Discussion
DISCUSSION

The orchid family of vegetable kingdom has been the largest assemblage of flowering plants known to science. One finds vast panorama of variation in this family. These inherently slow growing plants require a variety of factors including specific pollinators; appropriate fungal association and congenial climatic conditions for continued reproduction in nature. The extraordinary beauty of the orchid flowers naturally makes them the basis of a multimillion-dollar industry. They are most pampered and occupy top position among all the flowering plants valued for cut flower production and as potted plants.

Habitat destruction and overexploitation are the two important factors threatening the survival of orchids in India (Pradhan, 1985). The export of orchids is prevented and protected under the Committee for International Trade in Endangered Species of Wild fauna and flora (CITES). It is also included in the threatened plant list of India published by International Union for Conservation of Nature and Natural Resources (IUCN, 1991). Orchids are experiencing a steady decline in tropical countries due to destruction of natural forest areas. It is essential to take measures for the conservation and propagation of endangered orchid species (Sheelavantmath et al., 2000).

To prevent their possible extinction, a reliable propagation method is desirable (Lin et al., 2000). Plant tissue culture offers an opportunity to rescue and propagate a number of ill-fated botanicals. In cases where plants are threatened or endangered due to extreme human interference, micropropagation can provide an alternative source of species conservation (Wochok, 1981). Success achieved with the multiplication of
tropical orchids of conservation and horticultural interests are generally through seed cultures (Fay, 1988). This technique is also referred to as embryo/green pod or asymbiotic culture. In the present study, attempts were made to provide a suitable protocol for mass propagation and conservation of two unexplored epiphytic orchids namely *Coelogyne stricta* (Endemic) and *Eria bambusifolia* (Rare).

Seed germination and seedling development in orchids differs strikingly from other flowering plants. The orchid embryo is relatively undifferentiated when mature, with neither endosperm nor cotyledon. The small seed (usually < 1 mm long) swells before the embryo eventually breaks out of the coat. Further enlargement follows, forming a top-or cone-shaped seedling called a protocorm. Under natural conditions, the seedlings remains at this stage until its basal half is colonized by an appropriate endophytic fungus. After colonization, the protocorm apex produces the first leaf and roots are produced later. Grown aseptically the developing seedling must be supplied with a suitable carbohydrate source in order to progress beyond the protocorm stage (Harrison, 1977).

The nutrient requirement of the stages of the plant namely the seeds, protocorms and seedlings are not the same (Prasad and Mitra, 1975). The *in vitro* requirements of seeds/embryos are greatly influenced by the level of its maturity, genetic and ecological amplitude of the species (Anderson, 1991). Orchid seeds germinate on a wide variety of inorganic salt and ion concentrations. The nutritional requirement varies with genus, species and locality (Arditti *et al.*, 1982). Though standard media like Knudson’s (1946), Vacin and Went (1949), Raghavan and Torrey’s (1964) have been formulated for orchid
seed germination, several species show specific requirements (Mitra, 1971; Arekal and Anandakaranth, 1978). Varied media with different chemical composition have been formulated for general and specific uses (Knudson C, 1946; Vacin and Went, 1949; Thomale, 1954; Murashige and Skoog's 1962; Rosa and Laneri, 1977; Ernst, 1982). In the present study, the seeds of *E. bambusifolia* showed poor response (48 and 36%) for germination in both KC and MS media in contrast, MS medium was found to be the best (80%) for *C. stricta* seed germination and protocorm formation (Table 5 and 17). Earlier reports indicate that MS medium was unsuitable for seed germination in *Coelogyne prolifera*, *C. cristata*, *C. porrecta*, *Aerides multiflorum* (Sharma and Tandon, 1987) and *A. maculosum* (Kulkarni and Surwase, 1998).

The production of orchid seedlings from seeds involves sequential phases of germination, protocorm formation and seedling development. The same sequence of seedling development was also observed in *C. stricta* and *E. bambusifolia*.

Muralidhar and Mehta (1986) traced the sequences of histomorphological changes that take place from the onset of seed germination *in vitro*, through several stages prior to the formation of visible protocorm and the seedling formation. They concluded that the sequential histological changes from undifferentiated embryos leading to the seedling formation take two major lines of development in which embryo may directly give rise to a single seedling or the embryo proliferation may lead to twin embryos, mass of protocorm-like bodies and finally a bunch of plantlets. In the present study, the development of embryo to seedling followed the former pathway.
Germination of seed is a prelude to protocorm formation. Some of the epidermal cells of the protocorm develop rhizoids, which may be confined only to the basal regions of the protocorm as in *Calopogon, Dendrobium, Spathoglottis* and *Laeliocattleya*, or may cover the whole protocorm except the meristem region, as found in *Vanilla* (Mitra, 1986). In *C. stricta* and *E. bambusifolia* the rhizoids developed from all over the protocorms except the meristematic region. An interesting observation made in the present study was the development of a cluster of bulbous rhizoids from the protocorms in MS medium in *C. stricta* and long hairy, tubular, thread-like rhizoids in KC medium in *E. bambusifolia*. The presence of rhizoids on the protocorms is a good indicator of its predominant root character (Harvais, 1972).

The exact stage at which the germination entities develop chlorophyll however, varies with the species. The pre-protocorm development of chlorophyll is almost universal in epiphytic orchids (Vij *et al.*, 1981), which is in accordance with the present findings. For subsequent development of *in vitro* germinated seeds into seedlings, several modifications were made in the media by changing the ingredients and their quality and quantity. The most important development in the culture media was the incorporation of growth regulators like auxins, cytokinins and gibberellins. The discovery of various plant hormones led to their utilization in attempts to promote orchid seed germination and seedling growth. Growth hormones depending on type and concentration both inhibit and promote seed germination in orchids (Arditti *et al.*, 1981). The frequency with which embryos were activated to develop into protocorms and subsequent differentiation and growth of seedling was selectively affected when growth regulators were used singly or in combination in the medium.
Auxin was the first plant growth regulator used in the orchid seed culture. In majority of the cases, auxins (mostly NAA, IAA, IBA and 2,4-D) enhanced seed germination and seedling growth to some extent (Arditti, 1979). In *C. stricta*, among the auxins tested, IAA (1.0 mg/l) was found to be effective in promoting seedling growth, the same growth regulator enhanced root number and root length in *E. bambusifolia*. Similar stimulatory effect of IAA has been reported in *Cattleya* (Withner, 1951; Boesmann, 1962; Blowers, 1958; Hirish, 1959), *Vanda* (Rao and Avadhani, 1963) and *Spathoglottis plicata* (Chennaveeraiah and Patil, 1975).

**NAA stimulated germination and seedling growth in Epidendrum nocturnum** (Yates and Curtis, 1949), *Cymbidium* (Torikata *et al.*, 1965); *Cymbidium* and *Cattleya* (Strauss and Reisingar, 1976), *Vanda* (Mathews and Rao, 1980), *Dendrobium moschatum* (Sarma and Sarma, 1997) and *Arundina graminifolia* (Kaur and Sarma, 1997). Presence of NAA at lower concentration promoted normal development of the seedlings (Mitra, 1986). In *Sarcanthus scolopendrifolium*, seedling did not survive in the presence of NAA (Lee *et al.*, 1999). NAA favoured seedling growth in *E. bambusifolia*, and increased root number in *C. stricta*, as reported in *Bletilla* sp., *Cattleya aurantiaca* (Strauss and Reisingar, 1976) and Hong Kong orchids (Yam and Weatherhead, 1988). Beneficial effect of NAA on leaf and root development in *C. stricta* and *E. bambusifolia* are in line with the similar results of several orchids (Chung and Chun, 1983; Chung *et al.*, 1985). Inhibition of seedling growth in the presence of NAA was reported in a few cases. In *Dendrobium*, death occurred in the absence of NAA (Israel, 1963) and *Vanda tessellata* (Roy and Banerjee, 2002). NAA treatment differed from those of IAA treatments in the mode of the physiological action, which is in agreement with Paek and Yeung (1991) in
Cymbidium forrestii. In the case of auxin treatments (NAA and/or IAA) inverse relationship was observed between shoot length and multiple protocorms. An increase in shoot length was associated with decrease in multiple protocorms and root number in both the species investigated. This is in agreement with the results of Saiprasad et al. (2002) in Dendrobium, Oncidium and Cattleya. Growth promotion by auxins has sometimes been ascribed to their chelating properties (Harvais, 1972).

*In vitro* growth of seedlings of several species is enhanced by cytokinins. Growth in others is inhibited (Arditti and Ernst, 1984). Regarding germination of orchid seeds, in the presence of exogenous cytokinins three types of responses have been recorded: either germination has been improved or inhibited or there is no effect. In many terrestrial orchids such as Cymbidium reginae, C. calceolus and C. candidum a definite cytokinins preference for germination and protocorm growth has been reported in Geodorum densiflorum (Roy and Banerjee, 2001). Cytokinin treatments (BA and KN) resulted in more number of PLBs in Dendrobium, Oncidium and Cattleya (Saiprasad et al., 2002). In the present investigation, between the two cytokinins, KN proved to be effective for the formation of better seedlings in C. stricta whereas, BA (1 and 2 mg/l) proved to be beneficial for E. bambusifolia. Hadley (1970) also reported the promotive effect of kinetin with Coeloglossum viridae and Planthera bifolia. It’s inhibitory effect was observed in Dendrobium and Laeliocattleya (Kano, 1965). The stimulatory effect on Kinetin was reported in Coelogynce punctulata on both germination and seedling growth (Sharma and Tandon, 1986). Results presented in the table 6 and 18 establish the effectiveness of various growth regulators on seedling growth. BA at higher concentrations (3 and 4 mg/l) inhibited root formation in Cymbidium cv. Inmemoriamcyril Rucker (1974)
whereas, lower concentration proved inhibitory in *C. stricta*. BA promoted multiple protocorms in both *C. stricta* and *E. bambusifolia* and the same hormone were stimulatory in *Vanda coerulea* (Devi *et al.*, 1998) and *Anoectochilus sikkimensis, A. regalis* (Gangaprasad *et al.*, 2000).

The effects of GA$_3$ in orchid culture media could be expected to vary with the species and growth stage. Arditti and Ernst (1984) reported a negative effect of GA$_3$ on the growth of orchid seedlings. It seems that either orchid seedling synthesizes the required quantity of gibberellic acid and or the orchid seeds / seedlings have limited the ability to deactivate gibberellic acid. GA$_3$ at all three concentrations inhibited the growth of the seedlings both in *C. stricta* and *E. bambusifolia*. The same effect of the growth regulator was also reported in *A. ringens* (Jeyakodi, 2001), *Paphiopedilum spicerianum* (Sharma (1996). In contrary, the promotive or varied effect of the same hormone on seedling growth was reported in some orchids (Blowers, 1958). Saiprasad *et al.* (2002) obtained either positive or negative effect of GA$_3$ in the three orchids *Dendrobium, Oncidium* and *Cattleya*.

The synergistic effect of both auxin and cytokinin on seed germination and PLB proliferation has been reported in several orchids like *Vanda tessellata* (Roy and Banerjee (2002), *Geodorum densiflorum* (Roy and Banerjee, 2001), *Vanda’s* (Mathews and Rao, 1980) and *V. coerulea* (Seeni, 1988). In *C. stricta*, a combination of two auxins or cytokinins was ineffective in enhancing the growth of the seedlings whereas, in *E. bambusifolia* a combination of NAA and IAA favoured the development of the seedlings and BA and KN proved to be ineffective. Similar effect of the auxins was
observed in *Oncidium*, *Dendrobium* and *Cattleya* (Saiprasad et al., 2002). However, NAA + BA along with GA$_3$ proved to be the best combinations for inducing multiple shoots in *E. bambusifolia*. The same combination was also effective in *Cymbidium* spp. (Shimaski and Uemoto, 1990). These studies indicate that the growth regulators when used individually, promoted moderate growth of the seedlings and auxin, cytokinin and Gibberellic acid to be the best combination in enhancing the growth of the seedlings.

A large and bewildering array of complex additives (growth adjuvants) have been added to orchid seed and seedling culture medium. Many investigators (Vacin and Went, 1949; Kano, 1965; Prasad and Mitra, 1975) have stressed the effectiveness of organic compounds for growth of orchid seedlings. The commonly used compounds as source of organic nitrogen are urea, YE, CH and peptone. Incorporation of YE in MS medium favoured seedling development in *C. stricta* and *E. bambusifolia*. Likewise YE proved to obligatory for inducing proliferation in *Aerides multiflorum* and *Papilionanthe teres* cultures (Vij et al., 2000). On contrary, Kano (1965) and Kusumoto (1978) reported the inhibitory effect of YE in *Cymbidium* in *Brassolaeliocattleya* and *Dendrobium* respectively. YE, however, were detrimental to leaf and root growth in *Dendrobium nobile* (Devi et al., 2002). CH induced maximum multiple shoots in *C. stricta* and was ineffective in *E. bambusifolia*. Withner (1953) reported a harmful effect rather than stimulatory effect of CH on seedling growth in *Cypripedium*.

Ernst (1976) emphasized the importance of charcoal in asymbiotic culture of orchids. The addition of activated charcoal in the culture medium has helped in a rapid and better development of shoot and roots in seedlings of *Phalaenopsis* (Rosa and Laneri, 1977),
Cymbidium (Werckmeister, 1970) and Paphiopedilum (Ernst, 1974), Paphiopedilum ciliolar (Pierik et al., 1988), Cymbidium sinense (Li et al., 1997), Coelogyne nitida (Ashli, 1999), Dendrobium chrysanthum and Paphiopedilum spicerianum (Sharma, 1996) whereas, AC failed to promote the seedling development in both C. stricta and E. bambusifolia.

The enhancing effect of CW in certain terrestrial and epiphytic orchid cultures has been reported by Hegarty (1955), Lawrence and Arditti (1964), Mc Intyre et al., (1974) whereas in the present study, in C. stricta CW (10 and 20%) induced mean root length and root number and in E. bambusifolia it inhibited the root formation. Sharma (1996) observed similar inhibitory role of CW in Dendrobium chrysanthum and Kotomori and Murashige (1965) in Dendrobium sp. Letham (1974) attributed the beneficial effect of CW to its growth regulator contents, mainly cytokinins.

In C. stricta, gradual browning and death of the protocorms were observed in seed cultures. Protocorm death during early stages of the seed germination is a common phenomenon in orchid seed cultures (Harvais 1982; De Pauw et al., 1995). This phenomenon could be related to the lack of adequate nutrient conditions and/or essential growth stimulatory substances (Stoutmaire, 1974). In E. bambusifolia, in addition to the browning and death of protocorms extensive browning of the medium was also observed. Excessive phenolic production, possibly due to increased polyphenol oxidase and catalase activity triggered by certain cultural conditions (Harvais, 1982). The relative abundance of phenolics in E. bambusifolia and the negative effect of the phenolic oxidates on growth and differentiation of tissues in culture as evidenced from extensive browning of the medium and loss of cultures have been widely reported (Tanaka et al., 1988; Ernst, 1985).
Perennating organs (pseudobulbs, rhizomes, tubers) have been conventionally used to propagate orchids under field/greenhouse conditions, only a limited number of propagules could be generated, under a favorable season. However, their utility of these perennating structures in orchid micropropagation is being increasingly realized. Regeneration potential of pseudobulb explants has been positively tested in several orchids including *Cattleya, Miltonia, Cymbidium, Phaius* (Morel, 1964; Vajrabhaya, 1978), *Arundina* (Mitra, 1971), *Dendrobium* (Vij and Sood, 1982; Vij and Pathak, 1989), *Eria* and *Pholidota* (Pathak, 1989), *Mormodes* (Arditti and Ernst, 1993), *Bletilla* (Vij and Dhiman, 1997) and *Malaxis* (Vij and Kaur, 1998).

The regeneration competence of the pseudobulbs seems to be markedly influenced by physiological age of the mother plant, position of donor and growth stimulus in nutrient pool (Vajrabhaya, 1978). In the present investigation, regeneration capacity of pseudobulbs was tested only for *C. stricta*. The pseudobulb segments obtained from one year old in vitro cultures, responded readily on half strength MS medium supplemented with growth regulators. Individual treatment with BA (1 and 2 mg/l) and combined treatment with NAA resulted in production of direct development of shoot buds. The regenerative pathway and differentiation varied with quality, quantity and combination of growth adjuncts in the medium. In *Bletilla*, the pathway of regenerants was markedly influenced by the level of BA; 1 mg/l favoured callus mediated PLB development whereas, 2 mg/l favoured the development of multiple shoots. A combination of BA (1 mg/l), NAA (1 mg/l) and CH (1g/l) favoured PLBs in *Malaxis acuminata*. In *C. stricta*, irrespective of concentration of either BA or NAA favoured the development of multiple shoots as observed by Shimaski and Uemoto (1987) in
Cymbidium dayanum and Malaxis acuminata (Vij and Kaur, 1998). The ability of pseudobulb segments in orchids to regenerate multiple shoot buds and/or PLBs suggest that pseudobulb culture can be successfully employed for rapid multiplication by suitably adjusting the nutrient environment.

An imbalance between the efficiency of in vitro propagation and the delivery of regenerants poses certain limitations on the practical application of tissue culture technologies. In this connection the use of 'synseeds' (encapsulated propagules/ 'somatic seeds') as an efficient storage and delivery system is being increasingly realized. Synseeds were first prepared for alfalfa by Redenbaugh (1986); since then synseeds of many plant taxa including orchids have been prepared. So far synseeds for more than 20 orchid species have been prepared (Vij et al., 2001). Developing a synthetic seed system for orchids can obviate the routine high cost propagation methods (Rao et al., 2000). In orchids, the protocorm produced via seed and shoot tip cultures were found to be suitable for encapsulation (Singh, 1993). A variety of natural and synthetic polymers have been used as gelling agents for synthetic seeds (Redenbaugh, 1986). Polysaccharides such as alginate and carrageenin are promising due to their ready solubility at room temperature, non-toxicity, low cost and ability to form a completely permeable gel with CaCl₂. H₂O. Singh (1991) suggested sodium alginate to be the most suitable for encapsulation of PLB's in orchids.

A perusal of literature revealed considerable disparity in the concentration of alginate employed for preparing synthetic seeds. Variable requirement of sodium alginate (1-6%) seems to be related to quality of the compound and/or species specificity. In the
present study, 3% alginate formed ideal beads in both the species, but 4% alginate was found to be superior in *Cymbidium giganteum* (Corrie and Tandon, 1993), *Spathoglottis plicata* (Nayak et al., 1998b), *Geodorum densiflorum* (Datta et al., 1999), *Phaius tankervilliae* (Malemnganba et al., 1996), *Cymbidium longiflorum, Renanthera imschootiana, Agrostophyllum myrianum* (Devi et al., 1998) and 2.5% for *Aerides multiflorum* (Vij et al., 1992).

The conversion frequency and time taken for germination was found to vary with the species as well as the plating medium and the encapsulation matrix used. A comparative performance of synthetic seeds in the present study prepared in either distilled water or nutrient solution indicates better performance of the protocorms encapsulated with nutrient solutions.

The encapsulation matrix had a profound influence on the conversion frequency of synthetic seeds in *C. stricta* and *E. bambusifolia*. Protocorms encapsulated with either MS medium or MS nutrient medium supplemented with mannitol responded favourably for germination. Addition of nutrients, growth regulators and growth adjuvants in the encapsulation matrix and their influence in increasing the conversion rate has already been reported in orchid species such as *Dendrobium wardianum* (Sharma et al., 1992), *Cymbidium giganteum* (Corrie and Tandon, 1993), *Spathoglottis plicata* (Nayak et al., 1998b) and *C. odaratissima var. angustifolia* (Kamalakannan et al., 1999). In the present investigation, the nutrient medium supplemented with growth regulators such as BA (1.0 mg/l), GA3 (1.0 mg/l) individually, or NAA (1.0 mg/l) + BA (1.0 mg/l) and growth adjuvants like CH (200 mg/l), YE (200 mg/l) individually favoured the maximum
germination (100%) in *C. stricta* whereas in *E. bambusifolia*, in addition to the above growth regulators KN (1.0 and 2.0 mg/l) also favoured germination. YE (200 mg/l) AC (200 and 400 mg/l), CH (200 and 400 mg/l) to be the best for bead to plant conversion.

Though 100% germination was achieved in MS basal medium, addition of growth regulators or growth adjuvants are necessary for early germination and the growth of the seedlings in both the species. A similar effect of growth regulators was also reported in *Cymbidium giganteum* (Corrie and Tandon, 1993) and *Spathoglottis plicata* (Nayak et al., 1998b). The reason for higher growth rate and emergence of encapsulated shoot may probably be due to the availability of abundant nutrients and growth regulators in the encapsulating matrix and in the immediate cell surroundings.

The information available on the effect of growth adjuvants pertains to a reference made by Datta et al. (1999) in *Geodorum densiflorum*, where, a combination of growth regulators (NAA and BA) and growth adjuvants (CW and P) was found to yield higher percentage of germination (80%) and development of protocorms. Among the growth adjuvants, addition of YE in the plating medium enhanced the seedling growth in *C. stricta* and *E. bambusifolia*.

Orchids are endangered and require conservation through all possible means, *in vitro* storage is one component of an alternative conservation strategy, which also embraces propagation and movement of germplasm. The conversion frequency of synthetic seeds varies with passage of time, conditions of storage, and also with the nature of sowing substratum. Regeneration of plants after storage of encapsulated protocorm and protocorm-like bodies has been reported in orchids like *Cymbidium* sp.
(Talukdar and Ahmed, 2002), *Dendrobium densiflorum* (Vij et al., 2001), *Geodorum densiflorum* (Datta et al., 1999), *Phaius tankervilliae* (Malemnganba et al., 1996) and *D. wardianum* (Sharma et al., 1992). The encapsulated protocorms retained their viability up to 210 days in *C. stricta* and *E. bambusifolia* when stored at 4°C, however the germination percent declined with increase in storage time, which may be due to low metabolic rate at this temperature as suggested by Vij et al. (2001). These results are in agreement with the observation made in *Cymbidium* species (Talukdar and Ahmed, 2002), *Spathoglottis plicata* (Nayak et al., 1998 b), *Geodorum densiflorum* (Datta et al., 1999), *Phaius tankervilliae* (Malemnganba et al., 1996) and *Phalaenopsis* hybrid (Bhattacharjee et al., 1998 a, b). The germination percentage of synthetic seeds stored at ambient room temperature was always much lower in comparison with those stored at 4°C in both the species. Similar results have also been reported in *Geodorum densiflorum* (Datta et al., 1999), *Dendrobium wardianum* (Sharma et al., 1992), *D. densiflorum* (Vij et al., 2001) *Coelogyne mossiae* and *Eria reticosa* (Jeyakodi, 2001). The low conversion frequency after storage at room temperature may be due to desiccation. The differential conversion responses of the two orchid species investigated may be due to differences in their genetic make up.

Minimal growth conservation is the most direct way of restricting growth and development of *in vitro* materials, and is most frequently applied to differentiated plantlets and developing meristems cultures. Growth rate is reduced using sub optimal physical or nutrient conditions or by incorporating specific growth inhibitors into the medium (Naik and Sarkar, 2000). One of the approaches to reduce the growth rate is to increase the osmotic pressure of the medium by adding metabolically inactive sugar.
alcohols like mannitol or sorbitol. Addition of mannitol as an osmoticum to reduce plant growth has been tested in many crops (Ng and Ng, 1991; Westcott, 1981a), Xanthosoma sp. (Zandvoort et al., 1994) and potato (Naik and Sarkar, 2000). Osmotic stress is induced when sucrose or mannitol is seldom applied alone. In general, mannitol is applied in combination with other growth limiting treatments such as reduced temperature, low light intensity, varied photoperiods and sucrose combinations (Westcott, 1981a; Estrada et al., 1983; Siddiqui et al., 1996; Sarkar and Naik, 1998).

In C. stricta and E. bambusifolia, detailed studies were made to investigate the effect of mannitol and temperature on storage and conversion of synthetic seeds. Between the two different temperatures tried, low temperature (4°C) storage enhanced the viability of the protocorms. The percentage of conversion frequency declined with increase in storage time. A combined treatment of low temperature and inclusion of mannitol in the conservation medium increased the bead to plant conversion. A similar positive effect of inclusion of mannitol in the storage medium and conservation of microplants has been reported for potato (Sarkar and Naik, 1998; Lizarraga et al., 1989). The growth limiting effect of mannitol may be that it increases the osmotic pressure of the medium as a result, water availability to the growing cultures is reduced. Controlled osmotic stress in combination with other growth limiting treatments such as reduced temperature, low light intensity and varied photoperiods are primary requirements for devising most effective storage system. A combination of both factors in the media was found to be effective in reducing slow growth in Cassava (Unnikrishnan, 1996).

In order to induce effective rooting in the in vitro regenerated plants, half strength MS medium was used by incorporating auxins. NAA (2.0 mg/l) and IAA (2.0 mg/l)
proved to be effective for rooting in *C. stricta* and *E. bambusifolia* respectively. Similar effect of the growth regulators was also reported by Yunfang *et al.* (1999) in *Anoectochilus formosanus* and Forres and Mo Gollon (1998) in *Cattleya lueddemanniana* and in *Cymbidium ensifolium* rooting was achieved in different strengths of MS medium.

When orchids are cultivated artificially, they are put in a suitable growing media, which would provide mechanical support to plants, supply water and nutrients to the roots and at the same time provide good drainage and aeration. In the past, several investigations have been carried out to select suitable and inexpensive potting media for different type of orchids. An ideal medium should preferably be inert, resistant to organic decomposition, as well as porous enough to ensure adequate aeration, cost effective and easily available (Bose and Bhattacharjee, 1980). The regenerated plants of *C. stricta* and *E. bambusifolia* showed maximum survival of 76% and 60% respectively on the potting medium containing coconut husk, charcoal, brick pieces, broken tiles and perlite (2:1:1:1:1). Acclimatization of *in vitro* raised plantlets in both the species was essential thus supporting conclusions of Corner and Thomas (1981) and Dunstan and Turner (1984).

Orchid seeds are unique and depend on some external source of nutrients in order to make their undifferentiated embryos to develop into protocorms upon germination. The mycorrhizal fungi form a channel to supply the nutrients for the orchids under natural conditions. The orchids are heterotrophic and nourished by symbiotic fungi during the early stages of their establishment (Leake, 1994). The importance of mycorrhizal fungi in orchid seed germination was first established by Bernard (1911), which was isolated and identified as *Rhizoctonia* (Bernard, 1904). The symbiotic germination of orchid seeds and its critical analysis reveals a remarkable degree of control and regularity in the infection process.
by mycorrhizal fungus. Warcup (1973, 1981) assumed high level of specificity between host and fungus, however, Curtis (1939) has shown a lack of specificity implying that the fungal association was dependant on the fungal availability rather than the host species.

A major constraint in the large-scale application of tissue culture techniques in conservation related propagation is the high mortality experienced by the regenerants during and following their laboratory to land transfer. The tissue-cultured plantlets also lack sufficient resistance against abiotic and biotic stresses, particularly the soil microbes. The fungi endophytic with orchids are commonly considered as members of the form genus \textit{Rhizoctonia}. Mycorrhizal association has been found to enhance rooting and promote the growth of tissue-cultured plants. Studies were conducted to evaluate the effects of the orchid mycorrhiza \textit{Rhizoctonia solani} on micropropagated plants in the potting media containing coconut husk, charcoal, brick pieces, broken tiles and perlite. The survival percent of mycorrhizal plantlets was 84\% compared to non-mycorrhizal plantlets (76\%). The fungus when inoculated to tissue-cultured plants of \textit{C. stricta} influence and established structural and functional compatibility as evidenced by formation of pelotons. Senthilkumar (2001) suggested that there may not be any specificity of orchid and fungus at the seedling stage but there may be likelihood of specificity in the adult orchid under \textit{in situ} conditions (Masuhara and Katsuya, 1994). Smrecio and Currah (1989) opined that there might be succession of fungi involved in the association, from seed to mature plant.

\textbf{Antimicrobial activity and phytochemical analysis}

\textit{Rig} veda and the \textit{Atharvana} veda which are known to be the oldest books, provide inquisitive information about the medicinal value of the orchids. The Orchidaceae is a large family and many members of which contain phytochemicals. Related plant taxa
tend to produce similar chemical compounds (Harborne, 1984). The closer the taxonomic relationship, the better are the chances that similar compounds may occur in these taxa (chemical race). When such a compounds are of medical or pharmaceutical importance, attempts are made to search for similar or related compounds. *C. stricta* is used by rural Khasi and Jaintia tribes of Meghalaya as a source of medicine (Kharkongor and Joseph, 1981).

Testing of bioactive principles of *C. stricta* for antimicrobial activity showed varying degrees against bacterial (*Aeromonas hydrophila, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi, Vibrio cholerae*) and fungi (*Alternaria alternata, Aspergillus niger* and *Pyricularia oryzae*). The activity against these pathogens might be due to the presence of phytochemical constituents of *C. stricta*. Mostly pharmacological activity of medicinal plants resides in the so-called secondary metabolites, which are comparatively smaller molecules in contrast to the primary metabolites such as proteins, carbohydrates and lipids (Krishnakumar *et al.*, 1997). The *in vitro* and *in vivo* extracts of *C. stricta* showed higher inhibitory zone (20.8 and 12.8 mm) against *A. hydrophila* and *P. oryzae* at 1.0% concentration. Similar activity of the *in vitro* plant extracts was observed in *Amorphophallus smithsonianus* (Aravinthan, 2002), Marwani *et al.* (1997) in *Tectona grandis* against *Escherichia coli* and *Bacillus subtilis*. The *in vivo* extracts showed a slightly higher activity than *in vitro* against *B. subtilis* and *P. oryzae*. The antibacterial potential varied from species to species and the test organism (Ghanaksh and Kaushik, 1999a; Kaushik and Kishore, 1991). In the present study, the efficacy of the extract was however, directly proportional to its concentration, higher the level of its dilution lesser the effective zone of inhibition. This observation is in agreement with the findings of Ghanaksh and Kaushik, (1999b) in *Rhyncostylis retusa*. 
The presence of antimicrobial properties provides clues for identification of phytochemical compounds. Positive and promising laboratory test results for the extract provide a strong basis to identify the compounds through TLC.

Most of the orchid members screened showed the pyrrolizidine or dendrobine based alkaloids whereas, a few exhibited unrelated structures (Luning, 1964). In the *C. stricta*, the *in vitro* and *in vivo* samples showed the presence of the alkaloids berberine. The alkaloid berberine has a variety of unique pharmacological effects. Berberine containing plants are used medicinally in virtually all-traditional medicinal systems.

Berberine is an alkaloid present in a number of clinically important medicinal plants, including *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *B. vulgaris* (barberry) and *B. aristata* (tree turmeric). Berberine has demonstrated significant antimicrobial activity against bacteria, fungi, protozoans, viruses, helminthes and chlamydia. When antimicrobial activity of the extracts from *in vitro* and *in vivo* plantlets are concerned positive results were obtained. This may be due to the presence of berberine in the plant extracts. *In vitro* experimental and clinical results indicate that berberine as an excellent disinfectant for infective root canal deciduous teeth (Su, 1992). In addition to berberine, another oily alkaloid, conine, which reported to have insect disrupting property, was also traced out from the *in vivo* plant samples. The failure to produce secondary metabolites in *in vitro* may be due to special physiological and morphological properties of cultured tissues (Butcher, 1988).

In the present investigation, known flavonoids such as phloridzin, luteolin, hesperidin and dihydroquercetin were identified in both *in vitro* and *in vivo* samples. Similarly, the presence of luteolin was also reported in other orchids like *Listerva ovata*.
and Restrepia elegans (Williams, 1979). Luteolin has shown the ability to protect cells against the cancer and also to inhibit DNA oxidative damage, cardiotonic activity, inhibit enzyme tyrosin kinase on cell growth and metastasis, potential carcinogenic effects against estrogen-induced mammary carcinogenesis, potent antimitagenic action, anti-inflammatory and antioxidant properties.

Kandaswami et al. (1992) demonstrated their antiproliferative effect against three cell lines in tissue culture. Dihydroquercetin is effective for preventing osmotic stress in hyperglycemia (Haraguchi et al., 1997). Hesperidin is a bioflavonoid group of colored substances found in many fruits, and essential for the absorption and processing of vitamin C. Hesperidin shares left/right sided cell receptors and may be considered essential to human health. It has anti-inflammatory and analgesic effects.

Earlier reports indicated significant activity of flavonoids against several microorganisms (Brantner et al., 1996; Wang et al., 1989; Ismail and Alam, 2001). Flavonoids isolated from Sophora flavescens showed antifungal activity against Basidiomycetes and Phycomycetes (Yagi et al., 1989). Tereschuk et al. (1997) noticed several degrees of antimicrobial activities of flavonoids against gram-positive and gram-negative bacteria.

In addition to alkaloids and flavonoids, phenols and phenolics like gentisic acid, vanillic acid, phloroglucinol and 2-methyl resorcinol. Phloroglucinol is a non atropinic antispasmodic agent which preferentially used by intramuscular or intravenous infection in veterinary medicine for its spasmolytic properties against the urinary tract. The sugar rhamnose were detected in C. stricta whereas, Jeffrey et al. (1970) reported the presence of glucose, fructose, raffinose and sucrose from the orchid Coelogyne cristata.
In the present investigation, the phytochemical profiles of *in vitro* and *in vivo* plant samples varied, which may be due to the influence of cultural environment and also in the *in vitro* conditions the exogenous factors such as organic and inorganic components in the medium, growth hormones, light and temperature have strong influence on growth and secondary metabolism. Working with tissue cultures of *Catharanthus roseus*, Zenk (1978) observed that growth hormones added to the medium strongly influenced the production of phytochemicals. Manipulation of the plant cell culture environment and media can affect the rates of both cell growth and accumulation of secondary metabolites (Bhalsing and Maheshwari, 1998; Butcher, 1988).

The results of the present investigation and some earlier reports summarized above indicate a varied chemistry in the family of which only a small part have been investigated. Substantial information is available in the scientific literature regarding the folklore therapeutic uses and phytocconstituents of orchids, but biological evaluation of these plants or of their derived products has not been undertaken so far. The presence of the above mentioned medicinally important compounds confirm the fact that the plant under study is medicinally important.

The dire need of hour is to explore the potential metabolites of plant origin, which can mimic the effect of present drugs or can be supplemented with other drugs to make them more effective and easily available to mankind.