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Investigation on *In vitro* gametophyte development of selected fern species from Kolli hills, Tamil Nadu, India

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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* (BI.) 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 10 days, 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 kinetin (KIN) + 0.2 mg/l Gibberellic acid (GA3) 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 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 showed the maximum level of spore germination & protonema initiation (48%) and prothallus development (40%).

Keywords: ferns, *In vitro* culture, spore germination, protonema initiation, prothallus development, conservation

Introduction

The ferns can be propagated in huge numbers by applying the technique of micropropagation for the beneficiary purposes of ornamental, pharmaceutical industries and also for conservation. Under *in vitro* culture the ferns were regenerated using spores. (Xavier *et al*. 2015) [16]. In 2012 IUCN reported that, worldwide totally there were 12,000 species of pteridophytes, in that 167 species of the 311 evaluated species are threatened. (IUCN 2012). IUCN 2014 results shows that the Red List of Threatened Species was more than half of fern species and their allies are threatened to some degree. Conservation of fern biodiversity is the urgent need for further research in improving and implementing novel methods. In Western and Eastern Ghats of our subcontinent the population of pteridophyte is large. In that, 6% of pteridophytes and lycophyte species are threatened with extinction in worldwide and 22% are of elevated conservation concern (Threatened or Near Threatened); pteridophyte and lycophyte species were previously included on the Red List, in that 54% were considered as threatened (Brummitt *et al*. 2016) [2].

In a recent study, the high levels of primary and secondary antioxidant activities were found in *Pteris vittata* (Lai and Lim 2011) [7]. The whole parts of the plant was prepared as paste and applied on the affected area for wound healing. And the paste also mixed with pepper and taken orally to get relief from cold, cough and fever (Karthik *et al*. 2011) [6]. Extracts from *Pteris vittata* are used as demulcent, hypotensive, anti-viral, and antibacterial agents (Benjamin and Manickam 2007) [1]. Moreover, the plant fronds are used in worship at times of illness while the fronds are used as bedding in cattle sheds (Upreti *et al*. 2009) [13].

Leaves and roots of *Phymatosorous nigrescens* was prepared as decoction and to take two or three times per day for the remedy of weakness before and after childbirth (Weiner 1970) [14]. The pressed juice of the fronds is taken

to treat influenza in children, to get relief from healing of fractured bones, used to treat for pain in the lower chest, diarrhoea, stomachache and strained muscles. For relapsed illness the liquid pressed from the roots and leaves are used. Filtrate of stem is used for shortness of breath in the central chest cavity, for fish poisoning it is drunk and dripped into the ears and nose (Weiner 1984) [15]. The rhizome part of the plant is used for treating fish poisoning (Singh and Siwatibau 1977) [11]. The plant is reported to be used to get rid from migraine, stomach and body pains in new mothers, and to reduce swelling in the armpit (Jogia 1984) [5].

Hence this study focus 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., *Nephrolepis acutifolia* (Desv.) H., Chris *Phymatosorous nigrescens* (BI.) Pichi-Serm., were collected from Kolli hills, Namakkal district, Tamil Nadu. It extends to an area of about 503 Km^2 between 11˚10' - 11˚30' N latitude and 78˚15' - 78˚30' E longitude. The vegetation is prominently open evergreen, dense ever green and dry deciduous with patches of moist deciduous and evergreen forests. 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 of sporangia

The sporangia were surface sterilized using 35% (w/v) solution of Sodium hypochlorite (Suresh Scientific Co, Tiruchirappalli, Tamil Nadu, India) 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

Indole -3-acetic acid (IAA), Kinetin (KIN), Gibberellic acid (GA3) are the three hormones used for the culture of spores. 0.01g of each of IAA and KIN (Suresh Scientific Co, Tiruchirappalli, Tamil Nadu, India) are taken and dissolved in 1N NaOH (Suresh Scientific Co, Tiruchirappalli, Tamil Nadu, India). Similarly, 0.01g of GA3 (Suresh Scientific Co, Tiruchirappalli, Tamil Nadu, India) 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 (KC) medium (Suresh Scientific Co, Tiruchirappalli, Tamil Nadu, India) 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. 0.1N NaOH and 0.1N HCl (Suresh Scientific Co, Tiruchirappalli, Tamil Nadu, India) were used to determine the pH of the medium to 5.8. The mixture of medium with Agar powder (0.8%) was boiled till obtain the clear frothing solution. To avoid contamination the medium was autoclaved at 15 lbs/sq. inch of pressure for 20 minutes. In culture laboratory the culture bottles and tubes containing the medium were then allowed to solidify.

Inoculation of Sporangia

Under Laminar Air Flow Chamber the surface sterilized sporangia were inoculated in the culture bottles and tubes. The cultured sporangia were incubated in 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. 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 MS 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 (Table1). Muthukumar *et al*. (2013) reported that, in *Pteris vittata* the spore germination shows 40% and the mature prothallus (more than 90%) began to form 6 weeks after spore transformation in one-half strength MS medium with 20% (w/v) Sucrose in both transgenic and nontransgenic control samples. Ravi *et al*. (2014) results shows that, spores of *Pteris tripartita* were sprouted after 15 days of inoculation in MS basal culture medium. 84% of spore germination was observed with 2.3% of rhizoid formation in 70 g/L of sucrose.

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 micro propagation 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 *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 (Table 2). Sara *et al*. (1998) explained that, spores of *Nephrolepis multiflora* (Roxb.) sown in January using Knudson's C medium without sucrose with 0.8% agar was the most effective medium, that showed the germinating capacity (89%). The development of Prothallial was irregular in the latter. The mature gametophytes (12-week-old) with sex organs has been described using 6-furfuryl amino purine (kinetin) and benzyl amino purine (BAP).

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 *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 (Table 3). *Phymatosorus scolopendria*, the yellow coloured smooth walled spores was collected from the fronds which were inoculated in KC liquid medium, germinated on 20 days after spore sowing. The young cordate prothallus was developed after 60 days of growth and the apical meristematic cells were replaced by pluricellular meristem (Sujatha and Catharin 2016) [12].

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)		
1	Half MS medium	MS7	0.2	0.1	-	-	-
		MS8	0.4	0.2	-	-	-
		MS9	0.4	-	0.2	16	25
2	Fern micropropagation Medium (liquid)	F7	0.2	0.1	-	20	16
		F8	0.4	0.2	-	24	18
		F9	0.4	-	0.2	48	40

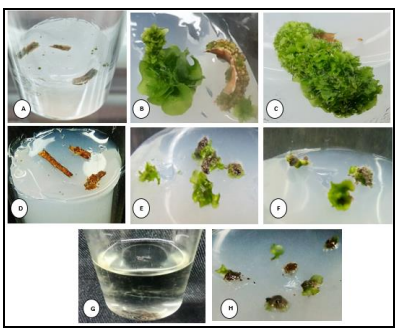

Fig 1: Spore germination, protonema development and prothallus stages of *Pteris vittata*, *Nephrolepis acutifolia* and *Phymatosorus nigrescens*

A. Spore germination and protonema development of *Pteris vittata* with Fern micropropagation medium contain the concentration of 0.4mg/l KIN + 0.2 mg/l GA3 (Scale bar – 3mm) *B &* C. Prothallus development of *Pteris vittata* with half strength Murashige & Skoog medium (MS) contain the concentration of 0.1 mg/l IAA + 0.4 mg/l GA3 and Fern micropropagation medium 0.4mg/l KIN + 0.2 mg/l GA3 (Scale bar – 1mm) D. Spore germination and protonema development of *Nephrolepis acutifolia* with Fern micropropagation medium contain the concentration of 0.4 mg/l KIN + 0.2 mg/l GA3 (Scale bar – 3mm) E & F. Prothallus development of *Nephrolepis acutifolia* with Fern micropropagation medium contain the concentration of 0.4 mg/l KIN + 0.2 mg/l GA3 and half strength Murashige & Skoog medium (MS) contain the concentration of 0.4 mg/l KIN + 0.2 mg/l GA3 (Scale bar – 2mm) G. Spore germination and protonema development of *Phymatosorus nigrescens* with fern micropropagation medium with 0.4 mg/l KIN + 0.2 mg/l GA3 (Scale bar – 3mm) H. Prothallus development of *Phymatosorus nigrescens* with fern micropropagation medium with 0.4 mg/l KIN + 0.2 mg/l GA3 (Scale bar – 2mm).

Conclusion

Pteris vittata, *Nephrolepis acutifolia* and *Phymatosorus nigrescens* showed 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 all the medium with all concentrations, especially in medium with IAA + GA3 combination. Not only has this plant all the ferns studied showed 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. This three ferns have many biological activities and economically important plants, so this is the time to conserve these ferns.

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