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In vitro Germination and Micropropagation of *Geodorum densiflorum* (Lam.) Schltr., an Endangered Orchid Species

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Abstract

Seeds of *Geodorum densiflorum* (Lam.) Schltr. were aseptically cultured on 0.8% (w/v) agar solidified Murashige and Skoog (MS) and Phytamax (PM) media. Seeds germinated and formed light green globular structures on both the media. These globular structures failed to produce seedlings on PM medium, where it proliferated and developed into irregular-shaped rhizomes with white hairy structures. On the other hand, on MS medium the globular structures directly produced tiny seedlings which underwent elongation in PGRs supplemented MS medium. A composition of 2.0 mg/l NAA and 2.0 mg/l BAP proved best for enhancing elongation. The elongated seedlings were grown on half and full strength MS medium with or without IAA and activated charcoal (AC). For sound development of the root system MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.1% (w/v) AC proved best. The irregular shaped rhizomes as developed on PM medium were further used for mass production of seedlings. Rhizome tips produced multiple shoot buds on PGRs (NAA, IAA, IBA, picloram, BAP and zeatin) supplemented media. The highest number of multiple shoot buds per segment was observed on 0.8% (w/v) agar solidified MS + 3% (w/v) sucrose + 2.0 mg/l BAP. The multiple shoot buds underwent elongation on the same media and the maximum rate of elongation was recorded on 0.8% (w/v) agar solidified MS + 3% (w/v) sucrose + 2.0 mg/l NAA + 2.0 mg/l BAP. The elongated seedlings were rooted in the rooting medium and the plantlets after acclimatization were established in pot.

Introduction

Geodorum densiflorum is one of the floriculturally and medicinally important ground orchids of Bangladesh. 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. Orchids also propagate vegetatively. But the conventional methods of propagation of orchids are very slow and labourious. For these reasons the price of orchids is very high. The discovery of *in vitro* germination and micropropagation contribute immensely to alleviate their scarcity. Sheelavantmath et al. (2000) reported a protocol for rhizome based propagation of *G. densiflorum*. It is important to study the different aspects of *in vitro* propagation using both seeds and explants. With this idea in mind the present investigation was undertaken with a view to developing an efficient *in vitro* cultural technique of germination and micropropagation of *G. densiflorum*, an endangered orchid species of Bangladesh.

Materials and Methods

Mature capsules of *Geodorum densiflorum* were surface sterilized by submerging them in a 0.2% (w/v/) $HgCl_2$ solution for 10 mins with occasional agitation followed by a dip in absolute ethanol for 12 - 15 sec. The sterilized capsules were then washed 5 - 6 times 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 medium in the culture vessel. All these operations were done in a laminar airflow cabinet. pH of the medium was adjusted between 5.4 and 5.8 prior to autoclaving at 121_C for 20 mins at 1.9 g/cm^2 pressure. Culture vessels with inoculated seeds were maintained in the culture room with 14/10 h light and dark cycle at 25 ± 2_C.

After 15 days of germination the tiny seedlings were aseptically taken out of the culture vessels and subcultured at a lower density on the same medium. Further subculturing was done at 20 - 25 days interval. In order to induce rapid growth, the seedlings were transferred to the elongation medium. Different types of 0.8% (w/v) agar solidified media fortified with different concentrations and combinations of PGRs (1.0 mg/l NAA + 0.5 mg/l BAP, 2.0 mg/l NAA + 2.0 mg/l BAP, 2.5 mg/l BAP + 0.1 % (w/v) AC, 1.0 mg/l Pic. + 2.0 mg/l BAP + 0.1% (w/v) AC, 1.0 mg/l IAA + 2.0 mg/l BAP) were used for the purpose. The elongated seedlings at a height of 3 - 4 cm were transferred to three kinds of 0.8% (w/v) agar-solidified rooting media viz. (i) half strength MS + 1.5% (w/v) sucrose, (ii) MS + 3% (w/v) sucrose + 0.5 mg/l IAA and (iii) MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.1% (w/v) AC. For micropropagation, tips

of seed-derived mini rhizomes were cultured aseptically on different 0.8% (w/v) agar solidified media supplemented with differnt PGRs. Activated charcoal was also added in some of the media as an additive. Culture vessels were maintained in the culture room under the same conditions as were used for germination. Within 25 - 33 days of culture, new shoot buds emerged from the rhizome tips and those underwent elongation on the same medium. When the multiple shoot buds reached a height of 3 - 4 cm, they were transferred to the rooting medium.

Results and Discussion

Seeds germinated on both PM and MS media and produced light green globular stuctures within 45 - 60 days of culture. The globular structures failed to produce seedlings on PM medium where it proliferated and developed into a irregular shaped rhizome with a white hairy structure (Fig. 1a). On MS medium these globular structures directly differentiated into seedlings (Fig. 1b). The finding indicates the differential reaction of a species to different media. It may be mentioned here that the main difference of these two germination media was enrichment of MS medium with sugar and macronutrients than PM. Therefore nutritional abundancy may be a cause of differentiation of these globular structures. Such differential response of a species to various media was also reported by Baker et al. (1987), Roy et al. (1993), Hoque et al. (1994), Oddie et al. (1994), Gupta and Bhadra (1998) and Barua and Bhadra (1999).

In order to induce rapid growth, the germinated seedlings were transferred to the shoot elongation medium. The highest elongation rate was recorded on MS medium supplemented with 3% (w/v) sucrose, 2.0 mg/l NAA and 2.0 mg/l BAP (Fig. 1c). The seedlings developed poor root system in both germination and elongation media. Thus for induction of strong and stout root system, 3 - 4 cm tall seedlings, were grown on three different rooting media viz. (i) half strength MS + 1.5% (w/v) sucrsoe, (ii) MS + 3% (w/v) sucrose + 0.5 mg/l IAA and (iii) MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.1% (w/v) AC. MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 01.% (w/v) AC was found best in terms of both greater number and lengths of roots (Fig. 1d). Activated charcoal has not only good adsorption properties but also creates partial darkness. Probably the activated charcoal-fortified media enhanced induction of roots. Such a stimulation by activated charcoal in the medium was also reported by Nagaraju et al. (2001).

For large scale micropropagation, tips of seed-derived 'mini' rhizomes were cultured on different 0.8% (w/v) agar solidified MS medium supplemented

with various kinds of PGRs such as auxins (NAA, IAA, IBA and picloram) and cytokinins (BAP and zeatin) at different concentrations. The explants produced multiple shoot buds through direct organogenesis on all the media (Table 1). The maximum number of adventitious shoot buds (4 - 5) was, however,

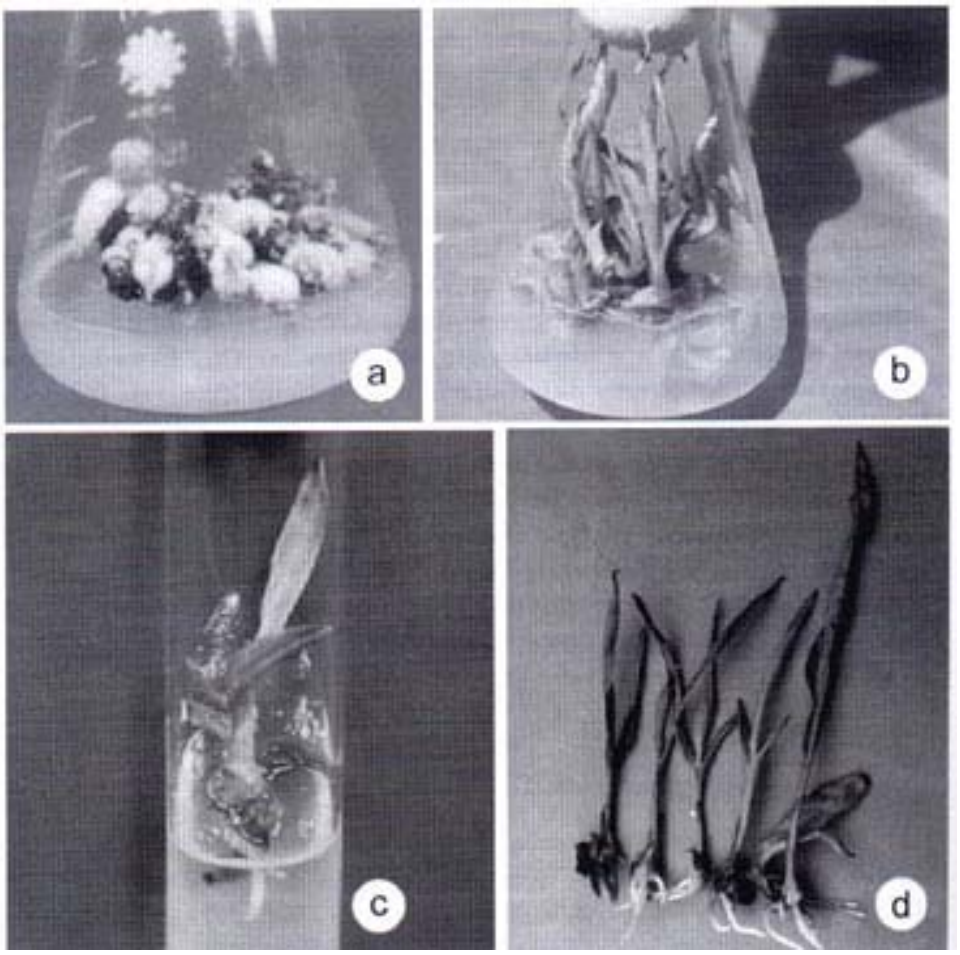

Fig. **1**. *In vitro* germination of *G. densiflorum*, (**a**) Irregular shaped rhizomes with hairy structure, (**b**) germinated seedlings, (**c**) elongation of individual seedling, (**d**) seedlings with well-developed root system.

observed on MS + 3% (w/v) sucrose + 2.0 mg/l NAA + 2.0 mg/l BAP and MS + 3% (w/v) sucrose + 2.5 mg/l BAP + 1% (w/v) AC (Fig. 2a). The findings indicate that BAP (2.0 - 2.5 mg/l) supplemented media is more effective towards induction of multiple shoot buds. Similar results were also reported by Sheelavantmath et al. (2000). Multiple shoot buds that developed in the

rhizome tips underwent elongation on the same medium. The highest rate of elongation was recorded on MS + 3% (w/v) sucrose + 2.0 mg/l BAP + 2.0 mg/l NAA (Fig. 2b, Table 2). When the multiple shoot buds attained a height of 3 - 4 cm, they were individualy grown on the rooting medium. MS + 3% (w/v) sucrose

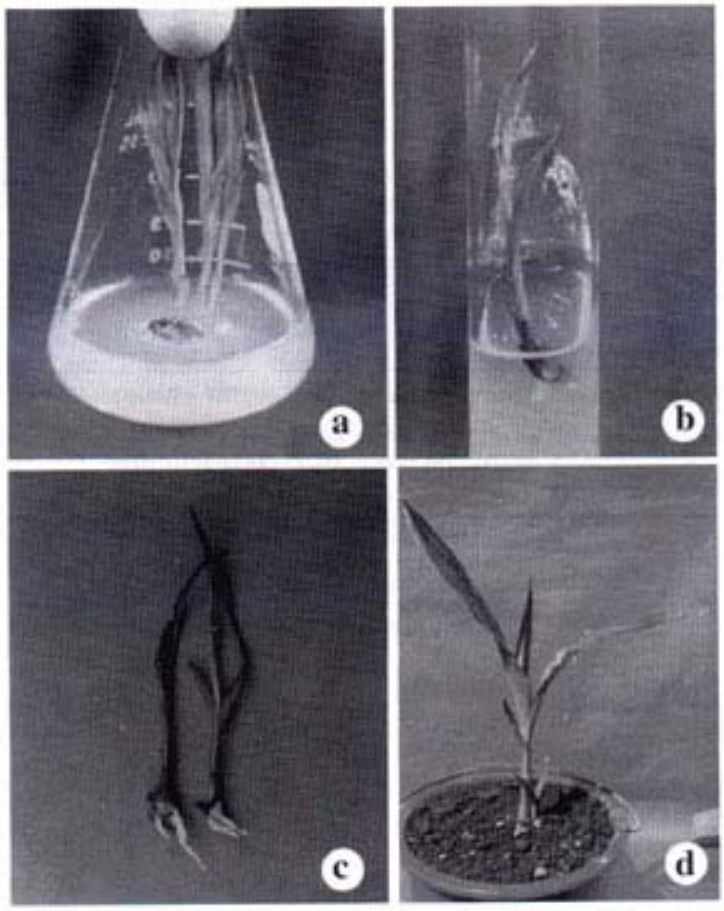

Fig. **2**. Micropropagation of *G. densiflorum*, (**a**) Development of multiple shoots from *in vitro* grown rhizome tips, (**b**) elongation of individual multiple shoot buds, (**c**) formation of root system in elongated multiple shoot buds, (**d**) a *in vitro* grown seedling established in pot outside the lab.

+ 1.0 mg/l IAA + 0.1% (w/v) AC was more effective for rooting (Fig. 2c). The plantlets with a strong root system were transferred from the culture room to outside pots containing loam soil through sequential steps of acclimatization. More than 80% of *in vitro* grown seedlings survived in the outside environment.

Table 1. Data on the development of multiple shoot buds from rhizome tips of *G. densiflorum*, when cultured on 0.8% (w/v) agar-solidified media supplemented with different PGRs.*

Culture media	Explants**	No. of days required for sprouting of multiple shoot buds	Av. number of shoot buds in each explant
MS + 3% (w/v) sucrose + 2.0 mg/l NAA + 2.0 mg/l BAP	RT	25 - 30	4.50
MS + 3% (w/v) sucrose + 2.5 mg/l BAP + 0.1% (w/v) AC	RT	25 - 30	3.85
MS + 3% (w/v) sucrose + 1.0 mg/l IBA + 1.0 mg/l zeatin	RT	35 - 40	2.25
MS + 3% (w/v) sucrose + 1.0 mg/l IBA + 1.0 mg/l BAP + 0.1% (w/v) AC	RT	25 - 30	3.02
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 g/l BAP	RT	35 - 40	2.00
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 2.0 mg/l BAP	RT	30 - 35	3.5

*Based on observations recorded from 20 cultured segments. **RT = Rhizome tip.

Table 2. Data on elongation of multiple shoot buds developed from rhizome tips of *G. densiflorum*, when grown on 0.8% (w/v) agar-solidified MS medium supplemented with different PGRs.*

Culture medium	Av. initial length of individual multiple shoot bud (cm)	Av. length of individual multiple shoot buds after 30 days of culture on elongation medium (cm)	Increase in length of shoot buds within 30 days of culture in elongation medium (cm)
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	2.05	3.52	1.47
MS + 3% (w/v) sucrose + 2.0 mg/l NAA + 2.0 mg/l BAP	2.10	4.58	2.85
MS + 3% (w/v) sucrose + 2.5 mg/l BAP + 0.1% (w/v) AC	1.95	4.80	2.48
MS + 3% (w/v) sucrose + 1.0 mg/l Pic. + 2.0 mg/l BAP + 0.1% (w/v) AC	2.03	3.98	1.95
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 2.0 mg/l BAP	2.15	3.57	1.42

*Based on observation recorded from 50 cultured shoot buds.

In this way a large number of seedlings could be established in the natural environment (Fig. 2d). Results of the present investigation indicate that, in vitro culture techniques could be formulated for germination and mass scale micropropagation of *G. densiflorum*. This strategy offers an opportunity for using the techniques in commercial nurseries as well as for *ex situ* conservation of this endangered orchid species.

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