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## *Research article*

# Spore Germination and Gametophyte Development of *Pleocnemia irregularis* (C.Presl) Holttum (Dryopteridaceae) Using Modified Culture Media

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## Abstract

*Pleocnemia irregularis* is an economically important fern, with no known literature on its developmental biology. Thus, this study was aimed to document various aspects of its developmental stages including its patterns of spore germination, the morphology of various developmental stages, type of gametophyte development and percentage of gametophyte survival using modified culture media as the substrate. Morphological observations highlighted the distinctive monolete-bilateral, ellipsoidal, and reticulated perispores, while the prominent protuberance of club-shaped unicellular hairs persisted continuously from the germ filaments to the late gametophytic stages. Spore germination follows the *Vittaria*-type while *Aspidium*-type for the prothallial development. Among the treatments, T0 (garden soil), T3 (ground adventitious roots of tree fern + ground clay pots), and T1 (pure ground adventitious roots of tree fern) demonstrated a consistently prolific performances, from spore germination until the late gametophyte stage with rapid development led by T0. Conversely, consistent delayed development was observed for spores sown in T2 (ground clay pots). Regarding gametophyte survival, T0 significantly yielded the highest percentage of gametophyte survival (94%), while T2 obtained the lowest number (48.33%). No performance variation was observed in T3 and T1. As a result, T0 served as the best culture medium for gametophyte coverage. The utilization of accessible and cost-effective growing media provides a conducive substrate for the successful developmental biological study of *P. irregularis.* The information provided in this paper will certainly be useful in studying the developmental biology of those often overlooked but economically important pteridophytes.

**Keywords:** culture; development; ferns; *Pleocnemia*; Tectariaceae

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## 1. Introduction

Pteridophytes are simple vascular plants that exhibit free-living sporophyte and gametophytic phases as patterns of their life cycle, commonly known as alternation of generations. These two phases are independent from each other, as these differ in functional, morphological, physiological, and preferred growth (Qiu et al., 2012; Sigel et al., 2018).

As the dominant phase in pteridophyte life cycles, the sporophyte has traditionally been the primary focus of studies on taxonomy and evolution in these plants (Nayar & Kaur, 1971). However, details on the gametophyte phase have also proven essential in studying the plant group's evolution and reproductive biology, and in phyletic studies (Nayar & Kaur, 1971; Chiou, & Farrar, 1997; Puspitasari et al., 2015). Analyzing gametophyte morphology including the patterns of spore germination, significant features in early development, the appearance of mature prothalli, and characteristics of sexual development, have proved crucial in defining taxonomic criteria in pteridophytes (Nayar & Kaur, 1971; Chen et al., 2008).

Nearly 1,100 species of pteridophytes are currently documented in the Philippines under 154 genera and 34 families (Amoroso et al., 2016; Coritico et al., 2020). Among the species, *Pleocnemia irregularis* (C.Presl) Holttum, which is terrestrial and pantropical, has a native distribution within the archipelago. This species was revealed to exhibit medicinal and ornamental value throughout Asia (de Winter & Amoroso, 2003; Kreier & Schneider, 2006).

To date, there is no existing literature on the developmental biology of *P. irregularis*. Developmental biological studies, especially by means of mass production, have been essentially helpful for ex-situ conservation (Akomolafe et al. 2015). Propagation in pteridophytes can be done either sexually or vegetatively. Unfortunately, the vegetative procedure of propagation has faced limitations, among which are sample availability and susceptibility towards stress upon handling and transplanting, making sexual technique through spores, in most cases, the preferred one (Apuan et al., 2016).

The use of agar supplemented with various sucrose concentrations served as the most popular composition in fern culture media. However, its availability is limited, it is impractical for commercial purposes, and it is simply expensive. Therefore, alternative growing media need to be assessed for their potency in the study of fern developmental and morphological characteristics and in the refining of the cost-effectiveness of mass propagation of the fern species.

Owing to these concerns, inexpensive and locally sourced materials were explored for the use as alternative growing media for fern developmental studies in the present investigation. Garden soil, pure ground adventitious roots of tree ferns, ground clay pots, and a combination thereof were evaluated for their influence on spore germination and gametophyte development. These readily available substrates have the potential to provide suitable growing conditions while reducing costs, promoting sustainable practices, and enabling research in resource-limited areas. The low organic content of ground clay pots and the inclusion of organic matter from garden soil and ground adventitious roots of tree ferns provide a balanced approach to assessing the substrate characteristics suitable for fern development.

The present study was undertaken with the following objectives: (1) to document the morphological characteristics and spore germination patterns of *P. irregularis*, (2) to document the gametophyte development, and (3) to determine the best culture medium in terms of the percentage survival of gametophytes through growth coverage. The findings

could generate valuable insights into the developmental biology of this economically important yet often overlooked fern species.

## 2. Materials and Methods

### 2.1 Letter of permission

Prior to conducting the study, a letter of permission was approved by the Director of the Natural Science Research Center (NSRC) at Central Mindanao University, granting the use of the equipment in the NSRC laboratory.

### 2.2 Place and duration of the study

The study was conducted at the Plant Tissue Culture Laboratory of the Natural Science Research Center, Central Mindanao University, Musuan, Maramag Bukidnon, during the months of April 2023 to mid-May 2023.

### 2.3 Collection of explants

The spores from the fertile fronds of *P. irregularis* were collected at the University Fernery on the campus. After that, an isolation method for the sporophylls was performed following the protocol of Jang et al. (2019). The sporophylls were isolated in newspaper bags and dried for one week, allowing the release of spores. Afterwards, spores were gathered, sieved, and placed in a plastic tube. Subsequently, the spores were stored at 4ºC and -20ºC for future use at the Spore Bank of the Natural Science Research Center (NSRC).

### 2.4 Spore morphological observations

The morphology of *P. irregularis* spore was examined under the Swift M10LB-S compound microscope at 1000x magnification. Water was used as the mounting medium.

### 2.5 Modified media

In this study, the methodology developed by Amoroso et al. (2021) was followed for improvised culture media, with modification. Using a weeding knife and a hammer, chopped adventitious roots of tree ferns, and garden clay pots, were thoroughly ground into smaller particles. Natural garden soil was obtained from the top 10-15 cm layer of the ground from a lower-elevation tropical woodland area that did not harbor the target *Pleocnemia* species. Visible debris from the soil was removed and the soil was thoroughly mixed to ensure homogeneity. Shortly, all modified media and materials were sterilized using an autoclave at 15 psi for 2 h. Subsequently, each medium was placed in a microwaveable plastic container, which served as the germination chamber. Afterwards, each medium was sprayed with distilled water before the spores were sown. The following treatments were used:

T0= Garden soil
T1= Pure ground adventitious roots of tree fern
T2= Ground clay pots
T3= Ground adventitious roots of tree fern + ground clay pots (1:1)

## 2.6 Sowing of spore

The spores were displaced into a small beaker filled with 15 mL of distilled water. Using a sterilized dropper, the spores dispersed in water were dispensed from the beaker and were sown onto ten distinct divisions on the surface of different spore germination-modified media contained in the microwaveable plastic containers. Subsequently, the plastic containers were properly labeled with the following: species name, treatment, replicate number, date of sowing, and name of person doing the culture. Then, the lids were closed properly.

## 2.7 Cultures in growth room

All cultures were incubated in the laboratory room at 18-22±ºC under 16 h of continuous light illumination.

## 2.8 Experimental procedure

Twelve transparent microwaveable plastic containers of 250 mL were prepared and divided into four groups; each consisted of an equal number of three replicates. Within the container, sampled specimens were isolated for the gathering of data. Each medium was constantly covered with a transparent lid after data gathering to avoid contamination (Figure 1).

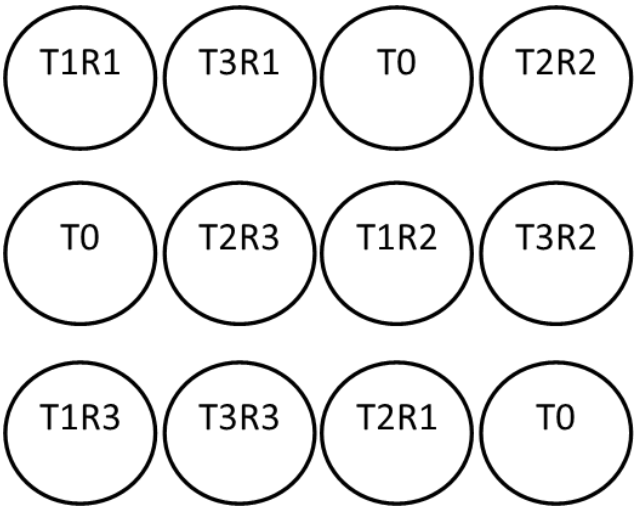


**Figure 1.** Experimental layout T0= Garden soil. T1= Pure ground adventitious roots of tree fern; T2= Ground clay pots; T3= Ground adventitious roots of tree fern + Ground clay pots

## 2.9 Monitoring of spore germination

In monitoring the germination of the spores, random samples were taken weekly from the culture container for each treatment. Specimens from the samples were dissected by a needle, placed on a glass slide with a drop of water, and examined under a compound

microscope. Several parameters were recorded, encompassing the initial stages of spore imbibition and swelling, extending to the earliest appearance of rhizoids.

## 2.10 Monitoring of gametophyte development

Gametophyte development was monitored twice a week over an 8-week period, following spore inoculation onto the different growing media. Appropriate materials, such as fine-end tweezers, were utilized to collect the sample specimens from the culture containers which were examined under the compound microscope. Ten gametophytes of each sample were randomly selected and observed. Several phases of gametophyte development were observed, and the duration in days for the appearance of each initial form was noted. The stages encompassed the germ filament formation, spatulate prothallial plate, lopsided prothallus, heart-shaped prothallus, and ultimately, the emergence of gametangia.

## 2.11 Percentage of gametophyte cover

The percentage of gametophyte cover was estimated by delineating each culture container into ten parts. The visible gametophyte cover per part of each treatment and the computed average were determined, as shown in Figure 2.

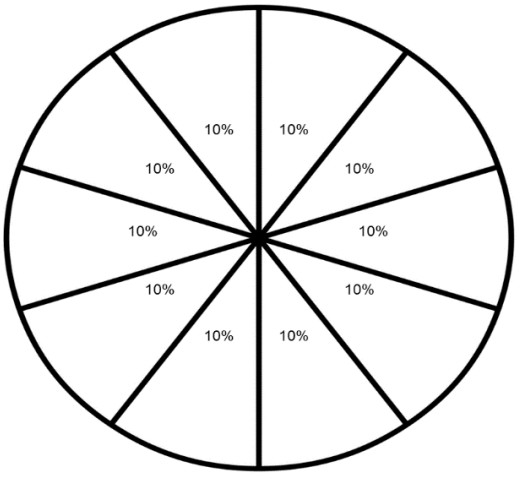


**Figure 2.** Pie-graph for the estimation of percentage gametophyte survival through growth coverage

## 2.12 Statistical analysis

The data gathered from the gametophyte growth coverage was analyzed using analysis of variance (ANOVA) and means were compared using Tukey's test at a 5% significance level.

### 2.13 Documentation

A Nikon D7500 DSLR camera with 18-140 mm was utilized to take photographs of the morphological developments, gametophyte growth coverage and processes involved in the study.

## 3. Results and Discussion

### 3.1 Spore morphology of the species

Spores of *P. irregularis* matched the description of Holttum (1974), certifying that the spores are monolete-bilateral and ellipsoidal. The spores are plano-convex in equatorial view, notably with an irregularly reticulated perispore and winged folds at the margins (Figure 3). Such spore characteristics were remarkable for tectarioid ferns.

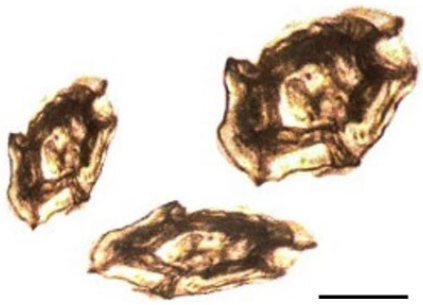

**Figure 3.** Spore morphology of *P. irregularis*. Scale bar: 50 µm

### 3.2 Spore germination

#### 3.2.1 Imbibition and swelling of the spores

Change in the original spore size and shape was first observed a few days after the spores were sown. This was due to imbibition of water where the spores got swollen as their volume increased. This noticeable swelling of the spores occurred during the third day after sowing, with a prolific imbibition of spores sown in the control group, T0, the medium which was composed only of garden soil. Among all the media, spores sown in T2 (ground clay) displayed a sluggish imbibition performance; the spores took 4-5 days to swell.

#### 3.2.2 Emergence of rhizoid

Considered the next stage of early development, the emergence of primary rhizoids indicated that spores sown successfully germinated. The species showed a *Vittaria*- type of spore germination. This pattern of spore germination is commonly observed among the tectariod ferns to which *P. irregularis* belongs (Kaur & Devi, 1976).

The primary rhizoid elongated during germination, and through a series of divisions, the uniseriate germ filament lengthened in the equatorial plane upwards (Figure 4C). The emergence of rhizoid was first observed simultaneously in all media at 6-7 days

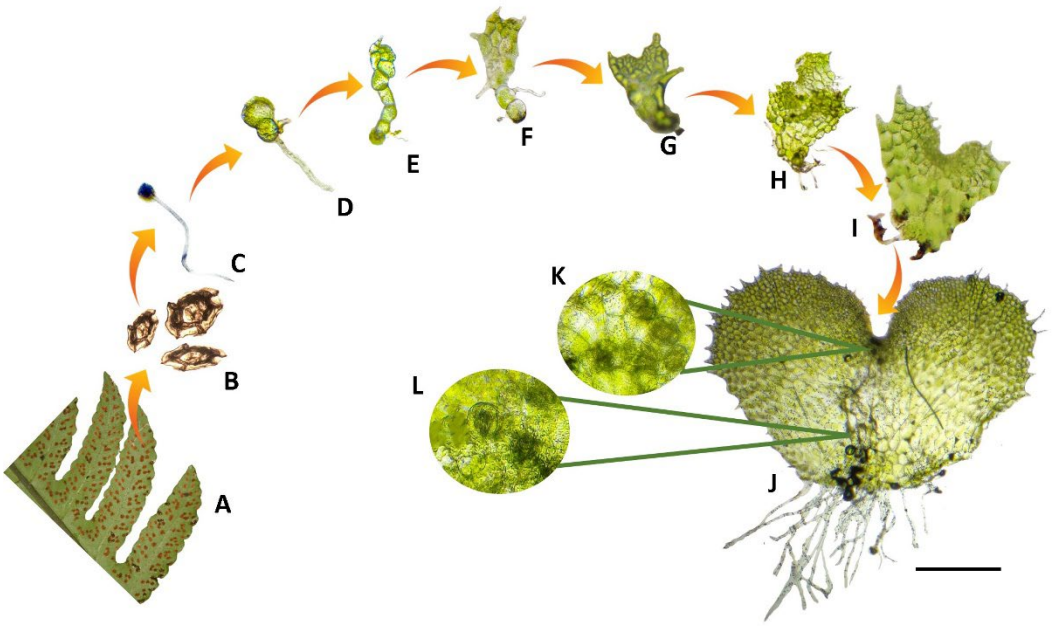


**Figure 4.** Spore germination and gametophyte development of *P. irregularis.* (A) fertile fronds of *P. irregularis*, (B) spores, (C) emergence of rhizoids, (D) formation of germ filaments, (E) 10-celled germ filaments, (F) early spatulate stage, (G) late spatulate stage, (H) lopsided prothallial plate, (I) asymmetrical cordate, (J) symmetrical cordate, (K) archegonia, (L) antheridia. Scale bar: (A) 8 cm; (B) 50 µm; (C-J) 100 µm; (K-L) 30 µm.

after sowing (DAS), except for the spores sown in T2, which developed rhizoids at 8 days after sowing. This successful germination rate achieved within one week was consistent with the findings reported by Guo & Liu, 2014.

## 3.3 Prothallial development

### 3.3.1 Germ filament formation

After the emergence of the rhizoids, a continuous division at the anterior end of the spore gives rise to a uniseriate germ filament composed of densely chlorophyllous cells with hairs at terminal ends (Figure 4D). *Pleocnemia irregularis* follows *Aspidium*-type of prothallial development, which is distinctively characterized by the margin of variability due to the protuberance of unicellular papillate hairs crowning at the terminal end (Nayar & Kaur, 1971).

This developmental stage arose the earliest in T3 (ground adventitious roots of tree fern + ground clay pots) and T0, at 9 DAS, and developed continuously into an average of 3-7-cells after 11 days. On the other hand, spores sown in T2 (ground clay pots) delayed the occurrence of the germ filaments compared to the other media.

### 3.3.2 Spatulate prothallium plate formation

In the next stage, a visibly green substrate surface became prolific as early as the second week after sowing. Following *Aspidium*-type prothallial development, the daughter cells divided longitudinally and transversely, producing a broad spatulate prothallium plate (Figures 4F-G).

This gametophyte development was first observed in T3 (ground adventitious fern + ground clay pots) and T0 (garden soil) 14 days after sowing. Prolific broad spatulate formation peaked at 21 DAS.

### 3.3.3 Lopsided prothallus formation

In this stage, active meristematic activity along the lateral position of the plate led to a gradual cell differentiation of the young asymmetrical prothallium plate. As the formation proceeded, the young thalli became lopsided, with one wing being larger than the other and persisting for a few days until meristematic activity formed near the apex. Marginal protuberance, such as the unicellular club-shaped hairs, was retained and became prolific as the prothallial stage proceeded (Figure 4H).

This stage of gametophyte development was first observed in T0 (garden soil) and T3 (ground adventitious roots of tree fern + ground clay pots) at 22 DAS, while the stage developed late in T1 and T2 at 25 and 29 DAS, respectively. Among all media, T0 had the most observable lopsided prothallus formation.

### 3.3.4 Cordate prothallus formation

Once the lopsided prothalli have differentiated for several days, further elongation results in the meristem being nearly apical and, eventually, the gradual development of symmetrically cordate prothallus. The cordate prothallus has a broad wing, notably with marginal unicellular club-shaped hairs. In addition, the thalli have a more pronounced notch at the anterior end and a distinct midrib, which bears a nonchlorophyllous rhizoid at the lower surface (Figures 4I-J).

This visibly green, heart-shaped structure was observed on the substrate surfaces of gametophyte in all modified media from the fourth to the fifth week after sowing. The earliest cordate gametophyte, which appeared 32 days later, was observed in all media.

Across the prothallium formations, T0 consistently produced the greatest number of observable cordate formations followed by T3, T1, with the least being seen in T1.

## 3.4 Gametangia

Hermaphrodite gametangia was observed and developed at different times. Antheridia, composed of small and irregular globoid cells appeared as early as 24 DAS, during the late lopsided prothallial stage, whereas the elongated and curved neck cells of archegonia occurred at 27 DAS. These sex organs developed in separate locations. The antheridia were located on the midrib region and in particular on the posterior part of the thallus while the archegonia occupied the anterior part near the apical notch (Figures 4K-L).

### 3.5 Percentage of gametophyte survival

At the end of the study, the gametophyte survival of each sample in different modified culture media (Figure 5) was assessed to determine which modified media performed better and yielded the highest number of gametophytes. The percentage of gametophyte survival was calculated by estimating growth coverage in the media (Table 1).

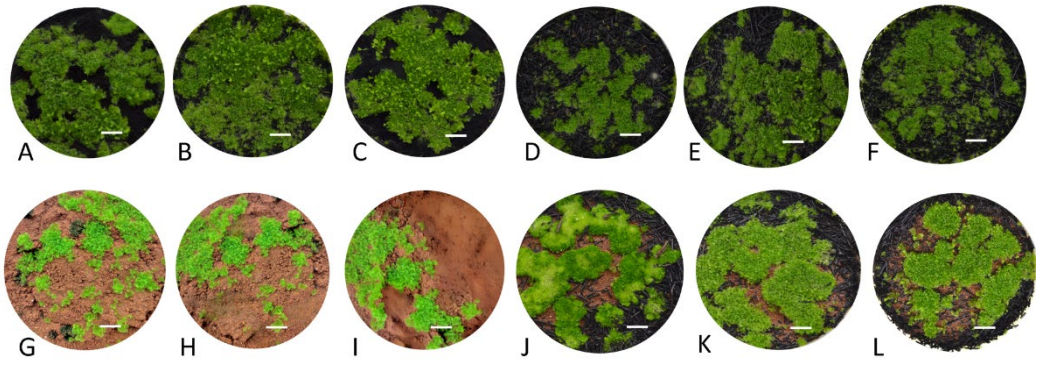


**Figure 5.** Gametophyte formation (based on growth coverage) of *P. irregularis* sown on modified media per replicate. (A-C) T0= Garden Soil, (D-F) T1= Pure ground adventitious roots of tree fern, (G-I) T2= Ground clay pots, (J-L) T3= Ground adventitious roots of tree fern + Ground clay pots. Scale bar: 10 cm.

**Table 1.** Percentage of gametophyte survival through growth coverage

| Treatment | Rep. 1 | Rep. 2 | Rep. 3 | Mean* |
|---|---|---|---|---|
| T0 (Garden soil) | 95 | 97 | 90 | 94.00[a] |
| T1 (Pure ground adventitious roots of tree fern) | 82 | 60 | 95 | 79.00[ab] |
| T2 (Ground clay pots) | 45 | 25 | 75 | 48.33[b] |
| T3 (Ground adventitious roots of tree fern + Ground clay pots) | 80 | 86 | 88 | 84.67[ab] |

*Means of different superscripts are statistically significant at a 0.05 level.

Among the modified culture media used, T0 yielded the highest average survival rate, which was 94%. This suggests that the garden soil was the most conducive substrate for gametophyte survival, making it an optimal choice for gametophyte cultivation. This was followed by T3, which had the second-highest mean survival rate of 84.67%. However, the survival rate dropped to 79% for treatment T1, which comprised purely ground roots of tree fern. Moreover, T2 composed of pure ground clay pots, displayed the lowest survival rate at an average of 48.33%.

Ferns have been naturally found growing in soils with varying organic matter content, ranging from rich humus and mineral-based substrates to xeric environments (Mehltreter et al., 2010). While the specific chemical and physical properties of the garden soil used in the study were not tested, the superior results observed with garden soil as a growing medium may be due to its near-neutral pH, moderate EC, nutrient availability

(Hoshizaki & Moran, 2001). Previous studies have concluded that these characteristics were often found in garden soils making it a suitable candidate for fern spore germination and gametophyte development (Ko, 2003; Cullina, 2008; Guo & Liu, 2014).

The mixtures of ground roots and ground clay pots (T3) did not significantly differ from the pure ground roots (T1), further confirming the potential beneficial effects of combining these two components. The adventitious roots of tree ferns are known to be rich in organic matter and can provide a nutrient-rich substrate when ground and used as a growing medium (Mishra & Behera, 2020). The combination of organic matter from ground adventitious roots and the mineral content and aeration provided by ground clay pots can create a balanced substrate for gametophyte development (de Winter & Amoroso, 2003).

While clay can provide good aeration and cation exchange capacity, the absence of sufficient organic matter may limit nutrient availability (Winch, 2007), resulting in the lowest gametophyte coverage. Furthermore, studies using spores of leptosporangiate ferns, where *P. irregularis* belongs, have displayed favorable germination rates at a slightly acidic to neutral pH (Miler, 1968; Fernández & Revilla, 2003). Pure clay substrates typically have a pH range of 7.5-10, which could influence the availability of essential nutrients for the gametophytes.

## 4. Conclusions

The study successfully documented the morphological characters and developmental stages of the economically important fern *Pleocnemia irregularis*, filling a gap in the literature on its developmental biology. Overall, garden soil (T0) proved to be the best culture medium for spore germination and gametophyte coverage of *P. irregularis*. The use of accessible and cost-effective growing media facilitated the successful study of the developmental biology of this economically important yet often overlooked pteridophyte species. The findings of this study provide valuable information to support further research on the developmental biology of *P. irregularis* and related ferns.

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## 6. Conflicts of Interest

The authors declare that there is no conflict of interest.

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