CHAPTER 2

Review of literature

The plants under study i.e. *Smilax glabra* Roxb., *Homalomena aromatica* (Roxb.) Schott., *Bulbophyllum caryanum* (Hook.) Spreng. and *Paphiopedilum spicerianum* (Rchb.f.) Pfitz. are the four important Non Timber Forest Products (NTFP) of Southern Assam, India. These four plant species are very important from the consideration of medicinal as well as commercial point of view. Once upon a time population of these three important plant species were widely distributed in the whole of the geographical area of this region, but now a days the population has declined due to over exploitation and some anthropogenic factors, i.e. conversion of wild habitat to agricultural field, deforestation etc.

2.1. METHODS OF VEGETATIVE PROPAGATION:

Vegetative propagation is an asexual technique which is very important for the preservation of the genetic integrity of the plant material for conservation (Matthews, 1999), easy and effective technique for multiplication and conservation of the plant species (Vashistha *et al*., 2009). There are many scientific methods for vegetative propagation.

2.1.1. Vegetative Propagation from stem Cutting:

The stem is the most common and important part of the plant, used for the vegetative propagation. The samples of specimens are collected from the strong, vigorous shoots. (Matthews, 1999). The common stem cutting techniques in the vegetative propagation are as follows:

2.1.1. 1. Hard wood cuttings:

This technique is relatively inexpensive and easy to execute, requiring little equipment. The sample cuttings are prepared by the following ways- a) Straight stems, b) Lateral shoots with a ‘heel’, which is a small portion of the older wood, c) Lateral shoots taken with a section of the previous year’s growth.

2.1.1. 2. Semi-ripe cuttings:

The sample of plant specimens are selected from the mature current year's shoots of the tested plants. Cuttings are done at the length of 5-15 cm and all the lower leaves are removed. Subsequently the lower ends of the samples are treated with a rooting hormone before inserting in the rooting medium.

2.1.1. 3. Semi-bud cuttings:

In this stem cutting technique, only one leaf and the axil bud are used with a short stem. This technique is mainly based on the maturity of tissue.

2.1.1. 4. Soft wood cuttings:

This is a very widely used technique of vegetative propagation. Here the plant tissue to be used is very soft and immature and the environment must be of high humid condition to minimize water loss from the cuttings. The sample specimen cuttings are collected mainly in the early morning hours from healthy, well-watered plants and selected specimens must be 10 cm long and cut below a node. The leaves are removed from the lower part of the cutting and growth hormone is added.

2.1.1. 5. Evergreen cuttings:

The technique is similar as in the soft wood cuttings. Here mainly vegetative shoots are selected at a length of 10 cm for longer leaf species and 5 cm for small leaved species.

2.1.1. 6. Layering:

This is a simple technique. The sample specimens (stem) are left attached to the mother plant during this process. After rooting the new plant is excised and planted.

2.1.2. Vegetative propagation by Grafting:

Grafting is a vegetative propagation technique of joining two pieces of living plant tissue together in such a manner that they will unite and subsequently grow and develop as one composite plant (Hartmann *et al*., 2007).

2.1.3. Vegetative propagation from the roots and rhizome:

Root cuttings provide a reliable propagation method for a number of plant species. The operations are best carried out during the dormant phase of the plant growth. The sample specimens (pencil thick) are cut into 5-10 cm long pieces. Subsequently cuttings are treated with fungicide and inserted into nearly full pots of the rooting medium, with the top of the cutting level on its surface. The pots are then filled with grit. After a few weeks new buds and roots are expected to regenerate. (Mathews, 1999).

The rhizome is a specialized modified stem. Mainly two types of propagation techniques are used, i.e. division of rhizome and culm cuttings. Division is done mainly in two ways. One is division of individuals at the point of attachment and the other is removal of single lateral offshoot. On the other hand the culm cuttings are applicable on the large rhizome bearing plants in which the entire aerial shoot is laid horizontally (Hartmann *et al*., 2007).

2.1.4. *In vitro* propagation by tissue culture method:

The tissue culture is a special type of vegetative propagation of plant cell and organs in aseptic *in vitro* condition. The sample specimens of explants' shoot tip, axillary bud and stem segment etc are used for culturing in the basal specific media fortified with the plant growth hormones like BAD, NAA and IBA in different concentrations and combinations. After the regeneration the specimens are transferred to the rooting media. Then the rooted plantlets are planted into the pots for hardening and transplant into the field (Wankhede *et al.*, 2007).

2.2. Vegetative propagation of *Smilax* species:

The protocol for the vegetative propagation of *Smilax* species is very rare. A few protocols have been developed for the production of large number of plantlets till date. Germination of seeds is difficult to achieve and their germinability is very low (Palhares *et al.*, 2009)

Smilax goyazana is an important species of *Smilax*. Palhares and Zaidan (2011) studied the vegetative propagation of *Smilax goyazana*. They have used the cuttings of the rhizomes in three ways, i.e. a) cuttings of the rhizome containing at least five nodes; b)

cuttings with 3 rhizome nodes and 2 aerial stem nodes; and c) cuttings from the hard tuberosity containing at least one root. The cuttings were allowed to grow in the cerrado and cultivated in moist sand for three months. All the cuttings had died by the end of the experiment. The subterranean system was composed of a hard tuberosity derived from the primordial node, which emitted few roots and one or more rhizomes. The rhizomes were sometimes branched; however, they did not produce adventitious roots and the roots were not branched. Thus, according to fractal geometry, the cuttings of this species were difficult to root because they were not sub-units of the adult plant.

Soars *et al*., (2011) developed the vegetative propagation of *Smilax fluminensis*. The stem cuttings, aerial and subterranean stems about 20cm long with two nodal regions were subjected to treatment with distilled water (control) and Indol butyric acid (IBA) at 100ppm. The best germination percentages obtained for *S. fluminensis* were 80% at 20-30ºC under light and 85% at 30ºC in the dark. Only subterranean stem cuttings showed significant difference concerning fresh and dry matter of roots with higher values in treatments with hormone compared to the control. *Smilax bona-nox* L., *S. glauca*, *S. laurifolia* L., *S. pumila* Walter, *S. pseudochina* L., and *S. smallii* Morong. All native North American species have been propagated vegetatively by dividing tubers into sections that contain at least one bud and some of the fibrous roots from the original tuber (Luna, 2008). In this method, the divided tubers must contain at least one node that exhibits active bud elongation or growth (apical dominance), because secondary lateral buds on the tuber may remain dormant. The rhizomes of *S. californica* (A. DC.) A. Gray, *S. coriacea* Spreng., *S. tamnoides* L., and *S. walteri* Pursh and *S. melastomifolia* Sm. are divided into sections, with each containing at least one bud and usually some roots, and then transplanted into the containers (Luna, 2008).

Cutting propagation of the Brazilian species, *S. fluminensis* Steud. was studied by Soares *et al.,* (2011). The aerial and subterranean stem cuttings with 20 cm (8 in) in length with 2 nodes were treated with 100 ppm Indolbutyric acid (IBA) or distilled water (control). The results showed that the subterranean stem cuttings generated more adventitious roots than the control and aerial stem cuttings.

2.3. VEGETATIVE PROPAGATION BY THE CUTTINGS OF RHIZOME:

Costus speciosus is an endangered medicinal plant under the family Zingiberaceae. Although the plant can be propagated from seeds, stem cutting and rhizomes; but commercially, it is being propagated only through rhizome cuttings. The rhizomes have a number of buds most of them being concentrated around the stem scars and the tips. According to Pandey *et al*., (2011) the cuttings of rhizome pieces for propagation should have at least 2 viable buds. Rhizome pieces weighing around 40 g are selected. About 2000-2400 kg of fresh rhizomes are required for planting one hectare of land.

Inula racemosa is an important and critically endangered medicinal plant species of North Western Himalayas. For its development, conservation and commercialization. Shabir (2010) developed a protocol for the vegetative propagation by rhizome splitting as a means of vegetative propagation. The collected rhizome portion was split longitudinally with a sterilized razor blade/knife into 2, 4 or 8 pieces, according to the size of the parental rhizome, with each piece having at least one vegetative bud. The Split rhizome cuttings were treated with varying concentrations of IAA, IBA and GA3 (25ppm, 50ppm and 100ppm) for 48hours. The shoot sprouting and percentage survival (rooting) were highest in 100ppm of IAA (88.89% and 77.78% respectively), as compared to control with 77.78% of sprouting and 44.45% of rooting, respectively.

Acorus calamus is an important Himalayan medicinal herb. Due to over exploitation and increasing demand in herbal market the species required immediate attention and priority for conservation. Keeping this in view, Bisht and Bhatt (2014) investigated to enhance the growth performance percentage of *Acorus calamus* by vegetative propagation. They collected the rhizomes from the wild habitat and divided them into segments of 2 cm. in length having at least two buds. The rhizome pieces were planted in various soil compositions with the treatment of different concentration of IAA and IBA under nursery condition. Treatment of rhizome by various concentrations of hormones and soil treatments (addition of litter) improved the growth performance. The maximum sprouting was recorded in IAA 100ppm being 63.67% ± 3.21 and minimum in IBA 200ppm (36.67%±0.00) followed by control (39.67%±0.58). On the other hand in case of rooting highest result was observed with IAA100ppm followed by IBA 50ppm (56.67 % ± 2.89), IBA 100ppm (55.67% ± 2.89) and in control (37.33% ± 0.58). Survival of root

cutting percentage was observed to be highest in IAA 100ppm (60.00% ± 1.00) followed by IBA 50ppm (55.00% ± 5.00), and lowest in control being 37.33% ± 0.58. Under nursery conditions with soil as rooting medium sprouting percentage and rooting percentage was found to be highest in litter (28.00% ± 0.00) followed by litter + sandy soil (18.00 ± 0.00). Survival percentage of rooted cuttings was observed to be maximum in litter (30.00 ± 0.00), while it was least in sand + litter (18.00 ± 0.00) treatments.

Boesenbergia siphonantha is a potential ornamental plant endemic to Andaman Island, found in the evergreen forests at an altitude of 5-45 m MSL. The plant is propagated through rhizomes and seeds. Prabhu Kumar *et al.,* (2013), reported the effect of temperature and light on the vegetative propagation of this species in their off season period. The experiment was started in the beginning of autumn season. The rhizomes with root tubers were treated with Copper oxychloride (COC) for 30 minutes. The COC treated rhizomes were stored at 15^0C, for 6-9 months from March-November. Observations were made at regular intervals to assess the condition of the rhizomes. The rhizomes were found to have started sprouting from August onwards. The sprouted rhizomes after chilling were planted at regular intervals with a gap of one month i.e., September, October and November. The rhizomes were planted in 8" earthen pots using potting mixture containing soil, sand and cow dung in 1:1:1 proportion. After sprouting each set were transferred to an experimental chamber setup in the green house. The night break treatments were carried out in the green house by exposing the plants to 2 hours additional illumination for 20 - 22 Hrs. using 100 Watt incandescent bulb controlled by an electronic timer. There were four treatments: T1 - night break supplied from sprouting of the first shoot until the floral spike emerged; T2 - the experiment continued until the first floret opened; T3 - plants without night break T4 - the control plants without chilling and night break. For each treatment 4 rhizomes were taken. From the results it has been observed that an induced off-season flowering is obtained in the Island Purple Ginger through low temperature and night break. There is an extension of flower production from December to March, when normal plants remain dormant under soil. T1 and T2 didn't show any significant variation in the growth performance, floral characters and flowering. No flower initiation was observed in T3 and growth pattern was found to be

similar to that of T1 and T2. The control T4 did not germinate and T1 and T2 showed better rate of growth and performance compared T3.

Angelica glauca Edgew., a high value medicinal, aromatic and edible plant species, is a native and endemic to the Himalayan region. The vegetative propagation of this plant is done by root cutting. According to Butola *et al*., (2010) vegetative propagation through root cutting is the most reliable method for successful establishment and early flowering of this plant. Apical parts of rhizome (2-3 cm) planted during April-March gave 100% rooting without any treatments. Rooting in lateral mature segments of the rhizomes can be increased by the treatment of IAA (0.25 mM or 1.25 mM).

Heracleum candicans Wall. (Apiaceae), an endangered Himalayan native medicinal herb, is commercially useful as a major source of Xanthotoxin. Butola *et al*., (2010) developed a simple, low cost and proven propagation protocol, agro-and post harvesting technologies for this species. In this plant species, seed germination was found to be higher as compared to vegetative propagation from the rhizome. Vegetative propagation is not preferred due to poor rooting, higher chances of decaying of roots and subsequent low yield. However, rooting of the rhizome was reported by them to have increased by the treatment of IBA (0.25 mM).

2.4. EFFECT OF ORGANIC MANURE:

Ayisha (1997), reported that uptake of major nutrients and yield was highest at 20 t FYM/ ha. Sadanandan and Hamza (1997), reported that the application of organic cakes increased the nutrient availability, improved the physical condition of the soil and increased the yield of ginger. *Kaempferia galanga* has been reported to have responded well to organic manuring and gave higher yields with 30 t/ ha of FYM (Thomas *et al*., 1997, 1998).

Menon and Potty (1998, 1999), reported that FYM application led to more balanced development of yield components in *Njavara* a variety of rice. Joy *et al.,* (1998) reported that application of FYM at 20 t ha-1 year-1 produced significantly higher rhizome yields in Alpinia.FYM application at 20 t ha-1 was the best for realising maximum yield of rhizome in *Curcuma zedoaria*. Joy and Thomas (1998) reported that organic manure application enhanced the growth, bloom and tuber formation in *Gloriosa superba*. Riba (2000), observed better growth of ginseng due to the addition of cow dung. Kasera and

Saharan (2001), reported that application of FYM at 8 t /ha was suitable for obtaining maximum plant growth and biomass in *Evolvulus alsinoides*.

Lohani *et al*., (2012) reported the effect of different organic amendments on the propagation of *Polygonatum verticillatum* to develop techniques for appropriate harvesting and cultivation practices for sustainable utilization of this plant. Three types of organic fertilizers namely*;* farmyard manure, forest litter and vermicompost were used to see their effect on the survival, growth and yield. The fertilizers at 60 qut/ha) were added in the beds in two doses, before planting (at the time of bed preparation) and after sprouting. The percentage of survival was observed to be 100% in all type of beds. The cultivation of *P. verticillatum* was found to be significant for all the pre-harvest and post-harvest agronomic characters viz. days to sprout, avg. height per plant (cm), average number of whorls per plant, fresh weight of rhizomes harvested (qut/ha), and dry weight of rhizomes obtained.

2.5. VEGETATIVE PROPAGATION OF ENDANGERED MEDICINAL PLANTS:

Berberis aristata DC. is a critically endangered species of Indian Himalaya due to its extensive collection of roots for its chemical Berberine alkaloid. Ali *et al*., (2008), conducted an experiment by taking different cutting portions, (viz., apical, sub-apical and basal, which were treated with various IBA concentrations viz., control, 2500, 5000 and 7500 ppm.). The results have shown that the apical cuttings when treated with 5000 ppm IBA concentration performed significantly better in sprouting (85%) and rooting percentage (50%) in comparison to other treatments. While the control treatment had shown no rooting in all types of cutting portions.

Sharma (2009), developed the standardize vegetative propagation techniques in selected RET medicinal plants *viz*., *Celastrus paniculata*, *Embelia tsjeriam-cottam* and *Premna integrifolia*. He used different types of stem and root cuttings with the treatment of different growth regulators at different concentration.. In root cuttings, better rooting was recorded (81 and 35%) at IBA 2000 ppm against control (52 and 15%) in *C. paniculata* and *E. tsjeriam-cottam*, respectively. The next best treatments were coumarin 1000 and IBA 1000 ppm in *C. paniculata*. In *E. tsjeriam-cottam*, IBA 1000 and 500 ppm were found to be the best next to IBA 2000 ppm. Among the different treatments, the field establishment percent was significantly higher (i.e. 95, 92.5 and 94 %) in *C. paniculata*

root, shoot cuttings and *E. tsjeriam-cottam* root cuttings treated with IBA 2000 ppm. In *P. integrifolia* maximum field establishment (96.5%) was observed under IBA 1000 ppm.

Podophyllum hexandrum Royle is an endangered medicinal plant of Western Himalayas. Kharkwal *et al*., (2008), studied the vegetative propagation of *Podophyllum hexandrum* Royle. The collected rhizomes were divided into transverse segments (2- to 3-cm long) and treated with indole-3-butyric acid (IBA), Indole- 3-acetic acid (IAA), or a-naphthalene acetic acid (NAA) (25–600 ppm each) and 25–400 ppm kinetin (KN) or 6-benzylaminopurine (BAP) for 3 h each. The treated segments were planted in 3-in. plastic sleeves containing sand: soil: farmyard manure (1:1:1) and kept under the greenhouse conditions. The result showed that bud emergence started 2 weeks after sowing. Auxin and cytokinins increased the number of bud emergence compared to the control but rooting was induced only after 6 weeks of sowing, and by 12 weeks, all the rhizome segments were able to root.

Pandey *et al*., (2011) developed the agrotechnique for *Costus speciosus* Koen ex. Retz, an important endangered medicinal plant. It is mainly propagated vegetatively through rhizome cutting. The cuttings of rhizome pieces for propagation should have at least 2 viable buds. Rhizome pieces weighing around 40 g should be selected. About 2000-2400 kg of fresh rhizomes are required for planting one hectare of land. The rhizome pieces are placed at a depth of 8-10 cm taking care to place the eye buds facing upwards, horizontally in rows 50 cm apart and covered with soil. Trials suggest that the optimum dose for obtaining maximum yield of Diosgenin is 45 Kg N, 30 Kg P2O5 and 30 Kg K2O along with 15 t/ha of FYM.

2.6. Vegetative propagation of *Homalomena aromatica* (Roxb.) Schott.

An agrotechnology was developed for the cultivation of *Homalomena aromatica* (Roxb.) Schott, by North Eastern Development finance Corporation Limited, Guwahati. (Ahmed, 2005). According to them the propagation of this species is by rhizome cutting during the month of April to June. The rhizome cuttings must be 2.5-3 cm size with active buds. On the other hand the appropriate method for the cultivation is ridge and furrow method.

Khan *et al.,* (2012) studied the agrotechnology of *Homalomena aromatica* (Roxb.) Schott, an aromatic plant of north east India. They cultivated the six genotypes of this

aromatic plant with different spacing with the application of NPK during two seasons, i.e. Kharif and Rabi. From this study they found that 45× 30 cm in plain areas and 60× 30 in hill slopes are the best spacing for the yield. While 100kg/h N and 80 kg/ha P_2O_5 are the best fertilizer application under the field condition.

2.7. CULTIVATION OF ENDANGERED MEDICINAL PLANTS:

Acorus calamus (Sweet flag) is one of the endangered medicinal plants mostly grown in the wild. Singh and Nongmaithem (2013) cultivated this medicinal plant for two consecutive years in the Kharif season of 2010 and 2011, to assess the possibility of optimizing rhizome yield of sweet flag (*A. calamus* L.) by maintaining different spacing under field conditions. The wider spacing 40 cm × 40 cm gave significant result in case of increase in rhizome length and weight; while in the closer spacing of 20 cm × 30 cm significantly higher rhizome yield of *A. calamus* was recorded followed by 30cm × 30 cm spacing.

Polygonatum cirrhifolium Royle (Meda.) is a highly demanded medicinal plant belonging to the family Liliaceae. Due to its great market potential, it is harvested and grown in an uncontrolled way; overexploitation has caused the decline of population of this herb from its natural habitat. A field experiment was undertaken by Lohani *et al*., (2011) to study the effect of different organic fertilizers and the nature of nursery beds on the survival, morphological growth and yield of this plant. The observations were recorded in 15-day interval. The results show that yield was lower in control beds when compared with the forest litter, farmyard manure and vermicompost treated plots in all type of beds prepared in rows, furrows and plain. The yield was highest at beds located at plantation with furrows which were supplemented with forest litter. Thus, it was concluded that the cultivation of *P. cirrhifolium* is optimal in beds with rows and furrows (plantation in furrows) and supplemented with forest litter. In addition, they reported that the composted organic material also improves both soil quality and fertility and regulates water.

2.8. MICROPROPAGATION:

Micropropagation is the production of new plants under ultra controlled environment within the culture vessel (i.e. glass bottles and tubes) (Hartmann *et al.*, 2007). It is one of

the important contributions of plant tissue culture to commercial plant propagation and has vast significance (Jha and Ghosh, 2005).

George Morel (1960) is the first researcher who first showed the potential of clonal propagation of *Cymbidium* sp. (Orchid) by *in vitro* technique in 1960. He used the shoot tip as explant (Jha and Ghosh, 2005).

Commercial micropropagation had begun in the United States during the year 1965 with orchid production. Commercial micropropagation emerged as an important method in horticultural field in the last quarter of twentieth century (Hartmann *et al.*, 2007).

The major credit goes to Toshibo Murashige for the establishment of micropropagation technique. He is one of the pioneering workers who demonstrated that many plants can be propagated *in vitro* (Hartmann *et al.*, 2007).

In 1997, it was estimated that the worldwide production of plants produced through micropropagation reached worth $15 billion dollar (Suman *et al.*, 1997).

Demand for micropropagated plants is high, but production is limited because of high labour costs (Hartmann *et al.*, 2007). Due to the labour costs, micropropagation in developed countries appears to have leveled off since the last 1980s (Suman *et al.*, 1997).

2.8.1. Asymbiotic seed germination and micropropagation of orchid:

Orchid is one of the highly evolved perennial plants in the plant kingdom. Now a days due to illegal threat and the anthropogenic pressure, most of the orchid species are categorized as threatened species. Therefore some urgent need for the multiplication and conservation including *ex situ* and *in situ* propagation must be taken up otherwise, this fascinated plants will be gone forever from its natural habitat.

The orchid seeds are poorly organized and they lack endosperm. They have undifferentiated embryos that require a fungal stimulus for germination in nature (Sagawa, 1963). But they have the ability to germinate *in vitro* under aseptic conditions with proper combination of artificial media and plant growth regulators.

Survey of the previous literature reveals that immature orchid seeds are better germinated than mature seeds. On the other hand the stage at which the embryos can be cultured successfully varied with the species, genus, hybrids, nutrient medium and culture condition (Arditti *et al.*, 1982).

The methods and Procedures for *in vitro* seed germination of one orchid species are not always applicable to other orchid species (Arditti, 1982).

The type and concentration of plant growth regulators play an important role during the *in vitro* propagation of orchids (Arditti and Ernst, 1993), because seed germination, shooting and rooting always require different plant growth regulators in different concentrations.

Bernard (1990) is one of the pioneering workers in the development of *in vitro* culture techniques of orchids. He successfully isolated the root infecting fungi helpful in orchid seed germination. On the other hand Knudson's work showed for the first time that germination of orchid seeds was possible *in vitro* without fungal association (Rao, 1998).

Many investigators and workers have used immature seeds from unripe pods for the seed germination of orchids in aseptic culture (Withner, 1995; Ito, 1995; Nimato and Sagawa, 1960; Rao and Avadhani, 1964; Teo and Teo, 1976; Mitra *et al*., 1976; Vij *et al*., 1981; Bopaiah and Jorapur, 1986)

Laelia speciosa is an endangered epiphytic orchid species. The seed germination of this orchid was studied by Avilla Diaz *et al*., (2009). They took 4, 7, and 9 months mature capsules which were hand-pollinated and its seeds were found to be germinated on Murashige and Skoog's (MS) media with 30 g l-1 sucrose and five concentrations of benzyladenine (BA) (0.0, 0.04, 0.22, 0.44, and 2.22 lM) under light and dark conditions. Gibberellic acid (GA3; 0.0, 0.29, 1.44, 2.89, 14. 43 and 28.87 lM) with naphthalene acetic acid (NAA; 0.0, 0.54, 1.34, 2.69, and 5.37 lM) were evaluated for *in vitro* sub cultivation. MS medium with 30 g l-1 sucrose was found to be the most effective for germination. The effects of BA and light on the germination of *L. speciosa* seeds differed with pod maturity. All the mature seeds germinated using 0.44 l M BA and light. The highest frequency of germinated seedlings (60%) was obtained using mature seeds grown on MS medium without BA and under light illuminated condition. For subculture, MS with 30 g l-1 sucrose, 2.69 lM NAA, and 0.29 lM GA3 was found to be effective. Plantlets of 5 cm in length were transplanted to the greenhouse, and a 77.5% of survival rate was obtained.

Mazumder *et al*., (2010) studied the *in vitro* seed germination of *Papilionanthe teres* (Roxb.) Schltr. which is a medicinal orchid found in all the reserve forests of Southern

Assam, India. From the mature pods, seeds of this orchid were inoculated in Murashige and Skoog's (MS), Knudson C (KnC), Commercial Orchid Maintenance (OMM) and Commercial Orchid Maintenance Replate (OMR) medium (HiMedia). Best germination and growth were observed in commercial orchid maintenance replate medium supplemented with 2mg\L IAA and 5mg\L KN followed by OMM and KnC. No germination was observed in MS media.

Bhattacharjee *et al*., (2010) established a protocol for the seed germination of *Dendrobium densiflorum*, a threatened orchid species. They inoculated the seed of eleven months mature capsules in Knudson C, MS, Nitsch and Mitra *et al*. medium. Maximum seed germination was recorded in Mitra et al media (92.1%) followed by MS (90.4%) and Nitsch (87.1%) respectively.

Dactylorhiza hatagirea (D. Don) Soo. is a critically endangered orchid species. The green pod of the orchid was studied by Giri and Tamta (2012). Four nutrient media: Knudson C (KC), Murashige and Skoog (MS), Vacin and Went (VW) and Vejsadova (VJ) were tested by adding different growth additives. MS medium supplemented with peptone (P) (1.0 g/L), morphoinoethane sulphonic acid (MES) (1.0 g/L) and activated charcoal (AC) (0.1%) were found to be the most effective medium for the development of protocorm like bodies (PLBs), development of chlorophyll and for the plantlet formation. To improve vegetative multiplication, tubers were treated with α- naphthalene acetic acid (NAA), indole-3- butyric acid (IBA) and Indole acetic acid (IAA) before planting. Rooting was observed in only apical segments. Maximum rooting (38.88%) was induced with 50.0 μM IBA treatments.

Arundina graminifolia (D. Don.) Hochr. Is an endangered orchid species popularly known as bamboo orchid. A protocol was developed by Das *et al.,* (2013) for the micropropagation of this species. Nodal segments were inoculated in MS media supplemented with different concentration of NAA and Kinetin (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) for shoot proliferation and NAA and IAA combination for rooting. MS medium supplemented with 1.0mg/L NAA+ 2.5mg/L KN showed highest shoot proliferation with root length of 3.50 cm. MS media when supplemented with 3.0mg/L IAA induced root with highest root length of 4.7cm. 87% micropropagated plants survived after field transfer.

An efficient protocol for seed germination and micropropagation of *Dendrobium chrysanthum* Wall ex Lindl. was established by Rao and Barman (2014). Four different nutrient media were used for seed germination and early protocorm development: Murashige and Skoog (MS), half –strength MS, Knudson 'C' (KC), and Vacin and Went (VW) supplemented with the combinations and alone with four plant growth regulators i.e. 6-benzylaminopurine (BAP), kinetin (KN), α-naphthalene acetic acid (NAA), and indole-3-butyric acid (IBA) were studied. MS medium was found to be the most ideal for seed germination (98±0.48) and lowest in VW (71.12 ±0.42). Subsequently 3 months old protocorm were sub cultured in fresh MS medium supplemented with different concentrations of BAP, KN, NAA, and IBA alone and in combination. After 30 days highest secondary protocorm development (21.25±0.63) were observed in MS medium containing BAP (4.0μM). MS medium supplemented with 8μM IBA induced the maximum root growth per shoot. After 16 days of transfer to green house the survival rate of these transferred plants were found to be 88%.

Many rare, vulnerable and endangered orchids are found in the North Eastern region. The propagation and seed germination of some of the economically and medicinally important orchids were done by different workers. Sharma and Tandon (1986, 1987, 1990), Kumaria and Tandon (1991), Kumaria (1991), Sharma (1993), Sharma and Chauhan (1995), Roy and Sharma (1992), Hazarika and Sarma (1995), Sarma (1998), Sarma and Sarma (1997), Kaur and Sarma (1995, 1996, 1997a, b), Devi *et al*., (1990), Mazumder *et al*., (2010),

2.8.2. Micropropagation of *Bulbophyllum* species:

Mazumder (2012) studied the *in vitro* culture of *Bulbophyllum careyanum* (Hook.) Spreng. a rare orchid species of Southern Assam, in three different media MS, B5 and White's media with different combination of the plant growth regulators viz. 2,4-D, NAA and IAA. The explants of his study were leaf tip. But the explants did not show any response.

Than *et al*., (2012) developed the reproducible protocols for the micropropagation of *B. auricomum* Lindl. They cultured the seeds from the three-month-old immature seed

capsules aseptically on Murashige and Skoog's (MS) and modified Knudson C (KC) medium. MS medium was found to be the most effective culture medium for seed germination, and a high rate of protocorm formation, multiplication, and differentiation into seedlings. The combination of 1.0 mg/L 6-Benzylaminopurine (BAP) with 0.5 mg/L α-Naphthalene acetic acid (NAA) induced maximum number of multiple shoots (15.0±1.7) and optimal rooting (3.0±0.7) of plantlets was obtained in 1.0 mg/L kinetin (KIN) and NAA 2.0 mg/L. *In vitro* flowering was observed within six months after seed sowing, with the highest percentage (50±15.8%) of flowering was observed in MS medium with 1.0 mg/L BAP + 0.5 mg/L NAA; with 2.0 mg/L BAP + 1.0 mg/L NAA or with 0.5 mg/L BAP + 2.0 mg/L NAA. *In vitro* developed flowers from different treatments were dissected and compared with flowers of the field grown plants.

2.8.3. Micropropagation of *Smilax* species:

Watanabe *et al*., (1990), tried to establish the *in vitro* propagation technique of *Smilax oldhami* Miq. According to him the whole plants were regenerated from stem, tendril and flower bud tissues of the species

In vitro propagation of *Smilax glabra* Roxb. was studied by Zeng *et al*., (2005). The results indicated that sterilized explants cultured on MS + 6-BA 1.0 mg/L + NAA 0.1 mg/L could lead to faster budding, the medium MS + 6-BA 1.0 mg/L + NAA 0.1 mg/L + 15% CM suitable for the proliferation, and 3/2MS + 6-BA 0.05 mg/L + 4% Sugar media in suitable for strong buds. Treatment on the medium H (modified) + NAA 0.5 mg/L was found to be best for rooting. The survival rate of the test-tube plantlets was 95% in transplanting on media contained turf-sand (1:1).

The protocol for the micropropagation through multiple shoot formation from nodal segments of *Smilax zeylanica* Vent. has been developed by Thirugnanasampandan *et al.*, (2009). The nodal explants were cultured on half strength MS medium containing BA (0.5 mg/l) and IAA (1 mg/l) with activated charcoal (100 mg/100 ml) and it produced single shoot within 7-10 days. The shoots were multiplied by using nodal segments of *in vitro* regenerated shoot in modified half strength MS medium supplemented with KIN (2 mg/l), L-Glu (0.5 mg/l) and activated charcoal (100 mg/100 ml). Two shoots were found to have formed. Rooting of the microshoots was achieved in half strength MS medium

fortified with IBA (1 mg/l) within three weeks time. The rooted plantlets were transferred to potting medium containing vermiculite, sand and coir pith (1:1:1). Survival of the plantlets under *in vitro* condition was recorded to be 70%.

An efficient method for the rapid propagation of *Smilax china* from the axillary buds was established by Song *et al.,* (2010). Axillary buds of *S. china* collected from the selected plants were cultured in various culture media (2MS, MS, 1/2MS, WPM, B5 and SH medium). Shoot was induced from axillary buds on MS basal medium after 4 weeks of culture. 1/2MS medium showed a higher growth rate compared to those of the others, while the lowest shoot growth was obtained in 2MS medium. Among the sucrose concentrations, 5% sucrose was the optimum level for the shoot growth from axillary buds. Among the cytokinins, 0.5 mg L -16-benzylaminopurine (BAP) treatment showed the best performance on shoot multiplication, yielding average shoot multiplication forming about 2.4. Rooting was induced directly near the base of the shoot on 1/2MS medium containing three-Auxin i.e. α-naphthalene acetic acid (NAA), Indole acetic acid (IAA) and β-Indolbutyric acid (IBA) (0.5 and 1.0 mg L -1). The 1.0 mg L -IBA treatment induced earliest rooting with maximum f root numbers and root growth. These rooted plantlets were successfully transferred to pots for 4 weeks hardening process, and were transferred to soil with above 90% survival rate.

A rapid multiplication through *in vitro* propagation of *Smilax corbularia* was developed by Jirakiattikul *et al*., (2013). The effect of MS (Murashige and Skoog) medium, salt strengths and plant growth regulators were investigated for shoot multiplication and root induction in their studies.. For shoot multiplication, single-node explants were cultured on half strength MS (½ MS) or full strength MS (MS) media supplemented with 15% coconut water (cw) in combination with either 0.5-1.0 mg l-1 BA (Benzyladenine) and 0-1.0 mg l-1 IAA (Indole-3-acetic acid), or 0.5-2.0 mg l-1 kinetin for six weeks *in vitro*. The results suggested that ½ MS medium supplemented with 15% Coconut water (CW) and 1 mg l-1 BA, and MS medium supplemented with 15% CW, 1 mg l-1 BA combination with or without 0.5-1.0 mg l-1 IAA gave the best shoot formations of 95-100%. The number of shoots and number of nodes per shoot obtained from these media were 1.0-1.3 and 4.4-5.0, respectively. For rooting, shoots were cultured on quarter

strength MS (¼ MS), ½ MS or MS medium supplemented with 0-2 mg l-1 NAA (α-Naphthalene acetic acid) for 12 weeks. The highest rooting percentages occurred on ¼ MS medium supplemented with 2.0 mg l-1 NAA (65.83%) and ½ MS medium supplemented with 0.5 mg l-1 NAA (66.67%), with the number of roots per shoot of 9.6 and 8.4, respectively. The rooted shoots were successfully transplanted with the survival rate of 80.81% in plastic pots containing soil, carbonized rice hull, decomposed rain tree leaf, manure and sand at the ratio of 0.5:0.5:0.5:1:1.

2.8.4. Micropropagation of *Paphiopedilum* species:

The effect of seed maturity, media type, carbon source, and organic nutrient additives on seed germination, protocorm development, and plant growth of *Paphiopedilum* var. *densissimum, P. insigne, P. bellatulum,* and *P. armeniacum* were investigated by Long *et al*., (2010). Micropropagation frequency was enhanced through the use of 200-day-old seed in Knudson C (KC) medium, with the presence of both glucose and coconut milk in the medium. The effect of various plant growth regulators on the frequency of shoot organogenesis in four *Paphiopedilum* species were also studied. The explants of *P. villosum* var. *densissimum* and *P. insigne* (Lindl.) Stein were incubated in the presence of 5 mg/l 6-benzyladenine (BA) with 0.5 mg/l a-naphthalene acetic acid (NAA) and 0.2 mg/l BA with 0.1 mg/l NAA, respectively, showed a twofold increase in the frequency of shoot organogenesis. On the other hand the explants of *P. bellatulum* (Rchb.f.) Stein and *P. armeniacum* S. C. Chen et F. Y. Liu, were incubated in combination of 5.5 mg l-1BA with 0.5 mg/l NAA and 4 mg/l BA with 0.1mg/l NAA, respectively, which resulted in the highest frequencies of shoot organogenesis.

Liao *et al.,* (2011) successfully established the induction of shoots and regeneration of plants from the flowering plants of a sequentially flowering *Paphiopedilum deperle* and a single floral *Paphiopedilum armeni* White. by using cross-sectioned flower buds (FBs). They found that in both the species, only sections that contained the base tissue of FBs were able to produce shoots and plants. They had also observed that the sections of FBs between 1.5 and 3.0 cm from *Paphiopedilum deperle* were able to produce shoots, but only sections of FBs >2.5 cm from *Paphiopedilum armeni* White were regenerable. The microscopic observations revealed that the small bract at the FB base harboured a new

miniature FB, which further harboured a primitive FB with dome-shaped meristem-like tissues that presumably led to the plant induction. The induction rates were 57–75%, and all the plants survived in a greenhouse. They suggested that this method is potentially applicable for the micropropagation and conservation of slipper orchids.

Wattanawikkit *et al*., (2011), studied the effect of cytokinins (BAP and TDZ) and Auxin (2,4-D) on the growth and development of *Paphiopedilum callosum.* The seedlings were grown on the half strength macro- and micro-elements of Murashige and Skoog (1962) (1/2 MS) medium supplemented with cytokinins 6-benzylaminopurine (BAP) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) alone or in combination with Auxin and 2,4-dichlorophenoxyacetic acid (2,4-D). Three months after incubation, shoots in the medium with 0.5 μM TDZ produced 1.6 ± 0.40 shoots /explant. It was also observed that 1/2 MS medium without plant growth regulators produced more roots (1.80 ± 0.20) per shoot and longer roots (30.40 ± 8.04 mm) than 1/2 MS with 0.5 μM TDZ combined with 50 μM 2, 4-D. The 1/2 MS medium with BAP at 10 and 50 μM resulted in 2.30 ± 1.42 and 2.20 ± 1.03 shoots/ explant, while 1/2 MS medium without plant growth regulators resulted in 100% root induction with an average of 3.70 ± 0.62 roots per explant and mean root length of 34.01 ± 4.87 mm respectively. Overall, BAP appeared to elicit the best shoot multiplication in response with *P. callosum* shoot explants compared with either 2,4-D or TDZ. The combined effect of TDZ and BAP may be worthwhile investigating in shoot proliferation experiments in future. Root induction appeared to be restorable if TDZ is removed and BAP is reduced, as the presence of BAP at the lower concentration tested did not appear to completely inhibit root induction (unlike TDZ).

The effect of *in vitro* cutting method and medium composition on efficient shoot multiplication of *Paphiopedilum* Hsinying Rubyweb was studied by Udomdee *et al*., (2012). Among the three different *in vitro* stem cutting methods, the vertical cutting was able to produce more new shoots than horizontal and cross cutting when cultured on Hyponex based medium. It was also observed after 12 weeks of culture, the plantlets regenerated from vertical cutting were able to produce new healthy and well rooted shoots better than without cutting on the same medium. However, the newly-formed shoots which were divided into single plantlets and sub cultured in the half-strength Murashige and Skoog (MS) medium without growth regulators could produce higher

shoot multiplication than in other media. The micropropagation procedure developed in this study provides a simple means to *in vitro* propagation of *Paphiopedilum* plantlets which are able to produce large number of uniform plantlets in a shorter time, compared to the conventional propagation method.

Thongpukdee *et al*., (2013) studied the multiple shoot formation of *Paphiopedilum* 'Delrosi' Shoots. The shoot with three leaves of this species were used as explants for multiple shoot induction on the Modified Hyponex medium , supplemented with Thiadiazuran (TDZ), N6 benzyladenine (BA) or kinetin (Kn) alone and in combinations with 2,4-dichlorophenoxyacetic acid (2,4-D). All the explants were cultured for 15 weeks. It was found that TDZ alone at the concentration of 0.45µM or in combination with 4.52µM 2,4-D and 8.88µM BA in combination with 13.56µM 2,4-D promoted multiple shoots. The highest shoot sprouting efficiencies (80.0, 90.0 and 80.0%) and new shoot numbers (1.5, 1.3 and 1.1) were obtained, and the highest numbers of new shoots per explants were observed from 0.45µM TDZ, the higher fresh weight of newly regenerated shoots and number of roots per new shoot were obtained from 0.45µM TDZ in the Multiple Shoot Formation of *Paphiopedilum* 'Delrosi'. However, among the three selected treatments that provided the higher regeneration of new shoots, the higher whole FWs (2129.3mg and 2714.7mg.), shoot FWs (1149.9mg and 1553.7mg.) of original shoot explants were found from the medium supplemented with 0.45µM TDZ in combination with 4.52µM 2,4-D and 8.88µM BA in combination with 13.56µM 2,4-D compared to whole FW (1942.5mg) and Shoot FW (936.1mg) from medium supplemented with 0.45µM TDZ with 4.52µM 2,4-D (210.8mg. and 1.3 roots) and 8.88µM BA in combination with 13.56µM 2,4-D (268.4mg. and 1.3 roots) than the whole fresh weights and number of root per new shoot from 0.45µM TDZ (109.9mg and 0.6 root). For other treatments of plant growth regulators, except for 2.32µM Kn in combination with 4.52µM 2,4-D, 10–60% of explants produced new shoots with 0.1–1.5 shoots per explants. There was no new shoot observed from the medium containing 2.32µM Kn in combination with 4.52µM 2,4-D. The medium without plant growth regulator (control) 30% of explants produced new shoots with 0.3 new shoot per explants and 15.8mg whole fresh weights of the new shoots with 0.2 roots per new shoot.

Paphiopedilum liemianum Fowlie., is a terrestrial orchid species and endemic in Northern Sumatra, Indonesia. The micropropagation was of this species was established by Utami *et al*. (2015) through *in vitro* seed germination for its conservation. Four months old seeds of *P. liemianum* were germinated on the five different basal media supplemented with 2.5 μM α-naphthalene acetic acid (NAA) and the cultures were incubated in the dark for 4 weeks followed by protocorm development in the condition of 16/8 h L/D photoperiod. Germination percentage was recorded to be 78.8% in Vacin and Went (VW) medium, which was significantly higher than the other basal media. To evaluate the effect of organic nutrient additives on the seed germination and protocorm development, the seeds were cultured on VW medium amended with different of organic nutrients. Additives, especially 10% Coconut Water (CW) to VW medium improved the protocorm development well, with 33.3% of the protocorm development to stage 5 (seedling). The seedlings were cultured on VW medium supplemented with different concentrations of Thiadiazuran TDZ (0.0, 1.0, 2.0, 3.0 and 4.0 μM). Healthy plantlets with developed leaves and roots were planted in pots with sphagnum moss grown under *ex situ* condition and the result was 76% survival rate after 4 weeks.

Paphiopedilum spicerianum (Rchb.f.) Pfitz. is listed as one of the country's Wild Plants with Extremely Small Population (PSESP). The procedures for the asymbiotic seed germination and *in vitro* seedling development aimed for producing seedlings for reintroduction were developed by Chen *et al.,* (2015). The 11month to one year old matured harvested seeds were tested in six basal medium (1) 1/2 strength modified MS; (2) 1/2 strength modified MS supplemented with 10% CW; (3) 1/4 strength modified MS; (4) 1/4 strength modified MS supplemented with 10% CW; (5) modified Robert Ernst with 1.0 g l^{-1} activated charcoal; (6) modified Robert Ernst with 1.0 g/l activated charcoal and 10% CW. The highest germination was achieved in Robert Ernst medium with the addition of coconut water with a 24 h dark cycle after pretreatment with 1% NaOCl for 40 min after 30 days from germination. However, these protocorms remained white and did not develop further. Although germination was lower under the same conditions in MSCW, it resulted in healthier and greener protocorm. The seedlings developed were further assessed for advanced seedling growth under six media conditions: (1) Modified MS with 0.2 mg/l NAA, 2.0 mg/l 6-BA and 2.0 g/l activated

charcoal; (2) 3.0 g/l Hyponex No 1, 0.5 mg/l NAA and 1.0 g/l activated charcoal; (3) 3.0 g/l Hyponex No 1, 2.0 mg/l 6-BA, 0.5 mg/l NAA and 1.0 g/l activated charcoal; (4) Modified MS with 0.2 mg/l IBA, mg/l 6-BA and 2.0 g/l activated charcoal; (5) 3.0 g/l Hyponex No 1, 3.0 g/l peptone, 0.2 mg/l NAA, 2.0 mg/l 6-BA and 1 g/l activated charcoal; and (6) 3.0 g/l Hyponex No 1, 1.0 mg/l NAA, 1.0 mg/l 6-BA, 0.5 g/l activated charcoal and 10% banana homogenate. Advanced seedling development was seen in all the six tested media during a 4 month growing period, with the highest leaf growth rate seen in the same media used for seedling formation, supplemented with 1.0mg/l. Leaf length growth rate was highest and leaves remained green in color in 3.0 mg/l Hyponex No 1, 1.0 mg/l NAA, 1.0 mg/l 6-BA, 0.5 g/l activated charcoal and 10% banana homogenate medium. However, this medium was not significantly different compared to 3.0 g l^{-1} Hyponex No 1, 3.0 g/l peptone, 0.2 mg/l NAA, 2.0 mg/l 6-BA and 1 g l^{-1} activated charcoal medium or compared to modified MS with 0.2 mg/l NAA, 2.0 mg/l 6-BA and 2.0 g l^{-1} activated charcoal medium. All other media were significantly different and gave lower leaf length growth rates compared to 3.0 g l^{-1} Hyponex No 1, 1.0 mg/l NAA, 1.0 mg/l 6-BA, 0.5 g l^{-1} activated charcoal and 10% banana homogenate medium (all $P<0.05P<0.05$). The first three media tested produced yellow leaves but plantlets remained alive even till + 6 months after growth in these media. Relative leaf width growth rate was not significantly affected by the growing medium.