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Explant-dependent high-frequency micropropagation and comparative plumbagin profiling of *in vitro*-rooted, field-grown tissue-culture-derived, and natural-source-derived field-grown *Plumbago indica*

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ABSTRACT

Plumbago indica L. is an important medicinal plant valued for plumbagin, but conventional propagation is limited by poor seed viability and slow multiplication. This study developed an explant-dependent micropropagation protocol and evaluated supporting phytochemical profiles among representative *in vitro*-rooted plantlets, field-grown tissue-culture-derived plants, and natural-source-derived field-grown plants. Direct regeneration was achieved from shoot-tip and nodal explants; nodal explants produced the highest response (36.70 ± 0.93 shoots/explant) on Murashige and Skoog medium supplemented with 1.5 mg l^{-1} 6-benzylaminopurine, 1.5 mg l^{-1} kinetin, and 0.5 mg l^{-1} α-naphthalene acetic acid, while shoot tips produced 24.70 ± 0.65 shoots/explant. Leaf explants produced maximum callus biomass (4.26 ± 0.14 g) and 9.0 ± 0.51 shoots/explant through indirect organogenesis. Regenerated plantlets rooted efficiently, and – 95%–100% survival was observed across nursery-scale acclimatization batches, although exact batch-wise survivor counts were not retained. In representative pooled ethanolic root extracts, the field-grown tissue-culture-derived sample showed the highest total phenolic content (80.50 ± 0.50 mg gallic acid equivalent (GAE)/g dry extract) and total flavonoid content (24.89 ± 0.27 mg QE/g dry extract), followed by the natural-source-derived field-grown and *in vitro*-rooted samples. High-performance liquid chromatography -based comparative peak profiling indicated a plumbagin-corresponding peak in all samples, with the field-grown tissue-culture-derived sample showing the highest relative peak response. The protocol supports rapid propagation, conservation, and production of quality planting material of *P. indica.*

1. INTRODUCTION

The genus *Plumbago indica* L. belongs to the family Plumbaginaceae and comprises — 10–20 species distributed across temperate and tropical regions worldwide. These species, including herbs and shrubs ranging from 0.5 to 2 m in height, are characterized by spirally arranged simple leaves and tubular flowers with five petal-like lobes in shades of white, blue, purple, red, or pink. Among these, *P. indica* L., originally native to Sikkim, India, has been widely cultivated across South Asia, the Philippines, and tropical Africa due to its significant medicinal value and pharmacological potential. *Plumbago indica* is recognized in traditional medicine systems for treating a wide range of ailments and has gained substantial attention for its rich phytochemical profile, particularly the presence of plumbagin, a naphthoquinone compound with demonstrated antimicrobial, anticancer, anti-inflammatory, and antioxidant properties [1,2]. Beyond plumbagin, *P. indica* roots and other plant parts are known to harbor alkaloids, flavonoids, glycosides, phenolics, tannins, and terpenoids, contributing to its therapeutic significance [1,2].

Despite its pharmacological importance, *P. indica* faces propagation challenges due to poor seed viability, low germination rates, and slow vegetative propagation, limiting its availability for medicinal, pharmaceutical, and conservation purposes. Consequently, there is a pressing need to develop efficient *in vitro* propagation strategies that ensure the rapid multiplication of disease-free and genetically uniform plantlets. Tissue culture approaches can overcome such constraints and have been successfully employed for several medicinal plants to support conservation, clonal multiplication, and sustainable utilization [3]. In medicinal plants such as *Plumbago*, micropropagation also has biotechnological importance because it enables the production of uniform planting material from selected donor plants and

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provides a controlled system for studying regeneration response and phytochemical variation.

Micropropagation through direct organogenesis from shoot tip and nodal explants has been extensively studied in *Plumbago* species, with reports demonstrating its effectiveness in producing multiple shoots [4,5]. In the present study, shoot tip and nodal explants were selected because they contain pre-existing meristematic tissues and are generally suitable for rapid direct shoot multiplication. In contrast, indirect regeneration via callus induction from leaf explants offers an alternative route, particularly valuable for generating morphogenic callus, studying regeneration potential, and supporting secondary metabolite-related tissue culture approaches [4,5]. Therefore, the use of shoot tip, nodal, and leaf explants allowed comparison of both direct and indirect regeneration pathways in *P. indica*.

Alongside propagation efforts, phytochemical profiling under different growth conditions is an important supporting consideration for medicinal plants such as *P. indica*. The biosynthesis and accumulation of secondary metabolites, including plumbagin, may vary between *in vitro*, field-grown, and natural-source-derived field-grown plants due to differences in physiological maturity, environmental exposure, and growth conditions [6,7]. *In vitro* propagated plantlets are maintained under controlled aseptic conditions, whereas field-established tissue-culture-derived plants and natural-source-derived field-grown plants are exposed to natural environmental factors that may influence phytochemical expression. However, most earlier reports on *Plumbago* have focused either on regeneration or phytochemical analysis separately, and limited information is available on the combined evaluation of explant-dependent regeneration with supporting comparative phytochemical and high-performance liquid chromatography (HPLC)-based plumbagin peak profiling across different plant sources.

Given this background, the present study was designed to develop and optimize a reproducible high-frequency micropropagation protocol for *P. indica* through both direct and indirect regeneration approaches. Additionally, preliminary phytochemical screening, estimation of total phenolic and flavonoid contents, and HPLC-based comparative plumbagin peak profiling were performed using representative root extracts from *in vitro* propagated plantlets, field-grown tissue-culture-derived plants, and natural-source-derived field-grown plants. The novelty of the study lies primarily in integrating explant-dependent regeneration, rooting, acclimatization, and supporting biochemical profiling, thereby contributing to rapid propagation, conservation, and quality planting material production of *P. indica*.

2. MATERIALS AND METHODS

2.1. Plant material, authentication, and surface sterilization

Healthy shoot cuttings of *P. indica L*. were collected from six-month-old mother plants maintained at Bhandimane Life Science Research Foundation (BLRF), Sirsi, Karnataka, India (GPS: JR4W+P56). Dr. Keshava H. Korse authenticated the species by morphological examination using Flora of the Presidency of Madras, Flora of Karnataka, and relevant taxonomic keys; nomenclature was cross-checked with current taxonomic databases. A voucher specimen was deposited in the BLRF Herbarium (BLRF-PI-2020-01).

Shoot tips, nodal segments, and young leaves were washed for 5–10 minutes and treated with 2% Tween-80 for 5 minutes. Shoot tips received 2% carbendazim (100 minutes), 1.5% streptomycin–tetracycline (60 minutes), 70% ethanol (30 seconds), 2% sodium hypochlorite (10 minutes), and 0.1% $HgCl_2$ (8 minutes). Nodal explants received the same treatments for 150 minutes, 90 minutes, 45 seconds, 15 minutes, and 10 minutes, respectively. Leaves were treated with 2% carbendazim (100 minutes), 1% streptomycin–tetracycline (50 minutes), 1% sodium hypochlorite (10 minutes), and 0.08% $HgCl_2$ (5 minutes), without ethanol. Explants were rinsed four to five times with sterile water, trimmed to 2–3 cm, and inoculated aseptically.

2.2. Culture medium and conditions

Murashige and Skoog (MS) medium [8] containing 3% sucrose and 0.8% agar was supplemented with 6-benzylaminopurine (BAP), kinetin (Kn), α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), or indole-3-butyric acid (IBA) as required. The pH was adjusted to 5.5 before autoclaving at 121°C and 15 psi for 15 minutes. Cultures were maintained at 25°C ± 2°C under a 14-hour photoperiod (–2,000 lux).

2.3. Direct and indirect regeneration

Shoot-tip and nodal explants were cultured on MS medium containing BAP (0–3 mg l^{-1}), Kn (0–2 mg l^{-1}), and 0.5 mg l^{-1} NAA. Shoot initiation was observed within about 15 days, and cultures were transferred to fresh medium every 3–4 weeks for up to 7–8 cycles. Data in Table 1 were recorded 75 days after inoculation; Figure 1 shows representative stages at –15, 45, and 90 days. Shoots were elongated on MS medium with 1.0 mg l^{-1} BAP, 2.0 mg l^{-1} Kn, and 0.5 mg l^{-1} NAA.

For indirect regeneration, leaf explants were cultured on MS medium containing NAA (0–2 mg l^{-1}) and 2,4-D (0–1.0 mg l^{-1}) either in darkness for 30 days or in darkness for 15 days followed by a 14-hour photoperiod for 15 days. Callus was transferred to MS medium with 2.0 mg l^{-1} BAP, 1.5 mg l^{-1} Kn, and 0.5 mg l^{-1} NAA for shoot regeneration, followed by the elongation medium described above.

2.4. Rooting and acclimatization

Elongated shoots were rooted on half-strength MS medium containing 0.5 mg l^{-1} BAP, IAA (0–2.5 mg l^{-1}), IBA (0–2.5 mg l^{-1}), and 2 g l^{-1} activated charcoal. Final rooting observations were recorded 45 days after transfer. Rooted plantlets were washed, treated with 1% Bavistin for 10 minutes, and hardened first in sterile coco peat for 30–45 days and then in polybags containing coco peat, red soil, and vermiculite (1:1:1) for 45–60 days before field establishment at BLRF.

2.5. Phytochemical analysis

2.5.1. Samples and extraction

Three pooled root samples were examined. Sample A comprised –500 g fresh roots pooled from *in vitro*-rooted plantlets maintained in about 100 culture bottles. Sample B comprised –1 kg roots pooled from 12 to 15 tissue-culture-derived plants grown at BLRF for 18–20 months. Sample C comprised –1 kg roots pooled from 12 to 15 field-grown plants established from root cuttings collected, with landholder permission, from naturally growing plants in a local Ayurvedic practitioner's field; the earlier provenance of the donor plants was not documented. Samples B and C were grown under comparable conditions without chemical fertilizers and were harvested after flowering in late January–early February 2025.

Each source represented one pooled composite biological sample. Roots were washed, shade-dried for 15–20 days, powdered, and stored dry. For phytochemical assays, 10 g powder was macerated with 100 ml absolute ethanol for 24 hours, filtered, dried at 40°C, and stored at 4°C. Qualitative screening for alkaloids, flavonoids, tannins, saponins, glycosides, phenolics, terpenoids, and quinones followed standard procedures [9,10].

2.5.2. Total phenolic and flavonoid contents

Dried extract was prepared as a 10 mg ml^{-1} ethanolic stock and diluted to 1 mg ml^{-1}. For total phenolic content (TPC), [11,12] 0.50 ml extract was mixed with 2.50 ml Folin–Ciocalteu reagent (1:10) and 2.00 ml 7.5% sodium carbonate, incubated for 30 minutes at 25°C in the dark, and read at 765 nm. Gallic acid standards (0–100 µg ml^{-1}) gave y = 0.010076x + 0.016857 (R^2 = 0.9971); results were expressed as mg gallic acid equivalent (GAE) g^{-1} dry extract.

For TFC, [12] 0.50 ml extract was mixed with 1.50 ml ethanol, 0.10 ml 10% aluminum chloride, 0.10 ml 1 M potassium acetate, and 2.80 ml water. After 30 minutes at 25°C, absorbance was read at 415 nm. Quercetin standards (0–50 µg ml^{-1}) gave y = 0.021820x–0.008111 (R^2 = 0.9933); results were expressed as mg QE g^{-1} dry extract. Three technical readings were obtained from one extract per pooled source; no inferential comparison among sources was made.

2.5.3. HPLC comparative peak profiling

For each source, 1.0 g powdered root was extracted overnight with 10 ml ethanol, filtered, evaporated under nitrogen, reconstituted at 1 mg/ml, and passed through a 0.22-µm filter. Analysis was performed using a Waters Alliance 2695 HPLC system with a Waters 2998 PDA detector and a HYDROSPHERE C18 column (250 x 4.6 mm, 5 µm) maintained at 35 °C. The flow rate was 1.0 ml/minute and the injection volume was 10 µl. The analytical gradient used acetonitrile (B) and 0.1% formic acid containing 5% acetonitrile in water (D), with A and C maintained at 0% throughout the run. The B/D program was 5/95 at 0–0.1 min, 30/70 at 10 minute, 60/40 at 14–16 minute, 80/20 at 24–32 minute, and 5/95 at 35–40 minute. PDA data were acquired over 190-800 nm, with absorbance channels at 220, 240, 280, 350, and 410 nm. A 1 mM plumbagin standard was used for retention-time comparison. Because calibration and replicate runs were unavailable, results were treated only as comparative peak profiles.

2.6. Experimental design and statistical analysis

Tissue-culture treatments included 10 independently maintained explants per run and were repeated in three independent runs. Each explant was an experimental unit; subcultures from the same explant were not counted as new replicates. Pooled data are presented as mean ± SE and were analyzed by one-way ANOVA followed by Tukey's HSD test at $p < 0.05$. TPC/TFC values are mean ± SD of three technical readings from one pooled extract per source. HPLC results represent one analytical run per pooled source and were not subjected to inferential analysis.

3. RESULTS

3.1. Direct shoot regeneration from shoot tip and nodal explants

Direct shoot regeneration was successfully achieved from both shoot tip and nodal explants of *P. indica*. However, the regeneration response varied significantly depending on explant type and plant growth regulator combination. Nodal explants showed a higher shoot multiplication response than shoot tip explants. The highest number of shoots from nodal explants was 36.70 ± 0.93 shoots/explant on MS medium supplemented with 1.5 mg l^{-1} BAP, 1.5 mg l^{-1} Kn, and 0.5 mg l^{-1} NAA. In shoot tip explants, the maximum shoot multiplication was 24.70 ± 0.65 shoots/explant on MS medium containing 2.0 mg l^{-1} BAP, 1.0 mg l^{-1} Kn, and 0.5 mg l^{-1} NAA.

The maximum shoot length from shoot tip explants was 3.02 ± 0.06 cm, recorded on MS medium supplemented with 1.5 mg l^{-1} BAP, 2.0 mg l^{-1} Kn, and 0.5 mg l^{-1} NAA. In nodal explants, the highest shoot length was 3.71 ± 0.10 cm on the same medium combination. The superior response of nodal explants indicates that the presence of axillary meristems may have enhanced shoot initiation and multiplication under cytokinin-rich conditions. The treatment means differed significantly at $p < 0.05$, as indicated by superscript letters (Table 1 and Fig. 1).

3.2. Indirect regeneration through callus induction from leaf explants

Leaf explants responded effectively to callus induction on MS medium supplemented with NAA and 2,4-D. Callus biomass varied significantly with auxin concentration and incubation conditions. The highest callus biomass of 4.26 ± 0.14 g was recorded on MS medium supplemented

Table 1. Effect of plant growth regulators on direct shoot regeneration from shoot tip and nodal explants of *P. indica*.

MS + growth regulators (mg l^{-1})			Shoot tip explant		Nodal explant	
BAP	Kn	NAA	Shoots/explant	Shoot length (cm)	Shoots/explant	Shoot length (cm)
1.0	0.0	0.5	7.60 ± 0.31 ^{i}	1.94 ± 0.05 ^{f}	14.20 ± 0.61 ^{j}	3.19 ± 0.08 ^{bcde}
1.5	0.0	0.5	9.90 ± 0.46 ^{hi}	1.83 ± 0.05 ^{fg}	18.60 ± 0.64 ^{i}	3.14 ± 0.04 ^{cde}
2.0	0.0	0.5	10.90 ± 0.43 ^{gh}	1.82 ± 0.05 ^{fg}	21.70 ± 0.68 ^{i}	2.90 ± 0.07 ^{efg}
2.5	0.0	0.5	13.90 ± 0.46 ^{ef}	1.64 ± 0.06 ^{gh}	26.00 ± 0.89 ^{efg}	2.67 ± 0.09 ^{fg}
3.0	0.0	0.5	11.60 ± 0.52 ^{fgh}	1.38 ± 0.06 ^{h}	23.60 ± 0.85 ^{gh}	2.50 ± 0.10 ^{g}
1.5	0.5	0.5	12.70 ± 0.65 ^{efg}	1.96 ± 0.07 ^{f}	28.60 ± 1.03 ^{def}	2.96 ± 0.10 ^{def}
1.5	1.0	0.5	14.30 ± 0.63 ^{e}	2.33 ± 0.07 ^{de}	32.00 ± 0.84 ^{bcd}	3.08 ± 0.08 ^{cdef}
1.5	1.5	0.5	17.00 ± 0.63 ^{d}	2.64 ± 0.07 ^{bc}	36.70 ± 0.93 ^{a}	3.35 ± 0.08 ^{abcd}
1.5	2.0	0.5	19.30 ± 0.47 ^{cd}	3.02 ± 0.06 ^{a}	34.90 ± 0.60 ^{ab}	3.71 ± 0.10 ^{a}
2.0	0.5	0.5	21.70 ± 0.62 ^{bc}	2.93 ± 0.04 ^{a}	32.70 ± 0.87 ^{bc}	3.59 ± 0.09 ^{ab}
2.0	1.0	0.5	24.70 ± 0.65 ^{a}	2.89 ± 0.05 ^{ab}	29.60 ± 0.75 ^{cd}e	3.47 ± 0.08 ^{abc}
2.0	1.5	0.5	22.10 ± 0.67 ^{ab}	2.56 ± 0.06 ^{cd}	28.00 ± 0.79 ^{ef}	3.36 ± 0.10 ^{abcd}
2.0	2.0	0.5	20.70 ± 0.47 ^{bc}	2.26 ± 0.06 ^{e}	25.20 ± 0.74 ^{fgh}	3.34 ± 0.10 ^{abcd}

Data were recorded 75 days after initial inoculation, following the initial establishment period and subsequent multiplication intervals. Values are pooled mean ± SE from three independent experimental runs, with 10 independently maintained explants per treatment in each run (n = 10 per run). Different letters indicate significant differences at $p < 0.05$ (Tukey's HSD test). BAP, 6-benzylaminopurine; Kn, kinetin; NAA, α-naphthalene acetic acid.

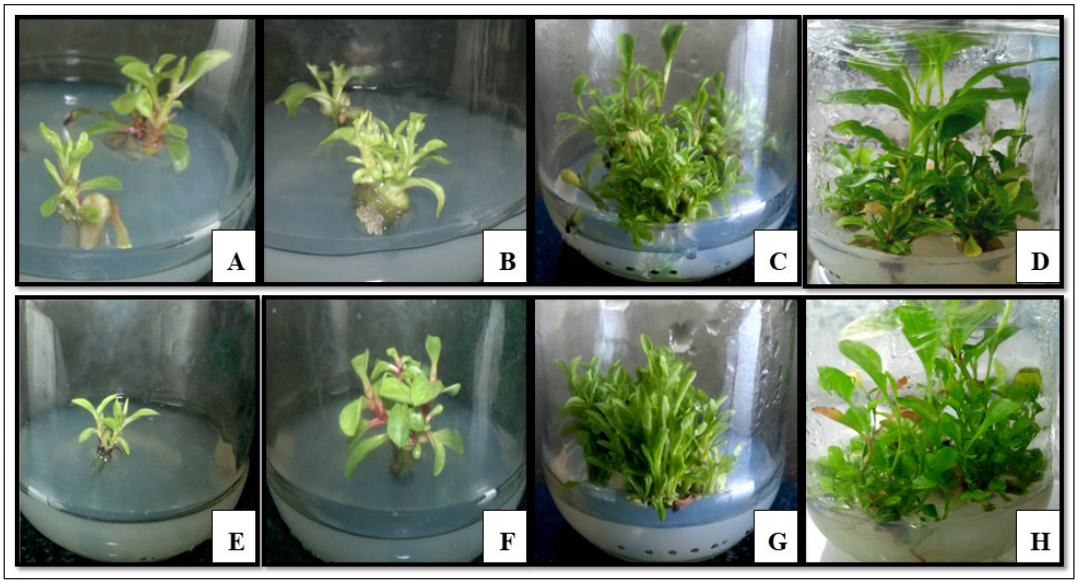

Figure 1. Stages of direct regeneration from shoot tip and nodal explants of *P. indica*. (A) Shoot-tip explant –15 days after inoculation. (B) Multiple-shoot development –45 days after initial inoculation from shoot-tip explants. (C) High-frequency shoot multiplication –90 days after initial inoculation from shoot-tip explants. (D) Elongation of shoot-tip-derived shoots after –30 days on elongation medium. (E) Nodal explant –15 days after inoculation. (F) Multiple-shoot development –45 days after initial inoculation from nodal explants. (G) High-frequency shoot multiplication –90 days after initial inoculation from nodal explants. (H) Elongation of nodal-explant-derived shoots after –30 days on elongation medium.

Table 2. Induction of callus from leaf explants on different nutrient media and under different culture conditions.

MS + growth regulators (mg l^{-1})		Callus biomass (g)	
NAA	2,4-D	30 days dark period	15 days dark + 15 days under a 14-hour photoperiod
0.0	1.0	0.14 ± 0.03 ^{e}	0.33 ± 0.08 ^{f}
0.2	0.9	0.23 ± 0.03 ^{e}	0.39 ± 0.09 ^{ef}
0.4	0.8	1.11 ± 0.32 ^{d}	0.82 ± 0.10 ^{de}
0.6	0.7	1.24 ± 0.10 ^{d}	1.06 ± 0.09 ^{d}
0.8	0.6	2.02 ± 0.14 ^{c}	1.65 ± 0.09 ^{c}
1.0	0.5	3.99 ± 0.08 ^{ab}	3.12 ± 0.10 ^{b}
1.2	0.4	4.26 ± 0.14 ^{a}	3.67 ± 0.10 ^{a}
1.4	0.3	3.92 ± 0.08 ^{ab}	3.20 ± 0.11 ^{ab}
1.6	0.2	3.66 ± 0.12 ^{ab}	3.17 ± 0.12 ^{b}
1.8	0.1	3.58 ± 0.11 ^{b}	2.96 ± 0.07 ^{b}
2.0	0.0	3.48 ± 0.13 ^{b}	2.99 ± 0.11 ^{b}

Data were recorded on the 30th day of inoculation/subculture. Values are pooled mean ± SE from three independent experimental runs, with 10 independently maintained explants per treatment in each run (n = 10 per run). Different letters indicate significant differences at $p < 0.05$ (Tukey's HSD test). NAA, α-naphthalene acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

with 1.2 mg l^{-1} NAA and 0.4 mg l^{-1} 2,4-D under complete darkness for 30 days. Under the alternate incubation condition of 15 days in darkness followed by 15 days under a 14-hour photoperiod, the same treatment produced 3.67 ± 0.10 g callus biomass.

The greater callus biomass under complete darkness indicates that dark incubation favored dedifferentiation and callus proliferation in leaf explants. Leaf-derived callus transferred to shoot regeneration medium showed organogenic response and produced a maximum of 9.0 ± 0.51 shoots/explant on MS medium supplemented with 2.0 mg l^{-1} BAP, 1.5 mg l^{-1} Kn, and 0.5 mg l^{-1} NAA. The highest shoot length from callus-derived shoots was 3.22 ± 0.13 cm on MS medium containing 1.5 mg l^{-1} BAP, 2.0 mg l^{-1} Kn, and 0.5 mg l^{-1} NAA. Treatment-wise differences were statistically significant at $p < 0.05$, as indicated in Tables 2 and 3 and Figure 2.

Table 3. Plant regeneration from callus derived from leaf explants.

MS + growth regulators (mg l^{-1})			No. of multiple shoots per explant	Shoot length (cm)
BAP	Kn	NAA		
1.00	2.50	0.5	5.0 ± 0.69 ^{c}	2.7 ± 0.11 ^{abc}
1.25	2.25	0.5	5.6 ± 0.63 ^{bc}	2.63 ± 0.09 ^{bc}
1.50	2.00	0.5	7.5 ± 0.45 ^{ab}	3.22 ± 0.13 ^{a}
1.75	1.75	0.5	7.9 ± 0.43 ^{ab}	2.81 ± 0.08 ^{ab}
2.00	1.50	0.5	9.0 ± 0.51 ^{a}	2.62 ± 0.09 ^{c}
2.25	1.25	0.5	7.8 ± 0.44 ^{ab}	2.24 ± 0.16 ^{c}
2.50	1.00	0.5	7.4 ± 0.49 ^{ab}	2.28 ± 0.12 ^{c}

*Data were recorded on the 30th day of inoculation/subculture. Values are pooled mean ± SE from three independent experimental runs, with 10 independently maintained explants per treatment in each run (n = 10 per run). Different letters indicate significant differences at $p < 0.05$ (Tukey's HSD test). BAP, 6-benzylaminopurine; Kn, kinetin; NAA, α-naphthalene acetic acid.

3.3. *In vitro* rooting and acclimatization

Regenerated shoots obtained from shoot tip, nodal, and leaf-derived callus explants rooted successfully on half-strength MS medium supplemented with IAA, IBA, BAP, and activated charcoal. Rooting response varied depending on the auxin combination and explant origin. Among shoot tip-derived shoots, the highest number of roots (12.1 ± 0.73 roots/shoot) and maximum root length (10.33 ± 0.12 cm) were recorded on half-strength MS medium supplemented with 1.5 mg l^{-1} IAA, 1.0 mg l^{-1} IBA, 0.5 mg l^{-1} BAP, and 2 g l^{-1} activated charcoal (Fig. 3; Tables 4A and B).

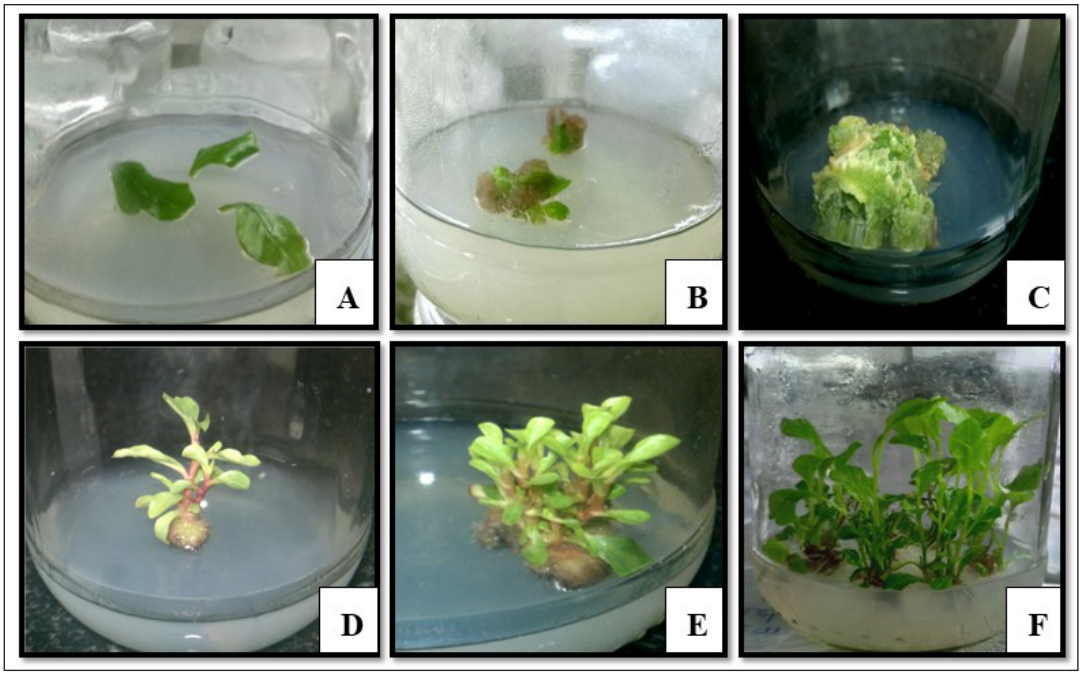

Figure 2. Different stages of indirect regeneration through callus induction in *P. indica*. (A) Aseptic inoculation of leaves (B) Induction of callus after 15 days in darkness followed by 15 days under a 14-hour photoperiod. (C) Induction of callus under dark treatment after 30 days. (D) Regeneration of shoots from callus after 30 days of subculture. (E) Multiple shoots after 45 days of subculture. (F) Shoot elongation after 30 days of subculture.

Table 4A. *In vitro* rooting from shoot tip and nodal explant-derived shoots of *P. indica*.

Half MS + growth regulators (mg l^{-1}) + activated charcoal (2 g l^{-1})			Shoot tip explants		Nodal explants	
IAA	IBA	BAP	No. of roots per shoot	Root length (cm)	No. of roots per shoot	Root length (cm)
0.00	2.50	0.5	6.7 ± 0.61 ^{c}	7.16 ± 0.09 ^{d}	6.2 ± 0.71 ^{c}	6.91 ± 0.14 ^{d}
0.50	2.00	0.5	8.0 ± 0.57 ^{bc}	8.35 ± 0.24 ^{c}	7.8 ± 0.89 ^{bc}	7.79 ± 0.10 ^{c}
1.00	1.50	0.5	10.7 ± 0.51 ^{a}	9.19 ± 0.16 ^{b}	11.4 ± 0.81 ^{ab}	8.07 ± 0.15 ^{bc}
1.50	1.00	0.5	12.1 ± 0.73 ^{a}	10.33 ± 0.12 ^{a}	14.3 ± 1.03 ^{a}	9.01 ± 0.12 ^{a}
2.00	0.50	0.5	10 ± 0.44 ^{ab}	9.69 ± 0.11 ^{b}	12.6 ± 0.87 ^{a}	8.53 ± 0.13 ^{ab}
2.50	0.00	0.5	9.8 ± 0.44 ^{ab}	9.42 ± 0.05 ^{b}	10.7 ± 0.88 ^{ab}	8.18 ± 0.10 ^{bc}

Table 4B. *In vitro* rooting from leaf-derived shoots of *P. indica.*

Half MS + growth regulators (mg l^{-1}) + Activated Charcoal (2 g l^{-1})				
IAA	IBA	BAP	No. of roots per shoot	Root length (cm)
0.00	2.50	0.5	8.30 ± 0.57^{c}	7.61 ± 0.10^{c}
0.50	2.00	0.5	10.1 ± 0.62^{bc}	8.01 ± 0.06^{c}
1.00	1.50	0.5	11.2 ± 0.61^{b}	9.39 ± 0.12^{b}
1.50	1.00	0.5	14 ± 0.93^{a}	9.95 ± 0.11^{a}
2.00	0.50	0.5	11.4 ± 0.61^{ab}	9.48 ± 0.11^{b}
2.50	0.00	0.5	9.7 ± 0.47^{bc}	9.24 ± 0.10^{b}

Half-strength MS medium was supplemented with 2 g l^{-1} activated charcoal in all treatments. Data were recorded 45 days after transfer to rooting medium. Values are pooled mean ± SE from three independent experimental runs, with 10 independently maintained explants per treatment in each run (n = 10 per run). Different superscript letters within the same column indicate significant differences at $p < 0.05$ according to Tukey's HSD test. BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid.

Rooted plantlets were transferred to trays containing coco peat for primary hardening –30–45 days and subsequently maintained in polybags for 45–60 days during secondary hardening. Approximately 500 plantlets from each of the three explant-derived groups—shoot-tip-derived, nodal-derived, and leaf-callus-derived—were subjected to acclimatization across nursery-scale batches. Approximately 95%–100% survival was observed across the primary and secondary hardening stages. Plantlets from all three regeneration pathways established successfully; however, the nodal-explant-derived protocol was subsequently selected for routine mass multiplication because of its superior shoot-production efficiency. Because exact batch-wise survivor counts were not retained, the survival range represents an approximate nursery-scale observation rather than a precisely enumerated experimental endpoint.

3.4. Preliminary phytochemical screening

The percentage recovery of the ethanolic extracts varied among the three pooled composite samples. The field-grown tissue-culture-

derived and natural-source-derived field-grown samples showed higher extractive yields (17.38%–17.78%) than the *in vitro*-rooted sample (6.34%). Thus, the two field-grown composite samples yielded approximately threefold greater extract recovery than the *in vitro*-rooted composite sample.

Preliminary phytochemical screening of ethanolic root extracts revealed variation in phytoconstituent profiles among the three plant sources. The tested phytochemical groups included alkaloids, flavonoids, tannins, saponins, glycosides, phenolics, terpenoids, and quinones. Field-grown tissue-culture-derived plants showed a comparatively stronger qualitative phytochemical profile than the *in vitro*-rooted plantlets and natural-source-derived field-grown plants.

Table 5. Qualitative phytochemical screening of *P. indica* from different sources.

Phytochemical group	*In vitro*-rooted plantlets	Field-grown tissue-culture-derived plants	Natural-source-derived field-grown plants
Alkaloids	+	++	++
Flavonoids	+	++	++
Phenols	+	++	++
Tannins	–	+	+
Saponins	+	++	+
Glycosides	–	+	+
Terpenoids	–	+	+
Quinones	+	++	++

–, indicates absence; +, indicates presence; and ++, indicates comparatively higher presence.

These qualitative results indicate differences among the representative pooled extracts but do not specifically identify plumbagin, because the general quinone test was not a plumbagin-specific assay (Table 5).

3.5. Quantitative estimation of total phenolic and flavonoid contents

Quantitative estimation of TPC and TFC showed descriptive variation among the pooled composite ethanolic root extracts prepared from the three plant sources. TPC ranged from 36.90 ± 0.55 to 80.50 ± 0.50 mg GAE g^{-1} dry extract. The field-grown tissue-culture-derived sample showed the highest TPC value (80.50 ± 0.50 mg GAE g^{-1} dry extract), followed by the natural-source-derived field-grown sample (72.76 ± 0.30 mg GAE g^{-1} dry extract), while the *in vitro*-rooted plantlet sample showed the lowest value (36.90 ± 0.55 mg GAE g^{-1} dry extract) (Table 6).

A similar descriptive trend was observed for TFC. The field-grown tissue-culture-derived sample showed the highest TFC value (24.89 ± 0.27 mg QE g^{-1} dry extract), followed by the natural-source-derived field-grown sample (20.37 ± 0.25 mg QE g^{-1} dry extract), whereas the *in vitro*-rooted plantlet sample showed the lowest value (8.33 ± 0.21 mg QE g^{-1} dry extract). The descriptive order for both assays was field-grown tissue-culture-derived plants > natural-source-derived field-grown plants >*in vitro*-rooted plantlets. Because the values were obtained from three technical readings of one extract prepared from one pooled composite biological sample per source, the results indicate analytical variation within the representative extracts and should not be interpreted as statistically generalizable differences among plant-source populations.

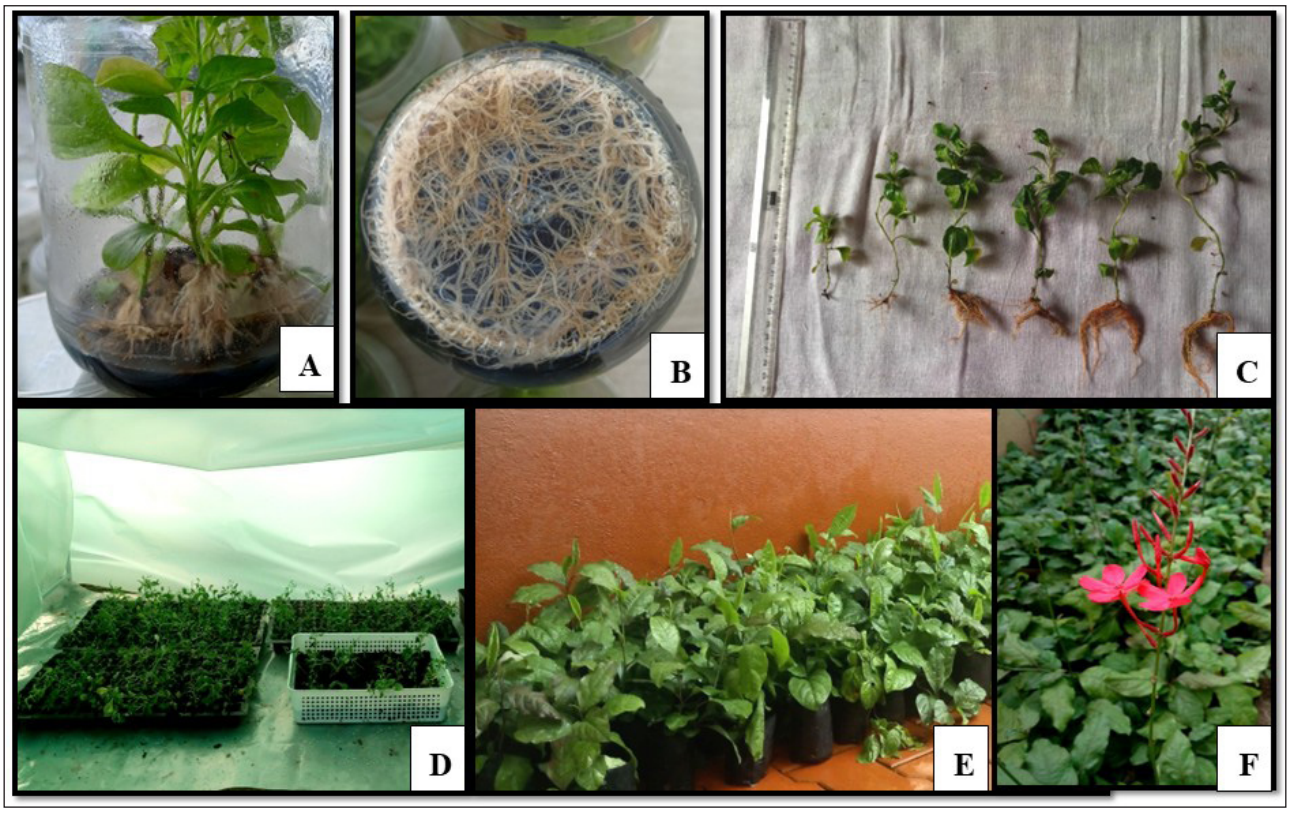

Fig ure 3. *In vitro* rooting and acclimatization of regenerated plants of *P. indica.* (A) *In vitro* rooting of nodal-explant-derived plantlets 45 days after transfer to rooting medium. (B) Profuse rooting. (C) Comparison of roots induced on different medium combinations shown in Tables 4A and 4B. (D) Primary-hardened plants in trays/cups containing coco peat after 30–45 days. (E) Secondary-hardened plants in polybags after 45–60 days. (F) Field-established *P. indica* showing characteristic red flowering.

Table 6. Total phenolic and flavonoid contents of ethanolic root extracts of *P. indica.*

Sample	Source of root extract	Total phenolic content (mg GAE/g dry extract)	Total flavonoid content (mg QE/g dry extract)
A	*In vitro*-rooted plantlets	36.90 ± 0.55	8.33 ± 0.21
B	Field-grown tissue-culture-derived plants	80.50 ± 0.50	24.89 ± 0.27
C	Natural-source-derived field-grown plants	72.76 ± 0.30	20.37 ± 0.25

Values are expressed as mean ± SD of three technical absorbance readings from one ethanolic extract prepared from one pooled composite biological sample per source. Samples A, B, and C were not independently biologically replicated. The data are therefore interpreted descriptively and not as inferential comparisons among plant-source populations. GAE, gallic acid equivalent; QE, quercetin equivalent.

Table 7. HPLC-based comparative plumbagin peak profile in ethanolic root extracts of *P. indica*.

Sample	Source of root extract	Retention time, Rt (min)	Peak area	Peak height	Area (%)	Interpretation
Standard	Plumbagin standard	18.52	4,488.11	23,588	50.88	Reference peak
A	In vitro-rooted plantlets	18.53	4,107.69	20,140	26.13	Plumbagin-corresponding peak detected
B	Field-grown tissue-culture-derived plants	18.53	8,254.27	43,870	41.64	Highest relative peak response
C	Natural-source-derived field-grown plants	18.53	5,869.61	30,843	39.23	Intermediate relative peak response

Rt, retention time. HPLC data represent single analytical runs used for comparative peak profiling. Area (%) indicates relative peak area within each chromatogram and should not be interpreted as absolute plumbagin concentration. Identification was based on retention-time agreement with the authentic plumbagin standard under the same chromatographic conditions. No calibration-curve-based absolute quantification, replicate analytical validation, PDA spectral overlay, or mass spectral confirmation was performed. Each source was represented by one pooled composite biological sample and one analytical run.

3.6. HPLC-based comparative plumbagin peak profiling

HPLC-based comparative peak profiling indicated the presence of a plumbagin-corresponding peak in ethanolic root extracts of all three representative *P. indica* sample sources. The authentic plumbagin standard showed a reference peak at Rt 18.52 minutes, while corresponding peaks in the sample extracts were observed at Rt 18.53 minutes under the same chromatographic conditions.

Among the three representative composite samples, the field-grown tissue-culture-derived sample showed the highest relative plumbagin-associated peak response, with a peak area of 8,254.27 and an area percentage of 41.64%. The natural-source-derived field-grown sample showed an intermediate relative peak response, with a peak area of 5,869.61 and an area percentage of 39.23%, whereas the *in vitro*-rooted sample showed the lowest relative response, with a peak area of 4,107.69 and an area percentage of 26.13%.

Since the HPLC data were obtained from single analytical runs and no calibration-curve-based quantification was performed, these results are interpreted only as comparative peak-profile data and not as absolute plumbagin concentration. The HPLC profiling is therefore used as supportive chromatographic evidence along with the micropropagation and phytochemical observations (Table 7 and Fig. 4). Because each source was represented by one pooled composite biological sample and one analytical run, differences in peak response should not be interpreted as evidence of source-wide or population-level differences.

4. DISCUSSION

The present study established an explant-dependent regeneration system for *P. indica* and descriptively evaluated phytochemical profiles among pooled composite samples prepared from *in vitro*-rooted plantlets, field-grown tissue-culture-derived plants, and natural-source-derived field-grown plants. Regeneration response differed among shoot-tip, nodal, and leaf explants, indicating that explant source and plant growth regulator balance strongly influenced morphogenic competence. Nodal explants produced the highest shoot multiplication response, followed by shoot tips and leaf-derived callus. This may be attributed to the presence of pre-existing axillary meristems in nodal explants, which are more readily activated under cytokinin-rich conditions than tissues requiring complete dedifferentiation and redifferentiation. Similar observations have been reported in earlier *Plumbago* micropropagation studies, where nodal explants were useful for rapid shoot multiplication due to their inherent meristematic activity and lower dependence on indirect organogenesis [4,5]. In a related species, *Plumbago auriculata*, Katoch *et al*. [13] also reported direct regeneration from nodal explants along with comparative plumbagin analysis, supporting the relevance of combining regeneration studies with marker-compound profiling in this genus.

The combined use of BAP and kinetin with low NAA was effective for shoot induction in the present study. BAP and kinetin are known to promote cell division, axillary bud break, and shoot proliferation, while low NAA supplementation helps maintain a favorable cytokinin–auxin balance for organogenesis. The higher response of nodal explants on MS medium containing 1.5 mg l^{-1} BAP, 1.5 mg l^{-1} Kn, and 0.5 mg l^{-1} NAA indicates that a balanced cytokinin combination was more effective for high-frequency regeneration than single cytokinin treatments. Shoot tip explants also responded well, but the comparatively lower shoot number may be due to apical dominance and fewer active meristematic sites than nodal segments. Since direct organogenesis reduces the risk of somaclonal variation, both shoot tip and nodal explants are useful for producing uniform planting material, with nodal explants being more suitable for large-scale multiplication.

Leaf explants showed successful callus induction and indirect shoot regeneration, demonstrating an alternative regeneration pathway in *P. indica*. The highest callus biomass under complete darkness on NAA- and 2,4-D-supplemented medium may be due to the favorable role of auxin-rich conditions in dedifferentiation and callus proliferation. Transfer of leaf-derived callus to cytokinin-rich medium promoted shoot regeneration, confirming the morphogenic potential of callus tissue. Although indirect regeneration produced fewer shoots than nodal

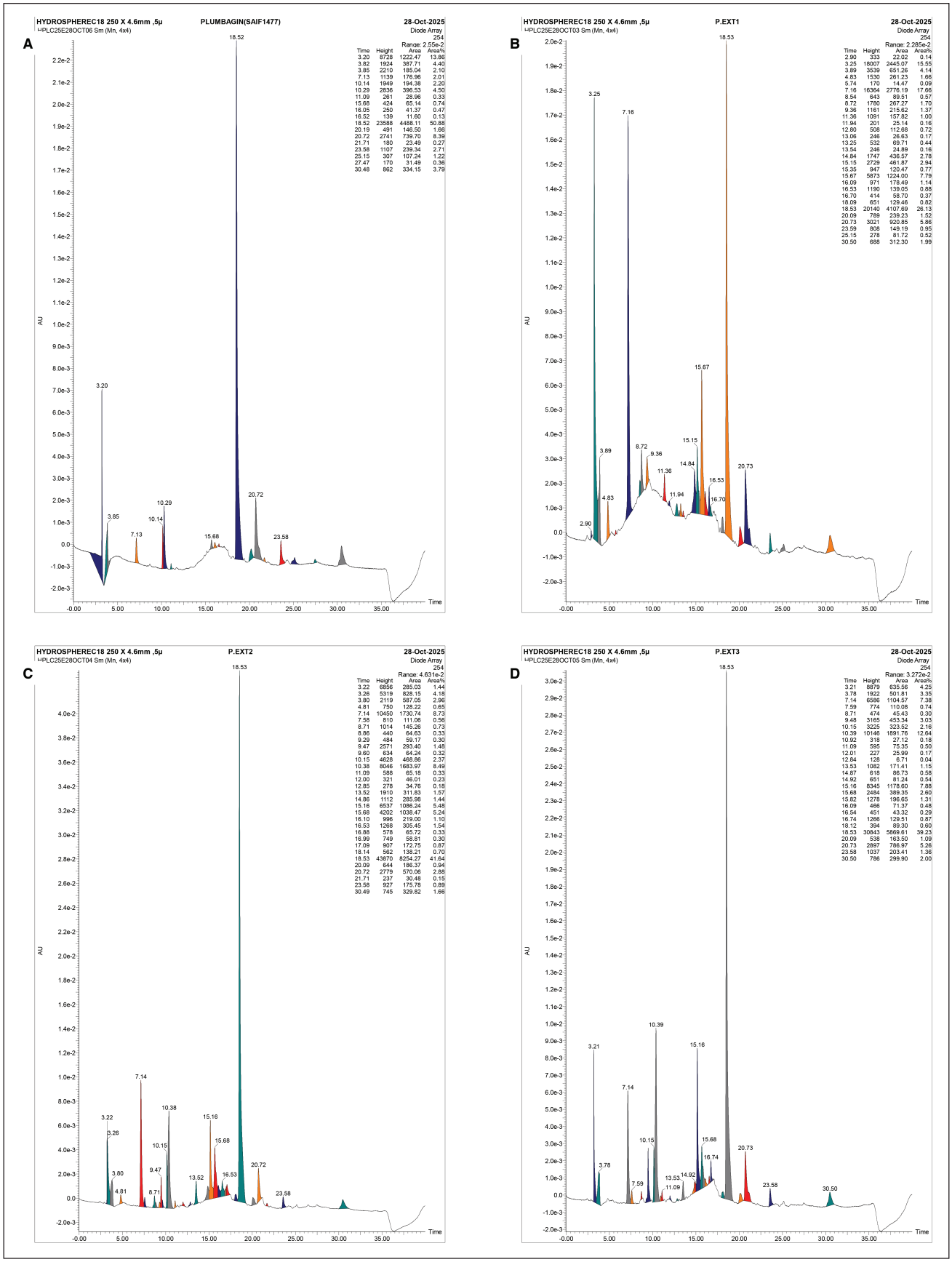

Figure 4. HPLC chromatograms of (A) plumbagin standard, (B) *in vitro* root extract (Sample A), (C) field-grown tissue-culture-derived root extract (Sample B), and (D) natural-source-derived field-grown root extract (Sample C) of *P. indica*, showing a plumbagin-corresponding peak at Rt ≈ 18.5 minutes under the same chromatographic conditions.

and shoot tip explants, it remains valuable for callus-based regeneration, secondary metabolite studies, and future elicitation or cell culture experiments. Previous phytochemical comparisons in *P. indica* have also included callus, tissue-cultured roots, conventionally propagated roots, and cell culture material, indicating the importance of evaluating different culture-derived sources for phytochemical potential [14].

Efficient rooting was obtained on half-strength MS medium supplemented with IAA, IBA, BAP, and activated charcoal. Half-strength MS medium may have reduced salt stress and favored root initiation, while IAA and IBA promoted adventitious root formation. Activated charcoal may have improved rooting by adsorbing phenolic compounds and residual plant growth regulators. Approximately 95%–100% survival was observed across nursery-scale acclimatization batches, indicating successful transition of regenerated plantlets from *in vitro* to ex vitro conditions. Nevertheless, this range should be interpreted as an approximate operational observation because exact batch-wise survivor counts were not retained.

Phytochemical screening showed qualitative variation among the representative pooled root extracts obtained from *in vitro*-rooted plantlets, field-grown tissue-culture-derived plants, and natural-source-derived field-grown plants. The *in vitro*-rooted composite sample showed a comparatively weaker qualitative response, whereas the two field-grown composite samples showed broader or stronger reactions for several phytochemical groups. These descriptive differences may be associated with physiological maturity and exposure to field conditions. Under controlled aseptic culture conditions, *in vitro* plantlets have limited exposure to natural light variation, soil-associated microorganisms, temperature fluctuation, and mild abiotic or biotic stress. In contrast, field-grown plants experience environmental cues after establishment that may influence pathways involved in the production of phenolics, flavonoids, quinones, and related compounds. Recent reviews on *P. indica* emphasize its pharmacological importance, conservation relevance, increasing demand for plumbagin, and the need for sustainable cultivation approaches [2]. Secondary-metabolite production in medicinal plants may also be influenced through engineered root cultures and elicitor-mediated approaches [6,7,15]. Because each source in the present study was represented by one pooled composite extract, these qualitative observations are descriptive only.

The reconducted TPC and TFC assays supported the qualitative phytochemical observations at the level of representative pooled-extract comparison. The field-grown tissue-culture-derived sample showed the highest TPC (80.50 ± 0.50 mg GAE g^{-1} dry extract) and TFC (24.89 ± 0.27 mg QE g^{-1} dry extract), followed by the natural-source-derived field-grown sample (72.76 ± 0.30 mg GAE g^{-1} and 20.37 ± 0.25 mg QE g^{-1} dry extract, respectively) and the *in vitro*-rooted sample (36.90 ± 0.55 mg GAE g^{-1} and 8.33 ± 0.21 mg QE g^{-1} dry extract, respectively). These descriptive differences may reflect physiological maturity and environmental exposure. However, because each source was represented by one pooled composite extract and replicate readings were technical rather than independent biological replicates, the observed pattern should not be interpreted as evidence of population-level differences.

HPLC-based comparative peak profiling provided supportive chromatographic evidence for a plumbagin-corresponding peak in all three representative composite samples. The authentic plumbagin standard showed a reference peak at Rt 18.52 minutes, whereas the sample extracts showed corresponding peaks at approximately Rt 18.53 minutes under the same chromatographic conditions. Among the extracts, the field-grown tissue-culture-derived composite sample showed the highest relative plumbagin-associated peak response, followed by the natural-source-derived field-grown and *in vitro*-rooted composite samples. However, because the HPLC analysis was based on one analytical run per pooled composite sample and did not include calibration-curve-based quantification, replicate analytical validation, PDA spectral overlay, or mass-spectral confirmation, the findings are interpreted cautiously as comparative peak-profile evidence rather than definitive evidence of enhanced plumbagin accumulation or absolute plumbagin concentration. HPLC and HPTLC have previously been used for plumbagin estimation and comparison among *Plumbago* samples [13,16].

Within the present single-composite comparison, the field-grown tissue-culture-derived sample showed the highest relative plumbagin-associated peak response. This observation may reflect differences in physiological maturity or field exposure; however, it cannot establish a consistent source-related effect because independent biological replication was not performed. Field conditions expose plants to natural light intensity, soil factors, microbial associations, temperature variation, and mild stress, all of which may act as signals influencing secondary metabolite biosynthesis. This interpretation is consistent with studies showing that biotic and abiotic elicitors such as chitosan, yeast extract, L-alanine, and methyl-β-cyclodextrin can influence plumbagin production in *P. indica* root cultures and regenerated shoots [6,15]. Nevertheless, the present HPLC data should be considered preliminary and supportive, and further validation using biological replicates, calibration-curve-based quantification, and spectral or mass confirmation would be required for definitive plumbagin quantification.

5. CONCLUSION

The present study established an explant-dependent micropropagation protocol for *P. indica* through direct and indirect regeneration pathways. Among the explants tested, nodal explants showed the highest shoot multiplication response, producing 36.70 ± 0.93 shoots/explant, followed by shoot-tip explants and leaf-derived callus cultures. Leaf explants also exhibited callus induction and indirect organogenesis, indicating their potential usefulness for callus-based regeneration and future secondary-metabolite studies. Regenerated shoots rooted efficiently on half-strength MS medium supplemented with IAA, IBA, BAP, and activated charcoal. Across nursery-scale acclimatization batches, an approximate survival range of 95%–100% was observed; however, exact batch-wise survivor counts were not retained.

Supporting biochemical profiling showed descriptive differences among the three pooled composite extracts examined. The field-grown tissue-culture-derived sample showed the highest TPC (80.50 ± 0.50 mg GAE g^{-1} dry extract) and TFC (24.89 ± 0.27 mg QE g^{-1} dry extract), followed by the natural-source-derived field-grown and *in vitro*-rooted samples. HPLC profiling similarly showed the highest relative plumbagin-associated peak response in the field-grown tissue-culture-derived composite sample. Because each source was represented by one pooled composite biological sample, the TPC/TFC replicate readings were technical, and HPLC was based on single analytical runs, these findings are interpreted as supportive descriptive profiling rather than as population-level evidence or absolute plumbagin quantification.

Overall, the study provides an efficient protocol for rapid multiplication, rooting, and acclimatization of *P. indica*. The integration of micropropagation, rooting, hardening, phytochemical screening, representative TPC/TFC estimation, and HPLC-based comparative peak profiling supports the practical value of this protocol for conservation, sustainable cultivation, and production of quality planting material. Further studies using biological replicates,

validated calibration-based plumbagin quantification, and spectral or mass confirmation would strengthen the biochemical conclusions.

6. ACKNOWLEDGMENT

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7. CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

8. FUNDING

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9. DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author on reasonable request.

10. ETHICS APPROVAL

Not applicable. The study involved plant tissue culture and phytochemical analysis and did not involve human participants or experimental animals.

11. AUTHOR CONTRIBUTIONS

Vinay Kumar Hegde: Conceptualization, methodology, investigation, data acquisition, data analysis and interpretation, and writing—original draft. Divya Kale: Statistical analysis. Keshav H. Korse: Conceptualization, data analysis and interpretation, taxonomic authentication, and writing—review and editing. Penna Suprasanna: Supervision and critical revision of the manuscript. Abhishek Guldhe: Conceptualization, data analysis and interpretation, statistical analysis, supervision, and writing—review and editing. All authors reviewed and approved the final manuscript.

12. DECLARATION ON THE USE OF ARTIFICIAL INTELLIGENCE TOOLS

ChatGPT, developed by OpenAI, was used solely for grammatical polishing, language refinement, and final editorial improvement of the manuscript. The experimental design, observations, laboratory work, data generation, data analysis, results, scientific interpretation, and original manuscript preparation were carried out by the authors.

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