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Stimulation of Micropropagation of the Medicinal Plant *Aristolochia indica* L. through Nodal Explants by Adenine Sulphate

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ABSTRACT

An efficient protocol for *in vitro* propagation of the medicinal plant *Aristolochia indica* L. (Aristolochiaceae) using nodal explants is presented. Kinetin (KN), gibberellic acid (GA_3), benzyl adenine (BA) and adenine sulphate (AdS) were used individually as well as in combination (KN+GA_3, KN+BA and KN+AdS) with 2.46 µM indole-3-butyric acid (IBA) incorporated in basal Murashige and Skoog (1962) medium. Shoot proliferation was promoted by AdS (27.1 µM) alone and also in combination with other plant growth regulators (PGRs). Compared to other combinations, KN (23.25 µM) and AdS (13.5 µM) evoked a high frequency of bud break. IBA resulted in enhanced root formation, while the inclusion of IAA rooting was induced on shoots with intervening callus formation at the basal end. Using our protocol, from one twig of *A. indica* (10 responsive nodal explants), within a period of three months, 10-12 plantlets could be raised with 80% transplantation success.

Keywords: axillary bud development, medicinal plant, plant regeneration
Abbreviations: AdS, adenine sulphate; **BA**, benzyl adenine; **GA_3**, gibberellic acid; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **2-iP**, 2-isopentenyl adenine; **KN**, kinetin; **MS medium**, Murashige and Skoog (1962) medium; **NAA**, α-napthaleneacetic acid; **PGR**, plant growth regulator

INTRODUCTION

Aristolochia indica L. belonging to family *Aristolochiaceae*, is a tropical twine with greenish-white grooved stem and ovate-oblong entire leaves. The whole plant contains 12-nonacosinic acid and its roots contain an essential oil having phenanthrene derivatives like aritolic acid, aristolochic acid, alkaloid 1-curine (the alkaloid aristolochin), sesquiterpenoids like ishwarene, ishwarone, aristolochene, naphthoquinone aristolindiquinone, steroids like β–sitosterol, sterol glucoside and others like *p*-coumaric acid, D-camphor, fixed oil having glycerides and sitosterol. Aristolochic acid is used for stimulating phagocytosis in infectious diseases in combination with antibiotics. The roots and rhizome are used as a gastric stimulant and bitter tonic. The juices of leaves are prescribed for cough and seeds in inflammation and biliousness. The leaves, seeds, roots and the whole plant are used against snake poisoning, worm infestation, intermittent fever, skin diseases, wounds and also spider poisoning (Yoganarasimhan 2000; Kumar *et al*. 2006; Samy *et al*. 2007). Further, the alkaloid aristolochin present in its roots causes cardiac and respiratory paralysis in frogs and mice, exerts some pressure action and increases the rate of respiration in rabbits (Chopra *et al*. 1996). The conventional method of propagation of *A. indica* through cuttings shows only poor response and low percentage of seed germination restricts its multiplication (Selvakumar and Balakumar unpublished data). Indiscriminate exploitation of this plant for diverse medicinal uses has threatened its survival even in the regions of its distribution. Moreover, the documented floristic survey on the Azhagar Hill (Eastern Ghats) Ranges located 22 km north-east of Madurai, India, which is a tropical dry evergreen forest, reveals that previously *A. indica* was widely distributed and abundant in these forests (Sriganesan 1984). Nevertheless, our survey in the same area conducted in 1997 indicates that within a short span of 13 years, the frequency and distribution of *A. indica* in the Azhagar Hills have dwindled significantly due to various biotic factors (Balakumar *et al.* 1998). This led to the realization that in the event of this species being left uncared for, it might face extinction from the local area. Although the technique of axillary bud micropropagation has been previously reported by Manjula *et al*. (1997), shoot multiplication of *Aristolochia* using benzyladenine (BA) and Soniya and Sujitha (2006) used 2-isopentenyl adenine (2-iP) for shoot induction. Here we report a stimulation of micropropagation of *A. indica* through nodal explants by adenine sulphate, which so far has not yet been attempted.

MATERIALS AND METHODS

A. indica (*ca*. one year-old) growing in the Azhagar Hills (Madurai, India) served as experimental material. Segments measuring 1.0–2.0 cm were excised from the actively growing twines, defoliated and individual dormant axillary buds were used as explants. The nodal segments were initially washed thoroughly in running tap water for 1 h followed by immersion in a 5% (v/v) solution of detergent (Labolene, Qualigens, India) for 5 min and in a 5% (w/v) solution of the fungicide Carbestin (Carbendazim 50% W.P) for 30 min on a rotary shaker (120 rpm). The explants were surface-decontaminated by immersing in 80% ethanol containing streptomycin, 2 mg ml^{-1} (Sigma, USA) for 30 s followed by 0.1% $HgCl_2$ for three to four min. The explants were than washed repeatedly with sterile distilled water and then inoculated on Murashige and Skoog (1962; MS) medium gelled with 0.8% (w/v) agar (Bacteriological Grade, Himedia, Mumbai, India) with 3% sucrose. The inclusion of activated charcoal (2%) and adjustment

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Short Communication

Table 1 Effect of different concentrations of KN, GA_3, BA, AdS individually and in various combinations, on bud break and shoot development of *Aristolochia indica* (IBA 2.46 µM was common to all treatments).

Treatment (µM)	Frequency of bud break (%)	Days to bud break	Shoot length (cm)	№ of leaves	№ of nodes
Control	-	-	-	-	-
Kinetin					
4.65	-	-	-	-	-
13.95	35	8	2.71 ± 0.06 c	2.62 ± 0.00 c	2.00 ± 0.75 c
23.25	45	8	2.21 ± 0.03 c	2.77 ± 0.07 c	3.25 ± 0.75 c
37.20	40	8	2.23 ± 0.19 c	2.92 ± 0.19 c	3.42 ± 0.68 c
GA_3					
5.78	-	10	1.12 ± 0.07 c	1.25 ± 0.05 c	2.00 ± 0.75 c
8.67	-	10	1.16 ± 0.19 c	1.25 ± 0.04 c	2.00 ± 0.32 c
14.45	35	8	1.96 ± 0.07 c	2.23 ± 0.10 c	2.25 ± 0.75 c
20.23	39	8	2.23 ± 1.90 c	2.69 ± 0.10 c	3.25 ± 0.75 c
28.90	55	8	2.30 ± 0.18 c	3.00 ± 0.18 bc	3.80 ± 0.75 c
34.68	47	8	2.11 ± 0.15 c	2.86 ± 0.26 c	3.25 ± 0.37 c
BA					
13.32	35	8	1.18 ± 0.20 c	1.32 ± 0.22 c	2.00 ± 0.51 c
22.20	40	6	1.83 ± 0.21 c	2.62 ± 0.21 c	2.00 ± 0.63 c
35.52	60	6	2.41 ± 0.25 c	3.00 ± 0.23 bc	3.80 ± 0.59 c
AdS					
5.42	63	5	4.85 ± 1.04 b	2.86 ± 0.28 c	3.86 ± 0.30 c
13.5	67	5	5.32 ± 0.58 b	3.70 ± 0.52 b	4.57 ± 0.75 b
16.26	77	5	5.50 ± 1.62 b	3.50 ± 0.41 b	4.50 ± 0.36 b
27.10	**93**	**5**	**8.91 ± 0.85 a**	**5.29 ± 0.53 a**	**8.54 ± 0.21 a**
40.65	73	5	7.20 ± 1.16 a	3.83 ± 0.25 b	4.83 ± 0.28 b
KN + GA_3					
13.95 + 14.45	52	8	2.53 ± 0.29 c	4.16 ± 0.28 b	4.10 ± 0.32 b
23.25 + 14.45	74	6	2.80 ± 0.36 c	4.40 ± 0.51 ab	5.40 ± 0.92 ab
37.20 + 14.45	67	8	2.62 ± 0.29 c	3.61 ± 0.62 b	3.50 ± 0.63 c
KN + BA					
13.95 + 22.2	46	8	2.21 ± 0.22 c	2.77 ± 0.31 c	3.25 ± 0.51 c
23.25 + 22.2	68	6	2.70 ± 0.32 c	4.16 ± 0.57 b	4.10 ± 0.32 b
37.2 + 22.2	56	8	2.50 ± 0.28 c	4.00 ± 0.39 b	4.00 ± 0.57 b
KN + AdS					
13.95 + 13.5	55	6	4.50 ± 0.58 b	3.00 ± 0.18 bc	3.86 ± 0.30 c
23.25 + 13.5	**82**	**6**	**6.58 ± 0.28 ab**	**4.70 ± 0.25 a**	**5.50 ± 0.36 a**
37.20 + 13.5	72	6	5.56 ± 1.22 b	3.50 ± 0.46 b	3.86 ± 0.30 c

Data are mean ± SE (n = 20) collected after 30th day from three independent experiments each with five replicates. Means followed by the same alphabets within the columns are not significantly different ($P < 0.05$) as tested by Duncan's Multiple Range Test (1955).

of the pH of the medium to 5.8 were done prior to autoclaving at 121°C for 20 min. The basal MS medium was supplemented with optimal concentrations of plant growth regulators (PGRs) according to experimental requirements established during initial trials. Routinely, 15 ml each of molten medium was dispensed into sterile 25 × 150 mm culture tubes. For each treatment, five replicates were used and all the experiments were repeated at least thrice. The cultures were incubated at 25 ± 1°C with 55-60% RH and a 16 h photoperiod supplied by cool white fluorescent tubes (PPFD 45 µE m^{-2} s^{-1}). At fortnightly intervals, the explants were transferred to fresh medium and sub-cultured.

Single stem segments were cultured on basal MS medium supplemented with 2.46 µM IBA and various concentrations of KN, GA_3, BA and AdS individually and also in combination to assess bud break and development of shoots (**Table 1**). Regenerated shoots measuring 2 cm in length were excised and planted on to rooting medium supplemented with IBA, IAA and NAA at various concentrations (**Table 2**). The rooted shoots were taken out of the culture vessels, washed thoroughly in sterile distilled water and the plantlets were transferred to plastic pots (5 cm diameter) containing autoclaved vermiculite. High (RH 75-80%) humidity was maintained by covering the pots with polythene covers. Potted plantlets were grown under the above-mentioned culture conditions and irrigated with sterile distilled water for four weeks. Established plants were repotted in clay pots (15 cm diameter) containing a mixture of soil and sand (1:1) and maintained in shade in the garden for another three weeks during which they were fortified with half-strength MS salt solution. After acclimatization, the plants were transferred to open light conditions in a botanical garden.

RESULTS AND DISCUSSION

A higher level of contamination (50-60%) was observed in the cultures, which must have probably emanated from the explants since they were collected from wild *A. indica* plants growing in the open forest. Nevertheless, treatment of the nodal segments with 5% of labolene and Carbestin reduced the contamination rate to about less than 10%. Basal medium failed to stimulate the growth of buds or the development of new shoots on nodal segments. On the contrary, depending on the PGR used in the medium, the explants showed shoot development after 5-10 days of incubation and bud break frequency varied from 35-93%. AdS was highly effective, more so than BA, GA_3 and KN. An increase in the concentration of KN (4.65 to 23.25 µM), GA_3 (5.78 to 28.9 µM), BA (13.32 to 35.52 µM) and AdS (5.42 to 27.10 µM) proportionally enhanced the frequency of bud break and number of nodes in each shoot; each implanted nodal segment developed into a single shoot. Shoot development was suppressed at 37.20, 34.68 and 40.65 µM of KN, GA_3 and AdS respectively. A similar response was noted during the micropropagation of *Morus australis* wherein higher concentration of cytokinins proved inhibitory to shoot elongation (Pattnaik *et al*. 1996). The best response was obtained when the medium was supplemented with 27.10 µM AdS and 2.46 µM of IBA. The combination of 2.46 µM IBA, KN+AdS (23.25+13.50 µM), brought about a higher level of bud break (82%) than other combinations. Our results are in agrrement with those of Malathy and Pai (1998) who also obtained a higher level of shoot differentiation only with AdS in *Ixora singaporensis*. These

Table 2 Effect of IBA and IAA on root induction in excised shoots of *A. indica*.

Auxins (µM)	Frequency of rooting (%)	Root length (cm)	Callus
Control	NR	NR	NR
IBA			
2.46	61.8 ± 2.85 a	3.72 ± 0.35 a	-
4.92	**83.5 ± 3.02 a**	**4.86 ± 0.29 a**	+
9.80	58.3 ± 2.92 b	2.83 ± 0.32 b	++
12.30	52.6 ± 2.85 b	2.63 ± 0.32 b	++
IAA			
1.44	51.8 ± 2.58 b	1.85 ± 0.38 b	++
2.85	53.0 ± 2.58 b	2.35 ± 0.34 b	+
5.70	-	-	+++
10.00	-	-	+++

+ denotes callusing: + little, ++ moderate, +++ enormous
NR, no response
Data are mean ± SE (n = 20) collected after 30th day from three independent experiments each with five replicates. Means followed by the same alphabets within the columns are not significantly different ($P < 0.05$) as tested by Duncan's Multiple Range Test (1955).

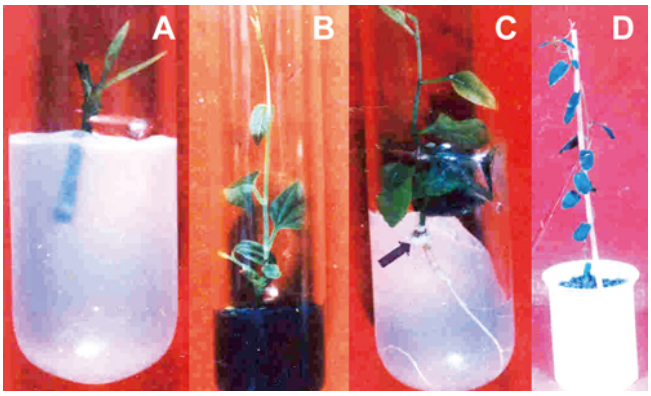

Fig. 1 ***In vitro*** **responses of nodal stem explants of field-grown twines of** ***Aristolochia indica*****.** (**A**) Nodal explants showing bud break (27.10 µM AdS + 2.46 µM IBA); (**B**) Development of shoot [medium containing activated charcoal (2%)]; (**C**) Induction of rooting (4.92 µM IBA); (**D**) A fully developed plantlet after four weeks of hardening.

researchers ascribed the better performance of AdS over KN, GA_3 and BA to its functional efficiency in promoting axillary bud development and differentiation. Premature leaf drop cultures on basal medium having KN, BA, and GA_3 was controlled by the addition of L-glutamine thiamine-HCl (100 mg l^{-1} and 10 mg l^{-1}, respectively). There was no leaf fall in cultures grown on medium containing PGRs with 2% activated charcoal and also increased subculture intervals.

Rooting did not occur on *in vitro* regenerated shoots cultured without PGRs (**Table 2**). In the presence of IBA and IAA rooting was induced on shoots with intervening callus formation at the basal end, while NAA failed to induce roots. The basal medium with 4.92 µM IBA showed 84% rooting and it also registered a higher extent of root elongation as compared to the lower concentrations of IBA tested. A similar role of IBA in rooting of shoots has been reported in *A. indica* (Manjula *et al*. 1997; Soniya and Sujitha 2006) and other plant species (Selvakumar *et al*. 2001; Zhang *et al*. 2003). A further increase in the concentration of IBA curtailed rooting. Plants with 6-8 fully expanded leaves and roots were successfully hardened in a growth chamber on vermiculite for four weeks and subsequently transferred to a mixture of soil and sand. All plants had normal leaf development and did not show any remarkable differences in morphological or growth characteristics as compared to the donor plants. Various stages of micropropagation of *A. indica* starting from nodal explant up to the potted plantlet growing in our garden are presented in **Fig. 1A-D**. Using our protocol, from one twig of *A. indica* (10 responsive nodal explants), within a period of three months, 10-12 plantlets could be raised with 80% transplantation success.

The procedure we present offers a simple and rapid protocol for the *in vitro* propagation of *A. indica* through nodal explants, which would be useful for conservation as well as large scale propagation of this important and over-exploited medicinal plant.

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