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Apogamous sporophyte development through spore reproduction of a South Asia's critically endangered fern: *Pteris tripartita* Sw.

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ABSTRACT

Objective: To develop an efficient reproducible protocol for a critically endangered fern, *Pteris tripartita* (*P. tripartita*) Sw. through spore culture. **Methods:** The spore derived gametophytes were grown in a half strength MS medium which sub-cultured after three months to MS medium containing growth regulators like BAP, Kinetin and GA3 with 3% sucrose. **Results:** The highest mean number of sporophytes (64.40) induced with 4.16 cm of length and also formed 1.51 cm of root length at 4 mg/L of BAP. On MS medium supplemented with KIN, 56.03 mean numbers of sporophytes with 2.61 cm along with 1.39 cm length of root were obtained in 4 mg/L of KIN. And, the highest mean numbers of juvenile sporophytes (59.66) were induced with 2.64 cm mean lengths of sporophytes with 2.23 cm of root length in 4 mg/L of GA3 in the MS culture medium. The *in vitro* developed plantlets were washed with distilled water and grown in small cups containing sterilized soil, sand and vermiculite (1:1:1). The acclimatized plantlet showed 90% of a survival rate. **Conclusions**: In this study, reproducible protocol could be useful for their rehabilitation and also to reduce the pressure on natural population in Asian habitat.

1. Introduction

Pteridophytes are having major advantages for their propagation and culture due to adaptation to various environmental conditions for a long time. Ferns are much easier to propagate and cultivate than other plants in general. In addition, they can be rapidly propagated using tissue culture techniques. For example, gametophytes are mass-propagated *in vitro* and then juvenile sporophytes are induced from *in vitro* cultured gametophytes in pots and produce huge biomass in short periods[1]. Application of *in vitro* culture methods could contribute to increase sporophyte production of desirable species. The formation of sporophytes in ferns is believed to be controlled in two possible directions: (a) from gametophyte to sporophyte, linked to the reproductive biology of the gametophytic phase; or (b) from preceding sporophyte to another sporophyte linked to the morphogenic potential of the sporophyte itself[2]. Due to unfavorable climate conditions, spore germination is very difficult and slow in sporophyte formation. At the same time, spore germination and conversion of gametophytic stage (haploid) into a sporophytic stage (diploid) are easily understandable under in vitro conditions. Thus, micropropagation can be applied to propagate ferns in large numbers for the benefit of ornamental, pharmaceutical industries and conservation purposes as well[3, 4]. Moreover, the regeneration of ferns was mostly obtained through its spore under *in vitro* conditions[5, 6]. In fern culture, formation of a sporophyte from a gametophyte occurs through sexual or asexual mechanisms. Biological factors such as growth rate and others concerning gametangia as the proportion of sexual phenotypes (male, female, hermaphrodites, and asexual) in the culture seem to have repercussions on sporophyte formation in the gametophytic phase[7−10]. The sporophyte formation in spore-derived gametophytes was raised in many ferns namely, *Polypodium cambricum* (*P. cambricum*), *Davallia canariensis* (*D. canariensis*) , *Dicksonia antarctica* (*D. Antarctica*) and *Asplenium ceterach* (*A. ceterach*)[2]. In our previous study, effects of MS medium, heavy metals, pH, sucrose, plant growth hormones like, 6- benzylaminopurine (BAP), Kinetin (Kin), Gibberellic acid (GA3)

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on spore germination and antioxidant activity of *Pteris tripartita* Sw. (*P. tripartita*) have been studied[11–13]. The main objective of this study was to develop a standardized protocol for the regeneration of South Asian's critically endangered fern, *P. tripartita* Sw.

2. Materials and methods

2.1. Spore collection and its culture

Matured spores of *P. tripartita* were collected from Alagar hills. Of them, only 5 mg of spores was sterilized in Sodium hypochlorite (0.5%) with double distilled water for about 10 minutes. Spores were rinsed with sterile double distilled water and centrifuged at 3 000 rpm for three minutes. Finally, spores were aseptically inoculated in 25 mL of culture tubes containing 10 mL of half strength MS basal medium to induce gametophyte and later cultured in MS ½ strength medium supplemented with plant growth regulators like BAP, Kinetin and GA3 (1, 2, 3, 4, 5 mg/L) along with 3% sucrose (30 g/L) and 0.7% agar-agar. The pH of the spore culture medium was adjusted to 5.7 with 0.1N NaOH and 0.1N HCl. The inoculated culture tubes were finally maintained at 25 ℃ under cool white fluorescent light (40 μmol $m^{-2}s^{-1}$) with 16 h artificial photoperiod (Philips, India) and with 55%–60% relative humidity (RH).

2.2. Statistical analysis

All values are the means of three experiments, and each contains ten replicates. Results were represented as Mean±Standard Error of triplicate values. Analysis of variance was carried out in SPSS software (version 17.0), and the data were analyzed by one-way ANOVA test with Duncan's multiple range tests (DMRT) along with $P < 0.05$ as the limit of significance.

3. Results

3.1. Development of juvenile sporophyte

Half strength MS medium was effectively used to induce gametophyte from the spores of *P. tripartita* Sw. and two months old gametophytes were given in Figure 1a. Three months old gametophytes were sub-cultured in MS medium supplemented with different concentrations of BAP, KIN and GA3 (Figure 1b). Juvenile sporophyte development was achieved after five months of sub-culture. A brown meristematic area developed near the apical notch of gametophyte and a single sporophyte produced between them. The sporophytes were measured after seven months to account their shoot numbers, both shoot and root lengths, respectively. Among five concentrations of BAP, highest numbers of sporophytes[64] were induced with 4.16 cm of height and 1.51 cm of root length at 4 mg/L (Figure 1d). In both 3 and 5 mg/L of BAP; 55 and 54 numbers of sporophytes were developed with 3.84 cm and 3.57 cm of shoot lengths along with 1.45 cm and 1.35 cm of root lengths, respectively (Table 1, Figure 1f). At 2 mg/L of BAP, 47.16 mean number of sporophytes formed along with 3.61 cm mean length and lowest length of root (1.15 cm) while 35.70 mean numbers of sporophytes were obtained with 2.66 cm of shoot length and 1.15 cm of root length at 1 mg/L.

The MS medium supplemented with Kinetin (4 mg/L) induced sporophytes (56.03 mean numbers) after five months (Figure 1c) and reached about 2.61 cm height along with 1.39 cm length of root. Similar mean numbers of sporophytes 44.83 and 44.66 were obtained with 2.42 cm and 2.70 cm length of shoots at 3 mg/L and 5 mg/L of Kinetin, correspondingly. Lowest mean numbers of juvenile sporophytes (30.00 and 37.76) were developed with 2.20 cm and 2.24 cm lengths at both 1 mg/L and 2 mg/L of Kinetin with root length of 0.96 cm and 1.14 cm, respectively. Among various concentration of GA3, highest mean number of juvenile sporophytes (59.66) were induced with 2.64 cm mean lengths of sporophytes

Table 1
Effects of plant growth hormones on apogamous sporophytes regeneration of *P. tripartita* from spore derived gametophytes.

Hormones (mg/L)	% of shoot formations/ experiment	BAP			KIN			GA3		
		Number of shoots/ culture	Shoot length	Root length	Number of shoots/ culture	Shoot length	Root length	Number of shoots/ culture	Shoot length	Root length
1	100	35.70±1.60^{d}	2.66±0.14^{c}	1.15±0.01^{c}	30.00±2.21^{d}	2.20±0.06^{c}	0.96±0.06^{c}	31.00±1.15^{e}	2.23±0.06^{b}	1.82±0.12^{c}
2	100	47.16±0.23^{c}	3.61±0.06^{b}	1.21±0.01^{c}	37.76±0.69^{c}	2.24±0.04^{bc}	1.14±0.02^{b}	42.66±0.06^{d}	2.27±0.02^{b}	2.04±0.12^{bc}
3	100	55.06±0.14^{b}	3.84±0.09^{b}	1.45±0.03^{ab}	44.83±1.40^{b}	2.42±0.02^{b}	1.23±0.01^{b}	50.90±0.92^{c}	2.27±0.14^{b}	2.39±0.09^{a}
4	100	64.40±0.65^{a}	4.16±0.06^{a}	1.51±0.07^{a}	56.03±1.18^{a}	2.61±0.09^{a}	1.39±0.05^{a}	59.66±0.18^{a}	2.64±0.02^{a}	2.23±0.07^{ab}
5	100	54.30±1.26^{b}	3.57±0.04^{b}	1.35±0.05^{b}	44.66±0.72^{b}	2.70±0.05a	1.50±0.03^{a}	53.60±0.92^{b}	2.36±0.04^{b}	2.13±0.03^{abc}

All values are expressed as Mean±SE of triplicate. Means followed by the same letter within columns are not significantly different at $P \leq 0.05$ by Duncan's Multiple Range Test (DMRT).

along with about 2.23 cm of root length at 4 mg/L of GA3 in MS culture medium. The lowest number of juvenile sporophytes were induced with 2.27 cm of length and along with 1.82 cm of root length at 1 mg/L of GA3. At both concentrations 2 and 3 mg/L of GA3, average mean number of juvenile sporophytes, (42.66 and 50.90) were obtained with 2.27 cm of lengths (Figure 1e). And, mean number (53.60) of juvenile sporophytes developed with 2.36 cm of sporophyte's length and root length (2.13 cm) were obtained from 5 mg/L of GA3. The MS solid medium containing GA3 induced sporophyte formation significantly. In our present investigation, half strength MS medium augmented with 30 g/L of sucrose induced juvenile sporophytes after 5 months. All the sporophytes were sub-cultured in their respective culture medium in every month for further shoot elongation. Each experiment was repeated three times with an interval of two weeks.

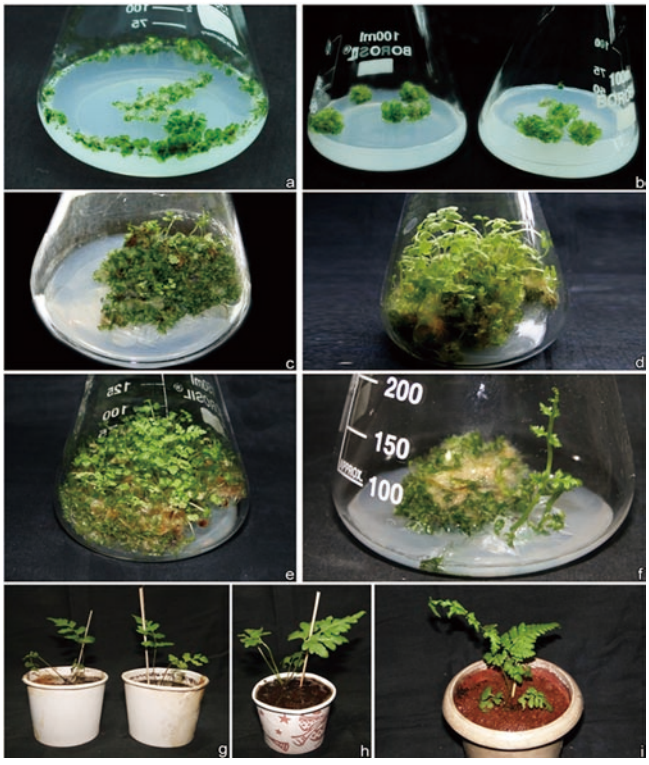

Figure 1. Influence of plant growth hormones on the apogamous sporophytes development of the spores of pteris tripartita SW.
a. Two months old gametophytes in half strength MS basal medium
b. Gametophytes colony after four months of spores sown
c. Juvenile sporophytes after five months at 3 mg/L of Kinetin
d. Apogamous sporophyte development at 4 mg/L of BAP
e. Apogaous sporophyte growth av 3 mg/L of GA_3
f. Sporophyte elongation av 3 mg/L of BAP
g. & h. Transplantation in caps

3.2. Hardening and acclimatization

For hardening, well-developed and rooted plantlets were taken out from culture vessels. Plantlets with roots were thoroughly washed with running tap water and followed by sterile distilled water in order to remove every traces of nutrient medium. Finally, treated plantlets were transferred to small cups containing a mixture of sterilized soil, sand and vermiculite (1:1:1) in growth chambers with controlled temperature and light (Figure 1g & h). Plantlets were sprayed regularly with water and covered with a porous polyethylene bag to maintain high humidity (90%–95%). The potted plantlets were kept under *in vitro* conditions and acclimatized with 90% of a survival rate (Figure 1 i).

4. Discussion

In fern culture, spores have been used as explants source for successful high-frequency reproduction. Although, *in vitro* regeneration of plants from spores is quite difficult, because the optimization of every step from initiation to acclimatization makes more feasible to produce *in vitro* plants from spores[14]. In fern's life cycle, two free-living generations, namely, the gametophyte and sporophyte are occurring in contrast. Sexual reproduction takes place in gametophyte for the development of sporophyte. After maturation, sporophyte produces spores later, which germinate in a suitable habitat patch to form a gametophyte[15]. Half-strength MS salt medium was used as experimental media in our present study and had been cited previously for other ferns as well[16–18]. Many reports indicated the application of growth regulators that enhance or suppress the plant growths and causes a direct effect on their multiplication rate[4]. In prior literature, hormones, sugar and casein hydroxylate are not only increasing fern spore sprouting capability and also involved in proliferation of fern sporophyte from its gametophyte[19, 20]. In fern's developmental biology, formation of a sporophyte from a gametophyte occurs through sexual or asexual mechanisms termed apogamy[6]. A similar development pattern was already observed in both, *Osmunda regalis* (*O. regalis*) and *Pteris ensiformis* (*P. ensiformis*). And also, the duration of development of the sporophyte from spore varied from one to eight months in many ferns[21]. Previous reports revealed that *Pteris vittata* (*P. vittata*), *P. ensiformis*, *Pteris excelsa* (*P. excels*), *Pteris fauriei* (*P. fauriei*), *Pteris finotii* (*P. finotii*) and *Pteris wallichiana* (*P. wallichiana*) were formed their first juvenile sporophyte after 5–8 weeks[22]. On the other hand, *Pteris cretica* (*P. cretica*) (3 months) and *Bolbitis portoricensis* (*B. portoricensis*) (6 to 8 months) showed first sporophyte formation after their spore inoculation in culture medium[23, 24]. The ample range of culture medium conditions like growth regulators, age of plant material and nutrients like sucrose level could also affect morphogenesis and regeneration of various ferns sporophyte[4, 25–32]. Earlier studies reported that cytokinin may also helped in both lipid metabolism and growth promotion of plants[33]. Similar results were also reported in other ferns namely, *Bolbitis costata* (*B. costata*), *Osmunda regalis* (*O. regalis*), *Asplenium nidus* (*A. nidus*) and *P. ensiformis*[16, 17, 34–36]. The regeneration of both aposporous gametophytes and sporophytes could be performed by cytokinin hormone, BAP[2]. In addition to that, culturing of gametophytes in the presence of a low auxin/ cytokinin ratio favored

sporophyte organization and gametophyte regeneration. Both, auxin and cytokinin were used for direct triggers of expression of sporophytic and gametophytic genes in the gametophytes of *Dryopteris affinis* (*D. affinis*)[4] and *Equisetum ravense* (*E. ravense*)[37]. Plants require both auxin and cytokinins could be supplied to the culture medium seems to be very important for multiplication[3, 21]. The choice of cytokinins in tissue culture is determined by its cumulative efficiency in inducing an acceptable rate of shoot multiplication. Cytokinins are well known to increase cell division, break apical dominance and release lateral buds from dormancy then promoting shoot formation and later for multiplication [38, 39]. An effective cytokinin hormone, BAP has been found to stimulate sporophyte development in several leptosporangiate ferns like, *Nephrolepsis cordifoli* (*N. cordifoli*), *Nephrolepsis exaldata* (*N. exaldata*), *Cyathea spinulosa* (*C. spinulosa*), *Cyclosorus dentatus* (*C. dentatus*), *Asplenium nidus* (*A. nidus*), *Rumhora* sp, *Blechnum spicant* (*B. spicant*), *P. ensiformis*, *P. vittata*, *Adiantum* sp, *Marsilea quadrifolia* (*M. quadrifolia*) , *Platycerium coronarium* (*P. coronarium*), and *Rumohra adiantiformis* (*R. adiantiformis*) by several authors[16, 40–47].

Maximum shoots, leaves numbers, stem height were raised in MS medium supplemented with BAP (2 mg/L) in *A. nidus* and Kinetin (3 mg/L) showed highest prothalli length, width and height[41]. Cytokinins are involved in many developmental processes in Planta, such as cell division, senescence, nutrient mobility, and endogenous cytokinin homeostasis are regulated by cytokinin oxidases. In addition to that, these enzymes are selectively degrade unsaturated N 6-isoprenoid side chain, whereas aromatic cytokinins are resistant to their cleavage, consequently causing a different strength in the cytokinin activity[48, 49]. Cytokinin oxidase activity is highly conserved, as it has been reported either in many higher plant species and in lower plant (moss), like *Funaria hygrometrica* (*F. hygrometrica*) Hedw[50]. However, the supremacy of BAP over Kinetin in producing huge number of juvenile sporophytes developed from their spore derived gametophytes of *P. tripartita* and that has also been documented in many flowering plants, *Eclipta alba* (*E. alba*)[51], *Stevia rebaudiana* (*S. rebaudiana*)[52] and *Acacia catechu* (*A. catechu*)[53]. Our results are in agreement with previous reports in *Dryopteris affinis* (*D. affinis*)[54]. According to Korpelainen[7], great number of sporophytes could be observed in gametophytes with either high or low growth rate. Kuriyama *et al.*[55] reported that the production of sporophytic plants of *Cyathea lepifera* (*C. lepifera*) was achieved in half strength MS medium without sugar. But, addition of sugar enhances growth and failed to produce sporophytes. An established protocol was developed for an efficient multiplication of a critically endangered fern, *P. tripartita Sw*. In this study, reproducible protocol could be useful for their rehabilitation and also to reduce the pressure on natural population in Asian habitat. More over, *P. tripartita Sw*. could be used for secondary metabolite characterization to enhance the human healthcare system.

Conflict of interest statement

We declare that we have no conflict of interest.

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