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IN VITRO EARLY ONTOGENIC STUDIES OF *ACTINIOPTERIS RADIATA* (SWARTZ) LINK. UNDER DIFFERENT MEDIUM

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ABSTRACT

Actiniopteris radiata (Swartz) Link. is a fern belonging to the family Pteridaceae. The spores collected from the pinnae of *A.radiata* was inoculated in Knudson's C and Knop's medium at pH 5.8. The ontogenic pattern such as germination type, prothallial development and micromorphological changes were studied in detail. The germination of the spore was *Anemia* type. The gametophyte development was *Ceratopteris* type. The growth area in Knudson's C medium(KC), Knop's (Kn) medium and brick pieces supplemented with Knudson's C medium showed different germination percentage, growth area and micromorphology. In micromorphology, the apical notch formation varied in different medium. Few antheridiate prothalli could be seen in Knop's medium.

KEYWORDS: ***Actiniopteris radiata*, *in vitro* spore germination, ontogenic stages of development.**

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INTRODUCTION

Ferns and their allies are a natural group of plants, descended from the oldest plants of the earth's history, being found as fossil, they date back nearly 400 million years. These pteridophytes have attracted the attention of botanists perhaps more than any other group of plants. But unfortunately, the study of pteridophytes has been sorely neglected in India[1]. Since ferns depend on the higher plants for their survival, any disturbance inflicted on the higher plants automatically destroys the ferns[2]. Homosporous gametophytes are free living and independent of sporophyte. Environment directly influences their demography because their distribution, development and mating systems are unique among the vascular cryptogams[3]. The fern gametophyte is a free growing entity with simpler organization devoid of protector devices such as cuticle**,** stomata etc, and hence extremely sensitive to environment. Gametophytes are also capable of indefinite growth and clonal expansion. Gametophyte morphology of ferns including types of spore germination, early development, mature form, trichomes and gametangia has been considered to be the significant, defining characteristics of fern taxa[4,5,6]. Spore propagation ensures genetic diversity of native fern species and provides a high volume of fern plants for gardens and environmental restoration. Among the various biotechnological options, propagation through tissue culture and *in vitro* spore germination are best applied and commercially exploited in fern species[7]. Application of this technology (*in vitro* spore germination) for conservation protocol through large scale multiplication of certain species of ferns from the Western Ghats has been demonstrated by various authors[8,9,10,11]. *Actiniopteris radiata* (Swartz) Link. is a terrestrial fern, found in an elevation of about 300meters at the foothills of Western Ghats. It belongs to the family Pteridaceae and commonly called peacock's tail and also known as Ray fern. The plants are 8-25cm height, rooting in the crevices of rock or moist and shady places. The rhizome is oblique to horizontal, 1.5 to 2.0cm in length, densely covered by scales and leaf bases. The young leaves (fronds) show circinate venation and lamina flabellate, semicircular or wedge-shaped. Fronds flabellate, dichotomously divided into linear segments, sometimes dimorphic with fertile and sterile fronds. According to the ayurvedic text it is used as styptic and anthelmentic[12,13], treatment of bronchitis and gynecological disorders, [14]tuberculosis, astringent, anti-inflammatory, antipyretic, alleviates vitiated blood, cough, asthma and bronchitis [15,16,17,18], also increases fertility in woman and in spermatorrhoea[19]. The plant is very much important for its ethnomedicinal values, but we couldn't find out any previous report regarding the regeneration and multiplication of this plant under *in vitro* conditions. This is the first report in germination and growth studies of gametophyte of *A. radiata*. The present work focuses on the morphology and *in vitro* development of gametophytes cultured in two different media. The main aim is to find out the optimum culture conditions and requirements for the healthy *in vitro* growth of gametophytes, which form a rich genetic diversity and provide micromorphological and sexual information which may pave the way for solving the taxonomical problems in pteridophyte systematics as well as for exploring multipurpose experiments or medicinal uses.

MATERIALS AND METHODS

Collection of plants and spores

The plants of *A. radiata* was collected from Sothuparai dam near Periyakulam, Madurai during the month of January 2012. The collected plants were grown in Holy Cross College (Autonomous), Tiruchirappalli- 620 002. Poor growth was observed under *in vivo* condition. The fronds bearing sori (Plate-1c) were kept upside down to release the spores on white sheet of paper near radiant sunlight. After the collection, the spores (Plate-1d) were stored in plastic vials at 4° C for further use. Voucher specimens are stored at Department of Botany, Holy Cross College (Autonomous), Tiruchirappalli for future reference.

Medium Preparation

Table 1
Stock solution of Kn (Knop's) and KC (Knudson's C) [20,21]

S.No	Salts	Kn salts conc. mg/l	KC salts conc. mg/l
1.	$MgSO_4.7H_2O$	200	250
2.	KH_2PO_4	200	250
3.	KNO_3	200	-
4.	$CaNO_2$	800	-
5.	$(NH_4)_2SO$	-	500
6.	$Ca(NO_3)_2\ 4H_2O$	-	1000
7.	$MnSO_4.7H_2O$	-	7.5
8.	$FeSO_4$	-	250

The liquid medium of KC and Kn and brick pieces supplemented with KC medium were adjusted for pH 5.8 to 6.0 was directly transferred to the glass wares like conical flask and test tubes. No carbon source was added in liquid medium. All the glass wares with medium were sterilized in an autoclave for 15 minutes at 15 lbs pressure.

Spore inoculation and culture conditions

After collection, immediately the spores were cultured in the liquid medium before its viability vanishes. Spores were sprinkled on the surface of liquid medium and kept undisturbed, untill the full growth of gametophytes was observed. All the procedures were carried out inside the laminar airflow chamber. Conical flask and other culture vessels were incubated in the culture room after inoculation and 12 hours photoperiod at 25±2°C was maintained. Spores germinated on the prescribed day were counted in a particular microscopic field and its percentage was calculated.

Measurement of gametophytes

A standardized measurement was carried out by adjusting the ocular meter and stage micrometer in a Weswox optik model TRHL – 66 –stereomicroscope[22]. After adjusting, the stage micrometer was removed and gametophytes to be measured are viewed and size of the gametophytes were measured. The 20, 40 and 60 days old gametophytes were isolated by using a sterile forceps or needle and the growth area was measured. The measurement was in micrometers. Micromorphological characters like apical notch, dermal hairs, marginal cells and sex organs were observed during 45-60 days period in KC, Kn and brick pieces supplemented with KC medium grown gametophytes. All the ontogenic and developmental stages were micro photographed using stereomicroscope fitted with Pentax camera. The calibration of size of the gametophytes were represented in scale bar as micrometer(µ). . The magnification was denoted as bar (1cm represents 1µm). Growth Area Index (GAI) was measured by calibrating its length and width of the prothalli. Total growth area of the prothallus is calculated as follows by using the formula, Length (µm) x Breadth (µm) = Growth area (mm^2).

RESULTS

Germination and developmental pattern of spores on KC medium

The germination was polar and wall formation was parallel to the equatorial plane of the spore. Elongation of primary rhizoid (Plate - 1e&f) and young prothallus was parallel to the polar axis of the spore. Germination of the spore was '*Anemia*' type. Linear row of filamentous growth with 5-7 cells were observed at 20 days time. This one dimensional filamentous growth (Plate- 2a&b) underwent anticlinal and periclinal divisions by the apical meristematic cells and initiated prothallial plate after 30 days (Plate 2c). This prothallial plate (Plate- 2d&e) showed pluricellular meristem unevenly on the spathulate prothalli. Unequal halves of prothallial plate showed pluricellular meristem at one end of the prothalli (Plate-2c; Table- 2). Therefore lopsided prothalli resulted. Rhizoids 5-7 numbers could be seen on the prothalli.

There was no hairs on the surface of the gametophytes. This type of gametophyte development was *Ceratopteris* type[5]. Apical notch formation was initiated during 60-70 days of culture.

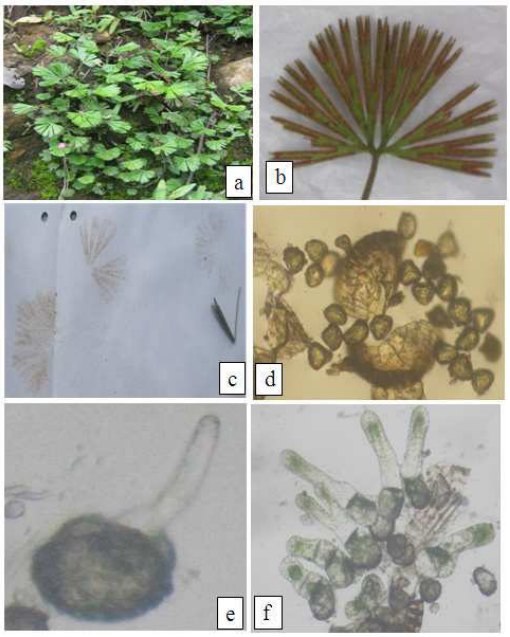


Plate 1

Showing the habit, spore morphology and germination of A. radiata

1a- Habit
1b- Frond bearing sporangia and spores on abaxial side.
1c- Spores dusted on white sheet of paper.
1d- Spores
1e- emergence of first rhizoid from spores on high power.
1f- Group of germinated spores.

Germination and developmental pattern of spores on Kn medium

The germination was polar and wall formation and elongation of primary rhizoid was parallel to the equatorial plane of the spore. Thus, like KC medium, Kn medium also showed '*Anemia*' type of spore germination. The next stage of filamentous growth resulted in about 10-12 cells of protonema. Very few apical meristematic cells were formed during 30-40 days. After 40 days, anticlinal and periclinal divisions resulted with the formation of spathulate prothalli. Prothallial plate was more elongated than the prothalli formed on Knudson's C medium (Plate-3b; Table-3). This elongated spathulate prothalli was enlarged due to anticlinal and periclinal divisions, but fails to produce any pluricellular meristem and apical notch (Plate-3b).

Germination and developmental pattern of spores on brick pieces supplemented with KC medium

The spores grown on brick pieces supplemented with KC medium showed the similar stages of prothalli and gametophytes that were grown on KC medium. The same germination type (*Anemia* type) and growth pattern (*Ceratopteris* type) of gametophyte was evidenced when spores grown on brick pieces supplemented with KC medium. Enormous amount of rhizoids were seen on the cultures grown on plastic cups containing brick pieces (Plate-3d; Table-4). Micromorphological details showed undulated blunt cells without any epidermal hairs and with distinct apical notch.

Growth area in different medium

The growth area was an exact measurement of the developed prothalli as well as

gametophyte of *A. radiata*. Table- 2,3,4 and Fig-1 showed the differences in the growth area of gametophytes grown on KC, Kn and brick pieces supplemented with KC medium. In KC medium, on 20th day, length, breadth and growth area was 47.9µm, 14.6µm and 716.7µm respectively. This has increased into 77.2µm, 59.8µm and 473.6µm in 40 days period and on 60th day the length attained 78.7µm and breadth increased up to 92.8µm and finally 6925.2µm of growth area (Plate-2; Table- 2).

Table-2
Germination percentage, length, breadth and growth area of prothalli of A. radiata in KC medium

Growth on days	Germination percentage (%)	Length (µm)	Breadth (µm)	Growth area (µm)
20 days	88	47.9 ± 10.2	14.60 ± 4.3	716.7 ± 27.4
40 days		77.2 ± 12.0	59.8± 23.0	473.6 ± 27.5
60 days		78.7 ± 5.1	92.8 ± 4.0	6925.2 ± 325.9

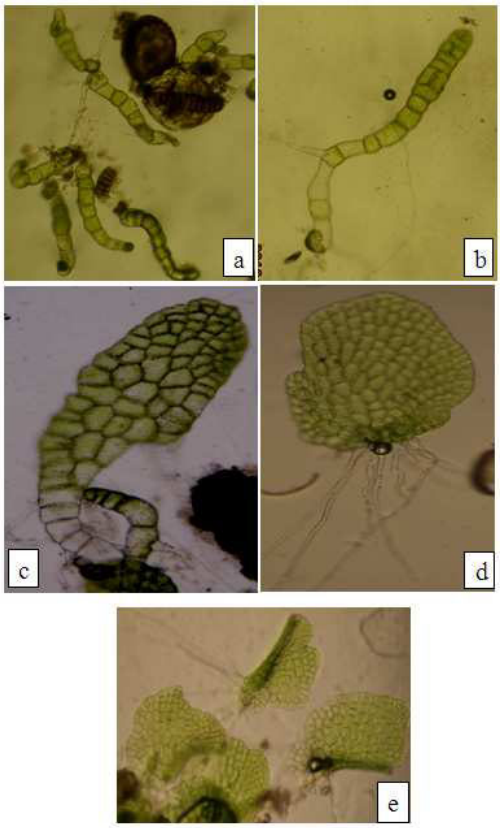


Plate 2
Showing different growth pattern of A. radiata prothalli on KC medium
2a- 10 days old filamentous prothalli.
2b- 25 days of old prothalli.
2c- 30-35 days of old prothalli showing few apical meristems.
2d- Lopsided prothalli with enormous rhizoids.
2e- Group of lopsided prothalli with enormous rhizoids.

Growth area in Kn medium shows 45.1µm, 10.1µm and 456.4µm as length, breadth and growth area on 20 days period. On 40th day, the length was 77.7µm; breadth was 29.5µm and the growth area was 2292.2µm and on 60th day, they were 78.2µm; 41.6µm and 5865.4µm respectively

(Plate-3; Table- 3). When compared to KC medium, Kn medium showed slightly reduced growth (Fig-1).

Table-3
Germination percentage, length, breadth and growth area of prothalli of A. radiata in Kn medium

Growth on days	Germination percentage (%)	Length (µm)	Breadth (µm)	Growth area (µm)
20 days	80	45.1 ± 7.4	10.1 ± 1.5	456.4 ± 11.4
40 days		77.7 ± 1.8	29.5 ± 12.2	2292.2 ± 21.9
60 days		78.2 ± 2.0	41.6 ± 2.1	5865.4 ± 2636.3

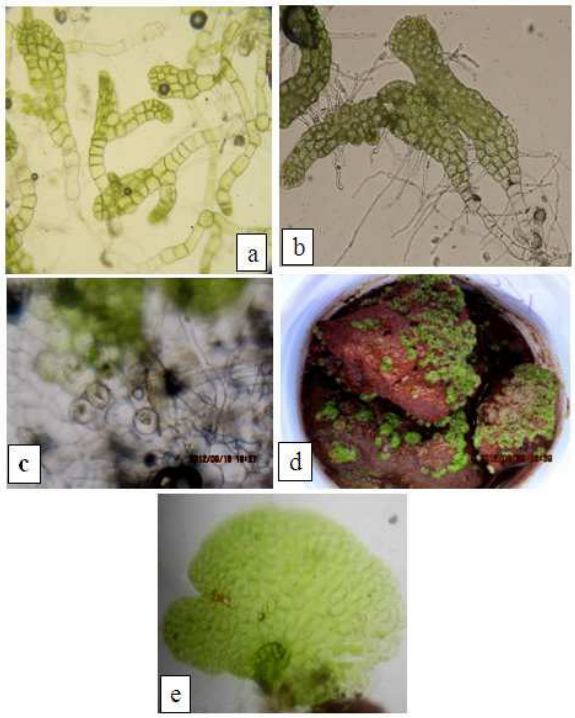
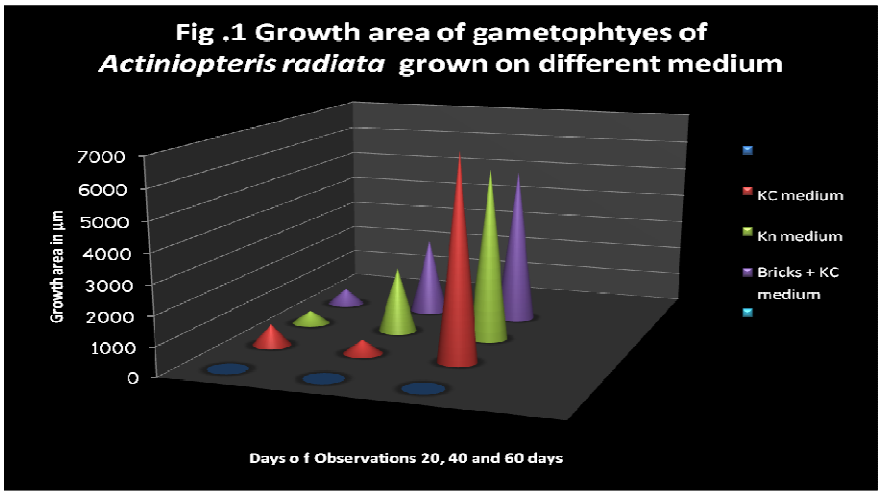


Plate 3
Showing different growth pattern of A. radiata prothalli on Kn medium and brick pieces supplemented with KC medium

3a- Strap shaped bifurcated prothalli on Kn medium
3b- Elongated strap shaped prothalli on Kn medium
3c- Antheridia of strap shaped gametophyte on Kn medium
3d- Gametophyte growing on brick pieces supplemented with KC medium
3e- Broad lopsided Ceratopteris type gametophyte grown on brick pieces supplemented with KC medium

Figure -1
Growth area of gametophytes of A. radiata grown on different medium



On brick pieces supplemented with KC medium, prothalli attained 15.1µm length, 37.7µm breadth and 569.3µm of growth area at 20 days time. During 40th day, the length was 542.7µm, breadth was 61.6µm and growth area leads to 2630.3µm. On 60th day, the growth area was attained 5317.1µm with a length of 53.6µm and a breadth of 99.2µm. Apart from the growth area, micromorphology also differs on brick pieces grown prothalli. The ecological niche of the plant in rock crevices and branches of trees and presence of more rhizoids is an important character that initiates the original niche of this plant in the cultures.

Table-4
Germination percentage, length, breadth and growth area of prothalli of A. radiata in brick pieces supplemented with KC medium

Growth on days	Germination percentage (%)	Length (µm)	Breadth (µm)	Growth area (µm)
20 days	85	15.1 ±0.7	37.7 ± 1.6	569.3 ± 1.1
40 days		42.7 ± 1.6	61.6 ± 2.1	2630.3 ± 3.4
60 days		53.6 ± 2.6	99.2 ± 2.1	5317.1 ± 5.5

Micromorphology of the gametophytes grown on various medium

Among the different medium used, typical micromorphological changes were observed in the formation of enormous rhizoids, chlorophyllous and spreaded lopsided cordate wings with apical notch in KC and brick pieces supplemented with KC medium grown cultures, whereas Knops medium grown cultures differed in morphology. From the results of the work, it was proved that vegetative growth was normal in KC and brick pieces supplemented with KC medium grown cultures. In Kn medium grown cultures, changes takes place in the growth of prothalli like bifurcation of the spathulate prothalli on 30 -40 days time. During 50-60 days time, prothalli grown on Kn medium attained elongated strap shaped gametophytes (Plate-3b). Irrespective of growth area of gametophyte in Kn medium grown cultures produced antheridia on 60th day (Plate-3c). Apical notch formation also differs in gametophyte grown on KC, Kn and brick pieces supplemented with KC medium. No hairs were observed in any of the cultures but the apical notch could be seen in KC medium and also in brick pieces supplemented with KC medium, whereas there was no definite pluricellular meristem formed on cultures grown on Kn medium.

DISCUSSION

The culture of gametophytes seems to be autotrophic in nature, because it doesn't need carbon source for the growth and no other additive or high osmoticum was needed for the germination or development or maintenance of the cultures in this present study. pH ranges from 5.8-6.0 and slightly it was acidic condition. The same result could be observed in various pteridophytic plants and the importance of low pH (pH 4.0) for the growth and development of *Ophioglossum palinatum* was also reported[23]. The A*nemia* type of spore germination and *Ceratopteris* type of gametophyte development was also evidenced in *Anemia rotundifolia*[24]. The preferable nature for liquid medium other than the agar based medium that prevailed in our approach was also supported and confirmed it for generating more tissues in *Pteridium aquilinum* and *Anemia phyllitidis* [25]. In the present study, medium seems to play a significant role in sex organ formation and other micromorphological changes of the gametophyte grown on Kn and KC medium. Growth area was high in KC than Kn medium. In Kn medium, there was decrease in the quantity of ammonium, potassium and iron content than KC medium. This shows that *A. radiat*a needs osm o ticum which provide nitrogen source (ammonium), potassium and iron for its germination and growth. The same condition was also observed in many ferns [26]. The study on early ontogenic development of the fern *A. radiata* is a first attempt on germination and gametophyte development and could be followed for further studies on new avenues later. The work is continuing for probing the complete reproductive biology of the species.

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