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AXENIC CULTURE OF *PHILONOTIS FALCATA* (HOOK.) MITT., AN ALTERNATIVE TO REDUCE THE IMPACT OF LARGE-SCALE COLLECTION FROM NATIVE HABITAT

Meenu Mathew, Abraham Mathew* and Sindu N

PG and Research Department of Botany, St. Peters College, Kolenchery, Kerala, 682311, India

*E-mail: abrphyton@gmail.com

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ABSTRACT

Axenic culture of *Philonotis falcata*, collected from Idukki district of Kerala was established. Spores were surface sterilized in sodium dichloroisocyanurate (NaDCC) and inoculated into half strength Hoagland's Basal media of pH 6.0. The inoculated tubes were incubated at 25°C at 18h light, 6h dark cycle for 30 days. The protonema developed were transferred to 30 ml fresh half strength media in conical flasks with different pH and kinetin concentrations and incubated for 45 days. Gametophyte proliferation, growth pattern and photosynthetic pigment content were estimated. Among the various media composition, pH5.0 with 0.5 mg/L kinetin supported maximum bud proliferation and growth. Pigment production was higher at pH 6.0, 0.5 mg/L kinetin. There seem to have interaction between pH and kinetin in growth, biomass production and pigment production. TLC plate analysis revealed similar banding pattern between wild and *in vitro* plant metabolites, indicating the possibility of using axenic plants in extraction of bioactive compounds thereby reducing the impact of collection from native habitat.

Keywords: Axenic culture, Bryophyte, *Philonotis falcata*, Hoagland's media, kinetin

INTRODUCTION

Bryophytes, commonly known as the amphibians of plant kingdom, are an assemblage of diverse taxa with an estimated number of 18,000 species. Considered to be the second largest group of terrestrial plants after angiosperms, bryophytes exhibit diverse morphology and ecology (Bagdatli and Erdag 2017). Though small in size, bryophyte play a great ecological role and have several economic importance. Lack of taxonomic knowledge, difficulty in sterilizing and establishing axenic culture and low availability of biomass have made studies in the group less interesting.

Raising an axenic culture of bryophytes is challenging due to delicate plant body, small size and close proximity of plant parts to the soil. Several studies have succeeded in raising axenic cultures in taxa like *Grimmia dissimulata*, *Syntrichia ruralis*, *S. laevipila* and *S. princeps* (Bagdatli and Erdag 2017), *Hyophilla nymaniana* (Mishra *et al.,* 2014), *Entosthodon hungaricus* (Sabovljevic *et al.,* 2012), *Marchantia linearis* (Krishnan and Murugan 2014), *Funariella curviseta*, *Orthotrichum handiense*, *Entosthodon commutatus* and *E. hungaricus* (Ros *et al.,* 2013), *Thamnobryum alopecurum* (Sabovljević *et al.,* 2012), *Anthoceros agrestis* (Szövényi *et al.,* 2015), *Marchantia polymorpha* (Vujicic *et al.,* 2010), *Pogonatum urnigerum* (Cvetic *et al.,* 2007), *Frullania ericoides* (Silva-e-costa *et al.,* 2017), *Amblystegium serpens* (Cvetić *et al.,* 2005), *Bryum argentum* and *B.capillare* (Sabovljević *et al.,* 2002), *Herzogiella seligeri* (Vujicic *et al.,* 2010), *Riccia billardieri* (Mahesh *et al.,* 2018), *Rhodobryum giganteum* (Chen *et al.,* 2009).

Genus *Philonotis* of the family Bartramiaceae consist of 169 species worldwide, with over 26 species in India (Nisha *et al.,* 2018). Axenic culture of *Philonotis thwaitesii* has been successfully raised from spores in eight different modified Knops media with varying combination and strength. Half strength Knops media devoid of sucrose in continuous light was the best for spore germination. Bud initiation occurred after 60 days of culture. Half strength Knops media supplemented with Nitsch's trace elements supported larger number of gametophytes but those supplemented with sucrose resulted with fewer but robust gametophytes (Awasthi *et al.,* 2012). In another study, axenic culture in *Philonotis falcata* was attempted in MS basal media. 3 % sucrose was found to stimulate spore germination. However protonema failed to produce buds and adult phase even after 80 days of culture (Nisha *et al.,* 2018).

Cytokinins are a class of phytohormones involved in cell division, cell growth and differentiation. They induce axillary bud growth and prevent senescence. Bryophytes possess Bryokinin, a type of cytokinin that chemically corresponds to N_6-γγ dimethylallyladenine. This hormone is found to be physiologically active at various stages of bryophyte development. It can also be used to replace kinetin as growth factor in tissue culture of vascular plants (Sabovljević *et al.,*2014). Phytohormone profiling among 30 bryophytes have revealed the presence of 26 isoprenoid cytokinins, ranging from few picomoles to hundreds of pico moles. Among the various forms, cis Z and iP types were more predominant (Drábková *et al.,* 2015). Cytokinin is found to have influence on protonemal proliferation and of bud induction including the number and position of buds in the caulonema (Vujičić *et al.,* 2012). Exogenous application of cytokinins like kinetin, 6- Benzyl aminopurine and thidiazuron on *Bryum argentum* had a positive effect on chlorophyll retention in both natural and

in vitro raised plants (Sabovljević *et al.,* 2010)they remain uninteresting for studying their chlorophyll level. The aim of this study was to compare the effect of different cytokinins on chlorophyll retention in moss B. argeteum gametophyte shoots grown in natural conditions with those grown in *in vitro* culture. Material and Methods: The effect of different cytokinins: kinetin (KIN.

The present study deals with developing a protocol for axenic culture of *Philonotis falcata* from spores and to study the effect of various pH and kinetin concentration on gametophyte growth and proliferation.

MATERIALS AND METHOD

Collection and identification of Specimen

Philonotis plant with sporophyte were collected during January from Marayoor region of Idukki District, Kerala. The plant was collected in sterile containers and brought to the lab and identified based on gametophytic and sporophytic characters

Media for axenic culture

Hoagland No. 2 basal salt mixture (Hogland and Arnon 1950) was used for the preparation of media. Half strength media was prepared and to solidify the media, 1.0 % agar was added. The media was poured into test tubes and autoclaved. Further studies were done using 30 ml of same media in conical flasks at different pH, with or without kinetin.

Surface sterilization of capsule and inoculation

Ripe capsules were separated from seta and surface sterilized using 0.1 to 2 % sodium dichloroisocyanurate (NaDCC) for 5 to 10 minutes. The capsule was washed well with sterile distilled water and was cut open to release spores. The spores were inoculated on to half strength Hoagland basal media (pH 6.0) with 1 % agar in test tubes. The inoculated tubes were incubated at 25 °C at 18 h light, 6 h dark cycle for 30 days.

Effect of pH and kinetin of gametophyte proliferation

After 30 days of culture, tubes that showed protonemal growth were selected and protonema along with agar was cut in to 3 mm square bits. These were used as the inoculum. Conical flasks (100 ml capacity) with 30 ml media solidified with 1 % agar was used. To study the effect of pH, media pH was adjusted to 5.0 to 8.0 before autoclaving. In order to study the effect of kinetin on growth, pigment production and retention, kinetin was added to the media at a concentration 0.1mg/L, 0.5 mg/Land 1.0 mg/L prior to autoclaving. The experimental design is as per Table 2. Each experimental run was done in triplicate. The nature of growth was evaluated after 45 days of inoculation.

Measurement of photosynthetic pigments

After 45 days of inoculation, the gametophyte from each conical flask were carefully collected, weighed and immersed in 10 ml DMSO at 60 °C for 3 hrs. The extract was centrifuged and chlorophyll a, chlorophyll b and carotenoid content were estimated (Alpert 1984).

Thin Layer Chromatography

One gram of gametophyte from naturally grown and *in vitro* raised *Philonotis* (pH 5.0, kinetin 0.5 mg/L) was ground using 5 ml of ethyl acetate. The extract was centrifuged, concentrated and spotted on TLC plate. The plate was run using chloroform as solvent and visualized under UV light.

RESULT AND DISCUSSION

Spores were brown in colour and elliptical in shape with a size of 22-24µm x 18-19 µm. Perispore was thick with high level of ornamentation. Spores showed germination within a week of inoculation. The germ tube protruded out of the spore and showed branching after 2 weeks of incubation. The protonema cells were 8-9 µm in width (Figure 1).

Among the various sterilization protocol used, best sterilization condition was 0.5 % NaDCC for 10 minutes. Higher concentration of 2 % NaDCC for 10 minutes was detrimental as only 2 of the 8 tubes inoculated showed protonemal growth. Lower concentration of sterilant and lower treatment duration showed fungal contamination (Table1).

The protonema developed from the sterilization condition of 0.5 % sterilant for 10 minutes was used for further studies. The protonemal bits placed in conical flasks with varying pH and kinetin concentrations showed bud initiation within 2 weeks of incubation, irrespective of the pH and kinetin concentration. Even in flasks that lack kinetin, protonema produced buds and adult gametophyte were seen emerging.

After 45 days of incubation, the flasks were taken out and growth nature was observed. All treatments showed gametophytic proliferation but the best growth noted was with pH 5.0 and 0.5 mg/L kinetin. Among the media pH tested for growth in the absence of kinetin, the best pH was 6.0. Greater proliferation of gametophytes were noted at this pH. With 0.1mg/L kinetin, media with pH 6.0 supported maximum growth. At 1 mg/L kinetin, pH 7.0 showed maximum growth (Plate1).

Though the best growth was seen with pH 5.0 and 0.5 mg/L kinetin, fresh weight seems to be high towards neutral and alkaline pH. Highest fresh weight was noted at pH 7.0 (Table 2).

Analysis of photosynthetic pigments revealed an interesting pattern. At pH 5.0, highest chlorophyll concentration was noted with gametophytes grown in media

Table 1 : Effect of sterilant concentration and time of treatment in surface sterilization of spore and protonemal growth (after 15 days of spore inoculation)

Sterilant Concentration (%)	Time of treatment (minutes)	Number of Tubes showing axenic growth	Number of tubes with contamination	Number of tubes with poor or no development of protonema
0.1	5	1	7	0
	10	3	5	0
0.5	5	6	2	0
	10	8	0	0
1.0	5	6	2	0
	10	7	0	1
1.5	5	5	2	1
	10	5	0	3
2.0	5	3	1	4
	10	2	0	6

Table 2 : Fresh weight of gametophyte

pH	Kinetin Concentration in mg/L	Fresh weight in mg
5	0	65.3 ± 1.4
	0.1	76.0 ± 5.0
	0.5	64.2 ± 0.8
	1.0	61.3 ± 3.3
6	0	78.0 ± 3.0
	0.1	78.0 ± 4.0
	0.5	66.2 ± 2.0
	1.0	63.2 ± 2.2
7	0	88.5 ± 5.5
	0.1	84.1 ± 3.2
	0.5	84.2 ± 4.2
	1.0	70.6 ± 3.9
8	0	79.9 ± 0.5
	0.1	75.4 ± 4.2
	0.5	86.3 ± 2.9
	1.0	72.4 ± 4.1

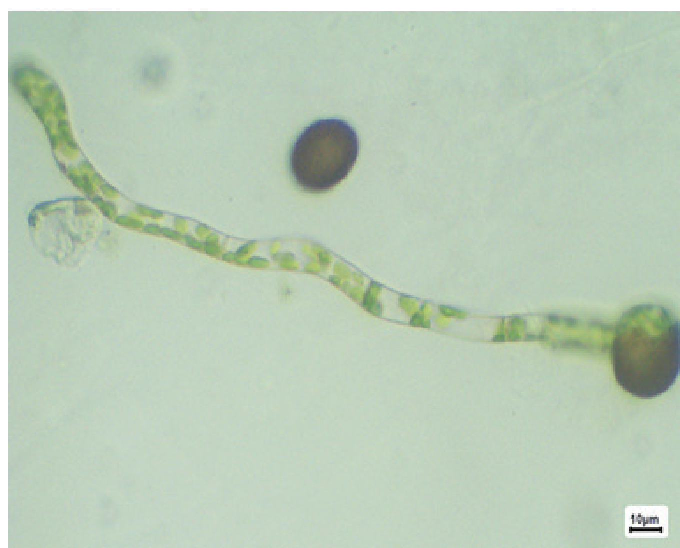
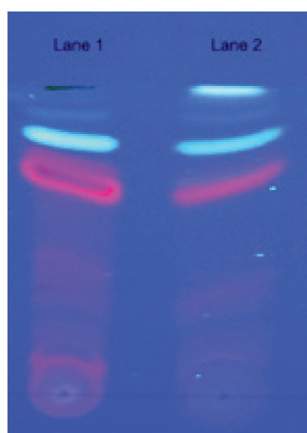


Figure 1: Protonemal growth from spore

Figure 3 : TLC plate of ethyl acetate extract viewed under UV light.

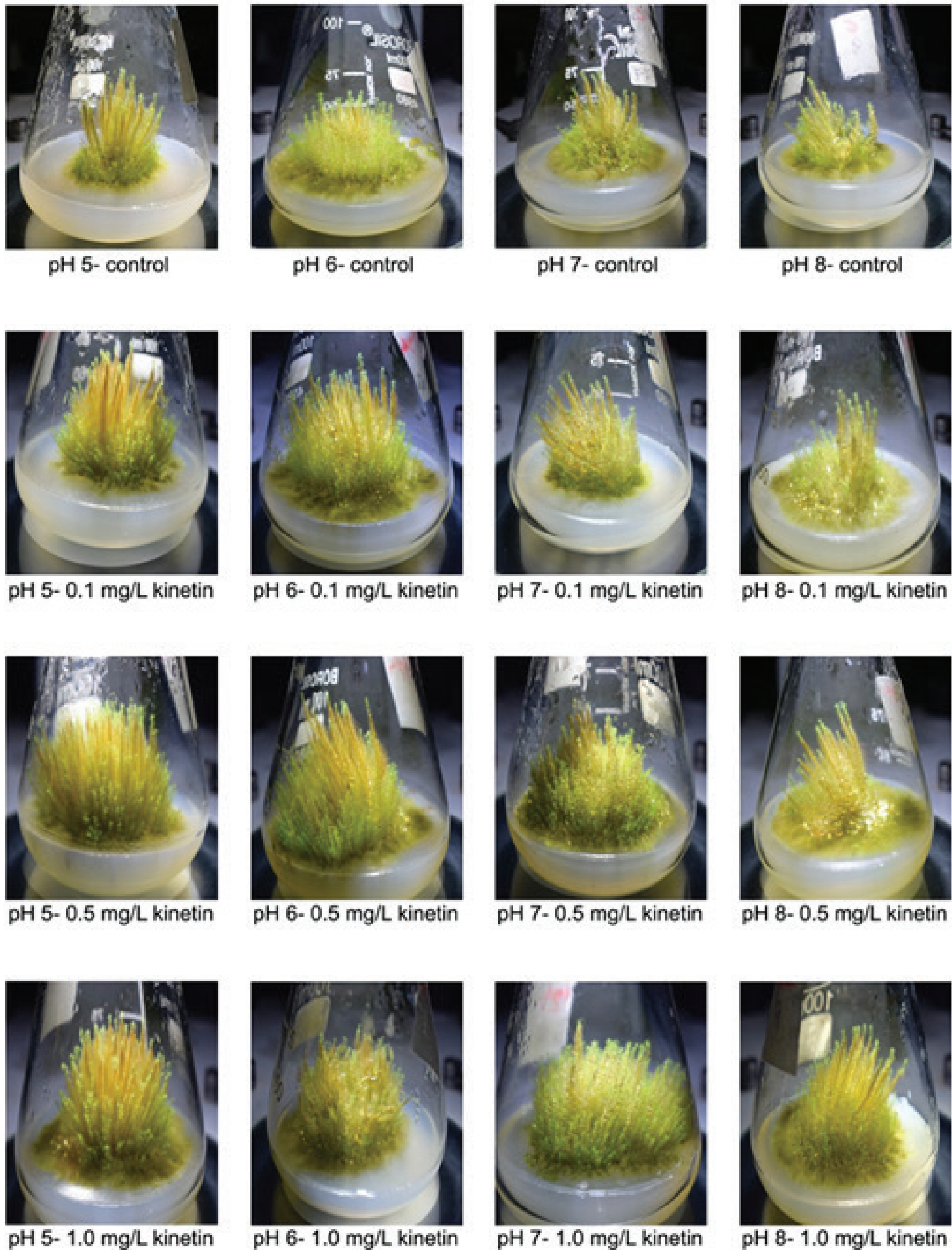


Lane 1: Naturally grown gametophyte; Lane 2: Axenically grown gametophyte

with 0.1 mg/L kinetin. At pH 6.0, it was with 0.5 mg/L kinetin. With pH 7.0, highest pigments were noted in flasks having no kinetin, while in pH 8.0, pigments were higher at 1.0 mg/L kinetin (Table 3). TLC analysis of metabolites from *in vitro* and natural plants revealed a similar banding pattern in UV light. Concentration of components varied, however presence and absence of several of them has to be studied with higher biomass (Figure 3).

Axenic culture of *Philonotis falcata*was successfully raised in half strength Hoagland's Media. The best sterilization condition was 0.5 % NaDCC for 10 minutes. NaDCC was successfully employed in sterilizing sporophyte of *Entosthodon hungaricus*. 3.0 % solution with a treatment time of 90 seconds produced a survival rate of 80 %, increasing the concentration or treatment time caused reduced survival (Sabovljevic *et al.,* 2012). NaDCC is considered as a good sterilant due to high levels of active chlorine at physiological pH and low plant toxicity. Among 5 bryophyte taxa spore sterilization was most effective at a concentration of 1 % for 3 minutes and among 13 species of bryophytestudied, 0.5 % concentration for 2 minutes was effective for sterilizing leafy gametophores (Rowntree

Figure 2-Effect of pH and kinetin on gametophyte growth and proliferation



2006).

Exogenous cytokinin is known to have impact on bud formation from protonema. When applied, cytokinin can inhibit the length wise growth of caulonema within 3 hours of application and tip growth restores when cytokinin is withdrawn(Bopp 1984). However in the current experiment, bud initiation and gametophyte growth was evident in all flasks, irrespective of pH and applied kinetin. This can be due to the sufficient endogenous levels of kinetin that can trigger bud initiation. An attempt of *in vitro* axenic culture of *P. falcata* was performed in MS basal media, but no bud initiation was noted(Nisha *et al.,* 2018). These indicate that media composition can have effect on bud initiation.

Best growth condition was pH 5.0 at 0.5 mg/L kinetin, however the biomass was low compared to many other experimental flasks.This can be due to the bud initiation ability of exogenous cytokinin that the caulonema produce profuse buds that develop into adult gametophyte. Since pH has an important role in nutrient

Table 3 : Effect of pH and kinetin concentration on chlorophyll and carotenoid content

pH	Kinetin Concentration in mg/L	Chlorophyll a µg/mg	Chlorophyll b µg/mg	Total chlorophyll µg/mg	Carotenoids µg/mg	Chlorophyll a/b
5	0	1.77 ± 0.15	0.73 ± 0.06	2.48 ± 0.21	0.19 ± 0.01	2.42
	0.1	2.04 ± 0.05	0.88 ± 0.11	2.92 ± 0.03	0.21 ± 0.09	2.28
	0.5	0.93 ± 0.07	0.55 ± 0.02	1.45 ± 0.07	0.17 ± 0.02	1.78
	1.0	0.73 ± 0.01	0.45 ± 0.02	1.17 ± 0.06	0.15 ± 0.03	1.68
6	0	2.21 ± 0.02	0.96 ± 0.07	3.19 ± 0.09	0.24 ± 0.06	2.25
	0.1	2.02 ± 0.20	0.90 ± 0.03	2.91 ± 0.12	0.21 ± 0.07	2.22
	0.5	2.43 ± 0.01	1.04 ± 0.05	3.50 ± 0.10	0.25 ± 0.02	2.32
	1.0	1.19 ± 0.04	0.61 ± 0.02	1.78 ± 0.09	0.18 ± 0.04	1.99
7	0	2.11 ± 0.08	0.95 ± 0.18	3.08 ± 0.18	0.23 ± 0.04	2.21
	0.1	1.96 ± 0.04	0.85 ± 0.06	2.81 ± 0.05	0.21 ± 0.04	2.35
	0.5	0.61 ± 0.05	0.44 ± 0.04	1.03 ± 0.17	0.17 ± 0.06	1.40
	1.0	0.45 ± 0.06	0.36 ± 0.03	0.78 ± 0.04	0.14 ± 0.04	1.20
8	0	1.70 ± 0.10	0.78 ± 0.10	2.51 ± 0.22	0.17 ± 0.02	2.17
	0.1	1.66 ± 0.14	0.76 ± 0.03	2.45 ± 0.06	0.18 ± 0.02	2.16
	0.5	1.64 ± 0.05	0.76 ± 0.11	2.42 ± 0.04	0.19 ± 0.02	2.12
	1.0	2.33 ± 0.17	1.00 ± 0.13	3.33 ± 0.16	0.24 ± 0.03	2.33

acquisition, this combination of pH and cytokinin favoured bud initiation but not biomass production. In *Calymperes erosum*, pH was found to have profound effect in gemmae germination and protonemal growth. This was attributed to the effect of media pH on solubility and availability of certain ions (Ogbimi *et al.,* 2014).

At pH 6.0, among various kinetin concentrations, 0.5 mg/L was found to have more effect on pigment content. However, at pH 5.0, the kinetin concentration of 0.1 mg/L and at pH 8.0, kinetin of 1.0 mg/L and at neutral pH, absence of kinetin had enhanced chlorophyll content. It seems that interaction of pH and kinetin concentration had effect on pigment production or retention. In *Bryum argentum*, exogenous kinetin applied to excised shoot enhanced chlorophyll content and there was a concentration dependent increase in pigment from 0-10 mM. Among the various cytokinins studied, kinetin was the most effective in chlorophyll retention (Sabovljević *et al.,*2010)they remain uninteresting for studying their chlorophyll level. The aim of this study was to compare the effect of different cytokinins on chlorophyll retention in moss B. argeteum gametophyte shoots grown in natural conditions with those grown in *in vitro* culture. Material and Methods: The effect of different cytokinins: kinetin (KIN.

Both natural and axenically grown plants showed a similar pattern of compounds under UV light indicating the possibility of axenic plants in replacing naturally collected plants as a source of bioactive compounds. By altering the media composition and pH, we can induce or enhance the production of secondary metabolites in axenic cultures that can pave way for large scale utilization of this group of plants in bioprospecting without extensive destruction of natural samples.

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