DISCUSSION
5. DISCUSSION

Presently there is a great awareness on the need to conserve natural plant resources world over. Studies undertaken during the last five decades on floras in several parts of the world have shown that many plant species are in danger of extinction while some have become extinct recently. Orchids are one of the very natural distinctive and highly advanced group of flowering plants exhibiting an incredible range of diversity in size, shape, structure, colour and number of flowers. The natural beauty of the orchids has made many of these plants vulnerable to extinction, because of destruction of natural forests and over collection of plants. Many of the orchids have become endangered and have entered the Red data book. Therefore, it is essential to conserve these valuable group of plants. One of the most desirable actions for safeguarding endangered/rare plants is to improve in any possible way the propagation techniques. During the last few years in vitro culture has emerged as a powerful tool for the effective propagation of many plant species. The reintroduction or restocking of orchid population in the wild has attempted on several occasions, with varying degree of success (Haeggstrom, 1992; Stewart, 1993; Warren and Miller, 1994; Mekendrick, 1995; Ramsay et al., 1994; Rubluo et al., 1989; Rubluo et al., 1993).

The Western Ghats (South India) having many useful wild species of high economic potential represent a great treasure of our country in their biological diversity. Despite of these facts, the hill tract stands ravaged, its wealth plundered, its regeneration capacity irreversibly
damaged in many places due to neglect in protecting/over exploitation. Nearly 410 species of flowering plants have been reported as endangered in Western Ghats (Nair, 1984) out of which 235 species are endemic. Keeping this in view the present work was undertaken to develop efficient methods of axenic seed propagation and/or micropropagation of four endemic species *Aerides crispum*, *A. maculosum*, *A. ringens*, *Luisia macrantha* and four popular wild species *Dendrobium barbatulum*, *D. crepidatum*, *D. macrostachyum* and *D. ovatum*.

5.1 Asymbiotic seed germination and seedling development:

Initiation of seed germination, protocorm formation and subsequent development of seedlings varied with species and medium employed. *Aerides maculosum* – VW medium proved to be the best for seed germination and 60% of seeds germinated in this medium (Table 3). Growth adjuncts CW (15%), YE (2.0 g/l), P (2.0 g/l), NA (0.5 mg/l) and GA3 (0.01 mg/l) have influenced seed germination and conversion of protocorms into seedlings (Table 4). VW medium with CW (15%) has yielded best results in which 95% of seeds transformed into protocorms and these protocorms developed leaves roots and reached seedling stage in 20 wks. *Aerides crispum* – optimum seed germination was observed in VW medium (Table 6). Addition of growth adjuncts (CW, CJ, YE and CH) have selectively accentuated the germination frequency (Table 7). Conversion of protocorms into seedlings and growth of seedlings was best in the VW medium with CW (10%) and CJ (10%). *Aerides ringens* – In KC medium 55% of seeds germinated to form protocorms in 4-5 wks (Table 8). These protocorms formed seedlings in 30-32 wks.
Addition of growth adjuncts CW (15%), CJ (10%), YE (1.0 g/l), CH (2.0 g/l) and P (2.0 g/l) have enhanced the process of germination and seedling growth (Table 9). *Luisia macrantha* - Embryos responded best in the KC medium and 45% of them germinated to form protocorms (Table 10). The germination frequency and development of shoots, leaves and roots from protocorms were enhanced when CW (10%), CJ (10%), YE (1.0 g/l), P (1.0 g/l) and GA₃ (0.001 mg/l) were supplemented in the KC medium (Table 11). *Dendrobium barbatulum* - KC medium was proved to be the best for seed germination and seedling growth of *D. barbatulum* (Table 12). Addition of growth adjuncts like CW (15%), CJ (15%), YE (2.0 g/l), CH (1.0 g/l), NAA (1.0 mg/l), KN (1.0 mg/l) and combination of growth adjuncts was most beneficial in inducing seed germination and seedling development (Table 13). KC medium with CW (15%) and combination of growth adjuncts CW (10%)+CJ (10%)+YE (200 mg/l)+P (200 mg/l)+NAA (0.5 mg/l) have induced multiplication of PLBs. *Dendrobium crepidatum* - Embryos of *D. crepidatum* responded very well to VW medium and 55% of them germinated to form protocorms (Table 14). The germination frequency and growth of protocorms was enhanced with addition of CW (15%), CJ (15%), YE (1.0 g/l), CH (1.0 g/l), P (2.0 g/l), NAA (0.5 mg/l) and KN (1.0 mg/l) (Table 15). *Dendrobium macrostachyum* - In KC medium 60% of seeds germinated to develop protocorms and protocorms differentiated to form seedlings (Table 16). Addition of growth adjuncts CW (15%), CJ (15%), P (2.0 g/l), YE (2.0 g/l), CH (2.0 g/l), NAA (1.0 mg/l), NAA+KN (0.5+0.5 mg/l) and combination of growth adjuncts CW (10%)+CJ (10%)+YE (200 mg/l)+P(200 mg/l)+NAA (0.5 mg/l) have enhanced seed germination and seedlings growth (Table 17). Best results
were on KC medium with CW (15%), wherein 90% of protocorms transformed into seedlings. *Dendrobium ovatum* – The embryos of *D. ovatum* responded better in KC medium and 61% of embryos germinated to form protocorms (Table 20). In order to invoke the germination frequency, further development and differentiation of protocorms into seedlings, various growth adjuncts were added to the medium. CW (15%), CJ (15%), YE (2.0 g/l), CH (2.0 g/l), P (2.0 g/l) and combination of CW (10%)+CJ (10%)+YE (200mg/l)+CH (200mg/l)+P (200mg/l)+NAA (0.5 mg/l) have favoured seed germination and seedling development (Table 21). MPLBs have formed in KC medium supplemented with CW (15%), NAA (1.0 mg/l) and combination of growth adjuncts.

The above results reveal that, KC and VW media have yielded excellent results in invoking embryo germination and seedling development of *Aerides, Luisia* and *Dendrobium* species. MS and N3f media however, have not influenced these processes. Many media, such as Pfeffer (Downie, 1940; Downie, 1943; Downie, 1949; Hadley and Harvis, 1968), Knudson’s C (Ernst, 1975; Hijner and Arditti, 1973; McIntyre et al., 1972; Stoutamire, 1963; Stoutamire, 1964), Thomale GD (Ernst, 1975), Heller (Pierik and Steegmans, 1972), Burgeff’s N3f (Reyburn, 1978; Stoutamire, 1963; Stoutamire, 1964) have been used for the axenic culture of terrestrial and epiphytic orchid seeds. These results have shown that depending on the orchid species to be germinated, the *in vitro* culture medium can be of extreme importance (Arditti and Ernst, 1984). The Knudson B or C culture media, or slight modifications of
either, have extensively used for orchid seed germination and seedling growth (Arditti, 1967).

Additional use of growth adjuncts in the media, selectively accentuated the germination frequency (Table 4, 7, 9, 11, 13, 15, 17 and 21) in accord with similar utility in orchid cultures (Arditti, 1982; Arditti and Ernst, 1984). The addition of CW and CJ invariably enhanced seed germination process, conversion of protocorms into seedlings and seedling development. CW not only enhanced the extent of protocorm proliferation but also consistently induced the formation of longer seedlings. Similarly, the enhancing effect of CW on orchid seed germination, protocorm multiplication had been studied by various Orchidologist (Vacin and Went, 1949; Kusumoto, 1980; Kusumoto and Furakawa, 1977; Kerbauy and Handro, 1981; Chung et al., 1985). CW though inhibitory during germination, it stimulates seedling growth in Dendrobium (Kotomori and Murashige, 1965). Withner (1959), Sheehan (1983), Singh and Prakash (1985), Mathews and Rao (1980) found enhanced effect of CW on seedling growth.

Addition of BE significantly reduced germination of Aerides, Luisia and Dendrobium species (Table 4, 7, 9, 11, 13, 15, 17 and 21). Similar inhibitory effect of BE on germination has been observed earlier in Paphiopedilum (Fast, 1971; Pierik et al., 1988). However, Ernst (1980) advised the addition of banana homogenate to the media for germination of Paphiopedilum.
In our experiments, addition of YE (upto 2.0 g/l) had beneficial effect on germination of seeds and further proliferation of PLBs and such effect was also observed in *Vanda* hybrids (Mathews and Rao, 1980).

Utilization of nitrogen compounds by orchids differs from species to species even in one genus (Arditti, 1979). Growth of a *Cymbidium* hybrids and *Epidendrum* species is stimulated by amino acids (Vacin and Went, 1949), but growth of *Cattleya amethystoglossa*, *C. molli*, *C. trianae* and *Epidendrum cochleatum* inhibited by amino acids added to nutrient media (Spoerl, 1948; Spoerl and Curtis, 1948; Raghavan and Torrey, 1964). Addition of CH (upto 2.0 g/l) have enhanced germination of embryos, proliferation of PLBs and enhanced seedling growth in the present study. Rubluo et al., (1989) have showed beneficial effect of CH during germination of *Bletia urbana*.

Peptone has activated germination process and seedling development in *Aerides*, *Luisia* and *Dendrobium* species. Similar stimulatory effect was shown by Fast (1971), Flamee (1978) and Thomale (1957).

The addition of vitamin Nicotonic acid has increased the germination process of embryos and proliferation of protocorms in *Aerides maculosum* and *Dendrobium ovatum*, but no enhancement in germination percentage and seedling development was observed in *Aerides maculosum*, *Dendrobium crepidatum* and *D. macrostachyum*. Likewise, the effect of vitamins during orchid seed germination, varies from beneficial (Hegarty, 1955; Arditti, 1963; Muralidhar and Mehta,
Among growth regulators tested (Tables 7,9,11,13,15,17 and 21), NAA has induced germination process and multiplication of PLBs in *Dendrobium barbatulum*, *D. macrostachyum*, *D. crepidatum* and *D. ovatum*, however, higher concentration was not beneficial. NAA showed deleterious effect on germination of *Aerides maculosum*, *A. crispum*, *A. ringens* and *Luisia macrantha*. Similarly Devi et al., 1990; Chung and Chun, 1983; Chung et al., 1985 have reported beneficial effect of NAA on leaf and root development. However, Nath et al., 1991; Vij et al., 1981 showed the deleterious effect of increased level of NAA on growth and differentiation of protocorms. The use of lower concentration of KN in the basal medium have enhanced seed germination and seedling development of *Dendrobium barbatulum*, *D. crepidatum* and *D. ovatum*. However, it showed negative effect in *Aerides maculosum*, *A. crispum*, *A. ringens*, *Luisia macrantha* and *Dendrobium macrostachyum*. These results are in accord with the observations of Rucker, 1974 and Fonnesbech, 1972.

Addition of GA$_3$ to the basal medium was beneficial in enhancing seed germination, growth of protocorms and seedlings in *Aerides maculosum*, *Dendrobium ovatum* and *Luisia macrantha*. On the other hand it has not influenced these processes in *Aerides crispum*, *A. ringens*, *Dendrobium barbatulum*, *D. crepidatum* and *D. macrostachyum*. In tissue culture along with many reports on the positive action of GA (Kusumoto, 1978 and Smith, 1968) and lack of response is also well
documented in literature (Singh et al., 1974 and Mathews and Rao, 1985).

The above findings have clearly demonstrated that the seeds of wild orchid species could be successfully germinated in vitro on a nutrient medium. Transplantable seedlings were obtained between 6 and 8 months after initial culture. The methods have therefore immense potential for direct use in the mass multiplication of wild species. Similarly, the asymbiotic germination potential of seeds, representing different developmental stages has been positively tested in several commercially viable and/or threatened Indian taxa (Bopaiah and Jorapur, 1986; Bose and Mukarjee, 1976; Chennaveeraiah and Patil, 1975; Hegde, 1990; Hegde et al., 1988, 1989; Krishnamohan and Jorapur, 1984, 1986; Muralidhar and Mehta, 1986; Nath et al., 1991; Pathak, et al., 1992; Prasad and Mitra, 1975; Rao, et al., 1998; Sharma and Tandon, 1986, 1987, 1990; Vij and Arora, 1988; Vij and Pathak, 1998a, 1988b and Vij et al., 1981, 1988).

5.2 Foliar explant cultures of Aerides maculosum:

The importance of foliar explants as an effective alternative to shoot meristems, for micropropagating orchids is being increasingly realized. They are easy to obtain, do not require the sacrifice of mother plant and offer exciting opportunities to raise large number of true to type of plants. Their regenerative potential has so far been successfully tested in many species and hybrids representing diverse taxonomic affinities, habits and habitats (Vij and Pathak, 1990). Presently, foliar explants
were successfully used for micropopagation of *Aerides maculosum* in *vitro*. The explants from mature leaves did not respond to any nutritional conditions employed. However, the juvenile explants responded well to the medium supplemented with NAA (0.5 mg/l), KN (1.0 mg/l), BAP (2.0 mg/l) and CW (15%) (Table 5) and developed PLBs from the basal end of the explants in 2-4 wks. On subculturing, these PLBs developed shoots and roots in 8-12 wks later.

The explants from mature leaves did not respond to any culture medium, while proliferation occurred in those from juvenile leaves developed PLBs. This differential response of the explants from mature and juvenile leaves under identical nutritional conditions seem to indicate the importance of their source and physiological age of an explant. Physiological age of an explant is an important factor for regeneration and in accord with the observations of Mathews and Rao, 1985; Vij *et al.*, 1986; Vij *et al.*, 1984 and Vij and Pathak, 1990.

In the present study regeneration of PLBs occurred only at the basal cut end of the explants. Similarly, in *Cattleya* (Champagnat *et al.*, 1970), *Vanda coerulea* (Seeni, 1988), *Neofinetia falcata, Satyrium nepalense, Vanda cristata and V. testacea* (Vij and Pathak, 1990) regenerative response remain restricted to the leaf base in accord with earlier suggestion of Zimmer and Peiper (1975) that leaf base is, generally meristematic in monocots and upon isolation and culture, it differentiates plantlets.
The efficiency of plant growth regulators in activating proliferative loci in foliar explants and regulating their subsequent development into plantlets is specific in orchids (Vij and Pathak, 1990). Combined effect of cytokinins and auxins proved useful in foliar cultures of hybrid *Vandas* (Mathews and Rao, 1985), *Vanda coerulea* (Seeni, 1988), *V. cristata* and *V. testacea* (Vij and Pathak, 1990). In the present cultures auxin (NAA) alone induced PLBs formation but it was inhibitory when used in combination with BAP and/or KN. Whereas, BAP (2.0 mg/l) and KN (1.0 mg/l) alone have induced PLBs in 80% of cultures. Explants have also developed PLBs in medium supplemented with CW (15%) and the utility of CW has already been demonstrated in promoting regeneration in *Phalaenopsis* leaf cultures (Tanaka and Sakanishi, 1977).

In many orchids, such as *Dendrobium* and *Epidendrum* (Churchill *et al.*, 1970, 1971), *Rhynchostylis retusa* (Vij *et al.*, 1984) including present species *Aerides maculosum* shoot regeneration from leaf explants has been reported to have occurred through the formation of callus or PLBs. However, in *Renanthera imschootiana* (Seeni and Latha, 1992), *Acampe praemorsa* (Nayak *et al.*, 1997) have reported shoot bud differentiation directly from the leaf bases without any intervening callus or PLB formation.

These results suggest that foliar explants effectively used for micropropagating *Aerides maculosum*. Juvenility of the tissue, and appropriate growth stimulus are, however important factor for the purpose. KN, BAP and CW were useful in initiating the cultures, for PLB multiplication and growth and development of plantlets.
5.3 Nodal explant cultures of *Dendrobium macrostachyum* and *D. ovatum*:

Use of shoot tip for clonal propagation of orchids endangers the original plants because meristem must be removed with no guarantee of success. Propagation methods using other explants would therefore be clearly advantageous. The utility of nodal explants for *in vitro* regeneration has been positively tested in a number of commercially significant orchid species (Arditti and Ernst, 1993). In the present study nodal explants of *Dendrobium macrostachyum* cultured on MS medium supplemented with BAP and KN (1.0 and 2.0 mg/l) have developed 2-4 axillary shoots. Where as the explants cultured on MS medium with NAA and BAP or KN combination have developed single axillary shoot. Addition of CW (5-15%) enhanced the process and 4-7 axillary shoots developed at the nodal region and eventually developed root initials after 10 wks of culture. The regenerated shoots developed on the other media were cultured on rooting medium (MS basal medium) where they developed roots in 4-8 wks.

Nodal explants of *Dendrobium ovatum* cultured on MS medium supplemented with BAP and/or KN (0.5-2.0 mg/l) and CW (5-15%) have developed multiple shoots at the nodal region in 5-6 wks. These shoots when subcultured in MS basal medium developed roots. These results in accord with similar earlier report in some orchid species and hybrids (Arditti, 1977; Arditti and Ernst, 1993 and Yoshiyuki et al., 1993).
In the present studies, an appropriate growth stimulus proved a major factor in controlling initiation and subsequent development of regenerative loci. Shoot buds were directly developed in the nodal region in cultures treated with growth adjuncts (BAP/KN/CW). BAP and CW are superior than KN in activation of shoot buds. Nearly 5-7 shoots could be obtained from each responding explants in 4 wks. Similarly, the role of cytokinins in induction of multiple shoots have been reported in *Cymbidium niveomarginatum* (Yoshiyuki et al., 1993) and *Cymbidium goerengii* (Wang et al., 1981), *Cymbidium pendulum* (Vij et al., 1994) and *Phalaenopsis* (Duan et al., 1996) from the nodal explants.