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In vitro seed germination of an Endangered Terrestrial Orchid Species *Geodorum densiflorum* (Lam.) Schltr.

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Abstract

Orchid seed is rather difficult to germination rather than other angiospermic seeds, because of lack endosperm, radical and leaf rudiments. Seed germination and protocorm like body (PBL) formation of *Geodorum densiflorum* (Lam.) Schltr. was performed. This study was conducted to determine the effects of coconut milk (CM), and BAP for optimal media culture. Seeds from sterilized capsules were cultured on two media (Knudson C orchid medium, KnC, and MS medium) containing 0 and 25% (v/v) CM and 0 and 5 mgl^{-1} BAP with 0.8% (w/v) agar as solidifying material. The cultures were maintained at 25±2°C with 12-hour illumination of 60 µmoles m^{-2} sec^{-1} light intensity provided by cool white florescent tubes. The highest germination percentage was observed in 15% (v/v), CM and 3 mg l^{-1} BAP both in MS and KnC media. Seeds germinated and formed light green globular structures on the medium after three weeks of culture. These globular structures produced Protocorm-like body (PLB) and proliferated and developed into irregular-shaped rhizomes with white hairy structures. Highest *in vitro* seed germination was found in KnC medium supplemented with 15% CM and 3 mgl^{-1} BAP about 95.31 % whereas in MS medium maximum germination reached at 79% with 15% CM and 3 mg l^{-1} BAP. Overall, this study showed that *Geodorum* seeds cultured on KnC medium containing CM and BAP can be used for clonal propagation.

Introduction

Orchids belonging to the family Orchidaceae are one of the largest and most evolved flowering plants. Orchidaceae includes about 800 genera and between 25,000 to 30,000 species distributed all over the world (Chowdhery, 2001). Orchids produce one of the most beautiful and enchanting flowers that have fascinated people of all ages. Besides being considered an ornamental treasure in the commercial market, orchids have important medicinal properties used in the preparation of herbal medicines in different parts of the world (Arditti, 1992). According to World Health Organization, 80% of people depend mainly on traditional remedies such as herbs for medicine (Kala, 2005), resulting in increasing demand for medicinal plants. Orchids are experiencing a steady decline in tropical countries due to destruction of natural forest areas. It is essential to take measures for the conservation and propagation of these endangered orchid species (Hossain, 2015; Hossain *et al.*, 2013; Hossain and Dey, 2013). *Geodorum densiflorum* (Lam.) Schltr. is an endangered terrestrial orchid appearing above the ground only during the rainy season. The introduction of an asymbiotic seed germination method by Knudson (1946) and shoot tip culture by Morel (1960) has helped in developing methods for orchid propagation. *In vitro* culture of seeds of *Geodorum densiflorum* (Lam.) Schltr. resulted in development of protocorms. *Geodorum densiflorum* is one of the floriculturally and medicinally important ground orchids. Rhizomes of *G. densiflorum* are used as medicine for the treatment of various diseases (Rao 1979). Because of damage of its natural habitats by continuous destruction of forest for land reclamation and indiscriminate collection by orchid lovers, this species has now become endangered. But the demand of such orchids is increasing day by day in local and foreign markets. As orchid seeds do not possess endosperm, their natural germination is limited and need a symbiotic association with specific mycorrhizal fungus. The discovery of *in vitro* seed germination and micropropagation contribute immensely to alleviate their scarcity. Sheelavantmath *et al.* (2000) reported a protocol for rhizome based propagation of *G. densiflorum*. Growth rate of the tissues can be increased by the addition of organic supplements and plant extracts. Many different

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organic additives like coconut water, banana pulp, peptone, tomato juice, slap honey, date palm syrup, corn extract, papaya extract and beef extract influenced medium culture and provide undefined mixture of organic nutrients and growth factors (Gnasekaran *et al.*, 2012 and Nambiar *et al.*, 2012; Shekarriz *et al.*, 2014). Coconut milk is the extract of white, solid endosperm of matured coconut after grinding and squeezing. This compound is often used when no other composition of known defined components produces the desire growth or development. Coconut milk (CM) is the colorless liquid endosperm of green coconuts (*Cocos nucifera*) which its liquid endosperm contains a number of amino acids, organic acids, nucleic acids, several vitamins, sugars and sugar alcohols, plant hormones (auxins, cytokinins), minerals and other unidentified substances, none of which alone is totally responsible for growth promoting qualities (Lu 2013).

The present investigation was undertaken with a view to developing an efficient *in vitro* cultural technique of germination, possibility of inducing PLBs from the seeds and micropropagation of *Geodorum densiflorum*, an endangered orchid species of North Bengal to help in *ex situ* conservation.

Materials and Methods

Mature capsules of *Geodorum densiflorum* were surface sterilized by submerging them in a 0.1% (w/v) $HgCl_2$ (mercuric chloride) solution for 8 mins with occasional agitation followed three washes in sterilized distilled water. Then dip in absolute ethanol for 20-25 sec. The sterilized capsules were then washed thrice with sterile distilled water. The capsules were then cut with a sterile surgical blade and the seeds were inoculated on to the surface of the Knudson C medium (Knudson, 1946) and MS medium (Murashige and Skoog, 1962) with growth regulators and adjuvants. All works were performed in a laminar airflow cabinet. The pH of the media was adjusted to 5.8 with 0.1N NaOH or 0.1N HCl prior to autoclaving and the medium was solidified with 0.8% agar. Media was autoclaved at 121°C for 15 mins at 15 psi. The cultures were maintained at 25±2°C with a cycle of 12/12 hour continuous light (illumination of 60 µmole $m^{-2}s^{-1}$) and dark conditions provided by cool white florescent tubes (Philips India). After germination of seeds, protocorms were subcultured at 25-day interval. Two different types of media were used in the present investigation KnC and MS media. Media were solidified with 0.8% (w/v) agar and fortified with different concentrations and combinations of PGRs (0-5 mgl^{-1} BAP and 0-25% coconut milk) were used for the purpose. Three replicates per treatment, arranged in a completely randomized design (CRD) were maintained. Survival percentage, germination percentage and protocorm like body formation were recorded.

Results and Discussion

Seed Germination and PLB formation

The seeds germinated on all the nutrient media used but germination percentages varied depending on the media composition. Maximum seed germination (95.31%) was recorded in KnC medium when fortified with 3.0 mgL^{-1} BAP + CM 15%. Species-specific media for seed germination have been reported in orchids (Arditti and Ernst, 1984). The effect of CM and BAP and media culture on seed germination

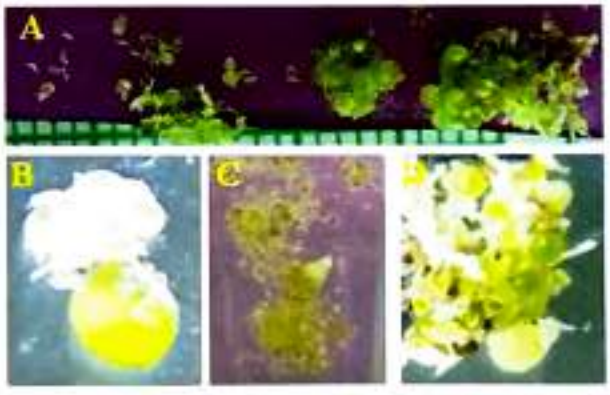

Fig. 1: In Vitro seed germination of one endangered orchid species *Geodorum densiflorum*. A. Seed swelling and embryo rupture from the seed coat, B. Globular structure before PLB formation, C. Protocorm like body formed (PLBs) and D. Secondary PLBs are formed

percentage is shown in Table 1. CM and BAP and media culture were significantly influenced germination percentage in *Geodorum*. Germination in presence of CM was better than

control. Germination increased when added coconut milk in both the media KnC and MS (Table 1). The specificity was reported even within species of the same genus, for example, Mitra *et al.* (1976) medium for *Cymbidium macrorhizon* (Vij and Pathak. 1988; Jamir *et al.*, 2002) medium for *C. iridioides* and Knudson C for *C. elegans* (Sharma and Tandon 1990). After 2 months, the embryos swelled and broke out of the testa, and then formed protocorm like-bodies (PLBs) (Figure 1) by 4 months. The protocorm enlarged, and produced rhizomes with multiple buds (Figure 1). The four month old PLBs were sub-cultured in the same medium for secondary PLBs formation for micropropagation purpose.

The protocorms proliferated in germination media but these did not develop seedlings in the culture media. Germination of orchid seeds followed a peculiar metamorphogenetic pathway (Figure 1); that is, undifferentiated embryos swelled up by absorbing water and nutrients from the media and developed a compact mass of parenchymatous cells called spherule which gradually develop protocorm, an intermediate structure between seed and seedling (Leroux *et al.*, 1997). At the initial stage of protocorm development (Figure 1), an appendicle, looking like a closed ridge, appeared at the upper part of the protocorms which leads to shoot formation while basal part escorts root development (Hossain and Dey, 2013). The protocorms became elongated and formed rhizome-like bodies (RLBs) with numerous hairs and some growth appendages on the body surface and a growing tip indicating the development of leafy shoots and the root initials, respectively. Formation of RLBs in *in vitro* protocorms has also been reported in a terrestrial orchid, *Geodorum densiflorum* (Bhadra and Hossain, 2003). Kanjilal and Datta (2000) reported that peptone (2 g l^{-1}) was effective in promoting the survival percentage of explants a terrestrial orchid *Geodorum densiflorum* (Lam) Schltr. but had no effect on PLB production. BAP is known to enhance germination frequency in *Cypripedium* spp., *Eulophia dabia*, and *Pachystoma senile* and stimulated protocorm multiplication as well as shoot formation in *Cymbidium pendulum* and *Cattleya aurantiaca*. This is a simple and efficient procedure for seed germination and PLB formation of *Geodorum densiflorum* (Lam.) Schltr. could be used for large-scale propagation and *ex situ* conservation of this endangered orchid species. PBL formation in different culture media significantly affected PBL formation in *Geodorum* orchid but CM had shown significant effect on PBL formation. The highest PBL number was showed with 15 % CM with 3 mgl^{-1} BAP in compare to control (0 mgl^{-1}). Between media culture, KnC medium showed the highest PBL formation (Table 1).

Table 1. Seed germination of one orchid species *Geodorum densiflorum*.

Medium	PGRs (mgL^{-1}) BAP	Additives (CM %)	Time (days)		Seed germination (%) (mean ± SE)
			Spherule	Protocorms	
MS	0.0	0	30–40	40–45	53.00 ± 1.34
	1.0	5	30–40	40–45	61.00 ± 2.30
	2.0	10	25–32	35–40	68 .00 ± 1.37
	3.0	15	20–25	30–35	79.00 ± 0.92
	4.0	20	25–35	40–45	65.00 ± 2.01
	5.0	25	30–40	40–45	55.00 ± 1.44
KnC	0.0	0	20–25	40–45	65.57 ± 1.22
	1.0	5	25–30	45–50	71.08 ± 1.58
	2.0	10	20–25	35–40	84.00 ± 1.09
	3.0	15	20–25	30–35	95.31 ± 0.08
	4.0	20	20–25	35-40	76.77 ± 1.99
	5.0	25	20–25	40-45	70.34 ± 2.03

Mean values of three replicates ± Sd.

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