Hindawi Publishing Corporation
Journal of Botany
Volume 2014, Article ID 949028, 8 pages
http://dx.doi.org/10.1155/2014/949028

Research Article

In Vitro Conservation of Some Threatened and Economically Important Ferns Belonging to the Indian Subcontinent

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Received 1 May 2014; Revised 17 June 2014; Accepted 26 June 2014; Published 10 July 2014

Academic Editor: Curtis C. Daehler

This study was undertaken to identify methods of mass multiplication for five ornamental, economically important ferns (*Nephrolepis biserrata* (Sw.) Schott., *N. cordifolia* cv. "*duffii*" (L.) Presl., *N. exaltata* cv. *bostoniensis* (L.) Schott., *Pteris vittata* L., and *Cyclosorus dentatus* Link.,) and three threatened ferns, namely, *Cyathea spinulosa* Wall. ex. Hook, *Pityrogramma calomelanos* (L.) Link., and *Microsorum punctatum* (L.) Schott., through *in vitro* techniques. Collections were made from different biodiversity zones of India including Northeast Himalayas, Kumaon Himalayas, and Western Ghat and successfully introduced and grown in a fern-house. Aseptic cultures were raised at the morphogenic level of callus, axillary shoot, multiple shoot, and rooted plants. An optimized medium is described for each fern species. Plantlets were also produced from spore culture of *Cyathea spinulosa* and successfully hardened under fern house conditions.

1. Introduction

Pteridophytes constitute an important part of the plant kingdom, as they are worldwide in distribution, with 12,000 species distributed in a wide range of habitats. Pteridophytes have enormous economic potentiality as medicine, sources of food, fodder, fibre, flavouring agents, aromatic oil, perfume, dyes, and folk remedies [1–3]. In recent times ferns have also proved themselves valuable as pollution indicators, as well as insect repellents, and some species have the ability to hyper-accumulate noxious metals and metalloids, making them good phytoremediators [4–6]. In addition to their popularity as ornamental plants in the cut flower industry, pteridophytes have been employed in Ayurvedic, Homoeopathic, and Unani systems of medicine since the times of Charaka and Sushruta. The aesthetic appeal and exquisite foliage patterns make them popular plants for landscaping. According to a recent agricultural and industrial survey [7], the world floriculture trade of cut flowers, green plants, and cut greens in 1995 was US $ 6.8 billion, of which 55 percent were cut flowers, 37 percent green plants, and 8 percent cut greens.

Due to overexploitation, habitat destruction, and other biotic interferences, numerous pteridophytic species are threatened and some are critically endangered. Bir [8] identified 100 species as threatened from throughout India, but little is known about the conservation status of many of the ferns, and only 33 of those species have been categorized in red books. Chandra and Khare [9] enumerated 58 species as threatened, out of which 18 taxa are endangered from the Kumaon Himalayas (India) region only.

Ferns are conventionally propagated both by sexual as well as vegetative methods. The sexual method of propagation involves raising plants from spores, whereas the vegetative method involves propagation of specialized vegetative organs, such as bulbils, proliferous frond tip, aerial growths, stolon and tubers, offsets, gemmae, stipules, and root buds. These methods require more time and are comparatively slow. In India, in comparison to angiosperms, few attempts

have been made on *in vitro* studies in ferns and fern-allies. Vascular cryptogams, especially ferns, have not been very favourable material for tissue culture because their vascular system is made up of highly differentiated tissues that are difficult to proliferate into cell masses capable of growth *in vitro* [10]. Although different workers have tried to initiate *in vitro* studies on ferns, very little work on tissue culture of pteridophytes is reported [11].

The present investigation deals with collection and *in vitro* and *ex situ* conservation of eight economically important and threatened taxa of pteridophytes belonging to different biodiversity zones of the Indian subcontinent. These ferns are economically important, and *C. spinulosa, P. calomelanos,* and *M. punctatum* are listed in Red Data Book as threatened species, so the work presented here would be beneficial for the biological conservation of threatened ferns as well as commercial production of ornamental ferns through *in vitro* techniques.

2. Materials and Methods

2.1. Collection of Plant Material and Type of Explants Used. *In vitro* studies were undertaken on the following fern species: *Pteris vittata L.*, *Nephrolepis biserrata* (Sw.) Schott., *N. cordifolia* cv. *duffii* (L.) Presl., *N. exaltata* cv. *bostoniensis* (L.) Schott., *Cyathea spinulosa* Wall. ex Hook., *Microsorum punctatum* (L.) Copel., *Cyclosorus dentatus* Link., and *Pityrogramma calomelanos* (L.) Link. Among these fern species, *P. vittata*, *N. biserrata*, *N. cordifolia* cv. *duffii*, *N. exaltata* cv. *bostoniensis*, *M. punctatum,* and *C. dentatus* were collected from different phytogeographic region of India and successfully grown in the Fern House of CSIR-NBRI, Lucknow. Spores of *C. spinulosa* and *P. calomelanos* were collected from Didihat, in the Pithoragarh district of Kumaon Himalayas (Uttaranchal), Northeast region and Kodaikanal, Tirunelveli Tamilnadu, respectively (Figure 1; Table 1).

In the present study, explants from leaf primordia (0.5–1 cm.) from the fresh plant of *P. vittata, N. cordifolia* cv. *duffii, N. exaltata* cv. *bostoniensis, M. punctatum,* and *C. dentatus* were used, whereas, in case of *C. spinulosa* and *P. calomelanos,* leaf primordium (0.5 cm) of *in vitro*-raised sporophyte was used as explants. In the case of *N. biserrata*, stolon explants obtained from the live plant population growing in the Fernery of CSIR-NBRI, Lucknow.

2.2. Raising Aseptic Cultures and Multiplication. Explants were first washed thoroughly for 1-2 hours under tap water. The explants were then disinfected with 50–75% ethanol for a few seconds (depending upon the nature of explant) and subsequently surface was sterilized with $HgCl_2$ solution (0.1% w/v). Finally, repeated rinsing of the explants was done with sterile double distilled water. Treatment time varied from plant to plant and from explant to explant. Stolon parts were treated for 5–10 min, spore, leaf primordium, circinate part of young fronds and leaf primordium, procured from *in vitro*-raised sporophytes, were treated for 2–5, 2–10, 5–10, and 1-2 min, respectively. The explants were then thoroughly washed with sterile double distilled water (3-4 times). Sterilized explants were inoculated into the P&T, MS, and KnD media with at full, half, and one-fourth strength with different combinations of growth hormones along with various percentages of agar and sugar. More than 50 media combinations were tested for raising aseptic cultures of all the fern species. According to the morphogenetic response required, medium recipes were made by using basal media fortified with different ranges of 2, 4-D, IBA, IAA, KN, and NAA. Three replicate cultures for each treatment were incubated in a culture room under 38 μ mol m^{-2} s^{-1} fluorescent light for 15 hrs per day. Temperature and humidity of the culture room were maintained at 25 ± 2°C and about 60–70 percent RH, respectively. The period of incubation usually varied from 30 to 45 days according to the need of the experiment. Cultures were raised in 19.5 cm long × 3.5 cm diameter and 15 cm long × 2.5 cm diameter culture tubes as well as in Petri dishes of 100 mm × 17 mm and 80 mm × 17 mm size.

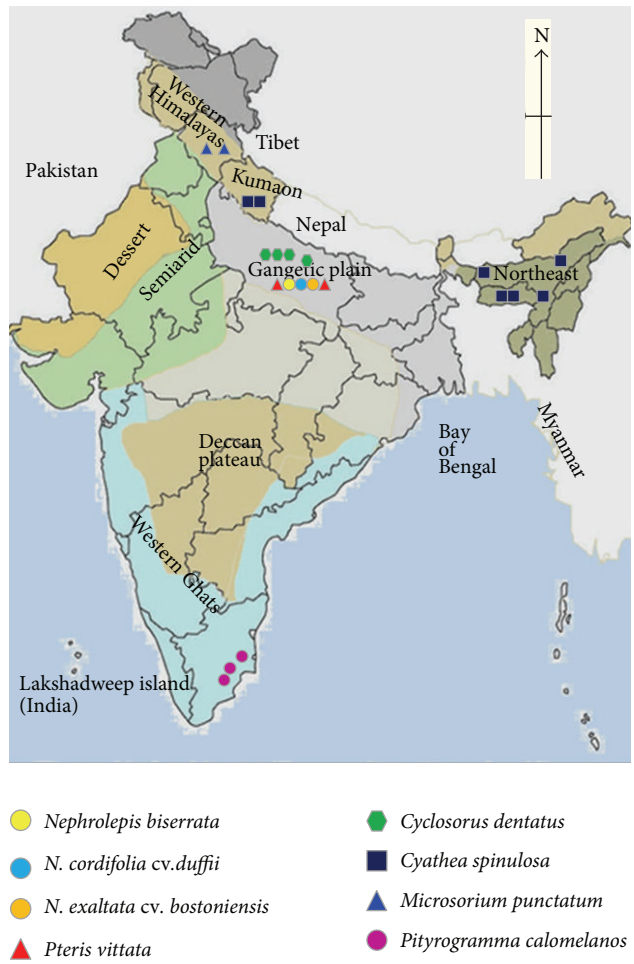

Figure 1: Different biodiversity zones of Indian subcontinent from where eight pteridophytic species were collected.

2.3. Fern House Hardening. *In vitro* regenerated plantlets, growing in aseptic cultures, were rooted successfully in the liquid P&T media [12]. The rooted plantlets were acclimatized

Table 1: Details of the pteridophytic plants collected, importance, and their site of collection for *in vitro* conservation.

S. number	Pteridophytic taxa	Family	Part used	Conservation status	Importance	Zone of collection
1	*Nephrolepis biserrata*	Nephrolepidaceae	Whole plant	—	Ornamental fern, much demand in nursery trait and cut flower industry. Cultivated in the botanical gardens as ornamentals and as potted plants for indoor decoration.	Gangetic plain
2	*N. cordifolia* cv. L *duffii*	Nephrolepidaceae	Whole plant, young leaves	—	Ornamental fern. Young leaves are cooked as vegetable by the tribals.	Gangetic plain
3	*N. exaltata* cv. *bostoniensis*	Nephrolepidaceae	Whole plant, young leaves	—	Ornamental fern, much demand in nursery trait and cut flower industry. Cultivated in the botanical gardens as ornamentals and as potted plants for indoor decoration.	Gangetic plain
4	*Cyathea spinulosa*	Cyatheaceae	Trunk and pith	Threatened	A threatened tree fern listed in Red-Data Book. Trunk is extensively using in the orchid cultivation. Pith from the trunks is used as a food product	Kumaon, Northeast Himalaya
5	*Pityrogramma calomelanos*	Hemioitidaceae	Whole plant	Threatened	A threatened tree fern listed in Red-Data Book. It is hyperaccumulator of arsenic	Tamilnadu
6	*Microsoium punctatum*	Polypodiaceae	Fronds	Threatened	A threatened tree fern listed in Red-Data Book and a popular houseplant. Much demand in nursery trait as an economically important fern due to its edible value in Asia and Pacific region. The cooked fronds of this fern are consumed in New Guinea.	Western Himalayas
7	*Pteris vittata*	Pteridaceae	Whole plant	—	An ornamental fern. Hyperaccumulator of arsenic. In Florida, USA it is using as a phytoremediator in arsenic-enriched soil.	Gangetic plain
8	*Cyclosorus dentatus*	Thelypteridaceae	Whole plant	—	An ornamental fern	Gangetic plain

in a solution consisting of only the inorganic salts of the same P&T media, before their final transplantation to potted soil. The plantlets along with their roots were carefully taken out of the Petri dishes with the help of forceps. 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. The nutrient solution was

Table 2: Morphogenic level along with the optimised medium and glasshouse hardening status of 8 plant species processed for *in vitro* conservation.

S. No	Plant species	Explant used	Callus (GR in mgL^{-1})	Cultures established at the level of axillary/multiple shoots (GR in mgL^{-1})	Rooted plants (GR in mgL^{-1})	Number of replicates	Time taken/ success rate during hardening
1	*Nephrolepis biserrata*	Stolon	—	P&T + 2,4-D (2.0) + NAA (2.0)	P&T + IAA (1.0)	10	2 wks/100%
2	*N. cordifolia* cv. *duffii*	Leaf primordium	—	P&T + 2,4-D (2.0) + BAP (2.0)	P&T + NAA (2.0) + Kn (1.0)	10	3-4 wks/60%
3	*N. exaltata* cv. *bostoniensis*	Leaf primordium	—	MS + 2,4-D (1.0) + BAP (0.5)	—	10	—
4	*Cyathea spinulosa*	Leaf primordium procured from *in vitro*-raised sporophytes	KnD + 2,4-D (2.0)	P&T + BAP (2.0) + NAA (1.0)	P&T + IBA (1.0)	10	3-4 wks/100%
		Spore	—	P&T basal media	P&T basal media	10	3-4 wks/80%
5	*Pityrogramma calomelanos*	Circinate part of young fronds	KnD + 2,4-D (1.0)	—	—	10	—
		Spore	—	P&T basal media	P&T basal media	10	5–7 wks/70%
6	*Microsorum punctatum*	Leaf primordium	1/4 strength of MS medium (Hormone- free)	—	—	10	—
		Spore	—	P&T basal media	P&T basal media	10	5–7 wks/50%
7	*Pteris vittata*	Leaf primordium	MS + 2,4-D (2.0) + BAP (0.5) GGBs- MS + 2,4-D (0.5) + BAP (0.5)	MS + IAA (0.5) + BAP (2.0)	P&T + NAA (1.0) + BAP (0.5)	10	3-4 wks/100%
8	*Cyclosorus dentatus*	Leaf primordium	—	P&T + BAP (1.0) + IAA (0.5)		10	

periodically changed every 5 days while the root system of plantlets was also thoroughly washed with double 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. The earthen pots along with the potting mixture were sterilized by autoclaving at 0.7 kg/cm^2 pressures for 10 min. before transplantation. The plants were initially covered with polyethylene 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 two species (*C. spinulosa* and *N. biserrata*) were later grown under Fern House conditions to observe their performance (Table 2).

3. Results and Discussion

Eight threatened and economically important ferns were successfully established *in vitro* at various morphogenic levels. Details pertaining to optimized media combination and morphogenic levels are given in Table 2. *C. spinulosa* and *P. vittata* showed both induction and proliferation of callus as well as shoots. *P. calomelanos* and *M. punctatum* were established only at the callus level, while *N. exaltata* cv. "*bostoniensis*" and *C. dentatus* were maintained only at shoot level. An efficient *in vitro* protocol has been standardized for the commercial production of an ornamental fern, *N. biserrata* through stolon explants. A threatened tree fern, *C. spinulosa,* has been multiplied by two methods: through caulogenesis from leaf primordium explants procured from *in vitro*-raised sporophyte and direct spore culture ([13]; Figure 2). Leaf primordium explants obtained from fresh plants growing in Fernery of NBRI, Lucknow, were used and evaluated for their regenerative potentiality *in vitro* in case of *P. vittata, M. punctatum,* and *C. dentatus.* In case of *P. vittata* leaf primordium explants showed better response for the callusing with GGBs and multiple shoots, which had been tried for the first time.

All the shoot cultures showed first bud break within 20–30 days, except for *C. spinulosa*, which required a longer time (three months) to show first bud emergence. Out of these cultures, friable callus of only *C. spinulosa* and *P. vittata* gave rise to differentiation of multiple shoots. Cultures of *N. cordifolia* cv. "*duffii*" and *N. exaltata* cv. "*bostoniensis*" showed excessive leaching of phenolics in the medium; therefore, they were subcultured frequently. Supplementation of 40 mg/l ascorbic acid in the respective media of *N. cordifolia* cv. *"duffii"* and *N. exaltata* cv. *"bostoniensis"* was able to control

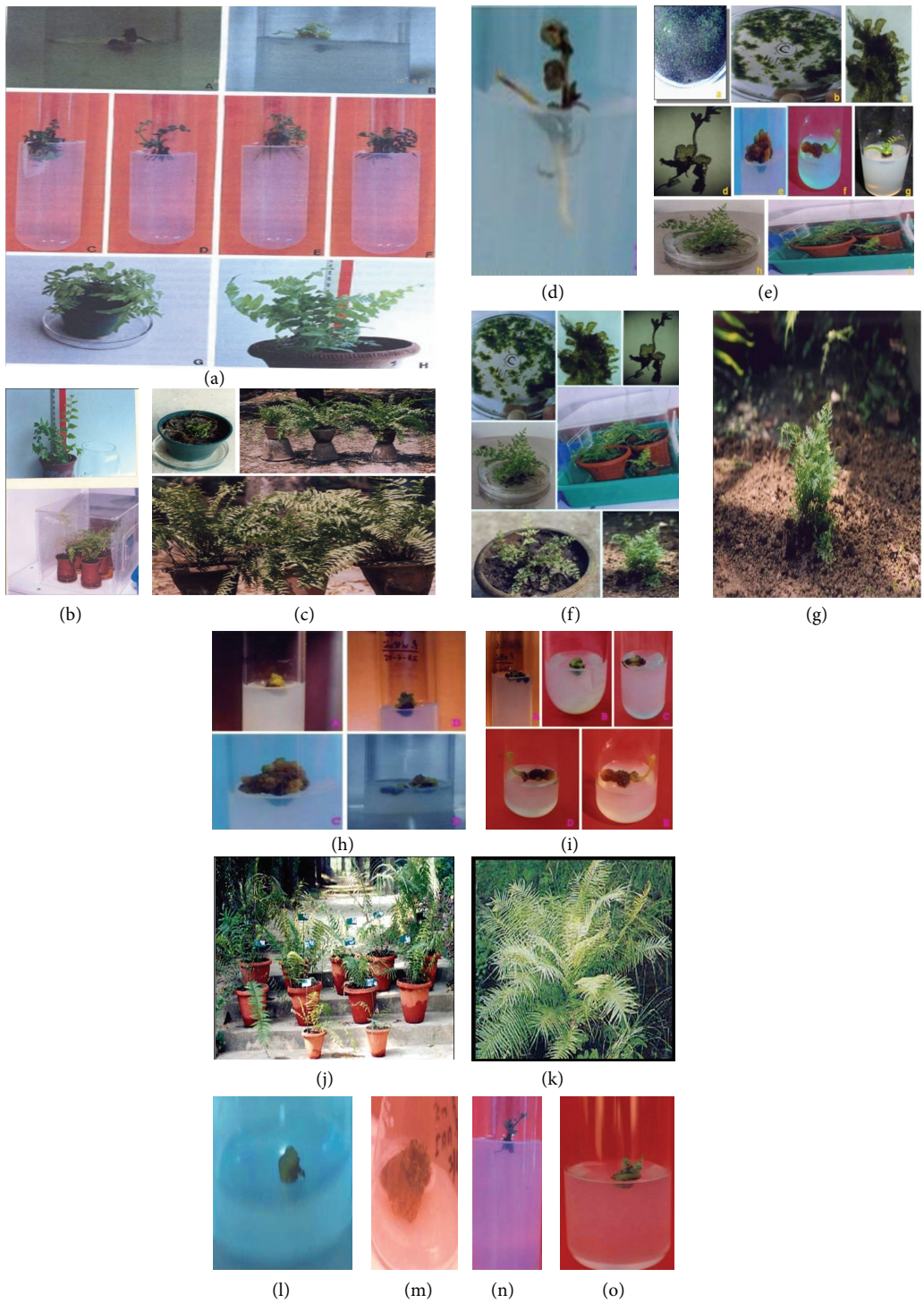

FIGURE 2: *In vitro* established cultures of some threatened and economically important ferns. (a) Regeneration of *Nephrolepis biserrata* through stolon explants, (b) acclimatization of the *in vitro*-raisedplantlets of *N. biserrata*, (c) transplantation of the plantlets of *N. biserrata* in fernery of CSIR-NBRI, Lucknow, (d) organogenesis in *N. cordifolia* cv. "*duffii*," (e) mass multiplication of *Cyathea spinulosa* through spore culture, (f) mass multiplication of *C. spinulosa* through spore culture via caulogenesis through excised pieces of sporophytes, (g) transplantation of *in vitro*-raised plantlets of *C. spinulosa*, in the fern house, (h) callus induction in *Pteris vittata*, (i) regeneration of leaflet in the callus culture of *P. vittata*, (j & k) *in vitro*-raised plantlets of *P. vittata* growing in the fern house condition, (l) GGBs in *P. vittata* culture, (m) callus culture of *Pityrogramma calomelanos*, (n) organogenesis in *Cyclosorus dentatus*, and (o) organogenesis in *Microsorum punctatum*.

the leaching and browning to certain extent, but frequent subculturing was the only feasible option observed in these plant species. Characteristic GGBs formation was observed within 15 days in *P. vittata* cultures.

The shoots of *N. biserrata, P. vittata,* and *C. spinulosa* were highly proliferating. *P. vittata* and *C. spinulosa* showed 100% germination rate of spore culture while only 50% and 70% spore germination was observed in *M. punctatum* and *P. calomelanos*, respectively. Rooting was observed in *N. biserrata*, *N. cordifolia* cv. "*duffii,*" *C. spinulosa,* and *P. vittata*. Rooted plants were successfully transferred to the fern house with a success rate of 60–80% (*N. cordifolia* cv. *"duffii"* and *C. spinulosa*) and 100% (*N. biserrata* and *P. vittata*) (Table 2).

In the present study, the morphogenetic potentialities of vegetative explants of three *Nephrolepis* species, namely, *N. biserrata, N. cordifolia* cv. *duffii,* and *N. exaltata* cv. *bostoniensis* were studied with a view to primarily develop methods of organogenesis and, subsequently, production of plantlets. In the case of *N. biserrata,* the explants of stolons were employed to induce differentiation of regenerants in them. In the course of investigation, the proper combinations and balances of growth hormones with appropriate inorganic salt media were enumerated for direct differentiation of regenerants from explants. Regeneration of plants from explants without intervening callus formation was reported to mostly produce genetically identical plants, in contrast with the formation of plants via callus, in which case, the genetically variable plants may be produced [14–17]. The three *Nephrolepis* species differed in their regenerative potentialities, which depended on the nature of explants used. The stolon explants used in the case of *N. biserrata* were highly regenerative, whereas leaf primordium explants of *N. cordifolia* cv. "*duffii*" and *N. exaltata* cv. "*bostoniensis*" were comparatively less regenerative. This may be due to higher meristematic tissue activity in stolon explants in comparison to leaf primordium explants. Survey of the literature revealed very few reports of economically important ferns that have been multiplied through *in vitro* method. The most prominent example of mass multiplication of an economically important fern is *N. exaltata* [18] in which callus induction was reported from provascular tissue of terminal and lateral buds of stolon tips. Some other reports are available on other species of *Nephrolepis, N. cordifolia* [19–21], and *N. exaltata* [14, 22–25], on morphogenetic studies pertaining to the micropropagation on a small scale. *In vitro*-regenerated plants had to be acclimatized in an inorganic salt solution for about two weeks in order to get 100 percent transplantation success in soil. The liquid culture phase for acclimatization of *in vitro*-raised plants was also necessary before transplantation in soil in case of *N. biserrata* [26], whereas in case of *Cheilanthes viridis* and *Diplazium cognatum* and *Matteuccia struthiopteris* direct plantlet transplantation in soil was successful [27, 28], which may be due to tropical rain condition of the area, which was not prevailing in other areas including the present study, where a liquid culture phase was essential for acclimatization. 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 other 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 [29]. These reports clearly indicated that Kn in combination with low concentration of NAA ($0.5–1\,mgL^{-1}$) produced shoots. The present investigation was carried out to identify the most appropriate culture media for *in vitro* mass propagation of three species of *Nephrolepis,* namely, *N. biserrata, N. cordifolia* cv. "*duffii,*" and *N. exaltata* cv. "*bostoniensis,*" because no such investigation has been reported to date. Some cultivars of this taxon are sterile [30], and economically very significant; thus, propagation through tissue culture technique is required.

Callus was induced from explants of leaf primordial of *P. vittata* in order to utilize it for morphogenetic studies. In ferns generally the nutritional requirements for callus induction and differentiation have been reported to be simple [31]. Callus production and its differentiation from leaf primordium is a comparatively difficult task because the percentage of contamination is high in the explants of young circinate fronds owing to their coiled pubescent nature. Kshirsagar and Mehta [32] reported induction and growth of callus from rhizome segments of *P. vittata* on "W" medium supplemented with 2, 4-D alone. In the present study, 2, 4-D used alone at various concentrations failed to induce callusing from explants of leaf primordia. However, 2, 4-D used with BAP was most effective for induction and fast growth of callus at its higher concentration, whereas its low concentration produced a green, compact, and globular callus indicating formation of GGBs. *In vitro* callusing was achieved in *in vitro*-raised frond tip explants of *P. calomelanos* and *M. punctatum* on full-strength of KnD medium supplemented with 2,4-D ($1\,mgL^{-1}$) and one-fourth strength of MS medium without hormone, but differentiation of callus was not achieved, whereas, in the cases of *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. During the course of investigation these taxa showed recalcitrant characteristics on *in vitro* studies (Figure 2).

4. Conclusion

In vitro studies on ferns and fern-allies are very meagre in comparison to angiospermic plants. Through the present study, economically valuable and endangered ferns were assessed for *in vitro* studies, which may help for mass-multiplication. Several pteridophytic plants are under the threat of overexploitation and biodiversity depletion. There is urgent need of their *ex situ* conservation. Collection and *in vitro* cloning and conservation of the 8 economically important ferns in the present study open fresh avenues towards the conservation and resource management of the overexploited pteridophytic plants.

Abbreviations

BAP: 6-Benzylaminopurine
CSIR: Council of Scientific and Industrial Research

2,4-D: 2,4-dichlorophenoxy acetic acid
GGBs: Green globular bodies
GR: Growth regulator
$HgCl_2$: Mercuric chloride
IAA: Indole-3-acetic acid
IBA: Indole-3-butyric acid
Kn: Kinetin
KnD: Knudson medium
MS: Murashige and Skoog medium
NAA: α Naphthalene acetic acid
NBRI: National Botanical Research Institute
P&T: Parker & Thompson's media
W: White medium.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Shastri P. Shukla conceptualized and wrote the first draft of the paper; P. B. Khare helped in maintaining *in vitro* cultures.

Acknowledgments

The authors thank the Director of CSIR-NBRI, Lucknow, for providing the necessary facilities to carry out this work. Shastri P. Shukla is particularly thankful to the Department of Biotechnology, Government of India, New Delhi, for providing the fellowship in the form of Senior Project Fellow during the course of study (2003–2006).

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