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Harnessing *in vitro* technologies for conservation of *Paeonia emodi*, a threatened medicinal and ornamental species

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Abstract

Paeonia emodi Wall. ex Royle, a valuable medicinal and ornamental species native to the north-western Himalayas, is threatened by overharvesting and habitat loss [1, 2]. This study presents a steadfast *in vitro* regeneration protocol for its conservation and mass propagation. Nodal explants were cultured on Murashige and Skoog's (MS) basal medium with various concentrations of plant growth regulators (PGRs). Among PGRs, 2.0 mg L^{-1} BAP and 2.5 mg L^{-1} zeatin, individually, induced the maximum shoot induction rates (70.44% and 59.66%) with 8.70 and 5.17 shoots per explant, respectively. A combination of 2.5 mg L^{-1} zeatin and 1.5 mg L^{-1} TDZ resulted in 66.20% shoot induction, improved shoot length (6.63 cm), and faster response (28.13 days). The highest rooting efficiency (48.22%) was obtained on half-strength MS medium containing 0.25 mg L^{-1} NAA and 0.5 mg L^{-1} IBA. Acclimatized plantlets showed 80–85% survival under greenhouse conditions. This simple and reproducible protocol supports large-scale propagation of *P. emodi*, and lays the groundwork for its conservation, genetic improvement, and commercial use.

Keywords Conservation, *In vitro* regeneration, Medicinal plant, *Paeonia emodi*, Plant growth regulators

1 Introduction

Paeonia emodi Wall. ex Royle, commonly known as the Himalayan peony, is an herbaceous perennial of the family Paeoniaceae, native to the temperate zones of the north-western Himalayas, including Kashmir, Himachal Pradesh, and neighbouring regions [3]. It is well recognized for its ornamental attributes, such as, large, fragrant white flowers and deeply lobed foliage [4]. However, despite its aesthetic appeal, the species remains underutilized in ornamental horticulture due to propagation challenges and the pressure of unsustainable medicinal harvesting. Ethnobotanically, *P. emodi* has a long history of use in traditional medicine systems, being employed to manage conditions like inflammation, digestive disorders, epilepsy, and gynaecological ailments [5]. Phytochemical investigations have confirmed the presence of bioactive compounds, -such as

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paeoniflorin, monoterpenes, triterpenoids, and phenolics that contribute to its pharmacological potential [6, 7].

Currently, *P. emodi* faces significant threats in its natural habitat due to anthropogenic pressures including habitat degradation, overexploitation, and its inherently poor seed viability [8]. Natural propagation is hampered by slow and erratic seed germination as well as laborious vegetative multiplication, necessitating urgent conservation measures. In this regard, biotechnological approaches such as *in vitro* propagation offer a promising alternative for the mass multiplication and *ex situ* conservation of rare and medicinally valuable plant species [9, 10]. Micropropagation allows the year-round production of genetically uniform, pathogen-free plants, independent of seasonal constraints. Although several *Paeonia* species have been studied for tissue culture applications [11, 12], research specifically targeting *P. emodi* remains scarce, limiting broader conservation and utilization initiatives. The present study aims to develop an efficient *in vitro* regeneration protocol for *P. emodi* by optimizing the concentration and combinations of plant growth regulators for callus induction, shoot proliferation, and root development. These insights are intended to support conservation efforts, promote its sustainable use in horticulture and medicine, and provide a foundation for future biotechnological interventions in *Paeonia* species (Fig.1)

2 Materials and methods

2.1 Source of plant material and explant preparation

Juvenile stock plants of *P. emodi* were collected in April 2023 from Aabnar Khag, Jammu and Kashmir, India (33°58′45.3″ N, 74°31′4.692″ E; 2211 m a.s.l.). The species was cultivated in the Botanical Garden of the University of Kashmir and authenticated at the Centre for Biodiversity and Taxonomy. A voucher specimen (Voucher No. KASH-9273) was deposited in the Kashmir University Herbarium (KASH) for future reference.

For *in vitro* culture, healthy explants (~ 1 cm^2 from 4-week-old plants) were excised and subjected to sequential sterilization: (1) 1% (v/v) Labolene (Hi Media Labs. Pvt. Ltd, Mumbai, India) detergent for 10 min, (2) 0.01% chemical sterilant mercuric chloride ($HgCl_2$) with 0.1% Tween-20 surfactant (SRL, Pvt. Ltd, Mumbai, India) for 10 min, (3) 70% ethanol for 1 min, and (4) 2% Bavistin fungicide (Carbendazim Powder, BASF India Ltd.) for 5 min [13]. Preliminary trials with sodium hypochlorite (NaOCl) for surface sterilization of *P. emodi* explants were ineffective, resulting in high contamination levels. A similar trend has been reported for other *Paeonia* species where NaOCl was less efficient than mercuric chloride ($HgCl_2$) in achieving aseptic cultures [14]. Consequently, mercuric chloride ($HgCl_2$) was adopted for its superior sterilization efficiency.



Fig. 1 Ornamental attributes and conservation concern of *Paeonia emodi* Wall. ex Royle: (**a, b**) plants showing characteristic large, fragrant white flowers and deeply lobed foliage; (**c**) habitat disturbance caused by unregulated construction activities threatening natural populations

All procedures involving $HgCl_2$ adhered to strict safety protocols, including the use of personal protective equipment and proper ventilation, and waste was disposed of following institutional hazardous waste guidelines. After each treatment, explants were rinsed thoroughly with sterile double-distilled water in a laminar airflow hood (Biobase). Sterilized explants were inoculated on Murashige and Skoog (MS) medium [15] supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} agar. The pH of the medium was adjusted to 5.7 ± 0.1 before autoclaving (121 °C, 15 psi, 20 min).

2.2 Culture medium composition and growth conditions

MS basal medium [15] was supplemented with 0–2.5 mg L^{-1} of auxins, including 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA), and with 0–3.5 mg L^{-1} of cytokinins, namely 6-benzylaminopurine (BAP), thidiazuron (TDZ), zeatin (Z), and kinetin (Kn) [16, 17]. MS basal media without PGRs was considered as control. Explants were incubated at 25 ± 2 °C under a 16/8 h light/dark photoperiod (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$, cool-white fluorescent light) and 55 ± 2% relative humidity. After four weeks, observations were recorded on callus induction frequency, callus morphology, shoot induction, rooting frequency, and mean shoot/root length.

2.3 Shoot initiation and direct regeneration

Shoot initiation was tested on MS basal medium supplemented with 0.5–3.5 mg L^{-1} of PGRs (BAP, NAA, 2,4-D, IAA, IBA, Kn, Z, TDZ) individually or in combination [16, 17]. The medium contained sucrose (3%) as carbon source, 0.8% agar (Hi Media Labs. Pvt. Ltd, Mumbai, India) as gelling agent, and polyvinylpyrrolidone (PVP, 0.5%) to reduce phenolic exudation [18]. The pH was adjusted to 5.7 ± 0.1 using 0.1 N NaOH or HCl. About 10–15 mL medium was dispensed into culture tubes (25 × 150 mm, Borosil) or 250 mL flasks (Borosil) and autoclaved at 121 °C for 15–20 min. Cultures were incubated for four weeks, after which shoot initiation, proliferation, and rooting responses were recorded. To enhance shoot multiplication, regenerated shoots were periodically subcultured onto fresh medium.

2.4 Shoot multiplication and elongation

Proliferated shoots were transferred to MS basal medium containing BAP alone or in combination with auxins (IAA, IBA, or NAA at 0.5–1.5 mg L^{-1}) to assess shoot multiplication and elongation. Subculturing was performed every four weeks for four cycles to test consistency of response.

2.5 Root Induction, Acclimatization, and hardening

For rhizogenesis, *in vitro* raised shoots (2–4 nodes) were transferred to half-strength MS medium with 3% sucrose, 0.8% agar (Hi Media Labs. Pvt. Ltd, Mumbai, India), and different auxins (IAA, IBA, NAA) [15, 19]. Cultures were initially maintained in the dark for 2 weeks and then shifted to standard growth conditions. Rooted plantlets were rinsed to remove agar and transplanted into plastic pots containing a sterilized mixture of peat moss, vermicompost, and perlite (1:1:1). Greenhouse conditions were maintained at 25 ± 2 °C with relative humidity 70–75%. Pots were covered with transparent polyethylene sheets for two weeks to maintain humidity. After 28 days of hardening in

the greenhouse, plantlets were transferred to earthen pots with garden soil: sand (1:1 v/v) and maintained under shade.

2.6 Experimental design and statistical analysis

The experiment was arranged in a completely randomized design (CRD) with 10 explants per treatment, replicated three times ($n = 30$ explants per treatment). Parameters recorded included callus induction (%), shoot induction (%), mean shoot number per explant, mean shoot length, rooting frequency, and root length. Induction frequency was calculated as: (responsive explants ÷ total explants) × 100. Morphological traits (color/texture) were observed under a stereo microscope (Nikon SMZ18). Normality and homoscedasticity were assessed using the Shapiro–Wilk and Levene's tests, respectively. Percentage data were arcsine square-root transformed prior to analysis. Data were analyzed using one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ to determine significant differences among treatments. Data analysis was carried out using IBM SPSS Statistics, Version 23.0.

3 Results

3.1 Callus induction efficiency

Stem explants of *P. emodi* were cultured on MS medium supplemented with varying concentrations of different plant growth regulators (Table 1). Callus induction from explants

Table 1 Effect of various concentrations of TDZ, zeatin, kinetin, NAA, IAA, and IBA on callus induction (%), days to callus initiation, and callus characteristics in *P. emodi*

MS medium + PGRs (mg L^{-1})						Response		
TDZ	Z	Kn	NAA	IAA	IBA	Callus induction (% ± SE)	No. of Days (mean ± SE)	Callus Colour, Texture
0	0	0	0	0	0	00.00 ± 0.00^{m}	00.00 ± 0.00^{o}	-
0.5	–	–	–	–	–	34.92 ± 1.00^{h}	43.20 ± 0.76^{efg}	Cream, Hard
1	–	–	–	–	–	38.74 ± 0.69^{g}	38.73 ± 0.37^{jkl}	Cream, Hard
1.5	–	–	–	–	–	44.81 ± 0.85^{ef}	38.60 ± 0.76^{jkl}	Green, Hard
2	–	–	–	–	–	46.73 ± 0.65^{cd}	45.60 ± 0.81^{d}	Green, Hard
2.5	–	–	–	–	–	56.66 ± 0.72^{a}	42.67 ± 0.71^{fgh}	Green, Hard
–	0.5	–	–	–	–	35.85 ± 0.50^{h}	41.20 ± 0.23^{hi}	Cream, Friable
–	1	–	–	–	–	57.21 ± 0.47^{a}	37.20 ± 0.35^{lm}	Green, Hard
–	1.5	–	–	–	–	47.63 ± 0.49^{c}	36.87 ± 0.37^{m}	Green, Hard
–	2	–	–	–	–	39.45 ± 0.31^{g}	38.00 ± 0.31^{klm}	Green, Friable
–	2.5	–	–	–	–	32.32 ± 0.49^{i}	34.80 ± 1.38^{n}	Green, Friable
–	–	0.5	–	–	–	31.62 ± 0.38^{j}	53.33 ± 0.55^{a}	Cream, Friable
–	–	1	–	–	–	43.58 ± 0.45^{f}	50.46 ± 0.47^{b}	Cream, Friable
–	–	–	0.5	–	–	29.74 ± 0.44^{l}	45.27 ± 0.29^{d}	Cream, Friable
–	–	–	1	–	–	35.15 ± 0.50^{h}	44.33 ± 0.37^{def}	Cream, Friable
–	–	–	1.5	–	–	45.99 ± 0.56^{de}	39.22 ± 0.39^{jk}	Green, Hard
–	–	–	2	–	–	40.09 ± 0.23^{g}	42.40 ± 0.64^{gh}	Green, Hard
–	–	–	2.5	–	–	32.61 ± 0.55^{i}	42.73 ± 0.37^{fgh}	Cream, Hard
–	–	–	–	1	–	34.71 ± 0.49^{h}	39.86 ± 0.63^{ij}	Green, Friable
–	–	–	–	1.5	–	51.37 ± 0.24^{b}	35.15 ± 0.23^{n}	Green, Hard
–	–	–	–	2	–	29.75 ± 0.15^{l}	43.38 ± 0.27^{efg}	Green, Friable
–	–	–	–	–	1	30.35 ± 0.35^{k}	44.77 ± 0.25^{de}	Green, Friable
–	–	–	–	–	1.5	43.43 ± 0.45^{f}	40.17 ± 0.21^{ij}	Green, Hard
–	–	–	–	–	2	55.81 ± 0.61^{cd}	37.33 ± 0.25^{lm}	Green, Hard
–	–	–	–	–	2.5	28.17 ± 0.33^{l}	47.99 ± 0.27^{c}	Green, Friable

was significantly influenced by varying concentrations and combinations of TDZ, Z, Kn, NAA, IAA, and IBA. Among the various plant growth regulator tested individually, the superior callus induction (57.21 ± 0.47%) was seen in the medium containing 1.0 mg L^{-1} zeatin followed by TDZ (2.5 mg L^{-1}) with callus induction 56.66 ± 0.72% with callus initiation achieved after 37.20 ± 0.35 and 42.67 ± 0.71 days, respectively. However, with different PGR combinations (Table 2), the highest callus induction (63.48 ± 0.38%) was recorded in the medium supplemented with TDZ (2.5 mg L^{-1}) and zeatin (2.0 mg L^{-1}), with callus initiation observed after 34.41 ± 0.63 days. This was followed by TDZ (2.5 mg L^{-1}) combined with IBA (2.0 mg L^{-1}), which yielded 56.71 ± 0.47% induction and exhibited the shortest callus initiation period (30.99 ± 0.11 days). Similarly, the combinations of TDZ (2.5 mg L^{-1}) with IAA (1.5 mg L^{-1}) and zeatin (1.5 mg L^{-1}) also showed high callus induction (55.14 ± 0.19% and 54.84 ± 0.25%, respectively), with relatively early responses. In terms of callus texture, most treatments involving high concentrations of TDZ, alone or in combination with cytokinins or auxins, resulted in green, hard callus. However, friable callus—preferable for regeneration—was induced with certain combinations such as TDZ (2.5 mg L^{-1}) with IBA (1.0 and 2.5 mg L^{-1}) and kinetin (2.0 mg L^{-1}), though the overall induction percentages were moderate. Notably, the use of 2,4-D in combination with BAP (0.5–1.5 mg L^{-1}) produced early but poor callus responses, with significantly lower induction rates (28.22–37.78%) and callus often appearing brown or cream in color. These findings highlight the superior efficacy of TDZ-based combinations, particularly with zeatin or IBA, for optimizing callus induction and response time in *P. emodi*. All results showed statistically significant differences ($p \leq 0.05$), as indicated by distinct superscript letters in the dataset.

Values are presented as mean ± SE. Different superscript letters within a column indicate significant differences at $p \leq 0.05$ according to Duncan's Multiple Range Test (DMRT). Callus color and texture are also noted for each treatment.

Table 2 Effect of various concentrations and combinations of TDZ, zeatin, kinetin, NAA, IAA, IBA, 2,4-D and BAP on callus induction (%), days to callus initiation, and callus characteristics in *Paeonia emodi*

MS medium + PGRs (mg L^{-1})								Response		
TDZ	Z	Kn	NAA	IAA	IBA	2,4–D	BAP	Callus induction (% ± SE)	No. of Days (mean ± SE)	Callus Colour, Texture
0	0	0	0	0	0	0	0	00.00 ± 0.00^{k}	00.00 ± 0.00^{h}	–
2.5	1	–	–	–	–	–	–	49.38 ± 0.21^{e}	36.83 ± 0.51^{cde}	Green, Hard
2.5	1.5	–	–	–	–	–	–	54.84 ± 0.25^{c}	35.74 ± 0.24^{de}	Green, Hard
2.5	2	–	–	–	–	–	–	63.48 ± 0.38^{a}	34.41 ± 0.63^{f}	Green, Hard
2.5	–	1	–	–	–	–	–	52.71 ± 0.18^{d}	37.21 ± 0.31^{bc}	Cream, Hard
2.5	–	–	1	–	–	–	–	50.35 ± 0.29^{e}	34.73 ± 0.52^{ef}	Green, Hard
2.5	–	–	1.5	–	–	–	–	45.54 ± 0.37^{g}	37.67 ± 0.48^{b}	Green, Hard
2.5	–	–	2	–	–	–	–	35.63 ± 0.45^{i}	40.99 ± 0.40^{a}	Cream, Friable
2.5	–	–	–	1	–	–	–	48.11 ± 0.44^{f}	36.16 ± 0.27^{cd}	Green, Friable
2.5	–	–	–	1.5	–	–	–	55.14 ± 0.19^{c}	32.09 ± 0.13^{g}	Green, Hard
2.5	–	–	–	–	2	–	–	56.71 ± 0.47^{b}	30.99 ± 0.11^{g}	Green, Hard
2.5	–	–	–	–	2.5	–	–	48.23 ± 0.17^{f}	34.64 ± 0.23^{ef}	Green, Friable
–	–	–	–	–	–	0.5	0.5	37.78 ± 0.53^{h}	31.33 ± 0.37^{g}	Brown, Hard
–	–	–	–	–	–	0.5	1	28.88 ± 0.13^{j}	32.00 ± 0.12^{g}	Cream, Friable
–	–	–	–	–	–	0.5	1.5	28.22 ± 0.33^{j}	37.06 ± 0.82^{bc}	Cream, Friable

Values are presented as mean ± SE. Different superscript letters within a column indicate significant differences at $p \leq 0.05$ according to Duncan's Multiple Range Test (DMRT) (Fig. 2)

3.2 Shoot induction efficiency

This study systematically investigated the effects of various plant growth regulators and their combinations on shoot organogenesis *in vitro*. The shoot induction response of *P. emodi* was significantly influenced by different combinations and concentrations of plant growth regulators. Our findings demonstrate that the highest shoot induction frequency (70.44 ± 0.44%) was observed in the medium supplemented with BAP (2.0 mg L^{-1}) alone, which also produced the maximum number of shoots per explant (8.70 ± 0.15) with a mean shoot induction time of 29.10 ± 0.12 days and a considerable shoot length (5.06 ± 0.12 cm). This was closely followed by zeatin (2.5 mg L^{-1}), which yielded 59.66 ± 0.43% induction and 5.17 ± 0.20 shoots. TDZ at 1.5 mg L^{-1} also showed good shoot proliferation (58.03 ± 0.27%) with 4.40 ± 0.21 shoots and good shoot length (6.03 ± 0.21 cm). Among the auxins, NAA alone induced shoot formation with the highest frequency (55.59 ± 0.91%) and moderate shoot length (3.76 ± 0.08 cm) at 0.5 mg L^{-1}; however, both the shoot number and length declined with increasing concentration. Kinetin, a cytokinin, showed improved responses at 2.0 mg L^{-1}, having induction frequency 55.96 ± 1.50% and yielding a shoot number of 3.33 ± 0.24 and a length of 5.71 ± 0.31 cm. TDZ, a phenylurea–type cytokinin, proved highly efficacious at 1.5 mg L^{-1}, producing the highest shoot induction frequency (58.03 ± 0.27%) and a substantial shoot number (4.40 ± 0.21), with elongated shoots (6.03 ± 0.21 cm) and early response (28.73 ± 1.12 days) (Table 3). Overall, TDZ and zeatin proved superior in promoting both the number and elongation of shoots when used independently, underscoring their potential in optimizing micropropagation protocols for *P. emodi*.

The synergistic effect of zeatin (2.5 mg L^{-1}) in combination with various concentrations of TDZ, 2,4–D, BAP, Kn, and NAA significantly influenced shoot induction responses in *P. emodi* (Table 4). Among all treatments, the highest shoot induction frequency (66.20 ± 0.31%) and substantial shoot number (4.97 ± 0.08) were observed in the

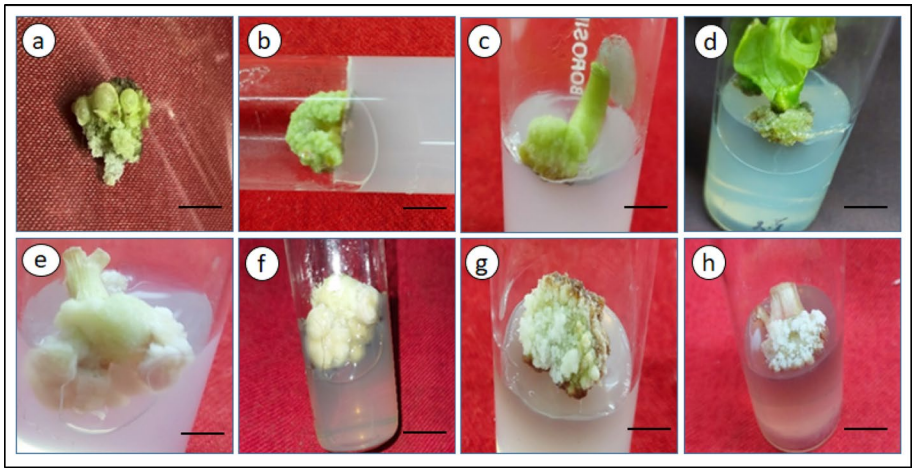


Fig. 2 *In vitro* callus induction and proliferation in *P. emodi*. Representative stages of callus development from explant initiation to active proliferation under controlled conditions. (**a– b**) 2.5 mg L^{-1} TDZ + 2 mg L^{-1} Z (**c**) 1 mg L^{-1} Z, (**d**) 2.5 mg L^{-1} Z (**e–g**) 2.5 mg L^{-1} TDZ + 1 mg L^{-1} Kn (**h**) 1 mg L^{-1} Kn Scale bar (**a–h**) represents 1 cm

Table 3 Influence of individual plant growth regulators (NAA, kinetin, zeatin, TDZ and BAP) at varying concentrations on shoot induction response in *P. emodi*

MS medium + PGRs (mg L^{-1})					Response			
NAA	Kn	TDZ	Z	BAP	Shoot induction frequency (% ± SE)	Shoot number/explant (Mean ± SE)	No. of days (Mean ± SE)	Shoot length (cm) (Mean ± SE)
0	0	0	0	0	00.00 ± 0.00^{j}	00.00 ± 0.00^{k}	00.00 ± 0.00^{m}	00.00 ± 0.00^{p}
0.5	–	–	–	–	55.59 ± 0.91^{cd}	1.37 ± 0.08^{j}	28.73 ± 0.17^{l}	3.76 ± 0.08^{ijk}
1	–	–	–	–	45.97 ± 0.98^{efg}	1.40 ± 0.17^{j}	28.06 ± 0.07^{l}	2.64 ± 0.21^{m}
1.5	–	–	–	–	43.96 ± 0.80^{f}	1.50 ± 0.21^{j}	30.20 ± 0.76^{ijk}	2.24 ± 0.02^{n}
2	–	–	–	–	35.07 ± 1.19^{h}	1.46 ± 0.21^{j}	34.47 ± 0.27^{fg}	1.60 ± 0.58^{o}
–	0.5	–	–	–	36.27 ± 0.49^{h}	1.32 ± 0.42^{j}	30.20 ± 0.23^{ijk}	3.65 ± 0.06^{jk}
–	1	–	–	–	46.25 ± 0.40^{efg}	1.44 ± 0.14^{j}	31.93 ± 0.99^{h}	4.85 ± 0.05^{de}
–	1.5	–	–	–	45.41 ± 0.26^{efg}	2.73 ± 0.13^{fg}	34.27 ± 0.35^{f}	5.58 ± 0.12^{bc}
–	2	–	–	–	55.96 ± 1.50^{cd}	3.33 ± 0.24^{e}	37.60 ± 0.12^{d}	5.71 ± 0.31^{ab}
–	2.5	–	–	–	36.01 ± 0.75^{gh}	1.77 ± 0.24^{ij}	34.73 ± 0.98^{efg}	4.01 ± 0.05^{hij}
–	–	0.5	–	–	44.74 ± 1.24^{f}	2.53 ± 0.14^{gh}	31.40 ± 0.40^{hi}	4.57 ± 0.16^{de}
–	–	1	–	–	53.79 ± 0.30^{d}	3.52 ± 0.19^{e}	30.40 ± 0.20^{ij}	5.23 ± 0.23^{cd}
–	–	1.5	–	–	58.03 ± 0.27^{bc}	4.40 ± 0.21^{d}	28.73 ± 1.12^{kl}	6.03 ± 0.21^{a}
–	–	2	–	–	38.32 ± 0.89^{g}	1.48 ± 0.17^{j}	44.60 ± 0.34^{a}	4.33 ± 0.06^{fgh}
–	–	2.5	–	–	35.37 ± 1.12^{h}	2.16 ± 0.27^{hi}	42.33 ± 0.47^{b}	4.13 ± 0.02^{ghi}
–	–	–	0.5	–	44.56 ± 0.40^{f}	2.47 ± 0.23^{gh}	39.73 ± 0.29^{c}	4.25 ± 0.02^{fgh}
–	–	–	1	–	47.59 ± 0.80^{d}	2.75 ± 0.14^{fg}	38.07 ± 0.26^{d}	4.32 ± 0.02^{fgh}
–	–	–	1.5	–	53.46 ± 1.12^{d}	3.23 ± 0.34^{ef}	35.67 ± 0.35^{ef}	4.42 ± 0.03^{fgh}
–	–	–	2	–	57.44 ± 1.00^{bc}	3.70 ± 0.57^{e}	33.40 ± 0.35^{g}	4.55 ± 0.02^{efg}
–	–	–	2.5	–	59.66 ± 0.43^{b}	5.17 ± 0.20^{c}	30.26 ± 0.47^{ijk}	4.60 ± 0.01^{f}
–	–	–	3	–	46.14 ± 0.80^{efg}	3.23 ± 0.04^{ef}	38.80 ± 0.35^{d}	3.77 ± 0.03^{ijk}
–	–	–	3.5	–	42.65 ± 0.52^{f}	2.33 ± 0.28^{gh}	36.60 ± 0.23^{e}	4.01 ± 0.14^{ghi}
–	–	–	–	1	35.70 ± 0.43^{h}	5.23 ± 0.15^{c}	36.10 ± 0.17^{e}	3.50 ± 0.23^{k}
–	–	–	–	1.5	55.87 ± 0.64^{cd}	7.10 ± 0.12^{b}	31.00 ± 0.23^{hi}	4.20 ± 0.06^{fgh}
–	–	–	–	2	70.44 ± 0.44^{a}	8.70 ± 0.15^{a}	29.10 ± 0.12^{jkl}	5.06 ± 0.12^{d}
–	–	–	–	2.5	29.16 ± 0.28^{i}	4.43 ± 0.18^{d}	33.96 ± 0.18^{g}	3.10 ± 0.12^{l}

Table 4 Effect of combined plant growth regulators (cytokinin and auxin) on shoot induction in *P. emodi*. treatments include various concentrations of BAP or TDZ in combination with NAA

MS medium + PGRs (mg L^{-1})						Response			
Z	2,4–D	TDZ	BAP	Kn	NAA	Shoot induction frequency (% ± SE)	Shoot number/explant (Mean ± SE)	No. of days (Mean ± SE)	Shoot length (cm) (Mean ± SE)
0	0	0	0	0	0	00.00 ± 0.00^{h}	00.00 ± 0.00^{h}	00.00 ± 0.00^{i}	00.00 ± 0.00^{g}
2.5	1	–	–	–	–	46.15 ± 0.80^{d}	1.33 ± 0.03^{g}	38.80 ± 0.35^{ab}	3.76 ± 0.03^{f}
2.5	1.5	–	–	–	–	42.65 ± 0.52^{e}	2.33 ± 0.28^{f}	36.60 ± 0.23^{c}	4.01 ± 0.14^{f}
2.5	2	–	–	–	–	50.54 ± 0.71^{c}	2.93 ± 0.47^{e}	34.40 ± 0.46^{c}	4.67 ± 0.17^{e}
2.5	2.5	–	–	–	–	57.10 ± 0.72^{b}	4.90 ± 0.21^{bc}	32.20 ± 0.58^{e}	5.04 ± 0.06^{d}
2.5	–	1	–	–	–	45.23 ± 0.17^{d}	3.57 ± 0.09^{d}	34.21 ± 0.18^{d}	4.63 ± 0.08^{e}
2.5	–	1.5	–	–	–	66.20 ± 0.31^{a}	4.97 ± 0.08^{b}	28.13 ± 0.11^{g}	6.63 ± 0.18^{a}
2.5	–	2	–	–	–	50.61 ± 0.33^{c}	4.57 ± 0.09^{bc}	30.30 ± 0.40^{f}	5.90 ± 0.20^{b}
2.5	–	2.5	–	–	–	23.43 ± 0.35^{g}	3.27 ± 0.12^{de}	39.74 ± 0.76^{a}	4.06 ± 0.09^{f}
2.5	–	–	1.5	–	–	45.62 ± 0.71^{d}	5.10 ± 0.11^{b}	26.90 ± 0.93^{g}	5.49 ± 0.15^{c}
2.5	–	–	2	–	–	65.41 ± 1.02^{a}	6.27 ± 0.12^{a}	24.40 ± 0.40^{h}	6.67 ± 0.19^{a}
2.5	–	–	2.5	–	–	35.71 ± 1.16^{f}	4.30 ± 0.21^{c}	30.83 ± 0.54^{f}	4.46 ± 0.15^{e}
–	–	–	–	1	0.5	35.46 ± 0.93^{f}	1.21 ± 0.10^{g}	39.60 ± 0.35^{ab}	5.62 ± 0.01^{bc}
–	–	–	–	1	1.5	47.45 ± 0.52^{d}	1.58 ± 0.27^{g}	38.32 ± 0.04^{b}	5.57 ± 0.01^{bc}

medium containing zeatin (2.5 mg L^{-1}) and TDZ (1.5 mg L^{-1}), with early shoot emergence at 28.13 ± 0.11 days and the longest shoot length of 6.63 ± 0.18 cm. A comparable response was seen with zeatin + BAP (2.0 mg L^{-1}), which yielded 65.41 ± 1.02% induction, the highest number of shoots (6.27 ± 0.12), and a slightly shorter induction period (24.40 ± 0.40 days). Treatments with zeatin in combination with 2,4–D (2.5 mg L^{-1}) and TDZ (2.0 mg L^{-1}) also showed enhanced shoot regeneration frequencies of 57.10 ± 0.72% and 50.61 ± 0.33%, respectively, accompanied by moderate shoot numbers and lengths. The longest shoots were generally observed in treatments combining zeatin with TDZ or kinetin, indicating their potential for effective shoot elongation. Overall, zeatin, when combined with optimal levels of TDZ or BAP, significantly enhanced both the shoot proliferation rate and quality, making these combinations highly suitable for micropropagation of *P. emodi*.

Data are presented as mean ± SE ($n = 3$). Parameters assessed include percentage of shoot induction, mean number of shoots per explant, and mean shoot length (cm). Means within columns followed by different letters are significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test (DMRT), indicating the effectiveness of specific hormone treatments in promoting i*n vitro* shoot proliferation.

Data represent mean ± SE ($n = 3$) for shoot induction percentage, number of shoots per explant, and shoot length (cm). Statistical analysis was performed using Duncan's Multiple Range Test (DMRT); means followed by different letters within the same column differ significantly at $P \leq 0.05$, demonstrating the synergistic impact of hormone combinations on shoot regeneration efficiency (Fig. 3)

3.3 Root induction efficiency

This research meticulously investigated the impact of three auxins – NAA, IBA, and IAA – on root development (Table 5), yielding three significant insights. Firstly, NAA at a concentration of 0.25 mg L^{-1} proved to be the most effective single treatment, resulting in the highest number of roots per explant (6.01 ± 0.17), the longest roots



Fig. 3 Stages of *in vitro* regeneration and acclimatization of *P. emodi*. (**a-b**) Multiple shoot induction on MS medium supplemented with 2.5 mg L^{-1} Z + 2 mg L^{-1} BAP; (**c**) Shoot elongation and development on 2.0 mg L^{-1} BAP; (**d**) *In vitro* root induction using 0.25 mg L^{-1} NAA enriched MS medium; (**e-f**) Healthy plantlets with established roots, ready for transplant; (**g-i**) Transplantation of rooted plantlets into pots containing a sterilized peat moss: vermiculite: perlite (1:1:1) mix under controlled humidity. Scale bar (**a-i**) represents 1 cm

Table 5 Effect of auxins (IAA, IBA, and NAA) on *in vitro* root induction in *P. emodi*. Different concentrations of individual auxins were evaluated for root induction percentage, number of roots per shoot, and mean root length (cm)

MS medium + PGRs (mg L^{-1})			Response		
NAA	IBA	IAA	Frequency of rooting (%±SE)	Root number (mean ± SE)	Root length (cm) (mean ± SE)
0	0	0	00.00 ± 0.00^{e}	00.00 ± 0.00^{f}	00.00 ± 0.00^{g}
0.25	–	–	46.49 ± 1.62^{ab}	6.01 ± 0.17^{a}	6.96 ± 0.84^{a}
0.5	–	–	36.69 ± 1.76^{cd}	4.38 ± 0.22^{bc}	6.53 ± 0.12^{b}
0.75	–	–	33.86 ± 0.99^{d}	4.22 ± 0.09^{c}	5.38 ± 0.05^{e}
–	0.25	–	43.66 ± 0.94^{b}	3.80 ± 0.08^{d}	5.75 ± 0.13^{c}
–	0.5	–	44.25 ± 1.97^{ab}	3.45 ± 0.15^{de}	5.50 ± 0.11^{cde}
–	0.75	–	35.74 ± 0.10^{cd}	3.17 ± 0.02^{e}	4.84 ± 0.07^{f}
–	–	0.25	38.47 ± 0.29^{c}	4.27 ± 0.27^{b}	6.55 ± 0.03^{b}
–	–	0.50	34.35 ± 1.95^{cd}	3.38 ± 0.04^{e}	5.46 ± 0.03^{de}
–	–	0.75	29.34 ± 2.44^{e}	3.23 ± 0.05^{e}	4.68 ± 0.17^{f}
0.25	0.5	–	48.22 ± 1.04^{a}	4.72 ± 0.11^{b}	5.74 ± 0.07^{cd}
–	0.5	0.5	42.67 ± 0.89^{b}	4.37 ± 0.21^{bc}	4.79 ± 0.09^{f}

(6.96 ± 0.84 cm), and a robust root induction rate (46.49 ± 1.62%). Secondly, a synergistic effect was noted when combining NAA (0.25 mg L^{-1}) with IBA (0.5 mg L^{-1}), leading to the highest root induction frequency (48.22 ± 1.04%), indicating that specific auxin combinations can outperform individual hormone applications in promoting rooting. Thirdly, auxin concentration was identified as a critical factor, with low concentrations (≤ 0.25 mg L^{-1}) maximizing rooting, while higher concentrations (≥ 0.5 mg L^{-1}) increasingly induced callus formation, with IAA exhibiting the most pronounced inhibitory effect on rooting at higher doses (29.34 ± 2.44% induction at 0.75 mg L^{-1}). Furthermore, the study highlighted that even moderate increases in IBA concentration (from 0.5 to 0.75 mg L^{-1}) led to a substantial reduction (24%) in root numbers.

Data are expressed as mean ± SE ($n = 3$). Significant differences among treatments were determined using Duncan's Multiple Range Test (DMRT); values with different letters in the same column are significantly different at $P \leq 0.05$, indicating differential auxin efficacy in promoting rhizogenesis.

3.4 Hardening and acclimatization

Successfully rooted plantlets were subjected to a two-phase hardening protocol. Initially, plantlets were transferred to a sterilized potting mix (peat moss: vermiculite: perlite = 1:1:1) and maintained in a high-humidity chamber for primary acclimatization. After two weeks, they were moved to greenhouse conditions for secondary hardening. Gradual exposure to ambient conditions ensured optimal adaptation. The survival rate recorded after four weeks was 80–85%, indicating a successful acclimatization protocol. This outcome is in agreement with studies on *Paeonia lactiflora* [20] and confirms the protocol's efficacy for field transfer and *ex vitro* survival.

4 Discussion

In the present study, callus induction in *Paeonia emodi* varied markedly with different combinations and concentrations of plant growth regulators. The highest callus formation (63.48 ± 0.38%) was achieved on MS medium supplemented with TDZ (2.5 mg L^{-1}) and zeatin (2.0 mg L^{-1}), where calli were compact and green, indicating high

morphogenic potential. In contrast, media with individual cytokinins or auxins produced comparatively lower callus percentages and slower responses, demonstrating that the TDZ + Z interaction is particularly efficient for inducing morphogenic callus in *P. emodi*.

The comparable efficacy of the TDZ-zeatin combination (63.48%) suggests a synergistic interaction between these cytokinins, possibly through complementary activation of cytokinin signaling pathways [21, 22]. This synergy may result from TDZ's ability to enhance endogenous cytokinin levels while zeatin directly activates cytokinin receptors, collectively promoting cell division and callus formation [23, 24]. TDZ, a phenylurea-type cytokinin, is known to stimulate endogenous cytokinin accumulation and has been effectively utilized in several herbaceous and woody plant species to induce morphogenic callus.

The early response and high induction rates observed with TDZ + IBA combinations, particularly at 2.0 mg L^{-1} IBA, suggest that auxins may enhance the competence of explants by promoting cell division and expansion at the wound site. The observed superiority of TDZ, particularly in combination with IBA, in promoting shoot induction in *P. emodi* is consistent with findings in other medicinal [25] and woody plant species [26]. For instance, TDZ has been reported to induce high-frequency shoot organogenesis in *Stevia rebaudiana* [27], *Vitex negundo* [28], and *Withania somnifera* [29], where its potent cytokinin-like activity enhances axillary shoot proliferation and adventitious shoot formation. Similarly, in *Paeonia suffruticosa* [30], TDZ alone or in combination with auxins effectively induced multiple shoots, highlighting the genus-specific responsiveness.

Variation in callus morphology—particularly the shift from green, compact callus in TDZ treatments to cream-colored, friable callus in auxin-containing combinations—suggests differential regulation of cellular differentiation pathways [31, 32]. This morphological spectrum likely reflects distinct auxin-cytokinin ratios activating alternative developmental programs. TDZ favours organized meristematic activity, whereas auxin-rich combinations promote undifferentiated proliferation. These differences offer options for downstream applications, with hard callus being more suitable for organogenesis and friable callus for suspension cultures or metabolite studies.

The rapid callus initiation observed in TDZ -IBA combinations aligns with studies showing auxin-cytokinin crosstalk in accelerating cell proliferation [33]. In contrast, the relatively poor performance of 2,4-D + BAP combinations, despite rapid initiation, suggests an initial burst of cell division without sustained morphogenetic potential—similar to patterns reported in other recalcitrant species [34, 35]. Overall, the results highlight the importance of precise hormonal balance in optimizing callus induction protocols, with TDZ-based combinations offering promising options for large-scale *in vitro* propagation of this endangered species.

The study also presents a comprehensive analysis of how different plant growth regulators (PGRs) influence shoot regeneration in *P. emodi*. Cytokinins, particularly BAP at 2.0 mg L^{-1}, were the most effective in inducing shoot proliferation, yielding the highest shoot induction frequency (70.44%) and maximum number of shoots per explant (8.70). This is consistent with earlier reports in *Paeonia suffruticosa* [36, 37]. Zeatin (2.5 mg L^{-1}) and TDZ (1.5 mg L^{-1}) also exhibited strong morphogenic potential, with zeatin

promoting balanced shoot formation and TDZ enhancing shoot elongation (6.03 cm) [38]. Similar results have been reported in *Paeonia lactiflora* [39].

Kinetin (2.0 mg L^{-1}) showed moderate efficacy (55.96% induction, 3.33 shoots), consistent with its known weaker cytokinin activity in *Paeonia* species [40]. Among auxins, NAA at 0.5 mg L^{-1} induced good shoot formation (55.59%) but became inhibitory at higher doses, matching patterns seen in other medicinal species [41]. The species-specific nature of PGR responses was evident, echoing findings in *Paeonia rockii*, where zeatin outperformed BAP in shoot elongation [11]. Similar findings have also been reported in related medicinal plant species, where *in vitro* approaches have been successfully used to enhance regeneration efficiency and support conservation efforts [42].

When TDZ was used alone, a clear dose-dependent trend emerged. Lower concentrations of TDZ (0.5–1.5 mg L^{-1}) resulted in fewer and shorter shoots, while moderate levels (2–2.5 mg L^{-1}) significantly improved proliferation, with diminishing returns at higher doses. Natural cytokinins like zeatin and kinetin showed lower induction rates, concurring with reports in *Paeonia lactiflora* and *Catharanthus roseus*, where these cytokinins promoted shoot elongation but were less efficient for shoot multiplication compared to synthetic cytokinins like TDZ or BAP [39, 43]. Each PGR displayed a distinct concentration optimum, emphasising the need for precise dosing and supporting emerging concepts such as the cytokinin threshold model and PGR-specific signalling pathways.

Auxin-mediated root induction also exhibited clear concentration-dependent patterns. NAA at 0.25 mg L^{-1} performed best across all rooting parameters, likely due to its stability and efficient transport [44, 45]. Higher NAA concentrations reduced rooting performance, consistent with reports in *Curculigo orchioides* [46] and *Withania somnifera* [47]. IBA at 0.25 mg L^{-1} also gave good results but declined at higher doses, echoing patterns seen in *Stevia rebaudiana* [48, 49]. IAA induced moderate rooting, peaking at 0.25 mg L^{-1}, similar to trends in *Peganum harmala* [50].

Combinatorial treatments, particularly NAA (0.25 mg L^{-1}) + IBA (0.5 mg L^{-1}), outperformed all single treatments, yielding the highest root induction frequency (48.22 ± 1.04%) and good root architecture (4.72 ± 0.11 roots per explant; 5.74 ± 0.07 cm in length). This synergistic effect mirrors similar interactions observed in *Bacopa monnieri* [51] and *Paeonia suffruticosa* [44]. The transition from rooting to callusing at higher auxin concentrations highlights the morphogen-like behaviour of auxins, with clear threshold concentrations (0.5 mg L^{-1} for NAA, 0.75 mg L^{-1} for IBA). The complete rooting inhibition by IAA at higher concentrations may reflect its dual role in both promoting cell division and activating stress responses [52].

In the present study, half-strength MS medium was used for rooting to reduce macronutrient levels, which can inhibit root initiation in woody and medicinal species. High salt concentrations in full-strength MS often cause excessive shoot growth and poor root differentiation due to osmotic stress. Reduced nutrient levels favour root primordia development and improve rooting efficiency, as shown in species such as *Aconitum heterophyllum* [53], *Panax ginseng* [54], and *Stevia rebaudiana* [48]. Our findings (46% rooting on half-strength MS with 0.25 mg L^{-1} NAA) support the suitability of diluted nutrients for effective rooting in *P. emodi*.

A major challenge in culturing *P. emodi* was phenolic exudation from wounded explants, which inhibits organogenesis through the formation of toxic quinones. To

counter this, 0.5% PVP was added to the medium. PVP binds phenolics and prevents oxidative browning, significantly improving explant survival [55]. Similar success has been documented in phenol-rich species such as *Tylophora indica* [56] and *Terminalia chebula* [57]. The use of half-strength MS further reduced stress and phenolic oxidation, consistent with findings in *Azadirachta indica* [58] and *Punica granatum* [59].

5 Conclusion

Optimized plant growth regulator regimes enabled significant improvements in shoot proliferation, root induction, and acclimatization, establishing a scalable framework for *ex situ* conservation, sustainable cultivation and providing a reliable strategy for germplasm preservation. Looking ahead, integrating this protocol with advanced approaches such as cryopreservation and metabolic profiling could further enhance conservation outcomes, offering a robust model for rescuing endangered species and advancing the frontiers of conservation biotechnology and phytopharmaceutical development.

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Author contributions
Munisa Manzoor: conceptualization, data curation, formal analysis, software, validation, visualization, and writing–original draft.Zahoor A Kaloo: conceptualization, investigation, methodology, supervision, validation, visualization, writing–original draft, and writing–review and editing.Nidha Zahoor Shahri: writing–eview and editing and data curation.Irshad Ahmad Bhat: writing–review and editing and data curation.

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Data availability
The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The collection of *Paeonia emodi* specimens complied with national and institutional guidelines for research involving plants. No special permissions were required under local regulations. The species was taxonomically identified by Akhtar H Malik, Jr Scientist, KASH-Herbarium, Centre for Biodiversity and Taxonomy (CBT), Department of Botany, University of Kashmir, Srinagar, Jammu and Kashmir, India. A voucher specimen of *Paeonia emodi* (Voucher No. KASH-9273) has been deposited in the Herbarium, Department of Botany, University of Kashmir, Srinagar, India.

Consent to publication
Not applicable.

Clinical trial
Not applicable.

Competing interests
The authors declare no financial or non-financial conflicts of interest. This study did not involve human participants or animals, so no informed consent was required. The plant material was collected from the wild and later established in the Botanical Garden of the University of Kashmir.

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