Discussion

Plant tissue culture techniques help in preservation and multiplication of desired genotypes through activation of resident or adventitious meristems in the explants. They are particularly useful in outbreeding taxa like Orchidaceae which generate a great deal of heterozygosity in the progenies and have added new dimensions to commercial cultivation of plant genetic resources. Identification of appropriate explants and growth stimulus are, however, important factors in the successful application of these techniques.

Presently, *in vitro* regeneration potential of 10 species and hybrids of epiphytic orchids with economically important and/or endangered genotypes, was tested using different explants. The results described in preceding pages, are discussed in light of available information in literature.

**Stem disc:**

Eversince Morel (1960) regenerated virus free *Cymbidium* clones using shoot apical meristem, the possibilities of using resident meristems (apical/axillary) for micropropagating orchids are being increasingly realised. Several species and hybrid commercialized through apical meristem (cf. Arditti and Ernst, 1993). *In vitro* culture of apical shoot meristem has, however, a limited utility in monopodial taxa, since their excision endangers the mother plant, periodic efforts have, therefore, been made to use the dormant (!, ) axillary shoot
meristems for the purpose, as a large number of explants can be obtained from the source plant. Axillary shoot meristem (uninodal stem explants) have been shown to regenerate in some sympodial [Arundina (Mitra, 1971), Dendrobium (Kim et al., 1970, Vij et al., 1994c), Vanilla (Duan and Hong, 1989)] and monopodial [Phalaenopsis (cf. Wang, 1989)] orchids.

Presently, uninodal explants were successfully used to regenerate Rhynchostylis retusa. Activation of resident and / or adventitious meristem was, however, markedly influenced by the quality of growth stimulus in the nutrient pool. BAP (1 mg/l) favoured a callus mediated regeneration from the resident meristem (in the nodal regions), whereas additional use of peptone (0.5 mg/l) proved useful in activating adventitious meristem in the adjoining internodal regions and direct development of PLBs therefrom. The efficacy of peptone was better expressed when the medium was either supplemented with AC / or an additional dose of calcium ions; nearly 80 PLBs were obtained through callus mediated development. In this connection it is worthwhile to mention that calcium ions are believed to favour the development of friable callus with a very high embryogenic competence (cf. Montora et al., 1993).

Pseudobulb:

The regeneration potential of pseudobulb segments was positively tested in Coelogyne cristata and C. ovalis. The juvenility of the tissues emerged as the major factor controlling cell proliferations, since the explants from well developed
pseudobulbs remained recalcitrant to regeneration and only those from freshly 
formed pseudobulbs reacted positively. The ability of the juvenile tissues to 
regenerate under controlled conditions has already been indicated in 
*Dendrobium chrysanthum* (Vij and Pathak, 1989).

In *C. ovalis* the explants responded to the basal medium whereas in *C. cristata* the response evocation was obligatory to a cytokinin treatment. Such a 
differential response may be attributed to their genetic and/ or source related 
physiological intricacies. In this connection, it is worthwhile to mention that *C. ovalis* explants were sourced from *in vitro* grown axenic cultures and *C. cristata* 
one from greenhouse grown plants. Incidentally, the activation of regeneration 
sites, in both the present taxa remained restricted to explants representing 
proximal and/ or distal segments of the pseudobulbs.

Like in pseudobulb derived cultures of *Dendrobium chrysanthum* (Vij and 
Pathak, 1989), the morphogenetic changes leading to plantlet formation, were 
also observed to vary with chemical stimulus in the present cultures. In *C. cristata*, a treatment with BAP/KN (10 mg/l), promoted direct development of 
shoot primordia. An additional treatment with NAA and AC in KN treated cultures 
proved ineffective but in BAP treated ones it favoured a switch in the 
regenerative pathway; the pathway was punctuated by a plb phase of 
development.
The regenerative pathway could be similarly modified in *C. ovalis* through a treatment with 1 mg/l either of BAP and KN. The treatment also proved useful in activating additional regenerative loci in the explants. Additional use of NAA countered Plb promotory effect of BAP/KN and regeneration occurred through direct development of shoot buds. The pathway was optimised by a combined use of BAP (3 mg/l), NAA (1 mg/l) as the chemical stimulus. Almost similar results were obtained by using 0.5 mg/l each of BAP and KN in medium bereft of NAA. Incidentally, NAA alone proved inhibitory to regeneration.

A perusal of literature reveals a positive micropropagation potential of pseudobulb segments in several orchid species and hybrids [*Arundina, Cattleya, Cymbidium, Miltonia, Phaius* (Morel, 1964, 1970); *Cymbidium* (Shimasaki and Uemoto, 1987); *Cattleya* (Vajrabhaya, 1978); *Dendrobium chrysanthum* (Vij and Pathak, 1989); *Dendrobium chrysanthum, Eria spicata and Pholidota articulata* (Pathak, 1989); *Thunia alba* (Arora, 1990); *Coelogyne rigida* and *C. viscosa* (Chand, 1991)]. In most of the earlier studies, KN alone or in combination with NAA proved useful in activating additional proliferative loci in the pseudobulb explants. In the present cultures a combination containing BAP and NAA was however, better used for the purpose.

Presently, the cytological status of pseudobulb derived plantlets could not be analysed but the reported chromosomal stability in *D. chrysanthum* plantlets.
raised from pseudobulb explants in vitro (Vij and Pathak, 1989) is significant and it suggests that the pseudobulbs can be effectively used.

In the recent years, orchid propagation through excised apical shoot meristems has gained popularity but the technique has a limited utility in monopodial taxa since it requires the sacrifice of the mother plant. With a view to find an equally effective explant whose exision is not detrimental to the survival of mother plant, organs other than apical shoot meristem (foliar, roots, and floral parts) was used as explants. The aim was to develop an efficient micropropagation system for each of the taxon.

**Foliar**:

The importance of foliar explants, as an effective alternative to shoot meristem, for micropropagating orchids is being increasingly realised. They are easy to obtain, do not require sacrifice of the mother plant, and offer exciting opportunities to raise a large number of true to type plants. The regenerative potential of foliar explants has so far been tested in nearly 60 orchid species and hybrids representing diverse habits, habitats and taxonomic groups (cf. Vij et al., 1994a).

The *in vitro* sourced explants regenerated frequently, whereas, those obtained from the greenhouse grown plants responded infrequently. In fact from the greenhouse sourced explants, only those belong to *Cattleya* cv. 'Almakee', *L. teretifolia*, *V. cristata* responded positively, the response was restricted to the explants from the unexpanded proximal parts of the youngest (juvenile) leaf as is also true in *Phalaenopsis* (Tanaka and Sakanishi, 1977, 1985). Such a differential response appears to be related to the extent with which the explants release autoinhibitory substances (Phenolics !) into the medium; the exudates were copiously released by the greenhouse source explants, and very poorly or none by the *in vitro* source explants. A poor regeneration capacity of the mature leaf segments was similarly attributed to their tendencies to turn brown and necrotic following the release of phenolic exudates in the medium (cf. Compton and Preece, 1986; Vij and Pathak, 1990).

In the present studies, juvenility of tissues emerged as the major factor controlling the activation of proliferative loci in the leaf explants. Since in the monocots, the cells in the proximal leaf segments retain their plasticity longer than in those with distal segments, the ability of very young leaves (<0.75 cm long) to proliferate all along the adaxial surface, and of somewhat older ones (>0.75 - 2cm long) to proliferate only along the basal region, is a point in favour of the above assumption. That the younger tissue with less rigid cell walls, are physiologically and biochemically more active, and show better morphogenetic potential has already been demonstrated in several plant groups including

The proliferative loci were invariably traced to the dermal (epidermis / hypodermis) cells. Their activation on both the abaxial and adaxial surfaces in *Luisia teretifolia*, *Rhynchostylis gigantea*, *R. retusa*, *Vanda cristata* and *V. teres*, is in accord with similar earlier findings in *Aranda Deborah* (Manorma et al., 1984), *L. teretifolia*, *R. retusa*, *V. cristata* and *V. teres* (cf. Vij and Pathak, 1990), and indicates that both the surfaces of bifacial leaves in these taxa are meristematically equipotential. However, the ability of only the adaxial surface to proliferate in *Vanda* hybrids (Mathews and Rao, 1985a; present study) may be related to hybridity of the genomes in this genus. Investigations in other hybrid vandas are suggested to test the validity of such a relationship.

Murashige (1974) opined that plant regeneration from cultures *in vitro* occurs frequently through adventitious shoot formation and rarely through somatic embryogenesis. Studies on *in vitro* proliferative potential of orchid leaves, however, reveal that they regenerate frequently through somatic embryogenesis (= Plbs) and less frequently through direct organogenesis. More frequently the plantlet formation follows either a Plb mediated development in *R. retusa* (Vij et al., 1984; Vij and Pathak, 1990), *V. testacea* (Vij et al., 1986), *Vanda* hybrid (Chaturvedi and Sharma, 1986) *Vanda coerulea* (Seeni; 1988) *Coelogyne speciosa* (Abdul Karim and Hairani, 1990)] or a callus-mediated
direct organogenesis [Epidendrum hybrid (Churchill et al., 1970, 1973); Laeliocattleya hybrid (Churchill et al., 1971, 1973)]. The embryogenetic (Plb) and organogenetic (through callusing) pathways of plantlet development appeared to be selectively influence by the chemical stimulus in Vanda hybrid (Mathews and Rao, 1985a) and Luisia trichorhiza, L. teretifolia, V. cristata, V. testacea (Vij and Pathak, 1990). Elimination of a callus phase and direct development of shoot buds in Neofinetia falcata, V. teres foliar explants (Vij and Pathak, 1990) may be a genetic attribute. In the present cultures, the plantlets were regenerated from the foliar explants through direct (Vanda Kasem's cv. Delight 'Tom Boykin') or callus mediated (Cattleya cv. 'Almakee', R. gigantea, V. teres) Plb development. Shoot buds were regenerated from the callused Plbs in Eria bambusifolia, R. retusa, whereas in L. teretifolia and V. cristata Plb development eluded the cultures and shoot buds were directly developed from callused cell proliferations depending upon the chemical stimulus in the nutrient pool.

The role of plant growth promotors (PGPS) in initiation and multiplication of meristemoids and regulation of their subsequent development into plantlet varies with the species in orchids (Vij et al., 1984, 1986, 1994q; Abdul Karim and Hairani, 1990), and the trend was confirmed in the present cultures. In this connection, a treatment with peptone was proved usefu. for initiating meristematic activity only in Cattleya cv. 'Almakee' and Vanda cv. Kasem's Delight 'Tom Boykin' explants, in accord with its efficacy in. R. retusa (Vij et al., 1984) and V. cristata and V. testacea (Vij and Pathak, 1990). According to Vij
and Pathak (1990), *V. teres* explants require a YE treatment for a positive response but in the present materials of the species, YE remained ineffective unless used with or replaced by a cytokinin (BAP). Such intraspecific variations in the requirements for PGPS may either be related with the intrinsic factors of the mother plants and/or the exact chemical composition of YE which is known to vary from batch to batch.

The PiIb (somatic embryo) production was significantly enhanced in most of the present cultures (Cattleya cv. 'Almakee', *E. bambusifolia*, *L. teretifolia*, *R. gigantea*, *Vanda* cv. Kasem's Delight 'Tom Boykin', *V. teres*) when subjected to a combined treatment with a cytokinin (BAP/KN) and an auxin (IAA/ NAA). In *R. gigantea*, the efficacy of NAA and BAP treatment was better expressed in the additional presence of AC; the PiIbs multiplied profusely through budding. The combination was similarly used advantageously in *Vanda* hybrid (Mathews and Rao, 1985a), *V. testacea* (Vij et al., 1986), *L. teretifolia*, *V. teres* and *R. retusa* (Vij and Pathak, 1990). In *R. retusa* cultures treated with 2 mg/l BAP and 1 mg/l NAA, shoot buds were directly developed but in the additional presence of AC their development was PiIb mediated. While AC was obligatory to shoot bud development in Cattleya cv. 'Almakee', a still higher dose of cytokinin (KN; 3 mg/l) was required in IAA (1 mg/l) enriched medium for similar results in *L. teretifolia*, indicating thereby that shoot bud initiation is markedly influenced by an appropriate balance between cytokinin and auxin in the nutrient pool and such a balance varies with the species. In Cattleya cv.
'Almakee', *E. bambusifolia*, *L. teretifolia*, *R. gigantea* and *V. teres*, the regenerants either remained arrested at the Plb stage of development or they took long to differentiate into plantlets, when auxins was eliminated from the combinations. On the other hand, regeneration eluded most of the cultures when cytokinis was eliminated instead. Such a differential behaviour may be explained on the basis of recent suggestions by Bellarosa *et al.* (1992) that Cytokinin helps in retaining the organisation of somatic embryos and the auxins in their differentiations.

In *V. cristata*, the explants failed to respond to a combined treatment of BAP (10 mg/l) and NAA / IAA (5 mg/l), unless the CuSO$_4$.5H$_2$O concentration was increased (2.2 mg/l) in the nutrient pool. A similar promotory role of higher doses of CuSO$_4$.5H$_2$O has already been recorded in several dicot and monocot plant (Purnhauser and Gyulai, 1993; Jumin, 1995). Since Cu$^{2+}$ is a component and/or factor of many important enzymes involved in electron transport, and protein and carbohydrate biosynthesis, Purnhauser and Gyulai (1993) attributed an important role to Cu enzymes in plant regeneration. In this connection, it is worthwhile to mention that zygograms of certain enzymes are considered as useful markers of morphogenesis in tissue raised cultures (cf. Purnhauser and Gyulai, 1993).

As already mentioned, the origin of the regenerants in the present cultures was invariably traced to the dermal (epidermal, hypodermal) cells. Since
the dermal cell are cytologically more stable, they are expected to generate cytologically uniform progeny (cf. Vij and Pathak, 1990), while the cytological status of the regenerants, could not be evaluated in the present effort, the phenotypic uniformity in the leaf regenerants was significant in *E. bambusifclia*, *L. teretifolia*, *R. gigantea*, *R. retusa*, *Vanda* cv. Kasem’s Delight ‘Tom Boykin’ and *V. teres* and it may be reflection of their cytological stability. That the leaf dermal plants are true to type was also apparent in *L. teretifolia* and *R. retusa* where uniformity was tested upto the flowering level. Earlier, phenotypic uniformity was observed in leaf derived plants of *Doritis pulcherrima* and several species and hybrids of *Phalaenopsis* (Tanaka, 1987).

**Foliar peel:**

Since the foliar explants invariably proliferate in their dermal cells (cf. Vij and Pathak, 1990; Vij et al, 1994q; present studies), an attempt was made to test the *in vitro* regenerative potential of foliar peels in several of the present taxa. The efforts proved successful in Cattleya cv. ‘Almakee’ and *Vanda* cv. ‘Kasem’s Delight ‘Tom Boykin’. An appropriate chemical stimulus, the physiological age of the donor leaf and the thickness of the peels, however, emerged as important factors controlling the proliferations.

Only the peels from juvenile leaves responded; the response was spread all along the adaxial surface in the younger peels (<0.75 cm long) and it was
restricted to the proximal parts of the relatively older peels (0.75 - 2 cm long) and due probably to the plasticity of cells in the younger tissues.

In the present studies, the uniseriate peels consisting only of epidermal cells did not respond and perished within 3 wks, but those with a pad of 2-3 underlying cell layers proliferated, indicating thereby the significance of subepidermal parenchyma in activating the proliferative loci in the epidermal cells. A similar nurse-mother role of the subepidermal parenchyma was earlier suggested in dermal cultures of *Daucus carota* (Kato, 1968), *Nautocalyx lynchei* (Tran Thanh Van, 1973a), *Nicotiana tabacum* (Tran Thanh Van, 1973b; Tran Thanh Van et al., 1974; Tran Thanh Van and Trinh, 1978), *Rhynchostylis retusa* and *Vanda coerulea* (Vij, 1994a).

The role of growth adjuncts in activation and multiplication of meristemoids in foliar peel cultures (cf. Vij, 1994a) was also apparent in the present investigation. The responsive peels (with a pad of 2-3 hypodermal cell layers) failed to develop neoformations unless treated with plant growth regulators (PGRs). The efficacy of the treatment was, however, hybrid specific.

Regeneration through direct development of Plbs in *Cattleya* cv. 'Almakee' dermal explants was obligatory to cytokinin treatment. While KN (0.5 mg/l) was effective individually; the efficacy of BAP at similar concentration was NAA (0.5 mg/l) dependent. The proliferative response was, however, suppressed in the additional presence of AC, unless the PGRs were used at
higher concentrations. In AC rich combination containing 1 mg/l each of KN and NAA, a callus mediated Plb development accounted for a high frequency of regeneration; nearly 60 plantlets could be harvested within 12 wks from each of the responding explant.

In Vanda cv. Kasem' Delight 'Tom Boykin' on the other hand, the regenerative loci could be activated by treating the explants with either of auxin (IAA/IBA) or cytokinin (KN). A treatment with KN (0.25 - 0.5 mg/l) yielded better results; nearly 25 plantlets/ explant could be obtained within 8-9 wks. The response frequency was somewhat impaired in the additional presence of IAA/IBA and it was completely suppressed when NAA/AC were used instead. These results conform to the earlier suggestions that the dermal peels are low in endogenous growth hormones due to reduced amount of parent tissues and are thus expected to be more sensitive to even the slightest changes including the quality and quantity of growth stimulus in the nutrient pool. Incidentally, IBA favoured callusing in Nicotiana tabacum (Tran Thanh Van et al., 1974) and Rhynchostylis retusa (Vij, 1994a) dermal peels, but in the present cultures of Vanda cv. 'Kasem' Delight 'Tom Boykin' it proved promotory to direct development of Plbs. Both these sets of observations would suggest that the effect of growth stimulants on the regenerative pathway (direct/ or callus mediated Plb development) is influenced by the genetic constitution of the mother plant.
Root:

Beechey (1970) suggested the possibility of using aerial roots for micropropagating orchids. Such possibility gained currency through the reported ability of orchid roots to spontaneously regenerate by callus/shoot primordia in some naturally grown species (Churchill et al., 1972). A limited morphogenetic ability of the root meristems of higher plants including orchids (cf. Kerbauy, 1991), notwithstanding, the utility of roots as explant source is being increasingly realised due to their round the year availability, low oxidation rate, and the ease with which they can be explanted. Incidentally, the regeneration potential of root explants has been positively tested in nearly 28 orchid species and hybrids and these include the present reports as well.

The explant from, well developed roots (from plant grown outdoors) exuded profusely and perished without showing morphogenetic changes. Those from the axenic culture in vitro behaved differentially depending upon the level of maturity of their tissues. The explants with intact and well developed root cap showed an extended root growth regardless of the chemical treatment in the nutrient pool; the growth was more pronounced in Cattleya cv. 'Almakee', Rhynchostylis gigantea and Vanda teres cultures. All efforts to induce Plb and/or shoot development in them failed. The explants from juvenile roots (<2cm long) with ill developed root cap, on the other hand, could be made to regenerate under certain selective PGP treatments; the efficacy of PGPs,
however, varied with the species/hybrids. These results are in accord with similar earlier reports in orchid literature (cf. Vij, 1994b) and are indicative of the importance of source and physiological age of root explants, and the chemical stimulus in guiding the development of neoformations.

A perusal of literature reveals that root growth is markedly influenced by the endogenous level of IAA which changes from sub-optimal to supra-optimal with age (cf. Philip and Nainar, 1988; Vij, 1994b). A supra-optimal level maintains root meristem whereas its sub-optimal level (brought about by excision of roots and degeneration of root caps) leads to a switch in the activities of the meristematic cells. Presently, the endogenous level of IAA could not be analysed in the root explants, but the regeneration potential of juvenile roots with ill-defined root caps and a recalcitrant behaviour of the mature ones with well-developed root caps seems to suggest the above contention since the root cap is an active site of IAA accumulation. However, as in Vanda cv. Kasem’s Delight ‘Tom Boykin’ juvenile one’s required an exogenous supply of IAA (1 mg/l) to induce neoformations (callus), it seems safe to assume that the supra-optimal level of IAA for maintaining root meristem varies with species/hybrids in orchids.

In the present studies, the root segments reacted positively depending upon the quality and quantity of cytokinins (BAP/KN) and the intrinsic factors of the mother plant. KN (1 mg/l) induced direct/callus mediated Plb generation in 25% explants each in Rhychostylis gigantea, R. retusa, Vanda cv. Kasem’s
Delight 'Tom Boykin' and *V. (=Papilionanthe) teres*. In *Cattleya* cv. 'Almakee', *R. gigantea*, *R. retusa* and *V. teres* the benign effect of KN was accentuated in the additional presence of NAA (1 mg/l), in accord with similar earlier reports in *R. retusa* (Sood and Vij, 1986; Vij, 1994b), *Cattleya* hybrid (Kerbauy, 1991). Replacement of NAA with IAA in the combination, however, proved less productive in *R. retusa* and *V. teres* cultures. BAP (1 mg/l) on the other hand, often proved inhibitory to organogenesis, unless used with NAA (1 mg/l). A similar BAP related root autonomy was reported in *Oncidium varicosum* (Kerbauy, 1984b), but a high organogenetic potential of *Catasetum* (hybrid) roots in a BAP regime suggests that BAP related root autonomy varies with the species in orchids. While the endogenous level of cytokinin could not be measured, the observation in *Cattleya* cv. 'Almakee', *R. gigantea*, *R. retusa* and *V. teres* root cultures are interesting since an exogenous supply of BAP (3 mg/l) favoured direct or callus mediated organogenesis in NAA supplemented medium due probably to a synergistic action between NAA and BAP. Incorporation of peptone (2 g/l) in the latter combination proved highly beneficial in *Vanda* cv. 'Kasem' s cultures; the number of plbs produced per explant was significantly enhanced. Peptone was also usefully employed in root cultures of *R. retusa* (Sood and Vij, 1986); *Vanda cristata* and *V. testacea* (Vij, 1994b). The dark green colour of the explants and regenerants in BAP supplemented media is in accord with similar earlier report in several plants including orchids (cf. Kerbauy, 1984b) and it seems to suggest the effect of cytokinins on chloroplast
development as has already been indicated by Stetler and Laetsch (1965). Further experiments are, however, suggested to assess the appropriate physiological age and nutritional requirements of orchid roots so these can be profitably used as alternative but equally effective explant to shoot meristem.

Floral organ Culture:

Ito (1961) tested the developmental potential of excised orchid ovaries whereas, Israel (1963), successfully cultured orchid flower in vitro, 1-6 wks after pollination on media with various concentrations of NAA. While Lim-Ho et al., (1984) cultured intact floral buds of Mokara and Spathoglottis in vitro and induced plb development therein. Kim and Kako (1984), cultured floral parts of a Cymbidium hybrid with a view to investigating the possibility of using floral organs as a source of explants for micropropagation purposes; the explants from column, ovary and flower stalk responded better than those from sepals, petals and lip. However, Suryowinoto and Sumaryo (1985) regenerate pollen tetrads of a Dendrobium hybrid.

Encouraged by the above possibilities, the regenerative potential of ovary (Coelogynae cristata), Perianth (C. ovalis, Vanda cristata) and Anther (Rhynchostylis relusa, Vanda cristata) was presently assessed. The proliferative potential was found to be obligatory to the use of an appropriate chemical stimulus, besides the proper staging of the explants.
Ovary:

A pollination stimulus appeared to be essential for invoking development and/or cell proliferations in the excised ovaries, since the unpollinated ovaries failed to respond and the pollinated ones responded to cell proliferations. A pollination stimulus related auxin stimulus is believed to activate organized ovule development in orchids. While the initial increase in the size of cultured ovaries in the present cultures could be related to above mentioned stimulus, the inability of ovules to develop may be attributed to self incompatibility problems. Nevertheless, the ovaries callused along their excised end when subjected to BAP (10 mg/l) and Peptone (2 g/l) treatment in the nutrient pool (MS medium). The callus was compact and chlorophyllous but its regeneration competence remained dormant unless it was segmented and subcultured in BAP and NAA supplemented medium (BM) under a low sucrose (0.5%) regime; several rhizogenic shoots could be obtained. Perusal of literature reveals a selective efficacy of BAP and NAA in invoking regeneration response in excised ovaries in vitro (Gray and Mortsen, 1987; Ichihashi and Kato, 1986). The ability of callus to organogenate under a low sucrose environment, in the present studies, hints at a negative correlation between the osmotic pressure of the medium and the organogenetic changes as has already been suggested by Ichihashi et al., 1986.
Perianth:

Regenerative competence of various floral appendages [sepal, petal] (Coelogyne ovalis, Vanda cristata) was tested at different stages of development.

The sepals invariably failed to respond whereas, from among the petals only the labellum responded and that too from buds 14 days prior to anthesis. Since the labellum with its relatively thicker and less delicate tissues is expected to better withstand the Sodium hypochlorite (surface sterilizing agent) induced cell injuries than the sepals and other petals. The studies suggest that besides proper staging, surface sterilization protocols also need to be perfected for invoking regeneration response in all the other perianth segments.

Ethylene is inhibitory to regeneration (Purnhauser et al., 1987; Chi et al., 1990 Chraibi et al., 1991) and it enhanced production in the flower from anthesis onwards (Zuping and Wei, 1994) is another factor favouring regenerative competent nature of juvenile tissues.

In the present taxa (Coelogyne ovalis, Vanda cristata) the cell proliferations in labellum explants were obligatory to a combined treatment with 5 mg/l each of cytokinin and an auxin, in accord with a similar BAP and NAA requirement for initiating cultures from floral appendages in a Cymbidium hybrid (Kim and Kako, 1984). While, in the present studies the cytokinins (BAP/ KN)
were more effective when used in a dose double than that of NAA, their efficacy varied with the species; KN was effective in *C. ovalis* cultures and BAP in those of *V. cristata*. Incidentally, the responding explants assumed an unevenly thickened appearance with the new growth invariably remaining confined within the epidermal limits suggesting thereby that only the inner tissues responded to cell proliferations. Differentiation eluded the neoformations unless removed from cytokinin influence and treated with 0.5 mg/l NAA under a low osmoticum in a nutrient pool.

**Anther:**

A perusal of literature reveals that studies related to embryogenic competence of anthers have been few and far between in orchids. Infact, prior to the present studies *Dendrobium 'Tomie white*', represents the only taxon where the totipotency of pollen tetrads was realized *in vitro* (Suryowinoto and Sumaryo, 1985). Based on a definite relationship between the size of the floral buds (in relation to that of bracts) and the stage of pollen development, Vij *et al.*, (1994b) suggested the importance of floral bud size in selecting responsive anthers for the purpose. Presently, the regenerative potential of excised anthers was positively tested in *Rhynchostylis retusa* and *Vanda cristata*. While in the former species, the responsive anthers were obtained from 1.25 - 1.35 cm long floral buds, in *V. cristata* these were obtained from those measuring between 1.50 - 1.65 cm. Suryowinoto and Sumaryo (1985) also obtained embryogenic anthers.
from young floral buds. While the authors (Suryowinoto and Sumaryo) failed to note the exact cytological stage of responsive microspores, late-uninucleate stage of microspores development was observed to be most appropriate for initiating cultures in the present materials. Since explants lacking an operculum invariably fail to regenerate (Suryowinoto and Sumaryo, 1985; present studies), the importance of operculum in guiding cell proliferations in anther explants is more than apparent.

Coconut water in Vacin and Went (1949, VW) medium was obligatory to regeneration in Dendrobium 'Tomie White', anther explants (Suryowinoto and Sumaryo, 1985), whereas in the present cultures, BAP (1 mg/l) in BM medium was effective singly (Vanda cristata) or in combination with Peptone (2g/l) in (Rhynchostylis retusa) to invoke cell proliferations in accord with similar earlier findings in these and some other orchid species (Vij et al., 1994b).

While the morphogenetic changes leading to plantlet development were not clearly described in Dendrobium 'Tomie White' (Suryowinoto and Sumaryo, 1985), these were invariably callus mediated in the present cultures. The callus was somewhat compact and achlorophyllous, it remained non-organogenetic in V. cristata but in R. retusa its embryogenic potential was obligatory to a subculture in KN (10 mg/l) and NAA (1 mg/l) supplemented medium. Upto 2 Plbs were obtained from each of the responding explant with 12 wks.
Incidentally, cytological status of the regenerants could not be studied, as the cultures got contaminated during the subculturing.

**Multiplication of Cultures:**

Eversince, Morel (1964) demonstrated the ability of protocorm slices to regenerate and suggested that up to $4 \times 10^6$ plants can be obtained within a year through repeated segmentation and culture of daughter P1bs. Protocorms/P1bs have been successfully used to mass multiply *in vitro* cultures of several orchid species and hybrids (cf. Mathews and Rao, 1985b; Amaki and Haruzo, 1989; Lam *et al.*, 1991; Kanase and Takano, 1995). Even the intact P1bs are capable of generating daughter P1bs directly through budding or indirectly through callusing (Vij *et al.*, 1981).

Since from amongst the present taxa *Coelogyne cristata* and *Eria bambusifolia* regenerated poorly and yielded only a limited number of regenerants (P1bs), the aforesaid proliferative potential of P1bs segments was successfully exploited to multiply their cultures.

The regeneration, in the present cultures, was invariably callus mediated; initiation, growth, and embryogenecity of the callus, however, varied with the species and it was markedly influenced by the chemical stimulus in the nutrient pool. *C. cristata* explants responded to the basal medium, whereas for those of *E. bambusifolia* a treatment with KN and/or Apeptone was obligatory for cell
proliferations. Incidentally, in the former species (C. cristata), an additional treatment with cytokinins (BAP/KN : 1 mg/l) yielded better results. BAP proved more effective (upto 38 Plbs could be obtained from each of the responding explants). Almost an identical number of regenerants (Plbs) could be obtained in the latter species (E. bambusifolia) as well, but by using 1mg/l IAA in the initiation medium (BM + KN). While, in most of the earlier studies, a callus mediated regeneration of multiple Plbs has been similarly recorded (cf. Fonnesbech, 1972; Morel, 1974; Mathews and Rao, 1985b), Plural Plbs have been directly obtained from auxin and cytokinin treated Plb segments in Cymbidium (cf. Fonnesbech, 1972) and Vanda (Mathews and Rao, 1985b) cultures. A treatment with 1 mg/l each of BAP and IAA/NAA is known to favour direct development of Plbs, whereas that with an increased dose of cytokinin, in auxins rich medium, stimulates callus punctuated Plb multiplication (Mathews and Rao, 1985b).

Based on their observation of direct development of Plbs in the apical segments and through callusing in the basal segments, Amaki and Haruzo (1989), considered the importance of explant source (position in the donor Protocorm Plb), in guiding the developmental pathway of the regenerants. Such a site specific regenerative pathway, however, eluded the explants in the present study, where the regeneration was invariably punctuated by a callus phase. In this connection, it is worthwhile to mention that presently, the explants were obtained only from very young Plbs.
In the present cultures, regenerations were invariably traced to the dermal cells in accord with similar earlier findings in *Cattleya* and *Cymbidium* Plb segment culture (cf. Morel, 1974; Kusumoto, 1984; Kanase and Takano, 1995). Earlier Vij and Pathak (1990), highlighted the cytological and phenotypic stability of dermal cell derived regenerants in several orchid species.

Regeneration of daughter Plbs from Plb segments within 2 and 1 wk(s) respectively in *C. cristata* and *E. bambusifolia*, is significant and it suggests the possibility of obtaining nearly $1.5 \times 10^6$ plantlets (within 6 months) through repeated segmentation and regeneration cycles, provided the health status of the cultures is properly managed. However, in the present experiments, browning of the tissues was frequently encountered (particularly in the liquid culture) and it impaired their proliferative potential. Almost similar problems of browning of tissues were encountered in *Cymbidium* (Wimber, 1963; Kusumoto, 1980a,b) and *Vanda* (Mathews and Rao, 1985) cultures. Further, studies are therefore, suggested to overcome the tissue senescence in later cycles of regeneration.

**Synthetic 'seeds':**

The possibility of preparing artificial 'seeds' by encapsulating embryos/meristem in a nutrient gel, first suggested by Murashige (1978), has been realized in several species of flowering plants including orchids.
Prior to the present efforts in *Coelogyne ovalis*, *Luisia teretifolia*, *Rhynchostylis gigantea*, *Vanda* cv. Kasem’s Delight ‘Tom Boykin’ and *V. teres*, synthetic ‘seeds’ were prepared in nearly a dozen of orchid species (cf. Vij et al., 1992). Such ‘seeds’ are easy to handle, and they ensure economy of space, medium and time during storage and lab to land transfer and transport of tissue culture raised genotypes. The utility of gelling agents, agar, polyox, alginate, agarose, carrageenan, polyacrylamide, nitrocellulose and ethylcellulose has been tested for encapsulation purposes, but Sodium alginate, due probably to its solubility at room temperature, non toxicity, low cost, and ability to form a completely permeable gel with CaCl$_2$.2H$_2$O, has proved most useful (cf. Wadhawa et al., 1989).

Presently, embryoids (Plbs) were used for encapsulation in Calcium alginate enriched (Sodium alginate + CaCl$_2$.2H$_2$O) nutrient matrix in accord with similar earlier attempts in *Vanda hookeriana* (Kadzimin, 1990), *Spathoglottis plicata* (Singh, 1991); *Dendrobium densiflorum* and *Phaius tankervillae* (Vij and Kaur, 1994). Sucrose (2%), NAA and either of BAP and KN (0.5 - 10 mg/l) were also supplemented in the matrix. The shape, size and firmness of the ‘seeds’ was directly related to concentrations of Sodium alginate and CaCl$_2$.2H$_2$O and subsequent complexation period. They were best formed by using 4% Sodium alginate and 100 mM CaCl$_2$.2H$_2$O allowed to complexate by subjecting them to complexation period of 40 minute in CaCl$_2$.2H$_2$O solution. Since in earlier studies, Sodium alginate and CaCl$_2$.2H$_2$O were used at 2.5% and 100 mM
respectively, it appears that the required concentrations may vary with quality of the chemicals, culture conditions and possible genotype as well. Incidentally, the conversion frequency of such 'seeds' was observed to vary with the passage of time and conditions of their storage and it was markedly influenced by the nature of the sowing substratum and intrinsic factors of the propagules. It was possible to achieve 30-50% conversion frequency after storage for 90 days at 4°C due probably to a low metabolic rate at lower temperature. The problems of susceptibility of seeds to bacterial and fungal contaminations (due to high nutritional status) under glass house or field conditions were minimised by using 100 mg/l each of Bavistin/ or Benomyl and Streptomycin in the nutrient matrix, but with a reduced conversion frequency. A similar reduction in conversion frequency in 'seeds' treated with antimicrobial agents is known in several species (cf. Redenbaugh et al., 1987; Ahuja et al., 1989; Bapat and Rao, 1990).

Since the encapsulated propagules invariably proliferate during germination, the production of synthetic 'seeds' is a novel technology representing natural extension of the somatic polyembryonic process. Moreover, they provide a significant alternative to the perpetual maintenance of live materials for preservation of the germplasm.

Perusal of literature reveals that the alginate capsules are difficult to store for long as they dry out quickly, thereby impairing the respiration process of the encapsulated propagules, unless kept in a humid environment and/ or coated
with a hydrophobic membrane. In this connection, presently the importance of powder talc coating is increasingly realised in the quickly drying alginate capsules. Consequently, protocols need to be developed for producing high quality and vigorous somatic seeds which can be stored for longer periods of time.