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Green Pod Culture and Rapid Micropropagation of *Dendrobium chrysanthum* Wall. – A Horticultural and Medicinal Orchid

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ABSTRACT

Present studies have demonstrated that immature seeds obtained from green pod of *Dendrobium chrysanthum* Wall., a pharmaceutically valuable, ornamental, epiphytic forest orchid having horticultural importance, can be germinated asymbiotically *in vitro* for rapid micropropagation. Knudson C (KnC) medium (1951) containing 0.1 mg L^{-1} NAA and 15% coconut water (CW) was found most the effective for high percentage (80-90%) of seed germination and seedling development. The rate of survival of plantlets after the transfer from culture vessel to natural condition was 98-99%. This method can be exploited for the rapid propagation and conservation of *Dendrobium chrysanthum*.

Abbreviation:

KnC – Knudson C medium
NAA – α-Naphthalene acetic acid
CW – Coconut water
PLB – Protocorm like body.

INTRODUCTION

Dendrobium chrysanthum Wall. (*Orchidaceae*) is a pharmaceutically valuable, ornamental forest epiphytic orchid of the temperate and subtropical regions (Sikkim and Darjeeling Hills, India) and is also precious for their long lived beautiful sweet scented flowers having horticultural importance. In traditional Chinese medicine, sun dried stem powder of *D. chrysanthum* is primarily used to replenish body fluids. Decoction of stem is commonly used as a tonic to moisten the stomach and lungs. It is very effective for treating conditions such dry mouth, stomach pain, mouth sores, sunstroke and other condition caused by dry weather, pollution or smoke. Additionally *D. chrysanthum* is used to enhance skin quality (Bensky and Gamble 1993). In traditional African medicine, it is used as an aphrodisiac (Sawandi 2002). On account of the excellent herbal medicinal value and horticultural importance, *D. chrysanthum* is becoming rare due to human exploitation. This plant 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 the flasking of mature seeds for micropropagation has some disadvantages because seedlings grow slowly and flowering plants are usually only 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 from the green pod which are obtained from the plant after fertilization but prior to dehiscence are cultured on nutrient medium. Fertilization of orchid ovules takes place long (50-80 days) after pollination. Successful germination of immature seeds depends on time after pollination when they are isolated 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 ½ to ⅔ in their development from pollination to maturity. There are several advantages in using immature seeds. 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 get aborted before reaching maturity. Fourthly, the chance of seed loss from ripe capsule by sudden natural dehiscence can also be lessened by green pod culture. Considering all the advantages, the present investigation is meant for rapid mass propagation of *Dendrobium chrysanthum* Wall. by asymbiotic *in vitro* green pod culture technique which, then, can be exploited for their conservation.

MATERIAL AND METHODS

Mature plants of *Dendrobium chrysanthum* Wall. (Fig. 1) were collected from the forest area of Sikkim Himalaya (latitude 27º36′ N longitude 88º30″ E and altitude 1524 m above sea level) and grown in the departmental experimental garden, Darjeeling Himalaya (latitude 27º3′57″N, longitude 88º15'45″E and altitude 2248.20 m above sea level). Reported time required for mature seed formation of *Dendrobium* (Nimoto and Sagawa 1961) is 100-140 days. The precise time for harvesting the immature seed is temperature and particularly species dependent. Therefore, roughly 70 days old green undehised pods were harvested because the seeds were approximately progressed to ½ to ⅓ in their development which was found suitable as explant for their culture in the preliminary experiment. The pods were cleaned thoroughly under tap water followed by washing with 5% Tween 20 (liquid detergent) for 10 minutes and finally with distilled water. They were surface sterilized with an aqueous solution of 0.1% mercuric chloride for 15 minutes and were subsequently rinsed 3 times in autoclaved distilled water. After surface sterilization, the capsules were taken in a sterile petridish containing filter paper to soak the surface water. The capsules were cut longitudinally and powdery mass of yellowish seeds were inoculated on the slant surface of 0.8% agar solidified Knudson C (KnC) nutrient medium (1951) supplemented with α-naphthalene acetic acid (NAA) at different concentrations and combinations with 15-25% coconut water (Table 1). PH of the media was adjusted to 5.4 and autoclaved at 15 kpa for 15 min at 121ºC. Five to six pods were the source of seeds for each kind of medium. The seeds (approximately 200 seeds per culture tube) were aseptically inoculated into fifty culture tubes for each kind (I – IX) of culture medium (each tube contains 20 ml of medium). Cultures of 450 tubes were then incubated at dark condition for 30 days at 20 ± 1°C and later cultures were kept on the same media without any subculture up to 45 days at 20 ± 1°C under 16 h

photoperiod from cool white-light giving 2659 µmol m^{-2} s^{-1} at culture level. The culture of one tube constitutes one replication and the whole experiments were repeated twice following the same methodology and keeping the same culture conditions. The germinating seeds from those media (III, V and VI) where germination was observed in large number, were subcultured into their respective media and were maintained for one month and another one month on these media which supported plantlet development (altogether two subcultures of one month duration). The plantlets obtained were next subcultured regularly four time at three weeks interval each times onto medium with halved concentration of nutrients, hormones and CW, so finally the plantlets were grown on very low concentration of nutrient ($1/10^{th}$ of original concentration of Knudson's medium) and hormone free agar gel. Each subculture consisted of 200 glass bottles (250 ml) with 30-40 plantlets. At the end about 150 micropropagated plantlets of 4-5 cm in length were taken out from the glass bottles (100 plantlets from the medium V and 50 plantlets from the medium VI) and washed in lukewarm water to remove agar gel sticking to them. These plantlets were first placed in a moist moss filled pot under the shady environmental condition of Darjeeling Hill {average daily temperature = 14.8°C, RH = average 90%, sun-shine brightness (hr/day) = 3.2} for 10-15 days, later they were tied gently with moist moss to the lower branches of a tree they were found to grow.

Seed structure and type of response of seeds in culture were examined frequently by microscopic observation.

RESULTS

Isolated immature seeds (Fig. 2) were extremely small (108.15 µm wide and 343.70 µm long). Out of nine tested media seed germination was observed in large number only on media III, V and VI, however medium supplemented with 0.1 mg L^{-1} NAA and 15% CW (medium V) was found the most effective and incidentally the highest percentage (80-90%) of seed germination and PLBs development was obtained in that case (Table 1). Swelling of seeds was the first visible change in culture when they were kept in dark. Development of a swollen spherical green corm-like embryo i.e. protocorm like body (PLB), one per explant (seed), was noted at the beginning of sixth week of culture when they were transferred under light. For latter developmental phases – shoot and plantlet development, light is essential. However PLBs did not undergo further development on medium III during subsequent subcultures and only on media V and VI they developed into plantlets of size suitable for potting (4-5 mm in length). PLBs first came out by breaking from the fine membranous seed coat (Fig. 3). During the normal process of germination, PLBs became covered with fine rhizoids (Figs 4-5). Typically

a seed gave rise to a PLB, which in turn developed into a seedling. Figs 6-7 show the development of a protuberance of shoot bud. The establishment of polarity of shoot protuberance was very prominent. Once lateral green protuberances formed on PLBs and subsequently began to produce shoot buds and subsequently seedlings (Figs 8-11), at this stage, lumps of PLBs (Fig. 12) from agar slope of culture tubes were sub-cultured to 250 ml glass bottles (Fig. 13) just to provide more space for the growth and development of plantlets (Fig. 14). The rate of survival of deflasked plantlets to the natural climatic condition after following the acclimatization process was about 98-99%.

Table 1. The effect of growth regulators and or growth adjunct on type of response of immature seeds of *Dendrobium chrysanthum* Wall. up to 60 days of culture

Medium	Media supplemented with / without growth regulators and or growth adjunct	Type of responds of immature seeds in culture		
		Stage I (Seed germination and development of PLBs)		Stage II (Development of shoot buds and seedlings from PLBs)
I	KnC + 15% CW	Swelling of seeds	No response	No response
II	KnC + 25% CW	Swelling of seeds	No response	No response
III	KnC + 0.1 mg L^{-1} NAA	Swelling of seeds	PLB initiation	No further growth
IV	KnC + 0.5 mg L^{-1} NAA	Swelling of seeds	No PLB initiation	No growth
V	KnC + 0.1 mg L^{-1} NAA + 15% CW	Swelling of seeds	80-90% seeds developed PLBs	Development of shoot buds from PLBs & young seedlings (approximately 4-5 mm in length)
VI	KnC + 0.1 mg L^{-1} NAA + 25% CW	Swelling of seeds	60-70% seeds developed PLBs	Development of shoot buds from PLBs & young seedlings (approximately 4-5 mm in length)
VII	KnC + 0.5 mg L^{-1} NAA + 15% CW	Swelling of seeds	No PLB initiation	No response
VIII	KnC + 0.5 mg L^{-1} NAA + 25% CW	Swelling of seeds	No PLB initiation	No response
IX	KnC + No supplementation	No swelling of seeds	No PLB initiation	No response

KnC = Knudson C nutrient medium, NAA = α-Naphthalene acetic acid, CW = coconut water, PLB = protocorm like body

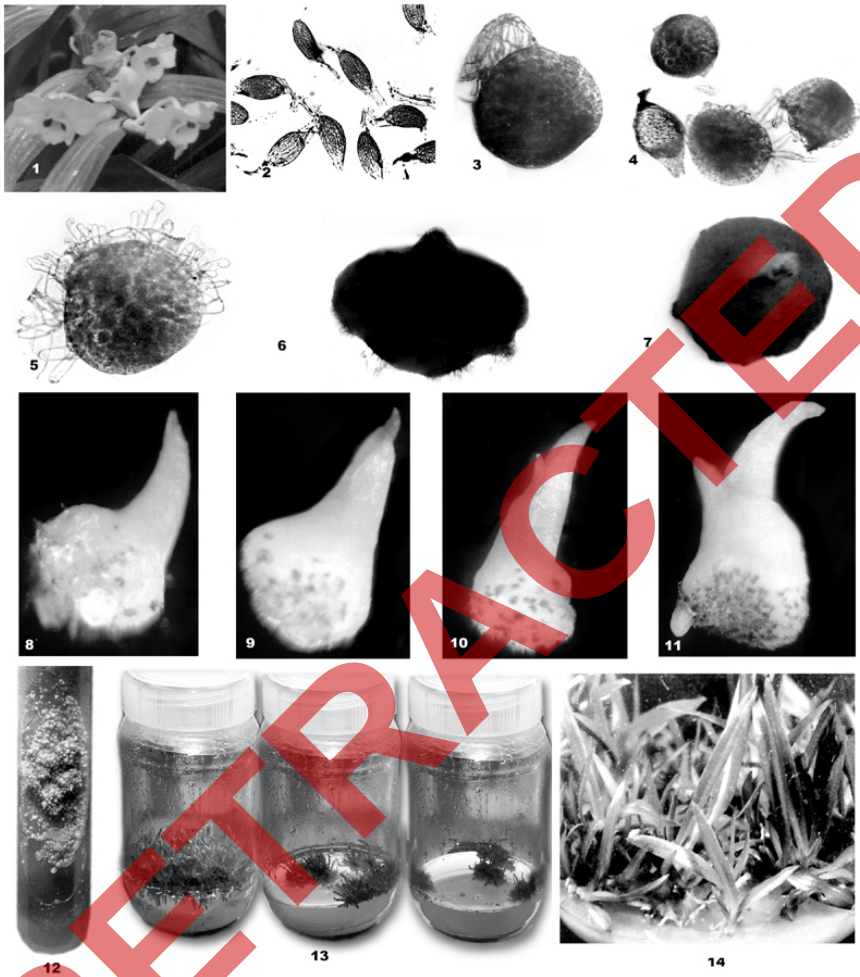

Figure 1. Flowering twig of *Dendrobium chrysanthum* Wall.
Figure 2. Isolated immature seeds (× 400)
Figures 3-4. Protocorm-like body (PLB) comes out by breaking the membranous seed coat on Knudson C medium supplemented with 0.1 mg L^{-1} NAA, 35 days after culture (× 400)
Figure 5. Protocorm-like body covered with fine rhizoids on Knudson C medium supplemented with 0.1 mg L^{-1} NAA and 15% CW, 40 days after culture (× 450)
Figures 6-7. Development of protuberance of shoot buds from PLB on Knudson C medium supplemented with 0.1 mg L^{-1} NAA and 25% CW, 10 days after first subculture (× 450)
Figures 8-11. Sequential stages of development of seedling from shoot buds on Knudson C medium supplemented with 0.1 mg L^{-1} NAA and 15% CW, 20-25 days after first subculture (× 600)
Figure 12. Seedlings on medium in culture tube on Knudson C medium supplemented with 0.1 mg L^{-1} NAA and 15% CW, 30 days after second subculture (× 1)
Figure 13. Seedlings growing on low nutrient and hormone free agar medium in jam bottle (× 1/2)
Figure 14. Development of plantlets

DISCUSSION

The process of development into complete plant from immature seed of *Dendrobium chrysanthum* Wall. through successive stages can be conveniently 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 under dark condition. Stage II represents the development of shoot buds and seedling from PLBs under light condition. It was observed that darkness prior to light condition was necessary; otherwise continuous dark or light conditions were inhibitory for immature seed germination and protocorm formation. Since the immature seeds retain a long period inside the tightly closed thick walled pod before reaching maturity under natural condition, hence it possibly needs an initial obligatory dark condition for the germination and development of PLBs *in vitro*. PLB formed *in vivo* became green and their growth relies partly on the supply of carbohydrates by a symbiotic fungus, and partly on photosynthesis but during *in vitro* germination, where sugars are provided by the medium, PLB development can take place in the dark (George 1996). It was also 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 *Dendrobium chrysanthum* at Stage I, whereas higher concentration of NAA (0.5 mg L^{-1}) showed inhibitory effect on immature seed germination and PLB formation. A perusal of literature reveals that in most of the orchids, germination of seeds *in vitro* takes place in presence of low concentration of NAA used as an auxin. NAA was successfully used also as auxin to initiate shoot bud in *Oncidium varicosum* Lindl. (Kerbauy 1984) *Bletilla striata* (Thumb.) Reichb., *Cleisostoma fordii* Hance. and *Pholidota chinensis* Lindl. (Yam and Weathrhead 1991) and *Cymbidium* sp. (Vij et al. 2004). 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 *Dendrobium 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 bring 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. 1997). Therefore, media (media IV, VII and VIII) containing 0.5 mg L^{-1} NAA were not conducive to PLB initiation and development. Presence of only CW in the medium (media I and II) was also not conducive to Stage I in case of immature seed. But there are many evidences that seed germination and seedling 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 a source of sugar, natural cytokinins and vitamins (Matthews and Rao 1980, Sarma 2002).

Sucrose (typically 20 g 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 sucrose (20 g L^{-1}) is desirable for the growth and development of protocorm and seedling (Sharma et al. 2005). It was revealed in the present experiment that when the seeds were grown in the medium containing only 0.1 mg L^{-1} NAA (medium III), the PLB initiation took place but no further growth, development and differentiation of PLB occurred. On the contrary, higher percentage of CW (25%) in presence of 0.1 mg L^{-1} NAA and sucrose (20 g L^{-1}) did not improve the percentage of seedling development (60-70%) from protocorm. The percentage of success standardized in the present study indicates that green pod culture of *Dendrobium chrysanthum* Wall. is a feasible method for rapid propagation and can be exploited as a part of their conservation.

CONCLUSIONS

1. Knudson C medium supplemented with 0.1 mg L^{-1} NAA and 15% coconut water was the most effective for high percentage (80-90%) of seed germination and seedling development.
2. During incubation of culture darkness prior to light condition is obligatory and conducive for PLB formation and seedling development.
3. The rate of survival of plantlets after the transfer from culture vessels to natural condition was 98-99%.
4. By the successful results obtained from green pod culture of *Dendrobium chrysanthum* Wall., many possibilities like preservation of germplasm, conservation and the creation of genetic variability for horticultural improvements can be done. This method of culture may become helpful to the orchid breeders also.

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