INTRODUCTION AND REVIEW OF LITERATURE.

Orchids stand supreme in the plant kingdom for their beauty. Their wonderful colours with a variety of colourful patterns, has enthused the admiration of the serious scientists, horticulturists and laymans alike. the Orchidaceae form the largest family of monocotyledonous plants, constituting about 7% of the angiosperms and nearly 40% of the monocots. It has 20,000 to 30,000 spp distributed over nearly 750 genera and more than 78,000 natural and man-made hybrids have been reported. More than 1,600 spp distributed over 220 genera are available in India (Hooker, 1890), out of which 600 spp. were reported to be distributed in North-East India. Orchids are cosmopolitan in distribution and found almost all over the world. While orchids are perennial herbs, some of them are epiphytic, some are saprophytic and some others are terrestrial (Holtum, 1953). Its vegetative parts show a great variation in their structure.

Most orchids, however, are grown for the intricate beauty of their flowers. The variety of flower form is far greater than that of plant habit, with almost every colour except black represented, even a true blue is found. Flowers may be spotted, blotched or striped with a contrasting colour. The lip is usually the most colourful and spectacular part of the flower and has often developed an intricate, occasionally even bizzer, shape. Moreover, orchid flower is scented too. In natural conditions, most of the flowers are not pollinated due their species specific requirement of pollinator. However, artificial pollination
can be made and thereby hybrids could be produced invariably to meet the increasing demand of orchid flowers. Orchid flowers can last for a few days up to 4 months, thereby they provide cut blooms for variety of flower arrangements.

Along with ornamental beauty, orchids are used in various purposes. The flowers of Aerides and Rhyncostyles were adorned by the ladies as a symbol of sanctity and womanhood, respectively (Hedge, 1984). Flowers of R. retusa (popularly known as 'Kopowlul' in Assamese) were used for adornment of ladies during popular 'Bihu' festival. Some orchid spp are of high medicinal value. Tubers of Hebenaria spp and Eulophia nuda were used as tonic for treatment of blood purification, leprocy and certain other purposes (Trivedi et al., 1961). Leaves and stems of Cleisostoma willamsonii were used for healing bone fractures in Arunachal Pradesh (Hedge, 1984). Different parts such as stems and roots were used as food in different countries. The tubers of Cyonoorchids flexusa were eaten in Eastern Madagasker (Usher, 1974). In N. E. India, local tribes used pseudo-bulbs of Cymbidium spp for food. The tubers of Orchis laxifolia L. were reported to be edible (Maheswari and Singh, 1965). Moreover, 'Salep' - a nutritive meal with starch or jelly was made from the dried tubers of the genus Orchis. The famous 'Vanillin' used for flavouring certain edible substances was extracted from green-pods of Vanilla planifolia.

Today, orchids constitute a flourishing trade in several countries and has become a multimillion dollar business. Introduction of multigeneric hybrids has helped commercial production of quality flowers. Presently, USA,
Thailand, Indonesia, Singapore, Malaysia are some of the countries exporting orchids.

In spite of their commercial value, the Indian orchids have not yet gained attention so far as international trade is concerned. Although N.E. India provides natural home for most of the economically important orchid genotypes, the orchid growers, traders generally depend on the natural forest to meet their demands. This has led to gradual depletion of orchid germplasm from the natural habitat. As a result, many valuable orchid species previously reported from N.E. India are now enlisted as endangered in IUCN red data book. This includes important orchid species like: *Acanthepipium sylhetense*, *Arundina graminifolia*, *Calanthe masuca*, *Cymbidium iridioides*, *Dendrobium crysotoxum*, *D. densiflorum*, *D. devonianum*, *Vanda coerulea*, *Phalaenopsis mannii*, *P. parishii*, *Phaius tankervillae*, *Paphiopedilum fairieanum*, *P. insignae*, *P. villosum* etc. Other important causes of decreasing orchid taxa particularly in N.E. India is the natural hazard like seasonal floods, landslides, erosion, shifting cultivation (Jhuming), forest fire, deforestation etc. (Arora, 1983, Kataki et al., 1984).

For a long time, the major means of clonally propagating orchids was through division of pseudobulbs. Orchid may be monopodial or sympodial, and the propagation method also differ accordingly. In monopodials, 'terminal cutting' methods are normally adapted as the side shoot occur infrequently. In the case of 'sympodials' separation of clumps was used for propagation. These traditional methods are time consuming and large-scale propagation was practically
impossible. Moreover, application of 'Keikis' is also very slow and only 2-3 plantlets can be obtained. Orchid seeds are tiny, powdery, varying in number from 1300-14,000,000 per capsule. Orchid seeds have relatively undifferentiated embryos, lacking endosperm and cotyledons (Maheswari and Narayanswamy, 1952). They require the association of an appropriate mycorrhizal fungus for supplying them with nourishment for proper seedling growth. Moreover, because of their specific mycorrhizal association less than 3-5% seeds are germinated in natural condition, thereby decreasing the population to rarity.

Orchid seed capsules, for their complete maturation and dehiscence, may require a few months up to 3 years depending on the sps. The seeds in most cases decrease their viability during storage. Further, mature seeds were hard to germinate due to presence of certain dormancy inducing factors. Eversince, the first successful experiments of Knudson (1946), the importance of asymbiotic germination of immature embryos was being realized in propagating orchids for commercial and conservation purpose. The culture of immature, embryos on the other hand, would be time saving and particularly helpful in understanding germination of those seeds in which pod development take several months. Therefore, it will be very convenient in understanding germination potentialities of immature embryos in artificial culture media for purpose of mass-propagation.

Propagation of orchids from seeds (immature or mature) involves three phases - germination, protocorm formation and seedling development in sequence. However, the requirement of nutrient composition of each phase
might be different considerably. The earliest stage at which embryos germinate in culture successfully varies with the genus, species and the environmental conditions (Arditti, 1979).

Reports on "green capsule" (immature) cultures (Knudson, 1950, Sagawa, 1963; Saudela, 1965; Davidson, 1965) have indicated that orchid embryos become viable and were capable of normal development before they become fully ripe. In V. joaquim, 12 week old pods germinated well although pod development completed in 16-17 weeks (Rao and Avadhani, 1964). The success of embryo culture has been reported in Dendrobium sps (Nimoto and Sagawa, 1961). Vanda (ong, 1969) Cymbidium (Kusomoto et al., 1977). The shortest period for in vitro embryo culture ranges from 40-85 days in various orchid sps. (Pierik and Steegman, 1972; Arditti, 1981; Kerbauy and Handro, 1981; Pack, 1987).

Chemically defined media were used for the induction of protocorms from embryos in various orchid sps. Knudson (1950) first used his medium B for induction of protocorms in Cymbidium. Hegarty (1955) used N medium for protocorm induction and seedling development after 6 mo in Vanda sps. In the case of R. gigantea the use of two media (EG-1 for protocorm formation and VW medium for seedling culture) coupled with frequent transflasking improved seedling growth (Kaewbambrung, 1967).

The induction of protocorms from Cymbidium sps were also reported (upto 60%) in KC and VW medium (Sawa and Namba, 1974; Ueda and...
Protocorm induction was reported in V.W basal medium (upto 50%) in *C. longifolium* within 40-50 days (Murulidhar and Mehta, 1986). Whereas, in the case of *C. aloifolium* Kc medium was found to be suitable for protocorm induction (Bopiah and Jorapur, 1986). Chennaveeriah and Patil (1973) used White's medium for immature embryo culture of *Spathoglottis plicata*.

Although success in the embryo culture in certain media were reported, their percentage was relatively lower. In many cases, protocorm development was observed but the seedling growth was hampered. Thus, addition of growth hormones, vitamins and complex additives were necessary not only for protocorm induction, but also for their growth and development. The growth promoting effect of CW in embryo culture experiments have been reported (Kerbauy and Handro, 1981). It enhanced both seed germination and seedling development in *Vanda* sps (Pages, 1971). However, CW inhibited *Dendrobium* seedling growth (Kotomori and Murshige, 1965) and induced abnormal proliferation in *Dendrobium* and *Vanda* seedlings (Rao and Avadhani, 1964). It was used along-with other additives like banana extract, pineapple extract, peptone in nutrient media for protocorm growth (Arditti, 1979). Strained apple juice along-with peptone was found to be effective on seedling development of *Paphiopedilum* and *Cymbidium* sps (Tuskamoto et. al., 1963). Banana extract, tomato juice and CW was found to be effective for seedling development in *Vanda* and *Phalaneopsis* sps (Valmayor, 1974). Caseine hydrolysate (CH) and yeast extract (YE) in Pfeffer's medium had improved germination in *Dactylorhiza purpurella* (Harvias, 1972). Peptone...
alone (Withner, 1955; Mathews and Rao, 1980), or along with banana extract improved germination percentage in A. japonicum (Chung et al., 1984). Peptone, CW, BE, thiamine HCl, niacin, glycine along with kin was found to have resulted healthy seedling growth of Cymbidium sps (Bopiah and Jorapur, 1986). The percentage of protocorm induction was higher in Kc medium containing potato extract in Cyperepedium sps (Harvias, 1982). Incorporation of vitamins to basal medium have improved protocorm induction and its subsequent development in certain orchid sps (Bahme, 1949; Rossifer, 1960; Lawrance and Arditti, 1964; Ueda and Torikata, 1972). Vitamins also played important role in protocorm development and seedling growth in Ophrys sphagoides (Lucke, 1971) and in certain other orchid sps (Mitra et al., 1976; 1987). Incorporation of growth hormones along with other additives have also been reported. Hegarty (1955) observed better protocorm development of Cyperepedium hybrids cultured in N3 medium along with vitamins and IBA or IPA at pH 6.0. Healthy protocorms were developed after 4-6 months in culture in this medium. Pffer's medium containing 1mg/l IAA or kin was found to have significant effect on protocorms of Coeloglossum viride (Hadely, 1970). Protocorms of Cymbidium proliferated when grown on medium containing IAA, whereas when 2, 4-D was used in higher concentration, it hampers protocorm growth (Fonnesbech, 1972). IAA at 0.1 mg/l concentration promoted protocorm development in C. mastersii (Prashad and Mitra, 1973); Similarly, the same auxin at 0.25 mg/l concentration enhanced vigorous protocorm growth in Vandaceous texa. The growth promoting effect of NAA or IBA on protocorm development in Cattleya, Phalanopsis and Vanda sps were reported
Bose and Mukherjee (1976) noted the beneficial effect of 2, 4-D in protocorm development in *C. giganteum*. However, combined effect of 2, 4-D and kinetin for uplift of protocorm development was also noticed in certain other spp (Kusomoto, 1978). Seed germination of terrestrial orchids were also promoted when supplemented with NAA in the culture media (Mathews and Rao, 1980; Vij. et al., 1981). The use of known quantities of growth hormone and vitamins in basal medium would be more helpful than complex additives in the embryo culture experiment as the later contains variety of substances.

In certain cases the embryoids, instead of direct protocorm formation proliferated to callus masses. Callus induction from embryoids will be useful to obtain increased number of seedlings in species with less seed production. The incidence of seed callus formation have been reported previously (Champagnat et al., 1968; Goh, 1970; Vij et al., 1981). For the induction of calli from embryoids external hormonal stimuli were essential. But, Mitra et al., (1976) observed seed calli in *D. fimbriatum* without addition of growth hormones. After separation and subculturing, many plantlets could be developed. Depending on the growth regulators in the medium, callus phase could be maintained.

Inclusion of sugar in defined culture media was essential for the induction of protocorms in either terrestrial or epiphytic orchid spp. As the orchid seeds are non-endospermic, they often depend on the external source of sugar for their energy source to initiate certain initial metabolic processes. Alternatively, orchid embryos were heterotrophic for carbohydrate source. The
suitability of using sugar for germination of orchid seeds was first reported by Bernards (1909). Thereafter, a number of workers had attempted to study the suitability of various sugars for protocorm formation and seedling growth in various orchid spp (Arditti et al., 1972; Raghavan, 1976; Harrison and Arditti, 1978).

Addition of sucrose in the culture medium has pronounced effect on chlorophyll formation and photosynthetic oxygen evolution. Sucrose supplemented cultures resulted higher percentage of germination in various orchid spp (Liddle, 1953; Karasawa, 1966; Usuto, 1975). Whereas, germination of a few terrestrial orchid spp reported to be greatly enhanced in addition of glucose or a mixture of glucose or fructose (Stoutamire, 1964). Germination and protocorm growth of Miltonia and Odontoglossum embryos in fructose was reported to be superior (Arditti, 1967). Growth of Dendrobium and Phalanopsis embryos greatly enhanced with addition of fructose (Ernst, 1967). Ferson (1969) suggested the inclusion of 0.63% glucose for protocorm multiplication and rhizoid formation in Cymbidium spp. The effect of maltose on seed germination and seedling growth of certain orchid spp were reported (Arditti, 1979). Protocorms of Odontoglossum and Miltonia spp could utilize maltose in the medium (Breddy, 1953; Arditti, 1967).

However, glucose and starch had resulted negative effect on the protocorm development and their subsequent growth in hybrid Cattleya spp. (Usuto, 1965). Corn starch in presence of tomato juice in the medium reported to have utilized by embryos of Miltonia and Odontoglossum (Ito, 1955).
Success of culturing axillary or apical meristems in sterile agarised medium has almost revolutionized the orchid industry. As a result, cut flowers of beautiful hybrid varieties were easily available. The orchid genera mericlone so far and a few including their hybrids were Aranthera (Rawati et al., 1977), Ascocenda (Fu, 1979), Holttumera (Teo and Wang, 1978), Renenthera, Vanda and Renantanda (Goh and Tan, 1978). It was first shown by Morel (1960) in his attempt to produce virus-free Cymbidium plant from diseased plants, by culturing shoot-apex in agarised Kc medium. He further reported that the colourless explant became green and enlarged slowly to form protocorms. These PLB's, when separated and subcultured, could regenerate large quantity of protocorms (Morel, 1964). Whimber (1963) used liquid medium along with trypone and sucrose for meristem culture of Cymbidium sps. Shoot bud cultures had produced shoots directly or they had proliferated to form calli. Upon subculturing, these calli developed to PLB's. When attempts were made to culture Vanilla planifolia nodal axillary buds in supplemented MS medium. The nodal explant instead of producing calli developed to shoots directly in presence of NAA (Kononowicz and Janick, 1984). Shoot tips of Cymbidium and Cattleya sps induced calli when cultured in MS medium (Ishii et al., 1974; Moral, 1974). Shoot buds of Paphiopedilum formed calli in presence of 2,4-D and BAP in MS medium (Steward and Button, 1975), whereas, induction of calli were not reported in Lindmann's medium. Intuwang and Sagawa (1975) and Singh (1976) had also reported the success of shoot-tip culture of Dendrobium sps. Hormone supplemented Knop's medium along with TCA produced shoot directly.
in Dendrobium sps (Singh and Prakash, 1984). Successes on shoot tip culture with Dendrobium Caeser Red Lip (Fernando, 1979), D. phalaneopsis (Kukulczanka and Wojciechowska, 1983; Kukulezanka, 1985) were also reported.

Subculturing of the shoot tips frequently had a pronounced effect on the induction of PLB's in certain orchid sps and their subsequent differentiation (Kako, 1973). Shoot tips culture of Dendrobium sps in VW medium containing 15% CW and 10 PPM NAA led to the rapid proliferation of PLB's (Sagawa and Shoji, 1967).

Although shoot tip culture is a well established technique, it requires sacrifice of a plant in monopodial taxa. Therefore, meristematic cells of other less valuable plant parts were attempted for clonal propagation. Attempts were also made utilizing vegetative shoots (Scully, 1967), lateral buds (Reinert and Mohr, 1967) and bud meristems (Moral, 1970) as explant for induction of PLB's to plantlet. Buds present in the tubers of Pachystoma senile could be used as potential source of explants (Vij et. al., 1983). The formation of calli to PLB's was enhanced in addition of IAA or Kin in Mitra's medium, whereas, NAA enhanced the formation of root, shoot-buds which directly initiated in various combinations of IAA/Kin in Pachystoma senile (Vij. et. al., 1983).

Leaf tissues of orchids appeared to be a potential source of explants. Whimber (1965) reported the differentiation of plantlets from leaf explant via PLB formation. Champagnat et. al., (1970) used seedling leaves of Cattleya hybrids to induce PLB's. That growth hormones and organic additives were essential
for regeneration of PLB's. Juvenile leaf tip of certain orchid sps viz. Dendrobium, Epidendrum and Leocattleya were used by Churchill et. al., (1971) to regenerate whole plant. Explants in H medium after 6-7 weeks formed small calli and after 10-12 weeks, small plantlets were developed. Subculturing in both agarised and liquid medium in appropriate time improved the regeneration of PLB's (Churchill, et. al., 1973). Steward and Button (1975) reported that only H medium containing 2, 4-D and BAP could regenerate callus in Paphiopedilum leaves, in dark. Cultures in MS, SH and Lindman's medium failed to induce calli. Leaf tissue of R. retusa Bl. induced to PLB's in Mitra's medium containing IAA, NAA, 2, 4-D in various concentrations and regenerated to plantlets after 4-5 weeks. (Vij et. al., 1984). Similar observation was also made by Chaturvedi and Sharma (1986) in hormone supplemented VW medium.

The orchids require a particular photoperiod for their growth and development. Moreover, light is directly related to the development of chlorophyll pigments in developing protocorms. The leaves adopted certain mechanism for reducing light absorption and consequent heat build up. A very frequent adaptation is the production of bluish pigment - anthocyanin in the hypodermal cells (Sanforth, 1974). Udea and Torikata (1972) have studied the effect of light on the adventitious root formation in Cymbidium sps. That different Orchids require various photoperiods for seed germination and protocorm development in certain orchid sps have been reported (Mukherjee, 1974; Stoutamire, 1974; Hasegawa et. al., 1978). It is clear from these reports that orchid protocorms
and developing seedlings vary in their response to light quality and/or photoperiod. Earlier workers have reported the use of white fluorescent light for the production of calli from various orchid leaves (Churchill, et. al., 1971). However, the effect of various light quality on the induction of calli in different orchids have not been studied so far.

Meristematic tissues excised from root of orchids could be used for in vitro propagation of plants like other dicot and monocot plants. Regeneration potentialities of orchid aerial roots has been studied by various workers. Churchill et. al., (1972) used supplemented H medium for root culture of Cattleya sps. The root sections formed PLBs via callusing and regenerated to plantlet lateron. Steward and Button (1978) obtained PLB's by culturing Epidendrum root sections. Kc medium along with NAA in various concentrations formed calli in Catasetum sps. Addition of 15% CW and 5 PPM NAA could help the regeneration process (Kerbauy, 1984). R. retusa root section also regenerated to plantlets in hormone-supplemented Mitra's medium (Chaturvedi and Sharma, 1986; Sood and Vij, 1986; Vij et. al., 1987). VW medium containing 15% CW was used by Sanchz (1988) for root tip culture of Cryopodium sps, however, depending on the concentration of NAA and BA, different morphological responses were observed. Although root meristems could be regenerated in vitro their potentiality was disappointingly low. Further, mycorrhiza often paused contamination problem in growing them in vitro.
In vitro propagation of orchid have certain advantages over in vivo methods. Maintenance of genetical make up, production of disease-free plants; maintenance space, breaking incompatibility barrier for hybrid production, and induction of desirable mutants, are some of the advantages for in vitro methods. Clonal propagation is particularly important for orchids because their genotypes are highly heterozygous.

The reduction of nitrate to nitrite is catalysed by nitrate reductase (NR EC 1.6.6.1) located in the cytosol. The most common form of enzyme in higher plants require NADH as reductant (Oaks and Hirels, 1985). Reduction of nitrate to nitrite is dependent on carbon metabolism in the following ways (Beever and Hageman, 1969; Beever and Hegeman, 1980, Stulen, 1986): i) for the provision of carbon skeletons and energy for the synthesis of enzyme; ii) for the reducing equivalents for functioning of the enzyme; iii) for providing carbon skeletons to accept reduced nitrogen. Nitrate reductase has been shown to vary in response to changes in environmental conditions such as light intensity, carbon-dioxide, oxygen levels, temperature, nitrogen source and other factors (Beever and Hageman, 1969, 1972). The seasonal variation in NR activity has been investigated in several crop species (DiMarco et al., 1986) and in forest crops (Kirby, 1989). Although NR activity of various other plants have been reported, the report is still lacking in orchids. The NR activity of in vivo and in vitro grown orchid seedling has not been compared as yet.
Works on various aspects of orchid tissue culture have been carried out in different laboratories abroad. In India emphasis on orchid tissue culture has been given only recently. Therefore, a very few accurate and comprehensive data on specific nutrient requirements during successive stages of embryo culture and their growth, regeneration of plantlets from leaf, root and shoot explant, of various Indian orchids, are available at present. Therefore, the experiments have been designed with the following objectives utilizing some of the rare and endangered orchid species of North-East India.

1. To determine the specific age of the embryos and the corresponding medium for regeneration of whole plants of a few orchid species.

2. To study the effect of various carbon sources on the growth and development of embryos.

3. To standardize media for induction of callus from axillary buds, leaves, roots to whole plants of a few orchid spp.

4. To study the effect of different light source for induction and subsequent growth of leaf calli of a few orchid species.

5. To study the effect of NR activity in the leaves of a few orchid plants grown in vitro and in vivo.

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