Research Paper

In vitro Propagation of the Medicinal Orchid *Dendrobium chrysanthum*

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An efficient system for propagation of *Dendrobium chrysanthum* Wall. ex Lindl., a medicinally important orchid, through *in vitro* asymbiotic seed germination has been established. Mature seeds from 8 months old green pods of *D. chrysanthum* were inoculated on different culture media *viz.*, Murashige and Skoog (MS), Nitsch and Nitsch (NN), Gamborg *et al*. (B_5) and Knudson C (KC) without phytohormone supplementation. MS medium proved to be the best as about 94% of the seeds gave complete plantlets with well-developed roots and shoots within 90 days. About 7 months-old plantlets hardened in a compost mixture comprising brick, charcoal, decaying litter in a ratio of 1:1:1 and a layer of moss on top showed survival percentage of 71.

Key Words: Asymbiotic Seed Germination; Endangered Orchid; *In vitro* Conservation

Introduction

Orchids produce many minute seeds that contain insufficient reserves for germination. The association of fungi, therefore, becomes indispensable for germination. Because of their particular fungal requirement, less than 5% seeds germinate in nature [1]. However, a very high percentage of seed germination could be achieved through asymbiotic germination in culture flasks or culture tubes. Different workers suggested various media for orchid seed germination [2-6]. But the responses of orchid seeds to nutrient media vary from species to species. Therefore, a thorough study on asymbiotic germination on different classes of orchid species is required.

Dendrobium chrysanthum Wall. ex Lindl. (Fig. 1) is an important medicinal orchid but is also valuable in floriculture industry. In traditional Chinese medicine, the stems of several species of *Dendrobium* are used as a tonic to nourish the stomach, promote the production of body fluid, and reduce fever [7]. "Shi-Hu", a famous tonic in traditional Chinese medicine lists *D. chrysanthum* as one of the original materials and it has been recorded in the Chinese Pharmacopoeia. Bensky and Gamble [8] reported that *D. chrysanthum* is used to enhance quality of skin. In the North East India, the natural habitats of *D. chrysanthum* are being affected due to over exploitation for commercial purposes, deforestation and urbanization leading to the rapid loss of this valuable orchid. *D. chrysanthum* figures in the list of endangered plants in CITES, therefore its propagation and conservation need immediate efforts to save it from extinction.

Materials and Methods

The plants of *D. chrysanthum* were collected from Nongpyiur forest, Upper Shillong, Meghalaya during June – July and grown in the glass house of the Department of Botany, North-Eastern Hill University, Shillong, India. Eight-months old pods of *D. chrysanthum* were used in the experiment. The pods were first washed thoroughly in running tap water. These were then sterilized by flaming 3-4 times after dipping in 70% alcohol. The pods were cut longitudinally under aseptic conditions to expose the seeds. Seeds were then scooped out and inoculated on different basal media *viz*., (MS) [9], (NN) [10], (B_5) [11] and (KC) [12]. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 15 mins. The culture tubes were kept at 25 ± 2°C with light intensity of 60 mmolesm^{-2}s^{-1} and 12 h photoperiod after initial incubation in dark for 2

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weeks. Each treatment had 10 replicates and the experiment was repeated. The percentage of germination was determined by examining the seeds under the microscope after 60 days. The seeds were considered as germinated upon emergence of the embryo from the testa. The developmental process of orchids from seeds to complete plantlets (with shoots and roots) can be categorized into four stages:

Stage I: Non-germinated seeds, embryo slightly swollen but still covered with its seed coat or testa;

Stage II: Germinating seeds, embryo greatly swollen forming ovoid tear drop shaped protocorm without seed coat or testa;

Stage III: Young protocorms showing vegetative apex and

Stage IV: Complete plantlets with shoots and roots. The formula for an oblete speroid $4/3\pi\ a^2b$, where 'a' and 'b' are the minor and major semi-axes, was used to determine the protocorm volume [5]. Seedlings after 120 days of culture were taken for observing growth parameters *viz*., shoot number (number of shoots/ protocorm), shoot length (length from the base of the shoot to the tip of the first leaf), number of leaves (number of leaves/seedling), root number (number of roots/seedling) and root length (length from the base of the shoot to the tip of the root) in the different media. The data was analyzed using one way ANOVA and Fisher's LSD test [13] and used for comparison among treatment means.

Plantlets with well-developed shoots and roots (4-5 cm) obtained after seven months of culture were washed with water to remove the adhering agar medium and transferred to clean thermocol pots of 8cm diameter containing different mixtures of compost *viz.*, (I) brick and charcoal pieces (1:1), (ii) brick pieces, charcoal pieces and coconut husks (1:1:1), (iii) brick pieces, charcoal pieces and decaying litter (1:1:1). These different compost mixtures were covered with a layer of moss on top. Single plantlet was potted in each pot and covered with holed polythene bags for about 2-3 weeks. The plantlets were carefully sprayed with water and shifted to the glass house at 18-25^{0}C temperature and 70-80% RH. The plantlets were watered on alternate days. The survivability of the transferred plantlets was recorded after 90 days of transfer.

Results and Discussions

Asymbiotic seed germination in sterile conditions is one of the methods of conservation and propagation of orchids [14]. The highest seed germination percentage was obtained in B_5 medium (96.7% ± 0.25) followed by NN (94.4% ± 0.30), MS (94.1% ± 0.33) and the least was recorded in KC (29.7% ± 0.46) (Table 1, Fig. 2). A balanced supply of both organic and inorganic nutrients is needed for the development of orchid seeds [15]. Plant growth regulators, i.e., cytokinins are reported to play an important role in orchid seed germination [16], however, in our study, the seeds of *D. chrysanthum* germinated in medium devoid of growth regulators. This could be due to the presence of sufficient endogenous growth regulators needed for the initial stages of germination [17]. The presence of nitrogen in the form of potassium nitrate in B_5 medium could have accounted for the high germination percentage of *D. chrysanthum* seeds. Also, the presence of the vitamin, thiamine in higher amount in B_5 might have influenced the germination of these seeds [18-19]. The largest protocorm volume was recorded in MS (29.95×10^{-4} mm^3) and the smallest in KC (1.8×10^{-4} mm^3). This might be because MS medium is rich in both micro and macro nutrients. The presence of nitrogen in the medium is also reported to influence the growth and differentiation of cells [20].

Further development of protocorms into seedlings can be attributed to the efficient assimilation and utilization of nitrogen in the form of ammonium nitrate present in the MS medium. The growth of the seedlings *viz*., shoot number (1.90 ± 0.11), shoot length (1.17 ± 0.04), number of leaves (2.80 ± 0.09), root number (3.05 ± 0.09) and root length (0.80 ± 0.009) was also found to be highest in MS medium (Table 2). In B_5 and NN media, the protocorms formed shoots and roots but growth stopped after 90 days. This might be attributed to the inhibitory influence of nitrogen in the form of ammonium sulphate in B_5 medium or mixtures of vitamins present in both B_5 and NN media on seedling growth [19, 21]. The poor response in terms of seed germination and growth of the protocorms in KC medium could have been due to the lower amount of nutrients and vitamins present in KC medium which were not sufficient for complete development of the seedlings [5]. However, Nongrum *et al*. reported enhanced seed germination of *Coelogynae ovalis* and *C. nitida* in KC medium [22]. Nath *et al.* reported a similar finding where *Vanda coerulea* failed to develop beyond the protocorm stage [23]. There are also similar reports of inhibition of seed germination of epiphytic orchids in KC

Table 1: Effect of different media on seed germination and development of protocorms of *Dendrobium chrysan*thum

Media	Germination %*	Protocorms				Volume (mm^3)**
		Development stage (weeks)				
		I	II	III	IV	
Murashige and Skoog	94.1 ± 0.33[b]	4	6	8	12	29.95 x 10^{-4a}
Nitsch and Nitsch	94.4 ± 0.30[b]	3	4	7	11	8.78 x 10^{-4c}
Gamborg *et al.*	96.7 ± 0.25[a]	4	6	8	13	13.61 x 10^{-4b}
Knudson C	29.7 ± 0.46[c]	6	8	12	-	1.80 x 10^{-4d}

*Data collected after 60 days of inoculation
**Mean of 30 values

Table 2: Effect of different media on growth and development of *Dendrobium chrysanthum* seedlings at 120 days from incubation of seeds for germination

Media	Shoot No.	Shoot length (cm)	No. of leaves	Root No.	Root length (cm)
Murashige and Skoog	1.90 ± 0.11[a]	1.17 ± 0.04[a]	2.80 ± 0.09[a]	3.05 ± 0.09[a]	0.80± 0.009[a]
Nitsch and Nitsch	1.26 ± 0.08[b]	0.63 ± 0.01[b]	2.55 ± 0.08[b]	2.41 ± 0.08[b]	0.50 ± 0.020[b]
Gamborg *et al.*	1.43 ± 0.09[b]	0.67 ± 0.02[b]	2.41 ± 0.07[b]	2.26 ± 0.07[b]	0.38 ± 0.013[c]

Values are mean±S.E. Means followed by same letter in the column are not significantly different as indicated by Fisher's LSD (p = 0.05)

medium [21, 24-25]. The differential response of orchid seeds to different nutrient media is due to specific requirement of the species. Plantlets were hardened in a compost mixture comprising brick, charcoal, decaying litter in a ratio of 1:1:1 and a layer of moss on top. During the process of hardening it was observed that the transferred plants initially shed their leaves then produced new leaves. According to Preece and Sutter plantlets when transferred, must produce new leaves to adjust to new conditions in order to enable effective photosynthesis and growth of the *in vitro* - raised plants [26]. Plantlets hardened in a compost mixture comprising brick, charcoal, decaying litter in a ratio of 1:1:1 and a layer of moss on top showed 71% survival (Fig. 3). The high moisture content retention ability of the layer of the moss on top proved to be beneficial for the successful transplantation of the *D. chrysanthum* plants.

The protocol developed in the present study can be used for *in vitro* mass scale propagation of *D. chrysanthum* through asymbiotic seed germination wherein a maximum of around 200 plants can be produced from 100 seeds in basal MS medium. Thus, the protocol is suitable for *ex situ* conservation and propagation of this threatened medicinally important orchid species.

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