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Cost effective medium for callus initiation from *Catharanthus roseus* leaves

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ABSTRACT - *Catharanthus roseus* (L.) G. Don. is well known for the anti-cancer agents vincristine and vinblastine, and anti-hypertensive agent ajmalicine. Low yields and high production cost of these alkaloids from intact plants motivated us to establish a cost effective medium for callus initiation. Healthy and friable callus is the prerequisite for any biotechnological investigation. Leaves of *C. roseus* were incubated in MS medium supplemented with different combinations of growth hormones. MSD medium containing 2,4 D (4.52 μM)+ Kn (4.65 μM) yielded good friable callus after five weeks of incubation. To economize the process, MS medium is prepared in normal water in stead of double distilled water, sucrose (3%) is replaced by market sugar (3%) and coconut water is added in place of growth hormones. Frequency of callus initiation is slightly improved by these modifications. Modified medium responded favorably for the production of good friable callus and high biomass in medium containing 10% coconut water after five weeks of incubation was observed. Higher concentration of coconut water however produced compact and hard callus.

KEY WORDS: *Catharanthus roseus*, anti-cancer, tissue culture medium, callus, sucrose, market sugar, coconut water

INTRODUCTION

Catharanthus roseus (L) G. Don, the Madagascar periwinkle, produces several commercially valuable alkaloids including the anti-cancer compounds vincristine, vinblastine and the anti-hypertensive compound ajmalicine (1). The low yield and high market value of these compounds have made *C. roseus* as a model system for plant biotechnology. Attempts have been made to obtain high value bioactive compounds vincristine and vinblastine from cell and organ cultures of *C. roseus*, but the content was much lower than in the natural grown plants (2, 3). Several strategies have been studied for the production of monomeric and dimeric indole alkaloids by cell and organ cultures of *C. roseus* (4), optimization of nutrient media (5, 6), growth regulators (7), chemical treatment (8), elicitation (9, 10), precursor feeding (11-13) and large-scale cultivation in pilot scale bioreactor (14 -18). Due to low productivity and high production costs of these alkaloids by cultures of *C. roseus*, efforts were made to minimize the costs by addition of market sugar in stead of sucrose, normal water in place of double distilled water and coconut water in place of growth hormones.

MATERIALS AND METHODS

Explants were collected form one-year-old plant of *C. roseus*. Leaves of plant have been used for initiation of cell cultures. Explants were kept under running tap water for 30 min and then washed with 10 % Dettol (Chloroxylenol 4.8 % w/v, Terpeniol 9 % v/v, and absolute alcohol 13.1 % v/v) for five min followed by thorough washing with distilled water to remove the traces of germicidal agent. The materials were then treated with 70 % ethyl alcohol for 2 min and subsequently with mercuric chloride (0.1 % w/v) solution for 2 min. Finally, the plant materials were thoroughly washed six times with sterilized distilled water. Disinfected explants were incised into small pieces (5-8 mm) and aseptically transferred to Murashige and Skoog's (MS) (19) medium supplemented with combinations of growth hormones stated in Table 1. The medium pH was adjusted to 5.8 ± 0.02 using 0.1N HCl or 0.1N NaOH prior to addition of Agar-Agar (8 g^{-1}) and autoclaved at 121^{0}C for 20 min. Cultures were kept on culture rack at 26 ± 2°C and maintained 16 h photoperiod with white fluorescent light. Cultures were incubated for 5 weeks and the percent induction was calculated using the following equation:

$$\textit{Frequency} = \frac{\text{No. of explants showing response}}{\text{Total No. of explants}} \times 100$$

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RESULTS

Figure 1. Illustrates the percent induction of callus from *C. roseus* leaves incubated for five weeks on MS medium supplemented with different combinations of growth hormones. Maximum percent (84 %) was observed on MSD medium supplemented with 2,4 D (4.52 µM) + Kn (4.65 µM) followed by MSC medium containing 2,4 D (9.05 µM) + Kn (4.65 µM) after same period of incubation.

The increase in dry weight of callus initiated from leaf explants of *C. roseus* on MS medium supplemented with different combinations of growth hormones is

Table 1. Combinations of growth hormones for initiation of C. roseus callus

Sl. No.	Medium	Growth hormones	References
1.	MSA	2,4 D (9.05 µM)+ Kn (0.93 µM)	(20)
2.	MSB	2,4 D (9.05 µM)+ Kn (2.32 µM)	(14)
3.	MSC	2,4 D (9.05 µM)+ Kn (4.65 µM)	(20)
4.	MSD	2,4 D (4.52 µM)+ Kn (4.65 µM)	(10)
5.	MSE	NAA (0.54 µM) + BAP (6.46 µM)	(21)
6.	MSF	NAA (10.74 µM) + BA (22.19 µM)	(21)

Table 2. Influence of coconut water on % callus initiation

Coconut water (%)	MS medium in double distilled water + 3 % sucrose	MS medium in normal water + 3 % market sugar
5 %	55	58
10 %	72	80
15 %	48	56
20 %	42	50

Table 3. Comparison of medium cost

Medium component & quantity	Conventional Protocol			Modified Protocol		
	Item	Rate	Cost (Rs.)	Item	Rate	Cost (Rs.)
Nutrients (As per MS medium)	Macro-Micro Nutrients	15/ lit.	15.00	Macro-Micro Nutrients	15/ lit.	15.00
Water (1.0 lit.)	Double Distilled Water	10/lit.	10.00	**Normal Water**	0.10/ lit.	0.10
Carbohydrate source (30 g)	Sucrose	300/Kg	9.00	**Market Sugar**	18/Kg	0.54
Hormones (0.5-2.0 ppm)	Auxin/ Kinetin	2/lit	2.00	**Coconut Water**	10/lit.	0.25
TOTAL COST			36.00/lit.			15.89/lit.

exhibited in Figure 2. The callus initiation was observed on 10th day of incubation on MSA, MSB, MSC and MSD media, whereas it was on 15th day of incubation on MSE and MSF media. Maximum (124 mg) dry weight of callus was obtained on MSD medium after 30 days of incubation [Plate 1] followed by 98 mg on MSC medium after same period of incubation. Dry weight biomass of callus increased upto 30th day of incubation on MSC, MSD and MSF media and reduced thereafter on further incubation.

To economize the medium cost, MS medium was prepared in normal water instead of double distilled water, sucrose (3%) was replaced by market sugar (3%) and coconut water is used in place of growth hormones. Both medium responded favorably for callus induction in coconut water. Table (2) represents that maximum frequency of callus induction was observed at 10% coconut water in both cases. The medium prepared in normal water and containing 3% market sugar showed better callus initiation as compared to medium prepared in double distilled water with 3% sucrose. Maximum frequency of callus initiation (80%) was observed in modified medium in normal water, 3% market sugar containing 10% coconut water. However, higher concentrations of coconut water affected

Figure 1. The frequency of callus initiation from leaves of C. roseus in different medium after three weeks of incubation.

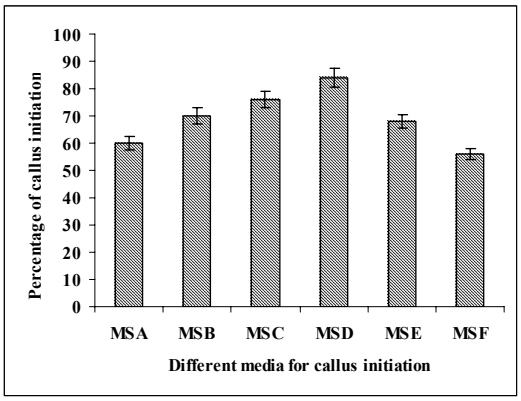
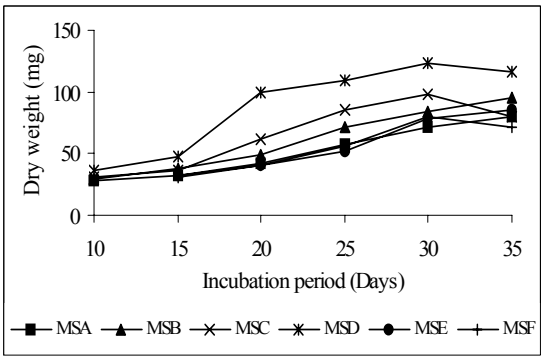


Figure 2. Increase in dry weight of callus initiated from leaf explants of C. roseus on MS medium supplemented with different combinations of growth hormones.



adversely in both cases. Callus obtained from modified medium was greenish, friable, fast growing. Thin layer chromatography (TLC) of alcoholic extract of dried callus shows the presence of similar concentrations of active constituents as showed by alcoholic extract of dried leaves of *C. roseus*. Table (3) shows the comparison between the cost of conventional medium/ protocol and the cost of modified protocol. It clearly indicates that if the cost of macro and micronutrients remains constant (Rs. 15.00), the cost of modified medium will reduce from Rs. 36.00 to Rs. 15.89, which is approximately 25 times cheaper than the conventional protocol using double distilled water, sucrose and growth hormones.

DISCUSSION

Healthy and fast growing friable callus is the prerequisite of different biotechnological investigations. Callus consists of undifferentiated masses of cells developed on a semi-solid medium. The maintenance of such cultures depends on an adequate supply of nutrients, growth hormones and controlled sterile environment. The cells, although undifferentiated, contain all the genetic information present in parent plant. By suitable manipulation of hormone and contents of the medium, it is possible to initiate the development of roots, shoots and complete plants from callus cultures (24). The nutritional requirements of plant cells and tissues vary from species to species and therefore a number of media have been devised for specific tissues by different workers (25, 26).

Sucrose is main constituent of the medium and contributes major costs of the product. Growth studies of *C. roseus* established in different concentrations of market sugar and analytical grade sucrose revealed that the cells grew uniformly for a period of 30 days and packed cell volume, fresh weight, dry weight and ajmalicine content were found maximum in medium containing 4% market sugar (27). Present investigation revealed that the growth pattern and ajmalicine production cell cultures in medium containing market sugar, normal water and coconut water were almost similar than those grown in similar concentrations of sucrose and distilled water. Cell cultures of *C. roseus* were grown in 20 l bioreactor containing Zenk's production medium with addition of market sugar and normal water produced ajmalicine that was also similar to the control medium supplemented with sucrose and distilled water (14). Similarly, large-scale cultivation of *Panax ginseng* was performed in a 2000 l bioreactor with normal water and market sugar (28). Reinhard *et al.*, (29) reported the biotransformation and production of cardenoloids in 300 l bioreactor containing medium with market sugar by *Digitalis lanata* cells.

Conclusion

The best combination (MSD) of growth hormones for maximum frequency (84%) of callus initiation was found to be 2, 4 D (4.52 μM) + Kn (4.65 μM). The cells of *C. roseus* responded favorably when grown in medium containing market sugar (3%), prepared in normal water and exposed to coconut water in place of growth hormones. However, this protocol reduced the cost of medium by about 25 times. Present work will not only motivate to work on callus initiation and further biotechnological aspects of other medicinal plants but will also provide an opportunity to conserve the endangered species through micropropagation.

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