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In Vitro gametophyte development of *Cyrtomium falcatum* (L.f.) C. Presl (Dryopteridaceae) using modified culture media

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Abstract

This study investigated the germination and gametophyte development of *Cyrtomium falcatum* (L.f.) C.Presl (Japanese holly fern) using modified culture media to optimize fern propagation, while exploring locally sourced materials as an alternative and sustainable growing medium for fern development. Mature spores from fertile fronds were sown under various treatment conditions: (T1) Garden Soil, (T2) Garden Soil with Ground Claypots, (T3) Garden Soil with Ground Adventitious Tree Fern Roots, and a (T4) combination of all three with 1:1 ratio in all treatments. Results revealed that the germination of *C. falcatum* follows the Vittaria type, while prothallial development conforms to the Aspidium pattern—progressing from uniseriate germ filaments to irregular and spatulate plates, and subsequently to lopsided plates in T2 within 31 days after sowing (DAS), with cordate prothalli observed in all treatments by 35 DAS. Fully matured gametophytes were observed at 73 DAS with folded margins. Leptosporangiate-reproductive organs such barrel-shaped antheridium manifested earlier into one of the germ filaments 17 DAS in T2, whereas the inverted round-bottomed flask archegonia were observed later in all treatments within 73 DAS, showing antheridiogen-influenced development. Gametophyte survival assessed by growth coverage varied significantly with (ANOVA, Tukey's HSD, $\alpha = 0.05$) T2 (91%) demonstrating the optimum medium for *C. falcatum* gametophyte *in vitro* propagation.

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Results in soil analysis supports that claypot-based media is favorable for *C. falcatum* cultivation, due to its near-neutral pH, high organic matter, and elevated phosphorus levels. Thus, this study shows that *C. falcatum* can be effectively propagated *in vitro* using modified media, especially those with ground clay pots.

Keywords: alternative media, fern gametophyte development, soil analysis

INTRODUCTION

Pteridophytes, usually known as ferns and lycophytes, defined as seedless vascular plants that reproduce via spores. In suitable environmental conditions, these spores germinate and develop into gametophytes, which can thrive in various habitats, including different soil types, tree branches, bryophyte mats, and low-light environments (Amoroso *et al*., 2015; Anjum *et al*., 2022).

Understanding the haploid phases of pteridophytes, specifically spores and gametophytes is essential to determine their dispersal mechanisms and environmental preferences. Spores function as the primary dispersal units, facilitating the growth of these plants in different environmental conditions (Farrar and Johnson, 2022), whereas gametophytes directly affect the spread of mature sporophytes (Quinlan *et al*., 2022). Gametophytes rely on certain environmental conditions for optimal growth and fertilization, as the early life stages are very sensitive to environmental variations (Wu *et al*., 2022). In light of this, *in vitro* spore germination has been established as a commonly employed technique for propagating ferns and examining their initial developmental stages, which are challenging to observe in natural environments due to the microscopic scale of their structures (Menéndez *et al*., 2010; Marcon *et al*., 2023). This technique serves as a recognized approach for ex situ conservation, especially in seed plants and more recently in ferns. Increasing studies have investigated the effectiveness of this technique in preserving economically significant fern species, as well as threatened (Amoroso and Amoroso 2003; Shukla and Khare 2014), rare, and endemic ferns (Pajo *et al*., 2024).

In the review of Singha *et al*. (2013), Murashige and Skoog (manuscript in preparation) medium as well as its modified variations has been the predominant choice for *in vitro* propagation of pteridophytes. Nonetheless, the lack of availability, high cost, and inaccessibility for extensive application indicate a necessity to investigate alternative growth media (Taer *et al*., 2025). The spore culture medium developed by Amoroso *et al*. (2021) (PH22021050310U1) presents a viable option, utilizing locally sourced materials as an alternative growing medium for fern development. Recent studies have successfully utilized this medium for spore germination and gametophyte development (Taer *et al*., 2025; Pajo *et al*., 2024). Moreover, these materials are easily accessible which may develop ideal growing conditions with reduced costs, promote sustainability, and facilitate investigations in environments with limited resources. However, specific soil parameters were not analyzed. Notably, the composition of the culture medium plays a crucial role in influencing the

development of spores, gametophytes, and sporophytes, as nutrient availability directly affects growth at each stage of the life cycle (Suo *et al*., 2015; Marcon *et al*., 2023).

Cyrtomium falcatum (L.f.) C.Presl, widely referred to as the Japanese Holly Fern, is classified within the Dryopteridaceae family and is primarily found in Japan, China and Korea (Raman and Park, 2016). Additionally, it has been introduced in various locations across the globe, such as the United States and Europe (Li *et al*., 2017). This fern is known for its glossy, rich green, pinnate fronds that feature holly-like pinnae, enhancing its appeal for both garden and indoor settings. Beyond its aesthetic appeal, *C. falcatum* exhibits medicinal benefits. Traditionally, the rhizomes have been utilized as an anthelmintic against tapeworms (Chopra *et al*., 1986). Recent studies have also reported its antioxidant, anti-inflammatory, and anti-melanogenesis properties, indicating its potential for application in natural therapeutics and skincare (Kim *et al*., 2021; Zhou *et al*., 2023). Given its significance in both medicinal and ornamental applications, understanding the development of its gametophyte may improve propagation techniques, particularly for extensive cultivation and conservation efforts. Thus, this study utilized a modified culture medium to investigate the gametophyte stages of the *C. falcatum* contributing further data to its previously limited developmental studies. Additionally, the culture medium has undergone soil analysis emphasizing the role of the selected soil properties in the gametophyte development of *C. falcatum*.

MATERIALS AND METHODS

Place and Duration of the Study

Prior to the conduct of the study, a permit was obtained from the Director of the Natural Science Research Center (NSRC), Central Mindanao University (CMU), authorizing the use of laboratory facilities and necessary equipment. The experiment on spore germination and gametophyte development of *C. falcatum* was conducted at the Plant Tissue and Spore Culture Laboratory, NSRC, CMU, Musuan, Maramag, Bukidnon, from March to May 2025.

Collection of Plant Samples and Matured Spores

The fertile fronds of *C. falcatum* were collected from the University Fernery of CMU, where the sporophylls were placed in a newspaper bag and underwent drying for 1 week at ambient temperature, allowing the release of matured spores. After drying, the abaxial surface of the sporophylls was brushed with a small clean paint brush to facilitate the release of the remaining spores from the sporangia. The released spores were placed in a clean white bond paper separating from the plant samples and then collected and sieved in a plastic tube.

Spore Morphological Observation

Matured spores of *C. falcatum* were examined under a OMAX M83E-Series compound microscope with 40x magnification using water as a mounting medium.

Preparation of Modified Culture Media

The preparation of the modified media in the study followed the methods developed by Amoroso *et al*., 2021 with some modifications. The adventitious roots of tree ferns were chopped into pieces and the broken clay pots were powdered thoroughly. The main substrate for the modified culture media in the study was garden soil collected from a residential area within the campus. Visible debris was removed from the soil and was thoroughly mixed to ensure its uniformity. Afterwards, all modified media and materials were sterilized using an autoclave at 15 psi for 2 hours (Taer *et al.,* 2025). There were three (3) treatment conditions mixed in a 1:1 ratio (i.e., T2- Garden Soil + Powdered Clay Pots, T3 Garden Soil + Ground adventitious Roots, T4- Garden Soil + Powdered Clay Pots + Ground adeventitious Roots) utilized in the study with 4 set ups in triplicate; where T1 (Pure Garden Soil) as a control. The garden soil and the mixed media were placed in a sterile growth chamber with a cover (90-mL (7 x 5 cm) Donewell/FAS Pack microwavable cup with transparent lid) in a flat manner with ≈2.54 cm depth.

Soil Analysis

Following the preparation of the modified culture media, representative samples from each treatment, weighing approximately 20 g, were collected and subjected to air-drying at ambient temperature for 7 days. The dried soil samples were sent to the Central Mindanao University Soil and Plant Analysis Laboratory (CMU-SPAL) for the analysis of the specified parameters, including soil pH, organic matter, extractable phosphorous, and exchangeable potassium.

Sowing of Spores

The collected spores were soaked in a beaker with 50 mL of dh2O. Using a sterile dropper, the spores were sown into 10 divisions of every growth chamber covering the gametophyte survival rate through growth coverage (Taer *et al*., 2025). The cultures were then incubated in the growth room at 18–22±ºC with continuous white, fluorescent light.

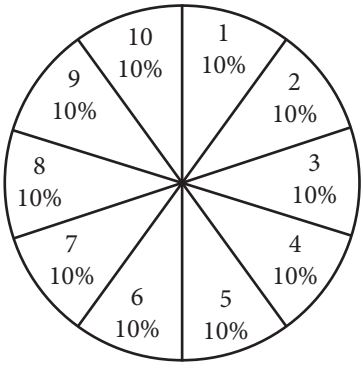

Figure 1. Illustration of the pattern of spores sowing, estimating the growth survival rate through growth coverage

Monitoring of Spore Germination and Gametophyte Development

The preliminary phases of spore germination were examined with a compound microscope, with random samples gathered biweekly from the growth chambers of each treatment. Samples were thereafter dissected using AmScope LED-144 stereo microscope to eliminate unwanted soil debris and placed on a glass slide with a drop of water. Multiple parameters were documented, encompassing the initial phases of spore germination, germ filament formation and mature gametophytes. Gametophyte development was monitored biweekly for a 10-week duration, starting after the spores were inoculated onto the various growth media. 5–10 gametophytes from each growth chamber were selected at random for examination. The various stages of gametophyte growth were monitored using stereo and compound microscopes, and the duration required for each step to manifest was documented. Identifiable stages consist of the development of germ filaments, spatulate prothallial plates, asymmetrical prothallus, heart-shaped prothallus, and ultimately, the emergence of gametangia.

Statistical Analysis

The data obtained from the gametophyte growth coverage underwent analysis of variance (ANOVA), and the means were compared using Tukey's test at a 5% significant level.

RESULTS AND DISCUSSION

Spore Morphology and Germination of *Cyrtomium falcatum*

The spores of *C. falcatum* observed in the study aligns with the species description previously delineated by Li *et al.* (2017). The spores are bilateral and perinate (Figure 2A). The outer layer features an oval, smooth structure on a regular, verrucate protuberance, as generally described within the polystichoid ferns by Chandra and Nayar (1970). The germination of spores follows the Vittaria type, producing a proximal rhizoid and a uniseriate germ filament in lateral position (Figure 2C) (Nayar and Kaur, 1971). The emergence of the rhizoid signified the successful germination of the spores (Figure 2B). Germination was observed in all media utilized in the study 11 days after sowing (DAS). In genus *Cyrtomium*, the terminal cell shows significant growth retardation when the germ filaments reach a length of five to eight cells (Figure 2C). Furthermore, the uniseriate germination filament becomes evident after a period of 12–30 days (Pérez-García *et al.*, 1998); consequently, the earliest germ filament observed in the study was in T2 (Garden Soil + Ground Claypots) 17 DAS.

Early Prothallial Developmental Stages

The prothallial development of *C. falcatum* conforms to the Aspidium type, with numerous reports supporting that the gametophytes of the species within the Dryopterida-

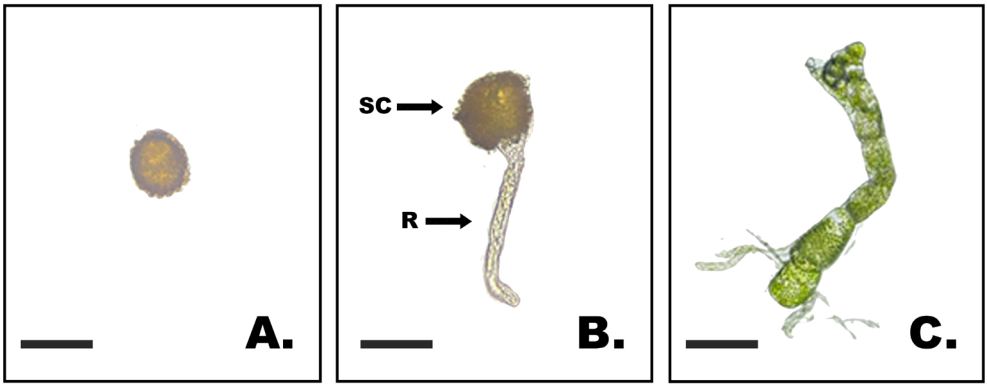

Figure 2. Spore morphology, germination, filamentous phase of *C. falcatum*: (A.) Spore of *C. falcatum* (B.) Germination with spore coat (SC) and Rhizoid (R), (C.) Earliest Germ Filament. Scale Bar: A-C= 20 µm

ceae family typically develop according to this model (Migliaro and Galán, 2012; Nayar and Kaur, 1971). Furthermore, the majority of the polystichoid ferns to which *C. falcatum* is associated have been documented following to this developmental pattern, exhibiting a broad range of variations common of this group (Chandra and Nayar, 1970). Figure 3 shows the early phases of gametophyte development in *C. falcatum*. Where, following 21 DAS (days after sowing) in T3 (Garden Soil + Ground Adventitious Roots of Tree Fern) and T2 (Garden Soil + Ground Claypots), germ filaments that serve as precursors of the prothallial plate were observed which initially originated from the cell located behind the quiescent apex of the germ filament (Figure 3A). Upon the development of the prothallial plate, *Cyrtomium* spp. germ filaments normally produce a unicellular papillate hair crowning their terminal cell (Figure 3C). With continuous development of the plate, more and more hairs were produced by division of the other prothallial cells. Consequently, glandular hairs (Figure 4E) were found on the margin and surfaces of the gametophytes, and were papillate to slender claviform (Zhang *et al.*, 2011). In *C. falcatum,* there is a notable likelihood for the marginal hair-bearing cells to protrude visibly.

Moreover, the cell subsequently undergoes longitudinal division, resulting in one daughter cell exhibiting a more pronounced expansion. The larger daughter cell undergoes an equal division into anterior and posterior daughter cells. The anterior cell underwent two successive oblique cell divisions and differentiates into a meristematic cell, whereas the posterior cells, including the basal cell of the young germ filaments undergo longitudinal division and form an irregular plate (Figure 3B–D). The presence of the irregular plate revealed that *C. falcatum* exhibits notable characteristics similar to those of polystichoid ferns, such as *Polystichum lonchitis* (Chandra and Nayar, 1970). This occurrence was observed in T4 (Garden Soil + Ground Claypots + Ground Adventitious Roots of Tree Fern) within 21 DAS following the emergence of the uniseriate germ filaments.

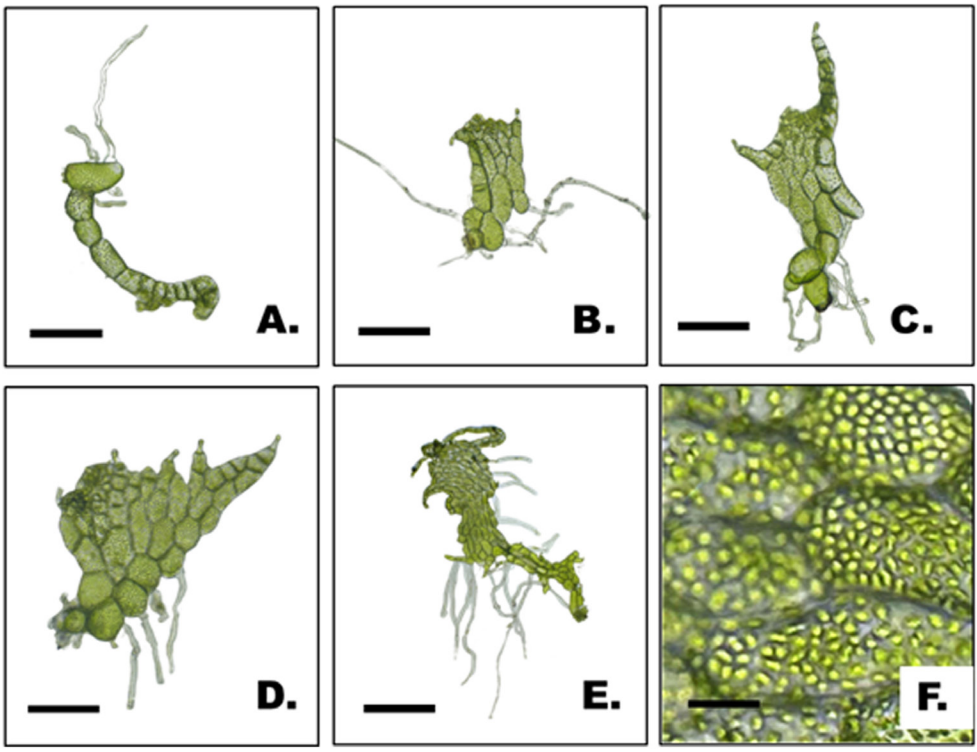

Figure 3. Early Stages of *C. falcatum* showing Aspidium type of Gametophyte Development in Modified Culture Media: (A) 11-celled uniseriate germ filament, (B)-(D) 21-43-celled Irregular-shaped germ filaments, (E) Spatulate plate, (F) Prothallial cells showing the chloroplasts Scale Bar: A-E = 20 µm, F= 150 µm

After 28 DAS, a spatulate prothallium plate (Figure 3E) was observed in T4 (Garden Soil + Ground Claypots + Ground Adventitious Roots of Tree Fern) and T2 Menénde(Garden Soil + Ground Claypots), transitioning from irregular germ filaments (Figure 3B–D) into a two-dimensional, spatula-shaped structure characterized by a broad, flat apical region and a narrower base (Raghavan, 1989). This phase generally encompasses cell division at the apical meristem, leading to the development of a heart-shaped prothallus precursor (Banks, 1999). The development of this stage appears to be relatively extended when compared to the findings of Taer *et al*. (2025) in *Pleocnemia irregularis* (Dryopteridaceae), which was observed at 21 DAS. Considering that both species exhibit similar prothallial development within the same family, these results suggest that they vary in their period of development in response to modified culture media.

Development of Mature Gametophytes of *C. falcatum*

As the gametophyte matures, the meristematic cell develops laterally in the prothallial plate, resulting in young thalli that are lopsided (Figure 4A) which was observed in T2 (Garden

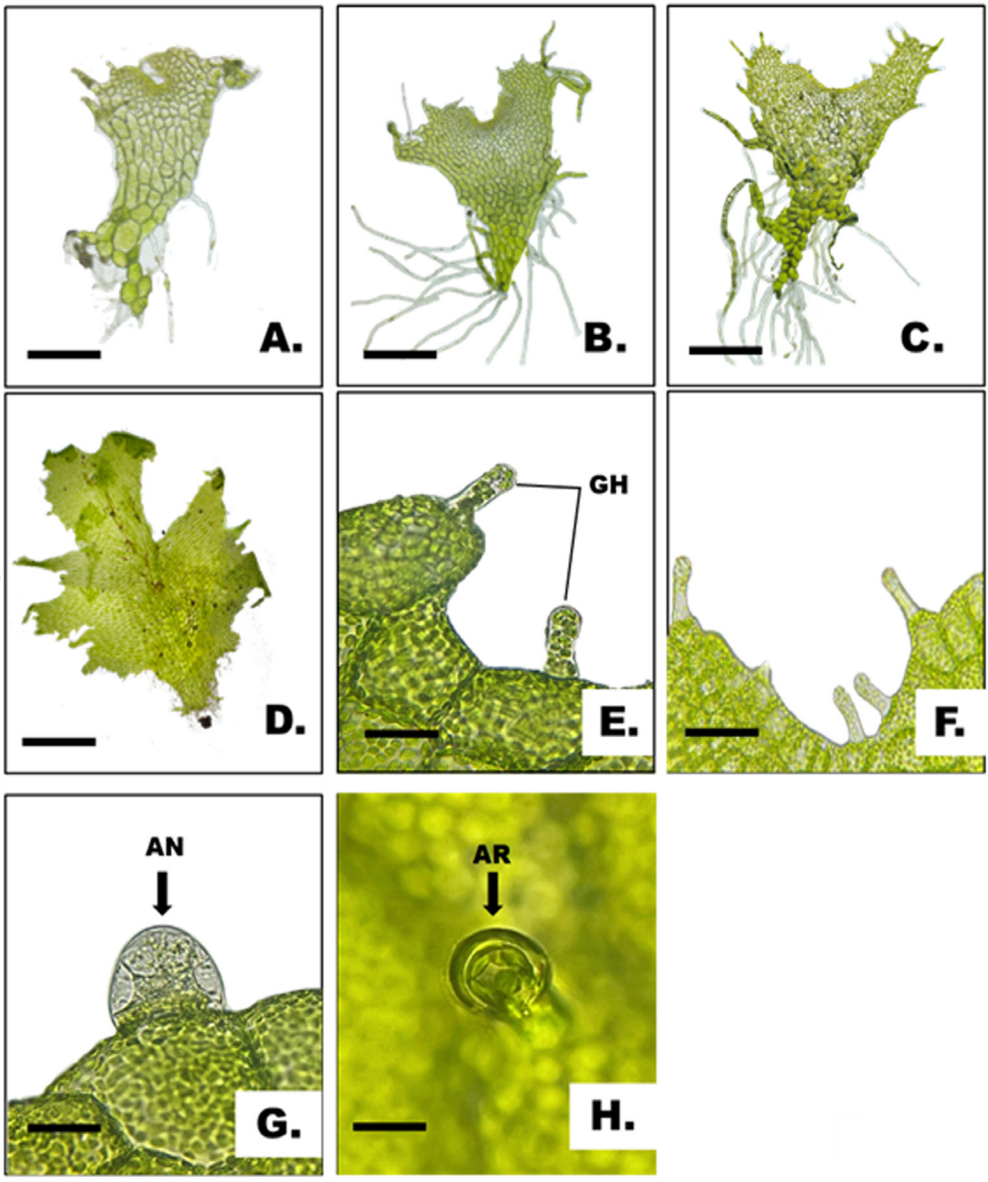

Figure 4. Developmental stages of the prothallus in *C. falcatum* in modified culture media: (A) Lopsided prothallial plate formation, (B) semi-heart-shaped young gametophyte, (C) heart-cordate prothallus formation, (D) Mature gametophyte with folded margins, (E) Detail of Marginal glandular hair (GH), (F) Anterior region of a young cordate prothallus, Reproductive Organs (G) Leptosporangiate-Antheridium (AN), (H) Archegonium (AR) Scale Bar: A-C= 30 µm, D-H = 150 µm

Soil + Ground Claypots) 31 DAS. Moreover, the asymmetry becomes more pronounced when the formation of a meristematic cell is further delayed. As the thalli develop, the asymmetry fades from lopsided plate 35 DAS, resulting in a cordate thallus (Figure 4B–C) which was observed from all the treatments, bearing a lot of superficial rhizoids. The fully matured gametophyte of *C. falcatum* (Figure 4D) was observed 73 DAS in all treatments, with more than one cordate meristematic apex, and irregular lobed and folded margin (Chandra and Nayar, 1970; Zhang *et al*., 2011).

The reproductive organs (Figure 4G–H) of *C. falcatum* are characterized as those Leptosporangiate-type considering that it is under the Dryopteridaceae, which is known to be one of the largest Leptosporangiate families (Raman *et al*., 2016; Nayar and Kaur, 1971). In the study, barrel-shaped antheridium of *C. falcatum* was observed into one of the germ filaments 17 DAS from T2 (Garden Soil + Ground Claypots). Notably, the antheridia of homosporous ferns typically develop preceding to the archegonia (Nayar and Chandra, 1970). The formation of antheridia in fern prothalli is shown to be specifically regulated by naturally occurring substances known as antheridiogens. However, the characteristics and processes involved remain understudied (Miller, 1968; Nayar and Kaur, 1971). Whereas the archegonium (Figure 4H) of *C. falcatum* was observed in the mature gametophytes positioned at the anterior region of the midrib in all treatments within 73 DAS. It is characterized as an inverted round-bottomed flask and consists of an axial row of three cells (a large basal egg; an ephemeral, small, ventral canal cell; an anterior, elongated, neck canal cell) surrounded by a jacket of one layer of cells (Nayar and Kaur, 1970). Consequently, the formation of archegonia in thalloid prothalli initiates when the process of antheridia formation ceases partially or completely. Thus, it is proposed that the sex organs of *C. falcatum* exhibit a protogynous pattern associated with sequential hermaphroditism (Schärer and Ramm, 2016).

Percentage of Gametophyte Survival

The gametophyte survival of *C. falcatum* in the various modified culture media was evaluated at the final stage of the study (Figure 5). The objective of this evaluation was to identify which modified culture media performed optimally and produced the highest number of gametophytes. The percentage of gametophytes that were able to survive was determined by assessing the amount of growth coverage observed in the medium.

The statistical analysis suggests notable variations in the effectiveness of the four modified media, as validated by the ANOVA and Tukey's HSD test ($\alpha = 0.05$). The combination of garden soil and ground claypots (91%) demonstrates the highest effectiveness, clearly surpassing all other treatments demonstrating claypots' importance in nutrient availability, water retention, and aeration. The combination of garden soil, claypots, and tree fern roots (67.67%) ranks as the second most effective, indicating that both additives have a synergistic but lesser effect, indicating that claypots exert a more significant influence. The combination

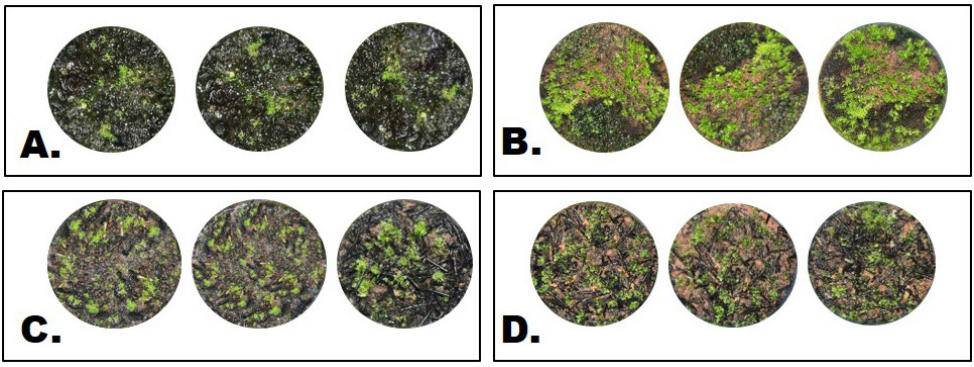

Figure 5. Gametophyte Growth Coverage of *C. falcatum* sown in (A) T1 Garden Soil, (B) T2 Garden Soil + Ground Claypots, (C) Garden Soil + Ground Adventitious Roots of Tree Fern, (D) Garden Soil + Ground Claypots + Ground Adventitious Roots

of garden soil and tree fern roots (40%) demonstrate a notable enhancement compared to garden soil alone (22.33%), which shows the least effectiveness.

Moreover, recent investigations have shown that claypot-based modified media exhibit a notable variation in their efficacy based on their application within the substrate and fern gametophyte species. The findings from Taer *et al*. (2025) in developmental studies of *Pleocnemia irregularis* reveal that pure ground claypots yielded the lowest gametophyte survival rate at 48.33%, suggesting that using only claypots as a growing medium is inadequate. This result indicates that although clay pots can offer advantageous physical characteristics like enhanced aeration and moisture retention, they probably do not supply the essential nutrients and organic matter needed for ongoing gametophyte growth when utilized solely. Thus, additional substrates such as claypots may be added to soil-based media, contributing to cation exchange capacity (CEC), which is essential for retaining nutrients and enhancing water-holding capabilities (Sellmer, 2023). In contrast, Pajo *et al*. (2024) illustrated the significant potential of claypots when incorporated into a composite medium in the gametophyte development of *Lecanopteris deparioides*. The combination of ground adventitious roots and ground claypots resulted in the highest gametophyte cover at 95.36%. This was closely followed by another mixture involving claypots, specifically garden soil combined with ground claypots, which demonstrated a robust performance with an 88.52% cover. The findings indicate that claypots serve as a significant additive, improving substrate structure, moisture balance, and potentially nutrient retention when integrated with organic materials such as tree fern adventitious roots or garden soil.

Soil Analysis

Table 2 presents the analysis of soil parameters in four modified culture media utilized in the study—T1 (Garden Soil), T2 (Garden Soil + Ground Claypots), T3 (Garden Soil +

Table 1. Survival Growth Percentage of *C. falcatum* sown in Modified Culture Media

MODIFIED MEDIA	R1	R2	R3	MEAN %
T1- Garden Soil	20	24	23	22.33[d]
T2- Garden Soil + Ground Claypots	89	90	94	91[a]
T3- Garden Soil + Ground Adventitious Roots of Tree Fern	43	40	37	40[c]
T4- Garden Soil + Ground Claypots + Ground Adventitious Roots of Tree Fern	68	65	70	67.67[b]

**groups that are not significantly different share the same letter

Ground Adventitious Roots of Tree Fern), and T4 (Garden Soil + Ground Claypots + Ground Adventitious Roots)—aimed at investigating gametophyte development in *Cyrtomium falcatum*. The gametophyte stage begins with the germination of spores into a prothallus and necessitates particular soil conditions, including pH, organic matter, phosphorus, and potassium, to facilitate growth and reproduction (Raven *et al*., 1999).

Results in the analysis revealed that T2 stands out as the most favorable treatment, attributed to its nearly neutral pH that maximizes nutrient availability, alongside elevated organic matter and phosphorus concentrations that improve moisture retention and facilitate

Table 2. Soil Analysis of the Modified Culture Media utilized in gametophyte development of *C. falcatum* encompassing different soil parameters

Parameter	T1	T2	T3	T4
Soil pH	5.71 (acidic)	6.76 (near-neutral)	5.85 (acidic)	6.16 (slightly acidic)
% Organic Matter	21.98%	25.39%	3.68%	11.81%
Extractable Phosphorus	27.63 ppm	111.29 ppm	49.44 ppm	112.27 ppm
Exchangeable Potassium	2.693 cmol/kg	1.604 cmol/kg	3.947 cmol/kg	3.792 cmol/kg

energy transfer for cell division (Brady and Weil, 2008). The incorporation of ground claypots in T2 possibly enhances soil structure and nutrient retention, although its reduced potassium levels may somewhat hinder water regulation, a drawback that could be alleviated by the elevated organic matter content (Clarkson, 1996). Whereas, T4 which combines garden soil with ground claypots and adventitious roots, effectively supports gametophyte development. Its slightly acidic pH, high phosphorus, and elevated potassium levels promote enzyme activation and water balance (Clarkson, 1996; Hoskins, 1997).

The presence of adventitious roots in T4 could contribute extra organic material; however, its lower organic matter relative to T2 indicates diminished moisture retention, which may require additional water management strategies. Recent findings indicate that mycorrhizal fungi can improve phosphorus absorption in ferns, potentially aiding gametophyte development in T4 by expanding hyphal networks, although this might come with a carbon cost to the plant (Martin *et al.*, 2024). The T1 (Garden Soil) offers moderate support owing to its substantial organic matter content. However, the acidic pH and low phosphorus levels could hinder nutrient absorption, potentially delaying prothallus development, especially considering that phosphorus availability diminishes in acidic soils (Brady and Weil, 2008). Furthermore, the reduced phosphorus levels in T1 could be exacerbated by the accumulation of phytate, a common organic phosphorus form in soils that binds tightly and restricts availability (Sun *et al.*, 2022).

Modified media enriched with tree fern roots (T3), tends to be the least promising option due to its significantly low organic matter content, which could result in desiccation, even with higher potassium and moderate phosphorus levels (Klekowski, 1969). Recent studies indicate that elevated potassium levels, such as those found in T3, may inhibit fine root growth in certain plants, which could impede the gametophyte's capacity to develop a strong prothallus (Kashem *et al*., 2024). In conclusion, T2 and T4 emerge as the most effective treatments for the development of *Cyrtomium falcatum* gametophytes, with T2 benefiting from its elevated organic matter content. However, additional experiments are necessary to investigate soil interactions and the role of microbial influences (Martin *et al*., 2024).

CONCLUSION

This study provides detailed insights into the spore morphology, germination, prothallial development, and gametophyte survival of *Cyrtomium falcatum* across four modified culture media. The results demonstrated that the addition of ground claypots into the culture medium significantly enhanced gametophyte development, with the addition of tree fern roots offering potential improvements in performance. These findings highlight the effectiveness of using claypot-based media for optimizing gametophyte survival and growth. Moreover, the use of locally available materials not only supplies essential nutrients but also offers favorable conditions for sustainable and efficient fern propagation.

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