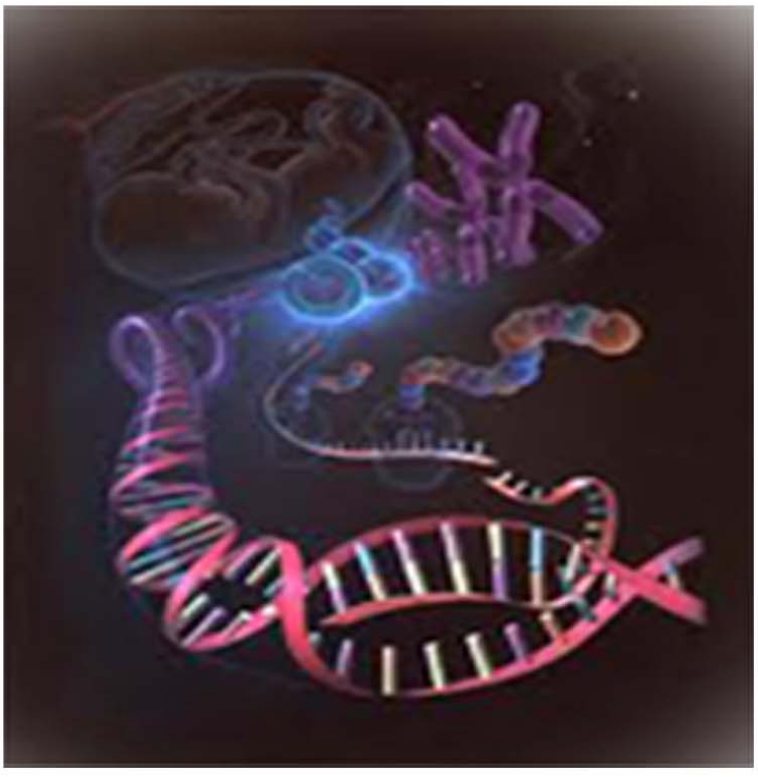
IJLBPR

ISSN 2250 - 3137
Vol1. 3, No. 4, October 2014

International Journal of

Life Sciences Biotechnology and Pharma Research



International Journal of Life Sciences Biotechnology and Pharma Research
Hyderabad, INDIA

www.ijlbpr.com

Email : editorijlbpr@gmail.com or editor@ijlbpr.com

ISSN 2250-3137 www.ijlbpr.com
Vol. 3, No. 4, October 2014

Research Paper

ESTABLISHMENT OF *IN VITRO* AND TEMPORARILY IMMERSED CULTURES USING RITA® OF *MARCHANTIA LINEARIS* LEHM. AND LINDENB. A LIVERWORT

Remya Krishnan[1] and K Murugan*[1]

Corresponding Author: **K Murugan ✉ harimurukan@gmail.com*

The use of *in vitro* techniques for conservation has been rising steadily since their inclusion in The Convention on Biological Diversity and The Global Strategy for Plant Conservation. Unfortunately, bryophytes are often overlooked in conservation initiatives, but they are important ecologically and medicinally. Axenic culturing of bryophytes seems to be so complicated due to possible interaction with other organisms in non axenic condition. *Marchantia linearis* Lehm. and Lindenb. a liverwort distributed in restricted scattered localities along the western Ghats and was massively exploited by the Kani tribes for curing many skin disorders. The objective of this study was to develop an efficient *in vitro* propagation protocol for *M. linearis*. Multiple leafy thalli were induced significantly on MS/5 medium containing 2 mg/L BA + 0.5 mg/L KIN +0.2 mg/L Naphthalene Acetic Acid (NAA) than other hormonal combinations. Rhizoid formation was significant with 0.2 mg/L indole-3-butyric acid (IBA) than NAA. Growth of the regenerated shoots in a temporary immersion bioreactor resulted in remarkable increase in fresh, dry mass, leafy shoot ratio. The regenerated shoots from the temporary immersion bioreactor formed roots when transferred onto a medium with IBA. The regeneration protocol developed in the present study provides a basis for germ plasm conservation and elucidation of phytochemicals for further therapeutic investigation of this liverwort species.

Keywords: Bioreactor culture, Germplasm conservation, *Marchantia linearis*, MS medium, Growth hormones, *In vitro* culture

INTRODUCTION

Axenic culturing of tissues and organs was first established and profitably employed in bryophytes especially mosses (Servettaz, 1913). However, bryophyte culturing did not gain much speed due to multiple reasons such as non availability of true type samples, genetic variability, contaminations with microbes and low level of species biology knowledge. Most of the tissue culture works are centralized on medicinal herbs (Duckett *et al.,*

[1] Plant Biochemistry and Molecular Biology Laboratory, Department of Botany, University College, Thiruvananthapuram, 695 034, Kerala, India.

2004). Research in bryophytes is useful for the elucidation of apogamy, apospory, stress-induced cellular responses in plants and protoplasmic fusion analysis (Cvetic *et al.*, 2005). Besides, axenical cultivation and propagation of bryophytes are significant for ex situ conservation of rare medicinal species and its reintroduction (Vujicic *et al.*, 2009; Rowntree *et al*., 2011).

Like other bryophytes, the liverworts are haploid-dominant plants and are generally with leafy thallus inhabiting in moist dampy areas so chances for contamination by xenic inhabitants such as bacteria, algae, protozoa, fungi and others. These features complicate the standard methodology for surface sterilization in connection with axenically culturing the bryophytes. Recently, bryophytes research received much attention with the discovery of more than 400 novel bioactive compounds and thus provide a considerable potential for biotechnological and biopharmaceutical applications (Asakawa *et al*., 2013). However, the problem for analyzing and/or certain identified substance production is often inadequate in the *in vivo* species. Similarly, there is evidence that, in general, crude extracts prepared from *in vitro* bryophyte cultures show higher therapeutic activity than extracts prepared from *in vivo* populations. In contrast, the antibacterial activity is higher in extracts from natural populations as compared to *in vitro* cultures. Based on the evidence presented, the production of secondary metabolites in bioreactors from plants, propagules, or spores may be possible through the establishment of culture of protoplasts or protonemata are challenging. In this scenario, axenically culturing of this group is much important to produce large quantity of biomass for chemical and pharmaceutical investigations, developing horticultural landscape and also as bioindicators to analyze pollution (Lara *et al.,* 2006). Awasthi *et al.*, established successful protocol for *in vitro* propagation of an endemic and threatened Indian liverwort *Cryptomitrium himalayense*.

M. linearis Lehm. and Lindenb. a thalloid liverwort with restricted distribution, has interesting ethnobotanical properties. It is a traditional medicinal liverwort of Kani tribe of South Kerala (Ramesh and Manju , 2009). In view of excessive utilization, limited distribution pattern and slow growth rate, the present study was undertaken to establish stable *in vitro* culture of the liverwort and massive multiplication through temporary immersed cultures using the Recipient for Automated Temporary Immersion system

MATERIALS AND METHODS

Plant Material

M. linearis specimens were collected from rocks along the riverine belts of Ponmudi hills, Trivandrum district (Kerala), India and the voucher specimen (UC DBH, 126) was kept in the Bryophyte Collection of University College Herbarium.

In Vitro Culture Protocol

All media and glasswares were sterilized by autoclaving at 15 lb/sq. in. for 15 min. After inoculation, three replicates of each medium were maintained under controlled and aseptic conditions, cultures were provided continuous illumination of 60 – 80 µmol/ m^2/s as well as alternate light and dark period of 16 h and 8 h, respectively with the help of a combination of fluorescent tubes. Temperature was maintained at 21 ± 2ºC.

Fresh, healthy leafy thalli were collected and screened carefully from the impurities, wrapped with cheese cloth, surface disinfected by dipping in 70% alcohol for 30 s, rinsed thrice with deionized water and kept in sterilized containers. Apical parts of gametophytes were further disinfected for 5 min with 2, 5, 10% or 15% solution of sodium hypochlorite (NaOCl), followed by rinsing repeatedly in sterile deionised water and finally, with propamidine and pentamisine (5 µg/mL) to remove the microbial contaminations.

Sterilized apical leafy thalli were inoculated into two types of culture media: half-strength Knop's and diluted MS/5 with different concentration (0.5, 1.0, 2.0, 3.0 mg/L) of BA, KIN alone or in combination of BA, KIN and NAA (0.2, 0.4 mg/L) with 0.25% w/v phytagel (Awasthi *et al*., 2011) for callus initiation. Percentage of callus formation is recorded after 4 weeks. Cultures were incubated in the growth room for 4 weeks at 24±2°C, at 60 µmol/ m^2/s for 16 h photoperiod. Cultures were incubated in the growth chamber for 4 weeks.

The regenerated calli were transferred into differentiation media - half-strength MS media with varying concentrations of NaH_2PO_4 (50 to 225 mg/L), containing growth regulators BA, KIN and NAA with the concentration of 2 mg/L + 0.5 mg/L + 0.2 NAA mg/L pH 5.75, and 0.25 w/v phytagel was added as a supporting material. Cultures were incubated in the growth chamber for four weeks.

Newly formed leafy thallus measuring 2-3 cm in length were excised individually from the mother explant and transferred to rooting media. Two types of rooting hormonal combinations were used in different concentration of NAA and IBA (0.1, 0.2, 0.5, 1.0 mg/L) in the MS semi-solid medium. The observations on development pattern of rhizoids were made throughout the entire culture period. Data were recorded after 7-8 weeks of culture.

Mixed Photo-Heterotrophic Growth

Mixed photo-heterotrophic growth of *M. linearis* was analyzed using supplements of 25, 50, or 100 mM D-glucose, or 25 and 50 mM sucrose in MS/5 medium.

Temporarily Immersed Cultures Using Rita®

Four culture methods for the regenerated thalli of *M. linearis* were compared: solid culture in magenta boxes, paper-bridge-support liquid culture in magenta boxes, suspended liquid-flask culture in 250 mL flasks, and periodical liquid-immersion culture in a temporary immersion bioreactor (RITA) with a 600 mL working volume. In the solid culture, 25 thalli were cultivated on 50 mL solid medium. In the paper-bridge-support liquid culture, a piece of filter paper over glass beads was used to support 25 thalli, and 50 mL liquid nutrient was added to the bottom of the magenta boxes. In the suspended liquid culture, 25 thalli were suspended in 50 mL liquid nutrient medium and culture flasks were kept on an orbital shaker at 80 rpm. Stabilization of pH in the culture medium was also evaluated by aerated liquid submersion cultures using diluted MS/5 medium supplemented with 2.5 to 10 mM concentrations of potassium citrate and potassium fumarate for 8 week period. For culture in the temporary immersion bioreactor, 150 thalli and 300 mL liquid medium were transferred in each bioreactor connected to an air supply of 0.25 vvm. The immersion cycle, set for 3 min every 60 min, was controlled by a timer.

Leafy thallus cultures from the temporary immersion bioreactors were transferred onto half strength MS medium supplemented with different concentrations of IBA (0.1, 0.2, 0.5) for inducing root formation. The culture conditions were same as described above for initial thallus regeneration. For fresh weight determination, the thallus cultures were gently pressed on filter papers to remove excess water and weighed. Then they were dried in an oven at 60 °C for 24 h for determining the dry weight. Leafy thallus multiplication ratio was calculated by the final thallus number after 30 days of culture compared to initial number of thalli.

STATISTICAL ANALYSIS

All treatments consisted of three replicates and the experiments were repeated twice. Data were analyzed using the Student-Newman-Keulls means separation test of SAS version 8.02.

RESULTS AND DISCUSSION

The leafy explants began to show the positive signs of compact primary callus proliferation after four weeks of culture on callus induction media. Calli were free from fungal as well as bacterial contamination. Apical part of leafy thallus were incubated on five different hormonal media of Knop and MS. Horizontally placed explants produced callus more effectively than the explant which were placed vertically. Varied frequency of callus formation was noticed in the different media (Table 1). After successful initiation of the culture (4-5 weeks culturing), newly formed calli were excised individually from the proliferated explants for subsequent sub culturing. Multiplication of calli was found best on MS/5 medium in combination of BA 2.0 mg/L, KIN 0.5 mg/L and NAA 0.2 mg/L. (Table 1 and Figure 1a,b,c) and the proliferation of leafy thalli took place in 2 weeks. In the differentiation media the percentage of differentiation and the number of thallus per explant was 80.6 and 5, respectably with 200 mg/L NaH_2PO_4 (Table 2). But the length of the thallus (4.5 cm) was maximum in MS medium supplemented with 150 mg/L NaH_2PO_4. Proliferating shoots obtained from shoot tip explants of *M. linearis* took maximum 8-9 weeks from the time of establishment to attain the size suitable for rooting (>2 cm). The percentage of shoots that induced rhizoids was observed in MS medium supplemented with IBA, followed NAA. The highest number of rhizoid per culture was found in MS medium supplemented with IBA 0.2 mg/L, followed NAA 0.4 mg/L (80.5 and 72.6%).

Plant growth of *in vitro* cultures using basal media is restricted through the photosynthetic capacity of the plants. Optimization of light intensity, composition regime and increased CO_2 levels have been reported to result in increased growth of plant cultures under photoautotrophic conditions (Dixon, 1985). Cultures supplemented with suitable carbon sources induce *in vitro* growth remarkably under mixed photo-heterotrophic conditions. In some cases, plant cultures even become independent from the photosynthetic process itself and grow in complete darkness under chemoheterotrophic conditions (Takio *et al.*, 1990). A growth promoting effect of carbohydrates in liverwort cultures has been reported with D-glucose and D-sucrose. In addition to plant growth, carbohydrate supplements also regulate secondary metabolism in terms of secondary metabolite synthesis have also been reported (Chopra and Sood, 1973; Morais and Becker, 1991; Takeda and Katoh, 1981). In the present study, for mixed photo-heterotrophic growth of *M. linearis*

Table 1: Effect of Different Concentration and Combination of Growth Regulators on Knop and Ms Medium Callus Proliferation From *In Vitro* Grown Apical Leafy Thallus Explants of M. Linearis After 4 Weeks

		Knop FS	MS/5
		% callus	% callus
BA	0.5	20±0.02	26±0.04
	1	55.4±0.05	65±0.01
	2	65.2±0.01	79±0.02
	3	52±0.03	62±0.03
KIN	0.5	16.3±0.04	23±0.05
	1	35.7±0.01	47±0.01
	2	63.4±0.03	74±0.06
	3	50.2±0.02	60±0.03
BA+ KIN	0.5+ 0.5	40.2±0.04	50±0.02
	0.5+ 1	35.4±0.05	43±0.04
	1+ 0.5	56.6±0.01	63±0.02
	1+1	46±0.04	56±0.03
	1+2	40.5±0.03	45±0.01
	2+0.5	69.89±0.05	89±0.05
	2+1	56.1±0.06	61±0.02
	3+1	45.8±0.02	58±0.04
	2+2	36.6±0.01	46±0.03
	1+3	40.7±0.05	47±0.01
	3+3	34.4±0.03	44±0.06
BA+ NAA	0.5+ 0.2	27.6±0.04	36±0.04
	1+ 0.2	45.9±0.02	59±0.01
	2+0.2	70.5±0.03	90±0.07
	0.5+ 0.4	45.3±0.01	55±0.02
	1+ 0.4	40.7±0.06	47±0.03
	2+0.4	30.8±0.04	38±0.01
	3+0.2	34.2±0.03	42±0.05
	3+0.4	28.93±0.01	33±0.04
BA+KIN+NAA	1+0.5+0.2	57±0.02	67±0.01
	2+0.5+0.2	74.9±0.05	94±0.03
	1+0.5+0.4	60.2±0.01	70±0.04
	2+0.5+0.4	52.0±0.06	60±0.05

Note: Values are mean ± SD. Significant at $P < 0.01$.

Figure 1: a, b and c *In Vitro* Propagation of *Marchantia Linearis*. Views a, Callus Induction b, Dark Greenish Calli c, Thallus Proliferaion in Differentiation Medium

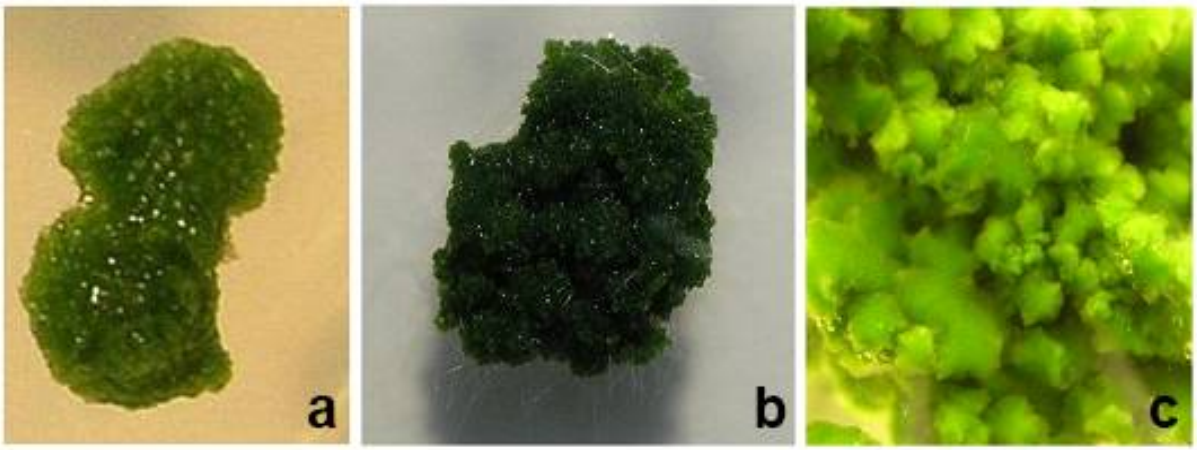


Table 2: Effect of NaH_2PO_4 on *In Vitro* Differentiation of *M. linearis*

NaH_2PO_4 (mg/L)	Number of Axillary Branches	Mean % of Differentiation
0	1±0.04	12±0.45
50	2±0.06	25±0.71
75	2±0.09	34±0.64
100	2±0.07	53±0.25
125	3±0.15	62±0.98
150	3±0.28	71.5±1.5
175	4±0.05	78±0.36
200	5±0.02	80.6 ±0.82
225	2±0.02	76 ±0.55

Values are mean ± SD. Significant at $P < 0.01$.

supplements of 25, 50, or 100 mM D-glucose, or 25 and 50 mM sucrose were found to be effective leading to increased growth rate. Differentiation of thalli was only observed with the lowest carbohydrate concentration of 25 mM D-glucose, whereas concentration above 50 mM resulted in dedifferentiation and callus formation.

M. linearis leafy thallus propagation was significantly affected by the culture regime. Overall growth of *M. linearis* on solidified media even under optimized mixed photo-heterotrophic conditions was slow, thus, making propagation extremely time-consuming. Leafy thallus cultures proliferated variously in the solid, paper-bridge-

support liquid, liquid-flask and temporary immersion bioreactor cultures.

Significantly higher levels of leafy thallus multiplication were observed in the temporary immersion system with an immersion cycle of 3 min every 60 min after 30 days of cultivation than in any other system (Table 3).

The *in vitro* derived leafy thalli from the temporary immersion bioreactor were separated and sub-cultured on MS medium with various levels of IBA. Rhizoid initials formed after 2 weeks of culture. The highest rate of rhizoid development was observed on half-strength MS medium with 0.1 to 0.2 mg/mL IBA after 21 days. The highest percentage for the rooting response was observed at 0.2 mg/mL IBA with 63.5% thalli forming about 3.8 rhizoids per thallus with an average length of 6.5 mm. However, the optimum concentration of IBA was 0.1 mg/mL IBA; 58.2% thalli developed rhizoids on this medium with > 4 rhizoids per thallus and an average length of 8.2 mm.

Liquid culture is ideal in micropropagation for reducing plantlet production costs and for automation. Many plants have been mass propagated in liquid medium using either flasks or bioreactors. However, morphological and physiological disorders such as hyperhydricity, are commonly observed in plants and shoots produced in liquid culture. These abnormalities might be related to stress induced by the environmental conditions (such as O_2, CO_2 and hydrodynamic forces) in the cultivation system. One interesting finding of this investigation was that the growth of *M. linearis* cultures was improved significantly in the temporary immersion bioreactor. In this system the thallus cultures were periodically immersed in liquid medium which was delivered uniformly. In other systems such as the paper-bridge-support liquid culture, the nutrients in the liquid medium were available indirectly and only the basal tissues of shoot cultures were in contact with the medium through the paper bridge. Shoots grown in the liquid-flask culture system displayed high hyperhydricity. This was not observed in the temporary immersion system, likely because the shoot cultures were only periodically exposed to the liquid phase. Temporary immersion culture systems offer several additional advantages including: (1) an efficient supply of nutrition and adequate levels of oxygen transfer; (2) reduced shear and hydrodynamic forces; and (3) automation at relatively low cost. Successful micropropagation of many plant species has been developed using

Table 3: Effect of Culture Regimes on Growth and Multiplication of *M. linearis* Thallus After 30 Days

	Fresh Mass (100g/L)	Dry Weight 10g/L)	Leafy Thallus Multiplication Ratio
Solid culture	1.1±0.03	1.2±0.12	3.4±0.05
Paper-bridge-support liquid culture	1.2±0.06	1.3±0.24	3.7±0.29
Suspended liquid flask culture	1.7±0.01	1±0.33	2.4±0.31
Temporary immersion	1.5±0.09	1.3±0.25	5.1±0.67

Note: Values are mean ± SD. Significant at $P < 0.01$.

temporary immersion culture systems (Ducos *et al.,* 2007). Further, the development of bioreactor propagation system allows the production of plant tissues for use in medicinal preparations. *In vitro* grown plants are free from the effects of seasonal variations, microbial infestations, and soil born contaminants that can affect the medicinal value of the harvested tissues (Saxena, 2001). Contamination of medicinal plant products with a range of environmental pollutants including heavy metals is a serious concern which compromises the safety and efficacy of plant based medicines. Goldenseal plants and products have previously been found to contain heavy metals (Liu *et al.,* 2004b). Although, the results obtained in the present study did not show much significant differences between the different culture methods, the time required for thallus regeneration, and development was reduced substantially using liquid culture media in RITA®. Establish an efficient and economic protocol for the *in vitro* multiplication of *M. linearis* in TIS, which is also applicable for a large scale production of plant material. Etienne and Berthouly reported that TIS generally improves plant quality and production. This is probably due to the fact that explants cultured in TIS are preferential with the availability of nutrients in the liquid. A frequency of four or eight immersions per day for one minute was suitable to stimulate thallus development and growth. Another advantage is that after immersion, enough liquid nutrient remains on the plant surface as a film, which also prevents desiccation during the non-immersed period. In contrast, propagation in solid medium also allows gas exchange, but nutrient uptake is limited to the explants basal surface, which may result in less thallus development and a slower growth rate. Gonzalez-Olmedo *et al.* (2005) in pineapple and Roels *et al.* (2005) in *Musa* successfully established increased shoot multiplication by TIS. However, immersion time is very important, since it determines nutrient and PGR uptake and can influence hyper hydricity of explants (Etienne and Berthouly, 2002).

In conclusion, the *in vitro* techniques reported here offer a simple efficient tool for mass-multiplication of lower non vascular plant species. The ability to rapidly deliver a large number of thalli is expected to help replenish and sustain decreasing populations in the natural environment. Further studies are planned to isolate and characterize the biological activities of the phytochemicals present in the liverwort.

REFERENCES

1. Asakawa Y, Ludwiczuk A and Nagashima F (2013), "Phytochemical and biological studies of bryophytes", *Phytochem.* Vol. 91, pp. 52-80.
2. Awasthi V, Nath V and Asthana AK (2010b), "*In vitro* propagation of the endemic and threatened Indian liverwort: *Cryptomitrium himalayense* Kash", *Curr. Sci.,* Vol. 98, pp. 1440-1441.
3. Awasthi V, Nath V, Pande N and Asthana A K (2011), "*In vitro* study on growth and gametangial induction in the male clone of *Marchantia papillata* Raddi subsp. *grossibarba* (Steph.) Bischl", *Int. J. Plant Repro. Biol*., Vol. 3, No. 2, pp. 99–104.
4. Chopra RN and Sood S (1973), "*In vitro* studies in Marchantiales. I. Effects of some carbohydrates, agar, pH, light and growth regulators on the growth and sexuality in *Riccia crystalline", Phytomorpho*., Vol. 23, pp. 230-244.

5. Cvetic T, Sabovljevic M, Sabovljevic A and Grubisic D (2005), "*In vitro* culture and apogamy - alternative pathway in life cycle of the moss *Amblystegium serpens* (Amblystegiaceae)", *Arch. Biol. Sci.,* Vol. 57, No. 3, pp. 267-272.

6. Dixon R A (1985), "Isolation and maintenance of callus and cell suspension cultures", In: Dixon R A (ed.), *Plant cell culture, a practical approach*. IRL Press, Oxford, Washington DC, pp. 1-21.

7. Duckett J G, Burch J, Fletcher P W, Matcham H W, Read D J, Russel A J and Pressel S (2004), "*In vitro* cultivation of bryophytes: a review of practicalities, problems, progress and promise", *J. Bryol.,* Vol. 26, pp. 3-20.

8. Ducos J P, Lambot C and Petiard V (2007), "Bioreactors for coffee mass propagation by somatic embryogenesis", *Int. J. Plant Develo. Biol*. ,Vol. 1, No. 1, pp. 1-12.

9. Etienne H and Berthouly M (2002), "Temporary immersion systems in plant micropropagation", *Plant Cell Tiss. Org. Cul.,* Vol. 69, pp. 215-231.

10. Gonzalez-Olmedo J L, Fundora Z, Molina L A, Abdulnour J, Desjardins Y and Escalona M (2005), "New contributions to propagation of pineapple (*Ananas comosus* L. Merr) in temporary immersion bioreactors", *In vitro Cellular Develop. Biol. Plant,* Vol.41, pp. 87-90.

11. Lara F, San Miguel E and Mazimpaka V (2006), "Mosses and other plants used in nativity sets: a sampling study in northern Spain", *J. Bryol.,* Vol. 28, pp. 374-381.

12. Liu C Z, Murch S J, Demerdash E L and Saxena P K (2004b), "*Artemisia judaica* L.: micropropagation and antioxidant activity", *J. Biotech*., Vol. 110, pp. 63-71.

13. Morais R M S C and Becker H (1991), "Growth and secondary product formation of *in vitro* cultures from the liverwort *Reboulia hemisphaerica*", *Zeitschrift für Naturforschung.,* Vol. 46c, pp. 28-32.

14. Remesh M and Manju C N (2009), "Ethnobryological notes from Western Ghats, India", *The Bryol.,* Vol. 112, No. 3, pp. 532-537.

15. Roels S, Escalona M, Cejas I, Noceda C, Rodriguez R, Canal M J, Sandoval J and Debergh P (2005), "Optimization of plantain (*Musa* AAB) micropropagation by temporary immersion system", *Plant Cell Tiss. Org. Cult. Vol.* 82, pp. 57-66.

16. Rowntree J K, Pressel S, Ramsay M M, Sabovljevic A and Sabovljevic M (2011), " *In vitro* conservation of European bryophytes", *In vitro Cell. Develop. Biol. Plant*, Vol. 1, No. 47, pp. 55-64.

17. Saxena P K (2001), "Preface to special issue on in vitro culture of medicinal plants", *Plant Cell Tiss. Org. Cult*., Vol. 62, pp. 167-171.

18. Servettaz S C (1913), "Reserches experimentales sur le development et la nutrition des mousses en milieux sterilise", *Ann. Sci. Nat. Bot. Biol. Veg.* Vol. 17, pp. 111-223.

19. Takeda R and Katoh K (1981), "Growth and sesquiterpenoid production by *Calypogeia granulata* Inoue. cells in suspension culture", *Planta*., Vol. 151, pp. 525-530.

20. Takio S, Akita C, Ngumi V W and Takami S

(1990), "Photosynthetic ability of dark-grown *Reboulia hemisphaerica* and *Barbula unguiculata* cells in suspension culture", *Plant Cell Repo.*, Vol. 8, pp. 575-578.

21. Vujicic M, Sabovljevic A and Sabovljevic M (2009), "Axenically culturing the bryophytes: a case study of the moss *Dicranum scoparium* Hedw. (Dicranaceae, Bryophyta)", *Botan. Serb.,* Vol. 33, No. 2, pp. 137-14.

International Journal of Life Sciences Biotechnology and Pharma Research
Hyderabad, INDIA. Ph: +91-09441351700, 09059645577
E-mail: editorijlbpr@gmail.com or editor@ijlbpr.com
Website: www.ijlbpr.com

ISSN 2250-3137