5. Discussion:

5.1. Asymbiotic seed culture:

Several investigators have worked on the asymbiotic seed germination of orchid seeds and formulated a considerable number of culture media, with an aim to improve seed germination and subsequent seedling growth (Wynd 1933a,b; Burgeff 1936; Curtis 1943; Yates and Curtis 1949; Vacin and Went 1949; Withner 1959; Raghvan and Torrey 1964; Kano 1965; Mitra et al. 1976; Clements and Ellyard 1979; Clements 1982; De Pauw and Remphrey 1993), followed by the pioneering works of Knudson (Knudson 1921, 1922, 1925 and 1946). It was found that various species often show differential response to the culture media depending on the specific concentrations and ratios of various inorganic salts (Arditti 1967, 1979, 1982; Oliva and Arditti 1984; Stoutamire 1974; Warcup 1975; Wrigley 1976; Fast 1982; Yadav and Bose 1989). Other workers have attempted to improve the culture response by the addition of various organic supplements and PGRs to the basal culture media. Among the most commonly used organic supplements had been different vitamins, amino acids, coconut water, urea, casein hydrolysate, yeast extract, peptone and banana extract (Arditti 1967; Mitra 1986; Yadav and Bose 1989).

In the present study, modified version of Knudson’s C was employed as basal medium for *in vitro* germination of seeds. This medium was modified by the addition of coconut water, peptone, various vitamins, myo-inositol and PGRs [details of medium modifications are shown in the Material and Method section].

The effects of basal medium:-

In the present study it was observed that the seeds of different species of orchids showed slightly better germination in the Knudson’s C basal medium than other basal medium types. But after germination, the protocorms grew equally well in same medium. However, regarding post-germination events, the protocorms of different species showed preferences for specific supplementation in the media. In the culture of different seeds, Knudson’s C medium proved the most efficient not only for prevention of a high frequency of protocorm death that occurred in Knudson’s C basal medium, but also for superior growth of seedlings. From these results it is
apparent that the nutritional requirements for initiation of germination vary considerably from that required for optimum growth of protocorms.

Earlier studies showed that seeds of many terrestrial orchids of temperate regions often exhibit stringent nutritional requirements for initiation of germination (Fast 1982; Van Waes and Debergh 1986; De Pauw et al. 1995).

In general, those orchid species prefer a comparatively low-salt medium and the source of nitrogen also seems to be vital for the germination. Mitra (1971) showed that seeds taken from dehisced capsules of Cattleya germinated within 18 days of inoculation in the media of Vacin and Went (1949) and Raghavan and Torrey’s medium (1963,1964) and took a much longer time to germinate (41 days). In contrast, Kanjilal et al. (1998a) showed that seeds of rare and endangered orchid species germinated within 20 to 25 days in both Knudson’s C and Vacin and Went media at quite high frequency. The significant differences observed in these studies, regarding the time required for germination of seeds could be due to the differences in the age of seeds (i.e. the degree of maturity), as it has been shown by Mitra (1971). However, the observation made by Mitra (1971) about the failure of seed germination in Knudson’s C medium is surprising and difficult to interpret.

Effect of peptone:

Peptone is a complex additive that contains many amino acids, several amides and a number of vitamins (Powell and Arditti 1975; Oliva and Arditti 1984). The reports of the application of peptone in orchid culture media are less common and its effects are not yet well established (Arditti and Ernst 1993; Mukhopadhyay and Roy 1994). The major amino acids that have been found in peptone are glycine (23%), glutamic acid (11%), arginine (8%), aspartic acid (59%), lysine (4.3%), leucine (3.5%), valine (3.2%), tyrosine (2.3%) and phenyl alanine (2.3%). Peptone also contains various minerals (e.g. P, K, Na, Mg, Ca, Cl, Mn, Pb, As, Cu and Zn) and vitamins (e.g. pyridoxine, biotin, thiamine, niacin and riboflavin).

Previous observations showed that the effects of peptone on orchid seed germination varied with the species. Peptone enhanced the rate of germination and growth of protocorms in Cattleya (Curtis 1943; Alberts 1953; Stoutamire 1964; Harvais 1973, 1974; Linden 1980; Oliva and Arditti 1984), Cypripedium (Liddell 1953), Dendrobium (Alberts 1953), Vanilla (Bouriquet
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1947) and various European terrestrial orchids. Stoutamire (1964) pointed out that seeds of *Spiranthes cernua* could germinate only in medium supplemented with peptone. According to Prasad and Mitra (1975) the use of 1% peptone proved effective for both seed germination and better growth of protocorms. Seeds of *Acampe praemorsa* showed better germination in presence of 0.05 and 0.1% peptone. Again for *Vanda*, peptone is ineffective for early stages of germination (Mathews and Rao 1980; Mitra 1986).

In the present study, beneficial effect of peptone, in the early phase of seed germination was noted in the culture of *Dendrobium*, but variation occurred among the different species. In case of *D. chrysanthum*, frequency of seed germination was improved at 0.4% peptone. In KC medium the effect of peptone on germination was either inhibitory or insignificant.

In case of *D. farmeri*, germination of seeds maximized with the application of 0.1% peptone. In *D. parishii*, the stimulatory effect of peptone was noted only at 0.2% level, while at 0.1% level it was less effective for germination. In case of *Hygrochilus parishii* seeds 0.4% peptone showed a insignificant stimulatory effect on germination. In *Thunia alba*, the application of 0.05-0.4% peptone showed no significant change on the percentage of seed germination.

Regarding the effects of peptone on the post-germination events, it was observed that the protocorms of different species were extremely sensitive to peptone and their growth rates could be enhanced considerably, if applied at appropriate concentrations and that the effective concentration varied with the species. In certain species, the effect of peptone also depended on the composition of the basal medium.

For seedling growth of *D. chrysanthum*, peptone was not stimulatory for production of leafy protocorms at any concentration. In case of *D. farmeri*, peptone exerted a significant effect on leaf formation as well as on root initiation, though at 0.4% concentration root induction was stunted. In *D. parishii* though peptone exerted no significant effect on rooting, but it was stimulatory for leaf formation as well as overall seedling growth and development, 0.4% being optimal. Similar improving effect of peptone on protocorm growth and development in *Dendrobium* (Alberts 1953, Morel 1974) and in *Vanda* (Lami 1927, Morel 1974, Mathews and Rao 1980; Mitra 1986). Growth of the protocorms of *H. parishii* remains restricted with the use of 0.05-0.4% peptone. In case of *Thunia alba*, though peptone exerted no significant effect on
rooting, it was stimulatory for leaf formation as well as overall seedling growth and development. Such promoting effects have also been reported on *Cyripedium* (Withner 1953), *Dendrobium, Cyripedium reginae, Cymbidium virescense, Cymbidium* hybrid, *Paphiopedium insignis* (Kano 1965) and *Phalaenopsis* hybrids (Ernst 1966b). These observations indicated that the growth of orchid embryos was more regular and generally better in presence of peptone and the present observation supports this.

It has been mentioned earlier that effects of peptone or other additives may vary according to the basal medium used depending on the nitrogen level in the basal solution (Kano 1965; Oyamada and Takano 1985; Ichihashi and Islam 1999). For example, in the basal medium of KC, the species of *Dendrobium, Hygrochilus parishii* and *Thunia alba* showed very high percentage of seed germination and a comparatively better growth of seedlings. The application of peptone in these media also improved the survival of protocorms considerably and also enhanced the growth rate of seedlings. The variable responses occurred may be due to nitrogen content of the culture medium. The total nitrogen content of KC (1946) medium is 224.7 mg/l and supplementation of 0.1% of peptone to this medium will provide additional 161.6mg/l nitrogen in the culture medium. However, other components of peptone could also be critical for the observed growth responses.

Protocorm necrosis is a very common phenomenon in asymbiotic seed culture of orchids. Variable rates of protocorm mortality in the basal media and their subsequent enhancement in presence of peptone indicated that the observed protocorm necrosis could be due to improper nutrition, as indicated by Stoutamire (1974). Addition of peptone (0.1% or more) in the basal media to some extent prevented such necrosis in *D. chrysanthum, D. farmeri* and *D. parishii*.

In seed culture of *D. chrysanthum*, the addition of .05%-0.4% peptone in KC medium proved beneficial, as shown by the reduction of protocorm necrosis and promotion of seedling growth. Therefore, it appears that the total nitrogen content of the media plays an important role in the survival of early protocorm as also observed in *Vanda tessellate* (Roy 2000).

**Effect of coconut water:**

Coconut water (CW), liquid endosperm of *Cocos nucifera* L. fruit, is an undefined complex organic substance. It has been successfully employed for the culture of different plant
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species (Arditti and Earnst 1993; Sutter 1996). It has been shown that, coconut water contains a large number of amino acids, like alanine, arginine, aspartic acid, glutamic acid, glycine, leucine etc. and other substances, like (e.g. citric acid and malic acid), vitamins (biotin, folic acid, niacin, pantothenic acid, riboflavin, pyridoxine and thiamine) and several plant hormones like auxins, cytokinins and gibberellins (Raghavan 1966, 1976; Arditti and Ernst 1993). Van Overbeek et al. (1941 and 1942) and Arditti and Ernst (1993) used coconut water for the culture of Datura embryos and showed its beneficial effects. Earlier Mariat (1948) used CW in an orchid culture medium, where it was shown to inhibit germination of Cattleya lawrenceoma seeds. In orchid seed culture the effect of CW was shown to be species specific. According to Kotomori and Murashige (1965), germination of Dendrobium seeds was unaffected by its application, but the growth of protocorms was inhibited by its presence. However, improved germination as well as growth was reported in Cymbidium mastersii (Prasad and Mitra 1975), Acampe praemorsa (Krishnamohan and Jorapur, 1986) and Spathoglottis plicata (Chennaveeraiah and Patil, 1975). In Arundina bambusifolia, rate of germination and growth of seedlings enhanced by the application of coconut water (Kanjilal et al. 1998a). Coconut water exerted a stimulatory effect in seed culture of Paphiopedilum, Vanilla and Cattleya (Hegarty 1955; Arditti 1967). For Cattleya, it was excellent when applied in a mixture with tomato and pineapple juice plus banana pulp and vitamin (Lawrence and Arditti 1964a, b). The effects of coconut water is species specific and in addition as it is a natural product, the constituents as well as activity of liquid endosperm may depend on factors like age, variety, seasons and means of in vitro sterilization (Arditti and Ernst 1993).

In the present study the effects of coconut water vary with the composition of basal medium as well as the presence of other organic substances. Regarding the growth rate of protocorms, the effect of coconut water was noted in KC medium, which corroborates the observation of Kanjilal et al. (1998a).

In D. chrysanthum significantly high frequency of seed germination was observed in KC medium, but in all the treatments growth of the protocorms remains arrested at leaf primordial stage. For D.farmeri and D. parishii, all concentrations of coconut water (5-40%) significantly enhanced the protocorm survival and also showed enhanced growth and development. Coconut water could not trigger root formation. This result is partially in line with the previous report on
Phalaenopsis seedlings that proliferated substantially in KC medium supplemented with 15% CW, whereas the organogenesis was retarded in presence of CW (Ernst 1967a; Mitra 1986). In H. parishii 5-10% coconut water showed growth promoting effect, but at 20% level it proved inhibitory. Therefore, it may be concluded that the individual effects of its constituents on germination or growth events of the species involved could not be inferred.

**Effects of yeast-extract:**

Yeast-extract (YE) contains water-soluble components of the yeast cell, like carbohydrates, amino acids, peptides vitamins and salts. In many orchid species yeast-extract has been successfully used for seed germination as well as in protocorm development (Curtis 1947a; Flamce 1978; Mariot 1948; Mathews and Rao 1980; Prasrad and Mitra 1975). In the present study germination of different species of *Dendrobium* was found to be stimulated by 0.05% YE, but remains unaffected with further increase in the concentration. Previously, it has been shown that the effect of YE on germination of *Dendrobium* could be both stimulatory as well as inhibitory (Kano 1965 and Ito 1955). This result partly contradicts the present finding on *Dendrobium* species. Schaffstein (1938) and Tonnier (1954a) also reported that application of YE in the media enhanced the germination and the present observation also contradicts this report.

It has been mentioned earlier that protocorm necrosis frequently occurs with orchid seed culture. Protocorm necrosis of *D.chrysanthum* was effectively overcome with the employment of 0.00625% YE, but not so, with further increase in its concentration from .0125%-0.05%. Growth and development of protocorm also became abruptly stunted with the application of YE. This result supports the observation of Kano (1965), where the effect of YE in the media was found to be inhibitory. In contrast in the present study, protocorm survival in *D.farmeri* and *D.parishii* was unaffected by YE. But for protocorm growth and development, YE were found to be beneficial as shown by higher frequency of protocorm producing leafy seedlings. Protocorm survival in *Hygrochillus parishii*, on the other hand is optimum in low concentration of YE. But protocorm necrosis could be completely overcome with the application of higher concentration of (.05%) YE. Moreover, the growth and development of protocorm was inhibited by the use of yeast extract. It should be mentioned that, YE has a high content of amino acids (Rasmussen 1995), which are essentially required for growth and development of the orchid protocorms, as
with other life forms. Thus it can be stated that regarding post germination events, nitrogen or the constituents of yeast extract either maintain or stimulate an unorganized growth of the seedlings. Therefore, the effects of YE on orchid seed germination and post-germination events varied greatly with the species. However, its effects are mostly dependent on the basal medium used. The total nitrogen content of KC medium (1946) is 224.7 mg/l; addition of YE to KC medium will provide excess nitrogen (Hadley and Ong 1978) that would promote the germination requirements of *Dendrobium* seeds, while supra-optimal levels of nitrogen obtained with addition of higher concentration of YE neither stimulated nor inhibited the germination process.

**Effects of Plant growth regulators (PGR) and their modulators:**

The use of different types of PGRs in orchid seed culture is very common and their results also vary considerably with the species. Among the different type of auxins; IAA, IBA, 2,4D and NAA have been most commonly used in orchid seed culture and their effects also varied with orchid species (Mitra 1986). However, in many orchids, endogenous auxins are sufficient for seed germination. So they do not require exogenous auxins for effective seed germination. In *Cymbidium mastersii* IAA promoted seed germination at very low concentrations (0.1 mg/l) (Prasad and Mitra 1975) but had no effect on protocorm growth. Whereas for Vandaceous taxa 0.25 mg/l IAA was effective for seed germination (Vij et al. 1981). NAA also promoted germination of seeds as well as proliferation of protocorms. In *Vanda* hybrid (Mathews and Rao 1980; Mitra 1986). Meyer and Pelloux (1948) stated that stimulation of both seed germination and protocorm growth of *Cattleya warneri* occurred by NAA and IBA, whereas in *Epidendrum nocturnum* the use of NAA promoted growth of roots and shoots (Yates and Curtis 1949). On the other hand 2, 4-D either inhibited germination or induced callusing response of the seeds (Mitra 1986). The use of IBA, along with other organic supplements, promoted excellent germination in *Phalaenopsis*, *Cattleya*, *Cymbidium*, *Oncidium* and *Vanilla planifolia* (Hegarty, 1955). It is generally used to initiate rooting of the seedling rather than seed germination studies.

In orchid seed germination studies, gibberellins are rarely used, whereas cytokinins are frequently used. Regarding the germination of orchid seeds in the presence of exogenous cytokinins three types of responses have been recorded: either germination is improved or inhibited or there is no effect (De Pauw et al. 1995; Roy and Banerjee 2001). The orchid seeds that do not require exogenous cytokinins for germination are known as cytokinin autonomous,
since they contain sufficient endogenous cytokinin (De Pauw et al. 1995; Mercier and Kerbauy 1991; Roy and Banerjee 2001). Such requirement for germination of orchid seeds is considerably related to the utilization of lipids that constitute primary storage material in most orchid seeds. It has been also observed that germination will not continue unless storage lipid is mobilized (De Pauw et al. 1995; Manning and Van Staden 1987; Roy and Banerjee 2001). In Cyripedium reginae, C. calceolus and C. candidum, a definite cytokinin preference for germination and protocorm growth has been reported (Harvais 1982; Van Waes and Debergh 1986; De Pauw et al. 1995). In the present study a preliminary investigation was performed on the effects of PGRs (auxin and cytokinins) on seed culture of D.aphyllum, D. parishii, D. farmeri, Hygrochilus parishii and Thunia alba.

From the study with D. aphylum it appears that this species is auxin autonomous so that no exogenous auxin was required to attain 100% germination. The use of different auxins produced no significant changes on the frequency of germination, but showed both beneficial and adverse effects on the growth of protocorms. Orchid seeds generally contain only traces or no endogenous auxin and Dendrobium seeds fall in the latter category (Poddelbnaya-Arnoldi and Zinger 1961), which contradicts the present result where endogenous auxins caused significant frequency of germination. Another negative aspect of 2, 4-D application was that it caused high protocorm mortality. This 2, 4-D induced protocorm necrosis being the result of nutrient depletion in the culture media caused by the fast growing protocorms could be overruled, which also opposed the suggestion made by Stoutamire (1974). In previous study the auxin-induced protocorm mortality has been reported in C. reginae (Harvais 1982). Seedling growth in D. aphylum was optimum on PGR-free medium, where 78.55% seedling reached upto stage 3. No significant changes occurred in auxin treated medium upto the production of leafy staged protocorms. Application of high concentrations of NAA (2-8µM) was found to be stimulatory for producing rooted protocorms.

From the study with D. chrysanthum it appears that this species is auxin and cytokinin autonomous (Mercier and Kerbauy 1991; De Pauw et al. 1995; Roy and Banerjee 2001) which did not cause any significant effect on germination. Moreover, application of exogenous BAP seems to be inhibitory for protocorm growth and almost no seedling reached stage 2. On the other hand, NAA caused protocorm necrosis at higher concentrations (2-4µM) which
corroborates with the suggestion made by Poddubnaya and Zinger (1961). However, high protocorm mortality in this species with high NAA might have been caused due to direct toxic effect of NAA. Here, NAA also had inhibitory effect on protocorm growth. Most of the seedling growths remain stunted at stage 1 and stage 2.

Seed germination in *D.farmeri* remained unaffected by application of exogenous auxin. So that it may be concluded that this species is auxin autonomous. Moreover, application of excess NAA (4µM) seems to be inhibitory for germination. Another negative aspect of NAA application was that it caused high protocorm mortality. This NAA induced protocorm necrosis may be the result of direct toxic effect of NAA. Since the protocorms showed certain level of differentiation, no phenolic substance was secreted and no correspondence of growth was observed with high NAA concentration with which necrosis resulted. None of the existing hypotheses, such as ‘non-viable’ embryo hypothesis (DePauw *et al.* 1993), ‘phenolics synthesis’ hypothesis (Harvais 1982) or improper nutrient supply hypothesis (Stoutamire 1964) could be taken into account. However with application of BAP, frequency of germination increased up to 74% compared to control and it completely hindered protocorm death. Seedling growth in *D.farmeri* was inhibited with BAP and mostly remained restricted at stage 1 or 2. On the other hand, NAA promoted seedling growth and development. Majority of the protocorms produced well developed leaves and roots.

Seeds of *D.nobile* proved to be auxin autonomous for germination. Like many other orchid species, seeds did not require exogenous auxin for optimum germination. 2-4D at 4-8µM impeded germination. On the other hand NAA and 2-4D caused protocorm necrosis at higher concentrations (2-8µM). These findings corroborate earlier reports. For optimal seedling growth, auxin was not required which is also in compliance with earlier reports on *Euanthe*, *Phalaenopsis*, *Vanda* (Burgeff 1936) with NAA. For *D.nobile*, 2-4D at 2-4 µM level hindered the seedling growth and 100% seedlings remain restricted to stage 1. For the production of leafy staged protocorm, IAA was better than NAA. NAA and BAP did not affect germination in *D.parishii*. Exogenous PGR did not cause any significant change in the frequency of germination. In contrast to NAA, BAP inhibited protocorm growth and development. Maximum seedling growth was stunted at stage 1 and stage 2. Although the inhibitory effects of BAP could be due to its particular level of application, it can be concluded that *D.parishii* has no cytokinin
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for which maximum protocorms could reach at stage 3 in the species, *D. primulinum* and *D. nobile*. It may be mentioned that $\rho$-coumaric acid is known to stimulate IAA oxidase activity and enhance break-down of endogenous IAA. Also, the inhibitors of polar transport of auxin, namely quercetin and TIBA were applied in the seed culture media of *D. primulinum*. Both the inhibitors had no effect on seedling growth. It indicates that, either endogenous IAA is not at all involved in protocorm growth, or these inhibitors in the experimental range were not sufficient to hinder auxin transport, so that the activity of endogenous IAA remained unaffected.

5.2. *In vitro* propagation:

A. Regeneration via shoot tip culture

During arising of orchid seedlings through *in vitro* seed germination technique, it was noted that the protocorms or even the leafy seedlings of all the species studied occasionally proliferated in the absence of exogenous PGRs. In most of the instances it occurred by means of adventitious PLB formation or in certain species like *D.aphyllum*, *D.parishii*, *D. nobile*, *D. farmeri* and *Hygrichilus. parishii* through adventitious shoot formation. The results have shown that the method could be successfully employed as an alternative to clonal propagation of seedlings.

Plants produced from shoot-tips come from essentially primordial stem tissue (Churchill *et al.* 1973). The development of plants from shoot-tips has been investigated for about 50 years (Morel 1960, 1963, 1964a, b, 1965, 1966; Champagnat 1965; Champagnat *et al.* 1966; Champgnat and Morel 1969). In the present study, shoot-tip culture was performed with *D.aphyllum*, *D.parishii*, *D.nobile* and *D.farmeri*.

For *D.aphyllum*, *D.nobile*, *D.parishii* and *D.farmeri*, an auxin (NAA), cytokinins (BAP and Kin) and a cytokinin like substance (TDZ) were used in different concentrations and combinations. Shoot-tip explants from *D.aphyllum* and *D.nobile* responded mostly via adventitious shoot formation and to a lesser extent via formation of direct PLBs. But in *D.farmeri* and *D.parishii* shoot-tip explants responded via two morphogenetic pathways, like direct adventitious shoot and PLB formation and indirect embryogenic callus induction.
The lethal browning of the shoot-tip explants is quite common and is often related to the degree of wounding (Compton and Preece 1986), relative size of the explants and differential sensitivity of various explants, all of which determine exudation and consequent explant survival (Seeni and Latha 1992). In *D.aphyllum*, both NAA and TDZ alone were inhibitory for seedling survival and increased the frequency of explant necrosis 16µM TDZ. However, their combination exerted a synergistic effect and a marked stimulation in seedling survival was evident. While in NAA-BAP combination both NAA and BAP had inhibitory effect on seedling survival.

In *D.nobile*, PGR-free control medium was optimum for seedling survival. Here, application of exogenous PGR increased the frequency of shoot-tip necrosis. Both kinetin and TDZ were inhibitory for seedling survival. Kinetin at 4µM caused 100% shoot-tip necrosis occurred. In *D.parishii* BAP and TDZ exerted positive response on survival of shoot-tip either alone or in combination with NAA, but Kinetin promoted shoot-tip necrosis though at 8-16 µM concentration it was quite well for survival of the shoot-tip. On the other hand, in *D. farmeri* cytokinin-like compound TDZ caused maximum shoot-tip necrosis. But kinetin and BAP either alone or in combination exerted a synergistic effect on survival of shoot-tip. On the other hand, NAA supported seedling survival unless its highest concentration (2 µM) was applied.

**B. Regeneration through adventitious shoot formation:**

In four species of *Dendrobium*, adventitious shoot formation occurred in the PGR-free medium very well. Such a technique was reported earlier by Thomas and Michael (2007) and Kishor and Sharma (2008) using agar solidified medium. This could be due to a relatively weak apical dominance of these plants *in vitro*. Previously it has been shown that a number of environmental factors, such as inorganic nutrients (especially nitrogen), water, light and gaseous atmosphere could affect the apical dominance phenomena (Phillips 1987; Rubinstein and Nagao 1976; Hillman 1987). However, it could also be noted that the *in vitro* branching habit of different species showed strong resemblance with their mode of growth *in situ*. In the control medium *in vitro* adventitious shoot was noted only in *D. aphyllum*, *D.farmeri*, *D.nobile* and *D. parishii*, which are characterized by their habitual lateral growth *in situ*. The formation of adventitious shoot was easily achieved by the application of auxin and cytokinin either alone or in combination.
In *D. aphyllum* most of the surviving explants propagated through adventitious shoot formation. Here, the frequency of axillary branching showed a direct correlation with the concentration of NAA. In the combined presence of BAP and TDZ, the enhancement of NAA sharply increased the rate of shoot formation. BAP alone exerted this adventitious shoot forming effect up to 4µM level. Further enhancement of BAP concentration (8-16 µM) proved stimulatory only in presence of 2 µM NAA. Maximum number of shoots was observed at 8 µM BAP+2 µM NAA forming 3 shoots/explant. On the other hand, TDZ was effective for shoot formation at 2 µM level combined with 1 µM NAA. The effect of NAA, when applied along with relatively low concentration of TDZ (below 2 µM), was best at 1 µM level. But in the presence of 2 µM NAA, the use of higher concentrations of TDZ proved ineffective for shoot formation. In *D. nobile*, adventitious shoot formation was maximum in presence of 1 µM and 2 µM TDZ along with 1 µM and 2 µM NAA. However, mean number of shoots per explant was optimum at 1 µM TDZ. Further enhancement of TDZ showed inhibitory effect. In contrast to NAA-TDZ combination, the frequency of shooting was also affected by the combined treatment of kinetin and NAA. Here, shoot promoting effect was best observed in 1 µM NAA without kinetin. Kinetin at 1-16µM concentration showed slightly inhibitory effect on this type of morphogenetic response.

In *D. parishii* adventitious shooting was optimum at 4 µM BAP alone. Application of NAA slightly lowered the frequency. Higher concentrations of BAP (8-16 µM) together with NAA also lowered the frequency of shooting. In contrast to NAA-BAP combination, combination of NAA and kinetin, induced a sharply increased formation of adventitious shoots. However, when kinetin was used alone, this shoot promoting effect did not prove satisfactory rather at higher level it proved inhibitory. The effect of kinetin when applied along with relatively low concentrations of NAA was best at 8 µM. But the combined use of TDZ and NAA, induced about 95% shooting response at 8 µM TDZ.

In *D. farmeri*, exogenous auxin (NAA) and two different cytokinin combinations proved satisfactory for shoot formation. But the combined use of BAP and NAA showed totally different results. BAP at 2-16 µM along with 1-2 µM NAA had an inhibitory effect on the frequency of shoot formation. Also, combined treatments of TDZ and NAA were not effective.
for shoot formation. The employment of comparatively high TDZ: NAA ratio tended to suppress the response.

It is generally known that in an intact plant the apical bud exerts an inhibitory influence on axillary buds, preventing their development into leafy shoots. According to Hillman (1987) this correlative inhibition of lateral buds is considered to be controlled by the endogenous auxin, cytokinin and under certain circumstances even by ethylene. However in a number of species, exogenous cytokinin could release lateral buds from the correlative inhibition and thereby promote lateral branching of the shoots (Wickson and Thimann 1958; Street and Opick 1986; Hillman 1987). This observation was later employed successfully in the \textit{in vitro} multiplication of a number of species by repeated formation of adventitious shoots (George 1993; Kane 1996). Beside the role of exogenous cytokinin in adventitious shoot proliferation, the application of auxins has also shown positive response in certain species. As for example, in \textit{Digitalis thapsi} L. the use of different auxins in the absence of cytokinins was able to induce shoot multiplication, although best results were obtained in the combined use of auxin and cytokinin (Herrera \textit{et al.} 1990). The synergistic action of an auxin (NAA) and a cytokinin (TDZ) in the formation of multiple shoots supports earlier reports on orchids. Ket \textit{et al.} (2004) showed that multiple shoot proliferation was induced by the application of exogenous cytokinins. Similar finding was also observed by Seeni and Latha (2000) in Blue Vanda. Similarly in the present study the use of both cytokinins (BAP and Kin), cytokinin-like compound (TDZ) and auxin (NAA) either alone or in combination promoted adventitious shoots in all the species. The optimum requirement of these PGRs varied from species to species. Furthermore, these results have shown that the effect of a particular PGR (BAP, Kin, TDZ or NAA) not only depended on its applied concentrations but also on the presence of other PGRs. However, observed plant response would be the result of a complex interaction between these PGRs and also the endogenous growth substances.

In case of shoot-tip culture of orchids, it appeared that the resident axillary meristem of the condensed nodes of the basal part of shoot apices relieved apical dominance by the defunct apical meristems and proliferated to produce callus-free shoot buds (Seeni and Latha 2000). In the present study it was noted that exogenous auxin along with cytokinin also suppressed apical dominance, which led to proliferation of adventitious shoots. The success of rapid and direct shoot regeneration without a callus phase or PLB formation from the shoot-tip explants also
showed a successful pathway for rapid regeneration and multiplication of shoots. Similar results have been reported earlier on *Dendrobium* hybrids (Martin and Madassery 2006). It is the most preferred pathway of micropropagation for the commercial production of majority of plant species, since it avoids undesirable somaclonal variations (Chu 1992; Suttle 1996; Kane 1996; Phillips and Hubstenberger 1996). It has been previously observed that smaller explants like apical meristems are much more dependent upon the exogenous PGRs in the culture medium for their survival and growth (Kane 1996). Whereas in the present study, the shoot meristems were found to be solely dependent on the endogenous hormones at the time of culture initiation and this might be crucial in retaining the highly organized structure of the meristems. The breaking of apical dominance and subsequent lateral growth of the seedlings must be the result of interplay between internal physiological status of the seedlings and the growth factors provided by the culture medium. So, far as the effectiveness of TDZ over BAP in the induction of multiple shoot is concerned, Sujatha and Kumari (2007) obtained a 2-fold increase in average number of shoots by 2mg/l TDZ over 4.4 µmol BAP on multiple shoot induction in *Artemisia vulgaris*. Vinocur *et al.* (2000) also reported the superiority of TDZ over BAP on shoot regeneration of root explants of *Populus tremula*. Such a superiority of TDZ may be attributed to its special properties which make it capable to fulfill both the cytokinin and auxin requirement of various regenerative responses of different plant species (Jones *et al.* 2007). The endogenous hormones in response to TDZ application include change in the levels of phytohormones such as cytokinins and auxins, cell signaling molecules, nutrients and markers of physiological stress (Murthy *et al.* 1998).

**Regeneration through PLBs:**

In the absence of exogenous PGRs protocorms derived from seed cultures or even leafy seedlings of various species occasionally proliferated by means of PLBs. With the application of exogenous PGRs, the rate of regeneration potential of shoot-tip could be enhanced considerably. Fonnesbech (1972a) while working with *Cymbidium* protocorms showed that the rate of PLB proliferation could be accelerated by exogenous PGRs. In the present study the use of cytokinin was found most effective in enhancing the rate of PLB formation. Addition of NAA also exerted a significant synergistic effect. For direct adventitious PLB (D-PLB) formation from shoot-tips of *D. aphyllum* BAP, Kin, TDZ and NAA exerted no significant effect, when applied
individually, but they exerted strong synergistic effect in combination. No D-PLB was formed in PGR-free control or with NAA alone. TDZ and NAA promoted a significant effect, only in some specific combinations. But the frequency of PLB formation was significantly low. The use of BAP alone or in combination showed no beneficial effect on the regeneration of PLB. It should be mentioned in this regard that many orchid species require auxin and/or cytokinin for neoformation of protocorm-like bodies (PLBs) and plantlet development (Van Le et al. 1999; Roy et al. 2003; 2007). But the ratio of auxin and cytokinin for PLB formation depends upon the species studied (Arditti and Ernst 1993). In the shoot-tip culture of D. nobile, Kinetin and NAA either alone or in combination exerted a completely inhibitory effect on PLB formation. But the presence of TDZ showed a significant change in the response. However, in presence or absence of NAA the use of higher concentrations of TDZ (4, 8, 16µM) showed stimulatory effects. In both D. nobile and D.aphyllum, failure of cytokinin for PLB regeneration could be attributed to the age of the seedlings. From previous studies it is known that the regeneration of PLBs or shoot buds from leaves or roots occurs primarily due to the meristimatic activity of the young tissues (Vij et al. 1984; Colli and Kerbauy 1993). In this context, it appears that the surface cells of the stem base (from where PLB formation was most frequently observed) which showed very limited growth in older seedlings, would lose their meristematic activity very soon and hence their regenerative potential. This could also explain to some extent why proliferation via PLB regeneration is more frequently found in the young protocorms rather than in the relatively older seedlings.

Exogenous cytokinin was found to be most effective for D-PLB formation from the shoot-tip explants of D. parishii, though in control medium also PLB formation occurred. The effective concentration required for optimum response was dependent upon the type of cytokinins used. TDZ was found to be more effective than other cytokinins. Kinetin alone showed no beneficial effect. But along with NAA (1or 2 µM) kinetin induced regeneration up to 4 µM level. Kinetin is also reported to act synergistically with NAA or IAA on the formation of PLBs in monopodial epiphytic species (Arditti and Ernst 1993). Lakshmanan et al. (1995) showed that in presence of coconut milk, NAA was more efficient than BAP for producing PLBs in Aranda. The present study showed that in NAA-BAP combination, the frequency of PLB regeneration was significantly affected by addition of BAP, depending on the concentration of accompanying NAA. BAP promoted PLB formation and in most instances the best results were
obtained at 2 µM level along with 1 µM NAA. At supra-optimal level (16 µM) BAP was found inhibitory. NAA is frequently used in combination with cytokinins such as BAP, in many monopodial orchid species (Goh and Wong 1990; Valmayer et al. 1986; Van Le et al. 1999; Kim and Kim 2003; Puchooa 2004). In *Rhynchostylis gigantea*, an auxin (0.1mg/l) enhanced regeneration via protocorms (Vajrabhaya and Vajrabhaya 1970). In *Vanda* the highest PLB production was obtained from shoot-tip explants using NAA and BAP (Valmayer et al. 1986).

In shoot-tip culture of *D. farmeri*, explant produced comparatively high frequency of PLBs. In this species, BAP and TDZ were more effective than kinetin either alone or in combination with NAA. TDZ at 1 µM and 16 µM levels gave optimum frequency of D-PLBs. However it was noted that this decline in PLB number coincides with an increase in callus formation at intermediary level of both BAP and TDZ. Therefore, the apparent drop in D-PLB formation might be due to this enhanced callus formation, which effectively reduces the available explants that could follow an alternative morphogenetic pathway, rather than a direct inhibitory effect at these concentrations. So, from the above findings it can be inferred that TDZ was quite an effective cytokinin for production of PLBs. It was first reported to mimic cytokinin activity by Mok et al. (1982) and since then it has been used as a more potent cytokinin for induction of both organogenesis as well as embryogenesis in several plants like *Hordeum vulgare* (Ganeshan et al. 2003), *Oryza sativa* (Gairi and Rashid 2004), *Hyoscyamus niger* (Uranbey 2005) and *Solanum tuberosum* (Sajid and Aftab 2009).

The present observations also revealed that the TDZ-NAA combinations enhanced PLB formation significantly, as reported earlier in leaf explants of *Dendrobium* (Chung et al. 2007), *Oncidium* (Chen and Chang 2001), *Doritaenopsis* (Park et al. 2003) and *Phalaenopsis amabilis* var. *Formosa Shimadzu* (Chen and Chang 2004). However, the optimum concentration required for direct adventitious PLB regeneration was efficient only at a low level of cytokinin which was previously noted in *D. chrysotoxum* (Roy et al. 2007) and *Phalenopsis* (Gow et al. 2009).

**Effect of polyamines on shoot tip culture of *D. farmeri* and *D. nobile*:**

In *D. farmeri* and *D. nobile*, shoot-tip culture was also performed with the application of exogenous polyamines like spermidine, spermine and putrescine. Polyamines play a significant role in plant growth, development and metabolic synthesis. The application of exogenous...
polyamines has been used to improve somatic embryogenesis in cultured carrot cells (Takeda et al. 2002) and the formation of multiple shoots from shoot-tip explants of cucumber (Vasudevan et al. 2008). Polyamines have been shown to be important for cellular differentiation and somatic embryogenesis (Chi et al. 1994; Bajaj and Rajam 1996; Rajam 1997) and have been suggested as regulators of somatic embryogenesis (Montague et al. 1979). Inhibitors of PA biosynthesis, such as α- difluoromethylornithine, block somatic embryogenesis in Coffee (Kumar et al. 2008), whereas in Circhorium (Helleboid et al. 1995) this effect is reversed by putrescine indicating a direct role of polyamines in somatic embryogenesis. But the exact mechanism by which PA exert their effect on somatic embryogenesis is not clear and still remains to be clarified (Niiemi et al. 2002). The results of the present investigation have demonstrated that exogenous polyamines are effective for the tridirectional micropropagation pathway in both the species of Dendrobium.

Polyamines have been shown to play a positive role in several tissue culture systems, such as morphogenesis in Helianthus tuberosus (Phillips et al. 1987), micropropagation of Asparagus (Fiala et al. 1991) and somatic embryogenesis in Hevea brasiliensis (El Hadrami et al. 1989). Callus induction response differs notably from earlier observation made on orchids, where development of callus from different types of explants, including shoot-tips was primarily dependent upon the exogenous auxin and cytokinin (Chang and Chang 1998; Lin et al. 2000; Roy and Banerjee 2003; Huan and Tanaka 2004; Roy et al. 2007). Among the polyamines, only spermidine had low degree of inducing effect in D. farmeri. In D. nobile, spermine at 0.2 and 0.4 µM level showed slight stimulatory effect for callus induction. The reduction in polyamine levels during callogenesis could be due to increased production of callus cells which are metabolically less active than the meristematic cells giving rise to somatic embryos. Thus, observed changes in PAs are related to the stage of development of somatic embryo (Minocha et al. 2004) and endogenous polyamine content in early induced embryogenic calli are higher than those in non-embryogenic and mature embryogenic calli, Exogenously added PAs can provide a strategy to study the regulation of endogenous PA pools, which have been correlated with embryo induction in plant species (Takeda et al. 2002). The inhibitory effect of spermine and putrescine on calli production in D. farmeri also supports the previous reports made in Coffee (Calheiro et al. 1994). In Mangifera indica Litz and Schaffer (1987) also found higher endogenous concentrations of polyamines in non-embryogenic than in embryogenic calli. From the above finding it could be stated that exogenously supplied putrescine suppressed the production of calli.
as well as was detrimental to PLB formation. Sanchez-Graz and Segura also (1988) demonstrated a dose-dependent inhibitory effect of exogenously supplied spermidine on calli production in *Sideritis angustifolia*. Effect of PAs on calli production was also studied in rice (Bajaj and Rajam 1995; 1996) and in sugar beet (Hagege *et al.* 1994).

An important observation of the present study is that the maintenance of embryogenic callus was possible in polyamines-free as well as PGR-free control medium for more than 18 months without any loss of embryogenic potentials. Therefore, it was evident that endogenous polyamines level was quite sufficient for the maintenance of callus in basal medium. From the perspective of propagation, this continuous proliferation of callus with side-by-side regeneration of PLBs shows great potential as an attractive method for mass production of plantlets. In the present study, it is also confirmed that necrosis of callus was quite low in polyamines-free control medium as compared to polyamines treatment.

Polyamines were found to be very effective for neoformation of PLBs and subsequent plantlet development, but the efficiency varied with the polyamines tested. All the putrescine concentrations yielded significantly higher number of PLBs, in comparison to control. This positive effect of putrescine also corroborates the previous observation of Scholten (1998) in cacti, Acacia, tomato and lilac. The present finding also agree with the earlier observation that precursors of putrescine enhance the plantlet formation from auxillary buds and PLBs in taro (Sabapathy and Nair, 1992). On the other hand, in all spermidine and spermine treatments, a significant production in PLBs was observed. At low concentration of spermine and spermidine, large numbers of dark green PLBs were observed in comparison to higher concentrations. In case of spermidine, there was an abrupt decrease in production at higher concentrations. Similar reports of high spermine levels causing inhibition of growth and plantlet formation in taro was made by Sabapathy and Nair (1992). It has been suggested that the differential response of tissue cultures to exogenous polyamines was related to the type specific metabolism and biosynthesis of polyamines that determine their biological activity.

In the present study on *D. farmeri* and *D. nobile*, it was noted that all the exogenously supplied polyamines suppressed apical dominance leading to adventitious shoot regeneration. Similar influence of polyamines on direct shoot-regeneration was observed in *Brassica campestris* (Chi *et al.* 1994) from cotyledonary explants and in Chinese radish from hypocotyl explants (Pua *et al.* 1996).
A number of reports are available on the multiplication of orchid species through shoot-tip culture (Dodson 1981, Goh 1990, Seeni and Latha 2000). The results observed herein have shown the potentiality of shoot-tip explants to produce phenotypically uniform, stable plants in intermediary callus-free state for conservatory significance. In case of shoot-tip culture of orchids, it appeared that the resident axillary meristems of the condensed nodes of the basal part of shoot apices relieved apical dominance by the defunct apical meristems and proliferated to produce callus-free shoot buds (Seeni and Latha 2000). The success of rapid and direct shoot regeneration without callus phase or PLB formation from the shoot-tip explants of *D. farmeri* also showed the successful pathway for rapid regeneration and multiplication of shoots. The fact that only 0.8 µM and 1 µM concentrations of spermidine assisted shoot initiation indicates that lower concentrations may be metabolically toxic.

The interplay between hormones and polyamines and their combined effects are probably complex. Sensitivity of intact plant tissues to growth substances is an important factor in understanding growth regulation by polyamines and hormones (Trewavas, 1982)

**Regeneration via foliar explants:**

Propagation of orchids through the culture of leaf tissues has been attempted for a number of species. Leaf tissue culture is advantageous, because it does not endanger the mother plant. In the present study, leaf cultures were done with *D.aphyllum, D. parishii, D.nobile, D.farmeri* and *Hygrochillus parishii*. In all cases, whole leaves were excised from 5-6 months old *in vitro* grown seedlings. While the foliar explants of *D.farmeri* satisfactorily responded within 3-4 weeks of inoculation, those of *D.aphyllum, D.nobile* and *D.parishii* failed to respond during the following 16 months and turned necrotic. Reports of successful plantlet regeneration from leaf culture of mature plants are rare and restricted to a few selected species, as for example, *Renanthera imschootiana* (Seeni and Latha 1992), *Renantanda* hybrid (Goh and Tan 1982), *Cattleya* (Churchill et al. 1971). On the other hand, leaf segments of relatively young seedlings have been found to be more responsive, e.g. *Rhyncostylis retusa* (Vij et al. 1984), *Cattleya* (Champagnat et al. 1970), *Epidendrum, Laeliocattleya* (Churchill et al. 1970, 1972, 1973), *Vanda* hybrid (Chaturvedi and Sharma 1986), *Aranda, Ascocenda* (Fu 1978, 1979), *Vanda coerulea* (Seeni and Latha 2000), *Doritaenopsis* hybrid (Park et al. 2002a), *Aerides crispum* (Sheelavanthmath 2005), *Dendrobium* hybrid (Martin and Madassery 2006) etc. This disparity in the response of leaf tissues from mature plant and seedlings is considered to be
related to the physiological age of the seedlings and also the retention of meristematic activity in young leaf tissues (Vajrabhaya and Vajrabhaya 1970; Vij et al. 1984). It has been observed in mature Cattleya leaf-tip explants that the regeneration potential of the explant lies in the presence of meristematic or undifferentiated tissues and the appearance of leaf-tip notch indicated that the leaves would no longer produce callus (Churchill et al. 1971; Arditti and Ernst 1993). Apart from this, the tissues of mature plants also release greater amount of auto-inhibitory phenolics that affect the survival and growth to a great extent (Vij et al. 1984; Compton and Precer 1986; Seeni and Latha 1992; Nayak et al. 1997a). This observation also corroborates with the result of Hygrochilus parishii where the medium become toxic due to exudation of phenolics.

In the present study, leaf segments started production of PLBs after about 3 weeks from beginning. It has been reported that in various species, the regenerative potential of the leaf is often restricted to a particular region. But the present study revealed that regeneration from the leaves of D. farmeri and H. parishii occurred from the basal wound region, leaf tips, along the leaf laminar margin and occasionally from the abaxial surface of the leaves. Our results agree with that of Vij et al. (1984) who reported that the PLB formation was initiated in the upper and lower epidermal cells near the cut ends of the explant and subsequently spread over the entire surface. However, restriction of regenerative potential to particular regions is well known among the orchids and may vary with the species, as shown in leaf tip of Epidendrum, Laeliocattleya (Churchill et al. 1970, 1972, 1973) and Vanda hybrid (Chaturvedi and Sharma 1986), leaf base of Cattleya (Champagnat et al. 1970), Aranda (Loh et al. 1975) Vanda hybrid (Mathews and Rao 1985), Renanthera imschootiana (Seeni and Latha 1992), Acampe praemorsa (Nayak et al. 1997a), Vanda coerulea (Seeni and Latha 2000) and Phalenopsis amabilis and P.Nebula (Gow et al. 2009). According to Vij et al. (1984) the restriction of the regenerative potential to a particular region of the leaves of some species could be related to the genetic makeup or the physiological age of explant. This also could be due to the presence or absence of meristematic tissue, as mentioned earlier. The results of H.parishii have shown that PLB formation depended on the application of cytokinin. However, the presence of NAA also influenced the frequency of regeneration and for each concentration of NAA, the optimum concentration of cytokinin varied.

Among the three different auxin-cytokinin combinations, the best results were obtained in the combined presence of Kin and NAA, showing a similarity with the reports of Vij et al. (1984),
where kinetin along with IAA or NAA improved PLB formation in the leaf culture of *Rhyncostylis retusa*. However, it was also found that induction of PLB could occur with the use of auxin alone which contradicts the present results. Further Seeni and Latha (1992) performed the foliar culture of *Renanthera imschootiana* and observed that the presence of both BAP and NAA was essential for induction of shoot buds, and a high BAP: NAA ratio gave optimum regeneration. Among the two responding species in the present study, the regenerative potential of *D. farmeri* in the basal medium was so vigorous that about 40% of the foliar explants regenerated even in absence of any exogenous PGR. The response could be further improved with inclusion of BAP, Kin or TDZ in the media. In case of *H. parishii*, BAP at intermediate concentrations showed stimulatory effect on PLB formation. But kinetin and TDZ showed no significant effect even at supra-optimal level. This species also responded through the production of a compact callus from where PLBs were regenerated. Auxin-cytokinin combination was beneficial for both the species, which corroborates with the earlier studies indicating that, the combined effect of NAA and BAP was stimulatory for *V. tessellata* and *Papilionanthe teres* (=*Vanda teres*) (Roy 2000).

The occurrence of callus initiation was very low in NAA-BAP and NAA-TDZ combinations. For such response kinetin showed a significant stimulatory effect. The regenerative potential of the callus was very slow and occasionally developed only few shoot buds and PLBs. For regeneration of direct PLBs from explants, different cytokinins (BAP, Kinetin and 2iPA) were stimulatory for *D. farmeri*. This is evident from the fact that sufficient amounts of explants formed direct PLBs with BAP, kinetin and 2iPA alone than with TDZ alone. Although a strong cytokinin-like compound TDZ was more efficient than BAP and kinetin in shoot-tip culture of *D. farmeri* and also in leaf culture of *Doritaenopsis* and *Phalaenopsis* (Chen and Piluek 1995; Ernst 1994), it did not favour somatic embryogenesis in *Oncidium* ‘Gower Ramsey’ callus (Chen and Chang 2000; Wu et al. 2004). In contrast to cytokinin, NAA alone inhibited induction of D-PLBs, in both the species. This result corresponds to the previous observation on *Oncidium* ‘Gower Ramsey’ and *Phalanopsis amabilis* (Chen et al. 1999; Chen and Chang 2001; Chen and Chang 2006) which revealed that embryo formation on leaf explants was retarded by all the auxins used (IAA, IBA, NAA and 2,4-D) and was promoted by all the cytokinins (2iP, zeatin, Kinetin, BA and TDZ). All the BAP concentrations (1-16µM) and 8-16 µM 2iPA showed stimulatory effect on callus induction. The differential effect of exogenous
cytokinin is attributed to the different types of cytokinins involved that might have triggered the type-specific metabolism resulting into differential biological activity (Horgan 1987; De Pauw et al. 1995).

For both the species, exogenous auxin (NAA) was either inhibitory or did not produce any effect on callusing when used alone, but exogenous cytokinin was stimulatory. Earlier observations involving different types of explants from many orchid species revealed that development of callus primarily depended upon the exogenous auxin (Smith and Krikorian 1990, 1991; Chang and Chang 1998; Ignacimuthu et al. 1999, Lin et al. 2000; Roy and Banerjee 2003; Huan et al. 2004; Huan and Tanaka 2004), all of which contradict the observation for both the species under study. In *D. faemeri* and *H. parishii*, callus formation occurred to some extent even in absence of any exogenous PGR, thereby indicating that the responding explants contained a fairly accurate endogenous balance of auxin and cytokinin of its own, which is necessary for regulation of cell division during callus neo-formation. The requirement for supplementary cytokinin for enhanced callusing is probably associated with the attainment of the appropriate balance between auxin and cytokinin so as to act synergistically in order to regulate cell division on callus formation (Johri and Mitra 2001; Roy and Banerjee 2003). The results of *D. farmeri* and *H. parishii* have also shown that there is a direct correlation between the concentrations of BAP and NAA in the culture medium and the necrosis of leaf explants. In both the species leaf necrosis was extremely high in the PGR-free treatments, but high concentrations of auxin and cytokinins reduced this growth considerably.

**Induction and maintenances of embryogenic callus:**

The results of the present investigation of shoot-tip culture as well as leaf culture demonstrated that the application of cytokinin to be the most effective means for induction of callus from shoot-tip explants of *D. farmeri* and *D. parishii* and leaf explants of *H. parishii*. On the other hand, NAA showed either inhibitory or stimulatory effect for callusing. For *D. aphyllum* and *D. nobile* no callus formation was found in all the three combinations of auxin and cytokinins. In both the species, most of the explants followed direct regeneration pathway. Such an obligatory requirement for a cytokinin and auxin combination for callus formation differs notably from earlier observations made on orchids, where development of callus from different types of explants including shoot-tips was primarily dependent upon exogenous auxin (Smith
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and Krikorian 1990, 1991; Chang and Chang 1998; Ignacimuthu et al. 1999; Lin et al. 2000; Roy and Banerjee 2003; Huan et al. 2004; Huan and Tanaka 2004). Application of auxin-cytokinin combinations for callus induction was also observed in D. officinale (Wei et al. 2010). Callus culture of most orchids also exhibits an absolute requirement of both auxin and cytokinin for their induction which was previously observed in different species like Paphiopedilum hybrid (Lin et al. 2000), Cypripedium formosum (Lee and Lee 2003) and in Cymbidium (Huan et al. 2004; Huan and Tanaka 2004; Tei-xeira Da Silva and Tanaka 2006). In D. farmer, the TDZ+NAA combination was found to be the most effective means for callus induction, while BAP failed to exhibit any promising result. This finding however does not tally with previous reports on D. fimbriatum and D. chrysotoxum (Roy and Banerjee 2003; Roy et al. 2007), where a purine type cytokine, 6-benzyl-αmino-purine (BAP) also showed promising effect for callus induction from shoot-tip explants. Generally cytokinin shows a very low callus-inducing effect, but it is generally required for overcoming callus necrosis and thus promotes growth particularly in members of orchidaceae (Lin et al. 2000; Roy and Banerjee 2003; Lu 2004; Chang and Chang 1998; Ishii et al. 1998; Ignacimuthu et al. 1999). TDZ combined with 2; 4-D also induced embryogenic calli from rhizomes of Cymbidium ensifolium var. misericors (Chang and Chang 1998) and longitudinally bisected segments of PLBs of Cymbidium hybrid (Huan et al. 2004). Roy and Banerjee (2003) suggested that the requirement for supplementary cytokinin for callusing might be related to the maintainance of a proper balance between auxin and cytokinin that acted synergistically to regulate cell division (Johri and Mitra 2001; Roy et al. 2007), which is essential for callus induction. It has also been suggested that the endogenous cytokinin content of initial explants might have an important role in callus initiation or embryogenic competence (Gaj 2004; Jiménez 2005). Therefore, it is reasonable to presume that for both D. farreri and D. parishii, a proper balance between auxin and cytokinin pre-existed that acted synergistically for regulation of cell division necessary for callus neo-formation as for which callus induction occurred even in PGR free control medium. On the contrary, exogenous auxin and cytokinins were inhibitory for callusing from shoot-tip explants of D.aphyllum and D.nobile. Such a species-specific requirement for a particular morphogenetic response is common among orchids. Therefore, complete absence of callus formation may also be related to the predominating trend of this explant to respond via direct organogenesis or continuation of axial growth which reduced
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the available explants to follow an alternative morphogenetic (callus-mediated) pathway, and in reality the requirement for the exogenous cytokinin may not be that much exacting.

Although the presence of exogenous PGRs was essential for callus induction, it could be possible to maintain this callus growth in PGR-free medium for more than 12 months. Hormone autonomous callus formation is a rare phenomenon among the orchids studied so far. Such an incidence was earlier reported only in some instances like *D. fimbriatum* (Mitra *et al.* 1976; Roy and Banerjee 2003), *Oncidium* ‘Gower Ramsey’ (Chen and Chang 2000a), *Cymbidium* hybrid (Huan and Tanaka 2004), *Dendrobium chrysotoxum* (Roy *et al.* 2007) and *Dendrobium formosum* (Nasiruddin *et al.* 2003). A similar PGR-free maintenance and proliferation of the cytokinin-induced callus from the foliar explants of *Hygrochilus parishii* has been observed which is mentioned in the respective section. In both the species, PLBs were regenerated from the surface of the callus. Zhao *et al.* (2008) reported that BAP-induced callus regenerated PLBs in PGR-free medium. Therefore, it was evident that an adequate endogenous hormonal system was responsible for the maintenance of callus in the PGR-free medium. On the contrary, the callus culture of most orchids exhibits absolute requirements of both auxin and cytokinins for their maintenances (Lin *et al.* 2000; Lee and Lee 2003; Huan *et al.* 2004; Wu *et al.* 2004). Unlike the beneficial effect of BAP, the presence of TDZ exerted an inhibitory effect on PLB formation which is in agreement with the reports on *Oncidium* ‘Gower Ramsey’ (Chen and Chang 2000a, b; Wu *et al.* 2004). A similar species-specific requirement for maintenance and proliferation of embryogenic callus has been previously observed in many orchid species (Smith and Krikorian 1990, 1991; Chang and Chang 1998). Such a differential response of cultured tissue to exogenous cytokinin is proposed to be related to the type-specific metabolism of cytokinins that determines their biological activity (Horgan 1987; De Pauw *et al.* 1995). The growth characteristic of the callus of *D. farmeri* which has been initiated in presence of plant growth regulators exhibits a similarity with that of the habituated callus (Hogan 1987; Meins 1989). But unlike the habituated callus that exhibits loss of morphogenetic potential (Choi *et al.* 2003), the callus of *D. farmeri* sustains its regenerative potential through continued production of PLBs for more than 12 months. This hormone-autonomous callus culture through PLB regeneration was earlier noted in *Ramex ginseng* and *Mammillaria gracillis* (Choi *et al.* 2003; Poljuha *et al.* 2003). Histological studies of PLBs showed that these are actual somatic embryos which strongly support previous report on *Cymbidium* (Da Silva and Tanaka 2006).
Necrosis of callus was a common occurrence during maintenance of callus in orchids. Similar observation was previously reported in several orchids including Paphiopedilum hybrid (Lin et al. 2000), Pleione formsana Hayata (Lu 2004) and Oncidium ‘Gower Ramsey’ (Wu et al. 2004).

5.3. Assessment of clonal fidelity:-

Several cytological studies have been reported to detect the variation and/or confirm the genetic fidelity in micropropagated plants (Vasil, 1985). In vitro regeneration via well developed seedlings of various orchids is a novel technique for their clonal propagation. Under the influence of high concentrations of different potent growth regulators, there are chances of getting somaclonal variation amongst the regenerants (Larkin and Srowcroft 1981) and they may be heritable (Breiman et al. 1989). Thus there arises the necessity to evaluate whether the regenerants are genetically identical with that of the mother plant. Many investigators have reported genetic stability of several micropropagated plants, viz., Picea mariana (Isabel et al. 1993), Pinus thunbergii (Goto et al. 1998), chestnut rootstock hybrid (Carvalho et al. 2004), Prunus dulcis (Martins et al. 2004), Swertia et al. (Joshi and Dhawan 2007), banana (Venkatachalam et al. 2007), etc. by using RAPD or ISSR or both.

PCR- ISSR analysis revealed that none of the primer showed any difference in the banding pattern, proving the genetic uniformity of the in vitro regenerated clones obtained through the present protocol. The clonal fidelity test using ISSR primers was successfully attempted in different micropropagated plant species (Gantait et al. 2008, 2009, 2010; Debnath et al. 2008; Bhatia et al. 2009). However, 2 out of 4 selected ISSR primers used in this study revealed discreet monomorphic bands when the mother and in vitro generated propaguls were subjected to clonality test. The limited number of bands produced by these ISSR primers would partially cover the genome. None of the primers exhibited any difference in banding pattern. Considering observed similar morphological response of the mother plant and micropropagated plants generated via in vitro as well as the displayed monomorphic banding pattern, it can be suggested that the in vitro regenerated clones maintained their clonal fidelity. It was evident from this experiment that, 1) the clones developed from in vitro direct organogenesis is the safest mode of micropropagation which comprises true-to-type progeny. The reports of Shu et al. (2003) and Martins et al. (2004) also cite similarity with these results.
RAPD profile obtained through amplification of genomic DNA of the in vitro regenerated plants and that of the source plant were similar and no polymorphic bands were seen. This clearly indicates the genetic integrity and true-to-type nature of the in vitro regenerated plants. RAPD is becoming a widely employed method in the detection of genetic diversity because it has the advantage of being technically simple, quick to perform and requires only small amounts of DNA (Williams et al. 1990). RAPD has been proven to be a suitable molecular technique to detect the variation that is induced or occurs during in vitro regeneration of plant species (Shu et al. 2003). This technique has already been applied in *Curcuma amada* (Prakash et al. 2004). RAPD technique was also applied by various researchers for testing the genetic fidelity of in vitro grown medicinal plants including *Drosera anglica* and *Drosera binata* (Kawiak and Lojkowska 2004), *Plumbago zeylanica* (Rout and Das 2002), *Zingiber officinale* (Rout et al. 1998), *Curcuma longa* (Selviet al. 2002), *Tylophora indica* (Jayanthi and Mandal 2001) and *Musa paradisiaca* (Venkatachalam et al. 2007).

In this study, both the RAPD primers (SB1 and SB2) did not exhibit any variation in banding profile. So it can be suggested that in vitro regenerated clones maintained their genetic integrity through RAPD analysis.