



Ex situ conservation of two threatened ferns of the Western Ghats through in vitro spore culture

Johnson Marimuthu¹ & Visuvasam Soosai Manickam²

¹ Department of Plant Biology and Biotechnology, ² Centre for Biodiversity and Biotechnology, St. Xavier's college (Autonomous), Palayamkottai, Tamil Nadu 627002, India
Email: ¹ptcjohnson@gmail.com (corresponding author), ²vsmanickam@gmail.com

Date of publication (online): 26 July 2011
Date of publication (print): 26 July 2011
ISSN 0974-7907 (online) | 0974-7893 (print)

Editor: V. Irudayaraj

Manuscript details:

Ms # o2687
Received 28 January 2011
Final received 08 June 2011
Finally accepted 27 June 2011

Citation: Marimuthu, J. & V.S. Manickam (2011). Ex situ conservation of two threatened ferns of the Western Ghats through in vitro spore culture. *Journal of Threatened Taxa* 3(7): 1919–1928.

Copyright: © Johnson Marimuthu & Visuvasam Soosai Manickam 2011. Creative Commons Attribution 3.0 Unported License. JoTT allows unrestricted use of this article in any medium for non-profit purposes, reproduction and distribution by providing adequate credit to the authors and the source of publication.

Author Details: see end of this article

Author Contribution: JM executed the work and contributed for paper writing; VSM was the principal investigator of the project and he only suggested the problem, given guidelines and contributed for paper writing.

Acknowledgements: The authors acknowledge the financial assistance from Ministry of Environment and Forests, Government of India, New Delhi, India.



Ministry of Environment and Forests
GOVERNMENT OF INDIA



OPEN ACCESS | FREE DOWNLOAD

Abstract: The present study was intended to produce a protocol for the conservation of two endangered ferns of southern Western Ghats of India using in vitro spore culture. In addition this study reports spore germination, gametophyte development, changes in the reproductive phases and sporophytes formation of the medicinally important ferns *Pronephrium triphyllum* (Sw.) Holttum and *Sphaerostephanos unitus* (L.) (Holttum). Matured spores of the two selected ferns were harvested, filtered through 40µM nylon membrane and sterilized with 0.1% mercuric chloride for 3 to 5 min and rinsed with sterile distilled water for 15 min showed less frequency of mortality and a high percentage of spore germinations. For *Pronephrium triphyllum*, the spores sown on the Knop's basal agar medium showed the highest percentage (38.3±1.13) of germination. Highest percentage (52.3±1.43) of sporophyte formation was observed in Knop's liquid medium. For *Sphaerostephanos unitus*, the highest percentage (36.8±1.31) of spore germination was observed in the Knop's basal agar. The highest percentage of sporophyte formation was observed only in Knop's medium (76.8±1.41), other media failed to induce sporophyte formation. The in vitro raised plantlets were hardened and established in the natural habitat and distributed to various botanic gardens as a part of ex situ conservation. Cytological and isoperoxidase analysis confirmed the genetic uniformity between mother plants and in vitro raised sporophytes / plants. The established protocol of the present study will be useful for the multiplication and conservation of the two threatened ferns of the Western Ghats. The same protocol may also be applicable to similar threatened ferns.

Keywords: Conservation, ex situ, ferns, isoperoxidase, *Pronephrium triphyllum*, *Sphaerostephanos unitus*, spore.

INTRODUCTION

The Western Ghats is one of the hotspots of the world and also one of the significant geographical regions. Around 233 species of ferns occur in southern India (Manickam & Irudayaraj 1992). In China, South Africa, USA, Europe and Canada, the ferns are used as medicines to cure diseases such as chest complaints, cancer, rheumatism, bowel disorder, ulcer, cough, fever and Alzheimer disease. In China alone, 401 kinds of pteridophytic medicines have been used for various ailments (Luo 1998). The economic value of the ferns has been enumerated by various authors from time to time (Kaur 1989). Today, the diversity of plant life is facing serious threats, largely due to habitat loss, habitat degradation and increasing exploitation of natural resources. It has been estimated that globally 30% of the flora is threatened (Raven 1999). The decline in the number and quality of the habitats is attributed to encroaching urbanization, growing industrialization, intensive farming and unsustainable harvesting of wild species. According to the World Resource Institute, India figures among 28 countries that are facing severe effects of increasing ecological imbalance if preservation is not taken on a war footing. The IUCN report says that in India 7.7% of the plants are under threat. In Western Ghats, a number

of epiphytic and lithophytic ferns are destroyed due to various deforestation activities. In the Western Ghats, 44 threatened ferns are facing extinction and the conservation of these species is a major concern of biologists (Manickam 1995). The establishment of plantations of cash crops like cardamom, coffee, rubber and tea is the main reason for the destruction of the evergreen forests and consequent demise of the ferns in the Western Ghats. A reduction in the anthropogenic pressure on natural populations would contribute to their conservation in nature. Among the various biotechnological options, also reported in other agri-horticultural crops, micropropagation through tissue culture and in vitro spore germination are best applied and commercially exploited in fern species (Fay 1994). Application of this technology (in vitro spore germination) for large-scale multiplication of certain species of ferns from the Western Ghats has been demonstrated (Sara et al. 1998; Manickam et al. 2003; Johnson et al. 2005; Sara & Manickam 2005; Johnson & Manickam 2006; Sara & Manickam 2007; Johnson & Manickam 2007; Johnson et al. 2008). The plant tissue culture as an effective tool to conserve plant genes and guarantee the survival of the endemic, endangered and over exploited genotypes is derived from the fact that it makes use of small units (cells and tissues) without losing the mother plant, takes pressure off the waning wild populations and makes available large numbers of plants for reintroduction and commercial delivery. Endangered ferns such as *Diplazium cognatum*, *Histiopteris incisa*, *Hypodematium crenatum*, *Thelypteris confluens*, *Athyrium nigripes*, *Pteris vittata*, *Metathelypteris flaccida*, *Pteris gongalensis*, *Pteris confusa*, *Cyathea crinita*, *Cheilanthes viridis*, *Pronephrium articulatum*, and *Nephrolepis multiflora* have been multiplied through in vitro spore culture as a part of ex situ conservation (Sara 2001; Johnson 2003; Manickam et al. 2003; Irudayaraj et al. 2003; Vallinayagam 2003). Based on this background, the present investigation was initiated to extend the good work already done in our laboratory to a few other equally endangered species. In the present study in vitro spore culture has been attempted as part of our continued efforts to conserve species of conservation importance and prospective economic value. Reintroduction of the plants so multiplied through spore culture in selected forest habitats hitherto untested in our centre has also been attempted.

MATERIALS AND METHODS

Two rare and endangered ferns from the Western Ghats were selected for the present study viz., *Pronephrium triphyllum* (Sw.) Holttum (Thelypteridaceae) and *Sphaerostephanos unitus* (L.) (Holttum) (Thelypteridaceae). Matured fertile fronds of the selected species were collected from the wild of the Western Ghats and established in the green house attached to the Centre for Biodiversity and Biotechnology, St. Xavier's College, Palayamkottai, India. The fronds were washed in running tap water for a few minutes. The fronds were cut into small pieces and dried over white absorbent paper at room temperature (25°C). After drying the fronds over the absorbent paper at room temperature for 24hr, the liberated spores were passed through 40mm nylon mesh to remove the sporangial wall materials and the clean spores were collected and stored in a refrigerator at 5°C (Images 1a & 2a). The spores were surface sterilized with 0.1% HgCl₂ solution for 5min and washed with sterile distilled water for 15min. The surface sterilized spores were inoculated onto different media viz., Knops (1906), Knudson (1946), Mitra et al. (1976), Moore's (1903), and Murashige & Skoog's medium (1962) devoid of sugar and plant growth regulators using sterile Pasteur pipettes and incubated at 25°C ± 2°C under 12hr photoperiod (1500 lux). The pH of the media was adjusted to 5.8 before adding agar 0.5% (w/v) and autoclaved at 121°C for 15min. Both liquid and agar nutrient media were used for spore germination and sporophyte formation. Gametophytes regenerated from spores were sub-cultured on different basal media (Knops, Knudson C, Mitra et al., Moore's and Murashige and Skoog's medium) for sporophyte formation. Germination percentage of the spores, growth area of the prothalli, and their development pattern were analyzed. Photomicrographs were taken with a labotriumph microscope. The culture tubes containing spore raised micropropagated plants of the two selected species were kept at room temperature (30–32 °C) for a week before transplantation. For acclimatization, the plants with well developed roots (5–8 cm) were removed from culture tubes, washed in running tap water to remove the remnants of agar and each group was planted separately onto a 10cm diameter polycup filled with different potting mixtures: river sand, garden soil

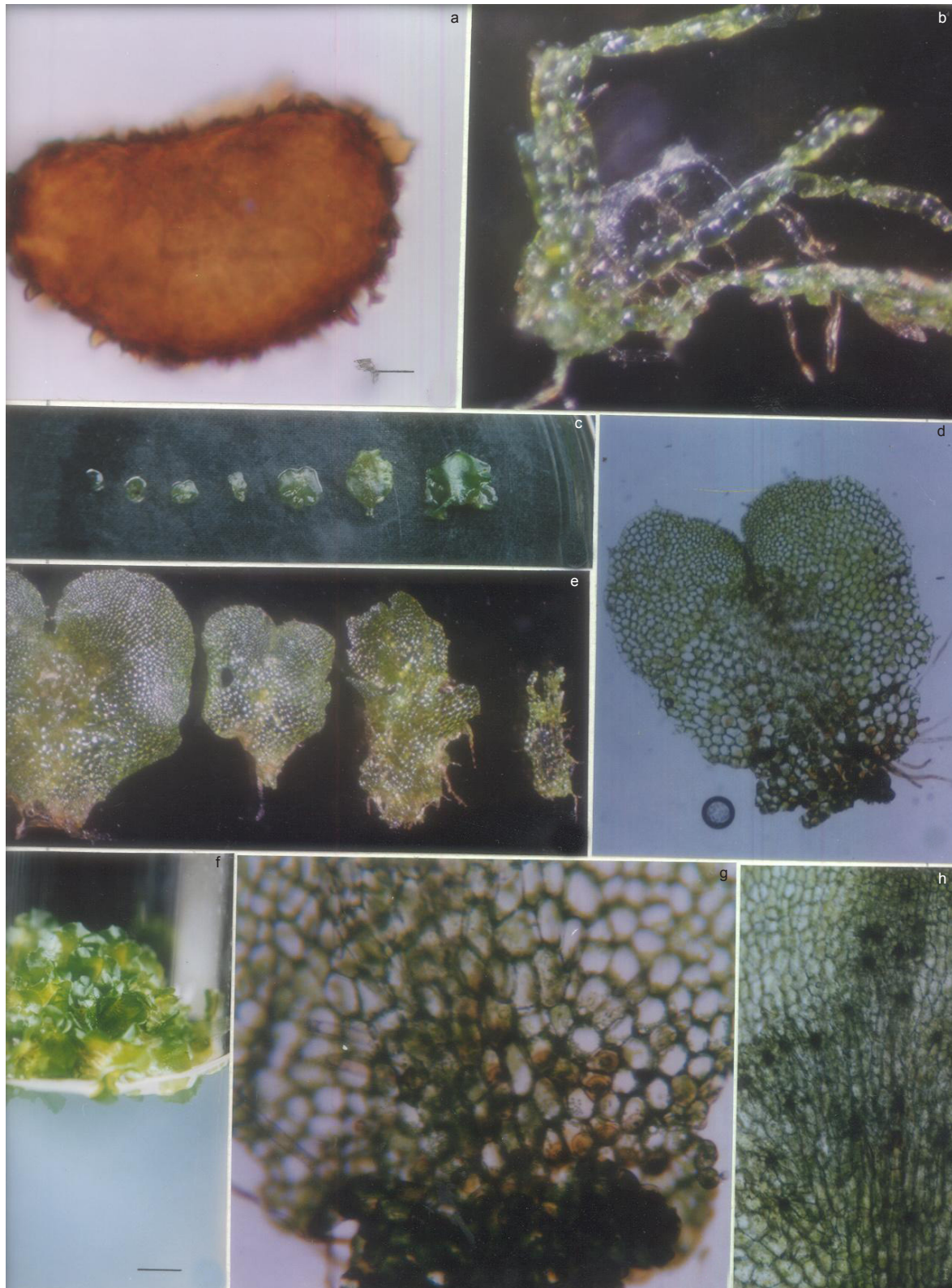


Image 1. In vitro spore culture: Different developmental stages of *Pronephrium triphyllum*

a - Spore (bar 1cm = 4 μ m); b - Filamentous stage (bar 1cm = 250 μ m); c - Different stages of prothalli - Camera view (bar 1cm = 250 μ m); d - Cordate prothallus (bar 1cm = 250 μ m); e - Different stages of prothalli - microscopic view (bar 1cm = 400 μ m); f - Matured prothalli (bar 1cm = 4mm); g - Cordate prothallus with male sex organs (bar 1cm = 400 μ m); h - Cordate prothallus with female sex organs (bar 1cm = 400 μ m)

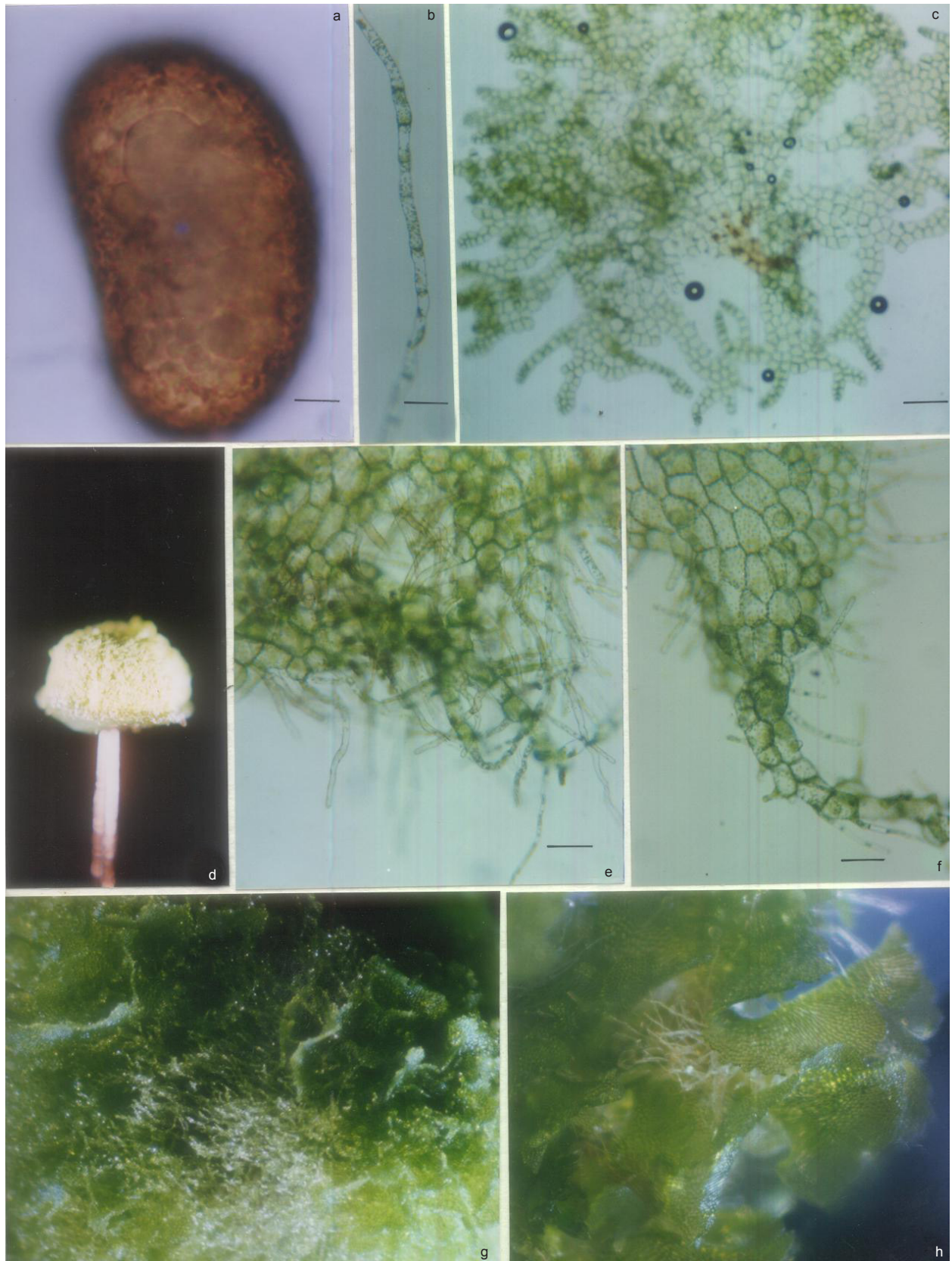


Image 2. In vitro spore culture: Different developmental stages of *Spherostephanous unitus*
 a - Spore (bar 1cm = 4 μ m); b - Filamentous stage (bar 1cm = 200 μ m); c - Different stages of spore germination (bar 1cm = 200 μ m);
 d - Gametophyte - filamentous stage; e - Gametophyte with rhizoids (bar 1cm = 250 μ m); f - Origin of the prothalli (spore - prothalli)
 (bar 1cm = 250 μ m); g - Surface view of gametophytes (bar 1cm = 600 μ m).; h - Cordate prothalli with rhizoids (bar 1cm = 600 μ m)

and farm yard manures (1:1:1) and sand and soil (2:1). The plants were kept in a mist chamber with a relative humidity of 70%. Plants were irrigated at 8hr intervals for 3–4 weeks and establishment rate was recorded. The plantlets established in community pots were transferred to a shade net house for 3–4 weeks and then repotted in larger pots (20cm diameter) with one plant in each pot.

For cytological analysis, the in vitro raised young sporophytes (croziers) and immature sporangia in fertile fronds were squashed in acetocarmine after being fixed in a 1:3:6 mixture of glacial acetic acid, chloroform and 100% ethyl alcohol for 24 hours and then preserved in 95% ethyl alcohol. Mitotic and Meiotic chromosomes were observed in several cells for establishing the correct counts.

For Peroxidase analysis, the explants were ground well in a mortar and pestle with phosphate buffer (pH 7.0) under ice cool condition. The resultant slurry was centrifuged at 10,000rpm for 10min at 4°C in a mikro 22R centrifuge and the supernatant was used as the enzyme source and stored in a 70°C deep freezer. Vertical discontinuous poly acrylamide gel electrophoresis (PAGE) was carried out for separation of isozyme. After the gel running, the gels were incubated in the dark with acetate buffer (pH 4.6) 85ml + Ethanol (10ml), O–Dianisidine (100mg) + 3% H₂O₂ (1ml) + 4ml distilled water for 30min for staining, 7% acetic acid was used to stop the reaction and fixed the gel (Smila et al. 2007). The Isoperoxidase profiles were documented using the Vilber Loubermet Gel Documentation system and the similarity between the mother plants and in vitro spore derived plants were calculated using the Biogene software (Vilber Loubermet, Germany).

RESULTS

Pronephrum triphyllum

Spores collected from the mature fronds showed a variety of contamination and survival rates. On treatment with 0.1% (w/v) HgCl₂ for 5min followed by washing with sterile distilled water for 15min showed 75–80 % of the spores free from the microbial contamination. The young spores showed a high percentage of mortality, even with a short duration of exposure to the sterilants. Spores were cultured

in liquid and solid basal media (Knudson, Knop's, Mitra, Moore's and Murashige and Skoog's). Microbial contamination was more in the liquid media compared to the solid media. Spore germination time and germination percentages were dependent on the composition of the media. The spores sown on the Knop's basal agar medium showed the highest percentage (38.3±1.13) of germination, followed in order, by the Moore's, Mitra and Knudson C media respectively (22.3±0.81, 21.3±0.83 and 15.3±1.21). The time taken for spore germination also varied. In Knop's medium spores germinated after 38 days, while in other media they took a much longer time for germination. The pattern of germination is of Vittaria type. After 3–4 weeks, repeated longitudinal and transverse division of the anterior cells of protonema and expansion of the resultant daughter cells formed the prothallial plate. The prothallus was cordate type. The prothallus development was Drynaria type. Highest percentage of prothallus (81.3±1.34) formation was observed in Mitra medium. The thallus was dioecious, dorsiventrally flattened which developed a midrib region with a cushion like structure and notched apical region.

The glandular hairs were present along the margin and the midrib regions. The sex organs and rhizoids originated from the midrib region. After 120 days, the male sex organs, antheridias were formed on the posterior end. After 160 days, the female sex organs, archegonias were formed on the anterior end (Images 1b–h). For sporophyte proliferation, the 180 day – old gametophytes were transferred to Knudson C, Knop's and Mitra liquid media. After 30 days, the sporophyte emerged from the midrib region on Knops liquid medium. After 15 days, the sporophytes were transferred to agar medium for sporophyte elongation. The highest percentage (52.3±1.43) of sporophyte formation was observed in Knop's liquid medium compared to the other two media [Knudson C (16.5±1.31) and Mitra (11.3±0.81)] (Table 1). After 15 days of rooting, the in vitro derived plantlets were washed thoroughly in running tap water to remove the pieces of agar adhering to the roots and implanted in the pots containing a mixture of (1:2:1) sterile soil: sand: farmyard manure irrigated with 10 x diluted Murashige and Skoog's / Knudson C liquid medium once a week. The pots were covered with poly bags to maintain the humidity. The plantlets were kept in a culture room

Table 1. Effect of medium on spore germination, development of gametophyte and sporophyte and field establishment in *Pronephrium triphyllum*

Medium & pH	% of Germination ± S.D.	% of Prothalli formation ± S.D.	% of Sporophyte formation ± S.D.	Mean no. of croziers/prothallus ± S.D.	Mean length of Sporophyte ± S.D.	% of establishment in polycups ± S.D.	% of establishment in Pots ± S.D.	% of establishment in field ± S.D.
KC Solid 5.8	15.3±1.27	67.3±1.31	-	-	-	-	-	-
KN Solid 5.8	38.3±1.13	72.3±1.08	-	-	-	-	-	-
Mi Solid 5.5	21.3±0.83	81.3±1.31	-	-	-	-	-	-
MO Solid 5.5	22.3±0.81	68.4±1.34	-	-	-	-	-	-
KC Liquid	-	-	16.5±1.31	1.78±0.34	1.12±0.16	73.5±1.24	78.4±1.31	69.3±1.21
KN Liquid	-	-	52.3±1.43	4.0±1.63	1.38±0.74	82.3±1.31	79.3±1.31	72.3±1.24

Table 2. Effect of medium on spore germination, gametophyte, sporophyte formation and field establishment of *Sphaerostephanos unitus*

Medium	% of Germination ± S.D.	% of Prothalli formation ± S.D.	% of Sporophyte formation ± S.D.	Mean no. of croziers/prothallus ± S.D.	Mean length of Sporophyte ± S.D.	% of establishment in polycups ± S.D.	% of establishment in Pots ± S.D.	% of establishment in field ± S.D.
KN Solid	36.8±1.31	73.8±1.31	76.8±1.41	3.2±1.13	6.3±1.38	79.8±1.34	83.1±1.21	74.8±1.31
KC Solid	26.3±1.30	74.8±1.28	-	-	-	-	-	-
MS Solid	-	-	-	-	-	-	-	-
Mitra	34.8±1.21	66.3±1.21	-	-	-	-	-	-

for 15 days. After that, they were transferred to a green house (R.H. 80%) under constant misting. After three weeks the plants were transferred to the field. The micropropagated plants showed 73.5±1.24% establishment during hardening and 72.3±1.24% establishment in the field at KBG. Subsequently the micropropagated plants were distributed to various botanic gardens for ex situ conservation. (Table 1) (Image 4c,d).

Sphaerostephanos unitus

Matured spores were used for culture initiation. The percentage of microbial contamination was less when the spores were treated with 0.1% HgCl₂ (w/v) for 5min and washed thoroughly using sterile distilled water for 15min. The survival of explants depended on the duration of the treatment with sterilants and the prolonged exposure (6–10 min) to 0.1% HgCl₂ resulted in high percentage mortality. The spores were cultured in hormone free liquid and solid media (Knudson C, Knop's, Murahige & Skoog's and Mitra). In the liquid media, the inoculated spores failed to germinate due to the high incidence of microbial contamination. After 35 days, the spores started to germinate in knops

agar medium. The germination pattern was Vittaria type. The prothallial plate was formed after 30 days of culture, due to the repeated divisions of the cells. The prothallus development was Drynaria type. The thallus was dorsiventrally flat with an apical notch. The prothalli were cordate type. A high percentage of prothalli formation (74.8±1.21) was observed in Knudson C medium. Glandular hairs were present on the margin and central areas of the gametophyte. The male and female sex organs formed on the midrib region. The male and female sex organs formed after 120 and 150 days respectively. The sporophyte emergence was noticed in the midrib regions after 180 days with the formation of sporophyte and root initials (Image 2b–h). The highest percentage (36.8±1.31) of spore germination was observed in the Knop's basal agar. Germination was not observed on the Murashige and Skoog's basal medium. The highest frequency of gametophyte formation and multiplication (74.8±1.28) were observed in Knudson C basal medium. The sporophyte formation was observed only in Knop's medium, that too at a high (76.8±1.41) percentage; there was no formation of sporophyte in other media (Table 2). Knop's medium also promoted the formation and

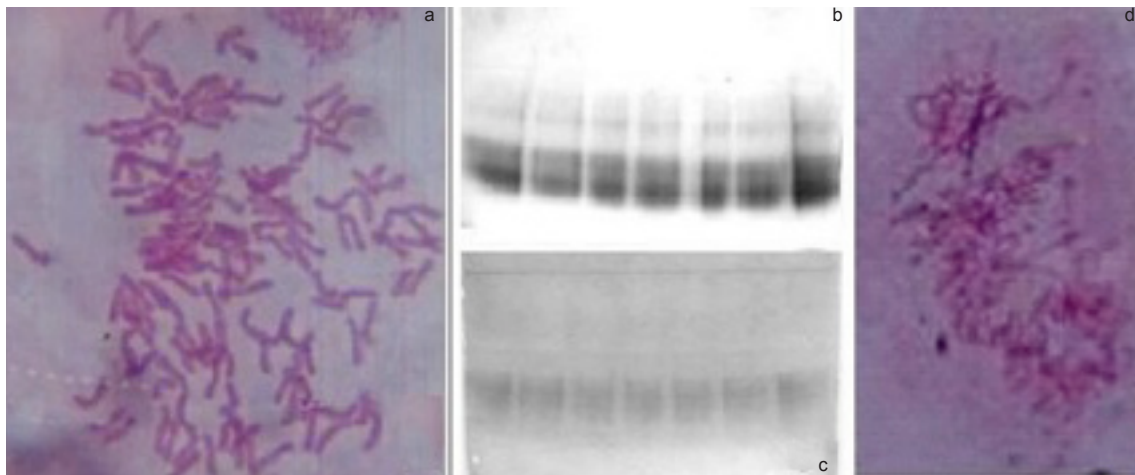


Image 3. Isoperoxidase profile and cytological studies on in vitro raised sporophytes and mother plants of *P. triphyllum* and *S. unitus*

a - *P. triphyllum* (Mitosis); b - *P. triphyllum* - Isoperoxidase Profile; c - *S. unitus* - Isoperoxidase Profile; d - *S. unitus* (Mitosis)



Image 4. Hardening and field establishment of in vitro raised plantlets of *P. triphyllum*, *S. unitus*

a - *S. unitus* - hardened plants; b - Micropropagated & hardened plants of *S. unitus* - reintroduced into KBG;
c - *P. triphyllum* - hardened plants; d - Micropropagated & hardened plants of *P. triphyllum* - reintroduced into KBG

elongation of rhizoids. After 30 days of rooting, rooted plants were hardened in polycups containing a mixture (1:2:1) of sand: garden soil: farmyard manure, covered with unperforated poly bags and irrigated with 10 x diluted MS liquid medium once a week. The plants

were kept in the culture room for 15 days. Seventy eight percentage of the plants were successfully established in poly cups. After 15 days, the hardened plants were transferred to 15cm diameter pots and kept in the green house. Eighty-five percentage of plants

Table 3. Growth of micropropagated plants of *P. triphyllum* and *S. unitus* re-established into Kodaikannal Botanic Garden at Kodaikanal, Tamil Nadu, India

Plant species	No. of plants transferred to polycups	% of establishing in polycup	No. of plants transferred to pots	% of establishment in pots	No. of plants transferred to field	% of establishment
<i>P. triphyllum</i>	375	73.5	275	77	120	85
<i>S. unitus</i>	300	79	235	73	120	75

were well established in the green house. After six months, the plants, 34cm in height, having 10 to 12 croziers were repotted, for distribution to various Botanic Gardens, such as the Calicut University Botanic Garden, Kozhikode; TBGRI, Trivandrum; Gurukula Botanic Garden, Wyanad and Genepool Garden, Gudalur for ex situ conservation. Many plants were also transferred to their natural settings in the Kodaikanal Botanic Garden, Kodaikanal for field establishment (Table 3) (Images 4a–d).

Isoperoxidase analysis revealed the genetic uniformity between the mother plants and in vitro spore raised sporophytes. The MW – Rf values and banding positions confirmed 100% genetic uniformity. In addition they provided the biochemical marker for the two selected species. *P. triphyllum* (Image 3b) showed three different bands in three different active regions (0.510, 0.664 and 0.764). *S. unitus* (Image 3c) showed only two bands in two different active regions (0.478 and 0.536). Cytological studies on root tips of ten randomly selected plants established in KBG revealed the presence of 144 chromosomes in *P. triphyllum* (Image 3a) and 72 chromosomes in *S. unitus* (Image 3d) confirming the mother plants chromosomes.

DISCUSSIONS

Spores are tiny objects which are used liberally by ferns for reproduction. A spore contains only half the normal chromosome number and no embryo. The single celled spores are excellent experimental material on par with pollen grains and isolated cells of higher plant species. Observations over the past twenty years reveal that spores are produced in huge numbers by nature, but the percentage of spore germination and their developmental physiology rate is very poor due to unfavourable conditions. Each and every species requires their unique environment for their growth and development; most of the rare and endangered

ferns failed to obtain the optimal growth condition for their development. The present study also confirmed the previous observations. However, there are a few published reports on successful germination of spores in vivo (Theuerkauf 1994). Under natural conditions, the percentage of spore germination is low due to the prevalence of unfavourable factors, both biotic and abiotic. It is not unusual that the spores are dispersed by wind to places unfavourable for their germination. The spores otherwise having little stored food materials, seldom germinate in the wild. The spores can germinate under in vitro conditions easily. The in vitro spore culture methods have advantages over soil based conventional methods. The in vitro culture techniques have been used to study different aspects of spore germination, growth and development of gametophytes and sporophytes in ferns (Nester & Coolbaugh 1986; Hickok et al. 1987; Miller & Wagner 1987; Melan & Whittier 1990). However there are number of factors such as temperature, humidity, light, and nutrient compositions (Raghavan 1989) which need to be addressed for successful in vitro spore culture. Tissue culture which tends to be more sophisticated than spore culture is also advocated and successfully explored for horticulture propagation of selected ferns (Hennen & Sheehan 1978; Padhya & Mehta 1981; Higuchi et al. 1986). Successful culture initiation, be it spore culture or tissue culture, depends on a number of physical and chemical factors. A number of workers have studied spore germination under the influence of various physiological and chemical parameters (Mehra & Palta 1971; Sharma & Vangani 1988; Sharma & Sharma 1991). In the present investigation also, the spores of all the selected species were cultured under varied conditions with the object of developing viable protocols for mass multiplication and conservation. In a comparative perspective, spore cultures are more desirable for rare species conservation than tissue culture as it retains the genetic variability inherent in the genetic make up of a species. Nowadays, accurate

recordings of the genetic uniformity and chemical characterization of the plants are needed to be verified before conservation. Analysis of isoenzyme banding profile is considered to be one of the best and cheapest system for the analysis of population structure, genetic uniformity and developmental pattern, due to its role in the metabolic pathway. It functions in harmony with other enzymes within the organizational framework of cells and Isoenzyme often exhibits tissue or cell specificity (Zeidler 2000). These banding profiles are useful in differentiating the selected species and the induced variants. In the present study also, Isoperoxidase studies revealed the biochemical uniformity between the mother plants and the in vitro spore raised plants. The earlier reports are directly consonant with the present study and strengthen the role of isoperoxidase in the study of genetic uniformity (Nair 2000; Johnson 2003; Nikhat 2004; Sonali 2004). Since the 1960s, Electrophoresis coupled with isoenzyme has been the tool of choice for studies of heritable variation by geneticist, systematist and population biologist (Zeidler 2000). The isoperoxidase profiles will be used as a taxonomic tool for the characterization of the two important ferns in the future. The present study completely demonstrated the life cycle and reproductive biology of the two rare and endangered ferns of the Western Ghats, India. The established protocol of the present study will be useful for the multiplication and conservation of the two threatened ferns of the Western Ghats. The same protocol may also be applied to similarly threatened conserved ferns.

REFERENCE

- Fay, M.F. (1994). In what situations is *in vitro* culture appropriate to plant conservation? *Biodiversity and Conservation* 3: 176–183.
- Hennen, G.R. & T.J. Sheehan (1978). *In vitro* propagation of *Platyserium stemaria* (Beauvois) Desv. *Hortscience* 13: 245.
- Hickok, L.G., T.R. Warne & M.K. Slocum (1987). *Ceratopteris richardii*: Applications for experimental plant biology. *American Journal of Botany* 59: 458–465.
- Higuchi, H., W. Amaki & S. Suzuki (1986). *In vitro* propagation of *Nephrolepis cordifolia* (L.) Presl. *Scientia Horticulturae* 32: 105–113.
- Irudayaraj, V., V.S. Manickam & M. Johnson (2003). Incidence of Apospory in *Pteris confusa* T. G. Walker. *Phytomorphology* 53(1): 73–77.
- Johnson, M. (2003). *In vitro* studies on some rare and endangered ferns of Western Ghats, south India. PhD Thesis. Manonmanium Sundarnar University, Tamil Nadu, India.
- Johnson, M., V.S. Manickam & V. Irudayaraj (2005). *In vitro* studies on the agamsporous fern (*Pteris gongalensis* T. G. Walker). *Ethiopian Journal of Science and Technology* 3(1): 1–8.
- Johnson, M. & V.S. Manickam (2006). Adventitious Proliferation of secondary and tertiary prothalli from the primary prothalli of *Pronephrum articulatum* (Houlst. & Moore) Holt. *Ethiopian Journal of Science and Technology* 3(2): 93–96.
- Johnson, M. & V.S. Manickam (2006). *In vitro* studies on normal and abnormal life cycle of *Metathelypteris flaccida* (Bl.) Ching. *Ethiopian Journal of Science and Technology* 4(1): 37–44.
- Johnson, M., V.S. Manickam, A. Benniamin & V. Irudayaraj (2008). Conservation of endangered ferns of Western Ghats through micropropagation, pp. 183–191. In: Verma, S.C., S.P. Khuller & H.K. Cheema (eds.). *Perspective in Pteridophytes*. Bishen Singh Mahendra Pal Singh, Dahrudun, India.
- Kaur, S. (1989). Economic exploitation and conservation emerging areas in the study. *Indian Fern Journal* 6: 23–29.
- Knops, N. (1885). Quantitative Untersuchungen über die Ernährungsprozesse der pflanzlichen Land wirtsch Vers, stn. 7: 93–107.
- Knudson, L. (1946). A nutrient solution for the germination of orchid seed. *Bulletin of American Orchid Society* 15: 214–217.
- Luo, G.H. (1988). Medicinal pteridophytes in China. pp. 309–312. In Singh, K.H. & K.C. Kramer (eds.). *Proceedings of the International symposium on systematic pteridology China Science and Technology Press, Beijing China*.
- Manickam, V.S. (1995). Rare and endangered ferns of the Western Ghats of South India. *Fern Gazette* 15: 1–10.
- Manickam, V.S. & V. Irudayaraj (1992). Pteridophyte flora of the Western Ghats - south India, B.I. Publications New Delhi, India.
- Manickam, V.S., S. Vallinayagam & M. Johnson. (2003). Micropropagation and Conservation of rare and endangered ferns of Western Ghats through in vitro culture. pp: 497–504. In: Chandra, S. & M. Srivastava (eds.). *Pteridology in the New Millennium*. Kluwer Academic Publishers, Netherlands.
- Medina, R., M. Faloci, M.A. Marassi & L.A. Mroginski (2004). Genetic stability in rice micropropagation. *Biocell* 28(1): 13–20.
- Mehra, P.N. & H.K. Palta (1971). *In vitro* controlled differentiation of the root callus of *Cyclosorus dentatus*. *Phytomorphology* 21: 367–375.
- Melan, M.A. & D.P. Whittier (1990). Effects of inorganic nitrogen sources in spore germination and gametophyte growth in *Botrychium dissectum*. *Plant cell and environment* 13: 477–482.
- Miller, J.H. & M.P. Wagner (1987). Co-requirement for

- Calcium and Potassium in the germination of spores of the fern *Onoclea sensibilis*. *American Journal of Botany* 74: 1585–1589.
- Mitra, G.C., R. N. Prasad. & R. Chowdhury (1976).** An inorganic salts and differentiation of protocornes, in seed callus of an orchid and correlated changes in free amino acids content. *Indian Journal of Experimental Biology* 14: 350–351.
- Moore, G.T. (1903).** Methods of growing pure cultures of algae. *Journal of Applied Microscopy* 6: 2309–2314.
- Murashige, T. & F. Skoog. (1962).** A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum* 15: 473–497.
- Nair, L.G. (2000).** Conservation through micropropagation restoration of selected woody medicinal plants. PhD Thesis. Kerala University, Thiruvananthapuram, Kerala, India
- Nester, J.F. & R.C. Coolbaugh (1986).** Factors influencing spore germination and early gametophyte development in *Anemia mexicanum* and *Anemia phyllitidis*. *Plant physiology* 82: 230–235.
- Nikhat, Y. (2004).** *In vitro* multiplication and electrophoretic studies on *Passiflora mollissima* H.B. K. Bailey. MPhil Thesis. Periyar University, India.
- Padhya, M.A. & A. R. Mehta. (1981).** Propagations of fern (*Nephrolepis*) through tissue culture. *Plant cell Reports*. 1: 261–263.
- Raghavan, V. (1989).** Developmental biology of fern gametophytes. Cambridge University press Cambridge, 27–53pp.
- Raven, P.H. (1999).** World's biodiversity becoming extinct at levels rivaling earth's past "mass extinctions". *Botanic Garden Conservation News* 3: 31–32.
- Sabu, K.K., P. Padmesh & S. Seeni (2001).** Estimation of active principle content and isozymes of *Andrographis paniculata* Nees (Kalmegh): An important medicinal plant of India. *Journal of Medicinal Aromatic Plant Science* 23: 637–647.
- Sara, S.C. (2001).** Conservation of selected rare and endangered ferns of the Western Ghats through micropropagation and restoration. PhD Thesis. Manonmaniam Sundaranar University, Tirunelveli, India.
- Sara, C.S. & V.S. Manickam (2005).** *In vitro* propagation of the critically endangered fern *Cyathea crinita* (Hook.) Copel. *Asian Journal of the Microbiology Biotechnology Environmental Science* 7: 527–536.
- Sara, C.S. & V.S. Manickam (2007).** *In vitro* developmental ontogeny and life cycle of a rare fern species - *Thelypteris confluens* (Thunb.) Morton. *Indian Journal of Biotechnology* 6: 372–380.
- Sara, S.C., V.S. Manickam & R. Antonisamy (1998).** Regeneration in kinetin - treated gametophytes of *Nephrolepis multiflora* (Roxb.) Jarret in Morton. *Current Science* 75: 503–508.
- Sharma, A. & B.D. Sharma. (1991).** Hitherto unreported methods for the multiplication of ferns. *Phytomorphology* 41: 271–274.
- Sharma, B.D. & P. Vangani (1988).** Effects of gibberellin (GA3) on development and sex expression in the gametophytes of *Cheilanthes farinos* (Forssk.) Klf. *Indian Fern Journal* 5: 1–4.
- Smila, H., M. Johnson & M. Rajasekarapandian (2007).** Studies on varietal difference, tissue specificity and developmental variation of esterase and peroxidase isozymes in pearl millet (*Pennisetum glaucum* (L.) R. Br.). *Indian Journal of Biotechnology* 6: 91–99.
- Sonali, D. (2004).** Micropropagation and *Intra* specific variation studies on *Vitex negundo* L. MPhil Thesis, Periyar University, India.
- Theuerkauf, W.D. (1994).** Preserving southern Indian pteridophytes. *Botanic Garden Conservation News* 2: 54–55.
- Vallinayagam, S. (2003).** Micropropagation of rare and endangered ferns of Western Ghats. PhD Thesis. Manonmaniam Sundaranar University, Tirunelveli, India.
- Zeidler, M. (2000).** Electrophoretic analysis of plant isozymes. *Acta Universitatis Palackianae Olomucensis, Facultas Rerum Naturalium Biology* 38: 7–16.

Author Details: DR. M. JOHNSON standardized the large scale multiplication protocol for thirteen rare and endangered ferns of the Western Ghats under the Ministry of Environment and Forest, New Delhi sponsored project. He has also published about 66 papers in national and international journals on large scale propagation, phytochemical, antimicrobial activity and isozymic profile of Indian medicinally important plants.

REV. DR. V.S. MANICKAM carried out several projects sponsored by DST, DBT, Moen, UGC. During the last twenty five years he has surveyed Pteridophytes of the Western Ghats and Angiosperms of Tirunelveli Hills. He coordinated the All India Coordinated Project on Taxonomy for Pteridophytes and Gymnosperms. He has completed several projects on ex situ conservation of several rare and endangered ferns and angiosperms. He has published six books and more than 300 research papers.

