Journal of Plant Biology, June 2005, 48(2) : 253-257 **SHORT COMMUNICATION**

Micropropagation of *Dendrobium fimbriatum* Hook. by Green Pod Culture

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Present studies have demonstrated that immature seeds obtained from green pod of *Dendrobium fimbriatum* Hook., an endangered epiphytic forest orchid having horticultural importance, can be germinated asymbiotically *in vitro* for rapid micropropagation. Vacin and Went medium containing 0.1 mg L^{-1} NAA and 15% coconut water was found most effective for high percentage (80-90%) seed germination and seedling development. This method can be exploited for their rapid micropropagation and conservation.

Keywords: Dendrobium fimbriatum, green pod culture, micropropagation

Dendrobium fimbriatum Hook. (Orchidaceae) is an endangered ornamental forest epiphytic orchid of the temperate and subtropical regions, which are valued for their long lived beautiful sweet scented flowers having horticultural importance. It is usually propagated by vegetative method, which is a very slow process. The pods of this orchid contain about million of tiny seeds that contain naked undifferentiated embryos composed of 80-100 cells without any functional endosperm. The dead seed coat is reduced to a membranous covering having longitudinal thickenings. Because of their specific symbiotic fungal requirement, seeds are rarely germinated in their natural environment. Knudson (1951) successfully germinated *Cattelya* seeds on nutrient medium under *in vitro* condition without the help of any symbiotic fungus. Since then extensive work has been initiated on micropropagation of many orchids from mature seeds and vast literatures have been accumulated in this area. But flasking of mature seeds for micropropagation has some disadvantages because seedlings grow slowly and flowering plants are usually obtained after several years (George, 1996; Sharma et al., 2004). A major advancement in orchid seed culture for micropropagation has been the development of green pod culture technique. In this technique, immature seeds within the green pod obtained from the plant after fertilization but prior to dehiscence are cultured on nutrient medium. Fertilization of orchid ovules takes place long (50 - 80 d) after pollination. However successful germination of immature seeds depends on selecting the correct time after pollination at which to harvest the green pod for culture. Sauleda (1976) published tables of optimal harvest times for selected orchids and hybrids of many genera. In general terms, immature seeds are removed from pod, which have progressed approximately 1/2 to 2/3 in their development from pollination to maturity. There are several advantages in using immature seeds such as; firstly, it increases the rate of orchid seed germination. Secondly, seedlings are given an earlier start by green pod culture and this can reduce the time from seed to flower. Thirdly, immature seed culture can assist in obtaining seedlings from wide crosses where embryos often abort before reaching maturity. Considering all the advantages, the present investigation has been carried out for rapid mass propagation of *D. fimbriatum* Hook. by asymbiotic *in vitro* green pod culture technique which can be exploited for their conservation.

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MATERIALS AND METHODS

Mature plants of *D. fimbriatum* Hook. were collected from the forest of Sikkim Himalaya (latitude 27°E 36'N, longitude 88°E 30"N, and altitude 1524 m above sea level) and grown in the departmental experimental garden, Darjeeling Himalaya (latitude 27° 3' 57"N, longitude 88° 15' 45"E, and altitude 2248 m above sea level). Reported time required for mature seed formation of *Dendrobium* (Nimoto and Sagawa, 1961) is 100-140 days. Therefore, 70 d old green undehisced pods from the experimental plants were harvested and used as explants. The pods were

Table 1. Effect of growth regulators and growth adjuncts on immature seeds of *D. fimbriatum* Hook.

SETS OF MIEDIUM	Media supplemented with/ without growth regulators and or growth adjunct	Type of response of immature seeds in culture		
		Stage I (Seed germination and development of PLBs)		Stage II (Development of shoot buds and seedling from PLBs****)
		Dark condition (up to 30 days)	Light condition (up to 45 days)	Light condition (up to 60 days)
I	VWM*+ 15% CW**	Swelling of seeds	No response	No response
II	VWM+25% CW	Swelling of seeds	No response	No response
III	VWM+0.1 mg L^{-1} NAA***	Swelling of seeds	PLB initiation	No further growth
IV	VWM+0.5 mg L^{-1} NAA	Swelling of seeds	No PLB initiation	No growth
V	VWM+0.1 mg L^{-1} NAA+ 15% CW	Swelling of seeds	80-90% seeds develop PLBs	Development of shoot buds from PLBs and young seedlings (approximately 4-5 mm length
VI	VWM+0.1 mg L^{-1} NAA+25% CW	Swelling of seeds	60-70% seeds develop PLBs	Development of shoot buds from PLBs and young seedlings (approximately 4-5 mm length
VII	VWM+0.5 mg L^{-1} NAA+15% CW	Swelling of seeds	No PLB initiation	No response
VIII	VWM+0.5 mg L^{-1} NAA+25% CW	Swelling of seeds	No PLB initiation	No response
IX	VWM+No supplement	No swelling of seeds	No PLB initiation	No response

*vacin and went medium; **coconut water; ***α-naphthalene acetic acid; ****protocorm like body.

cleaned thoroughly under tap water followed by washing with 5% Tween 20 for 10 min and finally with distilled water. They were surface sterilized with 0.1% mercuric chloride solution for 15 min and were subsequently rinsed 3 times in autoclaved distilled water. After surface sterilization, the capsules were taken in a sterile Petri dish containing filter paper to soak the surface water of the capsule. The capsules were cut longitudinally with a sharp sterilized surgical blade, and with a long spatula powdery mass of yellowish seeds were inoculated on the slant surface of solidified 0.8% agar Vacin and Went (1949) nutrient medium (VWM) supplemented with several concentrations and combinations of α-naphthalene acetic acid (NAA) and 15-25% coconut water (CW) as shown in Table 1. pH of the media was adjusted to 5.4 and autoclaved at 15 lbs/psi for 15 min at 121°C. After inoculating the seeds aseptically onto the culture medium, cultures were then transferred to dark condition for 30 d at 20 ± 1°C. After 30 d, cultures were maintained at 20 ± 1°C under 16 h photoperiod from cool white-light giving 2659 μmol $m^{-2}s^{-1}$ at culture level.

The results obtained from *in vitro* asymbiotic immature seed germination of *D. fimbriatum* in the medium containing different concentrations of NAA and coconut water are summarized in Table 1. Seed structure and type of response of seeds in culture were examined frequently by microscopic observation using 45X objectives.

RESULTS

Isolated immature seeds (Fig. 1) were extremely small (108.15 μm wide and 343.70 μm long). Among nine sets of media, 0.1 mg L^{-1} NAA and 15% CW (Set V) was found most effective and highest percentage (80 - 90%) of seed germination and seedling development were obtained. Swelling of seeds was the first visible change in culture when they were kept in the dark. Development of swollen spherical green corm-like embryo, i.e. protocorm like body (PLB), was progressed at the beginning of sixth week of culture when they were transferred to illumination. It was observed that darkness prior to light condition was necessary. Continuous dark or light conditions were inhibitory for immature seed germination and proto-

Figure 1. Isolated seeds of *D. fimbriatum* Hook. from green pod (X675).

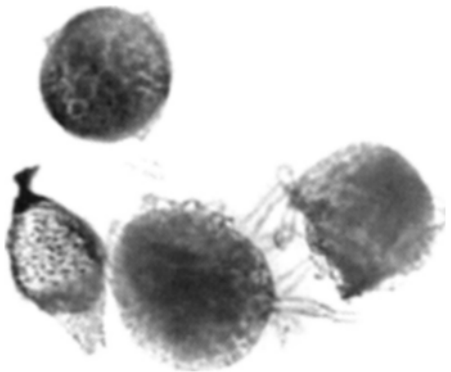

Figure 2. Development of fine rhizoid from the surface of PLBs (X675).

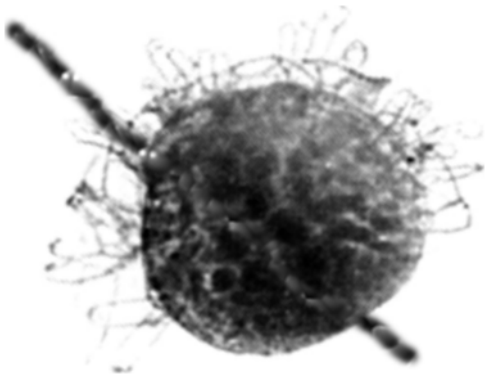

Figure 3. Development of fine rhizoid from the surface of PLBs (X675).

corm formation. Since the immature seeds retain long period inside the tightly closed thick walled pod before reaching maturity under natural condition, it possibly needs an initial obligatory dark condition for

Figure 4. Production of shoot bud from PLBs.

Figure 5. Development of rooted plantlets.

the germination and development of PLBs *in vitro* when they are exposed from the pod. But for latter developmental phases, light is essential. PLBs first came out by breaking from the fine membranous seed coat (Fig. 2). During the normal process of germination, PLBs became covered with fine rhizoids (Fig. 3). Typically a seed gave rise to a PLB, and in turn developed into a seedling. Division of very few percentages (5-6%) of PLB was also observed from which more than one plantlets were derived. The

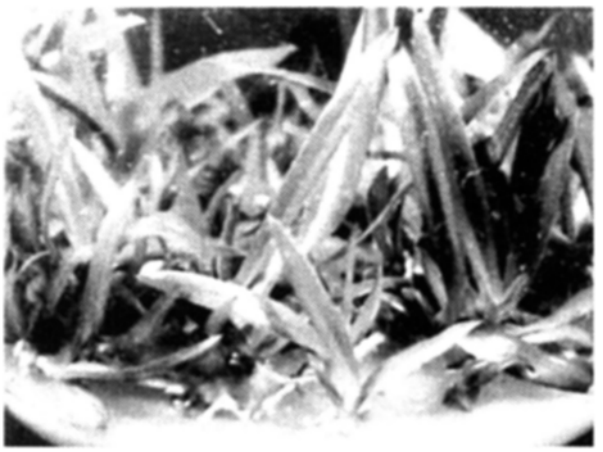

Figure 6. Development of rooted plantlets.

development of single protuberance of shoot bud took place from the PLBs. But the establishment of clear polarity was very weak. Once lateral green protuberances formed on PLBs and began to produce very small shoot buds (Fig. 4), lumps of PLBs from agar slope of culture tube were subcultured in a 250 ml culture bottle (Fig. 5) to provide more space for the development of plantlets (Fig. 6).

DISCUSSION

The process of development of complete plant from immature seed of *D. fimbriatum* through successive stages can conveniently be divided into two major stages, i.e., Stage I and Stage II. Stage I represents the seed germination and development of PLBs from the swollen seeds. Stage II represents the development of shoot buds and seedling from PLBs. It was observed that low concentration of NAA (0.1 mg L^{-1}) was essential in the medium for the initiation of PLBs from immature seeds of *D. fimbriatum* at Stage I, whereas higher concentration of NAA (0.5 mg L^{-1}) showed inhibitory effect on immature seed germination and PLB formation. Kumaria and Tandon (2000) studied the effect of growth regulators on peroxidase, polyphenol oxidase and IAA oxidase activities and phenolic content during protocorm initiation and development of *D. fimbriatum* var. *occulatum* Hk.F. They suggested that growth regulators at low concentration in the medium might act in a manner similar to symbiotic fungi and brought about the physiological changes for protocorm development. However, higher concentrations of growth regulators induced the increase of total phenolic content in the embryonic cells of seed. Polyphenolic oxidase oxidizes phenol and the oxidized products of phenol are inhibitory to plant cellular growth (Monaco et al., 1977). Therefore, media (Set IV, VII and VIII) containing 0.5 mg L^{-1} NAA were not conducive for PLB initiation and development. Presence of only CW in the medium (Set I and II) was also not conducive for Stage I in case of immature seed. But there are many evidences that seed germination and seed development of many orchids improved with the addition of coconut water to the medium (Lawrence and Arditti, 1964; McIntyre et al., 1974). However, Kotomori and Murashige (1965) observed that CW was not always suitable for the seed germination of *Dendrobium in vitro*. CW is generally added in orchid seed culture as source of sugar, natural cytokinins and vitamins (Matthews and Rao, 1980; Sarma, 2002).

Sucrose (typically 20 mg L^{-1}) and CW are added to promote protocorm differentiation, shoot bud formation and plantlet growth. Therefore, the necessity of sucrose is essential in the Stage II. Intuwong and Sagawa (1975) reported that protocorm differentiation and plantlet growth were improved when sugar concentration was reduced from the medium in case of *Dendrobium*. However, low percentage of CW (15%) in combination with 0.1 mg L^{-1} NAA and 20 g L^{-1} sucrose is desirable for the growth and development of protocorm and seedling. It was revealed in the present experiment that when the seeds were grown in the medium containing only 0.1 mg L^{-1} NAA (Set III), the PLB initiation took place, but no further growth, development and differentiation of PLB occurred. On the contrary, higher percentage of CW (25%) containing more sugar in presence of 0.1 mg L^{-1} NAA and 20 g L^{-1} sucrose did not improve the percentage of seedling development (60-70%) from protocorm. The percentage of success standardized in the present study by the manipulation of NAA and CW in the medium indicates that green pod culture of *D. fimbriatum* Hook. is a feasible method for rapid micropropagation and can be exploited as a part of their conservation.

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