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# Optimization of Symbiotic Seed Germination of an orchid *Calanthe sylvatica,* Using Mycorrhizal Fungi

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## ABSTRACT

*The present study aimed to find a suitable method for seed germination of Calanthe sylvatica orchid using fungi obtained from the roots and rhizosphere of the same plant. We collected mature seeds and isolated fungi from the soil collected from the roots and rhizosphere of C. sylvatica. Filter paper, Seed baiting, and oatmeal agar methods were used to find the symbiotic relationship between orchid seeds and isolated fungi. We noted 42% viability of C. sylvatica seeds. We monitored the growth of viable seeds for 60 days at an interval of 7 days. Among the three methods used for symbiotic orchid seed germination, oatmeal agar exhibited distinct patterns in their efficiency. Only 10% of seeds could not be germinated using this method. 57% of seeds attained stage 3, when they turned green, and protocorm development was started. Our study confirms that the OMA method of symbiotic seed germination can be useful in the conservation and/or production practices of Calanthe sylvatica and other terrestrial orchid species.*

**Keywords:** Mycorrhizal fungi, Oatmeal agar, Orchid, protocorm, symbiotic seed germination

## INTRODUCTION

Orchidaceae is one of the renowned family of angiosperms having more than 35,000 endangered or threatened species of orchids (Pujasatria *et al.,* 2022). Although orchids, known for their adaptability, thrive in a wide range of climates and ecosystems, spanning from sea level to tropical and temperate mountains, their survival is rare in nature (Khamchatra *et al.,* 2016). The reproduction process of orchids is very slow and difficult in nature because of the lack of nutritional reserves in seeds. So, involving the establishment of a symbiotic relationship with specific fungi known as orchid mycorrhizal fungi (OMF) is one intriguing aspect of orchids lies in their reproduction process (Selosse *et al.,* 2017). These mycorrhizal fungi are supposed to provide nutrients required for the germination of seeds and the development of protocorms (Jiang *et al.,* 2022). Earlier findings suggest that various mycobionts could play distinct roles in the growth and development of orchids (Sarsaiya *et al.,* 2019). Symbiotic seed germination method closely mimics natural conditions, contributing to enhanced seedling growth (Jiang *et al.,* 2022). Notably, the use of Symbiotic germination has proven advantageous even during the greenhouse acclimatization process, further underlining its potential as a promising alternative (Calevo and Duffy, 2023). Orchids, known for their strong reliance on mycorrhizal symbiosis, greatly benefited from this method (Phillips *et al.,* 2020). By allowing seeds to engage with fungi from the early developmental phases, an environment that closely mirrors the natural conditions essential for orchid growth can be created. This not only enhances the overall health and development of seedlings but also presents a promising strategy for successful greenhouse acclimatization (Li *et al.,* 2020).

In our current investigation, we employed a symbiotic germination approach, facilitating the interaction between orchid seeds and mycorrhizal fungi from the early stages of development. We included a terrestrial orchid species *Calanthe sylvatica,* which is widely distributed in the Western Ghats, especially at higher elevations (Ramesh *et al.,* 2022). We aimed to find a suitable method for seed germination using fungi which were obtained from their roots and rhizosphere.

## MATERIALS AND METHODS

### Plant material

*Calanthe sylvatica* seeds were collected from the Z-point of Chikmagalur district, Karnataka, a part of the Western Ghats. Their

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roots and rhizospheric soil samples were also collected from the same location. The delicate root system was carefully dug to avoid damage to the root system. The underground part (root) and rhizosphere soil samples were collected in clean polythene bags and labeled.

**Fungal isolation and purification**

Mycorrhizal fungi were isolated following conventional methods by plating segments of surface-sterilized roots or tubers on Potato Dextrose Agar (PDA) medium with 50 g/mL of streptomycin, as described by Jyothsna *et al.,* (2023). For further investigation, the cultures were stored on 1/5 PDA slants, at 15°C.

**Seed Viability test**

For germination investigations, mature capsules were stained with Triphenyl tetrazolium chloride (TTC) to determine the vitality of seeds (Srivastava *et al.,* 2015). Nonviable seeds remain unstained and viable seeds showed a pink color under the light microscope.

**Symbiotic seed germination**

The seeds were sterilized using 0.5% (v/v) NaOCl and sterile distilled water (Srivastava *et al.,* 2013). Sterilized seeds were sowed using a sterile bacterial inoculating loop so that each plate received between 20 and 25 seeds. Each mycobiont of fungal inoculum (1 $cm^3$ block) was added to each plate. As controls, three plates were left uninoculated. Three different methods were compared to study symbiotic seed germination under the laboratory conditions.

***Filter paper method***

In the filter paper method, surface sterilized seeds were placed on the center of the filter paper and a mycelial plug of the isolate was placed slightly away from the seeds in a Petri dish on two glass rods with 5ml of sterile distilled water.

***Seed baiting method***

This method attempted to imitate symbiotic seed germination close to natural conditions (Zi *et al.,* 2014). Seed packets (nylon mesh containing orchid seeds) were buried in autoclaved rhizosphere soil in sterile polythene bags under laboratory conditions and periodically observed for seed germination.

***Oatmeal agar (OMA) method***

*Seeds* were meticulously positioned on filter paper within a petri dish containing 20 ml of sterile Oat Meal Agar (OMA) medium (Mercado *et al.,* 2020). Each plate received a 10 mm diameter plug from fungal isolates sourced from freshly cultured specimens. To maintain experimental integrity, the plates were carefully enveloped in aluminum foil, effectively eliminating exposure to light. The entire setup was then stored in darkness at a constant temperature of 25°C for 60 days. Regular observations of seed germination and protocorm development were conducted using a stereomicroscope. Weekly inspections during dark maintenance were performed to detect signs of germination or contamination, with short periods (<10 min) of illumination used for examination. The plates were put back into the experimental settings following a visual examination.

**Data analysis**

The assessment of germination and developmental stages was conducted based on a modified scale derived from Zettler and McInnis (1993), utilizing a numerical scale ranging from 0 to 3. In this scale, 0 denoted an absence of germination, 1 represented the initiation of germination marked by swelling of seeds, 2 indicated the rupture of the seed coat due to the enlargement of the embryo, 3 indicated the development of the protocorm. These assessments were carried out periodically over the 60-day experimental period. The number of seeds that attended each specific development stage, divided by the total number of viable seeds utilized in the sample and multiplied by 100 was used to calculate the germination percentages.

## RESULTS AND DISCUSSION

**Isolation of Fungi**

The fungal isolates of the present study were obtained from the cortical cells of root/tuber that contained live pelotons. Colonies on PDA were initially white then turned into grey to dark grey at the centre. The mycelium grew up to 8.3±0.5 cm in diameter in 7 days (Figure 1a and b). These results are comparable with Jyothsna *et al.,* (2023) study.

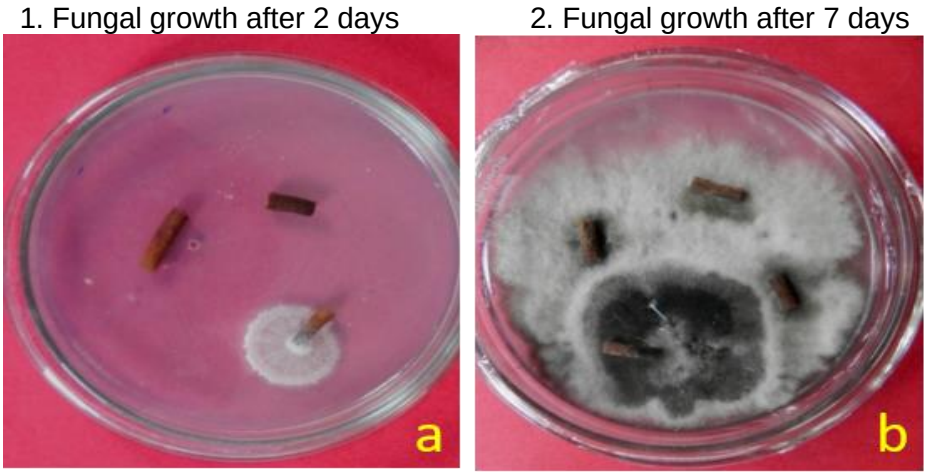


Figure 1: Isolation of fungi from roots of Calanthe *sylvatica*

**Seed Viability test**

Seed viability was determined to assess germination percentage. We noted 42% viability of *Calanthe sylvatic* seeds. Viable seeds were inspected by a dissection microscope, considering seeds having a rounded, distinct, and hyaline embryo (Figure 2). Similar viable seeds of orchids were seen in Srivastava *et al.* (2015) study.

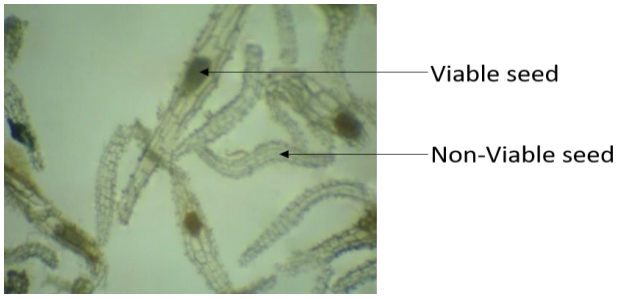


Figure 2: *Calanthe sylvatica* Seed viability test

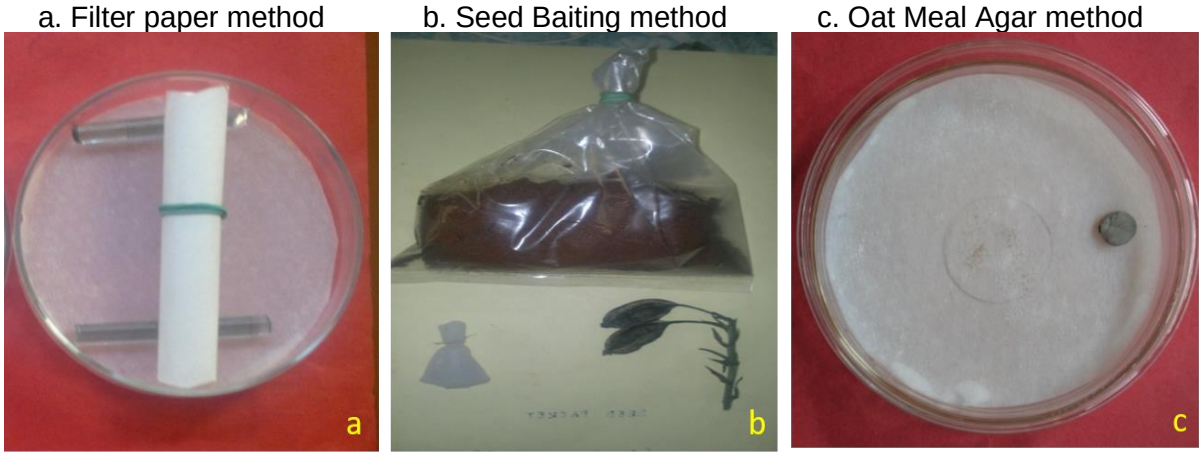


Figure 3: Symbiotic Seed germination of *Calanthe sylvatica* using three different techniques

**Study of seed germination**

OMA exhibited distinct patterns in their efficiency, and 57% of seeds reached stage 3 of germination. After 30 days of incubation, seeds on oatmeal agar (OMA) with a mycelium plug that was actively developing displayed the first visible signs of seed germination, including a noticeable rise in the embryo's translucency within the intact testa (Figure 4a). Fungal colonization was observed at the base of the embryo with many fungal hyphae entering the testa (Figure 4b). After 40 days of incubation, the embryo turned slightly green. This stage was followed by breakage of the seed coat and the release of the embryo. The protocorm development was observed in about 50 days of incubation (Figure 4c). Unlike other methods, here mycorrhizal fungi could exhibit a distinctive feature of OMF wherein hyphae form pelotons,

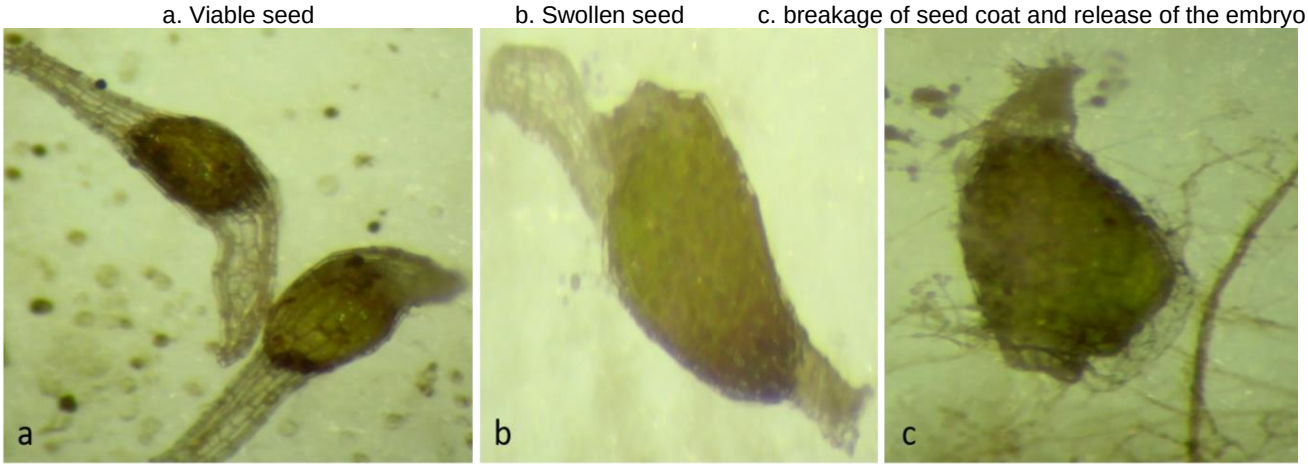


Figure 4: Germination of seeds using Oat Meal Agar

representing hyphal coils within host cells facilitating nutrient exchange which results in providing nutrients to germinating seeds (Yamamoto *et al.,* 2017). It could be due to the presence of oatmeal and agar around the seeds, which also helped fungi to survive. Colonization predominantly occurs at the basal part of the protocorm, and pelotons, temporary structures, undergo digestion by protocorm cells (Valadare *et al.,* 2020). No germination occurred in control on OMA without a fungal inoculum. Because the absence of endosperm in orchid seeds needs mycorrhizal symbiosis to acquire nutrients and initiate the process of germination (Yeh *et al.,* 2021).

**Comparison of developmental stages**

Among the three methods used for symbiotic orchid seed germination, only OMA was found suitable for symbiotic seed germination, which is similar to some studies by other workers using oatmeal agar on different orchid species to achieve advanced stages of germination (Chutima *et al.,* 2011, Mala *et al.,* 2017, Pujasatria *et al.,* 2020).

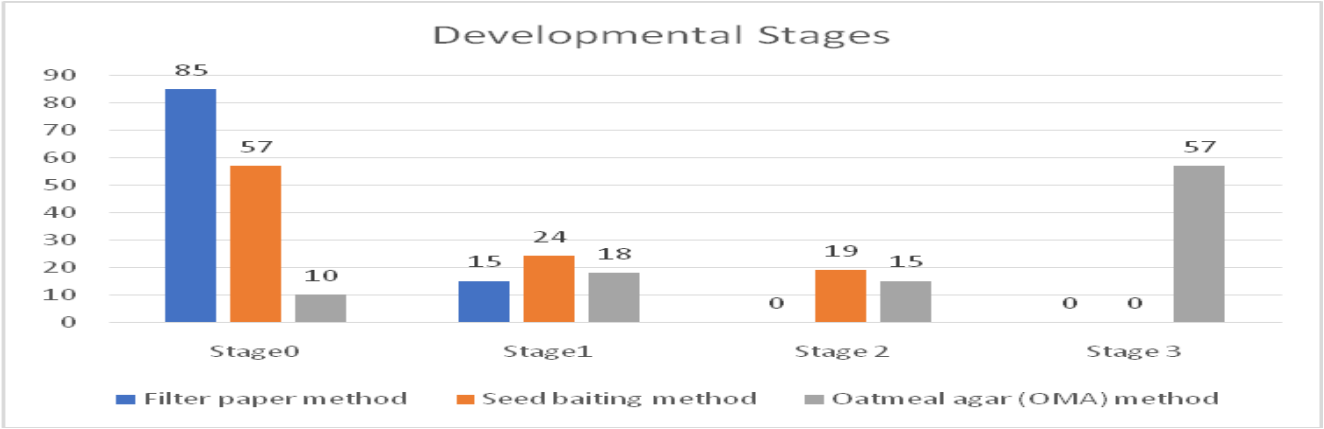


Figure 5: Percent germination of seeds on day 60

On day 60, we found that 85%, 57% and 10% of seeds could not germinate while using the Filter Paper method, Seed biting, and OMA method, respectively (Figure 5). In the filter paper method, 15% of seeds were readily swollen with globular embryos and showed initial stage 1 of seed germination with all the isolates on day 60. However, on further incubation, seeds failed to develop into the protocorm stage. This is probably due to a lack of significant penetration and formation of peletons in the embryo (Li *et al.,* 2020). The seed baiting method 24% of seeds could swell (stage 1), and 19% were imbibed water and the testa got ruptured (stage 2) by day 60 but did not grow further owing to insufficient or lack of suitable mycorrhizal inoculums in the collected soil sample and photo-period conditions given

(Wang *et al.,* 2011). No seeds of both Filter Paper and Seed Baiting could attain stage 3 in the study, whereas OMA exhibited a significant surge to 57%, showcasing its superior performance in promoting seed germination. This extended rate of germination in the OMA method could be seen because, in the presence of oatmeal media, a continual peloton formation and their digestion by seed was possible. It ensured a sustained nutrient supply to fungi and seeds until the protocorm became capable of photosynthesis (Pujasatria *et al.,* 2020). The present investigation has further scope in the multiplication, maintenance, and conservation of terrestrial orchids by making use of suitable mycorrhizal fungal partners. The mycobionts isolated can be beneficially used in the hardening of plants produced by the tissue culture method.

## CONCLUSION

This is the first attempt of symbiotic seed germination in *Calanthe sylvatica* using different methods. We noted seeds sown on OMA exhibited superior performance in promoting seed germination among the three methods tried.

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