International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 24-05-2026, Accepted: 23-06-2026, Published: 08-07-2026
Volume 11, Issue 3, 2026, Page No. 53-61

Establishment of an efficient *In vitro* seed germination and micropropagation protocol for *Spermacoce alata* Aubl. - A medicinally important plant species of chittagong Hill Tracts, Bangladesh

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Abstract
Spermacoce alata Aubl. (Rubiaceae) is a highly valued medicinal plant possessing significant therapeutic properties, including anti-leukemic and anti-diabetic activities. However, widespread habitat destruction and over exploitation necessitate the development of rapid, reliable conservation and multiplication strategies. Highest seed germination (90%) was achieved on MS medium with 1.0 mg/l 2, 4-D and 1.0 mg/l BAP while poorest (65%) result was recorded on MS + 1.0 mg/l BAP + 1.0 mg/l NAA. Natural nodal explants *via* direct organogenesis, exhibited the highest multiple shoot buds (MSBs) induction frequency (95%) when cultured on Murashige and Skoog (MS) medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, yielding an average of 4.87 ± 0.19 shoots per nodal explant within 20-23 days whereas, topmost MSBs induced (92%) on MS medium with 2.0 mg/l BAP and 1.0 mg/l NAA developed 4.77 ± 0.29 MSBs/ natural shoot apices explant within 24-28 days. During indirect organogenesis, MS medium with 1.0 mg/l 2,4-D and 0.5 mg/l BAP achieved the highest callus induction (94%) from *in vitro* raised nodal explant, while MS medium with 2.0 mg/l 2,4-D and 1.0 mg/l BAP facilitated maximum callus formation (88%) from *in vitro* developed leaf explant. For shoot elongation, the combination of 1.5 mg/l BAP and 1.0 mg/l NAA proved most effective, stimulating a maximum longitudinal increment of 4.32 ± 0.24 cm after 30 days of culture. Rhizogenesis was successfully induced *in vitro*, with half strength MS medium supplemented with 1.0 mg/l Indole-3-butyric acid (IBA) achieving the highest rooting frequency (92%), generating 2.18 ± 0.19 roots per shoot with an average root length of 3.80 ± 0.17 cm. Following a systematic hardening process in a 1:1 mixture of garden soil and compost, 85% of the *in vitro* regenerated seedlings were successfully acclimatized to *ex vitro* conditions. This protocol provides a robust foundation for the large scale commercial cultivation and systematic conservation of *S. alata*.

Keywords: Acclimatization, callus induction, organogenesis, rhizogenesis, *Spermacoce alata*

Introduction

The global reliance on botanical therapeutics has accelerated exponentially, with an estimated 80% of populations in developing nations depending on traditional phyto-medicine for primary healthcare (Hossain 2003, WHO 2002) [1-2]. Medicinal plants synthesize a vast and structurally diverse array of secondary metabolites including alkaloids, flavonoids, terpenoids and phenolic compounds that exert profound pharmacological effects (Dar *et al*. 2017, Ghani 2003) [3-4]. *Spermacoce alata* Aubl. locally known in Bangladesh as 'Ghuiojhil' or 'Madanghanta', is a pivotal medicinal herb belonging to the Rubiaceae family. While native to tropical America, it has naturalized extensively across South Asia, specifically within the fertile, moist ecosystems of the Chittagong Hill Tracts (Manandhar 1995) [5]. Ethno-pharmacological data and modern phytochemical analyses highlight the efficacy of *S. alata* in treating metabolic syndromes, diabetes and hepatic steatosis, while its extracts demonstrate potent antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*, alongside notable anti-leukemic and anti-malarial properties (Fan *et al*. 2024, Luo 2015, Saidu *et al*. 2023) [6-8].

Despite its immense pharmacological value, the natural populations of *S. alata* face severe threats. Driven by the surging pharmaceutical demand, unmanaged and indiscriminate harvesting from wild habitats has precipitated an alarming depletion of its natural reserves (Chen *et al*. 2016) [9]. Compounding this over exploitation is the progressive degradation of local ecosystems through agricultural encroachment and rapid urbanization, which threatens the localized extinction of numerous indigenous medicinal species in Bangladesh (Ghani 2003, Sridhar 2011) [3, 10]. Furthermore, traditional propagation methods reliant on seeds are often hindered by variable viability constraints, susceptibility to environmental pathogens and heterogeneous germination kinetics, rendering wild harvesting commercially unsustainable (Pence *et al*. 2011) [11].

To avoid these ecological bottlenecks, *in vitro* plant tissue culture technology offers an advanced, highly efficient alternative for the rapid mass clonal propagation and *ex situ* conservation of threatened medicinal flora (Debnath *et al*. 2006, Hasnain *et al*. 2022) [12-13]. Micropropagation ensures the continuous, year round supply of genetically uniform, disease free propagules independent of seasonal fluctuations, thereby mitigating wild harvesting pressures (Altpeter *et al*. 2016, Rout *et al*. 2000) [14-15]. While foundational *in vitro* protocols have been documented for related Rubiaceae taxa, such as *Paederia foetida* (Amin *et al*. 2003) [16] and *Ophiorrhiza* species (Biswas *et al*. 2007) [17], a standardized, highly reproducible direct and indirect micropropagation methodology for *S. alata* has not been thoroughly established.

Therefore, the present investigation was undertaken to address this critical research gap. The explicit objective of this study is to establish an optimized, highly efficient protocol for the *in vitro* seed germination, callus induction, multiple shoot buds (MSBs) proliferation, longitudinal elongation and rhizogenesis of *S. alata*. By meticulously evaluating the synergistic effects of various exogenous cytokinins and auxins, this research aims to provide a reliable biotechnological pathway for the mass multiplication and sustainable commercial utilization of this cherished medicinal species.

Materials and Methods

Source of explants and surface sterilization

Field grown specimens of *Spermacoce alata* Aubl. were collected from their natural habitats in the Chittagong Hill Tracts (CHT) region of Bangladesh. Seeds, intact shoot apices and nodal segments were excised and subjected to rigorous surface sterilization protocols under controlled aseptic conditions. The explants were initially washed thoroughly under continuous running tap water to remove superficial particulate matter. Subsequently, the tissues were treated with 1% Savlon (ACI Pharma, Bangladesh) enriched with a few drops of Tween-20 for 5-10 minutes with constant mechanical agitation. After rinsing 3-4 times with distilled water to eliminate detergent residues, the explants were transferred to a laminar air flow cabinet. The tissues were then briefly rinsed with 70% ethanol for less than 60 seconds and immediately submerged in a 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for species specific durations. To ensure the complete eradication of the sterilant, the explants were washed thoroughly with sterile, double distilled water over 4-5 successive changes.

Media preparation

The nutrient formulations utilized MS (Murashige and Skoog 1962) [18] basal medium. Media configurations were fortified with 3% (w/v) sucrose serving as the primary carbon source. Five distinct stock solutions encompassing macronutrients, micronutrients, iron-EDTA, vitamins and inositol were prepared using reagent grade chemicals. The pH of all media variants was digitally adjusted to 5.8 using a HANNA-30V pH meter *via* the drop wise addition of 1N NaOH or 1N HCl prior to the addition of 0.8% (w/v) agar as the gelling agent. Aliquots of the prepared media were dispensed into culture vessels, securely sealed and autoclaved at 121°C under 1.5 kg/cm² pressure for 30 minutes.

Plant growth regulators setup

To investigate morphogenic responses, the MS basal medium was supplemented with varying configurations of Plant Growth Regulators (PGRs). Cytokinins, including 6-benzylaminopurine (BAP) and Kinetin (Kn) and auxins, specifically α-naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D), were utilized. The experimental matrices involved individual and combined treatments for callus induction, direct MSBs induction, shoot elongation and rhizogenesis.

Culture conditions and acclimatization

All *in vitro* cultures were incubated in a growth room under a 14-hour continuous light and 10-hour dark photoperiod (2000-3000 lux) at 25 ± 2°C. Sub-culturing was executed aseptically at 15-20 day intervals. Well-rooted *in vitro* seedlings were transferred to ambient room temperature for progressive adaptation. Seedlings were eventually extracted, washed free of agar and potted in an autoclaved 1:1 mixture of garden soil and compost, pre-treated with 0.1% Agrosan fungicide and misted regularly to ensure successful *ex vitro* acclimatization.

Experimental design and statistical analysis

The experiments were arranged in a Completely Randomized Design (CRD) with five replicates per treatment. Quantitative metrics were recorded continuously and data were subjected to standard statistical computation (Mean ± SE).

Results

In vitro seed germination

Surface sterilized seeds of *S. alata* inoculated onto MS medium fortified with different kinds and combinations of PGRs successfully broke physical dormancy (Table 1). This provided a rapid, robust and contamination free pool of primary seedling explants utilized for subsequent *in vitro* morphogenic evaluations. From seed germination the highest percentage (90%) of whitish compact callus (WCC) was achieve on MS medium with 1.0 mg/l 2, 4-D and 1.0 mg/l BAP within 20-22 days of culture. Minimum percentage (65%) of seed germination was record on MS medium with 1.0 mg/l BAP and 1.0 mg/l NAA after 32-35 days of culture.

Direct MSBs induction from nodal and shoot apices explants

To promote direct organogenesis nodal and shoot apices of *S. alata* cultured on MS0 and MS media with varied PGRs combinations and concentrations (Table 2). The maximum shoot buds formation from natural nodal segment culture the highest frequency (95%) and number of MSBs per explant (4.87 ± 0.19 no.) were observed on MS medium containing 1.0 mg/l BAP + 1.0 mg/l NAA within 20-23 days of culture followed by PGRs free MS basal medium (65%, 2.07 ± 0.20 no., 35-38d). Whereas, in case of natural shoot apices culture the outmost percentage (92%) and number of MSBs (4.77 ± 0.29 no.) developed on MS medium with 2.0 mg/l BAP + 1.0 mg/l NAA within 24-28 days of inoculation followed by PGRs free MS basal medium (68%, 2.04 ± 0.20 no., 36-38d).

Callus induction from nodal and leaf explants

To evaluate indirect organogenesis, nodal and leaf segments were cultured on MS medium supplemented with various concentrations and combinations of PGRs (Table 3). Nodal segment produced the highest percentage (94%) of whitish compact callus (WCC) within 22-25 days of culture on MS medium containing 1.0 mg/l 2,4-D + 0.5 mg/l BAP followed by MS + 1.0 mg/l 2,4-D + 0.5 mg/l Kn (92%, 24-26d), while the minimum result (67%) was observed on MS medium fortified with 1.0 mg/l Kn alone.

Similarly, leaf segment produced the highest percentage (88%) of whitish brown Soft compact callus (WBC) within 24-28 days of culture on MS medium containing 2.0 mg/l

2,4-D + 1.0 mg/l BAP followed by MS + 2.0 mg/l 2,4-D + 1.0 mg/l IAA (85%, 25-28d), while the minimum findins (70%) was observed on MS medium fortified with 1.0 mg/l Kn alone.

***In vitro* MSBs development**

Direct organogenesis bypassing the intermediate callus phase was also successfully achieved and produced MSBs from *in vitro* callus culture (Table 4). Callus tissues successfully differentiated into multiple shoot buds (MSBs). *In vitro* raised callus tissue produced the maximum frequency of MSBs induction (95%) was record on MS medium supplemented with 0.5 mg/l 2, 4-D combined with 1.0 mg/l BAP. Under this optimal configuration, the highest mean number of shoot buds generated per explant was 3.86 ± 0.23 no, manifesting rapidly within 22-24 days of culture followed by MS medium containing with 1.0 mg/l 2, 4-D and 1.0 mg/l NAA (90%, 3.64 ± 0.22 no., 23-25d). Poorest responses (65%, 2.71 ± 0.37 no., 32-34d) was recorded on MS medium with 1.0 mg/l BAP.

Shoot elongation

To secure functional development, the induced MSBs were isolated and transferred to specific elongation media formulations (Graph 1). The synergistic application of BAP and NAA consistently outperformed other combinations. The maximum shoot elongation increment recorded was 4.32 ± 0.24 cm, achieved on MS medium supplemented with 1.5 mg/l BAP and 1.0 mg/l NAA followed by MS medium with 2.0 mg/l BAP and 1.0 mg/l NAA (3.32 ± 0.21 cm). In contrast, combinations utilizing IAA yielded comparatively stunted longitudinal growth, with a minimum recorded elongation increment of 2.35 ± 0.20 cm observed on MS medium with 0.5 mg/l BAP and 0.5 mg/l IAA.

Rhizogenesis

Elongated shoots (2.0-3.0 cm) were transferred to half strength MS medium supplemented with varying auxins to induce rooting. The highest percentage of root induction (92%) was achieved using half strength MS medium supplemented with 1.0 mg/l IBA (Graph 2). This medium supported optimal rhizogenic architecture, yielding a maximum of 2.18 ± 0.19 robust roots per micro shoot with an extensive average root length of 3.80 ± 0.17 cm. The lowest rooting frequency (67%) was recorded on PGR free half strength MS medium, resulting in a sparse 1.35 ± 0.23 roots per shoot and a shortened mean length of 2.10 ± 0.22 cm.

Acclimatization and survival

The ultimate validation of the germination and micropropagation protocol lies in *ex vitro* establishment. Upon the development of robust root architectures, the *in vitro* generated *S. alata* seedlings were subjected to sequential acclimatization in a 1:1 soil-to-compost substrate. Through meticulous misting and progressive environmental exposure, the seedlings transitioned smoothly to *ex vitro* autotrophy, culminating in a high final *ex vitro* survival rate of 85%.

Table 1: Effects of different concentrations and combinations of PGRs on *in vitro* seed germination of *Spermacoce alata* Aubl. on 0.8% (w/v) agar solidified MS medium with different PGRs

PGRs combination	Concentration (mg/l)	% of explants gave response	Time (d) required for induction of callus	Types of response
2, 4-D	1.0	70	29-32	WCC
	2.0	75	25-27	WCC
2, 4-D + BAP	0.5 + 1.0	85	26-28	WCC
	1.0 + 1.0	90	20-22	WCC
2, 4-D + Kn	1.0 + 0.5	75	30-32	WCC
	1.0 + 1.0	85	24-26	WCC
BAP + IAA	1.0 + 0.5	80	24-26	WCC
	1.0 + 1.0	85	25-28	WCC
BAP + IBA	1.0 + 0.5	75	28-30	WCC
	1.0 + 1.0	80	24-27	WCC
BAP + NAA	1.0 + 0.5	70	30-33	WCC
	1.0 + 1.0	65	32-35	WCC

*d = Days; WCC= Whitish compact callus;
**values are the means ± SE of each experiment consist of five replicates.

Table 2: Effects of BAP and Kn individually and BAP in combination with NAA or IAA for induction of MSBs from natural nodal segments and shoot apices of *S. alata*

Explants	PGRs combination	Concentration (mg/l)	% of explants produced MSBs	Time (d) require for induction of MSBs	Average* no. of MSBs per culture (mean ± SE)
Nodal Segments	MS0	-	65	35-38	2.07 ± 0.20
	MS + BAP	1.0	75	31-34	3.55 ± 0.35
		2.0	77	23-25	2.67 ± 0.26
	MS + Kn	1.0	77	23-25	3.33 ± 0.24
		2.0	75	25-27	2.56 ± 0.47
	MS + BAP + NAA	0.5 + 0.5	73	30-32	2.17 ± 0.32
		1.0 + 1.0	95	20-23	4.87 ± 0.19
		2.0 + 1.0	80	27-29	3.10 ± 0.17

	MS + BAP + IAA	0.5 + 0.5	78	27-29	2.60 ± 0.26
		1.0 + 1.0	78	23-25	3.44 ± 0.20
		2.0 + 1.0	80	26-28	3.30 ± 0.13
Shoot apices	MS0	-	68	36 - 38	2.04 ± 0.20
	MS + BAP	1.0	81	27 - 33	3.66 ± 0.26
		2.0	83	25 - 32	3.23 ± 0.15
	MS + Kn	1.0	71	34 - 36	2.12 ± 0.27
		2.0	79	33 - 37	3.18 ± 0.32
	MS + BAP + NAA	0.5 + 0.5	75	27-29	3.20 ± 0.27
		1.0 + 1.0	82	35 - 38	3.49 ± 0.33
		2.0 + 1.0	92	24 - 28	4.77 ± 0.29
	MS + BAP + IAA	0.5 + 0.5	78	27-29	3.55 ± 0.32
		1.0 + 1.0	85	33 - 38	4.13 ± 0.21
		2.0 + 1.0	80	34 - 38	4.39 ± 0.28

*d = days; MSBs = Multiple shoot buds;
**values are the means ± SE of each experiment consist of five replicates.

Table 3: Effects of different concentrations and combinations of PGRs on induction callus from *in vitro* raised nodal and leaf explant of *S. alata* on 0.8% (w/v) agar solidified MS medium

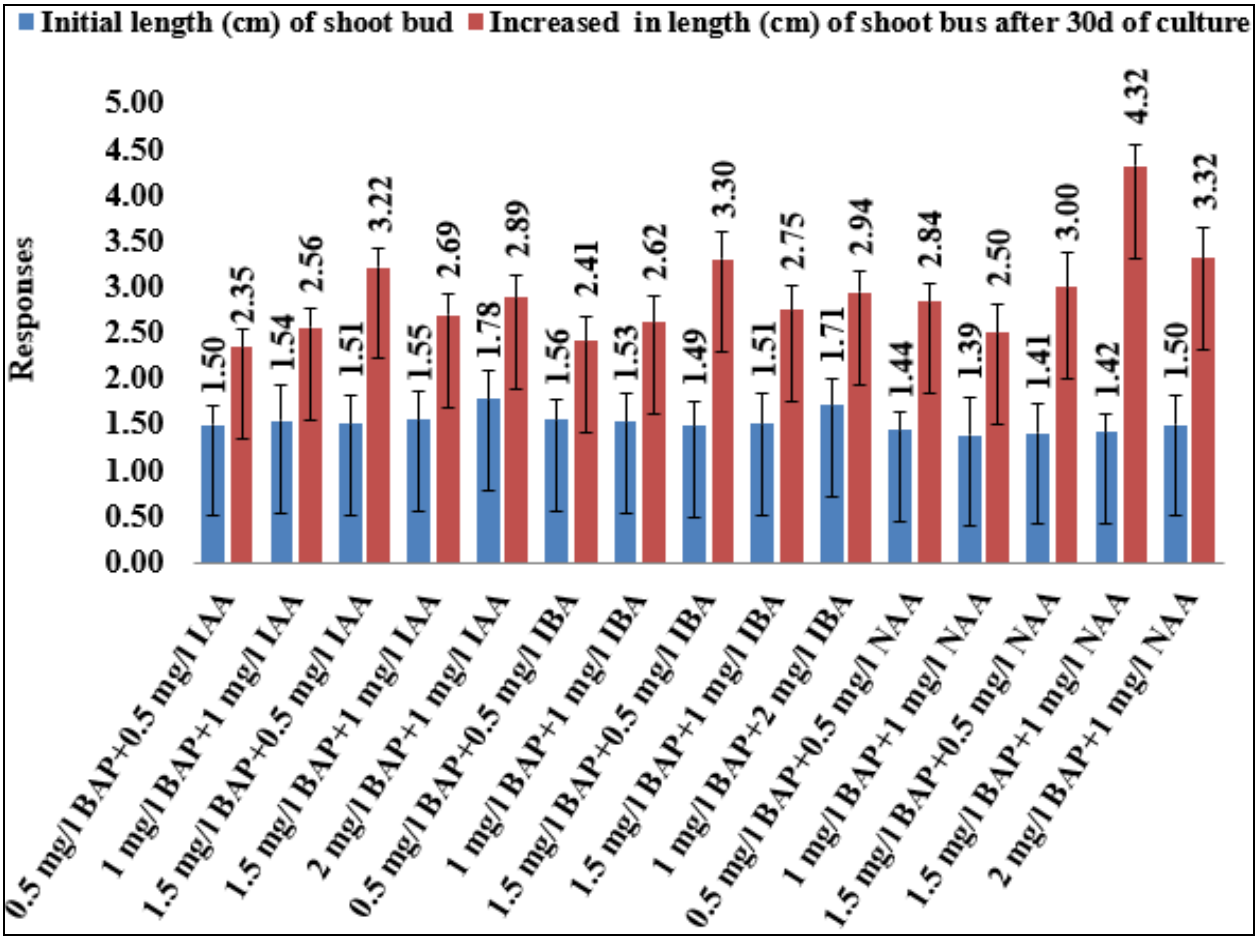
PGRs combination	Concentration (mg/l)	Type of explants	% of explants Produced callus	Time (d) required for Callus induction	Colour and texture of induced callus
Kn	1.0	NS	67	30-33	WCC
	2.0	LS	70	35-37	WBC
BAP	1.0	NS	78	28-30	WCC
	2.0	LS	83	30-33	WBC
2, 4-D	1.0	NS	77	28-30	WCC
	2.0	LS	82	29-31	WBC
2, 4-D + BAP	1.0 + 0.5	NS	94	22-25	WCC
	2.0 + 1.0	LS	88	24-28	WBC
2, 4-D + Kn	1.0 + 0.5	NS	92	24-26	WCC
	2.0 + 1.0	LS	81	30-33	WBC
2, 4-D + IAA	1.0 + 0.5	NS	73	29-32	WCC
	2.0 + 1.0	LS	85	25-28	WBC
2, 4-D + IBA	1.0 + 0.5	NS	80	28-31	WCC
	2.0 + 1.0	LS	77	33-35	WBC
2, 4-D + NAA	1.0 + 0.5	NS	85	26-29	WCC
	2.0 + 1.0	LS	75	34-37	WBC

*d = Days; WCC= Whitish soft compact callus; WBC = Whitish brown soft compact callus;
**values are the means ± SE of each experiment consist of five replicates.

Table 4: Development of MSBs on *in vitro* induced callus culture of *S. alata* on 0.8% (w/v) agar solidified MS medium with different concentrations and combinations of PGRS.

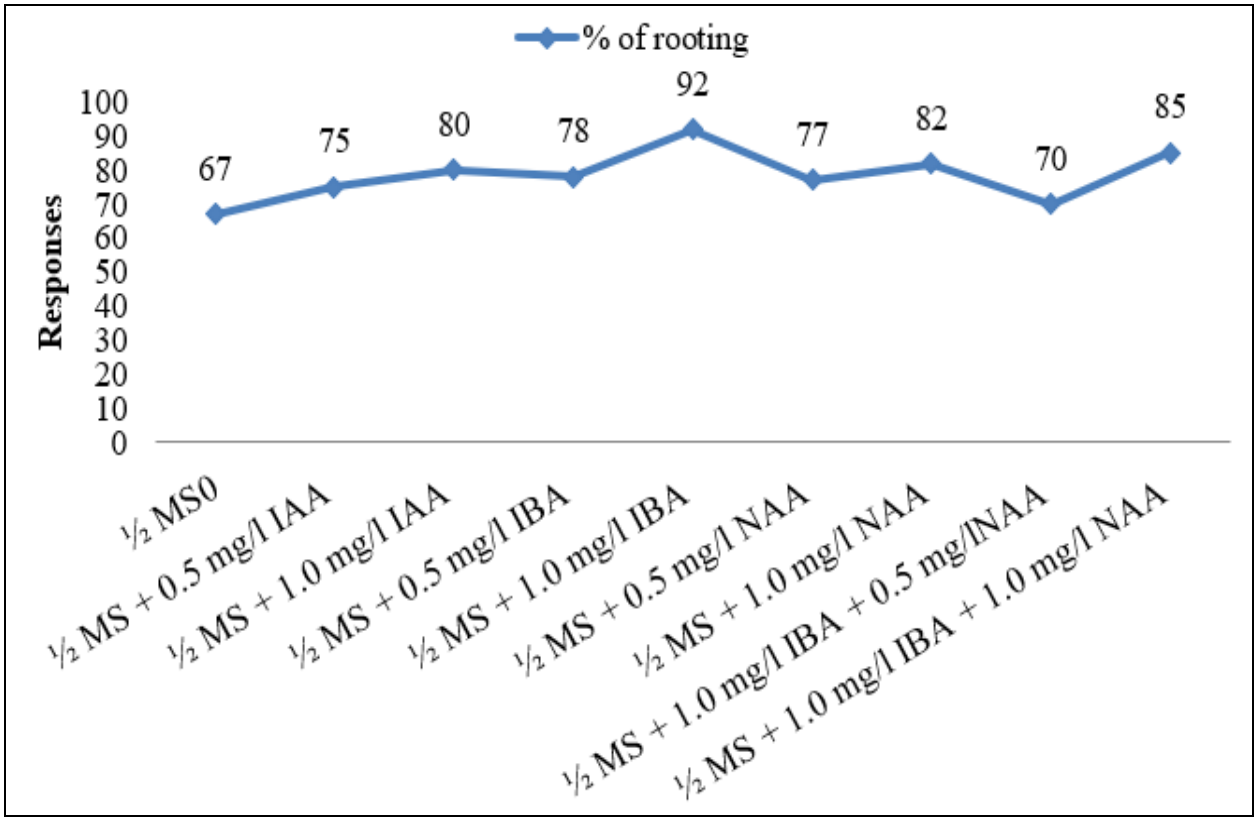
PGRs combination	Concentration (mg/l)	% of explants gave response	Time (d) required for induction of MSBs	Average* no. of MSBs per culture (mean ± SE)
BAP	1.0	65	32-34	2.71 ± 0.37
	2.0	70	30-32	3.16 ± 0.24
2, 4-D	1.0	75	28-30	2.98 ± 0.20
	2.0	80	25-27	3.40 ± 0.18
2, 4-D + BAP	0.5 + 1.0	95	22-24	3.86 ± 0.23
	1.0 + 1.0	85	24-27	3.10 ± 0.24
2, 4-D + Kn	0.5 + 1.0	75	30-32	3.18 ± 0.32
	1.0 + 1.0	80	26-29	2.90 + 0.22
2, 4-D + IAA	0.5 + 1.0	80	25-28	3.01 ± 0.19
	1.0 + 1.0	85	24-26	3.15 ± 0.24
2, 4-D + IBA	0.5 + 1.0	75	28-29	2.72 ± 0.28
	1.0 + 1.0	80	26-29	2.91 ± 0.22
2, 4-D + NAA	0.5 + 1.0	75	28-32	2.80 ± 0.17
	1.0 + 1.0	90	23- 25	3.64 ± 0.22

*d = Days; MSBs = Multiple shoot buds;
**values are the means ± SE of each experiment consist of five replicates.



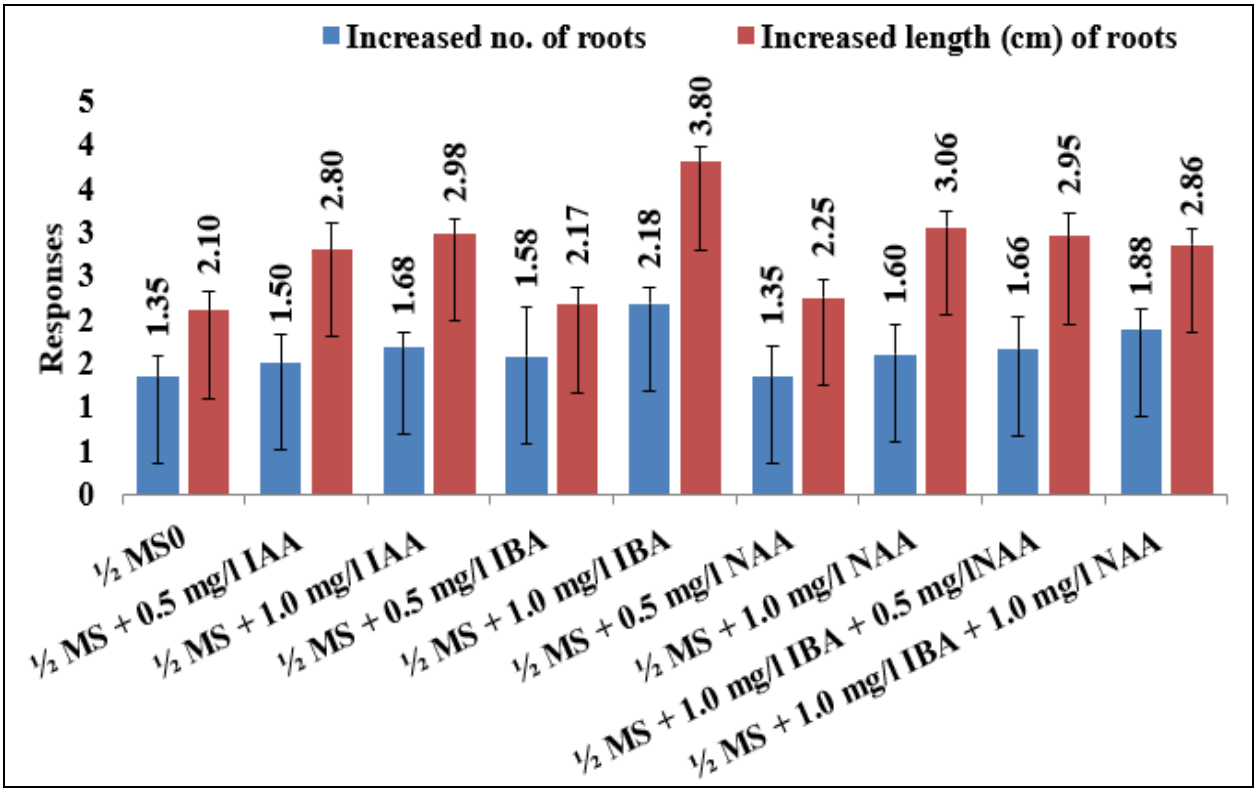
*d = days; MSBs = Multiple shoot buds; **values are the means ± SE of each experiment consist of five replicates.

Graph 1: Elongation of directly produced MSBs of *S. alata* on agar solidified MS medium supplemented with different PGRs.



*values are the means ± SE of each experiment consist of ten replicates.

Graph 2: Percentage of rooting in elongated shoot buds of *S. alata* on PGR free half MS and PGRs supplemented half strength MS medium.



*values are the means ± SE of each experiment consist of ten replicates.

Graph 3: Increased number and length of roots in *in vitro* elongated seedlings of *S. alata* on PGR free half MS and PGRs supplemented half strength MS medium.

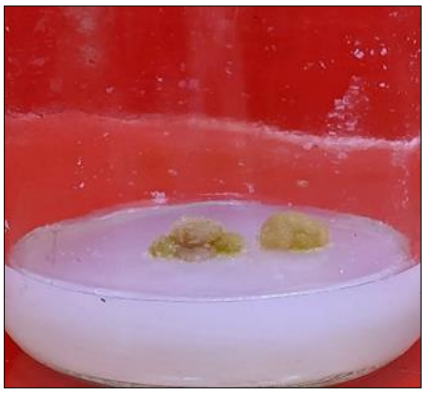


Fig 1: *In vitro* seed germination of *Spermacoce alata* on MS + 1.0 mg/l 2, 4-D + 1.0 mg/l BAP.

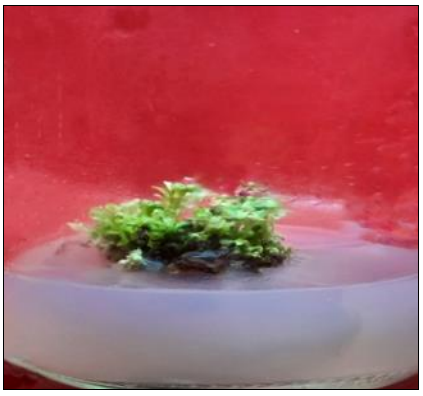


Fig 2: Development of MSBs from natural nodal segments of *S. alata* on MS + 1.0 mg/l BAP + 1.0 mg/l

Fig 3: Induction of MSBs from natural shoot apices of *S. alata* on MS + 2.0 mg/l BAP + 1.0 mg/l

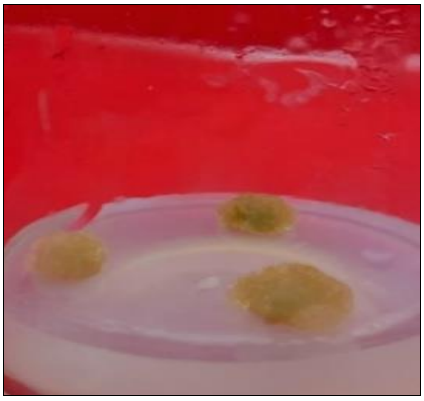


Fig 4: Callus initiation from natural leaf segment of *S. alata* on MS + 1.0 mg/l 2, 4-D + 0.5 mg/l Kn.

Fig 5: Development of MSBs derived from callus tissue culture of *S. alata* on MS + 0.5 mg/l 2, 4-D +

Fig 6: Elongation of shoot bud of *S. alata* on MS + 1.5 mg/l BAP + 1.0 mg/l NAA.

Fig 7: Elongation of shoot bud of *S. alata* on MS + 2.0 mg/l BAP + 1.0 mg/l NAA.

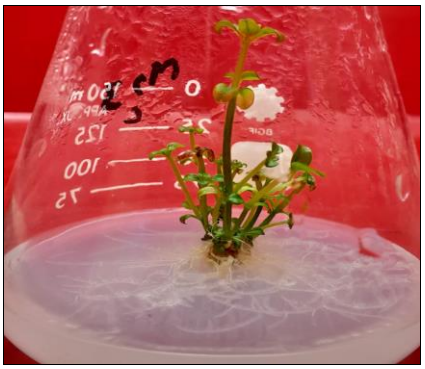


Fig 8: Induction of roots in elongated seedlings of *S. alata* on ½ MS + 1.0 mg/l IBA.

Fig 9: Transplantation of of *S. alata* in outside environment.

Discussion

The establishment of a highly reproducible *in vitro* propagation system for *Spermacoce alata* represents a critical advancement in the conservation and commercialization of this therapeutically potent species. The morphogenic sequence documented herein highlights the profound regulatory influence of exogenous plant growth regulators matrices on cellular totipotency and differentiation, heavily corroborating foundational theories of plant tissue culture.

The successful initiation of aseptic seeds on basal media circumvented common microbial contamination hurdles. Seed germination within the Rubiaceae family is often regulated by complex seed coat dormancies and the rapid *in vitro* hydration and nutrient assimilation provided by the basal MS salts accelerated radicle protrusion (Baskin and Baskin 1998, Hartmann *et al*. 2014) [19-20].

During indirect organogenesis, the synergy of 2,4-D and BAP effectively reprogrammed differentiated somatic cells into totipotent callus states, leading to successful MSBs formation. Similarly, during direct MSBs proliferation phase, explant origin profoundly influenced organogenic potential. Both nodal segments and shoot apices proved to be highly responsive meristematic hubs. The pronounced superiority of BAP over Kn in inducing MSBs aligns directly with established literature indicating BAP's enhanced metabolic stability and heightened affinity for cell division receptors in numerous medicinal taxa (Magyar-Tábori *et al*. 2010, Rout *et al*. 2000) [21, 15]. The physiological mechanism underpinning the massive shoot multiplication relies on the precise abrogation of apical dominance. Exogenous BAP fundamentally shifts the intracellular morphogenic gradients, signaling the rapid activation and division of quiescent axillary and adventitious meristems (Howell *et al*. 2003, Skoog and Miller 1957) [22-23]. Furthermore, the integration of a relatively moderate concentration of an auxin (NAA) synergistically modulated the cytokinin action. Auxins are critical for maintaining the mitotic cell cycle and mediating vascular tissue connectivity between the newly forming shoot primordia and the primary explant tissue (Perrot-Rechenmann 2010) [24]. Similar synergistic efficiencies of BAP and NAA driving organogenesis have been extensively reported in related medicinal plants, including *Boerhaavia diffusa* (Roy 2008) [25], *Ocimum sanctum* (Jamal *et al*. 2016) [26] and *Plumbago indica* (Bhadra *et al*. 2004) [27].

Longitudinal shoot elongation dynamics in *S. alata* explicitly required an altered plant growth regulators balance. The peak elongation observed with 1.5 mg/l BAP and 1.0 mg/l NAA indicates that while cytokinins sustain the cellular division required for continuous stem production, the augmented presence of NAA directly facilitates cellular expansion. Auxins stimulate the extrusion of protons into the apoplast, lowering the pH and activating expansin proteins, which loosen the cellulose micro-fibril network a physiological requisite for cell elongation known as the acid growth hypothesis (Majda and Robert 2018, Rayle and Cleland 1992) [28-29].

The rhizogenesis architecture generated during this study unequivocally demonstrated the superiority of IBA over IAA and NAA. The physiological rationale for IBA's dominance lies in its biochemical stability. IBA acts as an endogenous slow-releasing auxin depot; it is highly resistant to photo-degradation and enzymatic oxidation by IAA-oxidases, thereby ensuring a continuous, sustained exogenous auxin stimulus precisely at the cut surface of the micro shoot (De Klerk *et al*. 1999, Epstein and Ludwig-Müller 1993) [30-31]. Additionally, the strategic deployment of half strength MS basal medium lowered the ambient nitrogen load. Mild nutrient stress acts as a potent physiological trigger, forcing the plantlet to allocate carbon resources preferentially towards root biomass generation to expand its foraging surface area (Driver and Suttle 1987, George *et al*. 2008) [32-33]. This phenomenon mirrors the findings of Sivanesan and Jeong (2009) [34], who confirmed that diluted MS matrices combined with IBA universally optimize adventitious rooting in medicinal plant.

Finally, the 85% *ex vitro* acclimatization survival rate underscores the functional viability of the roots generated *in vitro*. *In vitro* environments, characterized by high humidity and low vapor pressure deficits, often yield seedlings with dysfunctional stomatal mechanics and impoverished epicuticular wax layers (Pospíšilová *et al*. 1999) [35]. The gradual hardening methodology employed here facilitated the successful physiological shift from heterotrophy to photoautotrophy, allowing the gradual deposition of cuticular waxes and the synchronization of stomatal closing mechanisms essential for terrestrial survival.

Conclusion

This investigation successfully established an exhaustive, highly efficient and reproducible protocol for the *in vitro* germination and micropropagation of *Spermacoce alata* Aubl., progressing from aseptic seed germination, direct and indirect organogenesis, elongation, rhizogenesis and successful field acclimatization. The optimal plant growth

regulators synergy of BAP and NAA for multiple shoot proliferation, coupled with IBA driven rooting, ensures maximum yield. This standardized methodology directly mitigates the ecological strain on wild populations caused by indiscriminate pharmaceutical harvesting, serving as a vital biotechnological tool for both large scale commercial cultivation and the strategic conservation of this valuable medicinal species.

Acknowledgement
The authors formally acknowledge the Plant Tissue Culture and Biotechnology Laboratory, Department of Botany, University of Chittagong, Bangladesh, for providing the essential infrastructural facilities, rigorous academic support and specialized resources that made this intensive botanical research possible. The authors thankfully acknowledge Ministry of Science and Technology, Bangladesh for the financial grant under special allocation to fruitfully completion this research work.

References

1. Hossain MZ. People's awareness about medicinal values of plants and prospect in Bangladesh. BRAC Research and Evaluation Division, Dhaka, 2003.
2. WHO. Traditional Medicine Strategy 2002-2005. World Health Organization, Geneva, 2002.
3. Dar RA, Shahnawaz M, Qazi PH. General overview of medicinal plants: A review. Journal of Phytopharmacology,2017:6(6):349-351.
4. Ghani A. Medicinal Plants of Bangladesh: Chemical Constituents and Uses (2nd Edition). Asiatic Society of Bangladesh, Dhaka,2003.
5. Manandhar NP. An inventory of some vegetable drug resources of Makawanpur district Nepal. Fitoterapia,1995:66:231-238.
6. Fan Y, Weng W, Ji S. Pharmacognostic studies on three species of *Spermacoce*. Journal of Holistic Integrative Pharmacy,2024:5(1):2-9.
7. Luo QL, Xu LM, Dong ZY, Zhou JW. Tan Triterpenic acids from *Spermacoce latifolia*. Journal of Tropical and Subtropical Botany,2015:463-468.
8. Saidu U, Ibrahim MA, De Koning HP, McKerrow JH, Caffrey CR, Balogun EO. Human Schistosomiasis in Nigeria: Present Status, Diagnosis, Chemotherapy and Herbal Medicines. Parasitology Research,2023:122:2751-2772.
9. Chen SL, Yu H, Luo HM, Wu Q, Li CF, Steinmetz A. Conservation and sustainable use of medicinal plants: problems, progress and prospects. Chinese Medicine,2016:11(1):1-10.
10. Sridhar TM. Studies on Plant Regeneration, Phytochemicals and Antimicrobial Activities of *Solanum nigrum* (L.). Ph.D. Dissertation, Sri Venkateswara University,2011.
11. Pence VC. Evaluating costs for the *in vitro* propagation and preservation of endangered plants. *In vitro* Cellular and Developmental Biology,2011:47(1):176-187.
12. Debnath M, Malik CP, Bisen PS. Micropropagation: a tool for the production of high quality plant-based medicines. Current Pharmaceutical Biotechnology,2006:7(1):33-49.
13. Hasnain A, Naqvi SA, Ayesha SI, Khalid F, Ellahi M, Iqbal S, Hassan MZ. Plants *in vitro* propagation with its applications in food, pharmaceuticals and cosmetic industries; current scenario and future approaches. Frontiers in Plant Science,2022:13:1-21.
14. Altpeter F, Springer NM, Bartley LE, Blechl AE, Brutnell TP, Citovsky V, Wang K. Advancing crop transformation in the era of genome editing. Plant Cell,2016:28(7):1510-1520.
15. Rout GR, Samantaray S, Das P. *In vitro* manipulation and propagation of medicinal plants. Biotechnology Advances,2000:18(2):91-120.
16. Amin MN, Rahman MM, Manik MS. Micropropagation of *Paederia foetida* L.: a rare and endangered medicinal plant of Bangladesh. Plant Tissue Culture,2003:13(2):117-123.
17. Biswas A, Roy M, Bari MA, Bhadra SK. *In vitro* propagation of *Abrus precatorius* L. - A rare medicinal plant of Chittagong Hill Tracts. Plant Tissue Culture and Biotechnology,2007:17(1):59-64.
18. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum,1962:15(3):473-497.
19. Baskin CC, Baskin JM. Seeds: Ecology, Biogeography and Evolution of Dormancy and Germination. Academic Press, San Diego, California,1998.
20. Hartmann HT, Kester DE, Davies FT, Geneve RL. Plant Propagation: Principles and Practices (8th Edition). Pearson, New Jersey,2014.
21. Magyar-Tábori K, Dobránszki J, Teixeira da Silva JA, Bulley SM, Hudák I. The role of cytokinins in shoot organogenesis in apple. Plant Cell, Tissue and Organ Culture,2010:101(3):251-267.
22. Howell SH, Lall S, Che P. Cytokinins and shoot development. Trends in Plant Science,2003:8(9):453-459.
23. Skoog F, Miller CO. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symposia of the Society for Experimental Biology,1957:11:118-130.
24. Perrot-Rechenmann C. Cellular responses to auxin: division versus expansion. Cold Spring Harbor Perspectives in Biology,2010:2(5):a001446.
25. Roy PK. Rapid multiplication of *Boerhaavia diffusa* L. through *in vitro* culture of shoot tip and nodal explants. Plant Tissue Culture and Biotechnology,2008:18(1):49-56.
26. Jamal MAHM, Sharif IH, Shakil M, Rahman ANMRB, Banu NA, Islam MR, Nazmuzzaman M. *In vitro* regeneration of a common medicinal plant, *Ocimum sanctum* L. for mass propagation. African Journal of Biotechnology,2016:15(24):1269-1275.
27. Bhadra SK, Akhter T, Hossain MM. *In vitro* clonal propagation of *Plumbago indica*. Plant Tissue Culture and Biotechnology,2004:14(1):25-32.
28. Majda M, Robert S. The role of auxin in cell wall expansion. International Journal of Molecular Sciences,2018:19(4):951-951.
29. Rayle DL, Cleland RE. The acid growth theory of auxin-induced cell elongation is alive and well. Plant Physiology,1992:99(4):1271-1274.
30. De Klerk GJ, Van der Krieken W, De Jong JC. The formation of adventitious roots: new concepts, new possibilities. *In vitro* Cellular and Developmental Biology,1999:35(3):189-199.

31. Epstein E, Ludwig-Müller J. Indole-3-butyric acid in plants: occurrence, synthesis, metabolism and transport. Physiologia Plantarum,1993:88(2):382-389.
32. Driver JA, Suttle GR. Nursery handling of propagules. In: Cell and Tissue Culture in Forestry. Springer, Dordrecht,1987:2:320-335.
33. George EF, Hall MA, De Klerk GJ. Plant Propagation by Tissue Culture (3rd Edition). Springer, Dordrecht, Netherlands,2008.
34. Sivanesan I, Jeong BR. *In vitro* propagation of *Sphagneticola trilobata* (L.). Propagation of Ornamental Plants,2009:9(1):10-15.
35. Pospíšilová J, Tichá I, Kadleček P, Haisel D, Plzáková Š. Acclimatization of plantlets to *ex vitro* conditions. Biologia Plantarum,1999:42(4):481-497.