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

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Abstract: The experiment was conducted at Plant Biotechnology Laboratoty of Institute of Biological Sciences, Rajshahi University, Bangladesh. Subsequent regeneration performance of two medicinal plants (*Aristolochia indica* Linn and *Hemidesmus indicus* R.B were studied throughout the experimental period. Different concentrations and combinations of growth regulators were used in MS medium to observe the callus induction, Callus regeneration and root induction. Among the different concentrations and combinations of growth regulators, the highest percentage of callus induction was 90.00% on MS medium supplemented with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP from *Aristolochia indica* where as 95.00% on MS medium supplemented with 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} Kn from *Hemidesmus indicus*. The highest percentage (85.00) of shoot regeneration was obtained in MS medium fortified with 1.0 mg L^{-1} BAP and 2.5 mg L^{-1} NAA from *Aristolochia indica* where as 95.00% on MS medium supplemented with 2.5 mg L^{-1} Kn and 1.0 mg L^{-1} NAA from *Hemidesmus 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, acclimatized in a culture room and finally 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 (Rahman, 2001). 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; 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, dyspepsia of children 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). Methyl ester of aristolic acid, a pure compound isolated from its roots was found to exert 100% abortifacient activity at single oral dose of 60 mg kg^{-1} b. wt when administered on 6th or 7th day of pregnancy; 25% abortifacient effect were observed at the same dose on day (Pakrashi and Shaha, 1978).

Hemidesmus indicus R. Br. (Anantamul) a member of the Asclepiadaceae, is also rare and endangered. It is climbing slender plant with twining woody stems and a rust-coloured bark, leaves opposite, petiolate, entire, smooth, shiny and firm, varying in shape and size according to their age.

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Flowers small green outside, deep purple inside, in axillary, sessile racemes, imbricated with flowers. The root is long, tortuous, cylindrical, little branched. It is used in Leprosy, Leucoderma, Itching, asthma, Bronchitis, Leucorrhoea, Desyntery, 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).

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 (Chatterjee *et al*., 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 pollutions 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; Sugandhi, 2000; Ghani, 2000).

The propagation of these two plants are mainly by seed, but seeds viability is limited to one year. These plants prefer light (sandy), medium (loamy) and heavy (clay) soils and requires well-drained soil. 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 plantchemovariability. It is hoped that a standard protocol to induce multiple shoots in culture may provide a more homogeneous source of plants. However, propagation protocols for these species (*Aristolochia indica* and *Hemidesmus indicus*) *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

This programme was carried out from June 2002 to June 2005. Explants were collected from *in vivo* grown medicinal plants *Aristolochia indica* and *Hemidesmus indicus* (L.) R.Br. and their nodal segments (1-2 cm) were used for establishing callus and 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 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. Then explants were transferred in 25×150 mm culture tubes with 15 mL basal media (MS) supplemented with different hormone (BAP, IBA, NAA, 2,4-D, Kn and NAA) concentrations for callus induction (Murashige and Skoog, 1962). Cultures were incubated at 25±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. The similar sterilization techniques were reported by Evans *et al*. (1983) Pierik (1987) Thorpe (1981) and Vasil (1984). Callus from these primary cultures was transferred to MS medium containing different concentration and combinations of BAP, 2,4-D, Kn, NAA and IAA for shoot differentiation and incubated in light. The hormonal concentrations were controlled by the addition of 1.0 mg L^{-1} PG (Phloroglucinol) to the callus induction medium (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

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

In case of *Aristolochia indicus* there was a wide range of variation in percentage of callus formation and average fresh weight of callus. Among the different concentrations and combinations of 2,4-D, IAA, NAA, BAP, Kn, 1.0 mg L^{-1} NAA + 2.0 mg L^{-1} BAP showed the highest percentage (90.00%) of callus formation which was followed by 78.00% callus induction in MS medium containing 2.0 mg L^{-1} NAA with 1.0 mg L^{-1} IAA from nodal explants. The lowest percentage of callusing was 12% in MS media containing 0.5 mg L^{-1} 2, 4-D (Table 1 and Fig. 1). Highest callus growth in terms of fresh weight (942±1.65 mg) was observed in MS medium fortified with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} BAP. The highest dry weight of calli (105±0.241 mg) was observed same concentration. Colour of calli was mostly light green to dark green. Callus induction was observed in MS media containing different concentrations and combinations of auxins (2,4-D, NAA and IAA) and cytokinins (BAP 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 (Phloroglucinol) to the callus induction medium Manjula *et al.* (2003). Induction of callus from nodal explants has been reported by Chandramu *et al.* (2003), Joshi and Dhar (2003) Siddique *et al.* (2002) and Manjula *et al.* (2003). Calli originating from nodal explants under went organogenesis to regenerate shoots whereas leaves 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 *Aristolochia indica* 2,4-D, IAA, NAA, alone produced callus but BAP in combination with NAA was more suitable than 2,4-D, IAA, NAA alone. Manjula *et al.* (2003) also used BAP and with NAA for callus induction. 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), *Artemisia judaica* (Liu *et al*., 2002). However, in the present investigation, a combination of NAA with BAP gave the best response.

In case of *Hemidesmus indicus*. Among the different concentrations and combinations of 2,4-D, IAA, NAA, BAP, Kn, 0.5 mg L^{-1} NAA + 2.0 mg L^{-1}Kn showed the highest percentage (95.00%) of callus formation which was followed by 79.00% callus induction in MS medium containing 2.0 mg L^{-1} NAA with 1.0 mg L^{-1} IAA from nodal explants. The lowest percentage of callusing was 11% in MS media containing 1.5 mg L^{-1} 2,4-D (Table 1 and Fig. 1). Highest callus growth in terms of fresh weight (944±1.67 mg) was observed in MS medium fortified with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} BAP. The highest dry weight of calli (102±0.244 mg) was observed same concentration. Colour of calli was mostly light green to dark green. It was observed that only light green calli produced shoot buds.

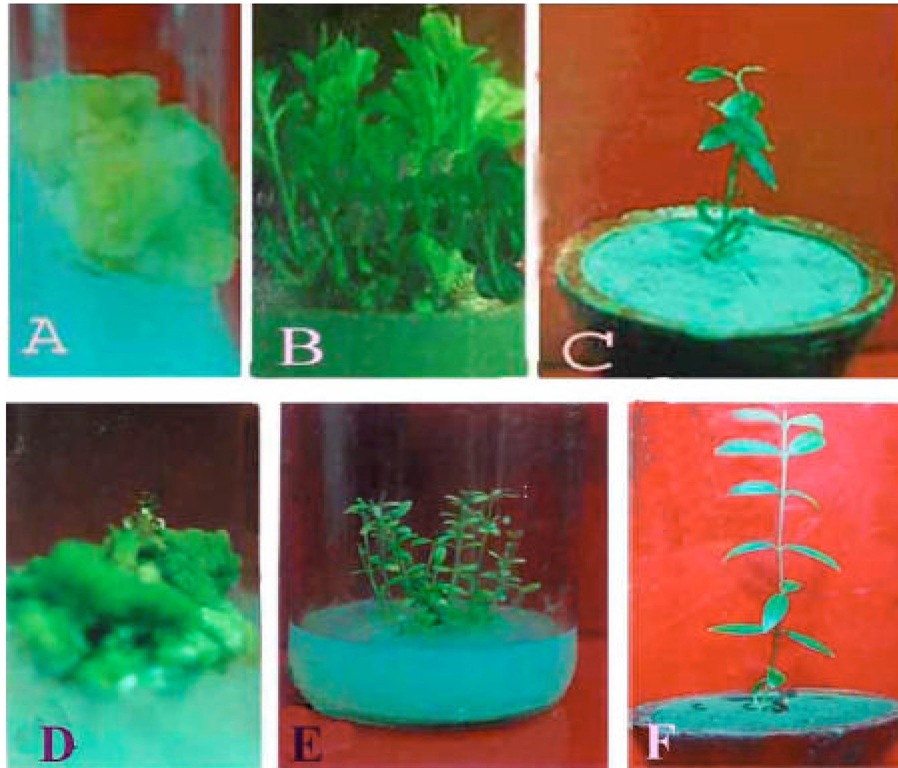

Fig. 1: Callus induction and shoot proliferation from axillary shoot explants of *Aristolochia indica* Linn and *Hemidesmus indicus* R.B. A. Induction of light green callus from nodal explants on MS + 1.0 mg L^{-1} NAA + 2.0 mg L^{-1} BAP+ 1.0 mg L^{-1} PG from *Aristolochia indica*. B. Induction of 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 nodal 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*

Proliferation of shoot buds was observed in MS+1.5 mg L^{-1} BAP +1.0 mg L^{-1} NAA and MS+2.5 mg L^{-1} Kn + 0.5 mg L^{-1} NAA. 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 was obtained in MS+2.0 mg L^{-1} BAP +1.0 mg L^{-1} NAA. In the present investigation, it was observed that for callus induction from different explants of *Hemidesmus indicus* 2,4-D, IAA, NAA, alone produced callus but Kn in combination with NAA was more suitable than 2,4-D, IAA, NAA alone. Patnaik and Debata (1996) also used Kn and with NAA for callus induction. 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*

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

	Aristolochia indica				
Treatments (mg L^{-1})	(%) Callus formation	Colour	Texture of callus	Fresh weight of callus (mg) $\bar{x}$±SE	Dry weight of callus (mg) $\bar{x}$±SE
2,4-D 0.5+PG1.0	12.00	LG	F	331±0.52	75±0.524
2,4-D 1.0+PG1.0	15.00	LG	F	320±0.75	81±0.351
2,4-D 1.5+PG1.0	21.00	LG	F	442±0.55	88±0.242
2,4-D 2.0+PG1.0	38.00	LG	F	523±1.52	75±0.243
2,4-D 2.5+PG1.0	22.00	Cr	F	441±1.32	64±0.374
2,4-D 3.0+PG1.0	15.00	Cr	F	321±1.21	65±0.241
IAA 0.5+PG1.0	35.00	G	C	455±1.20	75±0.234
IAA 1.0+PG1.0	70.00	Cr	C	681±1.32	89±0.641
IAA 1.5+PG1.0	60.00	DG	C	596±1.45	75±0.522
IAA 2.0+PG1.0	56.00	DG	C	528±1.42	71±0.341
IAA 2.5+PG1.0	43.00	G	C	441±1.65	54±0.452
IAA 3.0+PG1.0	28.00	Cr	C	374±1.56	43±0.351
NAA 0.5+PG1.0	25.00	G	F	551±1.25	51±0.452
NAA 1.0+PG1.0	55.00	G	F	641±1.42	61±0.468
NAA 1.5+PG1.0	60.00	DG	C	755±1.66	72±0.391
NAA 2.0+PG1.0	70.00	DG	C	797±1.57	85±0.385
NAA 2.5+PG1.0	62.00	DG	F	612±1.45	70±0.355
NAA 3.0+PG1.0	35.00	DG	F	637±1.55	62±0.284
NAA 0.5+Kn 0.5+PG1.0	30.00	LG	F	422±1.25	44±0.573
NAA 0.5+Kn 1.0+PG1.0	40.00	G	F	612±1.42	52±0.461
NAA 0.5+Kn 1.5+PG1.0	54.00	DG	C	625±1.22	60±0.355
NAA 0.5+Kn 2.0+PG1.0	65.00	DG	C	821±1.51	75±0.344
NAA 0.5+Kn 2.5+PG1.0	40.00	Cr	C	720±1.55	61±0.351
NAA 0.5+Kn 3.0+PG1.0	30.00	Cr	C	572±1.12	58±0.461
NAA 1.0+BAP 0.5+PG1.0	32.47	G	F	413±1.12	71±0.474
NAA 1.0+BAP 1.0+PG1.0	60.00	Cr	F	670±1.23	80±0.551
NAA 1.0+BAP 1.5+PG1.0	75.00	Cr	F	880±1.60	92±0.343
NAA 1.0+BAP 2.0+PG1.0	90.00*	LG	F	942±1.65*	105±0.241*
NAA 1.0+BAP 2.5+PG1.0	60.00	LG	F	815±1.82	68±0.351
NAA 1.0+BAP 3.0+PG1.0	30.00	LG	F	551±1.41	52±0.432
NAA 2.0+IAA 0.5+PG1.0	35.00	DG	C	410±1.52	82±0.470
NAA 2.0+IAA 1.0+PG1.0	78.00	DG	C	888±1.48	95±0.237
NAA 2.0+IAA 1.5+PG1.0	65.00	DG	C	752±1.56	88±0.451
NAA 2.0+IAA 2.0+PG1.0	50.00	DG	C	615±1.34	75±0.312
NAA2.0+IAA 2.5+PG1.0	42.00	DG	C	622±1.48	60±0.563
NAA 2.0+IAA 3.0+PG1.0	30.00	LG	C	421±1.51	62±0.525
2,4-D 2.0+Kn0.5+PG1.0	12.00	G	F	340±1.51	54±0.261
2,4-D 2.0+Kn1.0+PG1.0	36.00	LG	F	445±1.34	58±0.145
2,4-D 2.0+Kn1.5+PG1.0	45.00	DG	F	552±1.53	62±0.248
2,4-D 2.0+Kn2.0+PG1.0	50.00	DG	F	623±1.61	68±0.359
2,4-D 2.0+Kn2.5+PG1.0	40.00	LG	C	549±1.52	62±0.291
2,4-D 2.0+Kn3.0+PG1.0	32.00	LG	C	435±1.29	60±0.213

	Hemidesmus indicus				
Treatments (mg L^{-1})	(%) Callus formation	Colour	Texture of callus	Fresh weight of callus (mg) $\bar{x}$±SE	Dry weight of callus (mg) $\bar{x}$±SE
2,4-D 0.5+PG1.0	-	-	-	-	-
2,4-D 1.0+PG1.0	-	-	-	-	-
2,4-D 1.5+PG1.0	11.00	Cr	F	345±0.64	58±0.262
2,4-D 2.0+PG1.0	35.00	Cr	F	653 ±1.24	65±0.223
2,4-D 2.5+PG1.0	22.00	LG	F	564±1.28	64±0.372

Table 1: Continued

2,4-D 3.0+PG1.0	13.00	LG	F	451±1.23	63±0.364
IAA 0.5+PG1.0	30.00	G	C	463±1.24	78±0.332
IAA 1.0+PG1.0	76.00	DG	C	678±1.44	85±0.464
IAA 1.5+PG1.0	65.00	DG	C	565±1.35	78±0.356
IAA 2.0+PG1.0	50.00	DG	C	578±1.86	76±0.248
IAA 2.5+PG1.0	40.00	G	C	432±1.91	64±0.354
IAA 3.0+PG1.0	25.00	G	C	347±1.28	62±0.485
NAA 0.5+PG1.0	28.00	G	F	454±1.81	61±0.393
NAA 1.0+PG1.0	56.00	G	F	654±1.36	64±0.446
NAA 1.5+PG1.0	67.00	DG	C	723±1.55	78±0.326
NAA 2.0+PG1.0	72.00	DG	C	899±1.44	88±0.227
NAA 2.5+PG1.0	65.00	DG	F	795±1.47	75±0.427
NAA 3.0+PG1.0	35.00	DG	F	637±1.55	62±0.284
NAA 0.5+ Kn 0.5+PG1.0	38.00	LG	F	454±1.55	45±0.564
NAA 0.5+ Kn 1.0+PG1.0	45.00	LG	F	687±1.44	59±0.463
NAA 0.5+ Kn 1.5+PG1.0	54.00	DG	C	786±1.64	66±0.352
NAA 0.5+ Kn 2.0+PG1.0	95.00*	DG	C	845±1.75	78±0.334
NAA 0.5+ Kn 2.5+PG1.0	45.00	DG	C	776±1.84	69±0.325
NAA 0.5+ Kn 3.0+PG1.0	34.00	DG	C	673±1.24	58±0.342
NAA 1.0+ BAP 0.5+PG1.0	35.47	DG	F	453±1.48	74±0.483
NAA 1.0+ BAP 1.0+PG1.0	61.00	LG	F	678±1.57	86±0.554
NAA 1.0+ BAP 1.5+PG1.0	76.00	Cr	F	786±1.68	95±0.443
NAA 1.0+ BAP 2.0+PG1.0	77.00	Cr	F	944±1.67*	102±0.244*
NAA 1.0+ BAP 2.5+PG1.0	68.00	LG	F	798±1.28	67±0.354
NAA 1.0+ BAP 3.0+PG1.0	35.00	Cr	F	465±1.44	52±0.422
NAA 2.0+ IAA 0.5+PG1.0	38.00	Cr	C	456±1.48	83±0.457
NAA 2.0+ IAA 1.0+PG1.0	79.00	DG	C	876±1.54	96±0.328
NAA 2.0+ IAA 1.5+PG1.0	67.00	DG	C	769±1.59	88±0.354
NAA 2.0+ IAA 2.0+PG1.0	55.00	Cr	C	632±1.43	79±0.306
NAA 2.0+ IAA 2.5+PG1.0	47.00	DG	C	556±1.94	65±0.554
NAA 2.0+ IAA 3.0+PG1.0	35.00	LG	C	465±1.55	63±0.445
2,4-D 2.0+Kn0.5+PG1.0	25.00	LG	F	342±1.45	55±0.426
2,4-D 2.0+Kn1.0+PG1.0	35.00	LG	F	453±1.33	57±0.346
2,4-D 2.0+Kn1.5+PG1.0	49.00	LG	F	563±1.25	62±0.354
2,4-D 2.0+Kn2.0+PG1.0	50.00	Cr	F	674±1.66	67±0.253
2,4-D 2.0+Kn2.5+PG1.0	44.00	LG	C	598±1.48	64±0.492
2,4-D 2.0+Kn3.0+PG1.0	34.00	Cr	C	453±1.92	61±0.393

(Mauseth and Halperin, 1975) However, in the present investigation, a combination of NAA with Kn was proved as the most efficient medium composition for better callusing in *Hemidesmus indicus.*

For shoot differentiation light green compact calli were sudcultured on MS medium supplemented with different concentrations and combinations of BAP 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 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) and Cuenca *et al.* (1999) and were used BAP and Kn in combination with different concentrations of NAA and IAA.

Calli were subcultured with different concentrations of IAA and NAA (0.5-3.0 mg L^{-1}) alone failed to d ifferentiate any shoots. 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. 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}

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

	Aristolochia indica				*Hemidesmus indicus*			
	organogenic calli (%)		Number of shoot/callus	Length of shoot (cm)	organogenic calli (%)		Number of shoot/callus	Length of shoot (cm)
Treatments (mg L^{-1})	Root	Shoot	$\bar{x}$±SE	$\bar{x}$±SE	Root	Shoot	$\bar{x}$±SE	$\bar{x}$±SE
BAP 0.5	-	13.00	2.31±0.42	2.21±0.31	-	-	-	-
BAP 1.0	-	20.00	2.22±0.52	2.33±0.42	-	-	-	-
BAP 1.5	-	25.00	2.45±0.65	4.58±0.65	-	15.00	2.45±0.65	3.58±0.62
BAP 2.0	-	35.00	2.55±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	-	15.00	3.55±0.43	3.63±0.64	-	13.00	3.51±0.43	3.63±0.64
Kn 0.5	-	-	-	-	-	-	-	-
Kn 1.0	-	35.00	2.75±0.45	4.85±0.64*	-	38.00	2.78±0.44	3.85±0.64
Kn 1.5	-	64.00	3.65±0.35	3.78±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	2.35±0.95	3.65±0.55	-	44.00	2.32±0.91	3.64±0.54
Kn 3.0	-	25.00	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±0.93	-	25.00	2.54±0.82	3.61±0.93
BAP1.0+NAA 1.0	-	55.00	2.51±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	2.88±0.62	30	72.00	4.27±0.42	2.88±0.67
BAP1.0+NAA 2.5	20	85.00*	5.77±0.45*	3.75±0.77	-	65.00	3.95±0.45	3.75±0.77
BAP1.0+NAA 3.0	-	45.00	3.37±0.75	4.65±0.84	-	36.00	3.37±0.55	3.62±0.84
Kn2.5+ NAA0.5	-	68.00	3.55±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	2.59±0.63	35	95.00*	4.87±0.44	2.59±0.63
Kn2.5+ NAA 1.5	45	75.00	4.86±0.64	2.66±0.65	25	78.00	4.86±0.64	2.66±0.62
Kn2.5+ NAA 2.0	25	65.00	3.45±0.75	3.78±0.84	20	67.00	3.45±0.75	4.78±0.84*
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	2.75±0.94	3.58±0.85	-	34.00	2.73±0.24	3.58±0.89
BAP 1.0+ Kn 0.5	-	25.00	2.53±0.48	3.74±0.83	15	35.47	2.53±0.48	3.74±0.83
BAP 1.0+ Kn 1.0	22	45.00	2.78±0.57	3.86±0.50	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	2.95±0.43	13	80.00	4.42±0.65	2.15±0.43
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	-	35.00	3.65±0.44	3.55±0.67	-	35.00	3.65±0.44	3.52±0.67
BAP2.0+ IAA 0.5	-	35.00	2.55±0.48	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±0.88	-	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	3.35±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	-	25.00	2.42±0.45	3.45±0.75	-	25.00	2.42±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	2.65±0.95	-	44.00	2.98±0.48	2.64±0.92
Kn 2.0+ IAA 3.0	-	35.00	2.15±0.94	3.60±0.93	-	34.00	2.53±0.94	3.61±0.93

BAP with 2.5 mg L^{-1} NAA for *Aristolochia indica* and 1.0 mg L^{-1} Knwith 2.5 mg L^{-1} NAA for *Hemidesmus indicus* were found best for producing embryogenic callus. High frequency of embryogenesis was obtained after transferring the callus to MS medium supplemented with BAP and NAA

In case of *Aristolochia indica* the highest 85.00% of shoot regeneration was observed in 1.0 mg L^{-1} BAP with 2.5 mg L^{-1} NAA and number of shoots per callus was 5.77±0.45 and this was followed by 75.00% in 2.5 mg L^{-1} Kn with 1.5 mg L^{-1} NAA and number of shoots per callus was 4.86±0.64 from node derived calli. The lowest 13.00% of shoot regeneration was recorded in media having 0.5 mg L^{-1} BAP and number of shoots per callus was observed

(2.15±0.94) in media having 2.0g L^{-1} Kn with 3.0 mg L^{-1} IAA (Table 2 and Fig. 1). In the present investigation it was observed that BAP in combination with NAA was more suitable than BAP alone.

The percentage of explants for shoot induction and number of shoots per explant increased with increasing concentration of cytokynins (BAP, Kn) and auxin (NAA). Thiruvengadam and Jayabalan (2000) found similar effects in *Vitex negundo,* when nodal explants were cultured on the medium containing BAP and NAA. Studies of 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 BAP and NAA in shoot multiplication. Highest length of shoot 4.85±0.64 cm was recorded in 1.0 mg L^{-1} Kn from node derived calli which was followed by 4.58±0.65 in 1.5 mg L^{-1} and the lowest length of shoots 2.21±0.31 cm was recorded in 0.5 mg L^{-1} BAP from node derived calli.

In *Hemidesmus indicus*, 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. Highest 95.00% of shoot regeneration was recorded in 2.5 mg L^{-1} Kn with 1.0 mg L^{-1} NAA from node derived calli which was followed by 80.00% in 1.0 mg L^{-1} BAP with 2.0 mg L^{-1} Kn (Table 2 and Fig. 1). However, in the present investigation, 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 growth regulator in present protocol is an important factor to consider, as it minimizes the risk of producing genetically altered individuals (Edson *et al.*, 1996). The lowest 15.00% of shoot regeneration was recorded in media containing 1.5 mg L^{-1} BAP. The highest number of shoot per callus was 4.86±0.44 in media having 2.5 mg L^{-1}Kn with 1.5 mg L^{-1} NAA. The lowest number of shoot per callus was shoot 2.42±0.45 in media containing 2.0 mg L^{-1} Kn with 0.5 mg L^{-1} IAA. The highest length of shoot (4.78±0.84 cm) was recorded in 2.5 mg L^{-1} Kn with 2.0 mg L^{-1} NAA and lowest length of shoot was 2.49±0.63 in media having 2.5 mg L^{-1} Kn with 1.0 mg L^{-1} NAA from node derived calli.

Rooting of Shoots

For adventitious root formation, nodal segments of *Aristolochia indica* and *Hemidesmus indicus*, were excised and cultured on MS medium with different concentrations and combinations of auxin and cytokinins.

In case of *Aristolochia 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 lower concentration of NAA+ Kn induced 3-6 roots along with calli. On the other hand the treatment of IBA with Kn showed the highest rooting 85.00% inmedia having 2.5 mg L^{-1} Kn with 2.0 mg L^{-1} IBA and was followed by 80.00% in media 2.5 mg L^{-1} Kn + 1.5 mg L^{-1} IBA from node (Table 3 and Fig. 1). Similar observations were noticed in sweetgum by Kim *et al.* (1997).

In case of *Hemidesmus indicus*, IBA was found to be the more effective in the induction of roots without inducing callus. Thinvengadam and Jayabalan (2000) also reported similar observations in *Vitex negundo*. Siddique *et al.* (2003) in *Hemidesmus indicus.* Whereas the medium supplemented with NAA+IBA induced callus at the base of the shoots. On the other hand the treatment of Kn with IBA

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

	Aristolochia indica			
Treatments (mg L^{-1})	Frequency of root initiation	Average No. of roots/ shoot	Length of shoot (cm) $\bar{x}$±SE	Root morphology
IBA0.5	25.00	3.54±0.40	6.35±0.55	Fragile, long
IBA 1.0	35.00	4.75±0.44	4.85±0.65	Fragile, long
IBA 1.5	65.00	5.55±0.75	5.78±0.56	Thin, long
IBA2.0	76.00	3.78±0.76	4.76±0.45	Thin, long
IBA2.5	65.00	3.35±0.95	3.65±0.54	Thin, long
IBA 3.0	45.00	4.45±0.28	3.65±0.85	Thin, long
Kn2.5+ IBA0.5	45.00	6.55±0.85	3.65±0.95	Thick, long
Kn2.5+ IBA 1.0	75.00	5.50±0.75	4.65±0.45	Thick, long
Kn2.5+ IBA1.5	80.00	6.43±0.54	3.78±0.76	Thick, long
Kn2.5+ IBA 2.0	85.00*	7.27±0.45*	2.88±0.60*	Thick, short
Kn2.5+ IBA 2.5	75.00	3.95±0.40	3.75±0.75	Thick, short
Kn2.5+ IBA 3.0	65.00	3.35±0.55	4.65±0.85	Thick, short
BAP2.0+ IBA 0.5	35.00	4.55±0.48	3.83±0.55	Fragile, long
BAP 2.0+ IBA1.0	55.00	5.75±0.55	3.96±0.84	Fragile, long
BAP 2.0+ IBA 1.5	65.00	5.65±0.59	4.85±0.54	Fragile, long
BAP 2.0+ IBA 2.0	55.00	6.32±0.43	3.79±0.86	Fragile, long
BAP 2.0+ IBA 2.5	45.00	5.56±0.84	3.65±0.45	Thin, long
BAP 2.0+ IBA 3.0	35.00	4.65±0.55	3.63±0.45	Thin, long
IBA 2.0+NAA0.5	-	-	-	-
IBA2.0+NAA 1.0	35.00	3.55±0.35	4.55±0.56	Thick, long
IBA2.0+NAA 1.5	45.00	4.65±0.85	4.65±0.54	Thick, long
IBA2.0+NAA 2.0	55.00	3.74±0.66	3.65±0.55	Thick, long
IBA2.0+NAA 2.5	44.00	3.98±0.48	3.64±0.95	Thick, long
IBA2.0+NAA 3.0	35.00	2.55±0.94	3.65±0.95	Thick, long
Kn 2.5+NAA0.5	-	-	-	-
Kn2..5+ NAA1.0	-	-	-	-
Kn 2.5+ NAA1.5	25.00	3.42±0.65	4.55±0.65	Fragile, long
Kn 2.5+ NAA2.0	38.00	4.53±0.45	4.65±0.83	Fragile, long
Kn 2.5+ NAA2.5	25.00	3.64±0.54	4.64±0.75	Fragile, long
Kn 2.5+ NAA3.0	15.00	2.51±0.45	3.65±0.64	Fragile, long
Kn2.5+ IAA0.5	-	-	-	-
Kn2.5+ IAA 1.0	-	-	-	-
Kn2.5+ IAA 1.5	35.00	4.86±0.65	4.66±0.65	Thin, long
Kn2.5+ IAA 2.0	65.00	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
Kn2.5+ IAA 3.0	30.0	3.75±0.64	3.58±0.59	Thin, long

	Hemidesmus indicus			
Treatments (mg L^{-1})	Frequency of root initiation	Average No. of roots/ shoot	Length of shoot (cm) $\bar{x}$±SE	Root morphology
IBA0.5	42.00	3.92±0.61	4.33±0.53	Thin, short
IBA 1.0	72.00	6.51±0.73	3.41±0.88	Thin, short
IBA 1.5	78.00	3.45±0.65	2.58±0.62	Fragile, long
IBA2.0	72.00	7.53±0.45	4.65±0.83*	Fragile, long
IBA2.5	45.00	6.64±0.54	4.64±0.72	Fragile, long
IBA 3.0	43.00	4.51±0.43	3.63±0.64	Fragile, long
Kn2.5+ IBA0.5	25.00	3.58±0.42	3.35±0.67	Thin, long
Kn2.5+ IBA 1.0	38.00	4.78±0.44	4.85±0.64	Thin, long
Kn2.5+ IBA1.5	64.00	5.65±0.35	5.78±0.56	Thin, long
Kn2.5+ IBA 2.0	86.00*	8.78±0.86*	6.76±0.48	Thin, long
Kn2.5+ IBA 2.5	64.00	3.32±0.95	4.64±0.54	Thin, long
Kn2.5+ IBA 3.0	46.00	4.47±0.28	2.62±0.85	Thin, long

Table 3: Continued

BAP2.0+ IBA 0.5	45.00	6.54±0.85	3.61±0.93	Thick, short
BAP 2.0+ IBA1.0	65.00	5.54±0.35	4.64±0.46	Thick, short
BAP 2.0+ IBA 1.5	75.00	5.23±0.54	3.78±0.76	Thick, short
BAP 2.0+ IBA 2.0	76.00	5.51±0.32	2.88±0.65*	Thick, long
BAP 2.0+ IBA 2.5	75.00	3.95±0.45	3.75±0.75	Thick, long
BAP 2.0+ IBA 3.0	70.00	3.37±0.55	4.62±0.83	Thick, long
IBA 2.0+NAA0.5	35.00	4.56±0.48	3.83±0.57	Fragile, long
IBA2.0+NAA 1.0	55.00	5.76±0.55	4.96±0.84	Fragile, long
IBA2.0+NAA 1.5	67.00	5.69±0.59	4.88±0.54	Fragile, long
IBA2.0+NAA 2.0	55.00	6.32±0.43	3.79±0.86	Fragil, short
IBA2.0+NAA 2.5	45.00	5.56±0.94	3.65±0.55	Fragil, short
IBA2.0+NAA 3.0	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	45.00	6.63±0.25	4.62±0.54	Thick, long
Kn 2.5+ NAA2.0	55.00	7.74±0.66	3.67±0.55	Thick, long
Kn 2.5+ NAA2.5	44.00	3.98±0.48	3.64±0.95	Thick, long
Kn 2.5+ NAA3.0	35.00	3.53±0.94	3.61±0.93	Thick, long
Kn2.5+ IAA0.5	38.00	3.54±0.54	3.45±0.64	Thin, long
Kn2.5+ IAA 1.0	46.00	4.87±0.43	4.59±0.63	Thin, long
Kn2.5+ IAA 1.5	58.00	4.86±0.64	4.66±0.65	Thin, long
Kn2.5+ IAA 2.0	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
Kn2.5+ IAA 3.0	34.00	3.73±0.64	3.58±0.59	Thin, long

showed the highest rooting. 86.00% in 2.5 mg L^{-1} Kn + 2.0 mg L^{-1} IBA and this was followed by 78.00% in media having 1.5 mg L^{-1} IBA from node explants. Similar observations were noticed in sweetgum (Kim *et al*., 1997). The highest average number of roots per shoot was recorded (8.78±0.86) in media having 2.5 mg L^{-1} Kn + 2.0 mg L^{-1} IBA (Table 3 and Fig. 1). Similar observation was reported by Patnaik and Chand (1996) used IBA with Kn for root induction in *Hemidesmus indicus.* Generally shoots can be rooted very rapidly in medicinal plants and nearly all cases, roots were obtained in MS medium with auxin (Flick *et al*., 1989). After 12-15 days post transfer to rooting medium, roots appeared and by day 30, many were found to be 4.3-5.5 cm long. The planlets were transferred to pots containing a sand/soil mixture (1:1) initially covered with beakers.

From the forgoing discussion it can be concluded that among the two medicinal plants *Hemidesmus indicus* exhibited better response in shoot regeneration in different artificial media compositions than *Aristolochia indica*. In the present investigation significant variation for multiple shoot regeneration from nodal segments was also noted. The method can be usefully employed for mass propagation of endangered medicinal plants. Higher concentration of auxins or cytokinins took maximum period for shoot proliferation whereas lower concentration of auxins orcytokinins produced higher shoot length.

This two species are 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 plant chemovariability. It is hoped that a standard protocol to induce multiple shoots in culture may provide a more homogeneous source of plants.

In conclusion, it has been established an efficient and easy to handle protocol for micropropagation of three endangered medicinal plants *Hemidesmus indicus, Aristolochia indica)*. This protocol provides a successful and rapid technique that can be used for ex-situ 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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