

***In vitro* Studies on Gametophyte Development and Gametangial Ontogeny of *Athyrium falcatum* Bedd. (Woodsiaceae)**

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ABSTRACT

Athyrium falcatum Bedd. is an economically important fern that occur in different ecological niche. In the present work spores of *Athyrium falcatum* were cultured in P & T medium and 77.5% spore germination was recorded within a week of sowing. The viability of spores was lost in seven months, when kept in room temperature. Prothallial development was found to be 'Drynaria Type'. Antheridia appeared first after 85 days of spore sowing, archegonia were initiated after 95 days and bisexual stage appeared after 110 days of sowing. Fern habitats are under continuous threat due to anthropogenic factors and developmental activities which ultimately decrease the forest cover and fern vegetation. In vitro culture creates a new path for mass cultivation of ferns and will be very helpful for their restoration and conservation. The main aim of the present investigation was to record the peculiarities of the pattern of spore germination, sex ontogeny and gametophyte development in this terrestrial taxon.

Keywords: Conservation, Gametophyte development, Gametangial ontogeny, Prothallial development, Restoration, Spore germination.

Mathematics Subject Classification: 92C15, 92C80

Journal of Economic Literature (JEL) Classification: Q56

1. INTRODUCTION

Pteridophytes have proved to be phytoremediators, bio-indicators for pollution and bio-fertilizers, besides having an excellent ornamental potential. Ferns have high resilience and can maintain the productivity during perturbation as they have evolved a wide range of extraordinary ecological strategies like extensive underground rhizome, large leaves and their tolerance to desiccation. Ferns are now well recognized for landscaping, restoration of habitat, nursery material and medicinal purposes (Sharma, 2020) but much of the population is destroyed due to different developmental

activities. To fulfil the demand of plant resources for commercial and restoration activities, *in vitro* culture method creates new path in fern conservation (Srivastava, 2007).

During the development gametophyte undergoes dramatic changes in morphology including changing tissue infrastructure of newly differentiated cells. Different morphological stages like filamentous, spatulate and heart shaped stages can be seen in gametophyte development of ferns which follows a developmental progression to accumulate more and more photosynthates so as to prepare for the reproductive phase and to develop sporophyte. Gametophytes easily grow in the culture medium and the effect of the medium, growth regulators and culture conditions on spore germination and development of gametophyte can be easily recorded (Shukla, 2012). In this study we focused on the micropropagation of *Athyrium falcatum* Bedd. In this plant the fronds arise from black-scaly and erect rhizomes and it is commonly grown for the purpose of decoration. It is a large, feathery species of fern which is native throughout most of northern hemisphere and found in damp, shady woodlands. It is the commonly used medicinal fern in the Pacific region, smoked for colds, and used as an expectorant (Kachhiyapatel, 2019). Spore germination using tissue culture method permits to produce large population of gametophytes, where we can observe the developmental patterns and study the effect of growth regulators. The present study deals with the spore germination, gametophyte growth and differentiation and sex ontogeny of *Athyrium falcatum*.

2. MATERIALS AND METHODS

COLLECTION OF FERN SPECIMEN

Mature fertile fronds with ripen spores of *Athyrium falcatum* were collected in polythene bags from Mussoorie, Uttarakhand. The fronds were stored in brown spore packets in desiccators containing silica gel at room temperature.

STERILIZATION AND INOCULATION OF SPORES

The spores were kept in 2% sodium hypochlorite solution in water for two minutes. Spore sowing was done in the petriplates containing Parker's macro and Thompson's micronutrient culture medium (Klekowski et al. 1969).

SCANNING ELECTRON MICROSCOPE

Spore morphology was studied through Electron Microscope. Spores were dried and put on the stubs with the help of sticky tape and then placed in Sputter Coater (JFC-1600 Auto Coater, JEOL Japan) to scan at different magnifications.

3. RESULTS

Spore Germination

The spores of *Athyrium falcatum* were pale brown (Fig. 1 A, B) and $41.563 \times 29.868 \mu\text{m}$ in size. Spore germination was seen after 7-10 days of inoculation and 77.5 % of the spores were found germinated.

Spore germination was found to be polar and of 'Vittaria type' (Nayar and Kaur, 1971) and spore coat was seen to split at the laesura region. A series of cell divisions took place and proceeded by the chlorophyllous protonemal initial cell and first rhizoid (Fig.1, C). The protonemal initial cell divided and formed filamentous protonema (Fig.1, D) consisting of 5-6 cells. When kept at room temperature spore viability was totally lost within 200 days.

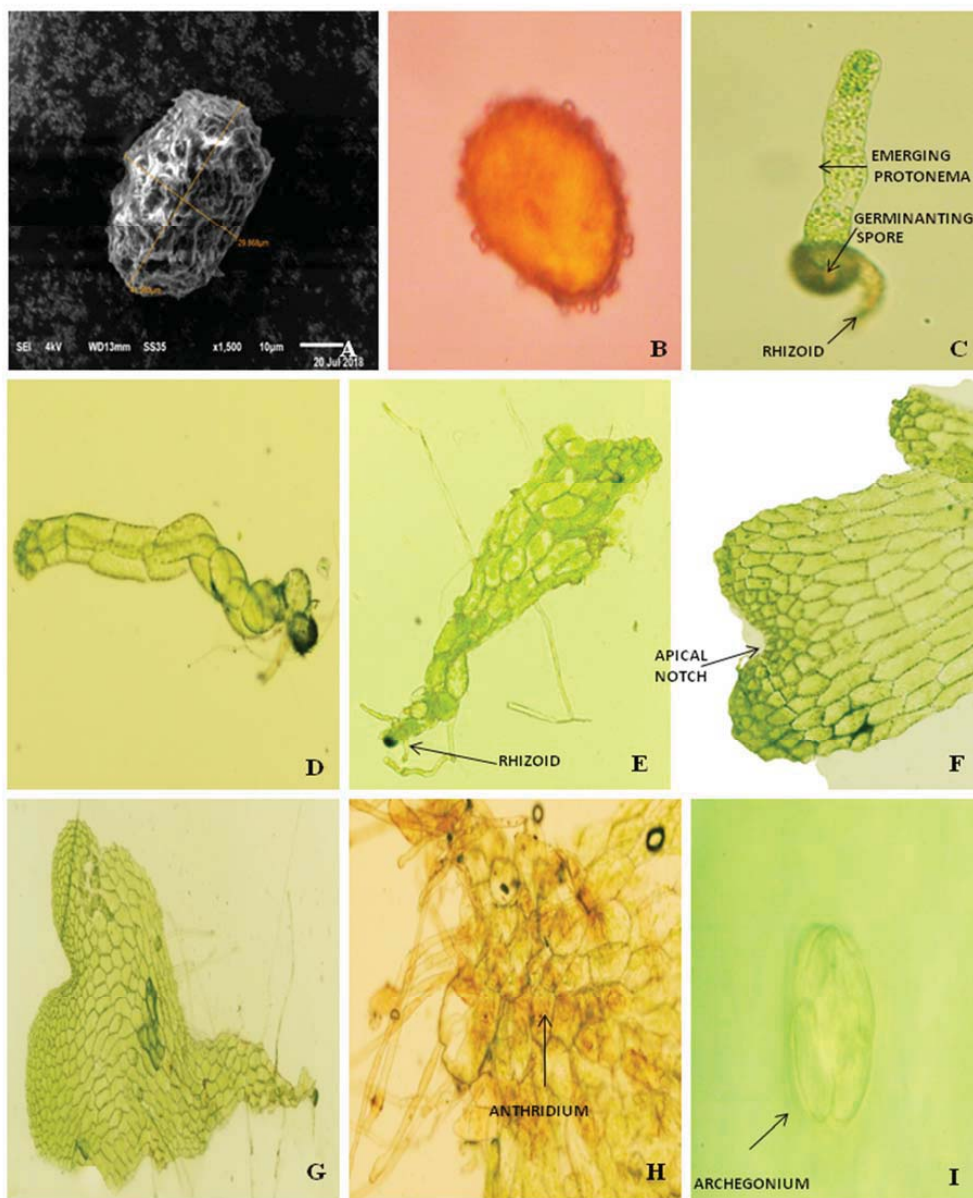


Fig.1. A, B: Spores, C: Emerging protonema, D: Filamentous stage, E: Spatulate stage, F: Pre-Cordate gametophyte, G: Cordate gametophyte, H: Antheridia in between rhizoid, I: Archegonia below apical notch

Prothallus Development

Two dimensional protonemal cells were seen after 14-16 days of sowing followed by spatulate stage after 21-25 days (Fig.1., E) and after 35-40 days cordate gametophytes developed (Fig.1., G). Prothallial development was found to be 'Drynaria type' (Nayar & Kaur 1971). The adult prothallus was cordate, thalloid, and massive, with thick midrib usually nearly as broad with spread-out wings. The gametophyte was symmetrical in nature with numerous brown coloured rhizoids and a shallow apical notch on its anterior region. Details of spore germination and gametophyte development are appended in Table 1.

TABLE: 1

Events of spore germination and gametophyte development of *Athyrium falcatum*

Days after sowing	Sample Size	% spore germination	% 2D-stage	Spatulate stage (%)	Cordate stage (%)
7	10	77.5±2.46	0	0	0
14	10	86.59±1.51	10±0.59	0	0
21	10	95.87±1.47	40±3.12	20±4.10	0
28	10	100	90±3.05	60±1.64	0
35	10	100	100	100	40±1.02
42	10	100	100	100	63±1.82
49	10	100	100	100	90±1.69
56	10	100	100	100	100
63	10	100	100	100	100
70	10	100	100	100	100

Gametangial Development

Cordate gametophyte after maturation remained vegetative for about 50 days and then initiation of gametangia started. Development of antheridia was seen after 85-90 days of spore germination on the posterior region in between the rhizoids on the ventral surface of thallus (Fig.1, H). Development of archegonia started after 95-110 days of spore germination on the ventral surface of thallus just below the apical notch along the anterior region of the midrib (Fig.1, I). Within four months the gametophyte become bisexual and maximum bisexual gametophyte were observed after 120 days of spore sowing. The detailed events of sex ontogeny are appended in Table 2.

TABLE: 2

Chronological changes in sex ratio in a composite culture of *Athyrium falcatum*

Days after sowing	Sample size	Neuter	Male	Female	Bisexual
80	20	17±0.85	0	0	0
90	20	19±0.72	2±0.79	0	0
100	20	15±1.41	7±0.91	4±0.72	0
110	20	9±0.85	6±0.79	6±0.85	3±0.79
120	20	4±0.85	0	14±0.79	8±0.85
130	20	8±0.91	0	16±0.85	4±0.79
140	20	9±0.79	0	12±0.91	0
150	20	5±0.91	2±0.85	0	5±0.91
160	20	7±0.91	2±0.85	1±0.85	6±0.85

4. DISCUSSION AND CONCLUSION

Though majority of ferns are listed in the International Red Book (Etter et al., 1993), they are collected from their natural habitat, resulting in their overexploitation and biodiversity depletion. With the application of *in vitro* culture of ferns, a system of plant multiplication is now developed by which young plant could be reintroduced to its natural habitat. In the recent years many aspects of fern development, including spore germination, gametangial division and differentiation, sex determination and fertilization have been described in details (Conway et al, 2020). In species like *Dryopteris affinis* vegetative multiplication of fern gametophytes through *in vitro* culture is clearly visible (Soare et al., 2010). This method is highly recommended for rapid multiplication of ferns in order to transplant the samplings in *ex situ* collection. These samplings can be used for the commercial purposes, experimental or fundamental research and multiplication of a number of endangered species (Golamaully et al., 2015). *In vitro* culture has created a new path for the conservation and restoration of ferns. The importance of gametophytes in tree ferns was emphasised, as it is useful in morphogenic studies of a haploid, single cell layer sheet that can be used for fern reproduction (Goller, 2007).

Several studies have reported allelopathy in gametophytes. Some ferns, such as *Thelypteris normalis*, secrete substances that inhibit cell division (Petersen and Fairbrothers, 1980; Chiou and Farrar, 1997). These growth-inhibiting substances inhibit the growth and development of neighbouring gametophytes. Differential growth patterns may be affected by the maturity level of the spores at the time of collection. The viability of spores at the time of collection may also account for the differential response of different fern species. The antheridium and archegonium were accounted for in the same week and hence it can be presumed that they appeared on the thallus at the same timeline. The antheridia were located a little

inward in the gametophytic thallus and the archegonia on the other hand were located on the outer side of the thallus.

A. falcatum showed 100% spore germination after 28 days of spore sowing which indicated that the spores were viable and media provided was good for the growth of ferns. It was seen that as the time increased number of rhizoidal and protonemal cells also increases with time. Rhizoidal cells function as roots and protonemal cells carry out photosynthetic activity. As seen via this experiment number of protonemal cells increased more compared to rhizoidal cells as the growth increased so does the photosynthetic activity. After almost eight weeks of sowing, it was seen that all the spores turned into cordate stage and sex organs starts to appear. After few weeks both male and female sex organs were seen. After 120 day of sowing maximum number of male and female sex organs appeared and after 150 day they start to reduce due to formation of sporophyte in ferns. Though, here *A. falcatum* did not show the formation of sporophytes although the mature antheridia and archegonia were obtained. The gametophytes showed functional archegonia but mostly spent up antheridia which could be due to the interference of antheridiogen mediation and this may have hindered the formation of sporophyte in *Athyrium falcatum* (Fernández and Revilla, 2003). Ferns require longer duration to produce sporophyte compared to other angiosperms because of low spore viability and other physiological reasons. At various points during the development it was seen that antheridia were absent but large number of functional archegonia were present. Various combinations of gametophytic lethal or semi-lethal genes can also explain lack of sporophyte formation as seen in *Osmunda regalis* population (Hedrick et al., 1987). This can also be explained by the fact that the gametophytes were hermaphrodite in nature so regardless other factors sporophyte formation was absent.

The gametophytic generation may also act as a useful indicator of phylogenetic relationships (Smith et al., 2006). Fern gametophytes possess evolutionarily conservative morphological characters that are useful in phylogenetic reconstruction which include spore germination, early development, form and origin of hairs, cellular structure of gametangia. Growth form of gametophytic thalli and gametangial position of fern gametophyte directly affects the survival of gametophytes.

In the present study antheridia were developed first, at least 10 days before the archegonia on the same prothallus to ensure cross fertilization and genetic variability in nature. In P&T nutrient medium the gametophyte take nearly three months to produce gametangia but the early production can also be managed by the manipulation of nutrients like Nitrogen and Carbon in the medium. This study provides a protocol for establishment of the population to a new site for ex-situ and in-situ conservation (Jang et al, 2019). In conclusion, many plants can be produced using this propagation method which is very practical and appropriate for field applications.

Gametophyte of fern has been the centre of interest for plant biologists for the past century because the structure and development are simple and amenable to investigation. Present study discusses the experimental research on the growth and development of fern gametophyte. Ferns have primitive features which can be easily exploited to study various aspects of plant structure and development

even though ferns are primitive plants and have less economic value. *Athyrium falcatum* spores are spherical in shape and have a distinct apical/basal polarity that is defined by monolete or trilete markings at the apex of the spore which acts as a mark for the germination of spores. The spore ruptures along these markings as it germinates. In these spores, neither cell differentiated a rhizoid, indicating that the first asymmetric division is a necessary condition for rhizoid differentiation. Protonema in ferns eventually give rise to photosynthetic prothallus and fern rhizoid is a non-photosynthetic, elongated and single celled cell which helps in anchoring and absorption of nutrients.

Helminthostachys zeylanica, a rare medicinal fern was mass cultivated and restored in southern assam via regeneration of sporophytes and this technique further helped in its phytochemical screening (Mazumder et al., 2010). Spore germination varies depending upon the taxonomy of fern species (Lloyd et al., 1970). Germinated spore give rise to a gametophyte which further produces a sporophyte after successful fertilization. Factors like dry weather, high temperature and low humidity are favourable for the release of matured spores as seen commonly in *Diplazium* family (Arosa et al., 2009). Germination of each spore produces one gametophyte that passes through filamentous stage to heart shaped stage. The culture of fern gametophytes provides a means of (in controlled conditions) studying their unique adaptive features and is also used as an experimental system to investigate cellular level growth and differentiation. Each gametophytic developmental stage can be controlled synchronously due to their high sensitivity towards light so proper light conditions must be maintained for the development of prothallus. It was observed that the percentage of spore germination is higher in *Athyrium falcatum* as compared to other taxon like *Pteris aspericaulis*, *Drynaria mollis*, *Onychium cryptogrammoides*, indicating the high adaptive significance and ability to recruit populations (Vaganov et al, 2017; Priti et al, 2016, Ravi et al, 2014; Cha-Cha et al, 2005; Quintanilla et al, 2002). In vitro culture can overcome difficulties like low germination rates, fungal and algal contamination, sporophyte formation and reduction in growth due to invasive species. Rare species *Cibotium schiedeii* (Central America) and *Angiopteris boivinii* (Seychelles) are seen to show successful growth through *in vitro* culture (Fay et al., 1992).

Not only does the gametophyte allow for sexual reproduction, but it also influences recruitment, species habitat selection, migration, and adaptation. The role of morphological and physiological diversity among gametophytes in habitat selection, time frame and method of development and maturation, breeding system and habitats contributing new recruits to sporophytic population, and other critical topics in gametophyte biology have received little attention. Fern gametophyte is haploid, simple in structure, mainly consisting of a single layer of cells. It lacks complex tissues and organs but it has great potential to yield significant insights and understanding of plant development. It is an ideal experimental system for the study of plant development and can be easily cultured and manipulated in a non destructive way.

Study of gametophytic development is necessary to understand and improve the growth and development of economically important ferns. Therefore, there is an urgent need to establish the *in vitro* regeneration protocol for the production of high-quality plantlets for commercial uses.

5. STATISTICAL ANALYSIS

All experiments were carried out with three independent replicates. Data are represented by their mean and standard deviations using descriptive statistical analysis tool.

6. ACKNOWLEDGEMENT

Authors are grateful to the Institute of Eminence, University of Delhi for financial assistance (IOE/FRP/LS/2020/27). Himani Yadav and Priti Giri also acknowledge University Grants Commission and CSIR for providing fellowship respectively.

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