

Micropropagation of *Adhatoda vasica* Nees—A woody medicinal plant by shoot tip culture

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Exudation was overcome during shoot tip (1 cm) culture of *Adhatoda vasica* by modifying the pH of the media and by lowering the concentrations of NH_4^+ and K^+ ions. Though shoot formation occurred in media supplemented with BA (2 mg L^{-1}), 96.67% of the shoots developed callus at the cut basal end and the explants turned brown and necrotic due to the phenolic exudates released into the medium. Development of callus tissue and browning was eliminated by culturing the shoot tip explants on primary MS medium supplemented with thiadiazuron (0.30 mg L^{-1}) and coconut milk (15%) which also promoted development of shoots but with stunted curled leaves. Shoots (5.2) with healthy leaves were obtained when these shoots were transferred to a secondary MS medium supplemented with 0.5 mg L^{-1} BA. Incorporation of coconut milk (15%) in the secondary MS medium had a growth promotory effect. High frequency rooting (9.33) was recorded in MS basal medium. The rooted plantlets were hardened and established at 85% rate in pots.

Keywords: thiadiazuron; *Adhatoda vasica*; phenolic exudates; shoot tip; micropropagation

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Introduction

Adhatoda vasica Nees (*Acanthaceae*), a perennial woody shrub is an important medicinal plant widely exploited for extraction of an alkaloid, vasicine, which is used in the preparation of 'vasaka', a well known drug in the Ayurvedic system of medicine. The drug is recommended for a range of ailments viz., bronchitis, asthma, jaundice, diseases of the respiratory system, diphtheria and gonorrhoea¹. The efficacy of vasicine as a uterotonic abortifacient has been proved². Alkaloids from *A. vasica* have pronounced protective activity against allergy induced bronchial obstructions³. Extract of the plant is also used as an effective Ayurvedic medicine in the treatment of tuberculosis. Bromohexine and ambroxol are the semi synthetic derivatives of vasicine reported to have inhibitory effect against *Mycobacterium tuberculosis*⁴. *A. vasica* is a potential candidate for investigations aimed at controlling resurgence of multidrug resistant tuberculosis (MDR-TB) and the recent pandemic human immunodeficiency virus (HIV) infections.

All over the tropics, large-scale destruction of natural habitat due to population pressure and

overexploitation have become a major threat to important bioresources. Development of viable micropropagation protocols is important for *ex situ* conservation and sustainable utilization of selected species. Though micropropagation of *A. vasica* through nodal segment culture has been reported earlier⁵, the cultures are often plagued with phenolic exudates, resulting in considerable loss of the shoots in cultures. This paper describes rapid and efficient propagation of *A. vasica* using shoot tip culture by overcoming exudation through amendments in the nutrient medium.

Materials and Methods

Plant Material

Plants of *Adhatoda vasica* Nees were collected from the campus of Tezpur University, Assam, India. Shoot tips (2-2.5.0 cm) were dissected out and washed thoroughly under running tap water for half an hour. The explants were trimmed further into smaller pieces (1 cm) and then immersed in tap water with 2-3 drops of Teepol (Sigma) for 5-10 min before sterilization in a solution of 0.01%(w/v) mercuric chloride (Hi Media, Mumbai) for 7-8 min. After thorough rinsing in autoclaved distilled water, the explants were transplanted onto the culture medium.

Culture Media and Conditions

The MS (Murashige and Skoog)⁶ medium with normal salts has been modified for achieving the

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normal growth and development of the shoot tip explants. These altered MS media are referred to as primary MS medium (MS_p), secondary MS medium (MS_s) and modified secondary MS medium (MS_m). The MS_m medium was used for achieving shoot elongation.

The MS_p medium contains 1% (w/v) sucrose, quarter strength NH_4NO_3 and KNO_3 together with normal concentrations of other salts. The pH of the medium was adjusted to 4.6 with 0.1N NaOH prior to autoclaving at 121°C under pressure of 15 lb per sq inch for 20 min. To this, filter sterilized thiadiazuron (TDZ, 0.30 mgL⁻¹) and coconut milk [CM, 15%(v/v)] was added. The CM was sterilized by boiling for 15-20 min before straining through muslin cloth. The secondary MS medium (MS_s) contains MS medium with 6-benzyladenine (BA, 1.0 and 0.5 mgL⁻¹) and the modified secondary MS medium (MS_m) contains MS_s medium with CM (15%, v/v). Rooting of elongated shoots (4.34±0.28 cm) was assessed by transferring them to full strength MS basal medium containing 0.5-0.65 g l⁻¹ activated charcoal (AC) and 2.0-3.0 mg l⁻¹ indole-3-butyric acid (IBA). The cultures were maintained under 16 h illumination at 25±1°C under cool fluorescent tube lights providing 3000 lux intensity.

Statistical Analysis

Data were scored after 14 and 30 days for multiple shoot initiation and growth in primary medium, modified secondary medium and rooting on basal medium. The data are presented in mean ± SD for shoot induction with 20 explants per treatment in six replicates and in mean ± SE for root induction with 10 explants per treatment in three replicates. Mean values with the same superscript were not significantly different ($p= 0.05\%$) according to Duncan's multiple range test (DMRT).

Establishment in Pots

After 4 weeks, the rooted micro shoots were transferred to full strength MS liquid medium for a week followed by transfer to half strength MS liquid medium for acclimatization for another week. The plantlets were then planted in pots containing sterilized mixture of sand and soil (3:1), irrigated and kept under fluorescent light (16 h photoperiod) at 25 ± 2°C. These plants were kept covered with polythene bags to maintain humidity for a week before transfer to the field.

Results and Discussion

Surface Decontamination

Initiation of shoot cultures in *A. vasica* appeared to be rather difficult initially due to heavy fungal and bacterial contamination. The method followed *viz.*, immersion in Teepol for about 10 min before treating with 0.01% $HgCl_2$ (w/v) for 5-7 min greatly helped to overcome this problem (90%). A quick dip of the explants in 70% ethanol followed by treatment with 0.01% $HgCl_2$ (w/v) for over 7-10 min resulted in tissue blackening and subsequent death. Alternatively, soaking the explants in tap water overnight helped to reduce the contamination percentage to 70-75% but blackening of the tissue occurred to the extent of 80%.

Shoot Initiation and Growth

The shoot tips were initially cultured on MS medium with different concentrations of BA (0.5, 1.0, 2.0 and 3.0 mg L⁻¹). After 2 weeks, average number of shoots (3.0±0.15) were more in the medium supplemented with 2 mg L⁻¹ BA, but 96.67% of the explants showed callus at the cut end (Table 1). The medium turned brown due to the release of the phenolic exudates and the leaves turned necrotic and fell off.

After 30 days of inoculation on the MS_p medium, young green shoots with curly leaves appeared. No phenolic exudation and callusing were seen (Table 1). To circumvent the problem of curly leaves, the explants were transferred to the MS_s medium. The shoots that appeared were stout, dark green, larger in size and without swelling at the base. The leaves were soft at the base and tended to fall off.

Though the number of leaves increased (5.2±0.20) (Table 2) the shoot elongation was less (Fig. 1A). For shoot elongation these were transferred to MS_m medium, which has the same composition as MS_s but additionally supplemented with 15% CM (v/v). Elongated shoots (4.34±0.28 cm) were obtained after 2 weeks (Table 2; Fig. 1B).

Modification of nutrient media has been recommended by various workers to overcome exudation in tissue cultures. Anderson suggested that by reducing the concentration of KNO_3 to half, browning could be prevented in *Rhododendron*⁷. In *Dioscorea opposita*, browning did not occur if callus cultures were initiated on MS medium with 10 mM NH_4NO_3 and KNO_3 ⁸. The activity of polyphenol oxidase also decreased at lower pH⁹. The results

obtained in the present study are in line with the findings of earlier workers but successful establishment of tissue cultures of *A. vasica* free of exudates is new.

Rooting of *in vitro* Derived Shoots and Plantlet Establishment

Shoot tip culture derived microshoots, 4.34 ± 0.28 cm long, were rooted on only full strength MS basal medium supplemented with different concentrations of AC (0.5-0.65 g L⁻¹) and IBA (2.0-3.0 mg L⁻¹). AC is reported to have several stimulatory effects on root growth¹⁰. At 3.0 mg L⁻¹ IBA, only 3.33 ± 1.29 number of roots were seen on an average. However, only 2.0 ± 1.29 number of roots on an average were observed when AC was used alone. Interestingly, there was total inhibition of rhizogenesis on shoots when the MS basal medium was supplemented with both AC and IBA (Table 3). AC and IBA together may have an

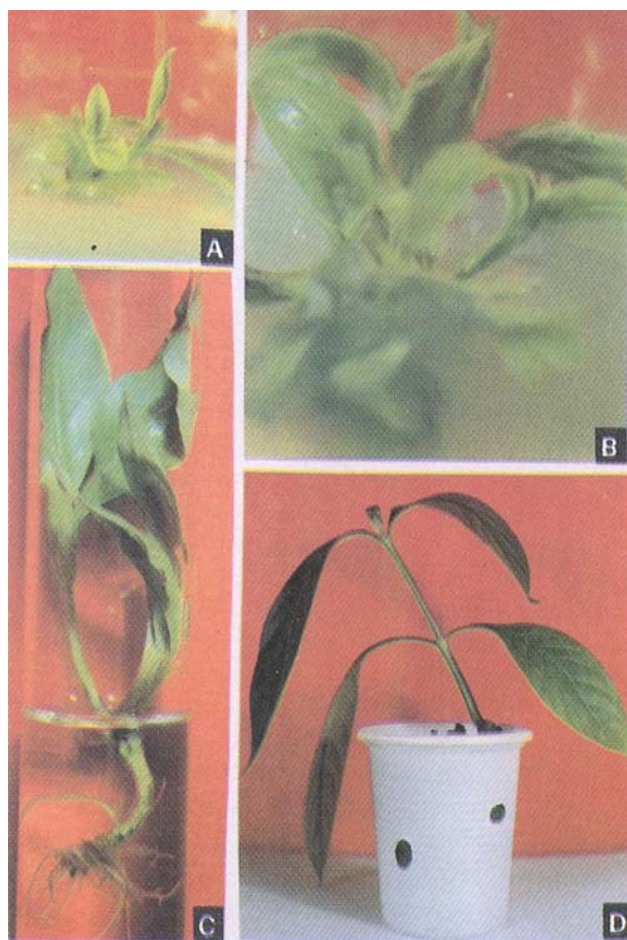


Fig. 1—Micropropagation of *A. vasica* through shoot tip explants: A & B, Multiple shoot proliferation after 30 and 14 days in MS_p & Modified MS_s media, respectively; C, *In vitro* rooted plantlet in liquid basal MS media for acclimatisation & D, Establishment of micropropagated plant (scale=10 cm in 250 ml flasks) in soil.

Table 1—Influence of BA, TDZ and coconut milk (15%) on multiple shoot induction in *A. vasica* after 30 days of inoculation on primary medium

Media	Growth regulator (mg L ⁻¹)	Shoot number per culture (Mean ± SD)	Shoots with basal callus (%)	Mean number of leaves per shoot (Mean ± SD)
MS	BA			
	0.5	$2.1 \pm .15^{bc}$	90.00	-
	1.0	$2.3 \pm .30^b$	93.33	-
	2.0	$3.0 \pm .15^a$	96.67	-
MSp	3.0	$2.1 \pm .15^{bc}$	90.10	-
	TDZ			
	0.30 + 15% CM	$1.73 \pm .21$	NB & NC	$3.93 \pm .46$

In each column, mean ± SD followed by the same letter were not significantly different ($p=0.05$) according to Duncan's multiple range test.

MS_p = primary MS medium

NB = no browning of the medium

NC = no callusing at the cut end

Table 2—Influence of BA and coconut milk on the growth and length of shoots of *A. vasica* in modified secondary medium after 14 days of inoculation

Media	Growth regulator (mg L ⁻¹)	Shoot number per explant (Mean ± SD)	Mean number of leaves per shoot (Mean ± SD)
MSs	BA		
	1.0	5.4 ± 1.03^a	3.8 ± 0.20^b
	0.5	5.2 ± 0.38^{ac}	5.2 ± 0.20^a
MSm	BA	Average length of shoot (cm) (Mean ± SD)	Average number of nodes per shoot (Mean ± SD)
	0.5 + 15% CM	4.34 ± 0.28^{bd}	3.2 ± 0.13^c

In each column, mean ± SD followed by the same letter were not significantly different ($p=0.05$) according to Duncan's multiple range test.

MS_s = secondary MS medium

MS_m = modified secondary MS medium

Table 3—Influence of IBA and activated charcoal on rooting of micropropagated shoots of *A. vasica*

Media	Growth regulator (g L ⁻¹ + mg L ⁻¹)	Root number per shoot (Mean ± SE)	Average length (cm) of roots per shoot (Mean ± SE)
MS	AC+IBA		
	0.5+2.0	0.00	0.00
	0.6+3.0	0.00	0.00
	0.0+3.0	3.33 ± 1.29^b	0.21 ± 0.21^b
	0.65+0.0	2.0 ± 1.29^c	0.60 ± 0.21^a
MSo	0.0 + 0.0	9.33 ± 1.29^a	0.60 ± 0.21^a

In each column, mean ± SE followed by the same letter were not significantly different ($p=0.05$) according to Duncan's multiple range test.

AC= Activated charcoal

MSo= MS basal medium

antagonistic influence nullifying the stimulatory affect of AC on root initiation. The observed better root initiation without either AC or IBA in the basal medium (9.33 ± 1.29) (Table 3), suggests that there is sufficient level of endogenous auxins available.

After 4 weeks, the rooted microshoots were maintained for one week in full strength MS liquid medium (Fig. 1C) followed by transfer for another one week to half strength MS liquid medium for acclimatization. The plantlets were finally transplanted to pots containing sterilized mixture of sand and soil (3:1) (Fig. 1D). Over 85% of the plantlets survived after transplantation to the field.

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