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In vitro propagation of threatened terrestrial orchids *Phaius tankervillieae* (L'Her) blume and *Geodorum densiflorum* (Lam.) schltr. via seed pod culture

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Abstract
The orchids belongs to orchidaceae family is the second largest and most diverse group of plants and it is widespread all over the globe, except for in Polar Regions. *Phaius tankervillieae* commonly known as Nun's hood Orchid is one of the most widely valued ornamental orchids in the world. *Geodorum densiflorum* is one of the floriculturally and medicinally important ground orchids. Orchid's seeds are small, numerous and non-endospermic. Germination rate is very slow and some needs fungal association for germination. Tissue culture has become the standard method of propagation for the conservation of orchids. In the present experiment, we have analysed the effect of various concentration of hormones in seed germination and protocorm formation of both species with MS media. The mature capsule of both the species were used as explant for present study. The seed germination rate of *Phaius tankervillieae* and *Geodorum densiflorum* were found to be variable in hormone free MS medium and MS medium supplemented with various concentration of hormones. Quickest germination of *Phaius tankervillieae* seed was observed on MS basal medium supplemented with 1mg/L kinetin + 1mg/L NAA which was also found to be the best for fastest protocorm formation. However MS medium when supplemented with 1.5mg/L kinetin + 1mg/L NAA produced maximum number of shoots per culture and maximum number of roots with shoots per culture. Fastest germination of *Geodorum densiflorum* seeds was observed on MS basal medium supplemented with 2mg/L NAA + 2mg/L BAP and 2.5mg/L BAP+ 0.1% AC which was also found to be the best for quickest protocorm formation.

Keywords: Orchid, MS media, hormone, protocorm, germination

Introduction

The Orchidaceae family, comprising approximately 28,484 species, ranks as the second-largest group among angiosperms (Govaerts *et al*., 2017) [8]. Orchid seeds are extremely small, resembling fine particles, abundant and lack endosperm. The process of germination is notably slow (taking 8-10 years) and in their natural environment, orchids require a mycorrhizal partnership to facilitate germination (Deb and Pongener, 2012) [6]. Within a solitary capsule, there exist millions of seeds that are microscopic in size (Dutta *et al*., 2011) [7]. The germination rate of orchids is comparably modest because of incomplete endosperm growth and absence of fungal infection. Moreover, the slender cuticle enveloping a diminutive embryo is insufficient in safeguarding it from drying out (Lee *et al*., 2006) [10]. Within *in vitro* conditions, non-symbiotic germination becomes achievable solely through tissue culture techniques, as it replaces fungal involvement with a nutritive medium (Pant *et al*., 2011) [12, 13]. *Phaius tankervilliea*, recognized as the Nun's Orchid or Lady Tankerville's Swamp Orchid, is categorized within the sub-family Epidendroideae. This substantial terrestrial orchid features captivating blossoms that grace the period between April and July (Cheng *et al*., 2012) [4]. Due to its significant value in terms of both aesthetics and potential medicinal use, there is unauthorized harvesting of this plant from its native habitat for commercial purposes. Additionally, the species is facing a consistent decrease in numbers due to the degradation of natural forest habitats. As a consequence, this particular orchid species has been classified as endangered according to the Environmental Protection and Biodiversity Conservation Act (Briggs and Leigh, 1996). Furthermore, it has drawn the attention of conservationists because of its heightened susceptibility to alterations in the environment (Rasmussen, 1995). Thus, it becomes imperative to implement appropriate strategies for the preservation and cultivation of these vulnerable orchid varieties. The tissue culture method has gained widespread acceptance for the large-scale propagation of numerous economically valuable orchids (Malabadi *et al*., 2005). *Geodorum densiflorum* holds significance in the realms of both horticulture and herbal medicine as a noteworthy terrestrial orchid (Rao 1979) [16]. The pseudo bulb of *Geodorum densiflorum* is employed in the management of diverse ailments. It is utilized topically for the healing of Carbuncles (Nath *et al*., 2011) [11], as well as to normalize the menstrual cycle (Dash *et al*., 2008) [5] and address diabetes (Patil *et al*., 2005) [14]. Continuous deforestation for land reclamation and unregulated human collection have led to the endangerment of this species through habitat degradation. The natural reproduction through vegetative methods is notably sluggish and demands significant time for generating a substantial number of orchid replicas. The *in vitro* approach holds the potential to accelerate the germination process and enable rapid large-scale multiplication, thereby mitigating the time constraints (Pradhan and Pant, 2009) [12, 13, 15].

Materials and methods

Source of Seeds

Materials used for the present experiment were the mature pods (capsules) of *Phaius tankervillieae* and *Geodorum densiflorum*. Materials were procured from Assam Bio-Resource Center, Baihata chariali, Assam.

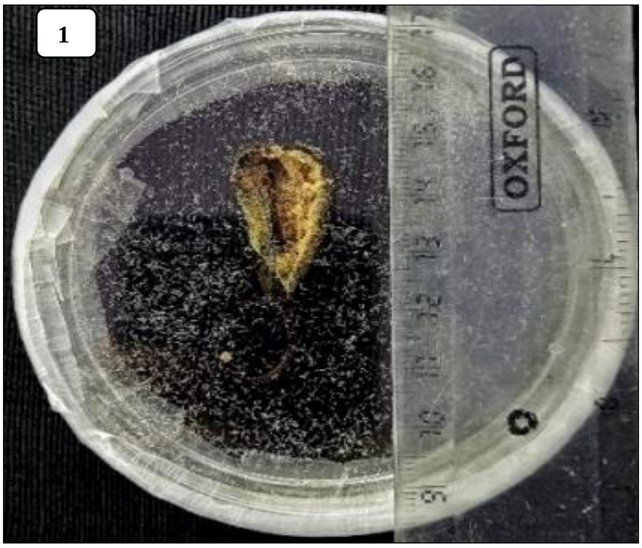


Fig 1: Capsule of *Phaius tankervillieae*

Fig 2: Capsule of *Geodorum densiflorum*

Culture medium preparation

In this experiment, MS full strength media with 3% sucrose, 0.8% agar and 0.01% meso-inositol without hormone is considered as control for both the species. The MS medium (Murashige and Skoog, 1962) was used as the basal medium (BM) supplemented with different growth regulators in different concentrations for this experiment. All media were adjusted to a pH of 5.7 ±0.02 with 0.5N HCl and 0.5N NaOH prior to autoclaving at 121°C at 15 lb pressure for 20 min. The medium was poured into autoclaved petriplates at the rate of about 30 ml per petriplates. Then, each petriplates was sealed with parafilm. All the cultures were incubated at 24 ±2°C under 350-500 lux illumination for 16-h photoperiod using white fluorescence tube lights (Baker *et al*., 2014) [2].

Sterilization of glasswares and metal instruments

Glasswares were sterilized using dry heat in a hot air oven at 155°C for 2-3 hours. Metal instruments were also sterilized using the same method. They were first wrapped in aluminum foil and then placed in the hot air oven for sterilization.

Culture medium and incubation for *Phaius tankervillieae* and *Geodorum densiflorum*

In this current study, the full concentration of MS media was employed, with the addition or absence of plant growth regulators (as a control), either individually or in conjunction with other hormones, to facilitate the processes of seed germination, protocorm establishment and seedling growth.

Table 1: MS media with hormonal combination for Phaius tankervillieae

Serial no.	Treatment combinations
1	MS (full strength media)
2	MS + 1mg/L kin
3	MS + 1mg/L IAA + 1mg/L NAA
4	MS + 1mg/L kin + 1mg/L NAA
5	MS + 1.5mg/L kin + 1mg/L NAA
6	MS + 1mg/L IBA + 1mg/L BAP
7	MS + 1mg/L 2,4-D + 1mg/L BAP
8	MS + 1mg/L BAP + 1mg/L IAA + 1mg/L 0.1% AC

Table 2: MS media with hormonal combination for *Geodorum densiflorum*

Serial no.	Treatment combinations
1	MS (full strength media)
2	MS + 1mg/L IAA + 1mg/L NAA
3	MS + 1.5mg/L kin + 1mg/L NAA
4	MS + 1mg/L IBA + 1mg/L BAP
5	MS + 1mg/L 2,4-D + 1mg/L BAP
6	MS + 2mg/L NAA + 2mg/L BAP
7	MS + 2.5mg/L BAP + 1mg/L 0.1% AC
8	MS + 2mg/L BAP
9	MS + 1mg/L BAP + 1mg/L IAA + 1mg/L 0.1% AC

Surface Sterilization of capsules

The culture tubes, vessels with media and all required materials for inoculation, were exposed to UV light for 45 minutes. After UV exposure, the blower was switched on to make the laminar air flow chamber ready for inoculation.

Sterilization for *Phaius tankervillieae*

The seed capsules underwent a thorough cleansing process with running tap water containing a few drops of Tween 20 for a duration of 30 minutes. Subsequently, the capsules were subjected to a treatment involving carbendazim 50 W.P. (0.1% bavistin) at a concentration of 1g/L for 30 minutes. This was followed by a 30-minute exposure to streptomycin sulphate (9%) and tetracycline hydrochloride (1%) at a concentration of 1g/L. The capsules underwent repeated washing using sterilized double-distilled water within a laminar airflow chamber. A sequential surface sterilization procedure followed, involving a 1-minute treatment with 70% ethyl alcohol, a 3-minute immersion in a 0.1% HgCl2 solution, and finally, thorough rinsing conducted 5-6 times using autoclaved distilled water (Thokchom *et al*., 2017) [18].

Sterilization for *Geodorum densiflorum*

The seed capsules underwent a 30-minute cleansing process using flowing tap water along with 3-4 drops of Tween 20. Afterward, the capsules underwent surface sterilization through immersion in a 0.2% HgCl2 solution with intermittent agitation, followed by a brief dip in absolute ethyl alcohol lasting 12-15 seconds. Subsequently, the capsules were thoroughly rinsed 5-6 times using sterile distilled water (Bhadra *et al*., 2003) [3].

Inoculation of *Phaius tankervillieae* and *Geodorum densiflorum* seeds

The orchid capsules, which had undergone surface sterilization, were moved to a Petri dish and sliced horizontally using a sterilized surgical blade. Following this, clusters of mature orchid seeds were introduced onto the surface of both MS medium alone and MS medium combined with various hormones. Sterilized forceps were employed to evenly disperse the seeds onto the agar medium. All these operations were done in a laminar air flow cabinet (Theng and Korpenwar, 2015) [17].

Fig 3: Cutting of sterilizedcapsules

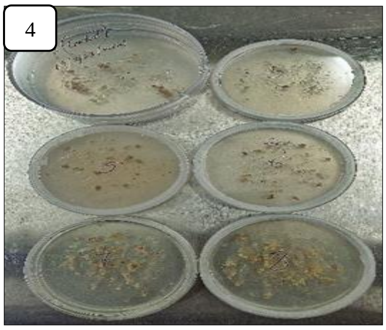


Fig 4: Seeds spreading of *Phaius tankervillieae*

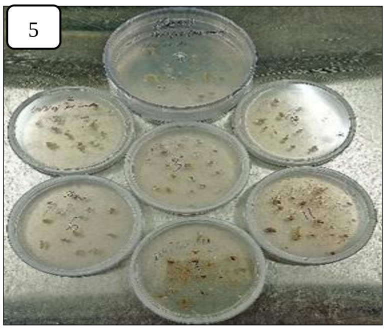


Fig 5: Seeds spreading of *Geodorum densiflorum*

Subculturing of *Phaius tankervillieae* and *Geodorum densiflorum*

After the development of protocorm-like bodies (PLBs), they were transferred to a new culture medium with the same hormonal concentration. The initiation germination rate of the seeds was measured and recorded. The entire experiment was conducted in an aseptic environment inside a laminar airflow hood to prevent any microbial contamination.

Results

For *Phaius tankervillieae (L'Her) Blume*

Culture of seeds and germination

The germination of *Phaius tankervillieae* seeds exhibited variability when subjected to both hormone-free MS medium and MS medium supplemented with varying hormone concentrations. The most rapid seed germination occurred in the presence of 1mg/L kinetin + 1mg/L NAA added to the MS basal medium, which also proved optimal for rapid protocorm development. While the hormone-free MS basal medium (control) facilitated seed germination, it required the longest duration for both germination and protocorm formation compared to the basal medium supplemented with hormones. However MS medium when supplemented with 1 mg/L kinetin + 1mg/L NAA showed fastest shoot initiation (44 days) and root initiation (53 days). Seeds were unable to progress into subsequent stages of differentiation within the hormone-free MS medium, as terrestrial orchids necessitate more controlled hormone conditions for the transformation of immature seeds into seedlings. The outcomes derived from the aforementioned discoveries indicated that the MS basal medium with hormone supplementation exhibited greater efficacy in facilitating the subsequent growth and maturation of seedlings. Table 3 provides details regarding the *in vitro* germination of premature seeds of *Phaius tankervillieae* (L'Her.) Blume in both the standard MS medium and the MS medium enriched with varying levels of hormonal concentrations.

Table 3: Impact of growth regulators within the MS medium on the germination of seeds and the growth of seedlings in *Phaius tankervillieae* (L' Her.) Blume

Serial no.	Treatment combinations	Initiation of germination (days)	Development of protocorm (days)	Capability of immature seeds forming PLBs
1	MS (full strength media)	47	60	+
2	MS + 1mg/L kin	30	49	++
3	MS + 1mg/L IAA + 1mg/L NAA	29	47	++
4	MS + 1mg/L kin + 1mg/L NAA	16	29	+++
5	MS + 1.5mg/L kin + 1mg/L NAA	21	36	+++
6	MS + 1mg/L IBA + 1mg/L BAP	33	52	++
7	MS + 1mg/L 2,4-D + 1mg/L BAP	35	53	++
8	MS + 1mg/L BAP + 1mg/L IAA + 1mg/L 0.1% AC	22	38	+++

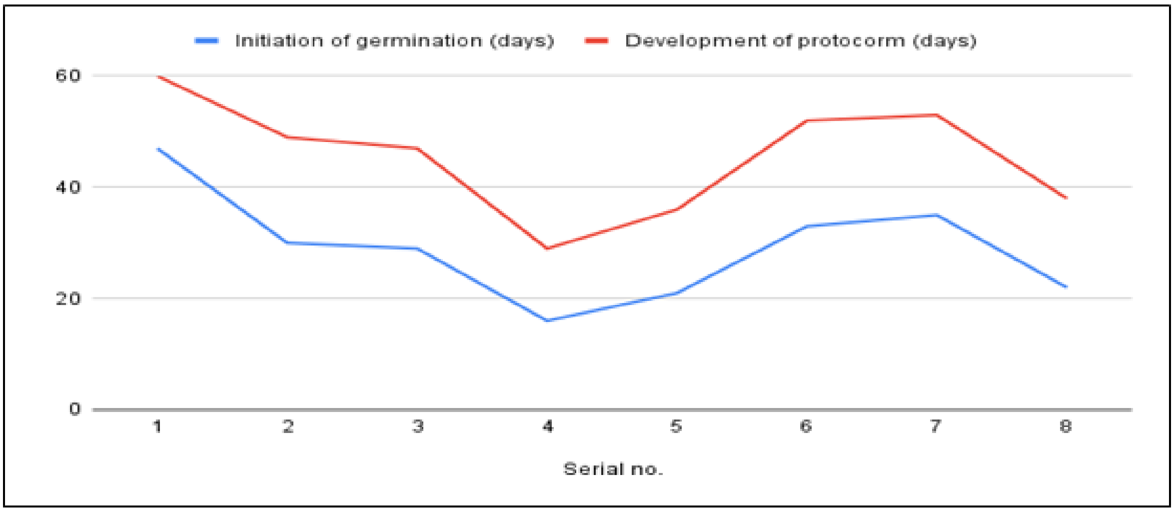
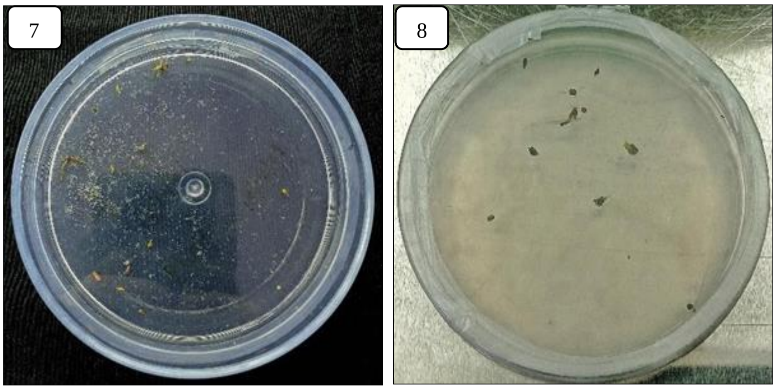


Fig 6: Graph showing influence of growth regulators within the MS medium on the germination of seeds and the development of protocorms in *Phaius tankervillieae*



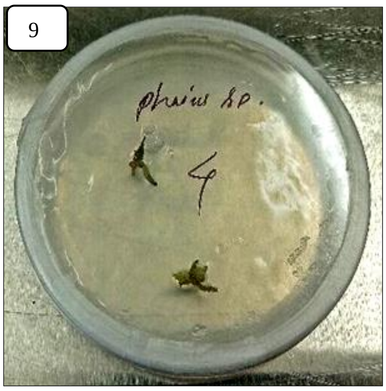


Fig 7&8: Protocorm like bodies (PLBs) of *Phaius tankervillieae*

Fig 9: Subculturing of *Phaius tankervillieae*

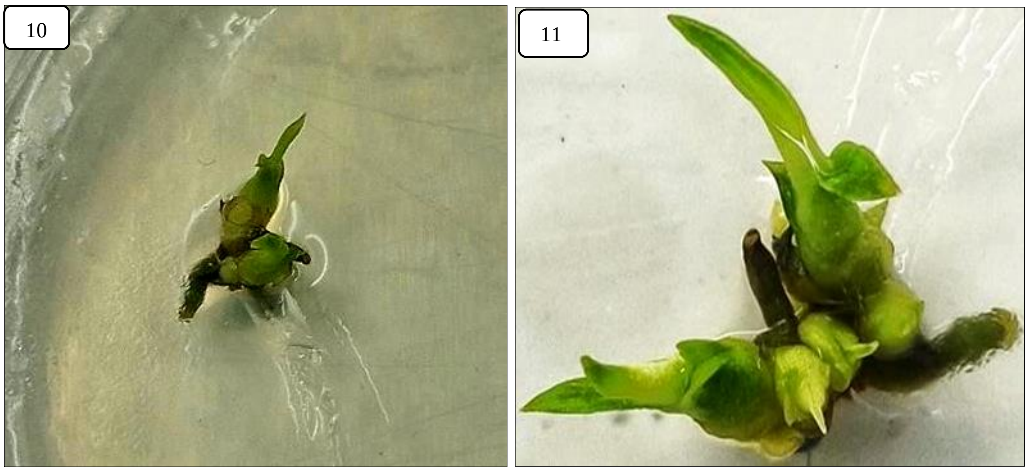


Fig 10&11: Growth and development of *Phaius tankervillieae* (initiation of shoots and roots)

Table 4: Impact of growth regulators within the MS medium on the initiation of shoots and roots in *Phaius tankervillieae* (L' Her.) Blume

Serial no.	Treatment combinations	Initiation of shoots (days)	Initiation of roots (days)
1	MS (full strength media)	82	95
2	MS + 1mg/L kin	60	69
3	MS + 1mg/L IAA + 1mg/L NAA	59	71
4	MS + 1mg/L kin + 1mg/L NAA	44	53
5	MS + 1.5mg/L kin + 1mg/L NAA	49	60
6	MS + 1mg/L IBA + 1mg/L BAP	68	76
7	MS + 1mg/L 2,4-D + 1mg/L BAP	69	76
8	MS + 1mg/L BAP + 1mg/L IAA + 1mg/L 0.1% AC	54	63

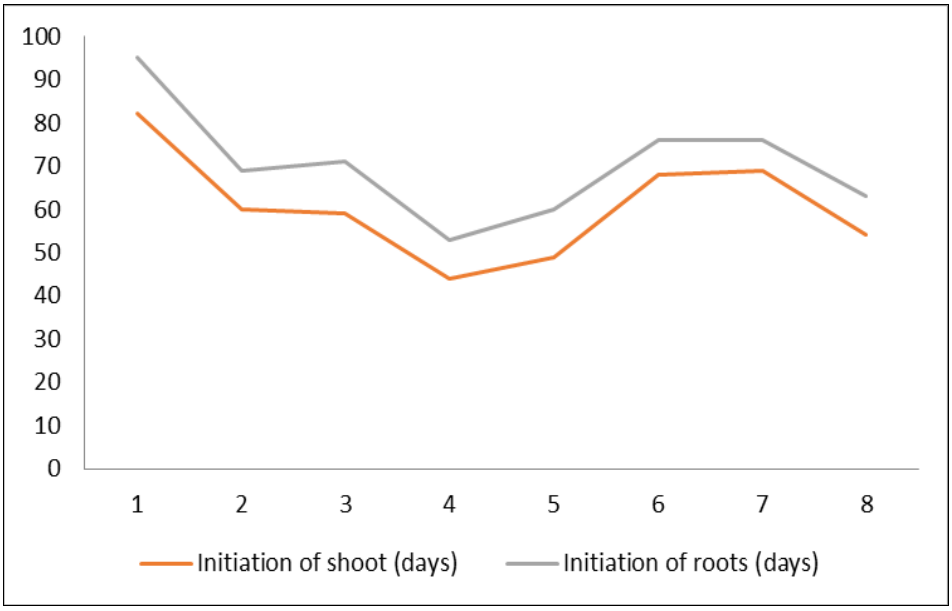


Fig 12: Graph showing influence of growth regulators within the MS medium on the initiation of shoots and roots in *Phaius tankervillieae* (L' Her.) Blume

***In vitro* germination of seeds**

The inoculation of seeds of *Phaius tankervilleae* (L'Her.) Blume. in MS medium and MS medium with different hormonal concentration is described below:

1. MS Basal Medium

Seed cultured in MS Basal media required more time for germination than in the hormone supplemented MS media. Germination was started after 47 days of seed inoculation. The development of PLB was observed after 60 days of seed inoculation and 13 days of seed germination. Initiation of shoot was observed after 82 days and initiation of root was observed after 95 days of inoculation.

2. Kinetin 1mg/L

Seed cultured in MS medium supplemented with Kinetin 1 mg/L required 30 days for seed germination. The development of PLB was observed after 49 days of seed inoculation and 19 days of seed germination. Initiation of shoot was observed after 60 days and initiation of root was observed after 69 days of inoculation.

3. IAA 1mg/L + NAA 1mg/L

Seed cultured in MS medium supplemented with IAA 1 mg/L+ NAA 1 mg/L required 29 days for seed germination. The development of PLB was observed after 47 days of seed inoculation and 18 days of seed germination. Initiation of shoot was observed after 59 days and initiation of root was observed after 71 days of inoculation.

4. Kinetin 1mg/L + NAA 1mg/L

Seed cultured in MS medium supplemented with Kinetin 1 mg/L + NAA 1 mg/L required 16 days for seed germination. The development of PLB was observed after 29 days of seed inoculation and 13 days of seed germination. Initiation of shoot was observed after 44 days and initiation of root was observed after 53 days of inoculation.

5. Kinetin 1.5mg/L + NAA 1mg/L

Seed cultured in MS medium supplemented with Kinetin 1.5 mg/L + NAA 1 mg/L required 21 days for seed germination. The development of PLB was observed after 36 days of seed inoculation and 15 days of seed germination. Initiation of shoot was observed after 49 days and initiation of root was observed after 60 days of inoculation.

6. IBA 1mg/L + BAP 1mg/L

Seed cultured in MS medium supplemented with IBA 1 mg/L + BAP 1mg/L required 33 days for seed germination. The development of PLB was observed after 52 days of seed inoculation and 19 days of seed germination. Initiation of shoot was observed after 68 days and initiation of root was observed after 76 days of inoculation.

7. 2,4-D 1mg/L + BAP 1mg/L

Seed cultured in MS medium supplemented with 2,4-D 1 mg/L + BAP 1 mg/L required 35 days for seed germination. The development of PLB was observed after 53 days of seed inoculation and 18 days of seed germination. Initiation of shoot was observed after 69 days and initiation of root was observed after 76 days of inoculation.

8. BAP 1mg/L + IAA 1mg/L + 0.1% AC 1mg/L

Seed cultured in MS medium supplemented with BAP 1 mg/L + IAA 1 mg/L + 0.1% AC 1 mg/L required 22 days for seed germination. The development of PLB was observed after 38 days of seed inoculation and 16 days of seed germination. Initiation of shoot was observed after 54 days and initiation of root was observed after 63 days of inoculation.

For *Geodorum densiflorum* (Lam.) Schltr.

Culture of seeds and germination

The germination rate of Geodorum densiflorum seeds exhibited variability when subjected to both hormone-free MS medium and MS medium supplemented with varying hormone concentrations. The most rapid seed germination occurred in the presence of 2mg/L NAA + 2mg/L BAP and 2.5mg/L BAP + 0.1% AC added to the MS basal medium, which also proved optimal for the fastest protocorm formation. The outcomes of this study suggest that it is possible to develop *in vitro* cultivation methods for both germination and large-scale micropropagation of *Geodorum densiflorum.*

Table 5: Impact of growth regulators within the MS medium on the germination of seeds and the development of seedlings in *Geodorum densiflorum* (Lam.) Schltr.

Serial no.	Treatment combinations	Initiation of germination (days)	Development of protocorm (days)	Capability of immature seeds forming PLBs
1	MS (full strength media)	50	67	+
2	MS + 1mg/L IAA + 1mg/L NAA	39	58	++
3	MS + 1.5mg/L kin + 1mg/L NAA	37	55	++
4	MS + 1mg/L IBA + 1mg/L BAP	33	52	++
5	MS + 1mg/L 2,4-D + 1mg/L BAP	31	48	++
6	MS + 2mg/L NAA + 2mg/L BAP	19	32	+++
7	MS + 2.5mg/L BAP + 1mg/L 0.1% AC	21	35	+++
8	MS + 2mg/L BAP	29	47	++
9	MS + 1mg/L BAP + 1mg/L IAA + 1mg/L 0.1% AC	27	41	++

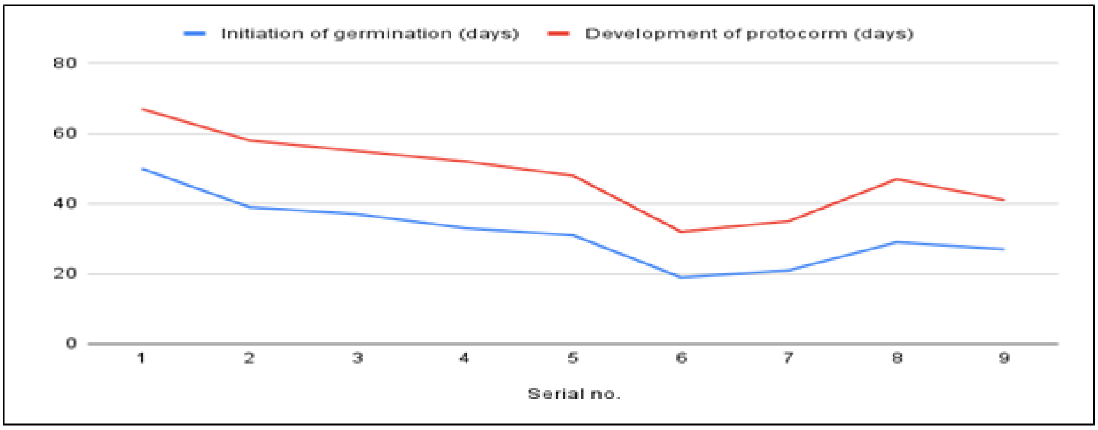


Fig 13: Graph showing Influence of growth regulators within the MS medium on the germination of seeds and the development of seedlings in *Geodorum densiflorum* (Lam.) Schltr

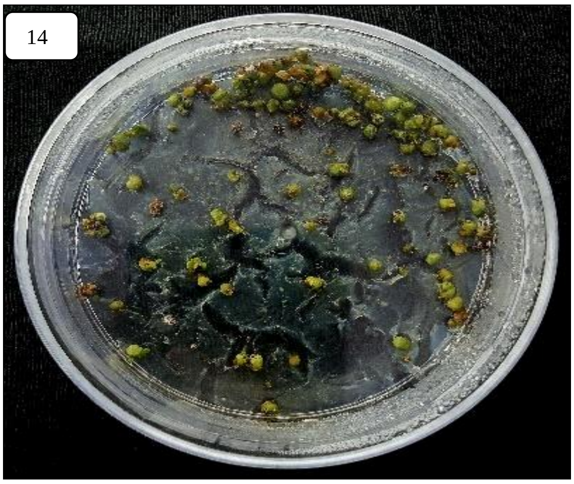


Fig 14: Protocorm like bodies (PLBs) *Geodorum densiflorum* (Lam.) Schltr

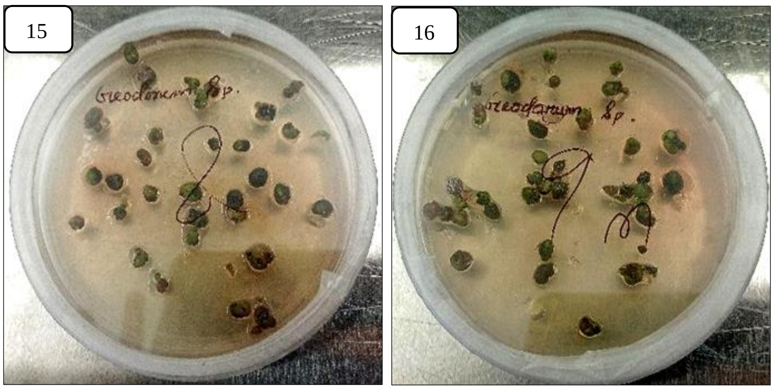


Fig 15&16: Subculturing of *Geodorum densiflorum* (Lam.) Schltr.

In vitro germination of seeds

The inoculation of seeds of *Geodorum densiflorum* (Lam.) Schltr. in MS medium and MS medium with different hormonal concentration is described below:

1. MS Basal Medium

Seed cultured in MS Basal media required more time for germination than in the hormone supplemented MS media. Germination was started only after 50 days of seed inoculation. The development of PLB was observed after 67 days of seed inoculation and 17 days of seed germination.

2. IAA 1mg/L + NAA 1mg/L

Seed cultured in MS medium supplemented with IAA 1 mg/L + NAA 1 mg/L required 39 days for seed germination. The development of PLB was observed after 58 days of seed inoculation and 19 days of seed germination.

3. Kinetin 1.5mg/L + NAA 1mg/L

Seed cultured in MS medium supplemented with Kinetin 1.5 mg/L + NAA 1 mg/L required 37 days for seed germination. The development of PLB was observed after 55 days of seed inoculation and 18 days of seed germination.

4. IBA 1mg/L + BAP 1mg/L

Seed cultured in MS medium supplemented with IBA 1 mg/L + BAP 1mg/L required 33 days for seed germination. The development of PLB was observed after 52 days of seed inoculation and 19 days of seed germination.

5. 2, 4-D 1mg/L + BAP 1mg/L

Seed cultured in MS medium supplemented with 2,4-D 1 mg/L + BAP 1 mg/L required 31 days for seed germination. The development of PLB was observed after 48 days of seed inoculation and 17 days of seed germination.

6. NAA 2mg/L + BAP 2mg/L

Seed cultured in MS medium supplemented with NAA 2 mg/L + BAP 2 mg/L required 19 days for seed germination. The development of PLB was observed after 32 days of seed inoculation and 13 days of seed germination.

7. BAP 2.5mg/L + 0.1% AC 1mg/L

Seed cultured in MS medium supplemented with BAP 2.5 mg/L + 0.1% AC 1 mg/L required 21 days for seed germination. The development of PLB was observed after 35 days of seed inoculation and 14 days of seed germination.

8. BAP 2mg/L

Seed cultured in MS medium supplemented with BAP 2 mg/L required 29 days for seed germination. The development of PLB was observed after 47 days of seed inoculation and 18 days of seed germination.

9. BAP 1mg/L + IAA 1mg/L + 0.1% AC 1mg/L

Seed cultured in MS medium supplemented with BAP 1 mg/L + IAA 1 mg/L + 0.1% AC 1 mg/L required 27 days for seed germination. The development of PLB was observed after 41 days of seed inoculation and 14 days of seed germination.

Discussion

Orchid seed germination deviates from the process of other seeds due to the lack of endosperm, radicle, and embryonic leaves. The embryo undergoes swelling, giving rise to a rounded, apex-shaped structure known as protocorms (Arditti *et al*., 1981) [1]. Germinating seeds of terrestrial orchids in a laboratory setting is comparatively more challenging than for epiphytic orchids. This heightened difficulty arises from their greater reliance on mycorrhizal fungi for germination compared to epiphytic orchids. In this investigation, the most rapid germination of *Phaius tankervillieae* seeds occurred on the MS basal medium enriched with 1mg/L kinetin + 1mg/L NAA, which also proved to be the optimal condition for achieving the swiftest protocorm formation. Nonetheless, the MS medium enriched with 1.5mg/L kinetin + 1mg/L NAA demonstrated the highest yield of shoots per culture and the greatest number of roots accompanying the shoots per culture. The most rapid germination of *Geodorum densiflorum* seeds occurred on the MS basal medium enriched with 2mg/L NAA + 2mg/L BAP and 2.5mg/L BAP + 0.1% AC, which also facilitated the optimal speed of protocorm formation. This finding aligns with the outcomes of studies conducted by Rocky Thokchom *et al*. in 2017 [18] and Bhadra and Hossain in 2003 [3], both involving *Phaius tankervilliae* and *Geodorum densiflorum* respectively. Pant and Gurung (2005) [12, 13] found that employing a blend of BAP (2.0 mg/l) and NAA (1.0 mg/l) enhanced seed germination and subsequent transformation into seedlings in Aerides odorata. Kabita and Sharma (2001) [9] noted that complete germination was achieved on MS medium supplemented with NAA (0.1 µg/ml) and Kn (1.0 µg/ml) in *Acampe longifolium* Lindl.

Summary and conclusion

In summary, this study presents a straightforward and effective methodology for the *in vitro* cultivation of seedlings from immature seeds of *Phaius tankervillieae* and *Geodorum densiflorum*. The most favorable hormonal combination for enhancing seed germination, protocorm formation, and the overall growth and development of *Phaius tankervillieae* seedlings was observed to be the judicious application of kinetin and NAA (1.0-1.5mg/L + 1.0mg/L) in conjunction with the MS basal medium. The addition of 1mg/L BAP + 1mg/L IAA + 1mg/L 0.1% AC to the MS medium also demonstrated positive outcomes. Meanwhile, the inclusion of 1 mg/L kinetin and 1mg/L NAA in the MS medium led to the most rapid initiation of both shoots and roots, occurring in 44 and 53 days respectively. The most effective hormonal combination for promoting seed germination, protocorm formation, and the comprehensive growth and development of *Geodorum densiflorum* seedlings was determined to be the judicious application of NAA and BAP (2mg/L) in conjunction with the MS basal medium. Additionally, the application of 2.5mg/L BAP + 0.1% AC to the MS medium also yielded positive results for *Geodorum densiflorum*. The findings from this current study suggest the feasibility of developing *in vitro* cultivation methods for the germination and large-scale micropropagation of *Phaius tankervillieae* and *Geodorum densiflorum*.

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Conflicts of Interest

Authors declare no conflicts of interest.

Authors Contribution

IK: conceptualization, literature research, visualization, experiment, writing and supervision; AB: literature research, visualization, experiment and writing; CB: Supervision.

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Ethical Statement

In this study, no animal or human have been harmed or subjected to any harmful activities.

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