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Influence of abiotic and biotic factors on developmental biology of four ferns of Western Ghats, India

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ABSTRACT

The present study examined the influence of age, season and pH on spore germination, early gametophyte development and sporophyte formation of four rare and endangered ferns, viz. *Cheilanthes viridis* (Forssk.) Swartz, *Phlebodium aureum* L., *Pronephrium triphyllum* (Sw.) Holttum and *Sphaerostephanos unitus* (L.) (Holttum) of Western Ghats of South India. Highest percentage of spore germination was achieved only in the matured spore inoculated media, whereas the young spores failed to germinate. Highest percentage of spore germination was achieved in the spores collected in the month of February for *Cheilanthes viridis*; July-*Phlebodium aureum*; March-*Pronephrium triphyllum* and January for *Sphaerostephanos unitus*. Germination of spores occurred in a wide range of pH from 4 to 8. For *Cheilanthes viridis*, highest percentage of spore germination (88.8±1.61) occurred on Knudson C Solid medium at pH 5.8. Mitra liquid medium at pH 5.5 showed highest percentage (81.3±0.84) of spore germination for *Phlebodium aureum*. *Pronephrium triphyllum* (38.3±1.21) *and Sphaerostephanos unitus* (36.8±1.31) showed highest percentage of spore germination on Knops solid medium at pH 5.8. There were no morphological differences among gametophytes grown under different pH optima. Gametophytes gave rise to new gametophytes and developed antheridia and archegonia after they were transferred to culture flasks. In *Cheilanthes viridis* (Forssk.) Swartz, high growth rate as well as high percentage (85.8±1.15) of sporeling formation were observed in KC medium at pH 5.8. In *Phlebodium aureum* L. highest growth area as well as highest sporeling emergence were recorded (33.4±1.21) in Mi medium at pH 5.5. In *Pronephrium triphyllum* (Sw.) Holttum, highest growth area and highest percentage (52.3±1.21) of sporeling emergence were observed in KN medium at pH 5.8. In *Sphaerostephanos unitus* (L.) Holttum, KN medium at pH 5.8 induced a maximum growth rate and high percentage (76.8±1.21) of sporeling emergence. The Isozyme profiles revealed the biochemical changes between the different developmental stages of selected ferns.

KEYWORDS

Abiotic;
Alternation of generations;
Gametophyte;
Sporophyte;
Isozyme;
Zymogram.

INTRODUCTION

Spores are tiny objects with which ferns reproduce themselves. A spore is actually a single vegetative structure, which contains only half the normal chromosomal complement. The propagation of ferns from the spores is the most efficient and economical means of raising large number of any species. Fern spores are excellent biological systems for the analysis of physiological and developmental criteria[1]. *In vivo* spore culture is restricted by a number of factors. Due to unfavorable environmental conditions, nearly 45 ferns were in the red status[2]. *In vitro* spore culture is a novel technique used to multiply the rare and endangered species. The age and viability of explants (spore), pH of the culture media and season of collection play a key role in successful culture initiation and establishment. The viability of spores of different ferns varies enormously with period ranging from a few days to a few years based on their dormancy. To break their dormancy suitable environmental conditions are required. The *in vitro* culture technique has been used to study different aspects of germination patterns leading to gametophyte and sporophyte development[3-27]. The advantages of artificial methods of spore and gametophyte culture over the conventional methods used by fern growers include the avoidance of contamination by potentially destructive microorganisms and the improved visibility of growth stages and sex organs. Spore germination and the resultant gametophyte and sporophyte development totally depend on cultural conditions (*in vitro*)/environmental conditions (*in vivo*)[28]. Major physical factors such as temperature, light, pH and density of cultures (spore and gametophytes) play a crucial role in spore germination and gametophyte development[6,29.31]. The effect of sterilization of spore was observed by many investigators viz., Simabukuro et al.[32]; Hamilton and Chaffin[33]; Camloh[8]; Miller[34]; Miller and Wagner[35]. Spiess and Krouk[36] and Hegde and D' Souza[37] observed the variation in the germination of spores under seasonal influence in *Polypodium aureum* and *Drynaria quercifolia*. Beri and Bir[9] have observed profound effect of temperature on fern spore germination. They also opined that *in vitro* culture can have major advantages over conventional propagation in the management of some species. The sexuality of the gametophyte is greatly influenced by the culture conditions, i.e. pH, light, sucrose and hormones[38]. Neutral or alkaline pH of the medium led to the development of female sex organs. Based on this background the present study was initiated to determine the effects of age of the spore, season and pH of the media on spore germination, development of gametophytes and sporophytes of *C. viridis, P. aureum, P. triphyllum* and *S. unitus*. In addition, the iso-peroxidase profiles of different developmental stages were studied.

MATERIALS AND METHODS

Spores of *Cheilanthes viridis* (Forssk.) Swartz were collected during June 2000 to May 2001. For *Phlebodium aureum* L, spores were collected during March 2001 to February 2002; for *Pronephrium triphyllum* (Sw.) Holttum during July 2000 to July 2001; and for *Sphaerostephanos unitus* (L.) Holttum during June 2000 to May 2001. The collected spores were passed through nylon mesh (40μm) to remove the sporangial wall materials, and cleaned spores were collected and used for culture initiation. They were surface-sterilized with 0.1% $HgCl_2$+0.1% sodium lauryl sulfate solution for 3-5min and washed with sterile distilled water for 15 min. For spore germination, gametophyte multiplication and sporophyte formation, spores of *C. viridis, P. aureum, P. triphyllum,* and *S. unitus* were cultured on liquid and agar (0.5%) gel with various pH (4.0, 4.5, 5.0, 5.5, 5.8, 6.0, 7.0 and 8.0). The pH of the medium was adjusted with 1N NaOH or 1N HCl. After inoculation, the cultures were incubated at 25±2°C under 70% relative humidity and 12 h photoperiod/day in a culture room. All the cultures were kept in 1200-1500lux light intensity provided by cool white fluorescent tubes (Phillips India Ltd, Mumbai). For further growth and development, one month-old gametophytes were sub-cultured in the optimal media. The culture tubes containing spore-derived micropropagated plants of *C. viridis, P. aureum, P. triphyllum* and *S. unitus* were kept at room temperature (30-32°C) for a week before transplantations. For acclimatization, the plants with well developed roots (5-8cm) were removed from culture tubes, washed in running tap water to remove the rem-

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nants of agar, and each group was planted separately onto 10cm dia polycup filled with different potting mixtures of river sand; garden soil and farm yard manures (1:1:1) and sand and soil (2:1). The plants were kept in mist chamber with a relative humidity of 70%. Plants were irrigated at 8h intervals for 3-4 weeks and establishment rate was recorded. The plantlets established in community pots were transferred to shade-net house for 3-4 weeks and then repotted in larger pots (20cm dia.) with one plant in each pot then transferred to its native habitat and also to the natural forest segment at KBG, Kodaikannal. For Iso-peroxidase analysis, the different developmental stages were harvested and washed once in de-ionized water and mashed in a pre-chilled mortar in 500µl of phosphate buffer (pH 7.0). The resultant slurry was centrifuged at 10,000 rpm for 10 min at 4°C in a Mikro 22R centrifuge and the supernatant was stored at -70°C until use. The method described by Manickam and Sadashivam[39] was followed for the activity staining of isoperoxidases. The banding profiles were recorded using Vilber Loubermat Gel documentation system and the banding uniformity was analyzed by Biogene software.

RESULTS

Influence of season

C. viridis

Spores were collected during July 2000 to June 2001 and cultured on both KC and KN media. High percentage of spore germination (84.7) was observed when the spores were collected in February 2001, and low percentage of spore germination (62.1) was recorded when the spores were collected in November 2000. Similarly high percentage (93.1) of gametophyte formation observed when the spores were collected in April 2001. High percentage (91.3) of sporophyte formation was observed when the spores were collected in November 2000 (TABLE 1).

TABLE 1: Influence of Season on spore germination, prothalli formation and Sporophyte formation

Species	*C. viridis*			*P. aureum*			*P. triphyllum*			*S. unitus*		
Month of spore collection	% of Germination	% of Prothalli formation	% of Sporophyte formation	% of Germination	% of Prothalli formation	% of Sporophyte formation	% of Germination	% of Prothalli formation	% of Sporophyte formation	% of Germination	% of Prothalli formation	% of Sporophyte formation
July, 2000	65.8	83.8	90.3	NP	NP	NP	NP	NP	NP	0.00	0.00	0.00
Sep, 2000	65.6	82.3	85.3	NP	NP	NP	NP	NP	NP	0.00	0.00	0.00
Oct, 2000	NP	NP	NP	NP	NP	NP	0.00	0.00	0.00	0.00	0.00	0.00
Nov, 2000	62.1	78.4	91.3	NP	NP	NP	0.00	0.00	0.00	13.3	78.3	76.8
Dec, 2000	NP	NP	NP	NP	NP	NP	0.00	0.00	0.00	NP	NP	NP
Jan, 2001	72.8	74.7	88.3	NP	NP	NP	NP	NP	NP	33.8	82.1	83.1
Feb, 2001	84.7	79.1	83.4	NP	NP	NP	24.3	78.3	34.3	NP	NP	NP
Mar, 2001	83.8	83.5	89.3	55.5	85.8	48.3	26.3	83.3	53.8	28.3	83.2	84.3
Apr, 2001	80.2	93.1	87.6	NP	NP	NP	18.3	79.8	68.3	22.3	80.3	82.1
May, 2001	74.8	78.9	89.8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
June, 2001	70.3	78.9	88.4	75.7	82.6	75.3	0.00	0.00	0.00	0.00	0.00	0.00
July, 2001	NP	NP	NP	81.7	88.3	78.3	0.00	0.00	0.00	0.00	0.00	0.00
Aug, 2001	NP	NP	NP	78.3	83.4	76.3	0.00	0.00	0.00	0.00	0.00	0.00
Sep, 2001	NP	NP	NP	74.3	89.3	75.3	0.00	0.00	0.00	NP	NP	NP
Oct, 2001	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
Nov, 2001	NP	NP	NP	73.8	87.3	79.8	NP	NP	NP	NP	NP	NP
Dec, 2001	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
Jan, 2002	NP	NP	NP	68.3	86.3	82.3	NP	NP	NP	NP	NP	NP
Feb, 2002	NP	NP	NP	65.3	88.3	79.8	NP	NP	NP	NP	NP	NP
Mar, 2002	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
Apr, 2002	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
May, 2002	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
June, 2002	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
July, 2002	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP

NP – Experiment not performed

0.00 – No Results (Germination, Prothalli formation and Sporophyte formation)

P. aureum

Mature spores were collected during March 2001 to March 2002. The spores were cultured on Mi, Mo, KC and KN media. High percentage (81.7) of spore germination observed when the spores were collected in the month of July. Spores collected in the month of May failed to germinate. High percentage (89.3) of gametophyte formation observed in the spores collected in the month of September. High percentage (82.3) of sporophyte formation was observed in the spores collected from the month of January 2002 (TABLE 1).

P. triphyllum

Spores were collected during October 2000 to September 2001. High frequency (38.3) of spore germination was observed when the spores were collected during the month of March 2001. The spores collected during February to April 2001 showed minimum amount of microbial contamination. High percentage (83.3) of gametophyte formation was observed when the spores were collected in the month of March 2001. Highest percentage (68.3) of sporophyte formation was observed when the spores were collected in the month of April 2001 (TABLE 1).

S. unitus

Spores were collected during the 12 months starting from July 2000 to June 2001. High percentage (33.8) of spore germination was observed when the spores were collected in January 2001 and the lowest percentage (13.3) was recorded in spores collected in the month of November 2000. Spores collected during the months of May, July and September failed to germinate. The highest percentage (83) of gametophyte formation was observed in the spores collected in the month of March 2001. High frequency (84.3) of sporeling formation was found in the spores collected during the month of March 2001 (TABLE 1).

Influence of pH

C. viridis

Both liquid and agar media were used for spore germination and prothalli development. The spores showed more or less same percentage of germination (83.8-88.8) and the difference observed between the media was negligible at pH 5.8. The highest rate of prothallial growth (88.3±0.94) was observed in KC medium with pH 5.8. The highest length (3.1±0.62 mm) and breadth (2.03±0.63 mm) of the prothalli were observed in KC (pH 5.5). In other pH ranges tested, the length and breadth of the prothalli were reduced to a varied extent. High percentage of sporophyte (86.3±1.21) emergence was observed in KC medium at pH 5.8.

P. aureum

The spores were cultured in hormone-free Mi basal liquid and solid medium with different pH ranges. High frequency (81.3±1.24 in liquid; 80.3±1.31 in solid) spore germination was observed in Mi medium at pH 5.5 and the lowest frequency (35.3±1.31 in liquid; 28.3±1.21 in solid) was recorded at pH 8.0. No germination was observed in Mi medium at pH 4.0 and 4.5. Highest rate of prothallus formation (78.3±1.31) was obtained in Mi medium at pH 5.5 and the highest percentage (33.4±1.21) of sporophyte emergence was observed in Mi medium at pH 5.5.

P. triphyllum

The spores were cultured on hormone-free basal media, viz. KC, KN and Mi with different pH ranges (4.5 to 7.0). The spores showed maximum germination percentage (38.3±1.21) and growth rate in KN solid medium with pH 5.8. The pH of media influenced the length and width of the prothalli, the highest length (2.03±0.83) and breadth (2.31±0.68) being recorded in KN medium (pH 5.8). The highest percentage (52.3±1.1) of sporophyte emergence was observed in KN liquid medium at pH 5.8.

S. unitus

Among the different media tested, KN medium was the best for spore germination and therefore it was selected to test the effect of pH on spore germination. Highest frequency (36.8±1.31) of germination was recorded in KN medium at pH 5.8. In the case of other media tested, spore germination percentage was reduced to a varied extent. The lowest frequency (18.3±0.81) was recorded in KN medium at pH 4.5. However, KN medium at pH 4.0 and 6.5 did not favour germination of spores. The length and breadth prothalli were measured after 30 days of culture at different pH

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levels. Among the pH ranges, pH 5.8 gave better results compared to other pH levels. Sporeling formation was also influenced by the pH of the media. Sporeling was advanced in KN medium at pH 5.8 compared to others.

Iso-peroxidase analysis of different developmental stages

C. viridis

Four zones of activity (Regions) were observed in *C. viridis*. Zone one contained a single band (PRX 1^1), whose position was very much restricted to the hardened sporophytes only, and other developmental stages failed to express. Similar to zone one, zone two contained two bands (PRX 2^2 and PRX 2^3), whose position also was restricted to sporophyte stage only, and early developmental stages failed to show the banding profiles. The third zone showed three bands at different positions (PRX 3^4, PRX 3^5 and PRX 3^6), whose positions were restricted to the sporophyte and matured gametophytes, and the protonema and filamentous stages failed to express. Similar to zone three, the fourth zone first band (PRX 4^7) was observed in matured gametophyte and hardened sporophyte plant, and this particular band was absent in the initial sporophyte stage. The second band (PRX 4^8) of the fourth zone also showed unique presence in the matured gametophyte stage (Gametophyte with male sex organ and Cordate matured gametophyte), and the second band was not expressed in the sporophyte and early stage gametophyte (Figure 1 B).

P. aureum

Multiple zones of activity were obtained for *P. aureum* PRX 1 to 4. Zone one contained two bands, PRX $1^1$and ^{2}. PRX 1^1 showed its presence in all developmental stages, PRX 1^2 was restricted to matured sporophyte (S2, S3) and hardened stage (S4), whereas the other stages failed to show their presence. Zone two contained bands in three different positions; the first band (PRX 2^3) showed its presence in 15 days (S2), 30 days (S3) and 3 months old sporophyte (S4). Initial stage of the sporophyte (S1) and different gametophyte stages (G1, 2 and 3) failed to express themselves. Second band (PRX 2^4) of the zone two also showed its restricted presence, the second band was present only in 30 days (S3) and 3 months old sporophyte (S4), while the other

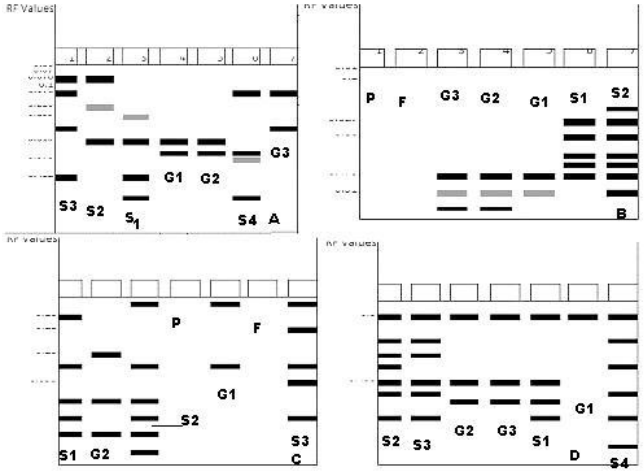

Figure 1 : Zymogram of Peroxidase banding pattern of different developmental stages of four rare and endangered ferns of Western Ghats

***Pronephrium triphyllum* (Sw.) Holttum (G1-Protonema stage; G2-Filamentous stage; G3-Cordate stage; S1-Sporophyte initial stage; S2-15 Day old sporophyte stage; S3-30 Days old sporophyte stage; S4-3 months old sporophyte stage)**

***Cheilanthes viridis* (Forssk.) Swartz. (P-Protonema stage; F-Filamenous stage; G1-Cordate stage; G2-Cordate with male sex organs; G3-Matured gametophyte; S1-Sporophyte initial stage; S2-30 Days old sporophyte stage)**

***Sphaerostephanos unitus* (L.) (Holttum). (P-Protonema stage; F-Filamenous stage; G1-Cordate stage; G2-Cordate with sex organs; S1-Sporophyte initial stage; S2-30 Days old sporophyte stage; S3-3 months old sporophyte stage)**

***Phlebodium aureum* L. (G1-Protonema stage; G2-Cordate with male sex organs; G3-Cordate with female sex organs; S1-Sporophyte initial stage; S2-15 Days old sporophyte stage; S3-30 Days old sporophyte stage; S4-3 months old sporophyte stage)**

stages were not expressed. The third band (PRX 2^5) showed its presence in all sporophyte stages and matured gametophytes, while early gametophytes (G1) failed to show its presence. Zone 3 contained bands in three different positions. The first band (PRX 3^6) showed its presence in 15 days, 30 days and 3 months old sporophytes. The second band (PRX 3^7) of Zone 3 was observed in cordate gametophyte with male (G2) and female sex organs (G3) and initial sporophytes (S1). PRX 3^8 was obtained in all sporophyte stages (S1, S2, S3 and S4). Zone 4 showed only one position which was restricted to 3 months old sporophyte (PRX 4^9), and the other developmental stages did not show any profiles in this region (Figure 1 D).

P. triphyllum

There were multiple zones of activity for *P. triphyllum* (PRX 1 –5). Zone 1 contained only a single band (PRX 1^1), which was restricted to 15 days (S2)

and 30 days (S3) old sporophytes. Zone 2 showed a single band in matured gametophyte (G3) and 30 days and 3 months old sporophyte (PRX 2^2). Zone 3 contained bands in three different positions; earlier stage gametophytes and matured sporophytes did not show the band in this region, while the other stages, viz. cordate stage gametophytes, 15 days, 30 days and 3 months old sporophytes showed variation in the banding position. PRX 3^3 was restricted to 30 days old sporophyte (S2), PRX 3^4 was specific to 15 days old sporophyte and PRX 3^5 was shared by cordate stage gametophyte and 30 days old sporophytes. Zone 4 contained bands in three different positions and the profiles showed their distribution specifically. PRX 4^6 showed its presence in early gametophyte stage, viz. protonema (G1) and filamentous stage (G2) and the early sporophyte stage also (S1 and S2). PRX 4^7 was observed in the G1, G2 gametophyte stages and 3 months old sporophyte stage (S4). PRX 4^8 expressed its unique presence with 3 month old sporophyte only. Zone 5 showed two bands in different positions (PRX $5^{9 \text{ and } 10}$). PRX 5^9 was specific to S1 and S3 sporophyte stages, PRX 5^{10} was specific to S1 and S4 sporophyte stages (Figure 1 A).

S. unitus

Five zones of activity were obtained in *S. unitus*. The first zone of activity was faint and disappeared within very short time (PRX $1^{1 \text{ and } 2}$). The first band (PRX 1^1) of zone one showed its presence in cordate stage of the gametophyte, 30 days (S2) and 3 months old (S3) sporophyte stages. The second band (PRX 1^2) was restricted to the initial stage of the sporophyte. The second zone of activity contained two bands in two different positions. The first band (PRX 2^3) was observed only in G2 stage of the gametophyte, but the second band (PRX 2^4) showed its presence in all sporophyte stages except 3 months old stage. Zone three contained two bands in two different positions; PRX 3^5 was expressed in G1 gametophyte stage and all sporophyte stages, but PRX 3^6 was restricted to S3 stage sporophyte. Zone four also showed two bands in two different locations; the first band (PRX 4^7) showed its presence in G2 gametophyte stage, S1 and S2 sporophyte stage. The second band (PRX 4^8) was expressed in all sporophyte stages, but was absent in the gametophytes. Zone five contained two bands in two different locations; PRX 5^9 was obtained in G2 gametophytes stage, S1 and S2 sporophyte stages. PRX 5^{10} was specific to S2 sporophyte stage (Figure 1C).

DISCUSSION

The age of explants plays a crucial role in culture initiation. Mature spores of the selected four species were by far the most preferred for in vitro multiplication. The mature spores germinated with ease compared to the younger ones. The young spores show high mortality rate when they are exposed to the surface sterilants. The influence of season is of paramount importance in the development of a successful conservation regime using the spore culture method. Each and every species has specific season for its growth and development. The viability and maturation of the spore is totally dependent on the season. Maturation of the spore is a dependent factor on the unfavourable season, leading to the release of immature spores by the sporangia that results in the vulnerable status of the plants. We observed the empty sporangia during the unfavourable seasons. In the present study, the viable spores were best obtained in the month of February for *C. Viridis*, July for *P. aureum*, March for *P. triphyllum* and January for *S. unitus*. These differences may be attributed to the dormant nature of the somewhat young spores and the nearly dead nature of the spores after maturity due to their inherent dehydrated nature and loss of viability. Previous workers have reported seasonal variation in spore germination in Polypodium aureum[37] Nephrolepis multiflora[26]; Drynaria quercifolia[38]; Thelypteris confluens, Cyathea crinita *and* Athyrium nigripes[27]; Diplazium cognatum, Hypodematium crenatum, Histiopteris incisa *and* Pteris vittata[40]. The present study shows some seasonal variation in spore germination, gametophyte development and sporophyte formation. The observations of the previous workers are in agreement with our observations. Spore germination is very sensitive to the pH of culture medium[6]. Spores of ferns are known to germinate well when the medium is slightly acidic to neutral with pH varying from 5-7[35]. In some cases, maximum germination was ob-

served at pH 4.0. Available evidences support Miller's statement that most of the fern spores germinated at pH 5-7[5,24,26-27,41]. In the present investigation also the spore germination percentage was high at pH 5.5-5.8, whereas in other pH ranges the germination rate was considerably reduced. While spores of *C. viridis, P. triphyllum* and *S. unitus* germinated well in a medium at pH 5.8, those of *P. aureum* germinated at pH 5.5. In addition to spore germination, the prothallial development and sporophyte formation also occurred well in the medium maintained at pH at 5.5-5.8.

The physical growth factors play a key role in *in vitro* development and organogenesis, of which pH is one of the most important factors recognized for *in vitro* growth. Information available is scarce on the influence of pH in a nutrient medium on *in vitro* growth, especially the sporophyte formation. The acidity or alkalinity of a medium is extremely important because it affects the growth of ferns (spore germination, gametophyte development and sporophyte formation). Page[41] pointed out that the optimal pH range was 5.5-7.5. The pH is not a universal character; each and every plant needs a different pH for its growth. Generally ferns prefer to grow in acidic habitats. There are a few reports available which show excellent spore germination and gametophyte development in acidic range[34]. Many plants, cells and tissues *in vitro* will tolerate pH in the range of about 4.0-7.2, and those tissues inoculated into media adjusted to pH 2.5-3.0/8.0 will probably die[42]. In a random sample of papers on spore germination and gametophyte development, the average initial pH adopted for several different media was found to be 5.8. In the present investigation spore germination percentage in all the four species was high at pH 5.5-5.8. Earlier reports lend support to the results of the present study. The present study reports the optimal pH for spore germination, gametophyte emergence and sporophyte formation for the endangered ferns. Whereas the pH of soil collected from the natural habitats of. *C. viridis* -6.8, *P. aureum* -4.5, *P.triphyllum* -5.0 and *S. unitus* -7.2 were varied from the *in vitro* optimized conditions. The present study reports that *C. viridis, P. triphyllum* and *S. unitus* require pH 5.8 and *P. aureum* 5.5 for spore germination, gametophyte and sporophyte development. This unfavourable natural condition is one of the important reasons for the red status or endangered ferns. Suitable environmental condition is one of the pre-requisites for the growth, development and establishment in natural habitats. The present investigation revealed that unfavourable environmental condition is one of the factors for the endangered status of these ferns. Hence, *in vitro* propagation method will provide an alternative tool for large-scale multiplication and aid for re-establishment in the wild. In addition, the present study determined the specific season for the selected four endangered ferns. Seasons also have their own role in fern spore germination and development. In the present study also supportive results have been obtained. From this study we found that optimization of age of explants, season of collection and pH of the culture media are pre-requisites for growth and development of any plant /fern.

Since 1930, electrophoresis joined with the zymogram technique has been the tool of choice for studies of heritable variation by geneticists, systematists, developmental biologists and population biologists. Isoenzymes are a powerful tool for gene variability within and between the populations of plants and animals, yet nowadays molecular techniques based on DNA are used. In contrast to DNA marker, isoenzyme analyses are widely used for their relative efficiency and cost-effectiveness, particularly in studies of intra- and interspecific variability[43-53] and varietal difference, tissue specificity and developmental variation[54]. In the present study also the iso-peroxidase revealed the biochemical differences between the developmental stages. The present observation was supported by observations of Smila et al.[54] on pearl millet and Johnson et al.[53] on *Rhinacanthus nasutus*. Morphological variation, a product of genotype and the environment, is an important parameter, but much diversity, which remains unexpressed morphologically, can be revealed by biochemical methods. The isozyme forms of this enzyme being the primary gene products can be used to deduce gene homology with precision by comparing variation in their expression patterns at different developmental stages of selected ferns as evident in the present study. However, appearance and disappearance of bands can be explained on the basis of gradual shifts of isozyme patterns in samples taken in the course of development due to differential activation of genes involved in synthesis of this enzyme at different stages of devel-

opment. Scandalios[55] has listed 46 isozyme systems, in which the pattern of gene expression varies with the developmental condition. In the present study also 9 to 10 iso-peroxidase systems were revealed. The changing banding profiles of isozymes during the development may be interlinked as evidence for differential timing of gene expression interpreted with the growth and development. Similar to this, Mehta and Ali[56] interpreted the changing pattern of isozymes with the physiological changes. Further, isozymes often exhibit tissue or cell specificity as observed in the present study. Modern molecular methods and isozyme sequencing are considered excellent for population structure analysis for sufficiently polymorphic taxa and developmental studies[57-58], but the data obtained through isozymes is relatively inexpensive compared to DNA. Large numbers of samples can be processed with far less training and time per sample; where as DNA analyses require more time and sophisticated instruments. Furthermore, in most cases, the new DNA- based markers provide the same type of information as isozymes.

The present study describes the requirements for *in vitro* spore germination, gametophyte development and sporophyte formation of *C. viridis, P. aureum, P. triphyllum* and *S. unitus*. The results may promote large-scale cultivation of selected four rare and endangered species to compensate their depletion in nature. In addition, the banding profile of different regeneration patterns revealed the biochemical changes during different developmental stages.

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