Chapter 1

General Introduction
Orchidaceae, a highly sophisticated family of distinction is one of the largest and includes diverse groups of flowering plants. Constituting a group of prized ornamentals, orchids culminate in one of the evolutionary lines of monocots and are still in the process of active speciation. These are botanically very interesting for their floral complexities, free gene flow across the specific barriers, minute seeds with undifferentiated embryos, suppressed endosperm formation and dependence on a suitable mycorrhizal association for germination. The uniqueness of the family is also reflected in its peculiar pollination contrivances and wide natural hybridization. Acclaimed all over the world for the bewitching beauty of its flowers, the family orchidaceae comprises of 20,000 - 23,000 species spread over 725 genera (Atwood, 1986). Besides, the exertions of man over the past 120 years have produced a parallel population of more than an equal number of artificial hybrids.

Orchids enjoy wide distribution, found in areas from sea-
level to snowline and are reported from all the continents except Antarctica. In India, the estimates of the number of orchid species vary from about 800-1300, but a fairly critical appraisal (Jain and Mehrotra, 1984) shows the presence of about 925 species. North-East India, including North-East Himalayas and Khasi and Jaintia Hills, forms the richest geographical region for orchids. About 700 species are reported to occur in terrestrial and/or epiphytic forms in the region (Hegde, 1984).

Phytogeographical studies have revealed that North-East region harbours about 50% of the total Indian flora (about 10,000 species). However, it has been recently observed that increasing biotic influences including socio-economic development and unrestrained commercial exploitation of forest wealth have threatened the survival of the genetic resources amounting to a great loss of natural heritage. The area of cultivation, distributional range/spread of such plants is shrinking in native habitats. On the other hand, for a number of taxa of this region, potentialities and desirable attributes are as yet not fully known and exploited. Therefore, the preservation of plant genetic resources of unknown promise as well as threatened types for posterity do need priority.

Orchids are perennial plants which grow either as epiphytes (growing on trees), lithophytes (growing on rocks), terrestrials (growing on the ground) or as saprophytes (leaf-less forms growing on decaying organic matter) and bloom annually. Depending on their mode of growth, orchids could be monopodial where a
single stem continues to grow from its apex year after year, producing new leaves continuously; sympodial, where each new shoot springing from the rhizomes of the previous growth is complete in itself and terminates in a potential inflorescence, or diapodials, where growths are built up in a similar way to the sympodials, but lack the characteristic feature, the pseudobulbs (swollen stems, providing the plant with a means of water storage).

In natural conditions, majority of the orchid flowers are not pollinated and their ovules not fertilized. As a consequence, capsules are rarely formed. Orchid seeds are extremely minute (0.3-2.0 mm, Stoutamire, 1964), and usually undifferentiated with the endosperm underdeveloped or completely lacking (Henrich et al., 1981). This insufficient nutrition results in the inefficiency of the orchid seeds to attain the autotrophic stage of development which could be provided under natural conditions by a mycorrhizal association. As such, less than 5% of orchid seeds are able to germinate in nature (Rao, 1977).

The pioneering work of Bernard (1909) laid the foundation of in vitro symbiotic cultures of orchids. Later Knudson (1922) showed that germination of orchid seeds was possible in vitro without fungal association by providing balanced organic and inorganic nutrition for the developing embryos. However, propagation of plants by seeds has a number of limitations viz.: a) viability of orchid seeds is remarkably less, b) seeds are available only for a limited period and
c) the regenerants are heterozygous.

While applying existing tissue culture techniques (White, 1951) to the study of virus transmission in Cymbidium, Morel (1960) noted that protocorm-like-bodies (plbs) developed around shoot tips cultured in vitro which eventually produced roots and shoots. Such plbs when cut into sections and transferred to new medium, multiplied in number. This process of protocorm multiplication could be repeated indefinitely and large tissue stocks of any one clone could be obtained within a relatively short period.

Clonal propagation, now a fairly common practice in orchid culture is particularly important as orchid genotypes are heterozygous. Furthermore, asexual propagation is essential for plants which may be completely sterile. Normally, members of a clone have identical genomes thereby exhibiting true-to-type characteristics. Since the initial publication (Morel, 1960), this technique and its modifications (Wimber, 1963; Kim et al., 1970; Intuwong and Sagawa, 1973; Lay, 1978; Sagawa and Kunisaki, 1982; Kukulczanka and Wojciechowska, 1983; Homma and Asahira, 1985; Sanchez, 1988; Kraus and Monteiro, 1989; Goh and Wong, 1990; Vij and Pathak, 1990; Shimasaki and Uemoto, 1991) have become important in the mass propagation of desirable varieties at rates which were undreamt of earlier.

Unlike other plant families, differences in requirements for propagation in vitro exist among the diverse genera, species and hybrids of orchidaceae. Nutrient formulations and steps that are
satisfactory for one may not be applicable to another (Huang, 1988). Growth as well as differentiation can be controlled by various media components including mineral nutrition. Several plant tissue and cell culture media are in use including formulations devised by Murashige and Skoog (1962), White (1963) and Gamborg et al. (1968) besides the more commonly used orchid culture medium of Vacin and Went (1949). Composition and components of culture media have been investigated and reviewed by various workers (Gamborg et al., 1976; Huang and Murashige, 1977). In the last three decades, many reports on the growth and development of various orchids as affected by different defined and undefined media have been made (Rao and Avadhani, 1963; Raghavan, 1964; Raghavan and Torrey, 1964; Arditti, 1966; Zeigler et al., 1967; Fonnesbech, 1972a, b; Harvais, 1973; Ernst, 1975; Mead and Bulard, 1979; Henrich et al., 1981; Krishna Mohan and Jorapur, 1984; Amaki and Higuchi, 1989). Pierik et al. (1988) have brought out a detailed report on the germination and further growth of Paphiopedilum, a genera considered to be difficult to propagate in vitro.

Besides nutritional requirements, other physico-chemical factors have been reported to influence the physiology and development of plants in culture. Orchids vary considerably in their requirements for light. Though seed germination can take place in light or total darkness, depending on the taxa involved, further development of seedling does require illumination (Stoutamire, 1974; Arditti, 1979). The effect of light, both
quantitative and qualitative including photoperiod on orchid seed germination and growth has been studied (Withner, 1959; Zeigler et al., 1967; Ueda and Torikata, 1972; Ernst, 1976; Hasegawa et al., 1978; Arditti et al., 1982; Zeigler et al., 1985; Pierik et al., 1988). Considerable differences exist in the light intensities used for orchid tissue culture. Light intensity has been shown to affect the type of growth in culture and higher light intensities have been reported to improve the survival of ornamental plants transferred to soil (Hughes, 1981). Photoperiod requirement of orchids has been found to vary from none at all (i.e. complete darkness, Morel, 1971) to continuous illumination (Wimber, 1963) ranging from 400 - 5000 lux (Werckmeister, 1970a,b,; 1971).

Although little information is available about the influence of pH of a culture medium on in vitro morphogenesis, the growth promoting properties and the selectivity of the culture medium are pH dependent (Sarma et al., 1990). Most tissue culture media are poorly buffered, and pH fluctuations that occur during culture may be detrimental to the growth and development. The majority of efficient nutrient solutions have pH values between 5.0-6.0 and these limits are associated with healthy growth of many plants. Though several studies have been conducted to investigate the effect of different physico-chemical factors on orchid seedling growth and development (Arditti et al., 1981), not much is known about the acidity effect on their asymbiotic cultures. The pH lower than 4.0 and higher than 8.0 has been
found to be inhibitory for seedling growth of orchids (Arditti et al., 1982).

Early studies on the growth of plant cells in cultures indicated that the optimum temperature range was between 26 and 28°C, but that species requirements differed considerably (Carew and Staba, 1965; Puchan and Martin, 1971). In practice, plant cultures are grown around 25°C and few studies investigating specific temperature requirements for various plant species are available (Hughes, 1981). Temperature between 20-25°C has been found to be suitable for the growth and development of most of the orchid species (Harvais, 1973; Stoutamire, 1974; Tanaka and Sakanishi, 1978; Arditti, 1982; van Waes and Debergh, 1986).

Results of experiments with auxins, cytokinins and gibberellins are inconsistent and consequently inconclusive with the responses on the germination and seedling growth of orchids differing from species to species (Wither, 1959, 1974; Arditti, 1977, 1982; Hadley and Harvais, 1968; Ueda and Torikata, 1969; Goh, 1970; Hadley, 1970; Fonnesbech, 1972a, b; Pierik and Steegmans, 1972; Harvais, 1973; Strauss and Reisinger, 1976; Tamanaha et al., 1979; Kusumoto, 1980; Nakamura, 1982; Sharma and Tandon, 1986; van Waes and Debergh, 1986; Huang, 1988). In most cases, auxins, mostly indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA) enhanced germination and/or seedling growth. Inhibition was reported in some cases (Arditti and Ernst, 1984). Auxin : cytokinin ratio may be important for growth in some instances (Hadley and
Harvais, 1968; Harvais, 1972; Fonnesbech, 1972a, b) and inhibitory (Kano, 1965) in others. High concentrations of 6-benzylaminopurine (BAP) resulted in formation of numerous plbs in Cattleya (Pierik and Steegmans, 1972), lily (Kawarabayashi and Asahira, 1988) and Phalaenopsis (Wang, 1989). Gibberellic acid (GA₃) has been found to inhibit seedling growth in Bletilla striata, Dendrobium (Kano, 1965) and Galeola (Nakamura, 1982). On the other hand, seedling growth in Cattleya and Cymbidium (Blowers, 1958; Hirsh, 1959; Harvais, 1982) was stimulated with GA₃ application. The suitability of some plant growth regulators over others and their inhibitory action in some thus suggest specific requirements of a particular system (Liu et al., 1988).

Nitrogen assimilation, both ammonium (NH₄⁺) and nitrate (NO₃⁻) ions, has an important role in plant growth and differentiation. Nitrogen is required for growth, production of proteins and formation of chlorophyll and cytochrome. The demand for nitrogen is closely related to the amount of growth and differentiation (Kramer and Kozlowski, 1979) and nitrogen deficiency is the most common limitation on growth after water stress. Of all the mineral nutrients, the form of nitrogen used is shown to affect the chemical composition of plant tissue as well as pattern of plant growth and differentiation (Ozias-Akins and Vasil, 1985). Number of studies have been conducted on the effect of different inorganic and organic nitrogen sources on orchid seed germination, growth and development (Withner, 1959; Raghavan, 1964, 1976; Raghavan and Torrey, 1964; Mitra, 1971; Mead and
Bulard, 1975, 1979; Ichihashi and Yamashita, 1977; Nakamura, 1982; van Waes and Debergh, 1986). Most of the orchid species are reported to be incapable of utilizing nitrate during the early stages of the growth and development and their ability to utilize nitrate parallels the appearance of nitrate reductase in plants (Raghavan and Torrey, 1964). Several workers have reported ammonium nitrate to be the most suitable form of nitrogen for the development of orchids in culture (Arditti and Ernst, 1984). Results with urea are contradictory even with species in the same genus. A culture medium for orchids formulated by Thompson (1977), however, contains only urea and ammonia nitrogen. Individual amino acids or mixtures of amino acids do not stimulate growth in orchids when added to a medium already supplying inorganic nitrogen. Some of the amino acids were observed to replace ammonium nitrate in orchid culture (Raghavan and Torrey, 1964; Mead and Bulard, 1975, 1979; Raghavan, 1976; van Waes and Debergh, 1986). However, results obtained from experiments with amino acids have been extremely variable (Arditti, 1967a, b, 1979, 1982; Nakamura, 1982).

Several enzymes are secreted by orchid seedlings into the culture medium and many additional enzymes probably are produced though very few have been studied (Arditti and Ernst, 1984). Alvarez (1968) and Alvarez and King (1969) found the peroxidase activity to be the exact reciprocal of IAA production by Vanda seedlings. Kumaria et al. (1990) studied the activities of some oxidative enzymes in Cymbidium giganteum Wall. as influenced by
different growth regulators. They have reported the specific activity of both peroxidase and polyphenoloxidase in the auxin and cytokinin treated protocorms to be slightly suppressed in comparison to the untreated controls. Varner and Ho (1977) dealt with the physiological and biochemical aspects by treating the best known response of plant hormones with regard to control of enzyme activity. It is accepted that changes in enzyme levels cause developmental changes and growth regulators bring about a transient change in the enzyme activity (Varner and Ho, 1977; Letham et al., 1978; Moore, 1980; Kumaria et al., 1990). Relations between growth regulators and nitrogen metabolism have been reported to be reciprocal. Not only do these hormones control certain phases of protein synthesis and degradation, but two of the three main classes of hormones, the auxins and cytokinins are themselves N-containing compounds whose production is invariably linked with the nitrogen metabolism of the plant (Luckwill, 1968). Though nitrogen assimilation in plants has been studied to a wide extent (Jackson et al., 1986), there is very little information on uptake, transport and storage of nitrogen in orchids (Hew et al., 1993).

Indiscriminate exploitation of natural resources, destruction of natural habitats and other unwarranted human activities have resulted in the deterioration of natural ecosystem. North-East India with its diverse topography, altitude, climate and rainfall plays a vital role in the occurrence of diverse orchid species, however, majority of
orchids of this region are faced with threats of extinction not only due to habitat destruction through deforestation and shifting agriculture (locally termed as 'jhum') which involves clearing up of vegetation, but also due to overexploitation for their ornamental value (Hore and Sharma, 1988). A large number of orchid species (e.g. *Paphiopedilum wardii*, *Dendrobium wardianum*, *Pleione lagevieria*, *Coelogyne assamica* etc.) have reached such a critical level that there is an urgent need for their preservation. *D. wardianum* Warner, a deciduous long-stemmed epiphyte, is found in isolated pockets of North-East India and Burma. A dendrobe of delicate, fragrant beauty with an exceptionally long period of blooming inflorescence of 29 days, it is in great demand in the South-East Asian cut flower industry. Once found in reasonable numbers, this splendid orchid has become endangered and now, is scarcely found in the wild (Kataki et al., 1984). Preservation of such endangered/rare plant species in vitro can be carried out by their rapid mass multiplication using tissue culture techniques for reintroduction into the wild and by preserving the germplasm using slow growth approaches (limiting the multiplication rate) or by cryo-exposure (arresting the metabolic activities). In vitro conservation refers to maintenance of germplasm in a relatively stable form under more or less defined nutrient conditions in an artificial environment (Withers, 1987). The major aim in developing in vitro storage methods is to reduce the frequent demands of subculturing and preserving the unique genetic constitution of the germplasm.
Freezing at liquid nitrogen (LN₂) temperature tends to suppress cell division, arrests growth and retains the cells in metabolically inactive state. The suspended animation prevents the cells from ageing and provides indefinite life-span with no genetic change. However, the technique is not yet applicable to many plant species. Hence, shoot cultures of many plant species have been stored under conditions in which growth is slowed down by use of a reduced culture temperature or by the application of osmotica or growth retardants (Mix, 1982, 1985; Monette, 1986; Staritsky et al., 1986; Love et al., 1987; Roca, 1990; Schoofs, 1990). Besides, mineral oil overlay (Crane and Hughes, 1990), reduced oxygen tension (Bridgen and Staby, 1981; Engelmann, 1990) and defoliation of shoots (Withers, 1987) have also been used for slow growth storage. In recent years, artificial seeds, consisting of somatic embryos enclosed in a protective coating, have been proposed as a low-cost, high volume propagation system (Redenbaugh, 1990). The inherent advantages of artificial seeds are the production of many somatic embryos and the use of conventional seed handling techniques for embryo delivery. Besides, artificial seeds can be stored for a considerable period at low temperature or by treating them with growth retardants.

Storage of alginate-encapsulated loblolly pine and Norway spruce somatic embryos have been reported by Gupta and Durzan (1986, 1987). Also, inhibited germination of alginate-encapsulated alfa-alfa somatic embryos for one week at 4°C was reported by Redenbaugh et al. (1986). Fujii et al. (1989) arrested the
germination of encapsulated alfalfa somatic embryos by treating them with abscissic acid (ABA), thus attaining maturation of the plants before transferring them to greenhouse conditions thereby enhancing the survival rate. Research on artificial seeds has increased significantly and various reports have been made (Kitto and Janick, 1985; Bapat et al., 1987; Mathur et al., 1989; Senaratna et al., 1990; Fernandes et al., 1992), however, the germplasm conservation reports in orchids remain scanty.

Plantlets cultured in vitro wilt rapidly on transfer to normal greenhouse or field conditions. Poor water uptake and excessive water loss (Grout and Aston, 1977) may lead to high rates of mortality unless plantlets are acclimatized by gradual stages to reduced humidity and increased light intensity (George and Sherrington, 1984). The problems of poor water relations are compounded by damage to shoots and roots during transplantation (Debergh and Maene, 1981). Thus, the establishment and healthy growth of in vitro cultured plants in the glasshouse require suitable conditions. Different potting mixtures, containers and composts influence the growth of orchids extensively and differ from genera to genera (Bose and Bhattacharjee, 1980; Stewart, 1988; Talukdar et al., 1988; Yadav et al., 1988; Cribb, 1990; Robbins and Bell, 1990). Water retaining capacity of sphagnum and osmunda moss makes them suitable for the initial establishment of the orchid plantlets. Addition of manure and fertilizers is considered beneficial and the amount as well as the type varies from one species to the other.
The present work on *D. wardianum* Warner was undertaken with the following main objectives:

1) development of a feasible protocol for the mass propagation and preservation,

2) study of enzymatic activities related to nitrogen assimilation and,

3) hardening and establishment of regenerants.