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Development of an efficient *in vitro* callus and multiple shoot buds regeneration system with comparative phytochemical profiling of *Hemidesmus indicus* (L.) R.Br. - An endangered medicinal plant of Bangladesh

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Abstract
Hemidesmus indicus (L.) R.Br. (Asclepiadaceae), commonly known as Anantamul, is an endangered, high value medicinal climbing shrub renowned for its therapeutic bioactives, including 2-hydroxy-4-methoxybenzaldehyde. Destructive wild harvesting and the inherent limitations of conventional seed propagation present severe challenges to its conservation, requiring the development of robust *in vitro* regeneration and biochemical evaluation protocols. The primary objective of this study was to establish an optimized, highly reproducible *in vitro* micropropagation protocol for *H. indicus* using varying explants, alongside a comparative qualitative phytochemical screening of *in vitro* regenerated plantlets versus naturally grown plants. Nodal, leaf and shoot apices segments were aseptically cultured on Murashige and Skoog (MS) media supplemented with varied combinations of auxins (2,4-D, NAA, IBA) and cytokinins (BAP, Kn). Qualitative phytochemical screening was performed on methanolic extracts of both *in vivo* and *in vitro* plantlets using standard colorimetric assays for alkaloids and ten other secondary metabolites. Optimal callus induction was achieved in nodal (96%) and leaf (95%) segments utilizing MS medium fortified with 1.0 mg/l 2,4-D or a combination of 1.5 mg/l BAP and 1.0 mg/l NAA. Direct multiple shoot regeneration from nodal segments peaked at 95% (yielding 5.44 ± 0.35 shoots/explant) on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l Kn. Shoot apices produced a maximum of 3.54 ± 0.29 shoots/explant on MS medium with 2.0 mg/l BAP and 1.0 mg/l Kn. The greatest shoot elongation (5.22 ± 0.15 cm) was achieved using 1.0 mg/l BAP and 0.5 mg/l NAA. Maximum rooting (85%; 5.52 ± 0.04 roots/shoot) was recorded on full strength MS medium supplemented with 1.5 mg/l IBA. Following hardening, *ex vitro* acclimatization achieved an 85% survival rate. Phytochemical profiling confirmed that *in vitro* regenerated clones accumulated substantially higher concentrations of coumarin, quinine and phlobatannin compared to naturally grown specimens, while consistently maintaining high levels of steroids, flavonoids and terpenoids. The optimized propagation protocol establishes a rapid, reliable and sustainable pathway for the mass multiplication of *H. indicus*. The enhanced accumulation of critical pharmacological metabolites in *in vitro* tissues confirms that tissue culture represents a superior strategy for both ecological conservation and commercial pharmaceutical exploitation.

Keywords: *Hemidesmus indicus*, micropropagation, phytochemical screening, secondary metabolites

Introduction

Hemidesmus indicus (L.) R.Br., widely recognized as Indian sarsaparilla, is a slender, twining, laticiferous shrub belonging to the family Asclepiadaceae. Indigenous to the Indian subcontinent and broadly distributed across the ecological zones of Bangladesh, the plant possesses an immensely valuable, highly aromatic root system (Kirtikar and Basu 1987, Ghani 2003) [1-2]. The characteristic sweet fragrance and primary therapeutic efficacy of *H. indicus* are attributed to the essential oil components, predominantly 2-hydroxy-4-methoxybenzaldehyde (HMB), alongside ledol and vanillin (Nagarajan *et al.* 2001) [3]. HMB has garnered profound scientific interest due to its potent antimicrobial, antioxidant and anti-biofilm properties (Cowan 1999) [4]. Historically, the roots and vegetative parts of *H. indicus* have been utilized extensively in traditional and modern pharmacopoeias as blood purifiers and in the treatment of asthma, bronchitis, leprosy, syphilis, diarrhea and severe skin diseases (Dev 1997, Patwardhan *et al.* 2005) [5-6].

Despite its vast pharmaceutical utility, *H. indicus* currently faces a severe conservation crisis. Unregulated, destructive wild harvesting by pharmaceutical industries, coupled with rapid habitat fragmentation, has decimated natural populations, categorizing the species as rare and endangered in the vascular flora of Bangladesh (Rahman 2001, Yazhni *et al.* 2021) [7-8]. Traditional propagation via seed germination is grossly inadequate due to low seed viability and poor germination percentages, while clonal stem cuttings are frequently restricted by low establishment rates and high susceptibility to environmental pathogens (Shanmugapriya and Sivakumar 2011, Saryam *et al.* 2012) [9-10].

To address these severe propagation bottlenecks, *in vitro* plant tissue culture has emerged as an indispensable biotechnological tool for rapid mass multiplication, disease free plantlet production and germplasm conservation (Purohit *et al.* 2014) [11]. Previous protocols for *H. indicus* micropropagation have demonstrated varying degrees of success using axillary buds, nodal segments and leaf explants (Siddique and Bari 2006, Shreekumar *et al.* 2000) [12-13]. However, achieving high-frequency morphogenetic responses relies strictly on the precision of exogenous plant growth regulator (PGR) application, which must be tailored to overcome the recalcitrance of regional ecotypes (Rout *et al.* 2000) [14].

Beyond mere vegetative multiplication, the biosynthesis and accumulation of plant secondary metabolites are of critical importance for pharmacological validation. Plants synthesize a structurally diverse array of secondary metabolites including alkaloids, flavonoids, tannins and terpenoids which act defensively *in natura* but serve as the backbone of herbal medicine (Oksman-Caldentey and Inze 2004, Ramachandra Rao and Ravishankar 2002) [15-16]. While the chemical constituents of wild-type *H. indicus* have been extensively studied, there remains a pronounced research gap concerning the comparative qualitative phytochemical profiling of *in vitro* regenerated clones against naturally grown plants (Edeoga *et al.* 2005, Bansode and Salarkar 2015) [17-18]. Evaluating these biochemical dynamics is essential to ascertain whether the artificial *in vitro* environment preserves, alters, or enhances the plant's native metabolic sinks (Sofowora 1993) [19].

Consequently, this research was undertaken with two explicit objectives: (1) to standardize a highly reproducible, large-scale *in vitro* organogenesis and propagation protocol for *H. indicus* utilizing nodal, leaf and shoot apices segments by optimizing varied PGRs concentrations and (2) to systematically conduct a comparative qualitative phytochemical screening of the secondary metabolites between the *in vitro* propagated plantlets and naturally grown wild type specimens, thereby validating the pharmaceutical potential of the micro-propagated clones.

Materials and Methods

1. Plant material and explant source

Field grown, mature, healthy plants of *Hemidesmus indicus* (L.) R.Br. were selected as the primary mother source. Actively growing nodal, shoot apices and leaf segments were meticulously excised and washed under continuous running tap water to eliminate superficial dust and microbial loads. The explants were subsequently surface sterilized by sequential immersion in 1% Savlon and a mild liquid detergent for 5 to 10 minutes with constant agitation. This was followed by 3-4 rinses in sterile distilled water. The tissues were then transferred to a laminar airflow cabinet, rinsed with 70% ethanol for less than 60 seconds and thoroughly sterilized using 0.1% (w/v) mercuric chloride ($HgCl_2$) for 7 minutes. To guarantee the complete removal of toxic sterilants, the explants were washed 4 to 5 times with sterile distilled water. Prior to inoculation, the sterilized explants were trimmed to 0.5-1.0 cm segments using a sterile surgical blade.

2. Media preparation and culture conditions

The basal nutrient medium employed for all *in vitro* organogenesis experiments was formulated according to MS medium (Murashige and Skoog 1962) [20]. For callus induction, multiple shoot buds (MSBs) induction and proliferation, the MS medium was fortified with 3.0% (w/v) bacteriological grade sucrose and solidified using 0.8% (w/v) agar (Himedia, India). The media pH was precisely adjusted to 5.8 utilizing 1N NaOH or 1N HCl prior to sterilization. The culture media were autoclaved at 121°C and 15 psi for 20 minutes. All inoculated culture vessels were incubated in a controlled growth chamber maintained at 25 ± 2°C under a photoperiod of 14 hours light and 10 hours dark, provided by cool white fluorescent tubes emitting a light intensity of 2000-3000 lux.

3. Callus induction and shoot proliferation treatments

For indirect organogenesis (callus induction), nodal and leaf segments were inoculated onto MS media supplemented with varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5-3.0 mg/l), 6-benzyl aminopurine (BAP) (0.1-2.0 mg/l) individually or combinations of BAP (1.0-1.5 mg/l) with α-naphthalene acetic acid (NAA) (0.5-1.0 mg/l).

For direct MSBs organogenesis, nodal and shoot apices segments were cultured on MS media supplemented with BAP (0.1-3.0 mg/l) or Kn (0.5-3.0 mg/l) individually and in precise combinations: BAP + NAA, BAP + Kn and Kn + NAA at specific concentrations ranging from 0.5 to 2.0 mg/l. Induced multiple shoot buds were sub-cultured into fresh differentiation media at 15-20 day intervals to maximize proliferation.

4. Shoot elongation and rooting

Proliferated microshoots were excised and transferred to designated elongation media consisting of MS basal salts augmented with combinations of BAP (1.0-2.0 mg/l) with either NAA (0.5-1.0 mg/l) or Kn (0.5-1.0 mg/l). For rhizogenesis, elongated microshoots measuring 4.0-4.5 cm were individually transferred to rooting media. Root induction was evaluated using half-strength and full-strength MS media fortified with 1.5% (w/v) sucrose, tested both without PGRs (control) and with varied concentrations of indole-3-butyric acid (IBA) (0.5-2.0 mg/l).

5. Acclimatization

Healthy *in vitro* plantlets demonstrating robust, well-developed root systems were carefully extracted from the culture vessels. The roots were gently washed under running tap water to remove the agar matrix completely without damaging the delicate root hairs. The plantlets were transplanted into small earthen/plastic pots containing a sterilized, homogenous mixture of garden soil and compost (1:1 v/v). The potted plantlets were maintained at room temperature for 3-5 days to facilitate gradual primary hardening prior to their successful transfer to the external natural environment for *ex vitro* establishment.

6. Phytochemical screening protocol

Qualitative phytochemical analyses were conducted utilizing methanolic extracts of both *in vitro* regenerated plantlets and naturally grown wild plants. The plant materials (25 g) were macerated, mixed with 50 ml methanol and shaken thoroughly for 30 minutes. Following overnight incubation, sonication and filtration via Whatman filter paper, the extracts were evaporated below 51°C and dried for testing (Harborne 1973) [21].

Alkaloid detection was comprehensively assessed using five distinct reagents: Dragendorff's (D), Hager's (H), Mayer's (M), Wagner's (W) and Tannic acid (T) reagents. The presence and relative accumulation of ten additional secondary metabolites (steroids, flavonoids, saponins, terpenoids, glycosides, tannins, phlobatannins, quinine, anthraquinone, coumarin and phenols) were evaluated via standard colorimetric reactions (Daniel 2006) [22]. Color intensity and precipitate formations were recorded categorically as heavy/high (+++), moderate (++), slight (+), or absent (-).

7. Statistical analysis

All tissue culture experiments were structured employing a Completely Randomized Design (CRD). Each distinct

treatment consisted of three replicates containing 15 explants per replicate. Quantitative experimental data, including callus induction frequency, shoot multiplication rate, shoot length, root number and root length, were recorded chronologically. All data were expressed as Mean ± Standard Error (SE).

Results

1. Callus induction

The morphogenetic response for callus induction from nodal (NS) and leaf segments (LS) of *H. indicus* exhibited significant variation based on the exogenous PGRs supplementation (Table 1). Callus initiation generally occurred within 15-30 days of inoculation. In nodal segments, the maximum callus induction frequency (96%) was recorded on MS medium supplemented with 1.0 mg/l 2,4-D (requiring 19-23 days, yielding yellowish green callus, YGC; Fig. 1) and on MS medium with 1.5 mg/l BAP and 1.0 mg/l NAA (requiring 19-23 days, yielding whitish green callus, WGC, Fig. 2-3). The lowest response (70%) for nodal segments was observed using 0.2 mg/l BAP. Correspondingly, leaf segments exhibited the highest callus induction (95%) under the identical optimized treatments of 1.0 mg/l 2,4-D (16-18 days, yielding whitish callus, WC; Fig. 4) and 1.5 mg/l BAP and 1.0 mg/l NAA.

2. Direct multiple shoot regeneration from nodal and shoot apices segments

Direct organogenesis from nodal and shoot apices explants bypassed the intermediary callus phase, demonstrating highly efficient, rapid shoot multiplication (Table 2). The peak explant response (95%) and the maximum absolute number of MSBs per explant (5.44 ± 0.35) were recorded on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l Kn (Fig. 5-6). This specific synergistic cytokinin formulation also expedited induction to merely 12-15 days. In stark contrast, the minimum morphogenetic response (65%) and the lowest average number of MSBs (1.16 ± 0.15) were observed on MS medium containing 1.5 mg/l BAP and 1.0 mg/l Kn (Fig. 6).

Shoot apices similarly exhibited robust direct MSBs induction across the tested PGRs formulations (Table 2). The most prolific regeneration occurred on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l Kn, resulting in a 90% explant response and yielding an average of 3.54 ± 0.29 MSBs per explant within 15-18 days (Fig. 7-8). The least effective treatment was MS medium augmented with 1.0 mg/l Kn and 0.5 mg/l NAA, culminating in a 63% induction response, delayed morphological emergence (21-25 days) and a minimal MSBs yield of 1.24 ± 0.40 (Fig. 8).

3. Multiple shoot buds regeneration from callus tissue

Sub-culturing the induced callus tissues onto differentiation media resulted in the proliferation of multiple shoot buds (MSBs) within 14-30 days (Table 3). The maximum shoot proliferation frequency from nodal derived callus (85%) was achieved on MS medium supplemented with 1.5 mg/l BAP and 1.0 mg/l NAA, which rapidly induced shoots (15-19 days) and produced the highest mean number of MSBs per vessel (3.46 ± 0.30, Fig. 9). Similar findings were also recorded on MS medium fortified with 2.0 mg/l Kn and 1.0 mg/l NAA. Conversely, the combination of 1.0 mg/l BAP and 1.0 mg/l NAA generated the lowest proliferation rate (72%) with only 1.18 ± 0.13 MSBs/vessel over 19-28 days.

The induced callus tissues were subsequently transferred to different differentiation media to evaluate their potential for shoot bud regeneration. Multiple shoot buds (MSBs) emerged from the leaf derived callus within 15-25 days of culture (Table 3), although the regeneration response varied considerably among the different PGRs combinations. The highest shoot proliferation frequency (90%) was obtained on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA (Fig. 10). This treatment also resulted in comparatively rapid shoot buds initiation (15-18 days) and produced the highest mean number of MSBs per vessel (3.54 ± 0.29). In contrast, the combination of 1.0 mg/l Kn and 0.5 mg/l NAA was comparatively less effective, resulting in the lowest shoot proliferation frequency (63%) and producing only 1.24 ± 0.40 MSBs per vessel after 19-28 days of culture.

4. Shoot elongation

Excised MSBs were cultured on specified elongation media for 30 days to facilitate continuous vertical growth (Table 4). The absolute maximum elongation (5.22 ± 0.15 cm, ascending from an initial length of 2.17 ± 0.16 cm) was recorded on MS medium fortified with 1.0 mg/l BAP and 0.5 mg/l NAA (Fig. 11-12). Conversely, the most suppressed elongation growth (2.54 ± 0.26 cm, ascending from 1.56 ± 0.45 cm) manifested when MSBs were cultured on MS medium containing 2.0 mg/l BAP and 1.0 mg/l Kn.

5. Root induction and development

The establishment of a vigorous *in vitro* root system dictates the absolute survival of propagated shoots *ex vitro*. Elongated shoots entirely failed to initiate rhizogenesis when cultured on half strength MS medium devoid of PGRs (Table 5). However, the specific inclusion of IBA drastically enhanced rooting dynamics. The demonstrably superior rooting response (85%) was realized on full strength MS medium supplemented with 1.5 mg/l IBA (Fig. 13-14). This optimized formulation induced rapid root emergence (10-11 days), generating an impressive average of 5.52 ± 0.04 roots per micro-shoot, with an extensive average root length of 4.54 ± 0.03 cm following 30 days of culture. By comparison, full strength MS medium supplemented with only 0.5 mg/l IBA yielded the minimum rhizogenic response (70%) with a mere 2.12 ± 0.02 roots per shoot, averaging 1.86 ± 0.05 cm in length.

6. Acclimatization

Following robust *in vitro* rhizogenesis, the complete seedlings were subjected to a rigorous sequential acclimatization protocol. Gradual hardening in plastic pots facilitated physiological adjustment to *ex vitro* humidity gradients. Following ultimate transfer to the outdoor field environment, an exceptional 85% overall survival rate was documented (Fig. 15). The acclimatized, field-established plants exhibited normal phenotypic characteristics devoid of observable somaclonal variations.

7. Comparative qualitative phytochemical screening

Analytical reactions employing five distinct alkaloid detecting reagents substantiated the biosynthesis of high alkaloid concentrations across both plant sources (Table 6). Dragendorff's (D) and Wagner's (W) reagents generated heavy (+++) precipitations uniformly for both *in vitro* and natural plants, while Hager's (H) reagent yielded moderate

(++) precipitations for both. Mayer's (M) reagent highlighted a distinct variance, detecting moderate (++) alkaloid levels in *in vitro* extracts versus slight (+) trace levels in natural plants. Tannic acid (T) reagent indicated slight (+) presence consistently across both sources.

Other Secondary Metabolites: Comprehensive qualitative screening for ten additional crucial secondary metabolites revealed significant pharmaceutical advantages within the *in vitro* generated clones (Table 7). Steroids, flavonoids, terpenoids, glycosides and tannins were confirmed present at uniformly high (+++) concentrations in both *in vitro* and natural plants. Anthraquinones and phenols were completely absent (-) in all samples evaluated. Crucially, *in vitro* developed plantlets accumulated higher relative concentrations of quinine and coumarin (+++) compared to their naturally grown counterparts (++). Phlobatannin was synthesized at uniquely high concentrations (+++) strictly within the *in vitro* plantlets, whereas it remained entirely undetected (-) in the naturally grown wild plants. Conversely, naturally grown plants exhibited slightly higher concentrations of saponins (+++) compared to the moderate (++) levels detected in the *in vitro* clones.

Table 1: Effects of 2,4-D and BAP individually and BAP in combination with NAA on induction of callus from natural nodal and leaf explants of *H. indicus*.

Plant growth regulators (mg/l)		Type of explants	% of explants produced callus	Time (d) required for callus induction	Colour and texture of induced callus
2,4-D	0.5	NS	75	15 - 26	YGC
		LS	85	17 - 30	WGC
	1.0	NS	96	19 - 23	YGC
		LS	95	16 - 18	WGC
	2.0	NS	83	15 - 22	YGC
		LS	75	15 - 26	WGC
	3.0	NS	79	15 - 18	YGC
		LS	77	15 - 19	WGC
BAP	0.2	NS	70	20 - 28	YGC
		LS	77	16 - 22	WC
	0.5	NS	83	20 - 23	YGC
		LS	82	16 - 30	WC
	1.0	NS	81	16 - 28	YGC
		LS	72	15 - 21	WC
	2.0	NS	81	17 - 21	YGC
		LS	74	17 - 22	WGC
BAP + NAA	1.0 + 0.5	NS	85	19 - 30	WGC
		LS	85	15 - 18	WC
	1.0 + 1.0	NS	80	20 - 23	WGC
		LS	75	18 - 23	WC
	1.5 + 0.5	NS	77	22 - 30	WGC
		LS	79	20 - 30	WC
	1.5 + 1.0	NS	96	19 - 23	WGC
		LS	95	16 - 18	WC

d = Days , NS= Nodal segment, LS= leaf segment, WGC= Whitish green callus, WC= Whitish Callus;
YGC= Yellowish green callus.

Table 2: Effects of BAP individually and in combination with NAA or Kn on induction of MSBs in natural nodal and shoot apices explants of *H. indicus*.

PGRs	Conc. (mg/l)	% of explants giving response	Time (d) require for induction of MSBs	Average no. of MSBs/explant ($\bar{x}$ ± SE)	% of explants giving response	Time (d) require for induction of MSBs	Average no. of MSBs/explant ($\bar{x}$ ± SE)
		Nodal segment			Shoot apices segment		
BAP	0.2	75	14 - 16	2.68 ± 0.49	77	16 - 21	2.39 ± 0.32
	0.3	77	13 - 15	3.52 ± 0.43	75	16 - 19	2.22 ± 0.25
	1.0	80	12 - 16	3.35 ± 0.79	77	17 - 22	2.32 ± 0.29
	2.0	82	13 - 17	3.53 ± 0.51	73	21 - 25	2.43 ± 0.24
Kn + NAA	1.0 + 0.5	76	17 - 21	2.49 ± 0.59	63	21 - 25	1.24 ± 0.40
	1.0 + 1.0	85	12 - 15	2.65 ± 0.84	73	21 - 25	2.25 ± 0.24
	2.0 + 0.5	77	20 - 22	3.73 ± 0.52	66	21 - 23	1.39 ± 0.41
	2.0 + 1.0	76	17 - 20	2.82 ± 0.60	75	20 - 22	2.17 ± 0.15
BAP + NAA	1.0 + 0.5	92	15 - 18	3.49 ± 0.51	65	15 - 18	1.37 ± 0.46
	1.0 + 1.0	78	13 - 17	2.50 ± 0.58	67	17 - 25	1.44 ± 0.36
	1.5 + 0.5	83	15 - 18	2.23 ± 0.21	86	15 - 18	2.38 ± 0.50
	1.5 + 1.0	81	12 - 22	3.72 ± 0.46	66	16 - 25	1.33 ± 0.32
	2.0 + 0.5	82	14 - 22	2.80 ± 0.63	85	16 - 20	2.28 ± 0.38

	2.0 + 1.0	80	16 - 20	2.40 ± 0.53	65	17 - 21	1.31 ± 0.29
BAP + Kn	1.0 + 0.5	80	17 - 19	3.63 ± 0.42	86	18 - 25	3.20 ± 0.18
	1.0 + 1.0	79	16 - 18	2.47 ± 0.63	76	17 - 22	2.26 ± 0.32
	1.5 + 0.5	77	18 - 20	2.19 ± 0.23	73	20 - 21	2.19 ± 0.19
	1.5 + 1.0	65	20 - 22	1.16 ± 0.15	73	21 - 25	2.24 ± 0.21
	2.0 + 0.5	95	12 - 15	5.44 ± 0.35	87	15 - 18	3.22 ± 0.19
	2.0 + 1.0	78	18 - 21	1.92 ± 0.19	90	15 - 18	3.54 ± 0.29

d = days; MSBs = Multiple Shoot Buds; Values are the means of 5 replicates.

Table 3: Effects of BAP individually and in combination with NAA or Kn in MS medium of induced callus tissue culture from nodal and leaf segments of *H. indicus*.

PGRs	Conc. (mg/l)	% of explants produced callus	Time (d) required for MSBs induction	No. of shoot buds/vessel ($\bar{x}$ ± SE)	% of explants produced callus	Time (d) required for MSBs induction	No. of shoot buds/vessel ($\bar{x}$ ± SE)
		Nodal segment			Leaf segment		
BAP	0.2	75	15 - 30	2.14 ± 0.12	77	16 - 21	2.39 ± 0.32
	0.3	78	14 - 23	2.29 ± 0.34	75	16 - 19	2.22 ± 0.25
	1.0	76	18 - 30	2.19 ± 0.15	77	17 - 22	2.32 ± 0.29
	2.0	76	18 - 26	2.18 ± 0.17	73	21 - 25	2.43 ± 0.24
Kn + NAA	1.0 + 0.5	72	19 - 28	1.18 ± 0.13	63	21 - 25	1.24 ± 0.40
	1.0 + 1.0	76	17 - 30	2.19 ± 0.14	73	21 - 25	2.25 ± 0.24
	2.0 + 0.5	82	19 - 30	3.37 ± 0.39	66	21 - 23	1.39 ± 0.41
	2.0 + 1.0	85	15 - 19	3.46 ± 0.30	75	20 - 22	2.17 ± 0.15
BAP + Kn	1.0 + 0.5	74	14 - 22	1.34 ± 0.19	65	15 - 18	1.37 ± 0.46
	1.0 + 1.0	74	15 - 19	1.32 ± 0.38	67	17 - 25	1.44 ± 0.36
	1.5 + 0.5	76	16 - 30	2.19 ± 0.17	86	15 - 18	2.38 ± 0.50
	1.5 + 1.0	80	15 - 26	3.24 ± 0.25	66	16 - 25	1.33 ± 0.32
	2.0 + 0.5	76	18 - 24	2.74 ± 0.12	65	17 - 21	1.31 ± 0.29
	2.0 + 1.0	74	16 - 20	1.92 ± 0.28	87	15 - 18	3.22 ± 0.19
BAP + NAA	1.0 + 0.5	76	17 - 30	2.19 ± 0.14	85	16 - 20	2.28 ± 0.38
	1.0 + 1.0	72	19 - 28	1.18 ± 0.13	90	15 - 18	3.54 ± 0.29
	1.5 + 0.5	82	19 - 30	3.37 ± 0.39	86	18 - 25	3.20 ± 0.18
	1.5 + 1.0	85	15 - 19	3.46 ± 0.30	76	17 - 22	2.26 ± 0.32
	2.0 + 0.5	76	19- 25	2.81 ± 0.32	73	20 - 21	2.19 ± 0.19
	2.0 + 1.0	74	18 - 24	1.84 ± 0.48	73	21 - 25	2.24 ± 0.21

d = days; MSBs = Multiple Shoot Buds; Values are the means of 5 replicates.

Table 4: Effects of different concentrations and combination of PGRs on the elongation of directly and indirectly produced multiple shoot buds of *H. indicus*.

PGRs	Conc. (mg/l)	Initial length (cm) of individual shoot buds ($\bar{x}$ ± SE)	Length (cm) of individual shoot buds after 30d of culture ($\bar{x}$ ± SE)
BAP + NAA	1.0 + 0.5	2.17 ± 0.16	5.22 ± 0.15
	1.0 + 1.0	1.21 ± 0.07	3.15 ± 0.09
	1.5 + 0.5	1.60 ± 0.41	2.57 ± 0.29
	1.5 + 1.0	2.15 ± 0.12	3.23 ± 0.05
	2.0 + 0.5	1.61 ± 0.20	4.25 ± 0.09
	2.0 + 1.0	1.07 ± 0.07	3.03 ± 0.04
BAP + Kn	1.0 + 0.5	2.64 ± 0.20	4.55 ± 0.02
	1.5 + 1.0	1.57 ± 0.44	2.65 ± 0.03
	2.0 + 0.5	1.63 ± 0.33	3.06 ± 0.05
	2.0 + 1.0	1.56 ± 0.45	2.54 ± 0.26
	2.5 + 0.5	2.65 ± 0.22	4.75 ± 0.03
	2.5 + 1.0	2.63 ± 0.22	4.83 ± 0.14

d = days; Values are the means of 5 replicates.

Table 5: Effects of different concentrations and combinations of PGRs on the development of roots in elongated MSBs of *H. indicus* on half and full strength MS medium.

PGRs	Conc. (mg/l)	Days to root induction	% of micro shoot produced roots	Average number of roots/ micro shoot ($\bar{x}$ ± SE)	Average length (cm) roots after 30d of culture ($\bar{x}$ ± SE)
½ MS0	0.0	---	---	---	---
½ MS + IBA	0.5	15 - 20	73	3.12 ± 0.01	3.03 ± 0.02
	1.5	14 - 19	75	3.13 ± 0.04	3.05 ± 0.03
	2.0	12 - 19	77	3.14 ± 0.05	3.08 ± 0.01

MS + IBA	0.5	16 - 20	70	2.12 ± 0.02	1.86 ± 0.05
	1.0	12 - 14	77	3.14 ± 0.02	4.03 ± 0.04
	1.5	10 - 11	85	5.52 ± 0.04	4.54 ± 0.03

d = days; --- = No response; Values are the mean of 5 replicates.

Table 6: Qualitative test for alkaloids of *Hemidesmus indicus* (L.) R.Br.

Plant sample used	Qualitative estimation of alkaloids by different reagents				
	D	H	M	W	T
In vitro developed plant	+++	++	++	+++	+
Naturally grown plant	+++	++	+	+++	+

Notes: Name of the reagents, D-Dragendroff's reagent, H-Hager's reagent, M-Mayer's reagent, W-Wagner's reagent and T- Tannic acid reagent.

Table 7: Qualitative test for ten secondary metabolites of *Hemidesmus indicus* (L.) R.Br.

Plant sample Used	Secondary metabolites(% of coloration)										
	Phl.	Flv.	Ter.	Str.	Gly.	Qui.	Cou.	Anthro.	Sap.	Tann.	Phe.
In vitro developed plant	+++	+++	++	+++	+++	+++	+++	+++	-	+++	-
Naturally grown plant	+++	+++	+++	+++	+++	+++	-	++	-	++	-

Notes: Phl.= Phlobatannins, Flv.= Flavonoids, Ter.= Terpenoids, Str.= Steroids, Gly.= Glycosides, Qui.= Quinine, Cou.= Coumarin, Anthro.=Anthroquinone, Sap.=Saponins, Tann.= Tannin, Phe = Phenol.

Fig 1: Induction of yellowish green callus on MS medium with 1.0 mg/l 2,4-D.

Fig 2: Induction of whitish green callus on MS medium with 1.5 mg/l BAP and 1.0 mg/l NAA.

Fig 3: Induction of whitish green callus on MS medium with 1.5 mg/l BAP and 1.0 mg/l NAA.

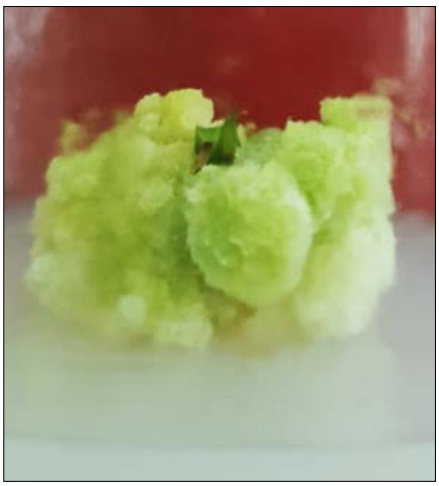

Fig 4: Induction of whitish callus on MS medium with 1.0 mg/l 2,4-D.

Fig 5: Induction of MSBs from nodal segment on MS medium with 2.0 mg/l BAP and 0.5 mg/l Kn.

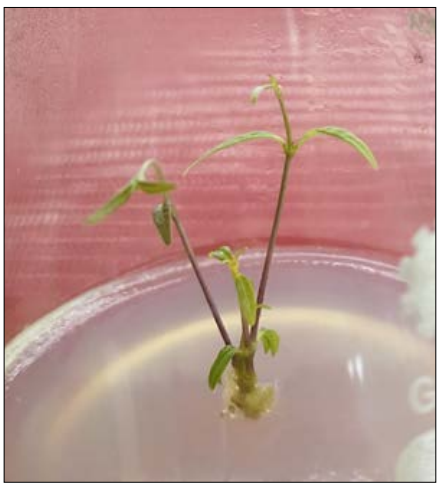

Fig 6: Proliferation of MSBs from nodal explant on MS medium with 2.0 mg/l BAP and 0.5 mg/l Kn.

Fig 7: Induction of MSBs from shoot apices on MS medium with 2.0 mg/l BAP and 1.0 mg/l Kn.

Fig 8: Proliferation of MSBs from shoot apices on MS medium with 2.0 mg/l BAP and 1.0 mg/l Kn.

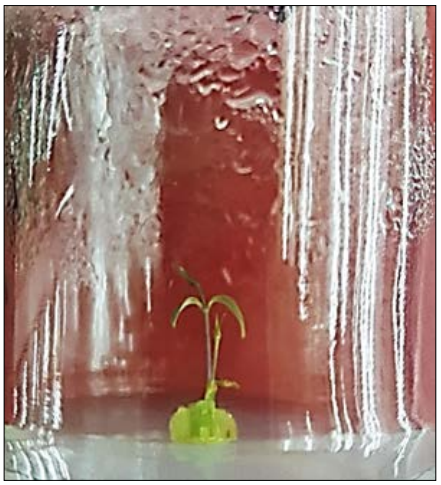

Fig 9: Initiation of MSBs from whitish green callus in MS medium with 1.5 mg/l BAP and 1.0 mg/l NAA.

Fig 10: Proliferation of MSBs from yellowish green callus in MS medium with 1.0 mg/l BAP and 1.0 mg/l NAA.

Fig 11: Elongation of MSBs on MS medium with 1.0 mg/l BAP and 0.5 mg/l NAA.

Fig 12: Maximum elongation of MSBs on MS medium with 1.0 mg/l BAP and 0.5 mg/l NAA.

Fig 13: Induction of root on full strength MS medium with 1.5 mg/l IBA.

Fig 14: Maximum number and length of roots on MS medium with 1.5 mg/l IBA.

Fig 15: *In vitro* grown seedling in plastic pot at outside environment.

Discussion

The establishment of a highly efficient *in vitro* regeneration system for *Hemidesmus indicus* holds immediate implications for both ecological conservation and the sustainable harvesting of pharmaceutical compounds (Shanmugapriya and Sivakumar 2011, Saryam *et al*. 2012) [9-10]. The morphogenetic plasticity observed in this study was heavily dictated by the precise concentrations and

combinations of exogenously applied plant growth regulators (PGRs).

Callus induction from nodal and leaf segments was most prolific utilizing the synthetic auxin 2,4-D (1.0 mg/l) or the combination of BAP and NAA (1.5 mg/l and 1.0 mg/l). Physiologically, 2,4-D acts as a potent stimulator of cellular dedifferentiation, reprogramming mature somatic cells to re-enter the cell cycle and rapidly divide into unorganized callus mass (Taiz and Zeiger 2006) [23]. Our findings heavily align with previous tissue culture research on *H. indicus*, which similarly reported vigorous callus proliferation utilizing synergistic auxin-cytokinin interactions (Siddique and Bari 2006) [12].

The critical transition from callus to organogenesis, as well as direct multiple shoot regeneration from intact nodal and shoot apex explants, demonstrated a profound dependency on cytokinin synergy. The maximum proliferation of multiple shoot buds (5.44 ± 0.35 buds per nodal explant) was achieved utilizing a high-cytokinin combination of 2.0 mg/l BAP and 0.5 mg/l Kn. Cytokinins operate fundamentally by overriding the endogenous hormonal suppressors that maintain apical dominance, thereby stimulating rampant cell division in axillary and adventitious meristems (Purohit *et al*. 2014) [11]. The superiority of BAP combined with Kn for generating high frequency shoot multiplication in Asclepiadaceae corroborates extensive reports demonstrating that optimal cytokinin ratios are vital for breaking recalcitrance in woody medicinal shrubs (Shreekumar *et al.* 2000, Shekhawat *et al.* 2015) [13, 24]. Furthermore, the *in vitro* conditions may mimic anatomical transformations, as observed in recent studies on *H. indicus* trichome variations between *in vivo* and *in vitro* states, where hormonal balance is critical to morphological expression (Deore 2021) [25].

Subsequent elongation of the induced microshoots required a distinct shift in the PGRs equilibrium. The specific combination of BAP and a low concentration of NAA (1.0 mg/l and 0.5 mg/l) facilitated the maximum linear vertical growth of the shoots. Physiologically, low auxin concentrations assist in cellular elongation and vascular tissue differentiation without triggering antagonistic basal callus formation a developmental bottleneck frequently documented during the micropropagation of climbing medicinal species.

For rhizogenesis, full strength MS medium supplemented with 1.5 mg/l IBA proved highly superior, yielding an 85% rooting response. IBA is a biochemically stable auxin that undergoes slow degradation by the plant's endogenous auxin oxidase system, ensuring a prolonged, localized physiological effect precisely at the basal cut end of the micro-shoot, thereby rapidly inducing vigorous adventitious root primordia. The complete absence of rooting in PGRs free control medium unequivocally highlights the strict requirement for exogenous auxin supplementation to trigger the rooting cascade in *H. indicus* (Shanmugapriya and Sivakumar 2011) [9].

The comparative phytochemical profiling yielded highly significant, novel findings for the pharmaceutical industry. The qualitative analysis confirmed that *in vitro* regenerated plantlets successfully biosynthesized and accumulated the core secondary metabolites (steroids, flavonoids, terpenoids) at concentrations completely equivalent to those found in naturally grown, mature plants (Harborne 1973) [21]. However, the *in vitro* clones exhibited a marked, demonstrable superiority in the accumulation of coumarins, quinine and uniquely, phlobatannins. Coumarins and phlobatannins possess potent anti-inflammatory, hepatoprotective, antioxidant and wound healing properties, which constitute the pharmacological hallmarks of the *H. indicus* root system (Webb 1949, Aplin and Cannon 1971) [26-27].

The elevated biosynthesis of these specific secondary metabolites *in vitro* can be mechanistically attributed to the controlled environmental stress inherent to the sealed culture vessels, alongside the direct elicitor-like action of the exogenous PGRs (Oksman-Caldentey and Inze 2004) [15]. Synthetic cytokinins (BAP) and auxins alter the biochemical pathways and transcriptomic profiles directly associated with secondary metabolism (Ramachandra Rao and Ravishankar 2002) [16]. Furthermore, the continuous, non-limiting availability of carbon sources (sucrose) and macronutrients within the MS media provides ample metabolic precursors, shifting the physiological cellular flux toward defense-related compound synthesis (Komes *et al.* 2011) [28]. This phenomenon definitively confirms that *in vitro* propagated *H. indicus* is not only a viable, sustainable conservation alternative, but it serves as a superior, highly concentrated metabolic sink for pharmaceutical bio-actives compared to wild harvested specimens (Edeoga *et al*. 2005, Bansode and Salarkar 2015) [17-18].

Conclusion

This research successfully establishes a highly reproducible, robust *in vitro* micropropagation protocol for the rapid mass multiplication of the critically endangered medicinal plant *Hemidesmus indicus* (L.) R.Br. Maximum direct shoot regeneration was efficiently achieved from nodal segments utilizing 2.0 mg/l BAP combined with 0.5 mg/l Kn, followed by rapid *in vitro* rooting facilitated by 1.5 mg/l IBA. The successfully acclimatized seedlings demonstrated an excellent *ex vitro* survival rate of 85%. Crucially, comprehensive qualitative phytochemical profiling validated that the *in vitro* generated plantlets function as a superior metabolic sink, accumulating equivalent or higher concentrations of vital therapeutic secondary metabolites specifically coumarin, quinine and phlobatannin compared to naturally grown plants. Consequently, this optimized protocol mitigates wild harvesting pressures, guarantees genetic and biochemical stability and establishes a highly reliable, controlled pathway for the commercial exploitation of *H. indicus* by the global pharmaceutical industry.

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