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In vitro Regeneration of Plantlets from Leaf and Nodal explants of *Aristolochia indica* L.- An Important Threatened Medicinal Plant

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ABSTRACT

Objective: An efficient reproducible protocol has been developed for in vitro regeneration of plantlets from leaf and nodal explants of *Aristolochia indica* L. **Methods:** Wild grown plants *Aristolochia indica* L. were collected and grown in the departmental garden. Leaf and nodal segments (0.5-1.0 cm) from young healthy plants were first washed thoroughly under running tap water for 15 - 20 minutes and then treated with liquid detergent [5% (v/v) Tween-20] for 5-10 minutes. Later these explants were washed with double-distilled water for 5 minutes. Subsequently, explants were immersed in 70% (v/v) ethanol for 2 - 3 minutes and washed with sterile glass double distilled water for 2-3 times. Eventually, the explants were treated with an aqueous solution of 0.1% (w/v) HgCl2 for 1 - 2 minutes and rinsed for two-to-three times in sterile ddH_2O to remove all traces of $HgCl_2$. The sterilized explants were inoculated aseptically onto solid basal Murashige and Skoog's medium with different concentrations and combinations of BAP and NAA for in vitro regeneration of plants. **Results:** Both leaf and nodal explants cultured on MS medium supplemented with 0.8 mg/L BAP developed into mass of callus. These calli were subcultured for the induction of shoots and roots. Shoots were induced from both calli on MS medium supplemented with 0.8 mg/L BAP+0.5 mg/L NAA. Roots were induced from in vitro shoots on MS medium supplemented with 0.8 mg/L NAA for 4 weeks. Nodal explants were more regenerative with 95 % response compared to leaf explants with 85%. Finally, these in vitro regenerated plantlets were hardened, acclimatised and successfully transferred to the field. **Conclusions:** The present protocol for in vitro regeneration of *Aristolochia indica* L. can be used to make this plant available throughout the year for traditional healers, pharmaceutical usages, germplasm conservation, commercial cultivation, and also for the production of secondary metabolites.

1. Introduction

Aristolochia indica L. is known by different vernacular names viz Ishwar balli (Kannada), Indian Birthwort (English), Ishar mul (Hindi) and Ishwari (Sanskrit). In Ayurveda, the leaves and roots are used for treatment of fever, insect bites, cholera, bowel troubles, ulcers, leprosy, poisonous bites (Krishnaraju *et al*.,and Kanjilal *et al*.,)[7,8], emmenagogue, abortifacient, antineoplastic, antiseptic, anti-inflammatory, antibacterial and phospholipase A2 inhibitor (Achari *et al*., 1981; Das *et al*.,)[2,4]. This plant also used as traditional medicine for postpartum infections and snakebite (Ramachandran *et al*) [17]. It is believed that the flowers of Aristolochia were thought to resemble a curved foetus or a snake. The Hindi name Ishwari suggests that the plant has the property of neutralizing snake poison.

Since the root of this plant is more valuable, people uprooting this plant for root drug from the wild population unscientifically. This resulted the plant to face the risk of depletion. Hence, the present study was aimed to produce an effective reproducible and simple and improved protocol for in vitro propagation by using leaf and nodal explants to make it available throughout the year for pharmaceutical use and also for conservation.

2. Materials and methods

2.1. Plant material and explants sterilization

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Aristolochia indica L. is a perennial herb with greenish, whitish stem. Leaves simple, alternate, entire, with undulate margins, acute; flowers greenish white, in axillary cymes; fruits rounded oblong, 6 chambered contain numerous winged compressed seeds. The flowering and fruiting period of this plant is between December and February (Neelima *et al*.,) [12].

Wild grown plants *Aristolochia indica* L. were collected and grown in the garden of the Department of Botany, Karnatak University, Dharwad, India for the source of explants. Leaf and nodal segments (0.5-1.0 cm) from young healthy plants were used for in vitro propagation. The suitable explants were first washed thoroughly under running tap water for 15 - 20 minutes and then treated with liquid detergent [5% (v/v) Tween-20] for 5-10 minutes. Later these explants were washed with double-distilled water for 5 minutes. Subsequently, explants were immersed in 70% (v/v) ethanol for 2 - 3 minutes and washed with sterile glass double distilled water for 2-3 times. Eventually, the explants were treated with an aqueous solution of 0.1% (w/v) HgCl2 for 1 - 2 minutes and rinsed for two-to-three times in sterile ddH2O to remove all traces of $HgCl_2$. The sterilized explants were inoculated aseptically onto solid basal Murashige and Skoog's medium [10] with different concentrations and combinations of BAP and NAA for in vitro regeneration of plants.

2.2. Culture media and growth condition

Basal MS medium with pH 5.6-5.8 containing 3 % (w/v) sucrose and solidified with 0.8 % agar (HI MEDIA, Laboratories, Pvt. Ltd., Mumbai, India) prior to autoclave at 1210C at 105 KPa for 15 - 20 minutes is used. Inoculations of explants into cultures tubes (150 X 25 mm) containing 20-25 ml MS medium and plugged tightly with non-absorbent cotton done under aseptic conditions in a laminar air-flow cabinet. All culture tubes were incubated in a controlled-environment chamber at 25 0 ±270 C under 16 h photoperiod at a light intensity of 50 μ mol m-2 s-1 provided by 40W white fluorescent tubes (Philips, Mumbai, India) and with a relative humidity of 55-60 %.

2.3. Induction of callus and regeneration of plantlets

Basal MS medium supplemented with different concentrations of Kinetin (Kn) (0.5, 0.8, 1.0 and 1.5 mg/L) and Benzylaminopurine (BAP) (0.5, 0.8, 1.0 and 1.5 mg /L) individually and in combinations with naphthaleneacetic acid (NAA) (0.1, 0.2, 0.5, 0.8, 1.0, 1.5 mg/L) and indole-acetic-acid (IAA) (0.1, 0.2, 0.5, 0.8, 1.0, 1.5 mg/L) were tested for the induction of callus and regeneration of shoot and root from leaf and nodal explants. Callus was induced from both explants when cultured on MS medium supplemented with 0.8 mg/L BAP.

All cultures with callus were sub-cultured after 2 weeks onto fresh MS medium supplemented with 0.8 mg/L BAP+0.5 mg/L NAA for 6 weeks to induce in vitro regeneration of shoot. The responses of each explant with regard to the induction of shoots, the length of shoot and the percentage of response were recorded after 6 weeks in culture.

2.4. In vitro rooting

In an aseptic chamber in vitro regenerated shoots were separated gently from the culture tubes and transferred to an other culture tubes containing MS medium supplemented with 0.8 mg/L NAA. The response of each explants with regard to the number of roots induced and root lengths per shoot after 4 weeks in culture were recorded.

2.5. Hardening and acclimatisation

In vitro grown plantlets were gently removed from culture tubes and washed with slightly warm (37 0C) sterile ddH2O to remove all traces of nutrient medium. They were transferred to polystyrene (50.28 cc) cups containing a 3:1 (v/v) mix of sterile vermiculite and sand. Initially, a high humidity (55-60 %) was maintained by covering the cups with punctured polythene bags. The plantlets were irrigated by sprinkling with 0.5X MS inorganic salts for three-to-four times per day for seven days and sterilized with ddH2O. The polythene bags were removed after seven days and plantlets were acclimatised for two weeks in an aseptic culture room under (16 h photoperiod at 28 0 ±2 0C; 8 h in dark at 25 0 ±20 C) conditions. Further, the plantlets were exposed gradually to sunlight for acclimatisation and were maintained in a garden.

2.6. Data collection and statistical analysis

Data for the percentage of response per explants with different concentrations and combinations of cytokinins and auxins with basal MS medium (shoot regeneration, shoot lengths, number of roots and root lengths) were recorded after 6 weeks of culture. Thus obtained data were analyzed statistically using SPSS.16 software (IBM Corporation SPSS, North America). The significance of difference among the means was calculated using Duncan's Multiple Range Test.

3. Results

Leaf and nodal explants of *Aristolochia indica* L. were cultured on MS medium supplemented with various concentrations of Kn and BAP individually and in combinations with NAA and IAA.

Leaf explants showed callus induction on MS medium supplemented with BAP (0.8 mg/L), but the growth of callus was slow & took nearly 35 days for complete proliferation into a rapid mass of callus (Table.1.Fig.A & B). Initially, callus was induced at the both ends of nodal explants and subsequently from entire surface of the each explant segments in 3 weeks. Further subculture of these little mass of callus were rapidly multiplied into a large mass of soft, green and friable callus on MS medium supplemented with 0.8 mg/L BAP (Table 1.and Fig. C & D). Indirect shoot organogenesis has been achieved from both leaf and nodal explant derived callus culture (0.8 mg/ L BAP +0.5 mg/L NAA). However, nodal segments were responded better compared to leaf explants for the induction of shoots (Table 1. Fig. D & E).

Shoot differentiation and regeneration was observed from the both leaf callus (85 %) and nodal callus (95%) after subcultured on MS medium supplemented with 0.8 mg /L BAP+0.5 mg /L NAA after six weeks of culture (Fig1).

However, in the present study, a combination of BAP (0.8 mg/ L) and NAA (0.5 mg/L) showed the best response than Kn and IAA individually and in combinations (Table 1).

Table 1

Morphogenic effect of Cytokinins and Auxins individually and in combinations for shoot induction from leaf and nodal callus of *Aristolochia indica* L. on MS medium after 6 weeks of culture.

Plant growth regulators (mg/L)				Leaf callus			Nodal callus		
Kn	BAP	NAA	IAA	(%) response	Number of shoots / explant (mean ±SE)	Shoot length/ explant (cm) (mean ±SE)	(%)response	Number of shoots/ explant (mean ±SE)	Shoot length/ explant(cm) (mean ±SE)
0.5				60	4.0±0.57ab	0.55±0.02b	65	5.1±0.08c	0.62±0.01b
0.8				65	5.3±.033a	0.74±0.04a	70	6.1±0.05a	0.74±0.01a
1.0				55	4.6±0.57a	0.48±0.032b	60	5.5±0.17b	0.46±0.02c
1.5				50	3.0±1.00b	0.32±0.025c	55	4.1±0.08d	0.35±0.02d
	0.5			70	6.1±0.16c	0.74±0.02b	75	8.2±0.14b	0.78±0.02b
	0.8			85	8.6±0.33a	1.06±0.06a	90	9.2±0.14a	1.08±0.01a
	1.0			70	7.3±0.16b	0.48±0.01c	75	7.2±0.14c	0.51±0.01c
	1.5			65	7.1±0.16b	0.42±0.014c	70	7.3±0.25c	0.44±0.02d
0.8	0.5			70	7.2±0.14b	0.69±0.01b	80	8.1±0.08a	0.68±0.01b
0.8	0.8			75	8.1±0.05a	0.90±0.02a	85	8.2±0.08a	1.03±0.08a
0.8	1.0			70	7.9±0.05a	0.62±0.02c	75	7.5±0.10b	0.62±0.01b
	0.8	0.5		85	14.5±0.28a	1.63±0.08a	95	16.4±0.23a	1.69±0.03a
	0.8	0.8		80	13.1±0.45b	1.00±0.05b	85	14.5±0.17b	1.23±0.14b
	0.8	1.0		70	12.0±0.28b	0.82±0.01b	80	12.4±0.23c	0.85±0.02c
	0.8		0.5	55	6.4±0.23a	0.25±0.02b	65	6.6±0.28a	0.29±0.02b
	0.8		0.8	40	4.9±0.06b	0.42±0.01a	60	4.9±0.05b	0.46±0.02a
	0.8		1.0	35	3.5±0.28c	0.35±0.03a	55	3.5±0.02c	0.32±0.01b

Each value represents the mean ±SE of ten replications. Mean values ±SE followed by the same letter within columns are not significantly different at $P \leqslant 0.05$ by Duncan's multiple range test.

Table 2

Morphogenic effect of NAA and IAA for root induction from in vitro shoots of *Aristolochia indica* L. on MS medium after 4 weeks of culture.

Auxins concentrations (mg/L)		Leaf callus			Nodal callus		
NAA	IAA	(%) response	Number of roots/ shoot(Mean±SE)	Root length/culture (cm)(Mean±SE)	(%)response	Number of roots/ shoot (Mean±SE)	Root length/culture (cm)(Mean ±SE)
0.1		25	2.0±0.57d	0.9±0.03c	45	1.6±0.66f	0.2±0.05b
0.2		30	6.3±0.88c	1.0±0.08c	50	2.3±0.33e	0.2±0.03b
0.5		45	9.0±0.57b	2.0±0.03b	65	8.0±0.57b	2.1±0.60ab
0.8		80	12.6±0.66a	2.3±0.08a	95	14.3±0.33a	2.6 ±0.24a
1.0		65	8.0±0.57b	1.0±0.06c	70	5.0±0.57c	1.4±0.30b
1.5		50	7.3±0.88b	0.9±0.03c	60	4.3±0.88d	0.1±0.04c
	0.1	15	1.3±0.33e	0.6±0.06c	30	2.0±0.57 b	0.1±0.03c
	0.2	25	4.6±0.88cd	0.9±0.05b	35	2.3±0.33 b	0.2±0.05c
	0.5	30	7.6±0.33b	1.0±0.05b	40	5.0±1.15b	0.9±0.04ab
	0.8	35	10.0±0.57a	1.9±0.06a	55	8.3±0.88a	1.2±0.37a
	1.0	30	6.0±0.57bc	0.9±0.03b	50	4.6±0.88b	1.4±0.30a
	1.5	25	3.0±0.57de	0.8±0.03b	35	2.0±1.00b	0.1±0.02b

Each value represents the mean ±SE of ten replications. Mean values ±SE followed by the same letter within columns are not significantly different at $P \leqslant 0.05$ by Duncan's multiple range test.

MS medium supplemented with BAP (0.8 mg/L) and NAA (0.5 mg/L) elicited the maximum shoot regeneration (14.5±0.28) and shoot elongation (1.63±0.08 cm) from leaf explants (Fig.1.B). Maximum shoot growth (16.4±0.23) and shoot elongation (1.69± 0.03 cm) was achieved on MS medium supplemented with BAP (0.8 mg/L) + NAA (0.5 mg/L) from nodal explants. (Fig. D, E).

In the present study, nodal explants showed better response than the leaf explants in induction of shoot and its elongation.

In vitro grown shoots of *Aristolochia indica* L. were separated and transferred to MS medium containing different concentrations of NAA and IAA individually for induction of roots. In vitro rooting was observed from leaf calli derived in vitro shoots (80%) on MS medium supplemented with 0.8 mg/L NAA and from nodal calli derived in vitro shoots (95%) on MS medium supplemented with 0.8 mg/L NAA. It is found that MS medium supplemented with 0.8 mg/L NAA was best for induction of in vitro roots. The maximum number of roots per shoot was 12.6±0.66 with a mean length of 2.3±0.08 cm from leaf callus and 14.3±0.33 with a mean length of 2.6 ±0.24 from nodal callus after 30-40 days (Table 2).

In the present study, it is observed that, the thick and long roots were developed from in vitro grown shoots on MS medium supplemented with NAA (0.8 mg/L) (Fig. C & D).

After 6 weeks, in vitro rooted plantlets were gently removed and washed with sterile water then transferred to polystyrene cups containing a 3:1 (v/v) mixture of sterile vermiculite and

sand. They were then transferred to earthen pots containing a 3:2:1 (v/v/v) mixture of garden soil, vermiculite and sand for hardening and acclimatisation (Fig. F). To prevent fungal growth, the in vitro plantlets were sprayed with 0.1% Bavistin (BASF Styrenics Pvt. Ltd, Mumbai, India) once in a week. Humidity was maintained by frequent spraying of water and covered by perforated polythene cover. After 35 days, the hardened plantlets were transferred to soil in the experimental garden of the Department. The in vitro regenerated plants were survived and showed vigorous growth with little morphological variation. Thus, the successful in vitro protocol for regeneration and transplantation of *Aristolochia indica* L. is achieved.

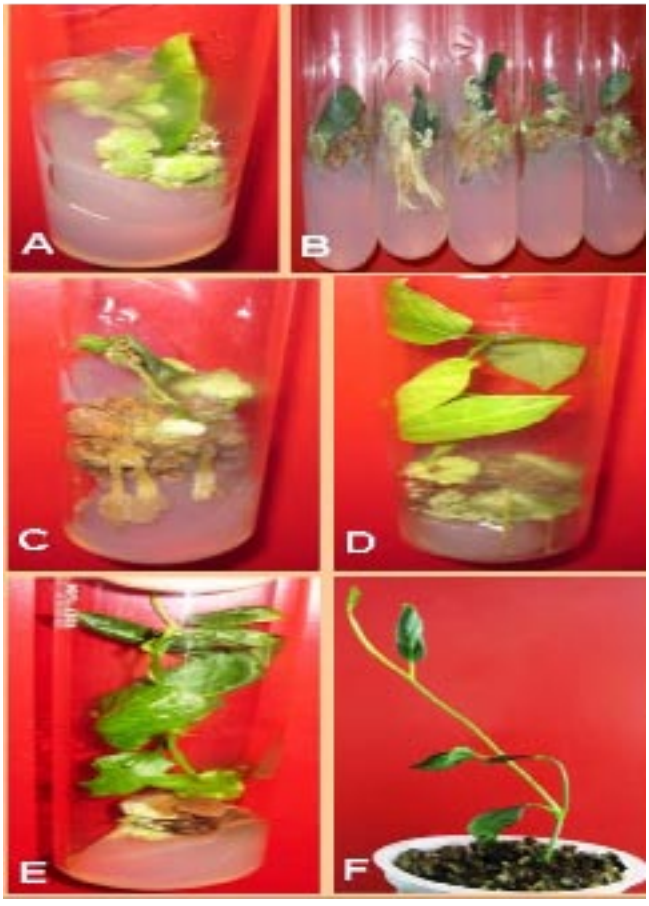

Figure 1. *In vitro* regeneration of Aristolochia indica L.

A. Callus induction from leaf explants in BAP (0.8 mg/L). B. Callus induction and initiation of shoot & thick roots from nodal explant. C. Regeneration of shoots and roots derived from leaf callus. D. Regeneration of shoot and roots from nodal callus. E. Shoot differentiation & root induced from nodal callus in MS+BAP (0.8mg/L)+NAA (0.5 mg/L). F. Acclimatised in vitro grown plantlet in greenhouse condition.

4. Discussion

Leaf and nodal explants of *Aristolochia indica* L. were cultured on MS medium supplemented with various concentrations of Kn and BAP individually and in combinations with NAA and IAA.

Leaf explants showed callus induction on MS medium supplemented with BAP (0.8 mg/L), but the growth of callus was slow & took nearly 35 days for complete proliferation into a rapid mass of callus. (Table.1. Fig. A & B). Similarly, Remashree *et al*.,[19] induced callus in 40 days from leaf explants of *Aristolochia indica* L. on MS medium supplemented with BAP (2 mg/L) + NAA (2 mg/L) and Ashish *et al*.,[3] induced callus from leaf explants of Abelmoschus moschatus on MS medium supplemented with BAP (0.3 mg/L) + NAA (2 mg/L).

Initially, callus was also induced at the both ends of nodal explants and subsequently from entire surface of the each explant segments in 3 weeks. Further subculture these explants with little mass of callus were rapidly proliferated into a large mass of soft, green and friable callus on MS medium supplemented with 0.8 mg/L BAP (Table 1.and Fig.C & D). Similarly, Siddique *et al*.,[22] induced callus from nodal explants of *Aristolochia indica* L. on MS medium supplemented with 2.0 mg/L BAP +1.0 mg/L NAA. Manjula *et al*.[9] used BAP (13.31 μM) and NAA (2.69 μM) for induction of callus from axillary shoot of *Aristolochia indica* L. with the addition of 1 mg/L of Phloroglucinol. High frequency callus mediated shoot morphogenesis has been reported in other medicinal plants like Phyllanthus amarus (Sen *et al*.,) [21], Phyllanthus niruri (Padmapriya and Mohammad) [13] and Scoparia dulcis L. (Premkumar *et al*.,)[16].

Plant regeneration via indirect shoot organogenesis has been achieved from both leaf and nodal explant culture. However, nodal segments were responded better compared to leaf explants for the induction callus and for further proliferation (Table 1. Fig. D & E).

Shoot differentiation and regeneration was observed from the leaf callus (85 %) and nodal callus (95%) after subcultured on MS with 0.8 mg /L BAP+0.5 mg /L NAA after six weeks of culture (Fig.C & D).

Siddique *et al*.,[22] obtained highest percent (85 %) of shoot regeneration from the nodal explants on MS medium supplemented with BAP (1.0 mg/L) and NAA (2.5 mg/L). Similarly, Siddique *et al*.,[23] obtained highest percent (95 %) of shoot regeneration from the axillary shoots on MS medium supplemented with Kn (2.5 mg/L) and BAP (1.0 mg/L).

Soniya and Sujitha [25] obtained multiple shoots from shoot tip and nodal explant derived callus of *Aristolochia indica* L. on MS medium supplemented with 2-iP (6 mg dm-3) and also direct regeneration of shoot from leaf and internodal explants in BAP (4 mg dm-3) and NAA (8 mg dm-3).

However, in the present study, a combination of BAP (0.8 mg/L) and NAA (0.5 mg/L) showed the best response than Kn and IAA individually and in combinations (Table 1).

Regenerated shoots from both explants were separated and subcultured repeatedly on fresh MS medium with 0.8 mg/L BAP where the number of shoots (85 %) increased up to 8.6 ±0.33 from leaf callus and (90%) with 9.2±0.14 from nodal callus per culture (Table 1).

MS medium supplemented with BAP (0.8 mg/L) and NAA (0.5 mg/L) elicited the maximum shoot proliferation (14.5±0.28) and

shoot elongation (1.63±0.08 cm) from leaf explants (Fig.1.B). Similar results have been also observed in leaf explants of in *Aristolochia indica* L. (Remashree *et al.*)[19] and Abelmoschus moschatus (Ashish *et al.*)[3].

Maximum shoot proliferation (16.4±0.23) and shoot elongation (1.69±0.03 cm) was achieved on BAP (0.8 mg/L) + NAA (0.5 mg/L) from nodal explants. (Fig.1. D & E).

In the present study, nodal explants showed better response than the leaf explants in induction of shoot and its elongation. The same effect of BAP and NAA on induction and elongation of shoots has also been reported from nodal explants of Enicostemma littorale (Nagarathnamma *et al.*)[11].

Pramod and Jayaraj[14] achieved shoot regeneration from nodal explants of Blepharis molluginifolia Pers. on MS medium containing 0.5 mg /L BAP + 0.5 mg /L IAA for 6 weeks of culture. In the same year, Hassan *et al.*[5] achieved shoot regeneration from nodal explants of Phlogacanthus thyrsiflorus Nees. on MS medium supplemented with 1.0 mg/L BAP + 0.5 mg/L NAA.

In vitro shoots of *Aristolochia indica* L. were separated and transferred to MS medium containing different concentrations of NAA and IAA individually (Table 2). In vitro rooting (80%) from leaf calli derived in vitro shoots and (95%) from nodal calli derived in vitro shoots and found MS medium supplemented with 0.8 mg/L NAA was best for induction of in vitro roots. The maximum number of roots per shoot was 12.6±0.66 with a mean length of 2.3±0.08 cm from leaf callus and 14.3±0.33 with a mean length of 2.6 ±0.24 from nodal callus after 30– 40 days (Table 2). However, in Mentha piperita L. (Sujana and Naidu) [26] and Sida cordifolia L. (Pramod and Jayaraj)[15] basal MS medium supplemented with NAA alone was found to be most suitable for the regeneration of roots.

In the present work, thick and long roots were developed from in vitro grown shoots on shoot inducing medium (MS with 0.8 mg /L BAP +0.5 mg/L NAA) after 35–45 days of subculture of in vitro shoots. Applications of auxins, individually or in combinations for rooting was also reported for many other medicinal plants like Carnation (Aamir *et al.*)[1] (1.0 mg/L NAA), Abelmoschus moschatus (Ashish *et al.*)[3] (2 mg/L NAA).

After 6 weeks, in vitro rooted plantlets were gently removed and washed with sterile water then transferred to polystyrene cups containing a 3:1 (v/v) mixture of sterile vermiculite and sand. They were then transferred to earthen pots containing a 3:2:1 (v/v/v) mixture of garden soil, vermiculite and sand for hardening and acclimatisation (Fig. F). To prevent fungal growth, the in vitro plantlets were sprayed with 0.1% Bavistin (BASF Styrenics Pvt. Ltd, Mumbai, India) once in a week. Humidity was maintained by frequent spraying of water and covered by perforated polythene cover. A similar method to maintain humidity was practiced for hardening of Banana (Jasrai *et al.*)[6] and Alpinia purpurata (Rolf and Ricardo)[18]. After 35 days, the hardened plantlets were transferred to soil in the experimental garden of the Department. The in vitro regenerated plants were survived and showed vigorous growth with little morphological variation. Thus, the successful in vitro protocol for regeneration and transplantation of *Aristolochia indica* L. is achieved.

Conclusion

Wild medicinal plants are being depleted rapidly due to over-exploitation and unscientific methods of collection. Hence, in the present work, a protocol for in vitro regeneration of the threatened medicinal plant species *Aristolochia indica* L. has been developed. This protocol can be used to make this plant available throughout the year for traditional healers, pharmaceutical usages, germplasm conservation, commercial cultivation, and also for the production of secondary metabolites.

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Conflict of interest statement

We declare that we have no conflict of interest.

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