CHAPTER - V

DISCUSSION
5. DISCUSSION - I

5.1. Standardization of medium:

Three media were selected for germination and growth of embryos and protocorms. Among these three media, KnC medium showed best results with maximum germination percent and better growth for all characters, when supplemented with IAA (0.2 mg/L) and KN (0.4 mg/L). The time required for germination was also found to be less in comparison to MS and VW medium. Bopaiah and Jorapur (1986) found KnC medium suitable for protocorm production for *Cymbidium longifolium*. Induction of protocorm from *Cymbidium* seeds were reported to be 60 per cent in KnC and VW medium (Sawa and Namba, 1974; Ueda and Torikata, 1976; Nagashima, 1978), whereas in present study 98 per cent germination of *S. plicata* seeds was obtained in KnC medium supplemented with IAA and KN. Hegde et al. (1988) also observed that modified KnC medium with 200 mg/L citric acid was suitable for germination and growth of *C. iridioodes* and *C. longifolium* hybrids. Yam and Weatherhead (1990) were successful in germinating seeds of *Hetera cristata* in KnC medium within two months. Immature embryos from unripe capsules of *Cattleya intermedia* were raised in KnC medium supplemented with 15% coconut milk. After 45 days of culture protocorms became green and then cultured in KnC medium containing banana pulp and complete plantlets developed (Kerbaux and Handro, 1981).

In contrary, Ismat et al. (1988) cultured 7 species of orchids including *S. Plicata* in KnC, MS and VW medium with different supplements. They observed that all the species of orchids except *C. bicolor* and *A. pramorsa* shoed best germination in VW medium supplemented with 1.0 mg/L NAA + 0.5 mg/L KN and 1.5 mg/L IAA + 1.5 mg/L BAP + 20 per cent coconut water. Supplementing VW medium with growth regulators only, they observed germination after 31-38 days, whereas in the present study germination was observed only within 16 days in KnC medium supplemented with 0.2 mg/L IAA + 0.4 mg/L KN.
Chennaveeriah and Patil (1973) used White’s medium for immature embryo culture of *S. plicata.*

Germination per cent and growth increased many fold due to interaction of IAA and KN. Because auxin alone leads only to cell enlargement while KN induce cell division only in presence of auxin (Steward and Shantz, 1955).

In basal medium some seeds germinate without addition of cytokinin and auxin. Perhaps due to presence of endogenous cytokinin and auxin but those seeds having inadequate levels did not germinate. Orchid seed that do not require any exogeneous cytokinin or auxin have high endogeneous level of these growth regulators *e.g.* *Epidendrum fulgens* (Mercier and Kerbauy, 1991).

KnC and VW medium contain identical qualities and quantities of most of macronutrients. However, KnC contain 1000 mg/L of Ca(NO₃)₂ *₄H₂O,* whereas VW medium differ in additional presence of 200 mg/L of Ca₃PO₄ and 525 mg/L of KNO₃. On the contrary MS medium contains very high nitrogen *i.e.* 1650 mg of (NH₄)₂NO₃ an 1900 mg/L KNO₃ along with 440 mg/L of CaCl₂. The seed germinated after 16, 29 and 24 days of inoculation, respectively. Moreover, 98, 64 and 83 per cent of seeds germinated, respectively in KnC, VW and MS media when supplemented with IAA and KN. As observed in the present study KnC medium appears to be appropriately balanced to support up to 40 per cent seed germination without any supplements.

5.1.2. Comparison of IAA and NAA on germination and growth:

Auxins such as IAA, IBA, NAA and 2, 4-D are most commonly used in orchid *in vitro* culture and their effects are variable. It was observed that, in comparison to NAA higher germination was obtained in medium supplemented with IAA. Out of three concentrations of both IAA and NAA, 0.2 and 0.3 mg/L of IAA showed maximum germination (97.9 and 93.3 per cent, respectively). Whereas, the same concentrations of NAA showed 89.9 and 82.3 per cent germination. It was also noticed that germination per cent decreased when NAA
concentration was increased. It was also observed that IAA (0.3 mg/L) gave maximum fresh weight. The fresh weight increased due to maximum number of roots and root length. This was may be due to maximum absorption of water (Reinder, 1938) or plasticity which is regulated by auxin because in the first phase auxin tends to accumulate in the interfibrillar spaces and cause swelling of the colloids which stretches the cell wall (Ruge, 1937a, b). On the other hand auxin induce entry of water and salts by increasing the permeability of the cell wall membrane (Bungenberg de Jong, 1935). Increase in fresh weight may be also due to lignification of cell wall or increased binding of pectin methyl esterase on the cell wall as a result of growth promoting action of auxin (Glassiou, 1957 and 1958). Shoot length and number of leaves were minimum in 0.3 mg/L IAA concentration. On the other hand 0.2 mg/L IAA produced balanced shoot as well as root growth which is most important for survival of seedlings in the natural environment and helps in acclimatization.

IAA at very low concentration (0.25 mg/L) promoted germination of seeds of Cymbidium mastersii upto 80 per cent (Prasad and Mitra, 1975). The same concentration was also effective for vandaceous taxa (Vij et. al., 1981). Cymbidium protocorms proliferated when grown in medium supplemented with NAA (Fonesbech, 1972a). NAA also promoted seed germination in some orchids (Mathew and Rao, 1980; Vij et. al., 1981). Addition of NAA to the basal medium produced similar effect in Cymbidium madidium and other species and hybrids of Bletilla and Vanda (Arditti, 1967; Fonesbech, 1972a; Straus and Resinger, 1976; Prasad and Mitra 1975; Mathew and Rao, 1980). The optimal concentration of NAA vary from 0.1 mg/L in Cattleya (Ichihasi and Kako, 1973) to 1.25 mg/L in Vanda (Payawal and Guzman, 1972). In the present study, 0.1 mg/L of NAA was found to produce optimal shoot and root growth. IAA was found to be more effective when compared after 3, 6 and 9 months of growth. However, its higher concentrations were inhibitory. Though, NAA did not significantly influence seed germination and seedling growth at lower concentrations but it markedly enhanced root growth development at concentration higher than 0.1 mg/L. It was evident that 0.2 mg/L of IAA was best for S. plicata seed germination and growth.
5.1.3. Comparison of KN and BAP germination and growth:

In general orchid seeds respond to cytokinins either by increase or decrease in germination or there is no effect (Arditti and Ernst, 1984). Between the two cytokinins (KN and BAP), KN showed better result in germination and growth. Germination generally increased with the concentration of KN and highest germination in 0.4 mg/L of KN. The exact function of cytokinin in germination is unknown but there is evidence that in seeds with high levels of storage lipids such as pecan nuts, cytokinin may play an important role in lipid mobilization (Dimalla and Van Staden, 1977). Orchid seeds have no endosperm and no cotyledons and lipid droplets in the embryo are the primary storage material (Arditti, 1979, Arditti and Ernst, 1984). The requirement for cytokinins in the germinating medium may thus be related to utilization of lipid. It has been shown that if storage lipids can not be utilized, germination will not continue (Manning and Van Staden 1987). This also suggested that KN causes the rupture of seed coats leading to germination. According to (Haber and Luippold, 1960), KN induces germination preceded with active mitotic activity and apparently results in cell enlargement.

In the present investigation increase in concentrations of both KN and BAP, the germination per cent and shoot growth increased. But at the same time root growth was insignificant. As such, KN as well as BAP promote shoot growth and are not effective for root growth. Harvis (1982) observed that aminopurines were the major growth regulators effecting germination and they play an important roles in development and morphogenesis of Cypripedium reginae. In his previous experiment he found that supplement of 1 mg/L KN to basal medium gave near about 100 per cent germination, while in the control without KN or on 10 mg/L KN was supra optimal. In his next experiment, he observed that seed germination was indeed very sensitive to the KN concentrations with the optimal around 0.5 to 1.0 mg/L. He also established the relative merits of three aminopurines, KN, BAP and YY (Y,Y-dimethyl allyl aminopurine) and observed that the best media for germination and early growth were YY at <0.5 mg/L → BAP at 0.25-0.5 mg/L → KN at >0.5 mg/L. However, after 7 months in the light, things becomes reverse, with less mortality after greening and best growth.
regulators in order was KN → BAP → YY. Pauw et al. (1995) also observed the effect of different cytokinins (BA, 2ip and KN) at increasing concentration on germination of *Cyperpidium caudatum*. Both BA and 2ip increased germination compared to control with no cytokinin. Germination in the presence of KN was not significantly different from control. Germination was not significantly different between concentrations but there was significant differences in germination between different group of cytokinins. So it is evident that particular response depends upon the genus and species Sharma and Tandon (1986) noted that germination and seedling growth of *Coelogyne punctulata* were much higher at 0.1 mg/L of KN in the medium. However, 0.5 mg/L of KN was optimum for both leaf and root development but higher concentrations were inhibitory. Pierik and Steegman (1972) also reported the stimulatory effect of KN on germination and growth in *Cattleya* but its inhibitory role was observed in *Dendrobium* and *Laeliocattleya* (Kano, 1965).

5.1.4. Effect of Auxin (IAA) + Cytokinin (KN) interaction on growth:

Plant growth regulators have different responses in orchid seed germination and seedling growth depending on the concentrations used. The result of the present investigation revealed that the effect of KN and IAA on seedling development alone was more pronounced than combined effect.

Keeping IAA concentration constant (i.e., 0.2 mg/L) when KN concentrations (0 1, 0 2, 0 3, 0 4, 0 5 & 0.6 mg/L) were increased, it showed plant height and shoot length equal to IAA alone at 0.4 and 0.5 mg/L. However, roots per plant and root length were found to decline considerably in these concentrations. Interacting influence of KN and auxin on shoot/root balance in orchids was reported by Hadley and Harvais, 1986; Pierick and Steegman, 1972; Rao, 1977; Harvais, 1982. In *C. punctulata* KN showed a pronounced stimulatory effect on both germination and growth (Sharma and Tandon, 1986), but its inhibitory role was observed in *Dendrobium* and *Laeliocattleya* by Kano, 1965. In *S. phcata* when KnC medium was supplemented with 0.4 mg/L of KN showed plant height and shoot
length growth equivalent to 0.2 mg/L of IAA but lower number of roots and root length after 3, 6 and 9 months of growth.

As IAA alone gave balanced shoot and root growth which is also advantageous for acclimatization and hardening, use of 0.2 mg/L of IAA for best shoot and root growth in *S. plicata* is recommended

5.2.1 Effect of different vitamins on growth:

Most of the researchers confirmed from their experiments that the seed of orchids failed to germinate in medium without vitamins. Similarly, *Downie* (1943), *Noggle* and *Wynad* (1943) reported that seeds germinate in medium containing sugar but failed to germinate in medium without vitamins or fungal extract. Vitamin deficiency was a limiting factor in orchid seed germination and growth. *Mitra et al.* (1976) observed the differentiation of protocorms in *Dendrobium fimbriatum* in medium containing vitamins but without containing hormones. In the present studies, all vitamins viz. Thiamine HCl, Pyridoxin HCl, Folic acid, Riboflavin and Biotin were incorporated in the KnC medium separately and together. Seeds of *S. plicata* germinated in both medium containing vitamins separately and together but showed difference in growth characters (viz. plant height, shoot and root length, number of leaves and roots and fresh weight). Medium containing Pyridoxin HCl showed best growth for all characters. However, except Biotin, all other vitamins supplemented separately showed better effect than the vitamins supplemented together for growth of the plants. The vitamins supplemented separately had better effect than in combination. Vitamins are necessary for germination and seedling development (*Boëtner* and *Axtman*, 1937; *Bonner* and *Bonner*, 1938). The positive growth effect of vitamins on orchids were reported in *Cattleya, Cymbidium* and *Laeliocattleya* by *Noggle* and *Wynd* (1943), *Withner* (1959), *Lawrence and Arditti* (1964), *Arditti* and *Bils* (1965) and *Ueda* and *Torikata* (1969a, b, 1972). Vitamins supplemented separately or together in the medium showed normal germination of seeds. *Krishnamohan* and *Jorapur* (1986) also observed that VW medium supplemented with thiamine HCl and niacin together as well as separately
induced highest percentage of seed germination and undergo organogenesis in *Acampe praemorsa*. At protocorm level it was difficult to differentiate the effect of different vitamins. The effect of each vitamin was more distinct after 3rd and 6th months of growth. Plants in the medium containing Pyridoxine HCl alone showed three times increase in fresh weight than any other vitamins supplemented separately in the same medium. This fresh weight was due to vegetative growth. During 9th month of growth this difference was more prominent. Murashig and Tucker (1969) systematically established optimum level of vitamins for the proliferation of *albedo*. They used myoinositol, pyrodoxin HCl, nicotinic acid, Thiamine HCl, ascorbic acid, biotin, calcium pantothenate, choline, folic acid and para-amino benzoic acid. Of these, only thiamine HCl appeared to be essential, while the other were inactive. Ascorbic acid was found to enhance the growth of *Citrus natsudaidai* plantlets (Ohta and Furusata (1957) and the stimulation of pseudobulbi! production in *Citrus senensis* (Button and Borhman, 1971 a). Bopaiah and Jorapur (1986) reported that KnC medium containing thiamine HCl, niacin and glycine was found most suitable for normal and healthy growth of *Cymbidium aloifolium*. It was observed that medium containing pyrodoxin HCl showed better fresh weight which was due to luxuriant growth of leaves, shoots and maximum plant height (Table-10) Harvais (1982) also reported that i-inosital improved germination, niacin and i-inositol helped in general health of protocorm and plantlets, while panthonic acid, thiamine HCl and pyrodoxin HCl improved shoot development and leaf broadening.

**DISCUSSION - II**

5.3.1 Regeneration of multiple shoots from *in vitro* cultured plantlet parts as explant

Three media (*viz*. KnC, MS and VW) were tested for regeneration of multiple shoots from different *in vitro* explants. From the observation it was confirmed that KnC and MS medium were suitable for regeneration of multiple shoots from *in vitro* explants (*viz*. Leaf basse, Node and Splitted pseudobulb). However, VW medium could not induce any shoot.
Between the two medium (viz. MS and KnC), MS medium was better than KnC for regeneration of multiple shoot. Weatherhead and Harberd (1980) cultured the shoots (1.0 to 1.5 cm long) in KnC, VW and Nitsch medium. They observed that KnC medium supplemented with activated charcoal gave rise to protocorms and recovery of shoot was 85 per cent. In other media, a few meristems developed very slowly but most of them degenerated. Vij et al. (1984) cultured young leaf segments of R. retusa on Mitra et al. (1976) medium and produced plbs from the explant. Griesbach (1983) used nodal section of Phalaenopsis in MS medium and was successful to regenerate multiple shoots from the same. Tanka et al. (1988) also regenerated multiple shoots from flower stalk section of Phalaenopsis using VW medium modified by replacing ferric tertrate with ferrous sulphate.

Out of the five explants (viz. leaf segment, leaf base, node, internode and splitted pseudobulb) only three explants e.g. leaf base, node and splitted pseudobulb responded and produced multiple shoots and roots. The best medium for leaf base was found to be MSb medium (supplemented with 0.2 mg/L IAA + 0.2 mg/L NAA + 0.5 mg/L KN) which induced 31 shoots and 35 roots after 6 months. In the same medium node produced 36 shoots and 40 roots, whereas splitted pseudobulb produced 24 shoots and 29 roots (Table-12a). It was observed that leaf base did not respond to KnCa, KnCd, and MSe medium. Node did not show any response in KnCe and MSe medium. Splitted pseudobulb did not show any response in MSa medium. So it is clear that production of multiple shoots depends on the concentrations of optimum exogenous auxins only. It was also evident from the studies that higher concentration of auxin (0.5 mg/L of IAA and NAA) as well as cytokinin (2.0 mg/L of KN) reduced the production of shoots and roots. The lower concentration of auxin (0.1 mg/L of IAA and NAA) was found to reduce shoot and root regeneration from different explants. Cytokinin did not play any significant role in shoot and root regeneration. Because 0.5 mg/L KN also used in MSb produced maximum shoot and root, whereas with lower concentration it did not show any such effect.

Shimaski and Uemoto (1991) cultured inflorescence segment of 5 cm long in MS medium supplemented with different concentration of BA and NAA. Shoot buds produced
rhizome mass  Shoots differentiated from rhizome apex segment but failed to produce roots. The optimum medium for plantlet regeneration from rhizome consisted of 0.1 mg/L BA + 1.0 mg/L NAA. However, they observed that both cytokinin and auxin had limited effect on induction of rhizome development and subsequent plantlet regeneration. Roy and Sharma (1992) observed that nodal segment of Arrundina gramnifolia produced 10-15 shoots when cultured in SH medium supplemented with KN (1 μg/ml) + NAA (2 μg/L). KN was also reported to be beneficial for shoot bud multiplication in Dendrobium species (Vij and Pathak, 1989). Vij et al. (1984) regenerated shoots from leaf explant of Rhyncostylis retusa in Mitra et al. (1976) medium supplemented with NAA, IAA and KN. They observed that out of these three hormones, NAA was most effective. High concentration of KN (5 mg/L) is required for shoot regeneration in urea supplemented media, whereas low concentration (1 mg/L) is necessary with yeast extract supplemented medium. Both KN and NAA or IAA, which act synergistically, were also effectively used for propagation through leaf tissue culture in Phalaenopsis species (Tanka and Sakanishi, 1977). Goh and Wong (1990) achieved the clonal propagation of orchid hybrid Aranda ‘Deborah’ through in vitro culture of inflorescence lip explants. The initiation medium consisted of KnC supplemented with coconut water and BA. Further growth and proliferation were better in VW medium. Plantlet developed was faster in VW agar medium supplemented with 1.0 mg/L BA + 1.0 mg/L KN. Kukulczanka et al. (1989) cultured shoot tip of Vuyistekeara ‘cambrica’ in MS medium and observed that the best regeneration and organogenesis, i.e. formation of numerous shoots and rooted plantlets of Vuyistekeara could be obtained when the medium was supplemented with cytokinin or 0.2 ppm BA and auxin 0.2 ppm of NAA. Enrichment of medium with exogenous auxin and cytokinin (NAA + IAA + KN) may promote the explants to produce multiple shoots. NAA and IAA stimulated the shoot growth and also accelerated root formation, whereas, KN may only stimulate growth. Torres and Carlisi (1984) observed in the same species the combination of auxin and cytokinin seems to be positive for growth of shoots. Whereas presence of KN complements auxins and stimulate shoot growth.
5.4 Introduction of callus from in vitro cultured explants and their regeneration.

5.4.1 Callus induction: Two media (KnC and MS) supplemented with different combinations of auxin and cytokinin were used for induction of callus. It was observed that only protocorms responded to KnC medium and induced callus. The percentage of callus induction was only 40 percent. Other explants did not produce any callus in KnC medium. On the other hand, most of the explants (viz. embryo, protocorm, node, pseudobulb, split pseudobulb, leaf base and roots) produced callus in MS medium with same supplementation. It is evident from the studies that for mass callus induction in S. plicata from different in vitro explants, MS medium was suitable than KnC medium. Other workers also confirmed this findings Champagnat et al. (1966); Champagnat and Morel (1969), Morel (1971) cultured the segmented tissues of protocorm of Miltonia, Odontoglossum, Cattleya and Vandea in MS and KnC media. In all such experiments MS medium was found to be more effective than KnC medium. Similarly, Dhanalaxmi and Lakshamanan (1992) developed callus from seedling roots on MS medium in Clitoria ternata. Yam and Weatherhead (1991) and Paek et al. (1990) also reported that MS medium is most suitable for development of callus from root tips of Bletilla striata, Cleiosostoma fordit, Pholidota canonensis and Cymbidium embryo.

Callus induction from different explants can be very useful to obtain an increased number of plantlet where the seed production is limited or the percentage of germination is low (Rao, 1963, Rao and Avadhani, 1964). So different combinations of auxin and cytokinin were tried for callus induction. Out of 10 combinations of 2, 4-D + KN used for callus induction, 3 mg/L of 2, 4-D + 0.2 mg/L KN + CH was found to be the best combination. About 96 percent of cultures produced callus in this combination from leaf base explant. This combination was also found to be better for other explants. It was noticed that with increase in 2, 4-D concentration (e.g. 5.0 mg/L) there was decline in callus initiation. 0.2 mg/L of KN was found to be more effective than any other concentrations of KN. Among the explants, leaf
base, splitted pseudobulb and pseudobulb were found to be better than any other explants. Kim and Kako (1982) also observed that 2, 4 - D (1.0 ppm) in combination with 0.1 ppm KN produced profuse callus. The level of 2, 4 - D and KN, the interaction of 2, 4-D and KN, the ratio of 2, 4-D and KN as well as total concentrations of these hormones had significant effect on growth of callus. Fresh weight of callus was observed to increase with the increase in 2, 4-D concentration. The increased fresh weight obtained with 2, 4 - D was friable type of callus. However, at 5 mg/L of 2, 4-D along with 0.2 mg/L as well as 0.4 mg/L of KN, fresh weight decreased. Kim et al. (1988) recorded higher callusing from corn explants in MS medium supplemented with 10 mg/L of 2, 4-D.

Ten combinations of 2, 4-D + BAP also showed variation in producing callus, time required for callus initiation as well as fresh weight of callus. It was observed that with increase in 2, 4-D and BAP concentrations, callus induction also increased but was limited up to 3 mg/L of 2, 4-D. In higher concentration callus induction percentage and fresh weight declined. Similarly, Mohanty and Ghosh (1988) observed callus initiation from leaf in MS medium supplemented with 2, 4-D (2 to 4 mg/L). Whereas, at 5 mg/L callus formation was suppressed.

In four different combinations of NAA along with KN or BAP was found to produce callus from different explants. 1.0 mg/L of NAA with 0.4 mg/L of KN was found to be optimum concentration for callusing but when the concentration of NAA was raised to 1.5 mg/L, percentage of callus initiation was reduced. Maximum callus was produced in 1.0 mg/L of NAA + 0.2 mg/L of BAP. It is evident that in S. plicata 1.0 mg of NAA is optimum for callus initiation and proliferation along with KN or BAP. Similarly, Barghchi and Alderson (1983) also observed that higher concentration of NAA (i.e. 1.0 to 4.0 mg/L) increase callus induction and callus growth in P. vera but Tusken et al. (1990) reported that maximum callus could be obtained from cotyledon of pine with 54 μM of NAA in GD medium. Haab et al. (1991) observed that NAA (0.2 to 12.0 mg/L) along with KN (0.1 to 3.5 mg/L) induced callus in Symphytum officinale. Keever et al. (1983) observed that petiole, leaf, stem and flower stalk produced callus in MS medium supplemented with 1 mg/L NAA + 1 mg/L BAP.
whereas, Bilkey and McCown (1979) used basal MS medium containing 0.1 mg/L NAA and 0.5 mg/L BA and observed best callus initiation in *S. plicata* between the auxins (2, 4-D vs NAA), 2, 4-D and between cytokinins (KN vs BAP) KN was found to be best for callus induction. Among the explants leaf base was found to be best explant in 2, 4-D + KN combinations, as well as in 2, 4-D + BAP combinations, whereas splitted pseudobulb and pseudobulb showed maximum callusing in NAA + KN and NAA + BAP, respectively. But internode, leaf and leaf segment failed to produce callus in any of these combinations. Differential response are due to the presence of endogenous auxin and cytokinins in various concentrations in different explants, where a specific exogeneous hormone concentration induces callusing. It may be due to lower concentration of endogeneous hormones in these explants (viz leaf segment, leaf and internode) which failed to initiate callus.

The level of auxin and cytokinin and their interaction have significant effects on the growth of callus. King and Morehart (1987) produced callus from shoot of red maple on MS basal medium supplemented with 0.5 mg/L NAA and 0.5 mg/L BA. They observed that in all experiments, callus medium lacking both BA and auxin (NAA or 2, 4-D) grew very little or lost volume. The peak growth of callus was at BA/NAA ratio of 1/3 and 1/1. Present study showed maximum growth of callus at KN/2, 4-D ratio of 1/15, BAP/2,4-D ratio of 1/3, KN/NAA ratio of 1/2.5 and BAP/NAA ratio of 1/5. Callus initiation, growth and development are influenced by a complex relationship between the explants used, the constituents of medium (including hormones) and the environmental conditions during culture (Akbar and Nabars, 1991). So gradient of hormones used play an important role. In the present study, it was observed that lower concentration of cytokinin (KN or BAP) and higher concentrations of auxin (NAA or 2, 4-D) was suitable for callus initiation. Similar fact was also established by Yam and Weatherhead (1991) in several species of orchids (viz *Bletilla striata, Clerisostoma fordtii, Pholidota cannzennis*). In callus formation type of explants and age of explants also play an important role. Mohanty and Ghosh (1985) observed a gradient of response of callus formation from the base of leaf to the apex. The meristamatic lower basal
segments were more responsive to lower concentrations of 2, 4-D, whereas higher concentration induced callus in more mature segments. In present study, this fact was also observed. A similar response was also reported in wheat leaves (Wernicke and Milkovits, 1984).

5.4.2 Shoot and root regeneration: In the present studies callus induced from different explants showed variation in shoot regeneration. The calli were treated with different combinations of BAP + KN + GA₃, which showed maximum shoot regeneration in 3 mg/L BAP + 0.4 mg/L KN + 1.0 mg/L GA₃. In regeneration of shoot, BAP was found to play a vital role. It was observed that with increase in BAP concentrations, regeneration percentage increased. Kukulczanka et al. (1989) observed that BA stimulated the induction of shoot formation in Vinylestekera. However, root formation was inhibited with 1.0 ppm of BA (Holter and Zimmer, 1990). Yam and Weatherhead (1991) used BA + NAA for shoot regeneration. In Cymbidium only 0.5 mg/L of BA gave better result in shoot regeneration (Pack et al., 1990). Khos-Khui and Sink (1982) and Davis (1980) observed regeneration in hybrid roses with 2.0 mg/L of BA, whereas, 4.0 mg/L concentration suppressed shoot production. However, Barghchi and Alderson (1983) observed rapid shoot proliferation with BAP and KN. In S. plicata, higher concentration of BA and lower concentration of KN together produced as much as 27 shoots from pseudobulb explant. 2, 4-D was eliminated from regeneration medium as it induced proliferation of callus and inhibit shoot formation. Similar fact was also observed by Wainwright and Hardwood (1985), Soediono (1983), Nadar et al. (1978) reported that in sugarcane, regeneration was not possible in medium containing 2, 4-D. Other auxins (NAA or IAA) was also eliminated from regenerating medium as it produced roots before shoots which obstructed in shoot formation.

GA₃ was observed to increase the globular structure from callus and also decrease regeneration time in all explant callus. Similarly, Richard et al. (1988) observed that GA₃ enhance shoot formation in tobacco callus. Roy and Sharma (1992) also reported that GA₃ along with BA promote in shoot development and shoot growth in Otchilus fusca. But Hasegawa (1979) reported that shoot proliferation was inhibited when GA₃ was used, while
Lane (1979) observed that GA₃ neither stimulated nor inhibited shoot multiplication in *Pyrus communis*.

For root regeneration IBA (2 to 4 mg/L) and IAA (0.2 to 0.4 mg/L) were used together. The number of roots increased with increase in auxin concentration. In case of *S. plicata* root regeneration was found to be much easier and quicker than shoot regeneration. Other workers also found that IBA and other auxins suitable for rooting in tropical orchids such as *Cymbidium*, *Cattleya*, *Phalaenopsis*, etc. (Fonnesbech, 1972; Morel, 1974; Kukulczanka, 1976; Arditti, 1977; Sood and Vij, 1986 and Holter and Zimmer, 1990).

*S. plicata* is an orchid whose natural germination as well as vegetative propagation is slow. So, successful callus initiation and regeneration of 27 shoots from each callus culture will be certainly helpful to produce large population from different explants of a plantlet.

**DISCUSSION - IV**

5.5 *In vitro* culture of buds obtained from natural pseudobulb:

Out of two media (viz. KnC and MS), KnC was found to be more suitable for direct development of plantlets from bud explant. About 95 per cent buds produced shoots and roots, while MS medium also showed 90 per cent development. In KnC and MS media it took respectively, 31 and 48 days, for development of plantlets. KnC medium saved time for complete regeneration of plantlets from aseptic culture of buds. The growth hormones used (viz. IAA, IBA and KN) showed variation in growth of plants. KnC medium supplemented with IAA and KN (0.5 mg/L each) showed best result, but MS medium when supplemented with 1.5 mg/L of IAA and 0.2 mg/L of KN gave better response. Other workers also reported *in vitro* culture of buds. Kim and Kako (1984), Shimaski and Uemoto (1991) reported that MS medium supplemented with 10 mg/L of NAA + 0.1 mg/L of BA and 0.1 ppm NAA + 1.0 ppm BA, respectively were found to be suitable for development of
Cyndndmm from floral buds. However, Hiroyki et al. (1991) reported that white's medium supplemented with 1.0 mg/L of BAP was best for culture of Podocarpus macrophyllus. The effect of medium and growth regulators was also clear from different growth characters. First 3 months better growth was observed in KnC medium, but growth after 9 months was found to be fairly good in MS medium.

5.5.1 Callus induction: In this study, it was observed that callus initiation and its proliferation from bud explant was maximum in MS medium supplemented with 2,4-D (3.0 mg/L)+ KN (0.4 mg/L). However, 2,4-D (3.0 mg/L) + BAP (1.0 mg/L) and NAA (1.0 mg/L) + BAP (0.4 mg/L) also produced callus. For producing more callus, 2,4-D (3.0 mg/L) was found superior to NAA. 6.0 mg/L 2,4-D with any other concentration of KN or BAP failed to produce callus. The failure to produce callus in 6.0 mg/L might be due to synthesis of phenolic substances which discoloured explant and further checked growth completely. Soediono (1983) also reported that excessive concentration of 2,4-D (above 10 mg/L) has deleterious effect in Dendrobium culture. Vij et al. (1984) observed no callusing with 1.0 mg/L of 2,4-D in leaf culture of Rhyncostylis retusa. But 2,4-D was successfully utilised by other workers for inducing callus in different explants (Wernicke and Mikovit, 1984; Ghosh and Mohanty, 1988; King and Morehart, 1987; Dass et al., 1989 and Bhansali, 1990).

5.5.2 Shoot and root regeneration: In case of shoot regeneration BAP (4 mg/L) + KN (0.25 mg/L) were found suitable. Moreover, it was noticed that with increase in BAP concentrations, shoot regeneration increased. But beyond 4 mg/L of BAP, shoot regeneration declined. Again 4 mg/L of BAP failed to produce any shoot from callus when it was combined with 0.4 mg/L of KN. It indicates that 0.4 mg/L of KN have antagonistic effect with 4 mg/L of BAP in shoot regeneration from bud callus. Many other workers also reported the regeneration of shoots with the help of BA and KN. Tanka and Sakanishi (1977) reported that BA and KN combined with coconut milk helped regeneration of callus. Loh et al. (1975), Kusumoto (1978) observed Plb formation from callus with KN only. Both KN or BAP and NAA or IAA was effective and favoured plb formation in R. retusa (Mroginski et al., 1981, Vij et al., 1984, Holter and Zimmer, 1990). Yam and
Weatherhead (1991) reported that 1 mg/L of BA alone can produce shoots in *Cleisosionoma fordit* in MS medium. Paek et al. (1990) observed that 0.5 mg/L of BA gave better shoot production in *Cymbidium*. Whereas, Waes et al. (1983) found 0.4 mg/L of BA + 0.1 mg/L of IBA to be better for shoot production in *Disa uniflora*. For root regeneration both IBA and IAA played important role. It was observed that increase in auxin concentrations stimulated root production from callus. Habb et al. (1991) observed that 0.4 to 4.0 mg/L IBA could produce roots. It was observed that root formation enhanced when BA and KN were excluded from medium and auxin level was increased. Similar fact was observed by Wainwright and Hardwood (1985).

It was noticed that when GA3 was added with BAP + KN there was reduction in shoot regeneration. It implies that for bud callus regeneration GA3 had antagonistic effect. Murashige (1961) observed the inhibitory effect of GA3 in organogenesis of Tobacco callus. Goh and Yang (1978) reported that plants of *Dendrobium* explants treated with GA3 did not initiate any shoot. Kevers et al. (1983) observed that MS medium supplemented with GA3 (1.0 mg/L) did not significantly modify the auxin effect. Increase in GA3 concentration (10-100 mg/L) reduced shoot proliferation. Murashige (1974) showed that GA3 (3,4,5,7,8 or 9 mg/L) were all equally effective inhibitor. Carlisi and Torres (1986) found that GA3 has no effect on the number of shoots obtained from primary nodal section explants of *C. japonica*. Hasegawa (1979) reported that shoot proliferation was inhibited when GA3 was used. However, Lane (1979) reported that GA3 neither stimulated nor inhibited shoot multiplication.

It was observed that the buds may be utilised for development of plantlets both directly or through callus. In direct development, only one plantlet is obtained from each bud, whereas via callusing as many as nine plantlets from each bud may be obtained. In case of monopodial orchids, excision of these explants may result in sacrificing the mother plant. Thus many attempts have been made for alternative explants such as inflorescence stalk, floral buds, buds produced on pseudobulb as well as root tip. In *Phalaenopsis* and *Doris* inflorescence stalk is preferred as explant (Rotor, 1949; Tse et al., 1971; Intuang et al.,
However, the success of these alternative explants in other monopodial orchids were limited e.g. young inflorescence of Ascofinitia (Intuang and Sagwa, 1973) and Renatenda (Goh and Tan, 1982), floral buds in Mokara (Lim-Hio et al., 1986) and Vanda (Valmayor et al., 1986). So it is evident that numerous plantlets can be obtained from a single plant using axillary buds developed from pseudobulb without injuring the mother plant.

DISCUSSION - V

5.6.1 Effect of gamma irradiation on embryo germination:

Irradiation of S. plicata embryos with different doses of gamma rays from 2 to 20 Krads were found to be lethal. In this investigation, it was observed that the irradiated embryos germinated, but not a single germinated embryo survived. It may tolerate much lower doses of gamma rays than 2 Kr. It was further investigated by irradiating the one month old protocorms with 0.5 to 5.5 Kr of gamma rays. In this investigation inverse relationship of survival per cent with doses were also observed. However, sufficient plants were available for studies in all the doses.

After the discovery of artificial induction of mutation by Muller and it has been possible with man's intervention to force an organism to change faster than when left to nature. Mutation breeding now has been recognised as an established method for plant improvement and this technique has been very successfully utilised for developing hundreds of improved novel varieties in sexually as well as asexually propagated plants. Wide variation may also be induced during in vitro culture of plant species, producing large number of mutants within a short period (Mitra, 1985 Evans et. al., 1986). In orchids it remains to be seen how far the direction of change of genetic architecture may be manipulated to provide inherited variability for screening useful mutants in a large population during in vitro culture.
Though production of induced mutations is non-directional, for measuring radio biological efficiency - the dose at which 50 per cent of the treated material is killed, its effect on morphological characters, chromosomal damage etc. in M₁ are commonly utilized. Sufficient literature is not available in radiation sensitivity studies in orchids. Nails (1966), Harn (1968) and Sauleda (1971) were of opinion that proliferated tissue resulting from either embryo or protocorm culture could be subjected to the influence of irradiation and mutagenic chemicals for redifferentiating them into better plants. Germination may be reduced due to damage of embryo because of increase of temperature during radiation. It may also be due to delay or inhibition of metabolic, physiological and biochemical activities essential for seed germination. In other monocot as well as dicot plant species germination percentage were found to be inversely proportional to doses.

5.6.2 Survivability of treated protocorms and effect of mutagens on growth:

Survivality and growth (plant height, leaf length, leaf breadth, No. of leaves, root length, No. of roots and fresh weight) of seedlings are widely accepted indices in determining radio sensitivity. These indices are closely interrelated phenomena. In the present study it was observed that in gamma treated embryos, green pigmentation developed late and also leaves and roots developed later than the control. It indicates that gamma irradiation obstructed development of green pigmentation as well as in root and leaf development also.

In S. plicata protocorms LD₅₀ was found in between 1.5 to 2.0 Kr when irradiated with gamma rays. Datta and Biswas (1984) reported LD₅₀ of different plant species. In EMS treated protocorms, it was observed to be at 0.3% for 30 minutes and 0.1% for 60 minutes treatment. Griesbach (1983) observed LD₅₀ in Phalaenopsis at much higher concentration i.e., 0.5% concentration for 30 minutes. However, in S. plicata LD₅₀ for colchicine treatment, it was found to be in between 0.35% to 0.40% for 30 minutes.

In the present investigation all the growth parameters except number of leaves and leaf breadth exhibited decreasing trend with the dose/concentrations, which is in agreement
with other workers. In different plant species, dose-dependent decrease in survivability and plant growth was also observed by Caldecott (1955), Scossiroli et al. (1961), Matsumura (1962), Gaul (1964), Purusuthaman (1967), Srivastava and Raina (1981), Kaicker and Dhyani (1983), Datta and Bhattacharjee (1984) and Puspalatha et al. (1992).

Survivability rate and reduction of seedlings growth were due to damaged cells, somatic lesions and disturbed biochemical and physiological functions induced by mutagens. As a result, a competition between these affected and normal cells begins during germination, provided it escape embryo damage. This may retard growth or even lead to mortality and delay in maturity. Mortality and slow growth rate were common during early stages than during latter growth stage. Chromosomal damage and mitotic inhibitions are also associated with seedling growth which may bring about such reduction (Sparrow et al., 1952). However, Evans and Sparrow (1961) were of opinion that radiation-induced growth inhibition was basically caused by genetic damages arising due to chromosomal aberrations. Physiological injury in the seed and seedlings may be considered as the main cause of reduction in mean height of M1 plants (Ignacimuthu and Babu, 1988). Sparrow et al. (1965) reported that such retardation may be due to DNA breakage and reduction in DNA content. But Gunkel and Sparrow (1961) opined that decrease of growth parameters were due to the effect of ionising radiations on auxin synthesis.

The EMS treated protocorms showed increase in number of leaves. Survived plants under lower concentrations gradually revived to normal leaf numbers. Appearance of higher number of leaves due to EMS treatment of protocorms were chimeric in nature.

5.6.3 Cytological studies:

Chromosomal abnormalities are the measure of the effect of mutagen and radiosensitivity of a species. Reduction in mitotic index as well as increase in chromosomal abnormalities with the increase of dose/concentration as observed in present investigation was also reported in other plant species. In all the three mutagen treatments chromosomal...
abnormalities were directly proportional to mitotic index i.e., with reduction in mitotic index (or increase in dose/concentration treatment) there was increase in abnormality percentage.

Several views have been put forward to explain the mechanism of chromosome breakage and production of aberration in mitotic cells. Different kinds of anomalies such as micronuclei, fragments, laggards, stickiness, bridge, scatterness etc. have been observed in the present study. Mitotic abnormalities were also reported by number of workers and found to be dose/concentration dependent increase (Swaminathan et al., 1962; Kallo, 1972; Ojomo and Chedha, 1971; Prasad and Das, 1980; Bandhyopadhyya and Bose; 1983; Gupta and Roy, 1985). Fragments, laggards and stickiness were found in higher percentage. These abnormalities observed in the present work was also observed by Prasad (1972), Kallo (1972), Shingh and Godward (1974), Srivastava and Raina (1981).

5.6.4 Chlorophyll mutation:

Altogether 8 types of viable and non-viable chlorophyll mutants were isolated in the present investigation. The frequency of chlorophyll mutants increased directly with increasing dose/concentration of mutagens. Dose dependent increase in the spectrum of chlorophyll mutation was also reported earlier in barely (Gaul, 1964), mung bean (Bahl and Gupta, 1982), in Lathyrus sativus (Marker, 1976, Prasad and Das, 1980), in Catharanthus (Venkataswarlu et al., 1988).

EMS was found to be more powerful than gamma rays and colchicine in producing chlorophyll mutation. Colchicine was observed to be less effective than other two mutagens. However, except viresence mutants, all other seven chlorophyll mutants were non viable. It is clear that different mutants obtained in this investigation were due to mutagenic effect.

5.6.5 Flower colour mutation:

Acclimatized plantlets started flowering after 27 to 30 months from the date of inoculation of embryos. Out of it one flower colour mutant was isolated. The mutant
showed light purple colour (or white) flower colour. Bracts and pedicels of the mutant was also light in colour. There was no change in flower shape or size.

The flower of normal (control) *S. plicata* under visible light are essentially monochromatic bluish purple in colour but mutants was uniformly light (white) purple in colour. *S. plicata* generally propagate from pseudobulbs and breed true to its flower colour. However, from this flower colour variant *i.e.*, light purple colour mutant it reveals that flower colour in *S. plicata* may be regulated by quantitative genes. Number of genes responsible for it is under study. From the available information, it may be explained that the genotype of the normal purple flower colour parent is homozygous dominant (Pp) and light to be heterozygous (Pp). Flower colour mutant may have appeared as a result of mutation in one of its dominant alleles *i.e.*, conversion of dominant allele to its recessive form. Occurrence of somaclonal variants *in vitro* cultured plants have been reported by Evans *et al.* (1986), Mitra (1985). Moreover, only one light purple flower colour mutant was observed in a population of about 350 plants. Test of probability was not possible, as many plants died during rearing and acclimatization. More investigation is needed to establish the genetic control of flower colour in *S. plicata*.

5.6.6 Study of stomata:

Increase in the size following colchicine treatment is a common phenomenon. Leaves of colchicine treated protocorms showed increase in breadth which had less number of larger size stomata but contained maximum number of chloroplastids. Results showed that size of the stomata increased with increase in colchicine concentration. Though concentration of colchicine was directly correlated with size but negatively related to frequency of stomata per unit area. On the other hand number of chloroplastids per guard cell of stomata increased directly with concentration. The mean difference between normal stomata containing number of chloroplastids versus treated showed significant difference at 0.001 probability level.
A positive correlation between number of stomatal chloroplastids and ploidy level has been analysed in tea (Ahmed and Shing, 1993), mulberry (Sikdar et al., 1986; Dwivedi et al., 1986), red clover, berseem and white clover (Najeevska and Speckmann, 1968) and in Gossypium spp. (Choudhury and Burrow, 1975, Krishnaswami and Andal, 1978 and Mehtrce, 1982). However, such relationship could not be derived in S. phcata or any other orchid species.

Grisbech (1983) also confirmed the induction of polyploidy in Phalaenopsis with colchicine treatment. Similar result was also observed by Chavedej and Becker (1984) in D. wallichii.

DISCUSSION - VI

5.7.1 Acclimatization of in vitro cultured platlets:

There was variation in the survivality rate of different group of plants raised on the basis of age and potting mixture. The control treatment (C-0) which contains only charcoal, brick bats and sand showed lower survivality rate, whereas plants grown in treatment mixture No 16 (C-16) showed cent percent survivality rate. This indicates that potting mixture has definite role in the survivality rate. In the present investigation, mixture of charcoal, brick bats, Farm Yard Manure (F Y M.) and sand showed best result. Other mixture viz. charcoal + brick bats + sand + water hyacinth, leaf mold + brick bats + charcoal + water hyacinth + sand and F.Y.M + Vermiculite + sand + leaf mold also showed encouraging survival rate. deFossard (1985) described that very successful acclimatization could be obtained by using peat alone. Vermiculite, loam and peat; perlite, pulverised pine bark and peat as well as mixture of peat, river sand and perlite; vermiculite and sand mixture also did well. Hegde et al. (1988) reported that a considerable population of Cymbidium hybrid could be acclimatized by transplanting the in vitro cultured seedling in the earthen pots containing
sterile mixture of humous and sandy soil in equal proportions alongwith brick bats. Sharma and Roy (1992) also tried a variety of organic materials for acclimatization of *Cymbidium aloifolium* such as 1) dried roots and leaves of water hyacinth, 2) dried roots and leaves of salvania, 3) dried rice straw and 4) dried foliage was mixed with charcoal, brick bats, sand and FYM. The optimum growth and development of seedlings was achieved in potting mixture comprising dried leaves and roots of water hyacinth, sand, brick pieces and charcoal.

The length and number of roots are also found to play an important role in acclimatization. The seedlings having maximum number and length of roots showed highest survivality rate. In the present investigation it was observed that, 6 months old platlets having average root length of 5.0 to 6.5 cm and 9.1 to 11 average root number showed cent per cent survivality in mixture No.16. So it is clear from the data and statistical analysis that age of the seedling potting mixture and length and number of roots have direct correlation with the survival percentage of *in vitro* cultured plantlets. Jagannathan (1985) also mentioned that length and number of roots before transfer to the community pots should be ensured by modifying the period of incubation in medium.

The transition of plantlets from nearly 100 per cent humidity in the test tubes to the low humidity under ambient conditions is of major importance for survival. In some species Jagannathan (1985) observed that low humidity can result in desiccation. So plants transferred to pots were covered with porous polythene bags. The bags were removed gradually when humidity was maintained at 80 to 90 per cent by spraying water at frequent intervals. He also emphasised that gradual increase in light intensity, a suitable photoperiod and optimum air and temperature are essential and require determination for each cultivar. It was observed that if plantlets were directly transferred to green house, survival percentage abruptly dropped down. But gradual transfer from culture laboratory (after one month) to glass house, from glass house to green house and then to field showed good survival of plantlets. Specific temperature (25° ± 1°C and 30° ± 1°C for winter and summer respectively) was maintained.
Higher phosphate containing fertilizer mixture has been claimed to give 50 fold increase in growth than other nutrients solution (Centro International de Agricultura Tropical, 1982). Therefore, spraying mixture of urea and superphosphate gave good result in the growth of seedlings. Damage of plantlets by different fungus species could be controlled by application of Broad Spectrum fungicides.

The length of inflorescence and number of flowers in each inflorescence emerged from acclimatized plants were observed to vary in treatment combination. As all the plants were treated with same light, temperature and nutrient solution and fertilizer, so this variation was due to difference in potting mixture.