Review of Literature
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Orchids form the world's largest family of flowering plants and have been marveled over for centuries. Inherently, they are slow growing plants. Consequently, the traditional propagation methods (cuttings, off-shoots, back bulbs, keikis and tubers) can hardly fulfill the need of the market. The reduction of natural habitats for orchid growth requires special method for species conservation. Embryo/seed culture is one such method. Embryo culture adds the possibility of preserving and enlarging the gene pool in apomictic and amphimictic species respectively.

Orchid seeds are unique in that they are minute in size and devoid of endosperm. Due to the non-endospermic nature of the orchid seed, their germination in nature is a unique process. In nature, the seeds depend on fungal infection for germination and development. The role of mycorrhizae in orchid seed germination discovered by Bernard (1899) formed the basis for in vitro symbiotic seed germination. Burgeff (1909) and Bernard (1909) established that a symbiotic relationship existed between the fungus and seed.

Attempts to propagate orchids from seeds, using symbiotic (mycorrhizal) fungi have been successful for species in Europe (Clements et al., 1986; Stewart and Mitchell, 1991), Australia (Clements and Ellyard, 1979; Clements, 1982; Dixon, 1987) and in North America (Anderson, 1991; Zettler and Mc Innis, 1992). Despite of advances in this area relatively few species have been propagated by this method. Since in the present investigation, seed/embryo culture studies were pursued using asymbiotic method, the literature pertaining to this aspect is reviewed in detail.
In 1922, Knudson introduced a new legendary means to cultivate many species of orchids particularly the epiphytic taxa, from seeds without the use of fungi. His technique of asymbiotic germination, involved sowing orchid seeds on a nutrient medium and carbohydrates within the medium would substitute for mycotrophy enabling orchid seeds to germinate. For the first time, many orchids could be grown with relative ease without involving a second organism, the fungus.

Seed germination, seedling development is strikingly different in orchids compared to other flowering plants due to the development of an intermediary cone-shaped structure, the protocorms, which has an inherent potential to multiply through budding in the superficial cell layers; the potential varies with species and medium (Withner, 1959) and can be exploited for culture multiplication.

Phase I: Seed germination

The nutritional requirement for the germination of seeds varies with the genus, species and locality (Arditti et al., 1982; Arditti and Ernst, 1984). Many media differing in chemical composition have been developed for general and specific uses.

Earlier, the medium used for orchid seed germination was a mixture of ground Ophrys tubers called salep and agar. Subsequent analysis of the above medium by Knudson (1922) proved that it contained 48 percent mucilage, 27% starch, 5% protein and trace of sugar and minerals.

Knudson (1922,1946) demonstrated that at least some orchids could germinate asymbiotically on suitable media. Since the advent of the Knudson’s medium a large
number of media were formulated for the culturing of orchid seeds. The Knudson C medium (1922) has been the basis for most of the macro salt solutions used to germinate orchid seeds. Others followed and tried to improve his media or design new ones to germinate additional species. Knudson's medium is suitable for most epiphytic and/or tropical orchids. However, some terrestrial species especially those from temperate regions, are most difficult to germinate. Consequently, additions, modifications or new media and procedures were employed for terrestrial species (Stoutamire, 1974; Hadley, 1970). Burgeff (1936) formulated N3f medium for general and specific uses and he provided considerable data regarding seed germination in about 25 genera, of which many species were hybrids.

Boeman (1958) compared the germination response of *Cypripedium* seeds in Knudson C, N3f and Thomale GD media. Plants grown on N3f medium along with ammonium sulphate showed better root development and dark green foliage with good percentage of germination. The seeds of *Cattleya* showed better germination on modified Steientz medium while *Ponthieva* germinated best in G&B mother flask medium (Baker et al., 1987).

Lucke (1971), established seed germination of 26 species of *Paphiopedilum* in Thomale's medium supplemented with either biotin or nicotinamide. A few species of *Dendrobium* showed satisfactory germination in the modified Knudson C medium (Mukherjee et al., 1973). Liquid culture proved to be unsuitable for growing *Cypripedium reginae* seeds (Harvais, 1973). Flamee (1978) tried a variety of media like Burgeff's N3F, Thomale GD and Knudson C to establish seed germination in *Paphiopedilum*, but significant results were obtained only on Thomale GD medium.
Stimart and Ascher (1981) tested four semisolid media viz., Burgeff EG-I, Thomale GD, Knudson C and Norstog for *in vitro* germination of *Paphiopedilum* seeds and suggested Norstog's medium to be effective. The seeds from immature capsules germinated faster and in higher proportions than those from mature capsules of *Calypso bulbosa, Epipactis atrorubens* and *Goodyera tesselata*. The most suitable media for orchid germination are those devised for the culture of barley embryos (Norstog) and modified Curtis medium containing urea and calcium carbonate, instead of ammonium nitrate and calcium nitrate (Arditti *et al.*, 1981). Meyers and Ascher (1982) germinated seeds of *Epipactis gigantea* and *Calopogon tuberosus* using Norstog's medium.

So and Lee (1985) reported that the seeds of *Cymbidium virescens* showed best germination in Kyoto 2 medium. However, addition of peptone (3g /l) in MS medium was better than Kyoto 2 medium. Singh and Prakash (1985) cultured the immature embryos from unripe capsules of *Epidendrum radicans* on both liquid and agar solidified Vacin and Went medium.

Van Waes and Debergh (1986) studied the effect of different dilutions of macro elements ranging from 0.47 to 97 mg of the basic medium containing Thomale's macro elements, Nitsch and Nitsch's micro elements, Fe EDTA solution and vitamins on 23 Western European orchids. They observed best germination in all species on the basal medium without macro elements.

*Cymbidium longifolium* seeds were successfully germinated on three basal media, KC, VW and RT. The rate of seed germination was assessed upto 30% on KC, 60% in VW and 35% on RT (Muralidhar and Mehta, 1986). Seeds of *Bletilla hyacinthina* (*B. striata*) took only 7 days for greening and germination in Vacin and Went medium (Nair *et al.*, 1986).
Sharma and Tandon (1987) incubated seeds of *Cymbidium elegans*, *Coelogyne prolifera*, *C. cristata*, *C. porrecta*, *Aerides multiflorum*, *Sarcanthus pellidus*, *Bulbophyllum comosus* and *Thunia alba* in Knudson C, Pfeffer or Vacin and Went nutrient media. The best germination for all the species was obtained in Knudson C medium and the maximum germination ranged from 80% in *A. multiflorum* to 14% in *C. prolifera*. The naked embryos from mature pods of *Dendrobium crepidatum* were cultured in four different media. Modified KC medium gave the quickest germination than KC, VW and MS basal media (Reddy et al., 1988).

Asymbiotic germination and growth response of *Dendrobium moschatum*, *D. farmeri*, *D. fimbriatum* var. *oculatum* and *D. primulinum* were tested using four different basal media. The seeds of *D. farmeri*, *D. primulinum* germinated better (50-60%) in VW and *D. fimbriatum* var. *oculatum*, *D. moschatum* on Nitsch media (Devi et al., 1990).

Seeds of *Spathoglottis plicata* responded favourably when cultured in MS medium and *Epidendrum radicans*, *Dendrobium crepidatum* and *Cymbidium aloifolium* to RL medium (Reddy et al., 1992). De Pauw and Remphrey (1993) cultured three species of *Cypripedium*, *C. candidum*, *C. reginae* and *C. calceolus* var. *parirflorum* on Harvais, Van Waes and Deberg and modified Norstog media. The germination was assessed at 4 weeks interval for 20 weeks. *Cypripedium reginae* germinated better than the other two species.

Wagner and Hansel (1994) studied the seed germination capacity of immature seeds of *Cypripedium calceolus* at different developmental stages on the culture medium of Van waes and Debergh. Seeds from green capsules of *Phaius tankervilliae* were cultured on Murashige and Skoog, Raghavan and Torrey's (RT) and Vacin and Went (VW) media and the germination was found to be best on VW medium (Nagaraju and Parthasarathi, 1994).
Immature seeds from unripe capsules of four species of Asiatic *Cypripedium* (*Cypripedium debile*, *C. henryi*, *C. japonicum* var. *C. formosanum* and *C. tibeticum*) were sown on Murashige and Skoog medium. Germination and protocorm development occurred within 3-5 months after sowing (Hoshi *et al.*, 1994). Seeds of *Habenaria radiata* were placed on Hyponex medium and incubated at 25°C under fluorescent light at an intensity of 2000 lux. Within a week, the seeds turned green and germinated (Nagayoshi *et al.*, 1996). The germination response of *Vanda* “John Club” embryos was tested in MSF. (MS medium – full concentration), MSH (MS medium – half concentration), VW and KC culture media (Bhaskar and Rajeevan, 1996).

Sharma (1996) tried a variety of nutrient media viz., Burgeff N3f, Fast, Knudson C, Murashige and Skoog, Robert Ernst, Thomale GD, Vacin and Went, Wolter and Skoog, Pfeffer and Zak media for the seed germination of *Dendrobium chrysanthum* and *Paphiopedilum spicerianum*. Both the species responded favourably in Thomale GD medium. Sharma (1998) studied the effect of age of the capsule of *Vanda* at various stages of development after pollination. Seeds obtained from pods formed 270 days after pollination exhibited the highest germination (90.13%) on Knudson C medium. Seeds were cultured on 4 different basal media. Knudson C medium with micronutrients (KC) promoted seed germination.

Seeds of *Eulophia cucullata*, *E. streptopetala* and *E. petersei* were germinated on Murashige and Skoog medium supplemented with 3% sucrose and 0.01% myo-inositol. *E. cucullata* and *E. petersei* germinated after 3 months, whereas, *E. streptopetala* took 6 months to germinate. *E. streptopetala* and *E. petersii* showed high percentage of seed
germination (70-80%) whereas, in *E. cucullata* only about 15 seeds germinated (Mc Alister *et al.*, 1998). Growth of *Cymbidium ensifolium* was enhanced in half strength MS liquid medium, at pH of 4.6-5.8 (Rizine *et al.*, 1998).

Bhattacharjee *et al.* (1999) studied the seed germination in the hybrid *Phalaenopsis* in MS, RL and KC media. Seed germination was faster on RL medium followed by MS. Seeds of *Aerides multiflorum* were cultured on MS, B5, KC and VW. MS medium was found to be the best for seed germination and development of plants, followed by B5 medium (Sarma and Sarma, 2001).

**Plant growth regulators**

The effect of different growth regulators on the germination of different orchids varies depending on the physiological requirements, hormone dosage, and different forms of hormones, culture condition and age of the plant.

Growth regulators proved to be effective in enhancing the germination of seed and subsequent growth of seedlings. Auxin was the first plant hormone added to the orchid seed culture (Burgeff, 1934; Arditti, 1967; Withner 1959; 1974). Seeds of *Cymbidium mastersii* gave more than 80% germination in Knudson C basal medium, containing amino acids, vitamin, purines or IAA, whereas seeds of *Cymbidium eburnum* did not germinate in any treatment (Prasad and Mitra, 1975). In majority of the cases auxins (mostly NAA, IAA, IBA 2,4-D) enhanced seed germination and seedling growth (Arditti, 1979). Naphthalene acetic acid (NAA) stimulated germination of *Corallorhiza innata* (Downie, 1943), *Cattleya harrisoniana*, *Epidendrum facestum* and *Oncidium varicosum* (Meyer, 1945). The optimal concentration of NAA for seed germination was
0.1 mg/l in *Cattleya* (Ichihashi and Kako, 1973) and 1.25 mg/l in *Vanda* (Payawal and Guzman, 1972). Strauss and Reisinger (1976), reported that higher concentration of NAA (1 mg/l) induced seed germination and more rapid growth in *Cattleya aurantica*, *Cymbidium madidum*, *Bletilia* spp, a hybrid *Chondrorhyncha discolor* and *Hycaste aromatica*. Das and Ghoshal (1989) studied the *in vitro* seed germination response of *Dendrobium chrysotoxum*, *D. pierardii*, *D. crepidatum*, *Aerides multiflorum* and *Cymbidium aloifolium* on Knudson C and Burgeff Eg-1 media. Both the media supplemented with NAA were found suitable for the development of seedling.

IAA had no effect on *Goodyera repens*, *Coeloglossum viride* and *Orchis purpurella* seeds (Hadley, 1970). IAA inhibited seed germination in *Vanda* cv. Miss joaquim (Goh, 1971). According to Mukherjee *et al.* (1973), IAA at 2 mg/l induced callus formation in *Dendrobium moschatum*. The germination of *Cymbidium mastersii* seeds increased at 1.0 mg/l of IAA to 80% as against 45-55% in control (Prasad and Mitra, 1975). Pierik *et al.* (1988) reported that IAA at 1 mg/l stimulated seed germination in *Paphiopedilum ciliolare*.

2, 4-D either inhibited germination or stimulated callusing of the seeds. Traces of 2,4-D have been detected in *Cyripedium* seeds but not in *Dendrobium* and *Calanthe* (Poddubnaya – Arnoldi, 1960; Poddubnaya Arnoldi and Zinger, 1961). According to Goh (1971), the germination of *Vanda* cv. Miss joaquim seeds was inhibited in the medium containing 2, 4-D at 0.1, 0.25, 0.5, 1, 2 and 5 ppm, while in *Spathoglottis plicata* 2,4-D (1 mg/l) induced seed germination (Chennavearaiah and Patial, 1975).
Sharma (1996) reported that auxin with the exception of 2,4-D, induced speedy and increased seed germination in *Paphiopedilum spicerianum* but inhibited seed germination, in *Dendrobium chrysanthum*. However, in both genera subsequent growth of the germinated seeds on the auxin-containing medium was inhibited. Nagabhusana (1981) reported that IBA at 2 and 4 ppm in VW media improved germination in *Spathoglottis plicata*. Growth and plant differentiations of *Cymbidium longifolium* were best on Nitsch medium containing 0.5 mg/l IBA (Siddique and Paswan, 1998).

Kinetin had pronounced effect on the germination of seeds in *Orchis purpurella* (Hadley and Harvais, 1968), while kinetin at 1-10 ppm retarded the germination of seeds in *Coelogyssum viride* and *Platanthera bifota* but increased the growth rate of protocorms (Hadley, 1970). Prasad and Mitra (1975) reported that kinetin at 0.3 mg/l promoted seed germination of *Cymbidium mastersii* to 85% against 45 percent in control. In *Cypripedium reginae*, Harvais (1982) reported 100% seed germination by addition of 1 mg/l kinetin against 5% germination in control. In *Dendrobium chrysanthum*, kinetin did not improve germination of seeds. In *Paphiopedilum spicerianum*, kinetin showed poor response (Sharma, 1996).

Van Waes and Debergh (1986) observed that BA at 0.88 μm improved the germination of seeds in *Epipartis helleborie*. Pierik *et al.* (1988) reported that BA at 0.001, 0.01, 0.1, 0.5 and 1.0 mg/l had no effect on seed germination in *Paphiopedilum ciliolare*.

The germination percentage of *Cattleya* was poor in the presence of GA₃ (Blowers, 1958; Hirish, 1959). In *Dendrobium nobile*, GA₃ at 1, 10 and 100 ppm promoted seed germination (Miyazaki and Nagamatsu, 1965). In *Cypripedium reginae*,
GA₃ upto 5 mg/l had no effect on germination and early development until shoot formation, but caused marked abnormal shoot elongation (Harvais, 1982). GA₃ reduced the germination of seeds in Dactylorhiza maculata and Listera ovata seeds (Van Waes and Debergh, 1986). In Cymbidium macrorhizon, gibberellic acid proved beneficial during early stages of seed germination but suppressed chlorophyll development (Vij and Pathak, 1988). GA₃ had an inhibitory effect on seed germination in Dendrobium chrysanthum and Paphiopedilum spicerianum (Sharma, 1996).

Harvais (1973) reported that KN and NAA in the ratio of 10:1 were very satisfactory for seed germination in Cypripedium reginae. Cymbidium goeringii when cultured in either MS or RM media containing 1 ppm NAA and 0.1 ppm kinetin produced maximum rhizome formation (Hasegawa and Gor, 1987). In Cymbidiums niveo and C. ensifolium × C. kanran cv Dohi a low kinetin: NAA ratio gave good rhizome growth and a high ratio gave good shoot formation and growth. Kinetin 3 mg/l and 0.3 mg/l NAA induced in vitro flowering from rhizomes (Paek et al., 1989).

In Bletilla striata, Gaya and Suner (1995) reported higher percentage of seed germination on Knudson C and Murashige and Skoog media supplemented with KN and BA. In Spiranthes spiralis, seed germination percentage was low on the above medium. An efficient procedure was developed by Nayak et al. (1998a) for the in vitro regeneration of Cymbidium aloifolium using rhizomes developed from seeds. MS medium containing NAA (5 mg/l) was effective for the proliferation of rhizomes. The highest frequency of shoot regeneration and the highest number of shoot bud formation were recorded in the medium supplemented with BA at 1.0 mg/l and NAA (0.1 mg/l). Moreover, NAA-BA combination induced rooting in regenerated shoots thereby producing complete plantlets in one step.
B5 and Murashige and Skoog (MS) culture media supplemented with BA, NAA and GA3 at 4.0, 0.3 and 0.3 mg/l, respectively, were most suitable for cluster bud regeneration and growth of Anoectochilus formosanus. One-tenth strength MS medium supplemented with sucrose at 20 g/l and NAA at 0.5 mg/l was most effective for rooting (Yunfang et al., 1999). Studies conducted by Zhu Yuqiv and Xuegen (1999) to optimize the culture medium and phytohormones of Bletilla ochracea revealed that half - strength MS medium supplemented with BA at 1.0 mg/l and NAA at 0.1 mg/l was favourable for proliferation of the original bulb.

Cymbidium aloifolium seeds were cultured on modified MS medium supplemented with various concentrations of kinetin alone or in combinations with NAA and IBA (Buzarbarua, 1999). The combined presence of growth hormones BAP and NAA was inhibitory to seed germination in Geodorum densiflorum (Roy and Banerjee, 2001). In Thunia venosa, Gurav and Dixit (2002) obtained 100% germination in Knudson medium.

Growth adjuvants

On the premise that orchid seeds may require supplements other than a carbon source and some of the known growth factors, various investigators have tested the effect of complex additives on the germination of orchid seeds.

A large number of complex additives have been used in orchid seed and seedling culture media. Some common additives are banana pulp (Arditti, 1968), tomato juice (Rao and Avadhani, 1963; Valmayer, 1974), pineapple juice (Arditti, 1967), potato extracts (Harvais, 1974), peptones (Withner, 1953; Tsukamoto et al., 1963), fish emulsion (Griffith and Link, 1957), honey (Thomale, 1954) and yeast extracts (Ito, 1955; Mathews and Rao, 1980; Prasad and Mitra, 1975).
Chung et al. (1984) found that the seed germination of *Aerides japonicum* was best in Hyponex medium containing peptone (3 g/l), banana homogenate (35 g/l) and activated charcoal (2 g/l), whereas, for *Cymbidium ensifolium* Hyponex medium supplemented with peptone (4 g/l) and four per cent sucrose at pH 4.5 was found to be the best (Chung et al., 1985a). Katiyar et al. (1987) reported asymbiotic seed germination and seedling development in *Coelogyne punctuata* and *Aerides multiflorum* on Knudson C medium supplemented with 100 gm/l ripe banana pulp. The percentage of seed germination at 90 days of culture was 81.4 for *C. punctuata* and 60.8 for *A. multiflorum*.

Soe and Jano (1988) reported Bayfolan medium to be the best and cheaper for *Dendrobium waltermoe* than Vacin and Went medium. The best root formation, shoot growth and fresh weight increments were obtained on the Bayfolan medium supplemented with CW, sugar, charcoal and fish emulsion. Sharma (1996) pointed out that incorporation of organic supplements like banana homogenate, apple juice and peptone was not required for the seed germination in *Dendrobium chrysanthum* and *Paphiopedilum spicerianum*. Siddique and Paswan (1998) studied the effect of organic supplements in differentiation of *Cymbidium longifolium*.

Withner (1953) reported that incorporation of mashed banana (40 g/l) in Knudson C medium or Burgeff N3F proved to be excellent for *Paphiopedilum* seed germination. Peptone enhanced germination of *Paphiopedilum insigne*, *Cymbidium virescense*, *Cypripedium reginae*, *Brassolaelio cattleya* and *Dendrobium* (Kano, 1965). According to Arditti (1966) tomato juice was not a good medium for orchid seed germination.
Tauge, a bean sprout product obtained from seedlings of *Vigna radiata* was incorporated into an orchid seed germination medium. The medium containing 100-150 g tauge, 20 g cane sugar and 13 g agar in 1 litre distilled water has been used successfully to germinate seeds of two species of *Dendrobium* (Soerohaldoko, 1980). *Aerides maculosum* seeds germinated readily on MS medium enriched with peptone.

The role of coconut milk has been variously assessed in orchid germination and growth. It promotes germination in several orchids including *Cattleya* (Kerbauy and Hando, 1981) and *Cymbidium* (Chung *et al.*, 1985b). Coconut water is commonly added to stimulate callus formation (Goh, 1970; Goh *et al.*, 1975). The range used was 10 to 25% (v/v). Singh and Prakash (1985) have confirmed that coconut water (150 ml) supplemented to both Knudson C and Vacin and Went media was most ideal for the germination of *Epidendrum radicans*. It has been established that the addition of coconut water (150 mg/l) to VW nutrient medium enhanced seedling growth in *Acampe praemorsa* (Krishna Mohon and Jorapur, 1986).

The first attempt to darken a culture medium used for orchid seed germination seems to have been made in an effort to germinate native American *Cypripedium* species (Curtis, 1943). The action of charcoal in tissue culture is uncertain; however, its ability to darken the medium and/or the absorb undesirable metabolic by-products might have contributed to the enhanced rooting performance of *Diuris longifolia* on the charcoal medium (Collins and Dixon, 1992). According to Devi *et al.*, (1990) addition of 15% CM and 5% each of banana extract (BE) and pineapple juice (PJ) in Vacin and Went medium, accelerated germination and favoured leaf and root growth in *Dendrobium farmeri* and *D. primulinum*. 
Phase II: Protocorm formation

Protocorm formation is a unique feature of the family Orchidaceae. Germination of seeds is a prelude to protocorm formation. The protocorm may be elongated as in Bromheadia, Taenophyllum, Vanilla or it may be trilobed as in Cypripedium or triangular and branched as in Cattleya and Dendrobium. Some of the epidermal cells of the protocorm develop into rhizoids, which may be confined only to the basal region of the protocorm as observed in Calopogon, Dendrobium, Spathoglottis and Laeliocattleya or whole protocorm except the meristem region as in Vanilla, rhizoids may be short or long, simple or branched.

Kerbauy and Hando (1981) reported that immature embryo from unripe capsules of Cattleya intermedia, C. gattata and Epidendrum mosenu developed protocorms in Knudson C liquid medium rather than solid medium. The germination response of Rhyncostylis retusa, Vanda coerulea embryo was tested in four different culture media. Vacin and Went medium proved to be the best for obtaining a better crop of protocorms (Nath et al., 1991). In Dendrobium simbriatum var. oculatum the seed germination was best in Nitsch's medium and protocorm development was better in Murashige and Skoog's medium (Kumaria and Tandon, 1991).

Orchis papilionacea seeds (ripe and immature) were cultured on modified double strength of Curtis medium. Seed germination (ripe) on both solid and in liquid medium was low and protocorms obtained in the solid medium developed into white callus and in liquid medium under light developed into minitubers (Pedrose and Pai, 1992). De Pauw and Remphrey (1993) cultured three species of Cypripedium, C. candidum, C. reginae
and *C. calceolus* var. *parirflorum* on Harvais, Van Waes and Deberg and modified Norstog media *Cypripedium reginae* germinated better than other two species. Subsequently, development of protocorms was superior in all cases on the modified Norstog medium.

MS with half concentration of inorganic salts proved to be the best for better production of protocorms in *Vanda* ‘John Club’ (Bhaskar and Rajeevan, 1996). In *Dendrobium moschatum* and *Cymbidium aloifolium*, Nitsch medium was found to be the best for the formation and proliferation of protocorm-like bodies (Devi et al., 1997). Leroux et al. (1995) observed that in *Cypripedium acaule*, seeds cultured on MS media supplemented with glucose showed protocorm formation, whereas in the medium devoid of sugar the seeds failed to germinate. Lucke (1971) observed that biotin induced better development of protocorms and increased development of chlorophyll in *Paphiopedilum*.

**Plant growth regulators**

In *Cymbidium*, NAA at 10 mg/l induced vigorous protocorms but affected growth of the roots and shoots (Fonnesbech, 1972a). In *Vanda*, the highest protocorm - like bodies were obtained with 100 μm IAA (Goh and Lie, 1978). Wang et al. (1993), reported the influence of plant growth regulators on seed germination in *Dendrobium candidum*. Protocorm formation was induced from seeds cultured on MS medium containing 1.5% sucrose and NAA (0.3 mg/l). Bose and Mukherjee (1976) revealed that 2,4-D at 1 mg/l stimulated protocorm formation in *Cymbidium giganteum* whereas it did not stimulate the germination of *Cyripedium reginae* seeds (Harvais, 1973). Addition of NAA to MS medium caused proliferation of the protocorms in *Dendrobium lindleyi* (Kaur and Sarma, 1997).
The cytokinin requirement for PLB production differs for each species. In *Cymbidium*, Fonnesbech (1972a) reported that kinetin (100 mg/l) supplemented in the solid medium induced growth of many small shoots in protocorms, but had no effect on the fresh weight whereas, in liquid medium it promoted callus formation and increased the fresh weight. Pierik and Steegmans (1972) reported that BA at lower concentrations had no effect on the formation of protocorms in unripe seeds of *Cattleya aurantiaca*. The number of roots, their fresh and dry weights decreased with increase in the concentration of BA whereas, the number of shoots increased at higher concentrations. Rucker (1974) revealed that 0.1 ppm BA retarded the development of protocorms and 1.0 ppm inhibited formation of root and root hairs in *Cymbidium cv. inmemoriamycril*.

In *Cymbidium*, BAP retarded the development and differentiation of protocorms (Singh, 1987). Vij and Dhiman (1997) reported that in *Bletilla striata* cultures MI (modified Ichihashi) medium supplemented with 2 mg/l BAP favoured development of multiple shoot buds whereas at BAP (1 mg/l) promoted callus mediated PLB formation. Fonnesbech (1972a) reported that gibberellic acid (1 mg/l) promoted shoot and root growth in *Cymbidium* protocorms. GA$_3$ at 0.01 ppm promoted shoot formation in *Cymbidium pumillium* protocorms (Ueda and Torikata, 1969).

In *Cymbidium* (Fonnesbech, 1972a) NAA (10 ppm) in combination with KN (1 ppm) resulted in optimal growth and best development of protocorms. Kusumoto (1979) studied the effects of growth regulator combination on the growth of *Cattleya* plantlets, *in vitro*. Best shoot formation was promoted in a medium containing 1.0 mg/l BA + 0.5 mg/l NAA or 0.1 mg/l KN and 0.1 mg/l 2,4-D. Protocorm proliferation was stimulated by
5.0 mg/l BA and 0.1 mg/l NAA. The medium containing 0.1 to 5.0 mg/l KN and 0.5 mg/l 2,4-D induced large number of protocorms around the apical and axillary meristems of the shoot. Sounderrajan and Lokeswari (1994) studied the multiplication of protocorms of *Dendrobium* cv ‘madame pampadour’ in liquid medium. They observed that BA (0.5 mg/l) and NAA (0.1 mg/l) supplemented in Knudson C medium was the best combination for multiplication of PLB’s.

The studies of Sobhana and Rajeevan (1993) on *in vitro* multiple shoot production in five species of *Dendrobium* revealed that irrespective of the media (MS, VW, KC and Morel), the combination of growth regulators namely NAA (1 mg/l) and BA (3 mg/l) was found to be effective in inducing multiple shoot production from the protocorms. KN also had a similar beneficial effect on the shoot proliferation. Lim *et al.* (1993) reported that IBA and kinetin gave good shoot production in *Dendrobium moniliformis*. IBA at 0.1 mg/l was best for producing many tall, rooted shoots. Devi *et al.* (1998a) reported that the differentiation of protocorms into seedlings varied with the concentration and combination of plant growth regulators.

The protocorms of *Cymbidium aloifolium* showed well developed root and a large number of shoot buds in the medium supplemented with 5 mg/l KN, NAA and IBA (Buzarbarua, 1999). Prasad and Verma (2001) found that protocorm multiplication in the hybrid orchid *Emken × Cymbidium* is faster on medium supplemented with NAA and KN.

Siddique and Paswan (1998) studied the effect of growth regulators in *Cymbidium longifolium*. The protocorms were cultured on Nitsch’s medium containing IBA, NAA and BA and indifferent combinations and concentrations. Growth and plant differentiation
was best on the medium containing IBA (0.5 mg/l). The protocorms were obtained in 8 weeks and for the differentiation of leaf and root primordia it took 10 and 12 weeks (Kulkarni and Surwase, 1998).

Protocorm segments were used for the multiplication of terrestrial orchid *Habenaria marginata* (Sheelavantmath and Murthy, 2001) and upto 22 PLB’s regenerated in Knudson medium containing BA (0.5 μM). Roy and Banerjee (2002) reported half strength MS medium was found to be the best for protocorm development in *Vanda tessellata*.

**Growth adjuvants**

Fonnesbech, (1972b) studied the effect of different organic supplements on the growth of protocorms in *Cymbidium*. CH and tryptone increased growth, while yeast extract was inhibitory and meat extract was not effective. Liquid endosperm from coconut water (10 to 15%) increased growth, while the liquid endosperm from *Aesculus hippocastanum* was inhibitory. Protocorms of *Acampe praemorsa* developed into seedlings with 2-3 leaves and 1-2 healthy roots, on VW medium supplemented with peptone, vitamins and CH (Krishna Mohan and Jorapur, 1986). In *Phalaenopsis*, the presence of potato extract improved the survival of the protocorms (Tsai *et al.*, 1993).

Gangaprasad *et al.* (1999) reported that the seeds of *Ipsea malabarica*, cultured in liquid nutrient medium supplemented with 0.05% (W/V) casein acid hydrolysate showed 90% germination and maximum growth of the protocorms in 60 days. In *Cattleya* 15-25 percent coconut milk was most effective for the development of protocorm (Sculty, 1967). Kusumoto (1980) reported that *Cymbidium* protocorms proliferated best on Knudson
C medium containing 15 gm/l agar and 10-25% coconut water. Malemnganba et al. (1994) formulated a low cost medium using commercial fertilizer, table sugar and 15% coconut water for the early protocorm development in Phaius tankervilliae, Dendrobium moschatum and D. fimbriatum var. oculatum.

Vacin and Went medium proved to be the best for obtaining protocorms in Rhyncostylis retusa and Vanda coerula. It favoured rapid proliferation of the embryo and accelerated differentiation of shoots and roots, when used along with coconut water (150 ml/l), banana and pineapple extracts (150 ml/l) and vitamin stock of Nitsch medium (Nath et al., 1991). Li et al. (1997) reported MS medium to be the best for protocorm growth and organogenesis in Cymbidium sinense. Protocorm growth was good in the presence of NAA (0.5 mg) and BA (1 mg/l). The addition of sucrose (1%) and activated carbon (0.1%) to the basal medium promoted growth of the protocorms.

Murashige and Skoog medium with half concentration of inorganic salts (MSH) proved to be the best for better production of protocorms in Vanda ‘John club’. Rapid proliferation of the embryo and accelerated differentiation of shoot and root was observed when the medium was supplemented with BA at 5 mg/l, NAA at 2 mg/l, 2,4-D at 1 mg/l activated charcoal, at 4 g/l and sucrose at 15 g/l (Bhaskar and Rajeevan, 1996).

**Phase III: Seedling development**

In orchids, the transformation of seedlings from the heterotrophic to the autotrophic mode during early stages of development is an unusual aspect of differentiation, since a reverse trend characterizes most of the other symbiotic systems. (Vij et al., 2000.).
The seeds of undehisced and dehisced capsules of *Arundina bambusifolia* germinated and developed into seedlings in Raghavan and Torrey medium (Mitra, 1971). Ichihashi (1979) studied the seedling growth of several orchids in Knudson C, modified Vacin and Went, Kano and Thompson media. It was observed that *Bletilla striata* made satisfactory growth on all the media except Knudson's medium. Seedlings of *Dendrobium nobile* and *Laelia anceps* grew normally on Knudson's medium but the germination was inhibited on Thompson's medium. Thiamine, nicotinic acid and biotin effectively promoted growth in *Cattleya* hybrid seedlings whereas riboflavin, pyridoxine or pantothenic acids were ineffective. In *Orchis laxiflora*, thiamine was essential for the formation and development of chlorophyll and its combination with pyridoxine, nicotinic acid and biotin favoured better germination and seedling growth (Mead and Bullard, 1975).

In the case of *Paphiopedilum insigne*, none of the media tested was found to be suitable. Stenberg and Kane (1998) developed a protocol for seed germination and seedling culture of *Encyclia boothiana* var. *erythronioides*. The best medium for *Sarcanthus scolopendrifolius* seedling growth was Murashige and Skoog basal medium supplemented with 5% sucrose and 0.7% agar at a pH of 4 (Lee et al., 1999).

Mitra (1971) reported that seeds obtained from dehisced and undehisced pods of *Arundina bambusifolia* germinated and developed into seedlings in Raghavan and Torrey medium. Sharma (1996) revealed that in *Dendrobium chrysanthum* and *Paphiopedilum spicerianum* calcium nitrate and ammonium sulphate was best for seed germination and seedling growth respectively.
Plant growth regulators

IAA was slightly effective in promoting seedling growth in Cattleya (Withner, 1951) and Vanda (Rao and Avadhani, 1963). In Cymbidium pumilum, addition of 0.1 mg/l NAA to Knudson C medium promoted shoot formation but the number of roots and shoots decreased with increasing concentration up to 0.6 mg/l (Ueda and Torikata, 1969). Mukherjee et al. (1973) revealed that NAA at 2 mg/l induced callus formation and reduced the percentage of normal seedlings in most of the Dendrobium seeds. NAA at 1 mg/l induced thick healthy roots between 15 to 18 days in Vanda roxburgii seedlings while it affected shoot growth (Bose and Mukherjee, 1974). NAA also stimulated root and shoot growth in Epidendrum nocturnum (Yates and Curtis, 1949) and Cymbidium seedlings (Torikata et al., 1965). IAA at 1 mg/l promoted leaf formation in 60 days old Vanda roxburgii seedlings (Bose and Mukherjee, 1974). IBA at 0.1 mg/l was best for producing many tall, rooted shoots in Dendrobium moniliforms (Lim et al., 1993).

Shoot formation was enhanced by the addition of 0.01 mg/l of 2,4-D to Knudson C basal medium in Cymbidium pumilum protocorms (Ueda and Torikata, 1969). In general, the effects of exogenous gibberellins on the growth of orchid seedlings are mostly negative. The growth of Cattleya seedlings improved 3-4 times under the influence of gibberellic acid (GA₃) when compared to control, but the germination percentage was poor (Blowers, 1958; Hirish, 1959). Rao and Avadhani (1963) reported that gibberellic acid at 500 ppm inhibited the seedling formation in Vanda cv Miss joaquim. Studies by Hadley and Harvais (1968) in Orchis purpurella revealed that presence of GA₃ in the medium enabled protocorm survival but caused abnormal elongation of the shoots. Mukherjee et al. (1973) observed that GA₃ induced callus
formation and reduced percentage of normal seedlings besides increasing the number and length of leaves in *Dendrobium*. GA$_3$ at 1 or 2 mg/l enhanced plant height in *Vanda roxburghi* (Bose and Mukherjee, 1974) and caused marked leaf elongation in the seedlings of *Cymbidium giganteum* (Bose and Mukherjee, 1976).

Matsui *et al.* (1970) reported an increase in the number of shoots in *Cymbidium* when cultured in a medium containing both NAA and BA. In *Dendrobium* cv ‘Madame pampadour’ medium containing GA$_3$ (0.02 or 0.05 mg/l) + BAP (0.05-0.5 mg/l) induced protocorms (Mujib and Jana 1994). Gaya and Suner (1995) reported that in *Bletilla striata* seedlings development was best in Murashige and Skoog medium supplemented with both KN and BA (5 ppm). In *Geodorum densiflorum*, the combined application of high BAP (2.0 mg/l) and low NAA (1.0 mg/l) showed high rate of seedling formation (Roy and Banerjee, 2001). Saiprasad *et al.* (2003) reported that NAA and IAA at all concentrations produced significantly more number of multiple shoots in *Dendrobium* ‘Sonia’.

**Growth adjuvants**

Addition of activated charcoal in the culture medium has helped in a rapid and better development of shoot and roots of seedlings of *Cymbidium* (Wreckmeister, 1970). Activated charcoal promoted rhizome growth in *Cymbidium forrestii* (Pack and Young, 1991), however shoot formation was inhibited in *Phalaenopsis* (Rosa and Laneri, 1977) and *Paphiopedilum* (Ernst, 1974). In *Diuris longifolia*, increasing the sucrose concentration in the root induction medium increased the rooting frequency and addition of activated charcoal to the culture medium influenced the rooting performance of *in vitro* generated plantlets or addition of 40 gm/l activated charcoal to the sucrose medium elicited a similar rooting response (Collins and Dixon, 1992).
Vij et al. (1994) reported that in *Cymbidium pendulum* the *in vitro* regeneration potential of nodal explants was best on MS basal medium. Shoot multiplication required BA and root development was best in the presence of activated charcoal. Addition of 0.4% activated charcoal increased rooting in *Eulophia petersii* and *E. streptopetala* in Murashige and Skoog medium (Mc Alister et al., 1998). MS medium supplemented with activated charcoal (500 mg/l) enhanced seedling growth and affected root growth in *Sarcanthus scolopendrifolius* (Lee et al., 1999).

Kotomori and Murashige (1965) reported that in *Dendrobium*, coconut water at 15, 30 and 40% inhibited root and leaf production in seedlings. It induced abnormal proliferation and retarded differentiation in *Phalaenopsis* and *Vanda* seedlings (Rao and Avadhani, 1964; Ernst, 1967; Ernst et al., 1970). Chung and Chun (1980) obtained 50 mm seedlings within 7 months when seeds of *Dendrobium* lady Hamilton were cultured in Vacin and Went medium containing 150 ml/l of coconut water.

Handique and Talukdar (1998) reported that Knudson C medium was quite effective in inducing seed germination in *Dendrobium aphyllum*. Both IAA and NAA significantly enhanced seedling differentiation and growth when added to the basal medium enriched with 15% coconut water and 6% banana extract. 2,4-D had negative effect as it induced callus formation instead of seedling differentiation. Immature embryos of *Vanda coerulea* were inoculated in both liquid and semi solid VW medium supplemented with vitamins, plant growth regulators and CW. Higher percentage of germination and a faster rate of germination were observed in VW liquid medium compared to VW solid medium.
Pathania *et al.* (1998) developed a protocol for the micropropagation of *Dendrobium* cv. sonia. Both VW and KC media favoured formation of PLB and subsequent development of plantlets. KC medium supplemented with BAP (1.5 mg/l), NAA (0.4 mg/l) and paclobutrazol (1 mg/l) was found to be the best for further multiplication of PLBs. All the media favoured rooting when supplemented with IBA (1 mg/l) or NAA (1.8 mg/l) and paclobutrazol (0.5 mg/l). In *Aerides multiflorum* the seedling showed enhanced growth on MS medium supplemented with KN (1 μg/ml), NAA (1 μg/ml) and CH (100 μg/ml) (Sarma and Sarma, 2001). Ananthan *et al.* (2003) studied the significant effect of CH and CW on the seedling development in *Coelogyne mossiae*.

**Pseudobulb segment culture**

Shoot tips remain the most commonly used explants for micropropagating orchids. But their utility is limited in monopodial orchids as their excision endangers the survival of mother plant. In later studies, the regenerative competences of different explants were tested (stem discs, rhizome, leaf, embryo, root, inflorescence, anther) for orchid micropropagation.

Perennating organs (pseudobulbs, rhizomes, tubers) have been conventionally used to propagate orchids under field/greenhouse conditions, but they generate only a limited number of propagules, and that too, under a favorable season. However, their utility for micropropagating orchids is increasingly being realized.

Eria and Pholidota (Pathak, 1989), Mormodes (Arditti and Ernst, 1993), Bletilla (Vij and Dhiman, 1997), and Malaxis (Vij and Kaur, 1998). Their regeneration competence seems to be markedly influenced by physiological age of the mother plant, position on the donor axis and growth stimulus in the nutrient pool as has also been suggested by Vajrabhaya (1978). In Bletilla cultures, the level of BAP in the nutrient pool markedly influences the developmental pathway in the regenerants; at 1 mg/l BAP favours callus-mediated PLB development whereas, at 2 mg/l it favours development of multiple shoots. Recently, Decruse et al. (2003) reported shoot multiplication from foliar meristems of Vanda spathulata.

Synthetic seeds

Synthetic seed technology forms an ideal system for propagation, conservation and exchange of plant material (Redenbaugh, 1990). This technology has been extended to an impressive number of horticulturally and economically important crops.

Initially, synthetic seed preparations were limited to somatic embryos (Redenbaugh et al., 1984). Presently, in addition to somatic embryos, a large number of vegetative propagules like axillary buds, adventitious buds, shoot-tips, cormlets, bulbs and protocorms were used for encapsulation. The encapsulation of protocorms in orchids would be very helpful in preserving tissue as well as transferring it from one place to another (Singh, 1991). So for synthetic seeds of more than 20 orchid species have been prepared.

Singh (1991) successfully encapsulated the protocorms of Spathoglottis plicata. The encapsulated protocorms retained their viability and showed a reduction in percentage after 180 days of storage. Vij et al. (1992) reported encapsulated PLB’s of
*Aerides multiflorum* in 2.5% of alginate matrix. In *Dendrobium wardianum*, the protocorm-like bodies were encapsulated and successfully regenerated after 120 days of storage at 4°C (Sharma et al., 1992).

Propagation of *Cymbidium giganteum* through high frequency conversion of encapsulated protocorms both under *in vivo* and *in vitro* conditions was reported by Corrie and Tandon (1993). Protocorm-like bodies of *C. giganteum* encapsulated in 4% alginate along with NAA and BAP (1.0 mg/l each) showed better conversion frequency. Tanaka et al. (1994) obtained successful regeneration of encapsulated protocorm-like bodies of *Phalaenopsis* species, after storing at 12°C for 12 weeks.

Malemnganba et al. (1996) attempted storage of encapsulated protocorms at 4°C in *Phaius tankervilliae* and their successful regeneration after 120 days. Protocorms (60-70 days old) of *Agrostophyllum myrianthum*, *Cymbidium longifolium*, *Phaius tankervilliae* and *Renanthera imschootiana* were encapsulated with sodium alginate and cultured in Nitsch medium containing IAA (0.1 mg/l) or on agar (0.7%) water medium. Regeneration was higher in the cases of encapsulated than non-encapsulated protocorms. Encapsulated protocorms of *Agrostophyllum myrianthum*, *Cymbidium longifolium*, *Phaius tankervilliae* and *Renanthera imschootiana* were stored at 3 different temperatures. Protocorms stored at room temperature showed better regeneration percentage (Devi et al., 1998b).

The protocorms of *Spathoglottis plicata* (Nayak et al., 1997) was encapsulated in 4% sodium alginate prepared in MS medium supplemented with NAA (2.0 mg/l) and BA (0.5 mg/l). High frequency of germination was achieved in MS medium along with NAA (2.0 mg/l) and BA (0.5 mg/l) combinations. Nayak et al. (1998b) developed a protocol for rapid and large-scale propagation of three cultivars of *Spathoglottis plicata*, through
high frequency plant regeneration from alginate-encapsulated protocorm-like bodies. The protocorm-like bodies (PLBs), developed from the shoots cultured in vitro, were encapsulated in alginate prepared in Murashige and Skoog medium (MS) augmented with BAP (2.0 mg/l) and NAA (1.0 mg/l).

*Spathoglottis plicata* seeds were encapsulated in alginate-chitosan or alginate-gelatin infected with mycorrhizal fungus *Rhizoctonia* AM9. The encapsulated seeds were placed directly on *Rhizoctonia* culture. About 66% of the seeds encapsulated in sucrose-free chitosan alginate established a symbiotic relationship with the mycorrhizal fungus after co-culturing for 2 weeks. The highest percentage of infection observed was about 84%. Addition of sucrose or the use of gelatin alginate for encapsulation reduced the percentage of infection by about half. The growth of *Rhizoctonia* AM9 in sucrose-free alginate, Chitosan and gelatin was found to be minimum (Tan *et al.*, 1998).

In *Coelogyne odoratissima* var. *angustifolia* encapsulated protocorms stored for 60 days at 25±1°C differentiated into complete plantlets within 15 days (100%) on MS medium whereas, there was a gradual reduction (88%) in the conversion frequency of the protocorms stored at 7°C (Kamalakannan *et al.*, 1999). Thirty days old PLB’s of *Geodorum densiflorum* were encapsulated in sodium alginate and germinated in modified Knudson C medium supplemented with 15% coconut milk (v/v), peptone (2 g/l), BA (2 mg/l) and NAA (1 mg/l) and obtained 88% germination. Artificial seeds showed 28% viability when transferred directly to non-sterile soil conditions (Datta *et al.*, 1999). Jeyakodi *et al.* (2000) reported 100% bead to plant conversion on MS medium supplemented with NAA (1 mg/l) and BA (0.5 mg/l). The protocorms retained their viability upto 210 days in *Coelogyne mossiae*. 
In *Dendrobium aqueum*, protocorms encapsulated in 2% alginate and N\textsubscript{6} medium showed 80% conversion rate (Baskar et al., 2000). Vij et al. (2001) reported the production of synthetic seeds by encapsulating PLBs of *D. densiflorum*. The conversion frequency of PLBs decreased with passage of every 15 days, which was more pronounced in beads stored at 25 °C. Pathak and Vij (2002) encapsulated PLBs of *Aerides multiflora* obtained from leaf cultures in 3.5% of alginate and recorded high percentage of regeneration after storage for 105 days at 4 °C. Talukdar and Ahmed (2002) reported a significant increase in the storage life of encapsulated PLB’s of *Cymbidium pendulum* and *C. aloifolium* stored at 4°C.

**Hardening**

Under natural conditions, terrestrial orchids are found growing in forest soils containing rich humus, accumulated from the falling leaves over a period of time. For potting medium stimulating this natural humus, a mixture of equal parts of leaf mould, garden soil and coarse river sand will be suitable. In many of the tropical countries, orchids have successfully been grown in coconut husk, pieces of brick and charcoal. Apart from these perlite, gravel, moss peat, saw dust, rice husks, stone chips, peanut shells, branches and twigs of various trees and several more have successfully been tried individually and in combination with other materials.

Many orchids are conveniently grown on wooden logs, tree fern blocks and charred wooden slabs (Bose and Bhattacharjee, 1972). Some terrestrial species of *Coelogyne* are better suited to grow in a mixture of equal parts of *Sphagnum* moss, leaf mould, white sand and loamy soil (Bhattacharjee, 1977b).
Most of the Eria spp. performs well in a potting media consisting of equal parts of *Osmunda* fibre, fibrous loam and *Sphagnum* moss (Bhattacharjee, 1978). While in cultivation, *Vanda* may be grown in the baskets containing chunks of hard wood charcoal alone (Bhattacharjee, 1979). Pine-bark, cork-bark, tree-fern pieces, *Osmunda* roots, *Sphagnum* moss, gravel and coconut-fibers are among the more commonly used potting materials for orchid seedlings (Fitch, 1981). In an experiment on two terrestrial species of *Cymbidium aloifolium* and *Phausis tankervilliae* with six different types of potting media, Bhattacharjee and Mukherjee (1981) reported that organic rich porous compost showed good response.

Tanaka (1988) observed that the growth of *Cattleya* in pumice and peat moss was better when mixed in the ratio of 1:1 than in the ratio of 2:1 or 3:1. Seeni and Latha (1990) reported a mixture of broken tiles, brick pieces, coconut husk, charcoal and moss as the more satisfactory potting medium for the growth of the axenic seedlings of *Phalaenopsis*. Koval’s Kaya and Zaimenko (1991) reported better growth of the seedlings of *Dendrobium* and *Phalaenopsis* in *Sphagnum* moss than in a combination of *Sphagnum* moss, charcoal, bark and broken tiles.

Seedlings of *Dendrobium wardianum* were potted in earthen pots containing charcoal pieces, brickbats, coconut fibers and a layer of moss at the surface and hardened under glasshouse condition (temperature 20° - 25°C, RH 80-90%). About 65% of the plantlets survived (Sharma *et al.*, 1992).

*Dendrobium fimbriatum*, *D. moschatum*, *D. farmeri* and *D. nobile* were cultured in a variety of media using charcoal, brick, gravel, coconut-fibre and coconut husk, in equal proportion and possible combination. A combination of bricks and gravel proved the
best medium with regard to its efficacy and cost effectiveness (Paul and Rajeevan, 1992). Acclimatization of *Dendrobium moniliforme* was best done in peat moss for improving stem diameter and plantlet height, although perlite was best for increasing root number (Lim *et al.*, 1993). The use of peat based soilless potting mixes, sometimes known as mud, for growing orchids was discussed by Brenneise and Halgren (1996).

Grove and Allikas (1998) studied the properties of potting materials for orchids. The potting materials include fir (conifer) bark, sponge rock (large particles perlite), larva rock, pumice rock, expanded clay pellet, peat, tree fern fibers, charcoal, *Sphagnum* moss, rockwool, coconut husk, chips, mixed coconut fiber, pro-mix (a soilless compost) and shredded tyre chips. In *Coelogyne odoratissima* var. *angustifolia*, the survival rate was 100% in potting medium containing brick pieces, vermiculate, charcoal and dry moss in the ratio of 1:1:1:1 (Kamalakannan *et al.*, 1999).

Seedlings of *Dendrobium nobile* were successfully grown for 3 years in different substrates like tree fern fiber, blocks of pressed coconut bark, bark of *Eucalyptus grandis*, mixtures with coconut bark blocks and eucalyptus bark and mixtures with the latter materials and charcoal (Dematti and Graziano, 2000). In *Aerides multiflorum*, the rate of survival was 90% and growth of the plantlets was better on the medium containing charcoal, brick chips, leaf mould and Farm Yard Manure in the ratio of 2:1:1:1 (Sarma and Sarma, 2001). Plantlets of *Doritaenopsis* hybrid survived in *Sphagnum* moss medium and produce normal plantlets (Park *et al.*, 2002).

**Orchid mycorrhiza**

Mycorrhizae represent ubiquitous associations (symbiotic) between the plant roots and soil-borne fungi (Varma, 1998, 1999). The most common of these associations,
involving arbuscular mycorrhizal fungi (AMF), play an indispensable role in promoting
growth, vigor and survival of plants by positively influencing their nutritional and hydratic
status, improving the health of their rhizosphere for better root performance, and providing a
natural defense against the pests and pathogens (Linderman, 1994; Varma, 1995). In fact, the
growth and development of a vast majority of autotrophic green plants is obligatory to the
establishment of a mycorrhizal association.

Orchid endophytes mostly belong to the imperfect genus *Rhizoctonia* (James et al., 1998). The orchid mycorrhizae differ from other types of mycorrhizae in several aspects and are considered unique. The importance of mycorrhizal fungi in orchid seed germination was not known to the scientific community until Bernard (1909) observed that orchid seeds successfully germinated only subsequent to colonization by suitable fungus which had been earlier isolated and identified as *Rhizoctonia* (Bernard, 1904). The perfect stages of Rhizoctonias are known to occur in both Basidiomycetes and Ascomycetes (Currah, 1987; Warcup and Talbot, 1967; 1980). Besides *Rhizoctonias*, several fungi belonging to the genera *Armillaria, Ceratobasidum, Ceratorhiza, Epulorhiza, Fomes, Marasmius, Thanatephorus* etc are important orchid symbionts (Saunders and Owens, 1998, Terashita and Chuman 1987).

Smith (1966) while commenting on the beneficial role of mycorrhizal in orchid life history, brought out the importance of a hyphal conduct for translocating nutrients into the infected seedling. Hadley and Pegg (1989) stressed the importance of mycorrhizae in the management and conservation of orchids. According to Stewart (1989) habitat protection and mass propagation (using conventional and tissue culture
techniques) are important conservation strategies for these plants. A major limitation in the large-scale application of tissue culture techniques in conservation related programmes is the high mortality experienced by the regenerants during and following their laboratory to land transfers. The tissue culture plants also lack sufficient resistance against the soil microbes, at least initially, and are prone to attacks by soil-borne fungal pathogens.

Janse (1897) made a detailed survey and recorded that a number of orchid had mycorrhizal association. According to Hijner and Arditti (1973) Rhizoctonia sp. is the most prevalent fungi involved in orchid mycorrhizae. The fungi isolated from Cymbidium roots apparently produced the pyrimidine moiety of thiamine - a compound which may enhance the growth of certain orchids, whereas, p-amino benzoic acid a constituent of folic acid, produced and released by the orchid tissue can in turn satisfy the vitamin requirement of the fungus. Niacin enhanced orchid seed germination and seed development more consistently than any other vitamin. This finding indicates that orchids may have a niacin requirement that mycorrhizal fungi satisfy in nature. This assumption is supported by evidence that niacin is released into culture media by Rhizoctonia strains from Dactylorhiza purpurella (Harvais and Pekkala, 1975) and Cymbidium (Hijner and Arditti, 1973).

The importance of mycorrhizal fungi in the adult stage of such autotrophic orchids is not yet clearly understood, although some supply soluble phosphates and at least some vitamins, if not all, to the host (Alexander et al., 1984). According to Vij and Sharma (1988) the root hairs were shown to serve as the sites of hyphal entry. Masuhara and Katsuya (1994) also have demonstrated that the specificity of orchid and fungus under in situ condition different from that of under in vitro conditions. Sarma and Kaur (1998)
reported enhanced survivability of the in vitro raised plantlets of *Arundina gramnifolia*. Blechert *et al.* (1999) reported significant enhanced growth of *Dactylorhiza maculata* with *Piriformospora indica*.

The fungi endophytic with orchids are commonly considered as members of the form genus *Rhizoctonia* (Richardson *et al.*, 1992). Azcon-Aguilar and Barea (1997) studied ex vitro mycorrhizal application at the rooting phase in vitro or at the beginning of acclimatization before the beginning of the hardening phase under green house conditions. *Piriformospora indica* promises to be an excellent material for biological hardening of micropropagated plantlets as the fungus rendered more than 90% survival rate (Sahay and Varma, 2000). Singh *et al.* (2001) suggested the possibility of using *Piriformospora indica*, an active root endosymbiont, as an effective alternative to arbuscular mycorrhizal fungi for orchids. Senthil kumar (2001) summarized the present status and problems and prospects of orchid mycorrhiza. Recently, Rasmussen (2002) emphasized the recent developments in the study of orchid mycorrhiza.

**Antimicrobial activity**

Plant derived compounds (phytochemicals) have been attracting much interest as natural alternatives to synthetic compounds. World Health Organization (WHO) studies indicate that over 30% of World’s plant species have at one time or another been used for medicinal purposes. Curative properties of the drug plants and their uses are mentioned in one of the oldest and holy books *Vedas*, which are considered to be the earliest literature on the earth (Kaushik, 1983, 1988, Kaushik and Kishore, 1991, 1995). The medicinal value of plants is due to the presence of certain secondary metabolites capable of curing diseases.
In ancient literature, orchids are mentioned as medicinal and ornamental plants. They have been used for their therapeutic value all over the world owing to their phytochemical constituents such as alkaloids, flavonoids, terpenes and glycosides. The orchids used in ayurveda known for the medicinal value are *Arethusa bulbosa*, *Cymbidium madidium*, *Orchis latifolia*, *Malaxis wallichii*, *Habenaria sp.*, *Vanda roxburghii*, *Dendrobium macraei*, *D. alpestre*, *Eulophia latifolia*, *Acampe papillosa* and *Liparis rostrata*. They have been used extensively to cure a variety of ailments (Lawler, 1984, Handa, 1986).

Several species of the orchids, such as *Dendrobium macraei*, *Orchis latifolia*, *Vanda roxburghii* and *Pholidata pallida* are widely used in the manufacture of ayurvedic medicines which are helpful in various types of human ailments (Maheshwari *et al.*, 1978; Hegde, 1984). Kiritikar and Basu (1918) reported that the entire plant of *Dendrobium ovatum* is quite useful in all kinds of stomachache in bile secretion and as a laxative. *Microstylis wallichii* is helpful in the treatment of tuberculosis.

*Eulophia campestris* tuber is used as an appetiser; stomachic, tonic, aphrodisiac, alternatige and purifies the blood in heart troubles (Kiritikar and Basu 1975). The root tubers of *Habenaria edgeworthii* form an important unit of ‘Astavarg’ group of drugs in ayurvedic system of medicine (Lal *et al.*, 1980). Trivedi *et al.* (1981) investigated the chemical constituents of thirty-six members of the Orchidaceae used as medicine in various diseases.

*Acampe papillosa* and *A. praemorsa* roots are used in rheumatism. Paste made from the tuber of *Anthogonium gracile* is used in joining broken articles and as glue. Pseudobulbs of *Bulbophyllum neilgherrens* are used for restoration of adolescence and as tonic.
Calanthe triplicata root extract is effective in diarrhoea and teeth cavities. Cleisostoma williamsonii is used for treating bone fractures. Dried pseudobulbs of Coelogyne punctulata are powdered and are applied to spots of burn injuries. It relieves pain immediately and helps in healing of the wound. Cymbidium aloifolium is used as purgative, emetic, tonic and in treating earache. Decoction of roots from C. ensifolium is used in curing gonorrhoea and decoction of flowers is useful in sore eyes. Tubers of Dactylorhiza hatagirea are used as expectorant, aphrodisiac and as an astringent. Dendrobium ovatum juice is used in curing stomachache, improving bile secretion and as laxative. D. nobile seeds are applied to the freshly cut wounds for early healing. Decoction of roots and leaves of Eria pannea is used in bone ache. (Chowdhery, 1998).

Juice from the entire plant of Dendrobium ovatum cures all kinds of stomachache, excites bile and acts as a laxative to the intestines (Kaushik, 1983). Das (1986) reported that the tubers as well as the whole plant of Satyrium nepalense act as an expectorant and astringent. Antiviral and anticancerous activites have been positively tested in Vanda parviflora (Rastogi and Dhawan, 1990) while mannose specific lectins from Cymbidium hybrids, Epipactis helleborine and Listera ovata have been reported to be selectively inhibitory to the AIDS virus (De Cleroq, 1994).

The alcoholic extract of *Aerides multiflora* was tested for antibacterial activity against *B. subtilis, Escherichia coli, K. pneumoniae, S. typhi* and *Staph. aureus* (Ghanaksh and Kaushik, 1999a). Antibacterial activity of *Rhynchostylis retusa* was positively tested against *Staph. aureus, E. coli, K. pneumoniae, S. typhi* and *B. subtilis* (Ghanaksh and Kaushik, 1999b). Senthil kumar et al. (2002) studied the antimicrobial activity of *Malaxis rheedii*.

**Phytochemical analysis**

Plant metabolites are major sources of pharmaceuticals, food additives, fragrances, pesticides (Sasson, 1992). Over four-fifths of about 30,000 known natural products are of plant origin. The so-called natural products are usually not involved in the main life processes of the cells (primary metabolism), but they are formed as peculiar offshoots along specific biogenetic pathways and referred to as secondary metabolites (Luning, 1974). Orchids are rich in alkaloids, flavonoids, phenols and other phytochemicals and hence they have high therapeutic value and have been extensively used in local system of medicine.

Early investigation on the alkaloid content of Orchidaceae were made on material in European orchid collections by de Wildemann (1892) and de Droog (1896) on *Dendrobium nobile* and *Phalaenopsis lueddemanniana*. Besides these findings, de Droog proved the occurrence of alkaloids in a number of *Catasetum* species. Luning in his survey (1964) reported a high alkaloid content in *L. loeselii*. Leander and Luning (1967) published the structure of malaxin from *Malaxis conesta*. Lawler and Slaytor (1969) screened 205 orchids for alkaloids in Australia. Nishikawa et al. (1969) isolated an alkaloid auriculine from *L. auiculata*. 
Phalaenopsin alkaloid was isolated from *Phalaenopsis ambilis* (Luning et al., 1966). Okamoto *et al.* (1966a) isolated denranine (6-hydroxydendrobine) and dendrine; dendroxine from *D. nobile* as well as 4-hydroxydendroxine (Okamoto *et al.*, 1966b). Yamamura and Hirata (1964) reported nobilie an open chain alkaloid related to dendrobine from *D. nobile*. In *D. hildebrondii*, Elander and Leander (1971) elucidated 6-hydroxynobilinone. In *D. wardianum*, a different quaternary alkaloid, dendrowardine was isolated by Blomqvist *et al.*, (1973). An optically active alkaloid pierardine was isolated from *D. pierardii* (Elander *et al.*, 1971). Biosynthetic studies of some orchidaceous alkaloids have also been undertaken (Rosenblom, 1975; Leete and George, 1976; Bodem, 1977). The available data on orchid alkaloids suggests that several species contain more than one in different species. It is worthwhile to mention that the structure of about 60% of the isolated orchid alkaloids has so far been established (Slaytor, 1977) and most of them are derived from aromatic aminoacids.

Many Erias, have shown an elevated alkaloid content during the screening tests, but so far alkaloids have been isolated only from *Eria jasensis*. These alkaloids are simple methylated phenethylamines, some of which, surprisingly, have not been observed previously in nature (Hedman *et al.*, 1969). Simple pyrolizidine derivatives have been found in many *Vanda* and *Vandopsis* species. *Vanda cristata*, *V. hindsii* contain laburnine acetate and *Vandopsis lissochiloides* and *V. gignatea* contain a mixture of laburnine and lindelozidine together with their acetates (Lindstrom and Luning, 1969). Floral anthocyanins were isolated from *Spathoglottis plicata* (Suryanarayana *et al.*, 1986). The quality and distribution of anthocyanins serve as important chemotaxonomic markers (Bate-smith and Swain, 1965).
Williams (1979) and Jorapur (1986) investigated a type of flavonoid from orchid leaves. Adunacin, a sesquiterpene related to picrotoxin has been isolated from *Dendrobium aduncum* (Crawell and Leander, 1976). Talapatra et al. (1982) isolated a new phenanthraquinone denbinobin from the whole plant of *Dendrobium nobile*. A new phenolic compound, coelonin isolated from *Coelogyne ochracea* and *C. elata* (Majumder, et al., 1982). Majumder and Datta (1984) elucidated a phenolic compound, Oxoflavidin from the Himalayan orchid *C. elata*. From the tubers of *Bletilla striata*, three new biphenanthrenes were isolated by Yamaki et al., (1989). Volucrin was isolated from the orchid *Lusia volucris* (Majumder and Lahiri, 1990). Rani and Singh (1995) studied the similarity of phenols between different taxa of *Herminium* by two-dimensional thin-layer chromatography. Singh and Rani (1996) assessed the leaf phenolics in 6 species of *Habenaria*.

Lin et al. (2000) isolated six novel dihydrophenanthrene derivatives, sinensols A-F (1-6) from the aerial parts of *Spiranthes sinensis*. Five phenanthrenes and a mixture Phytosterols were isolated from the roots of *Eulophia petersii* by Blitzke et al. (2000). Glucovanillin was extracted from green pods of *Vanilla* (Ruiz-Teran et al., 2001). Phenanthropyran derivatives, 3-methoxy-2, 7-dihydroxy-5H-phenanthro [4,5-bcd] and 2,3,7-trihydroxy-5H-phenanthro [4,5-bcd] pyran were isolated from *Phalaenopsis equestris* (Manako et al., 2001). Coeloginantheidin and Coeloginantherin were isolated from the *Coelogyne cristata* (Majumder et al., 2001).

Gastrol together with 10 phenolic compounds have been isolated from the MeOH extract of the rhizomes of *Gastrodia elata* (Hayashi et al., 2002). Ye and Zhao (2002),
obtained five new sesquiterpene glycosides with alloaromadendrane, cadinene and
cyclocopacamphane type aglycones, one new cyclocopacamphane type sesquiterpene,
two bibenzyls and eight known compounds have been obtained from *Dendrobium nobile*.

Two Phenanthrene derivatives, characterized as Erianthridin (9,10-dihydro-2, 7-dihydroxy-3, 4-dimethoxyphenanthrene) and gymnospusin (2,7-dihydroxy-3, 4,9-trimethoxyphenanthrene), were isolated from an extract of the orchid *Maxallaria densa* (Valencia-Islas et al., 2002). Recently, Kong et al. (2003) emphasized the use of orchids as herbal medicines.

Polysaccharides and oligosaccharides have been reported from various orchids (Mahmoud et al., 1963; David et al., 1967; Ernst et al., 1971). Sugars have been separated by TLC technique from orchid nectars (Jeffrey and Arditti, 1969). Jeffrey et al. (1970) studied the nectar sugars of several orchids. The pseudobulbs of *Microstylis wallichii*, on Indian drug used in ashtvarga, contain sugars besides basic compounds (Bhatnagar et al., 1971). Two glucosides cis and trans - crassinodin have been isolated from *Dendrobium crassinode* (Dahman et al., 1976). The well-known orchid glucoside loroglossin was isolated from many orchid species (Bourgmelot and Bridel, 1919) but its detailed structure could be worked out in 1976 and lately this glucoside has been reported from *Orchis papilionaceae* (Pagoni, 1982).