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# *IN VITRO* STUDIES ON NORMAL AND ABNORMAL LIFE CYCLE OF *METATHELYPTERIS FLACCIDA* (BL.) CHING

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**Abstract:** The present study was aimed to differentiate *in vitro* the normal and abnormal life cycle using the fern *Metathelpteris flaccida* (Bl.) Ching. For the normal life cycle the matured spores were used as experimental material and for the abnormal life cycle croziers and *in vitro* raised gametophytes were used. The spores were surface - sterilized with 0.1% $HgCl_2$ solution for 5 minutes and washed with sterile distilled water for 15 minutes and inoculated on the agar plates using sterile Pasteur pipettes and incubated at 25 ± 2°C under 12 h photoperiod (1500 lux). The Knudson's solid medium inoculated spores showed germination after 45 days. The prothalli development was drynaria type. The protnalli were dioecious, dorsoventrally flattened, with a midrib with cushion - like structure and notched at the anterior end. The sex organs and rhizoids emerged from the midrib of the ventral surface. The highest sporophyte formation (83.6 ± 2.37) was obtained on Knudson C basal medium. For apospory, croziers of *Metathelypteris flaccida* cultured in Knudson C medium supplemented with 3% sucrose and 2, 4, 5, Tri - chloro phenoxy acetic acid-1.0 mg/l and Chloro phenoxy acetic acid 1.5 mg/l produced the aposporous gametophytes on petiole of the young leaves after three months. The aposporous gametophytes and the normal spore - derived gametophytes of *Metathelypteris flaccida* are morphologically identical. For apogamy, the gametophytes were sub-cultured on the different media with and without sucrose for the sporophyte formation. The Knudson C medium with sucrose induced the sporophyte without sex organs (apogamous sporophyte). The maximum sporophyte percentage was observed on KC medium supplemented with 3% sucrose.

**Key words:** Aposporous gametophyte, Apogamy, *in vitro*, Plant growth regulators.

**Abbreviations:** 2, 4, 5, T - 2, 4, 5, Tri - chloro phenoxy acetic acid; 2, 4 – D - 2, 4 Di - chloro phenoxy acetic acid; BAP – Benzyl Amino Purine; CPA - Chloro Phenoxy Acetic acid; Kin –Kinetin; KC – Knudson C; KN – Knop's; Mi – Mitra; MS – Murashige and Skoog.

## Introduction

Pteridophytes, because of their distinct alternation of gametophytic and sporophytic generations have proved to be an ideal material for experimental studies (Bir and Anand, 1982). Ferns multiply either through spores or vegetatively by fragmentation of the rhizome. In some ferns, root buds or leaf buds help in multiplication (Sharma and Sharma, 1991). Agamosporous and aposporous modes of reproduction are also common in ferns. Apospory, the formation of gametophyte from the sporophyte was first reported in *Pteridium* (Farlow, 1889). Many attempts have been made to induce apospory in several members of the filicales with varying degree of success (Steil, 1939). In recent years there are many reports on induced apospory (Whittier, 1978; Sheffield and Bell, 1981; Raghavan, 1989; Kwa *et. al.*, 1991 and Fernandez *et. al.*, 1996). *in vitro* experiments have yielded useful information on many intricate problems of differentiation, development and life cycle in ferns (Loyal and Chopra, 1977). Based on this background, the present study was aimed to study the normal and abnormal life cycles of (induce apospory , apogamy in the normal) sexually reproducing fern *Metathelypteris flaccida* (Bl.) Ching and to determine the effect of plant growth regulators, sucrose and nutrients on phase change from gametophyte to sporophyte and from sporophyte to gametophyte.

## Material and Methods

Wild plant and mature fertile fronds of *Metathelypteris flaccida* (Bl.) Ching were collected from Kothayar, Tirunelveli hills of South Western Ghats, India and established in the greenhouse attached to the Centre for Biodiversity and Biotechnology, St. Xavier's College, Palayamkottai, Tamil Nadu, India for *in vitro* apospory production. Croziers of young plants were used as the source of explants for the apospory induction studies, and the matured spores were used for the *in vitro* life cycle study. Fertile fronds of *Metathelypteris flaccida* were collected from authentic mother plants at Kothayar hills (1500 m) of the South Western Ghats, India. After drying the fronds on absorbent paper at room temperature, the liberated

spores were passed through 40 µm nylon mesh to remove the sporangial wall materials and clean spores were collected and stored in refrigerator at 5°C. The spores were surface sterilized with 0.1% $HgCl_2$ solution for 5 min. and washed with sterile distilled water for 15 min. The surface sterilized spores were inoculated onto the media KN (Knop (1885), KC (Knudson, 1946) and Mi (Mitra *et. al.* 1976) using sterile Pasteur pipettes and incubated at 25 ± 2°C under 12 h photoperiod (1500 lux). The pH of the media devoid of sucrose was adjusted to 5.8 before adding agar 0.5 % (w/v) and autoclaved at 121°C for 15 min. Germination percentage of the spores, growth area of the prothalli and their development pattern were analyzed. Photomicrographs were taken with Labo triumph microscope. The *in vitro* spore - raised sporophytes were washed thoroughly in running tap water for few minutes to remove the agar ingredients. The washed sporophytes were transferred to polycups containing Sand: Soil: Farmyard manure (2:1:1) for hardening. After a few weeks, the hardened plants were transferred to field for re-establishment. Croziers with about 1 cm diameter were excised, washed in running tap water for 15 min, surface - sterilized with 0.1 % (w/v) mercuric chloride for 5 min and rinsed 3 to 4 times in sterile distilled water. They were cut into 1 cm long segments and implanted on Knudson C (1946) medium modified with Nitsch's trace elements and supplemented with 3% (w/v) sucrose and varied concentrations of plant growth regulators such as BAP, 2,4-D, 2,4, 5-T, 2 ip, Kin, CPA and IAA. The pH of the medium was adjusted to 5.8 before adding 0.5% (w/v) agar (Himedia, Mumbai, India) and steam-sterilized for 15 min at 121°C under 1.1 kg $cm^{-2}$ pressure. All the cultures were incubated at 24 ± 2°C under cool fluorescent white light (Phillips, India) (1200-1500 lux, 12 hr/d). The experiments were repeated twice, and each replicate comprised a minimum of 20 cultures. Gametophytes of *Metathelypteris flaccida* derived from spores and aposporous gametophytes were sub-cultured on different agar media (KC, KN, and MS) with and without sucrose for sporophyte formation.

## Results and Discussion

The Knudson's solid medium inoculated spores showed the germination after 45 days, and the germination percentage (36.8±1.31) was low compared to other

species. According to Fernandez et. al., (1996) ferns prefer low salt medium for their growth. Our results (Knudson C medium is one of the low salt media) are in consonance with the observation of Fernandez *et. al.*, (1996). The germination pattern was vittaria type (Nayar and Kaur, 1971). After 90 days, the complete prothalli were formed, which looks like cordate type (Sara *et. al.*, 1998). The prothalli development was drynaria type (Nayar and Kaur, 1971). The prothalli were dioecious, dorsoventrally flattened, with a midrib with cushion-like structure and notched at the anterior end. The sex organs and rhizoids emerged from the midrib of the ventral surface. Sex organs were formed, the antheridia were formed after 150 days and were restricted to the posterior end, and the archegonia were formed after 180 days and were restricted to anterior end of the prothallus. The glandular hairs were present in the gametophytes (Fig. 1 A - D). The gametophytes were sub-cultured on different media for gametophyte multiplication. The maximum percentage (75.5 ± 0.61) was observed on Knudson C medium. After 180 days, the gametophytes were transferred to liquid basal medium for fertilization for a few days then transferred to solid medium. On day 200, the sporophyte emerged was observed visibly in the culture. Since the sporophytes have sexual origin, they are attached by a support to the gametophyte. The highest sporophyte formation (83.6 ± 2.37) was obtained on Knudson C medium (Table – 1; Fig. 1 E).

Table 1: Effect of different media on spore germination, gametophyte and sporophyte formation of *Metathelpteris flaccida* Bl.Ching

| [illegible] | Germination period (Days) | % of germination | % of Gameto-phyte multipli-cation | % of sporophyte formation | Mean no. of crozier / tube sporophyte |
|---|---|---|---|---|---|
| [illegible] | 40 | [illegible] 1.31 | 75.5 ± 1.61 | [illegible] | 6.5 ± 0.31 |
| [illegible] | N | NIL | 67.8 ± 0.71 | N | NIL |
| [illegible] | NIL | NIL | 62.3 ± 0.57 | NIL | NIL |
| Modified B | NIL | NIL | 63.2 ± 0.54 | NIL | NIL |
| [illegible] | NIL | NIL | 54.4 ± 0.41 | NIL | NIL |

The result suggests that the Knudson C medium is suitable one for multiplication of the threatened species through *in vitro* spore culture. The *in vitro* raised sporophytes were transfered to the polycups for hardening, and 78.7 ± 1.34 percent of sporophytes were established in the polycups. The *in vitro* sporophytes were free from morphological variation. After one month, the hardened plants were transferred to the field for re-establishment, and 74.8±1.31% of plants were established in the field (Kodaikanal Botanic Garden, Eettipallam, Kodaikanal, TN. India; Fig. 1 F).

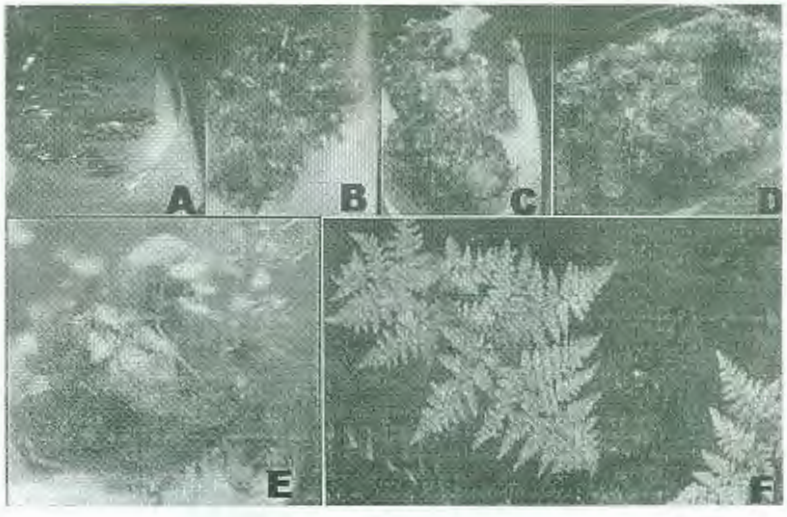


Figure 1. *In vitro* spore germination Different Stages of *Metathelpteris flaccida*
a - d: Gametophyte formation different stages
e: Sporophyte formation under *in vitro* conditions
f: *In vitro* raised plantlets established in the field

The *in vitro* raised 6 month old plants were transferred to pots and distributed to various botanical gardens for *ex situ* conservation. Initial attempts to culture the crozier segments of *M. flaccida* collected from the forests failed; nearly 90% explants were lost due to contamination, while the remaining did not respond. To overcome these problems, newly formed crozier segments from sporophytes adapted to greenhouse were used. The young crozier segments turned their colour to dark blackish brown. After 4 months, the croziers cultured on light showed early

stages of apospory (Whitier, 1978). Apart from light, several other factors also influence the induction of aposporous gametophyte from the crozier. The Knudson C supplemented with 3% sucrose and 2, 4, 5-T (1.0 mg/l) and CPA (1.5 mg/l) produced aposporous gametophytes (Fig.2 A). The Knops and Murashige and Skoog's media supplemented with 3% sucrose and different plant growth regulators at various concentrations failed to produce aposporous gametophytes. After 3 months of culture, 4-6 mm long, simple, dark green filamentous out-growths were seen on the petiole of the young leaves on the crozier (Table 2). After 6 months, the mature prothalli were formed (Fig. 2 B). They were about 1 cm wide, cordate and dark green in colour. The aposporous gametophytes were identical in morphology with the normal spore - derived gametophytes of *M. flaccida*.

Table 2: Effect of Plant Growth Regulators on croziers of *Metathelypteris flaccida* (Bl.) Ching.

| S. No. | KC (M)N Medium + PGR (mg/l) | Responses |
|---|---|---|
| 1. | 2,4-D (0.5) | No response |
| 2. | 2,4-D (1.0) | Dark green coloured calli formed on the petiole. |
| 3. | 2,4-D (1.5) | Dark green coloured calli formed on the petiole. |
| 4. | 2,4,5-T (1.0) | Green colour small outgrowth formed on the petiole (Filamentous stage aposporous gametophyte). |
| 5. | 2,4,5-T (1.5) | No response |
| 6. | Kin (1.0) | No response |
| 7. | CPA (0.5) | No response |
| 8. | CPA (1.0) | No response |
| 9. | CPA (1.5) | Green colour outgrowth formed on the petiole (aposporous gametophyte) |
| 10. | NAA (1.0) | No response |

The absence of vascular element and the stomata, which were typical characters of the sporophyte, morphological identity of aposporous derived gametophyte with spore derived gametophyte and the presence of rhizoid proved the gametophytic character of outgrowths on the petiole. The gametophytes were sub-cultured on different media (KC, KN, and MS) with and without sucrose for the sporophyte formation. The without sucrose (KC basal medium) inoculated medium induced the sporophyte with the presence of sex organs (4n). Other media failed to induce

sporophyte but with sucrose medium induced the sporophyte without sex organs (apogamous sporophyte (2n) Fig 2. D). The maximum sporophyte percentage was observed on KC medium supplemented with 3% sucrose. Above 3% sucrose level decreased the sporophyte formation percentage, and the optimal level was with KC medium supplemented with 3% sucrose. The very same result was observed in the normal gametophytes (spore - derived) and aposporous gametophyte also (Fig. 2 C). The conclusion drawn from this work is that the media and plant growth regulators are important to induce apospory in *M. flaccida.* Not only for *M. flaccida* but also for all plants the plant nutrients and growth regulators are very important for the normal or abnormal life cycle. Because *M. flaccida* is the normal life cycle fern, due to the changes in the medium and growth regulators the normal life cycle is altered and produced the apospory. Addition of sucrose leads to apogamy.

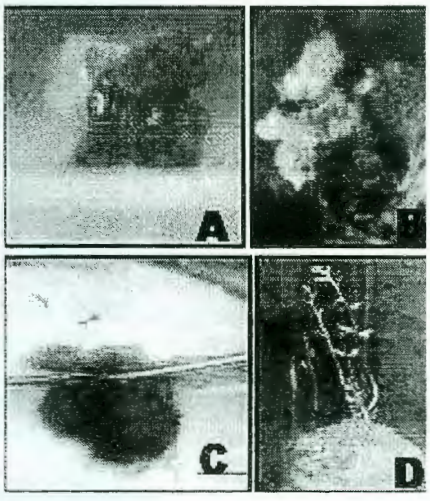


Figure. 2: Abnormal Life Cycle of *Metathelpteris flaccida*
A. Apospory Initiation on Petiole,
B. Apospory Matured Stage after 4 months,
C. Apospory Induced Gametophyte derived Sporophyte – in liquid medium,
D. Apogamous Sporophyte establishment–filamentous stage gametophytes

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