ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Fast In-Vitro Callus Induction in Catharanthus roseus - A Medicinally Important Plant Used in Cancer Therapy

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ABSTRACT

Secondary metabolites are produced by the plants besides primary metabolites, which are not absolutely essential for their survival. These secondary metabolites provide disease and stress resistance, help in pollination to the plants. The secondary metabolites are primarily used by medical sciences to control various diseases and ailments. Catharanthus roseus commonly called Sadabahar in India, belongs to the family apocynaceae, is an ornamental plant commonly seen growing in gardens. Catharanthus roseus or Vinca rosea contains some very useful secondary metabolites falling under the category of alkaloids. V. rosea contain three such important alkaloids viz., Vincristine, Vinblastine and Vindiscline which are used in the treatment of cancer. These alkaloids interfere with the mitotic cell division process of the cancerous cells. They stop formation of microtubules and thus chromosomes are unable to arrange on metaphase plate. Various explants from the plants were in vitro cultured to find out best hormonal combinations to produce good amount of callus. This callus can be used to extract the anticancerous metabolites which are otherwise largely extracted from the field grown plants. Naturally grown plants of Vinca rosea contain very low concentrations of the alkaloids. Present work suggests a quick protocol for good amount of callus production form Vinca rosea nodal explants. Murashige and Skoog's **(**MS) medium supplemented with Kinetin and BAP each with 1.0mg/l and 2, 4-D, IAA each 1mg/l combinations showed good callus production. Similarly MS + kinetin and BAP 2mg/l each; and MS + 2, 4-D, IAA 0.5 mg/l (each) combinations showed green and resin secreting callus. MS + BAP 2.0 mg/l, 2, 4-D 1mg/l produced best light green and resin secreting callus. When the leaf explants were cultured in MS + BAP 1mg/l, NAA 1mg/l, they showed large number of root formation. When combination of MS + kinetin (0.1mg/l), 2, 4-D (1.0mg/l); and MS + BAP 0.1 mg/l, IAA 1 mg/l were used quick process of callus induction was seen.

Key words: Vinca rosea, Callus Culture, Vincristine, Vinblastine, Ajmalicine, Secondary Metabolites, Alkaloids, Anti-cancer Drug

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ISSN: 0975-8585

INTRODUCTION

Catharanthus roseus [Madagascar periwinkle] which is synonymously called Vinca rosea can be commonly seen growing in gardens. Despite having common look it has very powerful medicinal properties which are due to the alkaloids present in it. Out of the several alkaloids found in it, vincristine and vinblastine are commonly used in cancer therapy whereas ajmalicine and serpentine are used as antihypertensive and sedative compounds [1]. Large population relies on pharmaceuticals derived from plants. Via tissue culture these compounds can be produced rapidly throughout the year without depleting the natural resources. Callus production and suspension culture are the techniques used by pharmaceutical companies to produce these chemicals. Objective behind the study was to try maximum possible hormonal combinations to develop a protocol for rapid in vitro cultivation of good amount of callus from the nodal and leaf explants so that the callus could be used for alkaloids production via cell suspension culture technique.

MATERIALS AND METHODS

Plant Material

Explants were taken from garden grown plants of Vinca rosea. Young branches from healthy plants were cut and washed in running tap water. They were further washed in 1% detergent for 1-2 minutes and then washed in tap water for 6-8 times. Finally 2 washing were done using sterilized distilled water. Explants [both leaves and nodes] were collected from third node from the apex. About 1.5-2cm long nodal segments were used for culture. Similarly leaves present at third nodes were used as explants. Small part of lamina was left intact with the nodal explants while culturing [Fig. 1D, 1F].

Table 1: Response of explants of Catharanthus roseus inoculated to Murashige and Skoog's [1962] medium supplemented with various hormonal combinations

Hormone used [in mg/l] along with MS medium	Response of Leaf and Stem Explants	Degree of Response
2,4-D 0.1	Nil	-
2,4-D 0.5	Nil	-
2,4-D 0.5	Nil	-
2,4-D 1.0	Nil	-
2,4-D 2.0	Nil	-
2,4-D 2.5	Nil	-
IAA 0.1	Nil	-
IAA 0.5	Nil	-
IAA 1.0	Nil	-
IAA 2.0	White callusing	++
BAP 2.0, 2,4-D 0.5	Callusing	++
BAP 0.1, 2,4-D 1.0	Light green callusing	+++

BAP 2.0, 2,4-D 1.0	Light green callusing with resin secretion	+++
BAP 2.0, 2,4-D 1.0	Callusing	++
BAP 2.0, IAA 0.5	White and Green callusing	+++
BAP 0.1, IAA 1.0	Fast callusing with profuse rooting	+++
BAP 0.1, IBA 1.0	White callusing	++
BAP 1.0, IBA 0.1	Good green callusing	+++
BAP 2.0, IBA 0.5	White callusing	+++
BAP 0.1, NAA 1.0	Good callusing with fast rooting	+++
BAP 1.0, NAA 0.1	Green callusing	+++
BAP 2.0, NAA 0.5	Green callusing	+++
Kinetin 0.5	Green callusing	+
Kinetin 1.0	Green callusing	+
Kinetin 1.5	Only swelling of explants	+
Kinetin 2.0	Only swelling of explants	+
Kinetin 2.5	Light green callusing	+
Kinetin 0.1 , 2,4-D 1.0	Fast white callusing with resin secretion	+++
Kinetin 0.1, IAA 0.5	Light green callusing	+++
Kinetin 0.1, IAA 1.0	Callusing	++
Kinetin 0.1, IBA 1.0	Nil	-
Kinetin 1.0, IBA 0.1	Green callusing	+++
Kinetin 2.0, IBA 0.5	Feeble callusing	+
Kinetin 0.1, NAA 1.0	Nil	-
Kinetin 1.0, NAA 0.1	Green callusing	+++
Kinetin 2.0, NAA 0.5	Green callusing	+++
Kinetin 2.0, 2,4-D 0.5	Light green callusing with resin secretion	++
Kinetin 2.0, 2,4-D 1.0	Nil	-
MS Without Hormone	Nil	-

- means Nil; + symbolizes feeble response; ++ is moderate response; and +++ means good response

Basal Medium and Culture Conditions

Murashige and Skoog's [MS] [2] basal medium containing 0.8% agar and sucrose [30.0 g l^{-1}] was used during the entire experiment. The pH of the medium was adjusted to 5.8. Growth regulators were supplemented at various concentrations [Table 1]. Routinely 30ml medium was dispensed in each flask [150ml capacity], and sterilized by autoclaving. The explants were then placed horizontally over the semisolid MS medium. During the experiments, a light regime of 18 hours with 100µmol m^{-2} s^{-1} light intensity provided by cool-white fluorescent tubes at 25 ± 2ºC followed by 6 hr dark period was provided to the cultures. 10 flasks [per hormonal combination] were used for culturing explants. Phenotypic changes taking place among the cultured explants were noted down every week.

ISSN: 0975-8585

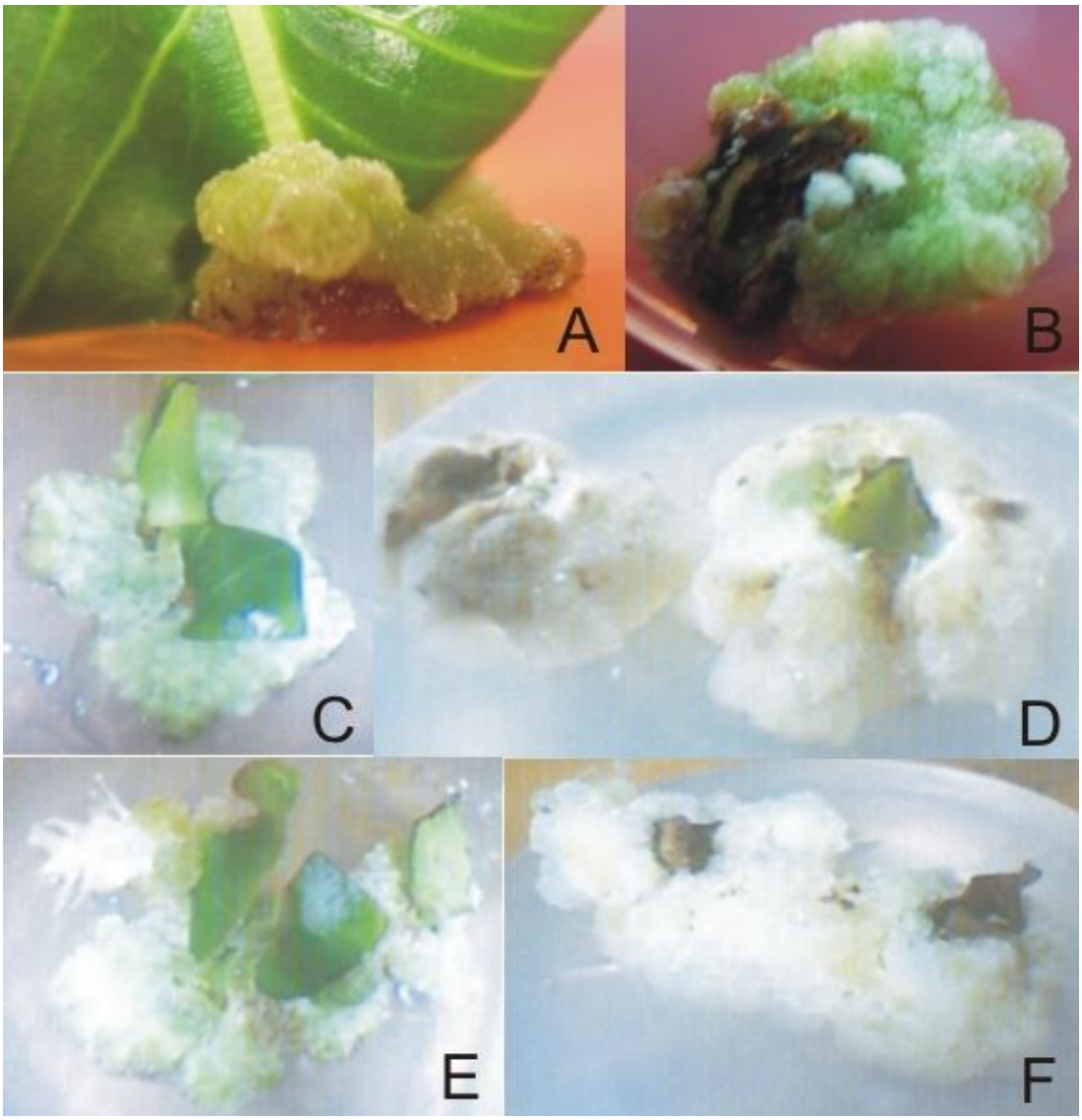

Figure 1: Callus induction in vitro cultured explants of Catharanthus roseus. A. Callus beginning from leaf surface Kn 0.1mg/l + IAA 1mg/l; **B**. Almost entire leaf gave rise to callus mass in MS + IAA 1mg/l + BAP 2mg/l; **C**. Nodal explants showing good callusing in MS + Kn 1mg/l + NAA 0.1mg/l; **D**. Two nodes entirely gave rise to callus mass in MS + BAP 2mg/l + 2,4-D 1mg/l; **E**. Nodal region showing profuse callusing and rooting in MS + BAP 0.1mg/l + IAA 1mg/l; and **F**. White callus mass generated from nodal regions of two explants in MS + Kn 0.1mg/l + 2,4-D 1mg/l, dried and brown coloured segment of lamina, initially attached with the nodal explants can be seen in the photograph.

ISSN: 0975-8585

Figure 2: Light green to yellowish friable callus produced in in vitro cultured leaf explants of Catharanthus roseus at MS medium with Kn 0.1mg/l and IAA 0.5mg/l hormonal combination.

RESULTS AND DISCUSSION

The nodal and leaf explants were selected and tried for best possible responses. Various hormonal combinations [Table 1] were tried along with the MS medium. These hormones used in the experiments included cytokinins and auxins used alone and in combinations [auxins with cytokinins]. Auxins except IAA at 2.0mg/l concentration, failed to trigger any response [Table 1]. IAA at 2mg/l concentration induced white callus in the explants. In contrast to auxins, cytokinin like kinetin alone could trigger swelling and callusing in both leaf and stem explants though at weak level. Increasing concentrations of kinetin from 0.5 to 2.5mg/l didn't have marked effect on the quantity of the callus thus produced.

BAP 0.1mg/l + 2, 4-D 1mg/l and BAP 2mg/l + 2, 4-D 1mg/l induced good amount of green callus [Fig. 1D]. Some resin droplets were seen shining over the callus surfaces in both the cases. Fast callusing with profuse rooting was observed in nodal explants cultured in MS + BAP 0.1mg/l and IAA 1mg/l combinations where callus initiation took place within 14-17 days time [Fig. 1E]. Fast callusing with resin secretion were also observed in explants cultured in MS + Kn 0.1mg/l and 2, 4-D 1mg/l [Fig. 1F]. Good amount of callus with rooting developed from nodal explants cultured in MS+BAP 0.1, NAA 1.0. Callusing was equally seen in same degrees in both stem and leaf explants [Figs. 1A-F].

Looking at the results, MS when supplemented with BAP 2mg/l and 2,4-D 1mg/l; BAP 1mg/l, IBA 0.1mg/l; Kinetin 0.1mg/l, IAA 0.5mg/l; Kinetin 1mg/l, NAA 0.1mg/l; Kinetin 2mg/l, NAA

0.5mg/l promoted generation of good amount of green callus [Figs. 1-2]. Green callus can be further used for cell suspension culture which is the base for secondary metabolite production at industrial level.

Catharanthus was found to contain a very large number of alkaloids, about 100 of which have been isolated so far [3, 4, and 5]. The root contains major alkaloids such as ajmalicine and serpentine, which are used in the treatment of circulatory diseases [6]. Besides other medicinal uses the importance of this plant is due to the presence of two bisindole antitumor alkaloids, vinblastine and vincristine. These two alkaloids can lower the number of white cells in blood. A high number of white cells in the blood are the indication for leukemia. So they are used as anti-cancer drug. Alkaloids from the Catharanthus are normally obtained from the field grown plants. It requires lots of space and infrastructure; in addition the raw material is season dependent and is affected by various fluctuating environmental risk factors. The antitumor alkaloids are produced in trace amounts [0.0003% dry weight]. Due to high prices of these anticancer products, ranging from $1 million to $3.5 million per kilogram, widespread research has been carried over the past 25 years in the development of alternative sources for the production of these compounds [7]. Tissue culture provides alternative, safe, cheap and constant methods of in vitro production of secondary metabolites.

Various workers in the past have tried in vitro culture of Catharanthus roseus. Carew and Krueger [8] reported number of as well as the growth factors such as 2, 4-D and IAA responsible for growth and alkaloid formation in Catharanthus roseus suspension cultures. They reported IAA at 0.5 and 2.0 mg/l in media produced tissue growth comparable to tissue receiving 1 mg/liter 2, 4-D. Agnieszka et al., [9] carried out in vitro culture of Catharanthus roseus for the production of secondary metabolites such as indole alkaloids and found that callus and cell suspension culture resulted into production of ajmalicine in unorganised tissue, catharanthine in the leaf and cell culture in the shake flask and vinblastine in shoots. Kalidass et al., [10] studied effect of auxin and cytokinin on vincristine production by callus cultures of Catharanthus roseus and found that MS medium supplemented with 2, 4-D [1µM] and 6-furfurylaminopurine [Kinetin] 1µM was useful to support the growth of callus cultures and the maximum amount of dry biomass [598.04 mg] was produced after seven weeks of culture. In our case also we have observed same concentrations of Kn and 2,4-D were effective in producing green callus which might be suitable for suspension culture for alkaloid production. As stated above we have reported additional combinations of auxins and cytokinins producing green callus in good amount [Table 1]. Kalidass et al., [10] also reported that the concentrations of the growth regulators such as alpha-naphthalene acetic acid [NAA] and kinetin played a critical role in the production of vincristine. They reported high content [maximum 20.38 mg/g] of vincristine in callus cultures and recommended that for further pharmacological exploitation of vincristine culture conditions of Catharanthus should be improved. In our case we found good amount of green callus in MS + Kinetin 1mg/l, NAA 0.1mg/l; and MS + Kinetin 2mg/l, NAA 0.5mg/l.

Saifullah and Khan [11] stated that for the cell suspension culture preparation MS medium along with 1.5 mg/l 2, 4-D and 0.5 mg/l Kn was best for friable callus production in Catharanthus roseus. In our case we observed MS + Kn 0.1mg/l and 2, 4-D 1mg/l triggering quick formation of white callus with resin secretion on its surface. We started this work in the year 2008, before than what few of the above workers did. In most of the cases one can see that our results matches with some of the workers mentioned here. Amount of callus produced in our case was comparatively better than some of the above workers. We got 8-17gm of callus after 2-3 months of culture. Saifullah and Khan [11] used shoot tips for callus induction but the amount of callus visible from their photographs appears far less than what we have reported here. Therefore we recommend leaf and nodal explants for quick and good amount of callus production. Additionally we can also correlate appearance of resin droplets on callus surface with alkaloid content by comparing our results with above workers' one. That means by simply observing resin droplets on callus surface one can directly comment on alkaloid producing quality of the callus.

We can conclude that leaf and nodal explants are good material for callus production. Separate hormonal combinations are responsible for production of white and green callus as discussed above. Finally by simply observing resin droplets on callus surface one can directly comment on alkaloid producing quality of the callus. Tissue culture can go a long way to produce such medicinally important alkaloids, saving both our time and money.

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