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A liquid culture system for shoot proliferation and analysis of pharmaceutically active constituents of *Catharanthus roseus* (L.) G. Don

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Abstract An efficient and cost effective micropropagation protocol using liquid medium was developed for *Catharanthus roseus,* a commercially important medicinal plant. Comparative analysis of shoot growth and proliferation in liquid Murashige and Skoog (MS) medium supplemented with different concentrations of cytokinins [6-Benzyladenine (BA), Kinetin (KN) and Thidiazuron (TDZ)] was conducted. Better response in terms of shoot proliferation, shoot diameter, number of leaves/shoot, number of branches/shoot, fresh weight and dry weight was observed in a liquid medium vis-à-vis solid medium. A sample of 20 ml of liquid medium supplemented with 5 μM of BA was optimized for propagation of *C. roseus* by a liquid culture system. Among various concentrations of auxins tried, 1-Naphthaleneacetic acid (NAA) 5 μM was found to be the best for root induction. Quantification of pharmaceutically important constituents (vincristine and vinblastine) and total alkaloid content of microshoots grown in solid and liquid medium as well as in vitro raised plants and mother plant was also conducted, hitherto unreported in this high-value medicinal plant. This work further lays the foundations for the shifting of plant production from small to commercial scale.

Keywords *Catharanthus roseus* · Liquid culture · Shoot multiplication · Rooting · Vincristine · Vinblastine

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Introduction

Catharanthus roseus (L.) G. Don, commonly known as Madagascar periwinkle, belongs to the family Apocynaceae and is distributed worldwide. The plant possesses 130 terpenoid indole alkaloids (TIAs) which are associated with anti-inflamatory, antimalarial, antihyperglycemic, antihypertensive, antihypercholesterolemic, antimutagenic, cardiotonic and antitumor properties. Natural dimeric TIAs, vinblastine and vincristine, are used in the treatment of Hodgkin disease and leukaemia and are expensive (€1,000,000–3,500,000 per kg) chemicals to manufacture due to their complex structure and difficulties in their chemical synthesis (Hedhili et al. 2006). At present, the plant also enjoys the status of a model medicinal plant due to the considerable advancement in understanding its underlying physiological and molecular mechanism of secondary metabolism (Mishra et al. 2001; Pomahacova et al. 2009).

A growing realization of mass multiplication of plants in vitro and the shifting of production from small to commercial scale has forced many researchers to think about important issues such as cost efficiency, higher productivity, automation and control, and optimization of the microenvironment. Hence, it is imperative to bring about further improvements in the existing tissue culture protocols for different plants. The introduction of a liquid culture system for in vitro mass propagation helps in the substantial reduction of plantlet production costs (Sandal et al. 2001) and is an important step towards automation (Aitken-Christie et al. 1995). Further, a liquid medium offers other advantages in the form of uniform culturing conditions, renewal of the media without changing the container, sterilization by microfilteration, and ease of cleaning containers. However, several reports indicate that

a liquid culture system promotes hyperhydricity (Aitken-Christie et al. 1995; Deterz et al. 1994) and is limited by a low oxygen content (Smith and Spomer 1995). Thus, optimization of protocols to circumvent these limitations and produce tissue-cultured plants in a cost effective manner is much warranted.

Although there are a few reports for in vitro propagation of *C. roseus* (Decendit et al. 1993; Hirata et al. 1990; Endo et al. 1987; Pietrosiuk et al. 2007; Zahid et al. 2010), there has been no study conducted on the comprehensive assessment of the potential of a liquid culture system for its mass propagation. Moreover, no information is available on the effect of a liquid culture system on alkaloid accumulation in this plant. Perusal of the literature indicates that pharmacologically active constituents, vinblastine and vincristine, are produced in trace amounts by the plant (Noble 1990), and there is an urgent need to develop strategies for the consistent production of improved quality plant material on a large scale for industrial and research applications. To address these challenges, the present work focuses on the study of various parameters associated with the development of an efficient liquid culture system for in vitro mass propagation of *C. roseus* and evaluates the status of pharmaceutically important constituents during various stages of micropropagation.

Materials and methods

Establishment of cultures

C. roseus (L.) G. Don plants used in the present study were collected from the experimental garden of Guru Nanak Dev University, Amritsar. Nodal segments (length, 9.0–10.0 mm; diameter, 1.0–2.0 mm) from actively growing branches of a selected plant were collected and used for inoculation. The nodal segments taken from field-grown plants were properly cleaned with Tween-80 using a sable hairbrush. Pre-sterilization was carried out with 0.4% (v/v) solution of sodium hypochlorite containing a drop of Tween-80 for 25 min on a gyratory shaker. Finally, surface sterilization of explants was done in a laminar flow cabinet with 0.04% (w/v) mercuric chloride solution containing a drop of Tween-80 for 7–8 min and, thereafter, washed repeatedly with sterile distilled water to remove all traces of sterilizing agents. Sterilized nodal explants were inoculated into different media. Cultures were incubated at photosynthetic photon flux density (PPFD) at 20 ± 5 μmol m^{-2} s^{-1} from cool white fluorescent lamps at 25 ± 2°C. A photoperiod of 14 h was maintained on a 24-h light/dark cycle.

Shoot proliferation

Effect of different concentrations of cytokinins on various growth parameters

In order to optimize the type and concentration of cytokinins for growth and shoot proliferation, liquid Murashige and Skoog (MS) medium containing sucrose (3.0%) supplemented with different concentrations of cytokinins viz. 6-Benzyladenine (BA) (0–15.0 μM), Kinetin (KN) (0–15.0 μM) and Thidiazuron (TDZ) (0–10.0 μM) were tested in glass jars (Kasablanka, Mumbai; 350 ml capacity). The growth parameters such as shoots produced per explant, shoot length and shoot diameter were recorded after 4 weeks of culture and analyzed statistically.

Comparative response of shoot proliferation in solid and liquid media

The comparative increments in shoot multiplication rates in both solid [Agar (0.8%) and Phytagel (0.25%)] and liquid basal MS medium supplemented with BA (5.0 μM) was recorded with respect to the initial explants in conical flask (Borosilicate Glass, Mumbai; 250 ml). Different growth parameters such as shoot length, shoot diameter, number of leaves/shoot, number of branches/shoot, fresh weight and dry weight (60°C) were recorded. Observations regarding shoot multiplication were recorded after 4 weeks of culture.

Optimization of liquid medium and shoot proliferation

In order to optimize the liquid medium required for growth and shoot proliferation, different volumes of basal liquid MS medium (10, 20, 30 and 40 ml) supplemented with BA (5.0 μM) and sucrose (3.0%) were tested in glass jars (Kasablanka; 350 ml capacity). Mean number of shoots produced per explant were recorded at regular intervals of 7, 14, 21 and 28 days.

In vitro rooting

For in vitro rooting of microshoots, multiple shoots raised in liquid culture were used. Microshoots of 2–4 cm length were inoculated in basal liquid MS medium containing sucrose (3.0%) and supplemented with Indole-3-acetic acid (IAA) (0–15.0 μM), Indole-3-butyric acid (IBA) (0–15.0 μM) and 1-Naphthaleneacetic acid (NAA) (0–10.0 μM). Various parameters like root induction period, root length and branching of root were recorded after 4 weeks of culture. The type and concentration of auxin optimized for rooting were used subsequently to

induce rooting in solid and liquid medium. The effect of the status of the medium on rooting performance was recorded after 4 weeks of culture.

Hardening

After 3 weeks, the rooted microshoots were transferred to pots containing a soil mix of sand:garden soil :: 1:1. The plantlets were initially covered with polythene bags and kept in the culture room at 25 ± 2°C under continuous light. After 7 days, the polythene bags were removed initially for a brief duration (to start with, 1 h only) and then reinserted. The exposure of the plants without polythene bags was increased day by day until they were eventually removed at the end of 20 days. The plants were then transferred to earthen pots containing only garden soil and kept in the greenhouse under high humidity for further hardening.

Analysis of pharmaceutically important constituents

For analysis of pharmaceutically important constituents such as vincristine, vinblastine and total alkaloids, multiple shoots grown in solid as well as liquid medium, in vitro-raised hardened plant, and mother plant were considered. The leaf sample (2 g) was harvested from these sources and subjected to alkaloid analysis. Initially the samples were dried under shade at room temperature (18°C) and then powdered using a pestle and mortar. Later on, they were defatted with n-hexane and then extracted with methanol in a soxhlet apparatus (Jain Scientific Glass Works, Ambala, Haryana, India) for 24 h. For quantification, these extracts were evaporated to dryness, dissolved in 1 ml of methanol, clarified using Millipore filter (0.22 μm) and subjected to HPLC analysis (Perkin-Elmer, UK). For both vincristine and vinblastin, HPLC was done at a flow rate of 1.0 ml min^{-1} employing LC_2 chromatographic column (actylsilane chemically bonded to porous silica particles, 5–10 μm in diameter). In the case of vincristine the detection was done at 297 nm using the mixture of methanol:1.5% v/v diethylamine (70:30) as elution solvent whereas, for vinblastine detection was performed at 262 nm using the mixture of methanol:1.5% v/v diethylamine acetonitrile (50:38:12) as elution solvent. In both cases, the solvent pH was adjusted to 7.0 using phosphoric acid. For quantitative analysis, peak areas were used to calculate the amount of vincristine and vinblastine. For total alkaloidal assay, comparative HPLC using working standards of indole alkaloids was used. Standard samples of vincristine and vinblastine were used to construct a calibrated graph plotting peak area versus amounts of vincristine and vinblastine.

Data analysis

The analysis was performed in triplicate (each containing 5 replicates) and values were presented as mean with their corresponding standard errors. Data were subjected to one-way analysis of variance (ANOVA) and means were compared using Tukey's HSD at significance level of $P < 0.05$ using Sigma Stat 3.5. However, to analyse the effect of volume of liquid medium and intervals of subculture on rate of shoot proliferation, two-way analysis of variance (ANOVA) using Tukey's HSD at a significance level of $P < 0.05$ was performed.

Results

Shoot proliferation

Effect of different concentrations of cytokinins on various growth parameters

Different cytokinins such as BA, KN and TDZ were tested for shoot proliferation. The intermediate concentration of different cytokinins such as BA (2.5, 5.0 μM), KN (5.0 μM) and TDZ (2.5 μM) were found to be most favorable for different growth parameters. Among different cytokinins used for shoot proliferation, TDZ (2.5), BA (2.5) and BA (5.0) were found to be significantly higher compared to control and other treatments. Upon statistical analysis, it was also found that the response of TDZ (2.5 μM) was at par with BA (5 μM) (Table 1). However, at a higher concentration of BA (15.0 μM), a sign of vitrification was clearly evident. A quantitative response of different cytokinins on shoot length and shoot diameter were also recorded (Table 1). The shoots treated with different cytokinins resulted in significant enhancement in their length and diameter as compared to the control. The result indicates that the lower concentrations of cytokinins have a promoting effect on shoot length; however, the higher concentrations were found to be associated with hyperhydricity. Although the number of shoots produced per explant was highest at TDZ (2.5 μM), the shoots were shorter and thinner as compared to shoots grown in medium containing BA (5.0 μM). Moreover, due to the higher cost of TDZ, BA (5.0 μM) was preferred for the subsequent experimentation.

Comparative response of shoot proliferation in solid and liquid medium

A comparative performance of shoot proliferation in solid [Agar (0.8%) and Phytagel (0.25%)] and liquid medium was recorded. After 4 weeks of subculture period, the

Table 1 Comparative response of different growth parameters of microshoots of *Catharanthus roseus* grown in liquid MS medium supplemented with different concentrations of cytokinins

Concentration (μM)	Mean number of shoots per explant	Shoot length (cm)	Shoot diameter (cm)
Control	1.73 ± 0.39 j	1.82 ± 0.22 l	0.11 ± 0.01 i
BA (2.5)	7.62 ± 1.20 a	5.58 ± 0.86 a	0.23 ± 0.01 e
BA (5.0)	7.87 ± 1.71 a	3.50 ± 0.21 e,g	0.26 ± 0.02 d
BA (10.0)	3.87 ± 0.83 d,e,f,g	4.46 ± 0.81 b	0.43 ± 0.05 a
BA[a] (15.0)	0.00 ± 0.00 k	3.84 ± 0.22 c,e,f	0.40 ± 0.04 b
KN (2.5)	3.42 ± 0.47 c,e,f	2.47 ± 0.22 i	0.22 ± 0.03 e
KN (5.0)	4.55 ± 0.75 b,e	4.10 ± 0.27 d,e	0.20 ± 0.04 f
KN (10.0)	2.77 ± 0.40 f,h,j	4.26 ± 0.30 b,c,d	0.14 ± 0.02 h
KN (15.0)	2.94 ± 0.24 f,i,j	4.40 ± 0.23 b,c,d	0.15 ± 0.02 g
TDZ (1.0)	4.67 ± 0.27 b,c,d	3.10 ± 0.36 g,h,i,j	0.20 ± 0.03 f
TDZ (2.5)	8.33 ± 0.88 a	3.26 ± 0.58 c,g,h	0.16 ± 0.03 g
TDZ (5.0)	5.53 ± 0.29 b	2.90 ± 0.48 g,h,i,j	0.25 ± 0.02 d
TDZ (10.0)	4.07 ± 0.70 d,e,f	2.64 ± 0.29 h,i,k	0.28 ± 0.02 c

Data recorded after 4 weeks of culture; results are mean ± SE of three independent experiments. The same letters within a column are not significantly different (Tukey, $P < 0.05$)

[a] Vitrified shoots

liquid medium showed significantly higher shoot proliferation (7.37 ± 1.06) compared to agar gelled medium (3.86 ± 1.44). Phytagel had an intermediate response (6.53 ± 0.74). Further, the shoots subcultured in liquid medium showed significantly better response in terms of shoot length (Fig. 1a), shoot diameter, number of leaves/shoot, number of branches/shoot, fresh weight and dry weight (Table 2). Shoots subcultured in agar gelled medium showed the symptoms of chlorosis and leaf yellowing at the end of subculture.

Optimization of liquid medium and shoot proliferation

Among different volumes of liquid medium used, 20 ml medium produced a significantly higher number of shoots (Table 3). It was further observed that increasing liquid medium to 40 ml promoted hyperhydricity while reducing it to 10 ml led to desiccation of shoots within 2 weeks of subculture. When the shoot proliferation was recorded after different intervals of subculture, it was found that there was a steady increase in the number of shoots produced per explant and the optimum was attained in 3–4 weeks. Prolonging the subculture period to 6 weeks in 20 ml medium did not affect the health of the microshoots (Fig. 1b).

Cost component analysis of solid and liquid medium

The cost of liquid and solid medium was calculated based on the use of gelling agent (Agar), the volume of the medium and the subculture period. It was estimated that the introduction of liquid culture medium leads to a 39-fold cost reduction compared to agar gelled medium (Table 4). As agar constitutes about 80% of the cost of media, the elimination of agar from the media reduces the cost up to 5.2-fold of the total cost. The use of 20 ml liquid medium compared to the normally used 100 ml solid medium leads to considerable reduction in the cost (5-fold). The cost is further reduced by 1.5-fold when the subculture period is extended to 6 weeks in the case of liquid medium as compared to the normal practice for *C. roseus* of 4 weeks in solid medium. Apart from these, the extended subculture period in liquid culture also saves the cost in terms of labor.

In vitro rooting response

Among various auxins used for rooting of microshoots, NAA (5.0 μM) was found to be the best with 92.0 ± 3.74 rooting response (Fig. 1c; Table 5). It also produced a significantly higher number of roots and induced a faster rooting (12–16 days) as compared to IBA (14–21 days) and IAA (21–25 days). Further, rooting was evinced at the lower concentration of NAA (2.5 μM) unlike other auxins such as IAA and IBA where rooting was induced at 5.0 μM and at higher concentrations. However, when root length was determined for different concentrations of auxins, it was found that IBA (10 μM) showed a significant increase in the length compared to other auxins. In control, where no auxins were applied, rooting was not observed, while a prolonged incubation of microshoots in the same medium resulted in rooting and also initiation of flower bud in vitro (Fig. 1d). The use of liquid medium for rooting provided a suitable microenvironment for rooting of microshoots and reduces the cost of production. In another experiment, rooting response of the microshoots in both solid and liquid medium was compared. It was observed that the percentage rooting, root number and root length were significantly higher in the case of liquid medium (Table 6). Further, the initiation of rooting response in liquid medium was faster (12 days) than the response in agar gelled medium (21 days).

Fig. 1 Micropropagation of *Catharanthus roseus*. **a** Shoot proliferation in agar gelled and liquid medium; **b** liquid medium showing proliferation of healthy shoots; **c** rooting in liquid medium; **d** development of flower bud during in vitro rooting; **e** hardened plants in pots; **f** tissue culture raised plants showing flowering

Hardening

The employment of a liquid culture system for rooting has minimized the damage to roots while transplanting. Further, the growth of new leaves was observed after 10–12 days of transfer to soil with a high rate of survival (Fig. 1e). The flowering of the tissue culture-raised plants was observed 3 months after transfer to the greenhouse (Fig. 1f).

Analysis of pharmaceutically important constituents

Analysis of total alkaloids and other pharmaceutical important constituents such as vincristine and vinblastine

Table 2 Comparison of different growth parameters in gelled [agar (0.8%) and phytagel (0.25%)] and liquid medium supplemented with sucrose (3.0%) and BA (5.0 μM)

Parameters	Agar	Phytagel	Liquid
Mean number of shoots per explant	3.86 ± 1.44 b	6.53 ± 0.74 a	7.37 ± 1.06 a
Shoot length (cm)	1.36 ± 0.26 c	1.75 ± 0.60 b	2.30 ± 0.19 a
Shoot diameter (cm)	0.21 ± 0.15 b	0.27 ± 0.05 a	0.28 ± 0.03 a
No. of leaves/shoot	4.00 ± 0.32 b	7.25 ± 1.31 a	8.33 ± 0.76 a
No. of branches/shoot	2.25 ± 0.63 c	3.00 ± 0.82 b	4.75 ± 0.48 a
Fresh weight (% w/w)	0.16 ± 0.03 b	0.16 ± 0.04 b	0.22 ± 0.17 a
Dry weight (% w/w)	0.014 ± 0.004 b	0.014 ± 0.002 b	0.018 ± 0.005 a

Data recorded after 4 weeks; results are mean ± SE of three independent experiments. The same letters within a row are not significantly different (Tukey, $P < 0.05$)

Table 3 Mean number of shoots produced per explant in different volumes of liquid MS medium supplemented with BA (5.0 μM)

Volume of medium (ml)	Mean number of shoots per explant during different days of subculture			
	7 days	14 days	21 days	28 days
10	2.57 ± 0.50 a,q	0.00[a] ± 0.00 b,r	0.00[a] ± 0.00 b,r	0.00[a] ± 0.00 b,r
20	3.25 ± 0.60 c,p	6.87 ± 1.73 b,p	7.62 ± 1.81 a,p	7.87 ± 1.71 a,p
30	2.64 ± 0.24 c,q	3.46 ± 0.25 b,q	4.39 ± 0.40 a,q	4.59 ± 0.38 a,q
40	1.38 ± 0.12 a,r	0.00[b] ± 0.00 b,r	0.00[b] ± 0.00 b,r	0.00[b] ± 0.00 b,r

Results are mean ± SE of three independent experiments. The same letters (a,b,c) within a row are not significantly different (Tukey, $P < 0.05$) and signify the effect of days of subculture on shoot proliferation. The same letters (p,q,r) within a column are not significantly different (Tukey, $P < 0.05$) and signify the effect of volume of liquid media on shoot proliferation

[a] Desiccated shoots

[b] Vitrified shoots

Table 4 Cost component analysis in liquid culture media

Components	Fold reduction in cost
Elimination of gelling agent (agar)	5.2
Reduction in volume of medium	5
Extended subculture period	1.5
Total cost reduction	5.2 × 5 × 1.5 = 39

indicates that there is a significant increase in the total alkaloid content in tissue culture-raised plants (0.888% w/w) compared to the mother plant (0.526% w/w). However, the concentration of vincristine (0.00028% w/w) and vinblastine (0.00032% w/w) did not change much (Fig. 2). In another experiment, multiple shoots grown in agar gelled and liquid medium were analyzed for vincristine, vinblastine and the total alkaloid content. It was observed that the total alkaloid content as well as vincristine and vinblastine were less in multiple shoots grown in liquid medium as compared to the agar gelled medium. Moreover, the total alkaloid content of multiple shoots raised in agar gelled medium was 1.5 times higher than multiple shoots grown in liquid medium (Fig. 2).

Discussions

Shoot culture system is the most attractive alternative compared to callus or cell suspension culture for the production of high value alkaloids (Mitra et al. 1997; Berkov et al. 2009). Keeping this in mind, multiple shoots were raised using nodal explants and were established in a liquid culture system using different cytokinins. The complete elimination of agar, an expensive ingredient, in the multiplication medium has helped in a substantial cost reduction (5.2-fold). A similar figure in the reduction of the cost by use of liquid medium has also been calculated and reported earlier (Sandal et al. 2001). Higher shoot proliferation and better response of other growth parameters, such as shoot length, shoot diameter, number of leaves/shoot, number of branches/shoot, fresh weight and dry weight in liquid medium makes the use of liquid medium an efficient system of choice for in vitro propagation of *C. roseus*. The reason for the better performance of liquid medium and its successful use in different crops has been highlighted in the literature (Pati et al. 2005; Simões et al. 2009).

The present report is the first to suggest the effectiveness of TDZ in shoot proliferation of *C. roseus.* However,

Table 5 In vitro rooting response of microshoots transferred to liquid MS medium supplemented with different concentrations of auxins

Concentration (μM)	Rooting (%)	Root number	Root length (cm)
Control	0 ± 0.00 l	0.00 ± 0.00 m	0.00 ± 0.00 f
IBA (2.5)	0 ± 0.00 l	0.00 ± 0.00 m	0.00 ± 0.00 f
IBA (5.0)	40 ± 3.33 f,h	4.00 ± 1.00 e,h,j	0.85 ± 0.05 c
IBA (10.0)	80 ± 3.33 a,b	7.00 ± 0.41 c	1.66 ± 0.14 a
IBA (15.0)	60 ± 6.66 c,d,e	6.30 ± 0.33 c,d	1.16 ± 0.03 b
IAA (2.5)	0 ± 0.00 l	0.00 ± 0.00 m	0.00 ± 0.00 f
IAA (5.0)	20 ± 6.66 k	2.04 ± 0.05 k,l	0.24 ± 0.32 e
IAA (10.0)	50 ± 5.77 d,f,g	3.00 ± 0.08 j,k	0.36 ± 0.22 e
IAA (15.0)	0 ± 0.00 l	0.00 ± 0.00 m	0.00 ± 0.00 f
NAA (1.0)	25 ± 8.33 j,k	5.00 ± 0.00 d,e,f,g	0.32 ± 0.10 e
NAA (2.5)	50 ± 5.77 e,g,h,i	4.50 ± 0.50 f,h,i	0.55 ± 0.15 d
NAA (5.0)	92.0 ± 3.74 a	17.25 ± 1.10 a	0.91 ± 0.60 c
NAA (10.0)	75 ± 4.41 b	10.60 ± 4.84 b	0.86 ± 0.51 c

Data recorded after 3 weeks of culture; results are mean ± SE of three independent experiments. The same letters within a column are not significantly different (Tukey, $P < 0.05$)

Table 6 Comparison of the extent of root formation in solid and liquid MS medium supplemented with NAA (5.0 μM)

Parameters	Agar gelled medium	Liquid medium
Rooting (%)	44.4 ± 11.10b	96.66 ± 3.33a
Root length (cm)	1.86 ± 0.80b	2.62 ± 0.23a
Root number	10.84 ± 0.23b	15.75 ± 1.88a

Data recorded after 3 weeks of culture; results are mean ± SE of three independent experiments. Rooting was induced in 12 days in liquid and in 21 days in agar gelled medium. *Rooting (%)* indicates the number of microshoots showing rooting response expressed as a part of total of 100. The same letters within a row are not significantly different (Tukey, $P < 0.05$)

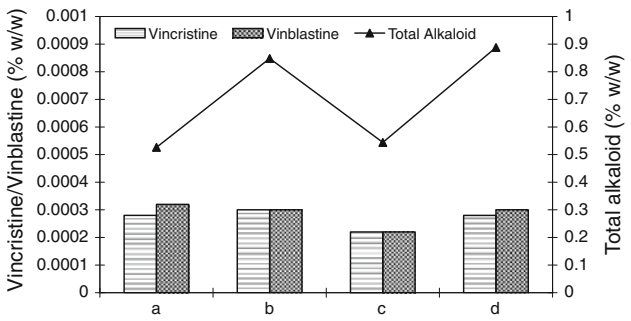

Fig. 2 Alkaloid analysis in *Catharanthous roseus.* **a** Mother plant; **b** microshoots grown in solid medium; **c** microshoots grown in liquid medium; **d** in vitro-raised hardened plant

perusal of the literature indicates the predominance of the use of BA for shoot multiplication (Endo et al. 1987; Mitra et al. 1997; Miura et al. 1988; Hirata et al. 1991; Yuan et al. 1994). Moreover, the stimulatory effect of TDZ on higher rates of axillary shoot proliferation and regeneration is well documented in the literature (Huetteman and Preece 1993; Jones et al. 2007; Yucesan et al. 2007).

The present data demonstrated that phytagel had a better response in terms of proliferation compared to agar. Agar and phytagel are natural polysaccharides with a high capability of gelation. The gels combine with water and absorb other compounds. Agar is most frequently used for solidification of plant culture media because of its desirable characteristics, such as clarity, stability, resistance to metabolism during use, and its inertness (Ibrahim 1994). Perusal of the literature indicates that the agar may chelate or adsorb nutrients ions making them unavailable to the explant, and may, depending on its source, add ionic impurities to the medium (Romberger and Tabor 1971; Debergh 1983; Conner and Meredith 1984; Bornmann and Vogelman 1984). Phytagel, the alternative gelling agent, is increasingly being used because it forms clear gels and contains no contaminants (Pierik 1987). The present response of higher number of shoots produced per explant in phytagel could be attributed to the above properties as a gelling agent.

The quantity of liquid medium was found to play an important role in shoot growth and multiplication. The lowest multiplication was recorded in 40 ml of medium, which could be due to hyperhydricity as a result of the lower availability of oxygen and submergence of shoots. Alternatively, 20 ml of medium was found to be most suitable with respect to optimal shoot proliferation as well as sustenance for up to a period of 6 weeks subculture. A similar response was also observed in the case of the rose (Chu et al. 1993; Pati et al. 2005), where a longer culture period in lower volume of liquid medium yielded best proliferation.

In the liquid medium, vitrification was clearly shown and led to pronounced elongation followed by translucence (increase of fresh weight/dry weight ratio) and eventually necrosis. In the present experiment, the fresh weight and dry weight in agar, phytagel and liquid medium were

determined. It was found that there was a proportional increase in dry and fresh weight in liquid medium. This suggests that the increase in biomass is not because of hyperhydricity and could be linked with better availability of nutrients.

Among different auxins used for in vitro rooting, NAA (5.0 μM) was found to be the best. However, perusal of the literature indicates that IBA or IAA could be effectively used for initiation of root culture in *Catharanthus roseus* (Endo et al. 1987). In the present study, the induction of rooting in liquid culture medium assumes greater significance as it saves tremendous costs by eliminating agar from the medium. Moreover, a significant difference in rooting response in liquid and agar gelled medium suggests that liquid medium is most suitable for in vitro root induction**.** In an earlier report (Pati et al. 2005), the major reason for the better response of in vitro rooting in liquid medium was attributed to the difference in osmotic potential of the two media.

The study of secondary metabolites in plant is of major interest in the areas of plant biotechnology and phytochemistry. Analysis of vincristine, vinblastine and total alkaloid of the mother plant and the tissue culture-raised plant is a step in this direction. The literature suggests that the content of pharmaceutically important constituents viz. vincristine and vinblastine in *C. roseus* is 0.00009–0.08000 and 0.0005–0.1066%, respectively (Mishra and Kumar 2000), while that of total alkaloid is 0.1–1.5% (Evans 1989). However, in the present experiment, high alkaloid accumulation was observed in in vitro-raised plants compared to the mother plant. The higher alkaloid content (0.888% w/w) of the tissue culture-raised plants substantiates the earlier report which suggests a higher amount of secondary metabolite accumulation in proliferated as well as differentiated tissue or organ (Rao and Ravishankar 2002). This finding could be ascribed to the use of various plant growth regulators (PGRs) during the micropropagation protocol (Di Cosmo and Towers 1984).

A significant finding of the present work has been the differential alkaloid accumulation in multiple shoots grown in liquid (0.544% w/w) and solid medium (0.848% w/w). The literature suggests that there is a positive correlation between alkaloid and water content of the tissue (Frischknecht et al. 1987). Smith et al. (1987) demonstrated that abscisic acid (ABA) stimulates intracellular accumulation of indole alkaloids when added to suspension cultures of *C. roseus.* Increased ABA biosynthesis in the whole plant in response to water stress is well documented (Walton 1980). Further, an experiment conducted in our laboratory where the osmotic potential of liquid and solid medium was measured clearly indicates that the availability of water is less in liquid medium (Pati et al. 2005). A similar finding was also reported earlier by Ghashghaie et al. (1991). In light of the above reports, the difference in alkaloid content of liquid and agar gelled medium in the present experiment could be easily understood.

In conclusion, the present study presents for first time a complete protocol for in vitro mass propagation of *C. roseus* in a liquid culture system and hence lays the foundations for automation of in vitro propagation of this important medicinal plant. It also highlights the enhanced metabolite production in culture conditions, which could be used to harvest vinblastine and vincristine on a commercial scale.

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