsequent developments. Data were recorded on the basis of plantlet development after 60 days of culture initiation. Means of 20 test tubes were taken for each treatment and all experiments were repeated three times.

5.1.2.2.4 Rooting and acclimatization

For root induction, six month old plantlets without roots were cultured on half-strength of MS medium supplemented with different concentrations of IAA and/or NAA (0.5, 1.0, 1.5 mg l⁻¹). The well developed rooted plantlets were hardened successfully in the pot containing different mixture of compost viz., (i) brick pieces, charcoal, decaying litter (1:1:1) and sphagnum moss, (ii) brick pieces, charcoal, decaying litter, saw dust (1:1:1:1) and sphagnum moss, (iii) brick pieces, charcoal, coconut husk (1:1:1) and (iv) brick pieces, charcoal, coconut husk (1:1:2) followed by the methods of Das et al. (2007). The plantlets were watered alternately in the evening and sprayed with MS nutrient solution (diluted 10 times) fortnightly for about a month.

5.1.2.2.5 Data recording and statistical analysis

Data about PLBs, its subsequent regeneration, shoot and root development was recorded. Each value represents an average of 20 replicates and each experiment was repeated three times. Data were subjected to analysis of variance and means were separated by Duncan's Multiple Range Test (DMRT).

5.1.3 Results

5.1.3.1 Seed germination and protocorm formation

In the present experiment, immature green pods were taken for *in vitro* culture and four types of basal media (MS, ½MS, PM and B₅) without PGRs were used to assess their effect on seed germination and protocorm formation. Percentage of seed germination was highest in MS (72.60%) followed by ½MS (60.60%), B₅ (55.20%) and PM (44.40%) (**Table 15**). Germination was marked by swelling and emergence of the embryo from the testa. Seed germination was first evident by swelling and within 7-8 weeks the undifferentiated embryos formed an irregular shaped cell mass as spherules. After 1-2 weeks, these spherules turned green and formed round structures as protocorms (**Fig. 22b**). Protocorms became visible after 11 weeks of culture initiation and showed at the vegetative apex (**Fig. 22c**). This was followed by the development of 2-3 leaf primordia when MS basal medium or half strength of MS medium or B₅ media were used (**Fig. 1d**). Out of four media MS showed most effective on the development of leaf primordia than others (**Table 15**). Protocorms cultured in PM medium did not develop beyond the vegetative apex stage, while half-strength of MS and B₅ supported only poor growth of protocorms in comparison with MS medium (**Table 15**).

Table 15: Comparative effect of four culture media on germination of seeds and development of protocorm in *Rhynchosstylis retusa*.

Medium	Time required (weeks)		% of seed germination (M $\pm$ S.E)	Protocorm development (% response)	
	Spherule formation	Protocorm formation		Stage-1	Stage-2
MS	7-8	9-10	72.60 $\pm$ 0.50	50.60 $\pm$ 0.67	39.40 $\pm$ 0.77
$\frac{1}{2}$ MS	7-8	9-10	60.60 $\pm$ 0.98	40.40 $\pm$ 0.94	33.20 $\pm$ 0.92
B ₅	8-9	10-11	55.20 $\pm$ 0.84	34.60 $\pm$ 0.67	30.40 $\pm$ 0.70
PM	8-9	10-11	44.40 $\pm$ 0.79	25.00 $\pm$ 0.58	-

MS = Murashige and Skoog (1962); PM = PhytamaxTM, P-1056, Sigma, USA; B₅ = Gamborg et al. (1968); – = no response. Stage 1: vegetative apex stage and Stage 2: 2–3 leaves stage.

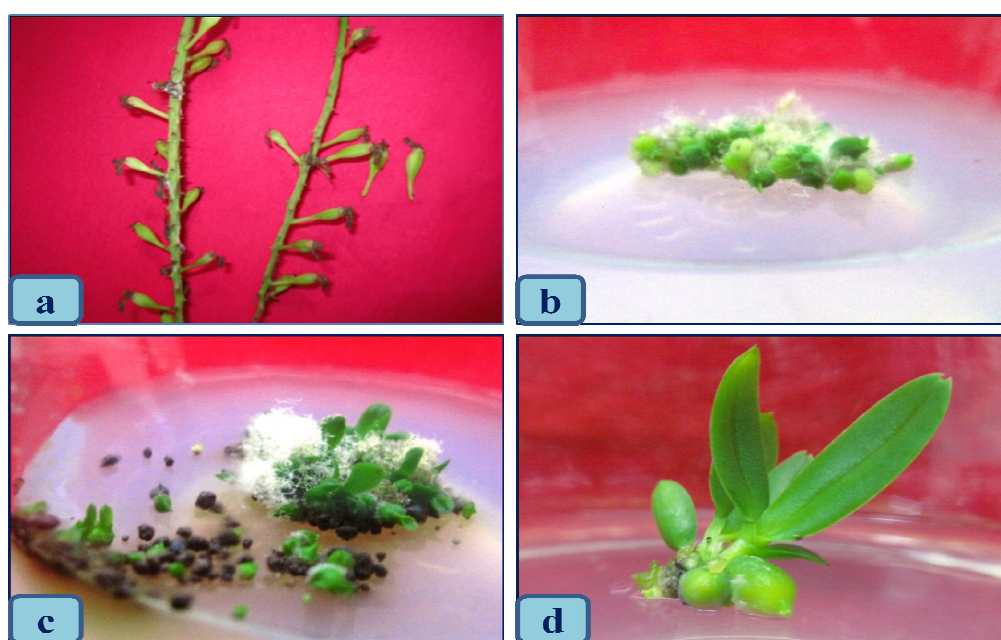


Fig. 22: Seed germination stages of *R. retusa*. a) Immature seeds, b) Rounded protocorms after 8 weeks of culture, c) Young protocorm with a vegetative apex at the upper side, and d) Complete plantlet with 2-3 leaves and 1-2 roots.

5.1.3.2 Production of secondary protocorms from primary PLBs

To assess the effectiveness of auxin and cytokinin for protocorm multiplication, 60 days old primary protocorms were sub-cultured on fresh MS medium supplemented with different concentrations of BAP (0.5, 1.0, 1.5, 2.0 mg l⁻¹), Kinetin (0.5, 1.0, 1.5, 2.0 mg l⁻¹), NAA (0.5, 1.0, 1.5, 2.0 mg l⁻¹), Picloram (0.5, 1.0, 1.5, 2.0 mg l⁻¹), IBA (0.5, 1.0, 1.5, 2.0 mg l⁻¹) and IAA (0.0, 0.5, 1.0, 1.5 mg l⁻¹) alone and in combination. This resulted in the generation of secondary protocorms (PLBs) instead of shoot formation directly (**Fig. 23 a,b**). Protocorm like bodies development initiated after 30 days of culture (**Fig. 23a**). The maximum number of PLBs (16.00 ± 0.41) per protocorm was observed after 60 days of culture in MS medium that contained BAP (1.0 mg l⁻¹) and NAA (1.0 mg l⁻¹), followed by BAP (0.5 mg l⁻¹) and NAA (0.5 mg l⁻¹) (Table 16). Almost all the PLBs were converted into plantlets (**Fig. 23 c,d**) in the following 20-30 days of the time period, giving rise to multiple numbers of shoots on the same media.



Fig. 23: Development of plants through PLBs of *Rhynchosstylis retusa*. a) Secondary protocorms (PLBs) developed from a single primary protocorms, b) Shoot formation from secondary protocorm, c) Plantlets developed from PLBs, d) Plants with good shoots and roots, e) Well-rooted plants ready for hardening, and f) Hardened plants in pots.

Table 16: Production of secondary protocorms in *Rhynchosstylis retusa* after 60 days of culture initiation in MS medium supplemented with different plant growth regulators (PGRs).

PGRs	Conc. (mg l ⁻¹)	No. of secondary protocorms M ± S.E
BAP + NAA	0.5 + 0.5	12.86 ^b ± 0.23
	1.0 + 1.0	16.00 ^a ± 0.41
	1.5 + 1.5	10.60 ^{cd} ± 0.23
	2.0 + 2.0	10.20 ^{cd} ± 0.38
BAP + IAA	0.5 + 0.5	10.40 ^a ± 0.28
	1.0 + 1.0	7.20 ^b ± 0.34
	1.5 + 1.5	5.00 ^{cd} ± 0.41
	2.0 + 2.0	4.00 ^{cd} ± 0.18
BAP + IBA	0.5 + 0.5	9.20 ^a ± 0.39
	1.0 + 1.0	8.40 ^a ± 0.26
	1.5 + 1.5	8.00 ^a ± 0.15
	2.0 + 2.0	7.20 ^b ± 0.15
BAP + Kinetin	0.5 + 0.5	8.20 ^b ± 0.28
	1.0 + 1.0	4.40 ^a ± 0.67
	1.5 + 1.5	3.80 ^a ± 0.42
	2.0 + 2.0	3.40 ^a ± 0.29
BAP + Picloram	0.5 + 0.5	10.40 ^{ab} ± 0.67
	1.0 + 1.0	10.00 ^a ± 0.37
	1.5 + 1.5	8.60 ^{bc} ± 0.35
	2.0 + 2.0	6.40 ^c ± 0.67

Secondary protocorms developed from each primary PLBs. Means followed by a common letter are not significantly different at 5% level.

5.1.3.3 Rooting and acclimatization

For increasing the number of roots and their length, plantlets were supplied with different concentration of auxins (**Fig. 24** and **25**). Half strength of MS medium supplemented with 1.0 mg l^{-1} IAA induced the most roots (7.00) per shoot (**Fig. 23e** and **24**), followed by 1.5 mg l^{-1} IAA. In comparison, NAA was less effective for root formation and promoting root extension. A significant difference was found on root length at different concentrations and combinations of growth regulators. The maximum root length (3.4 cm) was obtained $\frac{1}{2}$ MS medium that supplemented with 1.0 mg l^{-1} IAA followed by 1.0 mg l^{-1} NAA (**Fig. 25**). The different composts used for hardening of *in vitro* grown plantlets of *R. retusa* were found to be satisfactory for survivability and normal growth of the plantlets. The highest percentage of survivability (80%) with maximum length (5.00 cm) of *R. retusa* hardened plants was obtained on substratum containing brick pieces, charcoal, coconut husk (1:1:2) with a layer of moss (**Fig. 23f** and **Table 17**).

Table 17: *Ex vitro* establishment of *Rhynchosylish retusa* plantlets.

Substratum of compost	Survival (%)	Height (cm)
Brick pieces + charcoal + decaying litter (1:1:1) + moss (10 gm/pot)	69.20 ± 0.80	2.84 ± 0.07
Brick pieces + charcoal + decaying litter + saw dust (1:1:1:1) + moss (10 gm/pot)	53.80 ± 0.86	4.02 ± 0.18
Brick pieces + charcoal + coconut husk (1:1:1)	72.00 ± 0.80	2.98 ± 0.10
Brick pieces + charcoal + coconut husk (1:1:2)	80.00 ± 0.82	5.00 ± 0.04

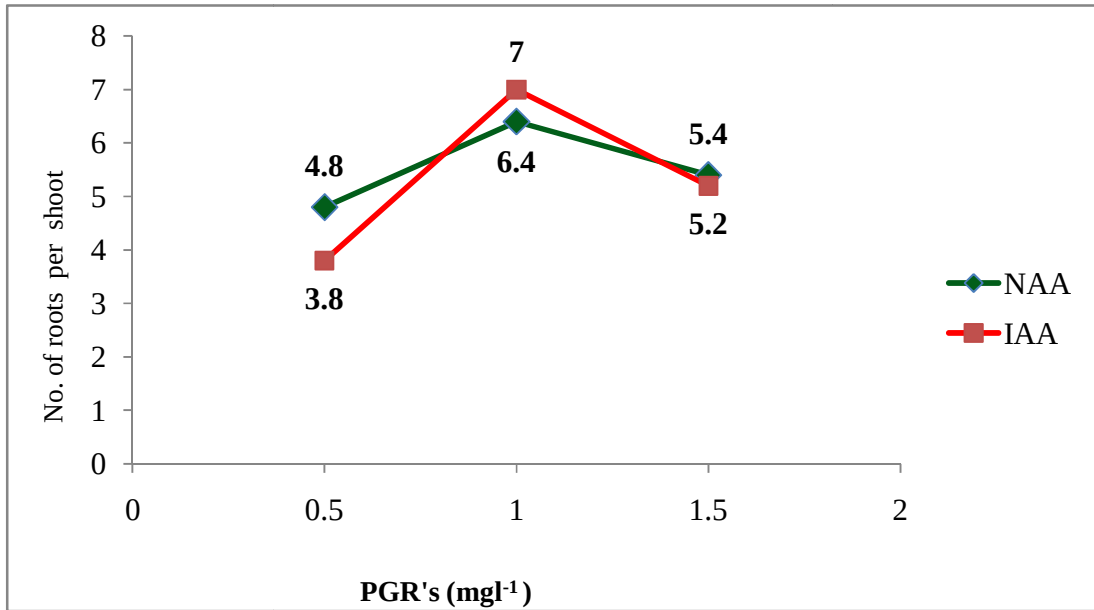


Fig. 24: Effects of different concentrations of NAA and IAA with $\frac{1}{2}$ MS medium on root induction per shoot of *Rhynchosytilis retusa*.

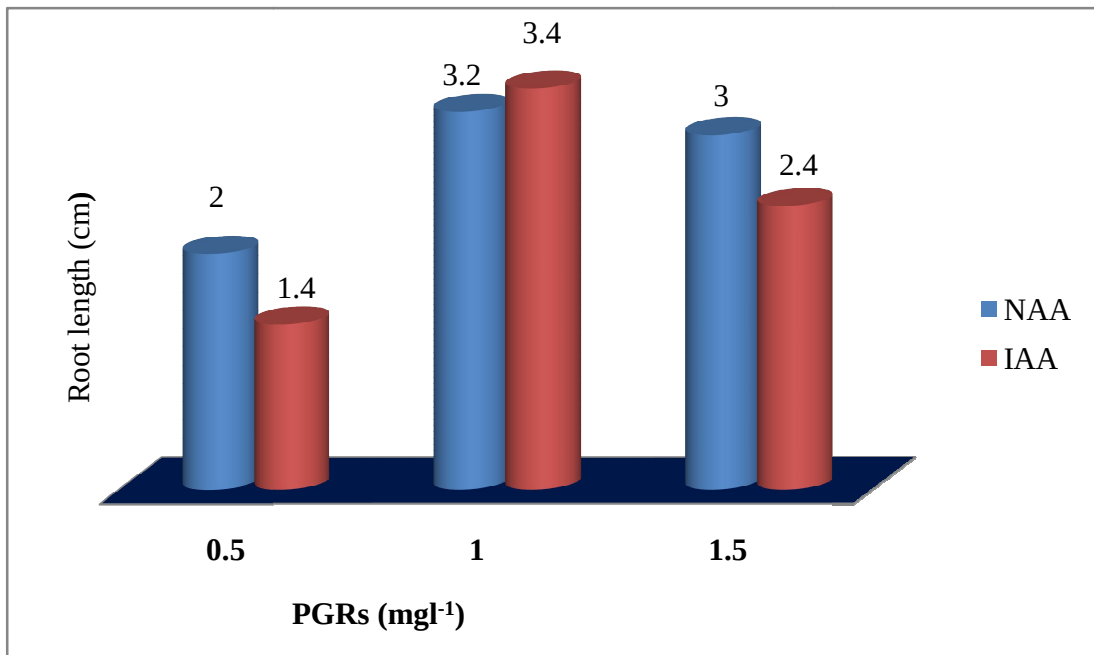


Fig. 25: Effects of different concentrations of NAA and IAA supplemented with $\frac{1}{2}$ MS medium on increase of root length of *Rhynchosytilis retusa*.

5.1.4 DISCUSSION

Germination and seedling development in orchids are strikingly different from other flowering plants. A single orchid capsule is estimated to contain millions of seeds, which lack endosperm. In spite of a very large number, only few seeds germinate in nature. Currently the horticultural trade depends on wild orchid population as a source of stock plants, but most are not propagated properly. Nutrient requirements for orchid seed germination are thought to be species specific (Arditti and Ernst, 1984; Kauth et al., 2008). It was therefore, not surprising that the various basal media used in the present study, with their different compositions and concentrations of mineral salts, organic supplements and vitamins, varied in their suitability for *in vitro* germination. In the present study found that the MS medium promoted seed germination. Nitrogen is very essential component for plant growth and its source has been shown to have an effect on the germination of different orchid species are reported by Stewart and Kane (2006). The MS medium contains nitrogen and ammonium stimulated seed germination (Popova et al., 2003). Under this study for *Rhynchostylis retusa* we observed that seed germination and PLB's formation was higher also in MS medium. The results are in agreement with the findings of Bhattacharjee and Islam (2014). On contrary, Hossain (2008) reported that 75% of the *Eulophia ibaguense* seeds germinated on MS medium supplemented with 1 mg l⁻¹ BAP, but with increasing concentrations of BAP, the seed germination percentage has been declined. In case of *Cypripedium* sp. as terrestrial orchid BAP enhanced seed germination (Depauw et al., 1995). The present study showed that primary protocorms responded significantly higher in combination with 1.0 mg l⁻¹ BAP and 1.0 mg l⁻¹ NAA. Highest yield of PLBs obtained in *C. lapine* by Fujii et al. (1999) and they suggested that a synergistic effect was also observed in the case of *Cymbidium nativity*. Kusumoto (1978) suggested that the negative effect of NAA on PLBs proliferation in case of *Cymbidiums* whereas using BAP in the medium showed positive effect on protocorm formation.

In case of rooting, IAA was found to be the most superior auxin for promoting root formation and increase of root length. The maximum number (7.00) of roots per shoot was recorded in the medium supplemented with 1.0 mg l⁻¹ IAA. Sunitibala and Kishor (2009) also reported that about the positive effects with IAA in the case of *Dendrobium transparens*. On contrary, Bhadra et al. (2002) reported that ½MS + 1.5% (w/v) sucrose without auxin showed better performance in case of root formation and increase of root length in *Dendrobium aphyllum*. However, the highest percentage of survivability of hardened plants showed in the compost of brick pieces, charcoal, decaying litter (1:1:1) with a layer of sphagnum moss. The layer of moss on top proved to be beneficial due to higher retention of moisture content. As reported earlier by Kumaria and Tandon (1994) in *D. fimbriatum* var. *oculatum*, feeding the plantlets with dilute MS nutrient salt solution was found beneficial to the developing hardened plantlets of *C. devonianum*. This study gives an effective protocol for seed germination, protocorm development and plantlet regeneration from immature seeds which can be used for establishing *Rhynchostylis retusa* populations in Bangladesh and elsewhere.

5.1.5 Conclusions

The efficient induction of PLBs and their proliferation from protocorms of *Rhynchostylis retusa* was achieved for large scale propagation. It was observed that MS medium was the best for seed germination and PLBs formation of *Rhynchostylis retusa* orchid. Similarly MS medium supplemented with BAP (1.0 mg l⁻¹) and NAA (1.0 mg l⁻¹) was found to be the most suitable for protocorm development. On the other hand, IAA was the most effective phytohormone for promoting root formation and increase of root length. Thus, the results clearly demonstrated that above culture condition was the most effective for mass multiplication of *Rhynchostylis retusa* and this protocol will be useful to commercial growers for mass multiplication. For further research on biochemical compound isolation for medicine, genetic transformation and conservation of endangered orchids this techniques and protocol will be helpful for researcher.

5.2 Plant Regeneration Through Somatic Embryogenesis from Leaf and Root Explants of *Rhynchosylis retusa* (L.) Blume

5.2.1 Introduction

Orchids are one of the most pampered plants and occupy top position among all the flowering plants for cut-flower production and as potted ornamentals (Parab and Krishnan, 2012). The plant is highly valued for its fantastic range of variation in colour, form and long-lasting flowers (Paudel and Pant, 2012). Many commercially important orchids are now artificially grown for their high value both in national and international markets (Kumar et al., 2002). However, some orchids face threat of extinction because of environmental disruption, anthropogenic activities and overexploitation for horticulture and ethno-botanical purposes (Coates and Dixon, 2007). *Rhynchosylis retusa* (L.) Blume is an endangered orchid in Bangladesh. The plant is a monopodial epiphytic orchid species with beautiful flowers arranged in racemose manner. This orchid species grows in moist areas and blossoms during monsoon (Bhattacharjee and Islam, 2015a). The plant is used to treat rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases, external inflammations (Kumar et al., 2012; Shanavaskhan et al., 2012). The plant leaf juice and aerial roots are used in ear pain and cleaning (Basumatary et al., 2004). In Kurigram district of Bangladesh, people use the leaves of this plant to cure rheumatic pain (Das et al., 2012). *R. retusa* roots are used to cure malarial fever (Tiwari et al., 2012, Radhika and Murthy, 2013). Juices of roots are applied to cuts and wounds while dried flowers are used as insect repellent and to induce vomiting (Subedi et al., 2013). The plant has shown significant antimicrobial activity against bacteria and fungi (Bhattacharjee and Islam, 2015b). However, these species need to be protected from the danger of extermination through reforestation (Bhattacharjee and Islam, 2015a). Therefore, rapid multiplication process of these species through micropropagation is the need of hour. The technique helps in rapid propagation of a large number of pathogen-free plants in a short time as well as helps in conservation of rare and endangered plant species (Kumar and Reddy, 2011).

Direct and indirect shoot organogenesis and somatic embryogenesis are most commonly used micropropagation techniques (Antony et al., 2014). Somatic embryogenesis in recent years has shown enormous potential in providing efficient clonal propagation system. The development of synthetic seed technology, plant transformation and their conservation systems are essential for large-scale clonal propagation of elite genotypes (Bomal and Tremblay, 2000; Bandyopadhyay and Hamill, 2000). However, plant regeneration through somatic embryogenesis is one of the main pre-requisites for potential use of clonal propagation in woody plants (Park et al., 1998). The phenomenon of somatic embryogenesis through *in vitro* callus development in orchids is rare as the success of callus formation is constrained due to its slow growth and tendency to become necrotic (Kerbaudy, 1984). Callus formation via leaf and root explants of orchids is rare and does not succeed in all cases. Even when plant regeneration from callus of orchids has been achieved, it usually occurs via protocorm like bodies (Begum et al., 1994; Roy and Banerjee, 2003; Huana et al., 2004). Plant regeneration through somatic embryogenesis has many advantages over other routes of *in vitro* plant production and appears to be the most promising area of research for large scale production, rapid plant propagation and conservation. Therefore, the success of somatic embryogenesis via callus culture is interesting in plant regeneration of orchids. The present study was focused on somatic embryogenesis and adventitious shoot formation from leaf and root explants of *Rhynchosyilis retusa* to develop efficient propagation and regeneration method for mass multiplication.

5.2.2 Materials and Methods

5.2.2.1 Plant materials, inoculation, medium and incubation

The seeds of *Rhynchosyilis retusa* were collected from Rajshahi, Bangladesh. The plants were grown through *in vitro* tissue culture systems. The young cotyledonous leaves and roots were cut into small pieces (approximately 1-2 cm) and then inoculated in sterile culture bottles containing 30 ml MS semi-solid medium (Murashige and Skoog, 1962). The MS medium was fortified with 3% (w/v) sucrose, 0.7% agar and with various combination and concentrations of 6-benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic

acid (2,4-D), α -naphthaleneacetic acid (NAA) and kinetin (Table 18). The pH of medium was adjusted to pH 5.8 and medium autoclaved at 121°C with 15 lb pressure for 15 min. Culture bottles were maintained at $25 \pm 2^\circ\text{C}$ with 16/8 h photoperiod using cool white florescent light in growth chamber. Sub-culturing was done after every 6 weeks of culture initiation. Observations were recorded at weekly interval upto 3 months. Each experiment was conducted in a completely randomized design (CRD) and each treatment replicated three times. Leaf and roots derived calli were multiplied and maintained on same medium and sub-cultured every month.

5.2.2.2 Shoot and root development and plant regeneration

For multiple shoot formation, the induced adventitious shoot were transferred to MS basal medium containing different concentration and combination of BAP, 2,4-D, NAA and kinetin. The cultures were maintained at $25 \pm 2^\circ\text{C}$ with 16 h photoperiod. For root induction, single shoots were separated from multiple shoot clusters and transferred to $\frac{1}{2}$ strength of MS medium supplemented with various plant growth regulators, viz., i) 0.5 mg l^{-1} IAA, ii) 0.5 mg l^{-1} NAA, iii) 0.5 mg l^{-1} IBA, iv) 0.5 mg l^{-1} IAA + 0.5 mg l^{-1} NAA, v) 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA and vi) 0.5 mg l^{-1} IAA + 0.5 mg l^{-1} IBA. Somatic embryos were isolated and transferred to MS and $\frac{1}{2}$ MS basal medium with various concentrations of BAP for germination and cultures were incubated at 16 h photoperiod.

5.2.2.3 Hardening and acclimatization

Well rooted plants were removed from the culture tubes, washed with running tap water to remove agar and transplanted into plastic cups containing small brick pieces (5-10 g each) and wood charcoal (1:3) with coconut husk. The young plants were maintained under 30-40% natural light, sprayed with water on every 2nd day and fertilized with full-strength macro-elements of MS medium after every 5th day. The acclimatized plants were eventually transferred to pot. The data were recorded with respect to per cent embryo induction, somatic embryos and multiple shoot developed after 4 weeks of culture initiation. Each experiment was repeated thrice.

5.2.2.4 Statistical analysis

The data was subjected to analysis of variance (Gomez and Gomez, 1976). The mean values were compared by least significant difference at 5% significance level using SPSS (version 16.0) for Windows 7.

5.2.3 Results and Discussion

In the present study direct somatic embryogenesis was induced from leaf and root explants of *Rhynchostylis retusa*. The explants (young leaf and root) when cultured on MS medium supplemented with different plant growth regulators showed highest response for direct somatic embryo induction in leaf (75%) and root (50.2%) after 25-30 days on MS medium supplemented with 1.0 mg l⁻¹ BAP and 1.5 mg l⁻¹ kinetin (**Table 18**). The results revealed that a combination of BAP and kinetin is essential for induction of somatic embryogenesis from leaf and root explants of *R. retusa*. Chung et al. (2007) reported that TDZ (thidiazuron) 0.3, 1.0 and 3.0 mg dm⁻³ induced 5-25% leaf tip segments in *in vitro* grown plants directly from embryos after 60 d of culture. In general, leaf explants produced significantly higher embryos than root explants. Somatic embryos were induced directly at the cut ends or surfaces of leaf and root explants (**Fig. 26** and **27**). The embryos were green, small and globular. The findings are in agreement with earlier reports on *Nothapodytes foetida* (Khadke and Kuvalekar, 2013) and *Sapindus emarginatus* (Devaraju and Reddy, 2013).

The frequency of somatic embryos was high when leaf explants was used. The somatic embryos were formed either individually or in groups directly on the cut ends or surface of explants without callus formation. Most of the globular embryos gradually developed as bipolar structures resembling heart, torpedo and cotyledon-stage embryos (**Fig. 26 b; 27c**). After 3-4 weeks of culture initiation, the cultures exhibited near-confluent growth which turned green with patches of developing embryos. The formation of somatic embryos around root segments was slower than leaf segment and more asynchronous. In another orchid *Oncidium* leaf tips reportedly showed highest ability to form embryos (Chen et al., 1999; Chen and Chang, 2001). Cordal et al. (2014) obtained somatic embryos directly from the cutting edge of blade and embryogenic callus (indirect way) from the midrib of *Phytolacca tetramera*.

Table 18: Effects of PGRs on embryos induction from leaf and root of *Rhynchosstylis retusa*.

Plant growth regulators	Concentration of PGRs (mg l ⁻¹)	Embryo induction (%) from explants	
		Leaf	Root
BAP + 2,4-D	0.5 + 1.0	40.00 ± 0.71 ^{cd}	0.00 ^a
	1.0 + 1.5	50.20 ± 0.73 ^f	45.40 ± 0.51 ^e
	1.5 + 2.0	38.80 ± 0.60 ^c	2.20 ± 0.33 ^b
	2.0 + 2.5	35.40 ± 0.33 ^b	0.00 ^a
BAP + NAA	0.5 + 1.0	0.00 ^a	0.00 ^a
	1.0 + 1.5	60.00 ± 0.84 ^h	45.00 ± 0.71 ^e
	1.5 + 2.0	40.80 ± 0.73 ^d	35.00 ± 0.45 ^d
	2.0 + 2.5	0.00 ^a	0 ^a
BAP + Kinetin	0.5 + 1.0	55.40 ± 0.51 ^g	0.00 ^a
	1.0 + 1.5	75.00 ± 0.71 ⁱ	50.20 ± 0.80 ^f
	1.5 + 2.0	45.00 ± 0.55 ^e	30.80 ± 0.66 ^c
	2.0 + 2.5	0.00 ^a	0.00 ^a

Values represent mean ± S.E. (standard error). Each treatment was repeated thrice. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$.

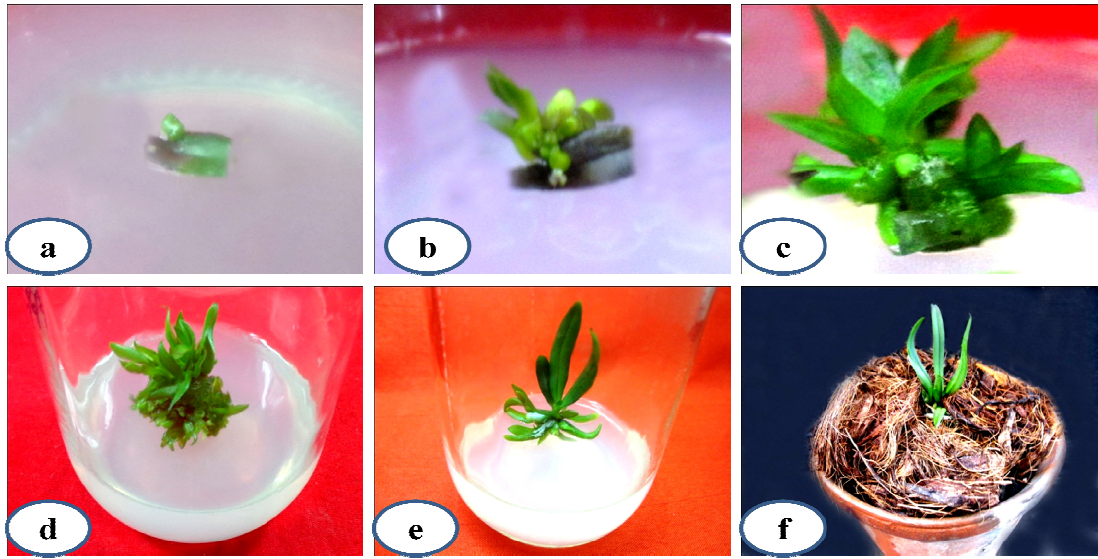


Fig. 26: Direct somatic embryogenesis from leaf explants of *Rhynchosstylis retusa* and subsequent plant regeneration, a) callus initiation from leaf surface, b) somatic embryos directly formed from the leaf surface, c-d) a cluster of embryos formed shoot, e) regenerated plantlet, and f) acclimatized plantlet.

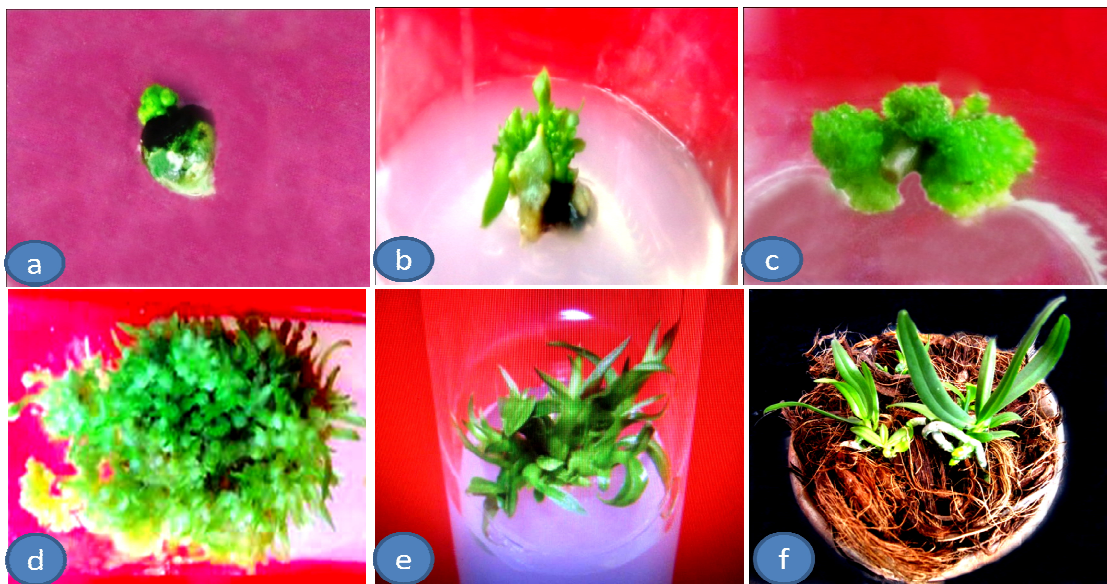


Fig. 27: Direct somatic embryogenesis from root explants of *Rhynchosstylis retusa* and subsequent plant regeneration; a-b) somatic embryos directly formed near the cut end of root; c) a cluster of somatic embryos directly formed vigorously from the cut end of root; d) a cluster of multiple shoots from embryo germination; e) regenerated plantlet; and f) acclimatized plantlets.

Table 19: Effects of different concentration of BAP on germination of somatic embryos.

Concentrations of BAP (mg l ⁻¹)	Germination (%)	
	MS (Mean ± S.E)	½ MS (Mean ± S.E)
0.5	0.00 ^a	0.00 ^a
1.0	35± 0.71 ^b	42± 0.71 ^c
1.5	50± 0.95 ^d	60± 0.95 ^d
2.0	30± 0.84 ^c	35± 0.55 ^b

‘0’ indicate no response. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$.

The germination of embryos occurred within 3 weeks after the transfer of embryos onto MS and ½MS medium supplemented with varying concentrations of BAP (Fig. 26c, d; 27d). The highest germination frequency (60.0 ± 0.95) of somatic embryos was observed in ½MS medium supplemented with 1.5 mg l⁻¹ BAP (Table 19).

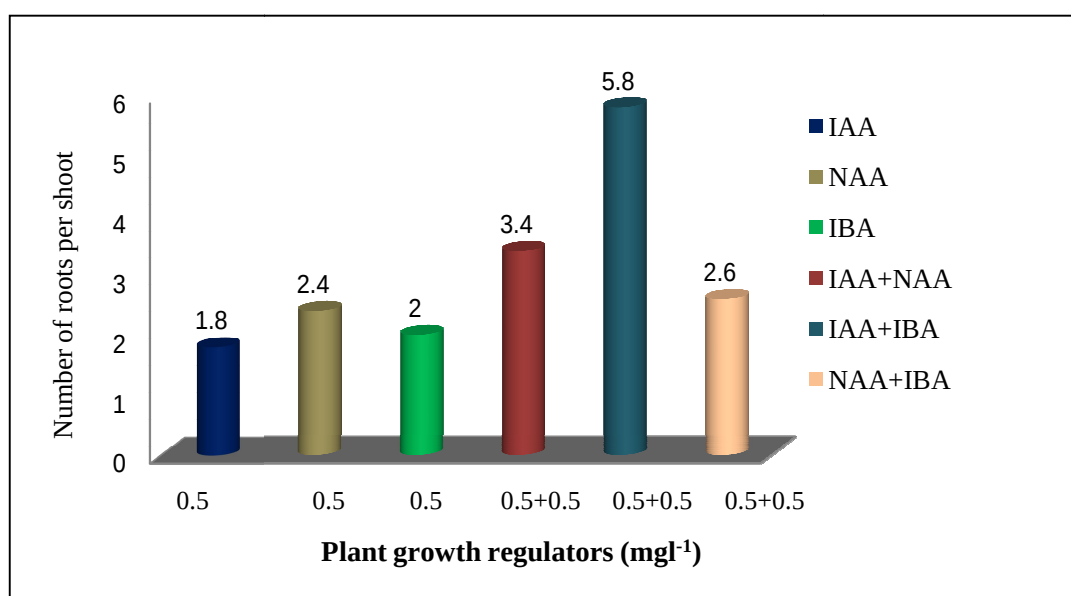


Fig. 28: Effects of different concentration and combination of PGRs with ½MS medium on induction of roots.

Adventitious shoot formation occurred 3 weeks after culture initiation. Plant regeneration with direct and indirect organogenesis from various explants sources of *N. foetida* have previously been reported (Thengane et al., 2001; Rai, 2002). Khadke and Kuvalekar (2013) reported that almost similar results with medicinal plant *N. foetida*, a critically endangered plant species, and suggested that this system may prove beneficial for *ex situ* conservation as well as for amenability of this plant for genetic manipulation. For multiple shoot formation, the induced adventitious shoots were transferred to MS medium containing different concentration and combination of plant growth regulators. These developed into multiple shoot clumps with maximum number of multiple shoots (10.20 ± 0.58) and shoot length (3.80 ± 0.37) noticed on MS medium fortified with 1.5 mg l^{-1} BAP + 1.0 mg l^{-1} NAA as compared to all other combination and concentrations of BAP, NAA, IAA and kinetin (**Table 20**).

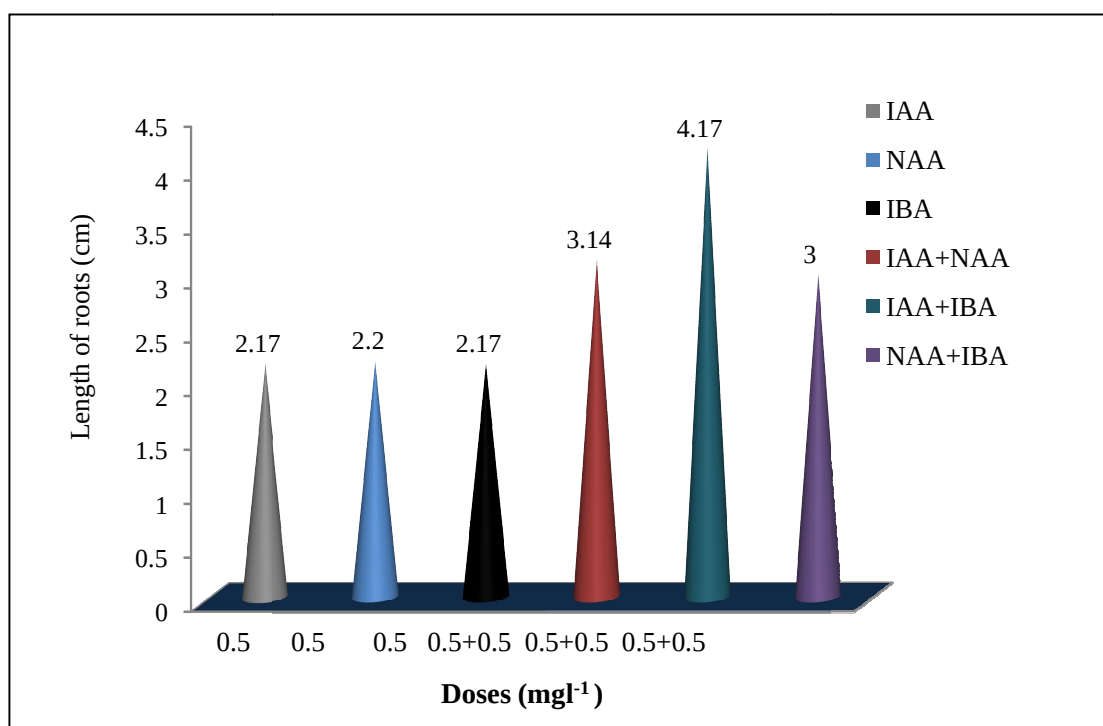


Fig. 29: Effects of different concentration and combination of PGRs with $\frac{1}{2}$ MS medium on root development.

To induce rooting, single shoots were separated from multiple shoot clusters (**Fig. 26e; 27e**) and transferred to rooting medium. Half strength MS medium supplemented with 3% sucrose + 0.5 mg l⁻¹ IAA and 0.5 mg l⁻¹ IBA showed best performance with regard to root formation (**Fig. 28, 29**). Well rooted plants were taken out and washed with sterile distilled water to remove agar and transplanted into plastic cups containing small brick piece (each 5-10 g) and wood charcoal (1:3) and coconut husk. The acclimatized plants were eventually transferred to their natural habitat. These plants depicted about 60% plants survival (**Fig. 26f; 27f**).

Table 20: Effects of different concentration and combination of PGRs on multiple shoot formation with shoot length of *R. retusa*.

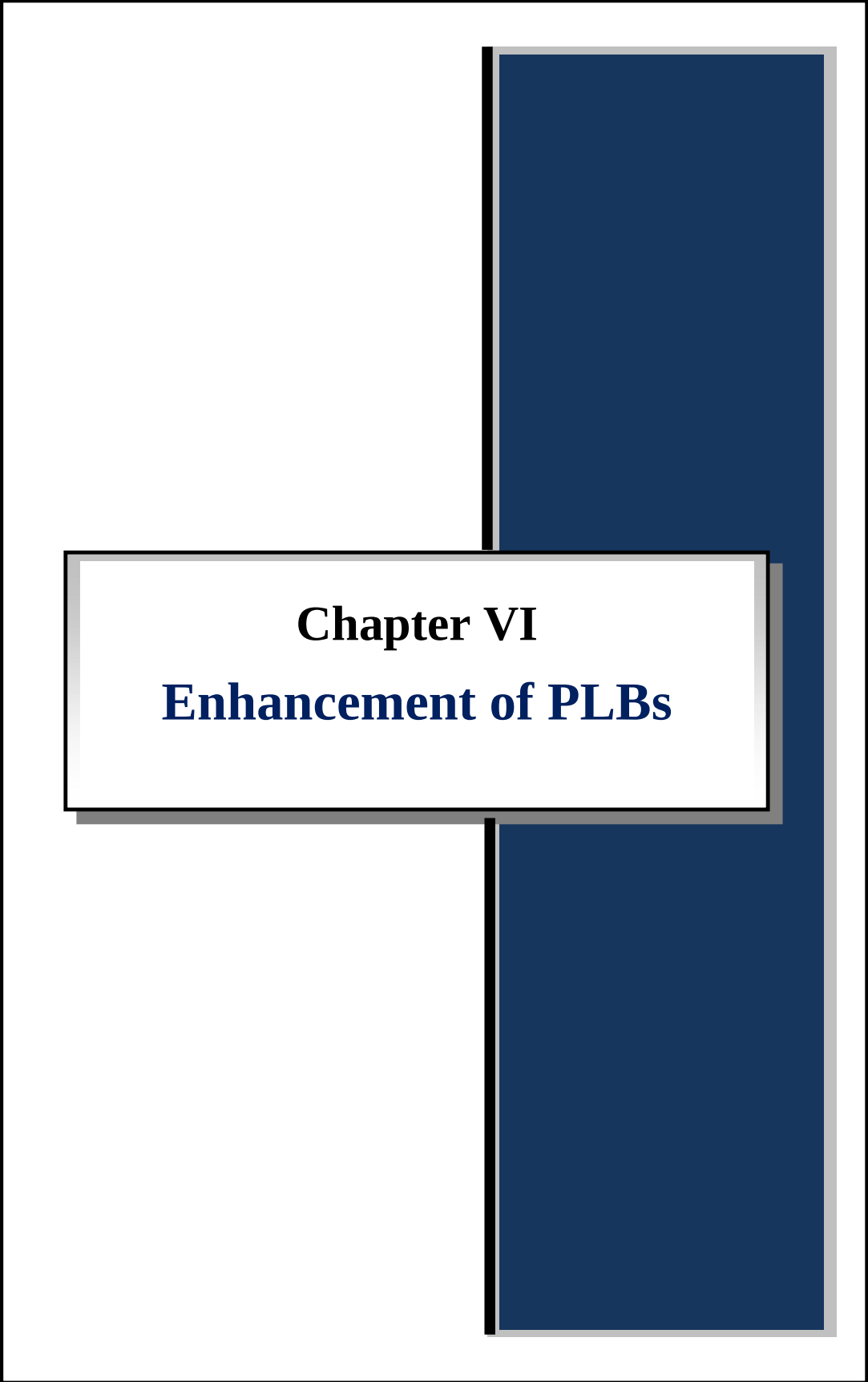
PGRs	Conc. of PGRs (mg l ⁻¹)	Average multiple shoots (No. explants)	Average length of shoots (cm)
BAP + NAA	0.5 + 0.5	2.80 ± 0.37 ^a	2.00 ± 0.31 ^{abc}
	1.0 + 0.5	4.80 ± 0.49 ^b	2.40 ± 0.51 ^{abc}
	1.5 + 1.0	10.20 ± 0.58 ^d	3.80 ± 0.37 ^d
	2.0 + 1.5	6.00 ± 0.55 ^{bc}	1.40 ± 0.24 ^a
BAP + Kinetin	0.5 + 0.5	2.60 ± 0.39 ^a	1.60 ± 0.40 ^a
	1.0 + 0.5	6.20 ± 0.73 ^c	2.60 ± 0.24 ^{bc}
	1.5 + 1.0	5.00 ± 0.32 ^{bc}	2.20 ± 0.37 ^{abc}
	2.0 + 1.5	1.80 ± 0.37 ^a	1.40 ± 0.24 ^a
BAP + IAA	0.5 + 0.5	2.00 ± 0.31 ^a	1.40 ± 0.40 ^a
	1.0 + 0.5	5.40 ± 0.51 ^{bc}	2.40 ± 0.51 ^{abc}
	1.5 + 1.0	6.20 ± 0.58 ^c	3.00 ± 0.44 ^{cd}
	2.0 + 1.5	2.80 ± 0.37 ^a	1.60 ± 0.24 ^a

Values represent mean $\pm$ S.E. (Standard Error). Each treatment was repeated thrice. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$.

In present study we developed a simple and efficient protocol for induction of direct somatic embryogenesis of *R. retusa* and successfully obtained plantlets from regenerated embryos. Regeneration system through direct somatic embryogenesis is suitable for further studying on physiology and morphology status of embryo development, mass propagation, genetic transformation and advance biotechnological work (Chung et al., 2007).

5.2.4 Conclusion

The technique standardized may be useful in the re-establishment of *Rhynchosylis retusa* in its natural habitat and for large scale multiplication for other commercial uses. The technique is efficient, quick and highly reproducible and can be used in advance biotechnological research not only for this specie but also for the micropropagation of other orchids.



Chapter VI

Enhancement of PLBs

6.1 Development of an Efficient Protocol for *In vitro* Germination and Enhancing Protocorm Like Bodies (PLBs) Development in Three Indigenous Orchid Species in Bangladesh

6.1.1 Introduction

Orchidaceae is the largest and most diverse family of flowering plants (Freudenstein and Rasmussen, 1997; Hajra, 2001; Singh et al., 2007; Bektas et al., 2013). Many orchid species have floricultural importance because of their attractive and long-lived flowers. Due to their popularity, orchids now occupy the top position among all flowering plants for cut flower production and as potted ornamentals. For this study three important orchid species were selected for this study for their beautiful flower and medicinal properties. *Acampe premorsa* is one of the most important epiphytic orchids; valued for its medicinal properties and beautiful flowers and to date there have been no reports of *in vitro* propagation of this orchid (Nayak et al., 1997). *Agrostophyllum khasianum* is an epiphytic but rarely found in lithophytic habitats, with a height of around 23-31 cm, flowers 4-5 mm across; sepals, petals and lip are cream-white, with patches of pale yellow on the mid-lobe of the lip (Yonzon et al., 2012). *Phalaenopsis cornorerris* is a monopodial epiphytic orchid and valued for its cut flowers and even more as a potted plant. Its flowers are large, colorful and durable and they are the most popular in the horticultural industry, though it can be difficult to propagate vegetatively (Košir et al., 2004). Unfortunately, many orchids are threatened with extinction because of environmental degradation, human occupation of natural habitats, and overexploitation for horticultural and ethnobotanical use (Coates and Dixon, 2007). Habitat destruction due to road construction, fire, urbanization, drainage, and other anthropogenic influences can also directly affect these plants (Cribb et al., 2003). 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). Careless

collection of these species has led to serious genetic and ecological erosion; many have already been listed as endangered species (Clements, 2003; Machaka-Houri et al., 2012). However, research into the production of unique and improved orchids may help create a competitive national and international floriculture market. *In vitro* propagation of orchids has always been an attractive prospect but a challenge since their nutrient requirements have not been fully understood (Tee et al., 2010; Bektas et al., 2013; Bustam et al., 2013; Poobathy et al., 2013). Orchid seeds do not possess endosperm, limiting germination in nature; although a very large number of seeds are produced, only 0.2 - 0.3% germinate (Singh, 1992). *In vitro* culture techniques have been developed for some important orchid species in Bangladesh by Bhadra and Hossain (2003). Saiprasad and Polisetty (2003) argued that *in vivo* vegetative propagation is undesirable due to the heterozygosity of seed, minute seed size and the presence of reduced endosperm. This necessitates the application of an *in vitro* seed propagation technique for orchid conservation (Stenberg and Kane, 1998; Gangaprasad et al., 1999; Subramaniam et al., 2011; Danial et al., 2013). Orchids can be propagated both asexually and sexually, but traditional asexual propagation is extremely slow and can give rise to only 2-4 plants per year (Nasiruddin et al., 2003). *In vitro* seed germination has been suggested as a suitable propagation method by several authors (Arditti et al., 1982; Bhattacharjee et al., 2014; Julkiflee et al., 2014). The effects of various basal media with different growth regulators on seed germination, development of protocorms and subsequent plantlet formation were studied in three important orchid species of Bangladesh extensively and within a short space of time. The present investigation on three indigenous orchid species was carried out with the following objectives: to standardize media for *in vitro* germination and protocorm regeneration from seeds; to accomplish seedling growth and development; and to achieve *ex vitro* establishment of plantlets for commercial use and proper conservation.

6.1.2 Materials and Methods

6.1.2.1 Plant materials

Capsules of *Acampe premorsa*, *Agrostophyllum khasianum* and *Phalaenopsis cornorerris* were collected from naturally grown mature plants found on trees in the hilly areas of Chittagong, Bangladesh, brought to the laboratory and stored at 4°C for 2 days. These capsules were used as seed sources for this study.

6.1.2.2 Sterilization of capsule and inoculation

Mature capsules of *A. premorsa*, *A. khasianum* and *P. cornorerris* were scrubbed with a soft brush and washed under running tap water with detergent. The washed capsules were treated with 0.2% (w/v) HgCl_2 solution for 10 min., dipped in 70% ethanol for 10-12 seconds, and finally washed with sterile distilled water. Sterilized capsules were dissected longitudinally using a sterile surgical blade, immature seeds scooped out, and around 200 mg of the aggregated seeds were inoculated in culture bottles containing 0.8% agar-solidified $\frac{1}{2}$ MS (Murashige and Skoog, 1962), PM (PhytamaxTM, Sigma, USA) and MVW (Modified Vacin and Went, 1949) basal medium to determine the most appropriate medium for seed germination and development. The basal media (MS and MVW) enriched with 3% (w/v) sucrose and the PM was enriched with 2% (w/v) sucrose. The pH of the media was adjusted to between 5.6 and 5.8 prior to autoclaving at 121°C for 15 minutes at 1.9 g/cm² pressure. Sterile petri dishes (90 mm) were used for seed inoculation with 25-50 mg per petri dish and maintained in a culture room with 16/8h light/dark cycle at 25 ± 2°C. Different types of plant growth regulators, namely 6-benzyl amino purine (BAP), α -naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D) and picloram were supplemented in different concentrations and combinations to study their effect on protocorm regeneration and seedling growth *in vitro*. Three basal media supplemented with different combinations of plant growth regulators (PGRs) were designated T₁ = MS + BAP (1.0 mg l⁻¹) + NAA (0.5 mg l⁻¹), T₂ = PM + BAP (1.0 mg l⁻¹) + NAA (0.5 mg l⁻¹) and T₃ = MVW + BAP (1.0 mg l⁻¹) + NAA (0.5 mg l⁻¹). Of these, T₃ was used for protocorm regeneration. The formation of globular structures as well as the

subsequent protocorm development rate (days) was recorded to analyze different stages of development, leading to seedling formation. After seedling formation, they were sub-cultured on MS medium supplemented with various phytohormones as previously described. Further sub cultures were conducted at 20-25 day intervals. In order to achieve rapid growth of the germinated seedlings, they were transferred to elongation medium. Twelve different concentrations of PGRs, namely BAP (0.5, 1.0, 2.0 mg l⁻¹), NAA (0.1, 0.5 mg l⁻¹), picloram (0.1, 0.5 mg l⁻¹) and 2, 4-D (1.0, 1.5 mg l⁻¹), 3% (w/v) sucrose and 0.8% (w/v) agar solidifying agent, were added to MS medium to assess the potentiality for seedling growth. The 2-4 cm tall elongated seedlings were transferred to three kinds of 0.8% (w/v) agar-solidified half strength or full MS medium with or without hormones, viz. M₁ = MS + 3% (w/v) sucrose + 0.5 mg l⁻¹ IAA, M₂ = ½MS + 1.5% (w/v) sucrose and M₃ = MS + 3% (w/v) sucrose + 1.0 mg l⁻¹ IAA. After development of strong and stout root systems plantlets were removed from culture vessels and rinsed thoroughly with luke-warm sterile water to remove agar and nutrients sticking to them and treated with 0.01% fungicide solution for 15-20 minutes. The plants were transferred to clay pots containing small (5-10 g) brick pieces, charcoal pieces and moss (1:1:1). The transplanted plants were finally kept in the shade house for further acclimatization. About 65-80% of in vitro grown seedlings were successfully grown in pots.

6.1.2.3 Statistical analysis

Data were recorded on the basis of different parameters subjected to analysis of variance (ANOVA) and mean values of treatments were compared by least significant difference (LSD). Calculations were done at the confidence level of P < 0.05 (Gomez and Gomez, 1976) and using SPSS (version 16.0) for Windows 7.

6.1.3 Results

The seeds of the three orchids species were aseptically grown on 0.8% (w/v) agar solidified viz. MS, PM and MVW. *A. premorsa* and *A. khasianum* germinated on all the media but *P. cornorerris* germinated only on PM (**Table 21**). In the case of *A. premorsa*, PM appeared to be better than MS or MVW because the time required for germination on

PM medium was less (33.60 ± 0.29 days) and the percentage of culture vessels with germinating seeds (96.80 %) was higher. The seeds of *A. khasianum* took less time (49 days) for germination on MS and MVW than on PM (52 days) medium. The seeds of *P. cornorerris* germinated only on PM medium and took 58.80 days. The seeds started swelling within 4-5 weeks which indicating the successful germination and subsequently protocorm formation (**Fig. 30, 31 and 32**). PM + BAP (1.0 mg l^{-1}) and NAA (0.5 mg l^{-1}) were the most efficient for the protocorms to directly differentiate into seedlings. Roots formed in a relatively short time of only 7-8 weeks (**Table 22**).

Table 21: Germination index of three orchid varieties.

<i>P. cornorerris</i>	<i>A.</i>	<i>A.</i>	Variety			
	<i>khasianum</i>	<i>premorsa</i>	RG (days)			
	45-59	25-38				
98.20 ± 0.28	38-52	25-38	GS (%)			
45.60 ± 0.77	85.40 ± 0.77	96.80 ± 0.56	(days)	TMinG	PM	GS (%)
49.60 ± 0.65	42.00 ± 0.37	25.00 ± 0.58		TRG (50%)		
58.80 ± 0.59	47.20 ± 0.50	30.2 ± 0.74		TMaxG		
	52.00 ± 0.37	33.60 ± 0.29				
-	97.60 ± 0.43	86.00 ± 0.61				
-	38.40 ± 0.91	32.80 ± 0.67	(days)	TMinG	MS	GS (%)
-	44.80 ± 0.59	34.60 ± 0.77		TRG (50%)		
-	49.00 ± 0.75	37.40 ± 0.39		TMaxG		
	97.20 ± 0.50	90.20 ± 1.02				
-	38.20 ± 0.59	29.60 ± 0.67	(days)	TMinG	MVW	GS (%)
-	43.40 ± 0.77	31.40 ± 0.92		TRG (50%)		
-	48.20 ± 0.38	35.20 ± 0.67		TMaxG		

PM = Phytamax medium; MS = Murashige and Skoog (1962) medium; MVW = Modified Vacein and Went (1949) medium. RG = Germination ranges; GS =Percentage of germinated seeds; TminG = Time to minimum germination; TRG = Time to reach germination (50%); TmaxG= Time to reach maximum germination. - = indicates no germination.

Table 22: Comparison of three culture media on protocorm development of three indigenous species of orchids.

Basal media + Plant Growth Regulator								
Species	T ₁			T ₂			T ₃	
	Spherule formation	Protocorm formation	Protocorm regeneration	Spherule formation	Protocorm formation	Protocorm regeneration	Spherule formation	Protocorm formation (days) (M ± S.E)
			(% response)			(% response)		
	Stg. 1	Stg. 2		Stg. 1	Stg. 2		Stg. 1	Stg. -2
<i>A. premorsa</i>	43.40 ± 0.62			38.20± 0.59			41.40 ± 0.92	
	49.20 ± 0.59			55.40±0.47			51.40 ± 0.79	
	71.80 ± 0.74 ^b			78.60 ± 1.07 ^b			65.40 ± 0.67 ^b	
	56.80 ± 0.56 ^b			69.60 ± 0.70 ^c			55.60 ± 0.79 ^b	
<i>A. khasianum</i>	54 ± 0.95			57.40 ± 0.65			55 ± 0.55	
	61.80 ± 0.74			66.60 ± 0.47			63.60 ± 0.98	
	64.80 ± 0.59 ^a			71.20 ± 0.46 ^a			51.20 ± 0.46 ^a	
	55.20 ± 0.82 ^a			56.60 ± 0.72 ^b			34.80 ± 0.62 ^a	
<i>P. cornorerris</i>	—			64.80 ± 0.62			—	
	—			75.40 ± 0.99			—	
	—			71.00 ± 0.75 ^a			—	
	—			49.80 ± 0.42 ^a			—	

T₁ = MS + BAP (1.0 mg l⁻¹) + NAA (0.5 mg l⁻¹),

T₂ = PM + BAP (1.0 mg l⁻¹) + NAA (0.5 mg l⁻¹) and

T₃ = MVW + BAP (1.0 mg l⁻¹) + NAA (0.5 mg l⁻¹)

Stage (1): Vegetative apex stage. Stage (2): 2-3 leaves stage, - = indicate no response

Values in a column with similar superscripts are not significantly different at p ≤ 0.05 levels.

Shoot elongation of *A. prernorsa* and *A. khasianum* took place on MS medium supplemented with 3% (w/v) sucrose + 1.0 mg l⁻¹ BAP + 0.1 mg l⁻¹ picloram than other selected combination media (Fig. 34). MS + 3% (w/v) sucrose + 1.0 mg l⁻¹ BAP + 0.1 mg l⁻¹ picloram was the best medium for growth of *A. prernorsa* and *A. khasianum* seedlings. On the other hand, *P. cornorerris* exhibited the highest elongation on MS + 3% (w/v) sucrose + 0.5 mg l⁻¹ BAP + 0.1 mg l⁻¹ NAA media (Fig. 35). Half strength MS with 0.8% (w/v) agar, and 1.5% (w/v) sucrose performed better than full strength MS basal medium both in terms of increased root length and greater number of seedlings (Fig. 30, 31 and 32).

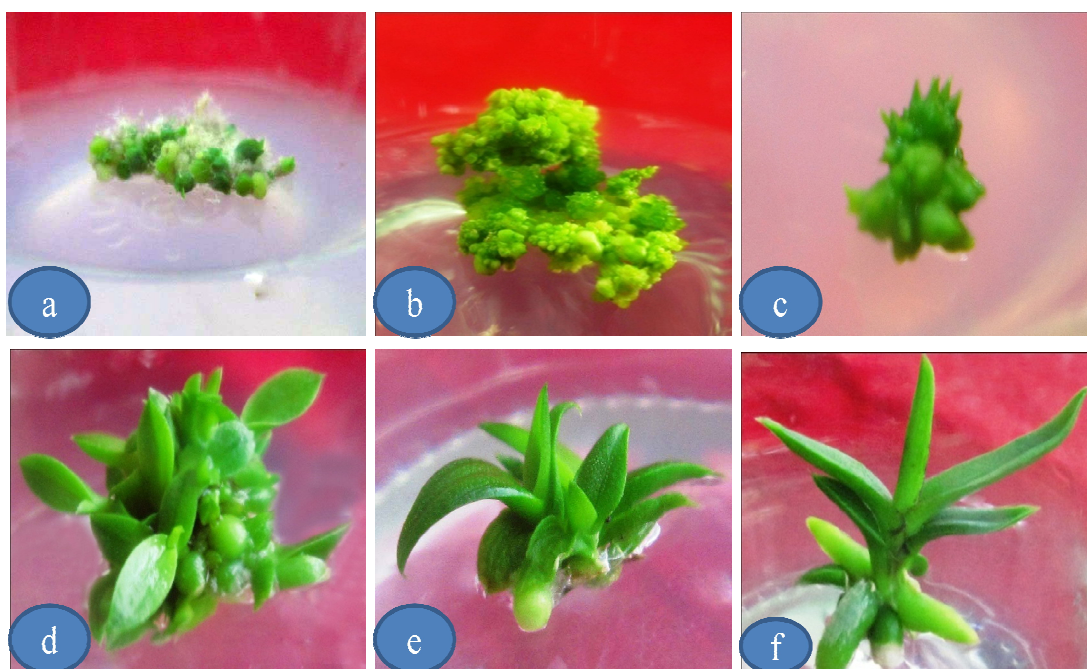


Fig. 30: Seed germination in *A. premorsa*, (a) seed culture after 4 weeks, (b) multiplication of protocorm, (c) initiation of shoot bud, (d) development of shoot and buds derived from PLBs, (e) growth of seedling, and (f) well rooted plantlet.



Fig. 31: Seed germination in *A. khasianum*; (a) seed culture after germination; (b) multiplication of protocorm; (c) shoot bud initiation; (d) protocorm developed shoot and bud; (e) seedling growth; and (f) well rooted plantlet.

Overall, $\frac{1}{2}$ MS + 1.5% (w/v) sucrose was found to be the best medium in terms of both greater numbers and lengths of roots. This result proves it to be more suitable for the establishment of a stout root system than auxin supplemented medium (**Table 23**). The plantlets with a strong root system were transferred to clay pots containing small brick pieces (5-10 g), charcoal pieces and moss (1:1:1) as potting mixture. The transplanted plants were acclimatized in the shade house: 65-80% of *in vitro* grown seedlings survived outside in pots.

6.1.4 Discussion

Three different basal media viz. PM, MS and MVW were used for *in vitro* germination of seeds of three species, *A. premorsa*, *A. khasianum* and *P. cornorerris*. of these, *P. cornorerris* germinated only on PM medium and *A. premorsa* and *A. khasianum*

germinated on all of the three media, suggesting the existence of species-medium specificity. Similar results have been observed with seeds of different orchid species (Hoque et al., 1994; Gupta and Bhadra, 1998; Gupta et al., 1998). The positive influence of basal MS medium on seed germination was also observed in *Vanda coerulea* (Murdad et al. 2010; Roy et al., 2011). Although the seeds of *A. premorsa* and *A. khasianum* germinated on all the three media, PM appeared to be better than MS and MVW (Gupta and Bhadra, 1998), further indicating the interaction between media and species in *C. aloifolium*. Seeds started swelling within 4-5 weeks indicating successful germination. The swelling was due to intake of water and nutrients and the embryos eventually underwent several divisions to produce irregularly shaped parenchymatous cell masses called spherules which emerged from the cracked seed coat. These globular spherules with hairy growth at the basal portion developed into protocorms which were achlorophyllous initially but subsequently turned green within 7-8 weeks. This phenomenon was also observed by Nongdam and Chongtham (2011) in *Cymbidium aloifolium*.

Gayatri et al. (2006) also reported that the protocorms of the terrestrial orchids of the well-drained, shady and seasonally-dry soils of open grassland are achlorophyllous. Gupta and Bhadra (1998) however reported direct seedling formation but the time required for seedling development was longer than in the present study. On the other hand, Jawan et al. (2010) reported that $\frac{1}{2}$ MS medium with 0.2% (w/v) yeast extract and 0.2% (w/v) sucrose was the best medium for the growth and development of protocorms in *Vanda dearei*.



Fig. 32: Seed germination in *P. cornorerris*; (a) seed culture forming protocorm, (b) multiplication of protocorm, (c) shoot bud initiation, (d) protocorm developed shoot and bud, (e) seedling growth, and (f) well rooted plantlet.

We noticed rapid seedling elongation of *A. prernorsa* and *A. khasianum* in MS medium supplemented with 3% (w/v) sucrose + 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} picloram whereas *P. cornorerris* showed better elongation in MS + 3% (w/v) sucrose + 0.5 mg l^{-1} BAP + 0.1 mg/l NAA media. Induction of the better number of shoots in response to a low concentration of BAP in the medium was also reported in *Cymbidium* and *Cattleya* by Nagaraju and Mani (2005) and *Vanilla planifolia* by Zuraida et al. (2013). Latha (1999) has also reported that the addition of 1.0 mg l^{-1} BAP to medium is effective in inducing a greater frequency of shoot formation in *Habenaria crinifera*. For induction of strong and stout root system, $\frac{1}{2}$ MS + 1.5% (w/v) sucrose was found to be best in terms of both a greater number and length of roots. This result proves that for establishment of stout root systems stress conditions were more suitable than auxin-supplemented medium. The present observation is collaborated by the earlier finding of Bhadra et al. (2002) and Gupta and Bhadra (1998). The results clearly demonstrated that the above mentioned culture conditions were effective for large scale seedling production of *A. premorsa*, *A. khasianum* and *P. cornorerris* and this protocol will be useful to commercial growers for mass propagation and for conservation of these orchid species.

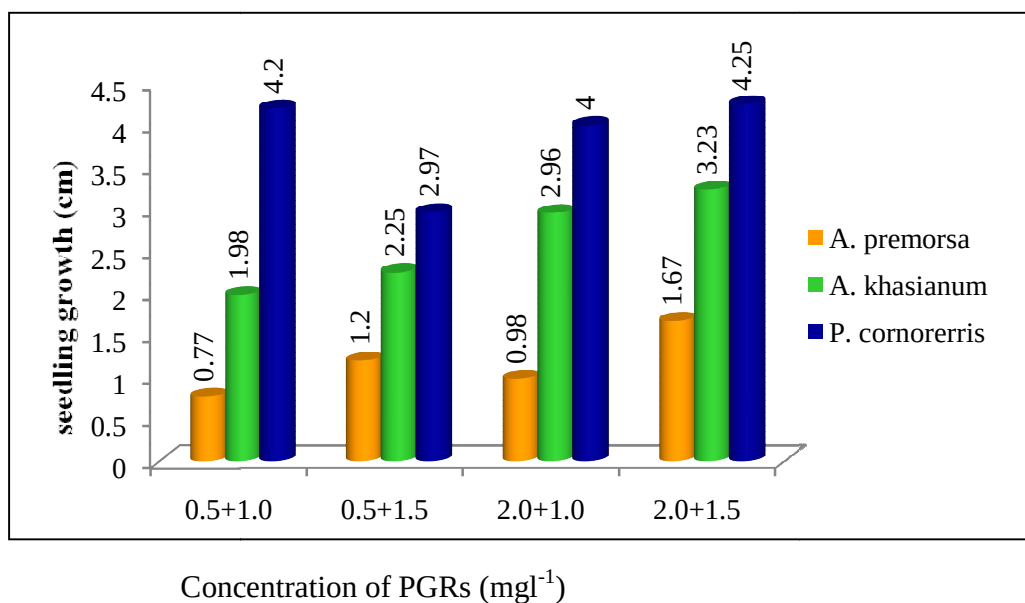


Fig. 33: The effect of BAP and 2,4-D on seedling growth of three orchid varieties.

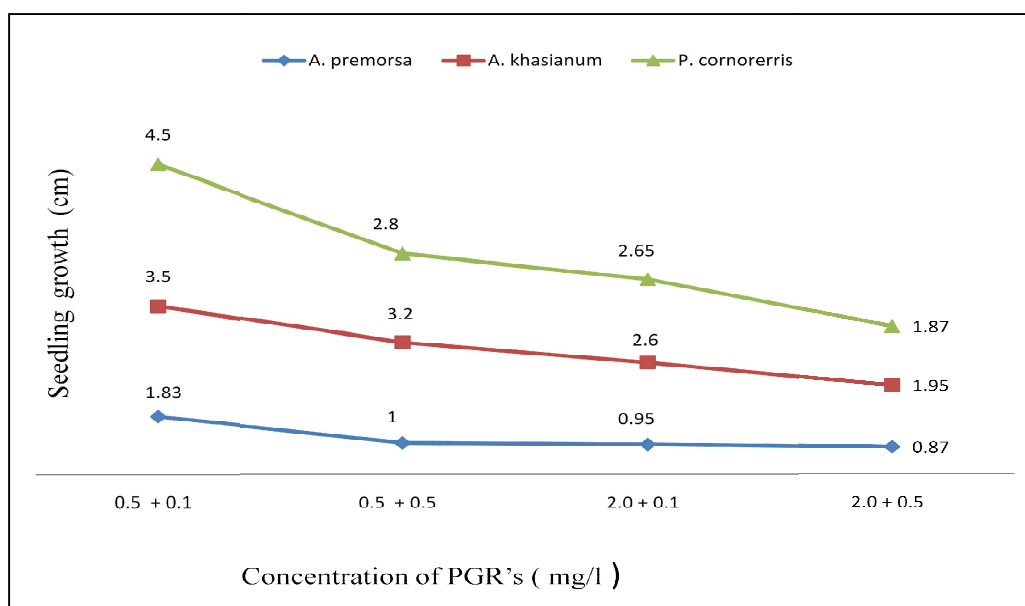


Fig. 34. The effect of BAP + picloram on seedling growth of three orchid varieties.

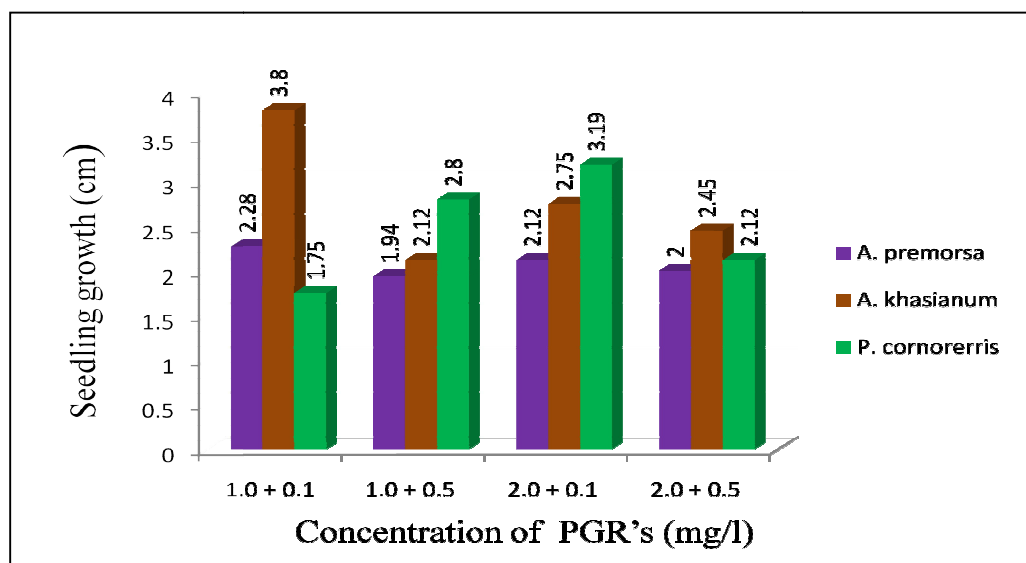


Fig . 35: The effect of BAP + NAA on seedling growth of three orchid varieties.

Table 23. Rooting and hardening in three orchid species.

Species	Medium	Initial No. of roots/seedling (Mean $\pm$ S.E)	Initial length of Roots (cm) (M $\pm$ S.E)	No. of roots/seedling (M $\pm$ S.E)	Length of roots (cm) (M $\pm$ S.E)	SR%
<i>A. premorsa</i>	M ₁	1.65 $\pm$ 0.52	0.65 $\pm$ 0.67	3.33 $\pm$ 0.57 ^{ab}	1.17 $\pm$ 0.85 ^a	80
	M ₂	1.54 $\pm$ 0.45	0.73 $\pm$ 0.39	4.5 $\pm$ 0.32 ^b	2.85 $\pm$ 0.45 ^b	
	M ₃	1.25 $\pm$ 0.57	0.45 $\pm$ 0.56	2.25 $\pm$ 0.67 ^a	0.92 $\pm$ 0.71 ^a	
<i>A. khasianum</i>	M ₁	2.52 $\pm$ 0.45	0.89 $\pm$ 0.43	5.75 $\pm$ 0.45 ^{bc}	1.5 $\pm$ 0.52 ^a	72
	M ₂	2.75 $\pm$ 0.34	0.76 $\pm$ 0.41	6.5 $\pm$ 0.62 ^c	2.89 $\pm$ 0.89 ^b	
	M ₃	2.25 $\pm$ 0.52	0.45 $\pm$ 0.57	4.2 $\pm$ 0.48 ^b	1.3 $\pm$ 0.53 ^a	
<i>P. cornorerris</i>	M ₁	4.2 $\pm$ 0.43	0.76 $\pm$ 0.45	9.5 $\pm$ 0.78 ^d	1.5 $\pm$ 0.54 ^a	65
	M ₂	3.51 $\pm$ 0.53	0.83 $\pm$ 0.48	9.67 $\pm$ 0.65 ^d	3.83 $\pm$ 0.35 ^c	
	M ₃	2.73 $\pm$ 0.62	0.72 $\pm$ 0.25	3.32 $\pm$ 0.56 ^{ab}	1.65 $\pm$ 0.5 ^a	

SR% =Percentage of survival rate of well rooted plantlet in natural condition. Each value represents an average of 10 replicates and each experiment was repeated at least thrice. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels.

6.1.5 Conclusion

The present investigation concluded that PM is the best medium for seed germination and protocorm formation from immature seeds in *A. premorsa*, *A. khasianum* and *P. cornorerris* in comparison to the results obtained on other media. PM medium supplemented with BAP (1.0 mg l^{-1}) + NAA (0.5 mg l^{-1}) was found to be the most suitable for development from protocorm regeneration to plantlet formation. This result suggests that the nutritional requirement in *in vitro* culture is species and medium-specific. Under this study we have successfully established a simple and an efficient protocol for *in vitro* seed germination, protocorm formation and mass propagation of three indigenous orchids. Using this protocol, it is possible to produce viable, uniform and healthy plants with a good survival rate that can be used for large scale cultivation. Furthermore, the protocol may facilitate conservation of these orchids from extinction in the natural population.

Chapter VII
**Antimicrobial activity and
Phytochemical screening**

7.1 Antimicrobial Activity and Phytochemical Screening of Whole Plant Extracts of *Vanda tessellata* (Roxb.) Hook. Ex. G. Don

7.1.1 Introduction

Vanda tessellata (Roxb.) Hook. Ex.G. is an epiphytic orchid, 30-60 cm high, with leafy stem. This plant has been used as indigenous medicine sources such as *Ayurveda* and local traditional medical practices (Chopra et al., 1956). In different countries plants are using for its medicinal value and an important source of many potent and powerful drugs (Srivastava et al., 1996). Medicinal plants represent a rich source of antimicrobial agents. So, a wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties (Mahesh and Satish, 2008). The roots of *Vanda tessellata* are antipyretic; useful in dyspepsia, bronchitis, inflammations, piles and hiccup. Externally the root is used in rheumatism and allied disorders and diseases of the nervous system. It is also remedy for secondary syphilis and scorpion-sting. Juice of the leaves is given in otitis and the paste as febrifuge. The roots possess significant anti-inflammatory activity. The plant contains an alkaloid, a glucoside, tannins, β - sitosterol, γ -sitosterol and a long chain aliphatic compound, fatty oils, resins and colouring matters. Roots contain tetracosyl ferrulate and β -sitosterol-D-glycoside (Ghani et al., 1990). A novel aphrodisiac compound isolated from the flowers of *Vanda tessellata* (Roxb.) Ex. Don which activates neuronal and endothelial, but not inducible (Subramoniam et al., 2013). Antibiotics are powerful medicines that fight bacterial infections and have greatly benefited the health related quality of human life since their introduction. Antibiotic resistance has become a global concern (Westh et al., 2004). One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents. Thus, drug resistance in human pathogens developed the necessity to search antimicrobial compounds as an alternative (Sandrasagaran et al., 2014). Therefore, screening of medicinal plants is vital to overcome these emerging problems

(Monica et al., 2013). On the contrary, the plant-derived antimicrobial agents are not associated with side effects and they have a prospective therapeutic benefit to heal many infectious diseases (Gulcin et al., 2004). Infectious diseases are the world's leading cause of premature deaths, killing almost 60000 people per day despite remarkable advances in medical research and treatment during the 20th century, infectious diseases remain among the leading cause of death worldwide (Agrawal et al., 2014). In the present scenario of emergence of multiple drug resistance to human pathogenic organisms, this has necessitated a search for new antimicrobial substances from other sources including plants. Phytochemical or secondary metabolites are chemical compounds formed during the plants normal metabolic processes and plants use them to protect themselves (Ning et al., 2009). Although the idea that those plant drugs are totally safe and free from the side effects, the erroneous, adverse effects of phototherapeutic agents are less common compared with synthetic drugs. The resistance that pathogens build against antibiotics and the oxidative stress caused by free radicals, has sparked interest in the search for new antibacterial and antioxidant compounds also from nature (Kokila et al., 2014). Natural crude drug extracts isolated from plant species can be prolific resources for such new drugs. Therefore, considering the medicinal properties of *Vanda tessellata* were evaluated under this study for their phytochemical analysis along with antimicrobial activities. The medicinal potential of this plant species would be validated which would help the scientific fraternity to formulate the ways to preserve this valuable endangered medicinal plant species and also will pave a way for further research on this plant species.

7.1.2 Materials and Methods

7.1.2.1 Materials

As plant materials the epiphytic orchid *Vanda tessellata* was collected from Rajshahi, Bangladesh. The whole plant parts were used as plant materials for the purpose. Solvents such as methanol, ethanol, chloroform and hexane were used of analytical grade and obtained commercially from Merck- Limited, Mumbai, India.

7.1.2.2 Preparation of extract

Different parts of *V. tessellata* were washed by running tap water and after excess water absorbing by tissue paper chopped it and make small pieces and then dried in incubator at 28°C (**Fig. 36 A, B**). Then they were coarse powdered by using a pulverizer (**Fig. 36 C**). The coarse powders were then subjected to successive extraction with organic solvents such as methanol, ethanol, chloroform and hexane by sonication method (Tiwari et al., 2011). The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitations. The extracts were then collected and distilled off on a water bath at atmospheric pressure and the last trace of the solvents was removed *in vacuo* and stored at 4°C (**Fig. 36 D**). Afterwards used it for antimicrobial activity and phytochemical analysis. To make stock solution of 10 mg/ml of each extract (crude drug) the appropriate amount is weighed and dissolved with DMSO (**Fig. 36 E**). The stock solution was passed through 0.2 µm pyrogenic filter to sterilize the solution and further concentrations of 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml was made by diluting with DMSO. Concentrations of extracts were prepared by filter paper disc method; discs with 5 mm diameter were prepared by using No. 1 Whatmann filter paper and sterilized by autoclaving. Then, the discs were impregnated with different concentrations of extracts.

7.1.2.3 Test of microorganisms

Clinical strains of bacteria isolated from patient sample were collected from the Popular Diagnostic Centre (Pvt.) Ltd. Rajshahi, Bangladesh. In the present study includes five different species of bacteria including both gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram negative (*Vibrio cholerae*, *Escherichia coli* and *Klebsiella pneumonia*) were evaluated. Cultures of these bacteria were grown in a nutrient broth (liquid) medium at 37°C for 24 hours. Pure culture of these bacteria was maintained at 4°C on nutrient agar medium. The fungal organisms which used for the present study were namely *Penicillium sp.*, *Rhizopus sp.* and *Aspergillus niger*.

7.1.2.4 Antibacterial activity

Twenty four hours old cultures of the organisms were used for observation of antibacterial activity. The nutrient agar medium plates were prepared by pouring 15 ml nutrient agar medium using 90 cm sterile petri dishes. The dishes were allowed to solidify for 5 minutes and 0.1% inoculum was inoculated. In agar disc diffusion method with 5 mm diameter were prepared using No. 1 Whatmann filter paper and sterilized by autoclaving (Bauer et al., 1966). Then the discs had been impregnated with various concentrations of the plant extracts. The plates were then incubated at 37°C for 24 hours. Ampicillin (10 mg/ml) was used as control against mentioned bacteria used under this study.

7.1.2.5 Antifungal activity

The extracts of *V. tessellata* plants were screened for antifungal activity by agar disc diffusion method (Chaitanya et al., 2013). The cultures of 48 hours old fungal culture grown on potato dextrose agar (PDA) were used for inoculation of fungal strain on PDA plates. An aliquot (0.02 ml) of inoculums was introduced to molten PDA and poured into petri dishes (90 cm). After solidification, the appropriate wells were made on agar plate by using cork borer. In agar well diffusion method different concentrations of extracts were introduced medium. Incubation period of 24 - 48 hours at 28°C was maintained for observation of antifungal activity of plant extracts. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the plant extracts. Diameter of the inhibition zones were measured with antibiotic zone scale in mm. The inhibition zones were recorded, the experiment was repeated thrice and the average values were calculated for antifungal activity. Ketoconazole (10 mg/ml) was used as control against fungi.

7.1.2.6 Phytochemical analysis

Phytochemical tests were carried out using various solvent extracts are described below. Methanol, ethanol, chloroform and hexane were subjected to routine qualitative chemical analysis to identify the nature of phytochemical constituents by standard procedures (Chandrashekar et al., 2013; Kalaiarasan et al., 2012; Harborne et al., 1998); Brindha et al., 1982).

7.1.2.7 Different phytoconstituents tested under this study

Terpenoids: 5 ml of the extract was mixed with 2 ml of chloroform and concentrated sulphuric acid to form a layer. A reddish brown coloration in the interface showed the presence of terpenoids.

Flavonoids: 5 ml of the diluted ammonia solution a portion of the aqueous extract was added, followed by addition of concentrated sulphuric acid. Appearance of yellow coloration indicates the presence of flavonoids.

Reducing sugars: 2 ml of test solution was added with a 2 ml Fehling's reagent A (or) B and 2 ml of water formation of reddish orange color indicates them presence of reducing sugar.

Phenols: Phenols are tested by adding 2 ml of ferric chloride solution to 2 ml of plant extract. Appearance of bluish green colour solution indicates the presence of phenols.

Alkaloids: 5 ml of extract and 5 ml of 2N HCl is added and boiled and then the mixture is filtered. To the filtrate a few drops of Mayer's reagent was added. A cream colour precipitate was produced immediately indicating the presence of alkaloids.

Saponins: Saponins are tested by boiling 5 ml of extracts in 10 ml of distilled water in a test tube and are shaken vigorously for about 30 seconds. The test tube is allowed to settle for half an hour. Formation of froth indicates the presence of saponins.

Tannins: Few drops of 1% lead acetate were added to 5 ml of plant extract. Formation of yellow precipitate indicates the presence of tannins.

Steroids: For testing the presence of steroids 1 ml extract was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added from the walls of the test tube. Appearance of red colour in the upper layer and yellow with green fluorescence indicates the presence of steroids.

Amino acids: 1 ml of the extract was treated with few drops of Ninhydrin reagent. Appearance of purple colour indicates the presence of amino acids.

Glycosides: 1 ml of the extract, 1 ml of alpha naphthol was added to which chloroform was added along the sides and it was looked for the development of color and the result was recorded. Development of violet color indicates the presence of glycosides.

Carbohydrates: Alcoholic solution of substance was added with 10% aqueous solution of alpha naphthali shaken and added concentrates H_2SO_4 along the side of the tube. Violet ring at the junction of two liquids shows the presence of carbohydrates.

7.1.3 Results

The antibacterial activity of different solvent (methanol, ethanol, chloroform and hexane) of whole plant extracts of *Vanda tessellata* was observed on both gram positive and gram negative bacteria. The results of antibacterial sensitivity of various solvent extracts were maximum (8.20 - 15.40 mm) with concentration of 10 mg l^{-1} chloroform extract of whole plants of *Vanda tessellata* and lowest (5.10 - 6.10 mm) with ethanol at the concentration of 1.25 mg l^{-1} . The diameter of inhibition zones for each of the samples were compared with standard antibiotic (**Fig. 37**). The chloroform extracts of *V. tessellata* exhibited highest inhibition against all the pathogenic bacteria (*E. coli*: 13.10 mm), (*V. cholera*: 8.20 mm), (*S. aureus*: 15.40 mm), (*B. subtilis*: 11.60 mm) and (*K. pneumonia*: 14.10 mm; **Fig. 36**). On the other hand, the hexane extract shows the second highest zone (8.10 - 9.60 mm) than other extract of *V. tessellata* (**Table 24**). Another experiment of antimicrobial activities the extracts (methanol, ethanol, chloroform and hexane) showed antimitotic activity against the tested fungal isolates (*Penicillium* sp., *Rhizopus* sp. and *Aspergillus niger*). The susceptibility of these fungi using *V. tessellata* whole plant extract showed significant results for most of these fungi have recently been implicated in case of immune-compromised patients who frequently develop opportunistic infections. The chloroform extract had highest activity against *Penicillium* sp., *Rhizopus* sp. and *Aspergillus niger* with inhibition zone of 17.20 mm, 16.10 mm and 16.70 mm respectively. This was followed by methanol, ethanol extract and least was observed in hexane extract (**Table 25**). The result of phytochemical activities showed that bioactive

compounds such as alkaloid, terpenoids, flavonoids, phenols, tannins, steroids and glycosides were present in the whole plant extracts of *V. tessellata*. Alkaloids and glycosides were detected in all types of *V. tessellata* extracts whereas carbohydrates, amino acids, saponins and reducing sugar were not detected in any of the plant extracts (Table 26).



Fig. 36: Procedure of extract preparation of *V. tessellata*. (A) Fresh chopped plant; (B) Dried chopped plant; (C) Coarse powdered by Pulverizor; (D) Crude extract and (E) Stock solution of extracts.

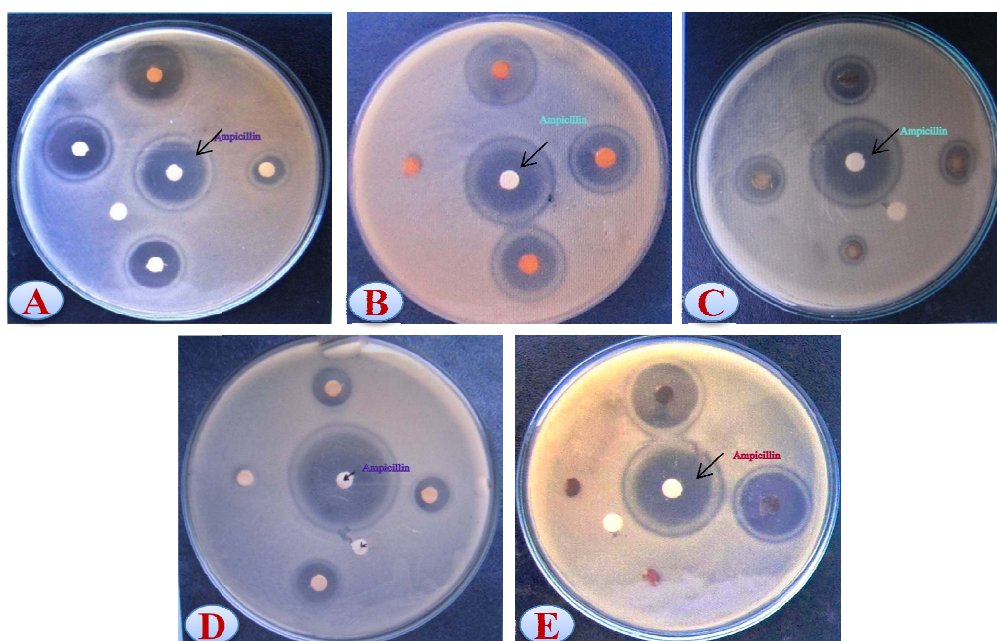


Fig. 37: Antimicrobial activities using plant extract effected of *V. tessellata*. (A) *Staphylococcus aureus*, (B) *Klebsiella pneumoniae*, (C) *Escherichia coli*, (D) *Bacillus subtilis* and (E) *Vibrio cholerae*.

Table 24: Inhibition zone of four different solvent extracts of *Vanda tessellata* against bacteria.

Solvent extracts	Conc. (mg /ml)	Inhibition zone diameter (mm $\pm$ S.E)				
		Bacteria				
		Gram positive		Gram negative		
		<i>S. aureus</i>	<i>B. subtilis</i>	<i>V. cholerae</i>	<i>E. coli</i>	<i>K. pneumonia</i>
Control	10.0	22.60 $\pm$ 0.39	20.80 $\pm$ 0.76	22.00 $\pm$ 0.76	25.20 $\pm$ 0.46	20.40 $\pm$ 0.65
Methanol	10.0	9.20 $\pm$ 0.22	9.00 $\pm$ 0.26	7.30 $\pm$ 0.36	8.00 $\pm$ 0.32	8.40 $\pm$ 0.29
	5.00	8.60 $\pm$ 0.29	8.80 $\pm$ 0.34	6.20 $\pm$ 0.35	6.40 $\pm$ 0.29	7.60 $\pm$ 0.23
	2.50	7.00 $\pm$ 0.18	7.00 $\pm$ 0.32	5.62 $\pm$ 0.18	-	5.20 $\pm$ 0.34
	1.25	-	-	-	-	-
Ethanol	10.0	7.35 $\pm$ 0.31	6.20 $\pm$ 0.31	7.10 $\pm$ 0.32	8.30 $\pm$ 0.31	6.50 $\pm$ 0.26
	5.00	6.60 $\pm$ 0.28	5.25 $\pm$ 0.16	6.30 $\pm$ 0.30	7.25 $\pm$ 0.27	-
	2.50	-	5.20 $\pm$ 0.34	5.10 $\pm$ 0.11	6.10 $\pm$ 0.21	-
	1.25	-	-	-	-	-
Chloroform	10.0	15.40 $\pm$ 0.40	11.60 $\pm$ 0.29	8.20 $\pm$ 0.28	13.10 $\pm$ 0.37	14.10 $\pm$ 0.32
	5.00	14.70 $\pm$ 0.56	11.20 $\pm$ 0.20	7.40 $\pm$ 0.14	12.20 $\pm$ 0.42	13.30 $\pm$ 0.38
	2.50	13.30 $\pm$ 0.38	5.20 $\pm$ 0.22	-	11.40 $\pm$ 0.29	12.50 $\pm$ 0.41
	1.25	12.60 $\pm$ 0.39	-	-	10.60 $\pm$ 0.39	-
Hexane	10.0	9.60 $\pm$ 0.39	9.30 $\pm$ 0.22	-	8.10 $\pm$ 0.19	8.60 $\pm$ 0.31
	5.00	7.70 $\pm$ 0.17	7.26 $\pm$ 0.16	-	6.20 $\pm$ 0.20	7.40 $\pm$ 0.21
	2.50	-	6.30 $\pm$ 0.35	-	6.00 $\pm$ 0.16	6.50 $\pm$ 0.26
	1.25	-	-	-	-	-

Control = Ampicillin (10 mg/ml), S.E = Standard error, *S. aureus* = *Staphylococcus aureus*, *B. subtilis* = *Bacillus subtilis*, *Vibrio cholerae* = *V. cholerae*, *E. coli* = *Escherichia coli*, *K. pneumonia* = *Klebsiella pneumonia*.

Table 25: Inhibition zone of four different solvent extracts of *Vanda tessellata* against three fungi.

Fungal organisms	Inhibition zone diameter in mm					
	Different solvent extract					
	Cont.1	Cont. 2	Methanol	Ethanol	Chloroform	Hexane
<i>Penicillium sp.</i>	-	25.00 ± 0.0	14.4 ± 0.25	13.5 ± 0.38	17.2 ± 0.34	14.0 ± 0.20
<i>Rhizopus sp.</i>	-	22.00 ± 0.0	14.5 ± 0.29	14.1 ± 0.32	16.1 ± 0.19	13.3 ± 0.38
<i>Aspergillus niger</i>	-	20.00 ± 0.0	15.9 ± 0.21	13.4 ± 0.36	16.7 ± 0.34	13.0 ± 0.27

Cont. 1 = Distilled water as a negative control, Cont. 2 = Ketoconazole (10 mg/ml) as a positive control, - = No result.

Table 26: Preliminary photochemical analysis of different extracts of *Vanda tessellata*.

Phytoconstituents	Solvent extracts			
	Methanol	Ethanol	Chloroform	Hexane
Terpenoids	+	+	+	-
Flavonoids	+	+	-	-
Reducing sugars	-	-	-	-
Phenols	+	-	-	-
Alkaloids	+	+	+	+
Saponins	-	-	-	-
Tannins	+	+	+	-
Steroids	+	+	+	-
Amino acids	-	-	-	-
Glycosides	+	+	+	+
Carbohydrates	-	-	-	-

+ = present, - = absent.

7.1.4 Discussion

Antimicrobial studies were conducted to investigate the antimicrobial potency of *V. tessellata* extracts against with few selected strains of bacteria and fungi are described briefly in the methods part. The present study showed that whole plant extracts of *V. tessellata* in chloroform provided a highest zone of inhibition while other three extracts were found to be less active against the tested organisms (**Table 24 and 25**). This difference is attributed to the solubility of the active component in different solvents. On the other hand, chloroform extract of *V. tessellata* leaves exhibited higher degree of inhibition against *Staphylococcus aureus* and *Escherichia coli* (11.00 mm and 11.00 mm) than *Vanda coerulea* leaves extract (2.6 mm and 4.4 mm) (Shanmuga et al., 2011). It is an indication of the broad spectrum antimicrobial potential of *V. tessellata* which make the plant a candidate for bioprospecting for antibiotic and antifungal drugs. The potential of compounds against the standard strains may be explored in order to develop therapeutics for microorganisms. The plant extracts revealed the presence of tannins, phenols, alkaloids, flavonoids, terpenoids, glycosides and steroids.

The presence of saponin, alkaloids and tannin enhanced the antimicrobial activity against the pathogenic microorganisms (Ndukwe et al., 2005). The secondary metabolites of various chemical types present in the plant species are known to possess antimicrobial activities. Flavonoids are found to be effective antimicrobial substances against a wide range of microorganisms, probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall; more lipophilic flavonoids may also disrupt microbial membrane (Tsuchiya et al., 1996). Phenolics and polyphenols present in the plants are known to be toxic to microorganisms (Mason and Wasserman, 1987). Besides, the presence of antimicrobial substances in higher plants is well established as they provided a source of inspiration for novel drug compounds as plants derived medicines have made significant contribution towards human health. Medicinal plants are important source for the development of potential new chemotherapeutic drugs and the *in vitro* antibacterial test form the basis.

7.1.5 Conclusion

Results showed that different extracts of those epiphytic orchids have good antibacterial and antifungal properties. This result reveals that chloroform extracts of *V. tessellata* shows the highest sensitivity to inhibit the growth of several bacterial and fungal species. The inhibitory effect of the extract on the growth of microorganisms could be attributed to the presence of some photochemical that were found present in the plant extract. It is an indication of the broad spectrum antimicrobial potential of *V. tessellata* which make the plant a candidate for bio-prospecting for antibiotic and antifungal drugs. The results of the study have justified the traditional use of the plants to curing the diseases. For further investigation of safe, potent and natural source of antimicrobial and phytochemical constituents of orchids and other plants, this technique and protocol will be helpful for researcher.

7.2 Assessment of Antibacterial and Antifungal Activities of the Extracts of *Rhynchosylis retusa* Blume – a Medicinal Orchid

7.2.1 Introduction

Various medicinal plants have always had important properties in the therapeutic armory of mankind (Velanganni et al., 2011). Medicinal plants would be the best source of drugs and therefore such plants should be investigated to understand better about their properties, safety and efficacy and major sources of obtaining antimicrobial drugs (Nascimento et al., 2000; Sofowora, 1986). Antibiotics are powerful medicines that fight against bacterial infections and have greatly benefited the health related quality of human life (Westh et al., 2004). One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents. Thus, drug resistance in human pathogens developed the necessity to search antimicrobial compounds as an alternative (Sandrasagaran et al., 2014). Therefore, screening of medicinal plants is very important to overcome these emerging problems (Monica et al., 2013; Bhattacharjee et al., 2015). *Rhynchosylis retusa* is an important medicinal orchid and was first described (Sibin and Gangaprasad, 2012). This plant is a monopodial, epiphytic orchid species with beautiful flowers arranged in racemose inflorescence and having a robust stem of about 25 cm long. Leaves are strap-shaped and about 25 cm long and the pendulous inflorescences are about 60 cm long which densely flowered. In Bangladesh, the plant grows naturally as epiphyte on the stem of big trees in the southern region, specially in Rajshahi, Patuakhali and Barisal districts. Due to lack of proper cultivation practices, destruction of plant habitats and illegal and indiscriminate collection of plants from natural habitats, many medicinal plants are threatened now (Bhattacharjee and Islam, 2014; Devendra et al., 2011).

The whole plant preparations of *Rhynchosylis retusa* is used to treat rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases and external inflammations (Kumar et al., 2012). It is also used as an emollient and in the

treatment of throat inflammation (Shanavaskhan et al., 2012). Leaf juice of this plant and aerial roots are used in ear pain and cleaning (Basumatary et al., 2004). *Rhynchosyilis retusa* roots are also used to cure malarial fever (Tiwari et al., 2012; Radhika and Murthy, 2013). In Kurigram district of Bangladesh, people use the leaves of this plant to cure rheumatic pain (Das et al., 2012). Juice of roots applied to cuts and wounds and dried flowers are used as insect repellent and to induce vomiting (Subedi et al., 2013). The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and disease preventive phytochemicals properties (Shakeri et al., 2012). Biologically active widespread from plant sources have always been of great interest to scientists working on infectious diseases. Over the past decade there has been an explosion of interest in the antimicrobial, particularly antibacterial and antifungal, activity of natural products (Clark and Hufford, 1993). Antimicrobial screening of plant extracts and phytochemical represents a starting point for antimicrobial drug discovery. Phytochemical studies have attracted the attention of plant scientists due to the development of new and sophisticated techniques. These techniques played a significant role in the search for additional resources of raw material for pharmaceutical industry (Shakeri et al., 2012; Mungole et al., 2010). In the present study the plants were checked for their phytochemical constituents and antimicrobial properties against some pathogenic microbes in humans. Different types of extracts using the whole plants of *R. retusa* were prepared that subjected to sensitivity analyses against some bacterial and fungal agent.

7.2.2 Materials and Methods

7.2.2.1 Materials

Healthy, disease free, epiphytic orchid *Rhynchosyilis retusa* was collected from Rajshahi, Bangladesh. Different parts from the whole plants were used as plant materials for this study. Solvents such as methanol, ethanol, chloroform and hexane were used (analytical grade) and obtained commercially from Merck- Limited, Mumbai, India as described previously.

7.2.2.2 Preparation of extracts

The whole plants of *R. retusa* were washed by running tap water and after excess water absorbing by tissue paper chopped it and make small pieces and dried by incubator at 28°C (**Fig. 38a**). Then they were coarse powdered by using a pulverizor (**Fig. 38b**). The coarse powders were then subjected to successive extraction with organic solvents such as methanol, ethanol, chloroform and hexane by sonication method (Tiwari et al., 2011). The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitations. The extracts were then collected and distilled off on a water bath at atmospheric pressure and the last trace of the solvents was removed *in vacuo* and stored at 4°C (**Fig. 38c**). Afterords used it for antimicrobial activity and phytochemical analysis.

7.2.2.3 Preparation of stock solution

To make stock solution of 10 mg/ml of each extract (crude drug) the appropriate amount was weighed and dissolved with DMSO (**Fig. 38d**). The stock solution was passed through 0.2 µm pyrogesic filter to sterilize the solution and further concentrations of 5 mg/ml and 2.5 mg/ml was made by diluting with DMSO. Concentrations of extracts were prepared by filter paper disc method; discs with 5 mm diameter were prepare by using No. 1 Whatmann filter paper and sterilized by autoclaving. Then, the discs were impregnated with different concentrations of extracts.

7.2.2.4 Phytochemical screening

To identify the constituents the phytochemical screening was carried out following standard procedure (Rani et al., 2013).

7.2.2.5 Test of different phytoconstituents

Alkaloids: 1 ml of extract was added with 2-3 drops of Mayer's reagent (dissolve 1.36 g of mercuric chloride in 60 ml of H₂O and pour into the solution of 5g of potassium iodide in 100 ml of H₂O). The appearance of cream colour precipitate or pale yellow colour precipitate indicates the presence of alkaloids.

Terpenoids (Salkowski test): 1 ml of the extract was mixed with 2 ml of chloroform and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface was formed to indicate positive results for the presence of terpenoids.

Flavonoids: 1 ml of extract was dissolved in diluted NaOH and then HCl was added. A yellow solution that turns colorless indicates the presence of flavonoids.

Tannins: 1 ml of extract was added to few drops of 1% lead acetate. A yellowish precipitate indicates the presence of tannins.

Phlobatanins: 1 ml of extract was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl solution. Red precipitate indicates the presence of phlobatanins.

Coumarins: 3 ml of 10% NaOH was added to 2 ml of aqueous extract formation of yellow colour indicates the presence of coumarins.

Steroids: 1 ml of the extract was dissolved in 10 ml of chloroform and equal volume of concentrated H_2SO_4 was added by sides of the test tube. The upper layer turns red and H_2SO_4 layer showed yellow with green fluorescence. This indicated the presence of steroids.

Phenols: Phenols are tested by adding 2 ml of ferric chloride solution to 2 ml of plant extract. Appearance of bluish green colour solution indicates the presence of phenols.

Saponins: Saponins are tested by boiling 5 ml of extracts in 10 ml of distilled water in a test tube and are shaken vigorously for about 30 seconds. The test tube is allowed to settle for half an hour. Formation of froth indicates the presence of saponins.

Amino acids: 1 ml of the extract was treated with few drops of Ninhydrin reagent. Appearance of purple colour indicates the presence of amino acids.

Glycosides: 1 ml of the extract, 1 ml of alpha naphthol was added to which chloroform was added along the sides and it was looked for the development of color and the result was recorded. Development of violet color indicates the presence of glycosides.

Carbohydrates: alcoholic solution of substance was added with 10% aqueous solution of alpha naphthol shaken and added concentrated H_2SO_4 along the side of the tube. Violet ring at the junction of two liquids shows the presence of carbohydrates.

7.2.3 Antimicrobial activity

7.2.3.1 Sample collection

Different microbes were isolated from patients sample were collected from the Popular Diagnostic Centre (Pvt.) Ltd., Rajshahi, Bangladesh.

7.2.3.2 Bacterial and fungal strains and its culture

Five different species of bacteria including both gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram negative (*Vibrio cholerae*, *Escherichia coli* and *Klebsiella pneumonia*) were evaluated. Cultures of these bacteria were grown in a nutrient broth (liquid media) at 37°C for 24 hours. Pure culture of these bacteria was maintained at 4°C on nutrient agar medium. Three fungal organisms which namely *Penicillium sp.*, *Rhizopus sp.* and *Aspergillus niger* were used under this study.

7.2.3.3 Antibacterial assay

Twenty four hours old cultures of the organisms were used for observation of antibacterial activity. The nutrient agar medium plates were prepared by pouring 15 ml nutrient agar media using 90 cm sterile petri dishes. The dishes were allowed to solidify for 5 minutes and 0.1% inoculum was inoculated. In agar disc diffusion method with 5 mm diameter were prepared using No. 1 Whatmann filter paper and sterilized by autoclaving. Then the discs had been impregnated with various concentrations of the plant extracts. The plates were then incubated at 37°C for 24 hours. Streptomycin (10 mg/ml) was used as control against mentioned bacteria used under this study.

7.2.3.4 Antifungal assay

The whole plant extracts of *R. retusa* were screened for antifungal activity by agar disc diffusion method. The cultures of 48 hours old fungal culture grown on potato dextrose

agar (PDA) were used for inoculation of fungal strain on PDA plates. An aliquot (0.02 ml) of inoculums was introduced to molten PDA and poured into petri dishes (90 cm). After solidification, the appropriate wells were made on agar plate by using cork borer. In agar well diffusion method different concentrations of extracts were introduced medium. Incubation period of 24 - 48 hours at 28°C was maintained for observation of antifungal activity of plant extracts. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the plant extracts. Diameter of the inhibition zones were measured with antibiotic zone scale in mm. The inhibition zones were recorded, the experiment was repeated thrice and the average values were calculated for antifungal activity. Nystatin (10 mg/ml) was used as control against fungi.

7.2.4 Results

The phytochemical analysis revealed the presence of secondary metabolites like alkaloids, terpenoids, flavonoids, tannins, coumerins, steroids, amino acids and carbohydrates were present in trace amount in the whole plant extract of *R. retusa* except phlobatanins, phenols, saponins and glycosides (**Table 27**). The antibacterial activity of different solvent (methanol, ethanol, chloroform and hexane) of whole plant extracts of *R. retusa* was observed on both gram positive and gram negative bacteria. The results of antibacterial sensitivity of various solvent extracts were maximum (10.00 - 12.30 mm) with concentration of 10 mg/ml chloroform extract of whole plants of *R. retusa* and lowest (5.00 - 7.52 mm) with methanol at the concentration of 2.50 mg/ml. The diameter of inhibition zones for each of the samples were compared with standard antibiotic streptomycin (**Fig. 39**). The chloroform extracts of *R. retusa* exhibited highest inhibition against all the pathogenic bacteria (*E. coli*: 10.20 mm), (*V. cholera*: 12.30 mm), (*S. aureus*: 10.00 mm), (*B. subtilis*: 10.10 mm) and (*K. pneumonia*: 10.70 mm) (**Fig. 39**). It was interesting that anyone of pathogenic bacteria did not show any inhibition zone with hexane extract. On the other hand, all the pathogenic bacteria show inhibition zone only the concentration of 10 mg/ml ethanol extract (**Table 28**). Another experiment of antimicrobial activities the extracts (methanol, ethanol, chloroform and hexane) showed antimitotic activity against the tested fungal isolates (*Penicillium* sp., *Rhizopus* sp. and

Aspergillus niger). The whole plant extract of *R. retusa* showed significant results for most of these fungi have recently been implicated in case of immune-compromised patients who frequently develop opportunistic infections. The chloroform extract had highest activity against *Penicillium* sp., *Rhizopus* sp. and *Aspergillus niger* with inhibition zone of 15.00 mm, 14.60 mm and 12.50 mm respectively (**Table 29**).

Table 27: Preliminary phytochemical analysis of different extracts of *Rhynchosyilis retusa*.

Phytochemicals	Test methods	Observation (colure/ precipitation)	Extracts	Result
Alkaloids	Mayer's test	Creamy colour precipitate	Hexane	+
			Ethanol	+
			Chloroform	+
			Methanol	+
Terpenoids	Salkowsky test	A reddish brown coloration of the interface was formed	Hexane	+
			Ethanol	+
			Chloroform	+
			Methanol	+
Flavonoids	Alkaline reagent test	A yellow solution that turns colorless	Hexane	+
			Ethanol	+
			Chloroform	+
			Methanol	+
Tannins	Lead acetate test	A yellowish precipitate indicates the presence of tannins	Hexane	+
			Ethanol	+
			Chloroform	+
			Methanol	+
Phlobatanins	General test	No red precipitate observed	Hexane	-
			Ethanol	-
			Chloroform	-
			Methanol	-
Coumerins	General test	Formation of yellow colour	Hexane	-
			Ethanol	-
			Chloroform	-
			Methanol	+
Steroids	Libermann-Burchard's test	Greenish colour	Hexane	+
			Ethanol	+
			Chloroform	+
			Methanol	+
Phenols	Ferric chloride test	No change is observed	Hexane	-
			Ethanol	-
			Chloroform	-
			Methanol	-
Saponins	Frothing test	No change is observed	Hexane	-
			Ethanol	-
			Chloroform	-
			Methanol	-
Amino acids	Ninhydrin test	Appearance of blue colour	Hexane	-
			Ethanol	+
			Chloroform	-
			Methanol	+
Glycosides	General test	Development of violet color	Hexane	-
			Ethanol	-
			Chloroform	-
			Methanol	-
Carbohydrates	Molisch Test	Violet ring at the junction of two liquids	Hexane	+
			Ethanol	+
			Chloroform	+
			Methanol	+

+ = Presence of constituents; - = Absence of constituents.

Table 28: Inhibition zone of four different solvent extracts of *Rhynchosyilis retusa* against the tested bacteria.

Bacteria	Con. (mg/ml)	Inhibition zone diameter (mm ± S.E)				Control (10 mg/ml)
		Methanol extracts	Chloroform extracts	Ethanol extracts	Hexane extracts	
Gram-positive						
<i>S. aureus</i>	10.0	8.40 ± 0.51	10.00±0.31	6.26±0.76	-	22.20±0.37
	5.00	7.20± 0.66	8.40±0.92	-	-	
	2.50	5.60± 0.81	6.40±0.62	-	-	
<i>B. subtilis</i>	10.0	8.80± 0.86	10.10±0.24	5.40±0.87	-	21.20±0.58
	5.00	6.60±0.82	8.20±0.86	-	-	
	2.50	5.40±0.67	6.10±0.67	-	-	
Gram-negative						
<i>V. cholerae</i>	10.0	10.60±0.60	12.30±0.49	6.00±0.54	-	25.00±0.77
	5.00	7.60±0.75	10.30±0.62	-	-	
	2.50	5.40±0.60	8.20±0.58	-	-	
<i>E. coli</i>	10.0	9.00±0.83	10.20±0.48	6.96±0.16	-	22.60±0.74
	5.00	8.20±0.58	8.30±0.70	-	-	
	2.50	5.00±0.80	6.30±0.48	-	-	
<i>K. pneumonia</i>	10.0	10.40±0.74	10.70±0.76	9.40±0.74	-	24.60±0.40
	5.00	8.60±0.40	7.10±0.40	-	-	
	2.50	7.52±0.28	-	-	-	

Control = Streptomycin (10 mg/ml), S.E = Standard error, *S. aureus* = *Staphylococcus aureus*, *B. subtilis* = *Bacillus subtilis*, *V. cholerae* = *Vibrio cholerae*, *E. coli*= *Escherichia coli* and *K. pneumonia* = *Klebsiella pneumonia*.

Table 29: Inhibition zone of four different solvent extracts of *R. retusa* against 3 fungi.

Treatments (Extracts/solvents)	Inhibition zone diameter in mm		
	Name of fungus		
	<i>Penicillium</i>	<i>Rhizopus</i>	<i>Aspergillus niger</i>
Cont. 1	-	-	-
Cont. 2	25.60 ± 0.67	22.20 ± 0.80	18.40 ± 0.68
Methanol	12.00 ± 0.63	12.60 ± 0.40	8.10 ± 0.55
Ethanol	10.40 ± 0.74	8.20 ± 0.80	8.30 ± 0.70
Chloroform	15.00 ± 0.31	14.60 ± 0.24	12.50 ± 0.94
Hexane	14.00 ± 0.31	10.40 ± 0.74	10.00 ± 0.57

Cont. 1 = Distilled water used for negative control, Cont. 2 = Nystatin (10 mg/ml) used for positive control, - = No result.

7.2.5 Discussion

The results of the phytochemical screening of *R. retusa* revealed the presence of alkaloids, terpenoids, flavonoids, tanins, coumarins, steroids, amino acids and carbohydrates in all the solvent extract (methanol, ethanol, chloroform and hexane) while coumarins were found only methanol extract and amino acid was absent in hexane and chloroform extract. On the other hand, phlobatanins, phenols, saponins and glycosides were not detected in any of the plant extracts. The presence of these secondary metabolites in *R. retusa* confirms their antibiotic properties and usefulness by the traditional medicine practitioners.

Flavonoids are also known to have a wide array of therapeutic activities as antihypertensive, anti-rheumatism, antimicrobial, diuretic and antioxidants (Burkill, 1988; Trease and Evans, 2002). Alkaloids are formed as metabolic byproducts and have

been reported to be responsible for the antibacterial activity (Doughari, 2006). Steroids are known for anti-inflammatory, lipolytic and anti-cholesteremic activities (Chawal et al., 1987). Tannins are reported to have various physiological effects like anti-irritant, antisecretolytic, antiphlogistic, antimicrobial and antiparasitic effects. Phytotherapeutically tannin-containing plants are used to treat nonspecific diarrhoea, inflammations of mouth and throat and slightly injured skins (Muthukrishnan et al., 2014).

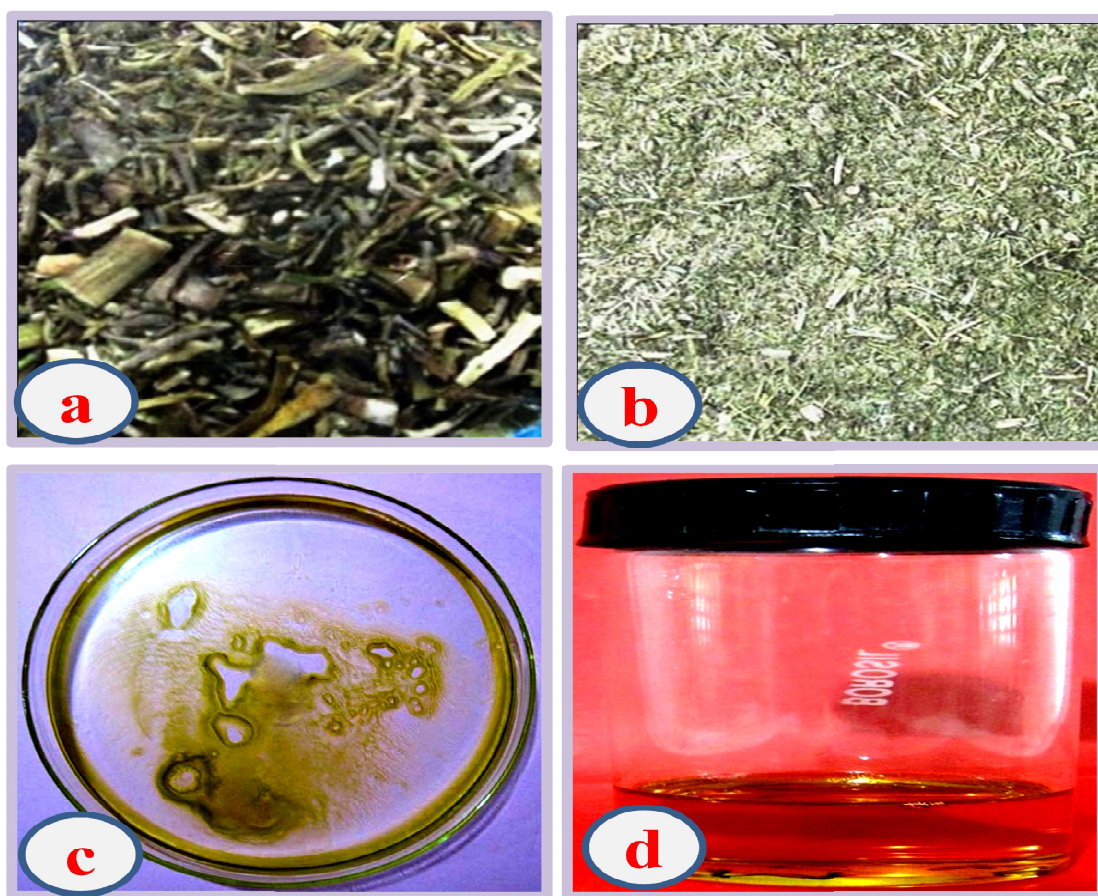


Fig. 38: Procedure of extract preparation of *R. retusa*. a) Dried chopped plant, b) Coarse powdered by Pulverizor, c) Crude extract, and d) Stock solution of extracts.

In the present investigation, the phytochemical constituents of *Rhynchosyilis retusa* was studied and further the antimicrobial activity of the plant extract was also tested against five potential clinical pathogenic bacteria namely *Staphylococcus aureus*, *Bacillus subtilis*, *Vibrio cholerae*, *Escherichia coli* and *Klebsiella pneumonia* and three fungal organism namely *Penicillium sp.*, *Rhizopus sp.* and *Aspergillus niger* at different concentrations of the plant extracts (10.0, 5.0, 2.5 mg/ml) to understand the most effective activity. The present study showed that whole plant extracts of *Rhynchosyilis retusa* in chloroform provided a highest zone of inhibition while other three extracts were found to be less active against the tested organisms. It is indicated that the plant extracts of *Rhynchosyilis retusa* contain compounds that largely inhibit the growth of gram positive and gram negative bacteria along with the fungal organism.

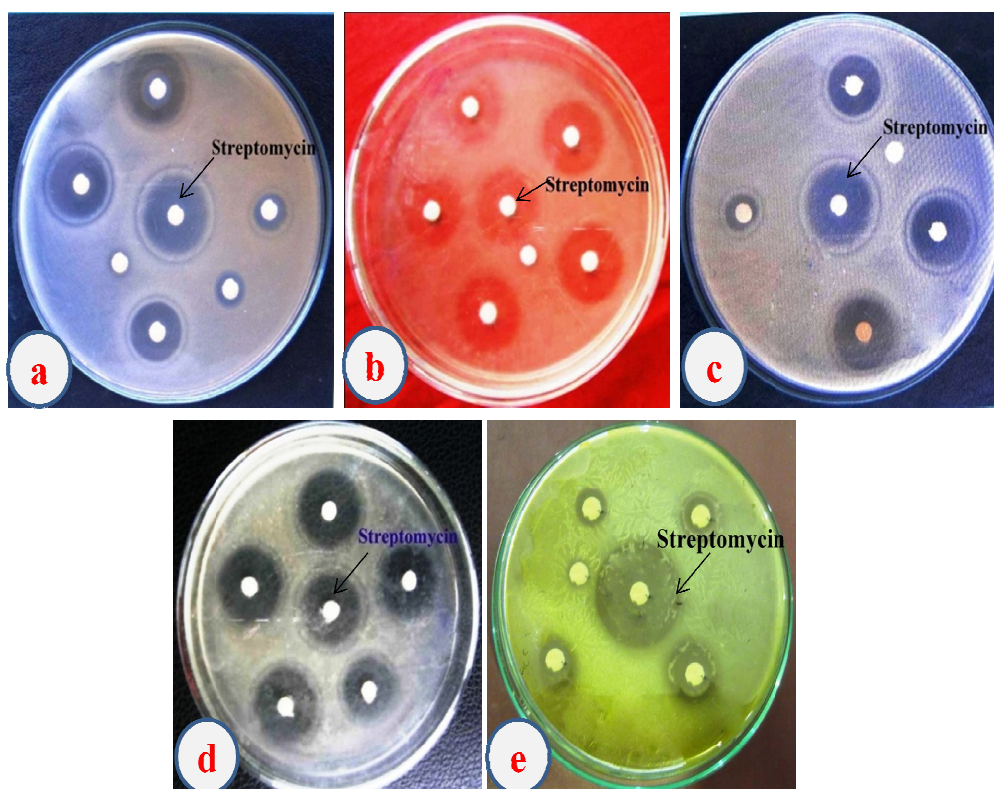


Fig. 39: Antimicrobial activities using plant extract effected of *R. retusa*. (a) *Staphylococcus aureus*, (b) *Klebsiella pneumoniae*, (c) *Escherichia coli*, (d) *Bacillus subtilis* and (e) *Vibrio cholerae*.

7.2.6 Conclusion

From the present study, it might be concluded that the chloroform extract was more active than others extract. This attest to the fact that *Rhynchosyilis retusa* contains bioactive compounds of potentially therapeutic significance and thus could be a promissory candidate for drug development and validates folkloric claim by the traditional healers.

Chapter VIII
Anther Culture

8 Anther Culture of Orchid

8.1 Introduction

The *in vitro* production of haploids through androgenesis (anther and microspore culture) is an efficient method for rapid production of fully homozygous line. Traditionally, plant breeders can achieve homozygosity by using self-fertilization, a time consuming process. By this method, complete homozygous plants can be produced within a year as compared to the long inbreeding method. Significant advantage is that these systems not only speed up the advance to homozygosity, but can also increase selection efficiency. In recent years, androgenic haploids have been produced in more than 50 genera, but major effect has been made to economically important plants such as cereals and vegetable crops (Cistué et al., 2006; Hoque and Islam, 2014; Kaushal et al., 2014). Anther culture could be as a method which can be utilized for advance biotechnological work. Moreover, it could be an approach for purification of superior genotypes or inbred lines in a species that is cross-pollinating or with a high cross pollinating rate (Wang and Campbell, 2006). Till now anther culture has proved to be an efficient and useful technique for plant breeding and biotechnological programme for various cereal crops (Islam et al., 2001; Islam, 2007; Suriyan et al., 2009; Khatun et al., 2010).

There are very few reports on anther culture in Orchids, but attempts to assess a similar competence of anther culture in monocots including Orchidaceae, have remained almost negligible because of the following reasons: (a) little success in inducing callus and maintaining proper growth, (b) difficult to obtain suitable size of homogenous tissue from monocotyledonous plants. As a result, orchid's improvement has taken a considerable interest of in different areas of biotechnology, such as optimizing the efficiency of anther and microspore culture.

8.1.1 The induction of haploids

The first successful reports on production of haploids through anther culture were done by Guha and Maheshwari (1967) in *Datura innoxia*.

8.1.2 Factors affecting haploid production

Many factors have been recognized as particularly important for successful anther and microscope culture such as growth conditions of donor plants, various pre-treatment factors, developmental stage of anther/microspores, culture media, etc. Anther and ovule culture have advantage of production of haploid plants and production of homozygous diploid lines through chromosome doubling, thus reducing the time required to produce homozygous lines. Haploids may be grouped into two broad categories: i) monohaploids (monohaploids) - which possess half the number of chromosomes from a diploid species, and ii) polyhaploids (gametophytic set) - which possess half the number of chromosomes from a polyploid species. However, the general term 'haploid' is applied to any plant originating from a sporophyte and containing half the number of chromosome (single set of chromosomes).

8.1.3 Different ways of haploids production

Haploids can result through natural parthenogenesis (development of haploid plants from unfertilized eggs) or androgenesis (development of haploid sporophytes from pollen). They can also be artificially induced through the culture of ovaries (Muren, 1989), ovules (Hansen et al., 1994), anthers (Foroughi-Wehr et al., 1982), microspores (Köhler and Wenzel, 1985; Islam, 2010b) and sexual hybridization e.g. cross between cultivated barley and the wild species (Kasha and Kao, 1970). However, anther or microspore cultures have been found to be the most efficient techniques for obtaining a large number of haploid plants (Buyser and Henry, 1980; Islam et al. 2013; Islam and Tuteja, 2012). There are many ways for the production of haploids is mentioned below:

8.1.4 Parthenogenesis

Parthenogenesis is a type of asexual reproduction. It has been possible to induce parthenogenesis by *in vitro* culture of unpollinated ovaries and ovules (Yang and Zhou, 1982). Attempts have been made to culture unfertilized ovules of ovaries in different plants species but growth of most of them stopped at the callus stages; only some crop species developed into haploid green plants, e.g. reported for *Hordeum vulgare* (San Noeum, 1976), *Triticum aestivum* (Zhu and Wu, 1979) and *Zea mays* (Ao et al., 1982). The practical application of parthenogenesis is rare because of the low frequency of haploid induction in most of the species except potato.

8.1.5 Genome elimination

Genome elimination occurs in intergeneric and interspecific crosses due to selective elimination of one of the parental genomes during the process of development after fertilization. Consequently, the embryo is formed with only one genome and the plant arising from such embryo is expected to be a haploid e.g. crosses between *Hordeum vulgare* and *H. bulbosum* results in the production of haploid *H. vulgare* (Jensen, 1977).

8.1.6 Gynogenesis

Development of haploid plants from the female gametophyte by culture of unfertilized ovules or ovaries is called gynogenesis. Since the first report of gynogenesis in barley by San Noeum (1976), this approach has been successfully applied to raise haploid plants of *Helianthus annuus*, *Beata vulgaris*, *Nicotiana tabacum*, *Oryza sativa*, *Triticum aestivum* and *Zea mays* (Yang and Zhou, 1982; Zhou et al., 1986).

8.1.7 In vitro androgenesis

The androgenic response is influenced by three genetic components: callus/embryoid induction, plant regeneration and green plant induction ability. During developing doubled haploid production into a routine technique, many problems have been solved (Lazar et al., 1984; Barnabás et al., 2000; Redha et al. 2000; Islam, 2010a). But still little information is available on the genetics of *in vitro* responses and on the combining ability

of doubled haploids in their crosses (Cseuz et al., 1990). Via androgenesis new varieties have been developed in a number of agriculture crops such as *Brassica* sp., tobacco, potato, asparagus, wheat, rice, maize, barley etc. (Bajaj, 1990). *In vitro* androgenesis is the most convenient and common technique used to produce DHs in orchids. Many factors influence androgenesis, including plant genotype, physiological state of the parent plant, pollen stage, pretreatment of flower buds and culture medium has been reported by Kumar et al. (2003). The current study was designed to test the effect of cold pretreatment, different carbon sources and culture media on androgenic response in *Vanda tessellata*.

Carbohydrates in the nutrient medium perform important functions as the main source of energy in plant tissues and provide the proper osmo-regulation. The composition of the culture media including carbohydrates and growth regulators play an important role for callus induction and regeneration in *Wedelia trilobata*, *Neoglaziovia variegata* and wheat (Keerthiga et al., 2012; Silveira et al., 2013; Asif et al., 2014). On the other hand, sucrose has been used as a major carbohydrate source in the induction medium. Sandhu et al. (1993) reported a high frequency regeneration of green plant in rice when lower concentration of sucrose was used (3% w/v) anther culture. Sucrose is often assumed to be the best choice of carbon source in tissue culture media, because it is the main sugar translocated in the phloem (Gauchan, 2012; Petrova et al., 2015; Chen, 1978). However, in some cases sucrose may not suitable for callus induction and plant regeneration and there are a number of plants that can grow using various carbohydrates. Maltose has been reported to be a superior source of carbohydrate than sucrose for androgenesis in several species, including cereals (Last and Brettel, 1990; Pande and Bhojwani, 1999; Shahnewaz and Miah, 2004). Orchid is a species for which there is no suitable method of haploid plant recovery yet (Sharma, 2012). The aim of the current research was to investigate the influence of various carbon sources, nutrient media, and stress treatments on callogenesis, embryogenesis and plant regeneration from anther cultures of orchid.

8.1.8 Problems associated with haploid induction

The genotype dependency is a critical factor affecting the variability of response in plant tissue culture. Success in anther culture is predominantly dependent on the genotype of the anther donor plant. Good tissue culture ability is equivalent to a good regeneration capacity under given culture conditions. Probably culture conditions could be optimized for each genotype, as proposed by Dunwell (1981), which is time consuming. Schaeffer et al. (1979) reported that the anther culture technique might not be efficient in all genotypes. Lazar et al. (1984) and Barnabás et al. (1989) also reported that the success of anther culture was strongly genotype dependent and it was under genetic control (Bullock et al., 1982). Hu et al. (1995) reported that out of five genotypes, one genotype gave the best culture response at all three stages, while in the other four genotypes the frequency of divided microspores was very low and no androgenic structure were formed. Other limitations of anther culture are despite the unavailable progress made in the field of anther and pollen culture the restricted application only to the members of the families, solanaceae, gramineae and cruciferae. The response of the legumes has been largely restricted to the formation of multicellular grains. Generally, about 5% of microspores in an anther undergo androgenic development (Raghavan, 1976; Sharma and Bhojwani, 1985) and a very small portion of these (androgenic structures) exhibit sustained development. Another problem often encountered in anther cultures is the emergence of plants of mixed ploidy due to the fusion of haploid nuclei at the initial stages of androgenesis or endopolyploidy (Sangwan-Norreel, 1983). Growth depression effects and the appearance of abnormalities with consecutive cycles of androgenesis have been observed in *Nicotiana sylvestris* (Paepe et al., 1983). This is accompanied by the increase in total DNA and increasing proportion of highly repeated DNA sequences (Dhillon et al., 1983).

The major problem mainly in cereal anther culture is the formation of large numbers of albino plantlets. Systematic studies of this problem should also contribute to the understanding of the genetics, physiology, and cytology of chloroplast development. Several physiological factors, such as anther culture medium, regeneration medium and high temperature can also increase albino frequency (Chuang et al., 1978; Buyser and

Henry, 1980). Molecular analysis of the chloroplast DNA (ctDNA) demonstrates the presence of deletion sometimes reaching 70% of the wheat or barley chloroplast genome in albino plants by Day and Ellis (1984). They also reported that prolonged callus culture appears to cause variants including albinos.

8.1.9 Physical treatment induced androgenesis

Some physical factors i.e. radiation, photoperiod, temperature, supply of water and nutrients growth regulators and pesticides applying to donor plants have a certain influence on the induction of androgenesis (Bajaj, 1983). It was reported that high light intensity before meiosis and low temperature, which lengthen the vegetative period, can enhance the induction of androgenesis. Different physical treatments such as cold, heat shock, drought, starvation, gamma radiation, osmotic pressure etc. have a great influence in androgenesis to produce a high number of embryos and green plantlets (Redha and Islam, 2010).

8.1.10 Cold treatment

In some cases, especially after low temperature pretreatment, symmetrically dividing microspores were found to be the main sources of androgenic haploids (Zheng and Ouyang, 1980; Islam and Tuteja, 2012; Khatun et al., 2012). Marsolais and Kasha (1983) also reported that cold pretreatment is not always necessary to obtain the higher rates of androgenetic plant. Uninucleate microspores from anther pretreated with cold (3°C) and assayed 3-5 days after inoculation had nuclei with DNA. Lazar et al. (1985) reported that variation in intensity and duration in cold pretreatment lead to significant differences in callus formation frequency. After cold treatment, analysis reveals that 5.0% of the microspores carry out DNA synthesis. Sharma (2012) observed that in case of orchid anther pretreatment has been shown to be more effective to enhance embryos development. He concluded that the most suitable culture temperature for anther culture of orchid at 4°C for 24 hours.

8.2 Materials and Methods

8.2.1 Plant Materials

Flower buds of *V. tessellata* were collected from natural grown plants at morning before 10:00 am were used as explants (anther) sources under this study (**Fig. 40a**). The developmental stages of the microspores were determined with 1% aceto-carmines (**Fig. 40f**).

8.2.2 Growth conditions of anther donor plants

Anther donor plants of *V. tessellata* were grown in natural environment in 10 cm diameter plastic pots filled with peat moss, charcoal piece and coconut husk in a pot.

8.2.3 Harvesting and pretreatment of flower bud with anthers and observation of microspore development

Cold pre-treatment retards ageing of the anther wall, allowing high proportion of microspore to change their developmental patterns from gametophytic to sporophytic. Certain pretreatment of the flower bud can have a positive effect on the development of microspores. In this case harvested flower buds were wrapped in aluminum foil and kept in a refrigerator at 4°C for up to 7 days to investigate the effect of cold pretreatment factors.

8.2.4. Surface sterilization and anther inoculation

Flower buds were surface sterilized with 70% ethanol and then with 0.1% HgCl₂ for one minute and thoroughly washed with sterilized distilled water for 4-5 times before inoculation (**Fig. 40**). After being removed aseptically, anthers from each bud were inoculated on callus induction medium. Plated anthers were incubated at 25°C ± 1°C chamber in dark for four to six weeks for callus induction and checked to record the response. For callus induction two categories medium were used. After being removed aseptically, anthers from each bud were inoculated on culture media.

8.2.5. Culture media

The culture medium is one of the important factors for the induction of androgenesis and the subsequent development of new structure and plants. In the present experiment four types of media viz. MS, $\frac{1}{2}$ MS, N₆ and B₅ were used for embryoids induction, differentiation and regeneration respectively for orchid anther culture. All media were categorized into two groups and the 1st group of media which consist of half strength of MS medium supplemented with different carbon sources such as sucrose (0-5%), maltose (0-5%) and glucose (0-5%) supplemented with BAP (10 mg l⁻¹), NAA (1.5 mg l⁻¹) and kinetin (1.0 mg l⁻¹) to evaluate the effect of carbon sources (**Table 32**). Another category which is consisted by mentioned all kinds of media (MS, $\frac{1}{2}$ MS, N₆ and B₅) supplemented with different concentration and combination of BAP (8.0 - 10 mg l⁻¹), NAA (1- 1.5 mg l⁻¹), kinetin (1.0 mg l⁻¹), 2,4-D (1.0 mg l⁻¹), peptone (2.0 mg l⁻¹) and CuSO₄.5H₂O (2.2 mg l⁻¹) (**Table 31**). As solidifying agent agar 8.0 gm/l were added in medium. The p^H of all media was adjusted to 5.8.

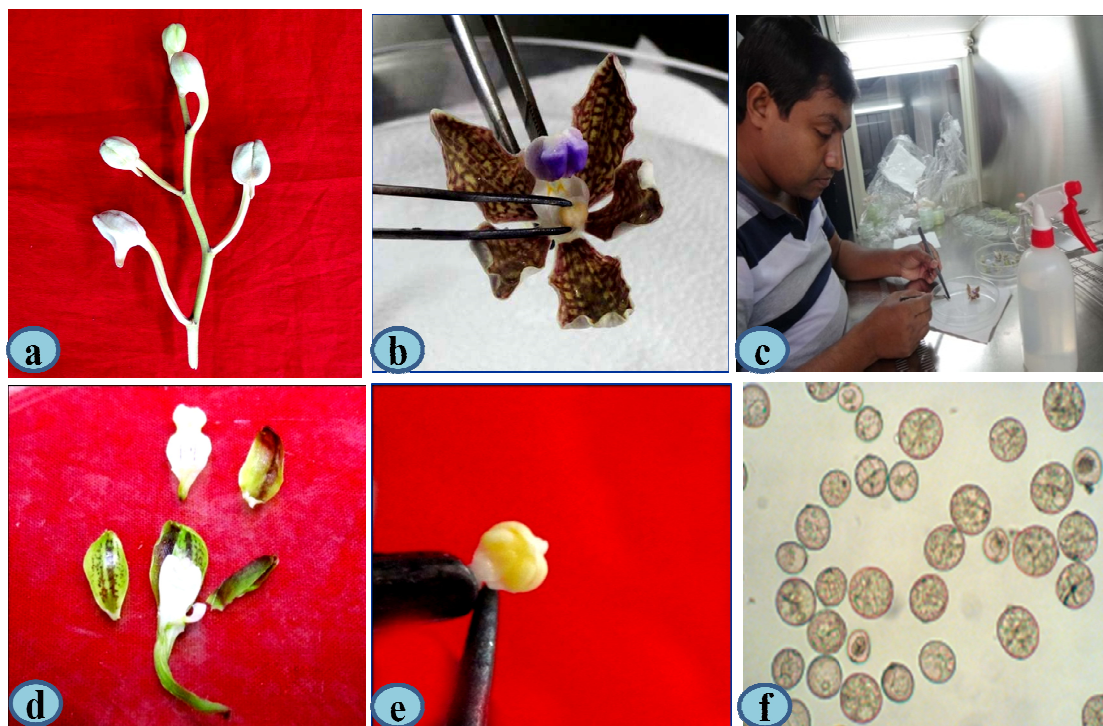


Fig. 40: Anther culture procedure from orchid flower buds: (a) young inflorescence; (b) indication of anther; (c-d) isolation of anther from bud and (e) excised anther; (f) the ideal microspore (late uni- and early binucleate) stage.

8.2.6. Media composition for anther culture of orchid

Chemicals	Media (mg l ⁻¹)		
	N ₆	MS	B ₅
Macronutrients			
NH ₄ NO ₃	-	1650.00	-
KNO ₃	2830.00	900.00	3000.00
(NH ₄) ₂ SO ₄	463.00	-	134.00
MgSO ₄ .7H ₂ O	185.00	370.00	500.00
CaCl ₂ .2H ₂ O	166.00	440.00	150.00
KH ₂ PO ₄	400	170.00	-
NaH ₂ PO ₄ .H ₂ O	-	-	150.00
Micronutrients			
KI	0.83	0.83	0.75
H ₃ BO ₃	1.60	6.20	3.00
MnSO ₄ .4H ₂ O	4.40	15.60	10.00
ZnSO ₄ .7H ₂ O	1.50	8.60	2.00
Na ₂ MO ₄ .2H ₂ O	-	0.25	0.25
CuSO ₄ .6H ₂ O	-	0.025	0.025
CoCl ₂ .6H ₂ O	-	0.025	0.025
Iron source			
Na ₂ EDTA	37.30	37.30	37.30
FeSO ₄ .7H ₂ O	27.80	27.80	27.80
Vitamins			
Myo-inositol	100.00	100.00	100.00
Nicotinic acid	1.00	5.00	1.00
Pyridoxine HCl	1.00	0.50	1.00
Thiamin HCl	1.00	0.50	1.00
Glycine	-	2.00	10.00

8.2.7 Steps of anther culture procedure in orchid

- Collected of flower buds : Flower buds were collected with anthers in different stages of development in the morning before 10 am and the microspores at the early to mid uninucleate stage (observed using 1% aceto-carmin under microscope).
- Cold treatment : Collected flower buds were wrapped in aluminum foil and kept in a refrigerator at 4°C for 1-7 days.
- Sterilization of flower buds : Flower buds were sterilized with 0.1% HgCl₂ for one minute in laminar air flow cabinet, then thoroughly washed with sterilized distilled water for 5-6 times.
- Inoculation of anthers : Anthers were removed by a fine forcep and incubated at 25°±1°C in dark chamber for 4-6 weeks for embryo formation.
- Transfer of callus : Callus was picked up from petri dishes and placed them to semi-solid ½MS medium and placed them in a growth chamber of 16/8h light/dark regimes for shoot regeneration.

8.2.8 Acclimatization of the plantlet

When the plantlets reached around 3-4 cm in length with sufficient root system then they were taken out from the culture bottles. Medium attached to the roots were gently washed out with running tap water. The plantlets were then transplanted to pots containing the peat moss, small bricks particles, coconut husk and charcoal pieces (1:1:1:1) mixture. After transplantation, the plants along with the pots were immediately covered with polythene bag to prevent desiccation. The bags were removed after 4 weeks and small plants in the pots were transferred from 90% shade to the sunlight.

8.2.9 Data collection and statistical analysis

Data on callus induction and the percentage of plant regeneration were subjected to analysis of variance (ANOVA) and mean values were evaluated at $p < 0.05$ level of significance using Duncans Multiple Range Test (DMRT).

8.3 Results

8.3.1 Effect of cold pretreatment on the callus formation from anthers

The effect of cold pretreatment evaluated by their response on anther culture is shown in **Table 30**. Thus maintaining anthers for periods ranging from 1-7 days at 4°C prior to culture stimulates anther culture responses in *V. tessellata*. The experimental result of callus induction shows that callus formation rate of *V. tessellata* increased after anthers pre-treated at 4°C condition for 3 days. The highest callus induction was found in 3-days induction period and the percentage of anthers response and callus induction was 23.63 and 10.63, respectively (**Table 30**).

Table 30: Effect of cold pretreatment (4°C) on callus induction and regeneration of anther culture of *V. tessellata*.

Duration (days)	Response of inoculated anthers (%) Mean $\pm$ SE	Callus formation (%) Mean $\pm$ SE	Regeneration (%) Mean $\pm$ SE	Survival rate (%) Mean $\pm$ SE
Cont.	3.20 $\pm$ 0.22	2.25 $\pm$ 0.25	2.00 $\pm$ 0.42	10.45 $\pm$ 0.35
1	7.25 $\pm$ 0.69	5.30 $\pm$ 0.72	4.45 $\pm$ 0.38	15.75 $\pm$ 0.22
2	15.38 $\pm$ 0.23	8.50 $\pm$ 0.41	9.38 $\pm$ 0.92	40.75 $\pm$ 0.34
3	23.63 $\pm$ 0.34	10.63 $\pm$ 0.15	11.38 $\pm$ 0.58	60.88 $\pm$ 0.26
4	11.25 $\pm$ 0.67	9.34 $\pm$ 0.70	8.75 $\pm$ 0.50	50.25 $\pm$ 0.79
5	10.90 $\pm$ 0.39	5.75 $\pm$ 0.28	8.50 $\pm$ 0.15	45.38 $\pm$ 0.98
6	5.60 $\pm$ 0.72	3.25 $\pm$ 0.56	4.50 $\pm$ 0.75	15.63 $\pm$ 0.52
7	2.65 $\pm$ 0.55	2.12 $\pm$ 0.62	2.42 $\pm$ 0.25	5.62 $\pm$ 0.56

Cont. = No cold pre-treatment, '–' = indicate no response.

It was observed that the efficiency of green plantlet regeneration and survival rate was 11.38 and 60.88, respectively (**Fig. 41 d,e**). In all treatments, there was significant increase in callus induction and regeneration (**Table 30**). We found that the pretreated anthers at 4°C for 3 days, frequency of callus induction, green plantlet regeneration and survival rate was better compared to all other treatments.

Table 31: Effects of different medium composition on embryo induction and regeneration in *V. tessellata* by anther culture.

No.	Media, PGRs and Carbon sources	% of anthers responded	Frequency of embryo induction (%)	Frequency of embryo regeneration (%)
i.	MS + 10 mg l ⁻¹ BAP + 1 mg l ⁻¹ NAA + 2 mg l ⁻¹ pepton + 3% (w/v) sucrose	-	-	-
ii.	MS + 2.2 mg l ⁻¹ CuSO ₄ .5H ₂ O + 10 mg l ⁻¹ BAP + 1.0 mg l ⁻¹ NAA + 1.0 mg l ⁻¹ Kin. + 1.0 mg l ⁻¹ 2,4-D + 2 mg l ⁻¹ pepton + 3% (w/v) sucrose.	3.88 ± 0.75	8.75 ± 0.88	10.38 ± 0.92
iii.	½ MS + 2.2 mg l ⁻¹ CuSO ₄ .5H ₂ O + 1.0 mg l ⁻¹ BAP + 1.0 mg l ⁻¹ NAA + 2 mg l ⁻¹ pepton + 3% (w/v) sucrose.	5.25 ± 0.54	10.34 ± 0.38	50.38 ± 0.58
iv.	½ MS + 2.2 mg l ⁻¹ CuSO ₄ .5H ₂ O + 10 mg l ⁻¹ BAP + 1.5 mg l ⁻¹ NAA + 2 mg l ⁻¹ pepton + 3% (w/v) sucrose.	10.00 ± 0.46	15.25 ± 0.45	75.25 ± 0.50
v.	½ MS + 8 mg l ⁻¹ BAP + 1.0 mg l ⁻¹ NAA + 1.0 mg l ⁻¹ Kin + 3% (w/v) sucrose.	4.20 ± 0.25	9.90 ± 0.22	45.50 ± 0.15
vi.	N ₆ + 10 mg l ⁻¹ BAP + 1.5 mg l ⁻¹ NAA + 1.0 mg l ⁻¹ Kin + 3% (w/v) sucrose.	-	-	-
vii.	N ₆ + 8 mg l ⁻¹ BAP + 1.0 mg l ⁻¹ NAA + 1.0 mg l ⁻¹ Kin + 3% (w/v) sucrose.	-	-	-
viii.	B ₅ + 10 mg l ⁻¹ BAP + 1.0 mg l ⁻¹ NAA + 2 mg l ⁻¹ pepton + 3% (w/v) sucrose	-	-	-

‘ - ’ = indicate no response.

8.3.2. Effects of the media

Four types of media such as MS, $\frac{1}{2}$ MS, N₆ and B₅ were used that supplemented with different concentration and combination of BAP (8-10 mg l⁻¹), NAA (1-1.5 mg l⁻¹), kinetin (1.0 mg l⁻¹), 2,4-D (1.0 mg l⁻¹), peptone (2.0 mg l⁻¹) and CuSO₄.5H₂O (2.2 mg l⁻¹) to evaluate then embryo induction and regeneration frequency. Among the eight media composition $\frac{1}{2}$ MS + 2.2 mg l⁻¹ CuSO₄.5H₂O + 10 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA + 2 mg l⁻¹ pepton + 3% (w/v) sucrose had a higher induction rate (15.25%) and maximum regeneration efficiency (75.25 %) followed by (10.34 % and 50.38% respectively) in $\frac{1}{2}$ MS + 2.2 mg l⁻¹ CuSO₄.5H₂O + 1.0 mg l⁻¹ BAP + 1.0 mg l⁻¹ NAA + 2 mg l⁻¹ pepton + 3% (w/v) (**Table 31**). It was observed that on MS medium, the androgenic response was very poor in comparison to $\frac{1}{2}$ MS medium. On the other hand, the anthers did not show any efficiency of embryo induction and regeneration in N₆ and B₅ media. On the basis of above results, it may be concluded that, out of four media, $\frac{1}{2}$ MS was the best induction medium for anther culture response in orchids.

8.3.3. Effect of different carbon sources on callus induction and regeneration

From the experiment we found that carbon sources were observed to be an essential component for induction of callus in anther culture of *V. tessellata* (**Fig. 41b**). A significant effect of different carbon sources in culture media on the frequency of callus formation and regeneration was noticed. Highest callus induction frequency (16.60%) was obtained in T₃ medium having 3% sucrose, and the frequency declined considerably at different concentration of glucose and maltose. In the present study, 3% sucrose, maltose and glucose were found suitable for callus induction and regeneration (**Table 32; Fig. 41 b,e**). Among the three different carbon sources (sucrose, maltose and glucose) sucrose had a higher induction rate (16.60%) and maximum regeneration efficiency (75 %) followed by maltose (3.45% and 50% respectively) and glucose (2.65% and 45% respectively) in T₃ medium which were significantly different (**Table 32**).

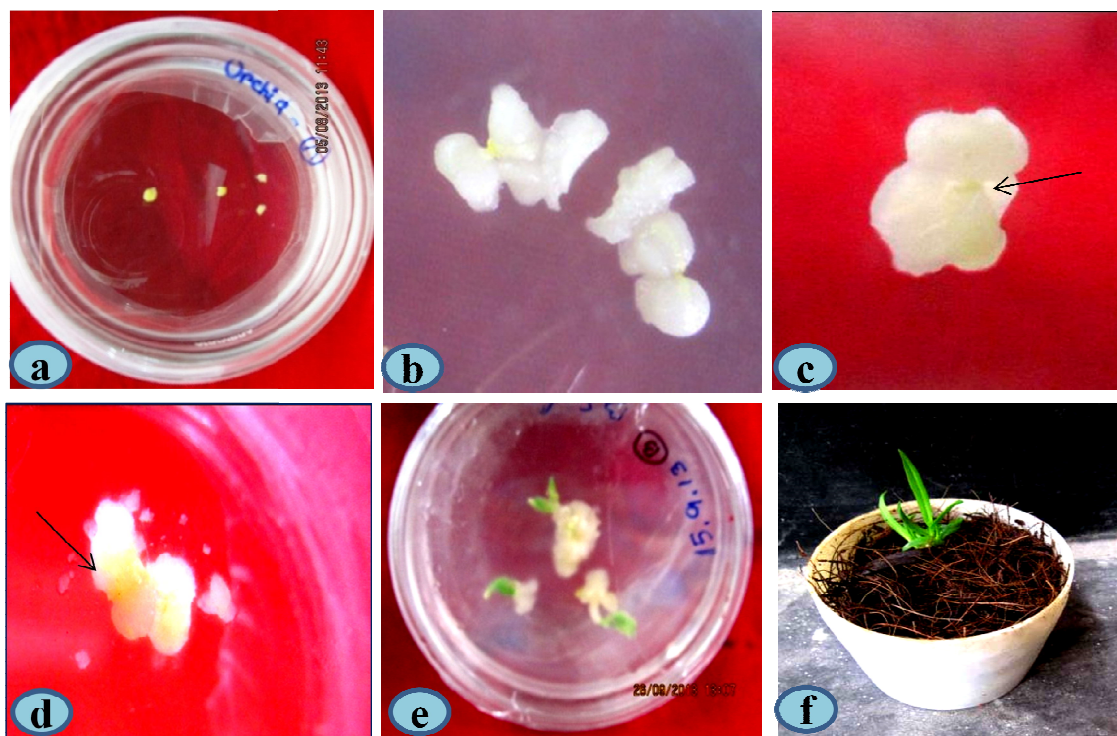


Fig. 41: Callus induction and its subsequent regeneration derived from anther of orchid. (a) inoculated anther; (b) callus initiation; (c-d) the callus was achlophyllous, compact and irregular in shape; (e) green plant regeneration, and (f) acclimatized plant.

Table 32: Effect of carbon sources on callus induction and regeneration through anther of *V. tessellata*.

Name of carbons	Treatments	% of responded anthers	Frequency of callus induction (%)	Frequency of callus regeneration (%)
Sucrose	Cont.	15.20 ± 0.55	2.25 ± 0.45	25.45 ± 0.32
	T ₁	35.25 ± 0.24	3.80 ± 0.62	45.0 ± 0.52
	T ₂	45.33 ± 0.31	5.25 ± 0.86	52.0 ± 0.45
	T ₃	50.63 ± 0.65	16.60 ± 0.34	75.0 ± 0.13
	T ₄	30.45 ± 0.35	8.20 ± 0.75	30.0 ± 0.24
	T ₅	26.62 ± 0.67	4.75 ± 0.22	15.0 ± 0.65
Maltose	Cont.	20.35 ± 0.75	2.00 ± 0.53	10.50 ± 0.63
	T ₁	30.35 ± 0.22	2.20 ± 0.33	20.0 ± 0.88
	T ₂	40.45 ± 0.43	2.69 ± 0.92	35.0 ± 0.76
	T ₃	48.53 ± 0.35	3.45 ± 0.74	50.0 ± 0.55
	T ₄	35.28 ± 0.83	2.88 ± 0.43	18.0 ± 0.62
	T ₅	25.35 ± 0.85	2.25 ± 0.37	8.0 ± 0.34
Glucose	Cont.	20.00 ± 0.35	1.75 ± 0.92	5.22 ± 0.79
	T ₁	35.65 ± 0.75	2.10 ± 0.78	22.0 ± 0.54
	T ₂	42.25 ± 0.43	2.25 ± 0.54	25.0 ± 0.85
	T ₃	45.65 ± 0.44	2.65 ± 0.63	45.0 ± 0.56
	T ₄	30.80 ± 0.18	2.42 ± 0.22	8.0 ± 0.75
	T ₅	20.20 ± 0.45	2.15 ± 0.36	4.0 ± 0.45

T₁ = ½ MS + 10 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA + 1.0 mg l⁻¹ Kin. + 1% sucrose / maltose/glucose;

T₂ = ½ MS + 10 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA + 1.0 mg l⁻¹ Kin. + 2% sucrose / maltose/ glucose;

T₃ = ½ MS + 10 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA + 1.0 mg l⁻¹ Kin. + 3% sucrose /maltose/glucose;

T₄ = ½ MS + 10 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA + 1.0 mg l⁻¹ Kin. + 4% sucrose /maltose/glucose;

T₅ = ½ MS + 10 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA + 1.0 mg l⁻¹ Kin. + 5% sucrose /maltose/glucose.

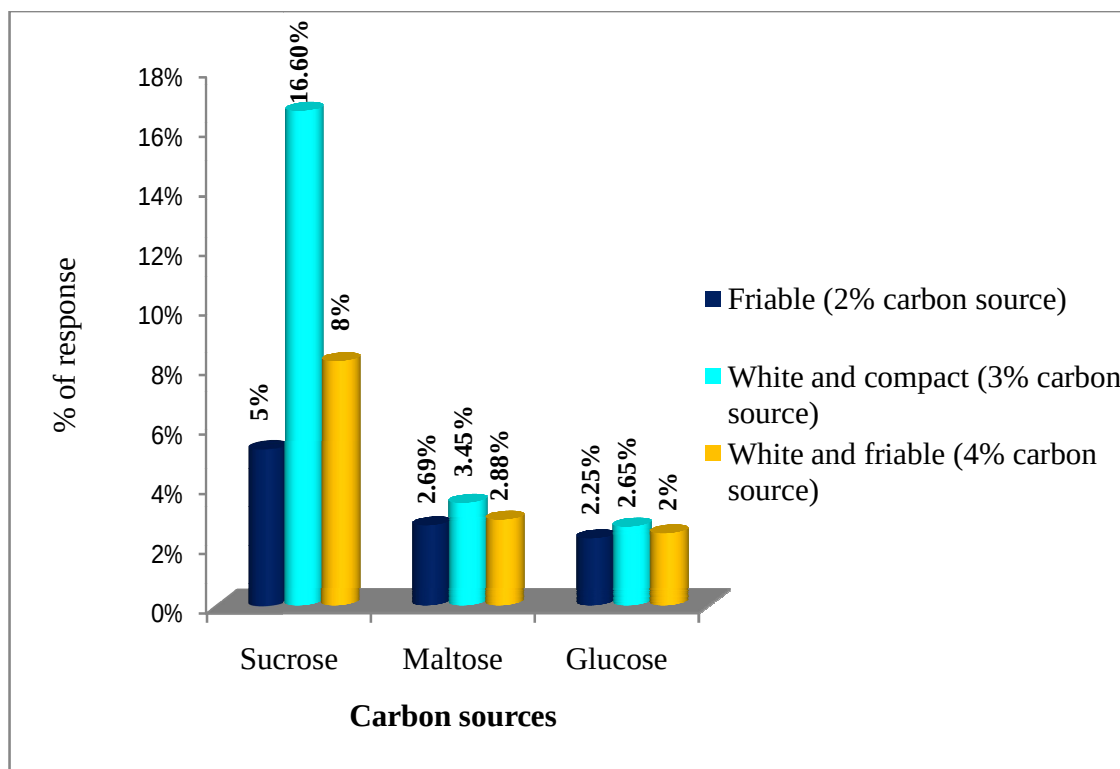


Fig. 42: Effect of carbon sources on nature of callus derived from anthers.

8.3.4. Acclimatization

Well developed rooted plants were transfer to pots containing the moss, small bricks particles, coconut husk and charcoal pieces (1:1:1:1) mixture. It was observed that the survival rate was 60% (**Fig. 41 f**).

8.4 Discussion

The induction of microspores to sporophytic instead of gametophytic pathway is strongly influenced by some kind of stress treatment of the anthers before culture. The response to chilling or heat treatment is also genotype dependent (Datta, 2005). However, a temperature shock has been reported to improve the androgenetic response in many plants (Kiviharju and Pehu, 1998; Xie et al., 1997). The pre-treatment of floral buds with

low temperature is well tried technique for enhancing androgenesis in cultured anthers of many species (Maheshwari et al., 1980; Sharma, 2012).

In this experiment, we found that best friable, heart and irregular shaped callus formed when anthers pre-treated at 4°C condition for 3 days. The result suggests that the cold pretreatment was found to have a positive influence on the callus induction frequency employed for 3 d period of cold shock at 4°C was observed to be optimum for callus induction and regeneration of *V. tessellata*. But while the anthers were pretreated for a long period (more than 3 days) then the responses would be decreased. The present results were in agreement with earlier studies (Lenka and Reddy, 1994) who also reported that the inhibitory effect of prolonged period of pretreatment. In terms of callus formation, there was an obvious trend that high temperature stress reduced anther response to induction in most of the treatments (Wang and Campbell, 2006). Similarly low temperature shock has been reported that to enhance the androgenic response in several species including rice (Ogawa et al., 1995; Silva and Ratnayake, 2009; Sen et al., 2011; Khatun et al., 2012). Some of the positive effects of cold pretreatment on callus induction include delay of anther wall senescence, increase of symmetric divisions of pollen grains and release of substances necessary for androgenesis, mainly amino acids and shock-thermic proteins (Kiviharju and Pehu, 1998).

On the other hand, effect of different carbon sources with various concentrations in culture medium on the frequency of callus induction and regeneration of plantlets from anther culture were investigated. Results revealed that 3% (w/v) sucrose was suitable for inducing high frequency (16.60%) callus induction and regeneration (75%) comparatively than any concentration of glucose and maltose. According to our findings, the callus was found white and compact when added on 3% sucrose in medium (**Fig. 42**). On the other hand, the highest concentration of carbon sources (3% above) in the culture medium not resulted in an increase in the percentage of callus formation. These findings are consistent with the previous reports (Chen, 1978; Chen et al., 1991; Sandhu et al., 1993; Pande and Bhojwani, 1999).

On the contrary, influence of different media on anther culture response was statistically significant in the present study. In this case four different media (MS, $\frac{1}{2}$ MS, N₆ and B₅) were used and found that $\frac{1}{2}$ MS + 2.2 mg l⁻¹ CuSO₄.5H₂O + 10 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA + 2 mg l⁻¹ pepton + 3% (w/v) sucrose showed highest efficiency in order to callus induction and regeneration. This combination and concentration were satisfied through an exogenous supply of peptone (2 mg l⁻¹), BAP (10 mg l⁻¹) and NAA (1.5 mg l⁻¹) $\frac{1}{2}$ MS medium (**Table 31**) along with higher dose of CuSO₄.5H₂O (2.2 mg l⁻¹). A similar promotory role of higher doses of CuSO₄.5H₂O has already recorded in several dicots and monocot plants (Sharma and Vij, 1997). In the experiment about 6 month was needed to form a complete plantlet. The plantlet was acclimatized and the survival rate was recorded 60%.

8.5 Conclusion

In conclusion, the above findings showed that 3d pretreatment at 4°C and $\frac{1}{2}$ MS medium slightly modified by adding CuSO₄.5H₂O with BAP (10 mg l⁻¹), NAA (1.5 mg l⁻¹), peptone (2.0 mg l⁻¹) and 3% sucrose as carbon sources was the best medium for callus induction. Thus the result of the study indicate that the cold pretreatment, different media composition and carbon sources and their interaction clearly influence the anther culture ability of *V. tessellata* and this protocol will be helpful to commercial growers for mass multiplication of orchids. For further research on biochemical compound isolation for medicine, genetic transformation and conservation of endangered orchids this techniques and protocol will be helpful for orchid development in Bangladesh.

Chapter IX
General Discussion

9. General Discussion

Orchidaceae is the largest and most diverse family of flowering plants, consisting of 30,000-35,000 species belonging to 600-800 genera (Hajra, 2001; Singh et al., 2007). The agro-climatic conditions of Bangladesh are congenial for natural orchid vegetation in different regions especially in Chittagong Hills tracks, Rangamati, Sylhet and Sundarbans. Taxonomical studies revealed that there are about 115 different varieties of local orchids in Bangladesh (Bhadra et al., 2002). But habitat destruction, deforestation, climate change, indiscriminate collection by hobbyists and traders are some reasons for the extinction of local orchids. Therefore, it is very important to collect and conserve the local orchid varieties that would be useful for the improvement of orchids in Bangladesh.

In the present study, five different orchid species namely *Vanda tessellata* (Roxb.), *Rhynchostylis retusa* (Blume), *Acampe premorsa* (Roxb.), *Agrostophyllum khasianum* L., and *Phalaenopsis cornorerris* were considered for this study. *Vanda tessellata* (Roxb.) is an epiphytic orchid and it is now listed as endangered in Bangladesh. It has a lot of economic importance such as medicinal, glycosidal, alkaloidal etc. The roots of *Vanda tessellata* are antipyretic; useful in dyspepsia, bronchitis, inflammations, piles and hiccup. Externally the root is used in rheumatism and allied disorders and diseases of the nervous system. It is also remedy for secondary syphilis and scorpion-sting. Juice of the leaves is given in otitis and the paste as febrifuge. The roots possess significant anti-inflammatory activity and the plant contains an alkaloid, glucoside, tannins, β -sitosterol, γ -sitosterol and a long chain aliphatic compound, fatty oils, resins and colouring matters. Roots contain tetracosyl ferrulate and β -sitosterol-D-glucoside (Ghani et al., 1990, Bhattacharjee and Islam, 2014). *Rhynchostylis retusa* is a monopodial, epiphytic orchid species with beautiful flowers arranged in racemose inflorescence. It is an endangered orchid species that grows in moist areas and blossoms during monsoon. The whole plant preparations of *Rhynchostylis retusa* is used to treat rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases and external

inflammations (Kumar et al., 2012, Bhattacharjee and Islam, 2015b). On the otherhand, *Acampe premorsa*, *Agrostophyllum khasianum* and *Phalaenopsis cornorerris* are the most important epiphytic orchid and valued for their medicinal properties and beautiful flowers (Bhattacharjee and Islam, 2014).

In spite of a very large number of seeds produced by the orchids in nature, only 0.2 - 0.3% germinates in nature (Singh, 1992). Hence, the development of efficient protocols for *in vitro* mass multiplication is essential for the conservation and commercial utilization of orchid species. Production of orchids through tissue culture forms the basis for the entire horticultural and floricultural trade. Asymbiotic seed germination has emerged as an important tool for propagating a large number of orchids and hybrids (Arditti et al., 1982). Germination of immature seeds of orchids in, *in vitro* is referred to as ovule/embryo/green pod/green fruit culture (Sagawa, 1963). Green pod culture technique has been considered as one of the major advancement in orchid seed culture (Sharma et al., 2005). In the present study, immature green pods were considered and four types of basal media (MS, ½MS, PM and B₅) without PGRs were used to assess their effect on seed germination and PLBs formation. Among the mentioned basal media tested, MS was the most effective for germination and protocorms development.

It was observed that the germinated seeds increased in size and highest protocorms was recorded in MS medium. Mohanty et al. (2012) reported that the MS medium was equally effective also for germination of *Cymbidium mastersii* species. Nutrient requirements for orchid seed germination are thought to be species specific (Arditti and Ernst, 1984; Kauth et al., 2008). It was therefore, not surprising that the various basal media used in the present study, with their different compositions and concentrations of mineral salts, organic supplements and vitamins, varied in their suitability for *in vitro* germination. In the present study found that the MS medium promoted seed germination. Stewart and Kane (2006) reported that nitrogen is very essential component for plant growth and its source has been shown to have an effect on the germination of different orchid species. The MS medium contains nitrogen and ammonium which stimulated seed germination

(Popova et al., 2003). Under this study it was observed that both of seed germination and PLBs formation was higher in MS medium. The results are in agreement with the findings of Bhattacharjee and Islam (2015a). On contrary, Hossain (2008) reported that 75% of the *Eulophia ibaguense* seeds germinated on MS medium supplemented with 1 mg/1 BAP, but with increasing concentrations of BAP, the seed germination percentage has been declined. In case of *Cypripedium* sp. as terrestrial orchid BAP enhanced seed germination (Depauw et al., 1995).

The present study showed that primary protocorms responded significantly higher in combination with 1.0 mg/1 BAP and 1.0 mg/1 NAA with MS medium. Highest yield of PLBs obtained in *Cymbidium lapine* by Fujii et al. (1999) and they suggested that a synergistic effect was also observed in the case of *Cymbidium nativity*. Kusumoto (1978) suggested that NAA showed the negative effect on PLBs proliferation in *Cymbidiums* whereas using BAP in the medium showed positive effect on protocorm formation. Results indicate that the combined effect of 1.0 mg^l⁻¹ BAP and 1.5 mg^l⁻¹ NAA with MS medium showed suitable results on PLBs proliferation. On contrary, Khatun et al. (2010) reported that 1.0 mg^l⁻¹ BAP + 1.0 mg^l⁻¹ IBA is the best for PLBs proliferation.

In case of protocorms regeneration, different combination and concentration of PGRs were used. The highest number of regenerated plantlets was obtained in the medium having 1.0 mg^l⁻¹ BAP + 1.5 mg^l⁻¹ NAA. Such results are in agreement with the findings of Khatun et al. (2010) where they reported the highest number of regenerated plantlet was found in 0.5 mg^l⁻¹ IAA + 1.0 mg^l⁻¹ IBA. The combined effect of different combinations and concentrations of growth regulators on shoot height showed significant difference. The highest shoot height was obtained from 1.0 mg^l⁻¹ BAP + 1.5 mg^l⁻¹ NAA in MS medium. Similar type of observation was done by Prasad et al. (2001). They found that the highest mean shoot height was obtained from 1.0 mg^l⁻¹ each of BAP + NAA on MS medium.

Bektas et al. (2013) reported that 0.25 mg^l⁻¹ 6-BA is suitable for shoot length and number and lengths of root development. Erisen et al. (2011) reported that TDZ was the most

effective plant growth regulator for *in vitro* shoot formation. The findings from the present study suggest that the combination of BAP + NAA is the best for protocorm regeneration and for shoot elongation as well as mass multiplication. They reported that single uses of NAA had no effect but the combination of BAP + NAA induced greatest effect on development and differentiation of PLBs in *Cymbidium*. It might be due to their effect on physiological processes or interaction between the two hormones.

Plant regeneration using different explants such as leaf, stem, roots, anther and immature seeds have been established in studied orchids. It was also reported that in orchids, the regeneration of plantlets from the callus usually occurred through PLBs formation involving somatic embryogenesis (Begum et al., 1994). Hence, attempt has been made in the present study for the callus induction using leaf, stem, roots, anther and immature seeds of these orchid species and also to develop and standardize the protocol for *in vitro* regeneration and mass multiplication via PLBs formation. To the best of my knowledge, there was no report of direct somatic embryogenesis from leaf and stem explants and subsequent plant regeneration of *V. tessellata*. It was observed that, somatic embryos formed directly at the cut edges of leaf sections and around the cut ends of stem explants. In another orchid, *Oncidium*, generally, leaf tips had the highest ability to form embryos (Chen et al., 1999). Somatic embryos germinated and developed on the induction medium and found embryogenesis was slower and asynchronous in leaf explants. Small, round, individual somatic embryos appeared 6-7 weeks after culture of initiation. The formation of somatic embryos around stem segments was slower and more asynchronous.

In order to adjust a protocol of somatic embryogenesis sections young leaves of *Phytolacca tetramera* obtained *in vitro* were placed in MS medium that supplemented with 2,4-D at different concentrations. Somatic embryos were obtained directly from the cutting edge of the blade and embryogenic callus (indirect way) from the midrib (Cordal et al., 2014). In this experiment, MS medium fortified with 0.5 mg/L BAP and 1.0 mg/L kinetin showed quick response to induce direct somatic embryos. On the other hand, thidiazuron (TDZ) at 0.3, 1.0 and 3.0 mg dm⁻³ induced 5-25% of leaf tip segments of *in*

vitro grown plants directly formed embryos after 60d of culture, and 1.0 mg dm⁻³ TDZ was the best treatment (Chung et al., 2007). In general, to produce significantly more embryos, leaf showed better than stem explants.

In the present study, maximum regeneration frequency of the somatic embryos was noticed on ½MS medium containing 1.0 mg l⁻¹ BAP. Although plant regeneration with direct and indirect organogenesis from various explants sources of *N. foetida* species have already been reported (Rai, 2002). Vij and Kaur (1992) reported that plant regeneration from callus culture in orchids via PLBs formation which is an intermediary somatic embryo phase. This type of morphogenetic development has been reported in a number of orchid species by many workers (Chen and Chang, 2000; Roy et al., 2007). It was reported that in orchids the differentiation of PLBs from callus occur in two different ways. In some orchid species, PLBs formation requires exogenous plant growth hormones while, in some species PLBs differentiation from the callus occurs in the absence of plant growth hormones (Roy and Banerjee, 2003). Anjum et al. (2006) reported that cytokinin and auxins ratio plays a vital role in the differentiation of callus into PLBs.

In the present study, it was observed that the callus differentiation into PLBs depends on concentration of cytokinins and auxins used. For multiplication, many plant species require specific *in vitro* conditions. A range of cytokinins (kinetin, BAP, 2-iP and zeatin) have been used for micropropagation studies (Bektas et al., 2013). But in this case, we found maximum number of multiple shoots in MS medium fortified with 3% (w/v) sucrose + 1.5 mg l⁻¹ BAP + 1.0 mg l⁻¹ NAA.

For increasing the number of roots and their length, plantlets were supplied with different concentration and combination of auxins. Here shows that half strength of MS medium supplemented with 1.0 mg/l IAA induced the most of the roots per shoot, followed by 1.5 mg l⁻¹ IAA. In comparison, NAA was less effective for root formation and promoting root extension. A significant difference was found on root length at different concentrations and combinations of growth regulators. The maximum root length (3.4

cm) was obtained in ½MS medium that supplemented with 1.0 mg/1 IAA followed by 1.0 mg/1 NAA.

Well developed plants with 3-4 leaves and 1-2 roots were used for the acclimatization process. For hardening of *in vitro* grown plantlets different composts were used. In cases of all studied orchids the substratum of compost were found to be satisfactory for survivability and normal growth of the plantlets. However, highest percentage survivability with maximum length of hardened plants was obtained on substratum containing brick, charcoal, decaying litter, coconut husk (1:1:1:1) with a layer of moss. The layer of moss on top proved to be beneficial to higher retention of moisture content. Feeding the plantlets with dilute MS nutrient medium found beneficial to the developing hardened plantlets. As reported earlier by Kumaria and Tandon (1994) in *D. fimbriatum* var. *oculatum*, feeding the plantlets with dilute MS nutrient salt solution was found beneficial to the developing hardened plantlets of *C. devonianum*. As precautionary measures, plants were sprayed with 0.1% bavistin and 2 ppm of Ceftriaxone as antibiotic once in 15 days to prevent fungal and bacterial infection. Similar to our treatment *in vitro* plantlets of *Zygopetalum intermedium* was given bavistin (0.2%) treatment for 1 h before acclimatization (Nagaraju and Mani, 2005).

Antimicrobial studies were conducted to investigate the bioactive compound and the antimicrobial potency of *V. tessellata* and *R. retusa*. To evaluate the antimicrobial activity and phytochemical analysis the various solvent extracts such as chloroform, methanol, ethanol and hexane of this orchid were considered for *in vitro* antimicrobial activity against five clinical pathogenic bacteria viz. *Staphylococcus aureus*, *Bacillus subtilis*, *Vibrio cholerae*, *Escherichia coli*, *Klebsiella pneumonia* and three fungi viz. *Penicillium* sp. *Rhizopus* sp. *Aspergillus niger* by disc diffusion method. A preliminary phytochemical analysis was performed for the detection of alkaloid, terpenoids, flavonoids, phenols, tannins, steroids and glycosides etc. All the extracts showed different degree of inhibitory that potential against tested bacteria under this study.

In case of *V. tessellata*, chloroform extracts exhibited higher degree of inhibition against *Staphylococcus aureus* and *Escherichia coli* (11.00 mm and 11.00 mm) than *Vanda coerulea* leaves extract (2.6 mm and 4.4 mm) (Shanmuga et al., 2011). It is an indication of the broad spectrum antimicrobial potential of *V. tessellata* which make the plant a candidate for bioprospecting for antibiotic and antifungal drugs. The potential of compounds against the standard strains may be explored in order to develop therapeutics for microorganisms. The plant extracts revealed the presence of tannins, phenols, alkaloids, flavonoids, terpenoids, glycosides and steroids. The presence of saponin, alkaloids and tannin enhanced the antimicrobial activity against the pathogenic microorganisms (Ndukwe et al., 2005). The secondary metabolites of various chemical types present in the plant species are known to possess antimicrobial activities. Flavonoids are found to be effective antimicrobial substances against a wide range of microorganisms, probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall; more lipophilic flavonoids may also disrupt microbial membrane (Tsuchiya et al., 1996). Phenolics and polyphenols present in the plants are known to be toxic to microorganisms (Mason and Wasserman, 1987). Besides, the presence of antimicrobial substances in higher plants is well established as they provided a source of inspiration for novel drug compounds as plants derived medicines have made significant contribution towards human health. Medicinal plants are important source for the development of potential new chemotherapeutic drugs and the *in vitro* antibacterial test form the basis.

On the other hand, The results of the phytochemical screening of *R. retusa* revealed the presence of alkaloids, terpenoids, flavonoids, tanins, coumerins, steroids, amino acids and carbohydrates in all the solvent extract (methanol, ethanol, chloroform and hexane) while coumerins were found only methanol extract and amino acid was absent in hexane and chloroform extract. On the other hand, phlobatanins, phenols, saponins and glycosides were not detected in any of the plant extracts. The presence of these secondary metabolites in *R. retusa* confirms their antibiotic properties and usefulness by the traditional medicine practitioners. Flavonoids are also known to have a wide array of

therapeutic activities as antihypertensive, anti-rheumatism, antimicrobial, diuretic and antioxidants (Burkill, 1988; Trease and Evans, 2002). Alkaloids are formed as metabolic byproducts and have been reported to be responsible for the antibacterial activity (Doughari, 2006). Steroids are known for anti-inflammatory, lipolytic and anti-cholesteremic activities (Chawal et al., 1987). Tannins are reported to have various physiological effects like anti-irritant, antisecretolytic, antiphlogistic, antimicrobial and antiparasitic effects. Phytotherapeutically tannin-containing plants are used to treat nonspecific diarrhoea, inflammations of mouth and throat and slightly injured skins (Muthukrishnan et al., 2014). In the present investigation, the phytochemical constituents of *Rhychostylis retusa* was studied and further the antimicrobial activity of the plant extract was also tested against five potential clinical pathogenic bacteria namely *Staphylococcus aureus*, *Bacillus subtilis*, *Vibrio cholerae*, *Escherichia coli* and *Klebsiella pneumonia* and three fungal organism namely *Penicillium* sp., *Rhizopus* sp. and *Aspergillus niger* at different concentrations of the plant extracts (10.0, 5.0, 2.5 mg/ml) to understand the most effective activity. The present study showed that whole plant extracts of *Rhychostylis retusa* in chloroform provided a highest zone of inhibition while other three extracts were found to be less active against the tested organisms. It is indicated that the plant extracts of *Rhychostylis retusa* contain compounds that largely inhibit the growth of gram positive and gram negative bacteria along with the fungal organism.

Androgenesis refers to the development of haploid plants derived from anther and microspore culture. The induction of microspores to sporophytic instead of gametophytic pathway is strongly influenced by some kind of stress treatment of the anthers before culture. The response to chilling or heat treatment is also genotype dependent (Datta, 2005). However, a temperature shock has been reported to improve the androgenetic response in many plant species (Kiviharju and Pehu, 1998; Xie et al., 1997). Recently, the pre-treatment of floral buds with low temperature is well tried technique for enhancing androgenesis in cultured anthers of many species (Maheshwari et al., 1980; Sharma, 2012). For androgenic response symmetrical division of microspores is very

important and which is the main source of androgenic haploids (Islam et al., 2013). Zheng and Ouyang (1980) found that spikes pretreated with low temperature might influence symmetrically dividing microspores. Lazar et al., (1985) found that variation in intensity and duration of cold pretreatment leads to significant differences in callus formation frequency. In the present investigation, cold pretreatment (4°C) of flower buds at different days (1-7 days) was applied prior to culture. The cold pretreatment of 2-6 days showed significant improvement of the induction efficiency. Three days of stress was found to be most effective of cold pretreatment. Croughan and Chu (1991) mentioned that cold pretreatment is very effective in enhancing callus formation and regeneration. In terms of callus formation, there was an obvious trend that high temperature stress reduced anther response to induction in most of the treatments (Wang and Campbell, 2006).

Different pre-treatment factors such as chemical and physical may influence the development of microspore condition, which is very important. In the experiment, the effect of different carbon sources with various concentrations in culture media on the frequency of callus induction and regeneration of plantlets from anther culture were investigated. Results revealed that 3% (w/v) sucrose was suitable for inducing high frequency (16.60%) callus induction and regeneration (75%) comparatively than any concentration of Glucose and maltose. According to our findings, the callus was found white and compact when anthers were culture on 3% sucrose. On the other hand, the highest concentration of carbon sources (6% above) in the culture medium not resulted in an increase in the percentage of callus formation. These findings are consistent with the previous reports (Chen, 1978; Chen et al., 1991; Sandhu et al., 1993; Pande and Bhojwani, 1999).

On the contrary, Influence of different media on anther culture response was statistically significant in the present study. In this study four different media (MS, ½ MS, N₆ and B₅) were used. Here we found that ½MS + 2.2 mg l⁻¹ CuSO₄.5H₂O + 10 mg l⁻¹ BAP + 1.5 mg l⁻¹ NAA + 2 mg l⁻¹ pepton + 3% (w/v) sucrose showed highest efficiency in order to callus

induction and regeneration. This combination and concentration were satisfied through an exogenous supply of Peptone (2 mg/l) in the BAP (10 mg/l) and NAA (1.5 mg/l) enriched with ½MS medium supplemented with higher dose of CuSO₄.5H₂O. A similar promotory role of higher doses of CuSO₄.5H₂O has already recorded in several dicots and monocot plants (Sharma and Vij, 1997). The reported results are very consistent with the present findings.

Chapter X
Summary

10. Summary

Considering the importance of rapid and mass propagation and medicinal value of indigenous orchids in Bangladesh this research programme has been under taken with a major view to develop a reproducible micropropagation technique for rapid multiplication, to estimate antimicrobial activity and to analyses of bioactive compounds of indigenous orchids. For this research programme five different orchid species namely *Vanda tessellata* (Roxb.), *Rhynchostylis retusa* (Blume), *Acampe premorsa* (Roxb.) Blatter., *Agrostophyllum khasianum* L., and *Phalaenopsis cornorerris* were selected.

The first experiment (Expt. 4.1) of this study was performed for rapid development of PLBs derived from immature seeds and mass multiplication of *Vanda tessellata* (Roxb.). *In vitro* micropropagation, hybridization and cryopreservation techniques are very essential tools to fulfil our commercial demand for orchid development and its conservation systems in Bangladesh. Under this study, it was observed that immature capsule showed better performance for germination (100.0%) and development of PLBs (90.0%). Around 45-60 days old PLBs were transferred to ½MS medium supplemented with different concentrations and combination of auxins and cytokinins. The highest number (90%) and weight of PLBs (5.53 g), best plant regeneration (16.50) and shoot length (3.25 cm) were recorded when the medium supplemented with BAP (1.0 mg l⁻¹) and NAA (1.5 mg l⁻¹). The highest number (5.0) and length (3.22 cm) of roots, plantlets (3.75) obtained with 1.5 mg l⁻¹ BAP and 1.0 mg l⁻¹ IAA. The maximum number of leaves (3.33) were recorded in 1.5 mg l⁻¹ BAP + 1.00 mg l⁻¹ IAA and highest length of leaves (2.59 cm) observed with the combination of BAP and IAA (2.0 mg l⁻¹) in medium. Well shoot and rooted plants were acclimatized in pots that contained two categories of composition substratum. Here, small brick particles + charcoal pieces + decaying litter + coconut husk (1:1:1:1) + moss (10.0 gm/pot) showed better performance on plant development.

An efficient protocol for mass production of protocorm like bodies (PLBs) were developed through leaf and shoot explants of *Vanda tessellata* as an endangered medicinal orchid in Bangladesh were described in Exp. 4.2. In this case somatic embryos were induced from leaf and shoot that cultured on MS medium supplemented with BAP, NAA and kinetin. Among them BAP (0.5 mg l^{-1}) and kinetin (1.0 mg l^{-1}) showed better performance on somatic embryogenesis and mostly it forms from near the cut ends of stem and leaf surfaces. Maturation of somatic embryos was accomplished with same induction medium. Germination and regeneration efficiency of somatic embryos were recorded as highest 60.83% in $\frac{1}{2}$ MS medium supplemented with 1.0 mg l^{-1} BAP. Maximum number of multiple shoots (10.80 ± 0.22) was recorded in MS medium with 1.5 mg l^{-1} BAP and 1.0 mg l^{-1} NAA. Individual shoots produced highest number of adventitious roots (5.0 ± 0.37) on $\frac{1}{2}$ MS medium supplemented with 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} NAA. This protocol is potentially useful for the micropropagation of this species. The results obtained could have enormous significance, since this is the first report of somatic embryogenesis for this endangered medicinal important orchid. So far as we know there is no report regarding the plant regeneration through somatic embryogenesis from leaf and stem explants of *V. tessellata*.

In Exp. 4.3., an efficient protocol for regeneration of endangered medicinal orchid of *Vanda tessellata* via multiple shoot has been established using shoot segments as explants. Under this study four culture media viz. PM, MS, $\frac{1}{2}$ MS and B5 were used. The most effective medium was MS where seed germination where recorded 80.0% .Twenty different plant growth regulators (PGRs) were tested for multiple shoot induction and its subsequent development. It was revealed that the combination of 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} BAP was proved to be the best combination for multiple shoot development and elongation. In this case the average number of induced shoots per explants was 7.52. The single shoots were separated from the multiple structures and sub-cultured in $\frac{1}{2}$ MS medium supplemented with NAA ($0.5 - 2.0 \text{ mg l}^{-1}$), IAA ($0.5 - 2.0 \text{ mg l}^{-1}$) and IBA ($0.5 - 2.0 \text{ mg l}^{-1}$) for root induction. Maximum root induction (6.1) and its growth (4.0 cm)

found in $\frac{1}{2}$ MS medium supplemented with 1.0 mg l^{-1} IAA. The well rooted plantlets were hardened successfully in the potting mixture containing coconut husk, charcoal, brick pieces in the ratio of 2:1:1 and eventually established under natural condition as described earlier.

Another Experiment (5.1) were conducted using different plant growth regulators (PGRs) were applied for *in vitro* development of protocorms, regeneration and mass multiplication derived from immature seeds of *Rhynchosstylis retusa* (L.) Blume. It is a monopodial, epiphytic orchid. Owing to its high commercial value in the floricultural industry, mass propagation provides an alternative means of satisfying the demand. Unfortunately, conventional propagation is slow and difficult, suggesting *in vitro* methods for mass multiplication may be more appropriate. In this case germination ability and early developments of PLBs in MS, $\frac{1}{2}$ MS, B5 and PM media were evaluated. In addition, different plant growth regulators viz. BAP, Kin, NAA and IAA were used either single or in combination. The maximum percentage of seed germination (72.60%) was recorded after 7-8 weeks of culture initiation in MS medium. Secondary protocorms were developed from primary PLBs on MS medium fortified with different concentrations and combinations of cytokinins (BAP and Kin) and auxins (NAA, Picloram, IBA and IAA). The highest numbers of secondary PLBs (16.0) were obtained from each of primary protocorms in MS medium supplemented with 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA. The maximum root induction (7.0 per shoot) was observed in $\frac{1}{2}$ MS medium that supplemented with 1.0 mg l^{-1} IAA. Plants were acclimatized successfully and survivable rate was 80%.

A reliable protocols for protocorm like bodies (PLBs) development and its regeneration of *R. retusa* through direct somatic embryogenesis was standardized and verified in the Experiment of 5.2. Somatic embryos were developed from leaf and roots and cultured in MS medium supplemented with different concentration and combinations of phytohormones either individually or in combination. The embryo induction was highest in leaf (75%) and root (50.2%) when 1.0 mg l^{-1} BAP + 1.5 mg l^{-1} kinetin was used.

Germination of somatic embryos was highest (60%) in $\frac{1}{2}$ MS medium with 1.5 mg l^{-1} BAP. The effect of different PGRs was observed on multiple shoot induction. Maximum number (10.2 ± 0.58) and highest length (3.8 cm) of multiple shoots was observed in MS medium + 1.5 mg l^{-1} BAP and 1.0 mg l^{-1} NAA. Highest number (5.8) and length (4.17 cm) of adventitious roots were seen in $\frac{1}{2}$ MS medium + 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} IBA.

Another investigation for *in vitro* germination and enhancing PLBs development in three indigenous orchids namely *Acampe premorsa*, *Agrostophyllum khasianum* and *Phalaenopsis cornorerris* were carried out in the Experiment of 6.1. Under this study three basal media were tested and among them PM was found to be the best because of the greatest rate of seed germination (98.20%), PLBs development and plant formation. PM supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l) gave a 78.60% PLBs formation rate. The MS with 1.0 mg/l BAP and 0.1 mg/l picloram was the most efficient medium for seedling growth of *A. premorsa* and *A. khasianum*. But in the case of *P. cornorerris* the best seedling growth was observed using medium supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA. The maximum number and length of roots and plantlets were observed in the $\frac{1}{2}$ MS medium with 1.5% (w/v) sucrose where no hormones were added. The well-rooted plantlets were successfully acclimatized, planted in pots and transferred to the shade house for establishment. This protocol may be applied for the mass propagation of other orchids for commercial use. It might be concluded that *in vitro* seed germination is technique with the highest potential for protocorm formation, multiplication and conservation of indigenous orchids.

In the Experiment of 7.1, antimicrobial activity and phytochemical screening of whole plant extracts of *Vanda tessellata* were studied. *Vanda tessellata* is an endangered and medicinal orchid that is effective to cure various diseases e.g. dyspepsia, bronchitis, inflammations, piles, hiccup etc. The purpose of this study was to determine phytochemical constitutes and antimicrobial activity using extracts from whole plant of *Vanda tessellata*. The various solvent extracts such as chloroform, methanol, ethanol and hexane of this orchid were considered for *in vitro* antimicrobial activity against five

clinical pathogenic bacteria viz. *Staphylococcus aureus*, *Bacillus subtilis*, *Vibrio cholerae*, *Escherichia coli*, *Klebsiella pneumonia* and three fungi viz. *Penicillium* sp., *Rhizopus* sp., *Aspergillus niger* by disc diffusion method. A preliminary phytochemical analysis was performed for the detection of alkaloid, terpenoids, flavonoids, phenols, tannins, steroids and glycosides etc. All the extracts showed different degree of inhibitory that potential against tested bacteria under this study. The antibacterial activity against all bacteria with the zone of inhibition ranging from 5 - 15 mm were observed and found that highest inhibition zone (14 - 15 mm) with the concentration of 10.0 mgL⁻¹ of chloroform extract. Chloroform extract showed significant antifungal activity against *Penicillium* sp., *Rhizopus* sp. and *Aspergillus niger* with the highest zone (16 - 17 mm) of inhibition. It might be concluded that antimicrobial activity was successfully investigated which could be further exploited for isolation and characterization of the novel phytochemicals effect on several infectious diseases especially in light of the emergence of drug resistant microorganisms and antimicrobial agents.

An assessment of antibacterial and antifungal activities of the extracts of *Rhynchosytilis retusa* was carried out in the Experiment of 7.2. The phytochemical screening and antimicrobial activities of different extract (methanol, ethanol, chloroform and hexane) of *Rhynchosytilis retusa* was applied on five different bacteria (gram positive: *Staphylococcus aureus* and *Bacillus subtilis*; gram negative: *Vibrio cholerae*, *Escherichia coli* and *Klebsiella pneumonia*) and three fungus species (*Penicillium* sp., *Rhyzopus* sp. and *Aspergillus niger*). The antimicrobial activity was assessed using disc diffusion method. A preliminary phytochemical screening was performed on the basis of detection of alkaloid, terpenoids, flavonoids, tannins, steroids, coumerins, amino acids and carbohydrates. Under this study methanol, ethanol and chloroform extracts from *Rhynchosytilis retusa* showed different degree of inhibitory that potential against tested bacteria while hexane extract not showed any inhibition zone on studied bacteria. The antibacterial activity against all bacteria with the zone of inhibition ranging from 5 - 12 mm were observed and found that highest inhibition zone (10 - 12 mm) with the

concentration of 10.0 mg/l of chloroform extract. Chloroform extract also showed significant antifungal activity against *Penicillium*, *Rhizopus* and *Aspergillus niger* with the highest zone (12 - 15 mm) of inhibition. The ability of the crude extracts of *Rhynchosyilis retusa* to inhibit the growth of bacteria and fungus is an indication of its broad spectrum antimicrobial potential which may be employed in the management of microbial infections.

Other attempts of this study were taken in Experiment 8.1, to enhance anther culture response of *Vanda tessellata* by applying physical and chemical pre-treatment factors including with different media. It was observed that 4°C for 3d pretreatment and ½MS medium slightly modified by adding CuSO₄.5H₂O (2.2 mg/l) with BAP (10 mg/l), NAA (1.5 mg/l), peptone (2.0 mg/l) and 3% sucrose as carbon sources was the best medium for callus induction. From the experimental findings it might be summarized that the cold pretreatment, different media composition and carbon sources and their interaction clearly influence the anther culture ability of *V. tessellata* and this protocol will be helpful to commercial growers for mass multiplication of orchids.

Chapter XI
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11. References

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Curriculum Vitae of Bakul Bhattacharjee

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B. Educational background

Examination	Year	Board / University	Division/Class
SSC	1990	Comilla Board	First
HSC	1992	Comilla Board	First
B. Sc (Hons) in Botany	1995	Chittagong University	Second
M. Sc (Thesis group)	1996	Chittagong University	First Class (First position)

C. List of Publications:

i) Published articles (07)

1. **Bhattacharjee B** and Islam SMS (2014) Effects of plant growth regulators on multiple shoot induction in *Vanda tessellata* (Roxb.) Hook. Ex G. Don an endangered medicinal orchid. Int. J. Sci. Nat. 5(4): 707-712.
2. **Bhattacharjee B**, Islam T, Rahman Z and Islam SMS (2015) Antimicrobial activity and phytochemical screening of whole plant extracts of *Vanda tessellata* (Roxb.) Hook. Ex. G. Don. World J. Phar. Pharmace. Sci. 4(1): 72-83.
3. **Bhattacharjee B** and Islam SMS (2015) The Effect of PGR'S on *in vitro* development of protocorms, regeneration and mass multiplication derived from immature seeds of *Rhynchostylis retusa* (L.) Blume. Global J. Biosci. Biotech. 4 (1): 121-127.
4. **Bhattacharjee B** and Islam SMS (2015) Antimicrobial activity and phytochemical screening of whole plant extracts of *Rhynchostylis retusa*. World J. Phar. Pharmace. Sci. 4(2). 74-87.
5. **Bhattacharjee B** and Islam SMS (2014) Development of an efficient protocol on *in vitro* germination and enhancing protocorm-like bodies using three indigenous orchid species in Bangladesh. As. Pac. J. Mol. Biol. Biotech. 22(3). 209-218.

6. Islam SMS and **Bhattacharjee B** (2015) Plant regeneration through somatic embryogenesis from leaf and root explants of *Rhynchostylis retusa* (L.) Blume. App. Biol. Res. 17(2): 158-165.
7. Islam SMS, Islam T, **Bhattacharjee B**, Mondal TK and Sreeramanan Subramaniam S (2015) *In vitro* pseudobulb based micropropagation for mass development of *Cymbidium finlaysonianum* Lindl. Emir. J. Food, Agric. 27(6): 469-474.

ii) Abstracts (Conference/Workshop/Symposium)- 07

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2. **Bakul Bhattacharjee** and S. M. Shahinul Islam - Rapid *in vitro* Development of Protocorms Derived from Immature Seeds, Regeneration and Mass Multiplication of *Vanda tessellata* (Roxb.) Hook. Ex. G. an Endangered Medicinal Orchids in Bangladesh, 7th Int. Plant Tiss. Cult & Biotech. Conf., 1-3 March, 2014, Org. by BAPTC&B, Bangladesh. Abst. 6, p 6.
3. M. Touhidul Islam, **Bakul Bhattacharjee**, Tushar Kanti Mondal and S. M. Shahinul Islam - *In vitro* Plant regeneration in Soybean [*Glycine max* (L.) Merr.] from Cotyledonary and Nodal Explants. 7th Int. Plant Tiss. Cult. & Biotech. Conf., 1-3 March, 2014, Organized by BAPTC&B, University of Dhaka, Bangladesh. Abst. 24, p 24.
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