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Full Length Research Paper

Micropropagation of the Indian Birthwort *Arsitolochia indica* L.

Syed Naseer Shah[1], Amjad M. Husaini[2]* and Fatima Shirin[1]

[1]Genetics and Plant Propagation Division, Tropical Forest Research Institute, Mandla Road, Jabalpur 482 021, India.
[2]Centre for Plant Biotechnology, Division of Biotechnology, SKUAST-K, Shalimar, Srinagar-191121, India.

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***Aristolochia indica* L. is a medicinal woody perennial climber plant of immense pharmaceutical value. The species is endangered with possible extinction due to its indiscriminate harvesting as raw material for pharmaceutical industry, to manufacture drugs against cholera, inflammation, biliousness, dry cough and snake bite. A rigorous attempt has been made for development of *in vitro* propagation procedure for this species, involving four steps, namely: culture establishment, shoot multiplication, rooting and hardening. Aseptic cultures were established by growing nodal segments (1 to 1.5 cm) as explants on Murashige and Skoog (MS) medium containing 5.0 µM N6-Benzyladenine (BA). Five nutrient media, MS, Woody Plant Medium (WPM), Gamborg Medium (B5), Nitsch and Nitsch Medium (NN), and Schenk and Hildebrandt Medium (SH) supplemented with different cytokinins and auxins at a concentration of 10.0 µM were used in this study. Ads at 10.0 µM proved optimum for *in vitro* shoot multiplication. The treatment resulted in 100% shoot number per explant at 15 days and 61.9% at 30 days on MS medium, 65.2% node number per shoot at 15 days and 196.2% at 30 days on WPM medium and 147.5 and 366.6% node number per explant at 30 days after inoculation on MS medium. The *in vitro* multiplied shoots were used for rooting experiment. Five nutrient media (MS, WPM, B_5, NN and SH) and three auxin sources 10.0 µM each (IBA, IAA and NAA). SH medium with 10.0 µM NAA induced 327.8% rooting at 21days and 654.8% at 28 days and root number per explant 4300% at 21 and 394% at 28 day after inoculation. The *in vitro* propagated hardened plants exhibited excellent growth on transfer to natural condition.**

Key words: *Aristolochia indica* L, *in vitro* propagation, N^6-Benzyladenine.

INTRODUCTION

Aristolochia indica L. (family- Asclepiadaceae.) is a perennial climber with greenish whitish woody stem growing throughout India especially in the tropical and sub-tropical regions. The active constituent “Aristolic acid” is potent drug used in Ayurvedic, Sidda and

Homeopathy systems of medicines. Roots are widely used in joint pains and seeds in inflammation, biliousness, dry cough and dyspepsia. The juice of leaves or roots is said to be a specific antidote for cobra poisoning (Kirtikar and Basu, 1987). The species is rare and endangered with extinction due to its indiscriminate collection and over exploitation from natural resources for commercial purpose by pharmaceutical industries (Rahman, 2001). The conventional propagation is hampered due to low seed viability and poor rooting of vegetative cuttings and emphasizes need for the alternative *in vitro* propagation method for large scale multiplication, improvement and conservation of the

*Corresponding author. E-mail: amjadhusaini@yahoo.com

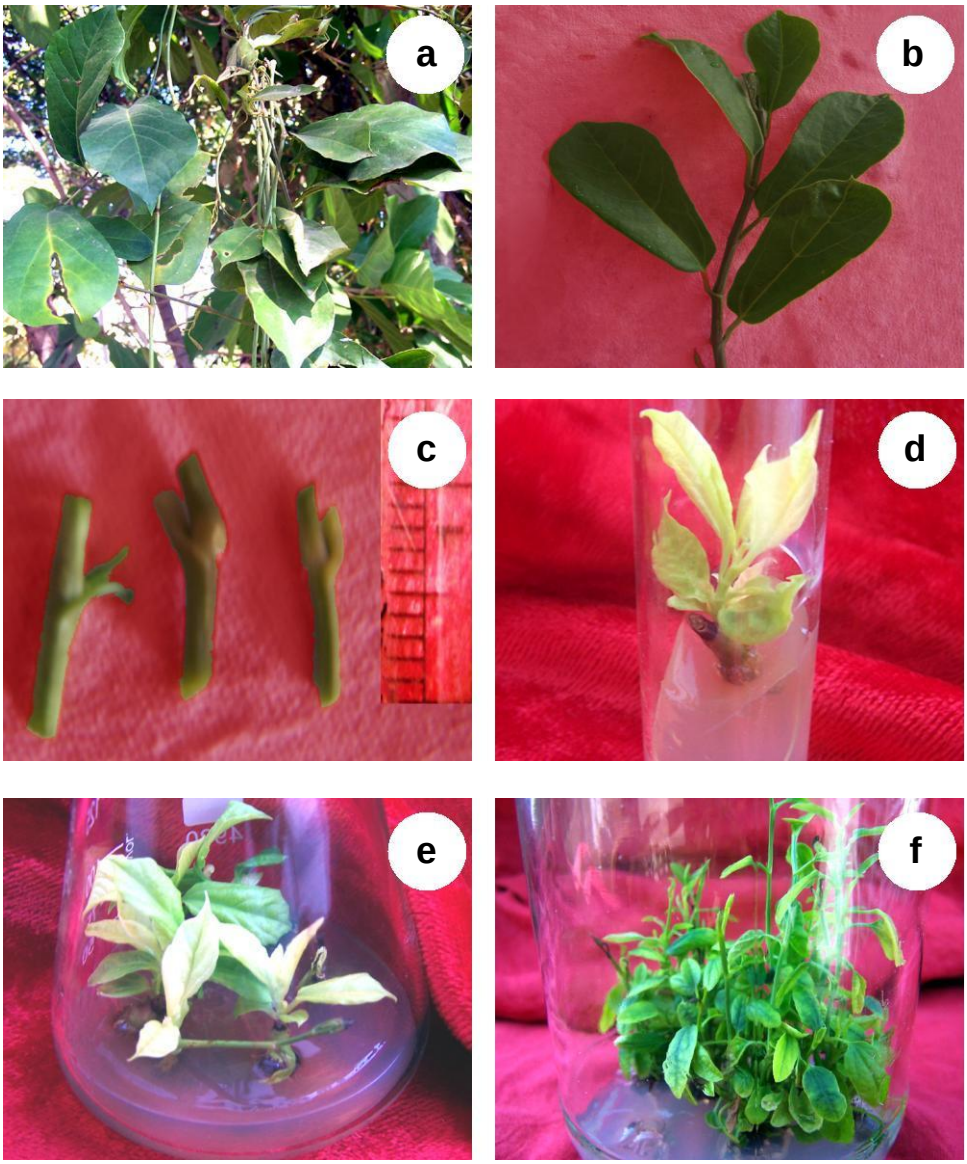

Figure 1. Explant collection, culture establishment and shoot multiplication in *Aristolochia indica* L.; (a) mother plant, (b) a twig, (c) nodal explants, (d) the *in vitro* culture establishment and (e-f) the *in vitro* shoot multiplication.

species. The objective of the study was to develop an efficient protocol for its micropropagation.

MATERIALS AND METHODS

The selected (mother) plant from Jabalpur area of Madhya Pradesh, India (Figure 1a) was used to collect twig (s) (Figure 1b), which were washed thoroughly for 15 min under running water for removing the surface debris. The washed twigs were defoliated and cut into nodal explants (approximately 1 to 1.5 cm long and 0.5 to 0.6 cm diameter) (Figure 1c). These explants were washed with 2% Cetrimide® and kept for 10 min with constant vigorous (shaking 150 rpm) on an orbital shaker incubator followed by rewashing 4 to 5 times with distilled water to remove traces of Cetrimide®. The washed explants were sterilized for 5 min with $HgCl_2$ (0.1%) and Bavistin® (1.0%) in the laminar flow cabinet. Finally, the surface sterilized nodal explants were rinsed 4 to 5 times with sterile distilled water and inoculated on MS medium (Murashige and Skoog, 1962) supplemented with 5.0 µM BA for culture establishment (Figure 1d).

The *in vitro* shoot multiplication (Figure 1e-f) was standardized through a factorial randomized experiment, using single nodal segments from established cultures. In this experiment we screened five nutrient media [MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1980), B_5 (Gamborg et al., 1968), NN (Nitsch and Nitsch, 1969) and SH (Schenk and Hildebrandt, 1972)] along with 10.0 µM each of three cytokinins (BA, TDZ, Ads), and their combinations on shoot number per explant, node number per shoot and node number per explant at 15 and 30 days after inoculation. In second experiment, five nutrient media (MS, WPM, B_5, NN and SH) and three auxins (IBA, IAA and NAA) at

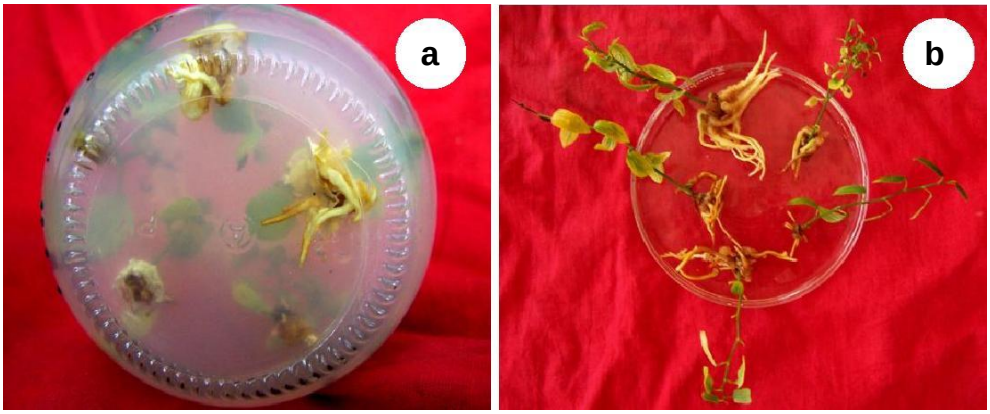

Figure 2. The *in vitro* induction of adventitious root in *Aristolochia indica* L. roots formation on semi-solid medium at (a) 21 and (b) 28 days after inoculation.

concentrations of 10.0 µM and their effect on rooting and root number was recorded at 21 and 28 days after inoculation (Figure 2).

Culture conditions

The inorganic salts used for preparation of culture medium were obtained from Qualigens Pvt. Ltd., India and phytohormones and B vitamins from Sigma Chemicals Pvt. Ltd., India. The medium contained 3% (w/v) sucrose, 0.8% (w/v) agar (Hi-Media chemical Ltd., India). The pH of the medium was adjusted to 6.0 before autoclaving for 15 min at 1.06 kg cm^{-2} (121°C). Explants were cultured in a 150 ml conical Borosil® flasks containing 40 ml semi-solid medium. For *in vitro* shoot multiplication and rooting experiment, the cultures were incubated at 25 ± 2°C under 16 h illuminations with fluorescent light (50 µmol Em^{-2} s^{-1}).

Hardening and transplantation

The *in vitro* raised plantlets were removed from rooting medium washed with distilled water and the plantlets were subsequently transferred to root trainers containing autoclaved soilrite (Figure 3a) and covered with perforated polythene to maintain humidity which were kept under culture room conditions for about 10 days. Subsequently, they were transferred to perforated polythene bags and kept initially in washing room for 5 days and finally transferred to natural condition (Figure 3b-d).

Statisticaly analysis

Each experiment had three replicates for *in vitro* shoot multiplication and rooting. Each replicate had 10 propagules. The data were subjected to two way (factor) analysis of variance for both the experiments with “F” test for ascertaining level of significance. If the data were found significant at $p \leq 0.05$, $LSD_{0.05}$ was computed for comparison of treatment means.

RESULTS

In vitro shoot multiplication

The effect of cultured media, cytokinin sources and their all possible combinations on shoot number per explant, node number per shoot and node number per explant at both the stages of sampling was recorded.

Shoot number per explant

SH medium produced maximum shoot number per explant at 15 days and MS medium at 30 days (Table 1).The enhancement of shoot number per explant in SH medium was 100% in comparison to B_5 medium at 15 days and 61.90% in MS medium at 30 days after inoculation in comparison to that in B_5 medium. Further BA had significantly maximum shoot number $explant^{-1}$ at both stages and was statistically equalled by Ads at 15 days. Shoot number per explant in BA was 1333%, 76% more than that of TDZ at 15 and 30 days, respectively. MS medium with 10.0 µM BA produced maximum shoot numbers per explant which was 189% at 30 days after inoculation.

Node number per shoot

SH medium produced maximum node number per shoot at 15 days and WPM medium at 30 days (Table 2). The enhancement of node number per shoot in SH medium was 65.2% more than that obtained in NN medium at 15 days and 139% at 30 days in comparison to that in B_5 medium which produced the lowest value for the parameter at both stages of sampling . BA induced maximum node number per shoot, which was enhanced by 61% at 15 days and 239% at 30 days in comparison with TDZ. Maximum node number per shoot was observed on SH medium with Ads, at 15 days and WPM 10.0 µM Ads at 30 days. NN medium produced minimum effect on node number per shoot at both the stages of sampling at 15 days and B_5 medium at 30 days.

Figure 3. Hardening and acclimatization of the *in vitro* raised plantlets of *Aristolochia indica* L. Plantlets transferred to root trainers (a) and covered with polythene (b) placed in the culture room, (c) hardened plantlets transferred to polythene bags and (d) growth of the plantlets in the open environment.

Table 1. Effect of culture media and different cytokinins on shoot number per explant in *Aristolochia indica* L. at two stages of sampling.

Cytokinin source (C)	Culture media(M)											
	Inoculation days											
	15 Days						30 Days					
	MS	**WPM**	**B5**	**NN**	**SH**	**Mean**	**MS**	**WPM**	**B5**	**NN**	**SH**	**Mean**
BA	1.00	1.00	0.39	0.56	1.11	**0.81**	**2.89**	1.73	1.06	1.67	1.45	**1.76**
TDZ	0.33	0.00	0.00	0.00	0.00	0.06	1.00	1.00	1.00	1.00	1.00	1.00
Ads	0.78	1.00	0.72	0.72	1.11	**0.86**	1.17	1.17	1.11	1.78	1.45	1.33
Mean	**0.70**	**0.66**	0.37	0.42	**0.74**		**1.70**	1.30	1.05	1.48	1.30	

	LSD (0.05)	
Variable	**15Days**	**30Days**
C	0.16	0.13
M	0.20	0.17
C *M	NS	0.29

Node number per explant

SH and MS medium induced maximum node number per explant at 15 days and at 30 days respectively (Table 3). The enhancement of node number per explant in SH medium was 147.6% at 15 days and 366.6% in MS medium at 30 days as compared to B_5 medium. BA and Ads had significantly maximum node number per explants at 15 and 30 days, respectively. BA enhanced node number per explants by 48% at 15 days and Ads by

Table 2. Effect of culture media and different cytokinins on node number per shoot in *Aristolochia indica* L. at two stages of sampling.

Cytokinin sources (C)	Culture media(M)											
	Inoculation days											
	15 Days						30 Days					
	MS	WPM	B5	NN	SH	Mean	MS	WPM	B5	NN	SH	Mean
BA	1.28	1.55	1.17	1.28	2.78	**1.61**	4.04	2.67	1.11	2.28	2.72	2.57
TDZ	1.00	1.00	1.00	1.00	1.00	1.00	1.78	1.06	1.00	1.00	1.06	1.18
Ads	1.56	**2.22**	1.06	1.17	1.83	1.56	3.50	**5.78**	1.11	4.28	5.32	**4.00**
Mean	1.28	1.60	1.80	1.15	**1.90**		3.10	**3.17**	1.07	2.52	3.03	

Variable	LSD (0.05)	
	15 Days	30 Days
C	0.19	0.47
M	0.25	0.61
C *M	0.43	1.06

Table 3. Effect of culture media and different cytokinins on node number per explant in *Aristolochia indica* L. at two stages of sampling.

Cytokinin sources (C)	Culture media(M)											
	Inoculation days											
	15 Days						30 Days					
	MS	WPM	B5	NN	SH	Mean	MS	WPM	B5	NN	SH	Mean
BA	1.28	1.56	0.81	0.67	**3.07**	**1.48**	11.72	4.33	1.36	3.97	4.27	5.13
TDZ	1.00	1.00	1.00	1.00	1.00	1.00	1.06	1.00	1.00	1.00	1.06	1.02
Ads	1.22	2.22	0.76	0.81	2.03	1.41	4.00	6.80	1.27	7.55	**7.73**	**5.47**
Mean	1.16	1.60	0.86	0.82	**2.03**		**5.60**	4.04	1.20	4.18	4.35	

Variable	LSD (0.05)	
	15 Days	30 Days
C	0.21	0.85
M	0.28	1.10
C *M	0.48	1.91

436% at 30 days in comparison to TDZ, which produced the lowest value for the parameter. As for interaction, SH medium with 10.0 µM Ads registered the highest value for the parameter at 30 days after sampling.

In vitro adventitious rooting

Auxin sources and their combinations with different media induced significant rooting and root number per explant at both the stages of sampling.

Percent rooting

SH medium produced significantly high percent of rooting. The enhancement of rooting in SH medium was 327.8% at 21 days and 655% at 28 days in comparison to MS medium. MS, B_5, NN and WPM produced minimum effect on rooting. NAA produced significantly maximum rooting (%), which was 228 at 21 days and 443.7% at 28 days after inoculation in compared to IAA producing minimum value for rooting. SH medium with10.0 µM NAA maximum rooting at both stages of sampling (Table 4).

Root number per explants

SH medium produced maximum root number per explant at both the stages of sampling. The enhancement of root number per explant was 4300% at 21 days and 394% at 28 days after inoculation in comparison with WPM, MS and NN medium. NAA was found to have significant effect on root number per explant at both stages of sampling and resulted in 800% at 21 days and 2900% at 28 days more than that obtained in IAA. SH medium

Table 4. Effect of culture media and different auxins on percent of rooting in *Aristolochia indica* L. at two stages of sampling.

Auxin sources (A)	Culture media (M)											
	Inoculation days											
	Rooting at % 21 Days						Rooting at % 28 Days					
	MS	WPM	B5	NN	SH	Mean	MS	WPM	B5	NN	SH	Mean
IBA	0 (4.16)	0 (4.16)	0 (4.16)	16.66 (24.06)	0 (4.16)	3.33 (8.14)	0 (4.16)	0 (4.16)	5.55 (10.79)	16.66 (24.06)	0 (4.16)	4.44 (9.47)
IAA	0 (4.16)	0 (4.16)	0 (4.16)	0 (4.16)	0 (4.16)	0 (4.16)	0 (4.16)	0 (4.16)	0 (4.16)	5.55 (10.79)	0 (4.16)	1.11 (5.49)
NAA	5.55 (10.79)	0 (4.16)	0 (4.16)	0 (4.16)	50 (45)	11.11 (13.65)	22.22 (24.4)	0 (4.16)	16.66 (24.06)	5.55 (10.79)	100 (85.84)	28.89 (29.85)
Mean	1.9 (6.4)	0 (4.16)	0 (4.16)	5.6 (10.8)	16.7 (17.8)		7.4 (10.9)	0 (4.16)	7.4 (13.00)	9.3 (15.2)	33.3 (31.4)	

Variable	LSD (0.05)	
	21 Days	28 Days
A	2.21	5.49
M	2.85	7.08
A*M	4.94	12.27

Table 5. Effect of culture media and different auxins on root number per explant in *Aristolochia indica* L. at two stages of sampling.

Auxin sources (A)	Culture media(M)											
	Inoculation days											
	Root number 21 Days						Root number 28 Days					
	MS	WPM	B5	NN	SH	Mean	MS	WPM	B5	NN	SH	Mean
IBA	0.00	0.00	0.00	0.16	0.00	0.03	0.00	0.00	0.28	0.39	0.00	0.13
IAA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.03
NAA	0.05	0.00	0.00	0.00	1.33	0.27	0.66	0.00	0.44	0.11	3.28	0.90
Mean	0.01	0.00	0.00	0.05	0.44		0.22	0.00	0.24	0.22	1.01	

Variable	LSD (0.05)	
	21 Days	28 Days
A	0.16	0.27
M	0.20	0.35
A*M	0.36	0.61

along with 10.0 µM NAA was found to have significant effect on root number per explant at 21 and 28 days after inoculation (Table 5).

DISCUSSION

The micro-propagation of *A. indica* comprises four steps, namely: establishment of culture from nodal explants, shoot multiplication, root induction and hardening and acclimatization. The present investigation was intended for the standardization of culture medium and plant growth regulators at second and third steps followed by hardening procedure. For shoot multiplications, the best *in vitro* combination was SH medium supplemented with 10.0 µM Ads. There is no published report on the *in vitro* shoot multiplication of *A. indica* using SH medium. The suitability of SH medium in the present study contrasts with earlier reports of micropropagation for this species wherein MS medium was found to be the most effective

(Siddique et al., 2006a; 2006b; Pattar and Jayraj, 2012). The results indicate that the species requires low amount of nitrogen for growth and differentiation of new shoots. Adenine sulphate was found as the most suitable cytokinin for shoot multiplication. Similar results have been reported in the medicinal plant *Cichorium intybus* also, where multiple shoots proliferation was observed on medium supplemented with BA, IAA and adenine sulphate (Nadagopal and Ranjitha Kumari, 2006).

For *in vitro* rooting also, better performance was obtained on SH medium. High concentration of thiamine (Vitamin B_1) included in SH medium seems to be synergistic with auxins for facilitation of rhizogenesis as reported in teak by Ansari et al. (2002). Of the various auxin treatments, NAA was found to be the best auxin for *A. indica.* Superiority of NAA for *in vitro* rooting may be attributed to its synthetic nature and stability. Further, NAA also eludes the auxin oxidizing/ degrading enzyme systems of the plants (Jacobs, 1972). IAA was found to be inferior to both NAA and IBA. In literature also there are reports of IBA and NAA being more effective than IAA, because of the instability of the latter (Gaspar and Coumans, 1987).

Conclusion

The study demonstrates successful development of *in vitro* propagation procedure for *A. indica*. The procedure offers a potential system for conservation and mass propagation using explants derived from mature plants. SH (medium supplemented with 10.0 µM Ads has been found the best for efficient and rapid multiplication of *in vitro* shoots, while SH medium supplemented with 10.0 µM NAA for optimum induction of *in vitro* adventitious roots. Further, the hardening procedure reported here ensures 70 to 80% field survival of micropropagated plants of *A. indica.*

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