J. Plant Biotechnology(2003) Vol. 5(4). pp. 239~244

Journal of Plant Biotechnology

Improvement in Clonal Propagation of *Hemidesmus indicus* R. Br. through Adenine Sulphate

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Abstract

A protocol has been developed for rapid large scale clonal propagation of an aromatic endangered medicinal plant, *Hemidesmus indicus* R. Br. with the elimination of the problems such as premature leaf fall and callus formation during caulogenesis and rhizogenesis. Multiple shoots were induced from shoot tip and nodal explants on Murashige and Skoog (MS) medium supplemented with 1 mg/L Benzylaminopurine (BAP) and 0.5 mg/L Napthaleneaceticacid (NAA). Addition of 15 mg/L adenine sulphate to the above medium checked leaf abscission completely, reduced the time required for caulogenesis and restored morphogenetic potential after several subcultures. The *in vitro* grown propagules were rooted in 1/2 MS medium supplemented with 2 mg/L Indolebutyric acid (IBA) + 1 mg/L NAA and sucrose 0.75% (w/v). Addition of charcoal at 100 mg/L to the rooting medium quickened root initiation with a complete check on callus formation. The effect of sucrose concentration on both caulogenesis and rhizogenesis was also studied. The resultant plantlets were acclimatized and grown in fields where ninety eight percent of the rooted shoots survived and grew normally. The estimation of the secondary metabolite content in the shoots of the regenerated plant and the mother plant indicated that the concentration of the three secondary metabolites lupeol, vanillin and rutin was similar.

Key words: Nodal segments, Shoot tips, *Hemidesmus indicus*, micropropagation

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Received Sep. 2, 2003; accepted Dec. 16, 2003

Introduction

Hemidesmus indicus R. Br. belonging to family Ascelpiadaceae is well known for its aromatic and medicinal properties. It has antimicrobial (Ahmad et al. 1999; Hiremath et al. 1997), anti-inflammatory (Alam and Gomes 1998), antipyretic (Alam and Gomes 1998), antioxidant (Alam and Gomes1998; Ravishankara et al. 2002) antidysentric (Ram and Saha, 1998), hepatoprotective (Prabhakaran et al. 2000), immunomodulatory (Atal et al. 1986), and antileprotic (Gupta 1981) action. Due to its multiple uses, the plant has been indiscriminately collected from its natural habitat. The entire plant is uprooted for its single long taproot which has led to scarcity of raw materials for the herbal drug industry, especially in the northern plains of India. Although *in vitro* propagation of *H. indicus* has been reported using nodal explants via organogenesis and somatic embryogenesis in callus cultures (Sarasan et al. 1994) and axillary bud culture (Patnaik and Debata 1996; Malathy and Pai 1998; Sreekumar et al. 2000) premature leaf fall and excessive callus formation during *in vitro* shoot formation and rooting leading to low survival rates during hardening has not been overcome and these procedures are also inadequate to meet the need in time. The present article reports a protocol for achieving rapid clonal propagation with the alleviation of above problems.

Materials and Methods

Plant materials and culture conditions

An elite plant of *H. indicus* growing in National Botanical Research Institute nursery was selected for this study. Nodal segments of 1 cm length were taken as explants and washed

in running tap water for 30 minutes. The explants were rinsed in 5% liquid detergent for 5 minutes and then washed again in running tap water. These were treated with 70% ethanol for 5 seconds followed by surface sterilization with 0.1% mercuric chloride for 5 minutes. They were then thoroughly washed with sterilized distilled water at least 5 times and inoculated on sterilized nutrient media in test tubes. The media and glass wares were sterilized at 15 lbs pressure at 121°C for 15 minutes, after adjusting the pH 5.7±0.1. All cultures were incubated under cool, white fluorescent light (photosynthetic photon flux density of 70 μmol m^{-2} s^{-1}, 16 h photoperiod) at 25°C±2°C temperature. For shoot proliferation, the basal medium consisted of MS salts and vitamins (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. Depending on the experiments, the basal medium was further supplemented with cytokinins (BAP or KN), auxins (NAA or IAA), adenine sulphate (5-20 mg/L) and sucrose (1-5%) in different concentrations and combinations. Well developed shoots (≥ 3 cm) were transferred to half strength MS medium supplemented with 0.75-6% (w/v) sucrose and 0.8% Agar. This medium was supplemented with charcoal (100-200 mg/L), IBA (1-2 mg/L) and NAA (1 mg/L). Regenerated plants with well-established roots were transferred to pots containing sand and manure (1:1 v/v) and were kept in moist chamber with 80-90 % relative humidity for 30 days before their transfer to glass house.

Estimation of secondary metabolites

The plant material to be analysed was completely dried in a hot air oven at 40°C for 24 h and powdered. 2 g of powdered material was extracted with ethanol. The extract was filtered and evaporated to dryness on a water bath.

Estimation of secondary metabolites was done through HPTLC (high-performance thin-layer chromatography) and densitometric scanning.

In the present work application of the sample was done with an automatic sample applicator (CAMAG Linomat IV, Switzerland). For this purpose a known quantity (1 mg) of the dried sample was dissolved in a known volume (1 mL) of the solvent and the sample (10 μL) was applied on precoated Silica gel Merck G60 F_{250} plate in the form of a band. Development of the chromatogram was affected in CAMAG twin trough chamber after the solvent of the applied sample completely evaporated. The plates were completely dried at room temperature and densitometric scanning was done with Camag TLC scanner 3 (CAMAG, Switzerland). Detection was done under UV_{254} (absorbance/reflectance mode) or UV_{366} (absorbance/reflec-tance mode) Post chromatographic derivatisation was done. CATS 4 software (CAMAG, Switzerland) was used for documentation of the results.

Lupeol was eluted in the solvent system of Toluene: Ethylacetate (9:1) and the spray reagent used for derivatisation was anisaldehyde sulphuric acid followed by heating the TLC plate at 100°C for 10 min. Lupeol appears as black spot (Rf=0.5). Detection was done at 600 nm. Vanillin was eluted in the solvent system of Chloroform: Acetic acid: Methanol: Water (60:32:12:8). Derivatisation was done by spraying the plate with anisaldehyde sulphuric acid reagent followed by heating the TLC plate at 100°C for 10 min. Vanillin appears as black spot under UV254 at which detection was done (Rf=0.6). For Rutin the solvent system used was Ethylacetate: Formic acid: Acetic acid: Water (100:11:11:27). Detection was done under UV at 366 nm. Post chromatographic derivatisation was done by spraying the plate with 5% methanolic ferric chloride solution (w/v) and allowing the plate to dry at room temperature. Rutin appears as green spot (Rf=0.4).

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for all the secondary metabolites in sample were confirmed by comparing the R_f values and spectra of the spot with that of standard.

Experimental design

All experiments were repeated three times with 15 replicates each. The data were subjected to Duncan's Multiple Range Test, using a SAS program (Version 8.1, SAS Institute Inc., Cary, NC, U.S.A).

Results and Discussion

Axillary bud multiplication

In a preliminary experiment, NAA along with cytokinin was found to be more suitable than IAA for shoot proliferation as excessive callus was formed in the presence of IAA. The response of the explants to various concentrations and combinations of NAA, BAP, KN and GA are presented in Table 1. Among the various combinations of hormones used, 1mg/L BAP with 0.5 mg/L NAA produced maximum number of shoots (4.82) with minimal callusing (Figure 1A). However it was observed that the condition of the shoots deteriorated due to excessive leaf fall and callus formation as reported earlier by Patnaik et al. (1996). Addition of KN to the medium containing NAA did not prove beneficial in contrast to Sarasan et al. (1994) as callus formation increased along with defoliation of shoots. When GA was added in combination with BAP and NAA, no new shoots were formed and also the explants turned yellowish. Premature leaf fall has been seen in some plants during *in vitro* shoot multiplication, and addition of adenine sulphate (Dhawan and Bhojwani 1985), cal-

Table 1. Effect of different growth hormones on shoot formation in cultured nodal explants of *H. indicus*. '+' sign denotes degree of callusing

Treatment mg/L	Average number of shoots	Average height of the proliferated shoots	Associated callus	General condition of the explant	Time taken in shoot proliferation and elongation (in days)
BAP 0.5+NAA 0.2	3.13c[z]	3.32b	-	Defoliated shoots	30
BAP 0.5+NAA 0.5	3.13c	3.50b	+	Defoliated shoots	30
Kn 0.5+NAA 0.2	3.27c	2.71d	+ +	Defoliated shoots	35
Kn 1.0+NAA 0.2	3.83b	3.03c	+ + +	Defoliated shoots	40
BAP 1.0+NAA 0.2	4.27a	3.13c	+	Defoliated shoots	30
BAP 1.0+NAA 0.5	4.82a	4.01a	+ +	Defoliated shoots	30
BAP 1.5+NAA 0.5	4.00b	3.03c	+ + +	Defoliated shoots	35
BAP 1.0+GA 0.2+NAA 0.2	-	-	-	Explants turned yellowish	-
BAP 1.0+GA 0.5+NAA 0.2	-	-	+	Explants turned yellowish	-

[z]Mean separation by Duncan's multiple range test at $p<0.05$. Values followed by the same letter within a column are not significantly different.

Table 2. Effect of Adenine sulphate concentration on shoot formation in cultured nodal explants of *H. indicus*.

Treatment (Conc. mg/L)	Average number of shoots	Average height of the proliferated shoots (in cms)	General condition of the explant	Time taken in shoot proliferation and elongation (in days)
BAP 1.0+NAA 0.5+AdS 5	4.80a[z]	5.38a	Shoots healthy	15
BAP 1.0+NAA 0.5+AdS 10	5.02a	4.13b	Shoots healthy with good leaf growth	10
BAP 1.0+NAA 0.5+AdS 15	5.13a	4.27b	Shoots healthy with good leaf growth	10
BAP 1.0+NAA 0.5+AdS 20	2.93b	2.91c	Shoots with yellow leaves	20

[z]Mean separation by Duncan's multiple range test at $p<0.05$. Values followed by the same letter within a column are not significantly different.

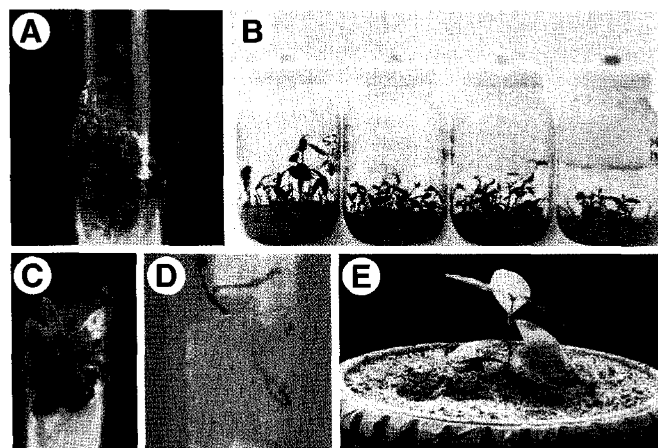

Figure 1. Clonal propagation of *Hemidesmus indicus*. (A) Proliferating shoots in MS+BAP 1mg/L+NAA 0.5 mg/L (B) (From Left to Right) Proliferating shoots in Adenine sulphate 5, 10, 15, 20 mg/L (C) Proliferating shoots in optimum medium (MS+BAP 1mg/L+NAA 0.5 mg/L), sucrose 4%, Adenine sulphate 15 mg/L. (D) Rooting of isolated shoot in 1/2 MS supplemented with 2 mg/L IBA+1 mg/L NAA+ Charcoal 100 mg/L and sucrose 7.5 g/L (E) Two months old *Hemidesmus indicus* plant in green house

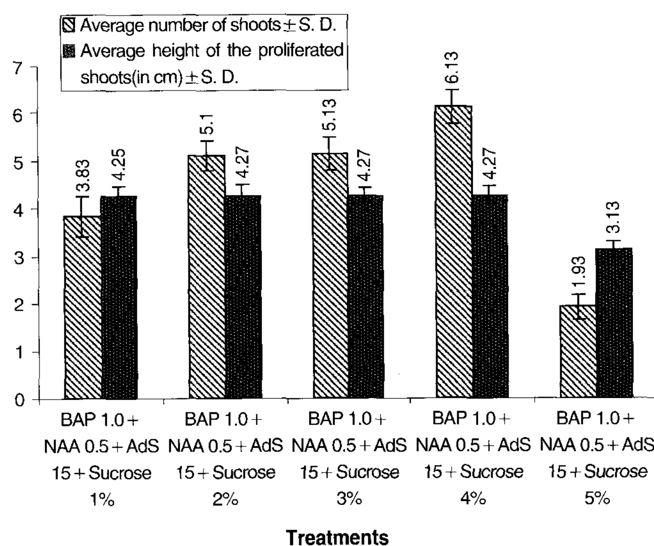

Figure 2. Effect of Sucrose concentration on shoot formation in cultured nodal explants of *H. indicus* (culture period-15 days).

cium salts or silver nitrate (Martin 2002) has been found to be beneficial. However, in case of *H. indicus*, addition of neither silver nitrate nor calcium chloride was found to be suitable as former induced yellowing of explants, whereas, latter induced shoot tip necrosis. In case of *H. indicus*, L-Phenylalanine and Shikimic acid, precursors of Phenyl propanoid pathway were found to support broad leaves with very good shoot proliferation, however, there was a concomitant increase in callus formation at the base and slight leaf abscission was visible (data not presented). When adenine sulphate was added to the shoot proliferation media, the rate of multiplication and growth of shoots enhanced as seen in Table 2, Figure 1B. With the addition of 15 mg/L adenine sulphate, time required for shoot proliferation and elongation was shorter (10 days) and the leaves formed were broad and healthy with a slight reddish tinge. Patnaik and Debata (1996) reported a reduction in shoot numbers of *H. indicus* by subsequent cultures. In accordance with this report, after several subcultures we found that the multiplication rate of the explants decreased though they were supplemented with optimum rate of growth hormones (MS+ BAP 1.0+ NAA 0.5). Addition of Adenine sulphate at 15 mg/L to the proliferation media was also found to restore the morphogenetic potential of these explants. The effect of graded concentrations of sucrose on shoot proliferation and elongation was also studied. Maximum shoot proliferation (6.13) was achieved at 40g/L sucrose supported with broad leaves (Figure 1C). In a very low (10g/L) or high (50g/L) sucrose concentrations both shoot multiplication and elongation declined (Figure 2).

Rooting *in vitro*

In a preliminary experiment for rhizogenesis, reducing MS salt concentration to half was found to be more effective than full strength and quarter strength MS. The promotory effect of reducing MS salt concentration has been reported on *in vitro* rooting of *Bauhinia vahlii* Wight and Arnott (Upreti and Dhar 1996). For rooting, to the 1/2 MS basal medium containing 3% sucrose (w/v) and 0.8% agar (w/v), individual auxin like IAA, IPA and NAA (1mg/L or 2mg/L) was added but found to be ineffective for root induction. A combination of KN (1mg/L) and NAA (1mg/L) in the basal medium was also ineffective for root induction. This was not expected as according to earlier published report (Malathy et al. 1998) which leads to root induction. Addition of other auxins like CPA or IPA to above mentioned medium also failed to induce roots. IBA (2.0 mg/L) when used singly induced root formation however callus was also formed alongwith roots as reported (Sreekumar et al. 2000). IBA (2 mg/L) when supplemented with NAA (1mg/L) induced thicker root formation, which definitely has the advantage of being able to withstand damage during planting out and a combination of two or more auxins is already known to be more effective in root induction (Yadav et al. 1990). But callusing at the end of the stem was visible as indicated in Table 3. To stop caulogensis in the optimum treatment (1/2 MS+IBA 2 mg/L+ NAA 1mg/L) addition of activated charcoal at 100 mg/L (w/v) proved to be highly beneficial. It not only stopped callus formation but also induced root formation within 10 days as seen in

Table 3. Rooting in cultured shoots of *H. indicus* (culture period- 30 days). '+' sign denotes degree of callusing

Treatment (mg/L)	Average numbe of roots	Length of roots (in cms)	Time taken for root initiation (in days)	% rooting	Callusing
BIBA 1.0	1.47c^{z}	1.52c	30	40	+
IBA 2.0	2.35b	3.50b	30	80	+
IBA 2.0+NAA 1.0	5.12a	3.70b	30	90	++
Charcoal 100+IBA 2.0+NAA 1.0	5.13a	4.83a	10	96	-
Charcoal 200+IBA 2.0+NAA 1.0	5.11a	4.82a	10	95	-

^{z}Mean separation by Duncan's multiple range test at p<0.05. Values followed by the same letter within a column are not significantly different.

Table 4. Effect of sucrose concentration on rooting in *H. indicus* (culture period- 30 days).

Treatment (g/L)	Average number of roots	Length of roots (in cms)	Time taken for root initiation (in days)	% rooting	General condition of shoots
Sucrose 7.5 g/L	5.20a^{z}	4.82a	15	98	Defoliation of shoots
Sucrose 15 g/L	5.16a	4.83a	15	94	Shoots were healthy but no new leave
Sucrose 30 g/L	5.13a	4.83a	15	94	Shoots were healthy with formation of new leaves
Sucrose 60 g/L	-	-	-	-	Browning of shoots

^{z}Mean separation by Duncan's multiple range test at p<0.05. Values followed by the same letter within a column are not significantly different.

Figure 1D. The beneficial effect of activated charcoal on rooting is well documented (Hussey 1986). However, doubling the charcoal concentration to 200 mg/L did not enhance this effect. (Table 3) and leaf drop was observed during root formation as reported (Patnaik and Debata 1996).

To study the effect of sucrose concentration on rooting, in the optimum medium derived sucrose concentration was varied at 0.75, 1.5, 3 and 6%. A sucrose concentration of 6% resulted in browning of the explants with no root formation. On reducing sucrose concentration to 1.5% number of roots formed were more as compared to sucrose at 3%. The beneficial effect of reduced sucrose concentration on root induction has been reported (Hussey 1986). Further reduction in sucrose concentration to 0.75% increased root induction but leaf fall also increased (Table 4). However, if shoots excised from adenine sulphate containing media were transferred to this rooting medium (containing sucrose at 0.75%) no leaf fall was observed. Thus adenine sulphate was found to be highly beneficial for *in vitro* propagation of *H. indicus*.

Acclimatization

A mixture of 1:3 soil and sand was not suitable in contrast to Sarasan et al. (1994) as all the plants transplanted in this mixture died after 15-20 days. On closely examining the roots it was found that while shoots were normal there was no root growth, which possibly resulted in death of plants. Even a combination of 1:1 soil and manure was not suitable for acclimatization. The rooted shoots were transplanted in a pot mixture of (1:1) sand and manure in which they grew normally. The essentiality of 1:1 sand and manure for acclimatization is possibly because the plant is found growing naturally in sandy soils of forests where the topsoil is littered with leaves. During acclimatization, maintaining the humidity at 80-90% for four weeks was essential. Thereafter, 500 potted plants have been transferred to glasshouse out of which 490 survived and they are growing normally (Figure 1E). Another interesting observation was that if the potted plants were transferred to the fields in hot and humid weather they had greater chance of survival (98%) as compared to cold weather (70%). This is probably because the plant is endemic to the southern region of India where a tropical climate is prevalent.

Estimation of secondary metabolites

The estimation of the secondary metabolites in the shoots of the regenerated plant and the mother plant indicated that the concentration of the three secondary metabolites lupeol, vanillin and rutin was similar (Table 5). Further the general HPTLC profiles of the two sources were found to be same indicating that the regenerated plants were clones of the parent plant in terms of their chemical constituents. Long term *in vitro* culture (3 years) had no deleterious effect on the homogeneity and secondary metabolite contents of the propagated plant (data not shown). This also indicates that the valuable genotype, *H. indicus* can be preserved for a long time even after several subcultures under *in vitro* conditions without any loss in secondary metabolite synthesis by the plant.

Table 5. Lupeol, Vanillin and Rutin content in parent plant and regenerated plants.

Source	Lupeol (mg/g DW)	Vanillin (μg/g DW)	Rutin (mg/g DW)
Parent plant (root)	0.419a[z]	2.339a[z]	Nil
Parent plant (shoot)	0.305a	6.821b	0.500a[z]
Regenerated plant (root)	0.423a	2.330a	Nil
Regenerated plant (shoot)	0.308a	6.794b	0.502a

[z]Mean separation by Duncan's multiple range test at $p<0.05$. Values followed by the same letter within a column are not significantly different.

Acknowledgments

The authors are thankful to the Director of the National Botanical Research Institute of Lucknow for providing necessary facilities for the above research.

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