Annals of Plant Sciences
ISSN: 2287-688X
OPEN ACCESS
www.annalsofplantsciences.com

Original Research Article

Induction of sporocarp development *in vitro in the* mosquito fern, *Azolla rubra* R. Br.

Sini S[1], Smitha RB[2] and PV Madhusoodanan[3*]
[1]Department of Botany, University of Calicut, Kerala, India
[2&3]Malabar Botanical Garden, Kozhikode – 673014, Kerala, India

Received for publication: October 15, 2014; **Accepted:** December 17, 2014.

Abstract: *Azolla rubra* R. Br. (Azollaceae: Pteridophyta), a popular biofertilizer applied in inundated paddy fields propagate usually through vegetative fragmentation only. Induction of sporocarp development, callus, and indirect organogenesis were accomplished. Influence of various factors such as agar, light, phosaphate, sucrose and pH on *in vitro* growth of *A. rubra* were also studied. Half strength MS medium with 2.26 µM 2,4-Dichlorophenoxyacetic acid and 6.6 µM 6-Benzyl Amino Purine was found best for callus induction. The callus transferred onto medium having 6.6 µM BA underwent indirect organogenesis. Shoot tips cultured in light on medium having 2.26 µM 2,4-Dichlorophenoxyacetic acid and 6.6 µM 6-Benzyl Amino Purine with 0.8% agar and 3 % sucrose at pH 5.8 was found best for induction of callus.

Key words: *Azolla rubra* R. Br., sporocarp induction, *in vitro* development

Introduction

Spectacular progress in our knowledge of plant sciences, including those relevant to agriculture and breeding, can be expected from the integration of the traditional sciences with molecular concepts and techniques. Tissue culture is one of the fabulous tools in biotechnology for the rapid propagation and improvement of crops, medicinal, RET and ornamental plants including ferns. Plant tissue culture techniques not only provide essential ways for the clonal propagation of many agriculturally important crops (e.g., ornamentals, medicinal and vegetables) but also for the production of transgenic plants, and are at the forefront of recent studies in plant physiology developmental biology, and biochemistry (Pereira and Carrapico 2009; Wang and Bhalla 2004; Lin *et al.,* 2004).

Fern tissue culture began with the culture of spores on artificial substrates, first under partially sterile conditions such as sterile sand banks and then under fully axenic condition on agar-plated petridishes. *In vitro* propagation strategies using sporophytic tissues facilitate low volume high production of true-to-type progenies, and are now used to scale up the production of ornamental plants to meet the growing demand in both domestic and export market. Commercially *Nephrolepis* cultivars have been propagated through *in vitro* culture. In the Netherlands, 11,194,000 plants were cloned by *in vitro* culture in1986 (Pierik 1987). According to Pierik (1991), 157 million plants *i.e.*75% of these, approximately 40 million plants were important pot plants.

Encapsulation of vegetative propagules *in vitro* has much pertinence due to its ease of production, handling, and direct delivery to field, and also as an efficient means for the conservation of rare endangered plants including ferns. *In vitro* culture opens new windows for the production of secondary metabolite production by cell culture methods, and makes possible for the production of valuable phytochemicals without distressing the wild plant resources. Considering our wealth of fern flora particularly the ornamental, endemic and endangered fern, tissue culture technology is an inevitable alternative to provide planting propagules *en masse* to meet the domestic and global demand. Biotechnology makes traditional agri/horticulture more productive and sustainable. The real driving force behind the success of applied plant biotechnology relies on plant protection based on the expression of several alien genes coding for different principles enabling the development of agri/horticultural crops resistant to pathogens, insect pests and herbicides, and with quality traits for survival in adverse conditions, improved pigments, and increased shelf life of fruits/flower/leaves.

The genus *Azolla* (commonly known as mosquito fern) has seven extant species, of which only *A. pinnata* R. Br. occurs naturally

***Corresponding Author:**
Prof. (Dr.) P.V. Madhusoodanan,
Emeritus Scientist,
Malabar Botanical Garden,
Kozhikode, Kerala, India.

in India, having wide distribution from temperate to tropical regions. *Azolla* spp. can be effectively utilized as a biofertilizer for nitrogen in the paddy fields. *Azolla rubra* R. Br., originated in China, has much relevance as a biofertilizer in rice fields of Kerala in having tolerance towards high temperature and high light intensity. This is mainly propagated by fragmentation of the plant body and is reported to have high N_2 content than other species (Sevichan 1994). The establishment and growth of *Azolla* are severely hampered by the growth of the weeds, and is difficult to tackle without damage to *Azolla*.

The major threat to *Azolla*, particularly in the tropics is the attack of insect pests. In the light of above, the present study focused callus induction of the selected plant *Azolla rubra* R. Br., induction of development of sporocarp, effect of sucrose, agar, pH and light in callus and sporocarp induction, induction of indirect organogenesis.

Materials and Methods

For the *in vitro* propagation studies *Azolla rubra* R. Br. were selected. The plant was originally collected from Central Rice Research Institute, Cuttack, India and maintained at the Calicut University Botanical Garden, Kozhikode in cement tanks with paddy field soil in 30 cm deep water with occasional supplement of organic manure (cow dung) (Fig. 1).

Fig. 1: *Azolla rubra* R. Br. Habit

Stock solutions for the preparation of media including growth regulators were made using tissue culture grade chemicals of Qualigens, Hi-Media and BDH. Tissue culture grade sucrose of BDH at 1-6% was used as the carbohydrate source. MS (Murashige and Skoog, 1962) medium was used as the basal medium for *in vitro* culture studies. Medium was modified by supplementing different growth regulators viz., NAA/IAA/IBA/2,4-D/BR/Kn at different concentrations either singly or in combination as given in Table 1. Half strength MS medium with the addition of different levels of phosphate and growth regulators (BAand 2,4-D) were used for the *in vitro* studies of *Azolla.*

Table 1: Concentration and types of growth regulators used for the present study

Plant	Explant	Basal Medium	Auxin	Conc. (mg/L)	Cytokinin	Conc. (mg/L)
Azolla rubra R. Br.	Shoot tips	MS	2,4 –D	2 4	BAP	0.5
			NAA	2 4	BAP	0.5

The stock solutions of macro and micronutrients, iron chelates, and vitamins of MS media were prepared separately using double distilled water. The different growth regulators separately dissolved in respective solvents were made up with double distilled water. All the stock solutions were stored in a refrigerator (4°C). Knop's solution and LB medium were prepared freshly according to the need. The effect of different pH regimes (4.5-7.0) was studied. Approximately 15 and 25 mL of the medium were dispensed into culture tubes and conical flasks (100 mL) respectively. The medium was gelled with different levels of agar (0.4-1.0%). Sterilization of the media was carried out at a pressure of 1.06kg cm^{-2} (temperature 121^0 C) in an autoclave for 20 minutes. Sterilized media were kept in the culture room.

Results

In vitro propagation studies were conducted on *Azolla rubra* R. Br., an economically valued fern, and the results are described below. *In vitro* studies of *Azolla rubra* R. Br. were carried out by culturing shoot tip explants on MS medium without or with the addition of various growth regulators. Different concentrations of mercuric chloride were used for the surface sterilization of explants. Of the different treatments, 0.05% for 5min. resulted establishment of 90% contamination-free cultures while high percentage of contamination (85%) was observed at low concentrations (0.01% for 7min.) of mercuric chloride. But mercuric chloride of 0.1% resulted in the death of the explants, nevertheless, it favored survival of 20% explants.

Shoot tip explants cultured on MS basal medium did not evoke callus initiation, however, it exhibited growth. Callus induction was attempted using MS medium modified with auxins alone or in combination with cytokinins (BA/Kn).

MS medium with NAA with or without BA/Kn

The explants cultured on medium having different concentration of NAA (2.69-10.74 μM) without or with BA (0.22-4.44 μM)/Kn (0.23-4.65 μM) did not induce callus. The explants showed vegetative growth, but died after 30 days.

MS medium with 2,4-D

Half strength MS medium fortified with various concentrations of 2,4-D (2.26-9.05 μM) induced callus. MS medium supplemented with 4.52 μM 2,4-D was the best concentration for induction and proliferation of callus (Table 2). The cream coloured callus initiation occurred from stem especially from axill region of the fronds (Fig. 2a) and showed initiation between 18 and 25 days (Fig. 2a). The culture explants exhibited progressive browning and death from the basal region, nevertheless, the callus showed proliferation (Fig. 2b).

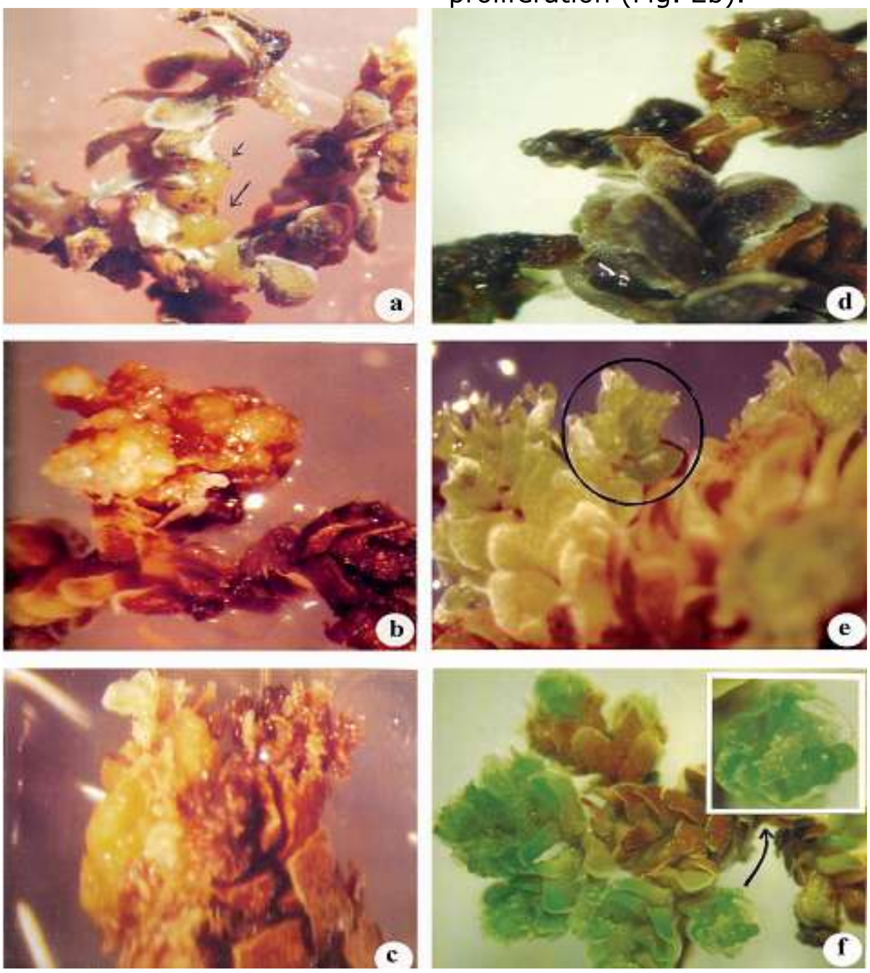

Fig. 2: Figure showing the callus and sporocarp induction from shoot tip explants of *Azolla rubra* R. Br. a. Callus developed from the axil of fronds on 1/2 MS medium with 4.52 μM 2,4-D, b. Proliferated callus on medium of a, c. callus developed on 1/2 MS medium with 4.52 μM 2,4-D and 2.32 μM Kn, d. callus developed on 1/2 MS medium with 4.52 μM 2,4-D and 6.66 μM BA, e. sporocarps developed on 1/2 MS medium with 2.26 μM 2,4-D and 6.66 μM BA and f. sporocarps developed on 1/2 MS medium with 2.26 μM 2,4-D and 8.87 μM BA (inset shows an enlarged view of shoot tip with sporocarps).

Table 2: Induction of callus from shoot tip explants of *Azolla rubra* on half MS medium with different growth regulators

Growth regulators (µM)			Explants inducing	Mean fresh
2,4-D	BA	Kn	callus (%)	weight of callus (mg)
2.26			75	189^{d}
4.52			85	212^{c}
6.78			60	182d^{e}
9.05			45	106^{h}
	2.22		70	125^{g}
	4.44		75	109^{gh}
	6.66		85	105^{h}
	8.87		65	91^{h}
2.26	2.22		60	206^{c}
2.26	4.44		70	201^{cd}
2.26	6.66		85	270^{a}
2.26	8.87		70	218^{c}
9.05	2.22		50	198^{d}
4.52	6.66		40	215^{c}
4.52			60	235^{b}
2.26		2.32	40	176^{ef}
2.26		4.65	50	158^{f}
2.26		6.97	55	142^{fg}

MS medium with 2, 4-D +BA/Kn

Different levels of 2,4-D in combination with various level of BA (2.22-8.87µM) or Kn (2.32-6.97µM) supplemented to half strength MS medium facilitated callus formation (Table 2). 2,4-D in combination with BA was superior to 2 ,4-D and Kn for the induction of callus (Table 2). Of the various concentrations, 2.26 µM 2,4-D and 6.66 µM BA was the best for callus induction and proliferation (Table 2). Before the formation of callus, the shoot tip showed vegetative growth and it initiated after 5 days of establishment. The growing shoot tip was green and the fronds were very close to each other. The callus developed from the stems of the initiated and growing shoot tips of the explants, especially from the axil of the fronds. The callus initiated after 21 days of explants culture. The callus on 2,4-D and Kn containing medium was cream coloured (Fig. 2c). Medium with 4.52 µM 2,4 –D and 6.6 µM BA, the callus was cream to pale green coloured, and appeared like globular structures (Fig. 2d). Increase or decrease of 2,4-D or BA from the optimal medium reduced the amount of callus (Table 2). Decrease of BA also reduced the vegetative growth of shoot tip explants. Besides the formation of callus, sporocarps were developed from the axils of the fronds (Fig. 2e). They were green in colour. The number of sporangia development also varied with the concentration of BA and the optimal callus induction medium developed a mean of nine sporocarps per shoot tip (Fig. 2e). While BA at 8.87 µM in combination with 2.26 µM 2,4-D developed a mean of twelve sporocarps per shoot tip (Fig. 2f). In the case of medium with higher concentration of BA and lower 2,4-D, the callus showed formation of shoot buds covered with small thin fronds (Fig. 3a).

MS medium with cytokinins (BA/Kn)

Addition of BA at different levels to half strength MS medium favored formation of sporocarps and little callus, however, it depended on BA concentration (Table 2; Fig.3b). Various concentrations of Kn (2.32-6.97 µM) supplemented to half strength MS medium did not facilitate callus or sporocarp induction, but favored vegetative growth for some time and died thereafter. BA enriched medium induced very little callus, which was pale green to green. Vegetative growth of the shoot tips was more pronounced on medium with BA. The fronds of growing tip was very close to each other and appeared like rosette especially on medium having BA higher than 8.87 µM. Medium with 6.66 µM BA induced a mean of nine sporocarps per shoot tip.

The sporocarps developed appeared in clusters from the axils of fronds (Fig. 3c & d). The sporocarps were of two types: large microsporocarps and small megasporocarps. The sporocarps were with a stalk like basal and bulbous tip region (Fig. 3c, d & e). In certain cases, the stalk was double to the length of the bulbous region (Fig. 3e) and occasionally, vice versa condition was also observed (Fig. 3e). The microsporocarps contained numerous sporangia and each microsporangium contained 64 microspores. The megasporangium contained only one large megapsorangium bearing a single megaspore. The distal region of some of the megasporocarps was beaked (Fig. 3e), while in the case of microsporocarps, the beak was small (Fig.3e). During maturation, the sporocarps turned brown (Fig. 3f) and the thick walled microspores contained prominent nucleus (Fig. 3f).

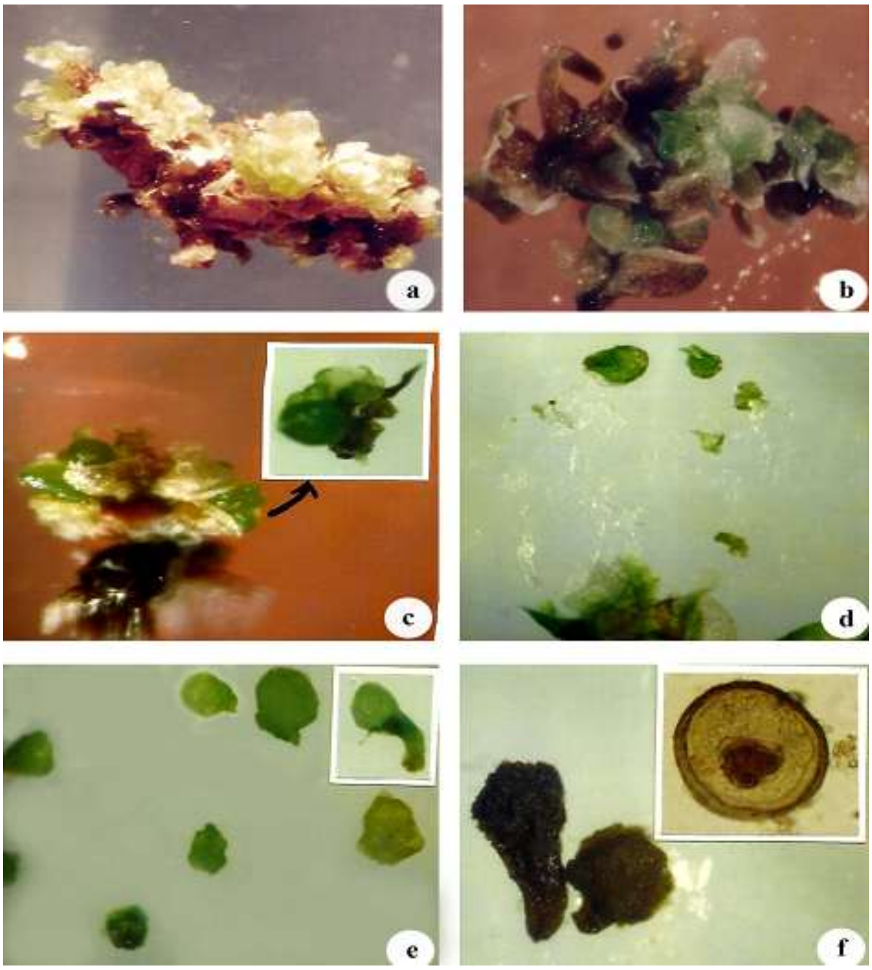

Fig. 3: Figure showing the induction of shoot bud and sporocarp on *Azolla rubra* R. Br. a. Small shoot developed on 1/2 MS medium with 2.26μM 2,4-D and 8.87 μM BA b. Sporocarps developed on 1/2 MS medium with 6.66μM BA, c. development of sporocarps from the axil of fronds in clusters (inset shows an enlarged view of isolated cluster of sporocarps, d. separation of sporocarps from the shoot tips, e. sporocarps of different sizes (inset shows sporocarp with stalk-like basal region and bulbous tip) and f. mature brown sporocarps (inset shows thick walled microspores with prominent nucleus).

Table 3: Percentage of shoot tip explants cultured on half strength MS medium fortified with 2.26 μM 2,4-D and 6.66 μM BA with different level of sucrose and agar in inducing callus and sporocarps. Data depicts the percentage of 20 explants per treatment for a growth period of 50 days.

Concentration (%)	Explants inducing callus (%)	Explants inducing sporocarps (%)
Sucrose (Agar 0.8%)	1	05
	2	65
	3	85
	4	75
	5	55
Agar (Sucrose 3%)	0.4	45
	0.6	65
	0.8	85
	1.0	80

Effect of sucrose

Different levels of sucrose (1.-5%) added to half strength MS medium with 2.32 μM 2,4-D and 6.66 μM BA exhibited significant difference in the percentage of explants inducing callus and sporocarps (Table 3). Medium with 3% was superior for the induction of callus and sporocarps.

Effect of agar

Different level of agar (0.4-1 %) used to solidify half strength MS medium with 2.32 μM 2,4-D and 6.66 μM BA showed significant difference in the percentage of explants inducing callus and sporocarps (Table 3). Of the different levels of agar, medium solidified with 0.8% agar showed the highest percentage (90%) of response (Table 3).

Effect of pH

Half strength MS medium having 2.32 μM 2,4-D and 6.66 μM BA with different regimes of pH (4.5-7.0) were tried to study the effect of pH. On the basis of percentage of explants inducing callus and sporocarps, pH 5.8 was superior (Table 4). Culture on medium with the optimal pH (5.8) exhibited faster growth compared to the lower and higher levels. Cultures grown on medium with pH 4.5 became brown after two weeks.

Table 4: Percentage of shoot tip explants cultured on half strength MS medium fortified with 2.26 μM 2,4-D and 6.66 μM BA with different levels of pH and phosphate in inducing callus and sporocarps. The data represents the percentage of 20 explants per treatment for a growth period of 50 days.

	Explants inducing callus (%)	Explants inducing sporocarp (%)
	4.5	55
	5.0	65
	5.3	75
pH level	5.5	80
(Sucrose3%, agar	5.8	90
0.8%)	6.0	85
	6.3	75
	6.5	70
	7.0	60
	1.25	45
Phosphate	1.50	50
concentration (mM)	1.70	60
(sucrose 3%,agar	2.00	55
0.8%,pH 5.8)	2.50	40
	3.00	20

Effect of light

Shoot tips cultured on half strength MS medium with 2.32 μM 2,4-D and 6.66 μM BA incubated in dark and light showed differences in response. Cultures in light exhibited faster growth and induction of callus and sporocarps. In dark, the culture did not induce callus and sporocarps (Fig. 4a & b). The cultures grown in light were dark green, while in dark, the shoots became yellowish. The cultures incubated in dark exhibited browning and tendency to die after 25 days.

Fig. 4: Effect of light and phosphate in the culture of *Azolla rubra* R. Br. on medium with 1/2 MS with 2.26 μM 2,4-D and 6.66 μM BA. a. effect of light on culture, b. an enlarged view of an explants from a and c. vegetative growth of the shoot tip on medium with 1.75 μM phosphate (inset shows the original size of c).

Effect of phosphate

Different levels of phosphate added to half strength MS medium with 2.32μM 2,4-D and 6.66μM BA (pH 5.8) displayed significant difference in the percentage of explants inducing callus and sporocarps (Table 4). Addition of phosphate at different levels did not exhibit induction of callus and sporocarps (Fig. 4c). Shoot tips on medium with 1.5 and 1.75 mM showed slightly increased vegetative growth. Compared to half strength MS medium with the optimal growth regulators (without the addition of extra phosphate), addition of phosphate was not beneficial for the induction of callus.

Subculture and indirect organogenesis

The callus developed on medium with either 2,4-D or in combination with BA facilitated proliferation upon transfer to medium with the same level of growth regulators. The subcultured callus showed slow proliferation. The shoots from the initiation culture subcultured on medium with BA (6.66 μM) showed fresh growth with an increased tendency to induce sporocarps. But at the same time, the older portions exhibited progressive browning. The callus obtained on different media upon subculture underwent formation of shoot buds with thin leaves when subcultured on half strength MS medium containing BA alone or combination with low levels of 2,4-D (0.26 μM). Half strength MS medium with 6.66 μM BA induced a mean of 9.2 shoots, and was superior for the induction of shoot buds (Fig. 4a & b).

Discussion and Conclusion

Ferns are unique in having the ability to develop diploid gametophytes from the sporophytic cells (apospory) without meiosis and vice versa (apogamy). Analogues to angiosperms, the balance between endogenous and exogenous auxins and cytokinins play significant role in *in vitro* morphogenesis. Efficacy of phytohormones in *in vitro* morphogenesis has been well demonstrated in ferns viz., *Pyrrosia piloselloides* (Kwa *et al.,* 1990), *Nephrolepis exaltata* (Byrne and Caponetti 1992), *Dryopteris affinis* (Fernandez *et al.,* 1996), *Asplenium nidus* (Fernandez *et al.,* 1997), *Diplazium congnatum* (Vallinayagam *et al.,* 2002) and *Platycerium bifurcatum* (Ambrožic–Dolinšek *et al.,* 2002). The key requirement for the elucidation of molecular mechanism involved in morphogenic process is the establishment of simple, reproducible regeneration system.

Surface sterilization of *Azolla* has been reported as difficult, because higher concentrations of sterilizing agents tend to kill the plants and lower concentrations of the agents were ineffective in disinfecting *Azolla* plants (Padhya 1989). Difficulty to obtain contamination-free culture has also been demonstrated in another aquatic fern *Salvinia* spp. (Seilheimer 1974). In the present study, mercuric chloride at 0.05% for 5 min. facilitated establishment of 95% contamination-free culture. According to Padhya (1989) sterilization using 10% hydrogen peroxide was effective for the establishment of 60% cultures of *Azolla pinnata,* and treatment of mercuric chloride and sodium hypochlorite were detrimental to *Azolla.* Use of penicillin as surface sterilizing agent has also been reported in *Azolla* (Nickell 1961).

Half strength MS medium was superior to full strength MS for the *in vitro* studies on *Azolla rubra*. According to Padhya (1989) and Watanabe *et al.,* (1977) this medium was optimal for the *in vitro* growth of *A. pinnata.* Sevichan (1994) has demonstrated simple Nitrogen containing medium for the *in vitro* growth of *Azolla* spp. According to him, *A. rubra* was superior in vegetation growth, when compared to *A. microphylla* and *A. pinnata*. Padhya (1989) and Sevichan (1994) observed only vegetative growth, and did not form callus and sporocarps.

Callus formation from *Azolla* has not been accomplished so far. The callus was induced only on medium enriched with 2,4-D either alone or in combination with BA or Kn. Medium with NAA singly or combination with BA or Kn was ineffective to evoke callus formation. 2,4-D is considered as the most trustworthy growth regulator for the induction of callus in many plants. High potency of 2,4-D in the induction of callus has been reported in several ferns *viz*., *Pteris vittata* (Bristow 1962), *Nephrolepis exaltata* (Byrne and Caponetti 1992) and *Platycerium coronarium* (Kwa *et al.,* 1997). Nevertheless, high efficacy of 2,4-5 trichlorophenoxyacetic acid over 2,4-D has been documented in *Diplazium congnatum* (Vallinayagam *et al.,* 2002).

In the present study, the culture explants exhibited progressive browning and death from the basal region. Retention of the callus without subculture resulted in the death of first formed callus. According to Padhya (1989), regular weekly renewal of the culture medium is mandatory to maintain continuous axenic cultures of *Azolla pinnata.* Similar results were also accomplished in other species of *Azolla* (Subudhi and Watanabe 1981). As opined by Salisbury and Ross (1986) this may be due to the change in the composition of the medium during the culture period because of the rapid absorption of some of the minerals and thereby its shortage. Decline of the pH as to the culture period goes on may also be a reason. Absence of death of the callus or shoots upon transfer to fresh medium up to a particular period fortifies this point. Medium supplemented with 2.26 µM 2,4-D and 6.66 µM BA was the best for callus induction and proliferation. Synergy of 2,4-D with BA or Kn in the induction of callus has reported in *Ophioglossum petiolatum* (Peterson 1967) and in flowering plants (Rani *et al.,* 2003; Wang and Bhalla 2004; Lin *et al.,* 2004). Induction of callus from shoot apices of the aquatic fern *Marsilea vestita* culture was noticed on Knop's medium having kinetin (Laetsch and Briggs 1961).

A. rubra is a non-sporocarp producing species under natural conditions and propagation is mainly by vegetative fragmentation. The best callus induction medium (2.26 µM 2,4-D and 6.66 µM BA) also developed sporocarps. Increase of BA increased the number of sporocarps. The formation of sporocarps in this non-sporocarp producing species may be due to the influence of growth regulators. The culture environment may also be favourable for the induction of sporocarps. The sporocarps developed in *vitro* on different medium in the present study were in clusters and contained numerous sporangia as in other *Azolla* species. All other characters of the *in vitro* developed sporocarps in *A. rubra* were similar to that described in other species of *Azolla* (Konar and Kapoor 1974; Calvert *et al.,* 1983). The microspores were thick walled contained prominent nucleus as described in *A. pinnata* (Konar and Kapoor 1974) and *A. mexicana* (Calvert *et al.,* 1983). Induction of spores, which forms the basis of sexual propagation in ferns, has great significance as it can increase genetic diversity. The culture of spores *in vitro*, which is difficult otherwise, facilitates development of gametophytes, and allows the studies at physiological, biochemical molecular level with respect to its development.

Sucrose at 3% was optimal for the development of callus and sporocarp. Efficacy of 3% sucrose in the induction of callus has been reported in *Nephrolepis exaltata* (Byrne and Caponetti 1992), *Platycerium coronarium* (Kwa *et al.,* 1997) and *Diplazium cognatum* (Vallinayagam *et al.,* 2002). Development of sporocarps/sporangia has not been reported in ferns till this time. In the present study, solidification of medium using 0.8% agar was superior for the *in vitro* studies on *A. rubra*. Though *Azolla* is a water fern lower percentage of agar was not beneficial for growth, and induction of sporocarps. Optimal water potential may be the reason behind better growth, induction of callus and sporocarp on medium with 0.8% agar.

Half strength MS medium with 2.32 µM 2,4-D and 6.66 µM BA having the pH 5.8 was optimal, and which exhibited faster growth compared to the lower and higher levels of pH. According to Padhya (1989), Watanabe *et al.,* (1977) medium with pH 5.5-6.5 was ideal for vegetative growth and development of *Azolla pinnata in vitro*. Medium with pH 7.5 has been observed as optimal for growth of different species of *Azolla* namely *A. pinnata, A.microphylla* and *A.rubra* (Sevichan 1994; Sevichan and Madhusoodanan 1996). The role of pH of the medium in mineral absorption has been emphasized. Cultures incubated in light on medium with 2.32 µM 2,4-D and 6.66 µM BA was superior to that in dark. Darkness did not facilitate induction of callus and sporocarps. As in the present study, presence of light, essential for callus induction has been reported in *Nephrolepis exaltata* (Byrne and Caponetti 1992).

Half strength MS medium having 2.32 µM 2,4-D and 6.66 µM BA (pH 5.8) with different levels of phosphate was not beneficial for the induction of callus and sporocarops. Influence of phosphate in vegetative growth of *Azolla* spp. *in vitro* has been emphasized (Sevichan 1994; Leena and Madhusoodanan 1996). According to them, phosphate at different levels did not influence the growth of *A. pinnata*. While *A. microphylla* and *A. rubra* exhibited significant increase in fresh weight at 2 mgI^{-1} and 1-10 mgI^{-1} phosphate, respectively. No significant increase in vegetative growth as to the increase of phosphate level in the present study may be due to the presence of growth regulators and its effect to induce callus and sporocarps.

References

1. Pereira AL, Carrapico F, Culture of *Azolla filiculoides* in artificial conditions, Plant Biosystems, 2009, 143, pp. 431–434.

2. Wang YH, Bhalla PL, Somatic embryogenesis from leaf explants of Australia lair flower, *Scaevola aemula* R. Br, Plant Cell rep, 2004, 22, pp. 408-414.

3. Lin CS, Lin CC, Chang WC, Effect of thidiazuron on vegetative tissue derived somatic embryogenesis and flowering of bamboo *Bambusa eludes,* Plant Cell Tissue Org Cult, 2004, 76, pp. 75 – 82.

4. Pierik RLM, Plant cell culture technology – a book review, Sci Horti, 1987, 33, pp. 308-309.

5. Pierik RLM, Commercial aspect of micro propagation. In: Prakash J & Pierik RLM (eds.) Horticulture – New Technologies and Applications, 1991, Kluwer Acad. Publ., Dordrecht, The Netherlands, pp. 141-153.

6. Sevichen PJ, Studies on *Azolla* species as biofertilizer for rice in Kerala, Ph.D thesis, Dept. of Botany, University of Calicut, Kerala, India, 1994.

7. Murashige T, Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol, 1962, 15, pp. 473-497.

8. Kwa SH, Wee YC, Loh CS, Production of first and second generation aposporous gametophytes from *Pyrrosia piloselloides* (L.) Price, frond strips cultured *in vitro,* Plant Cell Rep, 1990, 8, pp. 530 – 533.

9. Byrne TE, Caponetti JD, Morphogenesis in three cultivars of Boston fern. II. Callus production from stolon tips and plantlet differentiation from callus, Am Fern J, 1992a, 82, pp. 1-11.

10. Fernandez H, Bertrand AM, Tames RS, Influence of tissue culture conditions on apogamy in *Dryopteris affinis* sp, Plant Cell Tissue Organ Cult, 1996, 45, pp. 93-97.

11. Fernandez H, Bertrand AM, Tames RS, Plantlet regeneration in *Asplenium nidus* L and *Pteris ensiformis* L. by homogenization of BA treated rhizome, Sci Horti, 1997, 68, pp. 243-247.

12. Vallinayagam S, Manickam VS, Seeni S, Multiplication of *Diplazium cognatum* through *in vitro* culture, Phytomorphology, 2002, 52, pp. 23 – 27.

13. Ambrozic-Dolinsek J, Camloh M, Bohancec B, Zel J, Apospory in leaf culture of staghorn fern *Platyserium bifurcatum,* Plant Cell Rep, 2002, 20, pp. 791-796.

14. Padhya, Achievements and prospects of *in vitro* studies on *Azolla pinnata,* Indian Fern J, 1989, 6, pp. 73 – 77.

15. Seilheimer, AV, Axenic culture of the aquatic fern *Salvinia,* Am Fern J, 1974, 64, pp. 13 -14.

16. Nickell LG, Physiological studies with *Azolla* under aseptic conditions II. Nutritional studies and the effects of the chemicals on growth, Phyton, 1961, 17, pp. 49 – 54.

17. Watanabe I, Berja NS, Alimango VB, Utilization of *Azolla-Anabaena* complex as a nitrogen fertilizer for rice. Res Paper No 11, 1977, International Rice Research Institute.

18. Bristow JM, The controlled *in vitro* differentiation of callus derived from a fern *Pteris cretica* L. into gametophytic or sporophytic tissue, Develop Biol, 1962, 4, pp. 361 – 375.

19. Kwa SH, Wee YC, Lim TM, Kumar PP, Morphogenetic plasticity of callus reinitiated from cell suspension cultures of the fern *Platycerium coronarium,* Plant Cell Tissue Organ Cult, 1997, 48, pp. 37-44.

20. Subudhi BPR, Watanabe I, Differential phosphorous requirements of *Azolla* sp. And strains in phosphrous limited continuous culture. Soil Sci Plant Nutr, 1981, 27, pp. 237-247.

21. Salisbury FB, Ross CW, Plant Physiology. 3rd Edition, CBS publishers, New Delhi, India, 1986.

22. Peterson RL, Callus induction in *Ophioglossum petiolatum* hook. Can J Bot, 1974, 45, pp. 2225-2227.

23. Rani G, Virk GS, Nagpal A, Callus induction and plantlet regeneration in *Withania somniferra* (L.) dunal, In vitro Cell Dev Biol Plant, 2003, 39, pp. 468-474.

24. Laetsch WM, Briggs WR, Kinetin modification of sporelling ontogeny in *Marsilia vestiata,* Am J Bot, 1961, 48, pp. 369-377.

25. Konar RN, Kapoor RK, Embryology of *Azolla pinnata,* Phytomorphol, 1974, 24, pp. 228 -261.

26. Calvert HE, Perkins SK, Peters CA, Sporocarp structure in the heterosporous water fern *Azolla Mexicana,* Presi Scan Elect Microsco, 1983, 111, pp. 1499-1510.

27. Sevichan PJ, Madhusoodanan PV, Effect of *Azolla pinnata* R.Br. application as a biofertilizer for paddy in Kuttanad, Kerala. Geobios, 1996, 23, pp. 67-68.

28. Leena KR, Madhusoodanan PV, Effect of phosphorous on growth and nitrogen fixation of three *Azolla* species, Geobios, 1996, 23, pp. 238-240.

29. Paek KY, Lee C-H, Choi JK, Kwack BH, Mass propagation of *Nephrolepis exaltata* by runner tips *in vitro,* J Korean Soc Hort Sci, 1984, 25, pp. 313 – 321.

30. Camloh M, Gogala N, Ruzic R, The micro propagation of *Nephrolepis exaltata,* Boil Vest, 1989, 37, pp. 23-33.

31. Byrne TE, Caponetti JD, Morphogenesis in three cultivars of Boston fern. II. Callus production and plantlet differentiation from cell suspensions, Am Fern J, 1992b, 82, pp. 12-22.

Source of support: Nil
Conflict of interest: None Declared