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In Vitro Seed Germination and Protocorm Development of *Geodorum densiflorum* (Lam.) Schltr. An Endangered Terrestrial Orchid

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Abstract
The mature capsule of *Geodorum densiflorum* was used as explant for present study. Seeds obtained from mature capsule were successfully germinated on two basal media MS and Kn C by using different combination of growth hormones. The various concentration of BAP and NAA were supplemented to media for better germination of seed and development of protocorm. 0.1% activated charcoal also added to media with plant growth regulators. The seed germination was observed on MS media and Kn C media. The seed germination on MS and Kn C media was 95.60 % and 92.74 % respectively after 120 days of inoculation.
The protocorm formation was assessed at up to the highest seed germination and protocorm formation seen in MS medium. The spherule formation was observed in 9- 12 weeks on both media after germination of seed. The white colored spherule was turned into sturdy green color after 12-15 weeks. Whitish, light yellow, brown and green colored protocorms were observed in developmental stages of protocorms.

Keywords: *Geodorum densiflorum*, Spherule, Protocorm, Capsule.

Introduction
Orchids are second largest group of flowering plant belonging to family Orchidaceae. It comprising about 778 genera and 18,500 species distributed throughout the world (Mabberley, 1997) [3]. Terrestrial orchids (Orchidaceae) have become objects of concern to conservationists due to the plants high sensitive to changes in its environment (Rasmussen, 1995) [1]. Orchids during evolutionary process, adapted to distinct environments, so they can be epiphytic, terrestrial, saprophytes or lithophytes (Black, 1973). Generally orchids are propagated by vegetative means as well as through seeds. The rate of vegetative propagation is very poor i.e. 0.2 to 0.3% (Vij, 2002) [2].

Geodorum densiflorum is belongs into family Orchidaceae. *Geodorum densiflorum* is an endangered terrestrial orchid (Datta, 1999) [4]. *Geodorum densiflorum* is widely distributed in India, Nepal, Australia, Bangladesh, Srilanka, China, Bhutan, Papua New Guinea and Himalayas. It is estimated that about 1,300 species (140 genera) of orchids are found in India with Himalayas as their main home and other scattered in Eastern and Western Ghats (Deb, 2013) [5]. The pseudobulb of *Geodorum densiflorum* is ethno medicinally very important. It was used for the treatment of various diseases. The pseudobulb paste was applied externally to cure Carbuncles (Nath *et al.*, 2011) [7]. Used to regularize menstrual cycle (Dash *et al.*, 2008) [6]. For diabetes (Patil *et al.*, 2005) [8]. The aim of this study is to develop a suitable method for ex situ conservation of endangered terrestrial orchid *Geodorum densiflorum* through large-scale *in vitro* propagation.

Material and methods
Seed source and sterilization
The mature capsules of *Geodorum densiflorum* were collected from Amba Barwa forest, Jalgaon Jamod, Dist. Buldana (MS). The plant capsules were washed under running tap water. They were treated with liquid detergent laboline 5% (v/v) for 5-10 minutes followed by washing with autoclaved distilled water thrice. Further sterilization was done under aseptic conditions, inside the laminar flow cabinet. Mature capsules of *Geodorum densiflorum* were surface sterilized by submerging them in a 0.2% (w/v) $HgCl_2$ solution. 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 vessels. All these operations were done in a laminar airflow cabinet.

Seed Viability Test
Seed viability test was done by using TTC staining techniques with the help of TTC solution (1 gm in 100 ml of tris HCl buffer) and malachite green. Seeds overnight soaked in 10 % sucrose solution were transferred to 1 % aqueous solution of TTC (P^H 7). This solution was kept in darkness for 20- 24 hours at 30^o C. After that they were washed in distilled water and transferred to watch glass containing 0.01% aqueous solution of malachite green stain coated seeds were incubated for 45 minutes to 1 h at room temperature and then mounted in glycerin drop on a clean slide and sealed with sealing wax. Seeds were observed under light microscope. Swelled and intact red color embryo indicated viable seed, while seed with partially colored, white or brown embryos were assumed to be nonviable (Van Waes and Deberg, 1986) [9]. The viability percentage was calculated by following formula.

$$\text{Percent viability} = \frac{\text{Number of viable seeds}}{\text{Total number of seeds}} \times 100$$

Media Preparation

The germination media used were Knudson C (Kn C) and Murashige and Skoog (MS) medium for present investigation. In Kn C media 2 % sucrose and MS media 3 % sucrose served as carbon source. In addition, two different plant growth regulators (PGRs) NAA and BAP were supplemented to each type of medium in different combinations (Table: 2). Media also supplemented with 0.1% activated charcoal. Culture vessels with inoculated seeds were maintained in the culture room with 14/10 h light and dark cycle at 25 ± 2 ºC.

Study of seed germination and protocorm morphogenesis

Some seeds were scooped out and dispersed in one drop of water on a glass slide after 20 days of inoculation. These seeds were observed under a light microscope to find out the germination rate. All swollen seeds were considered to be germinated. Germination was calculated by using the formula:

$$\% \text{ of germination} \frac{\text{Total number of seed germinated}}{\text{Total number of seed observed}} \text{x } 100$$

Morphology and texture of germinated seeds were observed under a light microscope at the end of 30, 60, 90 and 120 days after inoculation of seeds to media.

Result and Discussion

Seed Viability Test

The seed viability test was taken by using TTC stain. The embryo of *Geodorum densiflorum* seeds turns red with triphenyl tetrazolium chloride (Fig. 2). The seed viability test confirmed the seed viability was about 96.59%. The percent of seed viability was reduces with increase time from harvesting.

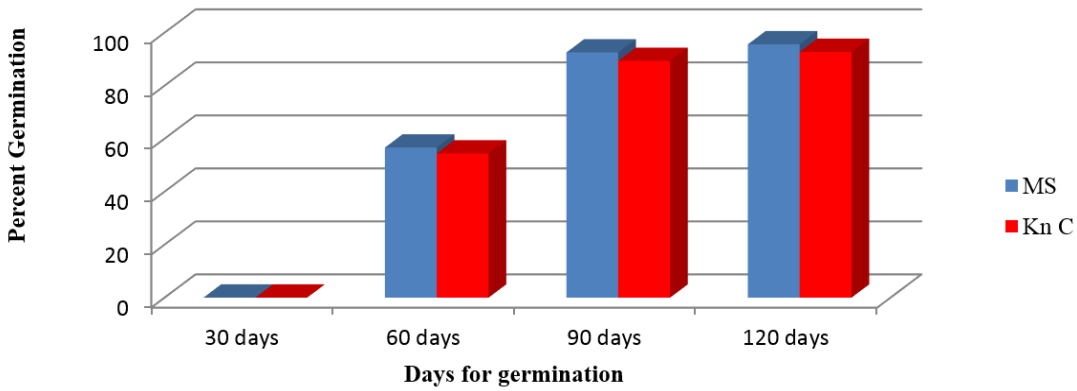
Seed germination

Seeds of *Geodorum densiflorum* are minute, dust like and fusiform in shape. The seed germination rate and duration varied with the medium used. Maximum germination % and the appearance of protocorm were achieved on MS medium. After some days, white nodular swelling of seed was observed. Seed germination began 5- 6 week after inoculation. Seeds initially showed swelling of the embryo (Fig: 3) then followed by rupturing of the seed testa and emergence of protocorm. Seed germination occurred on both MS and Kn C media produces globular structure within 50- 60 days after inoculation. These nodular swellings were subsequently converted into spherules. The percent seed germination was mentioned in table: 1 and graphical presentation in fig. 1.

Table 1: Percent seed germination in mean ± S.E

Media	30 days	60 days	90 days	120 days
MS	0.0 ± 0.0	56.65± 0.22	92.54± 0.12	95.60 ± 0.60
Kn C	0.0 ± 0.0	54.41± 0.18	89.33± 0.14	92.74± 0.12

Fig. 1: Seed germination potential of *Geodorum densiflorum* (Lam.) Schltr. in different medium (MS and Kn C).



MS (95.60 %) media shows maximum germination. Protocorm were formed on MS media were yellowish white in color. On Kn C media, germination rate was 92.74 %. Asymbiotic seed germination success depends on seed conditions such as seed capsule maturity and origin, as well as physical germination condition and the constituent in the growth media (Kauth *et al*., 2008 and Zeng *et al*., 2014) [12, 13]. Asymbiotic germination has many advantages including the capability to produce healthy seedlings at a frequency and rate far greater than achieved in nature (Nadarajan *et al*., 2011) [14].

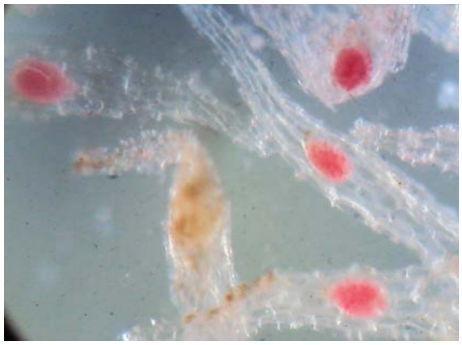


Fig 2: TTC stain viability test.

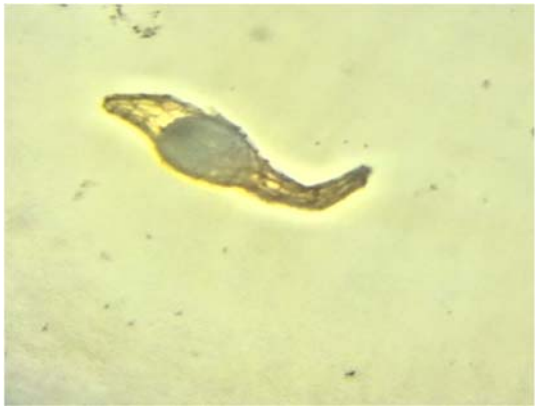


Fig.3: Swelling in seed (initiation of germination).

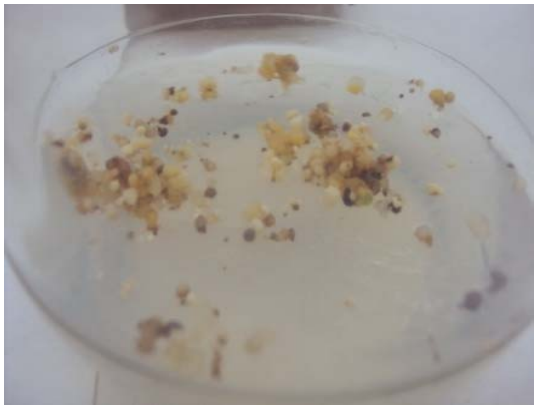


Fig. 4. White color protocorm formation on media.

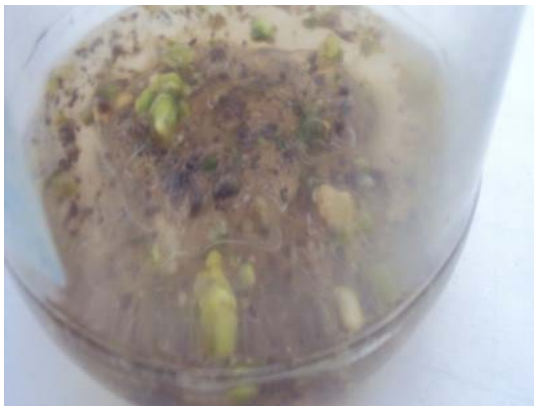


Fig. 5. Green color protocorm formation on media.

Fig. 6. Initiation of first leaf to protocorm.

Fig. 7: PLB (Rhizome) with multiple shoot buds.

Morphology, growth and development of protocorms:

The germinated seeds after emergence from seed coat initially appeared white with pointed basal end. The globular structure was formed from the inoculated seeds of *Geodorum densiflorum*. The swelling was observed after development of white colored embryo. The spherule formation was observed after 9- 12 weeks on MS and Kn C media. The white colored spherule was turned into sturdy green in color after 12- 15 weeks (Fig. 5).

After germination of seed, it was developed into globular structures called as protocorm (Morel, 1960) [15]. Shown in fig: 5. within a short period of time, a large number of secondary protocorms can be also obtained (fig. 4). Due to this reason, protocorm was preferred a great alternative for propagation of orchids. A protocorm is the tuber like swollen part of an orchid seed, which produced during early stage of germination. Whitish, light yellow and green colored protocorms were formed after seed germination (Roy and Banerjee, 2001) [16]. Beside these, brown color protocorms with hairy rhizoids were observed in PGRs supplemented culture media.

Table 2: Germination of seeds of *Geodorum densiflorum* on agar solidified media.

Medium	PGRs (mg/l)		Spherule formation time (in weeks)	Protocorm formation time (in weeks)
	BAP	NAA		
MS	1	3	11- 12	13- 14
	1.5	2.5	9- 10	12- 13
	2	2	10- 11	12- 13
	2.5	1.5	10- 11	12- 13
	3	1	11- 12	14- 15
Kn C	3	1	11- 12	13- 14
	2.5	1.5	11- 12	12- 13
	2	2	10- 11	12- 13
	1.5	2.5	9- 10	11- 12
	1	3	11- 12	13- 14

The sturdy green color protocorm form single leaf which are the initial stage in shoot development (fig. 6). The protocorm developed into large size with multiple buds called protocorm like bodies (PLBs), shown in fig. 7. The protocorm like bodies are somatic embryo in orchids (Lee *et al*., 2013) [13]. Similarly rhizome (PLB) was obtained from protocorm in *Geodorum densiflorum* reported by Sheelavantmath *et al*., 2000 [18]. The induction of multiple shoot buds from PLB was observed in 10-11 months after inoculation of seeds in culture medium. The protocorms continued develop and consequently formed shoots.

Conclusion

From the present study, it may be concluded that the requirement of nutrients for complete seedling development through asymbiotic seed germination vary at different stages of growth and development. The protocol developed in the present study can be used for conservation of this an endangered terrestrial orchid species through *in vitro* asymbiotic seed germination.

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