Eco. Env. & Cons. 28 (January Suppl. Issue) : 2022; pp. (S300-S305)
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ISSN 0971–765X

http://doi.org/10.53550/EEC.2022.v28i01s.042

Rejuvenation of Ecosystem using Axenic Culture of *Octoblepharum albidum* HEDW.

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(Received 15 August, 2021; Accepted 10 September, 2021)

ABSTRACT

Ecosystem is a delicate balance between living and non-living system and hence is prone to disturbances by natural calamities and human disturbances. Ecosystem tries to revive itself but in some cases, restoration of ecosystem has to be done. Re introducing native species or enhancing their numbers can substantially decrease the time required for ecosystem revival. Bryophytes form an important group of land plants that play a vital role in primary and secondary succession. Axenic culture of bryophytes can be a source of inoculum for ecosystem restoration as the material is available throughout the year and biomass can be scaled up to the required amounts. In the present work axenic culture of *Octoblepharum albidum* has been developed and was inoculated to rotting wood logs. *Octoblepharum* developed on these logs and were similar to the natural corticolous population. There was significant improvement in production of secondary metabolites in axenic plants when they were transplanted and grown in natural ecosystem.

Key words : *Ecosystem rejuvenation, Bryophyte, Axenic culture, Octoblepharum, Hoagland's media*

Introduction

Ecosystem all over the world is facing threat especially from human activities. The loss of delicate balance can have amplitude responses that can lead to species extinction. Ecosystem restoration projects are thus the need of the hour and has been successfully done in several cases. However the importance of bryophytes in ecosystem restoration is often overlooked and only vascular plants are considered as the key players. Bryophytes have a significant role in nature and are elemental for the sustainability of human civilization (Hallingback and Tan, 2010). Bryophytes are significant contributors in water recycling, carbon and nitrogen cycling and biomass production (Turetsky, 2003). Mosses can absorb nutrients from air, rain, wind and dust and can retain them for a long period in their undecomposed dead shoots. Their cell wall has net negative charge and hence can absorb cations (De Lucia *et al*., 2003). They can survive in extreme cold conditions and forms the staple food for animals and birds (Longton, 1992). Bryophytes play a key role in maintaining microclimate and thus sustain small insects, worms, protozoans that form the base line in several food chains. Disturbances in microclimate thus affect the higher trophic levels and can even lead to trophic cascades.

Octoblepharum albidum Hedw., is a tropical taxa found on tree trunks and fallen logs. Plant is small and greenish white with leaf costa having many layered leucocyst enclosing a single layer of chlorocyst. Capsule oval, erect and terminal with cucullate calyptra. *Octoblepharum albidum* has medicinal importance. It is used to treat urinary problems and is reported to ease urination (Krishnan *et al*., 2014). The taxa is used for growing ferns (Glime, 2007) and forms an important substrate for protozoans and other insects.

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Development of axenic culture of bryophytes requires a lot of effort due to their delicate plant body, difficulty in surface sterilization, small size, endophytic microbes and proximity to soil. Axenic culture has been successful in several taxa like *Syntrichia ruralis*, *S. laevipila, S. princeps* and *Grimmia dissimulata* (Bagdatli and Erdag, 2017), *Marchantia linearis* (Krishnan and Murugan, 2014), *Funariella curviseta*, *Orthotrichum handiense*, *Entosthodon commutatus* and *E. hungaricus* (Ros *et al*., 2013), *Thamnobryum alopecurum* (Sabovljevic *et al*., 2012), *Anthoceros agrestis* (Szovenyi *et al*., 2015), *Pogonatum urnigerum* (Cvetic *et al*., 2007), *Frullania ericoides* (Silva-e-costa *et al*., 2017), *Amblystegium serpens* (Cvetic *et al*., 2005), *Herzogiella seligeri* (Vujicic *et al*., 2010), *Marchantia polymorpha* (Vujicic *et al*., 2010), *Riccia billardieri* (Mahesh *et al*., 2018) and *Rhodobryum giganteum* (Chen *et al*., 2009).

Axenic culture can pay way for large scale production of biomass, all throughout the year. For bio monitoring studies, transplants from native habitats are not suitable and axenic *in vitro* raised plants provide standardized raw materials. For moss based pharmaceuticals, both natural and genetically engineered, photobioreactors are used. (Decker and Reski, 2020). In field studies with axenic and native moss species, superior metal uptake was reported by axenically grown *Sphagnum paulstre* than the native *Pseudoscleropodium purum* (Capozzi *et al*., 2017).

Axenic biomass can be used as source material for ecosystem restoration. Large scale collection from natural habitat can thus be minimized there by stabilizing the ecosystem. The present study deals with axenic culturing of *Octoblepharum albidum* from spores and attempts to introduce it on to decomposing wood logs.

Materials and Method

Collection and identification of Specimen

Octoblepharum albidum Hedw., plant with sporophyte were collected during March from 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 and mixture (Hoagland Arnon, 1950) was used for the preparation half strength media (pH 6.0). To solidify the media, 1.0 % agar was added. The media was poured into test tubes and autoclaved. For subsequent studies, 30 ml of same media in conical flasks were used.

Surface sterilization of capsule and inoculation

Ripe capsules were surface sterilized using 0.1 to 1.5 % sodium dichloroisocyanurate (NaDCC) for 5 to 10 minutes. The capsule was washed well with sterile distilled water and was transferred to a sterile centrifuge tube and 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. For every sterilization experiment, six tubes were used. The inoculated tubes were incubated at 25 °C at 18 h light, 6 h dark cycle for 45 days.

Sub culturing

After 45 days of culture, tubes that showed protonemal growth were selected and protonema were subcultured into sterile media in conical flasks.

Inoculation to field conditions

Flasks that showed adult gametophyte development were transferred to shade house and sterile soil water was added. The flasks were kept open in shade house and 2 ml of sterile soil solution were added every 3 days. Hardening was done for two weeks.

After hardening, the plants were taken out and used as the inoculum. Studies were conducted during rainy season (South West Monsoon period). Old wooden log was moistened with water and small holes were drilled into the wood. Adult gametophyte were placed in these pits and pressed. Water was sprayed over this for 2 weeks and there after left undisturbed. Growth was monitored every week and after 4 months, growth was analysed and photographs were taken.

Thin Layer Chromatography

One gram of gametophyte from axenic plants in conical flasks and reintroduced plants were ground using 5 ml of methanol and diethyl ether (1:1). The extract was centrifuged, concentrated and spotted on TLC plate. The plate was run using chloroform: ethyl acetate as solvent (1:1) and visualized under UV light.

Results

The plant collected was identified as *Octoblepharum albidum* based on leaf anatomy, gametophytic mor-

phology and nature of sporophyte. The spores were globose and light brown in colour. Spores showed germination after two weeks on inoculation. After 30 days, agar surface was covered with green protonema.

Among the various sterilization protocol used, best sterilization condition was 0.5 % NaDCC for 7.5 minutes. Higher concentration of 1.5 % NaDCC for 10 minutes was detrimental as all the six tubes inoculated showed no protonemal growth. Lower sterilant concentration and lower treatment duration showed fungal contamination (Table 1). Protonema showed high levels of branching and was seen embedded within the agar media (Figure 1 a).

The protonema developed from the sterilization condition of 0.5 % sterilant for 7.5 minutes was used for further studies. The protonemal bits were placed in conical flasks and they showed bud initiation within 2 weeks of incubation. After 30 days of incubation, the gametophytic plants were seen as fragile and thin with low green colouration and there after became little sturdier (Figure 1 b). From leaf tips new growth were seen to arise. In some plants, these developed into rhizoids (Figure 1 c), while in others a tendency to shoot development was seen (Figure 1 d).

After 45 days of incubation, the flasks were taken out and growth nature was observed. After 2 weeks of hardening, the plants became sturdy and green similar to the natural gametophyte. The plants bits inoculated on to wooden log (Figure 1 e) showed growth without much acclimatization period and within 2 weeks, side branches were seen arising. From protonemal branches from the base of the inoculated plants, new buds were initiated. After 6 months of inoculation, thick carpet like growth was noted which showed tendency to spread to regions not inoculated (Figure 1 f). A biomass enhancement from 1 g inoculum to 121 g in natural condition was noted after 4 months of growth. No rhizoidal or bud initiation from leaf apex were noted in natural conditions.

TLC analysis of metabolites from axenic plants in conical flasks and reintroduced plants revealed several similar banding pattern in UV light. More bands and fluorescence were seen in reintroduced plants. Concentration of components varied, however presence and absence of several of them has to be studied with higher biomass (Figure 2).

Discussion

Axenic culture of *Octoblepharum albidum* was successfully raised in half strength Hoagland's Media. The best sterilization condition was 0.5 % NaDCC for 7.5 minutes. NaDCC is considered as a good sterilant for plant axenic culture due to high levels of active chlorine at physiological pH and low toxicity to plant cells (Parkinson *et al*., 1996). Sabovljevic *et al*.(2012), studied the effect of sodium hypochlorite and NaDCC in sterilizing sporophyte of *Entosthodon hungaricus*. Survival rate for gametophytes without contamination was high when NaDCC was used, while sodium hypochlorite was better for sporo-

Table 1. Effect of sterilant concentration and time of treatment in surface sterilization of spore and protonemal growth (after 14 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	0	6	0
	7.5	1	5	0
	10	1	5	0
0.5	5	4	2	0
	7.5	6	0	0
	10	2	0	4
1.0	5	6	1	0
	7.5	5	0	1
	10	2	0	4
1.5	5	3	0	3
	7.5	2	0	4
	10	0	0	6

phyte sterilization. Studies by Rowntree (2006), revealed that, spore sterilization was most effective at a NaDCC concentration of 1 % for 3 minutes and for leafy gametophytes, 0.5 % concentration NaDCC for 2 minutes was effective.

Hormones play a major role in gametophytic and sporophytic development. Among these, cytokinin is found to have influence on protonemal proliferation and of bud induction, including the number and position of buds in the caulonema (Vujicic *et al*., 2012). Studies by Nisha *et al.* (2018), in axenic culture of *Philonotis falcata* resulted in spore germination and protonemal growth. However no bud initiation and adult gametophyte formation was noted. There could be several reasons both physiological and environmental factors which might have caused the failure in production of phytohormones in required quantities for bud initiation. In the present study,

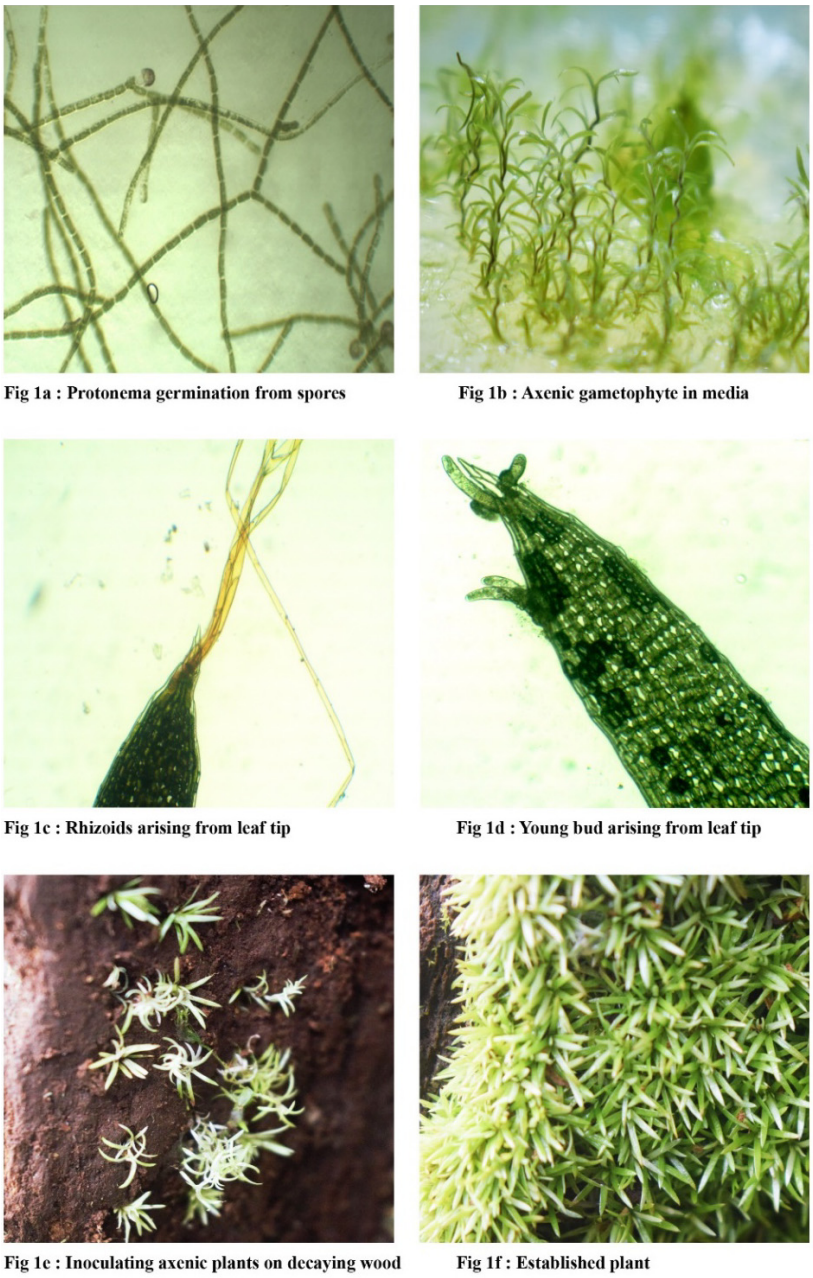

Fig. 1. Axenic culture and introduction of axenic plants on to wooden log

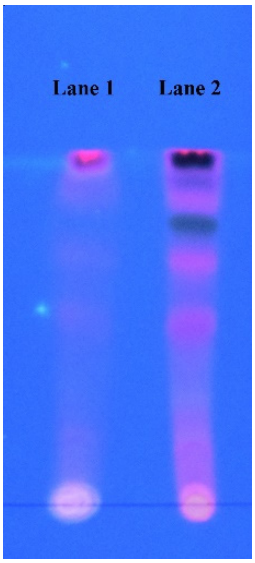

Fig. 2. TLC of crude extract viewed under UV Light
Lane 1- axenic plant; Lane 2- reintroduced plant

even without the addition of exogenous hormones, bud initiation were noted.

Hardening of explants is a prerequisite for better adaptation to external habitat. Plants enclosed in glass containers have the required nutrients and moisture and hence develop minimal water stress during their development. This will in turn make the plants fragile and weak and thus direct transplanting results in failure of establishment. Bryophytes are poikilohydric forms and hence gain or lose water easily in relation to the prevailing environmental conditions. Hardening of the axenic *Octoblepharum* made the plant more sturdy and green.

There are several methods of reintroducing bryophytes to donor sites. These includes the use of gametophyte fragments as propagules and transfer of bryophytes with green hay and raked material (Michalska-Hejduk *et al*., 2017). Studies by Schmalholz and Hylander (2009), revealed that bryophyte communities need over 40 years for complete restoration. In the present study, *Octoblepharum albidum* could grow well over the decomposing wooden log with a significant biomass increase over 4 months of growth. The vegetative propagule (gametophytic proliferation, bud initiation from protonema, multiplication of protonema and leaf buds) along with reproductive spores can result in fast spread of the species in the recipient site.

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.

Acknowledgement

Meenu Mathew is thankful to CSIR for providing fellowship in the form of JRF for carrying out her Doctoral studies. The Department of Botany, St. Peters College is thankful to DST FIST for providing grant in Aid for creating facilities for research.

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