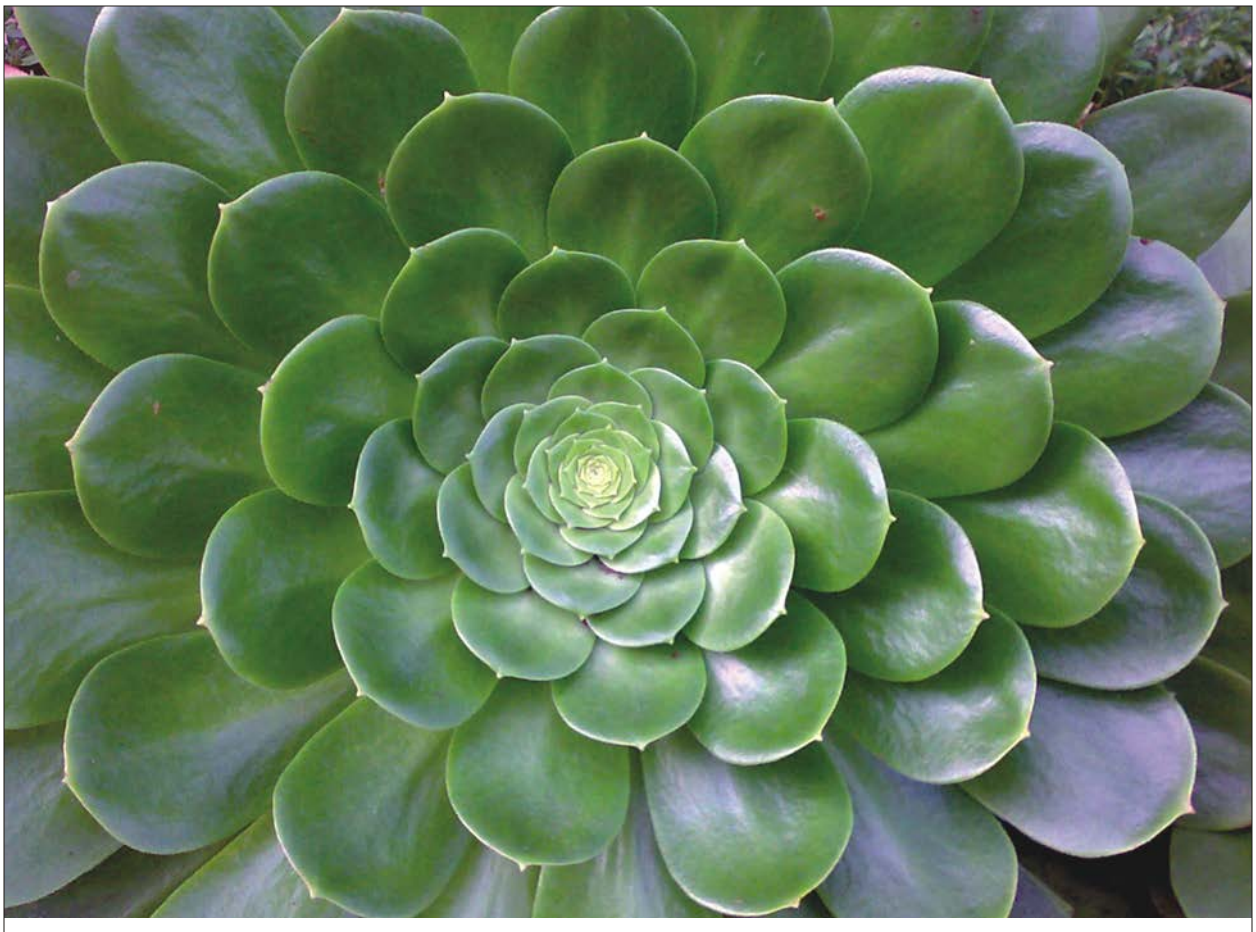

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Plant Regeneration from Axillary Shoot Segments Derived Callus in *Hemidesmus indicus* (L.) R. Br. (Anantamul) an Endangered Medicinal Plant in Bangladesh

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Abstract: Callus cultures were obtained from axillary shoots of *Hemidesmus indicus* on (MS) medium supplemented with 2,4-D, NAA, IAA and Kn. The highest frequency (85.00%) of organogenic callus induction was observed in MS medium containing 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn. Development of adventitious shoots occurred when the calli were subcultured in MS medium supplemented in the BAP, NAA, IAA and Kn. Shoots differentiated best (90.00%) from axillary shoot derived callus on MS medium containing 1.0 mg L^{-1} NAA and 2.5 mg L^{-1} Kn. Regenerated shoots rooted best in MS supplemented in the IBA and Kn. 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: Asclepiadaceae, aromatic and medicinal plant, organogenesis, callus, node

Introduction

Hemidesmus indicus R. Br. (Anantamul) a member of the Asclepiadaceae, is one of the most widespread used in Bangladesh. It is also rare and endangered (Rahman, 2001). 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. Flowers small green outside, deep purple inside, in axillary, sessile racemes, imbricated with flowers, followed with scale-like bracts. Fruit two long slender spreading follicles. The root is long, tortuous, rigid, cylindrical, little branched, consisting of aligneous centre, a brownish corky bark, furrowed and with annular cracks. Externally it has been applied as a poultice to boils, swellings and other painful parts. The root is harvested in the autumn and dried for later use. Huge quantities of plant materials and extracts are imported for the manufacture of Ayurvedic, Unani and Homeopathic Medicines (Chatterjee and Sastny, 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 these villages seems to be the cause of dependence on these medicinal plant species in addition to their prevent 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 pollutions that can affect the medicinal value of the harvested tissues (Geng *et al*.,

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2001). In addition, *in vitro* propagation methods offer powerful tools for germplasm conservation and the mass-multiplication of threatened plant species (Murch *et al*., 2000a). It is a widely used medicinal species useful in the treatment of inflammatory conditions. It is used in Leprosy, Leucoderma, Itching, Skin disease, asthma, Bronchitis, Leucorrhoea, Desyntery, Diarrhoea, Piles, Syphilis and Paralysis. It is said to promote health and cures all kinds of diseases caused by vitiated blood (Kirtikar and Basu, 1987). Propagation is mainly by seed, but seed viability is limited to one year. The plant prefers light (sandy), medium (loamy) and heavy (clay) soils and requires well-drained soil. The plant prefers acid, neutral and basic (alkaline) soils. It cannot grow in the shade. It requires moist soil. 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 plant chemo variability. The fragrant of roots contain 2-hydroxy-4-methoxybenzaldehyde (91%) and (-) ledol (4.5%), which are isolated in pure form, as the major constituents. The GC-MS analysis of the residual oil showed the presence of over 40 minor constituents. Among them nerolidol (1.2%), borneol (0.3%), linalyl acetate (0.2%), dihydrocarvyl acetate (0.1%), salicylaldehyde (0.1%), isocaryophyllene (0.1%), -terpinyl acetate (traces) and 1, 8-cineol (traces) are important as aromatic and bio-active principles (Nagarajan *et al*., 2001). Well identify endangered medicinal plant *Hemidesmus indicus* deserved research endeavored for establishing tissue culture protocol towards their conservation (Ghani, 1998; Mia, 1990). Secondary metabolites of pharmaceutical values can be harvested from medicinal plants by tissue culture and hemidesmic acid, smilasperic acid can be extracted from cell suspension culture (Nagarajan *et al*., 2001; Broun, 2004; Ramachadra Rao *et al*., 2002). It is hoped that a standard protocol to induce multiple shoots in culture may provide a more homogeneous source of plants.

Materials and Methods

This programme was carried out from June 2002 to June 2005. Explants were collected from *in vivo* grown medicinal plants *Hemidesmus indicus* (L.) R. Br. *(*Anantamul*)* and its axillary 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 seven 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 (2,4-D, Kn and NAA) concentrations for callus induction. 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, 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 with different concentrations of Kn, and IBA for adventitious root formation (Murashige and Skoog, 1962).

Results and Discussion

Callus induction was observed in MS media containing different concentrations and combinations of 2, 4-D, Kn and NAA. Within 10-12 days of incubation the nodal explants depending upon the

concentration and combination of hormones were induced calli. There was a wide range of variation in percentage of callus formation and average fresh weight of callus. The highest percentage of callus induction (85.00) was observed in MS medium containing 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn and followed by 78.00 percentage in MS medium containing 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} Kn (Table 1 and Fig. 1). Highest callus growth in terms of fresh weight (942±7.8 mg) was observed in MS medium fortified with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn. The highest dry weight of calli (125±0.281 mg) was observed in MS +1.0 mg L^{-1} NAA and 0.5 mg L^{-1} Kn. Colour of calli was mostly light green to dark green. It was observed that only light green calli produced shoot buds. Proliferation of shoot buds was observed in MS+1.0 mg L^{-1} Kn 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+2.5 mg L^{-1} Kn +1.0 mg L^{-1} NAA. Patnaik and Debata (1990) 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* (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 subcultured in MS medium supplemented with different concentrations of BAP or Kn alone or BAP incombination with NAA, BAP incombination with IAA, Kn incombination with NAA, Kn incombination with IAA and BAP incombination 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) and Cuenca *et al.* (1999) were used BAP and Kn in combination with different concentrations of NAA and

Table 1: Effect of different concentrations and combination of 2, 4-D, Kn and NAA on induction of callus from axillary shoot explants of *Hemidesmus indicus* (L.) R. Br. after four week 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	345±10.6	75±0.212
MS+2,4-D 2.0	12	21.00	DG	F	435±10.0	87±0.253
MS+ NAA 0.5	12	35.00	DG	F	567±10.7	85±0.321
MS+ NAA 1.0	12	70.00	LG	F	601±5.3	97±0.328
MS+ NAA 2.0	12	60.00	LG	F	892±4.9	87±0.315
MS+ Kn 0.5	12	56.00	LG	C	857±8.9	83±0.421
MS+ Kn 1.0	12	67.00	LG	C	897±8.9	84±0.335
MS+ Kn 2.0	12	72.00	LG	C	758±4.7	102±0.281
MS+ IAA 0.5	12	34.00	LG	C	436±9.8	78±0.223
MS+ IAA 1.0	12	45.00	LG	C	438±10.7	80±0.323
MS+ IAA 2.5	12	30.00	LG	C	344±8.7	72±0.352
MS+NAA 0.5 + Kn 0.5	12	46.00	DG	C	745±11.9	82±0.305
MS+NAA 0.5+ Kn 1	12	54.00	DG	C	857±10.5	97±0.305
MS+NAA 0.5+ Kn 2	12	67.00	LG	F	845±8.9	89±0.317
MS+NAA 1.0+ Kn 0.5	12	71.00	LG	F	759±9.7	125±0.281
MS+NAA 1.0+ Kn 1.0	12	76.00	LG	C	724±8.9	103±0.422
MS+NAA 1.0+ Kn 2.0	12	85.00	LG	F	942±7.4	109±0.372
MS+NAA 2.0+ Kn 0.5	12	67.00	LG	C	797±7.9	89±0.205
MS+NAA 2.0+ Kn1.0	12	78.00	LG	C	735±8.7	95±0.317
MS+NAA 2.0+ K n 2.0	12	49.00	DG	F	741±9.8	106±0.313

LG = Light Green, DG = Dark Green, C = Compact, F = Friable

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

Growth regulators (mg L^{-1})	% of organogenic calli		No. of shoot/callus	Length of shoot (cm)
	Root	Shoot		
BAP 0.5	-	-	-	-
BAP 1.0	-	42.00	2.35±0.49	3.21±0.95
BAP 2.5	-	55.00	2.42±0.42	3.33±0.82
BAP 3.5	-	40.00	2.53±0.47	3.15±0.56
BAP 2.5 + NAA 0.5	-	45.00	2.34±0.59	3.25±0.98
BAP2.5+ NAA 1.0	-	75.00	4.15±0.89	2.45±0.85
BAP2.5+ NAA 2.5	-	47.00	2.42±0.75	2.32±0.75
BAP2.5+ IAA 0.5	-	15.00	2.18±0.52	3.25±0.56
BAP2.5 + IAA 1.0	-	25.00	2.00±0.53	3.50±1.10
BAP2.5 + IAA 2.0	-	20.00	2.15±0.42	2.75±0.88
Kn 0.5	+	25.00	2.22±0.55	3.30±0.82
Kn 1.0	-	35.00	3.15±0.95	2.40±0.80
Kn 2.5	-	47.00	2.55±0.85	2.455±0.75
Kn 3.5	-	-	-	-
Kn 2.5 + NAA 0.5	+	45.00	2.00±0.52	2.50±0.93
Kn 2.5 + NAA 1.0	+	90.00	4.35±0.95	3.00±1.00
Kn 2.5 + NAA 2.5	-	70.00	3.34±0.87	3.21±0.82
Kn 2.5 + IAA 0.5	-	67.00	3.45±0.65	2.42±0.82
Kn 2.5 + IAA 1.0	-	32.00	3.10±0.55	2.47±0.71
Kn 2.5 + IAA 2.5	-	30.00	3.25±0.42	2.42±0.43
BAP 1.0+Kn 0.5	-	10.00	1.25±0.44	1.25±1.87
BAP 1.0+Kn1.0	-	35.00	2.25±0.54	2.25±1.09
BAP 1.0+Kn 2.5	-	-	-	-

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

Growth regulators (mg L^{-1})	Shoots rooted (%)	Root length (cm)	Root morphology
Kn 0.5	40.00	3.60±0.4	Fragile, long
Kn1.0	55.00	4.40±0.5	Fragile, long
Kn 2.0	50.00	3.25±0.9	Fragile, long
IBA 0.5	55.00	3.50±0.9	Thin, long
IBA 1.0	60.00	4.40±0.5	Thin, long
IBA 2.0	65.00	4.40±04	Thin, long
IBA 2.0+Kn 1.0	70.00	5.55±0.3	Thin, long
IBA 3.0+Kn 1.0	75.00	5.42±0.5	Thin, long
IBA 4.0+Kn 1.0	80.00	2.25±0.2	Thin
IBA 6.0+Kn 1.0	70.00	4.62±0.7	Thick, long

IAA. The highest 90.00% of shoot regeneration was observed in 2.5 mg L^{-1} Kn with 1.0 mg L^{-1} NAA and number of shoots per callus was 4.35±0.95 and this was followed by 75.00% in 2.5 mg L^{-1} BAP with 1.0 mg L^{-1} NAA and number of shoots per callus was 4.15±0.89 (Table 2 and Fig. 1). In the present investigation it was observed that Kn in combination with NAA was more suitable than BAP alone. Patnaik *et al.* (1996) used Kn with NAA for multiple shoot proliferation with 95.00% frequency. In the present investigation it was observed that when calli were sub cultured on media with different concentrations of Kn, BAP alone or BAP and Kn in combination with NAA and IAA, Only Kn 3.5 mg L^{-1}, BAP 0.5 mg L^{-1}, BAP 1.0 mg L^{-1}+Kn 2.5 mg L^{-1}, failed to produce any shoots. It was also observed that calli sub cultured on media with lower concentrations of Kn alone and NAA with Kn produced roots.

For adventitious root formation, axillary shoot excised and cultured on MS medium with different concentration of auxins and cytokinins. It was observed that IBA and Kn alone or combination in MS medium was the most effective for rooting of shoots in *Hemidesmus indicus* (L.) R. Br. (Table 3).

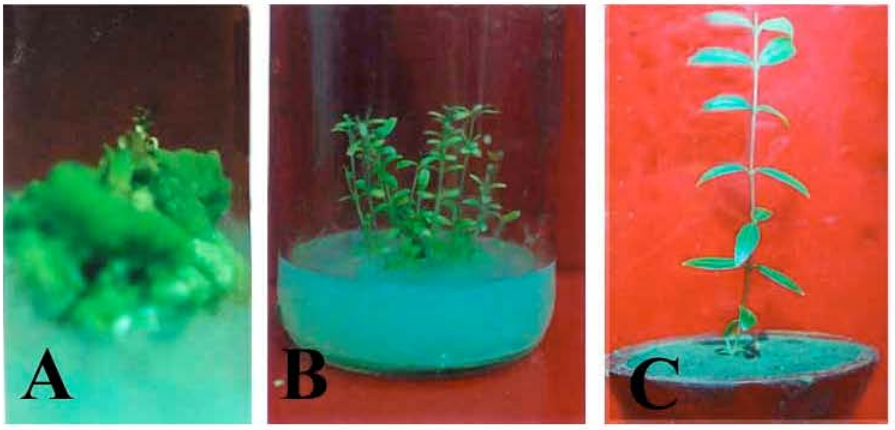

Fig. 1: Callus induction and shoot proliferation from axillary shoot explants *Hemidesmus indicus* (L.) R. Br.

A. Induction of light callus from nodal segments on MS+2.0 mg L^{-1} Kn and 1.0 mg L^{-1} NAA

B. Induction of multiple shoot buds from a shoots derived callus in MS+1.0 mg L^{-1} Kn and 1.0 mg L^{-1} NAA

C. A potted plant after 2 month of transplantation

After 12-15 days post transfer to rooting medium, roots appeared and by day 30, The highest 80.00% of root regeneration was observed in 4.0 mg L^{-1} IBA with 1.0 mg L^{-1} Kn. Patnaik *et al.* (1996) used IBA with Kn for root induction in *Hemidesmus indicus*. Many were found to be 4.3-5.5 cm. long. Then the planlets were transferred to pots containing sand/ soil mixture (1:1) initially covered with beakers.

In conclusion, we report an efficient and easy to handle protocol for micropropagation of the endangered medicinal plant (*Hemidesmus indicus)*. 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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