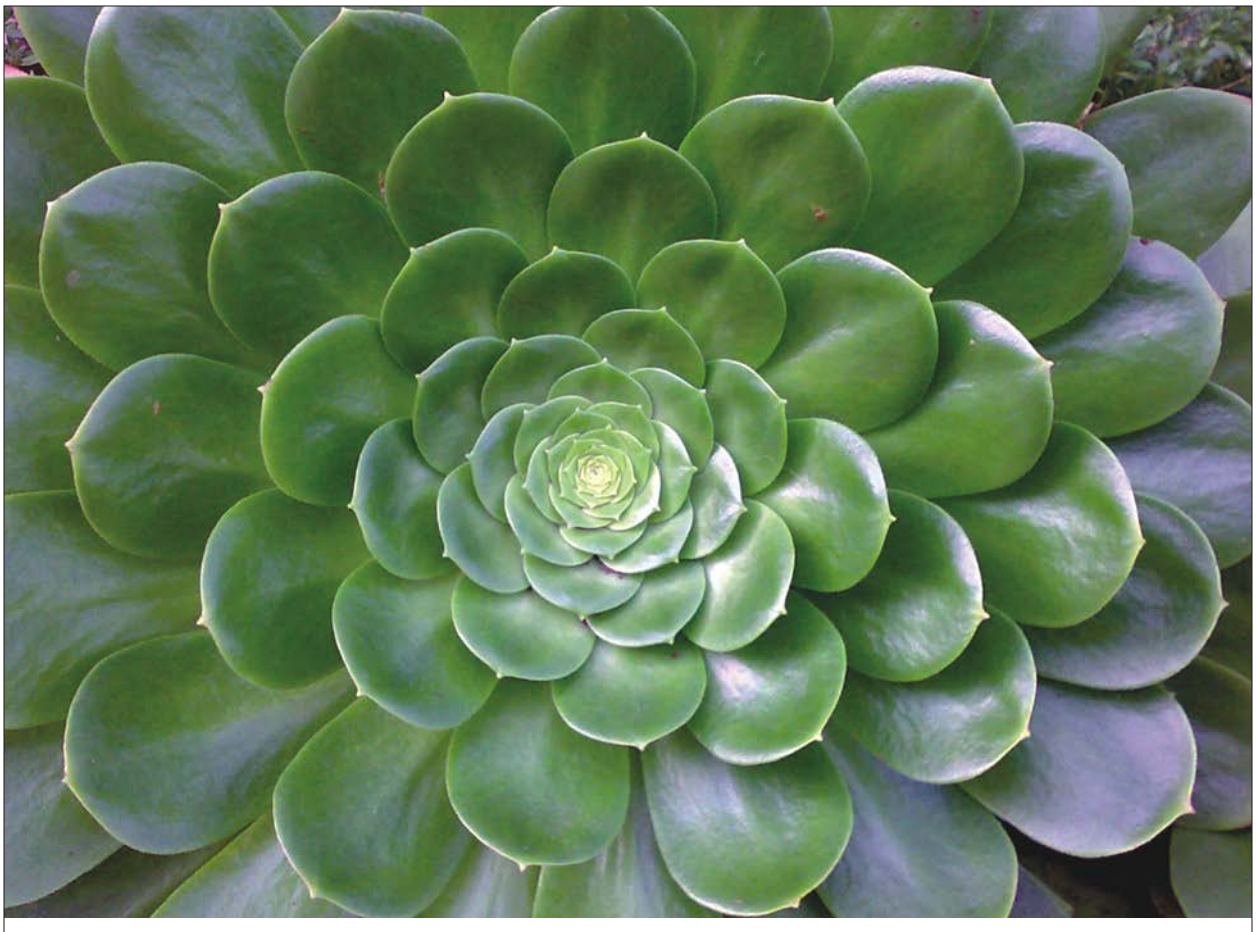

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Comparative *in vitro* Study of Plant Regeneration from Axillary Shoot Derived Callus in *Aristolochia indica* Linn. and *Hemidesmus indicus* (L.) R. Br-Endangered Medicinal Plants in Bangladesh

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Abstract: A procedure for rapid *in vitro* propagation of the aromatic and medicinal plants *Aristolochia indica* Linn and *Hemidesmus indicus* R.B (Family: Aristolochiaceae and Asclepiadaceae respectively) from axillary shoots is described. The highest percentage of callus induction was 92% on MS medium supplemented with 1.0 mg L^{-1} α-naphthalene acetic acid (NAA) and 1.0 mg L^{-1} 6-benzyladenine (BA) for *A. indica* while 98% was achieved on MS medium supplemented with 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} Kinetin (Kn) for *H. indicus*. The colour of the calli was mostly light to dark green. Development of adventitious shoots occurred when calli were subcultured on MS medium supplemented with BA and Kn alone or in BA combined with NAA and IAA, or NAA, IAA and BAP combined with Kn. The highest percentage (90%) of shoot regeneration in *A. indica* was obtained on MS medium fortified with 1.0 mg L^{-1} BA and 2.5 mg L^{-1} NAA, but a 98% rate on MS medium supplemented with 2.5 mg L^{-1} Kn and 1.0 mg L^{-1} NAA for *H. indicus*. Regenerated shoots rooted best on MS medium containing 2.5 mg L^{-1} Kn and 2.0 mg L^{-1} IBA. Plantlets were transferred to pots containing sand and soil mixture and acclimatized in a culture room. Finally rooted plants were transferred to soil.

Key words: Aromatic and medicinal plant, axillary shoots, callus, organogenesis, shoot

Introduction

Aristolochia indica Linn. (Isswar mul in Bengali) a member of Aristolochiaceae, is one of the most widely used in Bangladesh. It is also a rare and endangered medicinal plant (Rahman, 2001). It is a shrubby twiner; stems are long, slender and woody at the base, grooved, glabrous, with leaves variable. Flowers occur in few-flowered axillary racemes; bracts are small, ovate, acuminate and opposite the pedicels; seeds are deltoid-ovate, acute, flat and winged. Its roots are widely used in a pungent concoction to relieve joint pain while the seeds are useful in relieving inflammation, biliousness, dry cough and dyspepsia of children. The plant is also good for snake bite, while the juice of the leaves or roots of the plant are said to be a specific antidote for Cobra poisoning (Kirtikar and Basu, 1987). Methyl ester of aristolic acid, a pure compound isolated from its roots was found to exert 100% abortifacient activity at a single oral dose of 60 mg kg^{-1} b. wt when administered on the 6th or 7th day of pregnancy; a 25% abortifacient effect were observed at the same dose on the day of abortion (Pakrashi and Shaha, 1978).

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Hemidesmus indicus R. Br (Anantamul), a member of the Asclepiadaceae, is also a rare and endangered plant. It is a climbing, slender plant with twining woody stems and rust-coloured bark, with leaves opposite, petiolate, entire, smooth, shiny and firm, varying in shape and size according to their age. Flowers are small, green on the outside, deep purple on the inside, in axillary, sessile racemes, they are imbricated with flowers. The root is long, tortuous, cylindrical and little branched. It is used in the treatment of leprosy, leucoderma, itching, asthma, bronchitis, leucorrhoea, dysentery, diarrhoea, piles, sypilis and paralysis. It is said to promote health and cures all kinds of diseases caused by vitiated blood (Kirtikar and Basu, 1987).

Huge quantities of plant materials and extracts are imported for the manufacture of Ayurvedic, Unani and Homeopathic Medicines (Sudipto Chatterjeeand Sastry, 2000). Making health care and medical facilities available to the people is now a major concern of a large number of countries (Abdul Ghani, 2000). Due to the toxic and adverse reactions of synthetic and chemical medicines being observed around the globe herbal medicine has made a come back to improving the fulfillment of our present and future health needs. Religious-cultural faith, weak economic accessibility and consequently a lack of modern medicinal facilities in these villages seems to be the cause a dependence on these medicinal plant species in addition to their proven ameliorative effects (Sugandhi, 2000). 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. In addition, *in vitro* propagation methods offer powerful tools for germplasm conservation and the mass-multiplication of threatened plant species (Murch *et al*., 2000).

The propagation of these two plants is mainly by seed, but seed viability is limited to one year. These species are of economic interest for their wide ranging pharmacological activity and one of the major constraints in utilizing natural populations is the existence of plant-to-plant chemovariability. However, propagation protocols *in vitro* for both these species 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 rare cases, the 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 natural populations.

Materials and Methods

The experiment was conducted at the Plant Biotechnology Laboratory at the Institute of Biological Sciences, Rajshahi University, Bangladesh. The regeneration performance of two endangered medicinal plants, *Aristolochia indica* Linn and *Hemidesmus indicus* (L.) R.Br. was tested over a three year period. Explants were collected from *in vivo* grown plants and their axillary shoots (1-2 cm) were used as initial explants for establishing callus. They were washed thoroughly under running tap water, then treated with a few drops of Tween-80 and 1% Savlon for 10 min with constant shaking. This was followed by three successive washes with sterile distilled water to remove the savlon. Surface sterilization was carried out with 0.1% $HgCl_2$ for seven minutes followed by gentle shaking. After surface sterilization the segmented parts were thoroughly washed several times with sterile distilled water. Then explants were transferred to 25×150 mm culture tubes with 15 mL basal medium (MS) supplemented with different hormone (6-benzyladenine (BA), indolebutyric acid (IBA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D) and Kinetin (Kn)) concentrations on basal Murashige and Skoog (1962) (MS) medium for callus induction. Cultures were incubated at 25±2°C

under a warm fluorescent light whose intensity varied from 2000-3000 lux. pH was adjusted to 5.8 prior to autoclaving. Cultures were incubated at 25±1°C with a 16 h photoperiod. Similar sterilization techniques were reported by Evans *et al.* (1983), Pierik (1987) and Thorpe (1981), Vasil (1984) for plant A, plant B, plant C and plant D, respectively. Callus from these primary cultures was transferred to MS medium containing different concentrations and combinations of BA, 2,4-D, Kn, NAA and IAA for shoot differentiation and cultures were incubated in the light. Hormonal concentrations were controlled by the addition of 1.0 mg L^{-1} phloroglucinol (PG) to the callus induction medium, as suggested by Manjula *et al.* (2003).

Data on shoot proliferation efficiency were recorded after 8 weeks of culture. Proliferated shoots were transferred to MS with different concentrations of IAA, NAA, Kn and IBA for adventitious root formation.

Results and Discussion

In the present investigation it was observed that exogenous supply of an auxin and often in combination with a cytokinin to the medium is essential for callus induction but their requirement depends strongly on the genotype and endogenous hormone content of the explant. Pierik (1987) and Rao and Lee (1986) reported that an intermediate level of auxin and cytokinin in the medium usually promote callusing. However, many other factors like genotype, composition of the nutrient media and physical growth factors such as light, temperature, moisture etc. are important for callus induction (Pierik, 1987).

Callus Induction

In *A. indicus* there was a wide range of variation in the percentage callus formation and average fresh weight. Among the different concentrations and combinations of 2,4-D, IAA, NAA, BA and Kn, 1.0 mg L^{-1} NAA + 2.0 mg L^{-1} BAP showed the highest percentage (92%) callus formation, followed by 80% callus induction on MS medium containing 2.0 mg L^{-1} NAA with 1.0 mg L^{-1} IAA from axillary shoot explants. The lowest percentage of callusing was 15% on MS medium containing 0.5 mg L^{-1} 2,4-D (Table 1 and Fig. 1). Highest callus growth-in terms of fresh weight (952±1.60 mg) was observed on MS medium fortified with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} BA. The highest dry weight of calli (109±0.241 mg) was observed at the same concentration and whose colour was mostly light green to dark green. Callus induction was observed on MS media containing different concentrations and combinations of auxins (2,4-D, NAA and IAA) and cytokinins (BA and Kn). The cytokinin: auxin ratio for good callusing in medicinal plants was found to be 1:2. The hormonal concentrations were controlled by the addition of 1.0 mg L^{-1} PG to the callus induction medium Manjula *et al.* (2003). Induction of callus from axillary shoot explants has also been reported by Chandramu *et al.* (2003), Joshi and Dhar (2003), Siddique *et al.* (2002) and Manjula *et al.* (2003). Calli originating from axillary shoot explants underwent organogenesis to regenerate shoots whereas leaf portions led to rhizogenesis with no shoot formation. Pattnaik and Chand (1996), Sahoo and Chand (1998) and Thiruvengadam and Jayabalan (2000) observed somatic embryogenesis in callus cultures of medicinal plants. In the present investigation, it was observed that for callus induction from different explants of *A. indica,* 2,4-D, IAA, or NAA alone produced callus but BA in combination with NAA was more suitable than 2,4-D, IAA, or NAA alone. Manjula *et al.* (2003) also used BA and with NAA for callus induction. A similar, favourable combined effect of auxin and cytokinin have also been reported in *Adenophora triphylla* (Chen *et al.*, 2001), *Saussurea obvallata* (Joshi and Dhar, 2003) and *Artemisia judaica* (Liu *et al.*, 2002). However, in the present investigation, a combination of NAA with BA gave the best response.

Table 1: Effect of different concentrations and combinations of 2,4-D, Kn, BA and NAA on callus induction from axillary shoot explants of *Aristolochia indica* and *Hemidesmus indicus* after four weeks of culture

	Aristolochia indica					*Hemidesmus indicus*				
Treatments (mg L^{-1})	% of callus formation	Colour	Texture of callus	Fresh weight of callus (mg) ±SE	Dry weight formation (mg)±SE	% of callus formation	Colour	Texture of callus	Fresh weight of callus (mg) ±SE	Dry weight formation (mg)±SE
2,4-D 0.5+PG1.0	15.00	LG	F	341±0.52	74±0.524	-	-	-	-	-
2,4-D 1.0+PG1.0	18.00	LG	F	320±0.75	80±0.351	-	-	-	-	-
2,4-D 1.5+PG1.0	22.00	LG	F	442±0.55	88±0.242	18.00	Cr	F	345±0.64	58±0.264
2,4-D 2.0+PG1.0	38.00	LG	F	523±1.52	75±0.243	35.00	Cr	F	653±1.25	65±0.223
2,4-D 2.5+PG1.0	25.00	Cr	F	441±1.32	64±0.374	25.00	LG	F	564±1.28	66±0.372
2,4-D 3.0+PG1.0	17.00	Cr	F	321±1.21	65±0.241	15.00	LG	F	455±1.23	63±0.363
IAA 0.5+PG1.0	35.00	G	C	455±1.20	75±0.234	34.00	G	C	463±1.24	78±0.332
IAA 1.0+PG1.0	65.00	Cr	C	681±1.32	89±0.641	76.00	DG	C	678±1.44	85±0.464
IAA 1.5+PG1.0	60.00	DG	C	596±1.45	76±0.522	65.00	DG	C	564±1.35	79±0.356
IAA 2.0+PG1.0	56.00	DG	C	528±1.42	71±0.341	50.00	DG	C	578±1.86	75±0.248
IAA 2.5+PG1.0	43.00	G	C	431±1.65	54±0.452	45.00	G	C	432±1.91	64±0.354
IAA 3.0+PG1.0	28.00	Cr	C	374±1.56	43±0.351	25.00	G	C	345±1.28	63±0.485
NAA 0.5+PG1.0	35.00	G	F	551±1.25	50±0.452	28.00	G	F	454±1.81	61±0.393
NAA 1.0+PG1.0	55.00	G	F	641±1.42	61±0.468	55.00	G	F	654±1.36	64±0.446
NAA 1.5+PG1.0	60.00	DG	C	755±1.66	72±0.391	67.00	DG	C	725±1.55	78±0.326
NAA 2.0+PG1.0	70.00	DG	C	797±1.57	85±0.385	74.00	DG	C	899±1.44	88±0.227
NAA 2.5+PG1.0	62.00	DG	F	612±1.45	70±0.355	65.00	DG	F	798±1.47	75±0.427
NAA 3.0+PG1.0	44.00	DG	F	637±1.55	60±0.284	35.00	DG	F	637±1.55	62±0.284
NAA 0.5+Kn 0.5+PG1.0	32.00	LG	F	422±1.25	44±0.573	38.00	LG	F	454±1.55	45±0.564
NAA 0.5+Kn 1.0+PG1.0	42.00	G	F	612±1.42	52±0.461	45.00	LG	F	687±1.44	59±0.463
NAA 0.5+Kn 1.5+PG1.0	54.00	DG	C	625±1.22	63±0.355	84.00	DG	C	786±1.64	66±0.352
NAA 0.5+Kn 2.0+PG1.0	65.00	DG	C	821±1.51	75±0.344	98.00*	DG	C	845±1.75	78±0.334
NAA 0.5+Kn 2.5+PG1.0	40.00	Cr	C	720±1.55	61±0.351	75.00	DG	C	776±1.84	69±0.325
NAA 0.5+Kn 3.0+PG1.0	30.00	Cr	C	572±1.12	58±0.461	34.00	DG	C	674±1.24	58±0.342
NAA 1.0+BAP 0.5+PG1.0	32.47	G	F	418±1.12	71±0.474	35.47	DG	F	453±1.48	74±0.483
NAA 1.0+BAP 1.0+PG1.0	60.00	Cr	F	670±1.23	82±0.551	61.00	LG	F	678±1.57	86±0.554
NAA 1.0+BAP 1.5+PG1.0	75.00	Cr	F	880±1.60	92±0.343	76.00	Cr	F	786±1.68	95±0.443
NAA 1.0+BAP 2.0+PG1.0	92.00*	LG	F	952±1.60*	109±0.241*	77.00	Cr	F	934±1.68*	113±0.244*
NAA 1.0+BAP 2.5+PG1.0	60.00	LG	F	825±1.82	68±0.351	68.00	LG	F	798±1.28	67±0.354
NAA 1.0+BAP 3.0+PG1.0	30.00	LG	F	551±1.41	52±0.432	35.00	Cr	F	465±1.44	52±0.422
NAA 2.0+IAA 0.5+PG1.0	55.00	DG	C	414±1.52	82±0.470	68.00	Cr	C	456±1.48	83±0.457
NAA 2.0+IAA 1.0+PG1.0	80.00	DG	C	888±1.48	95±0.237	85.00	DG	C	876±1.54	96±0.328
NAA 2.0+IAA 1.5+PG1.0	65.00	DG	C	752±1.56	88±0.451	67.00	DG	C	769±1.59	88±0.355
NAA 2.0+IAA 2.0+PG1.0	55.00	DG	C	615±1.34	75±0.312	55.00	Cr	C	632±1.43	79±0.306
NAA2.0+IAA 2.5+PG1.0	42.00	DG	C	622±1.48	60±0.563	47.00	DG	C	556±1.94	65±0.554
NAA 2.0+IAA 3.0+PG1.0	35.00	LG	C	422±1.51	63±0.525	35.00	LG	C	465±1.55	63±0.445
2,4-D 2.0+Kn0.5+PG1.0	20.00	G	F	340±1.51	54±0.261	28.00	LG	F	342±1.45	55±0.426
2,4-D 2.0+Kn1.0+PG1.0	36.00	LG	F	445±1.34	58±0.145	35.00	LG	F	453±1.33	57±0.346
2,4-D 2.0+Kn1.5+PG1.0	45.00	DG	F	552±1.53	64±0.248	49.00	LG	F	563±1.25	62±0.354
2,4-D 2.0+Kn2.0+PG1.0	50.00	DG	F	623±1.61	68±0.359	50.00	Cr	F	674±1.66	67±0.253
2,4-D 2.0+Kn2.5+PG1.0	45.00	LG	C	549±1.52	62±0.291	44.00	LG	C	598±1.48	64±0.492
2,4-D 2.0+Kn3.0+PG1.0	32.00	LG	C	445±1.29	60±0.213	35.00	Cr	C	454±1.95	60±0.394

*G = Green, LG = Light green, DG = Dark Green, Cr = Creamy, C = Compact, F = Friable

In case of *H. indicus*, among the different concentrations and combinations of 2,4-D, IAA, NAA, BA and Kn, 0.5 mg L^{-1} NAA + 2.0 mg L^{-1} Kn showed the highest percentage (98%) callus formation, followed by 85% on MS medium containing 2.0 mg L^{-1} NAA with 1.0 mg L^{-1} IAA from axillary shoot explants. The lowest percentage of callusing was 18% on MS medium containing 1.5 mg L^{-1} 2,4-D (Table 1 and Fig. 1). Highest callus growth-in terms of fresh weight (934±1.67 mg) was observed on MS medium fortified with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} BA. The highest dry weight of calli (113±0.244 mg) was observed at the same concentration. The colour of calli was mostly light to dark green. It was observed that only light green calli produced shoot buds, whose proliferation was observed on MS + 1.5 mg L^{-1} BA +1.0 mg L^{-1} NAA and on MS + 2.5 mg L^{-1} Kn + 0.5 mg L^{-1} NAA. Shoot buds first appeared as nodular growths within 3-4 weeks of culture and at the end of 4 weeks this nodular growth increased in size and produced leaf primordia. The maximum number of shoot buds was obtained on MS + 2.0 mg L^{-1} BA +1.0 mg L^{-1} NAA. In the present study, it was observed that for callus induction from different explants of *H. indicus* 2,4-D, IAA, or NAA alone produced callus but Kn in combination with NAA was more suitable than 2,4-D, IAA, or NAA alone. Patnaik and Debata (1996) also used Kn and with NAA for callus induction in *H. indicus*. A similar, favourable combined effect of auxin (NAA) and cytokinin (Kn) have also been

reported in *Narcissus* sp. (Squires and Langton,1990), *Yucca glauca* (Bentz *et al*., 1988), *Capsicum frutescens* (Subhash and Christopher, 1988) and *Opuntia polyacantha* (Mauseth and Halperin, 1975). However, in the present investigation, a combination of NAA with Kn was proved to be the most efficient hormonal combination for better callusing in *H. indicus*.

Shoot Proliferation

For shoot differentiation, light green compact calli were subcultured on MS medium supplemented with different concentrations and combinations of BA and Kn alone and in combination with different concentrations of NAA and IAA. Such a combined effect has also been reported in *Petasites hybridus* of the family Asteraceae (Wildi *et al*., 1998). Significant improvement in shoot formation over controls has previously been achieved with the addition of cytokinins such as BA 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) and Cuenca *et al*. (1999) all used BA(P) and Kn in combination with different concentrations of NAA and IAA for the effective callusing. Calli were subcultured at different concentrations of IAA and NAA (0.5-3.0 mg L^{-1}), which when applied alone failed to differentiate into any shoots. The shoot buds first appeared as nodular growths within 3-4 weeks of culture and at the end of 4 weeks this nodular growth increased in size and produced leaf primordia. Calli produced shoots only when IAA and NAA was combined with cytokinins. For obtaining a high frequency of embryogenic cultures, growth regulator supplement and incubation conditions during the callusing phase were critical. In dark incubation MS medium supplemented with 1.0 mg L^{-1} BAP and 2.5 mg L^{-1} NAA for *A. indica* and 1.0 mg L^{-1} Kn with 2.5 mg L^{-1} NAA for *H. indicus* were found to be best for producing embryogenic callus. A high frequency of embryogenesis was obtained after transferring the callus to MS medium supplemented with BAP and NAA

In the case of *A. indica* the highest of shoot regeneration was observed in 1.0 mg L^{-1} l BA with 2.5 mg L^{-1} NAA and the number of shoots per callus was 5.77±0.45, followed by 85% at 2.5 mg L^{-1} Kn with 1.5 mg L^{-1} NAA, while the number of shoots per callus was 4.72±0.65 from axillary shoots derived from calli. The lowest (15%) percentage of shoot regeneration was recorded on media having 0.5 mg L^{-1} BA while 2.35±0.94 shoots per callus were observed on media having 2.0 mg L^{-1} Kn with 3.0 mg L^{-1} IAA (Table 2 and Fig. 1). In the present study it was observed that BA in combination with NAA was more suitable than BA alone.

The percentage of explants for shoot induction and the number of shoots per explant increased with increasing concentration of cytokinins (BA, Kn) and auxin (NAA). Thiruvengadam and Jayabalan (2000) found similar effects in *Vitex negundo,* when axillary shoot explants were cultured on medium containing BA and NAA. Studies by Mercier *et al*. (1992) in *Gomphrena officinalis*, Vincet *et al*. (1992) in *Kaempferia galangal* and Mathur *et al*. (1987) in *Rauvolfia serpentina* also revealed the enhancing effect of medium fortified with BA and NAA in shoot multiplication. Highest length of shoots (4.75±0.64 cm) was recorded at 1.0 mg L^{-1} Kn from axillary shoot-derived calli, followed by 4.55±0.66 at 1.5 mg L^{-1} while the shortest shoots (2.21±0.31 cm) were recorded at 0.5 mg L^{-1} BA from axillary shoot-derived calli.

In *H. indicus*, the shoot buds first appeared as nodular growths within 3-4 weeks of culture and at the end of 4 weeks this nodular growth increased in size and produced leaf primordia. Highest (98%) percentage of shoot regeneration was recorded in 2.5 mg L^{-1} Kn with 1.0 mg L^{-1} NAA from axillary shoot-derived calli, followed by 85% in 1.0 mg L^{-1} BA with 2.0 mg L^{-1} Kn (Table 2 and Fig. 1). However, in the present study, a combination of NAA with Kn gave the best response. Such a combined effect has also been reported by Sen and Sharma (1991). A reduction in shoot number with increasing cytokinin concentration has been reported by Patnaik and Debata (1996). Thus, the use of a comparatively lower concentration of plant growth regulators in our protocol is an important factor

Table 2: Effect of different concentrations and combinations of NAA, IAA, Kn and BAP on organogenesis of axillary shoot derived callus of *Aristolochia indica* and *Hemidesmus indicus* Data were recorded after four weeks of culture

	Aristolochia indica				*Hemidesmus indicus*			
	% of organogenic calli		No. of	Length of	% of organogenic calli		No. of	Length of
Treatments (mg L^{-1})	Root	Shoot	shoot/callus x±SE	shoot (cm) x±SE	Root	Shoot	shoot/callus x±SE	shoot (cm) x±SE
BAP 0.5	-	15.00	2.33±0.42	2.21±0.31	-	-	-	-
BAP 1.0	-	20.00	2.22±0.52	2.33±0.47	-	-	-	-
BAP 1.5	-	25.00	2.45±0.65	4.55±0.66	-	15.00	2.45±0.65	3.58±0.65
BAP 2.0	-	35.00	2.53±0.45	3.65±0.83	-	38.00	3.53±0.45	4.68±0.83
BAP 2.5	-	25.00	3.64±0.55	3.64±0.75	-	25.00	3.64±0.54	3.64±0.72
BAP 3.0	-	18.00	3.55±0.43	3.63±0.64	-	18.00	3.55±0.43	3.63±0.65
Kn 0.5	-	-	-	-	-	-	-	-
Kn 1.0	-	35.00	2.75±0.45	4.75±0.64*	-	38.00	2.75±0.44	3.85±0.64
Kn 1.5	-	64.00	3.63±0.35	3.74±0.56	-	64.00	3.65±0.35	3.78±0.56
Kn 2.0	-	56.00	3.78±0.86	3.76±0.48	-	56.00	3.78±0.86	3.76±0.48
Kn 2.5	-	44.00	2.33±0.95	3.65±0.55	-	45.00	2.35±0.91	3.64±0.54
Kn 3.0	-	30.00	2.47±0.28	3.62±0.85	-	26.00	2.47±0.28	3.62±0.85
BAP1.0+NAA 0.5	-	35.00	2.54±0.85	3.65±0.97	-	25.00	2.54±0.82	3.65±0.93
BAP1.0+NAA 1.0	-	55.00	2.54±0.35	3.64±0.45	-	55.00	2.54±0.35	3.64±0.46
BAP1.0+NAA 1.5	20	65.00	3.23±0.54	2.78±0.76	20	67.00	3.23±0.54	2.78±0.76
BAP1.0+NAA 2.0	30	72.00	4.95±0.45	2.86±0.62	30	72.00	4.27±0.42	2.88±0.67
BAP1.0+NAA 2.5	20	90.00*	5.70±0.55*	3.75±0.77	-	65.00	3.95±0.45	3.75±0.77
BAP1.0+NAA 3.0	-	65.00	3.37±0.74	4.45±0.84	-	36.00	3.37±0.55	3.62±0.84
Kn2.5+NAA0.5	-	68.00	3.54±0.75	3.45±0.64	22	68.00	3.54±0.55	3.45±0.64
Kn2.5+NAA 1.0	35	70.00	4.35±0.45	2.56±0.63	35	98.00*	4.87±0.44	2.51±0.65
Kn2.5+NAA 1.5	45	85.00	4.72±0.65	2.66±0.65	25	78.00	4.88±0.64	2.66±0.62
Kn2.5+NAA 2.0	25	65.00	3.45±0.74	3.78±0.84	20	67.00	3.45±0.75	4.88±0.85*
Kn2.5+NAA 2.5	-	45.00	3.74±0.84	3.69±0.75	-	45.00	3.76±0.84	3.69±0.75
Kn2.5+NAA 3.0	-	35.00	2.72±0.94	3.58±0.85	-	34.00	2.73±0.24	3.58±0.89
BAP 1.0+Kn 0.5	-	25.00	2.52±0.48	3.76±0.83	15	30.00	2.53±0.48	3.74±0.83
BAP 1.0+Kn 1.0	22	45.00	2.78±0.54	3.86±0.57	21	65.00	3.78±0.57	3.86±0.54
BAP 1.0+Kn 1.5	31	67.00	3.86±0.68	3.95±0.43	19	75.00	3.86±0.68	3.95±0.43
BAP 1.0+Kn 2.0	25	72.00	4.44±0.65	2.96±0.47	13	85.00	4.42±0.65	2.15±0.45
BAP 1.0+Kn 2.5	-	65.00	3.98±0.48	3.67±0.85	-	65.00	3.98±0.28	3.67±0.84
BAP 1.0+Kn 3.0	-	30.00	3.65±0.44	3.55±0.67	-	35.00	3.65±0.44	3.55±0.67
BAP2.0+IAA 0.5	-	35.00	2.55±0.44	3.83±0.57	-	38.00	2.56±0.48	3.83±0.57
BAP 2.0+IAA 1.0	-	55.00	2.76±0.54	3.96±0.87	-	58.00	2.76±0.54	3.96±0.88
BAP 2.0+IAA 1.5	-	67.00	3.69±0.57	2.88±0.54	-	67.00	3.69±0.59	2.88±0.54
BAP 2.0+IAA 2.0	-	55.00	3.32±0.43	2.79±0.86	-	55.00	3.32±0.43	2.79±0.86
BAP 2.0+IAA 2.5	-	45.00	2.56±0.94	3.65±0.54	-	45.00	2.56±0.94	3.65±0.54
BAP 2.0+IAA 3.0	-	35.00	2.65±0.55	3.65±0.45	-	35.00	2.65±0.55	3.63±0.45
Kn 2.0+IAA0.5	-	20.00	2.43±0.45	3.46±0.75	-	25.00	2.30±0.45	3.55±0.76
Kn 2.0+IAA 1.0	-	35.00	2.55±0.37	3.57±0.46	-	35.00	2.53±0.38	3.57±0.46
Kn 2.0+IAA 1.5	-	45.00	2.63±0.25	3.65±0.54	-	45.00	2.63±0.25	3.62±0.54
Kn 2.0+IAA 2.0	-	50.00	2.74±0.67	2.67±0.53	-	50.00	2.74±0.66	2.67±0.53
Kn 2.0+IAA 2.5	-	45.00	2.98±0.48	2.66±0.95	-	44.00	2.98±0.48	2.64±0.92
Kn 2.0+IAA 3.0	-	35.00	2.35±0.94	3.60±0.93	-	34.00	2.55±0.94	3.65±0.93

to consider, as it minimizes the risk of producing genetically altered individuals, or somaclonal variants (Edson *et al*., 1996). The lowest (15%) percentage of shoot regeneration was recorded on media containing 1.5 mg L^{-1} BA. The highest number of shoots per callus was 4.88±0.44 on media having 2.5 mg L^{-1} Kn with 1.5 mg L^{-1} NAA. Fewest shoots per callus was 2.30±0.45 on media containing 2.0 mg L^{-1} Kn with 0.5 mg L^{-1} IAA; longest shoots (4.88±0.85 cm) at 2.5 mg L^{-1} Kn with 2.0 mg L^{-1} NAA; shortest shoots (2.51±0.65) at 2.5 mg L^{-1} Kn with 1.0 mg L^{-1} NAA from axillary shoot-derived calli.

Rooting of Shoots

For adventitious root formation, axillary shoots of *A. indica* and *H. indicus* were excised and cultured on MS medium with different concentrations and combinations of auxin and cytokinins.

In the case of *A. indica*, Kn with IBA was found to be the most effective in the induction of roots without inducing callus. The medium supplemented with NAA+IBA induced callus at the base of the shoots. However a lower concentration of NAA + Kn induced 3-6 roots together with calli. On the

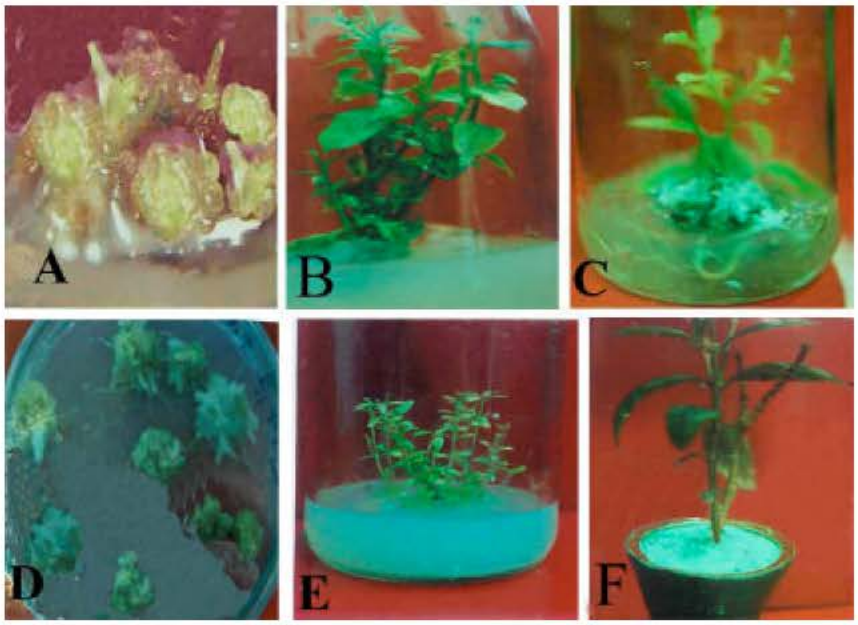

Fig. 1: Callus induction and shoot proliferation from axillary shoot explants of *Aristolochia indica* Linn and *Hemidesmus indicus* (L.) R. Br.

A: Induction of light green callus from axillary shoot explants on MS + 1.0 mg L^{-1} NAA + 2.0 mg L^{-1} BAP+ 1.0 mg L^{-1} PG from *Aristolochia indica*.

B: Induction of shoot from callus on MS + 1.0 mg L^{-1} BAP and 2.5 mg L^{-1} NAA from *Aristolochia indica*.

C: A potted plant after 2 month of transplantation in *Aristolochia indica*.

D: Induction of dark green callus from axillary shoot explants on MS + 0.5 mg L^{-1} NAA + 2.0 mg L^{-1} Kn +1.0 mg L^{-1} PG from *Hemidesmus indicus*.

E: Induction of shoot from callus on MS + 2.5 mg L^{-1} Kn + 1.0 mg L^{-1} NAA from *Hemidesmus indicus*

F: A potted plant after 2 month of transplantation in *Hemidesmus indicus*

other hand, treatment with IBA with Kn showed the highest rooting (87%) on media with 2.5 mg L^{-1} Kn and 2.0 mg L^{-1} IBA, followed by 83% on media with 2.5 mg L^{-1} Kn and 1.5 mg L^{-1} IBA, both from axillary shoots (Table 3 and Fig. 1). Similar results were observed in sweetgum by Kim *et al*. (1997).

In the case of *H. indicus*, IBA was found to be the most effective for root induction without inducing callus. Thinvengadam and Jayabalan (2000) also reported similar findings in *Vitex negundo* and Siddique *et al*. (2003) in *H. indicus*. Whereas the medium supplemented with NAA + IBA induced callus at the base of the shoots, treatment of Kn with IBA showed the highest rooting (90%) in 2.5 mg L^{-1} Kn + 2.0 mg L^{-1} IBA, followed by 85% in media having 1.5 mg L^{-1} IBA, both from axillary shoot-derived explants. Similar observations were noticed in sweetgum (Kim *et al*., 1997). The highest average number of roots per shoot (8.68±0.84) was recorded in media having 2.5 mg L^{-1} Kn + 2.0 mg L^{-1} IBA (Table 3 and Fig. 1). A similar observation was reported by Patnaik *et al*. (1996) who used IBA with Kn for root induction in *H. indicus*. Generally shoots can be rooted very rapidly in medicinal plants and in nearly all cases, roots were obtained on MS medium with auxin (Flick *et al*., 1989). After 12-15 days post-transfer to rooting medium, roots appeared and by day 30,

Table 3: Effect of different concentrations and combinations of, Kn, IBA, NAA and IAA on root induction from shoots obtained from axillary shoot explants of *Aristolochia indica* and *Hemidesmus indicus*. Data were recorded after four weeks of culture

	Aristolochia indica				*Hemidesmus indicus*			
Treatments (mg L^{-1})	Frequency of root initiation	Average No. of roots/shoot	Length of shoot (cm) ±SE	Root morphology	Frequency of root initiation	Average No. of roots/shoot	Length of shoot (cm) ±SE	Root morphology
IBA0.5	25.00	3.54±0.40	6.35±0.55	Fragile, long	45.00	3.92±0.61	4.33±0.55	Thin, short
IBA 1.0	35.00	4.75±0.44	4.86±0.65	Fragile, long	72.00	6.54±0.73	3.45±0.88	Thin, short
IBA 1.5	65.00	5.55±0.75	5.77±0.56	Thin, long	85.00	3.45±0.65	2.58±0.62	Fragile, long
IBA2.0	76.00	3.75±0.76	4.78±0.45	Thin, long	72.00	7.53±0.45	4.65±0.83*	Fragile, long
IBA2.5	65.00	3.35±0.95	3.65±0.54	Thin, long	45.00	6.64±0.54	4.64±0.72	Fragile, long
IBA 3.0	45.00	4.45±0.28	3.65±0.85	Thin, long	43.00	4.57±0.43	3.63±0.64	Fragile, long
Kn2.5+IBA0.5	45.00	6.55±0.85	3.65±0.95	Thick, long	25.00	3.58±0.42	3.45±0.67	Thin, long
Kn2.5+IBA 1.0	75.00	5.50±0.75	4.65±0.45	Thick, long	38.00	4.78±0.44	4.85±0.64	Thin, long
Kn2.5+IBA1.5	83.00	6.45±0.54	3.77±0.76	Thick, long	64.00	5.65±0.35	5.78±0.56	Thin, long
Kn2.5+IBA 2.0	87.00*	7.27±0.45*	2.88±0.60*	Thick, short	90.00*	8.68±0.84*	6.76±0.48	Thin, long
Kn2.5+IBA 2.5	75.00	3.95±0.40	3.75±0.75	Thick, short	64.00	3.32±0.95	4.64±0.54	Thin, long
Kn2.5+IBA 3.0	65.00	3.35±0.55	4.65±0.85	Thick, short	46.00	4.47±0.28	2.62±0.85	Thin, long
BAP2.0+IBA 0.5	25.00	4.55±0.48	3.85±0.55	Fragile, long	45.00	6.54±0.85	3.65±0.93	Thick, short
BAP 2.0+IBA1.0	55.00	5.75±0.55	3.96±0.84	Fragile, long	65.00	5.54±0.35	4.64±0.46	Thick, short
BAP 2.0+IBA 1.5	65.00	5.65±0.59	4.85±0.54	Fragile, long	75.00	5.23±0.54	3.78±0.76	Thick, short
BAP 2.0+IBA 2.0	55.00	6.32±0.43	3.79±0.86	Fragile, long	76.00	5.53±0.32	2.88±0.65*	Thick, long
BAP 2.0+IBA 2.5	45.00	5.56±0.84	3.65±0.43	Thin, long	75.00	3.95±0.45	3.75±0.75	Thick, long
BAP 2.0+IBA 3.0	35.00	4.65±0.55	3.64±0.45	Thin, long	70.00	3.37±0.55	4.65±0.83	Thick, long
IBA 2.0+NAA0.5	-	-	-	-	35.00	4.55±0.48	3.84±0.55	Fragile, long
IBA2.0+NAA 1.0	35.00	3.55±0.35	4.55±0.56	Thick, long	55.00	5.76±0.55	4.96±0.84	Fragile, long
IBA2.0+NAA 1.5	45.00	4.65±0.85	4.65±0.54	Thick, long	67.00	5.69±0.59	4.88±0.54	Fragile, long
IBA2.0+NAA 2.0	55.00	3.74±0.66	3.67±0.55	Thick, long	55.00	6.32±0.43	3.79±0.86	Fragile, short
IBA2.0+NAA 2.5	44.00	3.98±0.48	3.64±0.95	Thick, long	45.00	5.56±0.94	3.65±0.55	Fragile, short
IBA2.0+NAA 3.0	35.00	2.55±0.94	3.65±0.95	Thick, long	35.00	4.65±0.55	3.63±0.45	Fragile, long
Kn 2.5+NAA0.5	-	-	-	-	25.00	5.42±0.45	3.55±0.76	Thick, long
Kn2. 5+NAA1.0	-	-	-	-	35.00	5.53±0.35	4.57±0.56	Thick, long
Kn 2.5+NAA1.5	25.00	3.45±0.65	4.58±0.65	Fragile, long	45.00	6.63±0.25	4.62±0.54	Thick, long
Kn 2.5+NAA2.0	38.00	4.53±0.45	4.65±0.83	Fragile, long	55.00	7.74±0.66	3.67±0.55	Thick, long
Kn 2.5+NAA2.5	25.00	3.64±0.54	4.64±0.75	Fragile, long	45.00	3.98±0.48	3.64±0.95	Thick, long
Kn 2.5+NAA3.0	15.00	2.55±0.45	3.65±0.64	Fragile, long	35.00	3.53±0.94	3.61±0.93	Thick, long
Kn2.5+IAA0.5	-	-	-	-	38.00	3.55±0.54	3.45±0.65	Thin, long
Kn2.5+IAA 1.0	-	-	-	-	46.00	4.87±0.43	4.59±0.66	Thin, long
Kn2.5+IAA 1.5	35.00	4.86±0.65	4.66±0.65	Thin, long	58.00	4.86±0.64	4.66±0.65	Thin, long
Kn2.5+IAA 2.0	65.00	3.45±0.75	3.78±0.84	Thin, long	67.00	3.45±0.75	3.78±0.84	Thin, long
Kn2.5+IAA 2.5	45.00	3.76±0.84	3.69±0.75	Thin, long	45.00	3.76±0.84	3.69±0.75	Thin, long
Kn2.5+IAA 3.0	30.0	3.75±0.64	3.58±0.59	Thin, long	34.00	3.73±0.64	3.58±0.55	Thin, long

many were found to be 4.3-5.5 cm long. The plantlets were transferred to pots containing a sand : soil mixture (1:1) initially covered with beakers.

These two species are of economic interest for their wide-ranging pharmacological activity but one of the major constraints in utilizing natural populations is the existence of plant-to-plant chemovariability. It is hoped that a standard protocol to induce multiple shoots in culture may provide a more homogeneous source of plants.

From the foregoing discussion it can be concluded that among the two medicinal plants *Hemidesmus indicus* exhibited a better response to shoot regeneration in different artificial media compositions than *Aristolochia indica*. In the present study significant variation in multiple shoot regeneration from axillary shoots was also noted. The method can be usefully employed for mass propagation of endangered medicinal plants. A higher concentration of auxins or cytokinins took a maximum period for shoot proliferation whereas lower concentrations of either produced longer shoots. In conclusion, an efficient and easy-to-handle protocol was established for the micropropagation of two endangered medicinal plants, *Hemidesmus indicus* and *Aristolochia indica*. This protocol provides a successful and rapid technique that can be used for *ex situ* conservation. As part of a 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 Bangladesh.

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