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Research Article

Direct Organogenesis from Rhizome Explants in *Marsilea quadrifolia* L.: A Threatened Fern Species

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An efficient micropropagation protocol has been developed for *Marsilea quadrifolia* L. through direct organogenesis. The mature rhizomes were used as explants and successfully sterilized using 0.1% $HgCl_2$ for the establishment of cultures. The multiple shoots were differentiated from the explants on Murashige and Skoog (MS) medium augmented with 6-benzylaminopurin (BAP). Full strength MS medium was reported to be effective for the induction of sporophytes from the rhizomes after four weeks of inoculation. Maximum response (96%) with average of 6.2 shoots (2.72 cm length) was achieved on full strength of MS medium augmented with 0.5 mg/L BAP in culture initiation experiments. The cultures were further proliferated in clusters (79.0 ± 0.37 shoots per explant) with stunted growth on half strength MS medium supplemented with 0.25 mg/L BAP after four weeks. These stunted shoots were elongated (5.30 cm long) on half MS medium devoid of growth hormones. Root induction and proliferation (3.0–4.0 cm long) were observed after 4th subculture of sporophytes on hormone-free half strength MS medium. The rooted plantlets were hardened in the fern house for 4-5 weeks and transferred to the field with 92% survival rate. There were no observable differences in between *in vivo* grown and *in vitro* propagated plantlets in the field.

1. Introduction

The pteridophytes are fragile and vulnerable to anthropogenic disturbances and climate changes due to their microclimatic dependence and having a strong affinity on high moisture for sexual reproduction [1]. *Marsilea quadrifolia* L. (commonly known as water clover, four-leaf clover, or water shamrock) is an aquatic, perennial, and heterosporous fern that belongs to the family Marsileaceae (water clover family) and native to Europe and Asia [2]. It is widespread naturally in central and southern Europe, Caucasia, western Siberia, Afghanistan, southwest India, China, and Japan [3].

The plant body of *M. quadrifolia* is sporophytic with well branched slender creeping rhizome which is capable of indefinite growth. There are four leaflets of equal size at the tip of each petiole, hence popularly known as four-leaf clovers. The aerial or land leaves exhibit circadian and phototropic movements [4], which have trichomes on adaxial and abaxial surfaces. The leaves show circinate venation when they are young and arise alternately in two rows from the upper surface of the creeping rhizome [5].

M. quadrifolia is chosen for ecotoxicogenomic studies because it is sensitive to aquatic environmental toxicant exposure [6]. It is a high bioaccumulator of heavy metals with maximum bioconcentration factor for cadmium and chromium and could be used for phytosequestration of these metals from contaminated sites [7]. *Marsilea* was studied exhaustively for its valuable biochemical properties [8–10]. The fern has potential antibacterial, anti-inflammatory, diuretic, depurative, febrifuge, refrigerant, antiepileptic, antioxidant, and cytotoxic activities [11–13].

M. quadrifolia is facing major threats due to the climate aridisation, limited distribution of the species, drainage and drying up of the wetlands, redirection of the river beds, overgrowth of marshes, and water pollution [14]. This fern is classified as threatened species and enlisted in the IUCN Red Data Book [15, 16]. Due to the increasing human activities, all *Marsilea* species in Europe are considered as

rare, vulnerable, endangered, at extinction, or extinct in the wild [17]. As per the assessment of World Conservation Monitoring Centre [18], *M. quadrifolia* is threatened in 21 European countries, vulnerable in eastern Palaearctic region (ecozone), and known to be extinct in Germany, Poland, and Switzerland [19]. The National Biodiversity Act stressed on the conservation needs to protect *M. quadrifolia* in its natural habitats [20]. The fern is also ranked as endangered plant in eastern India [21].

Biotechnological approaches can be used in preservation of genetic material and explored for the reintroduction of the species in nature [22, 23]. *In vitro* culture of endangered and rare fern species at mass scale can be exploited as an alternative tool for *ex situ* conservation. *Nephrolepis exaltata bostoniensis* was the first fern in which the *in vitro* propagation protocol was developed for commercial exploitation [24, 25]. Sporocarps and spores were used as explanting materials in the previous attempts on micropropagation of *M. quadrifolia* [26, 27] which failed to respond under *in vitro* culture experiments [28]. This is an attempt to develop an efficient micropropagation protocol of aquatic fern *M. quadrifolia* using rhizomes as explants.

2. Materials and Methods

2.1. Plant Material and Explants Selection. The plant *M. quadrifolia* was collected from southeast India (swampy area near Villupuram, Pondicherry State) in the months of April-November 2013 and successfully grown in fern house. The rhizomatous nodal explants (stolons) of about 1-2 cm in length were harvested and brought to the laboratory in order to initiate the cultures *in vitro*.

2.2. Surface Sterilization of Explants. Since the stolons were used as explants, certain procedures followed to reduce the contamination in cultures: (1) Rhizomatous explants were rinsed with sterile running tap water for 15 min, (2) explants were rinsed with sterile double distilled water and centrifuged at 100 rpm for 5 min, (3) the explants were treated with 0.1% Bavistin (a systemic fungicide, BASF India Ltd. India) for 15 min, and then (4) surface sterilization was carried out with 0.1% $HgCl_2$ for 4-5 min which is followed by washing with sterile distilled water for 5 times under laminar air flow cabinet.

2.3. Culture Medium and Inoculation. Murashige and Skoog (MS) medium [29] was used for the establishment of cultures. The sterilized explants were inoculated aseptically on the half and full strength MS basal medium supplemented with additives (50 mg/L ascorbic acid, 25 mg/L each of citric acid, L-arginine, and adenine sulphate). The medium was augmented with 6-benzylaminopurine (BAP) and 6-furfuryl amino purine (Kinetin) in different concentrations ranging from 0.0 to 3.0 mg/L to induce shoots from the explants. The amendment of carbon source in the medium was 1.5% to 3% of sucrose as per MS salt concentrations, and the gel strength was maintained with the help of 0.8% agar-agar. The pH of medium was adjusted to 5.8 ± 0.02 with help of 0.1 N NaOH/KOH or HCl. The culture tubes (vessels) were finally incubated at 25 ± 2°C temperature under cool white fluorescent light (Philips, India) with 16 h artificial photoperiod (40 μmol m^{-2} s^{-1} Spectral Flux Photon Density, SFPD) at 55–60% relative humidity (RH).

2.4. Aseptic Culture Proliferation. MS basal medium supplemented with different concentrations of cytokinins (BAP and Kin) with varying concentrations (ranging from 0.0 to 1.0 mg/L) was used for multiplication of cultures of *M. quadrifolia*. The culture medium was changed as per the morphogenic response of the cultures using different concentrations of basal salts fortified with different ranges (0.0 to 1.0 mg/L) of growth hormones. The cultures were transferred to the fresh medium after 4-5-week intervals and the number and lengths of shoots and roots from rhizomatous sporophyte were evaluated after every 4 weeks of subculture.

2.5. Hardening and Acclimatization of Plantlets. *In vitro* raised plantlets were rooted simultaneously with culture proliferation stage on same medium combination. Hardening aimed at the introduction of micropropagated plantlets of *M. quadrifolia* into the greenhouse and finally to the field. The rooted sporophytes were separated from culture medium carefully and washed with distilled water to remove the remains of medium and transferred to sterile soilrite (in paper cups) moistened with one-fourth MS salts solution. The setup was maintained in the fern house for 10 days to ensure high humidity and open shade (diffused light) for hardening. After a week, the plants were shifted to nursery polybags containing garden soil and clay (1 : 1) to maintain aquatic environment. Finally the hardened plantlets were transferred to the aquatic, shade field.

2.6. Statistical Analysis. The values presented in each table are means of triplicates with ten replicates each. The results were presented as mean ± standard deviation (SD) of triplicates. The resulting data were analyzed by analysis of variance with Duncan's multiple range tests where the level of significance was $p < 0.05$ using SPSS software (version 16.0).

3. Results and Discussion

3.1. Establishment of Cultures. *M. quadrifolia* cultures were established on MS medium augmented with cytokinins in present study. Cytokinins regulate a considerable number of different developmental and physiological processes in aerial and subterranean organs, like cell divisions in the shoot and root meristems and chloroplast differentiation [30]. These also promote axillary buds proliferation by antagonizing the activity of auxins [31]. The effect of strength of MS medium and the different concentration of plant growth regulators were tested for multiplication and rooting of the shoots. Full strength MS medium was reported to be effective for the induction of fronds from the rhizome explants after four weeks of inoculation. Brownish green meristematic areas developed within two weeks around the notch (nodal part) of rhizome on full strength MS medium supplemented with

Table 1: Effect of different concentrations of cytokinins (BAP and Kin) on induction of shoots from rhizome explants of *M. quadrifolia*.

Conc. of cytokinins (mg/L)	Response (%)	Number of shoots (Mean ± SD)	Shoot length (cm) (Mean ± SD)
Control (0.0)	0.00	0.0 ± 0.00^{a}	0.00 ± 0.00^{a}
BAP			
0.5	96	6.2 ± 1.39^{h}	2.72 ± 0.40^{f}
1.0	69	3.7 ± 0.51^{f}	1.60 ± 1.25^{g}
1.5	73	4.8 ± 1.04^{g}	1.33 ± 0.18^{e}
2.0	61	3.0 ± 0.73^{de}	1.10 ± 0.12^{b}
2.5	48	4.8 ± 1.04^{g}	1.33 ± 0.18^{e}
3.0	22	2.5 ± 0.30^{cd}	0.44 ± 0.31^{c}
Kin			
0.5	57	3.3 ± 0.26^{ef}	1.31 ± 0.22^{e}
1.0	49	3.0 ± 0.14^{de}	1.08 ± 0.16^{d}
1.5	42	2.8 ± 0.40^{de}	0.85 ± 0.38^{f}
2.0	40	2.6 ± 0.39^{d}	0.93 ± 0.45^{h}
2.5	35	2.0 ± 0.81^{c}	0.49 ± 0.10^{d}
3.0	19	1.2 ± 0.22^{b}	0.32 ± 0.28^{c}

Note: mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

Table 2: Effect of growth hormones (BAP and Kin) on multiplication of sporophytes on agar gelled MS medium.

Conc. of BAP (mg/L)	Conc. of Kin (mg/L)	Number of shoots (Mean ± SD)	Shoot length (cm) (Mean ± SD)
0.00	0.00	79.0 ± 0.37^{k}	5.30 ± 0.51^{g}
0.10	—	54.3 ± 0.46^{fg}	4.13 ± 0.30^{e}
0.25	—	50.9 ± 0.23^{de}	4.08 ± 0.38^{e}
0.50	—	43.5 ± 0.51^{c}	3.20 ± 0.29^{b}
1.00	—	40.1 ± 0.26^{b}	3.06 ± 0.57^{b}
—	0.10	55.0 ± 0.71^{g}	3.74 ± 0.20^{cd}
—	0.25	49.5 ± 0.37^{d}	3.51 ± 0.35^{c}
—	0.50	44.2 ± 0.29^{c}	3.00 ± 0.62^{ab}
—	1.00	36.6 ± 0.18^{a}	2.79 ± 0.48^{a}
0.10	0.10	68.2 ± 0.49^{j}	4.78 ± 0.42^{f}
0.25	0.25	61.9 ± 0.35^{i}	4.22 ± 0.15^{e}
0.50	0.50	57.3 ± 0.26^{h}	3.80 ± 0.30^{d}
1.00	1.00	52.8 ± 0.40^{ef}	3.53 ± 0.41^{c}

Note: mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and the values represented in corresponding column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

0.5 mg/L BAP. The maximum response in culture initiation was achieved in presence of low concentrations of BAP (0.5 mg/L), and 96% of explants responded well with average of 6.2 shoots with 2.72 cm length on this combination (Figure 1(a), Table 1). Delayed response of the explants with less number of shoots was observed on half strength MS medium on same hormone concentrations. The shoots were induced after eight weeks of inoculation of explants on the medium. The response of explants with Kin was reported poor as compared to the BAP, and only 57% explants responded on full strength MS medium supplemented with Kin with 3.3 shoots per explant. The number of shoots was reported stagnant on MS medium supplemented with Kin (0.5 mg/L), even after four months of incubation.

Generally, ferns micropropagated using spores in most of the studies like *Salvinia natans* [32], *Dryopteris affinis* [33], *Equisetum arvense* [34], *Marsilea minuta* [35], and *Pteris tripartita* [36], but the shoots were induced from the nodal part of the rhizomes in present study. It has been reported that the supplementation of abscisic acid in the medium induced shoots, roots, and elongation of petioles of *M. quadrifolia* [37, 38], whereas the supplementation of auxins has no ameliorative effects on the regenerative response in this plant species [28]. The shoots were proliferated from the mother explants even in absence of abscisic acid and auxins in present study. Rolli et al. [27, 39] also studied the effect of plant hormones on *in vitro* propagation of *M. quadrifolia* using rhizomes as explants but less number of shoots was induced per explant.

3.2. Multiplication of Shoots In Vitro. The enhancement of shoots multiplication rate on various concentrations of cytokinins on culture medium was tested. The fresh shoots with mother explants were subcultured to the fresh medium after four weeks of incubation. Cultures were proliferated in clusters on half strength MS medium augmented with additives and 0.25 mg/L BAP with stunted growth (Figure 1(b)). Highest mean number of juvenile shoots (79.0 ± 0.37 shoots per explant) was regenerated on this concentration (Table 2). The further growth and elongation of shoots with large number of identical individuals from small parts of inoculums was achieved on half strength MS medium without any growth hormone in present study (Figure 1(c)).

The leaflets with long petiole arise solitarily from the nodes on the rhizomes. The response of the cultures under *in vitro* conditions was very slow, but frequent subculturing on fresh medium was found the only feasible option to multiply this plant species. It was observed that the shoots with mother explants failed to respond better if they were directly transferred to the hormone-free medium. Therefore, the cultures were first stabilized on 0.25 mg/L BAP and later transferred to the hormone-free medium. Higher concentrations of cytokinins (both BAP and Kin) showed stunted growth of sporophytic shoots and decreased rate of shoots multiplication. The elongated shoots (5.30 cm long) with good roots (3.0–4.0 cm length) were developed on MS medium without any growth hormones. In contrast to the present results the effects of cytokinins were found inhibitory on multiplication of cultures of *M. quadrifolia* by Rolli et al. [39]. Spore germination and conversion of gametophytic stage (haploid) into a sporophytic stage (diploid) could be exempted through micropropagation technique in *M. quadrifolia*. *M. quadrifolia* cultures were also established on

Figure 1: (a) Regeneration of leaflets from the rhizome explants on MS medium. (b) Early stage of multiplication (stunted growth of fronds). (c) Development of multiple sporophytes on half strength MS medium without growth hormones. (d and e) *In vitro* propagated sporophytes with fronds and roots. (f) Hardening of sporophytes of *M. quadrifolia* in soilrite. (g) Hardened sporophytes growing in the fern house.

Knop's nutrient solution and half strength MS medium by various researchers [28, 40].

3.3. In Vitro Rhizogenesis. Roots induction was observed after 4th subculture of sporophytic shoots on hormone-free half strength MS medium. The shoots were separated and transferred to the half strength MS medium, where number of shoots with thin and long creeping rhizomes was increased quantitatively in clusters. Best rooting frequency (100%) was achieved on hormone-free half strength MS medium (Figures 1(d) and 1(e)). The present results were also supported by Rolli et al. [39] and reported development of complete sporophytes on hormone-free medium. Banciu et al. [28] reported rooted sporophytes in Knop's liquid medium. Generally, auxins are used in rooting of the shoots in plant tissue culture experiments [41, 42] but in case of *M. quadrifolia* good rooting was reported without augmentation of any growth regulator with the MS medium. Breznovits and Mohay [43] also maintained *Marsilea* sporophytes on hormone-free half strength MS medium. Ravi et al. [36] grown spores derived gametophytes on half strength MS medium also support our results.

3.4. Hardening and Acclimatization of In Vitro Grown Sporophytes. The *in vitro* regenerated plantlets were acclimatized for conservative expression in different environmental conditions. The plantlets were taken out from the culture vessels and the traces of agar were removed using soft brush to avoid damage of the roots. Care has been taken while removing the agar, since the sporophytic roots were very soft and delicate. The plantlets were initially shifted to sterile soilrite containing paper cups (Figure 1(f)) and covered with transparent membrane and placed in the fern house to maintain high humidity. This setup was moistened after every three days to maintain semiaquatic environment for four weeks. Once the roots were adapted to external environmental conditions, these were shifted to nursery polybags, which contain clay and garden soil. Addition of clay with garden soil created semiaquatic environment, since, it has water holding capacity for long time. Rolli et al. [39] acclimatized plantlets by culturing on hormone-free MS medium and 2iP containing medium. It was reported that the hardened plants failed to survive in soil mixtures devoid of clay. The hardened plantlets produced new sporophytic shoots from their rhizomes (Figure 1(g)). The plantlets hardened were morphologically identical with the native plants in external features. About 92% of plantlets survived under the field conditions in present study.

3.5. Conclusion. An efficient micropropagation protocol has been developed for a threatened and economically important fern, *M. quadrifolia*. The aseptic cultures were raised at the morphogenic level of axillary shoots, multiple shoots, and rooted plantlets from the small portion of rhizome tissues. The developed plantlets can be reintroduced in the natural habitat or preserved in cells or tissue culture laboratories for future studies.

Disclosure

The present research work has not involved any human participants and/or animal.

Conflict of Interests

The authors report that there is no conflict of interests regarding the publication of this paper.

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