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Micropropogation and Organogenesis in *Adhatoda vasika* For The Estimation Of Vascine.

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ABSTRACT

Adhatoda vasika (Family: *Acanthaceae*) commonly known as vasaka has been used in traditional system of medicine for several respiratory tract ailments. Vasicine and vasicinone are the important alkaloids of vasaka having bronchodilatory and expectorant effect. In the present study we have tried to develop callus cultures of vasaka from leaf, petiole and nodes by using different plant growth regulator (PGR) combinations. The best PGR combination in terms of growth index, bioactive secondary metabolite content and repeatability to induce callus was determined. Secondary metabolite recovered from callus was identified using a standard sample of vasicine by chromatographic and spectroscopic techniques. MS medium prepared with 10.7µM NAA (Naphthalene acetic acid) and 2 .2µM 6BA (6 benzylaminopurine) showed 90% repeatability to induce callus with 7th day callus induction and secondary metabolite concentration of 3.2 % on gram dry weight basis. An attempt to increase secondary metabolite concentration using cell suspension culture was tried and a rise in alkaloidal content was obtained during a period of one month study. Organogenesis was established using nodes as an explant and shoots were observed after 18 days. The results of the present study revealed that the developed callus, shoot and root *invitro* cultures can be used as alternative source for production of vasaka alkaloids of pharmaceutical interest.

KEYWORDS: Callus culture, organogenesis, suspension culture and vasicine.

INTRODUCTION

Plant cell culture is viewed as a potential means of producing useful plant products so that conventional agriculture, with all its attendant problems and variables, can be circumvented. These problems include: environmental factors (drought, floods, etc.), disease, political and labour instabilities in the producing countries, uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, losses in storage and handling. Harvesting of plants can lead to its extinction from nature. Besides seasonal variations, the majority of plant based alkaloids can not be chemically synthesized. The tissue culture systems for a number of medicinal plants have been established, and this enables the synthesis of callus and cell suspension for presence of various secondary metabolites (1). Media optimization, differentiated cells, regeneration of plants, organogenesis are some of the strategies to improve secondary metabolite production through plant cell biotechnology (2).

Adhatoda vasika (Family: Acanthaceae) is an important medicinal plant found in India. It is wildly known as Adulsa and utilized in rural areas for several ailments. It is an evergreen perennial herb and grows to a height of 2m. Of these, the leaves, roots, and young plants of vasaka have been extensively investigated and the quinoline alkaloids vasicine, vasicinone, vasicinolone, vasicol, vasikoline have been reported (3, 4). In addition to this, extracts of leaves

are a commonly used medicine in India as an expectorant and a bronchodilator. It has also been claimed to be a uterine stimulant and abortifacient (5).

In the present study we have tried to develop callus cultures of vasaka from leaf and petiole by using different combinations of plant growth regulators (PGR). An attempt was made to increase the secondary metabolite using suspension culture. Secondary metabolite obtained from callus and cell suspension cultures was compared with standard vasicine. Root and shoot development through organogenesis was also carried out.

MATERIALS AND METHODS

Establishment of callus culture

Young leaves and petiole from plants of vasaka were collected from the botanical garden, S.V.B's College of Pharmacy, Dombivli (E), Thane and authenticated by Agarkar research institute, Pune. After thorough washing under running tap water and sterilized water, explants were sterilized in aseptic area with 0.3% cetrimide for 8min., 0.1% mercuric chloride for 3 min, 70% alcohol for 30 sec. and finally washed 2–3 times with sterilized distilled water. After each chemical treatment explants were washed with sterilized distilled water. For callus initiation explants were cultured on sterilized (autoclaved at 121°C and 15lbs pressure for 20 min.) solid Murashige & Skoog (MS) media (6) of pH 5.8 fortified with combinations of plant growth regulators in the ranges of 1.2–10.7 µM NAA (Naphthalene acetic acid); 2.2-4.4 µM 6BA (6 benzylaminopurine); 4.5–13.5 µM 2,4D(2, 4 dichlorophenoxy acetic acid)and 2.3–4.6 µM kinetin.

All cultures were maintained in 40 watt light with 18 hr photoperiod at 25± 2 °C. Each treatment was carried out with 10 replicates in test tubes and repeated thrice. The callus was observed for a period of 20 days and the obtained callus was estimated for the presence of vasicine after 2 months.

Establishment of suspension culture

MS media with PGR combination having highest % frequency of callus induction and highest % secondary metabolite production was selected for establishment of suspension culture. This media without agar was adjusted to pH 5.4 and autoclaved at 121°C and 15 lbs pressure for 20 min. Callus was sub-cultured in aseptic area into the sterilized liquid medium and the media was maintained in 40 watt light with 18 hr photoperiod at 25+ 2 °C and 100 rpm agitation. Two methods were developed for the study of increase in alkaloidal concentration.

1. Method A: Five identical flasks with 250 mg callus in 125 ml media were prepared. One flask each was evaluated for the alkaloidal content on 0^{th}, 7^{th}, 14^{th}, 21^{st}, & 28^{th} day of the study.

2. Method B: A single flask containing 500 mg callus in 250 ml media was prepared and maintained for a period of 35 days. Each day 2ml of the media was withdrawn and analyzed; simultaneously 2ml of fresh media was replaced into the flask.

Establishment of Organogenesis

Callus obtained as above along with fresh nodes from vasaka plant was selected as explants for shoot initiation. They were cultured on MS media fortified with auxin and cytokinin in the following ranges 5.3-13.2 µM NAA; 2.2-13.2 µM IAA (Indole acetic acid); 2.2-13.2 µM 6BA; 4.6-9.3 µM kinetin. For the development of roots; callus and the shoots obtained from the previous study was incubated in MS media containing high concentration of auxin and low concentration of cytokinin.

Isolation and purification of vasicine from vasaka leaves

Dried vasaka leaves were extracted with methanol by continuous maceration and percolation method. Then it was acidified with 2 % sulfuric acid. After removing the chloroform soluble residue acidic layer was basified with 10 % ammonia solution and the final aqueous layer was completely extracted in chloroform layer. Finally chloroform was recovered and the resulting solid was repeatedly crystalised with methanol to give long rods of vasicine crystals. The identity and purity of vasicine was confirmed by its melting point, λ_{max} and chromatographic studies (7, 8).

Estimation of vasicine

The quantitative estimation of alkaloids in the callus, suspension culture and petioles obtained by plant tissue culture technique was done by HPLC using vasicine as standard. HPLC was carried out in the mobile phase methanol: water = 2:3 with flow rate 0.7ml/min using C18 Spincotech column 5 µ(3.9mm × 15mm) and the absorbance was detected at λ_{max} 298nm. Vasicine (0.1 mg/ml) were prepared in methanol; 10 µl of which was injected into the column. The chromatograms of the methanolic extracts of the callus and standard vasicine were obtained and the relative amount of alkaloid in the callus was calculated using vasicine as standard.

Suspension culture was studied by two methods. In case of Method A whole culture was extracted in 10 ml chloroform. The chloroform extract was evaporated to dryness and 10 ml of methanol was added to it. 0.5

ml of the same was diluted to 30 ml with methanol and analyzed by HPLC. Method B was employed to study the growth curve of vasicine. Hence on each day 2 ml of suspension culture was extracted with chloroform and the solution obtained was analysed for vasicine content. The concentration of vasicine was also estimated in the methanolic extract of the petiole obtained by PTC technique.

RESULTS AND DISCUSSION

Vasicine isolated from vasaka leaves was analysed by HPLC; the standard curve of which is given in Fig. 1. The % frequency of callus initiation and the concentration of secondary metabolite in the callus of the leaf and petiole explant are presented in the Table 1. When the leaf and petiole explants were cultured on MS media supplemented with various combination of auxin and cytokinin, callus formation was observed from 7th day to 20th day (Fig. 2). Out of the two combination of media tested the frequency of callus initiation was 90% in MS media containing 10.7µM NAA and 2.2 µM 6BA (group2) with a secondary metabolite concentration of 3.24% with respect to standard vasicine. However the frequency reduced to 20% on 2.6µM NAA and 2.2 µM 6BA with secondary metabolite concentration of 1.03%. When NAA was replaced with 2, 4 D the response was observed on the 11th day with frequency of 30% and secondary metabolite concentration of 0.73% for 9.0 µM 2, 4 D and 4.4 µM 6BA media combination. All other media tested did not give appreciable alkaloidal concentration.

With an aim to increase the secondary metabolite concentration cell suspension culture was tried on MS media supplemented with 10.7µM NAA and 2.2 µM 6BA (group A) and 9.0 µM 2, 4 D and 4.4 µM 6BA (group B). The study was carried out for a period of one month. A Graphical plot for an increase in vasicine concentration

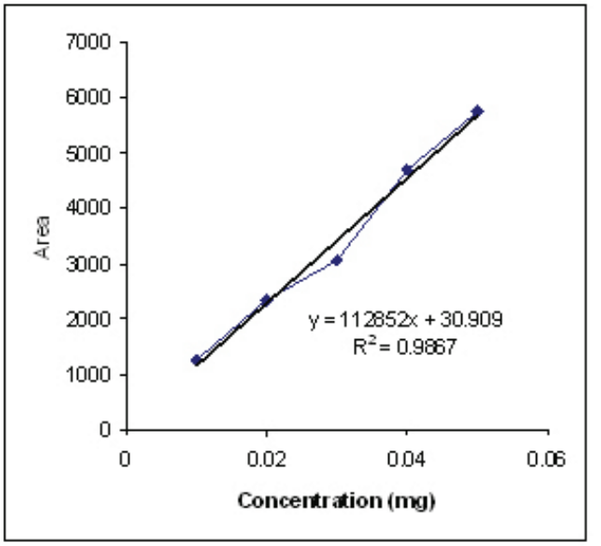
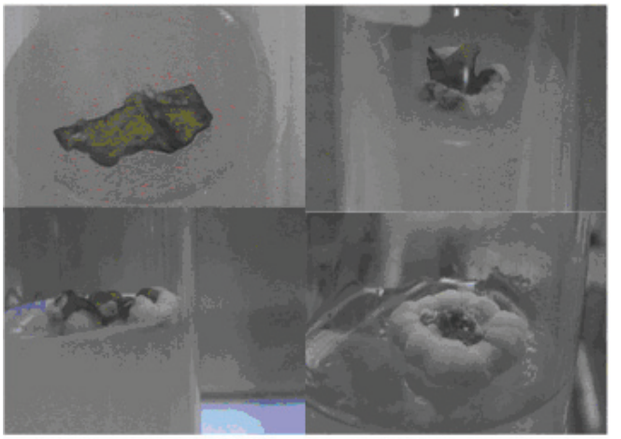


Figure 2: Stages showing callus development of vasaka plant.



a: callus initiation; b,c,d: callus development

Figure 1: Standard curve of vasicine obtained by RP-HPLC.

Table I: Effect of plant growth regulators on the leaves and petioles of vasaka plant:

		Plant growth regulator (µM)						%SM*	
Group	2,4D	NAA	6BA	Kinetin	Explant	DFCI	%FCI	HPLC	UV
1	-	2.6	2.2	-	L	7	20	1.03	1.01
2	-	10.7	2.2	-	L	7	90	3.24	3.18
3	-	1.3	4.4	-	P	12	20	0.000	0.000
4	9.0	-	4.4	-	L	11	30	0.73	0.69
5	13.5	-	4.4	-	P	9	30	0.13	0.15
6	9.0	-	8.8	-	L	11	20	0.19	0.19
7	9.0	-	2.2	-	L	20	20	0.015	0.015
8	4.5	-	-	2.3	L	15	10	0.000	0.000
9	9.0	-	-	4.6	L	14	20	0.000	0.000

DFCI: Day for callus induction, FCI: Frequency of callus induction, SM: Secondary metabolite,
*: % secondary metabolite was measured after two months.

for both the groups by Method A is shown in Fig. 3. It was observed that the vasicine content was 1.35 % & 0.875 % on 28th day as compared to 0.135 % & 0.119 % on the 0th day for Group A & Group B respectively. Method B studied the growth curve of vasicine from 1st day to 35th day through three phases (Fig. 4). Group A had lag phase from 1st to 4th day, linear exponential phase from 5th to 28th day and stationary phase from 29th day. Group B had 1st to 9th day lag phase, 10th to 25th day linear exponential phase and stationary phase 25th day onwords indicating cessation of alkaloidal production.

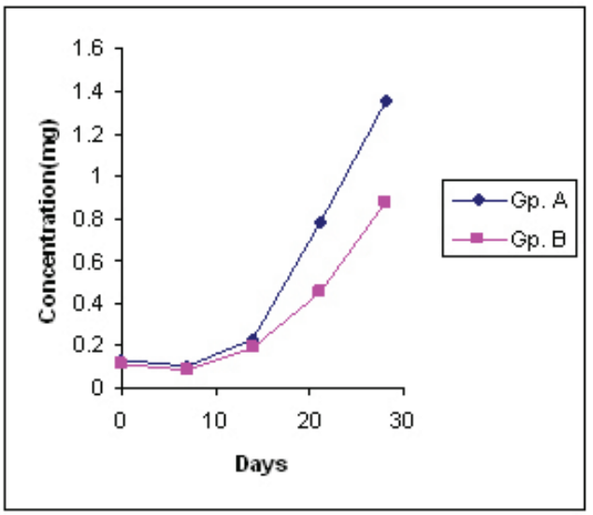


Figure 3: Graphical plot presenting increase in vasicine concentration by method A of cell suspension culture.

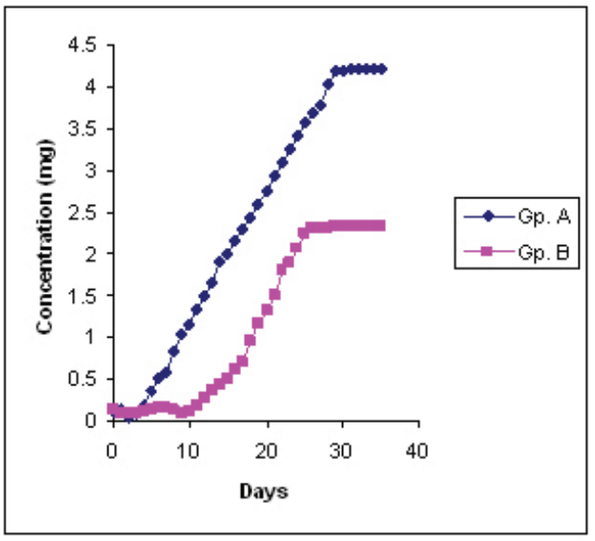


Figure 4: Growth curve of vasicine obtained by method B of cell suspension culture.

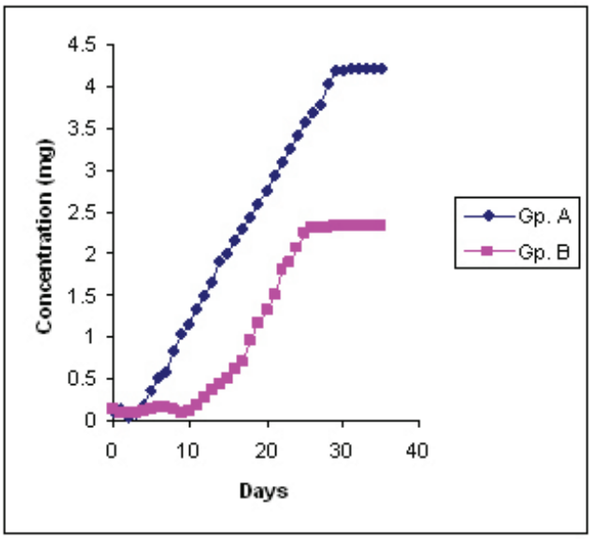


Figure 4: Growth curve of vasicine obtained by method B of cell suspension culture.

Organogenesis on MS media with various PGR combination resulted in the initation of both shoots & roots (Fig. 5). A single shoot was developed after 18th day from the node of vasaka in the media containing 5.3 µM NAA and 13.2 µM 6BA. The resultant shoot was then transferred to a media supplemented with 13.2 µM NAA and 5.3 µM 6BA and it developed roots within 30 days. Roots were also observed after 2 months from vasaka callus on MS media supplemented with 1.3 µM NAA and 4.4 µM 6BA. The concentration of vasicine in the petiole obtained by PTC technique was 24.76 ± 0.058 % as compared to petiole of the same weight from vasaka plant 1.12 ± 0.023 %.

Figure 6 gives the overlapping of the HPLC chromatogram of callus, suspension and petiole with respect to standard vasicine.

CONCLUSION

Vasicine was earlier isolated form whole plants of the vasaka. However this study was aimed at detection of quinazoline alkaloid on leaf a petiole derived callus cultures of *Adhatoda vasika*. Further investigation was done to enhance the vasicine content by cell suspension culture technique. Organogenesis was also developed through the plant issue culture technique. This study revealed that an increase in secondary metabolite content of vasaka was possible by the plant tissue culture technique. It also concluded that the developed callus, shoot and root *invitro*

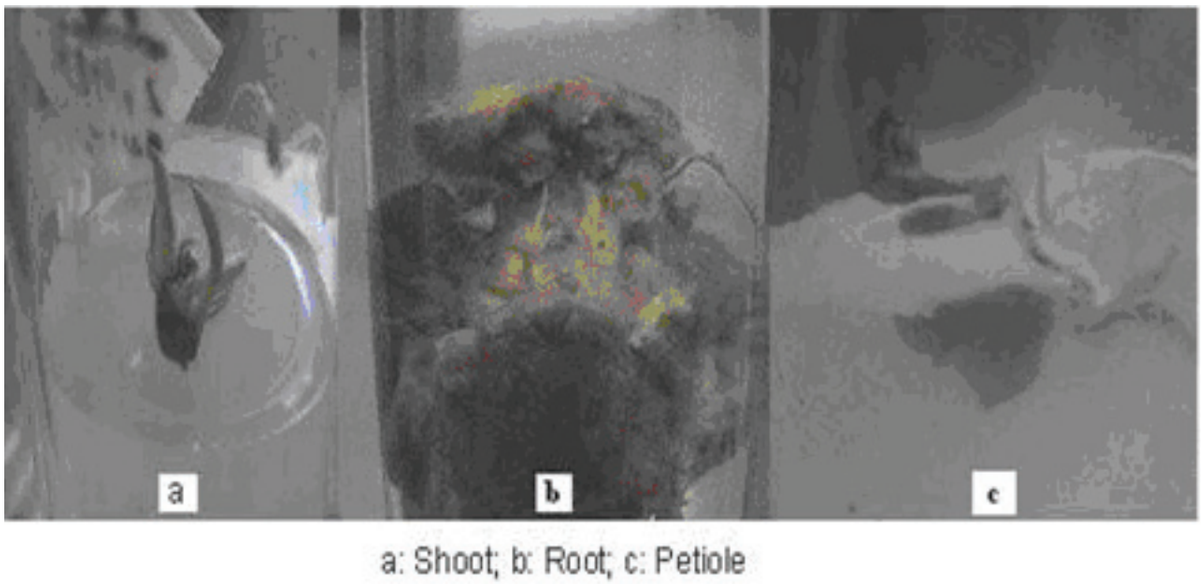


Figure 5: Organogenesis

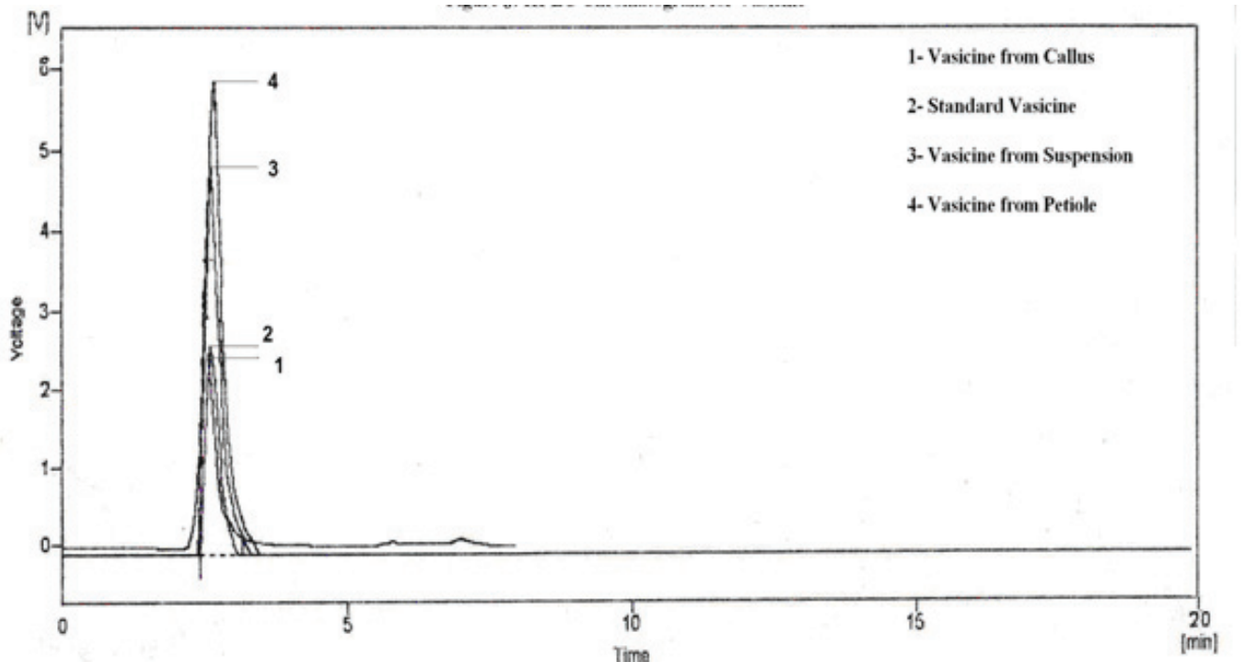


Figure 6: HPLC chromatogram of vasicine

cultures can be used as alternative source for production of vasaka alkaloids of pharmaceutical interest.

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