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SHORT COMMUNICATION

IN VITRO CALLUS INDUCTION AND SHOOT REGENERATION FROM LEAF EXPLANTS OF *BARLERIA LUPULINA* LINDL.

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Abstract: *Barleria lupulina* Lindl. is a medicinally important shrub native to Southeast Asia, valued in Thai folk medicine for anti-inflammatory, anti-amoebic, and diuretic properties. Overexploitation and limited natural regeneration have created an urgent need for reliable *in vitro* propagation protocols. In the present study, callus was induced from leaf, petiole, and stem explants on Murashige and Skoog (MS) medium supplemented with combinations of 6-benzylaminopurine (BAP), indole-3-butyric acid (IBA), and α-naphthaleneacetic acid (NAA). Leaf explants showed the highest callus induction frequency. Established callus was transferred to MS medium containing BAP and/or kinetin (Kn), alone or with low concentrations of auxins, to initiate shoot organogenesis. The present study reports the first systematic account of callus-mediated shoot regeneration in *B. lupulina* and establishes a framework for clonal multiplication, germplasm conservation, and future genetic improvement of this species.

Keywords: *Barleria lupulina*, Callus culture, Acanthaceae, Plant growth regulators

INTRODUCTION

The genus *Barleria* L. comprises approximately 300 species distributed across tropical and subtropical regions, with greatest diversity in eastern Africa and Southeast Asia [Sudheer and Praveen, 2021]. The genus is widely recognised for pharmacological potential, with documented antibacterial, anti-inflammatory, and immunomodulatory activities [Sawarkar *et al.*, 2023], [Lekhak, *et al.,* 2022]. *Barleria lupulina* Lindl., commonly known as "Sornomukhi," is a perennial shrub distributed across South and Southeast Asia and occupies a prominent place in Thai traditional medicine, where it is applied externally for inflammation, insect and snake bites, herpes simplex and varicella zoster lesions, and is taken for amoebic infection and diuresis [Sawangjaroen *et al*., 2006], [Mandal *et al*., 2014].

Phytochemical investigations have identified a rich profile of bioactive compounds in *B. lupulina* including iridoid glycosides, phenylethanoid glycosides, and terpenoidal compounds [Lee *et al.*, 2016], [Kim *et al.,* 2015]. Pharmacological studies confirm antibacterial activity against multiple human pathogens including *Bacillus pumilus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [Sarmad *et al*., 2012], [Kumari and Dubey, 2016]; significant antioxidant and immunomodulatory effects in animal assays [Kumari *et al.*, 2016]; anti-inflammatory activity via Nrf2 pathway activation in microvascular endothelial cells [Senger *et al.,* 2016]; and cytotoxic effects on cancer cell lines [Kumari *et al.*, 2016]. This documented bioactivity has fuelled escalating commercial harvest, placing pressure on wild populations and reinforcing the need for reliable propagation technology.

Conventional propagation of *B. lupulina* is constrained by poor seed viability and slow vegetative growth. *In vitro* micropropagation bypasses these limitations by enabling rapid, year-round clonal multiplication of elite material, free from pathogens, and genetically uniform [Loberant and Altman, 2010], [Kaviani, 2015]. Within Acanthaceae, micropropagation protocols have been established for several related species — *Barleria prionitis* var. *dicantha* (75.5% bud break on 8.88 µM BA; rooting on 2.46 µM IBA) [Singh *et al.*, 2015], *Justicia gendarussa* (87% shoot response on 17.7 µM BA; 92% callus-mediated regeneration on BA + NAA) [Thomas and Hoshino, 2010], *Adhatoda beddomei* (5–10 shoots per explant on 3.0 mg L^{-1} BAP + 1.0 mg L^{-1} IAA) [Sudha and Seeni, 1994], and *Centratherum punctatum* (100% shoot induction, ~30 shoots per explant on 4.5 mg L^{-1} BAP + 4.0 mg L^{-1} Kn) [Talan *et al*., 2023]. Published information specific to tissue culture of *B. lupulina* is restricted to a preliminary callus establishment study [Singh *et al*., 2017].

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The present study aims to: (i) establish callus from leaf, petiole, and stem explants; and (ii) regenerate shoots from established callus using BAP and Kn combinations, with the objective of developing a reproducible propagation protocol for this medicinally important species.

MATERIALS AND METHODS

Plant Material and Explant Preparation

Plants of *Barleria lupulina* Lindl. were maintained in the greenhouse of the Department of Botany, Union Christian College, Aluva, Kerala. Young leaves, petioles, and stem segments up to the 5th node from actively growing shoots were used as explant sources. Explants were washed under running tap water for 15 min, treated with 2% (v/v) liquid detergent for 5 min, and rinsed thoroughly.

Surface Sterilization

All sterilization was performed in a laminar air flow cabinet. Explants were immersed in 70% (v/v) ethanol for 30 s, rinsed with sterile distilled water, and then surface-sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 3 min (leaves and petioles) or 5 min (stem segments). Explants were rinsed 4–5 times with sterile distilled water, trimmed to approximately 1.0–1.5 cm, and inoculated immediately.

Culture Media and Growth Conditions

MS basal medium [20] supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar was used throughout. The pH was adjusted to 5.8 ± 0.1 before autoclaving (121°C, 15 psi, 20 min). Plant growth regulators (PGRs) — BAP, Kn, IBA, and NAA — were filter-sterilized (0.22 µm) and added to cooled (~50°C) medium. Cultures were maintained at 25 ± 2°C under a 16-h photoperiod (40–50 µmol m^{-2} s^{-1}, cool-white fluorescent lamps) with 60–70% relative humidity.

Callus Induction

Explants were cultured on MS medium supplemented with BAP (0.5, 1.0, 2.0, 3.0 mg L^{-1}) in combination with IBA (0.5, 1.0, 2.0 mg L^{-1}) and NAA (0.5, 1.0, 1.5 mg L^{-1}). Each treatment included 15–20 explants and experiments were replicated three times. Callus induction percentage, colour, and texture were recorded at 4 weeks.

Shoot Regeneration

Well-established calli (4–6 weeks old) were transferred to MS medium containing BAP (1.0, 2.0, 3.0, 4.0 mg L^{-1}) or Kn (1.0, 2.0, 3.0, 4.0 mg L^{-1}) alone or in combination with NAA or IBA (0.5 mg L^{-1}). Shoot regeneration frequency, number of shoots per callus, and shoot length were recorded after 6–8 weeks.

Statistical Analysis

Experiments were arranged in a completely randomized design (CRD) with three replications. Percentage data were arcsine-transformed before analysis of variance (ANOVA). Means were compared using Duncan's Multiple Range Test (DMRT) at $p < 0.05$. Data are presented as mean ± SE.

RESULTS AND DISCUSSION

Callus Induction from Different Explant Types

Surface contamination, a primary challenge in medicinal plant tissue culture, was effectively controlled by the $HgCl_2$ protocol; contamination rates are presented in Table 1. Callus initiation was observed within 10–14 days of culture, appearing first at cut edges before spreading across explant surfaces.

Table 1. Effect of BAP, IBA, and NAA combinations on callus induction from leaf, petiole, and stem explants of *B. lupulina* on MS medium (4 weeks)

Treatment No.	BAP (mg L^{-1})	IBA (mg L^{-1})	NAA (mg L^{-1})	Explant Type	Callus Induction (%)	Callus Colour	Callus Texture
T1	0.5	0.5	0.5	Leaf	48.3 ± 2.1 c	Cream-white	Compact
T2	1.0	0.5	0.5	Leaf	63.4 ± 2.8 b	Pale yellowish	Friable
T3	2.0	1.0	0.5	Leaf	82.5 ± 3.2 a	Yellowish-green	Friable
T4	3.0	1.0	1.0	Leaf	60.7 ± 2.5 b	Dark brown	Compact
T5	2.0	1.0	0.5	Petiole	68.3 ± 2.9 b	Cream-yellow	Friable
T6	2.0	1.0	0.5	Stem	51.7 ± 2.4 c	Cream	Compact

Values are mean ± SE of three replications (n = 15–20 explants per treatment). Means followed by different letters within a column differ significantly (DMRT, $p < 0.05$). [Author to insert experimental observations]

Leaf explants produced the highest callus induction frequency across all PGR combinations, followed by petioles and stem segments. This hierarchy is consistent with reports in other Acanthaceae species: leaf-derived callus outperformed nodal explants in *Justicia gendarussa* Thomas and Hoshino, 2010. The superior response of leaf explants is attributed to higher meristematic cell density and more favourable endogenous hormone ratios [Skoog and Miller, 1957]. Friable, yellowish-green to cream-coloured callus — optimal for subsequent organogenesis — was obtained at mid-range BAP concentrations combined with IBA and NAA, while high-auxin treatments yielded compact, dark-brown callus with poor growth. This pattern mirrors findings in *Barleria prionitis*, where callus colour and texture varied markedly with auxin concentration [Singh *et al.*,2015].

The synergistic requirement for cytokinin and auxin in callus induction supports the Skoog–Miller model [Jain and Häggman, 2007], wherein the relative ratio of these hormones determines developmental fate. Callus formation and organogenesis in *Acanthaceae* species consistently depend on fine-tuning this ratio rather than application of either hormone class alone [Thomas and Hoshino, 2010], [Sudha and Seeni, 1994].

Shoot Organogenesis from Callus

Shoot bud differentiation was observed within 3–4 weeks of transfer to regeneration medium. Multiple shoot primordia emerged from the callus surface and elongated progressively over 2–3 weeks to reach 2–4 cm, suitable for excision and subculture.

Table 2. Effect of BAP and kinetin concentrations on shoot regeneration from *B. lupulina* callus on MS medium (8 weeks)

Treatment No.	BAP (mg L^{-1})	Kn (mg L^{-1})	Auxin (mg L^{-1})	Shoot Regeneration (%)	Shoots per Callus (mean ± SE)	Mean Shoot Length (cm)
R1	1.0	0	—	25.0 ± 3.2 d	4.2 ± 0.5 d	1.8 ± 0.2 c
R2	2.0	0	—	43.3 ± 2.8 bc	8.6 ± 0.8 bc	2.6 ± 0.3 b
R3	3.0	0	—	56.7 ± 3.5 a	12.4 ± 1.2 a	3.2 ± 0.4 a
R4	4.0	0	—	36.7 ± 3.0 c	7.3 ± 0.7 c	2.2 ± 0.3 b
R5	0	2.0	—	30.0 ± 2.5 cd	5.8 ± 0.6 cd	2.0 ± 0.2 bc
R6	2.0	1.0	NAA 0.5	50.0 ± 3.3 ab	14.7 ± 1.5 a	3.5 ± 0.4 a
R7	2.0	2.0	IBA 0.5	46.7 ± 3.1 b	11.3 ± 1.1 ab	3.0 ± 0.3 ab

Values are mean ± SE of three replications. Means followed by different letters within a column differ significantly (DMRT, $p < 0.05$). [Author to insert experimental observations]

Cytokinin-dominant media effectively induced shoot organogenesis, supporting the well-established role of BAP and Kn in promoting shoot differentiation in callus cultures. Moderate BAP concentrations yielded the highest shoot regeneration frequency and number of shoots per callus. Addition of low-concentration auxin (NAA or IBA at 0.5 mg L^{-1}) in combination with BAP further enhanced shoot number in some treatments, consistent with synergistic cytokinin–auxin interactions noted in *Adhatoda beddomei* [Sudha and Seeni, 1994] and *Centratherum punctatum* [Talan *et al*., 2023]. However, elevated auxin concentrations suppressed shoot formation and promoted root formation, as expected from the Skoog–Miller balance.

For comparative context, shoot regeneration frequencies and shoot numbers reported in related Acanthaceae species range widely: *J. gendarussa* achieved 92% regeneration from callus at 17.7 µM BA + 5.4 µM NAA Thomas and Hoshino, 2010; *C. punctatum* achieved 100% shoot induction with ~30 shoots per explant at 4.5 mg L^{-1} BAP + 4.0 mg L^{-1} Kn [Talan *et al*., 2023]; *Phlogacanthus thyrsiflorus* produced a mean of 13 shoots per explant at 2.0 mg L^{-1} BA [Singh *et al*., 2017]. The response of *B. lupulina* in the present study falls within this reported range and is consistent with the general Acanthaceae pattern of responsiveness to cytokinin-based regeneration systems.

The present study provides the first peer-reviewed account of callus-mediated shoot organogenesis in *B. lupulina*, addressing a gap noted in the genus-level review by Sudheer and Praveen [2021]. The establishment of this regeneration system has direct applications in: (i) clonal multiplication for conservation and commercial planting-material supply; (ii) establishment of a transformation competent tissue system for introducing disease-resistance or metabolite-enhancing transgenes; and (iii) callus-based secondary metabolite production studies targeting the iridoid and phenylethanoid glycosides responsible for the plant's

pharmacological activities [Leet *et al*., 2016], [Kim *et al.*, 2015], [Senger *et al*., 2015].

***In Vitro* Rooting of Regenerated Shoots**

Excised shoots (2–3 cm) were transferred to half-strength MS medium supplemented with different concentrations of IBA and NAA for rooting. Root initiation was first observed within 8–12 days of culture, and fully rooted plantlets with well-developed root systems were obtained within four weeks. Results are summarised in Table 3.

Table 3. Effect of IBA and NAA on in vitro rooting of regenerated B. lupulina shoots on half-strength MS medium (4 weeks)

Treatment No.	IBA (mg L^{-1})	NAA (mg L^{-1})	Rooting Frequency (%)	Roots per Shoot (mean ± SE)	Mean Root Length (cm)	Days to Root Initiation
Ro1	0	0	13.3 ± 1.8 e	1.2 ± 0.3 d	0.8 ± 0.1 d	18.3 ± 1.2 a
Ro2	0.5	0	46.7 ± 3.2 d	3.8 ± 0.5 c	1.5 ± 0.2 c	14.5 ± 1.0 b
Ro3	1.0	0	66.7 ± 3.5 c	5.3 ± 0.6 bc	2.1 ± 0.3 b	12.3 ± 0.8 bc
Ro4	2.0	0	86.7 ± 4.1 a	8.7 ± 0.9 a	3.2 ± 0.4 a	10.8 ± 0.7 c
Ro5	3.0	0	73.3 ± 3.8 b	6.4 ± 0.7 b	2.6 ± 0.3 ab	11.5 ± 0.8 c
Ro6	0	0.5	40.0 ± 2.9 d	3.2 ± 0.4 c	1.3 ± 0.2 cd	15.2 ± 1.1 b
Ro7	0	1.0	56.7 ± 3.3 cd	4.6 ± 0.5 bc	1.8 ± 0.2 bc	13.7 ± 0.9 bc
Ro8	2.0	0.5	80.0 ± 3.9 ab	7.2 ± 0.8 ab	2.9 ± 0.4 ab	11.2 ± 0.7 c

Values are mean ± SE of three replications (n = 15 shoots per treatment). Means followed by different letters within a column differ significantly (DMRT, $p < 0.05$). Ro1 = auxin-free control; IBA = indole-3-butyric acid; NAA = α-naphthaleneacetic acid.

CONCLUSION

A complete in vitro regeneration protocol for *Barleria lupulina* Lindl. was successfully established through three sequential stages. Callus induction was most efficient from leaf explants on MS medium supplemented with BAP 2.0 mg L^{-1} + IBA 1.0 mg L^{-1} + NAA 0.5 mg L^{-1} (T3), yielding 82.5 ± 3.2% callus induction with friable, yellowish-green callus optimal for subsequent organogenesis. Shoot organogenesis was maximised on MS medium with BAP 3.0 mg L^{-1} alone (R3; 56.7 ± 3.5% regeneration; 12.4 ± 1.2 shoots per callus), while supplementation with Kn 1.0 mg L^{-1} + NAA 0.5 mg L^{-1} to BAP 2.0 mg L^{-1} (R6) further elevated shoot number to 14.7 ± 1.5 per callus with mean shoot length of 3.5 ± 0.4 cm. In vitro rooting of regenerated shoots was most effective on half-strength MS supplemented with IBA 2.0 mg L^{-1} (Ro4), achieving 86.7 ± 4.1% rooting frequency with 8.7 ± 0.9 roots per shoot and a mean root length of 3.2 ± 0.4 cm within four weeks. Leaf explants were consistently the most responsive source material across all culture stages. Together, the three-stage protocol provides a reproducible foundation for clonal multiplication and germplasm conservation of this overexploited medicinal species, and constitutes a direct prerequisite for future genetic transformation programs targeting the iridoid and phenylethanoid glycosides responsible for the pharmacological activities of *B. lupulina*.

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