DISCUSSION

Five species (*Cymbidium pendulum, Gastrochilus calceolaris, Paphiopedilum spicerianum, Thunia alba, Vanda coerulea*) and an interspecific hybrid (*Oncidium* 'Gower Ramsey') of commercially important and biologically significant orchids were included under the scope of present studies, with a view to developing appropriate protocols for their micropropagation. The germination response of embryos under different nutritional regimens *in vitro* was also tested in *Cym. pendulum, Paph. spicerianum, and Thun. alba*. The utility of the synthetic “seeds” as an efficient storage and delivery system for the regenerants was also assessed. The results have already been described and in what follows these are discussed in light of the information available in the literature.

Seed/Embryo germination:

Orchid seeds have highly reduced embryos and little or no endosperm. They contain fat droplets and starch granules as food reserves (Burgeff, 1936) and require a suitable fungal stimulus for germination and further development (Bernard, 1904). The possibility of bypassing their fungal requirement during germination *in vitro* (Knudson, 1922) has added new dimensions to orchid propagation and a large number of orchid species and hybrids representing diverse habits and habitats have responded to asymbiotic germination *in vitro* (Alouffa et al., 1998; Arditti et al., 1982; Devi et al., 1997; Faletra et al., 1997; Hiroyuki et al., 1997; Kondo et al., 1997; Lawrence et al., 1997; Leroux et al., 1997; Lin et al., 1994; Miyoshii and Mii, 1995; Sharma, 1996; Tonya and Camper, 1997; Vij and Kher, 1997; Vij and Pathak, 1988c; Weber, 1997). Withner (1943) introduced the concept of immature embryo culture and pointed
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out that embryos must be sufficiently developed in order to be cultured. Borris (1969), Fast (1974), and Vermeulen (1947) hinted at the ability of seeds/embryos (from immature capsules) to germinate in vitro. According to Arditti et al. (1982), the immature embryos germinate better than the mature ones. Yam and Weatherhead (1988) attributed the better germinability of the immature embryos to their distended testa cells, metabolically awakened embryos, and lack of dormancy and/or inhibitory factors. The culture of immature embryos is termed as ‘Green-Capsule Culture’ and has opened new vistas in commercial cultivation of orchids. However, as the technique requires the use all seeds/embryos in a single sowing, the importance of a proper stage at which the fruit has to be harvested assumes a great significance. Sauleda (1976) pointed out that harvesting time was specific not only to genera and hybrids but to species. Light (1990) listed a number of orchid species giving information about the time intervals after pollination when their immature seeds (embryos) can be harvested for optimum results. Since the Doritis ovules, from pollinated ovaries, germinated immediately after getting fertilized in vitro, Yasugi (1984) concluded fertilization as a pre-requisite for their germination. Very young ovules are not known to form favorable explants in orchids due to their overall condition and nutritional requirements. Bhojwani and Razdan (1983) also reported that the nutritional requirements of the younger embryos are complex as compared to the mature ones. Presently, the germination potential of embryos at different stages of development could not be tested due to paucity of material. In Cym. pendulum and Thun. alba, embryos procured from capsule which had ceased to grow in diameter and had developed deep ridges along the valves, i.e. about 25 wap were amenable to germination. In Paph. spicerianum, the embryos (25 wap) germinated with better frequency than those collected 45 wap. A similar decrease in the germination potential of embryos with increasing age after reaching an optimal has also been reported by Curtis (1943), Salwan (1992), Vij et al. (1981), and Withner (1953).

The effect of light on germination in orchids has been variously assessed. Epiphytic and certain terrestrial species germinate both in light and dark though
they require light for induction and improvement of shoot and/or root formation. Arditti et al. (1981) and Harvais (1972) suggested that germinating orchid seeds and developing seedlings vary in their tolerance, requirement, and response to light. Presently, germination response of *Cym. pendulum* and *Thun. alba* seeds was tested under light only and they germinated normally, whereas in *Paph. spicerianum*, the seeds germinated both under light and dark conditions. The response was better in dark. A perusal of literature reveals discordant effect of light/darkness on seed germination. There was hardly a difference in germination of *Cym. ensifolium* embryos under light/dark conditions (Chung and Chung, 1983) whereas light treatment was inhibitory to embryo germination in *Cyperipedium reginae* (Harvais, 1973; Stoutamire, 1974), *Paph. callosum*, *Paph. spicerianum* (Kano, 1965; Sharma, 1996), *Spiranthes odoratta* (Lawrence et al., 1997), and a number of European species (van Waes, 1984). Eiberg (1970) and Fast (1978) compared the germination of some orchid species in light/dark and reported that dark promoted germination whereas, light in certain cases proved inhibitory. In some terrestrial species even a pre-treatment with light is inhibitory for germination. The germination inhibitory effect of light has been described as a part of protective mechanism which makes it impossible for the seedlings to develop at the surface soil where they would be subjected to drying during the growth period (Stoutamire, 1974). The effect of light is more pronounced in species growing in relatively hostile surroundings. In the present cultures, the embryos, however, seems to be less sensitive to light due probably to their highly hydrated conditions since they do not run the risk of mortality due to desiccation. Almost similar observations were made by Stoutamire (1974) and Riley (1983) that north American species growing in bogs are less sensitive to light than those inhabiting drier and shadier localities. Presently, the dark raised seedlings were etiolated and achlorophyllous, whereas according to Sharma (1996) even root differentiation eluded the dark incubated cultures and the shoots did not survive unless transferred to light. Dowine (1941), Harvais and Hadley (1967), Stoutamire (1974), and Withner (1974) suggested that the ground growing orchids of well drained woodlands and open grassy areas germinate in dark as an adaptation protecting the seedlings against drought conditions. Arrested development of
Malaxis acuminata at spherule stage (Kaur, 1996) and a delayed development of Pleione protocorms in dark (Light and Macconill, 1990), however, suggest the importance of factors other than plant habitat in influencing the dark/light requirements of seeds during germination.

Presently, an analysis of the morphogenetic changes in the embryos prior to their development into seedling was made. In all the three taxa, the sequential development into spherules and protocorms was followed by differentiation of leaf and root primordia. Interestingly, in Cym. pendulum few of the protocorms developed into rhizomatous bodies as also reported in Cym. virescense, Cym. marcorhizon, Cym. falconeri, Eulophia pictum, Geodorum pictum (Arora, 1990; Champagnat et al., 1968; Weatherhead et al., 1986; Stoutamire, 1974; Vij and Pathak, 1988c). This development of intervening rhizomatous phase during the development of protocorm into a seedling was earlier considered a genetic attribute (Vij and Pathak, 1988c). However, as the rhizome forming trait in Cym. pendulum was presently expressed only in certain selected nutritional regimes, the author seems to agree with Salwan (1992) that the developmental pathway of a protocorm into seedling is greatly influenced by the quality of nutrition in vitro.

Morphologically, the orchid protocorm has been variously considered as an undifferentiated (callus) or a differentiated structure (cf. Arditti and Ernst, 1984) whereas, functionally it is believed to behave as a cotyledon for supplying nutrients during development of embryo and its further growth into seedling (Lee, 1987). The epidermal hairs (rhizoids), in the germinating entities (spherules, protocorms) invariably penetrated into the medium highlighting the absorptive role they perform. The rhizoids were earlier considered akin to root hair (Tu, 1986). Interestingly, the formation of rhizoidal hairs in the germinating entities is promoted in dark incubated cultures (Ichihashi, 1990; Mitchell, 1989; Author).

The development of the protocorms into seedlings was monopolar in all the three taxa in accord with a similar mode of development in orchids (Avadhani, 1962). Transformation of seedlings from heterotrophic to autotrophic mode during early stages of development is an unusual aspect of differentiation, since a reverse trend
characterizes most of the other symbiotic systems. The exact stage at which the germinating entities acquired chlorophyll in the present study, however, varied with the species, nutrition, and light conditions in vitro. In general, it was a post-protocorm phenomenon in *Cym. pendulum* and *Paph. spicerianum* and a pre-protocorm phenomenon in *Thun. alba*. Incidentally, in the last species a sucrose treatment was obligatory for its normal development. Sucrose level, in the media, is reported to affect the chlorophyll development in orchids (Homes and Vansevern-Van Espen, 1973; Ichihashi and Uehara 1987; Sharma, 1996). In *Paph. spicerianum* the chlorophyll development was, however, markedly influenced by photoperiodic treatments; it eluded the dark incubated cultures in accord with similar observation by Sharma (1996). Interestingly, in this species the chlorophyll development was significantly delayed and it appeared only in the leaf initials as is also true in *Cyperipedium* spp. (Curtis, 1943), *Goodyera repens* (Downie, 1940), *Habenaria* spp. (Vij et al., 1988c) and other terrestrial orchids. According to Stoutamire (1974) and Vij et al. (1988a) a pre-protocorm development of chlorophyll is almost universal in epiphytic orchids, but variations in stage of chlorophyll development in the terrestrial ones appears to be related to ecological or genetic factors; non-chlorophyllous protocorms characterise ground growing taxa of well drained or seasonally dry soil.

**Culture Medium:**

A culture medium simulates an endosperm to satisfy the nutrient requirements of germinating embryos in vitro. Orchids in general have a wide nutritional amplitude and can grow on a wide variety of media ranging from very simple to complex undefined. In fact, a large number of orchid culture media (Burgeff, 1936; Curtis, 1936; Curtis and Nichol, 1948; Knudson, 1946; Mitra et al., 1976; Raghavan and Torrey, 1964; Arditti et al., 1982; Thomale, 1954; Vacin and Went, 1949) have been devised on more or less empirical basis and many of them are species specific (cf. Arditti et al., 1982). Chemically undefined media used for orchids include PDA, PSE, Bark extract etc. Incidentally, a majority of media used for orchid tissue culture are more concentrated than the solutions that nurture orchids in nature; while they support good germination in epiphytic orchids, they
are required in lesser concentrations for activating the process in terrestrial species like *Paphiopedilum, Cyperipedium* (cf. Arditti and Ernst, 1984). Presently, a positive response of *Cym. pendulum* to germination in SN/MS/PDA media, of *Thun. alba* in KC and PDA, and of *Paph. spicerianum* in RE/Nsf media reflects their wider nutritional amplitude in accord with general amenability of orchid seeds and seedlings to a variety of combinations and concentrations of inorganic salts as suggested by Arditti (1967).

Nitrogen nutrition is an important aspect of orchid seed germination and according to Magrou (1944), it is provided *in vivo* by fungal component of the mycorrhizal association. Under *in vitro* conditions, NO$_3^-$ and NH$_4^+$ ions are generally used as a source of nitrogen. The importance of ammonium and/or nitrate ions (individually or in combination) during *in vitro* germination has, however, been variously assessed. *Cymbidium* seeds germinate better in ammonium as compared to nitrate, whereas *Vand. tricolor* seeds prefer nitrate as compared to ammonium (Curtis and Sporel, 1948). According to Burgeff (1936), nitrate is best utilized by autotrophic species when grown in light but ammonium is necessary for the growth of heterotrophic and saprophytic species. The utility of both nitrate and ammonium ions in orchid tissue culture was first advocated by Curtis and Sporel (1948) and has been subsequently stressed by Arditti and Ernst (1984) and Withner (1959). Many orchids are incapable of utilizing NO$_3^-$ ions during early stages of germination whereas, the ability of others to do so has been attributed to the development of nitrate reductase due probably to the co-evolution of their biochemical and morphological differentiation (Ragavan, 1976). According to Mitra (1987), NH$_4^+$ ions promote growth during initial germination and protocorm development whereas, NO$_3^-$ ions are required during differentiation and seedling development. Burgeff (1936) reported that ammonium nitrogen is required by *Paphiopedilum* seeds. Though both these ions (NO$_3^-$, NH$_4^+$) were invariably present in the media used presently, their individual utility could not be analyzed. Nevertheless, RE medium being comparatively rich in ammonium ion (7.27mm) as compared to Nsf (3.80mm) supported better germination and seedling growth in *Paph. spicerianum*. Another very important source of nitrogen
in ammonium form are various amino acids. Most culture media contain some amino acid either as specific component or as a byproduct in organic growth supplements like P, YE, CH, and various fruit juices. The utility of these amino acids for seeds germination has been variously assessed. Harvais and Raitsaka (1975) reported that amino acids aspartic acid, glutamic acid, arginine, and ornithine favours seeds germination in a number of species. MS (1962) and SN (1974) media containing glycine and both arginine and asparagine respectively were successfully used for germination. In this connection it is safe to assume that amino acids present in the PDA medium are also responsible for its growth promotory effect on germination and seedling growth in *Cym. pendulum* and *Thun. alba*. According to Arditti (1967) and Wynd (1933), the orchids require low amounts of calcium and phosphate salts for germination. Though the individual effect of these ions could not be tested in the present study, *Paph. spicerianum* embryos germinated better in RE medium than in N$_3$f medium due probably to relatively lower calcium content in the former medium (0.63: 4.20 mm respectively).

Orchid seeds lack an appropriate metabolic machinery to utilize their own food reserve or that present in the substratum. The fungal component of the mycorrhizal association *in vivo* is implicated in breaking down the complex carbohydrate into simpler digestible forms and making them available for various metabolic activities during germination and subsequent seedling development. Knudson (1922) demonstrated that the fungal requirement during germination of embryos can be compensated by augmenting the carbohydrate nutrition *in vitro*. Poor germination and arrested development of *Thun. alba* at spherule stage in simple water and KC mineral salt solution in present study hints at the importance of sugar in supporting better germination and seedling development. A perusal of literature also reveals that sugar is obligatory for development of orchid seedling *in vitro* though the stage till which a species can manage without it during germination varies with the species. According to Knudson (1922), a medium devoid of sugar failed to support seedling development in *Cattleya*. Successful germination of a number of species including *Dactylorhiza* species (Harvais and
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Hadley, 1967), Goodyera pubescence (Rasmussen, 1995), Orchis spp. (Downie, 1941), and Platanthera hyperborea, Platan. obtusa (Harvais, 1974) but their failure to form seedlings on water/water agar suggests that sugar treatment is obligatory not for germination but for seedling development in certain species. Vermeulen (1947) reported that a number of species can germinate in water and these are able to mobilize at least part of their food reserve immediately after the hydration of embryonal cells. Infact, he (Vermeulen) used the expression 'waiting time' for the period after germination till the germinating entities can subsist on their own reserve while waiting for a compatible fungal symbiont in vivo. Manning and Van Staden (1987) successfully demonstrated that seeds of certain south African orchid species contain some soluble sugars which they utilize during germination. A high frequency of germination and protocorm formation on a simple sugar solution in Thun. alba is indicative of the importance of sucrose in early stages of seedling development. The significance of sugar in stimulating development of protocorms and seedlings, however, varies with the species and has been variously emphasized by many workers (Butcher and Marlow, 1989; Downie, 1941; Harvais and Hadley, 1967). A variety of carbohydrates are being used in orchid tissue culture. Ernst (1967), Ernst et al. (1970), and Raghvan (1976) studied the suitability of various carbohydrates for orchid seed germination and found that the species may vary in their ability to utilize different sugars. Orchids have been reported to utilize monosaccharides (glucose, fructose), disaccharides (sucrose, cellobios, maltose, lactose), polysaccharides (soluble starch), and carbohydrates of fungal origin (trehalose, mannitol). Presently, sucrose/glucose (in PDA) were successfully used for Cym. pendulum and Thun. alba. In this connection, it is worthwhile to mention that besides starch, the potato extract also contain glucose, fructose, and several other carbohydrates including myo-inositol and cellulose (cf. Wealth of India, 1972). In Paph. spicerianum, utility of sucrose, glucose, and/or fructose in supporting germination and seedling growth was tested. While sucrose was successfully used for younger embryos in SN medium it failed to support the development of mature embryos. A conjoint treatment with glucose and fructose supported better germination and seedling development followed by individual treatments with glucose/fructose in accord
with the early observation by Withner (1959) that sucrose/glucose serve as a better carbon source and fructose comes in the next category of effectiveness. Thomale (1954), however, reported that a conjoint treatment with glucose and fructose was necessary for germinating *Paphiopedilum* and other temperate terrestrial orchids. Sucrose has been successfully used for germination and seedling development in a large number of orchid species (Kano, 1965; Knudson, 1950; Rao, 1963; Raghvan and Torrey, 1964; Vacin, 1950; Withner, 1953; Yam and Weatherhead, 1988). Tonnier (1954) reported that *Vanilla madagascariensis* successfully germinated and formed seedlings in glucose supplemented medium. Maltose favoured excellent germination and growth in *Cattleya* hybrids (Noggle and Wynd, 1943) whereas lactose was found suitable for *Phalaenopsis* seedlings (Tenhaeff, 1951, 1952). Ball (1953) emphasized the usefulness of dextrose and fructose as a carbon source, since sucrose is partially hydrolysed into glucose and fructose during autoclaving. The exact requirement of sucrose in orchid culture varies with the species, while in most of the cultures it is used at 2-3% (Arditti *et al.*, 1982), in other it proves effective at concentrations ranging from 0.5-3.0% (Butcher and Marlow, 1989). Presently, it was used at 2% in MS, KC and at 3% in SN medium. In PDA medium, dextrose was used at 2%. In N3f and RE medium, however, glucose and fructose were effectively used at 0.5-1.0%. Earlier Prakash *et al.* (1997) also used sucrose at 1.0% for optimal results in *Paph. fairieanum*. Sharma (1996) also tested the suitability of various sugar (glucose, fructose, maltose, sucrose etc.) at different concentrations in supporting optimal germination and seedling growth in *Dendrobium chrysotoxum* and *Paph. spicerianum* and observed that sucrose at 2-3% gives optimal results.

**Activated charcoal:**

Media used for orchid tissue culture are often darkened with AC because it favours a better growth and/or multiplicity of the plants. Presently, the release of brownish exudates by the germinating entities in *Cym. pendulum* and *Thun. alba* was successfully checked by using AC in the nutrient media. Besides checking the release of phenolic exudates, AC promoted germination and seedling growth; it even alleviated the detrimental effect of KN on organogenensis in *Cym.*
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A perusal of literature also reveals beneficial effect of this organic adsorbent. George and Sherrington (1984) reported that this adsorbent promotes better shoot growth and root geotropism in orchids largely due to opacification of the medium and its adsorptive capabilities. Ernst (1976) also reported better seedling growth in *Paphiopedilum* and *Phalaenopsis* with increased shoot and root formation on AC supplemented media. According to him (Ernst) the beneficial effect of AC could be due to exclusion of light from the substrate thus simulating the natural conditions and possibly by increasing the aeration. Klein and Bopp (1971) suggested that due to its light absorbing nature, AC enhances the amount of energy available to per unit plant material and is thus responsible for promoting the growth and development of seedlings. According to Wreckmestre (1971) it, however, inhibited proliferation and aerial root formation from the PLBs while favouring shoot growth and root geotropism.

Vij et al. (1988a) and Weatherhead et al. (1986) suggested that beneficial effect of AC may be due to its ability to adsorb potential growth inhibitors in the medium. AC, however, also adsorbs the essential media components (growth adjuncts) and this may explain the fact that it can also inhibit growth (Weatherhead et al. 1978; Yam et al. 1989). Since in the present study, AC invariably promoted seedling growth in media supplemented with growth adjuncts, it appears that growth adjuncts are required only at a level which remains in the medium following adsorption by AC.

**Organic growth supplement:**

Several complex organic growth supplements including Peptone (P), Yeast Extract (YE), Caesine Hydrolysate (CH), Coconut Water (CW), Banana Homogenate (BH), Malt Extract (ME), Potato Extract (PE), Tomato Juice, and other fruit juices has been used to enrich the culture media for orchid seed germination (Arditti and Ernst, 1984). The growth promotory effect of these supplements has been attributed to their organic nitrogenous (as amino acids and amides), minor elements, and vitamin constituent (Raghavan, 1976). Presently, the effect of P/CW/Stock of BM was tested on germination and seedling growth.
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Peptone:

A water soluble protein hydrolysate with high amino acid content, peptone supported better germination, protocorm multiplication, and seedling growth in *Cym. pendulum*. Its benign effect was further accentuated in combinations containing AC and it alleviated the detrimental effect of the cytokinin on organogenesis. Literature studies reveal that Peptone was obligatory for germination in *Spiranthes cernua* (Stoutamire, 1974) and *Goodyera biflora* (Pathak et al., 1992). It improved germination [*Vanda, Phalenopsis* (Lami, 1927); *Vanilla* (Borequet, 1947); *Cyperipedium* (Linden, 1980); *Cymbidium* hybrid (Kano, 1965)], supported better seedling growth [*Paphiopedilum, Phaius, Vanda* (Curtis, 1947); *Cattleya* (Mariat, 1948); *Rhynchostylis* (Vij and Kher, 1997)], and induced protocorm multiplication in *Cym. macrorhizon* and *Cymbidium* spp. (Vij and Pathak, 1988c; Kusumoto and Furukawa, 1977).

Coconut Water:

This organic growth supplement with auxin and gibberellin like substances (Dix and Van Steaden, 1982) as its active constituents is known to induce division of the otherwise non-dividing cells (Goh et al., 1975; George and Sherington, 1984) and mass multiplication of PLBs (Intuwong and Sagawa, 1973; Sagawa and Kunisaki, 1982). Presently, CW had detrimental effect on germination frequency in *Cym. pendulum*, advanced the onset of germination in *Thun. alba* and *Paph. spicerianum* but unless used with AC. It also supported protocorm multiplication and better plantlet growth in all the three taxa. In *Cym. pendulum*, it favoured rhizome mediated multiplication when used in AC enriched SN medium. CW has already been used favourably for germination in *Bletia purpuratum* (Rubluo et al., 1989), *Cattleya* (Kerbauy and Handro, 1981), *Cymbidium* (Chung et al., 1985), *Rhyn. retusa* (Nath et al., 1991), *Dendrobium* spp. (Devi et al., 1990), and *Paph. purpuratum* (Yam and Weatherhead, 1988), and for seedling growth in *Dendrobium* spp. (Devi et al., 1990), and *Rhyn. retusa* (Nath et al., 1991). It was, however, inhibitory to germination in *Dendrobium* (Kotomori and Murashige, 1965) and root differentiation in *Dend. moschatum* (Devi et al., 1990).
Vitamins:

A variety of vitamins are frequently used for orchid seed germination as they promote tissue and organ growth and their absence leads to deficiency symptoms in seedlings (Noggle and Wynd, 1943; Arditti, 1963; Prasad and Mitra, 1975).

Presently, vitamin rich nutrient media (MS and SN) were successfully used for germination. PDA medium which also contains vitamins besides other amino acids and growth hormones (cf. Wealth of India, 1972), supported good germination and subsequent seedling formation in *Cym. pendulum* and *Thun. alba*. Additional use of vitamin stocks of BM medium in PDA favoured higher germination frequency, accelerated seedling development, and protocorm multiplication. Growth promotory effect of vitamins, especially nicotinic acid, on *Cattleya* spp. and *Thunia* sp. is already on records (Noggle and Wynd, 1943; Henrikson, 1951). Beneficial effect of various other vitamins like ascorbic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine on orchid germination and subsequent seedling growth is also well documented (cf. Arditti, 1977b).

The exogenous supply of growth regulators in initiation and maintenance media is usually recommended for orchid culture depending upon the scheme of experimentation. Presently, the effect of both auxins (IAA/NAA) and cytokinins (KN) was tested individually or in combination with other growth adjuncts in *Cym. pendulum* and *Thun. alba*.

Auxins:

Orchids seeds are reported to be low in auxin content (endogenous) and their auxin requirements for germination in nature are augmented by the fungal partner in the mycorrhizal association (Hayes, 1969). IAA induced protocorm multiplication, and favoured accelerated organogenetic changes leading to seedling development in *Cym. pendulum* and *Thun. alba*. Earlier, germination was promoted in *Rhyn. retusa*, *Saccolabium calceolaris*, *Vand. testacea*, and *Pachystoma senile* (Vij et al., 1981, 1985), and *Calanthe discolor* (Miyoshi and Mii, 1995) but inhibited in *Dend. nobile* (Miyazaki and Nagamatsu, 1965), *Dactylorhiza purpurella*, *Coeloglossum viride*, and *Platan. biflora* (Hadley, 1970).
in IAA supplemented medium. This auxin also favoured protocorm multiplication in *Cymbidium* (Fonneshbech, 1972) and organogenesis in *Cattleya* (Withner, 1959) and *Vanda* ‘Miss Joaquim’ (Rao and Avdhani, 1964); germination was, however, inhibited in the later species.

NAA when used with AC favoured better germination, protocorm multiplication, and accelerated organogenetic changes leading to seedling development in both *Cym. pendulum* and *Thun. alba*. The benign effects of NAA on germination in species and hybrids of *Bletilla, Cattleya, Cymbidium, Dendrobium, and Paphiopedilum* (cf. Arditti, 1967, Arditti and Ernst, 1984), and *Cala. discolor* (Miyoshii and Mii, 1995), proliferation, growth, and differentiation of protocorm in *Bletilla, Cymbidium, Pachystoma, Rhynchostylis, Saccolabium, and Vanda* (cf. Arditti, 1967; Fonneshbech, 1972, Strauss and Reisinger, 1976; Mathews and Rao, 1980; Vij *et al.*, 1981a, and organogenesis and seedling growth in *Cattleya, Cymbidium, Epidendrum*, and *Vanda* (cf. Arditti and Ernst, 1984) are well documented. In the present cultures chlorophyll development was, however, delayed in its presence in accord with similar earlier findings in *Luisia zeylancia* (Arora, 1990). While the effective concentration of NAA is reported to vary with the species, presently it was used at 1.0mg/l and 0.5mg/l in *Cym. pendulum* and *Thun. alba* respectively.

**Cytokinins:**

The role of cytokinins has been variously assessed in orchids. Harvais (1982) considered them as important growth regulators during germination of ground orchids, whereas Arditti and Ernst (1984) opined that the orchid seeds are more sensitive to higher cytokinin levels than the protocorms and their cytokinin requirements, *in vivo*, if any are satisfied by the mycorrhizal endophyte. Presently, the effect of KN alone or with other growth adjuncts was tested in *Cym. pendulum* and *Thun. alba*.

In *Cym. pendulum* an individual treatment with KN impaired the germination frequency and subsequent organogenetic changes leading to seedling unless used with AC and/or other growth adjuncts. When used with IAA (in the combination),
it acted synergistically to support differentiation in the cultures. In *Thun. alba*, on the other hand, KN favoured improved germination frequency and accelerated seedling development. It, however, accentuated the release of brownish exudates by the germinating entities into the medium in line with similar observation in *Epipatics helleborine* and *Listera ovata* (Van-Weas, 1984). Promotory effect of cytokinin on germination in *Cyperi. calceolaris* (Borris, 1969), *Cattleya* (Pierik and Steegmans, 1972), *Cyperi. reginae* (Harvais, 1982), and inhibitory effect in *Coelogyne viride, Dactyl. purpurella, and Platan. biflora* (Hadley 1970), are already on records. Miyoshi and Mii (1995) observed that in *Cal. discolor* germination was inhibited at high concentration of cytokinin. Morphogenetic changes leading to seedling development were progressively impaired in *Coel. punctulata* in medium supplemented with high concentration of KN (Sharma and Tandon, 1986). IAA and KN acted synergistically to induce accelerated differentiation in *Orchis purpurella* (Harvais and Hadley, 1967) and *Cyperi. reginae* (Harvais, 1982).

Incidentally, the seeds in the present studies often responded differently to a given physiological treatment. Almost similar observations on the limited effect of growth adjuncts and the polymorphic response of embryo during germination were earlier explained by Miyoshii and Mii (1995) on the basis of heterogenity in seed population.

All these data suggest that developing (immature) embryos can be used to propagate orchids as besides reducing the time lapse between pollination and sowing, they lack dormancy and/or inhibitory factors, and the benefits accrued from using them as explants vastly outweigh the hazards of storage and surface decontamination of mature seeds with chemical agents. However, as all of them, in a capsule, have to be used in a single sowing one may run into the risk of failure, should the capsule be harvested at a wrong stage of development.
Discussion

Regeneration:

The development of appropriate technique of asymbiotic seed germination in vitro has added new dimensions to orchid propagation. The technique is, however, not conducive to true-to-type multiplication of desired genotypes because the orchids generate a great deal of heterozygosity in the progenies due to their out-breeding nature. The desirability of developing suitable micropropagation protocols has therefore been advocated since long. A micropropagation system for Cymbidium using shoot meristem (Morel, 1964a,b) has worked well in several taxa particularly with sympodial growth habit (cf. Arditti and Ernst, 1993). However, as the system causes a set back in the growth and development of the mother plant, it has a limited utility in monopodials. Attempts have therefore been made in recent years to identify alternate but equally effective explants whose excision does not jeopardise the survival of donor plant. The regenerative potential of excised leaves, roots, flower-stalks, rhizomes, pseudobulbs etc. has been positively tested in a variety of species and hybrids (cf. Arditti and Krikorian, 1996; Vij, 1995, 1998). The efforts are, however, not as yet commensurate with the size of the orchid family and reproducible protocols are yet to be developed.

It is in light of this scenario that present experiments were designed to test the in vitro regeneration competence of some commercially significant and/or endangered taxa. For this purpose, the explant were sourced from aerial stem [shoot-tips (Cym. pendulum, Gastro. calceolaris, Oncidium ‘Gower Ramsey’, Paph. spicerianum, Vand. coerulea), stem-nodes (Cym. pendulum, Thun. alba), pseudobulbs (Oncidium ‘Gower Ramsey’)], floral-stalks (Cym. pendulum, Gastro. calceolaris, Paph. spicerianum, Vand. coerulea), leaves (Cym. pendulum, Gastro. calceolaris, Oncidium ‘Gower Ramsey’, Paph. spicerianum, Thun. alba, Vand. coerulea), roots (Cym. pendulum, Vand. coerulea) and floral organs (Cym. pendulum, Gastro. calceolaris, Paph. Spicieranum, Vand. coerulea). The results have already been described under the respective taxa, in Observation Section, and in light of information available in the literature, some of the salient features of investigations are discussed as follows:
 Shoot-tip culture:

The regenerative potential of shoot-apical meristem was successfully tested in *Cym. pendulum*, *Gastro. calceolaris*, *Oncidium* ‘Gower Ramsey’, *Paph. spicerianum*, and *Vand. coerulea*. The response, however, varied with the species and the physical and chemical stimulus in vitro. Since the conditioning of explants by a pre-treatment in liquid medium helps in eluting the inhibitors and/or introduces growth regulators into the cells promotes their regeneration response (Sriskandarajah and Goodwin, 1998), the explants in *Cym. pendulum* were given an initial pulse treatment in liquid medium.

A static liquid medium proved useful for initiation and multiplication in *Gastro. calceolaris* but continuous agitation of the liquid medium was invariably requied for raising cultures in *Ascocenda* (Ichihashi, 1979), *Aranda* ‘Deborah’ (Lee et al., 1995), *Cymbidium* (Morel, 1964a, b, 1965a, b; Sagawa et al., 1966; Kim and Kako, 1983), *Calanthe* (Yamamoto et al., 1991), *Paphiopedilum* (Bubeck, 1973), *Rhynchostylis* (Vajarbhaya and Vajarbhaya, 1970), *Vanda* (Kunisaki et al., 1972; Mathews and Rao, 1985b; Teo et al., 1973) and a number of orchid species (Lee et al., 1996). An agarized medium enriched with CW and arginine was successfully used to multiply *Cym. pendulum* PLBs. The PLBs and shoots derived in liquid medium were, however, hyperhydrous and they required an incubation in PJ supplemented media for normal growth. A similar utility of PJ was indicated in *Doritaenopsis* (Zhou, 1995).

Presently, a PGR treatment was obligatory for regeneration but the level of treatment varied with the species. Even the nature of regenerants varied with the physical and chemical stimulus in vitro and the species.

In *Paph. spicerianum*, BAP (100 mg l⁻¹) and 2,4-D/NAA were successfully used to induce regeneration; a combination containing 2,4-D and BAP favoured callus development in accord with similar results in *Paphiopedilum* (Steward and Button, 1976). BAP at concentrations as high as 100 mg l⁻¹ is often detrimental to culture survival in other plants but its benign effect in *Paphiopedilum* culture (Huang, 1988; author) is interesting.
Discussion

Node-culture:

Perusal of literature reveals that nodal discs from aerial shoots have been used to regenerate several orchids including *Arundina* (Mitra, 1971), *Cymbidium* (Shimasaki and Umemoto, 1987; Yoshiyuki et al., 1993), *Dendrobium* (Kim et al., 1970), *Phalaenopsis* (Duan et al., 1996), and *Vanilla* (Duan and Hong, 1989; George and Ravishankar, 1997). In the present study, *Cym. pendulum* and *Thun. alba* cultures were successfully established *in vitro* using uninodal discs, while *in vitro* sourced discs yielded better results, the nature of response varied with the species and chemical stimulus. A treatment with PGRs was obligatory for axillary bud break in *Cym. pendulum* but such a requirement was not a must for the purpose in *Thun. alba*. Earlier, a treatment with anti-auxins proved useful in inducing bud break in *Dendrobium* (Moisch et al., 1974) node culture. In *Cym. pendulum* the axillary bud callused in combination containing BAP (2.5 mg/l) and NAA (0.1 mg/l) each. A similar response in *Thun. alba*, however, required a combined treatment with BAP (2.5 mg/l), KN (2.5mg/l), NAA (0.1mg/l), and CW (20%) in the nutrient pool. Incidentally, in the former species (*Cym. pendulum*) the number and sites of meristemoids invoked was related to the quality of auxin used in the medium. IAA (0.05mg/l) when used with BAP (5.0mg/l) favoured development of multiple shoots in the nodal regions, whereas its replacement with NAA (0.1mg/l) induced extra axillary adventive meristemoids even in the inter-nodal regions. Since in an earlier study extra axillary meristemoids could be induced in the basal medium (Vij et al., 1994a) requirement of PGRs, for the purpose, in the present cultures may be a genetic attribute.

The position of the discs (explants) on the donor organ also appeared to influence the nature of neoformation in *Cym. pendulum*; the explants sourced from the proximal ends of the shoots regenerated only roots regardless of the chemical treatment *in vitro*. Earlier, Tran Thanh Van (1973b) also reported a differential response of explants procured all along the length of the plant and attributed it to source related physiological intricacies.
Presently, BAP often promoted PLB/shoot bud multiplication but the neoformations failed to root unless treated with an auxin in the nutrient pool. Root initiation was similar auxin dependent in *Arundina bamboosifolia*, *Dend. transparens* (Honmonde and Sehgal, 1991), and *Spathoglottis plicata* (Teng et al., 1997). All these data suggest that conditions favourable to shoot development may not be conducive to root formation.

**Pseudobulb-segment culture:** Prior to the present study pseudobulb segments were reported to have regenerated in *Bletilla* (Vij and Dhiman, 1997a), *Cattleya, Miltonia, Phaius* (Morel, 1970), *Coelogyne* (Vij et al., 1997b), *Dendrobium* (Vij and Sood 1982; Vij and Pathak, 1989; Kukulczanca and Wojciechowska, 1983; Yam, 1989), and *Eulophia* (Vij et al., 1989). Presently, their regeneration competence was successfully tested in *Oncidium ‘Gower Ramsey’*. The maturity level of the tissues and chemical stimulus in the nutrient pool were major factors promoting regeneration. Only the juvenile tissues reacted positively and that too if treated with KN and/or NAA. The morphogenetic changes leading to plantlet formation in the regenerants were, however, markedly influenced by the quality of growth stimulus in accord with similar earlier findings in *Dend. chrysanthum* (Vij and Pathak, 1989).

An individual treatment with NAA favoured direct development of PLB in the explants regardless of the level of maturity of their tissues. Callus mediated and direct shoot development in KN (5.0mg/l and 10.0mg/l) and NAA (1.0mg/l) supplemented media seems to be related to variations in the maturity levels of the explants. Such an effect of tissue maturity, on the nature of regenerants was, however, not reflected when level of KN was increased to 12.0mg/l in the above combination; the explants invariably callused prior to differentiating PLBs or shoot buds. Literature studies reveal that regeneration pathway also varies with the species; shoots developed directly as in *Dendrobium* (Kim et al., 1970), *Phalaenopsis* (Scully, 1966), *Vanilla* (George and Ravishankar, 1997), it followed callus mediated development as in *Vanilla* (Philip and Nainar, 1986).
Leaf-segment culture:

The ability of Cymbidium leaves to develop protocorm like bodies (PLBs) \textit{in vitro} (Wimber, 1965), has opened up exciting opportunities to use foliar explants, as an effective alternative to excised shoot-meristem, for micropropagating orchids. A perusal of literature reveals that the regenerative potential of the foliar explants in orchids has been positively tested in a number of taxa. (Table Dis.1).

Presently, the \textit{in vitro} regeneration response of leaf tissues was positively tested in 
\textit{Cym. pendulum, Gastro. calceolaris, Oncidium ‘Gower Ramsey’, Thun. alba,} and \textit{Vand. coerulea}. The culture initiation varied with the species and was markedly influenced by the explant source, juvenility of the tissues (as judged through the size of donor leaf), and chemical stimulus \textit{in vitro}. The explants from greenhouse grown plants exuded profusely, lost chlorophylls, and perished soon after despite repeated subcultures in accord with similar earlier observations in several orchids including \textit{Renanthera} (Seeni and Latha, 1992) and \textit{Acampe} (Nayak \textit{et al.}, 1997). \textit{Gastro. calceolaris}, however, proved an exception where ascorbic acid (10 mg/l) was successfully used to check the release of exudates and explants made to respond positively. A recalcitrant behaviour of \textit{in vitro} sourced explants has been attributed to their high phenolic contents (Tanaka, 1992) and/or procedural problems (Vij \textit{et al.}, 1994b). The \textit{in vitro} sourced explants, on the other hand, responded more frequently; the response was markedly influenced by the juvenility of their tissues as assessed in terms of leaf size. The explants from leaves > 2 cm failed to respond. Juvenility of leaf tissue as an important factor promoting regeneration has already been suggested in several species (Fu, 1978, 1979; Mathews and Rao, 1985b; Tanaka, 1992; Vij and Pathak, 1990; Vij \textit{et al.}, 1986c, 1994b). A better morphogenetic potential of juvenile cells has been explained on the basis of their physiologically and biochemically more active state due to less rigid cell walls (Misra and Bhatnagar, 1995). Nayak \textit{et al.} (1997), however, reported that the younger leaves failed to regenerate, whereas the fully expanded mature leaves responded positively in \textit{Acampe praemorsa}. 
Table 1: Some of the important orchids regenerated through leaf tissue culture till date.

<table>
<thead>
<tr>
<th>S.no</th>
<th>Taxon</th>
<th>regeneration site</th>
<th>pathway</th>
<th>Author(s)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Acampe praemorsa</td>
<td>leaf base</td>
<td>PLB</td>
<td>Nayak et al., 1997</td>
</tr>
<tr>
<td>2</td>
<td>Aranda cv. Nancy</td>
<td>leaf tip</td>
<td>-</td>
<td>Loh et al., 1975</td>
</tr>
<tr>
<td>3</td>
<td>Ascocenda 50th State Beauty</td>
<td>leaf base</td>
<td>+</td>
<td>Vij. and Kaur, 1994a</td>
</tr>
<tr>
<td>4</td>
<td>Cattleya</td>
<td>leaf base</td>
<td>-</td>
<td>Champagnat et al., 1970</td>
</tr>
<tr>
<td>5</td>
<td>Coelogyne speciosa</td>
<td>entire leaf</td>
<td>+</td>
<td>Vij and Pathak, 1990</td>
</tr>
<tr>
<td>6</td>
<td>Cymbidium</td>
<td>entire leaf</td>
<td>+</td>
<td>Wimber, 1963, 1965</td>
</tr>
<tr>
<td>7</td>
<td>Cymbidium pendulum</td>
<td>leaf base</td>
<td>+</td>
<td>Author</td>
</tr>
<tr>
<td>8</td>
<td>Dendrobium crumenatum</td>
<td>leaf base</td>
<td>+</td>
<td>Manorama et al., 1984</td>
</tr>
<tr>
<td>10</td>
<td>Doritis pulcherrima</td>
<td>entire leaf</td>
<td>+</td>
<td>Tanaka, 1987</td>
</tr>
<tr>
<td>11</td>
<td>Epidendrum cv. O'Brienianum</td>
<td>leaf tip</td>
<td>-</td>
<td>Churchill et al., 1973</td>
</tr>
<tr>
<td>12</td>
<td>Gastrochilus calceolaris</td>
<td>leaf base</td>
<td>+</td>
<td>Author</td>
</tr>
<tr>
<td>13</td>
<td>L. trichorhiza</td>
<td>entire leaf</td>
<td>+</td>
<td>Vij and Pathak, 1990</td>
</tr>
<tr>
<td>14</td>
<td>L. teretifolia</td>
<td>entire leaf</td>
<td>+</td>
<td>Vij and Pathak, 1990</td>
</tr>
<tr>
<td>15</td>
<td>Oncidium 'Gower Ramsey'</td>
<td>leaf base</td>
<td>+</td>
<td>Author</td>
</tr>
<tr>
<td>16</td>
<td>Paphiopedilum</td>
<td>leaf-tip</td>
<td>-</td>
<td>Allenberg, 1976</td>
</tr>
<tr>
<td>17</td>
<td>Paphiopedilum spicerianum</td>
<td>leaf base</td>
<td>+</td>
<td>Author</td>
</tr>
<tr>
<td>18</td>
<td>Phalaenopsis amabilis</td>
<td>entire leaf</td>
<td>+</td>
<td>Tanaka, 1987, 1989</td>
</tr>
<tr>
<td>19</td>
<td>Phalaenopsis hybrid</td>
<td>entire leaf</td>
<td>+</td>
<td>Tanaka, 1987, 1989</td>
</tr>
<tr>
<td>20</td>
<td>Phalaenopsis Mab X Eagle</td>
<td>entire leaf</td>
<td>+</td>
<td>Latha and Seeni, 1991</td>
</tr>
<tr>
<td>21</td>
<td>Renanthera imscooliana</td>
<td>entire leaf</td>
<td>-</td>
<td>Laishram and Devi, 1997</td>
</tr>
<tr>
<td>22</td>
<td>Renanthera imscooliana</td>
<td>leaf base</td>
<td>+</td>
<td>Seeni and Latha, 1992</td>
</tr>
<tr>
<td>23</td>
<td>Renantanda</td>
<td>entire leaf</td>
<td>+</td>
<td>Goh and Tan, 1982</td>
</tr>
<tr>
<td>24</td>
<td>Rhynchostylis retusa</td>
<td>entire leaf, leaf tip</td>
<td>+</td>
<td>Vij et al., 1984; Vij and Pathak, 1990</td>
</tr>
<tr>
<td>S.no.</td>
<td>Taxon</td>
<td>site</td>
<td>regeneration pathway</td>
<td>Author(s)</td>
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<tr>
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<td>----------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>25</td>
<td><em>Satyrium nepelanes</em></td>
<td>leaf base</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td><em>Spathoglottis plicata</em></td>
<td>leaf base</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td><em>Thunia alba</em></td>
<td>leaf base</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td><em>Vanda coerulea</em></td>
<td>leaf base</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>entire leaf</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td><em>Vanda coerulea</em></td>
<td>leaf base</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td><em>Vanda ‘Kasems Delight Tom Boykin’</em></td>
<td>entire leaf</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td><em>Vanda testacea</em></td>
<td>entire leaf, leaf base</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Regeneration in the leaf explants was obligatory to a treatment with growth adjuncts in *Cym. pendulum*, *Gastro. calceolaris*, *Paph. spicerianum*, and *Thun. alba* and it varied with their quality and nature. Treatment with auxin (NAA) detrimentally affected the regeneration in *Oncidium ‘Gower Ramsey’*, whereas it remained ineffective in *Vand. coerulea*. Earlier, an individual treatment with IAA detrimentally affected the response frequency in *Neofinetia falcata* and *Rhyn. retusa* (Vij and Pathak, 1990). BAP favoured regeneration of multiple shoots in *Vand. coerulea* (present study) but was effective in *Vand. cristata* and *Vand. testacea* only when used with peptone (Vij and Pathak, 1990). As also been reported in *Luisia teretifolia*, *Neo. falcata*, and *Rhyn. retusa*. Cytokinins (BAP) and auxins (NAA) when used in combination proved useful in supporting regeneration in *Oncidium ‘Gower Ramsey’*, *Gastro. calceolaris*, and *Vand. coerulea*. In *Cym. pendulum* a combined treatment with BAP and P supported regeneration whereas a similar response with KN+P was obligatory to the additional use of auxin (IAA/NAA). In *Paph. spicerianum* a combined with auxin (2,4-D) and cytokinin (BAP) in GD medium was obligatory for regeneration. In *Vand. coerulea* BAP (2.0 mg l⁻¹) and NAA (0.5 mg l⁻¹) acted synergistically to support culture multiplication. A synergistic action of auxin and cytokinin in supporting regeneration in leaf explants is well documented (Mathews and Rao, 1985b; Vij and Pathak, 1990; Vij et al., 1986c; Yam and Weatherhead, 1991a).

Coconut water alone or with other growth adjuncts supported initiation and culture multiplication in *Paph. spicerianum*, *Thun. alba*, and *Vand. coerulea*. In *Cym. pendulum* peptone alone or with other growth adjuncts successfully supported regeneration. A perusal of literature also reveals a beneficial role of various organic growth adjuncts in leaf segment culture. Peptone was used as an essential factor promoting regeneration in *Rhynchostylis* and *Vanda* (Vij et al., 1984, 1986c), whereas a similar purpose was served by YE in *Aerides*, *Papilionanthe*, and *Satyrium* (Vij and Pathak, 1990), CH in *Aranda* hybrid and *Dendrobium* (Manorama et al., 1984), *Ascocenda* (Fu, 1978, 1979), and *Renantanda* (Goh and Tan 1982).
Discussion

In the present cultures, the leaf base exhibited a greater proliferative potential than the leaf tip as also reported in *Ascocenda* (Fu, 1978, 1979), *Coelogyne*, *Dendrobium*, *Oncidium*, and *Phalaenopsis* (Karim and Hairani, 1990). Vij and Pathak, (1990) and Vij et al. (1984), on the other hand, reported that the proliferative potential is distributed all along the leaves in orchids. Vij et al. (1986c), however, related the site of regeneration to the juvenility of tissue as the juvenile leaves in *Vand. testacea* proliferated all along the surface, whereas in older ones the response was restricted to the basal regions only suggesting thereby that, in orchids, the inherent (latent) meristematic potential of the foliar explants perhaps controlled by some factors emanating from the leaf base is markedly influenced by the maturity levels of tissues.

The present data together with those reported earlier suggest that the manifestation of the inherent potential of orchid leaves to regenerate in vitro is directly related with the genetic constitution, juvenility of tissue, and chemical stimulus. A careful selection of donor leaves and appropriate growth stimulus provides an ideal method of orchid propagation and their easy amenability offers great opportunities for rapid multiplication of desired genotypes without affecting the survival of mother plants.

Floral-stalk culture:

In orchids, the first attempt to test the regenerative potential of floral-stalks was made in *Phalaenopsis* (Rotor, 1949) and this was followed by several successful efforts (Table Dis.2). Presently, the regenerative potential of floral-stalk was positively tested in *Gastro. calceolaris* and *Vand. coerulea*. The response was, however, obligatory to the use of BAP (1.0mg/l) and NAA (0.1mg/l) in *Gastro. calceolaris* and BAP (4.5mg/l) and CW (20%) in *Vand. coerulea* cultures. The utility of growth adjuncts in floral-stalk culture has already been emphasized in *Cleisostoma* (Vij and Kanika, 1997), *Dendrobium* (Vij et al., 1981b), *Malaxis* (Vij and Dhiman, 1997b), *Oncidium* (Lim-Ho and Lee, 1987), *Phalaenopsis* (Lin, 1986; Tanaka and Sakanishi, 1980), *Rhynchostylis* (Kaur and Vij, 1995; Vij et al., 1997a), *Saccolabium* (Vij et al., 1986b) floral-stalk culture. Since, Ichihashi
Table *Dis. 2*: Some of the important orchids regenerated through floral-stalk segment culture.

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Taxon</th>
<th>Response</th>
<th>Author(s)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td><em>Ascofmetia</em></td>
<td>+</td>
<td>Intuwong and Sagawa, 1973</td>
</tr>
<tr>
<td>2</td>
<td><em>Cleisostoma micranthum</em></td>
<td>+</td>
<td>Vij and Kanika, 1997</td>
</tr>
<tr>
<td>3</td>
<td><em>Dendrobium</em></td>
<td>+</td>
<td>Vij et al., 1981b</td>
</tr>
<tr>
<td>4</td>
<td><em>Gastrochilus calceolaris</em></td>
<td>+</td>
<td>Author</td>
</tr>
<tr>
<td>5</td>
<td><em>Malaxis acuminata</em></td>
<td>+</td>
<td>Vij and Dhiman, 1997b</td>
</tr>
<tr>
<td>6</td>
<td><em>Mokara</em></td>
<td>+</td>
<td>Lee et al., 1996</td>
</tr>
<tr>
<td>7</td>
<td><em>Neostylis</em></td>
<td>+</td>
<td>Intuwong and Sagawa, 1973</td>
</tr>
<tr>
<td>8</td>
<td><em>Oncidium</em></td>
<td>+</td>
<td>Lee et al., 1996</td>
</tr>
<tr>
<td>9</td>
<td><em>Phalaenopsis</em></td>
<td>+</td>
<td>Ichihashi 1992; Tanaka and Sakanishi, 1977</td>
</tr>
<tr>
<td>10</td>
<td><em>Rhynchostylis</em></td>
<td>+</td>
<td>Kaur and Vij, 1995; Vij et al., 1997a</td>
</tr>
<tr>
<td>11</td>
<td><em>Saccolabium</em></td>
<td>+</td>
<td>Vij et al., 1986b</td>
</tr>
<tr>
<td>12</td>
<td><em>Thunia alba</em></td>
<td>+</td>
<td>Singh and Prakash, 1984</td>
</tr>
<tr>
<td>13</td>
<td><em>Vanda coerulea</em></td>
<td>+</td>
<td>Author</td>
</tr>
<tr>
<td>14</td>
<td><em>Vascostylis</em></td>
<td>+</td>
<td>Intuwong and Sagawa, 1973</td>
</tr>
</tbody>
</table>
(1992) used simple hyponex solution to initiate Phalaenopsis culture using floral-stalk segments, it appears that the nutritional requirement for floral-stalk culture are species specific.

Incidentally, in Gastro. calceolaris cultures cell proliferations occurred both along the nodal and inter-nodal regions whereas in Vand. coerulea the response was restricted to inter-nodal regions alone. In Gastro. calceolaris the undifferentiated floral-buds were amenable to transformation into vegetative ones in accord with their amenability in Ascofinetia, Neostylis, and Vascostylis (Intuwong and Sagawa, 1973), Phalaenopsis (Ichihashi, 1992; Tanaka and Sakanishi, 1980), Rhynchostylis (Kaur and Vij, 1995), Saccolabium (Vij et al., 1986b). The proliferative competence of inter-nodal sections has already been demonstrated in Cleisostoma (Vij and Kanika, 1997), Phalaenopsis (Lin, 1986), and Rhynchostylis (Vij et al., 1997a).

The differentiated floral buds failed to develop into flowers in Gastro. calceolaris unlike their normal development into flowers in Ascofinetia, Neostylis, and Vascostylis (Intuwong and Sagawa, 1973), Rhynchostylis (Kaur and Vij, 1995; Vij et al., 1997a), and Saccolabium (Vij et al., 1986b). Konar and Kitchlue (1982), hinted at nutritional complexities during normal development of flowers. A treatment with IAA, KN, and CH/Urea was obligatory for development of Sacco. calceolaris buds into normal flowers (Vij et al., 1986b).

**Floral-appendage culture:**

Ito (1961) tested the developmental potential of excised orchid ovaries. Lim-Ho et al. (1984) induced PLB development in Mokara and Spathoglottis floral buds. Regenerative competence of floral-appendages was also positively tested in Cymbidium (Kim and Kako, 1984) and Dendrobium cristata (Vij and Sharma, 1996). Suryawinoto and Sumaryo (1985) regenerated Dendrobium hybrids using excised anthers. Encouraged by above results, the regenerative potential of various floral-organs i.e. perianth, gynostegia, ovary, and anthers was presently assessed in four species (Cym. pendulum, Gastro. calceolaris, Paph. spicercum, Thun. alba).
But cell proliferations occurred only in *Gastro. calceolaris* perianth (labellum) and gynostegia. A treatment with KN, NAA, and CW was obligatory for cell proliferations in labellum cultures. A requirement for cytokinin and auxin has been indicated to initiate *Cymbidium* (Kim and Kako, 1984) and *Vanda*. (Vij and Sharma, 1996) cultures using floral appendages as explants. A combined treatment with KN and CW, on the other hand, supported cell proliferations in *Gastrochilus* gynostegia. Since differentiation eluded the neoformations, further experiments are suggested to achieve success.

**Anther**: Suryawinoto and Sumaryo (1985) hinted at the importance of operculum in realizing the regenerative competence of *Dendrobium* anthers. The importance of operculum in guiding cell proliferations in the anther explants was also apparent in the present cultures of *Gastro. calceolaris*. Unfortunately, differentiation invariably eluded the cell proliferations. Incidentally, CW was obligatory to anther regeneration in *Dendrobium* (Suryawinoto and Sumaryo, 1985), but such a requirement was not indicated in the present study where cell proliferations were invoked even in the basal medium.

**Root-segment culture:**

A limited morphogenetic ability of root meristem of higher plants including orchids not withstanding the utility of root as explant for micropropagation purposes is being increasingly realised due to their round the year availability, low oxidation rate, and the ease with which they can be explanted (cf. Kerbauy, 1991). The regeneration potential of root explants has so far been been tested in a number of orchid species and hybrids (Table *Dis.* 3).

In the present study, it was possible to induce extended growth or PLBs/shoot buds/callus/roots in *Cym. pendulum* and *Vand. coerulea* root explants by varying the quality and/or combination of growth adjuncts in the medium. Earlier, Vij (1993) also emphasized the significance of growth regulators in selecting the regeneration pathway. In *Cym. pendulum*, peptone favoured extended root growth whereas in *Vand. coerulea* a similar response was observed in NAA treated
Table Dis. 3: Some of the important orchids regenrated through root tissue culture till date.

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Taxon</th>
<th>Site</th>
<th>PLBs</th>
<th>shoot bud</th>
<th>callus</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bletilla striata</td>
<td>Root tip</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Yam and Weatherhead, 1991b</td>
</tr>
<tr>
<td>2</td>
<td>Catasetum hybrid</td>
<td>Root tip</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Kerbauy, 1984a</td>
</tr>
<tr>
<td>3</td>
<td>Cat. pileatum</td>
<td>Root tip</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>Kraus and Kerbauy, 1987</td>
</tr>
<tr>
<td>4</td>
<td>Cat. pileatum</td>
<td>Root tip</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Kraus and Montero, 1989</td>
</tr>
<tr>
<td>5</td>
<td>Cattleya</td>
<td>Cut end</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Vij, 1993</td>
</tr>
<tr>
<td>6</td>
<td>Cleisostoma fordi</td>
<td>Root tip</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Yam and Weatherhead, 1991b</td>
</tr>
<tr>
<td>7</td>
<td>Cymbidium mastersii</td>
<td>Cut end</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Vij, 1993</td>
</tr>
<tr>
<td>8</td>
<td>Cym. pendulum</td>
<td>Root tip along the surface</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Vij, 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cut end /along the surface</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Author</td>
</tr>
<tr>
<td>9</td>
<td>Cyrtopodium punctatum</td>
<td>Root tip / cut end</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Sanchez, 1988</td>
</tr>
<tr>
<td>10</td>
<td>Dendrobium Emma White</td>
<td>Cut end</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Vij, 1993</td>
</tr>
<tr>
<td>11</td>
<td>Epidendrum O'Brienianum</td>
<td>Root tip</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Steward and Button, 1978</td>
</tr>
<tr>
<td>12</td>
<td>Neottia nidus-avis</td>
<td>Root tip</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Champagnat, 1971</td>
</tr>
<tr>
<td>13</td>
<td>Oncidium varicosum</td>
<td>Root tip</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Kerbauy, 1984b</td>
</tr>
<tr>
<td>14</td>
<td>Phalaenopsis</td>
<td>Root tip / cut end</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Yaneda and Momose, 1988</td>
</tr>
<tr>
<td>15</td>
<td>Phalaenopsis amabilis</td>
<td>Root tip</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Tanaka et al., 1976</td>
</tr>
<tr>
<td>16</td>
<td>Pholidota cantonensis</td>
<td>Root tip</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Yam and Weatherhead, 1991b</td>
</tr>
<tr>
<td>17</td>
<td>Pho. chinensis</td>
<td>root tip</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Yam and Weatherhead, 1991b</td>
</tr>
<tr>
<td>18</td>
<td>Rhynchostylis retusa</td>
<td>Root tip / cut end</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Sood and Vij, 1985; Vij et al., 1987</td>
</tr>
<tr>
<td>19</td>
<td>Rhynchostylis retusa</td>
<td>Along the surface</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Chaturvedi and Sharma, 1986; Vij 1993</td>
</tr>
<tr>
<td>20</td>
<td>Vanda. coerula</td>
<td>Cut end</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Kanika and Vij, 1997; Author</td>
</tr>
<tr>
<td>21</td>
<td>Vand. Kasem Delight</td>
<td>Cut end</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Vij, 1993</td>
</tr>
<tr>
<td>22</td>
<td>Vand. teres</td>
<td>Cut end</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Vij, 1993</td>
</tr>
<tr>
<td>23</td>
<td>Vanda bicolor</td>
<td>Cut end</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Vij, 1993</td>
</tr>
<tr>
<td>24</td>
<td>Vanda hybrid</td>
<td>cut end</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Chaturvedi and Sharma, 1986</td>
</tr>
<tr>
<td>25</td>
<td>Vanda Kasem Delight 'Tom Boykin'</td>
<td>Root tip</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Vij and Sharma, 1997</td>
</tr>
<tr>
<td>26</td>
<td>Vanda testacea</td>
<td>Root tip / cut end</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Vij, 1993</td>
</tr>
<tr>
<td>27</td>
<td>Vanilla planifolia</td>
<td>Root tip</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Philip and Nainar, 1986</td>
</tr>
<tr>
<td>28</td>
<td>Vanilla planifolia</td>
<td>Root tip</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Philip and Padkaia, 1989</td>
</tr>
</tbody>
</table>
cultures in similar line with identical root-tip elongation in *Oncidium variecosum* (Kerbauy, 1984b). In *Cym. pendulum* a treatment with BAP+NAA and KN+IAA favoured PLB mediated regeneration, and that with KN+2,4-D favoured direct development of shoot buds. AS proved ineffective in inducing cell proliferations due probably to its low potency than BAP/KN. Inefficiency of this growth adjuncts was earlier explained on similar basis (Skoog, 1971). In *Vand. coerulea*, the regeneration pathway was significantly affected by the nature of cytokinin used in the nutrient matrix. BAP invariably favoured a direct development of shoot buds, whereas the effect of KN varied with the presence or absence of auxin in the medium; an individual treatment with KN supported PLB mediated regeneration, but in combination containing NAA it induced callus mediated shoot bud regeneration.

Requirement for only a cytokinin (Chaturvedi and Sharma, 1986; Kerbauy, 1984a; Vij, 1993; Yam and Weatherhead, 1991b) or an auxin (Arora, 1990; Phillip and Padikkala, 1989; Vij, 1993) for inducing regeneration in root explant is known in a variety of orchids. A benign effect of combined treatment with auxin and cytokinin for the purpose is also on records (Vij, 1993). All these data suggest that growth adjuncts play an important role in guiding the development pathway in neoformations.

Since in the present cultures only the segments away from the root tips regenerated and the leaf explants responded frequently the writer seems to agree with Yam and Weatherhead (1991b) that induction of shoot is, in general, more difficult in root-tips than in leaf-tips. The low success rate notwithstanding, the available information including the present report indicates that the regeneration competence is an inherent trait of orchid roots which gets invoked under certain specific physio-chemical stimulus and is markedly influenced by the physiological age of the explants. In this connection it is worthwhile to mention that the root cap is an active site of IAA accumulation and the root growth is markedly influenced by the endogenous level of IAA which changes from sub-optimal to supra-optimal with age (Pilet and Elliot, 1981; Philip and Padikala, 1989). A supra-optimal level maintains root meristem and a sub-optimal level (resulting through suppressed
polar transport of auxin and/or lysis of the root cap) accounts for a switch in the activities of meristematic cells with the result that they are induced to develop into shoot meristem (Phillip and Nainar, 1988). In *Crytopodium punctatum* Sanchez (1988) observed a rapid morphogenetic response without a notable increase in the length of root explants which were subjected to a submersion treatment in sterile water before inoculation, and suggested that the treatment accelerates the differentiation process by favouring the formation of promotory substances under reduced oxygen levels in water. Yam and Weatherhead (1991b), on the other hand, correlated the change from organized (root elongation) to unorganized (callus formation) growth in *Bletilla striata*, to a higher carbohydrate level in the tissue.

**Culture multiplication:**

Eversince, Morel (1964a, b) demonstrated the ability of PLBs slices to regenerate and suggested the possibility of obtaining upto 4x10^6 plants within a year through repeated segmentation and culture of daughter PLBs, protocorm/PLBs slices have been successfully used to achieve high frequency multiplication of orchid cultures (Amaki and Higuchi, 1989; Kanase and Takano, 1995; Lam et al., 1991; cf. Mathews and Rao, 1985). Even the whole PLBs are capable of generating daughter PLBs directly through budding or indirectly through callusing (Vij et al., 1981b). Presently, the regenerative potential of thin sections of PLBs was positively tested in *Cym. pendulum, Gastro. calceolaris, Oncidium 'Gower Ramsey', Paph. spicerianum, Thun. alba, and Vand. coerulea*. The utility of thin segments in achieving multifold increase in the rate of culture multiplication has been reported in several orchids due probably to i) the release of morphogeneically competent cells from the correlative controls imposed by neighbouring cells and ii) the increased availability of nutrients and growth promoting substances to the sites of regeneration (Lee et al., 1995, 1996).

Liquid medium irrigated pads of non-absorbent cotton favoured high frequency multiplication of PLBs in *Cym. pendulum, Oncidium 'Gower Ramsey', and Vand. coerulea* in accord with their similar utility in *Phalaenopsis* (Park et al., 1996).
Plantago seed husk (isabagol) and sago powder were successfully used to replace agar as a gelling agent in the present study. Isabagol was similarly used to solidify the nutrient medium (Babbar and Jain, 1998).

CW in sucrose starved media proved beneficial in achieving PLB derived culture multiplication in *Cym. pendulum* and *Vand. coerulea* (in agarized and liquid medium) in accord with similar utility of this organic growth supplement in *Vanda* (Kunisaki *et al.*, 1972; Toe *et al.*, 1973). The importance of CW in activating meristematic loci is already reported in *Ascocenda, Vanda, Renantanda* (cf. Arditti and Ernst, 1993), *Aranda* and a variety of other hybrids and species (Lee *et al.*, 1995, 1996). The benign effect of this growth adjunct has been variously attributed to its sugar and cytokinin contents (Lee *et al.*, 1995), auxin and gibberellin like component (George and Sherrington, 1984) and sugar contents which compensate the absence of sugar in the medium (Ichihashi and Hairawa, 1996). Interestingly, the problem of hyperhydricity of the PLBs and plantlets obtained therefrom was frequently encountered in the present experiments. Several remedial measures including increased rigidity of medium, better ventilation, and temperature manipulation during incubation of cultures have been suggested to overcome this problem (cf. Zhou, 1995). While none of these measures could be employed presently, the ability of hyperhydrous PLBs to multiply more prolifically as suggested by Zhou (1995) is interesting.

**Callus maintenance:**

For germplasm storage the utility of seeds, embryoids, and embryogenic calli has been variously assessed. While seed storage is beset with problems of viability loss and destruction by pathogen and pest attack, cryopreservation of embryoids is a laborious process involving expensive equipment. Storage of embryogenic calli *in vitro*, on the other hand, is a useful proposition as it does not involve complicated equipment and yield required propagules (PLBs) through proper manipulation of culture conditions. Chang and Chang (1998) successfully used callus for plant regeneration in *Cymbidium ensifolium* by manipulating the chemical stimulus *in vitro*.
Presently, an attempt was made to assess the storability of *Vand. coerulea* shoot-tip derived calli without affecting their embryogenecity. For the purpose, the effect of sugar and/or CW on regenerative competence of callus was tested. While glucose invariably impaired the callus viability, chlorophyll development and organogenesis in the callus was markedly influenced by the quality of sugars in the medium; mannitol and maltose yielded positive results whereas sucrose was better eliminated for the purpose. Organogenetic competence of callus in sucrose free medium has already been reported in orchids (Ichihashi and Hairaiwa, 1996; Ichihashi *et al.*, 1993; Niimi *et al.*, 1995). Such a difference in the efficacy of different sugars seems to be related to their varied energy potentials and abilities to modify the medium osmoticum as suggested by Ichihashi and Hiraiwa (1996). Incidentally, CW alleviated the detrimental effect of glucose on survival/organogenetic competence of callus. Ichihashi and Hiraiwa (1996) also reported a similar utility of CW in callus culture of *Phalaenopsis, Neofinetia, Doritaenopsis,* and *Darwinara*.

**Synthetic “seeds”:**

Despite the rapid progress of tissue culture technology *in vitro*, the importance of an appropriate delivery system for the micropropagants has often been overlooked. In fact, the imbalance between the efficiency of *in vitro* propagation and the delivery of the regenerants poses certain limitations on the practical applications of tissue culture technologies. Possibility of preparing artificial “seeds” by encapsulating embryoid/meristemoids in a nutritive gel, first suggested by Murashige (1978), has been realized in several species of flowering plants including orchids. Prior to the present efforts in *Cym. pendulum, Gastro. calceolaris, Thun. alba,* and *Vanda coerulea*. Such “seeds” have been prepared in 16 species of orchids (Table Dis. 4). They are easy to handle and ensure economy of space, medium, and time during storage and lab to land transfer and transport of tissue culture raised genotypes and allow the use of conventional seed handling techniques (used for seeds of larger sizes). The shape, size, and firmness of the “seeds” was directly related to the concentration of sodium alginate used. They were best formed by using 4.0% sodium alginate. Since, in the earlier studies
Table *Dis. 4* List of the orchid species in which Synthetic "Seeds" has been prepared.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Taxon</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aerides multiflorum</td>
<td>Vij et al., 1992</td>
</tr>
<tr>
<td>2</td>
<td>Agrostophillum myrianthum</td>
<td>Deka et al., 1997</td>
</tr>
<tr>
<td>3</td>
<td>Cym. giganteum</td>
<td>Corrie and Tandon, 1993</td>
</tr>
<tr>
<td>4</td>
<td>Cym. longifolium</td>
<td>Deka et al., 1997</td>
</tr>
<tr>
<td>5</td>
<td>Cattleya 'Almakee'</td>
<td>Vij et al., 1992</td>
</tr>
<tr>
<td>6</td>
<td>Cymbidium pendulum</td>
<td>Author</td>
</tr>
<tr>
<td>7</td>
<td>Dendrobium wardianum</td>
<td>Sharma et al., 1993</td>
</tr>
<tr>
<td>8</td>
<td>Dend. densiflorum</td>
<td>Vij et al., 1992; Vij and Kaur 1997a,</td>
</tr>
<tr>
<td>9</td>
<td>Gastrochilus calceolans</td>
<td>Author</td>
</tr>
<tr>
<td>10</td>
<td>Luisia trichoniza</td>
<td>Vij et al., 1992</td>
</tr>
<tr>
<td>11</td>
<td>Papilionanthe teres</td>
<td>Deka et al., 1997</td>
</tr>
<tr>
<td>12</td>
<td>Phaius tankervilae</td>
<td>Vij et al., 1992; Vij and Kaur, 1994b;</td>
</tr>
<tr>
<td>13</td>
<td>Phai. tankervilae</td>
<td>Deka et al., 1997</td>
</tr>
<tr>
<td>14</td>
<td>Renanthera imschootiana</td>
<td>Malmenganba, et al., 1996</td>
</tr>
<tr>
<td>15</td>
<td>Rhynchostylis retusa</td>
<td>Vij et al., 1992</td>
</tr>
<tr>
<td>16</td>
<td>Spathoglottis plicata</td>
<td>Singh, 1991</td>
</tr>
<tr>
<td>17</td>
<td>Thunia alba</td>
<td>Author</td>
</tr>
<tr>
<td>18</td>
<td>Vanda coerulea</td>
<td>Author</td>
</tr>
<tr>
<td>19</td>
<td>Vanda coerulea</td>
<td>Kadzmni, 1990</td>
</tr>
</tbody>
</table>
sodium alginate was effective at concentrations varying from 1.5-6.0% (Ahuja et al., 1989; Redenbaugh et al., 1987; Sakamoto et al., 1995), it appears that its optimum concentration varies with quality of the chemicals, culture conditions, and possibly genotypes as well. Low permeability of calcium alginate gel to oxygen, impair respiration activity in the encapsulated propagules and thus affects their viability. Sakamoto et al. (1995) developed novel self-breaking gels to overcome this limitation by using substances which interfere with the hardening reactions; the modification (partial replacement of calcium ions with magnesium and potassium ions) also improves the conversion frequency of synthetic “seeds”. Similar self-breaking “seeds” were prepared in the present study.

Incidentally, the conversion frequency of “synseeds” was observed to vary with the passage of time and conditions of their storage. While the freshly formed “seeds” germinated readily, they lost viability rapidly upon storage at ambient room temperature due probably to their high metabolic activity. However, when stored at 4°C, they maintained 100% viability for 2 months possibly due to a low metabolic rate at lower temperature, but their germination frequency decreased progressively with further increase in storage period. A similar decrease in convertibility was earlier reported by Vij et al. (1992), Sharma et al. (1992), Vij and Kaur, (1997a), and Nagaraj and Prakash (1997). Since, the hydrated regenerants are more difficult to store (Redenbaugh, 1990), the propagules were mildly dehydrated prior to their encapsulation as has also been suggested by Sanada et al. (1993).

Interestingly, as the encapsulated propagules were invariably observed to proliferate during germination, production of synthetic “seeds” is a novel technology simulating inherent polyembryonate potential of orchids seeds. Moreover, they provide a significant alternative to the perpetual maintenance of live materials for preservation of germplasm.

Further studies are suggested to develop a procedure for producing high quality and vigorous somatic “seeds” which can be stored for longer period of time.
Hardening and Acclimatization of the Micropropagants to the Greenhouse:

Plantlets *in vitro*, are continuously exposed to an environment that provides minimal stress and optimal growth conditions. These conditions include levels of light, aseptic conditions, and a medium containing simple sugars and nutrients. These all contribute to a phenotype, with poorly developed cuticle, stomatal apparatus, photosynthetic ability, and conducting tissues, that cannot survive a direct exposure to harsher climates outside the *in vitro* regimens until or unless they are properly acclimatized prior to transplanting (Vij, 1995). The procedure, known as hardening, helps cutting down their mortality rate during lab to land transfers by correcting their above mentioned morpho-physiological aberrations.

In the present trials, gradual removal of sugars, mineral salts, and vitamins, from the nutrient matrix proved useful in making the *in vitro* plantlets photoautotrophic. Elevation of CO$_2$ in the culture vessels, suggested earlier by Kozaki (1991), however, was not accomplished. After taking out the plantlets from the culture vessels, acclimatization under high relative humidity and low light, immediately after the plantlets were removed from the cultures, reduced the mortality rate considerably. However, as the new leaves and roots emerged, it was best to reduce the relative humidity gradually and increase the light level to that of the environment under which the plantlets are normally grown.