
Research Article

Studies of sterilization protocol development and calli induction of selected tropical mosses

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Abstract

Four tropical African mosses namely; *Racopilum africanum*, *Thuidium gratum*, *Archidium ohioense* and *Bryum coronatum*, were studied. This was with a view to developing a sterilization protocol and inducing calli from the gametophyte explants in vitro. The mosses were collected from their natural populations in the Central Campus of the Obafemi Awolowo University, Ile-Ife, Nigeria. Healthy stem tips from the gametophytes of each of the mosses were sterilized with JIK (containing 3.85 % M/V NaOCl) and then cultured on Murashige and Skoog (MS) 1962 full strength medium, which served as the control and a full strength MS medium supplemented with 5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D). Sterilization of the explants with 8 % JIK for 8 minutes was found to be most suitable. Only *Bryum coronatum* produced calli on hormone free medium, while the 2,4-D induced massive production of calli in *Bryum coronatum* and *Racopilum africanum*.

Keywords: Explant, Calli, Gametophyte, 2,4-D, Sterilization

Introduction

Bryophytes are the second largest group of plants, comprising 15,000 - 25,000 species (Crum, 2001; Gradstein et al., 2001) and occur on every continent in every location habitable by photosynthetic plants.

Propagation of bryophytes occurs both sexually and asexually (Makinde & Odu, 1993). Odu (1987), and Odu & Akinfenwa (1991) showed a great multiplicity of forms of vegetative propagules from which new generations of adult plants are often established on various substrates. Culture cells of bryophytes contain active chloroplasts and can grow under photoautotrophic conditions among green land plants (bryophytes, pteridophytes and seed plants) (Katoh, 1983).

Axenic culturing of bryophytes seemed to be so complicated that many investigators gave up their attempts and thus, bryophytes did not retain for long their rightful place as a highly favoured research object. For this reason, most studies of plant morphogenesis are now being done on vascular plants

(Milorad et al., 2010). Apart from axenic cultivation of bryophytes, developing methodology in propagation of bryophytes are significant in rare species conservation both for *ex situ* and reintroduction (Rowntree, 2006; Cvetic et al., 2007; Brezeanu et al., 2008; Vujcic et al., 2009).

All of the techniques that have been developed for tissue culture of seed plants have also been adapted for bryophytes, and these range from mere axenic culture to molecular farming. However, specific characteristics of bryophyte biology, for example, a unique regeneration capacity, have also resulted in development of methodologies and techniques different from those used for seed plants (Annette & Ralf, 2005). The induction of stably growing callus culture in bryophytes was first established by Allsop (1957). However, various bryophyte cultures have evolved as model systems in plant physiology (Wood et al., 2000). In August 2000, a pilot project for the *ex situ* conservation of endangered United Kingdom bryophytes was launched at the Royal Botanic Gardens, Kew. The project, a collaboration between the Royal Botanic Gardens and UK statutory conservation agencies (English Nature, Scottish Natural Heritage, Countryside Council for Wales), emphasized the development of *ex situ* techniques as a complement to, rather than a replacement for *in situ* conservation efforts (Ramsay & Burch, 2001). Most often, bryophytes grow in the field where they are exposed to varying contaminations. They also grow clumped together naturally in the field, as host to a wide variety of micro and macro-invertebrates (Peck & Moldenke, 1999). The disposal of some of the organisms living on the bryophytes is problematic in establishing their pure culture (Milorad et al., 2010).

In this study, we focused on four tropical African mosses; *Archidium ohioense* Schimp ex. C. Müll., *Bryum coronatum* Schwägr, *Racopilum africanum* Mitt. and *Thuidium gratum* (P. Beauv.) A. Jaeger; with a view to developing a protocol for sterilizing them and finally inducing callus from segments of their gametophytes. The mosses naturally grow at the Obafemi Awolowo University campus in very limited areas and very little information is available on their sterilization protocol and callus initiation. Calli induction is more amenable to molecular, physiological and biochemical studies than intact plants (Razdan, 2003).

Materials and Methods

Collection and identification of plant materials

The mosses investigated were collected from their natural populations in different areas on the Campus of the Obafemi Awolowo University, Ile-Ife, Nigeria, within Latitudes 7° 3' and 7° 34' N and Longitudes 4° 30' and 4° 32' E. These were identified at the Department of Botany of the same institution, and then grown in different pots for easy access and collection.

Preparation of culture medium

The basic nutrient medium consisted of MS (Murashige & Skoog) 1962, mineral salts; and vitamins. About 30g sucrose, 10mg ascorbic acid, 0.1g myo-inositol and 0.8g adenine sulphates were added. The pH was adjusted to 5.7 with 0.1 N HCl and/or 0.1 N NaOH after which 0.8 % agar was added. Following this, 20ml of the media was dispensed into 100ml flasks, each covered with non-adsorbent cotton wool wrapped with Aluminium foil before they were all autoclaved at 121 °C and 108 KPa for 15 minutes.

Sterilization experiment for callus induction

Healthy gametophyte tips from each species were selected and washed with distilled water several times in a stream of flowing water to scour off attached superficial materials. Then, in the Laminar flow chamber, the shoot tips were sterilized as follows with commercial bleach (JIK, containing 3.85 % M/V NaOCl),

- (i) 5 % JIK for 10 minutes,
- (ii) 8 % JIK for 8 minutes,
- (iii) 10 % JIK for 5 minutes.

The explants were rinsed 5 times after each case with sterile distilled water to eliminate traces of the disinfectant and then transferred to a sterilized Petri-dish containing wet filter paper.

Callus induction

The sterile gametophyte tips were cut into 5-10 mm lengths with a sterilized knife and the explants inoculated on the surface of prepared sterile culture media after which they were incubated at 25 ± 2 °C, 16 hours photoperiod, 30 - 40 $\mu\text{mol.m}^{-2}\text{s}^{-1}$ cool white fluorescent tube. The cultures were observed at regular intervals of 2 days for callus induction. For each treatment, there were 10 replicates and the experiment was replicated three times.

Results

The responses of the explants to disinfection with 5, 8 and 10 % commercial bleach (JIK) for the different times were quite different. While sterilizing with 5 % and 10 % JIK gave 25 % and 20 % success respectively, sterilizing with 8 % JIK for 8 minutes gave an 85 % success rate. Although there was still some contamination of the inocula cultured in the 8 % disinfectant concentration, when the concentration of the disinfectant was 5 %, it was observed that surface sterilization of the plant materials was not effective enough to kill the xenic organisms on the plants, as the explants were overgrown quickly with fungi, algae and bacteria. The attempt to leave the explants until the cultured plantlets overgrow the xenic organisms remained unrealizable. Meanwhile, at 10% concentration, surface sterilization killed the plant material or was not effective enough to kill the xenic organisms on the plants and not harm the plants at the same time.

Callus from *Bryum coronatum* was successfully induced after about two months of culturing (Figure 1A) on ordinary basal medium (i.e. hormone-free medium), though the calli produced were scanty. *Archidium ohioense*, *Racopilum africanum* and *Thuidium gratum* did not produce callus on the basal medium. Callus of *Bryum coronatum* and *Racopilum africanum* was successfully induced on MS medium containing 2,4-D (5 mg/L) after 12 days of incubation (Figures

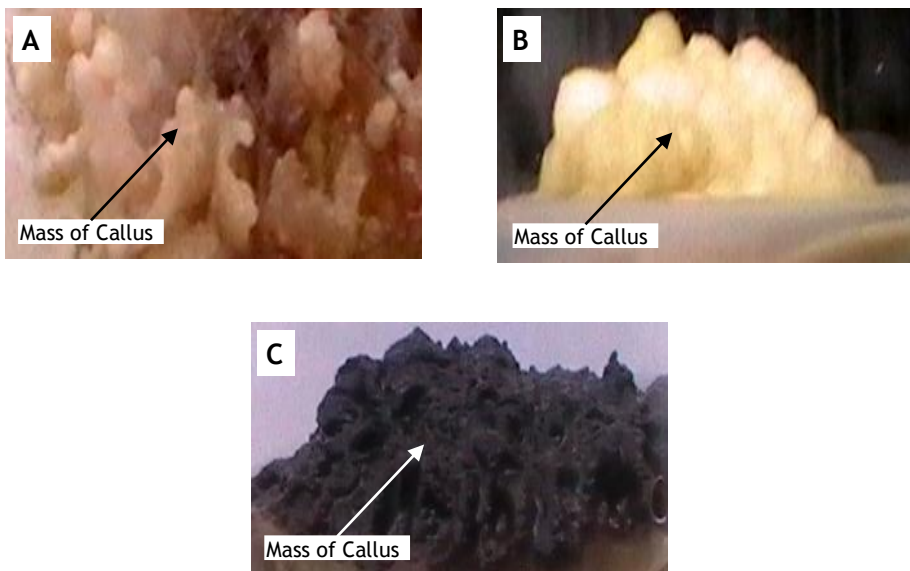


Figure 1. A Calli of *Bryum coronatum* cultured on a hormone free MS basal medium. B Calli of *Bryum coronatum* cultured on MS medium supplemented with 5 mg/L 2,4-D. C Calli of *Racopilum africanum* cultured on MS medium supplemented with 5 mg/L 2,4-D.

1B and 1C). The calli grew rapidly and massively and maintained the dedifferentiated state throughout the three month period of culture. The attempt to induce callus from *Archidium ohioense* and *Thuidium gratum* failed on both media, as no callus induction was observed throughout the period of the experiment.

Discussion

Axenic cultivation of bryophytes as well as developing methodology in the propagation of bryophytes are significant in rare species conservation both for *ex situ* and reintroduction (Rowntree & Ramsay, 2005, 2009; Cvetic et al., 2007; Brezeanu et al., 2008; Vujicic et al., 2009). Sterilization of explants before inoculation is no doubt of significant importance in tissue culture as contamination of plant tissue cultures by different sources, such as bacteria and fungi, reduces their productivity and can completely prevent their successful culture (Hatice et al. 2011). The use of commercial bleach (JIK containing 3.85 % M/V NaOCl) at 8 % concentration for these plants was found effective with a 85 % success rate. Other concentrations were either too weak for effective sterilization or too strong for the plant cells to retain their viabilities.

At the early research stage on bryophytes, most calli were induced from spores on culture media containing several organic additions, although factors affecting callus induction were not clear (Gang Yong-Yun et al., 2003). In this study, explants of *Bryum coronatum* in the absence of exogenous plant hormone produced callus which was greatly enhanced by the application of 2,4-D. In the case of *Racomitrium africanum*, callus was formed only in the presence of 2,4-D. Ono et al. (1988) reported that the addition of 10^{-6} mole/L 2,4-D to MS medium was necessary in inducing calli in *Polytrichum commune*, a moss and *Anthoceros punctatus*, an anthocerot. Earlier, they induced callus from spores of *Atrichum undulatum* using 2,4-D (Ono et al., 1987). Mixtures of 2,4-D and BA were also effective in callus induction in *Physcomitrella patens* (Chen et al., 2006) and *Cratoneuron filicinum* (Gao et al., 2003). This study recorded no callus induction from gametophyte explants of *Archidium ohioense* and *Thuidium gratum* in any of the media. However, various conditions have been reported for callus induction, depending on the species of the bryophytes (Glime, 1988). Several reports of production of callus at the cut surfaces of leaf explants as a consequence of wound reaction in hormone-free medium are also well known.

Conclusions

Sterilization of the mosses explants in 8% commercial bleach (JIK) is adequate for proper sterilization. Callus can be induced by 5 mg/L of 2,4-D in explants of *Bryum coronatum* and *Racomitrium africanum*, although, further work is required to determine the optimal concentration required for induction. The possibility of callus induction from explants of *Archidium ohioense* and *Thuidium gratum* should also be tried using other concentrations of 2,4-D and/or combination with other plant growth hormones such as cytokinins.

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References

- Allsop A. 1957. Controlled differentiation in culture of two liverworts. *Nature* 179:681-682
- Annette H, Ralf R. 2005. From axenic spore germination to molecular farming. One century of bryophyte *in vitro* culture. *Plant Cell Reports* 23:513-521
- Brezeanu A, Cogălniceanu G, Mihai R. 2008. Ultrastructural characterization of the *in vitro* gametophyte of *Bucegiario manica* Radian - a rare liverwort. *Romanian Journal of Biology - Plant Biology* 53(2):49-61
- Chen JW, Cao T, Shi DJ. 2006. The effect of plant hormones to calli inducement and dedifferentiation of *Physcomitrella patens* (Hedw) B.S.G. *Journal of Shanghai Normal University (Natural Science)* 35:70-74
- Crum H. 2001. *Structural Diversity of Bryophytes*. University of Michigan Herbarium, Ann. Arbor. Pp379
- Cvetic T, Sabovljević A, Sabovljević M, Grubišić D. 2007. Development of the moss *Pogonatum urnigerum* (Hedw) P. Beauv. Under *in vitro* culture conditions. *Archives of Biological Sciences* 59(1):57-61
- Gang Y-Y, Du G-S, Shi D-J, Wang M-Z, Li X-D, Hua Z-L. 2003. Establishment of *In vitro* Regeneration System of the *Atrichum* mosses. *Acta Botanica Sinica* 45(12):1475-1480
- Gao YC, Shaw W, Zhang H. 2003. Effects of different plant growth substances on callus induction of *Ceratoneuron filicinum*. *Plant Physiology Communication*. 39:29-31

- Glime JM. 1988. Methods in Bryology. Mainz: *Proceedings of Bryological Methods Workshop 1-16*: 99-105
- Gradstein SR, Churchill SP, Salazar AN. 2001. Guide to the bryophytes of tropical America. *Memoirs of the New York Botanical Garden* 86:1-577
- Hatice C, Ufuk K, Gulnur T. 2011. Influence of different sterilization methods on callus initiation and production of pigmented callus in *Arnebia densiflora* Ledeb. *Turkish Journal of Biology* 35:513-520
- Katoh K. 1983. Kinetics of photoautotrophic growth of *Marchantia polymorpha* cells in suspension culture. *Physiologia Plantarum* 59:242-248
- Makinde AM, Odu EA. 1993. Recolonisation of fire disturbed soil by mosses in a derived savanna. *Nigerian Journal of Botany* 6:217-221
- Milorad V, Tijana C, Aneta S, Marko S. 2010. Axenically Culturing the Bryophytes: A Case Study of the Liverwort *Marchantia polymorpha* L. ssp. *Ruderalis* Bischl. and Boisselier (Marchantiophyta, Marchantiaceae). *Kragujevac Journal of Science* 32:73-81
- Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* 15:473-497
- Odu EA. 1987. On the gemmae of West Tropical African Mosses. *Symposia Biologica Hungarica* 35:215-225
- Odu EA, Akinfenwa FO. 1991. Aerodiaspores and dispersal in some cryptogams. *Nigerian Journal of Botany* 4:107-114
- Ono K, Murasaki Y, Kawauchi K. 1987. Establishment and characteristics of cell suspension culture from a moss, *Atrichum undulatum*. *The Botanical Magazine Tokyo* 100:217-221
- Ono K, Murasaki Y, Takamiya M. 1988. Induction and morphogenesis of cultured cells of bryophytes. *Journal of the Hattori Botanical Laboratory* 65:319-401
- Peck JE, Moldenke A. 1999. *Describing and estimating the abundance of microinvertebrates in commercially harvestable moss*. Eugene District of the Bureau of Land Management, Eugene, Oregon.
- Ramsay MM, Burch J. 2001. *Ex situ* techniques in support of UK bryophyte conservation. *Novitates Botanicae ex Universitate Carolina* 15:27-33
- Razdan MK. 2003. *Introduction to Plant Tissue Culture* (2nd Ed) Science Publishers Inc. Enfield USA P.8.
- Rowntree JK, Ramsay MM. 2005. *Ex situ* conservation of bryophytes: progress and potential of a pilot project. *Boletín de la Sociedad Española de Briología* 26 - 27:17-22
- Rowntree JK, Ramsay MM. 2009. How bryophytes came out of the cold: successful cryopreservation of threatened species. *Biodiversity Conservation* 18:1413-1420
- Rowntree JK. 2006. Development of novel methods for the initiation of *in vitro* Bryophyte cultures for conservation. *Plant cell, Tissue and Organ Culture* 87:191-201

- Vujicic M, Sabovljević A, Sabovljević M. 2009.** Axenically culturing the bryophytes: a case study of the moss *Dicranum scoparium* Hedw. (Dicranaceae, Bryophyta). *Botanica Serbica* **33(2):137-140**
- Wood AJ, Oliver MJ, Cove DJ. 2000.** New frontiers in bryology and lichenology. Bryophytes as model systems. *Bryologist* **103:128-133**