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Plant Regeneration from Axillary Shoots Derived Callus in *Aristolochia indica* Linn. an Endangered Medicinal Plant in Bangladesh

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Abstract: A procedure for rapid *in vitro* propagation of the aromatic and medicinal plant *Aristolochia indica* Linn. (Family: Aristolochiaceae) from axillary shoots is described. The highest percentage of callus induction was (95.00) on Murashige and Skoog (15) (MS) medium supplemented with 2.0 mg L^{-1} Kn and 1.0 mg L^{-1} BAP. Colour of the calli were mostly light green to dark green. Development of adventitious shoots occurred when the calli were subcultured in MS medium supplemented with BAP and Kn alone or in BAP combination with NAA and IAA or NAA, IAA and BAP in combination with Kn. The Highest percentage (95.00) of shoot regeneration was obtained in MS medium fortified with 2.5 mg L^{-1} Kn and 1.0 mg L^{-1} BAP. The elongated shoots developed roots on a medium containing 1 mg L^{-1} Kn. The rooted plants were transferred to soil.

Key words: Aromatic and medicinal plant, organogenesis, shoot, callus, node

INTRODUCTION

Aristolochia indica Linn. (Isswar mul) a member of Aristolochiaceae, is one of the most widespread used in Bangladesh. It is also rare and endangered medicinal plant. It is shrubby twining: stems long, slender woody at the base, grooved, glabrous, leaves variable. Flowers in few-flowered axillary racemes: bracts small, ovate, acuminate, opposite the pedicels; predicels longs thickened above. Perianth greenish white with globose inflated base then bent at a right angle. Seeds deltoid-ovate, acute, flat, winged. Its roots are widely used in pungent, pains in the joints, the seeds are useful in inflammations, biliousness, dry cough, dyspnoea of children purgative and the plant is good for snake bit, the juice of the leaves or roots of the plant is said to be a specific antidote for Cobra poisoning (Kirtikar and Basu, 1987)

Bangladesh has a rich heritage of herbal medicine amongst the countries of South East Asia. Over the centuries, people here have depended on the nature around them. Huge quantities of plant materials and extracts are imported for the manufacture of Ayurvedic, Unani and Homeopathic Medicines (Chatterjee and Sastry, 2000). Making health care and medical facilities available to the people is now a major concern of a large number of countries (Ghani, 2000). Due to the toxic and adverse reactions of synthetic and chemical medicines being observed round the globe herbal medicine has made a come back to improving the fulfillment of our present and future health needs. Religious-cultural faith, weak economy in accessibility and consequently lack of modern medicinal facilities in the villages seems to be the cause of dependence on these medicinal plant species in addition to their proven ameliorative effects (Sugandhi, 2000). Methyl ester of aristolic acid, a pure compound isolated from the roots of *Aristolochia indica* Linn. was found to exert 100% abortifacient activity at single oral dose of 60 mg L^{-1} kg b.wt. when administered on 6th or 7th day of pregnancy; 20 and 25% abortifacient effect were observed at the same dose on day (Pakrashi and Shaha, 1978). For further research into the biochemical compositions and potential medicinal values of this plant, an efficient *in vitro* regeneration system for the production of plants is required because field – grown plants may be subject to seasonal and somatic variations, infestations of bacteria, fungi and insects as well as environmental pollution that can affect the medicinal value of the harvested tissues (Geng *et al*., 2001). In addition, *in vitro* propagation methods offer powerful tools for germplasm conservation and the mass-multiplication of threatened plant species (Murch *et al*., 2000; Roja *et al*., 1991). Due to the collection of large amounts of these plants, especially of the underground organ, with subsequent eradication of the natural populations, the natural habitats are rapidly decreasing. Thus, there is an urgent need for the domestication of this species. The application of *in vitro*

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propagation techniques might offer the possibility of producing large numbers of uniform plants for further field culture. In nature, the species propagates through seeds and vegetative perennial rootstock. However, propagation protocols for this species (*Aristolochia indica*) *in vitro* have not yet been reported. Based on results from preliminary investigations on propagation via seed, we concluded that specific habitat conditions for seedling survival and growth are required. Also, vegetative propagation was not possible as the rootstock degenerates very quickly or, in the rare cases and vegetative cutting is not an adequate solution to meet the demand for this wildflower. For this reason, the development of an *in vitro* protocol will be of great importance for production of planting material to conserve the species and to offset the pressure on the natural populations.

MATERIALS AND METHODS

Field grown medicinal plants **Issor mul** (*Aristolochia indica)* were collected from Barind Tract of Rajshahi region in Bangladesh. Axillary shoots arising from nodes of nodal segments were collected and were washed thoroughly under running tap water, then treated with a few drops of Tween-80 and 1% Savlon for 10 mins with constant shaking. This followed by successive three washing with distilled water to make the material free from savlon. Surface sterilization was carried out with 0.1% $HgCl_2$ for 7 min followed by gentle shaking. After surface sterilization the segmented parts were thoroughly washed for several times with sterile distilled water. The sterilized parts of the plants were taken in a sterilized petridish and nodal portions 1-2 cm were excised 7 mm above the bud and 7 mm below the bud. Then explants were transferred in 25×150 mm culture tubes with 15 mL basal media (MS) supplemented with different hormone (2,4-D, Kn and BAP) concentrations for callus induction. Cultures were incubated at 27+2°C under the warm fluorescent light with intensity varied from 2000-3000 lux. pH was adjusted to 5.8 prior to autoclaving. Cultures were incubated at 25±1°C with 16th photoperiod. Callus from these primary cultures was transferred to MS medium containing different concentration and combinations of BAP, Kn, NAA and IAA for shoot differentiation and incubated in light. Data on shoot proliferation efficiency were recorded after 8 weeks of culture. Proliferated shoots were transferred to MS media with different concentrations of IBA, Kn, IAA and NAA for adventitious root formation.

RESULTS AND DISCUSSION

Induction of callus was observed on to MS media containing different concentrations and combination of 2,4-D, Kn and BAP within 10-12 days of incubation of axillary shoot explants depending upon the concentration and combination of growth regulators. The highest percentage of callus induction (95.00%) was observed on MS medium containing 2.0 mg L^{-1} Kn and 1.0 mg L^{-1} BAP (Murashige and Skoog, 1962). The highest callus growth in terms of fresh weight (988±9.5 mg) was observed in MS medium fortified with 2.0 mg L^{-1} Kn and 1.0 mg L^{-1} BAP (Table1 and Fig. 1A). The highest dry weight of callus (145±0.345 mg) was observed in MS medium containing 2.0 mg L^{-1} Kn and 0.5 mg L^{-1} BAP. Colour of calli was mostly light brown to whitish green and light green. It was observed that only light green calli produced shoot buds. In the present investigation it was observed that Kn alone promoted root formation. Rani and Grover (1999) also used Kn and with BAP for induction of callus with 85.00% frequency.

Development of adventitious shoots occurred when the calli were subcultured in MS medium supplemented with BAP and Kn alone or in combination with NAA or IAA and BAP in combination with Kn. Such a combined effect has also been reported in *Petasites hybridus* of family Asteraceae (Wildi *et al.*, 1998). Significant improvement in shoot formation over control has previously been achieved with the addition of cytokinins like BAP and Kn in many composites. For example Conchou *et al.* (1992), Le (1994), Nin *et al.* (1994), Fauconnier *et al.* (1996), Wildi *et al.* (1998), Cuenca *et al.* (1999) were used BAP and Kn in combination with different concentrations of NAA and IAA. The highest percentage (95.00) of shoot regeneration was obtained in MS medium fortified with 2.5 mg L^{-1} Kn and 1.0 mg L^{-1} BAP. The shoot buds first appeared as nodular growth within 3-4 weeks of culture and at the end of 4 weeks this nodular growth increased in size and produced leaf primordial. Maximum number of shoot buds (4.35±0.95) was obtained in MS+2.5 mg L^{-1} Kn +1.0 mg L^{-1} BAP (Table 2 and Fig. 1B).The highest length of shoots was 3.50±1.10 cm. in media containing 2.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA. It was also observed that calli subcultured on media with lower concentrations of Kn alone produced roots.

For adventitious root formation, plantlets were subcultured in MS medium supplemented with different concentrations of IBA, Kn, IAA and NAA. Rani and Grover (1999) also used IBA, Kn, IAA and NAA for roots formation. After 12–15 days post transfer to rooting medium, roots appeared. The highest percentage of root formation (95.00%) was observed on MS medium containing 2 and 0 mg L^{-1} IBA and followed by 85.00% in MS medium containing 1.0 mg L^{-1} Kn (Table 3 and Fig. 1C).

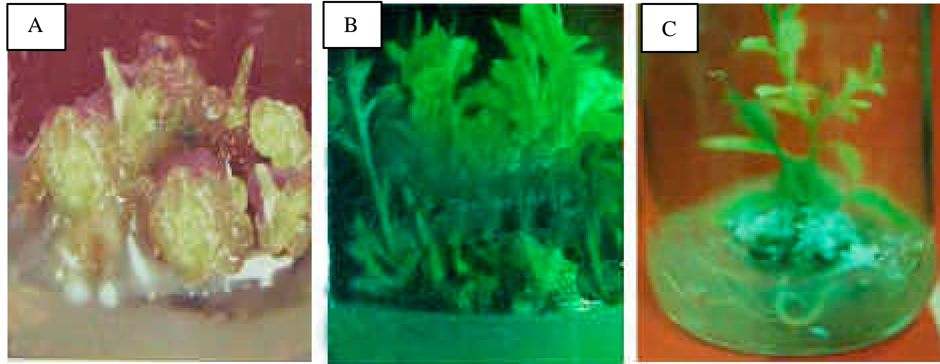

Fig. 1: Callus induction and shoot proliferation from axillary shoot explants of *Aristolochia indica* Linn.
A: Induction of light callus from axillary shoot buds on MS +2.0 mg L^{-1} Kn and 1 mg L^{-1} BAP
B: Induction of shoot from callus on MS +2.5 mg L^{-1} Kn and 0.5 mg L^{-1} BAP
C: Induction of root on MS +1.0 mg L^{-1} Kn

Table 1: Effect of different concentrations and combination of 2,4-D, Kn and BAP on induction of callus from nodal segment of *Aristolochia indica* Linn. after four weeks of culture

Treatments (mg L^{-1})	Days to callus initiation	% of callus formation	Colour	Texture of callus	Fresh weight of callus (mg)	Dry weight of callus (mg)
MS+2,4-D 0.5	12	-	-	-	-	-
MS+2,4-D 1.0	12	11.00	DG	F	384±10.6	78±0.222
MS+2,4-D 2.0	12	21.00	DG	F	445±10.0	87±0.253
MS+ BAP 0.5	12	41.00	DG	F	567±10.7	85±0.321
MS+ BAP 1.0	12	67.00	LG	F	601±5.3	87±0.328
MS+ BAP 2.0	12	70.00	LG	F	965±9.9	98±0.315
MS+ Kn 0.5	12	56.00	LG	C	888±8.9	133±0.421
MS+ Kn 1.0	12	75.00	LG	C	907±7.9	89±0.205
MS+ Kn 2.0	12	81.00	LG	C	808±5.7	110±0.281
MS+ Kn 0.5 + BAP 0.5	12	46.00	DG	C	705±10.9	123±0.305
MS+ Kn 0.5+ BAP 1.0	12	54.00	DG	C	982±9.8	97±0.305
MS+ Kn 0.5+ BAP 2.0	12	67.00	LG	F	875±8.9	89±0.317
MS+ Kn 1.0+ BAP 0.5	12	71.00	LG	F	789±8.7	125±0.281
MS+ Kn 1.0+ BAP 1.0	12	76.00	LG	C	764±8.9	103±0.422
MS+ Kn 1.0+ BAP 2.0	12	85.00	LG	F	835±9.7	98±0.372
MS+ Kn 2.0+ BAP 0.5	12	67.00	LG	C	897±7.9	145±0.345
MS+ Kn 2.0+ BAP 1.0	12	95.00	LG	C	988±9.5	95±0.317
MS+ K n 2.0+ BAP 2.0	12	68.00	DG	F	791±10.8	126±0.323

LG = Light green, DG = Dark green, C = Compact, F = Friable, ± :Mean ± SE

Table 2: Effect of BAP and Kn alone and BAP in combination with NAA and IAA or NAA, IAA and BAP in combination with Kn in MS medium on organogenesis of axillary shoots derived callus after 8 weeks of culture

	% of organogenic calli			
Growth regulars	Roots	Shoots	Number of shoot/callus	Length of shoot (cm)
BAP 0.5	-	-	-	-
BAP 1.0	-	20.00	3.75 ± 0.80	2.60 ± 1.17
BAP 2.5	-	50.00	2.25 ± 0.72	2.45 ± 1.15
BAP 3.5	-	20.00	2.65 ± 0.77	2.65 ± 1.09
BAP 2.5 + NAA 0.5	-	-	-	-
BAP2.5+ NAA 1.0	-	-	-	-
BAP2.5+ IAA 0.5	-	30.00	2.00± 0.53	3.50 ± 1.10
BAP2.5 + IAA 1.0	-	35.00	2.56±0.85	2.89±0.85
Kn 0.5	+	56.00	2.87±0.45	2.98±0.65
Kn 1.0	+	65.00	3.47±0.75	3.15±0.78
Kn 2.5	-	75.00	3.87±0.66	3.20±0.58
Kn 3.5	-	63.00	3.97±0.45	3.25±0.55
Kn 2.5 + NAA 0.5	-	-	-	-
Kn 2.5 + NAA 1.0	-	-	-	-
Kn 2.5 + IAA 0.5	-	35.00	3.58±0.54	2.75±0.83
Kn 2.5 + IAA 1.0	-	45.00	3.86±0.45	2.55±0.73
Kn2.5 +BAP0.5	-	45.00	2.00±0.52	2.50±0.90
Kn2.5 +BAP1.0	-	95.00	4.35±0.95	3.00±1.00
Kn2.5 +BAP2.0	-	35.00	2.25±0.54	2.25±1.09

Table 3:Effect of Kn, IBA, IAA and NAA alone or in combination in MS medium on rooting after 35 days of culture

Growth regulators	Shoots rooted (%)	Root length (cm)	Root morphology
Kn 1.0	85.00	4.6±0.5	Thin, long, Callus at base
Kn 2.0	77.00	4.3±0.4	Thin, long, Callus at base
Kn 4.0	70.00	3.25± 0.9	Fragile, long
IBA 0.5	75.00	3.5±0.9	Thin, long
IBA 1.0	90.00	4.4±0.6	Thin, long
IBA 2.0	95.00	4.8±0.4	Thin, long
IBA 2.0 + IAA2.0	80.00	5.5±0.4	Thin, long
IBA 4.0 + IAA 2.0	80.00	5.2±0.5	Thin, long
IBA 6.0+ IAA 2.0	80.00	2.25±0.2	Thin,
IBA 2.0 + NAA1	70.00	4.2±0.7	Thick, long
IBA 4.0+ NAA2	70.00	5.2±0.6	Thick, long
IBA 6.0+ NAA4	25.00	5.1±0.4	Thick, long

Many roots were found to be 4.3 –5.5 cm long after 30 days of subcultured. The planlets were transferred to pots containing a sand/soil mixture (1:1) initially covered with beakers. *Aristolochia indica* Linn. is normally propagated through seeds and being an open pollinated plant, the inherent variability attributable to recombination is expected. This species is of economic interest for its wide ranging pharmacological activity and one of the major constraints in utilizing natural populations is the existence of plant to plantchemovariability. It is hoped that a standard protocol to induce multiple shoots in culture may provide a more homogeneous source of plants.

In conclusion, we report an efficient and easy to handle protocol for micropropagation of the endangered medicinal plant (*Aristolochia indica* Linn*)*. This protocol provides a successful and rapid technique that can be used for *Exsitu* conservation. As a part of domestication strategy, these plants can be grown and further cultivated in fields. The application of this protocol can help minimize the pressure on wild populations and contribute to the conservation of the valuable flora of the Bangladesh.

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