Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences
ISSN- 0975-1491 Vol 6, Issue 9, 2014

Original Article

IN VITRO CULTURE OF MOSS *BRYUM CORONATUM* SCHWAEGR.*(BRYACEAE)* AND IT'S PHYTOCHEMICAL ANALYSIS

VIJAY KANT PANDEY[1], RASHMI MISHRA[1], RAMESH CHANDRA[1*]

[1]Department of Bio-Engineering, Birla Institute of Technology, Ranchi, India.
Email: rameshchandra@bitmesra.ac.in

Received: 25 Jul 2014 Revised and Accepted: 26 Aug 2014

ABSTRACT

Objective: The purpose of the present investigation was to establish *in vitro* conditions for moss *Bryum coronatum* Schwaegr. And to carry out preliminary phytochemical screening of *B. coronatum* leaf extracts in different solvents.

Methods: Fresh unopened, mature capsules were used as explant and surface sterilization of spores bearing capsule with different concentration of sodium hypochlorite (0.5, 1, 2, 4 and 8%) with different time duration. The Murashige and Skoog (MS) medium that contains different concentration(full,1/2, 1/4th, 1/8th strength) with different concentration of sucrose were used for culture this moss. Phytochemical screening were carried out using ethanol, methanol and ethyl acetate leaf extract of *B. coronatum* to identify various constitutes using the standard procedures.

Results: Surface sterilization of spores of this moss was most effective in 4% commercial bleach for 1 min sterilization. The optimum condition for germination of spores and for proper growth of gametophytes *B. coronatum* on MS/4 medium strength with sucrose (1.5%), at pH 5.8 and temperature 22±2ºC with 16/8h: light/dark condition. Phytochemical analyses revealed the presence of alkaloids, terpenoids and saponins in all extracts.

Conclusion: Four percentage NaOCl aqueous solutions are better for surface sterilization of moss sporophytes. MS/4 medium with 1.5% sucrose found the best medium for spore germination. Solvents extracts showed presence of alkaloids, terpenoids and saponins in all extracts.

Keywords: *In vitro*, *Bryum coronatum*, Spores, Alkaloids, Terpenoids, Saponins.

INTRODUCTION

Bryophytes (liverworts, mosses and hornworts) are non-vascular plants, with nearly 15,000 species worldwide of which approximately 8,000 are mosses, 6,000 are liverworts, and are 200 are hornworts [1], was probably reduced early during land plant evolution[2,3,4]. Bryophytes possess the following main characteristics: they have a very simple structure compare to higher plant. Dominant vegetative phase is haploid gametophytes and less chromosome number[5]. Ono et al (1988) reported that cell of bryophytes especially in suspension culture, as ideal materials for morphogenetic, genetic, biochemical, physiological and molecular studies. Bryophytes occur in nearly every ecosystem on earth and play a major role in the recycling of carbon and nutrients through growth and decomposition[6].

Nearly 40% of bryophytes are endangered at present and in urgent need of active protection and conservation. In nature, bryophytes propagated by means of asexual (by multiplication of gametophytes parts) and sexual (through spore) reproduction. Tissue culture methods of mosses offer an alternative mean of vegetative propagation. The pioneer example for a bryophyte especially moss developed into a scientific model organism was moss *Physcomitrella patens* Hedw. [7,8,9,10,11]. The main advantage of *P. patens* is the easy way of its axenic *in vitro* cultivation on solid mineral medium[12], in liquid culture in flasks and in bioreactor[13-14]. Unlike other higher plant, *in vitro* culture of mosses cannot be initiated by using leaf explants since surface sterilization causes cell death.

Phytochemicals are naturally occurring in plants, leaves and other vegetative parts and roots. These phytochemicals have a role in defense mechanism of plants and in protection of plants from various diseases. Chemical constituents and ethno-bryology of bryophytes are not elaborated very well [15-16]. Bryophytes are extremely rich in phenols (flavonoids and bibenzyl derivatives), terpenoids, glycosides, fatty acids and also some aromatic compounds. It also considered as a remarkable reservoir of new, natural products or secondary compounds, many of which have shown interesting biological activities. Phytochemicals are primary compound which included chlorophyll, proteins and common sugars and secondary compounds which have terpenoids, alkaloids and phenolic compounds [17]. Terpenoids exhibit various important pharmacological activities i. e., anti-inflammatory, anti-malarial, anticancer, inhibition of cholesterol synthesis, anti-viral and anti-bacterial activities [18]. Terpenoids are very important in attracting useful mites and consume the herbivorous insects [19].

Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. Alkaloids are used as anaesthetic agents and are found in various medicinal plants [20].

B. coronatum Schwaegr. belonging to family Bryaceae (Bryopsida) which are densely tufted, yellowish-green plant. It stem is short; 5-10 mm long, often branched. Stem leaves imbricate, lanceolate to ovatelanceolate, Setae 1-1.5 cm long, reddish-brown. Its capsules are pendulous, oblong and reddish-brown, the apophysis slightly wider than the urn and bulging, rounded-obtuse at base, warty when dry. This moss is widespread in tropical to warm-temperate regions Asia, Africa, North America, South America, Australia, and Oceanic islands. It habitat on soil, bricks, cemented bricks and rocks.

The aim of this paper is to report establishment of axenic culture of moss *B. coronatum* and to search for the conditions for full plant development. Such knowledge is essential for designing *ex situ* conservation schedules, for future introduction and reintroduction and for evaluationof the biotechnological potential. For development of protocol for the full gametophyte of *B. coronatum*. Our data will contribute future conservation biology of this moss as well as other related mosses. Preliminary phytochemical screening of *B. coronatum* leaf extracts in different solvents like ethanol, methanol and ethyl acetate is being reported.

MATERIALS AND METHODS

Fully developed plant of *B. coronatum* with sporophytes and gametophyte were collected from BIT, Mesra campus, Ranchi, India in spring of 2013. Fresh unopened, mature capsules were used as the starting plant material. Collected plant materials were identified by Botanical survey of India (BSI), Kolkata, India. For this voucher

specimen were deposited as plant herbaria in BSI, Kolkata and also in department of Bioengineering, BIT Mesra. Collected plants with healthy, immature sporophytes that contain spores wash them thoroughly in running tap water for 5 min. Immature capsules of *B. coronatum* with operculum or both with operculum and calyptra, and undamaged sporophytes were separated from the gametophytes and washed in distilled water. *In vitro* cultures of *B. coronatum* were initiated by surface sterilization of spores bearing capsule with different concentration of sodium hypochlorite (0.5, 1, 2, 4 and 8%) with different time duration. Then, rinsed them 3 times in cold, sterile distilled water for 5 min. Capsules were opened with a sterile needle and transferred onto solid medium. Capsules were opened aseptically under the laminar flow cabinet and spores were transferred with a sterile needle to petri dishes containing 20 ml solid medium (MS medium with different concentrations such as 1/2, 1/4th, 1/8th and its normal strength with and without sucrose. The basal medium (BM) contained MS(Murashige and Skoog)[21] mineral salts and vitamins, 100 mg/L myo-inositol, 0.70% (w/v) agar (Bacteriological) without any supplements of growth regulators. The medium pH was adjusted to 5.8 prior to autoclaving at 121ºC for 20 min. Cultures were grown at 22±2ºC under white fluorescent tubes at photon fluorescent rate of 47 µmol/m^{2}s and a day/night regime of 16/8h. Moss plants were sub-cultured after 1 month interval.

Preparation of plant extract

The leaves of *B. coronatum* was removed from the plants and then washed with distilled water to remove any adhering soil or extraneous material and were air dried inside the room at room temperature (28-32˚C) for 7day until water content of the sample become negligible and then grinded to coarse power by mechanical grinder. Methanol, ethanol and ethyl acetate extracts of this sample was prepared by soaking 2 g dried power sample in 20 ml of respective solvents for 24 hrs. Extracts were filtered and evaporated under reduced pressure.

Phytochemical screening

Chemical tests were carried out using ethanol, methanol and ethyl acetate leaf extracts of *B. coronatum* to identify various constituents using the standard procedures as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973).

Test for Alkaloid

Three ml leaf extractof moss was stirred with 3 ml of 1% HCl on steam bath. Mayer and Wagner's reagent was then added to mixture. Turbidity of the resulting precipitate was taken as an evidence for the presence of alkaloid.

Test for Tannins

About 2 ml leaf extract of moss was stirred with 2 ml of distilled water and few drops of $FeCl_3$ Solution were added. Formation of green precipitate was indication of presence of tannins.

Test for Saponins

5 ml of leaf extract of moss was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication of the presence of saponins.

Test for Flavonoids

To 1 ml of leaf extract of moss, 1 ml of 10% lead acetate solution was added. The formation of a yellow precipitate was taken as a positive test for flavonoids.

Test for Terpenoids

2 ml of leaf extract of moss was dissolved in 2 ml of chloroform and evaporated to dryness. 2 ml of concentrated sulphuric acid was then added and heated for about 2 min. Development of a greyish colour indicates the presence of terpenoids.

Tests for glycosides: Liebermann's test

2 ml of the leaf extract of moss was dissolved in 2 ml of chloroform and then 2 ml of acetic acid was added in it. The solution was cooled well in ice. Sulphuric acid was then added carefully. A colour change from violet to blue to green indicates the presence of a steroidal nucleus (that is, a glycone portion of glycoside).

Tests for steroids

i. A red colour produced in the lower chloroform layer when 2 ml of or ethanol, methanol and ethyl acetate leaf extracts of moss was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid was added in it, indicates the presence of steroids.

ii. Development of a greenish colour when 2 ml of the organic extract was dissolved in 2 ml of chloroform and treated with sulphuric and acetic acid indicates the presence of steroids.

RESULTS AND DISCUSSIONS

In vitro culture of profits usually started from sporophytes via spore germination [22]. However in some species like *Rhodobryum*,spores are not regularly available and also some species of bryophytes do not produce sporophytes regularly. For the establishment of axenic culture from gametophytes and sporophytes of bryophytes there is effective concentration of commercial bleach for surface sterilization which to kill the xenic organisms on the plants and not to harm the plants at the same time[22,23,24,25]. Surface sterilization of the sporophytes was more successful since we choose the almost mature but unopened capsules. The higher concentration of commercial bleach harms the spores which cause decrease in spores germination. Gametophytes of this moss are very delicate because of a very thin cuticle so it does not survive even in short time surface sterilization with commercial bleach at various concentrations. High concentration of commercial bleach is lethal for gametophytes of this moss.

For *in vitro* establishment of mosses, the first phase was multiplication and propagation, followed by reintroduction of the cultured specimens to native and potentially suitable habitats. It was found that surface sterilization of sporophytes by various concentration of commercial bleach was effective but comparatively less effective for the gametophytes. It was found that survival percentage was increasing with increase in commercial bleach concentration (0.5%-4%), then it deceased with further increase in commercial bleach concentration. Sporophytes of *B. coronatum* were treated for 1 min in all cases. However contamination percentage decreased with increase in commercial bleach. Maximum survival rate of sporophytes was found with 1 min treatment of 4% commercial bleach(Fig 1).

Asexual gametophytes were used as explant, although they were mostly vulnerable to disinfectants. The cutting treatment of protonema nearly doubles the biomass in short time [26], but sub-culture is necessary. The differentiation into gametophores and protonemal growth depend not only on internal factor transported from cell to cell, but also on interactions with substrate [27]. Spore germination in *B. coronatum* in *in vitro* cultures occurred after 5-6 days of establishment of axenic cultures of this moss species. The culture media for the germination of this moss was MS media that was solidified by 0.7% agar. The germination of moss was done in different concentration of MS media (Full, 1/2, 1/4 & 1/8) and also with different concentration of sucrose (0%, 1%, 1.5% & 3%). It was found that germination of spores of *B. coronatum* was good in 1/4 strength of MS medium with different concentration of sucrose. First the percent of germination increased from full strength to 1/4 strength then decreased in 1/8MS medium with different sucrose concentration. Maximum germination rate was found in 1/4MS with 1.5 % sucrose concentrations in continuous light. No spore germination was occurred in absolute dark.

The vegetative development of the gametophyte in mosses involved two stages: the filamentous protonema stage, and the budding gametophore stage that involves more complex cell differentiation. Spores of *B. coronatum* shown in figure 3. These spores turned bright green after 3–4 days of inoculation of spore on 1/4MS medium in continuous illumination as well as in alternate light and dark conditions and develop early filamentous protonema having chloronema (Fig. 3B) and after 7-8 days they germinated to produce branched chloronema with abundant rounded chloroplasts. After 10

days of spore germination, the distal end of chloronema started to become hyaline on account of disappearance of chloroplasts but only a few chloronemal filaments were differentiated into conspicuous caulonema (brown coloured filaments having a few spindle shaped chloroplasts and oblique septa).

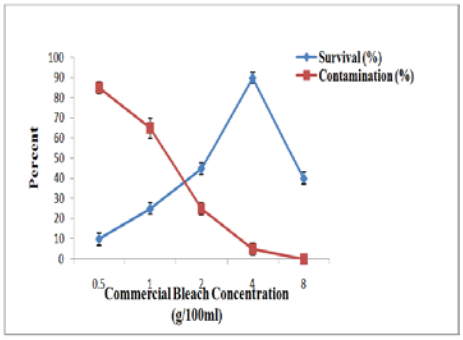

Fig. 1: Survival and contamination percentage of *B. coronatum* spores in culture.

Note: Error bar shows standard deviation.

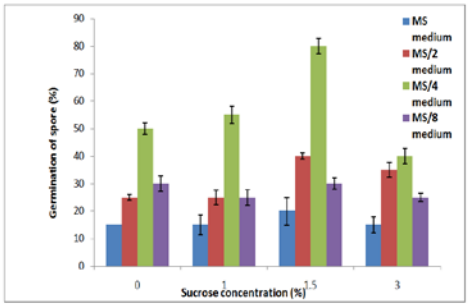

Fig. 2: Spore germination rate of *B. coronatum*in different MS media with different Sucrose concentrations after 5-6 day of inoculation.

Note: Error bar shows standard deviation

The culture contained mainly green chloronema and a few caulonema or hyaline chloronema are used for sub-culture on same media. Protonemal colonies were develop after subculture of protonema on 1/4MS medium after 30 day of inoculation (Fig. 3D). After 45 – 50 days of inoculation, protonemal buds were differentiated from the main chloronemal filaments or basal cells of the chloronemal branches and distributed throughout the protonemal patch in a scattered manner (Fig. 3E). Protonemal buds subsequently developed into young gametophytes (Fig. 3F) in form of erect main axis with numerous, more crowded erects leaves and brown colour rhizoids. 1/4MS medium with 1.5% sucrose gave abundant growth of protonema and rhizoids. In 65-70 day-old culture, well developed population of erect gametophytes developed (Fig. 3F). Rhizoids were morphologically similar to caulonema being brown in colour and with oblique septa but devoid of plastids. It has been reported previously for other bryophytes that the pattern of protonema development in *in vitro* culture varies depending on the mineral media[28]. *In vitro* culture of the moss *Bryum argenteum* was established from sterilized spores and its gametophytes grown in *in vitro* were used to investigate the influence of different substances on secondary protonema and on the growth and multiplication of the gametophytes [25]. Despite the importance of the mineral medium, interactions between its components and organic compounds, such as sugar, may affect culture development and growth [28-29]. Vitamins also appear to be important determinants of various physiological processes in higher plants [30]. A number of significant effects of sucrose on culture development were observed. Though, interpretation of these effects is complex. It is expected that sucrose have positive effects on biomass increase. As it does in bryophytes species and higher plant species[31-32] but in some species sucrose had very little and no effect on bud germination.

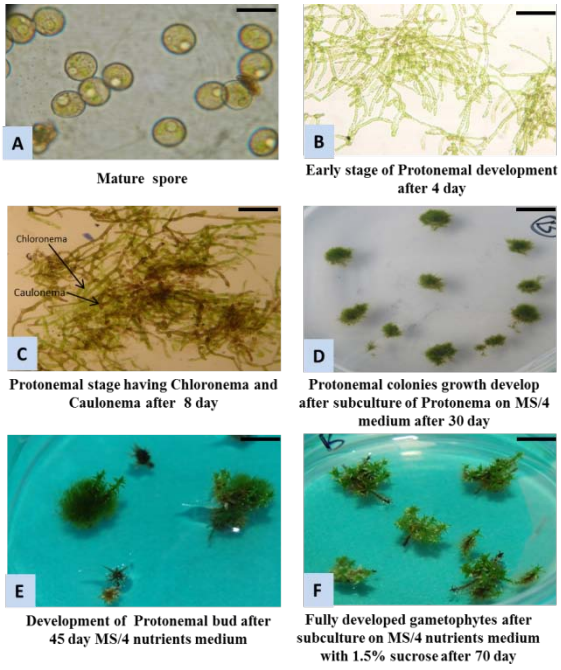

Fig. 3: *In-vitro* culture of *Bryum coronatum* Schwaegr.

(Note: Scale bar equals 100 µm in Fig A, B & C and 1 cm in Fig D, E & F)

Bryum species do not require any critical photoperiod for the onset of the reproductive phase[33] but while working on this *B. coronatum* it was found 16/8: day/dark periods. Kumar and Chopra (1983) reported that the response of *Bryum* species increase with increasing temperature upto 24°C although it was found that optimum temperature for growth of *B. coronatum* was 22±2C. Chopra and Kumra (1988) reported that some moss species could not germinate or even grow without a symbiosis with fungi and bacteria. In this experiment a very easy, effective and convenient method for surface sterilization and *in vitro* culturing of moss *B. coronatum* is shown. Further testing is needed, with more repetitions, with different range of growth regulator concentrations along with observation times, to determine what are the effect of the growth regulators for germination and differentiation of gametophytes.

The phytochemical characteristics of the leaf extract of *B. coronatum* investigated are summarized in table-1. Phytochemical analyses revealed the presence of alkaloids, terpenoids and saponins in all samples. Methanol and ethyl acetate extract of this moss plant also have steroid while in ethanol extracts doesn't have steroids. Ethanol, methanol extracts also have glucosides while this compound was absent in ethyl acetate extract. The alkaloids contained in plants are used in medicine as anaesthetic agents [34]. The presence of saponins in plants have been reported to be responsible for the tonic and stimulating activities observed in Chinese and Japanese medical herbs[35]. Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases. For example,alkaloids protect against chronic diseases. Saponins protect against hypercholesterolemia and antibiotic properties. Steroids and triterpenoids show the analgesic properties. The steroids and saponins were responsible for central nervous system activities. Steroidal compounds are of importance and interest in pharmacy and pharmaceutical science because of their relationship with compounds such as sex hormones [36].

Methanol and ethyl acetate extract of this plant also have steroid while in ethanol extracts doesn't have steroids. Ethanol, methanol extracts also have glucosides while this compound was absent in ethyl acetate extract. Previous reports convey that br yophytes possess medicinal value [37] and are good sources of antibiotics [38].

The preliminary result on moss plant under study (*B. coronatum*) has also shown antimicrobial activity and its detailed investigation is going on our lab.

Table 1: Phytochemical screening of *B. coronatum* Schwaegr. in different solvents extracts

Chemical Constitutent	Ethanol Extract	Methanol extract	Ethyl acetate Extract
Alkaloid	**+**	**+**	**+**
Tannins	**-**	**-**	**-**
Saponins	**+**	**+**	**+**
Flavanoid	**-**	**-**	**-**
Glycosides	**+**	**+**	**-**
Steroid	**-**	**+**	**+**
Terpenoids	**+**	**+**	**+**

Note: +: Present and **-:** Absent

CONCLUSION

Surface sterilization through commercial bleach (4% NaOCl) is easier to achieve on sporophytes than gametophytes. Maximum survival rate of sporophytes was found with 1 min treatment of 4% commercial bleach. The best condition for germination of spores and *in vitro* propagation of plant in *B. coronatum* on MS/4 medium strength with sucrose (1.5%), at pH 5.8 and temperature 22C with 16/8: light/dark condition. Higher sucrose concentrations (>3%) in MS medium tended to have a negative effect on germination of spores. Spore germination start in 4th day of inoculation and spore germinated to produce branched chloronema with abundant rounded chloroplasts. After 10 days of spore germination of chloronema started to become hyaline and disappearance of chloroplasts but only a few chloronemal filaments were differentiated into conspicuous caulonema which is brown coloured filaments. Sub-culturing of *B. coronatum* gave rise to new population via passing through chloronemal and caulonemal stage. Nearly after 2 months of subculture, gametophytes develop fully. The ethanol, methanol and ethyl acetate leaf extracts of *B. coronatum* were investigated for phytochemical screening. It analyses revealed the presence of alkaloids, terpenoids and saponins in all samples. Methanol and ethyl acetate extract of this plant also have steroid while in ethanol extracts doesn't have steroids. Ethanol and methanol extracts also have glucosides while this compound was absent in ethyl acetate extract.

CONFLICT OF INTERESTS

Declared None

ACKNOWLEDGEMENT

Authors acknowledge the support got from UGC MRP Project F No. 37-116/2009 (SR), Birla Institute of Technology, Department of Bio-Engineering and Centre of Excellence (COE) TEQUIP -II- for providing R&D facilities and Director, National Botanical Research Institute, Lucknow (CSIR)and Botanical survey of India (BSI), Kolkata, India for providing information related to identification process of the mosses.

REFERENCES

1. Hallingback T, Hodgetts N. Status survey and conservation Action plan for Bryophytes: mosses, liverworts and hornworts. IUCN/SSC Bryophyte Specialist Group. IUCN, Gland, Switzerland and Cambridge, UK; 2000. p. X+106.
2. Kenrick P, Crane PR. The origin and early evolution of plants on land. Nature 1997;389:33-9.
3. Graham LE, Cook ME, Busse JS. The origin of plants: body plan changes contributing to a major evolutionary radiation. Proc Natl Acad Sci USA 2000;97:4535-40.
4. Taylor TN, Kerp H, Hass H. Life history biology of early land plants: deciphering the gametophyte phase. Proc Natl Acad Sci USA 2005;102:5892-97.
5. Glime JM. Methods in Bryology. Mainz: Proc Bryol Meth. Workshop 1988;1-16:99-105.
6. Vanderpoorten A, Goffinet B. Introduction to bryophytes. Cambridge: University Press; 2009. p. 26-42, 185-213.
7. Reski R. Development, genetics and molecular biology of mosses. Bot Acta 1998;111:1-15.
8. Reski R. *Physcomitrella patens* as a novel tool for plant functional genomics. In: Vasil IK. (ed) Plant biotechnology 2002 and beyond. Kluwer Acad Publ; 2003. p. 205-9.
9. Cove DJ, Bezanilla M, Harries P, Quatrano RS. Mosses as model systems for the study of metabolism and development. Annu Rev of Plant Biol 2006;57:497-520.
10. Beike AK, Decker EL, Frank W, Lang D, Vervliet-Scheebaum M, Zimmer AD, Reski R. Applied Bryology Botechnology. Trop Bryol 2010;31:22-32.
11. Prigge MJ, Bezanilla M. Evolutionary crossroads in developmental biology: *physcomitrella patens*. Development 2010;137:3535-43.
12. Reski R, Abel WO. Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens,* using iso-pentenyladenine. Planta 1985;165:354-58.
13. Decker EL, Reski R. The moss bioreactor. Curr Opin Plant Biol 2004;7:166-70.
14. Hohe A, Reski R. From axenic spore germination to molecular farming: one century of bryophyte *in vitro* culture. Plant Cell Rep 2005;23:513-21.
15. Sabovljevic A, Sabovljevic M. Bryophytes, a source of bioactive and new compounds, in Recent Progress in Medicinal Plants Vol. 22:Phytopharmacology and Therapeutic values IV, Studium Press LLC, TX; 2008. p. 9-25.
16. Sabovljevic A, Sokovic M, Sabovljevic M, Grubisic D. Antimicrobial activity of *Bryum argenteum*. Fitoterapia 2006;77:144-45.
17. Krishnaiah D, Sarbatly R, Bono A. Phytochemical antioxidants for health and medicine: A move towards nature. Biotechnol Mol Biol Rev 2007;1:97-104.
18. Mahato SB, Sen S. Advances in triterpenoid research, 1990-1994. Phytochemistry 1997;44:1185-236.
19. Kappers IF, Aharoni A, Van Herpen TW, Luckerhoff LL, Dicke M, *et al*. Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. Sci 2005;309:2070-72.
20. Herouart D, Sangwan RS, Fliniaux MA, Sangwan-Norreel BS. Variations in the Leaf Alkaloid Content of Androgenic Diploid Plants of *Datura innoxia*. Planta Med 1988;54:14-7.
21. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 1962;15:473.
22. Sabovljevic M, Bijelovic A, Dragicevic I. *In vitro* culture of mosses: *Aloina aloides* (K. F. Schultz) Kindb, *Brachythecium velutinum* (Hedw.) B. S. &G. , *Ceratodon purpureus* (Hedw.) Brid. , *Eurhynchium praelongum* (Hedw.) B. S. &G. and *Grimmia pulvinata* (Hedw.) Sm. Turk J Bot 2003;27:441-46.
23. Vujicic M, Sabovljevic A, Sabovljevic M. Axenically culturing the bryophytes: a case study of the moss *Dicranum scoparium* Hedw. (Dicranaceae, Bryophyta), Botanica Serbica 2009;33(2):137-40.
24. Vujicic M, Cvetic T, Sabovljevic, Sabovljevic M. Axenically culturing the bryophytes: a case study of the liverwort *Marchantia polymorpha* L. ssp. Ruderalis Bischl. & Boisselier (Marchantiophyta, Marchantiaceae). Kragujevac J Sci 2010;32:73-81.
25. Sabovljevic A, Sabovljevic M, Grubisic D. Giberellin influence on the morphogenesis of the moss *Bryum argenteum* Hedw. *in vitro* conditions. Arch Biol Sci 2010;62(2):373-80.
26. Chen YY, Lou YX, Guo SL, Cao T. Successful tissue culture of the medicinal moss *Rhodobryum giganteum* and factor influencing proliferation of its protonemata. Ann Bot Fennici 2009;46:516-24.
27. Schween G, Hohe A, Koprivova A, Reski R. Effects of nutrients, cell Density, culture techniques on protoplast regeneration and

early protonema development in moss, *Physcomitrella patens*. J Plant Physiol 2003;160:209-12.

28. Welander T. Effects of nitrogen, sucrose, IAA and kinetin on *Beta vulgaris* grown *in vitro*. Physiol Plant 1976;36:7-10.
29. Custodio L, Martins-Loucao MA, Romano A. Influence of sugars on *in vitro*rooting and acclimatization of carob tree. Biol Plant 2004;48:469-72.
30. Vasudevan A, Selvaraj N, Ganapathi A, Kasthurirengan S, Ramesh Anbazhagan V, Manickavasagam M. Glutamine: a suitable nitrogen source for enhanced shoot multiplication in *Cucumis sativus* L. Biol Plant 2004;48:125-28.
31. Mehta P, Chopra RN. The effect of some chemical factors on growth and fertility in male clones of the moss *Microdus brasiliensis*. Cryptog Bryol Lichen 1991;12:55-62.
32. Sarla. *In vitro* studies on male clone of *Riccia discolor*: effect of sugars. J Hattori Bot Lab 1992;72:141-50.
33. Kumar P, Chopra RN. Effect of some physical factors on growth and gametangial induction in male clones of the three mosss grown *in vitro.* Bot Gaz 1983;144(4):533-39.
34. Pandey M, Debnath M, *et al*. Phytomedicine: an ancient approach turning into future potential source of therapeutics. J Pharmacognosy Phytother 2011;3(1):113-17.
35. Victor Njoku O, Chidi O. Phytochemical constituents of some selected medicinal plants, Afr J Pure and Appl Chem 2009;3(11):228-33.
36. Sofowora AA. Medicinal Plants and Traditional Medicine in Africa, 2nd Ed. Spectrum Books Ltd: Iba-dan; 1993. p. 81-5.
37. Cowman MM. Plant Products as Antimicrobial Agents. Clinic Microbiol Rev 1999;12(4):564-82.
38. Edeoga HO, Eriata DO. Alkaloids, Tannin and Saponin Contents of Some Nigerian Medicinal Plants. J Med Aro Plant Sci 2001;23:344-49.