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Liquid Culture System Stimulates *in Vitro* Growth and Shoot Multiplication in Four Medicinally Important Plants

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ABSTRACT

Agar is the most widely used gelling agent, and accounts for 10-20% of the cost of the culture medium. Besides this, agar can contain impurities leading to inconsistent responses. We show, in this study, that the *in vitro* propagation in liquid medium is an effective means for the establishment of cultures of some medicinally important plants of Aravallis in Rajasthan, so as to achieve stimulated growth and shoot multiplication. The shoots of *Chlorophytum borivilianum* grown in liquid culture medium supported by glass beads showed a much higher rate of multiplication (4.75-fold) than the control (solid medium). In the case of *Celastrus paniculatus*, a 7-fold rate of shoot multiplication was achieved on liquid medium compared to a 4.5-fold rate on 0.8 % agar-gelled solid medium. We could stimulate shoot growth with good leaf expansion in *Terminalia bellerica* and double the rate of shoot multiplication in *Boswellia serrata* as compared to control. In all plant species, the growth of shoots was improved on liquid medium more than on solid medium. Glass beads provided mechanical support to the multiplying shoots. No adverse effects such as hyperhydricity were observed in any of the cases under investigation and plants could be acclimatized easily.

Keywords: chlorophyll contents, fresh and dry weight, glass beads, solid medium
Abbreviations: BAP, 6-benzylaminopurine; **LM**, liquid medium; **NAA**, naphthalene-3-acetic acid; **SM**, solid medium

INTRODUCTION

Agar has become the most widely used gelling agent for plant tissue culture media since its first use by White (1939). The stability, high clarity, non-toxic nature and resistance to metabolism of agar has popularized its use during culture (McLachan 1985; Henderson and Kinnersley 1988). Several investigations have, however, raised doubts about its biological inertness and non-toxic nature (Babbar and Jain 1988). The number of reports on adverse effects of agar has been published including batch-to-batch variability, inhibition of growth, presence of impurities and impartment and impairment of hyperhydricity (Romberger and Tabor 1971; Debergh *et al.* 1981; Debergh 1983; reviewed extensively by Cameron 2008). Besides these, *in vitro* cultivation of plants on agar-gelled, solid media requires labour-intensive steps including repeated subculturing. Moreover, agar accounts for 10-20% of the cost of the culture medium (**Table 1**). To overcome these drawbacks of agar, it has become necessary to search for alternatives. One such approach is to use recent propagation techniques like temporary immersion systems or liquid culture system (Nandwani *et al.* 2004). Recently, methods of *in vitro* propagation in liquid medium have been attempted (Varshney *et al.* 2000; Aggarwal and Barna 2004; Cao *et al*. 2006). The use of liquid medium in tissue culture is often described as a means of reducing the cost of micropropagation (Alvard *et al*. 1992). The advantages include increased availability of water and dissolved substances to the explant and lower labour and production cost (Gupta and Timmis 2005; Mehrotra *et al*. 2007). However, liquid media provide a substantially different environment for the plantlets and its widespread use is hampered by several problems including hyperhydricity of the tissue, rapid spread of contaminants, plantlet asphyxiation and altered geotropy.

Notwithstanding the above problems, liquid culture system has been widely employed in a number of cases (Sood *et al*. 2000; Kim *et al*. 2003; Prathanturarug *et al*. 2005; Prasad and Gupta 2006; Maxwell *et al*. 2007).

Table 1 Comparative cost analysis of various agar brands used in tissue culture media.

Agar brand	Cost per Kg* (Indian Rupees)
Sigma	13,450/-
Merck	3,560/-
HIMEDIA	2,816/-
CDH	2,800/-

* Based on price list 2007-2008.

In the present communication, we report the stimulatory effects of liquid media supported by glass beads on the *in vitro* growth and shoot multiplication of four medicinally important plant species of Aravallis, Rajasthan: *Chlorophytum borivilianum* (Liliaceae), *Celastrus paniculatus* (Celastraceae), *Terminalia bellerica* (Combretaceae) and *Boswellia serrata* (Burseraceae).

MATERIALS AND METHODS

Establishment of *in vitro* culture

1. Chlorophytum borivilianum

C. borivilianum plants were collected from natural populations in Aravallis, Rajasthan along with root tubers to serve as explant sources. *In vitro* cultures were initiated from stem discs possessing shoot buds and young shoot bases as per the method described by Purohit *et al.* (1994). Explants were treated with sodium hypochlorite (1% (v/v) active chlorine) and 2-3 drops of Tween-20 (Loba Chemicals Company, India) for 15-20 min followed by surface disinfestation with autoclaved 0.1% (w/v) aqueous mercuric chloride solution for 7 min and rinsed several times with autoclaved double distilled water before inoculation. Surface sterilized ex-

Received: 30 April, 2008. Accepted: 19 September, 2008.

Original Research Paper

plants were inoculated on MS (Murashige and Skoog 1962) medium containing 5.0 mg l^{-1} BAP (6-benzylaminopurine; HIMEDIA, India) and supplemented with 0.8% agar (HIMEDIA, India) and 3.0% sucrose. The proliferated shoots were transferred without separating them into clusters for multiplication on the same fresh medium at every 21 days.

2. Celastrus paniculatus

Cultures were established using method developed in this laboratory and described by Bilochi (2001). Nodal explants obtained from mature field-grown plants of *C. paniculatus* were used to establish *in vitro* shoot cultures. The explants were washed in running tap water for about 30 min followed by treatment with 1.0% sodium hypochlorite for 10 min and subsequently washed with autoclaved double distilled water 4-5 times. The explants were surface sterilized with autoclaved solution of 0.1% $HgCl_2$ (w/v) for 10 min under a Laminar Flow Clean Air Bench. After 4-5 times thorough washing with autoclaved double distilled water, the nodal explants were placed vertically on the medium in culture tubes (25 × 150 mm, Borosil make) containing *ca*. 17 ml medium. The medium contained MS salts, 30 g l^{-1} sucrose, 0.5 mg l^{-1} BAP and gelled with 0.8% agar. The proliferated shoots along with mother explants and without separating them into clusters were further subcultured on the same fresh medium at every 21 days.

3. Terminalia bellerica

Fruits were collected from sexually mature field-grown *T. bellerica* plants to obtain seeds. After removing the seed coat, seeds were sterilized with autoclaved solution of 0.15% $HgCl_2$ (w/v) for 10 min and washed thoroughly with autoclaved double distilled water. Seeds were aseptically inoculated on 0.8% water agar and kept in dark for germination. Cotyledonary and epicotyledonary nodes obtained from 15-d-old seedlings were inoculated on medium containing SH salts (Schenk and Hildebrandt 1972) and 1.5 mg l^{-1} BAP. Shoots started to proliferate within 21 days. After their initial proliferation on SH medium containing 1.5 mg l^{-1} BAP shoots were further subcultured on MS medium containing 1.5 mg l^{-1} BAP at every 21 days without separating them into clusters as per the method described by Rathore *et al*. (2008).

4. Boswellia serrata

In vitro shoot cultures were established using cotyledonary node explants obtained from 15-d-old aseptically grown seedlings of *B. serrata.* Seeds were surface sterilized with autoclaved solution of 0.2% $HgCl_2$ (w/v) for 10 min under Laminar Flow Clean Air Bench. After 3-4 times thorough washing with autoclaved double distilled water the seeds were aseptically placed on MS medium containing $3/4^{th}$ strength of MS salts in culture tubes (25 × 150 mm, Borosil make) for their germination. Cotyledonary nodes obtained from 15-d-old *in vitro* grown seedlings were cultured on MS medium containing 3.0% sucrose, 0.8% agar and supplemented with 0.5 mg l^{-1} BAP and 0.05 mg l^{-1} NAA as described by Purohit *et al.* (1995). After proliferation, shoots were transferred without separating them into clusters for further multiplication on to the same fresh medium at every 15 days.

In vitro growth and shoot multiplication

In vitro multiplying shoot cultures were used as the source of explant (the inoculum) in present studies. Clusters having 4-5 shoots, harvested aseptically, were used as the explant in all the plant systems under investigation.

Aseptically harvested shoot clusters were inoculated on 35 ml of standard shoot multiplication medium of respective plant systems in 100 ml Erlenmeyer flasks (Borosil make) and stoppered with non-absorbent cotton plugs. The medium used was either gelled with 0.8% agar (solid medium) or by omitting it (liquid medium). In case of liquid medium, borosilicate glass beads (10 mm diameter locally fabricated at University Workshop) were used as support matrix. The pH of all the media was adjusted to 5.8 before autoclaving at 1.06 kg cm^{-2} for 15 min. All the cultures were maintained under controlled culture room conditions of temperature (28 ± 2°C), light (45 μmol m^{-2} s^{-1} for 16 h per day provided by white fluorescent tubes, Philips) and 50-60% air humidity. Shoot clusters were subcultured on same medium at every 3 weeks without harvesting the shoots and were maintained for 42 days under same incubation conditions. For each treatment, six replicates were used and each experiment was repeated thrice. The shoot cultures grown on both solid and liquid media were assessed and compared for their *in vitro* growth in terms of various growth parameters like rate of shoot multiplication (number of shoots produced per cluster divided by number of shoots initially inoculated), average shoot length, number of leaves per cluster, total fresh and dry weight, per cent water content and chlorophyll (Chl) contents. The fresh and dry weights were determined by weighing shoot cultures on a Top Pan Electronic Balance (Contech, India) wet and after drying overnight at 60°C temperature in a hot air oven, respectively.

The percentage water content was determined by the following formula:

$$\text{Water content (\%)} = \frac{FW - DW}{FW} \times 100$$

where, FW = fresh weight, DW = dry weight

Chl contents were determined spectrophotometrically according to Arnon's (1949) method. Leaves were ground in excess chilled 80% acetone. The homogenized mixture was filtered through filter paper. Absorbance of the filtrate was read at 645, 654 and 663 nm. The Chl contents were determined using the following formulae:

$$\text{Total Chl (mg g}^{-1}) = \frac{20.0 \times A_{645} + 8.02 \times A_{663}}{a \times 1000 \times W} \times V$$

$$\text{Chl } a \text{ (mg g}^{-1}) = \frac{12.7 \times A_{663} - 2.69 \times A_{645}}{a \times 1000 \times W} \times V$$

$$\text{Chl } b \text{ (mg g}^{-1}) = \frac{22.9 \times A_{645} - 4.68 \times A_{663}}{a \times 1000 \times W} \times V$$

where:
a = length of path of light in the cell
V = volume of the extract in ml
W = fresh weight of the sample in g.

Statistical analysis

The experiments were conducted in a completely randomized design. Data were presented as mean ± standard deviation and the difference between the culture systems (solid and liquid) was compared by the *t* test.

RESULTS AND DISCUSSION

In case of *C. borivilianum*, shoot cultures derived from stem discs of naturally growing plants, produced an average of more than 12 shoots on solid medium. The shoots attained an average height of 2.3 cm. The fresh and dry weight of each shoot cluster was 1.57 and 0.12 g, respectively as observed after 42 days of culture (**Table 2**). Significant improvement in *in vitro* growth of *C. borivilianum* was noticed on liquid culture medium. This medium showed good resultant growth in terms of shoot multiplication and elongation along with increase in total fresh and dry weight per cluster. On this medium, shoots multiplied at a rate of 4.7-fold producing more than 18 shoots per cluster. The shoots measured 3.33 cm in length. Increase in total fresh (7.16 g) and dry weight (0.376 g) with an increase in per cent water content was recorded when the shoots of *C. borivilianum* were grown on liquid medium.

The shoot cultures of *C. paniculatus* grown on agar-gelled standard shoot multiplication medium multiplied at a rate of 4.4-fold producing 22 shoots and 125 leaves per cluster. The shoots produced on this medium measured 3.0 cm in length with a total fresh weight of 1.46 g which was about 5-times the dry weight (**Tables 2, 3**). The cultures showed nearly 81% water content. A significant promotion in different growth parameters was observed when shoots of *C. paniculatus* were grown on liquid medium. The shoots of

Table 2 Effect of solid and liquid medium on *in vitro* growth and shoot multiplication in *C. borivilianum*, *C. paniculatus*, *T. bellerica* and *B. serrata.* (Observations recorded after 42 days of culture).

Plant	Media type	Average № of shoots	Average shoot length (cm)	Average № of leaves
C. borivilianum	SM	12.67 ± 2.07	2.33 ± 0.32	NR
	LM	18.83 ± 2.17	3.33 ± 0.21	NR
Significance (*t*-test)		**	**	**
C. paniculatus	SM	22.00 ± 1.41	3.07 ± 0.53	125.0 ± 19.33
	LM	36.67 ± 4.18	4.33 ± 0.21	231.0 ± 39.59
Significance (*t*-test)		**	**	**
T. bellerica	SM	9.67 ± 1.03	2.20 ± 0.20	44.0 ± 4.20
	LM	14.50 ± 2.35	3.80 ± 0.21	70.0 ± 5.44
Significance (*t*-test)		**	**	**
B. serrata	SM	10.0 ± 0.08	2.50 ± 0.11	55.0 ± 4.18
	LM	20.0 ± 1.41	2.80 ± 0.11	120.0 ± 4.02
Significance (*t*-test)		**	**	**

LM – liquid medium; NR – data not recorded; SM – solid medium
All values are the mean ± SD; *, ** Mean values of SM and LM of individual plants differ significantly at P ≤ 0.01 and P ≤ 0.05, respectively
NS – Mean values not differ significantly

Table 3 Effect of solid and liquid medium on some physiological parameters of *C. borivilianum*, *C. paniculatus*, *T. bellerica* and *B. serrata* during *in vitro* growth and shoot multiplication. (Observations recorded after 42 days of culture)

Plant	Media type	Fresh weight (g)	Dry weight (g)	Water content (%)
C. borivilianum	SM	1.57 ± 0.51	0.12 ± 0.02	92.00
	LM	7.16 ± 2.53	0.37 ± 0.08	94.00
Significance (*t*-test)		**	**	
C. paniculatus	SM	1.46 ± 0.36	0.27 ± 0.05	81.30
	LM	4.41 ± 0.34	0.48 ± 0.05	89.04
Significance (*t*-test)		**	**	
T. bellerica	SM	1.56 ± 0.04	0.24 ± 0.02	84.00
	LM	3.39 ± 0.59	0.34 ± 0.06	90.00
Significance (*t*-test)		**	*	
B. serrata	SM	2.42 ± 0.15	0.24 ± 0.01	90.00
	LM	6.77 ± 0.23	0.49 ± 0.03	92.70
Significance (*t*-test)		**	**	

LM – liquid medium; NR – data not recorded; SM – solid medium
All values are the mean ± SD; *, ** Mean values of SM and LM of individual plants differ significantly at P ≤ 0.01 and P ≤ 0.05, respectively
NS – Mean values not differ significantly

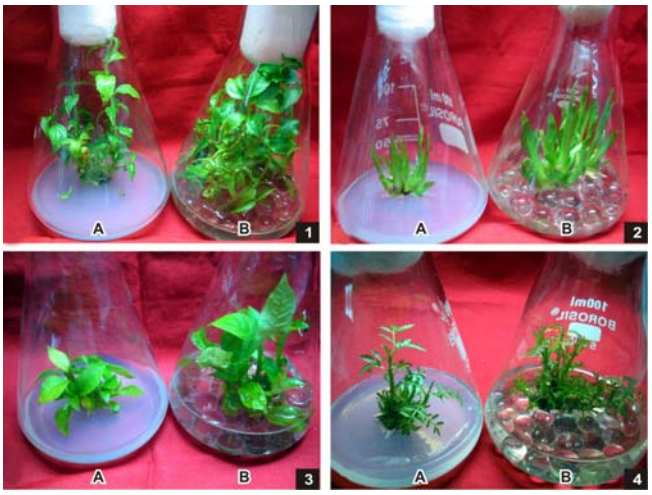

Fig. 1 *In vitro* growth and shoot multiplication in *C. paniculatus* (**1**), *C. borivilianum* (**2**), *T. bellerica* (**3**) and *B. serrata* (**4**) on solid (A) and liquid (B) medium.

C. paniculatus multiplied at a rate of 7.3-fold on liquid medium as observed after 42 days of culture. During this period more than 36 shoots were produced per cluster with an average length of 4.33 cm. A considerable increase in number of leaves (231 leaves) per cluster was recorded. The total fresh weight (4.41 g) per cluster was 9-times higher than the dry matter (0.48 g). The total fresh and dry biomass produced on liquid medium was 3 and 1.77 times higher, than the fresh and dry mass recorded on agar-gelled solid medium, respectively. An increase in per cent water content, with no hyperhydric symptoms, was observed. The leaves of shoots grown on liquid medium were dark green and broader than those observed on agar-gelled solid medium (**Fig. 1**).

Cotyledonary node derived shoot cultures of *T. bellerica* grown on solid medium multiplied at a rate of 2.4-fold producing more than 9 shoots per cluster. The shoots measured 2.2 cm in length. During multiplication, nearly 44 leaves per cluster were produced. The total fresh weight of the shoots was 1.56 g which was 6.5-times higher than the dry mass (0.24 g). The shoots grown on this medium showed 84% water content. A significant promotion of *in vitro* growth was observed when the shoots of *T. bellerica* were cultured on liquid medium. On this medium, the shoots of *T. bellerica* were multiplied at a rate of 3.6-fold which was 1.5-times higher than that was observed on solid medium. The shoots produced on this medium were more elongated measuring 3.8 cm in length. Such shoots produced more number (70 leaves) of leaves than those developed on agar-gelled solid medium. Significant increase in total fresh and dry weight per cluster was recorded when shoots were grown on liquid medium (**Table 4**). Increased shoot multiplication, number of leaves and shoot elongation on liquid medium resulted in considerable higher fresh and dry biomass per cluster. The total fresh and dry matter accumulated per cluster was 3.39 and 0.34 g, respectively. The shoots showed increment in per cent water content (90%) on liquid medium. However, none of the shoot cultures showed hyperhydric symptoms.

In the case of *B. serrata*, shoots cultured on agar-gelled solid medium multiplied with a rate of 2.5-fold where shoots attained an average height of 2.5 cm. On this medium, more than 55 leaves per cluster were recorded. The total fresh and dry weight per cluster was 2.42 and 0.24 g, respectively. The use of liquid medium in place of agar-gelled medium resulted in improvement in *in vitro* growth of *B. serrata*. On this medium, shoots multiplied at a rate of

Table 4 Effect of solid and liquid medium on chlorophyll contents of *C. borivilianum*, *C. paniculatus*, *T. bellerica* and *B. serrata* during *in vitro* growth and shoot multiplication. (Observations recorded after 42 days of culture)

Plant	Media type	Chlorophyll contents (mg/g fresh tissue)		
		Chl *a*	Chl *b*	Total Chl
C. borivilianum	SM	0.45 ± 0.02	0.32 ± 0.01	0.84 ± 0.03
	LM	0.32 ± 0.00	0.22 ± 0.02	0.58 ± 0.01
Significance (*t*-test)		*	*	**
C. paniculatus	SM	1.58 ± 0.02	1.07 ± 0.01	2.81 ± 0.05
	LM	1.62 ± 0.02	1.11 ± 0.01	2.98 ± 0.00
Significance (*t*-test)		NS	*	*
T. bellerica	SM	0.84 ± 0.20	0.64 ± 0.03	1.54 ± 0.37
	LM	0.66 ± 0.01	0.57 ± 0.03	1.27 ± 0.22
Significance (*t*-test)		**	*	NS
B. serrata	SM	0.59 ± 0.01	0.48 ± 0.02	1.02 ± 0.03
	LM	0.64 ± 0.02	0.50 ± 0.03	1.09 ± 0.03
Significance (*t*-test)		*	NS	*

LM – liquid medium; NR – data not recorded; SM – solid medium
All values are the mean ± SD *, ** – Mean values of SM and LM of individual plants differ significantly at $P \leq 0.01$and $P \leq 0.05$, respectively
NS – Mean values not differ significantly

5-fold producing 20 shoots per cluster. The shoots grown on this medium attained maximum average shoot length (2.8 cm) with considerable increase in total number of leaves (120) per cluster as compared to shoots grown on solid medium. The shoots developed on this medium produced a total fresh weight of 6.77 g, which was *ca.* 14-times higher than that of dry weight (0.49 g). However, no significant differences were observed in per cent water contents.

Besides other growth parameters, it was observed that liquid medium also affected Chl contents in *C. borivilianum*, *C. paniculatus*, *T. bellerica* and *B. serrata*. Increment in Chl *a*, Chl *b* and total Chl content was noticed when the shoots of *C. paniculatus* and *B. serrata* were grown on liquid medium as compared to the shoots grown on agar-gelled solid medium. However, higher contents of Chl *a*, Chl *b* and total Chl were obtained in the shoots of *C. borivilianum* and *T. bellerica* grown on solid medium.

It is well known that one of the advantages of liquid culture system lies in the higher multiplication rates when compared to agar-gelled solid medium. In the present investigation liquid medium stimulated *in vitro* growth and shoot multiplication in *C. borivilianum*, *C. paniculatus*, *T. bellerica*, and *B. serrata*. In the present case, the shoots of *C. borivilianum* multiplied at 2-times higher rate on liquid medium than on agar-gelled medium. Similarly, on this medium 2.0, 2- and 1.5-times higher shoot multiplication rates were observed in *C. paniculatus*, *B. serrata* and *T. bellerica*, respectively, as compared to the agar-gelled medium. Besides shoot multiplication, the liquid medium also promoted shoot elongation and accumulation of total fresh and dry weight per cluster. The shoots grown on this medium had more number of leaves with larger leaf area and expanded leaf lamina than the leaves produced on the shoots grown on solid medium. Rapid micropropagation using liquid culture system has been reported in several cases (Kim *et al*. 2003; Han *et al*. 2004; Parthanturarug *et al.* 2005; Wawrosch *et al.* 2005; Maxwell *et al*. 2007).

The increased *in vitro* growth and shoot multiplication on liquid medium, as observed in the present case, may be explained by the fact that shoots were bathed in nutrients, presenting a larger surface area for the rapid uptake of nutrients by cells and speedy nutrients replacement at the cell surface by diffusion and movement from outlying liquid (Gupta and Timmis 2005). It is always associated with the rapid intake of the growth regulators like cytokinins present in the medium to the cells, resulting in the formation of new buds and shoot elongation (Smith and Spomer 1994; Sandal *et al*. 2001). However, in agar-gelled medium this is a slow process, generating gradients of concentration for each nutrient in the zone of gel next to the cells, and slowing growth. In contrast to other reports (Le Roux and van Staden 1991; Debergh *et al.* 1981; Ziv *et al.* 1983), no abnormality such as hyperhydricity and fasciated shoots was observed on liquid medium in any of the plant systems under investigation.

ACKNOWLEDGEMENTS

The financial support provided by Council of Scientific and Industrial Research (CSIR), New Delhi, India to two authors (SV and MSR) is gratefully acknowledged.

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