Fundación Miguel Lillo
Tucumán
Argentina

In vitro development of gametophyte and sporophyte in the epiphytic fern *Phlebodium aureum* (Polypodiaceae)

Ontogenia in vitro del gametofito y esporofito del helecho epífito *Phlebodium aureum* (Polypodiaceae)

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Abstract

This paper presents the gametophyte development pattern of *Phlebodium aureum*, examined using light and scanning electron microscopy, covering spore germination, gametophyte development, and reproductive behaviour. Spores are homosporous and monolete, with a straight laesura. The percentage of spore germination is 43.33 ± 21.47%, following the *Vittaria*-type pattern. Prothallial development is *Drynaria*-type, yielding a laminar gametophyte within 21 days. The mature prothallus is broad, cordate-reniform, and develops by day 56. Antheridial initiation occurs at day 42, followed by archegonial formation at day 49 below the apical notch. Bisexual gametophytes appear by day 63. Sporophytes emerge in both composite populations and the isolated population within 90-120 days through intergametophytic as well as intragametophytic mating. A total of 80% of gametophytes in the composite population produced sporophytes, indicating that intergametophytic mating is predominant. A mixed mating system, low genetic load, and a perennial, clone-forming gametophyte growth habit interact to create effective breeding strategies for this epiphytic species.

Keywords: Epiphyte; genetic load; invasive; mixed mating type; prothallus.

➤ Ref. bibliográfica: Bhatia, M.; Kumari, P.; Yadav, A.; Uniyal, P. L. 2026. In vitro development of gametophyte and sporophyte in the epiphytic fern *Phlebodium aureum* (Polypodiaceae). *Lilloa 63* (1): 213-226. doi: https://doi.org/10.30550/j.lil/2346
➤ Recibido: 4 de febrero 2026 – Aceptado: 20 de mayo 2026 – Publicado: 7 de junio 2026.
➤ URL de la revista: http://lilloa.lillo.org.ar

Resumen

Este artículo presenta el patrón de desarrollo del gametofito de *Phlebodium aureum*, examinado mediante microscopía óptica y electrónica de barrido, abarcando la germinación de las esporas, el desarrollo del gametofito y el comportamiento reproductivo. Las esporas son homospóricas y monoletes, con una laesura recta. El porcentaje de germinación de las esporas es del 43,33 ± 21,47%, siguiendo el patrón de tipo *Vittaria*. El desarrollo protálico es de tipo *Drynaria*, dando lugar a un gametofito laminar en 21 días. El prótalo maduro es ancho, cordado-reniforme, y se desarrolla en 56 días. La iniciación de los anteridios ocurre a los 42 días, seguida por la formación de los arquegonios a los 49 días, por debajo de la escotadura apical. Los gametofitos bisexuales aparecen a los 63 días. Los esporofitos emergen tanto en las poblaciones compuestas como en la población aislada entre los 90 y 120 días, mediante apareamiento tanto intergametofítico como intragametofítico. El 80% de los gametofitos en la población compuesta produjeron esporofitos, lo que indica que el apareamiento intergametofítico es predominante. Un sistema de apareamiento mixto, una baja carga genética y un hábito de crecimiento del gametofito perenne y formador de clones interactúan para generar estrategias reproductivas eficaces para esta especie epífita.

Palabras clave: Carga genética; epífita; invasora; protalo; tipo de apareamiento mixto.

INTRODUCTION

Phlebodium aureum (L.) J. Smith (Polypodiaceae) is commonly referred to as the Golden Polypody or Goldfoot Fern, Blue Star Fern, Cabbage Palm Fern, Golden Polypody, Gold Foot Fern, Hare Foot Fern, Rabbits Foot Fern; and is a perennial herbaceous epiphytic fern.

Phlebodium aureum exhibits diversity in its growth forms, ranging from epiphytes, lithophytes, and terrestrial. It has a creeping rhizome 8–15 mm (rarely 30 mm) wide, covered with dense golden-brown scales. The leaves are large and pinnately compound (deeply lobed) with undulate margins; pinnae may be up to 35 in number. Sori is circular and present on both sides of the pinna midrib. It generally proliferates through the rhizome. Plants are adapted to various warm-temperate, subtropical, and tropical climates. It is native to tropical and subtropical regions of America, including the Caribbean, the southeastern United States, and parts of Central and South America. It has become an invasive species in some parts of South Africa. It is listed under the Alien and Invasive Species regulations of the National Environmental Management: Biodiversity Act (10/2004) (Jones *et al.*, 2020). In India, it is a common epiphytic species in the Western Ghats, especially in Tamil Nadu (Krishnan & Rekha, 2021), and is infrequently found in the Galikonda Hills, Andhra Pradesh, of the Eastern Ghats (Malati *et al*., 2022).

The plant is valued for its ornamental foliage, often with blue-green fronds, and is also used in herbal medicine. Traditionally, it is used to treat inflammations and some tumours by ethnic groups in Central America. In Mexico, the rhizome is used to treat cough and fever and is sudorific (Kumari *et al*., 2011; Singh, 1999). Dry rhizomes are used as phytocosmetic and Phytomedicines and are commercially sold in the domestic markets of Honduras and Guatemala (Cáceres & Cruz, 2019). Roots are also used for wound healing. Rhizomes and leaf extracts have been used to cure skin ailments in Europe and South America (Segars *et al*., 2021; Parrado *et al*., 2021). Castillo *et al*. (2023) reported that extracts of *P. aureum* show significant potential as an anti-vitiligo agent. Recently, it has been reported that silver nanoparticles synthesised from an aqueous extract of *P. aureum* exhibit promising anticancer activity (Marimuthu *et al*., 2021).

Ferns exhibit a remarkable alternation of generations as gametophyte and sporophyte, which differ in both form and function (Banks, 1999). The gametophytes are minute, haploid, one-celled in thickness, and free-living. These advantages make it a great tool for performing ontology, cytogenetic, morphological, ecological and physiological studies in vitro as well as in vivo. Their significant role in sustaining fern biodiversity, distribution patterns and limitations has been well established in many studies (Krieg & Chambers, 2022). Limited reports are available on the development of gametophytes and sporophytes of the *P. aureum*. The present study is focused on the ontogeny and reproductive behaviour of the *P.aureum* to evaluate the strategies that ensure its survival.

MATERIALS AND METHODS

Collection

Spores of *P. aureum* were collected from the Malabar Botanical Garden and Research Institute, Calicut, Kerala, in October 2015. Mature sporophylls were kept in brown paper packets and stored in a desiccator for the release of the spores.

Culture

The spores were surface sterilized with a 2% sodium hypochlorite solution for two minutes and rinsed three times with double-distilled water. Spores were sown on sterile Parker's macro and Thompson's micronutrient (P & T) culture medium solidified with 1% agar in Petri plates. The pH of the medium was maintained at 5.8. Cultures were kept in a culture room under controlled conditions of light intensity ranging between 47.3–56.8 μmol m^{-2} sec^{-1} at 23 ± 2ºC for a 16-hour light photoperiod followed by an 8-hour dark photoperiod (Klekowski, 1969).

Light Microscopy

Regular observations were made under an Olympus CX21 microscope and photographed using the Olympus camera EP-1 to document per cent spore germination, gametophyte growth, and gametangial ontogeny.

Scanning Electron Microscopy

Oven-dried spores were mounted on an aluminum stub using sticky tape and placed in a Sputter coater (JFC-1600 autofine coater, JEOL Japan) for gold palladium coating. Scanning was done under scanning electron microscope (JSM-6610LV-JEOL, Japan) at the University of Delhi.

Gametophytes were fixed in 2.5% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.2 and then dehydrated in a graded series of alcohol (80%, 95%, 100%). Subsequently subjected to critical point dryer using Polaron critical Point dryer (D-09-R-711/A) and mounted and coated with gold and palladium as done with spores.

Genetic Load

Cordate gametophytes were transferred to separate Petri plates containing P & T medium before the initiation of gametangia to study genetic load. The ratios of gametophyte-bearing male, female, bisexual, or neuter conditions were recorded.

Set 1: 25 Petri plates with a single gametophyte in each (isolate culture).

Set 2: 10 Petri plates with 25 gametophytes in each (composite culture).

After the initiation of gametangia in stock cultures, watering of all isolated and composite populations was done from above with sterile distilled water twice a week to facilitate fertilization. The percentage of sporophytes was recorded in both of the above-mentioned sets.

RESULTS

After inoculation of the spores on five Petri dishes (A1-A5), observations revealed that the spores adhered to the culture media and began germination.

Spore germination

The sporangium wall consisted of 28 annular cells (Fig. 1A). The spores were bilateral, ellipsoid, monolete, and golden in colour (Fig. 1B; Fig. 2A). The spore wall was double-layered, outer perispore and inner exospore. The perispore was dense and single-layered. The exospore showed two layers, the outer layer smooth and the inner layer verrucate to tuberculate.

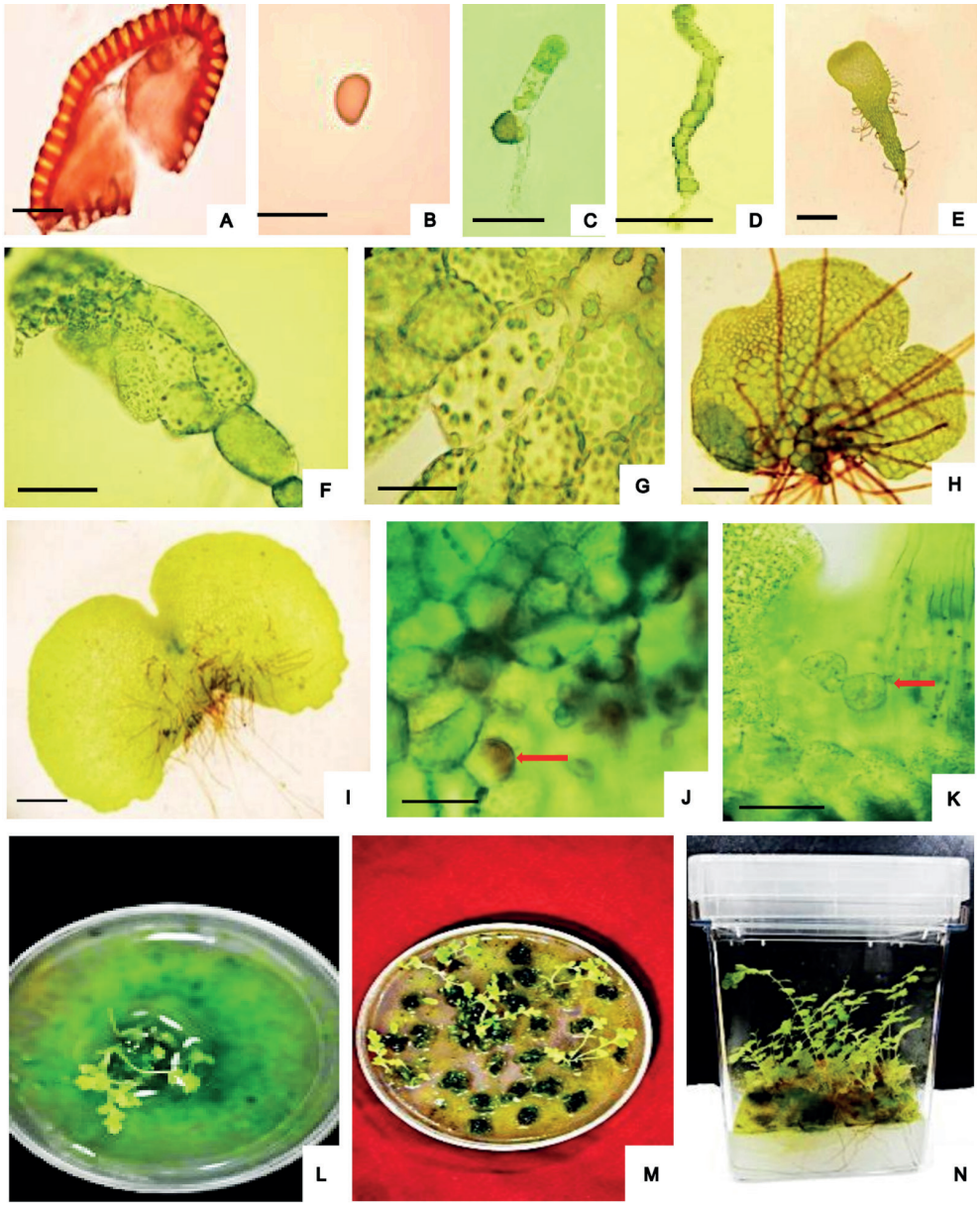

Fig. 1. Developmental stages of *Phlebodium aureum*. A) Sporangium showing annulus. B) Spore. C) First Prothallial cell and rhizoidal cell. D) Filamentous Prothallus. E) Laminar Prothallus. F) Spathulate prothallus with terminal Trichome. G) Apical notch. H) Formation of apical notch. I) Mature Bisexual Cordate Prothallus. J) Antheridia. K) Archegonia. L) Isolated population with sporophyte. M) Composite population with sporophyte. N) Sporophyte. Scale Bar = 1mm.

Fig. 1. Etapas de desarrollo de *Phlebodium aureum*. A) Esporangio que muestra el anillo. B) Espora. C) Primera célula protalial y célula rizoidal; D) Protalo filamentoso. E) Protalo laminar. F) Protalo espatulado con tricoma terminal. G) Muesca apical. H) Formación de la muesca apical. I) Protalo cordado bisexual maduro. J) Anteridios. K) Arquegonios. L) Población aislada con esporofito. M) Población compuesta con esporofito. N) Esporofito. Barra de escala = 1 mm

The average size of the spores was 60.219 × 37.392 μm (Fig. 2B). The laesura was short and straight, extending over half the length of the equatorial axis.

After analysing the spore germination in five Petri dishes (A1-A5) through 10 focuses (for each Petri dish), the average percentage of germination observed was 43.33 ± 21.47% in the first week after sowing. This reached 73.61 ± 10.92% in the second week. The germination pattern exhibited was of the *Vittaria* type, with deformed globules in the first prothallial cells. Spores possessed many oil globules before germination, which persisted in the filament stage. Rhizoids appeared within the first 3-5 days after sowing. The second division in the basal cell resulted in a protonemal cell with abundant chloroplasts. The rhizoid was long and clear, containing a large number of protoplasts.

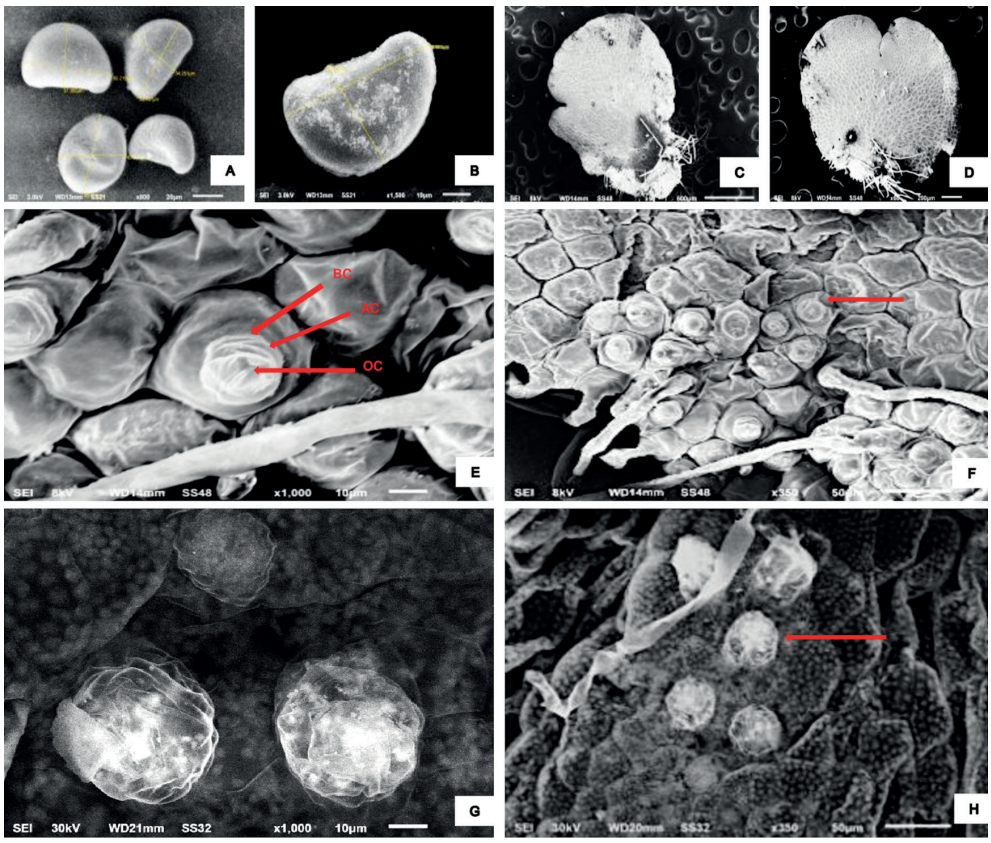

Fig 2. SEM of developmental stages of *Phlebodium aureum*. A) Spore. B) Spore in equatorial view. C) Spathulate prothallus. D) Cordate gametophyte showing development of apical notch. E) Single antheridium showing opercular cell (OC), antheridial cell (AC), and basal cell (BC). F) Antheridia group. G) Single archegonium. H) Archegonia group.

Fig. 2. Micrografías MEB de las etapas de desarrollo de *Phlebodium aureum*. A) Espora. B) Espora en vista ecuatorial. C) Protalo espatulado. D) Gametofito cordiforme que muestra el desarrollo de la escotadura apical. E) Anteridio individual que muestra la célula opercular (OC), la célula anteridial (AC) y la célula basal (BC). F) Grupo de anteridios. G) Arquegonio individual. H) Grupo de arquegonios.

Subsequent divisions in the protonemal cell produced a filament in 15 days. The filament was generally 2-6 cells long and had numerous chloroplasts and oil globules dispersed in the cytosol of each cell of the filament. The spore coat was persistent and remained adhered to the basal cell (Fig. 1C). One to three rhizoids were present. Rhizoids also developed from cells other than the terminal cells of the filament.

Prothallial development

The pattern of prothallus growth observed was of the Drynaria type. Generally, the apical cell and intermediary cell of the filament underwent a longitudinal division, resulting in a laminar gametophyte within 21 days (Fig. 1D). Further divisions in the filamentous prothallus resulted in a broad, spathulate prothallus in 28 days (Fig 1E). A terminal trichome may be present (Fig. 1F).

At the anterior margin, an obconical meristematic cell was divided by two oblique cuts at an early stage of the spathulate prothallus, which was soon replaced by the development of pluricellular meristematic tissue. The cells beneath the notch were elongated and wider, showing sparsely distributed chloroplasts (Fig. 1G). Within 40 days, a symmetric cordate thallus emerged. The young prothalli were strap-shaped, and the thallus broadened with the development of a cordate apex. In some spathulate prothalli, the meristem appeared laterally (Fig. 1H, Fig. 2C). Later, the wings became broader and developed a central cushion, leading to a cordate-shaped prothallus (Fig 2D). Rhizoids also formed on the wings as well as on both the ventral and dorsal sides of the midrib.

Mature gametophyte

The mature prothallus was cordate-reniform, thalloid, and generally broader than long (Fig. 1I). The thallus took 56 days to mature. The thalli were small, with an inconspicuous midrib and wide wings. Marginal cells in the cordate gametophyte remained small and symmetric, while the medial cells were comparatively large. Numerous hyaline rhizoids arose from the ventral side of the gametophyte. Later, both dorsal and ventral surfaces developed rhizoids along the midrib, as well as on the wings and margin, but fewer on the ventral side. The thallus lacked multicellular hairs. Fully grown gametophytes developed a few proliferations or branches similar to the prothallus, which later developed a pluricellular meristem. Multiple branching resulted in a mat of superimposed thalli. These branches are like gemmae and later produce gametes. This clonal growth led to a persistent gametophyte that continued its growth for over two years and produced multiple sporophytes.

Gametangia initiation

Antheridia appeared first at 42 days. They showed wide variation in their distribution. Generally, antheridia were present in groups intermixed with rhizoids at the basal portion of the gametophyte (Fig. 2F), or sometimes near the apical notch before midrib formation, or they may intermingle with archegonia in older prothalli on the ventral surface of the gametophyte. The antheridia were small, subglobose to hemispherical (Fig. 1J).

Each antheridium consisted of 3 cells: a flat to concave-shaped basal cell (BC) at the bottom, a middle ring cell (an annular cell, AC) containing the antheridial cavity, and an opercular cell (OC) at the top (Fig. 2E). They dehisced by the breakdown of the cap cell. Upon receiving moisture or a water drop, the opercular cap detached from the antheridium, resulting in the profuse release of multi-flagellate antherozoids. In the present study, male gametophytes were predominant in the culture.

Archegonia appeared in 49 days, just beneath the apical notch. Several one-celled, papillate, and secretory hairs also developed along with archegonia. The neck of the archegonia was small, slender, and bent away from the apical notch. The neck had four tiers of 5-7 cells (Fig. 1K). The neck canal cell was binucleate and inflated at its tip at maturity (Fig. 2H). Bisexual gametophytes started appearing 63 days after sowing. After 3 months, bisexual gametophytes became predominant. The details of sex ontogeny are given in Table 1.

Before mating, the neck of the archegonia became distinct with a slight expansion of the apical 4 cells forming the archegonial opening (Fig. 2G). During observations of bisexual gametophytes, it was noted that antherozoids were rapidly swimming, and some of them reached the archegonial openings. Despite multiple archegonia maturing simultaneously, only one sporophyte developed per gametophyte. Older gametophytes showed proliferation of the thalli, resulting in clone formation in the gametophyte, but no apogamous sporophyte was observed in composite population.

Sporophyte

After fertilization, the prothallus formed sporophytes (Fig. 1L, Fig. 1M). Sporophytes appeared 90 days after sowing in the composite population and 120 days in the isolated population. In the composite population, 80% of gametophytes produced sporophytes, while in the isolated population, only 20% did (Table 2).

A comparative study on the sporophyte production from 25 isolated gametophyte cultures revealed that the percentage of sporophyte production in isolated gametophyte cultures was very low. Out of the 25 isolated gametophyte cultures, only 5 gametophytes developed sporophytes, indicating that isolated gametophytes exhibiting a homozygous genotype have a 20% reproductive success rate and an 80% genetic load (Table 2).

Table 1. Chronological changes in the sex ratio of a composite culture in *Phlebodium aureum*.

Tabla 1. Cambios cronológicos en la proporción de sexos de un cultivo mixto de *Phlebodium aureum*.

Sample size	Days after sowing	Male	Female	Neuter	Bisexual
20	42	1	0	19	0
20	49	9	2	9	0
20	56	11	1	6	0
20	63	10	0	6	4
20	70	6	2	0	10
20	77	3	2	1	14
20	83	1	0	2	17
20	90	1	0	0	20

Table 2. Breeding behavior of different populations of *Phlebodium aureum.*

Tabla 2. Comportamiento reproductivo de diferentes poblaciones de *Phlebodium aureum.*

Sample size	Number of gametophytes observed	Number of sporophytes produced	% of sporophyte produced
Isolation	25	5	20
Composite	75	60	80

The first leaf appeared in 145 days after sowing. The first leaf was small, simple and lanceolate with a dichotomous venation system.

Later, it was dissected to form other pinnae (Fig. 1L). Leaves bear one-celled, small, capitate trichomes on the surface and along the margin. There was no sporophyte development in unwatered cultures, suggesting a sexual breeding system in this species.

Hardening, acclimatization and relocation in nature

Fully developed sporophytes (Fig 1N) were then transferred into pots filled with a mixture of soilrite, garden soil, and moss in a ratio of 1:1:1. They were watered and covered with polythene sheets. The pots were kept under controlled conditions in the greenhouse for hardening and acclimatization to natural conditions.

DISCUSSION

Spores are monolete, ellipsoid, and golden yellow, containing numerous fat globules, a typical characteristic of Polypodiaceae. Spore germination occurs within 3 to 5 days of sowing. Perez *et al.* (1998) reported germination taking 7 to 15 days in three species of *Phlebodium*. The exhibited germination pattern is *Vittaria-type* documented by Nayar & Kaur (1971). Deformed oil globules persisted during the filament stage. Some abnormally elongated filaments are observed in this study, which Nayar & Kaur (1971) also reported in *Tectaria*

polymorpha. A laminar gametophyte is produced in 21 days, a spathulate prothallus in 28 days, resulting in a symmetric cordate thallus in 40 days. The young prothalli are strap-shaped and become broad cordate with the development of an apical notch. A terminal hair may be present in some prothalli. Many studies have addressed the morphology of gametophytes (Nayar & Kaur, 1971; Chiou & Farrar, 1997; Perez *et al.*, 1998; Davíe, 1951; Hartman, 1931; Dassler & Farrar, 1997).

The pattern of gametangia initiation follows the formation of antheridia before archegonia. Ward (1954) reported a similar sequence of gametangia initiation, noting a protandrous condition that favours cross-fertilisation in a multispore culture. Klekowski (1969) stated that *P. aureum* exhibits the type 'D' sequence of gamete development, in which antheridia develop first, followed by a hermaphroditic phase, then an archegoniate stage, ultimately leading to hermaphroditism again. However, Chiou & Farrar (1997) reported that archegonia appeared first, and female gametophytes predominated in cultures that were 2-3 months old. Mature cordate gametophytes observed from composite cultures show a predominant bisexual tendency three months after sowing.

Leaf emerged in 120 days. Perez *et al.* (1998) reported the emergence of the first sporophytic leaf in 150-400 days in three different species of *Phlebodium*. Van Cotthem (1973) reported an anomocytic type of stomata on the abaxial surface.

The breeding test data for this species in the current study support the idea that the intergametophytic mating system is favoured. The observed gametangial sequence shows the emergence of antheridia followed by archegonia after one week, and later, all gametophytes became bisexual by 90 days. The interval between the emergence of both sexes is considered insignificant; within the next 40 days, all prothalli reached the bisexual stage, allowing ample time for both intergametophytic and intragametophytic mating. Therefore, a mixed mating system is favoured for this species. However, the intergametophytic mating type is predominant, as evidenced by the production of 80% sporophytes in a mixed population. *P. aureum* consists of diploids (n = 37) and tetraploids (n = 74; Evans, 1963). Polyploid plants of *P. aureum* favour outcrossing, while diploid plants favour inbreeding (Chiou *et al.*, 2002). The genetic load in the species is low, and no expression of the recessive lethal is seen, as no abortive sporophytes are observed in the study.

Sessa *et al.* (2016) reported that several fern species can produce both male and female gametangia, resulting in sporophyte development through gametophytic selfing in each prothallus. However, naturally occurring populations exhibit heterozygous sporophytes developed through crossing between the gametophytes (Ranker & Geiger 2008). Haufler *et al.* (2016) explored potential connections between gametophyte persistence and gametophytic mating systems.

Generally, intergametophytic mating is prevalent in homosporous ferns, with genetic load being the key controlling factor in determining the breeding system (Haufler & Welling, 1994). Dassler & Farrar (1997) reported that spore dispersal in this species is effectively separated, resulting in less interaction between neighboring gametophytes due to its epiphytic habit, which determines the species' natural distribution. Jones *et al.* (2019) conducted a global assessment of the invasiveness of ferns and identified 11 species of concern, including *Phlebodium aureum*.

The specialised branching morphology of epiphytic gametophytes serves as a critical adaptation for survival and reproduction in harsh canopy environments. By enabling sustained clonal growth and extended lifespans, it enhances the probability of outcrossing between distant individuals over time (Dassler & Farrar, 2001; Watkins & Cardelús, 2012). Furthermore, research shows that epiphytic ferns in subtropical and temperate montane ecosystems, such as the Himalayas, have evolved to occupy a wide variety of host trees while enduring diverse climatic regimes (Bhakuni *et al.*, 2021; Joshi *et al.*, 2020). Temperature and rainfall are critical to the establishment of the gametophyte. The three-dimensional architecture creates moisture-retaining crevices that slow desiccation, granting the epiphytic ferns superior drought tolerance compared to their terrestrial, heart-shaped counterparts (Watkins *et al.*, 2007).

Nevertheless, *P. aureum* can switch its habitat to epiphytic, epilithic, or terrestrial and has adapted to diverse climatic conditions, ranging from warm-temperate to subtropical and tropical (Randal, 2017). Additionally, highly dispersible spores, clonal growth in older gametophytes, and drought tolerance make the species robust and persistent, enhancing its opportunities to interact with later-established prothalli and facilitating outcrossing, which serves as an advantage to the survival strategies of this fern, leading to it becoming invasive in some parts of the world.

CONCLUSION

P. aureum is a perennial herbaceous epiphyte known for its low spore germination rate. Distant spore dispersal due to its epiphytic nature and low spore germination rate is the major constraint in its reproductive biology. The plants successfully proliferate in various habitats, ranging from epiphytic and epilithic to terrestrial environments and are well adapted to both tropical and temperate conditions. A mixed mating system, low genetic load, and a perennial, clone-forming gametophyte growth habit interact to create effective breeding strategies for this epiphytic species and facilitate its inhabitation to new sites.

ACKNOWLEDGEMENT

The Authors thank the University of Delhi, Delhi, for providing financial assistance through an R&D Grant (No. RC/2015/9677).

CONFLICTS OF INTEREST

The authors declared that they have no conflict of interest.

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