IN VITRO PRODUCTION OF PROTOCORMS AND PROTOCORM LIKE BODIES IN ORCHIDS - A REVIEW

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ABSTRACT

In vitro multiplication is the most important biotechnological tool being used in orchids, especially for large scale commercial propagation. Usually in vitro methods would be useful in a crop where in the existing conventional methods are too-slow, non-profitable and not successful. As orchid seeds lack a functional endosperm, the germination of seeds require a long period and any disturbance to the soil or physical environment destroy the whole population. In this context the in vitro multiplication provide an appropriate solution to this problem. So development of tissue culture protocol is an essential pre-requisite for any other biotechnological manipulations at cellular and molecular level.

Protocols for the establishment of cultures in vitro and direct formation of plants from these meristematic tissues are available for many orchid genera (Arditti and Ernst, 1993). Other tissues capable of plant regeneration like etiolated shoots are also suitable for Dendrobium hybrids (Kuehnle, 1997). Protocorms are derived from germinating seeds and protocorm like bodies (PLB’s) were obtained by culturing the shoot apices. Sterilisation prior to inoculation of orchid seeds is very important, as orchid seeds have to be cultured under completely aseptic conditions for the development of protocorms. Since mature orchid seeds have tough seed coats, chemical treatments for sterilization can be safely employed (Jordan, 1965).

PROTOCORMS

Surface Sterilisation of Pods

Green pod culture was proved to be the best, as the seeds directly transferred to the medium without outside exposure, germinated well and produced strong seedlings within eight to ten weeks (Rao and Avadhani, 1964). Mature seeds of Vanda Miss. Joaquim pretreated with 5.0 per cent Chlorox for ten minutes and rinsed with sterile water prior to inoculation produced seedlings in ten to twelve weeks whereas mature seeds without pretreatment were lost due to contamination. Mitra (1971) used chlorine water to sterilise capsules and seeds. Pods were dipped in absolute alcohol for 12 seconds and chlorine water for 45 minutes, whereas seeds folded in filter paper were dipped in chlorine water for ten minutes and rinsed with three changes of sterile water. Rosa and Laneri (1977) used 70 per cent ethanol for sterilising pods. Pyati and Murthy (1995) achieved pod sterilization in Dendrobium ovatum by dipping in alcohol followed by flaming. Pod sterilization of Vanda coerulea was effected by pretreatment in 0.1 per cent mercuric chloride for five minutes, followed by alcohol dip and flaming.

Seed Germination and Development in vitro

Knudson (1946) proved that the seeds of Cattleya, Laelia and Epidendrum germinated freely on sugar and mineral containing agar medium under aseptic conditions without fungal association. Arditti (1979) reported in four orchid genera including Dendrobium that only a few apical cells of protocorms divided to form a promeristem which gave rise to shoot apex and structures homologous to cotyledons. Mathews and Rao (1985) reported that the differentiated protocorms had to be subcultured within a period ranging from 70 to 80 days for proper in vitro growth. Overcrowding without transfer resulted in stunted growth. According to Yam and Weatherhead (1988), the seeds had germinated only when protocorms either green or white, were observed in cultures. Rubulo et al. (1989) defined germination as the presence of protocorms with one leaf primordium one month after culture.

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Pathak et al. (1992) in *Goodyera biflora* reported that the protocorms, on emergence from the testa were white and hairy. The first signs of chlorophyll development were apparent in leaf initials. Singh (1992) reported that depending upon their genotype, the seeds develop chlorophyll within 10-20 days on the nutrient medium.

Krishnan et al. (1993) observed the visible protocorm formation from the embryos by the second and third week of culture in *Spathoglottis plicata* and the first leaf primordium was initiated between the fifth and sixth week of culture. Nagashima (1993) studied the seeds of 47 orchid species and reported that the germination rate ranged from 0.8 to 100 per cent and the number of days from sowing to germination ranged between 3 to 305 depending on the stage of embryogenesis. Singh (1993) found that inoculation of seeds into a nutrient medium under *in vitro* conditions not only improves the per cent of germination, but also reduces the time for differentiation of orchid seeds, both biochemically and morphologically. Hazarika and Sarma (1995) reported that the immature seeds of *Dendrobium transparens* showed the signs of swelling, in 16-18 days after inoculation. Ninety per cent germination was observed after 25 days of inoculation. Lekharani (2002) reported that in *Dendrobium* the seed germination per cent ranged from 8.00 to 70.73.

**Effect of Culture Media on Seed Germination**

Many media have been used for the axenic germination of terrestrial and epiphytic orchids. However, none of these media is universal. The commonly used nutrient media for orchid seed culture are those proposed by Knudson (1946) (KC), Vacin and Went (1949) (VW) Murashige and Skoog (1962) (MS), Raghavan and Torrey (1964), Nitsch (1969), Mitra et al., (1976) and Rosa and Laneri (1977) (RL).

Seed germination and morphogenesis studies in *Epidendrum radicans* and *Dendrobium* Jaquelyn Thomas clearly indicated the superiority of MS medium over KC and VW media (Sangama, 1986). Reddy et al. (1992) observed that the South Indian orchids exhibited a significant interaction between the media and the species. *Dendrobium crepidatum* yielded better results in MS and RL media than in KC medium. Zhang et al. (1993) reported that since MS medium contained high ionic concentration of nutrient salts half-strength MS could adequately support rapid protocorm production in orchids. Hazarika and Sama (1995) conducted *in vitro* germination studies in *Dendrobium transparens* (Lindl) and reported that best growth of seedlings was obtained in supplemented MS medium.

Bhaskar (1996) found that supplemented quarter strength MS could produce seedlings with maximum number of shoots, leaves and roots in *Phalaenopsis* after a 12 week culture period. The basal medium MS half strength was found to be the best for early germination and rapid *in vitro* development as compared to MS quarter strength and MS, KC and VW full strengths (Lekharani, 2002). Xiang et al. (2003) reported that the best medium for *in vitro* regeneration of *Cymbidium sinensis* was MS medium supplemented with 4.0 mg BA + 1.0 mg NAA / l. Devi et al. (1990) pointed out that the preferred medium for *Dendrobium* seed germination varied with the species. *D. farmeri* and *D. Primulinum* gave 50-60 per cent higher germination on VW medium. Kumaria and Tandon (1991) were of opinion that high ionic concentration of nutrient salts and vitamins in the medium was inevitable for the germination of *Dendrobium fimbriatum* var *Oculatum* seeds. On inoculating four-month old seeds, the highest germination (91%) was obtained on Nitsch medium, followed by MS (85%).

**Effect of Organic Additives and Growth regulators on Protocorm Establishment**

The most frequently used complex organic additive in the production of orchid
protocorms is coconut water (CW), the liquid endosperm of coconut. It induces cell division in otherwise non-dividing cells and promotes morphogenesis and mass multiplication of protocorms in orchids (Intuwong and Sagawa, 1973).

Morel (1974) had enumerated the beneficial effects of coconut water in bringing about rapid protocorm multiplication in orchids. McIntyre et al. (1974) found that addition of coconut water (15%) to KC medium led to increased growth of both epiphytic and terrestrial orchids. Coconut water (10%) when added to KC medium along with micronutrients, gave satisfactory germination in five orchid genera (Rosa and Laneri, 1977). Sahid (1980) reported that growth rate of Dendrobium hybrids could be improved by adding potato and pea extracts to KC medium. Soediono (1988) found that supplemented VW medium (CW 15% + NAA 10 ppm) led to rapid protocorm proliferation followed by enhanced seedling growth in Dendrobium Jaquelyn Thomas. According to Rubalo et al. (1989) supplementing KC medium with 10 per cent coconut water gave the best germination in Bletia Urbana. Addition of 15 per cent CW and 5 per cent BP enhanced germination and accelerated seedling growth in Dendrobium farmeri and D. Primulinum (Devi et al., 1990). Immature seeds of Rhyncostylis retusa and Vanda Coerulea gave 20 per cent enhanced germination when VW media was supplemented with CW, banana pulp, pineapple juice and vitamin stock of Nitsch medium (Nath et al., 1991).

Sharon et al. (1992) used the basal medium supplemented with 15 per cent CW for raising protocorms of Dendrobium Snowfire from immature seeds. Enhanced growth in different orchids has been reported to occur in the presence of coconut water (CW), banana pulp, peptone, apple juice and peptone, fish extract and peptone, pineapple and tomato fruit (Arditti and Ernst, 1993). For Cattleya, Encyclia and Oncidium 25 per cent CW was the best additive (Villolobos and Munoz, 1994). Bhasker (1996) had pointed out the beneficial effects of peptone and CW on in vitro seedling growth in Phalaenopsis. Peptone 1000 mg l\(^{-1}\) along with BA 20 mg l\(^{-1}\) and NAA 1 mg l\(^{-1}\) maximised shoot leaf and root production after 12 weeks of culture. Foliar growth was enhanced by the addition of CW. Lekharani (2002) reported that coconut water 200 mg l\(^{-1}\) was the best for early protocorm differentiation and rapid seedling growth. According to Mathews and Rao (1980) yeast extract was successfully used for seed germination and protocorm proliferation in many orchid species.

**Effect of Charcoal on Protocorm Establishment**

Ernst (1974), Rosa and Laneri (1977) recorded that seedlings grew well on culture media to which activated charcoal was added. Activated charcoal adsorb the inhibitory phenolic and carboxylic compounds produced by the tissues in culture. The initial formulations of charcoal-containing medium for seed germination of Hong Kong orchids gained wide acceptance (Yam and weatherhead, 1988). Pierik et al. (1988) found that in Paphiopedilum ciliolare when activated charcoal 2 g l\(^{-1}\) was added to the medium after protocorm formation, induced significant increase of shoot and root development. But it was inhibitory during seed germination. According to Hinnen et al. (1989) activated charcoal strongly enhanced the growth and development of Phalaenopsis seedlings. Yam et al. (1990) observed that activated charcoal exerted a beneficial effect on culture media by adsorption and removal of phytotoxic metabolites. They further pointed out that it can also be detrimental due to the removal of additives such as auxins or cytokinins.

**Effect of Carbon Source on Seed Germination**

Orchids must have an external supply of carbohydrates to continue their growth and
differentiation. Orchid seeds and young seedlings have the ability to utilize various carbohydrates. However, different species have their own preferences (Arditti, 1967). Glucose, fructose or oligosaccharides containing these sugars alone could adequately satisfy the energy requirements of Phalaenopsis protocorms (Ernst et al., 1971). In Dactylorhiza purpurella, the results with dextrose and sucrose were essentially similar (Harvais, 1972). Of the sugars tested on the growth of Cymbidium protocorms, sucrose was better than maltose, glucose and fructose. The optimum concentration of sucrose ranged from 3.0 to 4.0 per cent (Fonnesbech, 1972). Harrison and Arditti (1978) found that sucrose induced germination and enhanced chlorophyll development in certain species that failed to germinate on sugar-free medium. Sucrose could be replaced by glucose. In hybrid Vanda, Mathews and Rao (1985) and in Cypripedium reginae, Bellard (1987) tested different carbon sources and found that 2.0 per cent sucrose was the best source. Absence of sucrose stopped the growth of protocorms and 10.0 per cent sucrose caused tissue necrosis. Pierik et al. (1988) concluded that in Paphiopedilum ciliolare an extraordinary low sugar concentration was optimal for germination, higher concentration being inhibitory.

High sucrose concentration (4.0) reduced germination in Bletia Urbana, but no significant difference could be observed in the response between 2.0 and 3.0 per cent sucrose (Rubulo et al., 1989). Sharma and Tandon (1990) reported that among the various carbon sources tested, sucrose, fructose and glucose at 2.0 to 3.0 per cent gave the best germination and seedling growth in Cymbidium elegans and Coelogyne sp. In sugar-free medium, the germination and growth were negligible. Lekharani (2002) observed that sucrose 30 g l⁻¹ showed significantly early development of first leaf and root primordia. Xiang et al. (2003) reported that 30g sugar/l was the optimum dose for the in vitro regeneration of Cymbidium sinensis.

Effect of pH of Media on Seed Germination

pH of the media play a major role on the germination of orchid seeds. Knudson (1951) noted the inability of Cattleya seeds to germinate if the initial pH of medium is below 4.5. Dendrobium nobile germinated better within a pH range of 4.0 to 5.0. (Ito, 1955) where as many other orchid species responded favourably to media with pH between 5.0 and 6.0 (Scott and Arditti, 1959; Kotamori and Murashige, 1965). Maintaining the pH at 5.2 to 5.5 was favourable for successful germination in Cymbidium mastersii (Prasad and Mitra, 1975). Rosa and Laneri (1977) observed that pH of 5.2 for Cattleya, and Phalaenopsis and 6.0 for Cymbidium and Paphiopedilum were satisfactory for germination. Reyburn (1978) recorded in Cymbidium that germination in the dark was optimal at pH 5.5 – 6.0 and a pH of 7.0 was strongly inhibitory. Orchid seeds germinated well within a pH range of 4.8 to 5.2 with germination commencing at pH 3.6 and tapering off at 7.6 (Arditti, 1979).

Maximum germination and optimal growth of protocorms at pH 5.0 was reported in Dendrobium chrysanthum and Sarcanthus pallidus (Raghuwanshi et al., 1986). Optimal germination of Paphiopedilum ciliolare occurred at a pH of 6.0 (Pierik et al., 1988). Ichihashi (1990) obtained good germination of Bletia striata seeds when the pH was adjusted to 5.1 ± 0.1. George (1997) found that optimal growth of protocorms in Dendrobium osterholti resulted when the pH was adjusted to 5.8.

PROTOCORM LIKE BODIES

Protocorm like bodies (PLBs) are obtained from the culture of shoot apices in vitro. Sagawa and Shoji (1967) opined that shoot tip cultures necessitated the sacrifice of the entire new growth or a whole plant for a procedure which at best might be successful with 66.7 per
percent of the explants. As with *Cymbidium* and other sympodial shoot tips remained the most commonly used explant (Goh, 1970; Teo et al., 1973). Stewart and Button (1975) reported that plantlets and callus which subsequently gave rise to plantlets could be differentiated from a single *Paphiopedilum* stem apex if bacterial-free cultures could be obtained. Shoot tip explants of *Dendrobium fimbriatum* produced a compact callus after two weeks of inoculation. Following transfer to plant growth regulator free medium, the callus further proliferated with side by side regeneration of PLBs (Jonojit and Nirmalya, 2003). The totipotent callus of *Cypripedium formosanum* an endangered slipper orchid was induced from the seed derived protocorm segments. The callus proliferated well and on an average 13 PLBs were obtained from a piece of 4 mm callus (Lee and Lee, 2003).

**Effect of Culture Media on the Development of PLBs**

The most commonly employed media for shoot tip culture are Knudson’s C (Knudson, 1946), MS (Murashige and Skoog, 1962), and VW (Vacin and Went 1949). Irawati et al. (1977) reported that the best growth and survival rates were obtained in *Dendrobium* when cultured in Knudson’s C medium. Shoot tip explants of *Dendrobium fimbriatum* produced PLBs when cultured on modified nutrient solution of Knudson’s C medium (Jonojit and Nirmalya, 2003). The totipotent callus of *Cypripedium formosanum* an endangered slipper orchid was induced from the seed derived protocorm segments. The callus proliferated well and on an average 13 PLBs were obtained from a piece of 4 mm callus (Lee and Lee, 2003).

**Effect of Plant Growth Substances on the Development of PLBs**

When excised apices of *Rhynocrystylis gigantea* were cultured on a composite agar medium supplemented with NAA and coconut milk, plantlets could be produced in three and half months (Vajrabhaya and Vajrabhaya, 1970). A higher concentration of NAA and BA induced maximum proliferation of shoots in *Cattleya* (Kusumoto, 1979). Addition of low concentration of NAA (below 0.1 mg / l ) promoted shoot formation (George and Sherington, 1984). Callusing was recorded in the presence of 0.5 mg/l NAA and 1 mg/l BAP. But the callus proliferated with side by side regeneration of PLB’s when it was transferred to plant growth regulator free medium (Jonojit and Nirmalya, 2003). Application of BA to the axillary buds increased the shoot proliferation in *Dendrobium antennatum* (Rukulczanka and Wójcieszewska, 1983). Shimasaki and Uemoto (1987) found that application of BA to axillary bud explants of *Calanthe* promoted shoot growth.

**Effect of Carbon Source on the Development of PLBs**

According to Hew et al. (1988) and Hew and Math (1989) when apical meristems of *Dendrobium* were cultured in a VW medium, fructose was more readily utilised than other sugars. Honjo et al. (1988) observed that the increase in fresh weight of PLB’s of *Cymbidium* was markedly affected by sucrose concentration. The beneficial effect of CO₂ enrichment was observed only in the case of low sucrose concentration. Sucrose [5.0% (w/v)] concentration was found to be the most effective
Effect of Organic Additives on the Development of PLBs

Coconut water has been proved to promote the growth and differentiation of excised tissues and organs of several crops. The beneficial effect of coconut water has been attributed to the presence of cytokinin like substances. Kim et al. (1970) found that the optimum concentration of coconut water in the medium was 10 - 15 per cent. It is added before autoclaving (Morel, 1965, Intuwong and Sagawa, 1973). Coconut water 15.0 per cent differentiated more number of plantlets within a short period in Dendrobium (Soediono, 1983). In Dendrobium fimbriatum modified nutrient solution of Knudson’s C supplemented with 10% V/V coconut water, 0.5 mg l⁻¹ niacin and 0.5 mg l⁻¹ pyridoxine HCl resulted in the production of compact callus which further proliferated into PLBs (Jonojit and Nirmalya, 2003).

Kusumoto (1979) reported that yeast extract retarded organogenesis and accelerated the production of protocorms in Cymbidium. Addition of 5.0 per cent pineapple juice to VW medium enhanced germination and accelerated leaf and root growth (Devi et al., 1990). Agarwal et al. (1992) found that in Vanilla walkeri MS medium supplemented with 0.5 mg l⁻¹ kinetin, 1.0 mg l⁻¹ BA and 1000.0 mg l⁻¹ caesin hydrolysate supported rapid proliferation of multiple shoots from stem node segments.

Effect of Gelling Agent on the Development of PLBs

Kusumoto (1980) reported that in KC basal solution containing 15 g l⁻¹ agar, Cymbidium protocorms proliferated best when 10 to 25 per cent coconut milk was added. PLBs from inflorescence tips of Mokara were cultured on solid Vacin and Went medium supplemented with 0.5 to 7.0 per cent agar. Increasing the agar concentration above 1.4 per cent resulted in reduction in the number of plantlets and leaves (Abdulkarim and Hairani, 1992). Xiang et al. (2003) reported that the best concentration of agar for in vitro regeneration of Cymbidium was 8 g l⁻¹.

Effect of pH of the Medium on the Development of PLBs

The pH in the medium greatly influences plantlet growth (Knudson, 1951). Vanda explants were grown best on White’s medium with pH 5.5 (Sagawa and Sehgal, 1967). Epidendrum leaf tips were best grown at pH 5.5 on modified MS medium (Churchill et al., 1970). Mosich et al. (1974) recommended a pH of 5.5 for Dendrobium. The same author reported a higher pH of 5.8 when modified MS medium was used for Dendrobium culture.

Epidendrum root tips were reported to grow on modified Ojima and Fujiwara medium with a pH of 5.0 (Churchill et al., 1972). Cattleya shoot tips when cultured on a solid medium turned brown and died eventually. Tests conducted on polyphenol oxidase activity showed that the leaves turned brown due to this activity. The activity was greatest at pH 6.5 and was inhibited at lower pH (Ichihashi and Kako, 1977).

CONCLUSION

In Dendrobium, the protocorm derived from the seeds, excised shoot tip, lateral buds, and protocorm-like bodies (PLB) derived from tissue explants are the tissues suitable for reliable orchid plant production. In vitro production and multiplication of orchids has revolutionized the commercial orchid growing and hybridization, as every viable seed can be turned into a new plant. This has put orchid growing on par with other commercial greenhouse crops and has become a promising industry in countries like Thailand, Malaysia and Singapore. Tissue culture method was commercially exploited by growers for rare orchids and hybrids, which could not be propagated by any other means, but
responded very well to in vitro multiplication at a rapid rate. The explants are cultured on desired simple medium viz, Knudson C, Vacin and Went and Murashige and Skoog medium. Both liquid and solid media are used. Besides, organic additives viz, coconut water, pineapple juice, apple juice, peptone, banana pulp, yeast extract and tomato fruit extract can be supplemented with the medium for better results and rapid proliferation. The inhibitory phenolic and carboxylic compounds produced by the tissues in culture can be adsorbed by activated charcoal. The meristem culture enables the production of virus-free plantlets and a much higher number of plantlets of the same clone in a short time. So thus the development of tissue culture protocols is the basic requirement for further biotechnological studies viz, meristem culture, embryo culture, molecular breeding and in vitro flowering studies.

REFERENCES
