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In vitro gametophyte development of *Pteris vittata* L., *Phymatosorous nigrescens* (Bl.) Pichi-Serm. and *Nephrolepis acutifolia* (Desv.) H. Chris

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Abstract

The *in vitro* technique is an art of growing plants in sterile condition to propagate and multiply the rare, endangered and endemic species. *Pteris vittata* L., *Nephrolepis acutifolia* (Desv.) H. Chris., *Phymatosorous nigrescens* (Bl.) Pichi-Serm. were collected from Kolli hills for *in vitro* multiplication. All these ferns are in under conservation status, so these ferns have to conserve for its biological activity and economical value. Matured spores of the 3 varieties of ferns were collected from air dried sporophylls and used as explant. Totally 3 nutrient medium were used such as Murashige & Skoog, 1962 (half strength), Fern micropropagation and Knudson C Orchid medium with different concentration of growth regulators. Spores were germinated after 10days, 30 days, 45 days from the day of inoculation respectively. The fern micropropagation medium shows best results and *Pteris vittata* fern shows high percentage of spore germination and prothallus development in all the medium used. In *Pteris vittata* the fern micropropagation medium with 0.4mg/l KIN + 0.2 mg/l GA3 (F2) shows the maximum level of spore germination & protonema initiation (90%) and shows 84% of prothallus development. In *Nephrolepis acutifolia*, the fern micropropagation medium with 0.4 mg/l KIN + 0.2 mg/l GA3 (F6) showed the maximum level of spore germination & protonema initiation that is 56% and shows 42% of prothallus development. In *Phymatosorus nigrescens*, the fern micropropagation medium with 0.4 mg/l KIN + 0.2 mg/l GA3 (F9) showed the maximum level of spore germination & protonema initiation (48%) and prothallus development (40%).

Keywords

Conservation, multiplication, prothallus, protonema, Spores.

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INTRODUCTION

Ferns can be propagated using tissue culture techniques. Micropropagation can be applied to propagate ferns in large numbers for the benefit of ornamental, pharmaceutical industries and also for conservation purposes. Regeneration of ferns can also be done using spores under *in vitro* condition [1]. In 2012, the International Union for Conservation of Nature (IUCN) estimated that, globally, there were 12,000 species of pteridophytes; 167 species of the 311 evaluated species are threatened [2]. The huge population of pteridophytes were present in

Western and Eastern Ghats. 6% of pteridophyte and lycophyte species are globally threatened with extinction and 22% are of elevated conservation concern (Threatened or Near Threatened); of species of pteridophytes and lycophytes previously included on the Red List, 54% were considered threatened [3]. The Red List of Threatened Species (IUCN 2014) clearly shows that more than half of fern species and their allies are threatened to some degree. Thus, there is urgent need for further research to improving and implementing novel methods for the conservation of fern biodiversity [4].

In a recent study, *Pteris vittata* was found to have high levels of primary and secondary antioxidant activities [5]. The whole plant parts are ground into paste and applied over the affected places for wound healing. The paste is mixed with pepper and taken orally to get relief from cold, cough and fever [6]. Extracts from this plant are used as demulcent, hypotensive, anti-viral, and antibacterial agents [7]. Moreover, leaves of this plant are used in worship at times of illness while the fronds are used as bedding in cattle sheds [8]. A decoction of the leaves and root of *Phymatosorous nigrescens* is take two or three per day to treat weakness before and after childbirth [9]. The pressed juice of the leaves is used to treat influenza in children, to aid the healing of fractured bones, and as a remedy for pain in the lower chest, diarrhea, stomachache and strained muscles. Liquid pressed from the roots and leaves is given for a relapsed illness. Filtrate from the stem is taken for shortness of breath in the central chest cavity and is drunk and dripped into the ears and nose to treat fish poisoning. Unnamed parts of the plant are utilized in the treatment of wounds which have healed on the outside but not on the Inside, intestinal muscle cramps, and severe chest pains [10]. The rhizome is said to be used for treating fish poisoning [11]. The plant is also reported to be used to treat migraine, stomach, and body pains in new mothers, and to alleviate swelling in the armpit [12].

Hence this study focuses to conserve the rare, endangered, near threatened, endemic and medicinally important fern species through *in vitro* micropropagation method.

MATERIALS AND METHODS

Collection and storage of explants:

Sporophylls of *Pteris vittata* L., *Phymatosorous nigrescens* (Bl.) Pichi-Serm., *Nephrolepis acutifolia* (Desv.) H. Chris were collected from Kolli hills, Namakkal district, Tamil Nadu. Matured spores of the 3 varieties of ferns were collected from air dried sporophylls. The collected spores were stored in glass vials under refrigeration.

Surface sterilization:

The spores were surface sterilized using 35% (w/v) solution of Sodium hypochlorite (4% active chlorine) for 30 minutes. Then wash the spores with sterile double distilled water for several times. After that filter the spores through autoclaved filter paper under laminar air flow chamber.

Preparation of growth hormones:

IAA, KIN, GA3 are the three hormones used for the culture of spores. 0.01g of each of IAA and KIN are taken and dissolved in 1N NaOH. Similarly, 0.01g of GA3 is taken and dissolved in 10ml ethanol.

Preparation of Culture Medium:

The nutrient medium used for sporangia inoculation was Murashige & Skoog (MS), 1962 (half strength) medium, Fern micropropagation medium and Knudson C Orchid medium with different concentration of growth regulators. The half MS medium with different concentrations of growth regulators were used for germination and development of protonema that include 0.2 mg/l KIN + 0.1 mg/l IAA, 0.4 mg/l KIN + 0.2mg/l IAA, 0.4 mg/l KIN + 0.2 mg/l GA3. The Fern micropropagation medium with 0.2 mg/l KIN + 0.1 mg/l IAA, 0.4 mg/l KIN + 0.2 mg/l IAA and 0.4 mg/l KIN + 0.2 mg/l GA3. Knudson C Orchid medium with 0.1 mg/l IAA + 0.4 mg/l GA3 were used. The pH of the medium was adjusted to 5.8 with 0.1N NaOH and 0.1N HCl. Agar powder (0.8%) was mixed with the medium and boiled until a clear frothing solution will obtain and then poured in culture tubes. Autoclave will be done at 15 lbs/sq. inch of pressure for 20 minutes to make the medium free of microbes. The culture bottles and tubes containing the nutrient medium were then allowed to cool for 24 hours in culture laboratory.

Inoculation of Spores:

The surface sterilized spores were inoculated in the culture tubes under Laminar Air Flow Cabinet to maintain the aseptic conditions. The cultured sporangia were incubated in culture room of the Tissue Culture Laboratory at 25˚C±1˚C, under 16-hour photoperiod and 8 hours darkness. And periodically took photographs for the development stages of the ferns for the observation.

RESULTS AND DISCUSSION

Germination of spores and development of protonema:

Spores of *Pteris vittata, Nephrolepis acutifolia* and *Phymatosorus nigrescens* were found to germinate after 10days, 30 days, 45 days respectively from the day of inoculation. All the germinated spores did not develop sporophytic bodies.

Table 1: Average Germination Percentage, Protonema and prothallus development of *Pteris vittata*

S. No.	Type of media	Media number	Concentration of Growth Hormones			Spore germination & Protonema initiation percentage	Prothallus Development Percentage
			KIN (mg/l)	IAA (mg/l)	GA3 (mg/l)		
1	Half MS medium	MS1	0.1	0.2	-	28	25
		MS2	0.2	0.4	-	46	39
		MS3	**-**	**0.1**	**0.4**	**80**	**70**
2	Fern micropropagation Medium	F1	0.2	0.1	-	66	58
		F2	**0.4**	**-**	**0.2**	**90**	**84**
		F3	0.4	0.2	-	74	67
3	Knudson C Orchid medium	K1	0.1	0.2	-	56	36
		K2	0.2	0.4	-	63	51
		K3	**-**	**0.1**	**0.4**	**70**	**62**

In *Pteris vittata* the fern micropropagation medium with 0.4mg/l KIN + 0.2 mg/l GA3 (F2) shows the maximum level of spore germination & protonema initiation (90%) and shows 84% of prothallus development. Next to this M.S medium with 0.1 mg/l IAA + 0.4 mg/l GA3 (MS3) shows 80% of spore germination & protonema initiation and 70% of prothallus development. Knudson C Orchid medium also responded well with the combination of 0.1 mg/l of IAA + 0.4 mg/l of GA3 (K3) in the spore germination & protonema initiation (70%) and development of prothallus (62%). After 30 days of growth, the young prothallus shows cordate shape of development. Half MS Medium with 0.1 mg/l KIN + 0.2 mg/l IAA (MS1) shows the lowest level of growth percentage. Compared to other two medium, the fern micropropagation medium with different growth hormones shows the highest rate of growth percentage.

Pteris vittata callus, sporophytes, and gametophytes all grew well under 1 mM of Arsenate and accumulated 1,250; 1,150 and 2,180 mg kg–1 dry weight as when grown on 2 mM Arsenate for 15 or 30 days [13]. Aposporous gametophytes and vegetative calli were produced at higher concentrations. The calli regenerated sporophytes when cultured on MS medium without growth regulators. The gametophytes grew vegetatively on MS medium but produced sporophytes when transferred into 0.1 strength MS medium [14]. Calli were induced on Murashige and Skoog's (MS) and parkers and Thompson's (P and T) media supplemented with different combinations of 2,4-dichloro phenoxyacetic acid (2, 4-D), 6-benzylaminopurine (BAP), α-naphthalene acetic acid (NAA) and Indole -3acetic acid (IAA). A combination of full-strength MS medium with 2, 4-D (2.6µM) and BAP (2.2µM) was found to be ideal for profuse callusing (80%) against other combinations [15].

Table 2: Average Germination Percentage, Protonema and prothallus development of *Nephrolepis acutifolia*

S. No.	Type of media	Media number	Concentration of Growth Hormones			Spore germination/ Protonema initiation percentage	Prothallus Development Percentage
			KIN (mg/l)	IAA (mg/l)	GA3 (mg/l)		
1	Half MS medium	MS4	0.1	0.2	-	-	-
		MS5	0.2	0.4	-	-	-
		MS6	0.4	-	0.2	32	28
2	Fern micropropagation Medium	F4	0.1	0.2	-	16	12
		F5	0.2	0.4	-	40	22
		F6	**0.4**	**-**	**0.2**	**56**	**42**

In *Nephrolepis acutifolia*, the fern micropropagation medium with 0.4 mg/l KIN + 0.2 mg/l GA3 (F6) showed the maximum level of spore germination & protonema initiation that is 56% and shows 42% of prothallus development. The fern micro propagation medium with 0.1 mg/l KIN + 0.2 mg/l IAA (F4) shows the minimum level of spore germination (16%) and prothallus development (12%). In half MS medium with 0.4 mg/l KIN+ 0.2 mg/l GA3 (MS6) shows the 32% of spore germination & Protonema initiation

and 28% of prothallus development. After 52 days of growth, the young prothallus shows cordate shape of development. In other concentrations of half MS media showed no results. From this observation, in all concentrations the Fern micropropagation media responded very well.

In *N. biserrata*, when NAA was substituted with BAP with 2,4-D (2 mgL−1), shoot regeneration in stolon explants was augmented, and this was also more effective than another synthetic auxin. Reports are available on the effects of Kn and NAA on *in vitro* shoot multiplication and rooting in *N. exaltata* cv. "bostoniensis" through runner tip culture [16]. *N. exaltata* cv. bostoniensis and *C. dentatus*, regeneration of shoots took place on full-strength MS medium supplemented with 2,4-D (2 mgL−1) and BAP (0.5 mgL−1) and full-strength P&T medium supplemented with BAP (1 mgL−1) and IAA (0.5 mgL−1), respectively [17].

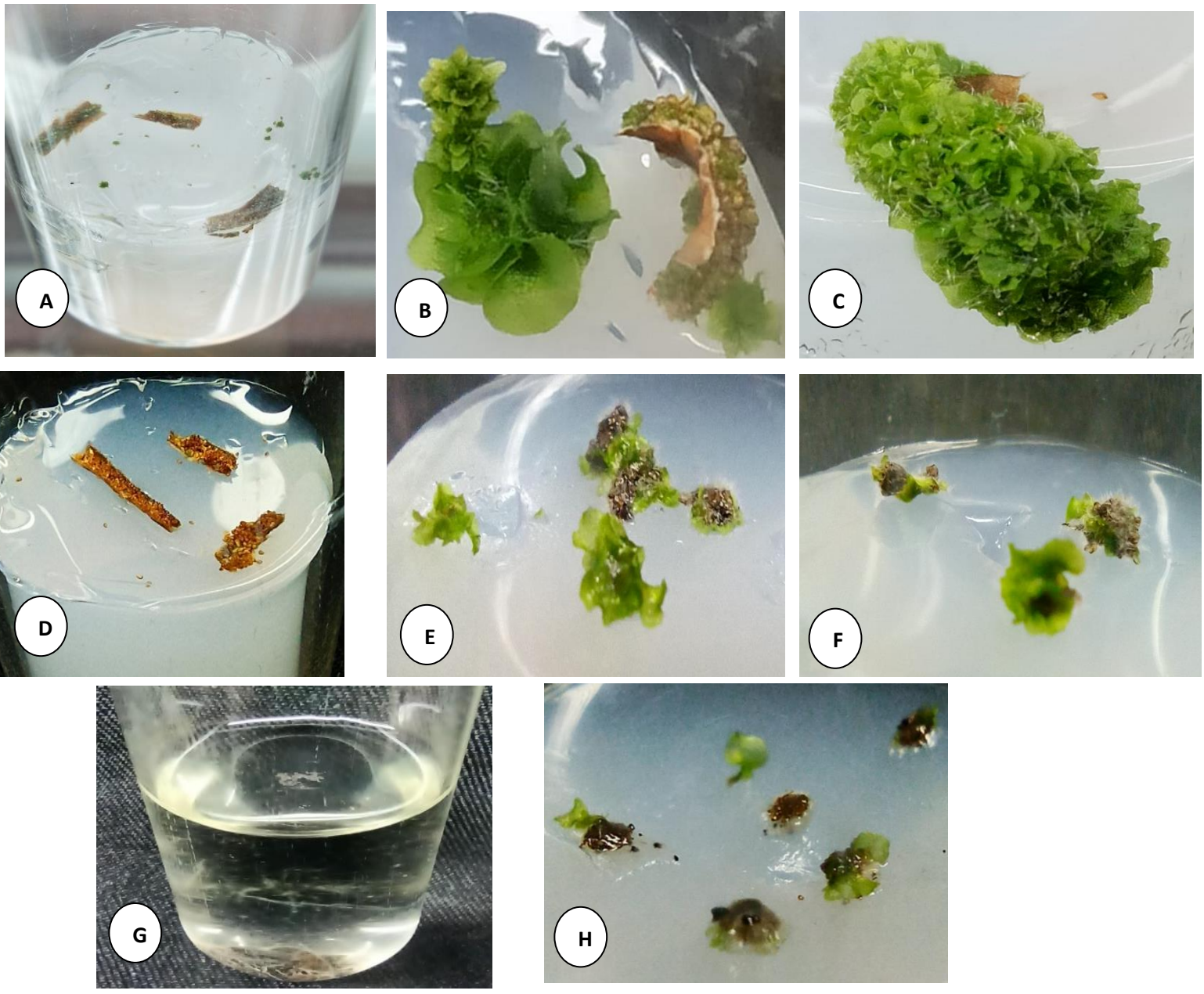
Table 3: Average Germination Percentage, Protonema and prothallus development of *Phymatosorus nigrescens*

S. No.	Type of media	Media number	Concentration of Growth Hormones			Spore germination & Protonema initiation percentage	Prothallus Development Percentage
			KIN (mg/l)	IAA (mg/l)	GA3 (mg/l)		
		MS7	0.2	0.1	-	-	-
1	Half MS medium	MS8	0.4	0.2	-	-	-
		MS9	0.4	-	0.2	16	25
	Fern	F7	0.2	0.1	-	20	16
2	micropropagation	**F8**	0.4	0.2	-	24	18
	Medium (liquid)	F9	**0.4**	**-**	**0.2**	**48**	**40**

In *Phymatosorus nigrescens*, the fern micropropagation medium with 0.4 mg/l KIN + 0.2 mg/l GA3 (F9) showed the maximum level of spore germination & protonema initiation (48%) and prothallus development (40%). The fern micropropagation medium with 0.2 mg/l KIN + 0.1 mg/l IAA (F7) shows the minimum level of spore germination & protonema initiation (20%) and prothallus development (16%). In half MS medium, 0.4 mg/l KIN+ 0.2 mg/l GA3 (MS9) shows the 16% of spore germination & protonema initiation and then shows 25% of prothallus development. After 60 days of growth, the young prothallus shows cordate shape of development. In other concentrations of half MS media showed no results. From this observation, in all concentrations the Fern micropropagation media shows better results.

The yellow-coloured smooth walled spores of *Phymatosorus scolopendria was* collected from the fronds which were inoculated in KC liquid medium, germinated on 20 days after spore sowing. After 60 days of growth, the young cordate prothallus developed and the apical meristematic cells were replaced by pluricellular meristem. A well-developed apical notch is formed with dermal hairs [18].

Figure 1: Spore germination, protonema development and prothallus stages of *Pteris vittata, Nephrolepis acutifolia* and *Phymatosorus nigrescens*



A. Spore germination and protonema development of *Pteris vittata* ***B* &** **C.** Prothallus development of *Pteris vittata* with different concentrations **D.** Spore germination and protonema development of *Nephrolepis acutifolia;* **E & F.** Prothallus development of *Nephrolepis acutifolia* with different concentrations **G.** Spore germination and protonema development of *Phymatosorus nigrescens* **H.** Prothallus development of *Phymatosorus nigrescens.*

CONCLUSION

Pteris vittata, Nephrolepis acutifolia and *Phymatosorus nigrescens* shows best growth rates in the fern microprogation medium than the M.S and Knudson C orchid medium. The high percentage of spore germination and prothallus development was seen in *Pteris vittata.* This fern responded very well in 3 medium with all concentrations, especially in medium with IAA + GA3 combination, Not only this plant all 3 ferns shows good growth percentage in this combination. *Phymatosorus nigrescens* shows no results in half M.S medium and it responded in very

low percentage of growth in other two medium. These 3 ferns have many biological activities and economically important plants, so this is the time to conserve these ferns.

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