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ORIGINAL ARTICLE

Elicitation of callus cultures of the medicinally important plant *Embelia ribes* Burm f. using biotic and abiotic elicitors for enhanced production of embelin

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Abstract

The berries of *Embelia ribes* Burm f. are a rich source of embelin, a compound known for its anthelmintic, antidiabetic, and anticancer activities. Due to over-exploitation, the natural habitat of *Embelia ribes* is now considered vulnerable. Traditional propagation methods are insufficient to meet current demands, necessitating alternative production approaches. This study was designed to explore in vitro culture as a viable alternative for the production of embelin. Although numerous studies have focused on extracting embelin from callus cultures, research on enhancing the embelin content in both organogenic and embryogenic callus cultures remains limited. The objective of this study was to enhance embelin production in callus cultures by using both biotic and abiotic elicitors. Additionally, efforts were made to optimize rapid in vitro shoot induction. We achieved the best response rate for the induction of organogenic and embryogenic calli using MS basal medium supplemented with BAP and TDZ in combination with 2-4-D. Among the various elicitors tested, chitosan at 200 mg/L was the most effective, yielding the highest embelin content, at 6.44% in embryogenic calli and 5.72% in organogenic calli. Subsequent subculturing enabled successful differentiation of callus cultures into shoot buds on MS medium supplemented with a combination of BAP (6-benzylaminopurine) and IAA (indole-3-acetic acid) at concentrations of 2.0 mg/L and 0.1 mg/L, respectively. An effective protocol has been developed for obtaining the highest embelin content from embryogenic and organogenic callus cultures, coupled with a high frequency of shoot multiplication. The protocol can be instrumental for large-scale embelin production, ex-situ conservation, sustainable utilization, and industrial applications.

Key message

Developed the first efficient protocol for enhancing embelin production in *Embelia ribes* through in vitro callus cultures, with chitosan as the most effective elicitor for large-scale production, ex-situ conservation, and sustainable use.

Keywords In vitro propagation · Sustainable cultivation · Ex-situ conservation · Plant growth regulators · Secondary metabolites · Organogenic callus · Embryogenic callus · Chitosan · Methyl jasmonate · Salicylic acid

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Introduction

Embelia ribes Burm f., a vulnerable medicinal liana of the family Primulaceae, is distributed across India, China, Malaya, and Sri Lanka and is typically found at altitudes ranging from 400 to 1200 m (Ravikumar and Ved 2000). Notably, the plant contains embelin, an active compound renowned for its anthelmintic properties and effectiveness in treating skin-related diseases (Githiori et al. 2003; Swamy et al. 2007). Additionally, *E. ribes* exhibits several other medicinal properties, including antifertility (Arora et al. 1971; Prakash 1981), antispermatogenic (Seth et al. 1982), antitumour, analgesic, anti-inflammatory (Handa et al. 1992; Chitra et al. 2004), antibacterial (Chitra et al. 2003), chemopreventive (Sreepriya and Bali 2005), antidiabetic (Bhandari et al. 2007) and cardioprotective (Bhandari et al. 2008) properties.

The red or black globular dried berries of *E. ribes* serve as the primary source of embelin. However, these berries often lack developed embryos and are frequently abortive. Unsustainable harvesting practices, driven by high market demand, further endanger the species survival. Traditional propagation methods, such as vegetative cuttings and seed germination, fall short of meeting production demands. Consequently, in vitro propagation has emerged as a promising strategy for large-scale production of *E. ribes* plant material (Raghu et al. 2006).

Organogenesis and embryogenesis represent common approaches for clonal propagation of medicinally important plant species (Gary and Brent 1986). Somatic embryogenesis has been instrumental in enhancing secondary metabolites, both in terms of quality and quantity, in several medicinal plants (Gastaldo et al. 1994). Biotic and abiotic elicitors play a crucial role in enhancing secondary metabolite production. Chitosan, widely used in drug delivery and biomedical sciences, has numerous applications (Desai et al. 2023). It is utilized in wound healing, drug delivery systems, food packing, cosmetics, dermatology, therapeutics, biomedicine, textiles, and agriculture (Azmana et al. 2021; Bayoumi et al. 2018; Morin-Crini et al. 2019; Rahman and Goswami 2021). Chitosan has also been shown to enhance phenolic content in the root callus culture of *Rumex hastatus* (Singh and Agrawal 2023), Additionally, methyl jasmonate and salicylic acid are well-known for increasing systemic resistance and promoting the production of secondary metabolites in plants (Jeyasri et al. 2023). For example, methyl jasmonate enhances the accumulation of polyphenols in the shoot culture of *Ruta graveolens* (Joshi et al. 2023). Previous attempts to isolate embelin from in vitro callus cultures of *E. ribes* have aimed to develop an efficient protocol for enhancing embelin content in vitro (Annapurna and Rathore 2010; Raghu et al. 2011). However, studies focusing on the elicitation of both embryogenic and organogenic calli in *E. ribes* are lacking. This study aimed to increase the embelin content in organogenic and embryogenic callus cultures of *E. ribes* using various elicitors, such as methyl jasmonate, salicylic acid, and chitosan. Additionally, we sought to develop an efficient in vitro shoot induction protocol for the rapid multiplication of *E. ribes*.

Materials and methods

Plant material and seed germination of *Embelia ribes*

Mature seeds of *E. ribes* were collected from Nagavelli, Karnataka, in June 2016. Initially, the seeds were shade-dried, rinsed in tap water 4 to 5 times, and then washed with a 5% v/v tween-20 sterilization solution for 10 min. Subsequently, the seeds were thoroughly washed with distilled water (4 to 5 times) to eliminate any detergent residues. Treated seeds were immersed in 0.1% $HgCl_2$ for 3 min, followed by additional rinses with distilled water. The prepared seeds were then placed on full- and half-strength liquid MS (Murashige and Skoog 1962) basal medium as well as liquid woody plant medium (Lloyd and McCown 1981) for germination. The germinated seeds were utilized for subsequent callus and shoot induction experiments (Fig. 1).

Callus induction

Leaves obtained from 40-day-old in vitro germinated seeds were inoculated onto MS medium supplemented with 3% (w/v) sucrose (Himedia, CAS: 57-50-1) and 0.8% Agar (Himedia, CAS No: 9002-18-0), with various concentrations of auxins and cytokinins, including 2,4-D (Himedia, CAS NO: 94-75-7), NAA (Himedia, CAS NO: 86-87-3), IAA (Himedia, CAS NO: 87-81-4), TDZ (Himedia, CAS: 51707-55-2), and BAP (Himedia, CAS NO: 1214-39-7) at concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L, individually and in combination. The concentration of 2,4-D (0.5 mg/L) was kept constant while the concentration of BAP and TDZ were varied (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L), to initiate callus formation. Proliferating callus was subcultured on the MS medium supplemented with the above-given concentrations at intervals of 21 to 30 days based on the rate of callus formation and shoot bud growth. The cultures were maintained at 25 ± 2 °C for 16 h of photoperiod with a light intensity of 35– 40 µmol m^{-2} s^{-1} irradiance (Philips India Ltd., Mumbai, India). Callus induction was carried out with a total of 200 replicates per concentration of each hormone used in the study. The callus response was quantified as a

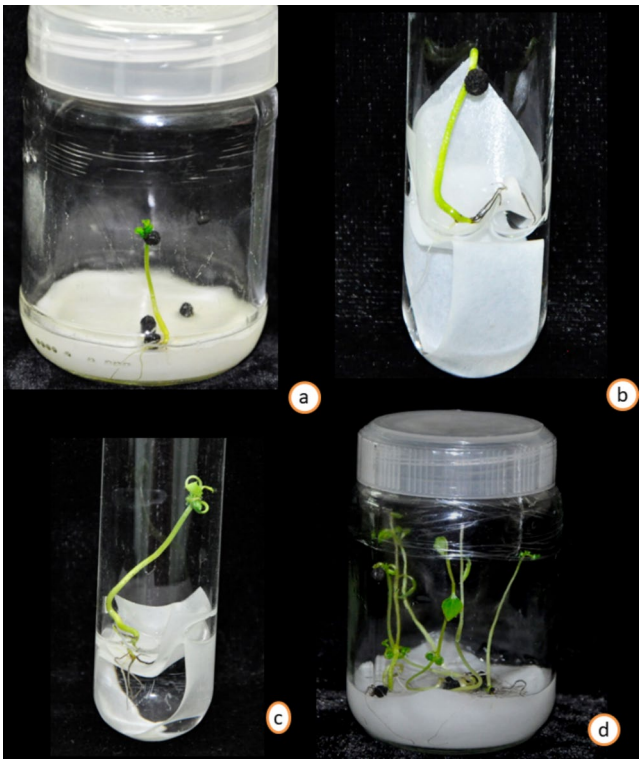

Fig. 1 In vitroseed germination in *Embelia ribes*. (**a**) In vitro seed germination on liquid MS medium, (**b**) In vitro seed germination liquid on Woody Plant medium, (**c**) well-grown seedling on full strength liquid MS medium, (**d**) well-grown seedling on full strength liquid Woody Plant medium

percentage of the total response, along with an assessment of its morphological characteristics.

Effect of elicitors

The elicitation study was conducted using both biotic (chitosan (Himedia, CAS No: 501-76-4)) and abiotic elicitors (salicylic acid (Himedia, CAS No: 69-72-7) and methyl jasmonate (Sigma Aldrich, CAS No: 39924-52-2)). Calli of both organogenic and embryogenic origins were incubated in liquid MS medium supplemented with 3% sucrose and various concentrations of chitosan, salicylic acid, and methyl jasmonate (50, 100, 150, 200, and 250 mg/L) for 20 days on a continuous rotatory shaker at 150 rpm. The cultures were maintained under 16 h with a light intensity of 35–40 µmol $m^{-2}s^{-1}$ irradiance at 25 ± 2 °C. Elicitation studies with biotic and abiotic elicitors were performed on 200 parallel replicates using different concentrations of elicitors. After the incubation period, the calli were analyzed for their embelin content.

In vitro shoot multiplication and root induction

Shoots excised from in vitro cultures were inoculated on MS medium supplemented with 3% sucrose and various concentrations of BAP, TDZ, KN, and IBA alone (0.5, 1.0, 1.5, 2.0, 2.5, and 3 mg/L). When used in combination, the concentration of IAA (0.1 mg/L) and NAA (0.1 mg/L) was kept constant while the concentration of BAP was varied (0.5, 1.0, 1.5, 2.0, 2.5, and 3 mg/L). Subculturing was performed every four weeks. The explant response, average number of shoots per explant, and average shoot length were recorded at each interval. Shoots measuring 3–4 cm in length were then inoculated on MS medium supplemented with various concentrations of auxins, such as NAA, IBA, and IAA (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L) for root induction. The average number of shoots per explant and the average root length were recorded every four weeks. The cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with a light intensity of 35– 40 µmol m^{-2} s^{-1} irradiance. Shoot multiplication and root induction were carried out with a total of 200 replicates per concentration of each hormone used in the study.

Hardening and acclimatization

The well-grown plantlets were subjected to a hardening process. The plantlets were removed from the culture tubes, and their roots were thoroughly washed under running tap water to remove traces of agar. The plants were then treated with 0.2% Bavistin for 1 min to prevent fungal infection. Subsequently, the plants were planted in sterilized coco peat mixed with sand and garden soil. The micropropagated plants were kept in a culture room for one week at 25 ± 2 °C under diffuse light conditions with 70 to 80% humidity. These potted plants were supplemented with half-strength liquid MS medium without 3% sucrose. After 30 days, the plants were transferred to a greenhouse at 28 ± 1 °C with 80% relative humidity and then were moved to a shade net with 50% light for two weeks for further growth and development.

Estimation of embelin by RP-HPLC and chromatographic conditions

Elicited organogenic and embryogenic calli were air-dried at 50 °C and then pulverized into a fine powder. This drying process ensures that the temperature, which is crucial for preserving secondary metabolites, is carefully controlled for reproducibility. One gram of the dried and powered organogenic and embryogenic callus, elicited with varying concentrations of chitosan, salicylic acid, and methyl jasmonate, was extracted separately in 10 ml of 95% methanol

(Sigma Aldrich, CAS NO: 67-56-1) for 90 s at 180 W using microwave-assisted extraction. The extracts were filtered through a 0.22 µm nylon filter (Axiva filters). The volume of the extract was then adjusted to 10 ml with 95% methanol and stored at 4 °C (Kamble et al. 2020). Standard embelin (Sigma Aldrich, CAS NO: 550-24-3) was weighed and dissolved in methanol to prepare a standard stock solution (mg/mL). This stock solution was serially diluted to concentrations of 250, 500, 750, and 1000 µg/mL to generate a calibration curve. RP-HPLC analysis was conducted following the method described by Kamble et al. (2020).

Statistical analysis

All data are presented as the mean ± standard deviation of the treatments. Differences among treatments were analyzed using analysis of variance (ANOVA), and statistical analyses were conducted with SPSS 22.0 (SPSS, Chicago, IL, USA). Post hoc tests were performed using Duncan's multiple-range test. A P-value of <0.05 was considered statistically significant, and different letters were used to denote significant differences ($P<0.05$).

Results

The combination of PGRs enhanced the percentage of callus induction

Callus proliferation was observed in all plant growth regulator (PGR) treatments. However, the frequency varied significantly with different concentrations and combinations of PGRs, especially 2,4-D, NAA, BAP, and TDZ, when added to the MS medium. Morphological differences were also observed in the callus color and texture. The greatest induction response was achieved with two specific combinations of PGRs. One combination of BAP (1.5 mg/L) with 2,4-D (0.5 mg/L) resulted in a 90% induction rate, resulting in the production of greenish, pigmented, hard, and organogenic calli (Figs. 2 and 3). The other combination, TDZ (2.0 mg/L) and 2, 4-D (0.5 mg/L), yielded an 85% induction rate with greenish, pigmented, semihard, embryogenic calli. Callus proliferation also occurred when each PGR was added separately to the medium, with the order of the callus induction rate being 2,4-D > TDZ > NAA > IAA (Figs. 2 and 3a–i).

Organogenesis induced in callus cultures using TDZ

After the fourth week of culture on medium supplemented with BAP (1.5 mg/L) and 2,4-D (0.5 mg/L), the calli were transferred to MS medium supplemented with 3% sucrose and 0.7% of agar with different concentrations of TDZ, such as 0.125, 0.25, 0.5, 1.0, 1.5 and 2.0 mg/L for shoot bud regeneration. The calli turned green, and the first vegetative bud was observed in the fifth week of culture. The average number of shoots per culture recorded was 69.41 ± 0.70, with an average shoot length of 16.3 ± 0.03 cm for 0.5 mg/L TDZ (Table 1; Fig. 3h).

Somatic embryo formation using a combination of TDZ and 2,4-D

Embryo differentiation began after six weeks of culture on media supplemented with TDZ (2.0 mg/L) and 2,4-D (0.5 mg/L). The somatic embryos appeared as whitish pro-embryogenic masses and underwent various stages of differentiation, including the globular, heart-shaped, torpedo, and cotyledonary stages. The embryos were then transferred to MS media supplemented with 3% sucrose and 0.7% of agar with different concentrations of TDZ for conversion into plants. Among the various concentrations of TDZ, 0.05 mg/L TDZ had the highest embryo conversion rate

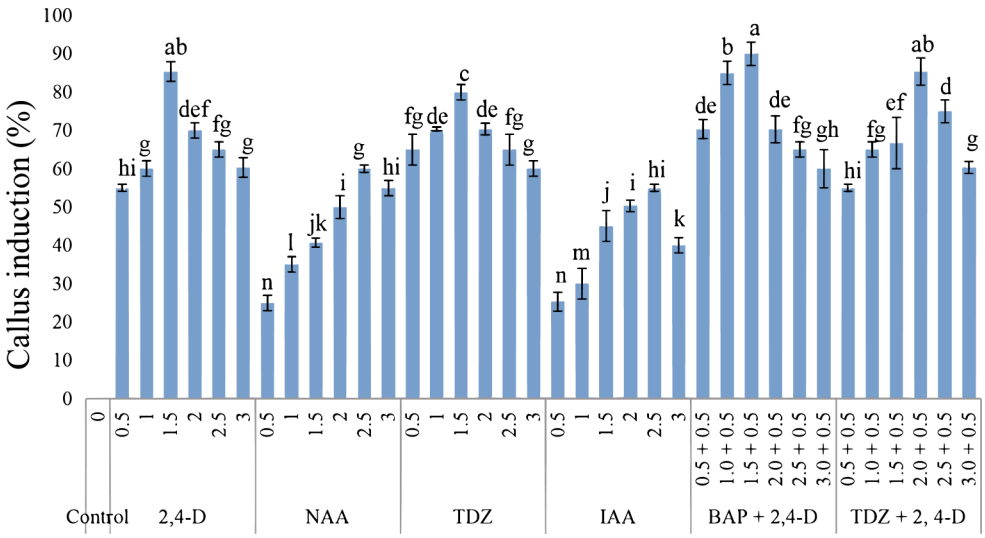

Fig. 2 Effect of plant growth regulators (PGR) on callus induction from leaf explants of *Embelia ribes* on MS basal medium supplemented with various concentrations of 2,4-D, NAA, TDZ, and BAP. Different letters indicate significant differences ($P<0.05$)

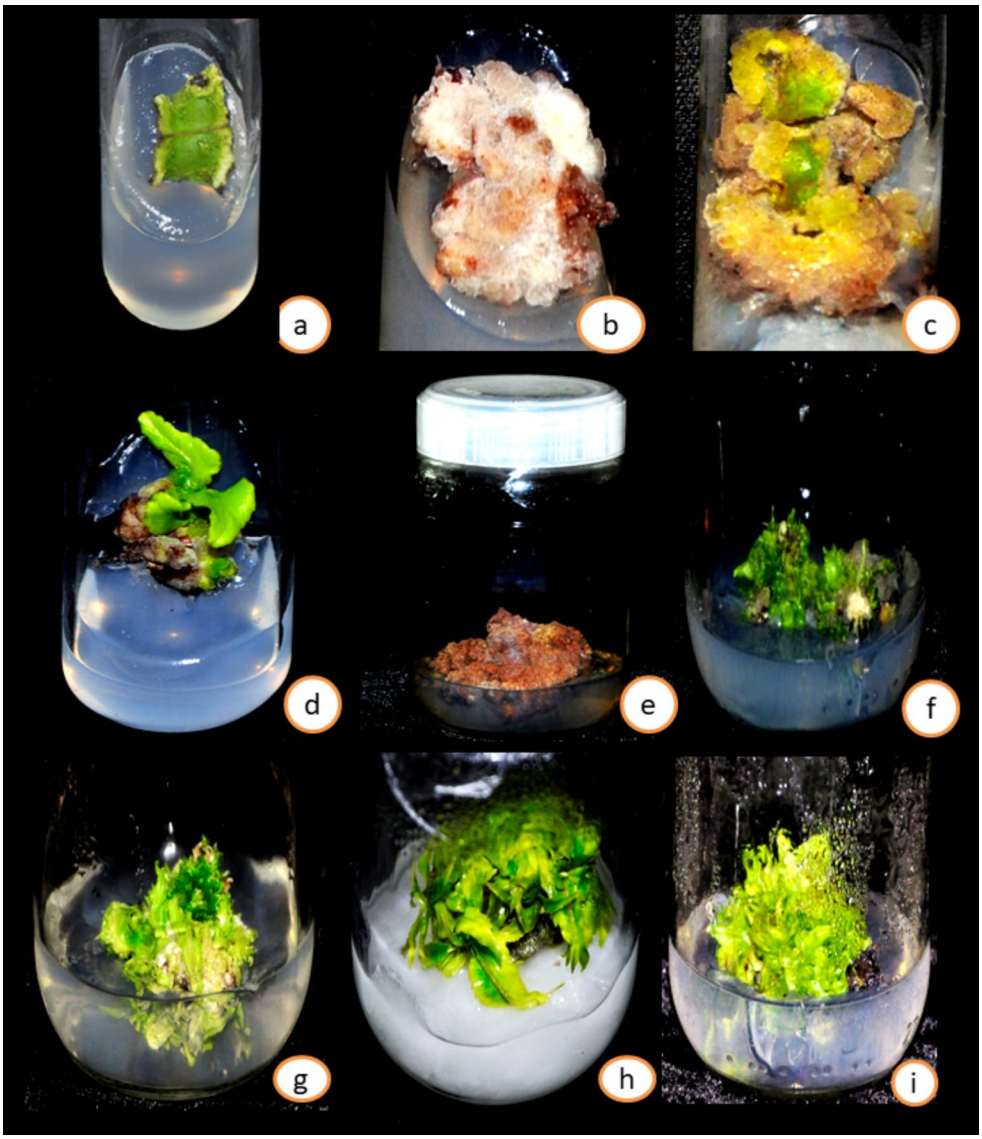

Fig. 3 Organogenic callus induction and proliferation from leaf explant of *Embelia ribes* (**a**) MS + 2,4-D (0.5 mg/L), (**b**) MS + NAA (2.5 mg/L), (**c**) MS + TDZ (0.5 mg/L), (**d**) MS + BAP + 2, 4-D (1.5 + 0.5 mg/L), (**e**) MS + 2,4-D (1.5 mg/L), (**f**) MS + TDZ (1.5 mg/L), (**g-i**) MS + TDZ + 2, 4-D (2.0 + 0.5 mg/ L)

Table 1 Effect of plant growth regulators (PGRs) on organogenic callus inducted from leaf explants of *Embelia ribes* on MS basal medium supplemented with various concentrations of TDZ for shoot multiplication. Values are the mean of 200 parallel replicates per concentration of TDZ (means ± SD). The superscripts with different letters indicate significantly different values (Duncan multiple range test, $p \leq 0.05$)

MS + PGR (mg/L) TDZ	Average number of shoots/1.0 g callus	Average shoot length(cm)
0.125	46.51 ± 0.69^{d}	09.18 ± 2.34^{f}
0.25	59.1 ± 2.96^{b}	11.32 ± 1.33^{e}
0.5	69.41 ± 0.70^{a}	16.3 ± 0.03^{a}
1.0	55.91 ± 2.7^{c}	15.4 ± 0.15^{b}
1.5	39.70 ± 0.03^{e}	14.6 ± 0.72^{c}
2.0	32.98 ± 1.70^{f}	14.03 ± 1.23^{d}

Table 2 Effect of TDZ on the conversion of embryoids into shoots of *Embelia ribes* on MS basal medium. Values are the mean of 200 parallel replicates per concentration of TDZ (means ± SD). The superscripts with different letters indicate significantly different values (Duncan multiple range test, $p \leq 0.05$)

TDZ (mg/L)	Conversion of Embryos (%)	Mean number of shoots/culture	Length of shoots (cm)
0.01	14.15 ± 0.48^{e}	16.70 ± 0.47^{e}	0.37 ± 0.03^{d}
0.03	44.33 ± 1.42^{d}	22.08 ± 0.98^{d}	0.45 ± 0.03^{c}
0.05	60.17 ± 1.06^{a}	36.15 ± 1.04^{a}	0.90 ± 0.13^{b}
0.08	54.29 ± 2.30^{b}	27.24 ± 0.44^{b}	1.27 ± 0.09^{a}
0.1	46.27 ± 1.07^{c}	23.73 ± 0.59^{c}	0.91 ± 0.07^{b}

(60.17 ± 1.06%) and shoot production (36.15 ± 1.04%) per culture, with an average shoot length of 0.90 ± 0.13 cm. (Table 2; Fig. 4).

Fig. 4 Induction of somatic embryos and conversion of embryoids into shoots of *Embelia ribes* (**a**) MS + TDZ (2.0 mg/L) + 2, 4-D (0.5 mg/L), (**b**) MS + TDZ (2.5 mg/L) + 2,4-D + 0.5 mg/L), (**c**) MS + TDZ (0.08 mg/L), (**d**) MS + TDZ (0.05 mg/L)

Table 3 Effect of different elicitors on embelin enhancement in organogenic and embryogenic calli. Values are the mean of 200 parallel replicates per concentration of different elicitors (means ± SD). The superscripts with different letters indicate significantly different values (Duncan multiple range test, $p \leq 0.05$)

Sr no	Biotic and abiotic elicitors	Concentration	% Embelin content in organogenic callus	% Eembelin content in embryogenic callus
1	Control	------	2.50^{e}	3.49^{f}
2	Chitosan	50 mg/L	2.88^{d}	3.58^{e}
3		100 mg/L	2.97^{c}	3.75^{d}
4		150 mg/L	3.50^{b}	6.02^{b}
5		200 mg/L	**5.72^{a}**	**6.44^{a}**
6		250 mg/L	3.53^{b}	4.03^{c}
7	Salicylic acid	50 µM	2.93^{d}	3.25^{e}
8		100 µM	3.28^{c}	3.43^{d}
9		150 µM	**5.30^{a}**	**5.63^{a}**
10		200 µM	3.55^{b}	3.83^{b}
11		250 µM	2.77^{e}	3.43^{c}
12	Methyl jasmonate	50 µM	1.88^{f}	2.03^{e}
13		100 µM	2.01^{e}	2.76^{d}
14		150 µM	2.35^{c}	3.67^{b}
15		200 µM	2.25^{d}	3.52^{c}
16		250 µM	**5.56^{a}**	**6.22^{a}**

Elicitation response of Chitosan, salicylic acid, and methyl jasmonate using RP-HPLC

Different concentrations of chitosan had different effects on embelin enhancement in both organogenic and embryogenic calli (Table 3). Overall, the embelin content increased with increasing chitosan concentration. The optimum concentration was 200 mg/L chitosan, which yielded 6.44% and 5.72% embelin content in embryogenic and organogenic calli, respectively (Table 3). Similarly, compared with those of the controls, the salicylic acid-treated calli showed increased embelin content, but higher salicylic acid concentrations were inhibitory. The maximum embelin content was achieved with 150 µM salicylic acid, resulting in 5.63% in embryogenic callus and 5.30% in organogenic callus. The lowest embelin content was obtained after treatment with 250 µM salicylic acid, with 3.43% in embryogenic calli and 2.77% in organogenic calli (Table 3). In contrast, a higher concentration of methyl jasmonate led to increased embelin content in both organogenic and embryogenic calli (Table 3). After 20 days of inoculation, the highest embelin content was recorded with 250 µM methyl jasmonate, at 6.22% in embryogenic callus and 5.56% in organogenic callus. The lowest embelin content was observed with 50 µM methyl jasmonate, with 2.03% and 1.88% in embryogenic and organogenic calli, respectively (Table 3, Supplementary Figs. S1–S18).

Cytokinin combination enhances the shoot induction response

Cytokinins significantly elevated shoot induction, although a gradual decline in the multiplication rate was observed after the third subculture. When used individually, BAP was superior to kinetin and TDZ in inducing shoot growth. Higher concentrations of BAP resulted in prolific shoot growth, while lower concentrations produced healthy shoot growth with fewer axillary buds. BAP at a concentration of 2.0 mg/L had the greatest effect (96%), with an average of 10.49 ± 0.2 shoots and an average shoot length of 6.38 ± 0.21 cm (Table 4; Fig. 5). The second highest response was recorded with 0.1 mg/L TDZ, which had an 85% response, with 18.39 ± 0.93 shoots and a shoot length of 1.98 ± 0.21 cm, followed by kinetin, which had a lower shoot multiplication performance in *E. ribes*, with a 68% response rate, 3.72 ± 0.29 shoots on average, and a shoot length of 2.20 ± 0.23 cm (Table 4; Fig. 5).

When used in combination with 2.0 mg/L BAP, 0.1 mg/L IAA had a 97% shoot yield, with an average of 16.1 ± 0.85 shoots and a maximum shoot length of 6.98 ± 0.78 cm, followed by an 89% shoot yield with 2.0 mg/L BAP and 0.1 mg/L NAA, resulting in 9.01 ± 0.23 shoots on average

Table 4 Effect of different plant growth regulators on bud breaking from nodal explant of *Embelia ribes* on MS medium. Values are the mean of 200 parallel replicates per concentration of different PGRs (means ± SD). The superscripts with different letters indicate significantly different values (Duncan multiple range test, $p \leq 0.05$)

Sr.No.	PGR	Concentration mg/L	Response %	Shoot number	Shoot length
1	MS basal	0	0	0	0
2	BAP	0.5	66^{hi}	4.29 ± 0.49^{f}	7.21 ± 0.14^{a}
3		1.0	76^{f}	$6.12 \pm 0.26e$	6.82 ± 0.34^{b}
4		1.5	85^{c}	7.69 ± 0.26^{d}	$6.61 \pm 0.24c$
5		**2.0**	**96^{a}**	**10.49 ± 0.21^{a}**	**6.38 ± 0.21^{d}**
6		2.5	83^{cd}	9.61 ± 0.42^{b}	5.87 ± 0.22^{e}
7		3.0	72^{g}	9.02 ± 0.21^{c}	4.76 ± 0.12^{f}
8	Kinetin	0.5	63^{i}	2.21 ± 0.24^{c}	1.62 ± 0.25^{c}
9		**1.0**	**68^{h}**	**3.72 ± 0.29^{a}**	**2.20 ± 0.23^{a}**
10		1.5	64^{i}	5.54 ± 0.46^{b}	1.99 ± 0.26^{b}
11		2.0	62^{i}	6.46 ± 0.63^{d}	1.71 ± 0.22^{d}
12		2.5	57^{k}	5.99 ± 0.69^{e}	1.43 ± 0.06^{e}
13		3.0	55^{kl}	5.12 ± 0.28^{f}	1.41 ± 0.08^{f}
14	TDZ	0.02	54^{l}	10.51 ± 0.41^{f}	1.23 ± 0.25^{f}
15		0.05	73^{g}	13.50 ± 0.55^{c}	1.45 ± 0.51^{c}
16		**0.1**	**85^{c}**	**18.39 ± 0.93^{a}**	**1.98 ± 0.21^{a}**
17		0.3	82^{de}	15.14 ± 0.96^{b}	1.32 ± 0.67^{b}
18		0.5	65^{i}	12.26 ± 0.98^{d}	1.08 ± 0.58^{d}
19		1.0	60^{j}	9.35 ± 0.76^{e}	0.95 ± 0.23^{e}
20	BAP + IAA	0.5 + 0.1	68^{h}	10.43 ± 0.81^{f}	7.51 ± 0.13^{a}
21		1.0 + 0.1	79^{e}	11.15 ± 0.63^{e}	6.73 ± 0.15^{c}
22		1.5 + 0.1	88^{b}	13.05 ± 0.72^{c}	6.65 ± 0.18^{d}
23		**2.0 + 0.1**	**97a**	**16.16 ± 0.85^{a}**	**6.98 ± 0.78^{b}**
24		2.5 + 0.1	85c	14.1 ± 0.89^{b}	5.35 ± 0.55^{e}
25		3.0 + 0.1	78e	12.22 ± 0.75^{d}	4.23 ± 0.43^{f}
26	BAP + NAA	0.5 + 0.1	65i	7.35 ± 0.11^{e}	5.35 ± 0.23^{e}
27		1.0 + 0.1	73^{fg}	8.71 ± 0.84^{c}	5.86 ± 0.21^{d}
28		1.5 + 0.1	80^{e}	8.98 ± 0.13^{b}	6.01 ± 0.56^{b}
29		**2.0 + 0.1**	**89^{d}**	**9.01 ± 0.24^{a}**	**6.23 ± 0.43^{a}**
30		2.5 + 0.1	79^{e}	7.45 ± 0.36^{d}	5.90 ± 0.12^{c}
31		3.0 + 0.1	71^{g}	6.37 ± 0.38^{f}	5.03 ± 0.15^{f}

and a shoot length of 6.23 ± 0.43 cm. The combination of 0.5 mg/L BAP and 0.1 mg/L NAA had the lowest response rate of 79%, with 7.35 ± 0.11 shoots on average and a shoot length of 5.35 ± 0.23 cm (Table 4; Fig. 5). (Table 4; Fig. 5).

Auxin IBA enhances the root induction response

Elongated shoots, 3–4 cm in length, were used for in vitro rooting on MS basal medium supplemented with 3% sucrose and 0.8% agar with various concentrations of auxins. Rooting was observed at all tested concentrations, with IBA being the most effective auxin, followed by NAA and IAA. The optimal response was achieved with 1.0 mg/L IBA, resulting in an average of 46.04 ± 0.58 roots and a root length of 6.57 ± 0.20 cm, with a 100% response rate. (Table 5; Fig. 6). The medium supplemented with 1.0 mg/L IAA showed an 86% response rate, producing 30.95 ± 0.40 roots per shoot and a root length of 5.60 ± 0.19 cm. Additionally, MS media supplemented with 0.5 mg/L NAA achieved a 100% response rate, yielding 41.98 ± 1.38 roots per shoot and a root length of 7.01 ± 0.14 cm (Table 5; Fig. 6).

Hardening and acclimatization of micropropagated plants of *Embelia ribes*

Hardening and acclimatization are crucial steps in plant tissue culture studies. Somaclonal variations in the morphology, anatomy, and physiology of plantlets obtained from in vitro culture can be addressed when transferred to ex-vitro conditions. During the hardening process, in vitro regenerated plantlets with well-developed shoots and roots were transferred to pots containing a mixture of garden soil, sand, and cocopeat (1:1:1). Initially, micropropagated plants were hardened in a culture room (25 ± 2 °C) for four days, followed by 3–4 weeks in the greenhouse at 28 ± 1 °C with 80% relative humidity and then transferred to a shade net with 50% light for two weeks. Subsequently, the plantlets were moved to an open nursery. One month later, the plantlet survival rate was 60%, with plants growing vigorously

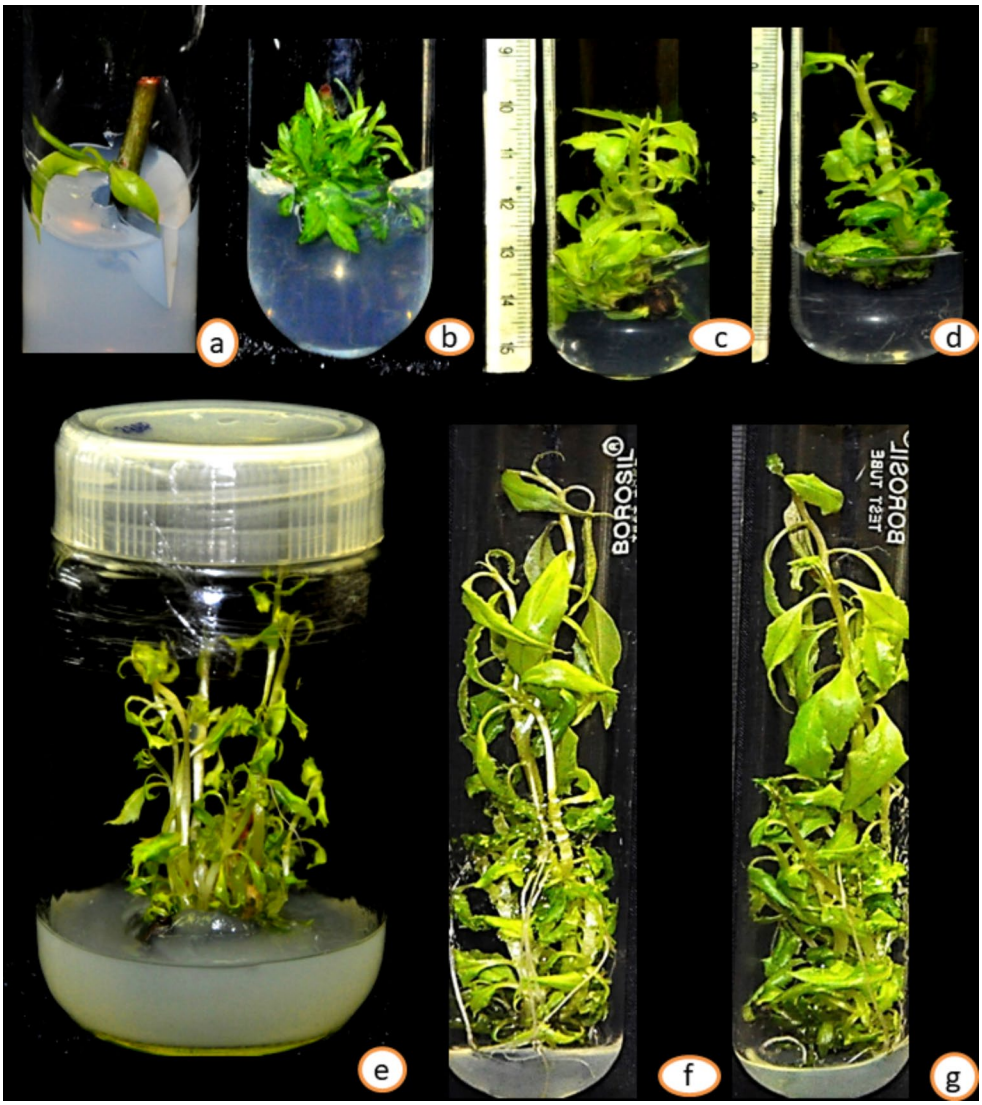

Fig. 5 Micropropagation of *Embelia ribes* (**a**) Induction of shoots (MS + BAP 2.0 mg/L), (**b**) Shoot multiplication (MS + BAP (2.0 mg/L) + IAA (0.1 mg/L), (**c**-**d**) First subculture (MS + BAP (2.0 mg/L) + IAA + (0.1 mg/L), (**e**) Second subculture (MS + BAP (2.0 mg/L) + IAA (0.1 mg/L), (**f** and **g**) In vitro rooting on ½ MS + NAA (0.5 mg/L)

and exhibiting true characteristics similar to those of field-grown plants. During the hardening process of *Embelia ribes*, high humidity and a mixture of garden soil, sand, and coco peat were found to be effective for the survival of cultured plantlets (See Fig. 7).

Discussion

Embelia ribes did not exhibit callus induction on basal MS media, highlighting the necessity of additional plant growth regulators (PGRs) for callus induction and proliferation. This observation aligns with previous studies that utilized PGRs for in vitro callus induction and proliferation in *Embelia ribes* (Raghu et al. 2011) as well as in other plant species, such as *Ceropegia juncea* (Nikam and Savant 2009), *Aponogeton madagascariensis* (Carter and Gunawardena 2011), *Mesua ferrea* (Saini et al. 2014) and *Pyrus malus* L. (Kumar et al. 2016). Callus formation devoid of exogenous PGRs can be induced through the wounding of explants, where cells at the cut ends undergo mitotic division to form calli (Kahl 1983). PGRs, particularly cytokinins, and auxins, are known to induce callus formation in cultured tissues (Skoog and Armstrong 1970; Akiyoshi et al. 1983). Auxins play a crucial role in promoting plant morphogenesis by influencing cell division, differentiation, and elongation. High concentrations of auxin can lead to intensive callusing (Evans et al. 1981). Both auxins and cytokinins are major growth regulators that profoundly affect cell division, callus proliferation, and regeneration (Tang et al. 2000).

In the present study, MS media supplemented with both auxins and cytokinins played a vital role in callus induction and proliferation. Successful callus induction in *Embelia ribes* was also reported by Raghu et al. (2011) using various concentrations and combinations of 2,4-D, NAA, IAA, BAP, and TDZ. This finding corroborates the observation

Table 5 Effect of different auxins on in vitro rooting in *Embelia ribes* on ½ strength MS medium. Values are the mean of 200 parallel replicates per concentration of different PGRs (means ± SD). The superscripts with different letters indicate significantly different values (Duncan multiple range test, $p \leq 0.05$)

Sr.No.	PGR	Concentration mg/L	Response %	Root number	Root length
1	Basal	-	-	-	-
2	IBA	0.5	96^{b}	34.86 ± 0.86^{d}	6.65 ± 0.23^{bc}
3		**1.0**	$\mathbf{100^{a}}$	$\mathbf{46.04 \pm 0.58^{a}}$	$\mathbf{6.57 \pm 0.20^{cd}}$
4		1.5	84^{c}	34.27 ± 1.36^{d}	5.71 ± 0.10^{fg}
5		2.0	72^{f}	28.85 ± 1.28^{f}	5.48 ± 0.07^{h}
6		2.5	68^{g}	24.43 ± 0.36^{g}	5.28 ± 0.25^{i}
7		3.0	63^{h}	20.78 ± 1.64^{h}	4.74 ± 0.21^{k}
8	IAA	0.5	82^{d}	24.88 ± 1.38^{g}	5.92 ± 0.07^{e}
9		**1.0**	$\mathbf{86^{c}}$	$\mathbf{30.95 \pm 0.40^{e}}$	$\mathbf{5.60 \pm 0.19^{gh}}$
10		1.5	70^{fg}	29.14 ± 0.63^{f}	5.33 ± 0.08^{i}
11		2.0	71^{f}	20.29 ± 0.49^{hi}	4.94 ± 0.04^{j}
12		2.5	63^{h}	19.47 ± 1.30^{i}	4.31 ± 0.18^{l}
13		3.0	58^{i}	16.61 ± 0.35^{j}	3.82 ± 0.13^{m}
14	NAA	**0.5**	$\mathbf{100^{a}}$	$\mathbf{41.98 \pm 1.38^{b}}$	$\mathbf{7.01 \pm 0.14^{a}}$
15		1.0	96^{b}	38.68 ± 0.45^{c}	6.77 ± 0.20^{b}
16		1.5	80^{d}	30.83 ± 0.65^{e}	6.43 ± 0.05^{d}
17		2.0	75^{e}	28.93 ± 0.58^{f}	5.82 ± 0.13^{f}
18		2.5	61^{h}	24.80 ± 1.25^{g}	4.94 ± 0.15^{j}
19		3.0	59^{i}	20.88 ± 1.67^{h}	4.64 ± 0.13^{k}

that auxins and cytokinins promote organogenesis in this species. Importantly, TDZ plays a key role in regulating morphogenesis and favours organogenesis in *E. ribes*. TDZ has also served as a substitute for auxin and cytokinin in organogenesis in numerous plant species (Raghu et al. 2006, 2011; Murthy et al. 1998).

The combination of auxin with cytokinin has shown promising effects on somatic embryogenesis (Ignacimuthu et al. 1999; Yancheva et al. 2003). Specifically, 2,4-D acts as an effective stressor, playing a key role in embryo development in cultured plant cells. In *E. ribes*, 2,4-D (0.5 mg/L) in combination with the cytokinin BAP (2.5 mg/L) proved useful for inducing embryogenic calli, resulting in a high frequency of somatic embryos. These findings are consistent with previous reports on the role of 2,4-D in somatic embryogenesis (Ammirato 1983; Flick et al. 1983; Krishna et al. 2004; Raghu et al. 2011). Recent advancements in callus morphogenesis involve significant factors such as hormonal signals, environmental conditions, and genetic factors. All these factors play a critical role in cell fate determination in callus tissues. Gene transformation techniques enhance the capacity of callus culture by allowing the introduction of desirable traits (Shobica et al. 2024).

Differences in embelin content were observed between organogenic and embryogenic calli and berries of *E. ribes*. Previous studies have shown that embryogenic calli contain a greater percentage of embelin than organogenic calli (Raghu et al. 2011). This is likely due to various changes during the development of embryogenic calli, leading to the production of higher quantities of secondary metabolites. The present findings also support that embryogenic callus culture is a promising method for producing high embelin content in *E. ribes* (Rao and Ravishankar 2002; Jain and Saxena 2009) However, our reproducible protocol for the species showed a higher embelin content (6.33%) than what is achieved in previous studies (Raghu et al. 2011).

This study is the first to report the elicitation of embelin in organogenic and embryogenic calluses using biotic and abiotic elicitors. A comparison of the elicited responses in both callus types with those in the untreated controls showed that different concentrations of chitosan significantly enhanced the embelin content, consistent with previous reports on phenolic enhancement using chitosan (Kim et al. 2005; Cai et al. 2012). For example, chitosan has been reported to increase secondary metabolite production in *Ocimum basilicum* and *Vitis vinifera* (Kim et al. 2005; Cai et al. 2012), and its concentration influences the response intensity in different plant species (Vasconsuelo and Boland 2007; Chang et al. 1998; Chakraborty et al. 2009). Similar increases in secondary metabolite production using chitosan have been observed in the present study confirming its role as an effective elicitor.

In this study, organogenic and embryogenic calli treated with salicylic acid had lower embelin contents than those treated with methyl jasmonate and chitosan, although the contents were greater than those in the controls. Salicylic acid acts as a signalling molecule in enzymatic pathways involved in polyphenol and alkaloid production (Van Loon 1997) and is also involved in plant defense mechanisms (Metraux et al. 1990). It has been used to enhance in vitro regeneration in several plant species (Luo et al. 2001; Hao et al. 2006) and is known to increase the production of various secondary metabolites in different plants (Mathur and Yadav 2011; Ajungla et al. 2009; Sudha and Ravishankar 2003; Satdive et al. 2007).

Methyl jasmonate, an elicitor, significantly enhanced embelin production in both the organogenic and embryogenic calli. Among the abiotic elicitors used, methyl jasmonate favoured higher levels of embelin production than salicylic acid. Methyl jasmonate is recognized as an important signaling compound for the hyperproduction of various secondary metabolites (Walker et al. 2002). It has been reported to enhance the production of bioactive compounds in several plant cultures (Sharma et al. 2013; Wu and Lin 2003; Yu et al. 2000, 2002; Wang and Zhong 2002). In the present study, similar results were observed for *E. ribes*. Overall, this study demonstrated the significant potential of using biotic and abiotic elicitors to enhance embelin

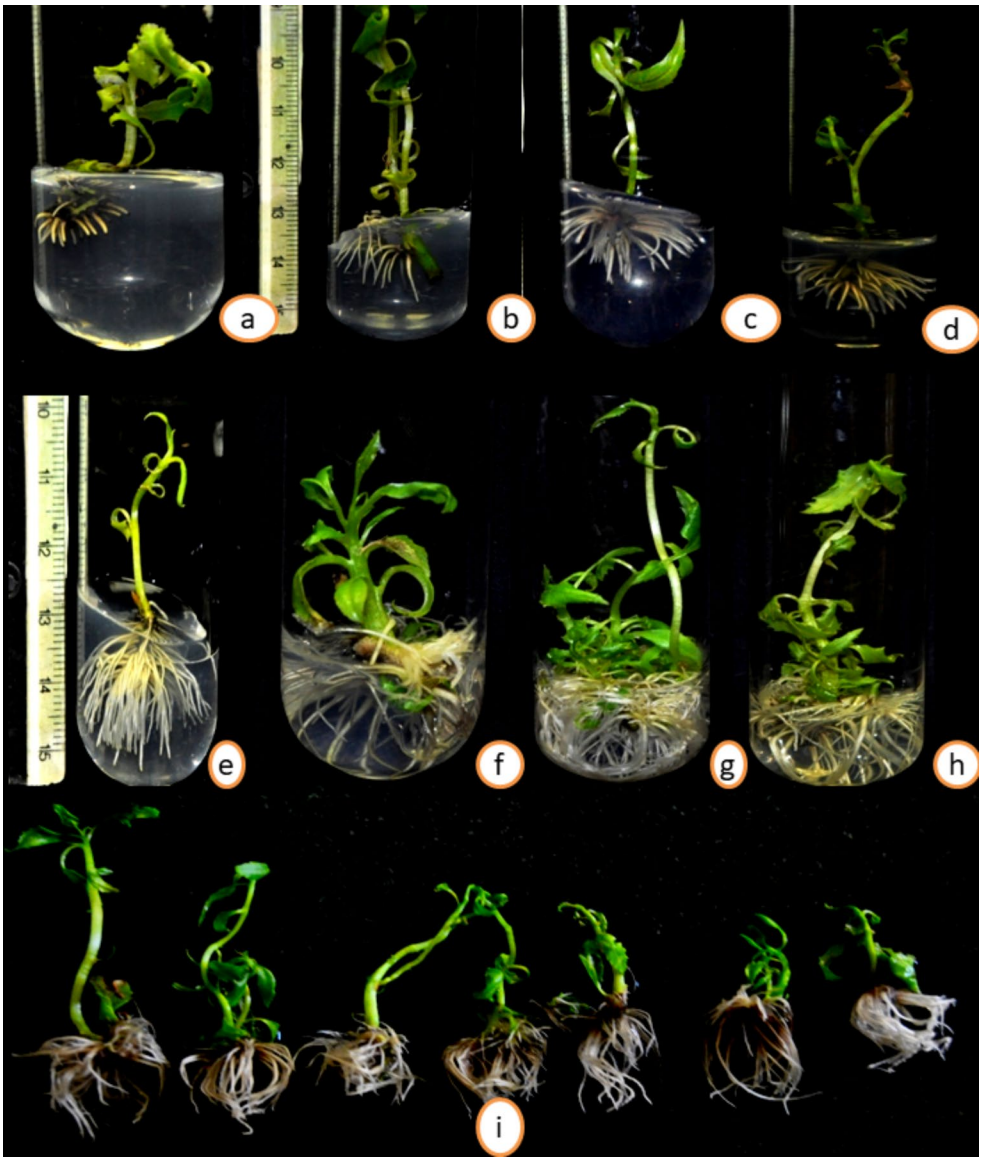

Fig. 6 In vitro rooting in *Embelia ribes* on (**a-d**) ½ MS + IAA (1.0 mg/L), (**e-f**) ½ MS + NAA (0.5 mg/L), (**g-f**) ½ MS + IBA (1.0 mg/L), (**i**) Rooted shoots on ½ MS + 1.0 mg/L IBA

production in *E. ribes* calli, providing a valuable method for the large-scale production of this important secondary metabolite.

The type of explants used for shoot induction plays an important role in establishing an efficient regeneration system (Koroch et al. 2002; Uranbey et al. 2005). Shoot induction in *E. ribes* using nodal explants on MS media supplemented with 0.5 mg/L BAP resulted in a mean number of 4.6 ± 0.57 shoots and a mean shoot length of 3.24 ± 0.02 (Preetha et al. 2012). Annapurna and Rathore (2010) reported 66.8 shoots from hypocotyl segments on MS media supplemented with 283.85 µM ascorbic acid, 118.96 µM CA, 142.33 µM cysteine, 684.22 µM glutamine, and 1.13 µM TDZ over 10 weeks in the same species. However, Raghu et al. (2006) reported 16–18 shoots per explant using seedling-derived leaf explants of *E. ribes* on MS media supplemented with 0.272 µM TDZ, noting that higher concentrations resulted in callusing. The results of the present study appear to be significantly higher than those achieved in Preetha et al. (2012) and Raghu et al. (2006) indicating that the nature of the explant has an impact on the morphogenic response and shoot induction. This observation aligns with the findings of the present study.

For rooting, IBA resulted in 100% rooting in half-strength MS media supplemented with 2.47 µM IBA, with an average of 10.67 roots per shoot and a maximum shoot length of 6.57 cm within 4 weeks of culture in the present study. In contrast, other studies on *E. ribes* used different auxin derivatives such as IAA, which resulted in an average of 1.6 ± 0.40 roots (Preetha et al. 2012). Half-strength MS basal media supplemented with 0.1 mg/L IBA resulted in an average of 9.2 roots per shoot, with a mean root length of 2.2 cm (Raghu et al. 2011). The present results suggest

Fig. 7 Hardening and acclimatization of micropropagated plants of *Embelia ribes*. (**a**-**b**) Hardening of in vitro regenerated plantlets with well-developed shoots and roots in pots containing a mixture of garden soil, sand, and cocopeat, (**c**-**d**) Hardening of micropropagated plants in the culture room, (**e**) Hardening of micropropagated plants in the greenhouse, (**f**) Hardening of micropropagated plants in shade net, (**g**) Plants growing in the open nursery

that IBA is an effective rooting hormone to be used in tissue culture to produce higher and thicker roots.

A sand and cocopeat mixture were successfully employed for hardening *E. ribes* Sand is effective during hardening and acclimatization for other plant species as well such as *Ceropegia* and ginger (Pawar 2009; Chavan et al. 2011). *E. ribes* also require high humidity during the initial stages of hardening. These observations align with similar findings observed in other plants such as in strawberry (Jofre-Garfias 2006).

Conclusion

In this study, we established a robust protocol to enhance embelin production in *Embelia ribes*, a valuable medicinal plant. Our investigation demonstrated that treatment with chitosan, salicylic acid, and methyl jasmonate significantly increased embelin production in both organogenic and embryogenic callus cultures. Notably, the highest embelin yield was achieved with a chitosan concentration of 200 mg/L, which resulted in a 6.44% embelin content in embryogenic calli and a 5.72% content in organogenic calli. Additionally, this study presents a straightforward and effective method for high-frequency multiple shoot formation from nodal explants of *Embelia ribes*. This protocol provides a valuable tool for genetic transformation studies and enables the large-scale propagation of *Embelia ribes* within its natural habitat. The enhanced production of embelin from both organogenic and embryogenic callus cultures holds significant promise for future applications in the pharmaceutical industry.

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Author contributions NBG designed the research, VVK performed all the experiments, SAR performed the data analysis, and SAR, VVK and NBG wrote the main body of the manuscript. SAR, MR, and BP edited the manuscript.

Data availability All the data generated during this study are included in this published article and its supplementary information files.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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