In-vitro Mass Multiplication of a Threatened Tree Fern, Cyathea spinulosa Wall. ex Hook

Shastri P. Shukla1* and P.B. Khare2

1Department of Plant Sciences, University of Hyderabad, Hyderabad-500 046, A.P., India
2Pteridology Laboratory, National Botanical Research Institute (CSIR, New Delhi) Lucknow -226 001, U.P., India
E-mail: kharepb@yahoo.com
*Corresponding Author E-mail: sp_shukla2002@yahoo.co.in

Abstract

In this study an attempt was made to standardize the in vitro protocol for the mass multiplication and conservation of a threatened tree fern Cyathea spinulosa Wall. ex. Hook employing leaf primordium explants excised from in vitro-raised sporophytes through spore culture. Calli were induced from the explants on Parker’s and Thompson (P&T) media supplemented with 8.87 µM 6-benzylaminopurine (BAP) and 2.21µM 2, 4-dichlorophenoxyaceticacid(2,4-D).Maximum multiple shoots (12.5±0.45) were differentiated from callus and elongated on P&T media with 4.52 µM BAP and 5.36µM α -naphthalene acetic acid (NAA). In vitro-raised shoots rooted on P&T with 2.24µM indole-3-butyric acid (IBA).The well rooted micropropagated plantlets were transferred in the fernery of NBRI, Lucknow.

Keywords: Cyathea spinulosa, conservation, micropropagation, tree fern.

Abbreviations
BAP-6-Benzylaminopurine, 2, 4-D- 2, 4-Dichlorophenoxyaceticacid, IBA- Indole-3-butyric acid,NAA-α-Naphthaleneaceticacid, NaOCl-Sodium hypochlorite, NBRI-National Botanical Research Institute, PGRs-Plant growth regulators, P&T -Parker’s &Thompson media, PVP-Polyvinyl pyrrolidone.

Introduction
Cyathea spinulosa is widely distributed and reported from North Western Himalaya.
(Chamoli), Eastern Himalaya (Darjeeling), North Eastern India and Central India (Pachmarhi, M.P. India). After extensive survey during 2003-2006, it has been noticed that now this species occur in scattered patches due to habitat destruction, urbanization, tourism including natural calamities and over-exploitation. It has been observed that mature plants are being cut and trunk of this species is extensively used to making of logs for the orchid cultivation and starch extraction.

In view of the over exploitation and its threatened state, it is paramount importance to conserve this taxon. Conventionally C.spinulosa is propagated by spores with major constraints like; low viability of the spores, slow and uneven growth and not hundred percent results oriented and reliable. The reproductive biology indicated that this species may be good colonizer but fertilization success is solely dependent on the prolonged availability of water at the time of maturity of sexes and raising of the plants through spores are depend upon several climatic factors. Keeping these in view, the investigation has been undertaken an investigation on formulating strategies for the conservation of C.spinulosa through in vitro mass multiplication.

Plant tissue culture has been used for off-site conservation and micropropagation but in comparison to higher plants very little work is reported on pteridophytic plants. Vascular cryptogams, especially ferns have not been very favorable material for tissue culture because its vascular system made up of highly differentiated tissues that are unlikely to proliferate into cell masses capable of growth in vitro and lack of secondary activities. Although many species of ferns have been successfully propagated by tissue culture techniques. Only a few studies on the genera Cyathea were reported and none on C.spinulosa. These studies also have not concentrated on efficient organogenesis and successful acclimatization of in vitro-raised plantlets. Present study indicated that plantlets of C.spinulosa can be obtained through in vitro culture of leaf primordium and propagation of this threatened fern is possible, independent of the season and climate. In this paper, we report regeneration of C.spinulosa through organogenesis in calli cultures from leaf primordium explants obtained through in vitro-raised sporophytes and conservational practices of this species in natural conditions.

The spores were collected from the plants growing in Pamtori locality of Kumaon Himalayas, Uttarakhal and stored at room temperature in a desiccator in laboratory. After drying, the spores were surface sterilized with 2% NaOCl for two min. and washed with sterile single distilled water for 3-4 times. Sterilized spores were sown onto P&T media in glass petridishes of size 80x70 mm. Media were solidified with 0.8% agar (HI MEDIA, Mumbai) and pH of the media was adjusted to 5.6 prior to autoclaving. Spore suspension was sown on the media using Pasteur pipettes and petriplates were sealed with Parafilm. When the gametophytes were spatulate shaped, they were picked up from the stock culture and 40-45 gametophytes were placed on the petridishes containing P&T media. After attainment of sexes in the gametophytes these populations were flooded with water to facilitate the fertilization and production of sporophyte scored out.

All the cultures were incubated in culture room at 22±2°C temp. under 16h/8h light/dark cycles (artificial light 47.3-56.8 µ mol m⁻²s⁻¹). Germination percentage of
the spores, sporeling emergence, growth of prothalli and developmental pattern were
analyzed at regular time intervals and photographs were taken using a Nikon camera
UP-II.

The circinate parts of the young leaf primordium (0.5 cm.) were excised from in
vitro-raised sporophytes of C. spinulosa. Explants were collected in a beaker
containing tap water and washed with running tap water for 10 min. and surface
sterilizes with 0.1 % HgCl$_2$ for 1 min. followed by 3-4 rinses in sterile distilled water.
Sterilized explants were placed on the P&T media with different combinations of 2, 4-
D (2.26-9.04µM) and BAP (0.44-4.43µM) alone and in combinations with 4%
sucrose also.

Experiments were performed with a minimum of 10 replicates and were repeated
thrice. After two weeks callusing observed on explants which was divided into two
equal halves and one half subcultured in the same medium (2, 4-D 9.04µM and BAP
2.21µM) while the other transferred to regeneration medium i.e.; P&T media fortified
with BAP (2.21—9.04µM) and NAA (5.36µM) alone or in a combinations for shoot
differentiation. The multiple shoots induced on BAP 4.43 µM and NAA 5.36µM
elongated well on the same medium. Well elongated shoots were shifted to half and
full strength of P&T media with IBA (1.12-8.96µM) alone or in combination with
BAP (0.44µM) for root induction.

After the acclimatization of the in vitro-raised plantlets in culture room transferred
to the fernery NBRI, Lucknow.Standard error is given to indicate the variation among
the means of three experiments based on 10 replicates for each treatment. Analysis of
variance (ANOVA) was done by completely randomized block design (CRBD) using
99 software for all the experiments.

Spores germinated at the frequencies of 50-65 % after 7 days on P&T media
(Fig.1 a). During the study it is observed that low concentrations of macro-and micro-
salts was suitable for spore germination and early gametophyte development as
reported earlier in Cyathea australis, C. schanschin and Dicksonia solviana$^{12,13,14}$.This
observation is consistent with the earlier report that high concentrations of macro-and
micro- salts retarded spore germination and sporophyte formation in Adainatum
raddianum cv.Tassel$^{15}$and contradict with Blechnum spicant spore culture$^{16}$.After
germination, the spores differentiated into a cordate shaped thallus within 30 days
after sowing. The mature gametophytes were spatulate thalloid, having multicellular
hairs on the midrib of the gametophyte (Fig.1 b&c) and due to proliferation, a
gametophyte in due course of time becomes a composite gametophytes, which
increases the chances of production of sporophytes (Fig.1 d). Freshly-harvested leaf
primordium excised from the in vitro-raised sporophytes was found suitable sources
of explants for culture establishment. Initially, due to leaching of phenolic compounds
into the medium from the cut ends of explants adversely affected the culture
establishment. To overcome this leaf primordium were treated with chilled
antioxidant solution consisting of citric acid and ascorbic acid.

Browning of the medium limiting both the growth and differentiation of the
shoots. Incorporation of 20 mg/l PVP, 100 mg/l ascorbic acid and 0.02% activated
charcoal in culture medium prevented the browning and necrosis of the cultures$^{17}$
.P&T media supplemented with 2, 4-D 9.04µM and BAP 2.21µM was found most
suitable for the induction of deep-yellow greenish callus from the cut surface of the leaf primordium explants excised from in vitro-raised sporophytes after two weeks (Fig.1 e, Table-1). The same media did not respond for shoot differentiation from the callus. For most of the studies on caulogenesis in ferns, 2, 4-D alone was reported effective for callus induction but in the present study 2, 4-D alone was found ineffective and in media devoid of growth regulators, explants remained fresh for one week and then turned necrotic. These findings are similar with the published reports on callus induction in a homosporous fern, Pteris vittata. Generally the nutritional requirements of ferns for callus induction and differentiation have been reported to be simple but present observations reveal that excellent growth and development of callus and their differentiation occurred on complex media like P&T supplemented with 2,4-D, BAP and NAA. The callus induction media (with 2, 4-D 9.04µM and BAP 2.21µM) promoted formation of meristemoid zones instead of shoot differentiation during subculture (Fig.1 f). After the subculturing of the pieces of calli on P&T media with different concentrations of 2, 4-D, BAP and NAA alone and in combinations, 12.5±0.45 shoots of length 8.3±0.6 cm were produced with BAP 4.43µM and NAA 5.36µM after four weeks as reported earlier on same combinations of growth regulators (Fig.1 g, Table-2). The developing shoots of more than 4.5 cm were excised and used for root induction. Interestingly it has been observed that full and half-strength of P&T media incorporated with IBA (1.12µM), BAP (0.44µM) alone and in combinations and without PGRs had no effect on root induction. Among all the used combinations, maximum (85%) root induction (10.5±0.7 roots of length 10.0±0.5mm) was observed on P&T media supplemented with 2.24µM IBA alone compared to the roots (8.50±0.5 roots of length 9.50±0.5mm) produced on the media containing BAP 0.44µM and IBA 2.24µM after two weeks (Fig.1 h, Table-3). On higher (more than 2.24µM) concentrations of IBA, the number of roots and length was reduced.

The rooted plantlets were acclimatized in a solution consisting of only the inorganic salts of P&T media before their final transplantation to potted soil. The plantlets along with their roots were carefully taken out of the petridishes. Roots of the plantlets were thoroughly washed under running tap water to remove the entire adhering nutrient agar. After rinsing with distilled water, they were acclimatized in inorganic salt solution at least for a period of ca. 20 days (Fig.1 i). The nutrient solution was periodically changed after every 5 days while the root system of plantlets was also thoroughly washed with distilled water.

The acclimatized plants were transplanted in small earthen pots containing a mixture of soil and leaf mould in the ratio of 2:1 (Fig.1 j). The earthen pots along with potting mixture were sterilized by autoclaving at 0.7-kg/cm² pressure for 10 min. before transplantation. The plants were initially covered with acclimatization chambers for a period of 4 to 8 days after transfer in liquid as well as in potted soil to prevent them from desiccation. Plants of C. spinulosa were later grown under Fern House conditions to observe their performance. Acclimatization process was done by exposing the plants to the Fern House condition. The acclimatization hood was removed periodically and after a month removed finally. The in vitro-raised plantlets showed more than 80% survival and normal growth under the fern house condition of
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NBRI, Lucknow (Fig.1 k). On the basis of the previous work on Nephrolepis biserrata27 carried out in same laboratory during the course of hardening a standardized acclimatization procedure (liquid culture phase) applied of all in vitro-regenerated plants of C.spinulosa in order to get 100 percent transplantation success in soil. The liquid culture phase for acclimatization of in vitro-raised plants had also been found necessary before transplantation in soil in case of Nephrolepis biserrata whereas in case of Cheilanthes viridis, Diplazium cognatum and Matteuccia struthiopteris direct plantlet transplantation in soil was successful28,29 which may be due to tropical rain condition of the area which was not prevailing in other areas including the present study where through liquid culture phase was reported to be essential for acclimatization. Thus in the present study efforts have been made for the ex situ conservation of this threatened tree fern.

Table 1: Effect of PGRs on P&T supplements on callus induction from in vitro-raised leaf primordium explants of Cyathea spinulosa after two weeks.

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Different concentrations and combinations of PGRs used (mg l⁻¹)</th>
<th>Percentage of response</th>
<th>Type of callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>P&amp;T</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>P&amp;T + 2,4-D 0.5 + BAP 0.1</td>
<td>60.2 ± 0.7</td>
<td>C,G,Md</td>
</tr>
<tr>
<td>3.</td>
<td>P&amp;T + 2,4-D 1.0 + BAP 0.1</td>
<td>65.3 ± 0.9</td>
<td>C,G,Md</td>
</tr>
<tr>
<td>4.</td>
<td>P&amp;T + 2,4-D 1.5 + BAP 0.5</td>
<td>75.2 ± 0.4</td>
<td>C,P,Md</td>
</tr>
<tr>
<td>5.</td>
<td>P&amp;T + 2,4-D 2.0 + BAP 0.5</td>
<td>95.5 ± 0.8</td>
<td>C,P,Md</td>
</tr>
<tr>
<td>6.</td>
<td>P&amp;T + 2,4-D 2.5 + BAP 1.0</td>
<td>66.2 ± 0.2</td>
<td>C,P,Md</td>
</tr>
<tr>
<td>7.</td>
<td>P&amp;T + 2,4-D 3.0 + BAP 1.0</td>
<td>72.2 ± 0.3</td>
<td>C,G,Md</td>
</tr>
<tr>
<td>8.</td>
<td>P&amp;T + 2,4-D 1.0 + BAP 1.0</td>
<td>65.6 ± 0.9</td>
<td>C,P,Lg</td>
</tr>
<tr>
<td>9.</td>
<td>P&amp;T + 2,4-D 1.5 + BAP 1.0</td>
<td>75.7 ± 0.5</td>
<td>C,G,Md</td>
</tr>
<tr>
<td>10.</td>
<td>P&amp;T + 2,4-D 2.0 + BAP 1.0</td>
<td>68.2 ± 0.4</td>
<td>C,P,Lg</td>
</tr>
</tbody>
</table>

C-Compact, G-Green, Md-Moderate, P-Pale green

Table 2: Effect of PGRs on differentiation of the shoots from leaf primordium calli of Cyathea spinulosa after four weeks.

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Different concentrations and combinations of PGRs used (µM l⁻¹)</th>
<th>Average No. of shoots</th>
<th>Average length of shoots developed (cm ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>P&amp;T+ BAP 2.21 + NAA 5.36</td>
<td>8.50 ± 0.32</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>2.</td>
<td>P&amp;T+ BAP 4.43 + NAA 5.36</td>
<td>12.5 ± 0.45</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>3.</td>
<td>P&amp;T+ BAP 6.64 + NAA 5.36</td>
<td>10.0 ± 0.35</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>4.</td>
<td>P&amp;T+ BAP 8.87 + NAA 5.36</td>
<td>9.50 ± 0.40</td>
<td>2.8 ± 0.5</td>
</tr>
</tbody>
</table>
Table 3: In vitro root induction in microshoots of Cyathea spinulosa after two weeks.

<table>
<thead>
<tr>
<th>PGRs (µM l⁻¹)</th>
<th>Rooting (%)</th>
<th>No. of roots/shoots</th>
<th>Root length (mm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>IBA</td>
<td>BAP</td>
<td></td>
</tr>
<tr>
<td>½ P&amp;T</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.12</td>
<td>0.44</td>
<td>35.5±1.7</td>
</tr>
<tr>
<td></td>
<td>2.24</td>
<td>0.44</td>
<td>65.0±2.0</td>
</tr>
<tr>
<td></td>
<td>4.48</td>
<td>0.44</td>
<td>60.0±2.5</td>
</tr>
<tr>
<td></td>
<td>8.96</td>
<td>0.44</td>
<td>30.7±2.7</td>
</tr>
<tr>
<td>P&amp;T</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.12</td>
<td>0</td>
<td>38.0±2.8</td>
</tr>
<tr>
<td><strong>2.24</strong></td>
<td>0</td>
<td><strong>85.0±6.0</strong></td>
<td><strong>10.5±0.7</strong></td>
</tr>
<tr>
<td></td>
<td>4.48</td>
<td>0</td>
<td>55.0±2.5</td>
</tr>
<tr>
<td></td>
<td>8.96</td>
<td>0</td>
<td>40.5±3.7</td>
</tr>
</tbody>
</table>

![Images of Cyathea spinulosa microshoots](image-url)
In-vitro Mass Multiplication

Figure 1(a–k): In vitro mass multiplication of a threatened tree fern, Cyathea spinulosa Wall.ex Hook. a. Spore germination b. Composite population of gametophytes c. Regeneration of spatulate prothallus d. Development of juvenile sporophyte from gametophyte e. Induction of callus from leaf primordium excised from juvenile sporophyte on P&T media with 2, 4-D 9.04µM and BAP 2.21µM after two weeks f. Differentiation of meristemoid zones from the callus masses g. Differentiation of multiple shoots from callus on P&T with BAP 4.43 µM and NAA 5.36µM after four weeks h. Rooting on P&T media with 2.24 µM IBA i. Hardening of the in vitro-raised plantlets of C.spinulosa in the culture room inside hood j. Transfer of the plantlet in earthen pot containing soil and leaf mould mixture (2:1) k. Transplantation of the plantlet in the Fern House, NBRI, Lucknow

Figure 2 (a&b): Effects of different concentrations of BAP on shoot differentiation in Cyathea spinulosa in presence of 0.1 mg l⁻¹ (5.36µM) NAA.

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References


