INTRODUCTION.
Orchids are no doubt the most beautiful and wondrous among all the flowering plants in the world. These extraordinary plants are economically important primarily due to their horticultural and floricultural appeal. Orchids as plants of ornamentation were known to ancient India. The flowers of *Aerides* and *Rhynchostylis* were used to be associated with the festivals and adorned by the ladies as "Draupti pushapa" and "Sheta pushapa" (Hegde, 1985). Some of the orchids were also used in "Ayurvedic" system of medicines, and also as food. Orchids, taxonomically represent the most highly evolved family among monocotyledons. They comprise of 800 genera and 35,000 species, distributed throughout the world world (Bose and Bhattacharjee, 1980). Perusal of literature reveals the existence of 925 Indian orchid species (Jain and Mehrotra, 1984). Out of 925 species, 284 are categorised as endemic, 30 have become extinct and 105 are endangered (Hegde, 1985). Six hundred orchid species are reported from the North-Eastern India, which represent about 65% of Indian and 1.7% of world orchid population.

Orchids are distributed in various forests, grasslands and agro-ecosystems. The North-Eastern region of India is rich in tropical wet evergreen, semi-evergreen, moist deciduous, subtropical broad leaved and alpine forest types, which provides conducive environment for the establishment and growth of epiphytic and terrestrial orchids due to varied climate, soil, vegetation and topography.
The North-East India is mainly inhabited by tribals, who commonly practise 'Jhum' cultivation. Besides, the increased population pressure coupled with other human activities, i.e. deforestation, construction of roads, dams, extraction of wood for fuel and furniture, have fastened the process of orchid depletion at a higher rate from their natural habitats.

Orchids are perennial with epiphytic or saprophytic habit in nature. The vegetative parts show great variations. The majority of flowers of orchids are not pollinated, their ovules are not fertilized and capsules are rarely formed. But where there is pollination a large number of seeds are produced which may range from 5000-1,00,000 per capsule. However, their germination is very poor (3-5%) under natural conditions, because of the particular fungal requirement.

Role of mycorrhizal fungi in orchids is emphasised in converting the complex reserve food in seed-coat into simpler forms (Harvais and Hadley, 1967b) which is made available to the germinating seeds and it helps in early development of seedlings (Arditti, 1967, 1979). In nature the growth of protocorms is arrested as long as infection by suitable mycobiont is achieved (Meyer, 1966). Efficiency of fungi in hydrolysing and mobilising the starch and other complex carbohydrates has been shown by some workers (Purves and
Hadley, 1975). The enhanced growth at early stage of seedling development is also assigned to its increased enzymatic activity in root region due to mycobiont which may also provide vitamins and other growth factors (Harvais and Pekkala, 1975).

Mycorrhizal fungi might produce some growth hormones like auxins, cytokinins, etc. It was postulated that such growth regulators might not only facilitate the infection of cells, but may also stimulate cell elongation, mobilization of sugars and even cell division (Hayes, 1969). Various media have been used for symbiotic germination and seedling growth of orchids. Clements (1979) found Oat medium most suitable for symbiotic seed germination. Linden (1980) studied a number of media for aseptic germination of seeds of orchids and found Burgeff's and Fast's media to support good germination and growth. The asymbiotic germination of seeds can take place if the medium is exogenously supplied with growth hormones, vitamins and trace elements (Harvais, 1982; Nakamura, 1982).

Harvais and Hadley (1967a) studied the symbiotic relationship between fungal symbiont and orchid hosts. Further, these authors (1967b) observed that the seedling development in Orchis purpurella was better in symbiotic cultures under low light and low temperature conditions than
in asymbiotic conditions.

A major benefit of fungus root association is the enhanced absorption of nutrients by the roots from non-fertile or moderately fertile soils. Absorption of phosphorus appears to be particularly enhanced by the mycorrhizal system. The fungal-symbionts capacity to produce acid phosphatase appears to be an important part of mycorrhizal absorption of phosphorus (Bowen, 1973). Gianinazzi (1978) has reported that mycorrhizal specific phosphatase activity is closely linked with the development of both mycorrhizal infections and the infected host plant. Infection of roots by mycorrhizal fungi may also be influenced by phosphatase concentration.

Orchid population is mainly influenced by the factors like temperature, light, pH of the soil and bark, amount of rainfall, humidity, type of soil and vegetation, etc. in the natural conditions (Hegde, 1985).

Most of the studies on orchids are related to the effect of growth hormones, vitamins, nitrogen sources, and complex additives (Knudson, 1922; Guednow, 1930; Meyer, 1943; Noggle and Wynd, 1943; Mariat, 1952; Withner, 1959; Fonnesbech, 1972; Mead and Bulard, 1975). However, work on the effect of some environmental factors, i.e., light, substrate pH, and temperature on seed germination of orchids, is meagre (Ueda and Torikata, 1972). And the role of mycorrhizal
fungi in phosphorus uptake and phosphatase metabolism in orchids has not been worked out in detail. Similarly, not much investigations have been done on the growth hormones production by mycorrhizal fungi of orchids. However, some information is available on the growth regulators production by some ectomycorrhizal fungi (Ulrich, 1960; Crafts and Miller, 1974; Ng et al., 1982) and by vesicular arbuscular mycorrhizal fungi (Barea and Aguilar, 1982).

Therefore, the following work was undertaken to study the effect of some eco-physiological factors on in vitro culture of orchids both in asymbiotic and symbiotic conditions and some aspects of their metabolism. The present investigations have been categorized as follows:

i. Studies on the effect of temperature, pH and light on asymbiotic seed germination and subsequent seedling growth of orchids.

ii. Studies on the effect of temperature, pH and light on symbiotic seed germination and subsequent seedling growth of orchids.

iii. Growth hormones production by mycorrhizal fungi of orchids.

iv. Phosphorus uptake and phosphatase activity in mycorrhizal seedlings of orchids.
REVIEW OF LITERATURE.
Orchidaceae, is one of the largest and most diverse of all plant families (Garay, 1960). Its representatives may be found from the Arctic to the Antarctic in bogs, deserts, valleys, plains, hills, mountains and even belowground (Hatch, 1953). Size of orchid plants varies from 3-4 mm to several meters, whereas flowers may range from 2-3 mm to 15-20 cm or more in diameter. Such wide variations necessitate numerous adaptive characteristics; for example certain orchids may contain little or no chlorophyll (Senn, 1927) and are therefore, saprophytic and parasitic (Burgeff, 1932; Hamada, 1939; Campbell, 1962, 1963; Hamada and Nakamura, 1963). The specialized flower structure has resulted in characteristic pollination mechanisms (Darwin, 1888) which include pseudo-copulation (Ames, 1948), self-pollination (Knudson, 1956) and cross pollination (Dunsterville and Garay, 1959). The most interesting adaptive features of the orchidaceae are those occurring in the physiology of their seed germination (Constantin, 1917).

At least three distinct periods may be delineated in the investigation dealing with the orchid seed germination. During the initial period, investigations were limited to the study of symbiotic relationship in orchids (Bernard, 1899, 1900, 1909; Ramsbottom, 1922a,b, 1929; Burgeff, 1936, 1959). In the early phases of the second period, which began with Knudson’s publications, the merits of symbiosis and asymbiosis
were argued (Bultel, 1924, 1925; Constantin, 1925, 1926; Knudson, 1924, 1930; Burgeff, 1936, 1959). Later on, the effects of various ions (La Garde, 1929; Wynd, 1953), sugars (Quednow, 1930; Wynd, 1933b), vitamins (Meyer, 1943; Noggle and Wynd, 1943; Bahme, 1949), hormones (Mariat, 1952; Withner, 1959d) and other complex organic additives (Knudson, 1922; Quednow, 1930) were examined.

Reissek (1847) first observed fungal association with roots in orchidaceae. Frank, afterwards coined the term "mycorrhiza" in 1885, to symbiotic association of fungi and roots. Wahrlich (1886) reported that the mycorrhizal association is a widespread phenomenon in most of the orchid species. Stahl (1900) described the mode of symbiosis with orchids and suggested the term "holomycotrophy" for the association (nourishment entirely through digestion of fungi; holes: total, mykes: fungus; trophe: nourishment).

First isolation of fungal endophyte from the germinated seeds and roots of orchids was done by Bernard (1903, 1904). The endophytes isolated from the Cattleya sp., Epipactis sp. and Cypripedium sp. were tested for their infection in the seeds of Odontoglossum sp.. He observed the lethal effect of fungi on the germinating seeds and thus concluded the association as parasitic instead of symbiotic. Bernard later on (1909) noticed that most of the orchid seeds
depended upon the mycorrhizal fungi for the germination and early growth under natural conditions, but they showed host specificity. He further (1911) observed the defense reaction of host to the invasion of fungal endophyte.

Burgeff (1909) observed fungal coil formation within the host cells. The coils contained protein, glycogen and fat. Host cells digest these coils through the 'tolyphagy' type of digestion. Based on the mode of fungal digestion, he (1959) categorised the digestion in orchids into three types, i.e., tolyphagy, ptyophagy, and themis-cophagy.

Kusano (1911) reported that heterotrophic orchid (Gastrodia eleta) may form symbiotic association with Armillaria millea. He also noticed that the bulb remained quiescent until the fungus attacked. Duggar (1915) gave detailed account of the genus Rhizoctonia. Duggar and Davis (1916) demonstrated nitrogen utilization of Phoma radicis, a mycorrhizal fungus with some orchids. The view that orchid seeds depend upon fungal association for germination was supported by Romsbottom (1922a). He again (1922b, 1927) advocated the specificity of the fungus in relation to the orchids. Noébacourt (1923) found that the growth of the fungus is inhibited by the antifungal substances present in the tubers of certain orchids.
In contrast to reports on fungal requirement of orchid seed germination, Knudson (1925) suggested that fungus is not necessarily required for seed germination, if the synthetic medium is supplemented with various substances like vitamins, amino acids, and growth hormones.

Rayner (1926) observed the presence of fungus in the seedlings of Goodyera procera. He opined that the association is a beneficial phenomenon. He (1929) further noticed that mycorrhiza of Neottia nidus-avis can fix the atmospheric nitrogen. While studying the germination of orchid seeds, Curtis (1936, 1937) noticed that some fungi were pathogenic. He (1939) also reported non-host specificity in case of some of the fungi. Downie (1943) isolated two strains of Rhizoctonia from the germinating seeds, and pointed out that soil can influence the distribution of endophytes in terrestrial orchids. She (1957) observed that Rhizoctonia constantly formed symbiotic association with orchids. Further, she reported (1959a,b) that fungal endophytes are capable of enhancing germination and provide some specific compounds like vitamins, etc. to the germinating seeds.

Gauman and Jaag (1945) isolated the endophytes from orchids and studied the defense reaction of Orchis militaris to the fungi using tissue culture techniques and observed anti-fungal substances in the host cell.
Slankis (1948) reported the production of auxins by the mycorrhizal fungus *Boletus variegatus*, but no identification of the auxins was made. Studies employing modern methods to characterize specific compounds have demonstrated the production of auxin by other fungi and bacteria. *Diplodia*, a member of the fungi imperfecti, grew uniformly on synthetic media and synthesized indoleacetic acid (IAA) both in the presence and absence of tryptophan (Gentile and Klein, 1955).

Ulrich (1960) screened many mycorrhizal fungi for the production of auxins, in the liquid culture medium and in the sporophores. He observed the production of indole compounds, i.e., IAA, indolebutyric acid (IBA), indoleglycolic acid (IGA), indolelactic acid (ILA) and indole pyruvic acid (IPyA) in most of the mycorrhizal fungi.

Gauman et al. (1960) studied the defense reaction of *Orchis militaris* to the fungi, using tissue culture techniques and observed the anti-fungal substances in the host cell. They (1960) also isolated the active substance and identified it as 'Orchinol'. Gauman and Hohl (1960) confirmed the production of orchinol in the living cells of orchid tubers *in vitro*.

Hadley and Perombelon (1963) observed the production of pectic enzymes by the *Rhizoctonia solani*, *R. repens* and *R. goodyera repens* *in vitro*. Harvais (1965) studied some
aspects of the symbiosis of *Orchis purpurella*.

Harvais and Hadley (1967a) suggested the symbiotic relationship between fungal symbiont and host, particularly in orchid mycorrhiza. They (1967b) observed that the seedling development in *O. purpurella* was better in symbiotic cultures under low light and low temperature conditions than in asymbiotic conditions. Zeigler *et al.*, (1967) studied the influence of various media and photoperiods on growth and aminoacid contents of orchid seedlings.

Gogala (1967, 1971) demonstrated that the ectomy- corrhizal forming fungus *Boletus elutis* var. *pinicola* synthesized several growth hormones. He observed that the fruiting bodies and the mycelia of this fungus in pure culture, as well as the culture medium in which the mycelia had grown for one month, contained three indole derivatives, corresponding to IAA, and a compound which seemed to be tryptophan and also growth hormones related to gibberellins and cytokinins.

Hadley and Harvais (1968) studied asymbiotic growth promotion in orchid-protocorms by certain growth hormones. Hadley (1969) found that cellulose promoted seed germination when supplied alongwith glucose both asymbiotically and symbiotically. He (1970) further noticed the non-aggressive nature of some endophytes and categorised them as
'Ubiquitous' endophytes. Hadley and Williamson (1971) found that symbiotically infected protocorms of *Dactylorhiza purpurella* showed linear increase in their length and width in contrast to non-infected ones. They (1972) also studied the influence of mycorrhizal infection and its intensity on the structure of nucleus of the host cells. Nuclear hypotrophy was observed in the host cells adjacent to the infected tissue.

Goh (1971) studied the effect of pH on the absorption of phosphate by the terrestrial roots of two orchid hybrids. He observed most efficient uptake in the range of pH 5.0 to 5.5 in *Vanda*, whereas in *Arachnis* pH 5.5 appeared to be the optimum. In both the cases, they showed the preferences for slightly acidic pH and uptake was much slower at alkaline pH. Ueda and Torikata (1972) investigated the effect of light and culture medium on adventitious root formation by *Cymbidiums* in aseptic culture and found no root formation in the shoots cultured in the dark, whereas white and blue light induced good root formation.

Smith et al. (1973) studied the uptake of glucose, trehalose and mannitol by the leaf slices of the orchid *Bletilla hyacinthina*, and found that the absorption of all three carbohydrates was reduced by low temperature.
Hijner and Arditti (1973) reported the production of vitamins by the orchid mycorrhizal fungi in the symbiotic conditions. The production of cytokinins: transzeatin and transribosyl zeatin in the culture medium of mycorrhizal fungus *Suillus punctipes* was reported by Crafts and Miller (1974). Similarly, they also found sufficient quantity of cytokinins in the culture medium of *Rhizogonum ochraceum*.

Hadley and Purves (1974) showed the movement of carbon\(^{14}\) from host to the fungus. Hadley (1984) explained the uptake of \(^{14}\)C glucose by asymbiotic and symbiotic protocorms of *Goodyera repens*.

Harvais and Pekkala (1975) found that the fungus could produce vitamins: nicotinamide, and thiamin in yeast extract supplemented liquid medium. The plantlets of *Orchis purpurella* in asymbiotic and symbiotic conditions were raised by Purves and Hadley (1975). They further (1976) suggested that the protocorms of *Goodyera repens* develop faster if infected with mycorrhizal fungi than the non-infected ones. Peschke and Volz (1978) observed the association of *Fusarium moniliforme* with various orchid species.

Clements and Ellyard (1979) studied symbiotic germination in the terrestrial orchids of Australia. They found stimulatory effect by most of the endophytes prior to seedling development. Vij and Datta (1981) investigated the
distribution of fungi in the roots of *Herminium angustifolium* (Benth). They isolated the fungal symbionts resembling with *Rhizoctonia sclerotina* (Burgeff). Vij and Sharma (1983) carried on a survey of mycorrhizal association in some terrestrial and epiphytic orchids of northern India.

Dexheimer and Serrigny (1983) studied the ultra-structure of endophyte of *Epidendrum ibaguense* and found more accumulation of alkaline and acidic phosphatase in infected cells.

Alexander and Hadley (1983) isolated endophyte from *Goodyera repens* and identified it as *Rhizoctonia goodyera repentis*. The symbiotic efficiency of the fungi with the germinating seeds of *G. repens* was also tested. The effect of fungicide on the mycorrhizal infection in *G. repens* (Alexander and Hadley, 1984a) and phosphate uptake (Alexander and Hadley, 1984b) have been studied. They (1985) also studied the movement of carbon between host and mycorrhizal endophytes at the time of *G. repens* development in the symbiotic conditions. Lin and Molnar (1983) observed the effect of photoperiod and high light intensity on the flowering of orchids.

Hills et al. (1984) studied the effect of different chemical compounds on the seedling development of *Cattleya aurantiaca*. Filipello et al. (1985) isolated some
endophytes from the orchids of Italy and carried morphological, cytological, and cytochemical studies. Williams (1985) observed that *Rhizoctonia* strains form association with orchids from the pot cultures of vesicular arbuscular mycorrhizal fungi.

Sharma and Tandon (1986) studied the influence of growth regulators on asymbiotic germination and early seedling development of *Coelogyne punctulata* Lindl.

Katiyar et al. (1986) reported the effect of organic supplements on the seedling growth of an endangered orchid species *Coelogyne punctulata*.

Raghuwanshi et al. (1983) reported the asymbiotic seed germination in epiphytic orchids. These authors further reported (1985) the effect of synthetic media on asymbiotic seed germination and seedling growth of *Dendrobium nobile* and *Sarcathus pallidus*, and found better germination and growth on Burgeff's and modified Kn C medium. Subsequently, they (in press) studied the effect of temperature on asymbiotic seed germination and seedling growth of orchids, and noticed better results between the temperature range of 20-30°C. Further, a pH range from 4.0 to 6.0 was found better for the seed germination and seedling growth (Raghuwanshi et al., 1986; in press).
CLIMATE AND VEGETATION OF COLLECTION SITE.
Survey of orchids was carried out from various grasslands, agricultural lands and forest of Khasi Hills, Garo Hills and adjacent areas of Meghalaya (Fig. 1). Plants of different epiphytic and terrestrial orchid species were collected and grown in charcoal and soil mixture, filled in earthen pots. Some epiphytic orchids were grown on old dead logs under semi-controlled conditions (in net houses) in Botany Department, School of Life Sciences, North-Eastern Hill University, Shillong (latitude, 25.34°N, longitude 91.56°E, altitude 1956 mm).

Soil

Shillong plateau has mild undulatous topography. The soil is laterite with reddish brown colour and the texture is sandy-loam at the surface end and silty-loam at deeper layers. It has originated from the hard rock representing gneses, schists and granites. Zimba (1977) has proposed that Shillong plateau and its surrounding hills might have uplifted from sea bed alongwith origin of great Himalaya during mesozoic and early tertiary times. The soil is rich in organic matters and nitrogen but acidic in reaction.

Vegetation

The vegetation of Meghalaya can broadly be
Fig. 1: Map of Meghalaya showing the places of orchid collection. (• = places of collection; θ = study site).
classified into:

1) Subtropical forest
2) The mixed evergreen forest
3) Temperate forest
4) The rolling grassland.

The sub-tropical pine forests represent biotic climax, dominated by Pinus kesiya along with some tree species like Alnus nepalensis, Schima spp., Quercus spp., Cedrus deodara, Cryptomaria japonica, etc. and some other trees, shrubs and herbaceous species like Symphyococcus spp., Rhododendron arboreum, Lantana camara, Eupatorium spp., Anaphilis spp. and Desmodium spp. etc. Mixed subtropical forests are confined to restricted areas and are much disturbed. These are dominated by Schima spp., Quercus spp., A. nepalensis, Erythrina arborescens and a number of rosaceae members.

Temperate forests are found from 1800 m and above. The true temperate vegetation represents the richest flora (in preserved/sacred forests) and gives an indication that probably the entire area was once covered by such type of dense vegetation but now has been disturbed due to human activities. In such forests the common trees namely Q. griffithii, Myrica esculenta, Betula alnoidus, R. arboreum, Castanopsis spp., Photinia rotondum etc., shrubs like, Daphne spp., Osbeckia spp. and beautiful orchids like
Dendrobium spp., Coelogyne spp., Cymbidium spp., Oberonia spp., Pleione spp., Pholidota spp., Eria spp., etc. are commonly encountered. The epiphytic plant species comprise of lichens, mosses, and ferns as dominant species on the tree trunks and branches of old trees in moist humid forests.

The grasslands represent most important vegetation type (ground flora) of this region. Most of the grasslands are in the different stages of sereal succession. The primitive agricultural practice, i.e., shifting cultivation locally known as 'jhumming' is commonly used for raising the agricultural crops in the North-Eastern region. The extensive use of 'jhumming' is main factor in resulting the genesis of grasslands in the region. Other biotic and human disturbances have also promoted the process. The grasslands consist of dominant species like Paspalum dilatatum, Pennisetum clandestinum, Imperata cylindrica, Pennicum brevifolium, Cyperus, spp., Fimbristylis spp., Arundinella spp., Trifolium repens, Cassia spp., Desmodium spp., etc. and some beautiful terrestrial orchids like Spathoglottis pubescens, Spirenthus spp., Herminium spp., Habenaria spp., Arundiana spp., Paphiopedilum spp. etc.

Climate

Shillong climate is very much influenced by the
Fig. 2: Meteorological data for minimum and maximum temperatures (°C), relative humidity (%) and rainfall (mm) for the year 1982-83 of the study site in Shillong.
south-west monsoon and north-eastern winds.

Therefore, the year can be divided into four seasons:

i) Spring season – March and April

ii) Summer (rainy) season – May to September

iii) Autumn season – October and November

iv) Winter season – December to February.

During spring season atmosphere gets warm gradually as compared to the preceding winter months. The maximum temperature reaches during the period of April to June-July. The average maximum temperature at Shillong recorded during the study period was 24.8°C and the average minimum temperature 12.5°C. Rain starts at the end of April and continues upto September. The monthly average rainfall normally is 212.0 mm. The average humidity (%) ranges between 61 to 88.5 (Fig. 2). October and November represent a typical autumn season with mild cold and usually with less rain. The winter season can be characterised by low temperature, from cool to cold one. The temperature drops down to a minimum of 1°C in the early period of January and occasional frost can appear. Sometimes there is rain during the month of March which is helpful for the germination and development of most of the trees, plants, and herbs.
Plate 1: *Cymbidium giganteum* (Fig. A) and *Cymbidium elegans* (Fig. B) showing epiphytic growth in nature.
Plate 2: *Thunia alba* showing epiphytic growth in nature (Figs. A and B)
Plate 3: Seedlings (150 day-old) of Cymbidium giganteum (Fig. A) and C. elegans (Fig. B), grown in Knudson 'C' Medium.
Plate 4: Seedlings (150 day-old) of *Thunia alba* (Figs. A and B), grown in Knudson 'C' Medium.
MATERIALS AND METHODS.
The orchids collected from forests were grown in charcoal and soil mixture, filled in earthen pots and were kept in net houses of the Botany Department of the North-Eastern Hill University, Shillong. *Cymbidium giganteum*, *C. elegans* and *Thunia alba*, were selected for the present study on the criteria that they are threatened species, have attractive flowers and show fast germination of seeds.

I. *Asymbiotic seed germination and seedling growth*

The unopened green capsules (approximately 3-5 months old) of *Cymbidium elegans*, *C. giganteum* and *Thunia alba* were collected and surface sterilized by dipping them in 5% (w/v) sodium hypochlorite solution for 10 minutes and then rinsing in absolute ethanol for a few seconds. The sterilized capsules were washed 2 to 3 times in sterilized distilled water and were cut into two half pieces with sterilized scalpel and the seeds were taken out with the sterilized needle. These seeds were then transferred to the slopes on modified Knudson 'C' medium (Bose and Bhattacharje 1980). All the inoculation processes were carried out under aseptic conditions using laminar flow chamber. The pH of the medium was adjusted to 5.0 by adding 1 N HCl and 1 N NaOH solution before autoclaving. Twenty five and 50 ml of sterilized medium was poured in culture tubes and conical flasks (25 x 150 mm and 100 ml), respectively and allowed to
solidify. Twenty five replicates of each species were incubated in B.O.D. incubators in dark conditions for two months and thereafter, under fluorescent light condition (1300 lux; 12 hr photoperiod) upto five months. Cultures were observed after 60 days and subsequently at 30 days intervals upto 150 days. Percentage of seed germination, average area of seedlings/plantlets, average number and area of leaf primordia/leaves, average number and area of rhizoids/roots and growth index were the parameters used to study germination and subsequent seedling growth. More than 100 seeds/protocorms were observed to calculate percentage of seed germination and seedling growth. The quantify normal growth of seedlings, seedling/plantlets were divided into six different developmental stages (Spoerl' 1948; Mariat, 1952; Ardi:ti, 1967b). Percentage of each developmental stage was multiplied by the developmental stage number and all sums so obtained were added. The total obtained was the growth index. Average area of seedlings/plantlets was measured by calculating average length and breadth of all the growth stages present and multiplied. Similarly, average number and area of rhizoids/roots and leaf primordia/leaves was also obtained for the different growth stages, during seed germination and seedling development of orchids. The effect of following treatments on seed germination and seedling growth of orchids was studied.
1) **Temperature treatments**

The seeds were incubated at different temperatures, viz., 20°C, 25°C, 30°C and 35°C as described above.

2) **pH treatment**

The seeds were sown in modified Knudson 'C' medium having different pH, viz., 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 10.0 adjusted by adding either 1 N HCl or 1 N NaOH before autoclaving it. The cultures were incubated at 25°C ± 2°C under 12 hr photoperiod of 1300 lux.

Composition of modified Knudson 'C' medium is given below:

Calcium nitrate 0.20 gm/l; monobasic potassium phosphate 0.15 gm/l; magnesium sulphate 0.25 gm/l, ammonium sulphate 0.10 gm/l; potassium nitrate 0.18 gm/l; disodium EDTA 74.6 mg/l; ferrous sulphate 25.0 mg/l; boric acid 6.2 mg/l; manganese sulphate 0.75 mg/l; zink chloride 3.9 mg/l; potassium iodide 0.80 mg/l; sodium molybdate 0.25 mg/l; copper sulphate 0.025 mg/l; cobalt chloride 0.025 mg/l; thiamin hydrochloride 0.30 mg/l; pyrodoxine hydrochloride 0.30 mg/l; riboflavin 0.30 mg/l; sucrose 20 gm/l; and agar 10 gm/l.

3) **Light treatment**

   a) **Light intensity**

   The cultures were incubated for 150 days,
under different light intensities, i.e., 1500, 3000 and 5000 lux, and in the dark conditions. Various light intensities were obtained by 40-Watt white fluorescent tubes.

b) **Light quality**

Light of different colours, viz., red, blue, green and white were provided by wrapping cellophane paper around the tube lights, in the B.O.D. incubators. One set of culture tubes was also kept under dark conditions.

c) **Photoperiod**

The seeds were sown on Knudson 'C' medium and were incubated at room temperature (20-25°C) under fluorescent light (3500-4000 lux). Photoperiods of 8, 12, 16, 20 and 24 hr were provided with the help of timers, using 40 watt fluorescent tubes.

II. **Symbiotic seed germination and seedling growth**

1) **Isolation and culture of endophyte**

The infected roots of the orchids were selected and cut, washed with tap water and surface sterilized with sodium hypochlorite solution (5%) for 10-15 minutes. The sterilized roots were washed several times in sterilized distilled water. Root sections of 1-2 mm thick were cut using sterile scalpel. Two media, i.e., Malt Extract Agar (MEA) (Booth, 1971) and Potato Dextrose Agar (PDA) (Hadley and Ong, 1978)
were used for the isolation of fungal endophyte from the roots. Chemical composition of the media is given below:

   a) **Malt Extract Agar**

   - Malt Extract: 20 g
   - Agar: 20 g
   - Distilled water: 1000 ml

   b) **Potato Dextrose Agar**

   - Potato Extract: 200 g
   - Dextrose: 15 g
   - Agar: 20 g
   - Distilled water: 1000 ml

Each nutrient medium was sterilized at 15 p.s.i. for 15 min. and poured into pre-sterilized petriplates. Sterilized root sections were teased and inoculated in centre of petriplate on the nutrient medium; one section was used for each petriplate. Twenty petriplates were used to isolate the fungi for each orchid species. The inoculated plates were incubated at 25 ± 2°C in a B.O.D. incubator. After one week, white mycelium appeared around the inoculated root sections. The fungus was retransferred onto freshly prepared PDA medium and reincubated at 25 ± 2°C in a B.O.D. incubator for one week. These fungi were sub-cultured on MEA medium in culture tubes for stock culture and kept at 4°C in the refrigerator. All the fungal isolates were grown on similar medium and those
varied in their morphology were considered as separate strains of *Rhizoctonia*. These fungi were represented by the code number RH and RA - (Rhizoctonia). Only those fungi were considered for symbiotic seed germination and seedling growth studies, which formed the symbiotic relationship with orchids. Fungal isolate number and their sources are mentioned below:

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Orchid source of the fungus</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA 4</td>
<td><em>Ione candida</em> (Lindl.)</td>
</tr>
<tr>
<td>RA 5</td>
<td><em>I. candida</em> (Lindl.)</td>
</tr>
<tr>
<td>RA 20</td>
<td><em>Cymbidium giganteum</em> (Wall.)</td>
</tr>
<tr>
<td>RA 40</td>
<td><em>Coelogyne punctulata</em> (Lindl.)</td>
</tr>
<tr>
<td>RH 15</td>
<td><em>Dendrobium longicornu</em> (Lindl.)</td>
</tr>
<tr>
<td>RH 36</td>
<td><em>Pleione maculata</em> (Lindl.)</td>
</tr>
<tr>
<td>RH 46</td>
<td><em>Coelogyne ocultata</em> (Hook.)</td>
</tr>
<tr>
<td>RH 51</td>
<td><em>C. prolifera</em> (Lindl.)</td>
</tr>
<tr>
<td>RH 54</td>
<td><em>Liparis distans</em> (Clarke.)</td>
</tr>
<tr>
<td>RH 61</td>
<td><em>Thunia alba</em> (Reichb. f.)</td>
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</table>

2) **Preparation of cultures**

The stock cultures of fungal isolates were maintained on PDA slopes at 4°C. Five isolates, viz., RA 20, RA 40, RH 15, RH 36 and RH 54 were selected and transferred to the fresh medium in the petriplates. The plates were incubated at 25 ± 1°C for 7 days.
Oat medium (Clements, 1979) was used to raise symbiotic seedlings in vitro. The nutrient medium contained 3.5 g powdered oats; 0.1 g yeast extract; and 10.0 g Agar, per litre. pH of the medium was adjusted to 5.0 by using digital pH meter. Medium was autoclaved for 15 minutes at 15 p.s.i. and poured in pre-sterilized culture tubes (25 x 150 mm). Seeds from the surface sterilized capsules of *Cymbidium elegans*, *C. giganteum* and *Thunia alba* species were sown on the agar slopes of oat medium, under aseptic conditions.

Different mycorrhizal fungi were also inoculated along with the seeds of each species separately at the time of sowing. Twenty replicates were made for each orchid species. The uninoculated (asymbiotic) and inoculated cultures were maintained under several combinations of illuminations, temperatures and pH, to investigate their influence on symbiotic culture of orchids in vitro.

3) **Incubation of cultures**

Fungal inoculated and control cultures were incubated under different culture condition such as temperature (20°C, 25°C, 30°C and 35°C), light (photoperiods 8, 12, 16, 20 and 24 hr), light intensities 0, 1500, 3000 and 5000 lux and light qualities (red, green, blue, white and dark) and pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 10.0) of the medium.
according to the details described above.

4) Observation of cultures

In general three replicate tubes were taken for each treatment. The seeds and protocorms (germinated seeds) were removed from each tube for microscopic examination and the percentage germination was determined. To study seedling growth, the protocorms/plantlets were divided into six different developmental stages and growth index was determined (Spoerl, 1948). Average area of seedlings/plantlets was observed by multiplying average length and width of all the growth stages occurred at the time of observation. Similarly, average number and area of rhizoids/roots and leaf primordia/leaves were also determined on the basis of developmental stages. Statistical analyses (standard error and analysis of variance) were carried out following the method of Croxton et al. (1975).

III. Detection and identification of growth hormones in mycorrhizal fungi of orchids

1) Culture of mycorrhizal fungi

Seven mycorrhizal fungi, i.e. RH 15, RH 36, RH 51, RH 46, RH 54, RH 61 and RA 40 were used to detect growth regulating substances in pure culture. The Hagem liquid medium (Fries, 1943) having the following composition per
litre: 5.0 g malt-extract; 5.0 g glucose; 1.0 g tryptophan; 0.5 g KH₂PO₄; 0.5 g MgSO₄·7H₂O; 0.5 g NH₄Cl; and 0.5 ml (1%) solution of FeCl₃ was prepared and autoclaved at 15 p.s.i. for 15 minutes and 100 ml of the medium was poured in 250 ml capacity conical flasks. Inoculum from pure culture of the fungus was inoculated in the nutrient medium under sterilized conditions. Cultures were incubated at 24 ± 1°C in the dark. After 10 days of growth, cultures were investigated for the presence of hormone-like substances, and further observations were made at 10 days intervals upto 30 days and then after 60 days.

2) Extraction of growth hormones from culture filtrates

The extraction, detection and identification of growth hormones were carried out following the method of Mahadevan et al. (1982) as described below:

(a) Auxins

The nutrient media in which mycorrhizal fungi had grown were centrifuged at 2,000 g in Remi centrifuge for 30 minutes, and the supernatant was acidified with 1N HCl to pH using a digital pH meter and extracted immediately with three aliquots of ethyl ether. The extracts were pooled and the volume reduced to about 25 ml. The ether fraction was partitioned with 25 ml of 5% sodium bicarbonate. This process was repeated three more times, each time with 25 ml of
bicarbonate. Bicarbonate fraction was acidified to pH 3.0 with 6 N HCl and extracted three times with 50 ml of ethyl-ether. Ethyl ether was evaporated and the residue was dissolved in 2-3 ml of methanol and stored in a vial at 0°C in the refrigerator.

(b) Gibberellins

Two hundred fifty ml of saturated NaHCO₃ solution was mixed with 100 ml of culture filtrate in a separatory funnel. It was partitioned twice with 300 ml of ethyl acetate and the organic phase was discarded. The aqueous layer was acidified to pH 2.5 using 5N HCl. Equal volume of ethyl acetate was added and shaken vigorously for 5 min in a separatory funnel. Extraction was done thrice with aqueous layer using 300 ml of the solvent for each extraction, and all the ethyl acetate fractions were pooled and evaporated to dryness. The residue was dissolved in 1-2 ml of methanol.

(c) Cytokinins

Culture filtrate was acidified to pH 3.0 with 0.1N HCl, and extracted with diethyl ether thrice using a separatory funnel to remove the auxins. pH of the aqueous phase was then adjusted to 7.8 with 0.1N NH₄OH, and extracted thrice with equal volumes of n-butanol. The butanolic fractions were combined and evaporated. The residue was dissolved in 10 ml of 0.1N HCl and passed through a column of
Dowex-50(H⁺ Cycle). Cytokinins were eluted with 200 ml to dryness and the residue was dissolved in minimum quantity (1-2 ml) of distilled water. This extract was used for chromatography.

3) Chemical detection of growth hormones

(a) Auxins

Ascending paper chromatography was used to separate the indole compounds extracted from the fungal medium. 100 ul of the extract was placed on spots along a line 2.5 cm from the bottom of a 20 cm square sheet of No. 1 Whatman filter paper. The spots were dried with warm air using hair dryer. Spots of known indole compounds were placed on the chromatograms for reference. The edges of the filter paper were then stapled to make a cylinder, which was placed into the solvent, in a airtight-wide-mouth jar. The jar was then placed at room temperature in the dark for approximately six hours. The solvent used for separating the indole compounds was isopropyl alcohol, ammonia, water in the proportion 8:1:1. The Salkowski spray reagent as modified by Gordon and Weber (1951) contained 0.01 M FeCl₃ in 35% perchloric acid and diluted with an equal volume of absolute ethyl alcohol, was used to detect indole compounds on the chromatogram. The chromatogram was dried at room temperature and examined within 30 minutes of spraying.
(b) **Gibberellins**

Spots of the extract of mycorrhizal fungi on Whatman No.1 paper were developed ascendingly in the solvent such as isopropanol-ammonium hydroxide-water (10:1:1, v/v/v) and chromatogram was dried at room temperature. The chromatogram was then sprayed with either ethanol-conc. sulphuric acid (95:5, v/v) or Water-conc. H$_2$SO$_4$ (30:70, v/v), and dried at 120°C for 10 min in a hot air oven and then examined under UV light for fluorescent spots (Macmillan and Suter, 1963). Spots of known gibberellins were also run along with unknown samples to help identification.

(c) **Cytokinins**

Ascending paper chromatography was used to separate the cytokinin extract. 100 ul drops of each extract were run, using the freshly mixed solvent of isopropanol-ammonia-water (10:1:1, by volume). Cochromatography with standard of authentic substances was done for the identification of cytokinins. Chromatograms were dried and viewed under UV light and fluorescing spots were marked. Colour intensity, size of the spot and Rf values were the parameters used to express the results, qualitatively.

4) **Quantitative measurement of growth hormones**

(a) **Auxins**

One ml of the extract containing indole-compounds
was taken in a test tube and 2 ml of Salper reagent (1 ml of 0.5 M FeCl₃ was mixed in 50 ml of 35% (v/v) perchloric acid) was added dropwise but rapidly with continuous agitation. The sample was incubated in the dark for 60 minutes. The absorbance of the sample was measured in a spectrophotometer at 535 nm against a solvent-reagent blank, and the quantity of auxins in the extract was estimated from a standard curve drawn from known concentrations of IAA.

(b) Gibberellins

Two ml of the zinc acetate solution was added to 1 ml of the extract containing GA. After 2 min, 2 ml of potassium ferrocyanide solution (Holbrook et al., 1961) was mixed. The mixture was then centrifuged at low speed for 15 min. To 3 ml of the supernatant solution, 3 ml of 30 percent HCl was added in a test tube and the mixture was incubated at 20°C for 75 min. The absorbance of the sample and blank was measured at 254 nm in a spectrophotometer, and the amount of GA was calculated in the extract from a standard curve prepared with known GA₃ and the results were expressed as GA₃ equivalents.

(c) Cytokinins

To one ml of the extract, 2 ml of sulphuric acid was mixed, and the samples were incubated at room temperature for 30 min. The absorbance of the mixture was taken in a
spectrophotometer at 254 nm against blank sample. The amount of cytokinins was quantified with known KI standard curve, and results were expressed as KI equivalents.

IV. Phosphorus uptake and phosphatase activity in orchids

(1) Raising of seedlings

Green pods of *Cymbidium elegans* and *C. giganteum*, were collected and surface sterilized by dipping them in absolute ethanol for a few seconds and followed by flamming (Linden, 1980). The seeds were then taken out in sterilized conditions and inoculated on the agar slopes of modified Knudson 'C' medium (Bose and Bhattacharjee, 1980), in 100 ml, 150 ml and 250 ml capacity conical flasks. Cultures were then placed at 25 ± 1°C, under 12 hr light and 12 hr dark conditions, in B.O.D. incubators. After 3 months of growth, germinated protocorms were again subcultured on freshly prepared nutrient medium, upto 9 months.

(2) Preparation of mycorrhizal inoculum

The orchid mycorrhizal fungus, i.e., RH 46, was cultured on Pfeffer mineral liquid medium (Hadley and Ong, 1978), in conical flasks and incubated at 25 ± 1°C, in the dark for 10-12 days. Fungus grown in liquid medium was filtered through Whatman filter paper No. 1. The mycelium of the fungus was homogenized. 20 ml of homogenate was
inoculated per pot to the roots of seedlings to produce mycorrhizal plants.

(3) Preparation of potting medium and treatments

The air dried cowdung, garden soil, and charcoal powder (1:1:0.4) were mixed to form basal substrate-medium for growing seedlings, in glass house conditions. Phosphorus as $\text{KH}_2\text{PO}_4$ was applied at five levels, viz., 1.68 mg, 3.36 mg, 33.6 mg, 67.2 mg and 0.00 mg in the 150 gm soil mixed thoroughly and filled in separate earthen community pots (10 cm diameter), having a hole at the bottom. 1-2 cm thick layer of sterilized stones was added, in all the pots before filling the mixture, the substrate filled pots were sterilized twice in autoclave for 30 min. at 15 p.s.i. 30 replicates were made for each treatment using each species.

(4) Transplantation of seedlings to earthen pots

Two to three leaf stage (9 months old) seedlings of each species were carefully removed from the flasks. The roots of the seedlings were gently washed in running tap water. Six-washed seedlings were then transplanted to each community pot. Earthen pots were kept at 16.5°C - 26°C range of temperature in the glass house. Seedlings were watered daily (50 ml/pot) with tap water. The mycorrhizal and non-mycorrhizal seedlings grown at different levels of phosphorus in the soil
mixture, were harvested at monthly intervals, and were used to study phosphorus uptake and phosphatase activity.

(5) **Determination of total phosphorus**

Phosphorus was determined using Vanadomolybdic Yellow colour method (Jackson, 1967). At monthly intervals, seedlings were harvested and washed carefully in running tap water, and the fresh and dry weight was calculated. Oven-dried ground plant samples were taken in 250 ml conical flasks and 10 ml of triacid mixture was added. The digestion was done using hot plate at 200 - 300°C for 1 - 2 hours, and the residue was diluted with little water. The residue was then filtered through ordinary filter paper, in 50 ml capacity volumetric flasks, and then volume was made to 50 ml with distilled water. To 40 ml of digested sample, 10 ml of ammonium vanado molybdate solution was mixed and the total volume was made to 50 ml. The mixture was kept for 30 min at room temperature to develop maximum colour and the absorbance was measured at 490 nm using spectrophotometer.

(6) **Quantitative analysis of phosphatases**

The soluble root enzymes were extracted by macerating the seedlings (2:1, w/v) in a mortar at 5°C using 0.1M borate buffer (pH 8.8) + 0.1% glutathione. The macerate was then centrifuged at 20,000 x g for 20 min and the acid and
alkaline phosphatase activity in the supernatant was determined quantitatively measuring the amount of p-nitrophenol (PNP) released by the enzyme from p-nitrophenyl phosphate (Gianinazzi et al., 1976).